PHYTOCHEMISTRY AND ANTIOXIDANT ACTIVITY OF *PLUCHEA INDICA*

ANCHALEE TRAITHIP

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PHYTOCHEMISTRY AND ANTIOXIDANT ACTIVITY OF *PLUCHEA INDICA*

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ABSTRACT

The leaves of Khlu (*Pluchea indica* (Linn.) Less) were extracted and tested for antioxidant activity using DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay. It was found that the leaf ethanol extract exhibited a strong antioxidative activity with an EC_{50} of 6.92 μg/ml.

The ethanolic extract of *Pluchea indica* leaf was further separated by Diaion HP20 column to give four fractions, i.e the H_2O fraction(EC_{50} = 16.02 μg/ml), the H_2O-MeOH fraction(EC_{50} = 2.65 μg/ml),the MeOH fraction (EC_{50} = 1.89 μg/ml) and the EtOAc fraction (EC_{50} = 12.08 μg/ml). The most active fraction was selected for further fractionation using silica chromatographic columns resulting in the isolation of compound A1. Compound A1 was identified by spectroscopic techniques as quercetin (5,7,3′,4′tetrahydroxyflavonol). Compound A1 is the major antioxidant component in the leaf extract of *P. indica* with an EC_{50} of 1.69 μg/ml .

KEY WORDS : *PLUCHEA INDICA* / ANTIOXIDANT ACTIVITY / DPPH / QUERCETIN

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พฤกษเคมีและฤทธิ์ต้านออกซิเดชันของขลู่ (PHYTOCHEMISTRY AND ANTIOXIDANT ACTIVITY OF *PLUCHEA INDICA*)

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บทคัดย่อ

เมื่อสกัดส่วนใบของขลู่(*Pluchea indica* (Linn.) Less.) และนำไปศึกษาฤทธิ์ต้านออกซิเดชั่นโดยวิธี DPPH scavenging assay พบว่าสารสกัดของใบมีฤทธิ์ต้านออกซิเดชั่นได้ดีโดยมีค่าEC_{50} = 6.92 ไมโครกรัม/มิลลิลิตร

เมื่อนำสารสกัดเอธานอลของใบขลู่ (*Pluchea indica*) มาแยกสารสำคัญโดยวิธี Diaion HP20 คอลัมน์ ได้ fraction ต่าง ๆซึ่งแสดงฤทธิ์การต้านออกซิเดชั่นดังนี้ fraction น้ำ (EC_{50} = 16.02 µg/ml), fraction น้ำ-เมทานอล(EC_{50} =2.65 µg/ml), fractionเมทานอล (EC_{50} =1.89 µg/ml)และfractionเอทิลอะซีเตท (EC_{50} = 12.08 µg/ml) นำ fraction เมทานอลซึ่งแสดงฤทธิ์ต้านออกซิเดชั่นที่ดีที่สุด มาแยกต่อด้วยซิลิกาเจลคอลัมน์โครมาโทกราฟฟี สามารถแยกได้สาร A1ซึ่งพิสูจน์โครงสร้างโดยวิธีสเปคโตรสโคปีพบว่าเป็น quercetin(5,7,3′,4′ tetrahydroxyflavonol) สาร A1 เป็นสารสำคัญชนิดหนึ่งในสารสกัดเมธานอลที่แสดงฤทธิ์ต้านออกซิเดชั่นโดยมีค่า EC_{50} = 1.69 ไมโครกรัม/มิลลิลิตร

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LIST OF ABBREVIATIONS

A_{ctr}	absorbance of control
A_{sample}	absorbance of sample
A_{std}	absorbance of standard
A_{test}	absorbance of test sample
$AlCl_3$	alumenium chloride
br	broad
^{o}C	degree celsius
CAT No.	catalog number
$CDCl_3$	deuterated chloroform
cm^{-1}	per centimetre
^{13}C-NMR	carbon-thirteen NMR
d	doublet
dd	doublet of doublet
DPPH	1,1-diphenyl-2-picrylhydrazyl
DMSO-d_6	deuterium dimethyl sulfoxide
EC_{50}	concentration that gave 50% effective
MS	mass spectrometry
$FeCl_3$	ferric chloride
FT-IR	fourier transform infrared spectroscopy
g	gram
H_3BO_3	borric acid
HCl	hydrochloric acid
^{1}H-NMR	proton nuclear magnetic resonance
Hz	hertz
$HO^{\bullet}$	hydroxyl radical
H_2O_2	hydrogen peroxide
IR	infrared
J	coupling constant

LIST OF ABBREVIATIONS (continue)

KBr	potassium bromide
KCl	potassium chloride
l	litre
LOH	hydroxy lipid
LOOH	lipid hydroperoxide
mg	milligram
M^+	molecular ion
MHz	megahertz
ml	millilitre
mmole	millimole
M.W.	molecular weight
NaCl	sodium chloride
NaOAc	sodium acetate
$O_2^{\bullet-}$	superoxide anion
O_2^{2-}	peroxide anion
O_2	molecular oxygen
$^1O_2$	singlet oxygen
O_3	ozone
$R^\bullet$	neutral radical
$R^{\bullet-}$	anion radical
$R^{\bullet+}$	cation radical
ROS	reactive oxygen species
SOD	superoxide dismutase
TLC	thin layer chromatography
UV	ultraviolet
ν	Stretching
δ	chemical shift
µg	microgram
%	percent

CHAPTER 1

INTRODUCTION

In recent years, the role of free radicals and reactive oxygen species (ROS) in human degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, brain dysfunction and immune-system decline has become apparent. Oxidants such as superoxide anion, hydrogen peroxide, hydroxyl, alkoxyl and peroxyl radicals, which are by-products of normal metabolism, can cause oxidative damage to macromolecules, particularly lipids, proteins and DNA (1).

The biological effects of dietary antioxidants have generated a lot of interest in recent years. Tissue lipid oxidation is efficiently inhibited by the synergistic action of various endogenous enzymes such as superoxide dismutase and glutathione peroxidase and various dietary antioxidants such as selenuim, ascorbic acid, tocopherols, β-carotene, flavonoids and glutathione. Extensive studies are underway to determine whether these dietary antioxidants can be used in preventive as well as therapeutic medicine in many diseases including cardiovascular diseases, cancer, and arthritis and even in the aging process (2).

Many plants have been studied for their antioxidant activities such as *Allium sativum, Centella asiatica,* and *Curcuma longa*. Plant derived compounds that are effective as antioxidants are allicin, epigallocathechin, oligomer procyanidins, quercetin and curcumin (1). There are many methods for determining the antioxidant activity such as DPPH scavenging assay, ABTS-metmyoglobin and electron spin resonance (3).

Pluchea indica (Khlu in Thai), with an English name of Indian March Flebane, is taxonomically classified in the family Compositae (Asteraceae). In Thailand, traditional medicinal system suggested the uses of many parts of this plant as therapeutic agents. The stems in decoction form are reported to be used for the treatment of kidney stones as a diuretic agent. A decoction form of the bark is used for

the treatment of hemorrhoid. The leaves are claimed to be used for the treatment of inflammation and also as nerve tonic. Fresh leaves are used in the form of poultice for gangrenous ulcers. The leaves and roots are suggested to possess antidysenteric, antipyretic and anti-inflammatory properties (4,5).

Recently,it has been reported that the antioxidant activity of *Pluchea indica* Root Extract (PIRE) included the scavenging of free radicals and the inhibition of 5-lipoxygenase (*in vitro)* (6). From these findings, it was inferred that the antioxidant activity was almost definitely a major contributing factor in its anti-inflammatory and antiulcer activity (6,7).

The chemical constituents found in *Pluchea indica* (*P. indica)* included terpenes, benzenoids, phenyl propanoids, lignans and steroids. Flavonoid compounds have been reported in certain *Pluchea spp*. (*P. sagitharis*) but as yet there has been no report of flavonoids in *P. indica*.

In this study we will try to verify the antioxidative activity of the leaf of *P. indica* by using DPPH scavenging assay. The active constituent(s) will be isolated and identified. Further development of *Pluchea indica* active compound(s) to be used as an antioxidant in the pharmaceutical or cosmetic industry may be of interest.

The objectives of this study are :

1. To determine the antioxidant activity of the extract of *Pluchea indica* using DPPH scavenging assay.
2. To isolate and identify chemical constituents from the active fractions of *Pluchea indica* and to study the antioxidant activity of the isolated compounds.

CHAPTER 2
LITERATURE REVIEW

1. Free radicals

1.1 Free radicals

A free radical is defined as any atom or molecule that possesses an unpaired electron. It can be anionic, cationic or neutral (8,9). A radical will thus be indicated, as recommended, by a superscripted dot at the right side of the formula in parenthesis so as to show that no judgement as to the location of the unpaired electron is made. The symbol **R** will be used throughout to represent an unspecified radical. Which may be positively charged (cation radial $(R)^{+\cdot}$), like the pyridinyl cation radical that forms on $(NAD)^{+\cdot}$; negatively charged (anion radical $(R)^{-\cdot}$, like the superoxide anion radical $(O_2)^{-\cdot}$; or neural (neutral radical $(R)^{\cdot}$,like the hydroxyl radical $(HO)^{\cdot}$, the alkoxyl radical $(alk\ O)^{\cdot}$, the alkylperoxyl radical $(alk\ OO)^{\cdot}$, the alkylthiyl radical $(alk\ S)^{\cdot}$.

Oxygen free radicals (OFRs) are potentially very toxic to cells. Due to their highly reactive nature, they can readily combine with other molecules, such as enzymes, receptor and ion pumps, causing oxidation directly, and inactivating or inhibiting their normal function. Some of the products of OFR attack of other molecules can interfere with nucleic acid function, generating alterations in the base sequence with potential for mutations, leading in extreme pathological situations to cancers or germ-line mutations. Changes in normal proteins and other structure by free radical species can also generate novel immunogenic structure.

1.2 Sources of free radicals

Sources of free radicals within calls can be divided into exogenous and endogenous sources (Table 1)

Table 1 Sources of free radicals within cells (10,11,12)

Exogenous sources
Randox-cycling substances (for examples, paraquat, diquat, alloxan, doxorubicin)
Drug oxidations (for examples, paracetamol, carbontetrachloride)
Ionizing radiation
Sunlight
Heat shock
Cigarette smoke
Substance that oxidize glutathione
Endogenous sources
Mitochondrial electron transport chain
Microsomal electron transport chain
Chloroplast clectron transport chain
Oxidant enzyme
Xantine oxidase
Tryptophan dioxygenase
Galactose oxidase
Cyclooxygenase
Lipoxygenase
Monoamine oxidase
Autooxidation reaction (for examples, Fe^{2+}, epinephrine)
Phagocytic cells (neutrophils, eosinophils, monocytes and macrophages)

2. Antioxidant of defense systems

An antioxidant is defined as a substance that when present at low concentration, compares with that of an oxidizable substrate, significantly delays or prevents the oxidation of that substrate (15). It can inhibit free radical production by;

1. Chelating the transitional metal catalysts, e.g. transferrin, heptoglobins, hemopexin and metallothionein.
2. Breaking chain reaction, e.g. alpha tocopherol, beta-carotene.
3. Reducing concentration of reactive oxygen species, e.g. glutathione.

4. Scavenging initiating radicals, e.g. superoxide dismutase, catalase and glutathione peroxidase.

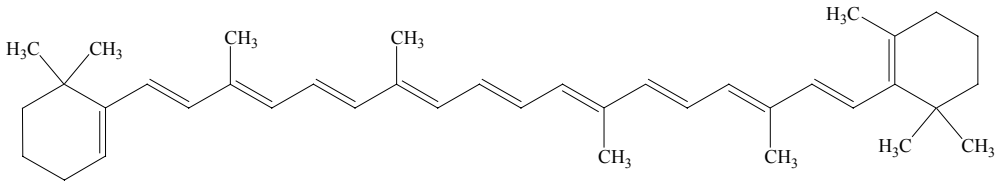
The antioxidant defense systems are usually divided into two groups: enzymatic and nonenzymatic antioxidant system (13).

2.1 Enzymatic antioxidants

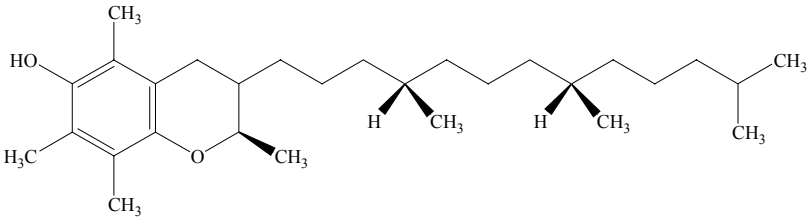
Antioxidant enzymes or scavenging enzymes that are directly involved in the detoxification of reaction of oxygen species are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX).

2.2 Nonenzymatic antioxidants

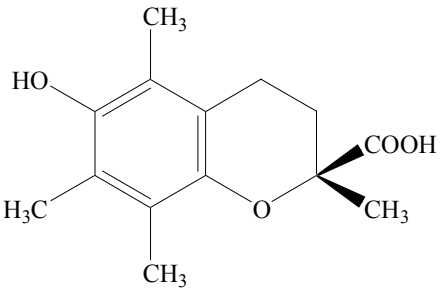
There are many nonenzymatic antioxidants. Such antioxidants include proteins in extracellular fluid i.e. transferrin, albumin etc. Other nonenzymatic antioxidants are small molecules consisting of water soluble compounds such as vitamin C and glutathione (GSH) and lipid soluble compounds such as, vitamin E, and beta-carotene.



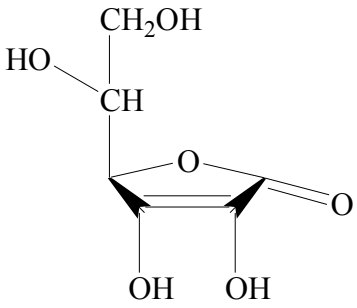
Beta-carotene



Vitamin E



Trolox



Vitamin C

Figure 1 Structures of some antioxidant agents (16)

3. Biological Effects of Free Radicals

Because of their very high chemical reactivity, free radicals have reasonably short lifetimes in biological system. Extensive studies with model systems and with biological materials in vitro, however, have clearly shown that reactive free radicals are able to produce metabolic disturbances and to damage membrane structures in a variety of ways. Free radicals seek out unsaturated sites in biomolecules for rapid attack. Such random attack may produce undesirable chemical modification of and damage to organic macromolecules such as proteins, carbohydrates, lipids, and nucleotides. Thus, if free radicals are produced during the normal cellular metabolism in sufficient amounts to overcome the normally efficient protective mechanisms, metabolic and cellular disturbances will occur.

Major disturbances induced by the free - radical reactions are shown in Fig 2. The reactive free radicals may cause cellular damage in a variety of ways (2). These include (i) covalent binding of the free radical to membrane enzymes and/or receptors, thereby modifying the activities of membrane components; (ii) covalent binding to membrane components, thereby changing cellular structure and affecting membrane function and/or antigenic character; (iii) disturbance of transport processes through covalent binding, thiol group oxidation, or change in polyunsaturated fatty acids (PUFAs)/protein ration; and (iv) initiation of lipid peroxidation of PUFAs with direct effects on membrane structure and associated influences of the products of peroxidation on membrane fluidity, cross-linking, structure, and function. The importance of the free-radical chain reactions and their cascading effects on a spreading network of disturbances are further illustrated in Fig 3.

Among the most deleterious effects of free radicals are damage to DNA and phospholipids. In vitro studies have clearly shown that the presence of a free-radical-generating system, such as phagocytosing lymphocytes or xanthine oxidase, leading in turn to harmful mutations and cytotoxic effects (17,18). The potential for such damage in the etiology of cancers and other diseases is therefore very large. It should, however, be remembered that the highly reactive free radicals are essentially trapped in the immediate vicinity of their site of formation as a consequence of their rapid interaction with neighboring molecules (19). Thus, in cellular terms, their radius of

diffusion is often very small. The reactive free radicals formed in the endoplasmic reticulum are therefore unlikely to diffuse far enough to react with DNA in the nucleus. These restrictions on the diffusivity of the highly reactive free radicals, especially the HO° radical, necessitate the generation of an intermediate chemical reactivity in order to interact directly with DNA (20). Thus, during "normal" cellular metabolism, highly reactive free radicals cannot diffuse far enough, whereas free radicals of very low reactivity, although able to diffuse farther, will not be reactive enough to produce significantly important covalent adducts responsible for the mutations and cytotoxic effects .

4. Protective Mechanisms Against Free Radical Induced Pathology

To circumvent the damaging effects of free radicals, humans have developed a three-tier defense strategy. The foremost defense is to prevent the generation of the reactive forms of partially reduced oxygen. This is accomplished by the cytochrome oxidase system of the electron transport chain operating in the mitochondria, which catalyzes the tetravalent reduction of oxygen without the release of a significant amount of any of these reactive intermediates.

The second line of defense is provided by enzymes that catalytically scavenge the intermediates of oxygen reduction. The intracellular enzymes superoxide dismutase (SOD) and catalases are extremely important in defusing free radical oxidants before they react with critical cellular materials. These enzymes are probably present intracellularly because cells contain iron and probably copperions in forms that can accelerate damaging free-radical reactions. Hence, rapid removal of both $O_2^{\bullet-}$ and H_2O_2 is essential before these species can come into contact with the intracellular metal ions. The enzymes glutathione peroxidase and glutathione reductase also play important protective roles in this regard.

The $O_2^{\bullet-}$ radical is eliminate by SOD, which dismutases $2O_2^{\bullet-}$ to H_2O_2 and O_2. The H_2O_2 is then reduced to water by the action of catalases. The removal of both $O_2^{\bullet-}$ and H_2O_2 prevents the formation of $HO^{\bullet}$ is impossible due to its extreme reactivity.

In contrast, the extracellular fluids contain little SOD or H_2O_2- scavenging enzymes, because at physiological generation rates, the $O_2^{\bullet-}$ to H_2O_2 are useful and thus should not be rapidly removed (2). However, it then becomes essential to prevent

them from interacting to form $HO^{\bullet}$ and other highly toxic species. This is achieved by making transition metal ions unavailable to catalyze reactions in extracellular fluids. Hence, there is three times as much transferrin iron-binding capacity in human plasma as iron needing to be transported, so there are essentially no free iron ions in plasma . Iron ions bound to transferrin cannot stimulate lipid peroxidation or the generation of free $HO^{\bullet}$ radicals, and the same is true of copper ions bound to the plasma proteins ceruloplasmin or albumin .

Finally, these two levels of enzymatic defenses are supplemented by a third level of biochemical defenses that include vitamin E (tocopherols), vitamin C (ascorbic acid), and other natural and diet-derived synthetic antioxidants or free-radical scavengers. Although the term *antioxidant* is implicitly restricted to chain-breaking compounds. To be effective against free-radical-mediated cell disturbances, the antioxidants or free-radical scavengers must have several important characteristics. These criteria are as follows.

The scavenger (antioxidant) must get to the right site within the cell of the relevant tissue in a concentration that is sufficient to allow effective competition with neighboring biomolecules.

The scavenger (antioxidant) must get to the right site at the right time in order to interact with transient damaging free radical species as they are formed.

The scavenger (antioxidant) must be able to interact with the toxic species sufficiently rapidly to ensure successful competition with biologically sensitive loci in the immediate vicinity of free – radical production.

The scavenger (antioxidant) must have acceptable biological properties, that is, its inherent toxicity must be low.

Finally, in summary, the scavenger (antioxidant) must get to the right site at the right time and in the right concentration; moreover, it must have acceptable low intrinsic toxicity for use under conditions in vivo.

Furthermore, their individual properties cannot be judged in isolation. Antioxidants are members of a rather large and actively cooperating family of chemical substances. For example, $\propto$ - tocopherol activity in humans is regenerated by ascorbic acid .

Antioxidants can act at different levels in the oxidative sequence as follows.

1. Decreasing localized oxygen concentrations.

2. Preventing chain initiation by scavenging initiating radicals such as $HO^{\bullet}$.

3. Binding metal ions in forms that will not generate such initiating species as $HO^{\bullet}$, ferryl, or $Fe^{2+}/Fe^{3+}O_2$ and/or willnot decompose lipid peroxides to peroxy and alkoxy radicals.

4. Decomposing peroxides by converting them to non – radical products, such as alcohols.

5. Chain-breaking, that is, scavenging intermediate radicals such as peroxy and alkoxy radicals to prevent continued hydrogen abstraction. Chain-breaking antioxidants are often phenols or aromatic amines.

Table 2 Examples of Some Disorders and Diseases Associated With Free-Radical Pathology

Cancers
Coronary heart disease/atherosclerosis
Diabetes
Cataract
Adverse drug reactions
Toxic liver injuries
CCl_4 and other halogenoalkanes
Bromobenzene
Allyl alcohol
Iron overload
Paracetamol
Alcohol
Redox cycling mechanisms
Quinones
Nitroimidazoles
Arthritis
Immune hypersensitivity
Inflammatory disorders
Reperfusion injuries
Thrombosis
Organ storage
Transplantation
Neurological degeneration
Aging
Traumatic inflammation

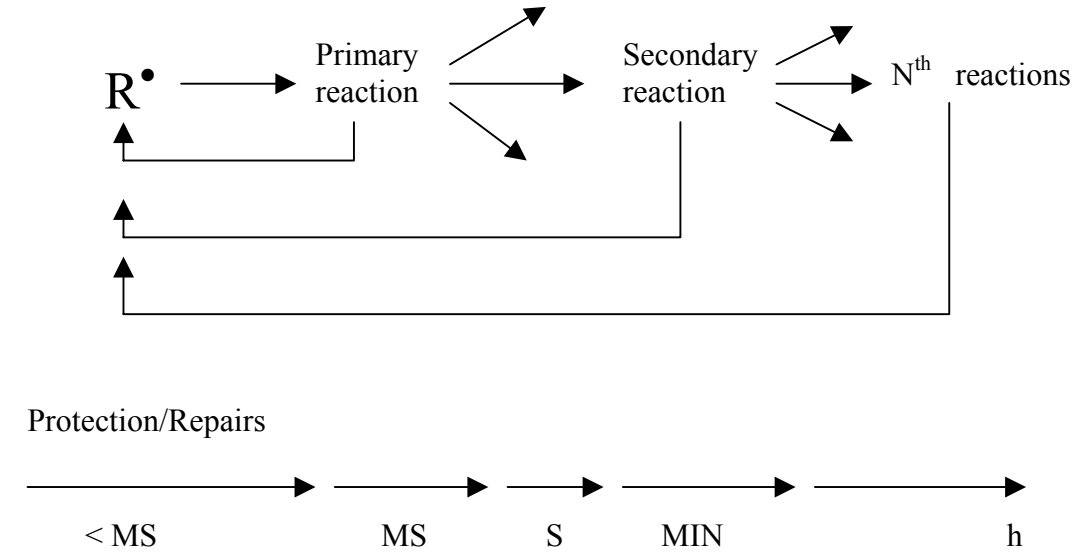


Figure 2 Free radicals and cellular injury. Major routes are shown in which a free radical (R•) can interact with neighboring components in cells to disturb their metabolic function(s).

