INTRODUCTION

*Abutilon indicum* Sweet or “giling-gilingan” in Filipino is a member species of the family Malvaceae and found throughout the Philippines in thickets and waste places at low and medium altitudes. It is a half-woody, erect, branched-plant 0.5 to 2.5 meters in height. The plant is a popular medicinal plant in the Philippines. The leaf decoction is used for cleansing wounds and ulcers, and for enemas or vaginal infection. The emollient leaf decoction is frequently used by Filipinos as demulcent, diuretic, sedative and aphrodisiac. It has also a long medical history of being used as an antidiabetic remedy. The diuretic property can be traced from its root extract and can be taken for the relief of hematuria. It is also effective in the treatment of leprosy. The seeds from this plant are considered to be aphrodisiac and can be used as a laxative for patients having hemorrhoids and in the treatment of coughs, puerperal disease, urinary disorders, chronic dysentery, and fever. Previous phytochemical studies reported the presence of fatty acids, carotenoid, sterols, triterpenes, sesquiterpenes, saponins, tannins, alkaloids,
antitubercular activity of Abutilon indicum

Plant material

The leaves of Abutilon indicum were collected from Laao City, Ilocos Norte, Philippines in April 2006. Voucher specimens were authenticated by Asst. Prof. Rosie S. Madulid of the Herbarium of the Research Center for the Natural and Applied Sciences, University of Santo Tomas – Manila (USTH5034).

Extraction, fractionation and isolation of compounds

The air-dried leaves of A. indicum (1.3 kg) were soaked in 1:1 DCM-MeOH (17 L) for three days, and then filtered. The filtrate was concentrated under reduced pressure to afford the crude extract (151.0 g) which was chromatographed in diethyl ether-ethyl acetate (20% gradients) to yield five fractions. Silica gel chromatography of the most active, sub-fraction one (7.0 g) was further chromatographed in diethyl ether-ethyl acetate (20% gradients) to yield five fractions. Silica gel chromato
graphy of the most active, sub-fraction one (7.0 g) in hexanes, and chloroform-acetonitrile-diethyl ether mixture (9:0.5:0.5) gave 1 (colorless solid, 104.2 mg), 2 (colorless oil, 11.3 mg) and a mixture of 3 and 4 (colorless crystals, 143.0 mg) (Figure 1).

Bacterial strains and growth conditions

M. tuberculosis H37Rv ATCC 27294 (H37Rv) obtained from the American Type Culture Collection (Rockville, Md.). For the first three (of four) replicate experiments, H37Rv inocula were first passaged in radiometric 7H12 broth (BACTEC 12B; Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) until the growth index (GI) reached 800 to 999. For the fourth replicate experiment, H37Rv was grown in 100 ml of Middlebrook 7H9 broth (Difco, Detroit, Mich.) supplemented with 0.2% (v/v) glycerol (Sigma Chemical Co., Saint Louis, Mo.), 10% (vol/vol) OADC (oleic acid, albumin, dextrose, catalase; Difco), and 0.05% (v/v) Tween 80 (Sigma). The complete medium was referred to as 7H9GC-Tween. Cultures were incubated in 500-ml nephelometer flasks on a rotary shaker (New Brunswick Scientific, Edison, N.J.) at 150 rpm and 37°C until they reached an optical density of 0.4 to 0.5 at 550 nm. Bacteria were washed and suspended in 20 ml of phosphate-buffered saline and passed through an 8-mm-pore-size filter to eliminate clumps. The filtrates were aliquoted, stored at 280°C, and used within 30 days.

Microplate Alamar Blue Assay (MABA)

Briefly, extracts, fractions and test compounds MICs against TB were assessed by MABA using rifampin as positive control. Compound stock solutions were prepared in DMSO at a concentration of 12.8 mM, and the final test concentrations ranged from 128 to 0.5 mM. 2-fold dilutions of compounds were prepared in Middlebrook 7H12 medium (7H9 broth containing 0.1% w/v casitone, 5.6 µg/mL palmitic acid, 5 µg/mL bovine serum albumin, 4 µg/mL catalase, filter-sterilized) in a volume of 100 mL in 96-well microplates (black viewplates). Mtb H37RV (100 mL inoculum of 2 to 105 cfu/mL) was added, yielding a final testing volume of 200 mL. The plates were incubated at 37°C. On the 7th day of incubation 12.5 mL of 20% Tween 80 and 20 mL of Alamar Blue (Trek Diagnostic, Westlake, OH) were added to the test plate. After incubation at 37°C for 16 to 24 h, fluorescence of the wells was measured (ex 530, 590 nm). The MICs were defined as the lowest concentration effecting a reduction in fluorescence of >90% relative to the mean of replicate bacteria only controls.

