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## Anti-Tubercular Flavonol Derivatives from *Uvaria rufa*.

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### ABSTRACT

Bioassay-guided chromatographic purification of the crude -methanolic extract of the Philippine medicinal plant *Uvaria rufa carteri* resulted in the isolation and identification of a mixture 1:1 the flavonols kaempferol (**1**) and quercitrin (**2**) on the basis of spectroscopic evidences (UV, IR, and NMR) and comparison with literature data. Microplate Alamar Blue assay showed moderately strong antitubercular activity for these flavonol derivatives.

**Keywords:** *Uvaria rufa*, flavonoids, kaempferol, quercitrin, antitubercular.

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## INTRODUCTION

The current first-line regimen for treating tuberculosis (TB) is considered old and prescribes rifampicin and isoniazid as component drugs. These antibiotics overtime effect the rise of multi-drug resistant (MDR) and extensively drug resistant (XDR) tuberculosis strains [1]. This increasing drug resistance incidence has led to an urgent need for the discovery of new plant natural products that may potentially eradicate TB. It has been claimed that several plant natural products inhibit several species of mycobacteria [2-3].

The Philippine medicinal plant *Uvaria rufa* Blume ("*susung kalabaw*" in Filipino) is a short climbing shrub found in low- and medium-altitude forests of Northern Luzon, Philippines. The alcoholic root extract is ethnomedicinally used to induce urine contractions (ecbolic) in pregnant women [4]. Triterpene, flavonoid and their glycosides, essential oils and a number of highly oxidized cyclohexene derivatives have been identified in samples growing in Asia [5-7]. Isoquercitrin and isoquercitrin-6-acetate, two flavonoids from Thai *U. rufa*, inhibit the formation of advanced glycation end-products (AGEs) [8]. In addition, the chloroform extract and fractions of *Uvaria rufa* have been reported to exhibit strong antitubercular activity. However, the major compounds isolated showed weak activity against *Mycobacterium tuberculosis*. As part of our continued interest to discover anti-TB phytochemicals from Philippine medicinal plants [9-17], we herein report the *in vitro* inhibitory activity of the methanol extract, fractions and flavonoids (**1** and **2**) from the leaves of *Uvaria rufa* Blume (Annonaceae) against *Mycobacterium tuberculosis* H<sub>37</sub>Rv.

## MATERIALS AND METHODS

<sup>1</sup>H and <sup>13</sup>C NMR were determined in MeOH-D<sub>4</sub> with TMS as internal standard at 300 MHz for <sup>1</sup>H and 75 MHz for <sup>13</sup>C. Column chromatography: silica gel 60 Merck Art. 7734 (230-400 mesh). TLC: Merck pre-coated silica gel plates 60 F<sub>254</sub> (0.25 mm thick). Color reactions were done by spraying TLC plates with vanillin/sulfuric acid, ferric chloride/potassium ferricyanide/HCl and Natural Product/polyethylene glycol reagents.

### Collection and identification of plant sample

*Uvaria rufa* Blume were collected at the lowland forests of Santa, Ilocos Sur, Philippines (April, 2004). Herbarium specimens (USTH4897) were deposited at the UST Research Center for the Natural and Applied Sciences Herbarium, Manila, Philippines. Plant identification was facilitated by Asst. Prof. Rosie S. Madulid.

