

**Evaluation of the effect of leaf development in
plectranthus amboinicus l. on antimicrobial activity
and virulence factors of pseudomonas aeruginosa
PA01 and staphylococcus aureus NCTC8325**

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1 **Essential title page information**

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3 **antimicrobial activity and virulence factors of *Pseudomonas aeruginosa* PA01 and**
4 ***Staphylococcus aureus* NCTC8325**

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19 **Evaluation of the effect of leaf development in *Plectranthus amboinicus* L. on**
20 **antimicrobial activity and virulence factors of *Pseudomonas aeruginosa* PA01 and**
21 ***Staphylococcus aureus* NCTC8325.**

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28 **Abstract**

29 *Plectranthus amboinicus* is widely recognized as a potential source of antimicrobial
30 compounds; due to the presence of bioactive components (essential oils) secreted by the
31 glandular trichomes borne on the leaves. As such, an understanding of the effect of leaf
32 development on the production of these essential oils (EOs) is of crucial importance to its
33 medicinal applications. The current study represents the first comparative investigation of
34 the effect of different stages of leaf development (lag, log, and stationary phase) upon the
35 yield and bioactivity of phytochemicals produced. The effects of leaf extracts on the
36 antimicrobial activity, cell surface hydrophobicity, biofilm formation, and motility of *P.*
37 *aeruginosa* and *S. aureus* were evaluated. Cryo-Scanning Electron Microscopy was used to
38 record the abundance and distribution of both glandular and non-glandular trichomes during
39 leaf development. Gas chromatography-mass spectrometry analysis revealed that the potent
40 phytochemical thymol is present primarily in log (30.28%) and stationary phase (20.89%)
41 extracts. Log phase extracts showed the lowest minimum inhibitory concentration (25mg/ml)
42 when compared to other phases of development. Stationary phase extracts were shown to
43 exhibit the highest biofilm dispersal activity against *P. aeruginosa* (80%), and log phase
44 extracts against biofilms of *S. aureus* (59%). Log phase extracts showed the highest biofilm
45 inhibitory activity against *P. aeruginosa* (66%) and *S. aureus* (63%). In conclusion, log phase

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leaf extracts of *P. amboinicus* exhibited a multimodal mechanism of action by displaying antimicrobial, antibiofilm activities and reducing the motility and hydrophobicity, which are important virulence factors in *P. aeruginosa* and *S. aureus* pathogenesis.

Keywords

Antimicrobial

Antibiofilm

Chromatography

Essential oil

Medicinal plants

Polyphenols

1. Introduction

According to the World Health Organization (WHO), 80% of the population living in developing countries, are dependent upon traditional remedies derived from plant-based medicines as their source of primary healthcare; due to their availability, cost-effectiveness, and minimal side effects [1–3]. Moreover, modern pharmacopoeia records 30-50% plant-derived drugs and several synthetic analogues, developed from compounds of plant origin [4, 5]. As such, plant-derived compounds (pharmacognosy) continue to play an important role in modern-day drug discovery [2, 6]. A holistic approach to drug discovery and development, which incorporates both pharmacognosy and recombinatorial chemistry, can provide an expansive and productive conduit, to future drug development.

In recent years, the World Health Organization (WHO) has recognized the marked increase in antimicrobial resistance (AMR) to antibiotics, as a major threat to public health [7]. This problem has been further exacerbated by improper prescription practices, restricted

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69 diagnostic resources, and a lack of appropriate and effective drug regulatory systems in many
70 developing countries [8]. According to one estimate, by 2050, the development of antibiotic
71 resistance in pathogenic bacterial species, may cause up to 10 million deaths per year, at a
72 cost of 100 trillion dollars [9, 10].

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73 Pathogenic bacterial species exhibit resistance to antibiotics because of innate/ intrinsic
74 resistance, such as limited permeability of bacterial outer membranes, combined with the
75 expression of β -lactamases and efflux pump systems. However, such microbes can also
76 acquire resistance by horizontal gene transfer, modification of antibiotic target sites,
77 overexpression of efflux pump systems and alterations in porin expression. Unfortunately,
78 these mechanisms can occur simultaneously, consequently presenting a multi-drug resistant
79 phenotype [11].

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80 *S. aureus* and *P. aeruginosa* biofilms have been the subject of extensive research in recent
81 years and many strategies have been devised to control or destroy such biofilms. These two
82 microorganisms are on the World Health Organisation's (WHO) priority pathogens list for
83 research and development of novel antimicrobial agents[12, 13]. In addition, they are part of
84 the ESKAPE pathogens group which have become multidrug-resistant and are now
85 responsible for causing major healthcare-associated infections which are difficult to control
86 and treat[14]. Therefore, these two bacterial species were selected for use in the current
87 study, as target microorganisms for the evaluation of the antimicrobial activity of the leaf
88 extracts. Considering the 'silent' pandemic of AMR, it is imperative to develop and evaluate
89 a variety of therapeutic agents to help alleviate the problems caused by these pathogens [9].

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90 In this regard, plant-derived phytochemicals and secondary metabolites have immense
91 potential for use as biocompatible therapeutics [9]. Considering which, the current study

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92 explored the potential application of leaf extracts of the herbaceous perennial species
93 *Plectranthus amboinicus*, as an antimicrobial agent/treatment.

94 More than 300 species belonging to the *Plectranthus* genus have been reported in Africa,
95 Asia, and Australia. The medicinal properties of species within this genus are well
96 documented and are known for their antimicrobial, antioxidant, and antiviral properties [15,
97 16].

98 *Plectranthus amboinicus* (Lour.) Spreng, is one of the more widely researched species within
99 the genus. It is a fleshy, aromatic, succulent herb, with distinctive soft, dense hairs covering
100 the leaves [17]. The current literature on this species is predominately focused on the
101 identification and quantification of various bioactive constituents found in leaf and stem
102 extracts of the plant. To date, a total of 76 volatile and 30 non-volatile phytochemicals have
103 been reported [18]. However, the biochemical composition of these bioactive chemicals and
104 their concentration varies, and is subject to changes in climate, geographical features, soil,
105 pH, humidity, and methods of extraction [16, 18].

106 Many classes of bioactive phytochemicals such as mono-, di-, tri- and sesquiterpenoids plus
107 flavonoids, phenolics, esters, alcohols, fatty acids, phenylpropanoids, and aldehydes are all
108 reported to be present in the leaves and stems of this species [18]. Both crude extracts and
109 essential oils obtained from the leaves of the plant have been shown to possess medicinal
110 properties [19]. However, relatively little is known with regards to the biosynthesis of these
111 compounds, and the regulation of their biosynthesis during leaf development.

112 Aerial plant organs, such as leaves, are often covered with both glandular and non-glandular
113 trichomes. Trichomes are epidermal appendages which occur in different forms and have
114 diverse functions [20]. Non-glandular trichomes are recognised by the absence of glandular
115 head morphology and a thin apex [20]. In contrast, glandular trichomes are further classified

116 as peltate, capitate, conoidal and digitiform types according to their base, stalk and head
117 morphology and the dimensions of these structures [21, 22]. It is the glandular trichomes that
118 are known to synthesize, store and secrete specialized secondary metabolites that gives the
119 leaves of many plants a unique fragrance. These specialised phytochemical metabolites
120 include those with antimicrobial properties such as those found in the leaves of *Plectranthus*
121 species [20, 23–25].

122 Currently, there is no literature available on trichome morphology or trichome distribution on
123 the leaf surface of *P. amboinicus*. Therefore, the main objective of the current study was to
124 investigate potential differences in antimicrobial activity of leaf extracts produced from
125 selected stages of leaf development (lag, log, and stationary phase) and whether any such
126 differences correlated with the abundance and distribution of glandular and non-glandular
127 trichomes present on the leaf surface.

128 **2. Methods and Materials**

129 **Plant Material**

130 The plant material used for the current study was *Plectranthus amboinicus* (Lour.) Spreng,
131 also known, amongst other names, as Indian borage, and was previously known as *Coleus*
132 *amboinicus* Lour.

133 **2.1 Propagation of *Plectranthus amboinicus***

134 Botanically authenticated plants of *Plectranthus amboinicus*, were vegetatively propagated
135 from stem cuttings of plants purchased from the National Herb Centre, Oxfordshire, United
136 Kingdom in September 2019 and were grown in the glasshouse facility based at the
137 University of Wolverhampton. Individual plants were repotted in a compost composed of
138 three parts of a specialist compost (Cactus and Succulent Focus repotting mix, Growth
139 Technology Ltd, UK) and one part of horticultural sand (Suregreen Melcourt, UK). The

140 ambient temperature in the glasshouse was maintained at 25°C +/- 5°C. Phytolux Attis LED
141 growth lights were used for supplemental lighting to maintain constant Photosynthetically
142 Active Radiation (PAR) during the daytime (12h).

143 **2.2 Growth Curve and Scanning electron microscopy**

144 The length of one hundred, immature leaves of *P. ambioincus*, of an initial length 6 mm were
145 measured daily, from the base of the petiole to the apex of the leaf, until fully mature. A leaf
146 growth curve was then plotted, using the mean values of these data points.

147 For Cryo – Scanning Electron Microscopy (Cryo – SEM), leaf specimens were excised using
148 a single-sided razor blade and cut into two segments. Cutting of the leaf material was
149 performed using a direct, downward force, rather than a slicing motion, to prevent the tearing
150 of the samples. Samples of both the abaxial (lower) and adaxial (upper) leaf surfaces were
151 mounted onto a Cryo Stage (Quorum Polar Prep S2000 Cryo Transfer System, Quorum
152 Technologies, Lewes, East Sussex, UK), and were rapidly frozen using liquid nitrogen slush,
153 at a temperature of -180 °C and sputter coated with platinum. For freeze fracture
154 observations, the leaf specimens were fractured horizontally and fixed to the Cryo Stage
155 vertically. The temperature for both the freeze fractured and non-fractured leaf material was
156 maintained at -95°C for 20 minutes and the samples were then transferred into the SEM
157 chamber [26].

