KJPR Korean J.Plant.Res. 10(3) 213~220(1997)

Screening for Cytotoxicity of Crude Extracts from Fruit on Leukaemia Cells in *Citrus* and Related Genera

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ABSTRACT

The present study has been undertaken to characterize availability of citrus as a medicinal plant with antineoplastic property. The crude extracts from 40 species of fruits with 12 species of the local *Citrus* in Cheju island were evaluated on their potential activities against mouse P388 lymphocytic leukaemia *in vitro*. The percent cytotoxicity varied from 25.40 to 97.94% at a concentration of 100㎍/mL. Among 40 spp., 8 species showed high toxicity more than 90% against P388 cells and Cheongkyool(*C. nippokoreana*) exhibited the most cytotoxicity as 97.94%(IC_{50}=20.2㎍/mL). Nine varieties of *C. junos* were showed insiginicant cytotoxicity. In trifoliate orange, immature fruit was stronger than mature and peel extract showed higher cytotoxicity(IC50=18㎍/mL) than the other tissues. Hexane fraction from methanol(MeOH) extract of trifoliate orange showed highly significant inhibition of cell growth(IC_{50}=3.9㎍/mL). In addition, its cytotoxicity increased remarkably from 3.95 to 0.40㎍/mL as exposure time lengthened. Cytotoxic activities of crude extracts were decreased considerably during a six months storage period. It was apparent that there is considerable variation in cytotoxicity, depending upon species, maturity and storage time of extracts. There was no meaningful cytotoxic difference between archicitrus and metacitrus in the genus *Citrus*.

Key words: *Citrus* spp., *Poncirus trifoliata*, Leukaemia cell, Cytotoxicity, MTT

INTRODUCTION

Fruits of the citrus plant have been used as a natural source of juice, seasoning, vineger as well as fresh fruit. Trifoliate orange(*Poncirus trifoliata*) and kumqual(*Fortunellar japonica*) are also known to be effective for treating pain, stomachache, hysteroptosis, stagnant blood, alcohol detoxification and cholesterol solubility. Unripe fruits of the genus *Poncirus* had been used as an important resource of the oriental medicine and folklore.

Studies of citrus plant found that a crude extract from various tissues exhibited a very diverse physiological activity such as antimicrobial activity(Mishra and Dubey, 1994), hypotensive effect(Akiyoshi and Yosharu, 1996), analgetic, antiphlogsitic, laxative property, sedative agent, and antitumor activity *in vivo, in vitro* (Edward et al.,1994). Their biological action is characterized moreover by compounds such as flavanone, glycosides, flavone, anthocyanin, and naringin. Citrus component associated with anticancer activity have recently been isolated and identified by Lam et al.(1994). These active substances have been isolating and analyzing in flower, leaves, and fruits. Although a large number of researcher have shown that citrus fruit is protective in a varity of human disease, more studies is needed specially for anticancer activity. Most of research has investigated the effect of unripe fruit against tumor cells, while there are only a few reports using ripe material. Recently we reported on cytotoxic activity against leukaemia cells(Lee et al., 1996).

Main purpose in our research is to characterize extensively citrus that might be involved in antitumor activity. Therefore, the present work investigated priliminarily the toxicity of 40 fruit extracts on leukaemia cell *in vitro* to prescreen the effective citrus species prior to carrying out assays for *in vivo* activity. To determine quality of crude extracts obtained from ripe fruits, we also investigated variability of cytotoxicity not during the storage period but with long duration of exposure to leukaemia cells.

