# Disentangling a complex genus: systematics, biogeography and bioactivity of the genus Phyllanthus L. and related genera of tribe Phyllantheae (Phyllanthaceae)

Bouman, R.W.

# CHAPTER 6

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Roderick W. Bouman, Hye Kyong Kim,
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**Chapter 6**

# Metabolic variation of selected *Phyllanthus* species and their correlation with antimicrobial activity

Short title: Metabolic variation in *Phyllanthus*

Roderick W. Bouman[1,2,3], Hye Kyong Kim[1,4*], Luis Francisco Salomé-Abarca[1,2], Young Hae Choi[1,4], Paul J.A. Kessler [1,2]

[1] Institute of Biology Leiden, Leiden University, PO Box 9500, 2300 RA Leiden, the Netherlands
[2] Hortus botanicus Leiden, PO BOX 9500, 2300 RA Leiden, the Netherlands
[3] Naturalis Biodiversity Center, P.O. Box 9517, 2300 RA Leiden, the Netherlands
[4] Natural Products Laboratory, Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands

**Abstract**
The profiling of medicinal plants has resulted in the finding of many bioactive compounds with possible application in medicine, thereby also often confirming the basis of their pharmacological effects. The genus *Phyllanthus* has a long history of therapeutic history in Asia, South America and Africa, but the therapeutical species are limited to a few, mostly focusing on their antimicrobial activity. In this study, the metabolic profile of *Phyllanthus* species selected from several lineages was compared with each other to elucidate their active compounds. By correlating antimicrobial activity with the results from our profiling using Proton Nuclear Magnetic Resonance (H-NMR) spectroscopy, the results suggested that active compounds were most likely plant phenolics. The result of antimicrobial activity in liquid suspension indicated that *P. arbuscula, P. muellerianus, P. tenellus and P. urinaria* have significant activity on several gram-negative bacterial. Furthermore, *Phyllanthus fraternus* and *P. glaucus* also showed activity in initial agar-based assay, but this could not be confirmed in the liquid suspension. To identify the phenolics in detail, subsequent investigation using a targeted approach with high performance thin layer chromatography (HPTLC) showed that the active species differed in the profile of plant phenolics.

**Keywords:** antimicrobial, Phenolics, Phyllanthaceae, *Phyllanthus*, high performance thin layer chromatography

## Introduction

Natural products have been for long time the most plentiful resource for bioactive or nutritive chemicals utilized in pharmaceutics, cosmetics, foods and agriculture

(Koehn & Garter 2005; Harvey 2008; Cragg & Newman 2013; Newman & Cragg 2016). While recently more diverse natural products are starting to be used as a resource for novel medicines, including microbes, insects and marine organisms (Cragg & Newman 2013), plants still represent the most sustainable options, particularly with a long history of their utilization. However, the use of natural products does present some difficulties, which include issues with sustainable access and supply, discussions on intellectual property rights, the difficulty of extracting a single active compound from the extracted mixtures and finally the slowness in getting new agents to the market (Koehn & Garter 2005; Harvey 2008; Wu & Chappell 2008; Yuliana et al. 2011). Botanical gardens harbour a large diversity of rare plants and are a valuable source for the plant sciences (Shan-An & Zhong-Ming 1991; Faraji & Karimi 2020). Based on the results of recently developed metabolomics, many influential factors on the metabolome could be deduced including the genotype, developmental stage and environmental or external factors (Jahangir et al. 2008; Kim et al. 2011). Not only chemical profiling but also, the correlation between metabolome and bioactivity against bacteria, fungi and viruses has received great attention in the field of metabolomics applications. Previously, there have been many similar approaches, a Mexican medicinal plant, *Gaphimia glauca* Cav., was investigated for its sedative triterpenoids (Cardoso-Taketa et al., 2008). In the study, a wide range of the accessions of the plants were measured for their sedative activity and correlated with the metabolome detected by $^{1}$H NMR. In addition, some methoxy flavonoids of *Orthosiphon stamineus* Benth. were found to be responsible for Adenosine A1 receptor binding activity by similar $^{1}$H NMR-based metabolomics (Yuliana et al., 2011).



*Phyllanthus* L. is a large genus with more than 800 species that occurs in all tropics and subtropics (Govaerts et al. 2000; Bouman et al. 2018). Several species have a long history in traditional medicine and are used for several purposes (see Unander et al. 1990; Calixto et al. 1999). Some species are well known, such as *P. emblica*, whose fruits are usually used for their high nutritional content like vitamin C (Masuma et al. 2014; Hasan et al. 2016; Yadav et al. 2017; Lanka 2018). However, most of them are common herbs that are often invasive and therefore have a wide distribution, which might pose interesting questions on differences in activity per locality (see Cardoso-Taketa et al. 2008). Not only are the species used in medicine or as healthy foods, but some species are also known to be used as fish poison in South America (Patiño 1967; van Andel 2000; Neuwinger 2004; Webster 2003).

