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Original Research Article

In Vitro Anticancer Activity of Hexane and Ethyl acetate Extracts of Leaf, Petiole and Rhizome of *Petasites japonicus*

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ABSTRACT

The aim of this study was to ascertain the effect of different solvents on extraction yield, antioxidant activity and cytotoxicity of extracts from *Petasites japonicus* (Siebold & Zucc.) Maxim. *P. japonicus*, widely distributed in Korea, Japan and China, is used as a culinary vegetable and a traditional medicine for asthma, migraines and tension headaches, and allergic rhinitis. In this study, twelve extracts from leaves, petioles and rhizomes of *P. japonicus* were evaluated for their antiproliferative activity against AGS, HepG2, HCT-116, and MCF-7 cancer cell lines. Plant extracts were fractionated using hexane, EtOAc, BuOH and distilled water, after which the antiproliferative activity was measured by MTT assay. Cell viability decreased in a dose dependent manner in response to the hexane and ethyl acetate extracts for all cell lines, with a significant decrease in cell viability being observed for 100 µg/ml and 75 µg/ml in the AGS and HepG2 cancer cell lines ($p < 0.05$). The leaf extract appeared to be more potent against HepG2, as indicated by IC_{50} values of 4.31 µg/ml for approximately 2.5- and 6.3-fold compared with rhizome and petiole extracts, respectively. The IC_{50} values of hexane extracts from leaves and rhizomes against AGS, HepG2, and MCF-7 human cell lines were greater than those of other extracts, while ethyl acetate extracts from petioles showed higher significant effect for all cell lines. Hexane and EtOAc extracts exerted their antiproliferative activity in four cancer cell lines. Overall, the results indicate that *P. japonicus* has the potential to be developed into selective anticancer nutraceutical and/or pharmaceutical treatments with low cost.

Keywords: Anti-cancer activity, *Petasites japonicus*, MTT assays, Pharmaceutical vegetable.

Introduction

Petasites japonicus MAX is cultivated as a culinary vegetable in Korea, Japan, and China, and *Petasites* species are used for the treatment of asthma, migraines and tension headaches, and allergic rhinitis in Europe.[1,2] Additionally, the extract of *P. hybridus* root have been used in the prophylactic treatment of migraines or gastric ulcers, and as an antispasmodic agent for asthma.[1-3] The bioactive compounds of *Petasites* species roots are sesquiterpenes, such as petasin or isopetasin.[4-7] A disadvantage of the extract of the plant root is that it contains a high level of toxic pyrrolizidine alkaloids.[8,9] However, the areal parts of *Petasites* species, which contain a negligible amount of pyrrolizidine,[8] have been reported to contain relatively lower polar compounds such as petasitin, S-petasin, *S*-petasitin, petasinol, and *S*-isopetasin.[7] Ethnobotanical and ethnopharmacological data describing plants with potential therapeutic activities are more economical and beneficial for identifying potential anticancer molecules than mass screening of plant species. Plants have long been used for their therapeutic values, and 85,000 plants have been documented for therapeutic use globally.[10] The World Health Organization (WHO) estimates that almost 80% of world' population has therapeutic experience with herbal drugs.[11]

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Cancer is one of the most dangerous diseases in humans; accordingly, there is considerable demand for discovery of new anticancer agents from natural products.[12-14]

The potential for using natural products as anticancer drugs was recognized in 1950 by the United States Natural Cancer Institute (NCI).[15] Since then, many naturally occurring anticancer drugs have been identified.[16,17]

Despite the recent dominance of synthetic chemistry as a method to discover and produce drugs, there is still enormous potential for bioactive plants or their extracts to provide new and novel products for disease treatment and prevention.[18,19] Plants have an almost unlimited capacity to produce substances that attract researchers in the quest for novel chemotherapeutics.[18] The continuing search for new anticancer compounds in plants and traditional foods is a realistic and promising strategy for its prevention.[20]

The objective of this study was to investigate the anticancer activity of the hexane, ethyl acetate, butanol and aqueous fractions of extract of *Petasites japonicus* Max plant parts against four human cancer cell lines.

