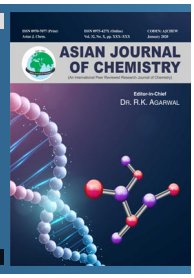
Asian Journal of Chemistry; Vol. 32, No. 5 (2020), 1197-1202

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2020.22591



Cytotoxic Sterols from Philippine Mushrooms

CONSOLACION Y. RAGASA[1,*], GLENN G. OYONG[2], MARIA CARMEN S. TAN[1], MARIQUIT M. DE LOS REYES[3] and MARIA ELLENITA G. DE CASTRO[4]

[1]Department of Chemistry, De La Salle University, 2401 Taft Avenue, Manila, Philippines
[2]Molecular Science Unit Laboratory Center for Natural Science and Environmental Research, De la Salle University, Manila, Philippines
[3]Department of Biology, De La Salle University, 2401 Taft Avenue, Manila, Philippines
[4]Department of Forest Biological Sciences, College of Forestry and Natural Resources (CFNR), UP Los Banos, Laguna

*Corresponding author: E-mail: consolacion.ragasa@dlsu.edu.ph

Received: 14 December 2019; *Accepted*: 3 February 2020; *Published online*: 29 April 2020; AJC-19856

Ergosterol peroxide (**1**) and ergosterol (**2**) were commonly isolated as the major compounds of Philippine mushrooms. Sterols **1** and **2** from the dichloromethane extract of *Geastrum triplex* and *Termitomyces clypeatus*, respectively, were evaluated for their cytotoxic activities against four human cancer cell lines, *viz.*, breast cancer (MCF-7), colon cancer (HT-29), leukemia (THP-1), and small lung cell carcinoma (H69PR), and a human normal cell line, human dermal fibroblast-neonatal (HDFn), using the PrestoBlue® cell viability assay. Compounds **1** and **2** exhibited the strongest activities against HT-29 with IC_{50} values of 1.79 and 2.98 µg/mL, respectively, while Zeocin gave an IC_{50} of 4.89 µg/mL. These compounds also exhibited strong antiproliferative effects against MCF-7 with IC_{50} values of 4.13 for **1** and 4.20 µg/mL for compound **2**, comparable to Zeocin with IC_{50} = 3.68 µg/mL. Only moderate cytotoxicity resulted when compounds **1** and **2** were tested against H69PR with IC_{50} values of 7.78 and 6.83 µg/mL, respectively, while Zeocin exhibited an IC_{50} of 9.81 µg/mL. Furthermore, compounds **1** and **2** showed no effects against THP-1 (IC_{50} > 100 µg/mL), while Zeocin showed an IC_{50} of 4.73 µg/mL. Although compounds **1** and **2** have been reported to exhibit different bioactivities in previous studies, the cancer cell lines tested and/or the polarities of the solvents for extraction varied. Therefore, comparisons of the cytotoxic activities of compounds **1** and **2** with earlier studies could not be made extensively.

Keywords: **Cytotoxicity, Ergosterol Peroxide, Ergosterol, Mushrooms.**

INTRODUCTION

This paper describes the results of present study on the chemical constituents of edible and toxic Philippine mushrooms. We commonly isolated ergosterol peroxide (**1**) as the major compound of the fruiting bodies of nine Philippine mushrooms namely, *Auricularia auricula-judae* [1], *Coprinus lagopus* [2], *Phellinus gilvus* (Schwein.) Pat. [3], *Geastrum triplex*, *Phellinus* sp., *Gymnopilus* sp., *Lepiota atrodisca, Marasmius scordinius* and *Pleurotus opuntiae.* On the other hand, ergosterol (**2**) was isolated as the major compound of the fruiting bodies of eight mushrooms namely, *Flammulina velutipes* [4], *Pleurotus eryngii* [4], *Lentinula edodes* [5], *Agaricus bisporus* [6], *Pleurotus djamor* [7], *Schizophyllum commune, Pleurotus citrinopileatus* and *Catharellus infundibuformis.* Both sterols (**1** and **2**) were isolated from the fruiting bodies of eight mushrooms namely, *Pleurotus florida* [8], *Pleurotus pulmonarius, Ganoderma lucidum, Auricularia polytricha, Cantharellus infundibuliformis, Lentinus sajor-caju, Termitomyces albuminosus* and *Termitomyces clypeatus.* To our best of knowledge, this is the first report on the isolation of compound **1** from *Geastrum triplex*, *Phellinus* sp., *Gymnopilus* sp., *Lepiota atrodisca, Marasmius scordinius, Pleurotus opuntiae*; compound **2** from *Schizophyllum commune, Pleurotus citrinopileatus, Pleurotus djamor;* and compounds **1** & **2** from *Pleurotus pulmonarius, Ganoderma lucidum, Auricularia polytricha, Cantharellus infundibuliformis, Lentinus sajor-caju, Termitomyces albuminosus* and *Termitomyces clypeatus.*

