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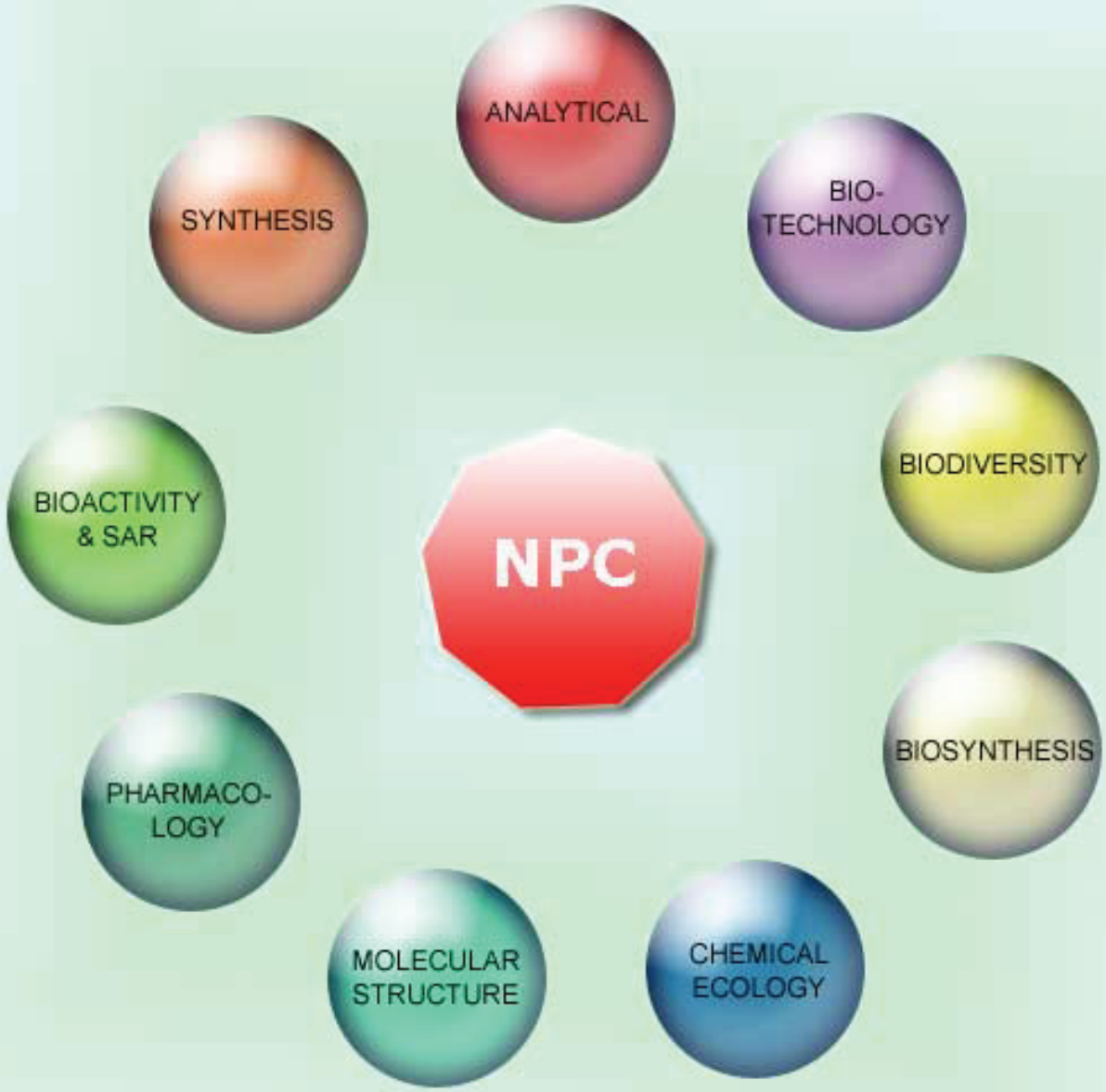

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Cytotoxic Evaluation of Compounds Isolated from the Aerial Parts of *Hedyotis pilulifera* and Methanol Extract of *Inonotus obliquus*

