BIOLOGICAL ACTIVITIES AND CHEMICAL CONSTITUENTS OF *Chromolaena odorata* (L.) King & Robinson

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ABSTRACT

Chloromolaena odorata was screened for its phytochemical properties and pharmacological activities. Phytochemical screening of *C. odorata* indicates the presence of terpenoid, flavonoid and alkaloid. GCMS analysis of the leaf extract of *C. odorata* shows four major compounds which are cyclohexane, germacrene, hexadecoic acid and caryophyllene. While, HPLC analysis has identify five peaks; quercetin-4 methyl ether, aromadendrin-4'-methyl ether, taxifolin-7-methyl ether, taxifolin-4'-methyl ether and quercetin-7-methyl ether, kaempferol-4'-methyl ether and eridicytol-7, 4'-dimehyl ether, quercetin-7,4'-dimethyl ether. By using the column chromatography, three compounds were isolated; 5,7-dihydroxy-2-(4-methoxyphenyl)chromen-4-one; 3,5-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-7-methoxy-chromen-4-one and of 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy-chromen-4-one. The toxicity evaluation and dermal irritation of the aqueous leaf extract of *C. odorata* verifies that it is non-toxic at the maximum dose of 2000mg/kg. For the formaldehyde induced paw oedema evaluation, it proves that the leaf extract of the plant is 80.24% (concentration of 100mg/kg) as effective as Indomethacine (standard drug). The methanolic extract (100mg/ml) of the plant shows negative anti- coagulant, as it causes blood clot in less than two minutes. Meanwhile, the petroleum ether and chloroform leaf extract shows negative anti-coagulant, as they prolong the blood coagulation from to two minutes to more than three minutes. For the result of anti-microbial evaluation, *C. odorata* shows medium inhibition on *S. aureus* ATCC 25293, weak inhibition on *S. aureus* ATCC 29213 and strong inhibition on *P. aeruginosa* but no inhibition on the fungi, *C. albicans*. The last evaluation which is wound healing evaluation certifies the rapid wound healing to four days (concentration of 300mg/kg) only from six days (using acriflavine lotion).

ABSTRAK

Penyelidikan telah dijalankan mengenai kandungan kimia dan aktiviti farmakologikal *Chloromolaena odorata* L.. Penskrinan fitokimia ekstrak daun *C. odorata* menunjukkan kehadiran terpenoid, flavonoid dan alkaloid. Analisis GCMS ekstrak daun *C. odorata* telah mengasingkan kompaun, germacrene, asid hexadekoik dan caryophyllene. Analisis HPLC pula telah menghasilkan lima puncak yang dikenalpasti sebagai quercetin-4 methyl ether, aromadendrin-4'-methyl ether; taxifolin-7-methyl ether; taxifolin-4'-methyl ether dan quercetin-7-methyl ether; kaempferol-4'-methyl ether dan eridicytol-7, 4'-dimehyl ether; quercetin-7,4'-dimethyl ether. Manakala kromatografi kolum telah mengasingkan tiga kompaun kristal, iaitu, 5,7-dihydroxy-2-(4-methoxyphenyl)chromen-4-one; 3,5-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-7-methoxy-chromen-4-one dan 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy-chromen-4-one. Selain itu, kajian toksiksiti dan keradangan kulit menunjukkan bahawa ekstrak daun *C. odorata* adalah selamat untuk digunakan sehingga dos maksimum 2000mg/kg. Di samping itu, kajian pembengkakan aruhan formaldehid menunjukkan ekstrak akues daun (kepekatan 100mg/kg) adalah 80.24% sama efektif seperti Indomethacine (dadah piawai). Kajian anti-koagulan ekstrak methanol pula menunjukkan keputusan negatif kerana ia mempercepatkan kadar koagulan kurang daripada dua minit. Manakala ekstrak petroleum eter dan kloroform menunjukkan keputusan positif anti-koagulan-ekstrak tersebut meningkatkan masa koagulasi darah melebihi tiga minit. Selain itu, kajian antimikrobial menunjukkan kesan rencatan sederhana terhadap bakteria *S. aureus* ATCC 25293, rencatan lemah terhadap *S. aureus* ATCC 29213 dan rencatan kuat terhadap *P. aeruginosa*. Tetapi tiada kesan rencatan terhadap kulat *C. albicans*. Akhir sekali, ujian luka menunjukkan bahawa kadar penyembuhan dipercepatkan (pada kepekatan 300mg/kg) kepada 4 hari berbanding 6 hari jika menggunakan Akriflavin (dadah piawai).

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ABBREVIATIONS

$CHCL_3$	-	chloroform
EtOAc	-	Ethyl acetate
EtOH	-	Ethanol
GCMS	-	Gas Chromatography Mass Spectrometry
HPLC	-	High Performance Liquid Chromatography
H_2SO_4	-	Sulphuric acid
HOAc	-	Acetic acid
HPLC	-	High performance liquid chromatography
I_2	-	Iodine
IR	-	Infra red
KBr	-	Potassium bromide
Kg	-	Kilogram
MeOH	-	Methanol
MS	-	Mass spectrometry
NMR	-	Nuclear Magnetic Resonance
PE	-	Light petroleum ether (b.p 40-60°C)
UV	-	Ultraviolet
Rf	-	Relative mobility
C	-	Celcius
cm	-	Centimeter
g	-	Gram
µg	-	Microgram
m	-	Meter
mm	-	Millimeter

Chapter One

INTRODUCTION

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1.1 General Introduction

Plants that are used for food flavouring and any medicinal values are known as herbs (Hornby and Parnwell, 1965). Herbalism is a traditional medicinal or folk medicine practice based on the use of plant or plant extracts. The scope of herbal medicine is extended to include fungi and bee products, as well as minerals, shells and certain parts of animals (Acharya *et al*., 2008). Herbs can be divided into four categories which are wild, ornamental, vegetable and spice (Arifin, 2005).

It is undeniable that herbs are the foundation of conventional drugs or modern medicine. Aspirin for example was used long ago by the traditional medicine practioners as a pain relief medicine (Arifin, 2005). Many plants substances that have been synthesized are useful to the health maintenance in human and other animals. The derived substances include aromatic compounds (mostly phenols or tannins) and many secondary metabolites, of which 12 000 have been isolated and reported (Lai, 2004). In many cases, these substances (particularly the alkaloids) serve as plant defence mechanisms against predation by microorganisms, insects and herbivores (Lai, 2004). Yet many of these herbs and spices are used by humans for food seasoning and they are found to yield useful medicinal compounds (Tapsell, 2008).

Herbs and medicinal plants are becoming popular these days as more people are using herbal remedies in their daily lives. Herbs are used as treatment for certain disease, for maintaining optimum health and also as aphrodisiac. It is believed that 80% of the outside industrialised countries rely on herbs for health (Mohd, 2003).

However, new scientific findings show that some flavonoids in plants and plant products such as herbal medicines and fruit juice are able to induce or inhibit cytochrome enzyme in human and animals. Cytochrome enzyme plays an important role in metabolising xenobiotics (foreign pollutants, drugs, chemicals and pesticides) that enters the human body. Over production or inhibition of the cytochrome enzyme may lead to cytoxicity or even death. For example, compounds such as genistein, daidzein and coumestrol can combine with the estrogen receptors and cause breast cancer in women (Mohd, 2003).

1.2 Traditional Medicine in Malaysia

In Malaysia, the herbal industry is set to grow 15% annually (Kaur, 2008). The market value of the Malaysian herbal supplements and cures industry is expected to increase from RM10 billion in 2008 to RM12 billion in 2009. This is due to acceptance of herbal medicine by the Malaysian consumers (Kaur, 2009).

The use of traditional preparation has been increased dramatically in the last two years. Medicinal plants such as 'tongkat Ali', 'kacip Fatimah', Noni fruit and juices (from Morinda cirifolia), ginseng, 'pegaga', animal products (gamat for example) and etc. Some of the natural products are also added to food and beverages such as coffee, tea, sweets and even cigarettes (Mohd, 2003).

Research by University of Malaya (UM) on effects of traditional preparation on modern medicine shows that there are many cases of abuse in the medicinal plants and consumer trust. The consumption of traditional preparation for a long term may lead to detrimental of the human body. This is due to the herbs inhibiting or increasing effects

of modern medicines. Some may even cause the modern medicine to be digested too fast before their effects can be seen. Thus, this may lead to toxicity and contraindication to the human body (Anonymous, 2003).

According to the UM research too, certain traditional preparations are added with established modern medicines such as dexamethasone (steroid), Betamethasone (steroid) glibenclamide and paracetamol. Generally, steroids are added for joint ache and arthritis preparation in traditional medicine. Glibenclamide, which is a modern drug for diabetic patients, is also found to be added to the traditional preparation for the diabetic medicine. Another example is paracetamol which is found in 'air badak', which is a popular water tonic (Anonymous, 2003).

However, there is no legal requirement for direct selling companies to conduct toxicity screenings on their own products. Therefore, the Ministry of Health and the Federation of Malaysian Traditional Medical Practioners are working on the guidelines to elevate the status of traditional medicine with scientific and safety measures (Mohd, 2003).

1.3 Family Compositae (Syn. Asteraceae)

The compositae or Asteraceae is the largest and most familiar family of flowering plants and consists of 1000 genera and 15000 species. Compositae is an interesting family because of its characteristic aggregation of reduced flowers into specialized inflorescences called capitula (Heywood *et al*., 1977).

Compositae can be found in a wide range of habitat except in Antarctica. Plants of compositae can be found from highly reduced, minute montane annuals (less than 1cm high, e.g; *Cuchanatanea* sp.) to relatively large tropical trees up to 20m tall, such as *Brachylaena* sp. The family is the most abundant in subtropical and tropical latitudes and is the most evidenced in mountainous areas such as the desert regions. The tribes Vernonieae and Eupatorieaehave are largely adapted to mainly tropical or subtropical habitats. And the Asteraceae, Inulaea and Lactuceae are adapted to mostly temperate and high latitudinal habitats ((Heywood *et al*., 1977).

Trees and tree like taxa can be found periodically in remote tribes, in regions where phyletic diversification is low, such as mid to low subtropical, slow growing, cold enduring, and hard wooden rainforest or in endemics where woodiness is clearly a secondary phenomenon (Wagnitz, 1976). Shrubs such as Artemisia occur along rapid growing, cold sensitive, herbaceous genera, which are common in warm climate as well as perennial, often succulent, saline taxa such as *Borrichia* sp and *Varilla* sp (Heywood *et al*., 1977).

Wholly aquatic genera for example, *Gymnocoronis* sp, *Trichoronis* sp and *Shinneria* sp and epiphytic taxa are usually found in Eupatoriaea. The extreme xerophytic and adaptation is more often found in Asteraea, Inuleae and Heliantheae (Heywood *et al*., 1977).

According to their habitat types, different dispersal mechanisms can be found amongst 5451 species of all tribes of the Compositae. Species which live in the plains and prairies tend to have less frequent capillary pappus compared to deciduous forests. On the other hand, species of mountainous regions have more frequent capillary pappus

but they have no obvious dispersal adaptation compared to the plants living in the same region but at lower allevation. The species of the desert regions have more adhesive structures and less wind dispersal pappus. While mesic, riverine and sea coast habitats have high percentage of species with no obvious modification of pappus for dispersal. From the annual to perennial shrubs, a concomitant increases in the proportion of capillary or plumose pappus (wind dispersal) a decreasing proportion of both adhesive or 'no obvious' dispersal adaptation (Heywood *et al*., 1977).

Table 1.1: The tribes of the Compositae *

Tribe	Name	Original spelling
1	Vernnonieae	Vernoniaceae
2	Eupatorieae	Eupatoriaceae
3	Asteraceae	Asteroideae
4	Inuleae	Inuloideae
5	Heliantheae	Helianthoideae
6	Helenieae	Helianthoideae
7	Anthemideae	
8	Senecioneae	Senecionideae
9	Calenduleae	Calendulaceae
10	Arctotideae	
11	Cynareae	Cynaroideae
12	Mustisieae	Mustisaceae
13	Cichorieae	Cichoriaceae

*** Heywood *et al*., 1977**

1.3.1 Chemical Constituent of Compositae

Compositae is very rich in both secondary compounds and also in the numbers of complex structures. The common chemical compounds found in compositae are terpenoid, sesquiterpene lactones, fatty acid derived from polyacetylenes and the polysaccharide fructans (Heywood *et al*., 1977). The chemical pattern found in the family compositae is shown in Table 1.2.

Common flavonoids in compositae are kaempferol and quercetin (flavonols), apigenin and luteolin (flavones). For example, tricin 5-glucoside is the main flavonoid found in *Cirsium arvense* L. Other flavonoids that can be found rarely in the compositae family are 6-hydroxylated compound (e.g. quercetagetin **(1)** and scutellarein **(2)**, methylation of flavonoids (e.g. artemitin, jaceidin, centaureidinm, eupatorin **(3)**, eupafolin **(4)**, eupatilin **(5)** and tetramethoxyflavone), chalcone glycosides (flower pigments, dehydro-para-asebotin, sakuranetin, isosakuranetin and 2-hydroxy-4',5',6',4-tetramethoxychalcone odoratin), flavanonols (e.g. Aromadendrin **(6)** and its 7-methyl-ether) and flavonolignans (Heywood *et al*., 1977).

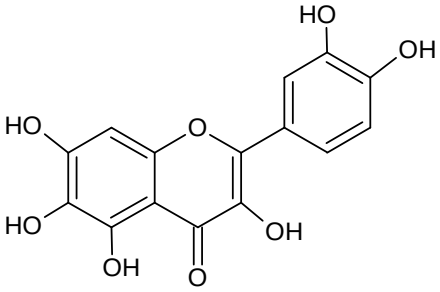
Phenolic monoterpenoid and large thymol derivatives (both free and esterified) are common constituents of certain genera in the compositae family, especially, Eupatorium, Inula, Helenium and Gaillardia. Reported volatile constituents found in this family are p-menth-3-ene-3, 6-diol (from *E.macrocephalum* Less), thymol derived dehydrobenzofuranoid (species Helenium), Furano sesquiterpenes (from Asteraceae), para-quinoid sesquiterpenoids perezone, hydroxyperezone and pipitzols (in Mutisieae) and irregular phenolic sesquiterpene (Heliantheae) (Heywood *et al*., 1977).

The seeds of members of the Compositae family are rich in linoleic acid and palmitic acid. Also found in the seeds are lenolenic acid, stearic acid and other minor fatty acids. Safflowers (75% linoleic acid and 15% oleic acid) and sunflower seed are commercially important for their oil (Heywood *et al*., 1977).

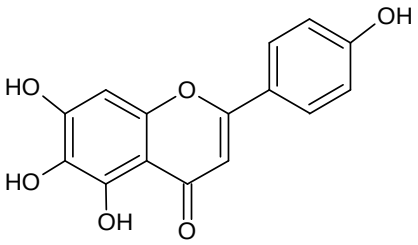
Table 1.2: Chemical Pattern of the Compositae * (Heywood *et al*., 1977)

Class of compound	Location and biological activity
Presents in all tribes	
1. Inulin-type fructans	In storage organ
2. characteristics fatty acids	In seed oils
3. Sesquiterpene lactones	Mainly in leaves (bitter taste)
4. pentacyclic triterpene alcohols	As in esters in fruit pericarp and in lipid
5. Caffeic acid esters	In leaves ; cynarin
6. Methylated flavonoids	In leaves and flowers (as yellow pigments)
Present in most tribes	
7. Acetylenic compounds	In roots and leaves
8. Essential oil (including phenolic monoterpenes)	In leaves and fruits
9. Cyclitols'coumarins	In leaves and flowers
Present in most tribes	
10. Rubber (polyisoprene)	In roots and stems
11. Pyrrolizidine alkaloids	In leaves
12. Triterpene acids	Free in flowers; combined with sugar (as saponin) in leaves
13. Diterpenes	In all tissues
14. Cyanogenic glycosides	In leaves and fruits
15. Anthochlor pigments	In yellow flowers
16. Chromenes	In leaves and roots
17. Fatty and amides	In roots

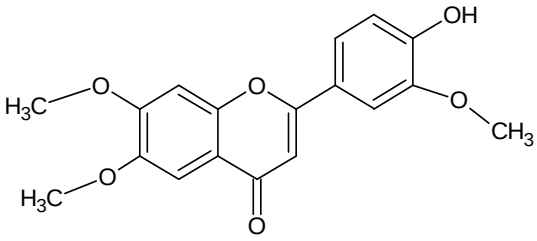
*** Heywood *et al*., 1977**



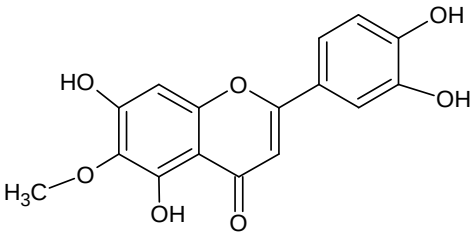
(1) Quercetagetin



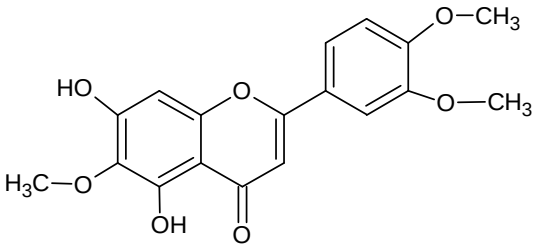
(2) Scutellarin



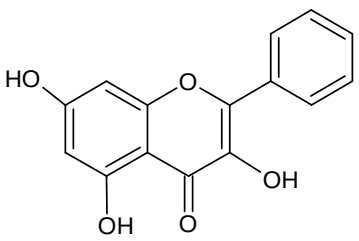
(3) Eupatorin



(4) Eupafolin



(5) Eupatilin



(6) Aromadendrin

1.3.2 Biological Activities of Compositae

Compositae is mostly toxic and rarely taken in human diet or animals'. However, it was reported that 25 species of the Compositae family have spasmolytic, choleretic, anti-hepatotoxic, anti-helmintic, anti-phloglistic and anti-microbial properties. The compositae family is also rich in terpenoid (essential oil), which can be used as food flavouring or liqueurs flavours. Phenolic substances are important too as preparation of modern medicines (Heywood *et al*., 1977).

Table 1.3: Some Important Members of the Compositae Family*

Species	Common name	Values
Achillea ptarmica L.	Sneezewort	Medicinal plant (sooth toothache)
Anacyclus pyrethrum DC		Insecticidal
Antennaria dioica L. Gaertner	Cat's foot	Medicinal plant
Arnica Montana L.	Arnica	Anti-inflammatory
Artemisia absinthium L.	Wormwood	Insecticides / liquor flavour
Artemisia dracunculus L.	Tarragon	Spice
Atrium lappa		Produce polysaccharide
Bellis parennis	Daisy	Wound healing properties
Calendula officinalis L.	Pot marigold	Ornamental plant
Carthamus tinctorius L.	Safflower	Produce dye and oil
Centaurea cyanus L.	Cornflower	Used as dye
Chamaemelum nobile L. All.	Chamomile	Medicinal plant
Chrysanthemum carinatum Schousboe	Annual chrysanthemum	Ornamental plant
Chrysanthemum cineraciae		Insecticides
Cichorium endivia Willd.	Endive	Food
Cichorium intybus L.	Chicory	Coffee substitute
Cirsum oleraceum (L.) Scop	meadow cabbage	Food
Cnicus benedictus L.	Blessed thistle	Medicinal plant

Table 1.3 : Continued

Cynora cardunculus L.	Cardoon	Food
Cynora scolymus L.	Globe artichoke	Food
Dahlia variabilis L.	Dahlia	Ornamental plant
Dendranthema indica (L.)	Autumn chrysanthemum	Ornamental plant
Echinacea anacyclus		Insecticides
Echinacea angustifolia DC		Insecticidal
Echinacea chrysanthemus		Insecticides
Echinacea heliopsis		Insecticides
Helianthus annus L.	Sunflower	Produce oil / anticancer properties
Helianthus tuberosus L.	Jurusalem artichoke	Food/animal feed/ coffee substitute/ Preparation of fructose
Ichthyothera terminalis		Fish poison
Inula helenium L.	Elecompane	Spice
Lactuca sativa L.	Garden lettuce	Food
Matricaria chamomilla		Antiiflammatory and wound healing properties
Parthenium argentatum Gray		Produce rubber
Petasites hybridus L. Gaertner	Butterbur	Anticonvulsive properties
Pulicaria dysenterica (L.) Bernh	Fleabane	Insecticidal
Scarzonera hispanica L.	Black salsify	Food
Silybum marianum L. Gaertner	Milk thistle	Medicinal plant
*Solidago virg*aurea L.	Golden rod	Used as dye
Tagetes indica L.	African marigold	Ornamental plant
Tagetes patula L.	French marigold	Ornamental plant
Tanacetum cinerariifolium (Trev.) Schultz Bip	Pyrethrum	Insecticidal
Tanacetum vulgare L.	Tansy	Spice / liquor flavour
Taraxacum bicorne L. Kok		Produce rubber
Taraxacum officinale	pissenlit	Diuretic properties
Tragopogon porrifolius L.	salsify	food
Tussilago farfara L.	coltsfoot	Medicinal plant

Vernonia sp.		Insecticidal

*****Heywood *et al*., 1977

1.4 General Description of *Chromolaena odorata* L.

Figure 1.1: *Chromolaena odorata*

Chromolaena odorata (L.) King & Robinson or formerly known as *Eupatorium odoratum* L. belongs to the Kingdom Plantae, subkingdom Tracheobionta (vascular plants), superdivision Spermatophyta (seeds plant), division Manoliophyta (flowering plant), class Magnoliopsida (dicotyledons), subclass Asterdae, order Asterales, family Compositae, genus *Chromolaena* DC (thoroughwort) and species *Chromolaena odorata* (L.). *C. odorata* is also known as *Eupatorium conyzoides* Vahl, *Eupatorium brachiatum* Sw. ex Wiestr, *Eupatorium atriplicifolium* Vahl and *Osmia odorata* (L.) Schultz-Bip. In English, it is known as Siam weed, triffed weed, bitter bush, Jack in the bush, Christmas bush, baby tea and so many other names in different countries with different languages (King and Robinson, 1970).

C. odorata is native from Florida through the West Indies and Texas through central and South America to Argentina (Lieogier, 1997). It has been accidentally or

deliberately introduced to the tropical countries, including Guam and Hawaii (King and Robinson, 1970).

C. odorata grows in well drained soil but they still can grow in other different type of soil. They grow in a range of vegetation types, e.g. forests (annual rainfall 1500mm), grassland and arid bushveld (annual rainfall less than 500mm). In arid areas, it is restricted to riverbanks. *C. odorata* grows from close to sea level to over 1000 meter in elevation. They are intolerant of frost and are limited by drought, about 90 mm of mean annual precipitation (Liogier, 1997). The seedlings of *C. odorata* grow well in the range of relative humidity from 60 to 70%. There are some experiments which show that the seedlings grow well at 30°C and even better on mulched soils at 25°C (King and Robinson, 1970).

The *C. odorata* plants are able to reach to 1 meter or more as free standing shrub and 4 meter or more when climbing into other trees or shrubs. Its stems can reach to 2cm in diameter, while the individual branches are long with relatively few branches. The plants are maintained by a system of abundant, yellowish, fine lateral roots. Besides, multiple sprouts arise from the crown root and lower stems.

The leaves are opposite, three nerved, are deltoid to ovate-lanciolate and are aromatic when crushed. The inflorescences are corymbs of cylindrical heads located on the terminal lateral branches. There are about 15 to 25 tubular florets per head, which are white, lavender, pink or blue in colour. The seeds have brownish gray to black achene that is 4mm long and a pale brown pappus 5 or 6mm long (Liogier, 1997).

C. odorata does not tolerate shade and flourish well in open areas. They can form dense stands and suppress the growth of other plants. This is due to the

competition and allelopathic effects. The plant will become invasive in the frost-free areas from medium to arid woodland which are not water-stressed in the growing season. When the plant is dry, it will promote wild land fires (Muniappan, 2000).

In north-eastern India, Siam weed is regarded as a nutrient-demanding early succession species. It takes advantage of the flush of soil that becomes available after a disturbance (such as fire or land clearing for agriculture) and exhibits relatively high foliar natrium, phosphorus and kalium contents (King and Robinson, 1970).

C. odorata blooms annually and is an abundant producer of seeds. Flowering and fruiting begins after the plants are one year old (Lieogier, 1997). The flowers are pollinated by wind, clinging to hair and clothing of animals and humans. The tiny seeds can occur contaminated in imported grass seeds (King and Robinson, 1970).

The individual stems of *C. odorata* will grow for about two years and die near the base of the plant and later will be replaced by new sprouts. The plants' survival skill is very strong-whenever they are being burnt or cut, they can grow back soon. The best current control method is mechanical or hand cutting followed by herbicide treatment. Partial control can be obtained through the use of aggressive cover crops (Muniappan, 2000).

1.4.1 Overture of *C. odorata* in Malaysia

Chromolaena odorata (L.) King & Robinson was first known in Malaysia during the war from year 1914 to 1918 (Henderson, 1974). *C. odorata* is locally known as 'pokok kapal terbang', 'pokok Jerman', 'pokok Jepun' and 'pokok siam' (Ling *et al*.,

2007c). *C. odorata* is considered a dangerous weed not only in Malaysia but also all over the tropical Asia, Africa, the Western Pacific and northern Australia (McFadyen and Skaratt, 1996). It is reported that *C. odorata* is highly allelopathic and suppresses the growth of other plants, especially vegetation and plantation crops, for example oil palm, coconut, cashew, rubber, citrus and teak (Azmi, 2000).

