

## Quantitative Phytochemical Analysis Reveals Significant Antibiofilm Activity in *Pleione maculata*, an Endangered Medicinal Orchid

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### Abstract

*Pleione maculata* has no scientific reports on quantitative phytochemical and antibiofilm activity till date. The objective of the study was to quantify and determine medicinally important bioactivity in *P. maculata* and analyse its anti-biofilm activity against clinical isolates *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Proteus mirabilis*. *P. maculata* exhibited the highest Total Antioxidant Capacity (TAC) about 193.98±0.1 mg, highest Total Phenolic Content (TPC) at 552±0.0 mg and Total Flavonoid Content (TFC) were observed highest at 879.5±0.2 mg. The acetone and ethyl acetate extracts of *P. maculata* pseudobulb showed distinct and significant zone of inhibition (ZOI) against drug-resistant *S. aureus* about 16±0.00 mm (MIC 0.875 mg/mL), ZOI of acetonitrile pseudobulb extract against *P. mirabilis* was 15.33±0.4 mm (MIC 1 mg/mL), ZOI of acetonitrile extracts of leaves and stem, ethyl acetate extract of pseudobulb was 12±0.0 mm, 12±0.14 mm, 12±2.8 mm against *K. pneumoniae* (MIC 1.8 mg/mL, 0.68 mg/mL and 3 mg/mL). Acetonitrile extract of pseudobulbs exhibited the highest Minimum Biofilm Inhibition concentration (MBIC) at 0.25 mg/mL against *S. aureus*, water root extract inhibited attachment of *K. pneumoniae* with lowest MBIC value 0.093 mg/mL, water and acetone extract of leaves inhibited cell attachment of *P. mirabilis* at lowest MBIC 0.117 and 0.171 mg/mL. The UV-VIS absorption band of *P. maculata* extracts ranges from 204-665 nm indicating the presence of phenolic and flavonoid compounds. The study indicates the potentiality of *P. maculata* as a rich source of medicinal active compounds as an antibiofilm agent against antibiotic-resistant clinical isolates.

**Keywords:** Quantitative analysis, Antibiofilm agent, UV-VIS, *Pleione maculata*, endangered orchid

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**Abbreviations:** DPPH - 2, 2, 1-diphenyl-1-picrylhydrazyl, IC<sub>50</sub> - Half maximal inhibitory concentration, TAC - Total antioxidant capacity, TAE - Total Ascorbic acid equivalent, TPC - Total phenolic content, TFC - Total flavonoid content, ZOI - Zone of inhibition, MIC - Minimum inhibitory concentration, MBIC - Minimum biofilm inhibitory concentration, DMSO - Dimethyl sulfoxide, GAE - Gallic acid equivalent, QE - Quercetin equivalent, LB - Luria Bertani. Solvents: A - Acetone, AN - Acetonitrile, C - Chloroform, ETH - Ethanol, EA - Ethyl acetate, M - Methanol, W - Water. Parts of *Pleione maculata*: L - Leaves, P - Pseudobulb, S - Stem, R - Roots.

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## INTRODUCTION

Orchids are the largest flowering plants and most threatened species in the plant kingdom, with 25,000 to 35,000 species<sup>1,2</sup>. Due to favourable climatic conditions and phytogeographical conditions, the North Eastern Region (NER) of India is abundant in orchid diversity<sup>3</sup> and commonly found in region viz. Meghalaya<sup>3</sup> Arunachal Pradesh<sup>4</sup> Manipur, Nagaland, and Sikkim<sup>5</sup>. The aesthetic aspects of orchids have been studied and exploited for centuries. But the rare and largely unexplored orchid flora of Northeast India is a potential goldmine from the view of medicinally important molecules. Urbanization, overexploitation, and illegal trade, orchids face climatic changes, biodiversity loss, habitat destructions, and extinction<sup>1</sup>. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) put all Orchidaceae species under Appendix II to conserve biodiversity. It ensures that their trade is only through permits of export<sup>6</sup>. One such unexplored, rare epiphytic orchid in the scientific research field belongs to the genus *Pleione*. Species of *Pleione* viz. *Pleione chunii*, *P. formosana*, *P. forrestii*, and *P. pleionoides* also appear in the IUCN list as endangered or vulnerable. *Pleione* is a small genus of orchids comprising of nineteen species only. The orchid is divided into two sections *Pleione Humiles* (autumn flowering) and *Pleione Pleiones* (spring flowering)<sup>7</sup>. *Pleione maculata* is a medicinally important epiphytic<sup>8</sup> rare species and is autumn flowering. In Nepal<sup>9</sup> and North Eastern states of India such as Sikkim and Arunachal Pradesh<sup>10</sup>, the rhizome of *P. maculata* therapeutic uses is for liver complaints and stomach ailments. *P. maculata* is one among several orchids species listed and notified as threatened under Schedule-38 of Biological Diversity Act, 2002<sup>11</sup>. Unbalances of highly reactive free radical molecules are causing various health problems across the world today<sup>12</sup>. Reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as hydrogen peroxide and nitrous acid, are examples of free radicals<sup>13</sup>. Free radicals develop many diseases such as cardiovascular diseases, cancer, aging, and diabetes due to imbalances in their electron molecules<sup>14</sup>. Plants include numerous phytoconstituents, such as phenolic compounds, flavonoids, vitamin C, vitamin E, and carotenoids, that generate and have

various biological effects, including free radical scavenging, antioxidant activity, and anti-tumor activity<sup>15</sup>.

Today, the science of dealing with the prevention and treatment of diseases caused by microorganisms has come down due to some antibiotic-resistant bacteria. The resistance may be due to the wide use of antibiotics as growth promoters in animal food and maybe overuse of antibiotics in humans<sup>1</sup>. Traditional treatment processes against bacterial infections depend on different antimicrobial compounds or antibiotics that can either inhibit or kill the growing microbial cells<sup>17</sup>. But pathogenic microorganisms can resist themselves against those inhibitory compounds by forming microbial communities termed biofilms<sup>16</sup>. Biofilms are the assemblages of microbial cells embedded in a matrix of self-produced polymeric substances consisting of polysaccharides, proteins, and DNA. The transcribed genes of biofilm-associated microorganisms differ from their planktonic cells<sup>18</sup>. Adhesion, followed by microcolony development, maturation, and dispersion, are the four distinct phases of microbial biofilm formation<sup>19</sup>. The dispersal mechanism is critical because it starts a new infection cycle by the colonization of a new surface, allowing microbial infection to spread quickly<sup>20</sup>. Biofilms adhere to biological and non-biological medical devices, urinary catheters, living tissue, dental and industrial setting surfaces<sup>21</sup>. The biofilm has a slower growth rate and secretes various surface chemicals and virulence factors, increasing their toxicity by hundreds of times<sup>22</sup>. Microbial biofilms are involved in several chronic infectious diseases in humans, an estimated 65% of all infections according to the Centre for disease control (CDC) and 80% according to the National Institutes of Health (NIH)<sup>16</sup>. Urinary tract infections, pneumonia, cystic fibrosis, periodontitis, endocarditis, osteomyelitis, rhinosinusitis are caused by bacterial biofilm<sup>24</sup>. Several bacteria are highly pathogenic to humans. *Staphylococcus aureus* is a pathogen causing various diseases on the human host through biofilm formation<sup>25</sup> and is multidrug-resistant<sup>26,27</sup>. *Klebsiella pneumoniae* isolated from urine, sputum, or wound swabs can form up to 40% biofilm. That can cause nosocomial infections such as urinary tract infections, septicemia, pneumonia, and tissue infections<sup>26</sup>.

The Gram-negative rod-shaped bacteria *Proteus mirabilis* is the most common cause of community and healthcare-associated illnesses. Multidrug-resistant (MDR) strains of *P. mirabilis* can cause severe complications in the survival of hospitalized patients<sup>28</sup>. Thus, effective strategies can combat these biofilm-linked diseases caused by different microbes. A phytochemical analysis can examine quantitative phytoconstituents in plants to assess plant characteristics. There are few techniques and give a brief idea for identifying the compounds. The most popular ones are spectroscopic and chromatographic techniques. A simple, cost-effective, and sensitive technique is UV-Visible spectroscopy<sup>29</sup>. There are no scientific reports on total antioxidant activity, total phenolics, and total flavonoid content of *Pleione maculata*, and the antibiofilm activity against clinical isolates.

