




RESEARCH ARTICLE

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Evaluation of Antioxidant and Antimicrobial Activity of Some Plants Collected from Malaysia

Sajna Keeyari Purayil¹, Chew Annley¹, Paulraj Ponnaiah¹, Sajeesh Pattammadath¹, Pazhayakath Thevarkattil Mohamed Javad¹, Jenifer Selvarani A.² , Raji P. , Thirumurugan R.³, Iyappan P.¹ and Antony V. Samrot^{1*} 

¹Department of Biomedical Sciences, MAHSA University, SP 2, Bandar Saujana Putra, Jenjarom, 42610, Malaysia.

²Department of Biotechnology, Sathyabama Institute of Science and Technology, Jeppiar Nagar, Chennai - 600 119, Tamil Nadu, India. ³Medical Laboratory Technologist, Department of Transfusion Medicine, JIPMER, Puducherry - 605 006, India.

Abstract

Five plant species namely, *Phyllanthus acidus*, *Piper aduncum*, *Pandanus amaryllifolius*, *Macaranga peltata* and *Acacia mangium* were analysed for their effective *in-vitro* bioactivity. The chloroform and aqueous extracted of the selected plants were subjected to TLC bioautography for antioxidant activity later all the extracted were subjected for DPPH assay where the chloroform extracts were found to express maximum antioxidant property. Amongst all the plants, *Macaranga peltata* accounted to 95% DPPH scavenging activity. The antimicrobial studies of the plant extracts were performed via agar well diffusion method, MIC determination, Biofilm inhibition assay in microtitre plate against clinical isolates like *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*. It was found that *Piper aduncum* (chloroform and aqueous extract) and *Macaranga peltata* (only aqueous) expressed antibacterial activity, of which only chloroform extract of *Piper aduncum* could show negative influence against the biofilm development of *P.aeruginosa*.

Keywords: DPPH scavenging, antibacterial activity, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

*Correspondence: antonysamrot@gmail.com

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INTRODUCTION

The ideology of utilising plants to treat diseases and to preserve human health dates back to ancient civilization which took its form into different system of medicinal practices namely Siddha, Unani and Ayurveda which are in practise till date. It is estimated that around 2,50,000 to 5,00,000 different types of plant source exist on earth but however, less than 11% are utilised as food or medicinal source by humans and other animals^{1,2}. In recent years, the advancements in technology and techniques had led to better understanding of plants components which helped in recognizing various plant species for their bioactivity³⁻⁵. Plants are believed to produce bioactive compounds in order to protect themselves from microbial invasion. The presence of phytochemicals especially secondary metabolites such as alkaloids, tannins, saponins, flavonoids, sterols, terpenoids are deemed to be the main supporting factor of the antimicrobial activity of the plant against invading pathogens⁶⁻¹³. Though the ancient system of medicine gives knowledge about treatment of various diseases using medicinal plants, only the *in-vitro* and *in-vivo* analyses of these herbal plants could possibly explain and describe the effect of bioactive compounds against the causing microbes. However, the microbial revolution has led to the rise of new resistant strains against the existing drug. Apparently, the formulation or discovery of a new effective drug against the pathogens is very essential. The broad range and diversity of phytomolecules with potent bioactivity remains as the promising source for drug synthesis and new formulation¹⁴. To discuss a few bioactive compounds, Tannins are found to possess antiviral, antineoplastic and antibacterial activity^{15,16}. Flavonoids are widely known for their antimicrobial activity, allergies, inflammation, prevent platelet aggregation¹⁷. Plant phenolics, vitamin C, vitamin E and carotenoids are known for their antioxidant activity¹⁵. Glycoside are reported to increase myocardial contraction¹⁸. Alkaloids are referred for range of pharmacological activities such as antimalarial, antineoplastic, antibacterial and anti-hyperglycaemic activities^{19,20}. Other alkaloids like cocaine, nicotine and caffeine are known as nervous system stimulants and psychotropic agents.

