PHYTOCHEMICAL, BIOLOGICAL AND TOXICOLOGICAL STUDIES OF THE EXTRACTS OF YOUNG TWIGS AND LEAVES OF GRAY NICKER NUT (*Caesalpinia bonduc* (Linn)) Roxb

OLUBANKE OLUJOKE OGUNLANA

MATRIC NO: CUGP070176

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CERTIFICATION

This is to certify that Mrs. OGUNLANA Olubanke Olujoke (Matric No: CUGP070176) carried out this research work in partial fulfillment of the requirements for the award of Doctor of Philosophy (Ph.D.) degree in Biochemistry of Covenant University, Ota, under our supervision.

Prof. J.A. Olagunju		4/06/2012
(Supervisor)	**Signature**	**Date**
Prof. A.A. Akindahunsi		4/06/2012
(Co-Supervisor)	**Signature**	**Date**
Prof. Ning-Hua Tan		4/06/2012
(CAS-TWAS Host Supervisor)	**Signature**	**Date**

DECLARATION

It is hereby declared that this research work titled "Phytochemical, Biological and Toxicological Studies of the Extracts of Young Twigs and Leaves of Gray Nicker Nut (*Caesalpinia bonduc* (Linn)) Roxb" was undertaken by Ogunlana Olubanke Olujoke. It is based on her original study in the Department of Biological Sciences, College of Science and Technology, Covenant University, Ota and the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, China, under the supervision of Prof. J.A. Olagunju, Prof. A.A. Akindahunsi and Prof. Ning-Hua Tan and that the ideas and the views of other researchers have been dully expressed and acknowledged.

Prof. J.A. Olagunju Signature and Date  4/06/2012
Supervisor

Prof. A.A. Akindahunsi Signature and Date  4/06/2012
Co-Supervisor

Prof. Ning-Hua Tan Signature and Date  4/06/2012
(CAS-TWAS Host Supervisor)

Prof. L.O. Egwari Signature and Date
Head of Department of Biological Sciences
Covenant Univeristy, Ota, Ogun State, Nigeria.

Prof. F.K. Hymore Signature and Date
Dean, College of Science and Technology,
Covenant University, Ota, Ogun State, Nigeria

Prof. C.O. Awonuga Signature and Date
Dean, School of Postgraduate Studies,
Covenant University, Ota, Ogun State, Nigeria

DEDICATION

This thesis is dedicated to God Almighty Who alone is worthy of all my praise and adoration and Whom I recognize as my divine source, from whom I tapped all the wisdom and favour for the completion of this work. I thank you, Lord.

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LIST OF ABBREVIATIONS

^{13}C NMR	Carbon Nuclear Magnetic Resonance
1-D NMR	One-Dimensional Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
2D-NMR	Two-Dimensional Nuclear Magnetic Resonance
APIs	Active Pharmaceutical Ingredients
APS	Adenosine-5'-phosphosulfate
AUD	African Union Draft
b	Broad
BIAs	Benzylisoquinoline Alkaloids
C4H	Cinnamate-4-hydroxylase
CC	Column Chromatography
COSY	Correlation Spectroscopy
d	Doublet
DEPT	Distortionless Enhancement by Polarization Transfer
DMAPP	Dimethylallyl Pyrophosphate
DSHEA	Dietary Supplements Health and Education Act
EI-MS	Electronic Impact Mass Spectrometry
ESI-MS	ElesctroSpray Ionization Mass Spectrometry
FDA	Food and Drug Administration
FPP	Farnesyl Pyrophosphate
GC-MS	Gas Chromatography-Mass Spectrometry
GPP	Geranyl Pyrophosphate

HMBC	Heteronuclear Multiple Bond Correlation
HMG-CoA	3-hydroxyl-3-methylglutaryl-Coenzyme A
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Pressure Liquid Chromatography
HR-ESI-MS	High Resolution ElesctroSpray Ionization Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
IC_{50}	Concentration causing 50% inhibition
IPP	Isopentenyl Pyrophosphate
IR	Infra Red
L-DOPA	L-3,4-dihydroxyphenylalanine
m	Multiplet
m. p	Melting point
MPLC	Medium Pressure Liquid Chromatography
MTD	Maximum Tolerated Dose
MVA	Mevalonic Acid
NCEs	New Chemical Entities
NDA	New Drug Administration
NMR	Nuclear Magnetic Resonance
ODS	OctaDecyl Silica gel
OTC	Over-the-counter
PAL	Phenylalanine-ammonia Lyase
PAPS	3'-phosphoadenosine-5'-phosphosulphate
PMT	Putrescine *N*-methyltransferase

ppm	Part Per Million
ROESY	Rotating Frame Overhausser Enhancement Spectroscopy
s	Singlet
SAM	S- adenosyl-methionine
t	Triplet
TCB	Tan *Caesalpinia bonduc*
TIAs	Terpenoid indole alkaloids
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
TPAs	Tropane Alkaloids

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ABSTRACT

This study involved the isolation, purification and characterisation of the bioactive phytochemicals from the ethanolic extract of young twigs and leaves of *C. bonduc*, the determination of the antimalarial activity of each isolated phytochemical, and the investigation of their *in vivo* toxicological effects. Further extractions were carried out using petroleum ether, ethyl acetate, butanol and water. Bioassay-guided fractionations of petroleum ether and ethyl acetate fractions were carried out with a series of chromatographic separation techniques. Structural elucidation of the compounds was done by spectroscopic methods. The *in vitro* antimalarial activity and selective indices determinations of the extracts and compounds were carried out on chloroquine sensitive strain of *Plasmodium falciparum* FCR-3 and mouse mammary tumor cells FM3A respectively. The anticancer activity of the extracts and compounds was carried out on BGC-823 and HeLa cell lines. *In vivo* toxicity studies of the ethanolic extract of the plant were also undertaken. Recovery was assessed 14 days after dosing. Biochemical, haematological and histopathological examinations were carried out. The percentage yield of The ethanolic, petroleum ether, ethyl acetate, butanol and water fractions were 12.7%, 13.4%, 10.7%, 15.1% and 56.3% respectively. Phytochemical screening revealed the presence of all major classes of phytochemicals except phlobatannins. A total of fourteen characterised compounds (1 - 14) and thirteen uncharacterised pure samples (TCB 28 - 45) were isolated from *C. bonduc*. Two new compounds, 12α-ethoxyl-1α,14β-dihydroxyl-cass-13(15)-en-16,12-olide and 1α,7α-diacetoxy-5α,6β-dihydroxyl-cass-14(15)-epoxy-16,12-olide, are reported for the first time. Eleven others are reported from *C. bonduc* for the first time. The antimalarial activity of the ethyl acetate and petroleum ether fractions showed moderate activities. Three compounds also showed antimalarial activities. Moderate anticancer activity against HeLa cell lines was

observed with the petroleum ether, water and ethyl acetate fractions. Six compounds showed various anticancer activities against HeLa cells. However, only two compounds showed high anticancer activity against BGC-823 cell lines. The 28 days toxicological assessment of the plant indicated that evaluated biomarkers remained unchanged in rats dosed with extract at 200 mg/kg body weight, while significant changes were observed in rats at extract doses of 400 mg/kg body weight and above. There were no noticeable histopathological alterations in the cellular architecture of the tested organs of the control rats. Similarly, there were no alterations at an extract dose of 200 mg/kg body. However, at extract doses of 400 mg/kg body weight and above, there were induced histopathological alterations in the cellular architecture of the liver and kidney. No significant change was observed in the tested groups and the recovery groups in the sub-acute toxicity study. In the acute toxicological investigation, there was no mortality in the experimental animals at all treatment doses. However, there were significant alterations in the biomarkers of toxicity and induced cellular damage to the liver. In conclusion, the ethanolic extract of *C. bonduc* could be toxic to selected organs in the rat body on continuous high dosage. Moreover, *C. bonduc* contains a wide range of bioactive flavonoids, most of which possess good anticancer activities; some have moderate inhibitory activities against *P. falciparum*, but have poor selectivity indices for the mouse mammary tumor cell line.

CHAPTER ONE

INTRODUCTION

1.1 Background

Areas of high biodiversity, including tropical rainforests, are domains of chemical warfare. In the battle for survival, plants have evolved with many chemical defences as means of survival to ward off attackers such as bacteria, insects, fungi, severe weather and, in some cases, mammals that may threaten their existence. Secondary metabolites, while not essential for growth and development, do promote the spread and dominance of plant species in an ecological setting (Fellows and Scofield, 1995). As a result of this and the reported therapeutic activities associated with different phytochemicals, they are therefore worth the effort in research into the discovery of new drugs or as a viable alternative to the existing drugs.

A disease can be defined as an abnormal condition affecting the body of an organism. It is any condition that causes pain, dysfunction, distress and/or death to an organism. Malaria, a vector-borne disease caused by protozoan parasites, is widespread in tropical and sub-tropical regions, including parts of America, Asia, and Africa. About 124 million people in Africa live in areas at high risk of seasonal epidemic malaria. There are many more in areas outside Africa where transmission is less intense (Hay and Snow, 2006). Malaria remains a major parasitic disease in many tropical and sub-tropical regions of the world (Frederich *et al.,* 1999; WHO, 2011a). It appears to be the most prevalent of human diseases; as such it constitutes a major health hazard. About 500 million malaria cases are reported annually, resulting in 1 - 2 million deaths (Bradley, 1995), the most vulnerable groups being pregnant women and children under the age of five living in sub-Saharan Africa (Tracy and Webster, 2001). In fact, malaria accounts for more than 90 % of deaths of children in Africa below 5 years of age (Sachs and Malaney, 2002).

As a result of its associated high morbidity and mortality, concerted research efforts are currently being channeled into the eradication of the disease across the globe (Good, 2001).

The challenge of malaria, especially to sub-Saharan African nations, continues to widen without easily defined limits as drug resistance to most antimalaria drugs, insecticide resistance in mosquitoes and other climatic and socio-cultural factors complicate malaria research (Krettli *et al*., 2001). The development of resistance to antimalaria drugs by malaria parasites is the most disturbing of these factors. Today, the malaria parasite has been confirmed to show notable resistance to inexpensive drugs like chloroquine, quinine, sulphadoxine/pyrimethamine and a number of other drugs in this category. Newer drugs, however, cost 7-60 times as much as these (Olliaro *et al.*, 1996).

The challenge of drug resistance is leading malaria researchers in the direction of antimalarial medicinal plant research. According to the World Health Organization (WHO), 80 % of the world's population uses medicinal plants in the treatment of diseases. In African countries this rate is much higher (Ajose, 2007). In recent years, medicinal plants have represented a primary health source for the pharmaceutical industry (Phillipson, 1991). No less than 400 compounds derived from plants are currently used in the preparation of drugs, such as vincristine and vinblastine used in the treatment of cancer (Ajose, 2007) and quinine and artemisinin used as antimalarials (Phillipson, 1991). Investigation of the chemical constituents of medicinal plants has become a celebrated research issue (Phillipson, 1991).

1.2 The Study Plant: *Caesalpinia bonduc* (Linn) Roxb

Caesalpinia bonduc (family: *Caesalpiniaceae*, genus *Fabaceae*), commonly known as Gray Nicker nut or Fever nut in *English*, Sèyó or Ayóo in *Yoruba* (Sonibare *et al.*, 2009) and Bois

canic in *French*, is a prickly shrub with grey, hard, globular shaped seeds with a smooth shining surface (Nadkarni, 1954) (Figure 1.1). It is a medicinal plant predominantly distributed in the tropical and sub-tropical regions of Africa, Asia and the Caribbean (Gupta *et al.,* 2003). It has a lot of applications in folk medicine. The pharmacological screening of the plant extract has revealed its anti-helminthic, anti-cancer, anti-malarial, anti-hyperglycemic, anti-inflammatory, anti-rheumatic, anti-measles and anti-pyretic activities (Chakrabarti *et al.*, 2003; Gupta *et al.*, 2004; Sonibare *et al.*, 2009). Its anti-diuretic and anti-bacterial (Neogi and Nayak, 1958), anti-convulsant (Adesina, 1982), anti-anaphylactic, anti-diarrheal and anti-viral (Dhar *et al.,* 1968) activities have also been reported. Additionally, its anti-asthmatic (Gayaraja *et al.,* 1978), anti-amoebic and anti-estrogenic activities (Gupta *et al.,* 2003) as well as it nematocidal (Kjuchi *et al.*, 1989) and abortifacient (Datte *et al.,* 1998) activities have been documented. Hepatoprotective and antioxidant properties of *C. bonduc* have also been reported (Gupta *et al.,* 2003). The phytochemical analysis of the plant shows that it contains saponins, alkaloids, flavonoids, triterpenoids, diterpenoids, tannins and steroids (Kumar *et al.,* 2005).

The isolation of *Caesalpin-A* to *-F* (Pascoe *et al.,* 1986; Peter *et al.,* 1997a), *Caesalpin -Y, -J, Caesalpinin -1* (Peter *et al*., 1997b), *Caesalpinins -C to -G and Norcaesalpinins -A to -E* (Banskota *et al.,* 2003; Linn *et al.,* 2005) has been reported in the literature. The characterisation of *Neocaesalpins -A to -D, -W* (Kinoshita *et al.,* 1996; Kinoshita, 2000; Wu *et al.,* 2007), *Caesalpinolide* -A to -C and -E (Yadav *et al.,* 2007; Yadav *et al*., 2009), *Caesaldekarin -A, -C, -H* to *-L* (Lyder *et al*., 1998), 17-hydroxy-campesta-4,6-dien-3-one, 13,14-*seco*-stigmasta-5,14-dien-3a-ol, 13,14-*seco*-stigmasta-9, (Udenigwe *et al.,* 2007) has also been reported. The reports on the isolation of Bonducellpins -A to -G, Caesalpin -R, (Peter *et al.*, 1997a; Pudhom *et al.*, 2007), ε-caesalpin, caesalpinins -K, -P and -C (Pudhom *et al.,* 2007), Caesalmins -C to -G (Jiang *et al.*,

2001), Caesalpinianone (Ata *et al.,* 2009) and 7- hydroxyl-4-methoxyhomoisoflavones (*bounducelline*) have also been reported (Kumar *et al.,* 2005).

The antibacterial and antifungal activities of bondenlide, a diterpene from the seeds of *C. bonduc,* have also been reported (Simin *et al*., 2001). The bioactive constituents of *C. bonduc*, caesalpinianone, 6-O-methylcaesalpinianone and hematoxylol with moderate glutathione-S-transferase activity and 6'-O-acetylloganic acid, 4'-O-acetylloganic acid and 2-O-β-D-glucosyloxy-4-methy benzenepropanoic acid with anti-candida albicans activity have been reported (Ata *et al.,* 2009). *Caesalpinolide* -A and -B (Yadav *et al.,* 2007), isolated from the seeds of *C. bonduc,* have been shown to have inhibitory activity against cancer cell lines. New cassane furanoditerpenoids from the seed kernel of *C. bonduc* with good antimalarial activity against multidrug-resistant K1 strain of *Plasmodium falciparum* has been isolated and reported (Pudhom *et al.,* 2007). Isolated diterpenoids from the seeds of *C. crista* showed significant dose-dependent *in vitro* inhibitory effects on the growth of *Plasmodium falciparum* FCR-3/A2 clone (Linn *et al.,* 2005). Diterpenoids with cytotoxic activities against HL-60 (Human promyelocytic leukemia) and HeLa (Human cervical carcinoma) have been reported (Das *et al.,* 2010).

Figure 1.1: Photograph of *Caesalpinia bonduc*

One of the basic criteria set by WHO for the use of herbs as medicines is that they should be shown to be non-toxic. Although the use of ethno-medicine is widespread in Africa, most of the plants have not been thoroughly investigated for their toxicities (Sowemimo *et al.,* 2007). Scientific studies show that some medicinal natural products are potent organ toxins and also possess anti-fertility properties. These properties have been documented for quinine and chloroquine (Sairam, 1978; Meisel *et al.,* 1993; Adeeko and Dada 1998). The anti-fertility activities of extracts of *Carica papaya*, *Quassia amara* and *Azaridiracta indica* have also been documented (Loyiha *et al.,* 1994; Raji and Bolarinwa 1997; Raji *et al.,* 2003). Since there is no scientific information on the substantial toxicity profile of *C. bonduc,* toxicity data are required to predict the safety and effects of long term exposure to the plant (Aniagu *et al.,* 2005). It has been discovered that the young twigs and leaves of *C. bonduc* are readily used in the Southwestern part of Nigeria as antimalarials. Thus, the need for safety and therapeutic evaluation pre-informed this study. Most of the studies previously carried out on this plant have been done on its seed extract. This study however reported detailed studies on the extracts from the young twigs and leaves of *C. bonduc.*

1.3 Justification/Rationale for the study

Malaria remains a major parasitic disease in many tropical and subtropical regions of the world (Frederich *et al.,* 1999). It appears to be the most prevalent human disease, especially in those areas, and constitutes a major health hazard. Its occurrences in 2006 were estimated in Africa (86%), followed by South-East Asia (9 %) and Eastern Mediterranean regions (3%) (Oliveira *et al.*, 2009). According to the World Health Organization (WHO), 80% of the world's population uses medicinal plants as the main primary health care source in the treatment of

diseases. In African countries the rate is much higher (Ajose, 2007). Limited scientific evidence regarding safety and efficacy to support the continued therapeutic application of some of these herbal remedies exists compared to such evidence for synthetically formulated drugs (Sowemimo *et al.,* 2007). The utilization of the medicinal plants is often based on ancestral experience. With the upsurge in the use of herbal remedies, there is a need for a thorough scientific evaluation to validate or disprove the supposedly therapeutic effects of some of these medicinal plants. Moreover, a number of compounds extracted from traditional plants have not been thoroughly studied for toxicity and efficacy. The leaves and young twigs of *C. bonduc* are used in Nigeria by herbal practitioners in the treatment of malaria infection together with other medicinal plants. There is therefore the need to study the toxicity of *C. bonduc*, and to determine its biological activities as well as its efficacy.

1.3 General Objectives

The current study aims at investigating *in vitro* the folkloric claims for the antimalarial activity of *C. bonduc* against *P. falciparium.* The research also aims at determining the phytoconstituents of the plant, as well as assessing the toxicity profile of its extracts.

1.4 Specific Objectives

The specific objectives of the study are to:

a) determine the phytochemical constituents and carry out partial characterisations of the extracts of the young twigs and leaves of *C. bonduc*;

b) determine the chemical structures of the isolated bioactive components;

c) investigate the *in vitro* antimalarial activity of the extracts; and

d) determine the toxic effects of the extracts of *C. bonduc* in albino rats using biochemical, haematological, and histological indices of toxicity.

CHAPTER TWO

LITERATURE REVIEW

2.1 Medicinal Plants

Plants provide a variety of resources that contribute to the fundamental needs of food, clothing and shelter. Among plants of economic importance are medicinal plants. Medicinal plants are groups of plants with vital roles in alleviating human suffering (Baquar, 2001). Plants have been utilized as therapeutic agents since time immemorial in both organized and unorganized forms (Girach *et al.,* 2003). The healing properties of many herbal medicines have been recognized in many ancient cultures.

2.1.1 History of Medicinal Plants in Phytotherapy

To trace the history of medicinal plants in phytotherapy is to trace the history of humanity itself. The discovery of the curative properties of certain plants must have sprung from instinct. Plants must have first been explored as sources of food. As a result of food ingestion, a link with some plant properties must have been established (Mendonça-Filho, 2006). Medicinal plants have been used for the treatment of various diseases for thousands of years. The use of terrestrial plants as medicines has been documented in Egypt, China, India and Greece from ancient times, and an impressive number of modern drugs have been developed from them. The first written records on the uses of medicinal plants are from Sumerians and Akkaidians, who described the well established medicinal uses of laurel, caraway and thyme; this record was dated about 2600 BC (Samuelsson, 1999).

The “Ebers Papyrus”, the best known Egyptian pharmaceutical record, which documented over 700 drugs, represents the history of Egyptian medicine dated from 1500 BC; garlic, opium,

castor oil, coriander, mint and other herbs have been listed as medicines. The Chinese manual, Materia Medica, "the Shenlong Bencao Jing", a documented record dating from about 1100 BC, also listed over 365 medicines, 252 of which were medicinal plants (Cragg *et al.*, 1997). The Ayurvedic system recorded the use of turmeric as medicine in Susruta and Charaka dating from about 1000 BC (Kappor, 1990). The Greeks also contributed substantially to the development of herbal drugs. Pedanius Dioscorides, the Greek physician (100 A.D.), described in his work "*De Materia Medica*" more than 500 medicinal plants (Samuelsson, 1999). The Pedanius documentation is considered to be the precursor to all modern pharmacopeias and it is considered one of the most influential herbal books in history.

Medicinal plants were the major sources of products used to sustain health until the nineteenth century. In 1828 the German chemist Friedrich Wohler, in an attempt to prepare ammonium cyanide from silver cyanide and ammonium chloride, accidentally synthesized urea. This was the first organic synthesis in history and it heralded the era of synthetic compounds (Mendonça-Filho, 2006).

2.1.2 Importance of Medicinal Plants in Drug Discovery

Numerous methods have been utilized in drug discovery, including isolation of compounds from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modeling (Ley and Baxendale, 2002; Geysen *et al.*, 2003; Lombardino and Lowe, 2004). Despite the recent interest of pharmaceutical companies and funding organizations in molecular modeling, combinatorial chemistry and other synthetic chemistry techniques, natural products (in particular, medicinal plants), remain an important source of new drugs, new drug leads and new chemical entities (NCEs) (Newman *et al.*, 2000; Butler, 2004). Between 2001 and

2002, approximately one quarter of the best-selling drugs worldwide were natural products or were derived from natural products (Butler, 2004). Approximately 28 % of NCEs that appeared between 1981 and 2002 were natural products or natural product-derived (Newman *et al.,* 2003). Another 20 % of NCEs that appeared during this time period were considered natural product mimics, implying that the synthetic compounds were derived from the study of natural products (Newman *et al.,* 2003). Combining these categories, research on natural products accounts for approximately 48 % of the NCEs reported from 1981-2002. In Table 2.1 is the summary of some of the most economically important pharmaceuticals and their precursors derived from plants.

Natural products provide a starting point for new synthetic compounds with diverse structures, and often with multiple stereo-centres that can be challenging synthetically (Clardy and Walsh, 2004; Nicolaou and Snyder, 2004; Peterson and Overman, 2004; Koehn and Carter, 2005). Many structural features common to natural products (e.g. chiral centres, aromatic rings, complex ring systems and degree of molecule saturation) have been shown to be highly relevant to drug discovery efforts (Lee and Schneider, 2001; Feher and Schmidt, 2003; Clardy and Walsh, 2004; Piggott and Karuso, 2004; Koehn and Carter, 2005). Furthermore, since the escalation of interest in combinatorial chemistry, with the subsequent realization that these compound libraries may not always be very diverse, many synthetic and medicinal chemists are exploring the creation of natural product and natural-product-like libraries that combine the structural features of natural products with the compound-generating potential of combinatorial chemistry (Hall *et al.,* 2001; Eldridge *et al.,* 2002; Burke *et al.,* 2004; Ganesan, 2004; Tan, 2004). Drugs derived from medicinal plants can serve not only as new drugs themselves but also as drug leads suitable for optimization by medicinal and synthetic chemists (Balunas and Kinghorn, 2005).

Table 2.1: Some of the most economically important pharmaceuticals and their precursors derived from plants

Plant names	Compounds	Class	Therapeutic use
Apocyanaceae, Rubiaceae spp.	Yohimbine	Indole alkaloid	Aphrodisiac
Artemisia annua L.	Artemisinin	Sesquiterpene lactone	Antimalarial
Camptotheca acuminata Dence	Camptothecin	Indol alkaloid	Antineoplastic
Capsicum spp.	Capsaicin	Phenylalkyl-amine alkaloid	Topical analgesic
Cassia angustifolia Vahl.	Sennosides A and B	Hydroxy anthracene glycosides	Laxatine
Catharanthus roseus L.	Vinblastin, vincristine	Bis-indole alkaloid	Antineoplastic
Cephaelis ipecacuanha (Brot.) A. Rich.	Ipecac	Mixture of ipecac alkaloids and other components	Emetic
Cephaelis ipecacuanha (Brot.) A. Rich.	Emetine	Isoquinoline alkaloid	Antiamoebic
Chondodendron tomentosum Ruiz, *Strychnos toxifera* Bentham	Tubocurarine	Bisbenzyl isoquinolone alkaloid	Skeletal muscle relaxant
Cinchona spp.	Quinine	Quinoline alkaloid	Antimalarial
Cinchona spp.	Quinidine	Quinoline alkaloid	Cardiac depressant
Colchium autumnale L.	Colchicine	Isoquinoline alkaloid	Antigout
Digitalis spp.	Digoxin, digitoxin	Steroidal glycosides	Cardiotonic
Erythroxylum coca Lamarck	Cocaine	Cocaine alkaloid	Local anesthetic
Leucojum aestivum L	Galanthemine	Isoquinoline alkaloid	Cholinesterase inhibitors
Nicotiana spp.	Nicotine	Pyrrolidine alkaloid	Smoking cessation therapy
Papaver somniferum L	Codeine, morphine	Opium alkaloid	Analgesic, antitussive
Physostigma venenosum Balfor	Physostigmine	Indole alkaloid	Cholinergic
Pilocarpus jaborandi Holmes	Pilocarpin	Imidazole alkaloid	Cholinergic
Podophyllum peltatum L.	Podophyllotoxin	Lignan	Antineoplastic
Taxus brevifolia Nutt.	Taxol and other taxoids	Diterpenes	Antineoplastic
Camptotheca acuminate Decne	Camptothecin	Diterpenes	Antineoplastic

(Adapted from van Agtmael *et al.,* 1999; Graul, 2001; Pirttila *et al.,* 2004; Butler, 2004; Cragg and Newman, 2004 and Ahmad *et al.,* 2006)

2.2 Traditional Systems of Medicine

2.2.1 Historical and Current Perspective

Herbs and herbal preparations have been used to treat ailments since pre-historic times, and the treatment of various diseases with plant-based medicines has remained an integral part of many cultures across the globe. Such medicines, derived directly or indirectly from plants, constitute over 25 % of the pharmaceutical arsenal (Ahmad *et al.*, 2006). Traditional medicine has attracted more attention worldwide since the latter part of the twentieth century. About 80 % of Africans have been reported to use traditional medicines to meet their health care needs (WHO, 2000). This is primarily due to the recognition of the value of traditional and indigenous pharmacopeias, the need to make health care affordable for all and the perception that natural remedies are somehow safer and more efficacious than remedies that are pharmaceutically derived (Murphy, 1999).

Over the past two decades, two apparently unrelated trends in the biomedical and biotechnological development of medicinal products have been described. There has been a rapid development of recombinant DNA technology and related procedures to provide biomedical proteins and related therapeutic drugs, prophylactic vaccines and diagnostic agents (Chan, 1996). At the same time, the growth in popularity of over-the-counter (OTC) health foods (nutraceuticals) and herbal products has taken a very large share of the health-care market (Raskin *et al.,* 2002). This entire trend has proven the need for continuous development of the knowledge base of the utilization of medicinal plants (Ahmad *et al.,* 2006).

2.2.2 Asian Traditional Medicine

The most established herbal therapeutic systems are Ayurveda, Unani and Siddha of Indian origin, WU-Hsing (China) and Kampo (Japan). China has possibly the greatest amount of documentation concerning herbal plants than any country in the world. The knowledge in Chinese medicine was accumulated over thousands of years and has been confirmed through both empirical experience and scientific evaluation (http://www.sahealthinfo.org/traditionalmeds/traditionalpart2.pdf). Most of the herbal remedies are mixtures of plants and, at times, in combinations with animal parts and minerals. Under ideal conditions, care is taken by traditionally trained practitioners to identify the ingredients, to harvest the plants at very specific times to ensure appropriate levels of bioactivity, to prepare the remedies under strict rules and to prescribe them to achieve an appropriate clinical response (Elvin-Lewis, 2001).

2.2.3 European Traditional Medicine

European traditional medicine has its roots mostly in ancient Mediterranean civilizations and in plants from the South. By the nineteenth century some of the medicinal plants had become part of the pharmacopeias of allopathy, naturopathy, and homeopathy. Usually when compounds are isolated and sometimes synthesized, their pharmaceutical uses are more carefully regulated (De Smet, 1997).

2.2.4 Neo-Western Traditional Medicine

In its totality, European traditional medicine has matured, along with American herbal medicine, into neo-Western herbalism. In this system, single plant preparations that have been

either selected from formulations found in ancient pharmacopeias, or derived from medicinal plants valued in other countries (including those of indigenous origin), are sold alone or as mixtures in an assortment of combinations (Lewis and Elvin-Lewis, 1977; De Smet, 1995; Elvin-Lewis, 2001).

2.2.5 African Traditional Medicine

In some African countries, traditional medicine is an integral part of the formal health system, on an equal status with modern medicine; but in others this is not the case. African traditional medicine, although important for individuals and communities, remains a form of private practice outside the formal health system, one that cannot be easily organized by the government. Current estimates suggest that, in many African countries, a large proportion of the population rely heavily on traditional practitioners and traditional medicines to meet their primary health-care needs (African Union Draft (AUD), 2007). Although modern medicine may be available in these countries, traditional medicine has often maintained its popularity for historical, holistic approaches and for cultural reasons. Some African countries have developed national policies on traditional medicine, which include a legal framework, and a code of ethics and conduct for the practice of traditional medicine. Some have put in place mechanisms for the regulation of traditional medicines practice: legislation, regulatory frameworks and institutional instruments for developing African traditional medicine and for locally producing commercial quantities of standardized African traditional medicines. Other countries have further moved towards integrating/institutionalising traditional medicine into the public health care system (African Union Draft, 2007).

2.3 Modern Phytomedicine

Phytomedicine is the emergence of phytotherapy as a separate therapeutic system of medicine based on the traditional usage of plants as drugs and the extraction of active substances from plants as adapted by some countries, such as German–speaking countries (Ahmad *et al.*, 2006). Phytotherapy may be divided into two, namely, rational phytotherapy (herbal medicinal products) and traditional phytotherapy. In rational phytotherapy, appropriate pharmacological investigations and clinical trials have documented the efficacy of the products employed, while in traditional phytotherapy the efficacy of phytopharmaceuticals or herbal teas has not yet been established (Ahmad *et al.,* 2006).

2.3.1 Prospects of Herbal Medicine

Herbal medicine and other plant-derived therapeutics or prophylactic products in various forms have been available for many hundreds of years for the treatment of diseases. More than 25 % of marketed orthodox pharmaceuticals are either derived from plant sources or from derivatives of secondary plant metabolites. In Table 2.1 is the list of some of the most economically important pharmaceutical and their precursors derived from plants (Bhattaram *et al.,* 2002; Ahmad *et al.,* 2006). The United States (US) Food and Drug Administration (FDA) has published guidelines for the standardization of the multicomponent plant extracts referred to as botanical drugs, thus making marketability possible under the New Drug Administration (NDA) approved process (Ahmad *et al.,* 2006). The following are common botanical dietary supplements sold in the United States of America: *Echinacea purpurea*, *Panax ginseng*, *Serono repens*, *Ginkgo biloba*, *Hypericum perforatum* (St. Johns wort), *Valeriana officinalis, Allium sativum*, *Hydrastis canadensis*, *Matricaria chamomilla*, *Silybum marianum*, *Trigonella foenum-graecum*,

Tanacetum parthenium, Ephedra sinica, and *Cimicifuga racemosa* (Raskin *et al.,* 2002). Presentyly, the basis for marketing these products in the US is the Dietary Supplements Health and Education Act (DSHEA) of 1994, which allows manufacturers to market products as dietary supplements without the rigorous testing required for other drug products (Ahmad *et al.,* 2006).

The approach of the Canadian Health Protection Branch with respect to herbal products is very similar to the FDA's, whereas several European countries have more advanced legislative regulations for herbal products (Ahmad *et al.,* 2006). Rapid growth has been seen in the herbal medicine market in recent years, as increasing numbers of consumers are persuaded by the benefits of plant extracts as alternatives to medicinal products with chemically derived Active Pharmaceutical Ingredients (APIs) (Greger, 2001). In 1999 the global market for herbal supplements exceeded US$15 billion, with a US$7 billion market in Europe, US$2.4 billion in Japan, and US$2.7 billion in the rest of Asia, and US$3 billion in North America (Glasser, 1999). It has been estimated that the market for branded non-prescription herbal medicine grew from $1.5 billion in 1994 to $4.0 billion in 2000 in the US alone. A similar trend is also being followed in European and African countries (De Smet *et al.,* 2000).

2.3.2 Constraints in Herbal Medicine

2.3.2.1 Reproducibility of Biological Activity of Herbal Extracts

A major constraint limiting the ready adoption of scientific reports on medicinal plants as pharmaceuticals discoveries is the lack of reproducibility of the activity of over 40 % of plant extracts (Cordell, 2000). This is a major problem in phytomedicine, as the activities detected in screens often are nogt reproduced when the same plants are re-sampled. This problem is largely due to differences in the biochemical profiles of plants harvested at different times and locations,

differences in variety, and variations in the methods of extraction and evaluations of biological activities. Furthermore, the activity and efficacy of plant extracts/medicines often result from additive interaction effects of the components (Ahmad *et al.,* 2006). Therefore, a strategy should be used to evaluate the qualitative and quantitative variations in the contents of bioactive ingredients of plant materials. It is also important to identify the different agro-climatic or stress locations, climate, microenvironmental, physical and chemical stimuli, often called elicitors, which quantitatively and qualitatively alter the content of bioactive secondary metabolites (Ahmad *et al.,* 2006).

2.3.2.2 Toxicity and Adverse Effects of Plants extracts

It is popularly believed that medicinal plants are safer than pharmaceuticals because they are of natural origin. However, recent scientific reports have demonstrated that several medicinal plants used in phytomedicine are potentially toxic, and some are even mutagenic and/or carcinogenic (De SaFerrira and Ferrago-Vargas, 1999). The toxicity for herbal drugs therefore depends on their purity, herbal combinations, absorption, bioavailability and reported adverse effects. Toxicity in phytomedicine may be due to the followings:

Dearth of reports on the side effect of medicinal plants;

Errors in botanical identification;

Accidental ingestion of cardiotonic plants,

Inappropriate combinations in phytotherapy; and

Interference of medicinal plants and conventional pharmacological therapy, such as plants containing coumarinic derivatives, a high content of tyramine, estrogenic compounds, plants causing irritation and allergic problems (Goldman, 2001; Wojcikowski *et al.,* 2004).

2.3.2.3 Adulteration and Contamination

Medicinal plant adulteration is the intentional inclusion of impurities or undesirable substances in medicinal plant combination while contamination is the mistaken inclusion of undesirable substances in medicinal plant combinations. This is common in countries that are lenient with regard to enforcement of their regulatory laws regarding purity. Adulteration in herbal medicine is particularly disconcerting because it is unpredictable. Often it remains undetected unless it can be linked to an outbreak of disease or epidemic. An example is the reported case of veno-occlusive disease due to the ingestion of plants containing pyrrolidizine alkaloids, which can be life threatening or fatal (Drew and Myres, 1997).

In many cases, contaminated or adulterated medicinal combinations can cause significant medical problems, especially in children (Ernst and Coon, 2001; Saper *et al.,* 2004). In a review on heavy metal poisoning in children consuming medicinal plant medicines, 13 cases were reported from Singapore, Hong Kong, the United States of America, the United Kingdom and the United Arabic Emirates from 1975 to 2002 (Ahmad *et al.,* 2006). The Indian Government has initiated a major programme under which pharmacopeia standards are developed for medicinal plants used in the *Ayurveda, Unani,* and *Siddha* systems of traditional medicine (Ahmad *et al.,* 2006). The resultant pharmacopeia will enhance a good knowledge of the constituents of herbal medicines and help to standardise the preparation of herbal drugs. The adulteration of most herbal preparations has been attributed to the improper identification of plants. This has resulted in a number of serious cases, primarily due to poisoning by digitalis and skullcap (Elvin-Lewis, 2001). In 1998, the California Department of Health, USA, reported that 32% of Asian patented medicines sold in the country contained undeclared pharmaceuticals or heavy metals (Ko, 1998;

Marcus and Grollman, 2002,). The Food and Drug Administration (FDA), USA and other scientific reports have also indicated or revealed the presence of prescription drugs, including glyburide, sildenafil, colchicines, adrenal steroids and alprazolam in medicinal products claiming to contain only natural ingredients (Ernst, 2002).

2.3.2.4 Herb–Drug Interactions

The dose of many medicinal herbs and pharmaceutical drugs is the major determinant of their therapeutic or toxic activity. Herbal medicines act through a variety of mechanisms to alter the pharmacokinetic profile of concomitantly administered drugs (Fugh-Berman, 2000). For example, *St John's wort*, has been reported to induce the cytochrome P450 isozyme CYP 3A4 and intestinal P-glycoproteins, accelerating the metabolic degradation of many drugs including cyclosporin, antiretroviral drugs, digoxin, and warfarin (Moore *et al.,* 2000). The interactions between concomitantly administered herbs may potentiate or antagonise a patient's metabolism, drug absorption, or elimination, thus interfering with the pharmacology or toxicology profile of the drug and herb. Synergistic therapeutic effects may also complicate the dosing of concomitantly administered herbs and drugs used in long-term medication. For example, herbs traditionally used to decrease glucose concentrations in diabetes could theoretically precipitate hypoglycaemia if taken for a long time in combination with conventional drugs (Bailey and Day, 1989).

Herbal medicines are ubiquitous; however, the dearth of reports on their adverse effects and interactions could probably be a reflection of a combination of under-reporting and the benign nature of most herbal products. Limited experimental data, unprescribed usage, lack of proper regulatory controls, especially in the developing world where they are locally used, may be

some of the factors responsible for the dearth of reports in this area. As a result, care should be taken to understand the effects of foods or herbal medicines in anticoagulant therapy, treatment of diabetes, depression, pain, asthma, heart conditions, or blood pressure disorders, and slimming (Elvin-Lewis, 2001). Scientific data on the interactions of various medicinal plants with drugs, their pharmacokinetics and bioavailability profiles should be evaluated (Bhattaram *et al.,* 2002).

Table 2.2: Documented reports on some medicinal plants and their adverse effects

Plant names	*Vernacular name*	*Part used*	*Common use*	*Adverse effect (in large doses)*
Aborus precatorius L.	Indian liquorice	Seed	Diarrhea, dysentery, paralysis and skin diseases, antiseptic, uterine stimulant and anticancerouss,	Abrin causes edema and ecchymosi inflammation antifertility activity, antiestrogenic activity, abortifacient and oxytocic activity
Aconitum casmanthum Stappex Holm	Aconite	Rhizome	Neuralgia, rheumatism, cardiac tonic and nerve poisons	Narcotic, powerful sedative, arrhythmia and hypertension
Gloriosa superba L.	Malanbar glory lily	Root	Anthelmintic, purgative, emetic, antipyretic, expectorant and toxic	Antifertility, vomiting, purging, gastrodynia and burning sensation
Croton tiglium L.	Croton	Seed	Abdominal disorders, constipation, helminthiasis, inflammation, leukoderma and oedema	Depressor responses and neuromuscular blockade
Cannabis sativa L.	Hemp	Leaf	Antidiarrhetic, intoxicating, stomachic and abdominal disorders,	Neurotoxic, respiratory arrest, nausea tremors, insomnia, sexual impotence and gastrointestinal disturbance
Datura metel L.	Thorn apple	Seed and leaf	Antihelminthic and anticancerous	Insanity
Euphorbia neriifolia	Milk hedge	Latex	Insecticidal and cardiovascular	Emetic, irritant, apnea and pathological changes in liver, heart and kidney
Papaver somniferum L.	Poppy	Exudate	Diarrhoea, dysentery, sedative, narcotic and internal hemorrhages	Highly narcotic
Semecarpus anacardium	Marking nut	Fruit	Antiseptic, cardiotoxic, anticarcinomic liver tonic and uterine stimulants	Abortive
Nerium indicum Mill	Oleander	Fruit and leaf	Antibacterial, ophthalmic and cardiotonices	Cardiac poison, paralysis and depressed respiration, gastrointestinal, neurological and skin rashes

(Adapted from Ahmad *et al.,* 2006)

Table 2.3 Herbal preparations - drugs interactions

Herb and drug(s)	Results of interaction	Comments
Betel nut (***Areca catechu***)		
Flupenthixol and procyclidine	Rigidity, bradykinesia, jaw tremor	Betel contains arecoline, a cholinergic alkaloid.
Fluphenazine	Tremor, stiffness	
Prednisone and salbutamol	Inadequate control of asthma	Arecoline challenge caused dose-related bronchoconstriction in six asthma patients
Chilli pepper (***Capsicum*** spp)		
ACE inhibitor	Cough	
Theophylline	Increased absorption and bioavailability	
Danshen (***Salvia miltiorrhiza***)		
Warfarin	Increased INR, prolonged PT/PTT	In rats, danshen decreases elimination of warfarin. Danshen is in at least one brand of cigarettes.
Devil's claw (***Harpagophytum procumbens***)		
Warfarin	Purpura	
Dong quai (***Angelica sinensis***)		
Warfarin	Increased INR and widespread bruising	Dong quai contains coumarins.
Eleuthero or Siberian ginseng ***(Eleutherococcus senticocus***)		
Digoxin	Raised digoxin concentrations	Herb probably interfered with digoxin assay, patient had unchanged ECG despite digoxin concentration of 5·2 nmol/L).
Garlic (***Allium sativum***)		
Warfarin	Increased INR	Postoperative bleeding and spontaneous pinal epidural haematoma have been reported with garlic alone. Whether garlic prolongs PT is unclear, but it does cause platelet dysfunction.
Ginkgo (***Ginkgo biloba***)		
Aspirin	Spontaneous hyphema	Ginkgolides are potent inhibitors of PAF

(Adapted from Ahmad *et al.,* 2006)

Table 2.3 contd Herbal preparations - drugs interactions

Herb and drug(s)	Results of interaction	Comments
Ginkgo (**Ginkgo biloba**)		
Thiazide diuretic	Hypertension	Ginkgo alone has not been associated with hypertension
Guar gum (***Cyamopsis tetragonolobus***)		
Metformin, phenoxymethylpenicillin, glibenclamide	Slows absorption of digoxin, paracetamol, and bumetanide; decreases absorption of metformin, phenoxymethylpenicillin, and some formulations of glibenclamide	Guar gum prolongs gastric retention.
Karela or bitter melon (***Momordica charantia***)		
Chlorpropamide	Less glycosuria	Karela decreases blood glucose concentrations
Liquorice (***Glycyrrhiza glabra***)		
Prednisolone	Glycyrrhizin decreases plasma clearance, orally increases AUC and increases plasma concentrations of prednisolone	
Oral contraceptives	Hypertension, oedema, hypokalaemia	Oral contraceptive use may increase sensitivity to glycyrrhizin acid.
Psyllium (***Plantago ovata***)		
Lithium	Decreased lithium concentrations	Hydrophilic psyllium may prevent lithium from ionising.
St John's wort (***Hypericum perforatum***)		
Paroxetine	Lethargy/incoherence	
Trazodone	Mild serotonin syndrome	A similar case is described with the use of St John's wort alone.
Sertraline	Mild serotonin syndrome	
Nefazodone	Mild serotonin syndrome	
Theophylline	Decreased theophylline concentrations	

(Adapted from Ahmad *et al.*, 2006)

Table 2.3 contd Herbal preparations - drugs interactions

Herb and drug(s)	Results of interaction	Comments
St John's wort (***Hypericum perforatum***)		
Digoxin	Decreased AUC, decreased peak and trough concentrations	Most studies indicate that St John's wort is a potent inhibitor of cytochrome P450 isoenzymes
Phenprocoumon	Decreased AUC	
Shankhapushpi (Ayurvedic mixed-herb syrup)		
Phenytoin	Decreased phenytoin concentrations, loss of seizure control	In rats, multiple coadministered doses (but not single doses) decreased plasma phenytoin concentrations; single doses decreased the antiepileptic effect of phenytoin. Shankhapushpi is used to treat seizures.
Sho-saiko-to or xiao chai hu tang (Asian herb mixture)		
Prednisolone	Decreased AUC for prednisolone	
Papaya (***Carica papaya***)		
Warfarin	Increased INR	
Tamarind (***Tamarindus indica***)		
Aspirin	Increased bioavailability of aspirin	Tamarind is used as a food and a medicine.
Valerian (***Valeriana officinalis***)		
Alcohol	A mixture of valepotriates reduces adverse effect of alcohol	
Yohimbine (***Pausinystalia yohimbe***)		
Tricyclic antidepressants	Hypertension	Yohimbine alone can cause hypertension, but at lower doses, can cause hypertension when combined with tricyclic antidepressants.
Ginseng (***Panax spp***)		
Alcohol	increased alcohol clearance	Increased activity of alcohol dehydrogenase.

ACE = angiotensin-converting enzyme; INR = international normalised ratio; PT = prothrombin time; PTT = partial thromboplastin time; ECG = electrocardiogram; PAF = platelet-activating factor; AUC = area under the concentration/time curve. (Adapted fron Ahmad *et al.*, 2006)

2.3.2.5 Standardisation of Medicinal Plant Products

Standardisation describes all measures taken during the manufacturing process and quality control, leading to a reproducible quality. Standardization of medicinal plants products is an important step in which the active constituents involved in the preparation are known. For many herbal products the active constituents are unknown or are undisclosed. Products may be standardised on the basis of the content of certain marker compounds. However, due to lack of scientific information on the product pharmacological profile, variability in the content and concentration of the constitutents of plant material, as well as the inconsistency in the extraction and processing procedures used by different manufacturers, herbal medicines rarely meet any laid down standard (Schulz *et al.,* 2000). The use of chromatographic techniques and marker compounds to standardize herbal preparations promotes batch-to-batch consistency but does not ensure consistent pharmacological activity or stability (Ahmad *et al.,* 2006).

Consistency in composition and biological activity are prerequisites for the safe and effective use of therapeutic agents (Goldman, 2001). But the standardisation of correct dosage forms is not always easy, especially in multi-herbal preparations or in the case of single plants that are not cultivated under controlled conditions.

2.3.3 Improving the Quality, Safety and Efficacy of Herbal Medicines

2.3.3.1 Encouraging Mediculture

Mediculture is defined as the cultivation of medicinal plants on a scientific basis. The cultivation of medicinal plants by mediculture should be encouraged. However, emphasis on genetic stability and uniformity of plant populations is important in order to ensure reproducible results. The concept of growing crops for health rather than for food or fiber is slowly changing

plant biotechnology and medicine (Ahmad *et al.,* 2006). The rediscovery of the connection between plants and health is responsible for launching a new generation of botanical therapeutics that include plant-derived pharmaceuticals, multicomponent botanical drugs, dietary supplements, functional foods and plant products, and recombinant proteins (Ahmad *et al.,* 2006).

2.3.3.2 Correct Identification of Plant Material

Classical methods of plant taxonomy for the identification of plant material provide an authentic and viable methodology. However, in many situations, when whole plants are not available to the taxonomist, a genetic approach will be more reliable (Ahmad *et al.,* 2006). DNA molecules are more reliable markers than chemicals based on proteins or caryotyping because the genetic composition is unique for each individual and it is not affected by age, physiological and environmental conditions. The DNA can be extracted from leaves, stems, and roots of herbal material. Thus, DNA fingerprinting can be a very useful tool to assess and confirm the species contained within a plant material of interest. However, developing nations lack expertise and equipment for this. Thus the correct identification of plant materials is still a very important and crucial issue.

2.4 Secondary Metabolites

Secondary metabolites are a wide range of organic compounds that are not essential for cell structure and maintenance of life but are often involved in plant protection against biotic or abiotic stresses (Weisshaar and Jenkins, 1998; Hättenschwiler and Vitousek, 2000). Unlike primary metabolites, the absence of secondary metabolites does not result in immediate death, but in the long-term impairment of the organism's survival/fecundity or aesthetics or, perhaps, in no

significant change at all (Fraenkel, 1959). Secondary metabolites are often restricted to a single species or a narrow set of species within a group, whereas primary metabolites are typically found throughout the plant kingdom (Kennedy and Wightman, 2011).

Secondary metabolites are involved in a series of ecological roles which include the following

protection against herbivores and infection by microorganisms;

aiding pollinators and seed-dispersing animals by serving as attractants in smell, colour, or taste; and

aiding in plant-plant competition (including allelopathy) and in plant-microbe symbioses (Kennedy and Wightman, 2011).

2.4.1 Classification and Biosynthesis of Secondary Metabolites

2.4.1.1 Flavonoids

Flavonoids constitute a relatively diverse family of aromatic molecules that are derived from phenylalanine and malonyl-coenzyme A via the fatty acid pathway. They are one of the major classes of phenolics, derived from a combination of the shikimic acid and malonic acid pathways (Winkel-Shirley, 2001). The general chemical structure is a combination of two aromatic C6 rings connected by a C3 bridge which may be cyclized into a benzopyrone ring (C6-C3-C6) (Figure 2.1). They often have glycoside substituents. Flavonoids perform major roles in plants such as, protection against ultraviolet radition, defence against pathogens and pests, pollen fertility, signaling with microorganisms, auxin transport regulation and pigmentation (Winkel-Shirley, 2001). More than 10,000 varieties of flavonoids have been identified (Dixon and Paiva,

1995; Tahara, 2007). Flavonoids are classified into various groups; some of which are discussed below.

2.4.1.1.1 Classes of Flavonoids

Anthocyanins: Anthocyanins are the largest group of water-soluble pigments in the plant kingdom (Kong *et al.*, 2003). A well-known physiological function of the anthocyanins is the recruitment of pollinators and seed dispersers to the plants (Kong *et al.*, 2003). These are coloured flavonoids that confer different colours as pigment in flowers, leaves and fruits. This tends to visually attract animals, birds and insects, making them agents of pollination. The pigments also protect leaves from photoinhibition arising from excess light and UV radiation. Examples and general structure of anthocyanins are illustrated in Figure 2.1.

Flavones and Flavonols: Flavones and flavonols also provide beautiful pigmentation for flowers, fruits, seeds, and leaves. They play key roles in signal transduction between plants and microbes (nectar guides) for insect pollination, in defence as anti-microbial agents and in ultraviolet radiation protection in the leaves of plants as sunscreens (Stafford, 1991). They have the general 2-phenylchromen-4-one backbone. They are secreted by legumes to establish a symbiosis relationship with nitrogen-fixing rhizobia e.g. apigenin, luteolin, quercetin, tangeritin and myricetin (Figure 2.1).

Isoflavones (Isoflavonoids): The phenyl group at the centre of this group is shifted to bridge the other two benzene groups. They have the general 3-phenylchromen-4-one backbone. They exhibit strong antioxidant, antimicrobial and anti-cancer activities. Stilbene in red wine has been reported to contribute to the reduction of heart disease (Manach *et al.*, 2004). Examples and the general structure of anthocyanins are illustrated in Figure 2.1.

Tannins: Tannins are located in the cell vacuoles or surface wax of plants; they are found in black tea, red wine, unripe and ripe fruits. They are phenolic compounds which serve a defensive role by reducing plant edibility. These are astringent polyphenols that bind or precipitate proteins nonspecifically either by hydrogen or covalent bonding to amino ($-NH_2$) group of proteins. Ingested tannins may decrease the digestibility of proteins and the bioavailability of metal ions. Animals, such as rodents, secrete a salivary proline-rich protein that improves tannin tolerance (Lamy *et al.*, 2010).

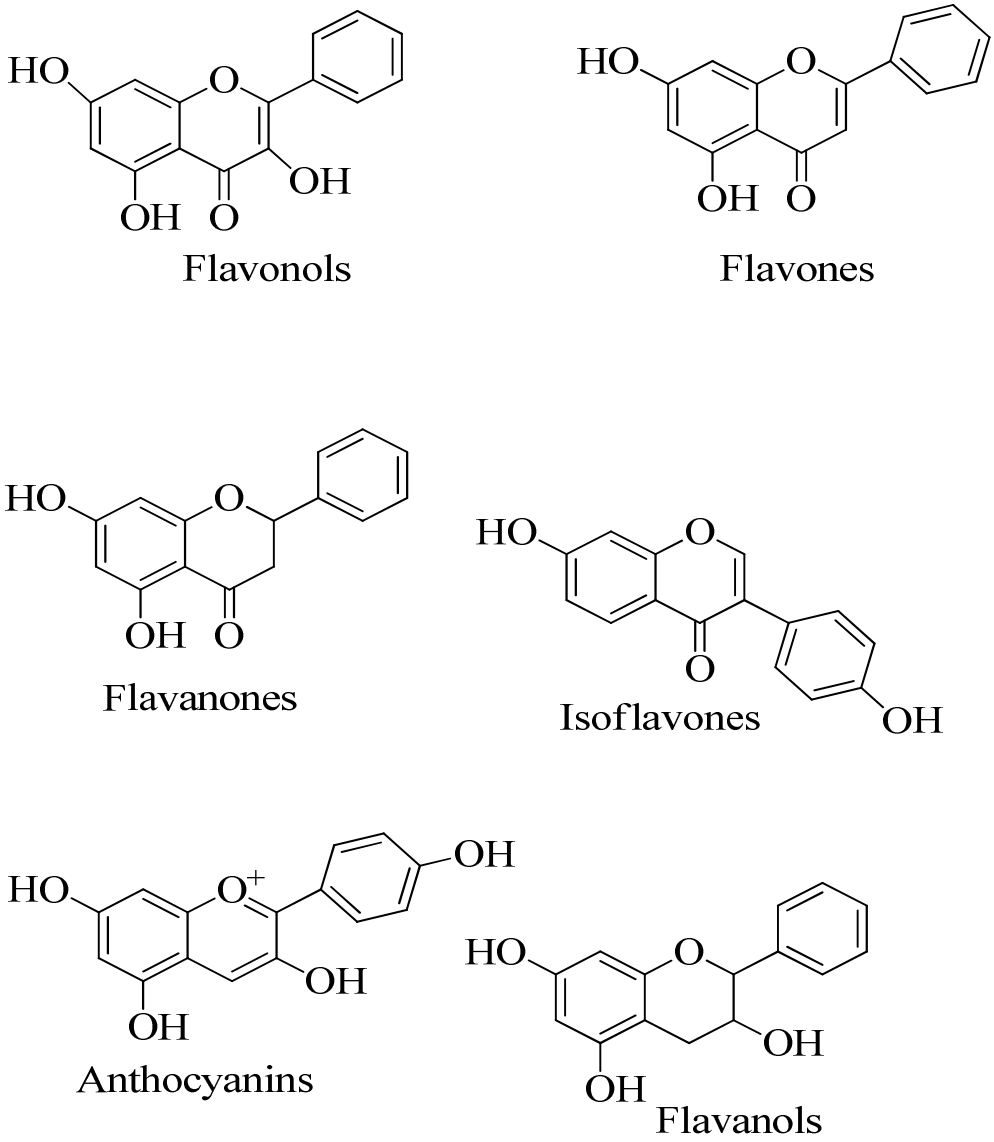


Figure 2.1 Structural Classifications of Flavonoids (Winkel-Shirley, 2001)

2.4.1.1.2 Chemistry of Flavonoids

The characteristic structural feature common to all flavonoids is a 15 carbon (15C) phenylchromane core, composed of a 6C-3C-6C backbone (Figure 2.1). The chromane (benzopyran) moiety is composed of two condensed rings: an aromatic A-ring (6C) and a heterocyclic (pyran) C-ring (3C) in association with another aromatic B-ring (6C). In the majority of flavonoids (flavonols, flavones, flavanones, isoflavones, anthocyanins and flavanols) the B-ring is attached at the 2-position of the benzopyran ring; in the relatively uncommon isoflavones, the B-ring is attached at the 3-position of the benzopyran ring. Various subclasses of flavonoids are distinguished by the degrees of saturation and oxidation of their C rings (Figure 2.1).

Each flavonoid subclass comprises numerous members, differing in the degree of hydroxylation or methoxylation of the A and B rings. Additionally, various glycosylation patterns further increase the potential number of flavonoids. In plant cells, flavonoids occur mostly as glycosides, reflecting a biological strategy apparently aimed at increasing their water solubility, at specifying their sub cellular localization and, most likely, at decreasing their propensity to interact with macromolecules (Winkel-Shirley, 2001).

2.4.1.1.3 Biosynthetic Pathways of Flavonoids

Flavonoids are formed through the phenylpropanoid pathway. The series of reactions involves the transformation of phenylalanine to 4-coumaroyl-CoA by the enzymes phenylalanine-ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumaroyl-CoA-ligase. It also involves the reaction of 4-coumaroyl-CoA and malonyl-CoA to form tetrahydroxychalcone, trihydroxychalcone and resveratrol by the enzymes chalcone synthase, chalcone reductase, and stilbene synthase (Figure 2.2). These reactions could lead to the formation of nine major

subgroups; the colourless flavonoids such as, the chalcones, the aurones, the isoflavonoids, the flavones, the flavonols, and the flavandiols and the others such as, the anthocyanins, the condensed tannins, and the phlobaphene pigments. The various other types of flavonoids are formed through modification reactions such as, isomerisation, reduction, oxidation and/or acetylation of terminal groups or by addition of sugar moiety (Winkel-Shirley, 2001).

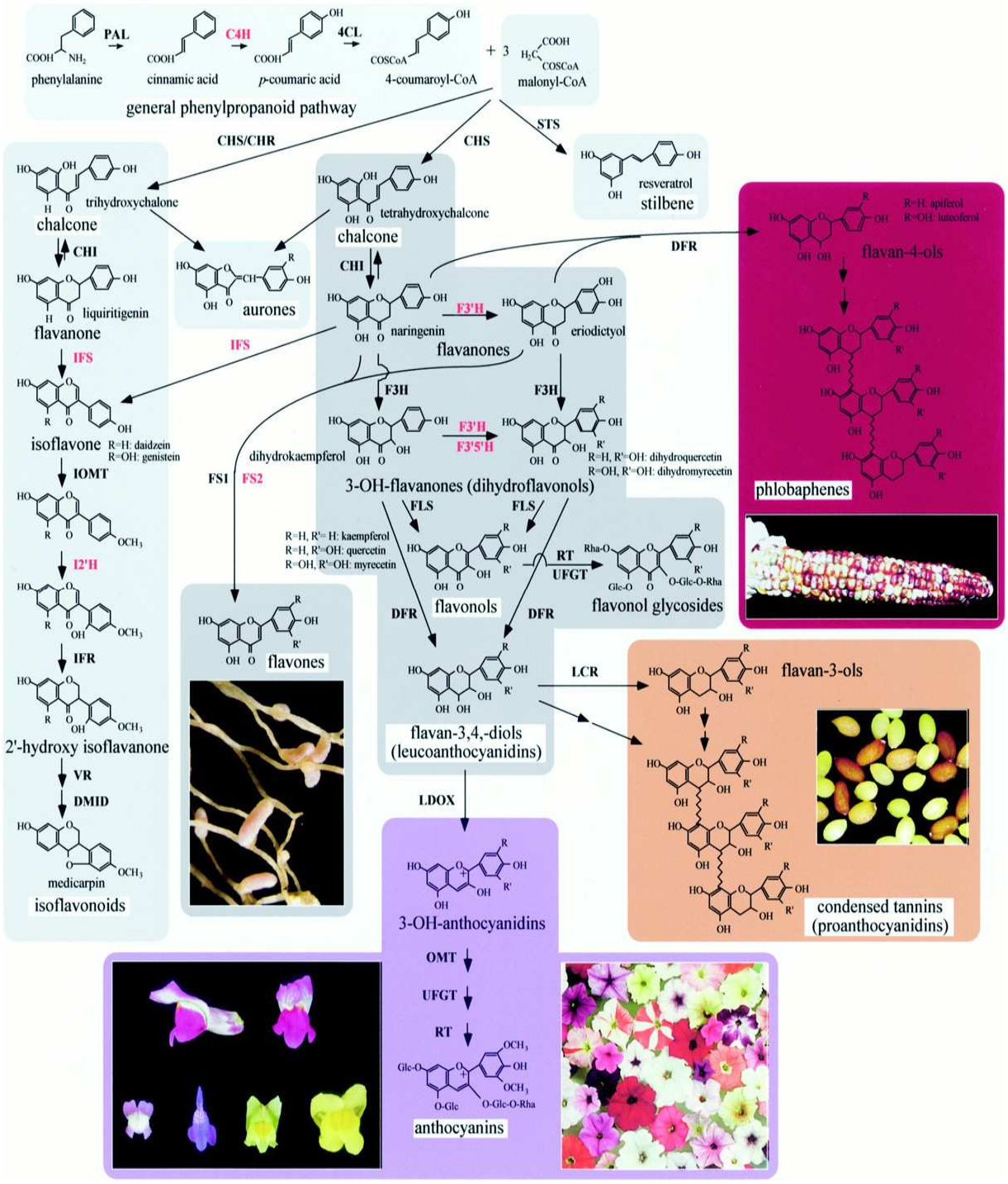


Figure 2.2: The schematic pathways of flavonoid biosynthesis. Enzyme names are abbreviated as follows: Phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumaroyl:CoA-ligase (4CL), ACCase, acetyl- CoA carboxylase, chalcone synthase (CHS), chalcone isomerase (CHI), chalcone

reductase (CHR), stilbene synthase (STS), dihydroflavonol 4-reductase (DFR), 7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase (DMID), flavanone 3-hydroxylase (F3H), flavone synthase (FSI and FSII), flavonoid 3'-hydroxylase (F3'H), flavonoid 3'5'-hydroxylase (F3'5'H), isoflavone *O*-methyltransferase (IOMT), flavonol synthase (FLS), isoflavone reductase (IFR), isoflavone 2'-hydroxylase (I2'H), isoflavone synthase (IFS), leucoanthocyanidin dioxygenase (LDOX), leucoanthocyanidin reductase (LCR), *O*-methyltransferase (OMT), rhamnosyl transferase (RT), UDPG-flavonoid glucosyl transferase (UFGT), and vestitone reductase (VR) (Winkel-Shirley, 2001).

2.4.2.1 Terpenoids

Terpenoids constitute the largest family of secondary metabolites, with over 30,000 members (Sacchettini and Poulter 1997; Dewick 2002). They are not only numerous but also extremely variable in structure, exhibiting hundreds of different carbon skeletons and a large assortment of functional groups. In spite of such a diversity, the simple unifying feature of all terpenoids is that they are derived from the simple process of assembly of a 5C unit, the isoprene. They are, thereby, classified by the homologous series of the number of five carbon isoprene units in their structure: hemiterpenes 5C (1 isoprene unit), monoterpenes 10C (2 isoprene units), sesquiterpenes 15C (3 isoprene units), diterpenes 20C (4 isoprene units), triterpenes 30C (6 isoprene units), tetraterpenes 40C (8 isoprene units) and polyterpenes $(5C)_n$ where 'n' may be between 9 and 30,000 (McGarvey and Croteau 1995). Figure 2.3 illustrates the basic structures of the Terpenoids.

Terpenoids play multifunctional roles in plants, human health and commerce. They have been reported as commercially viable medicinal products due to their wide number of industrial applications. They are the constituents of essential oils, flavouring and fragrance agents in foods, beverages, cosmetics, perfumes and soaps. They exhibit specific biological, pharmaceutical and therapeutical activities (Singh *et al.,* 1989; Martin *et al.,* 2003). In nature, they play significant roles in plants such as plant-to-plant communications and plant-to-insect/animal interactions (Pichersky and Gershenzon, 2002).

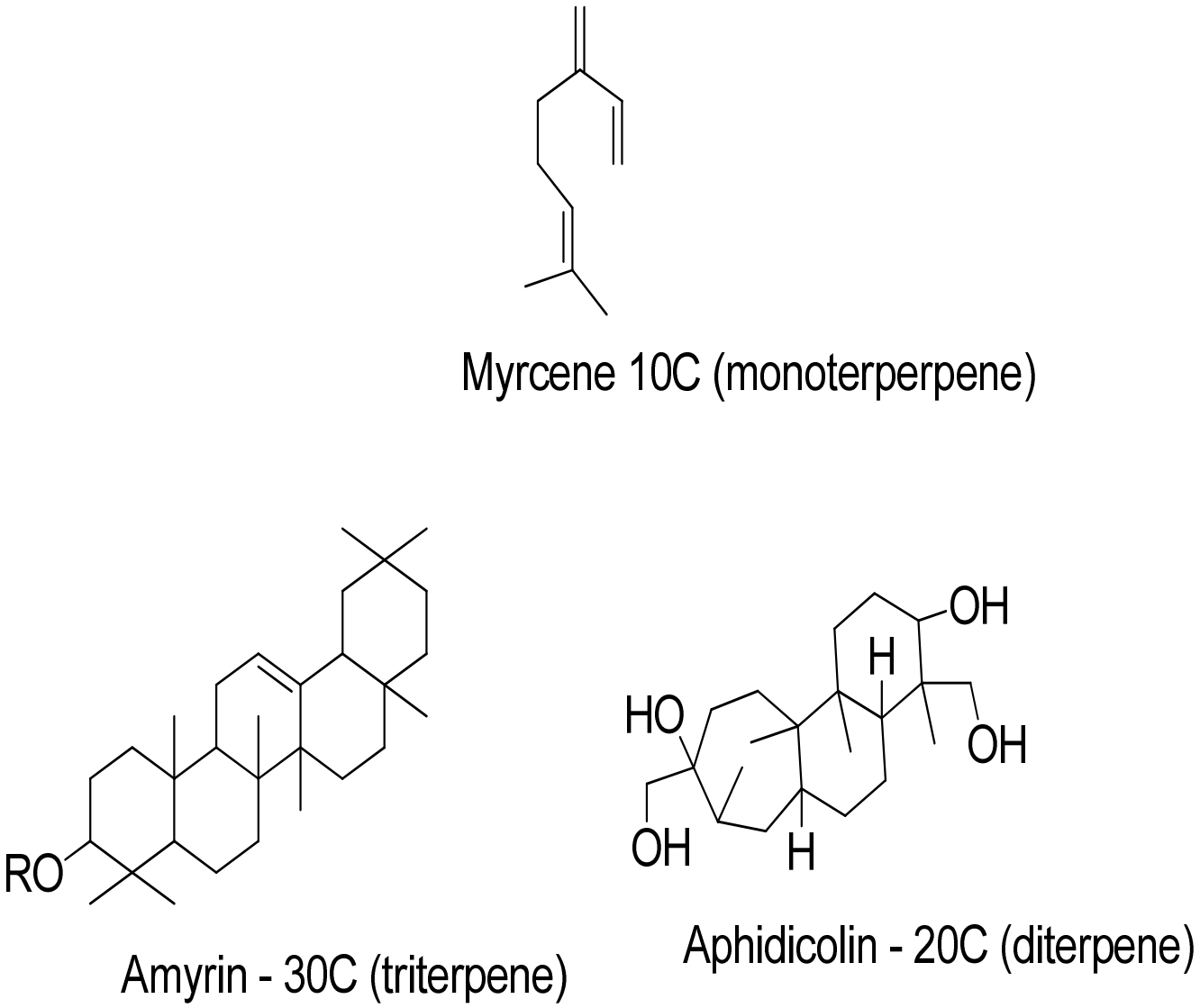


Figure 2.3: Basic Structures of Terpenoids (Pichersky and Gershenzon, 2002)

2.4.2.2 Biosynthesis of Terpenoids

The committed step in the biosynthetic pathway to terpenoids (Figure 2.3) involves the formation of isopentenyl pyrophosphate (IPP), the biological isoprene (5C) unit and its allyllic isomer, dimethylallyl pyrophosphate (DMAPP) (Carretero-Paulet *et al*., 2002). These can be synthesized by plants via either one of two routes: the well established acetate-mevalonate pathway or the newly discovered glyceraldehyde phosphate/pyruvate (non-mevalonate) pathway (Figure 2.4).

