ARTICLE

Cytotoxic property of *Streptocaulon baumii* extracts and their isolated compounds against different human cancer cell lines

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Investigation of the antiproliferative property of *Streptocaulon baumii* Decne. (Apocynaceae) leaves showed that the hexane fraction nonselectively inhibits cell proliferation of BJ, A549, HCT116 and MCF-7 cell lines using WST-1 cell proliferation assay. In a bioassay-guided scheme, loliolide (1), luteolin 3′-methoxy ether (2) and ursolic acid (3) were isolated. MTT assay indicated that ursolic acid has the highest cytotoxicity against HeLa, MDA-MB231, Cos7 and RAW264.7. The IC_{50} of the purified compounds against a panel of cell lines is in the low micromolar range. This is the first report on the cytotoxicity of *S. baumii* against cancer cells as well as the first report of the isolation of the three compounds from plants of the *Streptocaulon* genus.

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KEYWORDS

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INTRODUCTION

For decades, natural products have been the backbone of cancer chemotherapy. In the presence of emerging new technologies such as gene identification and targeted therapy (ASC 2016), a wider range of new and totally synthetic drugs is becoming available in the market. Nevertheless, natural products are still the source of lead compounds acting as templates in the construction of novel or new compounds that are modified to have optimum biological properties (Mann 2002).

Streptocaulon baumii is endemic to the Philippines and is locally known as hinngiu-kalabau, hinngiu-na-puti and sibot-sibotan. Except for a negative antioxidant activity (Jacinto et al. 2011), there is no published literature on its bioactivity and chemistry. By contrast, compounds isolated from *Streptocaulon juventas*

(Ueda et al. 2003), *Streptocaulon tomentosum* (Rashan et al. 2011), *Streptocaulon griffithin* (Luan et al. 2007) showed potential anticancer activity.

This study investigated the antiproliferative properties of *S. baumii* against human fibroblasts Bence Jones (BJ) cell, A549 (human lung cancer), HCT116 (human colon cancer) and Michigan Cancer Foundation–7 (MCF-7, human breast cancer) cell lines. The activity of the isolated compounds was further confirmed against human cervical carcinoma (HeLa cell line), human breast adenocarcinoma (MDA-MB231 cell line), monkey kidney fibroblasts (Cos7 cell line) and murine macrophage (RAW264.7 cell line).

MATERIALS AND METHODS

Bioassay-guided extraction, fractionation and isolation of S. baumii

Plant sample was collected from Sitio Tikal, Kaylawig, Catmon, Sta. Maria, Bulacan, Philippines (coordinates: 14.824704, 121.009830) and was authenticated against the same plant with an accession number of 3491 by the Dr. Jose Vera Santos Herbarium, Institute of Biology, University of the Philippines Diliman (UPD). A certificate was issued by the institute.

Extraction and solvent partitioning were performed at both the Institute of Chemistry, UPD (IC-UPD) and Academia Sinica, Taiwan (AS). Solvents used for extraction and fractionation for experiments performed in IC-UPD were single distilled, technical grade reagents (n-hexane, ethyl acetate and methanol). All the solvents used in AS Taiwan were purchased from Sigma-Aldrich (anhydrous n-hexane and ethyl acetate), J.T. Baker (High Performance Liquid Chromatography (HPLC) grade acetonitrile), Aencore (HPLC grade methanol), ECHO (American Chemical Society (ACS) grade acetone and methanol) and Uni Ward (neutral ethanol).

The leaves were air-dried and homogenized in methanol. The resulting extracts were filtered and concentrated *in vacuo*. Alcoholic extract was partitioned between hexane and distilled water (1:6 v/v). Aqueous extract was further partitioned with ethyl acetate (1:6 v/v). The resulting hexane and ethyl acetate extracts were concentrated *in vacuo*. The general procedure for the isolation of active compounds is shown in figure 1.

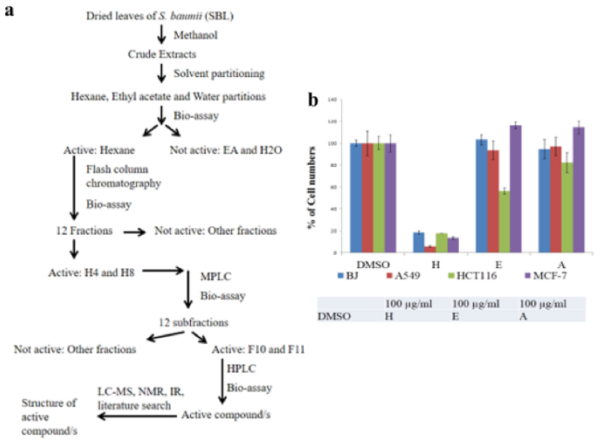

Figure 1: (a) Flow chart for the isolation of bioactive compounds; (b) Effect of various fractions on cell proliferation assay. BJ cell, A549, HCT116 and MCF7 cells were treated with different fractions, including 100µg/ml hexane (H), 100µg/ml ethyl acetate (E), and 100µg/ml aqueous (A) extracts for 48 hours.

