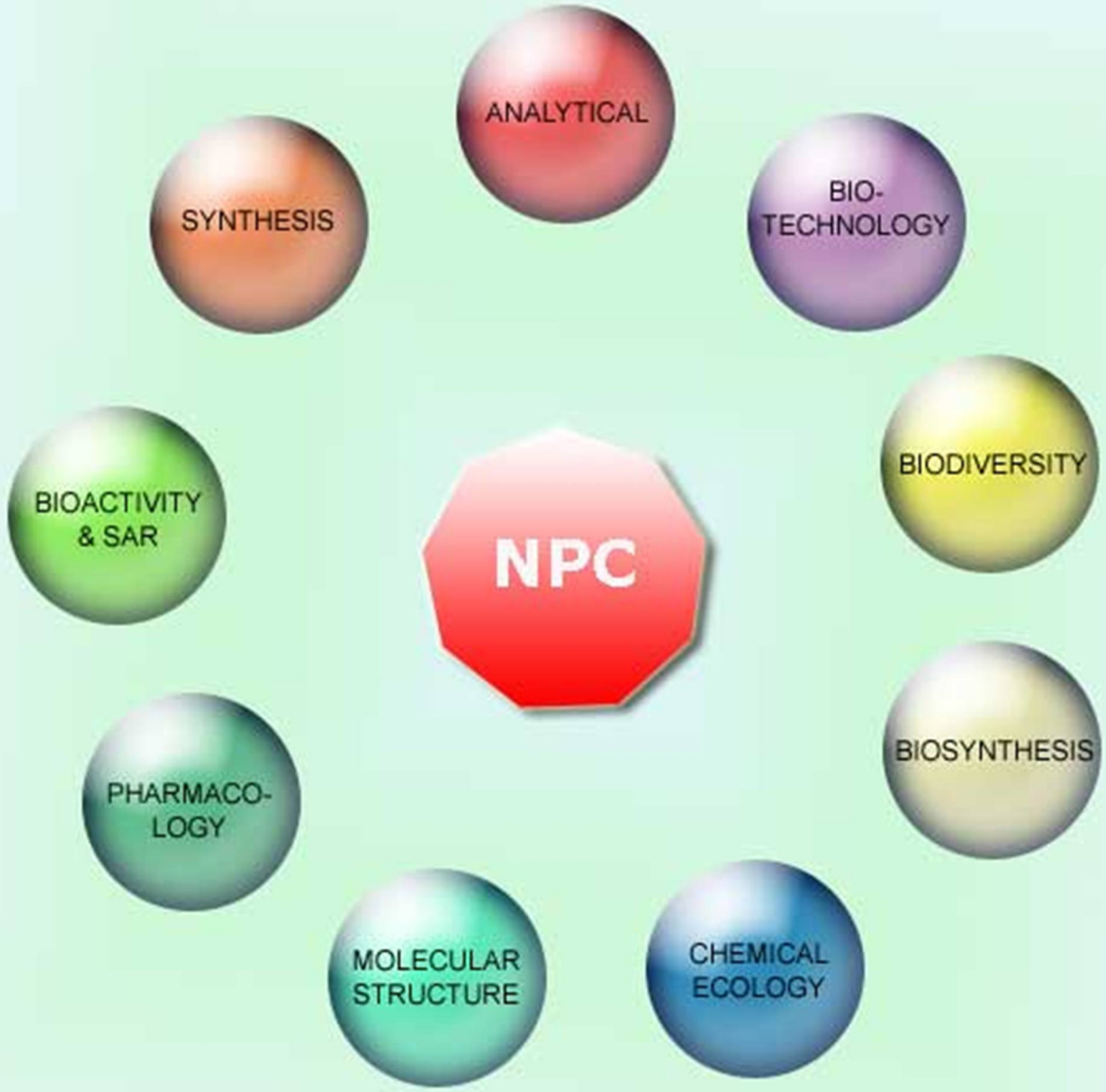
NATURAL PRODUCT COMMUNICATIONS

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



Volume 7. Issue 12. Pages 1557-1690. 2012
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

NPC Natural Product Communications

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NPC Natural Product Communications

