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ScienceDirect
Food Chemistry 108 (2008) 97–102
Food Chemistry
www.elsevier.com/locate/foodchem

Health-beneficial qualities of the edible mushroom, *Agrocybe aegerita*

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Received 19 June 2007; received in revised form 20 August 2007; accepted 18 October 2007

Abstract

The black poplar mushroom, *Agrocybe aegerita* is a popular edible mushroom with reported anti-tumor properties. A bioactivity-guided investigation gave positive results for ceramide (**1**), methyl-β-D-glucopyranoside and α-D-glucopyranoside, along with already reported linoleic acid and its methyl ester. The structure elucidation of the above was accomplished by NMR and mass spectral methods. The ceramide (**1**) inhibited cyclooxygenase enzymes, COX-1 and -2, by 43 and 92.3%, respectively at 25 μg/ml (34.4 μM). The 50% inhibition concentration (IC_{50}) of compound **1** against COX-2 was 5.3 μg/ml (7.3 μM). Similarly, its anti-cancer potential was investigated against five human cancer cell lines *in vitro* and it was found to inhibit the proliferation of stomach, breast and CNS cancer cell lines at 26.9, 23.2 and 39.1%, respectively, at 100 μg/ml (139 μM) concentration. This is the first report of the isolation of ceramide from *A. aegerita* and its COX and tumor cell proliferation inhibitory activities. This suggested that the consumption of *A. aegerita* would assist in alleviating inflammatory conditions, as well as reducing the development of the above cancers.

Keywords: Mushrooms; Ceramide; Anti-inflammatory; COX 1 and COX-2; Anti-cancer

1. Introduction

Mushrooms, due to their unique flavour and taste, are an attractive delicacy and were used by early civilizations. The knowledge of numerous health benefits that some of these edible mushrooms afford also dates back several centuries. In fact, several thousand years ago in eastern cultures, many edible and non-edible mushrooms were recognized for their potential health benefits (http://www. icnet.uk/labs/med_mush/med_mush.html). In Chinese medicine, the dietary supplements and nutraceuticals made from mushroom extracts are extensively used, along with various combinations of other herbal preparations, to treat a number of medical conditions. Many of these are employed as immunomodulators in cancer therapy (http://www.icnet.uk/labs/med_mush/med_mush.html).

The black poplar mushroom, *Agrocybe aegerita*, is an edible basidiomycete belonging to the order Agaricales. It is one of the constituents in the popular gourmet mix produced by mushroom cultivators and known to possess anti-fungal and anti-tumor properties. Previous studies of the fruiting body of this species have reported the presence of palmitic acid, linoleic acid, ergosterol, mannitol and

Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; ESIMS, electrospray ionization mass spectroscopy; COSY, correlation spectroscopy; FABMS, fast atom bombardment mass spectroscopy; HMBC, heteronuclear multiple bond coherence; TBHQ, t-butylhydroquinone; COX, cyclooxygenase.

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doi:10.1016/j.foodchem.2007.10.049

trehalose (Zang, Mills, & Nair, 2003). The genus *Agrocybe* is also reported to contain several bioactive metabolites, such as indole derivatives with free radical-scavenging ability (Kim et al., 1997), polysaccharides with hypoglycemic activity (Tadashi, Sobue, & Ukai, 1994) and agrocybin, a peptide with anti-fungal activity (Ngai, Zhao, & Ng, 2005).

Although several bioactive ceramides have been isolated and characterized from mushrooms (Gao et al., 2004; Tan & Chen, 2003; Zheng et al., 2006), only a few of them have been investigated for their potential to inhibit COX enzymes (Kang, Kim, Son, Kim, & Chang, 2001).