In the acetate-mevalonate pathway, the acetyl coenzyme A (acetyl-CoA) units are joined successively to form 3-hydroxyl-3-methylglutaryl-Coenzyme A (HMG-CoA). Thereafter, HMG-CoA is then reduced to mevalonate (MVA), which is subsequently phosphorylated, decarboxylated and dehydrated to form IPP. The first step in the non-mevalonate pathway is the condensation of glyceraldehyde-3-phosphate and pyruvate to form 1-deoxy-D-xylulose-5-phosphate, followed by a skeletal rearrangement coupled with a reduction step which yields a branched chain, 2-C-methyl-D-erythritol-4-phosphate (MEP). With series of other reactions and enzymes via nucleotide diphosphate intermediates, MEP is converted into its cyclic form, 2-C-methyl-D-erythritol-2,4-cyclo-pyrophosphate (MECP), which is further converted to 1-hydroxy-2-methyl-2-[E]-butenyl-4-pyrophosphate (HMBPP) and, with further reduction, converted to dimethylallyl diphosphate (DMAPP).

In the second step, the basic units condense by the addition of isopentenyl diphosphate (IPP, the active 5C isoprene unit) to its isomer dimethylallyl diphosphate (DMAPP), synthesizing geranyl diphosphate (GPP, 10C). Further, condensation of enzyme-bound geranyl diphosphate with additional IPP units forms successively larger prenyl diphosphates: farnesyl diphosphate (FPP, 15C), geranylgeranyl diphosphate (GGPP, 20C). With further condensation, GGPP might

form higher isoprene compounds. These general isoprene units: DMAPP, GPP and FPP can further undergo cyclization, coupling and/or rearrangement to produce the parent carbon skeleton of hemiterpenes, homoterpenes and sesquiterpenes respectively (Singh *et al.,* 1989; McGarvey and Croteau, 1995; Luthra *et al.,* 1999). Furthermore, FPP and GGPP dimerize by head to head condensation to form triterpenes and tetraterpenes as a parental precursors to the syntheses of other compounds (Figure 2.5). These parental precursors are subjected to structural modifications through oxidation, reduction, isomerization, hydration, conjugation and/or other transformations to give rise to a variety of terpenoids (McGarvey and Croteau, 1995).

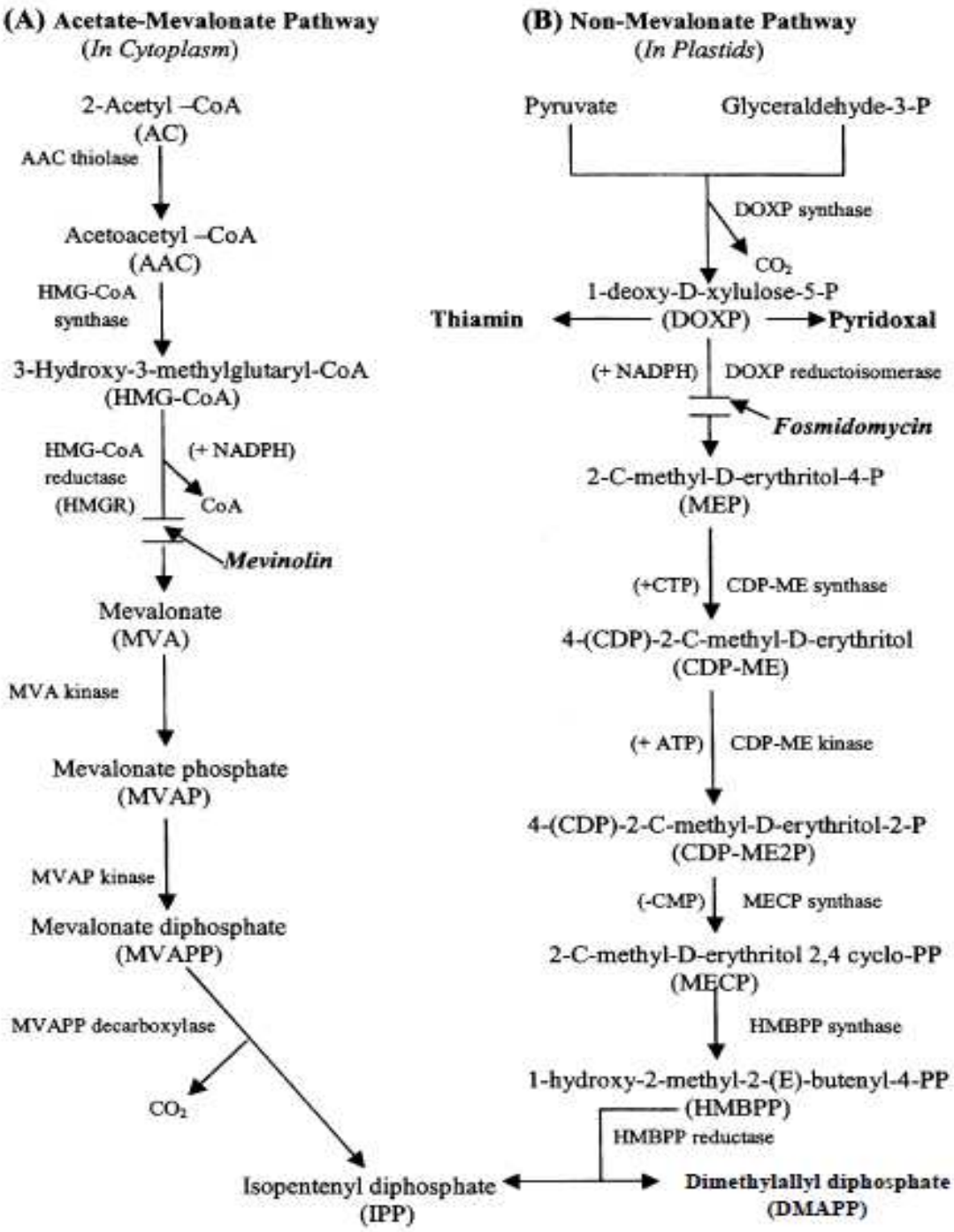


Figure 2.4: Two independent pathways for the biosynthesis of IPP and DMAPP in plants showing the role of DOXP in biosynthesis of thiamin (vitamin B1) and pyridoxal (vitamin B6) and the known inhibitors (mevinolin and fosmidomycin) of each pathway (Dubey, 2003).

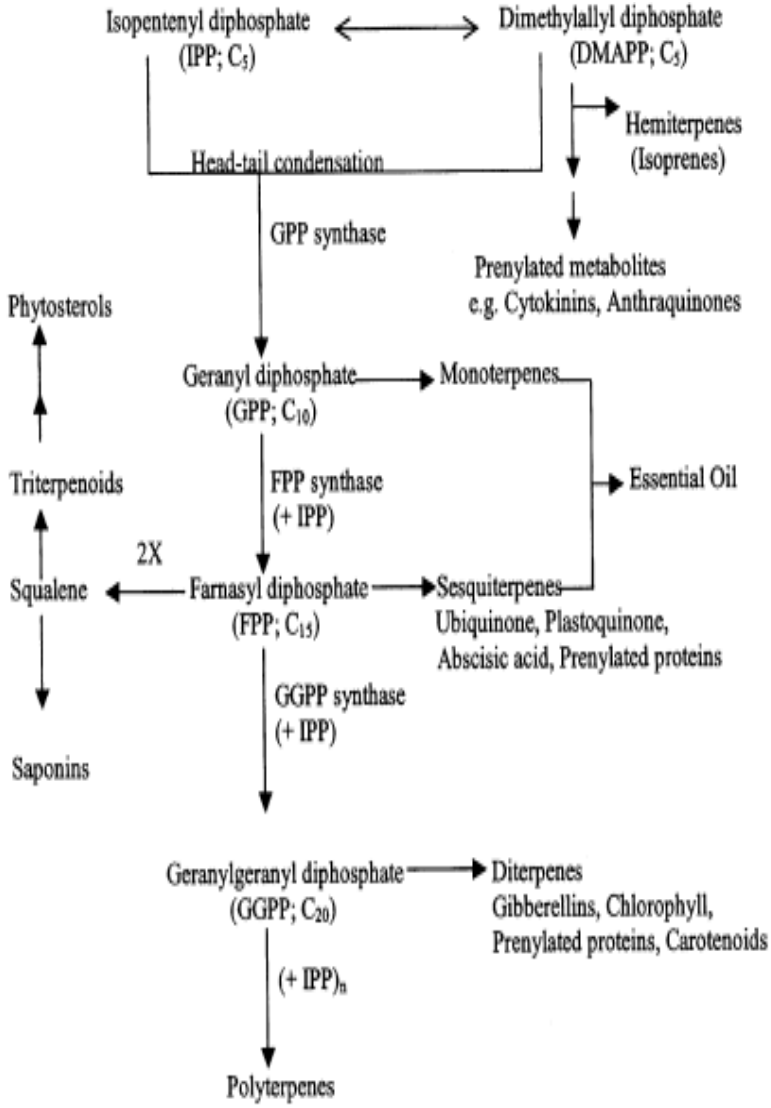


Figure 2. 5: Syntheses of various classes of terpenoids in plants (Dubey *et al*., 2003).

2.4.3.1. Saponins

Saponins are naturally occurring surface-active glycosides mainly produced by plants, lower marine animals and some bacteria (Riguera, 1997; Yoshiki *et al.*, 1998). Their names are derived from their ability to form stable, soap - like foams in aqueous solution. They consist of an aglycone (or sapogenin) and one or more sugar moieties. Depending on the structure of the aglycone, saponins can be classified into two types: (a) a triterpenoid and (b) a steroid (Figure 2.6). The most common sugar moieties are hexoses (glucose and galactose), 6-deoxyhexoses (furanose, quinovose, rhamnose), pentoses (arabinose and xylose) and uronic acids (glucuronic and galacturonic acids) (Akiyama *et al.*, 1972).

The aglycone (sapogenin) may contain one or more unsaturated C–C bonds. The oligosaccharide chain is normally attached at the 3C position (monodesmosidic), but many saponins have an additional sugar moiety at the 26C or 28C position (bidesmosidic). Figure 2.7 shows the nomenclature of a chemical structure of a sapogenin. The great complexity of saponin structure arises from the variability of the aglycone structure, the nature of the side chains and the position of attachment of these moieties on the aglycone. The saponins have various biological activites such as the expectorant, the diuretic and adaptogenic activities associated with them. They are also responsible for the characteristic bitter taste of most plants (Kensil, 1996; Barr *et al*., 1998; Sen *et al*., 1998; Yoshiki *et al*., 1998).

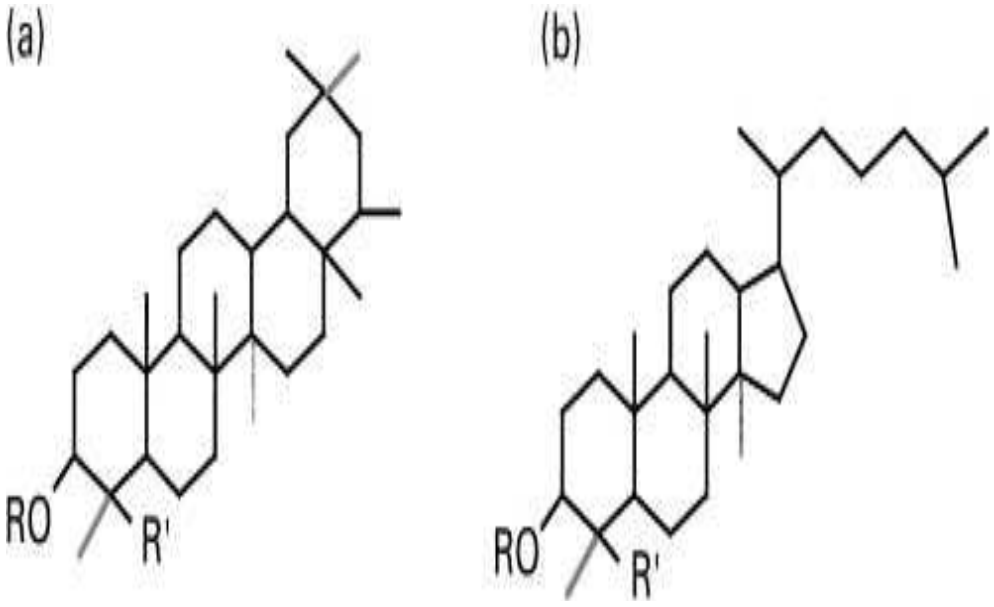


Figure 2.6: Basic structures of sapogenins: (a) a triterpenoid and (b) a steroid (Francis *et al.*, 2002)

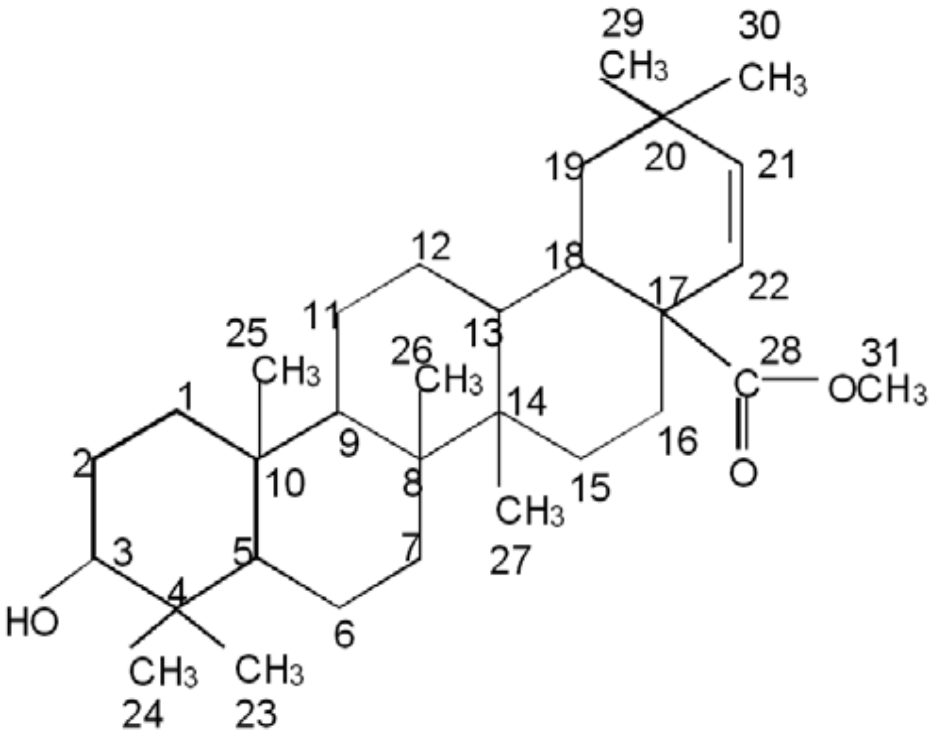


Figure 2.7: Nomenclature of a sapogenin (Francis *et al.*, 2002)

2.4.3.2 Biosynthesis of Saponins

Triterpenoid and steroidal saponins originate from the the 30C precursor (squalene). They are synthesised via the isoprenoid pathway (Figure 2.5 and 2.8). Squalene is oxidized to oxidosqualene, which is then converted to the cyclic derivatives, tripertenoid and saponin, depending on the cyclase enzyme involved in the formation (Figure 2.9) (Vincken *et al.,* 2007). The synthesis of sterols in plants involves the cyclization of 2,3-oxidosqualene from squalene , a reaction catalysed by oxidosqualene cyclase. This is followed by the formation of cycloartenol (the committed step in steroid synthesis), which is mediated by cycloartenol synthase (Crombie and Crombie, 1986; Crombie *et al.,* 1986; Papadopoulou *et al.,* 1999) (Figure 2.9). For triterpenoid saponin synthesis, 2,3-oxidosqualene is cyclized to one of a number of different potential products, the most common being β-amyrin, whose formation is catalysed by β-amyrin synthase (Figure 2.9) (Haralampidis *et al.,* 2001). Very little is known about the detailed enzyme and biological pathways involved in saponin biosynthesis (De-Geyter *et al.*, 2007).

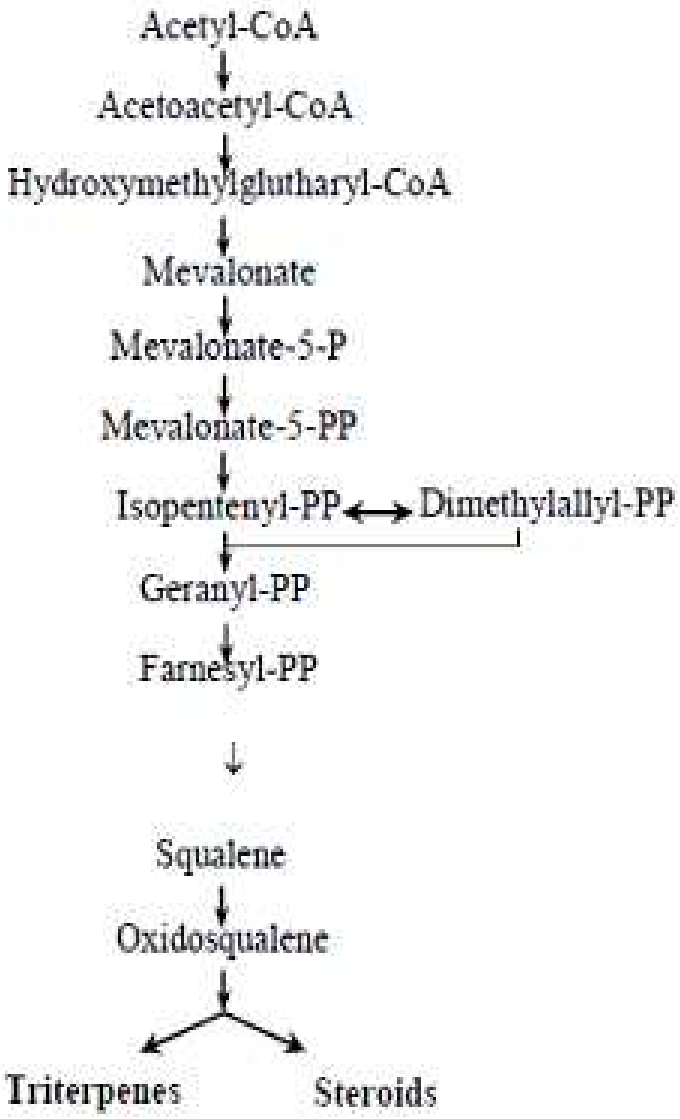


Figure 2.8: The biosynthesis pathway for the basic structures of sapogenins in plants: (a) a triterpenoid and (b) a steroid (Osbourn *et al*., 2003).

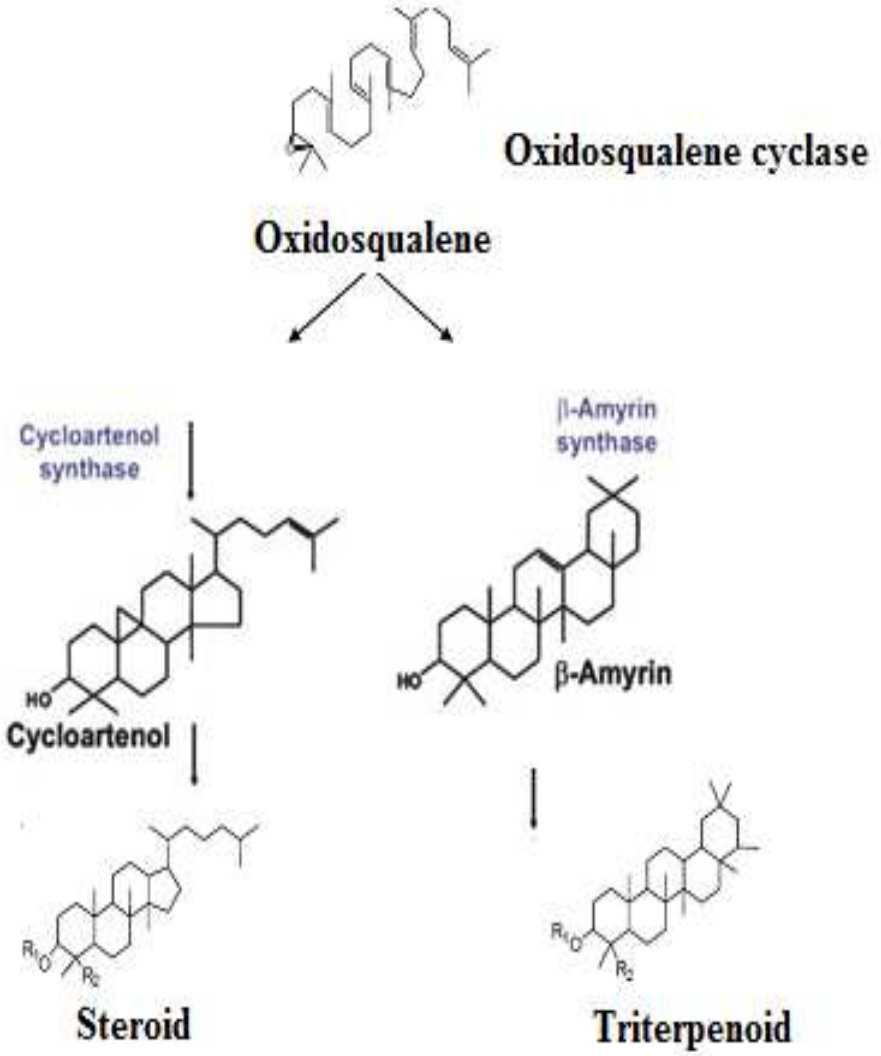


Figure 2.9: Structural biosynthetic pathways of a sapogenin (Osbourn *et al*., 2003).

2.4.4.1. Cardiac Glycosides

Cardiac glycosides comprise a large family of naturally derived compounds which represent a group of secondary metabolites that share the capacity to bind to the extracellular surface of the main ion transport protein in the cell, the membrane-inserted sodium potassium pump (Na^+/K^+-ATPase) (Xie and Askari, 2002). The biological importance of cardiac glycosides primarily is in the treatment of cardiac failure. They result in an increase in cardiac output by increasing the force of contraction as a result of their ability to increase intracellular calcium concentrations (Xie and Askari, 2002).

They show considerable structural diversity, with all members sharing a common structural motif. The core structure consists of a steroidal framework (Figure 2.10 and 2.11), which is considered the pharmacophore responsible for the biological activities of these compounds (Prassas and Diamandis, 2008). Chemically, glycosylated cardiac glycosides are compounds presenting a steroidal core as the steroid nucleus, with a lactone moiety (unsaturated butyrolactone or α-pyrone) at position 17 and a sugar moiety at position 3 (Figure 2.10). Cardiac glycosides having a terminal glucose are called primary glycosides whereas those lacking such a sugar moiety are termed secondary glycosides.

In the classification of cardiac glycosides, two main classes have been observed: the cardenolides (with an unsaturated butyrolactone ring) and the bufadienolides (with an α-pyrone ring) (Figure 2.11). The steroid nucleus has a unique set of fused ring systems that makes the aglycone moiety structurally distinct from the more common steroid ring systems. Cardenolides have a five-membered unsaturated butyrolactone ring, whereas bufadienolides contain a six-membered unsaturated pyrone ring (Figure 2.11). A wide variety of sugars are attached to natural cardiac glycosides, the most common are glucose, galactose, mannose, rhamnose and digitalose

(Mijatovic *et al*., 2007). Although sugars themselves have no activity, the addition of sugars to the steroid affects the pharmacodynamic and pharmacokinetic profile of each glycoside. For example, free aglycones are absorbed more rapidly and are metabolized more easily than their glycosylated counterparts. In addition, the type of sugar attached influences the potency of the compound. For instance, the addition of rhamnose has been shown to increase potency several times (6–35 times), whereas the addition of mannose had no significant effect (Melero *et al.*, 2000).

Based on this phenomenon, Langenhan and colleagues recently developed a powerful new tool, called neoglycorandomization, for the study of the relationship between attached sugars and biological activity. This high-throughput method allows the rapid conversion of a single aglycone molecule into a library of analogues with diverse sugar moieties (Langenhan *et al.*, 2005). Techniques such as this could facilitate the discovery of novel cardiac glycoside analogues with improved therapeutic properties (Prassas and Diamandis, 2008).

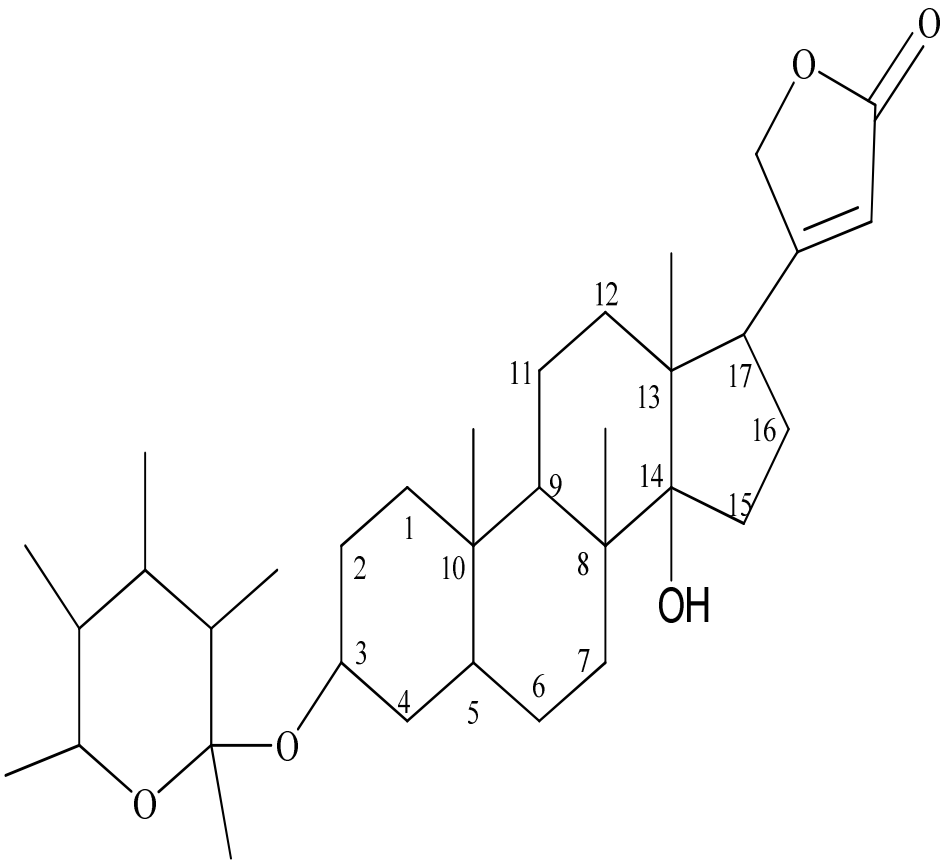


Figure 2.10 Nomenclature of the General Structure of Cardiac GlycosidesCardiac Glycosides

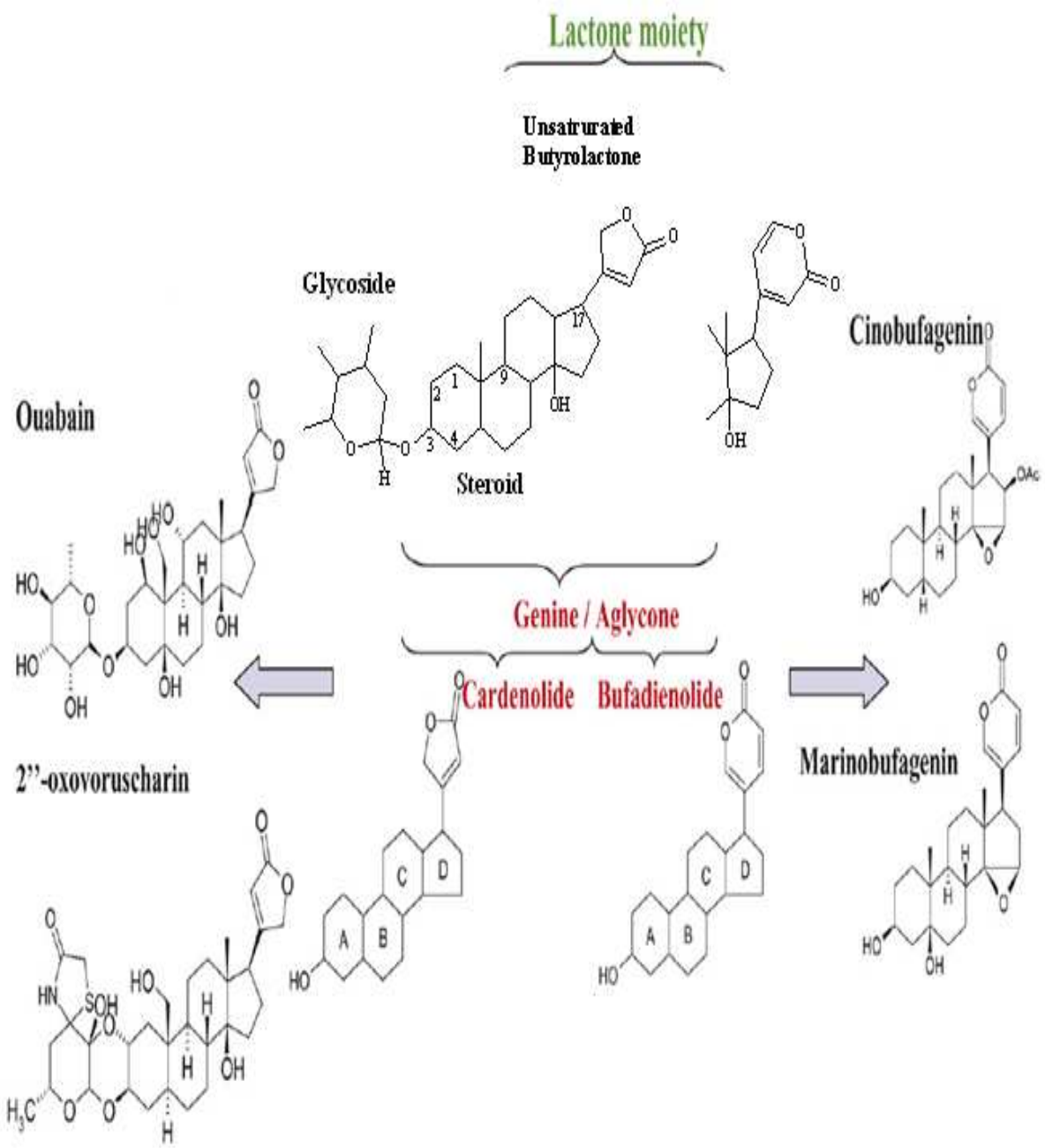


Figure 2.11: **C**ardiac glycosides with examples (Mijatovic *et al*., 2007).

2.4.4.2 Biosynthesis of Cardiac Glycosides

The actual biosynthetic pathways of cardiac glycosides have not been fully established. However, as a result of the steroidal core portion of the cardiac glycosides, the initial biosynthetic pathway was assumed and confirmed to be through mevalonic acid pathway (Kreis *et al.*, 1998). Their biosynthetic pathways from acetic acid to isopentenyl pyrophosphate (IPP); from IPP to squalene; from squalene to squalene 2,3- oxide; from squalene-2,3-oxide to lanosterol, cholesterol and pregnenolone are well established (Figures 2.4, 2.5, 2.8 and 2.12) (Kreis *et al.*,1998). Pregnenolone has been reported as a precursor of the cardenolides (Kreis *et al.*, 1998). For example, the conversion of pregnenolone into digitoxigenin (cardenolide) requires the inclusion of an acetate group, whereas in the biogenesis of scilliroside (bufadienolide), the α–pyrone is formed by the condensation of a pregnane derivative with one molecule of oxaloacetatic acid (Steyn and van Heerden, 1998).

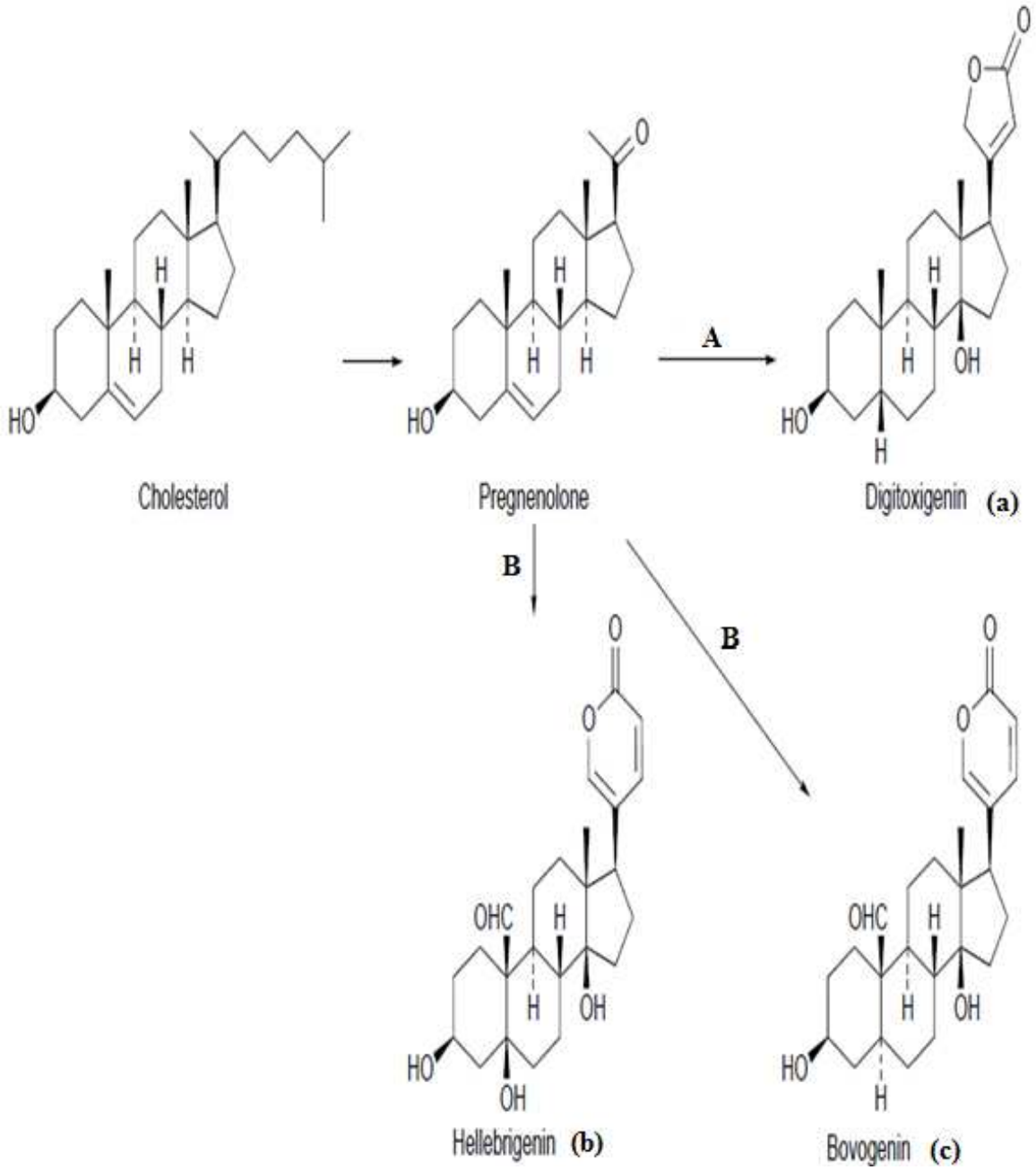


Figure 2.12 The putative biosynthetic pathway of cardiac glycosides in plants ((a) - cardenolide: ditoxigenin, which involves acetate as a precursor (A) and bufadienolides: hellebrigenin (b) and bovogenin (c), which involve oxaloacetate (B) as a precursor) in plants (Steyn and van Heerden, 1998).

2.4.5 Alkaloids

Alkaloids are a diverse group of low-molecular-weight, nitrogen-containing compounds found in about 20% of plant species (Caporale, 1995; Wink, 1999). The potent biological activities of some alkaloids have led to their exploitation as pharmaceuticals, stimulants, narcotics and poisons. Examples are (i) analgesics (morphine and codeine); (ii) anticancer agents (vinblastine and taxol); (iii) gout suppressant (colchicines); (iv) muscle relaxant ((+)-tubocurarine); (v) antiarrythmic (ajmaline); (vi) antibiotics (sanguinarine); and (vii) sedatives (scopolamine) (Facchini, 2001). Other important alkaloids of plant origin include caffeine, nicotine, cocaine and heroin. They are classified into several groups based on their highly diverse chemical structures; but the major classifications are terpenoid indole, benzylisoquinoline and tropane alkaloids (Facchini, 2001).

2.4.5.1 Terpenoid Indole Alkaloids

Terpenoid indole alkaloids (TIAs) comprise a family of greater than 3000, compounds which include the antineoplastic agents, vinblastine and camptothecin, the antimalaria drug quinine and the rat poison strychnine. It has been proposed that in plants some TIAs play a defensive role against pests and pathogens (Luijendijk *et al.*, 1996). TIAs consist of an indole moiety provided by tryptamine (from tryptophan) and a terpenoid component derived from the iridoid glucoside secologanin.

2.4.5.1.1 Biosynthesis of Terpenoid Indole Alkaloids

Tryptophan is converted to tryptamine by tryptophan decarboxylase (TDC). The first committed step in the terpenoid indole alkaloids pathways is the biosynthesis of secologanin,

which is the hydroxylation of geraniol to 10-hydroxygeraniol by the enzyme geraniol 10-hydroxylase (G10H), a P450 monooxygenase enzyme (Meehan and Coscia, 1973). The condensation of these two metabolites (tryptamine and secologanin) subsequently leads to the synthesis of strictosidine, the major precursor in the biosynthesis of all TIAs by the enzyme strictosidine synthase (STR) (Figure 2.13). Subsequent reactions lead to the formation of vindoline. Vinblastine, an anticancer drug, has been reported to be produced from a reaction between vindoline and catharanthine by a nonspecific peroxidase (Sottomayor *et al.*, 1998).

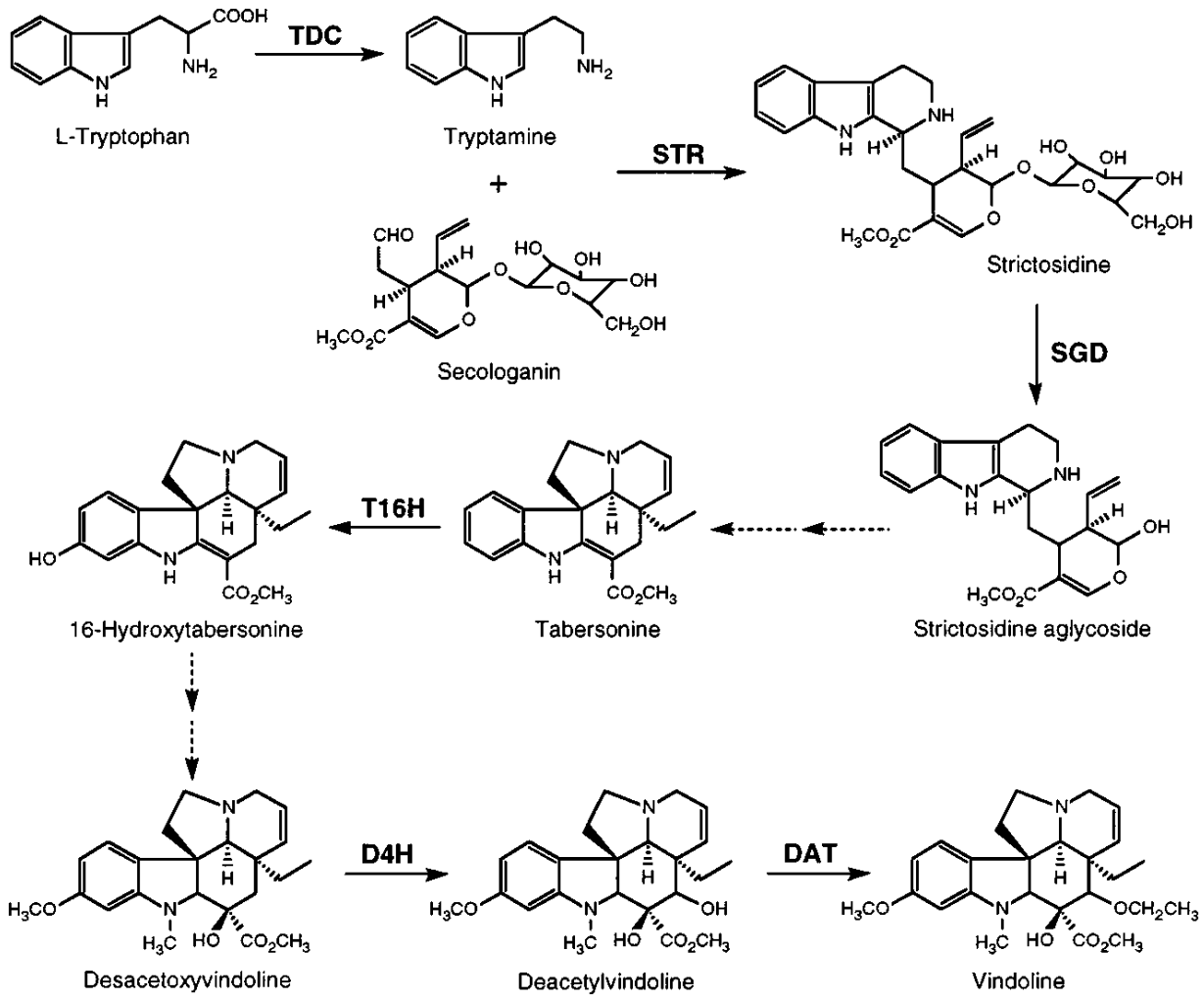


Figure 2.13 Biosynthesis of monoterpenoid indole alkaloid. Tryptophan decarboxylase (TDC), strictosidine synthase (STR), strictosidine-D-glucosidase (SGD), tabersonine 16-hydroxylase (T16H), desacetoxyvindoline 4-hydroxylase (D4H), deacetylvindoline 4-*O*-acetyltransferase (DAT) (Facchini, 2001).

2.4.5.2 Benzylisoquinoline Alkaloids

Benzylisoquinoline alkaloids (BIAs) are a large and diverse alkaloid group with greater than 2500 members (Facchini, 2001). The pharmacological activities of BIAs make many of them useful as pharmaceuticals and are often a clue to their biological role in the plant (Caporale, 1995). For example, the effectiveness of morphine as an analgesic, colchicine as a microtubule disrupter, and (+)-tubocurarine as a neuromuscular blocker suggest that these alkaloids function as herbivore deterrents (Caporale, 1995). The antimicrobial properties of sanguinarine reveal that it exerts protection against pathogens. The BIAs, berberine, sanguinarine, and palmatine were specifically shown to confer protection against herbivores and pathogens (Schmeller *et al*., 1997).

2.4.5.2.1 Biosynthesis of Benzylisoquinoline Alkaloids

The biosynthesis of BIA begins with a metabolic lattice of decarboxylations, *ortho*hydroxylations and deamination reactions that convert tyrosine to both dopamine and 4-hydroxyphenylacetaldehyde (Facchini, 2001). Tyrosine is converted to L-3,4-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase, with its subsequent decarboxylation to dopamine by the aromatic L-amino acid decarboxylase (TYDC); so also is tyrosine converted to tyramine by the aromatic L-amino acid decarboxylase (TYDC). Dopamine and 4-hydroxyphenylacetaldehyde are condensed by norcoclaurine synthase (NCS) to yield the trihydroxybenzylisoquinoline alkaloid (*S*)- norcoclaurine, which is the central precursor to all BIAs in plants (Figure 2.14).

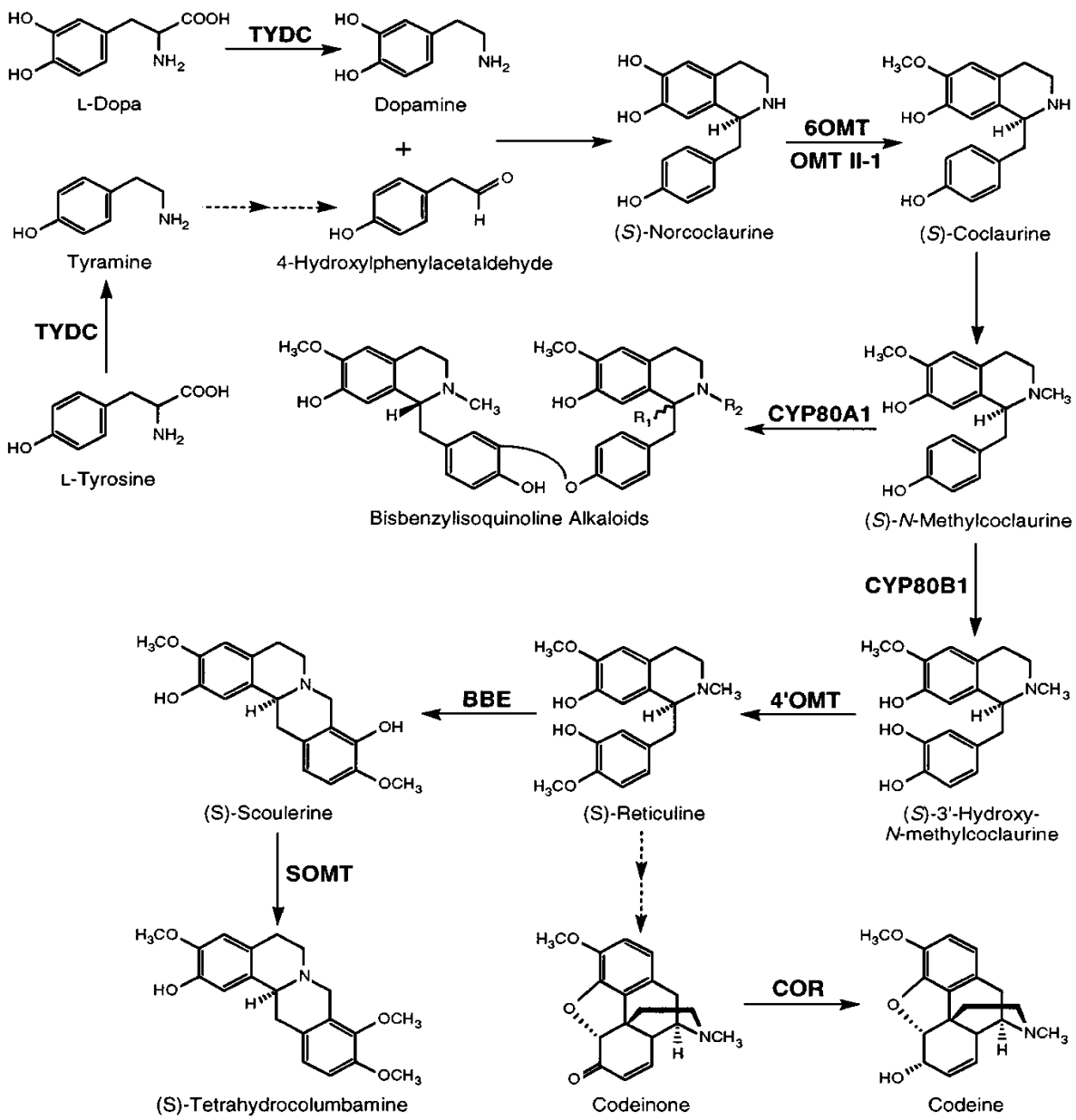


Figure 2.14: Biosynthesis of benzylisoquinoline alkaloid (BIA). Tyrosine/dopa decarboxylase (TYDC), norcoclaurine 6- *O*-methyltransferase (6OMT), 3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase (4'OMT), *O*-methyltransferase II-1 (OMT II-1), berbamunine synthase (CYP80A1), (*S*)-*N*-methylcoclaurine 3-hydroxylase (CYP80B1), berberine bridge enzyme (BBE), scoulerine *N*-methyltransferase (SOMT), codeinone reductase (COR) (Facchini, 2001).

2.4.5.3 Tropane Alkaloids and Nicotine

Tropane alkaloids (TPAs) occur mainly in the solanaceae and include the anticholinergic drugs atropine, hyoscyamine, and scopolamine and the narcotic tropical anesthetic cocaine (Dräger, 2002). Although nicotine is not a member of the tropane class, the *N*-methyl- Δ^1-pyrrolinium cation involved in TPA biosynthesis is also an intermediate in the nicotine pathway (Facchini, 2001).

2.4.5.3.1 Biosynthesis of Tropane Alkaloids and Nicotine

The biosynthesis of TPAs and nicotine begins with the decarboxylation of ornithine and/or arginine by ornithine decarboxylase (ODC) and arginine decarboxylase (ADC), respectively (Facchini, 2001). These enzymes are involved in the formation of putrescine, either directly through the decarboxylation of ornithine by ODC, or through the decarboxylation of arginine to agmatine by ADC, followed by the reaction of agmatinase on agmatine forming putrescine or that of agmatine iminohydrolase to give *N*-carbamoylputrescine and subsequently forming putrescine. ODC occurs in all living organisms, but ADC is not found in mammals and many lower eukaryotes (Facchini, 2001). Despite the existence of these two routes for the formation of putrescine, it has been suggested that arginine supplies most of the putrescine for alkaloid biosynthesis (Hashimoto and Yamada, 1992).

The first committed step in TPA and nicotine biosynthesis is the conversion of putrescine to M-methylputrescine which is catalysed by a S- adenosyl-methionine (SAM)-dependent putrescine *N*-methyltransferase (PMT). Subsequently, *N*-methylputrescine is oxidatively deaminated by a diamine oxidase to 4-aminobutanol, which undergoes spontaneous cyclization to form the reactive *N*-methyl-Δ^1-pyrrolinium cation (Hashimoto and Yamada 1994). The *N*-methyl-

Δ^1-pyrrolinium cation is thought to condense with acetoacetic acid to yield hygrine as a precursor of the tropane ring, or with nicotinic acid to form nicotine, although the enzymology of these steps is not known (Facchini, 2001). Tropinone is located at a branch point in the TPA pathway and is the first intermediate with a tropane ring. Two related dehydrogenases, tropinone reductase I (TR-I) and tropinone reductase II (TR-II), reduce the 3-keto group of tropinone to the 3α- and 3β- groups of the stereospecific alkamines tropine and Ψ-tropine, respectively (Figure 2.15). Hyoscyamine is produced by the condensation of tropine and the phenylalanine derived intermediate, tropic acid. This metabolite can be further hydroxylated by hyoscyamine 6β-hydroxylase (H6H) to form scopolamine (Figure 2.15).

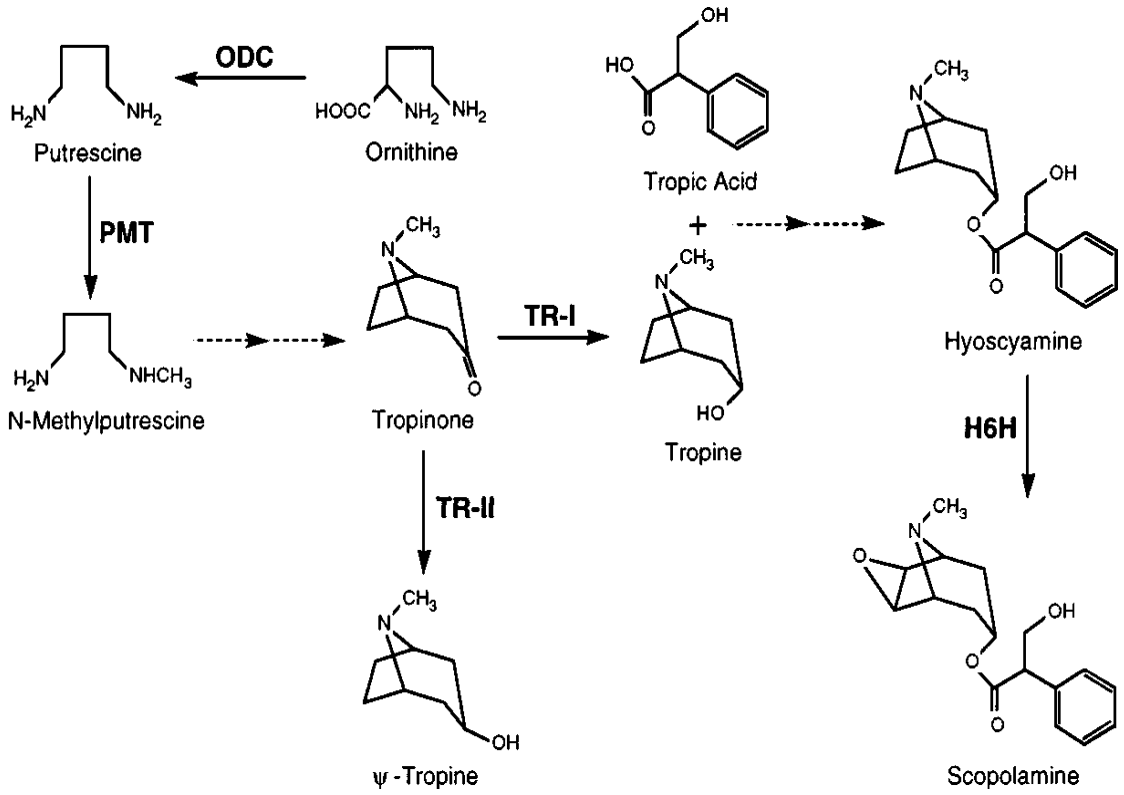


Figure 2.15: Biosynthesis of tropane alkaloid. Ornithine decarboxylase (ODC), putrescine *N*-methyltransferase (PMT), tropinone reductase-I (TR-I), tropinone reductase-II (TR-II), hyoscyamine 6β-hydroxylase (H6H) (Facchini, 2001).

2.5 Toxicology

Toxicology is the study of the interaction between chemicals and biological systems to determine the potential of chemicals or xenobiotics to produce adverse effects in a living system. It investigates the nature, incidence, mechanisms of production, factors influencing the development and reversibility of such adverse effects. Adverse effects are detrimental factors to the survival or the normal functioning of the orgainsm. The major evaluation points for toxicological assessments are the following:

the basic structural, functional and biochemical parameters (toxicological parameters) of injury;

the dose-response relationships of the agent of toxicity and toxicological parameters;

the mechanisms of toxicity (the fundamental biochemical alterations responsible for the induction and maintenance of the toxic response) and reversibility of the toxic effect; and

possible influencing factors with response modification, for example, route of exposure, species, and gender.

2.5.1 History of Toxicology

Toxicology is one of the oldest practical sciences ever known to human beings. From the primitive times, it has been documented in the Egyptian and Greek empires that humans ensured that various toxic chemicals in plants and animals were avoided (Dekant and Vamvakas, 2005a). Ebers Papyrus, an Egyptian papyrus dating from 1500 B.C., and the surviving medical works of Hippocrates, Aristotle, and Theophrastus, published at an early period, 400-250 B.C., all included some knowledge of poisons. The Greek and Roman civilizations intentionally applied the knowledge of poisons for hunting, warfare, suicide and murder (Dekant and Vamvakas, 2005a).

However, the principle and concept of modern toxicology gained prominence in the time of Paracelsus, when he clearly defined the toxicity of a given substance as having a dose-response relationship. His statement “All substances are poisons; there is none that is not a poison. The right dose differentiates a poison and a remedy” is properly regarded as a landmark in the development of the science of toxicology.

Since the 1960s, toxicology has entered a phase of rapid development and has changed from a science that was almost entirely descriptive to one in which the study of mechanisms has become the prime task. Some of the reasons for this included the development of new analytical methods since 1945, the emphasis on drug testing following the thalidomide tragedy (Dekant and Vamvakas, 2005a), the public concern over environmental pollution and disposal of hazardous waste.

2.5.2 Methods Employed in Toxicological Studies

2.5.2.1 Acute Toxicity Test Methods

Acute toxicity tests measure the adverse effects that occur within fourteen days after administration of a single dose of a test substance. This is performed principally on rodents (mice or rats) and is usually done early in the development of a new chemical or product to provide information on its potential toxicity. Traditionally, acute oral toxicity testing has focused on the immediate determination of the dose that kills half of the animals (i.e., the median lethal dose or LD_{50}), the timing of lethality following acute chemical exposure, as well as observing the onset, nature, severity and reversibility of toxicity. But in recent times, after the immediate observation is done, toxicological parameters (biochemical, haematological and histopathological) to assess potential adverse effects are carefully chosen and measured. The fixed single dose, at which signs

of toxicity but no deaths are detected is used to classify the test compounds according to their toxic potential.

The information obtained from acute toxicity studies in animals is aimed towards (i) understanding the possible health hazard that could result from a single exposure to a high dose of a particular chemical; (ii) protecting individuals who are working with new materials and developing standard procedures for handling, packaging, transporting and disposing of such chemicals; (iii) identifying the mode of toxic action of a substance; and (iv) providing information on doses associated with target organ toxicity and lethality. These then can be used to set doses for repeated-dose studies, extrapolation for diagnosis and treatment of toxic reactions in humans such as in the case of drug overdose or suicide attempts (Dekant and Vamvakas, 2005b).

2.5.2.2 Repeated-Dose Toxicity Studies: Sub-acute, Sub-chronic, and Chronic Toxicity

Repeated-dose toxicity studies assess the toxic effects resulting from the accumulation of a compound or its metabolites in an organism. Unlike acute studies, they are measures of toxic effects that appear with repeated doses of the toxicant; hence, continuous dosing is essential to assess the long-term toxicity effect of substances.

Chronic toxicity is usually performed on mice, rats, rabbit and guinea pigs for at least six months and on dogs for 12 months (Romero *et al*., 1997). The dose levels are usually selected on the basis of the results from acute and sub-acute toxicity studies. The highest dose applied should be toxic, i.e., should suppress the body weight by up to 10 % (maximum tolerated dose, MTD). The two other doses are usually 1/4 and 1/8 of the MTD. Xenobiotics showing no adverse effects in short-term studies are usually tested at doses which are 100–200 times higher than the expected human exposure. These studies are helpful in assessing the human risk resulting from frequent

exposure to household or workplace chemicals and from the intake of chemicals used for therapeutic purposes (Dekant and Vamvakas, 2005b).

2.6 Hepatotoxicity

The liver is the largest organ in the body and it consists of rows of hepatic cells (hepatocytes or parenchymal cells) perforated by specialized blood capillaries called sinusoids. The sinusoid walls contain phagocytic cells, called Kupffer cells, whose role is to engulf and destroy materials such as solid particles, bacteria, dead blood cells and others (Hodgson and Levi, 2004a). The hepatic portal vein is the main blood supply vessel to the liver. It empties its content from the intestinal vasculature, vessels from the spleen and stomach, into the sinusoids. The blood perfuses the liver and exits through the hepatic vein. The liver is the major organ actively involved in metabolism, biosynthesis and storage. It is the storage organ for glycogen, fat, fat-soluble vitamins and other nutrients. It is the site of metabolism of lipoproteins, functional proteins, such as enzymes and blood-coagulating factors, as well as xenobiotic (Hodgson and Levi, 2004a).

As a result of the strategic metabolic functions of the liver, it is often the targeted organ for chemically induced organ toxicities. Many factors have been reported to functionally and structurally contribute to the susceptibility of the liver to toxicity. These include the high perfusion of the liver to xenobiotics absorbed from the gastrointestinal tract and the high concentration of xenobiotic metabolizing enzymes (cytochrome P450-dependent monooxygenase system) in the liver. Chemical agents such as those used in laboratories and industries, as well as medicinal plants, are capable of inducing hepatotoxicity. More than 900 drugs have been implicated in liver injury. Drug related hepatotoxicity is an important cause of morbidity and

mortality. As such, it is the most common reason for withdrawing new drugs from circulation (Hodgson and Levi, 2004a). In Table 2.4 is the summary of the biochemical markers of liver injury, types and characteristics (Friedman *et al.*, 1996).

Table 2.4: Biochemical markers of liver injury

Type of liver injury	Marker	Characteristics
Cytotoxic	Serum aspartate aminotransferase	Low specificity for liver injury
	Serum alanine aminotransferase	Highly specific for acute hepatocellular injury
	Serum albumin/Serum total protein	Reliable marker of chronic hepatocellular injury
	Prothrombin time	Reliable marker of acute or chronic hepatocellular injury.
Cholestatic	Serum alkaline phosphatase	Highest increases occur with cholestatic injuries.
		It has poor specificity.
	Serum γ-glutamyltransferase	Correlates with alkaline phosphatase.
	Serum bilirubin	High increase indicates liver injury.
	Serum bile acids	Highly sensitive and specific for liver injury.

(Adapted from Friedman *et al.*, 1996)

2.6.1 Drug Induced Liver Injury (DILI)

DILI affect both the parenchymal and non-parenchymal cells of the liver, leading to a wide variety of pathological conditions, including acute and chronic hepatocellular hepatitis, fibrosis/cirrhosis, cholestasis, steatosis (fatty liver), as well as sinusoidal and hepatic artery/vein damage (Larrey, 2000). The predominant forms of DILI include steatosis, hepatitis, cirrhosis and cholestasis (Sturgill and Lambert 1997; Gunawan and Kaplowitz, 2004).

2.6.1.1 Steatosis (Fatty Liver)

Steatosis results from the abnormal accumulation of triacylglycerols within the hepatocytes (Hoyumpa *et al.,* 1975; Zimmerman and Maddrey, 1993). Macrovesicular steatosis is characterised by the preence of a single large cytoplasmic vacuole of triglyceride within the hepatocyte that displaces the nucleus peripherally. The etiology of macrovesicular steatosis is multifactorial, including increased mobilization of fatty acids, increased hepatic synthesis of fatty acids, increased synthesis of triglyceride from fatty acids and deficient removal of triglyceride from the hepatocyte via defective VLDL synthesis (Zimmerman, 1978; Salaspuro, 1991). Microvesicular steatosis is less common. It is a more severe variant, resulting primarily from deficient of mitochondrial β-oxidation of fatty acids and characterised by the presence of multiple small droplets of triglyceride within the hepatocyte, which do not displace the nucleus (Fromenty and Pessayre 1995; Pinto *et al.* 1995).

The β-oxidation of fatty acids is a critical process, because the resulting acetyl coenzyme A moieties are the primary sources of ATP in most cells. The disruption of this process promotes the esterification of fatty acids to triglyceride in the cytoplasm, robs the cell of energy, and leads to hyperammonemia via the inhibition of ureagenesis (Ide and Ontko, 1981; Corkey *et al.,* 1988).

Microvesicular steatosis can exhibit a diffuse or regional pattern and in severe cases is accompanied by inflammation and hepatocellular necrosis (Bass and Ockner, 1996; Fromenty and Pessayre, 1995).

Valproic acid has been established as a cause of microvesicular steatosis (Suchy *et al.*, 1979; Dreifuss *et al.*, 1989). In severe cases, the lesion is accompanied by inflammation, necrosis, and bile duct injury. Valproic acid-induced liver injury is thought to result from phase 1 bioactivation (Eadie *et al.*, 1988; Bass and Ockner, 1996). The cytochrome P450 enzymes mediate the production of D4-valproic acid, an oxidative metabolite capable of generating coenzyme derivatives. The production and accumulation of these derivatives may inhibit mitochondrial β-oxidation via the depletion of free coenzyme A and carnitine concentrations (Kesterson *et al.*, 1984; Zimmerman and Maddrey, 1993; Fromenty and Pessayre, 1995).

2.6.1.2 Necrosis

Hepatic cellular injury is a severe form of drug-induced liver injury characterised by thrombosis of the efferent hepatic venules, leading to centrilobular necrosis and liver outflow obstruction, which can progress to congestive cirrhosis (Zimmerman, 1986; Bras and Brandt, 1987; Zimmerman and Maddrey, 1993). These injuries are thought to result from bioactivation of xenobiotics to toxic metabolites (Zimmerman and Maddrey, 1993). Necrosis, usually an acute injury, may be localized and affect only a few hepatocytes (focal necrosis), or may involve an entire lobe (massive necrosis). Cell death occurs, along with the rupture of the plasma membrane. It is preceded by a number of morphologic changes such as cytoplasmic edema, dilation of the endoplasmic reticulum, disaggregation of polysomes, accumulation of triacylglycerols, swelling of mitochondria (with disruption of cristae), and dissolution of organelles and the nucleus. Some

of the biochemical events that may lead to these changes include the binding of reactive metabolites to proteins and unsaturated lipids (inducing lipid peroxidation and subsequent membrane destruction), the disturbance of cellular Ca^{2+} homeostasis, the interference with metabolic pathways, the shifts in Na^{+} and K^{+} balance, and the inhibition of protein synthesis (Zimmerman and Maddrey, 1993).

2.6.1.3 Apoptosis

Apoptosis is a process of programmed cell death that serves as a regulation point for biological processes. This selective mechanism is particularly active during development and senescence. Although apoptosis is a normal physiological process, it can also be induced by exogenous factors, such as xenobiotics, oxidative stress, anoxia and radiation. The suppression of apoptosis, if not carefully managed, can lead to the accumulation of cells, subsequently leading to the clonal expansion of malignant cells and tumor (Hodgson and Levi, 2004a).

2.6.1.4 Cholestasis

Drug induced cholestasis results from the disruption of bile production or flow and may have either intrahepatic or extrahepatic causes (Hodgson and Levi, 2004a). Hepatocanalicular (hypersensitivity) cholestasis is characterised by prominent monocytic portal inflammation and secondary damage to bile canaliculi, as in the case of chlorpromazine and its 7,8-dihydroxy and 7-hydroxy metabolites, which interfere with bile acid secretion via the disruption of canalicular membrane fluidity and Na^{+}/K^{+}-ATPase activity (Samuels and Carey, 1978; Elias and Boyer, 1979). The inhibition of phase II sulfation pathways increases the risk of liver injury (Watson *et*

al., 1988). The inflammation or blockage of the bile ducts results in the retention of bile salts as well as bilirubin accumulation, an event that leads to jaundice (Hodgson and Levi, 2004a).

2.6.1.5 Cirrhosis

Cirrhosis is a progressive liver disease characterised by the replacement of liver tissue by fibrous and scar tissues, leading to the loss of liver function (Hodgson and Levi, 2004a). In most cases, cirrhosis results from chronic chemical injury. The accumulation of fibrous material causes severe restriction of blood flow and of the normal metabolic and detoxication processes of the liver. This situation can, in turn, cause further damage and eventually lead to liver failure (Hodgson and Levi, 2004a).

2.6.1.6 Hepatitis

Hepatitis is a disease condition arising from the inflammation of the liver. It is characterised by the presence of inflammatory cells in the tissue of the organ. This disease condition can be self healing or can progress to fibrosis or cirrhosis (Hodgson and Levi, 2004a).

2.7 Nephrotoxicity

The major function of the kidney is the concentration and excretion of toxic metabolites and other foreign compounds. It is a site of drug toxicity. As a result of indiscriminate use of drugsand medicinal products, and the possibility of kidney damage from this indiscriminate use. The incidence of nephrotoxicity has increased from 8 to 18% between 1983 to 2002 (Hou *et al.*, 1983; Liano *et al.*, 1998; Nash *et al.*, 2002; Huistickle *et al*., 2005), and it has contributed significantly to all cases of in-hospital acute kidney injury (Zhang *et al.*, 2005). The renal system

consists of the kidneys and their vasculature and innervations. It is an essential part of the urinary system. Its functions include the elimination of endogenous and xenobiotic metabolic waste products, the regulation of extracellular fluid volume, electrolyte balance and blood pressure, the re-absorption of water, glucose and amino acids and the syntheses of various hormones influencing metabolism for example, 25-hydroxy-vitamin D_3 is metabolized to the active form, 1,25- dihydroxy-vitamin D_3; renin, a hormone involved in the formation of angiotensin and aldosterone, is formed in the kidney, as are several prostaglandins (Hodgson and Levi, 2004b).

Kidney drains its content through the ureter into a single median urinary bladder, and the latter drains to the exterior via a single duct called the urethra. The kidney has three major anatomical areas: the cortex, the medulla, and the papilla. The renal cortex is the outermost region of the kidney and contains glomeruli, proximal and distal tubules and peritubular capillaries. Cortical blood flow is high with the cortex receiving approximately 90% of the renal blood flow. Since blood-borne toxicants will be delivered preferentially to the cortex, they are more likely to affect cortical functions rather than those of the medulla or papilla. The renal medulla is the middle portion and contains primarily loops of Henle, vasa recta, and collecting ducts. Although the medulla receives only about 6% of the renal blood flow, it may be exposed to high concentrations of toxicants within tubular structures. The papilla is the smallest anatomical portion of the kidney and receives only about 1% of the renal blood flow. Nevertheless, because the tubular fluid is maximally concentrated and luminal fluid is maximally reduced, the concentrations of potential toxicants in the papilla may be extremely high, leading to cellular injury in the papillary tubular and/or interstitial cells (Hodgson and Levi, 2004b).