MATERIALS AND METHODS

Materials

Daidai(*C. aurantium*), Lime(*C. aurantifolia*), Kabosu (*C. sphaerocarpa*), Ponkan(*C. reticulata*), Manatsu(*C. natudaidai*), Grapefruit(*C. paradisi*), Bundan(*C. granddis*), Iyokan(*C. iyo*), Jaffa(*C. sinensis*), Valencia(*C. sinensis*), Sudachi(*C. sudachi*), Hassaku(*C. hassaku*), Lemon(*C. limon*), Dekopen(*C. dekopon*), Sanbokan(*C. sulcata*) were purchased during November and Feburary from vegetable market at Toyoda, Japan. Twelve spp. of the local citrus fruits were collected from cultivated areas of Cheju Citrus Research Institute Rural Development Administration(RDA) in August 1996. They were; Jikak(*C. tangerina*), Dangyooja(*C. grandis*), Kamja(*C. benikoji*), Pyunkyool(*C. tangerina*), Dongjeongkyool(*C. erythrosa*), Binkyool(*C. tachibana*), Yooja(*C. junos*), Byungkyool(*C. platymamma*). Nine varieties of *C. junos* collected in Wando Subtropical Plant Experiment Station, Chunnam Provincial R.D.A. are CH-1, CJ-1, Haenam, Haenambuckpyung, Haenamkekok, Jindo, Kangjin, Wando, and the others(Yuzu, Ayoyuzu) are collected at Japan. Trifoliate orange(*Poncirus trifoliata*) and Kumqual(*Fortunellar japonica*) harvested from Wando, and greenhouse in Cheju Do, Korea. The botanical identification was made by Dr.Hna-Yong Kim, Breeding Division, Cheju Citrus Research Institude R.D.A., Korea and Researcher Mun-Young Park, Wando Subtropical Plant Experiment Station Chunnam Provincial R.D.A, Korea. Whole fruit of trifoliate orange were dissected into three parts of peel, flesh and seeds.

Cell line used in this experiment is P388 murine leukaemia cell supplied by Dr. S.Tsukagoshi of Japan Foundation for Cancer Research(Koren et al., 1975).

Preparation of fruit extracts

Fifty grams of the fresh fruits was extracted with 500ml of 100% MeoH at40℃, respectively. The extract was filtered(Advantec Toyo: Filter paper No. 1) and the solvent was removed under reduced pressure(20mmHg). Each extract was freeze-dried, and stored at -20℃ until used. The MeoH extract of trifoliate orange was further extracted with n-hexane, ether, ethyl acetate, and water in sequence. All extracts were dissolved in DMSO(dimethyl sulfoxide) on the day of use.

In addition, to evaluate the change of toxicity of stored extract, freshly prepared extracts(Daidai, Yooja, Lime, Kumqual), stored at room temperature for 6 manths. Their cytotoxicity tested six time at 1 month intervals, respectively by the same method.

Cell culture

procedure for cytotoxic assay was carried out in a 96-well flat bottom(CORNING,6.4 × 11mm). Murine leukaemia P388 cells were maintained In a suspension culture with RPMI 1640 medium supplemented with 5% heat-inactivatrd fetal bovine serum and 0.1mg/mL kanamycin at 37℃ in a humidified atmosphere containing 5% CO_2 (Incubator, TABAI BIA-110). The cells were passaged twice each week. Each assay was conducted with cells (2×10^5 cells/mL) in an exponential growth phase. Numbers of viable cells were determined by counting the bright cells that excluded 0.3% trypan blue dye containing PBS.

Treatment of cells with crude extract

100 L of liquid media containing cells(3×10^4 cells/mL) was added to 93 well expect 3 well with only fresh media. After overnight culture at 37℃, extract solutions was added to each well to contain 100, 10, 3.0, 1.0, 0.3, and 0.1 ㎍/ml by serial dilution, respectively. The plates were mixed for 2 min on microplate mixer(IWAKIMPM-1). Control contained cultured cells with 5% DMSO in PBS

Table 1(A). Cytotoxicity of extract from mature fruits against mouse leukaemia cell. Each extract for cytotoxicity was examined at a final concentration of 100㎍/mL and the mean value of O.D. of 6-9 wells was used for calculating the % cytotoxicity. The equation is as follow: % cytotoxicity=(1-(O.D. treated well/O.D. control well)×100

Genus*	Subgenus	Species(common name)	%Cytotoxicity
Citrus	Archicitrus	*Citrus aurantium*(Daidai)	89.80
		C. aurantifolia(Lime)	96.60
		C. natudaidai(Amanatsu)	87.40
		C. paradisi(Grapefruit)	79.90
		C. grandis(Buntan)	65.70
		C. sinensis(Jaffa)	78.20
		〃 (Valencia);peel	74.95
		C. hassaku(Hassaku)	63.80
		C. limon(Lemon)	65.20
		C. sulcata(Sanbokan)	47.04
	Metacitrus	*C. sphaerocarpa*(Kabosu)	69.80
		C. sudachi(Sudachi)	63.40
		C. junos(Yooja)	93.10
		C. reticulata(Ponkan)	69.00
		C. ekopon(Dekopon)	70.10
Poncirus		*P. trifoliata*(Trifoliate orange) mature	94.70
		immature(August)	97.19
		〃 (September)	97.83
Fortunellar		*F. japonica*(Kumqual)	79.70
		〃 (Dajunkum) immature	96.66

*The nomenclature of the plants is based on Tanaka's classification.