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The aim of this study was to evaluate the inhibitory activity of compounds isolated from the aerial parts of *Hedyotis pilulifera* (Pit.) T.N.Ninh toward selected cancer cell lines. The isolated compounds were identified by analyzing their nuclear magnetic resonance spectral data and physical properties, and comparison of these with reported data. The sulforhodamine B assay was used for the cytotoxic evaluation of isolates. Among twenty-one compounds isolated from *H. pilulifera*, compounds **2**, **3**, and **4** showed moderate inhibitory effect on MCF-7 with IC_{50} values of 63.5, 59.4, and 52.7 μg/mL, respectively, while the other compounds exhibited no effect (IC_{50} values > 100 μg/mL). Further investigation using HT29, LU-1, HL-60, KB, Hep G2, and SK-Mel2 cancer cell lines showed the moderate cytotoxic activity of compound **3** (IC_{50} values ranging from 51.7 to 78.3 μg/mL) to all cells, while compound **4** showed selective effect only against HL-60 cells (IC_{50} 61.5 μg/mL). This is the first report of cytotoxic activity of pomolic acid 3β-acetate (**3**) toward all tested cancer cell lines, and also the first report of cytotoxicity of rotungenic acid (**4**) against LU-1, HL-60, KB, Hep G2, and SK-Mel2 cancer cell lines. The methanol extract of chaga mushroom {*Inonotus obliquus* (Ach. ex Pers.) Pilát} exhibited the strongest cytotoxic effects against HL-60 and LU-1 (32.2 and 38.0 μg/mL, respectively), and modest cytotoxic effects against SW480 (41.3 μg/mL), HepG2 (51.3 μg/mL), KB (57.0 μg/mL), and LNCaP (57.7 μg/mL). We conclude that compounds **3** and **4** from *H. pilulifera* may be useful in further investigation for anticancer agent discovery and chaga could be used as a natural anticancer remedy against promyelocytic leukemia and lung adenocarcinoma.

Keywords: *Hedyotis pilulifera*, Chaga, Pomolic acid 3β-acetate, Rotungenic acid, Leukemia, Adenocarcinoma.

According to the WHO report, approximately 14 million new cancer cases and 8 million cancer-related deaths occurred globally in 2012, making it a major cause of mortality [1]. Since the 1940s, the analysis of the sources of new and approved drugs indicated that about 47% of all approved antitumor drugs are either natural products or directly derived therefrom [2]. We have previously reported [3-4] phytochemicals and antibacterial activity of *Hedyotis pilulifera* (Pit.) T.N.Ninh collected in Vietnam. It is an herbal plant which has been used as a remedy for abdominal pain and osteoarthritis [5]. *Inonotus obliquus* (Ach. ex Pers.) Pilát (chaga mushroom) is a parasitic fungus found mainly on birch trees. The sterile black sclerotium has been considered for centuries as an anticancer material in Russia and other north European countries [6]. A lot of studies [7-10] have shown chaga's antitumor activity against different malignancies.

The aim of this study was to identify the cytotoxic compounds of *H. pilulifera* against seven cancer cell lines. The structure-activity relationships of the active compounds is also discussed. In addition, in this work our aim was to examine the potential action of chaga extract on eight cancer cells for evaluation of cytotoxic activity.

