ANTIOXIDANT ACTIVITY AND PROTECTIVE EFFECTS OF *GYMNEMA INODORUM* DECNE. ON RED BLOOD CELL HEMOLYSIS AND DNA DAMAGE IN TK6 HUMAN LYMPHOBLASTOID CELLS

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ABSTRACT

Epidemiological evidence indicates that diets high in fruits and vegetables are rich in many antioxidant nutrients, having capability of decreasing the risk of chronic degenerative diseases. The pathogenesis of these diseases is hypothesized to be the harmful effects of free radicals on DNA, protein, lipid, and small intracellular molecules. Recently, the search for natural antioxidants and other preparations of plants concerning the beneficial effect for good health has been intensified. Still, little information regarding free radical scavenging and/or antioxidant activity is available for a vast number of Thai local vegetables. *Gymnema inodorum* Decne. (Chiangda) is one of the Thai local vegetables that grows in the northern part of Thailand. It has been known to be effective for some diseases including diabetes mellitus, rheumatic arthritis, and gout. Interestingly, there are reports showing that Chiangda has many phytochemicals that act as antioxidants. Therefore, objectives of this study are to determine the antioxidant activity of various preparations of *Gymnema inodorum*.

Gymnema inodorum (GI) was prepared in four fractions. Edible parts of *Gymnema inodorum* were divided into two parts: fresh juice (GIJ) and dried forms, which were extracted with water (GIW), 50% ethanol (GIE50) and 95% ethanol (GIE95). The antioxidant activities were determined by DPPH radical scavenging assay, deoxyribose degradation assay, hemolysis assay, and comet assay. The total phenolic compounds were determined by folin-ciocalteu method.

The results showed that the GIJ fraction had the highest antioxidant activity in all tests as well as the highest content of vitamin E (22.6 mg/100 g freeze dry extract) and beta-carotene (25.9 mg/100 g freeze dry extract). The highest concentration of total phenolic compounds (3.95 g GAEs/100g freeze dry extract) was found in the GIE50 fraction. The GIE95 fraction had the lowest antioxidant activity and amount of total phenolic compound. In conclusion, this study reveals that juice of *Gymnema inodorum* has strong antioxidant activities including the highest protective effects on RBC hemolysis and DNA damage. In addition, juice of *Gymnema inodorum* has a high content of vitamins that act as antioxidants. This information can assist consumers in selecting a source of natural antioxidants for consumption.

KEY WORDS: *GYMNEMA INODORUM* DENCE./ TOTAL PHENOLIC COMPOUNDS/ ANTIOXIDANT ACTIVITY/ HEMOLYSIS/ DNA DAMAGE

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การศึกษาฤทธิ์ต้านอนุมูลอิสระของสารสกัดจากผักเชียงดาต่อการป้องกันการแตกตัวของเม็ดเลือดแดงและการเสียหายของดีเอ็นเอในเซลล์เม็ดเลือดขาวมนุษย์ชนิดTK6 (ANTIOXIDANT ACTIVITY AND PROTECTIVE EFFECTS OF *GYMNEMA INODORUM* DECNE. ON RED BLOOD CELL HEMOLYSIS AND DNA DAMAGE IN TK6 HUMAN LYMPHOBLASTOID CELLS)

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บทคัดย่อ

การศึกษาทางระบาดวิทยาพบว่าสารต้านอนุมูลอิสระที่มีอยู่ในผักและผลไม้มีคุณสมบัติในการลดโอกาสเสี่ยงที่จะเกิดโรคเรื้อรังและโรคที่เกิดจากความเสื่อมของอวัยวะต่างๆได้ โดยสมมุติฐานเกี่ยวกับพยาธิวิทยาของโรคเหล่านั้นเชื่อว่าเกิดจากการที่อนุมูลอิสระทำลายโมเลกุลของสารพันธุกรรม(ดีเอ็นเอ) โปรตีน ไขมัน และ สารโมเลกุลเล็กอื่นๆในเซลล์ของร่างกาย ปัจจุบันได้มีการศึกษาถึงคุณสมบัติของสารต้านอนุมูลอิสระจากธรรมชาติ โดยเฉพาะสารที่ได้จากพืชเพื่อใช้ในการรักษาและป้องกันการเกิดโรคเรื้อรังต่างๆเพิ่มมากขึ้น แต่จำนวนการศึกษาทางวิทยาศาสตร์เพื่อบอกถึงคุณสมบัติต้านอนุมูลอิสระของผักพื้นบ้านในประเทศไทยยังมีน้อยมาก ผักเชียงดาเป็นพืชพื้นบ้านของประเทศไทยที่นิยมปลูกในแถบภาคเหนือของประเทศ มีการศึกษาพบว่าผักเชียงดามีสารที่ออกฤทธิ์ต้านอนุมูลอิสระหลายชนิด ดังนั้นวัตถุประสงค์ของงานวิจัยนี้คือ เพื่อประเมินฤทธิ์ต้านอนุมูลอิสระของผักเชียงดาในรูปแบบการเตรียมต่างๆกัน รวมทั้งศึกษาผลต่อการป้องกันการแตกตัวของเม็ดเลือดแดงและการเสียหายของดีเอ็นเอ

ผักเชียงดาถูกเตรียมด้วยวิธีการ 4 แบบ ได้แก่ น้ำคั้นผักสด ส่วนที่ได้จากการสกัดด้วยน้ำ สกัดด้วยเอทานอลที่ความเข้มข้นร้อยละ 50 และ ที่ความเข้มข้นร้อยละ 95 การทดสอบคุณสมบัติการจับอนุมูลอิสระเพื่อป้องกันการทำลายสารชีวโมเลกุลใช้วิธี DPPH assay, deoxyribose assay, hemolysis assay และ comet assay จากการศึกษาครั้งนี้พบว่าน้ำคั้นผักสดมีคุณสมบัติต้านอนุมูลอิสระ และป้องกันการแตกตัวของเม็ดเลือดแดงและการเสียหายของดีเอ็นเอสูงสุด รวมทั้งมีปริมาณของวิตามินอี และเบต้าแคโรทีนสูงที่สุดด้วย ส่วนสารสกัดด้วยเอทานอลที่ความเข้มข้นร้อยละ 50 มีปริมาณของสารประกอบฟีนอลิกสูงสุด และสารสกัดด้วยเอทานอลที่ความเข้มข้นร้อยละ 95 มีฤทธิ์ต้านอนุมูลอิสระและปริมาณสารประกอบฟีนอลิกต่ำสุด การศึกษานี้แสดงให้เห็นว่าผักเชียงดาในรูปแบบของผักสดที่ไม่ผ่านขบวนการแปรรูปมีฤทธิ์ต้านอนุมูลอิสระได้ดีและยังมีปริมาณวิตามินที่มีคุณสมบัติที่เป็นสารต้านอนุมูลอิสระสูงอีกด้วย

ข้อมูลคุณสมบัติต้านอนุมูลอิสระของผักเชียงดาด้วยการเตรียมแบบต่างๆในงานวิจัยนี้น่าจะเป็นประโยชน์แก่ผู้บริโภค ในการพิจารณาเลือกบริโภคสารต้านอนุมูลอิสระจากธรรมชาติ

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AAPH	2, 2-azo-bis-(2-amidino-propane) dihydrochloride
CAT	Catalase
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-l-picrylhydrazyl
EtOH	Ethanol
Fe^{2+}	Ferrous
Fe^{3+}	Ferric
GAEs	Gallic acid equivalent
GSH	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
IC_{50}	50 percentage of inhibition concentration
$O_2^{\bullet -}$	Superoxide
$OH^{\bullet}$	Hydroxyl radical
RBC	Red blood cell
$ROO^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
rpm	Round per minutes
SOD	Superoxide dismutase
TBARS	Thiobarbitoric acid reactive substance
TD	Tail density
TL	Tail length
TM	Tail moment

CHAPTER I

INTRODUCTION

Most free radical reactions involve the formation of reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), and hydrogen peroxide (H_2O_2). These oxygen species can cause oxidative damage to several cell components and play an important role in various pathological conditions. The inflicted damage may contribute to chronic degenerative diseases such as brain dysfunction, cataracts, cancer, and cardiovascular disease (1-5). A high level of free radicals and low amount of antioxidants can lead to a condition of oxidative stress and chronic injury. However cells can develop antioxidant defense systems, including enzymatic antioxidants (superoxide dismutase (SOD), glutathione peroxidase (GSH), and catalase (CAT)) as well as nonenzymatic components (vitamin E, vitamin C, β-carotene, and phytochemicals), to protect molecules against toxic free radicals and other ROS (6). There is a considerable body of biological evidence that an imbalance between oxidants and antioxidant defense systems can cause damage to cells, contributing to cellular dysfunction and leading to chronic degenerative diseases (4-5). Therefore, the balance of free radical production and levels of antioxidants is essential for health.

Recently, the search for natural antioxidants and other preparations of plant origins that are beneficial for good health has been intensified. The potential of antioxidant constituents in plant materials has been overtly studied regarding the maintenance of health and protection from chronic degenerative diseases. One of the protective mechanisms is the inhibitory effects of biologically active phytochemical compounds in the plant foods on the initiation or propagation of oxidative chain reaction during the oxidation of lipids and other molecules (7-10).

In some cultures, particularly in the Orient, India and Africa, various plant-derived products have been used for centuries to treat several diseases. In the United States and Europe, the benefits of botanicals for humans are increasingly gaining recognition. It has been reported that 80% of the world population relies on plant-derived products for their primary health care need (11). Numerous Thai plants have been shown to have antioxidant activities such as free radical scavenging, lipid peroxidation inhibition, and antimutagenicity (12). However, little information is available for Thai local vegetables.

Gymnema inodorum Decne. (Chiangda) has been shown to be effective for treating some diseases such as diabetes mellitus, rheumatic arthritis, and gout. The extracts of *Gymnema inodorum* leaves has been found to suppress the intestinal smooth muscle contraction, to decrease the O_2 consumption, to inhibit the glucose and prevent the increased blood glucose level (13-14). The *in-vitro* studies have shown that *Gymnema inodorum* possesses a strong antioxidant activity. In addition, it has been reported that *Gymnema inodorum* consists of many phytochemical compounds that act as antioxidants (15). Since *Gymnema inodorum* contains varieties of antioxidant compounds, the assessment of its antioxidant properties is of great interest.

Therefore, I conducted this study to assess the antioxidant activities of *Gymnema inodorum* extracts and to determine whether or not *Gymnema inodorum* has a beneficial effect on ameliorating oxidative damage in human erythrocytes and lymphocyte DNA. This study should provide the information on the antioxidant properties of *Gymnema inodorum* in human. Moreover, the results of this study may shed some light on the use of this plant as dietary supplements to protect against oxidative stress.

OBJECTIVES

General objective

To determine the antioxidant capacity of *Gymnema inodorum Decne.*

Specific objective

1) To determine the antioxidant activities of various extracts of *Gymnema inodorum* edible portions

2) To determine the effects of *Gymnema inodorum* extracts on red blood cell hemolysis and DNA oxidative damage

CHAPTER II

LITERATURE REVIEW

2.1 FREE RADICAL AND OXIDATIVE DAMAGE

2.1.1 Free radical

A free radical is any chemical species that has an odd number of electrons. It contains one or more unpaired electron(s) which occupy an atomic or molecular orbit by itself. The presence of one or more unpaired electron(s) usually causes free radicals to be attracted slightly to magnetic field and sometimes makes them highly reactive (1-2).

Due to their high reactivity, free radicals such as reactive oxygen species are capable of causing either reversibly or irreversibly damage to compounds of all biochemical classes (Table 1), including nucleic acids, proteins and free amino acids, lipids and lipoproteins, and carbohydrates macromolecules (1-4).

2.1.1.1 Type of free radicals

There are numerous types of free radicals that can be formed in the biological system (Table 2) (1-2, 18). The major radical species of interest are those of oxygen centered free radicals or reactive oxygen species (ROS). The most common ROS include superoxide ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and peroxyl radical ($ROO^{\bullet}$). Hydroxyl radical is very active; therefore it is the most damaging radical in the body.