The study aims to evaluate the bioactive phytochemicals in *Pleione maculata*. Also, to assess the antibiofilm activity against select antibiotic-resistant isolates. The target isolates are *Staphylococcus aureus* (Gram-positive), *Klebsiella pneumoniae* (Gram-negative), and *Proteus mirabilis* (Gram-negative).

## MATERIALS AND METHODS

### Collection, preparation, and extraction of phytocompounds

*Pleione maculata* (Fig. 1.) was collected in Khliehriat, East Jaintia Hills District, Meghalaya, India, during the spring season (temperature was 19°C). For the plant sample, herbarium sheets were prepared. The leaves, pseudobulbs, stems, and roots of *P. maculata* were weighed individually after being surface sterilized with 70% ethanol. Plant parts were separated and dried in the shade for two weeks. Using a household grinder, the dry materials were ground into a fine powder and kept in microcentrifuge tubes at 4°C<sup>30</sup>. The powdered plant material was weighed and soaked in a 1:45 (w/v) ratio in several solvent systems (acetone, acetonitrile, chloroform, ethanol, ethyl acetate, methanol, water). The extracts were agitated for 48 hours at 37°C in an incubator shaker<sup>31</sup>. Extracts were filtered using Whatman filter paper No. 1 after incubation. For the remaining fractions, filtration was repeated. Evaporation at a constant temperature of 30°C concentrated the extracts.

The concentration of extracts obtained is listed in Table 1.

### Antioxidant activity

#### Determination of antioxidant activity by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Each extract (leaves, pseudobulb, stems, and roots) of *Pleione maculata* was tested to determine the radical scavenging activity<sup>36</sup>. For this, 2,2-diphenyl-1-picryl-hydrazyl (DPPH) (HiMedia)

**Table 1.** Stock concentration of *P. maculata* extracts

<i>Pleione maculata</i> extracts	Stock Conc. of extracts (mg/mL)
LA	22.5
PA	7
SA	31.25
RA	29.5
LAN	15
PAN	8
SAN	5.5
RAN	11
LC	13.5
PC	18.5
SC	17.25
RC	12
LE	35
PE	20
SE	43.75
RE	29.75
LEA	6.25
PEA	12
SEA	7
REA	13
LM	24.75
PM	16.75
SM	19.5
RM	24.75
LW	7.5
PW	8
SW	15.5
RW	6

Sample = Leaves (L), Pseudobulb(P), Stem(S), Root(R), solvent = acetone(A), acetonitrile(AN), chloroform(C), ethanol(E), ethyl acetate(EA), methanol(M), water(W)

solution was prepared at a concentration of 1 mg/mL with methanol. The mixture was set in the dark. A stock concentration (1mg/mL) of plant extracts was prepared. The plant extracts and the standard L-ascorbic acid (HiMedia) were diluted at different concentrations (20, 40, 60, 80, 100 µg/mL). A 0.1 mL methanolic DPPH solution was combined with varying concentrations of test extracts on a 96-well microtiter plate. The solution was mixed thoroughly and incubated in the dark at room temperature for 30 minutes. The colour reduction of DPPH was measured at 520 nm in BioEra Life Science Microplate reader Model Nova. A standard curve was plotted with absorbance against the concentration of extracts. The blank and control OD was measured. DPPH free radical scavenging activity can be determined using the formula:

$$\% \text{ DPPH scavenging activity} = \left( \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \right) * 100$$

The test was performed in triplicates and a graph was plotted with the percentage of DPPH radical scavenging activity against the concentration of samples extracts in µg/mL. IC<sub>50</sub> values were calculated using statistical functions in Microsoft excel 2010.

#### Determination of Total antioxidant capacity by Molybdate Assay

The total antioxidant activity was measured using a modified molybdate assay<sup>37</sup>. The test relies on the reduction of Mo (VI) to Mo (V) after the creation of a complex green-blue phosphate/Mo (V) complex at an acid pH. Molybdate reagent solution was prepared using the ascorbic acid standard solution at various concentrations (20, 60, 80, and 100 mg/mL). The plant extracts were made with dimethyl sulphoxide (DMSO) (HiMedia) at a concentration of 2 mg/mL. A 400µl of plant extract was pipetted out and diluted with methanol, 1.5 mL of molybdate reagent solution was added, and test tubes were sealed with aluminum foil before incubation in water bath at 95°C for 90 minutes. At room temperature, the mixture was allowed to cool. An SYSTRONICS PC-based double beam UV-VIS spectrophotometer-2202 was used to measure the absorbance at 695nm. The blank was made by combining 400 µl of the solvent used for sample extraction with 1.5 mL of molybdate reagent.

The total antioxidant capacity was measured in grams of ascorbic acid equivalents. The antioxidant activity was measured in milligrams of ascorbic acid equivalents (AAE) per milliliter of solution.

#### Determination of Total phenolic content by Folin Ciocalteu assay

Folin Ciocalteu test<sup>38</sup> with modification was used to measure the total phenolic content in each extract. Plant extracts were diluted in 3 mL of DMSO to achieve a concentration of 2 mg/mL. For the calibration curve, gallic acid (3, 4, 5-Trihydroxy benzoic acid - SRL) was collected at various concentrations (20, 40, 60, 80, 100, 120, 140, and 160 mg/mL)<sup>39</sup>. A 400µl of the test extracts (leaves, pseudobulb, stems, and roots) were mixed with 100µl ethanol and 0.2 mL of Folin Ciocalteu reagent (MERCK-DJ4D640513). The final volume was adjusted with distilled water. Then, 1 mL of 7% sodium carbonate solution was added. The tubes were vortexed and incubated in the dark at room temperature for 2 hours. Using an SYSTRONICS PC-based double beam UV-VIS spectrophotometer-2202, the mixture's absorbance was measured at 760 nm. The total phenolic content (TPC) was expressed in milligrams of Gallic acid equivalent per gram of *P. maculata* extracts (mg GAE/g of *P. maculata* extracts). The following formula was used to determine the total phenolic content of the extract with gallic acid equivalent<sup>12</sup>.



**Fig. 1.** *Pleione maculata* collected from Khliehriat, East Jaintia Hills (a) Habitat of *P. maculata* (b) *P. maculata* flowering during October.

$$TPC = Cx \frac{V}{M}$$

Where TPC is the Total Phenolic Content  
'C' is the concentration of phenolic calculated from the equation obtained from the calibration curve

'V' is the volume of the extracts added for the test (mL)

'M' is the weight of evaporated extracts (g)

Blank was prepared by adding only methanol in equal volume

#### **Determination of Total flavonoid content by Aluminium chloride method**

The  $AlCl_3$  technique with modification was used to determine the total flavonoid content of various plant extracts<sup>38</sup>. Plant extracts were prepared at a concentration of 2 mg/mL. A standard quercetin solution was prepared by pipetting 5 mL of stock and diluting it to make a final volume of 10 mL with 80 percent ethanol. For the calibration curve, a reference solution of quercetin (HiMedia- RM6191-25G) was obtained at various concentrations (20, 40, 60, 80, 100, 120, 140, and 160 mg/mL). A 400 $\mu$ L of the test extracts (leaves, pseudobulb, stems, and roots) and standard quercetin solution were diluted with ethanol. To this, 0.3 mL of 5%  $NaNO_3$ , 0.3 mL of 10% aluminium chloride solution, and 1M NaOH solution were added. The final volume was adjusted with distilled water. The tubes were vortexed and incubated at room temperature for 40 minutes. Using an SYSTRONICS PC-based double beam UV-VIS spectrophotometer-2202, the mixture's absorbance was measured at 510 nm. The total flavonoid content (TFC) of *Pleione maculata* extracts was measured in mg quercetin equivalent per gram of extracts (mg Quercetin/g of *P. maculata* extracts). The following formula was used to determine the total flavonoid content in the extract with quercetin equivalent<sup>12</sup>.

$$TFC = Cx \frac{V}{M}$$

Where TFC is the Total Flavonoid Content  
'C' is the concentration of quercetin calculated from the equation obtained from the calibration curve

'V' is the volume of the extracts added for the test (mL)

'M' is the weight of evaporated extracts (g)

#### **Collection of clinical isolates, sub-culturing, and Inoculum preparation**

A Gram-negative *Proteus mirabilis* from feces, a Gram-negative *Klebsiella pneumoniae* from a urine sample, and a Gram-positive *Staphylococcus aureus* from a urine sample were provided by Miss Bahunlang Susngi, Medical Microbiologist from Bethany Hospital Shillong, Meghalaya. These clinical isolates were transferred to the Microbiology laboratory of Assam Don Bosco University, Sonapur. Mannitol Salt Agar (HiMedia MH118-500MG) for clinical *Staphylococcus aureus* and Mac Conkey agar (HiMedia M081B-100G) for *Proteus mirabilis* and *Klebsiella pneumoniae* were used.

