Advance Journal of Food Science and Technology 11(5): 412-415, 2016
DOI:10.19026/ajfst.11.2651
ISSN: 2042-4868; e-ISSN: 2042-4876

Submitted: August 24, 2015 Accepted: September 11, 2015 Published: June 15, 2016

Research Article

Bioassay-guided Separation of Cancer Cells Inhibitory Constituents in *Aquilaria sinensis* Leaves: A New Food Raw Material

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Abstract: To explore the cancer cells inhibitory constituents in *Aquilaria sinensis* leaves, a new food raw material, applying bioassay-guided method by using cytotoxicity test to three human cancer cell lines, cancer cells inhibitory activity of different polar fractions extracted from wild and cultivated *A. sinensis* leaves by different solvents and twelve monomeric compounds were tested. It is disclosed that, cancer cells cytotoxicity of different polar fractions from wild *A. sinensis* leaves were better than that from cultivated *A. sinensis* leaves, no matter in what solvent extraction. The better cancer cells cytotoxicity lied in petroleum ether and ethyl acetate fraction extracted by 70% acetone from wild *A. sinensis* leaves, with the IC50 value low to 9.09±0.93 and 10.65±1.52 μg/mL to DU145 cell line. Twelve compounds were isolated though the bioassay-guided method, eleven of which exhibited slight to moderate inhibitory activities against human prostate cancer cell lines (PC-3 or DU145), while two compounds also exhibited moderate inhibitory activities against human hepatocellular cancer cell lines (HepG2). The best cancer cells cytotoxicity lied in Compound 9 with the IC50 value low to 11.05±2.04 μM to DU145 cell line.

Keywords: *Aquilaria sinensis* leaves, bioassay-guided method, cancer cells inhibitory constituents, cytotoxicity test, new food raw material

INTRODUCTION

The leaves of *Aquilaria sinensis* (Lour.) Gilg (Thymelaeaceae), a new food raw material, are abundant in southern China. Although the leaves are not the medicinal part of the plant, they are broadly used as a main component in several health foods including *A. sinensis* tea and flavor in China (Chen *et al*., 2013). *A. sinensis* leaves exhibited notable biological activities such as anti-inflammatory, antinociceptive, antioxidative, α-glucosidase inhibitory and laxative (Chen *et al*., 2013; Feng *et al*., 2011; Qi *et al*., 2009; Zhou *et al*., 2008).

Previous study indicated that the extract from *A. sinensis* leaves was confirmed to have anticancer activities, which pharmacodynamic material basis remain further investigation (Wang *et al*., 2008; Yu, 2007). Bioassay-guided method is a frequently-used and efficient separation method in natural product research (Wu, 2008), cancer cells inhibitory constituents of *A. sinensis* leaves were separated by bioassay-guided method in this study, to promote the industrial development and provide theoretical basis and technical support for high value-added utilization of *A. sinensis* leaves.

MATERIALS AND METHODS

Instruments and materials: A microplate reader (TECAN SpectraII Plate Reader, Research Triangle Park, N.C., USA).

Analytic grade ethanol, ethyl acetate (EtOAc), *n*-butanol, petroleum ether (b.p. 60-90°C), acetone, sodium nitrite, sulfanilic acid, N-ethylenediamine, citrate sodium, monosodium phosphate, dimethylsulfoxide, chloroform, methanol and muriatic acid were purchased from Guangzhou Chemical Reagent Co. (Guangzhou, China). All cell culture reagents were obtained from Invitrogen Co. (Carlsbad, CA, USA). Cell culture dishes and plates were purchased from Corning Inc. (New York, USA). 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich Co. (St. Louis, MO, USA). Human prostate cancer cell lines (PC-3 and DU145) and

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Human hepatocellular cancer cell lines (HepG2) were established and maintained in our laboratory.

The leaves of *A. sinensis* were collected from Zhongshan City, Guangdong Province, China. The plant material was botanically authenticated by Prof. Zhijian Feng in College of Forestry, South China Agricultural University.