Likewise, numerous new plants species were surveyed as crude extracts to report their *in-vitro* bioactivities. Analgesic, Anti-inflammatory, Antiviral, Anti-hepatotoxic and Anti-pyretic are the medicinal activities of *Phyllanthus spp.*^{21,22}. Fruits of *P. acidus* and *P. emblica* are proven as rich source of vitamin C hence they are often used to improve general body wellness as well as preventive measures against diabetes and to relieve coughing symptoms²¹. A wide variety of medicinal plants belonging to *Piperaceae*, *Moraceae*, *Euphorbiaceae*, *Clusiaceae*, *Berberidaceae*, *Asteraceae*, etc., were identified for its anti-HIV activity, which also serves as the base molecule to derive effective modern drugs against HIV²³. *P. granatum* and *C. cyminum* was seen effective in inhibiting growth of pathogenic bacteria such as *S. aureus*²⁴. Even plant derived silver nanoparticles too showed antibacterial activity^{25,26}.

This study deals with five plant species namely *Phyllanthus acidus*, *Piper aduncum*, *Pandanus amaryllifolius*, *Macaranga peltata* and *Acacia mangium*. The phytochemical separation was carried out by TLC bioautography for their crude extracts and their antioxidant property was analysed via TLC-DPPH bioautography and DPPH assay²⁷⁻³¹. These plant extracts are subjected for *in-vitro* studies against multidrug resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* to evaluate its antimicrobial and biofilm inhibition property.

MATERIALS AND METHODS

Collection of Plants

The selected plants namely *Phyllanthus acidus*, *Piper aduncum*, *Pandanus amaryllifolius*, *Macaranga peltata* and *Acacia mangium* were collected from Kuala Lumpur and Puchong, Malaysia. Fresh and healthy leaves were collected and cleansed in running tap water and left to shade dry for 1 - 2 weeks.

Preparation of Plant Extracts

The dried plant leaves were ground into fine powder by an electric blender. The plant extracts were prepared by maceration using solvents (chloroform and water) in the ratio 1:10 (g of powder: mL of solvent)³². 50g of each plant powder were mixed with 500ml of respective solvents (chloroform and water) separately and

placed under magnetic agitation for 30 min. The plant-solvent mixtures were allowed to react for a week and later vacuum filtered, followed by centrifugation at 8000 rpm for 5 min. The supernatant was lyophilized to obtain the solid residue of the crude extracts which was weighed and dissolved in respective solvents prior every analysis.

Phytochemical Screening

The plant extracts were screened for secondary metabolites such as Tannins, Phlobatannins, Saponins, Flavonoids, Terpenoids and Glycosides following Edeoga et al.³³.

TLC Bioautography

TLC Bioautography were performed for separation of individual plant component in the extract using silica plates (Merck, F245)³⁴. Each plant extract was loaded onto silica plates and the chloroform extracts were run having chloroform: benzene: acetonitrile: ethanol (3:2:0.5:0.5) and water extracts were run with the mobile phase of water: butyl alcohol: acetonitrile: ethanol: ethyl acetate (1:1:1:0.5:0.5) respectively. Thus, developed TLC plates were visualized under visible light, UV and iodine vapor. R_f (retention factor) were calculated from the below formula for each band developed under the different conditions.

$$R_f = \frac{\text{Distance travelled by sample}}{\text{Distance travelled by solvent}}$$

TLC DPPH Bio-autography for Antioxidant Activity

The plant extracts were screened for antioxidant property by a preliminary method of DPPH (1, 1- diphenyl-2-picrylhydrazyl) spray technique. The developed plates were dried and sprayed with DPPH (0.004 % w/v in 95% methanol) and observed for development of yellow spot²⁷.