Materials and Methods

Plant materials

Wild *Petasites japonicus* Max was collected in September 2019 from Chungdogun, Gyungbuk Province, Korea, and processed and divided into leaves, petioles, and rhizomes. The plant species was verified by Dr. Young Whan Choi, and a voucher specimen (No. PJR20190001) and deposited in the Natural Product Research Laboratory, Department of Horticultural Bioscience, College of Bioresources and Science, Pusan National University, Korea.

Reagents

Hexane, dichloromethane, chloroform, ethyl acetate, butanol, methanol and acetone were purchased from Fisher Scientific Korea, Ltd. (Kangnam-gu, Seoul, Korea). Sodium bicarbonate and dimethyl

sulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). RPMI-1640 medium, polyoxyethylene sorbitan monooleate (Tween 80), cell freezing medium-DMSO, trypsin-EDTA solution, and penicillin/streptomycin (P/S) solution were acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Promega Corporation (Madison, WI, USA). Fetal bovine serum (FBS) was purchased from Invitrogen Canada Inc. (Burlington, Ontario, Canada).

Extract preparation

The collected leaves, petioles, and rhizomes of wild *P. japonicus* were stored at -20°C until use. Briefly, 1 kg of frozen leaves, petioles and rhizomes were placed into separate 5,000 ml Erlenmeyer flasks containing 3,000 ml of 70 % ethanol (final concentration) and then ground into a powder using a blender (Hanil, Bucheon city, Korea. The flasks were then placed on a sonicator (Kodo, JAC-4020P, Hwaseong, Gyeonggi-do, Korea) and extraction was carried out for 2 hours three times. The 70 % ethanol extract of each plant part was then obtained by recovering the ethanol with an evaporator (Heidolph, Rotavapor 4000, 91126 Schwabach, Germany) at 45°C. The total extracts of leaves, petioles, and rhizomes were then successively fractionation with hexane, ethyl acetate, butanol and distilled water and the average yields were calculated on a fresh weight basis. Serial dilutions (3.125 µg/ml to 100 µg/ml) of the filtered crude extract stock were prepared in DMSO and used in an MTT assay. The extraction flow chart is shown in Figure 1.

Cell culture

AGS (human gastric carcinoma), HepG2 (human hepato cellular cancer), HCT-116 (human colorectal cancer), and MCF-7 (human breast cancer) cell lines were cultured with RPMI-1640 plus 10 % FBS and 1 % P/S. All cancer cell lines were obtained from the Korean Cell Line Bank. These cell lines were maintained in RPMI-1640 media supplemented with 10 % fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were grown in 25 cm^2 tissue culture flasks (SPL Life Sciences, Hwaseong, Gyenggi-do, Korea) and maintained in an incubator (Sanyo Electric Co., Ltd., Moriguchi, Osaka, Japan) under a 95 % humidified atmosphere and 5 % CO_2 at 37°C.

Cell viability assay

Each plant fraction of wild *P. japonicus* was added to culture medium in 96-well plates (SPL Life Sciences, Hwaseong, Gyenggi-do, Korea) to give different final concentrations (3.125, 6.25, 12.5, 25, 50, 100 µg/ml). The cells were then seeded at 10^4 cells/100 µl/well. All samples were analyzed in triplicate and the plates were incubated at 37°C for 24 hours, after which an MTT assay was performed according to the manufacturer's protocol. Briefly, 50 µl of MTT solution were added to each well, after which the samples were incubated for an additional 4 h and 100 µl of DMSO solution were added to each well. Finally, the absorbance of each well was recorded at a wavelength of 540 nm using a plate reader (BioTek, Epoch, Winooski, VT, USA). The percentage of surviving cells based on the control treatment (untreated) was then calculated and plotted against the extract concentrations and used to determine the concentration of *P. japonicus* extracts that induced 50 % inhibition of cancer cells (IC_{50} values). The values from three separate experiments were averaged and the mean IC_{50} and the standard deviation were determined for each extract.

Statistical analyses

All data were expressed as means ± standard deviation from three replications. IC_{50} values were determined by a polynomial regression equation composed of the logarithmic values of four graded concentrations and the viabilities of cells induced by the plant extracts of wild *P. japonicus*. Viability was calculated based on the formula: V (%) = T / C x 100, where V represents the viability or survival rate, T represents the absorbance of the extract-treated group, and C is the absorbance of the non-treated negative control group. The background MTT absorbance obtained from the blank control group was subtracted from all test groups.