Twenty-one compounds (**1**–**21**) were isolated from the aerial parts of *H. pilulifera* and identified as follows: oleanolic acid (**1**), betulinic acid (**2**), pomolic acid 3β-acetate (**3**), rotungenic acid (**4**), rotundic acid (**5**), daucosterol (**6**), octadeca-9*Z*,12*Z*-dienoic acid (**7**), (7*S**,8*R**,7′*R**,8′*S**)-icariol A2-9-*O*-β-xylopyranoside (**8**), 10-*O*-acetylborreriagenin (**9**), asperuloside (**10**), paederoside (**11**), asperulosidic acid (**12**), daphylloside (**13**), paederosidic acid methyl ester (**14**), adenosine (**15**), (2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxy-tetracosanoylamino)octadecane-1,3,4-triol (**16**), (2*S*)-2,3-*O*-dioctadeca-9*Z*,12*Z*,15*Z*-trienoylglyceryl-*O*-β-D-galactopyranoside (**17**), (2*S*)-2-*O*-octadeca-9*Z*,12*Z*-dienoyl-3-*O*-octadeca-9*Z*,12*Z*,15*Z*-trienoylglyceryl-*O*-β-D-galactopyranoside (**18**), (2*S*)-2,3-*O*-dioctadeca-12*Z*,15*Z*-dienoylglyceryl-*O*-β-D-galactopyranoside (**19**), (2*S*)-2-*O*-hexadecanoyl-3-*O*-octadeca-9*Z*,12*Z*,15*Z*-trienoyl-glycerylglyceryl-*O*-β-D-galactopyranoside (**20**) and (2*S*)-2-*O*-hexadecanoyl-3-*O*-octadeca-9*Z*,12*Z*-dienoylglyceryl-*O*-β-D-galactopyranoside (**21**). The isolation processes and structural elucidations of these compounds were reported in our previous publications [3-5,11].

All isolated compounds were tested for their inhibitory activity toward MCF-7 cells. The result showed that compounds **2** (betulinic acid), **3** (pomolic acid 3β-acetate), and **4** (rotungenic acid) possessed moderate inhibitory effect with IC_{50} values of 63.5, 59.4, and 52.7 μg/mL, respectively, while the other compounds exhibited no effect (IC_{50} > 100 μg/mL, Table 1). Compound **3** displayed moderate inhibitory activity toward all seven tested cell lines with IC_{50} values ranging from 51.7 to 78.3 μg/mL, and compound **4** showed moderate cytotoxicity toward HL-60 cells (61.5 μg/mL), but weak effect toward the other cancer cell lines (84.5-97.2 μg/mL), and no effect toward normal cells (3T3, IC_{50} > 100 μg/mL). To the best of our knowledge, this is the first report of cytotoxic activity of pomolic acid 3β-acetate. In addition, this is

Table 1: Cytotoxic activity of compounds **3** and **4** toward tested cell lines.

Cell lines	IC_{50} (μg/mL)		
	Compound **3**	Compound **4**	Ellipticine
MCF-7	59.4 ± 6.9	52.7 ± 4.0	0.3 ± 0.0
HT29	53.0 ± 3.9	84.8 ± 3.5	0.5 ± 0.1
LU-1	55.8 ± 5.9	95.3 ± 2.7	0.4 ± 0.0
HL-60	51.7 ± 2.1	61.5 ± 3.2	0.4 ± 0.1
KB	78.3 ± 3.3	97.2 ± 5.2	0.4 ± 0.1
HepG2	61.6 ± 5.3	84.5 ± 6.3	0.3 ± 0.1
SK-Mel2	66.3 ± 2.9	93.1 ± 4.3	0.4 ± 0.0
3T3	78.4 ± 6.0	>100	0.4 ± 0.0

Table 2: Cytotoxic activity of chaga extracts on various cancer cell lines.

Concentration (μg/mL)	IC_{50} (μg/mL)							
	LU-1		KB		MDA-MB-231		LNCaP	
	Chaga	Ellipticine	Chaga	Ellipticine	Chaga	Ellipticine	Chaga	Ellipticine
100	84.9	92.1	94.3	97.6	62.1	87.2	66.6	90.2
20	34.6	80.6	15.4	77.8	20.7	75.0	27.7	77.0
4	13.6	39.6	3.1	38.4	12.1	45.7	16.0	47.1
0.8	8.3	20.6	-1.9	3.0	5.1	6.9	5.0	10.8
IC_{50}	**38.0**	**0.4**	**57.0**	**0.6**	**79.2**	**0.5**	**57.7**	**0.4**
Concentration (μg/mL)	IC_{50} (μg/mL)							
	SW480		MKN7		HL-60		HepG2	
	Chaga	Ellipticine	Chaga	Ellipticine	Chaga	Ellipticine	Chaga	Ellipticine
100	89.2	95.7	56.6	81.2	95.9	99.1	92.3	99.8
20	27.9	78.9	30.3	69.7	37.8	79.9	20.2	82.6
4	12.2	43.2	0.5	42.7	11.5	44.1	1.6	43.0
0.8	-3.9	10.9	-5.4	15.4	1.5	19.8	-1.6	11.7
IC_{50}	**41.3**	**0.5**	**71.7**	**0.6**	**32.2**	**0.4**	**51.3**	**0.5**

