ANTIPLASMODIAL ACTIVITY OF EXTRACTS OF *CROTON MACROSTACHYUS* (EUPHORBIACEAE) AGAINST *PLASMODIUM FALCIPARUM* (D6) AND *PLASMODIUM BERGHEI* (ANKA)

BY

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DECLARATIONS

DECLARATION BY THE CANDIDATE

I declare that the content of this thesis is my original work and has never been presented for a degree in any other university.

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DEDICATION

I dedicate this thesi0s to my daughter Segrana, son James Jr., sister Dinahlee, brother-in-law Walter, brothers Octavius Jr., Lee, Wesley, Abayomi(late), father Octavius Sr, mother Mary (late) and husband James.

ABSTRACT

Medicinal plants have been used to cure malaria and parasitic diseases for centuries. This study was aimed at determining the effects of *Croton macrostachyus* (Euphorbiaceae) extracts *in vitro* using chloroquine sensitive *Plasmodium falciparum* (STRAIN D6) and *in vivo* using *Plasmodium berghei* (STRAIN: ANKA). Chemical analysis of the plant included extraction using methanol, ethyl acetate, butanol and water. Thin Layer chromatography and Column chromatography was used for chemical analysis of extracts. Structural elucidation was carried out using nuclear magnetic resonance (NMR) spectroscopy and Infrared(IR) spectroscopy . The *in vitro* study for *P. falciparum* included a growth inhibition assay and the minimum inhibitory concentration (IC_{50}) was determined.

An in vivo assay was carried out using BALB/c mice with *P. berhgei* (STRAIN ANKA). Thin blood films were prepared to determine the percent suppression of parasitaemia (PSP). An immunoprophylaxis assay was carried out to test the efficacy of the extracts as immune boosters. An acute toxicity experiment was carried out to determine the effect of the extract in clean, untreated BALB/c mice. Analysis for the *in vitro* study was done by Chemosen program to obtain a concentration-response data for a linear regression analysis. For the *in vivo* and immunoprophylaxis assays, analysis of variance (ANOVA) and tukey's test was used to analyze significance differences among and between mice treatment groups. Chemical analysis was carried out at The University of Eastern Africa, Baraton and University of Chicago, USA. The bioassays were carried out at the Kenya Medical Research Institute.

The *in vitro* IC_{50} were recorded as chloroquine (0.346±0.047), artemether (2.895±0.628), ethyl acetate extract (52.838±8.581), methanol extract (14.230±9.450) aqueous extract (43.446±2.218)butanol extract (26.163±5.487), pure compound F5(3.259±1.274) and pure compound $F5_2$(22.286±1.338). The *in vivo* treatment assay showed percent suppression of parasitaemia for ethyl acetate extract positive control (87.64±1.264), 500mg/kg(81.71±0.728), 250mg/kg(81.71±0.728) and 100mg/kg (61.82±0.571). The methanol extract showed percent suppression of parasitaemia for positive control (97.22±0.225), 500mg/kg(68.14±0.670), 250mg/kg (33.61±0.609) and 100mg/kg (27.44±0.443). The aqueous extract results were for positive control, 99.20±0.156, 500mg/kg, 71.85±0.447, 250mg/kg, 44.23±0.064 and 100mg/kg 24.36±0.447. The butanol extract give results for positive control as 100±0.000, 500mg/kg as 80.44±1.259, 250mg/kg as 60.66±0.445 and 100mg/kg as 72.69±0.306. The immunoprophylaxis or immune booster study showed that parasites were suppressed in the ethyl acetate experiment by chloroquine as 98.85±0.248, 500mg/kg as 99.88±0.058, 250mg/kg as 93.96±0.343 and 100mg/kg as 74.02±1.236. Using the methanol extract, percent suppression for the chloroquine control was 98.51±0.478, 500mg/kg was 83.04±0.635, 250mg/kg was 64.84±1.143 and 100mg/kg was 56.89±1.143. The butanol extract give percent parasitaemia for chloroquine control as 99.40±0.016, 500mg/kg as 92.07±0.294, 250mg/kg as 83.66±1.068 and 100mg/kg as 83.66±1.068. *C. macrostachyus* extract can serve as viable pharmaceutical agents for treatment and prophylaxis of malaria.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANKA	*Strain of Plasmodium berghei* parasite
ANOVA	Analysis of variance
HEPES	N-2-hydroxyethylpiperazine – N'-2- ethanesulfonic acid
HPLC	High performance liquid chromatography
IC_{50}	The minimum dose at which 50% percent of the parasite is inhibited
KEMRI	Kenya Medical Research Institute
MEM	Minimum essential medium
NMR	Nuclear magnetic resonance
PSP	percent suppression of parasitaemia
RPMI 1640	powdered malaria culture medium without folic or paraaminobenzoic acid
TLC	Thin layer chromatography
R_f	Refractive index
UNDP	United nations development program
UV	Ultraviolet spectroscopy
WHO	World Health Organization
STRAIN D6	Sierra Leonean Chloroquine sensitive strain of *Plasmodium falciparium*

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

Medicinal plants have been used to cure parasitic infections from time immemorial. It is estimated that the number of medicinal plants vary between 30,000 – 75,000 (Norman *et. al.,* 1985). For the past two decades there has be increased use of medicinal plants but little research has been carried out on them (WHO, 2009). The World Health Organization has compiled a list which contains 20,000 medicinal plants that are used all over the world. Approximately half of the world's flowering plants live in the tropics and about 50 drugs have been produced from tropical plants(Gurib-Fakim, 2006). Less than 30% of these registered plant species have been analyzed for potential medicinal properties.

A world health organization study has shown that 80% of the world's population relies solely upon medicinal plant as a source of remedies for the treatment of diseases, (Geoffrey, 1996). In China, India, Africa and Latin America, modern drugs are simply not available, or, if they are, they often prove to be too expensive, unavailable or inaccessible. Furthermore social factors may also make the application of "conventional" medical treatment difficult. The majority of drugs that are active against infectious agents are in fact developed from natural products ("leads"). As a source of novel drugs, plants remain grossly under-studied and under-used, especially in the developed world. Quinine, which was obtained from species of cinchona originating in South America, remains a vital drug in the treatment of malaria. Except for antifolate antimalarial drugs,

all the other commonly used antimalarial molecules are based upon plant-derived compounds (Geoffrey, 1996).

There is a new trend in the world today to turn back to natural substances due to the various side effects by some synthetic drugs (Huang *et al*., 1992). Production of unrefined drugs for export is also becoming very common in some countries. Examples include morphine from the opium poppy (*Papaver sominferum*), one of the best known pain killers. *Coca* leaves are also known to contain a strong stimulant (cocaine). Some steroid products with medicinal value have been isolated from plants; for example, diosgenin from *Dioscorea* species (Bergner, 2001) and stigmasterol from the soya bean (Carter *et. al.*, 2007). The study for herbal remedies in modern medicinal and agricultural practices is also being enhanced by pharmaceutical companies and research institutions in various countries where exploration of the medicinal plants is taking place (Hodgkin, 1991; Kofi-TseKpo, 1993a and 1993b). This will help in the management of forests and generating some foreign exchange to these countries. Plants also provide useful active compounds used to make insecticides, fungicides (pyrethrum plant) and industrial raw materials. The antimalarial activity of *Ajuja remota* has also been determined *in vivo* (Kuria *et. al.*, 2011).

1.2 Statement of the Problem

Malaria is one of the major parasitic infections in many tropical and subtropical regions, (Leonardo, *et al*., 1994; UNDP/WHO, 1990). *Plasmodium falciparum* is the most important agent of human malaria transmitted by the *Anopheles gambiae* mosquito in the human blood. It is a major cause of morbidity and mortality, claiming an estimated one to two million lives year in Africa alone (Geoffrey, 1996). The resistance of *P.*

falciparum to 4-aminoquinones (chloroquines, amodiaquine), antifols, and in some areas of Southeast Asia, the emergence of *in vitro* and *in vivo* resistance to aminoalcohols (quinine, mefloquine, halofantrine) have been reported (Leonardo *et al*, 1995). Problems associated with malaria control are mostly associated with growing drug resistance by the parasite. Some plant extracts used for the cure of malaria include *Enantia chlorantha* and *Rauwalfia vomitaria* (Agbaje and Elueze, 2006). The plant that was used in this study against *Plasmodium spp.* is *Croton macrostachyus*. It has been used traditionally to treat some diseases in Kenya (Kokwaro, 2009). There is need for more drug discovery for use in both complicated and uncomplicated cases of the disease and for prophylaxis purposes.

1.3 Objectives

1.3.1 General Objectives

1. To investigate compounds with *in vitro* and *in vivo* activities against *P. falciparum* from *Croton macrostachyus* bark extracts.

1.3.2 Specific objectives

1. To isolate and identify crude and pure compounds of *C. macrostachyus* bark extracts.
2. To determine *in vitro P. falciparum* parasite densities in erythrocytes of cultures treated with different extract dilutions and standard antimalarial drug.
3. To establish the *in vivo* efficacy of *C. macrostachyus* bark extracts against *P. berghei* infection in Balb/c mice.

4. To determine the *in vivo* immunoprophylactic properties and effects of *C. macrostachyus* crude bark extract in P. berghei infected Balb/c mice.

extracts *in vivo*.

1.4 Hypotheses

Null Hypothesis: The antimalarial and immunoprophylactic effects of *Croton macrostachyus* do not differ significantly from chloroquine or artemisinin.

Alternative Hypothesis: The antimalarial and immunoprophylactic effects of *Croton macrostachyus* are either significantly higher or lower than that of chloroquine or artemisinin.

1.5 Justification

Presently, multidrug resistance is a major obstacle to the control of *P. falciparum,* and its original modes of dissemination are imperfectly understood (Djimde *et. al.,* 2001). The first chloroquine resistance in *P. falciparum* was documented in 1960 in Venezuela but since then, advance studies have determined the genetics of drug-resistant *P. falciparum* in certain regions of Venezuela (Contreras *et. al.,* 2002). *In vitro* antifolate resistance was demonstrated in malaria strains from Brazil in the mid 1960s and recent studies have determined that the mechanisms of resistance is due to point mutations in dihydrofolate reductase and dihydropteroate synthase (Gregson and Plowe, 2005).

There has been an increase in the parasites, *Plasmodium falciparum* inclusive, developing resistance to some of the available medicines. For example, *Plasmodium falciparum* has been reported to being resistant to chloroquine which has recently activated a search for alternatives treatment from plant extracts and their compounds (Milhouse *et. al.,* 1985).

The purpose of this study was to look for an alternative drug for the treatment of malaria, and determine the biologically active compounds of *Croton macrostachyus*. These active compounds can be used to synthesize the analoques which could have enhanced activity and these can be mixed to determine the synergistic and wholistic effects on the parasite.

Protozoan infections is largely a problem of developing countries, thus, offers little commercial incentive for the pharmaceutical companies to develop cheap but effective antileishmanial drugs. Unlike pathogenic bacteria, protozoa share many biochemical pathways with the human host. It is therefore often difficult to identify compounds with sufficient selectivity to kill the parasite without damaging cells of the human host (Geoffrey, 1996). Because of the unavailability of effective and affordable drugs, many people in the parasite-endemic areas rely on traditional medical remedies for treatment. Therefore, it is possible that there are several medicinal plants which are yet to be identified and screened for the presence of new antiparasitic agents. The lack of effective drugs for the treatment of parasitic diseases has led to renewed interest in the re-evalution of ancient traditional remedies as sources of "leads" in the development of new antimalarial agents with better activity and low toxicity.

The main advantages associated with the exploitation of medicinal plants which have been used traditionally are:-

These plants provide locally accessible alternatives to imported drugs that will be accepted by the population both because of their cheapness and have been used for centuries.

They may be used together with western pharmaceutical products by being integrated within the framework of official health services in order to take advantage of the positive features of both traditional and modern medicines.

They will in the long run catalyze the establishment of a pharmaceutical industry based on local resources. This will be of benefit to the national economy and will provide a basis for the discovery of new substances that might be useful against intractable ailments.

CHAPTER TWO

LITERATURE REVIEW

2.1 Medicinal Plants Against Malaria and Other Parasitic Infections

Many plants have already been screened for antimalarial activity and have been shown to have activities against *Plasmodium falciparum* both *in vitro* and *in vivo* (Muthaura *et. al.,* 2007).

The antimalarial activity of boiled water extracts of *Cymbopogon giganteus* and *Enantia chlorantha* was assessed against chloroquine-resistant *Plasmodium yoeli nigeriensis* and it was shown that the two extracts cleared the infection in a dose dependant manner after treatment for three days with extract dilutions (Kimbi and Fagbenro-Beyioku, 1996). Four extracts with antimalarial activity were also tested *in vitro* using *P. falciparum* cultures and two of them, *Vernonia brasiliana* and *Acanthospermum australe*, were active. Extracts of *V. brasiliana* caused about 50% inhibition of parasite multiplication at relatively low doses (40 ng/ml) as compared to quinine (50 ng/ml).

In Serberida and Indonesia, medicinal plants are used mostly against malaria and fevers and a high frequency of the leaves of these plants may have a cultural phenomenon (Mahyar, *et. al*., 1991). Six different plant species including *Vernonia brasiliana* and *Eupatorium squalidum* were tested and demonstrated antimalarial activity *in vitro* (Cavalho *et. al*., 1991). In another study including fifty-eight Tanzanian medicinal plants screened, four of the plants (*Cissampelos mucronata, Maytenus senegalensis, Salacia madagascariensis, Zanthoxylum chalybeum*) showed strong antimalarial activity with IC_{50} values below 10 micrograms/ml (Gessler *et. al.,* 1994).

Knowledge about new malaria drugs and vaccine production has shown that there is prospect for the future. Multidrug resistance inhibitiors were produced and some showed no promise but one, WR268954 seems to be promising. One new vaccine that will be promising is the DNA vaccine. DNA fragment coding an appropriate immunogen can be incorporated to plasmid and injected intramuscularly, intranasally or with a transdermic gun. The plasmids go into the nucleus of the host cell and the corresponding mRNA allowsthe synthesis of immunogenic protein that is assembled by MHC-I molecules on T-cell surface receptor. This gives rise to receptor binding in a cellular cytotoxic immunity against Plasmodium parasites (Ambrose-Thomas, 1998).

Compounds such as furoquinolines and acridone alkaloids have been isolated from several plants in the Rutaceae family (Mitaku, *et al,* 1985). Plants such as *Geijera balansae, Sarcomelicope glauca,* and *Sarcomelicope dogniensis* have yielded furoquinolies and acridone alkaloids which have shown some antimalarial activities in-*vitro* and *in vivo* (Leonardo *et al.,* 1994). With the emergence of chloroquine-resistant *falciparum* malaria and reports of parasite resistance to alternative drugs, there has been renewed interest in antimalarial activity of the acridines (Figgitt *et al.,* 1992) and congeners including acridones (Leonardo *et al,* 1994) and dihydroacridinediones (Berman *et al*., 1994). Compounds like pyronaridine and mepacrine have shown high antimalarial activity against *P. falciparum,* (Elueze, *et al*., 1996). These compounds have been developed using the chemical structure of quinine.

Some of the plants from as many taxonomic families which have been shown to contain compounds with significant activity against malaria Africa are listed in Table 2.1.

The plant to be analyzed in for this study against malaria is *Croton macrostachyus* (Euphorbiaceae) (Kokwaro, 1976), which is not included in table 2.1. This plant has been used in various parts of Kenya for the cure of malaria but has not been analyzed for antimalarial active components and activity in Kenya.

Table 2.1: Some Medicinal Plants With Antimalarial Activity

1. Piper nigrum	*8. Cissampelos mucronata,*
2. Zanthoxylum chalybeum,	*9. Leonotis mollissima,*
3. Ximenia sp.	*10. Salacia madagascariensis*
4. Pavetta crassipes,	*11. Azadirachta indica,*
5. Margaritaria discoidea,	*12. Heinsia crinita (subsp.paruiformis),*
6. Erythrina sacleuxii,	*13. Maytenus senegalensis,*
7. Cussonia zimmermannii,	*14. Achyranthes aspera*

(Source : Gessler, *et al.*, 1995)

Several other plants have been tested and the structure of their active antiplasmodial ingredients elucidated. The study of the stem bark of Teclea aftzelii(Rutaceae) has resulted in isolation and characterization of four furoquinoline alkaloids, namely kokusaginine, tecleaverdoornine, masculine and montrifloline together with lupeol and β-sistosterol glucopyranoside (Wansi et. al., 2009). Another study by Ziegler (2009) proposed that incubation of lupeol from Rinorea ilicifolia with P. falciparum parasites grown in erythrocytes caused pronounced changes of the curvature of erythrocyte membrane towards stomatocytes and this correlated well with the observed IC_{50} value for inhibition. Emodin and lupeol were isolated from the ethyl acetate extract of Cassia siamea L. (Fabaceae) and were found to be the active principles responsible for the antiplasmodial property with IC_{50} values of 5μg/ml and 11.8ug/ml respectively (Ajaiyeoba et al., 2008).

In a study based on experiments employing parasite cultures synchronized by magnetic cell sorting (MACS), both lupeol and betulinic acid inhibited the proliferation of *Plasmodium falciparum* parasites by inhibition of the invasion of merozoites into erythrocytes (Ziegler *et. al.*, 2006). The evaluation of four Indian Diospyros species viz., *Diospyros melanoxylon*, *D. peregrine*, *D. sylvatica*, *D. tomentosa* for antiplasmodial activities against chloroquine-sensitive (3D7) and chloroquine-resistant (K1) strains of *P. falciparum* showed that six methanolic extracts were found to have significant activity, (IC_{50} =16.5 – 92.9 ug/ml) against 3D7 and five of these showed similar activities against strain K1 (IC_{50} = 20.5 -121.6µg/ml). *Diospyros sylvatica* was found to be the most active species (IC_{50} = 16.6 – 29.4 µg/ml) (Kantamreddi and Wright, 2007).

Ten triterpenoids were isolated and identified from the twigs of *Gardenia saxatilis* (Rubiaceae) and tested for their antiplasmodial activities. Of the ten, six compounds, namely lupenone, lupeol, betulinic acid, oleanolic, acid ursolic acid and winchic acid (27-O-feruloyloxybetulinic acid) were inactive in the assay. The other four compounds, messagenic acid A and messagenic acid B, the 27-O-p-(Z)- and 27-O-p-(E) coumerate esters of betulinic acid, and a mixture of uncarinic acid E (27-O-p-(E)-coumaroyloxyoleanolic acid and 27-O-p-(E)-coumaroylozyursolic acid exhibited antiplasmodial activity with the IC_{50} values of 1.5, 3.8 and 2.9 ug/ml respectively (Suksamram *et. al.* 2003).

In other malaria studies, ethanol extract from *Cassia occidentalis* leaves, *Euphorbia hirta* whole plant, *Garcinia kola* stem bark and *Phyllanus niruri* whole plant gave an IC_{50}<3 ug/ml (Tona *et. al.*, 2004). An ethnopharmacological investigation conducted among the Baka pygmies of Dja biosphere reserve in Cameroon showed that the stem barks of

Holarrhena floribunda had remarkable inhibitory activity against drug-resistant strains of *Plasmodium falciparum* at doses of 1.02 – 18.53 ug/ml when tested *in vitro* against two parasite clones designated against Indochina (W-2) and Sierra Leone (D6) (Fotie *et. al.*, 2005).

Menthol, a monoterpene (10 carbons) isolated from various mints is a topical pain reliever and antipuretic (relieves itching). Borneol, derived from pine oil, is used as a disinfectant and deodorant, camphor as a counterirritant, anesthetic, expectorant, and antipruritic (Miguel, 2010). Gossypol is a dimeric sesquiterpene which has been used clinically as a male contraceptive (Liu, 1981). Taxol has been isolated from *Taxus baccata* and is used as an anticancer drug (Malik *et. al.*, 2011). It acts to stabilize the mitotic apparatus in cells, causing them to act as normal cells rather than undergo rapid proliferation as they do in cancer. Triterpenes contain 30 carbons, derived essentially from coupling of two sesquiterpene precursors. Arbruside E., comes from *Arbrus precatorius* (jequirity) which has been used as an abortifacient and purgative (Zore *et. al.*, 2007). Steroids such as cortisone, are most often used as anti- flammatory agents, but many have other uses such as in birth control pills. Reduced sensitivity of *Plasmodium sp.* to formally recommended antimalarial drugs places an increasing burden on malaria control programs as well as on national health systems in endemic countries. Betulinic acid and its derivatives compounds, betulonic acid, betulinic acid acetate, betulinic acid methyl ester, and betulinic acid methyl ester acetate showed antiplasmodial activity against chloroquine-resistant *Plasmodium falciparum* parasites *in vitro*, with IC_{50} values of 9.89, 10.01, 5.99, 51.58, and 45.79 μM, respectively (de Sa *et. al.*, 2009). Mice infected with *Plasmodium berghei* and treated with betulinic acid acetate had a dose-

dependent reduction of parasitemia in the above-mentioned study. The *in vitro* antiplasmodial IC_{50} values of betulinic acid against chloroquine resistant (K1) and sensitive (T9-96) *Plasmodium falciparum* were found to be 19.6 micrograms/mL, and 25.9 micrograms/mL respectively (Steele *et. al,* 1999).

Betulinic acid seems to work by inducing apoptosis (programmed cell death) in cancer cells. Betulinic acid has also been found to retard the progression of HIV 1 infection, by preventing the formation of syncytia (cellular aggregates).In addition, betulinic acid has antibacterial properties and inhibits the growth of both *Staphylococcus aureus* and *Escherichia coli*. Betulinic acid`s anticancer property is linked to its ability to induce apoptotic cell death in cancer cells by triggering the mitochondrial pathway of apoptosis (Fulda, 2008). Normal cells and tissues are relatively resistant to betulinic acid, pointing to a therapeutic window (Yi Bi *et. al.,* 2006). Betulinic acid ester was tested for antitumor activity *in vivo* and it was found that it had a higher anti-tumor activity than 23-OH betulinic acid and had similar activity with cyclophosphamide and 5-fluorouracil (Yi Bi *et. al.,* 2006).

Most conventional drugs are used against malaria without achieving a complete cure of the infections. What is often achieved is a suppression of the symptoms of the disease with the parasite re-appearing in immunosuppressed subjects (Bartoloni and Zammarchi, 2012). Only a few alternative drugs such as artemesinin, its derivatives and atovaquone are under development (Leonardo, *et al.,* 1995). Atovaquones (coded 566c80), a hydroxynaphthoquinone, is a novel antiparasitic drug which as shown activities against leishmaniasis and malaria.

Scientific evaluation of medicinal plants used in the preparation of traditional medicine, has, in the past, provided modern medicine with very effective drugs for the treatment of parasitic diseases, (Iwu, 1994). Many of the antimalarial drugs in use at present were either obtained from plants (e.g. quinine and artemisinin), or have been developed using the chemical structure of quinine, or more recently the peroxide moiety of artemisinin as template (Wright and Phillipson, 1990; Warrell, 1993). Quinine, isolated from several species of *Cinchona* found in many tropical countries, was the first effective anti-malaria drug discovered. The drug is still used in clinical treatment of severe malaria due to chloroquine resistant *Plasmodium falciparum* (Alecrim *et. al.*, 2006). Endoperoxide sesqiterpene, artemisin, isolated from Chinese medicinal herb *Artemisia annua*, a novel antimalarial plant. Emetine, the amoebicidal drug, was obtained from *Cephaelis ipecacuanha*, a plant which has been used in traditional medicine in Asia for centuries (Iwu, 1994).

Table 2.2: Medicinal Traditional Uses of *C. macrostachyus*, the Plant That Was Tested Against Malaria

Botanical name	Ailment treated	Part of plant used	Family
Croton macrostachyus	Coughs	-leaves	Euphorbiaceae
	helminthes	-root	
	Skin Rashes	-Stem bark, roots	
	Wounds	-leaves	
	Malaria	-root	
	Venereal diseases	- root	
	Flu	-bark	

Adapted from Kokwaro (2009).

