Iron-Chelating, Free Radical Scavenging and Anti-Proliferative Activities of *Azadirachta indica*

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Background: *Excessive production of reactive oxygen species leads to oxidative stress which occurs in various diseases, such as diabetes, cancer, neurodegenerative diseases, and secondary iron overload in thalassemia. Antioxidants are compounds that inhibit the oxidative processes and delay or suppress oxidative stress. Phytochemicals in herbs are interesting sources of natural antioxidants with cancer preventive properties. The use of natural products is beneficial for the prevention and/or treatment of oxidative stress mediated diseases.*

Objective: *The study aimed to investigate the antioxidant and anti-proliferative properties of ethanolic extract from Azadirachta indica (neem) leaves.*

Material and Method: *Neem leaves were extracted by 80% ethanol (v/v). The ethanolic extract was tested for free radical scavenging activity by 2,2'-azino-bis-3-ethylbenzothiaziline-6-sulfonic acid (ABTS) and for the reduction of the power of ferric ion (Fe^{3+}) to ferrousion (Fe^{2+}) by ferric reducing antioxidant plasma (FRAP) assay. Furthermore, the ability of iron-binding activity was investigated by the spectrophotometry technique. The inhibitory effect on the growth of human promyelocytic leukemic cell line (HL-60 cells) was determined by MTT assay.*

Results: *The ethanolic extract from neem leaves exhibited free radical scavenging activities and reduced the power of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in dose responses. Furthermore, it was able to bind with iron rapidly within 5 minutes. Interestingly, this extract inhibited human promyelocytic leukemic cell line (HL-60 cells) growth in concentration response (0-500 μg/ml) for 24 hour treatment.*

Conclusion: *The ethanolic extract from neem leaves had strong antioxidant activity and an anti-proliferative effect on cancer cells.*

Keywords: *Antioxidant, Iron-chelating, Anti-proliferative, Reactive oxygen species, Free radicals*

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The metabolism in eukaryotic cells induces reactive oxygen species (ROS) such as electron transport chains in mitochondria, peroxisomal fatty acid metabolism, cytochrome P450 enzymes in microsome and flavoprotein oxidases[1]. ROS production has a beneficial effect in the physiological function of mammalian cells, occurring at low concentrations in cellular response to noxia, for example in defense against infectious agents. Oxidative stress is a term for a dangerous effect of free radicals that cause cellular and tissue damage[2,3]. Over production of ROS occurs in living organisms when there is a deficiency of enzymatic and non-enzymatic antioxidants. The excess of ROS can damage bio-molecules in the body, such as lipids, proteins, and DNA, inhibiting their normal functions leading to a number of human diseases. ROS causes cellular and tissue pathogenesis, leading to several chronic conditions, such as cancer, cardiovascular problems, diabetes mellitus, thalassemia, aging, and chronic renal disease[1,4-6]. Secondary iron overload in thalassemia is a chronic disease with oxidative stress because excessive iron catalyzes free radical generation via the Fenton reaction[7].

Antioxidant agents are a powerful defense system to prevent ROS induced-cell damage in various diseases. They act through one of three mechanisms to prevent oxidant-induced cell damage, reduction of the generation of ROS, scavenging ROS, or interference with ROS-induced alterations[8]. Medicinal plants are important sources of protective compounds against free radicals in chronic diseases, such as cancer[9,10] and secondary iron overload[11,12].

Nowadays, phytochemicals in herbs are

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interesting compounds for alternative medicine as they inhibit the damage of free radicals. Neem (*Azadirachta indica*; *A. indica*) is found extensively as a tropical evergreen in Thailand. Crude extracts from leaves, bark, roots, and oil in neem contain various biological properties and are used in the treatments of a number of conditions, such as cancer[13-15], oxidative stress[16-18] and diabetes[19,20]. The present study investigated the antioxidant and anti-proliferative effect of ethanolic extract from neem leaves, and iron-binding activities and free radical scavenging assays for antioxidant activity were determined. Furthermore, the anti-proliferative activity of neem leaves extract on human leukemic cell lines (HL-60) was investigated for the evaluation of its anticancer properties.

