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Synergistic Effect of Methanol Extract from *Kalopanax pictus* and Ascorbic Acid on Antioxidant, Anticancer and Immunomodulatory Activities

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The 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzthiazoline- 6-sulfonic acid (ABTS), nitric oxide (NO) scavenging activities and ferric- reducing/antioxidant power (FRAP) assay against extracts of *Kalopanax pictus* (KP) were measured. Radical scavenging and antioxidant activities were increased depend on the concentration and the effects were enhanced by ascorbic acid (AA). KP extracts and AA had a good anti-proliferating activity against HepG2 cells by MTT assay and induces cells apoptosis, which was demonstrated by flow cytometric analysis. KP extracts and AA caused the arrest of cell-cycle progression at either G0/G1-phase or G2/M-phase, which might be depending upon the KP extracts concentration. In addition, KP extracts and AA are effective in enhancing immunity and nitric oxide production by RAW 264.7 macrophages cells. KP extracts and AA inhibited tumor cell growth and exerted antioxidant effects as compared to controls. These results demonstrate that simultaneous AA and KP extracts treatment could be useful in preventing the oxidative damage and anti-proliferating HepG2 cells, and are effective in enhancing immunomodulatory and antioxidant activity.

Key words : Anticancer activity, antioxidant activity, NO scavenging activity, *Kalopanax pictus* (KP), ascorbic acid (AA)

Introduction

Traditional oriental plants contain many useful compounds, which can be used for the treatment of chronic disease. Many reports suggested that traditional herbs have potentials for preventing pathological outcome of oxidative stress. Utilization of phytochemicals, obtained from various kinds of plant, has increased all over the world. In East Asian countries, many researches have been conducted to find useful products, such as anticancer agents from traditional medicines. Compared to chemical synthesis, screening of natural plants has many advantages due to the long history of oriental medical treatments.

Kalopanak pictus Nakai (Araliaceae) is a deciduous tree, which is distributed in Oriental countries. The stem bark of *Kalopanak pictus* has been used in traditional medicine in rheumatic arthritis, wound and diarrhea [15]. Several phytochemicals, kalopanaxsaponins-A (KPS-A) and kalopanaxsaponins-I (KPS-I), have been isolated from the plant [5]. The biological activity of cortex was reported [11] and anti-diabetic activity of the constituents of the plant was also reported [18].

Ascorbic acid, a well noted antioxidant and free radical scavenger product is widely diffused in the plant kingdom, and it is a DPPH radical scavenging agent of medium strength [20] but its reaction is very fast with respect to other scavenging molecules, such as polyphenols [29].

Some plant extracts kill tumor cells by blocking metabolic pathways or inducing apoptosis in vitro [4,9], others have been shown to inactivate free radicals [22]. Oxidative stress, resulting from an imbalance between pro- and antioxidant systems in favor of the former, largely contributes to immune system deregulation and complications [1]. A diverse array of medicinal plants has traditionally been used in Korea to enhance innate immunity and treat cancers and preliminary studies have documented the metabolic effects of some of these plants. Dietary supplementation with KP extracts was reported to improve hepatitis and free radicals in diabetic rats [11]. However, few studies have characterized the effects of these medicinal plants on immunity and liver cancer. The present study deals with the antioxidant, anticancer and immune enhancement activity evaluation of the KP extracts and ascorbic acid. Furthermore we proposed to synergic effects of simulta-

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neous KP extracts and AA treatment on cytotoxicity in HepG2 cells. Therefore we suggest the potential possibility of good beneficial health effects of KP and AA.

Materials and Methods

Materials

The following reagents were used in this study: 2,4,6-tri-pyridyl-2,5,7,8- tetramethylchroman-2-carboxylic acid (TPTZ), sodium nitro-prusside (SNP), N-1-naphthyl-ethylen-diamine, lipopolysaccharide (LPS), avidin-peroxidase, 2'-AZINO-bis (ABTS) (Sigma, St. Louis, USA). TNF-α, IL-6 and GM-CSF (Pharmingen, San Diego, USA) levels in macrophage culture medium were determined by enzyme-linked immunosorbent assay. DMEM (Dulbeco's modified Eagle's medium), FBS (fetal bovine serum), 0.05% trypsin-0.02% EDTA and 100 units/ml penicillin-streptomycin were purchased from GIBCO Co. (Grand Island, NY, USA). RAW264.7 cells and HepG2 cancer cells were purchased from the Korean Cell Line Bank (Seoul, Korea).