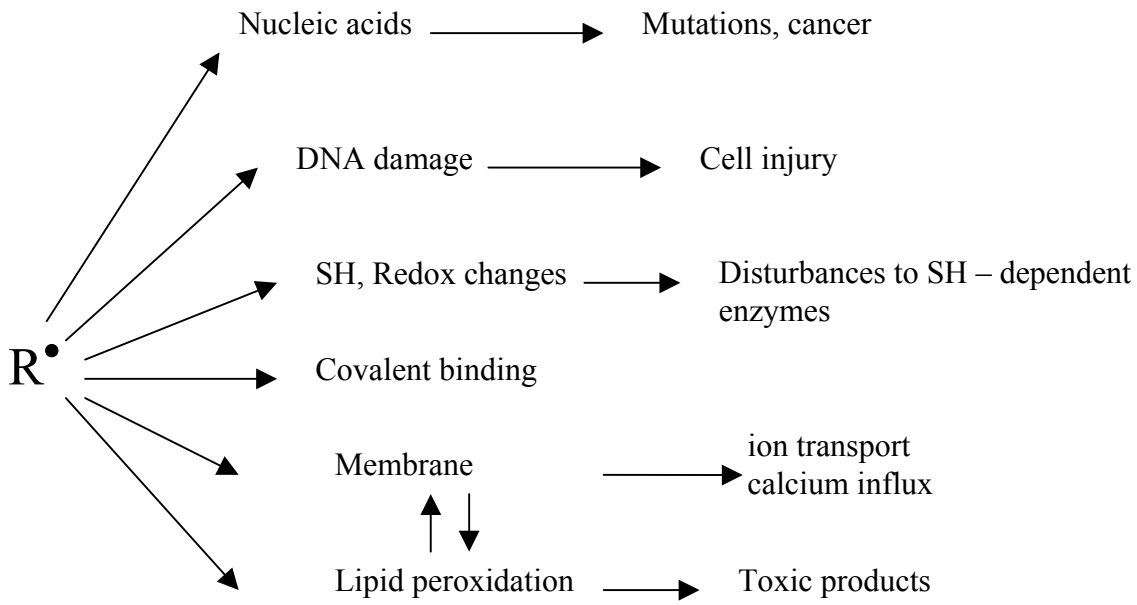


Figure 3 A representation of a free-radical-mediated disturbance producing a diverging set of metabolic perturbations, with some or al of these repaired or prevented by effective cell defenses involving free radical scavengers and antioxidants.

5. Other Antioxidants

Some antioxidants are synthesized such as Trolox. More importantly, a large number of antioxidative agents comes from natural sources such as curcumin, quercetin, caffeic acid.

5.1 Quercetin

Quercetin (3,5,7,3′,4′-pentahydroxyflavone) the major representative of the flavonol subclass of the flavonoids belongs to a large group of naturally-occurring flavonoid compounds found in plants, foods and beverages(22).

Characteristics of Quercetin (23)

- yellow needle
- UV_{max} (alc) 258,375 nm
- Insoluble in water
- mp. 314º c
- 1 g dissolves in 290 ml absolute ethanol

Quercetin possesses a variety of biological activities besides its antioxidative property. These include cytoprotective, anti-inflammatory, antihistaminic, anticarcinogenetic, etc.(Table 3).

Table 3 Biological activity of quercetin

Compound	Biological activity (24,25,26)
Quercetin	Cytoprotective Antihistamine Antispasmodic Antianxiety Antioxidant Anticarcinogenic Antiviral Antiinflammatory Inhibit platelet aggregation

6. Antioxidant Effects of Plant Phenolic Compounds

Phenolic compounds are widely distributed in plants. One of the major groups of phenolic compounds is the flavonoids, which are important in contributing to the flavour and color of many fruits and vegetables and products derived from them such as wine, tea and chocolate.

6.1 Antioxidant activity of flavonoids and phenolic acid

The term "phenolic compound" embrances a wide range of plant substances which possess an aromatic ring bearing one or more hydroxyl substituents. They frequently occur attached to sugars (glycosides) and as such tend to be water soluble. The flavonoids are the largest single group of phenolic compounds. Flavonoids are C15 compounds composing of two phenolic rings connected by a three-carbon unit (27). The flavonoids are biosynthetically derived from acetate and shikimate (28) such that the A ring has a characteristic hydroxylation pattern at the 5 and 7 position. The B ring is usually 4′,3′,4′ or 3′4′5′ hydroxylated. The major groups of flavonoid compounds compose of chalcone, flavonol flavone, isaflavone, flavanone, aurone and authocyanidin (Figure 5). Some major dietary sources of phenolic compounds are outlined in Table 4. The daily intake of flavonoids has been estimated at between 20 mg and 1 g (29). The flavonols, particularly catechin and catechin-gallate esters and the flavonol quercetin, are found in beverages such as green and black tea (27,30) and red wine (31). Quercetin is also a predominant component of onions, apples and berries. The flavanones, such as naringin, are mainly found in citrus fruits.

Flavonoids and phenolic acid can act as antioxidant by a number of potential pathways. The most important is likely to be by free radical scavenging in which the polyphenol can break the free radical chain reaction. A number of studies have been carried out on the structure-antioxidant activity relationships of the flavonoids. The main structural feutures of flavonoids required for efficient radical scavenging could be summarized as follows (27):

1. An ortho-dihydroxy (catechol) structure in the B ring for electron delocalization (Figure 6, a)
2. A double bond (2,3) in conjugation with a 4-keto function, provides electron delocalization from the B ring (Figure 6, b)
3. Hydroxyl groups at position 3 and 5, providing hydrogen bonding to the keto

group (Figure 6, c)

The structural features of flavonoids are illustrated in Fig. 4(32).

The phenolic acid may also be good antioxidants, paricularly those possessing the catechol-type structure such as caffeic acid. Recent studies have indicated that simple cell-derived phenolic acid such as hydroxyanthranilic acid may also be efficient co-antioxidants for α – tocopherol, able to inhibit lipoprotein and plasma lipid peroxidation in humans. The possible interaction between flavonoid and phenolic acids with other physiological antioxidants such as ascorbate or tocopherol is another possible antioxidant pathway for these compounds. The synergistic interaction of these antioxidants may be exemplificd by the enhancement of the antiproliferative effect of quercetin by ascorbic acid, possibly due to its ability to protect the polyphenol from oxidative degradation. In a similar manner, coincubation of low-density lipoprotein (LDL) with ascorbate and caffeic or coumaric acid resulted in a synergistic protection from oxidation promoted by apparent antioxidant action

Another pathway of apparent antioxidant action of the flavonoids, particularly in oxidation systems using transition metal ions such as copper or iron, is the chelation of the metal ions. Chelations of the catalytic metal ions may prevent their involvement in Fenton-type reactions which can generate highly reactive hydroxyl radicals.

Other biological actions of phenolic compounds have been noted which may be relevant to their effects on human health. For example, caffeic acid may have cytoprotective effects on endothelial cells related not only to its antioxidant action but also to its ability to block the rise in intracellular calcium in response to oxidized lipoproteins. Some phenolic compounds may also inhibit platelet aggregation. The ability of phenolic compounds to trap mutagenic electrophiles such as reactive nitrogen species may also protect biological molecules from damage (27).

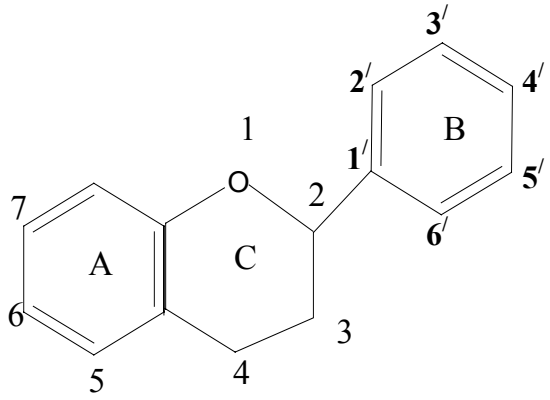
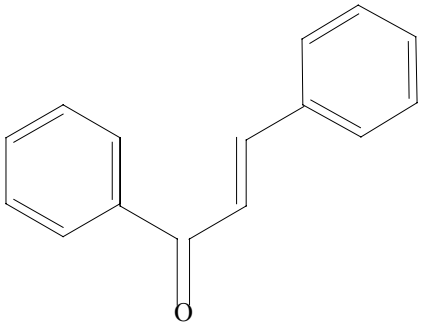


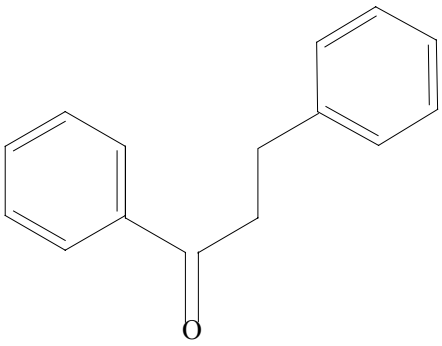
Figure 4 Flavonoid skeleton

Table 4 Some dietary sources of flavonoids and phenolic acids

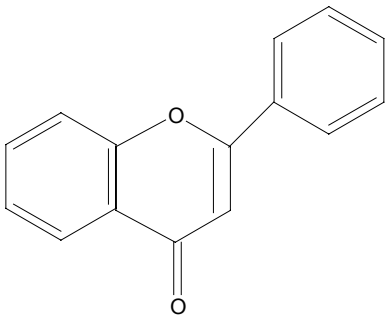
Flavonoid	Souce
Catechins	Tea, red wine
Flavonones	Citrus fruits
Flavonols (e.q. quercetin)	Onions, olives, tea, wine, apples
Anthocyanidins	Cherries, stawberries, graper, coloured fruits
Caffeic acid	Grapes, wine, olives, coffee, apples, tomatoes, plums, cherries



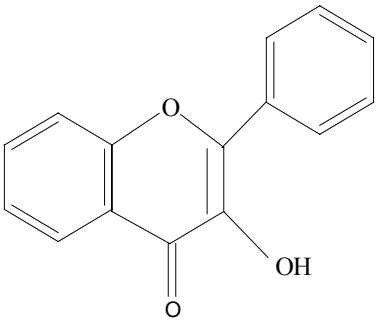
Chalcone



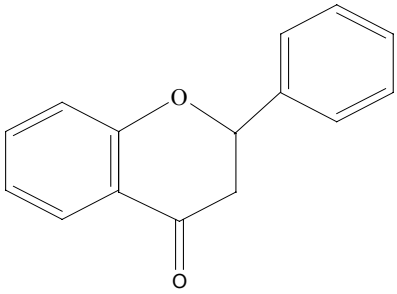
Dihydrochalcone



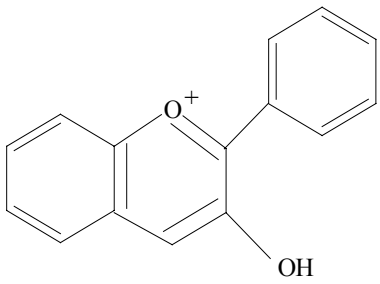
Flavone



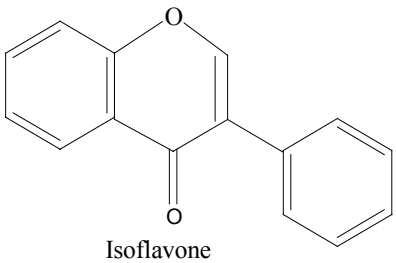
Flavonol



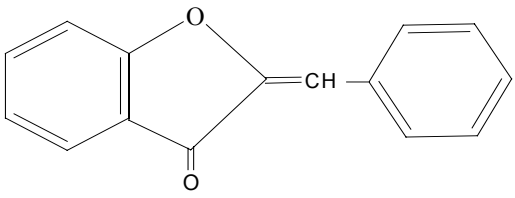
Flavanone



Anthocyanin



Isoflavone



Aurone

Figure 5 The major groups of flavonoids

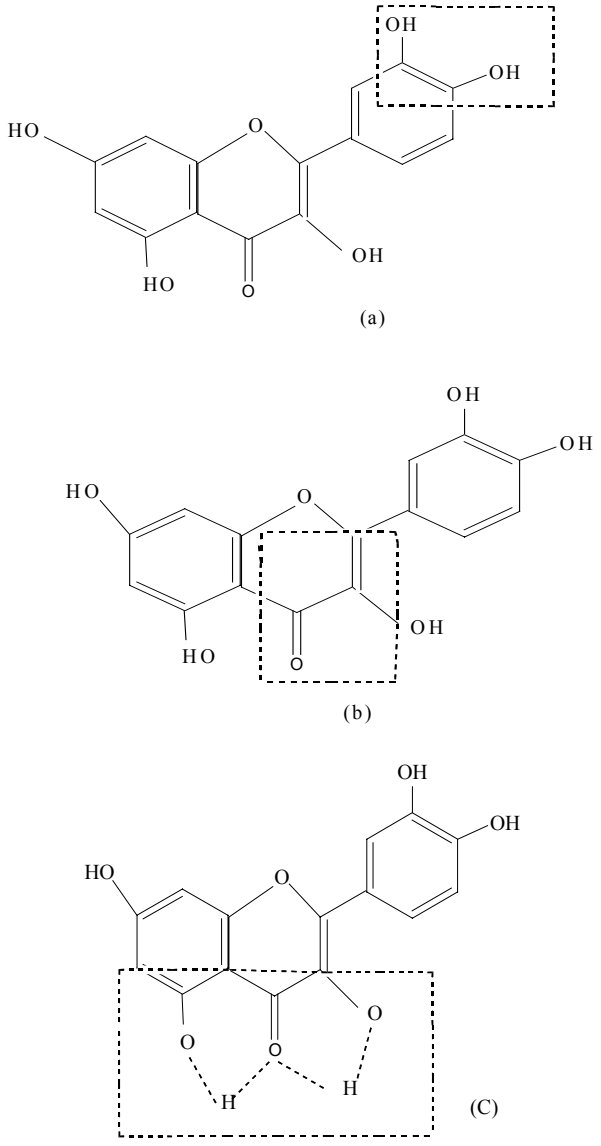


Figure 6 Structural group for radical scavenging

7. Antioxidant Activity Determination

The methods of detecting and quantitatively estimating synthetic and natural antioxidants have been detected by color reaction to semiquantitative and quantitative methods such as spectrophotometry ; voltametry ; polarography ; and chromatographic methods like paper, thin-layer, and column chromatography and the more advanced gas-liquid chromatography (GLC) and high performance liquid chromatography (HPLC).

There are many methods for the determination antioxidant activity depending on what we want to measure such as free radicals free radical products, antioxidant agents, metal chelating activity. We can choose the stable free radicals such as 1,1 – diphenyl -2-picrylhydrazyl (DPPH) radical for studying the antioxidant activity using method (3).

7.1 DPPH scavenging assay

1,1- Dipheny1-2-picrylhydrazyl (DPPH) radical is a very stable radical with a deep violet color. When DPPH radical receives a proton from the antioxidant then it converts to a colorless protonated DPPH molecule. The mechanism of the reaction of antioxidant with a DPPH radical is given below (33):

$$(DPPH)^{\bullet} + HO - R - OH \longrightarrow (DPPH): H + HO - R - O^{\bullet}$$

deep violet colorless

$$HO^{\bullet} - R - O + (DPPH)^{\bullet} \longrightarrow (DPPH): H + O = R = O$$

Colorless

Using this reagent, the free radical – scavenging ability of the antioxidant can be determined by spectroscope methods (32).

7.2 Total antioxidant determination by ABTS – metmyoglobin method

The ABTS-metmyoglobin assay for measuring total antioxidant activity is a measure of the collective hydrogen-donating abilities of the antioxidants in sample. It is based on the interaction between antioxidants in the samples with the ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)) radical monocation, which is highly chromogenic and has absorbance maxima at 600 nm. Trolox, water soluble vitamin E analogue, is used as an antioxidant standard, The mechanism of the reaction is given below.

$$HX\text{-}Fe^{3+} + H_2O_2 \longrightarrow X\text{-}[Fe^{+4} = O] + H_2O$$

$$ABTS + X\text{-}[O^{+4} = O] \longrightarrow ABTS^{\bullet+} + HX\text{-}Fe^{3+}$$

7.3 Lipid peroxidation

Tappel AL.(70) has defined lipid peroxidation as the oxidative deterioration of polyunsaturated lipid. Polyunsaturated fatty acids are essential biomolecules. They play an important role in cellular metabolism and cellular structure. They exist as free acid (e.g., arachidonic acid, the precursor of prostaglandin, leukotrienes, prostacycline), ad thioester (acetyl ScoA), but mainly as esters (e.g., triglycerides, phospholipid, sphingolipids and cholesteryl-esters). The natural unsaturated fatty acids have one or more cis-carbon-carbon double bonds starting from the ninth C upward, each double bond being separated from the other by an allylic methylene (CH_2) group. Because of their peculiar chemical structure (*cis* – double bonds separated by an allylic CH_2), unsaturated fatty acids can readily react with free radicals and undergo peroxidation.

Lipid peroxidation is a complex process that occurs in the presence of oxygen and transitional metal ions or enzymes. These are usually three stages in the oxidation process: initiation, propagation and termination. These processes can become autocatalytic after initiation and yield lipid peroxide, lipid alcohol, and aldehyde by – products.

Peroxidation of fatty acids contained three or more double bonds produce malondialdehyde (MDA). The production of malondialdehyde involves the formation of hydroperoxides, beta cleavage to yield hydroperoxyladehydes and finally *via* a

second beta scission. Both malondialdehyde and acrolein radicals are able to combine with hydroxyl radical ($HO^{\bullet}$) to form the enol.

8. Plants with Antioxidant Activity

8.1 Screening for antioxidant activity plants

Many plants are studied about their antioxidant activities such as *Allium satiam, Centella asiatica, Azadirachta indica A. J*uss,var, *siamensis* Valeton, *Ginkgo bilogba Linn. Curcuma longa* Linn. (34) It has been reported that natural antioxidants such as α - tocopherol (vitamin E), ascorbic acid (vitamin C), β - carotenids, flavonoids and polyphenolic compounds present in variety of vegetables and fruits.

9. Potential Sources of Natural Antioxidants (2).

In recent years, numerous report have been published on the identification of novel, naturally occurring antioxidants from plants, animals, microbial sources, and processed food products.Table 5 presents a list of plant antioxidant sources. Recent reports in this area discuss young green barley leaves (35); leaves of *Polygonum hydropiper*, a medicinal herb (36); pea bean (37); leaves of *Vernonia amygdalina* (38); and wild rice (39)

In addition to identification of the sources, numerous reports have appeared on further identification and isolation of the active compounds from various sources. Most natural antioxidants are phenolic compounds that, with the exception of the tocopherol, contain ortho-substituted active group, whereas the synthetic antioxidants, with the exception of the gallates, are para-substituted . Some of the major active compounds reported so far are flavonoids and related compounds in plant extracts; phenolics in spices and herbs; and proteins, protein hydrolysates, peptides, amino acids, and Maillard reaction products, This chapter presents a brief sumary of the important sources and compounds and some recent studies wherever possible.

9.1 Flavonoids and related compounds

Flavonoids are one of the most widely occurring groups of secondary metabolites in plants. They are found in almost all parts of the plant. The chemical structures are based on a C_6-C_3-C_6 skeleton. Various subgroups are classified on the basis of the substitution patterns of ring C and the position of ring B. The major subgroups are flavonols, isoflavones, catechins, proanthocyanidins, and anthocyanins (fig.8.). chalcones, flavanones, leucoanthocyanins, and dihydroflavonols are the common precursors for the different subgroups in the biosynthetic pathway. Cinnamic and phenolic acids are closely related to flavonoids, and some of them are precursors for the flavonoid biosynthetic pathway. Most of these compounds have shown marked antioxidant activity in model systems. The antioxidant properties reported for numerous extracts from leaves, seed hulls. Seeds, fruits, and stems are mainly due to flavonoids and cinnamic acids, Pratt and Hudson (40) conducted a general survey of the mechanisms and compounds involved and the structure-activity relationships of these compounds. Flavonoids function as primary antioxidants, chelators, and superoxide anion scavengers. The presence of hydroxyl groups at the 3´, 4´ and 5´ positions in the B ring enhances the antioxidant activity compared to that of a single hydroxyl group. Also, the presence of a 3 – hydroxyl group and the 2 – 3 double bond in the C ring seems to have an effect on the antioxidant properties.

3,4-Dihydroxychalcones such as butein and okanin are particularly effective antioxidants in the range of concentrations 0.025-0.1% in lard. They are more effective than the corresponding flavonones. Butein at 0.02% is twice as active as quercetin and ∝ - tocopherol and about six times as active as BHT in lard. It is effective in lard, cottonseed oil, and butter oil . Dihydroquercetin has been identified as one of the antioxidant compounds in peanuts .

The antioxidant activity of the flavones luteolin and isovitexin, a C-glycosyl flavone, have been reported. Luteolin is particularly active in lard and stripped corn oil . It has also been indentified as the active compound in peanut seed hulls and in the leaf extracts of *Vernonia amygdalina* . Igile et al. (38) showed that luteolin is a significantly more potent antioxidant than BHT and stronger than ∝ - tocopherol . Isovitexin was identified as the active component in the methanolic extracts of long – life rice hulls (*Oryza sativa* var. Katakutara). Isovitexin from young green barley

leaves has also been reported. Its antioxidant activity was almost equivalent to that of ∝ - tocopherol in an ethyl linoleate model system .