In Malaysia, an experiment done by The Rubber Research Institute has found that *C. odorata* suppresses the growth of rubber trees (Anonymous, 1967). In Nigeria, the Nigerian Oil Palm Research Institute has requested Commonwealth Institute of Biological Control (now known as CABI Bioscience) to conduct a biological control programme to overcome the growth of *C. odorata*. Efforts towards the biological research of *C. odorata* were started in the 1970s. Biological control experiment was tested using *Apion brunneanigrum* Beguin-Billecocq, which is a seed-eating beetle and the defoliating larva of a moth. But the experiment was unsuccessful and till now, no effective control method has been found yet (Ooi *et al*., 1998).

C. odorata is reported to grow along with other weeds for example *Melastoma malabathricum* L. (Melastomatacea), *Clidemia hirta* (L.) D. Don (Melastomatacea), *Diodia acimifolia* (Wild. Ex Roem & Schutt) Bremek (Rubiaceae), *Borreria latifolia* (Aubl.) Schum. (Rubiaceae), *Asytasia gangetica* (L.) T.Aderson (Acanthaceae) and *Dicranopteris linearis* Bp (Clecheniaceae). *C. odorata* is very common on the road side, open areas, foreast clearings, abandoned gardens and near the beach (Syed, 1979).

1.4.2 *C. odorata* in the Agricultural Sector

The threat of *C. odorata* to agriculture has been a global concern and studies have been done around the world to effectively control the menacing weed. However, an evaluation of the progress made has revealed that, through many years of research efforts that had been done, the problem of *C. odorata* has remained unsolved (Ambika and Jayachandra, 1980; Ooi *et al*., 1998).

Despite claims of *C. odorata* being a menacing weed, there are also some research that shows positive contribution of *C. odorata* to the agricultural sector. Research by Bomikole revealed that using *C. odorata* leaf meal in rabbits' diet has a nutrient profile that is similar to a concentrated feed. Therefore, the leaves of *C. odorata* can be used as an ingredient for formulating animal feed. The *C. odorata* leaf meal is reported to make the production of rabbits more economical and encourage production among farmers due to availability of this feed resource (Bamikole *et al*., 2004).

Another research by Apori *et al.,* also confirmed that the leaves of *C. odorata* have high nutritive values and have the potential to be used as protein supplements to ruminants. Their chemical analysis reported that the leaves of *C. odorata* are high in protein content with little or no presence of phenolic antinutritive factors (Apori *et al*., 2001).

A different study by Offor and Okonye shows that *C. odorata* is useful in soil fertility restoration by providing essential constituents needed for plant growth and protection. It is reported that the leaves of *C. odorata* have potentials for protecting and

maintaining optimum growth for plant in polluted environment (Offor and Okonye, 2006).

In Indonesia, a research by Kumalasari *et al*., reported that the mulch of *C. odorata* improves the growth of corn (*Zea mays* L.). Their research involved the application of *C. odorata* mulch on planting ground of corn. They concluded that the mulch of *C. odorata* improved the content of the mineral phosphorus and nitrogen in the soil.

1.4.3 *C. odorata* in Traditional Medicine

Leaf extracts of *C. odorata* added with salt is used as gargle for sore throat and colds. The leaves are also used to scent aromatic baths. By adding copious amounts of organic matter to soil, it is reported that it may reduced the population of nematodes (M'Boob, 1991). The leaves are also used as emergency medication. By pounding the leaves till fine and applied to the wound, it can stop the bleeding of wound. Furthermore, it can be used during emergency where we can crush the leaves by hand, mix with some saliva and applying on the wound (Muhamad and Mustafa, 2004).

In traditional medicine, a decoction of the leaves is used as a cough remedy and as an ingredient with lemongrass and guava leaves for the treatment of malaria. In Thailand, the juice of the leaf is used as a haemostatic on wounds and anti-inflammatory. While, decoction of the flowers is used as tonic, antipyretic and heart tonic (Bunyapraphatsara and Chokechajiaroenprom, 2000). In Vietnam, fresh leaves or decoction of the leaves have been used for the treatment of leech bite, soft tissue wounds, burnt wounds, skin infection and dento-alveolitis (Phan *et al*., 2001b; Ling *et al*., 2007c).

In some studies, it helps to reduce the desire of smoking, cures fever, coughing, jaundice and stomach ache. The fresh leaves and extract of *C. odorata* are used as traditional herbal treatment in developing countries for burns, soft tissue wounds and skin infections (Phan *et al*., 2001a). Other medicinal uses include antidiarrhoeal, astringent, antispasmodic, antihypertensive, antiinflammatory and diuretic (Iwu, 1993).

1.4.4 Chemical Constituents of *C. odorata*

Previous investigation of the leaves and stems of *C. odorata* has revealed the presence of essential oils, steroids triterpenes and flavonoids (Inya *et al*., 1987; Suksamrarn *et al*., 2004; Talapatra *et al*., 1974, Bose *et al*., 1994). Flowers of this plant species have been subjected to investigation for essential oils, fats alkaloids and favonoids (Baruah and Leclerq, 1993). The reported isolated compounds of this plant are shown in Table 1.4.

Table 1.4: Chemical Constituents in Extracts of *C. odorata*

Part of plant	Chemical constituent	References
Flower (aqueous extract)	• 3,5,4'-trihydroxy-7-methoxyflavanone • 5,7,3" " '-trihydroxy-5'-methoxyflavanone • 3,5,7-trihydroxy-4'-methoxyflavanone	Odunbaku and Ilusanya, 2008
Flower	• Akuranetin • Persicogenin • 5,6,7,4'-tetramethoxyflavanone • 4'-hydroxy-5,6,7-trimethoxyflavanone • 2'-hydroxy-4,4',5',6'-tetramethoxychalcone • 4,2'-dihydroxy-4',5',6'-trimethoxyflavanone • Acacetin • Luteolin	Suksamrarn *et al*., 2004; Pisutthanan *et al*., 2006

	• 5, 7-dihydroxy-6-4'-dimethoxyflavanone (Latest)	

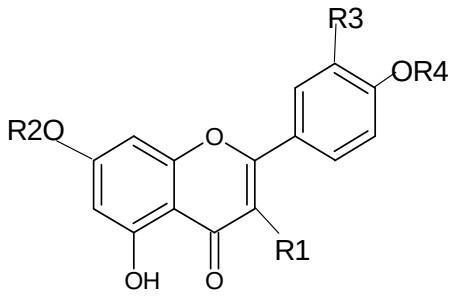
Table 1.4: Continued

Whole plant-above ground parts (dichloromethane extract)	• 2'-hydroxy-3,4,4',5',6'-pentamethoxy-chalcone • 2',4-dihydroxy-4',5',6'-trimethoxychalcone • Scutellarein tetramethyl ether • Sinensetin • 2'-hydroxy-4,4',5',6'-tetramethoxychalcone	Barua *et al*., 1978
Whole plant (ethanol and methanol extract)	• Aromadendrin 4' methyl ether • Eriodicytol 7,4'-dimethyl ether • Naringenin 4'-methyl ether • Taxifolin 4'-methyl ether ; taxifolin 7-methyl ether • Quercetin 7,4'-dimethyl ether • Kaempferol 4'-dimethyl ether • Quercetin 3-O-rutinoside • Quercetin 4'-methyl ether • Quercetin 7-methyl ether	Ling *et al*., 2007a; Suksamrarn *et al*,2004 ; Ling *et al*., 2007b
Leave (ethanol extract)	• Tamarixetin • Trihydroxymonomethoxyflavanone • Pentaethoxyflavanone • Dihydroxytrimethoxychalcone • Eupatillin ; 5,6,7,4'-Tetramethoxyflavanone • 5-Hdroxy6,7,3',4'-tetramethoxyflavone • Kaempferide • Protocatechuic acid • *p*-Coumaric acid • *p*-Hydroxybenzoic acid • Ferulic acid • Vanillic acid • Sinensetin • Rhamsetin • Tetrahydroxymonomethoxyflavanone	Phan *et al*., 2001b

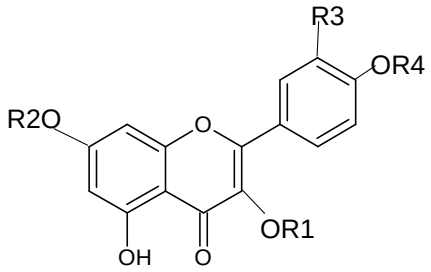
Table 1.4: Continued

Root and flower head (methanol extraction)	• 7-angeloylretronecine • 9-angeloylretronecine • Supinine • Intermidine • Lycopsamine • Rinderine • Echinatine • 3'-Acetylrinderine	Biller *et al*., 1994
Whole plant (aqueous extract)	Essential oils ; • Alpha-pinene • Limonene • *p*-Cymene • cadinene • Beta-caryophyllene • Camphor • Cardinal • Germacrene D	Dieneba *et al*., 1992
Whole plant (cold light petrol b.p. 60-80°)	• Lupeol • β-amyrin • Salvigenin	Talapatra *et al*., 1974

Figure 1.2: Chemical Structures of Flavonoids Isolated from the Leaves Extract of *C. odorata* *

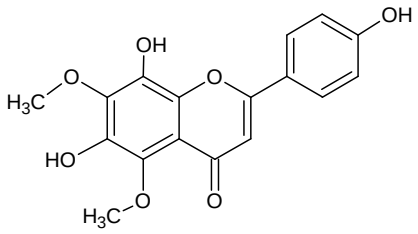


(7) Aromadendrin 4' methyl ether	R1=OH; R2, R3=H;R4=CH_3
(8) Eriodicytol 7,4'-dimethyl ether	R1=H; R2, R4= CH_3;R3=OH
(9) Naringenin 4'-methyl ether	R1,R2, R3=H; CH_3;R4=CH_3
(10) Taxifolin 4'-methyl ether	R1,R3=OH;R2=H; R4=CH_3
(11) Taxifolin 7-methyl ether	R1,R3=OH;R2= CH_3; R4= H

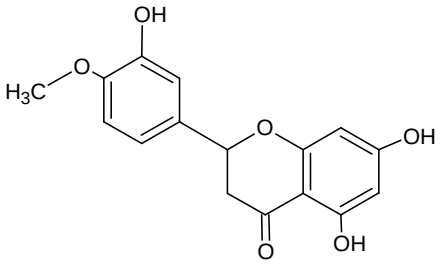


(12) Quercetin 7,4'-dimethyl ether	R1, R3=H;R2, R4=CH_3
(13) Kaempferol 4'-dimethyl ether	R1, R2,R3= H; R4=CH_3
(14) Quercetin 3-O-rutinoside	R1=glu(6-1)rham;R2, R4=H; R=OH
(15) Kaempferol 3-O-rutinoside	R1=glu(6-1)rham;R2, R3, R4=H
(16) Quercetin 4'-methyl ether	R1, R2=H;R3=OH;R4= CH_3
(17) Quercetin 7-methyl ether	R1,R4=H;R2= CH_3 ;R3=OH

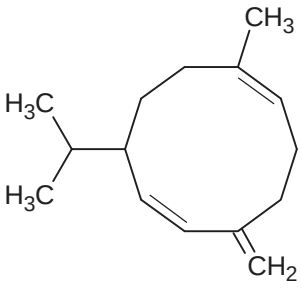
*** Ling *et al, 2*007b**



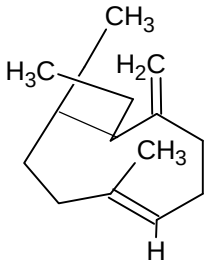
(18) 5,7-dihydroxy-6-4’-dimethoxyflavanone



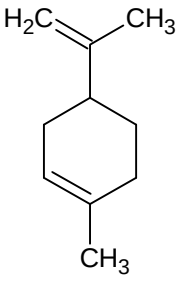
(19) 5,7,3” ” ’-trihydroxy-5’-methoxyflavanone



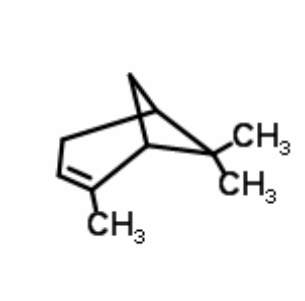
(20) Germacrene D



(21) Beta-caryophyllene



(22) Limonene



(23) Alpha pinene

1.4.5 Biological Activities of *C. odorata*

Several classes of flavonoids have been isolated from *C. odorata* extracts which are three flavanones and one flavone that proves to be responsible for blood coagulation. The extract of *C. odorata* is also reported to have strong anti oxidant effect (Phan *et al*., 2001b). It has also been reported to have anti-inflammatory, astringent, diuretic and hepatotropic activities (Watt and Breyer-Brandwijik, 1962). Some of the reported biological activities of *C. odorata* are shown in Table 1.5.

Table 1.5: Some Biological Activities of *C. odorata*

Part of plant	Biological Activity	Reference
Aerial part • Aqueous extract and essential oil	• Anti-malarial against • Anti-inflammatory • Antibacterial against	Pisutthanan *et al*., 2006
Whole plant extract of; • Ethanol, dichloromethane and methanol	• Analgesic • Anti-inflammatory • Antipyretic • Antibacterial against	Irobi, 1997 ; Odunbaku and Ilusanya, 2008; Oweyele *et al*., 2008
Leaf extract of; • Methanol, ethanol and aqueous	• Antioxidant • Anti-inflammatory • Wound healing properties • Anti-staphylococcal	Ling, 2006 ; Phan *et al*., 2001a ; Phan *et al*., 2001b ; Akinmoladun *et al*., 2007 ; Dieneba *et al*., 1992
Flower	• Antibacterial against Mycobacterium tuberculosis • Anticancer	Suksamrarn *et al*., 2004
Methanol extract of whole plant	• Platelet activating factor (PAF) receptor binding inhibitory activity	Ling *et al*., 2007b

1.5 Objectives of Present Study

The main objectives of this study are:

1. To screen the chemical constituents of *Chromolaena odorata* (L.)
2. To screen the bioactivity of *Chromolaena odorata* (L.)
3. To screen the wound healing activity of *Chromolaena odorata* (L.)
4. To isolate some flavonoid compound of *Chromolaena odorata* (L.)

Chapter Two

MATERIALS AND METHODS

CHAPTER TWO

MATERIALS AND METHODS

2.1 Plant Materials

In this study, the leaves of the plant *Chloromoena odorata* were investigated for its biological activities and chemical constituents. *C. odorata* was collected in University of Malaya, Kuala Lumpur and was authenticated by Prof. Dr. Muhamad bin Zakaria and Prof. Dr. Ong Hean Chooi. The leaves were carefully selected to avoid contamination by *Mikania* sp, which is seen to grow along side *C. odorata*. Then, the leaves were air dried for 14 days and after that ground into powder for experimental use.

2.2 Extraction of Plant Material

200g of the dried powdered leaves were extracted successively with different solvents of hexane, petroleum ether (b.p. 40-60C), chloroform and methanol. Then, each extracts were filtered and concentrated to dryness using a rotary evaporator at 50°C. The crude extracts were subsequently kept in the refrigerator for further studies.

For gas chromatography mass spectrum (GCMS) analysis, 250g of fresh leaves were collected and immediately immersed in 250 ml of HPLC grade solvents of hexane, chloroform and petroleum ether for 12 days. Then, they were filtered and evaporated using a rotary evaporator.

For the anti-inflammatory and toxicity evaluations, the powdered leaves were extracted using distilled water. The concentration of the leaf extracts were prepared according to the weight of the test subjects.

2.3 Instrumentation

Melting points were recorded on Optical Polarising Microscope with camera (Olympus BX51) equipped with Mettler Toledo FP82HT hot stage and the magnification is 20 times. The infrared spectra were taken in KBR. ^{1}HNMR, analyzed at 400MHz and ^{13}CNMR were analyzed at 100MHz. Both spectras were determined in deuterated methanol and chemical shifts are in δ units. The HPLC analysis was conducted using Shimadzu gradient HPLC. While GCMS analysis was performed on Hewlett–Packard Model 6890 combined with gas chromatography of Hewlett-Packard Model 6890. The isolation of compound was done by column chromatography.

2.4 Preliminary Evaluation (Biohealth Science Lab. Manual, 2003)

2.4.1 Preliminary Powder Extract Screening

One drop of 1mg/ml aqueous extract of *C. odorata* was added into test tubes with the following reagents:

- Dragendorf
- Meyers
- Anisaldehyde
- Potassium hydroxide
- Vanillin
- Ninhydrin

Changes of colour was observed and recorded according to Table 2.1.

Table 2.1: Standard Observation of Extract in Reagent

Component	Reagents						
	Dragendorft	**Meyer's**	**Anisaldehyde**	**Potassium hydroxide**	**Vanillin**	**Ninhydrin**	**Day light**
Alkaloid	Orange	White					
Terpenoid			Purple, blue, red		Purple, blue, grey		
Phenol					Purple/pink		
Flavonoid			Red/coloured				
Essential oil			Coloured				
Saponin			Black/blue				
Quinon				Purple/red			
Amino acid						Purple/Red	
Steroid			Green/gray				
Chlorophyll							Green
Carotene							Orange

2.4.2 Colour Test

Each 5 mg of blended sample was tested with 10 drops each of the following solvents:

- Concentrated ammonium sulphuric acid (H_2SO_4)
- Concentrated hydrochloric acid (HCl)
- 5 % natrium hydroxide (NaOH)
- 5% kalium hydroxide (KOH)
- 25% ammonia hydroxide (NH_4OH)
- 5% ferum chloric ($FeCl_3$)

Changes of colour was observed and recorded, according to Table 2.2.

Table 2.2: Standard Observation for Colour Test

Solvent	Colour	Compound detected
Sulphuric acid	Black	• Terpenoid
Hydrochloric acid	Green	• Flavonoid • Anthocyanin
Potassium hydroxide	• Purple • Red	• Parahydroxyqinon • Antraquinon
Ferric chloride	Blue or green	• Phenolic substances

2.5 Chromatographic Techniques

2.5.1 Thin Layer Chromatography (TLC)

2.5.1.1 TLC for Phytochemical Screening of Leaf Extracts

Commercial aluminum sheets Silica gel $60F_{245}$ of layer thickness 0.2 mm (Merck, Art. 5554) was used as TLC plates. The TLC strips were developed in several solvent systems to screen the secondary metabolites of the leaves. The extracts were diluted in different solvents and spotted onto the strips using fine glass capillaries to make fine spots.

The leaves extract was spotted 1.5 cm from the bottom of the strip and developed to 1 cm from the top of the TLC strip. The strips were developed in saturated chromatographic tank at room temperature. The spots were visualized by examination of strips under UV light, followed by staining with iodine or using other chromatographic reagents. The strips were sprayed with reagent, vanillin sulphuric and Anisaldehyde-sulphuric reagent.

2.5.1.2 Visualizing Reagents

(a) **Reagent Vanilin-Sulpuric acid-for detection of terpenoids**

1.5% by volume of concentrated sulphuric acid was added to a mixture of 1% vanillin in absolute ethanol. The TLC plates were heated after spraying of this reagent to develop colours.

(c) Reagent Anisaldehyde-Sulfuric acid-to detect flavonoid and volatile oil

Reagent is prepared by mixing 0.5ml of anisaldehyde with 10ml of glacial acetic acid followed by the addition of 85ml methanol and 5ml of concentrated sulphuric acid.

(d) Reagent Potassium Hydroxide-to detect quinon and quomarin

This reagent is also known as 5% ethanolic KOH. It is prepared by mixing 5g KOH with 100ml ethanol.

2.6 Column Chromatography

The leaves extracts were fractioned by column chromatography on silica gel 60 (Merck, Art. 7734, 70-230). The gel was made into slurry by different solvents depending on the solvents used to extract the leaves.

Table 2.3: Solvent System

Plant Extract	Solvent system
Hexane	• chloroform : hexane (80:20) • 100% chloroform
Methanol	• toluene : ethylacetate (70 : 30) • chloroform : petroleum ether (90 : 10)

The column used was glass column sizes 3 x 70cm and 2 x 60cm. Then, each packed column was left to equilibrate for 12 hours before used. The flow rate of the solvent systems was regulated manually by means of a stopcock at the rate of approximately 5ml/min. The eluates were collected and grouped into a series of fractions after screening by TLC.

2.7 High Performance Liquid Chromatography (HPLC) Analysis

1g of dried powdered sample was extracted overnight in 10ml HPLC grade methanol (Fisher Scientific). Water used was also HPLC grade and acidified to pH 3.0 with phosphoric acid. Qualitative analysis was made in step gradient mode, with the proportion of methanol and water 1:1 (analysed from 0 to 10 minutes) and 7:3 (analysed from 10 to 20 minutes) at a flow rate of 1mL/min. The injection volume was 10µL and the eluate was monitored at 250, 350 and 339nm. The filtered methanol extract was injected in Thermo ODS Hypersil C18 column (250 x 4.6 mm, 5µm).

2.8 GCMS Analysis

Fresh leaves of *C. odorata* was collected, cleaned and immersed into HPLC grade solvents of hexane, chloroform and petroleum ether. This is to ensure that the essential oil is not oxidized. The leaves were extracted in the solvents for 12 days. Then, they are filtered three times using Whatman (No. 1) filtering paper. Before injecting the sample into the GCMS, the samples were evaporated to approximately 5ml for each solvent. The sample was injected into the GCMS column using vial containing chloroform. One drop of each sample is mixed with approximately 3ml of HPLC grade chloroform. The GCMS machine used was Shimadzu QP-5000 combined with Shimadzu GC-17A**.**

2.9 Biological Activities Evaluations

2.9.1 Toxicity Evaluation

2.9.1.1 Acute Oral Toxicity Evaluation (Chan *et al*., 1982; Delporte *et al*., 2003)

Acute oral toxicity test was done according to Chan *et al*., 1982 and Delporte *et al*., 2003, with few modifications. The animals used were white albino mice, bought from the Institute of Medical Research (IMR). 0.05 ml of water extract of *C. odorata* was orally dosed using a feeding syringe to 25g fasted mice. The mice were given the maximum dosage of 2000 mg/kg of the leaves extract and 0.9% of saline was used as control.

2.9.1.2 Dermal Irritation Evaluation (Martin, 1982; Ilham *et al*., 1995)

Dermal irritation evaluation was done according to Martin, 1982 and Ilham *et al*., 1995 with few modifications and the OECD Guidelines for testing of chemicals (Guidelines number 404: Acute Dermal Irritation/Corrosion, Paris Cedex, May 12, 1981). The animals used were white albino mice, bought from Institute of Medical Research (IMR). 0.5 ml of water extract of *C. odorata* was used directly on $6cm^2$ of the shaven dorsal abdominal skin. The hair was carefully shaven to ensure that there was no abrasion and injury on the skin. Saline (0.9%) was also included as control negative.

The animals were separated in to two groups (n=3 for each group) and were stabilized for 24 hours after shaving prior to application of the substance. Then, any signs of colour changes or blister appearance on the skin were checked. Also, mortality and any abnormal responses were observed, such as, spontaneous activity, writhing, convulsion, gasping, hind limb placing, positional and traction. Irritation measured

according to Draize scale. Mortality and visible abnormal responses were observed through out the 14 days of evaluation.

Table 2.4: Characterization of erythema, eschar and oedema

Condition	Characteristic
Erythema	Erythema is an abnormal redness of the skin due to dilation of redness of superficial capillaries of the skin causing inflammation.
Oedema	Abnormal accumulation of fluid beneath the skin is known as oedema. This leads to puffy appearance often to a limb, most commonly the leg.
Eschar	Sloughed off dead tissues, caused by burn or cauterization.

2.9.2 Antimicrobial Activity Evaluation

2.9.2.1 Test organisms

The antimicrobial screenings consist of four bacterias and one fungi as test organisms. All species of the bacteria were obtained as clinical isolates from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya except for *Klebsella pneumonia*, which was obtained as laboratory isolates from the Department of Genetics and Cellular Biology, Faculty of Science, University of Malaya. The yeast sample was also obtained as clinical isolates from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya. All the organisms represent the different existing groups of human pathogenic bacteria and fungi as listed in Table 2.5.

Table 2.5: Test Organisms for Antimicrobial Activity Evaluation

Group	Species
Gram positive bacteria	*Staphylococcus aureus* ATCC 252593 *Staphylococcus aureus* ATCC 29213
Gram negative bacteria	*Escherichia coli* ATCC25922 *Escherichia coli* ATCC35213 *Klabsiella pneumonia* *Pseudomonas aeruginosa*
Yeasts	*Candida albicans*

2.9.2.2 Media for Microbial Cultivation and Maintainace

Four types of culture media were used (Table 2.6). Each medium was prepared to manufacturers' specification and adjusted to its appropriate pH before sterilized by autoclaving at 121ºC for 15 minutes. Then, about 20 ml of sterile agar media was poured into sterile petri dishes. The broth or liquid media were distributed to final containers before autoclaving.

Table 2.6: Culture Media for Microbial Growth

Medium	Purpose	Preparation
Mueller Hinton Agar (OXOID)	Semi-quantitative antibacterial test	38 g was suspended in 1 liter of distilled water and brought to boil to dissolve completely. pH = 7.4 ±2
Mueller Hinton Broth (OXOID)	Bacterial cultivation and quantitative antibacterial test	21g was dissolve to 1 liter of distilled water and distributed to final containers. pH 7.4 ±2
Brain Heart Agar	Fungal cultivation and maintaince. Semi quantitative antifungal test.	52g was suspended in 1 litre of distilled water and brought to boil to dissolve completely. pH: 7.4 ± 0.2
Brain Heart Broth	Fungal cultivation and quantitative fungal test.	37g was dissolve to 1 liter of distilled water and distributed to final containers. pH 7.4 ±2

2.9.2.3 Inoculums for Antimicrobial Evaluation

Cultures of bacteria were prepared one hour before antibacterial test were being done. One colony of each bacteria tested was mix in 5ml sterilized Mueller Hinton Broth. The density of each inoculum was standardized using the 0.5 McFarland standards. The concentrations of the resultant suspensions of bacteria are approximately 10^8 cells/ml.