Plant material and preparation of extracts

The stem bark of *Kalopanax pictus* Nakai was collected in Gyeongnam district of Korea and air dried in room temperature. The dried materials were cut in pieces and refluxed with methanol solution three times for 8 hr in water bath. The solution was filtered, evaporated under vacuum and lyophilized to give dried powder extract.

Cell culture

The human hepatocellular carcinoma cell line (HepG2) and RAW264.7 cells obtained from the Korean Cell Line Bank (Seoul, Korea), were grown at 37°C in DMEM medium containing 10% heat inactivated FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO_2. Cells were incubated with KP and AA at various concentrations.

DPPH assay

The volume of different KP and KP+AA was adjusted to 20 μl by adding MeOH. A 0.1 mM methanolic solution of DPPH was added (180 μl) to tubes, shaken vigorously and was standing at 27°C for 20 min. The control was prepared as above without any extract, and MeOH was used for the inhibition percentage and was calculated using the following formula: % radical scavenging activity = (control OD - sample OD/control OD) × 100, where OD is optical density at 517 nm [21].

$ABTS^+$ assay

This assay is based on scavenging of relatively stable blue/green $ABTS^+$ radical and converting it into a colorless product. $ABTS^+$ was generated by the incubation of 7 mM ABTS with 2.5 mM potassium persulfate in the dark for 16 hr. The samples (20 μl) were added into diluted ABTS solution (180 μl). The mixture was incubated at room temperature for 20 min and the absorbance was measured at 734 nm. The degree of the decolorization shows the antioxidant capacity [19].

FRAP assay

This method measures the ability of the antioxidants contained in the KP and KP+AA to reduce ferric-tripiridyl-triazine (Fe^{3+}-TPTZ) to a ferrous form (Fe^{+2}) which absorbs light at the main products of this reaction. FRAP level was calculated by plotting a standard curve of absorbance against μmol/L or μmol/g concentration of Fe^{2+} standard solution [2].

Scavenging of nitric oxide (NO)

Nitric oxide was examined by the Griess reaction and SNP was used as the NO generator [12]. The samples were mixed with an identical volume of 10 mM SNP and incubated at room temperature in the light. After 2.5 hr incubation, Griess reagent was added and the mixture was color-developed for 10 min. The absorbance was measured at 540 nm.

MTT assay for cell viability

Cytotoxicity studies performed in 96-well plate. HepG2 cells were mechanically scraped and plated at 1×10^5 cells/well in 96-well plates containing 100 μl of DMEM medium with 10% heat-inactivated FBS and incubated overnight. KP and AA were dissolved in methanol concentrations in all assays did not exceed 0.1%. After overnight incubation, the test material was added, and the plates were incubated for 24, 48 and 72 hr. Cells were washed once before adding 20 μl of FBS-free medium containing MTT 5 mg/ml. After 4 hr of incubation at 37°C, the medium was discarded and the formazan blue that formed in the cells was dissolved in DMSO 100 μl. The optical density was measured at 570 nm [23].

Flow cytometric analysis of cell cycle

HepG2 cells were plated on a 60-mm culture dish at a density of 1×10^6 cells/dish, and maintained for 24 hr. After exposing to the drug for 24 hr, the cells were harvested by trypsinization, rinsed with PBS, and then fixed with 70% ethanol for overnight. The fixed cells were incubated with propidium iodide (10 μg/ml) and RNase A (10 μg/ml) in PBS at room temperature for 30 min in the dark, and the DNA contents of these cells were analyzed using a flow cytometry (FAC-SCalibur, Beckton Dickinson, USA) [16].

Determination of suppressing effect on NO production by LPS-activated cells

In order to determine the effect of a KP and AA on NO production, 1×10^5 RAW264.7 cells were seeded into 96-well culture plates. RAW264.7 cells were cultured in DMEM medium supplemented with 10% FBS and 10 units of penicillin and 10 μg/ml streptomycin at 37°C in a humidified incubator containing 5% CO_2. The 100 μl of test extract was then added and serially diluted to give final concentrations. Cells were then stimulated with 200 U/ml of 10 μg/ml LPS for another 24 hr. The presence of nitrite, a stable oxidized product of NO, was determined in cell culture media by Griess reagent (1% sulfonamide and 0.1% N-(1-naphtyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4). The 100 μl of cell culture supernatant was removed and combined with 100 μl of Griess reagent in a 96-well plate followed by spectrophotometric measurement at 550 nm using a microplate reader (HT Biotec Ins, USA). Nitrite concentration in the supernatants was determined by comparison with a sodium nitrite standard curve [28].

