

# Antioxidant Activity and Cytotoxic Effect of *Ventilago denticulata* Willd Leaves Extracts

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**Background:** Oxidative stress is characterized by an imbalance between the antioxidant defense systems and the formation of reactive oxygen species (ROS). The excess of ROS can damage biomolecules and leading to several chronic conditions and diseases such as diabetes, atherosclerosis, ischemic injury, inflammation and carcinogenesis. Plant extracts and their constituents as a natural source of antioxidants have been extensively studied.

**Objective:** The study aimed to investigate the antioxidant and cytotoxicity of aqueous and ethanolic Rhang Dang (*Ventilago denticulata* Willd) leaves extract.

**Material and Method:** The aqueous and ethanolic extracts of Rhang Dang leaves were preliminary analyzed for their phenolic profile (total phenolics and total flavonoids). These extracts were evaluated for their antioxidant properties by different methods such as DPPH radical scavenging and peroxy radical scavenging activity generated by AAPH (2,2'-Azobis (2-methylpropionamide) dihydrochloride). Their cytotoxic effects on hepatocellular carcinoma cell line (HepG2) and peripheral blood mononuclear cells (PBMC) were determined by MTT assay. Anti-hemolytic activity was examined using spectrophotometrical method.

**Results:** The ethanolic extract from Rhang Dang leaves exhibited a strong antioxidant activity and prevented hemolysis. It showed the highest amount of phenolics ( $91.03 \pm 12.43$  mg of gallic acid equivalents/g extract) and flavonoid compound ( $69.76 \pm 10.84$  mg of catechin equivalents/g). Interestingly, this extract was more cytotoxic to HepG2 cells than PBMC.

**Conclusion:** The ethanolic extract from Rhang Dang leaves had strong antioxidant activity and cytotoxic effect on cancer cells.

**Keywords:** Antioxidant, Cytotoxicity, Reactive oxygen species, Free radical scavenging

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Oxidative stress is characterized by an imbalance between the antioxidant defense systems and the formation of reactive oxygen species (ROS)<sup>(1)</sup>. The excess of ROS can damage biomolecules and leading to several chronic conditions and diseases such as diabetes, atherosclerosis, ischemic injury, inflammation and carcinogenesis<sup>(2)</sup>.

Antioxidants are compounds that inhibit or delay oxidation of other molecules by inhibiting both initiation and propagation of oxidizing chain reactions. They protect organisms against radicals and are vital in neutralizing the destruction caused by radicals. Nowadays, there is an increasing interest in the biochemical functions of natural antioxidant extracts

from fruits, vegetable and medicinal plants, which contain a wide variety of bioactive compounds such as phenolic acids, flavonoids and tannins which possess antioxidant property<sup>(3,4)</sup>.

Rhang Dang (*Ventilago denticulata* Willd) which belongs to the family Rhamnaceae. It is found extensively as tropical evergreen in Thailand. The plant is rich in many pharmaceutical active ingredients. The stem bark contains friedel in and several anthraquinones that can be applied to treat skin diseases and sprains. The root contains anthraquinones, ventinones A and B, used for a tonic dyspepsia, mild fever and debility. The leaves give lupeol, beta-sitosterol and its glucoside<sup>(5)</sup>. The ethanolic extract of plant also shows anti-inflammation and anti-microbial activity<sup>(6)</sup>. Rhang Dang leaves are often used as tea products. Frequently drunk, it can help to diuretic cure, reduce cholesterol and blood sugar, serve as a relaxant, strengthen health, arthritis, reduce blood pressure and

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diet. The present study investigated the antioxidant and cytotoxicity effect of aqueous and ethanolic extract from Rhang Dang leaves.

## Material and Method

### Chemicals

Folin-Ciocalteu's phenol reagent, gallic acid (GA), 2,2-Diphenyl-1-picrylhydrazyl, AAPH (2,2'-Azobis (2-methylpropionamide) dihydrochloride) and ascorbic acid were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). All other chemicals and reagents used were of Anala R grade. Leaves of *Ventilago denticulata* Willd were collected in Chiang Rai Province.

### Preparation of plant extract

The dried powder of Rhang Dang leaves (100 g) were mixed with 1,000 ml of distilled water and ethanol (80% v/v) at room temperature for 24 hour. The supernatant was collected by filtration through filter paper No. 1. Activated charcoal was added to the filtrate for 10 minute, passed through a filter paper, and centrifuged. The supernatant was collected and concentrated by using rotary evaporator further lyophilized.