As a result of the high renal blood flow of the kidney and the increased concentrations of the excretory products after re-absorption of water from the renal tubular fluids, the susceptibility

of the kidney to nephrotoxicity from xenobiotics is highly enhanced. The biotransformation of chemicals to reactive and thus potentially toxic metabolites is also a key event leading to nephrotoxicity or kidney injury (Perazella, 2009).

2.7.1 Mechanism of Drug-induced Kidney Injury

Drugs may damage the kidney by several mechanisms. Understanding these mechanisms is the key to providing better therapeutic regimes and more efficacious preventive measures.

2.7.1.1 Glomerulonephritis

Also known as glomerular nephritis, this is a renal disease characterised by inflammation of the glomeruli or the small blood vessels in the kidneys. It may present with isolated haematuria and/or proteinuria.

2.7.1.2 Interstitial Nephritis

Interstitial nephritis is a form of nephritis affecting the interstitium of the kidney surrounding the tubules. This is mediated by inflammation of the interstitium and tubules. It has been associated with antibiotics (beta-lactams, quinolones [especially ciprofloxacin], rifampin, macrolides, sulfonamides, tetracyclines), most non-steroidal anti-inflammatory drugs (NSAID), diuretics (thiazides, loop diuretics, and triamterene), anticonvulsants (phenytoin), cimetidine, ranitidine, allopurinol, antivirals (acyclovir, indinavir), and cocaine (Kodner and Kudrimoti, 2003; Markowitz and Perazella, 2005).

2.7.1.3 Crystal Deposition

Ureterolithiasis (kidney stone) is a medical condition resulting from stones or renal calculi in the ureter. The stones are solid concretions or crystals formed in the kidneys from dissolved urinary minerals. The precipitation of crystals in the distal tubular lumen is mostly pH-dependent and explains the nephrotoxicity occurring with acyclovir, sulfonamide, methotrexate, indinavir, and triamterene (Perazella, 1999).

2.7.1.4 Osmotic Nephrosis

This is a non-inflammatory kidney damage due to osmotic pressure (Schetz *et al*., 2005). Hypertonic solutions may decrease the glomerular filtration rate (GFR) due to their effect on the glomerular filtration pressure or because of osmotically induced tubular damage. The uptake of non-metabolisable molecules into proximal tubular cells by pinocytosis generates an oncotic gradient with swelling and vacuolisation of tubular cells and tubular obstruction. Osmotic nephrosis is the mechanism of nephrotoxicity associated with high doses of mannitol (Visweswaran *et al.,* 1997), dextrans (Schwarz *et al*., 1984), and starches (Cittanova *et al.,* 1996; Schortgen *et al.,* 2001).

2.8 Metabolism of Xenobiotics

Xenobiotics are foreign substances that would normally accumulate in the body as a result of poor elimination and thus cause toxicity in the absence of metabolism. One of the most important determinants of xenobiotic persistence in the body, and subsequent toxicity to the organism, is the extent to which such xenobiotics are metabolized and excreted. Several families of metabolic enzymes, often with wide arrays of substrate specificity, are involved in xenobiotic

metabolism. Some of the more important families of enzymes involved in xenobiotic metabolism include the cytochrome P450 monooxygenases (CYPs), flavin-containing monooxygenases (FMOs), alcohol and aldehyde dehydrogenases, amine oxidases, cyclooxygenases, reductases, hydrolases, and a variety of conjugating enzymes such as glucuronidases, sulfotransferases, methyltransferases, glutathione transferases, and acetyl transferases. As a general paradigm, metabolism is the sum total of all the enzymatic reactions of the body. It is also the conversion of hydrophobic substances into derivatives that can easily be eliminated through the urine or the bile (Rose and Hodgson, 2004).

Most xenobiotic metabolism occurs in the liver, an organ devoted to the syntheses of many important biologically functional proteins and thus with the capacity to mediate chemical transformations of xenobiotics (Rose and Hodgson, 2004). Most xenobiotics that enter the body are lipophilic, a property that enables them to bind to lipid membranes and be transported by lipoproteins in the blood. After entrance into the liver, as well as in other organs, xenobiotics undergo one or two phases of metabolism. Xenobiotic metabolizing enzymes have been grouped into two classes: phase 1 and phase 2, depending on which phase of reactions they catalyse. Enzymes of phase 1 reactions are involved in oxidation, reduction, or hydrolytic reactions while enzymes of phase 2 reactions are involved in the conjugation of the products of phase 1 reactions. The phase 1 reactions lead to the introduction of polar functional groups while phase 2 reactions result in more polar products as a result of the conjugational modification of the phase 1 products (Rose and Hodgson, 2004).

2.8.1 Phase one reactions

Phase 1 reactions are classified as the functionalisation phase of drug metabolism. They are involved in the addition of polar constituents to the xenobiotics. The reactions carried out by phase 1 enzymes usually lead to the inactivation of an active site of a drug. In certain instances, metabolism, usually the hydrolysis of an ester or amide linkage, results in bio-activation of a drug. Inactive drugs may undergo metabolism to an active drug which are called pro-drugs. An example is the antitumor drug cyclophosphamide, which is bio-activated to a cell-killing electrophilic derivative. Phase 1 reactions include microsomal monooxygenations, cytosolic and mitochondrial oxidations, reductions, hydrolyses, and epoxide hydration. These reactions (with the exception of reduction reactions) involve the introduction of polar groups to the reactants, thus increasing the polarity of the products (Rose and Hodgson, 2004).

2.8.1.1 Monooxygenations

Monooxygenations, previously known as mixed-function oxidations, are those oxidations in which one atom of a molecule of oxygen is incorporated into the reaction substrate while the other is reduced to water. The reactions are catalysed either by the cytochrome P450 (CYP) - dependent monooxygenase system or by flavin-containing monooxygenases (FMO). These enzymes are located in the endoplasmic reticulum of the cell and have been studied in many tissues and organisms (Rose and Hodgson, 2004).

2.8.1.1.1 The Cytochrome P450 - Dependent Monooxygenase

Cytochrome P450 (CYP450) is a super-family of enzymes which contain a molecule of haem that is non-covalently bound to the polypeptide chain. They are highly expressed in the liver

and bound to the endoplasmic reticulum (Rose and Hodgson, 2004). CYP uses haem as the oxygen – binding moiety. Haem contains one atom of iron in a hydrocarbon cage that functions to bind oxygen in the CYP active site as part of its catalytic cycle. CYP uses O_2 as well as H^+ derived from the cofactor reduced nicotinamide adenine dinucleotide phosphate (NADPH) to carry out the oxidation of substrates. The metabolism of substrate by CYP consumes one molecular oxygen and produces an oxidized substrate and a molecule of water as a by-product (see equation of the reaction below).

$$\mathbf{H^+ + NADPH + RH + O_2 \rightarrow NADP^+ + H_2O + ROH}$$

(where R is substrate)

The reactions of CYPs are listed in Table 2.5 (Rose and Hodgson, 2004).

2.8.1.1.2 Flavin - Containing Monooxygenases

The flavin monooxygenases (FMOs) are another super-family of phase 1 enzymes involved in drug metabolism (Cashman, 2003). Similar to CYPs, the FMOs are expressed at high concentrations in the liver and are bound to the endoplasmic reticulum, a site that favours interaction with and metabolism of hydrophobic drug substrates. There are six families of FMOs, with FMO3 being the most abundant in liver (Motika *et al*., 2007). The reactions catalysed by FMOs are listed in Table 2.5.

Table 2.5: Some of the Oxidative and Reductive Reactions of Xenobiotics Metabolism

Enzymes and Reactions	Xenobiotics
Cytochrome P450	
Epoxidation/hydroxylation	Aldrin, benzo(a)pyrene, aflatoxin, bromobenzene
N-, *O*-, *S*-Dealkylation	Ethylmorphine, atrazine, *p*-nitroanisole, methylmercaptan
N-, *S*-, *P*-Oxidation	Thiobenzamide, chlorpromazine, 2-acetylaminofluorene
Desulfuration	Parathion, carbon disulfide
Dehalogenation	Carbon tetrachloride, chloroform
Nitro reduction	Nitrobenzene
Azo reduction	*O*-Aminoazotoluene
Flavin-containing monooxygenase	
N-, *S*-, *P*-Oxidation	Nicotine, imiprimine, thiourea, methimazole
Desulfuration	Fonofos
Alcohol dehydrogenase	
Oxidation	Methanol, ethanol, glycols, glycol ethers
Reduction	Aldehydes and ketones
Aldehyde dehydrogenase	
Oxidation	Aldehydes resulting from alcohol and glycol oxidations
Amine oxidases	
Oxidative deamination	P-chlorobenzylamine, cadaverine, putrescine
Esterases and amidases	
Hydrolysis	Parathion, paraoxon, dimethoate
Epoxide hydrolase	
Hydrolysis	Benzo(*a*)pyrene epoxide, styrene oxide

Adapted from (Rose and Hodgson, 2004).

2.8.1.2 Carboxylesterases and Amidases

Carboxylesterases and amidases catalyse the hydrolytic reactions of carboxylesters, carboxyamides and carboxythioesters. They are widely distributed in the body, occurring in many tissues both in the microsomal and soluble fractions.

$RC(O)OR' + H_2O \longrightarrow RCOOH + HOR'$ Carboxylester hydrolysis

$RC(O)NR'R'' + H_2O \longrightarrow RCOOH + HNR'R''$ Carboxyamide hydrolysis

$RC(O)SR' + H_2O \longrightarrow RCOOH + HSR'$ Carboxythioester hydrolysis

All the purified carboxylesterases have been observed to exhibit amidase as well as esterase activities. Thus, these two activities are now regarded as different manifestations of the same enzymes, The specificity of the enzymes depends on the nature of the R, R´, and R´´ groups and, to a lesser extent, on the constituents of atom (O, S, or N) adjacent to the carboxyl group (Rose and Hodgson, 2004).

2.8.1.3 Hydrolytic Enzymes (Epoxide Hydrolases)

Epoxide hydrolases are enzymes involved in the hydrolysis of epoxides produced by CYPs. There are two types of epoxide hydrolases: the soluble epoxide hydrolase (sEH), which is highly expressed in the cytosol, and the microsomal epoxide hydrolase (mEH), which is localized in the membrane of the endoplasmic reticulum (Morisseau and Hammock, 2005). Epoxides are highly reactive electrophiles that can bind to the cellular nucleophiles of proteins, RNAs, and DNAs, resulting in cell toxicity and transformation. Thus, epoxide hydrolases participate in the deactivation of potentially toxic derivatives generated by CYPs. The reactions and examples of drugs metabolized by epoxide hydrolases are listed in Table 2.5.

2.8.1.4 Non Microsomal Oxidation

In addition to the microsomal monooxygenases, other enzymes are involved in the oxidation of xenobiotics. These enzymes are located either in the mitochondria or in the soluble cytoplasm of the cell (Rose and Hodgson, 2004).

2.8.1.4.1 Alcohol Dehydrogenase

Alcohol dehydrogenases catalyze the conversion of alcohols to aldehydes or ketones:

$RCH_2OH + NAD^+ \rightleftharpoons RCHO + NADH + H^+$

This reaction is different from the monooxygenation of ethanol by CYP that occurs in the microsomes. The alcohol dehydrogenase reaction is reversible, with the carbonyl compounds being reduced to alcohols. This enzyme is found in the soluble fraction of the liver, kidney, and lung and is probably the most important enzyme involved in the metabolism of foreign alcohols (Table 2.5) (Rose and Hodgson, 2004). In mammals, six classes of the enzymes have been identified and characterised. They can use either NAD^+ or $NADP^+$ as a coenzyme, but the reaction proceeds at a much slower rate with $NADP^+$ (Rose and Hodgson, 2004).

2.8.1.4.2 Aldehyde Dehydrogenase

Aldehydes are generated from a variety of endogenous and exogenous substrates. Endogenous aldehydes may be formed during the metabolism of amino acids, carbohydrates, lipids, biogenic amines, vitamins, and steroids. The metabolism of many drugs and environmental agents produces aldehydes. Aldehydes are highly reactive electrophilic compounds; they may react with thiol and amino groups to produce a variety of effects. Some aldehydes produce therapeutic effects, but more often the effects are cytotoxic, genotoxic, mutagenic, and

carcinogenic (Rose and Hodgson, 2004). Aldehyde dehydrogenases catalyze the formation of acids from aliphatic and aromatic aldehydes, thereby making the acids available as substrates for conjugating enzymes.

$$RCHO + NAD^{+} \rightleftharpoons RCOOH + NADH + H^{+}$$

Other enzymes in the soluble fraction of the liver that oxidize aldehydes are aldehyde oxidase and xanthine oxidase. These are flavoproteins that contain molybdenum. Their primary role seems to be the oxidation of endogenous aldehydes formed as a result of deamination reactions.

2.8.1.4.3 Amine Oxidases

Amine oxidases are concerned with the oxidative deamination of both endogenous and exogenous amines. Monoamine oxidases are a family of flavoproteins found in the mitochondria of a wide variety of tissues (liver, kidney, brain, intestine, and blood platelets) (Rose and Hodgson, 2004). They are enzymes involved in the oxidative deamination of secondary, tertiary as well as, long chain amines. Whereas diamine oxidases are invoved in the oxidative deamination of primary diamines in which the chain length is four (putrescine) or five (cadaverine) carbon atoms (Table 2.5), diamines with carbon chains longer than nine will not serve as substrates but can be oxidized by monoamine oxidases (Rose and Hodgson, 2004).

2.8.2 Phase Two Reactions

The phase 2 reactions facilitate the elimination of drugs and the inactivation of electrophilic and potentially toxic metabolites produced by oxidation. While many phase 1 reactions result in the biological inactivation or activation of drugs, phase 2 reactions produce a

metabolite with improved water solubility and increased molecular weight and these serve to facilitate the elimination of the drug from tissues (Rose and Hodgson, 2004). Glucuronidation, sulfation, acetylation, methylation and conjugation with glutathione or amino acids are the major phase 2 reactions (Table 2.6) (Rose and Hodgson, 2004).

2.8.2.1 UDP – Glucuronosyltransferases (UGTs)

One of the major routes of inactivation and elimination of certain lipophilic substances, as well as some endogenous compounds, is the conjugation of xenobiotics with glucuronic acids (GA). Glucuronidation represents a major pathway which enhances the transformation of many lipophilic xenobiotics to compounds that are more water soluble (King *et al*., 2000). UDP-glucuronosyltransferases (UGTs) catalyse the glucuronidation of the glycosyl group of uridine-5'-diphosphoglucuronic acid (UDPGA) to an acceptor compound. The metabolised substrate from phase 1 reaction with one of many possible functional groups (R-OH, R-NH2, R-COOH), conjugate with the sugar moiety, resulting in a compound that is generally less biologically active and more polar. This characteristic facilitates their excretion in bile and urine (Siest *et al.,* 1987).

The mechanism involves the nucleophilic acceptor group on the substrate attacking the electrophilic group of the glucuronic acid. UDP - glucuronosyl transferase (UGT) is found in the microsomal fraction of liver, kidney, intestine, and other tissues. Glucuronide conjugation generally results in the formation of products that are less biologically and chemically reactive. This, combined with their greater polarity and greater susceptibility to excretion, contributes greatly to the detoxification of most xenobiotics (King *et al*., 2000).

Table 2.6: Some of the Phase two reactions of Xenobiotics Metabolism

Enzymes and Reactions	Xenobiotics
Sulfotransferases	
Conjugation of sulfate group	
UDP – glucuronosyltransferases (UGT)	
Conjugation of glucuronic acid	Coumarin, thiophenol, oxazapam, imipramine, 1-naphthol, 2-naphthylamine
Glutathione – S- transferases (GST)	
Conjugation of gluthathione	1-Naphthalene methyl sulfate, 1, 2 – epoxy-3-(p-nitrophenoxy)propane
N-acetyltransferases	
Acetylation	Isoniazid, sulfamethazine, hydralazine, procainamide, aminofluorene.
Methyltransferases	
Methylation	Epinephrine, norepinephrine, dopamine, and histamine

Adapted from (Rose and Hodgson, 2004)

2.8.2.2 Sulfotransferases

Sulfotransferases are the enzymes responsible for the conjugation of the sulphate group to xenobiotics. They are a family of soluble enzymes with different substrate specificities (Mulder and Jakoby, 1990). Sulphation is a major conjugation pathway for phenols. It also contributes to the biotransformation of xenobiotics with alcohol, amine, and thiol groups. It is important in the metabolism of endogenous compounds such as neurotransmitters and steroid hormones. The resulting compounds are generally less active, more polar, and more readily excreted in the urine. Sulphate conjugation is a multistep process. Inorganic sulfate is inert and must first be converted to adenosine-5' phosphosulfate (APS) and then to an activated 3'-phosphoadenosine 5' phosphosulfate (PAPS). The sulfuryl group from PAPS is then transferred to the acceptor molecule (xenobiotic), a reaction catalysed by sulfotransferase.

$ATP + SO_4^{2-}$ —ATP-sulfurylase→ APS + PPi

APS + ATP —APS-phosphokinase→ PAPS + ADP

R-OH + PAPS —Sulfotransferase→ $R\text{-}OSO_3H$ + 3´- phosphoadenosine 5' phosphate

The enzymes ATP-sulfurylase and APS-phosphokinase are present in the cytosol. PAPS is synthesised in all mammalian cells, with its concentration highest in the liver (Hazelton *et al.*, 1985), but the kidney also has significant amounts (Hjelle *et al.,* 1986). Sulfate conjugation is regulated by the sulfate concentration, the availability of inorganic sulfate and the synthesis of PAPS.

2.8.2.3 Methyltransferases

Methyltransferases are primarily involved in the metabolism of small endogenous compounds and macromolecules and in the biotransformation of certain drugs. Methylation

reactions lead to the formation of less polar compounds that may be less readily excreted from the body, unlike most conjugation reactions. N-, O-, and S-methyltransferases are present in the kidney. The cofactor S-adenosylmethionine (SAM) is required as a methyl donor in reactions catalysed by these enzymes. SAM is primarily formed by the condensation of ATP and L-methionine, and it is present at varying levels in different tissues (Eloranta, 1977).

2.8.2.4 Acetyltransferases

Acetyltransferases are the enzymes involved in the transfer of the acetyl group in phase 2 reactions. They are cytosolic enzymes and are known to exist in many metabolic organs, with the highest concentration in the liver (Weber and Glowinski, 1980). Acetyl-CoA is the acetyl donor for the acetylation reaction. Acetylation is the major metabolic route of arylamines such as isoniazid, sulfamethazine, *p*-aminobenzoic acid, hydralazine, procainamide, aminofluorene, and benzidine (Table 2.5) (Weber and Glowinski, 1980).

2.8.2.5 Glutathione-S-transferases (GSTs)

Glutathione-S-transferases (GSTs) catalyse the conjugation of metabolic substrates with electrophilic substituents from phase 1 reactions to reduced glutathione. Reduced glutathione (GSH) is synthesized from the amino acids glycine, L-cysteine, and glutamic acid and it is present at the highest concentrations in the liver, but is also found in the kidney at concentrations of 1 to 2 mmol/g tissue (Mohandas *et al.,* 1984). The concentration is higher in the cortex than in the medulla (Mohandas *et al.,* 1984). A sufficient supply of L-cysteine is essential for GSH synthesis. GSH is present in the blood at a concentration of approximately 20 mM (Anderson and Meister, 1980), and correlates with the liver concentration. GSH is degraded at the proximal tubule of the

kidney at both the luminal (Hahn *et al.,* 1978) and basolateral membranes (Abbott *et al*., 1984). Nearly all GSH filtered is reabsorbed from the lumen of the proximal tubule. GSH conjugation involves the formation of a thioether link between GSH and electrophilic compounds. This process usually facilitates detoxification, excretion and biosynthesis of certain compounds (Table 2.6).

2.9 Reactive Metabolites

From the time of ingestion into the systemic body to the time they are excreted from the body, many exogenous compounds (xenobiotics) undergo metabolism to highly reactive intermediates. These metabolites may interact with cellular constituents in numerous ways, such as binding covalently to macromolecules and/or stimulating lipid peroxidation. This biotransformation of relatively inert chemicals, drugs or secondary metabolites to highly reactive intermediary metabolites is commonly referred to as metabolic activation or bioactivation. Some toxicants are direct acting and require no activation, whereas other chemicals may be activated non-enzymatically (Rose and Levi, 2004).

Figure 2.16 depicts the overall scheme of metabolism of a potentially toxic xenobiotics. As illustrated, xenobiotic metabolism can produce not only non-toxic metabolites, which are more polar and readily excreted (detoxification), but also highly reactive metabolites, which can interact with vital intracellular macromolecules, resulting in toxicity. In addition, reactive metabolites can be detoxified, for example, by interaction with glutathione. In general, reactive metabolites are electrophiles, which can react with cellular nucleophiles such as glutathione, proteins, and nucleic acids. Other reactive metabolites may be free radicals or act as radical

generators that interact with oxygen to produce reactive oxygen species (ROS) that are capable of causing damage to membranes, DNA, and other macromolecules (Rose and Levi, 2004).

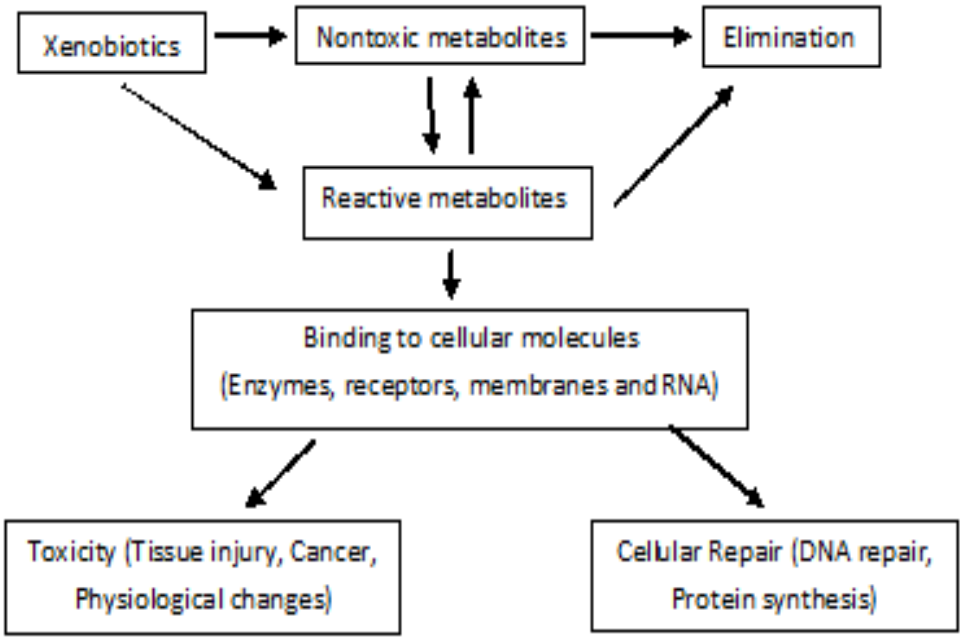


Figure 2.16 The relationship between metabolism, activation, detoxification, and toxicity of xenobiotics (Rose and Levi, 2004**)**

2.9.1 Nature and Stability of Reactive Metabolites

Reactive metabolites include such diverse groups as epoxides, quinones, free radicals, ROS and unstable conjugates. They are usually generated by the reaction of the enzymes involved in xenobiotic metabolism. The enzyme systems most frequently involved in the activation of xenobiotics are those which catalyze oxidation reactions, most eapecially cytochrome P450 monooxygenases (CYP), because of their ubiquitous nature. They are most abundant in the liver, as well as, in the kidney, skin, intestine, placenta, lung and nasal mucosa. As a result of their high reactivity, reactive metabolites are often considered to be short-lived. This is not always true, however, because reactive intermediates can be transported from one tissue to another, where they may exert their deleterious effects. Thus, reactive intermediates are usually divided into several categories, depending on their half-life under physiological conditions (Rose and Levi, 2004).

2.9.1.1 Ultra-short-lived Metabolites

These are metabolites that bind primarily to the parent enzyme. This category includes substrates that form enzyme-bound intermediates that react with the active site of the enzyme. Others also bind primarily to the activating enzymes or adjacent proteins, altering the function of the protein (Rose and Levi, 2004).

2.9.1.2 Short-lived Metabolites

These metabolites remain in the cell or travel only to nearby cells. In this case, covalent binding is restricted to the cell of origin and to adjacent cells. Many xenobiotics implicated in localized tissue damage occurring at the site of activation belong to this group (Rose and Levi, 2004).

2.9.1.3 Longer-lived Metabolites

These metabolites may be transported to other cells and tissues other than where they are produced. Reactive intermediates may also be transported to other tissues, not in their original form but as conjugates that can be released in their reactive form under the specific conditions in the target tissue. For example, carcinogenic aromatic amines are metabolized to their *N*-hydroxylated derivatives in the liver. Thereafter, following glucuronide conjugation, They are transported to the bladder, where the *N*-hydroxy derivative is released under the acidic conditions of urine (Rose and Levi, 2004).

2.9.2 Fate of Reactive Metabolites

Within the tissue a variety of reactions may occur, depending on the nature of the reactive species and the physiology of the organism. The following are the fates of the reactive metabolite *in-situ*.

2.9.2.1 Binding to Cellular Macromolecules

Most reactive metabolites are electrophiles that can bind covalently to nucleophilic sites on cellular macromolecules such as proteins, polypeptides, RNA, and DNA. This covalent binding is considered to be the initiating event for many toxic processes such as mutagenesis, carcinogenesis and cellular necrosis (Rose and Levi, 2004). For example, in the metabolism of acetaminophen at normal therapeutic doses, acetaminophen is safe, but at higher doses it has been reported to be hepatotoxic (Rose and Levi, 2004). In excessive consumption of acetaminophen, sulfate and glucuronide cofactors (PAPS and UDPGA) become depleted, thereby resulting in the

production of reactive metabolites (Figure 2.17) (Rose and Levi, 2004). These reactive metabolites can be detoxified, with the availability of glutathione. However, in cases of drug overdose or suicide attempts, the concentration of glutathione might be depleted, resulting in covalent binding to the sulfhydryl (thiol) groups of various cellular proteins which might eventually result in hepatic necrosis (Rose and Levi, 2004).

2.9.2.2 Lipid Peroxidation

Free radicals such as CCl_3^+, produced during the oxidation of carbon tetrachloride, may induce lipid peroxidation and subsequent destruction of lipid membranes. As a result of the critical nature of various cellular membranes, lipid peroxidation can be a pivotal event in cellular necrosis (Rose and Levi, 2004).

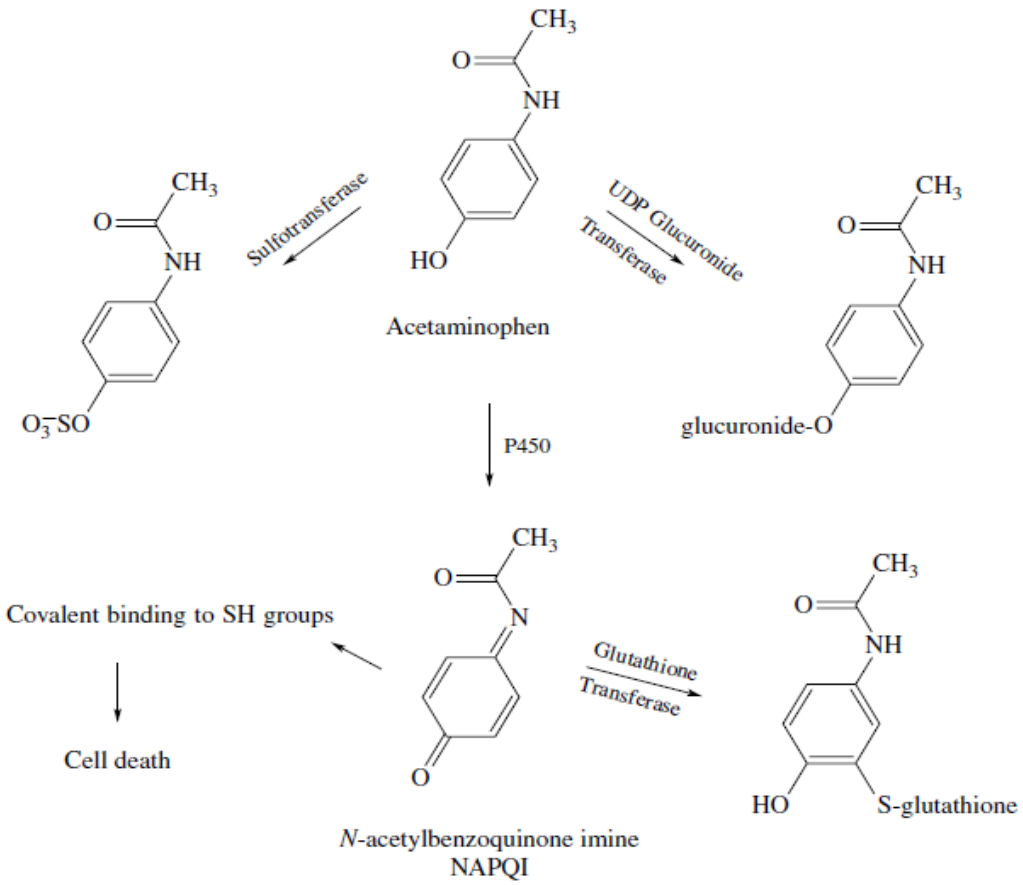


Figure 2.17 Metabolism of acetaminophen and formation of reactive metabolites (Rose and Levi, 2004).

2.10 Potential Toxicity of Dietary Flavonoid and Phenolics

Dietary flavonoids and phenolics have been shown to act as pro-oxidants in systems containing redox-active metals (Galati and O'Brien, 2004). In the presence of molecular oxygen (O_2), transition metals such as copper (Cu) and iron (Fe) can catalyse the redox cycling of phenolics, leading to the formation of reactive oxygen species (ROS) and phenoxyl radicals that can damage DNA, lipids, and other biological molecules (Li and Trush, 1994; Decker, 1997; Yamanaka *et al.,* 1997). It has been reported that exposure of DNA to dihydrocaffeic acid in the presence of Cu resulted in more DNA single- and double-strand breaks than exposure to caffeic acid, whereas chlorogenic acid caused only minimal damage even though these phenolics had similar structures and redox potentials (Galati and O'Brien, 2004). The inv investigators proposed that the initial oxidation of the catechols by Cu^{2+} generates a semiquinone that reacts with O_2 to form $O_2^{\cdot -}$, which then oxidises the catechol to regenerate the semiquinone and H_2O_2. H_2O_2 is then rapidly converted by Cu^{1+} to the $^{\cdot}OH$ radical in a Fenton-type reaction (Sakihama *et al.,* 2002)

Flavonols with pyrogallol or catechol B rings have also been shown to autoxidize in the presence of transition metals to produce ROS which accelerate low-density lipoprotein oxidation during the propagation phase (Ahmad *et al.,* 1992). However, *in vivo*, most transition metal ions are sequestered in forms unable to catalyse free radical reactions (Halliwell and Gutterridge, 1990). Very low levels of free copper ions may be released by tissue injury (e.g., atherosclerotic lesions) (Smith *et al.,* 1992) and possibly by hepatic Cu (II) overload diseases such as Wilson disease. The green tea catechin, epigallocatechin gallate (EGCG), has recently been shown to induce H_2O_2 generation and to cause subsequent oxidative damage to isolated and cellular DNA in the presence of transition metal ions (Furukawa *et al.,* 2003).

From the reports of Galati and O'Brien (2004), with particular focus on the peroxidase catalysed oxidation of phenol ring-containing flavonoids and other dietary phenolics to phenoxyl radicals, it has been shown that catalytic concentrations of flavonoids with a phenol B ring, upon oxidation by peroxidase/H_2O_2, formed phenoxyl radicals (Galati *et al.*, 1999; Chan *et al.*, 1999). Peroxidases are haem-containing enzymes that usually catalyse a one-electron oxidation of a variety of xenobiotics by hydrogen peroxide (Saunders, 1973). They have been reported to induce the oxidation of flavonoids, or their metabolites accumulating in the plasma or bone marrow, to their reactive metabolites (Galati and O'Brien, 2004). Myeloperoxidase, eosinophil peroxidase, and lactoperoxidase primarily found in granules (lysosomes) of neutrophils, eosinophils and secretory cells of the exocrine gland respectively, have been responsible for the development of secondary acute myelogenous leukemias occurring after cancer therapy with etoposide (a phenolic topoisomerase inhibitor). These have been attributed to DNA damage by pro-oxidant phenoxyl radicals formed by the peroxidase/H_2O_2 (Subrahmanyam *et al.*, 1991; Kagan *et al.*, 1999; Goldman *et al.*, 1999).

The peroxidase-mediated oxidation of catechol B ring-containing flavonoids has also been reported. This resulted in the formation of semiquinone- and quinone-type metabolites. These semiquinone- and quinone-type metabolites may act as electrophiles binding to cellular macromolecules such as DNA, lipids and proteins, and may also give rise to the production of ROS (Awad *et al.*, 2001). Quercetin, the most ubiquitous of the dietary flavonoids, contains a catechol B ring and has been shown to be oxidized by tyrosinase, peroxidases and hydrogen peroxide, to quinone/quinone methide intermediates, subsequent reactions with GSH resulting in quercetin glutathionyl adducts (Awad *et al.*, 2000; Galati *et al*. 2001).

The chemopreventive properties of flavonoids are generally believed to reflect their ability to scavenge endogenous ROS. However, the pro-oxidant action of plant-derived phenolics rather than their antioxidant action may be an important mechanism for their anticancer and apoptosis-inducing properties, as ROS can mediate apoptotic DNA fragmentation (Kaufmann, 1989; Rahman *et al.,* 1990; Hadi *et al.,* 2000;). Phenolic antioxidants can be both pro-oxidative and antioxidative (Figure 2.18).

This suggests that dietary flavonoids/phenolics could be potentially more of an oxidative risk than a benefit (Decker, 1997). The consumption of large amounts of flavonoids in the form of a concentrated supplement may not be safe until their *in vivo* potential for oxidative stress is evaluated (Galati and O'Brien, 2004).

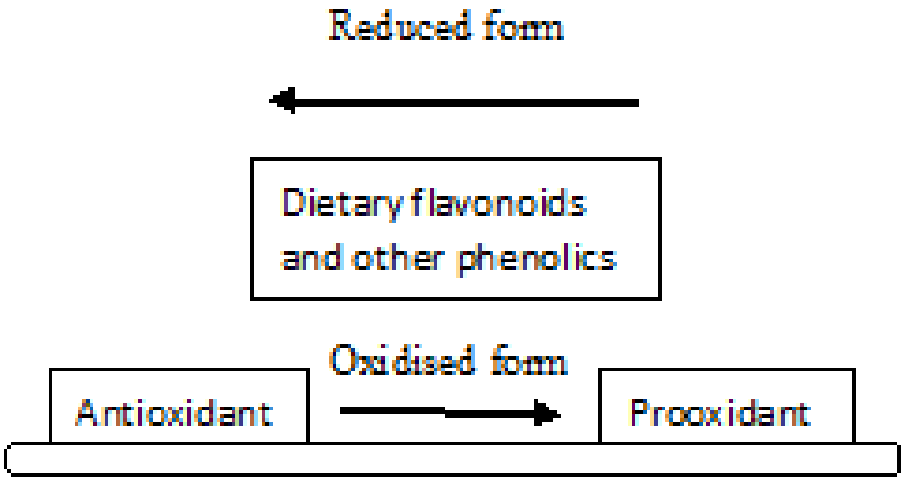


Figure 2.18: The representation of the balance between antioxidant and prooxidant characteristics of flavonoids and other dietary phenolics. The reduced forms of flavonoids or other dietary phenolics act as antioxidants; however, the oxidized forms (phenoxyl radicals or quinone/quinone methide intermediates) can have prooxidant activities (Galati and O'Brien, 2004).

2.11 Malaria

Malaria is caused by protozoan parasites of the genus *Plasmodium*. Four species (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) infect humans. Although malaria caused by *P. vivax* is the most common, malaria caused by *P. falciparum* is the most lethal (WHO, 2011b, http://www.searo.who.int/en/Section10/Section21/Section334.htm). Other species, such as *P. knowlesi*, *P. yoelii*, *P. berghei*, *P. chabaudi*, and *P. gallinaceum* infect a number of wild and domestic animals and are frequently used as models for the human parasites. Malaria parasites are transmitted by female anopheles mosquitoes. Figure 2.19 illustrates the malaria-endemic countries of the world. Once transmitted, the parasites begin to multiply in the red blood cells, eliciting symptoms of discomfort. Light headedness and breath shortage, both stemming from anaemia (caused by haemolysis), fever, chills, nausea, arthralgia (joint pains), vomiting, haemoglobinuria, convulsions, coma and death, are common symptoms noticed after the transmission of malaria parasites (Boivin, 2002).

The symptoms of malaria occurs in cyclic pattern such as sudden fever, rigour and sweating lasting between four to six hours every two days in *P. vivax* and *P. ovale* infections, three days in *P. malariae* infection, and 36 - 48 hours in *P. falciparum* infection (Boivin, 2002). Widespread anaemia and direct brain damage resulting from cerebral malaria, to which children are more vulnerable, have been reported (Boivin, 2002). Severe *P. falciparum* infection is associated with symptoms including an enlarged spleen (splenomegaly), cerebral ischemia, hepatomegaly, hypoglycemia, and haemoglobinuria, usually arising between 6 and14 days of infection (Kain *et al.*, 1998; Kain *et al.*, 1998). The life cycle of the malaria parasite is depicted in Figure 2.20.

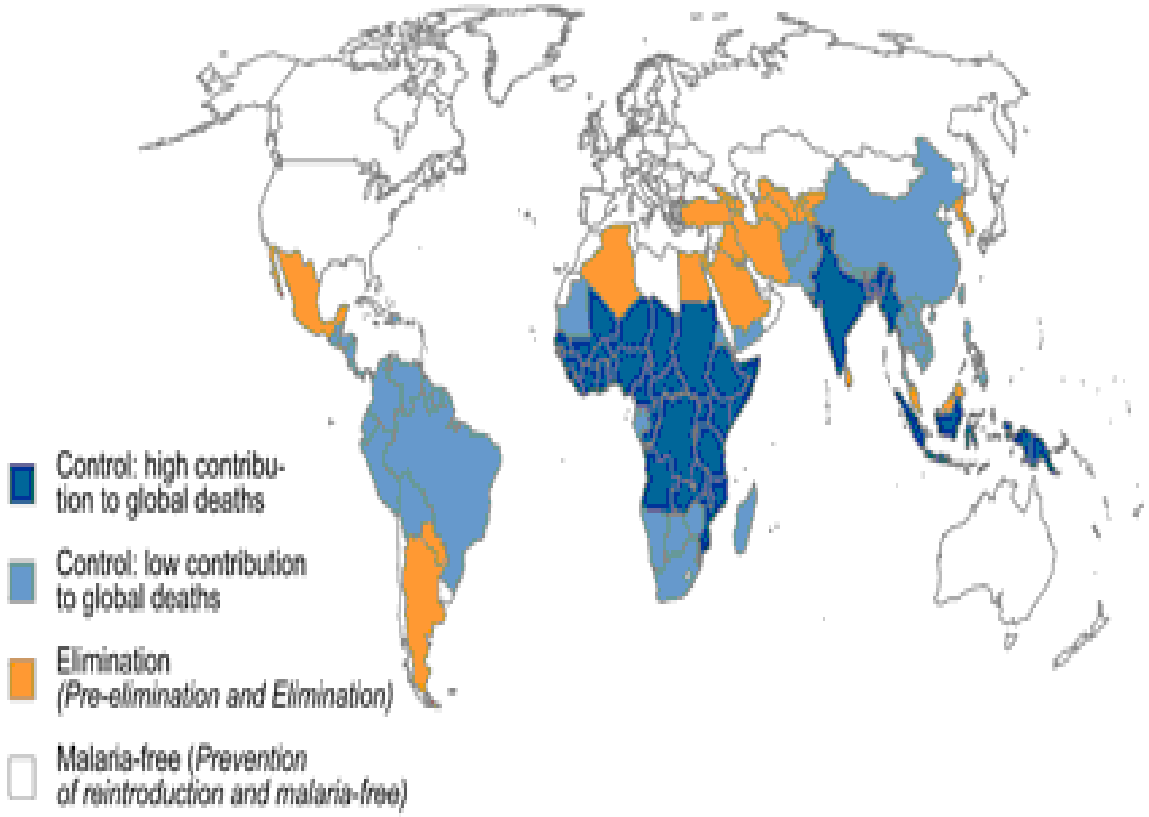


Figure 2.19: Malaria-endemic countries of the world (WHO, 2011c, http://www.rbm.who.int/endemiccountries.html)

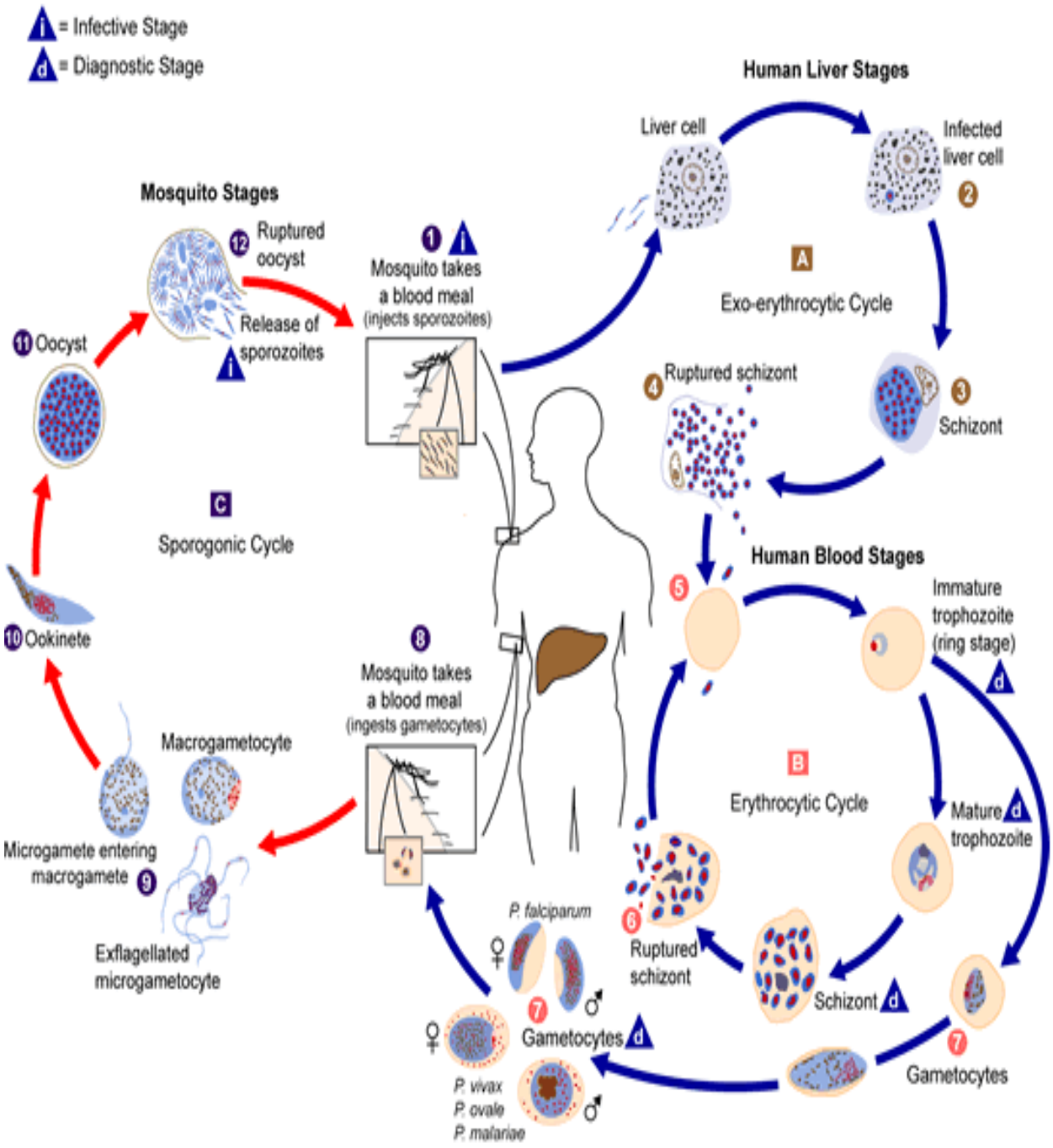


Figure 2.20: The life cycle of the malaria parasite. During a blood meal, a malaria infected female anopheles mosquito inoculates sporozoites into the blood stream of the human host❶. Within an hour after inoculation, sporozoites infect the hepatocytes❷ and mature into schizonts❸,

which rupture and release merozoites(4). In *P. vivax* and *P. ovale*, hypnozoites, a dormant form typically remain quiescent in the hepatocytes and can relapse after a while into the bloodstream but *P. falciparum* does not produce hypnozoites. Subsequently, after the initial exo-erythrocytic schizogony phase(A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony phase(B)), leading to the infection of the red blood cells(5) by the merozoites and the development of the ring stage, trophozoites, into mature schizonts. These thereafter, rupture releasing merozoites(6), the erythrocytic cycle continues with new daughter merozoites re-invading the reb blood cells. Some parasites differentiate into sexual erythrocytic stages (gametocytes)(7). The erythrocytic stage is responsible for the clinical manifetastion of malaria. The male (microgametocytes) and female (macrogametocytes) gametocytes are ingested by a female *Anopheles* mosquito during a blood meal(8). The parasites' multiplication in the mosquito is known as the sporogonic cycle(C). While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes(9). The zygotes become motile and elongated (ookinetes)(10) and which invade the midgut wall of the mosquito where they develop into oocysts(11). The oocysts grow, rupture, and release sporozoites(12), which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle(1) (Garcia, 2001; Centers for Disease Control and Prevention (CDC), 2011).

2.12 New Lead Antimalarial Compounds from Plants

The following are the criteria for the discovery of new lead antimalaria compounds from medicinal plants (Wright, 2010). Viable antimalaria compounds from medicinal plants must exhibit the following attributes:

potent antiplasmodial activity against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*;

high selectivity index (SI), selective toxicity to malaria parasites compared to mammalian cell lines;

complete eradication of malaria parasites in mice (i.e., suppress parasitaemia by close to 100%) without showing toxicity; and

oral administered with appreciable activity.

2.12.1 Compounds from Medicinal Plants with Potential Antimalarial Activities

Studies on isolated compounds from medicinal plants with moderate to appreciable activitites from different classes of phytochemicals have been documented (Wright, 2010). From various classes of alkaloids, dihydrousambarensine and isostrychnopentamine (Frederich, 2008; Frederich, *et al.,* 2004), constituents from *Strychnos usambarensis,* isosungucine (Frederich *et al.,* 2008; Phillippe *et al.,* 2007), from *S. icaja*, voacamine (Ramanitrahasimbola *et al.,* 2001), from *Tabernaemontana fuchsiafolia*, strychnobrasiline and malagashanine (Frederich, 2008; Rasoanaivo *et al.,* 1994; Ramanitrahasimbola *et al.,* 2006) from *S. myrtoides,* cryptolepine (Wright, 2007), from *Cryptolepis sanguinolenta*, dionchophylline C and dionchophylline A (Francois *et al.,* 1997), from *Triphophylum peltatum*, tazopsine and sinococuline (Bero *et al.,* 2009; Carraz *et al.,* 2008), from *Strychnopsis thouarsii*, have been reported to have moderate to

good antiplasmodial activities. But strychnobrasiline and malagashanine have also been reported to have weak antiplasmodial activities; however, in combination with chloroquine, they have been able to reverse chloroquine resistance in *P. falciparum* by stimulating the influx and reducing the efflux of chloroquine (Rasoanaivo *et al.,* 1994; Ramanitrahasimbola *et al.,* 2006; Frederich *et al.,* 2008), while, *in vivo* toxicity has been reported for cryptolepine (Wright, 2007).

Among the phenolic classes of phytochemicals, Lichochalcone A, a constituent of *Glycyrrhiza uralensis* (Chen *et al.,* 1994), (-)-Methyllinderatin, a prenyl-substituted dihydrochalcone from *Piper hostmannianum* (Portet *et al.,* 2007), 4-phenylcoumarins, 5-O-β-D-glucopyranosyl-7-methoxy-3',4'-dihydroxy-4-phenylcoumarin, from *Hintonia latiflora* (Argotte-Ramos *et al.,* 2006), stilbene glycoside, piceid-(1/6)-b-D-glucopyranoside, from *Parthenocissus tricuspidata* (Park *et al.,* 2008), have between reported to have moderate to good antiplasmadial activities both *in vivo* and *in vitro*. Lichochalcone A enhances the antiplasmodial action of artemisinin against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* (Mishra *et al.,* 2009). It has been suggested that chalcones have potential for the development of inexpensive antimalarials (Awasthi *et al,* 2009).

Among the terpenoids, a sesquiterpene lactone, ineupatorolide A, from *Carpesium rosulatum* (Chung *et al.,* 2008), with good antiplasmodial activity and Quassinoids an oxygenated, degraded diterpene constituent of Simaroubaceae family has antiplasmodial activity but exhibit toxicity in mice as a result of their effects on mammalian protein synthesis (Muhammad and Samoylenko, 2007). In addition, 3,15-dimethylcarbonate bruceolide and 3,15-diethylcarbonate bruceolide, from *Brucea javanica* also have good antiplasmodial activities with low toxicity in mice. More research into the antiplasmodial activities of quassinoids may be necessary so as to take advantage of their ability to inhibit protein synthesis. Since malaria parasites make their own

ribosomes, these are more susceptible to inhibition by quassinoids than the ribosomes of host cells (Muhammad and Samoylenko, 2007).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Collection and Identification of Plant

Young twigs and leaves of *C. bonduc* (CB) were collected from the Forest Research Institute of Nigeria (FRIN), Ibadan, Oyo State, Nigeria. Plant identification was done by Dr. Conrad Omonhinmi (Botanist), Department of Biological Sciences, College of Science and Technology, Covenant University, Ota, Ogun state. The authentication and voucher referencing were carried out at FRIN with voucher specimen no SHI108408 deposited in the FRIN Herbarium.

3.1.2 Purchase of Experimental albino Wistar Rats

Healthy adult female albino Wistar rats (n = 112) used in the in vivo and toxicological assessments were purchased from the National Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria.

3.1.3 Chromatography

The following chromatography materials and equipment were used during the study: pre-coated silica gel GF_{254} glass plates (Qingdao Marine Chemical, Ltd, Qingdao, China); Silica gels, 100-200 mesh, 200-300 mesh and 10-40 μl (Qingdao Marine Chemical, Inc, China); Lichroprep Reverse Phase gel RP-18, 40-63 μm (Merck, Darmstadt, Germany); MCI gel, 75-150 μm (Mitsubishi Chemical Corporation, Japan); Sephadex LH-20, 25-100 μm (Pharmacia Fine Chemicals Co., Ltd., Sweden); High Performance Liquid Chromatography, HPLC, HP Agilent

1100 (Agilent Technologies, USA); Medium Pressure Liquid Chromatography, MPLC (Agilent Technologies, USA).

3.1.4 *Plasmodium falciparum* and Cancer Cell Lines

BGC-823 (Human gastric carcinoma) and HeLa (Human cervical adenocarcinoma) cells were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China) and the Cell Culture Centre of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Peking, China). *Plasmodium falciparum* strain FCR-3 (ATCC 30932) and mouse mammary tumor FM3A cell line (wild-type, subclone F28-7) were obtained from the Japanese Cancer Research Resources Bank (JCRB), Japan.

3.1.5 Culture Media

A Rosewell Park Memorial Institute (RPMI) - 1640 medium (Gibco, NY, USA) and a mouse embryonic stem (ES) cell culture medium (Nissui Pharmaceuticals, Tokyo, Japan) were used for the experiments.

3.1.6 Standard Drugs

Taxol (Paclitaxel) (Sigma Chemical, St Louis, MO, USA), quinine hydrochloride, pyrimethamine and artemisinin (Sigma, St Louis, MO, USA) and Mefloquine (Roche LTD, Basel, Switzerland) were used for the study.

3.2 Methods

3.2.1 Preparation of Extraction of *C. bonduc*

The leaves and young twigs of *C. bonduc* were air-dried at room temperature (25^{o}C) and powdered. 8800 g of CB was extracted with 50 litres of 75% (v/v) ethanol by maceration using three successive cold (25^{o}C) extractions for 72 hours. The total filtrate was concentrated to dryness on a rotary evaporator at 50ºC. The percentage (%) yield of the dry residue was calculated (Pudhom *et al.,* 2007). The extract for *in vivo* study was re-suspended in 0.25 % w/v sodium carboxymethylcellulose and stored at -20ºC for further studies.

3.2.2 Differential Fractionation of the Ethanolic Extract of CB in Different Solvents

The dried ethanolic extract of CB (1120 g) was suspended in 10 litres of distilled water and partitioned in sequence with petroleum ether (10 litres), ethyl acetate (10 litres), and *n*-butanol (10 litres). The different solvent fractions were concentrated on a rotary evaporator to give a petroleum ether - soluble fraction (150 g), an ethyl acetate - soluble fraction (120 g), a *n*-butanol - soluble fraction (170 g), and a distill water - soluble fraction (630 g). Thin layer chromatography (TLC) assessments were made to qualitatively examine different phytoconstituents in each fraction.

3.2.3 Phytochemical Screening of the Plant

Phytochemical screening of the extracts was carried out by a procedure that was based on those earlier reports by Harborne (1973), Trease and Evans (1989) and Sofowora (1993).

3.2.3.1 Test for Tannins

Powdered leaves and twigs of the plant (0.5 g) was boiled in 20 ml of water in a test tube and then filtered. A few drops (5-6) of 0.1 % ferric chloride solution were added. The reaction mixture was observed for a brownish green or blue-black colouration for the confirmation of the presence of tannins.

3.2.3.2 Test for Phlobatannins

Powdered leaves and twigs of the plant (0.5 g) was boiled in 20 ml of water in a test tube and then filtered. An extract of the plant sample was boiled with 1 % aqueous HCl and then observed for the deposition of red precipitate for the confirmation of the presence of phlobatannins.

3.2.3.3 Test for Saponin

Powdered leaves and twigs of the plant (2 g) was boiled in 20 ml of distilled water in a test tube and then filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously and then observed for the formation of an emulsion, indicative of the presence of saponin.

3.2.3.4 Test for Steroids

Acetic anhydride (2 ml) and 2 ml of H_2SO_4 were added to 0.5 g ethanolic extract of the plant. The colour change from violet to blue or green in some samples is an indication of the presence of steroids.

3.2.3.5 Test for Terpenoids

The ethanolic extract (0.5 g) was mixed with 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration formed at the interface indicated the presence of terpenoids.

3.2.3.6 Test for Cardiac Glycosides

The ethanolic extract (0.5 g) was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was overlaid with 1 ml of concentrated H_2SO_4. A brown ring at the interface indicates the presence of a deoxysugar characteristic of cardenolides.

3.2.3.7 Test for Flavonoids

Powdered leaves and twigs of the plant (0.5 g) was boiled in 20 ml of water in a test tube and then filtered. 5 ml of dilute ammonia solution was added to a portion of the filtrate, followed by the addition of concentrated H_2SO_4. A yellow coloration was indicative of the presence of flavonoids.

3.2.4 Fractionation of *Caesalpinia bonduc* (CB)

From the phytochemical assessment of the different solvent fractions of CB, the ethylacetate and petroleum ether fractions (wt. 270 g) were pooled together and mixed with 400 g silica gel (100 - 200 mesh). A separating column was prepared with 3 kg silica gel (100 - 200 mesh). The eluent was a mixture of chloroform and methanol in different ratio; the starting eluent was in 100:1 mixture. Fractions with similar spots using thin layer chromatography examination were pooled together and these gave 20 different fractions labelled from C_1 to C_{20}.

Fraction labelled C_2 (25g) was mixed with 35 g silica gel (200 - 300 mesh) and layered on a silica gel column (200 - 300 mesh, 300 g). This was eluted with mixture of petroleum ether and ethyl acetate in different ratio, the starting eluent was in a 50:1 mixture. Fractions with similar spots using TLC examination were pooled together to give 4 fractions labelled C_2a_1 to C_2a_4. Fraction labelled C_3 (20 g) was mixed with 30 g of silica gel (200 - 300 mesh). This was layered on a silica gel column (200 - 300 mesh, 250 g) and eluted with mixture of petroleum ether and ethyl acetate in different ratio, the starting eluent was in a 30:1 mixture. Fractions with similar spots using TLC examination were pooled together to give 8 fractions labeled from C_3a_1 to C_3a_8. Fractions labelled C_3a_4 and C_3a_5 (2.5 g) were combined based on the result of the TLC examination and mixed with silica gel (200 - 300 mesh, 3.5 g). This was layered on a silica gel column (200 - 300 mesh, 4 g) and eluted with a mixture of petroleum ether and ethyl acetate in different ratio; the starting eluent was in a 20:1 mixture. Three fractions were produced and labelled from $C_3a_4b_1$ to $C_3a_4b_3$.

Fraction C_7 (10g) was mixed with 15 g silica gel (200 - 300 mesh) and layered on silica gel column (200 - 300 mesh, 120 g) and eluted with mixture of chloroform and acetone in different ratio; the starting eluent was in a 40:1 mixture. Five fractions were produced and labelled from C_7a_1 - C_7a_5. Fraction C_7a_4 (4g) was mixed with 6 g of silica gel (200 - 300 mesh) and was layered on a silica gel column (200 - 300, 50 g) and eluted with mixture of chloroform to acetone in different ratio; the starting eluent was in a 15:1 mixture. Four fractions were produced and labelled from $C_7a_4b_1$ to $C_7a_4b_4$. Fraction $C_7a_4b_3$ (3g) was mixed with 5 g of silica gel (200 - 300 mesh) and was layered on a silica gel column and eluted with mixture of chloroform and acetone in different ratio; the starting eluent was in a 10:1 mixture. Seven fractions were produced and labelled from $C_7a_4b_3c_1$ to $C_7a_4b_3c_7$. Fractions labelled $C_7a_4b_3c_4$ to $C_7a_4b_3c_6$ (2 g) were pooled

together and mixed with 3 g silica gel (200 - 300 mesh). This was layered on silica gel (10 - 40 μm, 25 g) and eluted with mixture of chloroform and acetone in different ratio; the starting eluent was in a 30:1 mixture. Seven fractions were produced and labelled from $C_7a_4b_3c_4d_1$ to $C_7a_4b_3c_4d_7$. Fraction labelled $C_7a_4b_3c_4d_5$, was layered on sephadex column (LH - 20) and eluted with mixture of chloroform and methanol in ratio 1:1. This produced seven fractions labelled $C_7a_4b_3c_4d_5S_1$ to $C_7a_4b_3c_4d_5S_7$. Fractions labelled $C_7a_4b_3c_4d_5S_5$ to $C_7a_4b_3c_4d_5S_7$ (0.5 g) were pooled together and mixed with 1 g of silica gel (200 - 300 mesh). This was layered on silica gel column (10 - 40, 10 g) and eluted with chloroform and acetone in different ratios; the starting eluent was in a 20:1 mixture. Three fractions were produced and labelled from $C_7a_4b_3c_4d_5S_5e_1$ to $C_7a_4b_3c_4d_5S_5e_3$. Fraction labelled $C_7a_4b_3c_4d_5S_5e_1$ (100 mg) was mixed with silica gel (200 - 300 mesh, 1 g) and layered on silica gel column (10 - 40, 10 g). The column was eluted with mixture of chloroform and acetone in different ratio; the starting eluent was in 15:1 mixture. Four fractions were produced and labelled from $C_7a_4b_3c_4d_5S_5e_1f_1$ to $C_7a_4b_3c_4d_5S_5e_1f_4$.

Fraction labelled C_6 (22 g) was mixed with 35 g of silica gel (200 - 300 mesh). This was layered on silica gel column (200 - 300 mesh, 250 g) and eluted with a mixture of petroleum ether and acetone in different ratio; the starting eluent was in 15:1 mixture. Sixteen fractions were produced and labelled C_6a_1 to C_6a_{16}. Fraction labelled C_6a_{13} (1 g) was mixed with 1.5 g of silica gel. This was layered on silica gel column (10 - 40, 10 g) and eluted with mixture of chloroform and ethyl acetate; the starting eluent was in 30:1 mixture. Six fractions were produced and labelled from $C_6a_{13}b_1$ to $C_6a_{13}b_6$. Fraction labelled C_6a_{14} (9 g) was mixed with 14 g of silica gel. This was layered on silica gel column (200 - 300, 100 g) and eluted with a mixture of chloroform and ethyl acetate in different ratio; the starting eluent was in 30:1 mixture. Five fractions were produced and labelled from $C_6a_{14}b_1$ to $C_6a_{14}b_5$. Fraction labelled C_6a_{16} (1.5 g) was mixed with 2.5

g silica gel (200 - 300 mesh). This was layered on silica gel column (10 - 40, 20 g) and eluted with the mixture of chloroform and methanol; the starting eluent was in a 150:1 mixture. Five fractions was produced and labelled from $C_6a_{16}b_1$ to $C_6a_{16}b_5$. Fraction labelled $C_6a_{16}b_2$ (50 mg) was layered on sephadex (LH-20) column and eluted with methanol and water to produce 5 fractions, labelled from $C_6a_{16}b_2S_1$ to $C_6a_{16}b_2S_5$. Fraction labelled $C_6a_{16}b_3$ (60 mg) was layered on sephadex (LH-20) column to produce 5 fractions labelled $C_6a_{16}b_3S_1$ to $C_6a_{16}b_3S_5$.

Fraction labelled $C_6a_{13}b_6$ (50 mg) was layered on sephadex (LH-20) column to give 3 fractions ($C_6a_{13}b_6S_1$ to $C_6a_{13}b_6S_3$). Fraction labelled $C_6a_{14}b_4$ (3 g) was mixed with silica gel (200 - 300, 4.5 g). This was layered on silica gel (200 - 300, 35 g) column and eluted with a mixture of chloroform and ethyl acetate in different ratio; the starting eluent was in 30:1 mixture. Four fractions were produced and labelled from $C_6a_{14}b_4d_1$ to $C_6a_{14}b_4d_4$. Fraction labelled $C_6a_{14}b_4d_4$ (200 mg) was mixed with silica gel (200 - 300 mesh, 250 mg). This was layered on a silica gel column (10 - 40, 2 g) and eluted with mixture of chloroform and ethyl acetate; the starting eluent was in 30:1 mixture. Five fractions were produced and labeled from $C_6a_{14}b_4d_4e_1$ to $C_6a_{14}b_4d_4e_5$.

Fraction labelled $C_6a_{14}b_4d_4e_5$ (100 mg) was mixed with silica gel (200 - 300 mesh, 150 mg). This was layered on a silica gel column (10 - 40, 1 g) and eluted with a mixture of chloroform and ethyl acetate; the starting eluent was in 25:1 mixture. Four fractions were produced and labelled from $C_6a_{14}b_4d_4e_5f_1$ to $C_6a_{14}b_4d_4e_5f_4$. Fraction labelled $C_6a_{14}b_4d_4e_5f_3$ (80 mg) was mixed with silica gel (200 - 300 mesh, 100 mg). This was layered on silica gel column (10 - 40, 1 g) and eluted with a mixture of chloroform and methanol; the starting eluent was in 150:1 mixture. Six fractions were produced and labelled from ($C_6a_{14}b_4d_4e_5f_3g_1$ to $C_6a_{14}b_4d_4e_5f_3g_6$). Fraction labelled $C_6a_{14}b_4d_4e_5f_3g_5$ (70 mg) was layered on Sephadex column (LH-20) and eluted with mixture of chloroform and methanol in ratio 1:1 to produce 5 fractions labelled from

$C_6a_{14}b_4d_4e_5f_3g_6S_1$ to $C_6a_{14}b_4d_4e_5f_3g_6S_5$. Fraction labelled $C_6a_{14}b_4d_4e_5f_3g_6S_2$ (50 mg) was layered on Sephadex (LH-20) column and eluted with mixture of chloroform methanol in ratio 1:1 to yield 3 fractions labelled from $C_6a_{14}b_4d_4e_5f_3g_6S_2h_1$ to $C_6a_{14}b_4d_4e_5f_3g_6S_2h_3$.

Fractions labelled C_{10} and C_{11} (32 g) were pooled together and mixed with silica gel (200 - 300, 48 g). This was layered on silica gel column (200 - 300, 550 g) and eluted with a mixture of chloroform and acetone in different ratio; the starting eluent was in 30:1 mixture. Twelve fractions were produced and labelled from $C_{10}a_1$ to $C_{10}a_{12}$. Fraction labelled $C_{10}a_5$ (2.5 g) was mixed with silica gel (200 - 300, 3.5 g). This was layered on silica gel column (200 - 300) and eluted with mixture of chloroform and methanol; the starting eluent was in 120:1 mixture. Six fractions were produced and labelled from $C_{10}a_5b_1$ to $C_{10}a_5b_6$. Fraction labelled $C_{10}a_5b_4$ (1 g) was mixed with silica gel column (200 - 300, 1.5 g). This was layered on silica gel column (10 - 40, 12 g) and eluted with mixture of chloroform and methanol; the starting eluent was in 100:1 mixture. Six fractions were produced and labelled from $C_{10}a_5b_4d_1$ to $C_{10}a_5b_4d_6$. Fraction labelled $C_{10}a_5b_5$ (1g) was mixed with silica gel (200 - 300, 1.5 g). This was layered on a silica gel column (10 - 40, 15 g) and eluted with a mixture of chloroform and methanol; the starting eluent was in 60:1 mixture. Six fractions were produced and labelled from $C_{10}a_5b_5d_1$ to $C_{10}a_5b_5d_6$. Fractions labelled $C_{10}a_5b_4d_5$, $C_{10}a_5b_4d_2$ and $C_{10}a_5b_5d_4$ were separately subjected to High Pressure Liquid Chromatography (HPLC, YMC-Pack ODS-A, 10 mm × 15 cm) column at a flow rate of 2 ml/min. The fractions, $C_{10}a_5b_4d_5$, $C_{10}a_5b_4d_2$ and $C_{10}a_5b_5d_4$ were eluted with Methanol (HPLC grade) and water at gradient of 60 to 40, 58 to 42 and 56 to 44 respectively for 20 mins, to produce different pure compounds.

Fractions C_{12} (12 g) was mixed with silica gel (200 - 300, 18 g). This was layered on silica gel (200 - 300, 135 g) and eluted with a mixture of chloroform and methanol in different ratio; the

starting eluent was in 200:1 mixture. Five fractions were produced and labelled from $C_{12}a_1$ - $C_{12}a_5$. Fraction labelled $C_{12}a_3$ (2 g) was mixed with silica gel (200 - 300, 3 g). This was layered on silica gel (10 - 40, 25 g) and eluted with a mixture of chloroform and methanol in different ratio; the starting eluent was in 200:1 mixture. Four fractions were produced and labelled $C_{12}a_3b_1$ to $C_{12}a_3b_4$. Fraction labeled $C_{12}a_3b_4$ (1 g) was mixed with silica gel (200 - 300, 1.5 g). This was layered on silica gel (10 - 40, 10 g) and eluted with chloroform amd methanol in different ratio; the starting eluent was in 40:1 mixture. Three fractions were produced and labelled from $C_{12}a_3b_4c_1$ to $C_{12}a_3b_4c_3$. Fraction labelled $C_{12}a_3b_4c_1$ (500 mg) was layered on Sephadex column and eluted with mixture of chloroform and methanol in ratio 1:1 to give three fractions labelled from $C_{12}a_3b_4c_1S_1$ to $C_{12}a_3b_4c_1S_3$. Fraction labelled $C_{12}a_4$ (1.5 g) was mixed with silica gel (200 - 300, 3 g). This was layered on silica gel column (10 - 40, 17 g) and eluted with a mixture of chloroform and methanol in different ratio; the starting eluent was in 40:1 mixture. Six fractions were produced and labelled from $C_{12}a_4b_1$ to $C_{12}a_4b_6$. Fraction labelled $C_{12}a_4b_5$ (800 mg) was mixed with silica gel (200 - 300, 1.2 g). This was layered on silica gel column (10 - 40, 10 g) and eluted with mixture if chloroform and methanol in different ratio; the starting eluent was in 30:1 mixture. Three fractions were produced and labelled from $C_{12}a_4b_5c_1$ to $C_{12}a_4b_5c_3$.

