

## Volatile Compounds and Antiproliferative Effects of *Dendropanax morbifera* on HepG2 Cells

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*Dendropanax morbifera* Lev. is known in Korea for its golden sap and medicinal properties. The many biological activities of the leaf and stem extracts suggest that this tree could be a valuable source of medicinal compounds for the treatment of various ailments such as dermatitis, migraines, dysmenorrhea, muscle pain, and infectious diseases. However, there is little information on the composition and biological activity of the volatile fraction of *D. morbifera*. Therefore, in this study, the volatile compounds in leaves, stems, and sap of *D. morbifera* were isolated using solvent and supercritical fluid extraction (SFE), and analyzed by gas chromatography/mass spectrometry to reveal their chemical composition and identify potential compounds of interest. Fifteen compounds were identified in the leaf extracts, whereas 29 and 3 compounds were identified in the stem and sap extracts, respectively. The volatile profiles obtained using solvent and SFE differed. Esters and aromatic hydrocarbons predominated in the solvent extract of leaves and SFE extract of stems, whereas the solvent extract of stems and SFE extract of leaves contained terpenoids. Limonene,  $\alpha$ -pinene, and  $\beta$ -myrcene were identified in the volatile extract of sap, with limonene representing 96.30% of the total peak area. In addition, the antiproliferative effects of the solvent extracts of leaves and stems were evaluated, revealing that these solvent extracts were particularly effective in decreasing the proliferation of HepG2 cells.

**Key words** : Antiproliferative effect, *Dendropanax morbifera*, GC/MS, HepG2 cells, volatiles

### Introduction

*Dendropanax morbifera* Lev. (Araliaceae) is a broad leaf evergreen tree species endemic in Korea, found in isolated pockets in the southwestern coastal areas of the country ranging from Jeju Island to Wando Island and Haenam [13]. *D. morbifera* is an economically important species traditionally used to extract a golden sap used as varnish in wood and metal, and is also used as an ornamental plant [2, 13]. Furthermore, the sap extracted from this tree, known as Hwangchil lacquer, contains benzoic acid and can be used as a sedative [2], and its leaves, stems, roots, and seeds are used in traditional medicine to treat skin diseases, migraines, dysmenorrhea, muscle pain, and infectious diseases [12, 18]. The antioxidant and anticancer activities of stem and leaf extracts [10], and the antidiabetic, anticomplement, anti-

atherogenic, hepatoprotective, and antiinflammatory activities [3, 4, 6, 11, 17] of leaf extracts of *D. morbifera* have been reported. Furthermore, the essential oil extracted from the flowers of this tree has been reported to have larvicidal effects against *Aedes aegypti*, the mosquito that acts as a vector of dengue fever [5].

The various biological activities of *D. morbifera* extracts suggest that this tree could be a valuable source of medicinal compounds for the treatment of various diseases. Nevertheless, previous reports have mainly concentrated on crude aqueous or alcoholic extracts [3, 10, 14]. Some compounds have also been identified, including an antiinflammatory triterpenoid [20], an anticomplement polyacetylene [18], and antiinflammatory phenolics [11]. However, there is little information on the composition and biological activity of the volatile fraction of *D. morbifera*, although it has been reported that the essential oil from its leaves has antiatherogenic effects [4]. Essential oils are of interest to the food and pharmaceutical industries because of their aroma and biological activities. Therefore, in this study, volatile extracts from stems, leaves, and sap of *D. morbifera* were obtained using solvent and supercritical fluid extraction (SFE), and analyzed to elucidate their chemical composition and identify poten-

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tial compounds of interest. Furthermore, the antiproliferative activities of the solvent extracts of leaves and stems were evaluated using human hepatocyte carcinoma cells.

## Materials and Methods

### Plant material

Dried leaves, stems, and sap of *D. morbifera* were obtained from Nowha Agricultural Cooperative Association (Wandogun City, Jeollanam-do, Korea) in April 2015.