(B) Cytotoxic activity of immature fruits of the local *Citrus* spp. in Cheju

Species	Common name	Maturity	%Cytotoxicity
Citrus aurantium	Jikak	Immature(August)	62.21
C. grandis	Dangyooja	〃	86.20
C. benikoji	Kamja	〃	69.90
C. tangerina	Pyunkyool	〃	69.97
C. erythrosa	Dongjeongkyool	〃	63.26
C. leiocarpa	Binkyool	〃	78.44
C. nippokoreana	Cheongkyool	〃	97.94
C. sunki	Jinkyool	〃	96.00
C. tachibana	Hongkyool	〃	96.08
C. junos	Yooja	〃	78.64
C. platymamma	Byungkyool	〃	91.24
C. paeudogulgul	Sadookam	〃	60.24

Table 2. Comparison of toxicity IC_{50} values of 9 species on P388 cells.

Species(common name)	Maturity		IC_{50}(μg/mL)	%Cytotoxicity
C. aurantium(Daidai)	M(December)		38.0	89.80
C. aurantifolia(Lime)	M(〃)		33.0	96.60
C. junos(Yooza;Ori)	M(〃)		32.0	93.10
C. nipplkoreana(Cheongkyool)	I(August)		20.2	97.94
C. platymamma(Byungkyool)	I(〃)		32.0	91.24
C. sunki(Jinkyool)	I(〃)		33.0	96.00
C. tachibana(Hongkyool)	I(〃)		23.0	96.08
P. trifoliata(Trifoliate orange)	M(December)	WF;	29.0	94.70
		Fl;	70.0	63.00
		Se;	23.0	81.60
		Pe;	18.0	95.20
	I(August)		10.0	97.19
	I(June)		22.5	97.83
F. japonica(Dajunkum)	I(〃)		40.0	96.66

IC_{50}; Values were average of double triplicate as a concentration to reduce viability by 50%.
WF;whole fruit, Fl;Flesh, Se;Seed, Pe;Peel

and was incubated for 48h in trifoliate orange, cells treated with four solvent fractions incubated for 48h and 60h separately to demonstrate changeability of cytotoxic activity by duration of reaction time to P388 cell. To evaluate the change of cytotoxicty of extracts in storage, one sample was treated immediately after extracting the cells and the other one assayed against cells after 6 months of storage.

MTT colorimetric assay

The MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay used modified method from Carmichael et al.(1987) and Twenty & Luscombe(1987). The blue formazan produced by the mitochondrial dehydrogenase of viable cells was measured spectrophotometrically in a 96-well plate. Fourty eight hour later 20μL of MTT solution(0.5% in PBS) is added to each well and agitated then on the plate mixer for 2 min. At this step four fractions from trifoliate orange were incubated 48h, 60h, respectively. After the plate incubated for 4h at 37 ℃, the resulting formazan with blue crystal was dissolved in 100μL of 10% SDS(sodium dodecyl sulfate) containing 0.01N HCl. Each well was mixed gently with a multipipet for 2 min and the plate was reincubated for 12h. The plate was read and analyzed using a microplate reader (Tosoh MPRA 4i) at 550-770nm with MASUDA 1. The growth rates were calculated in reference to the control contained cultured cells with 5% DMSO in PBS, without extract. Percentage cytotoxicty([1-O.D. 100μg treated well/ O.D. control well] $\times$ 100) is the mean value of O.D. of 9 well(Moritani et al., 1995). The IC50(μg/mL) value was defined as the concentration of sample that achieved a 50% reduction of viable cells with respect to the control.