Antioxidant DPPH assay

The free radical (DPPH) scavenging activity of the plant extracts were evaluated in terms of % by following Annegowda et al.³⁵ and Selvarani et al.³⁶. Ascorbic acid was used as the standard solution. Different concentrations of each plant extract such as 50, 100, 150, 200, 250 µg/ml were made up to 1 mL with methanol and added with 1 mL of DPPH solution (0.004% w/v in 95% methanol). The reaction mixture was incubated for 30 min at room temperature under dark. After incubation, the absorbance was read at 517nm against a reagent blank. The radical scavenging activity is calculated from the formula,

$$\% \text{ free radical scavenged} = \frac{(Ab_{\text{control}} - Ab_{\text{sample}})}{Ab_{\text{control}}} \times 100$$

Antimicrobial Activity

Agar well diffusion method

Clinical isolated *Staphylococcus aureus* and *Pseudomonas aeruginosa* was used for antibacterial activity studies. The antibacterial activity of the plant extracts against the isolated cultures was checked by agar well diffusion method on Muller Hinton Agar (MHA) plates³⁷. Stock solution of the plant extracts was prepared using DMSO as solvent for chloroform extracts and sterile water for aqueous extracts. Having their respective solvents as the negative control, the plant extracts were loaded in different concentrations such as 5 mg/ml, 10 mg/ml, 15 mg/ml and 20 mg/ml and positive control was maintained. Then, the plates were incubated at 37°C for 24 h.

Minimum Inhibition Concentration

The plant extract that showed positive

Table 1. Phytochemical screening from various qualitative biochemical tests

Phytochemicals	PLANT EXTRACTS									
	PA-C	PSPP-C	MP-C	AM-C	PA-W	PSPP-W	PDA-W	MP-W	AM-W	
Tannin	-	-	-	-	-	+	+	+	+	
Phlobatannins	-	-	-	-	-	-	-	-	-	
Flavonoids	-	-	-	-	+	+	-	+	+	
Terpenoids	-	-	-	-	-	-	+	-	+	
Glycosides	-	-	-	-	+	-	+	-	+	

(-) indicates the absence, and (+) indicates the presence. C- chloroform extract, W – aqueous extract. PA- *Phyllanthus acidus*, PSPP- *Piper aduncum*, PDA- *Pandanus amaryllifolius*, MP- *Macaranga peltata*, AM- *Acacia mangium*.

results for antibacterial activity are proceeded to determine the minimal concentration of inhibition. From the zone of clearance (if any) formed in antibacterial activity, the respective concentration was serially diluted and added to culture laden microtitre plate and the MIC was identified according to Mgbeahuruike et al³⁸.

Biofilm Inhibition Assay

To a sterile 96-well tissue culture plate, the plant extracts were pipetted in varied concentrations such as 4mg/mL, 8mg/mL, 12mg/mL and 16mg/mL in triplets and total volume of each well was made up to 100µL with Muller Hinton broth. Biofilm inhibition assay was performed as described earlier³⁹.

RESULTS AND DISCUSSIONS

Phytochemical Screening for Secondary Metabolites

The results obtained for qualitative phytochemical screening are tabulated as Table 1. According to Edeoga et al³³ the presence of phytochemicals were represented via colour

changes upon reaction with reagents. The chloroform plant extracts did not respond for presence of secondary metabolites but the aqueous extracts have projected the presence of several phytochemicals. The aqueous extracts of *Phyllanthus acidus* showed the presence of flavonoid and glycosides, *Piper aduncum* showed the presence of tannins and flavonoid while the methanol extract of *Piper aduncum* was reported to have other phytochemicals like alkaloids; triterpenoids, sterols, tannins, saponins and coumarins⁴⁰. Tannin, terpenoids and glycosides were present in *Pandanus amaryllifolius*, tannin and flavonoid were present in *Macaranga peltata* and *Acacia mangium* was identified with tannin, flavonoid, terpenoid and glycosides. Jain et al.⁴¹ has identified the presence of flavonoids, glycosides, phenolic compounds, proteins, amino acids, carbohydrates and saponins from the aqueous extract of *Phyllanthus acidus*. Al-Rifai et al⁴² has also seen with similar outcome where secondary metabolite compounds such as flavonoids are more favourably isolated from polar extract.

