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# 에탄올 농도에 따른 산딸나무 열매 추출물의 페놀 함량과 항산화 특성

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# Phenolic Content and Antioxidant Properties of *Cornus kousa* Fruit Extracts at Different Ethanol Concentrations

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## ABSTRACT

**Background:** The bioactive potential of *Cornus kousa* fruit was examined by assessing its phenolic and flavonoid contents and antioxidant capacity across extracts obtained using various ethanol concentrations.

**Method and Results:** Total phenolic content of *C. kousa* fruit extracts was measured using the Folin–Ciocalteu reagent, whereas total flavonoid content was determined using an aluminum chloride colorimetric assay. Antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl radical scavenging, ferric reducing antioxidant power, oxygen radical absorbance capacity, and Trolox equivalent antioxidant capacity assays. High-performance liquid chromatography was performed using a C18 column and gradient elution to detect the compounds at multiple wavelengths. As a result, the 60% ethanol extract showed the highest total phenolic (20.08 ± 0.20 ㎎·GAE/g) and flavonoid (0.86 ± 0.02 ㎎·QE/g) contents. It also exhibited superior antioxidant activity in all the assays. Catechin was identified as the predominant phenolic compound, and its concentration correlated well with the observed antioxidant capacity.

**Conclusions:** The 60% ethanol extract exhibited the strongest antioxidant properties, indicating its potential as a functional ingredient. Further research is required to explore its application in health-related formulations.

***Key Words***: *Cornus kousa,* Antioxidant Activity, Catechin, Ethanol Extract, Flavonoid, Functional Food, Polyphenol

## INTRODUCTION

Reactive oxygen species (ROS) are highly reactive molecules that can potentially cause cellular damage. They can be induced by external factors such as stress or increased metabolic activity. Excessive ROS can lead to oxidative stress, which is associated with cellular damage such as DNA damage, cell necrosis, aging, and cancer (Köksal *et al*., 2017).

According to Lee and Lee (2016), the body utilizes both enzymatic and non-enzymatic antioxidant systems to protect against ROS-induced damage. Enzymatic antioxidants, often referred to as antioxidant enzymes, include superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase.

Non-enzymatic antioxidants include not only synthetic compounds such as butylated hydroxyanisole and butylated hydroxytoluene, but also naturally occurring substances like phenolic acids, flavonoids, and vitamins. Natural non-enzymatic antioxidants, particularly phenolic acids and flavonoids, play a crucial role in neutralizing free radicals and inhibiting oxidative

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chain reactions.

These compounds can directly scavenge ROS, chelate metal ions that catalyze radical generation, and upregulate endogenous antioxidant defenses, thereby contributing significantly to cellular protection against oxidative stress (Huyut *et al*., 2017).

*Cornus kousa*, belonging to the Cornaceae family, is indigenous to the southern central part of Korea and Halla Mountain in Jeju. It is known for its resilience to environmental pollution and is commonly planted in urban landscapes, streets, and parks due to its ornamental flowers and fruit.

In traditional medicine, *C. kousa* fruit is known to be beneficial for digestion. Recent studies have reported a wide range of physiological activities, including the use of cosmetic ingredients for skin whitening and anti-wrinkle effects, pharmaceutical compounds for cancer, anti-inflammatory, and anti-gout treatments, as well as antioxidant activities (Kim *et al*., 2002; Kim *et al*., 2008; Lee *et al*., 2015).

Phytochemical analyses have identified various bioactive compounds in *C. kousa* fruit, including sterols such as β-sitosterol, stigmast-4-en-6β-ol-3-one, and daucosterol (Lee *et al*., 2006); anthocyanins, namely ideain and cyanidin 3-O-glucoside (Vareed *et al*., 2006); and flavonoids including myricetin 3-O-rhamnoside, astragalin, cornin, and stenophyllin (Vareed *et al*., 2007).

Phenolic compounds are a diverse group of secondary metabolites known for their strong antioxidant, anti-inflammatory, anticancer, and cardioprotective effects (de Araújo, 2021). Their biological activities are primarily attributed to their ability to scavenge reactive oxygen and nitrogen species, enhance antioxidant enzyme activities, and modulate cellular signaling pathways related to oxidative stress and inflammation. Flavonoids, a major subclass of phenolics, contribute significantly to these beneficial properties and are abundantly found in fruits and vegetables.