2012
Vol. 7
No. 12
1573 - 1574

ACAT Inhibitory Activity of Exudates from *Calocedrus macrolepis* var. *formosana*

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Received: May 28th, 2012; Accepted: October 17th, 2012

Cholesterol acyltransferase (ACAT) is an enzyme controlling cholesterol esterification in cells. Large amounts of cholesterol esters accumulate in macrophages and smooth muscle cells of blood vessel walls resulting in the initial stages of atherosclerosis. Thus, atherosclerosis might be inhibited through inhibition of the activity of ACAT. In the present study, we identified by spectral analysis and chromatographic quantification that ferruginol was the most abundant component of exudates secreted from the wounding site of *Calocedrus macrolepis* Kurz var. *formosana*. Results obtained from the cholesterol absorption assay revealed that ferruginol exhibited a significant inhibitory activity on cholesterol absorption in mice macrophages (RAW 264.7 cell). Based on the results from analyzing the ratio of cholesterol esterification, ferruginol dose-dependently suppressed cholesterol esterification and the IC_{50} value was 2.0 μg/mL. In conclusion, ferruginol revealed strong inhibitory activities that retarded the absorption and esterification of cholesterol in cells. Our finding indicates that ferruginol might possess a potential for development as a pharmaceutical product for preventing arteriosclerosis.

Keywords: Ferruginol, Arteriosclerosis, Acyl-CoA, Cholesterol acyltransferase, *Calocedrus macrolepis* Kurz var. *formosana*.

Calocedrus macrolepis Kurz var. *formosana* (Cupressaceae) is an endemic conifer in Taiwan. More than 100 compounds have been isolated from the plant, including monoterpenoids, diterpenoids, lignans and steroids [1-6]. The resin produced by plants, consisting mainly of susquiterpenoids and diterpenoids, is a vital defense system against insect and pathogen attacks [7]. In this study, we collected exudates from the bark surface of 20-year-old *C. macrolepis* and 7 diterpenoids, including ferruginol [8], *trans*-communic acid [9], isopimaric acid [10], isopimarol [11], 6α-hydroxysugiol, sugiol, and secoabietane dialdehyde [5] were identified using chromatographic separation and spectroscopic analyses. Ferruginol (Figure 1) was the most abundant compound identified in the exudates, forming approximately 77%, w/w, of the total.

ACAT is an enzyme which catalyzes the conversion of free cholesterol and fatty acyl CoA to a storage form of cholesterol, cholesteryl ester (CE) [12]. Atherosclerosis results from an excess accumulation of cholesterol and macrophages that deposit on the blood vessel wall and eventually narrow the artery [13]. In the present study, we utilized NBD-cholesterol, a fluorescent sterol analog, to mimic the absorption and esterification of native cholesterol in cultured cells [14].

RAW 264.7 cells pre-treated with ferruginol for 1 h significantly suppressed cholesterol absorption in a dose-dependent manner (Table 1). The inhibitory activity was 62.5% at a concentration of 20 μg/mL and the IC_{50} value was 9.5 μg/mL. Sandoz 58-035, a known inhibitor of cholesterol acyltransferase [15], showed 45.8% inhibition. Our data revealed that ferruginol has a higher potential for suppressing cholesterol absorption than Sandoz 58-035 and the working mechanism is worth further investigation.

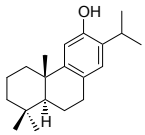

Figure 1: The dominant compound, ferruginol, in the exudates secreted from *Calocedrus macrolepis* var. *formosana*.

Table 1: Inhibition effect of ferruginol on cholesterol absorption

Compounds	Concentration (μg/mL)	Inhibition of cholesterol absorption (%)
Sandoz 58-035	10	45.8
Ferruginol	5	41.4
Ferruginol	10	52.5
Ferruginol	20	62.5

RAW 264.7 cells were incubated in medium containing ferruginol at indicated dosages for 1 h and further treated with 1 μg/mL NBD-cholesterol for 6 h. 10 μg/mL Sandoz 58-035 (Sigma St. Louis MO) was used as a reference compound. The inhibitory effect was compound-treated group compared with vehicle control (DMSO).

