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Antitubercular activity of the semi-polar extractives of *Uvaria rufa*

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ABSTRACT

Objective: To investigate the inhibitory activity of the chloroform extract, petroleum ether and chloroform sub-extracts, lead-acetate treated chloroform extract, fractions and secondary metabolites of *Uvaria rufa (U. rufa)* against *Mycobacterium tuberculosis (M. tuberculosis)* $H_{37}Rv$. **Methods:** The antituberculosis susceptibility assay was carried out using the colorimetric Microplate Alamar blue assay (MABA). In addition, the cytotoxicity of the most active fraction was evaluated using the VERO cell toxicity assay. **Results:** The *in vitro* inhibitory activity against *M. tuberculosis* $H_{37}Rv$ increased as purification progressed to fractionation (MIC up to 23 μg/mL). The chloroform extract and its sub-extracts showed moderate toxicity while the most active fraction from chloroform sub-extract exhibited no cytotoxicity against VERO cells. Meanwhile, the lead acetate-treated crude chloroform extract and its fractions showed complete inhibitions (100%) with MIC values up to 8 μg/mL. Phytochemical screening of the most active fraction showed, in general, the presence of terpenoids, steroids and phenolic compounds. Evaluation of the antimycobacterial activity of known secondary metabolites isolated showed no promising inhibitory activity against the test organism. **Conclusions:** The present results demonstrate the potential of *U. rufa* as a phytomedicinal source of compounds that may exhibit promising antituberculosis activity. In addition, elimination of polar pigments revealed enhanced inhibition against *M. tuberculosis* $H_{37}Rv$. While several compounds known for this plant did not show antimycobacterial activity, the obtained results are considered sufficient reason for further study to isolate the metabolites from *U. rufa* responsible for the antitubercular activity.

1. Introduction

Tuberculosis (TB) is a deadly infectious disease caused by various strains of mycobacterium, usually *Mycobacterium tuberculosis (M. tuberculosis)*. Tuberculosis has been considered to be a disease of poverty for many years with quite rare occurrence in the developed countries. With people in developed countries having their immune systems compromised by immunosuppressive drugs, substance abuse or AIDS, the situation has unfortunately been aggravated by contracting tuberculosis[1]. The sudden rise of tuberculosis was declared by World Health Organization (WHO) as a global emergency with 9 million new TB cases and two million deaths reported each year[2,3]; and about one-third of the world's population is currently reported to be infected with *M. tuberculosis*. Despite the efforts of academic institutions and the pharmaceutical companies engaged in the design, synthesis, and development of new antitubercular regimens, the current TB therapeutic arsenal is poor. It has been claimed that several plant natural products inhibit several species of mycobacteria[4, 5].

The tropical Philippine medicinal plant *Uvaria rufa (U. rufa)* Blume ("susung kalabaw" in Filipino) is a short climbing shrub that grows in low- and medium-altitude forests located in Northern Luzon to Palawan and Mindanao. Traditionally, the alcoholic root extract is introduced to laboring women to induce urine contractions (ecbolic)[6]. A triterpene, flavonoid and their glycosides and essential

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oils were previously described in *U. rufa*. In addition to these constituents, a number of highly oxidized cyclohexene derivatives have been identified in samples collected in Asia[7-9]. Isoquercitrin and isoquercitrin-6-acetate, two flavonoids from Thai *U. rufa*, were found comparable with quercetin in inhibiting the formation of advanced glycation end-products (AGEs)[10]. As part of an on-going search for anti-TB phytochemicals from Philippine medicinal plants[11-16], we report in this paper the *in vitro* inhibitory activity of the chloroform extract and its lead acetate - treated extract, petroleum ether and chloroform sub-extracts, their fractions and metabolites *1-7* (Figure 1) from the leaves of *U. rufa* Blume (Annonaceae) against *M. tuberculosis* $H_{37}Rv$.

2. Materials and methods

2.1. Plant collection

U. rufa was collected from Santa, Ilocos Sur, Philippines in April 2004. The voucher specimen (USTH4897) was deposited in the Herbarium of Plant Sciences Laboratory, Research Center for Natural and Applied Sciences. Taxonomic identification was done by Asst. Prof. Rosie S. Madulid.

2.2. Plant extraction and fractionation

Ground, air-dried leaf samples of *U. rufa* (3.34 kg) were extracted with chloroform ($CHCl_3$) to give a green (UrC, 482 g) syrupy extract. A portion of UrC (150 g) was subjected to solvent partitioning in water with 50% hexanes in MeOH, and $CHCl_3$ to give two sub-extracts namely, UrCP (15 g) and UrCC (106 g). Bioactive UrCC was subjected to gradient elution silica gel vacuum liquid chromatography using 20% increment of $CHCl_3$/acetone to furnish eight fractions, from which the most inhibitory fraction one was again chromatographed in silica gel with 20% gradients of petroleum ether/$CHCl_3$ and $CHCl_3$/acetone to afford five fractions.