Material and Method

Chemicals

1,1-diphenyl-2-picryl-hydrazyl, 2,2'- azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ascorbic acid were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). A stock of ferric nitrate (AAS iron reagent, 1,000 ppm, in 0.5% HNO_3; APS Finechem, Seven Hills, Australia) was used as the iron source for other preparations. Stock ferric nitrilotriacetate solution was prepared by consecutive mixing of ferric nitrate with the nitrilotriacetic acid (at a 1:5 molar ratio of Fe^{3+} to chelator). Various iron concentrations were freshly prepared in 10 mM MOPS buffer, pH 7.0, before use. All other chemicals and reagents used were of AnalaR grade. Leaves of *A. indica* were collected in Phayao Province.

Preparation of plant extract

The dried powder leaves of *A. indica* (100 g) were mixed with 1,000 ml of ethanol (80% v/v) at room temperature for 24 h. The supernatant was collected by filtration through filter paper No. 1. Activated charcoal was added to the filtrate for 10 min, passed through a filter paper, and centrifuged. The supernatant was collected and concentrated by using rotary evaporator further lyophilized (yield = 3.41%). The extracted powder was kept at -20°C until used.

ABTS free radical de-colorization assay

This assay assesses the total radical scavenging capacity based on the ability of a compound to scavenge the stable ABTS cationic radical ($ABTS^{\cdot+}$)[21]. ABTS was dissolved in de-ionized water to reach 7 mM concentration. $ABTS^{\cdot+}$ was produced by mixing the stock solution of ABTS with 2.45 mM potassium persulfate ($K_2S_2O_8$) solution, and allowing the mixture to stand in the dark at room temperature for 16-18 hours before use. $ABTS^{\cdot+}$ solution was diluted with de-ionized water with pH 7.4 to obtain an optical density (OD) value of 0.70 ± 0.02 at 734 nm using a spectrophotometer. In the assay, various concentrations of the ethanolic extract from *A. indica* (10 µl) or trolox (standard) solution were added to the 990 µl of diluted $ABTS^{\cdot+}$ solution. OD of the product was measured at 734 nm after 6 min against de-ionized water (blank). All determinations were carried out in triplicate.

Percentages of ABTS radical scavenging activity were calculated by the following equation: Inhibition (%) = $(OD_{control}-OD_{sample})/OD_{control}x100$. The IC_{50} value was defined as the concentration necessary to decrease free radicals by 50% as measured by the absorbance of ABTS.

Ferric reducing antioxidant plasma (FRAP) assay

The reducing power of the ethanolic extract from *A. indica* leaves was determined according to Benzie and Strain[22] with some modifications. The working solution was prepared with acetate buffer (300 mM, pH 3.6), 10 mM 2,4, 6-tripyridyl-s-triazine (TPTZ) and 20 mM ferric chloride ($FeCl_3$ or Fe^{3+}). The extract was mixed with a working solution for 10 minutes at 37°C and absorbance was measured at 593 nm by using a UV- spectrophotometer. The standard curve was linear between 100 and 1,000 µM $FeSO_4$ (Fe^{2+}). The concentration of $FeSO_4$ was in turn plotted against concentration standard antioxidant ascorbic acid. The assay was carried out in triplicate and the results were expressed as mean $\pm$ SD. Increases in the absorbance of the samples with concentrations indicated high reducing potentials of the samples.

Iron-binding assay

Spectral analysis

To determine the iron-binding activity, the ethanolic extract from *A. indica* (a final concentration of 1 mg/ml) was incubated with ferric nitrilotriacetate (Fe^{3+}-NTA) (0-200 µM at final concentrations), pH 7.0 at room temperature for 30 minutes. The absorbance of the resulting complex(es) was measured in the wave length range of 200-800 nm by the use of a scanning double-beam UV-VIS Spectrophotometer (Shimudsu, Japan). Equivalent concentrations of the ethanolic extract from the *A. indica* solution were used as the blank.

A stock solution of the ethanolic extract from

A. indica was freshly prepared by dissolving the extract powder in 50 mM 3-[N-morpholino] propanesulfonic acid (MOPS) (Sigma-Aldrich Co., St. Louis, MO, USA), pH 7.0 solution. A working Fe^{3+}-NTA solution was freshly prepared by mixing stock ferric nitrate solution (AAS iron reagent, 1,000 ppm in 0.5% HNO_3; APS Finechem, Seven Hills, Australia) with anitrilotriacetate (NTA) solution (a molar ratio of Fe^{3+}: NTA = 1: 5).

Time course binding

The ethanolic extract from *A. indica* (1 mg/ml at final concentration) was mixed with the solution of ferric nitrate solution (1-100 µM at final concentration), then incubated at room temperature for 0-900 minutes. The absorbance of the complex was measured at 416 nm against the ethanolic extract from the *A. indica* solution as a reagent blank.