RESULTS AND DISCUSSION

Cytotoxic effects of MeOH extracts of fruits against P388 leukaemia cells

We determined primarily percentage cytotoxicity of 40 species of citrus and related genera on leukaemia cell at final concentration(100μg/mL) and the results are given in Table 1(A), (B), 2. The assay was performed six times each sample. The percentage cytotoxic activity varied considerably with species and maturity of fruit, rainging from 26.58% to 97.94%. Among the these, unripe Cheongkyool(*C. nippokorreana*) a local species from Cheju

showed the strongest cytotoxicity(97.94%). Immature Dajunkum, Hongkyool, Jinkyool, Byungkyool and mature Lime, trifoliate orange, Yooja, Daidai also exhibited high toxicity 96.66, 96.08, 96.00, 91.24, and 96.60, 94.70, 93.10, 89.80%, respectively. It is suggested that these 9 extracts gave almost similiar toxicity on P388 cell. Also considerable cytotoxicity was not found based on different maturity in trifoliate orange. For Daidai(*C. aurantium*), it was previously reported that dried unripe fruit showed the antitumer activity *in vivo* and *in vitro* (Satoh et al., 1996). Besides, 10 spp.(ripe Amanatsu, Grapefruit, Jaffa, Valencia, Iyokan, Dekopon, Kumqual and unripe Dangyooja, Binkyool, Yooja) were exhibited toxicity greater than 70%. All of extracts however were found to reduce the living cell over 60% at 100㎍/mL.

Extracts of peel, flesh, and seeds separated from whole fruit were compared to their cytotoxicity. As shown in Table 2, peel extract exhibited 50% reduction in cell viability at a concentration of 18㎍/mL, whereas seeds and flesh exhibited IC50 23, 70㎍/mL, respectively. Citrus peel had been shown to posses polymethoxylated flavonoids involved antiproliferative and anticancer activity(Kan, 1979; Marc et al., 1994). Lam et al.(1994) reported that useful cancer-preventive agents present in seeds such as lemon, lime, and grapefruit. For peel and seed it is of interest to note that they are available as a source of a crude drug using byproducts removed in the process of manufacturing such as squash, juices, can and so on. More recently Satoh et al.(1996) reported that the albedo extracts of several citrus had strong inhibition activity against cyclooxygenase, lipoxygenase which are important in the process of skin tumor promotion(Bauer et al., 1986).

When nine spp. exhibited cytotoxicity of over 90% were compared with IC_{50} values, unripe trifoliate orange(10.0 ㎍/mL) showed the most high toxicity differs from percentage cytotoxicities in Table 1(B) while the others showed similar values such as 20.2(Cheongkyool), 23(Hongkyool), 32.0(Byungkyool), 33.0(Jinkyool), 32.0(Yooja), 33.0(Lime), and 38.0(Daidai) (Table 2). There was no considerable difference between the cytotoxicities on P388 cells of archicitrus and metacitrus which are a subgenus of Citrus.

Yooja together with Kabosu, and Sudachi of sour citrus are widely used as flavour enhancers because of their unique aroma. This experiment suggested that cytotoxicity of Yooja(93.10%) might be potential more than Kabosu(69.80%) and Sudachi(63.40%). Yooja cultivating or growing in Wando, Korea from old time, can be classified 3 type by different rootstock that is trees used Yooja itself(Y-Y) and used trifoliate orange(Y-P), as a rootstock respectively and original seedling(Ori). Thus we collected ripe fruit from each tree and their toxicity on P388 cell were compared as percentage cytotoxicity(data not shown). Fruit from Ori exhibited higher toxicity(93.10%) than the others showing 69.0%(Y-Y), 25.40%(Y-P), respectively but this result is further needed to be carefully retested.

Table 3. Cytotoxic variability of *C. junos* according to local varieties

Common name	Collecting place	Matruity	%Cytotoxictiy
CH-1	Korea*	I(August)	57.05
CJ-1	〃	I(〃)	53.47
Haenam	〃	I(〃)	58.93
Haenambuckpyung	〃	I(〃)	63.47
Haenamkekok	〃	I(〃)	26.58
Jindo	〃	I(〃)	51.20
Kangjin	〃	I(〃)	58.18
Wando	〃	I(〃)	70.16
Yuzu	Japan	M(February)	30.12
		I(September)	61.70

*9 varieties of *C. junos* collected in Wando Subtropical Plant Experiment Station Chunnam Provincial R.D.A.

Table 4. The change of cytotoxicity during storage of crude extracts

Common name	Fresh extract		Storaged extract(6 months)	
	%cytotoxicity	IC50(μg/mL)	%cytotoxicity	IC50(μg/mL)
Daidai	89.80	38.0	67.30	62.0
Yooja	93.10	30.0	80.30	52.0
Lime	96.60	33.0	74.60	54.0
Kumqual	79.70	64.0	54.60	85.0

Among 9 varieties of *C. junos*, Wando, and Haenambuckpyung showed cytotoxicity more than 60%. It was apparent that there is inconsiderably difference in cytotoxicity depending upon local varieties of *C. junos*(Table 3).