This paper also reports on the cytotoxic activities of the sterols (**1** and **2**) which were commonly isolated from 25 wild and commercially cultivated Philippine mushrooms.

EXPERIMENTAL

Sample Collection: *Geastrum triplex* and *Termitomyces clypeatus* used in this study were collected from Makiling Forest Reserve, University of the Philippines Los Baños, between the months of November 2018 to March 2019. Opportunistic method was used in the collection since most mushroom species grow and survive in any moist habitats like fallen foliage, beneath the canopy of a large tree, and even dumpsites or trash sites.

Isolation of sterols: The freeze-dried fruiting bodies of *Geastrum triplex* (18.49 g) were ground in a blender, soaked in CH_2Cl_2 for 3 days and then filtered. The solvent was evaporated under vacuum to afford a crude extract (0.0460 g) which was chromatographed using increasing proportions of EtOAc in petroleum ether at 5 % increment. The 20 % EtOAc in petroleum ether fraction was rechromatographed (3 ×) using CH_3CN:Et_2O:CH_2Cl_2 (1:1:8, v/v) to yield compound **1** (1.9 mg) after washing with petroleum ether.

The freeze-dried fruiting bodies of *Termitomyces clypeatus* (9.18 g) were ground in a blender, soaked in CH_2Cl_2 for 3 days and then filtered. The solvent was evaporated under vacuum to afford a crude extract (0.2896 g) which was chromatographed using increasing proportions of EtOAc in petroleum ether at 5% increment. The 15 % EtOAc in petroleum ether fraction was rechromatographed (2 ×) using 15 % EtOAc in petroleum ether to afford compound **2** (14 mg) after washing with petroleum ether.

Ergosterol peroxide (1): ^{1}H NMR (500 MHz, $CDC1_3$): δ 6.48 (d, *J* = 8.4 Hz, H-6), 6.22 (d, *J* = 8.4 Hz, H-7),5.12 (dd, *J* = 8.4 Hz,15.6 Hz, H-22), 5.20 (dd, *J* = 7.8 Hz, 15 Hz, H-23), 3.95 (m, H-3), 0.80 (s, Me-18), 0.86 (s, Me-19), 0.98 (d, *J* = 6.6 Hz, Me-21), 0.81 (d, *J* = 6.6 Hz, Me-26), 0.82 (d, *J* = 7.2 Hz, Me-27), 0.89 (d, *J* = 7.2 Hz, Me-28).

Ergosterol (2): ^{1}H NMR (500 MHz, $CDC1_3$): δ 5.57 (dd, *J* = 2.5 Hz, 5.5 Hz, H-6), 5.38 (dd, *J* = 2.5 Hz, 5.5 Hz, H-8), 5.22 (dd, *J* = 7.0 Hz, 15.5 Hz, H-23), 5.17 (dd, *J* = 7.5 Hz, 15.5 Hz, H-22), 3.63 (m, H-3), 1.03 (d, *J* = 7.0 Hz, Me-21), 0.94 (s, Me-19), 0.92 (d, *J* = 7.0 Hz, Me-28), 0.84 (d, *J* = 7.0 Hz, Me-26), 0.82 (d, *J* = 7.0 Hz, Me-27), 0.63 (s, Me-18).

Cytotoxicity tests: Compounds **1** and **2** from *G. triplex* and *T. clypeatus*, respectively were dissolved in DMSO to make a 4 mg/mL stock solution. Working solutions were prepared in complete growth medium to a final non-toxic DMSO concentration of 0.1 %.