158 The leaf specimens were examined under a XL30 Field Emission Gun Environmental
159 Scanning Electron Microscope (FEG-ESEM), FEI UK Limited, Cambridge, UK, and the
160 images were processed in Photoshop. This experimental work was performed in collaboration
161 with the Centre for Electron Microscopy, University of Birmingham, United Kingdom.

162 **2.3 Evaluation of the content and composition of phytochemicals at different stages of** 163 **leaf development**

164 Gas chromatography – Mass Spectrometry (GC-MS) analysis was performed on an Agilent
165 GC120 Auto sampler system, equipped with HP-5 capillary column (30m x 0.25mm; film
166 thickness 0.25 µm); the oven temperature was 50-325 °C heated at a rate of 4 °C/min. The
167 column's outlet pressure was 12.32 psi, with a linear velocity of carrier gas helium of
168 31.5cm/s, with an ionization energy 70eV and a scan time of 1s. The PAL sampler was the
169 injection source, with 1µl of sample injected into the GC instrument per cycle. The chemical
170 composition of the samples was identified by a comparison of the retention indices and mass
171 spectra of the samples with the controls and a compound reference library. The samples were
172 run on the GC-MS in triplicate, and the mean percentile values (retention time) were used for
173 the subsequent data analysis.

174 Leaves from the selected developmental stages i.e., lag, log, and stationary phase, were
175 harvested and analysed to determine whether the composition of phytochemicals present
176 varied and to correlate any observed differences in chemical composition with each extract's
177 antimicrobial activity.

178 The EOs from each of the developmental stages were extracted by a hydro-distillation
179 technique. Fifty grams of leaf material from each developmental stage were placed in a
180 round-bottom flask, containing 250ml of distilled water. Distillation was performed for 3
181 hours, and the steam condensed into a separating flask or a round-bottom flask. The top layer
182 of essential oil was then separated from the aqueous layer. Anhydrous sodium sulphate
183 (0.1mg/ml) was then added to remove traces of moisture and the resultant oil stored at -4 °C.
184 The EOs were then freeze-dried for 24h, to calculate the dry weight of samples and the yield
185 of essential oil. The yield was calculated using the following equation % yield= (M/Bm) x
186 100, where M is the mass of the essential oil (g) and Bm is the initial plant biomass (g).

187 Agilent glass vials were used for the GC-MS analysis. In addition, the following chemical
188 standards were also analysed p-cymene, thymol, carvacrol, eugenol, α -terpinene, β -
189 caryophyllene (Tokyo chemical industry UK Ltd), DMSO and acetone were also run for
190 comparison purposes.

191 **Solvent Extraction**

192 100 grams of leaves at each of the selected developmental stages were macerated in 250ml
193 70% acetone. The extracts were kept on a magnetic stirrer for 72 hours at 30°C. To obtain a
194 particle free filtrate, the extract was filtered using Whatman filter No. 1. The solvent present
195 in the extracts was evaporated, in a rotary evaporator, under vacuum at 25mm Hg at 40°C.
196 Finally, the extracts were weighed (into 10g aliquots) and dissolved in 0.1% DMSO to form
197 stock solutions of 400mg/ml - 1.56mg/ml.

198 **2.4 Determination of the minimum inhibitory concentration (MIC) and minimum lethal 199 concentration (MLC)**

200 Microdilution tests were performed to determine the minimum inhibitory concentration
201 (CLSI, 2009) in 96-well microplates (CoStar, UK). The microbial strains used for the study
202 i.e., *Staphylococcus aureus* NCTC8325, and *Pseudomonas aeruginosa* PA01, were obtained
203 from the University of Wolverhampton culture collection.

204 100 μ l Tryptic soy broth (TSB) (Sigma Aldrich, UK), plus 10 μ l of *S. aureus* and *P.*
205 *aeruginosa* inoculum at a concentration of 1×10^6 - 10^7 CFU/ml respectively and 100 μ l of
206 the lag, log, and stationary phase leaf extracts each ranging from 400mg/ml to 1.56mg/ml
207 were added to the 96 well plates. The plates were then incubated for 24 hours at 37°C. The
208 lowest concentration at which no visible growth (turbidity) was observed was taken as the
209 MIC. Samples from concentrations with no visible growth were plated onto tryptic soy agar
210 media and the MLC was determined as the lowest concentration showing no visible colony

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211 formation, after incubation for 18 h at 37°C. All experiments were performed in triplicate,
212 and the results shown represent the mean values. Ciprofloxacin (Sigma Aldrich, UK) at
213 concentrations ranging from 32- 0.015µg/ml was used as the reference antibiotic.

214 **2.5 Evaluation of the antimicrobial effect of *P. amboinicus* extracts on the** 215 **virulence factors of *S. aureus* and *P. aeruginosa*.**

216 **2.5.1 Effect of extracts on the hydrophobicity of bacterial cells**

217 *S. aureus* and *P. aeruginosa* overnight cultures were adjusted to 0.1 OD. Leaf extracts
218 prepared in 70% acetone were used for this experiment. 10ml cultures were incubated
219 with 5ml of lag, log, and stationary phase leaf extracts each (at the MIC value) for 24 hours at
220 37°C. After incubation, the samples were centrifuged at 2000g for 10 mins. The
221 samples were then washed thrice, in PBS pH 7. After the final centrifugation, the
222 samples were diluted with PBS to obtain an OD (measured at 470nm) of 1.0. PBS was used
223 as a diluent control for measuring the O.D. Untreated bacterial culture, 1% DMSO and 70%
224 acetone were used as solvent controls. Ammonium sulphate (Sigma Aldrich, UK), at
225 concentrations 0-3.0M was prepared in 10 ml distilled water. The controls and treated
226 suspensions (300ul) were then mixed with a series of dilutions of ammonium sulphate
227 (300ul) ranging from 0.2 to 3.0 M. The lowest concentration of ammonium sulphate at which
228 visible bacterial aggregation occurred was determined. The experiments were performed in
229 triplicate. If bacterial aggregation was visible, the salt aggregation test was identified as (+)
230 positive. If there was no aggregation, the test was negative (-). The concentration of
231 ammonium sulphate at which aggregation was visible was noted. The salt aggregation test
232 (SAT) titre can be defined as the lowest concentration of ammonium sulphate at which
233 bacteria displays evident cell aggregation. If aggregation takes place in the presence of
234 (NH₄)₂SO₄ at <0.2M = very strongly hydrophobic, 0.4-1.0M = strongly hydrophobic, 1.2-

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235 1.6M = hydrophobic, > 1.8M = hydrophilic. Aggregation at the above-mentioned molarity
236 displayed the hydrophobic or hydrophilic nature of the bacterial cells [27].

237 **2.6.2 Effect of extracts on the motility of *P. aeruginosa***

238 Bacterial cells were incubated in the *P. amboinicus* extracts and washed as described
239 previously in section 2.6.1. The final density of the bacterial suspension was adjusted to 0.5
240 O.D. 500µl of suspension was inoculated onto motility plates (1 % tryptone, 0.25 % NaCl,
241 and 0.3 % agar). Bacterial cells were also incubated with 1% DMSO and 70% acetone as
242 solvent controls. The plates were incubated at 37 °C for 24 h and the diameter of the
243 swarming zone was measured. The presented data are the mean of three independent
244 experiments.

245 **2.6 Screening for antibiofilm activity**

246 **2.6.1 Screening for the dispersal activity on preformed biofilms.**

247 Fresh isolates of *S. aureus* and *P. aeruginosa* were inoculated in sterile trypticase soy broth
248 and incubated in a thermostatically controlled orbital shaker at 37°C overnight. Overnight
249 culture's optical densities were then adjusted within the range of 0.04 to 0.06, at 600 nm.
250 0.5ml of each inoculum was then added to individual wells in a 24-well plate (CoStar, UK)
251 and incubated at 37 °C, for 72 hours in a static incubator. After incubation, the media from
252 the wells was removed, and the resultant biofilms were washed with PBS. After which, 0.5ml
253 (at MIC) of the leaf extracts was added. 0.5 ml of 70% Acetone, 0.1% DMSO and
254 Ciprofloxacin were added to the biofilms as controls. The plates were then incubated at 37 °C
255 for 72h in a static incubator. Following the incubation period, the media from the plate was
256 removed, by gentle inversion on a paper towel. The biofilms were washed 2-3 times with 1X
257 phosphate buffered saline (PBS pH 7.4, Thermofisher scientific, UK), and gently inverted on
258 a paper towel after each wash. The resultant biofilms were stained with 0.1 % crystal violet
259 (0.6 ml CV/well) for 30 minutes, and any excess crystal violet was removed by washing the

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260 plate with water. The plate was then dried, either for 2-3 hours, or overnight. After drying the
261 biofilm, the contents of each well were dissolved by adding 0.75 ml of acetic acid (30 %),
262 incubated for 15 minutes at room temperature, and transferred into a new plate. The optical
263 density of the biofilm was measured at 600 nm, using 30 % acetic acid as the blank (ESM
264 Fig. C1).

265 **2.7.2 Screening leaf extracts for biofilm inhibitory activity**

266 Overnight cultures were prepared as described in section 2.6.1 and 0.5ml of the leaf extracts
267 at MIC, were added to the wells with the diluted inoculum followed by incubation at 37 °C
268 for 72 hours. Similarly, 0.5 ml of 70% Acetone, 0.1% DMSO and Ciprofloxacin were added
269 to the biofilm as controls. After 72 hours incubation, biofilms were treated and quantified as
270 described in section 2.7.1.

271 The percentage reduction in biofilm density was calculated by the following equation.

$$272 \text{ \% Reduction in biofilm density} = (A_{OD600} - B_{OD600}) / A_{OD600} \times 100$$

273 A_{OD600} = (Control) Biofilm density before exposure, B_{OD600} = Biofilm density after exposure
274 to the extracts.

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276 **2.7.3 Statistical Analysis:** Analysis of variance (ANOVA) was used to determine the
277 significance ($P \leq 0.05$) of the data obtained from these antibiofilm screening tests.