### Extraction and purification

The air-dried leaves of *Uvaria rufa* (3.34 kg) methanol (MeOH) to give reddish-brown (UrM, 505 g) syrupy extract. An aliquot of UrM (200 g) was fractionated in water using ethyl acetate (EtOAc) and n-butanol to give sub-extracts, UrME (101 g) and UrMB (17 g). UrME was chromatographed initially by silica gel vacuum liquid column chromatography using EtOAc/MeOH gradients (20%) to give six fractions, from which the most active fraction UrMe3 (Table 1) was further chromatographed in silica gel 60 with 7:3:0.2 EtOAc/MeOH/formic acid to give fraction UrME32 (23.4 mg). This fraction was separated in Sephadex LH-20 (acetone/MeOH) to give a semi-pure flavone glycoside which was positive in Natural Product/polyethylene glycol and  $\alpha$ -naphthol/H<sub>2</sub>SO<sub>4</sub> sprays on TLC. Base-acid extraction (sodium acetate – 0.5M HCl) afforded a pale yellow solid (18.5 mg) which was identified with UV, IR and <sup>1</sup>H NMR experiments (1D and <sup>1</sup>H-<sup>1</sup>H correlation spectroscopy) as a 1:1 mixture of kaempferol (**1**) and quercitrin or quercetin-3-O- $\beta$ -D-glucoside (**2**) (Figure 1) [18-19].

Compound **1** and **2**. Yellow amorphous powder. <sup>1</sup>H NMR  $\delta$  (300 MHz, MeOH-*d*<sub>4</sub>). Kaempferol. 8.09 (2H, d, *J* = 8.8 Hz, H-2', 6'), 6.91 (2H, d, *J* = 8.8 Hz, H-3', 5'), 6.38 (1H, br.s, H-8), 6.13 (1H, br.s, H-6). All data were identical with that of kaempferol [18]. Quercitrin. 12.64 (1H, s, 5-OH), 7.59 (1H, d, *J* = 2.5 Hz, H-2'), 7.57 (1H, dd, *J* = 8.5, 2.5 Hz, H-6'), 6.84 (1H, d, *J* = 8.7 Hz, H-5'), 6.40 (1H, d, *J* = 2.5 Hz, H-8), 6.20 (1H, d, *J* = 2.5 Hz, H-6), 5.47 (1H, d, *J* = 6.9 Hz, H-1''), 5.4~3.0 (10H, m, glc). All data were identical with that of quercetin-3-O- $\beta$ -D-glucopyranoside [19].

### Microplate Alamar Blue Assay

The extracts, fractions and isolates were screened for inhibitory activity against *M. tuberculosis* H<sub>37</sub>Rv using a Microplate Alamar Blue assay (MABA) as previously described by Collins and Franzblau [20]. Extracts,

fractions and flavonol mixture (**1** and **2**, 1:1) sample dilutions were prepared in either dimethyl sulfoxide or distilled ionized water, and subsequent two-fold dilutions were performed in 0.1 mL of 7H9GC (no Tween 80) in the microplates. Wells were observed at 12 and 24 h for a color change from blue to pink and for a reading of > or equal to 50,000 fluorescence units (FU). Fluorescence was measured in a Cytofluor II microplate fluorimeter with excitation at 530 nm and emission at 590 nm. Visual MIC's were defined as the lowest concentration of drug that prevented a color change. Percent inhibition was defined as  $1 - (\text{test well FU} / \text{mean FU of triplicate B wells}) \times 100$ . The lowest drug concentration effecting an inhibition of > or equal to 90% was considered the MIC. For comparison purposes, rifampin was used as positive standard (98% inhibition at 0.125  $\mu\text{g}/\text{mL}$ ).

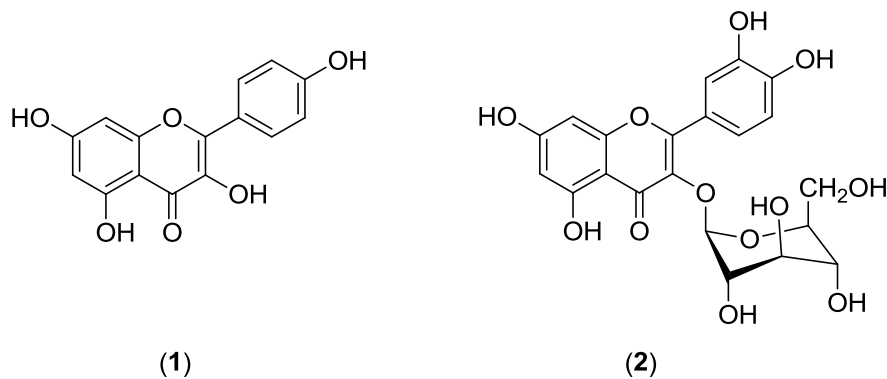


Figure 1: Antitubercular flavonoids from *Uvaria rufa*.