The interest in *Phyllanthus* has sparked a great number of studies, which has resulted in more than 500 reported compounds that have been extracted from *Phyllanthus* (Mao et al. 2016). *Phyllanthus* species have been found to be very rich in alkoloids, flavonoids, lignans, tannins and triterponoids (Calixto et al. 1999; Mao et al. 2016). Several studies have focused on testing the medicinal effects of *Phyllanthus* and most studies focus on anti-viral effects (Thyagarajan et al. 1988; Barrio & Parra 2000; Liu et al. 2001; Alvarez et al. 2009). The medicinal effects of

many *Phyllanthus* species have also been demonstrated in some studies that tested against bacteria (Bagalkotkar et al. 2006, Jagessar et al. 2008, Mehta et al. 2014) and fungi (Agrawal et al. 2004). The most interesting application is the activity against hepatitis B using *P. amarus* (Yeh et al. 1993; Thyagarajan et al. 1998; Patel et al. 2011) and *P. niruri* L. (Venkateswaran *et al.* 1987; Thyagarajan *et al.* 1988; Yeh *et al.* 1993; Wang et al. 1995; Lee et *al.* 1996; Wu *et al.* 2015; Kamruzzaman & Hoq 2016).

Closely related species of plants may generally have a similar chemical profiling that might results in similar pharmacological activity with some degree of difference (e.g. Beara et al. 2012). However, in the case of *Phyllanthus* species, compared with other species, the applications of individual species greatly differ from each other, which could propel a detailed study of chemical profiling to cover a broad range of metabolites and the metabolome.

In the past, with the advancement of metabolomics tools, many analytical platforms have been applied to the metabolomics of plants for many purposes e.g., deconvolution of physiological phenomena, chemical taxonomy, and agricultural applications (Choi et al. 2004; Kim et al. 2010a; Kim et al. 2010b). Moreover, a metabolomics tool has been applied in the chemical comparison of a few *Phyllanthus* species (e.g. Mediani et al. 2005; Wang et al. 2011), but has not yet been applied to a larger number of species. To study the metabolome of these species, $^{1}$H NMR was chosen to get information on more groups of metabolites. However, for longer term projects, a fingerprinting approach is required together with the identification of minor compounds.

Previous studies indicated that several species of Phyllanthus might be interesting for their antimicrobial activity. In this study we aim to compare the metabolic profile and potential anti-bacterial and anti-fungal activity of various *Phyllanthus* spp. from different lineages or geographical areas. Using a diverse selection of species ranging from herbs to shrubs and small trees, some of which with known traditional medical applications we hope to further explore the basis of their medicinal effects and to test these species for bioactive compounds.

## Methods

### Plant material

Fresh material of all plants used in this study were collected from the Hortus botanicus Leiden (Leiden, The Netherlands). The plants originated from different geographic areas, but were all grown at the botanical garden under similar growing conditions. Eleven species of *Phyllanthus* that cover a high degree of the variety within the genus were selected, including a few often used in traditional medicine. The age of the selected plants varied depending on the type of lifestyle such as annual herbs, shrubs and one aquatic species. Most of species were collected with three replicates to remove biological variation except for the large species of shrubs (e.g., *P. arbuscula*, *P. juglandifolius*, *P. glaucus*). Samples were taken several times during the span of a year. In total 27 samples from 11 species were selected for the

study (table 6-1).

**Preparation of plant samples for metabolomics analysis.**
Collected materials were stored in the freezer at -80°C before processing. Materials were ground to a fine powder with mortar and pestle under liquid nitrogen and then transferred to a 50 ml tube and subsequently placed in a freeze drier for 72 hours.