From an industrial perspective, phenolic compounds and flavonoids hold great promise for applications in the food, pharmaceutical, and cosmetic industries. They serve as natural preservatives, functional food ingredients, natural colorants, and bioactive agents in health-promoting products. Considering both their scientific relevance and commercial potential, this study focuses on evaluating the phenolic and flavonoid contents of *C. kousa* fruits to assess their biological activities and application possibilities.

Despite the well-documented physiological benefits of *C. kousa* fruit, research focusing specifically on optimizing the extraction conditions to maximize phenolic and flavonoid content and their antioxidant capacity remains insufficient.

Given that phenolic compounds and flavonoids are key contributors to antioxidant activity, and their extraction efficiency is highly dependent on solvent polarity, determining the optimal ethanol concentration is essential for enhancing bioactivity and yield (Ereifej *et al*., 2016). Therefore, it is necessary to determine the optimal ethanol concentration for maximizing the yield of phenolic compounds.

This study aims to investigate the effects of different ethanol concentrations used for extraction on the yield of phenolic compounds and antioxidant activity, providing foundational data for the potential functional food applications of *C. kousa* fruit.

## MATERIALS AND METHODS

### 1. Plant material

In this study, *Cornus kousa* fruits were harvested at full ripeness in October 2017. Immediately after harvesting, the fruits were carefully washed with distilled water to remove any surface contaminants. To preserve their freshness, the fruits were rapidly frozen at −80℃ within 2 hours post-harvest and stored under these conditions until further processing.

After storage, the frozen fruits were lyophilized for one week to ensure complete dehydration while maintaining phytochemical stability.

The lyophilized fruits were subsequently ground into fine powders using a pulverizer and stored at −20℃ in airtight containers until use for extraction.

### 2. Preparation of plant extracts

The solvents used were distilled water, 100% ethanol (EtOH), and EtOH-water mixtures (20%, 40%, 60%, and 80% EtOH).

The ethanol concentrations were selected to cover a wide polarity range for optimizing the extraction of diverse phenolic and flavonoid compounds, based on previous studies reporting solvent polarity as a critical factor influencing extraction efficiency (Gülçin, 2025).

Dried *C. kousa* fruit powders were mixed with each solvent at a fixed solid-to-liquid ratio of 1 : 20 (g/㎖), ensuring consistent sample concentration across all treatments. The mixtures were extracted by refluxing at 80℃ for 90 minutes using a heating mantle (MS-EAMD, Misung Scientific Co., Ltd., Seoul,

Korea). After extraction, the solutions were filtered, concentrated under reduced pressure, and subsequently lyophilized to obtain dry extracts.

The extraction yield (%) was calculated by dividing the weight of the lyophilized extract by the initial weight of the dried sample, then multiplying by 100. The dried extracts were stored at −20℃ until further analysis.

### 3. Total phenol content and Total flavonoid content

The total phenol and total flavonoid contents in the extracts were analyzed using the procedure described by Ko *et al*. (2017).

Total phenol content was determined by color development with Folin-Ciocalteu reagent and sodium carbonate ($Na_2CO_3$), and the results were converted into a calibration curve prepared using gallic acid as the standard substance (gallic acid equivalent, GAE).

Total flavonoid content was determined by color development with aluminum nitrate and potassium acetate ($CH_3COOK$), and the results were converted into a calibration curve prepared using quercetin as the standard substance (quercetin equivalent, QE).

### 4. Measurement of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH reaction mixture (0.1 mM DPPH solution 180 ㎕ + extract 20 ㎕) was incubated for 30 min, and the decrease in absorbance at 490 ㎚ was measured using microplate reader (i-Mark microplate reader 168-1135, Bio-Rad, Hercules, CA, USA).

The DPPH radical scavenging activity was calculated by comparing the absorbance change with that of the control reaction (0.1 mM DPPH solution 180 ㎕ + methanol 20 ㎕) (Ko *et al*., 2017). Scavenging activity was evaluated at various sample concentrations, and the concentration required to achieve 50% scavenging ($RC_{50}$) was calculated.

### 5. Trolox equivalent antioxidant capacity (TEAC)

The TEAC assay was performed based on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity (Zulueta *et al*., 2009).

A 7 mM ABTS solution was radicalized by reacting with 2.45 mM potassium persulfate, and the ABTS working solution was prepared by adjusting the absorbance to 0.7 at 734 ㎚ using UV spectrophotometer (UV-1800, Shimadzu Co., Kyoto, Japan).