Table 1. Targets and consequences of cellular free radical attack*

Targets	Consequences
Small molecules	
Nucleic acid bases	Cell cycle change, mutation
Carbohydrates	Cell surface reporter changes
Unsaturated lipids	Cholesterol and fatty acid oxidation
	Lipid cross-linking
	Organelle and cell permeability changes
Cofactors	Decreased nicotinamide and flavin-containing cofactor availability and activity, ascorbate oxidation, porphyrin oxidation
Neurotransmitters	Decreased neurotransmitter availability and activity, including serotonin, epinephrine
Antioxidant (including α-tocopherol and β-carotene)	Decreased availability
Macromolecules	
Protein	Peptide chain scission, denaturation
DNA	Strand scission, base modification
Hyaluronic acid	Change in synovial fluid viscosity

* Modified from reference 4

Table 2. Examples of free radicals*

Name	Formula	Example
Trichloromethyl	$CCl_3^{\bullet}$	A carbon-centered radical (the unpaired electron resides on carbon; formed during metabolism of CCl_4 in the liver and contributes to the toxic effects of this solvent; usually react rapidly with O_2 to make peroxyl radicals, e.g. $CCl_3^{\bullet}+O_2 \longrightarrow CCl_3O_2^{\bullet}$
Superoxide	$O_2^{\bullet-}$	An oxygen-centered radical
Hydroxyl	$OH^{\bullet}$	A highly reactive oxygen-centered radical; attacks all biomolecules
Thiyl/perthiyl	$RS^{\bullet}$/ $RSS^{\bullet}$	A group of radicals with unpaired electrons residing on sulphur
Peroxyl, alkoxyl	$RO_2^{\bullet}$, $RO^{\bullet}$	Oxygen-centered radicals formed (among other routes) during the breakdown of organic peroxides and reaction of carbon radicals with O_2 ($RO_2^{\bullet}$)
Oxides of nitrogen	$NO^{\bullet}$, $NO_2^{\bullet}$	Nitric oxide is formed *in vivo* from the amino acid L-arginine; nitrogen dioxide is made when $NO^{\bullet}$ reacts with $O_2^{\bullet-}$; both are found in polluted air and smoke from burning organic materials, e.g. cigarette smoke
Nitrogen-centered radical	$C_6H_5N{=}N^{\bullet}$	Formed during oxidation of phenylhydrazine by erythrocytes, e.g. phenyldiazine radical
Transition-metal ions	Fe, Cu	Ability to change oxidation numbers by one allows them to accept/donate single electrons; often powerful catalysts of free-radical reactions

* Modified from reference 1

The three most important oxygen-centered radicals ($O_2^{\bullet -}$, $OH^{\bullet}$, and H_2O_2) can be produced by the activity of a variety of oxidation at different sites in the cell including cytosol, mitochondria, lysosome, endoplasmic reticulum, and plasma membrane.

Superoxide ($O_2^{\bullet -}$)

The superoxide appears to play a central role for other reactive intermediates are formed from it. Superoxide is formed upon the one-electron reduction of oxygen mediated by enzymes such as NADPH oxidase or xanthine oxidase or from the respiratory chain. Phagocytic cells, including neutrophils and macrophages, defend against foreign organisms by generating $O_2^{\bullet -}$ and nitric oxide as a part of the killing mechanism (19).

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is a secondary product of the one-electron autoxidation, via spontaneously or enzymatically catalyzed dismutation of $O_2^{\bullet -}$ (1-2, 20). H_2O_2 is the natural primary product of miscellaneous oxidases, mainly the peroxisomal oxidase and some mitochondrial enzymes. H_2O_2 is also generated by granulocytes during phagocytosis (2, 21-22). The decomposition of hydrogen peroxide to water and oxygen can be catalyzed by catalase and glutathione peroxidase (1-2, 20-21).

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

$$H_2O_2 + 2GSH \xrightarrow{\text{glutathione peroxidase}} 2H_2O + GSSG$$

Hydroxyl radical ($OH^{\bullet}$)

The hydroxyl radical is the most reactive oxygen species. It is formed *in vivo* from the univalent reduction of hydrogen peroxide by five molecules/compounds as follows: (a) $O_2^{\bullet -}$ in the Haber-Weiss reaction; (b) semiquinone radicals; (c) Fe^{2+} in the Fenton reaction; (d) NO; and (e) the metal ion-catalyzed Haber-Weiss reaction (1-2, 20-21). The $OH^{\bullet}$-producing reactions are shown below:

a. Haber - Weiss reaction

$$O_2^{\bullet -} + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\bullet}$$

b. Semiquinone$^{\bullet}$ + $H_2O_2 \longrightarrow$ Quinone + OH^- + $OH^{\bullet}$

c. Fenton reaction

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

d. $NO + H_2O_2 \longrightarrow HNO_2 + OH^{\bullet}$

e. Metal ion-catalyzed Haber-Weiss reaction

$$Fe^{3+}\text{-complex} + O_2^{\bullet -} \longrightarrow Fe^{2+}\text{-complex} + O_2$$

$$Fe^{2+}\text{-complex} + H_2O_2 \longrightarrow Fe^{3+}\text{-complex} + OH^- + OH^{\bullet}$$

$$\text{Net} \quad O_2^{\bullet -} + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\bullet}$$

2.1.2 Oxidative damage

Although cells and organisms have taken advantage of the reaction potential of both the molecular oxygen and its reduction products, radicals and reactive oxygen species play roles in cell damage. (23-26).

Indeed, a life requires a balance between the production of such potentially damaging molecular species and the scavenging as well as the destruction of those molecules to keep them at the lowest toxic level. Toxicity and diseases may result from an imbalance of the system. The cause of an imbalance may be due to the overproduction of free radicals or the decrease in the capacity of the physiological defenses that cope with the stress produced by free radicals.

2.1.2.1 Oxidative DNA damage

DNA is probably the most biologically significant target of oxidative attack. ROS readily interact with cellular macromolecules and structures, resulting in changes in membrane permeability, the activation of proteases and nucleases, and altered gene expression (38-39). It is also well documented that the cellular changes induced by ROS lead to DNA damage in a variety of cell types (40).

It is widely thought that continuous oxidative damage to DNA is a significant contributor to the development of the major age-related cancers, such as those of the colon, breast, rectum, and prostate (41). Therefore, oxidative DNA damage is a 'biomarker' for identifying persons at risk for developing cancers. Agents that decrease oxidative DNA damage should thus decrease the risk of cancer development.

Aerobic cells become exposed to major risks of oxidative damage if the production of radicals and/or reactive oxygen species increases above a critical level In principle, two distinct approaches, which are the detection of purine base oxidation products and the measurement of DNA strand break, have been employed for investigation of oxidative damage to cellular DNA. Specific types of oxidative DNA damage, known as DNA adducts, can be assessed by chromatographic assay coupled with electrochemical detection. In such types of oxidative DNA damage, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) has been one of the most studied products because of its ease of formation and premutagenic potential (42). Another approach is to measure DNA strand breaks or groups of oxidatively modified DNA in terms of enzyme sensitive sites by the single cell gel electrophoresis (comet) assay, alkaline elution, or alkaline unwinding.

2.1.2.2 Cell membrane damage (hemolysis)

Reactive oxygen species have been reported to damage erythrocytes in patients with blood pathology (43). Erythrocytes are highly susceptible to oxidative damage due to the high polyunsaturated fatty acid content of their membranes and the high cellular concentrations of oxygen and hemoglobin, a powerful potential promoter of oxidative process (44). ROS in either aqueous or lipid phase can attack erythrocyte membranes and can induce the oxidation of lipid and proteins, triggering disruption in the membrane and hemolysis (45-47).

Numerous investigators have used erythrocytes as a model to study biomembrane oxidative damage (45-49). The erythrocyte has several membrane systems to protect itself against oxidation damage and hemolysis; these systems include superoxide dismutase, glutathione peroxidase, and catalase. In addition, water-soluble chain-breaking antioxidants can scavenge oxygen radicals residing in the aqueous phase; whereas lipid-soluble scavengers can scavenge radicals within the lipid region of erythrocyte membrane (47).

2.2 ANTIOXIDANT DEFENSE SYSTEM

2.2.1 Antioxidant

An antioxidant is defined as a substance that, when present at low concentrations, significantly delays and/or prevents the oxidation of a substrate (23). An antioxidant can inhibit the free radical production via many mechanisms as follows:

1. removing oxygen or decreasing local O_2 concentration
2. chelating the transitional metal catalysts
3. breaking chain reaction
4. reducing the concentration of ROS/RON such as $O_2^{\bullet -}$, H_2O_2, HOCl, singlet O_2, and $ONOO^-$
5. scavenging radicals such as $OH^\bullet$, $RO^\bullet$, and $RO_2^\bullet$

Cells have formidable defenses against oxidative damage; many of which may at first not be like antioxidants. Many antioxidants have more than one mechanism of actions. The following results occur as the effects of antioxidant sequences (25):

1. preventing a radical formation
2. intercepting formed radicals
3. repairing oxidative damage and increasing the elimination of damaged molecules
4. promoting the death of cells with excessively damaged DNA, thus preventing the accumulation of transformed cells

2.2.2 Antioxidant defense system

The harmful effects of ROS and/or RNS can be prevented by antioxidative defenses, which are usually divided into two groups: enzymatic and nonenzymatic antioxidants (Table 3) (1-2, 21, 25).

Antioxidants are synthesized within the body as well as are obtained from foods such as fruits, vegetables, seeds, nuts, meats, and oil. There are two lines of antioxidant defense within the cell. The first line, found in the fat-soluble cellular membrane, consists of vitamin E, β-carotene, and coenzyme-Q. The second line is oxygen scavengers present inside the cell wall, including vitamin C, glutathione peroxidase, superoxide dismutase, and catalase (23, 25-27).

Table 3. Some biologically important antioxidants and their actions*

	Mode of action
Enzymatic antioxidant	
Superoxide dismutase	Catalytic removal of $O_2^{\bullet -}$
Catalase	Catalytic removal of H_2O_2 at high concentrations (catalatic activity); peroxidatic activity when the electron donors are methanol, ethanol, formate, and nitrite
Glutathione peroxidase	Catalytic removal of H_2O_2 and lipid hydroperoxidases
Nonenzymatic antioxidants	
Vitamin E	Lipid soluble, chain-breaking antioxidant; protection of lipoproteins and lipids in the plasma
β-carotene	Singlet oxygen and $OH^{\bullet}$ radical scavenger; inhibitor of lipid peroxidation under certain conditions
Vitamin C	Free radical scavenger; singlet oxygen quencher; regeneration of vitamin E
Glutahione	Catalytic removal of hydrogen peroxide; hydroxyl radicals quencher; singlet oxygen quencher; regeneration of vitamin E and vitamin C
Transferrin	Binding ferric ions
Lactoferrin	Binding ferric ions and retaining them at low pH

Modified from reference 38

2.2.2.1 Natural antioxidants

Studies on the influence of diets on chronic diseases have linked the beneficial effects to the high consumption of mixed fruits and vegetables. Consuming a diet rich in plant foods provides a milieu of phytochemicals, which are nonnutritive substances in plants that possess health-protective benefits. Nuts, whole grains, and vegetables contain an abundance of phytochemicals such as phenolic compounds, terpenoids, carotenoids, xanthophylls, tannins, phytoesterols, and natural antioxidants. Those compounds have been associated with protection from and/or treatment of chronic diseases such as heart disease, stroke, hypertension, diabetes, cataracts, cancers as well as other medical conditions (29-30). The number of phytonutrients is very large, suggesting that there are a variety of compounds in foods that elicit an array of biologic activities that may be associated with reduction in rates of degenerative processes. Therefore, the growing concerns for general health, chronic disease prevention, and aging has fuelled consumers' interest in phytochemicals in plants.

2.2.2.2 Some of natural antioxidant agents

Vitamin E

Vitamin E is a lipid-soluble antioxidant, which is the major natural antioxidant in foods and is important for the stability of vegetable oils. It occurs in eight different forms including α-, β-, γ-, and δ-tocotrienols. The antioxidant efficacy of vitamin E isomers decreases in order as follows: $\delta > \gamma > \beta > \alpha$; whereas α-tocopherol is the most effective vitamin E (31-34).

Vitamin E is the principal component of the nonenzymatic defense mechanism against free radicals. In fact, it is the only natural physiological lipid-soluble antioxidant that can inhibit lipid peroxidation in cell membrane (1, 35).

Vitamin E is a potent peroxyl radical ($LOO^\bullet$) scavenger and can protect polyunsaturated fatty acids, within membrane phospholipids and in plasma lipoproteins, from oxidation. Under most conditions, the action of vitamin E in biological membranes is to donate the labile hydrogen to lipid peroxyl ($LOO^\bullet$) and alkoxyl ($LO^\bullet$) radicals. The termination of chain propagation is shown below (31, 33):

$$LOO^\bullet + \alpha\text{-tocopherol} \longrightarrow \alpha\text{-tocopherol}^\bullet + LOOH$$

$$LO^\bullet + \alpha\text{-tocopherol} \longrightarrow \alpha\text{-tocopherol}^\bullet + LOH$$

Vitamin E is reactive to abstract hydrogen radicals ($H^{\bullet}$) from oxidized membrane lipids, forming the tocopherol radical. The unpaired electron on the oxygen atom in the tocopherol radical can be delocalized into the aromatic ring structure, producing the non-radical α-tocopheryl quinone (32, 35). It has been reported that after the neutralization of free radicals and the termination of chain reaction, vitamin E can be regenerated by ascorbic acid and glutathione. (31-32).

Vitamin C (Ascorbic acid)

Vitamin C, which is a water-soluble antioxidant, interacts with free radicals ($OH^{\bullet}$, $LOO^{\bullet}$, H_2O_2) in the intracellular and extracellular fluid. It has a unique 2,3-enediol moiety in the five-member ring and possess a strong electron-donating ability. Donation of one electron by ascorbate gives the semidehydroascorbate radical; then it can be further oxidized to dehydroascorbate (34). The semidehydroascorbate radical is not particularly reactive and mainly undergoes a disproportionate reaction; two molecules of semidehydroascorbate yield ascorbate and dehydroascorbate. Dehydroascorbate is unstable and broken down rapidly in a very complex way; eventually, oxalic acid and L-threonic acid are produced (34).

