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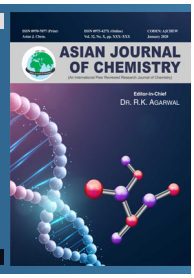

Sterols and Other Metabolites from Freshwater Microalga *Chlorococcum infusionum* (Schrank) Meneghini

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Chemical investigation of the dichloromethane extract of *Chlorococcum infusionum* afforded a mixture of chondrillasterol (**1**) and 22-dihydrochondrillasterol (**2**) in a 3:1 ratio as the major sterols, and lutein (**3**), triacylglycerols (**4**), fatty acids (**5**) and hydrocarbons (**6**) as the minor compounds. The structures of compounds **1** and **2** were elucidated by extensive 1D and 2D NMR spectroscopy, while compounds **3-6** were identified by comparison of their NMR data.

Keywords: *Chlorococcum infusionum*, Phytosterols, Chondrillasterol, 22-Dihydrochondrillasterol, Lutein, Triacylglycerols.

INTRODUCTION

An amazing diversity of phytosterols characterizes the algae in general. This is especially true among the green algae where even among species of the same genus show substantial differences in sterol profiles [1-4]. The genus *Chlorella* (class Trebouxiophyceae), for instance, form isolates rich with poriferasterol and 22-dihydrobrassicasterol, isolates with chondrillasterol and fungisterol and also isolates with ergosterol and fungisterol [4]. Sterol profiles of many freshwater chlorophyceans (class Chlorophyceae) show high sterol diversity with chondrillasterol, campesterol, corbisterol, ergosterol and fungisterol as dominant sterols [5]. Chondrillasterol, fungisterol and 22-dihydrochondrillasterol are dominant in *Scenedesmus obliquus* and *S. quadricauda* [4,6]; chondrillasterol, 22-dihydrochondrillasterol and fungisterol in *Ankistrodesmus fusiformis* [7]; ergosterol and 7-dehydroporiferasterol in *Chlamydomonas reinhardtii* strain and 22-dihydrochondrillasterol, fungisterol and chondrillasterol in *Monoraphidium obtusum* and *M. minutum* [4].

Studies on microalgae-derived phytosterols have grown tremendously over the past decades due to their potential for functional food and pharmaceutical applications [8], their implications to zooplankton nutrition [4] and their significance as chemotaxonomic biomarkers [5,9]. Yet less than 1% of microbial diversity including the approximately 200,000 to 800,000 species of microalgae has been examined for their chemical constituents [10,11]. This study hopes to contribute to the growing understanding on the chemical components in microalgae particularly among the microalgal chlorophyceans from freshwater habitats.

We report herein the isolation of chondrillasterol (**1**), 22-dihydrochondrillasterol (**2**), lutein (**3**), triacylglycerols (**4**), fatty acids (**5**) and long-chain hydrocarbons (**6**) from the freshwater microalga *Chlorococcum infusionum* (Schrank) Meneghini. This chlorophycean microalga, which is probably cosmopolitan in distribution, has also been found inhabiting neutral to acid soils and rocks. The structures of compounds **1** and **2** are shown in Fig. 1.

EXPERIMENTAL

NMR spectra were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR spectra. Column chromatography was performed with silica gel 60 (70-230 mesh). Thin layer chromatography was performed with plastic backed plates coated with silica gel F_{254} and the plates were visualized by spraying with vanillin/H_2SO_4 solution followed by warming.

Algal strain identification, cultivation and harvest: *C. infusionum* was collected from Carmona river, Laguna, Philippines, isolated and maintained at the MicroAlgae Systematics and Applied Phycology Research Unit (MSAPRU) of De La Salle University, Manila, Philippines. PCR amplification of the 18S rRNA gene fragment and subsequent DNA sequencing confirmed the isolate as *C. infusionum* based on the resulting 99.84% identity hit with Genbank accession number KF861549.1 (*C. infusionum* voucher CLS001) *via* nucleotide BLAST search (data not shown).