#### **Preparation of inoculums**

The overnight microbial culture was prepared using Luria Bertani broth (Miller-HiMedia M1245-1KG) at  $37 \pm 2^\circ C$ . A 40 $\mu$ L of the microbial culture was diluted in Eppendorf containing 1 mL of sterile double distilled water<sup>33</sup>. A 0.5 McFarland turbidity standard is a reference used to adjust the inoculum density at  $1.5 \times 10^8$  CFU/mL<sup>34</sup>.

#### **Antibacterial activity by Kirby-Bauer Disc diffusion susceptibility method**

Antibacterial activity was tested by the disc diffusion method using a 6mm disc. A Luria Bertani agar (Miller-HiMedia M1151-500G) was prepared and sterilized in an autoclave for 15 minutes at  $121^\circ C$ . The standard working inoculums were swab using a sterile L-shaped glass spreader at an angle of  $60^\circ C$  with three times rotation<sup>34</sup>. The discs were dipped in different extracts and dried, followed by inoculation in respected microbial plates. The plates were incubated at  $37 \pm 2^\circ C$  for 24 hours in an incubator. The zone of inhibition was observed the next day and measured in millimetres (including 6 mm diameter of the disc). The experiments were performed in duplicates. The ZOI was compared with standard reference antibiotic<sup>35</sup>.

#### **Broth micro-dilution method (MIC) with Resazurin indicator**

MIC is the lowest concentration of extract required to inhibit the growth of microbes in comparison with non-treated (negative) control. Overnight clinical isolates suspension was

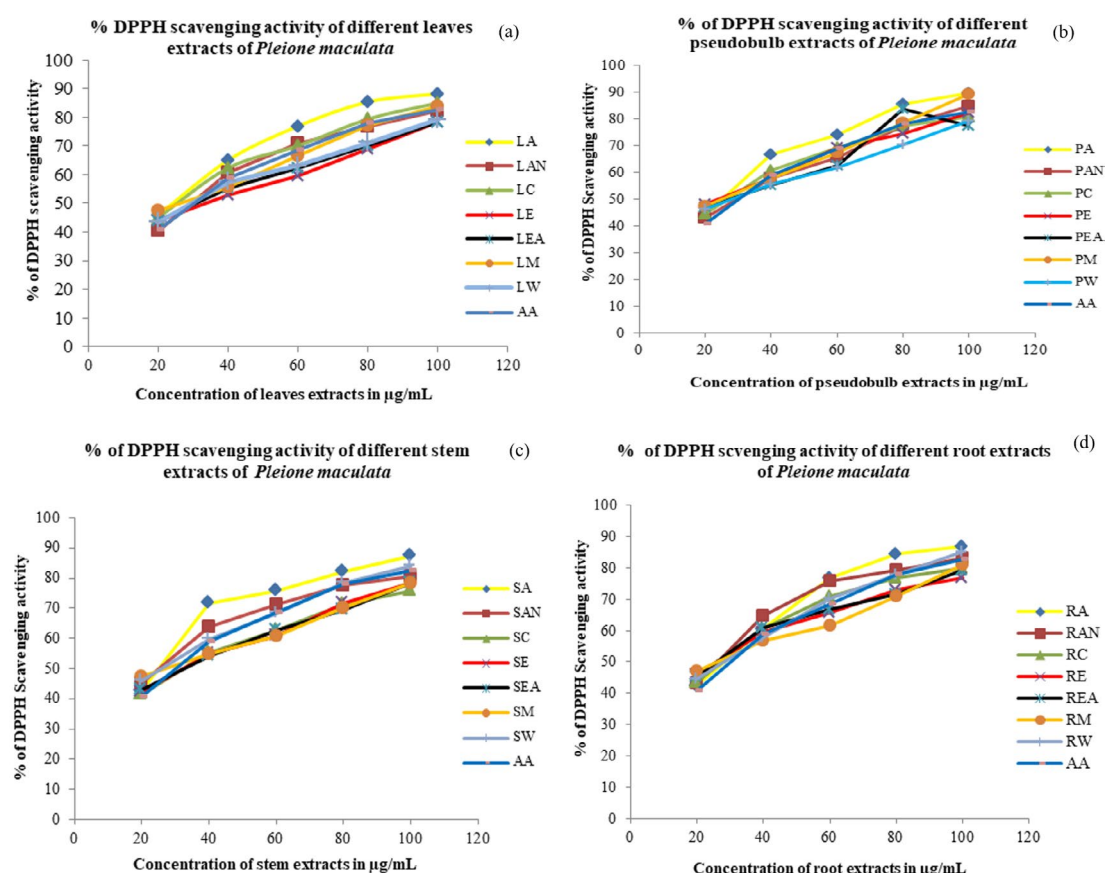


incubated at 37°C for 24 hours. Initial suspension was diluted (1:20 ratio) to adjust optical density with 0.5 McFarland's standards (0.5 OD). Luria Bertani broth was added to a sterile 96-wells microtitre plate containing test extracts followed by serial dilution. To 10µL of the inoculum in each well, 10µL of resazurin indicator (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) (HiMedia RM125-1G) was added. A negative control containing 10% DMSO with inoculated medium, and a positive control containing antibiotics with the inoculated medium were also prepared. The 96-well plates were sealed with parafilm and incubated at 37±2°C for 24 hours. The experiment was repeated in duplicates. The lowest concentration was the MIC for the extracts with inhibited growth of test organisms<sup>48</sup>.

## Antibiofilm assay

### Screening biofilm formation ability of the test organisms (Tube method)

Clinical isolates (*S. aureus*, *K. pneumoniae*, and *P. mirabilis*) were tested via biofilm screening conducted using the tube method with minor modifications<sup>47</sup>. To do so, 10 µl loopful of overnight bacterial cultures were added to tubes containing 5 mL of Luria-Bertani (LB) Broth and incubated at 37±2°C for 24–48 hours. After the incubation period, the tubes were washed off three times using sterile double-distilled water and dried prior to staining. The tubes were then stained with 0.1% (v/v) crystal violet and allowed to stand for 10 minutes. Subsequently, the tubes were washed off and the excess stain was removed, followed by drying at room temperature to observe biofilm



Solvents- A: Acetone; AN: acetonitrile; C: chloroform; E: ethanol; EA: ethyl acetate; M: methanol; W: water

AA: Ascorbic acid, Extracts - L: leaves; P: pseudobulb; S: stems; R: roots

Solvents- A: Acetone; AN: acetonitrile; C: chloroform; ETH: ethanol; EA: ethyl acetate; M: methanol; W: water

**Fig. 2.** A plot showing the percentage of DPPH radical scavenging activity of different (a) leave (b) pseudobulb (c) stem and (d) root extracts of *P. maculata* against Ascorbic acid standard.

formation. The stained was re-dissolved in 30% (v/v) glacial acetic acid and absorbance was measured at 492 nm using a SYSTRONICS PC-based double beam UV-VIS spectrophotometer-2202 with glacial acetic acid suspension.