### Colorimetric determination of total phenolics and total flavonoid content

Amount of total phenolics compound in the extracts were determined by using the Folin-Ciocalteu method with slight modification<sup>(7)</sup>. Briefly, 125 µl of crude extract were mixed with Folin-Ciocalteu reagent (125 µl), deionized water (2.0 ml) and 7% Na<sub>2</sub>CO<sub>3</sub> (1.25 µl). The mixture was incubated at room temperature for 30 minutes and measured optical density (OD) at 765 nm. The phenolic content was determined from a standard curve of gallic acid and expressed as milligrams of gallic acid equivalent per gram (mg GAE/g) of the extracts.

The total flavonoid compounds in each extract were determined according to Zhishen et al<sup>(8)</sup>. An aliquot (0.1 ml) of extract was added to 0.3 ml 5% (w/v) NaNO<sub>2</sub> and incubated for 5 minute. 0.3 ml 10% (w/v) AlCl<sub>3</sub> and 2 ml 1 N NaOH was added and the total volume was made up to 5 ml with distilled water. The absorbance was measured at 510 nm by using visible spectrophotometer. The results were expressed as mg catechin equivalents/g extract.

### Determination of antioxidant capacity

The antioxidant activity (free radical

scavenging activity) of the extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method developed by Wang et al<sup>(9)</sup>. In this experiment, 2.0 mg of each extract was dissolved in methanol. A solution of varying concentrations was obtained by a serial dilution technique. An aliquot of 2 ml of the extract in methanol was mixed with 3 ml of a DPPH-methanol solution (20 µg/ml) and was allowed to stand for 20 min for the reaction to occur. The absorbance was determined at 517 nm and from these values the corresponding percentage of inhibitions were calculated by using the following equation:

$$\% \text{ inhibition} = [1 - (\text{ABS}_{\text{sample}} / \text{ABS}_{\text{control}})] \times 100$$

Then % inhibitions were plotted against concentrations. The IC<sub>50</sub> was calculated by using ascorbic acid as a positive control. The experiment was carried out in triplicate and the Mean ± SD.

### Anti-hemolytic effect

The hemolysis in red blood cell (RBC) suspensions were induced by peroxy radicals generated by AAPH (50 mM) and the anti-hemolytic effect of different concentrations of Rhang Dang leaves extract (0.05-0.5 mg/mL) were evaluated according to Zou et al<sup>(10)</sup>. Scavenging of peroxy radical was measured by spectrophotometric method at 37°C. Absorbance of the reaction mixtures at 540 nm (absorbance of hemoglobin) was continuously monitored for 4 hour and percent of hemolysis was calculated. Anti-hemolysis effect Rhang Dang leaves extract was compared with quercetin.

### Cytotoxicity test

#### Cell culture

HepG2 cells (human hepatocellular carcinoma) cell line was obtained from Medical Molecular Biology Research Unit of National center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. Cells were cultured in DMEM medium containing 10% (v/v) FBS, 2 mmol/l L-glutamine and 1.0% penicillin-streptomycin at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

PBMC obtained from healthy volunteers were collected by venipuncture, transferred to 10 ml-heparin coated tubes, diluted with PBS (1:1, v/v), over-layered on to Histopaque®-1077 at a volume ration of 3:1, and centrifuged at 1,000 rpm for 30 minute. After centrifugation the PBMC were removed from the plasma and suspended in the density-gradient solution to

be isolated from erythrocytes and granulocytes. The PBMC layer was removed and washed twice with PBS. The supernatant was discarded and the cells were resuspended in RPMI 1640 culture medium supplemented with 10% FBS, 100 U/ml of penicillin, 0.1 mg/ml of streptomycin and an appropriate amount of sodium bicarbonate.

