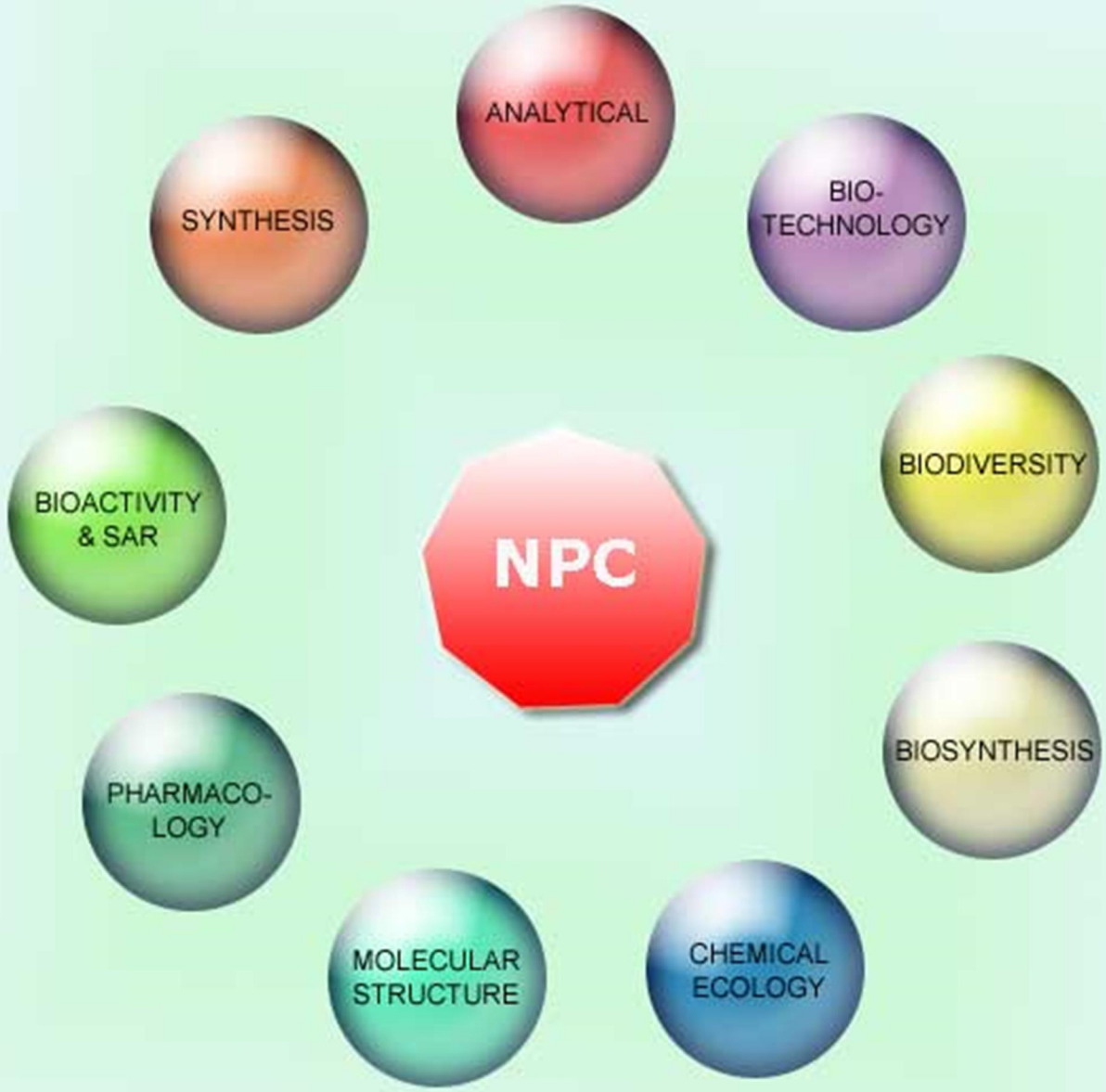
NATURAL PRODUCT COMMUNICATIONS

An International Journal for Communications and Reviews Covering all Aspects of Natural Products Research



Volume 8. Issue 6. Pages 689-846. 2013
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

NPC Natural Product Communications

INFORMATION FOR AUTHORS

Full details of how to submit a manuscript for publication in Natural Product Communications are given in Information for Authors on our Web site http://www.naturalproduct.us.

