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Cytotoxic and Antitumor Activity of some Coumarin Derivatives

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Several natural and synthetic coumarins were assayed against different cancer cell lines. Four of them have shown cytotoxicity against a panel of three human solid tumor cell lines (HeLa, T-47D, and WiDr) and a clearly activity/hydrophobicity relationship. Compound **13** proved to be the most active product in all cell lines tested, with values of 8.0 (± 0.38) μM against HeLa cells and also able to inhibit *Taq* DNA polymerase. This dual activity of **13** makes it a candidate to be considered as a "lead" compound in the search for novel antitumor drugs.

Keywords: Coumarins, Antitumor, Cell lines, Replication inhibition, Lipophilicity. Coumarins, Antitumor.

Cancer is still the second leading cause of death worldwide. Numerous experiments have been going on to develop compounds having minor or no side effects; and coumarins were reported to exhibit these properties [1].

Synthetic or natural coumarins are a versatile class of aromatic heterocycles and constitute a relevant class of pharmacological agents presenting a wide range of different functionalizations [2]. Their antitumor properties include the inhibition of the cell cycle, thus conferring chemoprevention of cancer [3, 4]. The antitumor activities of coumarins and its known metabolite, umbelliferone (7-hydroxycoumarin), were tested against three human carcinoma cell lines {colon-carcinoma cell line (Caco-2), a hepatoma-derived cell line (Hep-G2) and a lymphoblastic cell line (CCRF cem)} [5]. Coumarins, represented by 2,4-diaryl-4H,5H-pyrano[3,2-c]benzopyran-5-ones, have displayed strong anti-proliferative activities in MCF-7 breast carcinoma cells by a mechanism that remains to be determined [6].

The search for new leads can also be made by combinatorial chemistry or semi-synthesis reactions. Although it is possible with these techniques to produce quickly new collections of compounds of various size and composition which increase molecular diversity, the generation of new defined stereogenic centers is an arduous labor [7].

In this work we report the cytotoxic and antiproliferative activities of a small set of natural and synthetic coumarin derivatives. Some of them have shown DNA polymerase inhibition previously [8, 9]. In order to perform structure-activity relationship (SAR) studies against DNA replication and antitumor activity, we envisioned different points of modification on the molecular scaffold.

The *in vitro* antiproliferative activity of twenty compounds was evaluated using the National Cancer Institute (NCI) protocol, after 48 h of drug exposure using the sulforhodamine B (SRB) assay, including the commercial drugs coumarin (**18**), 4-hydroxy (**19**), and 6-hydroxycoumarin (**20**) [10-12]. The results expressed as 50% growth inhibition (GI_{50}) are shown in Table 1. From the GI_{50} values, the structure-activity relationships clearly show a direct relationship between lipophilicity and activity [13].

Compounds **1** and **2** were isolated from plants belonging to the *Pterocaulon* genus (Asteraceae) and **3** was obtained using NBS in

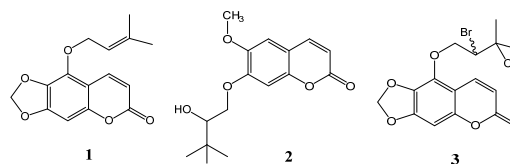


Figure 1: Natural coumarins from *Pterocaulon* sp. and halohydrin derivative assayed.

CH_2Cl_2 (Figure 1). Product **3** was able to inhibit *Taq* DNA polymerase, but did not show antitumor activity. Its loss of action could be attributed to its remarkably polarity. This halohydrin has a particular structural feature in its side chain, showing polar groups (alcohol and halogen) able to form an intramolecular hydrogen bond with a partial five membered cycle.

Using 4-hydroxycoumarin as starting material and either allyl or γ,γ -dimethylallyl bromine, we could obtain compounds **4-9**. The derivatives **4** and **9** were liquid oils, due to their low polarity. Thus, but not surprisingly, only these two bi-substituted products exhibited inhibition of growth and cell division. Once again, their potential permeability through membranes seems to be crucial for their activity.

Cytotoxic drugs continue to play a crucial role in cancer therapy. Certain cytotoxic agents have been improved via pro-drug approaches. Improvements have been decidedly specific for drugs and disease, but have drawbacks with respect to the accuracy of cellular uptake and drug release. Among the strategies proposed to enhance the passive internalization of drugs into cells, increasing their lipophilicity has often been validated as a successful approach [14].

Taking this into account, we envisaged the preparation of more hydrophobic derivatives using 6-hydroxycoumarin as starting material. Compounds **10** and **11** were obtained via silicon protecting groups, and **12** in a classical acetylation reaction. The less polar of these products (**10**) showed *in vitro* antiproliferative activity against representative human solid tumor cell lines. Thus, functionalized chemical groups containing silicon atoms might just provide lipophilicity to the drug, permitting it to pass through the cell membrane by passive diffusion. This strategy has been successfully implemented in the antitumor drug analogs silaplatins (cisplatin analogs) [15,16] and silatecans (silicon-containing camptothecins) [17].