Statistical analysis of data

The experiments shown are a summary of the data from at least three experiments and are presented as the mean±SD. Analysis of variance (ANOVA) was used for all data analysis, followed by Dunnett's test for multiple comparisons. Statistical significance was defined as $p<0.05$.

Results

DPPH and ABTS$^+$ assay was used to evaluate the ability of antioxidants to scavenge free radicals. As shown in Table 1, the scavenging activities of KP and KP+AA on DPPH and ABTS$^+$ radicals were compared. KP extract had a good scavenging activity on the DPPH and ABTS$^+$ radicals and the effect enhanced with increasing concentration in the range of 500~1,000 μg/ml. KP extract exhibited the highest scavenging activity (81.16% and 86.31%, respectively). Fig. 1 shows differences in total antioxidant capacity measured by the FRAP method on KP, KP+AA and control. The FRAP value was found within the range 10~380 μM. FRAP assay demonstrated the higher antioxidant activity for KP+AA. Related FRAP values ranged from 287.77 to 378.68 μM L^{-1}. Therefore values of FRAP were significantly different, all studied concentrations showed high ferric reducing ability.

It is well-known that nitric oxide (NO) has an important role in various types of inflammatory processes in the animal

Table 1. Free radical scavenging activity of KP and KP+AA at various concentrations

Sample (mg/ml)		DPPH	ABTS
		Scavenging activity (%)	
KP	0.5	65.68±6.34[a,1]	73.84±0.59[a]
	1	81.16±3.95[b]	86.31±0.34[b]
KP+AA[2]	0.5	88.34±4.94[c]	90.24±3.67[b]
	1	96.98±5.83[d]	94.28±4.93[c]

[1]The values given are the mean±SD of at least three independent experiments, each performed in triplicate.
[2]KP+AA (0.5, 1 mg/ml) indicate the mixture of KP (0.25)+AA (0.25 mg/ml) and KP (0.5)+AA (0.5 mg/ml), respectively.
[a-d]Means with different letters within the same column are significantly different at $p<0.05$ by Dunnett's test.

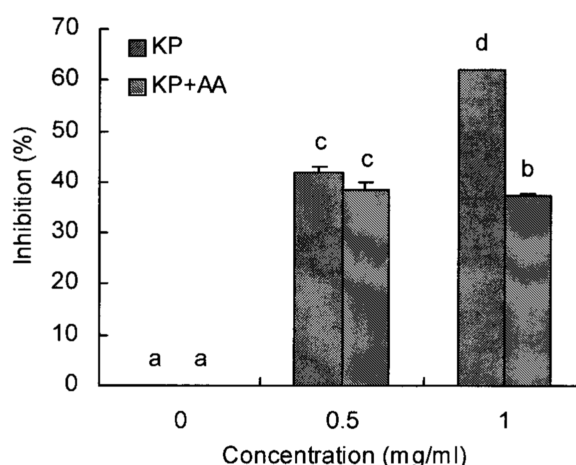


Fig. 1. Effect of concentration on ferric reducing ability of KP and KP+AA. The values given are the mean±SD of at least three independent experiments, each performed in triplicate. KP+AA : refer to Table 1. [a-e]Means with different letters are significantly different at $p<0.05$ by Dunnett's test.

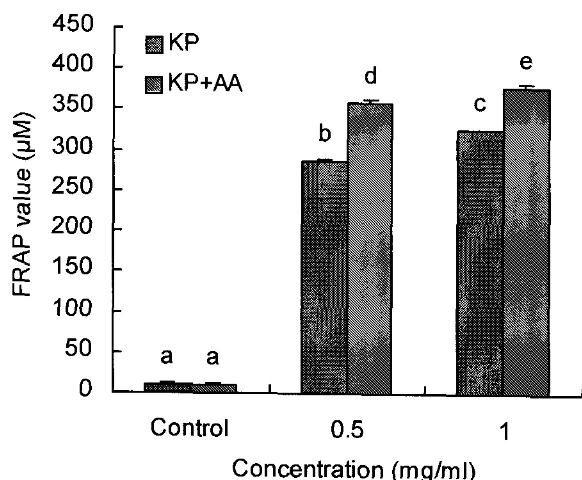


Fig. 2. In vitro NO-scavenging activity of KP and KP+AA. The values given are the mean±SD of at least three independent experiments, each performed in triplicate. KP+AA : refer to Table 1. [a-d]Means with different letters within the same column are significantly different at p<0.05 by Dunnett's test.

body. The scavenging NO activity may help arresting the chain of reactions initiated by excess generation of NO that are detrimental to human health [24]. The scavenging activities of KP and KP+AA on NO radical were evaluated, respectively. As shown in Fig 2, KP+AA had a stronger nitric oxide scavenging activity at 500~1,000 μg/ml. The inhibition effect enhanced with increasing concentration. KP+AA extract exhibited 37.34% of NO radical scavenging activity as 1,000 μg/ml was used.