Ellipticine was used as a positive control, at 10, 2, 0.4 and 0.08 μg/mL

also the first report of cytotoxicity of rotungenic acid against LU-1, HL-60, KB, Hep G2, and SK-Mel2 cancer cell lines. Against MCF-7 and HT-29 cells, rotungenic acid showed percentage cell proliferation inhibitions of 2% and 9%, respectively, at the test concentration of 10 μM [12].

In our previous report [4], the structure-activity relationship between rotungenic acid (**4**) and rotundic acid (**5**) was deduced from the sole difference in absolute configuration at C-4. In particular, rotugenic acid with a 4*S*-configuration was considered to be more active than rotundic acid with a 4*R*-configuration. Interestingly, our current study resulted in a similar conclusion in which the inhibitory effect toward MCF-7 cells of the 4*S*-configuration was significantly stronger than those of 4*R* (IC_{50} 52.7 and >100 μg/mL, respectively). Furthermore, rotungenic acid exhibited selective activity toward MCF-7 and HL-60 cell lines, whereas it showed weak effect toward the rest of the cancer cell lines and no inhibitory activity toward normal fibroblasts (3T3) supporting its safety in future applications. Among the isolated compounds, betulinic acid (**2**) has attracted increasing attention of scientists over the last few years because of its biological and medicinal properties. The review of Moghaddam *et al*. [13] has summarized the inhibitory effect of betulinic acid toward many cancer cell lines. Recently, abundant studies have shown the potential of betulinic acid to become an important agent in cancer therapeutics [14]. The continuing study [4] on the biological activities of the twenty-one compounds isolated from the aerial parts of *H. pilulifera* led to the elucidation of inhibitory activities of the well-documented betulinic acid (**2**) toward MCF-7 cells.

The strongest effects of methanol extracts of chaga were found against promyelocytic leukemia (HL-60) (IC_{50} 32.2 μg/mL) and lung adenocarcinoma (LU-1) (38.0 μg/mL) cells; the effect of this extract on colon adenocarcinoma (SW480) cells was somewhat weaker (41.3 μg/mL) (Table 2). The cytotoxic IC_{50} activity of chaga on other cancer cell lines studied has been between 51.3-79.2 μg/mL.

Generally, the long experience of ethnomedicine should not be underestimated and our previous results [6, 15-18] demonstrated that some popular plants of Estonian and Asian folk medicine showed cytotoxic effect. Anticancer activity of chaga extracts *in vitro* on HL-60 and HepG2 cells has also been shown by other authors [19-20]. In this study the anticancer action of extracts prepared from chaga sclerotium on human lung carcinoma (LU-1), colon adenocarcinoma (SW480), and oral epidermoid carcinoma (KB) is described for the first time. We conclude that chaga could be used as a natural anticancer remedy, especially against promyelocytic leukemia and lung adenocarcinoma.

Experimental

Cell culture: The cell lines used in this study were: ER-positive breast cancer (MCF-7), ER-negative breast cancer (MDA-MB-231), colon cancer (HT29), lung adenocarcinoma (LU-1), leukemia (HL-60), oral epidermoid carcinoma (KB), liver hepatocellular carcinoma (HepG2), melanoma (SK-Mel2), fibroblast (3T3), prostate cancer (LNCaP), gastric carcinoma (MKN7), colon adenocarcinoma (SW480), and promyelocytic leukemia (HL-60). The cells (except HL-60) were cultured as monolayers in either Dulbeco's Modified Eagle Medium (DMEM) or RPMI-1640 (depending on the cell lines) with ingredients including 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and supplemented with fetal bovine serum (FBS) 10%. The cells were sub-cultured after 3-5 days at a ratio of 1:3 and incubated at 37°C in 5% CO_2 and 100% humidity.