**$^{1}$H NMR experiments**
For $^{1}$H NMR screening, 20 mg of freeze-dried plant material was extracted with 1 mL of a mixture of $CH_3OH$-$d_4$: $KH_2PO_4$ buffer in $D_2O$ (1:1, v/v, pH 6.0) containing 0.29 mM trimethylsilane propionic acid sodium salt (TMSP). The extracts were vortexed and subsequently sonicated for 20 minutes and then centrifuged at 13,000 rpm for 10 minutes. A volume of 300 µl of the supernatant of each extract was transferred to 3mm-NMR tubes. $^{1}$ H NMR spectra were recorded at 25 $^{o}$C on a 600 MHz Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany) equipped with cryo-probe operating at a proton NMR frequency of 600 MHz. The methyl signal of $CH_3OH$-$d_4$ was used as the internal lock. Each $^{1}$H NMR spectrum consisted of 64 scans requiring 5 min acquisition time with the following parameters: 0.25 Hz/ point, pulse width (PW) = 30◦ (10.8 s), and relaxation delay (RD) = 1.5 s. A pre-saturation sequence was used to suppress the residual $H_2O$ signal with low power selective irradiation at the $H_2$ O frequency during the recycle delay. Free induction decays (FIDs) were Fourier transformed with Line Broadening (LB) = 0.3 Hz and the spectra were zero-filled to 32 K points. The resulting spectra were manually phased and baseline corrected and calibrated to Theory of Spectroscopy and Molecular Properties (TMSP) at 0.0 ppm, using TOPSPIN (version 3.0, Bruker).

**High performance thin layer chromatography (HPTLC) and Liquid chromatography–mass spectrometry (LC-MS)**
The metabolites of *Phyllanthus* species were further analysed by high performance thin layer chromatography (HPTLC). The results were compared between active and nonactive samples and used in further antimicrobial activity testing against *Staphylococcus aureus* Rosenbach. HPTLC chromatographic separation was performed on 20 x 10 cm HPTLC silica gel $F_{254}$ plates (Merck, Darmstadt, Germany) and samples were applied using an automatic Thin Layer Chromatography (TLC) sampler (CAMAG, Muttenz, Switzerland). For chemical profiling, we applied 15 µl while 35 µl was applied for the bioautography tests from 2 mg/ml methanol extracts. The band length was 6 mm for each sample, and they were spaced 10 mm from the bottom of the plate and 20 mm from the left and right border of the plate. The distance between tracks was 18 mm allowing for 9 samples to be spotted on each plate. To separate non-polar compounds, the mobile phase consisted of toluene-ethyl acetate (8:2, v/v). For polar compounds separation, a mixture of ethyl acetate-formic

**Table 6-1.** List of samples of each species included in this study, for each species the subgenus, habit and life stage is noted along with the Registration number of the Hortus botanicus Leiden.

| Sample nr | Species | Subgenus | Habit | life stage | Hortus number |
|---|---|---|---|---|---|
| ARB01 | *P. arbuscula* (Sw.) J.F.Gmel | *Xylophylla* | Shrub | Non-flowering | HBLA00587-02610 |
| ARB02 | *P. arbuscula* (Sw.) J.F.Gmel | *Xylophylla* | Shrub | Non-flowering | HBLA00587-02610 |
| FLU01 | *P. fluitans* Benth. Ex Müll.Arg. | *Isocladus* | Aquatic herb | Non-flowering | HBL20150681 |
| FLU02 | *P. fluitans* Benth. Ex Müll.Arg. | *Isocladus* | Aquatic herb | Non-flowering | HBL20150681 |
| FLU03 | *P. fluitans* Benth. Ex Müll.Arg. | *Isocladus* | Aquatic herb | Non-flowering | HBL20150681 |
| FRA01 | *P. fraternus* G.L. Webster | *Swartziani* | Herb | Flowering | HBL20160134 |
| FRA02 | *P. fraternus* G.L. Webster | *Swartziani* | Herb | Flowering | HBL20160134 |
| FRA03 | *P. fraternus* G.L. Webster | *Swartziani* | Herb | Flowering | HBL20160134 |
| GLA01 | *P. glaucus* Jabl. | *Kirganelia* | Shrub | Non-flowering | HBL20160136 |
| JUG01 | *P. juglandifolius* Willd. | *Xylophylla* | Shrub | Non-flowering | HBL20170041 |
| MIR01 | *P. mirabilis* Müll.Arg. | *Phyllanthodendron* | Shrub | Non-flowering | HBL20090748 |