The cycloxygenase enzymes, COX-1 and -2 are enzymes involved in the process of inflammation. They exist as two distinct but similar isozymes and are responsible for the biotransformation of arachidonic acid to prostanoids. The prostaglandins formed by the enzymatic activity of COX-1 are primarily involved in the regulation of homeostatic functions throughout the body, whereas the prostaglandins formed by COX-2 primarily mediate pain and inflammation (Blobaum & Lawrence, 2007). With the enormous influences that the COX enzymes have over metabolic functions, it has been very difficult to design drugs to regulate inflammation without effecting normal metabolic processes. In addition, several studies have revealed that selective COX-2 inhibitors can prevent colorectal cancers. The COX-2 enzyme is proven to play a key role in the development of such cancers via promotion of angiogenesis, invasiveness and anti-apoptotic effects (Blobaum & Lawrence, 2007). Hence, naturally occurring selective COX-2 inhibitors are of immense significance, as they can be consumed as supplements with few side effects, and they may prevent the formation of colorectal polyps.

The preliminary assay of the methanolic extract of *A. aegerita* showed good anti-oxidant and anti-inflammatory activities. Therefore, in this paper, we report the chemical constituents of *A. aegerita* and the cyclooxygenase enzyme and human tumor cell proliferation inhibitory activities of one of the compounds, the ceramide (**1**), present in this mushroom.

2. Materials and methods

2.1. General experimental procedure

The NMR spectra were obtained on Varian INOVA 300 and VRX 500 instruments in the Max T. Rogers NMR facility at Michigan State University. The spectra were recorded in DMSO-d_6 and $CDCl_3$. The ^1H and ^{13}C chemical shift values were presented in ppm, based on the residual chemical shift δ values, for DMSO-d_6 at 2.50 and 39.95 and for $CDCl_3$ at 7.24 and 77.23 ppm, respectively. The mass spectral data were obtained at the Michigan State University mass spectrometry facility. The FABMS were acquired by a JEOL JMS HX 110 mass spectrometer (Peabody, MA). ESIMS were acquired by a Q-TOF Waters mass spectrometer by flow injection analysis. Medium pressure liquid chromatography (MPLC) was carried out on silica gel 60 and preparative TLC was performed with silica gel GF (G stands for gypsum included in the adsorption formulation of silica and F indicates the presence of a short wave UV indicator that is fluorescent at 254 nm) glass. Analytical TLC was visualized by spraying 10% H_2SO_4 in MeOH. Positive controls, t-butyl hydroquinone (TBHQ), butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT), used in the anti-oxidant assay, were purchased from Sigma Chemical Company.

2.2. Plant material

A. aegerita, a commercial strain, was originally obtained from Fungi Perfecti, LLC (Olympia, WA). The culture was maintained on PDA (potato dextrose agar) Petri plates cultured at 18 °C. Mushrooms were grown in polypropylene bags (Unicorn Imp & Mfg. Corp., Commerce, TX) filled with 3 kg of hydrated soy-supplemented oak sawdust and oak woodchips. The bags were inoculated with grain spawn of *A. aegerita* and incubated in the dark for 56 d at 20 °C and 82% RH. Once fully colonized, the polypropylene bag was removed and the block culture was incubated at 18 °C, 95% RH and 1000 lux light for 35 d. Two flushes of mushrooms were harvested at 10–14 d apart. The mushrooms were commercially cultivated at Diversified Natural Products Inc. Scottsville, Michigan.

2.3. Extraction and islolation

The *A. aegerita* fruitbodies (1 kg) were blended with MeOH (1 l) (8 h X 3) at 25 °C and the methanolic extract was evaporated under reduced pressure. The resultant MeOH extract was partitioned between water and EtOAc. The EtOAc-soluble fraction was separated and dried to obtain a brownish gummy solid (1 g). The water-soluble fraction, upon freeze-drying, yielded a golden brown solid (6 g).

