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Cytotoxic triterpenoids from the mushroom *Clavulina cinerea* (Bull) J. Schröt (cantharellaceae)

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ABSTRACT

C. cinerea (Bull) J. Schröt (Lyophyllaceae) is among the many edible mushrooms in Kenya and is also traditionally regarded as a complementary medicine for chronically-ill people. The use of these mushrooms in the East African prompted this investigation in which the phytochemistry and potential anti-cancer activity was studied. Chemical constituents of *C. cinerea* were isolated using chromatographic techniques and structures were determined using NMR spectroscopic methods. The NCI 60 human cancer cell line panel was used to evaluate the cytotoxicity of the compounds isolated at 10μM. Three triterpenes, ergosta-7,22-dien-3β-ol (**1**), 5α,6α-epoxyergosta-8(14),22-dien-3β,7α-diol (**2**) and ergosta-7,22-dien-3β,5α,6β,-triol (**3**) and pentacyclic triterpenoids β-amyrin (**4**) were isolated. The compounds were found to possess moderate toxicity against most of the cancer cell lines.

Keywords. Antiproliferative, c*lavulina cinerea*, cytotoxic, ergostane, triterpenoids.

INTRODUCTION

Higher fungi are a rich source of various natural compounds with a wide range of interest in pharmaceutical and healthcare industries. They are able to synthesize diversified functional secondary metabolites due to their survival in unfavorable environments. Ectomycorrhizal fungi are a diverse group of mutualistic root symbionts that receive carbon from their host plants and in return provide enhanced nutrient uptake and resistance to stress and disease (Smith et al., 2010). *Clavulina* species also known as *Clavulina coralloides* are a widely distributed genus of ectomycorrhizal fungi that form fleshy, predominantly coralloid basidiomata. They are particularly important for the decomposition of plant material, due to their production of a wide range of lignocellulolytic enzymes (Osono, 2007). They are traditionally distinguished from other coral fungi by the presence of two-spored basidia with cornuted sterigmata (Corner, 1950; Thacker and Henkel, 2004; Uehling et al., 2012). The

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species habitat is on deciduous and mixed woods. They are characterized by branched basidiomata which are distinctive coral mushroom recognized in the field by its colours and cristate branch tips. *Clavulina* species has a worldwide distribution, with maximum diversity primarily in the tropics. It displays a high morphological variability and its irregular branches grow from a common base (Henkel et al., 2005). Extracts from powdered basidiomata of *C. cinerea* (Bull) J. Schröt have been used as insecticides and have been found to be toxic to *Drosophila melanogaster* (Mier et al., 1996). They are also used as antioxidant with ascorbic acid as the main ingredient and are reported to be a rich source of protein (Agrahar and Subbulakshmi, 2005). *C. cinerea* has been traditionally regarded as a complementary and alternative medicine for chronically-ill people due to their rich nutritive value. The widespread use of these mushrooms in the diet prompted this investigation in which the phytochemistry and cytotoxicity was studied.

MATERIALS AND METHODS

General experimental procedures

NMR analysis was performed on a Bruker 500 MHz NMR spectrophotometer and spectra were recorded in $CDCl_3$. ESIMS were recorded on a Finnigan MAT 95 XP High Resolution Double Focussing MS at the University of Surrey. Structures of compounds isolated were confirmed by comparison of NMR and MS data against literature values as referenced in figures.

Material

The mushroom was collected from the Kerio valley, Elgeyo Marakwet County in Kenya. Kerio Valley is situated in a narrow, long strip that is approximately 80 km by 10 km wide lies between the Cherangani hills and the Tugen Hills. They were collected in July 2013, when the rains had ended and the temperatures were not excessive but with high humidity. The identification of the species through morphological examination tends to be straightforward. *Clavulina* species are separated on the basis of the overall branching pattern, colour, cristation, and acuteness of the apices, as well as the size of the basidiospores (Corner, 1950; Knudsen and Vesterholt, 2012). *C. cinerea* differs from the other species by virtue of moderate branching, dark grey fungus with cristate apices ranging from acute to blunt. Mushroom specimens were authenticated by Dr. Leung Siu Han from Mushroom Initiative, Hong Kong. The mushroom was kept as herbarium voucher specimen number JO 13032 in Integrated Biotechnology Research Laboratory at Egerton University.