Cancer cells inhibitory activity test: Cancer cells cytotoxicity test was evaluated using MTT assay, which was performed as described previously (Mosmann, 1983) with paclitaxel, 5-Fluorouracil and Doxorubin (Dox) served as the positive control. Briefly, cells were plated on 96-well plates at 3×10^3 cells per well for human prostate cancer cell lines (PC-3 or DU145) or human hepatocellular cancer cell lines (HepG2). After 48 h of exposure, the cells were stained with MTT. Absorbance at 570 nm was used to measure with a multiplate reader.

Cancer cells inhibitory activity of different polar fractions extracted from wild and cultivated *Aquilaria sinensis* leaves by different solvents: Oven-dried leaves of wild and cultivated *A. sinensis* leaves were extracted with methanol, ethanol and 70% acetone for 5 h under percolation method. The filtered extract was condensed under vacuum to give a syrupy extract, which was then partitioned successively with petroleum ether, EtOAc and *n*-butanol. The combined layers of each organic solvent were evaporated in vacuo to yield a petroleum ether-soluble fraction, an EtOAc-soluble fraction and a *n*-butanol-soluble fraction. All the fractions were conducted cancer cells cytotoxicity test according to the cancer cells cytotoxicity test described previously using human prostate cancer cell lines (PC-3 and DU145) and the fractions with the higher cancer cells inhibitory activity were used to carry out follow-up experiments.

Bioassay-guided separation of cancer cells inhibitory constituents in wild *Aquilaria sinensis* leaves: According to the experimental results of the previous section, wild *A. sinensis* leaves and 70% acetone were chosen as experimental materials and extraction solvent, petroleum ether and ethyl acetate fractions were used for follow-up separation and purification to yield 12 compounds. The separation, purification and and structural identification process of the 12 compounds were performed as described previously (Yang *et al*., 2014, 2015). All the compounds were conducted cancer cells cytotoxicity test according to the cancer cells cytotoxicity test described previously using human prostate cancer cell lines (PC-3 and DU145) and human hepatocellular cancer cell lines (HepG2).

RESULTS AND DISCUSSION

As is well-known, plants grow in wild environment often show different characteristics or biological activities comparing with their corresponding cultivated plants and the separation efficiency and quality of active constituents can be affected by extraction solvent. Cancer cells inhibitory activity of different polar fractions extracted from wild and cultivated *A. sinensis* leaves by different solvents were investigated in this study at first.

Cancer cells cytotoxicity of different polar fractions extracted from wild and cultivated *Aquilaria sinensis* leaves by different solvents: The results of cancer cells cytotoxicity of different polar fractions extracted from wild and cultivated *A. sinensis* leaves by different solvents were listed in Table 1.

In Table 1, Cancer cells cytotoxicity of different polar fractions from wild *Aquilaria sinensis* leaves were better than that from cultivated *A. sinensis* leaves, no matter in what solvent extraction. In general, the better cancer cells cytotoxicity lied in petroleum ether and ethyl acetate fraction extracted by 70% acetone from wild *A. sinensis* leaves, with the IC_{50} value low to 9.09±0.93 and 10.65±1.52 μg/mL to DU145 cell line.

Table 1: Cancer cells cytotoxicity of different polar fractions extracted from wild and cultivated *Aquilaria sinensis* leaves by different solvents

Different polar fractions of wild *A. sinensis* leaves	DU145 IC_{50}[a] (μg/mL)	PC-3IC_{50} (μg/mL)	Different polar fractions of wild *A. sinensis* leaves	DU145IC_{50} (μg/mL)	PC-3IC_{50} (μg/mL)
AP[b]	9.09±0.93 [a]	13.26±1.85	AP	>50	>50
AE	10.65±1.52	12.03±1.60	AE	>50	>50
AB	>50	>50	AB	>50	>50
MP	8.49±0.89	28.76±2.39	MP	36.20±3.46	>50
ME	14.91±1.91	29.12±2.56	ME	27.14±2.24	>50
MB	>50	>50	MB	>50	>50
EP	15.16±1.98	>50	EP	>50	21.48±2.50
EE	9.27±0.98	20.18±2.34	EE	>50	>50
EB	23.27±2.44	44.17±4.65	EB	>50	>50
Paclitaxel	0.043±0.007	0.037±0.008	Paclitaxel	0.043±0.007	0.037±0.008

[a]: IC_{50}(half maximal inhibitory concentration) data expressed as mean±SEM (standard error of mean) of three observation per sample.