In a study by Steel *et al*,. (1999), when lupane-type triterpene betulinic acid was isolated from an ethanol extract of the root back of the Tanzanian tree *Uapaca nitida* Mul-Arg. (Euphorbiaceae) Matheus *et al.* (2009) stated that reduced sensitivity of P*lasmodium sp.* to formally recommend antimalarial drugs places an increased burden on malaria control program as well as on national health systems in endemic countries. The methalonic extract of the rhizomes parts of *Angelica purpuraepolia* was investigated for its activity against chloquine-sensitivity stains of *Plasmodium falciparum* using the parasite lactate dehydrogenase easy method. Two natural khellactone, (+)-4' Decanoyl-cis-khellactone and (+)-3'-Decanoyl-cis-khellactone were isolated from the rhizomes parts of *A. purpuraefolia*. Two compounds were evaluated for *in vitro* antiplasmodial activities and well as their cytotixic potential on SK-OV-3 cancer cell line cells. Compounds 1, 2 showed notable growth inhibitory activity against chloroquine-sensitive strains of *Plasmodium falciparum* with IC_{50} values from 1.5 and 2.4 µM. This compound showed no significant cytotoxicity (IC_{50} > 100 µM) evaluated using SK-OV-3 cancer cell in cells (Ill-Min *et al,* (2009).

Phytochemicals investigation of the hexane and CH_2CL_2 extracts of *Erythrina stricta* roots and *E. subumbrans* stems led to the isolation of six pterocarpans, one flovanone, one isoflavone, two alkaloids, five triterpenes, six steroids and alkyl trans-ferulates. The structures of all known compounds were determined on the basis of spectroscopic evidence. Sophodadial, a mixture of stigmast-4-en-3-one and stigmasta-4-22-dien-3-one and 3β-hydroxystigmast-5,22-dien-compounds were evaluated for antiplasmodial activity, antimycobacterial activity and cytotoxicity. Among the tested compounds, 5-hydroxysophoranonne exhibited the highest antiplasmodial activity against *Plasmodium*

falciparum (IC_{50} 2.5 µg/mL). Compounds 8, erystagallin A , erycristagallin and erysubin F showed the same level of antimycobacterial activity against mycobacterium tuberculosis (MIC 12.5 .5 µg/mL). For cytotoxicity, erybraedin A showed the highest activity against the NCI-H187 and BC cells (IC_{50} 2.1 and 2.9 µg/mL, respectively), whereas 10 exhibited the highest activity against the KB cells (IC_{50} 4.5 µg/mL). It was noted that, for pterocarpans 2 and 4, the position of prenyl group at C-4 in 2 played an important to enhance all bioactivity potency (Thitima *et al.*, 2007).

An ethnomedicinal study conducted by Haile *et. al.*, (2008) to document the indigenous medicinal plants knowledge and use by traditional healers in southwestern Ethiopia from December 2005 to November 2006, identified sixty-seven ethnomedical plant species used by traditional healers to manage 51 different human ailments. Higher degree of consensus was observed among traditional healers in treating tumor, rabies, and insect bite. The study revealed that leaves of *C. macrostachyus* and *Cyathula uncinulata* (Shrad) Schinz were crushed in fresh condition, mixed with water and very small amount of it (only base of cup) was reported to be drunk to treat this ailment. On the method of preparation for remedies, it was reported that medical plant parts were mostly to be crushed (35%), squeezed (27%) and powdered (12%). It further indicates that mixture of different species were used to treat most of the ailments than the use of a single species. About 65%, 41% and 27% of the reported ailments were treated with remedies prepared by crushing, squeezing and powdering, respectively. Traditional healers also indicated that their remedies were devoid of any adverse effects. However, some mild adverse effects like abdominal pain, diarrhea, inflammation, vomiting, unconsciousness and high rates of breathing were reported (Haile *et. al.*, 2008).

Fotie *et al.* (2005), in their study mentioned that bioassay-guided fractionation of the neutral fraction of the crude extract of *Holarrhea floribunda* bark led to the isolation of *lupeol* and its three new long-chain fatty acid ester derivatives namely, hydroxyeicosanoy, hydroxypropanoy and hydroxypropanoy which displayed some *in vitro* inhibition activity against the chloroquine-resistant strain of *P. falciparum*. WR243251, a dihydroacridinedione , was another active antimalarial compound which had been tested for activity against *Plasmodium sp* (Berman, 1994).

A study conducted by Suksamrarn *et al.* (2003), showed that ten triterpenes were isolated and identified from the *Gardenia saxatilis* and subjected to antiplasmodial evaluation against the parasite *Plasmodium falciparum.* The first six compounds; lupenone, lupeol, betulinic acid, oleonolic acid, ursolinic acid, and winchic acid were inactive. while the remaining four; messagenic acid A, messagenic acid B, the 27-O-p-(Z)-and 27-O-p-(E)-coumarate esters of betulinic acid, and a mixture of uncarinic acid E (27-O-p-(E)-coumaroyoxyoleanolic acid) and 27-O-p-E coumaroyloxyursolic acid exhibited antiplasmodial activity, with the CI_{50} values 1.5, 3.8, and 2.9 µg/ml, respectively. It can be concluded that the p-coumarate moieties at the 27-positions, both the *cis* and *trans* isomers, contributed to antiplasmodial activity.

Bergia suffruticosa (Elatinaceae family) is an important Indian medicinal plant hitherto unexplored for its chemical constituents and its pharmacological activity. When thin layer chromatography densitometric methods was used, those were found to be precise with RSD for intra-day in the range of 0.61–1.83, 1.14–1.57, 0.38–0.52 and 0.15–0.52 and for inter-day in the range of 0.97–1.45, 0.58–1.27, 0.42–0.50 and 0.26–0.61 for different concentrations of gallicin, gallic acid, lupeol and β-sitosterol compounds and the average

percentage recoveries obtained were 99.89, 100.58, 99.79 and 100.11 Instrumental precision was 1.05, 1.20, 0.65 and 0.85 (% RSD) for gallicin, gallic acid, lupeol and β-sitosterol. B. suffruticosa sample was found to contain 0.34% w/w of gallicin, 0.288% w/w of gallic acid, 0.064% w/w of lupeol and 0.034% w/w of β-sitosterol (Sheetal *et. al.*, 2007).

Cassia siamea L. (Fabaceae) was identified from the southwest Nigerian ethnobotany as a remedy for febrile illness. This led to the bioassay-guided fractionation of stem bark of the plant extract, using the parasite lactate dehydrogenase assay and multi-resistant strain of *Plasmodium falciparum* (K1) for assessing the *in vitro* antimalarial activity. Emodin and lupeol were isolated from the ethyl acetate fraction by a combination of chromatographic techniques. The structures of the compounds were determined by spectroscopy, co-spotting with authentic samples and comparison with literature data. Both compounds were found to be the active principles responsible for the antiplasmodial property with IC50 values of 5 μg/mL and 11μg/ml respectively (Ajaiyeoba, 2008).

The study of the chemical constituents of the stem bark of *Teclea afzelii (*Rutaceae) has resulted in the isolation and characterization of four furoquinoline alkaloids, namely kokusaninine tecleaverdoornine , masculine and montrifolin together with lupeol and b-ssitosterol glucopyranoside. The structure of the isolated compounds were elucidated based on spectroscopic studies. The antimalarial activity of compounds 1-4 against *Plasmodium falciparum in vitro* show partial suppression of parasitic growth (Wansi *et. al.*, 2010).

Research conducted by Teresa *et al.* (2009), used two strains of *Plasmodium*, a CQ-sensitive strain and a CQ-resistant strain. The activities of the crude extract were

grouped. Thus, any extracts that showed an IC_{50} for antiplasmodial activity of less than 5 µg/mL were considered active, while those that had CI_{50}s' of over 10 µgmL were considered inactive. The activities obtained indicated that there are differences in the strains' sensitivities to the extracts. The hydrophilic MeOH extracts of *P. linearifolia, M. pyrifolia* and *L. javanica* showed high anti-parasitic activity against the CQ-sensitive strain and inactive against both strains of *P. falciparum*. However, the methanoic exract from *S. hennignsii* was active against CQ-sensitive strain and inactive against the CQ-resistant strain of *P. falciparum*. The $CHCl_3$ extracts from *M. heterophylla* had moderate activity towards both strains, but the $CHCl_3$ extracts from *S. henningnsii* were only moderately active against the CQ-sensitive *P. falciparum* strains. All of the EtOAc extracts were inactive, as well as the extracts from this specie's root back (RB) which were moderately active against the D-6 strain of *P. falciparum,* but were inactive against the W-2strain.

Many species of *Croton* have been studied and showed various medicinal properties. *C. lechleri* mature leaves is used as a purgative. *C. malambo* Karst, grown in Venezuela is used in traditional medicine for the treatment of diabetes, rheumatism, gastric ulcer and as anti-inflammatory and anagelsic. *C. arboreous* is a native plant in Mexico and it is used as a beverage and is anti-inflammatory in respiratory ailments. More species of *Croton* that have proved to have medicinal value include *C. eluteria*, used to treat bronchitis, diarrhea and dysentery in the West Indies and northern South America. *C. palanostigma* is a popular plant in South American tropics and is used as a wound healer (Salatino, 2007).

C. macrostachyus, the plant that was studied has also shown some activity against malaria parasites both *in vitro* and *in vivo.* According to Bantie *et. al.,* (2014), C. macrostachyus has been used in Ethiopian folklore medicine to treat malaria. Another study by Bantie, (2015), showed that *C. macrostachyus* was used to treat malaria, gonorrhea, diabetes, wounds, fungal and helminth infections. Analysis of *C. macrostachyus* has shown that the fruit of the plant contains alkaloids, saponins, phenolic compounds, tannins, flavoinoids and terpenoids. The root of the plants also contains alkaloids, saponins, phenolic compounds, tanins, terpenoids, anthraquinones and phlobatamins. Studies have also indicated that *C. macrostachyus* possesses antifungal activity with minimum fungicidal concentration of 25ug/ml, 30ug/ml and 20 ug/ml against three species of Trichophyton (Tengwa *et. al,* 2013). Several of the compounds described in *C. macrostachyus* are agents with anitimalarial activity. The antimalarial activity of *C. macrostachyus* was tested *in vivo* in Swiss albino mice and 600mg/kg extract dose showed 50.53% suppression of parasitaemia (Mohammed *et. al.*, 2014).

Our study has shown that *C. macrostachyus* possesses both *in vitro* and *in vivo* activity against *Plasmodium* species and immunoprophylactic efficacy. This work is meant to show that *C. macrostachyus* possesses properties in malaria disease prevention *in vivo* that no other work has shown.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

C. macrostachyus bark was obtained from The Nature Preserve of The University of Eastern Africa, Baraton, a Seventh-day Adventist university located in Nandi County of Kenya. It is located 0^0 15'20"N and $35^0$04'57"E. The University Campus covers an area of 339 acres of 1.37km^2. Baraton is 10 km from Kapsabet, the headquarters of Nandi Central District, in the highlands of Kenya and has a cool climate. Tea is grown as a cash crop and maize on a small scale and subsistence level of farming. Highland malaria is seasonal, with epidemic cases observed mainly from May to August. This is when the rainy season is at its peak and hence malaria transmission is optimum. Sample of plant were deposited at the Herbarium in the Department of Biological Sciences, University of Eastern Africa, Baraton, for identification. Assays were carried out at the Kenya Medical Research Institute (KEMRI) department of malaria. Phytochemical analysis of the plant was done at the University of Eastern Africa Baraton, Department of Chemistry and the University of Illinois at Chicago.

Plate 3.1: *Croton macrostachyus* plant, Baraton University Compound (Source : Author, 2015)

Plate 3.2: A closer view of *C. macrostachyus* leaves. (Source : Author, 2015)

3.2 Preparation of Plant for Extraction

The plants were selected based on ethnobotanical information and literature compiled from communication with local herbalists.

Two kilograms of fresh *Croton macrostachyus* bark was obtained from the study site. It was cut into small pieces using a pen knife. It was then placed in a cool cupboard and allowed to air dry for one week in the absence of sunlight. The dried bark was ground using a grinder. The ground bark was soaked in methanol and water at a ratio of 9:1. The residue was extracted with ethyl acetate (EtOAc) five times and filtered. The residue containing mainly polysaccharide was kept for further analysis. The filtrate was evaporated using a rotary evaporator (Rotavapor) to dryness and kept as the ethyl acetate extract that contained neutral extract of fats and waxes. Half of the filtrate from the methanol was labeled and kept as methanol extract. The other half of the filtrate from the methanol extract was evaporated to 1/10 vol at below 40^0C. It was then acidified to $2MH_2SO_4$ and extracted with chloroform three times. The chloroform extract was labeled and kept then later evaporated to dryness. The aqueous acid layer was basified to pH10 with NH_4OH. It was then extracted with chloroform ($CHCl_3$) – Methanol (MeOH) at a ratio of 3:1 twice and then chloroform once. The $CHCl_3$-MeOH extract was evaporated to dryness at below 40^0C. The aqueous basic layers were extracted with methanol to give a methanol extract and then an aqueous extract. Each was then evaporated to dryness and labeled. The crude methanol extract previously kept was extracted with n-butanol. The lower layer was kept as butanol extract while the upper layer was kept as methanol extract.

The total weight of the crude extract was obtained. The weight of each solvent extract was also obtained after removal of the solvent by evaporation. The percent yield of each extract was calculated as the weight of each extract divided by the weight of the crude extract times a hundred.

3.3 Chemical Analysis by Thin Layer Chromatography and Column Chromatography

3.3.1 Thin Layer Chromatography

The ethyl acetate extract was dissolved in n-hexane and diethyl ether at a ratio of and spotted on the TLC plate. Ethyl acetate extract was also dissolved in Toluene-MeOH at a ratio of 9:1 and spotted on commercial TLC plates. The crude methanol extract and separated methanol extracts were dissolved in n-BuOH-HOAc-H_20 at a ratio of 4:1:5 upper layer and spotted on TLC plates. The same compounds were also dissolved in $CHCl_3$-MeOH at a ratio of 4:1 and toluene-MeOH at a ratio of 9:1 so that the best solvent could be chosen for use in column chromatography. The butanol extract was also dissolved in n-BuOH-HOAc-H_2O at a ratio of 4:1:5 ratio upper layer and spotted on TLC plate for separation of extract components.

A pencil line was drawn near the bottom of the plate and a small drop of a solution of the dye mixture placed on it. Any labeling on the plate to show the original position of the drop was also drawn in pencil. If any of this was done in ink, dyes from the ink would also move as the chromatogram developed.

When the spot of mixture got dry, the plate was placed vertically in a shallow layer of solvent in a covered jar. The solvent level was below the line with the spot on it.

The jar containing the solvent and plate was covered to make sure that the atmosphere was saturated with solvent vapour. The jar was lined with some filter paper soaked in solvent. Saturating the atmosphere in the beaker with vapour stopped the solvent from evaporating as it rises up the plate.

As the solvent slowly traveled up the plate, the different components of the extract traveled at different rates and the components were separated into different coloured spots. The solvent was allowed to rise until it almost reached the top of the plate. That gave the maximum separation of the extract components for this particular combination of solvent and stationary phase.

3.3.1.1 Measuring R_f values

Measurements were taken from the plate in order to aid in identification of the compounds present in the extracts. These measurements are the distances travelled by the solvent, and the distance travelled by individual spots. When the solvent front got close to the top of the plate, it was removed from the jar and the position of the solvent marked with another line before it evaporated.

The R_f value for each spot was then worked out using the formula:

R_f = Distance traveled by component /Distance traveled by solvent, where R_f is the retention or retardation factor.

In cases where the substances were colourless, fluorescence by ultraviolet light was used. The stationary phase on a thin layer plate often had a substance added to it which

fluoresced when exposed to UV light. That glow was masked at the position where the spots were on the final chromatogram even if those spots were invisible to the naked eye. That meant that if UV light was shone on the plate, it glowed apart from where the spots were. The spots showed up as darker patches. While the UV light was still shining on the plate, the positions of the spots were marked by drawing a pencil circle around them. Another method used in the study was allowing the chromatogram to dry and then placing it in an enclosed container along with a few iodine crystals. The iodine vapour in the container either reacted with the spots on the chromatogram, or simply stuck more to the spots than to the rest of the plate. The substances showed up as brownish spots.

3.3.2 Column Chromatography

The same principles used in thin layer chromatography was applied on a larger scale to separate mixtures in column chromatography. Column chromatography was used to purify compounds made in the lab. The column that was used in column chromatography was prepared using silica gel. Column chromatography works on a much larger scale by packing the same materials into a vertical glass column. Various sizes of chromatography columns including sand, silica gel and cotton wool were used to make the column in a laboratory burette. A concentrated solution of the extract was first made in the solvent selected that was used in the column.

The tap of the column was opened to allow the solvent already in the column to drain so that it was level with the top of the packing material. The concentrated extract solution was carefully added to the top of the column. The tap was carefully opened again so that the coloured mixture was all absorbed into the top of the packing material. Fresh solvent

was added to the top of the column, without disturbing the packing material. The tap was opened for the solvent to flow down through the column, collecting it in a beaker or flask at the bottom. As the solvent ran through, fresh solvent was added to the top so that the column never dried out. The compounds were more spread out the further down the column they traveled.

The unknown solid (compound F5), which was obtained after drying the collections of column chromatography, was dissolved in $CDCl_3$ and transferred into a 5 mm NMR tube. Subsequent NMR analysis (appendices 1-40) were carried out at RT on Bruker Avance AV400 spectrometer with 5 mm ATM BBO probe, including ^{1}H and ^{13}C experiments. All experiments were referenced to the solvent peak.

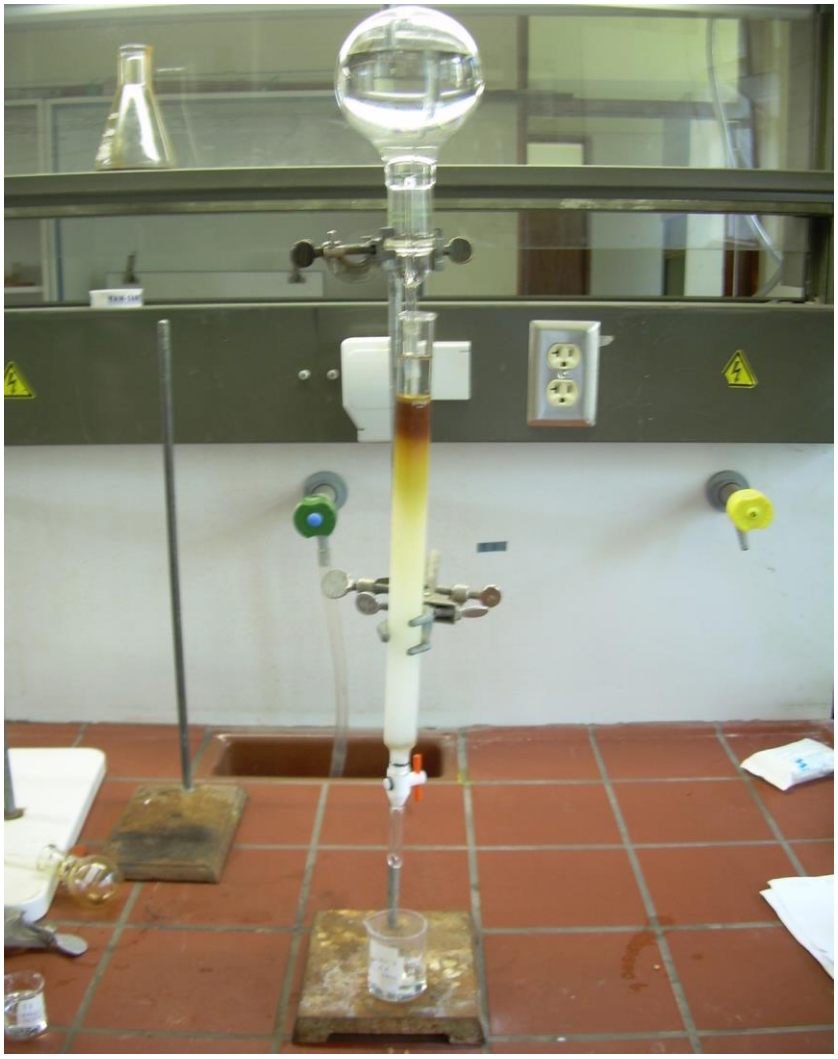


Plate 3.3: Column chromatography set up using silica gel –Ethyl acetate extract compounds are being separated at University of Eastern Africa, Baraton, Chemistry Laboratory.
(Source : Author, 2015)

The above color plate shows the column chromatography procedure being carried out

The Chemistry Department, University of Eastern Africa, Baraton.

3.3.3 Nuclear Magnetic Resonance (NMR) analysis and mass spectrometry for structural elucidation of pure compound

The Ethylacetate extract was analysed and a pure compound obtained. The pure compounds were collected using column chromatography and appeared as fractions no.

5, labeled F5 and fraction number $F5_2$. These pure compounds were sent to Youngstown University, Ohio, USA for chemical analysis and possible elucidation of the structure. They were later forwarded to the University of Illinois, at Chicago for final analysis and structural elucidation. COSY and DEPT NMR procedures were carried out on the compounds. Mass spectrometry was also performed on the compounds.

3.3.3 Nuclear Magnetic Resonance(NMR) Analysis of unknown sample

Unknown solid was dissolved in $CDCl_3$ and transferred into a 5 mm NMR tube. Subsequent NMR analysis was carried out at RT on Bruker Avance AV400 spectrometer with 5 mm ATM BBO probe, including ^{1}H and ^{13}C experiments. All experiments were referenced to the solvent peak.

Carbon data are was obtained and tabulated. Numbering used was that of triterpene framework. Comparison of chemical shifts showed that the unknown sample corresponds to triterpene lupeol. Literature reported chemical shifts were taken from Reynolds *et al*, 1986.

3.4 *In vitro* Assay against *Plasmodium falciparum* Strain D6

3.4.1 Media Preparation for *In Vitro* Assay

5.94 g of powdered HEPES acid was weighed onto a clean weigh boat and added to the cylinder containing 100 ml of autoclaved distilled water. A l liter packet of powdered RPMI 1640 was emptied into the cylinder containing the HEPES acid. Approximately 200ml autoclaved tissue culture distilled water was added to the cylinder to the 960 ml mark. Gentamycin was added to a final concentration of 10µg/ml. The combination was mixed well until the powder dissolved. Approximately 200 ml of the medium was

poured into a 0.2 um filter unit and filtered with the aid of a vacuum line. In a laminar flow hood, the filtered medium was poured into a sterile 500ml plastic flask. The procedure was repeated until all media were filtered and transferred into two flasks. The medium was left in the incubator overnight at 37^0C and observed the following morning to see if any traces of contamination were present. If there was no contamination, it was stored at 4^oC. 2.5 g of tissue culture sodium bicarbonate was weighed onto a weigh boat and poured into a 50 ml sterile plastic centrifuge tube. Autoclaved tissue culture water was added to the 50 ml mark on the tube and mixed well until all powder dissolved. A 20 ml syringe and Millipore "millex-GS" 0.22 um filter unit was used to sterilize the mixture directly into a 25 cm^2 flask.

To add serum to the culture medium, the frozen serum was first thawed out in a 30^0C incubator. It was then brought to room temperature as soon as it was thawed. The correct amount of RPMI 1640/HEPES, 5% bicarbonate and 10% serum were calculated. For 50 ml volume, these amounts were 43.20, 1.80 and 5 ml respectively. The RPMI1640/HEPES and 5% bicarbonate were removed from the refrigerator and placed under hood with the thawed serum. The RPMI1640/HEPES was added to a flask. With 5% bicarbonate being added, it was mixed carefully until the color was uniform. The serum was mixed by aspirating and expelling from pipette 5 times. The desired amount of serum was pipetted and transferred to the flask containing RPMI 1640/HEPES and bicarbonate and mixed by aspirating and expelling 10 times. The flask was flushed with 5% O_2, 3%$CO_2$and 92%N_2. This was done by opening the main stopcock. The can of the plugged Pasteur pipette was also opened carefully. The needle valve was opened

slowly so that gas flowed at a rate of 2.5 l/min and allowed to flush the tubing for 5 seconds. The tubing was kinked to stop the gas flow.