Table 1: %Inhibition and MIC of the methanolic extract, sub-extracts and UrME fractions vs. *M. tb. H<sub>37</sub>Rv*.

Extract/Fractions	Test Concentrations		MIC ( $\mu\text{g}/\text{mL}$ )
	128 $\mu\text{g}/\text{mL}$	64 $\mu\text{g}/\text{mL}$	
UrM	49	21	>128
UrME	49	22	>128
UrMB	45	28	>128
UrME1	47	24	>128
UrME2	58	38	>128
UrME3	99	96	64
UrME4	42	41	>128
UrME5	38	25	>128
UrME6	27	17	>128

Standard drug: Rifampin (98% @ 0.128  $\mu\text{g}/\text{mL}$ )

## RESULTS AND DISCUSSION

Initial screening for susceptibility against *Mycobacterium tuberculosis* H<sub>37</sub>Rv using the colorimetric Microplate Alamar Blue assay (MABA) showed the crude methanolic extract (UrM), ethyl acetate sub-extract (UrME) and *n*-butanol sub-extract (UrMB) to be weakly inhibitory with >128  $\mu\text{g}/\text{mL}$  minimum inhibitory concentration (Table 1). Interestingly, silica gel fractionation of the ethyl acetate sub-extract yielded a fraction with MIC value 64  $\mu\text{g}/\text{mL}$ . After tedious purifications, the antitubercular principles, a 1:1 mixture of flavonoids **1** and **2** (MIC=64  $\mu\text{g}/\text{mL}$ ) were identified spectroscopically and through comparison with literature data. This is the first report on the occurrence of these flavonoids from *U. rufa*.

Flavonoids are natural products that form a major group of bioactive polyphenolic secondary metabolites. Recently, it has been reported that several derivatives of these compounds inhibit the growth of *M. bovis* BCG [21]. Moreover, their data suggest that flavonoids are inhibitors of mycobacterial FAS-II and Rv0636 which are involved in mycolic acid biosynthetic pathways of mycobacteria. In a separate study, the antitubercular activity of several flavonoid derivatives and their structure–activity relationships have been studied against *Mycobacterium tuberculosis* H<sub>37</sub>Rv radiometrically using the BACTEC 460 assay which led to the identification of five flavonoids (luteolin, baicalein, quercetin, myricetin and hispidulin) as new antitubercular natural products [22]. For significant antitubercular activity, hydroxyl substitutions at C-5 and C-7 in the flavonoidal structures leads to inactivity, whereas hydroxyl substitutions at C-5, C-6, C-7 (a trihydroxy) or C-3',

C-4' (dihydroxy) are of particular importance for antitubercular activity of a flavonoid – an observation which corroborates well with the present antitubercular activity of **1** and **2**. The presence of methyl and glycosidic residues in these substitution patterns may lead to inactivity against the test organism. Based on the results presented in this study, it may be possible to conclude that the mechanism underlying the inhibitory activity of **1** and **2** follow those of the reported, related compounds.

This paper reports for the first time the potential use of *U. rufa* as source of antitubercular agents. Furthermore, this study contributes to a number of compounds, in particular flavonoids, which may exhibit inhibitory activity against enzymes that participate in the anabolism of mycobacterial cell wall.

### CONCLUSION

The focal point of the current study was the identification of a flavonoid as synergistic antitubercular principles from the Philippine medicinal plant, *Uvaria rufa*. Consequently, the degree of activity by these flavonol compounds make these compounds a privileged structure to discover more active derivatives in ongoing studies.

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