In Vitro Free Radical Scavenging and Cell-Based Antioxidant Activities of Kheaw-Hom Remedy Extracts and Its Plant Ingredients

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Background: *The oxidative stress (OS) and antioxidants play a key role in the pathogenesis of inflammatory diseases such as fever which is promoted by the production of reactive oxygen species and impaired antioxidant defense mechanisms. The Kheaw-Hom remedy is popularly used as anti-pyretic drug in Thai traditional medicine.*
Objective: *To investigate antioxidant activity of the ethanolic and aqueous extracts of Kheaw-Hom remedy and its plant ingredients by three assays such as DPPH, ABTS radical scavenging assays and NBT dye reduction assay.*
Material and Method: *The extract procedures were maceration method with 95% ethanol, dried by an evaporator, or the decoction by boiling in water, filtrated, dried by lyophilizer. In the preliminary studies, all extracts were evaluated for antioxidant activity through two chemical assays: DPPH radical-scavenging and ABTS radical-scavenging assay, as well as through cell-based assay: scavenging capacity of intracellular ROS in HL-60 cells using the NBT reduction.*
Results: *The ethanolic extract of Khaew-Hom remedy showed higher antioxidant activity using DPPH and ABTS assays but it had no antioxidant activity using cell-based assay (EC_{50} = 16.96, 30.91 and IC_{50} >100 μg/mL, respectively). The ethanolic extract of Cyathea gigantea and Tacca chantrieri showed the highest antioxidant activity using DPPH assay with EC_{50} = 7.55 and 8.00 μg/mL, respectively. The ethanolic extract of Dracaena loureiri and Globba malaccensis exhibited the best antioxidant activity using ABTS radical scavenging with EC_{50} = 7.88 and 8.06 μg/mL, respectively. For the NBT dye reduction assay, only the ethanolic and aqueous extracts of Tacca chantrieri were effective having IC_{50} = 63.38 and 70.65 μg/mL, respectively.*
Conclusion: *The ethanolic of Khaew-Hom showed antioxidant activity only with chemical based assay but both ethanolic and aqueous extracts of Tacca chantrieri (rhizome) showed high antioxidant activities on chemical-based and cell line-based assay. Thus, this plant should be developed to be health products in the future.*

Keywords: *Antioxidant activities, Kheaw-Hom, DPPH, ABTS, NBT assay, HL-60*

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Free radicals are products from the metabolism of various substances in the liver. The stimuli of free radicals production are pollution, dust, cigarette smoke, unsaturated fat food, sunlight, heat, chemicals and medicine[1]. They can stimulate immune system to produce radical for eliminate the disguise. The downside of free radicals are, if there are too many free radicals, they destroy tissue proteins and cells, including white blood cells[2]. On the opposite side, benefit of free radicals is killing of antigens such as bacteria, virus or parasite and it implicate in inflammation[3,4]. Inflammation is immune exhibition and appears in many diseases such as fever, recurrent aphthous stomatitis[5]. For example, exanthematous fever, the cause of this fever could be viral, bacterial infection and allergic medicine[6]. The inflammatory condition can cause progessive disease. There is support evidence that ROS are essential second messengers in innate and adaptive immune cells and excessive of ROS within immune cells can result in

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hyperactivation of inflammatory responses[7]. So that, if ROS decreased, the inflammation would be reduced.

In Thai traditional medicine, there are many antipyretics, one of them is Kheaw-Hom remedy. It is antipyretic for exanthematous fever in Thai traditional medicine. It has been published in National List of Essential Herbal Medicines, Ministry of Public Health, 2013[8]. This remedy consists of eighteen Thai herbs shown in Table 1 with each herb in equal amount. Some herbs in remedy have been previously studied for antioxidant activity such as *Mimusops elengi* Linn[9], *Mesua ferrea* Linn[10], *Mammea siamensis* Kosterm[11], *Nelumbo nucifera* Gaertn[12], *Sophora exigua* Craib[13], *Vetiveria zizanioides* (L.) Nash ex Small[14], but Kheaw-Hom remedy has not been studied. So that, the objective of this study was to investigate the antioxidant activity of the ethanolic and aqueous extracts of Kheaw-Hom remedy and its plant ingredients.