A pipette was removed, touched with the fingers by only the one removed and not allowing the distal half of the pipette to touch the sides of the can or any other pipette. The pipette was attached to gas line tubing. The pipette tubing was held in the right hand and the distal half of the pipette back moved back and forth in full flame for 4 seconds. The cap was removed from the culture flask using the little finger of the other hand and the pipette was inserted onto the flask. The tubing was unlinked and the gas was allowed to flush the flask for 5 seconds. The cap was placed and it was sealed tightly. The needle valve was then closed and the pipette removed from the tubing and discarded.

3.4.2 Preparation of Microtitration Plates

The microtiter plate consisting of 96 flat-bottomed wells was used for the *in vitro* culture. The plates were prepared and the parasites harvested by use of microdrop to place 25µ1 of the culture medium in each well. Known concentration of the standard drug, different concentrations of plant extract or the medium alone was added to the cultured cells in 96 – well flat-bottom microtiter plates. 25µl of the compound solution was added to each of two adjacent wells in the second row of the plate. After the addition of the compounds to the wells of row two, an Automatic Diluter was used to make two-fold dilutions across the plate in each column.

3.4.3 Preparation of Parasites and *In Vitro* Experiment

Blood stage parasites of the malaria *Plasmodium falciparum*, within erythrocytes was maintained in culture at KEMRI, Nairobi, Kenya after collection from malaria-infected patients in Kenya. The *in vitro* assay system described by Le Bras and Beloron, (1983) was used in this study. The infected erythrocytes was resuspended in RPMI 1640 supplemented with 10% human serum and buffered with 25 $mmol^{-1}$ of HEPES (N-2-hydroxyethylpiperazine – N'-2- ethanesulfonic acid) and 25 $mmol^{-1}$ of $NaHCO_3$ to obtain a haematocrit of 2.5%. The suspension (700µl/well) was distributed in 96-well plates pre-coated with the test compounds and incubated at 37^{o}C in an atmosphere of 5% O_2, and 90% N_2 for 42 hours. G-^{3}H]hypoxanthine (specific activity 4.3 Ci $mmol^{-1}$, 1µCi/well) was added 18 hours after initial incubation was used as an index of parasite growth. After the incubation period, the plates were collected on to filter disc with cell harvesters. The discs were dried, and each placed in a scintillation vial containing 1.5ml of a toluene-based scintifluor and counted in a liquid scintillation spectrophotometer. The resulting concentration-response data was interpreted by linear regression analysis using the Chemosen Program. The 50% inhibitory concentration (IC_{50}) was recorded as the drug concentration giving 50% of the parasite uptake of [G-^{3}H] hypoxanthine found in drug-free control wells.

3.4.3.1 Preparation of Radio Label

The radiolabel used for the *in vitro* chemosensitivity test was triatiated hypoxanthine (^{3}H-hypoxanthine). ^{3}H hypoxanthine is incorporated by *P. falciparum* into RNA and DNA.

When a culture was harvested by filtration through a glass fibre filter, the macromolecules, including DNA and RNA were adsorbed onto the fibres; incorporated label was washed through and the amount of label in the macromolecules measured by liquid scintillation counting. Each micro-culture of 225ul was labeled by the addition of 0.5µCi (microcuries) of ^{3}H-hypoxanthine after incubation of parasitized RBCs with drug. The test relied on the ability of the viable parasites to incorporate label, whereas non-viable parasites did not take up the label. A vial of freeze-dried ^{3}H-hypoxanthine was removed from -20^{o}C storage. The two strengths were 1 and 5 mCi (millicuries).
The vial cap was removed. The aluminium seal was removed under the aseptic hood, and the underlying rubber seal was loosened. Sterile distilled water was added to produce a mCi/ml solution (i.e either 1ml or 5ml mCi). The vial cap (only) was replaced and the contents were swirled to dissolve the solids and stored frozen at -20^{o}C until used. ^{3}H-hypoxanthine that is already in solution form was used.

3.4.3.2 Dilution of Radiolabel and Addition to Test Cultures

The vial of stock ^{3}H-hypoxanthine was removed from -20^{o}C storage and allowed to thaw. To label a complete 96-well microculture plate, 2.8ml diluted label (25 µl per well). 2.74 ml CMS was pipetted into a 7ml bijou. 56 µl of H-hypoxanthine (1 mCi/ml) was added and mixed by gentle inversion. This solution contained 0.5 µCi label per 25 µl.
Using an Eppendorf multipipette plus 4981, the diluted label was aspirated to fill the tip. The button was pressed once, with the tip against the wall of the bijou to engage the rachet. The dispenser was then ready. The wells were labeled in order; Unparasitised RBC control (URBC), parasitized RBC control (PRBC), then drug containing wells, from

the lowest concentration to the highest. (NB. To avoid cross-contamination of wells, measures were put in place to avoid the sides of the well or the well contents being touched by the tip of the fingers.

3.4.3.3 Harvesting of Test Parasites From Plates

Eighteen hours after the addition of the radiolabeled hypoxanthine, the parasites in the test culture plate was harvested using the "HARVESTER 96". After 18 hours of radio label incorporation, the test plate was removed from the gas box at 37^{o} incubator. The test plate was wrapped with a parafilm. The test plates were put in the -86^{o}C freezer for about 2 hours or until the test culture was frozen. The test plate was removed from freezer and placed in the 37^{o}C incubator to allow culture to thaw. When all the culture was thawed, the plate was harvested. If test plate was not to be harvested immediately after the 18 hours of radio label incorporation, the test plate was removed from the gas box at 37^{o}C incubator and wrapped with parafilm.

3.4.3.4 Harvesting the 96 Well Test Plate With the Harvester 96

The pump connected to the Harvester 96 was switched on from the main socket. The small white pump connected to the auto Trap 24 was switched on. The Harvester 96 was switched on. Ensuring that the pin on the auto trap is in the "Trap" position, the pressure indicated on the pump connected to the auto trap was raised to more than 20 in Hg (approximately 22 in Hg). In the case of harvesting 96 well plates for determining hypoxanthine incorporation "pulse wash 5 cycles" program was selected. A wash plate was placed onto the "microplate stage" and brought to the correct position. A test check was done by pressing "RUN" to let the pulse wash program to start running. This was to

ensure that there was no blockage and the system was running well. The microplate stage was lowered and the wash plate removed and replaced with a test plate containing culture to be harvested. Once the plate was mounted onto the stage the lid was removed. The lid was preserved since it has the plate number written on it. The head clamp of the harvester was lifted and placed on a glass fiber filter mat onto which the test plate that was to be harvested. The glass fibre filter mats were appropriately labeled to enable one to differentiate the various test plates that were harvested. Labeling the plates with a pen was avoided as the ink could have reacted with the liquid scintillation fluid. A better way was to cut out one of the numbers indicated on the glass fibre filter mat corresponding to the test plate harvested. With the "pulse wash" program selected, the "RUN" label was pressed for the program to start and when the program's cycles were through which also included drying the mat, the microplate stage was lowered and observed if there were any traces of parasitized cells remaining in the test plate.

If there were any cells present, the pulse wash program was repeated again until there were no traces of cells remaining. Once no cells were traced then the test plate was discarded. The head clump was lifted and the glass fiber filter mat removed. The glass fiber filter mat was placed on a hand paper towel and put into the incubator at 37^{o}C until it dried. When dry, the glass fiber filter mat was placed into a plastic sample bag. Five ml liquid scintillation fluid was added and the excess liquid scintillation fluid was removed by rolling a serological pipette on the outside of the sample bag and letting the excess scintillation fluid soak onto a hand paper towel. The open end of the sample bag was sealed using the wallac heat sealer. The edges of the sample bag were cut appropriately to ensure that the bag fit into the cassettes. The packed filter mat was

placed onto a cassette in readiness for counting of parasitized erythrocytes in the microbeta counter. The procedure was repeated for all the plates to be harvested.

Plate 3.4:The Microbeta Trilux Liquid Scintillation and Luminescence Counter used for determination of 3Hypoxanthine uptake by *Plasmodium falciparum* (D6) at the International Lifestock Research Institute (ILRI) Laboratory, Nairobi. (Source: Author, 2015)

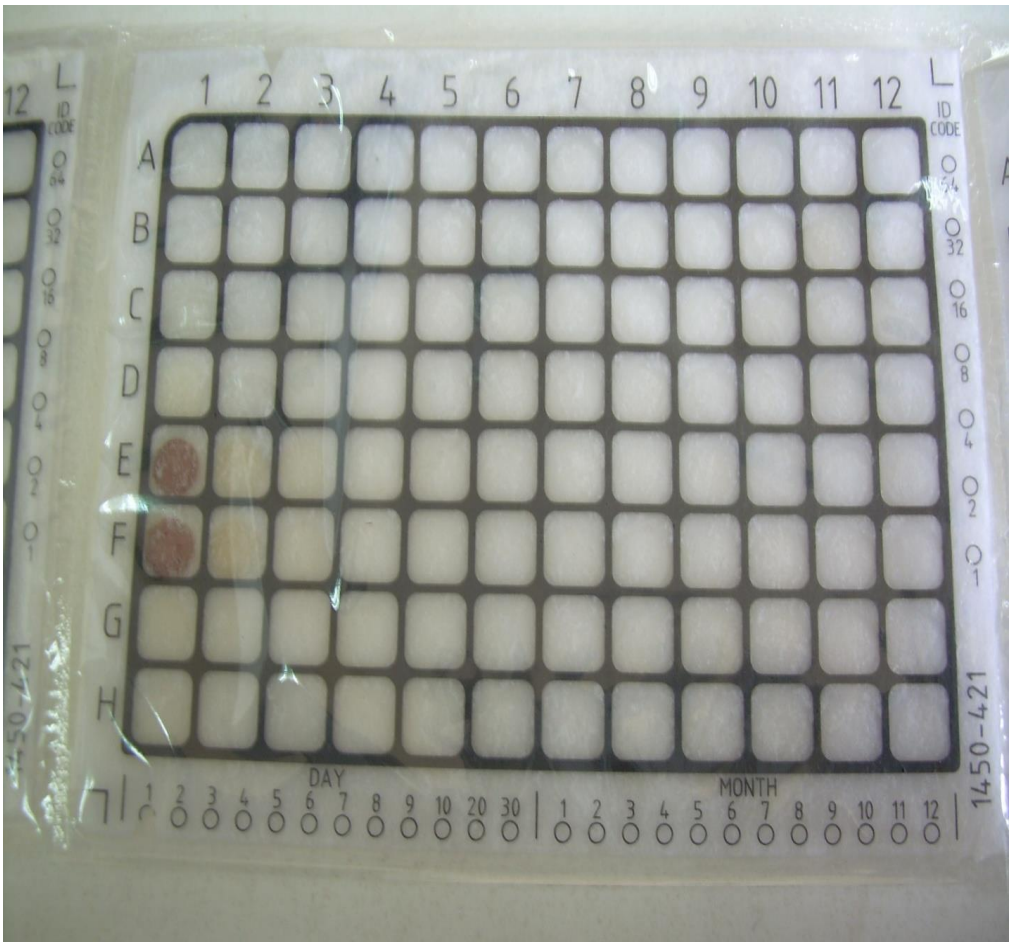


Plate 3.5: Plate with 96 well micro test plate for drug testing after harvesting 3Hypoxanthine-labeled *Plasmodium falciparum* (D6) from counter. (Source: Author, 2015)

3.4.4 Acute Toxicity Experiment

This test was carried out to ascertain the safety of the extract in clean, uninfected BALB/c mice. Five groups of six clean uninfected BALB/c mice each were used in the toxicity test. The groups of six mice were given an oral dose 0.2ml per animal of either 500mg/kg, 250mg/kg or 100mg/kg body weight doses of the extract. One group was given 0.2ml per animal of 10% Tween and another group was given artemether positive control. The weights of all animals were taken before and after the experiment. The

animals in each group were observed for any change in physical activity and signs of abnormal growth or disease condition. These included observations of mortality, hair erection, tremors, lacrimation, convulsions, salivation, diarrhea, and abnormal features in organs and blood for 14 weeks.

3.4.5 *In vivo* Infection Experiments with *Plasmodium berghei* ANKA

BALB/c male mice 6-8 weeks old weighing 20 ± 2g were selected for the *in vivo* study. Each individual in a group of 30 BALB/c mice was infected by injecting 2 x 10^7 erythrocytes parasitized with *Plasmodium berghei* strain ANKA intraperitoneally. For each extract, 6 animals were selected to be tested as positive controls, negative controls, 500, 250 and 100 ug/kg body weight respectively. One group of mice was infected but not treated and served as untreated controls (NC). One group was treated with artemether, and therefore served as treated or positive control group (PC). The other groups were treated with the crude ethyl acetate, butanol, methanol and aqueous extracts at 500, 250, and 100 mg/kg body weight. The plant extracts were dissolved in 10% w/v Tween 80 with the aid of ultrasonication and was further diluted with distilled water to achieve an end concentration of 500 mg/kg body weight (Gessler, *et. al.,* 1995). This concentration was then used to make 250 and 100mg/kg body weight for each of the extract. The animals were treated orally once on days 0, 1, 2, and 3 with a volume of 0.02 ml/g body weight. The mice received NAFAG pellets (9009 PAB – 45) as diet and were held at room temperature (Peters, *et al.,* 1995). The survival of the mice in all the groups was checked twice a day. Parasitized erythrocytes was counted in Giemsa stained thin films from tail blood on day 4. The percentage suppression of parasitaemia for each plant extract or fraction was calculated as:

PSP = $\frac{100 - 100x \text{ (mean\% parasitaemia in treated mice)}}{\text{mean\% parasitaemia in control mice}}$

(Source: Gessler *et. al.*, 1995).

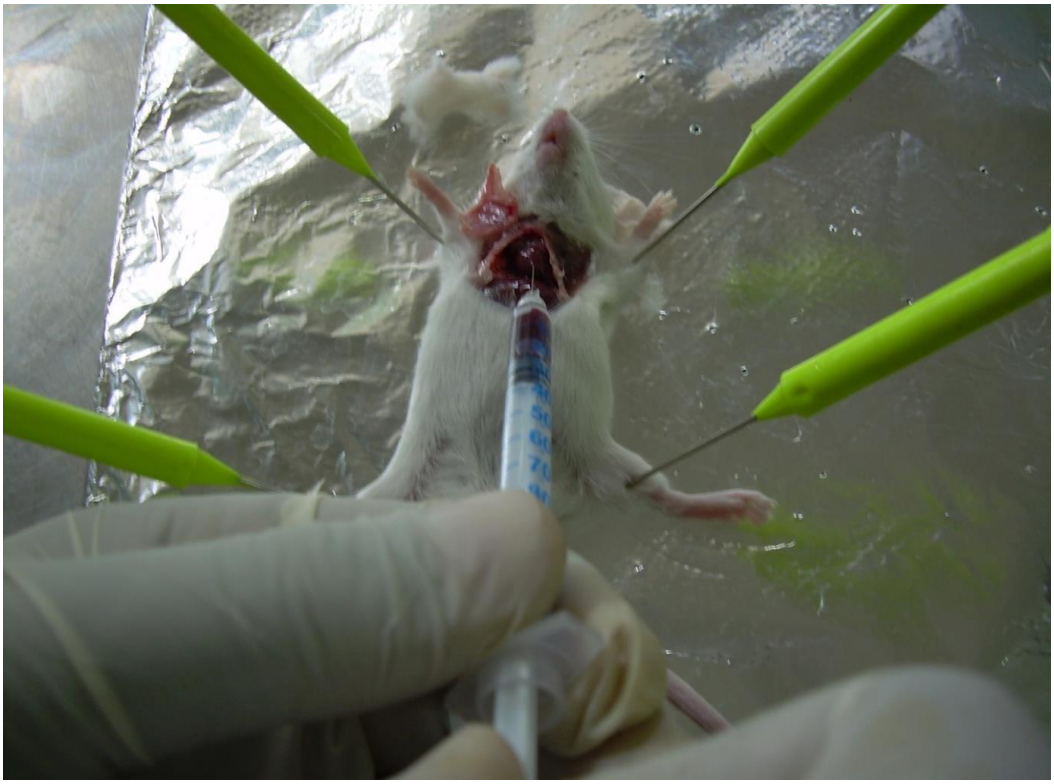


Plate 3.6: Obtaining parasite from a donor mouse for infection by cardiac puncture (Source: Author, 2015)

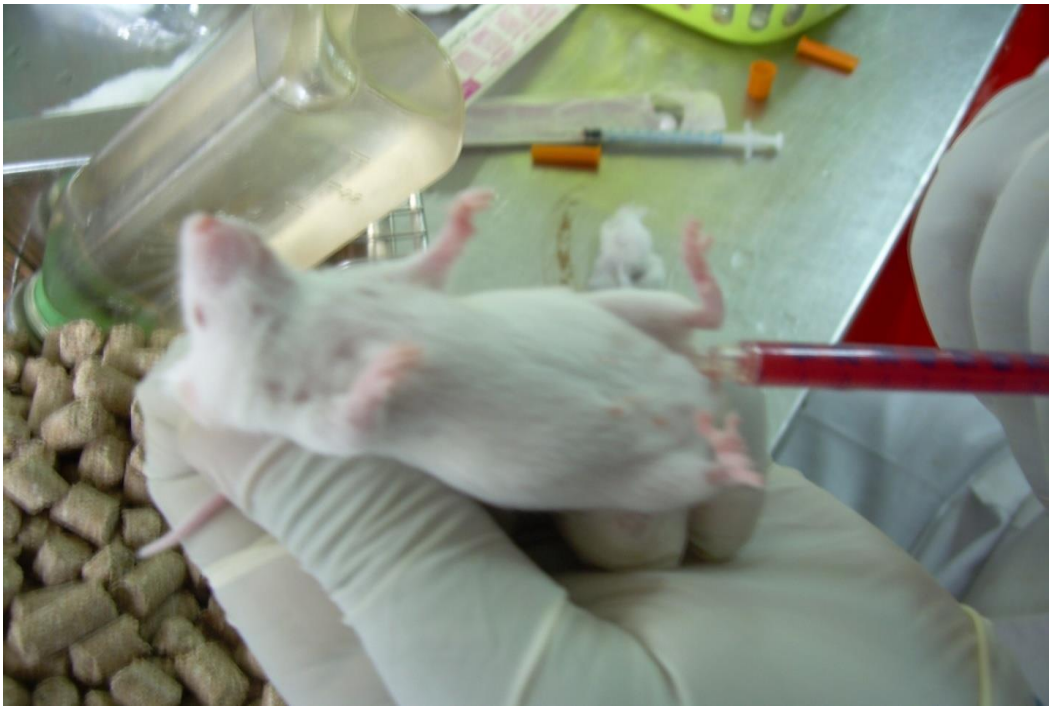


Plate 3.7: Infection of mouse with 2 x 10^7 erythrocytes parasitized with *P. berghei* strain ANKA
(Source: Author, 2015)

3.5 Procedure for Immunoprophylaxis Assay

BALB/c male mice 6-8 weeks old weighing 20 ± 2g were selected for the immunoprophylaxis study. For each extract, 6 animals were selected to be tested as positive controls, negative controls, 500, 250 and 100 ug/kg body weight respectively. The animals were treated with the extract concentrations before they were infected. Each individual in a group of 30 BALB/c mice was infected by injecting 2 x 10^7 erythrocytes parasitized with *Plasmodium berghei* strain ANKA intraperitoneally. One group of mice was infected but not treated and served as untreated controls (NC). One group was treated with artemether, and therefore served as treated or positive control group (PC). The mice received NAFAG pellets (9009 PAB – 45) as diet and were held at room temperature (Peters, *et al.*, 1995). The survival of the mice in all the groups was checked twice a day.

Parasitized erythrocytes was counted in Giemsa stained thin films from tail blood on day 4. The percentage suppression of parasitaemia for each plant extract or fraction was calculated.

3.6 Statistical Analysis

The results from the *in vitro* experiments on parasite density were generated using the program Chemosen. This is a computer generated program that calculates IC_{50} after parasite density has been calculated by the beta-scintillation counter. The acute toxicity experiment results were recorded as adverse reactions of mice to the plant extract. The percent parasitaemia and percent suppression of parasitaemia was assessed by using analysis of variance (ANOVA) (SPSS 20.0 for Windows) to compare variation among the treatment group and the untreated control groups. ANOVA was also used to compare the immunoprophylactic results for the study. Further comparison between individual groups and control was carried out by the Tukey's multiple comparison test.

CHAPTER FOUR

RESULTS

4.1 Chemical Analysis

4.1.1 Extract Yield

The percent yield of the various extract components are indicated below (table 3), with the methanol extract having the highest yield, since it was the first crude extract from which the others were extracted.

Table 4.1: *C. macrostachyus* extract yield after extraction of crude product.

Extract	Percent Yield (Grams)
Ethyl acetate	12.6
Methanol	15.4
Butanol	11.2
Aqueous	12.5

4.2 Thin Layer Chromatography Results

The ethylacetate extract of *C. macrostachyus* showed retention factor ranging from 0.18 to 0.75. The results obtained from the TLC plate showed that the extracts that were isolated from the ethyl acetate solvent filtrate were mostly fats and waxes. These fats and waxes are compounds among which the triterpenoids are found. The butanol extract of *C. macrostachyus* gave retention factors ranging from 0.12 to 0.22 on three different plates. This indicated that the compounds present in the butanol extract were main polar compound since they did not travel far up the TLC plates and were close to the spotting point due to retention by the polarity of the stationary phase. The aqueous extract, on the other hand showed retention factor values ranging from 0.54 to 0.80. This indicates that

the aqueous extract contained moderately polar and nonpolar compounds. The aqueous extract contained mostly the quarternary alkaloids and N-oxides.

The methanol extract of *C. macrostachyus* contained mostly alkaloids. The compounds were separated on TLC and they showed retention factors ranging from 0.24 to 0.0.73. This indicates that the methanol extract contained a wide variety of active components ranging from highly polar to highly non-polar compounds.

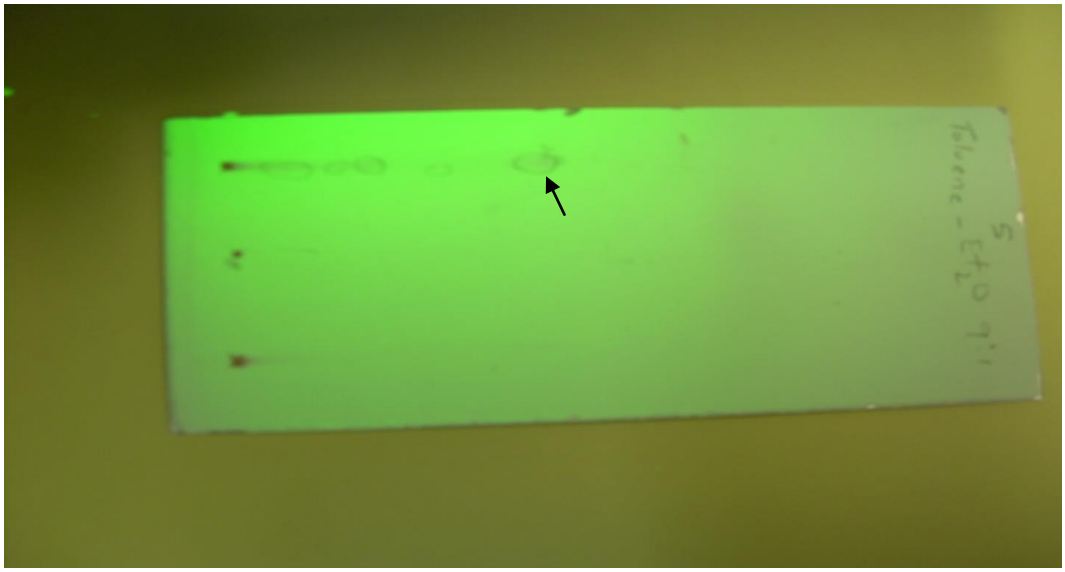


Plate 4.1: EtOAc, MeOH and BuOH extracts spotted on a TLC plate in toluene-Et_2O 9:1
(Source: Author, 2015)

The thin layer chromatography (TLC) plate in the above photograph showed compounds from ethyl acetate, methanol and butanol extracts dissolved in toluene-diethyl ether at a ration of 9:1. The first spot had some nonpolar compounds that traveled up the plate due to the nonpolar eluent toluene with a high retention factor. The other two spots had

mainly polar compounds that did not travel from the spot because the stationary phase SiO_2 retained the polar compounds.