Extraction and isolation

The quantity of the mushroom collected was about 857.3 g. They were air dried, macerated and the sample weighed 326.7 g. The sample was extracted in 100% methanol for 72 hrs and the extract concentrated *in vacuo* to give a gummy mass of 5.7 g. The obtained crude extract was suspended in distilled water (2 l) and was then extracted with ethyl acetate thrice to give an EtOAc-soluble crude extract. The extract was purified using column chromatography with silica gel (0.063–0.200 mm, Merck 9385). Purity was checked using TLC plates (Merck Art 554, 20 cm x 20 cm, silica gel 60 F_{254}-coated). The fractions were collected after gradient elution from 100% hexane to 100% dicloromethane (DCM) and thereafter step addition of ethyl acetate (EtOAc) to achieve a 100% ethyl acetate. This was followed to step addition of methanol to achieve EtOAc:MeOH ratio of 95:5, as shown in Figure 1, to obtain 60 fractions of 75 ml each. The first twenty fractions were fatty acids and the remaining forty fractions were combined as shown in Figure 1. The combined fractions were further fractionated using small column with DCM and diethylether and five compounds were isolated.

Antiproliferative assay

The sulphorhodamine B (SRB) assay was used for assessing the cytotoxicity of test agents in a panel of 60 cell lines (Boyd and Paull, 1995). The compounds were evaluated by using the US National Cancer Institute (NCI) Drug Screen Program of 60 human tumour cell lines in the USA. Briefly, the human cancer cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% FBS and 2 mM L-glutamine. For a typical screening experiment, cells were inoculated into 96-well microtiter plates in 100 µl of medium at plating densities depending on the growth characteristics of specific cell lines. After cell inoculation, the microtiter plates were incubated at 37 ^{o}C, 5% CO_2, 95% air and 100% relative humidity for 24 hrs prior to addition of experimental compounds. Following compound addition, the plates were incubated for 48 hrs and change in protein stain optical density allowed the inhibition of cell growth to be analysed.