Conidial suspensions of the test fungi were also prepared one hour before the antifungal test. The tested fungi was also standardized using McFarland but then diluted

to 10^7cells/ml by adding 225ml of sterile Brain Heart broth to 25 ml of suspensions of the test fungi.

2.9.2.4 Paper Disc Diffusion Assay (Bauer *et al*., 1966; Brown and Blowers, 1978)

The bacterial suspension (refer table 2.5) were evenly spread on the surface of 4 mm thickness of Mueller Hinton Agar (MHA) plates and the fungi suspensions on Brain Heart Agar (BHA). Sterile cotton swabs were used to produce uniform growth organisms and Kirby-Bauer swabbing technique was used.

Petroleum ether, chloroform and methanol extracts of the leaves of *C. odorata* was used as test extracts. Concentrations used for each solvent were 100, 200, 300, 400, 500, 600, 700 and 800mg/ml. Each extract was then loaded on a 6mm diameter disc (Whatman No.1 filter paper). Each disc was loaded with 10µl of extract and then evaporated. After evaporation of the solvents, the discs were placed in good contact on the seeded agar plates.

Chloramphanicol and 5-fluorocystina with the concentration of 1 mg/ml was used as reference antibiotic for bacteria and fungi respectively. Empty disc and disc with solvents were also used as negative references.

Incubation was done at 37˚C for 24 hours. Each extract and control was employed in triplicate for each organism. The diameter of clear zones produced around the discs (if present) was measured after the incubation time.

2.9.3 Anti-Inflammatory Studies (Suleyman *et al.*, 1999); Suleyman and Emin, 2001; Suleyman *et al*., 2002)

2.9.3.1 Formaldehyde-Induced Paw Edema in Rats

White albino mouse (120±2g) was bought from Institute of Medical research (IMR). Female and male mouse were separated in different cages and acclimatized to the new environment for one week. Before anti-inflammatory evaluation, the animals were fasted for 12 hours. Aqueous extract of *C. odorata* was used in doses of 50, 100, 300, 500 and 1000mg/kg. Tween 80 was used as vehicle to dilute the plant extracts. Saline water with Tween 80 and 10mg/kg of Indomethacine were included as control negative and positive respectively. The animal was separated into seven groups, according to different extract concentrations, saline solution and Indomethacine drug. Each group consisted of three animals.

Next, each mouse was administered orally with the extracts using a feeding syringe. Before the injection of formaldehyde, the hind paw of each mouse was measured. One hour after the final drug administration, formaldehyde, 0.2ml (1% w/v) was subcutaneously injected into the plantar surface of the right hand paw. The volumes of the hind paws were measured after injection at third, sixth and 24^{th} hour, till the inflammation disappeared (original paw volume obtained). The edema was expressed as a percentage of pre-injection right paw volume. Finally the anti-inflammatory effects of animals that received *C. odorata* extracts were compared with those in the control group and indomethacine administered group.

The ratio of anti-inflammatory activity was calculated by the following equation : anti-inflammatory activity (%)=(1-D/C) x 100, Where D represents the percentage of difference of the paw volume after the administration of extract and C represents the percentage difference of the paw volume in the control group

2.9.4 Blood Coagulation Evaluation (Mary *et al*., 2003)

Fresh blood from rats was used for this evaluation. First, 10 drops of fresh blood was dropped onto a white plate. Then, leaves extracts of *C. odorata* was added on the blood. PE, $CHCL_3$ and MEOH extract was used at the concentration of 100, 300 and 500mg/ml each. Saline, PE, $CHCL_3$ and MEOH solvents were used as control. The time for the blood drop to coagulate was recorded and compared between solvents, saline water and leaf extracts. This evaluation was done in triplicate.

2.10 Dermal Wound Healing Evaluation (Villegas *et al*., 1997; Rashed *et al*., 2003; Gomez-Beloz *et al*., 2003)

White albino mice (weighed 25g) from University Malaya animals' house were used. Before the wound incision was made, the hair on the dorsal back of each animal was removed by shaving and ethanol (70%) was used as an antiseptic. Then, the animals were fainted using diethyl ether. An incision wound was made on the dorsal side of each mouse vertically by using a shaving razor with the length of 10mm on the skin from a predetermined shaved region. The wound was left undressed to the open environment and no local or systemic anti-microbial agents were used. The mice were distributed in groups randomly and each mouse was placed in separate cage.

After that, aqueous leaf extract of *C. odorata* was applied on the wound of each animal using a cotton bud. The concentration used was 100, 300 and 500mg/ml. Saline water (0.9%) and Acriflavine lotion was also included as negative and positive control respectively. Observation was done for 16 days, till the last animal healed from the wound. Inflammation, scabbing, scarring and animal behaviour were checked and recorded throughout the evaluation.

Chapter Three

RESULTS AND DATA ANALYSIS

CHAPTER 3

RESULTS AND DATA ANALYSIS

3.1 Phytochemical Studies

3.1.1 Preliminary Evaluation

The preliminary evaluation was done using aqueous leaves extract of *C. odorata*. Each drop of the extract was added into test tubes containing 1ml of the dragendorf, Meyer's, potassium, anisaldehyde, vanillin and ninhydrin reagents respectively. The observation of this evaluation is based on the changes of colour of each reagent. The colour changes was then compared to standard observation of the reagent (please refer to Table 2.1, page 26).the observation for the aqueous leaves extract of *C. odorata* is shown in Table 3.1.

Table 3.1: Observation of Preliminary Evaluation

	Reagent	Observation	Remark
1.	Dragendorff	Orange with precipitate	Presence of alkaloid
2.	Meyer's	White with precipitate	Presence of alkaloid
3.	Potassium	No changes	No presence of quinone
4.	Anisaldehyde	dark green	Presence of essential oil
5.	Vanillin	Brown	Presence of flavonoid
6.	Ninhydrin	No changes	No presence of amino acid

From Table 3.1, it is known that there was presence of flavonoid, alkaloid and essential oil in the crude extract of *C. odorata*. But presences of amino acid or quinone were not detected in the crude extract.

3.1.1.2 Colour Evaluation

The colour evaluation was done by adding 10 drops of each of the prepared solvent (as listed in Table 3.2) to 5mg of the blended leaves sample of *C. odorata*. Again, changes of colour of the blended sample of the leaves of *C. odorata* was observed and recorded. The changes of colour was compared to standard observation of each of the solvents, according to Table 2.2 (please refer page 27). The observation of the colour evaluation is as shown in Table 3.2.

Table 3.2: Results of Colour Evaluation

Solvent	Observation of colour	Remark
H_2SO_4	Green to dark brown	Positive presence of terpenoid, phenilpropane etc
HCl	Green to yellowish green	Positive presence of antocyanin and flavonoid
KOH	Green to bright greenish yellow	Negative presence of presence quinone
$FeCl_3$	Dark green	Positive presence of phenolic substances and tannin

According Table 3.2, it is known that there are presences of terpenoid, flavonoid and phenolic substances in the blended leaves sample of *C. odorata*. It is also known that there is no presence of quinone.

3.1.2 Compound Identification of the Crude Extract

3.1.2.1 Gas chromatography Mass Spectrum (GCMS) Analysis

The crude Petroleum ether, hexane and chloroform extracts of *C. odorata* were subjected to GCMS analysis. GCMS analysis was performed on Hewlett–Packard Model 6890 combined with gas chromatography of Hewlett-Packard Model 6890. The isolation of compound was done by column chromatography. The solvent extraction of the fresh leaves was done in 12 days. The extracts were then filtered thrice to ensure that there was no presence of impurities. After that, the extracts were evaporated to 10ml and only compounds with SI (Standard Index) higher than 80% were recorded, so that accuracy was ensured.

The GCMS analysis of the essential oil from the hexane extraction had detected three compounds which are caryophyllene **(20)**, germacrene D **(21)** and hexadecoic acid **(24)**. Meanwhile, the petroleum ether extraction detected three compounds which are germacrene D **(21)** and hexadecanoic acid **(24)**. Lastly, the chloroform extract detected only one compound and that was germacrene D **(21)**. It was noticed that the compound Germacrene D **(21)** was found in all three solvent extractions. The compounds detected by the GCMS analysis are shown in Table 3.3.

Table 3.3: Detected Compounds of GCMS Analysis

Extraction solvent	Peak No.	SI (%)	Compound (Name and Synonym)	Mol. form	Mol. Weight (g/mol)
Hexane	7	89	• Germacrene D • 1, 6-cyclodecadine, 1-methyl05methylene-8methylethyl	$C_{15}H_{24}$	204
	14	94	• Hexadecanoic acid • Pentadecanecarbocyclix acid • Palmitic acid	$C_{16}H_{32}O_2$	256
	18	90	• Caryophyllene	$C_{15}H_{24}$	206
Petroleum ether	8	91	• Hexadecanoic acid • Pentadecanecarbocyclix acid • Palmitic acid	$C_{16}H_{32}O_2$	256
	11	87	• Germacrene D • 1, 6-cyclodecadine, 1-methyl05methylene-8methylethyl Germacrene D	$C_{15}H_{24}$	204
Chloroform	5	88	• Germacrene D • 1, 6-cyclodecadine, 1-methyl05methylene-8methylethyl	$C_{15}H_{24}$	204

3.1.2.2 HPLC Analysis

The HPLC system consisted of a pump and a diode array detector. The system was controlled by Shimadzu LC Solution. The column used was thermo ODS hypersil C18, sized 250 x 4.6 mm with 5µm particle size. Solvents used were HPLC grade bought from Fisher Scienctific. Water was also HPLC grade and acidified to pH 3.0 with phosphoric acid. The crude extract of *C. odorata* was extracted from methanol.

The qualitative analysis was made in step gradient mode, with the ratio of methanol: water, 1:1 (0 to 10 minutes) and 7:3 (10 to 35 minutes) at a flow rate of 1mL/min. The injection volume was 10 µl and the eluate was monitored at 250nm, 350nm ad 339 nm. Identification of the compound detected was done by comparison with Ling *et al*., 2007a. The HPLC analysis was able to detect five peaks and is shown in Table 3.4.

Table 3.4: Compound Detected by HPLC Analysis

Peak no.	Retention time (Minute)	Compound detected
17	15.71	Quercetin-4 methyl ether
18	16.23	Aromadendrin-4'-methyl ether
20	17.20	Taxifolin-7-methyl ether; taxifolin-4'-methyl ether and quercetin-7-methyl ether
22	19.92	Kaempferol-4'-methyl ether and eridicytol-7, 4'-dimehyl ether
23	21.19	Quercetin-7,4'-dimethyl ether

3.1.3 Column Chromatography

The methanol crude extracts of *C. odorata* were subjected to column chromatography. The column sizes used were 2 x 60cm, 3x 70cm and 5 x 90cm. For each sample, the concentration used was 3mg of extract for 50 g of silica gel (Merck, F_{124}). Meanwhile, the fractions were collected 5ml for each eluant vial. Then, fractions collected were grouped in series of fractions after monitoring with TLC and the Anisaldehyde-sulphuric spray reagent. Each series were then treated separately to isolate and purify the compounds present by TLC.

The petroleum extract (column size, 2x 60 cm) yield 39 vials of eluant with 18 vials that showed single compound, tested through TLC and the anisaldehyde spray reagent. The colour observed was purple (6 vials), red (8 vials), yellow (2 vials), orange (1 vial) and blue (1 vial). Initially, this showed that there are presence of essential oil, flavonoid and terpenoid.

For the column size of 3 x70cm (also the petroleum extract), 85 vials were yield and 20 vials showed single compound through TLC. The anisaldehyde spray reagent showed 5 vials of purple coloured compound and 32 vials of red coloured compound. Preliminary, these showed that there are presences of terpenoid and flavanoid compounds. But no pure crystal was obtained from all the eluant collected.

The chloroform extracts on the other hand, showed presence of flavanoid, terpenoid and essential oil. But no pure compound crystal was yield. The 2 x 60cm column yields 39 vials of eluant. All eluant were tested with TLC and sprayed with anisaldehyde spraying reagent. Also for the chloroform extract, no eluant re-crystallised after evaporation.

As for the of methanol extract of *C. odorata* with the column size of 3 x 70cm, 150 vials of 1ml were collected. And 13 vials re-crystallised after evaporation. The TLC evaluation showed that there were only three different single compounds. Therefore, the compounds which are the same were combined together. Thus, three different single compounds were isolated and labelled as compound A, compound B and compound C. Evaluation with the Anisaldehyde spraying reagent also proved that the compounds were flavonoids. Evaluation of the pure flavonoid compounds was continued with structure elucidation of the three crystal obtained.

3.1.4 Compound Elucidation

3.1.4.1 Compound A

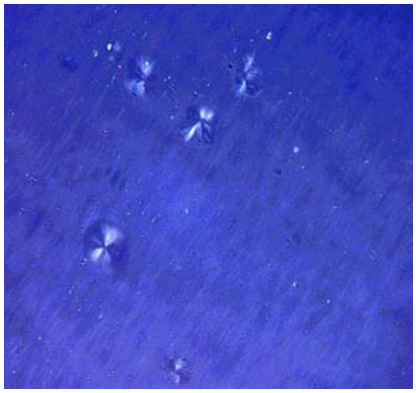


Figure 3.1: Compound A

Compound A was seen as crystals at room temperature with fan shaped crystals (seen as white spots on Figure 3.1) and in homeotropic alignment. Its melting point was recorded at 71ºC. Mass [M^+]: 284; IR (KBr) cm^{-1}: 3426 (OH), 2961 (OCH_3), 2855, 2726, 1764,1714 (C=O), 1539, 1449, 1376, 1309, 1207, 1162, 1087, 1019; ^{1}HNMR (400MHz, DMSO) δ: 83.87 (3H, s, OCH3-4'), 6.18 (1H, d, H3'), 6.17 (1H, d, H5'), 6.38 (1H, d, H6'), 6.39 (1H, d, H2'), 11.91 (1H, s, OH-5), 7.03 (1H, d, H6), 7.7 (1H, d, H8) ; ^{13}CNMR δppm : 168.52 (C-2), 165.69 (C-3), 177.42 (C-4), 147.39 (C-5), 147.47 (C-5a), 125.45 (C-6), 137.61 (C-7), 130.68 (C-8), 147.39 (C-8a), 94.43 (C-1'), 99.28 (C-2'), 112.2 (C-3'), 121.48 (C-4'), 115.66 (C-5'), 104.55 (C-6').

3.1.4.2 Compound B

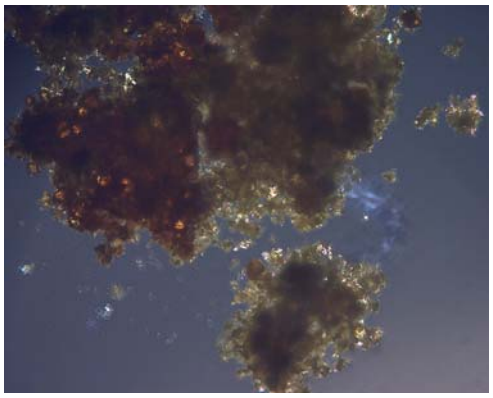


Figure 3.2: Compound B

Compound B was seen as granule shaped crystals at room temperature. Its melting point was recorded at 184ºC. EIMS m/z (% intensity): 330 [M^+] (6), 314 (6), 285 (6), 274 (6), 153 (6), 128 (6), 69 (6) ; IR (KBr) cm^{-1}: 3307 (OH), 2917 (OCH_3), 1654 (C=O), 1617,1594, 1556, 1510, 1441, 1372, 1338, 1305, 1258, 1235, 1201, 1164, 1111, 1090, 1022; ^{1}HNMR (400MHz, DMSO) δ: 3.3 (1H, d), 3,89 (3H, s, OCH_3-7), 3.3 (3H, s, OCH_3-3'), 3.03 (2H, dd), 11.95 (1H, s, OH-5), 6.3 (1H, d, H-6), 6.1 (1H, d, H-8), 7.03 (1H, d, H-2'); ^{13}CNMR δppm: 165.62 (C-2), 162.52 (C-3), 177.42 (C-4), 150.73 (C-5), 125.41 (C-6), 137.62 (C-7), 130.72 (C-8), 94.50 (C-1'), 99.35 (C-2'), 112.25 (C-3'), 121.62 (C-4'), 115.72 (C-5'), 104.67 (C-6').

3.1.4.3 Compound C

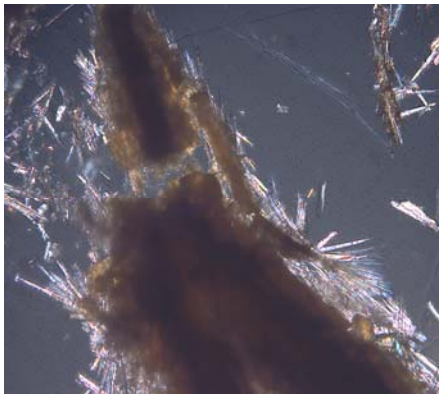


Figure 3.3: Compound C

Compound C was seen as yellow needlelike crystal at room temperature. Its melting point is recorded at 202ºC. EIMS m/z (% intensity): 344 [M^+] (11), 180 (11), 166 (11), 122 (11) ; IR (KBr) cm^{-1}: 3436 (OH), 3286, 3119, 2917 (OCH_3), 2848, 1369 (C=O), 1615, 1511, 1465, 1402, 1370, 1309, 1292, 1250, 1170, 1139 ; ^{1}HNMR (400MHz, DMSO) δ:3.8 (3H, s, OCH_3 -7), 3.86 (3H,s, OCH_3 -3'), 3.88 (3H, s, OCH_3 -4'), 6.18 (1H, d, H-8), 7.0 (1H, d, H-6), 7.03 1H, d, H-2'), 7.4 (1H, d, H-3').

3.2 Toxicity Evaluation

3.2.1 Dermal Irritation Evaluation

The mice were gauzed with equivalent dosage of 2000mg aqueous leaves extraction/kg body weight. For the control, the animal was gauzed with 0.9% of saline solution. The area of evaluation is on the dorsal abdominal skin of the mice. The area was shaven and checked for any injury or abrasion before the evaluation was started.

After the application of both the leaves extract of *C. odorata* and the control of saline solution, any abnormal skin irritation was observed and characterisation of the skin irritation is as describe in Table 2.4 (please refer page 32). Besides that, abnormal responses were also observed and the mortality of all the animals. Observation is shown in Table 3.5.

Table 3.5: Observation of the Dermal Irritation Evaluation

Observation	**Treated**			**Control**		
(Female mice 25± 2g)	**1**	**2**	**3**	**1**	**2**	**3**
Increased motor activity	N	N	N	N	N	N
Tremor	N	N	N	N	N	N
Chronic convulsion	N	N	N	N	N	N
Tonic extension	N	N	N	N	N	N
Muscle spasm	N	N	N	N	N	N
Lose of righting reflex	N	N	N	N	N	N
Decreased motor activity	N	N	N	N	N	N
Ataxia	N	N	N	N	N	N
Sedation	N	N	N	N	N	N
Analgesia	N	N	N	N	N	N
Hypnosis	N	N	N	N	N	N
Anaesthesia	N	N	N	N	N	N
Arching and rolling	N	N	N	N	N	N
Ptosis	N	N	N	N	N	N

Table 3.5: Continued

Salivation	N	N	N	N	N	N
Writhing	N	N	N	N	N	N
Breathing depression	N	N	N	N	N	N
Breathing stimulation	N	N	N	N	N	N
Breathing failure	N	N	N	N	N	N
Muscle relaxation	N	N	N	N	N	N
Piloerection	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N
Blister appearance	N	N	N	N	N	N
Colour changes for skin	N	N	N	N	N	N

Legend: N = Normal ; A = Abnormal

Table 3.6: Grading of Skin Reactions

Erythema and Eschar Formation	**Grade**
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4
Oedema Formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Slightly oedema (edges of area well defined rising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm) and extending beyond area of exposure	4

Table 3.7: Skin Reaction on Dermal Irritation Test Mice No.1

Mice no.1 (treated)	**Weight (Kg)**	**Hours**								**Remarks**
		1	**24**	**48**	**72**	**96**	**120**	**144**	**168**	
Erythema and Eschar Formation	25± 2	0	0	0	0	0	0	0	0	Normal, no changes observed.
Oedema formation	25± 2	0	0	0	0	0	0	0	0	Normal, no changes seen.

Table 3.8: Skin Reaction on Dermal Irritation Test Mice No.2

Mice no.2 (treated)	Weight (Kg)	Hours								Remarks
		1	24	48	72	96	120	144	168	
Erythema and Eschar Formation	25± 2	0	0	0	0	0	0	0	0	Normal, no changes observed
Oedema formation	25± 2	0	0	0	0	0	0	0	0	Normal, no changes observed

Table 3.9: Skin Reaction on Dermal Irritation Test Mice No.3

Mice no.4 (Control)	Weight (Kg)	Hours								Remarks
		1	24	48	72	96	120	144	168	
Erythema and Eschar formation	25± 2	0	0	0	0	0	0	0	0	Normal, no changes observed
Oedema formation	25± 2	0	0	0	0	0	0	0	0	Normal, no changes observed

Table 3.10: Skin Reaction on Dermal Irritation Test Mice No.4

Mice no.4 (Control)	Weight (Kg)	Hours								Remarks
		1	24	48	72	96	120	144	168	
Erythema and Eschar formation	25± 2	0	0	0	0	0	0	0	0	Normal, no changes observed
Oedema formation	25± 2	0	0	0	0	0	0	0	0	Normal, no changes observed

Legend of severity:

1 = very slight

2 = well define

3 = moderate

4 = severe

3.2.2 Acute Dermal Irritation Evaluation

Acute dermal irritation evaluation was done on white albino mice. This evaluation used the maximum dosage of 2000mg/kg of the aqueous extraction of *C. odorata*, directly on the shaven skin of the dorsal side of each mouse. While saline (0.9%) was used as control. Observation was then made on the first hour and continuously until the seventh day. Any signs of colour changes, blister appearance or inflammation on the skin were checked. The result of the acute dermal irritation evaluation is shown in Table 3.11.

Table 3.11: Acute Dermal Irritation and Inflammation Test of Anique MMF

No. of mice	**Average weight (Kg)**	**Dosage**	**Observed dermal irritation and inflammation**								**Remarks**
			1	24	48	72	96	120	144	168	
3 Female (treated)	25± 2	2000mg/ kg	0	0	0	0	0	0	0	0	Normal, no changes observed
1Female (control)	25± 2	2000mg/ kg	0	0	0	0	0	0	0	0	Normal, no changes observed

The dermal irritation and acute dermal irritation evaluation showed that the extract of *C. odorata* did not cause any deterioration on the skin of the mice. No erythema, eschar or oedema was formed. Also, throughout the entire evaluation, there were no changes of behaviours observed on the tested animal. All the animals reacted normally until their fur grows back on the shaved skin. The maximum dosage was given to each animal and no negative effects were seen.

3.2.3 Oral Toxicity Evaluation

Each mouse was dosed once orally with equivalent dosage of 2000 mg/kg body weight, i.e. 0.05 ml per animal.

Table 3.12: Observation of Oral Toxicity Evaluation on Female Mice

Observation **Female mice 25±2g**	**Treated**			**Control**		
	1	**2**	**3**	**4**	**5**	**6**
Increased motor activity	N	N	N	N	N	N
Tremor	N	N	N	N	N	N
Chronic convulsion	N	N	N	N	N	N
Tonic extensor	N	N	N	N	N	N
Piloerection	N	N	N	N	N	N
Muscle spasm	N	N	N	N	N	N
Loss of righting reflex	N	N	N	N	N	N
Decreased motor activity	N	N	N	N	N	N
Ataxia	N	N	N	N	N	N
Sedation	N	N	N	N	N	N
Analgesia	N	N	N	N	N	N
Hypnosis	N	N	N	N	N	N
Anaesthesia	N	N	N	N	N	N
Arching and rolling	N	N	N	N	N	N
Ptosis	N	N	N	N	N	N
Salivation	N	N	N	N	N	N
Writhing	N	N	N	N	N	N
Breathing depression	N	N	N	N	N	N
Breathing stimulation	N	N	N	N	N	N
Breathing failure	N	N	N	N	N	N
Muscle relaxation	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N

Note : Mice remained active during the whole experiment

N = Normal A = Abnormal

Table 3.13: Observation of Oral Toxicity Evaluation on Male Mice

Observation **Male mice 25±2g**	**Treated**			**Control**		
	1	**2**	**3**	**4**	**5**	**6**
Increased motor activity	N	N	N	N	N	N
Tremor	N	N	N	N	N	N
Chronic convulsion	N	N	N	N	N	N
Tonic extensor	N	N	N	N	N	N
Piloerection	N	N	N	N	N	N
Muscle spasm	N	N	N	N	N	N
Loss of righting reflex	N	N	N	N	N	N
Decreased motor activity	N	N	N	N	N	N
Ataxia	N	N	N	N	N	N
Sedation	N	N	N	N	N	N
Analgesia	N	N	N	N	N	N
Hypnosis	N	N	N	N	N	N
Anaesthesia	N	N	N	N	N	N
Arching and rolling	N	N	N	N	N	N
Ptosis	N	N	N	N	N	N
Salivation	N	N	N	N	N	N
Writhing	N	N	N	N	N	N
Breathing depression	N	N	N	N	N	N
Breathing stimulation	N	N	N	N	N	N
Breathing failure	N	N	N	N	N	N
Muscle relaxation	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N

Note : Mice remained active during the whole experiment.

N = Normal A = Abnormal

3.2.3 Acute Oral toxicity Evaluation

The toxicity test and acute toxicity test were done on both male and female mice. The mice were fed orally using a feeding syringe water extract of *C. odorata* and at the maximum dosage of 2000mg/kg. Whereas the control mouse was given distilled water only. Then, observation was done on both behaviour and mortality of all the mice for 14 days.