An aliquot of 50 ㎕ from each extract was combined with 1 ㎖ of the ABTS working solution, and the mixture was incubated for 5 min. The absorbance at 734 ㎚ was then measured. TEAC values were determined and presented as the concentration of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) equivalents (mM·TE/g) per gram of dry sample, based on a calibration curve constructed using trolox as a reference standard.

### 6. Ferric reducing antioxidant power (FRAP)

The FRAP measurement was determined based on ferric ion reduction (Guo *et al*., 2003).

The FRAP working solution was prepared by adding 1 ㎖ of 10 mM 2,4,6-tripyridyl-s-triazine and 1 ㎖ of 20 mM ferric chloride to 10 ㎖ of 300 mM sodium acetate buffer (pH 3.6). A mixture of 150 ㎕ of the FRAP working solution and 50 ㎕ of each extract was incubated at 37℃ for 15 min, and the absorbance was then measured at 595 ㎚ using microplate reader (i-Mark microplate reader 168-1135, Bio-Rad, Hercules, CA, USA).

FRAP values were determined and presented as the concentration of $FeSO_4$ equivalents (mM·FE/g) per gram of dry sample, based on a calibration curve constructed using $FeSO_4$ as a reference standard.

### 7. Oxygen radical antioxidant capacity (ORAC)

The ORAC measurement was based on the principle of fluorescence decrease induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (Cao *et al*., 1993).

A mixture of 50 ㎕ of each extract and 150 ㎕ of 78 nM fluorescein was incubated at 37℃ for 10 min to stabilize the temperature. Following this, 221 mM AAPH was added to initiate the fluorescence quenching reaction, and fluorescence was measured every min under excitation at 485 ㎚ and emission at 535 ㎚ using (SpectraMax i3, Molecular Devices, San Jose, CA, USA).

ORAC values were determined and presented as the concentration of trolox equivalents (mM·TE/g) per gram of dry sample, based on a calibration curve constructed using trolox as a reference standard.

### 8. ROS scavenging activity in HepG2 cells

The cell lines used in this experiment were HepG2 cells derived from human hepatoma. Dulbecco's Modified Eagle Medium (DMEM) containing 5% fetal bovine serum (FBS), penicillin 100 units/㎖, and streptomycin 50 ㎍/㎖ was used

and cultured in a T-shaped flask.

The inhibition of ROS production was measured using the following method. HepG2 cells were seeded at a density of 1 × $10^5$ cells/well and cultured for 24 hours. After removing the old DMEM, the cells were treated with fresh DMEM containing the extracts at concentrations of 5, 10, and 20 ㎍/㎖. After 12 hours of incubation, 50 μM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was added to each well and incubated for 30 min. Subsequently, 100 μM tert-butyl hydroperoxide (TBHP) was added, and the cells were incubated for an additional 90 min.

Fluorescence was measured in each well using excitation at 485 ㎚ and emission at 530 ㎚ using SpectraMax i3 (Molecular Devices, San Jose, CA, USA). ROS inhibition was expressed as a multiple of the control value, based on the fluorescence intensity of the control.

### 9. High performance liquid chromatography (HPLC) analysis

The extracts were analyzed by HPLC (LC-20A, Shimadzu Corp., Kyoto Japan) using gradient elution.

Two solvents were used: (A) water (0.1% trifluoroacetic acid) and (B) acetonitrile (0.1% trifluoroacetic acid). The concentration of solvent B was programmed as follows: 10% to 20% for the first 5 min, held at 20% for 20 min, increased to 25% over 15 min, further raised to 60% in the next 5 min, then increased to 80% over the following 5 min, and maintained at 80% for 5 min. The mobile phase was then returned to the initial condition (10% solvent B) over 3 min, followed by re-equilibration for 5 min before the next injection.

A YMC-Triart C18 column (250 ㎜ × 4.6 ㎜, YMC, Kyoto, Japan) was used for the analysis with a flow rate of 1.0 ㎖/min and a column temperature of 40℃. Absorbance was recorded at 245 ㎚, 260 ㎚, and 320 ㎚ during each run.