Plant material: The aerial parts of *H. pilulifera* were collected in Quang Tri province (1702'15.2"N 10703'55.9"E), Vietnam in August, 2014. Plant identification was conducted by Dr Nguyen The Cuong, Institute of Ecology and Biological Resources, VAST, Vietnam. A voucher specimen (VL01) was deposited at the Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Vietnam. The chaga commercial sample was obtained from a retail pharmacy in Tartu, Estonia. The plant material was collected from the herb farm Kubja Ürditalu, Estonia, N59.054344, E25.963234 and was identified by specific morphological profile. The commercial sample (FB01) is deposited at the Institute of Pharmacy, University of Tartu, Estonia.

Extraction of chaga: The commercial sample of chaga was crushed to a fine powder, which was extracted with methanol, 3 times (48 h per time), at room temperature (20°C). The extracts were evaporated under reduced pressure in a rotary evaporator to obtain crude extracts for cytotoxic assays. Methanolic extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare 4 mg/mL stock solutions that were later mixed with cell culture medium to achieve the desired final test concentrations of 0.8, 4, 20 and 100 μg/mL.

In vitro cytotoxic assay: The effects of the compounds isolated from *H. pilulifera* on viability of cells were determined by sulforhodamine B cytotoxic assay [21]. Cells were grown in 96-well microtiter plates containing 190 μL of medium in each well. After 24 h, 10 μL of the test samples dissolved in DMSO was added to each well. The one plate with no samples served as a day 0 (time-zero) control. The cells were continuously cultured for additional an 48 h, fixed with trichloroacetic acid and stained with sulforhodamine B, followed by determination of optical density at 515 nm using a Microplate Reader (BioRad). DMSO 10% was used as a control sample while ellipticine was used as positive control.

The percentage of growth inhibition (GI %) was calculated by the following equation GI % = 100% − [(OD_t − OD_0)/(OD_c − OD_0)] × 100, in which: OD_t: the average optical density value at day 3; OD_0: the average optical density value at time-zero; OD_c: the average optical density value of the DMSO control sample.

Statistical analysis: Cytotoxicity data for compounds from *H. pilulifera* were calculated and expressed as half-maximal inhibitory concentrations (IC_{50} values ± SD). All experiments were carried out in triplicate and TableCurve 2Dv4 software was used to calculate IC_{50} values.

