Table 1: Percent inhibitory activity of the extracts, sub-extracts and fractions of *A. purpurata* at 64 µg/ml against *M. tuberculosis* $H_{37}Rv$

Sample	% Inhibition
Crude ethanolic extracts	
Flowers	30
Leaves	62
Rhizome	34
Leaf sub-extracts	
Hexane	64
DCM	72
n-Butanol	35
Hexane fractions (APH)	
APH 1	47
APH 2	38
APH 3	55
APH 4	48
APH 5	61
APH 6	74
APH 7	73
APH 8	70
APH 9	74
APH 10	46
APH 11	23
DCM fractions (APD)	
APD 1	65
APD 2	58
APD 3	68
APD 4	60
APD 5	69
APD 6	66
APD 7	46
APD 8	50
APD 9	64

Control: rifampin, 99% at 0.18 µg/ml

fraction two of the hexane sub-extract, which afforded a white amorphous solid after crystallization. This compound was distinctly identified by the ^{1}H-NMR and ^{13}C-NMR spectra to be a fatty alcohol, but the LR-EIMS spectrum otherwise showed it to be a mixture of fatty alcohols. By careful analysis of the *m/z* values in the mass spectrum, it could be claimed that it is composed of montanyl alcohol (C28:0), melissyl alcohol (C30:0, major component) and domelissyl alcohol (C32:0), based on several characteristic peaks due to fragment ions of $[M^+-H_2O]$.[11]

The dichloromethane extract was likewise subjected for further investigation owing to its interesting phytochemical profile. Nine fractions were obtained after VLC from which fractions three and four yielded three solid compounds **1–3**.

Compound **1**, a yellow crystalline substance (13.0 mg), was purified from fraction three after recrystallization. It was found to be a flavonoid after treating its thin layer chromatograms with $FeCl_3$-$K_4Fe(CN)_6$ and 10% $SbCl_3$ in chloroform, as shown by a blue-green spot and red-orange fluorescence, respectively, under UV (530 nm).[12] In addition, major absorptions at 269 nm (band I) and 352 nm (band II) and a weak shoulder at 303 nm, which are typical for flavones, were observed in its UV spectrum.[13,14] The structure, and degree and pattern of oxygenation in the flavonoid structure were examined by studying the effect of several wavelength shift reagents, NaOMe, NaOAc, NaOAc-1% aq. H_3BO_3, $AlCl_3$ and $AlCl_3$-HCl in the UV spectral region of **1**. With NaOMe, a 44-nm bathochromic shift and a significant increase in absorbance intensity were noted for band II. This indicates the presence of a C-4' hydroxyl group. Treatment with NaOAc gave no observable change in the spectrum, which shows that an alkoxy substituent is present in the C-7 position of the flavone nucleus. Addition of 1% boric acid produced no change in the spectrum, which is symptomatic of the absence of ortho-hydroxyphenolic functionalities. This was also substantiated by the result of adding 0.1 M HCl/$AlCl_3$ in a separate experiment. The appearance of four absorption peaks (278, 304, 352 and 399 nm) after the complexation of $AlCl_3$ with **1** is a clear indication that a 5-hydroxyl moiety is present.[14]

The IR spectrum of 1 showed the presence of an enone (1665 cm^{-1}) and phenolic OH's (3243 cm^{-1}). The base peak at *m/z* 314 in the LR-EIMS mass spectrum was designated the molecular-ion peak. In the 500-MHz ^{1}H NMR spectrum, two sets of AA´BB´-protons belonging to a para-substituted benzenoid moiety, two methoxy protons and meta-coupled protons were noted. In the proton-decoupled ^{13}C and DEPT-135 NMR spectrum, a total of 18 carbon atoms were accounted for **1**, from which a conjugated ketone, six oxygenated olefinic/aromatic carbons, six aromatic methines and two methoxy carbons were deduced. The gross structure of the compound which is analogous to kumatakenin[15,16] was elucidated via an HMBC experiment. Key ^{1}H-^{13}C correlations are shown in Figure 2.

It is noteworthy to report the isolation of kumatakenin (**1**) from *A. purpurata*. This rare ethyl ether flavonol was first isolated from the seeds of *Alpinia japonica*[17] and *Alpinia kumatake*.[18] Hence, the identification of **1** strengthens the chemical link of *A. purpurata* with the other species of *Alpinia*.