Fractions labelled $C_{12}a_3b_4c_1S_1$ and $C_{12}a_4b_5c_1$ were separately subjected to HPLC (YMC-Pack ODS-A, 10 mm × 15 cm) column at a flow rate of 2 ml/min. Fractions $C_{12}a_3b_4c_1S_1$ and $C_{12}a_4b_5c_1$, were eluted with Methanol (HPLC grade) and water at gradients of 60 to 40, 50 to 50 respectively for 20 mins to produce different pure compounds.

Fraction labelled C_{14} (6 g) was mixed with silica gel (200 - 300, 9 g). This was layered on silica gel column (200 - 300, 70 g) and eluted with a mixture of chloroform and methanol in different ratio; the starting eluent was in 200:1 mixture. Seven fractions were produced and

labelled from $C_{14}b_1$ to $C_{14}b_7$. Fraction labelled $C_{14}b_3$ (3.5 g) was layered on Sephadex (LH-20) column and eluted with a mixture of chloroform and methanol in ratio 1:1 to produce four fractions labelled from $C_{14}b_3S_1$ to $C_{14}b_3S_4$. Fraction labelled $C_{14}a_3S_3$ (2 g) was mixed with silica gel (200 - 300, 3 g). This was subjected to silica gel (10 - 40, 25 g) column and eluted with a mixture of chloroform and methanol in different ratio; the starting eluent was in 40:1 mixture. Three fractions was produced and labelled from $C_{14}a_3S_3b_1$ to $C_{14}a_3S_3b_3$. Fraction labelled $C_{14}a_3S_3b_2$ was subjected to Medium Pressure Liquid Chromatography (MPLC, lichroprep reverse phase gel RP-18, 40 - 63 µm) column, at flow rate of 15 ml/min and pressure of 15 MPa. This was eluted with methanol and water in different ratio starting from 10: 90 to give a compound.

Fractions labelled C_{16} and C_{17} were pooled together (4 g) and layered on Sephadex (LH-20) column. This was eluted with a mixture of chloroform and methanol in ratio 1:1 to produce five fractions labelled from $C_{16}S_1$ to $C_{16}S_5$. Fraction labelled $C_{16}S_5$ (42.9 mg) was mixed with silica gel (200 - 300, 75 mg). This was layered on silica gel (10 - 40, 1 g) column and eluted with a mixture of chloroform and methanol in different ratio; the starting eluent was in 30:1 mixture. Three fractions were produced and labelled from $C_{16}S_5b_1$ to $C_{16}S_5b_3$. Fractions labelled $C_{16}S_5b_1$ and $C_{16}S_5b_2$ were pooled together (0.103 g) and subjected to HPLC (YMC-Pack ODS-A 10 mm × 15 cm) column, at a flow rate of 2 ml/min, and eluted with mixture of methanol and water, at gradient of 35 to 65 for 10 min and 20 to 80 for additional 10 min. This produced a pure compound. Fraction labelled $C_{16}S_4$ (115.7 mg) was mixed with silica gel (200 - 300, 0.2 g). This was layered on silica gel (10 - 40, 2 g) and eluted with a mixture of chloroform and methanol in different ratio; the starting eluent was in 30:1 mixture. Three fractions were produced and labelled from $C_{16}S_4a_1$ to $C_{16}S_4a_3$. F0raction labelled $C_{16}S_4a_2$ (65.9 mg) was mixed with silica gel (200 - 300, 0.15 g). This was layered on silica gel (10 - 40, 1 g) column and eluted with a mixture of

chloroform and methanol in different ratio; the starting eluent was in 20:1 mixture. Three fractions were produced and labelled from $C_{16}S_4a_2c_1$ to $C_{16}S_4a_2c_3$. Fraction labelled $C_{16}S_4a_2c_1$ (15.5 mg) was separated by HPLC (YMC-Pack ODS-A, 10 mm × 15 cm) column at a flow rate of 2 ml/min and eluted with mixture of methanol and water at gradient of 47 to 53 % for 20 min to produce a pure compound. Fraction labelled $C_{16}S_1$ was subjected to MPLC, MCI gel (75-150 µm) column and eluted with methanol and water in gradient starting from 20:80 % to produce two fractions labelled from $C_{16}S_1a_1$ to $C_{16}S_1a_2$. Fraction labelled $C_{16}S_1a_2$ was subjected to MPLC (RP-18) column and eluted with methanol and water in gradient starting from 10:90 % to give three fractions labelled from $C_{16}S_1a_2b_1$ to $C_{16}S_1a_2b_3$. Fraction labelled $C_{16}S_1a_2b_3$ was mixed with silica gel (200 - 300). This was subjected to silica gel (10 - 40) column and eluted with a mixture of chloroform and methanol in different ratio; the starting eluent was in 15:1 mixture. Four fractions were produced; one resulted in a pure compound.

Fraction labelled C_{15} (3.5 g) was layered on Sephadex (LH-20) column and eluted with a mixture of chloroform and methanol in ratio 1:1 to produce eleven fractions labelled $C_{15}S_1$ to $C_{15}S_{11}$. Fraction labelled $C_{15}S_6$ (2.8 g) was mixed with silica gel (200 - 300). This was layered on a silica gel (10 - 40) column and eluted with a mixture of chloroform and methanol in different ratio; the starting eluent was in 20:1 mixture. Three fractions were produced and labelled $C_{15}S_6a_1$ to $C_{15}S_6a_3$. Fraction labelled $C_{15}S_6a_1$ (18.8 mg) was separated using HPLC (YMC-Pack ODS-A, 10 mm × 15 cm) column at a flow rate of 2 ml/min, and eluted with methanol and water in gradient of 33 to 67 for 20 min to produce pure compounds.

Fractions labelled C_8 and C_9 were pooled together (13 g), and mixed with silica gel (200 – 300). This was layered on silica gel (200 - 300) column and eluted with a mixture of chloroform and acetone in different ratio; the starting eluent was in 20:1 mixture. Six fractions were produced

and labelled from C_8a_1 to C_8a_6. Fraction labelled C_8a_4 (6.5 g) was mixed with silica gel (200 - 300). This was layered on silica gel column (200 - 300) and eluted with a mixture of chloroform and methanol in different ratio; the starting eluent was in 100:1 mixture. Six fractions were produced and labelled from $C_8a_4b_1$ to $C_8a_4b_6$. Fraction labelled $C_8a_4b_4$ (6.0 g) was mixed with silica gel (200 - 300). This was layered on silica gel column (10 - 40) and eluted with a mixture of chloroform and acetone in different ratio; the starting eluent was in 20:1 mixture. Six fractions were produced and labelled $C_8a_4b_4c_1$ to $C_8a_4b_4c_6$. Fraction labelled $C_8a_4b_4c_5$ was mixed with silica gel (200 - 300). This was layered on silica gel column (10 - 40) and eluted with a mixture of petroleum ether and acetone in different ratio; the starting eluent was in 6:1 mixture. Three fractions were produced and labelled from $C_8a_4b_4c_5d_1$ to $C_8a_4b_4c_5d_3$. Fraction labelled $C_8a_4b_4c_5d_1$ was layered on Sephadex column (L-H 20) and eluted with chloroform and methanol in ratio 1:1 to produce two fractions labelled from $C_8a_4b_4c_5d_1S_1$ to $C_8a_4b_4c_5d_1S_2$. Fraction labelled $C_8a_4b_4c_5d_1S_1$ was subjected to MPLC (RP-18) and eluted with methanol and water in gradient of 20 to 80 % to produce pure compound.

Fraction labelled C_{19} (8 g) was layered on Sephadex column (LH-20) and eluted with mixture of methanol and water in ratio 1:1 to produce three fractions, labelled from $C_{19}S_1$ to $C_{19}S_3$. Fraction labelled $C_{19}S_2$ was mixed with silica gel (200 - 300). This was layered on silica gel (10 - 40) and eluted with a mixture of chloroform and methanol in different ratio; the starting eluent was in 15:1 mixture. Three fractions was produced and labelled from $C_{19}S_2a_1$ to $C_{19}S_2a_3$. Fraction labelled $C_{19}S_2a_1$ (52.9 mg) was separated by HPLC (YMC-Pack ODS-A, 10 mm × 15 cm) column, at a flow rate of 2 ml/min, and eluted with methanol and water in gradient of 47 to 53 % for 20 min. This produced 3 pure compounds. Fraction labelled $C_{19}S_2a_3$ (172.6 mg) was separated by

HPLC (YMC-Pack ODS-A, 10 mm × 15 cm) column, at a flow rate of 2 ml/min, and eluted with methanol and water in gradient of 50 to 50 % for 20 min. This produced 4 pure compounds.

Fraction labelled C_5 (10 g) was layered on Sephadex (LH-20) column and eluted with mixture of chloroform and methanol to produce two fractions. Fraction C_5S_1 was subjected to MPLC (MCI) column and eluted with methanol and water in gradient of 10:90 % to produce three fractions. Fraction labelled $C_5S_1a_1$ was mixed with silica gel (200 - 300). This was layered on silica gel column (10 - 40) and eluted with a mixture of petroleum ether and ethyl acetate in different ratio; the starting eluent was in 20:1 mixture. Seven fractions were produced and labelled from $C_5S_1a_1b_1$ to $C_5S_1a_1b_7$. Fraction labelled $C_5S_1a_2$ was mixed with silica gel (200 - 300). This was subjected to silica gel column (10 - 40) and eluted with a mixture of petroleum ether and ethyl acetate in different ratio; the starting eluent was in 20:1 mixture. Four fractions were produced and labelled from $C_5S_1a_2b_1$ to $C_5S_1a_2b_4$. Fraction labelled $C_5S_1a_2b_1$ was subjected to MPLC (RP-18) column and eluted with methanol and water in gradient of 10-90 % to give four fractions.

Fraction labelled $C_5S_1a_2b_1c_4$ was mixed on silica gel (200 - 300). This was layered on silica gel (10 - 40) column and eluted with a mixture of chloroform and acetone in different ratio; the starting eluent was in 70:1 mixture. Four fractions were produced and labelled from $C_5S_1a_2b_1c_4d_1$ to $C_5S_1a_2b_1c_4d_4$. Fraction labelled $C_5S_1a_2b_2c_1$ was mixed with silica gel (200 - 300). This was layered on silica gel (10 - 400) column and eluted with a mixture of chloroform and methanol in different ration; the starting eluent was in 400:1 mixture. Three factions were produced and labelled from $C_5S_1a_2b_2c_1d_1$ to $C_5S_1a_2b_2c_1d_3$. Fraction labelled $C_5S_1a_3$ was mixed with silica gel (200 - 300). This was layered on silica gel column (10 - 40) and eluted with a mixture of petroleum ether and ethyl acetate in different ratio; the starting eluent was in 20:2 mixture. Four

fractions were produced and labelled from $C_5S_1a_3b_1$ to $C_5S_1a_3b_4$. Fraction labelled $C_5S_1a_3b_2$ was mixed with silica gel (200 - 300). This was layered on silica gel column (10 - 40) and eluted with a mixture of chloroform and methanol in different ratio; the starting eluent was in 400:1 mixture. Three fractions were produced and labelled from $C_5S_1a_3b_2c_1$ to $C_5S_1a_3b_2c_3$. Fraction labelled $C_5S_1a_3b_2c_2$ was mixed with silica gel (200 - 300). This was layered on silica gel (10 - 40) column and eluted with a mixture of chloroform and acetone in different ration; the starting eluent was in 70:1 mixture. Three fractions were produced and labelled from $C_5S_1a_3b_2c_3d_1$ to $C_5S_1a_3b_2c_3d_3$.

Fraction labelled C_{20} (8g) was layered on Sephadex (LH-20) column and eluted with a mixture of chloroform and methanol in ration 1:1 to produce three fractions. Fraction labelled $C_{20}S_2$ was subjected to MPLC (RP - 18) column and eluted with methanol and water in gradient of 10-90 % to produce 3 fractions. Fraction labelled $C_{20}S_2a_3$ was subjected to HPLC (YMC-Pack ODS-A, 10 mm × 15 cm) column, at a flow rate of 2 ml/min, and eluted with methanol and water in gradient of 60:40 % for 20 min to produce a pure compound.

Thin layer chromatographic (TLC) assessment in different solvent systems was used to determine the purity of each fractions and samples. Pre-coated glass plates with silica gel GF_{254} were used. Spots were visualized and detected under UV light or by spraying with 10 % H_2SO_4 in 95 % ethanol, followed by heating at 70^{o}C.

3.2.5 Spectral Studies

3.2.5.1 Nuclear Magnetic Resonance (NMR) Analysis

Structural elucidations of all isolated and purified phytochemicals were carried out by 1-Dimensional Nuclear Magnetic Resonance spectroscopy methods (1-D NMR) which include: ^{13}C-, DEPT and ^{1}H- NMR analyses. 2-D NMR spectroscopy (COSY, HMBC, HMQC and HSQC) was

also carried out for the newly isolated compounds. 1-D and 2-D NMR spectra were recorded on Bruker AM-400 MHz, Bruker DRX-500 MHz and Avance III-600 MHz NMR spectrometers. Tetramethylsilane (TMS, $SiCH_3$) was used as the internal standard.

3.2.5.2 Mass Spectroscopy (MS) Analysis

Positive Electrospray Ionization Mass Spectroscopy (ESI-MS) analyses were conducted for each isolated compound to determine its appropriate chemical formula and molecular weight. High Resolution Electrospray Ionization Mass Spectroscopy (HR-ESI-MS) analyses were carried out only on new compounds. ESI-MS and HR-ESI-MS were conducted using an API Qstar-time-of-flight pulsar instrument (Applied Biosystems, USA).

3.2.5.3 Other Spectroscopic Methods

Infra red (IR) spectra of new compounds were obtained on a Bruker Tensor 27 spectrophotometer with samples in KBr pellet. Ultraviolet (UV) spectra were measured on a Shimadzu UV-240 1PC spectrophotometer (Shimadzu Coorporation, Tokyo, Japan). Optical rotation was measured with a Horbia SEAP-300 automatic polarimeter (Horiba, Tokyo, Japan). Melting points were performed on melting point apparatus, XRC-1 (Sichuan University, Sichuan, China).

3.2.6 *In vivo* Antioxidant Assessment of *Caesalpinia bouduc*

3.2.6.1 Experimental Design for *in vivo* Antioxidant Assay

Healthy adult female albino Wistar rats (n = 42) were used in the *in vivo* antioxidant study. The rats were housed in standard cages in the Animal House, Covenant University, Ota, Ogun

State. They were allowed to acclimatise for two weeks and were given food and water *ad libitum*. The cages were cleaned daily and the animals were treated according to standard ethical guidelines. The weights of the rats were recorded before treatment started. The rats weighed between 60 and 100 g. The rats were divided into seven groups, and each group contained 6 rats. The dosage with the CB extract was based on the body weight of each rat. Groups I to IV had the following dosages of CB extract: 50 mg/kg bwt, 100 mg/kg bwt, 150 mg/kg bwt, 200 mg/kg bwt. , 10 mg amodiaquine/kg bwt was used as the negative control; 10 mg vitamin C/kg bwt was used as the positive control; and 2 ml distilled water/kg bwt was employed as the normal control. (Farombi, 2000; Tafazoli and O'Brien, 2009).

On the 15th^{h} day, the rats were placed under light ether anasthesia after an over night fast and blood samples were collected by cardiac puncture into heparinised tubes. Whole blood collected were kept in the bio-freezer (-20^{o}C) until analysed.

3.2.6.2 Antioxidant Assays

3.2.6.2.1 Assay of Catalase Activity

Catalase activity was assayed according to the modified method that was based on those of Claiborne (1985) and Aebi (1974) in which the disappearance of hydrogen peroxide was monitored spectrophotometrically at 240 nm. One unit of catalase activity was defined as the amount of protein that causes one micromole of hydrogen peroxide to decompose per minute under specified conditions at 25^{o}C in a regulated water bath.

Into two cuvettes labelled test and control, 1 ml of diluted whole blood (0.02 ml blood in 10 ml 0.05 M phosphate buffer (pH 7.0)) was added to test while 1ml of 0.05 M phosphate buffer (pH 7.0) was added to the control tube. To each cuvette, 0.5 ml of substrate (30% H_2O_2 in

phosphate buffer) was added. The contents of each tube were mixed thoroughly and the blank tube was used to set the spectrophotometer to zero. The decrease in the absorbance of the mixture in the test cuvette was monitored at 240 nm for 70 seconds at 10 seconds interval. An extinction coefficient of 43.6 $M^{-1}cm^{-1}$ was assumed for H_2O_2 at 240 nm.

$$\text{Catalase activity} = \frac{\Delta A_{sample}}{43.6}$$

(where ΔA_{sample} is the change in absorbance of sample after 10 sec)

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

3.2.6.2.2 Assay of Peroxidase Activity

The activity of peroxidase was assayed as reported by Wever *et al.* (1980). The reaction mixture consisted of 3.0 ml of pyrogallol in 0.1 M phosphate buffer, pH 7.0 and 0.5 ml of 1 % (v/v) H_2O_2. To this was added 0.1 ml of the sample, and the change in absorbance was measured at 430 nm at 30 sec intervals for 2 min. The peroxidase activity was calculated using molar extinction coefficient of 4.5 $M^{-1}cm^{-1}$ for oxidised pyrogallol.

$$\text{Peroxidase activity} = \frac{\Delta A_{sample}}{4.5}$$

(where ΔA_{sample} is the change in absorbance of sample after 30 sec)

$$H_2O_2 + \text{Pyrogallol} \xrightarrow{\text{peroxidase}} 2\ H_20 + \text{Purpurogallin}$$

3.2.6.2.3 Determination of Thiobarbituric Acid Reactive Substances (TBARS) Concentration

The blood concentration of thiobarbituric acid reactive substances (TBARS) is an index of lipid peroxidation and oxidative stress.

The concentration Thiobarbituric acid reactive substances (TBARS) were determined by the modified method of Buege and Aust (1978). 0.1 ml of blood in 0.04 M Tris-HCl buffer, pH 8.3 was treated with 2.0 ml of a 1:1:1 TBA-TCA-HCL, 1:1:1 (thiobarbituric acid (TBA) 0.37 %; 15 % (w/v) TCA and 0.25M HCl). The mixture was incubated at 95^{o} C for 15 mins. The tube was then cooled on ice and centrifuged. The absorbance of the clear supernatant was measured 535 nm against a blank. The TBARS content was determined using an extinction coefficient of 155 $nM^{-1}cm^{-1}$.

$$\text{TBARS concentration} = \frac{A_{sample}}{155}$$

(where A_{sample} is the absorbance of sample)

3.2.7 Toxicological Evaluation of Ethanolic Extract of *C. bonduc*

The toxicological assessment of *C. bonduc* was divided into two phases: acute and sub acute (28 days) toxicicological investigations. Acute toxicity study is a measure of the interaction of xenobiotic with biomolecules after a single administration within fourteen days while sub-acute toxicity study is a measure of the interaction of xenobiotic with biomolecules after repeated administration within 28 days.

3.2.7.1 Grouping and Treatment of Experimental Rats for Sub-acute study

This study was carried out according to the Organisation for Economic Cooperation and Development (OECD) guidelines (OECD, 1995). Fifty female albino Wistar rats were selected by stratified randomization for the sub-chronic toxicity study. They were divided into six groups. Four groups (Grps 3 to 6), each containing ten rats, were treated with the CB extract. In each

group five rats were used for the actual test and five for the recovery test. The five animals in Grp 1 were used for normal control; the five in Grp 2 were used for the vehicle control.

The rats were weighed before the commencement of treatment. Thereafter, they were weighed weekly throughout the duration of the study. The rats were grouped as follows: Groups 1 to VI - normal control (distilled water), vehicle control (Sodium carboxymethyl cellulose), 200 CB mg/kg bwt, 400 CB mg/kg bwt, 800 CB mg/kg bwt, and 1600 CB mg/kg bwt respectively. The animals were dosed daily by gastric intubation. The physical appearances and the daily activities of the rats, such as eating patterns, were observed and recorded. Signs of abnormalities were observed and recorded. On the 29th day, after treatment with CB, the animals were put under light ether anaesthesia (Muto *et al.*, 2003). The recovery groups were kept alive and left untreated for additional 14 days and were later sacrificed. Blood was collected by cardiac puncture into heparinised and EDTA bottles, followed by centrifugation at 3000 rpm for plasma preparation. The plasma samples were collected and kept in a bio-freezer (-20°C) until they were analysed for the biochemical indices of toxicity. The blood samples collected into EDTA bottles were analysed immediately for haematological indices. The kidney, liver, heart and spleen were also collected from the animals and washed in normal saline, weighed and stored in 10 % formalin in plastic bottles. The biochemical, haematological and histopathological parameters of organ toxicity were evaluated in the treated animals and compared with controls. The relative organ weights was also calculated and recorded.

3.2.7.2 Grouping and Treatment of Experimental Rats for Acute study

The female albino Wistar rats (n = 20) were used for the acute toxicity study. The plant extract at fixed doses of 0, 2000, 4000 and 6000 mg/kg body weight were administered to four

groups, each containing rats. The animals in Grp 1 served as the control and received 1 ml of distill water while groups II to IV were given ethanolic extract of CB at doses of 2000, 4000 and 6000 CB mg/kg bwt by gastric intubation. The animals were observed for signs of morbidity and mortality at 1, 2, 4 and 6 hr after treatment and subsequently on the 7th and 14th days after treatment. This weight of experimental rats were measured and recorded on Days 1, 7 and 14 respectively. The study was carried out in accordance with the Organisation for Economic Co-operation and Development (OECD) guidelines No. 423 (2001). The biochemicals, haematological and histological parameters of the experimental animals were evaluated after 14 days.

3.2.7.3 Biochemical Analyses

3.2.7.3.1 Determination of Concentration of Plasma Total Protein

A total protein test kit (Randox Laboratories Ltd, Crumlin, UK), was used for the estimation of plasma total protein. **C**upric ions in alkaline medium interact with protein peptide bonds, hence, resulting in the formation of a coloured biuret complex (Weichselbaum, 1946).

The content of the test kit is R1a (10 ml of bottled R1 (biuret reagent) in 40 ml distilled water (dH_2O)), R21 (10 ml of bottled R2 in 40 ml dH_2O), and protein standard. The reaction mixturte contained 1.0 ml of R1a and 0.02 ml of plasma for sample's test tubes, while blank and standard test tubes contain 1.0 ml of R1a and 0.02 ml of dH_2O or 0.02 ml of standard respectively. Each test tube was mixed and incubated at 25^{o}C for 30 min. The absorbance of the standard $A_{standard}$ and samples A_{sample} were measured against reagent blank at 546 nm and protein concentration is calculated as follows:

$$\text{Total protein concentration} = \frac{A_{sample}}{A_{standard}} \text{ x Standard concentration}$$

(where standard protein concentration = 5.85 g/dl)

3.2.7.3.2 Determination of Plasma Urea Concentration

A urea test kit (Randox Laboratories Ltd., Crumlin, UK) was used for this assessment. Urea in serum/plasma is hydrolysed to ammonia in the presence of urease. The ammonia is measurable photometrically by Berthelot's reaction (Weatherburn, 1967).

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{hypochlorite} + \text{phenol} \longrightarrow \text{indophenol (blue compound)}$$

The content of the kit is R1a (Sodium nitroprusside and urease solution), R2a (40 ml of R2 (phenol) in 280 mls of dH_2O), R3a (10 ml of R3 (Sodium hypochlorite) in 340.9 mls of dH_2O) and standard. 10 µl of each sample was mixed with 100 µl of R1a, while 10 µl of standard and 10 µl of dH_2O with 100 µl were mixed for standard and blank respectively, these were incubated for 10 min at 37^{o}C. Thereafter, 2.50 ml of R2a and 2.50 ml of R3a were added and incubated for additional 15 mins. Absorbance of sample A_{sample} and standard $A_{standard}$ were read against the blank. Urea concentration was calculated as follows:

$$\text{Urea concentration} = \frac{A_{sample}}{A_{standard}} \text{ x Urea standard concentration}$$

(where Urea standard concentration = 80.5 mg/dl)

3.2.7.3.3 Determination of Plasma Creatinine Concentration

A creatinine test kit (Randox Laboratories Ltd., Crumlin, UK) was used for this assessment. Creatinine, in alkaline solution, reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration (Bartels *et al.*, 1972).

The content of kit is R1a (picric acid), R1b (sodium hydroxide) and creatinine standard. The working reagent contained equal volumes of R1a and R1b. 100 µl of plasma, 100 µl of creatinine standard and 100 µl of dH_2O were separately placed in different test tubes and 1000 µl of working reagent was added to each tube. The content of each tube were mixed gently and absorbance $A1_{sample}$ and $A1_{standard}$ were read at 492 nm against blank after 30 sec. Thereafter, 2 min of initial reading, Absorbance $A_{2sample}$ and $A_{2standard}$ were also read. The concentration of creatinine was calculated as follows:

$$\text{Creatinine concentration} = \frac{\Delta A_{sample}}{\Delta A_{standard}} \text{ x Creatinine standard concentration}$$

where $\Delta A\text{sample} = A2_{sample} - A1_{sample}$;

$\Delta A_{standard} = A2_{standard} - A1_{standard}$; and

(creatinine standard concentration = 1.97 mg/dl)

3.2.7.3.4 Determination of Plasma UricAacid Concentration

A uric acid test kit (Linear Chemicals, Barcelona, Spain) was used for this assessment. Uric acid is oxidized by uricase to allantoin with the formation of hydrogen peroxide. In the presence of peroxide, a mixture of dichlorophenolsulphonate (DPCS) and 4- aminoantipyrine (4-AA) is oxidized by hydrogen peroxide to form a quinoneimine dye proportional to the concentration of uric acid in the sample (Barham and Trinder, 1972).

$$\text{Uric acid} + O_2 + 2\,H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + H_2O_2$$

$$\text{4-AA} + \text{DCPS} \xrightarrow{H_2O_2} \text{Quinoneimine} + 4\,H_2O$$

The content of kit: R1 (Monoreagent solution) and uric acid standard. Into each test tube, 1.00 ml of R1 was pipeted and 25 µl of plasma, 25 µl of standard and nothing were added for sample, standard and blank tubes respectively. The mixtures were incubated for 10 min at room

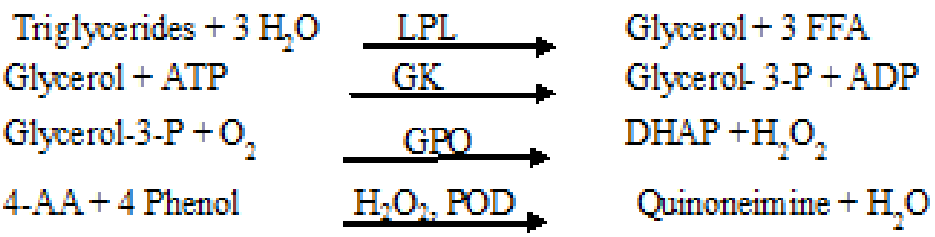
temperature. The absorbance (A_{sample} and $A_{standard}$) were read at 520 nm against reagent blank. The concentration of Uric acid in plasma samples was calculated as follows:

$$\text{Concentration of uric acid} = \frac{A_{sample}}{A_{standard}} \text{ x Concentration of standard uric acid}$$

(where concentration of standard uric acid = 6 mg/dl)

3.2.7.3.5 Determination of Plasma Triglyceride Concentrations

A triglyceride test kit (Linear Chemicals, Barcelona, Spain) was used for this assessment. This is based on the enzymatic hydrolysis of plasma triglyceride to glycerol and free fatty acids (FFA) by lipoprotein lipase (LPL). The glycerol is phosphorylated by adenosin triphosphate (ATP) in the presence of glycerolkinase (GK) to form glycerol-3-phosphate (G-3-P) and adenosine diphosphate (ADP). G-3-P is oxidized by glycerophosphate oxidase (GPO) to form dihydroxyacetone phosphate (DHAP) and hydrogen peroxide (H_2O_2). A red chromogen is produced by the peroxidase (POD) catalysed coupling of 4-aminoantipyrine (4-AA) and phenol with hydrogen peroxide (H_2O_2), proportional to the concentration of triglyceride in the sample (Fossati and Prencipe, 1982).



The content of kit is R1 (Monoreagent) and triglycerides standard. Each tube contained 1.00 ml of R1 and 10 µL of plasma or 10 µL of standard or nothing for sample or standard or blank tube respectively. These tubes were mixed gently and incubated at room temperature for 15

min and the absorbance (A_{sample} and $A_{standard}$) were read at 500 nm against reagent blank. The colour was protected from light. Triglyceride concentration was calculated as follows:

$$\text{Concentration of triglyceride} = \frac{A_{sample}}{A_{standard}} \text{ x } 200 \text{ mg/dl}$$

200 mg/dl = Concentratio of triglyceride standard concentration

3.2.7.3.6 Determination of Plasma Glucose Concentration

A glucose test kit (Cypress Diagnostics, Vlaams-Brabant, Belgium) was used for this assessment. Glucose is oxidized by glucose - oxidase (GOX) to gluconic acid and hydroxide peroxide. The formed hydrogen peroxide (H_2O_2), is detected by a chromogenic oxygen acceptor, phenolaminophenazone in the presence of peroxidase (POD). The intensity of the colour formed s proportional to the glucose concentration in the sample (Trinder, 1969).

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOX}} H_2O_2 + \text{Gluconic acid}$$

$$H_2O_2 + \text{Phenol} + \text{4-AP} \xrightarrow{\text{POD}} \text{Quinone} + 4H_2O$$

Where 4-AP is 4-aminophenazone

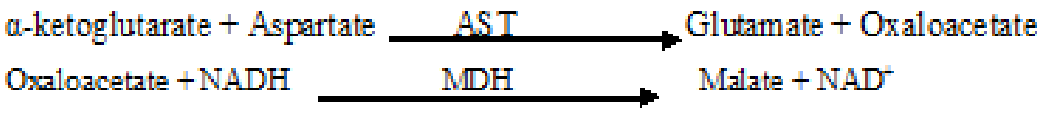
The content of kitc is R1 (Tris buffer PH 7.4 and Phenol), R2 (GOD, POD and 4-AP) and glucose standard. Working reagent was prepared by mixing 100 ml of R1 with 10 ml of R2. Each tube contained 1.00 ml of working reagent and 10 µL of plasma or 10 µL of standard or nothing for sample or standard or blank tubes respectively. These tubes were mixed gently and incubated at room temperature for 20 mins. Absorbance (A_{sample} and $A_{standard}$) were read at 505 nm against blank. The colour was protected from light. Glucose concentration was calculated as follows:

$$\text{Glucose concentration} = \frac{A_{sample}}{A_{standard}} \text{ x Glucose standard concentration}$$

(where glucose standard concentration is 100 mg/dl or 5.55 mmol/L)

3.2.7.3.7 Assay of Plasma Aspartate Aminotransferase Activity

Aspartate aminotransferase UV kinetic test kit (Cypress Diagnostics) was used for this assessment. The principle of this assessment was based on the following equations:



Malate dehydrogenase (MDH) catalyzes the conversion of oxaloacetate to malate in the presence of NADH. The rate of NADH consumption is determined photometrically and is directly proportionally to the aspartate aminotransferase (AST) activity in the sample (Bergmeyer et al., 1986a).

The content of kit is R1 (Tris buffer pH 7.8 and aspartate), R2 (NADH, MDH and α - ketoglutarate). The working reagent was prepared by dissolving substrate R2 into 15 ml of R1. 1.00 ml of working reagent was pipetted into each test tube with the addition of 0.10 ml of plasma. The mixture was mixed gently, initial absorbance (A_1) taken at 1 min and absorbance was taken every minute for additional 3 mins at 340 nm at 25^{o}C. The difference between absorbances (ΔA) and the average absorbance differences per minute (ΔA/ min) were calculated.

Concentration of AST (U /I) = ΔA (min^{-1}) x 1750

One International Unit (IU) is the amount of enzyme that transforms 1 µmol of substrate per minute, in standard conditions. The concentration is expressed in units per litre of sample (U/I).

3.2.7.3.8 Assay of Plasma Alanine Aminotransferase Activity

Alanine aminotransferase UV kinetic test kit (Cypress Diagnostic) was used for this assessment. The principle of this assessment was based on the following equations:

$$\alpha\text{ - ketoglutarate + alanine} \xrightarrow{ALT} \text{Glutamate + pyruvate}$$

$$\text{Pyruvate + NADH + H}^{+} \xrightarrow{LDH} \text{Lactate + NAD}^{+}$$

Lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate in the presence of NADH. The rate of NADH consumption is determined photometrically and is direct proportional to the alanine aminotransferase (ALT) activity in the sample (Bergmeyer *et al*., 1986b).

The content of kit is R1 (Tris buffer PH 7.8 and L-alanine), R2 (NADH, LDH and α - ketoglutarate). The working reagent was prepared by dissolving substrate R2 into 15 ml of R1. 1 ml of working reagent was pipetted into each test tube with the addition of 0.10 ml of plasma. The mixture was mixed gently, initial absorbance (A_1) taken at 1 min and absorbance was taken every minute for additional 3 min at 340 nm at 25^{o}C. The difference between absorbances (ΔA) and the average absorbance differences per minute (ΔA / min) were calculated.

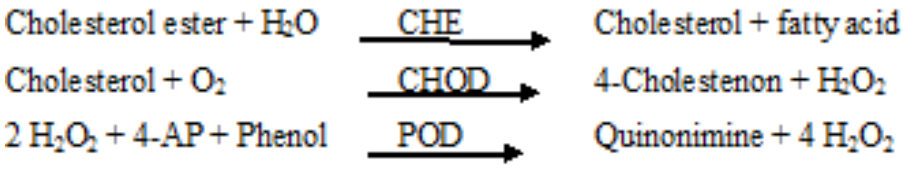
Concentration of ALT (U /I) = ΔA (min^{-1}) x 1750

One International Unit (IU) is the amount of enzyme that transforms 1 µmol of substrate per minute, in standard conditions. The concentration is expressed in units per litre of sample (U/I).

3.2.7.3.9 Determination of Plasma Cholesterol Concentration

A cholesterol test kit (Cypress Diagnostics, Vlaams-Brabant, Belgium) was used for this assessment. Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase (CHE) hydrolyses the esters and H_2O_2 is formed in the subsequently enzymatic oxidation

of cholesterol by cholesterol – oxidase (CHOD). The formed hydrogen peroxide (H_2O_2), is detected by a chromogenic oxygen acceptor, phenolaminophenazone in the presence of peroxidase (POD). The intensity of the colour formed is proportional to the cholesterol concentration in the sample (Zoppi and Fellini, 1976).



where 4-AP is 4-Aminophenazone

The content of kit is R1 (Pipes PH .9 and phenol), R2 (CHE, CHOD, POD and 4-AP) and cholesterol standard solution. Working reagent was prepared by mixing 100 ml of R1 with 10 ml of R2. Each tube contained 1.00 ml of working reagent and 10 µL of plasma or 10 µL of standard or 10 µL of distilled H_2O for sample or standard or blank tubes respectively. These tubes were mixed gently and incubated at 25^{o}C for 10 mins. Absorbance (A_{sample} and $A_{standard}$) were read at 505 nm against blank. The concentration of cholesterol in sample was calculated as follows:

$$\text{Cholesterol concentration} = \frac{A_{sample}}{A_{standard}} \text{ x Cholesterol standard concentration}$$

(where cholesterol standard concentration is 200 mg/dl)

3.2.7.3.10 Determination of Plasma Bilirubin Concentration

A bilirubin test kit (Randox Laboratories Ltd., UK) was used for this assessment. Direct bilirubin reacts with diazotized sulphanilic acid in alkaline medium to form a blue coloured complex. Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotized sulphanilic acid (Jendrassik and Grof, 1938).

The content of the kit is R1 (Sulphanilic acid and HCl), R2 (Sodium nitrite), R3 (Caffeine) and R4 (Tartrate and NaOH). 200 µl of R1, 1000 µl of R3 and 200 µl of sample were pipetted into each test tube. 50 µl of R2 was added to each sample tube while 50 µl of distilled H_2O was added for the corresponding blank tube. This was done for each sample. The test tubes were mixed and incubated for 10 mins at 25^oC and 1000 µl of R4 was added, mixed and incubated for further 30 min at 25^oC. Absorbance of the sample (A_{sample}) was read at 578 nm against sample blank. Total bilirubin was calculated as follows:

Total bilirubin concentration (mg/dl) = $10.8 \times A_{sample}$

Calculation of Plasma Direct Bilirubin Concentration

200 µl of R1, 2000 µl of normal saline (0.9% NaCl) and 200 µl of sample were pipetted into each test tube. 50 µl of R2 was added to each sample tube while nothing was added for the corresponding blank sample tube. This was done for each sample. The test tubes were mixed and incubated for 10 min at 25^oC. Absorbance of the sample (A_{sample}) was read at 546 nm against sample blank. Direct bilirubin concentration was calculated as follows:

Direct bilirubin concentration (mg/dl) = $14.4 \times A_{sample}$

3.2.7.4 Evaluation of the Haematological Parameters

Blood samples were collected by cardiac puncture into ethylenediaminetetraacetic acid (EDTA) bottles. The following haematological parameters; packed cell volume (PCV), white blood cells (WBC), neutrophil (N), eosinophil (E), lymphocyte (L) and monocyte (M) were evaluate according to the methods of Dacie and Lewis (1984).

3.2.7.5 Histopathological Studies of the Organs of Experimental Animals

The histopathological analysis of the kidney, the liver, the spleen and the heart excised from the experimental animals were carried out in the histopathology laboratory of the Lagos University Teaching Hospital (LUTH), Idi Araba, Lagos, Nigeria. The various organs were cut and placed in embedded cassettes. Thereafter, they were fixed with 10% formalin for 1 hour and afterwards dehydrated with methanol (70, 90 and 100%) at different concentration in ascending concentration and different time in order to remove water from the tissues. Thereafter, clearing with xylene was done for 2 to remove alcohol and prepare the tissue for waxing. Embedding was done using paraplast wax by impregnating cassettes with molten wax at 60 ^{o}C for 3 h. Slicing was done at 5 microns using a microtome. The slide was dyed for 20 min on hot plate. Afterwards, dewaxing and hydration were done using xylene and various percentage of alcohol respectively. Thereafter, staining was done with cole's hematoxylin for 10 min to stain the nucleus while eosin was used to stain the cytoplasm for 3 min.

Dehydration was once again carried out in alcohol and alcohol cleared with xylene. A mounting medium, dibutylphthalate xylene (DPX) was dropped on the tissue section and they were viewed through the microscope.

3.2.8 Evaluation of *in vitro* Antimalarial Activities and Selectivity Determination of Extract of CB

3.2.8.1 Culture of Malaria Parasites

P. falciparum (ATCC 30932, FCR-3 strain) was used in this study. It was cultivated by a modification of the method of Trager and Jensen (Trager and Jensen, 1976; Jensen and Trager 1977, Ogunlana *et al.*, 2009). A 5 % hematocrit of type A human red blood cells suspended in

RPMI 1640 medium, and supplemented with heat-inactivated 10 % type A human serum was used. The plates were placed in a CO_2 - O_2 - N_2 incubator (5% CO_2, 5% O_2, 90% N_2 atmosphere) at 37 °C, and the medium was changed daily until 5% parasitemia was attained (which means the existence of 5 parasite-infected erythrocytes in every 100 erythrocytes).

3.2.8.2 Culture of Mammalian Cells

Mouse mammary tumor FM3A cells (wild-type, subclone F28-7) were maintained in a suspension culture at 37 °C in a 5% CO_2 atmosphere in culture bottles containing mouse embryonic stem (ES) cell culture medium supplemented with 2% heat-inactivated fetal bovine serum (Gibco, NY, USA) (Yoshioka *et al*., 1987).

3.2.8.3 Evaluation of *in vitro* Antimalarial Activity of Various Fractions of CB

Various concentrations of extracts and isolated compounds including positive control samples (quinine hydrochloride, pyrimethamine, artemisinin and mefloquine) were prepared in dimethyl sulfoxide (DMSO, Sigma, St Louis, MO, USA)) and water (H_2O). 10 µL of each solution was added to individual wells of a 24-well multi-dish. Erythrocytes (10 µL) with 0.3 % parasitaemia were added to each well containing 990 µL of culture medium to give a final hematocrit level of 3 %. The plates were incubated at 37 °C for 72 h in a multigas incubator (5 % CO_2, 5 % O_2, 90 % N_2 atmosphere). To evaluate the antimalarial activity of samples, thin blood films from each culture were prepared and stained with Giemsa solution. A total of 10000 erythrocytes per one thin blood film were examined under a microscope. All the tested samples were assayed in duplicate at each concentration. Drug-free control cultures were run simultaneously. The level of parasitemia in control was between 4 - 5 % at 72 h (Kim *et al.*, 1999).

Values were presented as IC_{50}, which is the concentration of sample necessary to inhibit the increase in parasite density at 72 h by 50 % of the control.

3.2.8.4 Evaluation of Toxicity of Various Fractions Against Mouse Mammalian Cell Line

Mouse mammalian cell line, FM3A cells grew with a doubling time of about 12 h. Prior to exposure to drugs, the cell density was adjusted to 5×10^4 cells/ml. A cell suspension of 990 µL was dispensed to the test plate and 10 µL of the samples at various concentrations suspended in DMSO or H_2O were added to individual wells in a 24-well multi-dish. The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. Triplicate assays were made for each concentration of the drugs/*C. bonduc* extract. Cells were counted using a cell counter CC-130 (Kim *et al.,* 1999). The values were presented in IC_{50}, which refers to the concentration of the sample necessary to inhibit by 50% the increase in cell density of the control at 48 hrs. Selectivity values for the tested drug/compounds and extracts were calculated as follows:

Selectivity = IC_{50} value of FM3A cell / IC_{50} value of *P. falciparum*

The antimalarial and cytotoxicity assays were carried out at the Department of Pharmaceutical Information Science, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama, Japan.

3.2.9 *In vitro* Cytotoxic and Anti-microbial Activities of Various Fractions of CB

3.2.9.1 Culture of Cancer Cell Lines

Cancer cell lines, BGC-823 (gastric carcinoma) and HeLa (cervical carcinoma) (BGC - 823, HeLa) were maintained in a suspension culture at 37 °C in a 5% CO_2 atmosphere in plastic

bottles containing RPMI - 1640 medium (Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 10% heat - inactivated fetal bovine serum albumin (Gibco, NY, U.S.A.). Prior to exposure to drugs, the cancer cell lines were cultured in a CO_2 incubator for 48 hours and the cell density was adjusted to 5×10^4 cells/well.

3.2.9.2 Preparation of Samples

Compounds (2.5 mg/ml) were prepared according to their different solubilities either in DMSO or H_2O. 2 and 8 µl of the sample (2.5 mg/ml) was dispensed into Eppendorf tubes containing 48 µl of complete medium (RPMI 1640 containing 10% Fetal Bovine Serum) to make a final concentration of 100 and 400 µg/ml for compounds and extracts respectively.

3.2.9.3 Cell growth Inhibition Assay (Pre-test and Evaluation Assessment)

The sulphorhodamine B (SRB) assay was adopted for a quantitative measurement of cell growth and viability (Tang *et al*., 2010). Cultured cancer cells in RPMI 1640 medium (Sigma, St Louis, MO, USA), were seeded in aliquots of 90 µl in 96-well flat-bottomed microtiter plates (Greiner). The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. Twenty four hours later, 10 µl of samples (pure compounds and solvent fractions) were added to make final concentrations of 10 and 40 µg/mL respectively. All of the tested samples were assayed in duplicate. After incubation at 37^oC and in an atmosphere of 5% CO_2 for 48 h, cells were fixed by the addition of 25 µl of 80 % ice-cold trichloroacetic acid (CCl_3COOH, TCA) per well, incubated for 5 mins and refrigerated at 4^oC for 1 h. Thereafter, the plates were rinsed in excess cold water and dried on absorbent paper. After washing, air-drying and staining for 15 min with 100 µl of Sulforhodamine B (SRB) (0.4% SRB in 1% glacial acetic acid), which ensures the full staining of

cellular proteins (Skehan *et al.*, 1990), excessive dye was removed by washing with 1% glacial acetic acid and dried on absorbent papers. 100 µl of 10 mM Tris was added to each well and placed in a plate shaker for 10 mins to solubilize the SRB stain bound to the cellular protein.

The absorbance of the plates was measured at 560 nm using a microplate reader (Molecular Devices, SPECTRAMAX 340, USA). Further assessment was carried out with four diluted concentrations (dilution ratio 1:2), if the inhibition was up to 50 % at its pre-test assessment. Cell growth inhibition values were expressed as IC_{50} (50% inhibitory concentration) for evaluation assessments and I % (Percentage inhibition) for pre - test assessment. Taxol, clinically used as an anticancer drug, was used as a positive control (Tang *et al*., 2010). I % was calculated in relation to the mean of negative control.

Percentage inhibition (I %) was calculated by the following equation:

$$I\ \% = \frac{Cc - Cs}{Cc} \times 100$$

Cc = viable cell counts of negative control

Cs = viable cell counts of sample

IC_{50} was calculated by the following equation:

$$\text{Log}_{10}(IC_{50}) = \frac{\text{Log}_{10}(C_L)(I_H - 50) + \text{log}_{10}(C_H)(50 - I_L)}{I_H - I_L}$$

$IC_{50} = 10^{(\text{Log}_{10}(IC_{50}))}$;

I_H: I % above 50%;

I_L: I % below 50%;

C_H: high drug concentration; and

C_L: low drug concentration

3.2.9.4 Anti-microbial Assay

The anti-microbial experiment was conducted according to the turbidimetric method on the bacterium *Candida albicans* (Tang *et al*., 2010). *C. albicans* was inoculated in *Mueller Hinton Broth* (Oxiod, CM0405, Hampshire, England) to McFarland 0.5 and diluted with medium to 1×10^6 CFU/ml. Aliquots of 90 µL were filled in 96-well U-bottomed microplate. Samples, dissolved in DMSO as decribed previously and diluted with the medium to a total volume of 10 µl, were dispensed in the wells to final concentrations of 10 and 40 µg/ml. After culturing at 37^{o}C for 24 h, absorbance was recorded at 620 nm with the aforementioned microplate reader. The level of inhibition was calculated as the percentage of maximum absorbance (negative control) to the absorbances of the samples. Miconazole Nitrate (Keygen, China) was used as positive control.

3.2.10 Statistical Analyses

Inhibition data were expressed as percentage inhibition (I %) and 50% inhibitory concentration (IC_{50}) values. Other data were expressed as mean ± standard error of mean (SEM). The statistical analysis of the results was carried out by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS), version 15.0 (SPSS Inc., Chicago, IL, USA). The least significant difference (LSD) was used to compare the difference between the means of the groups of the *C. bonduc*-treated animals and the recovery group, as well as between the *C. bonduc*-treated animals and the control groups. The test for statistical significance was carried out at the 95 % confidence limit.

CHAPTER FOUR

RESULTS

4.1 Quantitation of Yields of *C. bonduc*

The yield of CB (8800 g) from 75% v/v ethanolic solution was 1120 g and the % yield was 12.7 %. The yields (% yields) of the petroleum ether - soluble fraction, the ethyl acetate - soluble fraction, the nbutanol - soluble fraction and the water - soluble fraction were 13.4 %, 10.7 %, 15.2 % and 56.3 % respectively.

4.2 Qualitative Phytochemical Assessment of *C. bonduc*

In table 4.1 is the summary of the phytoconstituents of twigs and leaves of CB. The ethanolic exztract gave positive (+ve) reaction tests for the presence of key metabolites except phlobatannins.

Table 4.1: Phytochemical Constituents of *C. bonduc*

Phytochemicals	Results
Tannins	+
Flavonoids	+
Saponin	+
Steroids	+
Phlobatannins	-
Terpenoids	+
Cardiac glycosides	+
Glycosides	+

+ represents a positive result and - represents a negative result

4.3 Quantitation of Isolated Compounds from the Petroleum Ether and Ethyl Acetate Fractions of *C. bonduc*

TCB 1 (319.3 mg) was collected as a yellow crystalline solid from $C_6a_{14}b_4$. It was soluble in methanol. Its structure was elucidated and it was designated as compound 1. TCB 2 (25.9 mg) was collected from $C_3a_4b_2$ as a colourless crystalline solid, soluble in chloroform. Its structure was elucidated and it was designated as compound 2. TCB 3 was obtained as a colourless crystalline solid from $C_6a_{16}b_2S_4$. It was soluble in chloroform. Its structure was elucidated and it was designated as compound 3.

TCB 4 was obtained impure from $C_6a_{16}b_3S_2$ as a yellow amorphous solid soluble in acetone. TCB 5 (15 mg) was obtained from $C_6a_{13}b_6S_2$ as a colourless crystalline solid soluble in chloroform. Its structure was partially elucidated and it was designated as compound 4. TCB 6 was obtained from $C_7a_4b_3c_4d_5S_5e_4$ as a colourless crystalline solid soluble in chloroform. Its structure was partially elucidated and it was designated as compound 5. TCB 7 (5 mg) was obtained impure from $C_6a_{14}b_4d_4e_5f_3g_6S_2h_1$ as a white amorphous powder soluble in chloroform. TCB 8 (4 mg) was obtained impure from $C_7a_4b_3c_4d_5S_5e_1f_4$ as a white amorphous powder soluble in chloroform.

TCB 9 (15.60 mg) was obtained from fraction $C_{10}a_5b_4d_5$ as yellow powder soluble in methanol. Its structure was elucidated and it was designated as compound 6. TCB 10 was the same compound as in TCB 9. TCB 11 (10.4 mg) was obtained from fraction $C_{10}a_5b_4d_2$ as a yellow powder soluble in methanol. Its structure was elucidated and it was designated as compound 7. TCB 12 was the same compound as in TCB 11.

TCB 13 (3.4 mg) was obtained from fraction $C_{10}a_5b_5d_4$ as a brown powder soluble in methanol. TCB 14 (800 mg) was obtained from $C_{14}a_3S_3b_2$ as a white amorphous solid soluble in pyridine. Its structure was elucidated and it was designated as compound 8. TCB 15 (12.8 mg) was obtained from $C_{12}a_4b_5c_1$ as a yellow crystal soluble in methanol. Its structure was elucidated and it was designated as compound 9. TCB 16 (7.2 mg) was obtained from $C_{12}a_4b_5c_1$ as a yellow crystal soluble in methanol. Its structure was elucidated and it was designated as compound 10. TCB 17 (227 mg) was obtained from $C_{12}a_3b_4c_1S_1$ as a yellow crystal soluble in methanol. Its structure was elucidated and found to be the same as TCB 16. TCB 18 (3.4 mg) was obtained from $C_{16}S_5b_1$ as brown powder soluble in methanol. TCB 20 (12.8 mg) was obtained from $C_{12}a_4b_5c_3$ as a yellow crystal soluble in methanol. Its structure was elucidated and found to be the same as TCB 15. TCB 21 (5.2 mg) was obtained from $C_{15}S_6a_1$ as a white crystalline solid soluble in methanol. Its structure was elucidated and assigned as compound 11.

TCB 22 (11.4 mg) was obtained from $C_8a_4b_4c_5d_1S_1$ as white powder soluble in pyridine. TCB 23 and TCB 24 were obtained from fractions $C_8a_4b_4c_5d_2S_1$ and $C_8a_4b_4c_5d_2S_1$ respectively. They were isolated as white powder soluble in pyridine. Their structures were the same as in TCB 22. TCB 25 was obtained from fraction $C_7a_4b_3c_4d_4S_5e_4$ as a white crystal soluble in chloroform. Its structure was the same as in TCB 6. TCB 26 was obtained impure from fraction $C_{16}S_2a_2c_1$ as a white powder soluble in pyridine. TCB 27 (24.4 mg) was obtained from $C_{16}S_4a_2c_1$ as a brown gel soluble in methanol. TCB 28 (6.6 mg), TCB 29 (5.9 mg) and TCB 30 (5.3 mg) were all obtained from fraction $C_{19}S_2a_1$ as a white powder soluble in methanol. TCB 31 (36.1 mg), TCB 32 (7.2 mg), TCB 33 (9 mg) and TCB 34 (24.9 mg) were obtained from $C_{19}S_2a_3$ as a yellow crystalline solid soluble in methanol. TCB 35 (21.78 mg), TCB 36 (22.78 mg) and TCB 37 (10.30 mg) were obtained from $C_{16}S_1a_2b_3$ as a white powder soluble in pyridine. TCB 38 (12.97 mg) was obtained

from $C_5S_1a_1$ as a white powder soluble in pyridine. TCB 39 (64.19 mg) was obtained from $C_5S_1a_2b_1$ as a white powder soluble in pyridine. TCB 40, 41, 42, 43 and 44 (79.5 mg) were obtained from $C_{20}S_2a_3$ as a yellow amorphous solid soluble in methanol. They were identified as the same. TCB 45 (17.6 mg) was obtained from $C_5S_1a_2b_2c_1$ as a white powder soluble in chloroform. TCB 46 (4.8 mg) was obtained from $C_5S_1a_3b_2c_2$ as a white powder soluble in chloroform.

4.4 Physical Properties, Spectra Assignments and Structural Elucidation of Pure Compounds from *C. bonduc*

TCB 1 has a melting point of 208^{o}C (McPherson *et al.*, 1983). Its molecular formula was determined as $C_{17}H_{14}O_4$, on the basis of the molecular ion peak of positive ESI-MS *m/z* 305 $[M+Na]^+$ (See Appendix 3). The ^{1}H and ^{13}C NMR spectra revealed the following: ^{1}H NMR (DMSO-d_6, 500 MHz): δ 5.35 (2H, d, J = 1.5 Hz, H-2), 7.73 (1H, d, J = 8.5 Hz, H-5), 6.54 (1H, dd, J = 8.5, 2.0 Hz, H-6), 6.31 (1H, d, J = 2.0 Hz, H-8), 7.63 (1H, br s, H-11), 7.39 (2H, d, J = 8.5 Hz, H-2', H-6'), 7.04 (2H, d, J = 8.5 Hz, H-3', H-5'), 3.81 (3H, s, 4'-OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 67.5 (t, C-2), 126.5 (s, C-3), 179.5 (s, C-4), 129.4 (d, C-5), 111.1 (d, C-6), 164.6 (s, C-7), 102.4 (d, C-8), 162.5 (s, C-9), 114.2 (s, C-10), 135.2 (d, C-11), 128.8 (s, C-1'), 132.2 (d, C-2', C-6'), 114.3 (d, C-3', C-5'), 160.3 (s, C-4'), 55.3 (q, 4'-OCH_3) (See Appendix 1 and 2). The data are in agreement with the literature (Purushothaman *et al.*, 1982; McPherson *et al.*, 1983) and the structure of TCB 1 was identified as 7-hydroxy-4'-methoxy-3,11-dehydrohomoisoflavanone (bonducellin) (Figure 4.1).

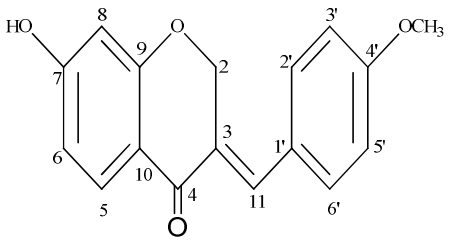


Figure 4.1: Chemical Structure of TCB 1, (1) - 7-hydroxy-4'-methoxy-3,11-dehydrohomoisoflavanone - $C_{17}H_{14}O_4$ (M. wt.: 282)

The molecular formula of TCB 2 was determined as $C_{28}H_{58}O$ on the basis of the molecular ion peak of positive ESI-MS *m/z* 411 $[M+H]^+$. The ^1H and ^{13}C NMR spectra revealed the following: ^1H NMR ($CDCl_3$, 500 MHz): δ 0.88 (3H, t, J = 6.48 Hz, H-1), 3.64 (br s, 28-OH), 3.49 (2H, d, J = 2.55 Hz, H-28), 1.59 (2H, br s, H-27), 1.55 (2H, m, H-26), 1.26 (2H, d, H-26); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.1 (s, C-1), 22.6 (d, C-2), 31.9 (d, C-3), 29.3 (d, C-4), 29.6 (d, C-25), 25.6 (d, C-26), 32.2 (d, C-27), 62.8 (d, C-28). The data are in agreement with the literature (Yadava and Nigam, 1987). The structure of TCB 2 was identified as 1-octacosanol (Figure 4.2).

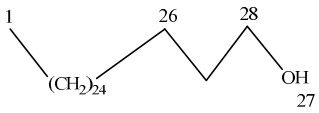


Figure 4.2: Chemical Structure of TCB 2, (2) - 1-octacosanol - $C_{28}H_{58}O$ (M. wt.: 410)

The molecular formula of TCB 3 was determined as $C_{22}H_{26}O_8$ on the basis of the molecular ion peak at positive ESI-MS *m/z* 441 $[M+Na]^+$, 859 $[2M+Na]^+$. The ^1H and ^{13}C NMR spectra revealed the following: ^1H NMR ($CDCl_3$, 500 MHz): δ 3.10 (2H, br s, H-1, H-5), 4.73 (2H, d, *J* = 3.0 Hz, H-2, H-6), 3.90 (2H, overlap, H-4a, H-8a), 4.28 (2H, m, H-4b, H-8b), 6.58 (4H, s,

H-2', H-2", H-6', H-6"), 3.90 (12H, s, 3'-OCH_3, 3"-OCH_3, 5'-OCH_3, 5"-OCH_3), 5.54 (2H, s, 4'-OH, 4"-OH); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 54.3 (d, C-1, C-5), 86.0 (d, C-2, C-6), 71.7 (t, C-4, C-8), 132.0 (s, C-1', C-1"), 102.6 (d, C-2', C-2", C-6', C-6"), 147.1 (s, C-3', C-3", C-5', C-5"), 134.2 (s, C-4', C-4"), 56.3 (q, 3'-OCH_3, 3"-OCH_3, 5'-OCH_3, 5"-OCH_3). The data are in agreement with the literature (Garnier *et.al.*, 1975; Shu *et al.*, 2007). The structure of TCB 3 was identified as (+)-Syringaresinol (Figure 4.3).

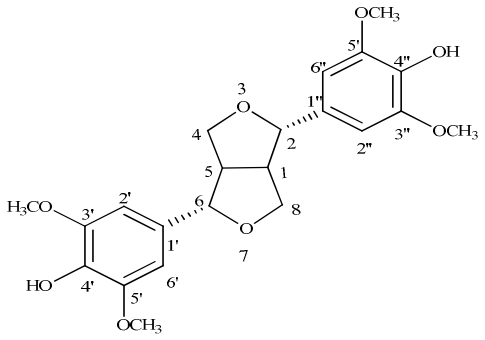


Figure 4.3: Chemical Structure of TCB 3, (3) - (+)-Syringaresinol - $C_{22}H_{26}O_8$ (M. wt.: 418)

TCB 5 has a melting point of 236 ^{o}C. Its molecular formula was determined as $C_{25}H_{34}O_{11}$ on the basis of the molecular ion peak at positive ESI-MS m/z 533 $[M+Na]^+$, 1043 $[2M+Na]^+$ (See Appendix 12) and HRESI-MS as m/z 533.2142 $[M+Na]^+$ (calculated as $C_{25}H_{34}O_{11}Na$, 533.2142) (See Appendix 13). It has the following chemical properties: Optical rotation $[\alpha]_D^{11}$ +33.5 (*c* 0.12, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 203 (3.68) nm; IR (KBr) ν_{max} 3572, 3440, 2950, 1788, 1736, 1441, 1401, 1368, 1234, 1166, 1062, 1027, 856 cm^{-1} (See Appendices 14, 15 and 16). The ^{1}H and ^{13}C NMR data are assigned in agreement with the general structures of the cassane diterpenes (Wu *et al.*, 2010) (Table 4.2 and Appendices 4, 5, 6, 7, 8, 9 and 10). TCB 5 (compound 4) was partially elucidated as 1α,7α-diacetoxy-5α,6β-dihydroxyl-cass-14(15)-epoxy-16,12-olide (Figure 4.4).

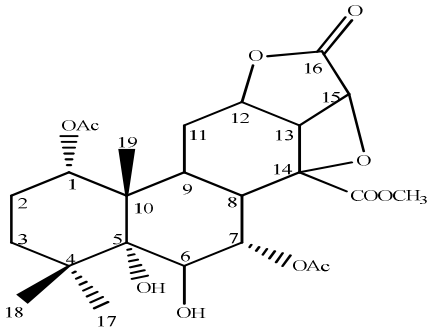


Figure 4.4: Chemical Structure of TCB 5, (4) - 1α,7α-diacetoxy-5α,6β-dihydroxyl-cass-14(15)-epoxy-16,12-olide (M. wt.: 310)

TCB 6 (compound 5) has a melting point of 243^{o}C. Its molecular formula was determined as $C_{26}H_{38}O_9$ on the basis of the molecular ion peak at positive ESI-MS m/z 517$[M+Na]^+$ (See Appendix 27), 1011 $[2M+Na]^+$ and HRESI-MS as m/z 517.2402 $[M+Na]^+$ (calculated as $C_{26}H_{38}O_9Na$, 517.2413 without adjustment) ((See Appendix 26)). It has the following chemical properties: Optical rotation $[\alpha]_D^{18}$ -74.7 (*c* 0.13, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 219 (3.98) nm (Figure 4.37); IR (KBr) ν_{max} 3569, 3541, 3439, 2981, 2939, 1745, 1649, 1369, 1335, 1259, 1226, 1170, 1065, 1041, 951, 935, 902 cm^{-1} (See Appendices 28, 29, 30); ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, (Table 4.3 and See Appendices 19, 20, 21, 22, 23, 24 and 25). The ^{1}H and ^{13}C NMR spectra data are similar to those of 12α-ethoxyl-1α,6α,7β-triacetoxy-5α,14β-dihydroxy-cass-3(15)-en-16, 12-olide (Wu *et al.*, 2010), with the similar carbon skeleton and the presence of ethyl signals at δ_H3.52 (1H, m, 12-OCH_2-a), δ_H3.17 (1H, m, 12-OCH_2-b), δ_H1.13 (3H, overlapped, 12-CH_2CH_3), δ_C58.7 (t, 12-OCH_2CH_3) and δ_C14.8 (q, 12-OCH_2CH_3) and the absence of acetoxyl signals at 6- and 7- positions. The structure of TCB 6 was elucidated as 12α-ethoxy-1α,14β-diacetoxy-2α,5α-dihydroxy-cass-13(15)-en-16,12-olide (Figure 4.5).

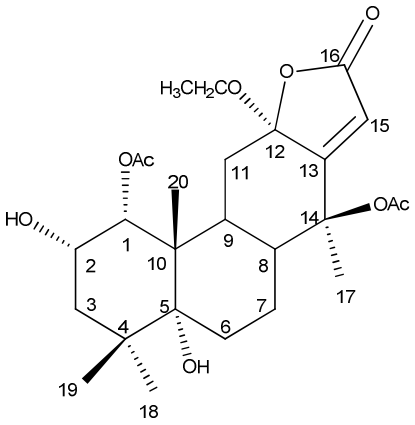


Figure 4.5: Chemical Structure of TCB 6, (5) - 12α-ethoxy-1α,14β-diacetoxy-2α,5α-dihydroxy-cass-13(15)-en-16,12-olide (M. wt.: 494)

The molecular formula of TCB 9 was determined as $C_{16}H_{14}O_4$ on the basis of the molecular ion peak at positive ESI-MS m/z 271 $[M+H]^+$ and 293 $[M+Na]^+$ (See Appendix 35). The ^1H and ^{13}C NMR data revealed the following: ^1H NMR (Methanol-d_4, 400 MHz): δ 7.50 (2H, d, J = 8.4 Hz, H-2, H-6), 6.82 (2H, d, J = 8.4 Hz, H-3, H-5), 7.56 (1H, d, J = 15.6 Hz, H-7), 7.41 (1H, d, J = 15.6 Hz, H-8), 6.51 (1H, br s, H-3'), 6.45 (1H, br d, J = 8.4 Hz, H-5'), 7.57 (1H, d, J = 8.4 Hz, H-6'), 3.88 (3H, s, 2'-OCH_3); ^{13}C NMR (Methanol-d_4, 100 MHz): δ 128.0 (s, C-1), 131.4 (d, C-2, C-6), 116.9 (d, C-3, C-5), 161.2 (s, C-4), 144.2 (d, C-7), 125.1 (d, C-8), 193.2 (s, C-9), 121.8 (s, C-1'), 162.5 (s, C-2'), 100.1 (d, C-3'), 164.5 (s, C-4'), 108.9 (d, C-5'), 133.7 (d, C-6'), 56.1 (q, 2'-OCH_3) ()See Apendices 33 and 34) (Namikoshi *et al.*, 1987a; Liu *et al.*, 2009; Fu *et al.*, 2008). The ^{13}C and ^1H NMR data correlated with the signals for Isoliquiritigenin except for the additional signal at δ_C56.1, δ_H3.88 (3H, *s*, 2'-OMe) for the methoxy group at C-2' (Hwang *et al.*, 1998). The structure of TCB 9 was identified as 4,4'-dihydroxy-2'-methoxy-chalcone (2'-methoxyisoliquiritigenin) (Figure 4.6).

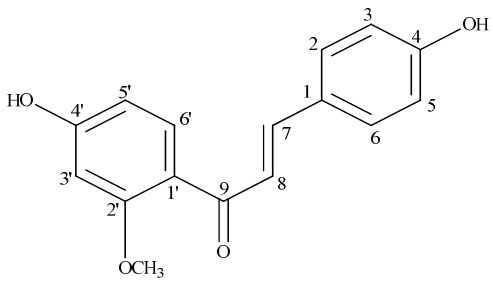


Figure 4.6: Chemical Structure of TCB 9, (6) - 4,4'-dihydroxy-2'-methoxy-chalcone - $C_{16}H_{14}O_4$ (M. wt.: 270)

TCB 11 (compound 7) has a melting point in range of 248 to 249^{o}C (Namikoshi *et al.*, 1987b). Its molecular formula was determined as $C_{16}H_{12}O_4$ on the basis of the molecular ion peak of positive ESI-MS *m/z* 269 $[M+H]^+$(See Appendix 38). The ^{1}H and ^{13}C NMR spectra revealed the following: ^{1}H NMR (Methanol-d_4, 400 MHz): δ 5.35 (2H, br s, H-2), 7.80 (1H, d, J = 8.0 Hz, H-5), 6.52 (1H, br d, J = 8.0 Hz, H-6), 6.31 (1H, br s, H-8), 7.71 (1H, br s, H-11), 7.25 (2H, d, J = 7.6 Hz, H-2', H-6'), 6.88 (2H, d, J = 7.6 Hz, H-3', H-5'); ^{13}C NMR (Methanol-d_4, 100 MHz): δ 69.0 (t, C-2), 127.1 (s, C-3), 183.1 (s, C-4), 130.7 (d, C-5), 112.2 (d, C-6), 166.6 (s, C-7), 103.6 (d, C-8), 164.8 (s, C-9), 115.9 (s, C-10), 138.2 (d, C-11), 129.6 (s, C-1'), 133.5 (d, C-2', C-6'), 116.7 (d, C-3', C-5'), 160.5 (s, C-4') (See Appendices 36 and 37). The NMR spectra are very similar to those of TCB 1. The data are in agreement with the literature (Namikoshi *et al.*, 1987b). TCB 11 was identified as 7,4'-dihydroxy-3,11-dehydrohomoisoflavanone (Figure 4.7).