Ascorbic acid ($AscH^{-}$) reacts rapidly with hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($LOO^{\bullet}$), and superoxide radical ($O_2^{\bullet -}$), and give the semidehydroascorbate radical ($Asc^{\bullet -}$) and dehydroascorbate (DHA) as shown below (31-33):

$$AscH^{-} + OH^{\bullet} \longrightarrow H_2O + Asc^{\bullet -}$$

$$AscH^{-} + O_2^{\bullet -} + H^{+} \longrightarrow H_2O_2 + Asc^{\bullet -}$$

$$AscH^{-} + LOO^{\bullet} \longrightarrow LH + Asc^{\bullet -}$$

$$AscH^{-} + H_2O_2 + H^{+} \longrightarrow 2H_2O + DHA$$

Carotenoids

The carotenoids, including β-carotene, γ-carotene, and lycopene, are lipid-soluble antioxidants (31, 34). β-Carotene is the most prominent representative of this lipophilic class of compounds. It is referred to as a pro-vitamin A because of its ability to be metabolized in animals to vitamin A. β-Carotene is effective as an antioxidant by quenching singlet oxygen and/or free radicals that are formed during lipid oxidation (36-37) and by scavenging of reactive oxygen species (e.g. oxyhalides, sulfite, and fenton -reaction-generated radicals) (32-33, 35).

In addition, β-carotene is efficient in a chain termination at low partial oxygen pressures. In the presence of peroxyl radicals, β-carotene produces a carbon-centered carotenyl radical (β-car)$^{\bullet}$, which in the absence of oxygen, is an efficient chain terminator (1-2).

$$\beta\text{-carotene} + ROO^{\bullet} \longrightarrow ROO\text{-}\beta\text{-car}^{\bullet}$$

$$ROO\text{-}\beta\text{-car}^{\bullet} + ROO^{\bullet} \longrightarrow \text{inactive products}$$

Phenolic compounds

Phenolic compounds, which are widely distributed in plants, are important in contributing to flavors and colors of many fruits and vegetable products. The term phenoloic compound embraces a wide range of substances, which possess an aromatic ring bearing one or more hydroxyl substiuents. They frequently attach to sugar (glycosides) and methoxyl groups (29-30).

Many polyphenols, other than vitamin E, exert a powerful antioxidant effect *in vitro*, inhibiting lipid peroxidation by acting as a chain-breaking peroxyl-radical scavenger. Like β-carotene, many plant phenolics are good inhibitors of lipid peroxidation. Phenols can also directly scavenge ROS, such as $OH^{\bullet}$, $LOO^{\bullet}$ and $O_2^{\bullet-}$. Phenols with two adjacent OH groups or other chelating structures can also bind transition metal ions (especially iron and copper) to form less active free-radical promoters. The chelating ability of polyphenol can interfere with metal absorption in the diet.

2.2.3 Measurement of antioxidant activity

Antioxidant activity (either capacity or potential) is widely used as a parameter, together with others, to characterize different plant materials such as fruits, vegetables, wines, teas, and oil. Such activity is related with compounds that are capable of protecting a biological system against the harmful oxidation (50). Several methods are used to measure the antioxidant activity of biological materials. The most common methods used for their ease, speed and sensitivity are those involving chromogen compounds of a radical having a nature to simulate RONS. The presence of antioxidants leads to the disappearance of the radical chromogens (50).

A variety of experiments (Figure 1) can be performed to examine the antioxidant ability *in vitro* (52). Antioxidant indices based on the ability to scavenge peroxyl radicals provide a support for the antioxidant efficacy *in vitro* (52). However, demonstrating the antioxidant efficacy *in vivo* requires the use of valid *in vivo* models since results from the *in vivo* methods enable an assessment of the potential *in vivo* efficacy.

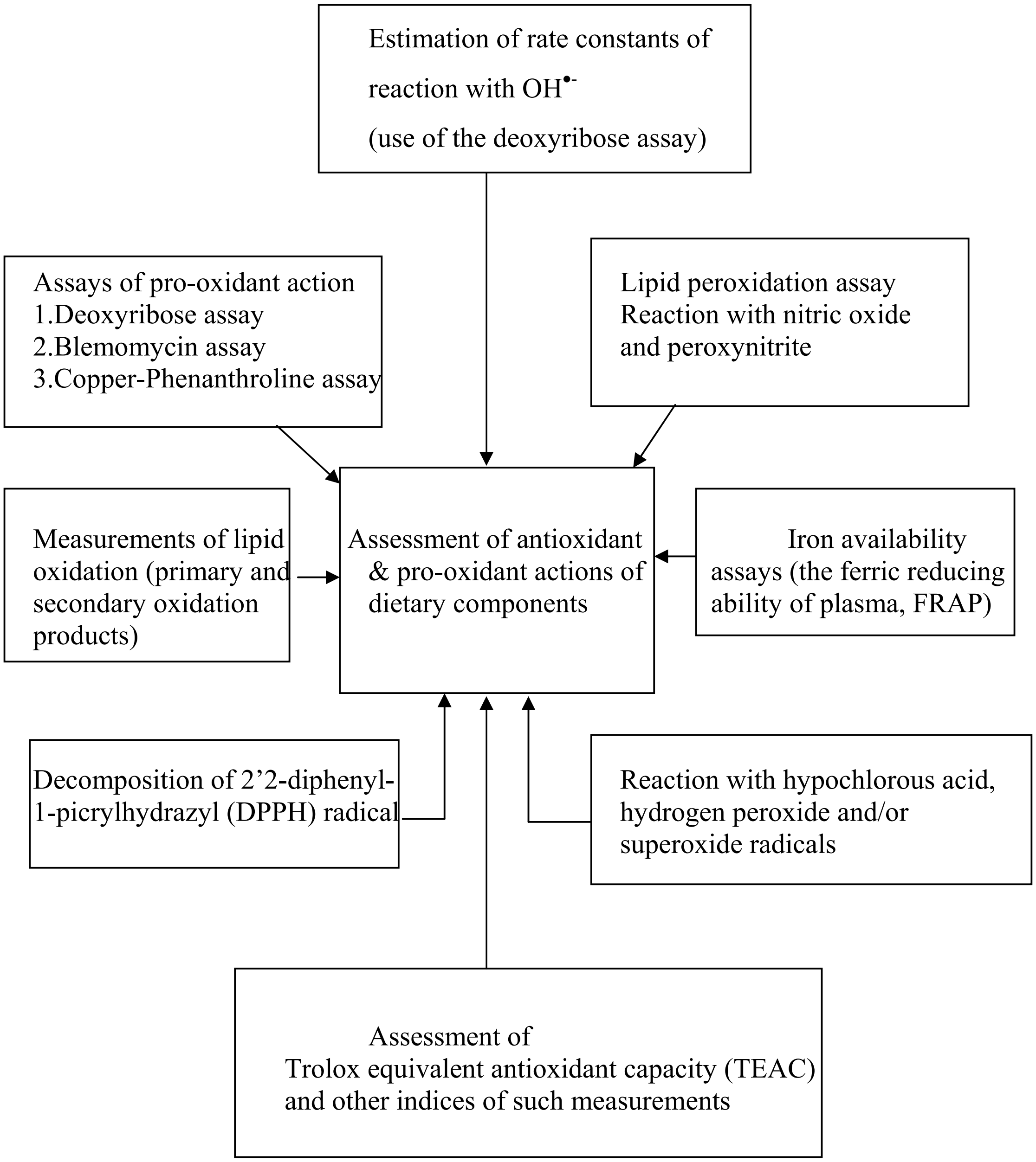


Figure 1. Profile of various analytical methods (modified from reference 50)

DPPH assay

This method is based on the scavenging of the stable radical of 2, 2-diphenyl-l-picrylhydrazyl (DPPH$^{\bullet}$). It has been used extensively to predict the antioxidant activities of phenols, catechols, and aromatic amines. The accepted mechanism of reaction is abstracting a hydrogen atom from a phenol donor to give diphenyl picrylhydrazine and a phenoxyl radical. The reaction (Figure 2) involves the change of color from violet to yellow (51).

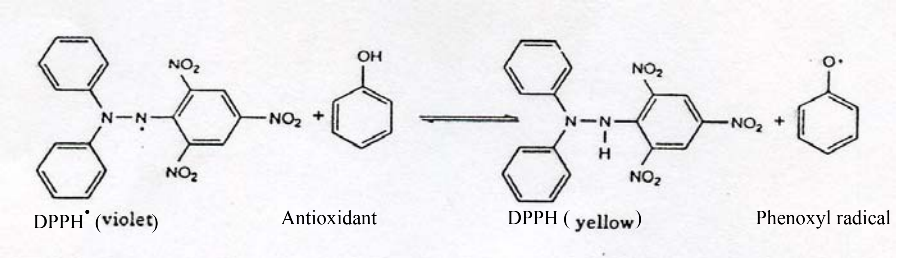


Figure 2. Reaction of DPPH radical scavenging

The reduction in the concentration of the DPPH$^{\bullet}$ is assessed by monitoring the decrease in its absorbance at a characteristic wavelength during the reaction. The color of DPPH$^{\bullet}$ is absorbed at 515 nm; but upon reduction by an antioxidant or a radical species (R$^{\bullet}$), the color disappears. The antiradical activity is defined as the amount of an antioxidant necessary to decrease the initial DPPH$^{\bullet}$ concentration by 50% (52).

Deoxyribose assay

The pro-oxidant actions in the deoxyribose system rely on the ability of the compounds to promote reduction of Fe^{3+} to Fe^{2+}; thereafter, $OH^{\cdot}$ is produced in the presence of H_2O_2. The deoxyribose assay therefore allows the determination of rate constants of reactions with $OH^{\bullet}$ as well as the assessments of abilities to exert a pro-oxidant action and abilities to chelate metal iron.

When iron is added to the assay mixture, some of the Fe^{3+} ions bind to deoxyribose and cause damage to the sugar since the $OH^{\bullet}$ formed by bound iron ions immediately attacks the deoxyribose. However, iron ions are equally available to both the deoxyribose and the compounds under test. Thus, compounds that are able to preferentially chelate iron can protect deoxyribose against damage in the presence of H_2O_2, producing metal complex in a less redox-active form. In addition, substances that have the antioxidant activity are able to bind iron ions strongly enough to remove iron ions from deoxyribose. Therefore, the deoxyribose assay can be used to determine whether or not molecules can chelate iron ions in a way that prevents them from catalyzing $OH^{\bullet}$ formation. The oxidation degradation can be detected by heating the products with 2-thiobarbituric acid (TBA) under the acid condition, a pink chromogen (thiobarbituric acid reactive species, TBARS) is produced in the reactions. The ability of a substance to inhibit deoxyribose degradation under these reaction conditions is measured by its ability to interfere with the site-specific Fenton chemistry (52, 55).

$$\begin{array}{lcl} Fe^{2+} + H_2O_2 & \xrightarrow{\qquad\qquad} & Fe^{3+} + OH^{\bullet} + OH^{-} \\ \text{Deoxyribose} + OH^{\bullet} & \xrightarrow{\qquad\qquad} & \text{Oxidized products} \\ \text{Oxidized products} + \text{TBA} & \xrightarrow[\Delta]{100^{o}C} & \text{TBARS} \end{array}$$

Hemolysis

Biological membranes are the targets for free radical damage due to their high content in polyunsaturated fatty acids and proteins. The membrane of intact erythrocytes is one of the examples, containing high concentrations of polyunsaturated fatty acids together with molecular oxygen, iron ions in the ligand state and antioxidant system (47-49). Isolated red blood cells can be readily stressed by various pro-oxidant species such as azo-compounds under thermal decomposition; those azo-compounds generate peroxyl radicals ($ROO^{\bullet}$) that are the main oxidant species responsible for cell hemolysis via both lipid peroxidation and protein oxidation (49):

$$\begin{array}{lcll} R\text{-}N = N\text{-}R & \xrightarrow{\qquad} & N_2 + R^{\bullet} + R & \text{carbon-centred radicals} \\ R^{\bullet}\cdot + O_2 & \xrightarrow{\qquad} & ROO^{\bullet} & \text{peroxy radicals} \end{array}$$

Finally, the end point of such oxidative attack can be determined by the extent of hemolysis and the quantitative measurement of hemoglobin released from damage cells.

Single cell gel electrophoresis (comet assay)

The comet assay, so called the single cell gel assay (SCG) or microgel electrophoresis (MGE), was first introduced by Singh et al (56) as a microelectrophoretic technique for the direct visualization of DNA damage in cells (57-58). A small number of irradiated cells suspended in a thin agarose gel on a microscope slide are lysed, electrophoresed, and stained with a fluorescent DNA binding dye. The electric current pulls the charged DNA from the nucleus that is relaxed and broken DNA fragments migrates further. The resulting images, which are subsequently named for their appearance as “comet”, are measured to determine the extent of DNA damage. Ostling and Johanson (57) observed that the extent of DNA liberated from the head of the comet during electrophoresis was a function of the dose of irradiation.