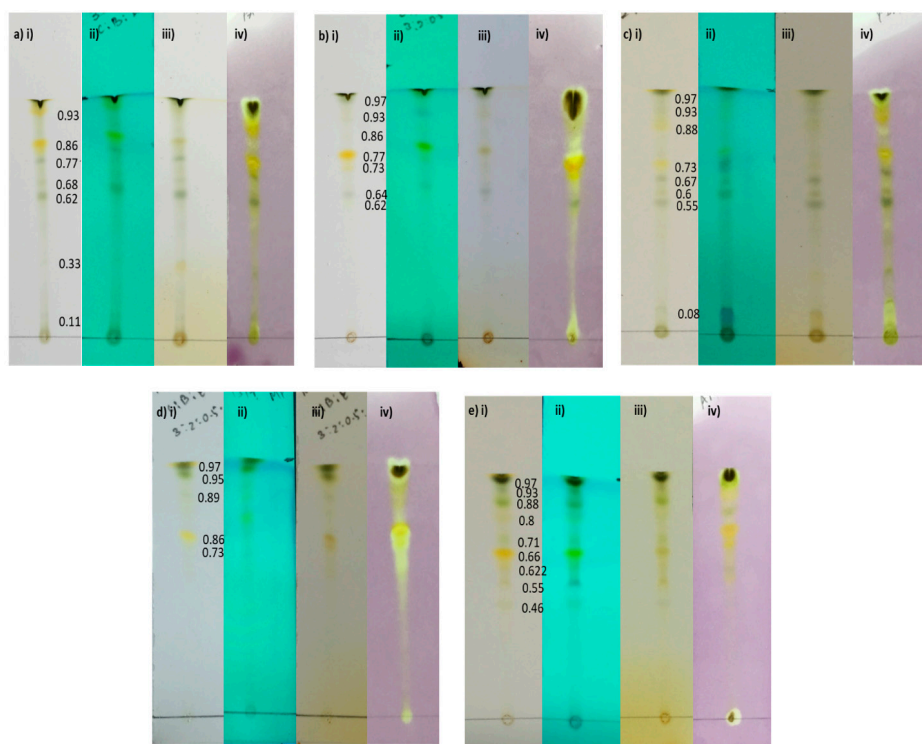


Fig. 1. TLC and TLC DPPH Bio-autography results of chloroform extracts of a) *Phyllanthus acidus*, b) *Piper aduncum*, c) *Pandanus amaryllifolius*, d) *Macaranga peltata*, e) *Acacia mangium*. i) Visible light ii) UV iii) Iodine vapour and iv) DPPH Sprayed

TLC and TLC- Bioautography

The chloroform extracts of *P. acidus*, *P. aduncum*, *P. amaryllifolius*, *M. peltata* and *A. mangium* were run with chloroform, benzene, acetonitrile and ethanol in the ratio 3:2:0.5:0.5 respectively and their respective water extracts were run with water, butyl alcohol, acetonitrile, ethanol and ethyl acetate in the ratio 1:1:1:0.5:0.5. And the plates were exposed to UV and iodine vapours to visualize the other possible components. On comparing the TLC results in Fig.

1 & 2, the chloroform extracts resolved better to water extracts with maximum number of bands. The R_f of each band was calculated and tabulated in Table 2 and Table 3. DPPH was sprayed on to the TLC developed plates for chloroform and water extracts in order to identify the antioxidant property. The scavenging activity of DPPH as yellow spots was observed over the resolved bands as seen in Fig. 1 (a-e iv) and Fig. 2 (a-e iv). This confirmed the presence of potent antioxidant phytochemicals. TLC Bioautography method is

Table 2. R_f value of compounds separated from chloroform plant extracts

<i>P. acidus</i>	<i>P. aduncum</i>	<i>P. amaryllifolius</i>	<i>M. peltata</i>	<i>A. mangium</i>
0.11	0.62	0.08	0.73	0.46
0.33	0.64	0.55	0.89	0.55
0.62	0.73	0.60	0.95	0.62
0.68	0.77	0.67	0.97	0.66
0.77	0.86	0.73		0.71
0.86	0.93	0.88		0.80
0.93	0.97	0.93		0.88
		0.97		0.93
				0.97