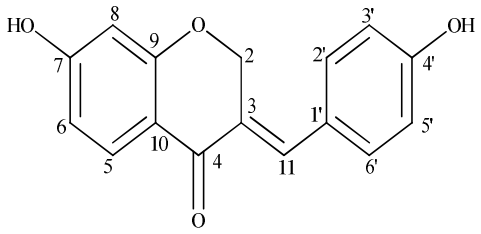


Figure 4.7: Chemical Structure of TCB 11, (7) - 7,4'-dihydroxy-3,11-dehydrohomoisoflavanone - $C_{16}H_{12}O_4$ (M. wt.: 268)

The molecular formula of TCB 14 was determined as $C_{35}H_{60}O_6$ on the basis of the molecular ion peak of positive ESI-MS *m/z* 576 $[M+Na]^+$. The ^1H and ^{13}C NMR spectra revealed the following: ^1H NMR (Pyridine-d_6, 500 MHz): δ *1.38* (2H, t, *J* = Hz, H-1), 1.53 (2H, m, H-2), 2.72 (1H, d, *J* = 12.84 Hz, H-3), 2.13 (2H, d, *J* = 11.48 Hz, H-4) 1.73 (1H, d, *J* = 11.30 Hz, H-7), 2.05 (1H, m, H-7), 1.42 (1H, s, *J* = 18.78 Hz, H-8), 1.50 (2H, m, H-11), 1.59 (2H, m, H-12), 1.24 (2H, d, *J* = 6.82 Hz, H-19), 1.24 (2H, d, *J* = 6.82 Hz, H-20), 1.55 (2H, m, H-27), 0.97 (3H, d, *J* = 6.25 Hz, H-29), 0.92 (3H, s, H-25), 0.92 (3H, s, H-26), 0.91 (3H, s, *J* = 5.76 Hz, H-28), 5.04 (1H, d, *J* = 7.68 Hz, H-1'), 3.94 (1H, s, H-2'), 3.58 (1H, s, H-3'), 3.97 (1H, s, H-5'), 3.98 (2H, s, H-6'); ^{13}C NMR (pyridine-d_6, 125 MHz): δ 37.0 (d, C-1) *29.6* (d, C-2), 78.7 (t, C-3), 39.9 (d, C-4), 141.03 (q, C-5), 122.0 (t, C-6), 32.2 (d, C-7), 30.4 (t, C-8), 50.46 (t, C-9), 37.6 (q, C-10), 21.6 (d, C-11), 39.9 (d, C-12), 42.6 (q, C-13), 56.9 (t, C-14), 26.5 (d, C-15), 25.8 (d, C-16), 56.2 (t, C-17), 19.3 (s, C-18), 12.1 (s, C-19), 36.5 (t, C-20), 34.3 (d, C-21), 26.5 (d, C-22), 46.2 (t, C-23), 30.1 (t, C-24), 21.4 (s, C-25), 20.1 (s, C-26), 23.5 (d, C-27), 12.3 (s, C-28), 19.5 (s, C-29), 102.7 (t, C-1'), 75.4 (t, C-2'), 78.3 (t, C-3'), 62.9 (t, C-4'), 78.5 (t, C-5'), 71.8 (d, C-6'). The data are in agreement with the literature (Chen and Yang, 2008; Shu *et al.*, 2008). TCB 14 was identified as Daucosterol (Figure 4.8).

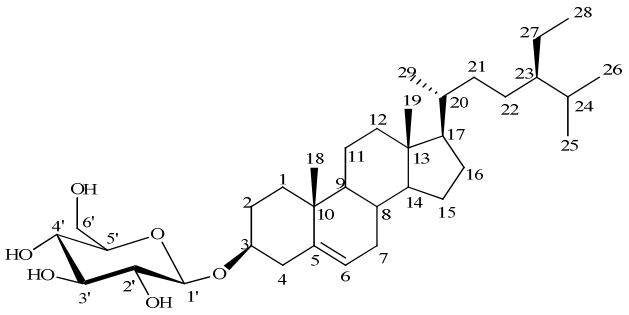


Figure 4.8: Chemical Structure of TCB 14, (8) - Daucosterol - $C_{35}H_{60}O_6$ (M. wt.: 576)

The molecular formula of TCB 15 was determined as $C_{15}H_{10}O_6$ from its molecular ion peak at positive ESI-MS *m/z* 287 $[M+H]^+$ (See Appendix 41). The ^1H and ^{13}C NMR revealed the following: ^1H NMR (Methanol-d_4, 400 MHz) (See Appendices 39 and 40): δ 6.54 (1H, s, H-3), 6.20 (1H, d, J = 1.2 Hz, H-6), 6.44 (1H, br s, H-8), 7.38 (1H, overlap, H-2'), 6.90 (1H, d, J = 8.4 Hz, H-5'), 7.38 (1H, overlap, H-6'); ^{13}C NMR (Methanol-d_4, 100 MHz): δ 166.0 (s, C-2), 103.8 (d, C-3), 183.8 (s, C-4), 163.2 (s, C-5), 100.1 (d, C-6), 166.3 (s, C-7), 95.0 (d, C-8), 159.4 (s, C-9), 105.3 (s, C-10), 123.6 (s, C-1'), 114.1 (d, C-2'), 147.0 (s, C-3'), 151.0 (s, C-4'), 116.7 (d, C-5'), 120.3 (d, C-6'). The data are in agreement with the literature (Wagner and Chari, 1976; Suarez *et al.*, 1984). TCB 15 was identified as 5,7,3',4'-tetrahydroxy-flavone (Luteolin) (Figure 4.9).

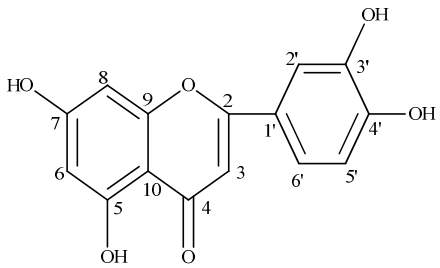


Figure 4.9: Chemical Structure of TCB 15, (9) - 5,7,3',4'-tetrahydroxy-flavone - $C_{15}H_{10}O_6$ (M. wt.: 286)

The molecular formula of TCB 16 was determined as $C_{16}H_{12}O_7$ on the basis of the molecular ion peak at positive ESIMS *m/z* 317 $[M+H]^+$ (Appediced 44). The ^1H and ^{13}C NMR spectra revealed the following: ^1H NMR (Methanol-d_4, 400 MHz) (See Appendices 42 and 43): δ 6.20 (1H, d, *J* = 2.0 Hz, H-6), 6.39 (1H, d, *J* = 2.0 Hz, H-8), 7.63 (1H, d, *J* = 2.0 Hz, H-2'), 7.53 (1H, dd, *J* = 8.4, 2.0 Hz, H-5'), 6.90 (1H, d, *J* = 8.4 Hz, H-6') , 3.78 (3H, s, 3-OCH_3); ^{13}C NMR (Methanol-d_4, 100 MHz): δ 158.0 (s, C-2), 139.5 (s, C-3), 180.0 (s, C-4), 163.1 (s, C-5), 99.7 (d, C-6), 165.9 (s, C-7), 94.7 (d, C-8), 158.4 (s, C-9), 105.8 (s, C-10), 122.9 (s, C-1'), 116.4 (d, C-2'), 146.5 (s, C-3'), 150.0 (s, C-4'), 116.4 (d, C-5'), 122.3 (d, C-6'), 60.5 (q, 3-OCH_3) (Jurd and Horowitz, 1957; Nguyen *et al*., 2007). The data correlated with the signals for quercetin except for the presence of a methoxyl signal at δ_C60.5, δ_H3.78 (3H, *s*) (Tachakittirungrod *et al*., 2007). TCB 16 was identified as 5,7,3',4'-tetrahydroxy-3-methoxyflavone (quercetin-3-methyl ether) (Figure 4.10).

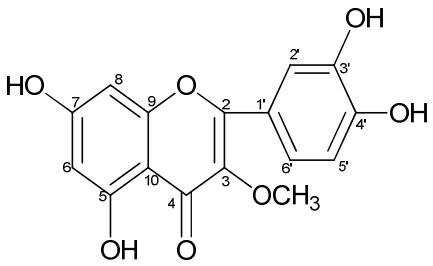


Figure 4.10: Chemical Structure of TCB 16, (10) - 5,7,3',4'-tetrahydroxy-3-methoxyflavone - $C_{16}H_{12}O_7$ (M. wt.: 316)

The molecular formula of TCB 21 was determined as $C_7H_6O_4$, on the basis of the molecular ion peak at positive ESI-MS *m/z* 155 $[M+H]^+$. The ^1H and ^{13}C NMR spectra revealed the following: ^1H NMR (Methanol-d_4, 500 MHz): δ 6.79 (1H, d, *J* = 8.0 Hz, H-6), 7.42 (1H, overlap, H-3), 7.42 (1H, overlap, H-5); ^{13}C NMR (Methanol-d_4, 100 MHz): δ 151.5 (s, C-1),

146.1 (s, C-2), 115.8 (d, C-3), 123.2 (s, C-4), 123.9 (d, C-5), 117.8 (d, C-6), 170.2 (s, 4-COOH). The data are in agreement with the literature (Perkin, 1897). TCB 21 was identified as Protocatechuic acid (Figure 4.11).

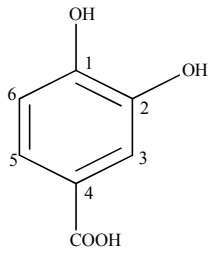


Figure 4.11: Chemical Structure of TCB 21, (11) - Protocatechuic acid - $C_7H_6O_4$ (M. wt.: 154)

The molecular formula of TCB 22 was determined as $C_{19}H_{38}O_4$ on the basis of the molecular ion peak at positive ESI-MS *m/z* 353 $[M+Na]^+$. The ^1H and ^{13}C NMR spectra revealed the following: ^1H NMR (pyridine-d_6, 500 MHz): δ 4.61 (1H, dd, *J* = 11.0, 6.0 Hz, H-1a), 4.68 (1H, dd, *J* = 11.0, 4.5 Hz, H-1b), 4.41 (1H, m, H-2), 4.09 (2H, d, *J* = 5.5 Hz, H-3), 2.31 (1H, m, H-2'), 1.60 (1H, m, H-3'), 1.19 (24H, overlap, H-4' - H-15'), 0.82 (1H, m, H-16') ; ^{13}C NMR (pyridine-d_6, 125 MHz): δ 66.8 (t, C-1), 71.0 (d, C-2), 64.3 (t, C-3), 173.8 (s, C-1'), 14.3 (q, C-16'). TCB 22 was identified as 1-O-Hexadecanolenin (Figure 4.12)**.**

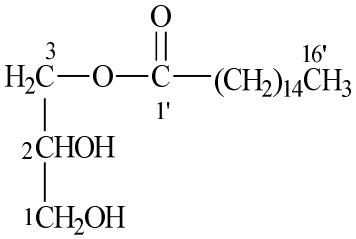


Figure 4.12: Chemical Structure of TCB 22, (12) - 1-*O*-Hexadecanolenin - $C_{19}H_{38}O_4$ (M. Wt.: 330)

The molecular formula of TCB 27 was determined as $C_{20}H_{18}O_{10}$ on the basis of the molecular ion peak at positive ESI-MS *m/z* 441 $[M+Na]^+$. The ^1H and ^{13}C NMR spectra revealed the following: ^1H NMR (Methanol-d_4, 400 MHz): δ 6.18 (1H, br s, H-6), 6.37 (1H, br s, H-8), 8.01 (2H, d, J = 8.4 Hz, H-2', H-6'), 6.86 (2H, d, J = 8.4 Hz, H-3', H-5'), 5.16 (1H, d, J = 6.8 Hz, H-1''), 3.74 (1H, d, J = 4.79 Hz, H-2''), 3.48 (1H, t, J = 5.77 Hz, H-3''), 3.41 (1H, d, J = 8.21 Hz, H-4''), 3.76 (2H, s, J = 4.60 Hz, H-5''); ^{13}C NMR (Methanol-d_4, 100 MHz): δ 158.4 (s, C-2), 135.3 (s, C-3), 179.4 (s, C-4), 163.0 (s, C-5), 99.9 (d, C-6), 166.0 (s, C-7), 94.8 (d, C-8), 158.9 (s, C-9), 105.6 (s, C-10), 122.6 (s, C-1'), 132.2 (d, C-2', C-6'), 116.1 (d, C-3', C-5'), 161.6 (s, C-4'), 104.6 (d, C-1''), 75.3 (d, C-2''), 77.5 (d, C-3''), 71.0 (d, C-4''), 67.2 (t, C-5'') (Kruglii and Glyzin, 1968). Additional signals at $\delta_{C1''}$104.6, $\delta_{H1''}$5.16; $\delta_{C2''}$75.3, $\delta_{H2''}$3.74; $\delta_{C3''}$77.5, $\delta_{H3''}$3.48; $\delta_{C4''}$71.0, $\delta_{H4''}$3.41; $\delta_{C5''}$67.2, $\delta_{H5''}$3.76 were ascribed to the xylose moiety attached to the C-3 of the flavonol A-ring. The data are in agreement with the literature (Kruglii and Glyzin, 1968). TCB 27 was therefore identified as kaempferol-3-*O*-β-D-xylopyranoside (Figure 4.13).

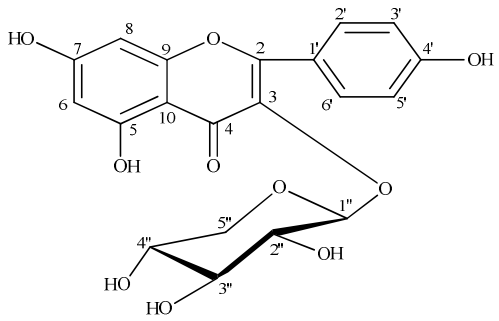


Figure 4.13: Chemical Structure of TCB 27, (13) - kaempferol-3-*O*-β-D-xylopyranoside - $C_{20}H_{18}O_{10}$ (M. Wt.: 418)

The molecular formula of TCB 40 was determined as $C_{26}H_{28}O_{14}$ on the basis of the molecular ion peak at positive ESIMS *m/z* 565 $[M+H]^+$. The ^1H and ^{13}C NMR spectra revealed the following: ^1H NMR (Methanol-d_4, 500 MHz): δ 6.06 (1H, br s, H-6), 6.24 (1H, br s, H-8), 7.92 (2H, d, *J* = 8.5 Hz, H-2', H-6'), 6.82 (2H, d, *J* = 8.5 Hz, H-3', H-5'), 5.53 (1H, d, *J* = 7.0 Hz, H-1''), 5.21 (1H, br s, H-1'''); ^{13}C NMR (Methanol-d_4, 125 MHz): δ 158.1 (s, C-2), 134.3 (s, C-3), 179.1 (s, C-4), 162.9 (s, C-5), 99.8 (d, C-6), 165.5 (s, C-7), 94.7 (d, C-8), 158.5 (s, C-9), 105.8 (s, C-10), 122.9 (s, C-1'), 132.0 (d, C-2', C-6'), 116.1 (d, C-3', C-5'), 161.2 (s, C-4'), 101.2 (d, C-1''), 79.3 (d, C-2''), 77.9 (d, C-3''), 72.2 (d, C-4''), 66.9 (t, C-5''), 102.5 (d, C-1'''), 71.3 (d, C-2'''), 72.3 (d, C-3'''), 74.0 (d, C-4'''), 70.0 (d, C-5'''), 17.7 (q, C-6'''). The data are in agreement with the literature (Moon et al., 2010; Cui *et al.*, 2003). TCB 40 was identified as Kaempferol-3-*O*-α-L-rhamnopyranosyl-1→2)-β-D-xylopyranoside (Figure 4.14).

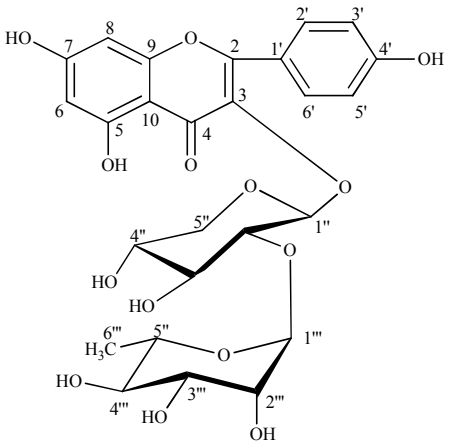


Figure 4.14: Chemical Structure of TCB 40, (14) - kaempferol-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-xylopyranoside - $C_{26}H_{28}O_{14}$ (M. Wt.: 564)

Table 4.2: ^{1}H, ^{13}C-NMR and DEPT Spectral Data Assignment for TCB 5

^{1}H NMR and ^{13}C NMR data for compound TCB 5 in MeOH (δ in ppm, J in Hz)		
No.	δ_C	$\boldsymbol{\delta_H}$
1	76.3 (d)	3.86 (1H, s)
2	23.0 (t)	1.80 (1H, overlapped H-2a), 1.55 (1H, m)
3	32.5 (t)	1.25 (1H, overlapped H-3a), 1.61 (1H, overlapped, H-3b)
4	38.9 (s)	-
5	80.5 (s)	-
6	78.0 (d)	3.70 (1H, s, J = 7.30 Hz)
7	75.5 (d)	4.73 (1H, s)
8	41.7 (d)	2.94 (1H, m)
9	30.8 (d)	1.52 (1H, m)
10	54.3 (s)	-
11	33.8 (t)	1.77 (1H, d, J = 3.55 Hz, H-11a), 1.55 (1H, m H-11b)
12	77.0 (d)	3.88 (1H, s, J = 9.55 Hz)
13	42.8 (d)	3.90 (1H, s, J = 10.05 Hz)
14	141.5 (s)	-
15	115.3 (d)	5.56 (1H, s)
16	178.1 (s)	-
17	24.3 (q)	1.15 (3H, s)
18	24.3 (q)	1.14 (3H, s)
19	16.2 (q)	1.19 (3H, s)
1-O*C*OCH$_3$	169.5 (s)	-
1-OCOCH$_3$	21.0 (q)	2.02 (3H, s)
7- OCOCH$_3$	171.7 (s)	-
7- OCOCH$_3$	20.8 (q)	2.10 (3H, s)
14-COOCH$_3$	173.2 (s)	-
14- COOCH$_3$	52.3 (q)	3.69 (3H, s)

^{1}H NMR and ^{13}C NMR were recorded at 500 and 125 MHz respectively in $CDCl_3$, Rotating-frame Overhauser Effect Spectroscopy (ROESY) (Figure 4.21), Heteronuclear Multiple Bond Coherence (HMBC) (Figure 4.20), Heteronuclear Multiple Quantum Coherence (HMQC) (Figure 4.19) and Correlation Spectroscopy (COSY) (Figure 4.18) were recorded at 600 MHz.

Table 4.3: ^{1}H, ^{13}C-NMR and DEPT Spectral Data Assignment for TCB 6

^{1}H NMR and ^{13}C NMR data for compound TCB 6 in MeOH (δ in ppm, J in Hz)		
No.	δ_C	$\boldsymbol{\delta_H}$
1	74.3 (d)	5.21 (1H, s)
2	67.0 (d)	5.28 (1H, m)
3	35.7 (t)	1.94 (1H, overlapped H-3a), 1.40 (1H, overlapped, H-3b)
4	40.1 (s)	-
5	76.5 (s)	-
6	25.1 (t)	1.76 (1H, m, H-6a), 1.54 (1H, m, H-6b)
7	19.1 (t)	1.88 (1H, overlapped, H-7a), 1.70 (1H, m, H-7b)
8	47.4 (d)	1.54 (1H, overlapped)
9	34.2 (d)	2.45 (1H, t, J = 13.0 Hz)
10	45.0 (s)	-
11	40.1 (t)	2.11 (1H, d, J = 13.0 Hz, H-11a), 1.28 (1H, t, J = 13.0 Hz, H-11b),
12	106.9 (s)	-
13	173.1 (s)	-
14	74.8 (s)	-
15	115.1, (d)	6.02 (1H, s)
16	168.7 (s)	-
17	20.2 (q)	1.41, (3H, s)
18	28.1 (q)	1.09, (3H, s)
19	25.6 (q)	1.16 (3H, s)
20	16.8 (q)	1.10 (3H, s)
1-O*C*OCH$_3$	168.9 (s)	-
1-OCO*CH$_3$*	20.8 (q)	2.17, (3H, s)
12-O*CH$_2$*CH$_3$	58.7 (t)	3.52 (1H, m, 12-OCH$_2$-a), 3.17 (1H, m, 12-OCH$_2$-b)
12-OCH$_2$*CH$_3$*	14.8 (q)	1.13, (3H, overlapped)
14-O*C*OCH$_3$	170.3 (s)	-
14-OCO*CH$_3$*	20.9 (q)	1.98, (3H, s)

^{1}H NMR and ^{13}C NMR were recorded at 500 and 125 MHz respectively in $CDCl_3$, Rotating-frame Overhauser Effect Spectroscopy (ROESY) (Figure 4.34), Heteronuclear Multiple Bond Coherence (HMBC) (Figure 4.33), Heteronuclear Multiple Quantum Coherence (HMQC) (Figure 4.31) and Correlation Spectroscopy (COSY) (Figure 4.32) were recorded at 600 MHz.

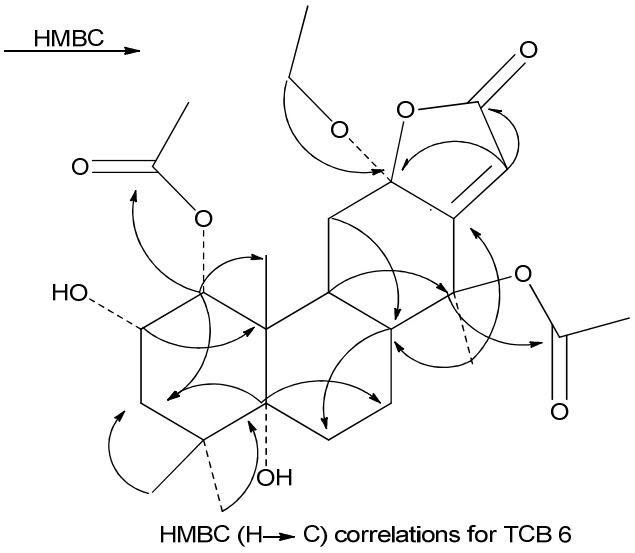


Figure 4.15: Major HMBC (H→C) Correlations for TCB 6

4.5 *In vivo* antioxidant activity of the crude extract of *Caesalpinia bonduc*

The antioxidant activities of *C. bonduc* were studied by assaying for antioxidant enzymes. Compared with the controls, there were significant increases in the peroxidase and catalase activities of the extract treated groups at all doses. Compared with the positive and negative controls (rats treated with 10 mg vitamin C/kg bwt and rats treated with 10 mg amodiaqiune/kg bwt respectively), these increases are highly significant in rats treated with 150 and 200 mg *C. bonduc*/kg bwt (Figures 4.16 and 4.17). The localization of radical formation resulting in lipid peroxidation, measured as the concentration of malondialdehyde (MDA) and TBARS, was significantly increased in negative amodiaquine control and decreased in graded doses in extract and vitamin C treated rats compared with normal control (Figure 4.18).

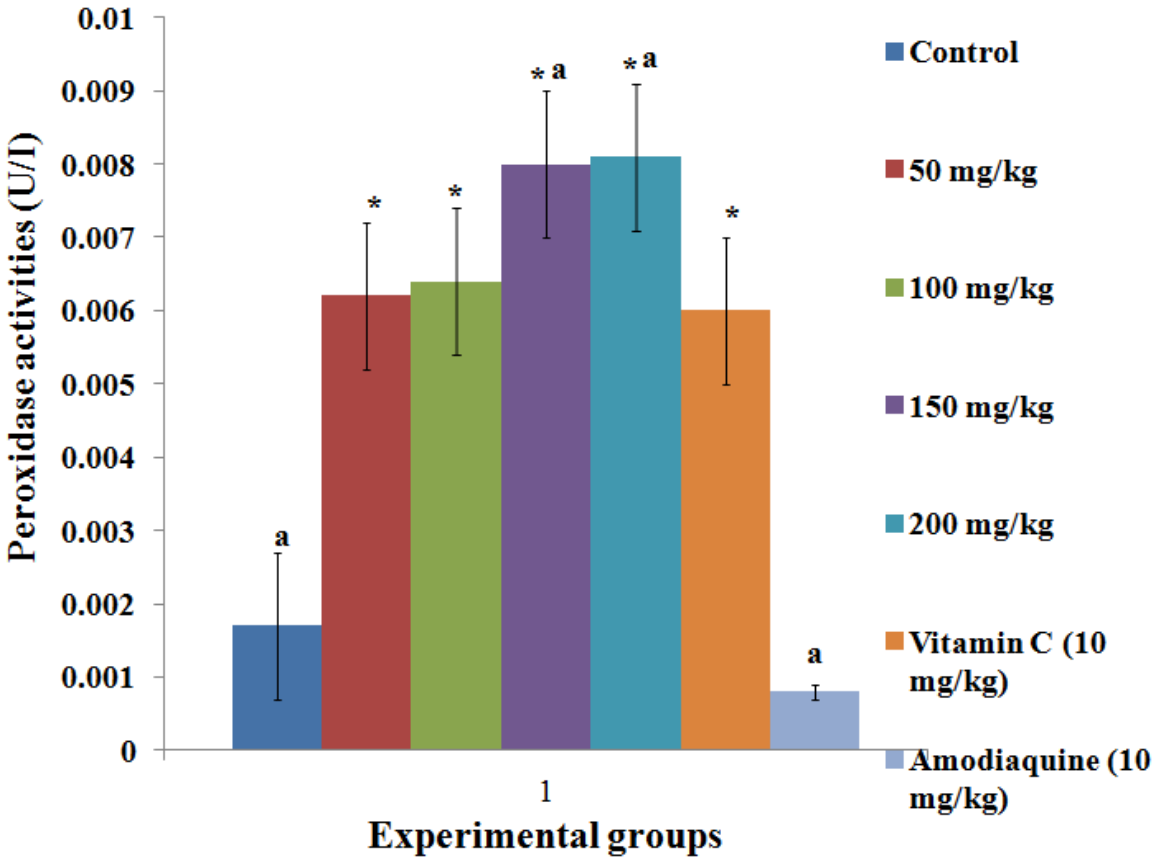


Figure 4.16: Bar Chart Showing the Effect of the Ethanolic Extract of *C. bonduc* on the Peroxidase Activity in Experimental Rats. * means that the difference between the control and the treated groups is significant at $p < 0.05$. [a] means that the difference between vitamin C and the other treated groups is significant at $p < 0.05$. Values are presented as mean ± SEM of six replicates.

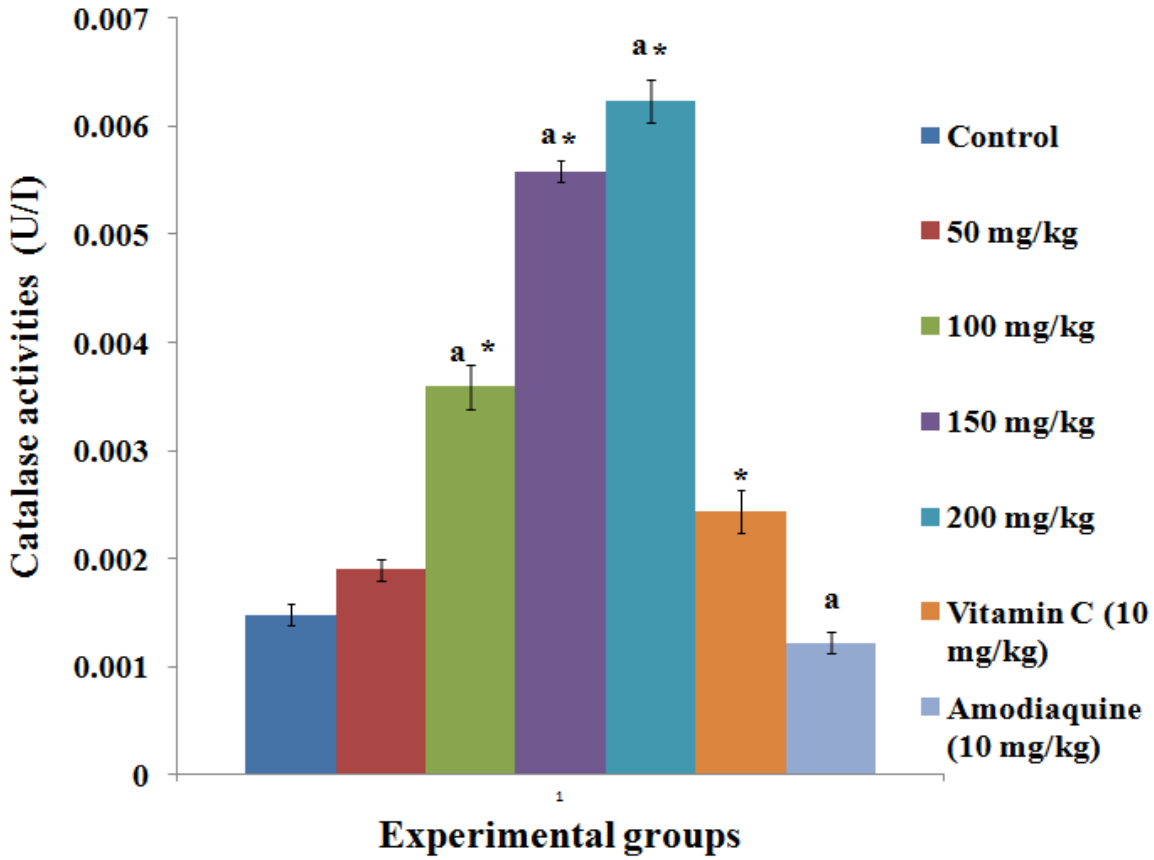


Figure 4.17: Bar Chart Showing the Effect of the Ethanolic Extract of *C. bonduc* on the Catalase Activity of the Experimental Rats. * means that the difference between the control and the treated groups is significant at $p < 0.05$. [a] means that the difference between vitamin C and the other treated groups is significant at $p < 0.05$. Values are presented as mean ± SEM of six replicates.

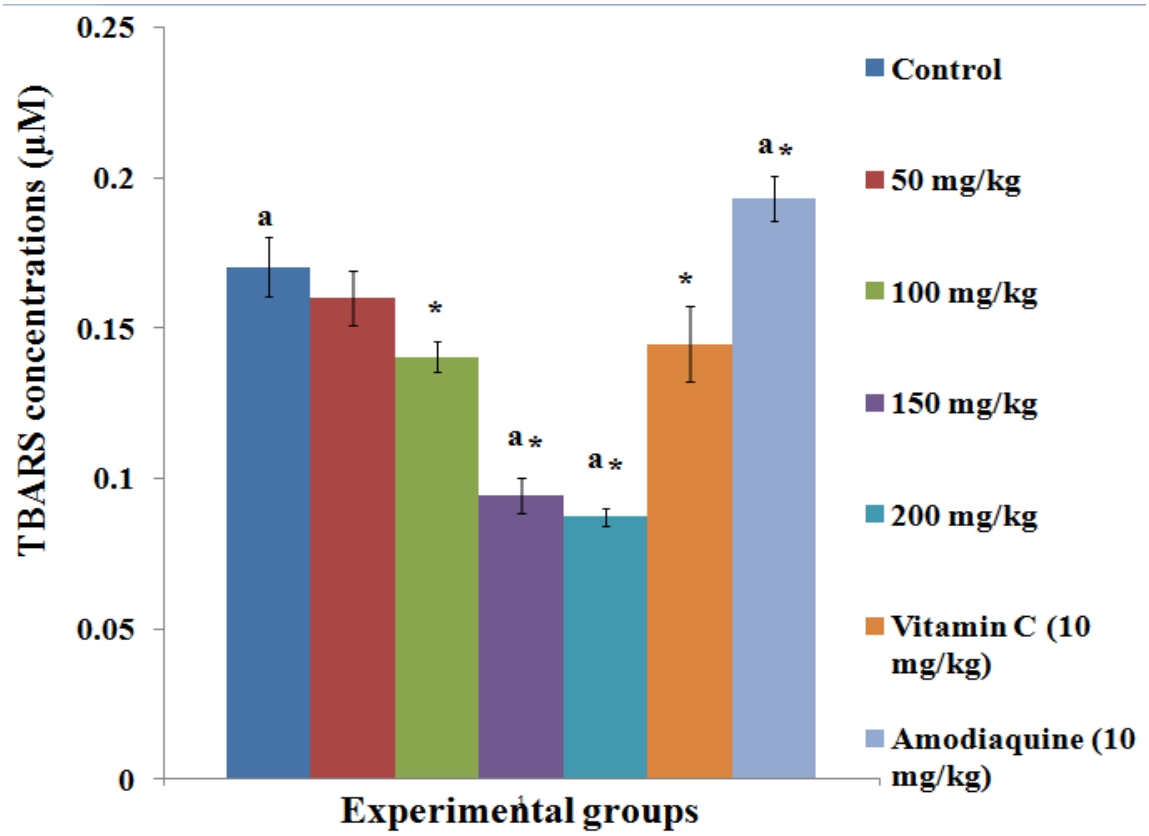


Figure 4.18: Bar Chart Showing the Effect of the Ethanolic Extract of *Caesalpinia bonduc* on TBARS Concentration of the Experimental Rats. * means that the difference between the control and the treated groups is significant at $p < 0.05$. [a] means that the difference between vitamin C and the other treated groups is significant at $p<0.05$. Values are presented as mean ± SEM of six replicates.

4.6 Sub-acute Toxicological Evaluation of CB on Food Intake, Body and Relative Organ Weights of Experimental Rats

The water and food consumptions of the animals treated with the extract at doses of 200 and 400 mg CB/kg bwt did not differ with controls A and B. However, the consumptions of the other groups (800 and 1600 mg CB/kg bwt) were reduced compared to control A. Nevertheless, the body weights of all the experimental animals were observed to increase progressively throughout the duration of the experiment (Figure 1.19). No significant changes in the relative organ weights were observed in rats treated with 200 mg CB/kg bwt compared with the control. However, there were significant increases in organ weights in rats treated with higher doses of CB and in the recovery test groups (Table 4.4).

4.7 Sub-acute Toxicological Evaluation of the Ethanolic Extract of CB on Biochemical Parameters of Experimental Rats

There was no significant alteration in the plasma biochemical parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB), direct bilirubin (DB), indirect bilirubin (IB), cholesterol (PC), urea (PU), triglyceride (PTG), total protein (TP) and creatinine (PCT)) in rats treated with 200 mg CB/kg bwt in comparison with rats treated with distill water in control A. However, there was a significant reduction in the plasma glucose (PG) concentration and increase in the plasma uric acids (PUA) in rats treated with 200 mg CB/kg bwt (Tables: 4.5, 4.6 and 4.7). Plasma enzymes levels (AST and ALT) and some plasma biochemical parameters (PTG, TB, PU, PUA, PC) were significantly increased in rats treated with higher 400, 800 and 1600 mg CB/kg bwt in comparison with control A and the extract treated dose of 200 mg/kg body weight. The levels of glucose, total plasma protein, indirect bilirubin, direct bilirubin

and creatinine were slightly altered at extract doses of 400, 800, 1600 mg/kg body weights of experimental Wistar rats.

It was also observed that there was no significant alteration in the biochemical parameters of the extract treated test groups (200, 400, 800, 1600 mg/kg body weight) compared with the extract treated recovery groups (200R, 400R, 800R and 1600R).

4.8 Haematological Evaluation of Sub-acute Toxicological Effect of the Ethanolic Extract of CB on Experimental Rats

There was no significant change in the number of white blood cells, neutrophils, lymphocytes, monocytes and packed cell volume of the extract treated rats at 200mg/kg body weight compared with the control. However, these haematological parameters were significantly altered at extract doses of 400, 800 and 1600 mg/kg body weight compared with the control and with the dose of 200 mg/kg body weight, with a significant increase in lymphocytes counts and a decrease in white blood cells, neutrophil and monocyte counts (Table 4.8).

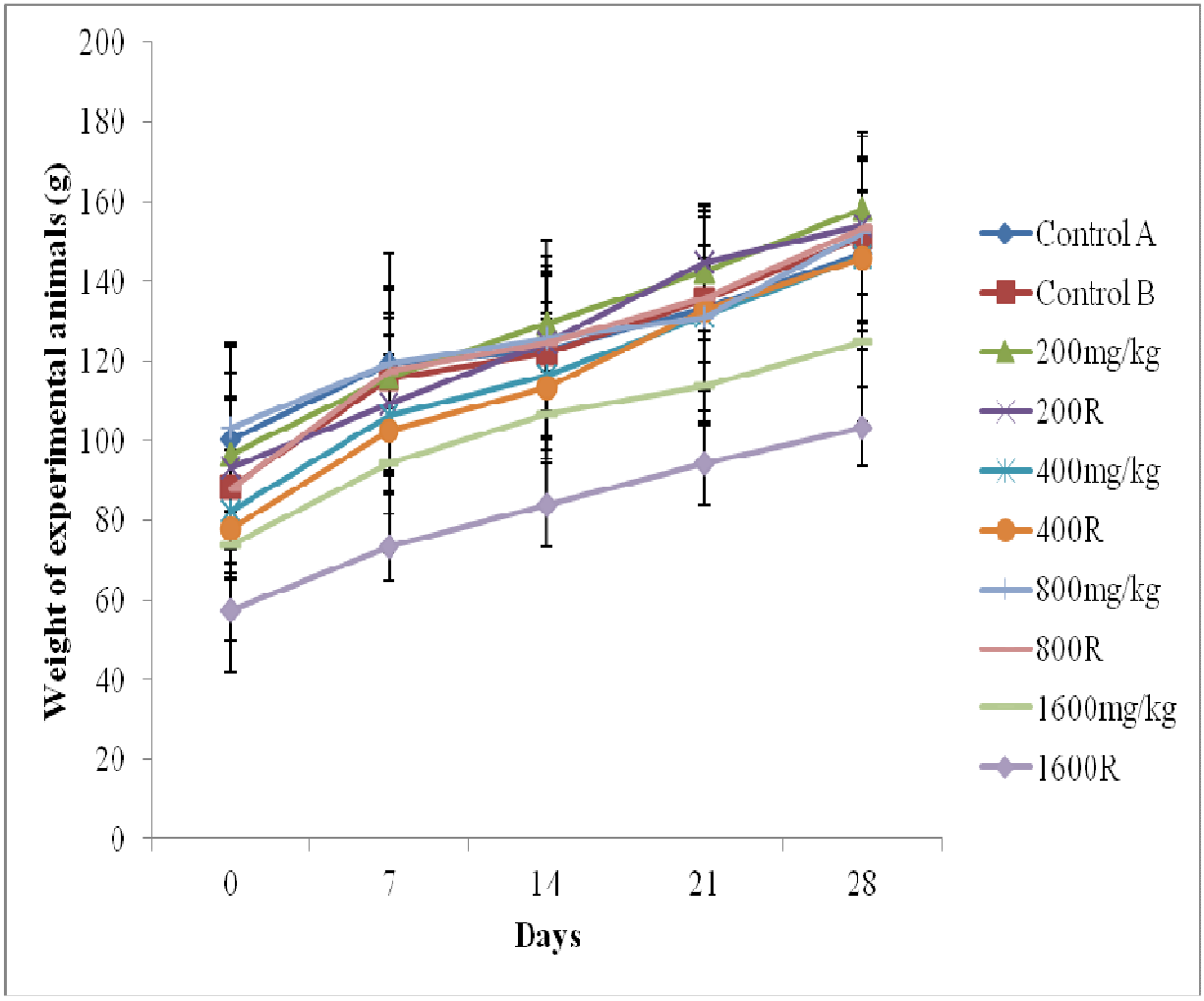


Figure 4.19: Changes in Body Weight of Experimental Animals for Sub-acute Study (See Appendix 45)

Table 4.4: Relative Organ Weights of Animals in the Sub-acute Toxicological Evaluation

Groups	liver	Heart	Kidney	spleen
Control A	0.033±0.00	0.003±0.00	0.003±0.00	0.003±0.00
Control B	0.031±0.00	0.003±0.00	0.003±0.00	0.003±0.00
200 mg/kg	0.035±0.01	0.003±0.00	0.003±0.00	0.004±0.00
200R	0.051±0.08*	0.004±0.00	0.004±0.00	0.004±0.00
400 mg/kg	0.036±0.01	0.003±0.00	0.003±0.00	0.003±0.00
400R	0.033±0.00	0.004±0.00	0.005±0.00*	0.003±0.00
800 mg/kg	0.043±0.01*	0.004±0.00*	0.004±0.00*	0.006±0.00*
800R	0.049±0.00*	0.003±0.00	0.004±0.00	0.005±0.00*
1600 mg/kg	0.039± 0.00	0.004±0.00	0.003±0.00	0.003±0.00
1600R	0.054±0.00*	0.004±0.00*	0.004±0.00*	0.004±0.00

Values are presented as mean ± SEM (n = 5 readings). Values marked with * are significantly different at $p < 0.05$ compared with control. Control A, Control B, 200 mg/kg, 200R, 400 mg/kg, 400R, 800 mg/kg, 800R, 1600 mg/kg and 1600R represent groups of rats treated with distill water; rats treated with sodium caboxylmethyl cellulose; rats treated with 200 mg CB/kg bwt; recovery group of rats treated with 200 mg CB/kg bwt; rats treated with 400 mg CB/kg bwt; recovery group of rats treated with 400 mg CB/kg bwt; rats treated with 800 mg CB/kg bwt; recovery group of rats treated with 800 mg CB/kg bwt; rats treated with 1600 mg CB/kg bwt and recovery group of rats treated with 1600 mg CB/kg bwt respectively.

Table: 4.5: Effect of Sub-acute Toxicological Evaluation of CB on Liver Function Markers of the Animals

Groups	ALT (U/I)	AST (U/I)	TB (mg/dl)	DB (mg/dl)
Control A	243.06±23.7	21.19±2.8	0.17±0.0	0.09±0.0
Control B	246.46±51.0	25.08±0.6	0.11±0.0	0.06±0.0
200 mg/Kg	242.86±44.5	25.67±1.2	0.17±0.0	0.07±0.0
200R	247.60±28.9	28.00±1.0	0.22±0.0	0.12±0.0
400 mg/kg	334.83±11.7*^{c}	32.67±1.8*	0.19±0.0	0.05±0.0
400R	323.17±12.6*^{c}	34.22±3.4*	0.21±0.0	0.06±0.0
800 mg/kg	320.83±19.8*^{c}	33.25±0.6*	0.23±0.0*	0.11±0.0
800R	310.92±20.3	33.06±1.6*	0.21±0.0	0.15±0.0*^{c}
1600 mg/kg	320.06±13.1*^{c}	31.31±2.5*	0.27±0.0*^{c}	0.11±0.0
1600R	304.31±20.1	32.96±0.2*	0.30±0.0*^{c}	0.09±0.0

Values are presented as mean ± SEM (n = 5 readings) (ALT, AST, TB, DB represent Alanine aminotransferase activity; aspartate aminostrasferase activity; total bilirubin concentration and direct bilirubin concentration respecvtively). Values marked with * are significantly different at $p < 0.05$ compared with control A while values marked with superscript c 'c' and d 'd' are significantly different at $p < 0.05$ compared with dose at 200 mg/kg body weight of experimental animals and recovery groups respectively. Control A, Control B, 200 mg/kg, 200R, 400 mg/kg, 400R, 800 mg/kg, 800R, 1600 mg/kg and 1600R represent groups of rats treated with distill water; rats treated with sodium caboxylmethyl cellulose; rats treated with 200 mg CB/kg bwt; recovery group of rats treated with 200 mg CB/kg bwt; rats treated with 400 mg CB/kg bwt; recovery group of rats treated with 400 mg CB/kg bwt, rats treated with 800 mg CB/kg bwt, recovery group of rats treated with 800 mg CB/kg bwt, rats treated with 1600 mg CB/kg bwt and recovery group of rats treated with 1600 mg CB/kg bwt.

Table: 4.6: Effect of Sub-acute Toxicological Evaluation of CB on Kidney Function Markers of the Animals

Groups	PUA (mg/dl)	PU (mg/dl)	PCT (mg/dl)	PTG (mg/dl)
Control A	0.39±0.0[c]	93.92±7.1	1.08±0.1	64.18±3.5
Control B	1.50±0.2[c]	84.53±4.0	0.88±0.1	72.42±3.2
200 mg/kg	4.56±0.4*	85.87±14.2	0.94±0.1	71.95±17.2
200R	6.60±0.4*	83.18±11.7	0.89±0.3	123.01±26.2
400 mg/kg	6.89±0.9*[c]	112.70±12.3*	1.08±0.1	116.90±30.4
400R	9.00±0.4*[c]	107.33±7.1	1.15±0.0	123.01±4.4
800 mg/kg	8.56±0.9*[c]	112.70±4.7*	1.07±0.2	144.13±37.9*[c]
800R	11.60±0.4*[c]	118.07±2.7	1.10±0.0	149.26±8.6*[c]
1600 mg/kg	10.00±1.4*[c]	185.15±44.8*[c]	1.23±0.3	154.46±42.4*[c]
1600R	13.60±0.8*[c]	187.83±9.7*[c]	1.16±0.1	141.59±3.9*[c]

Values are presented as mean ± SEM (n = 5 readings); PUA, PU, PCT and PTG represent uric acid concentration, urea concentration, creatinine concentration and triglyceride concentration. Values marked with * are significantly different at $p < 0.05$ compared with control A while values marked with superscript c 'c' and d 'd' are significantly different at $p < 0.05$ compared with dose at 200 mg/kg body weight of experimental animals and recovery groups respectively. Control A, Control B, 200 mg/kg, 200R, 400 mg/kg, 400R, 800 mg/kg, 800R, 1600 mg/kg and 1600R represent groups of rats treated with distill water; rats treated with sodium caboxylmethyl cellulose; rats treated with 200 mg CB/kg bwt; recovery group of rats treated with 200 mg CB/kg bwt; rats treated with 400 mg CB/kg bwt; recovery group of rats treated with 400 mg CB/kg bwt, rats treated with 800 mg CB/kg bwt, recovery group of rats treated with 800 mg CB/kg bwt, rats treated with 1600 mg CB/kg bwt and recovery group of rats treated with 1600 mg CB/kg bwt.

Table: 4.7: Effect of Sub-acute Toxicological Evaluation of CB on Other Biochemical Markers of the Animals

Groups	TP (g/dl)	PG (mg/dl)	PC (mg/dl)	IB (mg/dl)
Control A	9.04±1.5	354.87±5.9	54.44±4.0	0.08±0.0
Control B	9.56±2.2	365.00±1.9	50.64±10.2	0.04±0.0
200 mg/kg	8.62±1.7	222.82±55.3*	60.53±1.1	0.10±0.0
200R	7.38±0.1	183.61±10.9*	66.27±3.7	0.10±0.1
400 mg/dl	8.45±0.6	418.97±41.4[c]	70.35±4.1	0.14±0.0
400R	7.67±0.2	292.22±14.2	70.59±13.6	0.15±0.1
800 mg/dl	6.51±0.4*	323.59±58.9	77.71±4.1*	0.12±0.0
800R	7.02±0.1	273.06±126.5	74.12±4.1*	0.06±0.0
1600 mg/dl	6.65±0.5	314.17±12.1	85.89±7.1*[c]	0.16±0.0*
1600R	7.23±0.1	314.17±12.1	71.37±8.2	0.21±0.1

Values are presented as mean ± SEM (n = 5 readings); TP, PG, PC, IB represent total protein concentration, glucose concentration, cholesterol concentration and indirect bilirubin concentration.Values marked with * are significantly different at $p < 0.05$ compared with control A while values marked with superscript c 'c' and d 'd' are significantly different at $p < 0.05$ compared with dose at 200 mg/kg body weight of experimental animals and recovery groups respectively. Control A, Control B, 200 mg/kg, 200R, 400 mg/kg, 400R, 800 mg/kg, 800R, 1600 mg/kg and 1600R represent groups of rats treated with distill water; rats treated with sodium caboxylmethyl cellulose; rats treated with 200 mg CB/kg bwt; recovery group of rats treated with 200 mg CB/kg bwt; rats treated with 400 mg CB/kg bwt; recovery group of rats treated with 400 mg CB/kg bwt, rats treated with 800 mg CB/kg bwt, recovery group of rats treated with 800 mg CB/kg bwt, rats treated with 1600 mg CB/kg bwt and recovery group of rats treated with 1600 mg CB/kg bwt.

Table: 4.8: Effect of Sub-acute Toxicological Evaluation of CB on Haematological Markers of the Animals

Groups	PCV ($\times 10^{12}$/L)	WBC ($\times 10^{12}$/L)	L (%)	N (%)	M(%)
Control A	38.33±5.4	9.63±1.7	45.67±2.9	53.00±3.2	1.67±0.3
Control B	44.50±6.5	10.00±2.0	42.00±4.0	57.00±3.0	2.00±0.0
200 mg/kg	42.33±4.7	9.13±3.1	47.67±2.6	51.00±2.7	1.67±0.3
200R	41.00±1.2	4.03±0.8*^{c}	56.33±5.9	48.67±4.1	1.67±0.3
400 mg/kg	45.67±1.5	8.47±0.1	64.33±12.3*	35.67±12.3*	1.33±0.3
400R	46.00±1.2	4.37±1.3*^{c}	60.00±5.8*	40.00±2.9	1.33±0.3
800 mg/kg	42.33±2.6	5.87±1.5*	59.33±11.1*	39.33±11.5*	1.33±1.3
800R	39.00±0.6	3.47±0.5*^{c}	63.33±6.0*	35.00±5.8*	0.67±0.7
1600 mg/kg	44.00±3.2	4.23±0.6*^{c}	74.33±2.9*^{c}	25.67±2.9*^{c}	0.67±0.3
1600R	38.67±2.0	4.80±0.6*^{c}	70.33±5.8*^{c}	29.00±1.2*^{c}	0.67±0.3

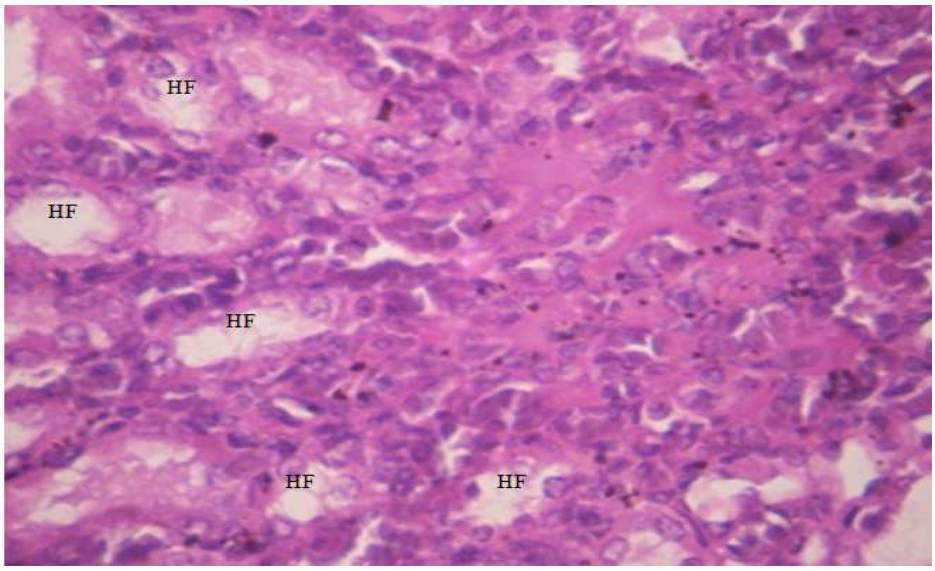
Values are presented as mean ± SEM (n = 5 readings); PCV, WBC, L, N, M represent packed cell volume, white blood cell count, percentage lymphocyte count, percentage neutrophil count, percentage monocyte count. Values marked with * are significantly different at $p < 0.05$ compared with control A while values marked with superscript c $^{'c'}$ and d $^{'d'}$ are significantly different at $p < 0.05$ compared with dose at 200 mg/kg body weight of experimental animals and recovery groups respectively. Control A, Control B, 200 mg/kg, 200R, 400 mg/kg, 400R, 800 mg/kg, 800R, 1600 mg/kg and 1600R represent groups of rats treated with distill water; rats treated with sodium caboxylmethyl cellulose; rats treated with 200 mg CB/kg bwt; recovery group of rats treated with 200 mg CB/kg bwt; rats treated with 400 mg CB/kg bwt; recovery group of rats treated with 400 mg CB/kg bwt, rats treated with 800 mg CB/kg bwt, recovery group of rats treated with 800 mg CB/kg bwt, rats treated with 1600 mg CB/kg bwt and recovery group of rats treated with 1600 mg CB/kg bwt.

4.9 Effect of Sub-acute Ethanolic Extract of CB on Histopathology of the Organs of the Animals

Compared with the control, no remarkable changes on the morphology of organs of the experimental rats treated with extract at 200 mg/kg body weight were noticed on gross examinations. The histologic section of the liver showed a preserved hepatic architecture, with hepatocytes arranged in plates with no vascular congestion; there were no areas of necrosis or haemorrhage, fatty change or fibrosis. The kidney showed cellular turfs of glomeruli surrounded by bowman's spaces with disposed background that contains tubules cut in varying planes. The heart muscles, myocytes were composed of interlacing fascicles of myocardial cells which are elongated with spindle nuclei while the spleen showed periarteriolar cuffing composed of lymphocytes, histiocytes and red blood cells.

However, the livers of animals treated with higher doses of extract showed noticeable cellular alterations such as the presence of; (i) lipid-filled hepatocytes (hepatic fatty changes) in rats treated with 400 mg CB/kg body weight (Plate 4.1), (ii) prominent hepatic sinusoids which are engorged with red blood cells (sinusoidal congestion), central hepatic venous congestion which are engorged with fatty vacuoles (fatty congestion) in rats treated with 800 mg CB/kg bwt (Plate 4.2); and aggregation of dead cells, inflammatory cells and amorphous debris (hepatic fatty necrosis) in rats treated with 1600 mg CB/kg body weight (Plate 4.3).

A gross examination of the kidney showed kidney tubular necrosis and glomerular congestion (gross dilatation, engorgement or distension of blood vessels by the blood) at extract doses of 400, 800 and 1600 mg/kg body weight of the experimental animals (Plate 4.4) while there were no noticeable histopathological alterations on the gross examinations of the spleen and the heart at extract doses of 400, 800 and 1600 mg/kg body weight.

A 

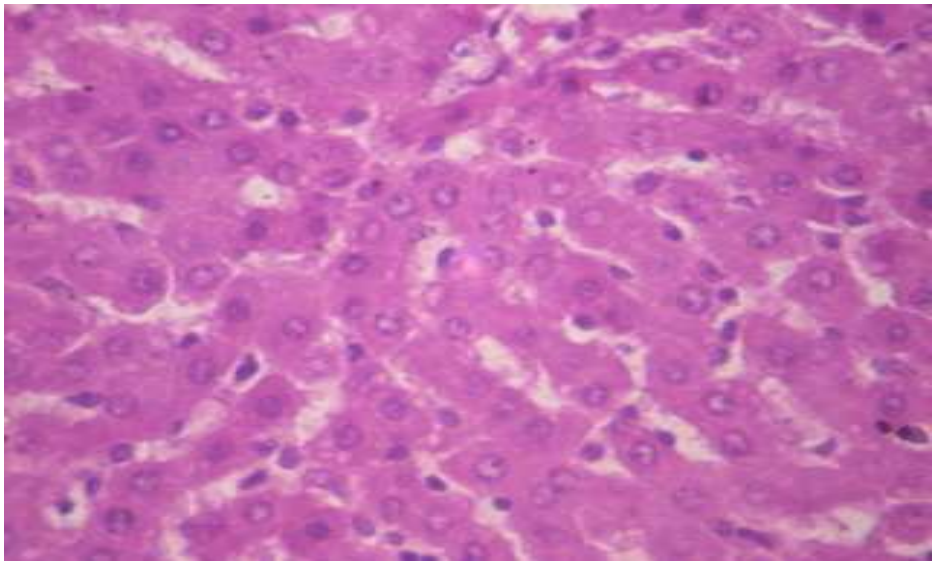
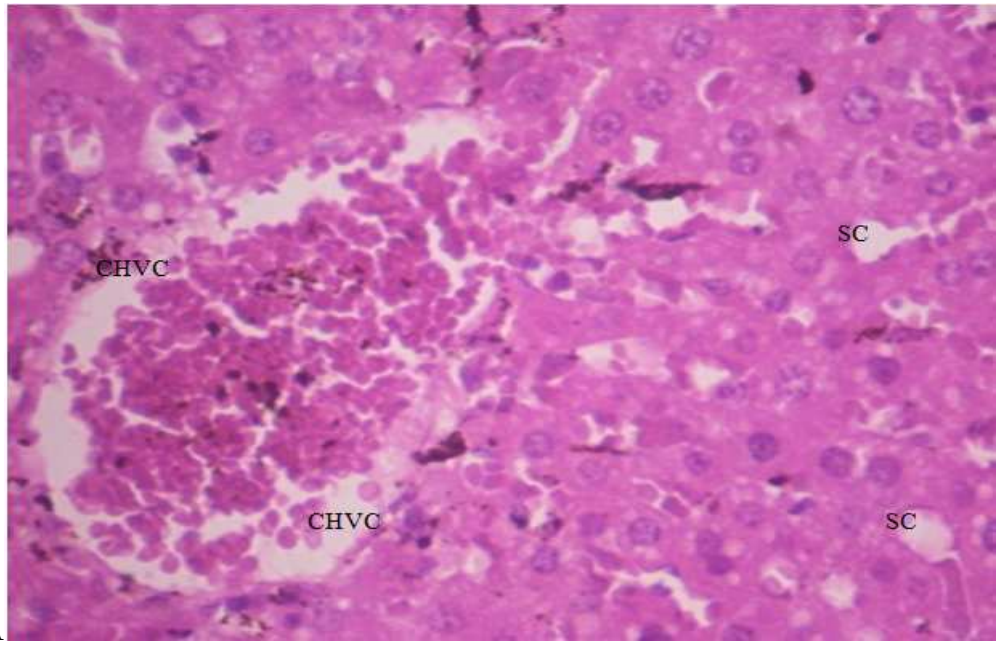
B 

Plate 4.1: A Cross-sectional View of the Rat Liver of a Group Orally Administered 400 mg/kg of *C. bonduc* for 28 days, showing areas of gross hepatic fatty changes (HF) (Plate 4.1A) compared with normal liver architecture (Plate 4.1B) (Magnification, × 400).

A 

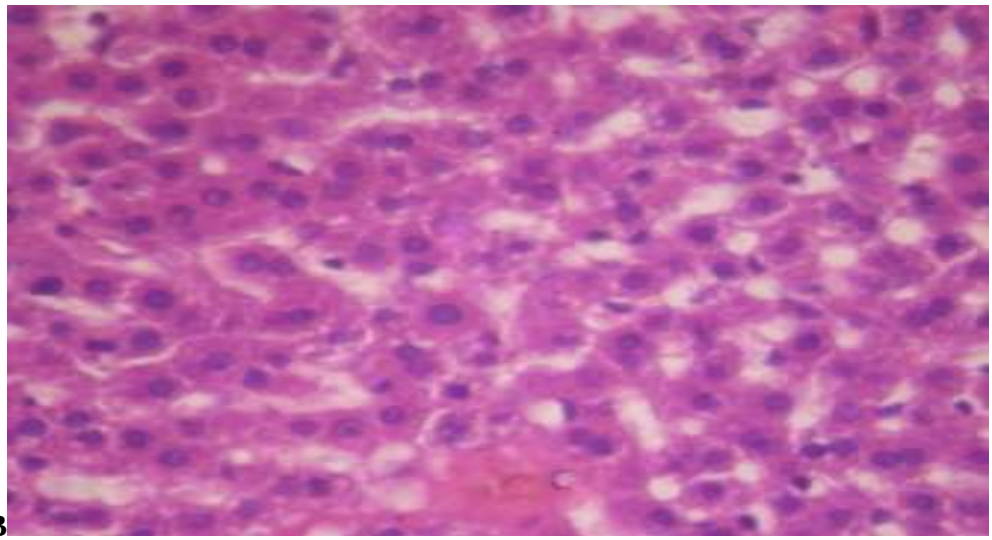
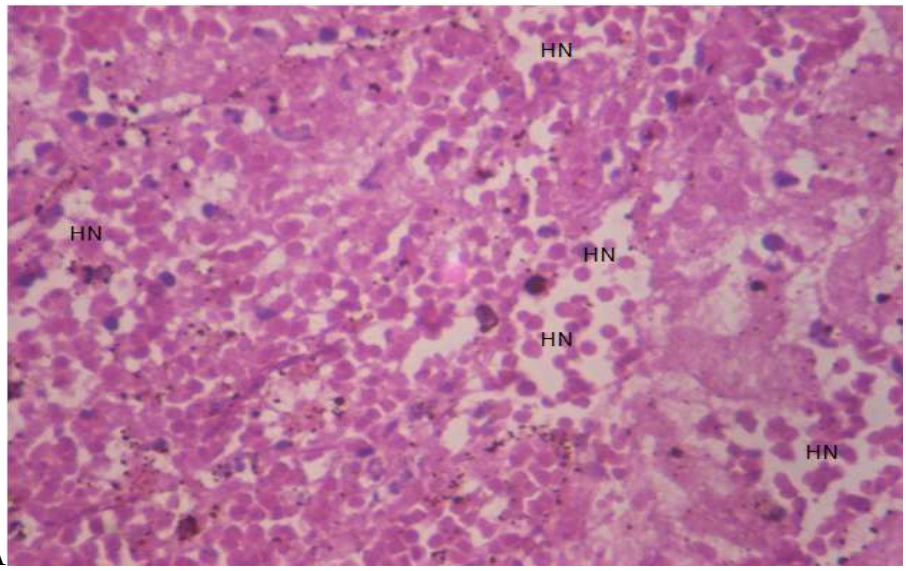
B 

Plate 4.2: A Cross-sectional View of the Rat Liver of a Group Orally Administered 800 mg/kg of *C. bonduc* for 28 Days, showing areas of central hepatic venous congestion (CHVC) and sinusoidal congestion (SC) (Plate 4.2A), compared with normal liver architecture (Plate 4.2B) (Magnification, × 400).

A



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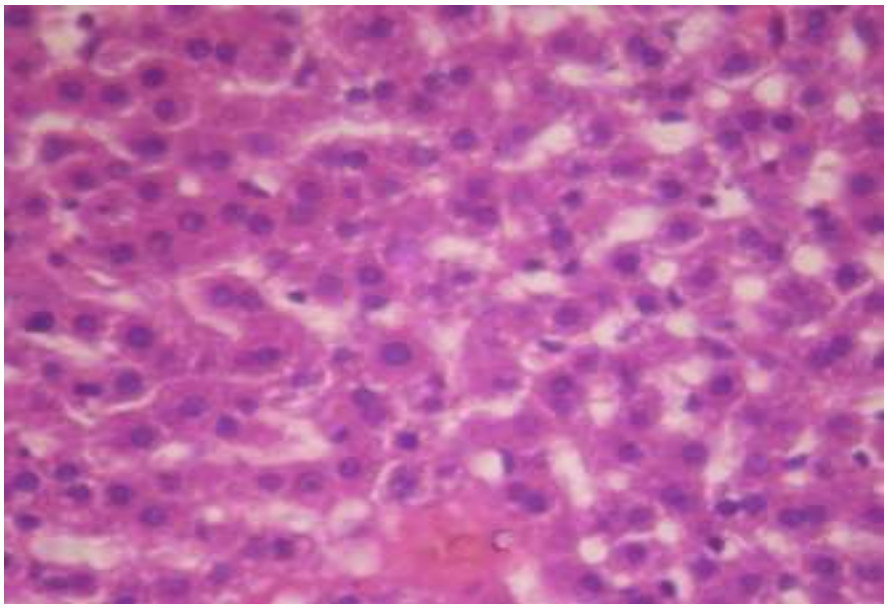


Plate 4.3: A Cross-sectional View of the Rat Liver of a Group Orally Administered 1600 mg/kg of *C. bonduc* for 28 Days, showing areas of hepatic necrosis (HN) (Plate 4.3A), compared with normal liver architecture (Plate 4.3B) (Magnification, × 400).

A

B

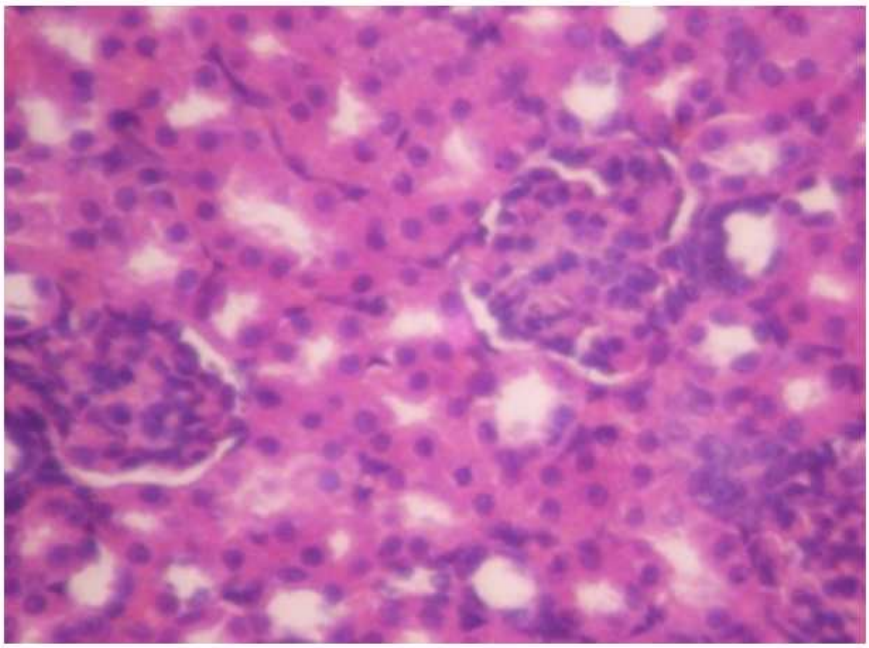


Plate 4.4: A Cross-sectional View of the Rat Kidney of a Group Orally Administered 1600 mg/kg of *C. bonduc* for 28 Days, showing areas of tubular necrosis (TN) and glomerular congestion (GC) (Plate 4.4A), compared with normal liver architecture (Plate 4.4B) (Magnification, × 400).

4.10 Effect of Acute Toxicological Evaluation of the Ethanolic Extract of CB on Food Intake, Body and Relative Organ Weight and Biochemical Parameters

The body weights of all tested groups increased progressively throughout the duration of the experiment (Figure 4.20). An effect of the extract in causing drowsiness in all the treated groups was observed for the first 1 hr after dosing, compared with control. No mortality was recorded for any treated groups throughout the duration of the experiment. There were no significant changes in the relative liver and heart weights of the experimental rats at any dosage however, there were significant decreases in the relative kidney and spleen weights at each extract dosage (Tables 4.9).

There were significant changes in the plasma liver and kidney function makers as well as other biochemical toxicological parameters with significant changes in cholesterol (PC), glucose (PG), triglyceride (PTG), urea (PU), uric acids (PUA), creatinine (PCT) and aspartate aminotransferase activity (AST) while insignificant increase was observed with the activity of alkaline aminotransferase (ALT) and decrease in concentration of total plasma protein (TPP) (Table 4.10).

There were significant changes in the haematological parameters of organ toxicity. There were decrease in WBC counts, PCV and Neutrophil counts while there was significant increase in lymphocyte counts in all extract treated groups (Table 4.11).