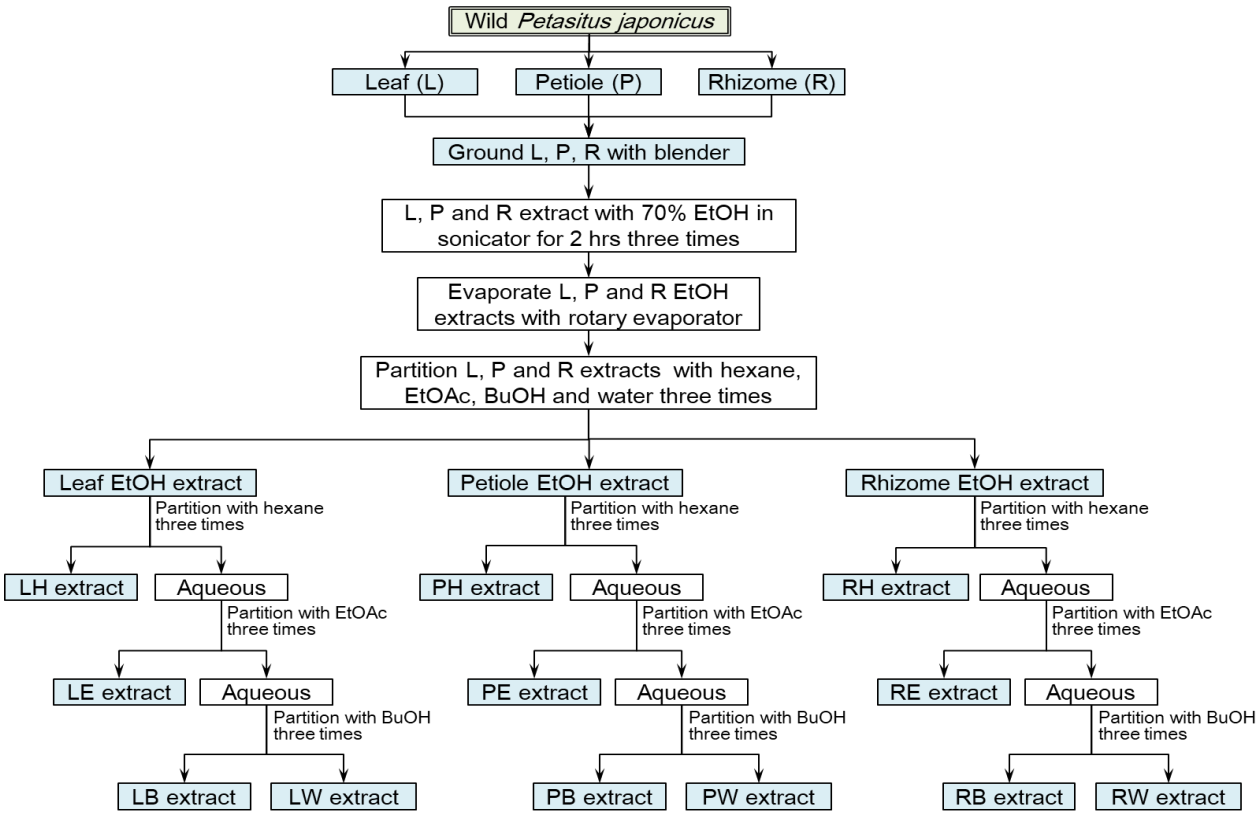

Figure 1: Systematic extraction of leaf, petiole and rhizome parts of *Petasites japonicus* Max with 70 % ethanol and successive fractionation with hexane (H), ethyl acetate (E), butanol (B) and distilled water (W).

Results and Discussion

Yield

The yields of the total extracts (weight of dried total extract/weight of fresh plant parts) were 38.17 g, 33.55 g and 53.33 g for the leaf, petiole and rhizome total extracts, respectively (Table 1). The yields of the LH (leaf hexane fraction), PH (petiole hexane fraction) and RH (rhizome hexane fraction) were 13.06 g/kg, 0.99 g and 1.03 g, respectively. The yields of EtOAc extracts from leaves, petioles and rhizomes were almost the same, whereas the BuOH and aqueous extracts showed higher yields for rhizome extracts, followed by petiole and leaf extracts.