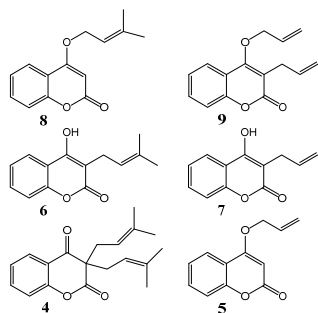


Figure 2: Synthesized allyl coumarins assayed.

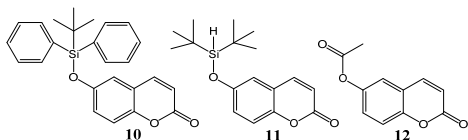


Figure 3: 6-Hydroxycoumarin derivatives evaluated.

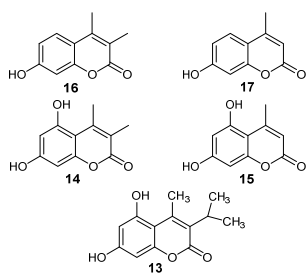


Figure 4: Assayed coumarins synthesized using Pechmann reaction

In order to increase the number of compounds for biological tests we prepared different semi-synthetic coumarins using the von Pechmann reaction variant [8] in the presence of ionic liquids. Products **13-17** show some degree of polarity, attributable to the presence of phenolic moieties. Compound **13** (the most hydrophobic of these five products) was active against all the tested cell lines and against DNA replication.

This dual activity of **13** makes this coumarin a good candidate to be considered as a “lead” product in the search of novel antitumor drugs, and its target of action could be nuclear DNA polymerases.

Coumarins target a number of pathways in the cancer field, such as kinase inhibition, cell cycle arrest, angiogenesis inhibition, heat shock protein (HSP90) inhibition, telomerase inhibition, antimetabolic activity, carbonic anhydrase inhibition, monocarboxylate transporters inhibition, aromatase inhibition and sulfatase inhibition [1]. DNA polymerase inhibition affects the unlimited replicative potential of cancer cells by inhibiting DNA replication, and consequently antitumor activity, in leukemic and colon cancer cell lines for instance. There is a close evolutionary relationship between DNA dependent enzymes (such as human DNA polymerases) and *Taq* DNA polymerase. These structures resemble a right hand domain and conserve the amino acid sequence inside the catalytic region [18, 19]. It is very likely that the majority of effects observed on cellular divisions can be mediated by the reaction of DNA related enzymes with biomolecules such as proteins, peptides, and, at higher concentrations, DNA or chemical drugs.

In summary, the effects over cell viability were tested *in vitro* on several tumor cell lines, as well as in previous work against *Taq* DNA polymerase. Four products from a family of twenty coumarins

Table 1: *In vitro* antiproliferative activity against representative human solid tumor cell lines.^a

Compounds	Cell line			cLogP ^b
	HeLa	T47D	WiDr	
1	na	na	na	3.32
2	na	na	na	1.01
3	na	na	na	2.64
4	17.6 (±6.4)	26.0 (±5.1)	21.60 (±5.9)	5.15
5	na	na	na	2.22
6	na	na	na	3.21
7	na	na	na	2.28
8	na	na	na	3.27
9	17.5 (±1.5)	54.3 (±4.7)	21.6 (±7.6)	3.38
10	24.1 (±4.1)	26.6 (±2.3)	32.2 (±1.8)	5.49
11	na	na	na	4.19
12	na	na	na	1.48
13	8.0 (±3.9)	27.0 (±7.4)	22.6 (±3.6)	2.00
14	na	na	na	1.32
15	na	na	na	0.88
16	na	na	na	1.67
17	na	na	na	1.23
18	na	na	na	nc
19	na	na	na	nc
20	na	na	na	nc

^a Values, expressed as GI₅₀ (50% growth inhibition), are given in μ M and are means of three to five experiments; standard deviation is given in parentheses.

^b The detected cLog P values were implemented using ALOGPS 2.1 program that is publicly available.

have shown cytotoxic and antitumor activity in μ M concentrations. Furthermore, compound **13** was able to inhibit both DNA replication and cell division in the three cancer cell lines assayed.

Experimental

General procedures: All the chemicals used were of analytical grade. Reactions requiring anhydrous conditions were performed under nitrogen. Dichloromethane and diethyl ether were distilled from CaH₂ and Na/benzophenone, respectively, under N₂ prior to use. TLC was carried out on Merck aluminum sheets coated with silica gel 60 F₂₅₄. Plates were visualized by use of UV light and/or sodium permanganate 20% solution without heating. NMR spectra were measured at 500, 400 or 200 MHz (¹H NMR) and 100 or 50 MHz (¹³C NMR) in either CDCl₃ or MeOD, and chemical shifts are reported relative to internal Me₄Si (*d* = 0).