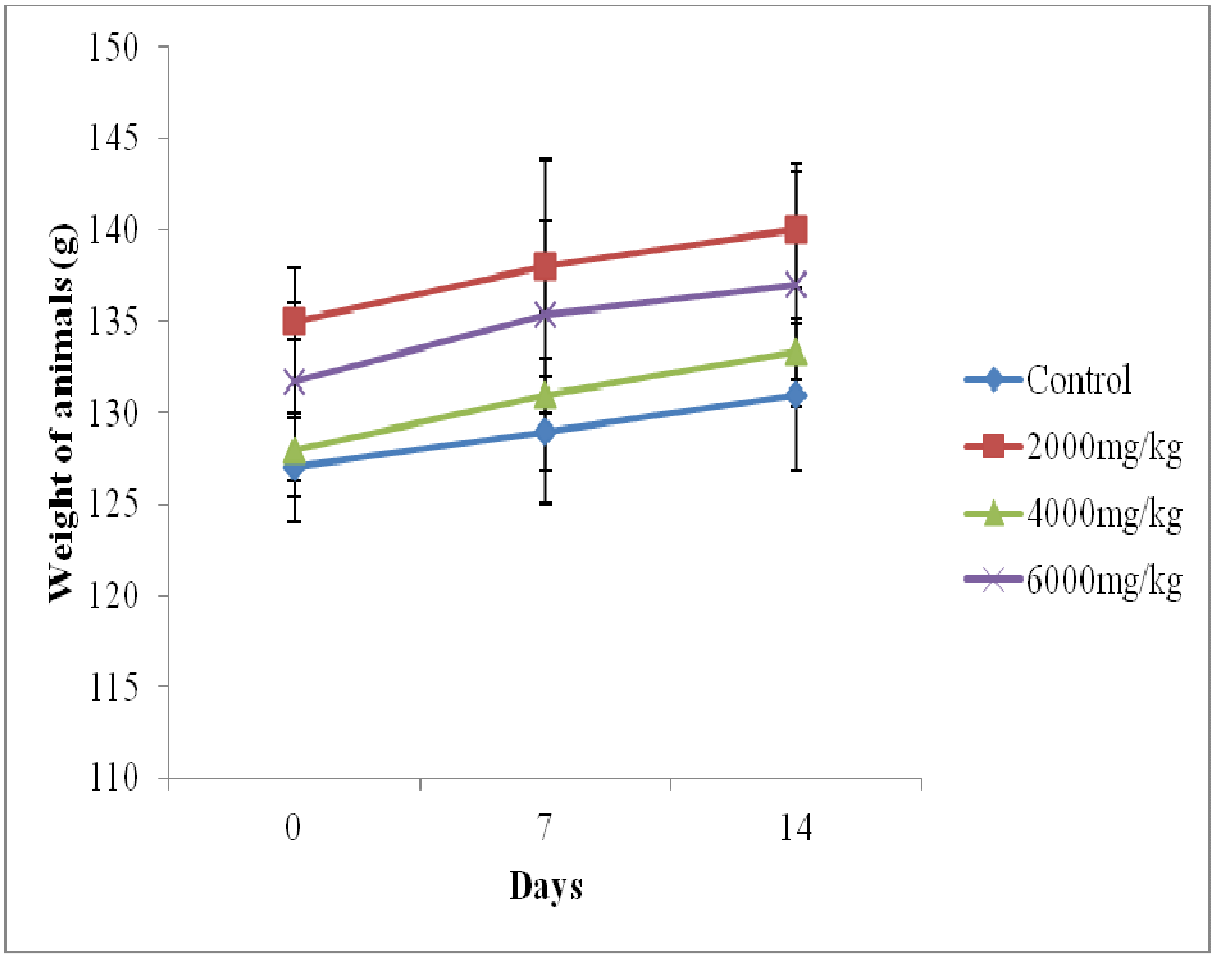


Figure 4.20: Changes in Body Weight of Experimental Animals for Acute Study (See Appendix 46)

Table 4.9: Relative Organ Weights in Acute Toxicological Evaluation of Experimental Animals

Groups	Liver	Heart	Kidney 1	Spleen
Control	0.04±0.0031	0.005±0.0005	0.009±0.0001	0.005±0.0001
2000 mg/kg	0.04±0.0038	0.004±0.0008	0.006±0.0006*	0.003±0.0009*
4000 mg/kg	0.04±0.0178	0.005±0.0009	0.006±0.0015*	0.003±0.0008*
6000 mg/kg	0.05±0.0068	0.005±0.0011	0.007±0.0011	0.002±0.0014*

Values are presented as mean±SEM. Values marked with * are significantly different at $p<0.05$ compared with control.

Table 4.10: Effects of Acute Toxicological Evaluation of the Ethanolic Extract of CB on Biochemical Parameters

Groups/ Biomarkers	Control	2000 mg/kg	400 mg/kg	6000 mg/kg
ALT (U/I)	84.58±6.1	87.11±7.3	93.53±13.1	161.78±64.7
AST (U/I)	11.47±2.4	11.67±2.1	17.89±4.1	119.77±23.5*
PUA (mg/dl)	7.24±1.7	9.04±1.6	14.80±1.7	54.57±28.20*
PU (mg/dl)	144.90±4.7	122.09±3.6	136.85±4.7	461.53±46.8*
PCT (mg/dl)	0.80±0.1	0.62±0.1	0.73±0.0	1.79±0.21*
TP (g/dl)	5.65±0.7	5.43±0.9	4.93±0.1	4.06±0.6
PC (mg/dl)	78.43±5.49	55.69±3.06*	34.90±3.06*	131.65±12.0*
PG (mg/dl)	131.89±1.7	274.17±16.7*	309±58.3*	412.50±52.2*
PTG (mg/dl)	117.11±16.8	100.89±7.4	104.12±5.6	188.20±13.4*

Values are presented as mean ± SEM (n = 5 readings); ALT, AST, PUA, PU, PCT, TP, PC, PG, PTG, represent alanine aminotransferase activity, aspartate aminotransferase activity, uric acid concentration, urea concentration, creatinine concentration, total protein concentration, cholesterol concentration, glucose concentration, triglyceride concentration. Values marked with * are significantly different at $p < 0.05$ compared with control. Control, 2000 mg/kg, 4000 mg/kg and 6000 mg/kg represent groups of rats treated with distill water; rats treated with 2000 mg CB/kg bwt; rats treated with 4000 mg CB/kg bwt and rats treated with 6000 mg CB/kg bwt.

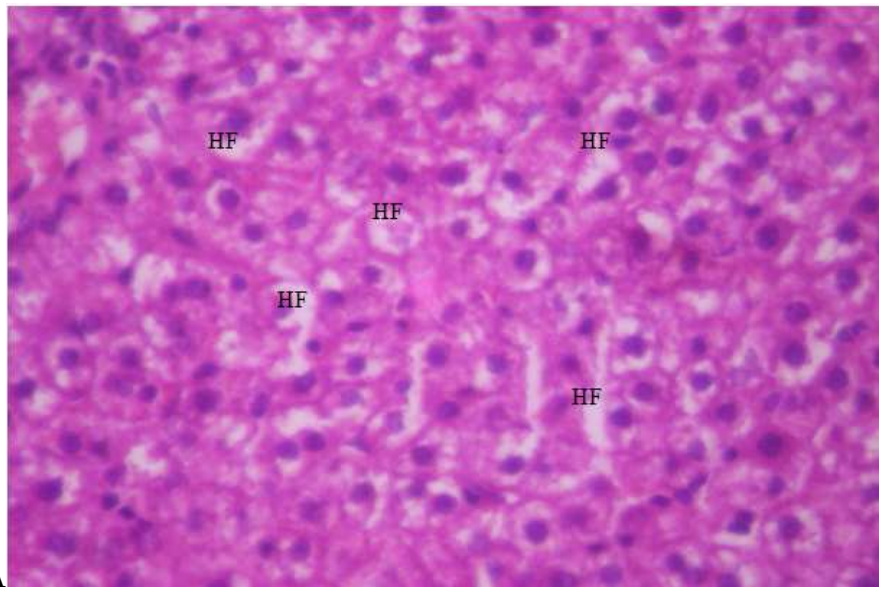
Table 4.11: Effects of Acute Toxicological Ethanolic Extract of CB on Haematological Parameters

Groups	PCV ($\times 10^{12}$/L)	WBC ($\times 10^{12}$/L)	L (%)	N (%)
Control	39.00±1.0	6.90±0.5	45.00±7.6	55.00±7.6
2000 mg/kg	31.33±1.8	4.27±0.8	75.6 7±3.4	22.67±1.8
4000 mg/kg	30.00±6.1	3.87±0.6	75.33±3.2	24.67±3.2
6000 mg/kg	20.33±2.6	5.63±1.2	72.67±1.5	47.67±6.1

Values are presented as mean ± SEM (n = 5 readings). PCV, WBC, % L, % N represent packed cell volume, white blood cell count, percentage lymphocyte count and percentage neutrophil count. Values marked with * are significantly different at $p < 0.05$ compared with control. Control, 2000 mg/kg, 4000 mg/kg and 6000 mg/kg represent groups of rats treated with distill water; rats treated with 2000 mg CB/kg bwt; rats treated with 4000 mg CB/kg bwt and rats treated with 6000 mg CB/kg bwt.

4.11 Acute Toxicological Effect of Ethanolic Extract of CB on Histopathology of the Organs of the Animals

There were no obvious histopathological alterations or remarkable changes in the internal organs of rats in the control group and in all the extract treated groups, except in the liver. The liver showed hepatic fatty changes at the extract dose of 2000 mg/kg bwt and hepatic fatty congestion at extract doses of 4000 and 6000 mg/kg body weight. All the other organs showed no remarkable changes compared with the control (Plates 4.5; 4.6).

A 

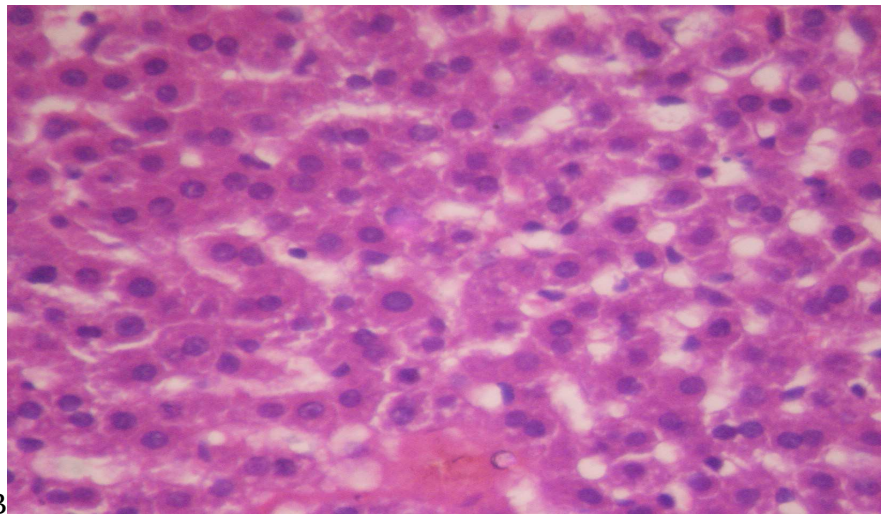
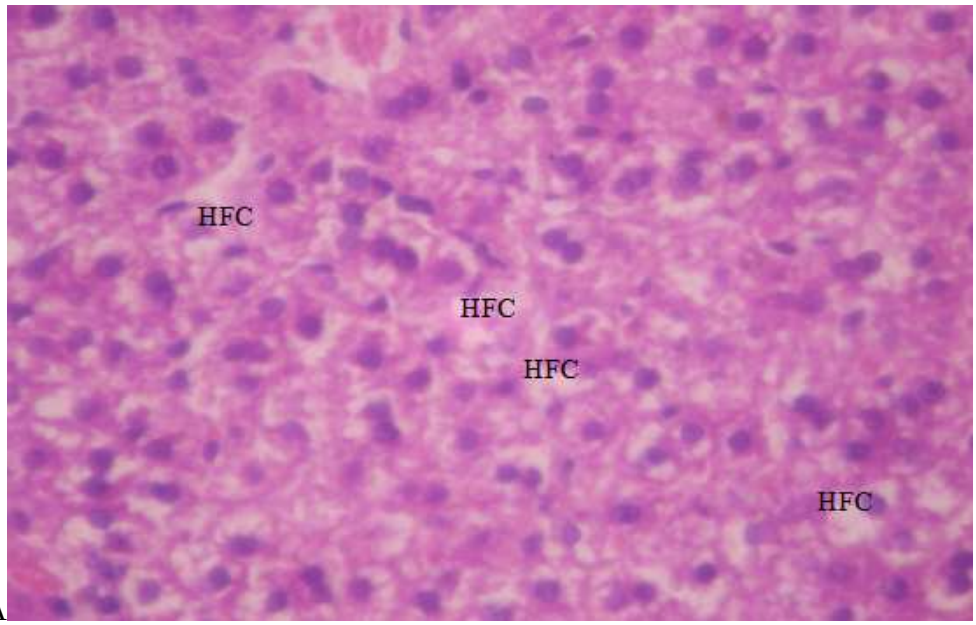
B 

Plate 4.5: A Cross-sectional View of the Rat Liver of a Group Orally Administered 2000 mg/kg of *C. bonduc* (single dose), showing areas of hepatic fatty changes (HF) (Plate 4.5a), compared with normal liver architecture (Plate 4.5b) (Magnification, × 400).

A 

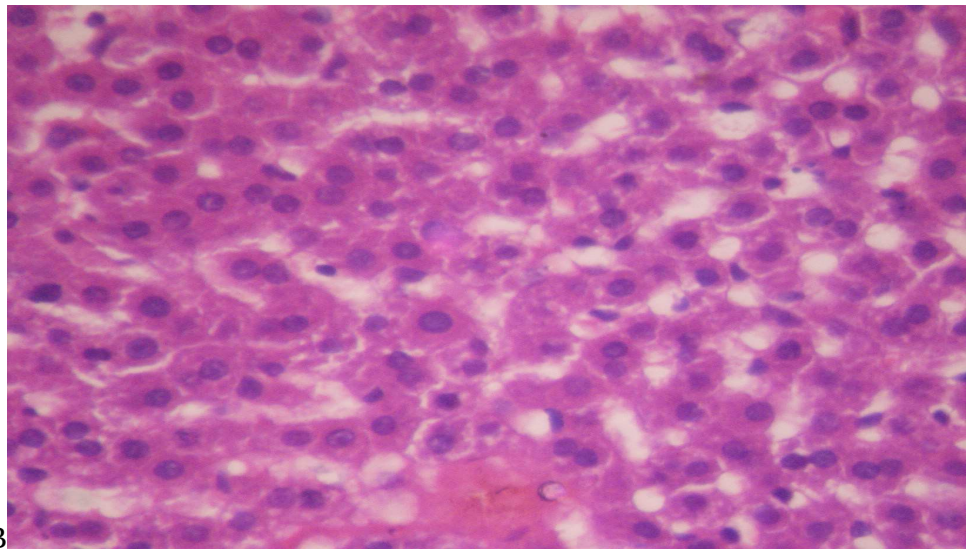
B 

Plate 4.6: A cross-sectional view of the rat liver of a group orally administered 6000 mg/kg of *C. bonduc* (single dose), showing areas of hepatic fatty congestion (HFC) (Plate 4.6A), compared with normal liver architecture (Plate 4.6B) (Magnification, × 400).

4.12 *In vitro* Antimalarial, Selectivity and Cytotoxic Activities of Extracts and Compounds Isolated from *C. bonduc*

The results of the *in vitro* antimalarial and selectivity index determinations of the ethanolic extract, solvent fractions and compounds isolated from *C. bonduc* against the chloroquine sensitive strain FCR-3 of *P. falciparum* and mouse mammary tumor FM3A cells are illustrated in Tables 4.12, 4.13 and 4.14. When compared with standard antimalaria drugs (quinine, mefloquine, pyrimethamine and artemisinin) the petroleum ether and ethyl acetate solvent fractions exhibited moderate antimalarial activities, with IC_{50} values of 18 and 16 µg/ml and selectivity indices of 0.29 and 0.69 respectively (Table 4.12). TCB 9 (6) and TCB 31 exhibited moderate antimalarial activities, with IC_{50} values of 33 µM and 10 µg/ml and selectivity indices of 0.33 and 0.022 respectively. By contrast, TCB 29 exhibited good antimalarial activities with IC_{50} values 4.6 µg/mL and selectivity index of 0.26 (Table 4.13 and 4.14).

The ethanolic extract and the ethyl acetate fraction of *C. bonduc* exhibited moderate antiproliferation activities against mouse mammary tumor FM3A cells, with IC_{50} values of 36 and 11 µg/ml respectively; the petroleum ether fraction had a good antiproliferation activity, with an IC_{50} value of 5.2 µg/ml (Table 4.12). The characterised pure compounds, TCB 1, 9, 11, 15 and 16 exhibited various antiproliferation activities, with IC_{50} values of 8.8, 11, 11, 5.4, 0.56 µM respectively (Table 4.13). In addition, the uncharacterised compounds TCB 28, 29, 30, 31, 33 and 38 also exhibited cytotoxic activities, with IC_{50} values of 1.9, 1.5, 1.2, 1.3, 0.22, 2.2, 3.4 µg/ml respectively (Table 4.14). In comparing the antiproliferation activities of samples against *plasmodium falciparum* (Chloroquine sensitive strain (FCR-3) and mammalian tumor FM3A cells, the cytotoxicity selectivities of samples were established. TCB 9 and 11 have moderate cytotoxic activities with poor selectivity indices of 0.33 and 0.41 respectively (Table 4.13).

Table 4.12: Antimalarial and Selectivity Assay Results for Ethanolic Extract and Solvent Fractions of *C. bonduc*

Samples	[a]IC_{50} (µg/mL)	[b]IC_{50} (µg/mL)	[c]Selectivity
Et. ext.	> 92 (51 %)*	[d]36	0.39
Pet. Ether	[d]18	[#]5.2	0.29
Ethyl ac.	[d]16	[d]11	0.69
Buthanol	> 90 (76 %)	> 90 (80 %)	1
Water	> 62 (82 %)	> 62 (68 %)	1

* > 92 (51 %) means IC_{50} is greater than 92 µg/ml: there is 51 % growth at 92 µg/ml. "[a]" represents chloroquine sensitive strain (FCR-3) of *P. falciparum*, "[b]" represensts mouse mammary tumor FM3A cells representing a model of host, "[c]" represent selective toxicity = IC_{50} value for FM3A/IC_{50} for *P. falciparum*. "[#] and [d]" represent good and moderate *P. falciparum* and mammalian cell antiproliferation activity. Et. ext., Pet. and Ethyl ac. represent ethanolic extract, petroleum ether fraction and ethyl acetate fraction of *C. bonduc*.

Table 4.13: Antimalarial and Selectivity Assay Results of Structurally Characterised Compounds from *C. bonduc*

Samples	[a]IC_{50} (µM)	[b]IC_{50} (µM)	[c]Selectivity
TCB 1 (compound 1)	> 27 (64%)*	[d]8.8	0.33
TCB 2 (compound 2)	> 10.2 (89%)	> 10.2 (100 %)	1
TCB 3 (compound 3)	> 14 (81%)	> 14 (96 %)	1
TCB 5 (compound 4)	> 13 (96%)	> 13 (100 %)	1
TCB 6 (compound 5)	> 17 (98%)	> 17 (99 %)	1
TCB 9 (compound 6)	[d]33	[d]11	0.33
TCB 11 (compound 7)	> 27 (61%)	[d]11	0.41
TCB 14 (compound 8)	> 1.8 (76%)	> 1.8 (91%)	1
TCB 15 (compound 9)	> 25 (58%)	[#]5.4	0.2
TCB 16 (compound 10)	> 9.8 (99%)	[##]0.56	0.06
TCB 22 (compound 12)	> 5.5 (100%)	627.0	0.35
TCB 27 (compound 13)	> 9.7 (100%)	> 9.7 (88 %)	1
TCB 40 (compound 14)	> 5.7 (95%)	> 5.7 (97 %)	1
Quinine	[##]0.2	100	500
Pyrimethamine	[##]0.001	[#]0.1	100
Mefloquine	[##]0.032	[#]2.8	88
Artemisin	[##]0.01	[d]9.0	900

* > 27 (64%) means IC_{50} is greater than 2.7×10^{-5} µg/mL: there is 64 % growth at 27 µM. "[a]" represents chloroquine sensitive strain (FCR-3) of *P. falciparum*, "[b]" represents mouse mammary tumor FM3A cells representing a model of host, "[c]" represents selective toxicity = IC_{50} value for FM3A/IC_{50} for *P. falciparum*. "[##], [#], [d] and [e]" represent very good, good, moderate and weak antiproliferative activity.

Table 4.14: Antimalarial and Selectivity Assay Results of Uncharacterised Compounds from *C. bonduc*

Samples	[a]IC_{50} (µg/ml)	[b]IC_{50} (µg/ml)	[c]Selectivity
TCB 24	> 7.85 (95%)*	> 7.85 (100 %)	1
TCB 28	> 4.1 (96%)	[#]1.5	0.37
TCB 29	[#]4.6	[#]1.2	0.26
TCB 30	> 4.9 (97%)	[#]1.3	0.27
TCB 31	[d]10	[##]0.22	0.022
TCB 32	> 5.7 (91%)	> 5.7 (60%)	1
TCB 33	> 9.6 (70%)	[#]4.2	0.44
TCB 34	> 6.0 (100%)	> 6.0 (99 %)	1
TCB 35	> 9.6 (100%)	> 9.6 (100 %)	1
TCB 36	> 4.0 (95%)	> 4.0 (100 %)	1
TCB 38	> 8.2 (100%)	[#]3.4	0.41
TCB 45	> 5.8 (62%)	> 5.8 (82 %)	1
TCB 46	> 9.5 (89%)	> 9.5 (93 %)	1

"> 7.85 (95%)" means IC_{50} is greater than 7.85 µg/mL: there was 95 % growth at 7.85 µg/mL, "[a]" represents chloroquine sensitive strain (FCR-3) of *P. falciparum*, "[b]" represents mouse mammary tumor FM3A cells representing a model of host, "[c]" represents Selective toxicity = IC_{50} value for FM3A/IC_{50} for *P. falciparum*. "[##]", [#], [d] and [e]" represent very good, good, moderate and weak antiproliferative activity.

4.13 Antibacterial Activity of *Caesalpinia bonduc* Samples Against *Candida albicans*

All tested samples isolated from *C. bonduc* had no inhibitory activity against the growth of *Candida albicans* when compared with the standard antifungal drug, miconazole nitrate, as the positive control (Tables 4.15 and 4.16).

Table 4.15: Anti-fungal Pre-test Assessment Results for Ethanolic Extract and Solvent Fractions of *C. bonduc*

Samples	Concentration (µg/ml)	*Candida albicans* (I %)
75 % Ethanol	10	1.92
Petroleum ether	10	0.027
Ethyl acetate	10	2.45
Buthanol	10	0.90
Water	10	1.81
MICO	10	97.91

MICO - antibacterial control - miconazole nitrate.

Table 4.16: Anti-fungal Pre-test Assessment for Pure Compounds from *C. bonduc*

Samples	Concentration (µg/ml)	*Candida albicans* (I %)
TCB 1	10	15.46
TCB 2	10	3.61
TCB 3	10	- 12.32
TCB 5	10	4.56
TCB 6	10	- 9.04
TCB 9	10	-12.01
TCB 11	10	- 2.27
TCB 14	10	0.34
TCB 15	10	- 2.70
TCB 16	10	- 1.31
TCB 22	10	4.615
TCB 24	10	- 4.54
TCB 27	10	- 8.64
TCB 28	10	5.78
TCB 29	10	5.89
TCB 30	10	6.09
TCB 31	10	3.00
TCB 32	10	7.90
TCB 33	10	4.00
TCB 34	10	10.6
TCB 35	10	- 4.9
TCB 36	10	-5.00
TCB 38	10	3.00
TCB 40	10	4.90
TCB 45	10	-5.80
TCB 46	10	0.90
Mico (Positive standard)	10	97.91

Mico - miconazole nitrate

4.14 Cytotoxic activities of *Caesalpinia bonduc* samples against HeLa and BGC - 823 cells

The ethanolic extract, solvent fractions and all compounds isolated from *C. bonduc* were studied for cytotoxicity against the cancer cell lines HeLa and BGC - 823. Tables 4.17, 4.18, 4.19, 4.20 and 4.21 summarise these results. The results reveal that several of the compounds and solvent fractions are active against the two cancer cell lines. The petroleum ether, water and acetyl acetate fractions, as well as the third, fourth, fifth and eighth fractions from the first column separation, exhibited moderate activities against the HeLa cell lines, with percentage inhibitory concentrations (I %) of 84.55, 80.39, 59.00, 82.00, 60.67, 55.78 and 60.34 (Table 4.17) and IC_{50} values of 32.00, 30.14, 35.80, 19.50, 32.04, 32.00 and 30.78 µg/mL respectively (Table 4.18).

The result of the pre-evaluation cytotoxic test of isolated compounds showed that TCB 1 (compound 1), TCB 9 (compound 6), TCB 11 (compound 7), TCB 15 (compound 9), TCB 16 (compound 10) and TCB 30 have percentage inhibitory activity (I %) values higher than 50 % against HeLa cell lines (Table 4.19) from which their IC_{50} values were calculated. By contrast, only TCB 11 (compound 7) and TCB 45 have inhibitory activity (I %) values with more than 50 % against BGC - 823 cell lines from which their IC_{50} values were also calculated (Table 4.19).

TCB 1, 9, 11, 15, 16 and 30 exhibited high to very high cytotoxic activities, with IC_{50} values of 5.88, 8.69, 5.91, 5.91, 0.81 and 8.79 µg/ml against HeLa cancer cells (Table 4.21), TCB 11 and 45 exhibited good cytotoxic activities of 6.45 and 5.55 µg/ml against BGC - 823 cancer cells (Table 4.20).

Table 4.17: Cytotoxicity Pre - test Results for Ethanolic Extract, Solvent Fractions and First Column Fractions of the Petroleum Ether and Ethyl Acetate Fractions of *C. bonduc* on HeLa Cells

Samples	Concentration (μg/ml)	HeLa cells (I %)
75 % Et. ext.	40	10.05
Pet. ether	40	84.55
Ethyl ac.	40	59.00
Butanol	40	10.07
Water	40	80.00
C_3	40	82.00
C_4	40	60.67
C_5	40	55.78
C_8	40	60.34
Taxol	40	61.72

I (%) is percentage inhibition; C_3, C_4, C_5 and C_8 were third, fourth, fifth, and eighth fractions of the first column separation respectively. Et. ext., Pet. ether, Ethyl ac. represent ethanolic extract, petroleum ether fraction and ethyl acetate fractionof *C. bonduc* respectively.

Table 4.18: Cytotoxicity Evaluation for Ethanolic Extract, Solvent Fractions and First Column Fractions of the Petroleum Ether and Ethyl AcetateFractions of *C. bonduc* on HeLa Cells

Samples	I (%) at Conc. (40 μg/ml)	I (%) at Conc. (20 μg/ml)	I (%) at Conc. (10 μg/ml)	I (%) at Conc. (5 μg/ml)	IC_{50} (μg/ml)
Pet. Ether	84.55	22.73	7.24	9.194	[d]32.00
Water	80.39	6.00	-2.68	-1.54	[d] 30.14
Ethyl ac	59.00	21.74	2.78	2.00	35.80
C_3	82.00	50.87	27.25	12.194	[d] 19.50
C_4	60.67	27.35	9.35	4.45	[d] 32.04
C_5	55.78	20.04	12.04	0.86	[d] 32.00
C_8	60.34	33.16	18.67	6.79	[d] 30.78

I (%) is percentage inhibition; C_3, C_4, C_5 and C_8 are first column fraction 3, 4, 5 and 8 respectively. [d]denotes moderate cytotoxicity activities of samples respectively.

Table 4.19: Cytotoxicity Pre - test Results of Compounds Isolated from *C. bonduc*

Samples	Concentration (μg/ml)	BGC - 823 cells (I %)	HeLa cells (I %)
TCB 1 (compound 1)	10	44.94	69.20
TCB 2 (compound 2)	10	12.91	-26.17
TCB 3 (compound 3)	10	6.57	-31.02
TCB 5 (compound 4)	10	10.88	-6.60
TCB 6 (compound 5)	10	- 0.12	-12.49
TCB 9 (compound 6)	10	48.15	54.91
TCB 11 (compound 7)	10	70	69.62
TCB 14 (compound 8)	10	14.02	- 4.78
TCB 15 (compound 9)	10	9.20	70.68
TCB 16 (compound 10)	10	46.89	72.30
TCB 22 (compound 12)	10	6.010	- 6.29
TCB 24	10	15.42	- 24.80
TCB 27 (compound 13)	10	8.73	-8.13
TCB 28	10	23.23	23.33
TCB 29	10	42.16	44.13
TCB 30	10	16.17	53.71
TCB 31	10	8.13	-16.06
TCB 32	10	11.67	-14.67
TCB 33	10	9.40	18.11
TCB 34	10	3.32	-22.1
TCB 35	10	15.05	-15.75
TCB 36	10	18.97	-15.95
TCB 38	10	22.14	-11.96
TCB 40 (compound 14)	10	14.98	-13.38
TCB 45	10	78.03	2.99
TCB 46	10	5.43	16.74
Taxol	10	76.69	61.72

Table 4.20: Cytotoxicity Evaluation of Pure Compounds of *C. bonduc* Using BGC-823 Cells

Samples	I (%) at Conc. (10 μg/ml)	I (%) at Conc. (2 μg/ml)	I (%) at Conc. (0.4 μg/ml)	I (%) at Conc. (0.08 μg/ml)	IC_{50} (μg/ml)
TCB 1 (1)	44.94	2.20	- 4.73	-8.55	NC
TCB 9 (6)	48.15	0.66	- 1.79	- 1.81	NC
TCB 11 (7)	70.95	- 5.79	- 9.61	- 10.07	$^{\#}$6.45
TCB 16 (10)	35.47	20.76	- 3.05	- 7.34	NC
TCB 24	15.42	5.29	6.11	2.66	NC
TCB 30	16.17	5.10	- 4.69	- 7.28	NC
TCB 34	3.32	- 6.21	- 7.33	- 9.02	NC
TCB 40 (14)	14.04	- 1.22	- 6.32	-8.41	NC
TCB 45	78.03	1.39	-5.44	- 7.92	$^{\#}$5.55
Taxol	76.69	76.22	73.18	26.20	$^{\#\#}$0.15

NC – Not calculated because I_H (inhibition above 50 %) could not be determined. $^{\#\#}$ and $^{\#}$ signify very good and good cytotoxicity activity.

Table 4.21: Cytotoxicity Evaluation of Pure Compounds Isolated from of *C. bonduc* Using HeLa cell

Samples	I (%) at Conc. (10 µg/ml)	I (%) at Conc. (2 µg/ml)	I (%) at Conc. (0.4 µg/ml)	I (%) at Conc. (0.08 µg/ml)	IC_{50} (µg/ml)
TCB 1 (1)	69.20	10.97	- 4.77	- 6.38	#5.88
TCB 9 (6)	54.91	-1.50	- 9.27	- 12.48	#8.69
TCB 11 (7)	69.62	9.63	6.37	- 6.50	#5.91
TCB 15 (9)	70.68	18.73	9.32	- 5.52	#5.27
TCB 16 (10)	69.43	58.99	42.89	- 7.34	##0.81
TCB 30	53.71	7.40	- 6.47	- 4.32	#8.79
Taxol	61.72	60.36	52.68	- 14.34	##1.13

##, # and denote very good and good cytotoxicity activity.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Phytochemical Investigation of *C. bonduc*

The phytochemical assessment of the young twigs and leaves of *C. bonduc* revealed the presence of major classes of phytochemicals, except phlobatannins. The presence of these phytochemicals in *C. bonduc* could be responsible for the various reported biological activities of the plant. A bioassay-guided fractionation of the petroleum ether and ethyl acetate fractions of the ethanolic extract of *C. bonduc* led to the isolation of two new diterpenoids, 12 known compounds and 13 unknown pure samples.

From previous reports, TCB 1 (bonducellin) was isolated from *C. bonduc* (Purushothaman *et al.*, 1982); TCB 14 (Daucosterol) was isolated from *C. millettii* (Chen and Yang, 2008); and TCB 15 (Luteolin) was isolated from *C. gilliesii* (Suarez *et al.*, 1984). The following compounds isolated from C. sappan: TCB 2 (1-octacosanol) (Yadava and Nigam, 1987); TCB 3 ((+)-Syringaresinol) (Shu *et al.*, 2007); TCB 9 (4,4'-dihydroxy-2'-methoxy-chalcone) (Liu *et al.*, 2009) and TCB 11 (7,4'-dihydroxy-3,11-dehydrohomoisoflavanone) (Namikoshi *et al.*, 1987a). These reports are corroborated by the phytochemical study of the young twigs and leaves of *C. bonduc* carried out in the present work.

5.2 Antioxidant Evaluation of *C. bonduc* Extracts

The production of free radicals has been associated with various physiological and pathological events such as inflammation, aging, mutagenicity and carcinogenicity (Ogunlana and Ogunlana, 2008). Antioxidants are vital substances with the ability to protect the body from the damage caused by free radical-induced oxidative stress (Ozsoy *et al.*, 2008). Natural antioxidants

present in medicinal and dietary plants have been implicated in the prevention of oxidative damage (Silva *et al.,* 2005). Hence, the antioxidant activity of the ethanolic extract of *C. bonduc* was investigated by measuring the activities of the antioxidant enzymes (catalase and peroxidases). The extent of lipid peroxidation was gauged by measuring the concentration of TBARS in rats treated with different doses of the extract. There was graded increase in catalase and peroxidase activities and decrease in concentration of TBARS in treated rats in comparison with the controls. This increase in antioxidant enzymes was significant in rats treated with 150 and 200 mg of extract/kg bwt in comparison with rats treated with 10 mg of vitamin C/kg bwt. A significant antioxidant activity against Ehrlich ascites carcinoma (EAC) in mice has been reported for the methanolic extract of the leaves of *C. bonduc* (Gupta *et al*., 2004). Significant i*n vitro* antioxidant activities have been reported for protosappanin A, protosappanin B and brazilein (Jun *et al*., 2008). These are phenolic compounds isolated from *C. sappan*.

The present study and that of Gaur *et al*. (2008) have revealed the presence of alkaloids, flavonoids, glycosides, saponin, tannins and terpenoids in *C. bonduc* by phytochemical analysis. Flavonoids are phenolic compounds with potent metal chelating and free radical scavenging activities (Middleton *et al.,* 2000). Along with other natural antioxidant, vitamins and enzymes, they provide protection against free radicals by acting as antioxidants involved in scavenging reactive oxygen species (Varalakshmi *et al.,* 2011). Previous studies have reported the presence of the triterpenoid lupeol in *C. bonduc*. Lupeol protects cells and tissues from oxidative stress by increasing the activity of catalase (Liby *et al.,* 2007).

The *in vitro* antioxidant activity of the methanolic extract of the root of *C. digyna* was highly comparable with reference antioxidants (ascorbic acid and rutin) (Srinivasan *et al*., 2007). This correlated with the estimate of the total phenolic compounds in *C. digyna*. Polyphenolic

compounds with catechol structures are oxidised in neutral and alkaline pH environments and form dimerised products which possess higher superoxide radical scavenging activities and iron chelating properties (Spencer, 2003). The antioxidant activity of the extracts of *C. bonduc* seeds, measured by their DPPH, hydroxyl, nitric oxide and super oxide radical scavenging activities, has a significant linear correlation with their total phenolic content (Shukla *et al*., 2009). The high antioxidant activity exhibited by the extract of *C. bonduc* might be due to the presence of phenolic components such as flavonoids.

Phenols are very important plant constituents because of their radical scavenging ability due to the presence of hydroxyl groups (Hatano *et al*., 1989). The phenolic compounds may contribute directly to antioxidative action (Duh *et al*., 1999). The phenolic compounds of the extract of *C. bonduc* leaves and twigs could be major contributors to its antioxidant activity.

5.3 Toxicological Evaluation of *C. bonduc* Extracts

The ethanolic extract of the leaves and young twigs of *C. bonduc* at the 200 mg/kg body weight dose did not elicit deleterious effects during 28 days treatment in rats while higher treatment doses of 400 to 1600 mg/kg body weight were toxic to experimental animals. The behavioural profile of all the tested animals revealed that the animals were alert and responded to pain and touch. The animals showed no signs of depression or restlessness. Feron *et al*. (1973) reported that in sub-acute toxicity experiments, the relative organ weight (the ratio of the organ to the whole body weight) is a useful index of toxicity. Monitoring the body weight during treatment provides an index of the general health status of the animals; such information may be important for gauging their health (Sharma *et al.*, 2009). There was a progressive increase in the body weight of all animals throughout the duration of the experiment. At the highest treatment dose,

there was a relative increase in the organ weight compared to the control. The ethanolic extract of *C. bonduc* did not induce toxic changes in the liver and other organs at a dose of 200 mg/kg body weight. However, it induced toxic effects on some organs at higher doses. These results are in agreement with the results of Gupta *et al*. (2004) and Kumar *et al*. (2005). These authors reported that there was no significant change in the organ weights of animals treated with 100 and 200 mg/kg body weight; but there was a significant increase in organ weight at a dose of 400 mg/kg body weight after 14 days of treatment. The liver, being the detoxification organ of mammals, and the kidney, being the most important excretory organ in the body, might be susceptible to the toxic effects of extracts of *C. bonduc* at higher doses. This has been supported by the report of Kumar *et al*. (2005), who reported the associated toxicity of anticancer agents and plants rich in flavonoids on liver and kidney (Kumar *et al.,* 2005).

The 28 days toxicological assessment of the ethanolic extract of *C. bonduc* indicates that ALT, AST, TB, TP, DB, IB, PTG, PC and PCT remain unchanged at an extract dose of 200 mg/kg body weight. The plasma enzymes (ALT and AST) and the other biochemical parameters mentioned above were significantly increased at the higher treatment doses of 400, 800 and 1600 mg/kg body weight. The depletion in the total plasma protein level has also been observed in the present assessment. This may be due to impaired protein synthesis in the damaged liver or to the altered nutritional status of the animals (Sharma *et al.,* 2009). It has been reported that the synthesis of plasma proteins in the liver was quantitatively and qualitatively affected during liver damage (Kumar *et al.,* 2005). The decrease in plasma albumin or in the total plasma protein indicated hepatocellular dysfunction or liver disease (Kumar *et al.,* 2005). Elevated levels of ALT and AST may be due to pathological changes such as necrosis of hepatocytes, which causes an increase in the permeability of the cell membranes, resulting in the release of aminotransferases

into the blood stream (Ali *et al.*, 2008). ALT has been reported to be a marker with a high specificity for acute hepatocellular injury (Friedman *et al.*, 1996).

A high level of plasma cholesterol has been reported in obstructive jaundice or in chronic hepatitis (Albrink *et al.*, 1950). There was a significant increase in the plasma cholesterol of animals given *C. bonduc* extract dosages of 800 and 1600 mg/kg body weight. The kidney eliminates the waste products of metabolism from the body. In renal failure, there is an increase in metabolic waste products, especially nitrogenous substances like plasma urea and uric acid. An elevated plasma urea level has been linked to elevated non-protein nitrogen in diseases associated with nephrotoxicity (Varley *et al.*, 1980). There was no observable changes in plasma urea concentration at an extract dose of 200 mg/kg body weight in rats compared with the control. The plasma urea concentration was significantly increased at higher doses: 400, 800 and 1600 mg/kg body weight.

The occurrence high levels of plasma triglycerides have been reported as a useful biomarker in the prediction of renal dysfunction (Muntner *et al.*, 2000). There was a significant increase in plasma triglyceride in the rats at extract dosages of 800 and 1600 mg/kg body weight. The increase in creatinine concentration, the least variable nitrogenous constituent of the blood, was found to be insignificant. Nevertheless, increased plasma creatinine has been reported in renal injury subsequent to trauma or anuria, in traumatic injuries to the muscle and in muscular dystrophy (Srisawat *et. al.*, 2010). The findings in this work corroborate the report of Gupta *et al*. (2004) on the 14 days interperitoneal toxicity of the methanolic extract of *C. bonduc* with a significant increase in plasma urea, ALT and AST of the experimental animals.

Blood is a sensitive index of the physiological changes in an animal in response to any environmental pollutant, and it has been documented that toxicant or potentially toxic substances

induce conspicuous and significant changes in the haematological parameters (Jain *et al.*, 2009). In the 28 days toxicological assessment, the white blood cell count and the differential counts (lymphocyte and neutrophil counts) were not significantly altered at a dosage of 200 mg/kg body weight. But there were significant decreases in the neutrophil and white blood cell counts and an increase in the lymphocyte count at extract dosages of 400, 800 and 1600 mg/kg body weight. However, the PCV value did not show any dose graded response. The results of the findings were in agreement with those of Sagar and Vidyasagar (2010b) and Gupta *et al*. (2004) on the effects of ethyl acetate and methanolic extracts of *C. bonduc* on treated animals.

Kramp *et al*. (1974) opined that functional studies in toxicology should be coupled with the appropriate histological studies because appropriate morphological studies are useful, especially during the anatomical localization of the action of a toxin. Based on this, a histological study of the effect of ethanolic extract of *C. bonduc* was conducted. It was observed that the extract (at a dose of 200 mg/kg body weight) and the control groups caused no histopathological alterations in the cellular architectures of the liver, heart, kidney and spleen. However, at higher extract doses (400, 800 and 1600 mg/kg body weight), there were noticeable histopathological alterations in the cellular architectures of the liver and kidney. The liver, at the higher doses of 400 mg/kg and above, showed hepatic degeneration due to the lipid-filled hepatocytes, sinusoidal congestion, fatty congestion, aggregation of dead cells, inflammatory cells and amorphous debris (fatty necrosis) (Plate 4.1 to 4.3). At these higher doses (400, 800 and 1600 mg/kg body weight), the kidney showed gross alterations in the cellular architecture, such as necrosis and congestion (Plate 4.4). The above histopathological damages at the increased extract doses are probably responsible for the alterations in the biochemical and haematological markers of the liver and kidney functions. These are markers of induced organ toxicity or injury. This finding corroborates the finding of Sagar and Vidyasagar (2010b) who observed slight changes in cellular architecture of the

livers of animals treated with 250 mg/kg body weight of an ethyl acetate extract of *C. bonduc.* On the other hand, the methanolic extract of *C. bonduc* seeds orally administered to mice at a dosage of 400 mg/kg body weight for 28 days has been reported to be non-toxic to the experimental animals (Pillaia and Suresh, 2011).

In the recovery groups (200R, 400R, 800R and 1600R mg/kg body weight), no significant changes were observed in the biochemical, haematological and histopathological assessment of actual tested and recovery groups. The induceable alterations in the markers of toxicity caused by the extract on rats might be irreversible two weeks after the end of dosing.

In the investigation on the acute toxicological effects of *C. bonduc* in albino Wistar rats, there was no mortality in the experimental animals at all the extract treatment doses. However, there were significant alterations in the haematological and biochemical markers of toxicity at all extract doses. These are decreases in white blood cell and neutrophil counts, increases in lymphocyte counts and in cholesterol, glucose, uric acid, triglyceride, urea and creatinine concentrations and in alanine aminotransferase levels. There was also induced cellular damage to the liver in all extract administered rats in all treatment groups (Plate 4.5 and 4.6). Although the LD_{50} of the *C. bonduc* extract at all the tested doses could not be determined from this study, the markers of toxicity showed that *C. bonduc* was toxic to all experimental animals at all tested doses.

5.4 Antimalarial and Antiproliferation Activities of *C. bonduc*

Malaria is a globally recognized serious public health issue, mainly in the tropical and sub-tropical regions of the world. *Plasmodium falciparum*, the most widespread etiological agent of human malaria, is becoming increasingly resistant to conventional antimalarial drugs. This increased resistance of malarial parasite to exsiting drugs has presented a major obstacle to

antimalarial chemotherapy. The *in vitro* bioassay study of the ethanolic extract and solvent fractions of *C. bonduc* revealed its antimalarial and cytotoxic activities. The petroleum ether and ethyl acetate fractions had moderate antimalarial activities with IC_{50} values of 18 and 16 µg/ml respectively, compared with the crude fraction and other solvent fractions that had no antimalarial activities. However, a poor selectivity was observed in the antiproliferation activities of the petroleum ether and the ethyl acetate fractions; with selectivity indices were 0.29 and 0.69 respectively. Low selectivity indices of samples have been reported as an indication of normal cellular damage (Wright, 2010). A bioassay guided fractionation of *C. bonduc* led to the isolation of antimalarial compounds with various activities. TCB 9 (4,4'-dihydroxy-2'-methoxy-chalcone), TCB 29 and 31, were compounds isolated from *C. bonduc* with considerable antimalarial activity but with low selectivity indices.

The *in vitro* antiplasmodial activities of the flavonoids, exiguaflavanone A and B, from *Artemisia indica* have been reported (Chanphen *et al.*, 1998). The *in vitro* antimalarial activity of (−)-*cis*-3-acetoxy-4′,5,7-trihydroxyflavanone, isolated from *Siparuna andina* has also been reported (Jenett-Siems *et al.*, 2000). The antimalarial activities of 6-Hydroxyluteolin-7-*O*-(1″-α-rhamnoside) against K1 and NF54 strains of *P. falciparum,* acacetin against poW and Dd2 strains of *P. falciparum* (Bringmann *et al.*, 2000) and calycosin and genistein, first isoflavones to possess antiplasmodial activity, against poW and Dd2 strains of *P. falciparum* **(**Kaur *et al.*, 2009**)** have been reported. Elford *et al.* (1987) demonstrated that *in vitro* the methoxylated flavonones, artemetin and casticin, act synergistically with artemisinin against *P. falciparum*. Although, the exact antiplasmodial mechanism of action of flavonoids is unknown, they have been shown to inhibit the influx of L-glutamine and myoinositol into infected erythrocytes (Elford, 1986). It has also been speculated that their mode of action is linked to their unusual antioxidant pathway. The

ability of flavonoids to form reactive oxygen species has also been linked to their antimalarial activities (Iwu *et al*., 1986). A lack of defence mechanisms against oxidative stress in *P. falciparum* makes the parasite susceptible to drugs with the ability of generating reactive oxygen species (Ribeiro *et al*., 1997).

Structure-activity relationship analyses of the antimalarial flavonoids isolated in this investigation, together with those of Kim *et al*. (2004), suggest that the introduction of a hydroxyl group at the 7 position and *O*-methylation at the 5 position of the A ring might be responsible for the antimalarial activity of methoxy-chalcone.

5.5 Antifungal Evaluation of the Extracts of *C. bonduc*

There was no inhibitory activity against the growth of *Candida albicans* for all the tested samples. Young twigs and leaves of *C. bonduc* obtained from Nigeria may have no inhibitory activity against *C. albicans*. This corroborates the findings of Woldemichael *et al*. (2003), which reported that compounds isolated from *C. paraguariensis* have no inhibitory activity against *C. albicans*. However, a good inhibitory activity has been reported for α-(2-hydroxy-2-methylpropyl)-ω-[2-hydroxy-3-methylbut-2-en-1-yl] polymethylene isolated from the leaves of *C. bonduc* in India (Sagar and Vidyasagar, 2010a).

5.6 Cytotoxic Activity of *C. bonduc*

Cancer is a major public health burden in both the developed and developing countries of the world, with over six million estimated deaths in the year 2002 (Parkin *et al*., 2005). It is one of the major causes of death in the United States (Hoyert *et al*., 2005), where one in four deaths is due to cancer. Plants have long been used in the treatment of cancer (Hartwell, 1982). Drugs from

medicinal plants have played an important role in the treatment of cancer, and medicinal plants have accounted for about 40 % of the anticancer drugs produced between 1940 and 2002 (Newman *et al*., 2003). A number of active compounds have been shown to possess anticancer activity; these include flavonoids, diterpenoids, triterpenoids, and alkaloids (Han *et al*., 2008). The cytotoxic activity of flavonoids in malignant cells is characterised by an increased generation of reactive oxygen species (ROS) (Wang *et al*., 1999), accompanied by a decrease in the level of redox active proteins, superoxide dismutase and thioredoxin which are crucial for maintaining the cellular redox balance (Lu *et al*., 2006).

Reactive oxygen species (ROS) are released from normal oxidative metabolism in eukaryotic cells and cellular antioxidants, such as superoxide dismutase and thioredoxin which carry out the detoxification process in these species. However, an overproduction of ROS or a decrease in the antioxidative capacity of cells can cause oxidative stress (Halliwell, 1992). Almost all cancer cells are under an oxidative stress associated with increased metabolic activity; hence they have an increased production of ROS (Szatrowski and Nathan, 1991). The increased basal oxidative stress in transformed cells makes them highly dependent on their antioxidant systems to counteract the damaging effect of ROS; this makes them susceptible to further oxidative stress (Schumacker, 2006). ROS-generating agents can effectively kill cancer cells by increasing the intracellular ROS to a toxic level (Schumacker, 2006). Human cancer cells with an intrinsic oxidative stress are highly sensitive to ROS stress (Huang *et al.*, 2000; Zhou *et al.*, 2003). Promoting ROS generation in mitochondria can effectively kill them (Pelicano *et al.*, 2003).

The anticancer activity of medicinal plants has been associated with their possession of flavonoids among their secondary metabolites. They anticancer activity of flavonoids in malignant cells is characterised by an increased generation of reactive oxygen species (ROS)

(Wang *et al.*, 1999) accompanied by decrease in the level of redox active proteins, superoxide dismutase and thioredoxin which are crucial for maintaining the cellular redox balance (Lu *et al.*, 2006). The cancer chemopreventive activity of kaempferol, a flavonoid, has been linked to the induction of apoptosis in glioma cells arising from the elevation of intracellular oxidative stress (Sharma *et al.*, 2007). Flavonoid-generated induced apoptosis is associated with membrane hyperpolarisation, leading to a decrease in the mitochondrial membrane potential and to an alteration in the plasma membrane potential (Trachootham *et al.*, 2006). High levels of ROS can cause apoptosis by triggering a mitochondrial permeability transition pore opening and the release of proapoptotic factors (Brenner and Grimm, 2006).

The results of the present investigation are consistent with the reports of Kawaii *et al*. (1999) and Rubio *et al*. (2006) on the cytotoxic activity of quercetin-3-methyl ether (TCB 16), luteolin (TCB 15) and other flavonoids. Kaempferol-3-methyl ether has a cytotoxic activity with an IC_{50} value of 35 µM on HeLa cells (Rubio et al., 2006); its glycones (TCB 27 and 40) lack cytotoxic activity. It is suggested that the lack of cytotoxic activity might be due to the additional sugar moiety attached at position 3 of the C-ring; this increases their polarity and limits their cellular permeability (Spencer, 2003). Samples isolated from *C. bonduc* showed good cytotoxicity against HeLa and BGC - 823 cancer cell lines.

Structure-activity relationship (SAR) analyses of flavonols and flavones isolated from *C. bonduc* suggest that the introduction of a hydroxyl group at the 3' position of the B ring might cause an increase in cytotoxicity; on methlylation of the 3-hydroxyl group on the C ring, there is an enhancement of cytotoxicity. *O*-methylated flavonoids obtained from flavonoid metabolism in the small intestine have been shown to possess enhanced cytotoxicities while their glucuronides, especially at the 5 and 7 positions, have been shown to inhibit cytotoxicity (Spencer, 2003).

A number of bioactive flavonoids as well as other phytochemicals with anticancer activity have been isolated from *C. bonduc* (Han *et al*., 2008) However, this thesis is the first report on the isolation, purification and structural elucidation of cytotoxic compounds (TCB 9, 11, 15 and 16) from *C. bonduc*. Since flavonoids display a wide array of cellular activities, several mechanisms have been proposed to account for their cytotoxicity. These include the inhibition of DNA topoisomerase I/II activity, an increased generation of reactive oxygen species (Wang *et al*., 1999), a decrease in the level of redox active proteins (Lu *et al*., 2006), DNA oxidation and fragmentation, regulation of heat-shock-protein expression, cell cycle arrest, modulation of survival/proliferation pathways and activation of proapoptotic cellular factors (Ramos, 2007).

5.7 CONCLUSION AND RECOMMENDATION

From the phytochemical assessment of the young twigs and leaves of *C. bonduc*, two new cassane diterpenoids (one fully structurally characterised and the other partially characterised), seven known flavonoids, one lignan, four different plant phytochemicals were isolated and their structures elucidated and identified. Furthermore, 13 uncharacterised compounds were also isolated separated from the young twigs and leaves of *C. bonduc* obtained from Ibadan, Oyo state, Nigeria, West Africa. Their antimalarial and cytotoxicity activities were evaluated. The cytotoxic activity of *C. bonduc* was linked to the presence of bioactive flavonoids with a B-ring as part of their phytoconstituents. The petroleum ether and ethyl acetate fractions showed moderate antimalarial activity. But these activities are not comparable to those of the available antimalarial drugs. Some solvent fractions and compounds of *C. bonduc* showed good anticancer activities. These activities are comparable with those of the existing anticancer drugs. In the present investigation, the 28 days of repeated doses, cellular alterations observed at the higher doses of extract (400, 800 and 1600 mg/kg body weight) corroborate the biochemical and haematological alterations found in the investigated experimental animals. Similarly, in the acute toxicological investigation, fixed extract doses of 2000 mg/kg and above caused cellular alterations which corroborated the biochemical and haematological alterations. It can be concluded that the daily administration of crude ethanolic fractions of *C. bonduc* at a dose of 400 mg/kg body weight for 28 days is toxic to experimental animals. Similarly, the fixed dose of *C. bonduc* at 2000 mg/kg body weight and above is also toxic to experimental animals, even though, no mortality was observed at all the tested doses.

Based on the present assessments and investigations, the daily use of concentrated extracts of the young twigs and leaves of *C. bonduc*, as is done in the southwestern part of Nigeria, should

be carefully considered in view of the observed toxicities recorded in this investigation. It is clear form this study and from another (Kumar *et al.,* 2005) that potential toxicity might arise from the continuous intake of plants such as *C. bonduc* which are rich in flavonoids. The phenolic ring containing flavonoids, upon oxidation by peroxidases, yields the phenoxyl radical. This radical is cytotoxic, co-oxidizes unsaturated lipids, GSH and nucleic acids and causes ROS formation and mitochondrial toxicity. The antiproliferation and antimalarial activities of *C. bonduc*, as well as the indepth understanding of their mechanism of action should be further explored. This can help in exploring the possibility of using flavonoids, after structural modifications, as both anticancer and antimalarial agents. The i*n vivo* prophylactic and chemotherapeutic activities of the extracts of *C. bonduc* will further validate its folkloric use as an antimalarial. This might constitute a topic for future research focus.

5.8 CONTRIBUTIONS TO KNOWLEDGE

This research work has contributed the following to the body of scientific knowledge:

1. The discovery of two new compounds, a fully structurally characterised one, TCB 6 (12α-ethoxyl-1α,14β-diacetoxy-2α,5α-dihydroxyl-cass-13(15)-en-16,12-olide) and a partially characterised compound, TCB 5 (1α,7α-diacetoxy-5α,6β-dihydroxyl-cass-14(15)-epoxy-16,12-olide) from the young twigs and leaves of *C. bonduc*.
2. The isolation and structural elucidation of eleven compounds from the young twigs and leaves of *C.bonduc* which are being reported for the first time. These include: 1-octacosanol, (+)-Syringaresinol, 4,4'-dihydroxy4,4'-dihydroxy-2'-methoxy-chalcone, 7,3'-dihydroxy-3,11-dehydrohomoisoflavanone, Daucosterol, 5,7,3',4'-tetrahydroxy-flavone, 5,7,3',4'-tetrahydroxy-3-methoxyflavone, Protocatechuic acid, 1-O-Hexadecanolenin, kaempferol-3-*O*-β-D-xylopyranoside and Kaempferol-3-*O*-α-L-rhamnopyranosyl-1→2)-β-D-xylopyranoside.
3. The moderate antimalarial activity of 4,4'-dihydroxy4,4'-dihydroxy-2'-methoxy-chalcone, isolated from *C. bonduc* is reported for the first time.
4. The ethanolic extract and some compounds isolated from *C. bonduc* have antimalarial and cytotoxic activities.
5. The ethanolic extract and compounds isolated from the young twigs and leaves of *C. bonduc* have no inhibitory activity against *Candida albicans.*

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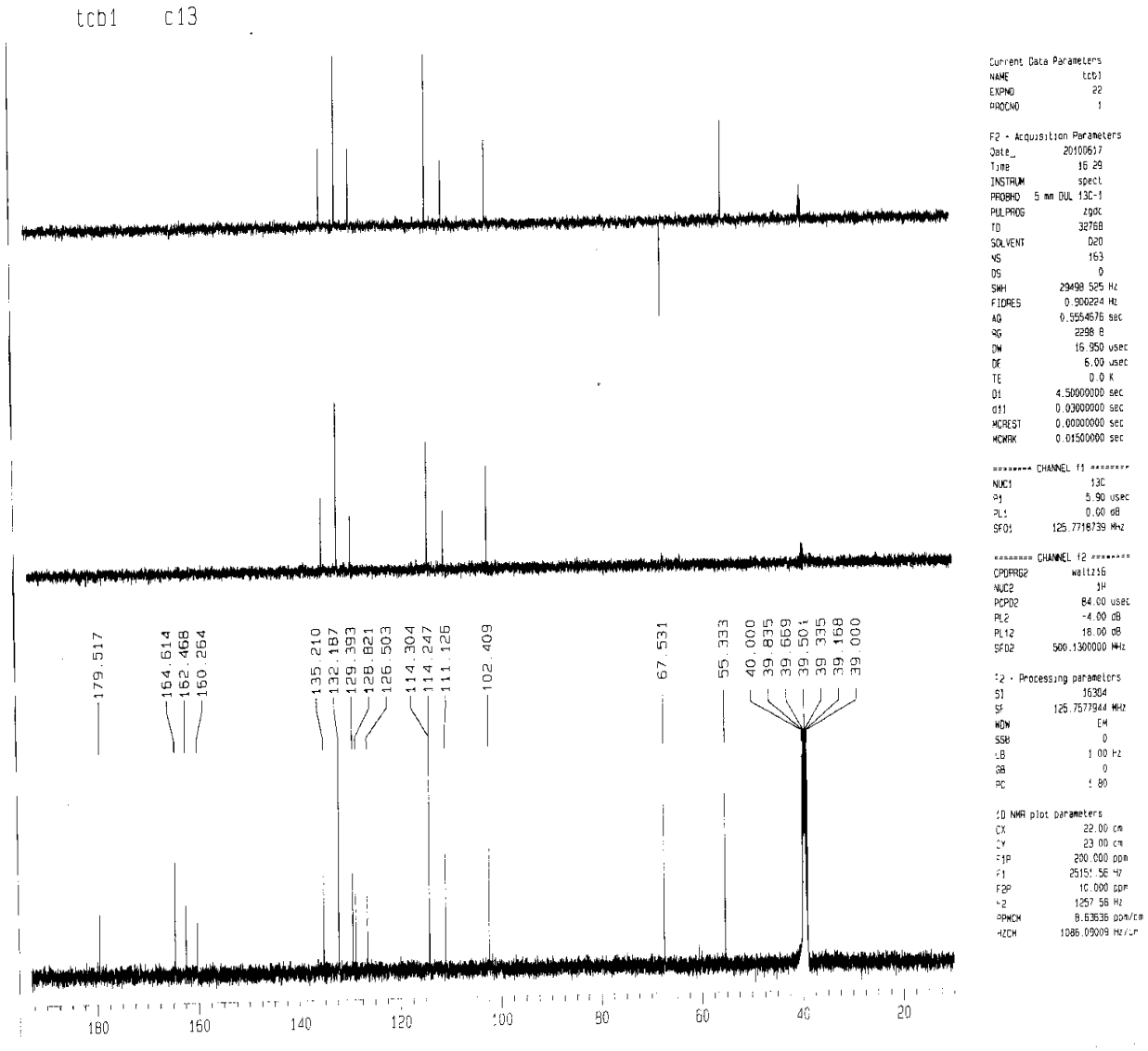
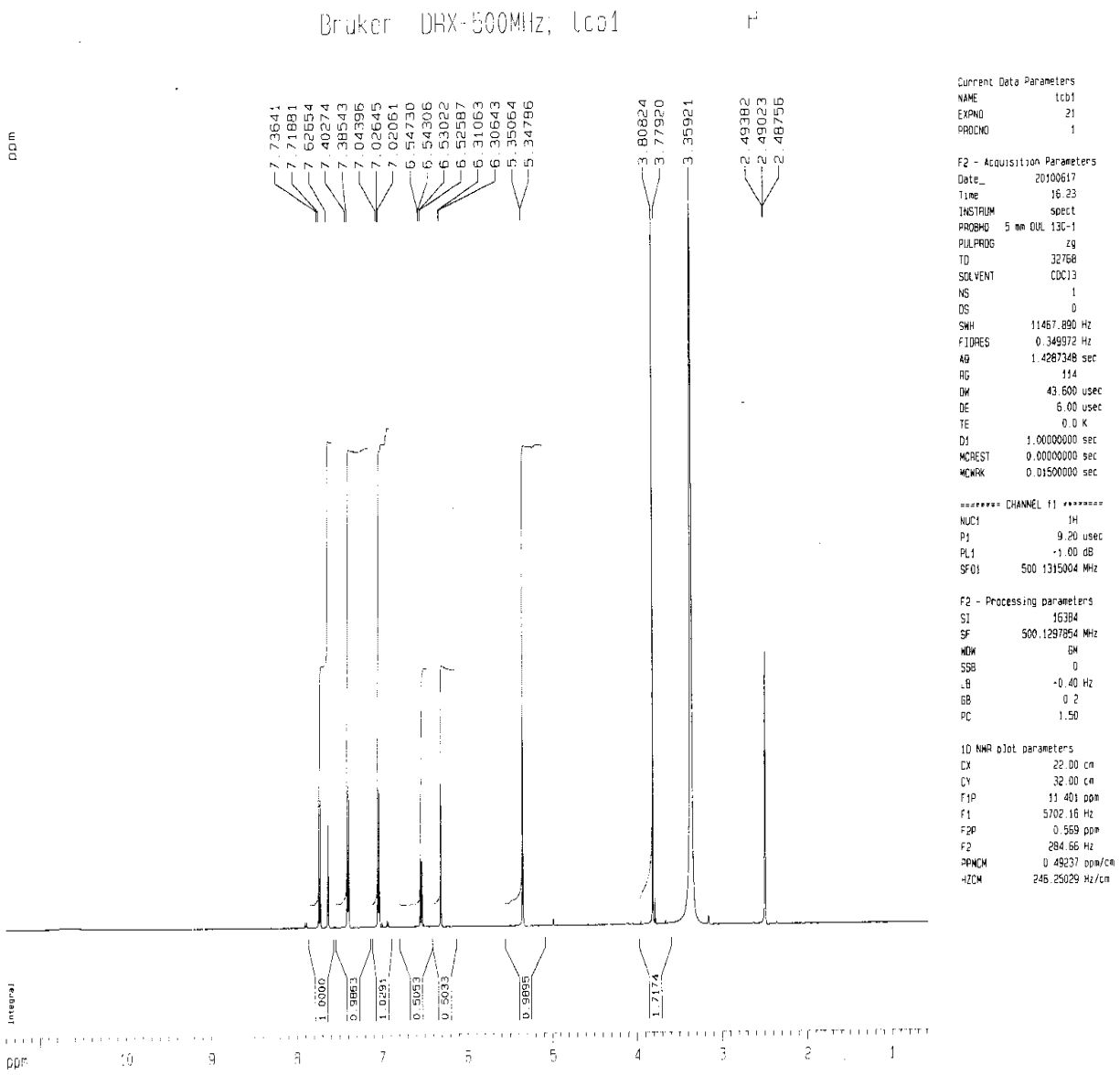
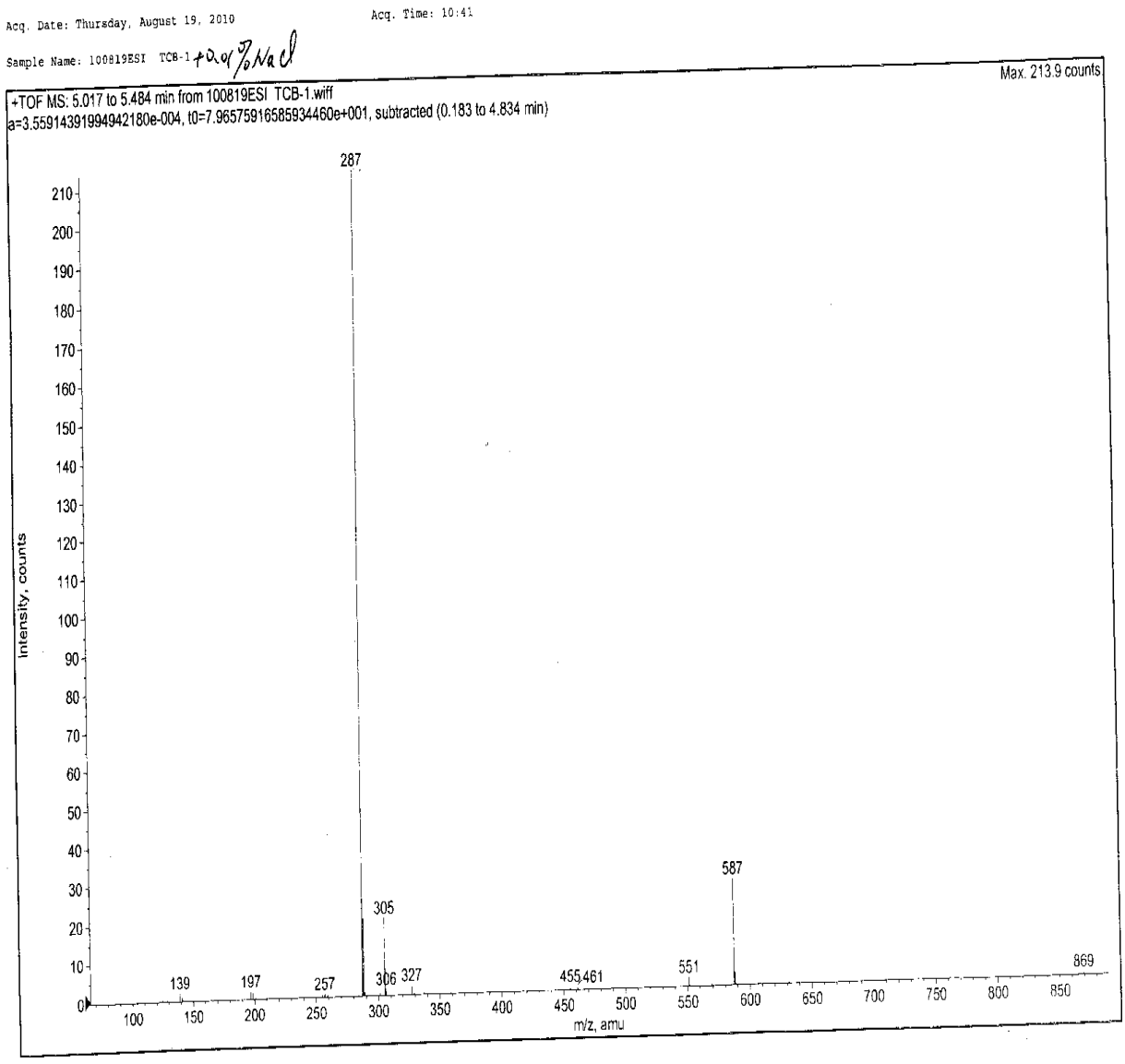
APPENDICES