Catechin standard (≥ 98% purity, Sigma-Aldrich, St. Louis, MO, USA) was used for quantification. Calibration curves were constructed using five different concentrations of catechin to ensure linearity ($R^2 > 0.99$). Quantification was based on the external standard method by comparing the peak areas of the samples with those of the catechin standard. The HPLC method and quantification approach were adapted and modified from the procedures described by El-Shahawi *et al*. (2012).

A total of nine phenolic compounds—gallic acid, catechin, chlorogenic acid, caffeic acid, syringic acid, *p*-coumaric acid, protocatechuic acid, myricetin, and quercetin—were analyzed using HPLC based on previous methodologies (Kim *et al*., 2010; de Araújo *et al*., 2021). Among these, five compounds—gallic acid, catechin, chlorogenic acid, syringic acid, and protocatechuic acid—were positively identified by matching their retention times with those of authenticated standards under identical chromatographic conditions.

Furthermore, the peaks corresponding to these compounds were selectively confirmed by comparing their UV-Vis absorption spectra obtained through a diode array detector (DAD) with the reference spectra of standard substances. This dual validation approach ensured accurate identification of the major phenolic constituents present in the *C. kousa* extracts.

### 10. Statistical Analysis

The data are presented as means ± standard deviation, and statistical significance was determined using Duncan's Multiple Range Test (DMRT, $p < 0.05$). Pearson's correlation analysis was conducted to assess correlations between variables All statistical analysis were performed on SPSS (Ver. 18.0 SPSS Inc., Chicago, IL, USA).

## RESULTS AND DISCUSSION

### 1. Total phenol and flavonoid content of *C. kousa* fruit extract

Polyphenols have a high antioxidant activity and are secondary metabolites of plants contained in many foods, including phenolic acid, flavonoid, stilbene, and lignan. Flavonoids have a carbon structure of C6-C3-C6 and are classified as flavonol, flavone, isoflavone, anthocyanidin, flavanone, and flavanol compounds such as quercetin, luteolin, and myricetin have been identified (Del Rio *et al*., 2013).

The structural features of flavonoids have strong antioxidant activity against lipid peroxidation. Total phenol and flavonoid content of the *C. kousa* fruit extracted in different ethanol concentrations is as follows (Table 1).

The lowest total phenol content was found in the water extract (11.93 ± 0.13 ㎎·GAE/g), while the highest content was observed in the 60% EtOH extract (20.08 ± 0.20 ㎎·GAE/g). Regarding total flavonoid content, the lowest value was found in the water extract (0.09 ± 0.01 ㎎·QE/g), and the highest was in the 60% EtOH extract (0.86 ± 0.02 ㎎·QE/g). As the ethanol concentration increased from water to 60%, both total phenol and total flavonoid contents increased. However, ethanol concentrations above 80% were not favorable for extraction. The total phenol content was highly correlated with total

**Table 1.** Total phenol and flavonoid content of the *C. kousa* fruit extracted in different ethanol concentrations.

| Extracts | | Total phenol (㎎·GAE/g) | Total flavonoid (㎎·QE/g) |
|---|---|---|---|
| Distilled water | | 11.93±0.13$^{f}$ | 0.09±0.01$^{f}$ |
| Ethanol concentration (%) | 20 | 15.80±0.18$^{d}$ | 0.50±0.01$^{c}$ |
| | 40 | 19.22±0.07$^{b}$ | 0.72±0.02$^{b}$ |
| | 60 | 20.08±0.20$^{a}$ | 0.86±0.02$^{a}$ |
| | 80 | 16.08±0.20$^{c}$ | 0.44±0.02$^{d}$ |
| | 100 | 12.49±0.12$^{e}$ | 0.40±0.01$^{e}$ |

Data represent the means ± SD (standard deviation) (n=3) with varying letters (a - f) in the same column are significantly different at Duncan Multiple Range Test (DMRT, $^{*}p < 0.05$).

flavonoid content (Table 2), and the content extracted from *C. kousa* fruit is similar according to the concentration of the ethanol solvent.

In addition, the extracts mixed with water and ethanol showed higher total phenol and flavonoid contents than the separate water or 100% ethanol extracts combined with a single solvent.

When extracting using a mixture of water and ethanol, the various physiologically active substances of the water-soluble and lipid components contained in the plant are extracted together (Hwang *et al*., 2015). This result mirrored those found in a study in which the extraction yield of the polyphenols of mulberry fruits was the highest when utilizing a 60% ethanol concentration of the solvent (Cha *et al*., 2004). Therefore, 60% ethanol is considered an advantageous solvent for extracting *C. kousa* fruit due to its ability to yield higher total phenol and flavonoid contents.

