Original Article

Chemical Constituents of *Phyllanthus acidus* (L.) Skeels

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ABSTRACT

Background: This study describes the isolation of chemical constituents from the leaves and bark of *Phyllanthus acidus*, a medicinal plant widely used for the treatment of several ailments. **Materials and Methods:** The crude extract of *P. acidus* was chromatographed on a gravity column dry packed with silica gel and was fractionated by increasing proportions of acetone in CH_2Cl_2. The purified isolates were subjected to NMR for structure elucidation. Their structures were identified mainly by using ^{1}H-NMR and by comparing current NMR data with those reported in the literature. **Results:** The results showed that the air-dried leaves extracts of the plant afforded mixture of β-sitosterol and stigmasterol while the bark yielded mixture of phyllanthol, α-amyrin, β-amyrin and lupeol. **Conclusion:** The air-dried of DCM extract of *P. acidus* resulted in the isolation of the mixture of phyllanthol, α- amyrin, β-amyrin and lupeol from the bark and mixture of β-sitosterol and stigmasterol from the leaves of the plant. It is important to conduct detailed pharmacological and toxicity investigations as well as clinical studies using different doses or concentrations to verify its traditional uses.

Key words: *Phyllanthus acidus,* Karamay, Phyllanthol, Lupeol, Amyrin, Sitosterol, Stigmasterol.

INTRODUCTION

Phyllanthus acidus (L.) Skeels or gooseberry, locally known as karamay in Ilocos region, Northern Philippines, is one of the trees with edible small yellow berries fruit in the Euphorbiaceae family. It is an indigenous plant in the country and frequently used in folk medicine for the treatment of several diseases such as cough, fever, bronchitis, asthma, respiratory disorders, hypertension, diabetes, rheumatism, pain, psoriasis and other skin disorders.[1,2] The unripe fruit is also used as sour flavouring and for making vinegar, jams and jellies.

Phytochemical studies on the genus *Phyllanthus,* revealed the presence of lignin,[3-5] terpenes, sterols,[6,7] polyphenolic compounds, tannins,[8-10] flavonoids,[11,12] glycosides[13] and alkaloids.[14] Biological investigations have also shown antihepatotoxic,[15,16] antidiabetic,[17] antioxidant,[12,15,18,19] diuretic,[20] anticancer,[21,22] antimicrobial[23] and anti-inflammatory properties.[24]

Here in the present report, two known substances, phyllanthol (1) and (2) α-amyrin have been identified from the mixture of the DCM extract of bark of *P. acidus*. The mixture also contained β-amyrin (3) and lupeol (4) (Figure 1). The isolation of mixture of closely related compounds, beta-sitosterol (5) and stigmasterol (6) (Figure 2) of the DCM extract of the leaves of *P. acidus* are also described in the study.

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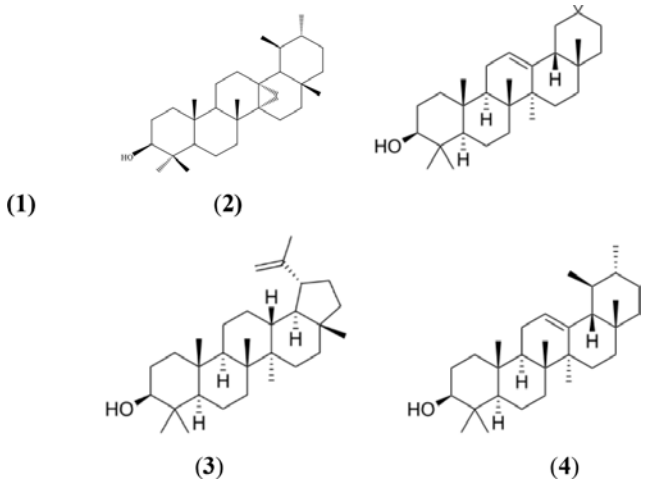

Figure 1: Chemical Constituents of *Phyllanthus acidus* Bark.

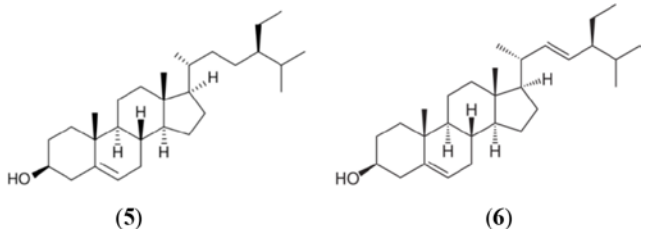

Figure 2: Chemical Constituents of *P. acidus* Leaves.

