In vitro antibacterial, antioxidant, total phenolic contents and anti-HIV-1 reverse transcriptase activities of extracts of seven Phyllanthus sp

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Abstract

Phyllanthus species has long been used in folk medicine in many countries as antimicrobials and/or antioxidants. Eighty percent methanol extracts obtained from seven Phyllanthus sp. were evaluated for antibacterial activity using the broth micro-dilution assay, anti-HIV-1 reverse transcriptase (RT) activity using the HIV-RT assay, antiradical scavenging effects and phenolic contents using the DPPH assay and Folin–Ciocalteau colorimetric method, respectively. Best antibacterial activity as indicated by the minimum inhibitory concentration (MIC) values was obtained by Phyllanthus amarus against Staphylococcus aureus (Gram-positive) with a MIC value of 17.7 μg/ml. Phyllanthus myrtifolius and Phyllanthus urinaria inhibited growth of Pseudomonas stutzeri (Gram-negative) with MIC values of 78 μg/ml and 117 μg/ml, respectively. A strong inhibition of HIV-RT was obtained by Phyllanthus pulcher (IC\textsubscript{50} 5.9 μg/ml) followed by P. urinaria and P. myrtifolius (IC\textsubscript{50} of 10.4 and 12.7 μg/ml, respectively). A remarkable DPPH scavenging effect was observed with P. myrtifolius, Phyllanthus reticulatus and P. urinaria (IC\textsubscript{50} of 10.2, 10.8 and 17.4 μg/ml, respectively). Highest total phenolic contents were recorded for P. myrtifolius and P. urinaria (207 and 205 mg/GAE/g respectively). With the exception of P. amarus, Phyllanthus debilis and P. pulcher, total phenolic contents correlated with DPPH radical scavenging activity. Our findings support the uses of the Phyllanthus species in traditional medicine. The interesting biological activities obtained by P. myrtifolius, P. urinaria and P. pulcher need to be further investigated to isolate active agents and to study their mechanism of action.

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1. Introduction

Plants have provided mankind with useful drugs for centuries. Despite the availability of different approaches for the discovery of therapeutics, natural products still remain as one of the best reservoirs of traditional and/or orthodox medicine (Cappasso et al., 2003; Hostettmann et al., 1995). Infectious diseases are the leading cause of death worldwide. The emergence of multidrug resistant pathogens threatened the clinical efficacy of many existing antibiotics. This situation has been recognized globally as a serious concern and justifies further research to discover antimicrobial agents from natural origins including plant extracts (Alviano et al., 2008; Eldeen et al., 2005; Nguyen and Graber, 2009).

Human immunodeficiency virus (HIV-1) is the causal agent of AIDS. HIV-1 (RT) catalyzes the synthesis of double-stranded DNA from the genomic RNA (Andréola, 2009). Various combinations of anti-HIV-RT drugs known as highly active anti-retroviral therapy (HAART) are proved to be effective in suppressing viral replication. However, new sources of anti-HIV-1 agents with possible unique mechanism of action are urgently needed due to a rising concern of issues such as drug resistance, viral reservoirs, high dosages and costs (Huang et al., 2005; Park et al., 2009).

Oxidative stress induced by oxygen radicals is believed to be a primary factor in various degenerative diseases such as cancer and gastric ulcers. Scientific evidence suggests that plant-derived materials possess biological activities that may protect tissues against oxidative stress (Aherne et al., 2007). Many of these agents have been identified as free radicals or active oxygen scavengers (Kumaran and Karunakaran, 2007).
The genus *Phyllanthus* (Euphorbiaceae) are widely distributed throughout most of tropical and subtropical countries (Edeoga et al., 2006). Pharmacological properties of *Phyllanthus* species has recently become a focal point of several studies due to their broad therapeutic use in folk medicine and their wide distribution as well as their diverse secondary metabolite entities (Kalidas and Mohan, 2009). A decoction of *Phyllanthus amarus* is used for kidney and liver troubles in Asia from Hainan to Indonesia and also used for venereal diseases, chest pain and smallpox in Nigeria and for diabetes in Tanzania (Schmelzer and Gurib-Fakim, 2008). Different parts of *Phyllanthus acidus*, *Phyllanthus debilis*, *Phyllanthus pulcher*, *Phyllanthus reticulatus*, *Phyllanthus urinaria* and *Phyllanthus myrtifolius* are reported uses traditionally in Malaysia, Southern Africa, India and South America for cough, jaundice, gonorrhoea, dysentery, diabetes, skin ulcers, headache, stomach-ache, eye wash, sore throat, dysentery and dressing of wounds (Calixto et al., 1998; Hutchings et al., 1996; Schmelzer and Gurib-Fakim, 2008). Some of these ailments are related to microbial causing diseases and/or associated with oxidative stresses (NIAID, 2006).