The flavonols quercetin, myricetin, robinetin, and gossypetin also have potent antioxidant properties. Quercetagetin, gossypetin, 3, 5, 8, 3´, 4´- pentahydroxyflavone, and 3, 7, 8, 2´, 5´ - pentahydroxyflavone are the most potent antioxidants reported in nonaqueous systems . Quercetin has been effective in inhibithing copper – catalyzed oxidation of lard and in dry milk products and potato flakes . Quercetin. 7, 4´ - dimethylquercetin, 3´- methylquercetin, and isoquercitrin (quercetin glucoside) were identified as the active compounds in the leaf extracts of *polygonum hydropiper*, a medicinal herb . The acetone extracts of red onion skin have antioxidant properties that can be attributed to the presence of quercetin (2.5 – 6.5 % as aglycone).

Isoflavones from soybeans consist primarily of 7-*O*-monoglucosides of genistein, daidzein, and glycitein. The aglycones have comparable antioxidant properties. Studies by Dziedzic and Hudson (2) showed a pronounced synergism between genistein and phosphatidylethanolamine in lard. Both the 4´-and 5-hydroxy groups are needed for significant activity. In general, isoflavones show a relatively low order of antioxidant activity compared to the flavonols, flavones, flavonones, and chalcones .

Of the various leaf extracts reported, green tea extracts have the potential for large – scale application as natural antioxidants. The ethanol and acetone extracts of green tea are highly effective in soybean, corn, palm, and peanut oils and lard. The major active compounds in green tea are catechins with the following order of activity: epigallocatechin gallate>epigallocatechin>epicatechin gallate>epicatechin. They were superior to BHA and ∝ - tocopherol in lard and salad oil. Epigallocatechin gallate also showed synergism with ascorbic acid, ∝ - tocopherol, citric acid, and tartaric acid .

The antioxidant activity of water – soluble tannins from *Osbeckia chinensis* has been reported . Two proanthocyanidin dimers B-1 and B-3 having a higher antioxidant activity than ∝ - tocopherol in a linoleic acid-β-carotene-water system were isolated from red beans . Esters of gallic acid, the main constituent of tannins, are being used as food antioxidants. Proanthocyanidins from fruits such as grapes, black currants, and

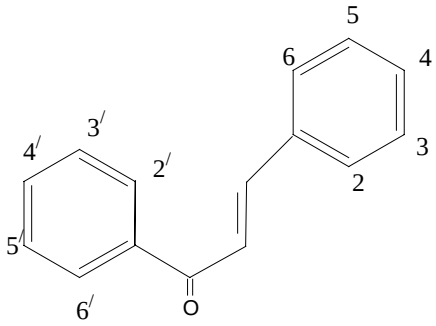
bilberries function as superoxide anion scavengers in model systems. Proanthocyanidins are effective in salas oil, frying oils, and lard.

Anthocyanins, the major coloring pigments in higher plants, also have antioxidant activity. The antioxidant properties of the major pigment in grapes, malvidin 3, 5-diglucoside, have been reported. Igarashi et al. (2) also reported the antioxidant properties of nasunin, the acylated anthocyanin of egg-plant. The higher antioxidant activity of nasunin compared to the corresponding delphinidin aglycone was attributed to the presence of *p*-coumaric acid moiety in nasunin. Recently, Tsuda et al.(2) reported the antioxidant activity of anthocyanins cyanidin, pelargonidin, and delphinidin-3-glucosides from the seed coat of pea bean.

Phenolic acids with antioxidant activity occur widely in oil seeds and leaf extracts. The various active components identified in mustard seeds and repeseeds include cinnamic, ferulic, caffeic, prototocatechuic, sinapic, salicylic, and vanillic acids. Phenolic acids with antioxidant properties have also been reported in cottonseed flour, peanuts, and peanut flour. The antioxidant activity is determined by the numbwer of hydroxyl groups in the molecule. The antioxidant properties of germinated oat (*Avena sativa*) grains or their aqueous extracts have been attributed to the presence of dihydrocaffeic acid and phospholipids . Recently, Ohta et al. (41) identified diferulic acid and ferulic acid sugar esters as active components in corn bran hemicellulose fragments. Ohta et al. (41) also identified ferulic acid glycosides as active compounds in sake.

Table 5 Some Potential Sources of Natural Antioxidants from Plants.

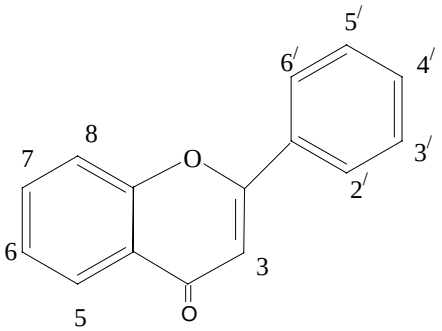
Apple cuticle	Oats
Birch bark	Olives
Carob pod	Oregano
Chia seed	Peanut seed coat
Cloves	Pepper
Cocoa shells	Red onion skin
Garlic	Rice hull
Korum rind	Rosemary
Leaf lipids	Sesame seed oil
Licorice	*Silybium marianum* seed oil
Mustard leaf seed	Tea
Myristica fragrans	Wheat gliadin
Nutmeg	



Chalcones

Butein 2´= 4´ = 3 = 4 = OH

Okanin 2´ = 3´ = 4´= 3 = 4 = OH

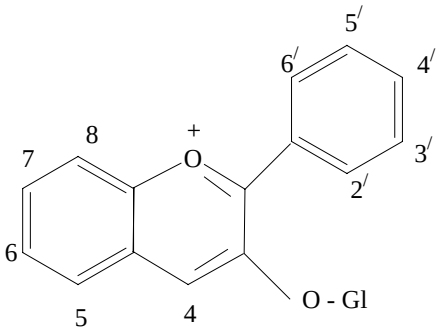


Flavones

Luteolin 5 = 7 = 3´= 4´ = OH

Isovitexin 4´ = 5 = 7 = OH, 6 = Glucose

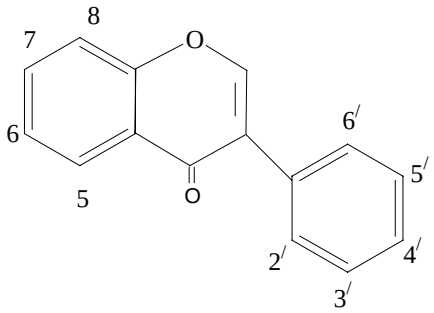
Figure 7 Some antioxidant flavonoids and related compounds



Anthocyanins

Cyanidin-3-glucoside 5′= 4′= 5 = 7 = OH

Malvidin-3-glucoside 5 = 7 = 4′= OH, 3′ = 5′ = OCH_3

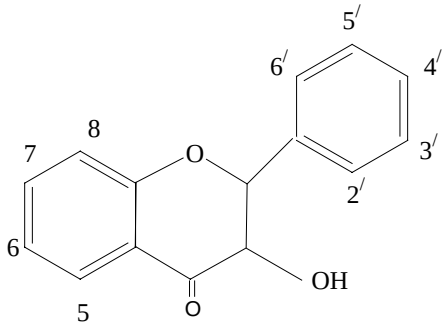


Isoflavones

Daidzein 7 = 4′ = OH

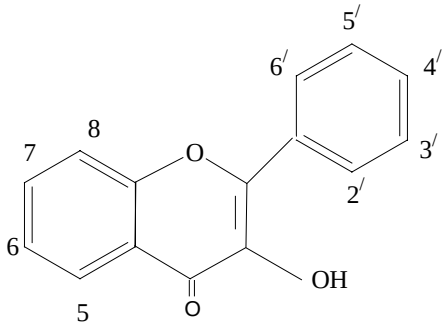
Genistein 5 = 7 = 4′= OH

Figure 7 (Continued)



Dihydroflavonols

Dihydroquercetin 3 = 5 = 7 = 3′ = 4′ = OH

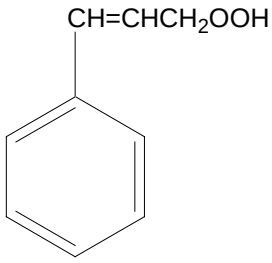


Flavonols

Quercetin 3 = 5 = 7 = 3′ = 4′ = OH

Myricetin 3 = 5 = 7 = 3′ = 4′= 5′ = OH

Gossypetin 3 = 5 = 7 = 8 = 4′= 5′ = OH

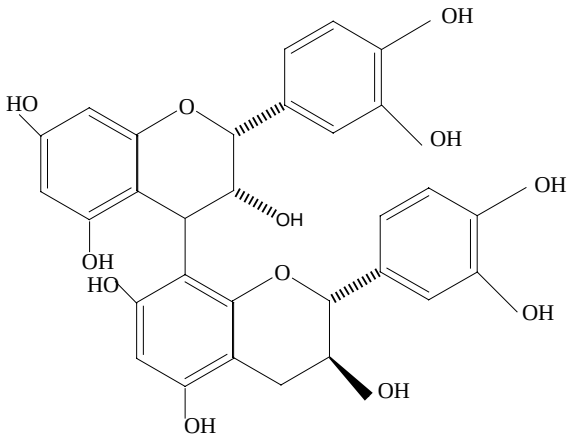


Cinnamic acids

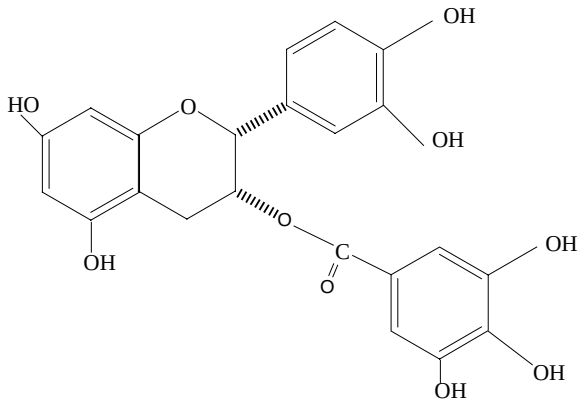
Ferulic acid 4 = OH, 3 = OCH_3

Caffeic acid 3 = 4 = OH

Figure 7 (Continued)



Procyanidin B – 1



(-) – Epigallocatechin gallate

Figure 7 (Continued)

9.2 Natural antioxidants from plant sources.

Many plants have been tested for antioxidant activity using the DPPH scavenging assay, Trolox equivalent antioxidant activity (TEAC) or inhibition of lipid peroxidation. The antioxidant activity of crude extracts from some plants and the pure compounds using differented method are shown in Table 6.

Table 6 Antioxidant Activity of Some Plant Extracts and Plant Constituents

Compounds/Extract	Plants	Antioxidant activity determination methods			Referrences
		EC_{50} (ug/ml)	TEAC***	%DPPH decoloration	
Methanolic extract	*Piper chaba*	47.8*	NA	NA	42
Methanolic extract	*Piper longum*	45.1*	NA	NA	42
Methanolic extract	*Piper nigrum*	48.7*	NA	NA	42
Aqueous extract	*Piper chaba*	57.6*	NA	NA	42
Aqueous extract	*Piper longum*	69.4*	NA	NA	42
Volatile oil	*Piper chaba*	229.5*	NA	NA	42
Volatile oil	*Piper longuni*	115.5*	NA	NA	42
Ethanolic extract	*Illicium vernum*	54.7*	NA	NA	44
Water extract	*Illicium vernum*	42.7*	NA	NA	44
Methanol extract	*Illicium vernum*	49.8*	NA	NA	44
Butanol extract	*Illicium vrernum*	38.8*	NA	NA	44
Methnol extract	*Hedyotis corymbosa*	64*	NA	NA	44
Methanol extract	*Hemidesmus indicas*	18.87*	NA	NA	44
BHT	-	18.2*	NA	NA	42

Table 6 Antioxidant Activity of Some Plant Extracts and Plant Constituents (continue)

Compounds/Extract	Plants	Antioxidant activity determination methods			Referrences
		EC_{50} (ug/ml)	TEAC***	%DPPH decoloration	
Gallic acid	-	2.6*	NA	NA	43
Vitamin C	-	18.2*	NA	NA	43
(+)-Epicatechin	-	0.8*	NA	NA	44
(+)-Catechin	-	0.8*	2.4	NA	44, 46
Vitamin E	-	7.75*	NA	NA	44
Pyrogallol	-	1.62*	NA	NA	45
Quercetin	-	1.36*	4.7	58	46, 47,48
Epigallocatechin gallate	-	NA	4.8	NA	46
Epigallocatechin	-	NA	3.8	NA	46
Taxifolin	-	NA	1.9	NA	46
Luteolin	-	NA	2.1	59	46, 47
Rutin	-	9.77**	2.4	36	46, 47

Table 6 Antioxidant activity of Plant extracts and Plant constituents(continue)

Compounds/Extract	Plants	Antioxidant activity determination methods			Referrences
		EC_{50} (ug/ml)	TEAC***	%DPPH decoloration	
Kaemferol	-	NA	1.3	37	46, 47, 48
Apigenin	-	NA	0	-	47

TEAC*** = Trolox equivalent antioxidant activity
* = DPPH scavenging assay
** = Inhibition of lipid peroxidation
NA = Not available

10. Genus *Pluchea*

Genus *Pluchea* contain 80 species in family Compositae (Asteraceae)

Distribution : Asia, America, Africa and Australia

The ethnomedicinal of species of genus *Pluchea* such as diuretic from *P. indica*, anti-inflammatory from *P. sagittalis* are shown in Table 7.

Table 7 Ethnomedicinal of species of genus *Pluchea*

Species	Part used	Ethnomedicinal Uses
P. indica	aerial	eye diseases, itchy skin, diuretic, dysentery, scabies, rheumatism, anti – inflammatory
P. lanceolata	leaves, flowers and stem	rheumatoid arthritis
P. symphytifolia	leaves	diarrhea, gartrointestinal parastes stomach pain, infectious of the ear, diarrhea, stomach ache
P. odorata	entire plant	convulsion, emmenagogue, tooth aches, body aches, diarrhea, rheumatism, fever,grippe stomach ache, stomach cramp, skin rash

Table 7 Ethnomedicinal of species of genus *Pluchea* (Continue)

Species	Part used	Ethnomedicinal Uses
P.odorata	leaves	stomach pain, cough, vertigo, edema, pallor
P. sagittalis	aerial	antisepic, antipyretic, stomach ache, antitussive, digestive, antiinflammatory, gastrointestinal disorder, cholagogue
	entire plant	stomach ache,
	leaves	diuretic
	root	a tonic

Table 8 Some biological activity of genus *Pluchea*

Speices	Part used	Compounds/ Extracts	Biological activity
P. indica	aerial	Freeze dry aqueous extract in capsule	Diuretic
	aerial	Decoction	
	aerial	Water extract	Anti-HIV1
	root	Methanol extract	Anti-inflammatory
	root	Petroleum ether	Analgesic activity
	root	Petroleum ether	Barbiturate-potentiaton
	root	Methanol extract	Anti-ulcer
	root	Methanol extract	Antioxidant
P. lanceolata	aerial	Ethanol extrat	Antiinflammatory
	aerial	Water extract	Antiarthritic
P. symphytifolia	aerial	Water extract	Antibacterial
	aerial	Water extract	Antisecretory
	aerial	Caffeoylquinic acid	Antibacterial
	aerial	Chloroform extract	Antinematodal
P. odorata	entire	Methanal extract	Insecticide
P. sagittalis	aerial	Water extract	Antiinflammatory

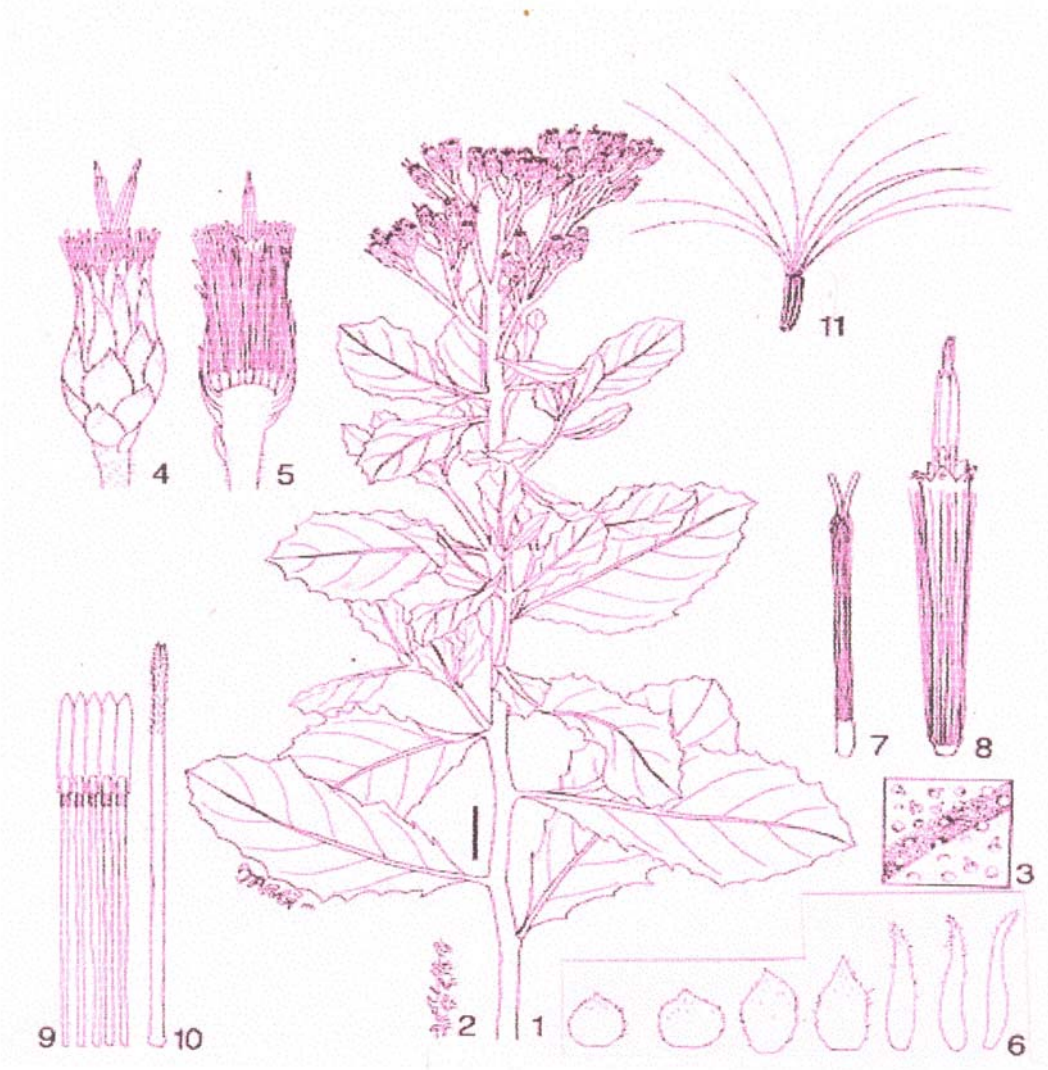


Figure 8 ***Pluchea indica*** **Less.** 1: Habit; 2: Pubescence of a branch; 3: Portion of leaf, adaxial surface; 4: Capitulum; 5: Capitulumn, longitudinal section; 6: Involucral bracts; 7: Outer floret; 8: Central floret with rudimentary ovary; 9: Stamens; 10: Style of Central floret; 11:Achene. All from Chen 215 (HAST). Bar = 1 cm**.**

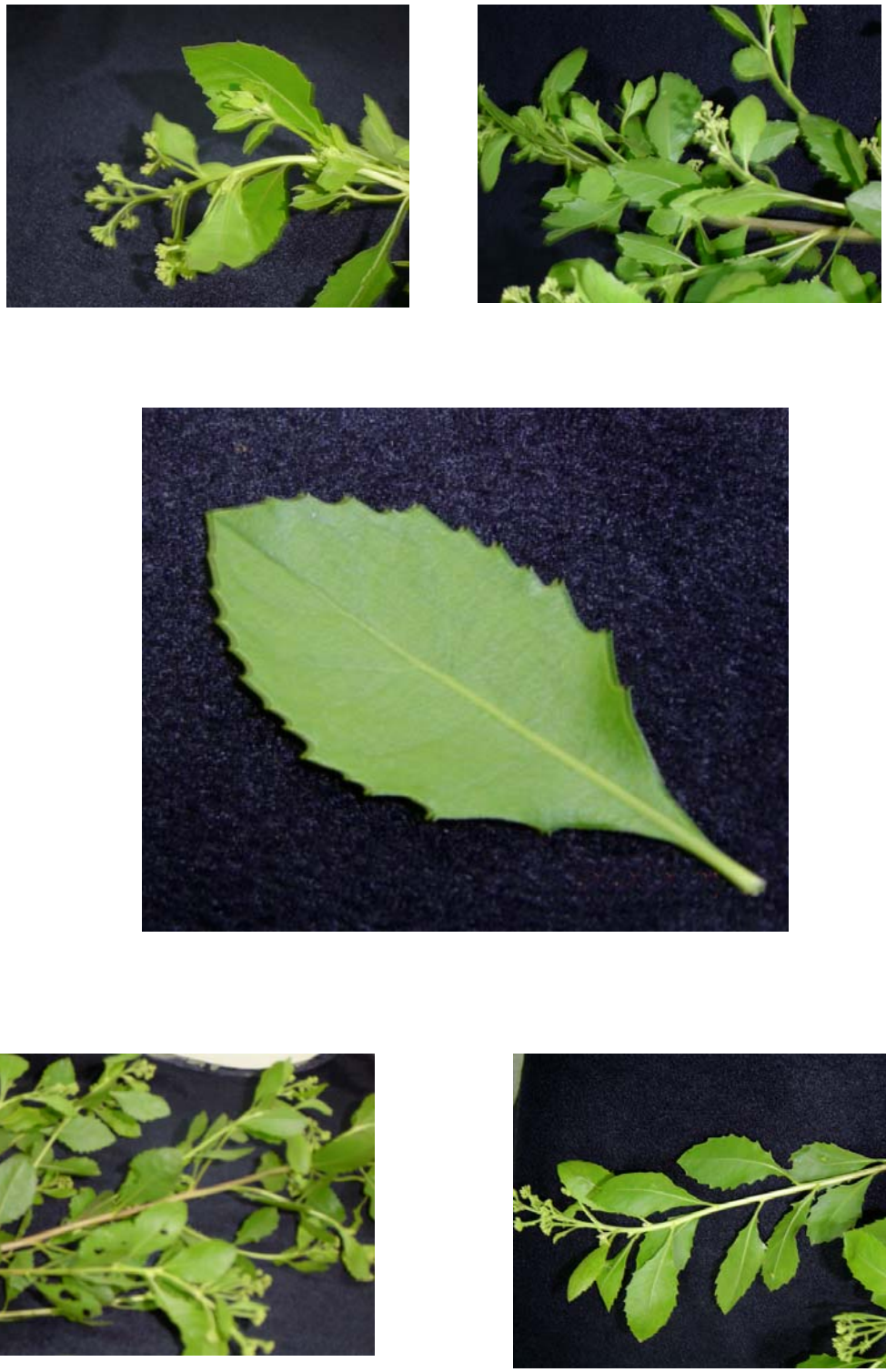


Figure 9 ***Pluchea indica*** **(Linn.) Less.**

10.1 *Pluchea indica* (Linn.) Less.