Table 3.14: Mice Acute Oral Toxicity Test Aqnique MMF

No. of mice	Average Weight (g)	Dosage (mg/kg)	Mortality/ no. of animals (Hrs)			Remarks
			24	48	96	
3 Females	25± 2	2000	N	N	N	No changes
Female (control)	25± 2	0	N	N	N	No changes
3 Males	25± 2	2000	N	N	N	No changes
Female (control)	25± 2	0	N	N	N	No changes

Table 3.14 shows that throughout the evaluation all the mice survived and did not show any changes in their behaviour or any deterioration in health. Both male and female showed the same results. No mortality was observed throughout 14 days after dosing.

3.3 Pharmacological Activities

3.3.1 Anti-microbiology Evaluation

3.3.1.2 Disc diffusion Evaluation

Extracts of *C. odorata* were tested at various concentrations, ranging from 100mg/ml to 800mg/ ml. Samples were extracted from three solvents which are petroleum ether, chloroform and methanol. Bacteria strains used were *Staphylococcus aureus* ATCC 25293 (SA 25293) *Staphylococcus aureus* ATCC 25923 (SA 25923), *Erischia cocci* ATCC 25922 (EC 25922) *Erischia cocci* ATCC 35213 (EC 35213), *Klebsila pneumonia* (KP) and *Pseudomonas aeruginosa* (PA).

The extracts of *C. odorata* were loaded to 6mm diameter disc. The discs were prepared by using Whatman No. 1 paper and then sterilised. After sterilisation the discs were loaded with different concentration of the extract of *C. odorata*. The discs were loaded with 10µl of leaves extracts in three separate portions so that the discs absorb all fluid. The discs were then left to evaporate in a sterile environment.

Culture media was prepared according to manufacturer's specification (please refer page 34). Then, about 20ml of the sterile agar media was poured into sterile Petri dishes. The broths were distributed to final containers before autoclaving at 121°C for 15 minutes.

Cultures of bacteria were prepared one hour before the evaluation was started. One colony of bacteria tested was mixed in 5ml of sterilesed Mueller Hinton broth. The density of each inoculums was standardised according to McFarland 0.5 standards.

The bacterial suspensions were evenly spread on the surface of 4mm thickness of the Mueller Hinton Agar plates. Sterile cotton swabs were used to produce uniform growth organisms and Kirby-Bauer swabbing technique was used.

Each Petri dish was divided into four sections according to different concentration of the loaded discs. Each section was labelled and then placed with the different concentration of prepared discs. The discs were placed in good contact on the seeded agar plates. This method was repeated three times for each bacterial suspension. The results for disc diffusion evaluation of the *C. odorata* extracts were compared to the standard antibiotic of Chloramphenicol with the concentration of 1mg/ml. The observation of the antibacterial activities is shown in Table 3.15 to 3.20 and Figure 3.1 to 3.3.

Table 3.15: Antibacterial Activity of Methanol Extract of *C. odorata*

Extract conc. (mg/ml)	**Diameter of inhibition zone (mm)**					
	SA 25293	SA 29213	EC 25922	EC 35213	KP	PA
100	4±2	0	0	0	0	11±2
200	4±2	2±2	0	0	0	11±2
300	5±2	2±2	0	0	0	13±2
400	5±2	2±2	0	0	0	13±2
500	6±2	2±2	0	0	0	15±2
600	8±2	2±2	0	0	0	16±2
700	8±2	2±2	0	0	0	16±2
800	9±2	3±2	0	0	0	16±2

Inhibition strength indicator**:**

1) Diameter of inhibition zone ≥ 15 mm: Strong
2) Diameter of inhibition zone 10 mm -14.5 mm: Medium
3) Diameter of inhibition zone ≤ 9 mm: Weak
4) Diameter of inhibition 0 mm: No activity

Table 3.16: Antibacterial Activity of Chloroform Extract of *C. odorata*

Extract conc. (mg/ml)	**Diameter of inhibition zone (mm)**					
	SA 25293	SA 29213	EC 25922	EC 35213	KP	PA
100	-	0	0	0	0	14±2
200	4±2	0	0	0	0	14±2
300	4±2	0	0	0	0	15±2
400	4±2	0	0	0	0	15±2
500	6±2	2±2	0	0	0	15±2
600	6±2	2±2	0	0	0	15±2
700	6±2	4±2	0	0	0	15±2
800	6±2	4±2	0	0	0	15±2

Inhibition strength indicator**:**

1) Diameter of inhibition zone ≥ 15 mm: Strong
2) Diameter of inhibition zone 10 mm -14.5 mm: Medium
3) Diameter of inhibition zone ≤ 9 mm : Weak
4) Diameter of inhibition 0 mm : No activity

Table 3.17: Antibacterial Activity of Petroleum Ether Extract of *C. odorata*

Extract conc. (mg/ml)	**Diameter of inhibition zone (mm)**					
	SA 25293	SA 29213	EC 25922	EC 35213	KP	PA
100	0	0	0	0	0	0
200	0	0	0	0	0	0
300	0	0	0	0	0	0
400	0	0	0	0	0	0
500	0	0	0	0	0	0
600	0	0	0	0	0	0
700	0	0	0	0	0	0
800	0	0	0	0	0	0

Inhibition strength indicator**:**

1) Diameter of inhibition zone ≥ 15 mm: Strong
2) Diameter of inhibition zone 10 mm -14.5 mm: Medium
3) Diameter of inhibition zone ≤ 9 mm: Weak
4) Diameter of inhibition 0 mm: No activity

Table 3.18: Antibacterial Activity of Standard Reference

Extract conc. (mg/ml)	**Diameter of inhibition zone (mm)**					
	SA25293	SA29213	EC 25922	EC 35213	KP	PA
Empty disc	0	0	0	0	0	0
Empty disc with MeOH solvent	0	0	0	0	0	0
Chloramphenicol (1mg/ml)	27±2	26±2	30±2	24±2	30±2	24±2

Inhibition strength indicator**:**

1) Diameter of inhibition zone ≥ 15 mm: Strong
2) Diameter of inhibition zone 10 mm -14.5 mm: Medium
3) Diameter of inhibition zone ≤ 9 mm: Weak
4) Diameter of inhibition 0 mm: No activity

3.3.1.3 Anti-fungal Evaluation

Methanol, chloroform and petroleum ether extracts (concentration 100mg/ml to 800mg/ml) of *C. odorata* were tested on the fungi *Candida albicans*. However, all the extracts of *C. odorata* did not inhibit the growth of *C. albicans*. Since no inhibition was observed, test on anti fungal was stopped. This is because *C. albicans* is a very sensitive fungal. Inability to inhibit *C. albicans* means, there are no property of anti-fungal in *C. odorata.* The observations of the antifungal activity are shown in Table 3.19 to 3.22.

Table 3.19: Antifungal Activity of Methanol Extract of *C. odorata*

Extract conc. (mg/ml)	Diameter of inhibition zone (mm)					
	SA 25293	SA 29213	EC 25922	EC 35213	KP	PA
100	0	0	0	0	0	0
200	0	0	0	0	0	0
300	0	0	0	0	0	0
400	0	0	0	0	0	0
500	0	0	0	0	0	0
600	0	0	0	0	0	0
700	0	0	0	0	0	0
800	0	0	0	0	0	0

Inhibition strength indicator**:**

1) Diameter of inhibition zone ≥ 15 mm: Strong
2) Diameter of inhibition zone 10 mm -14.5 mm: Medium
3) Diameter of inhibition zone ≤ 9 mm: Weak
4) Diameter of inhibition 0 mm: No activity

Table 3.20: Antifungal Activity of Chloroform Extract of *C. odorata*

Extract conc. (mg/ml)	Diameter of inhibition zone (mm)					
	SA 25293	SA 29213	EC 25922	EC 35213	KP	PA
100	0	0	0	0	0	0
200	0	0	0	0	0	0
300	0	0	0	0	0	0
400	0	0	0	0	0	0
500	0	0	0	0	0	0
600	0	0	0	0	0	0
700	0	0	0	0	0	0
800	0	0	0	0	0	0

Inhibition strength indicator**:**

1) Diameter of inhibition zone ≥ 15 mm: Strong
2) Diameter of inhibition zone 10 mm -14.5 mm: Medium
3) Diameter of inhibition zone ≤ 9 mm: Weak
4) Diameter of inhibition 0 mm: No activity

Table 3.21: Antifungal Activity of Petroleum Ether Extract of *C. odorata*

Extract conc. (mg/ml)	Diameter of inhibition zone (mm)					
	SA 25293	SA 29213	EC 25922	EC 35213	KP	PA
100	0	0	0	0	0	0
200	0	0	0	0	0	0
300	0	0	0	0	0	0
400	0	0	0	0	0	0
500	0	0	0	0	0	0
600	0	0	0	0	0	0
700	0	0	0	0	0	0
800	0	0	0	0	0	0

Inhibition strength indicator**:**

1) Diameter of inhibition zone ≥ 15 mm: Strong
2) Diameter of inhibition zone 10 mm -14.5 mm: Medium
3) Diameter of inhibition zone ≤ 9 mm: Weak
4) Diameter of inhibition 0 mm: No activity

Table 3.22: Antifungal Activity of Standard Reference

Extract conc. (mg/ml)	Diameter of inhibition zone (mm)					
	SA25293	SA29213	EC25922	EC35213	KP	PA
Empty disc	0	0	0	0	0	0
Empty disc with Methanol solvent	0	0	0	0	0	0
5-fluorocystina (1mg/ml)	15±2	18±2	18±2	19±2	20±2	15±2

Inhibition strength indicator**:**

1) Diameter of inhibition zone ≥ 15 mm: Strong
2) Diameter of inhibition zone 10 mm -14.5 mm: Medium
3) Diameter of inhibition zone ≤ 9 mm: Weak
4) Diameter of inhibition 0 mm: No activity

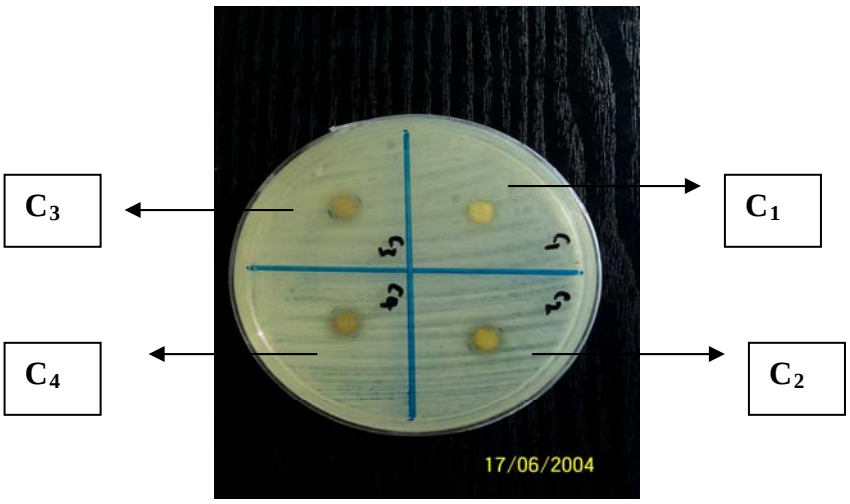


Figure 3.4: Antimicrobial activity of *S. aureus* ATCC 25293 against chloroform extract of *C. odorata* at concentration of 100mg/ml (C_1), 200mg/ml (C_2), 300mg/ml (C_3) and 400mg/ml (C_4).

Figure 3.5: Antimicrobial activity of *P. aruginosa* against chloroform extract of *C. odorata* at the concentration of 100mg/ml(C_1), 200mg/ml (C_2), 300mg/ml (C_3) and 400mg/ml (C_4).

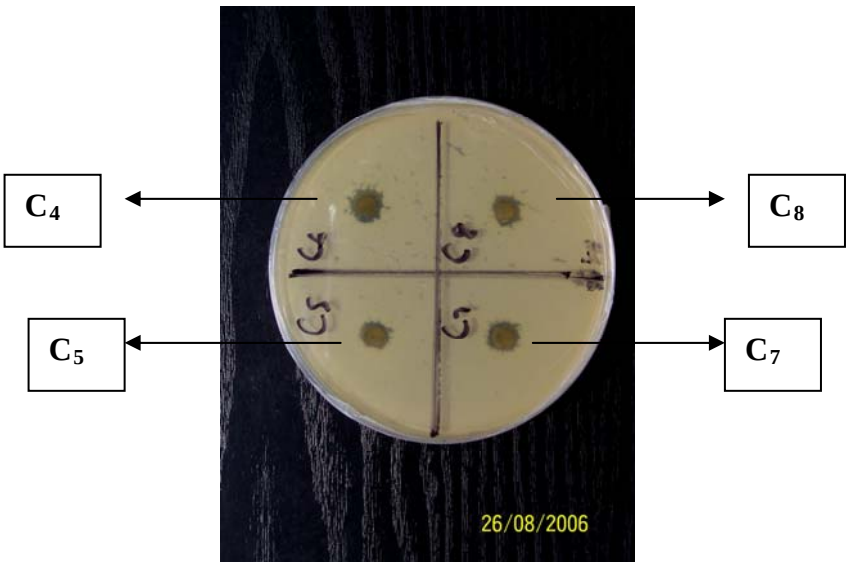


Figure 3.6 : Antimicrobial activity of *P. aruginosa* against chloroform extract of *C. odorata* at the concentration of 500mg/ml (C_5), 600mg/ml (C_6), 700mg/kg (C_7) and 800mg/ml (C_8).

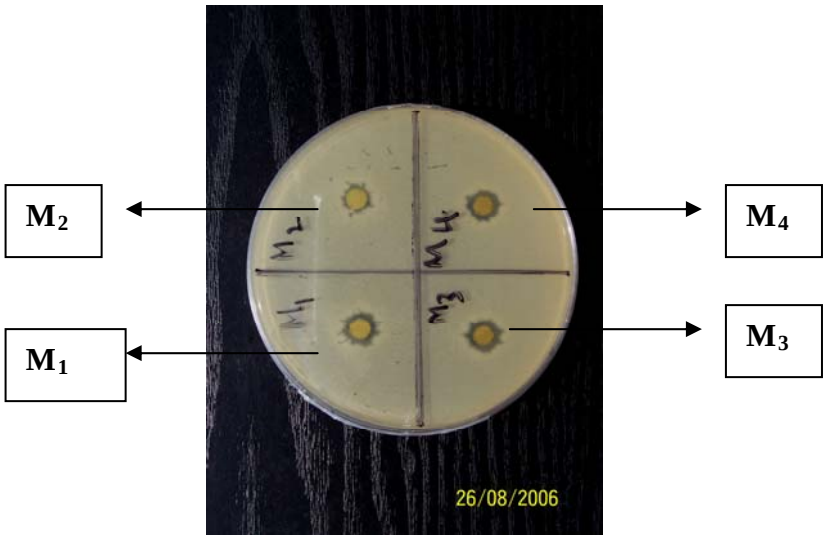


Figure 3.7: Antimicrobial activity of *P. aruginosa* against methanol extract of *C. odorata* at the concentration of 200mg/ml (M_2), 300mg/ml (M_3) and 400mg/ml (M_4).

Figure 3.8: Antimicrobial activity of *S. aureus* ATCC 29213 against methanol extract of C. *odorata* at the concentration of 100mg/ml (M_1), 200mg/ml (M_2), 300mg/ml (M_3)and 400mg/ml (M_4).

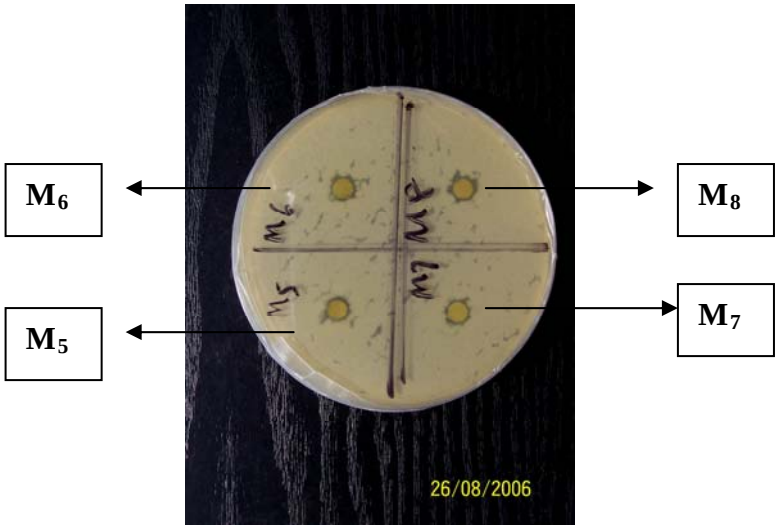


Figure 3.9: Antimicrobial activity of *S. aureus* ATCC 29213 against methanol extract of *C. odorata* at the concentration of 500mg/ml (M_5), 600mg/ml (M_6), 700mg/ml (M_7) and 800mg/ml (M_8).

3.3.2 Blood Coagulation Evaluation

The samples used were petroleum ether, chloroform, methanol and aqueous extract of *C. odorata*. The concentrations prepared were ranging from 100mg/ml to 500mg/ml. The control was drops of blood (BD) only. During the evaluation, the room temperature was 27°C. Blood was taken from adult rat.

Ten drops of fresh blood was dropped onto white plate. Then, the leaves extacts of *C. odorata* was added on the blood. Saline was used as standard control and drops of blood only (without addition of any solvents or saline) were also used as control. Observation was made immediately and time was measured using a stop watch.

According to Table 3.23, all the extracts of *C. odorata* did not increased time of blood coagulation, except for the methanol extract. The extracts were observed to prolong the coagulation time. The coagulation time was compared to the control (normal blood drop-BD), which only took 2 minutes to coagulate.

Table 3.23: Influence of *C. odorata* on Blood Coagulation Time

Extract concentration (mg/ml)	Coagulation Time (minutes)				
Solvents	PE	CHCl3	MeOH	H2O	BD
0 (solvent only)	3.00	3.21	Immediately	2.00	2 .00
100	3.77	6.19	Immediately	3.28	
300	2.94	7.19	Immediately	Negative	
500	2.94	7.82	Immediately	Negative	

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3.3.3 Anti-inflammatory Evaluation

The anti-inflammatory evaluation used the aqueous leaves extract of *C. odorata* with the concentration of 50, 100, 300, 500 and 1000mg/kg. tween 80 was used as vehicle to administered the plant extract into the mice. Also included in this evaluation were Tween 80, saline water and 10mg/kg of Indomethacine (standard drug) which were used as control.

Each mouse was administered orally with the extracts and control using a feeding syringe. And before the injection of formaldehyde (inflammatory agent), the hind paw of each mouse was measured. One hour after the final drug administration, 0.2ml (1% w/v) formaldehyde was subcutaneously injected into the plantar surface of the right hand paw. The volumes of the hind paws were measured after injection at third, sixth and 24^{th} hour, till the inflammation disappeared (original paw volume obtained). The edema was expressed as a percentage of pre-injection right paw volume. Finally the anti-inflammatory effects of animals that received *C. odorata* extracts were compared with those in the control group and indomethacine administered group.

The ratio of anti-inflammatory activity was calculated by the following equation ; anti-inflammatory activity (%)=(1-D/C) x 100, Where D represents the percentage of difference of the paw volume after the administration of extract and C represents the percentage difference of the paw volume in the control group

Table 3.24: Effects of *C. odorata* on Formaldehyde induced Paw Oedema in Rats

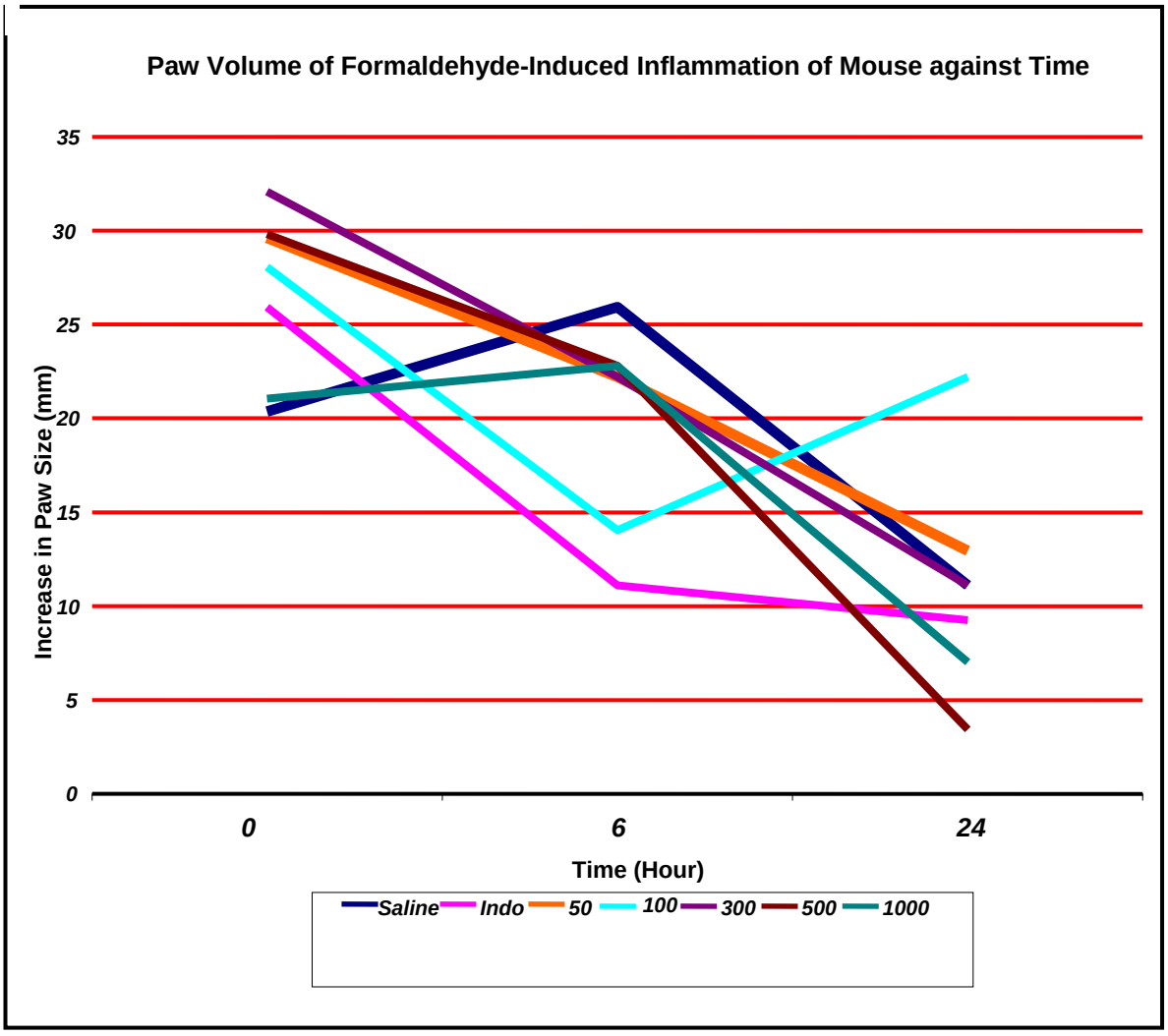
Drugs (mg/kg) / Time (hour)	Saline	Indomethacine	50	100	300	500	1000
Normal	0.54	0.54	0.54	0.57	0.54	0.57	0.57
0	0.65	0.68	0.70	0.73	0.70	0.74	0.69
1	0.65	0.54	0.69	0.70	0.70	0.60	0.80
3	0.64	0.68	0.67	0.73	0.78	0.70	0.64
6	0.68	0.60	0.66	0.65	0.66	0.70	0.70
24	0.60	0.59	0.61	0.63	0.60	0.63	0.61
48	0.57	0.54	0.50	0.57	0.53	0.57	0.58
72	0.54	Normal	Normal	Normal	Normal	Normal	Normal

Table 3.25: Effects of *C. odorata* on Formaldehyde induced Paw Oedema in Rats after 24 hours

Dose (mg/kg)	Saline	Indo	50	100	300	500	1000
Animals	3	3	3	3	3	3	3
Paw volume (%) at 0 hour	20.37	25.93	29.63	28.07	32.08	29.82	21.06
Differences in volume of paw (%) after 6 hours	25.93	11.11	22.22	14.04	22.22	22.81	22.81
Differences in volume of paw (%) after 24 hours	11.11	9.26	12.96	10.5	11.11	3.42	7.02
Anti-inflammatory effects (%)	-	57.14	14.31	45.85	14.31	12.03	12.03

After injecting the formaldehyde, inflammation can be seen on all of the animals. The anti-inflammatory activity also can be observed on all animals, except for the animal that was given the saline solution. All the paw volume of the animal returned to normal after 24 hour, except for the mice given with saline solution. But the percentage of suppression was measured on the 6th hour.

Figure 3.10: Paw Volume of Formaldehyde-Induced Inflammation of Mouse Against Time



3.3.4 Wound Healing Studies

Crude extracts of *C. odorata* were subjected to wound healing studies which was done on white albino mice weighed 25g, obtained from the animal house of Biological Science Institute, University of Malaya. Observation was done throughout 16 days. All experimental tools and their living cages were disinfected with 70% of alcohol to prevent infection and ensure cleanliness. They were provided with food and water ad libitum throughout the evaluation.

The animals were left unrestrained and their hair was shaved on the dorsal side. Ethanol 70% was used as antiseptic for the shaved region. An incision was made on each mice vertically using a sharp shaving razor with the length of 1cm. The wound was left undressed to the open environment and no local antimicrobial agents were used. The animals were kept in separate cages according to the concentration of extracts. The control animal was swab with acriflavine lotion.