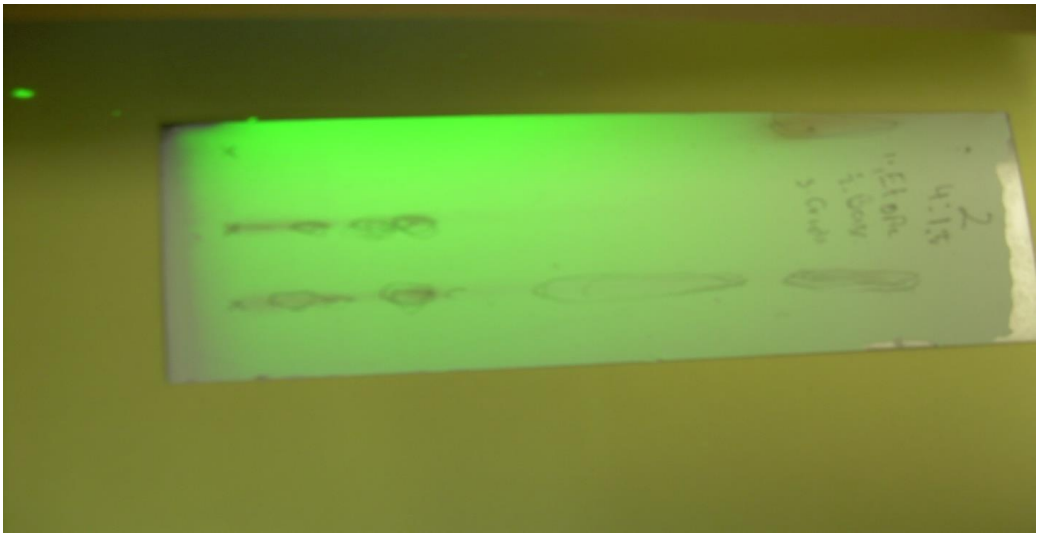


Plate 4.2: EtOAc, MeOH and BuOH extract spotted on TLC plate using butanol, acetic acid and H_2O at a ratio of 4:1:5.
(Source: Author, 2015)

The ethyl acetate extract spotted on TLC plate showed movement of mainly one compound far from the spotted point, indicating the that compound was highly nonpolar. The retention factors calculated for the compounds were 0.05, 0.18, 0.45 and 0.75 respectively away from the original position of spotting of the extract. The butanol extract showed spots representing compounds with R_f values of 0.12, 0.12, 0.22 and 0.22 respectively up the chromatogram. The methanol extract showed one compound that traveled far up the chromatogram with an R_f value of 0.70.

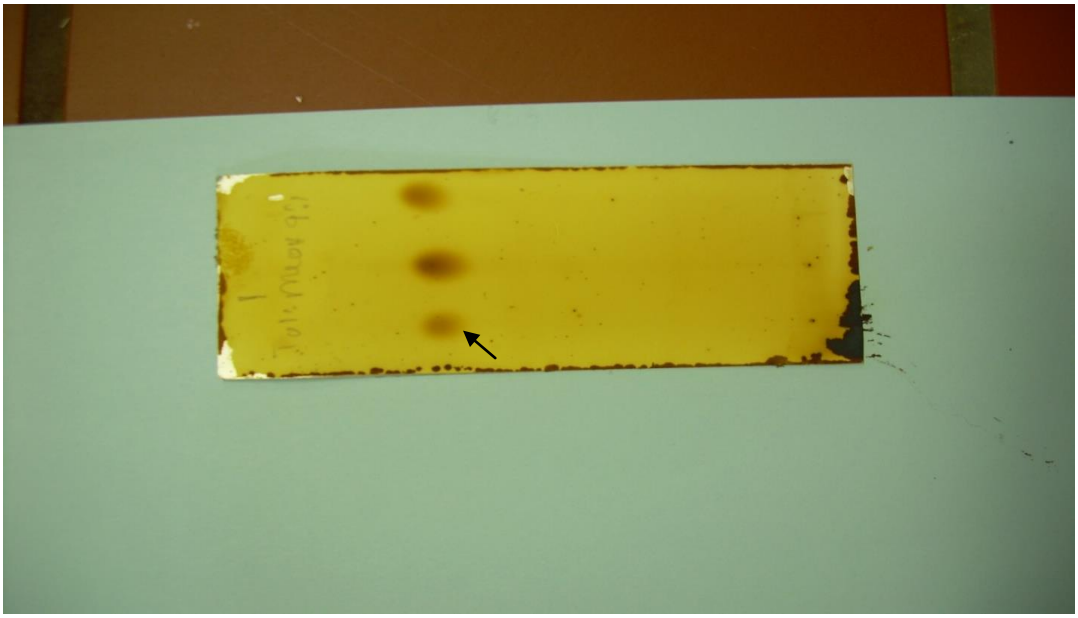


Plate 4.3: Methanol extract spotted on TLC plate with solvent Toluene-MeOH 9:1 in Iodine bath.
(Source: Author, 2015)

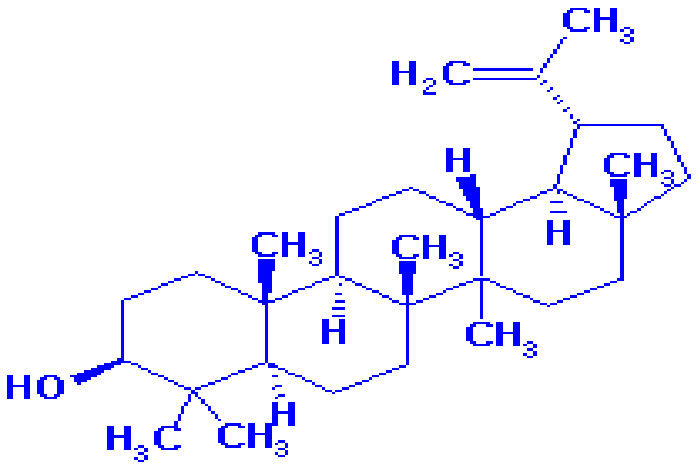
Plate 4.3 shows compounds traveled from the mixture of compounds in the methanol extract of *C. macrostachyus*. This preliminary identification shows the compounds must be highly nonpolar due to the distance travelled by the compounds. The retention factor (R_f) values calculated were 0.72, 0.72 and 0.73 for the three spots shown above. The results indicate that the compounds in the fraction were nonpolar compounds. The common classes of nonpolar compounds from plant sources found in methanol extracts are quarternary alkaloids and N-oxides (Harbourne, 1998).

4.2.1 Column Chromatography Results

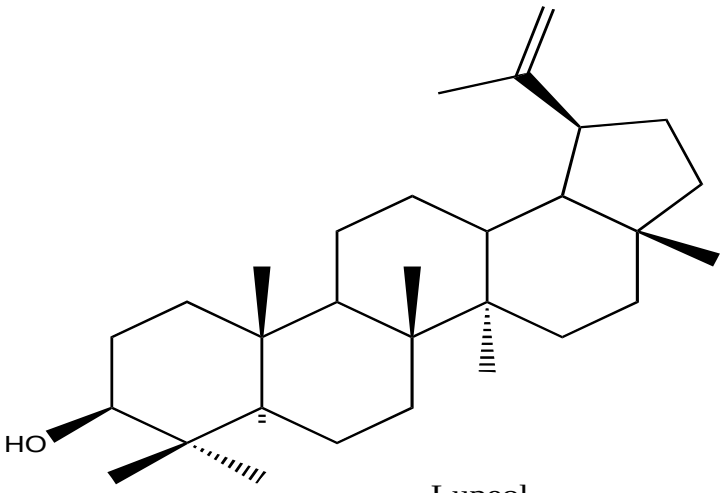
After column chromatography was performed on the ethyl acetate extract, two fractions were isolated from the extract. One fraction was a white fraction(F5) and the other a brown fraction ($F5_2$). The fractions were sent to Chicago, Illinois in the United States of America for further analysis. The compound found to be present in the white fraction

(f5) was lupeol. The $F5_2$ could not be analysed because it was too volatile and disappeared from all the flasks during the analysis. The F_5 compound had an R_f value of 0.65. The results from the analysis showed a positive comparison between the unknown compound and lupeol. The unknown compound was concluded to be lupeol according to ^{1}H and ^{13}C NMR analysis as indicated in fig. 4.1 and table 4.2.

(a)



(b)



Lupeol

Fig 4.1: Lupeol, the active ingredients isolated from the ethyl acetate extract of *C. macrostachyus;* (a) NMR result for F5 and (b) standard lupeol backbone structure

Table 4.2: ^{1}H and ^{13}C Nuclear Magnetic Resonance Results for Lupeol compared to F5

lupeol	Unknown, (F5)	Carbon#
17.97	18.22	28
150.88	151.20	20
109.31	109.54	29
78.94	79.23	3
42.95	43.22	17
55.25	55.51	5
50.38	50.65	9
47.94	48.20	19
48.24	48.51	18
42.78	43.04	14
40.78	41.04	8
38.81	39.07	4
38.67	38.92	1
38.00	38.26	13
39.96	40.20	22
34.23	34.49	7
35.54	35.80	16
27.41	27.66	15
29.80	30.06	21
27.95	28.20	23
27.35	27.62	2
37.11	37.38	10
25.08	25.35	12
20.89	21.14	11
19.28	19.52	30
18.28	18.50	6
15.94	16.19	26
16.09	16.33	25
15.35	15.59	24
14.51	14.76	27

Carbon data provided in table 4.2 for the unknown compound matched that of lupeol . Numbering used was that of triterpene framework. Comparison of chemical shifts showed that the unknown sample corresponded to triterpene lupeol. Literature reported chemical shifts were taken from Reynolds *et al*, (1986).

4.3 *In Vitro* Results on Parasite Density and Hypoxanthine *Intake*

The *in vitro* results showed IC_{50} were recorded as chloroquine (0.346±0.047), artemether (2.895±0.628), ethyl acetate extract (52.838±8.581), methanol extract (14.230±9.450) aqueous extract (43.446±2.218)butanol extract (26.163±5.487), fraction F5(3.259±1.274) and fraction $F5_2$(22.286±1.338).

Table 4.3: Average IC_{50} ±S.E. (µg/ml)in an *In Vitro* Hypoxanthine Intake Assay on Parasite Density of Chloroquine-sensitive Strain of *Plasmodium falciparum* (D6) After Treatment With Different Crude Extracts and Pure Compounds from *Croton macrostachyus*.

Treatment	CQ	ART	EtOAc	MeOH	Aqua	BuOH	F5	$F5_2$
1	0.331	1.731	67.570	32.772	43.547	15.283	5.310	20.259
2	0.274	3.033	37.847	8.127	39.554	30.440	0.925	21.786
3	0.434	3.891	53.097	1.791	47.238	32.772	3.542	24.812
Average	0.346 ±0.047	2.885 ±0.628	52.838 ±8.581	14.230 ±9.450	43.446 ±2.218	26.165 ±5.487	3.259 ±1.274	22.286 ±1.338

The table 4.3 shows that the controls chloroquine and artemether had the lowest parasite density after the in vitro study. Among the extract, the pure compound F5 caused the lowest parasite density at the end of the assay. This was followed by the methanol extract, $F5_2$, butanol extract, aqueous extract and then the ethylacetate extract.

Analysis of variance showed that there were significant difference among the change in parasite density of the various control and extract treatment groups, $p<0.001$, $F=15.072$.

Table 4.4: Tukey's honestly significant differences in parasite density between various control and extract treatment for the *in vitro* study.

Comparison	p-value	Significance
CQ vs ART	1.000	NS
CQ vs EtOAc	0.000	S
CQ vs MeOH	0.537	NS
CQ vs Aqueous	0.000	S
CQ vs BuOH	0.036	S
CQ vs F5	1.000	NS
CQ vs $F5_2$	0.099	NS
ART vs EtOAc	0.000	S
ART vs MeOH	0.745	NS
ART vs Aqueous	0.001	S
ART vs BuOH	0.070	NS
ART vs F5	1.000	NS
ART vs $F5_2$	0.182	NS
EtOAc vs MeOH	0.001	S
EtOAc vs Aqueous	0.877	NS
EtOAc vs BuOH	0.029	S
EtOAc vs F5	0.000	S
EtOAc vs $F5_2$	0.010	S
MeOH vs Aqueous	0.014	S
MeOH vs BuOH	0.699	NS
MeOH vs F5	0.774	NS
MeOH vs $F5_2$	0.939	NS
Aqueous vs BuOH	0.290	NS
Aqueous vs F5	0.001	S
Aqueous vs $F5_2$	0.120	NS
BuOH vs F5	0.077	NS
BuOH vs $F5_2$	0.999	NS
F5 vs $F5_2$	0.198	NS

Key: CQ=chloroquine; ART= artemether; EtOAc=ethyl acetate extract; MeOH=methanol extract; Aqueous=aqueous extract; BuOH=butanol extract; F5=pure compound 1; $F5_2$=pure compound 2

The tukey's multiple comparison test showed that the parasite density was significantly lower for the chloroquine control group compared to the ethyl acetate and aqueous extract groups ($p<0.001$) and butanol extract group ($p<0.05$), but not significantly different from the artemether, methanol extract, F5 and $F5_2$ treatment groups ($p>0.05$).

The artemether control parasite density was significantly lower than those of the ethyl acetate ($p<0.001$) and aqueous ($p<0.05$) extract groups, but not significantly different from those of the methanol, butanol, F5 and $F5_2$ groups ($p<0.05$). The ethyl acetate treatment parasite density was significantly higher than those of the methanol, butanol and $F5_2$, $p<0.05$, and F5, $p<0.001$. The ethyl acetate extract was not significantly different from the aqueous treatment group, $p>0.05$. The methanol extract treatment group recorded parasite density significantly lower than the aqueous extract, $p<0.05$, but not significantly different from the butanol extract, F5 and $f5_2$ treatment groups, $p>0.05$. The butanol extract treatment parasite density was not significantly different from the F5 and $F5_2$ treatment groups, $p>0.05$. The F5 treatment group parasite density was not significantly different from the $F5_2$ treatment parasite density after hypoxanthine uptake by the parasites.

4.4 *In Vivo* Results of Study on the Extract Effect on *P. berghei* in BALB/c Mice

4.4.1 Acute Toxicity Results

The results from the toxicity experiment showed that all animals tested with the ethyl acetate group appeared normal at the end of fourteen weeks. No adverse effect was observed in this group. The animals were normal during observations and at the end of the study period. A similar observation was made for the methanol and water extracts. The butanol extract however appeared to have induced some toxicity. The animals appeared withdrawn on the third day and moved toward a secluded part of the cage. They did not move around to play as usual. On a closer observation, the fur seemed thin and slightly colored unlike the usual white fur and were erected. When given the butanol extract, the animal scratched the mouth for a prolonged period as opposed to the other groups that only scratched their mouth right after oral administration of treatment and stopped. No animal experienced salivation, lacrimation, diarrhea or convulsions. The animals that received butanol extract experienced hair erection and tremors and two animals died after one week.

Table 4.5*:* Mean Percent(%) parasitaemia of mice treated with various concentrations of the ethylacetate extract of *C. macrostachyus* post infection.

	PC	NC	500	250	100
M1	1.18	16.77	2.59	6.82	7.45
M2	2.07	17.75	2.08	6.95	5.05
M3	2.72	17.10	4.81	7.82	8.51
M4	2.45	18.55	3.86	8.23	6.91
M5	2.12	17.21	2.36	7.54	6.87
M6	2.37	16.96	3.15	6.17	5.24
MEAN	2.15	17.39	3.18	7.26	6.64

The lowest percent parasitaemia counted in the ethyl acetate treatment group was that of the positive artemeter control (2.15%). The next lowest was that of the 500mg/kg body weight dose, followed by 100mg/kg and then the 250mg/kg dose. These results show that decrease in parasite by the ethyl acetate extract was not dose dependant, because the 100mg/kg dose destroyed more parasites on average than the higher 250mg/kg dose.

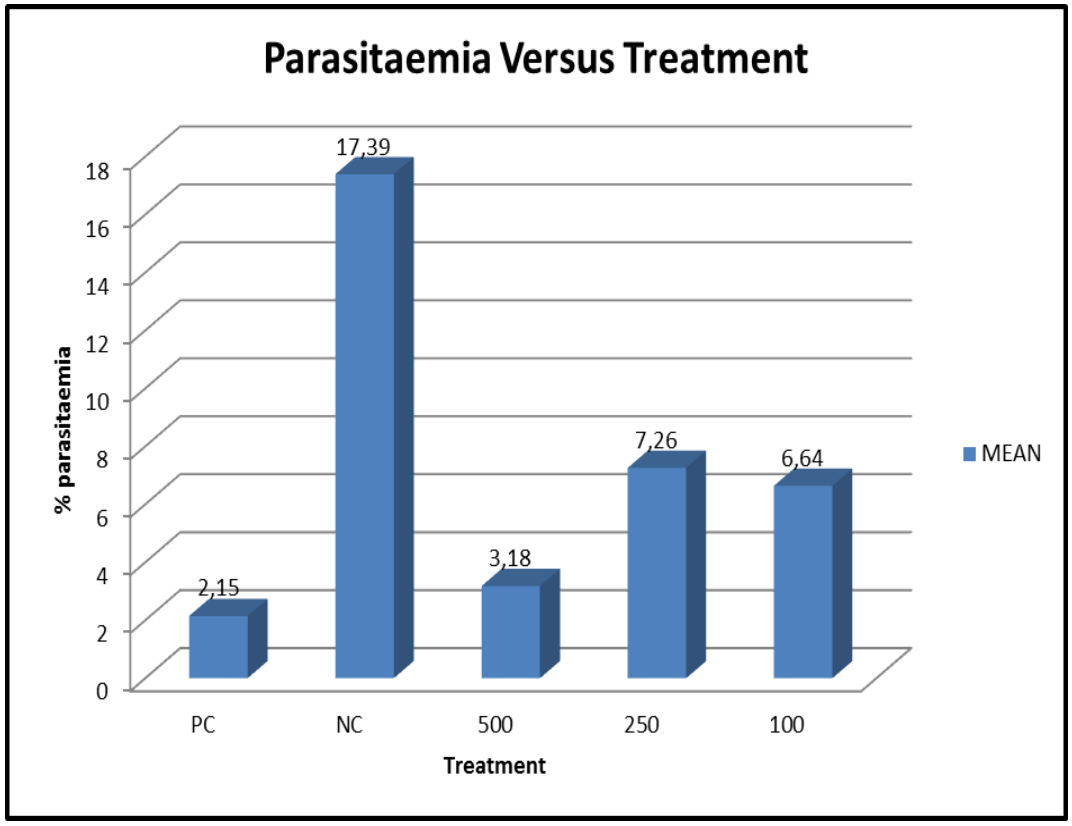


Fig. 4.2: Mean percent parasitaemia of mice treated with various concentrations of the ethyl acetate extract of *Croton macrostachyus* post-infection.
Key: PC= positive artemether control, NC= negative untreated control, 500= 500mg/kg extract dose; 250= 250mg/kg extract dose; 100= 100mg/kg extract dose

The above figure clearly reveals that the ethyl acetate treatment was not dose dependent and the negative control did not destroy the parasites at the same rate as the other treatment.

Table 4.6: Average percent parasitaemia in BALB/c mice after treatment with the methanol extract of *C. macrostachyus*

	PC	NC	500	250	100
M1	0.42	18.5	4.30	13.90	12.24
M2	0.25	17.5	6.38	10.34	13.86
M3	0.93	19.0	5.31	10.60	14.18
M4	1.43	17.0	7.41	11.58	14.56
M5	0	18.5	6.34	12.46	10.44
M6	0	19.5	5.28	14.12	14.54
MEAN	0.51	18.33	5.84	12.17	13.30

The average percent parasitaemia calculated after treatment with the methanol extract shows that the effect of the extract was dose-dependant. From the results in table 4.6, it is observed that the highest dose of 500mg/kg body weight caused a lower percent parasitaemia than the 250mg/kg dose which had a lower percent parasitaemia than the 100mg/kg dose. The negative 10% Tween 80 (NC) control had the highest percent parasitaemia and the artemether positive control (PC) had the lowest percent parasitaemia for the experiment.

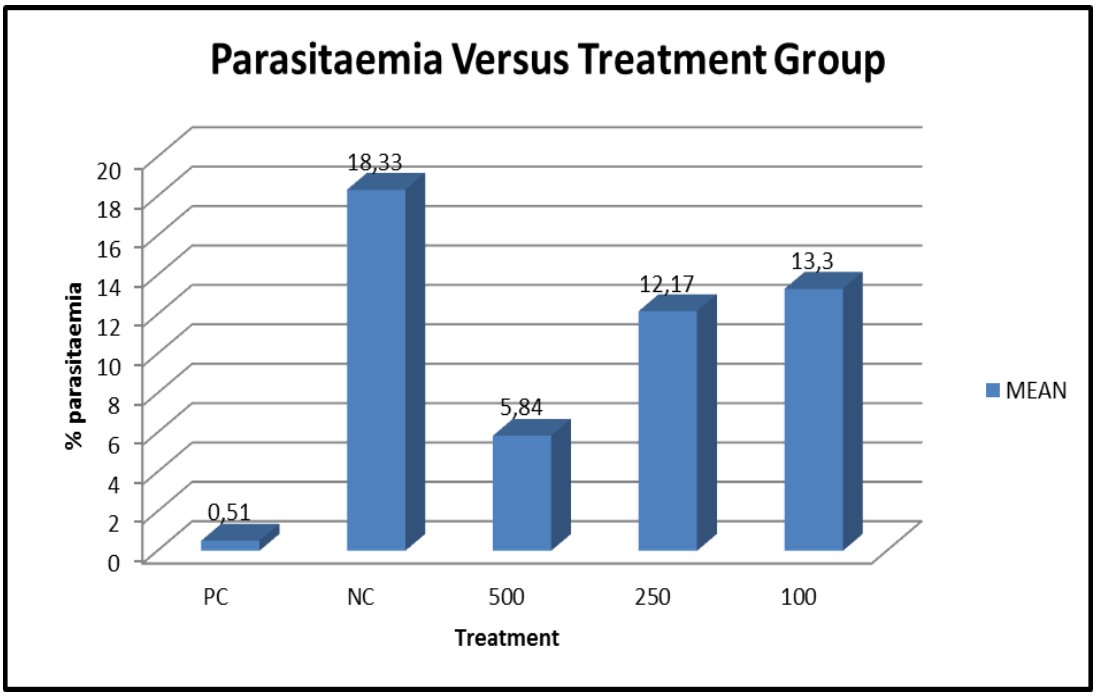


Fig. 4.3: Mean percent parasitaemia of mice treated with various concentrations of the methanol extract of *Croton macrostachyus* post-infection.

Figure 4.3 shows that the treatment with the methanol extract was dose dependent. It also revealed that the artemeter positive control had the lowest percent parasitaemia and the negative control had the highest.

Table 4.7: Percent parasitaemia calculated for aqueous extract of *C. macrostachyus* in *P. berghei* infected BALB/c mice.

	PC	NC	500	250	100
M1	0	18.72	6.11	10.80	12.75
M2	0.35	16.24	5.23	11.02	14.30
M3	0.52	18.80	5.44	9.76	15.31
M4	0	21.42	4.82	8.23	15.16
M5	0	20.86	3.76	10.46	15.23
M6	0	16.26	6.28	12.34	12.18
MEAN	0.15	18.72	5.27	10.44	14.16

The table above shows that all animals in the experiment survived. It also reveals that the positive control had percent parasitaemia of zero in some of the animals.