#### Minimum Biofilm Inhibitory Concentration (MBIC) of initial cell attachment assay

The minimum inhibitory concentration of test plant extracts was estimated by ten-fold serial dilution in 96-well polystyrene plates. From fresh LB broth, 100 µl was pipetted to each well of a microtiter plate, and 50 µl of overnight cultured biofilm-forming isolates added. Positive control contained bacteria with the reference antibiotic, and negative control was bacteria without antibiotics prepared for each isolate for comparison. The plates were then sealed with a parafilm and kept for 24–48 hours incubation at 37±2°C to check the potentiality of test plant extracts to inhibit initial cell attachment of biofilm-forming clinical isolates. The plates were washed and stained with 0.1% (v/v) crystal violet

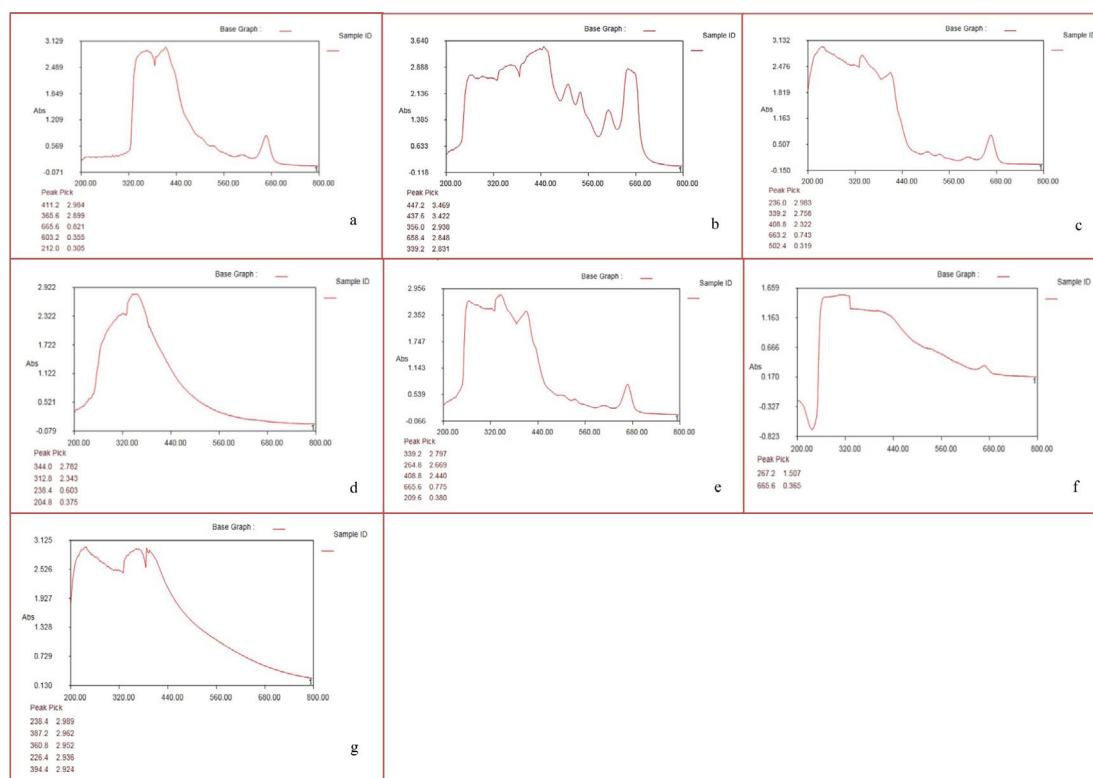
for 10 minutes, and the excess stain was removed with double distilled water and dried at room temperature. Absorbance was measured at 492nm using a BioEra Life science Microplate reader Model Nova. The percentage of biofilm inhibition was compared with untreated control.

Percentage biofilm inhibition was calculated using the following formula:

$$\% \text{ biofilm inhibition} = \frac{(\text{OD of untreated control}) - (\text{OD of treated sample}) \times 100}{\text{OD of untreated control}}$$

#### Preparation of extracts for UV-VIS spectroscopy analysis

Dried plant samples weighing 0.25g were soaked in 5mL different solvents (acetone, acetonitrile, chloroform, ethanol, ethyl acetate, methanol, and water)<sup>40</sup>, and were incubated in a shaker for 24 hours. The extracts were centrifuged at 3000 rpm for 10 minutes and filtered using Whatman filter paper No. 1



**Fig. 3.** UV-VIS spectrogram peaks of different extracts {(a) acetone (b) acetonitrile (c) chloroform (d) ethanol (e) ethyl acetate (f) methanol and (g) water} of *P. maculata*

qualitative circle 125mm diameter. The filtrates were scanned ranging from 200–1100nm in a spectrophotometer<sup>29</sup>.

### Statistical analysis

Experiments were conducted in duplicates, triplicates, and experimental data were presented as mean values  $\pm$  SD. The concentration of IC<sub>50</sub> that could inhibit 50% DPPH free radical was calculated using statistical Microsoft excel 2010. Using IBM SPSS Ver 20 Multivariate statistical analysis viz. Hierarchical cluster analysis (HCA) was performed. The relationship among different samples was presented as clades of a dendrogram which measured Squared Euclidean distance (clustered by the average linkage between groups)<sup>41</sup>.

## RESULTS

The finely powdered sample of *P. maculata* was solvent extracted. The antioxidant activity, quantitative phytochemical analysis, and antibiofilm activity were determined using the solvent extracts. Preliminary compounds were identified using SYSTRONICS PC-based double beam UV-VIS spectrophotometer-2202.

### The fresh and dry weight of different parts of *P. maculata*

The length of *P. maculata* leaves measured 15.5 cm in matured leaves with a surface area of 2.3 cm width; pseudobulb length measured 2.3 cm with green and purplish colour. It gave an aroma of locally prepared medicine. Table 2 lists the differences between the fresh and dry weights. For instance, fresh pseudobulb weighed about 200.59 grams, whereas 27.6 grams in dry weight. It indicates high water storage and mineral content in pseudobulb.

### Antioxidant activity

Antioxidant activity using DPPH radical  
The extracts of *P. maculata* were able to decolorize the deep purple color of DPPH radical to yellow color. This indicates the capability of extracts to scavenge and neutralize free radicals. Acetone and acetonitrile are suitable solvents for the extraction of antioxidant compounds present in *P. maculata*. The capability to neutralize DPPH harmful free radicals indicates extracts' potentiality as an antioxidant source attributed to the presence of a high amount of polyphenols<sup>42</sup>. The standard ascorbic acid showed no significant difference

between the extracts of *P. maculata*. The different solvent extracts of *P. maculata* can be a promising source of antioxidant compounds with the highest scavenging activity of about 89.583% observed in acetone extract of pseudobulb, 89.216 % in methanol extract of pseudobulb, 88.235% in acetone extract of leaves, 82.598% in reference ascorbic acid standard as presented in Fig. 2

The IC<sub>50</sub> value calculated was found lowest in acetone extract of leaves (20.76 $\pm$ 0.00  $\mu$ g/mL), acetonitrile extract of the stem (22.30 $\pm$ 0.00  $\mu$ g/mL), acetone extract of pseudobulb (23.03 $\pm$ 0.00  $\mu$ g/mL) which is lower than ascorbic acid standard (30.80 $\pm$ 0.01 $\mu$ g/mL). The lower the IC<sub>50</sub> concentration value than the ascorbic acid standard, the higher the antioxidant activity of samples. The observed IC<sub>50</sub> value of different extracts of *P. maculata* was lower in acetone extracts than other solvent extracts represented in Table 3.