To Subscribe: Natural Product Communications is a journal published monthly. 2013 subscription price: US$2,395 (Print, ISSN# 1934-578X); US$2,395 (Web edition, ISSN# 1555-9475); US$2,795 (Print + single site online); US$595 (Personal online). Orders should be addressed to Subscription Department, Natural Product Communications, Natural Product Inc., 7963 Anderson Park Lane, Westerville, Ohio 43081, USA. Subscriptions are renewed on an annual basis. Claims for nonreceipt of issues will be honored if made within three months of publication of the issue. All issues are dispatched by airmail throughout the world, excluding the USA and Canada.

NPC Natural Product Communications

2013
Vol. 8
No. 6
703 - 705

Constituents of the Indonesian Epiphytic Medicinal Plant *Drynaria rigidula*

Ari S. Nugraha[a,b], Rachada Haritakun[c] and Paul A. Keller[a*]

[a]*School of Chemistry*, *Faculty of Science*, *University of Wollongong*, *Wollongong*, *NSW*, *Australia 2500*
[b]*Department of Pharmaceutical Chemistry*, *Faculty of Pharmacy*, *University of Jember*, *Indonesia 68121*
[c]*Bioresources Technology Unit (BTU)*, *National Center for Genetic Engineering and Biotechnology (BIOTEC)*, *Pathumthani*, *Thailand 12120*

keller@uow.edu.au

Received: February 28th, 2013; Accepted: March 19th, 2013

The rhizome of *Drynaria rigidula* has been traditionally claimed to have anti-infective properties. A phytochemical study conducted on the rhizome successfully isolated two new aromatic glycosides as well as three known terpenoids, two benzoic acid derivatives and two known flavonoid glycosides. The rhizome methanol extract and its fractions were tested against *Mycobacterium tuberculosis*, *Plasmodium falciparum*, herpes simplex virus and KB-oral cancer cell; insignificant activity was found.

Keywords: Epiphytic medicinal plant, Indonesia, Bondowoso, *Drynaria rigidula*, Terpenes, Flavonoids, Glycosides, Anti-cancer, Anti-malaria, Anti-TB, Anti-HSV.

Since early Indonesian civilization, medicinal plants have been used to treat ailments from generation to generation with knowledge of their healing properties being passed through verbal and non-verbal records. Throughout the archipelago, more than 9,000 plants have been recorded to have medicinal properties. However, a recent study [1] concluded that only a small percentage of them have been studied – this includes the particularly neglected Indonesian epiphytic plants, both parasitic and non parasitic varieties.

Epiphytes occupy more than 10% of the world's total plant species and the majority exists in the Magnoliophyta division [2]. Currently, our laboratory is developing a record of epiphytic medicinal plants that covers the ethnopharmacology, ethnopharmacy, phytochemistry and pharmacology of 260 epiphytic medicinal plants.

Our study started in early 2010, with field trips in the Malabar Forest, East Java, Indonesia where we surveyed, selected and collected epiphytic medicinal plants used traditionally for infection therapy. In this paper, we report the phytochemical study of *Drynaria rigidula*, used traditionally as an anti-viral and anti-inflammatory agent, and to treat eye infections and gonorrhea.

Drynaria species are common epiphytes in tropical forests. The genus is composed of at least 16 species with only 5 being claimed to have medicinal properties: *D. fortunei, D. quercifolia, D. propinqua, D. sparsisora* and *D. rigidula*. *D. fortunei* is highly prescribed in China for osteoporosis therapy with recent findings revealing that the flavonoids present significantly suppress osteoclast activities. Phytochemical studies have been performed on the first three species, with 30 compounds being isolated, including steroids, flavonoids and their glycosides [3-7].

D. rigidula is an epiphytic bird's-nest fern locally named as *simbar layangan* or *pasilan kelapa*. It has fertile and sterile leaves. The species is distributed throughout the Indonesian Islands as well as southeast Asia to Polynesia (Fiji) and Australia. To the people of Java, the rhizome of *D. rigidula* has been used in traditional medicine, but, to date, there has been no phytochemical investigation of this species. We report here the results of our study into the bioactive constituents of this plant [8].