A portion of the EtOAc extract (800 mg) was fractionated on a silica gel MPLC under gradient conditions by using hexane/EtOAC as the mobile phase. The fractions were collected by gradient elution of hexane/EtOAc (h/E, v/v): h/E 100:0, 100 ml; h/E 95:5, 100 ml; h/E 90:10, 100 ml; h/E 85:15, 100 ml; h/E 80:20, 100 ml; h/E 70:30, 100 ml; h/E 60:40, 100 ml; h/E 50:50, 100 ml; h/E 40:60, 100 ml; h/E 20:80, 100 ml; h/E 0:100, 100 ml. Following the investigation by TLC, the above fractions were combined to yield fractions A (220 mg), B (28 mg), C (200 mg) and D (105 mg).

Further purification of fraction A by PTLC (hexane: EtOAc, 85:15, v/v) afforded the methyl ester of linoleic acid (150 mg) as a pale yellow oil. Fraction C, upon further purification by PTLC (hexane: EtOAc, 80:20, v/v), afforded linoleic acid (10 mg) in the form of a white amorphous solid. Chemical identity of linoleic acid and its methyl ester isolated from these fractions were confirmed by NMR experiments and by comparison with published data (http://www.lipidlibrary.co.uk/nmr/1NMRdbs/index.htm). Frac-

tion D was purified further by chromatography on silica gel with $CHCl_3$/MeOH, resulting in compound **1** as a white amorphous solid (12 mg).

A portion of the water-soluble fraction (3 g) was fractionated by using a C-18 column (2.1 × 30 cm) and eluted with MeOH/water. The fractions were collected under gradient elution by using MeOH/water (v/v): 7:3, 500 ml; MeOH/water 1:1, 500 ml and MeOH (500 ml) as mobile phases. Based on TLC observations, two major fractions, E (150 mg) and F (50 mg), were selected for further purification. The fraction E upon separation by PTLC ($CHCl_3$/MeOH 85:15, v/v) gave methyl-β-D-glucopyranoside (54 mg). Purification of the latter fraction by PTLC (MeOH/0.01 M CH_3COOH H_2O 6.5:3.5:0.5, v/v/v) afforded α-D-glucopyaronoside (12 mg). The identities of these compounds were established by comparison of their NMR spectral data with those reported in the literature (Breitmaier & Voelter, 1987; Hobley, Howarth, & Ibbett, 1996).

Ceramide (**1**)

^{1}H NMR (DMSO-d_6, 500 MHz) δ 0.85 (tr, 6H, H_3-18 and H_3-20′), 1.52 (s, 3H, H_3-19), 1.92 (m, 1H, H-10a 7H-10b), 1.94 (m, 2H, H-6a and H-6b), 1.97 (m, 2H, H-7a and H-7b), 2.95 (m, 1H, H-2″), 3.52 (m, 1H, H-1a), 3.81 (m, 1H, H-2 and H-2″), 3.92 (m, 1H, H-1b), 3.98 (m, 1H, H-3), 4.11 (d, $J = 8$ Hz, H-1′), 5.09 (m, 1H, H-8), 5.38 (m, 1H, H-4), 5.58 (m, 1H, H-5), 7.38 (d, $J = 10$Hz, NH), ^{13}C NMR (DMSO-d_6, 125 MHz) δ 68.65 (C-1), 52.82 (C-2), 70.53 (C-3), 130.96 (C-4), 131.01 (C-5), 32.10 (C-6), 27.32 (C-7), 123.44 (C-8), 134.83 (C-9), 39.80 (C-10), 13.86 (C-18/C-16′), 22.05(C-17/ C-15′), 15.66 (C-19), δ 173.71 (C-1′), 71.00 (C-2′), 103.45 (C-1″), 73.35 (C-2″),76.84 (C-3″), 76.52 (C-4″), 69.99 (C-5″), 61.04 (C-6″), (Gao et al., 2004).