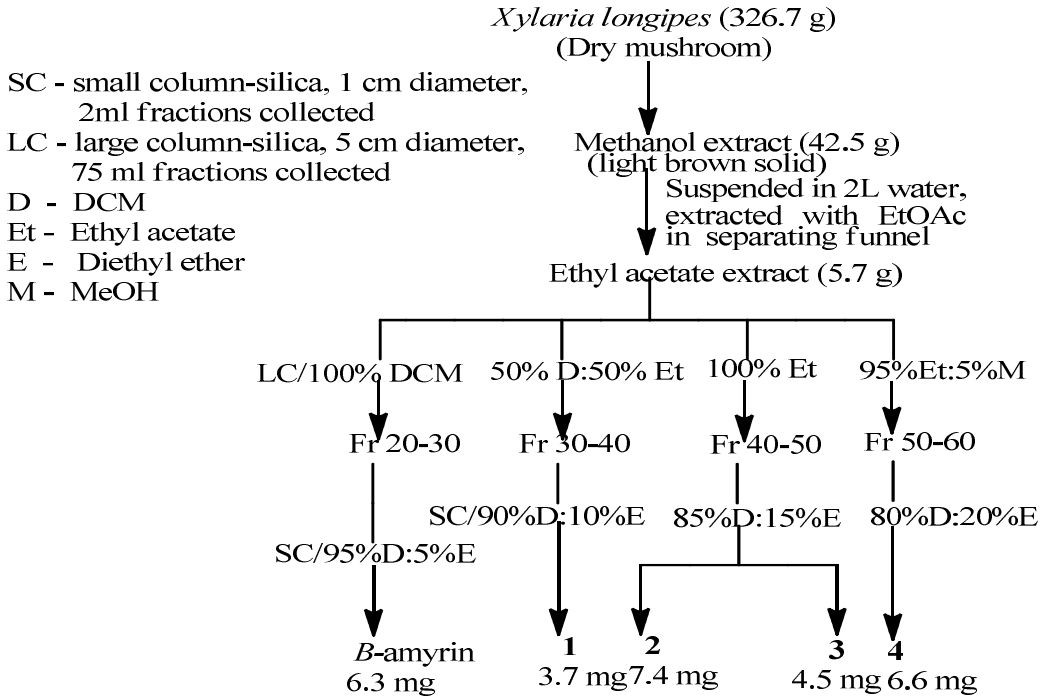

Figure 1: Flow chart showing the isolation of compounds from *C. cinerea.*

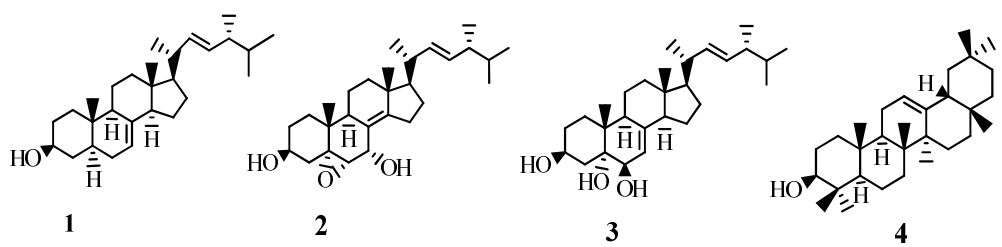

Figure 2: Structures of compounds **1-4.**

RESULTS

Four compounds were isolated from the mushroom as shown in Figure 2. They are namely ergosta-7,22-dien-3β-ol (**1**), 5α,6α-epoxyergosta-8(14),22-dien-3β,7α-diol (**2**) and ergosta-7,22-dien-3β,5α,6β,-triol (**3**). While the sterols are the most common eukaryotic biochemical consequence of squalene cyclization, this fungi produced a second cyclization product, β-amyrin (**4**). The pentacyclic triterpene is commonly found in plants, but rarely reported in fungi. This is the first report of this compound in the *clavulina* species. Compound **1** was obtained as a colourless amorphous powder. The ^{13}C-NMR of compound **1** showed a total of 28 carbon signals containing and one oxygenated carbon signal confirming the structure (Table 1). Compound **2** was isolated as white crystalline needles. Observation of ^{13}C NMR spectra indicated the presence of 28 carbons of a sterol molecule with four oxygenated carbons as shown in Table **1**. Compound **3** was isolated as colourless crystals. The ^{13}C NMR in Table **1** demonstrated 28 carbon signals comprising of three oxygenated carbons. The ^{13}C NMR spectra of compound **4** displayed thirty carbon resonances including one oxygenated carbon, which indicated that this compound was pentacyclic triterpenoid derivative.

Table 1: The ^{13}C NMR spectra of compounds 1-4.

Position	Comp 1 (δ_C)	Comp 2 (δ_C)	Comp 3 (δ_C)	Comp 4 (δ_C)
1	37.4	32.4	33.1	38.6
2	28.3	31.3	31.1	27.9
3	71.3	68.9	67.9	79.3
4	38.2	39.8	40.6	38.8
5	40.4	68.0	76.1	55.4
6	31.6	61.5	73.7	18.6
7	117.7	65.3	117.5	32.6
8	139.8	125.7	144.0	40.7
9	49.7	38.9	43.9	47.7
10	34.4	36.0	37.4	36.9
11	21.8	19.2	22.3	23.3
12	39.7	36.6	39.4	122.9
13	43.5	44.7	43.7	143.7
14	55.3	153.0	54.9	42.2
15α	23.1	25.2	23.1	27.4
16α	29.8	27.4	27.9	27.2
17	56.2	57.1	56.0	33.3
18	12.3	18.3	12.3	53.0
19α	13.2	16.7	19.1	46.0
20	40.7	39.4	39.7	31.3
21α	21.3	21.5	21.3	32.9
22α	135.9	135.4	135.4	36.9
23	132.1	132.5	132.2	28.4

24	43.0	43.0	43.0	15.7
25	33.3	33.3	33.3	15.5
26	20.1	20.2	20.1	17.3
27	19.9	19.9	19.9	26.1
28	17.8	17.8	17.9	28.4
29				33.3
30				21.4

Solvent: CD_3OD; δ in ppm, 125 MHz.