#### Cell viability assay

The HepG2 cells ( $3 \times 10^3$  cells/well) and PBMC ( $100 \times 10^3$  cells/well) were added to 96-well culture plate and treated with the extracts (0-400  $\mu\text{g/ml}$ ) at  $37^\circ\text{C}$  for 24 hour. Then, the cells were added to a serum-free medium (100  $\mu\text{l}$ /well). Finally, the treated cells determined their viability using the MTT method. The MTT solution (15  $\mu\text{l}$ ) was added to the cells and incubated at  $37^\circ\text{C}$  for 4 hour. The resulting purple formazan product was extracted with DMSO solution (100  $\mu\text{l}$ ) and OD of the solution was read at 540/630 nm using a microplate reader. The number of viable cells was calculated from the untreated cells and the data were expressed as % cell viability<sup>(11)</sup>.

#### Statistical analysis

The results were expressed as Mean  $\pm$  SD.

### Results

#### Total phenolics content and total flavonoid content

Total phenolics contents and total flavonoid content of the extracts are present in Table 1. Total phenolic content of aqueous and ethanolic extract of Rhang Dang leaves were  $21.88 \pm 1.9$  and  $91.03 \pm 12.43$  mg of gallic acid equivalents/g extract, respectively, and the corresponding flavonoid contents were  $16.25 \pm 4.95$  and  $69.76 \pm 10.84$  mg of catechin equivalents/g aqueous and ethanolic extract.

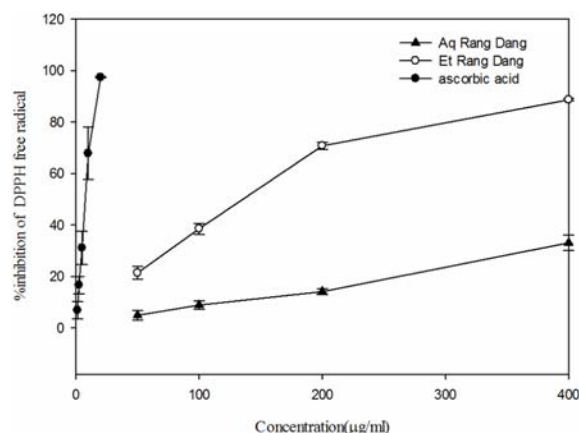
#### Antioxidant activity

Many methods have been used to determine the antioxidant activity of natural products<sup>(12)</sup>. The DPPH is one of the methods widely used to assess antioxidant activity and we used this method to screen

the antioxidant activity. Antioxidant activity measured in the ethanol and aqueous extracts of Rhang Dang leaves compares to vitamin C. The results are show in Fig. 1. The percent of DPPH free radical scavenging activity was increased in a concentration-dependent manner. The  $\text{IC}_{50}$  values (the concentration causing 50% free radical inhibition) of ethanolic and aqueous extract for DPPH radical scavenging activity were  $0.16 \pm 7.6$  and  $0.62 \pm 4.86$  mg/ml, respectively, which showed a marked difference with ascorbic acid standard ( $\text{IC}_{50} = 10.14 \pm 0.78$   $\mu\text{g/ml}$ ).

#### Inhibitory effect of RBC hemolysis

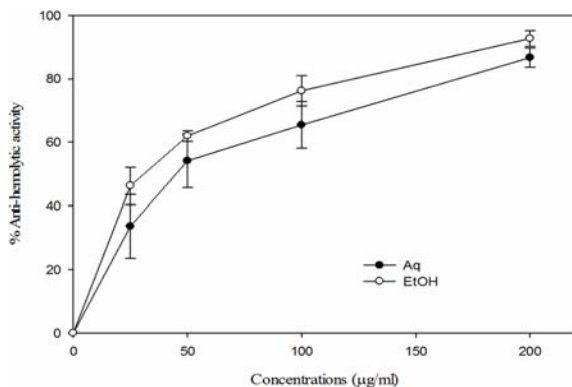
Scavenging of peroxy radicals by different concentrations of Rhang Dang leaves extract were evaluated by its inhibitory effect on RBC hemolysis induced by peroxy radicals. The results showed inhibitory effect of Rhang Dang leaves extracts on AAPH-induced RBC hemolysis in concentration-dependent manner. The extracts of Rhang Dang leaves