| | | | | | |
|---|---|---|---|---|---|
| MIR02 | *P. mirabilis* Müll.Arg. | *Phyllanthodendron* | Shrub | Non-flowering | HBL20090749 |
| MUE01 | *P. muellerianus* (Kuntze) Exell | *Kirganelia* | Herb | Non-flowering | HBL20160132 |
| MUE02 | *P. muellerianus* (Kuntze) Exell | *Kirganelia* | Herb | Non-flowering | HBL20160132 |
| MUE03 | *P. muellerianus* (Kuntze) Exell | *Kirganelia* | Herb | Non-flowering | HBL20160132 |
| PEN01 | *P. pentandrus* Schumach. & Thonn | *Tenellanthus* | Herb | Budding flowers | HBL20160133 |
| PEN02 | *P. pentandrus* Schumach. & Thonn | *Tenellanthus* | Herb | Budding flowers | HBL20160133 |
| POL01 | *P. polyspermus* Schumach. | *Kirganelia* | Herb | Non-flowering | HBL20160135 |
| POL02 | *P. polyspermus* Schumach. | *Kirganelia* | Herb | Non-flowering | HBL20160135 |
| POL03 | *P. polyspermus* Schumach. | *Kirganelia* | Herb | Non-flowering | HBL20160135 |
| TEN01 | *P. tenellus* Roxb. | *Tenellanthus* | Herb | Flowering | HBL20140316 |
| TEN02 | *P. tenellus* Roxb. | *Tenellanthus* | Herb | Flowering | HBL20140316 |
| TEN03 | *P. tenellus* Roxb. | *Tenellanthus* | Herb | Flowering | HBL20140316 |
| URI01 | *P. urinaria* Beille | *Emblica* | Herb | Flowering | HBL20140356 |
| URI02 | *P. urinaria* Beille | *Emblica* | Herb | Fruiting | HBL20140356 |
| URI03 | *P. urinaria* Beille | *Emblica* | Herb | Fruiting | HBL20140356 |

acid-acetic acid-water (100:11:11:27, v/v/v/v) was used. A saturation time of 20 min was set for all chromatographic separations and the solvent migration distance spanned 85 mm from the application point. The plate images were recorded using a TLC visualizer (CAMAG) under 366 nm UV light.

The samples were analysed using liquid chromatography mass spectrometry (LC-MS) using a UHPLC-DAD-QTOF, Thermo Scientific (Dreieich, Germany) UltiMate 3000 system coupled to a Bruker (Bremen, Germany) OTOF-Q II spectrometer with electrospray ionization (ESI). The mass spectrometer parameters were set as follows: nebulizer gas 2.0 bar, drying gas 10.0 mL/min, temperature 250°C, capillary voltage 3500 V. The mass spectrometer was operated in positive mode with a scan range of 100 – 1650 m/z, and sodium formate was used as a calibrant.

**Bioassays**

We screened for general antifungal activity for each plant species and this a was conducted by paper diffusion assay against *Fusarium oxysporum*. To produce spores, two weeks old plates were filled with 25 mL of sterile physiological solution (PS), then the media plates was rubbed with a sterile cotton swab to transfer spores to the PS. After that, the liquid containing the fungal structures was recovered with a sterile pipet and filtered through two layers of sterile miracloth and transferred into a sterile 50 mL-centrifuge tube. The volume was adjusted to 30 mL with sterile water, vortexed for 15 seconds and centrifuged at 4000 rpm for 10 minutes. This process was repeated three times. The supernatant was discarded, and the pellet was re-suspended in 30 mL of sterile PS. After, spore concentration per milliliter was quantified in a cell counter apparatus (Bio Rad). Potato dextrose agar (PDA) was sterilised and cooldown to 60 °C. Then 49 mL of PDA were inoculated with 1 mL of the spore solution and softly homogenized. The final spore concentration in the medium was 2.5 x $10^5$ spores/ml. The treatments were sterile 6 mm paper discs loaded with 500 µg of methanol extract. In order to eliminate the methanol from the discs, after loading they were dried at room temperature for 5 min. The negative control consisted of a sterile disc paper with and without methanol processed in the same way of the plant methanol extracts. The plates were incubated at 28 °C for 39 hours and the diameter of inhibition zones were recorded.

Each sample showed mild activity against *Fusarium oxysporum*, but effectivity was low in general, so we opted to do further testing for antibacterial effects instead. For the first test, one hundred mg of dried material was extracted with 10 ml of methanol using ultrasonicator for 30 minutes at room temperature. 10 ml of *n*-hexane was added to remove chlorophyll which may hinder the biological activity. After hexane fraction was discarded, remaining extracts were further evaporated using rotary evaporator. Extracts were transferred to 1.5mL-microtube tubes after re-dissolving them in 1 mL MeOH, and dried completely by Speed-vac.