2.4. Lipid peroxidation inhibitory assay

The MeOH extract of *A. aegerita* and compound **1** were tested *in vitro* for their potential to inhibit the oxidation of large unilamellar vesicles (LUVs) (Jayaprakasam, Vanisree, Zhang, Dewitt, & Nair, 2006). The phospholipid, 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphatidylcholine (SLPC) (1 mg), in $CHCl_3$ and fluorescence probe, 3-[p-(6-phenyl)-1, 3, 5-hexatrienyl] phenylpropionic acid (DPH-PA) (1 mg), in dimethylformamide (DMF) (1 ml) were mixed and evaporated under reduced pressure to yield a residue. It was then freeze-thawed in morpholinic propane sulfonic acid buffer (MOPS) buffer and homogenized with an extruder (Avestin Inc., Ottawa, ON, Canada) to yield large unilamellar vesicles (LUVs). The final assay volume was 2 ml, consisting of 100 μl of HEPES buffer (50 mM HEPES and 50 mM TRIS), 200 μl of 1 M NaCl, 1.64 ml of N_2-purged water, 20 μl of test sample or DMSO and 20 μl of liposome suspension. The peroxidation was initiated by the addition of 20 μl of $FeCl_2 \cdot 4H_2O$ (0.5 mM). The fluorescence was monitored at 0, 1, 3 and every 3 min up to 21 min, using a Turner Model 450 Digital Fluorometer. The decrease of relative fluorescence intensity over time was used to determine the rate of peroxidation (Jayaprakasam et al., 2006). The percentage of inhibition was calculated with respect to DMSO control. The MeOH extract was tested at 250 μg/ml, whereas the pure compound **1** was tested at 25 μg/ml. The positive controls, BHA, BHT, and TBHQ, were tested at 10 μM.

2.5. Cyclooxygenase inhibitory assay

The COX inhibitory assays of the extracts and compound **1** were performed by using COX-1 and COX-2 enzymes according to the previously published procedures (Jayaprakasam et al., 2006). The initial rate of oxygen consumption during the initial phase of the enzyme-mediated reaction, with arachidonic acid as substrate, was measured using a Model 5300 a biological oxygen monitor (Yellow Spring Instrument Inc., Yellow Spring, OH). An aliquot of 10 μl of test compounds or standards in DMSO was added to the reaction chamber containing 0.6 ml of 0.1 M Tris buffer (pH 7), 1 mM phenol and hemoglobin (17 μg). COX-1 or -2 enzymes (10 μl) were added to the chamber and incubated for 3 min. The reaction was initiated by the addition of arachidonic acid (10 μl of 1 mg/ml solution). Instantaneous inhibition was measured by using Quick Log Data acquisition and control computer software (Strawberry Tree Inc., Sunnyvale, CA, USA). The extracts were tested at 250 μg/ml and the compound **1** at 25 μg/ml (34.4 μM). The dose-response study for COX-2 was performed with a series of concentrations from 25–1.56 μg/ml (34.4–2.1 μM). The percent inhibition was calculated with respect to DMSO control. Each sample was assayed in duplicate and the standard deviation was calculated for $n = 2$. Non-steroidal anti-inflammatory drugs (NSAIDs), aspirin (60 μM), Celebrex™ (26 nM) and Vioxx® (32 nM) were used as positive controls. Adriamycin (doxorubicin) was used as a positive control.

2.6. Tumor cell proliferation assay

Human tumor cells MCF-7 (breast), SF-268 (CNS), NCI-H460 (lung), HCT-116 (colon) and AGS (gastric) were maintained in our laboratory and were cultured in RPMI-1640 medium containing penicillin–streptomycin (10 units/ml for penicillin and 10 μg/ml for streptomycin) and 10% FBS. The cells were grown in a humidified incubator (37 °C, 5% CO_2), counted and plated in 96-well plates (Jayaprakasam et al., 2006). The compound **1** was prepared in DMSO to afford 139 and 34.4 μM concentrations and the final DMSO concentration at 0.2%. At 24 h of incubation, test samples (139 and 34.4 μM) were added to the wells containing appropriate tumor cells and incubated for a further 48 h. MTT [3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg) was dissolved in PBS (phosphate-buffered saline; 1 ml) and aliquots of 25 μl were added to each well. The cell viability was determined according to the published procedure (Jayaprakasam et al., 2006). The samples were assayed in triplicate.