### Reagents

Fetal bovine serum (FBS), minimal essential medium (MEM), and other cell culture reagents were obtained from Gibco BRL (Grand Island, NY, USA). The lactate dehydrogenase (LDH) assay kit was from BioVision (Mountain View, CA, USA). Earle's basal salt solution (EBSS), trypsin solution, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], Triton X-100, t-BHP, CCl<sub>4</sub>, silymarin, and all other reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

### Preparation of solvent extracts of *D. morbifera*

Ground leaves and stems of *D. morbifera* were extracted 10-fold (w/v) with 70% ethanol at room temperature for 24 hr. After filtering, the extracts were concentrated in a rotary vacuum evaporator at 55°C and freeze-dried. Sap was extracted in the same way, but was sonicated before filtering. The crude extracts were successively partitioned in hexane: water (1:1), and the hexane fractions were evaporated. Prior to GC/MS analysis, the concentrate was dissolved in hexane at a concentration of 300 µg/ml.

### Supercritical fluid extraction of leaves and stems from *D. morbifera*

A Waters AD-RC08 apparatus (Milford, MA, USA) was used to perform the SFE of leaf and stem samples. The supercritical fluid was CO<sub>2</sub>, and ethanol (96% purity) was used as a co-solvent. Briefly, *D. morbifera* leaves or stems were placed in the extraction vessel of the SFE equipment and extraction was performed statically at a total flow rate of 50 ml/min (CO<sub>2</sub>: ethanol flow rate = 47.5:2.5 ml/min), pressure of 40 MPa, and temperature of 50°C for 120 min. The extracts were stored in the dark at 4°C.

### GC/MS analysis

A Shimadzu QP2010 Plus gas chromatograph/mass spectrometer (Kyoto, Japan) fitted with a DB-5 column (0.25-mm i.d. ×30-m length, 0.25-µm film thickness; J&W Scientific, Folsom, CA, USA) was used for the analysis. Helium was used as a carrier gas, pumped at a flow rate of 1.0 ml/min, and a split ratio of 10:1 was used. The oven temperature was maintained at 40°C for 10 min, then increased to 220°C at a rate of 4°C/min, and held at 220°C for 5 min. The temperatures of the injector, ion source, and interface were 280, 250, and 280°C, respectively. Total ion current (TIC) chromatograms were recorded in a range of 60-600 *m/z*. Compounds were identified by comparison of their mass spectra with those in the NIST and WILEY libraries of the GC-MS system, as well as those available in the literature.

### Cell culture

HepG2 cells (KCLB No. 88065) were purchased from the Korean Cell Line Bank and cultured in MEM (Gibco BRL) with 10% heat-inactivated FBS (Gibco BRL) and 1% streptomycin/penicillin at 37°C in a humidified incubator with an atmosphere of 5% CO<sub>2</sub> in air.

### Determination of cell viability

Cell viabilities were determined using the MTT assay and by measuring LDH leakage [15] in supernatants, using LDH kits (BioVision). MTT and LDH assays were both performed according to the manufacturer's instructions. HepG2 cells were seeded at a density of 2×10<sup>4</sup> cells/well in 96-well microplates. The next day, cells were pretreated with various leaf and stem solvent extract concentrations (0, 10, 100, 500, and 1,000 µg/ml) at 37°C for 24 hr.

### Statistical analysis

For cell viability, the assays were performed in triplicate. Data are expressed as the mean ± standard error of the mean (SEM). Significant differences were assessed using the Student's *t* test for each paired experiment; the level of significance was set at *p*<0.05.

## Results and Discussion

### Composition of leaf extracts

The composition of the solvent and SFE extracts of leaves is summarized in Table 1. The compounds are listed in their order of elution from the DB-5 column. The yield of ex-

Table 1. Volatile compounds in solvent and SFE extracts of leaves

Compound	RT (min)	Peak area (%)	
		Solvent	SFE
Ethyl propanoate	3.333	15.38	
Propyl acetate	3.400	3.52	
<i>m</i> -Ethyltoluene	15.933	1.80	
<i>o</i> -Ethyltoluene	16.050	1.42	
Pseudocumene	17.742	5.30	
$\gamma$ -Muuroolene	36.650		10.01
$\alpha$ -Amorphene	37.208		2.31
$\alpha$ -Selinene	37.317		4.23
$\alpha$ -Muuroolene	37.408		4.03
$\gamma$ -Cadinene	37.842		10.24
$\delta$ -Cadinene	37.950		18.53
Fonenol	41.108		38.03
Cubenol	41.267		3.70
Calarene	41.683		3.07
$\alpha$ -Cadinol	42.058		5.85

traction was low, being 0.51% for solvent extraction and 0.018% for SFE. Ten compounds were detected in both the solvent and SFE extracts of leaves; however, the chemical profiles obtained using these techniques were different and no common compounds were present.