In addition, five polyphenols—gallic acid, catechin, chlorogenic acid, syringic acid, and protocatechuic acid—were selected for HPLC analysis based on their high antioxidant activities and their common presence in plant materials, as reported in previous studies (Tashtoush *et al*., 2016).

Gallic acid and chlorogenic acid are well-known for their strong free radical scavenging effects, while catechin, a representative flavonoid, exhibits significant lipid peroxidation inhibitory activity. Syringic acid and protocatechuic acid have also been recognized for their potent antioxidant and anti-inflammatory properties.

Thus, the selection of these compounds enabled a comprehensive evaluation of the antioxidant potential of *C. kousa* fruit extracts. In addition to these compounds, a broader range of phenolics including protocatechuic acid, syringic acid, *p*-coumaric acid, gallic acid, caffeic acid, catechin, chlorogenic acid, and quercetin were analyzed through HPLC. Among these, quercetin and chlorogenic acid were identified as the major polyphenols present in *C. kousa* fruit extracts, highlighting their significant contribution to antioxidant activity. Notably, syringic acid was exclusively detected in *C. kousa* extracts.

The samples, including leaves, branches, and fruits of *C. kousa*, were extracted using a pressurized liquid extractor with 40% ethanol as the extraction solvent to ensure efficient recovery of both hydrophilic and lipophilic polyphenols (Kim *et al*., 2010).

### 2. Antioxidant activity of the *C. kousa* fruit extract

This study investigated the antioxidant activity of the *C. kousa* fruit extract according to the DPPH, TEAC, FRAP, and ORAC methods (Table 2). The range of the DPPH $RC_{50}$ value

**Table 2.** Correlation analysis between total phenol and flavonoid and antioxidant activities of the *C. kousa* fruit extracted in different ethanol concentrations.

| | Phenol | Flavonoid | DPPH[1)] | TEAC[2)] | FRAP[3)] | ORAC[4)] | ROS[5)] |
|---|---|---|---|---|---|---|---|
| Phenol | 1 | 0.935$^{***}$ | –0.875$^{***}$ | 0.940$^{***}$ | 0.971$^{***}$ | 0.870$^{***}$ | –0.832$^{***}$ |
| Flavonoid | | 1 | –0.678$^{**}$ | 0.774$^{***}$ | 0.831$^{***}$ | 0.685$^{**}$ | –0.857$^{***}$ |
| DPPH | | | 1 | –0.979$^{***}$ | –0.955$^{***}$ | –0.933$^{***}$ | 0.560$^{*}$ |
| TEAC | | | | 1 | 0.988$^{***}$ | 0.932$^{***}$ | –0.658$^{**}$ |
| FRAP | | | | | | 0.932$^{***}$ | –0.756$^{***}$ |
| ORAC | | | | | | 1 | –0.634$^{**}$ |
| ROS | | | | | | | 1 |

Significant differences are indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ($n = 3$). [1)]DPPH; 2,2-diphenyl-1-picrylhydrazyl, [2)]TEAC; trolox equivalent antioxidant capacity, [3)]FRAP; ferric reducing antioxidant power, [4)]ORAC; oxygen radical absorbance capacity, [5)]ROS; reactive oxygen species. Principal component analysis (PCA) was performed using SPSS based on Pearson's correlation matrix to assess the relationships among total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activities.

of extracts by ethanol concentration was 283.64 to 1190.30 ㎍/㎖. When using a range of 0 to 60% ethanol concentration, the activity increased in a concentration-dependent manner.

The DPPH $RC_{50}$ value in the 60% ethanol showed the highest scavenging activity as 283.64 ㎍/㎖. This suggests that many phenolic hydroxyl (OH) groups of 60% ethanol extracts may affect the reducing activity of free radicals. As a result, correlation analysis with total phenol content showed $r = -0.875$, $p < 0.001$ (Table 2).

In addition to DPPH, antioxidant activities measured by TEAC, FRAP, and ORAC were also quantified based on standard compounds: TEAC and ORAC results were expressed as mM trolox equivalents per gram of dry extract (mM·TE/g), and FRAP values were expressed as mM $FeSO_4$ equivalents per gram (mM·FE/g). The TEAC values of the extracts ranged from 42.20 to 119.40 mM·TE/g, and they gradually increased with the ethanol concentration, which was highest in the 40 and 60% ethanol extracts, whereas these values decreased in the 80 and 100% ethanol extracts.