In the past ten year, there has been increasing interest in the comet assay because the unique of the comet assay provides direct determination of the extent of DNA damage in individual cells. Heterogeneous response by cells during treatment can aid in the prediction of tumor response to specific treatment protocols since it is possible to identify small populations of cells which may be resistant. The single cell gel assay has also been used to examine DNA damage and repair under a variety of experimental conditions. The technique may prove to be valuable in the dissections of the mechanisms of genotoxicity and DNA repair (58).

Procedures for measuring DNA strand breaks are generally based upon the principle that strand-breaking agents reduce the size of the large duplex DNA molecules. Assays that are sensitive to detect DNA strand breaks generally require unwinding of the double-stranded DNA molecules. A high pH (>13) is generally utilized to facilitate denaturation, unwinding, and expression of single-stranded breaks (so-called alkali labile lesions) (58).

Comets from the broken ends of the negative-charged DNA molecules become free to migrate in the electric field towards the anode. The ability of DNA to migrate is a function of both the size of the DNA and the number of broken ends which may be attached to large pieces of DNA but still be able to migrate a short distance from the comet head. The tail length initially increases with damage but reaches a maximum that is largely defined by the electrophoresis condition, not the size of the fragments. At low damage levels, stretching of attached strands of DNA, rather than migration of individual pieces, is likely to occur. With increasing numbers of breaks, DNA pieces migrate freely into the tail of the comet, and at the extreme (the apoptotic cell), the head and tail are well separated. The intensity of the fluorescence in the tail relative to the head provides information about the numbers of strand breaks. These two concepts, stretching and migration of strands, are generally accepted to explain the DNA migration patterns observed in the comet assay (58).

2.3 *GYMNEMA INODORUM* DECNE.

Gymnema inodorum Decne. (Chiangda) is a type of Thai local vegetables that grows in the northern part of Thailand. *Gymnema inodorum* belongs to the Ascalepiadaceae Family (Figure 3).

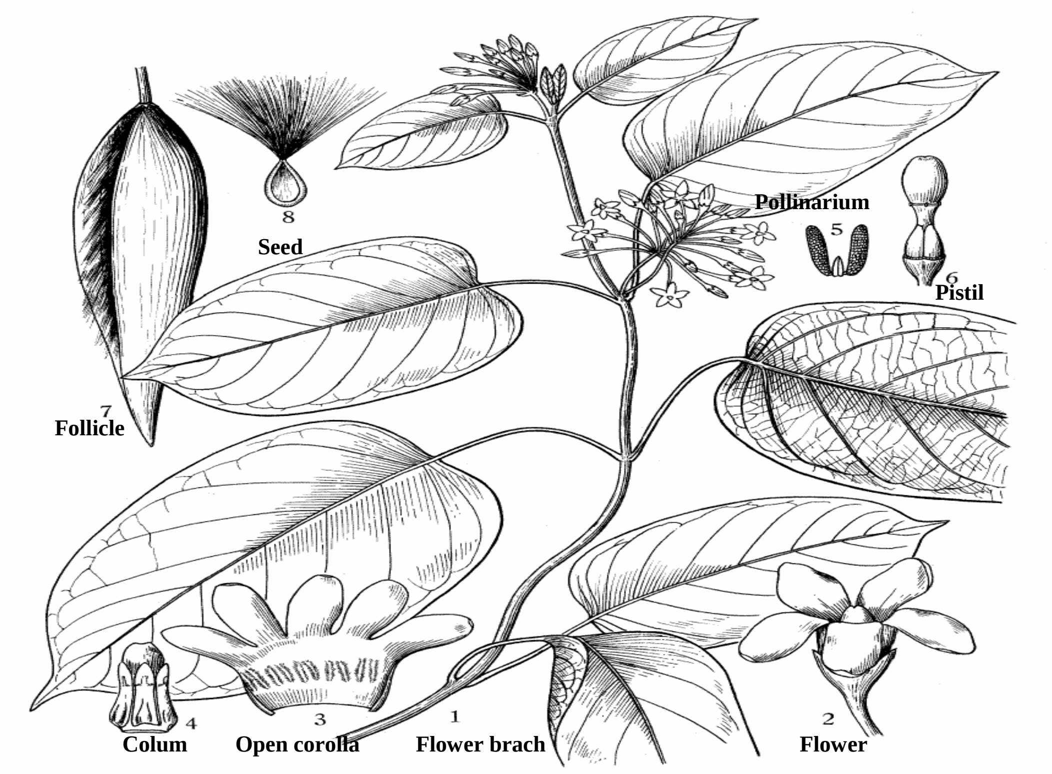


Figure 3. *Gymnema inodorum* Decne.

It contains many nutrients and chemical constituents such as gymnemic acid (12-14), β-carotene, vitamin C, vitamin E, tannin, xanthophyll, and phenolic substances (Table 4) (15). It has been known that this vegetable is effective for treatment of several diseases including diabetes mellitus, rheumatic arthritis, and gout. The extracts of *Gymnema inodorum* leaves suppress the intestinal smooth muscle contraction, decrease the O_2 consumption, inhibit the glucose absorption as well as prevent the increase of blood glucose level (12-14). In Japan, *Gymnema inodorum* leaves are used for drinking by adding in foods and beverage for diabetes prevention and for blood sugar reduction in diabetic patients (13-14). Besides diabetes prevention and blood sugar reduction, the *Gymnema inodorum* roasted tea is shown to be rich in vitamins (especially vitamin C, vitamin E, and β-carotene), amino acids, and essential minerals necessary for the adjustment of biological functions (Table 5) (17).

Table 4. Nutritive values of *Gymnema inodorum* Decne.*

Nutrients	**Nutrient composition (per 100 g edible portion)**
Proximal composition (grams)	
Protein	5.4
Fat	1.5
Carbohydrate	8.6
Dietary fiber (Crude fiber)	2.5
Minerals (milligrams)	
Calcium	78.0
Phosphorus	98.0
Iron	2.3
Vitamins (micrograms)	
Beta-carotene	5905.0
Total vitamin A	984.0
Thiamin	0.12
Riboflavin	0.35
Niacin	1.0
Vitamin C	153.0

* Modified from reference 16

Table 5. **Nutrient content in *Gymnema inodorum* roasted tea***

Nutrient	**Content (per 100 g dry weight)**
Vitamin (milligrams)	
Beta-carotene	6.09
Vitamin B_1	0.11
Vitamin B_2	1.10
Vitamin C	42
Vitamin E	36.5
α-tocopherol	13.2
β-tocopherol	18.5
γ-tocopherol	0.9
δ-tocopherol	3.9
Nicotinic acid	8.8
Essential amino acids (milligrams)	
Lysine	30
Phenylalanine	26
Leucine	33
Isoleucine	28
Valine	60
Tryptophan	17
Minerals (milligrams)	
Phosphorus	394
Iron	59.8
Calcium	1.75
Sodium	11.6
Potassium	4.01
Magnesium	544
Copper	7.00
Zinc	34.2
Manganese	163
Nickel	2.37
Selenium	0.19
Aluminium	432
Boron	52

* Modified from reference 17

It has been reported that *Gymnema inodorum* has a high antioxidant index (15) and high content of β-carotene (16).Likewise, *Gymnema inodorum* has been found to have a strong antioxidant activity in *in vitro* analysis. However, the antioxidant effects of *Gymnema inodorum* on biological samples including erythrocytes and DNA oxidation have never been studied. The damage of erythrocytes and DNA may induce the deterioration of functions in many organs leading to diseases in human.

CHAPTER III
MATERIALS AND METHODS

3.1 SAMPLE PREPARATION

3.1.1 Vegetable preparation

Edible parts of *Gymnema inodorum* (GI) were obtained from Chiangda Farm in Nayaiarm district, Chantaburi province of Thailand, harvested in April 2004 and identified by Department of Agriculture, Ministry of Agriculture and Cooperatives.

3.1.2 Sample preparation

Edible parts of *Gymnema inodorum* (GI) including leaves were washing three times by tap water. After that, they were separated into two parts. One was prepared as juice by homogenizing 500 gram of fresh GI with 500 mL of deionized water at 8,000 rpm for 20 min. The supernatant was filtered through Whattman No. 1 filter paper to obtain the crude juice, and then freeze-dried into powder (**GIJ**). The other part, weighed 5 kg, was dried at 50°C in a cross-flow air drier for 20 hours. The dried preparation (1.5 kg of GI parts remained) was grounded in a multimil and passed through a 0.5-mm sieve, then the 60-g preparation was extracted with 3 L of boiling deionized water for 2 hours. The extract was then filtered through Whattman No.1 filter paper and freeze–dried into powder (**GIW**). As for organic solvent extraction, the 60 g of dry-grounded GI was extracted with 3 L of 50% ethanol and 95% ethanol for 2 hour**,** and followed by filtration and evaporation at approximately 60°C. The organic extracts were named as **GIE50** and **GIE95**, respectively. The yields of 4 extracted fractions (GIJ, GIW, GIE50, and GIE95) were 27.00%, 10.80%, 14.50% and 5.25%, respectively. All of the extracts were sealed in foil and stored at -4°C until analysis.

3.2 DETERMINATION OF BIOACTIVE COMPOUNDS

3.2.1 Determination of the level of total phenolic compounds

Total phenolic compounds in the extracts were determined by the Folin-Ciocalteu method with some modification (53). Two hundred and fifty mg of GI parts were put in a 25-mL volumetric flask and 15 mL of 40% ethanol was added, then mixed and sonicated for 10 min. Afterwards, the mixtures were cooled down to room temperature and adjusted volume with 40% ethanol. The mixtures were filtrated with No.1 Whattmam filter paper; and finally, about 10 mL of clear solution from each mixture was obtained. One mL of each solution was drawn into a 100-mL volumetric flask containing HPLC grade water; the contents were mixed by swirling, and 5 mL of Folin-Ciocaiteu's phenol reagent was added. After 1 min, 15 mL of 2% sodium carbonate solution was added, and make the volume up to 100 mL with HPLC grade water. After 2 hours (time zero when 2% sodium carbonate was added), the UV absorption was recorded at 760 nm using a spectrometer (UV-Visible Spectrometer GBC Cintra 4.0).

Gallic acid was used as standard and the total phenolic content of the samples was expressed as gallic acid equivalents (GAE); therefore, the phenolic content was reflected as the amount of gallic acid (mg) in 100 g of sample.

3.2.2 Determination of the levels of antioxidant vitamins

Vitamin E and beta-carotene levels were determined using the method of Official Method of Analysis of AOAC International by High Performance Liquid Chromatography (75).

3.3 DETERMINATION OF ANTIOXIDANT ACTIVITY

3.3.1 Scavenging activity using the diphenyl-1-picrylhydrazyl (DPPH) radical assay

The DPPH-radical scavenging activity was assessed using the method described by Yamaguch et al (60) with some modifications. An 50-µL aliquot of 2 mM DPPH radical solution dissolved in methanol was mixed with 0.5 mL of sample extracts at various concentrations and DPPH in methanol was used as control. The decrease in absorbance at 515 nm was monitored continuously by capturing at 30-sec intervals until 15 min (constant absorbance) with a spectrophotometer (UV-Visible Spectrometer GBC Cintra 4.0) and trolox was used as standard. All measurements were performed in triplicate. The DPPH scavenging capacity of GI extracts were expressed as percentage of inhibition (% inhibition) using the following equation:

$$\%\ \text{inhibition} = \{1-(\text{Ab}_{515}\ \text{sample}/\text{Ab}_{515}\ \text{control})\} \text{ x } 100$$

Percentage of fall in absorbance against concentration was plotted and extrapolated to find the inhibition concentration of samples at the 50% fall in absorbance of DPPH (IC_{50} values).

3.3.2 Inhibitory activity on deoxyribose degradation (the deoxyribose assay)

The deoxyribose assay was used to evaluate the antioxidant activity against hydroxyl radicals ($OH^{\bullet}$). Hydroxyl radicals was generated by reacting ferrous salt (Fe^{3+}) and H_2O_2 with some modification (55).

$$\begin{aligned}
&Fe^{2+} + H_2O_2 \xrightarrow{\qquad\qquad} Fe^{3+} + OH^{\bullet} + OH^{-}\\
&\text{2-Deoxyribose} + OH^{\bullet} \xrightarrow{\qquad\qquad} \text{Oxidized product}\\
&\text{Oxidized product} + \text{TBA} \xrightarrow[\triangle]{100^{\circ}C} \text{TBARS*}
\end{aligned}$$

After mixing 200 µL of 5 mM 2-deoxy-D-ribose with 10 mM phosphate buffer (pH 7.4), and adding various concentrations of GI extracts (3-0.015 mg), the mixture was at the final volume of 1 mL. The $OH^{\bullet}$-radical generating system (50 µL of 200 µM ferrous salts and 50 µL of 100 µM H_2O_2) was then added to the mixture. The reaction mixture was mixed, and then incubated in a water bath (EYEL 4 water bath) at 37°C for 30 min to increase the reaction. After incubation, 500 µL of 2.8% trichloroacetic acid (TCA) (w/v) and 500 µL of 2% thiobarbituric acid (TBA) (w/v) were added into the reaction mixture, followed by heating in a water bath at 100°C for 15 min. The developed chromogen was determined using a fluorescence spectrometer (Luminiescence Spectrometer LS 55 Perkin Elmer) at 533 nm emission and 515 nm excitation.