Material and Method

Chemicals and reagents

95% ethanol (CMJ Anchor company, Thailand), Distilled water (Milford, USA), 2, 2-diphenyl-l-picrylhydrazyl (Fluka, Germany), Butylated hydroxytoluene (BHT) (Fluka, Germany), 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-ALDRICH, USA), 6-hydroxy-2, 5, 7, 8-tetramethyl chroman-2-carboxylic acid (Trolox) (Sigma-ALDRICH, USA), Potassium sulfate (Sigma-ALDRICH, USA), Dimethyl sulfoxide $(CH_3)_2SO$ (DMSO) (RCI Labscan, Thailand), Nitroblue tetrazolium chloride (NBT) (Sigma, USA), Phorbol myristate acetate (PMA) (Sigma, USA), Thiazolyl blue tetrazolium bromide (MTT) (Sigma, USA).

Cell lines and culture conditions

Human leukemia cell line HL-60 cell was purchased from ATCC. The cells were maintained by twice weekly passages in RPMI-1640 medium supplemented with 10% FBS, 1% Penicillin-Streptomycins and incubated at 37°C in a 5% CO_2.

Plant materials

The parts of 18 plants in Kheaw-Hom remedy were collected from several regions of Thailand in 2015, with voucher specimen numbers shown in Table 1. The voucher specimens were carried out at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

Preparation of crude extracts

All plants were washed, sliced to small pieces, dried in an oven at temperature 50°C and ground to powder and extracted by maceration with 95% ethanol and boiling in water as ethanolic and aqueous extract, respectively. The ethanolic extract was prepared by maceration of Kheaw-Hom remedy (1,170 grams), and where each of its herbal ingredients (65 grams of each) was macerated with 95% ethanol for 3 days. Filtrate was obtained using Whatman No. 1 filter paper and concentrated to dryness by an evaporator (Rotavapor R-205, Germany). The aqueous extract of Kheaw-Hom remedy (1,170 grams) and its herbal ingredients (65 grams of each) were prepared by boiling in distilled water. The duration of decoction was 15 min. The extracts were filtered through a Whatman No. 1 filter paper and dried by lyophilization. The crude extracts were kept at -20°C untill used.

Determination of antioxidant activity

DPPH radical scavenging assay[15]

The antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH). Sample was dissolved in absolute ethanol or distilled water in various concentrations including 100, 50, 10 and 1 µg/mL. 100 µL of samples were transferred into a 96-well microplate. Then 100 µl of 6×10^{-5} M DPPH (in absolute ethanol) was added into each well. After incubation for 30 min in the dark at room temperature, the absorbance was measured at 520 nm, where BHT was used as a positive control. The concentration of antioxidant needed to decrease the initial DPPH concentration (EC_{50}) by 50% is a parameter widely used to measure the antioxidant activity. The scavenging activity was calculated as percentage inhibition in the formulae below:

$$\% \text{ Inhibition} = ((\text{Mean of } OD_{Control} - \text{Mean of } OD_{sample})/\text{Mean of } OD_{Control}) \times 100$$

Effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50}) was obtained by linear regression analysis of the dose-response curve of % inhibition versus concentration, and EC_{50} is calculated using prism program. All determinations were carried out in triplicate.