Cytotoxic effects of fractions

The IC_{50} values and viability curves of leaf, petiole and rhizome extracts for AGS, HepG2, HCT-116 and MCF-7 cancer cells are presented in Table 2 and Figure 2. Decreased cell viability was observed in response to hexane and ethyl acetate extracts for all the cell lines in a dose dependent manner, with a significant decrease in cell viability of AGS and HepG2 being observed for 100 µg/ml and 75 µg/ml ($p < 0.05$). The IC_{50} values for hexane and ethyl acetate extracts against four human cancer cell lines were higher than those of the butanol and aqueous extracts. When the hexane extracts of leaves, petioles and rhizomes were compared, the leaf extract appeared to be more potent, as indicated by IC_{50} values of 4.31 µg/ml for approximately 2.5-fold and 6.3-fold compared with rhizome and petiole extracts for HepG2, respectively. Among the four cancer lines, the IC_{50} values of hexane extracts from leaves and rhizomes were higher for AGS, HepG2, and MCF-7, but the IC_{50} values of the ethyl acetate extracts from petiole were higher than hexane extracts for all cell lines (Table 2).

The top five fractions (LH, PH, PE, RH and TE) with the highest cytotoxicities against AGS, HepG2 and MCF-7 cancer cells and IC_{50} values lower than 63.20 ± 0.38 µg/ml were selected from the 12 fractions by *in vitro* bioassay. All five fractions concentration-dependently reduced the viabilities or survival rates of the four cancer cell lines tested. The minimum survival rates of the AGS, HepG2, HCT-116 and MCF-7 cancer cells induced by LH at 100 µg/ml were 7.25 ± 0.48, 2.45 ± 0.53, 75.35 ± 1.11 and 7.47 ± 1.04 % with IC_{50} values of 27.56 ± 0.36, 4.31 ± 0.15, >100 and 21.53 ± 1.05 µg/ml, respectively. With PE at 100 µg/ml, the survival rates were 0.37 ± 0.28, 0.10 ± 1.03, 0.93 ± 0.21 and 1.26 ± 0.22 % for AGS, HepG2, HCT-116 and MCF-7 with IC_{50} values of 16.50 ± 0.57, 16.66 ± 0.88, 13.51 ± 0.62, and 14.06 ± 0.23 µg/ml, respectively (Figure 2 and Table 2). Wild *P. japonicus* is cultivated as a culinary vegetable in Korea, Japan and China[2] and extract from the roots of *Petasites* species has been used therapeutically in Europe.[1,2] This plant has also been screened for identification of pharmaceutically important compounds.[7]

Table 1: Yields of hexane, ethyl acetate (EtOAc), butanol (BuOH), and aqueous extracts of leaves, petioles, and rhizomes of *Petasites japonicus* Max.

Plant parts	Contents of extracts (g/fresh kg)				
Plant parts	Hexane	EtOAc	BuOH	dH_2O	Total
Leaf	13.06	1.90	4.61	18.60	38.17
Petiole	0.99	0.33	6.186	26.05	33.55
Rhizome	1.03	1.75	9.24	41.31	53.33

One kg aliquots of fresh leaves, petioles and rhizomes were extracted with 70 % ethanol for 2 hours three times. The total extracts of each part were then further fractionated with hexane, ethyl acetate, butanol and water. Finally, the extracts were obtained by recovering the solvents in an evaporator at 45°C.

Table 2: IC_{50} values of AGS, HepG2, HCT-116, and MCF-7 cancer cell lines induced by hexane, EtOAc, BuOH, and aqueous extracts obtained from leaves, petioles, and rhizomes of *P. japonicus* Max.

Plant parts	**Solvents**	**Abbreviation**	**IC_{50} (µg/ml)**			
			AGS	**HepG2**	**HCT-116**	**MCF-7**
Leaf	Hexane	LH	27.56 ± 0.36	4.31 ± 0.15	>100	21.53 ± 1.05
	EtOAc	LE	>100	94.57 ± 2.71	>100	99.36 ± 1.71
	BuOH	LB	>100	>100	>100	>100
	dH_2O	LW	>100	>100	>100	>100
Petiole	Hexane	PH	30.94 ± 0.24	27.31 ± 1.42	39.67 ± 1.92	31.56 ± 0.81
	EtOAc	PE	16.50 ± 0.57	16.66 ± 0.88	13.51 ± 0.62	14.06 ± 0.23
	BuOH	PB	>100	>100	>100	>100
	dH_2O	PW	>100	>100	>100	>100
Rhizome	Hexane	RH	21.94 ± 0.30	10.69 ± 0.35	63.20 ± 0.38	20.47 ± 2.17
	EtOAc	RE	44.89 ± 0.22	58.57 ± 2.81	61.39 ± 1.23	57.26 ± 1.48
	BuOH	RB	>100	>100	>100	>100
	dH_2O	RW	>100	>100	>100	>100