To asses the anticancer activity properties of KP and KP+AA, we used cancer cell line, HepG2 cells. In Table 2, anticancer activity was observed in HepG2 cells. Treatment

Table 2. Cytotoxicity of HepG2 by KP and KP+AA at various concentrations after 24, 48 and 72 hr of treatment, as determined by MTT assay

Sample (mg/ml)		Cytotoxicity (%)		
		24 hr	48 hr	72 hr
KP	0.5	53.574±1.36[a,1]	65.061±1.66[ab]	69.458±0.83[c]
	1	56.466±2.42[a]	68.836±1.67[c]	70.996±0.42[c]
KP+AA[2]	0.5	58.594±4.71[a]	79.478±5.65[d]	82.771±1.10[d]
	1	63.815±2.98[ab]	87.309±3.48[d]	91.366±0.56[e]

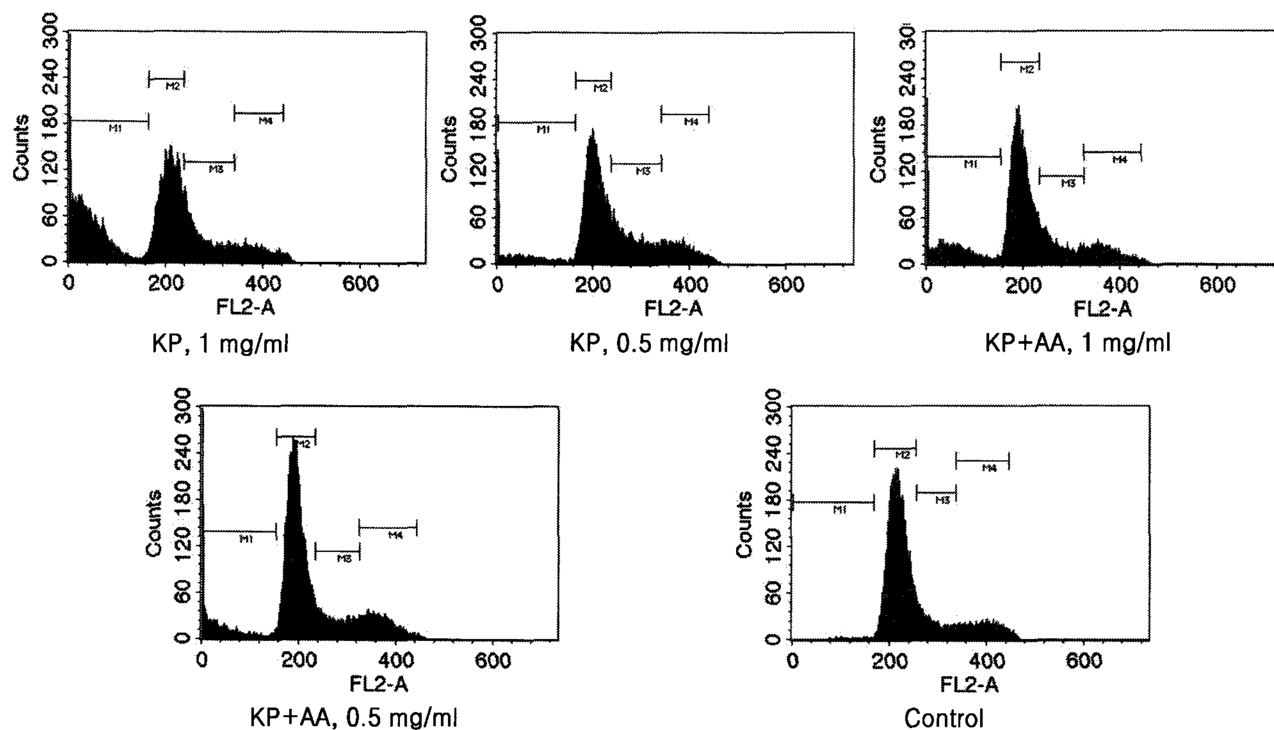
[1]The values given are the mean±SD of at least three independent experiments, each performed in triplicate.
[2]KP+AA (0.5, 1 mg/ml) indicate the mixture of KP (0.25)+AA (0.25 mg/ml) and KP (0.5)+AA (0.5 mg/ml), respectively. [a-d]Means with different letters within the same column are significantly different at p<0.05 by Dunnett's test.