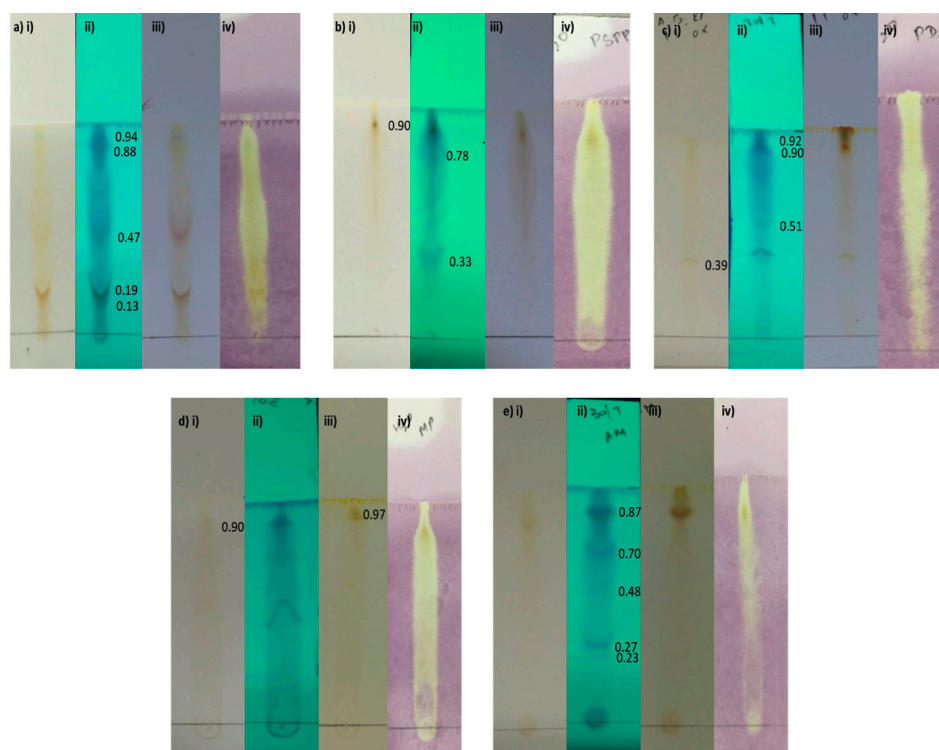


Fig. 2. TLC and TLC DPPH Bio-autography results of aqueous extracts of a) *Phyllanthus acidus*, b) *Piper aduncum*, c) *Pandanus amaryllifolius*, d) *Macaranga peltata*, e) *Acacia mangium*. i) Visible light ii) UV iii) Iodine vapour and iv) DPPH Sprayed

rather an easy way to screen plant extracts for its antioxidant activity, where Wang et al⁴⁴ was able to identify three antioxidant compounds with the incorporation of this technique alongside with UV, MS and NMR spectra.

DPPH assay

Having ascorbic acid as the standard sample, the % of DPPH scavenged by the plant extracts were calculated and compared. On relating with the aqueous extracts in Fig. 3b, the chloroform extracts were found to show better radical scavenging activity where the maximum inhibition was expressed by *Macaranga peltata* (Fig. 3a). With increase in concentration of plant extracts, the percentage of DPPH scavenged was found to increase, reaching a maximum inhibition of 80 % - 95 % at 250µg/mL by all the chloroform extracts. The IC 50 of chloroform extracts were as follows, *Acacia mangium* at 75µg/mL, *Phyllanthus*

acidus at 120µg/mL, *Pandanus amaryllifolius* at 125µg/mL and *Piper aduncum* at 170µg/mL. The aqueous extracts showed a constant inhibition of 35% to 50% with increase in concentration and the maximum scavenging activity was expressed by *Phyllanthus acidus* (80 %). 50% of DPPH inhibition was noticed in *Phyllanthus acidus*, *Piper aduncum*, *Pandanus amaryllifolius* whereas *Macaranga peltata* and *Acacia mangium* showed a maximum of 35% and hence the IC 50 of *Phyllanthus acidus*, *Piper aduncum*, *Pandanus amaryllifolius* were 70µg/mL, 50µg/mL and 50µg/mL respectively.