MATERIALS AND METHODS

Methodology

Sample Collection and Identification of *P. acidus*: The bark and leaves samples were collected at Brgy. Cabaroan Daya, Vigan, Ilocos Sur in January 2017. The bark and leaves were identified as *P. acidus* (L) Skeels at the National Museum of the Philippine, and stored at the Research Center Laboratory, Cavite State University. Voucher specimens were deposited as #CVSU-PAL-001 and #CVSU-PLB-001 for leaves and bark of *P. acidus*, respectively.

Sample Preparation and Extraction: The bark and leaves of *P. acidus* were air-dried for two months and one month, respectively, without exposure to sunlight. The bark and leaves samples were homogenized into fine, ground pieces using an osterizer. The air-dried powdered bark samples 0.85 kg and 1.45 kg were soaked in CH_2Cl_2 for three days. The CH_2Cl_2 extract were both filtered using cheese cloth and each residue was washed several times with recovered CH_2Cl_2 and then filtered. The filtrate from 0.85 kg of bark and 1.45 kg of leaves were concentrated in vacuo to afford the crude extracts 45.10 g and 0.93 g, respectively.

Isolation: Both crude extracts of *P acidus*, 45.10 g and 0.93g were chromatographed on a gravity column (Internal diameter: 2 inches) dry packed with silica gel (100-230 mesh) and was fractionated using increasing proportions of acetone in CH_2Cl_2 at 10% increment. Eleven (11) 100 ml fractions were collected for each crude extract. The 30% and 40% acetone in CH_2Cl_2 from the bark of *P. acidus* were combined and rechromatographed using 20% EtOAc in petroleum ether, followed by 15% EtOAc in petroleum ether and rechromatographed (6x) to afford 10 mg mixture of (1), (2), (3) and (4). While, the 30% acetone in CH_2Cl_2 fraction from the leaves of *P. acidus* was rechromatographed using 15% EtOAc in petroleum ether followed by 10 % EtOAc in petroleum ether and rechromatographed (5x) to afford 11 mg mixture of (5) and (6). Fractions were monitored by TLC which was performed with plastic-backed plate coated with silica gel F_{254}. The spots were visualized by spraying with vanillin-H_2SO_4 followed by warming.

Structure Elucidation: The TLC isolates were sent to National Research Institute of Chinese Medicine, Taiwan for NMR analyses. The NMR were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR spectra.

RESULTS AND DISCUSSION

^{1}H-NMR OF MIXTURE OF (1), (2), (3) AND (4)

The mixture of (1), (2), (3) and (4) isolated from CH_2Cl_2 extract of the air-dried bark of *P. acidus* was obtained as white solid and produced a blue-violet TLC spot when warmed with vanillin-sulfuric acid visualizing agent. It has an R_f value of 0.58 when developed with 20% EtOAc in petroleum ether.

Looking at the ^{1}H-NMR spectra suggested that it was mixture of four compounds based on the integrals and resonance intensities. These compounds which include phyllanthol (1), α-amyrin (2), β-amyrin (3) and lupeol (4) were identified based also on the specific characteristics of each signals appeared in the spectra and by comparing them with spectral data reported in the literature. The presence of the first compound in the mixture, phyllanthol was attributed due to the signals at δ 0.00 and δ 0.63 which correspond to the methylene protons (H-27) of the cyclopropyl ring. In addition to these, ^{1}H-NMR spectra also showed resonances at δ 3.20, an indicative of oxygenated proton (H-3) and seven methyls at δ 0.77, 0.86, 0.87, 0.89, 0.92, 0.95 and δ 1.11. These assignments were found to be consistent with the reported literature[7] as shown in Table 1.

Table 1: Comparison of the ^{1}H-NMR Spectral Data of the mixture with Phyllanthol in $CDCl_3$.