278 279 **3 Results**

280 **3.1 Leaf growth curve**

281 Leaf growth was shown to display an exponential growth curve, which comprised of three
282 main developmental stages: a lag phase, a log phase, and a stationary phase. Leaves harvested
283 from each of these stages were selected for use in the subsequent experiments. The mean
284 lengths of leaves at the three developmental stages were as follows: lag phase (10mm), log

285 phase (20mm) and stationary phase (30mm) (ESM Figure A1.). From here onwards, the
286 three selected stages of leaf development will be referred to as lag, log, and stationary phase
287 leaves.

288 **3.1.1 Cryo - Scanning Electron Microscopy and Freeze fracture techniques**

289 **Morphology and distribution of glandular and non-glandular trichomes**

290 Leaves of *P. amboinicus* bear a smooth, dense, pubescent indumentum (covering of
291 trichomes) on both the abaxial and adaxial surfaces. Our data demonstrated that the size and
292 composition of both glandular and non-glandular trichomes present on both leaf surfaces
293 varied according to the stage of leaf development observed.

294 **Adaxial leaf surface**

295 On the adaxial surface during the lag phase of leaf development, the density of non-glandular
296 trichomes was noticeably higher than observed at the log and stationary phases (ESM Fig.
297 A2: a-c). By the log phase (ESM Fig. A2: b), the frequency of these trichomes had already
298 noticeably decreased and by the stationary phase (ESM Fig. A2: c), relatively few non-
299 glandular trichomes were observed when compared to the lag phase.

300 In terms of glandular trichomes, those observed were primarily of three types namely:

301 capitate, conoidal and digitiform. On the adaxial leaf surface, the number of short-stalked,
302 capitate trichomes observed to be present increased as the leaf developed from the lag phase
303 through to the stationary phase of leaf development. (ESM Fig. A2).

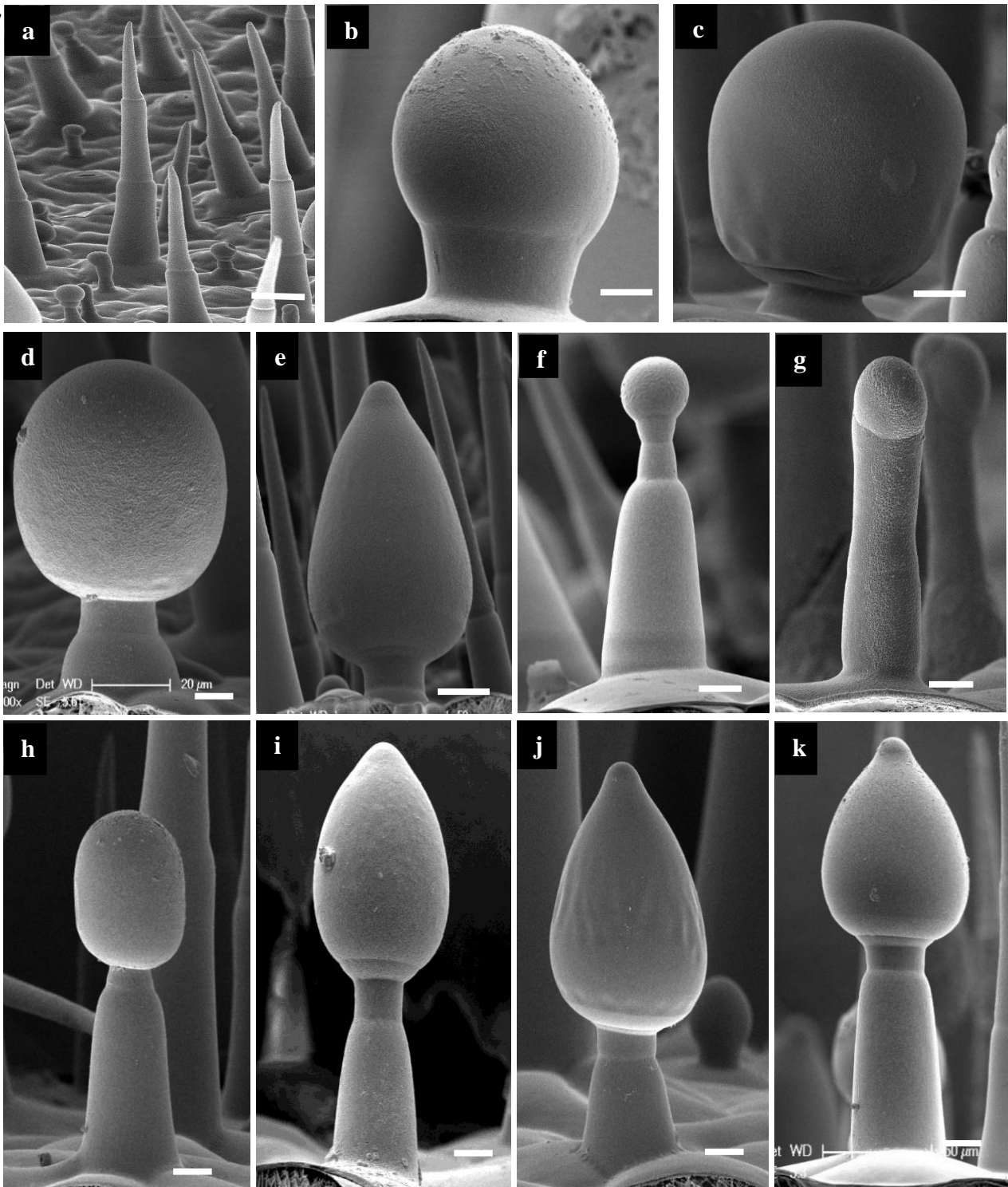
304 In terms of long-stalked capitate and conoidal trichomes, relatively few were observed on the
305 adaxial surface during the lag phase, the distribution of which was not uniform across the leaf
306 surface (ESM Fig. A2: a). However, during both log (ESM Fig. A2: b) and stationary phase
307 (ESM Fig. A2: c), the frequency of both categories of glandular trichomes markedly
308 increased.

309 **Abaxial leaf surface**

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8 312 Fig A3: a-c). As noted previously, on the adaxial leaf surface (ESM Fig. A2), the non-
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10 313 glandular trichomes presented a long-stalked morphology (ESM Fig. A3), however, on the
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12 314 abaxial leaf surface the non-glandular trichomes were predominately short-stalked (ESM Fig.
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14 315 A3). In terms of glandular trichome distribution, the frequency of both short-stalked and
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16 316 long- stalked capitate trichomes present on the abaxial leaf surface was shown to increase as
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18 317 the leaf matured. At both the lag and log phase, conoidal trichomes displaying rostrum-like
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20 318 projections were observed (ESM Fig. A3: a-b).

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25 319 In addition to the low magnification images of the overall distribution of both glandular and
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27 320 non-glandular trichomes on the adaxial and abaxial leaf surfaces (ESM Fig. A2 and A3);
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29 321 higher magnification images of the various categories of trichomes were taken from freeze
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31 322 fractured leaf material (Fig. 1) to obtain a more accurate comparison of the size and
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33 323 micromorphology of these structures. From these images, it can be observed that the non-
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35 324 glandular trichomes (Fig.1: a) are tall, hair-like, single, uniseriate, multicellular structures
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37 325 with lengths of between 50- 200 μ m, with a thin apex comprising three to seven cells
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39 326 supported by a cellular base. Whereas the short stalked, capitate glandular trichomes, were
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41 327 shown to display bicellular globoid heads, with a short unicellular base or stalk (Fig.1: b-e).
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43 328 The long-stalked capitate trichomes consisted of a long, narrow neck stalk, with a glandular
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45 329 head and slightly expanded base (Fig.1: f). The digitiform trichomes, when observed under
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47 330 higher magnification, were shown to consist of a rounded glandular head (apex), with a
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49 331 narrow subcuticular space and a stalk (Fig.1: g). The long-stalked capitate trichomes,
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51 332 consisted of an ovoid shaped unicellular head, with a single basal cell and two to three cell
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333 long stalks of varying lengths (Fig.1: h). The conoidal trichomes were comprised of a long,
334 glandular conical shaped head, and a bicellular stalk of distinct lengths (Fig.1: i-k).
335 The presence of these non-glandular and glandular trichomes on the leaf surface gives the
336 leaves, their characteristic verrucose surface morphology.



339 **Figure 1: High magnification Cryo-SEM freeze fracture micrographs, of *Plectranthus amboinicus***
340 **glandular and non-glandular trichomes.**

338 **GC-MS analysis of leaf extracts**

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2 339 The chemical composition of *P. amboinicus* has been studied extensively, however, no
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4 340 reports have included the isolation and evaluation of the bioactive constituents of *P.*

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7 341 *amboinicus* leaves harvested at different stages of development. The current study documents
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9 342 the phytochemicals produced at different stages of leaf development using GC-MS to obtain
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11 343 a better understanding of their antimicrobial properties and biological significance.

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14 344 The essential oil yields extracted from lag, log, and stationary phase leaves were 0.05%,
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17 345 0.1%, and 0.1% respectively. The essential oil obtained from the lag phase of leaf
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20 346 development revealed the presence of 18 compounds. This oil contained the following major
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22 347 chemical components, phenol (20.5%), p-cymene (17.5%), trans-Chrysanthenyl acetate
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24 348 (16.42%), linalool (9.26%) and benzene (9.43%). Also present were fatty acids such as
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27 349 phenylpropanoids, terpenes such as 1,3-Hexadiene (1.06%), trans-1,4-Hexadiene (3.13%), 4-
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29 350 Acetylanisole (4.93%) (ESM Table A1). The chemical constituents of the oil extracted at lag
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31 351 phase differed from that extracted at log phase.

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35 352 The oil extracted at log phase contained 19 chemical constituents including thymol (30.28%),
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37 353 benzene (17.61%), linalool (8%), p-cymene-2,5-diol (7.73%), fomepizole (3.4%), santolina
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39 354 alcohol (3.09%) and other fatty acids, esters and phenylpropanoids (ESM Table A2).