Compound **2** was obtained as a white amorphous solid (8.4 mg). Thin layer chromatograms of the isolate sprayed with Liebermann-Burchard and Molisch reagents[12] suggested a steroidal glycoside structure. The IR spectrum showed the presence of hydroxyl (3439 cm^{-1}) and ester (1738 cm^{-1}) functionalities. The molecular ion peak was not observed in the LR-EIMS spectrum. Instead, fragment ions corresponding to sitosterol (*m/z* 414, $C_{29}H_{50}O$) and a

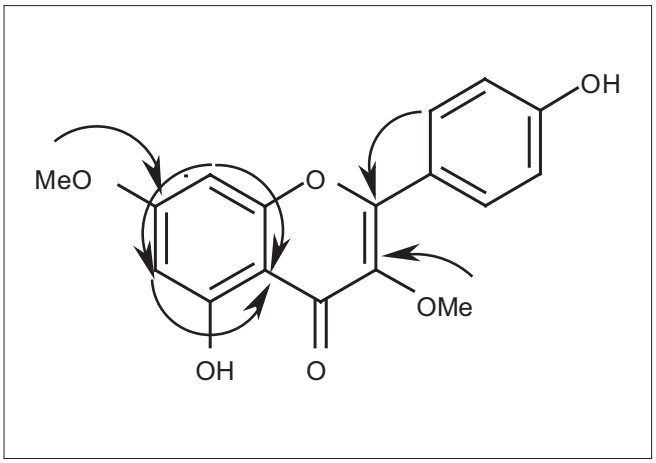

Figure 2: Key ^{1}H-^{13}C HMBC correlations in **1**

palmitoxy group (*m*/*z* 256, $C_{16}H_{31}O_2$) were noted. Signals characteristic of sitosterol resonances, i.e., C-6 olefinic proton (δ 5.36), methyls associated with the cholestane skeleton (δ 0.69–0.79), glucose (δ 4.34–4.38) and a palmitoyl group (δ 0.84, 1.26, 2.34) were evident in the 500-MHz ^{1}H NMR of 2. A total of 51 carbon atoms, i.e., 7 CH_3, 25 aliphatic CH_2, 7 aliphatic CH, 2 aliphatic quaternary C, 1 oxygenated CH_2, 6 oxygenated CH and one each olefinic CH and C, were found in the 125 MHz ^{13}C NMR spectrum. HMBC correlations, which were instrumental in finding the correct identity of **2** as sitosteryl-3-O-6-palmitoyl-β-d-glucoside, are shown in Figure 3. The NMR values of **2** are well in agreement with those reported for sitosteryl-3-O-6-palmitoyl-β-D-glucoside by Pei-Wu *et al*.,[19] Gomes *et al*.,[20] and Shaiq Ali *et al*.[21]

Metabolite **3** was obtained as white crystalline flakes (4.1 mg). The partial identity of **3** was revealed to be a steroidal glycoside as in **2** using the same phytochemical experiments. Only the occurrence of hydroxyl functionalities was inferred this time from the IR spectrum. The presence

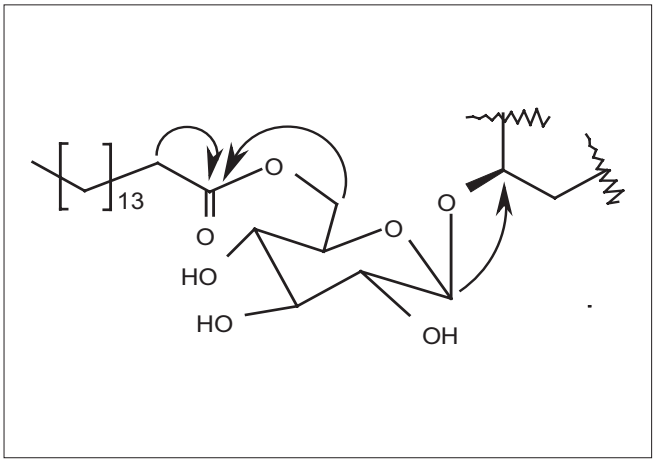

Figure 3: Key ^{1}H-^{13}C HMBC Correlations in **2**

of a sitosterol fragment was ascertained in the LR-EIMS spectrum, which was also verified by the ^{1}H and ^{13}C NMR spectra. The sugar moiety was deduced to be galactose upon comparison of the ^{1}H-NMR and ^{13}C-NMR values with those in literature.[22-24] Hence, the identity of **3** was established.