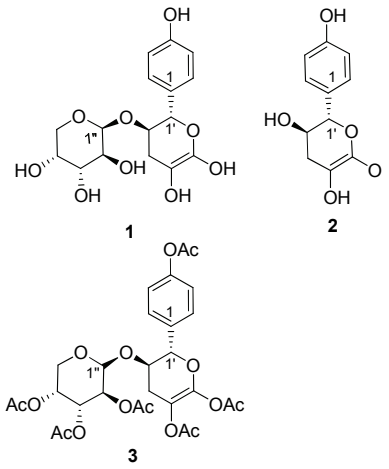


Figure 1: Aromatic glycosides isolated from *D. rigidula* rhizome. Compound **3** was obtained from acetylation of **1**.

The crude extract of the rhizomes was fractionation into *n*-hexane, dichloromethane, ethyl acetate and residual fractions. Column chromatography of the *n*-hexane and dichloromethane fractions, followed by preparative thin layer chromatography, produced three isolated compounds. EIMS and nuclear magnetic resonance spectral analyses suggested these were the known compounds fern-9(11)ene [9], hop-22(29)-ene [10], and γ-sitosterol [11]. These terpenes are common in ferns, but are reported here for the first time from *D. rigidula*.

The ethyl acetate and residual fractions produced similar HPLC profiles; therefore, our study only focused on the ethyl acetate

fraction. The first two peaks with t_R of 6 and 8 minutes were identified from subsequent NMR spectral comparison as the known benzoic acids, 3,4-dihydroxybenzoic acid and 4-hydroxybenzoic acid. Two other known glycosidic flavonoids, kaempferitrin [12] and 3,5-dihydroxy-flavone-7-*O*-*β*-rhamnopyranosyl-4'-*O*-*β*-glucopyranoside [13], came with t_R of 35 and 42 minutes, respectively. For two previously unreported compounds, isolated with t_R of 14 and 17 minutes, we propose the glycoside structures **1** and **2** (Figure 1).

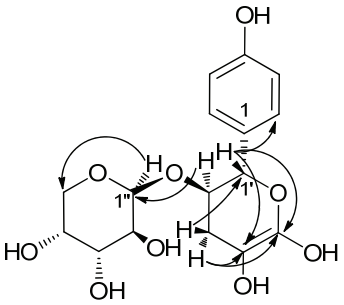


Figure 2: Selected proton-carbon correlations in compound **1**.

Compound **1**, with a t_R of 14 minutes, was a UV-active brown solid. ^{1}H NMR and ^{13}C NMR analyses indicated a glycoside molecule with an aromatic aglycon and two sugar moieties, for which gCOSY and TOCSY spectral analysis clearly indicated one aromatic proton system (aglycon) and two proton systems (two sugar units). The aglycon (proton system) was assigned as a 1,4-disubstituted benzene ring with the quaternary carbon at 158 ppm assigned as attached to an -OH substitution (C4). The COSY and TOCSY spectra clearly indicated proton H1' to H3' of the first sugar unit. Through gHMBC spectral analysis, two quaternary alkene carbons, δ 100 (C4') and 156 (C5'), were assigned to be components of the first sugar ring system.

The chemical shift of the anomeric carbon C1' at 80 ppm was lower than that of the common anomeric sugar carbon chemical shifts. This suggested a C-C bond which, through gHMBC spectral analysis, clearly indicated a C-C bond bridge between C1' and C1. COSY and TOCSY spectral analyses were also able to assign proton H1" to H5" of the second sugar ring system, with coupling constant patterns of $^3J_{H1\text{-}H2}$= 7.5 Hz, $^3J_{H2\text{-}H3}$ = 9.0 Hz, $^3J_{H3\text{-}H4}$ = 9.0 Hz, and $^3J_{H4\text{-}H5}$ = 5.5 Hz, which suggest proton-proton configuration patterns of axial-axial, axial-axial, axial-axial, and axial-equatorial, respectively [14]. In combination, this suggested an arabinopyranose moiety, which was confirmed by a MS/MS experiment, which showed a fragment loss of 132 a.m.u. The coupling constant of the anomeric proton ($^3J_{H1\text{-}H2}$ = 7.0 Hz, H1") suggested a *β* configuration. The anomeric carbon chemical shift at 104 ppm of the second sugar clearly indicated a C-O bond bridge as the gHMBC was able to allocate a C-O-C bond between C2' and C1". The proposed molecular structure of **1** is illustrated in Figure 2, along with selected H-C correlations.