In the solvent extract, esters predominated, with ethyl propanoate and propyl acetate representing almost 19% of the total peak area. Both esters have fruity odorous notes, with ethyl acetate being described as having a pineapple smell [1]. Aromatic hydrocarbons were also present in the solvent extract, with pseudocumene representing approximately 5% of the total peak area.

By contrast, the SFE extract of leaves was composed of sesquiterpenoids, with fonenol (38.03%),  $\delta$ -cadinene (18.53%),  $\gamma$ -cadinene (10.24%), and  $\gamma$ -muuroolene (10.01%) having the highest concentrations as % of peak area. Sesquiterpenoids are known for their herbal and spicy odorous notes and biological activities [9, 16, 22]. Terpenoids are also an important component in the essential oil of other medicinal Araliaceae species including *Panax ginseng*, *Schefflera* spp., and *Eleutherococcus* spp. [8, 19]. The alpha isomer of selinene was present in the SFE extract of *D. moribifera* leaves, whereas the beta isomer of this compound was reported to be present in the flowers, whose essential oil has larvicidal properties [5].

#### Composition of stem extracts

The composition of the solvent and SFE extracts of stems is summarized in Table 2. The compounds are listed in their

order of elution from the DB-5 column. The yield of extraction was low, being 0.41% for solvent and 0.19% for SFE. Twenty compounds were detected in the solvent extract and 10 compounds were detected in the SFE extract.

Monoterpenoids predominated in the solvent extract, representing more than 97% of the total peak area. Menthol (37.74%), *p*-menthone (16.55%), isomenthone (8.60%), and limonene (7.58%) were the compounds with the highest concentrations. In addition, two sesquiterpenoids (*trans*-caryophyllene and germacrene D) and an alcohol (3-octanol) were found, although at low concentrations. Menthol, *p*-menthone, and isomenthone have mint-like aromas, whereas limonene is described as citrus and minty. *Trans*-caryophyllene and germacrene D have woody and spicy notes [1].

The SFE extract contained mainly esters and aromatic hydrocarbons, which were not present in the solvent extract.

Table 2. Volatile compounds in solvent and SFE extracts of stems

Compound	RT (min)	Peak area (%)	
		Solvent	SFE
Ethyl propanoate	3.325		46.10
Propyl acetate	3.400		9.35
$\alpha$ -Pinene	14.167	2.02	
<i>m</i> -Ethyltoluene	15.925		6.01
<i>o</i> -Ethyltoluene	16.058		4.69
Hemellitol	16.425		3.35
Sabinene	16.617	1.54	
$\gamma$ -Pinene	16.767	2.25	
1-Ethyl-4-methylbenzene	16.867		2.34
$\gamma$ -Myrcene	17.725	1.11	
Pseudocumene	17.733		16.64
3-Octanol	18.233	0.32	
Cumene	19.108		3.53
Limonene	19.625	7.58	
Eucalyptol	19.800	0.66	
Isopregol	25.050	1.85	
<i>p</i> -Menthone	25.367	16.55	
Isomenthone	25.750	8.60	
Neomenthol	25.958	5.24	
Isopulegone	26.142	0.31	
Menthol	26.283	37.74	
Neoisomenthol	26.658	0.75	
$\alpha$ -Terpineol	27.008	0.42	
<i>cis</i> -Isopulegone	28.533	4.57	
Piperitone	29.167	1.31	
Menthol acetate	30.483	5.46	
<i>trans</i> -Caryophyllene	34.848	1.05	3.92
Germacrene D	36.825	0.67	
Caryophyllene oxide	39.933		4.07

Table 3. Volatile compounds in solvent extract of sap

Compound	RT (min)	Peak area (%)
$\alpha$ -Pinene	14.200	0.82
$\gamma$ -Myrcene	17.733	2.88
Limonene	19.642	96.30

Ethyl propanoate and ethyl acetate represented more than half of the total peak area of the SFE extract. Both esters have fruity smells, with ethyl acetate being described as pineapple-like [1]. Only one sesquiterpenoid (caryophyllene oxide) was detected in the SFE extract of stems. Furthermore, only *trans*-caryophyllene was detected in both the solvent and SFE extracts with concentrations of 1.05% and 3.92%, respectively.