Sitosteryl glycosides **2** and **3** have been isolated from other several plant sources.[19,21] These metabolites are also present in plant species belonging to the family Zingiberaceae.[25]

The fatty alcohols showed an MIC value of 64 μg/ml and proved most active compared to the flavonoid kumatakenin and the steroidal glycosides previously reported to have MIC values >128 μg/ml. Long chain alcohols show growth inhibitory activity to Gram-positive organisms including *Staphylococcus aureus* and *Propionibacterium acnes*.[26] Moreover, a study done by Togashi and co-workers in 2007 further supported the antibacterial activity of long chain aliphatic alcohols that had bactericidal activity and membrane-damaging activity on *S. aureus*. Experimental results indicate that the antibacterial activity of long chain alcohols is mediated by damage to cell membranes, which allows leakage of K^+ ions, with subsequent reactions that induce further leakage.[27] Thus, the ability of the isolated fatty alcohols to inhibit the growth of *M. tuberculosis* $H_{37}Rv$ gives further credence on the antibacterial activity of long chain alcohols, particularly those bearing more than 20 carbon chains.

The activity of kumatakenin confirms the study by Murillo *et al*.[28] on its action against *M. tuberculosis* $H_{37}Rv$. This compound has antiviral activity against HIV,[29] the virus that aggravates the problem on tuberculosis (TB) due to susceptibility to the lung pathogen. Plant sterols, particularly β-sitosterol and its glucosides, have been investigated as immune regulators of T-cell activity[30] and as agents in maintaining the CD_4^+ count in the absence of anti-retroviral therapy in HIV-infected patients.[31] Plant sterols were effective in patients treated for pulmonary TB, causing an increase in their peripheral blood lymphocytes and eosinophil counts.[32]

To date, this is the first report on the compounds **2** and **3** from the genus *Alpinia*. More importantly, this article cites for the first time the isolation of all compounds from *A. purpurata*. Furthermore, this study demonstrates the promise of this plant as a source of phytomedicinals that can fight TB.

ACKNOWLEDGMENTS

This study was supported by the Philippine Council for

Advanced Science and Technology Research and Development, Department of Science and Technology, Manila, and by the Department of Chemistry, College of Science, University of Santo Tomas, Manila. The authors thank Dr. Yuehong Wang for the MABA, Ms. Bernadette Macalino and Mr. Emerson Espadero for initial studies, and Prof. Mary Garson for useful discussions.