Throughout the evaluation, there were no changes of behaviours in all the animals. There were also no signs of infection or irritation. It took 10 days for the treated animals to heal and to grow back their fur. However it took 15 days for the control animals to heal and there was also presence of scab observed. Moreover, inflammation was seen very obvious on the control animals. As for the treated animals, the wound healed faster and no presence of scab was seen. Though scaring was obvious on both control and treated animals.

Table 3.26: Observation of the Wound healing Studies

Dosage (mg/kg)	**Acriflavine lotion (control)**	***C. odorata* Leaves Extract Concentration (mg/kg)**		
		100	**300**	**500**
Day 0	Fresh open wound, bleeding	Fresh open wound, bleeding	Fresh open wound, bleeding	Fresh open wound, bleeding
Day 1	Inflammation (++) Red (+++) Blood coagulation on open wound	No inflammation Red (++) Open wound	No inflammation Red (+) Open wound	No inflammation, no redness, open wound
Day 2	Inflammation (+++) Redness (+++) Scab formed	No inflammation Redness (+) Wound starts to close/heal (+)	No inflammation Redness (+) Wound starts to close/heal (+++)	No inflammation No redness Wound starts to close/heal (+++)
Day 3	No inflammation Redness (+) Scab seen	No inflammation Redness (+) Wound starts to close/heal (+)	No inflammation No redness Healing (++)	No inflammation No redness Healing (++)
Day 4	No inflammation No redness Scab observed	No inflammation No redness Healing (+)	No inflammation No redness Healing (++)	No inflammation No redness Healing (++)
Day 5	No inflammation No redness Scab observed	No inflammation No redness Healing (+)	No inflammation No redness Healing (+)	No inflammation No redness Healing (+)
Day 6	No inflammation No redness Scab observed	No inflammation No redness Healed Scar (+++)	No inflammation No redness Healed Scar (++)	No inflammation No redness Healed Scar (+)

Table 3.26: Continued

Day 7	No inflammation No redness Scab observed	No inflammation No redness Scar (++)	No inflammation No redness Healed Scar (+)	No inflammation No redness Healed No scar
Day 8	No inflammation No scab Redness (+)	No inflammation No redness Scar (+) Hair starts to grow	No inflammation No redness Healed No scar	No inflammation No redness Healed No scar
Day 9	No inflammation No scab Redness (+) Healing (+)	No inflammation No redness Healed No scar	Normal Hair starts to grow	Normal Hair starts to grow
Day 10	No inflammation No scab Redness (+) Healing (++)	Normal	Normal	Normal
Day 11	No redness Healing (+++)	Normal	Normal	Normal
Day 12	Healing (+++)	Normal	Normal	Normal
Day 13	Healing (++++)	Normal	Normal	Normal
Day 14	Healed Scar (++)	Normal	Normal	Normal
Day 15	Scar (++)	Normal	Normal	Normal
Day 16	Scar (+)	Normal	Normal	Normal

Legend:

\+ Vague

++ Evident

+++ Obvious

++++ Very obvious

Chapter Four

DISCUSSION

CHAPTER 4

DISCUSSION

4.1 Phytochemical Analysis

4.1.1 Preliminary Analysis

From the preliminary analysis and colour test, it was found that the leave powder extract of *C. odorata* contains terpenoid, flavonoid and alkaloid. These compounds are common in the compositae family (Heywood *et al.*, 1977). Alkaloid and flavonoid are compounds which have medicinal properties and are widely used. This result is also parallel with the phytochemical studies of the plant done by Afolabi *et al*., 2007.

To isolate the flavonoid compound, trial and error method was used to obtain the suitable solvent system of extraction and to find suitable solvent of extraction. It was found that methanol extract isolated the flavonoid compound best and this is accordance with Ling *et al*., 2007c and Afolabi *et al*., 2007.

4.1.2 Crude extract

The crude extract of *C. odorata* was subjected to HPLC and GCMS analysis. The fresh leaves were extracted using petroleum ether, hexane and chloroform. The extraction was done in 12 days and then filtered for three times. After the filtration, two layers of liquid were obtained. The essential oil was seen on the top layer and was then subjected to GCMS analysis. Meanwhile, the crude solvent extract was subjected to HPLC analysis.

4.1.2.1 GCMS Analysis

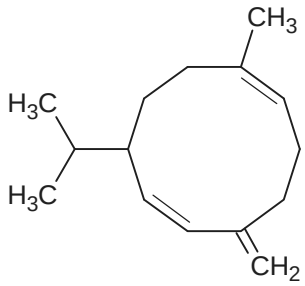
One drop of each solvent extract of the leaf was diluted in a small vial containing chloroform. Chloroform was used as solvent vehicle to prevent the column of the GCMS from clogging. The injection into the GCMS was done in triplicate and each analysis was run in 20 minutes.

The GCMS analysis detected three major compounds which are caryophyllene **(20)**, germacrene D **(21)** and hexadecoic acid **(24)**. This result of the isolated compound of the GCMS analysis was also reported by Dieneba *et al*., 1992.

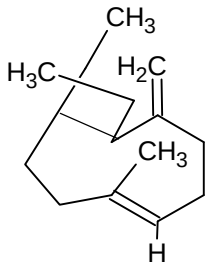
The first compound-caryophylene **(20)** is a mixture of hydrocarbons, primarily sesquiterpenes. So far there are three hydrocarbons derived from caryophylene **(20)**. They are alpha, beta and γ-caryophyllenes, which own individual properties. Generally, caryophylene **(20)** is reported to be non steroidal anti-inflammatory agent. Complementary to its anti-inflammatory agents, it is reported that they have analgesic, antipyretic, and platelet-inhibitory actions. Caryophyllene **(20)** is also reported used primarily in the treatment of chronic arthritic conditions and certain soft tissue disorders associated with pain and inflammation (Heywood *et al*., 1977).

The second compound is Germacrene **(21)** or occasionally germacrane which refers to a subset of volatile organic hydrocarbons, specifically sesquiterpenes. There are two major molecules of germacrene **(21)** which are germacrene A and germacrene D. Germacrenes **(21)** are normally produced in a number of plant species for their antimicrobial and insecticidal properties. It also functions as insect pheromones (Heywood *et al*., 1977).

The last compound which is hexadecanoic acid **(24)** or palmitic acid occurs in oil of vetiver, Canadian snakeroot, pimento, myrrh, cascarilla, in the seed oils of ambrette, celery, anise, parsley and carrot, as well as in expressed almond oil. Palmitic acid is present in 11 percent of all commercial fixed oils and it is insoluble in water (Heywood *et al*., 1977). Meanwhile, hexadecanoic acid **(24)** is reported to be an enzyme inhibitor. It is an agent that combines with an enzyme in such manner as to prevent the normal substrate-enzyme combination and the catalytic reaction. The World Health Organization (WHO) claims there is convincing evidence that dietary intake of palmitic acid increases risk of developing cardiovascular diseases (WHO/FAO Consultant Organisation, 2003).



(20) Germacrene D



(21) Caryophylene

(24) Hexadecanoic acid

4.1.2.2 HPLC Analysis

HPLC chromatogram (please refer to appendix) indicated five major peaks. The peak numbers identified were peak numbers 17 (retention time 15.71), 18 (retention time 16.23), 20 (retention time 17.20), 22 (retention time 19.92) and peak number 23 (retention time 21.19). This result is as shown by Ling *et al*., 2006. The flavonoids isolated by HPLC are also parallel with Ling *et al*., 2007a; Ling *et al*., 2007b and Suksamrarn 2004.

Peak number 17 was assigned to quercetin-4 methyl ether and peak number 18 is aromadendrin-4'-methyl ether. As for peak number 20, there were three compound identified, taxifolin-7-methyl ether, taxifolin-4'-methyl ether and quercetin-7-methyl ether. Peak number 22 was assigned to two compounds which are kaempferol-4'-methyl ether and eridicytol-7, 4'-dimehyl ether. Lastly, peak number 23 was assigned to quercetin-7,4'-dimethyl ether.

4.1.3 Column Chromatography

The column chromatography method was used to isolate flavonoid compound of the methanolic leaf extract of *C. odorata*. It was done using the 2cm x 60cm, 3cm x 70cm and 4cm x 80cm column sizes. Slurry method was used for packing the silica gel in the entire column. The slurry method is the best column packing method but it must be done carefully and using the suitable solvent.

The most important aspect of packing the column is creating an evenly distributed and packed stationary phase. Cracks, air, bubbles and channelling will lead

to poor separation and not to mention the expensive wastage of chemical solvents and materials.

When adding sample into the packed column, it must be done cautiously so that the top column can be remained even. After adding the sample, some white sand is sprinkled on top so that it remained even when solvent is added. It is also very important not to let the column run dry at any time as this will allow air enter into the column, which can result in uneven bands and poor separation.

Small vials were used to collect the eluent with approximate volume of 5ml. This was to ascertain that the compounds were separated carefully. Then, collected the eluent was evaporated to about 1ml and spotted on a TLC plate to determine the presence of compounds. A single spot would indicate a single compound. A developed TLC plate that showed the same colour after spraying with Anisaldehyde reagent and with the same Rf value would be combined. As these would indicate that the same compound were collected in different vials.

Crystal of pure compound was obtained after evaporation of the solvents. If an oily residue was obtained, the vials were scratched to induce crystallization. The crystals were then weighed and its physical characteristic (shape and colour) noted. The structure elucidation was done through determination of the melting point, NMR, IR and the MS analysis.

4.1.3.2 Isolated Compound

There were 11 flavonoids present in the leaves of *C. odorata* that had been identified and reported by Ling *et al*., 2006. The targeted compound flavonoid is a class of diverse biological properties. It has been reported as anti-oxidant, anti-inflammatory, anti-allergic, anti-carcinogenic, vitamin C sparing (Kuhnau, 1976) as well as chelate metals. It also stimulates the immune system and also reduces allergic response, prevents formation of carcinogens, impedes cancer growth and protection against bacteria and viruses (Ling *et al*., 2007a).

Through the column chromatography, three flavonoid compounds were able to be isolated and identified. The structures of the isolated compounds were elucidated according to ^{1}HNMR, ^{13}CNMR, IR, and MS data. The compounds isolated were labelled as Compound A, Compound B and Compound C.

4.1.3.2.1 Compound A

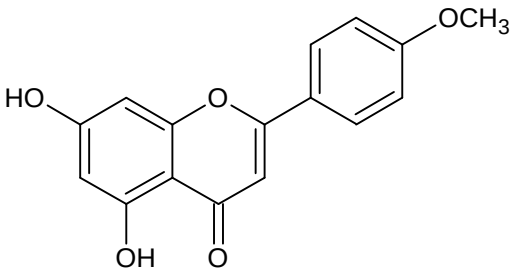


Figure 4.5: Chemical Structure of Compound A

The mass spectrum revealed that the molecular weight for Compound A is [M^+] 284 fit for $C_{16}H_{12}O_5$. The IR spectrum showed a hydrogen bonded signal at 1714cm^{-1} which confirmed the presence of hydrogen bonded carbonyl at the C-4 to C-5 position. Signals at 2961cm^{-1} and 3426cm^{-1} confirmed the presence of methoxyl and hydroxyl bonded hydrogen respectively.

The ^{1}HNMR suggested the presence of one methoxy with the signal at δ3.89, positioned at C-4'. The other proton signals can be assigned as follow; δ6.39 (C-2'), δ6.18 (C-3'), δ6 .17 (C-5'), δ6.38 (C-6'), δ7.03 (C-6) and δ7.70 (C-8).

^{13}CNMR showed the carbon signal for carboxyl at 177.42 which can be assigned to position at C-4. While the methoxyl signal was at 56.40 and could be assigned to C-4'. The other carbon signals were as follow; δ168.52 (C-2), δ165.69 (C-3), δ177.42 (C-4), δ147.39 (C-5), δ 147.47 (C-5a), δ125.45 (C-6), δ137.61 (C-7), δ130.68 (C-8), δ147.39 (C-8a), δ94.43 (C-1'), δ99.28 (C-2'), δ112.2 (C-3'), δ121.48 (C-4'), δ115.66 (C-5'), δ104.55 (C-6').

The Compound A was assigned to a structure of 5,7-dihydroxy-2-(4-methoxyphenyl)chromen-4-one. This compound had been isolated previously and known acacetin or apigenin-4'-methyl ether (Suksamrarn *et al*., 2004). It had been isolated from the aqueous flower extract of *C. odorata*. Through my literature review, this is the first time the compound is isolated from the leave extract of *C. odorata*. Compared to the 11 flavonoid compound which was isolated from the leave extract of *C. odorata* by Ling *et al.,* 2007a where it has not mentioned anything about the compound.

4.1.3.2.2 Compound B

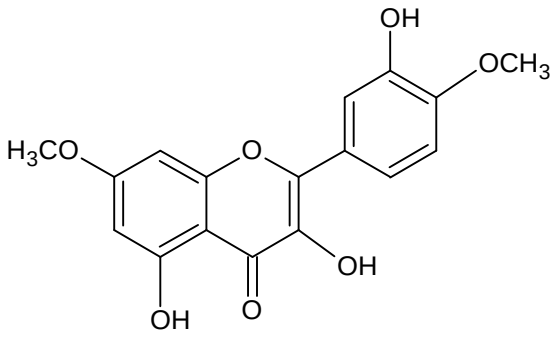


Figure 4.6: Chemical Structure of Compound B

The mass spectrum showed that Compound B was having [M^+] 330, fit for $C_{17}H_{14}O_7$. The IR spectrum indicated a hydrogen bonded carbonyl signal at 1654cm^{-1} which confirmed the presence of a carbonyl at the C-4 to C-5 position and had a typical structure of a flavone. Signals at 3306cm^{-1} and 2917cm^{-1} also confirmed the presence of a hydroxyl and methoxyl functional group respectively.

The position of ^{1}H NMR suggested the presence of two methoxy with the signal at δ3.89 and could be assigned to C-7 and C-4' as compared to Ling *et al*., 2007b. The other proton signals were 7.7 (d, C-3'), 7.3 (d, C-2'), 6.1 (d, C-8) and 6.3 (d, C-6) which could be assigned to aromatic protons. The mass spectrum fragmented into methoxyl (OCH_3) with peak 330MHz.

The presence of OH at C-5 position was confirmed from the ^{1}HNMR by the signal at δ 11.95 typical of hydroxyl bonded hydrogen. Based on the proton signal for C-2', C-3 and C-6', the hydroxyl signal on the Ring B could only be placed at the C-5' position.

^{13}CNMR showed the carbon signal for carboxyl at 177.5 that could be assigned at C-4 position. It also proved that the methoxy signal at 56.4 and could be assigned to the position of C-7. The other carbon signals were as follow; δ165.62 (C-2), δ162.52 (C-3), δ177.42 (C-4), δ150.73 (C-5), δ125.41 (C-6), δ137.62 (C-7), δ130.72 (C-8), δ94.50 (C-1'), δ99.35 (C-2'), δ112.25 (C-3'), δ121.62 (C-4'), δ115.72 (C-5'), δ104.67 (C-6').

Compound B could be assigned to the structure of 3,5-dihydroxy-2-(3-hydroxy-4-methoxy-phenyl)-7-methoxy-chromen-4-one. This compound had also been previously isolated and known as ombuine; flavone, 4',7-dimethoxy-3,3',5-trihydroxy or 7,4'-Di-O-methylquercetin (Ling *et al*., 2007a; Pisutthanan *et al*., 2006). It was also found in the ethanol extract of the leaf of *C. odorata* (Phan *et al*., 2001a).

4.1.3.2.3 Compound C

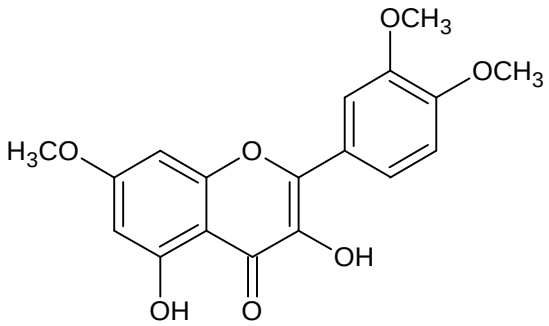


Figure 4.7: Chemical Structure of Compound C

The mass spectrum showed that Compound C was having [M^+] 344, fit for $C_{18}H_{16}O_7$. Meanwhile, the IR spectrum showed a hydrogen bonded signal at 1639cm^{-1} which confirmed the presence of carbonyl at the C-4 to C-5 position. Signals at 3436cm^{-1} and 2917cm^{-1} confirmed the presence of hydroxyl and methoyxl respectively.

The position of the ^{1}HNMR suggested the presence of three methoxy with the signal at δ 3.89. This signals could be assigned to C-7, C-4' and C-3'. The other proton signals were at 7.03 (C-2'), 7.4 (C-3'), 7.0 (C-6) and 6.2 (C-8). The mass spectrum fragmented into methoxyl (OCH_3) with the peak 344MHZ.

The presence of OH at C-5 position was confirmed from the ^{1}HNMR by the signal at δ11.95, typical of a hydrogen bonded hydroxyl. Based on the proton signal for C-2', C-3', C6 and C-8, then the methoxy signal could only be placed at C-3' and C-4' position.

Compound C could be assigned to the structure of 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy-chromen-4-one. From my literature review, this compound has not been mentioned in the plant extract of *C. odorata*.

4.2 Pharmacological Analysis

4.2.1 Toxicity Evaluation

4.2.1.1 Oral Toxicity Evaluation

Toxicity test was done using two-month-old white albino mice. Before the evaluation, the mice were kept in four separate cages, separating the males from the females and left to acclimatize to the new surrounding for three days. They were fed with food and water. Their cages were also cleaned and sanitized regularly. 12 hours prior to the experiment, all the mice were weighed and then fasted. The water extracts of *C. odorata* were prepared according to their weight. The concentration was 2000mg/kg, which was the maximum dosage for oral toxicity test.

Each animal was force fed with 1ml of the extract using a feeding syringe. This was to ensure that the extract was taken by the animals as it would be directly inserted into their trachea. This had to be done carefully as it may cause harm to the animal and even death if done improperly. The animals were observed through out the 14 days. Their eating habit, their alertness and their behaviours had been carefully observed.

During the whole extend of the oral toxicity evaluation, there were no abnormal behaviours. The animals remained active and normal with their usual weight and eating habit. Acute oral toxicity test was also done with the maximum dosage of 2000mg/kg.

The mortality of the animals was observed for three consecutive days. At the end of the third day, no fatality was seen.

The dosage of 2000mg/kg is the limit dose stipulated by Organization for Economic Cooperation and Development (OECD). The limit test is primarily used in situation where the investigator has information indicating that the test material is likely to non toxic or has low toxicity (Etuk *et al*., 2006). Overall the aqueous leave extract of *C. odorata* did not caused any side effects on the animals. The result of acute oral toxicity study at the limit dose had also shown there was no toxicity. Therefore, the aqueous leave extract of this plant is non toxic and it is safe for oral formulation.

Based on my literature reviews, no proper toxicity evaluation had been done on the crude aqueous extract of *C. odorata* on life subjects. Cytoxic effect which was done by Ling *et al*., 2007c, was on the flavonoid of the plant extract and it was done on the liver and kidney cells only.

4.2.1.2 Dermal Irritation Evaluation

Dermal irritation test was done on white albino mice. As mentioned earlier, *C. odorata* is usually used directly on the skin. Therefore, it is important to determine whether it will cause any type of skin irritation. The dermal irritation test was done by shaving a 1cm x 1cm area on the dorsal side of the mice. After shaving, the area was checked for any skin abnormalities or cuts to avoid interruption of the test observation. The shaven area is cleaned and disinfect with alcohol. Then, the prepared aqueous extract of *C. odorata* (2000mg/kg) was applied three times on the skin. Dark green

colour of the extract can be seen on the applied area. The extract was applied once daily and was observed for two weeks.

During the whole experiment, all the animals behaved normally. They did not seem to be uncomfortable nor scratch the shaven area. No swelling or redness on the shaven area of the animals during the observation. By the end of the two weeks observation, the whole shaven area was covered back with hair. Therefore, no adverse side effects were seen on the experimented animals and this proved that the extract is safe to be used directly on the skin.

Since the poultice of the leave of *C. odorata* is used directly on the skin, it is important that dermal irritation evaluation is looked upon. As for what I've known, there was no reported dermal irritation evaluation which had been done on this plant before.

4.2.2 Antiinflammatory Study

Effects of drugs and acute phase of inflammation can be studied by models induced with inflammatory agents such as carrageenan, dextrane, formaldehyde, serotonin, histamine and bradykinin in rat paws. Chronic inflammation models by implanting the foreign body under the skin are used to study the effects of the drug on the proliferation phase of inflammation (Suleyman *et al*., 2002).

It is known that formaldehyde-induced inflammation usually involves two distinct phases. It has been proposed that the first phase reflects direct stimulation of

noci-receptors, while the latter phase maybe associated with inflammation mediators (Suleyman *et al*., 2002).

4.2.2.1 Formaldehyde Induced Paw Oedema Evaluation

To induce oedema or inflammation, formaldehyde was used as an inflammation agent. The injection of formaldehyde will increase the production of bradykinin, which is an inflammation mediator (Fereira *et al*., 1992). The role of bradykinin in the pathogenesis of formaldehyde-induced inflammation was reported by Kulkarni, 1986.

For the anti-inflammatory result, normal is the time when the paws of the mice were measured before any injection of extract administered to them. Zero hour is the measurement of the paw immediately after the injection of formaldehyde on the sub plantar paw hind leg of the mice. Then, the measurement of the paw was taken on the first, third and sixth hour. Measurement of the paw was then taken daily until they reach normal or the original paw measurement obtained (Suleyman *et al*., 1999).

After the injection of formaldehyde, the average percentage of inflammation of all the mice was 26.71%. This means that formaldehyde had successfully induced inflammation. The result of this evaluation showed that all paw measurements were reduced to normal on the second day, except for the saline solution.

Observation on the first hour showed the reduction of inflammation on all animals, except for the saline solution. The standard drug Indomethacine showed immediate inflammation reduction of 25.93%. The leaf extract of the plant decreased to 1.43% (50mg/kg), 4.29%, (100mg/kg) and 18.92% (500mg/kg). As for the 1000mg/kg,

the inflammation increased to another 15.94% for the first hour. The inflammation started to decrease on the third hour until normal paw measurement was achieved on the third day.

On the sixth hour, the lowest percentage on paw measurement was Indomethacine (11.11%) and followed by extract concentration of 100mg/kg (14.04%). The percentage of paw measurement of the concentration 50, 300, 500 and 1000mg/kg were relatively the same that was 22%.

On the 24th hour, the extract concentration of 100mg/kg showed the reduction of measurement to 10.5%. As for the concentration of 50, 300, 500 and 1000mg/kg, the percentage of paw oedema had reduced to 12.96%, 11.11%, 3.42% and 7.02% respectively. And on the 48th hour, all mice had reached their original paw measurement.

The percentage of anti-inflammatory effect wad 57.14% for Indomethacine, 45.85% for extract concentration of 100mg/kg, 14.31% for both 50 and 300mg/kg and 12.03 for both 500 and 1000mg/kg extract concentration.

On the second day, all the extracts given reached normal paw volume. Therefore, the result on the sixth and the 24th hour were used for comparison. This is to determine which extract will reduce inflammation in a better and faster way. It can be deduced that the concentration of 100mg/kg showed the best result. Although the effect of anti-inflammation was slow at first, but after the sixth hour the effect was rapid. And compared to the standard drug Indomethacine, the effect of extract concentration of 100mg/kg is 80.24% as effective as Indomethacine.

To my knowledge, this is the first formaldehyde induced paw oedema evaluation done with *C. odorata* leaf extract. This evaluation validate the anti-inflammatory activity of this plant as mentioned by Ling *et al*., 2006; Phan *et al*., 2001a; Phan *et al*., 2001b; Akinmoladun *et al*., 2007 and Dieneba *et al*., 1992.

4.2.3 Blood Coagulation Test

Blood coagulation is part of an important host defence mechanism termed hemostasis. Upon vessel injury, platelets adhere to macromolecules in the sub endothelial tissues and then aggregate to form the primary hemostatic plug. The platelets stimulate the local activation of plasma coagulation factors, leading to generation of a fibrin clot that reinforces the platelet aggregate. Later, as wound healing occurs, the platelet aggregate and fibrin clot are broken down. Mechanisms that restrict formation of platelet aggregates and fibrin clots to sites of injury are necessary to maintain the fluidity of the blood. Normal blood coagulation time for human is 190-300 seconds (Mary *et al*., 2003).

Platelets are the major constituents of thrombus and a source of powerful vasoconstrictors that can cause vasospasm and enhance coagulation by diminishing blood flow (Rodger, 1988). The antiplatelet therapy is one of the revolutionise mechanism related to atherogenesis (Cimmniello and Toschi, 1999).

All the extracts of *C. odorata* in the observation of blood coagulation showed a positive anti-coagulation mechanism at the concentration of 100mg/ml, except for the methanol extract. The plant extracts prolong coagulation time compared to the control

normal blood drop. It was also observed that as concentration increased, time taken for the blood to coagulate was also increased.

However, for the methanol extract (concentration of 100mg/ml), immediate blood clot was seen after adding the extract to the blood drop. This may due to active flavonoid compounds in the methanol extract. As reported by Oweyele *et al*, 2008, the anti-inflammatory of *C. odorata* is related to the flavonoid compound of the plant. This result also validates the wound healing (Phan *et al*., 2001b) activity as rapid blood clot helps to increase wound healing process.