DISCUSSION

Compound **1** has earlier been isolated from the fruit bodies of *Ganoderma lucidum* and no activity has been reported (Seo et al., 2009). Compound **2,** 5α,6α-epoxy-(22*E*,24*R*)-ergosta-8(14),22-diene-3β,7α-diol, has been reported from *Tricholoma portentosum* (Yasunori et al., 1999), the mangrove fungus *Aspergillus awamor*i (Gao et al., 2007), and the fungus *Lactarium volemus* (Yue et al., 2001). Compound **3**, (22*E*,24*R*)-ergosta-7,22-diene-3β,5α,6β-triol, has been earlier isolated from *Agaricus blazei* (Ueguchi et al., 2011), mangrove fungus *Aspergillus awamor*i (Gao et al., 2007), and a liquid culture of *Ganoderma applanatum* (Lee et al., 2011). β-amyrin has been isolated from stems, barks, leaves and seeds of many plants such as leaves of *Melastoma malabathricum* L (Sirat et al., 2010), methanol extract of the stem bark of *Poncirus trifoliate* (Feng et al., 2010), chloroform extract of aerial parts of the plants of *Euphorbia tirucalli* L. (Uchida et al., 2010), seed oil of *Capparis spinosa* (Tlili et al., 2011) among many others. β-amyrin has antihyperglycemic and hypolipidemic effects (Santos et al., 2012) and as potential therapeutic drug for treating asthma owing to its anti-inflammatory, anti-lipoxygenase and antioxidant activities (Bulani et al., 2011).

Antiproliferative activity

Only compounds **1, 2** and **3** were selected for screening against NCI 60 cancer cell lines screen since antiproliferative activity has not been reported (Table 2). The β-amyrin showed inhibition activity against A 549 human lung carcinoma of an IC_{50} of 46.2 μM and MCF-7 human breast cancer of 38.6%. It also inhibited the growth of HCT 116 human colon cancer cells and PC-12 adrenal pheochromocytoma cells at micromolar concentrations (Ono et al., 2004). It exhibited weak cytotoxic activities against NTUB1 cells, a human bladder cancer cell (Lin et al., 2010) and against A549 and HL-60 cancer cell lines with IC_{50} values of 46.2 and 38.6 μM, respectively (Thao et al., 2010). Compound **1** inhibited leukemia MOLT-4 cancer cell line of 12.6%, non-small cell lung cancer A-549/ATCC and NCI-522 of 24.64% and 28.09% respectively. It also inhibited HT-29 colon cancer of 13.36%, UACC-257 melanoma cancer of 14.44%, UO-31 renal cancer of 21.72% and T-47D breast cancer of 12.25%. Compound **1** was screened against 58 cell lines and only inhibited 39 cell lines with a mean graph midpoint (MG_MID) of 98.46% (Table 3). Compound **2** was screened against 58 human cancer cell lines and only 40 gave positive results with a (MG_MID) of 96.80% as seen in Table 3. It was effective against most of non-small lung cancer cell lines especially A-549/ATCC of 24.03% and NCI-H522 of 27.63%. It's also effective against melanoma UACC-257 and 62 of 11.28% and 12.68% respectively, renal cancer UO-31 of 18.23%, Prostate PC-3 of 25.11% and breast cancer MDA-MB-468 of 15.84%. Compound **3** was screened against 58 cell lines and only

24 cell lines inhibited with a (MG_MID) of 100.96% (Table 3). It exhibited inhibition against leukemia CCRF-CEM and K-562 human cancer cell line of 10.19% and 13.79% respectively, non-small lung cancer cell line A-549/ATCC and NCI-H522 of 18.54% and 17.81% respectively. It also inhibited melanoma UACC-62 of 14.25% and prostate PC-3 of 14.78% (Table 2). Among the three compounds, 5α,6α-epoxy-(22*E*,24*R*)-ergosta-8(14),22-diene-3β,7α-diol (**2**), was most active giving the highest number of cell lines inhibited. The hydroxy group at C-3 of the triterpene has been suggested as a possibly important moiety for the activity but addition of functional groups increases of activity. The epoxy ring increases the activity of 5α,6α-epoxyergosta-8(14),22-dien-3β,7α-diol (**2**). The activity of these compounds against cancer cell lines is being reported for the first time.

Table 2: Growth percentage of compound **1-3** in the NCI in vitro 60-cell Drug Screen Program.