Since hexane extract of *S. baumii* leaves (SBL-H) was found to be the active fraction, it was fractionated by using normal phase flash chromatography. Silica gel (Silica gel 60 Merck) with pore size of 0.040-0.063mm and mesh of 230-400 was used (60.3grams). The final dimension of the column is 18.5cm long and 3.5cm in diameter. Gradient system of hexane, ethyl acetate and methanol was used (100% hexane: 0% ethyl acetate to 0% hexane: 100% ethyl acetate and from 100% ethyl acetate: 0% methanol to 0%ethyl acetate: 100% methanol). After combining fractions with the same Thin Layer Chromatography (TLC) profiles (fig. S1), 12 fractions were obtained (namely, SBL-H1 to SBL-H12).

WST (Water-soluble tetrazolium salt) -1 cell proliferation assay against MCF-7 cells of the 12 fractions gave the results shown in figure 2. Figure 3 compares the two most active fractions, SBL-H4 and SBL-H8.

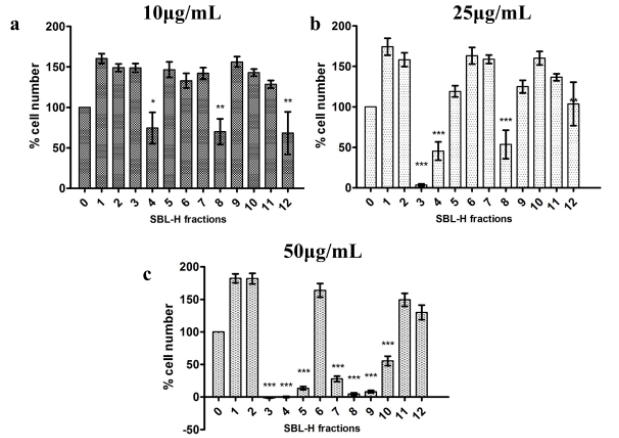

Figure 2: WST-1 Cell Proliferation Assay against MCF-7 cell lines of SBL-H subfractions. Comparing the activity among the subfractions at (a) 10 µg/ml, (b) 25 µg/ml and (c) 50 µg/ml. ***P< 0.001, **P< 0.01, and *P<0.05 when compared to the blank solution (0 µg/ml).

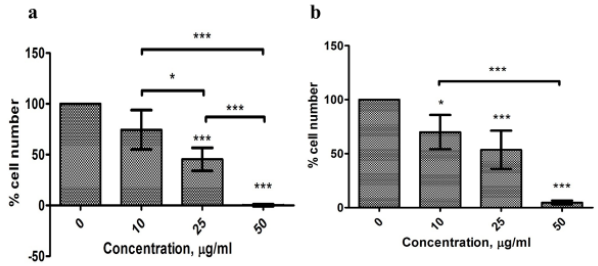

Figure 3: The concentration dependence of the cytotoxic activity of (a) SBL-H4 and (b) SBL-H8 against MCF-7 cell lines. ***P<0.001, **P<0.01, and *P<0.05.

Since SBL-H8 (~ 1g) was prioritized, it was subjected to reverse phase medium pressure liquid chromatography (RP-MPLC) (Biotage Isolera One). The column used was Biotage SNAP Cartridge KP-C18-HS packed with 120 g of C18 stationary phase. The gradient system used is summarized in table S1. The flow rate employed is 30.0mL/min and the UV wavelengths used as detector are 230 and 280nm.

WST-1 cell proliferation assay against MCF-7 cell lines of SBL-H8-F1 to F12 gave the results shown in figure 4.

Combination of similar fractions resulted in 12 subfractions of SBL-H8 (SBL-H8-F1 to SBL-H8-F12). SBL-H8-F10 and F11 were subjected to reverse phase high pressure liquid chromatography (RP-HPLC). Analytical column (Luna 5µ C18 (2) 100A 250 x 4.60mm) was used in the optimization of chromatogram of the two active sufractions with a flow rate of 0.75ml/min and UV wavelengths of 230, 240, 254, 280 and 330 nm. Sample concentration prepared was 10mg/ml and injection volume was 20µl. The optimized gradient system for peak collection for both subfractions is summarized in table S2. Peak

collection was performed using semipreparative column (Luna 5µ C18 (2) 100A 250 x 10mm) with a flow rate of 1.63mL/min and an injection volume of 100µl (10mg/ml sample concentration) using the gradient system in table S2.

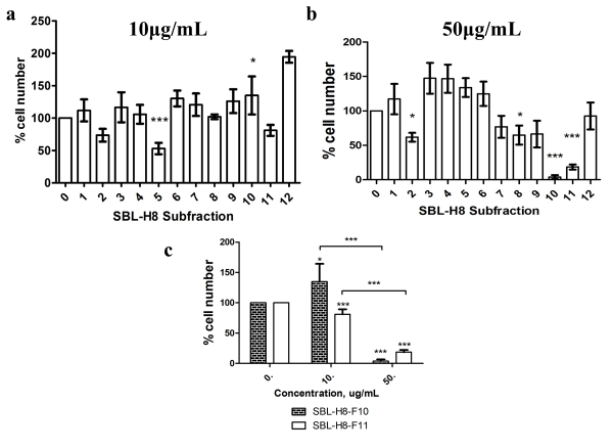

Figure 4: WST-1 cell proliferation assay against MCF-7 cell lines of SBL-H8 subfractions. Comparing the activity among the subfractions at (a) 10 µg/ml and (b) 50 µg/ml where ***P< 0.001, **P< 0.01 and *P<0.05 when compared to the blank solution; (c) shows the concentration-dependent activity of SBL-H8-F11.