3. Results and discussion

The strong anti-inflammatory and anti-oxidant activities demonstrated by the MeOH extract of *A. aegerita* prompted us to perform a comprehensive chemical investigation of the bioactive metabolites present. In order to separate the salts and water-soluble material in the methanolic extract of *A. aegerita*, it was partitioned with water and EtOAc. The purification of the EtOAc extract yielded compound **1** as a white amorphous solid.

The structure elucidation of compound **1** was accomplished by extensive NMR spectral experiments. The N–H proton of **1,** observed at δ 7.38 as a doublet ($J = 10$ Hz) in the ^{1}H NMR spectrum, exchanged with D_2O. This signal showed a heteronuclear multiple bond coherence (HMBC) resonance with a quaternary carbon at δ 173.71 (C-1′) and the methine at δ 52.83 (C-2). In addition, the H-2 (δ 3.81) signal showed two-dimensional correlation spectroscopy (COSY) coupling with the doublet at δ 7.38 and this further confirmed the presence of an amide moiety in compound **1**. Further, the H-3′ showed HMBC correlation with C-1 and a hydroxyl methine at δ 71.00. Based on the HMBC correlation of H-2′ with C-1 and the COSY coupling observed between H-2′ and H-3′, this hydroxyl methine was assigned to the α-position of the carbonyl. The absence of any other COSY correlations for H-2′ was consistent with this assignment.

In addition, the ^{13}C NMR signal of C-2 (δ 52.82) showed HMBC correlations with H-1a (δ 3.52), H-1 b (δ 3.92) and H-3 (δ 3.98). The HMBC resonance of H-1 with the anomeric carbon at (δ 103.45) of the sugar moiety indicated the linkage of the sugar unit at C-1. The coupling constant and chemical shift of the anomeric proton observed at δ 4.12 (d, $J = 8$ Hz) were consistent with a β-configuration of the glucose linkage. The identity of the sugar was established as D-glucopyranoside by comparison of ^{13}C and ^{1}H NMR data (Hobley et al., 1996).

The hydroxymethine signal (δ 70.53) in the ^{13}C NMR spectrum of **1** was assigned next to C-2, based on its HMBC and COSY correlations observed with H-2. The olefinic proton at δ 5.38 (H-4) displayed a distinct HMBC coupling with a methylene at C-6 and C-3, defining the further elongation of this side chain with a trans-double bond at C-5 and C-4. The trans- geometry of this double bond was supported by the chemical shift value of C-6 at δ 32.10. The carbon signals next to a double bond with trans-configuration usually appear around 32–33 ppm (Dong, Li, & Zhang, 2005). Based on the HMBC correlations of H-4 and H-5 at δ 5.58 with C-6 (δ 32.10) and that of H-6 and H-7 with C-7 and C-8, two methylenes were assigned between this double bond and the trisubstituted double bond at C-9 and C-8. These assignments were consistent with the COSY correlations observed between H-8, H-7, H-6 and H-5. The ^{13}C NMR shift of 19-Me at δ 16.40 was in agreement with the assignment of a trans-double bond (Gao et al., 2004). The side chains extending from C-9 and C-1′ gave rise to two strands of linear alkanes with overlapping NMR signals. The terminal methyls in the molecule were observed at δ 0.85 as a triplet (6H, $J = 6.9$ Hz).