RESULTS

Silica gel fractionation of the DCM-MeOH (1:1) crude extract of the leaves of A. indicum (MIC >128 µg/mL versus Mycobacterium tuberculosis H37Rv) (Table 1) afforded four fractions with the first fraction being the most inhibitory according to the colorimetric Microplate Alamar Blue assay (MABA) (MIC = 64 µg/mL) (Figure 1). For comparison purposes, the known TB drug rifampin was used as positive drug standard for comparison purposes (MIC = 0.125 µg/mL). Further chromatographic separation of this fraction resulted into five fractions with MIC values up to 64 µg/mL. Chromatographic purification of the major constituents of the first sub-fraction gave β-aminor 3-palmitate (1), squalene (2) and a 1:1 mixture of the sterols β-sitosterol (3) and stigmastanol (4).
The structures of 1–4 were established through NMR spectroscopic evidences and confirmed by comparison of their MS and NMR ($^1$H, $^{13}$C) spectral data with those reported in the literature for $\beta$-amyrin 3-palmitate,[18] squalene[19] and mixture of $\beta$-sitosterol and stigmasterol[20]. The MABA assay revealed no significant antitubercular activity for all isolated compounds (MIC = >128 $\mu$g/mL). The result may indicate synergistic action of the constituents for marked antitubercular activity. To the best of our knowledge, this is the first report on the isolation of 1 and 2 from $A$. indicum.

Table 1: Minimum inhibitory concentration (MIC) values vs. $M$. tuberculosis $H_37$Rv of $A$. abutilon fractions and isolated compounds 1–4

<table>
<thead>
<tr>
<th>Fraction</th>
<th>MIC ($\mu$g/mL)*</th>
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<tbody>
<tr>
<td>Fraction 1</td>
<td>64 (91)</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>&gt;128 (58 @ 128)</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>&gt;128 (-24 @ 128)</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>&gt;128 (15 @128)</td>
</tr>
<tr>
<td>Fraction 1.1</td>
<td>64 (93)</td>
</tr>
<tr>
<td>Fraction 1.2</td>
<td>64 (94)</td>
</tr>
<tr>
<td>Fraction 1.3</td>
<td>&gt;128 (82 @ 128)</td>
</tr>
<tr>
<td>Fraction 1.4</td>
<td>128 (90)</td>
</tr>
<tr>
<td>Fraction 1.5</td>
<td>128 (96)</td>
</tr>
<tr>
<td>Compound</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&gt;128</td>
</tr>
<tr>
<td>2</td>
<td>&gt;128</td>
</tr>
<tr>
<td>3 &amp; 4 (1:1)</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.125</td>
</tr>
</tbody>
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*% Values in parentheses denote % inhibition.

DISCUSSION

Tuberculosis (TB) has re-emerged as the deadly plague by recent reports of outbreaks of drug-resistant cases. Mortality rates have escalated in number due to the HIV-1 epidemic and the inconsistent use of antibiotics, which has led to a rise of drug-resistant TB in areas of the world. This growing global health concern has led to renewed interest in natural product-inspired derivatives with promising activity against Mycobacterium tuberculosis. Literature reviews indicate natural products possess marked growth inhibitory activity towards $M$. tuberculosis and some have been selected as prototype molecules for anti-TB drug development.[21–23] Relevant to this study, pentacyclic terpenoids, sterols and polyenes have been reported as promising inhibitors of $M$. tuberculosis $H_37$Rv.[24] Previous studies on $A$. indicum indicate the presence of several non-polar secondary metabolites viz. acyclic and pentacyclic triterpenes and sterols. An independent study on the methanolic extract of $A$. indicum showed activity against bacteria.[3] Based on our results, activity against $M$. tuberculosis is ascribed to the non-polar constituents of $A$. indicum in fraction one. However, purification of the major constituents yielding compounds 1–4 did not result into the isolation of the active constituents (MIC's >128 $\mu$g/mL). While a separate report indicates that squalene (2) is active against $M$. tuberculosis, our anti-TB assay data for 2 interestingly agreed with the result of Noro and co-workers.[25] Further purification of the antimycobacterial principles of $A$. indicum appears to be significant in order to clarify the bioactive components responsible for antitubercular activity.

CONFLICTS

All authors have none to declare.

REFERENCES

1. Quisumbing E. Medicinal plants of the Philippines, Quezon City. JMC Press. 1978; p. 1–977.