10.1.1 General information (49)

Pluchea indica (Khlu in Thai) is taxonomically classified in family Compositae (Asteraceae). Its common name is Indian Marsh Fleabane. The plant is an evergreen shrub of about 0.5-2.0 meters in height with small branches, glabrous leaves of about 1-5 cm. in length. Terminal organs of young plant are fine hairy. It has simple leaves, sessile, alternate, oboral leave shape. Leave apex are acute, margin base entire slightly serrate at the apex, 1-5 cm. width, and 2-9 cm, length. The cylindrical inflorescence consist of raceme 2-3 cm. length. Flowering at terminal or axillary, each rachilla composes of much perfect violet-white flowers. It has small fruits. It can grow in every season. The plant is widely found in Thailand (4).

10.1.2 Cultivation

Pluchea indica is cultivated in many tropical and warm temperature regions such as India, Mexico ,Java and Thailand (4, 50).

10.1.3 Medicinal Uses

In India, traditional medicinal system suggests a variety of therapeutic uses of this plant. The methanolic root extract has been reported for its anti-inflammatory and anti-ulcer activities. The root extract significantly inhibited mediator induced-edema, turpentine-induced joint edema, and carragenin-induced granuloma (50).

In Thailand, traditional medicinal system suggested the use of many parts of this plant as therapeutic agents. The stem, in decoction form is said to be useful in the treatment of kidney stones as diuretic agent. A decoction form of the bark is used for the treatment of hemorrhoid. Leaves are claimed to be useful for the treatment of inflammation and also used as nerve tonic. Fresh leaves are used in the form of poultice for gangrenous ulcer. Leaves and roots are suggested to posses antidysentery, antipyretic, and anti-inflammatory properties (4, 5, 51, 52). Cigarettes prepared from the chopped stem bark are smoked to relieve the pain of sinusitis (53). The leaves and young shoots crushed, mixed with alcohol are applied to the back area in cases of lumbago and also are used for rheumatic pains and in baths to treat scabies (54).

10.1.4 Chemical components

The chemical constituents of *P. indica* can be divided into 5 groups including terpene, benzenoids, phenylpropanoids, lignans and steriods . The chemical composition and quantity and structure of each chemical constituent are shown in Table 9 and Figure 10.

Table 9 Chemical composition and quantity of *P. indica* (55,56)

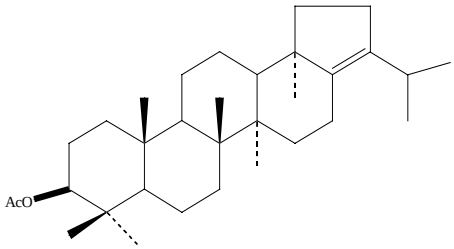
Chemical group	Chemical composition	Quantity	Part use
1.Terpenes	Boehmeryl acetate	0.00088%	root
	HOP-17 (21)-en 3β - acetate	0.0008%	root
	Linaloyl glucoside	0.000516%	aerial
	Linaloyl apiosyl glucoside	0.00031%	aerial
	Linaloy –hydroxy glucoside	0.00053%	aerial
	Plucheoside C	0.00033%	root
	Cuauhtemone,3-(2´-3´-diacetoxy-2´ methyl-butyryl)	0.00022%	entire
	Plucheol A	0.0004%	root
	Plucheol B	0.00023%	root
	Plucheoside A	0.00015%	root
	Plucheoside B	0.00023%	aerial
	Plucheoside E	0.00046%	root
	Pterocarptriol	0.00008%	root
2.Benzenoids	Benzyl glucoside	0.00053%	aerial
	Phenyl-ethyl-glucoside	0.00008%	aerial
	Methyl salicylate glucoside	0.00018%	aerial
3.Phenylpropanoids	eugenyl glucoside (Citrucin C)	0.00358%	aerial
	1,2-bis-(4-hydroxy-3-methoxyphenyl-propane-1-3-diol)	0.00016%	aerial

Table 9 Chemical composition and quantity of *Pluchea indica* (continue)

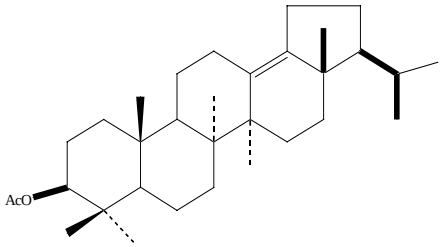
Chemical group	Chemical composition	Quantity	Part use
4.Lignans	Hedyotisol A	0.00013%	aerial
	Hedyotisol B	0.00021%	aerial
	Pinoresionol monoglucoside	0.00011%	aerial
	Plucheoside D-1	0.00031%	root
	Plucheoside D-2	0.00053%	root
	Propane-1-3-diol,1-9-4-hydroxy-3-phenyl)-2[2-2 Methoxy-4-(1-trapropene-3-ol)-phenoxy]	0.0002%	aerial
5.Steroids	Stigmasterol glucoside	0.00046%	leave

1. Terpene

1.1 Boehmeryl acetate



1.2 HOP-17 (21)-en-3β-acetate Triterpene ; root



1.3 Linaloyl Apiosyl glucoside Monoterpene ; Aerial parts

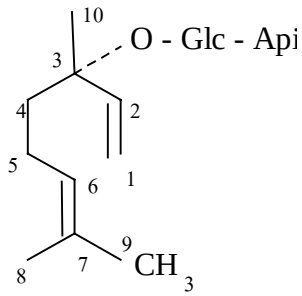
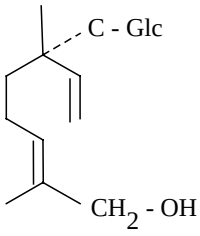
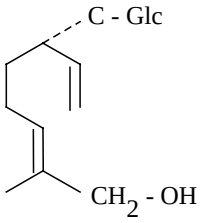


Figure 10 Structure of chemical constituents of *Pluchea indica* (55,56,57,58)

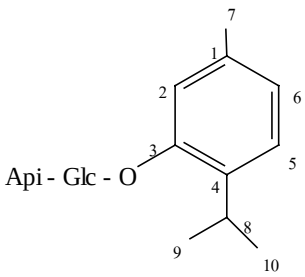
1.4 Linaloyl glucoside Monoterpene ; Aerial parts



1.5 Linaloyl-hydroxyl glucoside Monoterpene ; Aerial parts

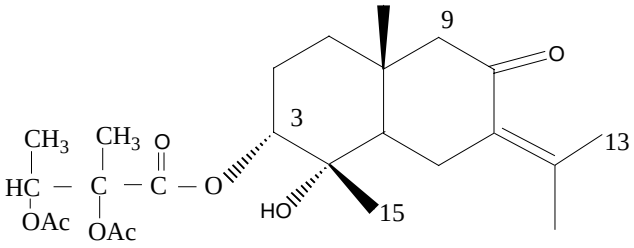


1.6 Plucheoside C Monoterpene ; Aerial parts

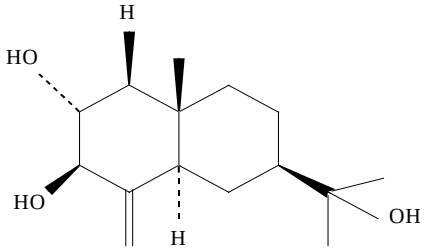


1.7 3-(2´,3´-Diacetoxyl-2´-methyl-butyryl)- cuauhtemone

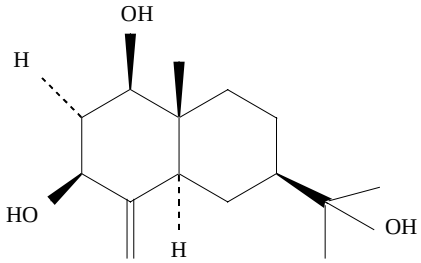
Sesquiterpene : leaf



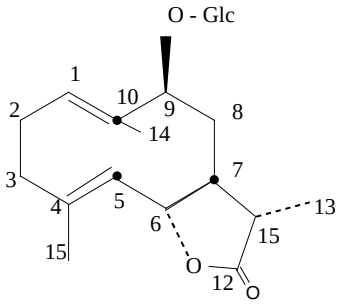
1.8 Plucheol A : Sesquiterpene ; root



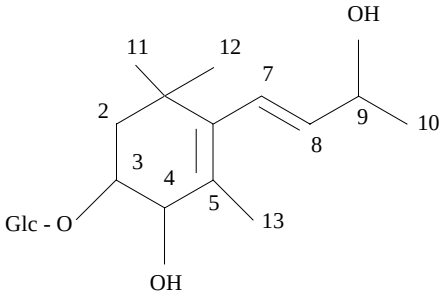
1.9 Plucheol B : Sesquiterpene ; root



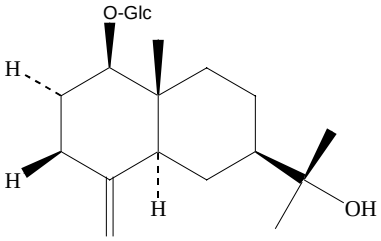
1.10 Plucheoside A : Sesquiterpene ; Aerial parts



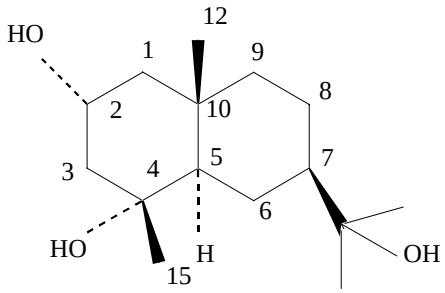
1.11 Plucheoside B : Sesquiterpene ; Aerial parts



1.12 Plucheoside E : Sesquiterpene ; root

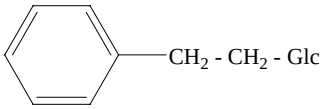


1.13 Pterocarptriol : Sesquiterpene ; root

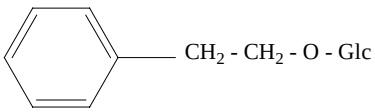


2. Benzenoid

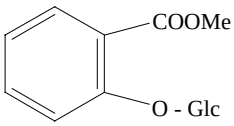
2.1 Benzyl glucoside ; aerial parts



2.2 Phenyl-ethyl-glucoside ; aerial parts

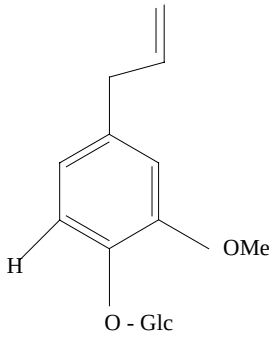


2.3 Methyl salicylate glucoside

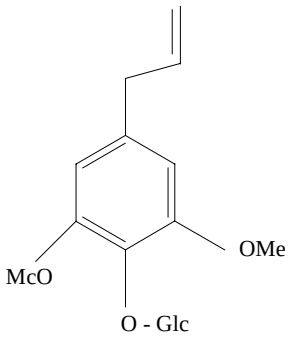


3. Phenylpropanoid

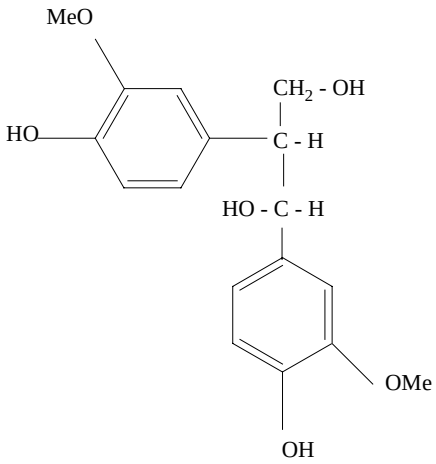
3.1 Eugenyl glucoside (Citrucin c); aerial parts



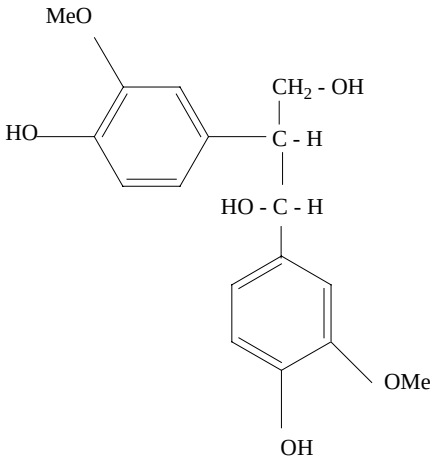
3.2 Phenol,4-allyl-2-6-dimethoxy glucoside; aerial parts



3.3 Propane-1-3-diol,1-2-bis (4-hydroxy-3-methxy-phenyl) ; aerial parts

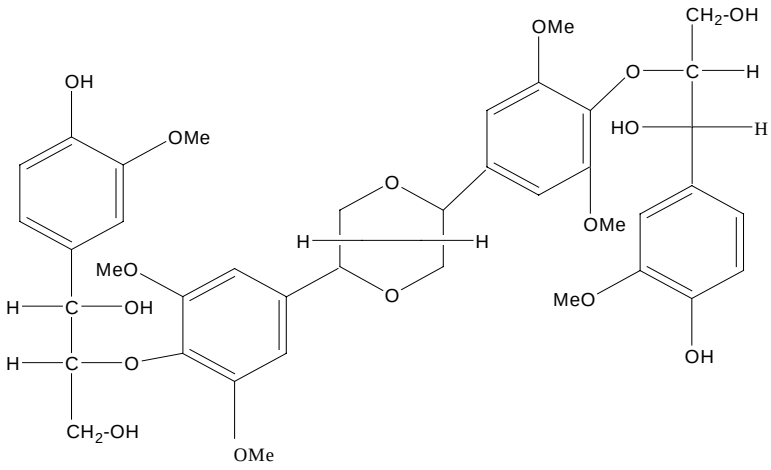


3.4 Propane-1-3-diol,1-2-bis-(4-hydroxy-3-3methoxy-phenyl) ; aerial parts

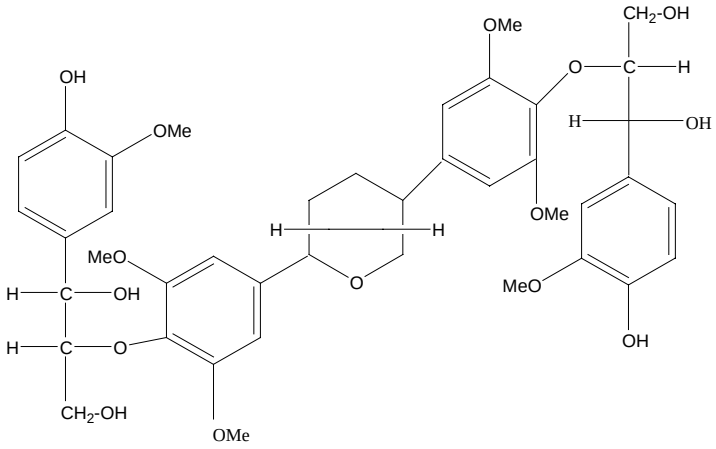


4. Lignans

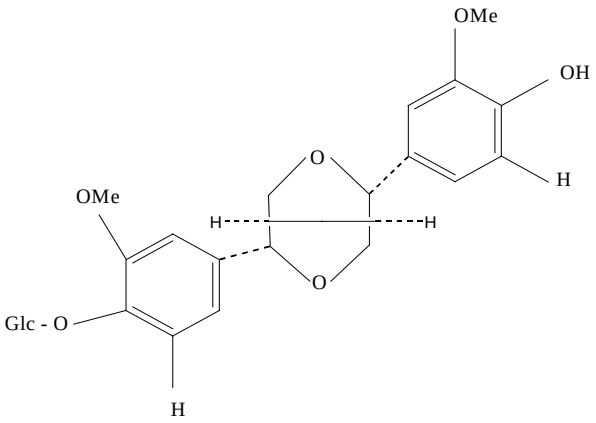
4.1 Hedyotisol A ; aerial parts



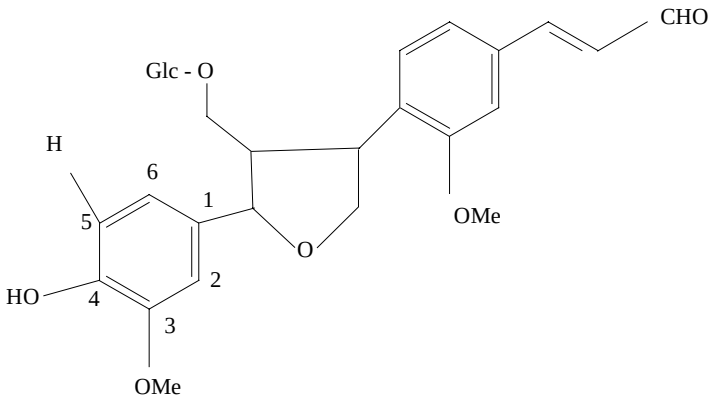
4.2 Hedyotisol B



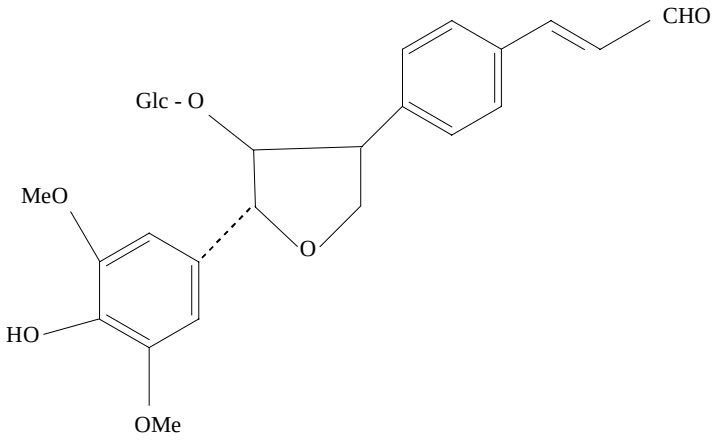
4.3 Pinoresinol monoglucoside ; aerial parts



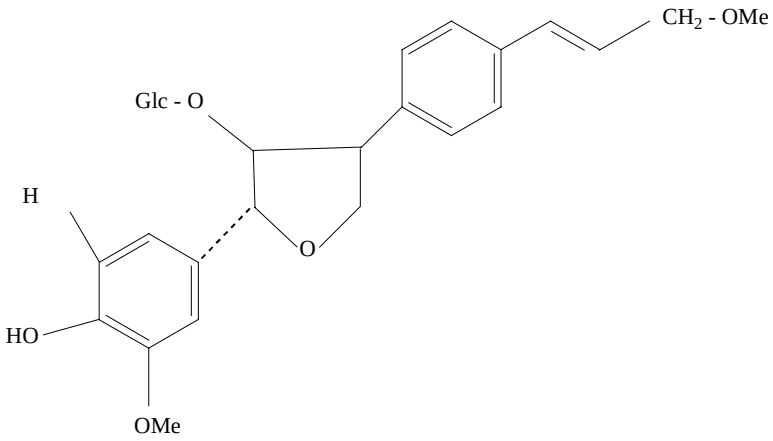
4.4 Plucheoside D – 1 ; root



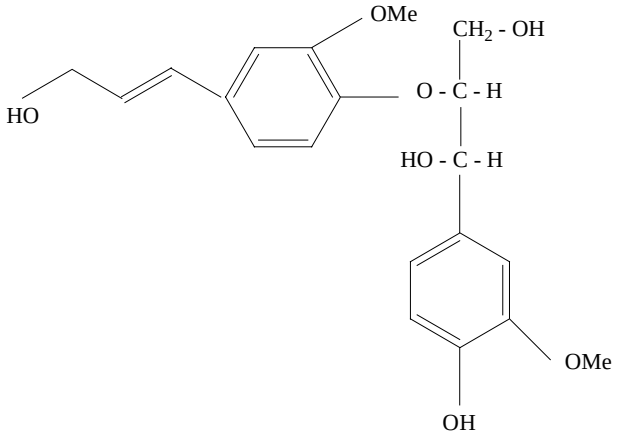
4.5 Plucheoside D – 2 ; root



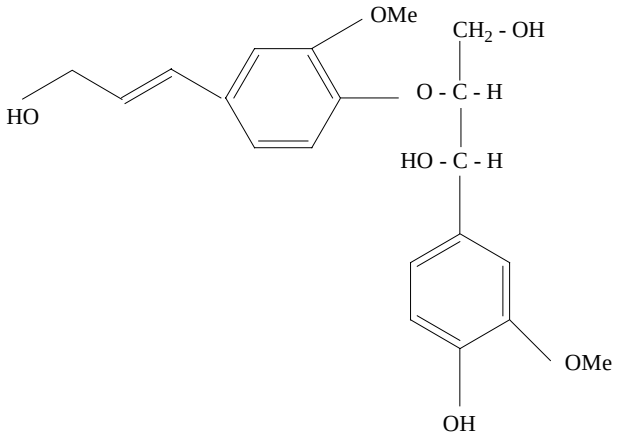
4.6 Plucheoside D – 3 ; root



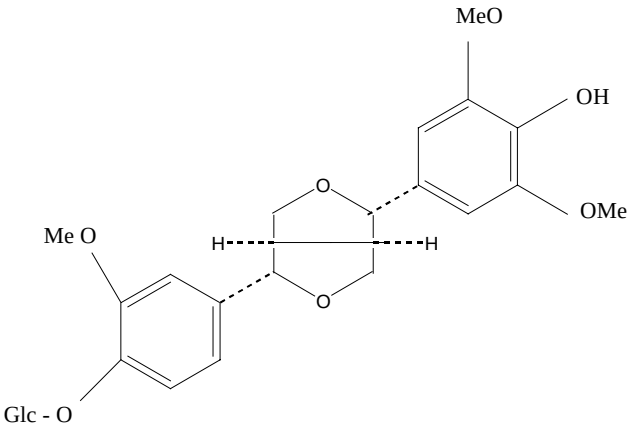
4.7 Propane-1-3-diol, 1(4-hydroxy-3-phenyl)-2-[2-methoxy-4- (1-trans-propene-3-ol) – phenoxy] ; erythro ; aerial parts