with KP and KP+AA caused a dose-dependent reduction in cell numbers of cancer cells. KP+AA was more effective in inhibiting the growth of these cancer cell lines than KP at 1,000 μg/ml. The observed inhibition rate of KP and KP+AA was 71.0% and 91.37% at 1,000 μg/ml, respectively. The effect of KP and KP+AA on cell cycle progression on HepG2 cell was determined by flow cytometry. As shown in Fig 3, showed slightly S phase block by decreasing the population of the S phase from 23.17% to 8.26% (KP), and 23.17% to 13.92% (KP+AA) at 1,000 μg/ml, respectively. The changes of population of the G2/M phase from 3.37% to 20.64% (KP) and 3.37% to 18.55% (KP+AA) compared with that of the control groups.

As shown in Table 3, KP and KP+AA could stimulate the production of NO RAW264.7 macrophages were stimulated with LPS (1 μg/ml) for 24 hr to evoke NO synthesis. NO was measured as the accumulation of its stable metabolite, nitrite, in the supernatant. Incubation of cells with the KP and KP+AA and LPS resulted in dose-dependent reduction of NO production.

Discussions

Plant extracts contain phenolic compounds. Total phenolic content of a plant has been usually found to correlate highly with $ABTS^+$, DPPH and O_2^- scavenging activities of that plant, and therefore, phenolics were considered as the main contributors responsible for the free radical scavenging activity of a plant [10,17,27]. In accord with the observations of these workers, it was observed that a significant correlation existed between the total phenolics content of a plant and its free radical scavenging ability. Flavonoid is essentially required for excellent NO scavenging ability [7] and that flavonoid linked to flavan-3-ol also plays an important role in the NO scavenging [14]. Ascorbic acid in present study was simultaneously used for increasing the stability of reaction mixture and synergic effects of some biological activities such as antioxidant, anticancer and immune enhancement of the KP extracts. KP and KP+AA quenched RNS effectively. Therefore, in this study, NO scavenging activity correlate with total phenolics content, this suggested that KP and KP+AA have the critical ability for the NO scavenging activity. A correlation was found for the total phenolic content and the FRAP assays and this is in correspondence with Schlesier et al. [20] and also values of FRAP were significantly different, all studied



Items	Concentration (mg/ml)				
	Control	KP		KP+AA	
	0	1	0.5	1	0.5
G0/G1	71.85±0.35	37.84±0.36	54.69±0.51	50.50±0.11	61.46±0.31
S	23.17±0.12	8.26±0.14	13.86±0.46	13.92±0.28	13.46±0.06
G2/M	3.37±0.09	20.64±0.37	13.55±0.25	18.55±0.12	13.34±0.15

Fig. 3. Effect of KP and KP+AA on cell-cycle distribution in human liver carcinoma cells. HepG2 cells were exposed to various concentrations of KP and KP+AA for 24 hr, and the cell-cycle distribution was analyzed as described in the text. Results were expressed as the percentage of total cell number. The values given are the mean±SD of at least three independent experiments, each performed in triplicate. KP+AA : refer to Table 1.

samples showed high ferric reducing ability.

Many of the biological effects of antioxidants appear to be related to their ability not only to scavenge deleterious free radicals but modulate cell-signalling pathways [13]. Thus the modulation of cell signaling pathways by antioxidants could help prevent cancer by preserving normal cell cycle regulation; inhibiting proliferation and inducing apoptosis. As an antioxidant, it may be important to support the antiproliferative effect of KP and KP+AA is correlated with its scavenging ROS. Further investigation are needed to determine whether KP and KP+AA induced apoptosis in HepG2 liver cancer cell lines via modulate the intracellular redox status. As for inhibitory effect of KP and KP+AA on cell proliferation were found to be active against the cancer cell lines and showed a concentration-dependent affectivity. The inhibitory effects of KP+AA appeared to be more than those of KP. These inhibitory effects were due to antioxidative effects because KP and KP+AA - treated cells were affected at the doses used in this study.