^{1}H shift, δ of Mixture	^{1}H shift, δ of phyllanthol
3.20	3.18
0.00 0.63 (d, J = 5.4 Hz)	0.00 0.66 (d, J = 5.55 Hz)
1.11 (3H, s)	1.11 (3H, s)
0.77 (3H, s)	0.77 (3H, s)
0.95 (3H,s)	0.96 (3H,s)
0.86 (3H, s)	0.86 (3H, s)
0.89 (3H,s)	0.89 (3H,s)
0.87 (3H, d, J = 5.6 Hz)	0.87 (3H, d, J = 5.6 Hz)
0.92 (3H, d, J = 6.0 Hz)	0.93 (3H, d, J = 6.0 Hz)

Table 2: Comparison of the ^{1}H-NMR Spectral Data of Mixture with α-Amyrin in $CDCl_3$.

^{1}H shift, δ of Mixture	^{1}H shift, δ of α-amyrin
3.49	3.23
5.10	5.10
0.85(3H, s)	0.85 (3H, s)
0.84 (3H, s)	0.84 (3H, s)
0.95 (3H, s)	0.96 (3H, s)
0.98 (3H, s)	0.98 (3H, s)
1.05 (3H, s)	1.04 (3H, s)
0.78 (3H, s)	0.78 (3H, s)
0.77 (3H, d, J = 5.0 Hz)	0.77 (3H, d, J = 6.0 Hz)
0.83 (3H, d, J = 5.0 Hz)	0.83 (3H, d, J = 6.0 Hz)

Table 3: Comparison of the ^{1}H-NMR Spectral Data of Mixture with β-Amyrin in $CDCl_3$.

^{1}H shift, δ of Mixture	^{1}H shift, δ of β-amyrin
3.20	3.20
5.16	5.16
0.92 (3H, s)	0.92 (3H, s)
0.94 (3H, s)	0.94 (3H, s)
0.98 (3H, s)	0.98 (3H, s)
1.11 (3H, s)	1.11 (3H, s)
0.85 (3H, s)	0.85 (3H, s)
0.85 (3H, s)	0.85(3H, s)
0.81 (3H, s)	0.81 (3H, s)
0.77 (3H, s)	0.77 (3H, s)

Table 4: Comparison of the ^{1}H-NMR Spectral Data of Mixture with Lupeol in $CDCl_3$.

^{1}H shift, δ of Mixture	^{1}H shift, δ of lupeol
3.20	3.20
4.55	4.55
4.68	4.70
0.77 (3H, s)	0.77 (3H, s)
0.78 (3H, s)	0.79 (3H, s)
0.85 (3H, s)	0.85 (3H, s)
0.94 (3H, s)	0.94 (3H, s)
0.95 (3H, s)	0.97 (3H, s)
1.05 (3H, s)	1.05 (3H, s)
1.65 (3H, s)	1.65 (3H, s)

For the second compound in the mixture, the ^{1}H-NMR spectra gave resonances for an olefinic proton and oxygenated proton at δ 5.10 and δ 3.49, respectively. Presence of eight methyls were also detected, of which, six were singlets at δ 0.85, 0.84, 0.95, 0.98, 1.05 and δ 0.78 and two doublets at δ 0.77 and δ 0.83. These obtained data were typical for α-amyrin and disclosed a total match with this compound (Table 2) when compared in the literature.[25]

The spectra also implied the presence of β-amyrin due the signals that appeared at δ 5.16 and δ 3.20 which correspond to the olefinic proton and oxygenated proton, respectively. Eight methyl singlets were also observed at δ 0.92, 0.94, 0.98, 1.11, 0.85, 0.85, 0.81 and δ 0.77. These spectral data were also typical for β-amyrin, a closely related compound of α-amyrin. Putting the spectra of the mixture along with the spectra of β-amyrin matched in all essential respects[25] as shown in Table 3.

Lastly, the mixture also contained lupeol which was the first compound isolated from the genus *Phyllanthus*. The ^{1}H-NMR spectra indicated the presence of olefinic protons at δ 4.68 and δ 4.55 which are the distinctive characteristics for lupeol. In addition to these, seven methyl singlets also appeared at δ 0.77, 0.78, 0.85, 0.94, 0.95, 1.05 and δ 1.65 and an oxygenated proton at δ 3.20. These data obtained were similar with the reported literature shown in Table 4.[26]

^{1}HNMR of Mixture of (5) and (6)

The mixture of (5) and (6) isolated from CH_2Cl_2 extract of the air-dried leaves of *P. acidus* was obtained as white solid. It produced a blue-violet TLC spot when warmed with vanillin-sulfuric acid visualizing agent. It has an R_f value of 0.49 when developed with 15% EtOAc in petroleum ether.