4.8 Propane-1-3-diol, 1(4-hydroxy-3-phenyl)-2-[2-methoxy-4- (1-trans-propene-3-ol) – phenoxy] ; Threo

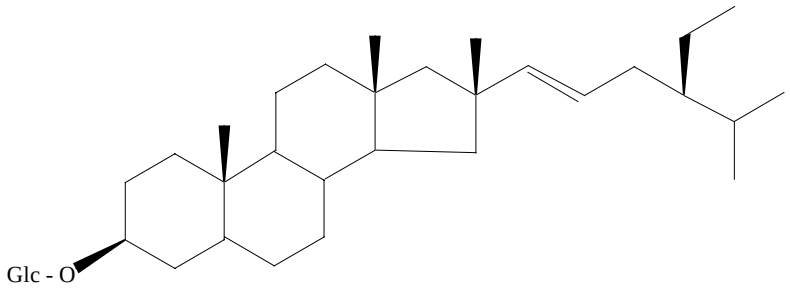


4.9 Syringaresinol monoglucoside ; aerial



5. Steroids

5.1 Stigmasterol glucoside ; Leaf.

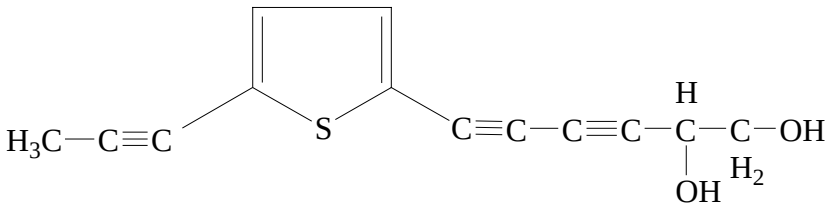


6. Miscellaneous

6.1 Hex – 3 – CIS – enyl glucoside

Glc – O – CH_2 – CH_2 – CH = CH – CH_2 – CH_3

6.2 Thiophene, 2-(prop-1-1ynyl) – 5 – (5-6-dihydroxy-hex a-1-3-diynyl ; root



6.3 thiophene, 2 - (Prop-1-ynyl) – 5 – (6 – Acctoxy – 5 - hydroxy – hex a – 1 – 3 – diynyl) ; root

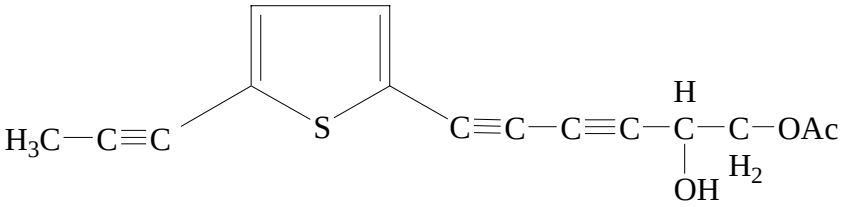


Figure 10 Structure of chemical constituents of *Pluchea indica* (55,56,57,58)

10.1.5 Biolgical activity of *Pluchea indica*

Table 10 Biological activity of *Pluchea indica*

Biological activity	Part use	Compound/extract	Experiment
Diuretic	aerial	Freeze dry aqueous extract in capsule	Clinical (59)
	aerial	Decoction	Clinical (60)
Anti-HIV1	aerial	Water extract	*in vitro* (61)
Anti-inflammatory	root	Methanol extract	*in vivo* (50)
Analgesic activity	root	Petroleum ether	*in vivo* (62)
Barbiturate-potentiation	root	Petroleum ether	*in vivo* (62)
Anti-ulcer	root	Methanol extract	*in vivo* (63)
Antioxidant	root	Methanol extract	*in vivo* (6)

CHAPTER 3
MATERIALS AND METHODS

Part I : Extraction and antioxidant activity determination of selected Thai herbs & vegetables

1. Extraction of selected Thai herbs & vegetables

Nine common Thai herbs and vegetables contained *Pluchea indica*, *Oenanthe stolonifera*, *Polygonum odoratum*, *Centella asiatica*, *Eryngium foetidum*, *Acacia penata*, *Hibiscus sabdariffa*, *Piper darmentosum* and *Marsilea crenata* were extracted in methanol. The result solution was evaporated and tested for antioxidant activity using DPPH scavenging assay.

2. Screening for antioxidant activity with DPPH scavenging assay.

2.1.1 DPPH scavenging assay (10,11)

DPPH solution was prepared in absolute ethanol to give a 1.52 x 10^{-4} M solution (Appendix B) and 5 mg/ml stock sample solution was prepared to make a serial dilution from 5 to 20 µg/ml. The DPPH scavenging reaction was performed when DPPH solution was added to the sample solution in the same volume (1 ml).The absorbance at 518 nm after 30 minutes of the reaction was recorded and % inhibition calculated from the formula below.

$$\text{Inhibition} = \frac{A_{ctr} - A_{sample}}{A_{ctr}} \times 100$$

when A_{ctr} = Absorbance of control

A_{sample} = Absorbance of sample

Positive control = Vitamin C

EC_{50} value (the concentration at 50% inhibition) was determined from the curve between %inhibition and concentration.

Part II : Extraction and antioxidant activity determination of ethanolic extract from the leaf of *P. indica*

1. Materials

1.1 Plant materials

Leaves of *Pluchea indica* were collected from Nakhon pathom provine, Thailand in March 2001.The plant was identified by Mrs.Vacharee Prachasaisoradej, an agricultural scientist 8 at Plant Variety Protection Division research unit of Princess Sirinhorn Plant Herbarium Building, Bangkok, Thailand. A vocher specimen BK 63510 was deposited at the same place.

1.2 Chemicals

Hexane AR grade

Chloroform AR grade

Methanol AR grade

Ethyl acetate AR grade

95% Ethanol (commercial grade)

Formic acid

1,1–diphenyl-2-picrylhydrazyl radical (DPPH)

1.3 Equipments

Analytical balance

Autopipette

Centrifuge

Spectrophotometer

Water bath

1.4 Spraying reagent (Appendix A)

DPPH (1,1 Diphenyl-2-picrylhydrazyl radical) spraying reagent

1% Vanillin HCl spraying reagent

10% $FeCl_3$ spraying reagent

Natural product/ Polyethlene glycol (NP/PEG)

1.5 Solvent systems for thin-layer chromatography

Solvent system I: $CHCl_3$: EtOAc: Formic acid (1:1:0.1)

Solvent system II : EtOAc: Distilled water: Formic acid (5:0.5:1)

1.6 UV detection

TLC chromatograms were detected under UV light at wavelength 254 and 366 nm.

1.7 Standard drug

Quercetin (Sigma)

2. Methods

2.1 Extraction of the leaf of *P. indica*.

2.2 Antioxidants activity determination of ethanolic extract of the leaf of *P. indica* .

2.3 Thin-layer chromatography analysis of crude extracts from leaf of *Pluchea indica*. (Figure 14)

2.1Extraction of the leaf of *P. indica*

2.1.2 Plant material preparation and extraction

Leaves of *Pluchea indica* were cleaned and air dried in a hot air oven (50°C) and then grounded into a powder. The powdered drug (800 g) was extracted in a soxhlet apparatus with 95% ethanol for 72 hours. The resulted solution was evaporated using of rotary evaporator to give a dark brown sticky mass which was further fractionated to give the active compound.

2.2 Antioxidants activity determination of the ethanolic extract of the leaf of *P. indica*

Antioxidant activity of the ethanolic extract of the leaf of *P. indica* was determined by DPPH scavenging assay .

2.3 Thin-layer chromatography analysis of crude extract from leaf of *Pluchea indica* (Figure 14,15,17)

Crude extract from leaf of *Pluchea indica* were analyzed by thin- layer chromatography using solvent system I ($CHCl_3$-EtOAc- formic acid 1:1:0.1) with four spray reagents; DPPH spraying reagent, 1% Vanillin HCl spraying reagent 10% $FeCl_3$ spraying reagent and NP/PEG spraying reagent for detecting antioxidant components, flavonol and polyphenolic compounds, respectively.

Part III : Isolation and identification of chemical constituents from the active fraction of *P. indica*

1. Chemicals and equipments

1.1 Chemicals

Hexane, AR grade

Chloroform, AR grade

Ethylacetate, AR grade

Methanol, AR grade

Silica gel 60 F_{254} , pre-coated on TLC aluminium sheets 20x20 cm, layer thickness 0.25 cm

Diaion HP20, size 75-150 μm

1.2 Equipments

Melting point apparatus

UV spectrophotometer

FI-IR spectrophotometer

NMR spectrometer

Mass spectrometer

Rotary evaporator

Hot air oven

1.3 Solvent system

Solvent system I; Chloroform: Ethyl acetate : formic acid (1:1:0.1)

1.4 Spraying reagent (Appendix A)

1%Vanillin HCl spraying reagent

10%$FeCl_3$ spraying reagent

$AlCl_3$ spraying reagent

DPPH spraying reagent

Natural product/Polyethylene glycol (NP/PEG) spraying reagent

1.5 UV detection

TLC chromatograms were detected under UV light at wavelengths of 254 nm and 366 nm .

2. Methods

2.1 Fractionation of the ethanolic extract from the leaf of *P. indica.*

The ethanolic extract was fractionated using Diaion HP20 column chromatography. Diaion HP20 (equivalent to 200 ml) was wet packed using water methanol (1:1) into a glass column (5 cm,idx45cm, length) and equilibrated with 1,500 ml of water before use.

The ethanolic extract (30g) was dissolved in 300 ml distilled water and sonicated in an ultrasonic bath. The suspension obtained was centrifuged at 3,000 rpm for 30 minutes. The supernatant was put onto the Diaion HP20 column chromatography, and the column was eluted with distilled water (600 ml).to give fraction 1 (10.73 g) The water insoluble residue was dissolved in water-methanol (1:1), and the supernatant was put also onto the column using aqueous methanol as solvent system. The process was repeated with the methanol insoluble components. Finally, the column was washed with 500 ml of ethyl acetate. After the removal of the solvents in vacuo, it afforded the water fraction, 11.73 g (Fr.1), water-methanol fraction 6.86 g (Fr.2), methanol fraction 10.30g (Fr.3) and ethyl acetate fraction 1.60 g(Fr.4). The scheme of the fractionation is presented in Figure10.

2.2 Antioxidant activity determination of fractions from ethanolic extract of *P. indica*

All fractions from Diaion HP20 column were tested for antioxidant activity using DPPH scavenging assay as mentioned before. The most active fraction was then selected for the separation of chemical constituents.

2.3 Thin-layer chromatography analysis of fractions from the ethanolic extract of *P. indica*.

All fractions from Diaion HP20 column chromatography were also analyzed by thin layer chromatography using solvent system I ($CHCl_3$:EtOAc:formic acid,1:1:0.1) with DPPH spraying reagent, 1% vanillin spraying reagent, 10% ferric chloride spraying reagent, aluminium chloride spraying reagent and NP/PEG spraying reagent.

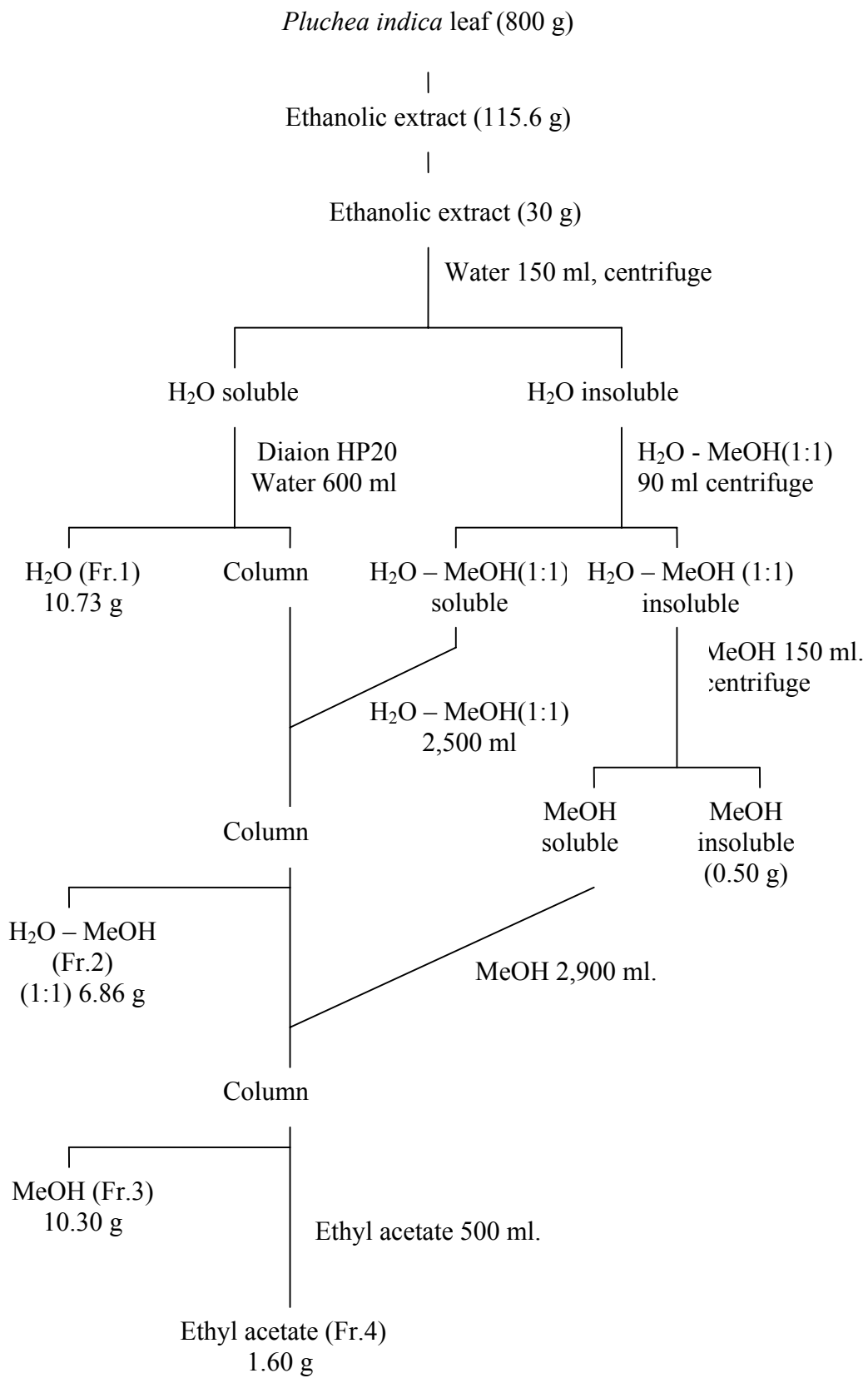


Figure 11 Extraction and fractionation of *P. indica* leaf by Diaion HP20 column chromatography

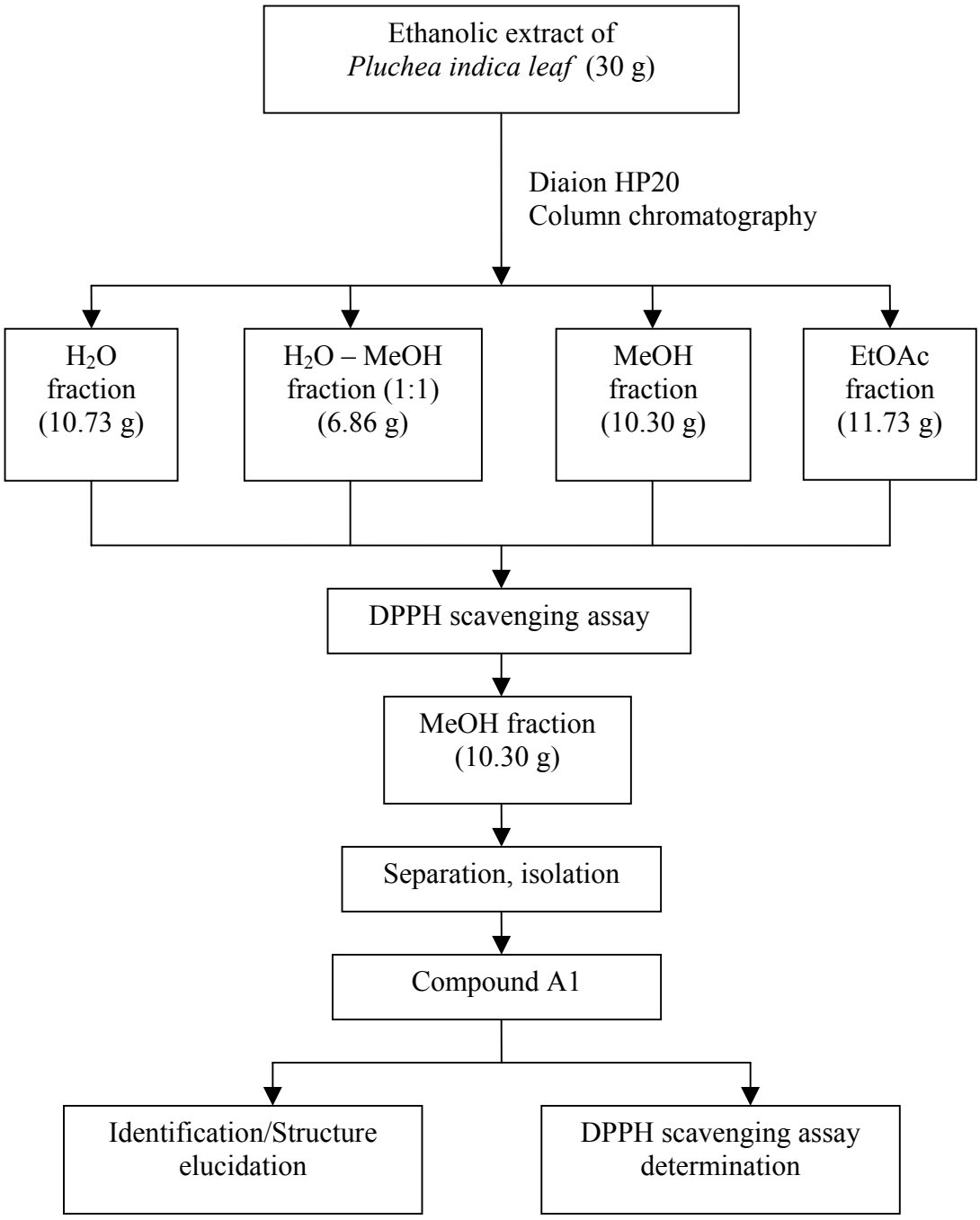


Figure 12 Diagram showing the procedures for the separation, antioxidant activity determination and isolation of the active compounds from the ethanolic extract of *P. indica*

2.4 Isolation and identification of chemical compounds from leaf ethanolic extract of *P. indica*

2.3.1 Isolation of compound A1

MeOH fraction (fr. 3,3.05 g) was submitted to separation by column chromatography using silica gel GF_{60} 63-200 μm(75 g) eluted with hexane, chloroform and methanol gradiently, fractions of 50 ml were collected . TLC analysis of fractions was performed on precoated silica gel G_{60} plates using solvent system I. Detection of TLC was performed under UV light. Similar fractions were combined to obtain fractions Q_1-Q_6 Q1(1-15),Q2(16-22),Q3(23-28),Q4(30-32),Q5(precipitate from 33-35),Q6 (36-40). On standing fraction Q_5 (0.0165 g) furnished yellow precipitates which showed positive antioxidant to DPPH spraying reagent. TLC analysis of precipitate from Q_5 fraction was performed on precoated silica gel G_{60} plates using solvent system II to furnish the band of Rf value 0.47. After evaporation and recrystallization from chloroform and methanol, compound A1(16 mg) was obtained. The diagram of isolation of compound A1 was shown in Figure 13.

2.5 Thin-layer chromatography analysis of the isolated compound.

The isolated compound A1 was analysed by thin layer chromatography using solvent system I and different spraying reagent such as DPPH spraying reagent, 1% vanilin HCl spraying reagent, 10% $FeCl_3$ spraying reagent, $AlCl_3$ spraying reagent and NP/PEG spraying reagent and also UV light detection. Authentic quercetin was used as a reference compound.

2.6 Antioxidant activity determination of isolated compound.

Compound A1 was tested for antioxidant activity using DPPH scavenging assay determination.

2.7 Identification of the isolated compound.

The isolated compound was structurally identified using its UV, IR, MS and NMR spectral data in comparison with authentic quercetin.

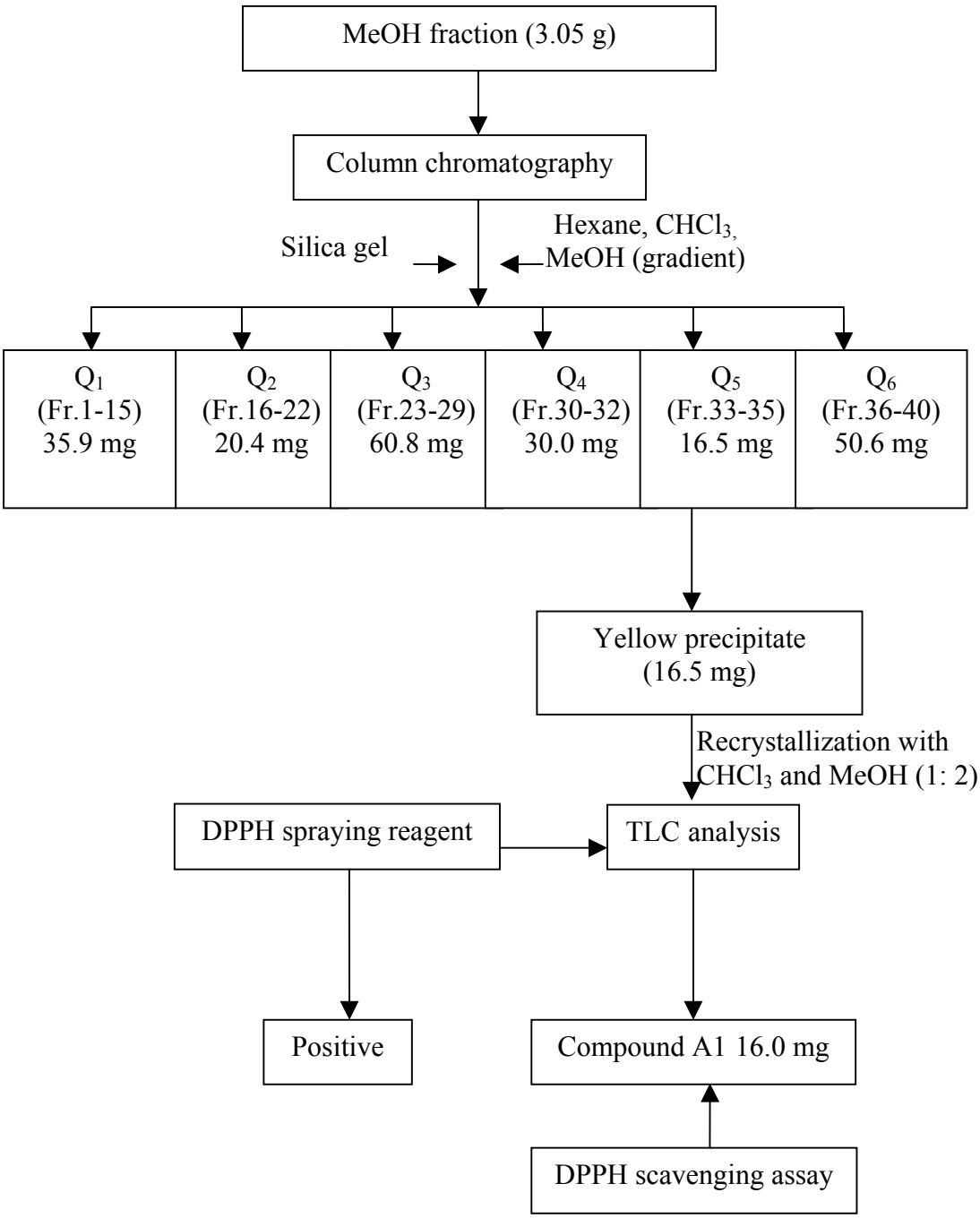


Figure 13 Diagram of the isolation of compound A1

CHAPTER 4
RESULTS

Part I : A Preliminary screening of some Thai Plants for Antioxidant Activity

1. Screening for antioxidant activity

Nine common Thai herbs and vegetables were tested for their antioxidant activity using DPPH scavenging assay. The results are shown in Table 11.

