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PHYTOCHEMICAL ANALYSES, ANTI-BACTERIAL AND ANTI-BIOFILM ACTIVITIES OF MANGROVE-ASSOCIATED *Hibiscus tiliaceus* EXTRACTS AND FRACTIONS AGAINST *Pseudomonas aeruginosa*

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Abstract: The search for novel biological activities from plant-based natural products is gaining traction due to the high abundance and accessibility of the plants, with consequent lower cost for discovery, and lesser side effects and toxicity on human health and the environment. This study focused on the phytochemical analyses and the potency of mangrove-associated *Hibiscus tiliaceus* extracts and fractions as anti-bacteria and anti-biofilm agents against *Pseudomonas aeruginosa*. The bacterial strain contributes towards biofilm formation of various infectious diseases such as cystic fibrosis in lung, and causes blockage in urinary catheter. It is also an initiator of biofouling in shipping and maritime facilities. The methanol extracts of each part of fruits, leaves, and twigs were fractionated into chloroform, ethyl acetate, and methanol fractions using column chromatography. Crystal violet assay was carried out for anti-biofilm activity in a 96 well-plate to evaluate the bacterial inhibition and biofilm formation. Phytocemical analyses suggested the presence of protein, carbohydrate, phenols, tannin, flavonoids, saponins, glycoside, steroids, terpenoids, and alkaloids in *Hibiscus tiliaceus*. The strongest anti-bacterial and anti-biofilm activities were exhibited by the chloroform fraction of fruits (HFC). The methanol crude of fruits (HFMc), methanol fraction of fruits (HFM), and chloroform fraction of twig (HTC) showed more than 80% inhibition as compared to the control.The results suggested that *Hibiscus tiliaceus* had a good potential to be developed as anti-bacterial and anti-biofilm agents.

Keywords: *Hibiscus tiliaceus, Pseudomonas aeruginosa*, phytochemicals, anti-biofilm, anti-bacteria.

Introduction

The search for anti-bacterial properties and biofilm prevention from plant-based natural products has been spurred by the facts that the phytochemicals are less toxic to environment. The use of iron-based paint to prevent anti-biofilm and anti-fouling has adversely affected the marine eco-system (Yebra *et al*., 2004). *Pseudomonas aeruginosa* is known to be a virulent and resilient bacteria capable of surviving in a harsh environment such as lack of nutrition and persist in communityin marine facilities and hospitals equipment by producing biofilms (Lister *et al*., 2009). This adaptibility may have conferred its multi-drug resistance properties.

Hibiscus tiliaceus from the Malvaceae family is mainly found in the tropics and is native to Eastern and Northern Australia, Oceania and South-East Asia.It can be used as an alternative, natural source for antibacterial activity against *P. aeruginosa* (Hemaiswarya *et al*., 2009), and also reportedly rich in phenolics, flavonoids, vitamin E and several stigmasterol derivatives (Zhang *et al*., 2011). The flavonoids isolated from *Hibiscus* sp. have been identified as kaemperol, kaemferol-G-Rha, quercetin, and rutin (Zhen *et al*., 2016). The phenolics, kaemferol and the derivatives have also shown anti-bacterial activity against *P. aeruginosa* (Tatsimo *et al*., 2012; Plyuta *et al*., 2013). There is a high distribution of *H. tiliaceus* around the mangrove and coastal area of Terengganu. It

has great potential to provide new compounds that could inhibit the formation of biofilm by *P. aeruginosa*.

The objectives of the study were to carry out the phytochemical analyses and to investigate the potency of mangrove-associated *H. tiliaceus* extracts and fractions as anti-bacteria and anti-biofilm agent against *P. aeruginosa*. There has been no report on the antibiofilm activity of leaves, fruits and twigs (Figure 1) from *H. Tiliaceus* extracts and fractions.