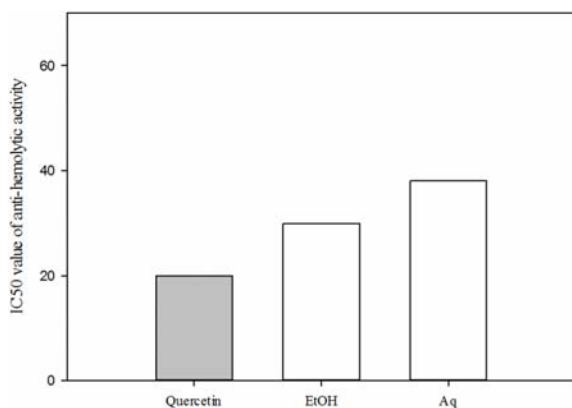
**Fig. 1** DPPH free radical scavenging activity of the aqueous and ethanolic extract from Rhang Dang (*V. denticulata* Willd) leaves compared to ascorbic acid. Data were obtained from triplicate results of three independent experiments and shown as Mean  $\pm$  SD.

**Table 1.** Total phenolic and flavonoid content of Rhang Dang leaves

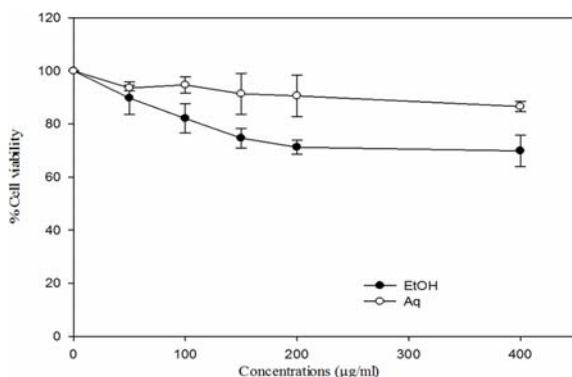
Sample	Total phenolic content mg GAE/g extract	Total flavonoid content mg C/g extract
Ethanol extract of Rhang Dang leaves	$91.03 \pm 12.43$	$69.76 \pm 10.84$
Aqueous extract of Rhang Dang leaves	$21.88 \pm 1.9$	$16.25 \pm 4.95$



**Fig. 2** Anti-hemolytic activity of aqueous (Aq) and ethanolic (EtOH) extracts from Rhang Dang leaves. Data were expressed as Mean  $\pm$  SD.

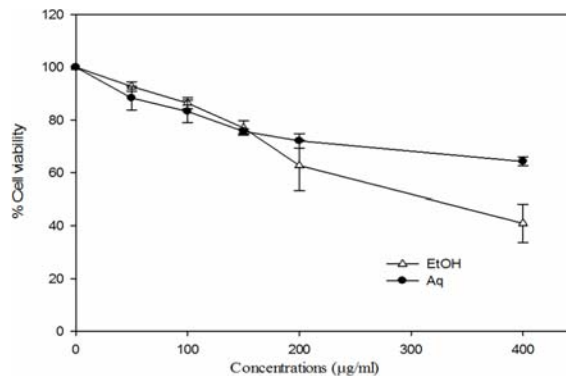


**Fig. 3** Anti-hemolytic activity of aqueous (Aq) and ethanolic (EtOH) extracts from Rhang Dang leaves shown in term of inhibitory concentration at 50% (IC<sub>50</sub>).



**Fig. 4** Cytotoxic effect of aqueous (Aq) and ethanolic (EtOH) extract from Rhang Dang leaves on PBMC. Data were expressed as Mean  $\pm$  SD.

could inhibit the hemolysis of red blood cells in a dose-dependent manner. In comparison, the ethanolic



**Fig. 5** Cytotoxic effect of aqueous (Aq) and ethanolic (EtOH) extract from Rhang Dang leaves on HepG2. Data were expressed as Mean  $\pm$  SD.

extract was a bit more effective in the inhibition than the aqueous extract. Inhibitory concentration at 50% hemolysis (IC<sub>50</sub>) was determined and shown in Fig. 2, 3. Considerably, the ethanolic extract from Rhang Dang leaves showed the IC<sub>50</sub> value of 16 µg/ml, which was the lowest concentration that showed the most protective effect against the AAPH-induced RBC hemolysis.

#### Cytotoxicity on PBMC and HepG2

The results of the toxicity of the extracts against PBMC and HepG2 cell are shown in Fig. 4, 5. None of the extracts from the Rhang Dang leaves behaved as a toxic to the PBMC culture even high concentrations up to 400 µg/ml. Higher concentration of the ethanolic extract from Rhang Dang leaves significantly decreased the viability of HepG2 cell (Fig. 5). The IC<sub>50</sub> of the ethanolic extract in HepG2 cell after a 24-hour period was 300 µg/ml.