In the first test, the antimicrobial activity was tested using the disc-

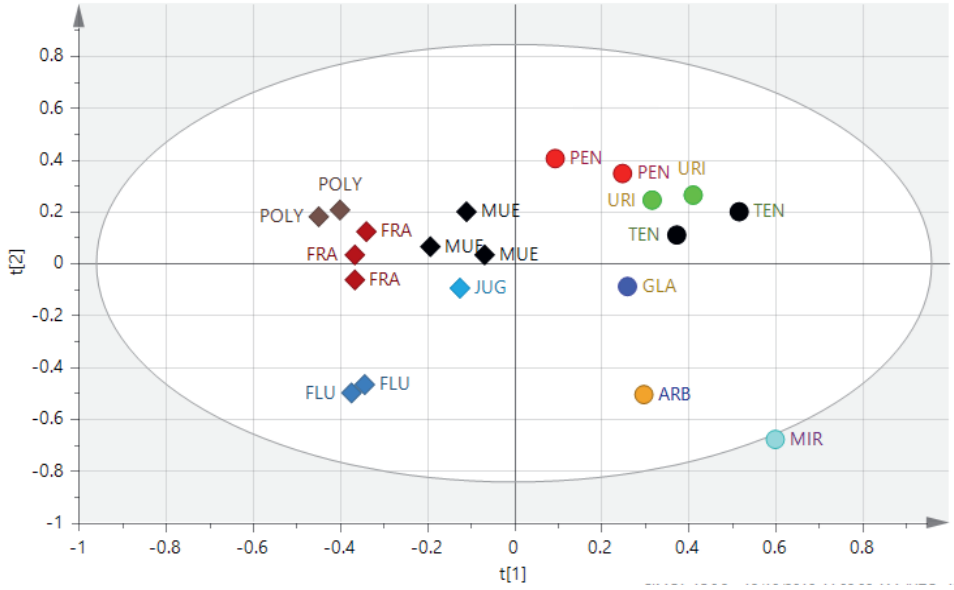


**Figure 6-1.** PCA score plot of NMR data, Sample names are shorted from table 1.

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diffusion methods against *Bacillus subtilis* (gram positive) and *Escherichia coli* (gram negative). Unfortunately, only 20 of the extracts had sufficient material after $^{1}$H NMR screening for the bioassay test. The test was performed as described in previous studies (Abreu et al. 2014). All extracts were prepared in MeOH. Each extract was added to previously autoclaved and cooled LB agar in the amount calculated to obtain the required final concentration of 500 ug. Then, 20 mL of medium was poured into 90 mm Petri dishes. The bacterial suspensions were adjusted to 0.5 McFarland standard and seeded over hardened LB agar Petri dishes using a sterilized cotton swab. Sterile blank discs (6 mm diameter; Oxoid) were placed on the agar plate seeded with the respective bacteria. The plates were incubated at 37 °C for 24 h. After incubation, each inhibition zone diameter (IZD) was recorded and analyzed according to CLSI guidelines (reference above). The results were correlated with the NMR spectra as described below to determine the class of compounds we should focus on.

In the second test, we tested one sample of each species in concentrations of 125, 250 and 500 µg/ml against *B. cereus* strain NCCB 75009, *B. subtilis* strain *168* and *S. aureus* strain ATCC29213 to corroborate the bioactivity of each species of *Phyllanthus*. This was done using concentrations of 125, 250, 500 or >500 µg/ml of dried extracts in 100% MeOH (MIC testing). Dissolved extracts from each concentration was incubated in a liquid medium containing the tested bacteria. To determine the specific compounds underlying the bioactivity of the species

| Nr | Sample name | Bacteria Gram + | Bacteria Gram - | Fungal |
|---|---|---|---|---|
| 1 | ARB01 | | | |
| 2 | URI01 | + | | |
| 3 | JUG01 | | | + |
| 4 | FRA03 | ++ | ++ | + |
| 5 | FLU03 | | | |
| 6 | MUE02 | + | | |
| 7 | TEN03 | ++ | | + |
| 8 | URI02 | + | | + |
| 9 | ARB02 | | | + |
| 10 | MIR02 | | | |
| 11 | MUE03 | + | | |
| 12 | GLA01 | ++ | + | + |
| 13 | FRA01 | | | + |
| 14 | POLY01 | | | |
| 15 | URI03 | + | ++ | |
| 16 | FRA02 | | | + |
| 17 | TEN02 | + | | |
| 18 | FLU02 | | | + |
| 19 | MUE01 | ++ | ++ | |
| 20 | POLY02 | | | |