4.2.4 Antimicrobial Evaluation

The antimicrobial consists of antifungal and antibacterial evaluation. Both these evaluations are important to identify the antimicrobial properties of leave extract of *C. odorata*. There are two sensitivity methods which are suitable, they are disc diffusion and a dilution technique. For the disc diffusion technique, a disc of blotting paper is used on a plate of sensitivity testing agar (inoculated with test organisms). Then observation is made after 24 hours by examining the areas of growth around the disc agar. The dilution sensitivity test involves the measurement of the minimum inhibitory concentration (MIC). This can be measured by the concentration of bactericidal or lowest concentration of antimicrobial. This method involves dilution of an antimicrobial to a broth or agar medium using extracts and antibiotics by comparison (Bauer *et al*., 1966; Brown and Blowers, 1978).

The antifungal evaluation may also be tested using the same method; disc diffusion or dilution technique. But the medium (agar and broth) is replaced with

suitable medium. Antifungal that can be used are nystatin, flucytosine or imidazole agents (Bauer *et al*., 1966; Brown and Blowers, 1978).

4.2.4.2 Disc Paper Disc Diffusion Assay

The antibacterial evaluation of *C. odorata* showed positive effect of inhibition against gram positive bacteria, (*S. aureus*) gram negative bacteria *and P. aeruginosa* bacteria). Though negative inhibition was shown against bacteria *K. pneumonia,* the antibacterial result showed that methanolic extract of *C. odorata* at the concentration of 800mg/ml was the most efficient to inhibit *S. aureus* ATCC 25923. The percentage of inhibition was 33.33% compared to Chloramphenicol. This result is considered medium inhibition activity as the inhibition zone was only 9mm.

Meanwhile, for the second strain of *S. aureus* ATCC 29213, the most efficient plant extract was chloroform ($\mathbf{C}_{800}$) with the concentration of 800mg/ml. The percentage of inhibition by $\mathbf{C}_{800}$ was 15.38% compared to Chloramphenicol. It was considered as weak inhibition activity as the inhibition zone was only 4mm.

P. aeruginosa was inhibited the highest by the methanol leaves extract of *C. odorata* with the concentration up to 800mg/ml. The percentage of inhibition was 62.50% compared to Chloramphenicol. The inhibition of the extract against *P. aeruginosa* was considered as strong inhibition as the inhibition zone was 16 mm. There was no inhibition observed for both strain of *E. coli* ATCC 25922 and *E. coli* ATCC 35213 and *K. pneumonia*.

As for the anti-fungal test which had been shown in the result, no positive observation was obtained. *Candida albican* is a low strength fungal which can be easily inhibited (Viejo-Diaz *et al*., 2004). Since the extract did not show any inhibition, therefore, *C. odorata* is not an anti-fungal agent.

Overall, the result of gram negative bacteria showed only a weak to mild activity of the leave extract of *C. odorata*. Strong activity was seen on the gram negative bacteria, but no activity was observed on the fungal strain.

Antimicrobial evaluation has also been done by other authors which shows the effects of antimicrobial activity of *C. odorata*. Yet, there is no author who has specifically done the antimicrobial evolution on the methanol leaf extract of this plant. Evaluations by Odunbaku and Ilusanya, 2008 used the whole plant methanolic extract. Suksamrarn *et al*., 2004 and Ling *et al*., 2007c emphasize only on the antimicrobial of the flavonoid extract of the plant.

4.2.5 Dermal Wound Healing Evaluation

Wound healing is the process of repair that follows injury to the skin and other soft tissues. Wounds may result from trauma or from surgical incision. In addition, pressure ulcers (also known as decubitus ulcers or bed sores), a type of skin ulcer, might also be considered wounds. Following the injury, an inflammation response occurs and the cells below the dermis (the deepest skin layer) begin to increase collagen (connective tissue) production. Later, the epithelial tissue (the outer skin layer) is regenerated (Bert *et al*., 1996).

The process of wound healing is a complex and encompassing a number of over-lapping phases, including inflammation, epithelialisation, angiogenesis, matrix deposition and tissue remodelling. These processes are essential to restore the structure and functional integrity of the damaged tissue. During inflammation, the formation of blood clot re-establishes haemostasis and provides provisional matrix for cell migration (Gopinath *et al*., 2004).

The result for wound healing test was positive. Crude water extract of *C. odorata* healed the wound faster compared to acriflavine lotion. The healing of wound was also cleaner with no inflammation and no formation of scab observed.

Both the acriflavine lotion and the extract of *C. odorata* were applied as soon as the incision was made. Incisions were made at the back of the mice so that they will not be able to scratch the wound. This is because scratching will worsen the wound and prolong the healing process. Incision on the back of the mice was also easier to be observed.

It was observed that concentration of 300mg/kg and 500mg/kg showed rapid wound healing. Scaring was observed on all tested mice. As for the concentration of 300 and 500mg/kg extract, scaring was seen on the sixth day and seventh day only. On the eighth day, the scar was gone and the hair stated to grow on the ninth day.

A number of wound healing or wound healing related studies were done for this plant. But most of the studies done were using the fractionation extract of the leaf or whole plant only and the evaluation was done through in vitro methods. Phan *et al*., 2001b emphasizes the phenolic wound healing compounds of the leaves of *C. odorata*

on cultured skin cells. Another study by Phan *et al.*, 2001a uses antioxidant evaluation on human cells, in vitro. In Vietnam, clinical trial was done on burn victims on the stimulatory effect of *C. odorata* extract on the formulation of granulation tissue and wound re-epthelialization was proven clinically and histoogically (Phan *et al*., 2001a and Ling *et al***.**, 2007b). Again, this clinical trial used Eupolin ointment, a compound isolated from the leaf extract of *C. odorata*.

Based on my literature reviews, an evaluation of wound specifically on the crude extract of the leaf of *C. odorata* has never been reported. An open wound incision has also never been done on life subject using the crude leaf extract of this plant. This evaluation is important as traditional preparation use the poultice and fresh leave of this plant (Ling *et al*., 2007c). Therefore, this is the first open wound incision evaluation of the methanol leave extract of *C. odorata*.

Generally the pharmacological studies show that the leaves extract of *C. odorata*;

(i) is non toxic and do not cause any type of dermal irritation

(ii) has an anti-bacterial properties

(iii) has anti-inflammatory properties

(iv) has an anti-coagulant properties and

(v) promote wound healing.

Chapter Five

CONCLUSION

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CONCLUSION

Chromolaena odorata of the family Compositae was investigated for its chemical constituents and pharmacological properties. *C. odorata* was reported to be used as wound healing plant locally. Therefore, the pharmacological activities investigated were biological activities that were related to wound healing activity.

Premilinary phytochemical analysis revealed that, *C. odorata* consists of terpenoid, flavanoid, alkaloid and essential oil. The major compound of the plant is terpenoid as it is the major compound of the Compositae family. The targeted compound to be isolated for the present study was therefore flavonoid.

Through the literature reviews, it was known that there are 11 known compound of flavonoids reported for *C. odorata*. However through this study, only three compounds were successfully extracted through column chromatography. The three compounds extracted were identified as flavonoid.

GCMS analysis revealed three compounds from the hexane extraction. They were caryophyllene **(20)**, germacrene D **(21)** and hexadecoic acid **(24)**. The HPLC analysis had identified seven compounds from the methanol extract of this plant, which are all identified as flavonoid. They were aromadendrin-4'-methyl ether; taxifolin-7-methyl ether; taxifolin-4'-methyl ether; quercetin-7-methyl ether; kaempferol-4'-methyl ether; eridicytol-7, 4'-dimehyl ether and quercetin-7,4'-dimethyl ether.

The pharmacological analysis revealed that *C. odorata* is safe to be applied directly on the skin and is safe to be consumed as there was no adverse effect during the toxicity and dermal irritation evaluation. Meanwhile, for the wound healing study, the extract of *C. odorata* took only seven days to healed wound compared to the Acriflavine lotion. There was no scars observed after the ninth day and the fur of the mouse starts to grow on the tenth day. Acriflavine lotion took 14 days to heal and the scar still can be seen after the 16th day. It is obvious that *C. odorata* helps to heal wound faster.

Wound is also shield from infection as *C. odorata* also shows positive activity of antimicrobial. Though, only weak activity recorded on antibacterial activity on gram positive bacteria (*S. aureus*) and mild antimicrobial activity on gram negative bacteria (*P. aeruginosa*). And no antifungal activity was found on *C. albican*. Still, this antibacterial activity will assist in the wound healing process.

Anti-inflammatory evaluation proved that the extract of *C. odorata* reduced inflammation at the concentration of 100mg/kg and is 80.24% as effective as the standard drug indomethacine. Meanwhile, the petroleum ether and chloroform extract of the leaf at the concentration of 100mg/ml showed positive anti-coagulant activity. However, the methanol extract, at concentration of 100mg/ml, showed negative anti-coagulant activity. Anti-coagulant properties open the possibilities of isolating drugs for arthrosclerosis condition. At the same time as the methanolic extract support the ability of *C. odorata* as a wound healing plant.

Although the use of herbal medicines has been widely accepted, they are still unable to be correlated to the clinical practice and diagnostic and therapeutic. In herbal medicines, the classification of diseases is based on symptoms. Therefore, it becomes difficult to define with certainty, the pharmacological activity to be evaluated with the respect to the nature of traditionally prescribed use of an indigenous drug. Nevertheless, presumption based on nature of traditional use and evaluation of specific beneficial activity of indigenous drugs have been found to be successfully approach in medicinal plant research.

It is hope that the findings and results from this study will help to enlighten us more on the benefits of *C. odorata*, besides proving its usefulness as claimed by the locals of its wound healing property. The plant is easily available but known as harmful weed all over the world. At the moment, people around the world are very keen in medicinal herbs and many foresee that medicinal herbs and plants are the trend of tomorrow medicines.

Since the *C. odorata* is proved to help in wound healing activities, my suggestion is to bring the research further for clinical trials. *C. odorata* is proven save for consumption, therefore its ability as wound healing agents on superficial and internal wounds, such as gastric ulcer should be analyze on.

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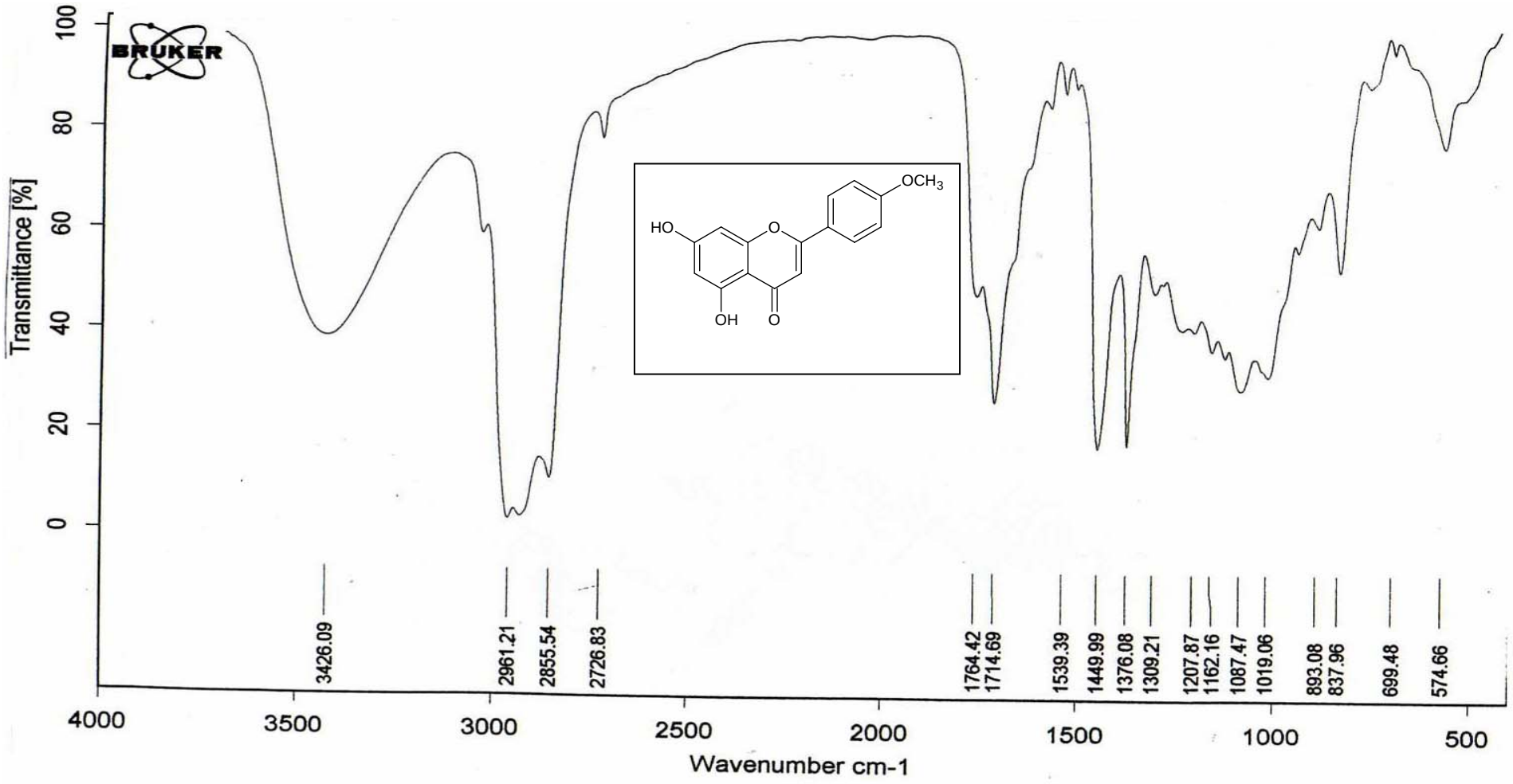
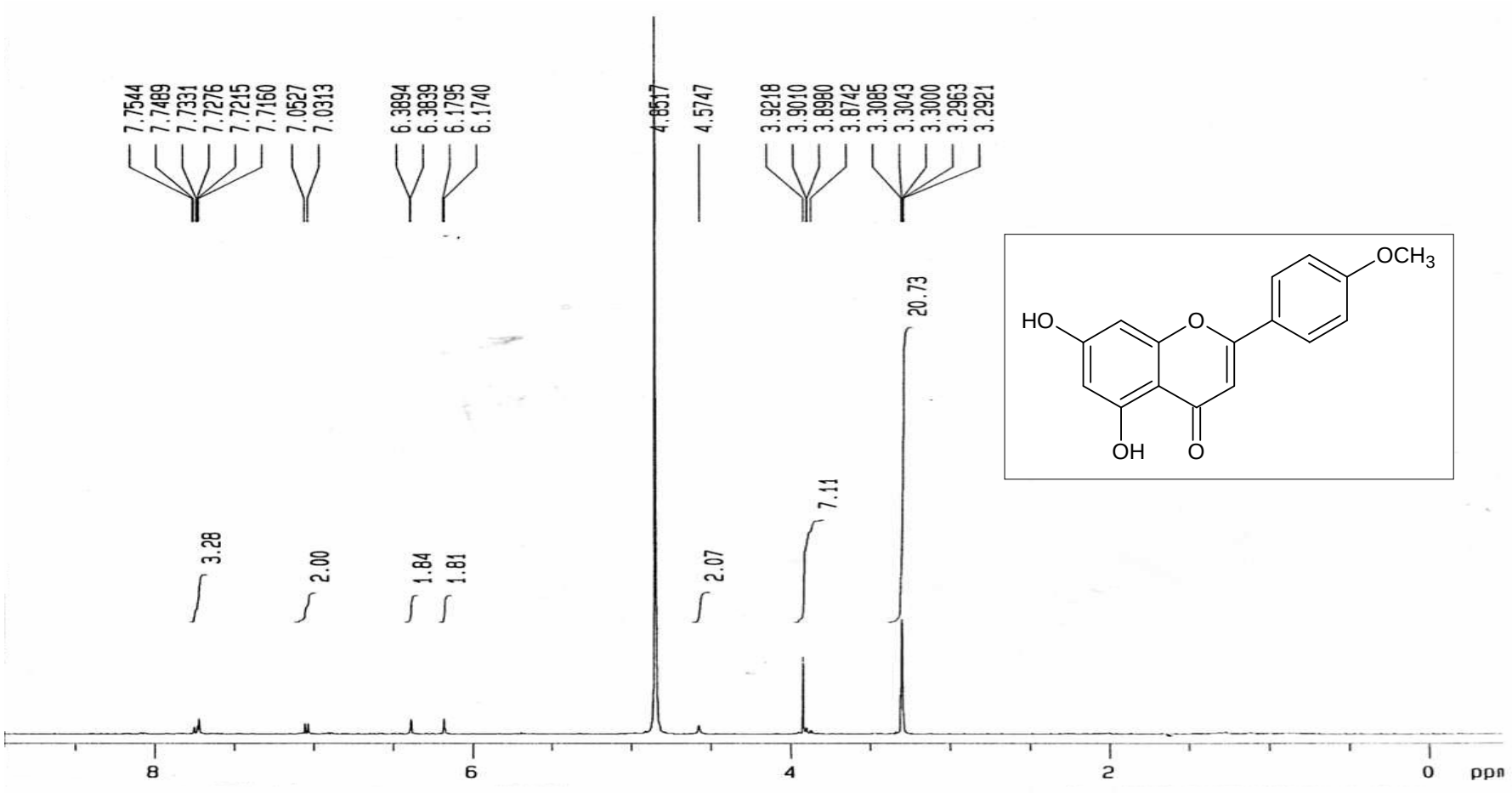
APPENDICES