Cytotoxicity test

Cell culture

HL-60 cells (Human promyelocytic leukemic cell line) were maintained in RPMI 1640 medium (Gibco™, Life Technologies, USA) supplemented with 10% fetal bovine serum (Gibco™, Life Technologies, USA), 100 U/ml penicillin, and 100 U/ml streptomycin, and incubated at 37°C under a normal humidified atmosphere (95% air and 5% CO_2).

Cell viability assay

The HL-60 cells ($1x10^4$cells/well) were incubated with the ethanolic extract from *A. indica* (0-500 µg/ml at final concentration) in 96-well culture plates at 37°C for 24 hours. The MTT test[23] was used to investigate the cytotoxic effect of the ethanolic extract from *A. indica* on the cells. MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5 diphenyltetrazolium bromide) (15 µl) was added to the cells and further incubated for 4 hours. The reaction product, blue-colored formazan, was extracted with dimethyl sulphoxide (DMSO) (200 µl) and the absorbance 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.

Statistical analysis

The results were expressed as mean ± SD.

Results

Free radical scavenging activity

Many methods have been used to investigate the antioxidant activity of natural products[24]. The ABTS free radical de-colorization method was utilized to measure the antioxidant activity of the ethanolic extract from *A. indica* and compared to trolox, a water soluble derivative of vitamin E. The results are shown in Table 1 and Fig. 1. The percentage of ABTS free radical scavenging activity was increased in a concentration-dependent manner in the ethanolic extract from *A. indica* leaves (3-25 µg/ml). The extract from *A. indica* leaves and trolox showed 50% inhibition of ABTS free radical activity (IC_{50}) at 5.2 and 3.08 µg/ml, respectively.

Reducing activity

The reducing power of the ethanolic extract from *A. indica* was determined by FRAP assay, which depends upon the reduction of ferric tripyridyltriazine (Fe(III)-TPTZ) complex to ferrous tripyridyltriazine (Fe (II)-TPTZ) by a reductant at low pH. Fe (II)-TPTZ has

Table 1. Percentage inhibition of ABTS free radicals in the ethanolic extract from *A. indica* leaves. Data were obtained from triplicate results of three dependent experiments and shown as mean ± standard deviations (SD)

Ethanolic extract from *A. indica* leaves (µg/ml)	% Inhibition of ABTS free radicals
3.0	40±2
6.0	52±3
12.5	74±5
25.0	94±5

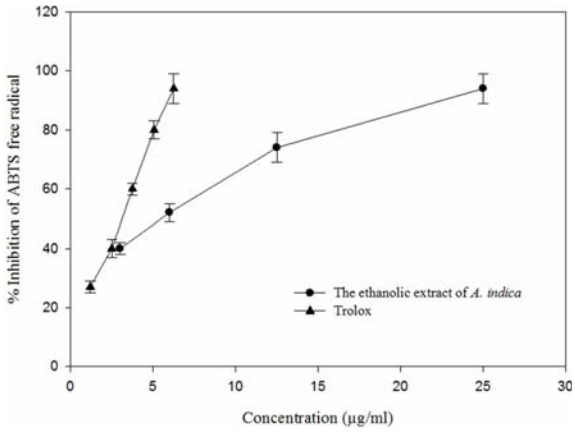

Fig. 1 ABTS free radical scavenging activity of the ethanolic extract from *A. indica* compared to trolox. Data were obtained from triplicate results of three dependent experiments and shown as mean ± standard deviations (SD).

an intensive blue color and can be monitored at 593 nm. The reducing power of the ethanolic extract from *A. indica* leaves at concentrations 20, 40, and 60 µg/ml were equivalent to ascorbic acid at 6.1±0.2, 14.4±0.8, and 20.3±0.1 µM, respectively (Fig. 2). The reducing property of the extract equivalent to ascorbic acid was 30.07±2.25 µg/ml.

Iron-binding activity

Spectral analysis of the ethanolic extract from *A. indica* was determined by measuring OD at the wavelength 200-800 nm against 20 mM MOPs buffer solution as a reagent blank. The spectrums of the ethanolic extract from A. *indica* alone exhibited maximum absorption at 265 and 337 nm (Fig. 3). The reactivity of ferric ion with the ethanolic extract from *A. indica* formed a colored complex(es) and exhibited the new maximum absorption at 416 and 570 nm, the respective major and minor peaks (Fig. 4). The OD of complex(es) was increased dose dependently (0-200 µM ferric ion). The time course of the complex formation at 416 nm was rapid and reached saturation within 5 minutes of incubation time (Fig. 5). The ethanolic extract from *A. indica* formed to complex(es) in a dose-dependent pattern.