	COMPOUNDS/GROWTH PERCENTAGE[a]		
Panel/cell lines	**1** NSC785141	**2** NSC777926	**3** NSC777924
Leukemia			
CCRF-CEM	104.39	98.86	89.81
HL-60(TB)	97.68	106.48	106.38
K-562	103.64	106.65	97.82
MOLT-4	87.5	93.48	86.21
RPMI-8226	104.78	99.53	95.06
SR	96.05	97.98	94.03
Non-Small Cell Lung Cancer			
A549/ATCC	75.36	75.97	81,46
EKVX	97.16	94.05	95.19
HOP-62	90.69	89.96	97.50
HOP-92	103.49	90.95	106.29
NCI-H226	96.28	94.55	97.83
NCI-H23	92.14	92.69	100.10
NCI-H322M	95.87	93.19	94.57
NCI-H460	106.15	106.82	109.38
NCI-H522	71.91	72.37	82.19
Colon Cancer			
COLO 205	101.13	110.03	116.68
HCC-2998	100.26	99.59	125.56
HCT-116	100.8	94.02	93.57
HCT-15	104.9	98.81	104.36
HT29	86.64	88.10	100.03
KM12	102.39	87.51	106.44
SW-620	100.91	106.12	102.12
CNS Cancer			
SF-268	94.91	100.20	100.28
SF-295	100.82	96.71	108.64

SF-539	102.37	99.99	110.22
SNB-19	104.7	98.34	98.08
SNB-75	98.74	104.63	NT
U251	93.96	88.31	89.95
Melanoma			
LOX IMVI	96.64	96.57	97.18
MALME-3M	101.47	93.46	106.11
M14	100.79	95.39	103.12
MDA-MB-435	109.89	111.16	110.43
SK-MEL-2	94.49	97.26	115.34
SK-MEL-28	108.74	113.43	116.72
SK-MEL-5	100.09	99.59	98.81
UACC-257	85.56	88.72	85.75
UACC-62	95.85	87.32	98.20
Ovarian Cancer			
IGROV1	109.18	99.30	110.33
OVCAR-3	110.13	117.50	117.01
OVCAR-4	109.33	99.80	107.06
OVCAR-5	99.08	105.76	98.56
OVCAR-8	100.51	89.52	100.50
NCI/ADR-RES	97.65	97.93	102.89
SK-OV-3	94.58	100.47	99.79
Renal Cancer			
786-0	107.45	103.91	107.02
A498	NT	NT	NT
ACHN	105.71	98.07	105.53
CAKI-1	93.94	92.96	94.18
XF 393	NT	NT	NT
SN12C	98.85	98.33	100.09
TK-10	96.5	88.92	100.23
UO-31	78.28	81.77	89.95
Prostate Cancer			
PC-3	92.73	74.89	85.22
DU-145	108.32	112.19	105.41
Breast Cancer			
MCF7	98.49	96.76	97.79
MDA-MB-231/ATCC	105.29	103.44	103.44
HS 578T	98.98	96.61	101.54
T-47D	107.05	105.45	92.15
MDA-MB-468	87.75	84.16	102.39
BT-549	100.24	99.54	105.15

[a]Data obtained from NCI *in vitro* 60-cell drug screen program at 10^{-5} M concentration.

Table 3: Overview of the results of the *in vitro* single high dose (10^{-5}M) antitumor screening for compounds **1-3**[a].

Compounds	No. studied[c]	No. giving positive results[c]	Growth percent[b] MIG_MID[d]
1	58	39	98.60
2	58	40	96.80
3	58	24	100.96

[a]Data obtained from the NCI's in vitro disease-oriented human tumour cells screen.
[b] Grow percent is the percent of grow inhibition at the single high dose of 10^{-5} M
[c] Refers to the number of cell lines.
[d]MG_MID = mean graph midpoint = arithmetical mean value for all tested cancer cell lines

Conclusion

The aim of the study was to search for anticancer compounds from *C. cinerea*. The compounds isolated were mainly Ergostanes, which are the major antitumor sterol present in this edible mushroom. From the antiproliferative essay results, the compounds have moderate activity against most of the cancer cell lines. Although the molecular mechanism by which this cell death is induced remains to be clarified, they permit to confirm the use of mushrooms as the origin of compounds to be used as novel therapeutic agents for cancer treatment, or as models for molecules more active and selective.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

AUTHORS' CONTRIBUTIONS

AWN was the principal investigator; JOO, PKC and AWW contributed fully to the work. All authors read and approved the manuscript.

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