APPENDIX 1

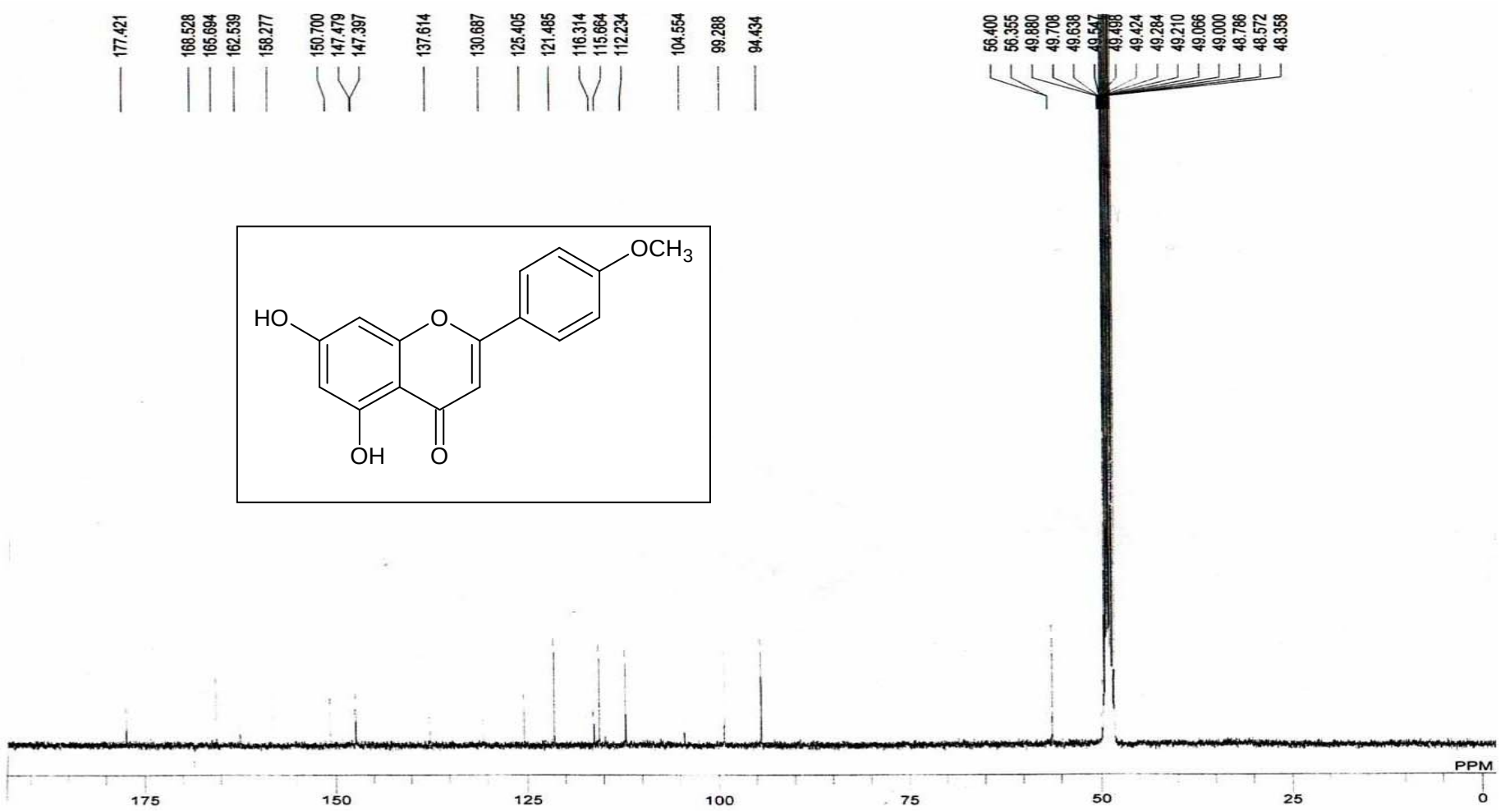
A) IR Spectrum of Compound A



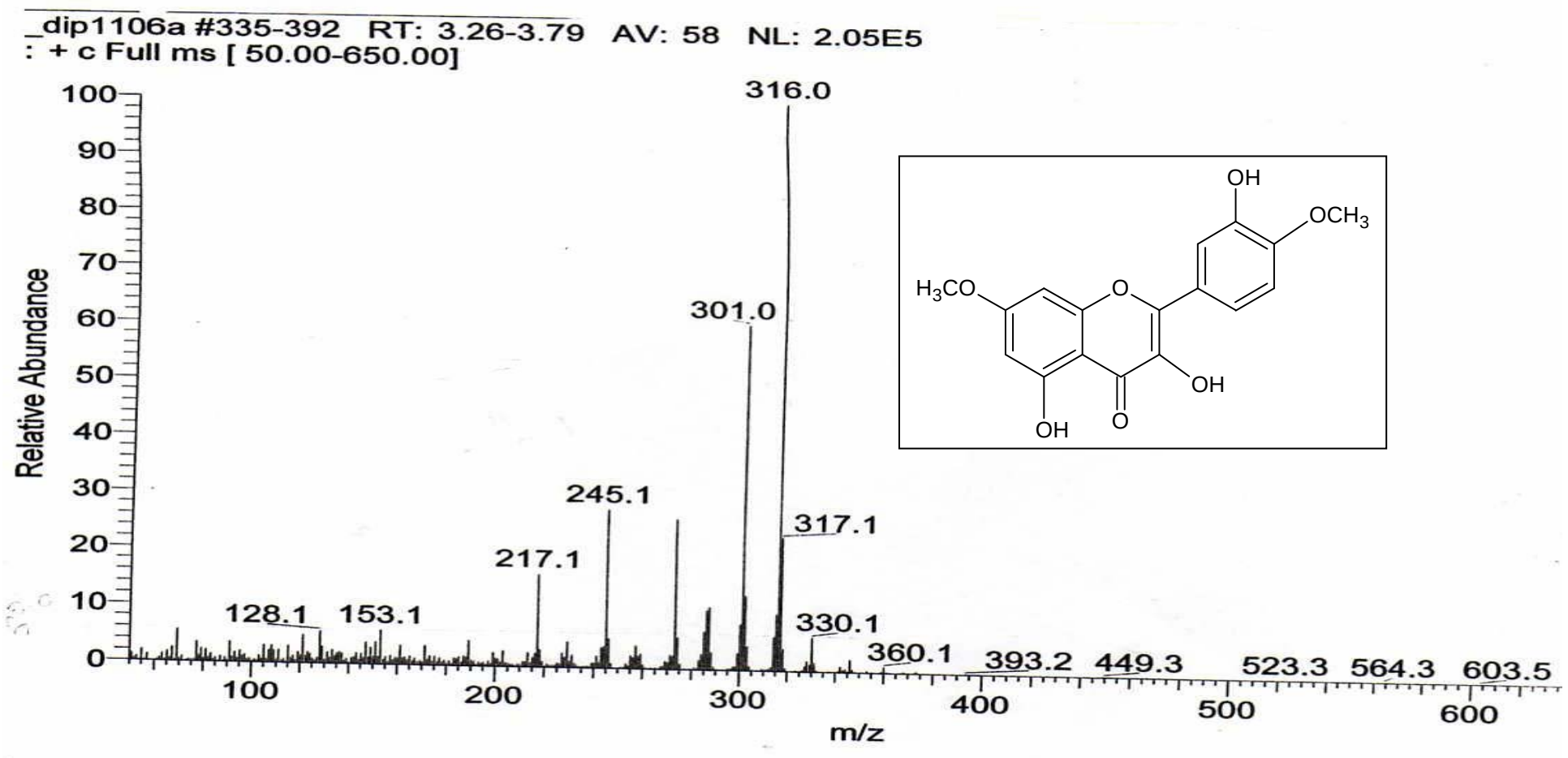
B) ^{1}HNMR Chromatogram of Compound A



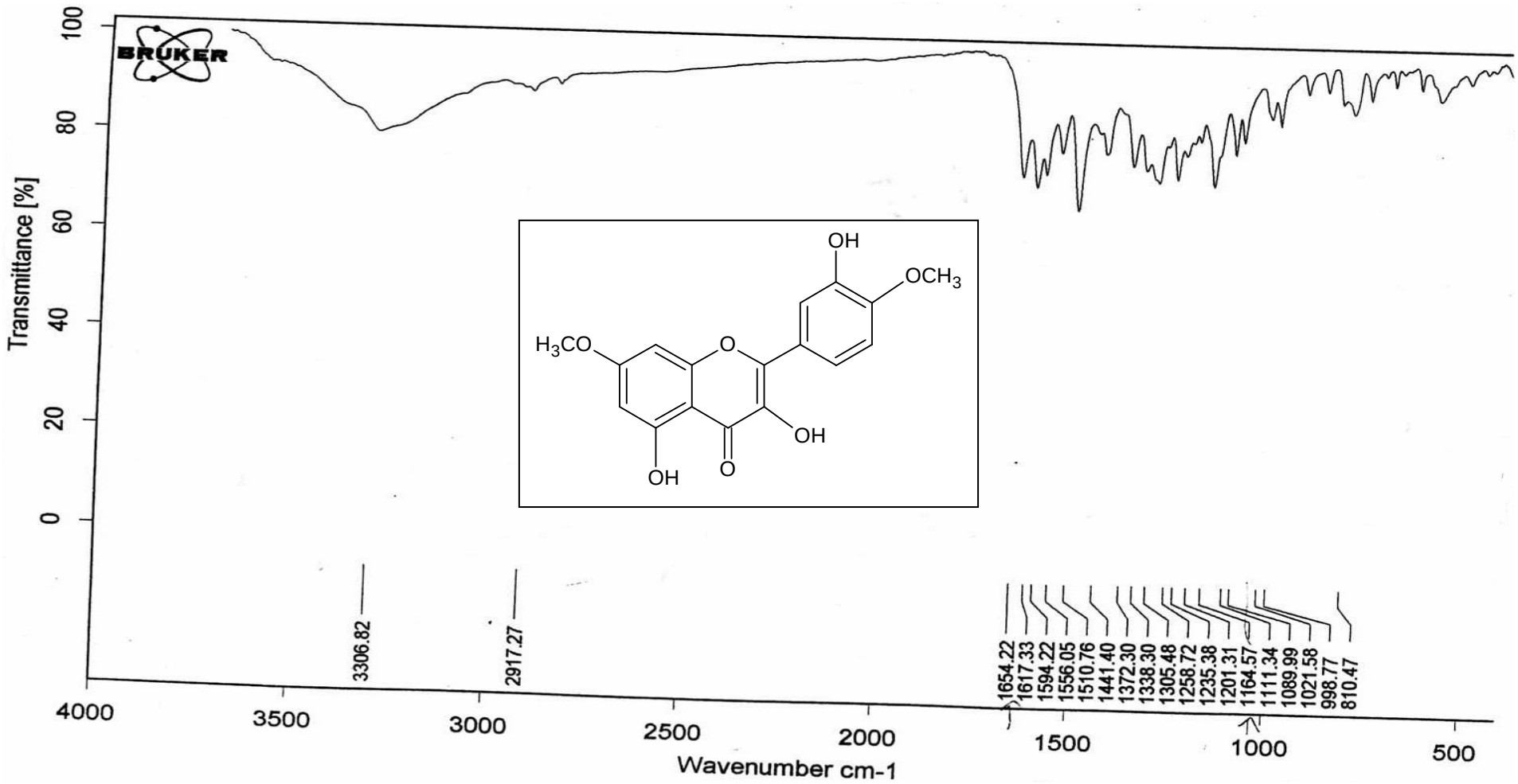
C) ^{13}CNMR Spectrum of Compound A



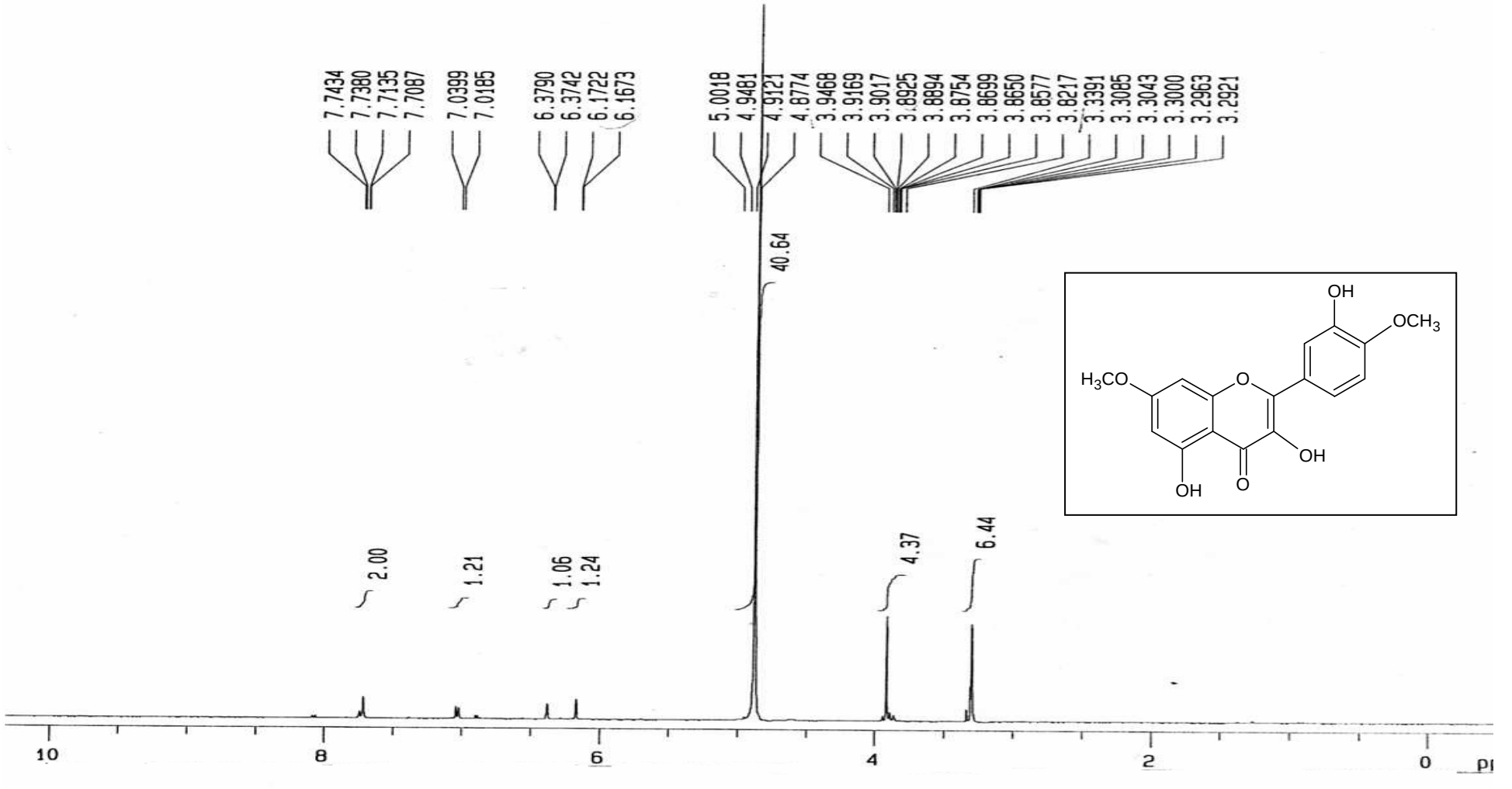
D) MS Chromatogram of Compound B



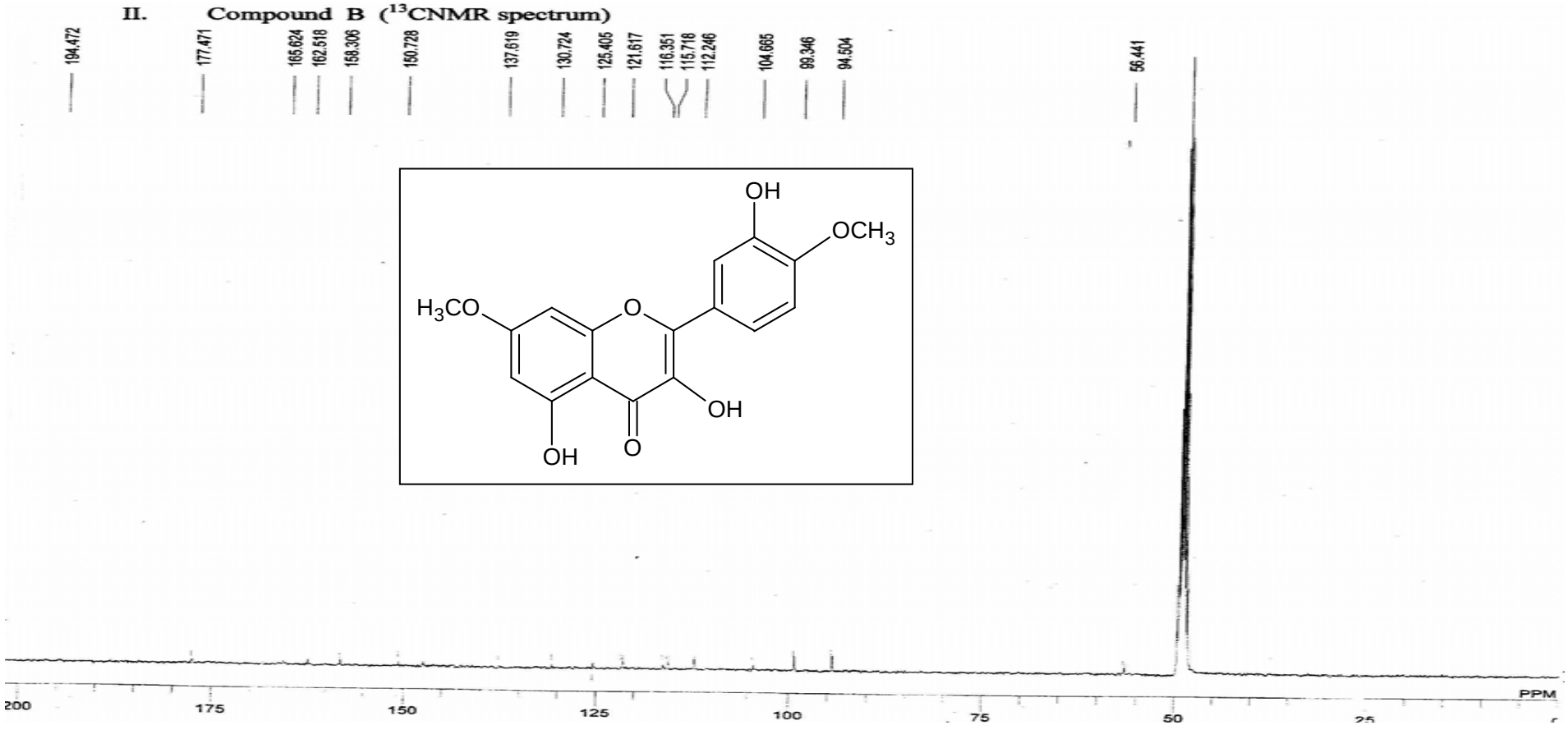
E) IR Spectrum of Compound B



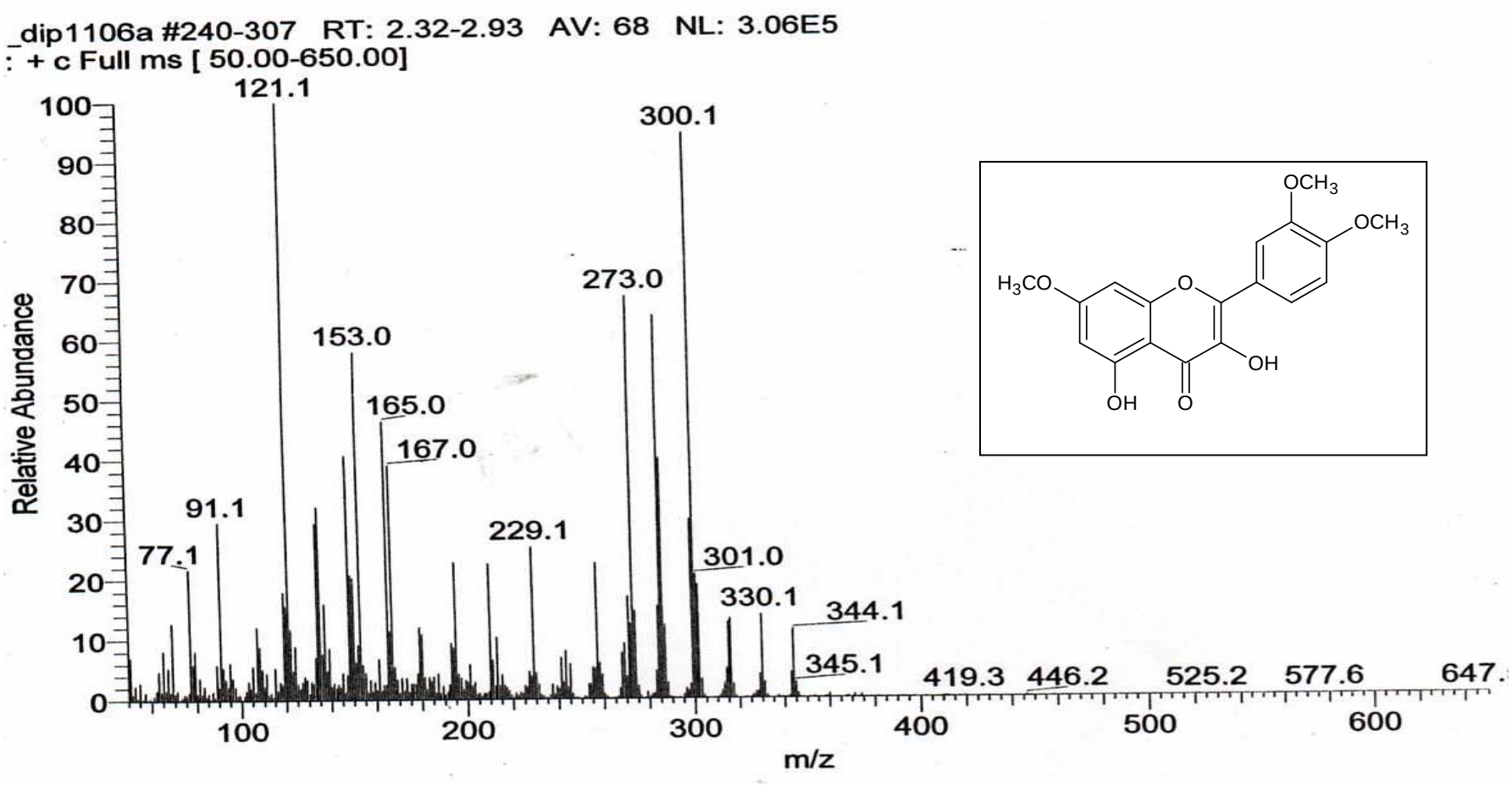
F) [1]HNMR Chromatogram of Compound B



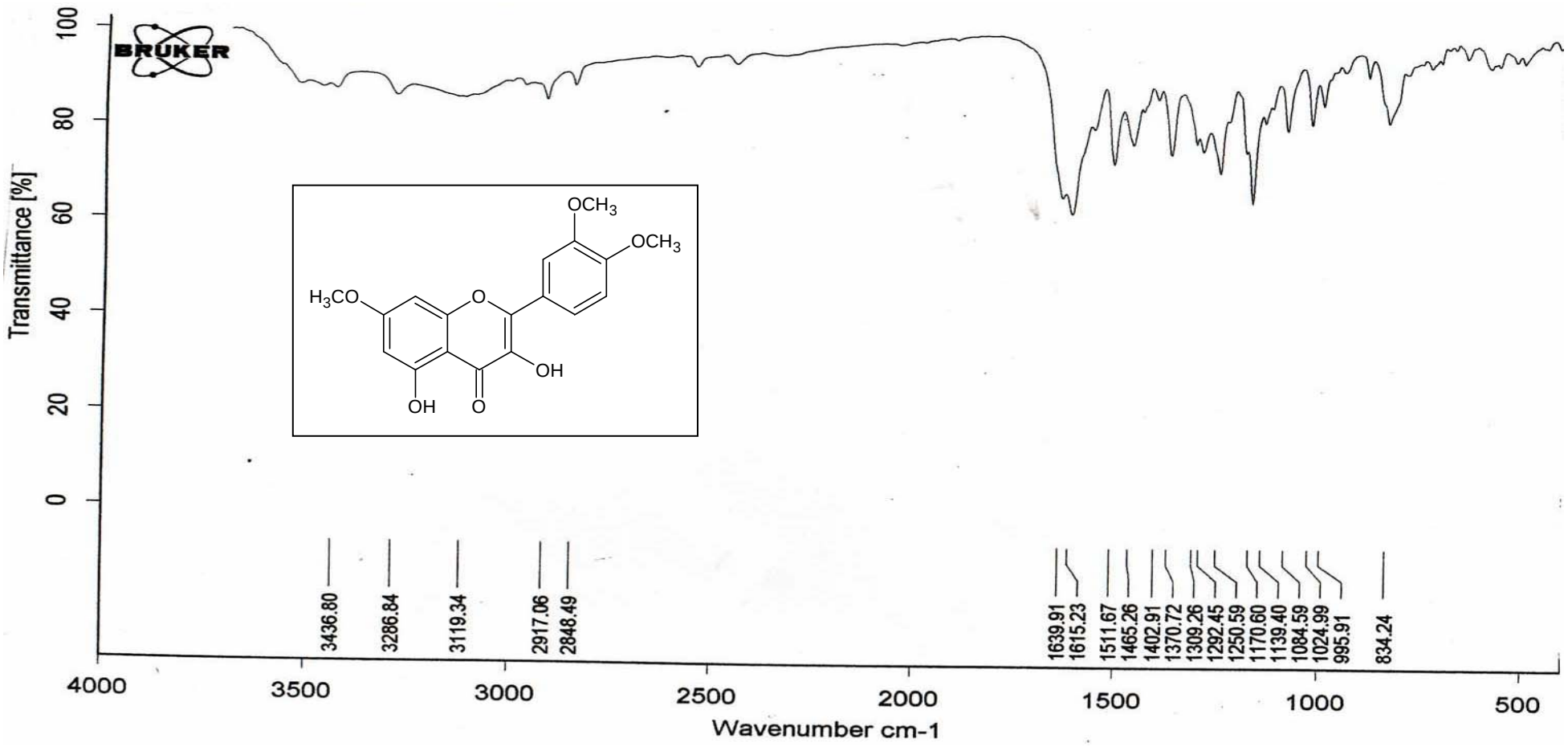
G) ^{13}CNMR Spectrum of Compound B



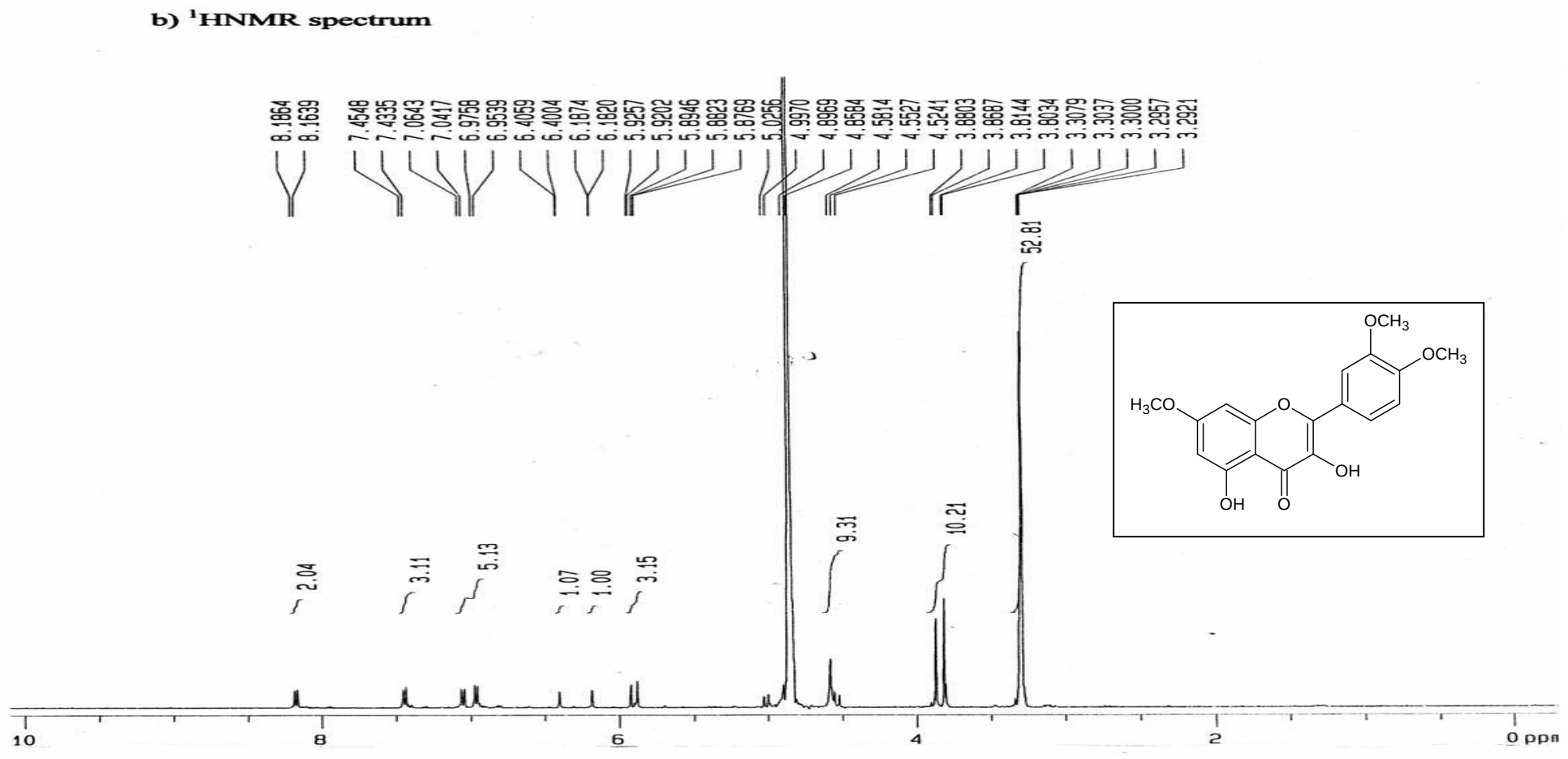
H) MS Chromatogram of Compound C



I) IR spectrum of Compound C



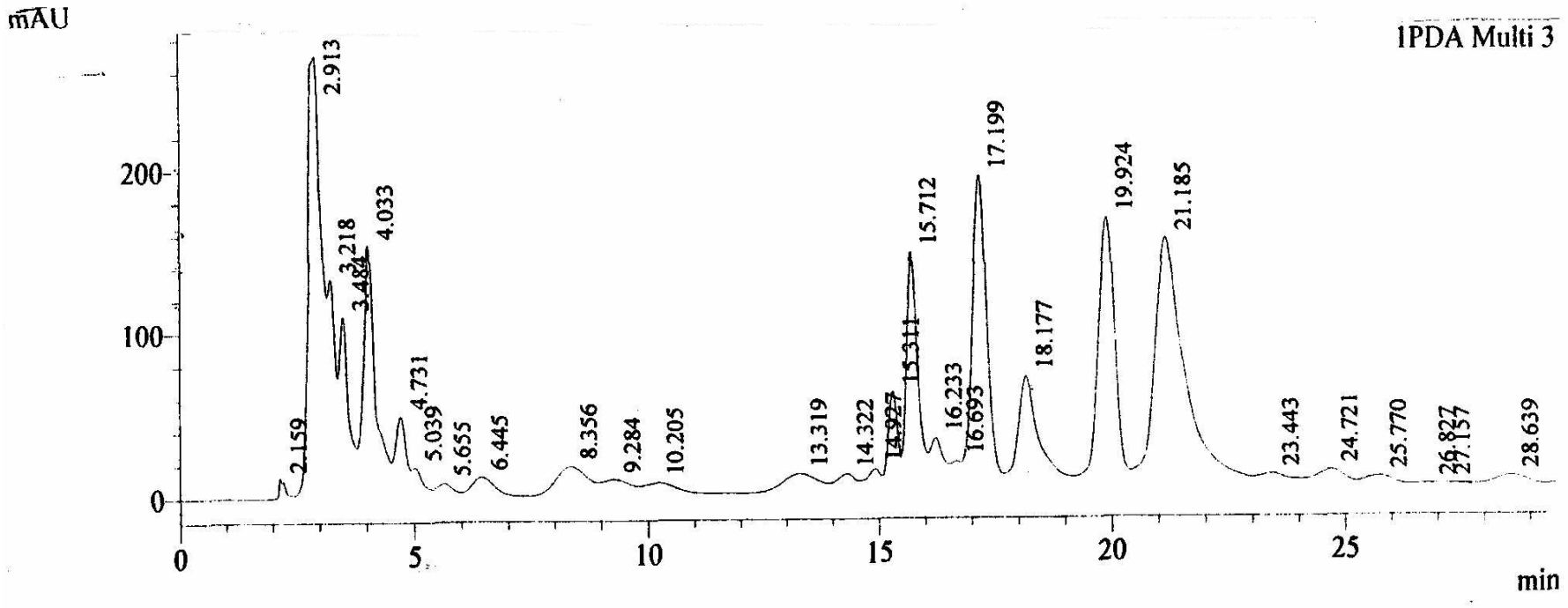
J) ^{1}HNMR of Compound C



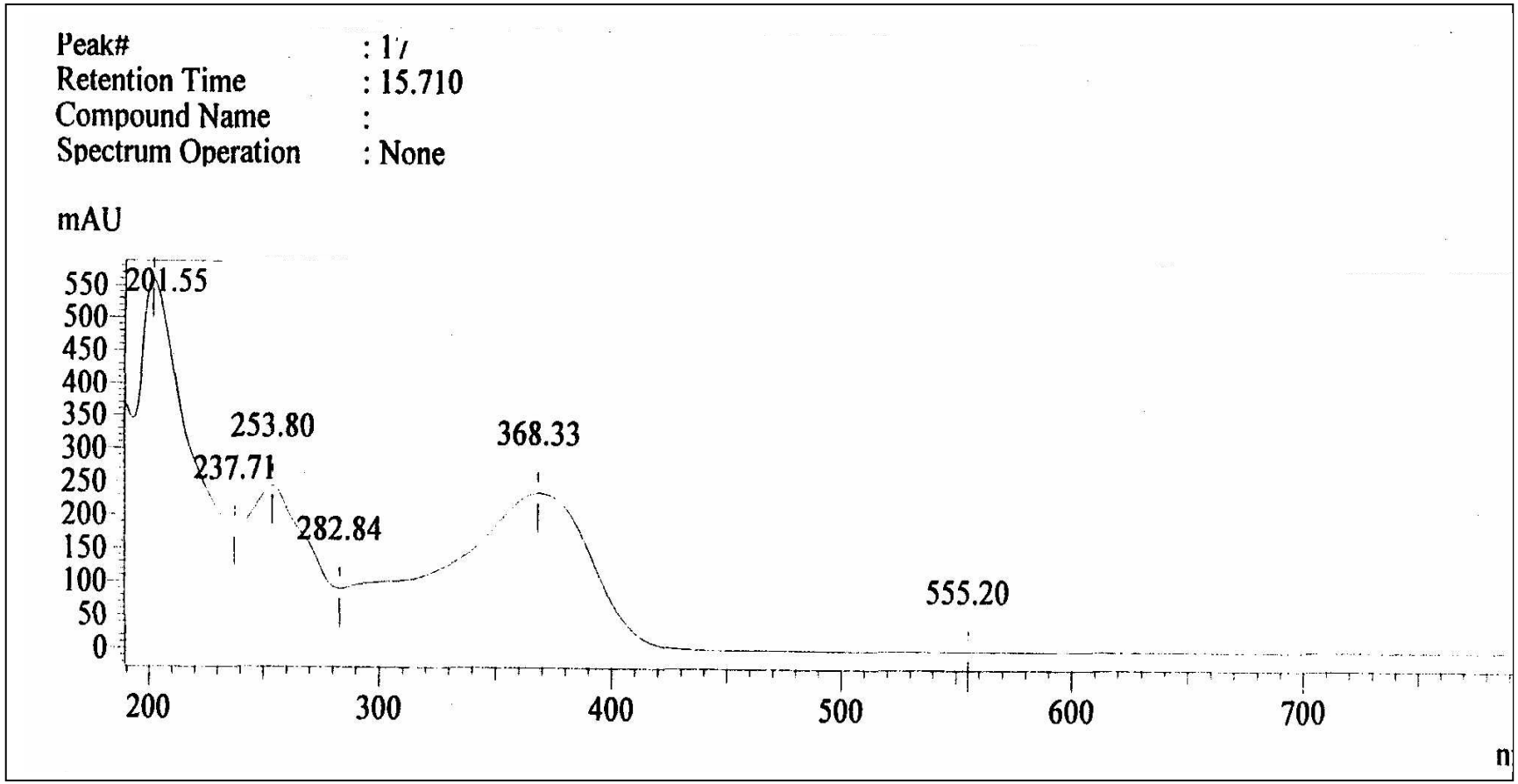
H

APPENDIX 2

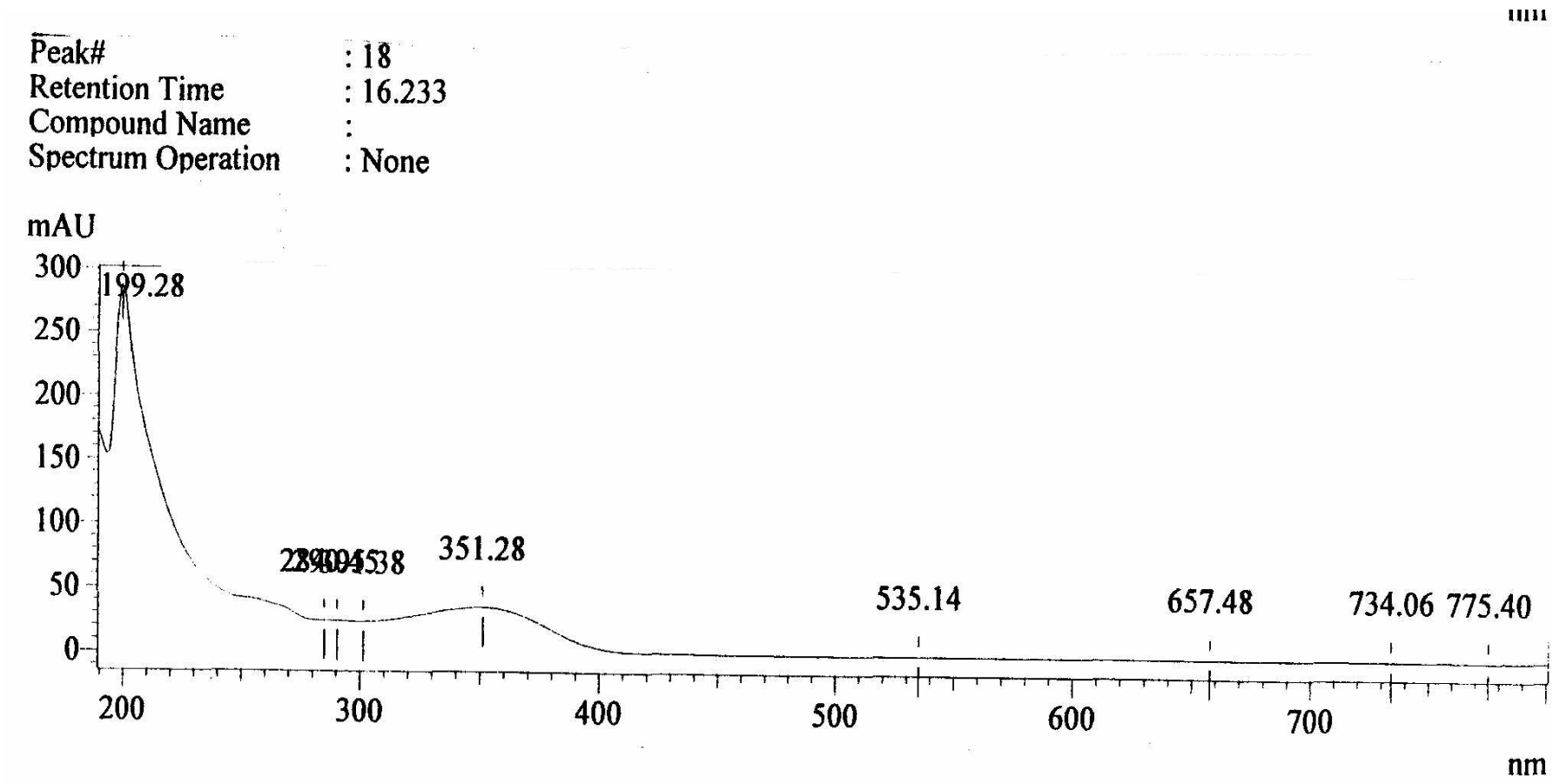
HPLC spectrum of the methanolic leaf extract of *Chromolaena odorata*.