Antibacterial Activity by agar well diffusion method

The zone of clearance was observed (Table 4 & 5) for the aqueous extracts of *Piper aduncum* and *Macaranga peltata* against *Staphylococcus aureus* (MRSA) and by the chloroform extract of *Piper aduncum* against MDR - *Pseudomonas*

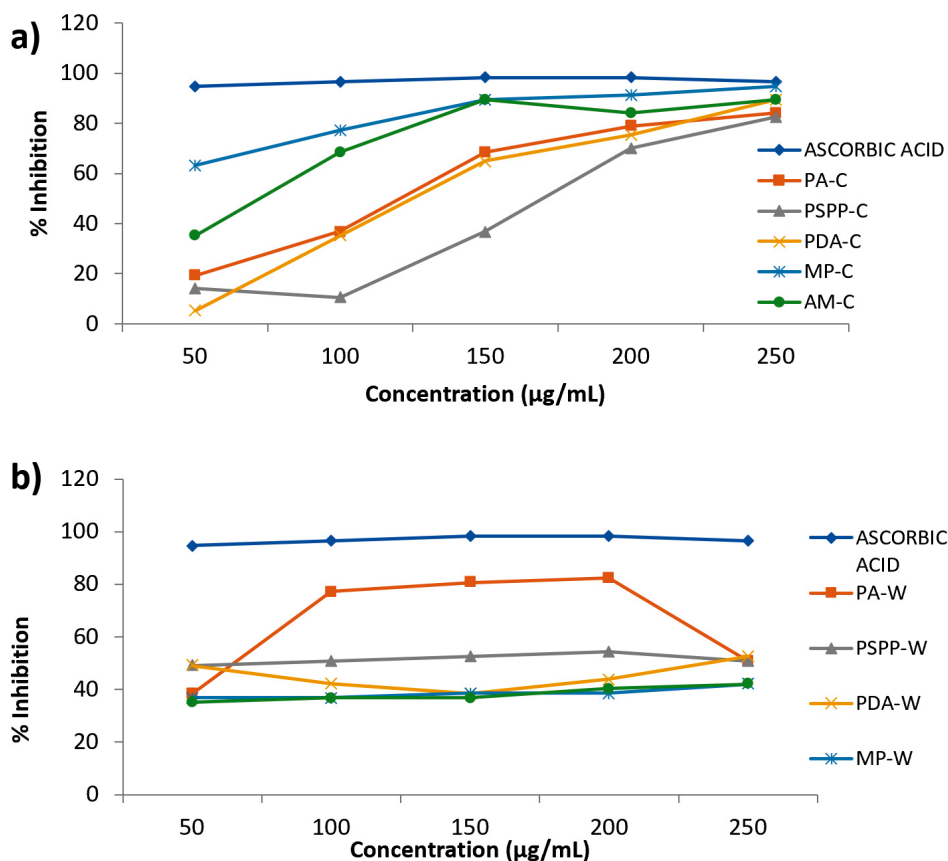


Fig. 3. Antioxidant activity by DPPH assay a) Chloroform extracts and b) Aqueous extracts
PA- *Phyllanthus acidus*, PSPP- *Piper aduncum*, PDA- *Pandanus amaryllifolius*, MP- *Macaranga peltata*, AM- *Acacia mangium*

aeruginosa. No zone of clearance was noticed for other plant extracts. This confirms the antibacterial activity of *Piper aduncum* (chloroform & aqueous extract) against both the isolated cultures and only the aqueous extract of *Macaranga peltata* to have antibacterial activity against *Staphylococcus aureus*. A similar antimicrobial activity of *P. aduncum* have been reported against its inhibitory actions against *S. aureus*⁴⁵. Likewise, the methanolic extract of *P. aduncum* was reported to exhibit antimicrobial action against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella sonnei*, *Klebsiella pneumoniae*⁴⁰. *M. peltata* are reported to aid in wound healing as its antimicrobial effect studied

against various wound pathogen⁴⁶. The results are supported by the believed potential of *Piper spp.* against pathogenic Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* that have been investigated and reported from published researches⁴³.