Methanolic and aqueous extracts obtained from *Phyllanthus muellerianus* and *Phyllanthus discoides* were previously reported potent antibacterial activity (Mensah et al., 1990; Doughari and Sunday, 2008). Inhibition of HIV-1 RT and antioxidant properties of some *Phyllanthus* species was also previously highlighted (Garg et al., 2008; Unander et al., 1995).

This work aimed to investigate antibacterial, anti-HIV-1 RT and antioxidant activity of seven *Phyllanthus* species grown in Malaysia. These species included: *P. amarus*, *P. acidus*, *P. debilis*, *P. myrtifolius*, *P. reticulatus*, *P. pulcher* and *P. urinaria*. The report also highlighted total phenolic contents of the investigated plants.

2. Material and methods

2.1. Plant materials

Plant materials including whole plant of *P. urinaria*, *P. debilis* and *P. amarus*, and leaf of *P. pulcher*, *P. acidus*, *P. reticulatus* and *P. myrtifolius* were collected from the Herbal Garden, School of Biological Sciences, University Sains Malaysia in July 2009. Voucher specimens were deposited in the Herbarium. The collected materials were cleaned, dried in an oven at 60 °C, powdered and extracted using 80% methanol (20% water). The extracts were filtered using Whatman No. 1 filter paper. A rotary evaporator was used for removal of the solvent. Residues obtained were dried at room temperature prior to further tests.

2.2. Antibacterial test

The serial dilution technique described by Eloff (1998), using 96-well microplates to determine the minimum inhibitory concentration (MIC) of antibacterial activity was used. Two ml cultures of three Gram-positive bacteria: *Bacillus licheniformis* (ATCC12759), *Bacillus spizizenii* (ATCC6633) and *Staphylococcus aureus* (ATCC12600) and three Gram-negative: *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (ATCC13883) and *Pseudomonas stutzeri* (ATCC17588) were prepared and placed in an incubator overnight at 37 °C. The overnight-cultures were diluted with sterile MH broth (1 ml bacteria/50 ml MH) to yield density of bacterial cells between 10⁶ and 10⁸ cell/ml. The extracts were re-suspended to a concentration of 10 mg/ml with ethanol to yield a final concentration of 2.5 mg/ml in the assay. For each of the six bacteria used, 100 μl of redissolved extract were two-fold serially diluted with 100 μl sterile distilled water in a sterile 96-well micro-plate. A similar two-fold serial dilution of tetracycline (1 mg/ml) was used as a positive control against each bacterium. One hundred μl of each bacterial culture was added to each well. The plates were covered and incubated overnight at 37 °C. To indicate bacterial growth, 50 μl of 0.2 mg/ml p-iodonitrotetrazolium violet (INT) was added to each well and the plates incubated at 37 °C for 30 min. Bacterial growth in the wells was indicated by a red color, whereas clear wells indicated inhibition by the tested substances. This assay was repeated three times.

2.3. Antioxidant activity: DPPH free radical scavenging assay

The potential antioxidant activity of the studied plant materials were assessed on the basis of the free radical scavenging activity of the *Phyllanthus* extracts against the stable 2, 2-diphenyl-1-picrylhydrazil (DPPH) free radical. The assay was performed according to the method described by Brand-Williams et al. (1995) with slight modification. A total 200 μl of a reaction mixture was prepared in 96 microwell plates. Firstly, the reaction mixture consisted of 50 μl of extracts and positive control (quercetin) (after dilution to a final concentration of 250 μg/ml for both extract and quercetin) and 150 μl of 0.3 mM DPPH ethanolic solution was incubated at 37 °C for 30 min. Then, the decrease in absorbance value was measured at 515 nm using microplate reader (Thermo, Multiskan Ex). Obtained absorbance value was then converted into the percentage of radical scavenging activity using the following equation:

\[
\text{Radical scavenging activity (%) = } 100 - \left( \frac{AS - AC}{AS} \right) \times 100
\]

where AS: absorbance of the sample; AC: absorbance of the negative control (ethanol). For IC₅₀ determination, the extracts were serially diluted to six different concentrations (from 250 to 7.81 μg/ml). IC₅₀ values were obtained from the graph of radical scavenging percentage against log concentration, at 50% of radical scavenging.