Further purification of the collected peaks was performed by using Shimadzu HPLC with DGU-20A5 as the degasser, LC-20AP and AT as the solvent pump, CBU-20A as the controller, SPD-M20A as the PDA detector and SIL-10AP as the injector. LabSolutions is the software used in processing HPLC profiles for the Shimadzu HPLC. Tables S3, S4 and S5 summarize the solvent system used in these purifications.

Structure elucidation of the compounds of *S. baumii*

Nuclear magnetic resonance (NMR) and mass spectroscopy (MS) data were acquired from the University of Utah NMR Spectroscopy Core. Inova 600MHz NMR Agilent Spectrometer equipped with hydrogen cyanide (HCN) cryogenic probe was used. Agilent Q-TOF 6550 equipped with electrospray ionization (ESI) positive scan was used for the MS direct infusion experiments. For MS sample preparation, compounds **1** and **3** were dissolved in 5.0% dimethyl sulfoxide (DMSO) and 95% methanol while compound **2** was dissolved in methanol. The dissolved sample (1µl) was mixed with 100µl of 70% methanol and 0.1% formic acid. Compound **3** was rerun in the University of Connecticut by using Applied Biosciences API2000 ESI-MS. It was resuspended in DMSO, then diluted in acetonitrile 1:1000 and directly injected manually. It was analyzed by using Analyst v1.5.2. Data gathered were compared to the literature (tables S6, S7, and S8).

Compound **1**. (-)-loliolide or (7aR)-6-hydroxy-4,4,7a-trimethyl-6,7-dihydro-5H-1-benzofuran-2-one (CID 100332). White powder. ^{1}H NMR (MetOH-d4, 600MHz) δ 5.76 (1H, s, H7), 4.23 (1H, dt, J= 7.02, 3.37, 3.30 Hz, H3), 2.43 (1H, dt, J = 13.68, 2.40 Hz, H4b) 2.00 (1H, dt, J = 14.00, 2.40, 0.54 Hz, H2b') 1.76 (3H, s, 11-CH_3) 1.76 (1H, dt, J = 13.68, 3.78, 0.54 Hz, H4a) 1.55 (1H, dd, J = 14.00, 3.78 Hz, H2a') 1.48 (3H, s, 9-CH_3) 1.29 (3H, s, 10-CH_3); ESI(+)MS *m/z* 219.0997 $[M+Na]^+$ (calcd for $C_{11}H_{16}O_3$ is 196.24294 g/mol).

Compound **2**. Luteolin 3′-methoxy ether or 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl) chromen-4-one (CID 5280445). Yellow orange powder. ^{1}H NMR (MetOH-d4, 600MHz) δ 7.45 (1H, dd, J = 8.50, 2.22 Hz, 6'H), 7.37 (1H, d, J = 2.22 Hz, 2'H) 7.07 (1H, d, J = 8.50 Hz, 5'H) 6.44 (1H, s, 3H), 6.21 (1H, d, J = 1.95 Hz, 8H), 6.02 (1H), d, J = 1.95, 6H) 3.94 (3H, s, -OCH_3); ESI(+)MS *m/z* 301.0707 $[M+H]^+$ (calcd for $C_{16}H_{12}O_6$ is 300.262889 g/mol).

Compound **3**. Ursolic acid or (1S,2R,4aS,6aR,6aS,6bR,8aR,10S,12aR,14bS)-10-hydroxy-1,2,6a,6b,9,9,12a-heptamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid (CID 64945). White powder. ^{13}C NMR (DMSO, 500MHz) δ 179.02 (C, C-28) 138.88 (C, C-13) 124.78 (CH, C-12) 77.30 (CH, C-3) 55.24 (CH, C-5) 52.92 (CH, C-18) 47.49 (CH, C-9) 47.29 (C, C-17) 42.09 (C, C-14) 39.53 (C, C-8) 39.01 (CH, C-19) 38.98 (CH, C-20) 38. 83 (C, C-4) 38.68 (CH_2, C-1) 36. 98 (C, C-10) 36.86 (CH_2, C-22) 33.18 (CH_2, C-7) 30.76 (CH_2, C-21) 28.71 (CH_3, C-23) 28.04 (CH_2, C-15) 27.42 (CH_2, C-2) 24.36 (CH_2, C-16) 23.73 (CH_3, C-27) 23.30 (CH_2, C-11) 21.60 (CH_3, C-30) 18.46 (CH_2, C-6) 17.53 (CH_3, C-29) 17.44 (CH_3, C-26) 16.54 (CH_3, C-25) 15.68 (CH_3, C-24); APcI ESI(+)MS *m/z* 553.500 $[M+DMSO+H_2O+H]^+$, (calcd for $C_{30}H_{48}O_3$ is 456.463500g/mol).

The structure of the three compounds are shown in figure 5.