ABTS radical scavenging assay[16]

The antioxidant activity was determined 2.45 mM $ABTS^{\bullet+}$ solution was prepared using potassium persulfate diluted with DI water to get the absorbance

Table 1. Plants and part of plant components in Kheaw-Hom remedy

Scientific Name	Family Name	Thai Name	Part used	Flavor	Voucher number	Ratio (%)	Thai traditional used
Angiopteris evecta (G. Forst.) Hoffm.	Marattiaceae	Wan keep rat	Rhizome	Flavorless	SKP110-10105 01	5.56	Reduce fever, use as astringent
Cordyline fruticosa (L) Goeppert. (Green leaves)	Asparagaceae	Mak mia	Leaf	Flavorless	SKP005030601	5.56	Reduce fever, treat exanthematous fever and itch
Cordyline fruticosa (L) Goeppert (red leaves)	Asparagaceae	Mak phu	Leaf	Flavorless	SKP005030601	5.56	Reduce fever, treat exanthematous fever and itch
Cyathea gigantean Holtt.	Cyatheaceae	Ma has sa dam	Stem	Cool	SKP059030701	5.56	Reduce fever and pain, treat cough
Dracaena loureiri Gagnep.	Dracaenaceae	Chan deang	Stem	Bitter	SKP065041201	5.56	Reduce fever, scurvy and abscess
Eupatorium stoechadosmum Hance	Compositae	San phra hom	Leaf	Cool& Flavorless	SKP051051901	5.56	Treat fever, use as astringent
Globba malaccensis Ridl.	Zingiberaceae	Wan ron thong	Rhizome	Hot& Fragrant	SKP206071301	5.56	Use as antiallergic, insect bites
Kaempferia galanga Linn	Zingiberaceae	Proh hom	Rhizome	Hot& Fragrant	SKP206110701	5.56	Treat cold, use as carminative
Kheaw-Hom	-	-	-	Bitter& Cool	-	100	Treat fever, measles, chickenpox and aphthous ulcers
Limnophila rugosa Merr	Scrophulariaceae	Phak krachom	Leaf	Cool& Fragrant	SKP177121801	5.56	Treat exanthematous fever
Mammea siamensis Kosterm.	Guttiferae	Sa ra phi	Flower	Cool& Fragrant	SKP083131901	5.56	Cardiac tonic, treat vertigo
Mesua ferrea Linn.	Guttiferae	Bun nak	Flower	Cool& Fragrant	SKP083130601	5.56	Cardiac tonic, treat vertigo
Mimusops elengi Linn.	Sapotaceae	Phi kul	Flower	Cool& Fragrant	SKP171130501	5.56	Cardiac tonic, blood tonic
Myristica fragrans Houtt	Myristicaceae	Chan thet	Stem	Hot& Fragrant	SKP121130601	5.56	Reduce fever, use as carmina carminative
Nelumbo nucifera Gaertn.	Nelumbonaceae	Bua luang	Pollen	Astringent& Fragrant	SKP125141401	5.56	Cardiac tonic, treat vertigo
Pogostemon cablin (Blanco) Benth.	Labiatae	Phim sen thon	Leaf	Cool& Fragrant	SKP095160301	5.56	Reduce fever, use as carminative
Sophora exigua Craib	Fabaceae	Phit sa nat	Trunk	Bitter	SKP072190501	5.56	Reduce fever, increase breast milk
Tacca chantrieri Andre	Taccaceae	Nae ra phu sri	Rhizome	Astringent	SKP189200301	5.56	Reduce fever, use as astringent
Vetiveria zizanioides (L.) Nash ex Small	Gramineae	Faek hom	Root	Cool& Fragrant	SKP081222601	5.56	Use as diuretic and carminative

of 0.68-0.72 at 734 nm before use. Each extract (20 µL) at the same concentration range mentioned above was mixed with $ABTS^{\bullet+}$ solution (180 µL) and incubated at room temperature for 6 min. The absorbance of these concentrations was measured at 734 nm. The percent of $ABTS^{\bullet+}$ scavenging activity in this concentration range was calculated, and EC_{50} (µg/mL) was determined using the method described above. Trolox was used as a positive control. Experiments were done in triplicate. The calculation of percent scavenging activity is by the following formula:

$$\% \text{ Inhihibition} = ((\text{Mean of } OD_{Control} - \text{Mean of } OD_{sample})/ \text{Mean of } OD_{Control}) \times 100$$

Effective concentration of sample required to inhibited ABTS radical by 50% (EC_{50}) was obtained by linear regression analysis of the dose-response curve of % inhibition versus concentration, and EC_{50} is calculated using prism program. All determinations were carried out in triplicate.