REFERENCES

1. Chopra I, Khajuria B, Chopra C. Antibacterial properties of volatile principles from *Alpinia galanga* and *Acorus calamus*. Antibiot Chemother 1957;7:378-83.
2. Lee S, Shin H, Hwang H, Kim J. Antioxidant activity of extracts from *Alpinia katsumadai* seed. Phytother Res 2003;17:1041-7.
3. Madulid DA. A Pictorial Cyclopedia of Philippine Ornamental Plants. Manila: Domingo A. Madulid and Bookmark, Inc; 1995.
4. Ali M, Banskota A, Tezuka Y, Saiki I, Kadota S. Antiproliferative activity of diarylheptanoids from the seeds of *Alpinia blepharocalyx*. Biol Pharm Bull 2001;24:525-8.
5. Zoghbi M, Andrade E, Maia JG. Volatile constituents from the leaves and flowers of *A. speciosa K. Schum*. and *A. purpurata* (Vieill.) K. Schum. Flavour Fragrance J 1999;14:411-4.
6. Shimoda H, Shan S, Tanaka J, Okada T, Murai H. Anti-inflammatory agents from red ginger. U.S.: Pat Appl Publ; 2007. p. 24.
7. Shimoda H. Plant materials for bone and joint diseases: Citrus unshiu and red ginger. Food Style 21. 2008;12:74-5.
8. Aguinaldo AM. Selected Zingiberaceae species exhibiting inhibitory activity against *Mycobacterium tuberculosis* H_{37}Rv: A phytochemical profile. Gardens' Bull Singapore. 2007;59:13-22.
9. Mandap K, Marcelo R, Macabeo AP, Yamauchi T, Abe F, Franzblau SG, *et al*. Phenyldecanoids from the antitubercular fractions of the Philippine ginger (*Zingiber officinale*). ACGC Chem Res Comm 2007;21:20-2.
10. Collins LA, Franzblau SG. Microplate Alamar Blue Assay versus BACTEC 460 System for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob Agents Chemother 1997;41:1004-9.
11. Kanya, Sindhu TC, Jao JL, Sastry SM. Characterization of wax esters, free fatty alcohols and free fatty acids of crude wax from sunflower seed oil refineries. Food Chem 2007;101:1552-7.
12. Guevara B. A guidebook to plant screening: Phytochemical and biological. Manila: University of Santo Tomas; 2004.
13. Harbourne JB, Baxter H. Phytochemical dictionary: A handbook of bioactive compounds from plants. London: Taylor and Francis; 1993.
14. Martin T, Fabon C, Hernandez H. Ultraviolet spectral analysis of flavonoids. Proceedings of the Sub-Regional Workshop on Plant Glycosides I. U.P. Los Banos, Laguna: Regional Network for Chemistry of Natural Products in Southeast Asia; 1988.
15. Wang Y, Hamburger M, Gueho J, Hostettman K. Antimicrobial flavonoids from *Psidia trinervia* and their methylated and acylated derivatives. Phytochemistry 1989;28:232-7.
16. Urbatsch L, Mabry T, Miyakado M, Ohno N, Yoshioka H. Flavonol methyl ethers from *Ericameria diffusa*. Phytochemistry 1976;15:440-1.
17. Kimura Y, Takido M, Takahashi S. Studies on the constituents of the seeds of *Alpinia japonica*. Yakugaku Zasshi 1967;87:1132-3.
18. Kimura Y, Takido M, Takahashi S, Kimishima M. Studies on the constituents of the seeds of *Alpinia kumatake*. Yakugaku Zasshi1967;87:440-3.
19. Pei-Wu G, Fukuyama Y, Rei W, Jinxian B, Nakagawa K. An acylated sitosterol glucoside from Alisma plantago-aquatica. Phytochemistry 1988;27:1895-6.
20. Gomes D, Alegrio L. Acyl steryl glycosides from *Pithecellobium cauliflorium*. Phytochemistry 1998;49:1365-7.
21. Shaiq Ali M, Saleem M, Ahmad W, Parvez M, Raghav Y. A chlorinated monoterpene ketone, acylated β-sitosterol glycosides and flavanone glycoside from *Mentha longifolia* (Lamiaceae). Phytochemistry 2002;59:889-95.
22. Ahmad V, Aliya R, Perveen S, Shameel M. A sterol glycoside from marine green alga *Codium iyengarii*. Phytochemistry 1992;31:1429-31.
23. Ahmed W, Ahmad Z, Malik A. Stigmasteryl galactoside from *Rhynchosia minima*. Phytochemistry 1992;31:4038-9.
24. Crouch NR, Langlois A, Mulholland DA. Bufadienolides from the southern African Drimia depressa (Hyacinthaceae: Urgineoideae). Phytochemistry 2007;68:1731-4.
25. Ayimele GA, Tane P, Connolly J. Aulacocarpin AB, nerolidol and β-sitosterol glucoside from *Aframomum escapum*. Biochem Syst Ecol 2004;32:1205-7.
26. Kubo I, Muroi H, Himejima H, Kubo A. Antibacterial activity of long-chain alcohols: The role of hydrophobic alkyl groups. Bioorg Med Chem Lett 1993;3:1305-8.
27. Togashi N, Shiraishi A, Nishizaka M, Matsuoka K, Endo K, Hamashima H, *et al*. *Antibacterial activity* of long chain fatty alcohols against *Staphylococcus aureus*. Molecules 2007;12:139-48.
28. Murillo J, Encarnacion-Dimayuga R, Malstrom J, Christophersen C, Franzblau SG. Antimycobacterial flavones from *Haploppapus sonorensis*. Fitoterapia 2003;74:226-30.
29. Fukai T, Sakagami H, Toguchi M, Takayama F, Iwakura I, Atsumi T, *et al*. Cytotoxic activity of low molecular weight polyphenols against human oral tumor cell lines. Anticancer Res 2000;20:2525-36.
30. Bouic PJ, Etsebeth S, Liebenberg RW, Albrech CF, Pegel K, Van Jaarsveld PP. Beta-sitosterol and beta-sitosterol glucoside stimulate human peripheral blood lymphocyte proliferation: Implications for their use as an immunomodulatory vitamin combination. Int J Immunopharmaco 1996;18:693-700.
31. Breytenbach U, Clark A, Lamprecht J, Bouic P. Flow cytometric analysis of the Th1-Th2 balance in healthy individuals and patients infected with human immunodeficiency virus (HIV) receiving a plant sterol/sterolin mixture. Cell Biol Int 2001;25:43-9.
32. Donald PR, Lamprecht JH, Freestone M, Albrecht CF, Bouic PJ, Kotze D, *et al*. A randomized placebo-controlled trial of the efficacy of beta-sitosterol and its glucoside as adjuvants in the treatment of pulmonary tuberculosis. Int J Tuberc Lung Dis 1997;1:518-22.

Source of Support: Philippine Council for Advanced Science and Technology Research and Development, Department of Science and Technology, Manila, and by the Department of Chemistry, College of Science, University of Santo Tomas, Manila., **Conflict of Interest:** None declared