Data for assayed coumarins are listed below and in the previously publications [8, 9].

5-(3',3'-Dimethylallyloxy)-6,7-methylenedioxy coumarin (1)

¹H NMR (200 MHz, CDCl₃): δ 1.78-1.73 (6H, bs, Me), 4.83 (bs, 1H, H-1'a), 4.87 (1H, d, *J* = 7.2 Hz, H-1'b), 5.47 (1H, bs, H-2'), 6.01 (2H, s, O-CH₂-O), 6.19 (1H, d, *J* = 9.6 Hz, H-3), 6.52 (1H, s, H-8), 7.96 (1H, d, *J* = 9.6 Hz, H-4)

¹³C NMR from HSQC (50.6 MHz, CDCl₃): δ 17.00 (CH₃), 29.30 (CH₃), 69.00 (CH₂), 92.52 (CH-8), 101.60 (CH₂), 111.88 (CH-3), 119.53 (CH₂), 139.30 (CH-4).

7-(3'-Methyl-2',3'-dihydroxybutoxy)-6-methoxycoumarin (2)

¹H NMR (200 MHz, CDCl₃): δ 1.34-1.26 (6H, bs, Me), 3.89 (1H, bs, H-2'), 3.92 (3H, s, OMe), 4.30-4.15 (2H, bs, H-1'), 6.25 (1H, d, *J* = 9.8 Hz, H-3), 6.85 (1H, s, H-5), 6.91 (1H, bs, H-8), 7.62 (1H, d, *J* = 9.8 Hz, H-4)

¹³C NMR from HSQC: δ (50.6 MHz, CDCl₃) 26.00 (CH₃), 27.50 (CH₃), 68.40 (OMe), 70.60 (CH₂), 74.80 (CH-2'), 97.60 (CH-5), 103.40 (CH-8), 110.99 (CH-3), 142.30 (CH-4).

5-(2'-Bromine-3'-hydroxy-3'-methyl-butyloxy)-6,7-methylenedioxy coumarin (3)

¹H NMR (200 MHz, CDCl₃): δ 1.44-1.25 (6H, bs, Me), 3.34 (1H, bs, OH), 4.22 (1H, dd, *J* = 3.2; 5.6 Hz, H-2'), 4.50 (1H, dd, *J* = 8.8; 2.8 Hz, H-1'b), 5.02 (1H, dd, *J* = 3.2; 8.0 Hz H-1'a) 6.05 (2H, s, O-

CH₂-O), 6.22 (1H, d, *J* = 9.8 Hz, H-3), 6.56 (1H, s, H-8), 8.12 (1H, d, *J* = 9.8 Hz, H-8)

¹³C NMR from HSQC: δ (50.6 MHz, CDCl₃) 30.00 (CH₃), 64.20 (CH-2'), 74.10 (CH₂), 93.22 (CH-8), 101.90 (CH₂), 111.92 (CH-3), 139.80 (CH-4).

3,3-Bis(3-methylbut-2-en-1-yl)chromane-2,4-dione (4)

¹H NMR (200 MHz, CDCl₃): δ 1.68-1.51 (12H, s, Me), 2.75 (4H, bs, H-1'), 4.90 (2H, bs, H-2'), 7.25 (1H, bs, H-6), 7.52 (1H, bs, H-8), 7.63 (1H, dd, *J* = 1.8; 7.2 Hz, H-7), 7.90 (1H, dd, *J* = 1.6; 7.6 Hz, H-5)

¹³C NMR (50.6 MHz, CDCl₃): δ 25.93-18.07 (CH₃), 37.55 (CH₂), 62.46 (C), 117.5 (CH-8), 117.64 (CH-2'), 124.84 (CH-6), 126.84 (CH-5), 137.02 (CH-7), 137.23 (C), 155.04 (C), 170.69 (C=O), 194.72 (C=O).

4-Allyloxy-coumarin (5)

¹H NMR (200 MHz, CDCl₃): δ 4.70 (2H, dd, *J* = 1.2; 5.0 Hz, H-1'), 5.48 (2H, bs, H-3'), 5.69 (1H, s, H-3), 6.13 (1H, m, H-2'), 7.27 (1H, bs, H-6), 7.32 (1H, bs, H-8), 7.52 (1H, dd, *J* = 1.6; 7.2 Hz, H-7), 7.86 (1H, dd, *J* = 1.4; 7.8 Hz, H-5)

¹³C NMR from HSQC (50.6 MHz, CDCl₃): δ 69.00 (CH₂), 91.00 (CH-3) 117.05 (CH-8), 119.00 (CH-3'), 123.00 (CH-5), 124.05 (CH-6), 131.00 (CH-2'), 134.00 (CH-7).