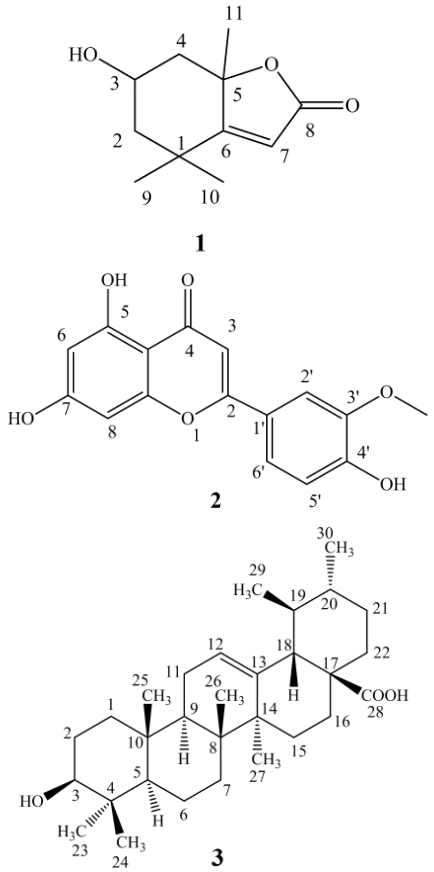

Figure 5: Structures of (-)-loliolide (1), 3'-methoxy luteolin (2) and ursolic acid (3).

Cell proliferation assay

WST-1 assay was used for the bioassay-guided fraction. The cells were prepared by following the method for cell subculture. Enough amount of 1.0×10^4 cells/ml solution was prepared and 100µl was placed in each well in a 96-well plate (Corning Incorporated). Human fibroblasts BJ cell, A549 (lung cancer), HCT116 (colon cancer) and MCF-7 (breast cancer) cells were utilized. Sample extract/fraction/isolate was dissolved in enough amount of DMSO by obtaining a final concentration of 10 mg/ml. From this stock solution, desired sample concentrations were prepared by pipetting out an appropriate amount of sample and diluting it with a fresh prewarmed medium.

After 24 hours of incubation, the medium was removed from the well leaving the adherent cells. One hundred microliters of the prepared sample in the medium was added into each well. DMSO (1µl in 1000µl of medium), puromycin (1µl of 10mg/ml diluted to 1.0ml with medium) and medium only were prepared and added as controls. The plate was incubated for another 72 hours before the addition of 100µl WST-1 dye (Roche) solution (prepared by mixing 10µl of dye with 100µl of medium). The plate was read by using Synergy H1 microplate reader (Biotek Instruments) and Gen5 software version 2.01.14 with a 440nm wavelength after 3 hours of incubation. Percentage viability was calculated by using the following formula:

$$\%viability = \frac{Abs_{sample} - Abs_{control}}{Abs_{DMSO} - Abs_{control}} \qquad \text{(Eq. 1)}$$

where Abs_{sample}, $Abs_{control}$ and Abs_{DMSO} are the absorbance of sample extract, medium control and DMSO, which serves as the blank or 0µg/mL solution.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity assay was performed on the isolated pure compounds using 5000 cells each of Human Cervical Carcinoma (HeLa cell line), Human Breast Adenocarcinoma (MDA-MB231 cell line), Monkey Kidney Fibroblasts (Cos7 cell line) and Murine Macrophage (RAW264.7 cell line). The compounds, being resuspended in DMSO while noting that the highest final DMSO concentration was 2.5%, were diluted in a growth medium. Cells were exposed to a twofold serial dilution series (starting at 125 µg/ml) and incubated for 24 hours. Following incubation with the compound, media were aspirated and the cells were washed with prewarmed sterile phosphate buffered saline (PBS) twice. Culture medium containing 500 µg/ml MTT was then added. Color development was allowed to occur for 4 hours. Absorbance at 595 nm was recorded using a Molecular Systems FlexStation 3 Plate Reader.

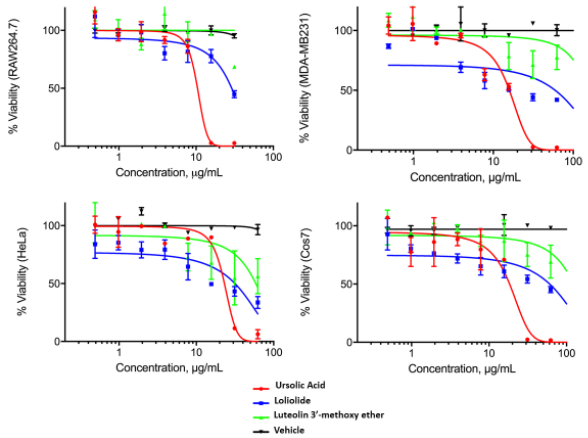

Figure 6: Cytotoxicity assay result against (A) HeLa; (B) MDA-MB231; (C) Cos7 and (D) RAW264.7.

Table 1: IC_{50} of (-)-loliolide (1), luteolin 3'-methoxy ether (2) and ursolic acid (3).

Compounds	IC_{50}, µM			
	HeLa	MDA-MB231	Cos7	RAW264.7
1	59.82#	130.45#	225.03#	145.02#
2	75.50#	OR	OR	OR
3	52.57	22.29	46.29	25.86

*OR = out of range of the concentration used, #P<0.05 when compared to IC_{50} of **3** since no chemotherapeutic drug was available. Ursolic acid is already a known cytotoxic compound (Ma et al. 2005).