Cytotoxic changeability during storage of extracts

For the quality control of citrus extracts as a crude drug, their cytotoxic changeability of during storage in RT were investigated. The results are given in Table 4. Significant difference was found between the immediately treated sample after extraction and 6 months later treated extracts. In the case of Lime, fresh extract with 96.60% markedly decreased to lower cytotoxicity(74.60%) after 6 months.

Its cytotoxicity however remained unchanged for the duration of 4 months while that started to decline after 5 months of storage. It was suggested that extracts have duration-dependent decrease of cytotoxicity. This change might be associated with the decrease of the some characteristic odour or loss of volatile components which are effective as a cytotoxic factor in the extract. The persistence of cytotoxicity in the extract for 5 months indicated that even stored extract may be used as a crude medicine as well as source for isolating some compounds associated with cytotoxicity. For dried peels of citrus Tosa et al.(1988) and Ishihara et al.(1990) demonstrated that content of some components did not decrease during two year storage period contributing to biological activity. We found therefore that better citrus tissue for natural medicine is intact part as storage rather than solvent extract from it.

Cytotoxic activity in relation to exposure time of solvent fractions on cells in Trifoliate orange(*P. trifoliata*)

Trifoliate orange had been used not as oriental medicine, folklore but various citrus of rootstock in China, Japan, and Korea(Lee et al., 1986; Ahn et al., 1986). Thus MeOH extract of it was further fractionated by n-hexane(He), ether(Et), ethyl acetate(EA), and water(Aq) in sequence and cytotoxic fractions of these was He extract that showed IC_{50} value in Table 5. The most cytotoxic fractions of these was He extract that showed IC_{50}=3.9μg/mL against P388 cell. In addition, both He and Et fractions appeared to be the complete lethality against living P388 cell at final concentrations of 100μg/mL. It is suggested that

Table 5. Increased inhibition of extracts on cell viability by elongation of exposure time. Used extracts are fractionated to hexane, ether, ethyl acetate and aquous phase from crude MeOH extract respectively. Abbreviation : fr, fractions

P. trifoliata	Incubation time IC_{50}(μg/mL)	
	48h	60h
Hexane fr	3.95	0.40
Ether fr	10.94	3.05
Ethyl acetate fr	49.75	30.05
Aquous fr	>100	>100

He fr from MeOH extract contain the cytotoxic principle that acts strongly against leukaemia cell. Other three fractions showed IC_{50} values of 10.9(Et), 49.7(EA), and $>100\mu g$ /mL(Aq) respectively. Aq fr had little or no antileukaemic activity.

To evaluate the variability of cytotoxicity by duration of exposure time of fractions on P388 cell, cells were incubated more lengthenly 12h from 48h of which original reaction time and then cytotoxic differences were compared with each other. As shown in Table 5, prolongation of the exposure time to cells increased their cytotoxicities from IC_{50} value 3.9(He fr),10.9(Et fr) and 49.7(EA fr) to o.4, 3.0, and $30.5\mu g$/mL in 48, 60h, respectively. Aq fr however was scarcely shown cytotoxicity on leukaemia cell ($IC_{50}>100\mu g$/mL) in duration of incubation time as well. Thus our result has also demonstrated that crude such as He fr inhibits 50% of the viability of leukaemia cell even at relatively low concentration($0.4\mu g$/mL). Ikegawa and Ikegawa(1994) isolated antitumer substance from *P. trifoliata*, an original plant of the traditional Chinese medicine "Gou Ju". It has been known to be effective for treating pain, stomachache and as a detoxicant of wine poisoning(Nogata et al., 1996).

If ripe citrus fruits have also certain cytotoxicity on different leukaemia cells with P388, these will be comsumed on relatively high concentration by human since it is a natural food source as well as less toxic than unripe one. On the basis of this evidence several compounds associated with antileukaemic activity should be furthermore isolated from various of citrus and we are separating some principles with strong toxicity.

ACKNOWLEDGEMENT

This research was finacially supported by Chosun University(1995), Korea. We wish to thank Dr. H. Y. Kim, Cheju Citrus Research Institude R.D.A. and researcher M.Y.Park, Wando Subtropical Plant Experiment Station Chunnam Provincial R.D.A. for their valuable advice and assistance.

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