### Volatile compounds in sap

Three monoterpenoids were detected, namely  $\alpha$ -pinene,  $\beta$ -myrcene, and limonene. Limonene represented 96.30% of the peak area. Limonene has a citrus, minty odor, whereas  $\alpha$ -pinene has a pine-like smell, and  $\beta$ -myrcene has a balsamic aroma [1]. The volatile profile of the sap reported here differed from that reported in a previous study, in which  $\alpha$ - and  $\beta$ -selinene, germacrene D,  $\delta$ -cadinene and  $\beta$ -elemene were found [2]. Nevertheless, we detected  $\alpha$ -selinene and  $\delta$ -cadinene in leaves and germacrene D in stems of *D. moribifera*. This result suggests that the composition of sap may differ according to the individual trees, region, and

season.

The most abundant volatile in sap, limonene, was also one of the major compounds in the solvent extract of *D. moribifera* stems, and has been reported to have antimicrobial and anticancer activities [7, 21].

### Antiproliferative activity of solvent extracts

A previous study evaluated the antioxidant and anticancer activities of *D. moribifera*, and reported that methanol extracts of stems and leaves exhibited high antioxidant and anticancer activities, which were associated to the presence of phenolic compounds [10]. Another study evaluated the immune activation activity of ethanol and aqueous extracts of *D. moribifera* leaves [14]. Ethanol is less toxic and easier to dispose of than methanol; thus, in this study we prepared solvent extracts of the leaves and stems of *D. moribifera*, using ethanol and hexane, and evaluated their antiproliferative activities on liver cancer cells.

To test the effects of the crude solvent extracts on the cell viability of HepG2 liver cancer cells, an MTT assay was performed. HepG2 cells were cultured in medium containing 10% FBS with or without the extracts for 24 hr. As a result, a dose-dependent decrease in cell proliferation was observed when the cells were treated with the solvent extracts (0, 10, 100, 200 and 400  $\mu$ g/ml) (Fig. 1). The antiproliferative effect of the leaf extract on HepG2 cells was substantially stronger than that of the stem extract. The  $IC_{50}$