The values from three experiments were averaged and the mean IC_{50} and standard deviation were calculated for each extract.

Although many compounds have been identified as possessing medicinal properties,[3] the anticancer effects of extracts from the leaves, petioles and rhizomes of *P. japonicus* have not yet been validated *in vitro*. In this study, different fractions were extracted from wild *P. japonicus* and their anticancer effects were tested on human cancer cell lines. The results revealed that the hexane and ethyl acetate fractions had the highest anticancer potency. The highest yield among these fractions from each extract was obtained from LH.

A smaller IC_{50} value indicates a greater anticancer effect. Comparison of the extracts revealed that PE had a greater effect against all four cell lines investigated herein than PH, LH, LE, RH and RE. Moreover, the most effective fraction was LH for anti-HepG2, PE for anti-AGS, PE for anti-HCT-116, and PE for anti-MCF-7 cancer cells. Conversely, the butanol and aqueous extracts from leaves, petioles and rhizomes exerted no cytotoxicity against the four cancer cell lines at 100 µg/ml. These findings agree with those of many previous studies that reported the bioactivity of non-polar principles in plants such as *Typhonium flagelliforme* and *chisandra sphenanthera*.[21,22] The yield from the total extract for LH was also about ten times higher than that for PH and RH (Table 1).

This is the first study to report the antiproliferative activity of *P. japonicus*. The hexane and ethyl acetate extract of *P. japonicus* plant parts inhibited the proliferation of different cancer cell lines and showed selective toxicity toward HepG2 cells (Table 2). Fractions of some parts exhibited different activity against different cell lines. For example, the IC_{50} (µg/ml)) values of the hexane fraction of the *P. japonicus* rhizome were 21.94 ± 0.30 for AGS, 10.69 ± 0.35 for HpeG2, 63.20 ± 0.38 for HCT-116 and 20.47 ± 2.17 for MCF-7 (Table 2), but the IC_{50} of hexane and EtOAc from petioles and EtOAc from rhizomes were almost the same. This selectivity could be due to sensitivity of the cell line to the active compounds in the extract or to tissue specific response.[23]

The refined preparation (Ze 339) of the ethyl acetate or ethanol extract of *P. hybridus* root has been used in prophylactic treatment of migraines or gastric ulcers and as an antispasmodic agent for asthma.[1] The main bioactive constituents in the ethyl acetate extract of *P. hybridus* rhizome are petasin derivative sesquiterpenes,[7] eremophilane sesquiterpenes[24] and bakkenolides sesquiterpenes.[25,26] A disadvantage of the ethyl acetate extract of the plant root is that it contains a high level of toxic pyrrolizidine alkaloids.[7] However, the leaves of *Petasite* species contain a negligible level of pyrrolizidine.[1,7] Based on our results, the cytotoxicity estimates of leaf, petiole and rhizome extracts indicate that *P. japonicus* is a repository for anticancer compounds, and the fact that the IC_{50} values are significantly lower than the NCI criteria indicates its potential for cancer drug discovery programs.[27]

As the global scenario is now changing towards the use of nontoxic plant products with traditional medicinal uses, development of modern vegetables from medicinal plants should be emphasized for the control of various diseases, including cancer. The hexane and ethyl acetate extracts from the leaves, petioles and rhizomes of *P. japonicus* have high anticancer activity, and the procedure for LH extraction was simple and afforded high yields.