3-(3',3'-Dimethylallyl)-coumarin (6)

¹H NMR (200 MHz, CDCl₃): δ 1.80-1.71 (6H, s, Me), 2.65 (2H, d, *J* = 7.2 Hz, H-1'), 5.04 (1H, bs, H-2'), 7.36 (1H, bs, H-8), 7.41 (1H, bs, H-6), 7.64 (1H, bs, H-7), 7.86 (1H, dd, *J* = 1.4; 7.8 Hz, H-5).

4-Hydroxy-3-allyl-coumarin (7)

¹H NMR (200 MHz, CDCl₃): δ 3.50 (2H, d, *J* = 6.8 Hz, H-1'), 5.27 (2H, bs, H-3'), 6.03 (1H, bs, H-2'), 7.27 (2H, bs, H-6; H-8), 7.51 (1H, dd, *J* = 1.6; 7.2 Hz, H-7) 7.81 (1H, d, *J* = 8.0 Hz, H-5)

¹³C NMR from HSQC (50.6 MHz, CDCl₃): δ 29.00 (CH₂), 116.15 (CH-8), 117.20 (CH-3'), 121.89 (CH-5), 123.05 (CH-6), 134.23 (CH-7), 135.76 (CH-2').

4-(3',3'-Dimethylallyloxy)-coumarin (8)

¹H NMR (200 MHz, CDCl₃): δ 1.84-1.78 (6H, s, Me), 4.68 (2H, d, *J* = 7.4 Hz, H-1'), 5.51 (1H, bs, H-2'), 5.68 (1H, s, H-3), 7.25 (1H, bs, H-6), 7.33 (1H, bs, H-8), 7.54 (1H, dd, *J* = 1.8; 7.2 Hz, H-7), 7.83 (1H, dd, *J* = 1.6; 8.0 Hz, H-5).

4-Allyloxy-3-allyl-coumarin (9)

¹H NMR (200 MHz, CDCl₃): δ 3.40 (2H, d, *J* = 6.2 Hz, H-1'), 4.63 (2H, d, *J* = 5.6 Hz, H-1'), 5.07 (2H, bs, H-3') 5.37 (2H, bs, H-3') 5.99 (1H, bs, H-2'), 6.08 (1H, bs, H-2'), 7.30 (1H, bs, H-6), 7.36 (1H, bs, H-8), 7.50 (1H, dd, *J* = 1.6; 7.6 Hz, H-7), 7.70 (1H, dd, *J* = 1.6; 8.0 Hz, H-5).

6-(*t*-Butyldiphenylsilyloxy)-coumarin (10)

¹H NMR (200 MHz, CDCl₃) δ 1.11 (9H, s, *t*-Bu), 6.31 (1H, d, *J* = 9.6 Hz, H-3), 6.79-7.72 (14H, aromatic).

6-(Di-*t*-butylsilyloxy)-coumarin (11)

¹H NMR (200 MHz, CDCl₃): δ 1.06 (18H, s, *t*-Bu), 4.45 (1H, s, H-Si), 6.41 (1H, d, *J* = 9.6 Hz, H-3), 6.98-7.22 (3H, bs, H-5, 7, 8), 7.62 (1H, d, *J* = 9.6 Hz, H-4).

6-Acetoxy-coumarin (12)

¹H NMR (200 MHz, CDCl₃): δ 2.33 (3H, s, Ac), 6.46 (1H, d, *J* = 9.6 Hz, H-3), 7.28 (3H, bs, H-5, 7, 8), 7.66 (1H, d, *J* = 9.6 Hz, H-4).

3-Isopropyl-4-methyl-5,7-dihydroxycoumarin (13)

¹H NMR (400 MHz, MeOD): δ 1.3 (6H, d, *J* = 9.0 Hz, 2Me), 2.65 (3H, s, Me), 3.30 (1H, m, CH), 6.18 (1H, d, *J* = 2.0 Hz, H-8), 6.23 (1H, d, *J* = 2.0 Hz, H-6).

¹³C NMR (100 MHz, MeOD): δ 17.52 (CH₃), 18.82 (2CH₃), 27.37 (CH-2', *i*-Pr), 94.15 (CH-8), 99.16 (CH-6), 103.37 (C), 124.20 (C), 149.96 (C), 155.04 (C), 157.66 (C), 160.07 (C), 161.16 (C=O).

3,4-Dimethyl-5,7-dihydroxycoumarin (14)

¹H NMR (400 MHz, MeOD): δ 2.08 (3H, s, Me), 2.58 (3H, s, Me), 6.19 (1H, d, *J* = 2.0 Hz, H-8), 6.22 (1H, d, *J* = 2.0 Hz, H-6).