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43 355 Similarly, the oil obtained from the stationary phase leaf material revealed the presence of 23
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45 356 compounds. The major chemical constituents identified were thymol (20.89%), linalool
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47 357 (18.70%), p-cymene (9.38%), benzene (8.4%), carvacrol (5.79%), phenol (3.36%), eucalyptol
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49 358 (3.01%), cis-verbenol (2.8%) (ESM Table A3). The most common compounds present in the
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51 359 essential oils extracted at all three developmental stages, were phenol, benzene, linalool, and
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55 360 p-cymene (Table 1).

361 The GC-MS analysis demonstrated an increase in the percentage of oxygenated monoterpene,
 362 linalool from 9.2% (lag phase) to 18.70% (stationary phase). However, there was a drop in
 363 the percentage of p-cymene from 17.5% (lag phase), to 7.73% (log phase) and 9.38%
 364 (stationary phase). 30.28% and 20.899% thymol were detected in the log phase oil extract
 365 (ESM in Table A2) and stationary phase extract (ESM in Table A3) respectively, whereas
 366 carvacrol (5.79%) was solely identified in the stationary phase extract.

367 These data indicate that the essential oil extracts obtained from the three selected stages of
 368 leaf development had a marked variation in their essential oil content and chemical
 369 composition [2, 3, 16, 18].

370 **Table 1: GC-MS Peak Report of essential oils extracted at three different leaf**
 371 **developmental stages**

Sr no.	COMPOUNDS	Lag phase (%)	Log phase (%)	Stationary phase (%)
1	(z)-1,3-hexadiene	1.067	3.187	-
2	p-cymene	10.91	-	-
3	Pyrimidine	2.928.44	-	-
4	Linalool	9.26	8	18.70
5	(+)-2-Bornanone	1.29	1.23	-
6	Benzene	9.43	17.61	8.44
7	Phenol	20.57	2.034	3.36
8	Trans-Chrysanthenyl acetate	16.42	-	-
9	p-cymene-2,5-diol	6.64	7.738	-
10	Fomepizole	-	3.42	-

11	Santolina alcohol	-	3.09	-
12	5-Chlorovaleric acid	-	1.762	-
13	Thymol	-	30.28	20.89
14	Thiophene	-	2.94	-
15	Cis-Verbenol	-	-	2.887
16	Eucalyptol	-	-	3.017
17	Carvacrol	-	-	5.79

3.2 Determination of the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC):

Table 2: Minimal inhibitory concentration of *P. amboinicus* extracts against *S. aureus* and *P. aeruginosa*. Values represent the mean of three independent experiments with lag, log, and stationary phase extracts with ciprofloxacin used as the control antibiotic.

Extracts	<i>S. aureus</i>	<i>P. aeruginosa</i>
Lag phase	100mg/ml	100mg/ml
Log phase	25mg/ml	25mg/ml
Stationary phase	50mg/ml	50mg/ml
Ciprofloxacin	0.5µg/ml	1µg/ml

The antimicrobial activity of acetone leaf extracts obtained from the selected stages of development was studied at various concentrations (400mg/ml- 1.56mg/ml). Table 2 shows the MIC established for the planktonic cells of *S. aureus* and *P. aeruginosa* isolates treated with the extracts obtained at lag, log, and stationary phases.

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387 The results revealed that the MIC of lag phase extracts, against both *S. aureus* and *P.*
388 *aeruginosa*, was 100mg/ml. Whereas, the MIC for the log phase extract was 25mg/ml and
389 50mg/ml for the stationary phase extract. These data indicate that the acetone extracts at lag,
390 log, and stationary phases presented considerable antibacterial activity. However, the log
391 phase extracts were shown to display the greatest antimicrobial activity.

392 **3.3 Effect of extracts on the virulence factors affecting *S. aureus* and *P.*** 393 ***aeruginosa* biofilm formation.**

394 **3.3.1 Effect of extracts on the hydrophobicity of bacterial cells**

395 Table 3 illustrates the effect of the extracts on the cell surface hydrophobicity of *P.*
396 *aeruginosa* and *S. aureus*. From literature, it is known that the adherence of a developing
397 biofilm to a surface is stabilized and assisted with non-specific hydrophobic binding [28–31].
398 Any disruption in these bonds, affects the capacity of bacteria to bind to surfaces [27].
399 Additionally, the disruption of hydrophobic bonds causes the bacterial cell to turn hydrophilic
400 making them prone to precipitation by ammonium salts. The dissolved ammonium salts
401 compete for the limited water molecules, causing the bacterial proteins to fold tighter and
402 decrease their interaction with water, thereby causing them to aggregate [32, 33].
403 The data presented from the current study showed that the surface hydrophobicity of *P.*
404 *aeruginosa* and *S. aureus* changed after exposure to the log phase and stationary phase leaf
405 extracts. The bacterial cell surface was deemed to have become hydrophilic, because the cells
406 had aggregated at concentrations of ammonium sulphate above 1.8M. The untreated, 70%
407 acetone and 1% DMSO treated samples of *P. aeruginosa*, were shown to possess a strongly
408 hydrophobic cell surface as they demonstrated salt aggregation at ammonium sulphate
409 concentrations as low as 0.6M. Similarly, the untreated, 1% DMSO and 70% acetone treated
410 samples of *S. aureus*, showed salt aggregation at 0.8M ammonium sulphate indicating the
411 very strong bacterial cell surface hydrophobicity. The *P. aeruginosa* and *S. aureus* cells

412 treated with the lag phase extract showed visible aggregation at 1.8M and 2.0M respectively,
 413 indicating the marginal hydrophilic nature of the cells. However, the samples of *P.*
 414 *aeruginosa* and *S. aureus* treated with log and stationary phase extracts revealed the
 415 hydrophilic to strongly hydrophilic nature of cells as they were shown to aggregate at 2.4M
 416 and 2.8M ammonium sulphate respectively.

417 **Table 3: Effect of extracts on the *P. aeruginosa* and *S. aureus* cell surface hydrophobicity**

Samples	The final molar concentration of ammonium sulphate causing visible bacterial aggregation	
	<i>P. aeruginosa</i>	<i>S. aureus</i>
Untreated sample	0.6M (strongly hydrophobic)	0.8M (strongly hydrophobic)
70% acetone treated	1.0M (strongly hydrophobic)	0.8M (strongly hydrophobic)
1% DMSO treated	1.0M (strongly hydrophobic)	1.0M (strongly hydrophobic)
Lag phase	1.8M (hydrophilic)	2.0M (hydrophilic)
Log phase	2.4M (hydrophilic)	2.6M (hydrophilic)
Stationary phase	2.8M (strongly hydrophilic)	2.8M (strongly hydrophilic)

3.3.2 Effect of extracts on the motility of *P. aeruginosa*

420 The evident effects of lag, log, and stationary phase extracts upon the swarming motility of
 421 motile *P. aeruginosa* were recorded (Table 4). The swarming motility of the bacteria was
 422 compared by observing the swarming zone diameters (cm). The log and stationary phase
 423 extracts were shown to radically reduce the swarming zone diameters, thus displaying its
 424 effect on bacterial motility. The observed motility ranged between 1.3cm and 1.5cm
 425 respectively for *P. aeruginosa* cells treated with the stationary, and log phase extracts
 426 respectively (Fig. 2.e and 2.f). The swarming zone diameter of *P. aeruginosa* cells incubated
 427 in the presence of lag phase extracts (1.9 cm) was only slightly reduced in comparison to the

428 control sample (2.7cm). The 1% DMSO (2.5cm) and 70% acetone (2.5cm) treated sample,
 429 showed slight changes in bacterial motility. These data demonstrate that all the acetone leaf
 430 extracts reduced the bacterial motility of *P. aeruginosa*.

431 **Table 4: Effect of extracts on swarming motility of *P. aeruginosa*. Results displaying the motility**
 432 **zone are the mean values of swarming zone diameter from triplicates (\pm SD: Standard**
 433 **deviations).**

Samples	Untreated samples	1% DMSO treated	70% Acetone treated	Lag phase	Log phase	Stationary phase
Swarming zone diameters (cm)	2.7cm (\pm 0.05)	2.5cm (\pm 0.05)	2.5cm (\pm 0.03)	1.9cm (\pm 0.08)	1.3cm (\pm 0.02)	1.5cm (\pm 0.03)