Another portion of UrC (150 g) was subjected to lead (II) acetate treatment[17], to give a brown syrup (UrCLa, 15 g) which was fractionated in silica gel column with 10% gradients of $CHCl_3$/acetone to give eight fractions.

2.3. Isolation of compounds 1-7

Further purification and identification of **1-7** from these fractions are reported elsewhere[8,9].

2.4. Microplate alamar blue assay (MABA)[18]

Briefly, the test compound MICs against TB were assessed by the MABA using INH, Moxifloxacin and PA824 as positive controls. Compound stock solutions were prepared in DMSO at a concentration of 12.8 mM, and the final test concentrations ranged from 128 mM 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 mg/mL palmitic acid, 5 mg/mL bovine serum albumin, 4 mg/mL catalase, filter-sterilized) in a volume of 100 mL in 96-well microplates (black viewplates). *M. tuberculosi* $H_{37}RV$ (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 ℃. 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 ℃ for 16 to 24 h, fluorescence of the wells was measured (ex 530, 590 nm). The MICs are defined as the lowest concentration effecting a reduction in fluorescence of >90% relative to the mean of replicate bacteria only controls.

2.5. Cytotoxicity assay

Vero cells (ATCC CRL-1586) were cultured in 10% fetal bovine serum (FBS) in minimum essential medium Eagle. J774A.1 cells were cultured in 10% FBS in Dulbecco's modified Eagle'smedium(DMEM). The cells were incubated at 37 ℃ under 5% CO_2 until confluent and then diluted with phosphate-buffered saline to 106 cells/mL. In a transparent 96-well plate (Falcon Microtest 96), threefold serial dilutions of the macrolide stock solutions resulted in final concentrations of 102.4 to 0.42 mM in a final volume of 200 mL. After incubation at 37 ℃ for 72 h, medium was removed and monolayers were washed twice with 100 mL of warm Hanks' balanced salt solution (HBSS). One hundred microliters of warm medium and 20 mL of freshly made MTS-PMS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium and phenylmethasulfazone] (100:20) (Promega) were added to each well, plates were incubated for 3 h, and absorbance was determined at 490 nm.

3. Results

Results of antitubercular activity of the *U. rufa* extracts and fractions based on data provided by the Microplate Alamar Blue assay (MABA)[18] are shown in Table 1. Generally, the results showed that antimycobacterial activities of the chloroform extract (UrC) and its sub-extracts (UrCP and UrCC: *P* = petroleum ether solubles; C = chloroform solubles) and the fractions increased with increasing concentration and by further separation and purification. The chloroform sub-extract (UrCC) was observed to be the most inhibitory extract (MIC = 128 μg/mL). Chromatographic separation of the constituents by increasing solvent polarity in silica column afforded fractions namely, UrCC1, UrCC2 and UrCC4 with enhanced inhibitory rates. Further purification of fraction UrCC1 afforded UrCC12 with an MIC of 24 μg/mL. Thin-layer chromatographic phytochemical analysis of the latter fraction revealed the presence of steroids, terpenoids

and flavonoidal constituents. The fraction was found to have an IC_{50} greater than 102.4 μg/mL against VERO cells.

The known cyclohexenoids **1–5** were isolated from fraction UrCC12, UrCC13 and UrCC14[7,8]. With MICs greater than 128 μg/mL, all compounds were considered inactive.

The UrC extract treated with lead (II) acetate (UrCLa) was also subjected to the MABA assay. Interestingly, extract UrCLa (MIC = 24 μg/mL) and its chromatography were observed to have significantly higher inhibitory activity against *M. tuberculosis* H_{37}Rv compared to its parent UrC extract. The fractions obtained after silica gel fractionation possessed lower MIC values ranging from 8–29 μg/mL against the test organism. Likewise, the major compounds **6** and **7** previously isolated and purified from fraction UrCLa3 (MIC = 8 μg/mL) did not show significant activity against *M. tuberculosi* H_{37}Rv (MIC = >128 μg/mL).

Table 1
% Inhibition and MIC of extracts, sub-extracts and fractions *vs.* *M. tuberculosis* H_{37}Rv.

Crude extracts	Test concentrations		MIC (μg/mL)
	128 μg/mL	64 μg/mL	
UrC	46	18	>128
UrCP	85	70	>128
UrCC	94	70	128
UrCC fractions			
UrCC1	95	72	128
UrCC2	90	49	128
UrCC3	71	47	>128
UrCC4	99	43	128
UrCC5	53	50	>128
UrCC6	61	55	>128
UrCC7	69	56	>128
UrCC8	74	73	>128
UrCC1 fractions			
UrCC11	73	52	>128
UrCC12	99	93	24
UrCC13	99	71	128
UrCC14	91	66	128
UrCC15	87	67	>128

Table 2
%Inhibition and MIC of the lead acetate-treated chloroform extract and its fractions *vs.* *M. tuberculosis* H_{37}Rv.