* Thiobarbituric acid reactive substance

All determinations were performed in triplicate. The hydroxyl-radical scavenging capacity of GI extracts was expressed as percentage of inhibition (% inhibition) which was calculated using the following equation:

$$\% \text{ inhibition} = \left\{ 1-(\text{Em}_{533}\text{Ex}_{515}\text{sample}/\ \text{Em}_{533}\text{Ex}_{515}\text{control}) \right\} \times 100$$

The decrease in the pink chromogen indicated the decreased oxidation of deoxyribose. The percentage of inhibition of deoxyribose degradation was plotted against concentrations and extrapolated to find the concentration of samples at the 50% inhibition (IC_{50} values).

3.3.3 Inhibitory effect on red blood cell hemolysis

Blood samples from healthy volunteers were obtained by venipuncture. Blood was collected in heparinized tubes and centrifuged at 2500 rpm for 10 min (Refrigerate KUBOTA 5900 centrifuge); then, plasma and buffy coat were removed by aspiration. The erythrocytes (RBC) were washed three times with 10 mM phosphate buffer (PBS) at pH 7.4; after that RBC were 10% suspension in PBS solution.

The oxidative hemolysis in RBC was induced by a peroxyl-radical initiator, 2, 2’-azo-bis-(2-amidino-propane) dihydrochloride (AAPH) (47). AAPH added to a suspension of RBC causes the oxidation of lipids and proteins in the cell membrane and thereby induces hemolysis. The AAPH-induced hemolysis in RBC is a function of the incubation time and is proportionate to the concentration of free radicals. The inhibitory effect against RBC hemolysis is also proportionate to the concentration of antioxidants in the mixture. One hundred μL of PBS solution containing various amounts of GI extracts (0.015-3 mg) was mixed with 100 μL of RBC suspension; then 100 μL of 200 mM AAPH was added in to the mixtures. The reaction mixture was shaken gently while being incubated at 37°C for 3 hour in a water bath (EYEL 4 water bath). After incubation, 4 mL of PBS solution was added into the reaction mixture followed by centrifugation (Refrigerate KUBOTA 5900 centrifuge) at 3000 rpm for 10 min. The absorbance of supernatant was read at 540 nm using a spectrophotometer (UV-Visible Spectrometer GBC Cintra 4.0). Similarly, the reaction mixture was treated with 4 mL of distilled water to achieve the complete hemolysis; and the absorbance of supernatant obtained after centrifugation was measured at 540 nm. The inhibition percentage of hemolysis was calculated using the following equation:

$$\% \text{ inhibition} = (1\text{-}A_{\text{control or antioxodant}} / B_{\text{complete hemolysis}}) \times 100$$

The percentage of inhibition was plotted against concentrations and extrapolated to find the concentration of samples at the 50% inhibition (IC_{50} values).

3.3.4 Effect on H_2O_2-induced oxidative DNA damage using the comet assay

Hydrogen peroxide (H_2O_2) has been a well known chemical for studying oxidative damage in cells. It can penetrate through cell membrane and reacts with cellular molecules, particularly DNA in the nucleus. The comet assay is the method to detect the migration of damaged DNA in individual cells under electrophoretic conditions. Under a microscope, a cell has the appearance of a comet with a head (the nuclear region) and a tail containing DNA fragments migrating in the direction of the anode.

3.3.4.1 Cell line and culture procedure

The TK6 human lymphoblastoid cell line (ATCC CRL-8015) was purchased from the American Type Culture Collection (USA). The TK6 cells were cultured as suspension in RPMI-1640 media supplemented with 10% heated inactivated horse serum (HS). The cells were grown in a 75-cm^2 culture flask and incubated at 37°C in a incubator with 5% CO_2 in air atmosphere. They were maintained by addition of fresh media or replacement of fresh media. Alternatively, the cell deusity was established by centrifugation with subsequent resuspension at 1-2 x 10^5 viable cells/mL. The cell density between 10^5-10^6 viable cells/mL was used for experiments.

3.3.4.2 Slide preparation for comet assay

The procedure for slide preparation performed using the standard technique was described by Singh et al (56) with some modifications. Regular slides were coated with 1% normal melting point (NMP) agarose gel in phosphate buffered saline (PBS) and allowed to dry overnight.

3.3.4.3 Cell treatment and determination of viability

After the overnight culture, cells were adjusted to the density of 4 x 10^5/mL in fresh media. One mL of the cell suspension was put in an eppendorf tube and centrifuged at 3000 rpm for 2 min to remove the media. Afterwards, cells were treated in the media at a total volume of 1 mL containing different concentrations (0.2, 0.5, and 1 mg/mL) of GI extracts whereas the control contained only media. The treated cells were incubated at 37°C in the incubator with 5% CO_2 for 1 hour. Then the cells were harvested after centrifugation at 3000 rpm for 2 min. The supernatant was removed and cells were resuspened in 1 mL of the medium containing 50 µM H_2O_2 and incubated at 4°C for 5 min to allow the induction of oxidative damage. The treated cells were collected after centrifugation at 3000 rpm for 2 min and then washed twice with fresh media to remove the residual H_2O_2. To distinguish from cytotoxicity effect, the viability of TK6 cells was checked during the experiments using trypan blue stain. In this study, the viability rate of above 70% was accepted for the comet assay.

3.3.4.4 Comet slide preparation

Following treatment, cells were resuspened in 75 µL of 0.5% low melting point (LMP) agarose in PBS, and the cell mixture was immediately layered onto a prepared glass microscope-slide precoated with 0.75% NMP agarose as described. The slides were gently covered with the coverslips and placed on a cold flat surface to allow the agarose to solidify for about 5 min. The coverslips were gently removed by sliding them sideways from the slides, and 80 µL of 0.5% LMP agarose was spread on glass slides, recovered with the coverslips, and left on cold surface for agarose to solidify.

3.3.4.5 Lysing, Unwinding and Electrophoresis

The coverslips were gently removed and slides were submerged into freshly prepared lysis solution (2.5M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10; (4°C) for 2 hour. After lysis, the slides were equilibrated in the freshly prepared electrophoresis buffer containing alkaline buffer (300 mM NaOH, 1 mM EDTA, pH > 13; (4°C) to allow unwinding of double-stranded DNA for approximately 20 min. The slides were then transferred into an elctrophoresis unit with the same buffer and subjected to an electrophoretic field at

300 mA and 25 V at 4°C for 20 min. The level of the electrophoresis buffer was adjusted in order to achieve 300 mA.

3.3.4.6 Neutralization and DNA staining

Following electrophoresis, the slides were neutralized in 0.4M Tris (pH 7.5) for 5 min three times. After removing the neutralization buffer, the slides were washed with cold water and allowed to dry at room temperature. The DNA was stained with 50 µL of 0.2% ethidium bromide.

3.3.4.7 Comet scoring

Each slide was covered with a coverslip and visualized under a fluorescence microscope (Olympus BX51, Japan). Comet images were acquired with a CCD camera connected to a computer and was analyzed using the automated comet analysis imaging system (Comet III analysis software, Perceptive Instruments, Halstead, UK). In each experiment, at least 50 randomly selected cells from each slide were analyzed. The DNA damage in comet cells was expressed as the tail length (TL, the distance of DNA migration measured from the center of the nucleus towards the end of the tail), tail density (TD, the percentage of DNA in tail) and tail moment (TM, a measure of tail length x % of DNA in tail).

3.4 STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS for window version 11.0 software program (SPSs Inc., USA.). The mean values were analyzed using one-way analysis of variance (one-way ANOVA); and Tukey multiple comparison was used to detect a significant differences between the means. The significant difference between means at the level of 0.05 (p-value < 0.05) were considered as significance. Correlation were analyzed by Spearman' correlation coefficient in bivariate correlation

CHAPTER IV

RESULTS

4.1 CONTENTS OF BIOACTIVE COMPOUNDS

4.1.1 Total phenolic compounds content and yields of *Gymnema inodorum* extracts

The content of phenolic compounds in *Gymnema inodorum* extracts are expressed as grams of gallic acid equivalents (GAEs)/100 g of freeze-dry extract using the regression equation of calibration curve ($y = 2.0163x+0.0029$, $R^2 = 0.9953$). The total phenolic contents in various fractions of *Gymnema inodorum* extracts are shown in Table 6. The concentrations of total phenolic content in the GIJ and the GIW were not significantly different (3.43 ± 0.011 and 3.35 ± 0.02 g GAEs/100 g, respectively). The highest value of phenolic contents was found in GIE50 (3.95 ± 0.01 g GAEs/100 g) ($p<0.05$), whereas GIE95 had the lowest one (2.44 ± 0.005 g GAEs/100 g).

4.1.2 The content of antioxidant vitamins in *Gymnema inodorum* extracts

In this study, the contents of antioxidant vitamins are expressed as unit of mg vitamin content per 100 g freeze-dry extract (Table 7). The GIJ contained the highest levels of vitamin E and β-carotene (22.6 and 25.94 mg/100 g freeze-dry extract, respectively). The GIW contained the lowest levels of vitamin E (1.6 mg/100 g freeze-dry extract) and the lowest level of β-carotene are in the GIE50 0.009 mg/100 g freeze-dry extract).

Table 6. Total phenolic contents in *Gymnema inodorum* (GI) extracts

Extract	**Total phenolic content** [1] (gGAEs/100 g of freeze-dry extract)
GIJ	3.43 ± 0.011^a
GIW	3.35 ± 0.020^a
GIE50	3.95 ± 0.011^b
GIE95	2.44 ± 0.005^c

[1] Total phenolic content is expressed as gallic acid equivalents (GAEs)
Each value is the mean ± SD of triplicate measurements.
Values within a column with different letters (a-c) differ significantly ($p<0.05$; Tukey test)
GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract

Table 7. Contents of vitamin E and β-carotene in *Gymnema inodorum* (GI) extracts*

Extract	**Vitamin E** (mg/100 g freeze-dry extract)	**β-carotene** (mg/100 g freeze-dry extract)
GIJ	22.6	25.944
GIW	1.6	2.979
GIE50	2.1	0.009

Each value is the mean of duplicate measurement
GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract
*The data for the GIE95 is missing for the amount of extract was insufficient for analysis

4.2 ANTIOXIDANT ACTIVITY

4.2.1 DPPH radical scavenging activity

The DPPH radical scavenging activity of different fractions of *Gymnema inodurum* extracts is shown in Table 8. The DPPH radical scavenging activity of all extracts at various concentrations (0.015 to 0.5mg/mL) increased in a dose-dependent manner. The highest activity was found in the GIJ; whereas the GIE95 had the lowest activity at all concentrations. In addition, the GIJ had the significantly highest value of IC_{50} for DPPH radical scavenging (0.25 ± 0.017 mg/mL). Moreover, the GIJ at the amount of 1 mg had the antioxidant activity equivalent to trolox at the amount of 0.2 µmole (Table 10).

Table 8. DPPH radical scavenging activity (%) of *Gymnema inodurum* (GI) extracts

Conc. (mg/mL) / **Extract**	**Scavenging activity (%)**[1] 0.015	0.15	0.3	0.5
GIJ	14.00 ± 0.27^a	39.48 ± 0.44^a	61.90 ± 0.40^a	81.02 ± 0.66^a
GIW	5.89 ± 0.56^b	22.54 ± 0.52^b	44.44 ± 0.29^b	56.64 ± 2.33^b
GIE50	6.06 ± 0.74^b	36.25 ± 1.38^c	52.34 ± 1.08^c	63.59 ± 0.36^c
GIE95	1.39 ± 0.57^c	19.46 ± 1.09^d	34.00 ± 0.33^d	50.10 ± 0.13^d

[1] Values are expressed as mean ± SD of 3 measurements
Values within a column with different letters (a-d) differ significantly ($p<0.05$; Tukey test)
GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract

Table 9. IC_{50} values of *Gymnema inodorum* (GI) extracts and trolox using the DPPH assay

Extract	IC_{50} (mg/mL)[1]
GIJ	0.25 ± 0.017^a
GIW	0.40 ± 0.024^b
GIE50	0.33 ± 0.042^c
GIE95	0.48 ± 0.031^b
Trolox	0.0125 ± 0.012^d

[1] 50% inhibition concentration: concentration of antioxidant required to quench 50% of stable free radicals

Values are expressed as mean ± SD of 3 measurements. Values within a column with different letters (a-d) differ significantly ($p<0.05$; Tukey test)

GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract

Table 10. Activity of *Gymnema inodorum* (GI) extracts on DPPH radical scavenging expressed as trolox equivalent

Extract	Trolox equivalent (µmole)[1]
GIJ	0.20
GIW	0.13
GIE50	0.15
GIE95	0.10

[1] Trolox equivalent: 1 mg extract equal to trolox concentration in molecular weight

GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract

4.2.2 Inhibitory effect of deoxyribose degradation (deoxyribose assay)

The inhibitory effect of GI extracts on deoxyribose degradation is shown in Table 11. The inhibition activity of all extracts increased in a dose-dependent manner. The GIJ exhibited the highest activity. At the same concentrations ranged from 0.15 to 0.5 mg/mL, the inhibition activity in the GIW and the GIE50 was not significantly different. Moreover, three extracts (GIJ, GIW and GIE50) had significantly higher activity than the GIE95. The values of the 50% inhibition concentrations (IC_{50}) of *Gymnema inodorum* extracts and trolox using deoxyribose assay are shown in Table 12. In addition, the GIJ at the amount of 1 mg had the inhibition activity equivalent to trolox at the amount of 0.19 µmole (Table 13).