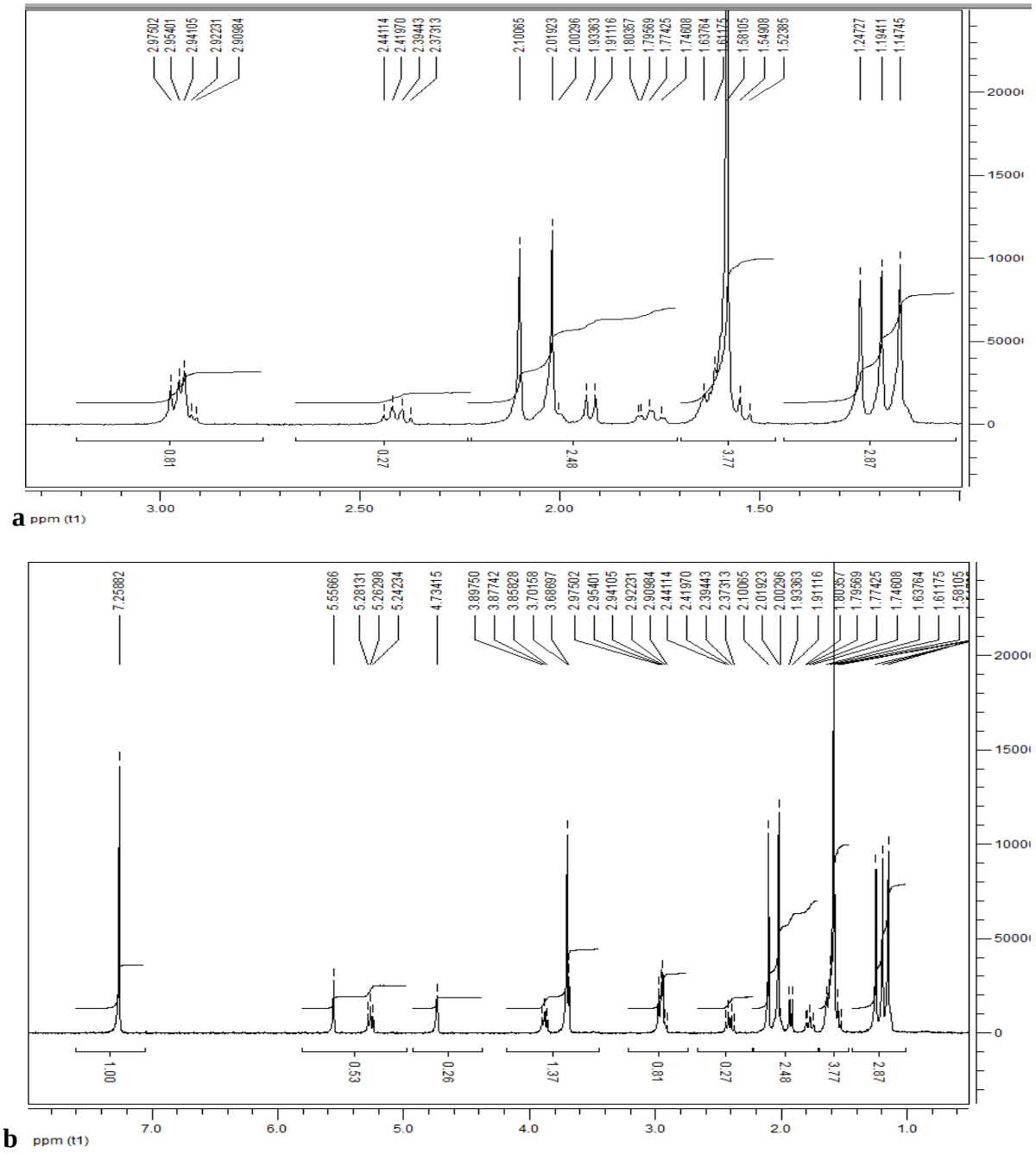
Appendix 1: ^{13}C NMR Spectrum of TCB 1



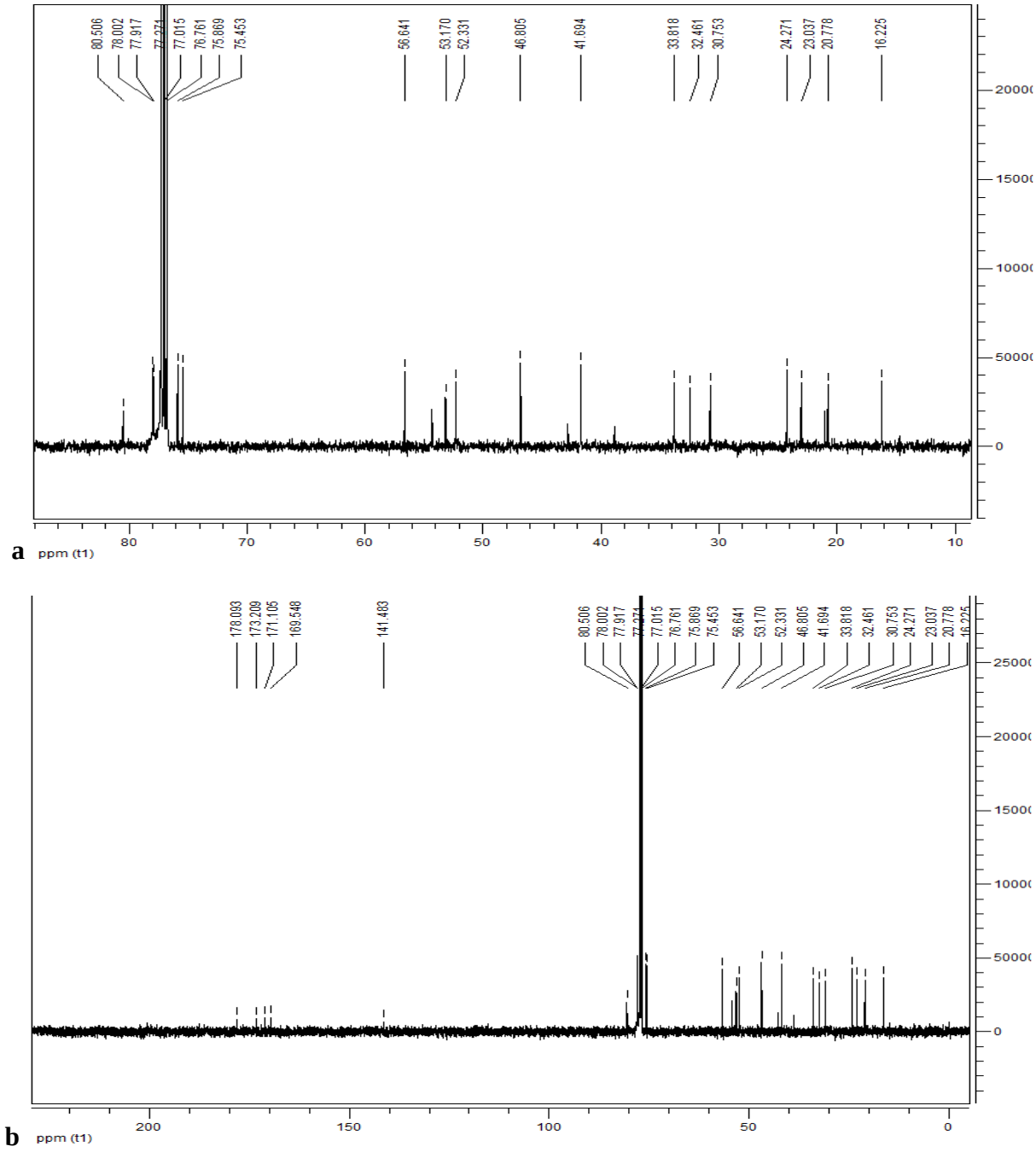
Appendix 2: ^{1}H NMR Spectrum of TCB 1



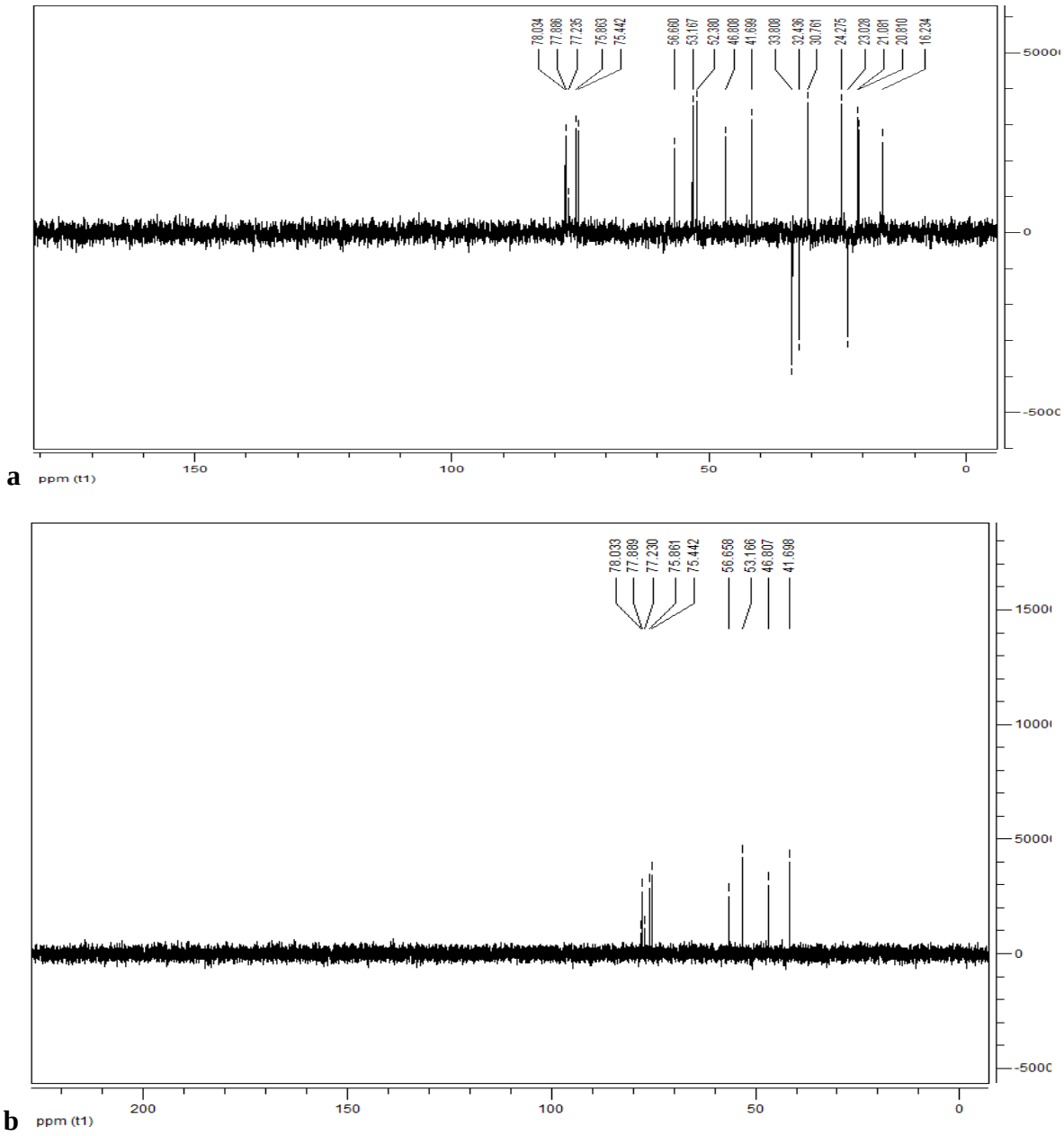
Appendix 3: ESI-MS Spectrum of TCB 1



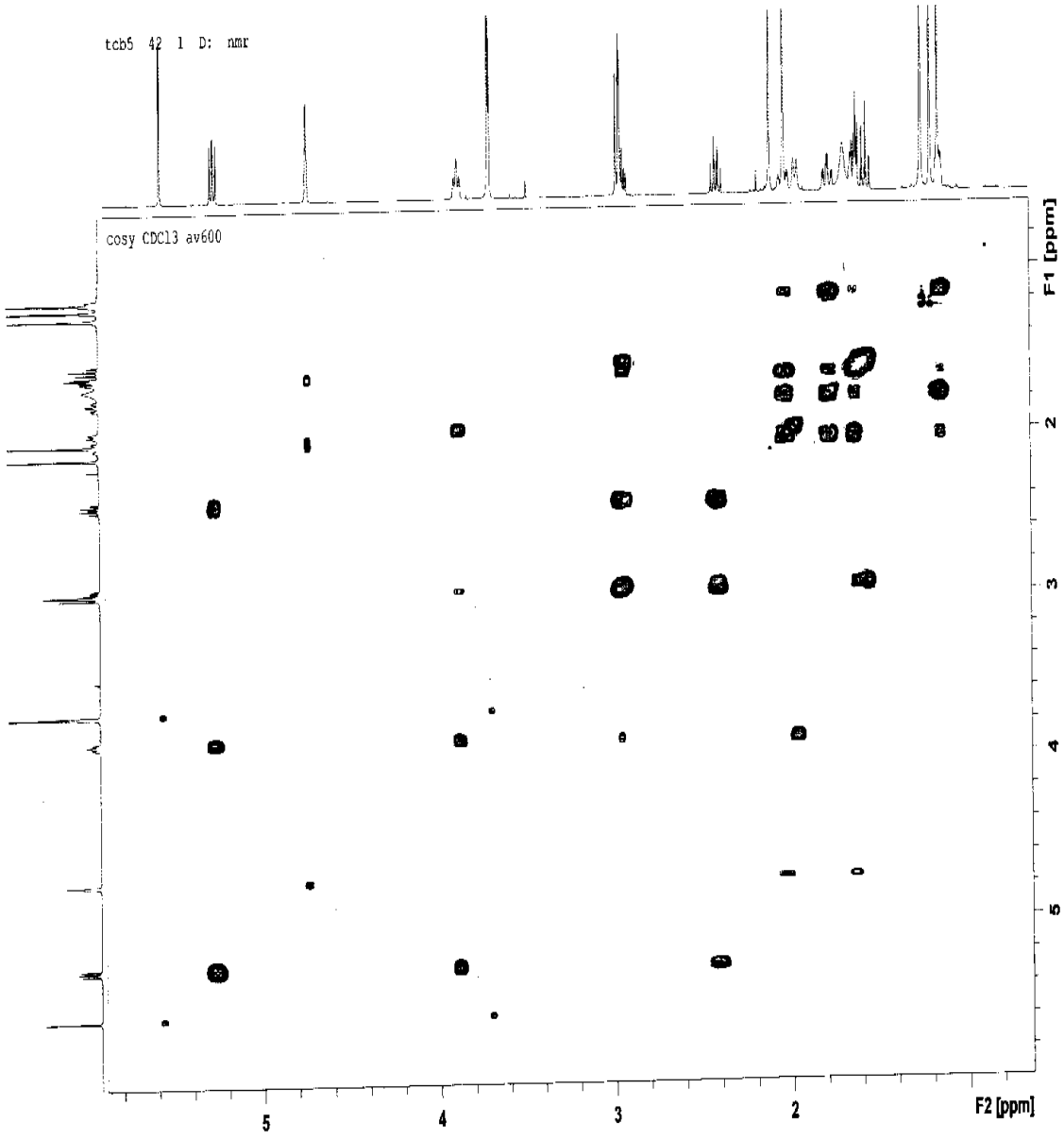
Appendix 4 ^{1}H NMR Spectral of TCB 5 (Compound 4) recorded at 500 MHz in deuterated chloroform ($CDCl_3$) with **a:** showing the extended spectrum of **b** between 0 to 3.0 ppm. The chemical shifts for the spectral were assigned in ppm.



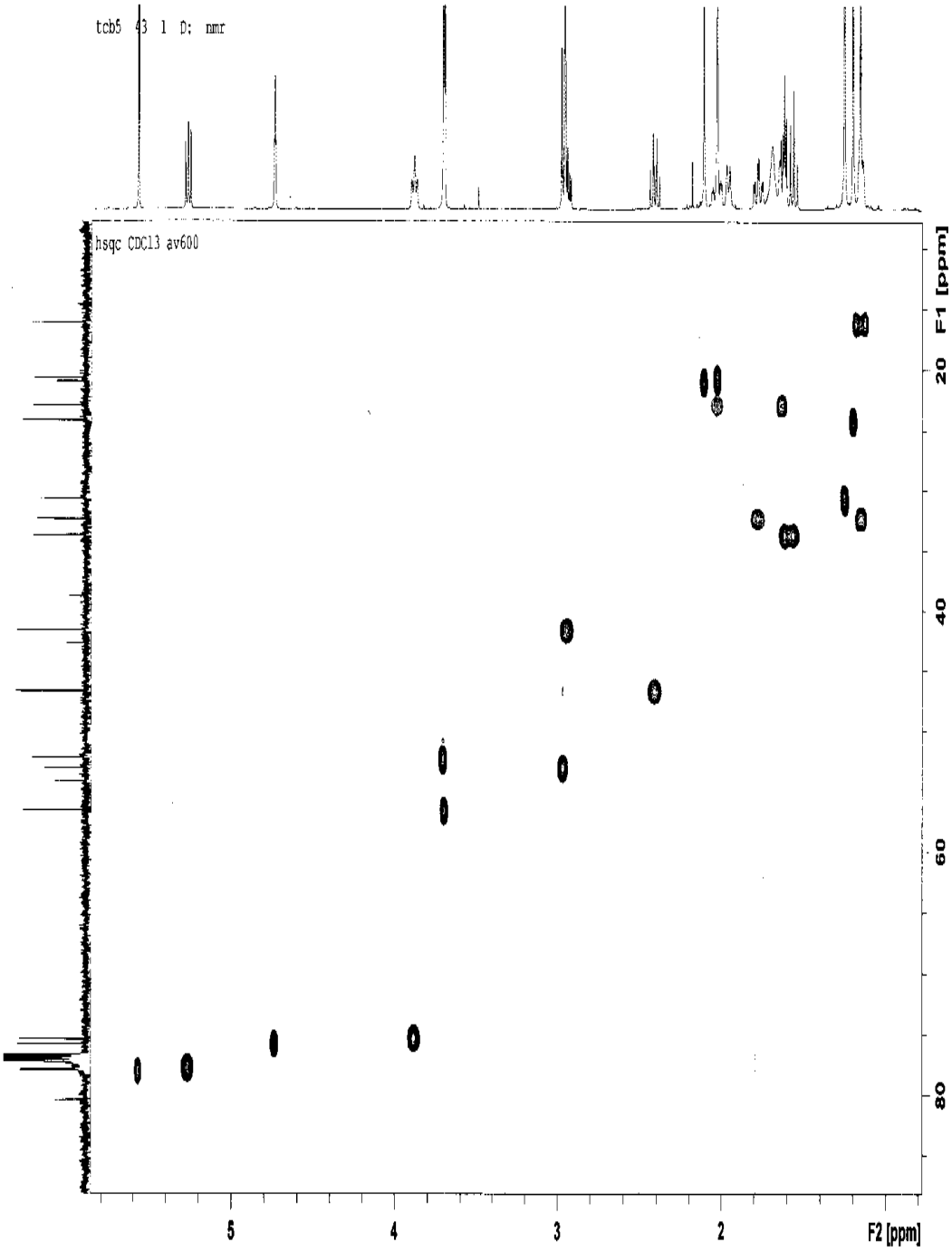
Appendix 5 ^{13}C NMR Spetral of TCB 5 (Compound 4) recorded at 125 MHz in deuterated chloroform ($CDCl_3$) with **a**: showing the extended spectrum of **b** between 10 to 81 ppm. Chemicaal shifts were assigned in ppm.



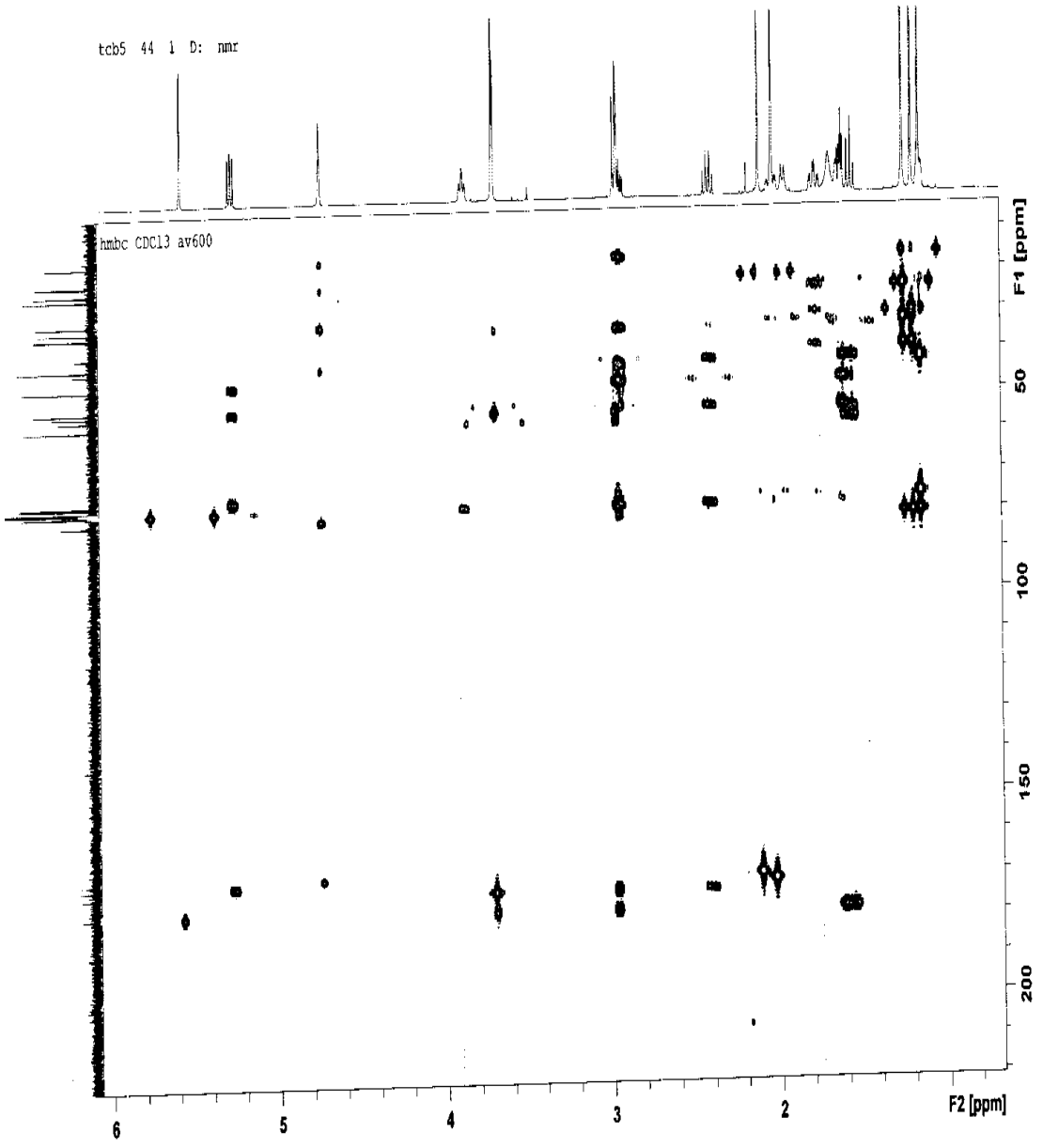
Appendix 6 ^{13}C NMR DEPT (**D**istortion-less **E**nhancement by **P**olarization **T**ransfer) Spectral of TCB 5 (Compound 4) recorded at 125 MHz in deuterated chloroform ($CDCl_3$) with **a**: showing the shift due to the presence of CH and CH_3 in a phase opposite to CH_2 and **b**: showing shift due to only CH groups. Chemicaal shifts were assigned in ppm.



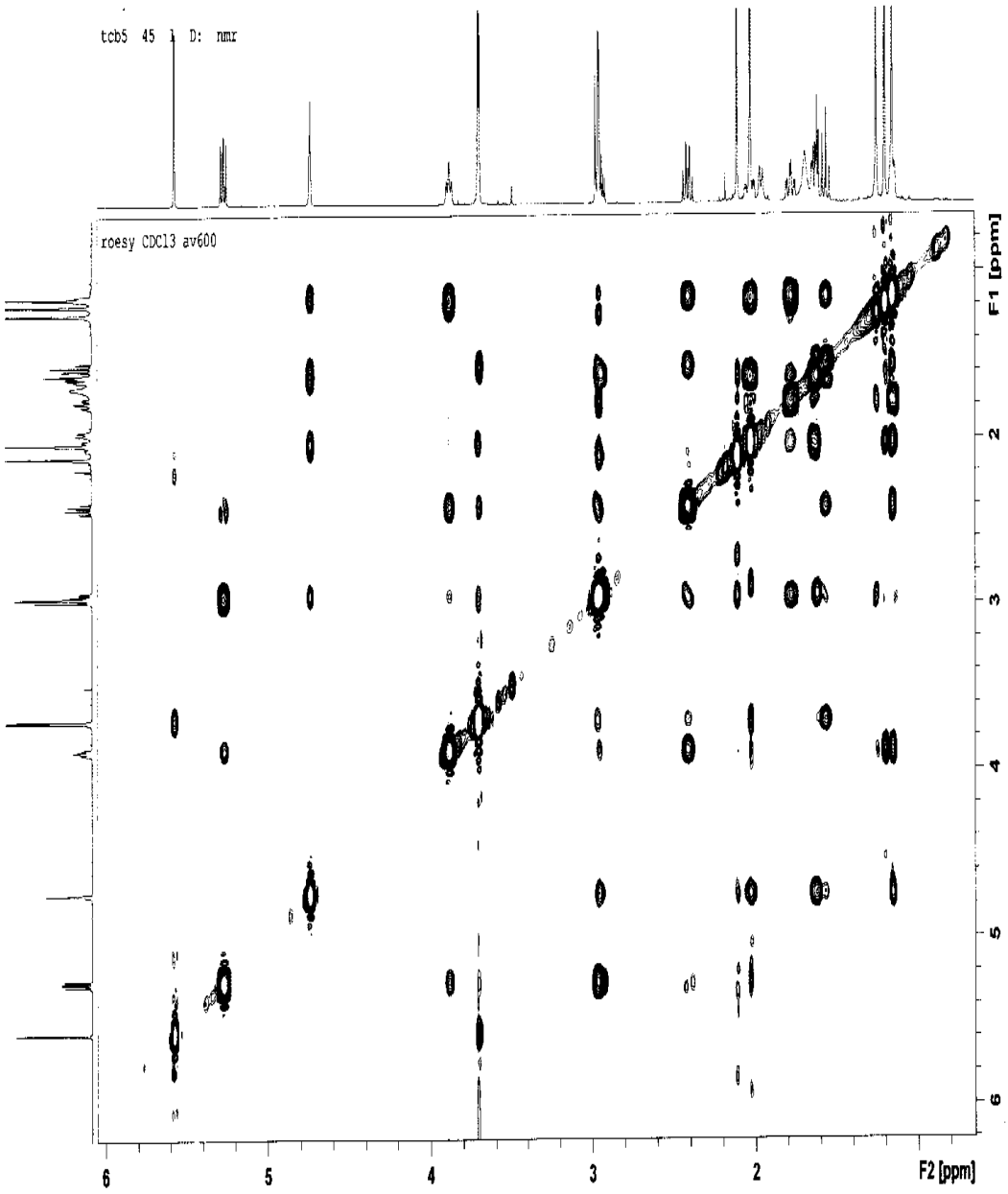
Appendix 7 Correlation Spectroscopy (COSY) Spectrum of TCB 5 recorded at 600 MHz in deuterated chloroform ($CDCl_3$).



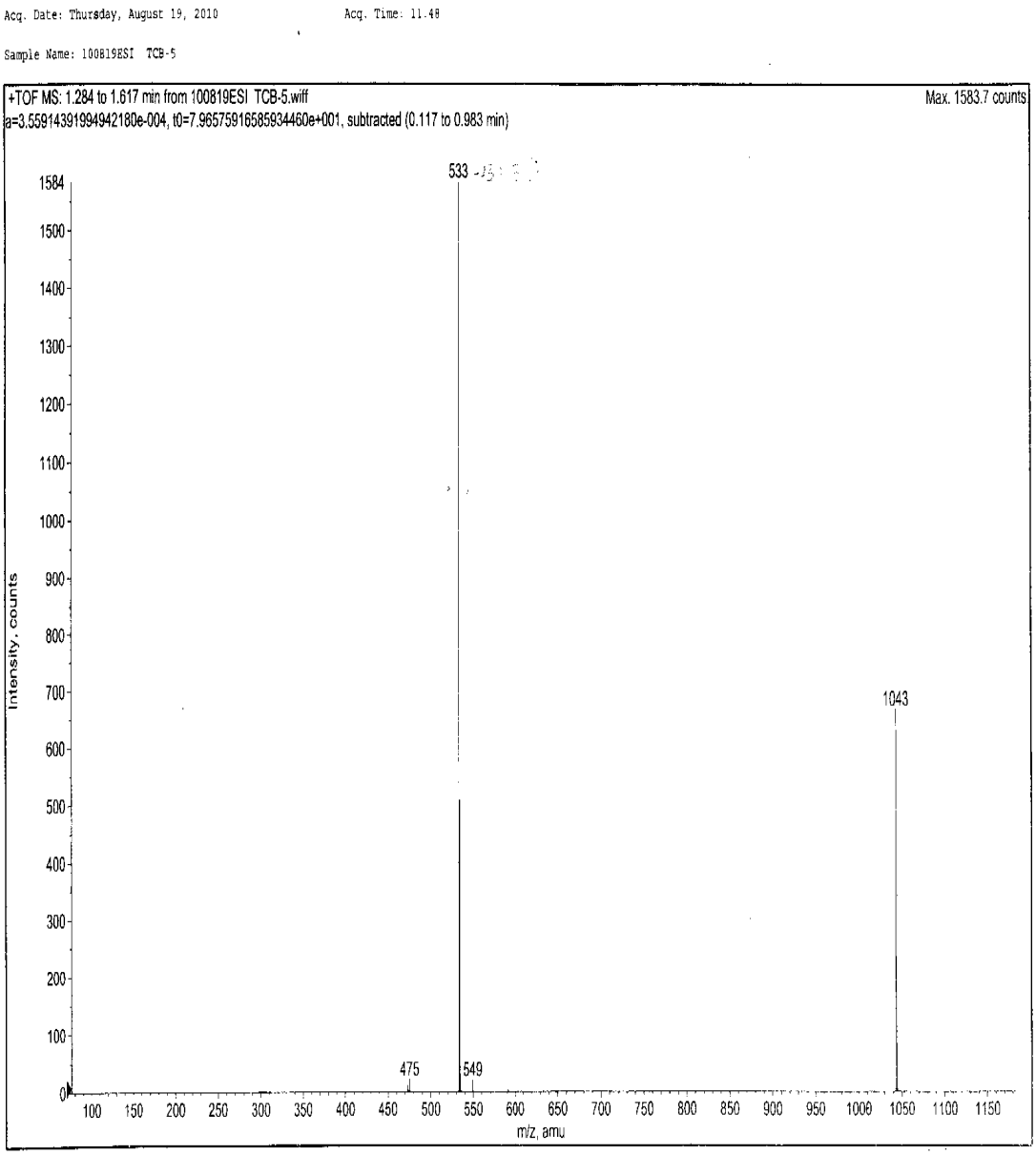
Appendix 8 Heteronuclear Multiple Quantum Coherence (HMQC) Spectrum of TCB 5 recorded at 600 MHz in deuterated chloroform $CDCl_3$.



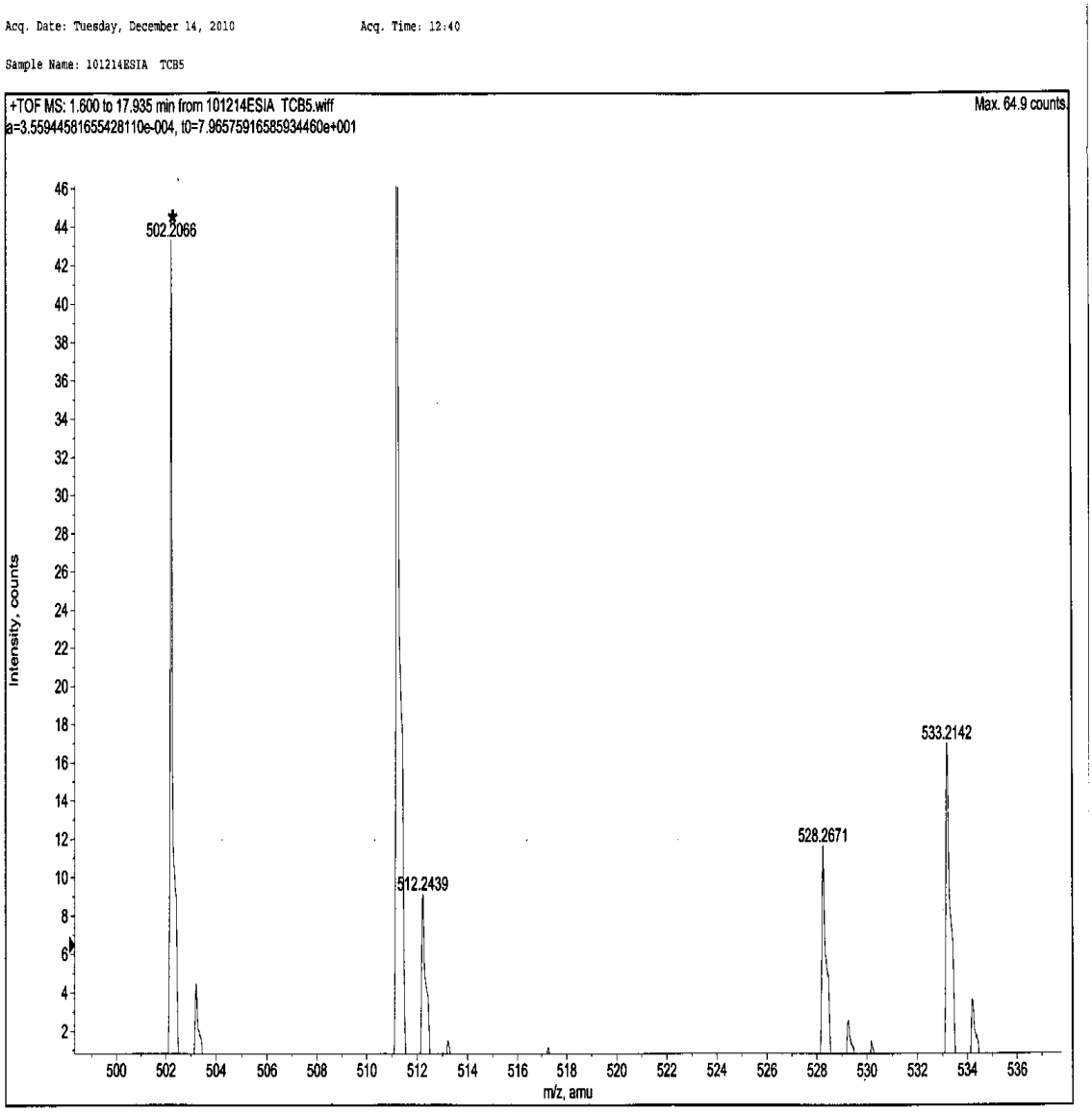
Appendix 9 Heteronuclear Multiple Bond Coherence (HMBC) Spectrum of TCB 5 recorded at 600 MHz in deuterated chloroform $CDCl_3$.



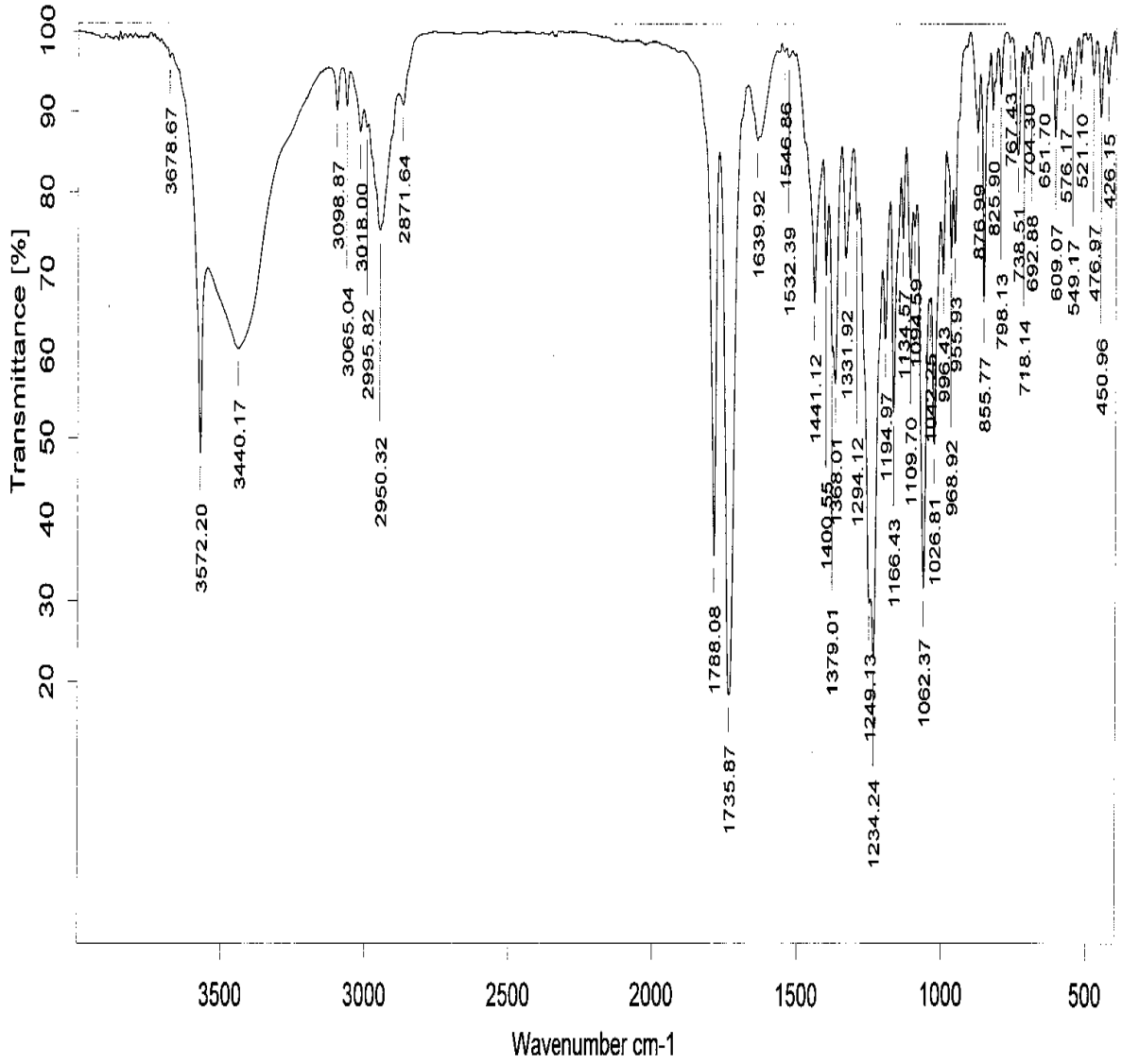
Appendix 10 Rotating-frame Overhauser Effect Spectroscopy (ROESY) Spectrum of TCB 5 recorded at 600 MHz in deuterated chloroform $CDCl_3$.



Appendix 11 Electrospray Ionization Mass Spectroscopy (ESI-MS) Spectrum of TCB 5

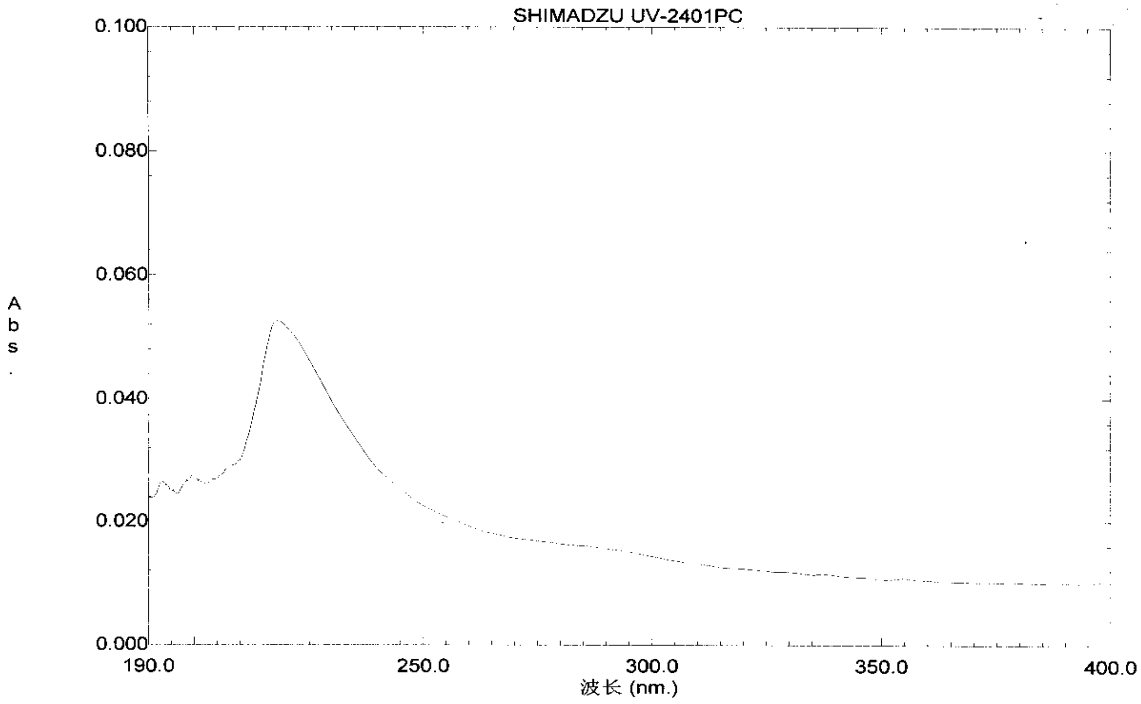


Appendix 12 High Resolution Electrospray Ionisation Mass Spectroscopy (HR - ESI – MS) Spectrum of TCB 5



Sample : TCb 5	Frequency Range : 399.271 - 3996.57	Measured on : 16/12/2010	
Technique : KBr压片	Resolution : 4	Instrument : Tensor27	Sample Scans : 16
Customer : 101216IR5	Zerofilling : 2	Acquisition : Double Sided,For	

Appendix 13 IR Spectrum of TCB 5



文件名: TCB5

TCB5 --------

样品浓度：0.0585mg/ml
溶剂：MeOH

创建于: 15:42 10-12-15
数据: 原始

测量模式: Abs.
扫描速度: 中速
狭缝: 5.0
采样间隔: 0.2

否.	波长 (nm.)	Abs.
1	218.40	0.0525
2	199.40	0.0275

Appendix 14 UV Spetrum of TCB 5

Optical rotation measurement

Model : P-1020 (A06040638)

No.	Sample	Mode	Data	Monitor Blank	Temp. Cell Temp Point	Date Comment Sample Name	Light Filter Operator	Cycle Time integ Time
No 1	2 (1/3)	Sp.Rot	32.8810	0.0194 0.0000	11.1 50.00 Cell	Thu Dec 16 15:11:22 2010 0.00118g/mlCHCl3 TCB5	Na 589nm	2 sec 10 sec
No 2	2 (2/3)	Sp.Rot	35.9320	0.0212 0.0000	11.0 50.00 Cell	Thu Dec 16 15:11:35 2010 0.00118g/mlCHCl3 TCB5	Na 589nm	2 sec 10 sec
No.3	2 (3/3)	Sp.Rot	31.5950	0.0187 0.0000	11.1 50.00 Cell	Thu Dec 16 15:11:49 2010 0.00118g/mlCHCl3 TCB5	Na 589nm	2 sec 10 sec

+33.702°

Appendix 15 Optical Rotation Measurement of TCB 5

Acq. Date: Tuesday, December 14, 2010 Acq. Time: 12:40

Sample Name: 101214ESIA TCB5

Elemental composition calculator

Target m/z: +533.2142 amu
Tolerance: +110.0000 ppm
Result type: Elemental
Max num of results: 1000
Min DBE: -10.0000 Max DBE: +60.0000
Electron state: OddAndEven
Num of charges: 0
Add water: N/A
Add proton: N/A
File Name: 101214ESIA TCB5.wiff

	Elements	Min Number	Max Number
1	Br	0	0
2	C	0	200
3	Cl	0	0
4	F	0	0
5	H	0	400
6	K	0	0
7	N	0	0
8	Na	1	1
9	O	8	10
10	Pt	0	0

Appendix 16 Elemental Composition Calculation for TCB 5

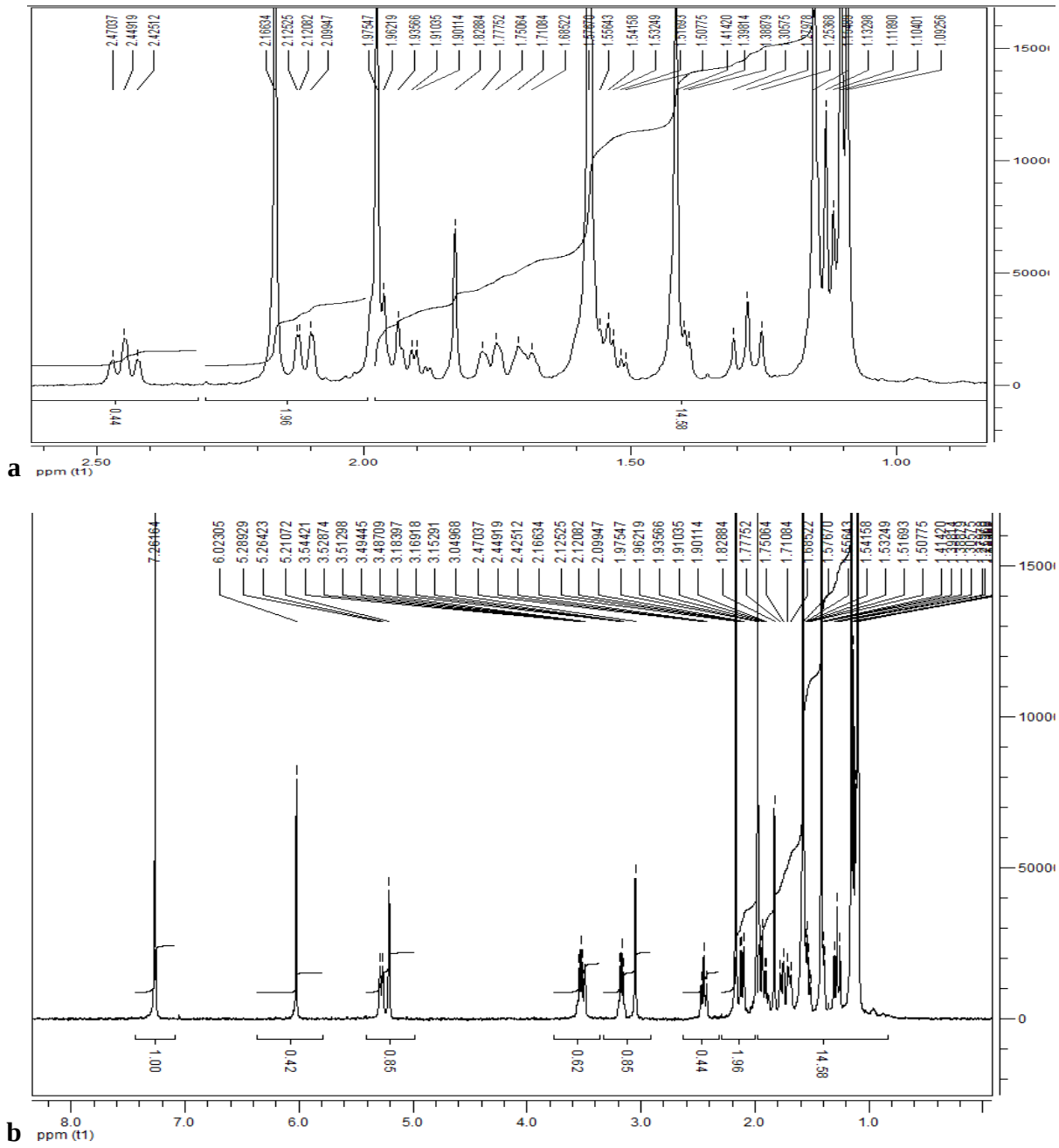
Acq. Date: Tuesday, December 14, 2010 Acq. Time: 12:40

Sample Name: 101214ESIA TCB5

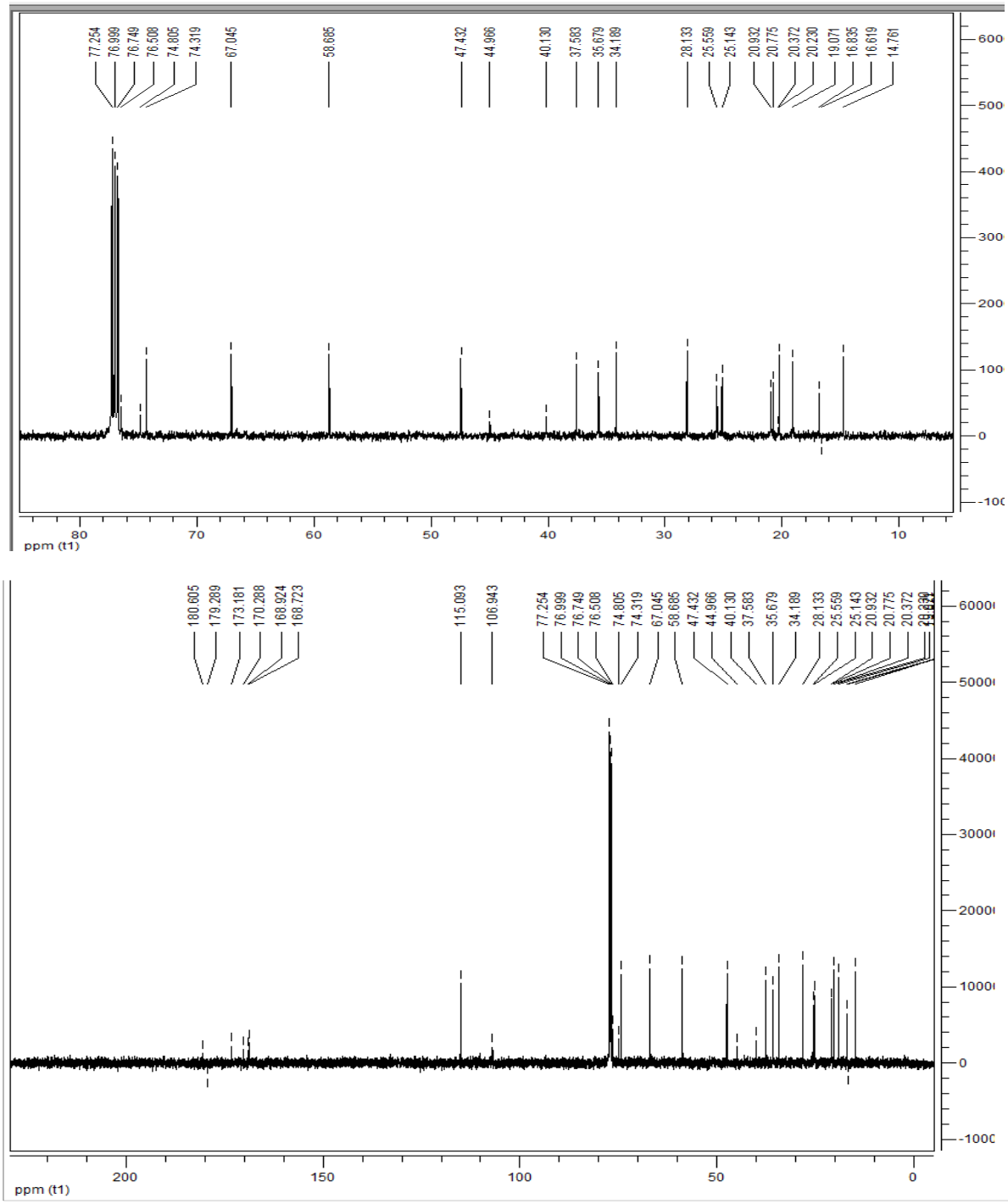
	Elements	Min Number	Max Number
11	S	0	0
12	Si	0	0

	Formula	Calculated m/z (amu)	mDa Error	PPM Error	DBE
1	C29 H34 O8 Na	533.2151	-0.9381	-1.7594	12.5
2	C26 H38 O10 Na	533.2362	-22.0676	-41.3860	7.5
3	C28 H30 O9 Na	533.1787	35.4473	66.4786	13.5
4	C27 H42 O9 Na	533.2726	-58.4531	-109.6241	6.5

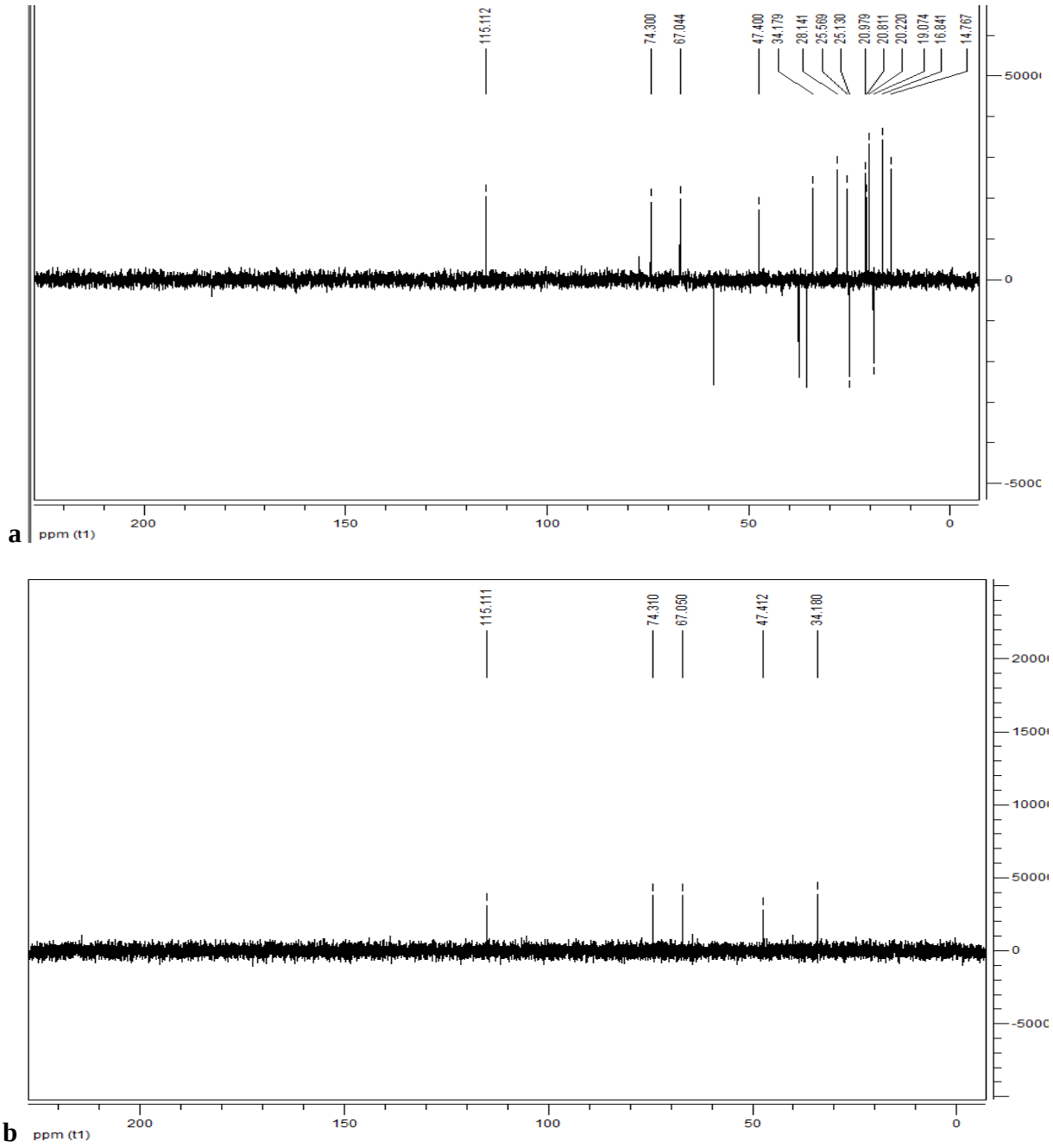
Appendix 17 Elemental Calculation for TCB 5 Continued



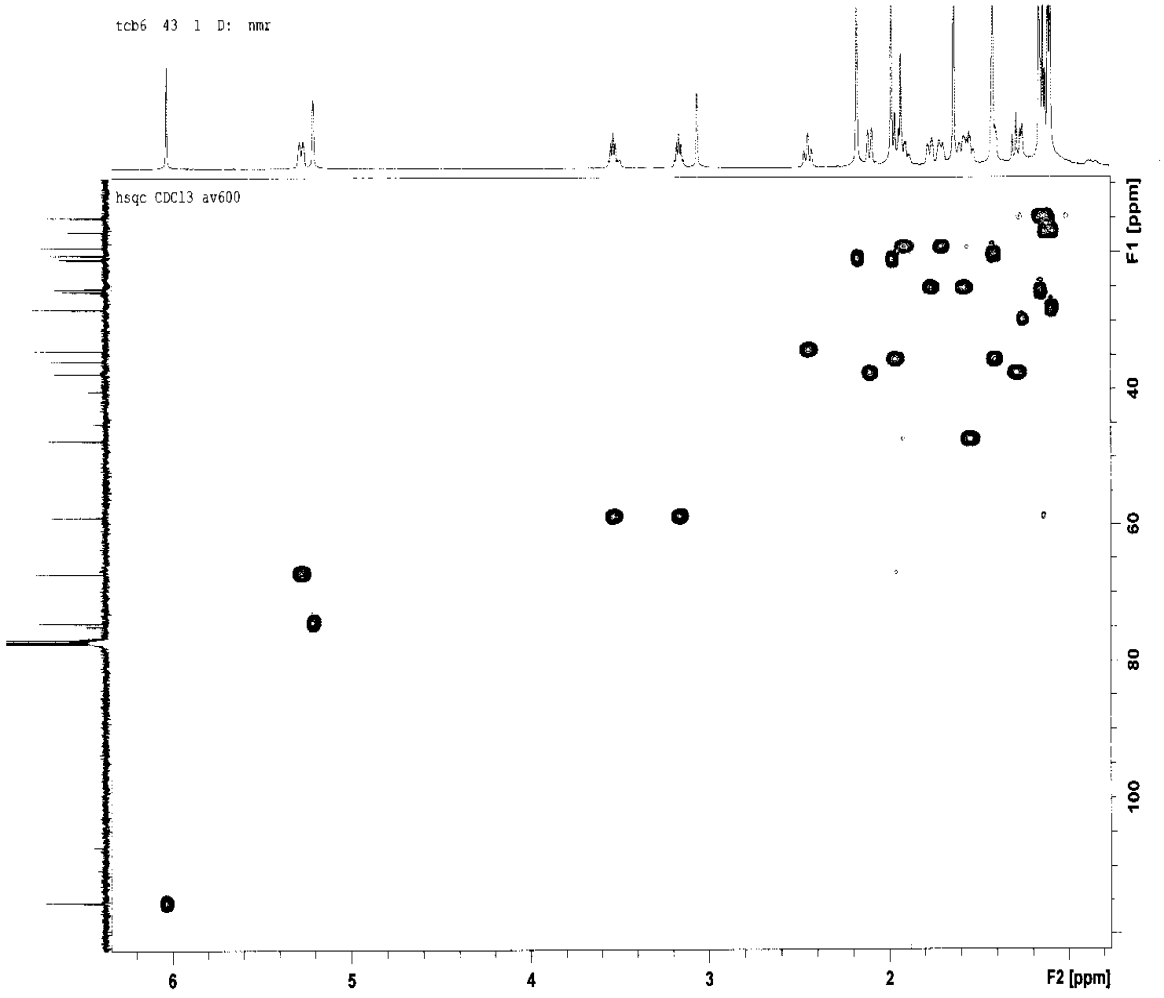
Appendix 18 ^{1}H NMR Spectral for TCB 6 (Compound 5) recorded at 500 MHz in deuterated chloroform ($CDCl_3$) with **a:** showing the extended spectrum of **b** between 0 to 3.0 ppm. The chemical shifts for the spectral were assigned in ppm.



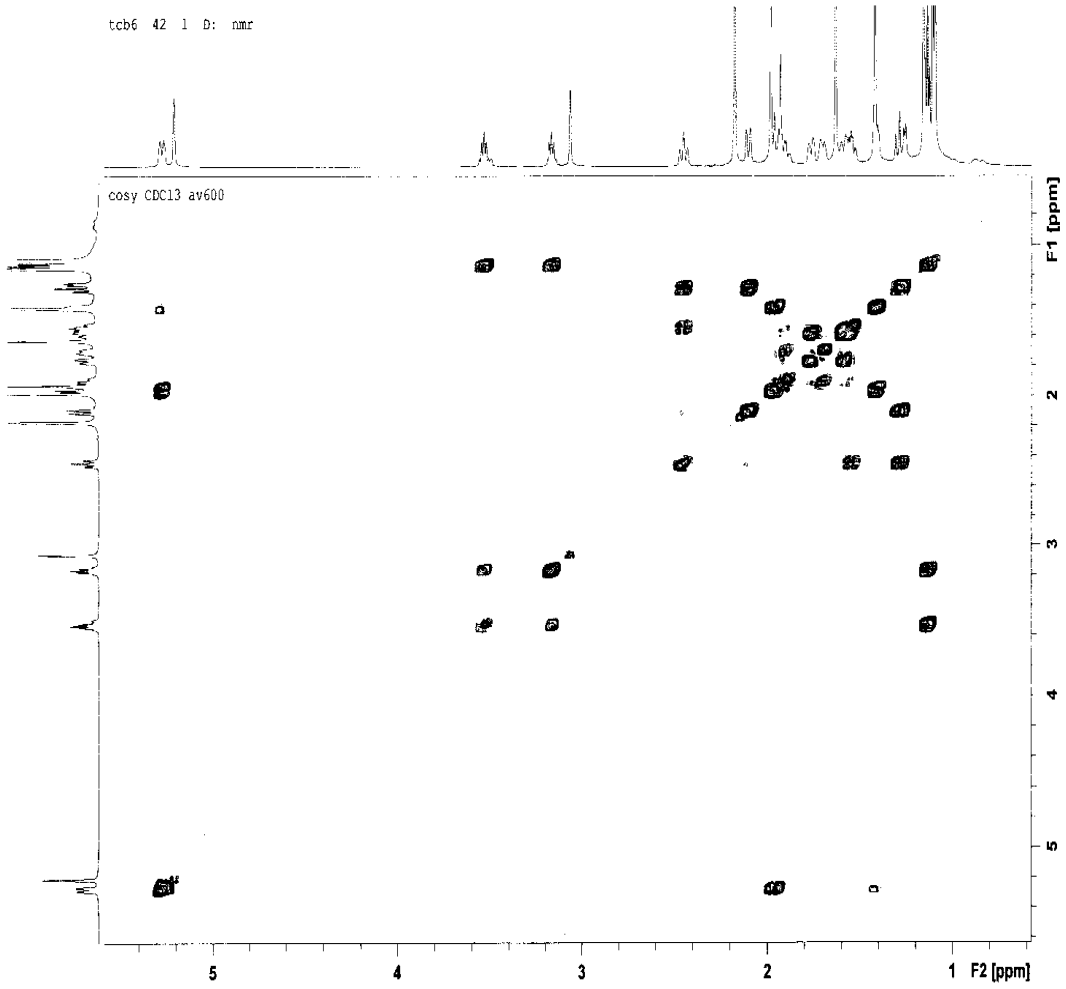
Appendix 19 ^{13}C NMR Spectral of TCB 6 (Compound 5) recorded at 125 MHz in deuterated chloroform ($CDCl_3$) with **a**: showing the extended spectrum of **b** between 10 to 80 ppm. Chemicaal shifts were assigned in ppm.



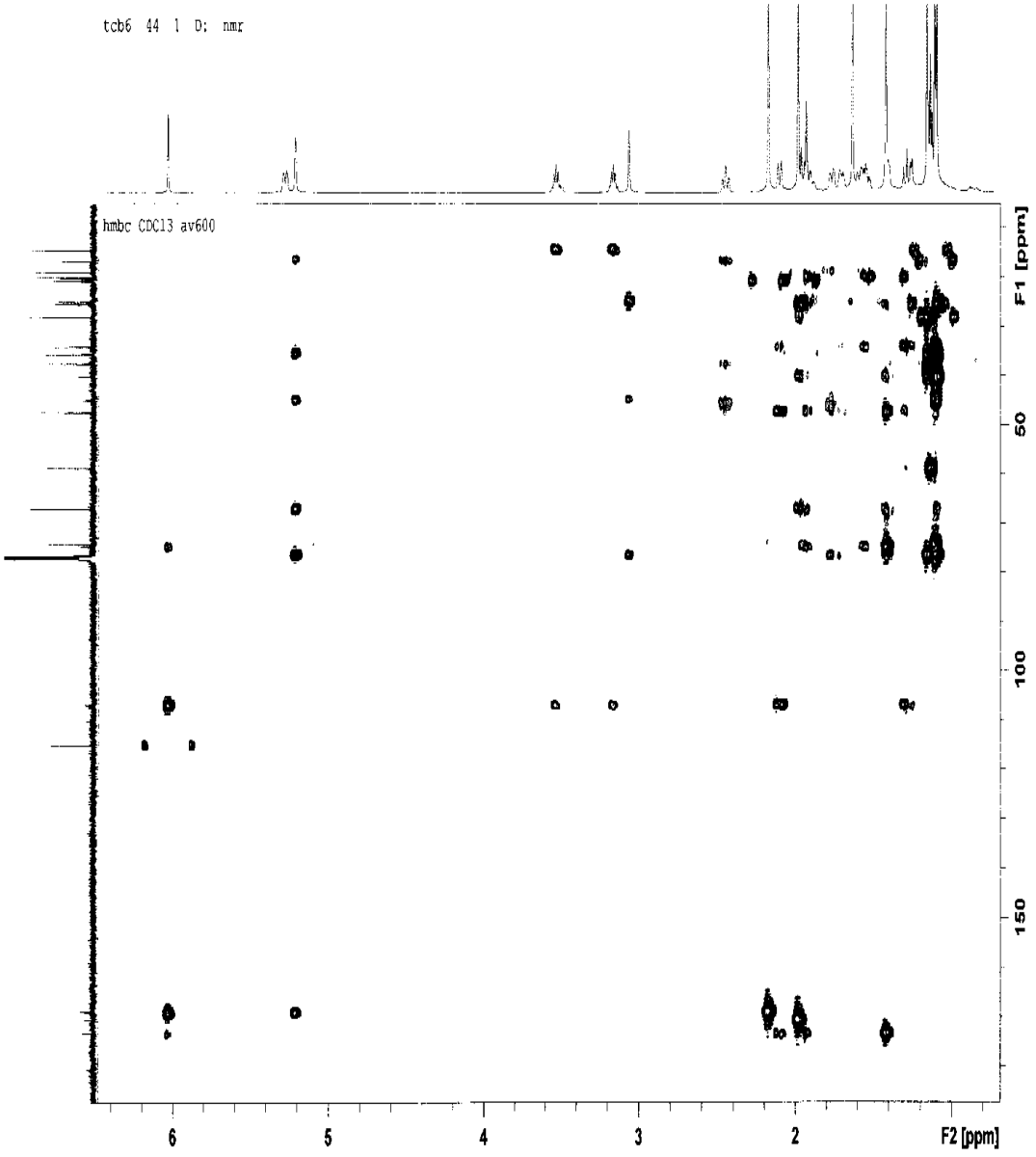
Appendix 20 ^{13}C NMR DEPT (**D**istortion-less **E**nhancement by **P**olarization **T**ransfer) Spectral of TCB 6 (Compound 5) recorded at 125 MHz in deuterated chloroform ($CDCl_3$) with **a**: showing the shift due to the presence of CH and CH_3 in a phase opposite to CH_2 and **b**: showing shift due to only CH groups. Chemicaal shifts were assigned in ppm.