Intracellular superoxide anion scavenging assay (NBT assay)[17]

Phorbol 12-myristate 13-acetate (PMA) was used to stimulate differentiated HL-60 cells to generate superoxide anions ($O_2^{\bullet-}$) via respiratory burst, which then reduced the nitroblue tetrazolium (NBT) solution to blue formazan. Prior to performing the NBT assay, the optimal concentration of sample with no cytotoxic effects on HL-60 cells was determined using the MTT assay. In NBT assay, differentiated HL-60 cells ($1x10^6$ cells) in Hank's buffered salt solution (HBSS) (200 µl) was incubated with each sample (500 µl) at the optimal concentration for 15 min 37°C in a 5% CO_2 atmosphere. Next, the mixture was incubated for 60 min with a final concentration of 250 ng/ml PMA and 0.625 mg/ml NBT in HBSS. The reaction was stopped by adding 2 ml of 1 M HCl and centrifuged at 4,000 rpm for 10 min to collect cell pellet containing formazan, which was then dissolved in DMSO (300 µl). The absorbance was determined at 572 nm. The $O_2^{\bullet-}$ scavenging activity of the extract at the optimal concentration was calculated:

$$\% \text{ Inhibition} = \frac{(\text{Mean of } OD_{control} - \text{Mean of } OD_{baseline}) - (\text{Mean of } OD_{extract} - \text{Mean of } OD_{baseline})}{\text{Mean of } OD_{control} - \text{Mean of } OD_{baseline}}$$

Statistical analysis

All experiments were carried out in triplicate. Statistical analysis was performed using Prism Software.

Results

Antioxidant activity

The effect of the ethanolic and aqueous extracts of Kheaw-Hom remedy and each of its herbal components were studied using DPPH radical scavenging assay, ABTS radical scavenging assay and NBT assay. IC_{50} values are summarized in Table 2, 3.

Discussion

Kheaw-Hom remedy was used in Thai traditional medicine as antipyretic for exanthematous fever such as measles and chickenpox. Kheaw-Hom remedy was reported in the previous studies on several biological activities such as antiviral, anti-inflammatory and antimicrobial. Firstly, antiviral activity against enterovirus 71 (EV71) which cause hand, foot and mouth disease that its aqueous extract at concentration of 400 µg/ml inhibited EV71 concentrate 25TCID50[18]. Secondly, anti-inflammatory activity by inhibiting nitric oxide release in RAW 264.7 that the aqueous and ethanolic extracts had weak activity showed IC_{50} value of 48.86 and 59.77 µg/mL, respectively[18]. Lastly, the ethanolic extract had antimicrobial activity against three gram-positive bacteria of skin infection complications in exanthematous fever include *Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus* (MRSA), *Staphylococcus epidermis* with an inhibition zone of 7.33, 7.00, 8.00 mm, respectively; yet showed no inhibition on fungus (*Candida albicans*)[19]. This study is the first report on antioxidant activity of Kheaw-Hom remedy using DPPH and ABTS radical scavenging assays that the ethanolic extract had strong activity in DPPH radical scavenging assay and had weak activity in ABTS radical scavenging assay. The aqueous extract had weak activity in ABTS radical scavenging assay and had no activity on DPPH radical scavenging assay. All these results could support use of Kheaw-Hom remedy related with Thai traditional medicine used for exanthemathous fever.