Materials and Methods

Sample Collection and Extraction

The collection of leaves, fruits and twig parts of *H. tiliaceus* was carried out at the mangrove sites, near to the School of Marine and Environmental Sciences, Universiti Malaysia Terengganu (UMT) in September 2014. The voucher specimen (H09092016) was deposited in the Institute of Marine Biotechnology, UMT, Malaysia. The extraction of ground samples in powder form was done by cold maceration using methanol at room temperature for 48 hours. The soaked samples were then filtered to collect the soluble extract using Buchner vacuum filter set with whatman filter paper no.2 (100 Ø). The process was repeated until clean filtrate was obtained and the filtrate was then evaporated under a reduced pressure at 40 ^{o}C using a rotary evaporator to yield methanol crude extract. The methanol extracts from the part of fruits (HFMc), leaves (HLMc) and twigs (HTMc) were fractionated using column chromatography to attain chloroform (HFC, HLC, HTC), ethyl acetate (HFE, HLE, HTE), and methanol fractions (HFM, HLM, HTM). All samples were used for anti-bacteria and anti-biofilm assay.

Phytochemical Analyses

Phytochemical analyses of phenolics, tannins, flavonoids, terpenoids, alkaloids, steroids, saponins, protein, carbohydrate and glycoside were adapted from Yadav & Agarwala (2011).

Anti-bacterial and Anti-biofilm Activities

Antibacteria and antibiofilm assays were modified from Musken (2010). Samples were prepared by dissolving 30 mg of each extract or fraction in 100 µL dimethyl sulfoxide (DMSO) and 900 µL Muller Hinton Broth (MHB), mixed and then vortexed at 600 rpm for 5 minutes.

Anti-bacterial Assay

P. aeruginosa was sub-cultured on Muller Hinton Agar (MHA) and incubated for 24 h at 37 ^{O}C. After 24 hours, the bacteria was transferred into MHB by inoculating loop, and incubated for further 24 h at 37 ^{O}C and shaken vigorously, until the growth reached an OD of 0.2 as determined by spectrophotometer at 595 nm. Later, 100 µL of each sample stock and 100 µL of MHB-grown bacteria were added into each well and incubated for 24 h at 37 ^{O}C for antibacteria and antibiofilm assay. The concentration was then determined by the absorbance reading of enzyme-linked immunosorbent assays (ELISA)

Leaves **Twigs** **Fruits**

Figure 1: Leaves, twigs and fruits of *H. tiliaceus*

plate reader at 595 nm for three replicates of each sample.

For bacteria or biofilm control, 200 µL of MHB-grown bacteria was aliqouted into the 96 well-plate without sample addition, while for anti-bacteria or anti-biofilm control, 100 µL of MHB-grown bacteria and 100 µL of DMSO (50% total volume) was aliqouted into the well without sample addition. The anti-bacterial activity of the sample was given as the percentage of inhibition and calculated as follows:

$$Inhibition\ (\%) = \frac{[(OD\ bacteria\ control - OD\ blank) - (OD\ sample - OD\ blank)]}{(\text{OD bacteria control} - \text{OD blank})} \times 100\%$$

Equation 1

Anti-biofilm Assay

The same plate for anti-microbial assay was used for anti-biofilm assay. The culture media were first removed from the plate and dried in an oven for 30 minutes, before 200 µl of 1% crystal violet was added into each samples well and retained for 5 minutes. The crystal violet was then removed from the well and rinsed with distilled water. The well-plate was dried in an oven for 24 h at 60 °C. Ethanol (70% v/v) was used to dissolve the crystal violet in each well prior to reading the concentration of the biofilm with ELISA reader. The anti-biofilm activity of samples was given as the percentage of inhibition as follows:

$$Inhibition\ (\%) = \frac{[(OD\ biofilm\ control - OD\ blank) - (OD\ sample - OD\ blank)]}{(\text{OD biofilm control} - \text{OD blank})} \times 100\%$$

Equation 2

Statistical Analysis

All the experiments were conducted in triplicate and the data are presented as mean values ± standard deviation.

Results and Discussion

Phytochemical Analyses

Phytochemical studies on the methanol extracts of *H. tiliaceus* leaves, fruits and twigs indicated the presence of protein, carbohydrate, phenols, tannin, flavonoids, saponins, glycoside, steroids, terpenoids, and alkaloids chemical constituents (Table 1). Alkaloids and saponins were found only in the twigs and fruit extracts, respectively. The *H. tiliaceus* leaves were reportedly rich in the phenolics and flavonoids which is in agreement with our study (Zang *et al.,* 2011). The presence of tannins in the leave and bark has been reported for the methanol extracts of *H. tiliaceus* from Bangladesh, while the alkaloids and reducing sugar are found in the methanol extract of *H. tiliaceus* barks (Abdul-awal *et al*., 2016). Any differences may be a result of geographical location of the samples which may affect the chemical constituents.