2.4. Total phenolic content: Folin–Ciocalteau colorimetric method

Total phenolic concentration in the extracts was determined spectrophotometrically according to the Folin–Ciocalteau method (Singleton et al., 1999), with slight modification using a 96 microwell plate (Dicko et al., 2002). The total phenol concentration was calculated from the calibration curve using gallic acid as a standard and the results were expressed as milligram of gallic acid equivalents per gram dry weight of extract (mg GAE/g). Determination of total phenolics in this
assay was based on their chemical reducing capacity relative to an equivalent reducing capacity of gallic acid (Katalinic et al., 2006). The sample was prepared 1 mg/ml using ethanol. The same solvent was used to prepare gallic acid standard solutions. Briefly, 25 μl of Folin–Ciocalteu’s reagent (50% v/v) was added to 10 μl of 1 mg/ml (w/v) sample. After 5 min incubation at room temperature, 25 μl of 20% (w/v) sodium carbonate was added. One hundred forty microliters of water was then added to yield a final volume of 200 μl per well. Water was used as blank (negative control). After 30 min of incubation the absorbance was read at 760 nm. All assays were carried out in triplicate. Gallic acid was used as standard and results were expressed as mg gallic acid equivalent per gram sample.

2.5. HIV-1 reverse transcriptase (RT) assay

The dried extracts of Phyllanthus species were re-suspended (500 μg/ml) and two-fold serially diluted to a final concentration of 7.81 μg/ml using dimethylsulphoxide (DMSO). A reverse transcriptase assay using the colorimetric kit produced by Roche Molecular Biochemicals was applied in this experiment. Azido-deoxynucleoside-triphosphate (AZT151TP) was used as a positive control. The serially diluted extracts and positive control were mixed with the enzyme HIV-1 RT (5 ng) and added to a reaction mixture of nucleotides dioxigenin (DIG)-dUTP, dTTP and the template/primer hybrid polyA oligo (dT)15 (750 mA260 nm/ml) into a streptavidin precoated microtiter plate. The plate was then incubated at 37 °C for 2 h. The solution was removed and was rinsed with washing buffer. Then, anti-DIG-peroxidase solution (40 mU) was added into each well of the microtiter plate. The mixture was incubated at 37 °C for 1 h. After the removal of the solution, the plate was rinsed with washing buffer. The ABTS substrate solution (1 mg/ml) was added to the mixture. The optical density of the colorimetric reaction was measured at 405 nm using a microplate reader (Thermo, Multiskan Ex) with reference wavelength of 490 nm. The signal intensity was obtained and recorded as absorbance and was directly proportional to the actual RT activity. The test was carried out in duplicate. Percentage of inhibition of the RT was calculated as the following:

\[
\text{Inhibition} (\%) = 100 \left( \frac{\text{Abs}_{\text{ctl}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{ctl}} - \text{Abs}_{\text{n}}} \right)
\]

Abs ctl = absorbance of positive control; Abs n = absorbance of negative control; and Abs sample = absorbance of sample.

IC50 values were obtained from the graph of percentage of inhibition against log concentration, at 50% of inhibition.

3. Results and discussion

3.1. Antibacterial activity

Antibacterial activity of the seven Phyllanthus species investigated in this study was indicated by minimum inhibitory concentration (MIC) values. Six bacterial strains were employed. Different levels of activities were observed by the plant extracts against both Gram-positive and Gram-negative bacteria tested (Table 1). The lowest MIC value (17.7 μg/ml) was obtained by P. amarus against S. aureus (Gram-positive) followed by P. myrtifolius against B. spizizenii (MIC=20 μg/ml) and B. licheniformis (MIC=75 μg/ml) (Gram-positive) and P. stutzeri (MIC=78 μg/ml) (Gram-negative). P. urinaria possessed the lowest MIC value (156 μg/ml) against K. pneumoniae and E. coli (Gram-negative).

Compounds belonging to various chemical groups such as alkaloids, tannins, flavonoids, lignans, phenols and terpenes have been isolated and identified from different Phyllanthus species (Kalidas and Mohan, 2009). It is worth mentioning that these chemical groups are the most abundant compounds determined in the genus Phyllanthus (Calixto et al., 1998).