TAC was calculated using the linear equation obtained by plotting the absorbance value of standard ascorbic acid against the concentration. The equation was  $y = 0.0241x + 0.1176$  with  $R^2 = 0.9677$  where 'y' is absorbance of extracts, and 'x' is Total Ascorbic acid equivalent (TAE) in mg per grams of extracts. Total antioxidant capacity was highest in ethanol, ethyl acetate, methanol, and acetone extracts of *P. maculata* (Table 4). The TAC of ethyl acetate extract of leaves was 193.98 $\pm$ 0.1 mg of TAE/gram of extracts, 198.14 $\pm$ 0.3 mg of TAE/grams of ethanol extract of leaves. The least antioxidant capacity was 2 mg of TAE/grams of water extracts of roots.

### Quantitative phytochemical analysis

The concentration of phenolics in *P. maculata* extracts was expressed as gallic acid equivalent per gram of the extract using the gallic acid standard calibration curve. The equation obtained by plotting a graph with the absorbance

**Table 2.** Fresh and dry weight of different parts of *P. maculata* (in grams)

Samples of <i>P. maculata</i>	Fresh weight (in grams)	Dry weight (in grams)
Leaves	150.50	55.9
Pseudobulb	200.59	27.6
Stem	26.55	13.4
Roots	65.21	41.55



value of gallic acid standard on the y-axis against concentration on the x-axis was  $y = 0.0096x + 0.0966$  with the  $R^2 = 0.9898$ .

Phytochemical screening was performed for the seven solvents extracts of *P. maculata*. The solvents were acetone, acetonitrile, chloroform, ethanol, ethyl acetate, methanol, and water. LEA showed the highest phenolic content with  $552 \pm 0.0$  mg of GAE/ grams of extracts listed in Table 5. PAN extracts yielded  $509.375 \pm 0.0$  mg of GAE/ grams of extracts. Total phenolic content was lowest in chloroform (SC) extracts of the stem with  $25.48 \pm 0.0$  mg of GAE/ grams of extracts. Chloroform may not be an ideal solvent choice for phenolic extraction of *P. maculata*. The concentration of flavonoids in various extracts of *P. maculata* was expressed as Quercetin equivalent per gram of the extracts using the quercetin calibration curve equation obtained by plotting a graph with absorbance value of the standard quercetin on the y-axis against concentration on the x-axis. The equation obtained was  $y = 0.0046x + 0.0819$  with the  $R^2 = 0.9733$ .

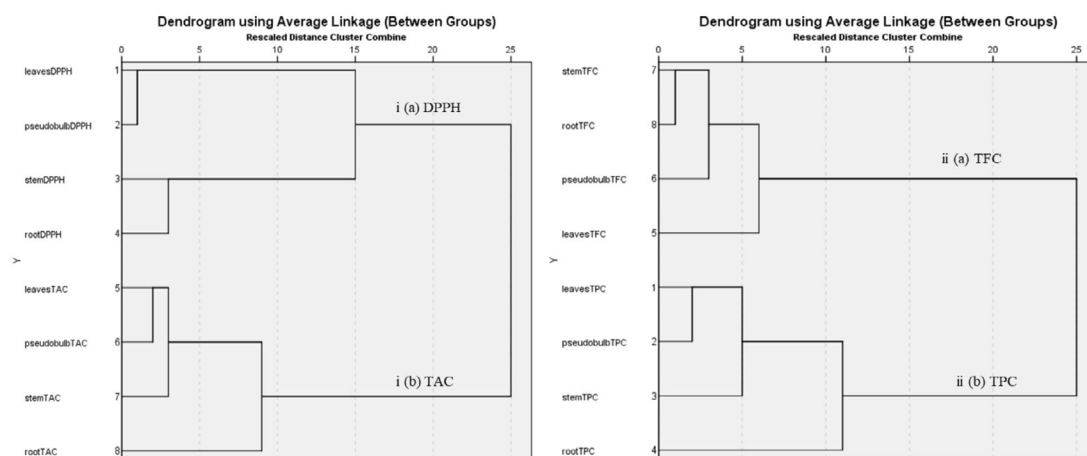
The total flavonoid content was highest in LA extracts with  $879.5 \pm 0.2$  mg of QE/ grams, and PAN showed  $845.2 \pm 0.2$  mg of QE/ grams as listed in Table 6. Total flavonoid content was the least in SW extracts with  $20.40 \pm 0.0$  mg of QE/ grams of extracts. Leaves and pseudobulb of *P. maculata* showed better results for antioxidant capacity, phenolics, and flavonoid content.

### Effect of *Pleione maculata* against clinical isolates

*P. maculata* extracts were effective against all three clinical isolates. Table 7 presents the effective zone of inhibition, and Table 8 presents the MIC value of all the tested extracts. The acetone and ethyl acetate extracts of pseudobulb showed the highest diameter of inhibition ( $16 \pm 0.00$  mm) against *S. aureus* with a MIC value of 0.875 mg/mL for PA. Acetonitrile extracts of leaves and stem, and ethyl acetate extract of pseudobulb showed inhibition of  $12 \pm 1.00$  mm against *K. pneumoniae* with MIC value of 1.375 mg/mL for LAN, 0.6875 mg/mL for SAN and, 3 mg/mL for PEA. The acetonitrile extracts of pseudobulb showed a zone of inhibition (ZOI) of about  $15.33 \pm 0.33$  mm with a MIC value of 1 mg/mL against *P. mirabilis*. Amoxicillin, a reference antibiotic, showed a ZOI of  $11.33 \pm 0.00$  mm against *S. aureus* and a ZOI of  $10.66 \pm 0.00$  mm against *P. mirabilis* with a MIC value of 1mg/mL. Amoxicillin did not inhibit *K. pneumoniae*. Inhibition by amoxicillin was less effective as compared to acetone pseudobulb extract of *P. maculata*.

### Screening of biofilm formation on the selected clinical isolates

The clinical isolates screened for biofilm formation were observed to be positive for the three isolates. Gram-positive bacteria *S. aureus* showed a slightly stained film on the end walls of the test tubes, gram-negative bacteria *K. pneumoniae* formed a very thick stained film on



**Fig. 4.** Hierarchical cluster analysis between (i) DPPH Scavenging activity and TAC, and (ii) TFC and TPC

the sides of the test tubes, and gram-negative bacteria *P. mirabilis* formed a moderately stained film on the end walls of the tubes.

### Effect of *P. maculata* on the inhibition of initial biofilm attachment

The Minimum Biofilm Inhibition Concentration (MBIC) for all extracts is presented in Table 9. Among *Pleione maculata* extracts, the pseudobulb acetonitrile extract of *P. maculata* exhibited the highest inhibitory activity against initial cell attachment of clinical isolates at its highest dilution,  $10^{-5}$ , with 87% inhibition of *S. aureus* cell attachment and the lowest MBIC at 0.25mg/mL. At a  $10^{-6}$  dilution water extract of root and leaves, pseudobulb acetonitrile extracts inhibited 97%, 90%, and 87% attachment of *K. pneumoniae* with MBIC values of 0.093, 0.117, and 0.125 mg/mL, respectively. Water extract of leaves, acetone extract of leaves, water root extracts, ethyl acetate leaf extracts, and pseudobulb acetonitrile extracts inhibited 100%, 91%, 89%, 87%, and 86% attachment of *P. mirabilis* at the lowest dilution,  $10^{-6}$ , with MBIC values of 0.117, 0.171, 0.090, 0.097, and 0.125 mg/mL, respectively. These results indicate that *P. maculata* is as effective as many known medicinal plants and antimicrobial agents.