#### Discussion

Plant phenolics and flavonoids are considered as potent free radical scavengers. The moderate concentration of total phenolics and flavonoids in Rhang Dang leaves indicated a notable antioxidant activity. Many studies strongly suggest that amount of polyphenol content should be considered as an important feature of herbal drugs. Some of its pharmacological effects like antioxidant, anti-inflammatory, anticancer and diuretic activity can be attributed to the presence of these valuable constituents of many<sup>(13,14)</sup>. Leaves of *V. denticulate* willd have been report to contain numerous active substances, especially polyphenolics such as flavonoids, lupeol,  $\beta$ -sitosterol and glucoside. The ethanolic extracts of *V.*

denticulate willd leaves have strong anthelmintic<sup>(15)</sup> and antifungal activity<sup>(6)</sup>.

The present study demonstrated the pharmacological potential of Rhang Dang (*V. denticulate willd*) leaves extracts. The extract contains phenolic and flavonoid substances clustered as antioxidant compounds. The antioxidant activity of the Rhang Dang leaves extract was evaluated through its ability to quench the synthetic DPPH radical. The ethanolic leaf extracts possessed very good reductive ability and it showed an increment with increase in concentration of extracts, which indicated its potent antioxidant capability. It may predict that the anti-oxidative capacity would depend on the phenolic constituents or the persisting flavonoid, or both.

In red blood cell hemolysis assays, AAPH is used as a peroxy-radical initiator that, by its thermal decomposition, generates free radicals that attack the erythrocytes to induce chain oxidation of lipid and protein, disturbing membrane organization and eventually leading to hemolysis<sup>(16)</sup>. Many studies have focused on the free-radical-initiated peroxidation of membrane lipid, associated with a variety of pathological events. Both natural and synthetic antioxidants have been used to trap peroxy radicals and other radicals to protect the membrane lipids against free radical chain reactions<sup>(17)</sup>. Hence, the AAPH-induced hemolysis provides a good approach to determine the free-radical-induced membrane damage. Anti-hemolytic effects of Rhang Dang leaves extract indicate higher activity of peroxy radical scavenging. The results showed the protective effect of Rhang Dang leaves extract on the oxidative damage of cell membrane in a dose-dependent manner. Interestingly, the ethanolic extract of Rhang Dang leaves has strong inhibiting hemolysis (IC<sub>50</sub>) with a concentration of 30 µg/ml.

The highest antioxidant and cytotoxic activity were found on ethanol extract. Ethanol has been known to be more effective in dissolving active compounds in cells. Hence, it was easier to penetrate the cellular membrane to extract the intracellular ingredients from plant materials. Phenolics and flavonoids mostly found in plants are reported to have numerous biological effects including antioxidant, anti-neovascularization, antiproliferation and anticarcinogenic properties and are therefore considered for their important dietary roles as antioxidants and chemoprotective agents<sup>(18)</sup>. Recently, intensive research has been focused on studying the naturally occurring phenolics and flavonoids that are able to decrease the generation of reactive oxygen species (ROS) in biological system<sup>(19)</sup>.

Oxidative stress contributed by ROS plays a critical role in the pathologies related with chronic disease such as cancer and excessive vascularization. ROS-induced development of cancer involves malignant transformation due to DNA mutations and altered gene expression through epigenetic mechanisms, which in turn leads to the uncontrolled proliferation of cancerous cells<sup>(20)</sup>. Further, high levels of ROS are observed in various cancerous cells and a number of accumulating evidences suggest that ROS function as key signaling molecules to stimulate various growth-related responses that eventually initiate angiogenesis and tumorigenesis. Several studies demonstrated a significant role of phenolics in growth inhibition of breast, colon, prostate, ovary, endometrium and lung cancer cells. The present study confirmed that the ethanolic extracts of Rhang Dang leaves demonstrated selective cytotoxicity towards Human hepatocellular carcinoma while being less cytotoxic against the normal cells (PBMC). Such selective cytotoxic activity suggested that the active substances interact with special cancer-associated receptors or cancer cell special molecule, thus triggering some mechanisms that cause cancer cell death.