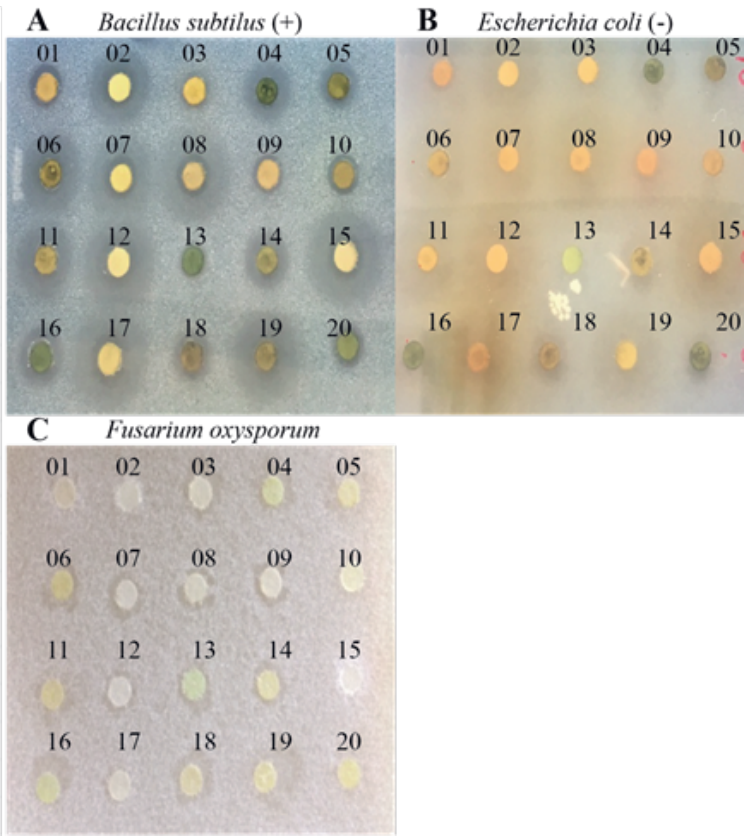


**Figure 6-2.** Bioactivity testing of various *Phyllanthus* spp. Extracts listed on the left by sample names with results from bioactivity screening against a gram positive bacteria, gram negative and a fungus strain, activity is only shown when mild (+) or strong (++) activity was recorded. On the right is shown the results from the disc diffusion method for A) against the gram positive bacteria *Bacillus subtilis* (+), B) against *Escherichia coli* and C) against *Fusarium oxysporum*.

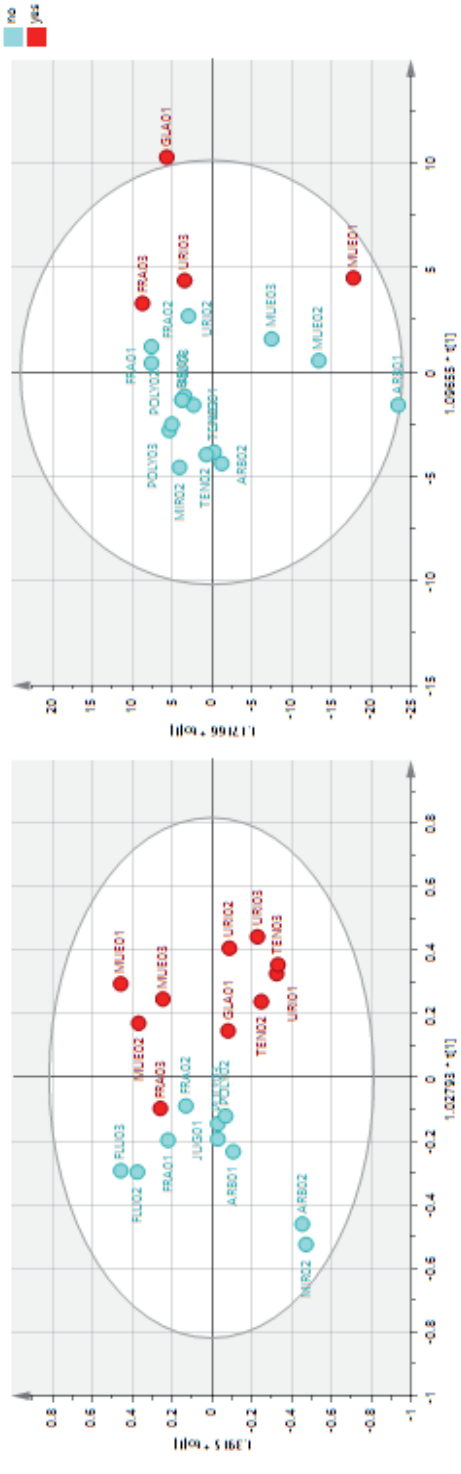


**Figure 6-3.** OPLS-DA plot of activity test against *Bacillus subtilis* (left, Q2 is 0.526, CV-ANOVA: P=0.0183117) and *Escherichia coli* (right, Q2: -0.308, CV-ANOVA: P=1).