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References

[1] Forman D, Ferlay J. (**2014**) The global and regional burden of cancer. In *World Cancer Report 2014*. France, World Health Organization, France. 16-53.
[2] Newman DJ, Cragg GM. (**2007**) Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products*, ***70***, 461-477.
[3] Nguyen TH, Ho VD, Nguyen DQP, Kodama T, Ito T, Morita H. (**2016**) A new iridoid from the aerial parts of *Hedyotis pilulifera*. *Natural Product Communications*, ***11***, 365-367.
[4] Nguyen HT, Ho VD, Vo QH, Le TA, Nguyen MH, Kodama T, Ito T, Morita H, Raal A. (**2017**) Antibacterial activities of chemical constituents from the aerial parts of *Hedyotis pilulifera*. *Pharmaceutical Biology*, ***55***, 787-791.
[5] Boissière M, Basuki I, Koponen P, Wan M, Sheil D. (**2006**) Biodiversity and local perceptions on the edge of a conservation area, Khe Tran Village, Vietnam Bogor. Center for International Forestry Research (CIFOR), Indonesia.
[6] Sak K, Jürisoo K, Raal A. (**2014**) Estonian folk traditional experiences on natural anticancer remedies: From past to the future. *Pharmaceutical Biology*, ***52***, 855-866.
[7] Lee SH, Hwang HS, Yun JW. (**2009**) Antitumor activity of water exract of a mushroom, *Inonotus obliquus*, against HT-29 human colon cancer cells. *Phytotherapy Research*, ***23***, 1784-1789.
[8] Lemiezek MK, Langner E, Kaczor J, Kandefer-Szerszen M, Sanecka B, Mazurkiewicz W, Rzeski W. (**2011**) Anticancer effects of fraction isolated from fruiting bodies of chaga medicinal musroom, *Inonotus obliquus* (Pers.:Fr) Pilat (*Aphyllophoromycetideae*): *In vitro* studies. *International Journal of Medicinal Mushrooms*, ***13***,131-143.
[9] Nomura M, Takahashi T, Uesugi A, Tanaka R, Kobayashi S. (**2008**) Inotodiol, a lanostane triterpenoid, from *Inonotus obliquus* inhibits cell proliferation through caspase-3-dependent apoptosis. *Anticancer Research*, ***28***, 2691-2696.
[10] Won DP, Lee JS, Kwon DS, Lee KE, Shin WC, Hong EK. (**2011**) Immunostimulating activity of polysaccharides isolated from fruiting body of *Inonotus obliquus*. *Molecules and Cells*, ***31***, 165-173.
[11] Tran TTL, Vo QH, Ho VD, Nguyen TH. (**2016**) Initial study on chemical constituents of *Hedyotis pilulifera* (PIT.) T.N.Ninh. *Journal of Medicinal Materials*, ***21***, 185-189.
[12] Cheng J-J, Zhang L-J, Cheng H-L, Chiou C-T, Lee IJ, Kuo Y-H. (**2010**) Cytotoxic hexacyclic triterpene acids from *Euscaphis japonica*. *Journal of Natural Products*, ***73***, 1655-1658.
[13] Moghaddam MG, Ahmad FBH, Samzadeh-Kermani A. (**2012**) Biological activity of betulinic acid: A review. *Pharmacology & Pharmacy*, ***3***, 119-123.
[14] Ali-Seyed M, Jantan I, Vijayaraghavan K, Bukhari SNA. (**2016**) Betulinic acid: Recent advances in chemical modifications, effective delivery, and molecular mechanisms of a promising anticancer therapy. *Chemical Biology & Drug Design*, ***87***, 517-536.
[15] Raal A, Nguyen TH, Ho VD, Do TT. (**2015**) Selective cytotoxic action of Scots pine (*Pinus sylvestris* L.) needles extract in human cancer cell lines. *Pharmacognosy Magazine*, ***11***, 290-295.
[16] Sak K, Nguyen TH, Ho VD, Do TT, Raal A. (**2017**) Cytotoxic effect of chamomile (*Matricaria recutita*) and marigold (*Calendula officinalis*) extract on human melanoma SK-MEL-2 and epidermoid carcinoma KB cells. *Cogent Medicine*, ***4***, 1333218, 1-7. https://doi.org/10.1080/2331205X.2017.1333218
[17] Nguyen TH, Ho VD, Do TT, Bui HT, Phan VK, Sak K, Raal A. (**2014**) A new lignin glycoside from the aerial parts and cytotoxic investigation of *Uvaria rufa*. *Natural Product Research*, ***28***, 1091-1098.
[18] Ho VH, Hoanh TNH, Vo QH, Nguyen MH, Raal A, Nguyen TH. (**2017**) A new triterpene ester and other chemical constituents from the aerial parts of *Anodendron paniculatum* and their cytotoxic activity. *Journal of Asian Natural Products Research, doi.org/10.1080/10286020.2017.1336163*, *1-7.*
[19] Kim YJ, Park J, Min BS, Shim SH. (**2011**) Chemical constituents from the sclerotia of *Inonotus obliquus. Journal of the Korean Society for Applied Biological Chemistry*, ***54***, 287-294.
[20] Tanaka R, Toyoshima M, Yamada T. (**2011**) New lanostane-type triterpenoids, inonotsutriols D, and E, from *Inonotus obliquus. Phytochemistry Letters*, ***4***, 328-332.
[21] Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, Gray-Goodrich M, Campbell H, Mayo J, Boyd M. (**1991**) Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. JNCI: *Journal of the National Cancer Institute*, ***83***, 757-766.

Accounts/Reviews

Natural Product Communications
2018
Volume 13, Number 8

Contents

Continued inside backcover