There are nine plant ingredients in Kheaw-Hom remedy that were reported in the previous study on antioxidant activity using DPPH radical scavenging assay including *P. cablin, C. fruticosa, V. zizanioides, M. fragrans, G. malaccensis, C. gigantean, M. elengi, M. siamensis* and *N. nucifera.* Firstly, the previous study demonstrated the antioxidant activity using DPPH radical scavenging assay and the methanolic extract of *N. nucifera*[12] and vetiver oil of *V. zizanioides*[14] dissolved in methanol had strong activity and, this present study showed that the aqueous extract of *N. nucifera* had strong activity but,

Table 2. Antioxidant activities on DPPH scavenging assay, ABTS scavenging assay and NBT dye reduction assay of ethanolic extract of Kheaw-Hom remedy and its plant ingredients

Botanical name	Code	Antioxidant activity		
		DPPH scavenging assay (EC_{50} ± SEM) µg/mL	ABTS scavenging assay (EC_{50} ± SEM) µg/mL	NBT dye reduction assay (IC_{50} ± SEM) µg/mL
Angiopteris erect (G. Forst) Hoffm.	AEE	42.95±4.24	>100	>100
Cordyline fruticosa (L.) Goeppert. (green leaves)	CGE	>100	>100	>100
Cordyline fruticosa (L.) Goeppert. (red leaves)	CRE	47.55±4.448	>100	>100
Cyathea gigantea Holtt.	CyGE	7.55±0.893	20.09±2.960	>100
Dracaena loureiri Gagnep.	DLE	13.89±1.138	7.88±0.650	>100
Eupatorium stoechadosmum Hance	ESE	50.97±1.187	>100	>100
Globba malaccensis Ridl.	GME	61.29±4.982	8.06±0.53	>100
Kaempferia galanga Linn.	KGE	>100	>100	>100
Kheaw-Hom remedy	KHE	16.96±1.214	30.91±1.530	>100
Limnophila rugosa Merr.	LRE	>100	>100	>100
Mammea siamensis Kosterm	MSE	36.60±5.030	71.86±3.250	>100
Mesua ferrea L.	MeFE	37.40±1.954	63.15±4.033	>100
Mimusops elengi L.	MEE	53.89±0.645	>100	>100
Myristica fragrans Houtt	MFE	>100	>100	>100
Nelumbo nucifera Gaertn.	NUE	>100	>100	>100
Pogostemon cablin (Blanco)Benth.	PCE	90.90±5.029	>100	>100
Sophora exigua Craib	SEE	9.42±2.107	>100	>100
Tacca chantrieri Andre	TCE	8.00±2.368	14.84±0.48	63.38±3.290
Vetiveria zizanioides (L.) Nash ex Small	VZE	>100	>100	>100
BHT		13.40±0.266	-	-
Trolox		-	5.850±0.730	-
Propyl gallate			-	14.87±0.02

Table 3. Antioxidant activities on DPPH scavenging assay, ABTS scavenging assay and NBT dye reduction assay of aqueous extract of Kheaw-Hom remedy and its plant ingredients