Table 11. Inhibitory effect (%) of *Gymnema inodorum* (GI) extracts on deoxyribose degradation

Conc. (mg/mL) / **Extract**	**Inhibitory effect (%)[1]**			
	0.015	0.15	0.3	0.5
GIJ	21.50 ± 1.84^a	59.48 ± 1.15^a	86.83 ± 1.57^a	97.58 ± 2.08^a
GIW	11.96 ± 0.86^b	45.97 ± 2.93^b	56.39 ± 2.59^b	65.73 ± 0.49^b
GIE50	17.96 ± 1.57^c	41.62 ± 2.33^b	55.19 ± 0.49^b	67.94 ± 2.16^b
GIE95	9.83 ± 0.71^b	30.21 ± 1.95^c	43.53 ± 2.40^c	53.34 ± 2.80^c

[1] Values are expressed as mean ± SD of 3 measurements
Values within a column with different letters (a-c) differ significantly ($p<0.05$; Tukey test)
GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract

Table 12. IC$_{50}$ values of *Gymnema inodorum* (GI) extracts and trolox on deoxyribose degradation inhibition.

Extract	**IC$_{50}$ (mg/mL)[1]**
GIJ	0.15 ± 0.075^{a}
GIW	0.29 ± 035^{b}
GIE50	0.27 ± 0.049^{b}
GIE95	0.42 ± 0.076^{c}
Trolox	0.007 ± 0.022^{d}

[1] 50% inhibition concentration: concentration of antioxidant required to inhibit 50% of activity
Values within a column with different letters (a-c) differ significantly ($p<0.05$; Tukey test)
GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract

Table 13. Activity of *Gymnema inodorum* (GI) extracts expressed as trolox equivalent using the deoxyribose assay

Extract	**Trolox equivalent (μmole)[1]**
GIJ	0.19
GIW	0.09
GIE50	0.1
GIE95	0.06

[1] Trolox equivalent: 1 mg extract equal to trolox concentration in molecular weight
GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract

4.2.3 Inhibition effect of red blood cells hemolysis

The percentage of hemolysis inhibition, as a result of protection against the oxidative damage induced by AAPH in cell membranes of erythrocytes, is shown in Table 14. The protective effect of the *Gymnema inodorum* extracts against erythrocyte oxidative damage was increase in all extracts in a dose-dependent manner. The GIJ had a significantly higher inhibitor effect than other extracts at concentrations ranged from 0.01 to 0.5 mg/mL, indicating that the GIJ has the greatest protective effect against hemolysis of erythrocytes. On the other hand, the GIE95 had the lowest inhibitory effect on hemolysis compared with other extracts. The values of the 50% inhibitory concentration (IC$_{50}$) of *Gymnema inodorum* extracts and trolox to inhibit hemolysis is shown in Table 15. The GIJ at the amount of 1 mg had the activity to inhibit RBC hemolysis equivalent to trolox at the amount of 0.26 µmole (Table 16).

Table 14. Inhibitory effect of *Gymnema inodorum* (GI) extracts on AAPH-induced hemolysis

Conc. (mg/mL)	**Hemolysis inhibition (%)**[1]			
Extract	0.01	0.05	0.1	0.5
GIJ	9.96 ± 6.70^{a}	33.47 ± 3.34^{a}	66.90 ± 4.15^{a}	79.50 ± 4.39^{a}
GIW	5.11 ± 2.04^{ab}	28.21 ± 4.31^{ab}	58.92 ± 7.63^{ab}	71.80 ± 7.50^{a}
GIE50	3.35 ± 2.43^{b}	22.65 ± 4.08^{b}	54.06 ± 6.18^{b}	72.30 ± 4.39^{a}
GIE95	0.64 ± 1.07^{b}	11.98 ± 6.07^{c}	44.30 ± 2.95^{c}	51.62 ± 3.96^{b}

[1] Values are expressed as mean ± SD of 6 experiments
Values within a column with different letters (a-d) differ significantly ($p<0.05$; Tukey test)
GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract

Table 15. IC$_{50}$ values of *Gymnema inodorum* (GI) extracts and trolox on RBC hemolysis inhibition

Extract	**IC$_{50}$ (mg/mL)[1]**
GIJ	0.15 ± 0.23^{a}
GIW	0.24 ± 0.37^{b}
GIE50	0.29 ± 0.21^{b}
GIE95	0.71 ± 0.1^{c}
Trolox	0.01 ± 0.12^{d}

[1] 50% inhibition concentration: concentration of antioxidant required to inhibit 50% of activity
Values within a column with different letters (a-d) differ significantly (p<0.05; Tukey test)
GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract

Table 16. Activity of *Gymnema inodorum* (GI) extracts on hemolysis inhibition expressed as trolox equivalent

Extract	**Trolox equivalent (µmole)[1]**
GIJ	0.26
GIW	0.16
GIE50	0.13
GIE95	0.05

[1] Trolox equivalent: 1 mg extract equal to trolox concentration in molecular weight
GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract

4.2.4 Inhibition of oxidative DNA damage induced by H_2O_2 (comet assay)

The viability rate of TK6 cells after incubated with 200 μg/mL of various extracts of *Gymnema inodorum* was above 70% for all treatments. The measurements of oxidative damage in comet cells expressing as tail length (TL), tail intensity (TI) and tail moment (TM) in the study are shown in Table 17. It was found that the cells, treated with 50 μM of H_2O_2 for 5 min, produced a marked damage which was indicated by the values of TL and TM parameters. Interestingly, pre-treatment of cells with *Gymnema inodorum* extracts at the concentrations of 200 μg/mL prevented cells from H_2O_2–induced DNA damage. The inhibitory effect of GI extracts on H_2O_2-induced DNA damage in TK6 cells was expressed in percentage as shown in Table 18. The highest inhibitory effect on the comet assay was found with the treatment of trolox (93.8%) whereas the order of the inhibitory effect of GI extracts using the comet assay was as follows: GIJ > GIE50 > GIW > GIE 95.

Table 17. Values of tail length (μm) and tail moment (%) in comet assay of TK6 cells treated with *Gymnema inodorum* (GI) extracts followed by H_2O_2 exposure[1]

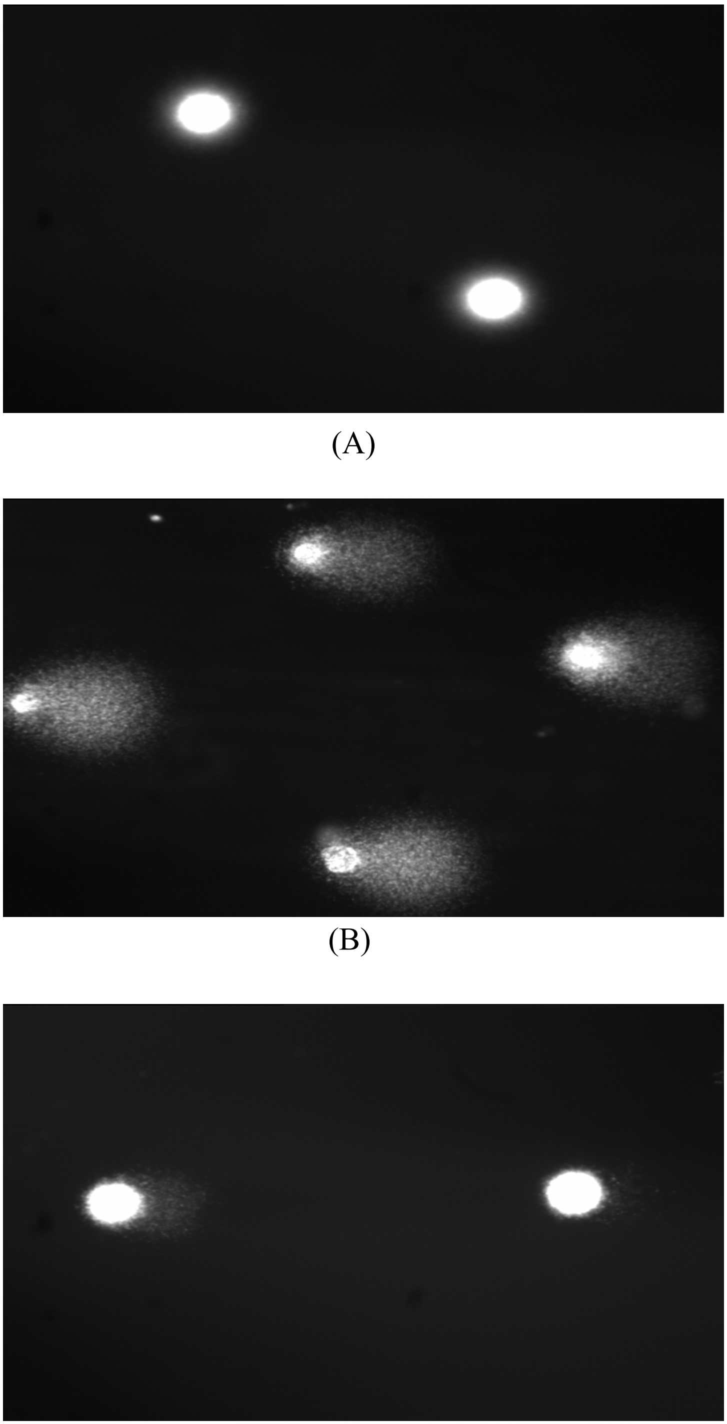
Treatment	Tail length (μm)	Tail moment (%)
H_2O_2	32.89 ± 0.73	5.50 ± 0.45
GIJ + H_2O_2	19.95 ± 3.24	1.19 ± 0.17*
GIW + H_2O_2	26.63 ± 3.44	3.30 ± 0.63
GIE50 + H_2O_2	23.86 ± 0.44	2.72 ± 0.46*
GIE95 + H_2O_2	33.64 ± 0.89	5.06 ± 0.45
Trolox + H_2O_2	12.88 ± 0.74	0.34 ± 0.45*

[1] Values are means ± SD; calculated on 50 cells/ slide/ treatment from 3 independent experiments
* Significant difference from treatment with H_2O_2
GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract

Table 18. Inhibitory effect of *Gymnema inodorum* (GI) extracts and trolox on H_2O_2-induced DNA damage in TK6 cells using comet assay

Treatment	Concentration	Inhibition of DNA damage (%)[1]
H_2O_2	50 µM	0
GIJ + H_2O_2	200 µg/ml + 50 µM	78.02*
GIW + H_2O_2	200 µg/ml + 50 µM	40.38
GIE50 + H_2O_2	200 µg/ml + 50 µM	50.54*
GIE95 + H_2O_2	200 µg/ml + 50 µM	8.68
Trolox + H_2O_2	75 µg/ml + 50 µM	93.8

[1] Damage is given in % inhibition of DNA damage
Values are expressed as means from 3 independent experiments
* Significant difference from treatment with H_2O_2
GIJ: juice; GIW: water extract; GIE50: 50% ethanol extract; GIE95: 95% ethanol extract

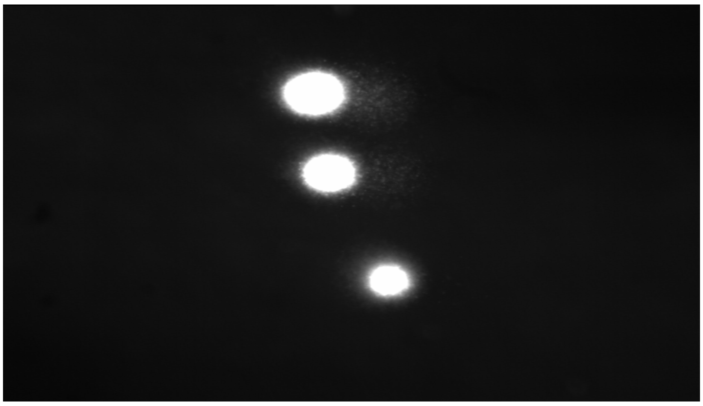


(A)