It can be concluded that the ethanol extract of Rhang Dang (*V. denticulata willd*) leaves show good antioxidant activity. These activities might be due to the presence of phenolic and flavonoid compounds with various appreciable amounts. The cytotoxicity activity indicated that the ethanol extract of Rhang Dang leaves could have anti-proliferative effect in the hepatoma cancer cell line (HepG2). The antioxidant and cytotoxicity of Rhang Dang leaves may be useful in the treatment and prevention of a number of oxidative stress disorders, and the anti-proliferative properties of *V. denticulata willd* should be further elucidated.

#### **What is already known on this topic?**

Previous studies, Rhang Dang leaves extract have been studied of active ingredient pharmacological and activities, including anti-inflammation and anti-microbial activity, but the anti-oxidative and anti-proliferative activity remains unknown.

#### **What this study adds?**

The study investigated the antioxidant and cytotoxicity of aqueous and ethanolic Rhang Dang (*V. denticulata willd*) leaves extract by preliminary analysis for their phenolic profile (total phenolics and total flavonoids) and evaluation of their antioxidant properties by different methods such as DPPH radical

scavenging and peroxy radical scavenging activity generated by AAPH (2,2'-azobis (2-amidinopropane) dihydrochloride. The inhibitive effect on growth of hepatocarcinoma cancer cell lines (HepG2) was determined by MTT assay.

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#### Potential conflicts of interest

None.

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## การศึกษาฤทธิ์ในการต้านอนุมูลอิสระและความเป็นพิษต่อเซลล์ของสารสกัดจากใบรางแดง

อนุสรฯ พงศ์จันทา, กาญจนา แปงจิตต์, สมเดช ศรีชัยรัตนกุล

**ภูมิหลัง:** สภาวะเครียดในร่างกายเกิดจากการไม่สมดุลกันระหว่างอนุมูลอิสระและสารต้านอนุมูลอิสระที่ร่างกายสร้างขึ้นโดยสารอนุมูลอิสระที่สร้างมากเกินไปจะไปทำลายสารชีวโมเลกุลภายในร่างกายและเหนี่ยวนำให้เกิดโรคต่างๆ ตามมา เช่น โรคเบาหวาน, โรคหลอดเลือดอุดตัน, การอักเสบ และโรคมะเร็ง การศึกษาหาสารจากพืชธรรมชาติจึงเป็นแหล่งของสารต้านอนุมูลอิสระที่สำคัญ

**วัตถุประสงค์:** ศึกษาคุณสมบัติการต้านอนุมูลอิสระและความเป็นพิษต่อเซลล์ของสารสกัดใบรางแดงด้วยน้ำและเอทานอล

**วัสดุและวิธีการ:** นำสารสกัดหยาบใบรางแดงด้วยน้ำและเอทานอลมาทำการวิเคราะห์ปริมาณสารฟีนอลิกรวมและปริมาณฟลาโวนอยด์รวม จากนั้นทำการวิเคราะห์ฤทธิ์ในการต้านอนุมูลอิสระโดยวิธี DPPH และการจัดข้อมูลเปอร์ออกซิโดยใช้สาร AAPH วิเคราะห์ความเป็นพิษต่อเซลล์เม็ดเลือดขาวปกติ (PBMC) และเซลล์มะเร็งตับ (HepG2) ด้วยวิธี MTT

**ผลการศึกษา:** สารสกัดหยาบใบรางแดงด้วยเอทานอลมีความสามารถในการเป็นสารต้านอนุมูลอิสระที่สูง และป้องกันการแตกของเซลล์เม็ดเลือดแดง โดยการแสดงออกของค่าการยับยั้งที่ต่ำและยังมีปริมาณสารฟีนอลิกรวม และปริมาณฟลาโวนอยด์รวมในระดับที่สูง และเป็นที่น่าสนใจว่าสารสกัดจากใบรางแดงด้วยเอทานอล และสามารถยับยั้งการเจริญเติบโตของเซลล์มะเร็งตับ (HepG2) ในลักษณะที่ขึ้นกับความเข้มข้นของสารสกัด (0-400  $\mu\text{g/ml}$ ) ภายใน 24 ชั่วโมง

**สรุป:** สารสกัดหยาบใบรางแดงด้วยเอทานอลและมีฤทธิ์ในการต้านอนุมูลอิสระสูงและยังสามารถยับยั้ง การเจริญเติบโตของเซลล์มะเร็ง

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