Chlorococcum infusionum inoculum was maintained in TMRL medium at 22 ºC under 40 µmol m^{-2} s^{-1} light intensity, and agitated regularly by manual shaking. TMRL medium contained $FeCl_2$ (3 g/L), Na_2SiO_3 (1 g/L), Na_2HPO_4 (10 g/L) and KNO_3 (100 g/L). Mass production of this alga was done in an indoor 40 L bag photobioreactor containing twenty percent (20%) mother inoculum and an initial 50% volume of TMRL. The remaining 25% culture medium was then added to the system at regular interval for a month until the full 40 L volume was attained. The whole set-up was maintained at 30 ºC with continuous aeration and light provision of 40 µmol m^{-2} s^{-1} at 12/12 light/dark cycle.

The algal biomass was harvested after 20 days using an algae cream separator and later centrifuged at 4000 rpm for 10 min. The collected biomass was freeze dried for 48 h prior to analysis.

Extraction and isolation: Freeze-dried *Chlorococcum infusionum* sample (31.89 g) was grounded in a blender, soaked in CH_2Cl_2 for 3 days and filtered. The filtrate was concentrated under vacuum to afford a crude extract (96.6 mg) which was chromatographed by gradient elution using increasing proportions of acetone in CH_2Cl_2 at 10% increment by volume. The CH_2Cl_2 fraction was rechromatographed using petroleum ether to afford compound **6** (0.6 mg) after washing with petroleum ether. The 10% acetone in CH_2Cl_2 fraction was rechromatographed using 10% EtOAc in petroleum ether to provide compound **4** (8.2 mg). The 20% EtOAc acetone in CH_2Cl_2 fraction was rechromatographed using 10% EtOAc in petroleum ether to afford a mixture of compounds **1** and **2** (20 mg) after washing with petroleum ether. The 30% acetone in CH_2Cl_2 fraction was rechromatographed using 15% EtOAc in petroleum ether to yield compound **5** (1.5 mg). The 40% acetone in CH_2Cl_2 fraction was rechromatographed using CH_3CN:Et_2O:CH_2Cl_2 (0.5:0.5:9, v/v) to yield compound **3** (1.8 mg) after washing with petroleum ether followed by Et_2O.

Chlorococcum infusionum cultures afforded chondrillasterol (**1**), 22-dihydrochondrillasterol (**2**), lutein (**3**), triacylglycerols (**4**), fatty acids (**5**) and long-chain hydrocarbons (**6**).

Chondrillasterol (1): ^1H NMR (600 MHz, $CDCl_3$): δ 3.58 (m, H-3), 5.14 (br s, H-7), 0.53 (s, H3-18), 0.80 (s, H3-19), 1.01 (d, *J* = 6.6 Hz, H3-21), 5.15 (dd, *J* = 8.4, 15 Hz, H-22), 5.02 (dd, *J* = 9, 15 Hz, H-23), 0.85 (d, *J* = 6.6 Hz, H3-26), 0.84 (d, *J* = 6.6 Hz, H3-27), 0.83 (t, *J* = 7.2 Hz, H3-29); ^{13}C NMR (150 MHz, $CDCl_3$): δ 37.1 (C-1), 31.5 (C-2), 71.1 (C-3), 38.0 (C-4), 40.3 (C-5), 29.6 (C-6), 117.5 (C-7), 139.6 (C-8), 49.5 (C-9), 34.2 (C-10), 21.5 (C-11), 39.8 (C-12), 43.3 (C-13), 55.1 (C-14), 23.0 (C-15), 28.4 (C-16), 55.9 (C-17), 12.1 (C-18), 13.0 (C-19), 40.8 (C-20), 21.3 (C-21), 138.1 (C-22), 129.5 (C-23), 51.2 (C-24), 31.8 (C-25), 21.3 (C-26), 19.0 (C-27), 25.4 (C-28), 12.4 (C-29).