Preparation of cell lines for cytotoxicity tests: The effects on the cell proliferation of compounds **1** and **2** from dichloromethane extracts of *G. triplex* and *T. clypeatus* were tested on the following human cell lines: breast cancer (MCF-7), colon cancer (HT-29), leukemia (THP-1), small lung cell carcinoma (H69PR) (all from ATCC, Manassas, Virginia, USA) and human dermal fibroblast-neonatal (HDFn; Invitrogen Life Technologies, USA), which were routinely maintained at the Cell and Tissue Culture Laboratory, Molecular Science Unit, Center for Natural Science and Environmental Research, De La Salle University, Manila, Philippines. Following standard procedures followed previously [9], cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, USA) containing 10% fetal bovine serum (FBS, Gibco®, USA) and 1× antibiotic-antimycotic (Gibco®, USA) and kept in an incubator (37 ºC, 5 % CO_2, 98 % humidity). After reaching about 80 % confluence, the cells were prepared for cell counting and inoculation. The cells were washed with phosphate-buffered saline (PBS, pH 7.4, Gibco®, USA), trypsinized with 0.05 % Trypsin-EDTA (Gibco®, USA), and resuspended with fresh complete media as defined above. Cells were counted following standard trypan blue exclusion method [10], using 0.4% Trypan Blue Solution (Gibco®, USA). Cells were then seeded in 100 µL aliquots into a 96-well microtiter plate (Falcon™, USA) with a final inoculation density of 1×10^4 viable cells/well. The plates were further incubated overnight (37 ºC, 5 % CO_2, 98 % humidity) until cell attachment has been achieved. These monolayer cultures were used for the cytotoxicity studies described below.

Cell viability assay: The cytotoxicity of compounds **1** and **2** from the dichloromethane extracts of *Geastrum triplex* and *Termitomyces clypeatus* crude extracts was determined in an *in vitro* cell viability test using PrestoBlue® (Molecular Probes®, Invitrogen, USA), a resazurin dye which is a cell permeable redox indicator used to monitor viable cells. Only viable cells with the active enzymes, mitochondrial reductases of the electron transport chain, are able to convert the blue and non-fluorescent, resazurin dye, to the pink and highly fluorescent, resorufin product. The amount of resorufin produced is proportional to the number of metabolically active cells and quantified using a microplate reader capable of either absorbance or fluorescence measurements. To the previously prepared monolayers in the microtiter plate, 100 µL of filter-sterilized compounds **1** and **2** were added to corresponding wells at two-fold serial dilutions to make final screening concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 µg/mL, respectively [11]. Wells with no compound added served as negative controls, wells with Zeocin™ (Gibco®, USA) served as positive controls, and wells containing only cell culture medium were used to correct for background color. The cells were further incubated (37 ºC, 5 % CO_2, 98 % humidity) for 4 days before the addition of 10 µL of PrestoBlue® per well, afterwhich, the cells were incubated for another 2 h under the same conditions. Absorbance was measured using a BioTek ELx800 absorbance microplate reader (BioTek® Instruments, Inc., U.S.A.) at 570 nm and normalized to 600 nm values (reference wavelength). Absorbance readings were used to calculate for the cell viability for each compound concentration following the equation below:

$$\text{Cell viability (\%)} = \frac{A_{\text{Treated sample}} - A_{\text{Blank}}}{A_{\text{Negative control}} - A_{\text{Blank}}} \times 100$$

For each cell line, the computed cell viability is used to plot viability index as a function of treatment concentration. Non-linear regression and statistical analyses were done using GraphPad Prism 8.1.2 (Graphpad Software, Inc, La Jolla California, USA) to extrapolate the half maximal inhibitory concentration, IC_{50}, which is the concentration of the compound that resulted in 50% reduction in viability index. Hence, the cytotoxicity (antiproliferative potential) of compounds **1** and **2** was expressed as IC_{50} values. All tests were performed in triplicates and data were expressed as mean ± SD. One-way ANOVA was used to determine differences in IC_{50} under different treatments, followed by Tukey's multiple comparison post

hoc test, to evaluate differences between data pairs. The level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Compounds **1** and **2** from dichloromethane extracts of *Geastrum triplex* and *Termitomyces clypeatus* mushrooms, respectively, were evaluated for their cytotoxic activities against four human cancer cell lines, breast cancer (MCF-7), colon cancer (HT-29), leukemia (THP-1) and small lung cell carcinoma (H69PR), and a human normal cell line, human dermal fibroblast-neonatal (HDFn). The % viability index per cell line, as a function of the logarithmic values of treatment concentration, is shown in Fig. 1. The effects of the known anticancer drug, Zeocin and DMSO are also shown. Overall, the curves follow the typical sigmoidal curve which is characteristic of an inhibitory dose-response relationship between treatments and cell viability. The extrapolated IC_{50} values are summarized in Table-1 and compared in Fig. 2 for the four cancer cell lines tested.