Interestingly, we have assigned **1** as the ene-diol and not the corresponding keto molecule. There was no stretch in the IR spectrum typical of a carbonyl bond, and in the ^{13}C NMR spectrum, there were no signals that could be assigned to a carbonyl. Furthermore, there were chemical shifts at δ 156.5 (C5') and δ 100.7 (C4') assigned to the alkene attached to 2 x oxygen substituents (i.e. OH) in the pyran ring system. Therefore, the NMR analysis suggests an unusual ene-diol structure instead of the 'keto' form.

In order to confirm the assignment of **1**, we undertook exhaustive acetylation of the isolated natural product. Analysis of the ESI MS of this product revealed a peak at *m/z* 681, an extra 252 a.m.u over **1**, corresponding to the addition of six acetyl groups, confirming that six hydroxyl groups had been acetylated. Based on the NMR spectral analysis we found 16 carbons, 14 hydrogens and the carbon chemical shift analysis indicated 3 oxygens and 5 hydroxyl groups. Therefore, **1** had a molecular formula of $C_{16}H_{20}O_9$, with molecular weight of 356 a.m.u.

Spectral analysis of compound **2** indicated that the compound was identical to compound **1**, but without the second sugar moiety.

Biological testing: The anti-microbial activity of the crude rhizome extract, as well as the *n*-hexane, dichloromethane and ethyl acetate fractions were screened for activity against *Plasmodium falciparum*, *Mycobacterium tuberculosis*, and herpes simplex virus, and also for their cytotoxicity against KB-oral cavity, breast and lung cancers. No activity was detected, which suggests that the isolated compounds do not possess significant biological activity.

Experimental

Plant material: *Drynaria rigidula* (Sw.) Bedd. (Polypodiaceae) rhizomes were collected from Malabar forest, Bondowoso, Indonesia and identified at the School of Pharmacy, University of Jember, Indonesia, where sample vouchers are kept under accession codes DRR. The rhizomes were cleaned, washed, sliced, sun-dried and then powdered.

Extraction and fractionation: A suspension of the above powder (500 g) in methanol (5.0 L) was stirred for 48 h at room temperature. The mixture was then filtered and the supernatant vaccuum dried to produce the rhizome extract (24.07 g). Liquid-liquid fractionation of the rhizome extract produced *n*-hexane (10.44 g), CH_2Cl_2 (0.93 g), and EtOAc (4.33 g) fractions, along with a residue (8.22 g).

Isolation: Part of the *n*-hexane fraction (1501.8 mg) was subjected to flash silica gel CC (2 cm diameter, 40 cm length) and elution with a *n*-hexane to ethyl acetate gradient, followed by an ethyl acetate to acetonitrile gradient, followed by acetonitrile-methanol (9:1). This produced 115 fractions. Crystal needles were obtained from fractions 5-9 (116.4 mg), and 21-24 (11.5 mg). Separately, these fractions were subjected to preparative TLC with *n*-hexane as the developing solvent to obtain needle crystals of fern-9(11)ene (75.2 mg) and hop-22(29)-ene (5.1 mg) from fraction 5-9 and 21-24, respectively. Pale yellow needle crystals were also collected from fraction 50 - 59 (30.5 mg). Fraction 50 - 59 was subjected to preparative TLC with 20% EtOAc in *n*-hexane as the developing solvent, which produced *γ*-sitosterol (12.7 mg).

The dichloromethane fraction (698.2 mg) was subjected to silica gel CC and elution with a gradient solvent system from *n*-hexane to ethyl acetate, and then ethyl acetate to acetonitrile, produced 71 fractions. Needle crystals were collected from fractions 2 – 3, which contained fern-9(11)ene, and fractions 22 – 23, which contained *γ*-sitosterol. These compounds were identified using EIMS analysis followed by database searching.

The ethyl acetate fraction (500 mg) was subjected to silica gel CC (2 cm x 5 cm). Elution with ACN: MeOH: H_2O (8:1:1) produced 200 mL of solution, which was reduced to 10 mL. This was injected into 8 blocks for preparative HPLC (19 x 150 mm, 5 µm, Sunfire^{TM} C_{18}) with gradient elution from 88% to 80% solvent A (0.1 % TFA

in H_2O) in 40 min (15 mL/min) with solvent B (0.1 % TFA in ACN) to produce 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, kaempferitrin, 3,5-dihydroxy-flavone-7-*O*-*β*-rhamnopyranosyl-4'-*O*-*β*-glucopyranoside and compounds **1** and **2**.