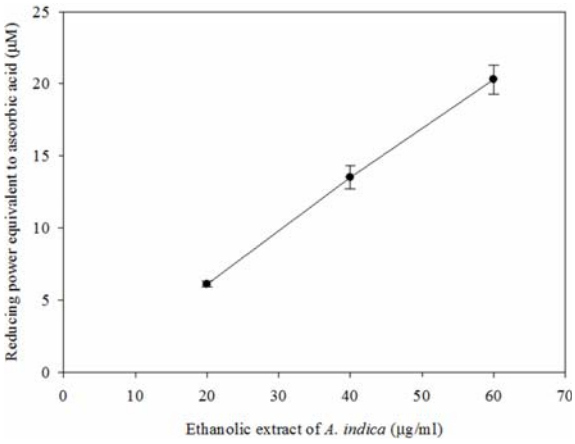

Fig. 2 Reducing activity of the ethanolic extract from *A. indica* leaves equivalent to the concentrations of ascorbic acid. Data were obtained from triplicate results of three dependent experiments and shown as mean ± standard deviations (SD).

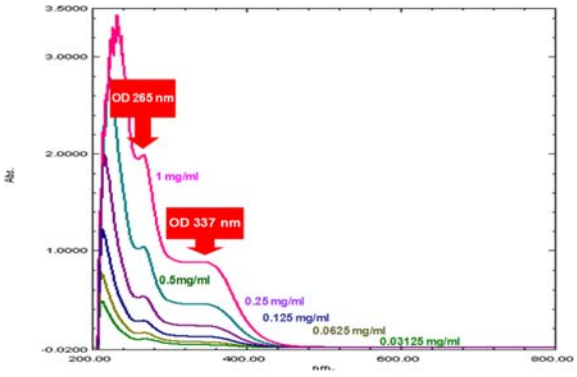

Fig. 3 Spectral analysis of the ethanolic extract from *A. indica* leaves (0-1 mg/ml at final concentration).

Cytotoxicity on HL-60 cells

Higher concentration of the ethanolic extract from *A. indica* leaves significantly decreased the viability of HL-60 cells (Fig. 6). The IC_{50} of the ethanolic extract in HL-60 cells after 24 hours period was 100 µg/ml.

Discussion

Alternative medicine is becoming increasingly interesting, especially that involving medicinal herbs to reduce dosages and the cost of synthetic drugs, and to increase the use of dietary supplements to

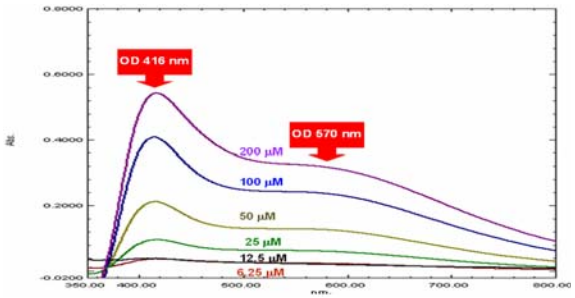

Fig. 4 Spectral analysis of iron complex(es). 1 mg/ml ethanolic extract from *A. indica* leaves was incubated with various concentrations of ferric ion (0-200 µM).

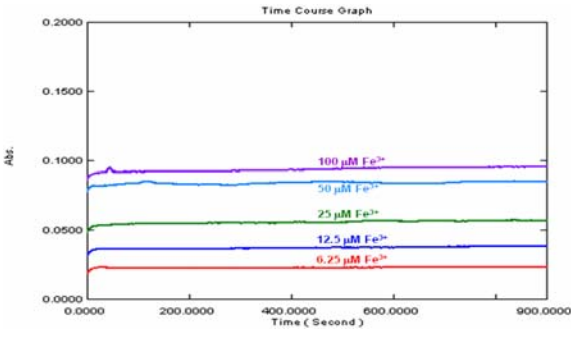

Fig. 5 Time course binding of ferric ion (6.25-100 µM) to the ethanolic extract from *A. indica* leaves (1 mg/ml).