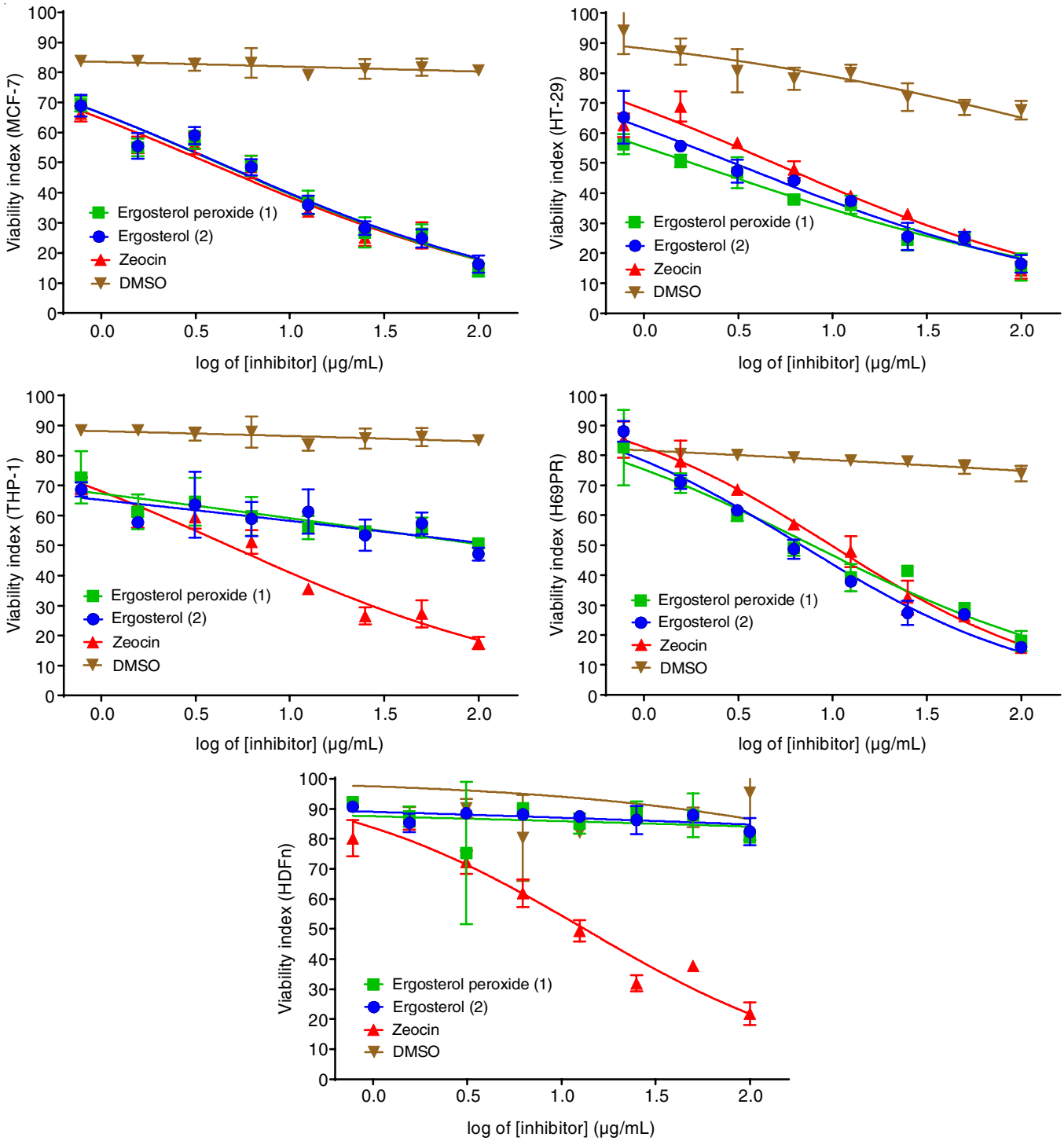


Fig. 1. Dose-response curves showing the cytotoxic activities of ergosterol peroxide (**1**) from *G. triplex* and ergosterol (**2**) from *T. clypeatus*, respectively, and Zeocin and DMSO, on the cell viability of breast (MCF-7), colon (HT-29), leukemia (THP-1), small lung cell carcinoma (H69PR) and human dermal fibroblast neonatal (HDFn)