Many of these constituents are known to have antibacterial effects (Bruneton, 1995; Eldeen et al., 2006; Hutchings et al., 1996). Antibacterial activity observed in this study may be due to the presence of these agents or similar constituents. Obtained results indicated a lack of activity against Gram-negative bacteria when compared to Gram-positive. This may be due to the lipopolysaccharides in the outer membrane of Gram-negative bacteria which render them impermeable to certain compounds (Clements et al., 2002). Inhibition of P. stutzeri (Gram-negative) with MIC values of 78 and 117 μg/ml by P. myrtifolius and P. urinaria respectively are interesting exceptions and may indicate the presence of promising antibacterial agents that need to be further investigated. Antibacterial activities of P. discoideus and P. muellerianus previously reported by Mensah et al. (1990) and Doughari and Sunday (2008), respectively, were much weaker when compared to the activities observed by P. myrtifolius and P. urinaria against similar bacterial strains. This could be attributed to variations in chemical compositions among Phyllanthus species and/or differences in the concentration of bioactive agents in these extracts as a result of using different extraction methods. Our findings supported the previously reported uses of Phyllanthus species in the treatment of microbial related diseases.

3.2. Antioxidant activity and total phenolic contents of the Phyllanthus extracts

The antioxidant activity of the Phyllanthus species was determined using the DPPH scavenging assay. The radical scavenging activity of the extracts (250 μg/ml) was expressed as percentage inhibition and IC50 values (Table 2). Most of the tested extracts showed scavenging activity above 80% with IC50 values ranging between 10.2 and 128 μg/ml (exceptions were P. debilis and P. acidus). The lowest IC50 values were obtained by P. myrtifolius and P. reticulatus. Both P. acidus and P. debilis showed very low or no activity as indicated by their high IC50 values.

Total phenolic contents of the tested Phyllanthus extracts were determined using the Folin–Ciocalteu colorimetric method by manipulation of the regression equation of gallic acid calibration curve \((y=1.509x+0.077, r^2=0.9779)\). The amount of phenolics per each extract was expressed as gallic
Table 1
Yield percentage and minimum inhibitory concentration (MIC) values (μg/ml) of the 80% methanolic extract of seven *Phyllanthus* species and tetracycline standard (control) as detected using the microdilution assay. Results expressed as means±SD.

| Sample          | Yield (%) of 100 g dried materials | MIC values μg/ml | Bacteria tested |
|----------------|----------------------------------|------------------|----------------|
|                 |                                  |                  | Bs | Bl | Ec | Kp | Ps | Sa |
| *P. urinaria*   | 10.3                             | 79±2.0           | 154±3.5 | 185±4.0 | 156±0.0 | 117±0.0 | 39±0.0 |
| *P. debilis*    | 6.5                              | 83±1.7           | 297±2.2 | 311±3.5 | 301±1.9 | 125±5.7 | 137±1.7 |
| *P. amarus*     | 11.5                             | 101±3.8          | 157±2.0 | 255±4.2 | 305±6.1 | 137±6.6 | 17.7±1.9 |
| *P. pulcher*    | 9.8                              | 462±0.0          | 459±7.3 | 623±2.5 | 625±0.0 | 281±9.8 | 185±7.3 |
| *P. acidus*     | 8.7                              | 307±5.2          | 350±6.6 | 625±2.0 | 315±3.8 | 156±0.0 | 156±0.0 |
| *P. reticulatus*| 8.2                              | 84±4.9           | 164±6.1 | 312±0.0 | 312±0.0 | 180±8.3 | 79±1.2 |
| *P. myrtifolius*| 11.4                             | 20±1.1           | 75±5.3  | 306±6.9 | 178±1.4 | 78±1.5  | 40±3.0 |
| Tetracycline (control) | 1.8±0.1                        | 1.0±0.1          | 15.6±0.0 | 3.7±0.2 | 8.1±0.1 | 7.7±0.1 |

Bs = *Bacillus spizizenii*; Bl = *Bacillus licheniformis*; Ec = *Escherichia coli*; Kp = *Klebsiella pneumoniae*; Ps = *Pseudomonas stutzeri*; and Sa = *Staphylococcus aureus*.