In the DPPH assay, the scavenging activity of free radicals was evaluated, while the ABTS assay measured the scavenging activity of cation radicals. Despite the different approaches, both assays exhibited similar trends and a strong correlation (Table 2).

Polyphenols in the extracts were reported to significantly influence DPPH and ABTS radical scavenging activities (Cha *et al*., 2004). Furthermore, the results showed similar findings to those of Min and Jhoo (2013), where intermediate concentrations of ethanol extracts exhibited higher DPPH and ABTS radical scavenging activities compared to distilled water and 100% ethanol.

Statistical analysis (Duncan's Multiple Range Test, $p < 0.05$) showed that the antioxidant activities differed significantly between extracts prepared with different ethanol concentrations, especially highlighting the superior activity of the 60% ethanol extract in all assays.

The FRAP value of the extracts ranged from 10.98 to 187.81 mM·FE/g, and the activity was dependent on the range of 0 to 60% ethanol concentration. The FRAP value of the 60% ethanol extract was highest at 187.81 mM·FE/g, whereas the FRAP values decreased in the 80 and 100% ethanol extracts.

According to a study by Pulido *et al*. (2000), polyphenols extracted from plants display antioxidant activity, with an emphasis on redox reactions. Therefore, the high FRAP value of the 60% ethanol extract is considered closely related to the content of phenol compounds.

The ORAC assay produces peroxyl radicals by AAPH, which is eliminated by antioxidant substances and, thus, can be used to measure the inhibitory activity of free radicals by fluorescence. It also has a wide range of applications because it can respond to both hydrophobic and hydrophilic components contained in plants. The ORAC values showed an increasing trend up to a 60% ethanol concentration, with the highest values observed in the 40% and 60% ethanol extracts, recorded at 662.55 mM·TE/g and 720.67 mM·TE/g, respectively. There was no significant difference between these two values. On the other hand, activity decreased at the 80 to 100% ethanol concentrations. When each extract was treated with 10 ㎍/㎖ concentration, it was found that in 100% ethanol extract, the ORAC index could not be measured.

The overall trend that antioxidant activities peaked at 60% ethanol extraction is consistent with findings from Cha *et al*. (2004) and Min and Jhoo (2013), supporting the conclusion that mid-polarity solvent mixtures more effectively extract bioactive compounds than water or absolute ethanol alone. The ORAC assay has been reported to be most correlated with clinical trials in the antioxidant *in vitro* assays. There was a high correlation in each of the antioxidant experiments performed in this study (Table 2). In all experiments, the 60% ethanol extract showed the best antioxidant activity.

### 3. ROS scavenging activity in HepG2 cells

When the nonpolar DCFH-DA enters the cell through the membrane, the intracellular esterase enzyme acts to decompose it into 2',7'-dichlorodihydrofluorescein (DCFH). DCFH is converted to fluorescent 2',7'-dichlorofluorescein (DCF) by reacting with ROS produced by tert-butyl hydroperoxide (TBHP).

Therefore, when the amount of ROS produced is large, the fluorescence value is increased. Greater ROS in the body causes oxidative stress and damage to cells and tissues. Therefore, sufficient control of ROS in intracellular metabolism is required because it directly affects chronic diseases, such as aging-related denaturation, inflammation, and diabetes (Tak *et al*., 2014).

The inhibition of ROS production was measured by the decrease in the fluorescence value when reacted with the ROS-inhibiting component. Intracellular ROS scavenging activity of the extracts of the *C. kousa* fruit in different ethanol concentrations is as follows (Table 3).

**Table 3.** Intracellular reactive oxygen species (ROS) scavenging activity of the *C. kousa* fruit extracted in different ethanol concentrations.