As shown in Fig. 3, cells treated with the KP and KP+AA for 24 hr slightly decreased in the number of cells in S phase whereas accumulated in the G2/M and M1 phase of the cell cycle. The results indicated that the KP and KP+AA could suppress HepG2 liver cancer cell lines proliferation via the cell cycle blockage.

Many anticancer agents from plants that have been prescribed for treating malignancies nowadays inhibit cancer cell growth through cell cycle regulation including the G2/M accumulation [26]. It is well known that agents affect the G2/M phase cell cycle arrest interact by targeting tubulin or disrupting the tubulin-microtuble equilibrium

Table 3. Effect of KP and KP+AA on LPS-induced NO production in RAW 264.7 macrophages

Sample	Nitrite (μM)	
	KP	KP+AA[2]
Control	9.12±0.75[c,1]	10.11±1.43[c]
Control+0.5 mg/ml	2.34±0.01[a]	2.71±0.78[a]
Control+1.0 mg/ml	2.14±0.11[a]	2.06±0.44[a]
LPS	15.12±0.13[d]	18.26±0.41[e]
LPS+0.5 mg/ml	9.96±0.54[c]	10.65±0.25[c]
LPS+1.0 mg/ml	5.87±0.81[b]	8.54±0.11[c]

RAW264.7 cells (5×10^5 cells/well) were stimulated with LPS or KP and KP+AA for the production of nitric oxide. After 24 hours of culture, the amounts of NO production were measured by the Griess method as described under Materials and Methods. [1]The values given are the mean±SD of at least three independent experiments, each performed in triplicate. [2]KP+AA (0.5, 1 mg/ml) indicate the mixture of KP (0.25)+AA (0.25 mg/ml) and KP (0.5)+AA (0.5 mg/ml), respectively. [a-e]Means with different letters within the same column are significantly different at $p<0.05$ by Dunnett's test.

[8]. According to our results, KP and KP+AA exhibited the antiproliferative effect on HepG2 liver cancer cells via G2/M cell cycle arrest, thus it should interact with plant derived chemotherapeutic agents. However, the antiproliferative activities of this plant might be possibly dependent on cell types including the culture conditions.

Host immune function is critically important in the response to tumorigenesis [6]. Our results demonstrated that the antitumor effects of the KP and KP+AA were similar to that of TNF-α, one of the most potent antitumor molecules known [25]. In present study, KP and KP+AA was found to inhibit tumor cell growth and also showed potential as an anticancer agent.

In this study, KP and KP+AA decreased NO production. NO has been identified as a major effecter molecule produced by macrophages and is involved in the regulation of apoptosis and in host defenses against microorganisms and tumor cells [3]. In conclusion, KP and KP+AA exhibited a good antioxidant activity in four models studied and showed a good immuno-modulating effect. In addition, our results suggested that cell cycle arrest at G2/M phase and induce apoptosis as a mechanism by which KP and KP+AA an antiproliferative effect. However, further investigation at molecular level is required to identify the active components that could induce growth inhibition and establish the possible correlation among the mentioned activities of the KP and KP+AA. Also, more detailed work is required on the protective effects against cancer cell and immuno-modulating effects to know their exact mechanism of action.

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초록 : 음나무(*Kalopanax pictus*) 추출물과 비타민 C의 항산화, 항암 및 면역활성 상승효과

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음나무 (*Kalopanax pictus*, 이하 KP)의 추출물과 ascorbic acid(AA)의 DPPH와 ABTS 라디칼, FRAP 및 NO 소거능 상승효과를 조사하였다. 라디칼 소거능과 항산화능은 농도에 비례하여 증가하였으며, AA 첨가에 의해서 그 활성이 향상되었다. 인간 간암세포주에 대한 KP 추출물+AA의 항암능은 MTT법에서 우수한 효과를 나타내었으며 세포 사멸을 유도하였다. 또한 KP 추출물+AA는 세포주기의 G0/G1-phase 또는 G2/M-phase에 영향을 미쳤으며, 농도 의존적인 효과를 나타내었다. 그리고 KP 추출물+AA는 대식세포주를 이용한 NO생성과 억제의 면역활성 영향을 나타내었다. 결론적으로 KP 추출물의 항산화, 항암 및 면역조절 효과는 KP 추출물 단독으로 처리할 때보다, KP 추출물과 AA를 동시에 처리한 경우가 더욱 효과적이었다.