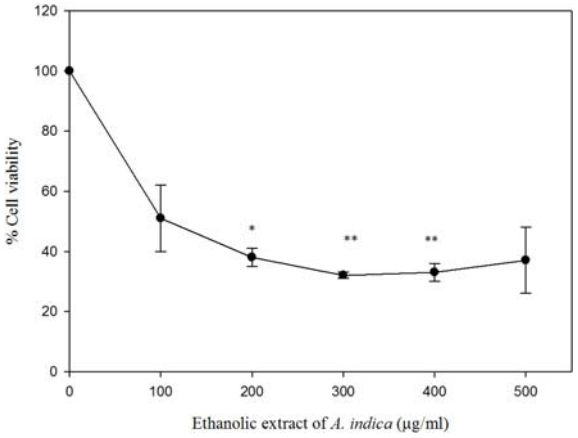

Fig. 6 Cytotoxic effect of the ethanolic extract from *A. indica* leaves (0-500 µg/ml at final concentrations) on cultured HL-60 cells for 24 hours. Data were obtained from three time independent experiments and expressed as mean ± SD, * $p<0.05$, ** $p<0.01$ as compared to control.

improve health. Medicinal herbs have been used in the treatment of several diseases such as diabetes mellitus[25], Alzheimer's disease[26], Parkinson's disease[27], cancer[28,29], and oxidative stress in secondary iron overload[12,30]. More attention has been focused on antioxidative stress properties in natural products. Phytochemicals, especially polyphenols which are derivatives and/or isomers of flavones, isoflavones, flavonols, catechins, and phenolic acids, possess antioxidant properties[31]. Leaves of *A. indica* have been reported to contain numerous active substances that have potent antioxidant and anticancer activities, especially polyphenolics such as flavonoids and their glycosides, dihydrochalone, cumarin, and tannin. β-carotenes, ascorbic acid, terpenoids, limonoids and flavonoids in *A. indica* leaves have a potent antioxidant and anticancer presence[32]. The radical scavenging properties of 70% ethanolic *A. indica* leaf extract reduced lipid peroxidation by enhancing the activity of antioxidant enzymes, superoxide dismutase (SOD), and catalase (CAT) in N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced rats[33,34].

The present study investigated the antioxidant activity of the ethanolic extract from *A. indica* leaves by ABTS free radical de-colorization, ferric reducing antioxidant potential (FRAP), and iron-binding assay. De-colorization of ABTS cations has been extensively used to determine antioxidant capacity[35]. The ABTS free radical de-colorization method has proved the antioxidant potential in comparison with the reference antioxidant, vitamin E (trolox). The results showed that the extract from *A. indica* possessed ABTS free radical scavenging activity in a concentration-dependent manner (3-25 µg/ml). The extract and trolox exhibited antioxidant activity with IC_{50} value at 5.2 and 3.08 µg/ml, respectively. The reducing power assay by FRAP was quick and simple and the reaction was related to the amount of antioxidant present. The reducing power of *A. indica* leaf extract was increased related to the dose response and equivalent to the concentration of ascorbic acid. The results from ABTS free radical de-colorization and FRAP assay suggest that the extract of *A. indica* had a potent antioxidant activity.

ROS are generated by iron and are potentially damaging to cellular organism. The finding of active substances that can bind to iron molecules leads to the inhibition of the generation of ROS. Interestingly, the *A. indica* leaf extract exhibited rapid iron-binding activity within 5 minutes of the incubation time. This activity may be due to the component of the extract. A previous study reported the ability of flavonoids to chelate iron[36] and copper ions and its dependence of structure (hydroxyl and the oxo groups) and pH[37]. Moreover, the functional carbonyl group of flavonoids (apigenin, luteolin, kaempferol, quercetin, myricetin, and naringenin) was able to chelate iron and copper[38]. In the present study that is the subject of this paper, the extract from the *A. indica* leaves showed iron chelating activity, and it may reduce ROS generated from iron via Fenton reaction. An active compound in *A. indica* leaves, quercetin, has been demonstrated to inhibit cancer cell growth in many malignant cell lines and animal models as well as in humans[39,40]. Moreover, β-carotene and vitamin C inhibit tumor development by their radical scavenging properties[41,42].