Statistical analysis

Comparisons between multiple groups were performed by using Tukey's Multiple Comparison Test of One Way analysis of variance (ANOVA) unless indicated otherwise. P < 0.05 (*), < 0.01 (**) or < 0.001(***) was considered to be statistically significant between control and treatment groups.

Selectivity index

To know the cytotoxic selectivity of the compounds isolated, the selectivity index (SI) was determined. The following equation was used:

$$SI = \frac{IC_{50}^{NCC}}{IC_{50}^{CC}} \qquad \text{(Eq. 2)}$$

where IC_{50}^{NCC} is the IC_{50} of the compound in normal cell lines, while IC_{50}^{CC} is the IC_{50} in cancer cell line. SI was determined against Cos7. An SI ≥10 was considered to belong to a selective compound (Peña-Morán et al. 2016). Table 2 summarizes the SI of the compounds.

Table 2: Selectivity index of isolated compounds.

Compounds	HeLa	MDA-MB231	RAW264.7
1	3.76	1.73	1.55
2	5.51*	NA	NA
3	0.88	2.08	1.79

*Calculated by using 416.30 µM as the IC_{50} of the compound in Cos7 cells (the maximum concentration used in the experiment). This was performed because the compound has an out-of-range IC_{50} for Cos7.

RESULTS AND DISCUSSION

Bioassay-guided fractionation and isolation of bioactive compounds

Hexane, ethyl acetate, and water extracts of *S. baumii* leaves (SBL) were initially tested for inhibitory activity against a panel of cells by using WST-1 cell proliferation assay (fig. 1). Hexane extracts of SBL (SBL-H) decreased the cell density of all cells to less than 20%. Among the 12 subfractions from flash chromatography (fig. 2), SBL-H4 and H8 gave a dose-dependent cytotoxicity (fig. 3) when tested using WST-1 assay against MCF-7 cell line. SBL-H8 exhibited high potency at 10 µg/ml (P < 0.05) compared to SBL-H4 (fig. 4). When compared to the puromycin, which serves as the positive control, SBL-H8 showed almost the same activity as puromycin (P<0.05) unlike SBL-H4 (P<0.01) (data not shown). With this and its potency at low concentration, SBL-H8 was prioritized. RP-MPLC of SBL-H8 gave 12 fractions which were tested against MCF-7 cells (fig. 4). SBL-H8-F10 and F11 have a significant activity at 50 µg/ml (P < 0.001). SBL-H8-F11 showed dose-dependent cytotoxicity and significant activity at 10 and 50 µg/ml (P < 0.001). Purification and characterization using HPLC (tables S3. S4, and S5), MS and NMR (tables S6. S7, and S8) of the SBL-H8-F11 resulted in the identification of three compounds: (-)-loliolide (1), 3′-methoxy luteolin (2) and ursolic acid (3) (fig. 5) with percent yields of 0.00617%, 0.00370% and 0.0136%, respectively.

Cytotoxic effects of compounds loliolide, luteolin 3′-methoxy ether and ursolic acid

The cytotoxic effects of the compounds loliolide, luteolin 3′-methoxy ether, and ursolic acid were evaluated using the MTT assay on a panel of cell lines that included HeLa, MDA-MB231, Cos7, and RAW264.7 (fig. 6, table 1). Ursolic acid and loliolide showed nonselective cytotoxicity (table 2).

Ursolic acid has the most potent nonselective activity against all four cell lines (table 2). It is ubiquitous (Babalola and Shode 2013, Becker et al. 2005, Nascimento et al. 2014, Ringbom et al. 1998) and its cytotoxic activity has been reported against HL-60, BGC, Bel-7402, and HeLa (Ma et al. 2005). Another *in vitro*

cytotoxicity assay was performed in ursolic acid and derivatives by Yaqui Meng et.al. (Meng et al. 2010) showing its antitumor activity against HeLa, SKOV3, and BGC-823 cell lines. Ester derivatives of ursolic acid can exert antiproliferative activity against lung, colon, breast and renal cancers, melanoma and leukemia cell lines with IC_{50} ranging from 1.2 µM to 1.1 µM (Neto 2011). However, these papers do not mention the cytotoxic activity of ursolic acid against normal human cell lines. Hence, based on this study, ursolic acid can be considered toxic even to normal cell lines. The mechanism of action of ursolic acid with regards to its cytotoxic activity has been studied. Łukasz Woźniak et al.'s review (Woźniak et al. 2015) summarizes the different mechanisms of action of the compound with different cancer cell lines. Further investigation of ursolic acid's structure modification to enhance selectivity is a prospect of our paper. This is the first time that ursolic acid is reported to be isolated from *S. baumii*.

Luteolin 3′-methoxy ether is the least active (IC_{50} of 75.50 µM) but is a potential antitumor drug because of its selectivity against HeLa cells (table 2). The mechanism of action of this compound can be different from ursolic acid and loliolide since it is not active against MDA-MB231. It is not toxic compared to ursolic acid and loliolide since it did not affect the cell proliferation of Cos7 and RAW264.7 normal cell lines. Further experiments are now ongoing regarding its mechanism of action. Luteolin 3′-methoxy ether has antioxidant (Mishra et al. 2003), chemoprotective (Takemura et al. 2010), and antiproliferative effects in MCF-7 cells (Takemura et al. 2010). This is the first time that luteolin 3'-methoxy ether was isolated from *S. baumii*.