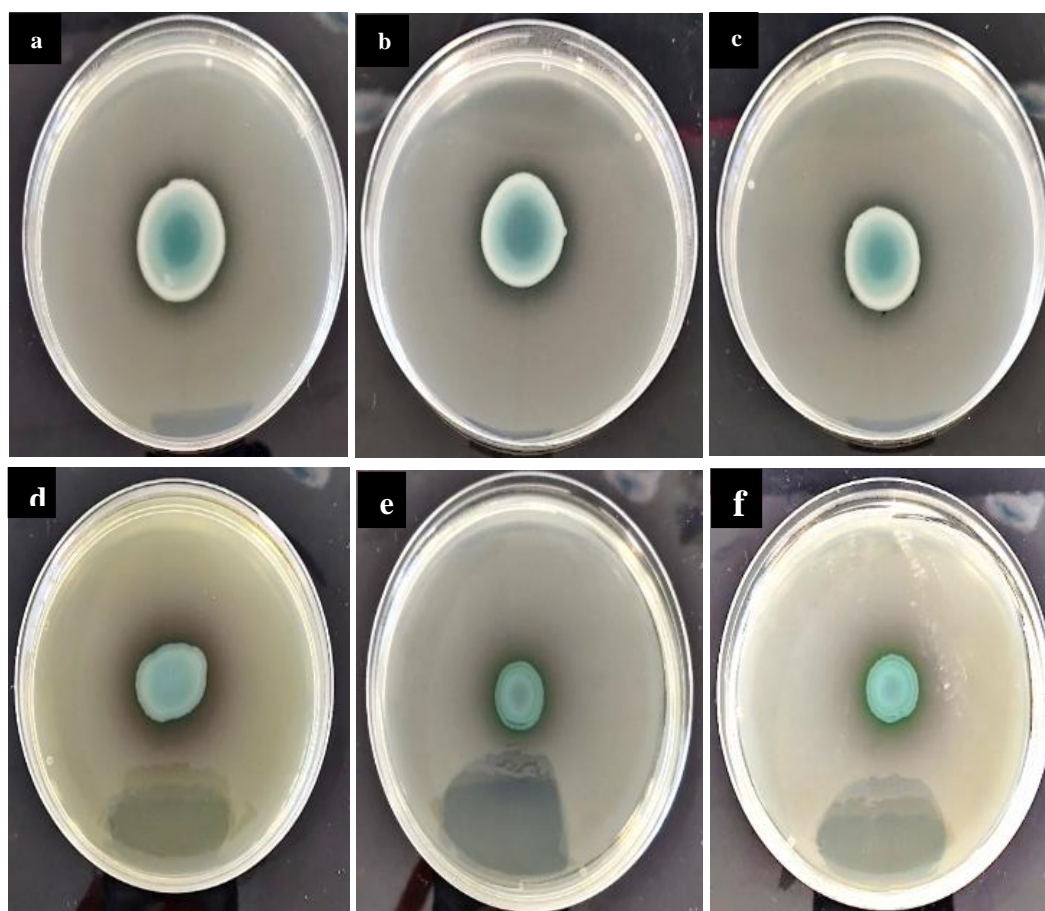
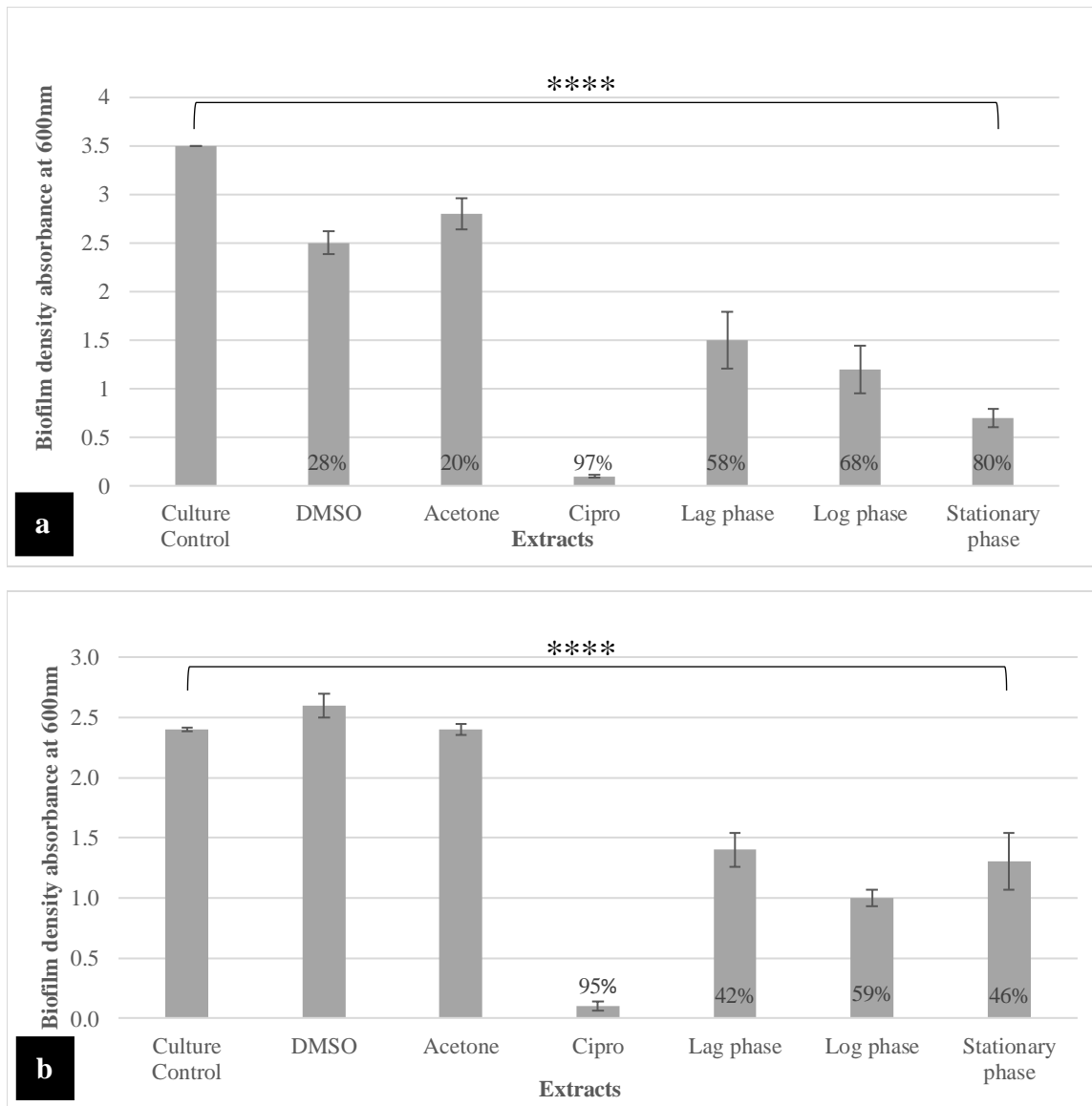


Figure 2: Effect of extracts on swarming motility of *P. aeruginosa*.

440 **3.4 Screening for antibiofilm activity**

441 **3.4.1 Screening for the biofilm dispersal activity**

442 For this study, acetone extracts obtained from leaves of the selected developmental stages
443 were tested against preformed biofilms to screen for their dispersal activity.



444 **Figure 3: The dispersal activity of *P. amboinicus* extracts from different developmental stages**
445 **on a) *P. aeruginosa* biofilm b) *S. aureus* biofilm**

446 Stationary phase extracts were shown to exhibit a maximum reduction in biofilm density
447 (80%) of *P. aeruginosa* (Fig. 3.a), followed by log phase (68%) and lag phase (58%) extracts
448 (ESM B1). Similarly, when used to treat, *S. aureus* biofilms (ESM B2) the log phase extracts
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1 450 (59%) were observed to produce the highest dispersal activity followed by stationary phase
2 451 (46%) and lag phase (42%) extracts (Fig. 3.b).
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5 452 **3.4.2 Screening for the biofilm inhibitory activity**

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8 453 Figures 4.a and 4.b depict the biofilm inhibitory activity of the leaf extracts on *P. aeruginosa*
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10 454 and *S. aureus* respectively. Results displayed in the ESM Table C1 and C3 are the mean
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12 455 values of three replicates. Log phase leaf extracts displayed a maximum reduction in biofilm
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14 456 density of both *P. aeruginosa* (66%) and *S. aureus* (63%) biofilms. Lag phase (54%) and
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16 457 stationary phase (28%) extracts showed strong inhibitory activity against the *P. aeruginosa*
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18 458 biofilms (Fig. 4.a). Likewise, stationary phase (50%) and lag phase (37%) extracts displayed
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20 459 moderate inhibitory action on *S. aureus* biofilms (Fig. 4.b). The solvent controls, 1% DMSO
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22 460 and 70% acetone showed no evident effect on biofilm development. The log phase extracts
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24 461 inhibited 63% of biofilm formation in *S. aureus*. These values are comparable to the
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26 462 inhibitory effect of ciprofloxacin (68%) on *S. aureus* biofilms.
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33 463 ANOVA analysis confirmed that the extracts obtained at different developmental stages had
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35 464 significant variation in their dispersal activity ($P < 0.0001$) against *P. aeruginosa* (ESM Table
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37 465 B2) and *S. aureus* biofilms (ESM Table B4) in comparison to the controls. Similarly, the
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39 466 extracts displayed significant ($P < 0.0001$) biofilm inhibitory activity against *P. aeruginosa*
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41 467 (ESM Table C2) and *S. aureus* (ESM Table C4). These findings support the conclusion that
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43 468 the extracts derived at the three leaf developmental stages such as lag, log and stationary
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45 469 phase had a substantial effect on biofilm formation.
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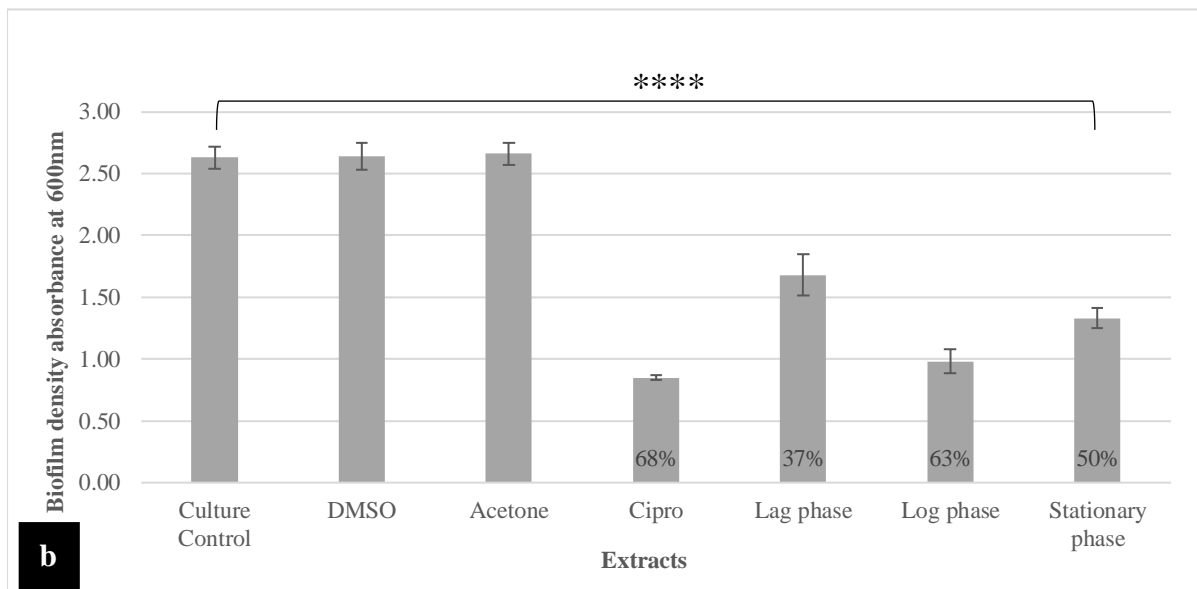
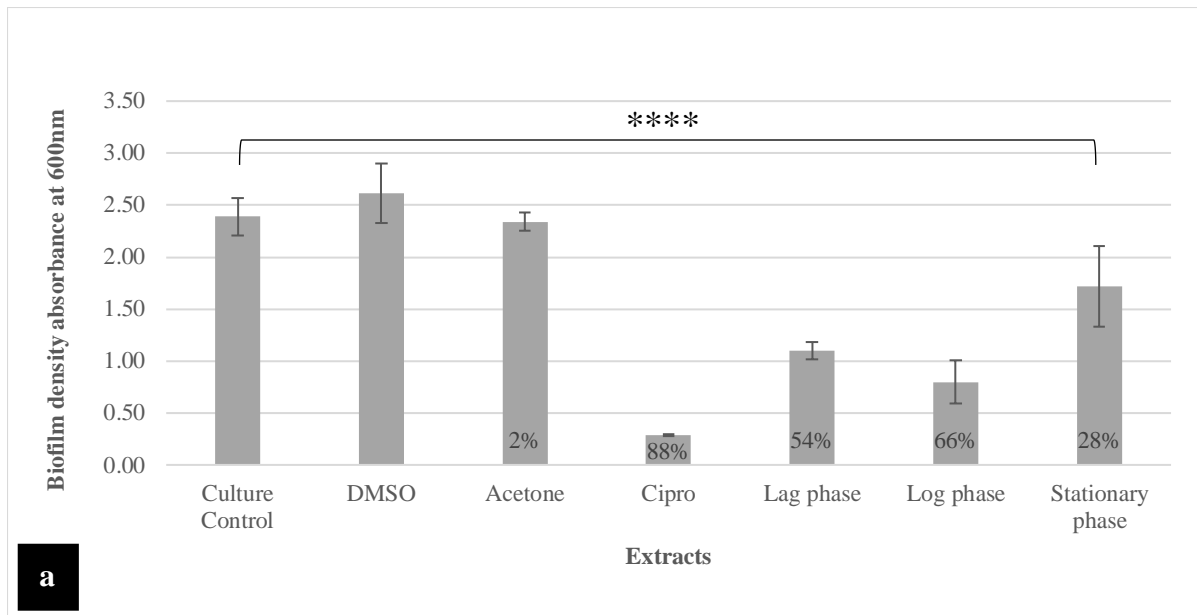