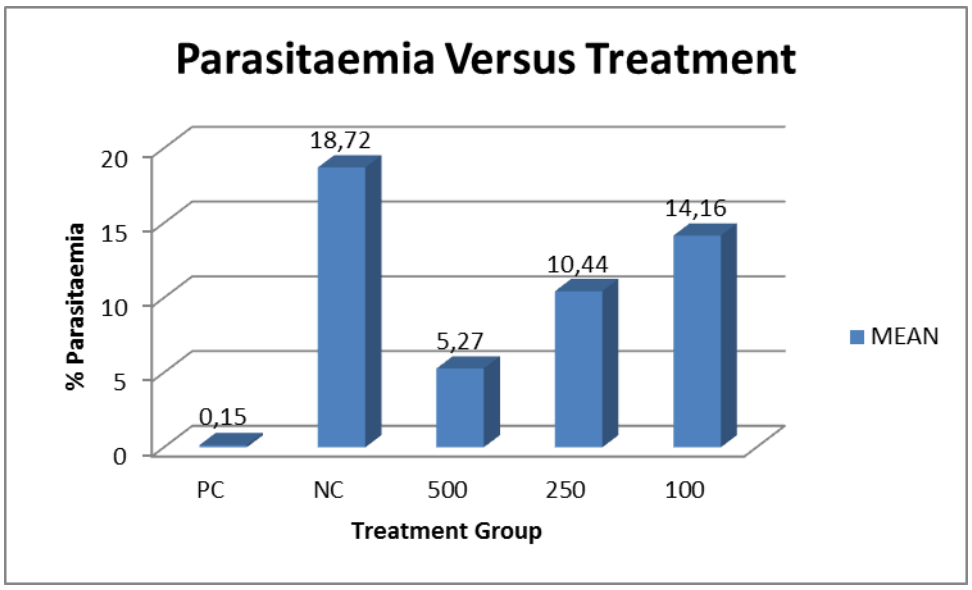


Fig. 4.4: Mean percent parasitaemia of mice treated with various concentrations of the aqueous extract of *Croton macrostachyus* post-infection.

The aqueous extract treatment was also dose dependent on percent parasitaemia in the study. The negative control recorded the highest parasitaemia and the artemeter positive control recorded the lowest parasitaemia.

Table 4.8: Average Percent parasitaemia of *P. berghei*-infected BALB/c mice after treatment with the Butanol extract of *C. macrostachyus*.

	PC	NC	500	250	100
M1	0	21.60	2.10	0	2.79
M2	0	18.80	-	2.15	6.26
M3	0	16.60	3.5	6.72	0.28
M4	0	15.80	3.7	-	7.74
M5	0	-	-	5.80	9.54
M6	0	-	4.90	5.60	0.46
MEAN	0	18.20	3.56	7.16	4.97

Results from the butanol extract treatment group showed that there was no parasitaemia after treatment with the positive artemeter control. The negative Tween 80 control had the highest parasitaemia. Among the extract doses, the 500mg/kg had the lowest parasitaemia, followed by the 100mg/kg treatment and then the 250mg/kg treatment group. This showed that the percent parasitaemia was not dose-dependent for the aqueous extract treatment. Some of the animals died during the study, hence no parasitaemia was recorded.

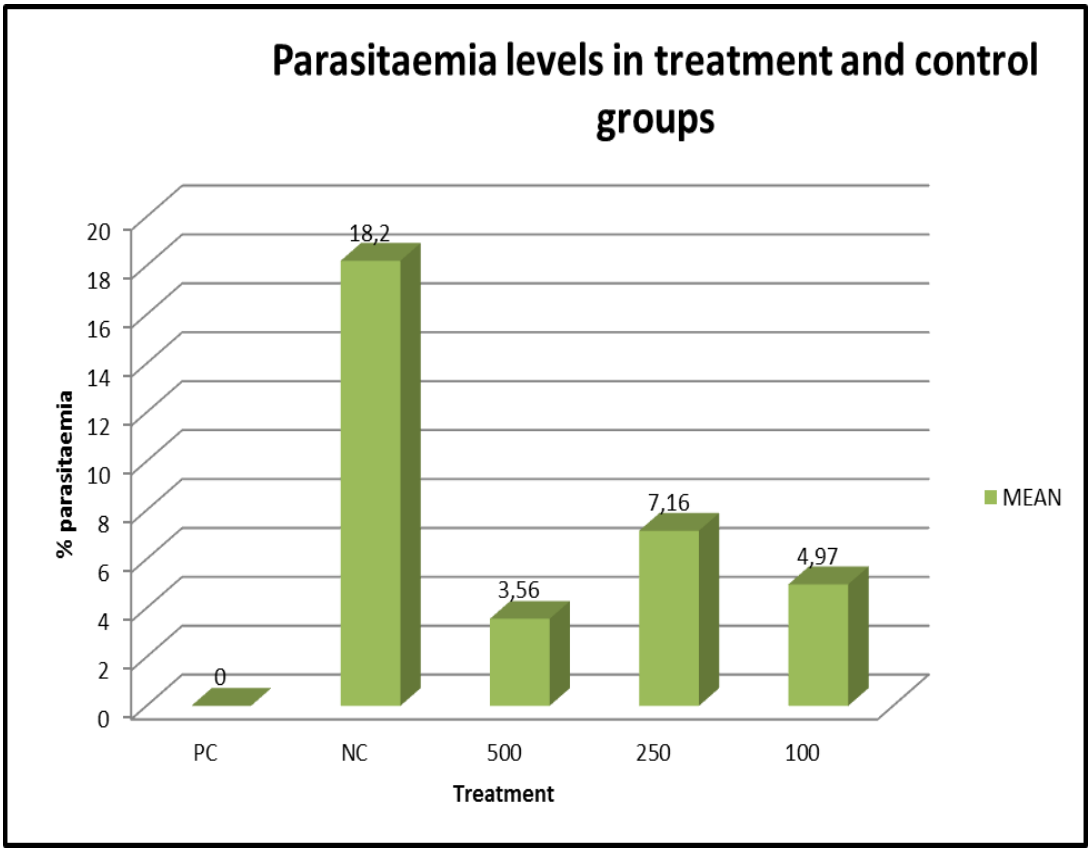


Fig. 4.5: Mean percent parasitaemia of mice treated with various concentrations of the Butanol extract of *Croton macrostachyus* post-infection

Figure 4.5 shows that the butanol extract of *C. macrostachyus* caused reduction in parasitaemia from only the the 250mg/kg dose to the 500mg/kg dose. The same was not observed from the 100mg/kg dose to the 250 mg/kg dose.

The effect of the extracts of *C. macrostachyus* on non-infected animals before exposure to infection was different from that of the *in vivo* study in which animals were infected before exposure to treatment. This is evident by the results obtained in each treatment group after the mice were exposed to extract treatment before infection, and then later checked to determine if the parasitaemia had increased or decreased.

Table 4.9: Immunoprophylactic effect of the Ethyl acetate on BALB/c mice

	PC	NC	500	250	100
M1	0	16.19	0	0	2.79
M2	0.39	18.97	0	4.23	6.26
M3	0.82	18.86	0	1.13	0.28
M4	0	19.50	0	1.56	7.74
M5	0	14.08	0	0.44	9.54
M6	0	16.58	0.10	0	0.46
MEAN	0.20	17.36	0.02	1.23	4.51

The immunoprophylactic effect of the ethyl acetate extract showed that the response to mean parasitaemia reduction was dose-dependent. The lowest recorded parasitaemia was 0.02% for the 500mg/kg dose, 1.23% for the 250 mg/kg dose and 4.51% for the 100mg/kg dose. The positive artemeter control group had an average parasitaemia of 0.20% which was higher than that of the 500mg/kg dose group. There were more animals with 0% parasitaemia in the 500mg/kg dose of butanol than in the positive artemeter control.

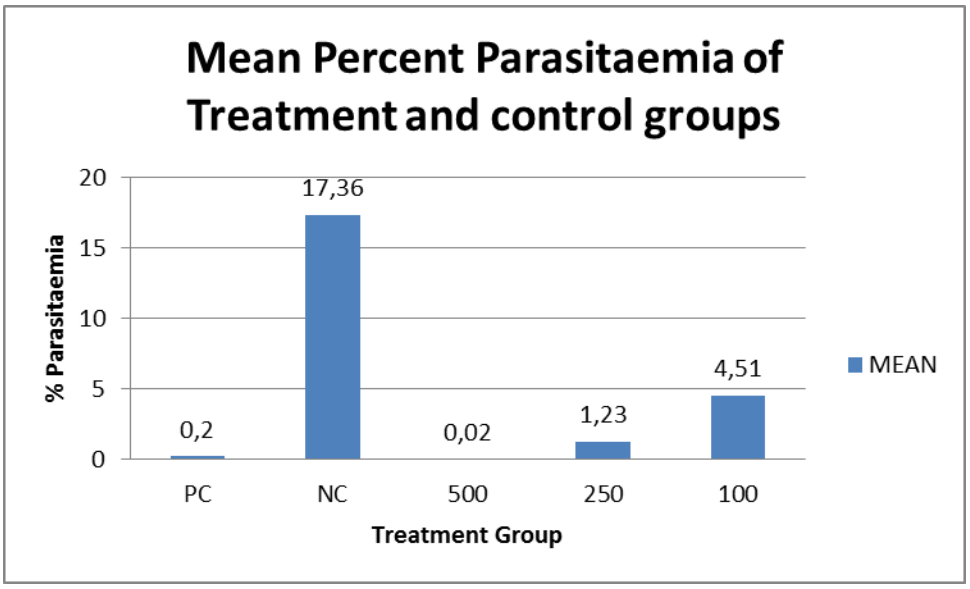


Fig. 4.6: Immunoprophylactic effect for percent parasitaemia of mice treated with various concentrations of the ethyl acetate extract of *Croton macrostachyus* before infection.

The bar graphs above demonstrate that the ethyl acetate extract immunoprophylactic effect was stronger than the *in vivo* treatment effect.

Table 4.10: Immunoprophylactic effect of the aqueous extract on BALB/c mice.

	PC	NC	500	250	100
M1	0.56	19.68	17.30	16.30	13.26
M2	4.88	27.29	0	11.20	14.57
M3	0	11.10	0	0	15.63
M4	10.20	12.70	0.11	21.20	20.45
M5	0	18.70	8.81	0	16.39
M6	0	17.10	0	17.70	19.24
MEAN	2.54	17.19	4.16	11.35	16.41

There were some animals that did not have parasites due to the immunoprophylactic effect of the aqueous extract. These animals were in the groups that received artemeter, 500mg/kg and 250 mg/kg doses of the extract.

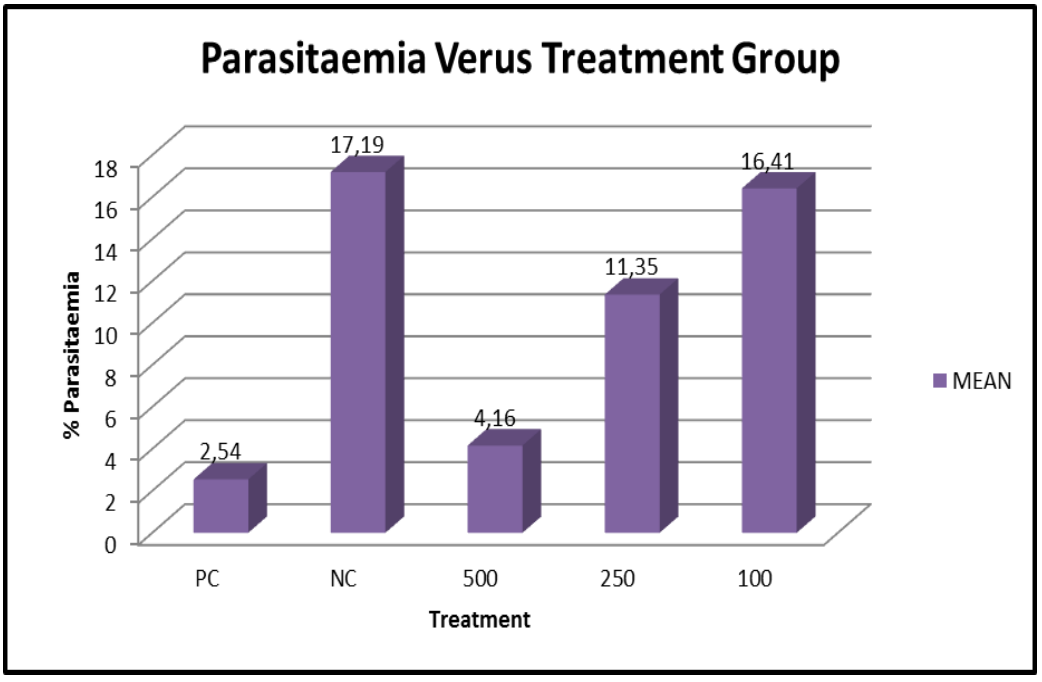


Fig. 4.7: Immunoprophylactic effect for percent parasitaemia of mice treated with various concentrations of the aqueous extract of *Croton macrostachyus* before infection.

The effect of the aqueous extract on *Croton macrostachyus* was dose-dependent. The higher doses decreased the percent parasitaemia. The negative control had the highest percent parasitaemia while the positive control had the lowest percent parasitaemia.

Table 4.11: Immunoprophylactic effect for percent parasitaemia of the methanol extract of *C. macrostachyus* on *P. berghei* in BALB/c mice.

Mice#/Treatment	PC	NC	500	250	100
M.1	0	14.23	2.04	6.24	6.86
M2	1	16.15	2.46	5.04	6.38
M3	0.42	15.78	3.12	5.36	7.46
M4	0	18.36	2.56	6.78	7.48
M5	0	15.96	3.04	4.15	6.68
M6	0	-	3.14	6.36	6.79
MEAN	0.24	16.10	2.73	5.66	6.94

One animal in the negative control group died. Four animals in the positive artemeter control group did not have parasites at the end of the experiment. Most of the animals in the extract treatment groups recorded percent parasitaemia less than 8.

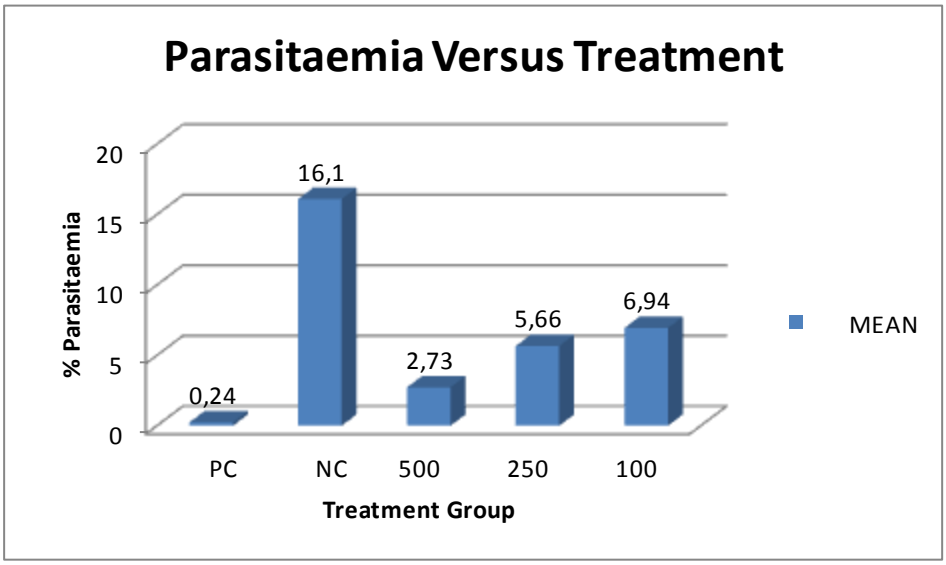


Fig. 8: Immunoprophylactic effect for percent parasitaemia of mice treated with various concentrations of the methanol extract of *Croton macrostachyus* before infection.

Table 4.12: Immunoprophylactic effect for percent parasitaemia of mice treated with various concentrations of the butanol extract of *Croton macrostachyus* before infection.

Mice#/Treatment	PC	NC	500	250	100
M.1	0.46	17.42	2.86	2.15	1.64
M2	0	16.48	1.28	2.24	3.12
M3	0	-	0.76	2.79	2.14
M4	0	16.15	0.42	-	2.68
M5	0.14	16.42	-	2.82	3.46
M6	0	17.36	-	3.68	1.44
MEAN	0.1	16.77	1.33	2.74	2.41

The butanol extract was toxic to the animals and caused death in the negative control group, the 500mg/kg and 250 mg/kg doses treatment groups. Eventhough the extract was highly effective in lowering the parasitaemia in the experiment, it was more toxic than all other extracts.

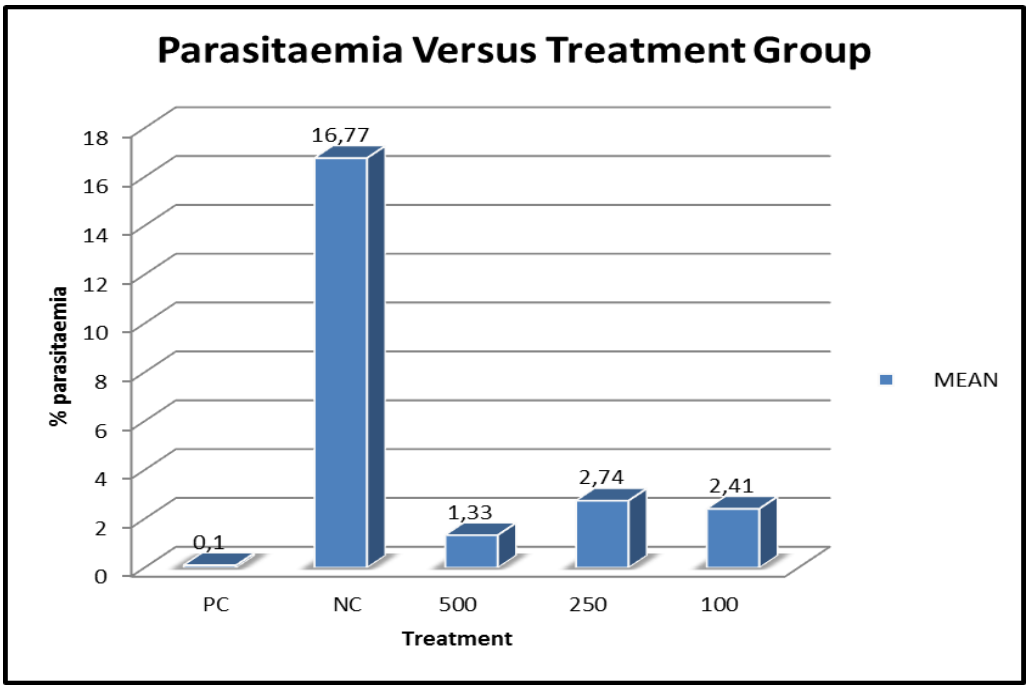


Fig. 4.9: Immunoprophylactic effect for percent parasitaemia of mice treated with various concentrations of the butanol extract of *Croton macrostachyus* before infection.

Figure 4.9 shows that the immunoprophylactic effect of the butanol extract is not dose dependant. The lowest dose of 100 mg/kg body weight has a lower percent parasitaemia than the higher dose of 250 mg/kg body weight dose.

Table 4.13: Mean ± S.E. of percent parasitaemia for the *in vivo* experiment after infected BALB/c mice were treated with different extract concentrations and a positive control.

Extracts / Treatments	EtOAc	MeOH	Aqueous	BuOH
PC	2.15±0.22	0.51± 0.23	0.15±0.09	0.00±0.00
NC	17.39±0.27	18.33±0.38	18.72±0.90	18.20±1.30
500	3.18±0.42	5.84±0.45	5.27±0.38	3.56±0.57
250	7.26±0.31	12.17±0.66	10.44±0.56	7.16±1.28
100	6.64±0.54	13.30±0.67	14.16±0.56	4.97±1.59

The results of treatment with ethyl acetate showed that the 500mg/kg dose give an average percent parasitaemia of2.15±0.22, the500mg/kg 3.18±0.42, the 250mg/kg 7.26±0.31 and the 100mg/kg 6.64±0.54. The methanol extract give percent parasitaemia of artemether control as 0.51±0.23, 500mg/kg as 5.84±0.45. This was the best result because the other concentrations showed higher values(Table 13). The methanol extract also showed that 500mg/ml

had an average percent parasitaemia of 5.84±0.45, 12.17±0.66 and 100mg/kg 13.30±0.67. The aqueous extract experiment had a artemether control group of 0.15±0.09, a 500mg/kg group of 5.27±0.38, 250mg/kg of 10.44±0.56 and 100mg/kg of 14.16±0.56. The butanol extract had parasitaemia for the positive control group as 0.00±0.00, 500mg/kg as 3.56±0.57, 250mg/kg as 7.16±1.28 and 100mg/kg as 4.97±1.59. For all the negative control groups, percent parasitaemia were not less than 17 % (Table 4.13).

Table 4.14: Tukey's multiple comparison test for the effect of extracts of *C. macrostachyus* on *P. berghei* parasitaemia in BALB/c mice.

Comparison	EtOAc		MeOH		Aqueous		BuOH	
	p-value	sig	p-value	sig.	p-value	sig	p-value	sig
PC vs NC	0.000	S	0.000	S	0.000	S	0.000	S
PC vs 500	0.346	NS	0.000	S	0.000	S	0.246	NS
PC vs 250	0.000	S	0.000	S	0.000	S	0.110	NS
PC vs 100	0.000	S	0.000	S	0.000	S	0.046	NS
NC vs 500	0.000	S	0.000	S	0.000	S	0.000	S
NC vs 250	0.000	S	0.000	S	0.000	S	0.000	S
NC vs 100	0.000	S	0.000	S	0.000	S	0.000	S
500 vs 250	0.000	S	0.000	S	0.000	S	0.998	NS
500 vs 100	0.000	S	0.000	S	0.000	S	0.977	NS
250 vs 100	0.797	NS	0.519	NS	0.001	S	0.998	NS
ANOVA	F=262.258		F=187.620		F=168.168		F=32.243	

Key: PC=positive artemether control; NC=negative tween 80 control;
EtOAc=ethylacetate extract; MeOH= methanol extract; Aqueous = aqueous extract;
BuOH= butanol extract; S=significant; NS=not significant

The tukey honestly significant difference test showed that there were significant differences in percent parasitaemia between the positive control and negative control for all the *in vivo* extract treatement experiments, $p<0.001$. The percent parasitaemia for the positive controls were significantly lower than that of the 500mg/kg treatment groups in the methanol and aqueous treatment groups, $p<0.001$, while there was no significant

difference ($p>0.05$) for the ethyl acetate and butanol treatment groups. The percent parasitaemia for the positive controls were all significantly lower than the 250 mg/kg group in the ethyl acetate, methanol and aqueous extract groups, but was not significantly different for the butanol extract treatment group, $p>0.05$. The mean percent parasitaemia of the artemeter positive control was also significantly lower than that of the 100mg/kg dose group for the ethyl acetate, methanol and aqueous extract treatment groups ($p<0.001$), but not significantly different for the butanol group ($p>0.05$). The mean percent parasitaemia of the negative control group was significantly higher than the 500mg/kg, 250mg/kg and 100mg/kg group for all the extract treatments ($p<0.001$). The mean percent parasitaemia for the 500mg/kg dose treated animals was significantly lower than the 250mg/kg dose in the ethyl acetate, methanol and butanol extract treatment groups ($p<0.001$) and not significantly different in the butanol extract treatment group. The percent parasitaemia of the 250mg/kg dose was not significantly different from the 100mg/kg dose in the ethyl acetate, methanol and butanol extract treatment groups but they were significantly different in the aqueous extract treatment groups ($p<0.05$) (Table 4.13).

Table 4.15: Mean Percent Supression of Parasitaemia(%)± S.E. of Mice Treatment Group and Positive Control.

Extracts / Treatments	EtOAc	MeOH	Aqueous	BuOH
PC	87.64±1.264	97.22±0.225	99.20±0.156	100±0.000
500	81.71±0.728	68.14±0.670	71.85±0.447	82.62±1.259
250	58.25±0.635	32.61±0.609	44.23±0.064	60.66±0.445
100	61.82±0.571	27.44±0.433	24.36±0.447	73.69±0.306

The *in vivo* treatment assay showed percent suppression of parasitaemia for ethyl acetate extract positive control (87.64±1.264), 500mg/kg(81.71±0.728), 250mg/kg(81.71±0.728) and 100mg/kg (61.82±0.571). The methanol extract showed percent suppression of parasitaemia for positive control (97.22±0.225), 500mg/kg(68.14±0.670), 250mg/kg (33.61±0.609) and 100mg/kg (27.44±0.443). The aqueous extract results were for positive control, 99.20±0.156, 500mg/kg, 71.85±0.447, 250mg/kg, 44.23±0.064 and 100mg/kg 24.36±0.447. The butanol extract give results for positive control as 100±0.000, 500mg/kg as 80.44±1.259, 250mg/kg as 60.66±0.445 and 100mg/kg as 73.69±0.306.