¹³C NMR (100 MHz, MeOD): δ 11.24 (CH₃), 18.07 (CH₃), 94.05 (CH-8), 99.13 (CH-6), 103.19 (C), 114.83 (C), 150.67 (C), 154.84 (C), 157.43 (C), 160.09 (C), 163.24 (C=O).

4-Methyl-5,7-dihydroxycoumarin (15)

¹H NMR (400 MHz, MeOD): δ 2.60 (3H, s, Me), 5.85 (1H, s, H-3), 6.23 (2H, bs, H-6-8).

¹³C NMR (100 MHz, MeOD): δ 22.72 (CH₃), 94.41 (CH-8), 98.91 (CH-6), 102.71 (C), 108.22 (CH-3), 156.67 (C), 156.89 (C), 158.18 (C), 161.56 (C), 162.64 (C=O).

3,4-Dimethyl-7-hydroxycoumarin (16)

¹H NMR (400 MHz, MeOD): δ 2.15 (3H, s, Me), 2.41 (3H, s, Me), 6.69 (1H, d, *J* = 2.4 Hz, H-8), 6.82 (1H, dd, *J* = 2.4; 8.8 Hz, H-6), 7.60 (1H, d, *J* = 8.8 Hz, H-5).

¹³C NMR (100 MHz, MeOD): δ 11.53 (CH₃), 13.68 (CH₃), 101.72 (CH-8), 112.71 (CH-6), 113.02 (C), 117.04 (C), 125.65 (CH-5), 147.93 (C), 153.43 (C), 160.29 (C), 163.19 (C=O).

4-Methyl-7-hydroxycoumarin (17)

¹H NMR (400 MHz, MeOD): δ 2.42 (3H, s, Me), 6.09 (1H, s, H-3), 6.69 (1H, bs, H-8), 6.82 (1H, bs, H-6), 7.57 (1H, bs, H-5).

¹³C NMR (100 MHz, MeOD): δ 17.28 (CH₃), 102.08 (CH-8), 109.81 (CH-3), 112.41 (C), 112.94 (CH-6), 125.95 (CH-5), 154.49 (C), 155.08 (C), 161.57 (C), 162.46 (C=O).

Biological studies: All starting materials were commercially available, research-grade chemicals, and were used without further purification. RPMI 1640 medium was purchased from Sigma-Aldrich (St Louis, MO, USA), fetal bovine serum (FBS) from Nataclor (Nataclor-Argentina), trichloroacetic acid (TCA) and dimethylsulfoxide (DMSO) from Merck (Darmstadt, Germany), and penicillin G-streptomycin, sulforhodamine B (SRB) and glutamine from Sigma-Aldrich (St Louis, MO, USA). Pure compounds were initially dissolved in DMSO at 400 times the desired final maximum test concentration, that is, 100, 10 and 1 μM.

Antiproliferative assay: The human solid tumor cell lines HeLa (cervix), T-47D (breast) and WiDr (colon) were used in this study. Cells were maintained in 25 cm² culture flasks in RPMI 1640 supplemented with 5% FBS and 2 mM L-glutamine in a 37°C, 5% CO₂, 95% humidified air incubator. Exponentially growing cells were trypsinized and re-suspended in antibiotic containing medium (100 U penicillin G and 0.1 mg of streptomycin per mL). Single cell suspensions displaying >97% viability by trypan blue dye exclusion were subsequently counted. After counting, dilutions were made to give the appropriate cell densities for inoculation onto 96-well microtiter plates. Cells were inoculated at 100 μL per well at densities of 1.5 x 10⁴ (HeLa and T-47D), and 2 x 10⁴ (WiDr) cells per well, based on their doubling times. Each agent was tested at least in triplicate at different dilutions in the range of 1–100 μM. The drug treatment was started on day 1 after plating. Drug incubation time was 48 h, after which time cells were precipitated with 25 μL ice-cold TCA (50%, w/v) and fixed for 60 min at 4°C.

Then the SRB assay was performed. The optical density (OD) of each well was measured at 492 nm, using BioTek's PowerWave XS Absorbance Microplate Reader. Values were corrected for background OD from wells only containing medium.

The percentage of growth (PG) was calculated with respect to untreated control cells (*C*) at each of the drug concentration levels based on the difference in OD at the start (*T*₀) and end of drug exposure (*T*), according to the National Cancer Institute (USA) formulas 0. Therefore, if *T* is greater than or equal to *T*₀ the calculation is $PG = 100 [(T-T_0)/(C-T_0)]$. If *T* is less than *T*₀ denoting cell killing the calculation is $PG = 100 [(T-T_0)/(T_0)]$.