| Extracts | | Control | TBHP | Concentration (㎍/㎖) | | |
|---|---|---|---|---|---|---|
| | | | | 5 | 10 | 20 |
| Distilled water | | 1.00±0.02$^{a*}$ | 4.83±0.03$^{e}$ | 3.79±0.36$^{dC}$ | 3.44±0.16$^{cE}$ | 2.41±0.04$^{bB}$ |
| Ethanol concentration (%) | 20 | | | 3.62±0.39$^{dBC}$ | 3.08±0.03$^{cD}$ | 2.26±0.36$^{bB}$ |
| | 40 | | | 3.10±0.13$^{dAB}$ | 2.51±0.08$^{cB}$ | 1.89±0.13$^{bA}$ |
| | 60 | | | 2.96±0.18$^{dA}$ | 2.23±0.04$^{cA}$ | 1.66±0.07$^{bA}$ |
| | 80 | | | 3.54±0.17$^{dBC}$ | 2.48±0.01$^{cB}$ | 1.73±0.08$^{bA}$ |
| | 100 | | | 3.53±0.38$^{dBC}$ | 2.91±0.04$^{cC}$ | 2.52±0.15$^{bB}$ |

*Data represent the means ± SD (standard deviation) (n = 3) with varying letters (a - e) in the same row are significantly different at Duncan Multiple Range Test (DMRT, $p < 0.05$). Means with varying letters (A - E) in the same column are significantly different at Duncan Multiple Range Test (DMRT, $p < 0.05$).

**Table 4.** Antioxidant activity of the *C. kousa* fruit extracted in different ethanol concentrations.

| Extracts | | DPPH[1)] $RC_{50}$[5)] (㎍/㎖) | TEAC[2)] (mM·TE/g) | FRAP[3)] (mM·FE/g) | ORAC[4)] (mM·TE/g) |
|---|---|---|---|---|---|
| Distilled water | | 783.22±5.19$^{e}$ | 67.31±1.35$^{c}$ | 46.00±2.34$^{e}$ | 169.10±46.90$^{c}$ |
| Ethanol concentration (%) | 20 | 550.05±6.64$^{c}$ | 91.93±1.67$^{b}$ | 108.67±1.92$^{d}$ | 429.44±127.56$^{b}$ |
| | 40 | 410.84±12.26$^{b}$ | 117.13±1.03$^{a}$ | 173.79±4.16$^{b}$ | 662.55±7.36$^{a}$ |
| | 60 | 283.64±25.60$^{a}$ | 119.40±0.96$^{a}$ | 187.81±4.75$^{a}$ | 720.67±50.50$^{a}$ |
| | 80 | 596.71±10.76$^{d}$ | 90.88±1.50$^{b}$ | 124.78±2.48$^{c}$ | 512.34±79.83$^{b}$ |
| | 100 | 1190.30±19.27$^{f}$ | 42.20±1.06$^{d}$ | 10.98±0.86$^{f}$ | ND |

[1)]DPPH; 2,2-diphenyl-1-picrylhydrazyl, [2)]TEAC; trolox equivalent antioxidant capacity, [3)]FRAP; ferric reducing antioxidant power, [4)]ORAC; oxygen radical absorbance capacity, [5)]$RC_{50}$; amount of the sample sufficient to elicit 50% reduction of the initial DPPH concentration. ND; None detected. *Data represent the means ± SD (standard deviation) (n = 3) with varying letters (a - f) in the same column are significantly different at Duncan Multiple Range Test (DMRT, $p < 0.05$).

When the amount of ROS in the control without any treatment was 1, the amount of ROS produced by TBHP alone increased about 4.83 times. All extracts exhibited an inhibitory effect on ROS generation, with the effect becoming stronger as the concentration of the extract increased. In addition, the inhibitory activity of ROS formation at 5 ㎍/㎖ and 10 ㎍/㎖ was highest in 60% ethanol extracts compared to others, and 40 to 80% ethanol extracts showed the highest activity at 20 ㎍/㎖ concentration.

In conclusion, *C. kousa* fruit extract demonstrated antioxidant activity by inhibiting ROS generation induced by TBHP in HepG2 cells. ROS scavenging activity was highly correlated with phenol and flavonoid content (Table 4). This indicates that the phenol and flavonoid contents of the *C. kousa* fruit extract effectively inhibited ROS in HepG2 cells.

### 4. Polyphenol content of *C. kousa* fruit extract

The polyphenols in *C. kousa* fruit extract were analyzed using HPLC, and the results are summarized in Table 5.

Five major compounds were identified: gallic acid, catechin, chlorogenic acid, syringic acid, and protocatechuic acid. These compounds are known polyphenols with strong antioxidant activities (Papadopoulos and Boskou, 1991; Kono *et al*., 1997; Yilmaz and Toledo, 2004).