Anti-bacterial and Anti-biofilm Activities

The results on anti-bacterial and anti-biofilm activities of extracts and fractions from leaves, fruits, and twig of *H. tiliaceus* against *P. aeruginosa* are shown in Table 2 and Figure 2. Both studies used 50% DMSO (100 µL) as a positive control since preliminary study (data not shown) had found that DMSO at > 10% concentration could kill most of the bacteria. The strongest anti-bacterial and anti-biofilm activities were exhibited by the chloroform fraction of fruits (HFC). The methanol crude of fruits (HFMc), methanol fraction of fruits (HFM), and chloroform fraction of twig (HTC) showed more than 80% inhibition as compared to the control. The anti-bacterial activity of HFC resulted in 87.2% inhibition, while the

Table 1: Phytochemical analyses of *H. tiliaceus* from the leaves, fruits and twigs part

Chemical constitutents	Phytochemical result		
	Leaves	Fruits	Twigs
Protein	+	+	-
Carbohydrates	+	+	+
Phenolics and tannins	+	+	+
Flavonoids	+	+	+
Saponins	-	+	-
Glycosides	+	+	+
Steroids	+	+	+
Terpenoids	+	+	+
Alkaloids	-	-	+

NOTE: +, chemical constituent present in the sample.

lowest was from HTE at 0.3%. Some fractions from HLC, HLM, HFE, HTM and HTMc also showed weak anti-bacterial activity.

The highest anti-biofilm activity was exhibited by HFC with 94.7% inhibition, while the lowest was by HLM (53.8%). The results can be further divided into three categories:- samples with anti-bacteria and anti-biofilm activities; samples with anti-biofilm but without anti-bacterial activities; and samples with anti-bacterial but without anti-biofilm activities. Samples which showed both activities included HLC, HLM, HFC, HFE, HTE, HTM, and HTMc. Generally, biofilm formation is reduced due to the reduction of viable bacteria. There appears to be a linear correlation between the ability to inhibitthe biofilm formation with the ability to inhibit the bacterial growth. Samples with anti-biofilm but without anti-bacteria such as HTM and HFM probably possess specific anti-biofilm compound which targets the formation of biofilm by either disrupting the extracellular polysaccharides (EPS) matrix, disturbing the quorum-sensing (QS) mechanism or contaminating the nutrient source for biofilm formation without affecting the bacterial growth (Srinandan *et al*., 2012; Sanchez *et al*., 2013; Lee *et al*., 2006).

Several compounds were detected in fruits, leaves and twigs of *H. tiliaceus* (Table 1). Gossipol, reported to have anti-bacterial activity against gram negative and gram positive bacteria, could be one of compounds responsible for anti-bacterial activity of HFC. Gossipol is a non-polar phenolic aldehyde compound that has been isolated from *H. tiliaceus* fruits (Subramanian & Nair, 1973). Other chemical constituents such as the phenolics, flavonoids, triterpenoids, and steroids have been recorded as possessing anti-microbial activity (Thaleb-Contini *et al*., 2003; Riihinen *et al*., 2014) and anti-biofilm activity (Agrawal, 2011; Plyuta *et al*., 2013; Maryam *et al*., 2013; Awolola *et al*., 2014).

The tannins and alkaloids present in the methanol extract of *H. tiliaceus* barks have shown anti-bacterial activity against *Staphylococcus aureus* and *Staphyloccous epidermidis*, but not against other tested bacteria (*Plesiomonas shigelloides*, *Shigella dysenteriae*, *Vibrio cholera*, *Salmonella typhii*, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*, *Staphylococcus saprophyticus*, *Streptococcus pyogenes*). However, contrary to our findings, both the methanol extracts of *H. tiliaceus* leaves and barks containing the tannins do not show any anti-bacterial activity against *P. aeruginosa* (Abdul-awal *et al*., 2016). This difference in biological activity suggests the importance of considering the geographical location and the eco-system of *H. tiliaceus* samples as all these have direct bearings on the phytochemical constituents and the ensuing biological activities.