Minimal Inhibition Concentration (MIC)

From the antibacterial results of *Piper aduncum* and *Macaranga peltata*, the minimum inhibition concentration (MIC) of the plant extracts was determined. It was found that the aqueous extract of *Piper aduncum* and *Macaranga peltata*

Table 3. R_f value of compounds separated from aqueous plant extracts

<i>P. acidus</i>	<i>P. aduncum</i>	<i>P. amaryllifolius</i>	<i>M. peltata</i>	<i>A. mangium</i>
0.13	0.33	0.39	0.90	0.23
0.19	0.78	0.51	0.97	0.27
0.47	0.90	0.90		0.48
0.88		0.92		0.70
0.94				0.87

Table 4. Zone of Inhibition by plant extracts against *Staphylococcus aureus*

<i>S. aureus</i> Plant Extract	Zone of Inhibition (mm)									
	PA-C	PA-W	PSPP-C	PSPP-W	PDA-C	PDA-W	MP-C	MP-W	AM-C	AM-W
5mg/ml	0	0	0	12	0	0	0	6	0	0
10mg/ml	0	0	0	15	0	0	0	7	0	0
15mg/ml	0	0	0	17	0	0	0	8	0	0
20mg/ml	0	0	0	20	0	0	0	10	0	0

C- chloroform extract, W – aqueous extract.

PA- *Phyllanthus acidus*, PSPP- *Piper aduncum*, PDA- *Pandanus amaryllifolius*, MP- *Macaranga peltata*, AM- *Acacia mangium*

Table 5. Zone of Inhibition by plant extracts against *Pseudomonas aeruginosa*

<i>P. aeruginosa</i> Plant Extract	Zone of Inhibition (mm)									
	PA-C	PA-W	PPSP-C	PPSP-W	PDA-C	PDA-W	MP-C	MP-W	AM-C	AM-W
5mg/ml	0	0	15	0	0	0	0	0	0	0
10mg/ml	0	0	17	0	0	0	0	0	0	0
15mg/ml	0	0	19	0	0	0	0	0	0	0
20mg/ml	0	0	22	0	0	0	0	0	0	0

C- chloroform extract, W – aqueous extract. PA- *Phyllanthus acidus*, PSPP- *Piper aduncum*, PDA- *Pandanus amaryllifolius*, MP- *Macaranga peltata*, AM- *Acacia mangium*.

inhibited the bacterial growth of *Staphylococcus aureus* (MRSA) at 1.25 mg/mL and 0.1563 mg/mL respectively. And a minimal concentration of about 1.25 mg/mL was required by chloroform extract of *Piper aduncum* to inhibit the growth of MDR - *Pseudomonas aeruginosa* (Table 6).

Antibiofilm Activity

On 48 h of observation, it was found that the aqueous extracts of *Piper aduncum* and *Macaranga peltata* did not seem to affect the biofilm formation of *Staphylococcus aureus* (Fig. 4a and b). The bacteria maintained its growth and prevailed the whole incubation period. However, the population of treated culture was minimal at the highest concentration (20 mg/mL of plant extract) when compared with the control. While

treating *Pseudomonas aeruginosa* with chloroform extract of *Piper aduncum*, the biofilm formation decreased gradually with increase in concentration and the density of culture remained the same as in 12h with no further growth till the lasting incubation period (48 h) (Fig. 5).

Table 6. MIC for plant extracts with Antibacterial activity

S.No.	<i>S.aureus</i> MIC (mg/ml)		<i>Pseudomonas</i> <i>aeruginosa</i> MIC (mg/ml)
	PSPP-W	MP-W	PSPP-C
1	1.25mg/ml	0.1563mg/ml	1.25mg/ml