Loliolide inhibits the cell proliferation of cancer cells in the same selectivity as ursolic acid (table 2). Like luteolin 3′-methoxy ether, its mechanism of action had not been reported before. Loliolide's mechanism of action will be different from that of ursolic acid because of their noticeable difference in structure and functional groups (fig. 5). Likewise, experiments are already ongoing for the determination of its mechanism of action. Loliolide is reported to possesses vasodilatory, anti-inflammatory, antipyretic, antitumor, antifungal and antibacterial properties. It may also be used to treat wound, dysentery, and diarrhea (Grabarczyk et al. 2015). It was reported to be inactive against MCF-7 cells (Pan et al. 2009). This is the first time that loliolide is reported to be isolated from *S. baumii*.

CONCLUSION

This study shows the cytotoxic activity of SBL-H extract of *S. baumii* leaves and the isolation of ursolic acid, luteolin 3′-methoxy ether, and loliolide as cytotoxic compounds. These three compounds will be further assessed using confirmatory and orthogonal assays.

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REFERENCES

American Cancer Society. Targeted Cancer Therapy. December 11 2016. Available from https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types/targeted-therapy/what-is.html

Babalola IT, Shode FO. Ubiquitous ursolic acid: A potential pentacyclic triterpene natural product. J. Pharmacogn and Phytochem. 2013; 2:214−22.

Becker H, Scher JM, Speakman J-B, Zapp J. Bioactivity guided isolation of antimicrobial compounds from Lythrum salicaria. Fitoterapia. 2005; 76:580−84.

Faidi K, Hammami S, Salem AB, Mokni RE, Garrab M, Mastouri M, Gorcii M, Ayedi MT, Ayedi MT, Taglialatela-Scafati O. Polyphenol derivatives from bioactive butanol phase of the Tunisian narrow-leaved asphodel (Asphodelus tenuifolius Cav., Asphodelaceae). J Med Plants Res. 2014; 8:550−57.

Grabarczyk, M., Wińska, K., Mączka, W., et al. Loliolide - the most ubiquitous lactone. Folia Biologica et Oecologica. 2015;11: 1−8.

Hiraga Y, Taino K, Kurokawa M, Takagi R, Ohkata K. 1997. (−)-Loliolide and Other Germination Inhibitory Active Constituents in Equisetum Arvense. Natural Product Letters. 1997/07/01;10:181−86.

Jacinto S. D., Siguan, A.P.T, Canoy R.J.C. Determining the antioxidant property of plant extracts: a laboratory exercise. Asian J Biol Educ. 2011; 5:22−25.

Luan L-J, Wang Y-F, Zhang L, Wu Y-J. [Effect of griffithin on anticancer activity and apoptosis of cancer cells in vitro]. Acta Pharm Sinica. 2007: 42:104−07.

Ma C-M, Cai S-Q, Cui J-R, Wang R-Q, Tu P-F, Hattori M, Daneshtalab M. The cytotoxic activity of ursolic acid derivatives. Eur J Med Chem. 2005: 40:582−89.

Mann J. Natural products in cancer chemotherapy: past, present and future. Nat Rev Cancer. 2002; 2:143−48.

Meng Y, Song Y, Yan Z, Xia Y. Synthesis and in vivo cytotoxicity of novel ursolic acid derivatives. Molecules.2010: 15:4033−40.

Miguel OG, Santos AR, Calixto JB, Delle Monache F, Yunes RA. Antinociceptive activity of the natural piperidine alkaloid hydrochlorides from Syphocampylus verticellatus. Z Naturforsch C Bio Sci. 2001; 57:81−84.

Mishra B, Priyadarsini KI, Kumar MS, Unnikrishnan MK, Mohan H. Effect of O-glycosilation on the antioxidant activity and free radical reactions of a plant flavonoid, chrysoeriol. Bioorg Med Chem. 2003; 11:2677¬−85.

Nascimento PGGd, Lemos TLG, Bizerra AMC, Arriaga AMC, Ferreira DA, Santiago GMP, Braz-Filho R, Costa JGM. Antibacterial and antioxidant activities of ursolic acid and derivatives. Molecules. 2014; 19:1317−27.

Neto CC. Ursolic acid and other pentacyclic triterpenoids: anticancer activities and occurrence in berries. In: Seeram N., Stoner G. (eds) Berries and Cancer Prevention. Springer, New York, NY. 2011: 41−49.

Pan L, Sinden MR, Kennedy AH, Chai H, Watson LE, Graham TL, Kinghorn AD. Bioactive constituents of Helianthus tuberosus (Jerusalem artichoke). Phytochem Lett. 2009; 2:15−18.

Peña-Morán OA, Villarreal ML, Alvarez-Berber L, Meneses-Acosta A, Rodriguez-Lopez V. Cytotoxicity, post-preatment pecovery, and selectivity analysis of naturally occurring podophyllotoxins from Bursera fagaroides var. fagaroides on breast cancer cell lines. Molecules. 2016; 21: 1420−3049.