The highest PSP for the positive artemether control was by the butanol extract group, followed by the aqueous extract, methanol and ethyl acetate group respectively. The highest percent suppression of parasitaemia for the 500mg/kg dose was for the butanol extract, then followed by the ethyl acetate, aqueous and methanol extracts respectively. The PSP for the 250mg/kg dose followed the same pattern as the 500mg/kg dose. The PSP of the 100mg/kg dose was highest for butanol extract, followed by ethyl acetate, methanol and aqueous extracts respectively (table 4.15).

Table 4.16: Mean ± S.E. percent parasitaemia of the immunoprophylactic treatment result of BALB/c mice with different extract concentrations and positive control after infection with *P. berghei.*

Extracts / Treatments	EtOAc	MeOH	Aqueous	BuOH
PC	0.20±0.14	0.24± 0.17	2.54±1.71	0.10±0.08
NC	17.36±0.86	16.10±0.66	17.19±02.35	16.77±0.26
500	0.02±0.02	2.73±0.18	4.16±2.96	1.33±0.54
250	1.23±0.65	5.66±0.40	11.36±3.74	2. 74±0.27
100	4.51±1.59	6.94±0.18	16.41±1.12	2.41±0.33

Results obtained from the *in vivo* study showed that the treatment that controlled the parasite the most for the ethyl acetate extract was 500mg/kg dose (0.02±0.02), followed by the positive control (0.20±0.14), 250mg/kg (1.23±0.65), then 100 mg/kg (4.51±1.59). For the methanol extract, the lowest percent parasitaemia was recorded for the positive control group (0.24± 0.17), then 500mg/kg2.73±0.18, 250mg/kg (5.66±0.40) and then 100mg/kg (6.94±0.18). The aqueous extract treatment group artemether control showed reduced parasitaemia(2.54±1.71), followed by the 500mg/kg(4.16±2.96), the 250mg/kg(11.36±3.74), and the 100mg/kg doses (16.41±1.12). For the butanol extract, it was artemether positive control(0.10±0.08), followed by the 500mg/kg(1.33±0.54), 100mg/kg and then the 250 mg/kg(2. 74±0.27) doses. In all of the four groups, the negative control (NC) groups give the highest percent parasitaemia.

Table 4.17: Tukey's multiple comparison test for the immunoprophylactic effect of extracts of *C. macrostachyus*.

Comparison	EtOAc		MeOH		Aqueous		BuOH	
	p-value	sig	p-value	sig.	p-value	sig	p-value	sig
PC vs NC	0.000	S	0.000	S	0.002	S	0.000	S
PC vs 500	1.000	NS	0.000	S	0.998	NS	0.074	NS
PC vs 250	0.915	NS	0.000	S	0.163	NS	0.000	S
PC vs 100	0.013	S	0.000	S	0.006	S	0.000	S
NC vs 500	0.000	S	0.000	S	0.008	S	0.000	S
NC vs 250	0.000	S	0.000	S	0.364	NS	0.000	S
NC vs 100	0.000	S	0.000	S	0.997	NS	0.000	S
500 vs 250	0.856	NS	0.000	S	0.364	NS	0.042	S
500 vs 100	0.009	S	0.000	S	0.018	S	0.139	NS
250 vs 100	0.084	NS	0.084	NS	0.551	NS	0.934	NS
ANOVA	F=72.140		F=285.608		F=7.326		F=508.779	

Key: PC=positive artemether control; NC=negative tween 80 control; EtOAc=ethylacetate extract; MeOH= methanol extract; Aqueous = aqueous extract; BuOH= butanol extract; S=significant; NS=not significant

The tukey's honestly significant difference test was performed on the percent parasitaemia for the immunoprophylaxis experiment. The results showed that the mean percent parasitaemia for the artemether and 10% Tween 80 controls were all significantly lower compared to the ethyl acetate extract treatment group ($p<0.001$), methanol extract treatment group ($p<0.001$), butanol extract treatment group ($p<0.001$) and the aqueous extract treatment group ($p<0.05$). The percent parasitaemia for the positive artemeter

control was significantly higher than the 500mg/kg in the methanol treatment group ($p<0.01$), but they were not significantly different in the ethyl acetate, aqueous and butanol extract treatment groups ($p>0.05$). The percent parasitaemia for the artemeter control was significantly lower than those of the250mg/kg treatment dose for the methanol and butanol extract groups ($p<0.001$) and not significantly different from those of the ethyl acetate and aqueous extract groups ($p>0.05$). The percent parasitaemia for the positive artemeter control was significantly lower than the 100mg/kg dose for the ethyl acetate and aqueous extract experiments, $p<0.05$, but significantly lower for the methanol and butanol experiment at $p<0.001$. The negative 10% Tween 80 group of animals had parasitaemia significantly higher than the 500mg/kg dose for the ethyl acetate, methanol and butanol extract treatment groups ($p<0.001$) and for the aqueous extract at $p<0.05$. The negative control animals parasitaemia was significantly higher than the parasitaemia of the 250mg/kg and 100mg/kg animals for all extract treatments ($p<0.001$) except for the aqueous extract that did not show significant difference in parasitaemia ($p<0.05$). The percent parasitaemia for the 500mg/kg treatment groups were significantly lower that the 250mg/kg treatment groups for the methanol extract group, $p<0.001$ and the butanol extract group, $p<0.05$. No significant differences were observed between the 500 and 250mg/kg dose groups for the ethyl acetate and aqueous treatment, $p<0.05$. The percent parasitaemia of the 500mg/kg treatment was significantly lower than the 100mg/kg treatment for the ethyl acetate and methanol extract ($p<0.001$) and the aqueous extract ($p<0.05$), but not significantly different for the butanol extract ($p>0.05$). Mean percent parasitaemia for the 250mg/kg dose were not significantly different from those of the 100mg/kg dose for all the extract treatment groups ($p>0.05$) (Table 4.17).

Table 4.18: Mean Percent Supression of Parasitaemia (%) ±S.E. for Immunoprophylaxis Study for Mice Treatment Group.

Extracts / Treatments	EtOAc	MeOH	Aqueous	BuOH
PC	98.85±0.248	98.51±0.476	85.32±0.404	99.94±0.016
500	99.88±0.058	83.04±0.635	75.78±0.958	92.07±0.294
250	92.91±0.343	64.84±1.143	33.97±0.450	83.66±1.068
100	74.02±1.236	56.89±1.143	3.96±0.490	85.63±0.618

The immunoprophylaxis or immune booster study show that parasites were suppressed in the ethyl acetate experiment by chloroquine as 98.85±0.248, 500mg/kg as 99.88±0.058, 250mg/kg as 93.96±0.343 and 100mg/kg as 74.02±1.236. Using the methanol extract, percent suppression for the chloroquine control was 98.51±0.478, 500mg/kg was 83.04±0.635, 250mg/kg was 64.84±1.143 and 100mg/kg was 56.89±1.143. The butanol extract give percent parasitaemia for chloroquine control as 99.40±0.016, 500mg/kg as 92.07±0.294, 250mg/kg as 83.66±1.068 and 100mg/kg as 85.63±0.618.

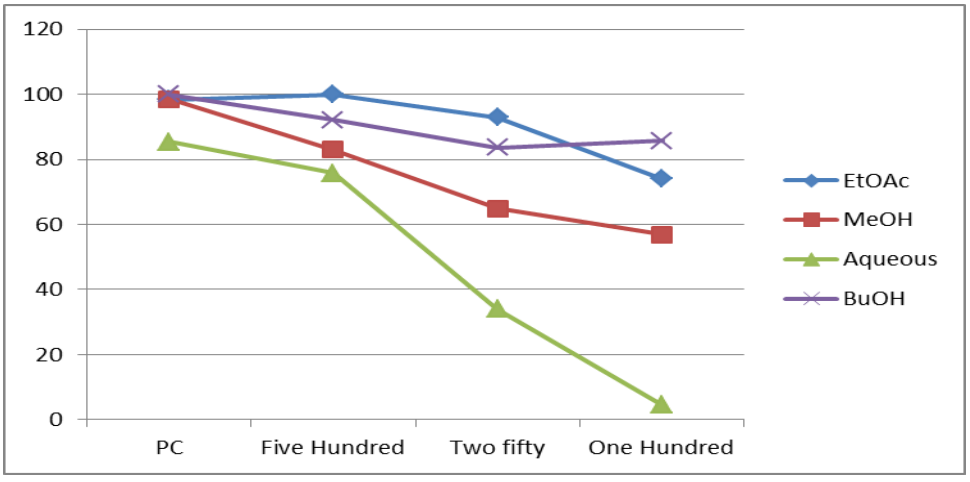


Fig. 4.10: Overall Percent Suppression of Parasitaemia for the Immunoprophylaxis Study

The percent suppression of parasitaemia for the immunoprophylaxis study did not follow the same pattern like that of the *in vivo* drug testing study. The PSP was highest for the

500mg/kg dose for the butanol extract, followed by the ethyl acetate, methanol and aqueous extracts respectively. For the 500mg/kg dose, the highest was that of the ethyl acetate extract, followed by the butanol, methanol and aqueous extracts. The 250 mg/kg dose followed the same pattern for the 500mg/kg dose. The highest PSP for the 100mg/kg dose was for the butanol, ethyl acetate, methanol and then the aqueous extracts respectively (Figure 4.10).

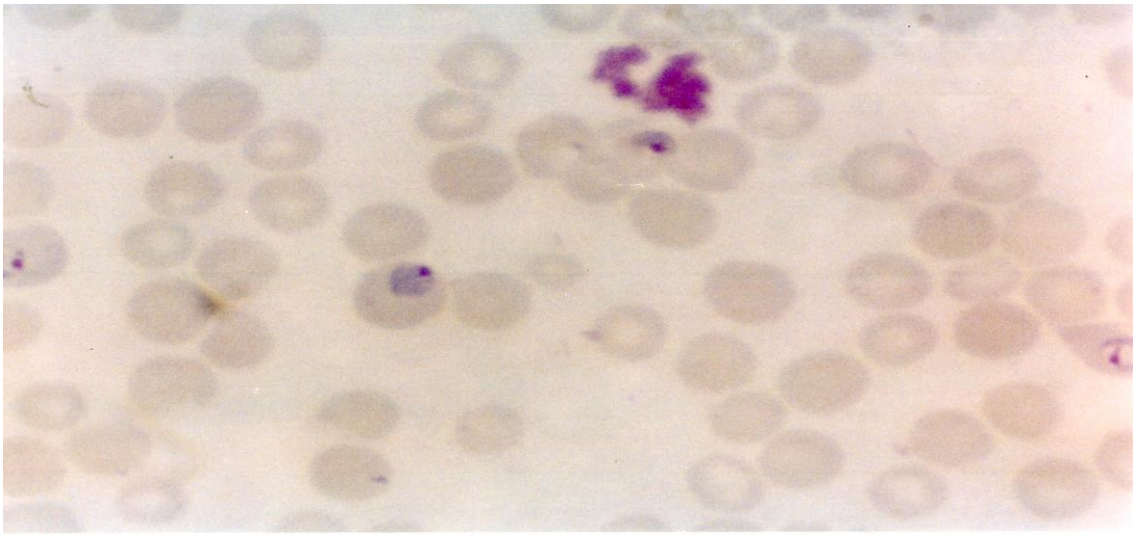


Plate 4.4: Ethyl acetate(EtOAc) extract, positive control group treated with artemeter.
(Source: Author, 2015)

The slide in the above plate shows a mouse blood film made after treatment with the positive artemeter control. The blood cell morphology were mostly normal with some cells having hypochromia. Few intracellular parasites were observed on the film.

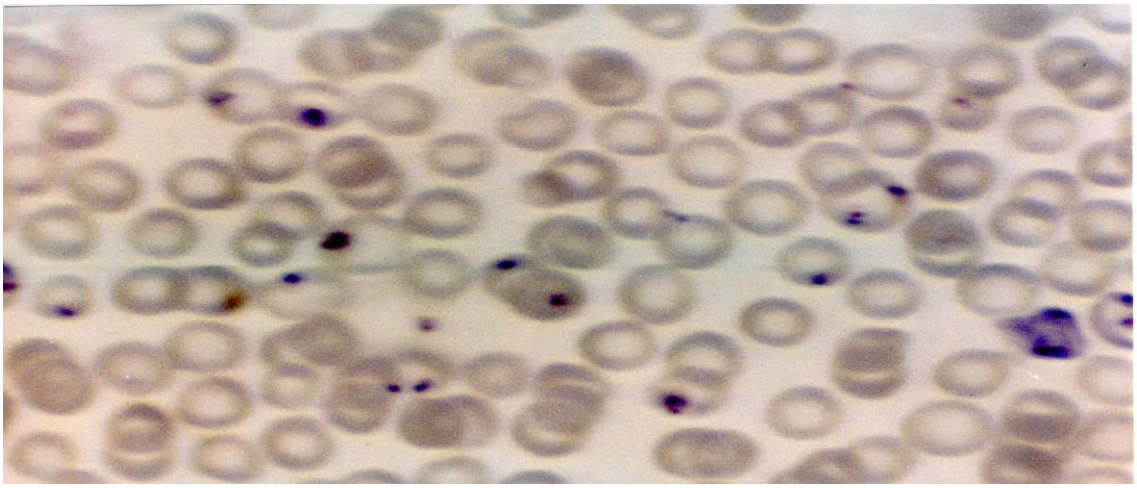


Plate 4.5: Blood film from mouse treated with 100mg/kg dose of ethyl acetate extract
(Source: Author, 2015)

The parasites were mainly intracellular and were all trophozoites of *P. berghei.* Red blood cells were mostly normocytic and hypochromic.

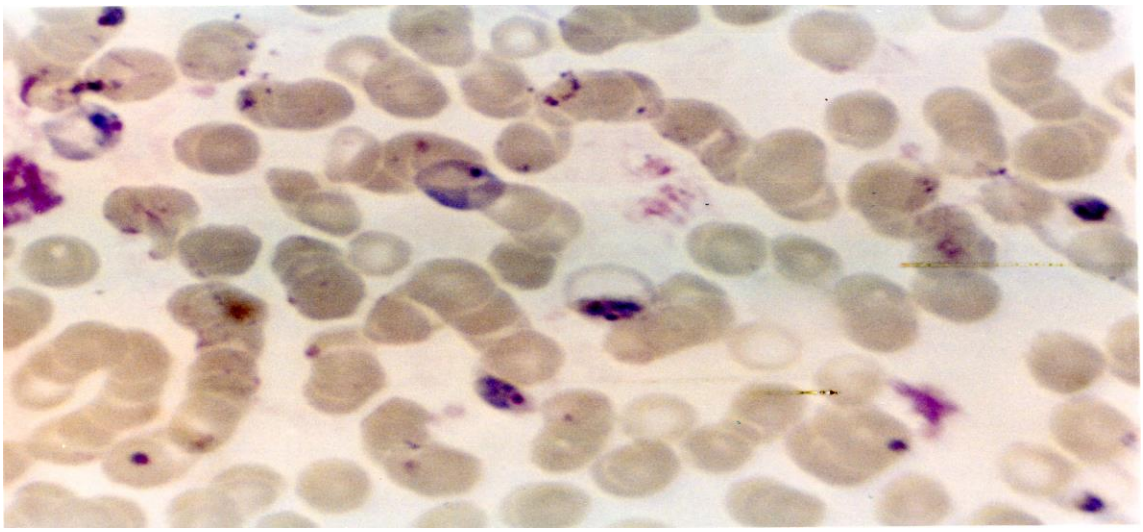


Plate 4.6: Blood film from mouse treated with 250mg/kg dose of ethy acetate extract (Source: Author, 2015)

The parasites were few on the above plate and red blood cells were mostly normocytic.

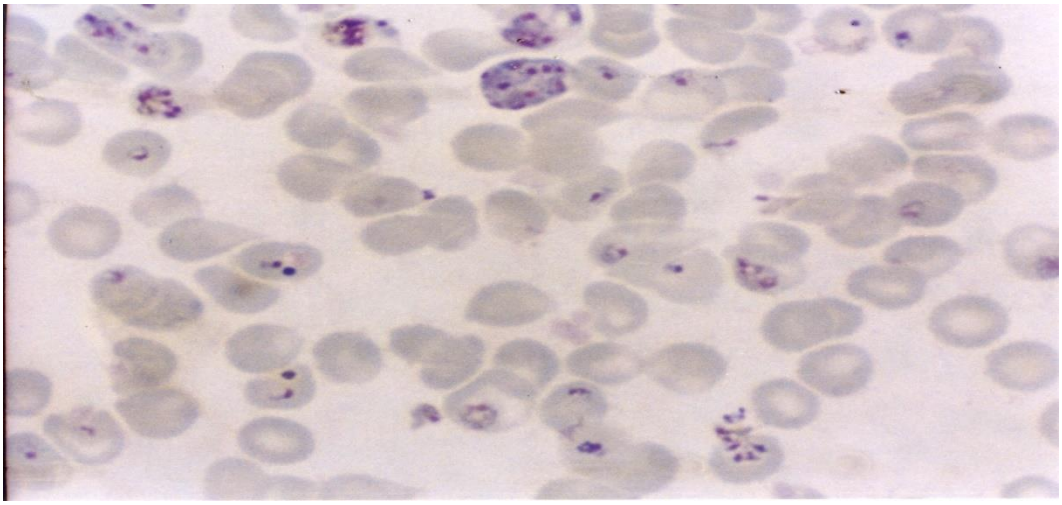


Plate 4.7: Mouse bloodfilm in negative control group in ethyl acetate experiment that received placebo treatment with Tween 80
(Source: Author, 2015)

Several red blood cells in the film for the negative control group were infected with *P. berghei.*

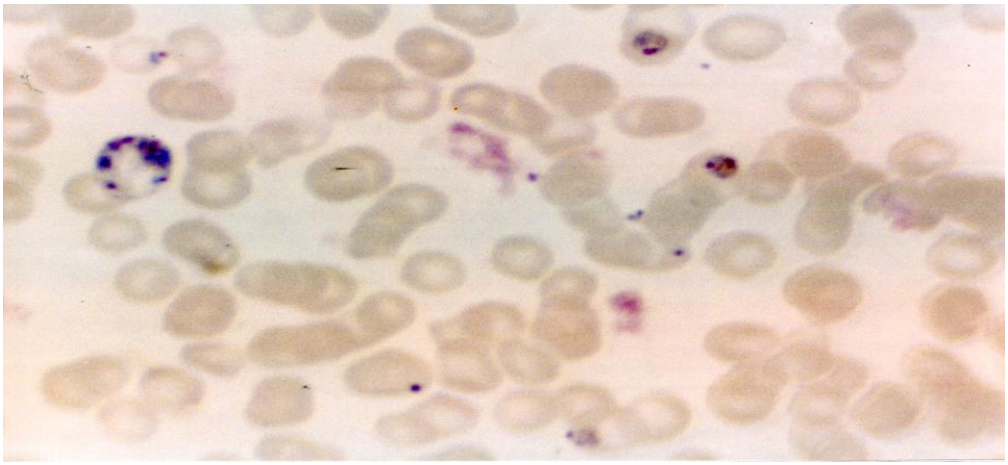


Plate 4.8: Mouse blood film treated with 500mg/kg dose of aqueous extract. (Source: Author, 2015)

The 500mg/kg of aqueous extract reduced the parasitaemia, as indicated in plate 4.8. The red blood cells were both normal and hypochromic in color and normocytic.

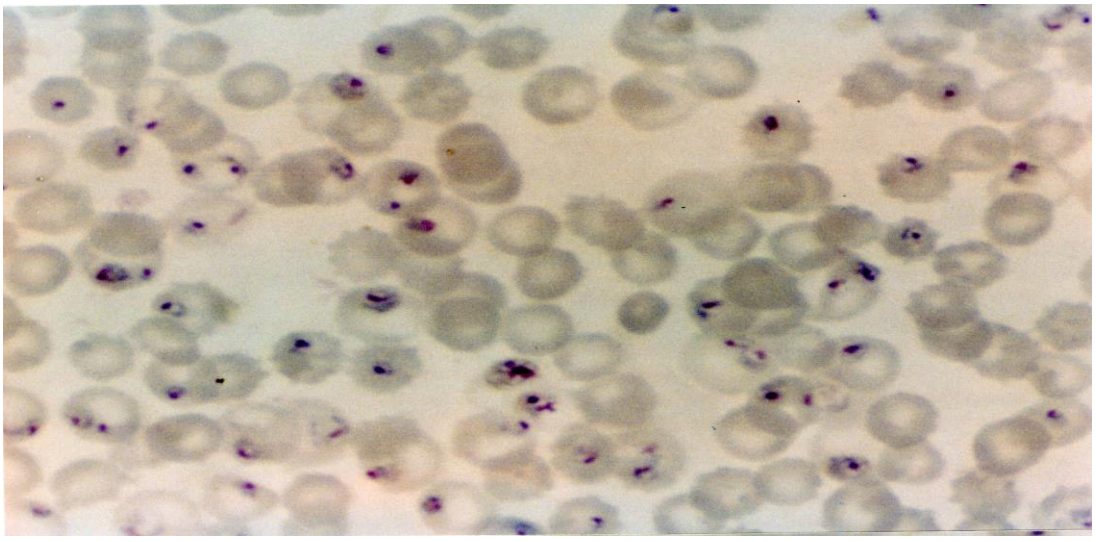


Plate 4.9: Aqueous extract: mouse treated with 10% Tween 80, negative control (NC) group.
(Source: Author, 2015)

Several multiple infections were noted in the red blood cells in the film above. Some of the red blood cells were normocytic while other were microcytic. Hypochromia was observed.

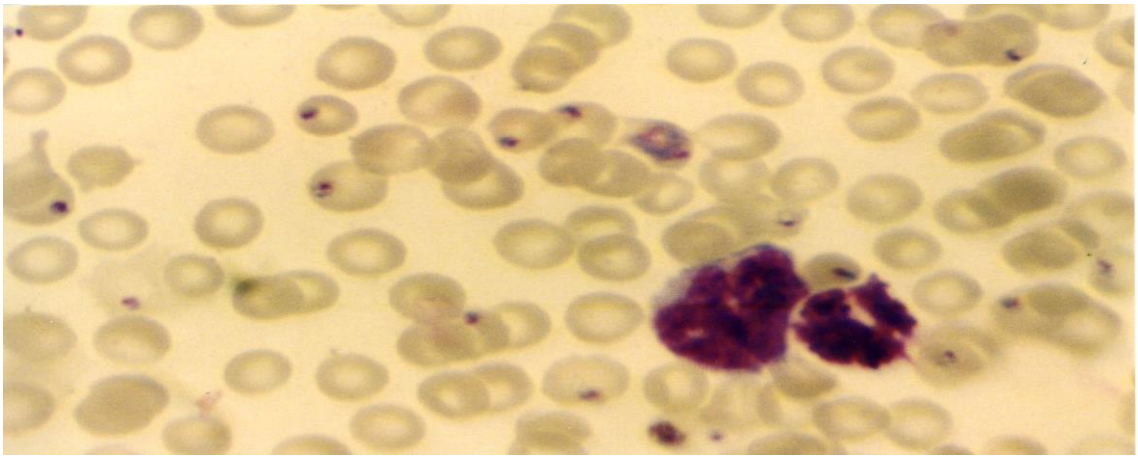


Plate 4.10: Blood film from mouse treated with 250mg/kg of aqueous extract. (Source: Author, 2015)

Some parasites were observed in the plate 4.10 film. The plates showed that white blood cells were also present. No multiple infection was observed in the film.