TABLE-1
CYTOTOXIC ACTIVITIES (IC_{50}) OF COMPOUNDS **1** AND **2** FROM *G. triplex* AND *T. clypeatus*

Treatment	IC_{50}* (µg/mL)				
	MCF-7	HT-29	THP-1	H69PR	HDFn
1	4.13	1.79	> 100	7.78	> 100
2	4.20	2.98	> 100	6.83	> 100
Zeocin	3.68	4.89	4.73	9.81	13.3
DMSO	> 100	> 100	> 100	> 100	> 100

*IC_{50} values were extrapolated from dose-response curves generated from nonlinear regression analysis performed using GraphPad Prism 8.1.2. For each cell line, one-way ANOVA was conducted, with Tukey's multiple comparison post hoc test, to determine differences between data sets (treatments). Treatments: ergosterol peroxide (**1**), ergosterol (**2**), Zeocin, and DMSO. Cell lines: human cancer cell lines, breast (MCF-7), colon (HT-29), leukemia (THP-1), and small lung cell carcinoma (H69PR), and, a normal cell line, human dermal fibroblast neonatal (HDFn).

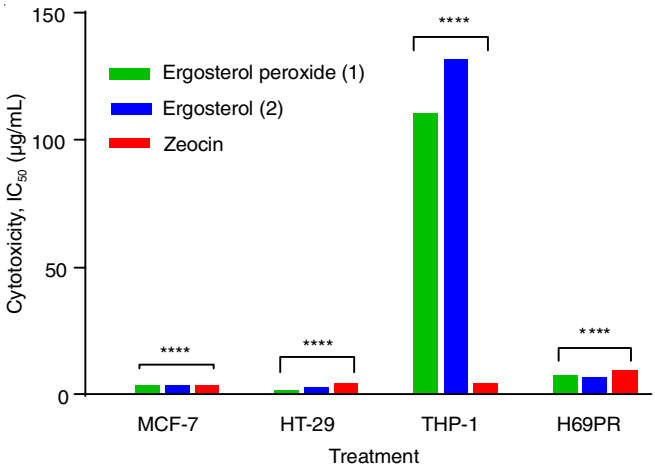


Fig. 2. Comparative cytotoxic activities (IC_{50}) of **1** and **2** from *G. triplex* and *T. clypeatus*, respectively and Zeocin, against breast (MCF-7), colon (HT-29), leukemia (THP-1) and small lung (H69PR) cancer cells

Ergosterol peroxide (**1**) and ergosterol (**2**) from *Geastrum triplex* and *Termitomyces clypeatus*, respectively, exhibited strong antiproliferative effects against the breast cancer (MCF-7) cell line, with IC_{50} values of 4.13 and 4.20 µg/mL, respectively, comparable to the level of effect of Zeocin at 3.68 µg/mL. One-way ANOVA, followed by Tukey's multiple comparison post hoc test, revealed that there is no significant pairwise difference between compounds **1** and **2** ($p = 0.8548$). When compounds **1** and **2** were each compared against Zeocin, $p = 0.0005$ and $p < 0.0001$ were obtained, respectively, implying that Zeocin exhibited the strongest cytotoxicity against MCF-7 cells.

For all the tests conducted, ergosterol peroxide (**1**) and ergosterol (**2**) gave the strongest activities against colorectal cancer cell cells (HT-29) with IC_{50} values of 1.79 and 2.98 µg/mL, respectively. Zeocin exhibited the lowest bioactivity with IC_{50} = 4.89 µg/mL. One-way ANOVA, followed by Tukey's post hoc test, showed significant differences between all data pairs ($p < 0.0001$).

For all the cytotoxicity tests done, both compounds **1** and **2** showed no effects when leukemia cells (THP-1) were used ($IC_{50} > 100$). This is clearly seen in Fig. 2, where, compared to the other treatments including the known anticancer drug, Zeocin, there is no cytotoxicity exhibited by compounds **1** and **2**. The antiproliferative effect of Zeocin (IC_{50} = 4.73 µg/mL) was comparable to the results obtained using MCF-7 and HT-29 cells. A pairwise comparison revealed a significant difference between compounds **1** and **2** ($p < 0.0001$). Leukemia, in general, is hard to treat because of the known drug resistance of several chemotherapy drugs [12].