With these calculations, 3 levels of effect could be determined; 50% growth inhibition (GI₅₀), total growth inhibition (TGI), and 50% cell killing (LC₅₀) that represent the concentration at which PG is +50, 0, and -50, respectively. Thus, a PG value of 0 corresponds to the amount of cells present at the start of drug exposure, while negative PG values denote net cell kill.

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Effect of Auxins on Anthocyanin Accumulation in Hairy Root Cultures of Tartary Buckwheat Cultivar Hokkai T10 Chang Ha Park, Aye Aye Thwe, Sun Ju Kim, Jong Seok Park, Mariadhas Valan Arasu, Naif Abdullah Al-Dhabi, Nam Il Park and Sang Un Park	1283
A New Coumarin from the Stems of <i>Pterocarpus indicus</i> Jirapast Sichaem, Suttira Khumkratok, Pattara Sawasdee and Santi Tip-pyang	1287
Cytotoxic and Antitumor Activity of some Coumarin Derivatives Hugo A. Garro, Guillermo F. Reta, Osvaldo J. Donadel and Carlos R. Pungitore	1289
New Chromone, Isocoumarin, and Indole Alkaloid Derivatives from three Sponge-derived Fungal Strains Ling-Hong Meng, Hui-Qin Chen, Imke Form, Belma Konuklugil, Peter Proksch and Bin-Gui Wang	1293
Two Pyrones with Antibacterial Activities from <i>Alpinia malaccensis</i> Tita Juwitaningsih, Lia D Juliawaty and Yana M. Syah	1297
Acetophenones from <i>Acronychia pedunculata</i> and their Cancer Chemopreventive Activity Chihiro Ito, Mari Hosono, Harukuni Tokuda, Tian-Shung Wu and Masataka Itoigawa	1299
Inhibitory Effects of Echinochrome A, Isolated from Shell of the Sea Urchin <i>Anthodiaris crassispina</i>, on Antigen-Stimulated Degranulation in Rat Basophilic Leukemia RBL-2H3 Cells through Suppression of Lyn Activation Tomohiro Itoh, Azusa Fujiwara, Masayuki Ninomiya, Toshimichi Maeda, Masashi Ando, Yasuyuki Tsukamasa and Mamoru Koketsu	1303
Preparative Synthesis of Spinochrome D, a Pigment of Different Sea Urchin Species Olga P. Shestak, Nadezhda N. Balaneva and Vyacheslav L. Novikov	1307
Structural Elucidation and Free Radical Scavenging Activity of a new <i>o</i>-Orsellinic Acid Derivative Isolated from the Lichen <i>Cladonia rappii</i> Tiago C.A. Lage, Livia P. Horta, Ricardo M. Montanari, Jefferson G. Silva, Ângelo de Fátima, Sergio A. Fernandes and Luzia V. Modolo	1311
Phenanthrenes in Chinese Yam Peel Exhibit Antiinflammatory Activity, as Shown by Strong <i>in Vitro</i> Cyclooxygenase Enzyme Inhibition Qian Li, Chuan-Rui Zhang, Amila A. Dissanayake, Qun-yu Gao and Muraleedharan G. Nair	1313
Bis-bibenzyls from the Cameroon Liverwort <i>Marchantia debilis</i> Kenneth Yongabi Anchang, Miroslav Novaković, Danka Bukvički and Yoshinori Asakawa	1317
<i>In vitro</i> Antigonorrhoea Activity of the Aerial Part of <i>Asparagus suaveolens</i> <i>n</i>-Hexane Fraction and Palmitone as a Bioactive Compound Mutendela Tabize Olivier, Freddy Munyololo Muganza Leshweni Jeremia Shai and Stanley Sechene Gololo	1319