Figure 4: The biofilm inhibitory activity of different developmental stages of *P. amboinicus* leaves on a) *P. aeruginosa* biofilm b) *S. aureus* biofilm

4 Discussion

The objective of the current study was to perform an investigation of the effect of leaf development on the phytochemical composition and antimicrobial activity of *P. amboinicus*

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3 478 leaf extracts. The aim was to identify the stage of leaf development at which to harvest leaves
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5 479 for large-scale commercial production of the antimicrobial plant extract.

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7 480 The indumentum of both the abaxial and adaxial leaf surfaces was shown to consist of a
8 481 mixture of both glandular and non-glandular trichomes (ESM Fig. A2 and Fig. A3).

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11 482 Non-glandular trichomes are known to protect plants against both abiotic and biotic stress,
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13 483 which explains the high number of non-glandular trichomes observed on both the abaxial and
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15 484 adaxial leaf surfaces of the majority of flowering plant species [34, 35].

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19 485 In the current study, non-glandular trichomes were observed to be more abundant than
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21 486 glandular trichomes on the adaxial leaf surface at the lag phase of leaf development (ESM
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23 487 Fig. A2: a). Then, as the leaf surface area enlarges (during the log phase of growth), the
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25 488 observed abundance of non-glandular trichomes was shown to decrease relative to the
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27 489 abundance of glandular trichomes.

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32 490 The function of glandular trichomes, is to synthesize, store and/or release bioactive
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34 491 compounds [36]. It has been reported previously, that in young leaves of *Plectranthus* species
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36 492 non-glandular trichomes present strong morphological resemblances to glandular ones, such
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38 493 as the swollen apical cells [34]. However, as leaves mature, the glandular trichomes are seen
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40 494 to develop a more defined structure, such as a head and base morphology which is not
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42 495 observed in the non-glandular trichomes [34].

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47 496 It has been reported in previous studies of *Plectranthus* and other genera within the
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49 497 *Lamiaceae*, that the presence of glandular leaf trichomes is a distinctive phenotypic feature
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51 498 [37–39]. Within this family, the capitate trichomes observed, have been shown to be variable
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53 499 in terms of their morphology and dimensions. In previously studied *Plectranthus* species, in
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55 500 the short-stalked capitate trichomes the phytochemicals present have been shown to be stored
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501 in the cuticular space and secreted through micropores. However, in the long-stalked capitate
502 trichomes, the secretory substances are released through cuticular rupture [37].

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503 The previous studies of trichome distribution on the leaf surfaces of *Plectranthus ornatus* and
504 *Plectranthus madagascariensis* are in broad agreement with our data [37, 40]. The uni- or
505 bicellular globoid or bulb-shaped glandular heads (of diameter 20-25µm), and varying stalk
506 lengths observed in the current study, are very similar to those reported in other species of
507 *Plectranthus* [41]. The long-stalked capitate trichomes recorded in our study also bear a
508 strong resemblance to those observed in *P. grandidentatus*[41]. However, it is of interest to
509 note that we did not observe the presence of the numerous peltate, glandular trichomes, as
510 previously reported in *P. grandidentatus* [41].

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511 Digitiform glandular trichomes were first recorded to be present in the genus *Plectranthus* on
512 the floral organs of *P. ornatus* [40]. This type of trichome was later reported to be present on
513 the leaves and stems of *P. grandidentatus* [41].

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514 Digitiform trichomes were originally incorporated in Werker's type II capitate trichome
515 category and were first recorded to be present on leaves of *Calmintha menthifolia* [42]. In
516 the current study, digitiform trichomes were observed, but they were not shown to be
517 abundant in any of the selected stages of leaf development. Most of this type of trichome
518 were shown to display an atypical stalk and head morphology (Fig.1: g).

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519 Previously, conoidal trichomes have been reported as abundant on the leaf surfaces of *P.*
520 *grandidentatus*, *P. ornatus*, but much less abundant on leaves of *P. madagascariensis* [37,
521 39, 41]. In the present study, abundant conoidal and short-stalked capitate trichomes, were
522 observed on the adaxial leaf surface (ESM Fig. A2) and conoidal, short and long-stalked
523 capitate trichomes were also observed in high numbers on the abaxial surface (ESM Fig. A3).

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524 In summary, the current study of glandular and non-glandular trichome distribution, on both
525 the adaxial and abaxial leaf surfaces of *Plectranthus amboinicus*, during the selected phases
526 of leaf development; has shown that the trichomes observed are in broad agreement with
527 earlier studies of species within the genus, but with some species-specific differences.

528 As reported in the previous studies[21, 37, 39], digitiform, capitate and conoidal trichomes
529 were all observed to be present. However, the peltate trichomes observed in all other
530 *Plectranthus* species studied to date were not observed in the current study.

531 Furthermore, the non-glandular trichomes were observed to be most abundant at the lag phase
532 of leaf development, whilst the glandular trichomes were observed to be most abundant
533 during the log phase of development. These data are consistent with a biological function for
534 the non-glandular trichomes to physically protect the leaf during the early stages of
535 development and for the glandular trichomes to produce phytochemicals to chemically
536 protect the leaf/plant in the later stages of leaf development. These observations also correlate
537 with the recorded increase in antimicrobial activity of the leaf extracts of log and stationary
538 phase leaves in comparison to those extracted from leaves at the lag phase of growth, as is
539 described below.

540 Essential oil composition:

541 To date, there are no reports in the literature which describe the effect of leaf development on
542 the composition or presence of bioactive constituents present in the leaves of any
543 *Plectranthus* species.

544 In the current study, the essential oil obtained from lag, log, and stationary phase leaves,
545 revealed the presence of non-volatile components such as flavonoids, phenolic acids,
546 oxygenated monoterpenes, monoterpene hydrocarbons and esters. The details of these

1 547 chemical constituents and their relative percentage composition are shown in ESM Table A1,
2 548 A2, and A3.
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5 549 Thymol is reported as one of the major components present in these extracts. This component
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7 550 was detected in both log phase and stationary phase extracts, whereas carvacrol, was
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9 551 identified as the dominant component in stationary phase oil only. The absence of these
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11 552 components in the early stages of leaf development suggests that the chemical variation in
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13 553 phytochemicals is influenced by the sequence of events that occur during leaf growth and
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15 554 development. It was also noted that there was a variation in the aroma and colour of the oil
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17 555 obtained from the different leaf stages, which was most likely a result of the qualitative and
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19 556 quantitative differences in chemical composition between the EOs.
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25 557 According to previous studies, essential oil obtained from *Plectranthus amboinicus* is rich in
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27 558 phenolic monoterpenes such as thymol, which corresponds to the data presented [43–45].
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30 559 The compound 1,3- Hexadiene previously reported to be present in an essential oil extracted
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32 560 from *P. amboinicus* obtained from Martinique, France [46] was shown to be present in the
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34 561 current study in the essential oils obtained from lag and log phase leaves.
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38 562 The GC-MS study of the lag phase EO identified compounds such as pyrimidine, (+)-2-
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40 563 Bornanone, carbonic acid and trans- Chrysanthenyl acetate (ESM Table A1) to be present.
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43 564 Other compounds such as (+)-2-Bornanone, santolina alcohol, thiophene and 5-chlorovaeric
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45 565 acid were identified in the log phase EOs. (ESM Table A2). Other chemical components
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47 566 observed in the stationary phase EO included cis-Verbenol, Eucalyptol and
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49 567 phenylpropanamide (ESM Table A3).
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53 568 Previous studies suggest that acetone extracts of leaves had significantly higher phenolic
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55 569 contents than hydroalcoholic extracts [47]. Moreover, acetone extracts displayed better
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1 570 antibacterial activity and high antioxidant activity [47] , as a result, 70% acetone was used in
2 571 the present study for solvent extraction of the *P. amboinicus* leaves.