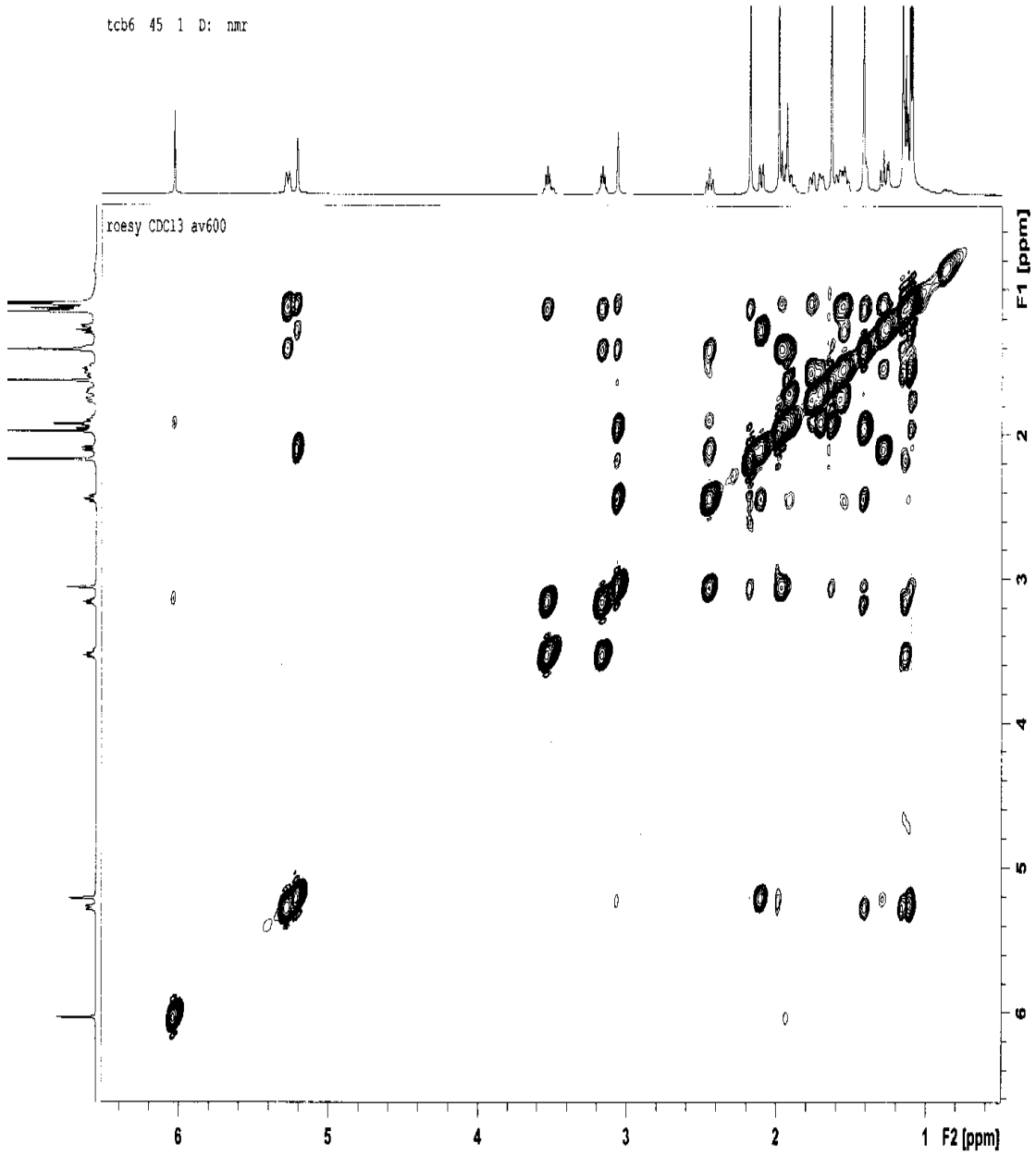
Appendix 21 Heteronuclear Multiple Quantum Coherence (HMQC) Spectrum of TCB 6 recorded at 600 MHz in deuterated chloroform $CDCl_3$.



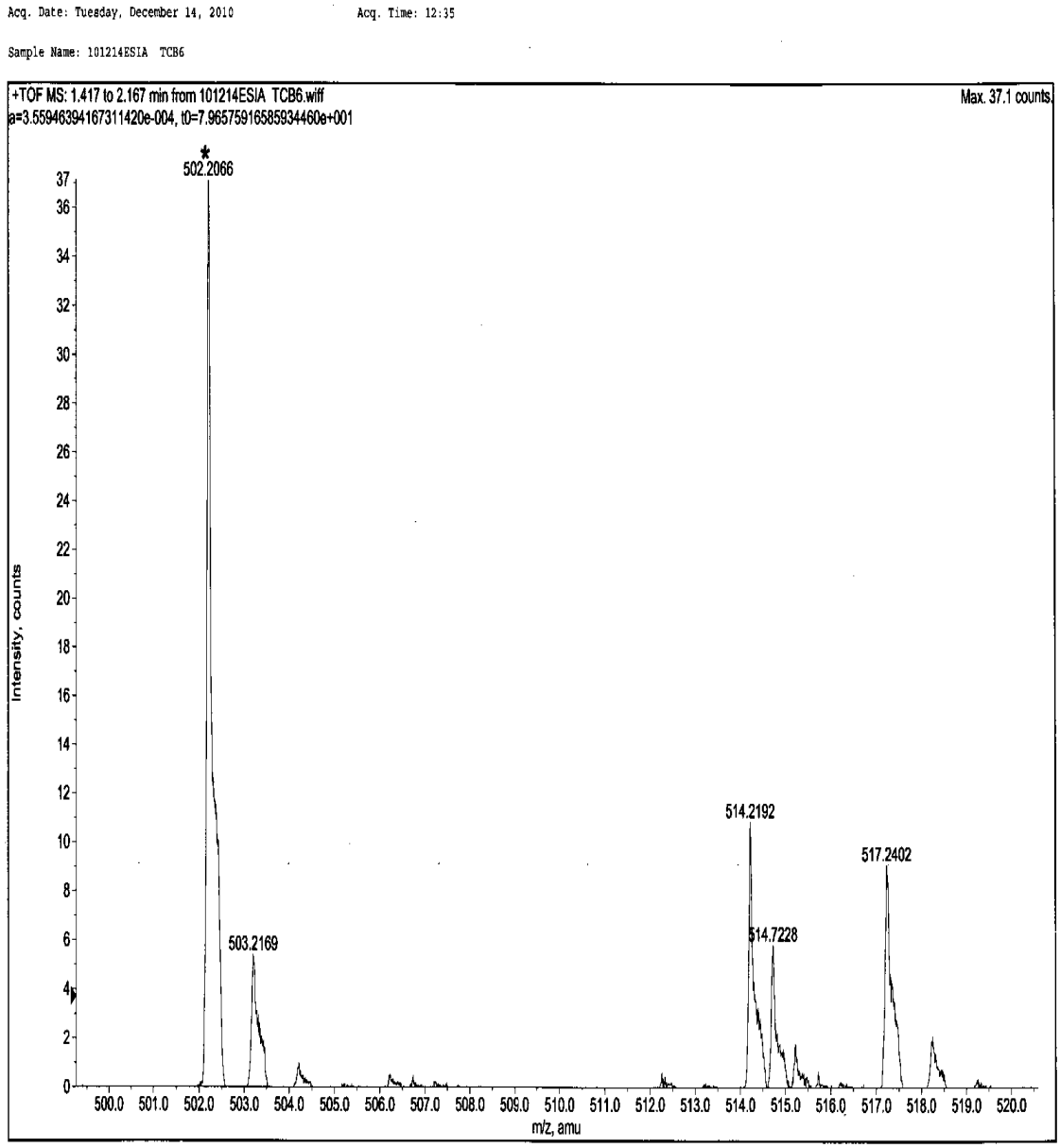
Appendix 22 Correlation Spectroscopy (COSY) Spectrum of TCB 6 recorded at 600 MHz in deuterated chloroform ($CDCl_3$).



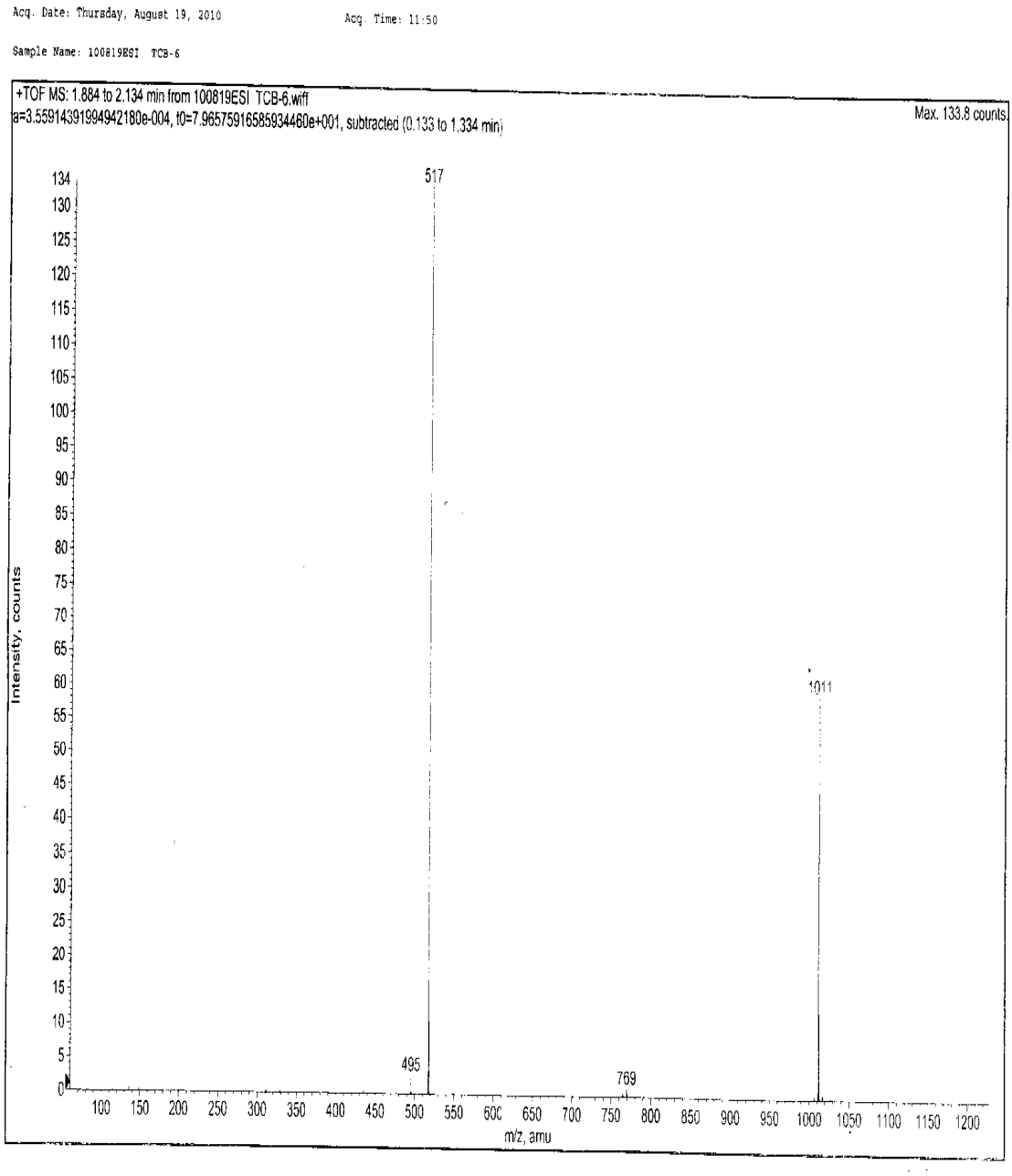
Appendix 23 Heteronuclear Multiple Bond Coherence (HMBC) Spectrum of TCB 6 recorded at 600 MHz in deuterated chloroform $CDCl_3$.



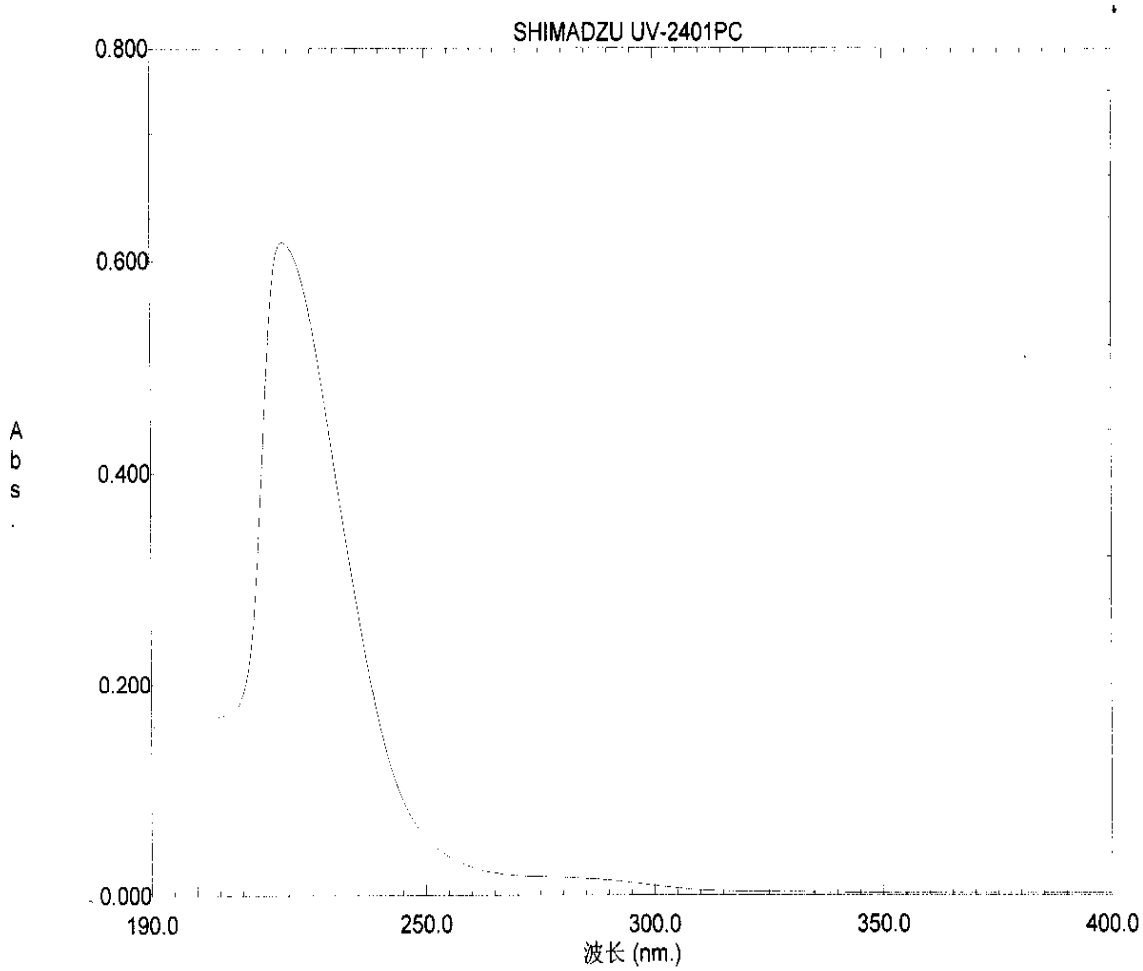
Appendix 24 Rotating-frame Overhauser Effect Spectroscopy (ROESY) Spectrum of TCB 6 recorded at 600 MHz in deuterated chloroform $CDCl_3$.



Appendix 25 High Resolution Electrospray Ionisation Mass Spectroscopy (HR - ESI – MS) Spectrum of TCB 6



Appendix 26 Electrospray Ionization Mass Spectroscopy (ESI-MS) Spectrum of TCB 6



文件名: TCB6

TCB6

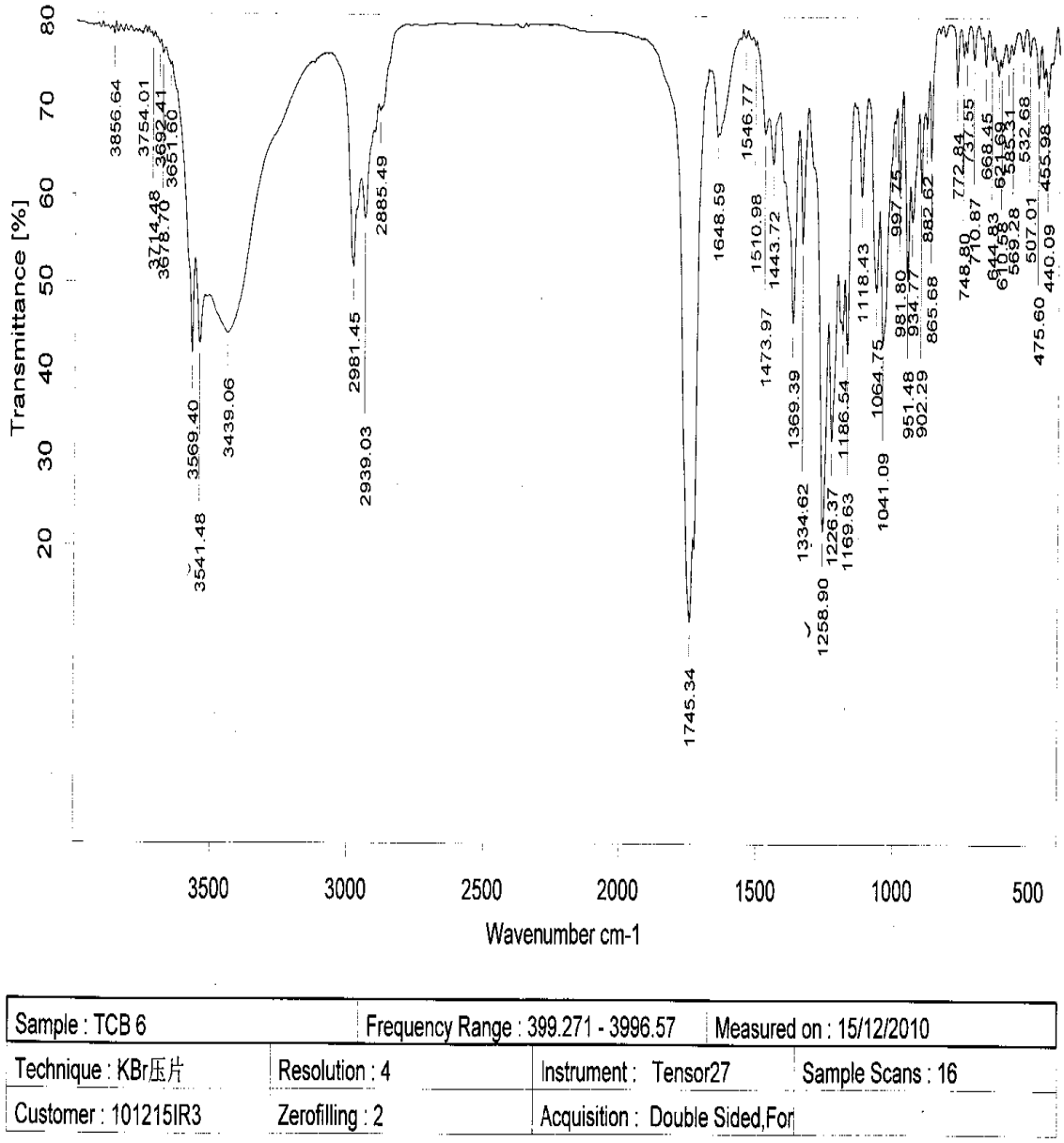
样品浓度：0.0320mg/ml
溶剂：MeOH

创建于: 16:15 10-12-13
数据: 原始

测量模式: Abs.
扫描速度: 中速
狭缝: 5.0
采样间隔: 0.2

否.	波长 (nm.)	Abs.
1	218.80	0.6170

Appendix 27 UV Spetrum of TCB 6



Sample : TCB 6	Frequency Range : 399.271 - 3996.57	Measured on : 15/12/2010	
Technique : KBr压片	Resolution : 4	Instrument : Tensor27	Sample Scans : 16
Customer : 101215IR3	Zerofilling : 2	Acquisition : Double Sided,For	

Appendix 28 IR Spectrum of TCB 6

Optical rotation measurement

Model : P-1020 (A060460638)

No.	Sample	Mode	Data	Monitor Blank	Temp. Cell Temp Point	Date Comment Sample Name	Light Filter Operator	Cycle Time Integ Time
No.1	13 (1/3)	Sp.Rot	-73.1250	-0.0468 0.0000	18.1 50.00 Cell	Tue Dec 14 16:00:49 2010 0.00128g/mlCHCl3 TCB6	Na 589nm	2 sec 10 sec
No.2	13 (2/3)	Sp.Rot	-74.6880	-0.0478 0.0000	18.1 50.00 Cell	Tue Dec 14 16:01:03 2010 0.00128g/mlCHCl3 TCB6	Na 589nm	2 sec 10 sec
No.3	13 (3/3)	Sp.Rot	-76.4060	-0.0489 0.0000	18.1 50.00 Cell	Tue Dec 14 16:01:16 2010 0.00128g/mlCHCl3 TCB6	Na 589nm	2 sec 10 sec

Appendix 29 Optical Rotation Measurement of TCB 6

Acq. Date: Tuesday, December 14, 2010 Acq. Time: 12:35

Sample Name: 101214ESIA TCB6

Elemental composition calculator

Target m/z: +517.2402 amu
Tolerance: +10.0000 ppm
Result type: Elemental
Max num of results: 1000
Min DBE: -10.0000 Max DBE: +60.0000
Electron state: OddAndEven
Num of charges: 0
Add water: N/A
Add proton: N/A
File Name: 101214ESIA TCB6.wiff

	Elements	Min Number	Max Number
1	Br	0	0
2	C	0	200
3	Cl	0	0
4	F	0	0
5	H	0	400
6	K	0	0
7	N	0	0
8	Na	1	1
9	O	8	10
10	Pt	0	0

Appendix 30 Elememental Calculation for TCB 6

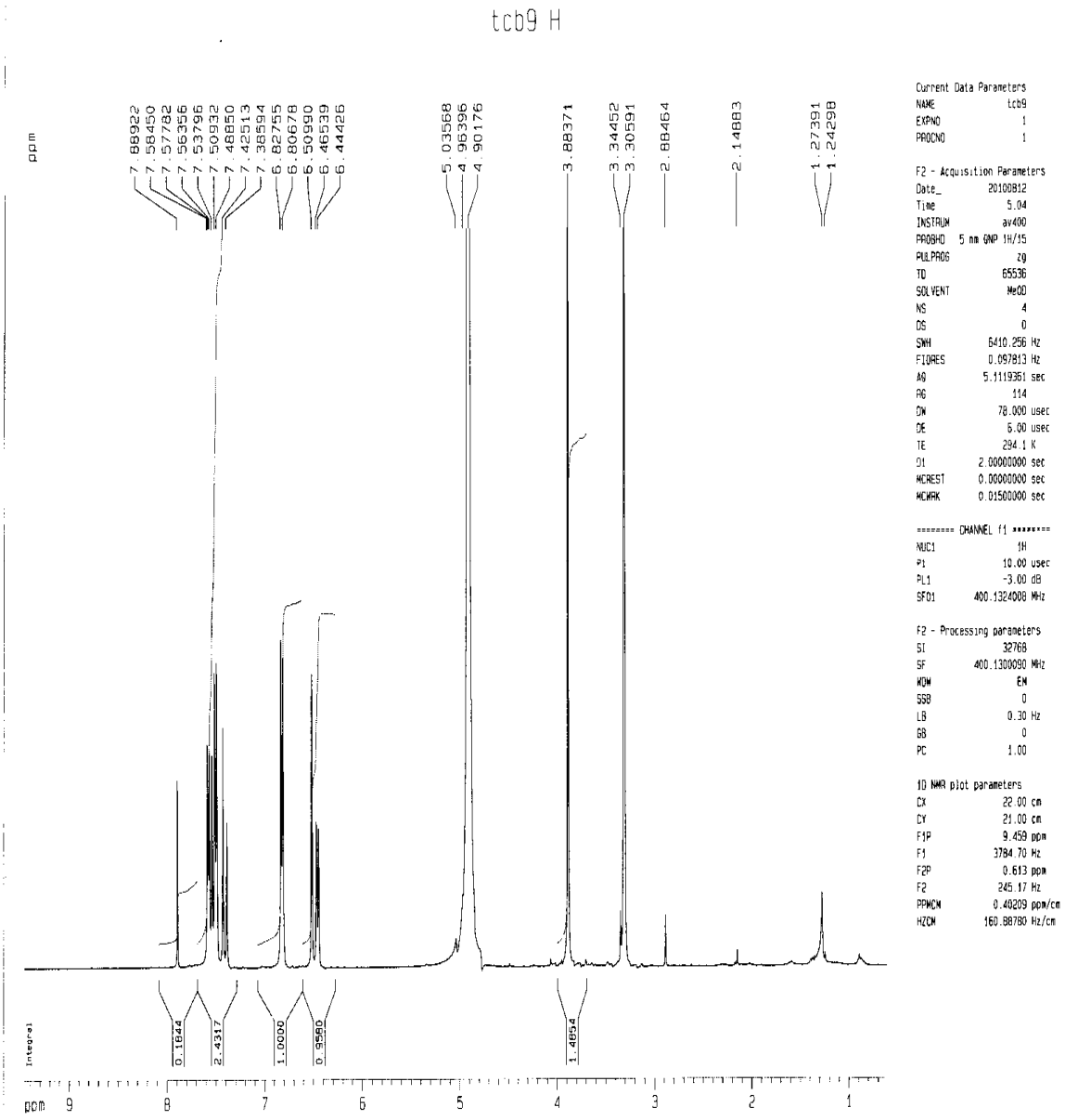
Acq. Date: Tuesday, December 14, 2010 Acq. Time: 12:35

Sample Name: 101214ESIA TCB6

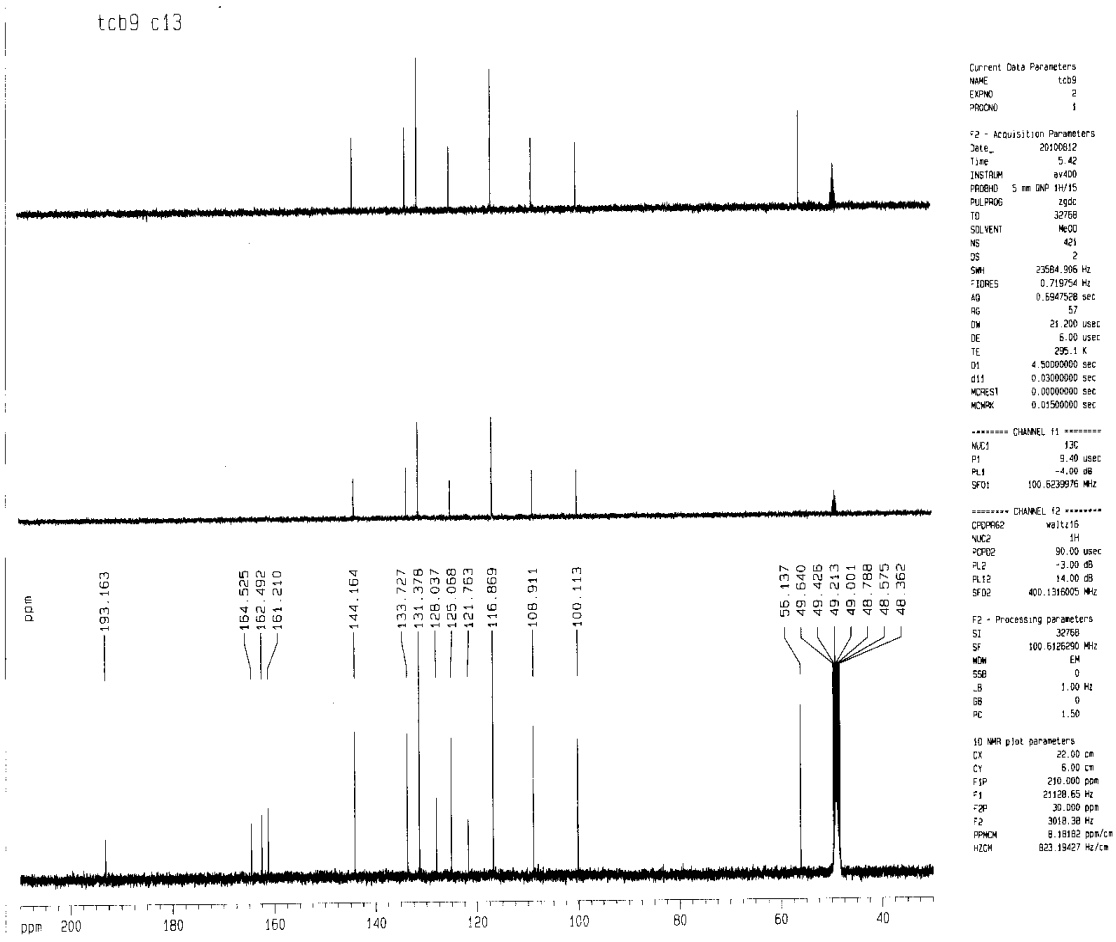
	Elements	Min Number	Max Number
11	S	0	0
12	Si	0	0

	Formula	Calculated m/z (amu)	mDa Error	PPM Error	DBE
1	C26 H38 O9 Na	517.2413	-1.1529	-2.2290	7.5

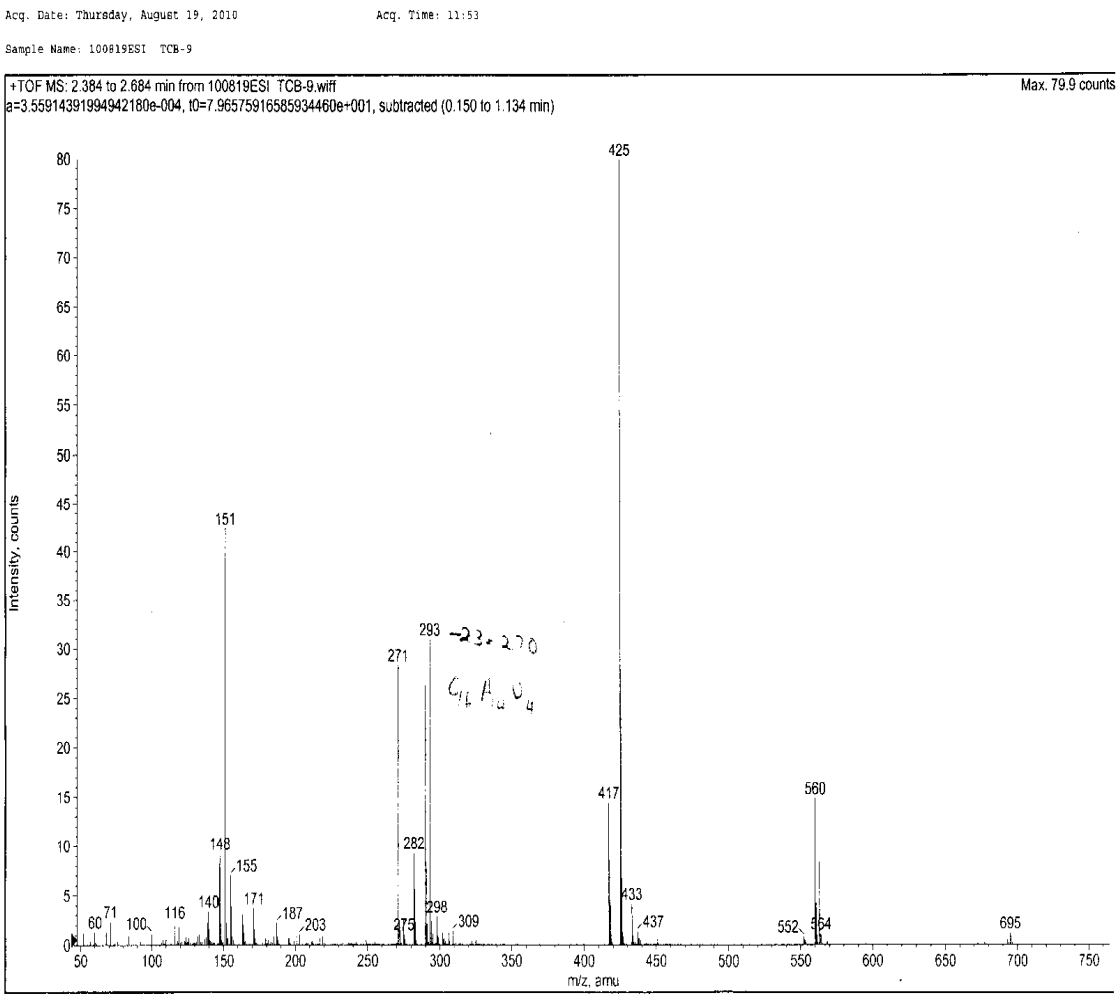
Appendix 31 Elemental Calculation for TCB 6 Continued



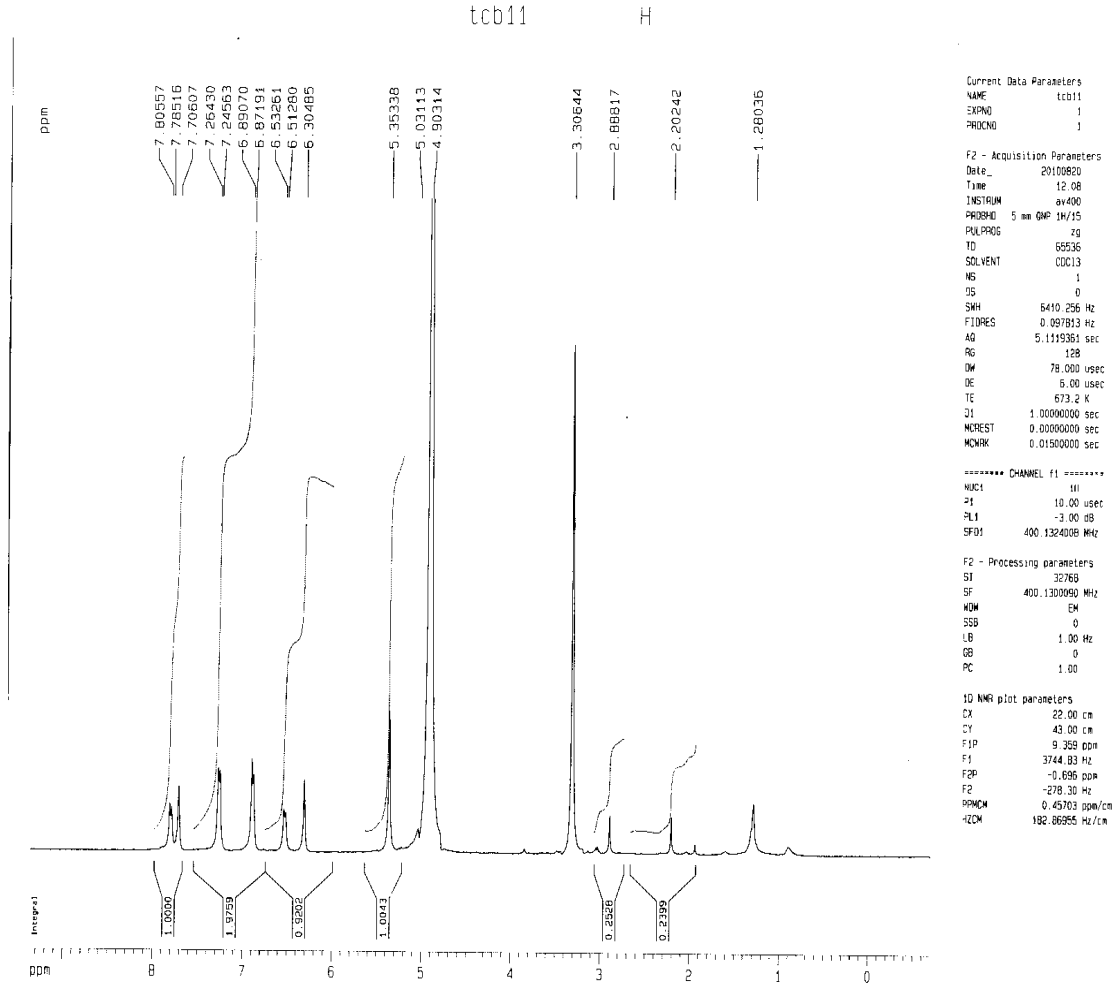
Appendix 32 ^{1}H NMR Spectrum of TCB 9



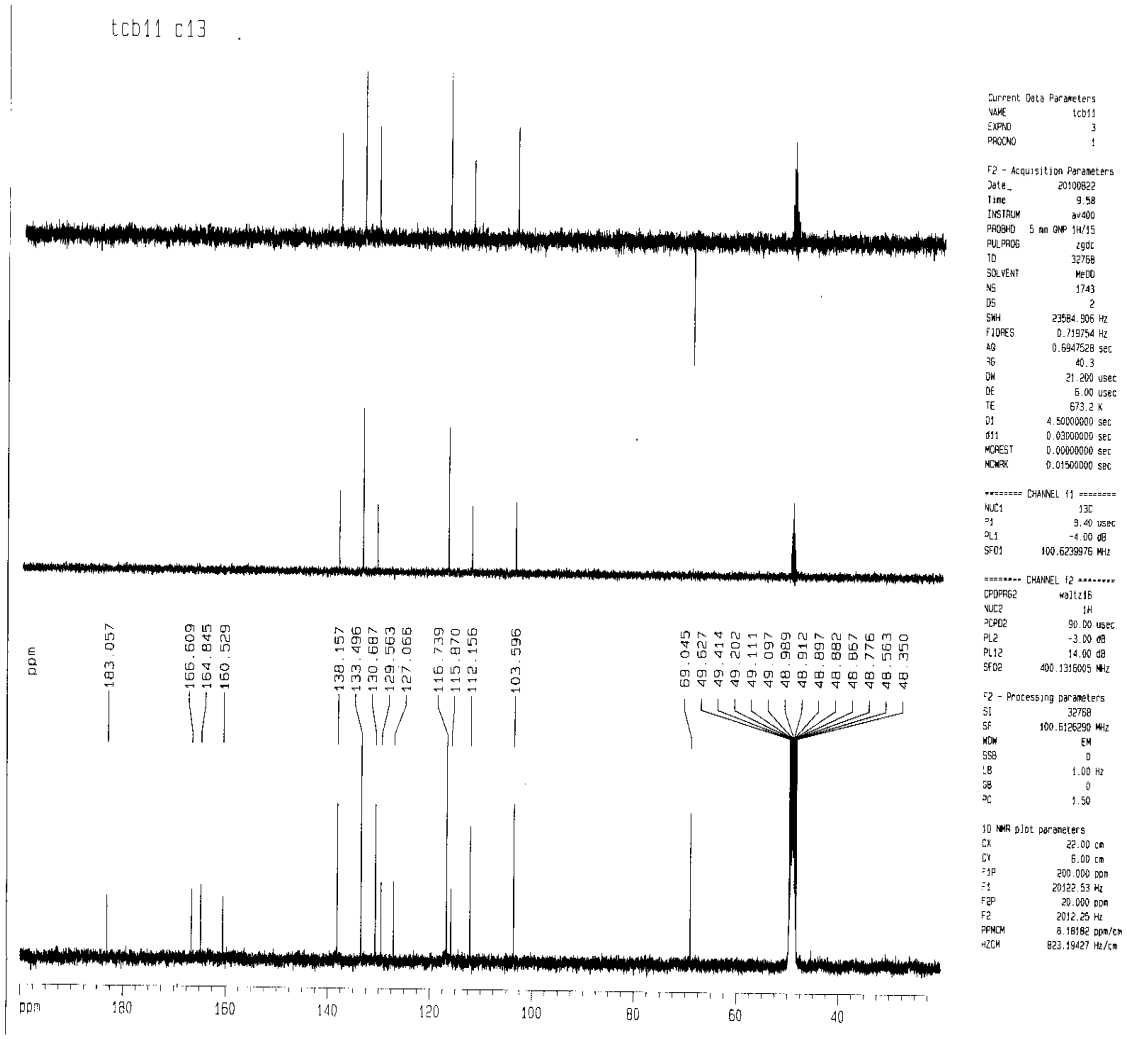
Appendix 33 ^{13}C NMR Spectrum of TCB 9



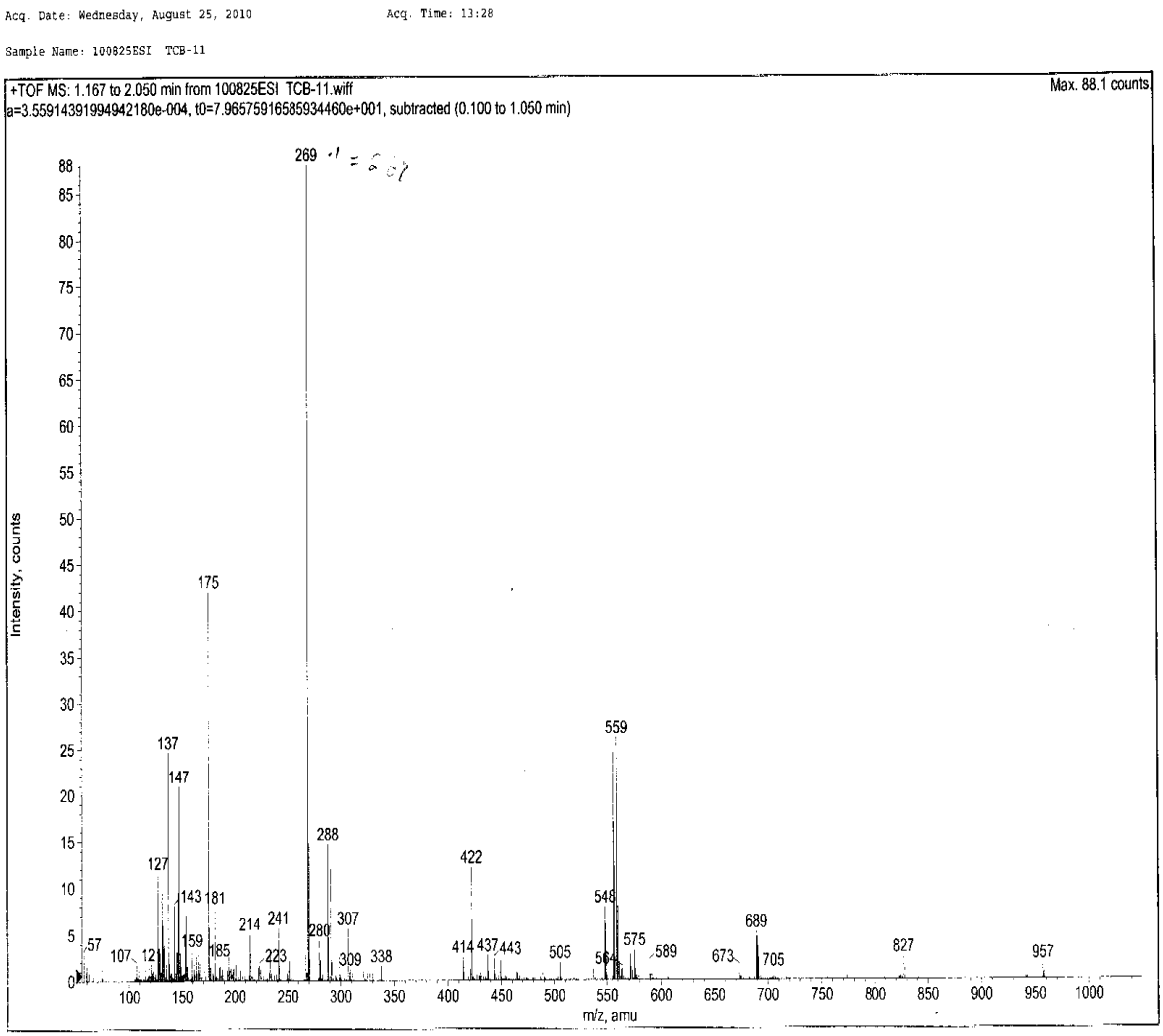
Appendix 34 ESI-MS Spectrum of TCB 9



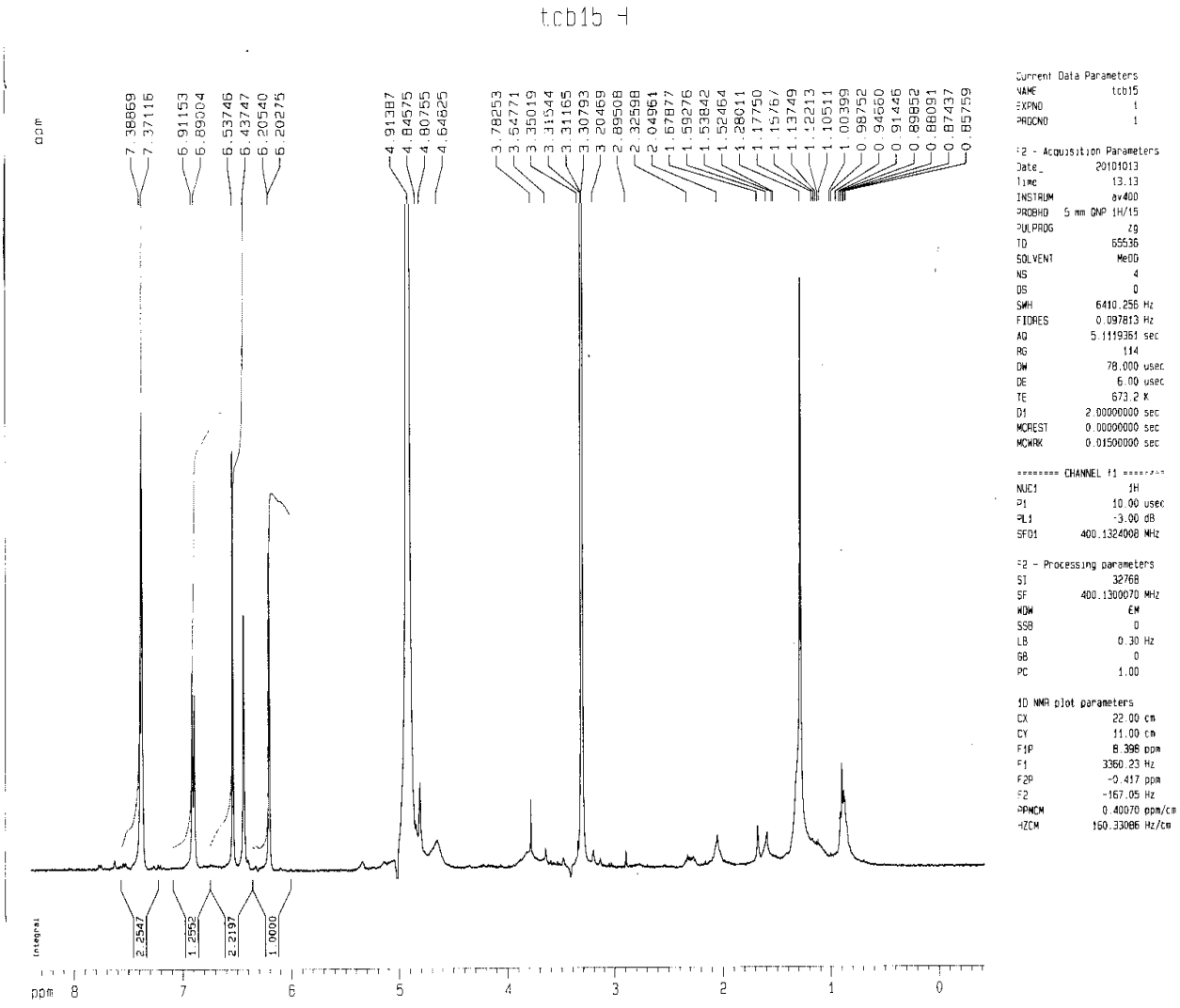
Appendix 35 ^{1}H NMR Spectrum of TCB 11



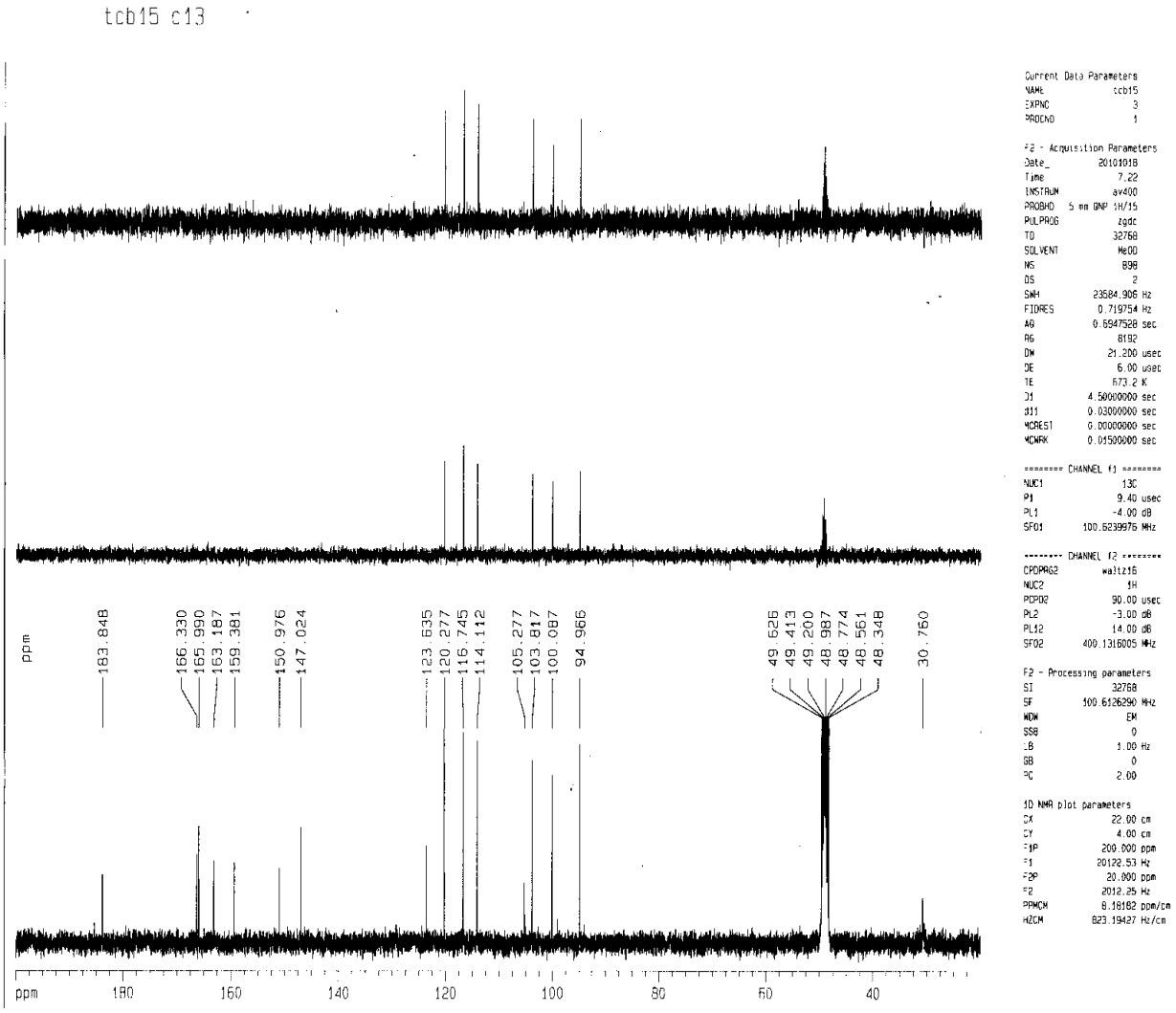
Appendix 36 ^{13}C NMR Spectrum of TCB 11



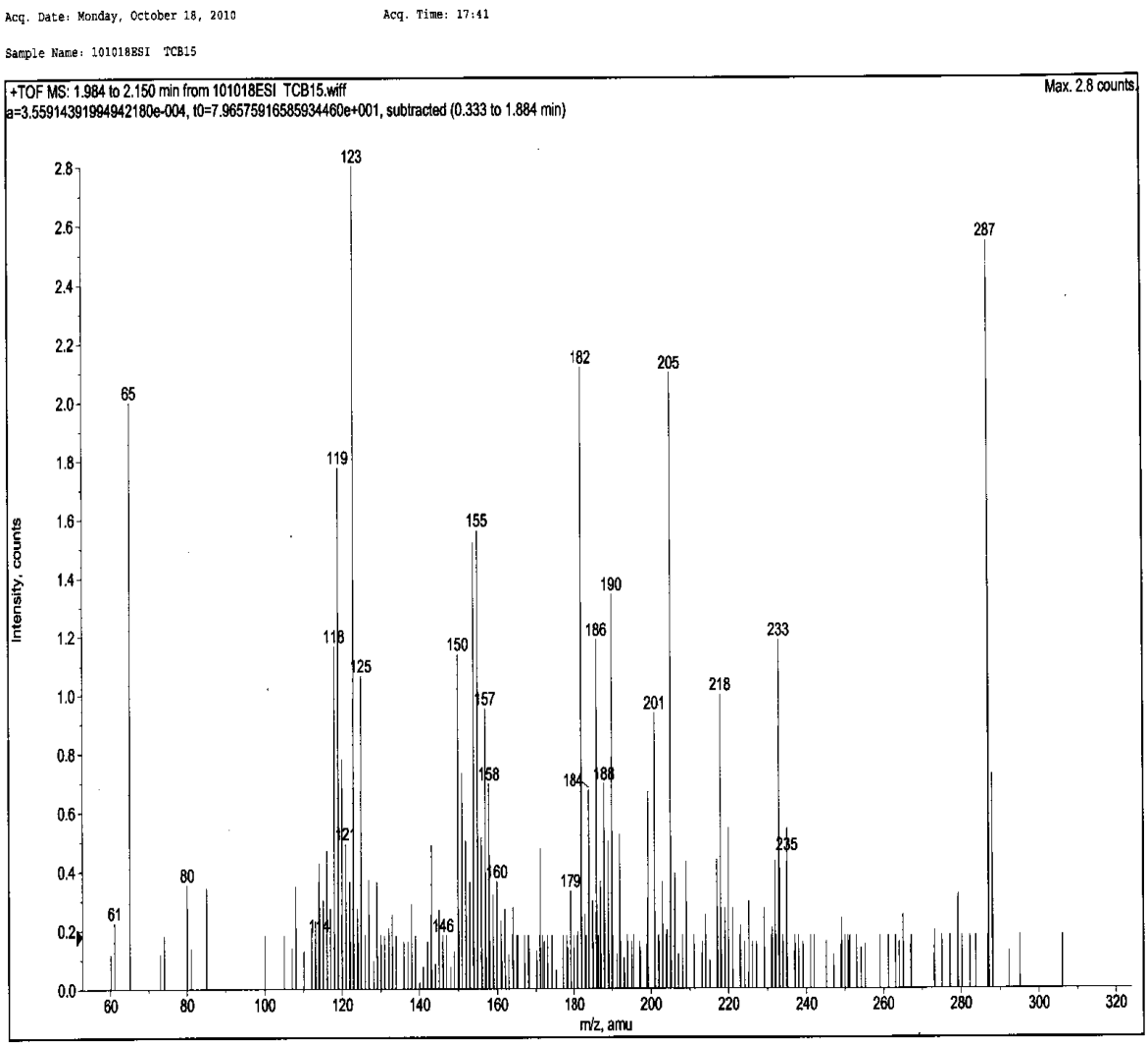
Appendix 37 ESI-MS Spectrum of TCB 11



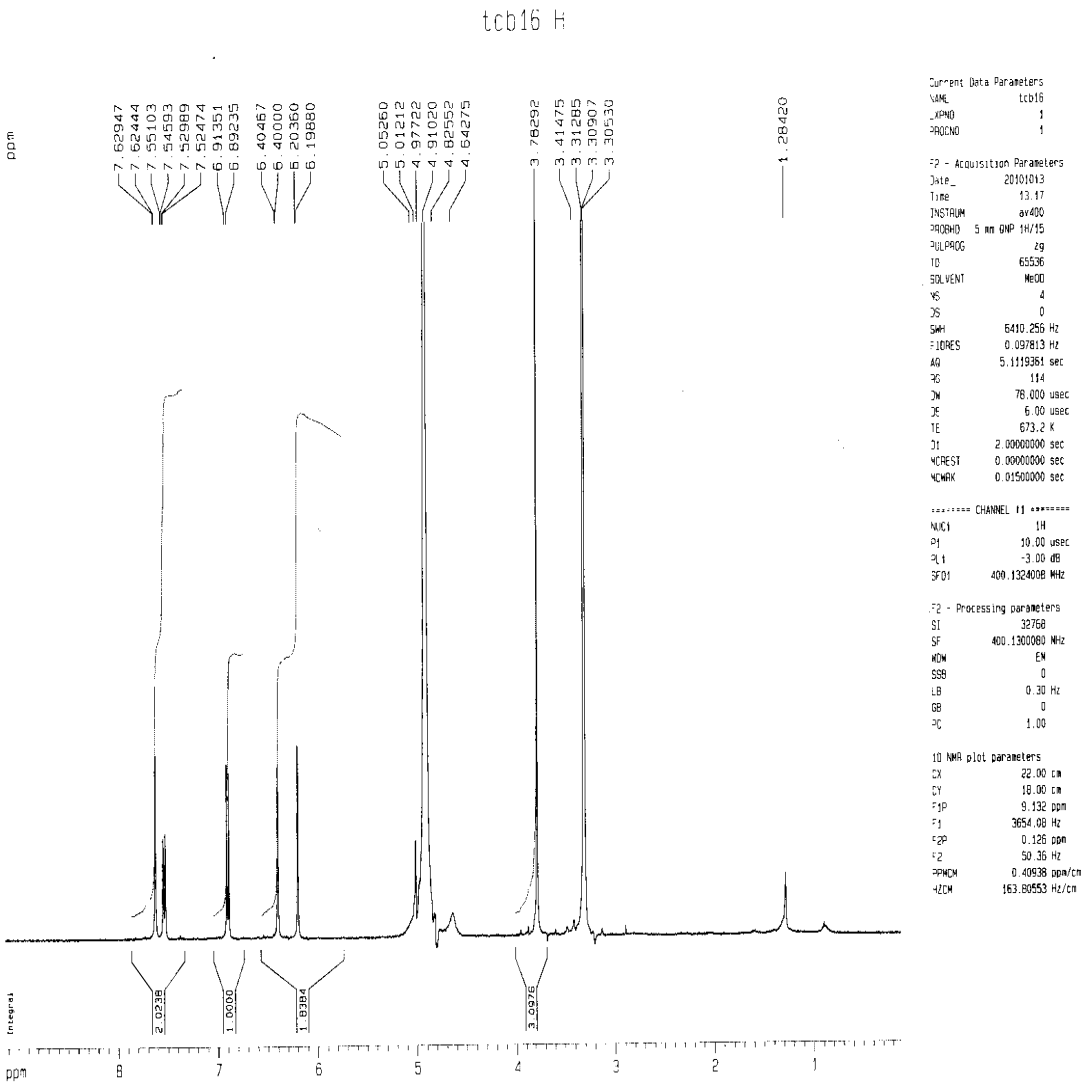
Appendix 38 ^{1}H NMR Spectrum of TCB 15



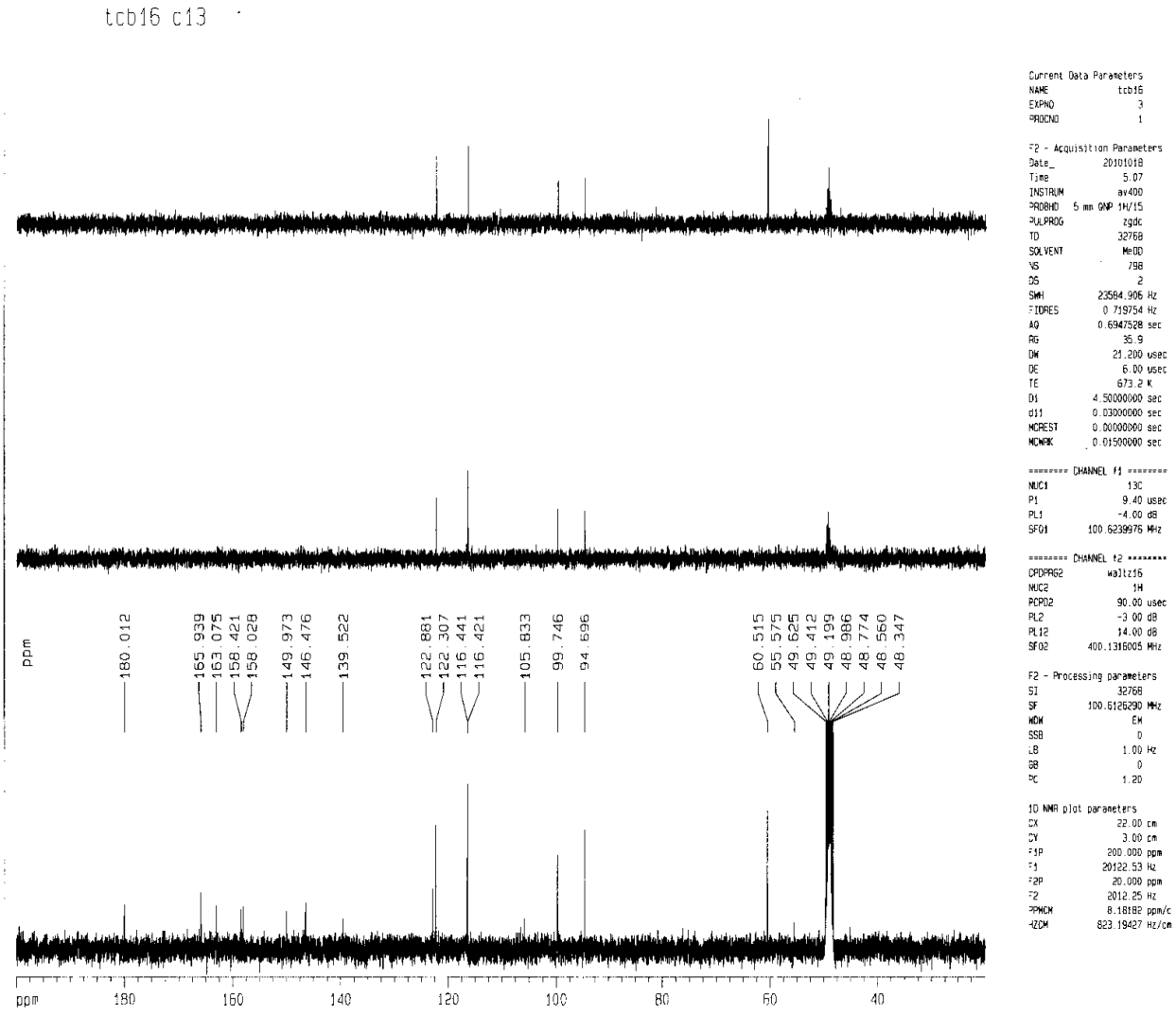
Appendix 39 ^{13}C NMR Spectrum of TCB 15



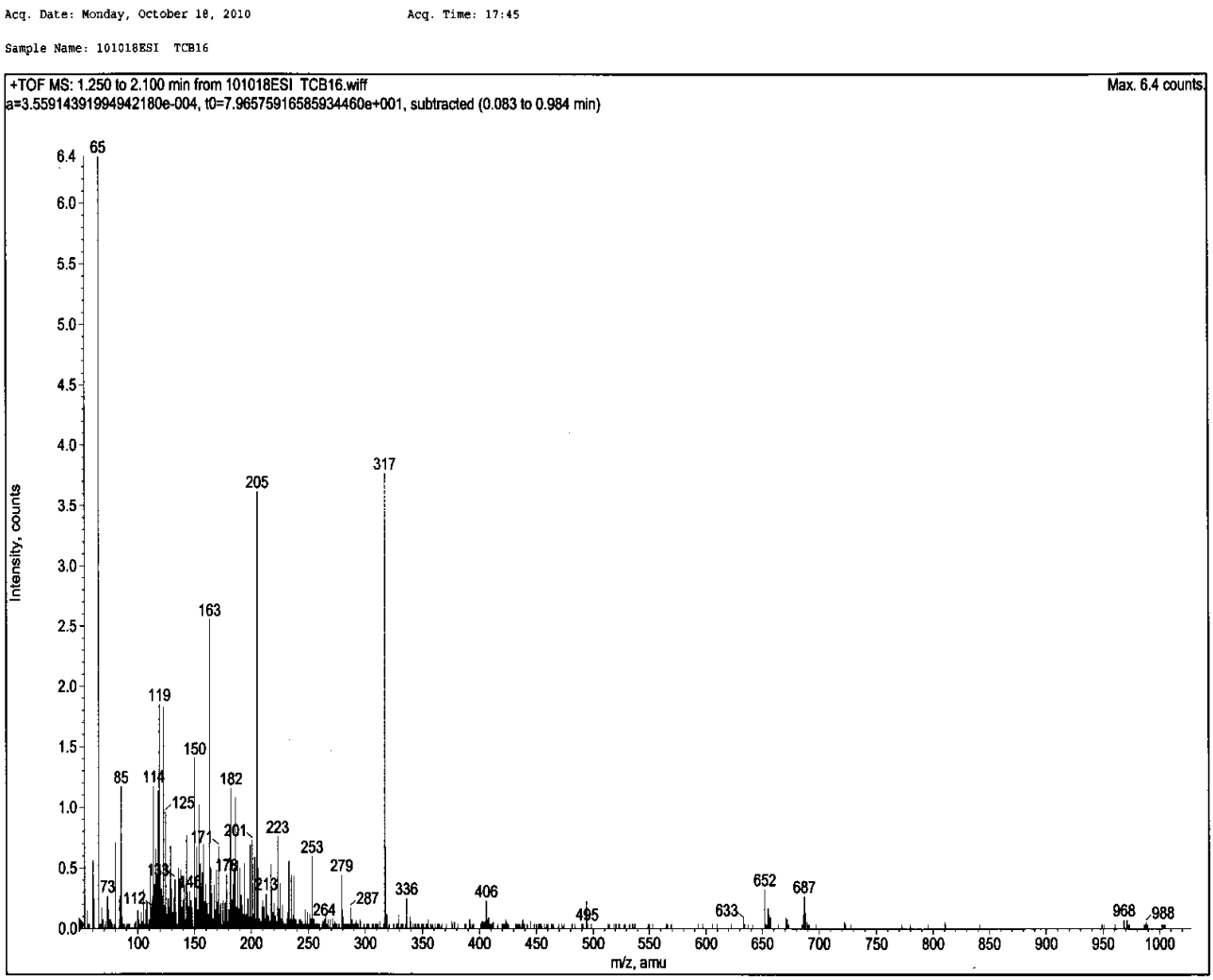
Appendix 40 ESI-MS Spectrum of TCB 15



Appendix 41 ^{1}H NMR Spectrum of TCB 16



Appendix 42 ^{13}C NMR Spectrum of TCB 16



Appendix 43 ESI-MS Spectrum of TCB 16

Appendix 44: Changes in weight of Experimental Animals for Sub-acute study

Groups	Weight 1(g)	Weight 2 (g)	Weight 3 (g)	Weight 4 (g)	Weight 5 (g)
Control A	100.40±32.5	119.40±27.7	122.80±27.5	133.2±25.9	146.80±29.8
Control B	88.40±21.8	115.60±23.3	122.00±24.3	135.20±22.6	151.20±26.4
200 mg/kg	96.57±14.6	116.00±15.0	129.43±11.9	142.29±16.9	158.00±12.9
200R	93.33±23.7	109.33±22.7	124.67±17.2	144.67±11.4	154.00±17.1
400 mg/kg	82.29±16.9	106.29±15.1	116.29±15.9	131.14±17.6	146.29±16.1
400R	78.00±12.5	102.67±15.3	113.33±12.2	132.67±13.0	146.00±16.4
800 mg/kg	103.43±21.1	119.43±18.7	125.71±18.3	130.86±26.9	152.00±24.6
800R	88.00±8.0	117.33±9.2	124.67±9.9	135.67±8.1	153.33±9.5
1600 mg/kg	73.67±23.9	94.33±22.4	106.67±23.9	113.67±20.1	125.00±20.3
1600R	57.33±15.3	73.33±8.3	84.00±10.4	94.33±10.6	103.33±9.9

Values are presented as mean ± SEM (n = 5 readings). Weight 1, weight 2, weight 3, weight 4 and weight 5 represent weight of rats on day 0, weight of rats on day 7, weight of rats on day 14, weight of rats on day 21 and weight of rats on day 28 respectively. Control A, Control B, 200 mg/kg, 200R, 400 mg/kg, 400R, 800 mg/kg, 800R, 1600 mg/kg and 1600R represent groups of rats treated with distill water; rats treated with sodium caboxylmethyl cellulose; rats treated with 200 mg CB/kg bwt; recovery group of rats treated with 200 mg CB/kg bwt; rats treated with 400 mg CB/kg bwt; recovery group of rats treated with 400 mg CB/kg bwt, rats treated with 800 mg CB/kg bwt, recovery group of rats treated with 800 mg CB/kg bwt, rats treated with 1600 mg CB/kg bwt and recovery group of rats treated with 1600 mg CB/kg bwt.

Appendix 45: Changes in weight of Experimental Animals for Acute study

Groups	Week 1	Week 2	Week 3
Control	127.00±3.00	129.00±4.00	131.67±4.16
2000 mg/kg	135.00±1.00	138.00±2.52	140.33±3.21
4000 mg/kg	128.00±1.73	131.00±1.00	133.33±1.53
6000 mg/kg	131.67±7.23	135.33±8.50	137.00±9.64

Values are presented as mean±SEM.