### UV-VIS spectrophotometer

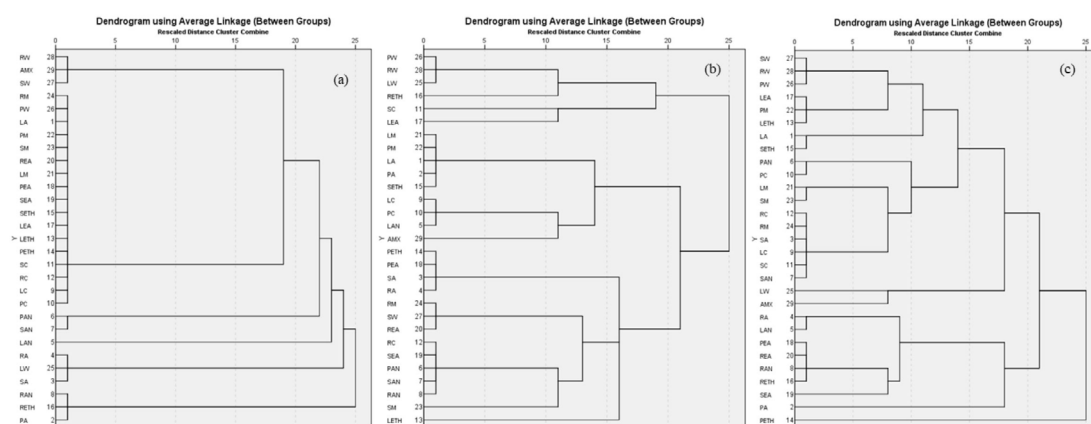
The results of UV-VIS spectrogram of seven solvents (acetone, acetonitrile, chloroform, ethanol, ethyl acetate, methanol, and water) extracts of *P. maculata* exhibited absorption band ranging from 204- 665 nm, as shown in Fig. 3. The UV-VIS spectrogram results of *P. maculata* extracts

indicate phenolic compounds such as flavonoids, terpenoids, and tannins in the plant extracts. The absorption band ranging from 204–665 nm is a characteristic feature of flavonoids and their derivatives<sup>44,35</sup>. The absorption of flavonoids ranges from 230–285 (Band I) and 300–350 (Band II)<sup>45,35</sup>. There are two absorption bands of flavonoid and terpenoids that range from 230–290 nm (Band I) and range from 400–550 nm (Band II), whereas the range between 600–700 nm represents the absorption of chlorophyll<sup>44</sup>.

### Hierarchical cluster analysis (HCA)

#### HCA between DPPH and Total antioxidant capacity

Hierarchical Cluster Analysis (HCA) is a multivariate analysis that identifies natural clusters without prior knowledge about the data to provide useful information such as the graphical representation of the resulting partitions in the form of a hierarchy or dendrogram revealing more information<sup>50</sup>. HCA was performed to determine the similarity or dissimilarity of clusters between various extracts of *P. maculata* to determine the robustness of the analysis. The horizontal axis of a dendrogram indicates the distance or dissimilarity between clusters whereas the vertical axis represents object clusters. In the HCA dendrogram, six individual clusters were grouped as shown in Fig. 4. to form two grand clusters based on two different methods used for the experiments. In Fig. 4. (i)(a), the rows of leaves of DPPH and pseudobulb DPPH are remarkably similar and have low dissimilarity as they are closely clustered at a rescaled distance of 1 unit



**Fig. 5.** Hierarchical cluster analysis of antibiofilm activity between different extracts of *P. maculata* against (a) *S. aureus* (b) *K. pneumoniae* and (c) *P. mirabilis*.

from each other as compared to the stem DPPH, which also shows some minute similarities at a rescaled distance of 14 units. The root DPPH shows a completely different clade from the leaves DPPH, pseudobulb DPPH, and stem DPPH. In Fig. 4 (i)(b), the rows of leaves TAC and pseudobulb TAC have low dissimilarity as they are closely clustered at a rescaled distance of 2 units from each other and from stem TAC, which also shows some minute dissimilarity at a rescaled distance of 1 unit. The root TAC shows a completely different clade from the leaves TAC, pseudobulb TAC, and stem TAC.

#### HCA between TFC and TPC

Fig. 4 illustrates two grand clusters based on two different methods used for the experiments. Six individual clusters were grouped together. In Fig. 4 (ii) (a), the rows of stem TFC and root TFC are very similar as they are closely clustered at a rescaled distance of 1 unit as compared to the pseudobulb TFC, which also shows similarities at a rescaled distance of 3 units. The leaves TFC shows a completely different clade with few similarities in the pseudobulb, stem and

root TFC at a rescaled distance of 3 units. In Fig. 4 (ii) (b), the rows of leaves TPC and pseudobulb TPC have low dissimilarity as they are clustered at a rescaled distance of 2 units from each other and from stem TPC, which also shows some minute dissimilarity at a rescaled distance of 3 units. The root TPC shows a completely different clade from leaves TPC, pseudobulb TPC, and stem TPC at a rescaled distance of 6 units.

#### HCA of antibiofilm activity

Fig. 5 illustrates the extracts cluster based on similarities in the presence of antibiofilm activity in a series of dilution factors. In Fig. 5 (a), there are five grand clusters of extracts: RW, AMX, and SW form a single cluster with a similar dilution factor  $10^{-1}$  at a rescaled distance of 1 unit, PAN and SAN form one cluster with a similar dilution factor  $10^{-5}$  at a rescaled distance of 1 unit, and LAN forms one grand cluster with a dilution factor  $10^{-4}$ , which indicates large differences with other extracts at a rescaled distance of 23 units. In Fig. 5 (b), the HCAs of various extracts against *K. pneumoniae* form two grand clusters. Each grand cluster is further divided

**Table 3.** IC<sub>50</sub> of DPPH radical scavenging activity of *Pleione maculata* extracts

Plant extracts	IC <sub>50</sub> (μg/mL)						
	Acetone	Acetonitrile	Chloroform	Ethanol	Ethyl acetate	Methanol	Water
Leaves	20.76 ± 0.00	30.37 ± 0.01	23.60 ± 0.00	34.86 ± 0.01	31.63 ± 0.00	25.86 ± 0.00	30.45±0.00
Pseudobulb	23.03 ± 0.00	30.13 ± 0.00	25.47 ± 0.00	22.26 ± 0.00	31.07 ± 0.02	25.51 ± 0.00	29.32±0.00
Stem	23.9 ± 0.00	22.30 ± 0.00	34.16 ± 0.00	34.45 ± 0.00	33.36 ± 0.12	28.23 ± 0.00	24.34±0.00
Root	26.87 ± 0.00	24.82 ± 0.01	26.71 ± 0.01	24.22 ± 0.0	24.44 ± 0.11	27.48 ± 0.00	26.92±0.00
Standard Ascorbic acid	30.80						

**Table 4.** Total antioxidant capacity (TAC) of *P. maculata* by Phosphomolybdenum assay

Plant extracts	Leaves TAC (mg of TAE/ g of extracts)	Pseudobulbs TAC (mg of TAE/ g of extracts)	Stems TAC (mg of TAE/ g of extracts)	Roots TAC (mg of TAE/ g of extracts)
Acetone	167.0 ± 0.5	129.68 ± 0.2	47.59 ± 0.1	74.56 ± 0.7
Acetonitrile	62.27 ± 0.1	75.30 ± 0.1	52.89 ± 0.0	53.56 ± 0.1
Chloroform	39.54 ± 0.1	114.05 ± 0.9	49.16 ± 0.3	69.49 ± 0.7
Ethanol	193.14 ± 0.3	190.57 ± 0.3	184.76 ± 0.3	177.96 ± 0.1
Ethyl acetate	193.98 ± 0.1	190.57 ± 0.2	184.85 ± 0.4	177.88 ± 0.3
Methanol	165.68 ± 0.3	177.29 ± 0.5	161.94 ± 0.3	64.887 ± 0.0
Water	142.94 ± 0.5	73.81 ± 0.2	109.60 ± 0.2	2.85 ± 0.0

TAE; Total ascorbic acid equivalent (mg of TAE/ g of extracts ± SD)

**Table 5.** Total Phenolic content (TPC) of different parts of *P. maculata* by Folin Ciocalteu reagent

Plant extracts	Leaves TPC (mg of GAE/ g of extracts)	Pseudobulbs TPC (mg of GAE/ g of extracts)	Stems TPC (mg of GAE/ g of extracts)	Roots TPC (mg of GAE/ g of extracts)
Acetone	429.166 ± 0.0	372.54 ± 0.0	336.11 ± 0.1	315.6 ± 0.0
Acetonitrile	207.77 ± 0.0	509.375 ± 0.0	378.18 ± 0.0	203.59 ± 0.0
Chloroform	149.8 ± 0.0	111.88 ± 0.2	25.48 ± 0.0	67 ± 0.0
Ethanol	127.58 ± 0.1	272.2 ± 0.0	108.73 ± 0.0	111.94 ± 0.0
Ethyl acetate	552 ± 0.0	228.45 ± 0.0	457 ± 0.0	282.61 ± 0.0
Methanol	238.299 ± 0.0	351.49 ± 0.0	227.135 ± 0.0	166 ± 0.0
Water	495 ± 0.0	288.67 ± 0.0	214.51 ± 0.0	66 ± 0.0