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5 572 Minimum inhibitory concentration: The antibacterial activity of the different extracts was
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8 573 evaluated by the microdilution method against *S. aureus* (ESM Fig. B1) and *P. aeruginosa*
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10 574 (ESM Fig. B2) and expressed as MIC, in mg/ml. The MIC of extracts against these pathogens
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13 575 was shown to vary depending upon the leaf stage (Table 1). *S. aureus* and *P. aeruginosa* were
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15 576 the most sensitive to the log phase extracts and exhibited the least MIC value against this
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18 577 extract. The MIC value of lag phase extracts was the highest among all the extracts tested
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20 578 against the cultures.

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23 579 The antibacterial action of these extracts is most likely due to their phytochemical content.
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26 580 Earlier research has reported that high concentrations of thymol and carvacrol present in plant
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28 581 extracts and EOs can inhibit pathogenic bacteria [48]. The low MIC value recorded using the
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31 582 log phase leaf extracts (Table 1) against both bacterial species is most probably due to the
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33 583 high concentration of thymol (30.28%) present in this extract (Table 1). Moreover, the
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35 584 absence of thymol in lag phase leaves may also explain the high minimal inhibitory
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38 585 concentration (100mg/ml) of this extract against both microorganisms. Linalool,
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40 586 monoterpene alcohol has previously been shown to exhibit various biological properties such
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43 587 as antimicrobial, anticancer, and anti-inflammatory activities [49]. Thymol and carvacrol are
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45 588 active components in the tissues of the *Lamiaceae*, and as mentioned previously these
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48 589 phytochemicals have proven antibacterial and antibiofilm effects [50].

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51 590 The presence of thymol and carvacrol in the essential oil shows synergistic effects with *p*-
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53 591 cymene as their precursor, which can disrupt the bacterial cytoplasmic membrane by altering
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56 592 its' permeability for protons and ions. The integrity of the impaired membrane compromises
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58 593 cell function [51]. The presence of high levels of carvacrol, *p*-cymene, linalool, and thymol,

594 therefore, explains the observed minimum inhibitory concentration of the stationary phase
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3 595 extracts (Table 1).
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5 596 Effect of extracts on the hydrophobicity of bacterial cells: Bacterial cell surface
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8 597 hydrophobicity is one of the most crucial aspects that regulate bacterial adhesion to
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10 598 air/oil/water interfaces, teeth, biomaterials, activated sludge, animal cells and a range of solid
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13 599 surfaces. The salting out aggregation technique was used to measure the bacterial cell surface
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15 600 hydrophobicity. Both *P. aeruginosa* and *S. aureus* have previously been shown to possess
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17 601 hydrophobic surfaces which facilitate biofilm formation [30].
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20 602 The current study showed that the leaf extracts affected the cell surface hydrophobicity of
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22 603 both *P. aeruginosa* and *S. aureus*. It was observed that *P. aeruginosa* and *S. aureus* gave a
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25 604 positive SAT reaction at lower concentrations of ammonium sulphate (<0.2M) revealing the
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27 605 hydrophobic surface of both bacterial species. Both species were treated with extracts
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30 606 obtained at lag, log and stationary phases which revealed aggregation at a higher molar
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32 607 concentration of ammonium sulphate, thus, expressing the SAT titre. The hydrophobic
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35 608 properties of both microorganisms were studied using SAT on untreated, 70% acetone and
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37 609 1% DMSO treated samples, which showed aggregation in the concentration range of 0.6M-
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39
40 610 1.0M. However, the hydrophobicity of *P. aeruginosa* and *S. aureus* was modulated by
41
42 611 acetone extracts obtained at lag, log, and stationary phases of leaf development.
43
44 612 Extracts containing EOs may interact with or influence the plasma membrane of the bacterial
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46
47 613 cells, thus, impeding energy production and respiratory chain activity [52–54]. Extracts
48
49 614 containing EOs are known to inhibit the bacteria through distinct modes of action.
50
51
52 615 Components such as carvacrol and thymol present in the extracts may possibly enhance
53
54 616 antibiotic intake by acting as membrane permeabilizers [55]. They ally with fatty acids to
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56
57 617 improve the passive permeability of the cytoplasmic membrane which leads to enhanced
58
59 618 antibiotic uptake [15]. Thymol and its isomer carvacrol trigger perturbation of the lipid
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619 fraction in the bacterial plasma membrane causing leakage of intracellular components [56].

620 The inactivation of bacterial enzyme systems could possibly be one of the potential modes of
621 action [15]. It was noted that incubation of *E. coli* O157:H7 with thymol and carvacrol at
622 sublethal concentrations caused negative regulation of *tnaA* and *bssS*, genes responsible for
623 biofilm formation and upregulation of *marA* and *acrB*, genes associated with multidrug efflux
624 pumps [57].

625

626 Effect of extracts on bacterial motility: The swarming zone diameters recorded in our study
627 revealed that extracts obtained from different developmental stages reduced the motility of
628 bacteria, however, log phase and stationary phase extracts showed more prominent reduction
629 in the motility when compared to lag phase extracts (Fig 2). As there are very few studies
630 reporting the effect of extracts on bacterial motility, and none on *P. amboinicus* plant
631 extracts, these findings are noteworthy. Motility is a significant feature of bacterial
632 physiology [58]. The membrane motor responsible for rotating the flagella, is driven by the
633 proton gradient across the membrane and hence, considered a measure of membrane integrity
634 [58]. It has also been suggested that motility plays a crucial role in virulence [59]. When
635 treated with extracts, bacterial cells possibly lost their motility due to altered membrane
636 integrity. The extracts could be further studied as potent bacterial motility inhibitors.

637 Therefore, the antimotility activity of the extracts obtained from the log phase and stationary
638 phase leaves might play an essential role in the inhibition of bacterial pathogenesis.

639

640 Effect of extracts on preformed biofilm: Dispersal activity is the ability of bacteria to disperse
641 from the biofilm and disseminate the organism. It is assumed that the dispersed bacteria are
642 analogous to their planktonic forms both in terms of their physiology and virulence.

643 Therefore, making it easier for antimicrobial drugs to inhibit or kill microbes. Dispersal

1 644 activity is seen on a mature biofilm where cells have already attached, formed microcolonies
2 645 and are enclosed in the EPS matrix. The dispersed cells no longer reside within the biofilm
3
4 646 and are easier to eradicate in comparison to biofilms [60].
5
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7 647 The developmental trajectory of lag, log and stationary phase leaves can be correlated to their
8
9 648 antibiofilm activity. The log phase extracts exhibited the highest biofilm dispersal activity
10
11 649 against *S. aureus* (59%) and *P. aeruginosa* (68%) biofilms. Followed by stationary phase
12
13 650 extracts, which were shown to display a noticeable biofilm dispersive activity against *P.*
14
15 651 *aeruginosa* biofilms. However, lag phase leaves extracts showed comparatively low activity
16
17 652 against biofilms formed by both the microbes (ESM Table B1 and B3).
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22 653 The log phase extracts resulted in a considerable decrease in biofilm density when compared
23
24 654 with the standard drug, ciprofloxacin. These results provide an insight into the most
25
26 655 biologically active stage of leaf growth, which can be further exploited in harvesting leaves at
27
28 656 this specific leaf length to extract active constituents and maximise their yield and biological
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30 657 activity.
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35 658 Screening for the biofilm inhibitory activity: The extracts were tested on *S. aureus* and *P.*
36
37 659 *aeruginosa* biofilms to determine their inhibitory action (ESM Table C1 and C3). The
38
39 660 acetone extract of the leaves was shown to have an antibiofilm activity on *S. aureus* and *P.*
40
41 661 *aeruginosa*. The anti-biofilm effect of the extracts observed could be due to inhibition of
42
43 662 processes such as cell attachment or adhesion, generation of extracellular matrix, production
44
45 663 of virulence factors and disruption of the quorum sensing network [61]. The effect of the log
46
47 664 phase extract on *S. aureus* biofilms (63%) was comparable to the effect of the drug
48
49 665 ciprofloxacin (68%). Biofilm density of *S. aureus* (Fig. 4.b) in the presence of the log phase
50
51 666 extracts was 0.98 (absorbance at 600nm), in comparison to the culture control (2.63), there is
52
53 667 a substantial difference revealing a 63% reduction in biofilm density. In case of *P.*
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55 668 *aeruginosa* biofilms, the biofilm density declined from 2.39 to 0.80, in presence of log phase
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1 669 extracts showing a 66% reduction in biofilm density (Fig. 4.a). Earlier reports had
2 670 documented the antibiofilm and antibacterial properties of *P. amboinicus* against
3
4 671 *Staphylococcus aureus* [15, 62]. However, these workers failed to show any inhibitory effects
5
6
7 672 of *P. amboinicus* on *P. aeruginosa* biofilm development. The current study provides a
8
9 673 detailed analysis of the inhibitory and dispersal effect of *P. amboinicus* extracts on *P.*
10
11 674 *aeruginosa* biofilms. *Pseudomonas aeruginosa* is considered as a model organism for the
12
13 675 study of Gram-negative biofilms and *Staphylococcus aureus* for Gram-positives, therefore,
14
15 676 the antibiofilm activity of these extracts indicates its pharmacological potential against both
16
17 677 Gram-positive and Gram-negative microorganisms.

18
19 678 Recent research showed the potential of thymol in association with rifampicin on *S. aureus*
20
21 679 biofilm by preventing the formation of persister cells. The potential of thymol to improve
22
23 680 biofilm eradication efficacy, makes *P. amboinicus* a potential therapeutic candidate and a
24
25 681 major source of phenolic compounds [63].

26
27 682 The antibacterial activity of extracts is potentially due to the cumulative effect of polyphenol
28
29 683 adsorption onto bacterial membranes, leading to disruption of the membrane and leakage of
30
31 684 cellular contents [64]. These phenomena support the findings of the present study, the
32
33 685 superior antibacterial and antibiofilm activity of *P. amboinicus* extracts obtained from leaves
34
35 686 in the log phase in comparison to other phases is likely due to the presence of high
36
37 687 concentration of thymol, which is a phenolic compound.