1-Acyl-3-<i>O</i>-[β-glucopyranosyl-(1''\rightarrow6')-β-glucopyranosyl]-glycerols and Cordyceptide B and C, New Metabolites from <i>Bacillus pumilus</i> Hongpeng Wang, Frederike Drawert, Michael Steinert, Stefan Schulz and Hartmut Laatsch	1323
Glucosinolate Profiling of <i>Calepina irregularis</i> Marina Zekić, Ani Radonić and Zvonimir Marijanović	1329
Management of Diabetic Bacterial Foot Infections with Organic Extracts of Liverwort <i>Marchantia debilis</i> from Cameroon Kenneth Anchang Yongabi, Miroslav Novaković, Danka Bukvički, Catherine Reeb and Yoshinori Asakawa	1333
Biological Activities of Extracts from Different Parts of <i>Cryptomeria japonica</i> Hiroki Horiba, Toshinori Nakagawa, Qinchang Zhu, Ahmed Ashour, Atsushi Watanabe and Kuniyoshi Shimizu	1337
Inhibition of Spore Germination and Appressorium Formation of Rust Species by Plant and Fungal Metabolites Eleonora Barilli, Alessio Cimmino, Marco Masi, Marco Evidente, Diego Rubiales and Antonio Evidente	1343
Antibacterial Activities of Endophytic Fungi Isolated from <i>Mentha cordifolia</i> Leaves and Their Volatile Constituents Sakon Monggoot, Jariya Burawat and Patcharee Pripdeevech	1349
Compositional Variability and Toxic Activity of Mugwort (<i>Artemisia vulgaris</i>) Essential Oils Asta Judzentiene and Rasa Garjonyte	1353
Antioxidant Activities and Reduced Amyloid-β Toxicity of 7-Hydroxycalamenene Isolated from the Essential Oil of <i>Zelkova serrata</i> Heartwood Pei-Ling Yen, Sen-Sung Cheng, Chia-Cheng Wei, Huan-You Lin, Vivian Hsiu-Chuan Liao and Shang-Tzen Chang	1357
Composition, <i>in vitro</i> Anti-inflammatory, Antioxidant and Antimicrobial Activities of the Leaf Essential Oil of <i>Machilus konishii</i> from Taiwan Yu-Chang Su, Kuan-Ping Hsu and Chen-Lung Ho	1363
Chemical Composition and Anti-phytopathogenic Activity of the Essential Oil of <i>Beilschmiedia miersii</i> Marcela A. Carvajal, Alejandra P. Vergara, Rocío Santander and Mauricio E. Osorio	1367
<u>Accounts/Reviews</u>	
Terpenoids with Special Pharmacological Significance: A Review Rolf Jaeger and Eckehard Cuny	1373
Recent Advances of Total Syntheses of Diterpenoids Starting from Carvone Hisahiro Hagiwara	1391
A Survey of the Chemical Compounds of <i>Piper</i> spp. (Piperaceae) and Their Biological Activities Cai-Peng Xiang, Yan-Ni Shi, Fang-Fang Liu, Hai-Zhou Li, Ying-Jun Zhang, Chong-Ren Yang and Min Xu	1403
Integration of Traditional and Western Medicine in Vietnamese Populations: A Review of Health Perceptions and Therapies Sabrina Adoriso, Alessandra Fierabracci, Arielle Rossetto, Isabella Muscari, Vincenza Nardicchi, Anna Marina Liberati, Carlo Riccardi, Tran Van Sung, Trinh Thy Thuy and Domenico V. Delfino	1409