[b]: The first letter of each code represents extraction solvent and the second letter represents partitioned extraction solvent; Specifically, A represents 70% acetone, M represents methanol, E represents ethanol (at the first letter) or ethyl acetate (at the first letter), B represents *n*-Butanol and P represents petroleum ether

Table 2: Cancer cells cytotoxicity of 12 monomeric compounds

Samples	Cell line	IC50 (μM)[a]	Cell line	IC50 (μM)	Cell line	IC50 (μM)
Compound 1[C]	DU 145	44.31±4.21	PC-3	38.24±1.90	HepG2	-
Compound 2		>100		>100		-
Compound 3		86.97±8.28		>100		>100
Compound 4		61.85±6.08		>100		>100
Compound 5		73.24±1.88		68.58±7.46		>100
Compound 6		94.85±8.54		95.27±7.87		-
Compound 7		18.43±3.01		50.92±3.92		-
Compound 8		13.48±5.42		33.32±1.17		-
Compound 9		11.05±2.04		36.70±7.96		>100
Compound 10		35.88±5.84		42.23±3.14		>100
Compound 11		>100		76.17±8.01		12.54±1.37
Compound 12		76.64±7.27		85.47±6.50		38.63±4.05
Paclitaxel		0.043±0.007		0.037±0.008		-
5-Fluorouracil		31.63±3.80		40.53±6.781		-
DOX		-[b]		-		0.17±0.03

[a]: IC_{50} data expressed as mean±SEM (standard error of mean) of three observation per sample; [b]: - *means no data*; [C]: Compound 1: Mangiferin; Compound 2: Ethyl linoleate; Compound 3: apiolin-7, 4'-dimethyl ether; Compound 4: luteolin-7, 3', 4'-trimethyl ether; Compound 5: Genkwanin; Compound 6: Ergosta-5,7,22-trien-3-one; Compound 7: Quercetin-3-O-*β*-D-galactopyranoside; Compound 8: Stigmasterol; Compound 9: 5,4'-Dihydroxy-7-Methoxyflavanone; Compound 10: 5-Hydroxy-7, 2', 4', 5'- Tetramethoxyflavone; Compound 11: Quercetin; Compound 12: Kaempferol

Cancer cells cytotoxicity of monomeric compounds test: The result of scavenging rates of cancer cells cytotoxicity of monomeric compounds were listed in Table 2.

In Table 2, the present study indicated that, except for Compound 2, monomeric compounds exhibited slight to moderate inhibitory activities against human prostate cancer cell lines (PC-3 or DU145), while Compound 11 and Compound 12 also exhibited moderate inhibitory activities against human hepatocellular cancer cell lines (HepG2). Among the active compounds which exhibited inhibitory activities against human prostate cancer cell lines, the best cancer cells cytotoxicity lied in Compound 9 and Compound 8, with the IC50 value low to 11.05±2.04 and 13.48±5.42 μM to DU145 cell line.

CONCLUSION

According to the experimental result of this study, cancer cells cytotoxicity of different polar fractions from wild *A. sinensis* leaves were better than that from cultivated *A. sinensis* leaves, no matter in what solvent extraction. The better cancer cells cytotoxicity lied in petroleum ether and ethyl acetate fraction extracted by 70% acetone from wild *A. sinensis* leaves.

Twelve compounds were isolated though the bioassay-guided method, eleven of which exhibited slight to moderate inhibitory activities against human prostate cancer cell lines (PC-3 or DU145), while two compounds also exhibited moderate inhibitory activities against human hepatocellular cancer cell lines (HepG2). These active compounds can be used as candidate lead compounds in human prostate cancer or hepatocellular cancer drug research and development.

ACKNOWLEDGMENT

This research was financially supported by the National Natural Science Foundation of China (No. 81172982), the Guangdong Provincial Project of Science and Technology (No. 2013B020411003 and 2010A030100006) and the Science and Technology Plan of Zhongshan City (2013A3FC0324, 20101H006 and 20123A276).

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