The ESIMS (+) spectrum of compound **1** showed peaks at *m/z* 728 and 750, representing the $[M + H]^+$ and $[M + Na]^+$, respectively. Also, the ESIMS (-) data displayed a peak at *m/z* 726 and indicated the presence of a $[M - H]^+$ ion. Therefore, the molecular formula of compound **1** was assigned as C_{41} H_{77} N O_9. Based on the NMR and mass spectral data, the identity of compound **1** was established as 1-O-β-D-glucopyranosyl-(4E, 8E)-2-[-2-hydroxyhexadecanoylamino]-9-methyl-4,8-octadecadiene-1,3-diol, a known ceramide (Gao et al., 2004) (Fig. 1). However, this is the first report of the isolation of this ceramide from *A. aegerita* fruitbody.

The methanolic extract of *A. aegerita* inhibited lipid peroxidation by 95% at 250 μg/ml. However, compound **1**, the ceramide, did not inhibit lipid peroxidation and this suggested that the observed activity for the extract was probably due to the presence of linoleic acid and its methyl ester present in it. These unsaturated fatty acids were reported to possess anti-oxidant activity (Zang et al., 2003) and were present in the extract at a relatively higher percentage (20%). Linoleic acid, a fatty acid belonging to the category of omega six fatty acids, is an essential dietary requirement (Sanders, 1999).

The methanolic extract also inhibited COX-1 and -2 enzymes, by 31.8 and 61.4%, respectively, at 250 μg/ml concentration. Although linoleic acid and its derivatives are known for their anti-inflammatory properties (Zang et al., 2003), compound **1** also showed significant inhibition of COX-1 and -2 enzymes, by 43 and 92.3% at 25 μg/ml (34.4 μM), respectively (Fig. 2). The 50% COX-2 enzyme

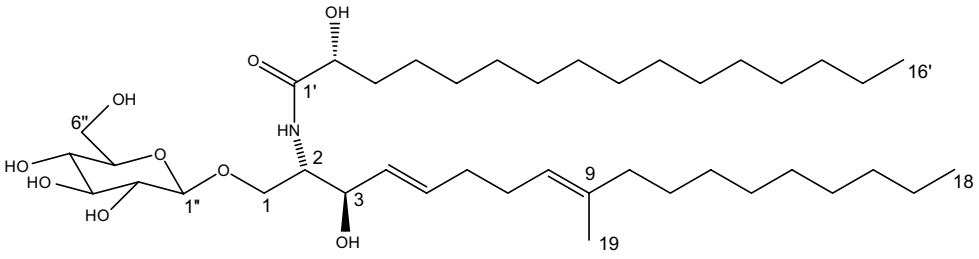

Fig. 1. Structure of compound **1**.

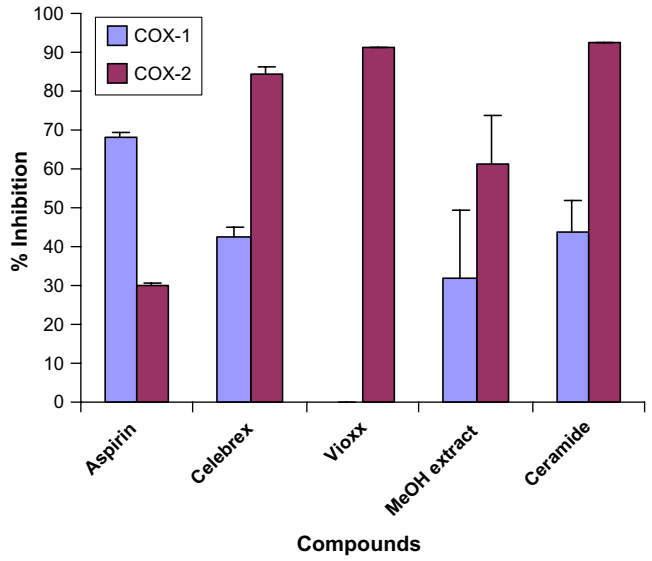

Fig. 2. COX-1 and COX-2 inhibitory activities of the methanolic extract of *Agrocybe aegerita* and compound **1**. Positive controls aspirin, Celebrex and Vioxx, were assayed at 60 μM, 26 nM and 32 nM, respectively, in order to obtain COX enzyme inhibitions >50%. Vertical bars represent the standard deviation.