Table 11 Antioxidant activity of selected Thai Plants using DPPH scavenging assay

Plant	Thai name	Antioxidant Activity EC_{50} (μg/ml)
Pluchea indica	ขลู่	6.92
Oenanthe stolonifera	ผักชีล้อม	43.72
Polygonum odoratum	ผักไผ่	1.11
Centella asiatica	บัวบก	114.71
Acacia pennata	ชะอม	472.76
Eryngium foetidum	ผักชีฝรั่ง	57.13
Hibiscus sabdariffa	กระเจี๊ยบ	219.98
Piper sarmentosum	ชะพลู	139.81
Marsilea crenata	ผักแว่น	419.08

Of those tested, only two plant samples showed significant antioxidative activity. These were *Polygonum odoratum* (EC_{50}=1.11 μg) and *Pluchea indica* (EC_{50}=6.92 μg). *P. indica* was chosen for detail study on the basis of its previous antioxidant activity report (6).

Part II Extraction and antioxidant activity determination of ethanolic extract from the leaf of *P.indica*

1. Extraction of crude *P.indica* leaf

Table 12 The yields (%) (w/w) of the crude extract from *P.indica*

Plant extract	Crude drug (g)	Dried extract (g)	% yield (w/w)
Ethanolic extract	800	115.6	14.45

2. Antioxidant activity determination of crude extracts of *P. indica*

2.1 DPPH scavenging assay

Table 13 Antioxidant activity of the crude extract from *P. indica* leaf by DPPH scavenging assay

Plant extract	Antioxidant activity EC_{50} (µg/ml)
Ethanol extract of *P. indica*	6.92
Vitamin C	1.49

3. Thin layer chromatography analysis of crude extract from *Pluchea indica*

Thin layer chromatography of crude extract from *Pluchea indica* (branches and leaves) with solvent systems I and II . TLC chromatograms were detected with four spraying reagents, DPPH spraying reagent, 1% vanillin HCl spraying reagent ,10% ferric chloride and NP/PEG spraying reagents. TLC chromatograms of crude extract from *P. indica* are shown in Figures 14, 15.

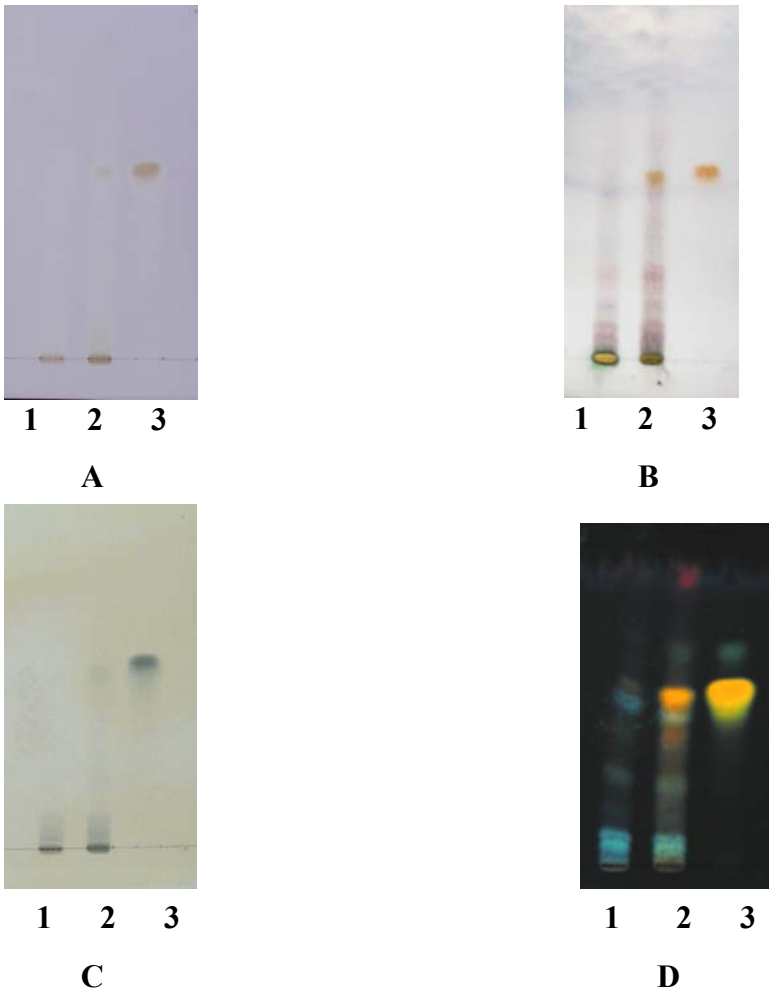


Figure 14 Thin-layer chromatography of crude extract from *Pluchea indica*

Track: 1 = branch ethanol extracts

2 = leaf ethanol extract

3 = authentic quercetin

Solvent system I : Chloroform-ethyl acetate-formic acid (1:1:0.1)

Spraying reagents :

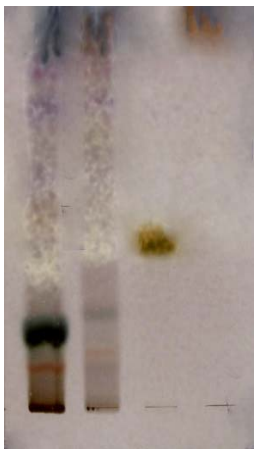
A = DPPH spraying reagent

B = 1% vanillin HCl spraying reagent

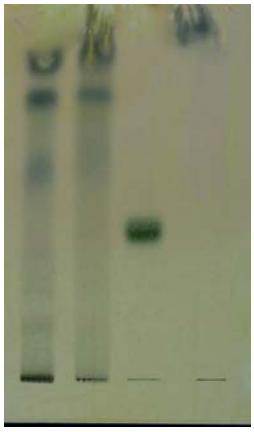
C = 10% ferric chloride spraying reagent

D = NP/PEG spraying reagent

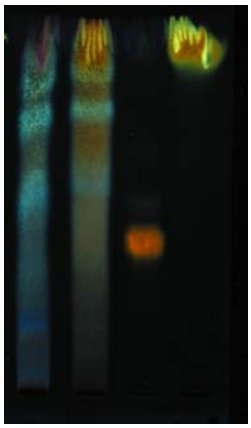
1 2 3 4
A



1 2 3 4
B



1 2 3 4
C



1 2 3 4
D

Figure 15 Thin-layer chromatography of crude extract from *Pluchea indica*

Track: 1 = branch ethanol extracts

2 = leaf ethanol extract

3 = authentic rutin

4 = authentic quercetin

Solvent system II : Ethyl acetate-distilled water - formic acid (5:0.5:1)

Spraying reagents :

A = DPPH spraying reagent

B = 1% vanillin HCl spraying reagent

C = 10% ferric chloride spraying reagent

D = NP/PEG spraying reagent/UV_{366}

Part III : Isolation and identification of chemical constituents from the active extract.

1. Separation of fractions from leaf ethanol extract of *P. indica*

Table 14 The yields of fractions from Diaion HP20 column chromatography of ethanolic extract from *P. indica* leaf (30 g)

Fraction	Dried weight (g)	% yield(w/w)
H_2O fraction (Fr.1)	11.73	39.10
H_2O – MeOH (1:1) (Fr.2)	6.86	22.87
MeOH fraction (Fr.3)	10.30	34.33
Ethyl acetate fraction (Fr.4)	1.60	5.33

2. Antioxidant activity determination of fractions from Diaion HP20 column chromatography of ethanolic extract from *P. indica* leaf

Table 15 Antioxidant activity of fraction by DPPH scavenging assay

Fraction	Antioxidant activity EC_{50} (µg/ml)
Fraction 1	16.02
Fraction 2	2.65
Fraction 3	1.89
Fraction 4	12.08

3. TLC chromatogram of fractions from leaf ethanol extract of *P. indica*

Thin-layer chromatographic investigation of fractions from Diaion HP20 column chromatography of ethanolic extract from *P. indica* leaf. H_2O fraction (Fr. 1), H_2O-MeOH (1:1) (Fr.2), MeOH fraction (fr.3) and Ethyl acetate fraction (Fr.4) was carried out using solvent system I. TLC chromatograms were detected with five spraying reagents, namely, DPPH spraying reagent, 1% vanillin HCl, 10% $FeCl_3$, $AlCl_3$ and NP/PEG spraying reagent. TLC chromatograms of fractions from *P. indica* are shown in Figure 16.

TLC chromatograms of fractions Q1-Q6 from column chromatography and authentic quercetin using solvent system I and detecting with four spraying reagents, DPPH, 1% vanillin HCl, 10 % ferric chloride and NP/PEG spraying reagent are shown in Figure 17.

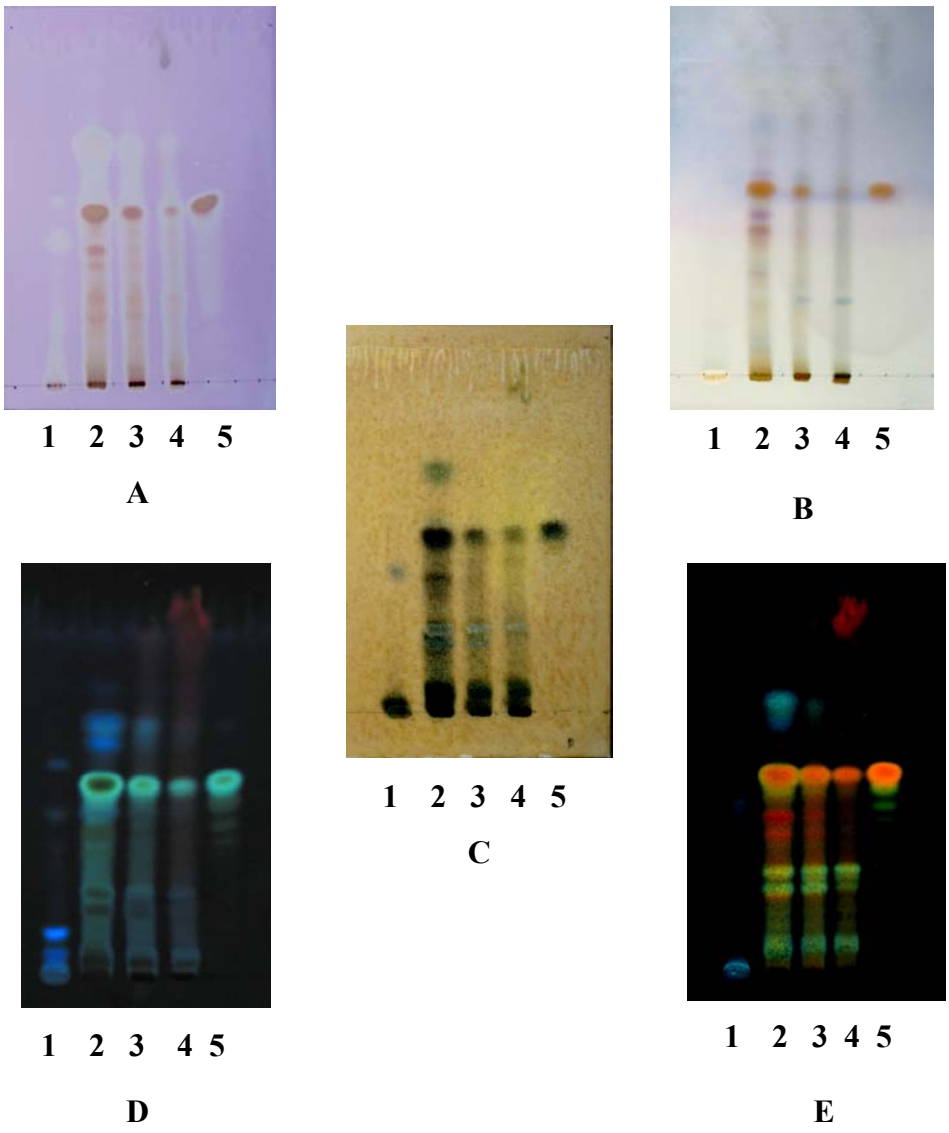


Figure 16 Thin-layer chromatography of fractions from Diaion HP20 column chromatography of *Pluchea indica*

Track: 1 = Water fraction, 2 = Water-MeOH fraction, 3 = MeOH fraction,
4= Ethyl acetate fraction 5 = authentic quercetin

Solvent system I : chloroform: ethyl acetate: formic acid 1:1:0.1

Spraying reagent: A = DPPH spraying reagent
B = 1% vanillin HCl spraying reagent
C = 10% $FeCl_3$ spraying reagent
D = $AlCl_3$ spraying reagent/UV_{366}
E = Natural product/Polyethylene glycol (NP/PEG) spraying regent/UV_{366}

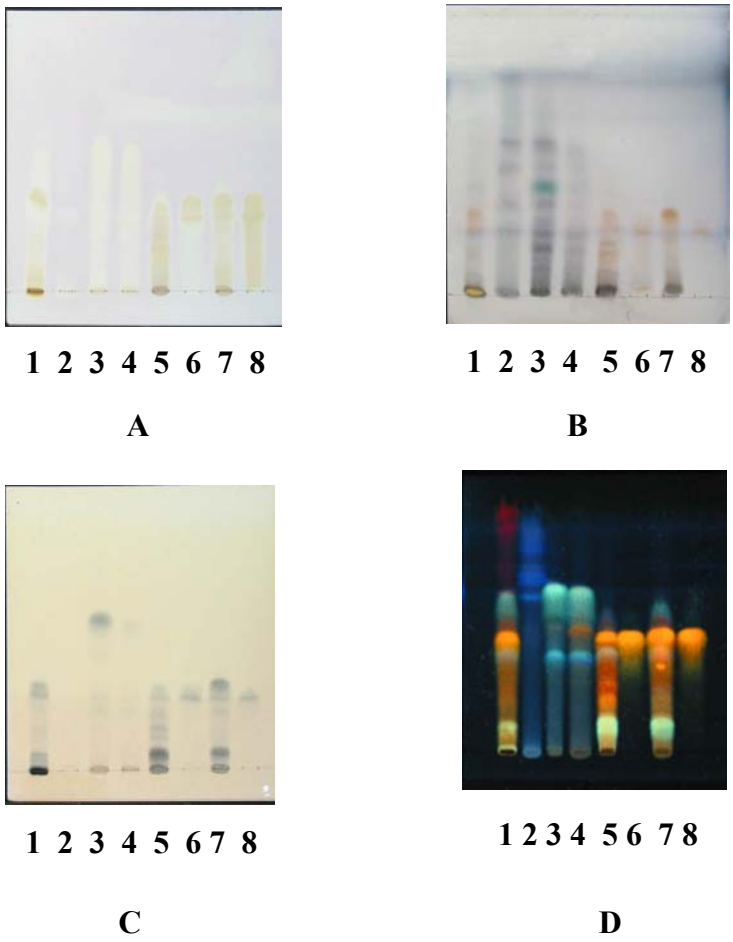


Figure 17 Thin layer chromatography of fractions from silica gel column Chromatography of fraction 3 (MeOH fraction) from Diaion HP20 column

Track: 1 = leaf ethanol extract

2 = Q1 fraction (fractions 1-15)

3 = Q2 fraction (fractions 16-22)

4 = Q3 fraction (fractions 23-29)

5 = Q4 fraction (fractions 30-32)

6 = Precipitate from Q5 fraction (fractions 33-35)

7 = Q6 fraction (fractions 36-40)

8 = authentic quercetin

Solvent system I : Chloroform-ethyl acetate-formic acid (1:1:0.1)

Spraying reagents : A = DPPH spraying reagent

B = 1% vanillin HCl spraying reagent

C = 10% ferric chloride spraying reagent

D = NP/PEG spraying reagent/UV_{366}

4. Isolation of chemical compounds from ethanolic extract of *P. indica* leaf.

4.1 Isolation and identification of compound A1

4.1.1 Compound A1

The methanol fraction was selected from the Diaion HP20 column chromatography because it was the most active fraction in the DPPH scavengting assay. The MeOH fraction was submitted for separation by column chromatography on silica gel GF_{60} eluting with hexane, chloroform and methanol in a gradient and fractions of 50 ml collected. Fraction Q5(Fr. 33-35) furnished yellow precipitates which on recrystallization with chloroform and methanol (1:2), compound A1 was obtained as a yellow amorphous compound soluble in methanol.

4.1.2 Thin-layer chromatographic Analysis of Compound A1 from *P. indica*

TLC analysis of compound A1 from *P. indica* with solvent system I. TLC chromatograms were detected with five spraying reagents (DPPH, $AlCl_3$, 1% vanillin HCl, 10% ferric chloride and NP/PEG spraying reagents), it showed a single spot with a Rf of 0.47 and giving a dark green color with 10% $FeCl_3$ spraying reagent, a red color with vanillin HCl spraying reagent, green fluorescence under UV 366 nm to $AlCl_3$ spraying reagent, yellow fluorescence under UV 366 nm with NP/PEG spraying reagent and a pale yellow spot in purple background to DPPH spraying reagent.

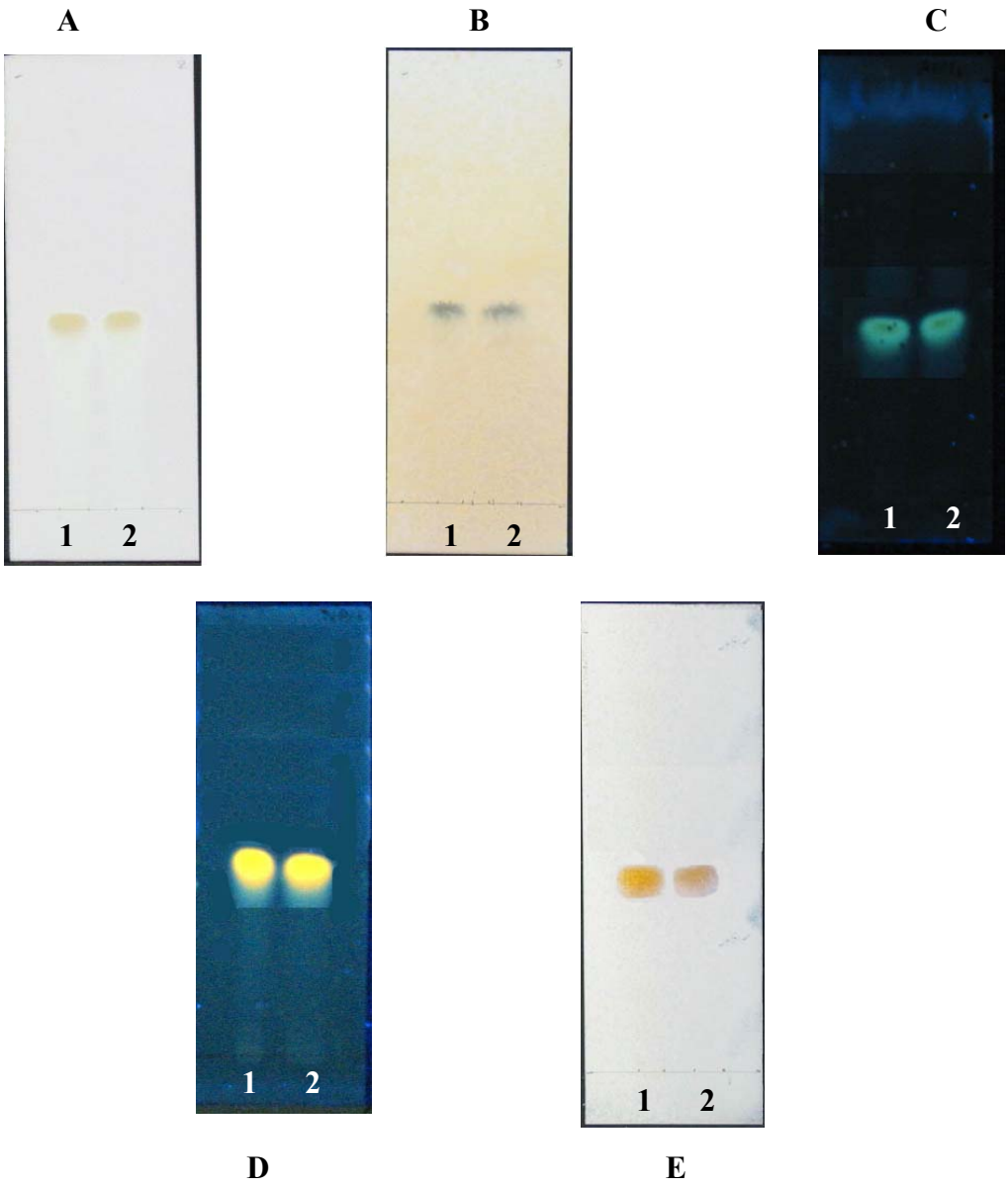


Figure 18 Thin-layer chromatography analysis from compound A1 and authentic quercetin

Track: 1 = Authentic quercetin, 2= compound A1

Solvent system: solvent system I (chloroform: ethyl acetate: formic acid 1::0.1)

Spraying reagents : A = DPPH spraying reagent

B = 10% $FeCl_3$ spraying reagent

C = $AlCl_3$ spraying reagent/UV_{366}

D = Natural product/Polyethylene glycol (NP/PEG) spraying regent/UV_{366}

E = 1% vanillin HCl spraying reagent

4.1.3 The melting point of compound A1

The melting point of compound A1 was 285-287 ºC (Literature 285-287 ºC) and mixed melting point with authentic quercetin was 285-287ºC.