Among these, gallic acid, catechin, and syringic acid were present in all extracts, whereas chlorogenic acid and protocatechuic acid were undetected in both distilled water and 100% ethanol extracts. The 60% ethanol extract exhibited the highest overall polyphenol content compared to other solvent concentrations.

Specifically, catechin, chlorogenic acid, and syringic acid were most abundant in this extract, with concentrations of 287.01 ㎍/g, 4.71 ㎍/g, and 24.03 ㎍/g, respectively. Catechin, the predominant polyphenols in *C. kousa* fruit, ranged from

**Table 5.** Polyphenols content of the *C. kousa* fruit extracted in different ethanol concentrations.

| Extacts | | Polyphenols content (㎍/g) | | | | |
|---|---|---|---|---|---|---|
| | | Gallic acid | Catechin | Chlorogenic acid | Syringic acid | Protocatechuic acid |
| Distilled water | | 12.02±6.50[a] | 145.64±1.53[f] | ND | 10.76±0.33[d] | ND |
| Ethanol concentration (%) | 20 | 14.65±0.01[a] | 207.14±2.92[d] | 1.33±0.08[c] | 16.37±0.07[c] | 19.79±0.12[c] |
| | 40 | 10.92±0.28[a] | 242.56±3.01[c] | 1.03±0.01[d] | 16.03±0.24[c] | 33.42±0.86[ab] |
| | 60 | 11.20±0.37[a] | 287.01±2.38[a] | 4.71±0.01[a] | 24.03±0.51[a] | 31.53±0.16[b] |
| | 80 | 12.25±0.27[a] | 276.04±1.46[b] | 1.71±0.02[b] | 17.99±0.25[b] | 37.53±4.12[a] |
| | 100 | 1.09±0.09[b] | 181.48±0.33[e] | ND | 10.07±0.24[d] | ND |

[**]Data represent the means ± SD (standard deviation) (n = 3) with varying letters (a - f) in the same column are significantly different at Duncan Multiple Range Test (DMRT, $p < 0.05$). ND; not detected.

145.64 ㎍/g to 287.01 ㎍/g across all extracts.

According to Grzesik *et al*. (2018), catechin exhibits the highest ABTS radical scavenging activity among common antioxidants and demonstrates the greatest $Fe^{3+}$-reducing capacity in the FRAP assay. Additionally, catechin has been shown to effectively protect against AAPH-induced erythrocyte hemolysis. Given its high catechin content, the strong antioxidant activity observed in the 60% ethanol extract is likely attributed to this compound.

This study extracted *C. kousa* fruit using different ethanol concentrations and measured the total phenol and total flavonoid contents of the extracts. In addition to evaluating antioxidant properties through various methods, the ROS scavenging activity in HepG2 cells was assessed, providing foundational data for the application of functional foods. The results demonstrated a strong correlation between total phenol and flavonoid contents and antioxidant activity, with both parameters reaching their highest levels in the 60% ethanol extract. Additionally, intracellular ROS scavenging activity was also maximized in this extract.

Catechin was identified as the major polyphenols in *C. kousa* fruit, with its highest concentration (287.01 ㎍/g) observed in the 60% ethanol extract. Given the well-documented antioxidant properties of catechin, its abundance likely contributes to the superior antioxidant activity of this extract. Therefore, *C. kousa* fruit appears to be most effectively utilized as a functional food material when extracted with 60% ethanol.

To further enhance the applicability of *C. kousa* fruit as a raw material for health functional foods, future studies should focus on optimizing extraction conditions by investigating variables such as extraction time, temperature, and solvent ratio. Establishing optimal extraction parameters would maximize the yield of bioactive compounds, thereby increasing the functional potential of *C. kousa* fruit in nutraceutical applications.

Moreover, considering the abundant antioxidant properties demonstrated in this study, *C. kousa* fruit extracts hold significant potential for broader industrial applications, including their use as natural antioxidants in functional foods, active ingredients in cosmetic formulations targeting skin aging and pigmentation, and as supportive compounds in pharmaceutical products for managing oxidative stress-related diseases.

Previous studies have highlighted the antioxidant and anti-inflammatory properties of *Cornus* species extracts; however, detailed investigations optimizing ethanol extraction conditions to maximize phenolic and flavonoid yields are limited. Compared to earlier works, this study provides a novel and practical approach by systematically analyzing extraction efficiency across varying ethanol concentrations, thus offering valuable baseline data for industrial scaling and product development.

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