GAE; Gallic acid Equivalent (mg of GAE/g of extracts ± SD)

**Table 6.** Total flavonoid Content (TFC) of different parts of *P. maculata* by Aluminium Chloride method

Plant extracts	Leaves TFC (mg of QE/ g of extracts)	Pseudobulbs TFC (mg of QE/ g of extracts)	Stems TFC (mg of QE/ g of extracts)	Roots TFC (mg of QE/ g of extracts)
Acetone	879.5 ± 0.2	548.56 ± 0.0	552.62 ± 0.1	547.72 ± 0.4
Acetonitrile	516.014 ± 0.3	845.2 ± 0.2	676.08 ± 0.0	224.40 ± 0.1
Chloroform	234 ± 0.1	293.78 ± 0.0	246.44 ± 0.1	154 ± 0.0
Ethanol	159.03 ± 0.1	218.53 ± 0.0	84 ± 0.0	97.58 ± 0.1
Ethyl acetate	179.30 ± 0.0	104.25 ± 0.0	406.98 ± 0.1	75.33 ± 0.0
Methanol	263.109 ± 0.2	270.671 ± 0.1	152.78 ± 0.0	153.31 ± 0.1
Water	127.68 ± 0.0	277.31 ± 0.0	20.40 ± 0.0	175.90 ± 0.0

QE; quercetin equivalent (mg of QE/g of extracts ± SD)

into two grand clades based on the similarities in their dilution factors. Extracts LM, PM, LA, PA, and SETH fall under the same clade, which indicates similarities in the presence of antibiofilm activity with the same dilution factor  $10^{-6}$  at a rescaled distance of 1 unit. LETH extracts fall under one sub-clade alone because of their dissimilarity with other extracts at a rescaled distance of 17 units. In Fig. 5 (c), the HCAs of extracts against *P. mirabilis* reveal two grand clusters representing two grand clades, with four sub-clades. Extracts PW, SW, and RW are remarkably similar as they belong to the same leaf in a sub-clade with a dilution factor of  $10^{-6}$  whereas extract PETH exhibits a single separate grand cluster, which indicates high dissimilarity with other extracts at a rescaled distance of 25 units.

## DISCUSSION

Preliminary qualitative phytochemicals were earlier reported in *P. maculata* collected

from Meghalaya, which showed the presence of secondary metabolites such as terpenoid, phenol, tannin, saponin, coumarin, and cardiac glycosides in the leaves, pseudobulb, and seeds<sup>51</sup>. In vitro pseudobulb explants regeneration was conducted for the conservation of the rare and therapeutically important *P. maculata*<sup>5</sup>. *P. maculata* collected from Arunachal Pradesh also reported the presence of phytochemicals such as flavonoids, steroids, cardiac glycosides, and alkaloids<sup>52</sup>. The bioactivity of *P. maculata* was compared mainly with the closely related genus *Coelogyne* and its various species. A species of *Coelogyne stricta* exhibited 93% antibiofilm activity<sup>53</sup>. The aqueous leaf extracts of *Coelogyne nervosa* yielded the best DPPH radical scavenging activity, with IC<sub>50</sub> values of 126 µg/mL and 206 µg/mL for ethanol leaves and 312 µg/mL for ethyl acetate leaf extracts<sup>54</sup>. The ethyl acetate extracts of *Coelogyne stricta* exhibited a good DPPH scavenging activity with an IC<sub>50</sub> value of 47.0 µg/mL. The IC<sub>50</sub> values of

**Table 7.** Antibacterial screening of different parts of *P. maculata* against some clinical isolates

Solvents	Parts of <i>P. maculata</i> used	Zone of inhibition (in mm $\pm$ SD)		
		<i>Staphylococcus aureus</i>	<i>Klebsiella pneumoniae</i>	<i>Proteus mirabilis</i>
Acetone	Leaves	N	N	7.98 $\pm$ 0.9
	Pseudobulb	16 $\pm$ 0.0	10 $\pm$ 0.00	10.33 $\pm$ 0.4
	Stem	N	N	7.75 $\pm$ 1.7
	Root	N	N	8.655 $\pm$ 0.4
Acetonitrile	Leaves	N	12 $\pm$ 0.00	9.665 $\pm$ 0.9
	Pseudobulb	12 $\pm$ 1	11 $\pm$ 1.4	15.33 $\pm$ 0.4
	Stem	N	12 $\pm$ 1.4	10.33 $\pm$ 1.8
	Root	N	N	8.5 $\pm$ 0.7
Chloroform	Leaves	N	N	N
	Pseudobulb	10 $\pm$ 2	9 $\pm$ 1.4	9.2 $\pm$ 1.1
	Stem	N	N	N
	Root	N	N	N
Ethanol	Leaves	N	N	N
	Pseudobulb	N	N	N
	Stem	N	N	N
	Root	N	N	N
Ethyl acetate	Leave	N	8 $\pm$ 1.4	N
	Pseudobulb	16 $\pm$ 0.5	12 $\pm$ 2.8	N
	Stem	N	10.5 $\pm$ 0.7	N
	Root	N	10.5 $\pm$ 0.7	N
Methanol	Leaves	N	10 $\pm$ 0.00	8.1 $\pm$ 1.2
	Pseudobulb	N	11 $\pm$ 0.00	11.1 $\pm$ 0.8
	Stem	N	10.5 $\pm$ 0.70	N
	Root	N	10.50 $\pm$ 0.70	N
Water	Leaves	N	N	N
	Pseudobulb	N	N	N
	Stem	N	N	N
	Root	N	N	N
Amoxicillin (1mg/mL)			11.33 $\pm$ 0.00mm	No Inhibition
10.66 $\pm$ 0.00mm (turbid)				

N: No inhibition

methanol and water extracts were 72  $\mu$ g/mL and 112  $\mu$ g/mL, respectively 54. Antioxidant activity was also reported in extracts of *Coelogyne breviscapa*, with an IC<sub>50</sub> value of 71.91  $\mu$ g/mL<sup>55</sup>. The total phenolic content (TPC) of *Coelogyne brevis* was 0.081 mg/mL<sup>43</sup> and that of *Coelogyne stricta* was estimated to be a maximum of 0.090 mg/gram of dry extract<sup>53</sup>. In Moin et al., 2012, ethyl acetate extracts of *Coelogyne stricta* had the highest phenolic content of 90  $\mu$ g GAE/mg of the sample and 39.20  $\mu$ g GAE/mg in methanol

extracts. The total flavonoid content was higher in ethyl acetate at 37.25  $\mu$ g QE/mg of sample<sup>55</sup>. They also reported the TPC of *Coelogyne breviscapa*, which yielded 25.00 mg of GAE/gram of extracts. *Coelogyne brevis* TFC was observed to be less than 0.0625 mg/mL<sup>43</sup>. *Coelogyne stricta* reported a TFC of about 0.037mg/gram of dry extract<sup>53</sup>.