Table 2: Samples from the fractionation of methanol extract of fruits, leaves and twigs

Sample		Labels	Antibacterial activity (OD)	Antibiofilm activity (OD)
Fruits	Methanol extract	HFMc	1.309±0.03	0.398±0.02
	Chloroform fraction	HFC	0.157±0.03	0.140±0.07
	Ethyl acetate fraction	HFE	1.097±0.07	0.885±0.03
	Methanol fraction	HFM	1.644±0.13	0.526±0.10
Leaves	Methanol extract	HLMc	1.031±0.04	3.771±0.30
	Chloroform fraction	HLC	0.909±0.04	0.807±0.22
	Ethyl acetate fraction	HLE	0.879±0.17	2.971±0.47
	Methanol fraction	HLM	1.218±0.03	1.222±0.05
Twigs	Methanol extract	HTMc	0.873±0.06	0.441±0.16
	Chloroform fraction	HTC	1.241±0.01	0.373±0.05
	Ethyl acetate fraction	HTE	1.226±0.03	0.408±0.03
	Methanol fraction	HTM	1.169±0.02	0.425±0.07
200 µL MHB + Bacteria		Bacteria control	1.230±0.02	-
200 µL MHB + Bacteria		Biofilm control	-	2.650±0.98
100 µLMHB content bacteria + 100 µL DMSO		Antibacteria control	0.081±0.003	-
100 µLMHB content bacteria + 100 µL DMSO		Antibiofilm control	-	0.199±0.03

NOTE: Each value was expressed as the mean ± SD (n=3).

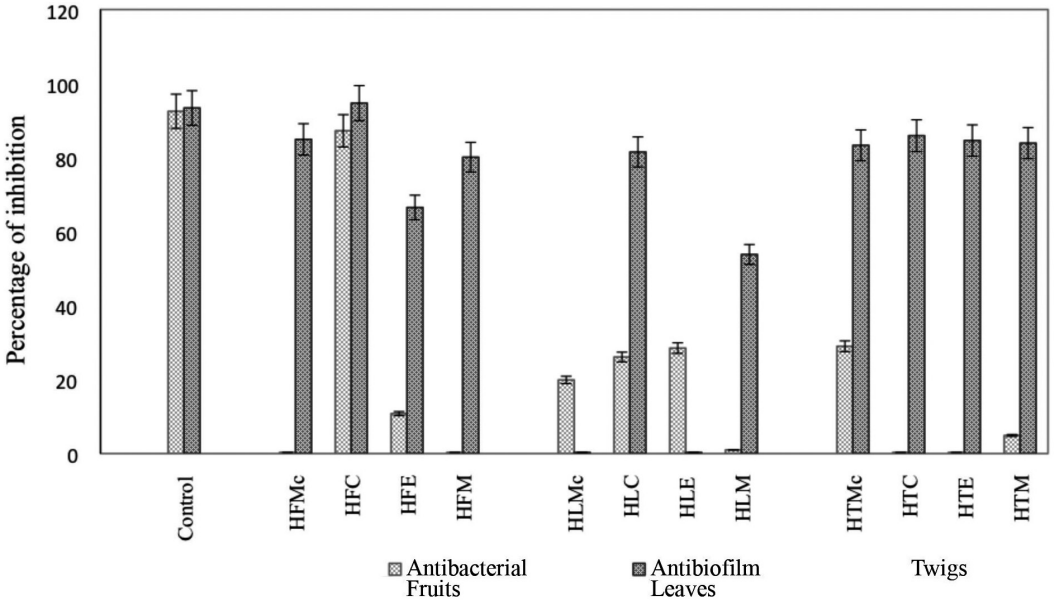

Figure 2: Antibacteria and antibiofilm properties of extracts and fractions from *H.tiliaceus* from leaves, fruits, and twig against *P.aeruginosa.*. The results are interpreted as follows:
< 50% - weak activity; 50-70% - mild activity; >70% - strong activity

Conclusion

The extracts and fractions of leaves, fruits and twig parts of *Hibiscus tiliaceus* had exhibited anti-bacterial and anti-biofilm activities against *P. aeruginosa*. The chemical constituents present in *H. tiliaceus* were found to vary between the plant parts. The flavonoids, phenolics, steroids and terpenoids were the major compounds that may contributetowards these activities. *H. tiliaceus* could therefore be used as an alternative commercial source of anti-bacterial and anti-biofilm agents.

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