Only moderate cytotoxicity was obtained when compounds were used against small lung cell carcinoma (H69PR) with IC_{50} values of 7.78 and 6.83 µg/mL for compounds **1** and **2**, respectively. Zeocin gave an IC_{50} = 9.81 µg/mL which is the weakest activity for all the cell lines tested. Pairwise comparisons verified significant differences between all pairs of treatments ($p < 0.0001$).

For normal cell line, HDFn, compounds **1** and **2** did not elicit any cytotoxic effect ($IC_{50} > 100$ µg/mL). Zeocin against HDFn (IC_{50} = 13.3 µg/mL) gave the weakest cytotoxic effect against all the cell lines used, implying that only cancer cells are responsive to this drug. This was expected as the normal cell line should be the least affected by most of the anticancer treatments. The vector, DMSO, has no effect on any of the cell lines tested (IC_{50} >100 µg/mL). Again, the data revealed that ergosterol peroxide (**1**) and ergosterol (**2**) are most effective against the colorectal cancer cells (HT-29) since the lowest IC_{50} values were obtained using these compounds, meaning, the lowest concentration of these compounds are needed to cause a 50% drop in viability index. Compounds **1** and **2** also showed strong cytotoxicity against breast cancer cells (MCF-7), followed by a moderate activity in small lung cell carcinoma cells (H69PR). A known anticancer drug, Zeocin, showed moderate to strong antiproliferative activities as expected. The US National Cancer Institute has defined the active cytotoxic limits of natural products as 20 µg/mL or less for crude extracts and 4 µg/mL or less for pure compounds [13]. Pure compounds that exhibit active cytotoxicity may have some potential for further drug development [14]. Using this as standard, both ergosterol peroxide (**1**) and ergosterol (**2**) from the mushrooms, *Geastrum triplex* and *Termitomyces clypeatus*, respectively, can be further evaluated for their potential antiproliferative effects, especially against HT-29, MCF-7, and H69PR cells.

A comprehensive review of anticancer compounds from nine commercially grown and wild Philippine mushrooms revealed that the active bioactive compounds present in the organism include ergosterol and ergosterol peroxide [15]. These two compounds have been reported to exhibit anticancer properties and this can possible explain the moderate to strong antiproliferative effects observed in the study conducted here using *Geastrum triplex* and *Termitomyces clypeatus*. While there are limited studies discussing the anticancer properties of *G. triplex*, there are some reports indicating the anticancer activities in *Termitomyces clypeatus*.

Ergosterol peroxide (**1**), a sterol derived from mushrooms, has been found to have a wide range of bioactivities. Compound **1**, purified from the fermentation broth of *Paecilomyces cicadae*, significantly inhibited the growth of human renal cell carcinoma (RCC) cells *in vitro*, suppressing migration and invasion, triggering apoptosis, and modulating the cell cycle of the cells, all in a dose-dependent manner [16]. Ergosterol peroxide (**1**) showed antitumor effects against ovarian cancer cells

(OVCAR-3, CAOV3) by inhibiting the oncogenic signaling mediated by β-catenin and STAT3 pathways [17]. The same compound isolated from *Inonotus obliquus*, inhibited cell proliferation and likewise suppressed clonogenic colony formation in human colorectal cancer (CRC) cells such as HT-29 and HCT-116, by inhibiting the nuclear levels of β-catenin which ultimately led to a reduced transcription of c-Myc, cyclin D1, and CDK-8 [18]. Compound **1** also suppressed cell growth and STAT1-mediated inflammatory activities in colon cancer (HT-29) cells [19]. In another study, compound **1**, isolated from *Ganoderma lucidum* extract, decreased the migratory and invasive effects of inflammatory breast cancer (IBC) cells while inhibiting the expression of total AKT1, AKT2, BCL-XL, Cyclin D1, and c-Myc; likewise inducing the formation of reactive oxygen species that compromise cellular fate [20]. Compound **1**, also isolated from *G. lucidum*, exhibited higher bioactivity in inducing the death of miR-378-transfected MT tumor cells that initially exhibited multiple drug resistance [21]. Similar antitumor activities were exhibited against human mammary adenocarcinoma, human gastric tumor (SNU-1), human hepatoma (SUN-354), human colorectal tumor (SUN-C4), multiple myeloma U266 cells, murine sarcoma-180 cell lines, and Walker carcinosarcoma [22]. The results shown in the present study agree with the findings above that ergosterol peroxide (**1**) exhibit anticancer properties.