The ^{1}H-NMR spectra indicated a mixture of two compounds that were closely related consisting of

β-sitosterol as the major component and stigmasterol. The identification of these compounds in which they were present approximately in ratio of 6:1 were deduced from the integrals and resonance intensities. The following assignments for β-sitosterol and stigmasterol that were derived from the signals that appeared in the spectra as shown in Tables 5 and 6 were found to be consistent with the reported literatures.[27,28]

Table 5: Comparison of the ^{1}H-NMR Spectral Data of Mixture with β-sitosterol in $CDCl_3$.

^{1}H shift, δ of mixture	^{1}H shift, δ of β-sitosterol
3.51	3.52
5.33	5.36
0.99 (3H, s)	1.01 (3H, s)
0.66 (3H, s)	0.68 (3H, s)
0.90 (3H, d, J = 6.6 Hz)	0.93 (3H, d, J = 6.6 Hz)
0.82 (3H, d, J = 6.6 Hz)	0.82 (3H, d, J = 6.6 Hz)
0.79 (3H, d, J = 7.20Hz)	0.81 (3H, d, J = 7.20Hz)
0.83 (3H, t, J = 6.5Hz)	0.84 (3H, t, J = 6.5Hz)

Table 6: Comparison of the ^{1}H-NMR Spectral Data of Mixture with stigmasterol in $CDCl_3$.

^{1}H shift, δ of Mixture	^{1}H shift, δ of stigmasterol
3.51	3.51
5.33	5.36
5.14	5.21
5.10	5.10
0.99 (3H, s)	1.03 (3H, s)
0.68 (3H, s)	0.71(3H, s)
0.90 (3H, d, J = 6.6 Hz)	0.91 (3H, d, J = 6.6 Hz)
0.82 (3H, d, J = 6.6 Hz)	0.82 (3H, d, J = 6.6 Hz)
0.79 (3H, d, J = 7.20Hz)	0.81 (3H, d, J = 7.10Hz)
0.83 (3H, t, J = 6.5Hz)	0.83(3H, t, J = 6.2Hz)

CONCLUSION

The air-dried of DCM extract of *Phyllanthus acidus* resulted in the isolation of the mixture of phyllanthol, α-amyrin, β-amyrin and lupeol from the bark and mixture of β-sitosterol and stigmasterol from the leaves of the plant. The identification of these components was characterized on the basis of ^{1}H-NMR and comparison with their literature data. Since the plant is widely used in traditional herbal medicine for treating several ailments, it is important to conduct detailed pharmacological and toxicity investigations as well as clinical studies using different doses or concentrations to verify its traditional uses.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

P. acidus: *Phyllanthus acidus*; **NMR:** Nuclear Magnetic Resonance; **TLC:** Thin Layer Chromatography; **R_f value:** Retention Factor value; **EtOAc:** Ethyl Acetate; **DCM:** Dichloromethane.

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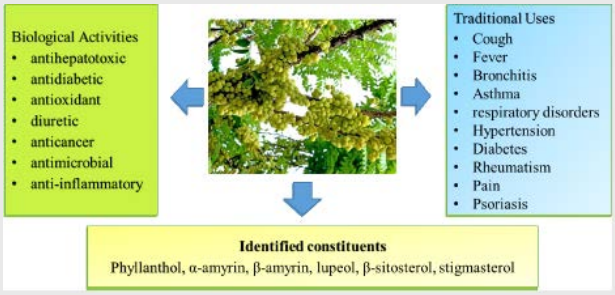
PICTORIAL ABSTRACT



SUMMARY

This study describes the isolation of chemical constituents from the leaves and bark of *Phyllanthus acidus*, a medicinal plant widely used in traditional medicine for the treatment of several ailments. The air-dried of DCM extract of *P. acidus* resulted in the isolation of the mixture of phyllanthol, α- amyrin, β-amyrin and lupeol from the bark and mixture of β-sitosterol and stigmasterol from the leaves of the plant. It is important to conduct detailed pharmacological and toxicity investigations as well as clinical studies using different doses or concentrations to verify its traditional uses.

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