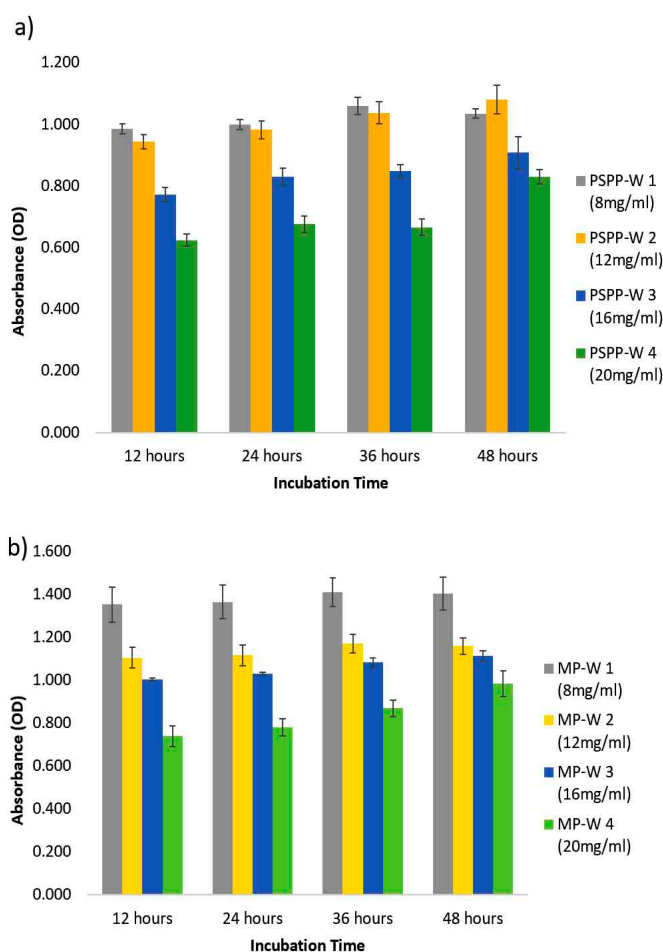


Fig. 4. Antibiofilm activity of aqueous extracts of a) *Piper aduncum* and b) *Macaranga peltata* against *Staphylococcus aureus*

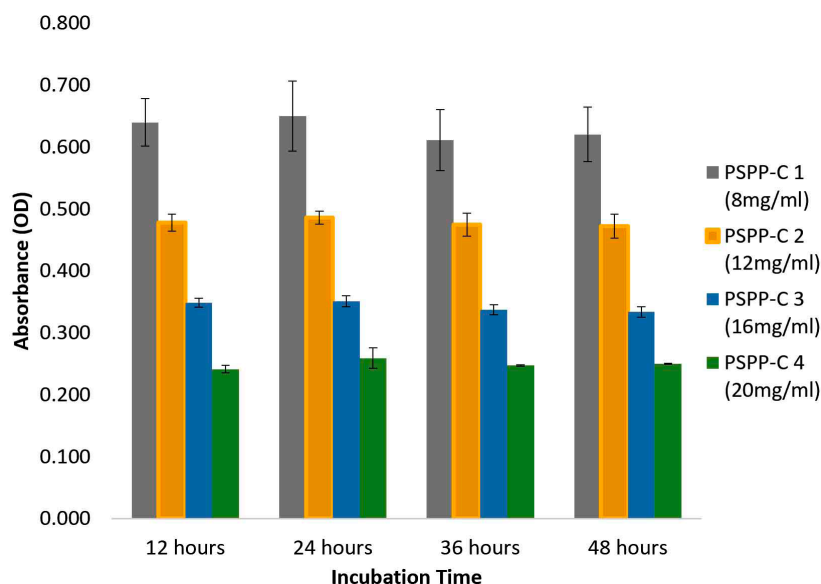


Fig. 5. Antibiofilm activity of chloroform extract of *Piper aduncum* against *Pseudomonas aeruginosa* over 48 hours of incubation.

CONCLUSION

It can be concluded that the chloroform and aqueous extracts of the selected plants, i.e. *Phyllanthus acidus*, *Piper aduncum*, *Pandanus amaryllifolius*, *Macaranga peltata* and *Acacia mangium* were found to possess antioxidant property which was confirmed by TLC-DPPH bioautography and DPPH assay. All the plant extracts were examined against the isolated cultures, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, where, the aqueous extract of *Macaranga peltata*, *Piper aduncum* and chloroform extract of *Piper aduncum* showed antibacterial activity and their respective minimum inhibition concentration was determined. And only the chloroform extract of *Piper aduncum* was found to have impact on biofilm formation of *P.aeruginosa*.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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None.

AUTHOR'S CONTRIBUTION

All the authors have made direct contribution on idea creation, research work and editing of the manuscript.

DATA AVAILABILITY

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

ETHICS STATEMENT

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

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