Natural Product Communications

2016

Volume 11, Number 9

Contents

Original Paper

- A Computational Examination of the Uncatalyzed Meinwald Rearrangement of Monoterpene Epoxides**
William N. Setzer 1207
- The Iridoid Myodesert-1-ene and Elemol/Eudesmol are found in Distinct Chemotypes of the Australian Aboriginal Medicinal Plant *Eremophila dalyana* (Scrophulariaceae)**
Nicholas J. Sadgrove, Timothy L. Collins, Sarah V.A.-M. Legendre, Julian Klepp, Graham L. Jones and Ben W. Greatrex 1211
- Harpagoside Content in Devil's Claw Extracts**
Narasimharao Kondamudi, Matthew W. Turner and Owen M. McDougal 1215
- A Study on the Lipase-catalysed Acylation of 6,7-Dihydroxy-linalool**
Stefano Serra and Davide De Simeis 1217
- Enhanced Production of δ -Guaiene, a Bicyclic Sesquiterpene Accumulated in Agarwood, by Coexpression of δ -Guaiene Synthase and Farnesyl Diphosphate Synthase Genes in *Escherichia coli***
Takahiro Kato, Jung-Bum Lee, Futoshi Taura and Fumiya Kurosaki 1221
- Germacrene Analogs are Anti-androgenic on Androgen-dependent Cells**
Jukkarin Srivilai, Nantaka Khorana, Neti Waranuch, Wudtichai Wisuitiprot, Nungruthai Suphrom, Apichart Suksamram and Kornkanok Ingkaninan 1225
- Terpenoids from the Fermented Broths of *Coprinellus radians***
Ming-Shian Lee, Che-Jen Hsiao, Yu-Ming Ju, Yueh-Hsiung Kuo, Ruo-Kai Lin and Tzong-Huei Lee 1229
- On the Allylic Oxidation of *Ent*-kaurenic Acid Methyl Ester with Lead Tetra-acetate**
Julio Rojas, Rosa Aparicio and Alfredo Usubillaga 1231
- Antioxidants from Annatto Seeds as Possible Inhibitory Agents of the Hepatotoxicity Induced by the Antitumor Agent Cisplatin**
Lucécia Fátima Souza, Niara da Silva Medeiros, Paula Cilene Pereira dos Santos, Carlos Henrique Pagno, Cleice Dalla Nora, Erna Vogt de Jong and Alessandro de Oliveira Rios 1233
- Cytotoxic Activity of Some Lupeol Derivatives**
Barbara Bednarczyk-Cwynar, Tomasz Więcaszek and Piotr Ruskowski 1237
- In vitro* Anticancer Activities of Some Triterpene Glycosides from Holothurians of Cucumariidae, Stichopodidae, Psolidae, Holothuriidae and Synaptidae families**
Sergey N. Fedorov, Sergey A. Dyshlovoy, Alexandra S. Kuzmich, Larisa K. Shubina, Sergey A. Avilov, Alexandra S. Silchenko, Ann M. Bode, Zigang Dong and Valentin A. Stonik 1239
- Regulosides A, B, and C, Three New Polyhydroxysteroid Glycosides from the Starfish *Pentaceraster regulus***
Alla A. Kicha, Natalia V. Ivanchina, Timofey V. Malyarenko, Anatoly I. Kalinovskiy, Pavel S. Dmitrenok, Evgeny A. Pisyagin and Ekaterina A. Yurchenko 1243
- Aphelasteroside F, a new Asterosaponin from the Far Eastern Starfish *Aphelasterias japonica***
Roman S. Popov, Natalia V. Ivanchina, Anatoly I. Kalinovskiy, Sofiya D. Kharchenko, Alla A. Kicha, Timofey V. Malyarenko, Svetlana P. Ermakova and Pavel S. Dmitrenok 1247
- Unusual Steroid Constituents from the Tropical Starfish *Leiaster* sp.**
Timofey V. Malyarenko, Alla A. Kicha, Natalia V. Ivanchina, Anatoly I. Kalinovskiy, Pavel S. Dmitrenok and Valentin A. Stonik 1251
- Absolute Configuration and Body Part Distribution of the Alkaloid 6-*epi*-Monancherin from the Marine Polychaete *Chaetopterus varioepedatus***
Larisa K. Shubina, Tatyana N. Makarieva, Vladimir A. Denisenko, Pavel S. Dmitrenok, Sergey A. Dyshlovoy, Gunhild von Amsberg, Valery P. Glazunov, Artem S. Silchenko, Inna V. Stonik, Hyi-Seung Lee, Yeon-Ju Lee and Valentin A. Stonik 1253
- N*-Demethylaaptanone, A new Congener of Aaptamine Alkaloids from the Vietnamese Marine Sponge *Aaptos aaptos***
Natalia K. Utkina and Vladimir A. Denisenko 1259
- Influence of the Metabolites of the Marine Algicolous Fungus *Penicillium* sp. on Seedling Root Growth of Agricultural Plants**
Mikhail M. Anisimov, Elena L. Chaikina, Olga F. Smetanina and Anton N. Yurchenko 1261
- Gramine-derived Bromo-alkaloids Activating NF- κ B-dependent Transcription from the Marine Hydroid *Abietinaria abietina***
Alla G. Guzii, Tatyana N. Makarieva, Sergey N. Fedorov, Vladimir A. Denisenko, Pavel S. Dmitrenok, Aleksandra S. Kuzmich, Vladimir B. Krasokhin, Hyi-Seung Lee, Yeon-Ju Lee and Valentin A. Stonik 1263
- Inhibitory Effect of Three Diketopiperazines from Marine-derived Bacteria on Secretory Group IIA Phospholipase A2**
Hyukjae Choi, Sae-Kwang Ku and Jong-Sup Bae 1267
- Multifunctional Monoamine Oxidases and Cholinesterases Inhibitory Effects, as well as UPLC-DAD-MS Chemical Profile of Alkaloid Fractions Obtained from Species of the Palcoureeae Tribe**
Luiz Carlos Klein-Júnior, Carolina dos Santos Passos, Juliana Salton, Fernanda Gobbi de Bitencourt, Luís Funez, Jean Paulo de Andrade, Jonathan Parra Villalobos, Sérgio Augusto de Loreto Bordignon, André Luís Gasper, Yvan Vander Heyden and Amélia Teresinha Henriques 1271
- Flavonoids from the Bark of *Artocarpus integer* var. *silvestris* and their Anti-inflammatory Properties**
Masuri Kama Kamaruddin Shah, Hasnah Mohd Sirat, Shajarahtunnur Jamil and Juriyati Jalil 1275
- A Novel Biflavonoid from *Rhus leptodictya***
Tshifhiwa Matamela, Ivan R. Green and Fanyana M. Mtunzi 1279
- A New Flavonoid from *Camellia sinensis* Fermented Tea**
Ayaka Usui, Azusa Nakamura, Manami Era, Yosuke Matsuo, Takashi Tanaka and Kanji Ishimaru 1281

Continued inside backcover