Ergosterol (**2**), another sterol found in medicinal mushrooms, has also been reported to provide anticancer and antioxidant properties. The ergosterol contents of brown and white button mushrooms were found to be associated to their antioxidant activities [23]. Compound **2** also provided protection against the onset of bladder tumor resulting from various environmental promoters [24]. In an *in vivo* experiment with mice injected with murine cancer cell line B16, compound **2**, isolated from *Amauroderma rude* mushroom, suppressed cell viability through apoptosis and up-regulation of the expression of the tumor suppressor, Foxo3 [25]. In another study, ergosterol, isolated from *Ganoderma lucidum* extract (GLE), inhibited tumor growth rate in Hepa1-6-bearing C57 BL/6 mice through the up-regulation of the expression levels of PGR and ESR1, and the down-regulation of NR3C2 and AR [26]. Different ergosterol derivatives were isolated from GLE and many were found to display both antitumor and antiangiogenesis activities against human tumor cells and human umbilical vein endothelial cells (HUVECs) *in vitro* [27]. The results shown in the present study also agree with the reports above that ergosterol (**2**) exhibit anticancer properties.

Although compounds **1** and **2** have been reported to exhibit different bioactivities in previous studies, the cancer cell lines tested and/or the type and polarities of the solvents for extraction varied. Therefore, comparisons of the cytotoxic activities of compounds **1** and **2** with earlier studies could not be made extensively.

Conclusion

Ergosterol peroxide (**1**) and ergosterol (**2**) from dichloromethane extracts of *Geastrum triplex* and *Termitomyces clypeatus*, respectively, exhibited the strongest cytotoxic activities against colon cancer cells (HT-29) (IC_{50} = 1.79 and 2.98 μg/mL for compounds **1** and **2**, respectively). A strong antiproliferative effect was also seen against breast cancer (MCF-7) (IC_{50} = 4.13 and 4.20 μg/mL for compounds **1** and **2**, respectively). A moderate activity was observed using small lung cell carcinoma (H69PR) (IC_{50} = 7.78 and 6.83 μg/mL for compounds **1** and **2**, respectively). Both compounds did not exhibit any cytotoxicity against the normal cell line, human dermal fibroblast-neonatal, HDFn cells (IC_{50} > 100 μg/mL). Zeocin, as expected, exhibited moderate (IC_{50} = 9.81 μg/mL for H69PR) to strong (IC_{50} = 4.89 and 3.68 μg/mL for HT-29 and MCF-7, respectively) activities. The results suggest that ergosterol peroxide (**1**) and ergosterol (**2**) from the dichloromethane extracts of *G. triplex* and *T. clypeatus*, respectively, can be further evaluated for their potential use as anticancer agents.

ACKNOWLEDGEMENTS

Research grants from the CHED-PHERNet and the De La Salle University for this work are gratefully acknowledged.