PubChem Open Chemistry Database. Compound Summary for CID 64945: Ursolic Acid. Available at https://pubchem.ncbi.nlm.nih.gov/compound/64945

PubChem Open Chemistry Database. Compund Summary for CID 5280666: Chrysoeriol. Available at https://pubchem.ncbi.nlm.nih.gov/compound/5280666

PubChem Open Chemistry Database. Compound Summary for CID 12311356: (-)-loliolide. Available at https://pubchem.ncbi.nlm.nih.gov/compound/12311356

Rashan LJ, Franke K, Khine MM, Kelter G, Feibig HH, Neumann J, Wessjohann LA. Characterization of the anticancer properties of monoglycosidic cardenolides isolated from Nerium oleander and Streptocaulon tomentosum. J Ethnopharmaco.2011; 134:781−88.

Ringbom T, Segura L, Noreen Y, Perera P, Bohlin L. Ursolic acid from Plantago major, a selective inhibitor of cyclooxygenase-2 catalyzed prostaglandin biosynthesis. J Nat Prod. 1998; 61:1212−15.

Takemura H, Nagayoshi H, Matsuda T, Sakakibara H, Morita M, Matsui A, Ohura T, Shimoi K. Inhibitory effects of chrysoeriol on DNA adduct formation with benzo[a]pyrene in MCF-7 breast cancer cells. Toxicol. 2010; 274:42−48.

Takemura H, Uchiyama H, Ohura T, Sakakibara H, Kuruto R, Amagai T, Shimoi K. A methoxyflavonoid, chrysoeriol, selectively inhibits the formation of a carcinogenic estrogen metabolite in MCF-7 breast cancer cells. J Steroid Biochem and Mol Biol. 2010; 118:70−76.

Ueda JY, Tezuka Y, Banskota AH, Tran QL, Tran QK, Saiki I, Kadota S. Antiproliferative activity of cardenolides isolated from Streptocaulon juventas. Biological & pharmaceutical bulletin. 2003:1431−35.

Valdes LJ. Loliolide from Salvia divinorum. J Nat Prod. 1986; 49:171.

Wozniak L, Skapska S, Marszalek K. Ursolic acid−a pentacyclic triterpenoid with a wide spectrum of pharmacological activities. Molecules. 2015; 20: 20614−41.

Yang HH, Hwangbo K, Zheng MS, Cho JH, Son JK, Kim HY, Baek SH, Choi HC, Park SY, Kim JR. Inhibitory effects of (-)-loliolide on cellular senescence in human dermal fibroblasts. Arch Pharm Res. 2015: 876−84.

Yang JS, Su YL, Wang YL. Studies on the chemical constituents of Desmodium styracifolium (Osbeck) Merr. Yao xue xue bao = Acta Pharm Sin. 1993; 28:197−201.

Yang X, Kang M-C, Lee K-W, Kang S-M, Lee W-W, Jeon Y-J. Antioxidant activity and cell protective effect of loliolide isolated from Sargassum ringgoldianum subsp. coreanum. Algae. 2011; 26:201−08.

SUPPLEMENTARY INFORMATION

A. TLC results for SBL-H extract

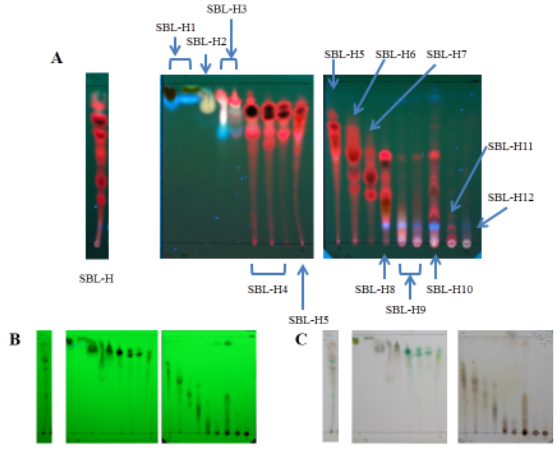

Figure S1: Normal phase TLC profile of SBL-H sub-fractions H1-H12. (A) Viewed under 365nm UV, (B) viewed under 254nm UV and (C) viewed under normal light after treatment of 10% H2SO4 in methanol which serves as charring reagent detecting organic compounds.

B. RP-MPLC of SBL-H8

Table S1: Gradient system used in RP-MPLC experiment for SBL-H8

	Solvent Mixture	Time, mins
Equilibrium	20% H_2O: 80% methanol	13.0
1	20% H_2O: 80% methanol	10.0
2	20% H_2O: 80% methanol to 10% H_2O: 90% methanol	15.0
3	10% H_2O: 90% methanol to 0% H_2O: 100% methanol	32.0
4	0% H_2O: 100% methanol	30.3

C. TLC profile of SBL-H8 fractions

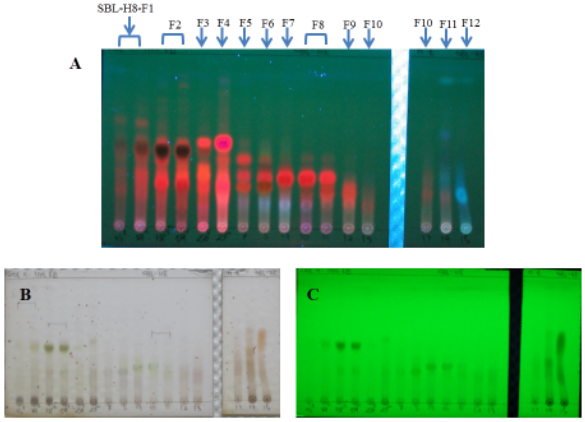

Figure S2: Normal phase TLC profile of SBL-H8 fractions F1-F12. (A) Viewed under 365nm UV, (B) viewed under normal light after treatment of p-anisaldehyde solution, which is used for the detection of phenols, sugars, steroids and terpenes and (C) viewed under 254

D. RP-HPLC gradient system for SBL-H8-F10 and F11

Table S2: Gradient system used for peak collection of SBL-H8-F10 and F11 subfractions.