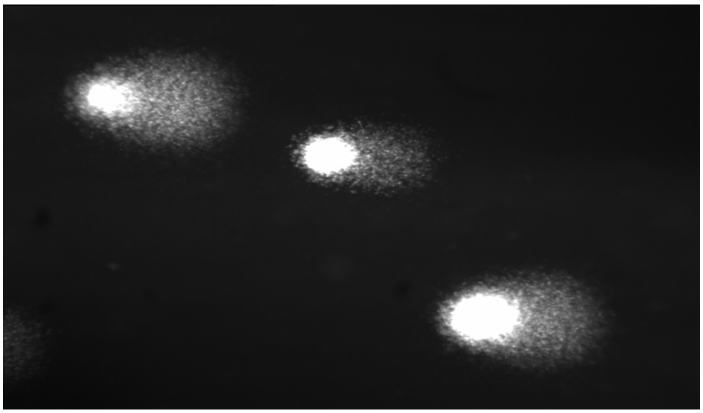
(B)

(C)

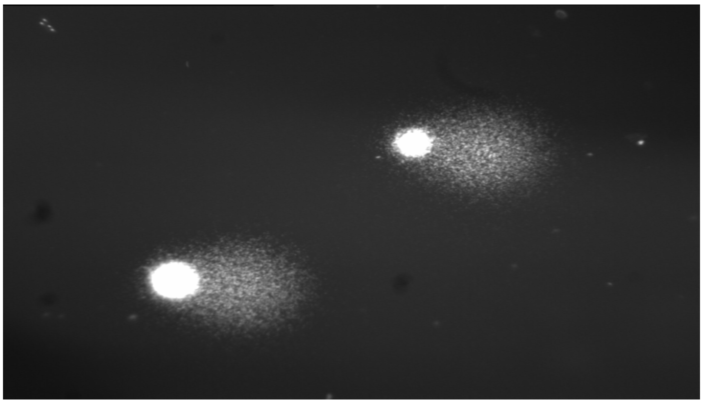
Figure 4. Fluorescence photos of whole DNA of TK6 cells viewed by Olympus BX51 (magnification 200x): (A) non-treated, (B) 50µM H_2O_2, (C) 200µg/ml GIJ + 50µM H_2O_2



(D)



(E)



(F)

Figure 4 (cont.) Fluorescence photo of whole DNA of TK6 cells viewed by Olympus BX51 (magnification 200x): (D) 200μg/ml GIE50 fraction + 50μM H_2O_2,
(E) 200μg/ml GIW fraction + 50μM H_2O_2,
(F) 200μg/ml GIE95 fraction + 50μM H_2O_2

4.3 CORRELATION BETWEEN ANTIOXIDANT ACTIVITY OF *GYMNEMA INODORUM* EXTRACTS AND BIOACTIVE COMPOUNDS

4.3.1 Correlation between antioxidant activity of *Gymnema inodorum* extracts and total phenolic content

For IC_{50} values of DPPH assay, deoxyribose assay, hemolysis assay and tail moment (%) of DNA damage were compared with corresponding to total phenolic compound. There was no significant difference between IC_{50} values of antioxidant activity in all extracts to total phenolic content. However, negative correlation (Spearman's correlation r_s = -1.00) was found among dry preparation condition between GIJ, GIW and GIE50 in DPPH, deoxyribose, and comet assay (Figure 5). The results indicated that antioxidant activity of *Gymnema inodorum* extracts in dry preparation were strong correlate with total phenolic content.

4.3.2 Correlation between antioxidant activity of *Gymnema inodorum* extracts and antioxidant vitamins

The IC_{50} values of *Gymnema inodurum* extracts in all experiment and % tail moment were compared with corresponding to β-carotene and vitamin E content. The IC_{50} values of *Gymnema inodorum* extracts were found negative correlation (Spearman's correlation r_s = -1.00) with β-carotene in hemolysis assay only (Figure 6). Negative correlation between IC_{50} values and % tail moment with vitamin E content were found in DPPH deoxyribose, and comet assay (Figure 7).

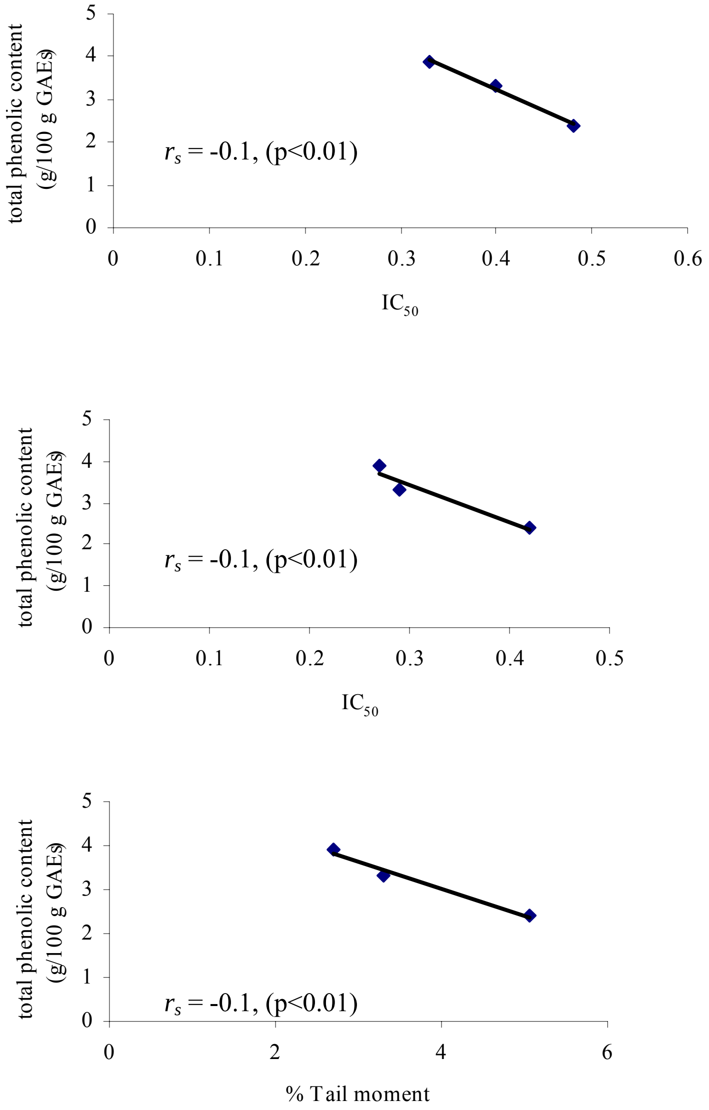


Figure 5. The correlation between antioxidant activities of *Gymnema inodorum* extracts (dry preparation) and total phenolic content in DPPH, deoxyribose, and comet assay

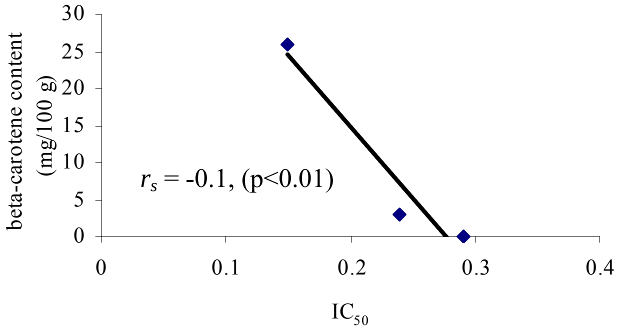


Figure 6. The correlation between antioxidant activities of *Gymnema inodorum* extracts and β-carotene content in hemolysis assay

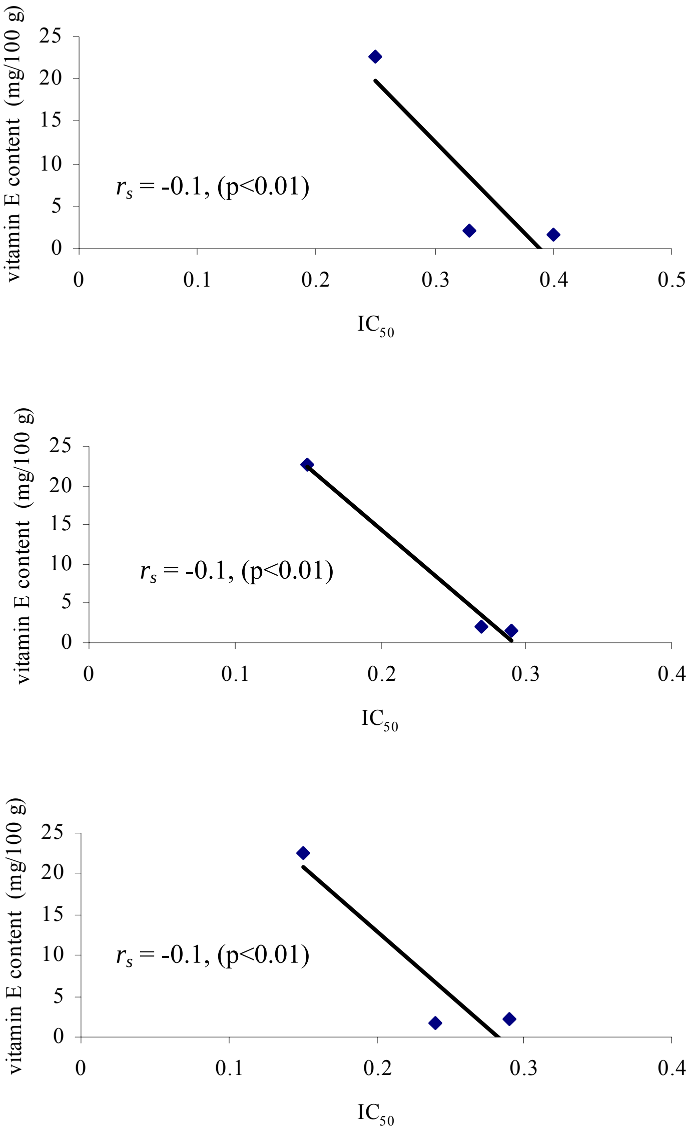


Figure 7. The correlation between antioxidant activities of *Gymnema inodorum* extracts and vitamin E content in DPPH, deoxyribose, and comet assay

CHAPTER V

DISCUSSION

Vegetables are good sources of natural antioxidants and other useful substances necessary for good health. Antioxidants in vegetables can scavenge free radicals and inhibit cell membrane and DNA oxidative damage. Thus, the consumption of dietary antioxidants from vegetable is beneficial in preventing chronic diseases. Fortunately, Thailand has a variety of local vegetables and herbs as sources of natural antioxidants. *Gymnema inodorum* (chiangda) is a Thai local vegetable, which is widely consumed in the northren of Thailand, has currently been found to possess the antioxidant activity and have many bioactive compounds such as β-carotene, vitamin E, vitamin C, xanthophyll, and tannin (15).

There are many usual preparations of *Gymnema inodorum* for consumption such as fresh, tea and extracts, depending on the propose of consumers. Therefore, in this study edible part of *Gymnema inodorum* was prepared in different conditions to determined antioxidant activities using chemical and biological tests. Edible part of *Gymnema inodorum* were divided into two parts; fresh (juice, GIJ) and dry parts. The latter was prepared in three fractions using different solvents including water (GIW), 50% ethanol (GIE50) and 95% ethanol (GIE95). The aim of this study was to compare the different methods of *Gymnema inodorum* preparation in term of the antioxidant activity of *Gymnema inodorum* extracts using four different tests in order to find the most valuable fraction for health benefit. This study was the first to evaluate the antioxidant activity of *Gymnema inodorum* extracts in a comprehensive manner employing a variety of *in vitro* methods.

The antioxidant activity of *Gymnema inodorum* was assessed using the DPPH radical assay, which primarily evaluates the proton radical-scavenging ability. DPPH is one of the compounds that possess a proton-free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (60). It is well accepted that the DPPH free radical-scavenging by antioxidants is due to their hydrogen-donating ability (73-75). In the present study, *Gymnema indorum* extracts showed a concentration-dependent DPPH radical scavenging ability, which may be attributable to its hydrogen-donating property. However, the comparison of IC_{50} values of DPPH radical scavenging between *Gymnema inodorum* juice extract and other antioxidants (54) that had lower activity than trolox, vitamin C, and BHA about 20, 40, 80 folds. All of fractions of *Gymnema inodorum* extracts were effective to prevent the degradation of deoxyribose in a dose-dependent manner. Thus, the present study suggests that the *Gymnema inodorum* extracts is able to prevent deoxyribose damage associated with the binding of iron to deoxyribose and the subsequent attack by $OH^{\bullet}$ radical generated via the Fenton-reaction (55). The ability of the *Gymnema inodorum* extract to scavenge reactive oxygen species (ROS) and free radicals was further confirmed by using RBC hemolysis assay to show the protection of RBC membrane and using H_2O_2-induced DNA damage assay. In RBC hemolysis assay, AAPH is used as a peroxyl-radical initiator that, by its thermal decomposition, generates free radicals that attack the erythrocytes to induce chain oxidation of lipid and protein, disturbing the membrane organization and eventually leading to hemolysis (47-49). The result showed the protective effect of *Gymnema inodorum* extracts on the oxidative damage of cell membranes in a dose-dependent manner.