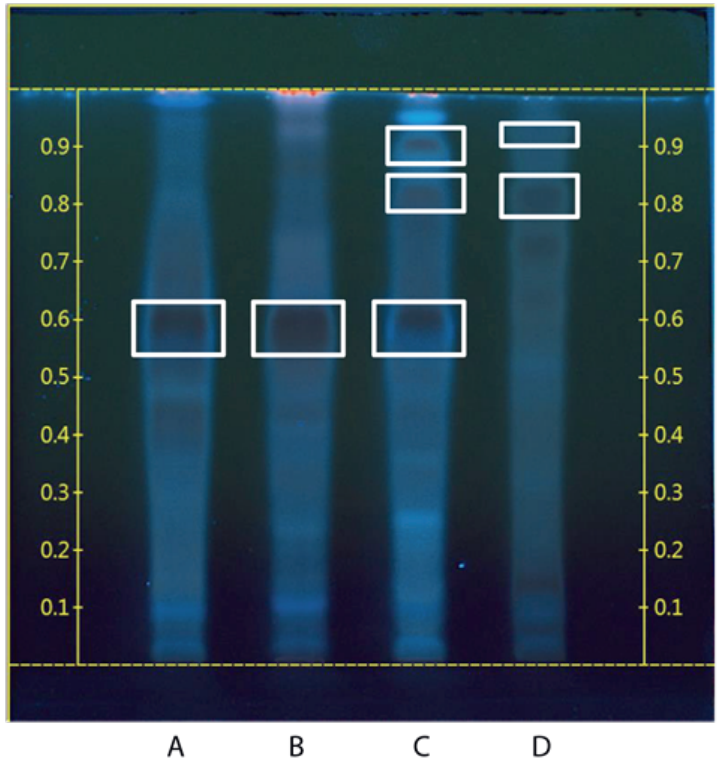


**Figure 6-4.** Bioautography with the HPTLC spectra of selected species of *Phyllanthus* screened for activity against *S. aureus*. From left to right, the species is *P. urinaria* (A), *P. muellerianus* (B), *P. tenellus* (C) and *P. arbuscula* (D), active bands extracted for LC-MS in the spectra are highlighted with white boxes.

resulting from test two, we applied in the third step direct testing with a 0.5 McFarland solution of *S. aureus* that was sprayed on a HPTLC plate following preparations described below. The plates were incubated for 24 hours and checked afterwards for activity under UV light.

**Data processing and multivariate data analysis for metabolome and activity correlation**

Spectral data was bucketed using the AMIX program ((Bruker Biospin Corp., Billerica MA), bucket size was 0.04 ppm and the areas were normalised to total intensity. Intervals for methanol and water signals were deleted from the bucket data. Multivariate data analysis was performed using SIMCA-P V.14.1 (Umetrics, Umeå, Sweden). Differentiation of the various *Phyllanthus* species was shown using Principal Components Analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA) and of the $^{1}$H NMR data. The samples were divided in two classes for Y-variables in OPLS modeling. PCA and OPLS-DA analysis data was scaled using

the Pareto and unit-variance (UV) scaling method.

**Results**

**Principal component analysis (PCA)**

Principal component analysis (PCA) showed a good separation for each species (Fig. 6-1), but the closeness between species did not follow the previous morphological and genetic data. The top-right corner does show three of the four herbaceous species, but *P. fraternus* places more closely to some shrubs and small trees from Africa and South America. No clear pattern related to evolution, morphological adaptation or habitat in the greenhouses are evident from the PCA plot.

**Biological activity testing**

Initial activity testing for anti-fungal properties against *F. oxysporum* showed low activity for most species, but none of the samples showed a particular strong reaction against the fungi. Strongest activity was seen in all tested samples of *P. fraternus*, but testing of other species like *P. urinaria* only showed mild activity in one of the samples. As our anti-fungal testing were largely inconclusive with only mild activity in some species, we opted to do further screening for antibacterial testing instead.