Compound 1

Brown powder, 0.12 mg/g dried sample.
IR (cm^{-1}) υ_{max}: 3333 (m), 2926 (w), 1672 (m), 1611 (m), 1202, (m) 1140 (m), 1037 (s), 820 (w).
^{1}H NMR (CD_3OD, 500 MHz): 7.21 (d, ^3J= 8.5, 2H, H2, H6), 6.76 (d, ^3J= 8.5, 2H, H3, H5), 4.87 (d, ^3J= 7.0, 1H, H1'), 4.10 (dd, ^3J= 7.0, 5.5, 1H, H2'), 4.06 (d, ^3J= 7.5, 1H, H1''), 3.84 (dd, ^2J= 11.5, ^3J= 5.5, 1H, H5''$_A$), 3.44 (ddd, ^3J= 9.0, 5.0, 5.0, 1H, H4'') 3.18 (dd, ^3J= 9.0, 9.0, 1H, H3''), 3.12 (dd, ^2J= 11.5, ^3J= 5.5, 1H, H5''$_B$), 3.08 (dd, ^3J= 9.0, 7.5, 1H, H2''), 2.77 (dd, ^2J= 16.5, ^3J= 5.5, 1H, H3'$_A$), 2.67 (dd, ^2J= 16.5, ^3J= 5.5, 1H, H3'$_B$).
^{13}C NMR (CD_3OD, 125 MHz): 158.3 (C4), 156.5 (C5'), 131.3 (C1), 129.2 (C2, C6), 116.1 (C3, C5), 104.6 (C1''), 100.7 (C4'), 80.3 (C1'), 77.1 (C4''), 76.5 (C2'), 75.7 (C2''), 71.1 (C4''), 66.6 (C5''), 27.0 (C3').
ESI-MS: *m/z* 405 $[M-H]^-$, 407 $[M+H]^+$, 429 $[M+Na]^+$.
ESI-MS2: *m/z* 405 $[M-H]^-$, 273 $[M-H-132]^-$.

Compound 2

Brown powder, 0.03 mg/g dried sample.
IR (cm^{-1}) υ_{max}: 3336 (m), 2928 (w), 1675 (w), 1611 (m), 1201 (s), 1140 (s), 1096 (s), 800 (m).
^{1}H NMR (CD_3OD, 500 MHz): 7.22 (d, ^3J= 8.0, 2H, , H2, H6), 6.79 (d, ^3J= 8.0, 2H, H3, H5), 4.60 (d, ^3J= 8.0, 1H, H1'), 3.99 (dd, ^3J= 8.0, 6.0, 1H, H2'), 2.86 (dd, ^2J= 16.0, ^3J= 6.0, 1H, H3'$_A$), 2.50 (dd, ^2J= 16.0, ^3J= 6.0, 1H, 3'$_B$).
^{13}C NMR (CD_3OD, 125 MHz): 158.3 (C4), 156.9 (C5'), 131.5 (C1), 129.7 (C2, C6), 116.0 (C3, C5), 100.9 (C1'), 82.8 (C4'), 68.9 (C4'), 28.8 (C3').
ESI-MS: *m/z* 275 $[M+H]^+$.

Acetylation of 1: Compound **1** (1.5 mg) was stirred with acetic anhydride (0.25 mL) and pyridine (0.25 mL) under nitrogen for 24 h. The reaction was then quenched by the addition of distilled water (1.0 mL) and the aqueous mixture extracted with ethyl acetate (2 x 5 mL). A sample of the ethyl acetate layer was added to methanol (2 mL), which was then used for mass spectrometric analysis. A peak at *m/z* 681 $[M+Na]^+$ was assigned to **3**.

Bioactivity testing: Anti-malarial activity was determined against *Plasmodium falciparum* K1 based on the microculture radioisotope technique [15]. Cytotoxicity was tested against KB-oral cancer cell, lung cancer cell NCI-H187 and breast cancer cell based on the resazurinmicroplate assay. Anti-TB was tested against *Mycobacterium tuberculosis* H37Ra strain using a green fluorescent protein microplate assay. Anti-viral activity was tested against herpes simplex virus type 1 using a green fluorescent protein assay [16].