Interestingly, the GIJ and the GIE50 showed an inhibitory effect on H_2O_2-induced oxidative DNA damage in TK6 cells analyzed by comet assay. Thus, one of the mechanisms for the antioxidant activity of *Gymnema inodorum* extracts may be via H_2O_2 scavenging ability.

In hemolysis and deoxyribose assay, results were different from the other assay the inhibitory effects of the GIW and the GIE50 were not significantly difference. This may be described that, these assay were complex reactions and not specific for type of antioxidant compounds. Base on these reasons, the GIE50 had highest total phenolic compounds but not highest in antioxidant activity.

According to 4 tests, the highest antioxidant potential was observed in the GIJ. This extract possessed the highest level of vitamin E and β-carotene content but its total phenolic content did not highest. Therefore, the reason for the high activity of the GIJ may be due to high content of vitamin E and β-carotene or quality of phenolic compounds. Vitamin E is the natural physiological lipid-soluble antioxidant that can inhibit lipid peroxidation in cell membrane (1, 63-64). β-carotene is the most prominent representative of this lipophilic class of compounds. It is effective as an antioxidant by quenching singlet oxygen or free radicals that are formed during lipid oxidation, such as the lipid radicals formed by hydrogen abstraction from an allylic CH_2 group, the peroxy and the hydroxyl radicals (64).

The bioactivity of phenolic may be related to their ability to chelate metals, inhibit lipid peroxidation and scavenging free radicals (65-67). The processing may result in changing the quality of the phenolic compounds. Franke et al (65) showed that fresh juice orange had the highest antioxidant effect although it had a lower concentration of phenolic compounds when compared with processed orange juice. The difference in the antioxidant potential may partially be attributed to qualitative variations of the phenolic compounds as the structural differences may modify the antioxidant potential of phenolic acid (66). Likewise, Jimenez-Escring et al (68) reported that the radical scavenging activity of brown alga *Fucus* was decreased by drying at 50°C for 48 hour. In case of honshi-nori, a dried product of red alga *Porphyra*, not only ascorbic acid but also carotenoids and chlorophylls were decreased (69). The temperature, during drying and extraction, might pay an important role on the stability of these compounds due to chemical and enzymatic degradation. It has been suggested that the loss of activity by volatilization or thermal decomposition is the main mechanism of the reduction in polyphenol contents. Also, the prolonged exposure at moderate temperatures caused phenolic degradation during their enzyme-assisted extraction from grape pomance for 48-hour hydrolysis, whereas at 1-8 hour, no degradation was observed (70). In addition, Miillard and Berest (71) observed 20% reduction in the antioxidant activity of bound and free polyphenols during kilning of barley and malt at 90°C. Futhermore, Larranri et al (72) found a significant reduction in extractable polyphenols and condensed tannins when red grape pomace peels were dried with air at 100°C or higher. In the present study, the GIW and the GIE50 exhibited a lower content of vitamins compared to that of the GIJ, therefore this may affect their antioxidant activities.

Antioxidant activity comparison of total phenolic compounds content in dry preparation (GIW, GIE50, and GIE95), implies that the slight decrease in antioxidant activity may result from solubility of the active components. Antioxidant activities in DPPH and comet assay were decreased in the same order as total phenolic content, which can be explained by the solubility of phenolic compounds. Several solvents such as ethanol, methanol, and water are more frequently used for isolation of antioxidants. Antioxidant activities of extracts are strongly dependent on the polarity of the solvent.

Ethanol and water are the most widely employed solvents for hygienic and convenient reasons. Since the activity depends on the polyphenol compounds and the antioxidant assay, comparative studies for selecting the optimal solvent providing maximum antioxidant activity were done. Manuel et al (71) recommends the commercial alcoholic preparations with 44 % alcohol. The GIE50 used 50% ethanol for extraction which was close to recommendation. Thus, result in this study indicated that stronger antioxidant activity of the GIE50 could be enhanced by cooperative of polar and non-polar antioxidant compounds as well as the synergistic effects among all antioxidants (combinations of different compounds).

Interestingly, the antioxidant activity of fresh preparation (GIJ) was different from dry preparation (GIW, GIE50, and GIE95) for less than 10-folds in all experiment. While its vitamin E and β-carotene content was over 20-folds compare to other extracts, suggesting that the synergistic effect of the dry preparation may condense some bioactive compounds and provide enhancing activity. Heating may induce increasing in other secondary and/or unidentified bioactive compound (74). Maria et al (74) reported that maillard reaction on tomato as a consequence of heating minimizes the loss of natural antioxidants (Vitamin C), but enhancing the overall antioxidant properties of the product. Thus, antioxidant activity that present in dry preparation may have the other phenolic compounds which act as strong antioxidant activity or different bioactive compounds from fresh juice extract.

From the results of this study are the primary data that determine the antioxidant activity of *Gymnema inodorum* extracts. Thus, the results may not exactly suggest for the mechanism of antioxidant activity. Therefore, further research should be done to characterize individual phenolic compounds of the extracts of *Gymnema* *inodorum* in order to assign particular antioxidant effects to individual compounds of the extracts. It would be desirable to employ experimental conditions that can more specifically reflect the various gastric/intestinal microenvironments that are pertinent to the uptake of plant secondary metabolites in the human system.

CHAPTER VI

CONCLUSION

1. Yields of *Gymnema inodorum* freeze-dry extracts were in a range of 5.25% to 27% (w/w) of fresh weight. The juice fraction obtained the greatest yield.
2. The contents of total phenolic compounds content range from 2.44 to 3.95 mg gallic acid equivalent (GAEs)/100g freeze-dry extract. The highest content of total phenolic compounds was in the fraction extracted with 50% ethanol
3. Antioxidant activities of *Gymnema inodorum* extracts were correlated with the levels of vitamin E and β-carotene.
4. The antioxidant activites of dried preparation (GIW, GIE50 and GIE95) were found to be positively correlated with the total phenolic content.
5. The most potent antioxidant activity in all experiments and the highest vitamin content was found in the juice of *Gymnema inodorum.*
6. The 95% ethanol extract of *Gymnema inodorum* had the lowest antioxidant activity and total phenolic content.
7. The 50% ethanol extract of *Gymnema inodorum* had stronger activities than the water extract and 95% ethanol extract regarding DPPH scavenging, inhibition of deoxyribose degradation, and inhibition of DNA damage.
8. In hemolysis inhibition assay, the water extract had stronger activity than the 50% ethanol and 95% ethanol extracts.

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APPENDIX

APPENDEX A

Chemical

The 6-Hydroxy-2,5,7,8-tetramethyl-chro-man-2-carboxylic acid (Trolox), 1,1-diphenyl-2picryl hydrazyl (DPPH), 2-deoxy-D-ribose, 2-thiobabituric acid (TBA), tricholroacetic acid 2,2’-azo-bis-(2-amidinopropane)dihydrochloride (AAPH), gallic acid, Hydrogen peroxide (H_2O_2), *N*-Lauroyl sarcosinate, ethidium bromidine (EtBr), Triton X-100, disodium EDTA, trizma base from Aldrich-Sigma Chemical Co. Ltd.(Thailand), dimethyl sulfoxide (DMSO), sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), sodium bicarbonate (Na_2CO_3), sodium chloride (NaCl), sodium hydroxide (NaOH), from Merck Co. Ltd.(Thailand), normal melting agarose (NMA), low melting agarose (LMPA) RPMI-1640 medium, horse serum from GibcoBRL

Chemical preparation of comet assay

Agarose

- 1% normal melting agarose (NMA) (w/v) in PBS	1g in 100ml
- 0.5% low melting agarose (LMA) (w/v) in PBS	0.5g in 100ml

<u>PBS, $Mg^{2+}$and Ca^{2+} free</u>

- NaCl	80g
- KCl	2g
- $Na_2HPO_4.2H_2O$	11.5g
- KH_2PO_4	2g

set to pH 7.2, q.s. to 1 liter with DI water, autoclave store at RT

APPENDEX A (cont.)

Lysis solution

-

- 2.5M NaCl	146.1g
- 100mM $Na_2EDTA.2H_2O$	37.2g
- 10mM Tris-base	1.4g

set to pH 10 with 10N NaOH, q.s. to 890mL with DI water and final lysis solution add fresh 1%Triton X-100 and 10%DMSO refrigerate (4°C) for 60 min before use

Electrophoresis solution **300mM NaOH, 1mM Na_2EDTA pH>13**

Stock solution	
- 5N NaOH	100g in 500mL DI water
- 200mM $Na_2EDTA.2H_2O$	14.89g in 150 mL DI water, set pH to 10 with NaOH, q.s. to 200ml, store at RT
Fresh preparation	
- 5N NaOH	60mL
- 200mM Na_2EDTA	5mL

q.s. to 1000ml with DI water check pH >13, chill 4°C before use

Neutralizing buffer

- 400mM Tris-base	48.5g

set to pH 7.4 with conc.HCl, q.s. to 1000 with DI water

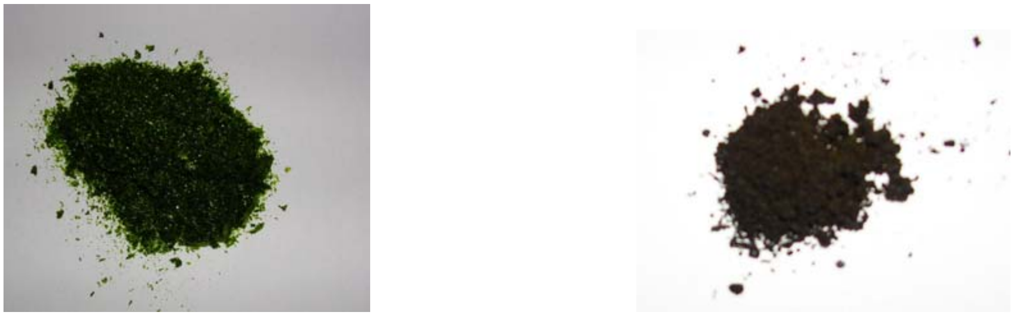
Fluorescent staining solution **20ug/mL**

- Stock solution 2 mg/mL of ethidium bromide and dilute 100X when staining

APPENDIX B

Figure 8. Edible parts of *Gymnema inodorum*

APPENDIX C



G. inodorum juice extract

G. inodorum 95% ethanol extract

G. inodorum water extract

G. inodorum 50% ethano extract

Figure 9. Characteristics of *Gymnema inodorum* freeze-dry extracts

APPENDIX D

Table 21. % of Moisture content of *Gymnema inodorum* (GI) extracts

Sample	Moisture content (%)
Gymnema inodorum	
GI juice extract	2.92
GI water extract	1.1
GI 50%EtOH extract	1.25
GI 95% EtOH extract	0.66

Table 22. Comparison of IC $_{50}$ values of various compounds and herbs for DPPH radical scavenging (54)

Plants	IC_{50} values (mg/mL)
BHA	0.0031
Ascorbic acid	0.0062
Trolox = Vitamin E	0.0125
G. inodorum juice extract	0.25
Holy Basil (กระเพรา)	0.09
Phyllanthus amarus (ลูกใต้ใบ)	0.16
Cassis alanta (ชุมเห็ดเทศ)	0.17
กวาวเครือขาว	0.19

APPENDIX E

Table 23. % of viability of TK6 cells after incubated with *Gymnema inodorum* extracts

Treatment	Number of viability cells*	% of viability cells
Untreatment	1.318×10^5	100
Trolox (50 µM)	1.175×10^5	89.15
GIJ extract 200 µg/mL	1.162×10^5	88.16
GIW extract 200 µg/mL	1.272×10^5	96.50
GIE50 extract 200 ug/mL	1.145×10^5	86.87
GIE95 extract 200 ug/mL	1.139x105	86.41

*Value is expressed as mean of 2 experiments

APPENDIX F

The amount of fresh leaves for antioxidant activity equal vitamin E 400 mg

(Recommended the level of the antioxidant daily dose of vitamin E at 400 mg)

Scavenging DPPH radical

Gymnema inodorum juice extract = 0.029 kg of fresh leaves
Gymnema inodorum water extract = 0.118 kg of fresh leaves
Gymnema inodorum 50% EtOH extract = 0.072 kg of fresh leaves
Gymnema inodorum 95% EtOH extract = 0.292 kg of fresh leaves

Calculation

The DPPH activity of GIJ 0.25 mg/ml = activity of trolox 0.0125 mg/ml

Trolox (vitamin E) 0.0125 mg/ml = 0.25 mg/ml of GIJ freeze-dry extract

$$\text{Vitamin E 400 mg/daily} = \frac{0.25\text{x}400\text{ mg}}{0.0125}$$

Vitamin E 400 mg/daily = 8000 mg of GIJ freeze-dry extract

Yield of GIJ = 27% per 100 g freeze-dry (w/w)

27 mg of extract from 100 mg of fresh leaves

$$\text{8000 mg of GIJ freeze-dry extract} = \frac{100\text{x}8000\text{mg}}{27}$$

= 29629.6mg

= 29.6g

Fresh leaves = 0.029kg

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