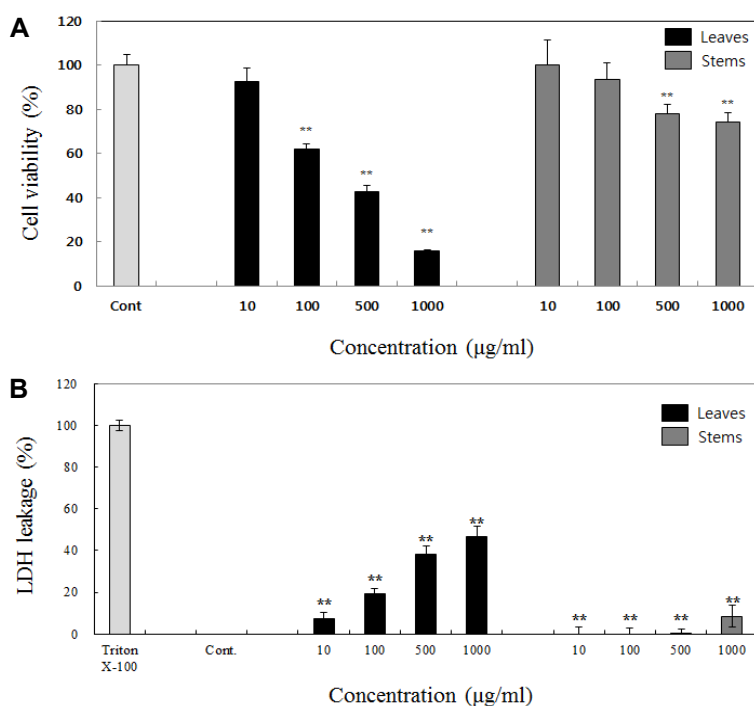


Fig. 1. Antiproliferative effect of *D. moribifera* solvent extracts of leaves and stems on HepG2 cells. The cell death (A) and LDH leakage (B) in HepG2 cells were measured by the MTT assay and LDH assay. Values shown are means  $\pm$  SEM of three independent experiments, and the error bars represent the standard deviation; \* $p$ <0.05, \*\* $p$ <0.01.

value of the leaf extract on HepG2 cells was 345.48 µg/ml, and that of the stem extract was 1,136.30 µg/ml. Furthermore, pretreatment with leaf extract effectively increased the leakage of LDH from HepG2 cells (Fig. 1).

Hyun et al. [10] evaluated the antioxidant activity and anticancer potential of methanol extracts of five *D. morbifera* parts against various tumor cell lines (COLO-205, HOS, SNU-245, -308, and Huh-BAT, -7), and their results suggested that the extracts of debarked stems, green leaves, and yellow leaves were a potent source of anticancer compounds, particularly against Huh-7 cells. Hyun et al. [10] also indicated that rutin and rosmarinic acid were the major bioactive compounds in leaves and debarked stems, respectively. However, in contrast with their results, the solvent extract of stems used in our experiment exhibited only a slight anticancer activity. A possible explanation for this outcome could be the difference in the solvent and cancer cell lines used. Nevertheless, the solvent extract of leaves analyzed here exhibited a high antiproliferative activity against HepG2 cells, which agreed with its reported bioactivity.

In conclusion, the volatile compounds of leaves, stems, and sap of *D. morbifera* were extracted using solvent and SFE and analyzed by GC/MS, revealing that terpenoids were the predominant compounds in the extracts. Evaluation of the antiproliferative activity showed that the solvent extracts of leaves had a stronger antiproliferative effect against HepG2 cells than that of the stem extracts. This result suggests that *D. morbifera* can be a source of anticancer compounds, and further analysis of the leaf extracts using HPLC is planned for identifying their bioactive compounds.

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### 초록 : 황칠나무의 휘발성 화합물 분석 및 HepG2 세포의 증식 억제 효과

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황칠나무(*Dendropanax morbifera* Lev.)는 황금빛의 수액과 약리효과로 한국에서 알려져 있으며, 잎 및 줄기 추출물의 다양한 효능은 피부질환, 편두통, 월경통, 근육통 및 전염성 질환 등의 질병을 개선하는 약리 성분을 공급하는 우수한 공급원이 될 수 있는 것을 시사한다. 그러나, 황칠나무 추출물의 효능에 관해서는 다양한 연구가 보고되어 있으나, 부위별 휘발성 성분의 조성에 대한 보고는 전무한 상황이다. 따라서, 본 연구는 황칠나무의 잎, 줄기 및 수액의 주요 휘발성 성분을 규명하기 위하여, 유기용매 및 초임계유체추출법을 이용한 추출물을 가스크로마토그래피-질량분석법으로 분석하였다. 잎 추출물에서는 15가지 화합물이 검출되었으며, 줄기 및 수액에는 각각 29가지 및 3가지 성분이 확인되었다. 또한 용매와 초임계유체추출법을 사용하여 얻은 휘발성 성분의 프로파일은 다르게 나타났다. 잎의 용매 추출물과 줄기의 초임계유체 추출물에서는 에스테르와 방향족 탄화수소가, 잎의 초임계유체 추출물 및 줄기의 초임계유체 추출물에는 테르페노이드가 주요 성분으로 나타났다. 한편, limonene (96.3%),  $\alpha$ -pinene, 그리고  $\beta$ -myrcene은 수액 용매 추출물의 휘발성 성분으로 확인되었다. 잎 및 줄기의 용매 추출물의 암세포 증식 억제 효과를 평가한 결과, 잎의 용매 추출물이 HepG2 세포의 증식을 유의적으로 감소시키는 것으로 나타났다.