Acknowledgments - ASN would thank to the University of Wollongong for a scholarship and research support and the University of Jember for facilitating the collection of sample.

References

[1] Nugraha AS, Keller PA. (**2011**) Revealing indigenous Indonesian traditional medicine: anti-infective agents. *Natural Product Communications*, ***6***,1953-1966.
[2] Benzing DH. (**1990**) *Vascular epiphytes: General biology and related biota*. Cambridge University Press, Cambridge, 1-354.
[3] Khan A, Haque E, Mukhlesur Rahman M, Mosaddik A, Rahman M, Sultana N. (**2007**) Isolation of antibacterial constituent from rhizome of *Drynaria quercifolia* and its sub-acute toxicological studies. *Daru-Journal of Faculty of Pharmacy, Tehran University of Medical Science,**15***, 205-211.
[4] Liu S, Xiao Z, Feng R. (**1994**) A flavanol glycoside from *Drynaria propinqua. Phytochemistry*, ***35***, 1595-1596.
[5] Ramesh N, Viswanathan MB, Saraswathy A, Balakrishna K, Brindha P, Lakshmanaperumalsamy P. (**2001**) Phytochemical and antimicrobial studies on *Drynaria quercifolia. Fitoterapia*, ***72***, 934-936.
[6] Wang XL, Wang NL, Gao H, Zhang G, Qin L, Wong MS, Yao XS. (**2010**) Phenylpropanoid and flavonoids from osteoprotective fraction of *Drynaria fortunei*. *Natural Product Research*, ***24***, 1206-1213.
[7] Wang XL, Wang NL, Zhang Y, Gao H, Pang WY, Wong MS, Zhang G, Qin L, Yao XS. (**2008**) Effects of eleven flavonoids from the osteoprotective fraction of *Drynaria fortunei* (Kunze) J. SM. on osteoblastic proliferation using an osteoblast-like cell line. *Chemical and Pharmaceutical Bulletin*, ***56***, 46-51.
[8] Giesen W, Wulffraat S, Zieren M, Scholten L. (**2006**) *Mangrove guidebook for Southeast Asia*. FAO and Wetlands International, Bangkok, 1-769.
[9] Ageta H, Shiojima K, Arai Y, Suzuki H, Kiyotani T. (**1994**) NMR spectra of triterpenoids. II. Hopenes and migrated hopenes.*Chemical and Pharmaceutical Bulletin*, ***42***, 39-44.
[10] Ageta H, Shiojima K, Suzuki H, Nakamura S. (**1993**) NMR spectra of triterpenoids. I. Conformation of the side chain of hopane and isohopane, and their derivatives.*Chemical and Pharmaceutical Bulletin*, ***41***, 1939-1943.
[11] Balamurugan R, Stalin A, Ignacimuthu S. (**2012**) Molecular docking of *γ*-sitosterol with some targets related to diabetes. *European Journal of Medicinal Chemistry*, ***47***, 38-43.
[12] Lin SSC, Lu TM, Chao PC, Lai YY, Tsai HT, Chen CS, Lee YP, Chen SC, Chou MC, Yang CC. (**2011**) *In vivo* cytokine modulatory effects of cinnamaldehyde, the major constituent of leaf essential oil from *Cinnamomum osmophloeum* Kaneh. *Phytotherapy Research*, ***25***, 1511-1518.
[13] Rao GV, Rao PS. (**1985**) A new flavonol glycoside from the flowers of *Crotalaria verrucosa. Fitoterapia*, ***56***, 175-177.
[14] Duus J, Gotfredsen CH, Bock K. (**2000**) Carbohydrate structural determination by NMR spectroscopy: modern methods and limitations. *Chemical Reviews*, ***100***, 4589-614.
[15] Desjardins RS, Canfeld CJ, Haynes JD, Chulay J.D. (**1979**) Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrobial Agents and Chemotherapy*, ***16***, 710-718.
[16] Haritakun R, Sappan M, Suvannakad R, Tasanathai K, Isaka M. (**2010**) An antimycobacterial cyclodepsipeptide from the entomopathogenic fungus *Ophiocordyceps communis* BCC 16475. *Journal of Natural Products*, ***73***, 75-78.

Natural Product Communications
2013
Volume 8, Number 6

Contents

Australasian Natural Product Studies
(Guest Editor: Sylvia Urban)

Continued Inside backcover