Table 2
Total phenolic contents, free radical scavenging activity and inhibition of HIV-1 RT by extracts from the seven *Phyllanthus* species investigated and the positive controls. Results are expressed as means±SD and IC₅₀ values.

| Sample          | Total phenols mg GAE/g | Free radical scavenging activity % Inhibition (μg/ml) | Inhibition of HIV-1 RT IC₅₀ values | IC₅₀ value μg/ml |
|----------------|------------------------|-----------------------------------------------------|----------------------------------|----------------|
| *P. urinaria*   | 205.3±21.3             | 88.6±0.7                                            | 17.4                             | 10.49          |
| *P. debilis*    | 98.2±13.1              | 45.6±0.7                                            | >250                             | 67.88          |
| *P. amarus*     | 21.3±3.6               | 89.4±0.9                                            | 128                              | 57.60          |
| *P. pulcher*    | 35.5±3.7               | 85.2±0.7                                            | 35.8                             | 5.90           |
| *P. acidus*     | 0.16±0.38              | 31.3±0.3                                            | >250                             | >500           |
| *P. reticulatus*| 127.8±11.0             | 88.6±0.5                                            | 10.8                             | 131.17         |
| *P. myrtifolius*| 207.8±5.0              | 89.3±0.8                                            | 10.25                            | 12.77          |
| Quercetin       | nt                     | 91.4±0.3                                            | 8.01                             | nt             |
| AZT-TP (control)| nt                     | nt                                                  | nt                               | 1.63           |

Quercetin and AZT-TP were used as positive controls for the DPPH and HIV-RT assays, respectively. nt = not determined.

Acid equivalent (mg GAE/g dry extract). Results obtained from the assay expressed as means±standard deviation of triplicate analyses are presented in Table 2. Highest total phenolic contents were recorded for *P. myrtifolius* and *P. urinaria*. The lowest amount was obtained by *P. acidus*. With the exception of extracts from *P. amarus*, *P. debilis* and *P. pulcher*, total phenolic content correlated with DPPH radical scavenging activity. This may indicate the involvement of some phenolic compounds in the antiradical activity observed by the extracts. On the other hand lack of correlation between antioxidant capacity and total phenolic contents observed may suggest the presence of non-phenolic compounds with possible antiradical effects in these extracts. A similar observation was also previously reported (Sengul et al., 2009). The total phenols recorded for *P. debilis*, *P. amarus* and *P. urinaria* were relatively low when compared to the previous report of Kumaran and Karunakan (2007). This may be due to the drying process used. Some phenolic compounds decompose rapidly in direct sunlight or relevant temperature (Lim and Murtijaya, 2007). The drying process in some cases may result in a depletion of naturally occurring antioxidant and phenols in plant materials as most of these agents are unstable. However, these processes cause little or no changes in other cases (Tomaino et al., 2005).

3.3. Inhibition of HIV-1-RT

Inhibition of reverse transcriptase (RT) by the plant extracts was determined using the reverse transcriptase assay. Inhibition of the HIV-1 RT enzyme by the plant extracts was evaluated based on their IC₅₀ values. Obtained results are shown in Table 2. The lowest IC₅₀ value was shown by extracts from *P. pulcher* followed by *P. urinaria*, *P. myrtifolius* and *P. amarus*. The methanolic extract from *P. pulcher* exhibited the best anti-HIV-1-RT activity.

The process by which viruses evade the immune system is not fully elucidated. However, it is generally believed that the structural features of a glycoprotein on the virus’s outer coat block antibody access and inhibited the immune system from generating an equivalent defense response (Schaeffer and Krylov, 2000). Chang et al. (1995) reported that phyllamycin B and retrojusticidin B isolated from *P. myrtifolius* showed inhibitory effect against HIV-1 RT. Inhibition of the enzyme obtained by *P. myrtifolius* in this study may be due to the presence of similar or related class of these compounds. Differences in the IC₅₀ values of the studied *Phyllanthus* species might be due to the variation in quantity and quality of their active constituents. This preliminary finding suggested further study to isolate bioactive compounds from the biologically active extracts.

In general, our results support the reported traditional uses of *Phyllanthus* sp. for the treatment of ailments of microbial origin. Antimicrobial effects observed by *P. myrtifolius* and *P. urinaria* need to be further investigated in order to isolate active compounds that may be responsible for such activities. Results of antioxidant activity and total phenolic contents in this study suggest that some extracts with low total phenols still may have antiradical activity. Variation in the results obtained for total phenols and DPPH radical scavenging activity in this study compared to previous reports may be due to effects of drying and extraction procedures used.
The extract from *P. pulcher* showed interesting activity against HIV-1 RT with an IC_{50} value of 5.9 \mu g/ml. This work contributes positively to the efforts towards scientific evaluation of the uses of *Phyllanthus* species in traditional medicine. Results obtained in this study could be used for further investigation to isolate active constituents that might be responsible for the observed activities and to study their mechanism of actions.

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