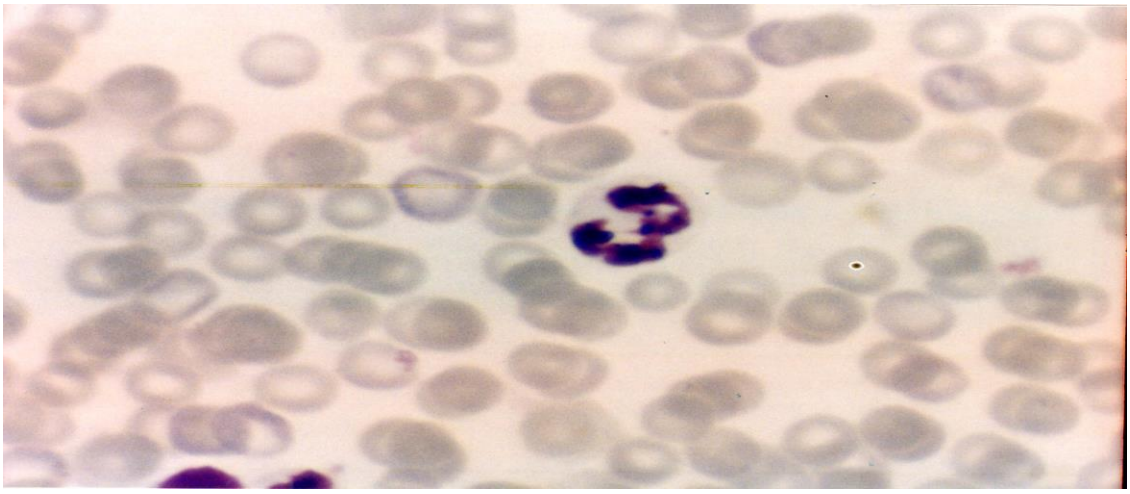


Plate 4.11: Methanol Extract: Blood slide of mouse treated with artemether (positive control).
(Source: Author, 2015)

The positive control drug destroyed all the parasites as demonstrated in the above slide. Hypochromia was observed and some neutrophils were also observed.

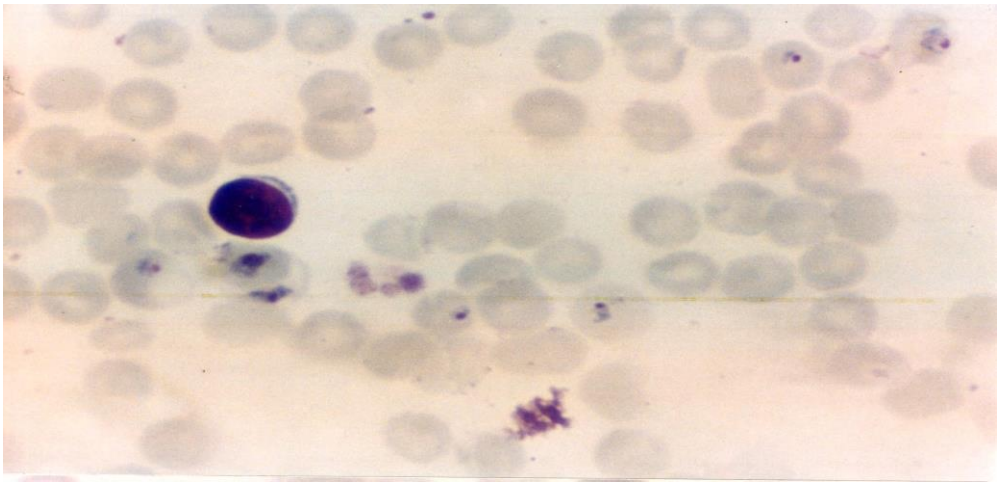


Plate 4.12: Methanol Extract- Blood slide of mouse treated with 500mg/kg dose. (Source: Author, 2015)

Few parasitized red blood cells were observed on the blood film. A lymphocyte was also observed on the blood film.

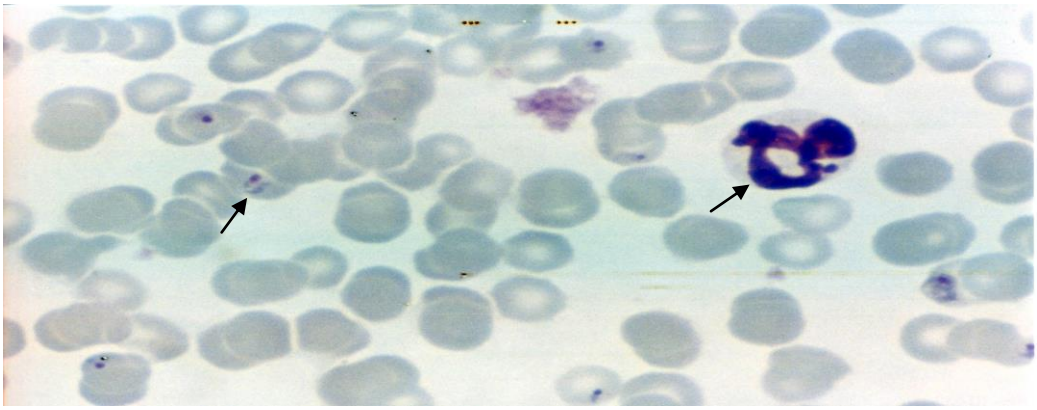


Plate 4.13: Methanol Extract: Mouse treated with 250mg/kg body weight. (Source: Author, 2015)

Plate 4.13 shows a neutrophil (arrow). It also has some parasitized red blood cells (arrow). Many unparasitized red blood cells were observed in the film. Some platelets were seen.

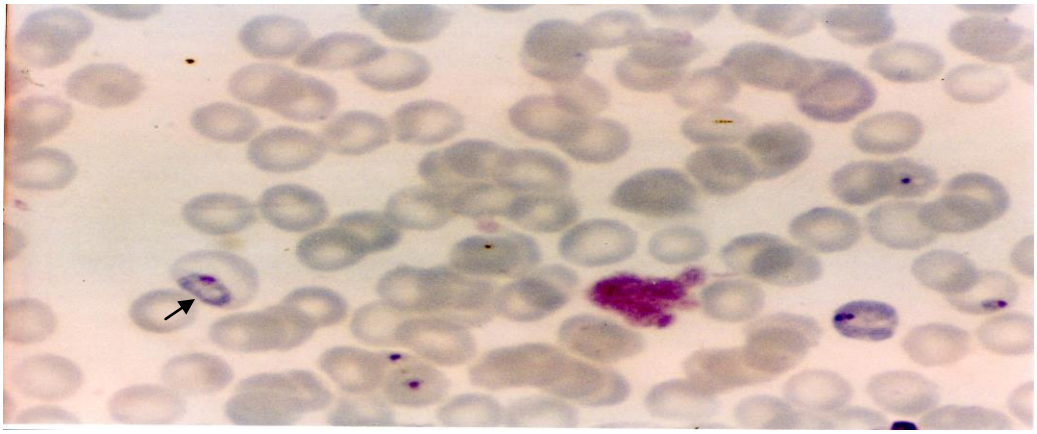


Plate 4.14: Methanol Extract: Mouse treated with 100mg/kg body weight (Source: Author, 2015)

The parasites observed in this film were both small and large (arrow). The red blood cells were normocytic and hypochromic.

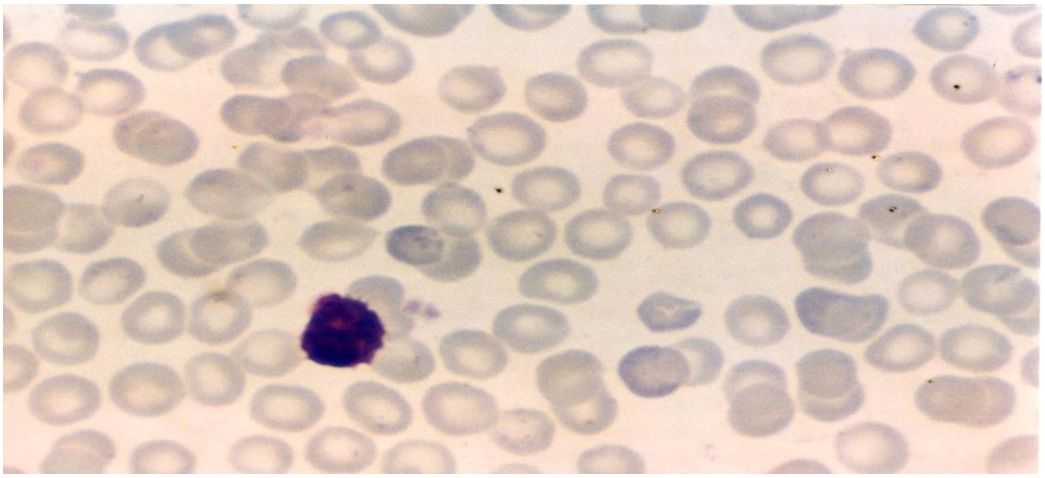


Plate 4.15: Butanol Extract: Mouse treated with artemether (positive control). (Source: Author, 2015)

The blood film prepared from mice in the positive control group had no parasites at all. The percent parasitaemia was 0.00. The control drug was highly effective in destroying the parasites in this experimental group.

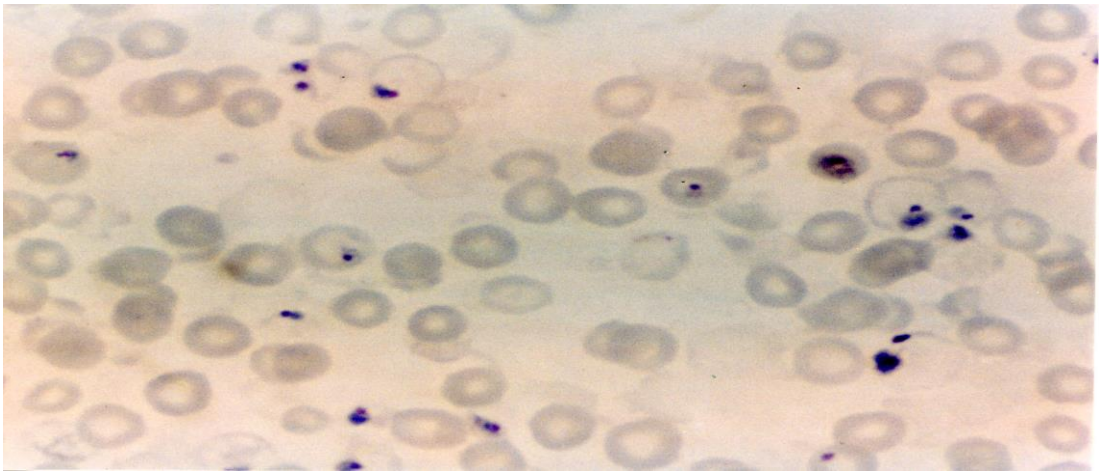


Plate 4.16: Butanol Extract: Mouse treated with 10% Tween 80 , Negative control (NC) group.
(Source: Author, 2015)

A negative Tween 80 control in the butanol extract experiment showed parasitaemia higher than all the other groups. No extract treatment was administered was administered to the group. The average parasitaemia at the end of the experiment was

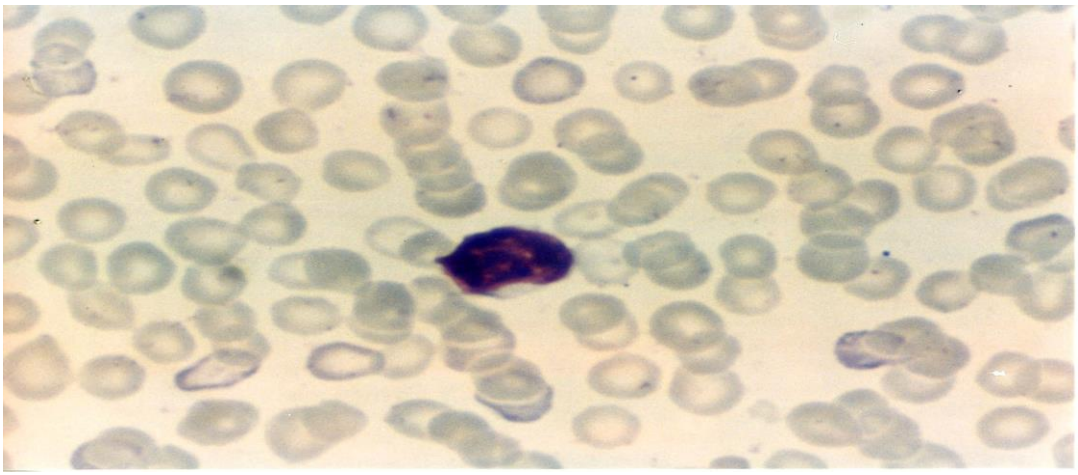


Plate 4.17: Butanol Extract: Mouse treated with 500mg/kg body weight of extract.
(Source: Author, 2015)

The film has no parasites. Mouse was treated with a high dose of the extract. A white blood cell is observed in the middle of the film.

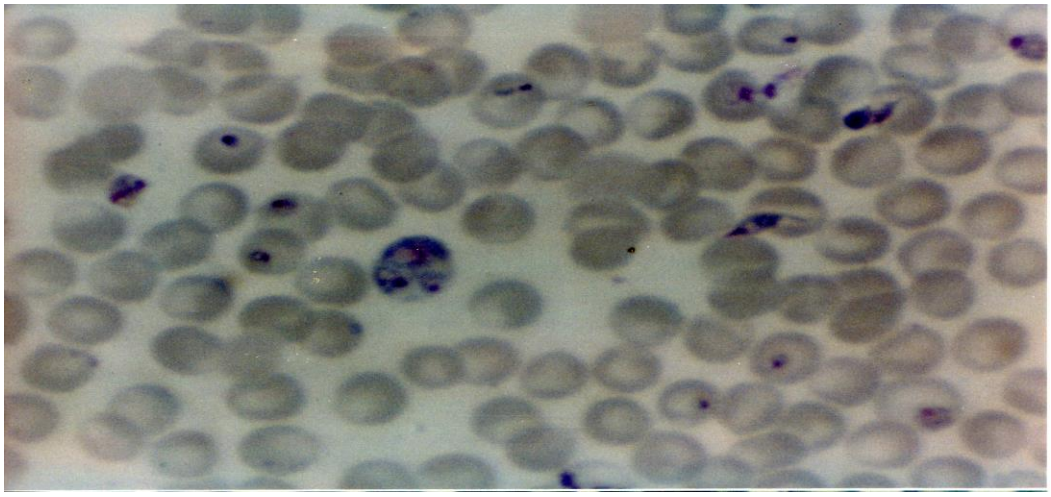


Plate 4.18: Butanol Extract: Mouse treated with 250 mg/kg body weight of extract. (Source: Author, 2015)

One cell has more than two parasites in it. Most cels are uninfected. Excellular parasites are noted in the film.

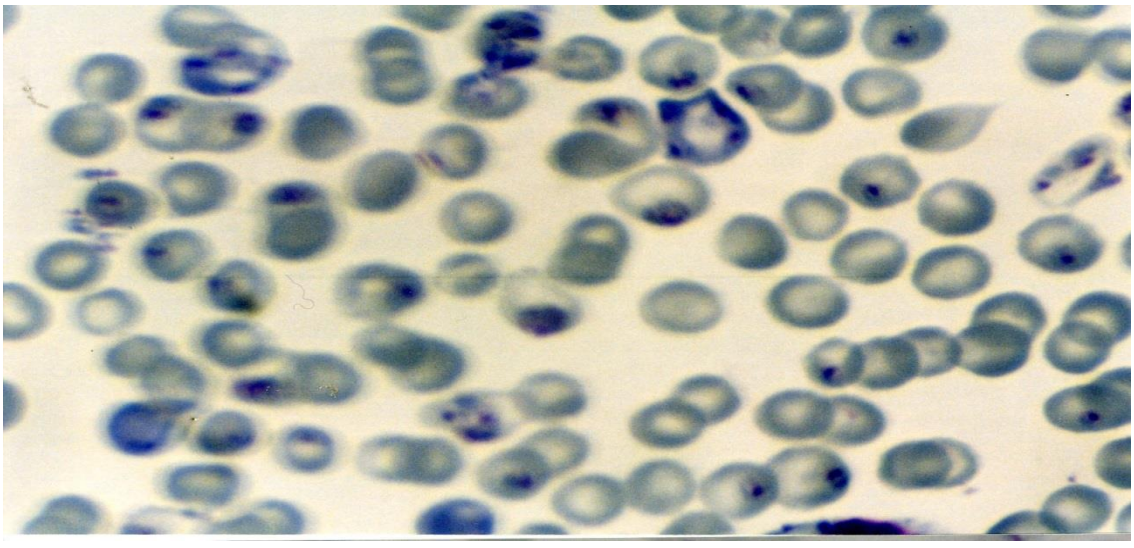


Plate 4.19: Butanol Extract: Mouse treated with 100mg/kg of extract (Source: Author, 2015)

The film has several infected red blood cells. There are many multiple infections in cells.

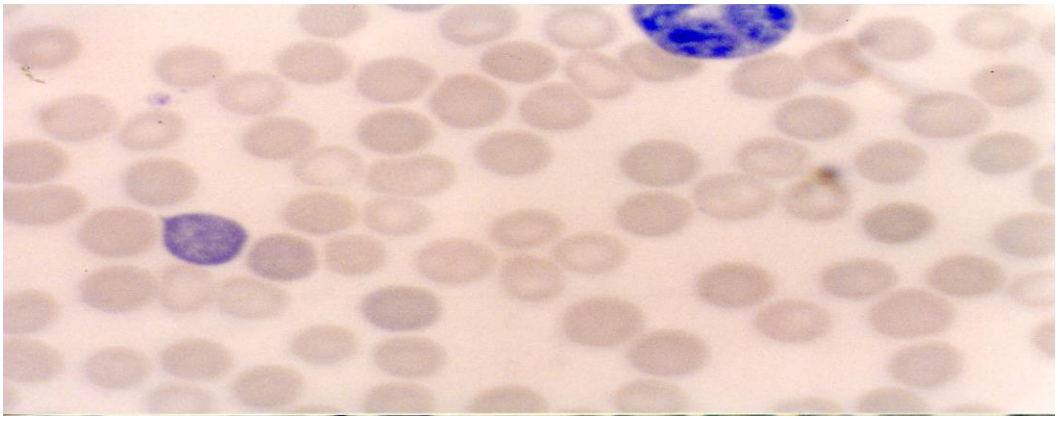


Plate 4.20:Aqueous Extract: Mouse treated with artemether, positive control(PC) group.
(Source: Author, 2015)

The red blood cells are normal in size and shape and have pigment. No parasites are in the film.

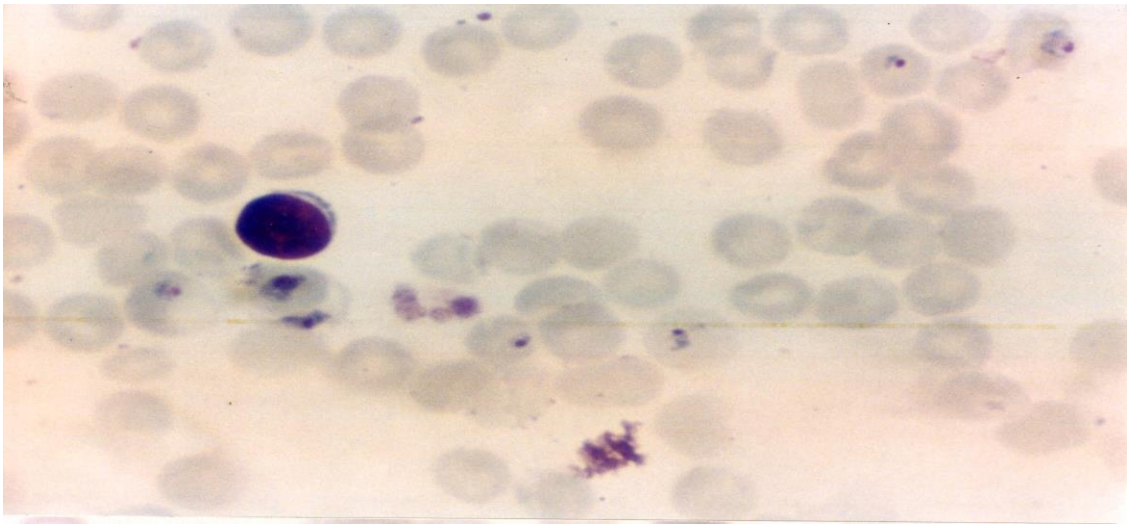


Plate 4.21:Aqueous Extract: Mouse treated with 500mg/kg body weight dose of aqueous extract:
(Source: Obey, 2015)

This red blood cells on this slide appear normal both in size and shape. They maintained their pigment. There are few parasites and a white blood cell.

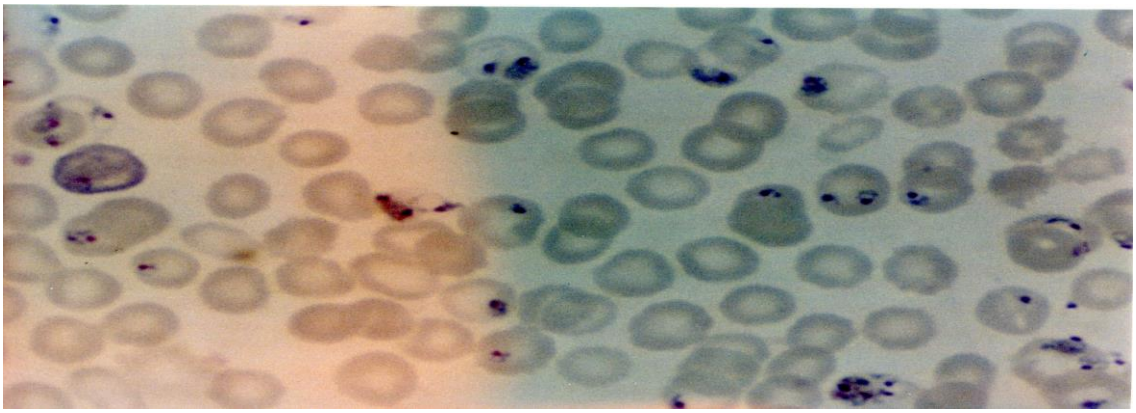


Plate 4.22:Aqueous extract: Mouse treated with 250mg/kg body weight dose of aqueous extract.
(Source: Author, 2015)

Red blood cells in the above film are hypochromic and multiple infections are noted.

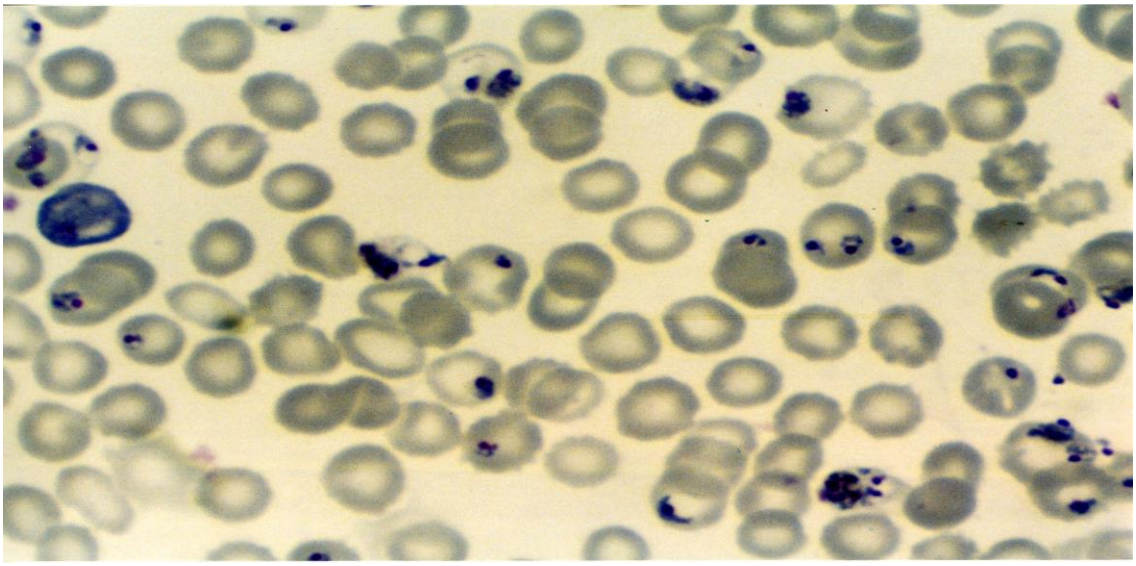


Plate 4.23 :Aqueous Extract: Mouse treated with 100mg/kg body weight of extract.
(Source: Author, 2015)

Multiple infection of red blood cells with two and more parasites are noted in the film.

Several cells are hypochromic but normocytic.