38
39 688 Plant volatile oils extracted from various aromatic herbs were tested against 25
40
41 689 microorganisms, volatile oils with the broadest spectrum of activity showed the presence of
42
43 690 thymol, carvacrol, linalool, terpenoids etc [65]. EOs from *Satureja subspicata* Vis showed
44
45 691 antimicrobial activity against 13 bacterial and 9 fungal strains. The essential oil showed the
46
47 692 presence of carvacrol, p-cymene, thymol, and γ -terpinene linking its biological activities with
48
49 693 the presence of phenolic compounds [66].

694 p-Cymene, a major component found in *P. amboinicus* extracts, exhibits synergistic
695 antibacterial efficacy when linked with γ -terpinene and thymol. On the other hand, minor
696 bioactive components present in extracts and EOs work synergistically to exhibit stronger
697 antimicrobial activity, which supports the significance of all the major and minor
698 phytochemicals in relation to the bioactivities of the extracts [67, 68].

699 To choose an appropriate treatment, it is necessary for clinicians to identify the mechanisms
700 of resistance used by the pathogen against a specific antibiotic. However, several classes of
701 antimicrobials are known to exhibit a single mode of action against bacteria, which limits the
702 activity of antibiotics, thus leading to the development of resistance. Therefore, to overcome
703 resistance, novel antimicrobials are needed, that exhibit multimodal mechanisms of action
704 which act upon more than one target in the bacterial cell. The present study has demonstrated
705 the effect of extracts of *P. amboinicus* on planktonic as well as biofilm forms of Gram-
706 negative and Gram-positive bacteria. It has also revealed the prominent impact of the extracts
707 on the virulence factors such as hydrophobicity and motility which are essential for bacterial
708 attachment and biofilm formation. Moreover, the dispersal properties of the extracts on a pre-
709 formed biofilm and inhibitory activity at the initial stages of biofilm formation show potential
710 to study the mechanistic action of these extracts against the resistance mechanisms.

711 The current research also addressed the link between the phytochemical yield and the stages
712 of leaf maturation and its impact on biological activity. Our understanding of the role of
713 specific types of trichomes in the production of specific phytochemical components is still in
714 its infancy. However, the Cryo-SEM images obtained in the current study provide an
715 improved understanding of the categories of glandular trichomes present at the three selected
716 stages of leaf development. The presence of capitate trichomes was observed to be more
717 prominent on log phase and stationary phase leaf surfaces which may explain the relative
718 thymol content observed in the EOs at these leaf lengths, followed by the presence of

1 719 carvacrol in stationary phase EO. This trend strengthens the findings with respect to log and
2 720 stationary phase extracts demonstrating exceptional results in comparison to lag phase
3
4 721 extracts.

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8 722 This study also represents the first report on the effect of leaf developmental stages upon
9
10 723 trichome micromorphology, glandular trichome distribution and a comparison of the
11
12 724 antimicrobial effect of extracts of leaves harvested at different phases of development.

13
14
15 725 Our future studies will involve a quantitative analysis of both glandular and non-glandular
16
17 726 trichomes on the leaf surface and histochemical studies of resin-embedded leaf material
18
19 727 which will improve our understanding of the specific categories of phytochemicals present in
20
21 728 the various types of glandular trichomes present on the leaf surface.

22 23 729 **5 Conclusion**

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29 730 The Cryo-SEM study revealed the presence of both non-glandular and glandular trichomes on
30
31 731 the leaf surfaces of *P. amboinicus*. The GC-MS analysis of EOs obtained from the selected
32
33 732 stages of leaf development presented different classes and compositions of phytochemicals at
34
35 733 specific developmental stages. The extracts obtained from the log phase of leaf development
36
37 734 revealed the greatest antibacterial and antibiofilm activity. The extracts obtained from all
38
39 735 three leaf stages were shown to modulate the bacterial cell surface hydrophobicity in *S.*
40
41 736 *aureus* and *P. aeruginosa*. These extracts were observed to have the ability to alter the
42
43 737 swarming motility of *P. aeruginosa*. The log phase of leaf development is likely to be the
44
45 738 most biologically active and potent developmental stage in relation to antimicrobial,
46
47 739 antibiofilm effects and enhanced yield of phytochemicals. In addition, mechanistic studies
48
49 740 exploring the effect of these extracts upon EPS matrix components and physiology of
50
51 741 persister cells is currently underway.

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Figure captions

Fig. 1 High magnification Cryo-SEM freeze fracture micrographs, of *Plectranthus amboinicus* glandular and non-glandular trichomes.

a: Long, hair-like non-glandular trichomes amidst shorter glandular trichomes. Scale bar = 50µm. b-d: Short-stalked glandular capitate trichomes. Scale bars = 20µm. Image c and d show enlarged globoid glandular cells produced due to the accumulation of phytochemicals within the subcuticular space. e: Short-stalked conical glandular trichome. Scale bar = 50µm. f: Long-stalked capitate glandular trichome with a spherical head. Scale bar = 20µm g: Digitiform trichome with an indistinct head and stalk. Scale bar=20µm h: A bulb-shaped capitate glandular trichome with mature long stalk. Scale bar = 100µm. i-k: Conoidal glandular trichomes with mature stalks. Scale bars = 50µm

Fig. 2 Effect of extracts on swarming motility of *P. aeruginosa* a: Untreated sample b: 1% DMSO. c: 70% acetone. d: lag phase extracts. e:

log phase extracts. f: stationary phase extracts. Swarming motility seen in *P. aeruginosa* is one of three distinct motility modes which requires flagellar motility for movement across the semisolid surface, in this case, it was the motility plates with 0.3% agar. The untreated samples showed a swarming zone diameter of 2.7cm, followed by samples treated with 1% DMSO and 70% acetone displayed 2.5 cm of swarming motility. Samples treated with log phase extracts displayed swarming zone diameter of 1.3 cm. The bacterial motility of samples treated with lag and stationary phase extracts showed swarming zone diameters of 1.9 cm and 1.5 cm respectively

Fig. 3.a The dispersal activity of *P. amboinicus* extracts from different developmental stages on *P. aeruginosa* biofilms. The bar graph

indicates the biofilm density of *P. aeruginosa* determined by crystal violet assay in presence of lag, log, and stationary phase extracts obtained from different leaf developmental stages. Cipro: ciprofloxacin drug control. DMSO/Acetone: Solvent controls. Data labels inside the bars denote the % change in biofilm density. The vertical bars represent mean values based on three replicates with error bars representing standard deviation. Among the three extracts, stationary phase extracts (80%) showed maximum reduction in biofilm activity, followed by log phase (68%) and lag phase extracts (58%). The antibiotic control, ciprofloxacin showed 97% reduction in biofilm density. The solvent controls,

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985 1%DMSO and 70% acetone showed 28% and 20% of dispersal activity. The P-value denoted by asterisks reveals that the leaf extracts had a
986 significant ($P < 0.0001$) antibiofilm effect on *P. aeruginosa* biofilm as compared to the controls

987 **Fig. 3.b The dispersal activity of different developmental stages of *P. amboinicus* leaves on *S. aureus* biofilm.** The bar graph indicates the
988 biofilm density of *S. aureus* determined by crystal violet assay in presence of lag, log, and stationary phase extracts obtained from different leaf
989 developmental stages. Cipro: ciprofloxacin drug control. DMSO/Acetone: Solvent controls. Data labels in the bars denote the % change in
990 biofilm density. The vertical bars represent mean values based on three replicates with error bars representing standard deviation. Among the
991 three extracts, log phase extracts (59%) showed maximum reduction in biofilm activity, followed by stationary phase (46%) and lag phase
992 extracts (42%). The antibiotic control, ciprofloxacin showed 95% reduction in biofilm density. The solvent controls, 1%DMSO and 70% acetone
993 showed no evident dispersal activity. The P-value denoted by asterisks reveals that the leaf extracts obtained from different developmental stages
994 had a significant ($P < 0.0001$) antibiofilm effect on *S. aureus* biofilm in comparison with the controls

995 **Fig. 4.a The biofilm inhibitory activity of different developmental stages of *P. amboinicus* leaves on *P. aeruginosa* biofilm.** The bar graph
996 indicates the biofilm density determined by crystal violet assay of *P. aeruginosa* in presence of lag, log, and stationary phase extracts obtained
997 from different leaf developmental stages. Cipro: ciprofloxacin drug control. DMSO/Acetone: Solvent controls. Data labels inside the bars denote
998 the % change in biofilm density. The vertical bars represent mean values based on three replicates with error bars representing standard
999 deviation. Log phase (66%) extracts displayed maximum biofilm inhibitory activity followed by lag phase (54%) and stationary phase extracts
1000 (28%). The antibiotic control, ciprofloxacin showed 88% antibiofilm activity. Solvent controls, 1% DMSO and 70% acetone showed no evident
1001 effect on biofilm inhibition. The P-value denoted by asterisks reveal that the leaf extracts had a significant ($P < 0.0001$) biofilm inhibitory effect
1002 on *P. aeruginosa* biofilm compared to the controls

1003 **Fig. 4.b The biofilm inhibitory activity of the *P. amboinicus* leaves at different developmental stages on *S. aureus* biofilm.** The bar graph
1004 indicates the biofilm density of *S. aureus* determined by crystal violet assay in presence of lag, log, and stationary phase extracts obtained from
1005 different leaf developmental stages. Cipro: ciprofloxacin drug control. DMSO/Acetone: Solvent controls. Data labels inside the bars denote the

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1006 % change in biofilm density. The vertical bars represent mean values based on three replicates with error bars representing standard deviation.
1007 Log phase (63%) extracts displayed maximum biofilm inhibitory activity followed by stationary phase (50%) and lag phase extracts (37%). The
1008 antibiotic control, ciprofloxacin showed 68% antibiofilm activity. Solvent controls, 1% DMSO and 70% acetone showed no evident effect on
1009 biofilm inhibition. The P-value denoted by asterisks reveal that the leaf extracts obtained at three developmental stages had a significant ($P <$
1010 0.0001) biofilm inhibitory effect on *S. aureus* biofilm.