	Solvent Mixture	Time, mins
1	50% H_2O: 50% Methanol	0.0
2	20% H_2O: 80% Methanol	20.0
3	20% H_2O: 90% Methanol	25.0
4	1.8% H_2O: 98.2% Methanol	28.0
5	1.8% H_2O: 98.2% Methanol	50.0
6	1.7% H_2O: 98.3% Methanol	55.0
7	0% H_2O: 100% Methanol	60.0

E. RP-HPLC Gradient system for the purification of isolated compounds.

Table S3: Gradient system used for the purification of ursolic acid.

	Solvent Mixture	Time, mins
1	10% H_2O: 90% Methanol	0.01
2	2% H_2O: 98% Methanol	40.0
3	0% H_2O: 100% Methanol	40.1
4	0% H_2O: 100% Methanol	53.0
5	10% H_2O: 90% Methanol	53.1
6	10% H_2O: 90% Methanol	65.0

Table S4: Gradient system used for the purification of luteolin 3'-methoxy ether.

	Solvent Mixture	Time, mins
1	50% H_2O: 50% Methanol	0.0
2	20% H_2O: 80% Methanol	20.0
3	20% H_2O: 90% Methanol	25.0
4	1.8% H_2O: 98.2% Methanol	28.0
5	1.8% H_2O: 98.2% Methanol	50.0
6	1.7% H_2O: 98.3% Methanol	55.0
7	0% H_2O: 100% Methanol	60.0
8	0% H_2O: 100% Methanol	65.0

Table S5: Gradient system used for the purification of loliolide.

	Solvent Mixture	Time, mins
1	70% H_2O: 30% Methanol	0.01
2	60% H_2O: 40% Methanol	10.0
3	50% H_2O: 50% Methanol	30.0
4	30% H_2O: 70% Methanol	31.0
5	20% H_2O: 80% Methanol	40.0
6	5% H_2O: 95% Methanol	50.0
7	0% H_2O: 100% Methanol	60.0
8	0% H_2O: 100% Methanol	80.0
9	70% H_2O: 30% Methanol	81.0
10	70% H_2O: 30% Methanol	95.0

F. Literature comparison of NMR data of the isolated compounds

Table S6: ^{1}H NMR reported for loliolide.

Position	Chemical Shift, δ, ppm				
	in MeOH-d4 (present study)	in DMSO-d6 (Yang et al. 2011)	in CDCl3 (Hiraga et al. 1997)	in CDCl3 (Valdes 1986)	in Methanol-d4 (Yang et al. 2015
H2a'	1.55	1.27	1.53	1.535	1.55
H2b'	2.00	1.87	1.98	1.981	1.96
H3	4.23	3.97	4.33	4.338	4.22
H4a	1.76	2.08	1.79	1.785	1.72
H4b	2.43	2.33	2.46	2.466	2.43
H7	5.76	5.80	5.70	5.697	5.74
9-CH_3	1.48	1.22	1.47	1.472	1.45
10-CH_3	1.29	1.19	1.28	1.276	1.26
11-CH_3	1.76	1.51	1.79	1.787	1.75

Table S7: ^{1}H NMR chemical shifts of luteolin 3'-methoxy ether as reported in the literature.

Position	Chemical Shift, δ, ppm			
	in MetOH-d4 (in this study)	in acetone-d6 (Yang et al. 1993)	in MetOH-d4 (Faidi et al. 2014)	in C_5D_5N (Miguel et al. 2002)
-OCH_3	3.94	4.00	3.98	3.83
3 H	6.44	6.69	6.62	7.00
6 H	6.02	6.25	6.20	6.88
8 H	6.21	6.55	6.45	6.78
2' H	7.37	7.63	7.50	7.62
5' H	7.07	7.00	7.52	7.29
6' H	7.45	7.60	6.95	7.66

Table S8: Comparison of ^{13}C NMR chemical shifts for ursolic acid as reported in the literature.

Position	in MetOH-d4 (in this study)	As reported (Babalola and Shode 2013)
C-1	38.68	38.4
2	27.42	28.1
3	77.30	78.1
4	38.83	38.4
5	55.24	55.8
6	18.46	18.8
7	33.18	33.6
8	39.53	40.0
9	47.49	48.3
10	36.98	37.4
11	23.30	23.6
12	124.78	125.6
13	138.89	139.7
14	42.09	42.5
15	28.04	28.7
16	24.35	24.9
17	47.29	48.0
18	52.92	53.5
19	39.01	39.5
20	38.98	39.1
21	30.76	31.1
22	36.86	37.3
23	28.71	28.8
24	15.68	15.7
25	16.54	16.6
26	17.44	17.4
27	23.73	23.8
28	179.02	180.0
29	17.53	17.5
30	21.6	21.4