Botanical name	Code	Antioxidant Activity		
		DPPH scavenging assay (EC_{50}±SEM) µg/mL	ABTS scavenging assay (EC_{50}±SEM) µg/mL	NBT dye reduction assay (IC_{50}±SEM) µg/mL
Angiopteris evecta (G.Forst) Hoffm.	AEW	>100	>100	>100
Cordyline fruticosa (L.) Goeppert. (green leaves)	CGW	>100	>100	>100
Cordyline fruticosa (L.) Goeppert. (red leaves)	CRW	>100	>100	>100
Cyathea gigantea Holtt.	CyGW	14.40±2.07	20.16±1.51	>100
Dracaena loureiri Gagnep.	DLW	10.00±0.88	15.31±4.09	>100
Eupatorium stoechadosmum Hance	ESW	>100	82.06±3.17	>100
Globba malaccensis Ridl.	GMW	>100	>100	>100
Kaempferia galanga Linn.	KGW	>100	>100	>100
Kheaw-Hom remedy	KHW	>100	64.89±0.82	>100
Limnophila rugosa Merr.	LRW	>100	>100	>100
Mammea siamensis Kosterm	MSW	11.45±1.24	23.10±1.20	>100
Mesua ferrea L.	MeFW	15.91±4.69	18.30±1.27	>100
Mimusops elengi L.	MEW	32.56±2.25	24.24±2.58	>100
Myristica fragrans Houtt	MFW	25.81±0.37	21.21±1.17	>100
Nelumbo nucifera Gaertn.	NUW	15.68±2.46	19.33±0.97	>100
Pogostemon cablin (Blanco) Benth.	PCW	18.13±2.35	37.31±1.31	>100
Sophora exigua Craib	SEW	>100	74.07±4.30	>100
Tacca chantrieri Andre	TCW	9.46±1.48	16.00±0.44	70.65±1.28
Vetiveria zizanioides (L.) Nash ex Small	VZW	>100	>100	>100
BHT		13.40±0.266	-	-
Trolox			5.85±0.73	-
Propyl gallate			-	14.87±0.02

that of *V. zizanioides* had no activity. Secondly, the ethanolic extract of *P. cablin*[20], the ethanolic and aqueous extracts of *M. fragrans*[21], the ethanolic extract of bark of *C. gigantean*[24] and methanolic extract of flower of *M. elengi*[9] had moderate activity and, in this present study the ethanolic extract of *P. cablin* had weak activity, only the aqueous extract of *M. fragrans* had moderate activity, the ethanolic extract of *C. gigantean* had strong activity and the aqueous extract of *M. elengi* had moderate activity. Finally, the aqueous extract of *P. cablin*[20], the methanolic extract of *C. fruticosa*[23] and the ethanolic extract of *G. malaccensis*[24] had weak activity, and aqueous extract of *P. cablin* had strong activity, the ethanolic extract of of *C. fruticosa* had moderate activity and the ethanolic extract of *G. malaccensis* had weak activity. The results showed different values may be from the usage various solvent in extraction and source of plants lead to the chemical constituents of plants were different.

Interestingly, the ethanolic and aqueous extracts of *T. chantrieri* showed strong efficacy in DPPH and ABTS radical scavenging assays and had weak efficacy in NBT assay. *T. chantrieri* was reported in the previous study, ABTS radical scavenging assay that ethanolic extract had moderate efficacy[25], but for DPPH radical scavenging assay and NBT assay have never been reported.

Conclusion

To the best of our knowledge, the best antioxidant activity using DPPH scavenging assay are that of ethanolic extract of *C. gigantean*, *T. chantrieri* and *S. exigua* having EC_{50} ±SEM values of 7.55±0.89, 8.00±2.37 and 9.42±2.11 µg/mL, respectively. The best antioxidant activity using ABTS scavenging assay are that of ethanolic extract of *D. loureiri*, *G. malaccensis* and *T. chantrieri* having EC_{50} ±SEM values of 7.88±0.65, 8.06±0.53 and 14.84±0.48 µg/mL, respectively.

The best antioxidant activity using NBT dye reduction assay are that of ethanolic and aqueous extracts of *T. chantrieri* have IC_{50} ±SEM values of 63.38±3.29 and 70.65±1.28 µg/mL, respectively.

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Potential conflicts of interest

None.