Table 16 The melting point of compound A1 and authentic quercetin

	Compound A1 (A1)	Authentic Quercetin (A.Q)	Mixed A1+A.Q
Melting point	285-287 ºC	285-287 ºC	285-287 ºC

4.1.4 Mass spectrum of compound A1

Time of flight (TOF mass) mass spectrum exhibited the molecular ion peak at m/z 303.34 [M^+H] indicating an aglycone mass of compound A1 without sugar moiety(Figure 19).

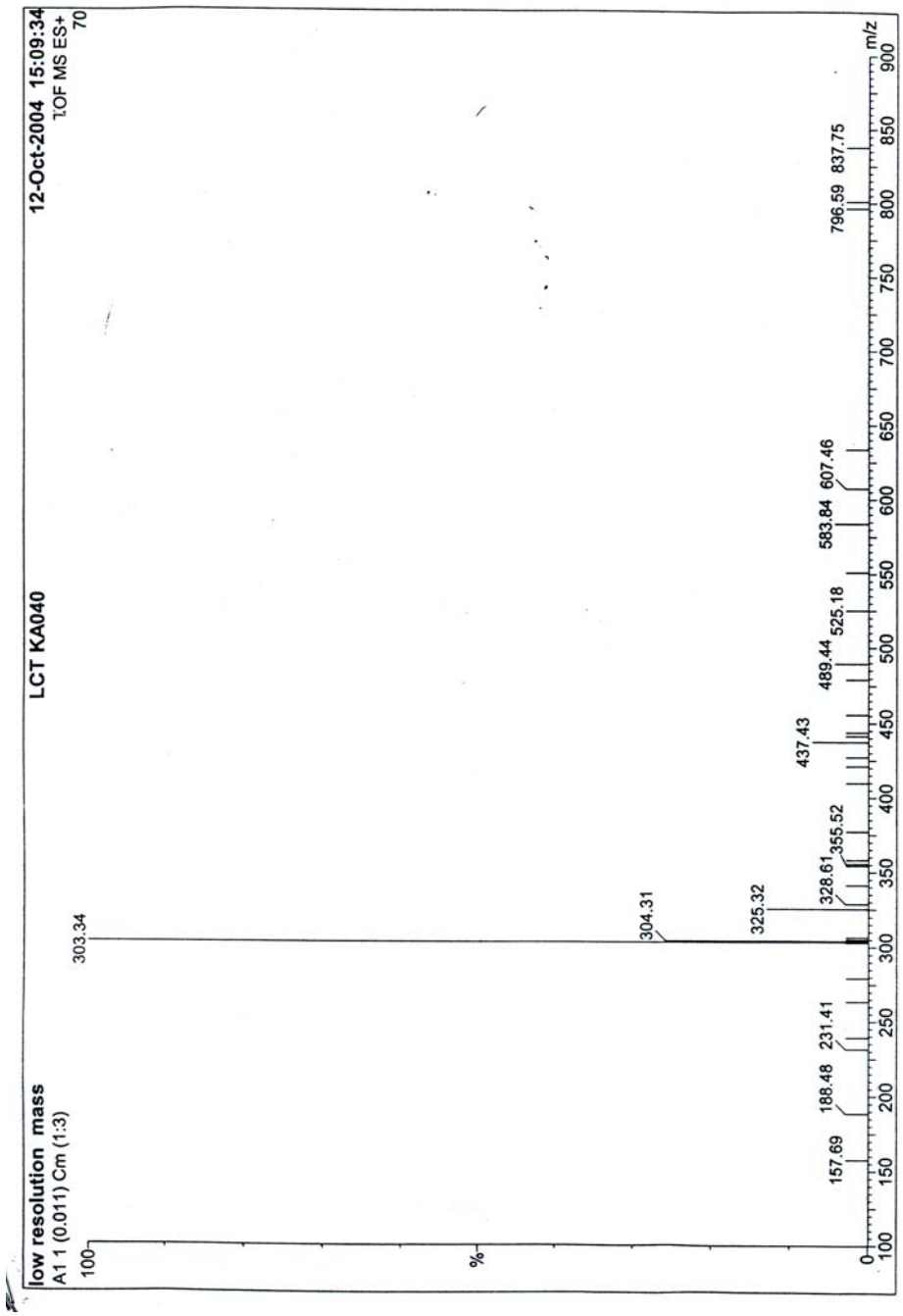


Figure 19 Mass spectrum of compound A1

4.1.5 Ultraviolet absorption of compound A1

The UV spectrum of compound A1 in methanol showed two major absorption bands at 255.32 nm (band II) and 372.77 nm (band I) which are typical of flavonol.

Table 17 Ultraviolet absorption of compound A1 and authentic quercetin (57)

Shift reagent	λmax (methanol)			
	Band I (nm)		Band II (nm)	
	A1	Authentic quercetin	A1	Authentic quercetin
No shift reagent	372.27	372.27	255.32	255.35

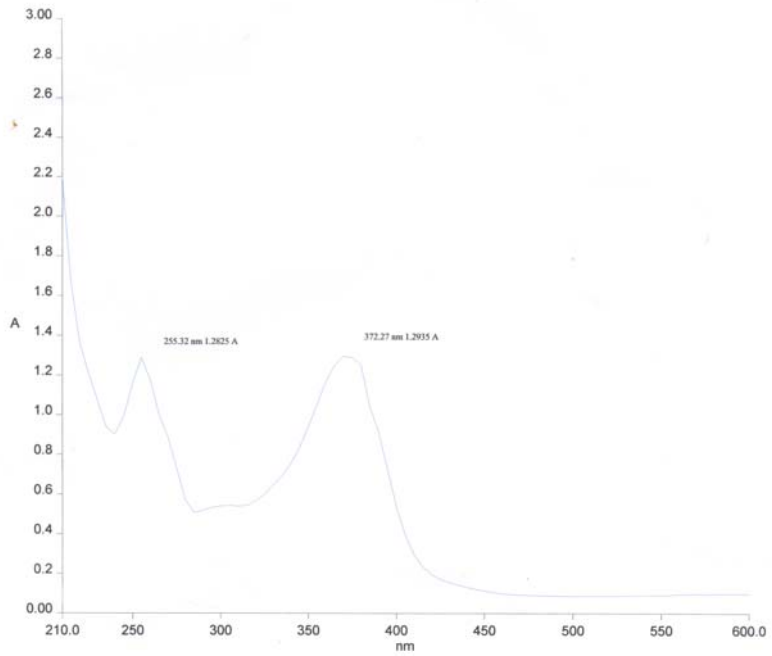


Figure 20 UV spectrum of compound A1

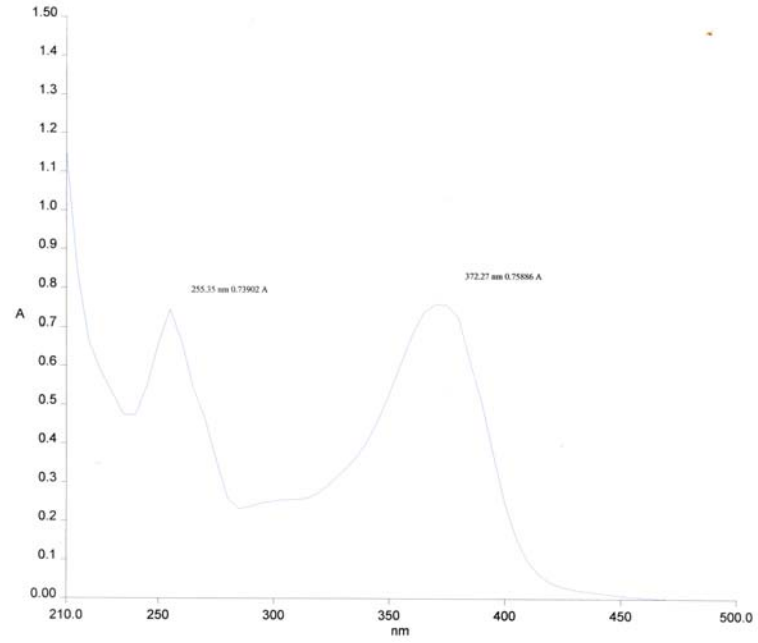


Figure 21 UV spectrum of authentic quercetin

4.1.6 IR spectrum of compound A1

The IR spectrum (KBr disc) showed absorption bands at 3414.3(v O-H), 1663.6 (v C=O), 1380(v C=C), 1255.6-1005.5(v C-O) and 669.8(benzene ring).

Table 18 IR spectrum of compound A1 and Authentic Quercetin

	Compound A1	Authentic quercetin
IR spectrum (cm^{-1}) (KBr)	3414.3, 1663.6, 1380, 1255.6, 827.8, 669.8	3414.3, 1670.2, 1380.6, 1163.4, 821.2, 683.0

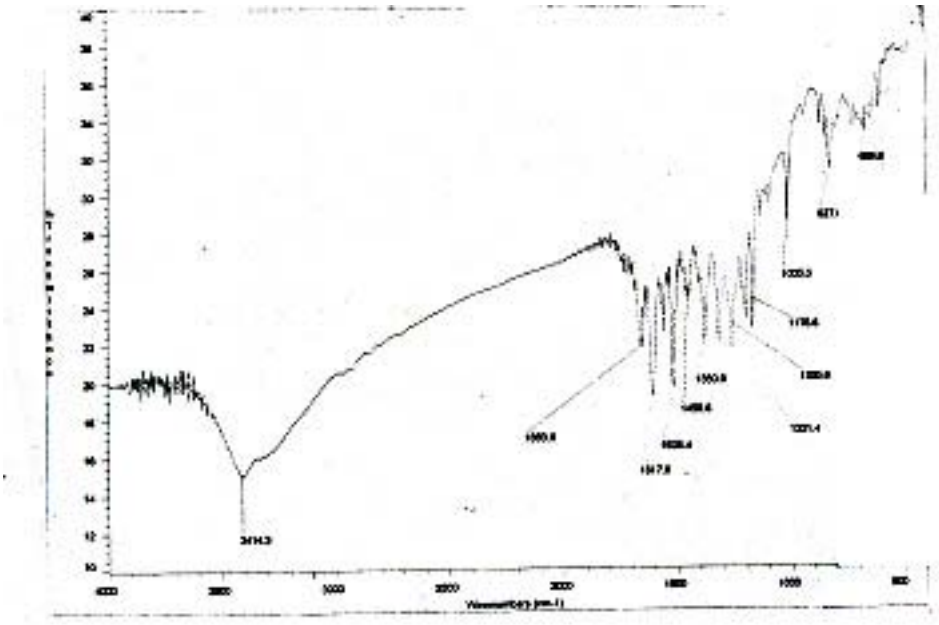


Figure 22 IR spectrum of compound A1

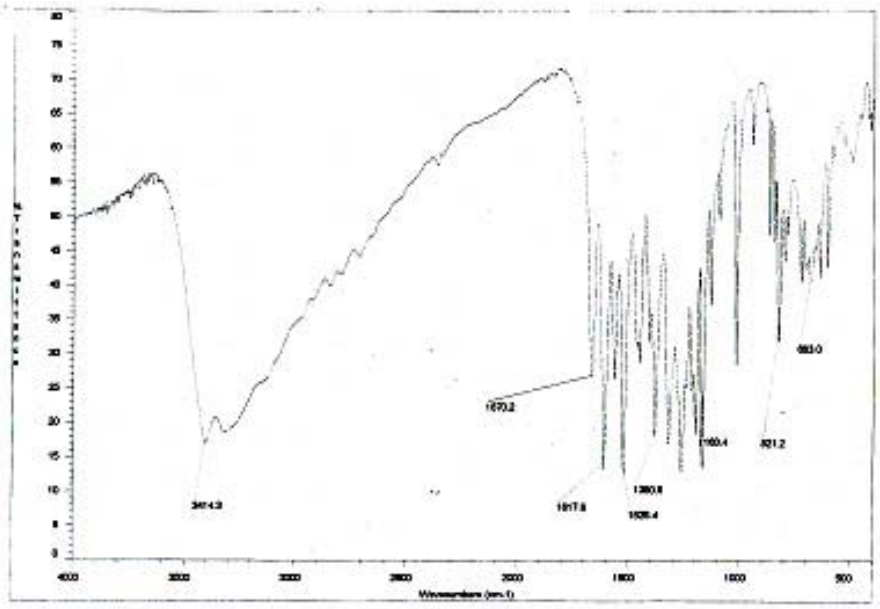


Figure 23 IR spectrum of authentic quercetin

4.1.7 ^{1}H-NMR and ^{13}C-NMR of compound A1

The ^{1}H-NMR data were assigned on the basis of the chemical shifts and splitting patterns. The signals at 6.88, 7.65 and 7.76 ppm belonged to 5′-H, 6′-H and 2′-H of the ring B, respectively. The two aromatic proton signal at 6.18 and 6.38 were attributed to the 6-H and 8-H, respectively. The proton signals were also in accordance with the proton signals of quercetin in the literature. The ^{13}C-NMR of compound A1 spectrum also confirm carbon 15 signals of the flavonoid skeleton in the region 90-180 ppm and compared well with literature report of quercetin.

Table 19 ^{1}H-NMR of compound A1($CDCl_3$) and literature values (58)

Proton	^{1}H-NMR ($CDCl_3$)	
	Compound A1 (500 MHz)	Literature values (400 MHz)
H-2′	7.76, d, J = 2.1	7.78, d, J = 1.8
H-6′	7.65, dd, J =2.2,2.2	7.65, dd, J = 9,1.8
H-5′	6.88, d, J = 8.5	6.95, d, J = 9
H-8	6.38, d, J =2.1	6.41, d, J = 2
H-6	6.18, d, J = 2.1	6.26, d, J = 2

Table 20 ^{13}C-NMR data of A1 ($CDCl_3$) (59)

C	A1 δ (ppm)	Liturature values (DMSO)(ppm)
2	148.78	146.90
3	137.24	135.50
4	177.35	175.80
5	162.53	160.70
6	99.26	98.20
7	165.66	163.90
8	94.43	93.30
9	158.26	152.20
10	104.52	103.10
1′	124.16	122.10
2′	116.00	115.30
3′	146.24	145.00
4′	148.01	147.60
5′	116.24	115.60
6′	121.68	120.00