CONFLICT OF INTEREST

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

REFERENCES

1. C.Y. Ragasa, M.C.S. Tan, M.E.G. De Castro and C.-C. Shen, *Der Pharm. Chem.*, **8**, 168 (2016).
2. C.Y. Ragasa, M.C.S. Tan, M.E.G. De Castro, J. Perez and C.-C. Shen, *Int. J. Toxicol. Pharmacol. Res.*, **8**, 421 (2016).
3. C.Y. Ragasa, M.C.S. Tan, M.E.G. De Castro and I.A. van Altena, *Der Pharm. Chem.*, **8**, 222 (2016).
4. C.Y. Ragasa, M.C.S. Tan, R. Brkljaèa and S. Urban, *Int. J. Curr. Pharm. Rev. Res.*, **7**, 334 (2016).
5. C.Y. Ragasa, J.M.A. Reyes, M.C.S. Tan, R. Brkljaèa and S. Urban, *Int. J. Pharm. Clin. Res.*, **8**, 1451 (2016e).
6. N.G.U. Resurreccion, C.-C. Shen and C.Y. Ragasa, *Der Pharm. Lett.*, **8**, 117 (2016).
7. C.Y. Ragasa, M.C.S. Tan, J. Ting, R.G. Reyes, R. Brkljaèa and S. Urban, *Der Pharm. Chem.*, **8**, 343 (2016).
8. C.Y. Ragasa, V.D. Ebajo Jr., R.G. Reyes, R. Brkljaèa and S. Urban, *Der Pharm. Chem.*, **7**, 331 (2015).
9. M.M. De Los Reyes, G.G. Oyong, V.D. Ebajo Jr., C.-C. Shen and C.Y. Ragasa, *Asian J. Chem.*, **30**, 613 (2018); https://doi.org/10.14233/ajchem.2018.21053
10. R.I. Freshney, Culture of Animal Cells: A Manual of Basic Techniques. Wiley-Liss, Inc.: New York, U.S.A. (2000).
11. M.M. De Los Reyes, G. Oyong, V. Jr, V. Ng, C.-C. Shen and C. Ragasa, *J. Appl. Pharm. Sci.*, **5**, 23 (2015); https://doi.org/10.7324/JAPS.2015.501104
12. J. Zhang, Y. Gu and B. Chen, *OncoTargets Ther.*, **12**, 1937 (2019); https://doi.org/10.2147/OTT.S191621
13. R.L. Geran, N.H. Greenberg, M.M. McDonald, A.M. Schumacher and B.J. Abbott, *Cancer Chemother. Rep.*, **3**, 17 (1972).
14. S.D. Jacinto, E.A.C. Chun, A.S. Montuno, C.-C. Shen, D.L. Espineli and C.Y. Ragasa, *Nat. Prod. Commun.*, **6**, 803 (2011); https://doi.org/10.1177/1934578X1100600614
15. C.Y. Ragasa, *Manila J. Sci.*, **11**, 42 (2018).
16. L. He, W. Shi, X. Liu, X. Zhao and Z. Zhang, *Int. J. Mol. Sci.*, **19**, 3935 (2018); https://doi.org/10.3390/ijms19123935
17. W. Tan, M. Pan, H. Liu, H. Tian, Q. Ye and H. Liu, *OncoTargets Ther.*, **10**, 3467 (2017); https://doi.org/10.2147/OTT.S139009
18. J.-H. Kang, J.-E. Jang, S.K. Mishra, H.-J. Lee, C.W. Nho, D. Shin, M. Jin, M.K. Kim, C. Choi and S.H. Oh, *J. Ethnopharmacol.*, **173**, 303 (2015); https://doi.org/10.1016/j.jep.2015.07.030

19. A. Russo, V. Cardile, M. Piovano, S. Caggia, C.L. Espinoza and J.A. Garbarino, *Chem. Biol. Interact.*, **184**, 352 (2010); https://doi.org/10.1016/j.cbi.2010.01.032
20. M.M. Martínez-Montemayor, T. Ling, I.J. Suárez-Arroyo, G. Ortiz-Soto, C.L. Santiago-Negrón, M.Y. Lacourt-Ventura, A. Valentín-Acevedo, W.H. Lang and F. Rivas, *Front. Pharmacol.*, **10**, 115 (2019); https://doi.org/10.3389/fphar.2019.00115
21. Q.P. Wu, Y.Z. Xie, Z. Deng, X.M. Li, W. Yang, C.-W. Jiao, L. Fang, S.-Z. Li, H.-H. Pan, A.J. Yee, D.Y. Lee, C. Li, Z. Zhang, J. Guo and B.B. Yang, *PLoS One*, **7**, e44579 (2012); https://doi.org/10.1371/journal.pone.0044579
22. Y.H. Rhee, S.J. Jeong, H.-J. Lee, H.-J. Lee, W. Koh, J.H. Jung, S.-H. Kim and K. Sung-Hoon, *BMC Cancer*, **12**, 28 (2012); https://doi.org/10.1186/1471-2407-12-28
23. S. Shao, M. Hernandez, J.K. Kramer, D.L. Rinker and R. Tsao, *J. Agric. Food Chem.*, **58**, 11616 (2010); https://doi.org/10.1021/jf102285b
24. Y. Yazawa, M. Yokota and K. Sugiyama, *Biol. Pharm. Bull.*, **23**, 1298 (2000); https://doi.org/10.1248/bpb.23.1298
25. X. Li, Q. Wu, Y. Xie, Y. Ding, W.W. Du, M. Sdiri and B.B. Yang, *Oncotarget*, **6**, 17832 (2015); https://doi.org/10.18632/oncotarget.4026
26. R.-L. Zhao and Y.-M. He, *J. Ethnopharmacol.*, **210**, 287 (2018); https://doi.org/10.1016/j.jep.2017.08.041
27. S. Chen, T. Yong, Y. Zhang, J. Su, C. Jiao and Y. Xie, *Front Chem.*, **5**, 1 (2017).