Activity testing against the gram-negative bacteria *E. coli* and the gram-positive bacteria *Bacillis subtilis* (Fig. 6-2a and b) showed more active species against the latter (Fig. 6-2a and b). During testing against pathogenic fungi, *P. fraternus* showed a mild activity against *Fusarium*. Some other species showed some activity against *Fusarium*, but this was not always consistent for each extract of a specific species. As the plate did not show particularly strong activity as it did not show a significant correlation in the OPLS-DA (not shown here), we continued with the anti-bacterial bioactivity in the following steps. Extracts were divided into two classes, active and non-active, to discriminate metabolites, that were more involved in the antibacterial or antifungal activity. Correlating antimicrobial activity with the $^{1}$H NMR data using OPLS-DA was not significant against *B. subtilis* strain *168*, but not validated (P=0.0183117; Q2 = 0.526). The significant p-value indicates some biological effect, but to improve the Q2 value, we would need to increase the sample size. Figure 6-3 shows a good separation of active (red) and non-active (blue) species. In the case of *E. coli*, the extracts of *P. urinaria* and *P. muellerianus* showed some inhibition, but the separation between active and non-active was not validated by OPLS-DA (Fig. 6-3 right). Subsequent testing against more gram-positive bacteria showed the strongest activity against *S. aureus* and this was selected for further testing using HPTLC to isolate the compounds that could possibly have caused the anti-microbial effects.

**Targeted approach (HPTLC/LC-MS) for compound identification**
Samples showed a good separation of bands on the HPTLC profile which were largely similar, but only the extracts run on a polar phase showed some reaction to *S. aureus*. Activity was tested with both TLC plates using the direct spray method. The non-polar compounds did not show any activity, but some polar compounds showed antimicrobial effects (Fig. 6-4). Due to unavailability of more material from test 1, only a limited number of species could be tested with the HPTLC and four are shown in figure 6-4. We targeted these for identification using HPTLC coupled with LC-MS, but only one compound could be identified. The highest peak in the LC chromatogram profile of *P. tenellus* were cross referenced with Buckingham (1993) and indicated flavan-like compounds, but we could not identify other possible fragments due to the low concentration of the extracts.

**Discussion**
The medicinal value of *Phyllanthus* has become a field of high interest (Kuttan & Harikumar 2011; Mao et al. 2016). Many species were already known to have some medicinal effect from traditional medicines (Unander et al. 1990, 1991, 1992, 1995). In this study, several methods were combined to elucidate the potential antimicrobial effect of a few species commonly found in botanical gardens or as invasives. These were also aimed at sampling a larger morphological variety within *Phyllanthus* instead of focusing on any specific taxon. Proton NMR is a strong tool to study the metabolome and it indicated that phenolics were an important class of compound when it was correlated with activity (Fig. 6-2). Our correlation of activity and the NMR data was used as a tool to predict the active compounds as it has been used before (Eriksson et al. 2006). Accessional differences as shown by figure 6-2 were present within our samples, as antimicrobial activity was not always at the same level between extracts. This was also indicated in the initial general antifungal screening, but the effects were quite low. Our study did not manage to identify all compounds attributed to the antimicrobial effects detected in some species. Only small indications were found for flavan in *P. tenellus*, which has been found before in this species (Buckingham 1993). Several issues could underlie our inability to identify the remaining compounds. This includes a strong adherence to the plate silica that prevented them from separating well in the TLC, or it is a matter of concentration of the extracts. The strength of activity against *S. aureus* (Fig. 6-4) was also different from the initial tests with *E. coli* (Figure 6-2), possibly due to differences in testing method, solvent, or the age of the extracts. The results presented here are quite fragmented as material was not available for all experiments, so future studies would need to gather more fresh material for a more thorough screening of *Phyllanthus*.

While our approach was unable to identify the antimicrobial compounds in this study, similar research has generated a wealth of knowledge on the bioactivity of other species. Ghafar et al. (2020) similarly used H-NMR coupled liquid

chromatography in *P. acidus* to identify almost 80 compounds, some of which were involved with antioxidant, anti-diabetic and anti-inflammatory reactions (see also Muthusamy et al. 2017). Of the species from this study, especially the three annual herbs *P. urinaria*, *P. fraternus* and *P. tenellus* are good candidates for their medicinal value due to their rapid growth. Over 90 naturally occurring compounds have been reported for *Phyllanthus urinaria* (Fang et al. 2008; Geethangili & Ding 2018), many of which were shown to have an antioxidant effect.

While the genus *Phyllanthus* with almost 900 species (Bouman et al. 2018) has a long history in traditional medicine, the majority of studies have focused on a few select species, especially more common herbaceous species like *P. urinaria* (Geethangili & Ding 2018), *P. niruri* (Kamruzzaman & Hoq 2016) and *P. amarus* (Patel et al. 2011). In this study we attempted to include a broader variety of species of various habits, countries of origins and across the phylogeny of the genus. Unfortunately, due to sampling issues and inconsistent results of bioactivity screening, we were unable to elucidate any compounds with antimicrobial activity. Some activity was found in a few species and future studies might improve upon our work to find those compounds of interest for their medicinal value.