At present, several microbes that cause severe diseases affecting people around the globe have become strongly resistant to prescribed antibiotics, so new antibiotics need to be discovered



**Table 8.** MIC value of the effective extracts of *P. maculata* against the clinical isolates

<i>Pleione maculata</i> extracts	Conc. of extracts showing minimum inhibition against the microbes (mg/mL)		
	<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>
LA	N	1.375	2.75
PA	0.875	0.875	0.875
SA	N	N	3±0.0
RA	N	N	2.60
LAN	N	1.875	1.875
PAN	1	2	1
SAN	N	0.6875	0.6875
RAN	N	N	2.75
LC	N	N	N
PC	2.25	4.5	N
SC	N	N	N
RC	N	N	N
LE	N	N	N
PE	N	N	N
SE	N	N	N
RE	N	N	N
LEA	N	0.78	1.56
PEA	1.5	3	3
SEA	N	1.75	1.75
REA	N	3.25	3.25
LM	N	N	N
PM	N	N	N
SM	N	N	N
RM	N	6.1875	6.1875
LW	N	N	N
PW	N	N	N
SW	N	N	N
RW	N	N	N

Sample = Leave(L), Pseudobulb(P), Stem(S), Root(R), solvent = acetone(A), acetonitrile(AN), chloroform(C), ethanol(E), ethyl acetate(EA), methanol(M), water(W), antibiotic = AMX(Amoxicillin), N= Negative

to combat them effectively. *Pleione maculata*, an epiphytic rare medicinal orchid containing numerous effective secondary metabolites, was evaluated to gauge its antimicrobial and antibiofilm activity<sup>51</sup>. The ethanolic pseudobulb extract of *Coelogyne speciosa* exhibited strong antibacterial activity against *S. aureus* (ZOI was 19 mm) and methanolic pseudobulb extracts (ZOI was 8 mm), whereas ethyl acetate extracts showed no bacterial inhibition<sup>56</sup>. The extracts of *Coelogyne stricta* showed no antibacterial activity against *S. aureus*. The ethanolic extracts of *Coelogyne brachyptera* showed good antibacterial activity

with a ZOI of 20 mm against gram-positive *S. aureus*<sup>56</sup>. It has also exhibited antibacterial activity against *S. aureus* with a strong ZOI of 17±0.6 mm<sup>52</sup>. *Coelogyne stricta* exhibited antibacterial activity at a ZOI of 8 mm against *K. pneumoniae* and 14 mm against *S. aureus*, whereas *Coelogyne cristata* showed an inhibition of about 12 mm against *S. aureus* while its MIC was 31.25 mg/mL 34. *P. maculata* extracts also showed a good ZOI ranging from 7.75–16 mm in diameter and a very low MIC value ranging from 0.875–14.875 mg/mL. This result indicates that *Pleione maculata* is as effective as many other medicinal plants and antimicrobial agents. *P. maculata* and its closely related genus *Coelogyne* have not been the subjects of existing antibiofilm research reports.

## CONCLUSION

Oxidative stress causes imbalances of oxygen species in the body. It results in health problems such as cancer, cardiovascular disease, neurological disorders, liver malfunction, and other complications caused by DNA damage. *Coelogyne* and its related genus, *Pleione*, have previously been studied for their antioxidant and antibacterial properties. However, there were no antibiofilm investigations reported thus far. *Pleione* and its members, on the other hand, remain an unknown orchid genus. There is a lack of documentation of its antibiofilm efficacy and quantifiable TPC, TFC, or antioxidant activity. As a result, this study aimed to determine the bioactivity of *Pleione maculata*. *P. maculata* has potent antioxidant and antibiofilm properties attributed to secondary metabolites such as phenolics and flavonoids. Antibiotic-resistant pathogens forming biofilms are a global concern. Primarily due to the treatment failures for infectious diseases. *Pleione maculata* exhibits antibacterial and antibiofilm action against *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. Future research can focus on developing unique and broad-spectrum antimicrobial compounds. Further studies are required to understand how the extracts work. This study demonstrates the therapeutic potential of *Pleione maculata*. It establishes the importance of this epiphyte in pharmaceutical and nutraceutical companies. Besides, creating awareness for preventing its extinction and overexploitation.

**Table 9.** Minimum Biofilm Inhibitory Concentration (MBIC) of *Pleione maculata* based on highest dilution to show inhibition against tested clinical isolates

Solvents	Parts of <i>P. maculata</i> used	Minimum biofilm inhibitory concentration (MBIC) of <i>P. maculata</i> extracts with highest dilution factor showing good inhibitory percentage against attachment of biofilm forming microbes to glass surfaces					
		<i>Staphylococcus aureus</i>		<i>Klebsiella pneumoniae</i>		<i>Proteus mirabilis</i>	
		dilution	Concen. (mg/mL)	dilution	Concen. (mg/mL)	dilution	Concen. (mg/mL)
Acetone	L	10 <sup>-5</sup>	0.344	10 <sup>-6</sup>	0.171	10 <sup>-6</sup>	0.171
	P	10 <sup>-3</sup>	1.218	10 <sup>-6</sup>	0.152	10 <sup>-6</sup>	0.152
	S	10 <sup>-2</sup>	3	10 <sup>-2</sup>	3	10 <sup>-6</sup>	0.187
	R	10 <sup>-1</sup>	5.25	10 <sup>-2</sup>	2.625	10 <sup>-6</sup>	0.164
Acetonitrile	L	10 <sup>-4</sup>	0.93	10 <sup>-6</sup>	0.234	10 <sup>-6</sup>	0.234
	P	10 <sup>-5</sup>	0.25	10 <sup>-6</sup>	0.125	10 <sup>-6</sup>	0.125
	S	10 <sup>-5</sup>	0.171	10 <sup>-6</sup>	0.085	10 <sup>-6</sup>	0.085
	R	10 <sup>-3</sup>	1.375	10 <sup>-6</sup>	0.171	10 <sup>-6</sup>	0.171
Chloroform	L	10 <sup>-2</sup>	3.375	10 <sup>-6</sup>	0.21	10 <sup>-6</sup>	0.210
	P	10 <sup>-2</sup>	4.625	10 <sup>-6</sup>	0.289	10 <sup>-6</sup>	0.289
	S	N	N	10 <sup>-6</sup>	0.269	10 <sup>-6</sup>	0.269
	R	N	N	10 <sup>-6</sup>	0.187	10 <sup>-6</sup>	0.187
Ethanol	L	N	N	10 <sup>-6</sup>	0.546	10 <sup>-6</sup>	0.546
	P	N	N	10 <sup>-2</sup>	5	10 <sup>-5</sup>	0.625
	S	N	N	10 <sup>-6</sup>	0.68	10 <sup>-6</sup>	0.683
	R	10 <sup>-2</sup>	7.44	10 <sup>-3</sup>	3.718	10 <sup>-6</sup>	0.464
Ethyl acetate	L	N	N	10 <sup>-6</sup>	0.097	10 <sup>-6</sup>	0.097
	P	N	N	10 <sup>-2</sup>	3	10 <sup>-6</sup>	0.187
	S	N	N	10 <sup>-6</sup>	0.109	10 <sup>-6</sup>	0.109
	R	N	N	10 <sup>-6</sup>	0.20	10 <sup>-6</sup>	0.203
Methanol	L	N	N	10 <sup>-6</sup>	0.386	10 <sup>-6</sup>	0.386
	P	N	N	10 <sup>-6</sup>	0.2617	10 <sup>-6</sup>	0.261
	S	N	N	10 <sup>-6</sup>	0.304	10 <sup>-6</sup>	0.304
	R	N	N	10 <sup>-6</sup>	0.386	10 <sup>-6</sup>	0.386
Water	L	10 <sup>-2</sup>	1.875	10 <sup>6</sup>	0.117	10 <sup>-6</sup>	0.117
	P	N	N	10 <sup>-6</sup>	0.125	10 <sup>-6</sup>	0.125
	S	10 <sup>-1</sup>	7.75	10 <sup>-6</sup>	0.242	10 <sup>-6</sup>	0.242
	R	10 <sup>-1</sup>	3	10 <sup>-6</sup>	0.093	10 <sup>-6</sup>	0.09
AMX (control)		10 <sup>-1</sup>	1	10 <sup>-1</sup>	1	10 <sup>-1</sup>	1

Sample = Leaves (L), Pseudobulb (P), Stem (S), Root (R), antibiotic = AMX (Amoxicillin), N= Negative

**SUPPLEMENTARY INFORMATION**Supplementary information accompanies this article at <https://doi.org/10.22207/JPAM.15.3.51>**Additional file:** Additional Figures and Table.**ACKNOWLEDGMENTS**

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and in the supplementary file.

## ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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