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ฤทธิ์ต้านอนุมูลอิสระของสารสกัดตำรับเขียวหอมและสมุนไพรในตำรับ

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ภูมิหลัง: อนุมูลอิสระและสารต้านอนุมูลอิสระมีบทบาทสำคัญในโรคที่เกิดจากการอักเสบ เช่น ไข้ ซึ่งขณะการเกิดไข้มีการสร้างอนุมูลอิสระขึ้นจำนวนมาก และสารต้านอนุมูลที่ร่างกายที่สร้างขึ้นไม่เพียงพอสำหรับการกำจัดอนุมูลอิสระที่เกิดขึ้น ซึ่งยาตำรับเขียวหอมเป็นยาแก้ไข้ที่นิยมใช้ในศาสตร์การแพทย์แผนไทย

จุดประสงค์: เพื่อศึกษาฤทธิ์ต้านอนุมูลอิสระของสารสกัดแอลกอฮอล์และสารสกัดน้ำของตำรับยาเขียวหอมและสมุนไพรในตำรับ ด้วยใช้วิธีการ DPPH radical scavenging, ABTS radical scavenging และ NBT dye reduction assay

วัสดุและวิธีการ: สารสกัดชั้นเอทานอล สกัดโดยการหมักสมุนไพรกับ 95% เอทานอล ระเหยแห้งด้วยเครื่อง rotary evaporator สารสกัดชั้นน้ำใช้วิธีการสกัดโดยการต้มและกรอง สุดท้ายนำไปทำให้แห้งด้วยเครื่อง lyophilizer ในการศึกษาเบื้องต้น นำสารสกัดทั้งหมดไปทดสอบฤทธิ์ต้านอนุมูลอิสระโดยวิธี DPPH radical scavenging, ABTS radical scavenging และวิธีการที่ทดสอบกับเซลล์ คือ NBT dye reduction assay

ผลการศึกษา: สารสกัดชั้นเอทานอลของตำรับยาเขียวหอมมีฤทธิ์ต้านอนุมูลอิสระดี โดยวิธี DPPH radical scavenging และ ABTS radical scavenging แต่ไม่มีฤทธิ์ต้านอนุมูลอิสระโดยวิธี NBT dye reduction (EC_{50} = 16.96+1.21, 30.91+1.53 และ IC_{50} >100 μg/ml ตามลำดับ) และจากวิธี DPPH radical scavenging พบว่าสมุนไพรที่มีฤทธิ์ต้านอนุมูลอิสระดีที่สุด 2 อันดับแรก ได้แก่ สารสกัดชั้นเอทานอลของมหาสดำ ตามด้วยสารสกัดชั้นเอทานอลของเนระพูสี (EC_{50} = 7.55±0.89 และ 8.00±2.36 μg/mL) จากวิธี ABTS radical scavenging พบว่าสมุนไพรที่มีฤทธิ์ต้านอนุมูลอิสระดีที่สุด 2 อันดับแรก ได้แก่ สารสกัดชั้นเอทานอลของจันทร์แดง ตามด้วยสารสกัดชั้นเอทานอลของว่านร่อนทอง (EC_{50} = 7.88±0.65 และ 8.06±0.53 μg/mL) และจากวิธี NBT dye reduction พบว่าสมุนไพรที่มีฤทธิ์ต้านอนุมูลอิสระชนิดเดียวคือ เนระพูสี โดยพบว่าสารสกัดชั้นเอทานอลมีฤทธิ์ต้านอนุมูลอิสระดีกว่าชั้นน้ำ (EC_{50} = 63.38±3.29 และ 70.65±1.28 μg/mL)

สรุป: สารสกัดชั้นเอทานอลของตำรับยาเขียวหอมมีฤทธิ์ต้านอนุมูลอิสระ มีฤทธิ์เฉพาะในการทดลองด้วยสารเคมี แต่ไม่มีฤทธิ์ในการทดลองแบบจำลองร่างกาย และสมุนไพรที่มีฤทธิ์ต้านอนุมูลอิสระ ทั้ง 3 วิธี คือ เนระพูสี ซึ่งสามารถยับยั้งอนุมูลอิสระทั้งวิธีใช้สารเคมีและแบบการจำลองในร่างกาย ดังนั้นควรมีการพัฒนาสมุนไพรชนิดนี้เพื่อง่ายต่อการบริโภคต่อไป