Extract/Fractions	Test concentrations		MIC (μg/mL)
	128 μg/mL	64 μg/mL	
UrCLa	100	100	24
UrCLa1	100	99	13
UrCLa2	100	100	11
UrCLa3	98	100	8
UrCLa4	99	100	14
UrCLa5	99	99	29
UrCLa6	87	37	>128
UrCLa7	99	74	128
UrCLa8	94	74	128

Standard drug: Rifampin (98% @ 0.128 μg/mL).

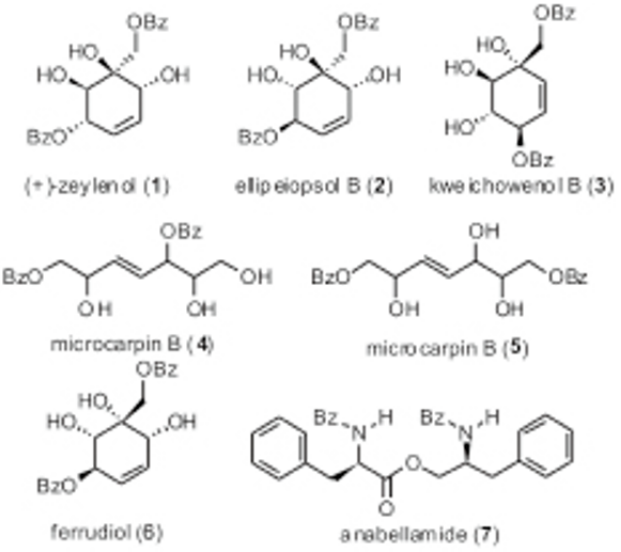

Figure 1. Aromatic constituents from *U. rufa*.

4. Discussion

Ethnomedically, hundreds of plants worldwide are used in traditional medicine as treatments for bacterial infections. Some of these have also been directed to *in vitro* bioassay screening but the effectiveness of such herbal medicines has seldom been severely tested in controlled clinical trials. Conventional drugs usually provide efficient antibiotic therapy for bacterial infections but there is an escalating problem of antibiotic resistance and a continuing need for new medical strategies. Although natural products are not necessarily safer than synthetic antibiotics, most patients prefer to use herbal medicines.

Although much has been reported on the phytochemistry of *U. rufa*, yielding flavonoids and oxidized aromatic constituents as the title compounds, little is known about the pharmacologic promise of its phytometabolites. The ethyl acetate extract of the leaves of *U. rufa* and its flavonol glycoside constituents were previously observed to possess advanced glycation end-products (AGEs) inhibitory activity[10]. The antimycobacterial activity against *M. tuberculosis* H_{37}Rv is a new addition to the activities reported for this plant. Conventional extraction makes use of water as solvent but in our study and experience, it was indisputably established that extraction with organic solvents was more efficient in solubilizing potent antituberculosis compounds[11-16]. Noteworthy, various extracts and fractions obtained by chloroform extraction of the air-dried leaves including a lead-acetate treated extract, exhibited potent antiproliferative property against *M. tuberculosi* H_{37}Rv. The latter chemical treatment was performed to eliminate chlorophyll and other polar pigments[17]. Most phytochemical investigations pertaining to anti-TB assay guided isolations

have yielded (mostly) compounds of weak to strong polarity i.e. steroids, terpenoids, flavonoids, alkylated aromatics and lactones, acetylenics and alkaloids among others[4,5]. Although no considerable antitubercular activity was observed for the compounds known for this plant (**1–7**) [7,8], it is worth noting that many other constituents may be isolated from the antitubercular fraction UrCC12 which was also observed to be non-cytotoxic to VERO cells. The toxicity assessment against VERO cells may model toxicity to mammalian cells. Thus, with potent antimycobacterial activity, fraction UrCC12, the cytotoxicity test result indicate that it maybe a good source of anti-TB compounds with selective activity. In addition, thin-ayer chromatographic analysis indicated the presence of phytochemical constituents such as flavonoids that may demonstrate a promising antitubercular activity. It has been reported that flavonoids exhibit antimycobacterial activity by inhibiting enzymes involved in the fatty acid and mycolic acid biosynthesis[19]. Furthermore, several flavonoids were also observed to have isoniazid (INH) modulating activities and thus could be administered with anti-TB treatment to prevent or counter resistance to INH[20]. Therefore, further investigations warrant the isolation of bioactive principles which may become future alternative therapeutic agents for tuberculosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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