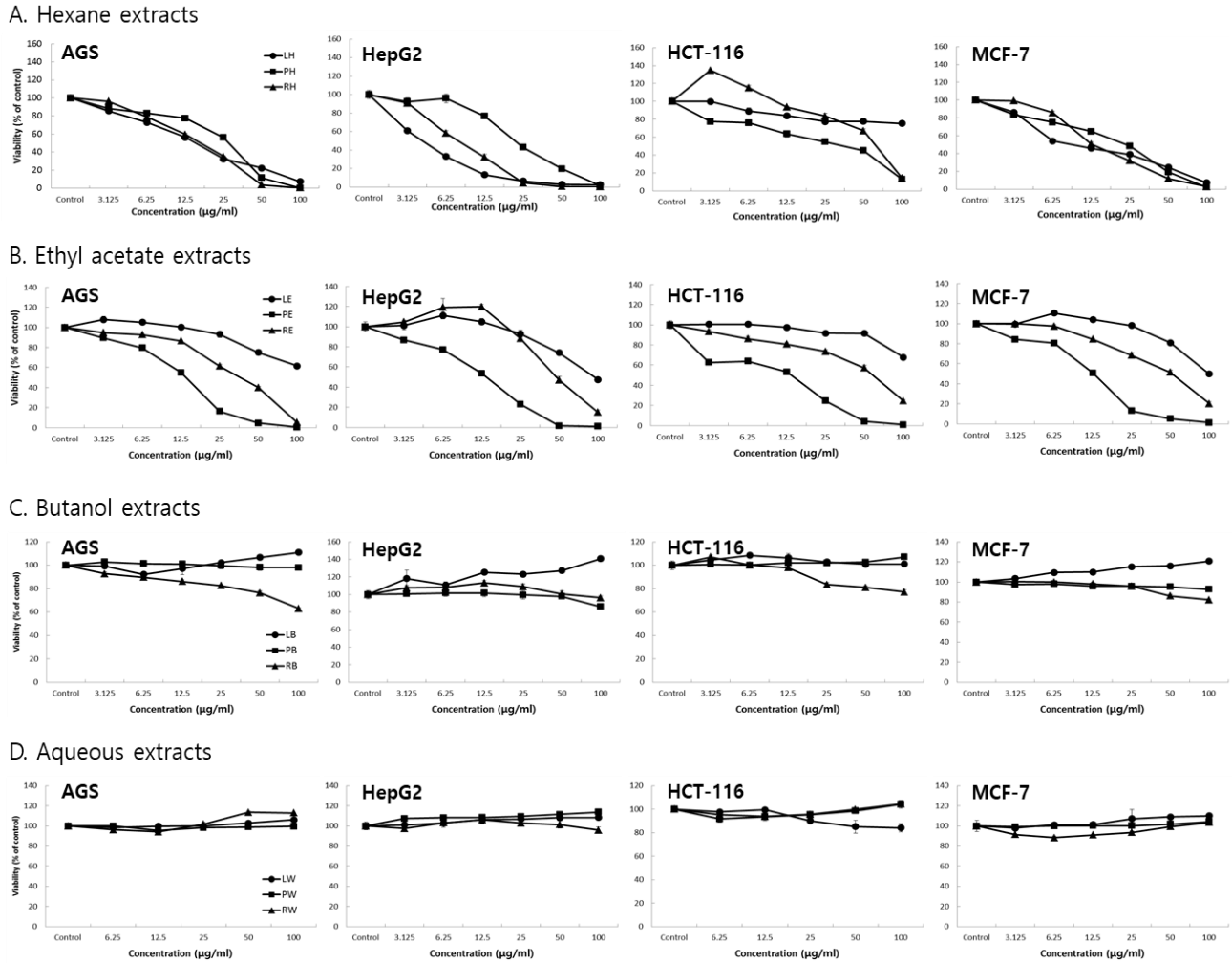

Figure 2: Viabilities of AGS (human gastric carcinoma), HepG2 (human hepato cellular cancer), HCT-116 (human colorectal cancer), and MCF-7 (human breast cancer) cell lines in the presence of hexane (A), ethyl acetate (B), butanol (C) and aqueous (D) fractions of *P. japonicus* Max extract.

Conclusion

From these results, we can conclude that hexane and ethyl acetate extract of the roots of *P. japonicus* have potential anticancer activity. The results of the present study clearly indicate the anticancer potential of *P. japonicus* extract, validating its pharmaceutical vegetable use. However, further study is necessary to elucidate the chemical nature and active compounds of the hexane extract from leaves that are responsible for the anticancer activity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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