22-Dihydrochondrillasterol (2): ^1H NMR (600 MHz, $CDCl_3$): δ 3.58 (m, H-3), 5.14 (br s, H-7), 0.51 (s, H3-18), 0.80 (s, H3-19), 0.91 (d, *J* = 6.6 Hz, H3-21), 0.82 (d, *J* = 6.6 Hz, H3-26), 0.82 (d, *J* = 6.6 Hz, H3-27), 0.83 (t, *J* = 7.2 Hz, H3-29); ^{13}C NMR (150 MHz, $CDCl_3$): δ 37.1 (C-1), 31.5 (C-2), 71.1 (C-3), 38.0 (C-4), 40.3 (C-5), 29.7 (C-6), 117.4 (C-7), 139.6 (C-8), 49.5 (C-9), 34.2 (C-10), 21.5 (C-11), 39.6 (C-12), 43.3 (C-13), 55.1 (C-14), 23.0 (C-15), 27.9 (C-16), 56.0 (C-17), 11.8 (C-18), 13.0 (C-19), 36.6 (C-20), 19.0 (C-21), 33.7 (C-22),

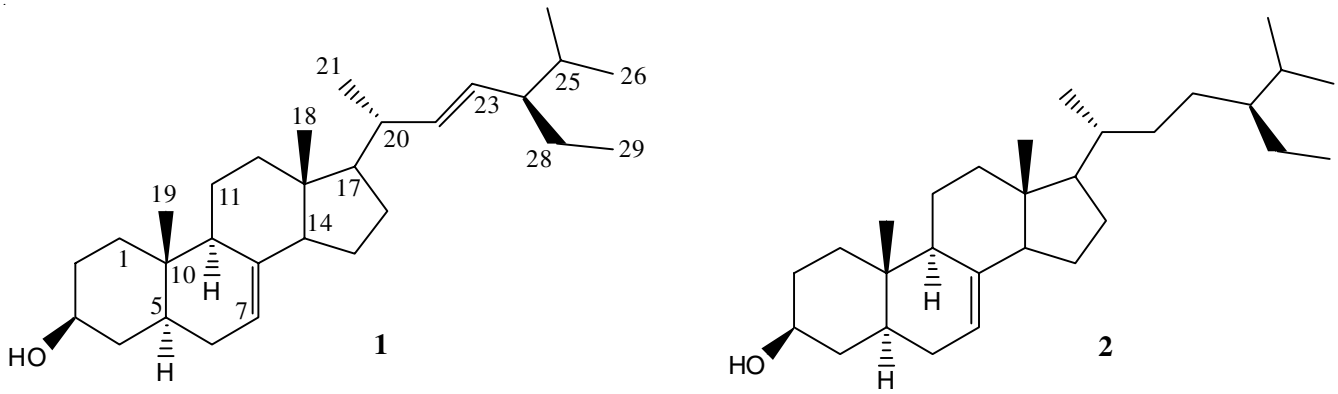

Fig. 1. Chemical structures of chondrillasterol (**1**) and 22-dihydrochondrillasterol (**2**) from *C. infusionum*

26.5 (C-23), 46.0 (C-24), 29.0 (C-25), 19.0 (C-26), 19.6 (C-27), 23.0 (C-28), 12.4 (C-29).

RESULTS AND DISCUSSION

Silica gel chromatography of the dichloromethane extract of *Chlorococcum infusionum* afforded chondrillasterol (**1**), 22-dihydrochondrillasterol (**2**) as the major sterols, and lutein (**3**), triacylglycerols (**4**), fatty acids (**5**) and hydrocarbons (**6**) as the minor compounds. The NMR data of compound **1** are in accordance with the data reported in the literature for chondrillasterol [12,13]; compound **2** for 22-dihydrochondrillasterol [13]; compound **3** for lutein [14]; compound **4** for triacylglycerols [15], compound **5** for fatty acids [16] and compound **6** for long-chain hydrocarbons [17].

The major sterols found in *Chlorococcum infusionum* concur with those mainly constituting several freshwater chlorophytes such as *Chlorella*, *Monoraphidium*, *Ankistrodesmus* and *Scenedesmus* (=*Tetradesmus*) [4-7]. Chlorophytes and rhodophytes commonly contain lutein as a photosynthetic pigment [18]. In general, microalgae contain hydrocarbons at < 5% level along with fatty acids and esters of glycerol of which triacylglycerols are the most common storage lipids [19].

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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