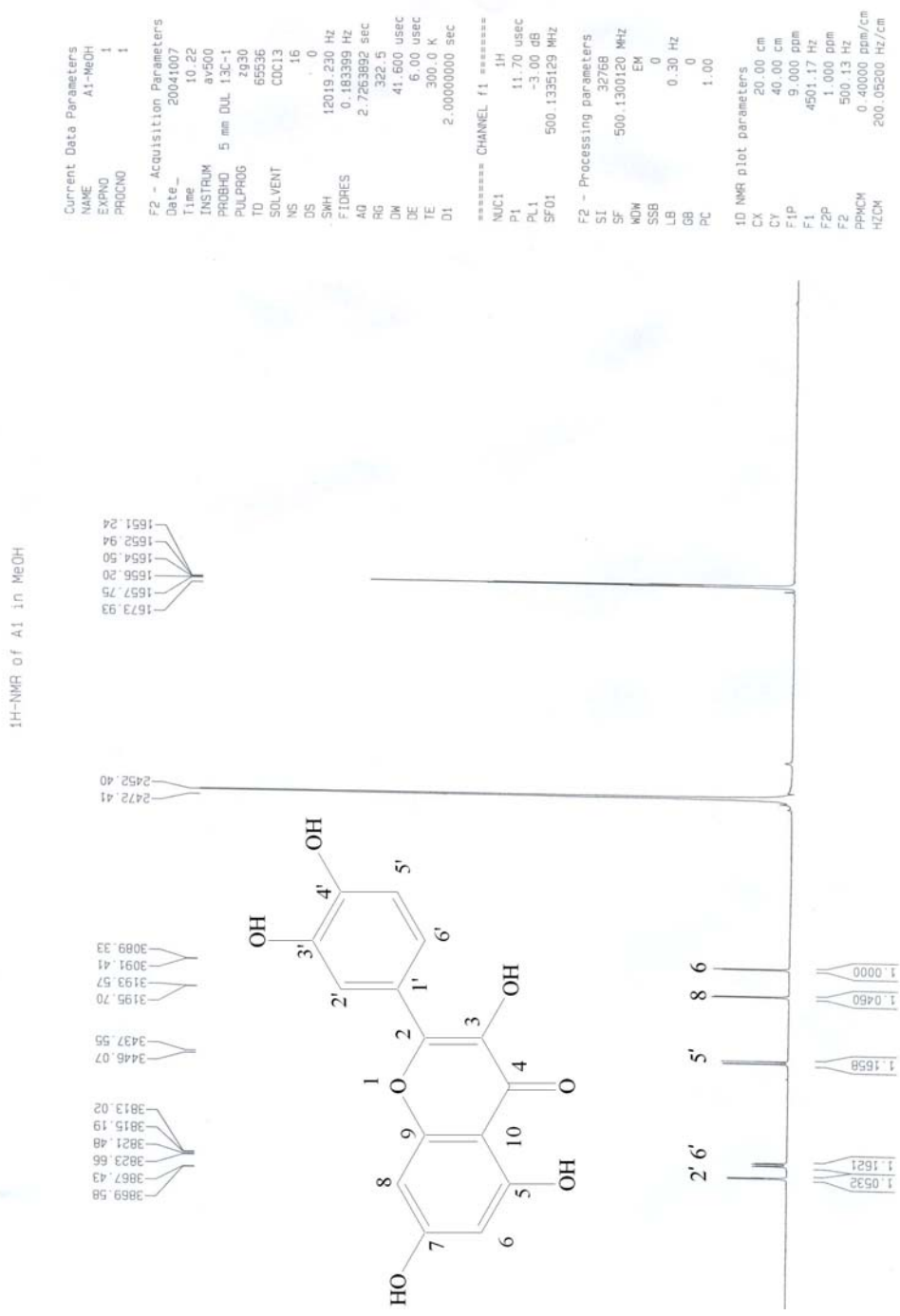


Figure 24 ^{1}H-NMR spectrum ($CDCl_3$) of compound A1

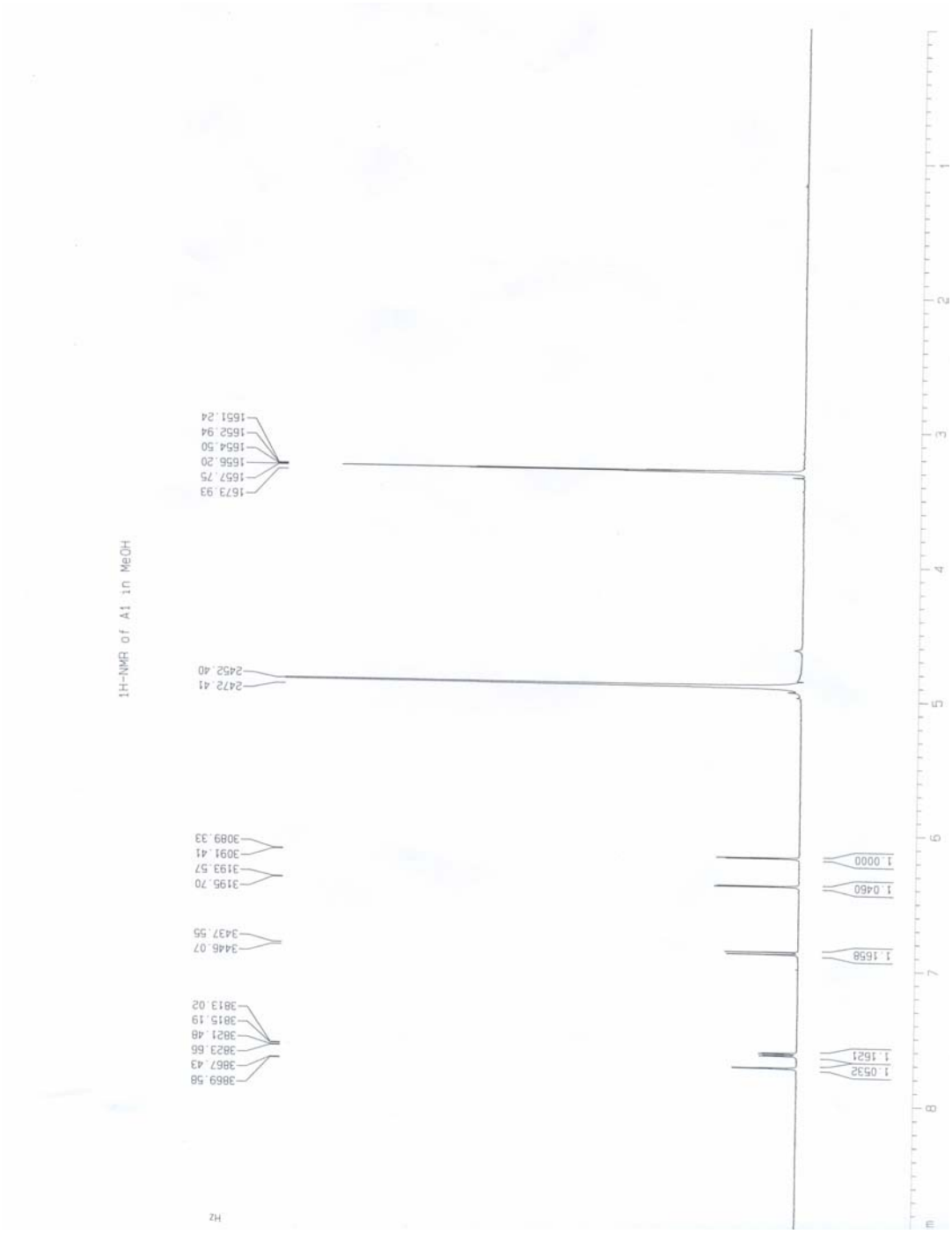


Figure 25 ^{1}H-NMR expanded spectrum ($CDCl_3$) of compound A1

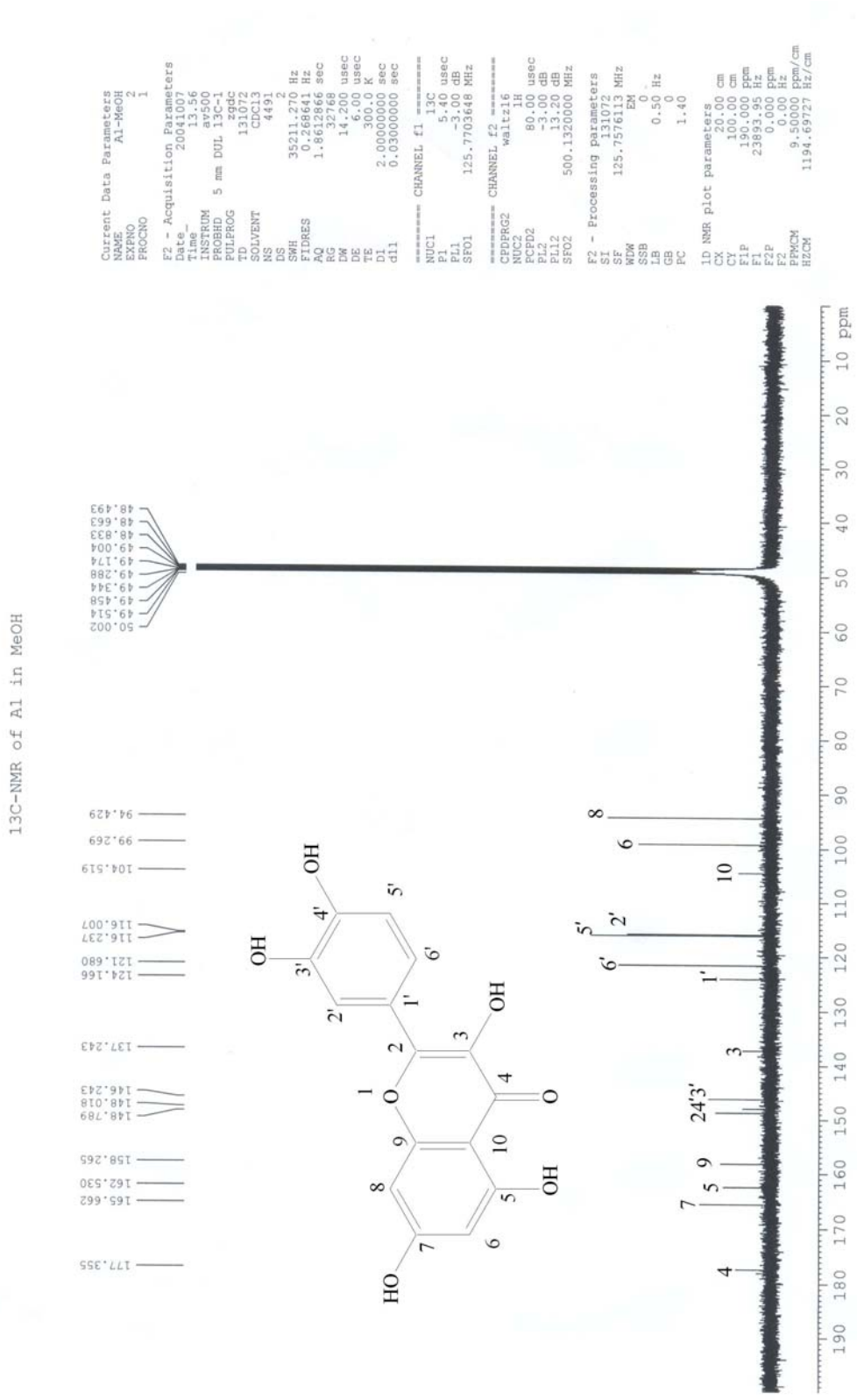


Figure 26 ^{13}C-NMR spectrum of compound A1

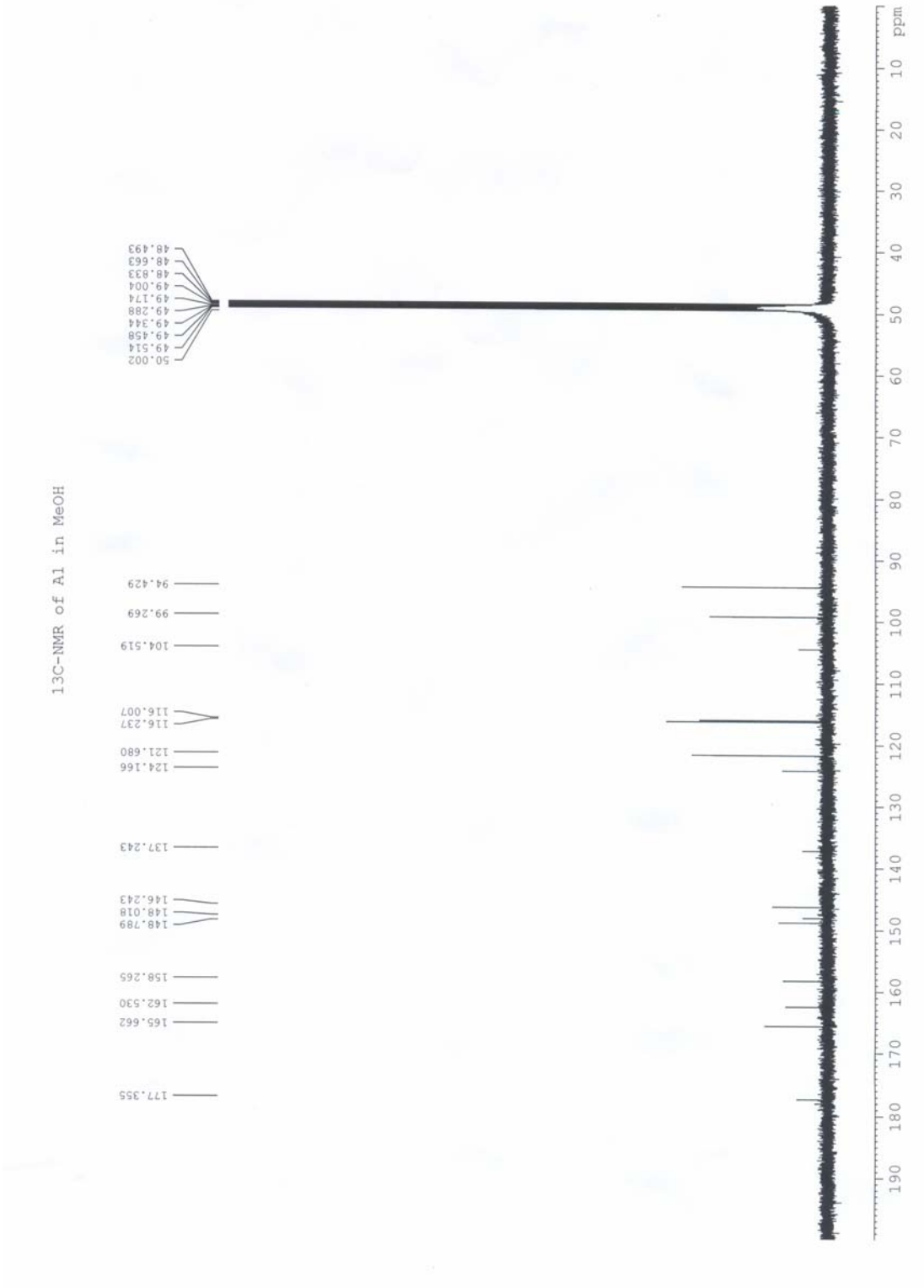


Figure 27 ^{13}C-NMR spectrum of compound A1

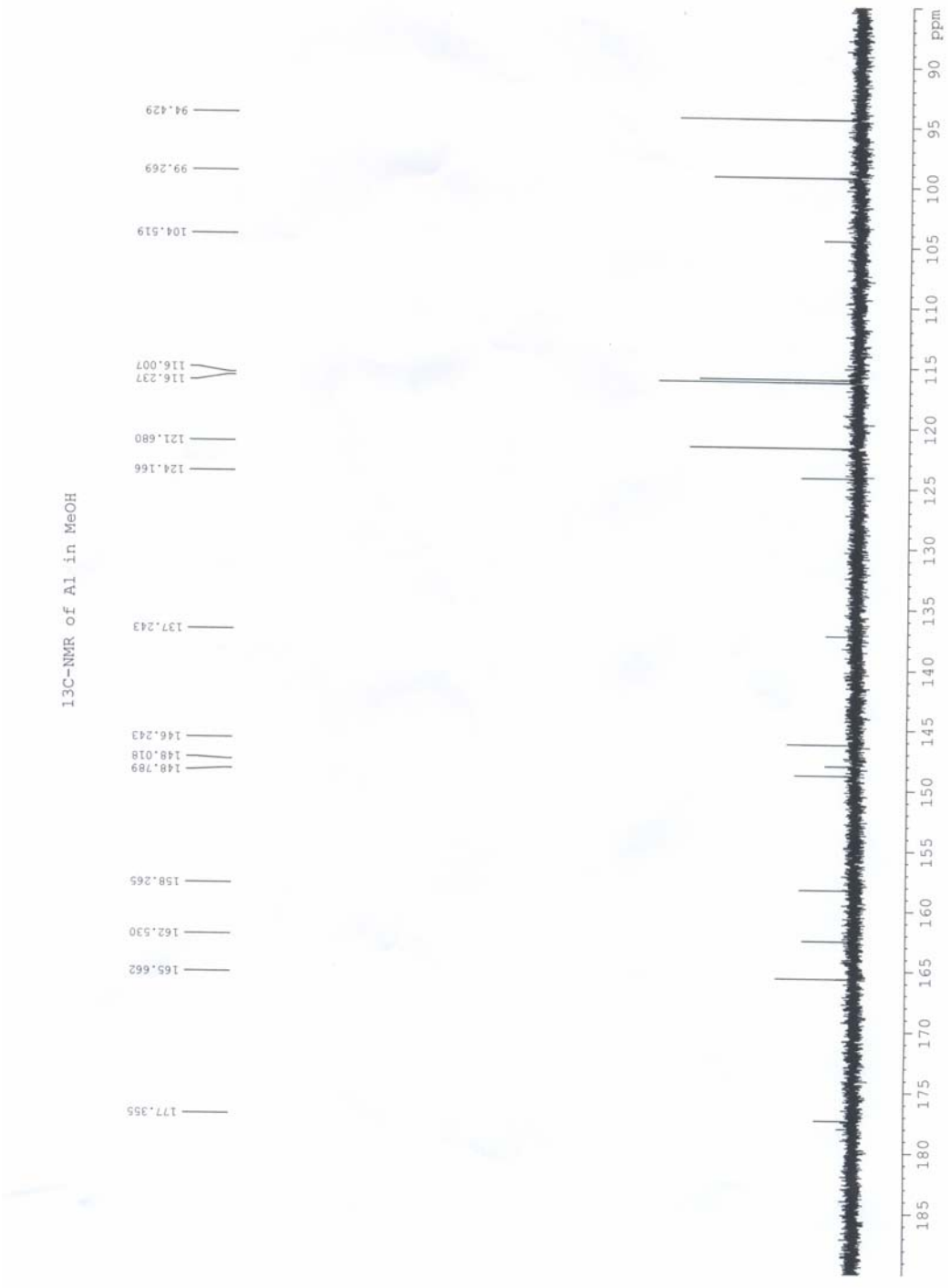


Figure 28 ^{13}C-NMR expanded spectrum of compound

4.2 Antioxidant activity of compound A1

Table 21 Antioxidant activity of compound A1 and authentic quercetin

Compound	Antioxidant activity DPPH scavenging activity EC_{50}(μg/ml)
Compound A1	1.69
Authentic quercetin	1.71
Vitamin C	1.49

This table shows the antioxidant activity of compound A1 compare with authentic quercetin and vitamin C. The antioxidant activity using DPPH scavenging assay of compound A1 shows high activity similar that of to authentic quercetin.

CHAPTER 5

DISCUSSION

A preliminary screening for the antioxidant activity of nine selected Thai herbs and vegetables, revealed that *Polygonum odoratum* and *Pluchea indica* had significant free radical scavenging. *P. indica* was selected for further study since there was a previous report of an antioxidant activity of the MeOH extract of its roots (6).

In this study the ethanolic extract of the *P. indica* leaf was found to have antioxidant activity (EC_{50} = 6.92 µg/ml) when compare with standard drug (vitamin C) EC_{50} = 1.49 µg/ml .

Comparing between the antioxidant activity of four fractions from Diaion HP 20 column chromatography by using DPPH scavenging assay, it was found that the activity of MeOH fr. (Fr.3) > H_2O-MeOH (1:1)fr. (Fr.2) > EtOAc fr. (Fr.4) > H_2O fr. (Fr.1),(EC_{50}= 1.89, 2.65, 12.08, 16.02 µg/ml, respectively)

Thin-layer chromatography analysis of the ethanolic extract of the *P. indica* leaf showed active spot activity when detecting with DPPH spraying reagent. By using vanillin HCl spraying reagent, extract clearly showed the red spots that might be flavonoids. When using $FeCl_3$ spraying reagent, the extract showed dark green spots that might be polyphenolic compounds (15).

TLC analysis of the four fractions from Diaion HP20 column showed the MeOH fraction had the highest antioxidant activity when tested by DPPH scavenging assay (EC_{50}=1.89 µg/ml) and gave positive spot with DPPH spraying reagent. The active component was likely to be a flavonoid due to a red spot with 1% vanillin HCl spraying reagent. With $FeCl_3$ spraying reagent, the MeOH fraction also showed dark green spots indicating the presence of polyphenolic compounds and showed yellow spot indicating the presence of flavonol when detected with NP/PEG spraying reagent gave a yellow spot with the same Rf value as that of authentic quercetin. These reactions indicated that the compound A1 may be flavonol-type compound.

The time of flight (TOF) mass spectrum of compound A1 indicated that the molecular ion peak at 303.34 [M^++H] should be that of a free flavonol without sugar

moiety. Electrospray can also be used in the case of molecules without any ionizable sites, through the formation of sodium, potassium, ammonium, or other adducts. This enables sensitivities in the range of a few hundred moles to be achieved. Electrospray mass spectra normally correspond to a statistical distribution of multiply charged molecular ions obtained from protonation $(M+nH)^{n+}$, while avoiding the contributions from dissociations or from fragmentations. Computer-based algorithms have been developed to allow the determination of the molecular mass through the transformation of the multiply charged peaks in the ES spectrum into singly charged peaks. TOF mass (Time-of-flight) analyzers are positive analyze ions by bombardment of the sample with brief pulses of electrons. TOF mass spectrometers have some notable advantages, namely simplicity, virtually unlimited mass range, high scan rated and very short ion-formation times. The IR spectrum of compound A1 indicated the characteristic υ O-H group at 3413.3 cm^{-1}, υ C=O at 1663.6 cm^{-1}, υ C=C at 1380 cm^{-1}, υ C-O at 1255.6-1005.5 cm^{-1} and the fingerprint of benzene ring at 669.8 cm^{-1}.

The ^{1}H-NMR spectrum of compound A1 also confirmed the flavonol structure and displayed the presence of H-6, H-8, H-2′, H-5′ and H-6′ protons. The PMR spectrum of this compound in MeOH showed two distinctive resonance groups. Its displayed two doublets at δ7.76 (1H, J = 2.1 Hz) and δ6.88 (1 H, J = 8.52 Hz) and one doublet of doublet at δ7.65 (1H, J = 2.2, 2.2 Hz), characteristic of a 1,2,4-trisubstituted benzene ring (the ABC system with ortho and meta coupling, the B-ring of the flavonol) and two doublet at δ6.38 (1H, J = 2.1 Hz) and δ6.18 (1H, J = 2.1 Hz) characteristic of a 1,2,3 ,5-tetrasubstituted benzene ring (the AB system with meta coupling, the A-ring of the flavonol). The combination of the substitution patterns of the A, a rings suggested that the compound could be 3′,4′,5,7 tetrahydroxyflavonol (12,13,14). The ^{13}C-NMR of compound A1 spectrum also confirm carbon 15 signals of the flavonoid skeleton in the region 90-180 ppm. A4-carbonyl signal was seen at 176 ppm indicating 2,3 unsaturated 4-keto flavonoid nucleus. The carbon signals were in accordance with literature quercetin(69). The UV spectrum of the compound A1 in methanol showed two major absorption bands at 255.32 nm (band II) and 372.77 nm (band I) which are typical for flavonol. The presence of a shoulder in band II (255.32 nm) was an evidence of the 3′,4′-ortho-dihydroxy system shifted 5 nm indicating ring

B ortho-di-OH(15). The shifts of UV spectrum of compound A1 in methanol with addition of shift reagents are shown in Table 16.

According to the above spectral data, compound A1 is proposed to be quercetin (5,7,3′,4′-tetrahydroxyflavonol). The spectroscopic data including UV and IR of compound A1 compared with authentic quercetin are shows in Tables 15,16. The ^{1}H-NMR and ^{13}C-NMR spectroscopic data of compound A1 compared well with those of quercetin from literature as shown in Tables.17,18 (66).

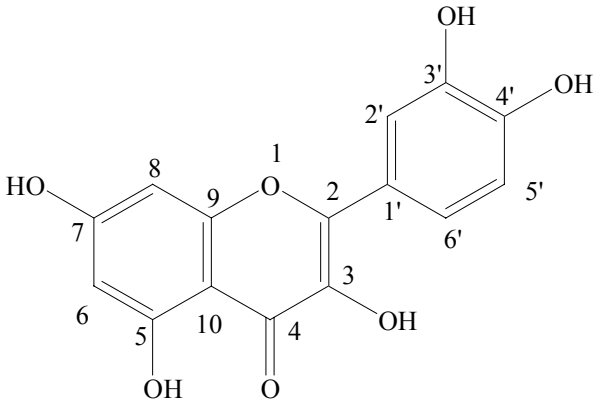


Figure 29 Structure of compound A1

CHAPTER 6
CONCLUSION

Pluchea indica (Linn.) Less is taxonomically classified in the family Compositae (Asteraceae). Thai traditional medicinal system indicated the use of this plant as a diuretic, treatment for the treatment of sinusitis and as an anti-inflammatory.

In this study the ethanolic extract of the leaf of *P. indica* showed a positive tested for antioxidant activity using DPPH scavenging assay with an EC_{50} = 6.9195 μg/ml. TLC analysis using specific spraying reagents of the crude extract and fractions of the leaf extract of *P. indica* from column chromatography showed the presence of some flavonoids and polyphenolic compounds.

From the result of an antioxidant activity determination by DPPH scavenging assay, the activity of four fractions from Diaion HP20 column chromatography were : Methanol fraction > water-methanol fraction > ethyl acetate fraction > water fraction. (EC_{50} = 1.89, 2.65, 12.08 and 16.02 μg/ml, respectively). These results suggested the most active fraction is the methanol fraction. This fraction showed positive test flavonoids and polyphenolic components after TLC analysis.

Further separation of the methanol fraction by silica gel column chromatography. According to spectroscopic data, compound A1 was identified as quercetin(5,7,3´,4´tetrahydroxyflavonol). Compound A1 showed antioxidant activity using DPPH scavenging assay with an EC_{50} of 1.69 μg/ml. The antioxidant activity of this compound is as strong as the activities of authentic quercetin and vitamin C (EC_{50} of 1.17 and 1.49 μg/ml, respectively)

In conclusion compound A1, which was identified as quercetin, was responsible for the antioxidant activity of the ethanolic extract from the leaf of *Pluchea indica*. This compound is one of the major active components present in this extract.

The finding of quercetin in the leaf of *Pluchea indica* is the first report of this compound in this plant. Quercetin, a known antioxidant, has also been identified as one of the main contributing factors in the antioxidant activity of *P. indica.* For further work, the development of an alternative source of quercetin and/or quercetin-enriched extracts from the leaf of *Pluchea indica* for use as dietary supplements or in cosmetics may be of some interest.

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APPENDIX

APPENDIX A
SPRAYING REAGENTS

DPPH spraying reagent

1,1 Diphenyl – 2 – picrylhydrazyl (DPPH) radical	6	mg
Methanol to	100	ml

6 mg of DPPH is dissolved in methanol and adjusted to 100 ml

1% vanillin HCl spraying reagent

Vanillin	1	g
Hydrochloric acid (conc.HCl)	1	ml
95% ethanol to	100	ml

1 g of vanillin is dissolved in 1 ml of hydrochloric acid then adjusted to 100 ml with 95% ethanol

After spraying with vanillin HCL spraying, TLC plate is heat for 5 minutes at 100 °C

10% ferric chloride spraying reagent

Ferric chloride ($FeCl_3$)	10	g
Distilled water to	100	ml

10 g of ferric chloride is dissolved in distilled water and adjusted to 100 ml

Aluminium chloride spraying reagent

Aluminium chloride ($AlCl_3$)	1	g
Ethanol to	100	ml

1 g of aluminium chloride is dissolved in ethanol and adjusted to 100 ml. TLC plate was evaluated under UV 366 nm.

Natural product/poly ethylene glycol (NP/PEG)

Spraying neagent

1% Natural product spraying reagent		
Diphenylburyloxyethylamin (NP)	1	g
Methanol to	100	ml

1 g of NP is dissolved in methanol and adjusted to 100 ml

5% Poly ethylene glycol spraying reagent		
Poly ethylene glycol spraying - 4λ 000 (PEG)	5	g
Ethanol to	100	ml

5 g of PEG is dissolved in ethanol and adjusted to 100 ml

TLC plate is sprayed with 1% NP spraying followed by 5% PEG reagent and evaluated under 366 mm.

APPENDIX B
DPPH SCAVENGING ASSAY

DPPH solution

1,1 Diphenyl – 2 – picryl hydrazyl radical	3	mg
Absolute ethanol to	50	ml

1,1 Diphenyl – 2 – picryl hydrazyl (DPPH) radical was purchased from Sigma, Thailand (lot No. 50 k/482). 3 mg of DPPH radical was transferred to 50 ml volumetric flask, added absolute ethanol to the volume. The solution was then solicited for 2 min, kept in the refrigerator and protected from light.

BIOGRAPHY

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