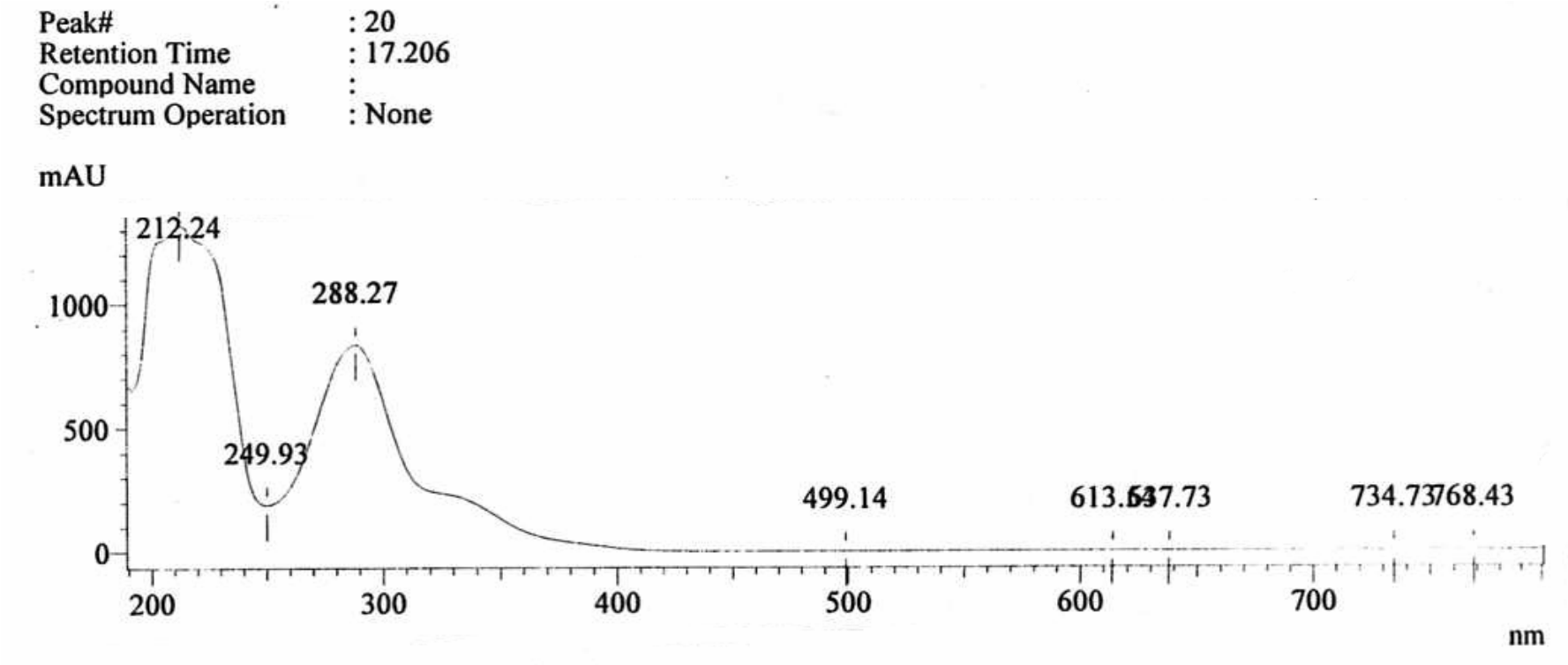
B) HPLC spectrum of peak No. 17 of the methanol extract of *C. odorata*



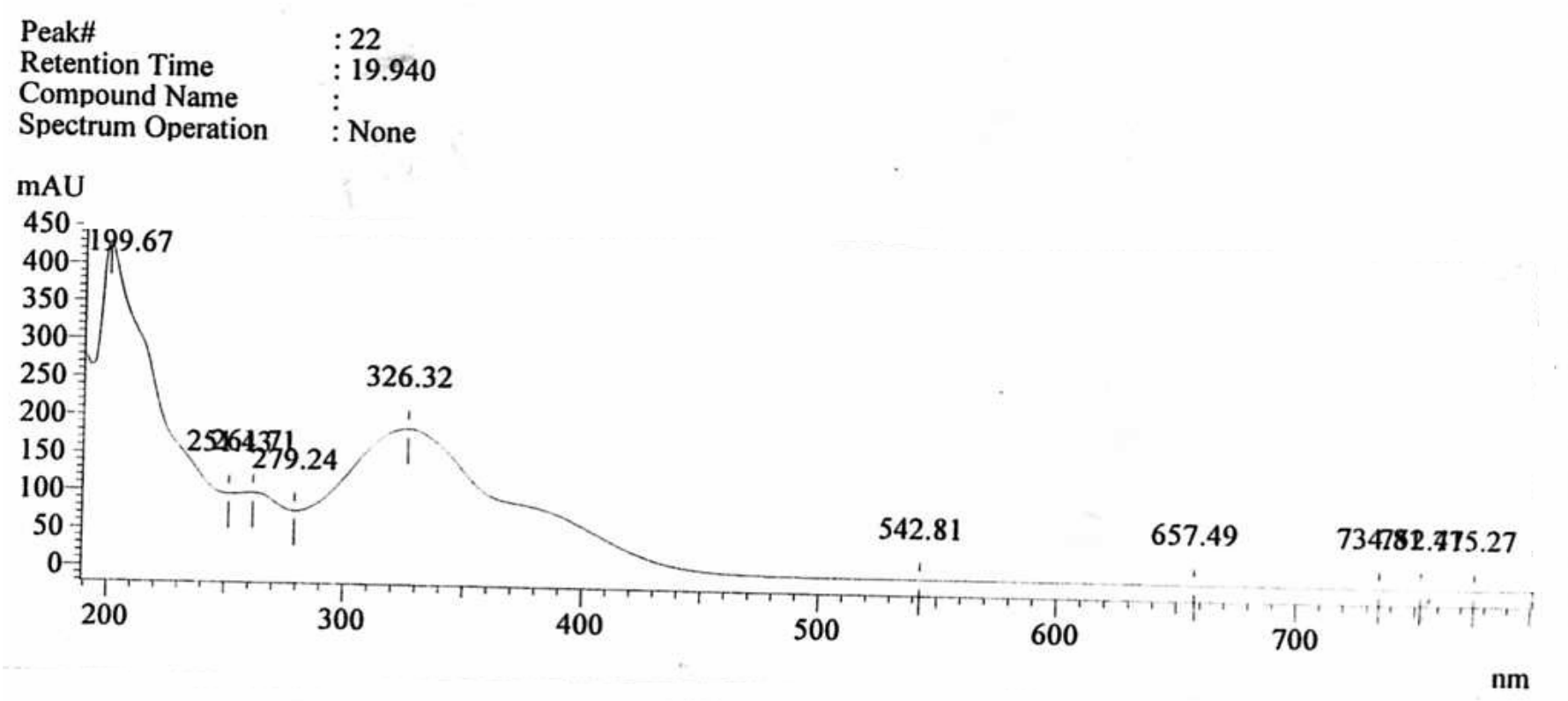
C) HPLC spectrum of peak No. 18 of the methanol extract of *C. odorata*



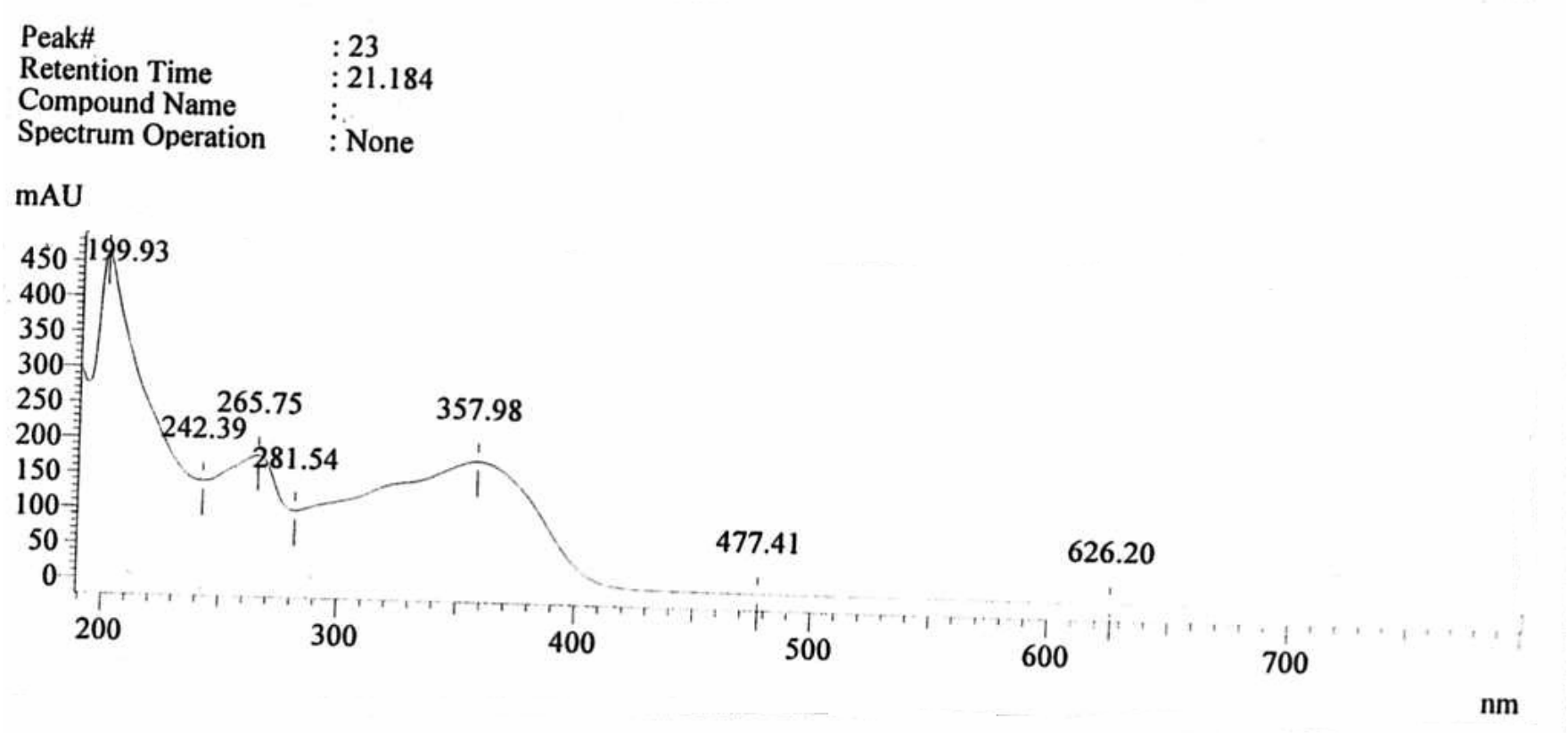
D) HPLC spectrum of peak No. 20 of the methanol extract of *C. odorata*



E) HPLC spectrum of peak No. 22 of the methanol extract of *C. odorata*

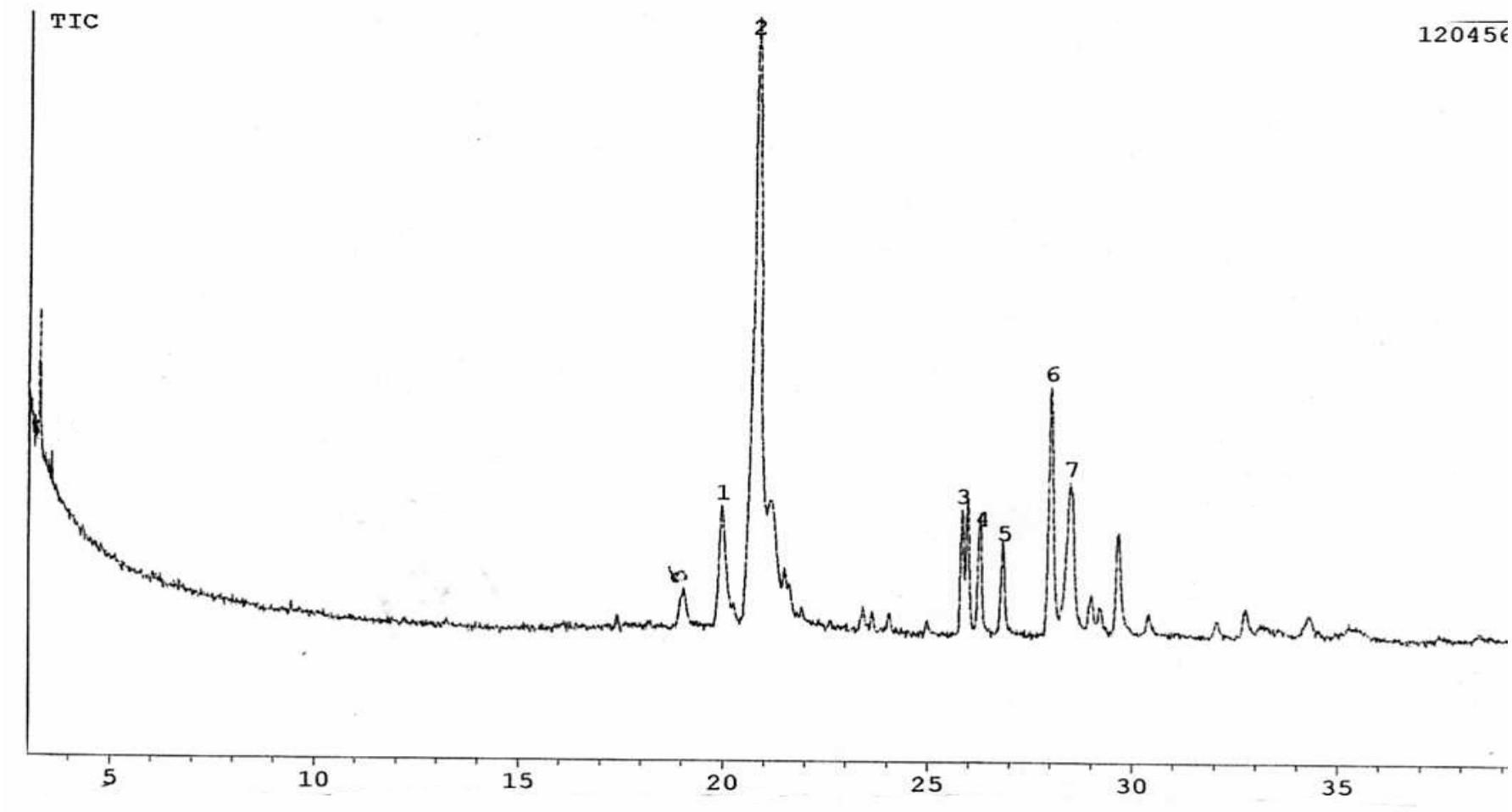


F) HPLC spectrum of peak No. 23 of the methanol extract of *C. odorata*

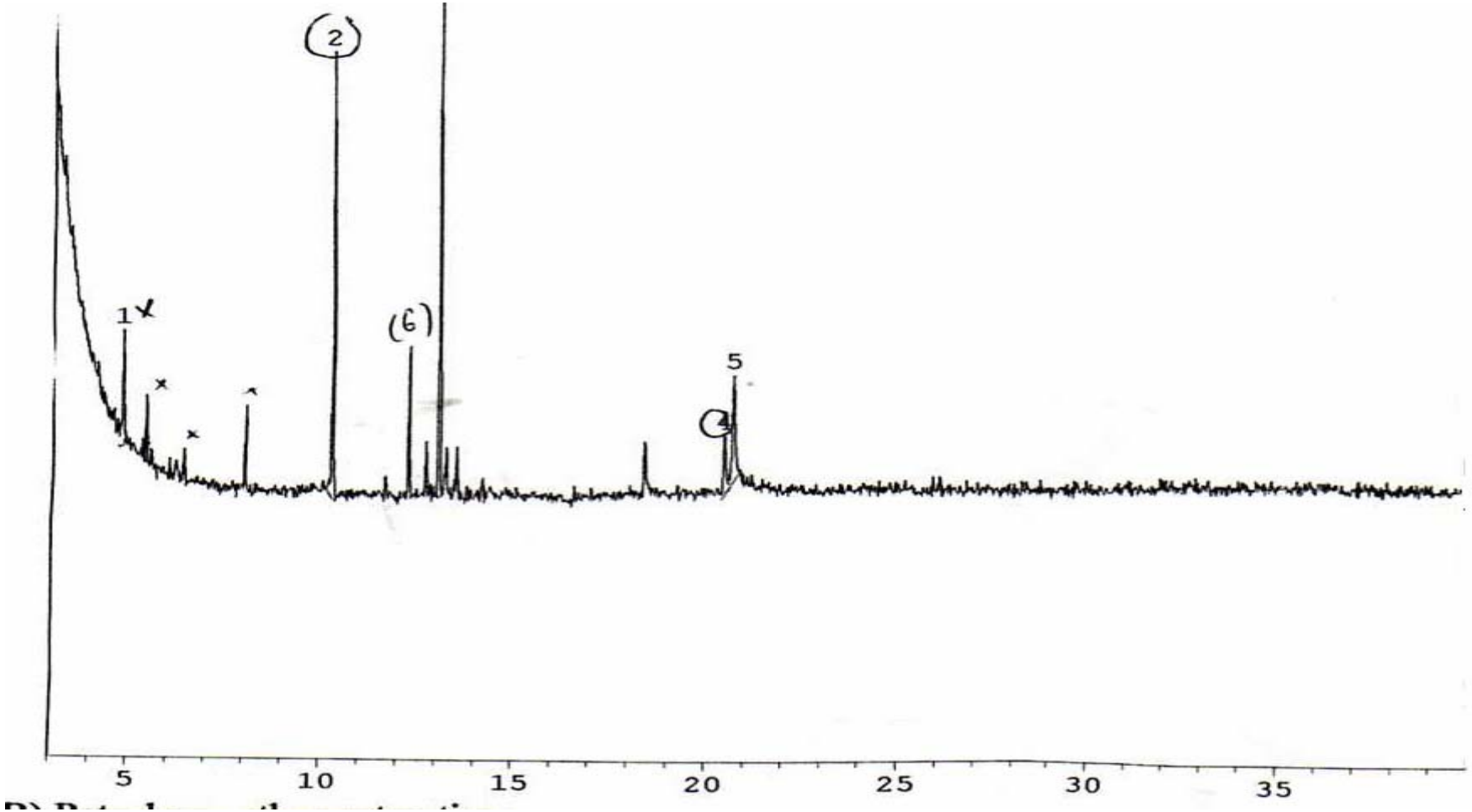


APPENDIX 3

A) GCMS Chromatogram of Chloroform Extract of the leaves of *Chromolaena odorata*



B) GCMS Chromatogram of Hexane Extract of the leaves of *Chromolaena odorata*



C) GCMS Chromatogram of Petroleum Ether Extract of the leaves of *Chromolaena odorata*