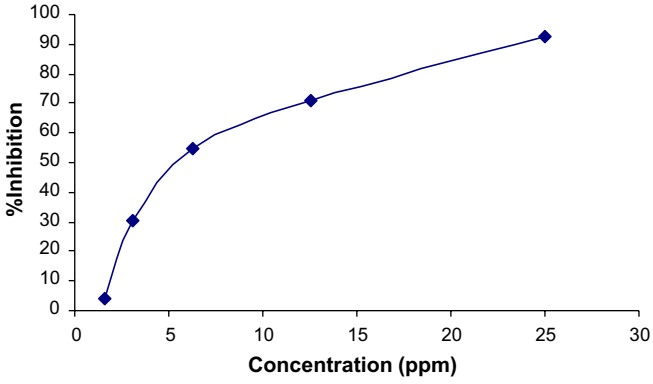

Fig. 3. Dose-response curve for compound **1** against COX-2 enzyme. DMSO was used as solvent control. Percent inhibition was calculated with respect to DMSO control and the vertical bars represent average of two experiments ± SD.

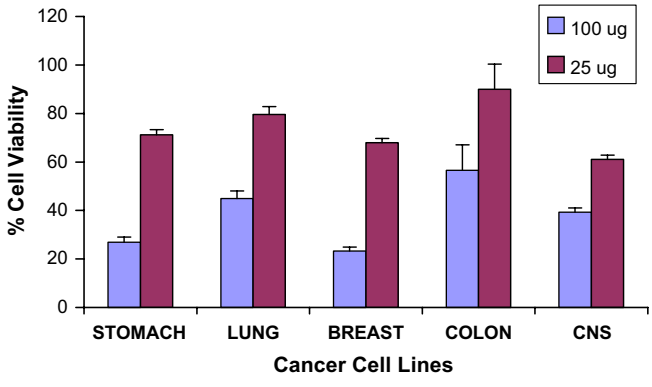

Fig. 4. Percentage cell viability displayed by compound **1** against five different human cancer cell lines at 100 and 25 μg/ml concentrations.

inhibitory (IC_{50}) concentration of compound **1** was at 5.3 μg/ml or 7.3 μM (Fig. 3).

The *in vitro* tumor cell proliferation inhibition of compound **1** was determined against human cancer cell lines: stomach, lung, breast, colon and CNS, at 100 (139 μM) and 25 μg/ml (34.4 μM) concentrations, respectively. It displayed a profound tumor cell proliferation inhibition against stomach, breast and CNS cancer cell lines, as indicated by the percentage cell viability values at 26.9, 23.2 and 39.1%, respectively, when tested at 100 μg/ml (139 μM, Fig. 4). Similarly, the percent cell viabilities of this compound at 25 μg/ml or 34.4 μM against the same cancer cell lines were 71.1, 68.0, and 61.1%, respectively, (Fig. 4). Adriamycin (doxorubicin) was used as a positive control.

In conclusion, the ceramide isolated from *A. aegerita* showed significant inhibition against COX-2 enzyme and the growth of stomach, breast and CNS cancer cell lines. This suggested that *A. aegerita* is a functional food. Furthermore, it provided evidence for the ability of COX-2 inhibitory food components to function as inhibitors of tumor cell proliferation. This is the first report of COX and tumor cell proliferation inhibitory effects of ceramide **1** and its isolation from *A. aegerita*. An efficacious dose of this compound in fresh or dried *A. aegerita* has the potential to offer useful health benefits. In other words, our data indicate that a regular consumption of the edible mushroom *A. aegerita* may alleviate inflammatory conditions with fewer side effects and reduce the occurrence of stomach, breast and CNS cancers.

Acknowledgement

This is a contribution from the Michigan State University Agricultural experiment station.

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