Iron is an essential trace element important for biological processes including cell growth and differentiation. It is also required for cancer cell growth because of its catalytic effect on the formation of hydroxyl radicals, suppression of the activity of host defense cells, and promotion of cancer cell multiplication[43,44]. Iron accumulation in many organs is correlated with the process of carcinogenesis[44]. The selectivity of iron chelators for malignant cells arises from an increased requirement of iron for DNA synthesis and metabolism in rapidly growing tumor cells when compared to more slowly growing normal cells. Deferrioxamine was found to be anti-proliferative against liver cancer cells but not in normal proliferating human diploid cells[45,46]. Iron depletion by various iron

chelators has shown anticancer activity in tumor cells[46-48]. The experiment that is the subject of this paper found that the ethanolic extract from *A. indica* leaves could inhibit HL-60 cells growth, indicating that the *A. indica* leaf extract has anti-proliferative properties due to its ability to deplete cancer cells of iron.

It can be concluded that the ethanolic extract from *A. indica* leaves could have antioxidant activity, iron chelating properties, and an anti-proliferative effect in the human leukemic cell line (HL-60). The antioxidant and iron chelating properties of the *A. indica* leaf extract may be useful in the treatment and/or prevention of a number of oxidative stress disorders, and the anti-proliferative properties of *A. indica* should be further elucidated.

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Potential conflicts of interest

None.

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การศึกษาฤทธิ์ในการจับธาตุเหล็ก ลดอนุมูลอิสระ และยับยั้งการแบ่งตัวของเซลล์มะเร็ง ของสารสกัดจากใบสะเดา

กาญจนา แปงจิตต์, พยุงศักดิ์ ตันติไพบูลย์วงศ์, วิทวัส สัจจาพงศ์, สมเดช ศรีชัยรัตนกูล

ภูมิหลัง: *การสร้างอนุมูลอิสระที่มากเกินนำไปสู่ภาวะเครียดในร่างกายพบในโรคต่าง ๆ มากมาย เช่น โรคเบาหวาน โรคมะเร็ง ความเสื่อมถอยของเซลล์ประสาท และภาวะเหล็กเกินในผู้ป่วยธาลัสซีเมีย สารต้านอนุมูลอิสระสามารถยับยั้งหรือชะลอขั้นตอนการเกิดภาวะเครียดต่าง ๆ ในร่างกายได้ สารประกอบสำคัญที่พบในพืชหรือสมุนไพรต่าง ๆ เป็นที่น่าสนใจในการช่วยยับยั้งอนุมูลอิสระและป้องกันการเกิดมะเร็ง การค้นคว้าหาสารสกัดจากพืชธรรมชาติเป็นประโยชน์ในการช่วยป้องกันและรักษา สภาวะเครียดจากอนุมูลอิสระที่เกิดจากโรคเรื้อรังต่าง ๆ ได้*

วัตถุประสงค์: *ศึกษาคุณสมบัติในการต้านอนุมูลอิสระและยับยั้งการแบ่งตัวของเซลล์มะเร็ง ของสารสกัดจากใบสะเดา*

วัสดุและวิธีการ: *สกัดสารสำคัญจากใบสะเดาโดยสารละลายเอธานอล 80% จากนั้นนำมาทดสอบฤทธิ์ในการกำจัดอนุมูลอิสระโดยวิธี ABTS และทดสอบคุณสมบัติในการรีดิวซ์ธาตุเหล็กจากเฟอร์ริกไอออน เป็นเฟอร์รัสไอออนของสารสกัดโดยวิธี FRAP นอกจากนั้นศึกษาคุณสมบัติในการจับธาตุเหล็กโดยเทคนิคทางสเปกโตรโฟโทเมทรี ศึกษาฤทธิ์ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งเม็ดเลือดขาว (HL-60) โดยวิธีเอ็มทีที (MTT assay)*

ผลการศึกษา: *สารสกัดจากใบสะเดามีฤทธิ์ในการกำจัดอนุมูลอิสระและฤทธิ์ในการรีดิวซ์เฟอร์ริกเป็นเฟอร์รัสไอออน ในลักษณะที่ขึ้นกับความเข้มข้นของสารสกัด นอกจากนี้ยังสามารถจับกับธาตุเหล็กได้อย่างรวดเร็วภายในเวลา 5 นาที และยับยั้งการเจริญเติบโตของเซลล์มะเร็งเม็ดเลือดขาว (HL-60) ในลักษณะที่ขึ้นกับความเข้มข้นของสารสกัด (0-500 μg/ml) ภายใน 24 ชั่วโมง*

สรุป: *สารสกัดจากใบสะเดาด้วยเอธานอลมีประสิทธิภาพในการยับยั้งอนุมูลอิสระและยับยั้งการแบ่งตัวของเซลล์มะเร็ง*