To decipher whether the inhibitory effect of cholesterol absorption by ferruginol is correlated with ACAT activity, we isolated the lipid contents from ferruginol treated cells and detected the levels of NDB-cholesterol and NBD-cholesterol ester, respectively, using high performance liquid chromatography (HPLC). Our data showed that ferruginol dose-dependently suppressed the level of NBD-cholesterol ester, which dramatically decreased from 25.3% to 1.4%

at a concentration of 10 μg/mL (Table 2). By comparison with control, cells treated with 10 μg/mL ferruginol suppressed 94.4% of cholesterol esterification. From these findings we suggest that ferruginol efficiently inhibits the ACAT activity of converting free cholesterol to CE by macrophages. In conclusion, ferruginol revealed a strong retarding activity for the absorption and esterification of cholesterol in cells. Our finding indicates that ferruginol might possess the potential to be developed as a pharmaceutical product for preventing arteriosclerosis.

Table 2: Inhibitory activity of cholesterol esterification by ferruginol.

Ferruginol dosages (μg/mL)	Ratio of NBD-cholesteryl esters	Inhibition of cholesterol esterification (%)
Control	25.3	0.0
1.0	20.2	20.4
2.5	9.2	63.6
5.0	3.4	86.7
10.0	1.4	94.4

RAW 264.7 cells were incubated in medium containing ferruginol at indicated dosages for 1 h and further treated with 1 μg/mL NBD-cholesterol for 6 h. Isolated lipid contents and detected the levels of NBD-CE and NBD-cholesterol respectively using HPLC. The inhibitory effect was ferruginol-treated group compared with control.

Experimental

Materials: The exudates secreted from the wounding site of 20-year-old *C. macrolepis* were dissolved in ethyl acetate and separated by HPLC using a Luna silica column (250 × 10 mm; 5 μm, Phenomenex, Torrance, CA) eluted with *n*-hex/EtOAc (*n*-hex/EtOAc = 88/12, flow rate = 2.5 mL/min). Compounds **1** to **7** were obtained at the retention times of 10.8, 16.0, 19.3, 21.6, 24.6, 33.5, and 38.3 min, respectively. The structures of the compounds were elucidated using spectroscopic analysis. The amount of the major compound (ferruginol) in exudates was further analyzed by the same HPLC system. The peak area of ferruginol in the chromatogram of the exudates (with known loading concentration) was then defined and its content in the exudates calculated based on the quantity calibrated from the standard calibration curve.

Cell culture: RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (GIBCO Carlsbad, CA) at 37°C in a 5% CO_2 humidified incubator.

Cholesterol absorption assay: RAW 264.7 cells were plated in 24-well culture plates at a density of 1×10^6 cells/well and allowed to recover for 24 h. Cells were incubated in medium containing ferruginol at various concentrations (5, 10 and 20 μg/mL) for 1 h and further treated with 1 μg/mL NBD-cholesterol for 6 h. 10 μg/mL Sandoz 58-035 (Sigma St. Louis MO) was used as a reference compound. After incubation, the cells were washed thrice with phosphate buffered saline (PBS). Fluorescence was detected using a Chameleon V Multilabel Microplate Reader (Hidex) with 485 nm excitation and 535 nm emission filters [16].

Cholesterol esterification assay: RAW 264.7 cells were plated in 6 cm culture dishes at a density of 5×10^6 cells/well and allowed to recover for 24 h. Cells were incubated in medium containing ferruginol at various concentrations (1, 2.5, 5 and 10 μg/mL) for 1 h and further treated with 1 μg/mL NBD-cholesterol for 6 h. Cells were collected and the lipid contents isolated using *n*-hex/2-propanol (3:2) solution. The ratio of NDB-cholesterol and NBD-cholesterol ester was determined from the absorbance at 443 nm using HPLC (Luna Silica 250 mm × 10 mm, 5 μm).

$$\text{Inhibition\%} = \left(1 - \frac{\text{ratio of NBD-CE}_{\text{sample}}}{\text{ratio of NBD-CE}_{\text{control}}}\right) 100$$

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Natural Product Communications
2012
Volume 7, Number 12

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