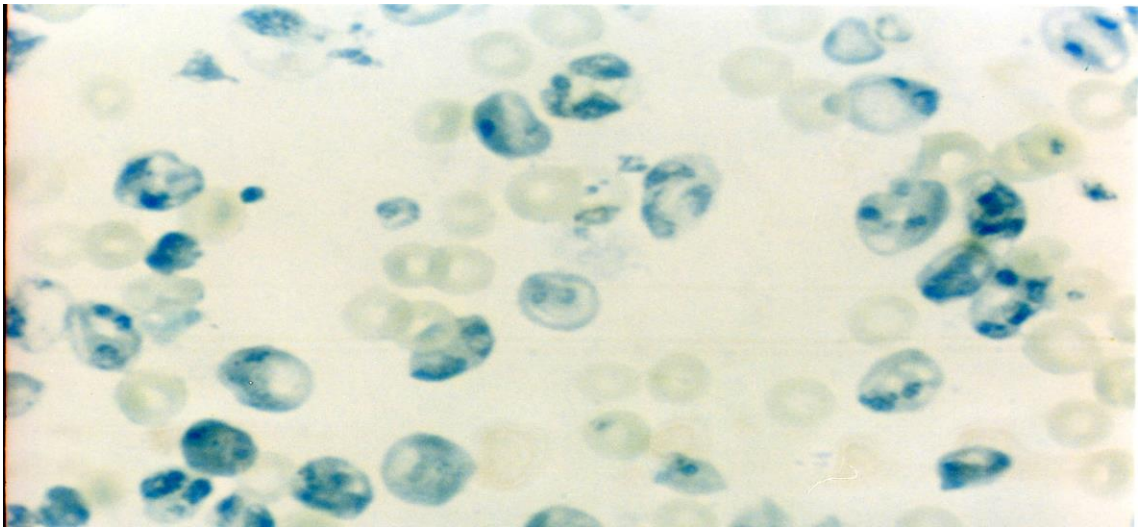


Plate 2.24: Slide prepared from mice given a prophylaxis of 250mg/kg Aqueous Extract of *C. macrostachyus* before infection.
(Source: Author, 2015)

The film above also show that none of the red blood cells were infected. The red blood cells were larger than normal. They are stomatocytes.

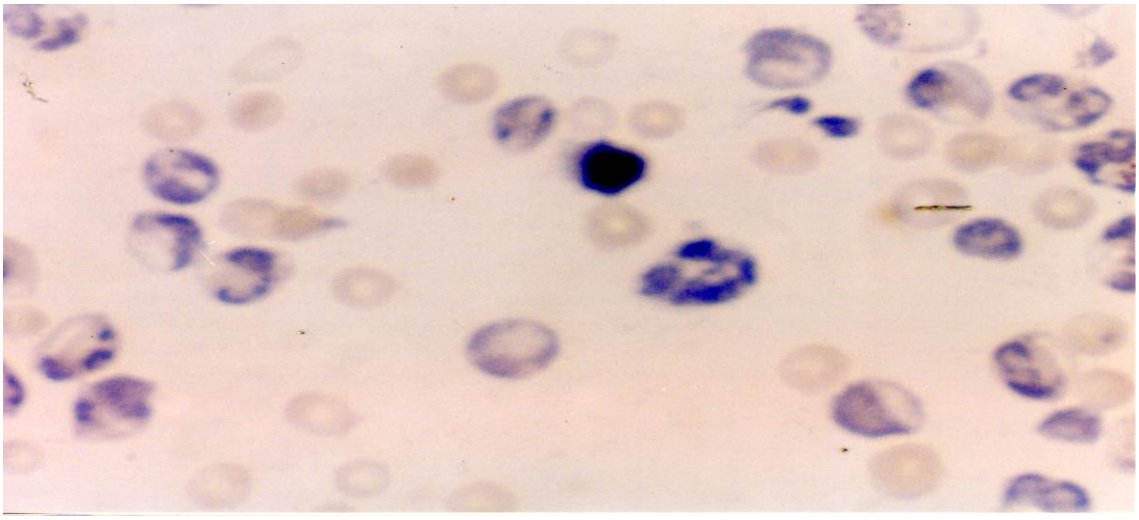


Plate 4.25: Slide prepared from Immunoprophylactic experiment using 250mg/kg body weight of butanol extract.
(Source: Author, 2015)

The blood film has no parasites due to immunoprophylaxis, though the animal was infected. Red blood cell morphology are likened to those of stomatocytes. There is a lymphocyte in the film.

The red blood cells observed in this slide had swollen ends. Several of them can be likened to stomatocytes. There were no parasites observed in plate 4.25.

The clean mice treated with the extract concentrations all showed protective immunity to the extracts regardless of treatment group and type of extract (aqueous, ethyl acetate, methanol or butanol). When treated with the lowest concentration as an immune booster, the ethyl acetate give a percent parasitaemia of 0.20±0.14% for the positive control group, 0.02±0.02% for the 500mg/kg body weight treatment group, 1.23±0.6% for the 250mg/kg group and 4.51±1.59% for the 100mg/kg group. The methanol extract gave 0.24±0.17%, 2.73±0.18%, 5.66±0.44% and 6.94±0.18% for the artemether control, 500mg/kg, 250mg/kg and 100mg/kg respectively. The aqueous extract yielded 2.54±1.71%, 4.16±2.96%, 11.36±3.74 and 16.41±1.12% for artemether control, 500mg/kg, 250mg/kg and 100mg/kg respectively. The butanol extract gave 0.10±0.08%, 1.33±0.54%, 2.74±0.27% and 2.41±0.33% the artemether control, 500mg/kg, 250mg/kg and 100mg/kg respectively. This amazing result as reflected in plate 4.24 was similar for all the animals at the end of the immunoprophylaxis assay. After infection, no animal developed malaria. The main activity of the extract responsible for it immune booster efficacy is due to red blood cell membrane curvature and changes due to stomatocyte formation, making it difficult for parasite survival.

On close observation, it was realized that all the animals treated with the placebo (which was 10% Tween 80 solution) did not respond to treatment well. A slight drop in parasitaemia occurred but the animals had parasitaemia much higher than the treatment groups. For all the extracts, the higher dose treatment controlled parasite growth, therefore the blood films showed few parasites on the film. However, the average of the 6 slides for each concentration was taken from three different experiments. The general trend seen was decrease in parasite density as the dose became higher. This did not rule out the fact that some animals treated with 100mg/kg body weight had lower parasite density than the higher extract dose of 250mg/kg body weight dose in the same experimental group.

The animals that received treatment with Butanol showed the best overall results in the study as far as destruction of the parasites was concerned. The butanol extract was very potent and destroyed the parasites as seen in the films and from the results for the percent parasitaemia in the tables. The animals however, did not survive the butanol treatment. Two of the animals in the butanol group treated with 500mg/ml and 250 mg/ml were dead before the completion of the treatment period of two weeks. In very low concentration, eg 100mg/ml, all the animals survived the treatment.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

Chemical analysis of the crude extracts of *C. macrostachyus* showed that active compounds were terpenes belonging to various general classes of phytochemicals. The methanol extracts contained alkaloids and the ethylacetate extract contained terpenoids, while the aqueous extracts contains n-oxides and quarterinary alkaloids. In other studies done on *C. macrostachyus,* terpenes have been isolated from *C. macrostachyus* stem bark by ethyl acetate and by other organic solvents like dichloromethane (Yibraligm, 2007). Compounds that have been isolated from extracts of *C. macrostachyus* include cromatocrine from the fruits (Yibraligm, 2007) crotepoxide from the fruits(Gelaw *et. al.,* 2012) , 3β-acetoxy-tetraxer-14-en-28-oic acid, trachyloban-19-oic acid, trachyloban-18-oic acid, noeclerodan-5-,10-en19,6β-20,12-diolide, 3α, 19-dihydroxy trachylobane, 3α-, 18,19-trihydroxy trachylobane and lupeol (Yibraligm, 2007).

Results obtained from the spectroscopic analysis at the University of Illinois at Chicago showed that the ethyl acetate pure compound F5 was lupeol (Fig 1) and corresponded well with the nuclear magnetic resonance and carbon-13 spectroscopic analysis of lupeol as indicated in table 4.2 (Reynolds et. al., 1986). The systematic name for the compound lupeol is: 3β-lup-20(29)-en-3-ol. Lupeol is isomorphic with the pentacyclic triterpene 3β,30-dihydroxylup-20(29)-ene, which differs from lupeol due to the presence of an additional hydroxy group. The crystal packing is stabilized by van der Waals interactions

and intermolecular O-H. . .O hydrogen bonds, giving rise to an infinite helical chain along the *c* axis. The chemical formula of lupeol is $C_{30}H_{50}O$. This compound was responsible for the parasitaemia suppression of F5. The lupeol molecule consists of four six-membered rings (adopting chair conformations) and one five-membered ring (with an envelope conformation), all fused in *trans* fashion (Fotie *et. al.,* 2005). Changes in the red blood cell membrane curvature takes place and this changes the shape of the erythrocytes, making them stomatocytes, which are unsuitable for parasite growth (Ziegler, 2002).

In the *in vitro* assay, the chloroquine sensitive strain D6 of *Plasmodium falciparum* was highly susceptible to the pure compound F5 isolated from the ethyl acetate extract of *C. macrostachyus.* The anti-plasmodial activity for the extract was classified as high for $IC_{50} \leq 10\mu g/ml$, moderate for IC_{50} 10-50µg/ml, low at IC_{50} 50-100 and inactive at $IC_{50} >$ 100µg/ml. The IC_{50} values of the controls chloroquine and artemether were the lowest recorded. However, the pure compound F5 extracted from the ethyl acetate showed the lowest IC_{50} *in vitro* and this must have been because it was a pure compound. Before Ziegler, (2002), no mechanism of the *in vitro* activity of lupeol had been reported. The mode of action of lupeol, a pure compound isolated from *C. macrostachyus* ethyl acetate extract, is that it transforms human erythrocytes shape towards those of stomatocytes. It hence causes membrane curvature changes. Lupeol is said to be incorporated into erythrocyte membrane irreversibly. This stomatocyte transformation of host cell membrane leads to an environment unsuitable for parasite survival and is therefore not due to the toxic effects of the plant extract (Ziegler *et. al.,* 2002). Analysis of variance showed significant difference in the IC_{50} for the control drugs, crude extracts and pure

compounds ($p<0.001$, $F=15.072$). A tukey's multiple comparison test on the results showed that the IC_{50} obtained for the control drugs were similar to those of the methanol extract, butanol extract. he pure compounds F5 and $F5_2$ ($p>0.05$). This means that *in vitro*, these extracts and pure compounds were active. Studies have shown that stomatocytogenic compounds act generally as *in vitro* antiplasmodial agents with moderate to high activity(Ziegler *et. al.*, 2002).

Several mechanisms have been proposed for the activity of the extracts from *C. macrostachyus* against *P. berhei in vivo.* According to Kokwaro, (2009), *C. macrostachyus* crude extract has been used traditionally to treat malaria, mumps, diarrhea and other ailments. The *in vivo* treatment assay showed a decrease in parasite density for all the extract groups after treatment. The best result obtained for the percent suppression of parasitaemia was for the aqueous extract (99.20±0.156). The activity of *C. macrostachyus* extracts *in vivo* is comparable to results from studies in which antiplasmodial activity has been related to a range of several classes of secondary plant metabolites including alkaloids and sesquiterpenes, triterpenes, flavonoids, inonoids, quassinoids (Salatino, 2007). These compounds are mostly amphiphiles and are said to protect erythrocytes against hypotonic hemolysis (Hagerstrand and Isomaa, 1994).

In this study using *C. macrostachyus* crude extracts, it was found that the best result for percent suppression of parasitaemia was obtained by the 500mg/kg dose of the ethyl acetate extract (99.88±0.058). Immunoprophylaxis study has been conducted on *Mastomys coucha* using Withanferin A isolated from *Withania somnifera* and this protected the rodents from infection with *Brugia malayi*, a filarial worm (Kushwahaa *et.*

al., 2011). The results from *C. macrostachus* using BALB/c mice proved that the mice developed immunity to *P. berghei* and were able to destroy the parasites *in vivo*. Stomatocytes were observed in the blood film and parasitaemia decrease was probably due to membrane curvature changes that were unsuitable for the parasite survival (Ziegler, 2002). They seem to have been responsible for the protective immunity. On several slides, only few intact parasites could be seen. This shows that extracts from *C. macrostachyus* could be formulated into powerful immune booster drinks against malaria. The results obtained from the prophylaxis studies showed that all the extracts obtained from the *C. macrostachyus* plant can serve as excellent immune boosters for malaria. This finding shows that drugs prepared from *C. macrostachyus* can serve as both an antimalarial drug and can also be used for immunoprophylaxis for travelers to malaria endemic areas.

The chemistry responsible for the mode of action of these species of *Croton* probably lies in the fact that they have been shown to produce secondary metabolites, chiefly diterpenes belonging to the cembranoid, clerodane, neoclerodane, halimane, isopimarane, kkaurane, secokaurane, labdane, phorbol and trachylobane skeletal types (Tene *et. al.*, 2009). The triterpenoids are either pentacyclic or have steroid structure and can even present themselves as shikimate-derived compounds once in a while. Some classes of alkaloids have been found in *Croton* species. *C. macrostachyus* possess these alkaloids in various parts of the plant and have been tested both *in vitro* and *in vivo* (Salatino, 2007). Various other medicinal plants showing antiplasmodial activity in Africa include

Cassia sp., *Lantana sp*. *Kigelia sp*. among others. If studied in more details, these plants can provide a lead to the discovery of several other antimalarial compounds.

Other compounds found in *C. macrostachyus* have suppressed parasitaemia *in vivo* in various situations. Crotepoxide, another active ingredient found in *C. macrostachyus* fruits have been shown to possess antileishmanial acitivity (Gelaw *et. al.,* 2012). The epoxide structure found in crotepoxide is similar to that of artemisinin, a novel antimalarial agent(Gelaw *et. al,* 2012). Another compound found in the fruit of *C. macrostachyus* is cromatocrine, a new clerodane diterpene (Tane *et. al.*, 2004), showed antiplasmodial activity both *in vitro* and *in vivo* situations.

5.2 Conclusion

Four crude extracts were isolated from *C. macrostachyus*. They were the methanol, aqueous, ethyl acetate and butanol extracts. Two fractions were isolated from the ethyl acetate extract. All the crude extracts and fractions showed activity against *P. falciparum* (D6) *in vitro* and *P. berghei* (ANKA) *in vivo.* On further analysis with spectroscopic techniques, the ethyl acetate extract produced a pure compound call lupeol.

The parasite density in infected erythrocytes was reduced in culture in the *in vitro* assay. The results obtained from this study has shown that it is possible to control the growth and destroy *Plasmodium falciparum in vitro* using extracts and fractions of *C. macrostachyus.* The *in vitro* study showed that the *P. falciparum* D6 parasite density decreased when various doses and pure compounds from the *C. macrostachyus* plants were used to control growth *in vitro*.

The *in vivo* assay showed that the extracts of *C. macrostachyus* were able to significantly suppress parasites in infected Balb/c mice. The tukey's test showed that some of the extract suppression of parasitaemia were comparable to artemether, the control drug.
The methanol, aqueous and ethyl acetate extracts of *Croton macrostachyus* have shown potential as antimalarial agents. They are able to indirectly control *Plasmodium* parasites multiplication through reversible erythrocyte membrane modification to form stomatocytes that will destroy malaria parasites and later on transform to normal red blood cells.

The aqueous extract suppressed parasitaemia the highest in the immunoprophylaxis study. The butanol extract proved to be very effective in the treatment of mouse malaria, but was lethal in high doses. It should be prepared in extremely low doses if it is to be considered as a potential antimalarial drug. At 100mg/kg body weight, all the extracts were potent as immunoprophylactic agent. All animals given the doses before infection and treatment survived and had low or no parasitaemia level at the end of the study period. *Croton macrostachyus* extracts had active immunoprophylactic properties against *P. berghei*. It can therefore be concluded that the extracts of *C. macrostachyus* should be tested on a larger sample in animals for possible clinical trials to be conducted for approval for human use.

5.3 Recommendations

This study has produced results that have proved that the compounds found in *C. macrostachyus* extract can be further studied for antimalarial drug preparations. The aqueous and methanol extract components have promise to form antimalarial drug due to their very low toxicity. The butanol extract, regardless of its high efficacy, requires to be studied in lower doses for better results because some animals died in the *in vivo* studies in the butanol extract treatment groups. Immunopropylactic studies are very significant for all the extract components in low doses, therefore, this study should be taken to phase III trials in a bigger group and in *P. falciparum* if the required ethical approval can be obtained. Presently, the study was able to obtain pure compound of only the ethyl acetate extract. More studies must be carried out to obtain pure compounds for each extract of *C. macrostachyus.* This will facilitate determination of the activity of the pure compounds singly or in combination with other components.

REFERENCES

Agbaje, E.O. and Elueze, N.R. (2006). Antimalarial Activities of *Enantia chlorantha* and *Rauwalfia vomitaria* Extracts in Rodents Malaria. *Tropical Journal of Medical Research;* 10 (1): 11-14.

Ajaiyeoba E.O., Ashidi J.S., Okpako L.C., Houghton P.J., Wright C.W., (2008). Antiplasmodial compounds from *Cassia siamea* stem bark extract. *Phytotherapy Research*, 22(2), 254-255.

Alecrim, M.G., Lacerda, M.W., Mourao, M.P., Alecrim, W.D., Padilha, A., Cardoso, B.S., Boulos, M. (2006). Successful treatment of *Plasmodium falciparum* malaria with a six-dose regimen of artemether-lumefantrine versus quinine-doxycline in the Western Amazon region of Brazil. *American Journal of Tropical Medicine and Hygiene;* 74(1): 20-25.

Ambroise-Thomas P.,(1998). New Drugs and Vaccination Against Malaria. Present Status and Perspectives. *Research and Reviews in* Parasitology ; 58(3-4) :237-241.

Anandjiwala S., Honnegowda S., Rajani M., (2007). Isolation and TLC Densitometric Quantification of Gallicin, Gallic Acid, Lupeol and β-Sistosterol From *Bergia suffruticosa*, a Hitherto Unexplored Plant. *Chromatographia;* 66(9-10) 725-734.

Ayuko T. A., Richard N, N., Wanjala C., Nyangasi L. and Isaih O.N., (2009). *In vitro* antiplasmodial activity and toxicity assessment of plant extracts used in

traditional malaria therapy in the Lake Victoria Region. *Mem Inst Oswaldo Cruz, Rio de Jeneiro.* 104(5), 689-694.

Bartoloni, A. and Zammarchi, L.(2012). Clinical Aspects of Uncomplicated and Severe Malaria. Mediterranean Journal of Hematology and Infectious Diseases; 4(1): Open Access logo PLoS Transparent svg.

Berman, J.I., Brown, R., Miller, S.L., Andersen, P., MeGreevy, Schuster, B.G. (1994). Antimalarial activity of WR243251 a dihydroacridinedione. *Antimicrobial Agents and Chemotherapy,* 38 1753-6.

Bergner, P. (2001). Discorea: Wild Yam and Hormonal Synthesis; *Medical Herbalism, Journal for the Clinical Practioner;* 4(4): 9.

Bjorkman, A., and P.A. Phillips-Howard, (1990). Drug resistant malaria: mechanisms of development and inferences for malaria control. *Amer. Journal of Tropical Medicine and Hygiene*, 84: 323-324.

Carter, B.A., Taylor, O.A., Prendergast, D.R., Zimmerman, T.L., Von Furstenberg, R., Moore, D.D., Karpen, S.J. (2007). Stigmasterol, a Soy Lipid-Derived Phytosterol, Is an Antagonist of the Bile Acid Nuclear Receptor FXR; *Pediatric Research*, 62: 301-306.

Carvalho LH. Brandao MG. Santos-Filho D. Lopes JL. Krettli AU.(1991) Antimalarial activity of crude extracts from Brazilian plants studied *in vivo* in *Plasmodium berghei*-infected mice and *in vitro* against *Plasmodium falciparum* in culture. *Brazilian Journal of Medical & Biological Research*. 24(11):1113-23.

Carvalho LH. Krettli A.U., (1991) Antimalarial chemotherapy with natural products and chemically defined molecules. Memorias do Instituto Oswaldo Cruz. 86(2):181-4.

Chen, M., S.B. Christensen, T.G. Theander and A. Kharazmi, and L. Zhai (1994B). Licochalcone A. A new antimalaril agent, inhibits *in-vitro* growth of the human malaria parasite *Plasmodium falciparum* and protects mice from *Plasmodium yoelii* infection. *Antimicrobial Agents chemotherapy*, 38 1470 – 1475.

Contreras, C.E., Cortese, J.E., Caraballo, A., Plowe, C.V. (2002). Genetics of Drug-Resistant *Plasmodium falciparum* Malaria in the Venezuelan State of Bolivar. *American Journal of Tripical Medicine and Hygiene;* 67(2): 400-405.

Craker, L.E. (2007). Medicinal Plants and Aromatic Plants-Future Opportunities. ASHS Press, Alexandria, VA.

de Sa, M.S., Costa, J.F., Krettli, A.U., Zalis, M.G., Maia, G.L., Sette, I.M., Camara, C.A., Filho, J.M., Giulieti-Harley A.M., Ribeiro Dos Santos, R., Soares, M.B. (2009). Antimalarial Activity of Betulinic Acid and Derivatives *In Vitro* Against *Plasmodium falciparum* and *In Vivo* in *P. berghei*-infected Mice. *Parasitology Research;* 105(1): 275-279.

Djimde, A., Doumbo, O.K., Cortese, J.F., Kayentao, K., Doumbo, S., Diourte, Y., Coulibaly, D., Dicko, A., Su, X.Z., Nomura, T., Fidock, D.A., Wellems, T.E., Plowe, C.V. (2001). A Molecular Marker For Chloroquine-resistant falciparum Malaria. *New England Journal of Medicine;* 344(4): 257-63.

Dzubak P., Hajduch M., Vydra D., Hustova A., Kvasnica M., Biedermann D.,

Markova L., Urban M. Sarek J. (2006). Pharmacological activities of natural triterpenoids and their therapeutic implications; Royal Society of Chemistry, *Natural Product Reports,* 23: 394-411.

Elueze, E.I., S.L. Croft and D.C. Warhurst, (1996). Activity of pyronaridine and mepacrine against twelve strains of *Plasmodium falciparum in-vitro*. *Journal of Antimicrobial Chemotherapy,* 37 511-518.

Fernández MA[1], de las Heras B, García MD, Sáenz MT, Villar A.(2001). New insights into the mechanism of action of the anti-inflammatory triterpene lupeol. *J Pharm Pharmacol.* 53(11):1533-9.

Figgitt, D., W. Denny, P. Chavalitshewinkoon, P. Wilairat and R. Ralph, (1992) *In-vitro* study of anticancer acridines as potential antitrypanosomal and antimalarial agents. *Antimicrobial Agents and Chemotherapy*, 36 1644-7.

Fotie J., Bohle D.S., Leimanis M.L., Georges E., Rukunga G., Nkengfack A.E., (2005). Lupeol Long-Chain Fatty Acids Esters With Antimalarial Activity From *Holarrhea floribunda.* Journal of Natural Products. 69 (1), 62-67.

Fournet, A., V. Munoz, A. Angelo, M. Aguilar (1990). Plants medicinales Boliviennes antiparasitaires. International congress of parasitology, A29 Paris.

Fulda, S., (2008). Betulinic Acid for Cancer Treatment and Prevention. *Internal Journal of Molecular Sciences*, 9, 1096-1107.

Gelaw H., Adane L.; Tariku Y., Hailu A.,(2012). Isolation of Crotepoxide From Berries of *Croton macrostachyus* and Evaluation of Its Anti-Leishmanial Activity; *Journal of Pharmacognosy and Phytochemistry;* 1(2): 15-24.

Geoffrey, C.K., (1996). Medicinal plants and the control of parasites. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 90: 605-609.

Gessler M.C., Nkunya MH. Mwasumbi LB., Heinrich M., Tanner M.; (1994) Screening Tanzanian medicinal plants for antimalarial activity. *Acta Tropica*. 56(1):65-77.

Gessler M. C., M. Tanner, J. Chollet, M.H.H. Nkunya, M. Heinrick, (1995). Tanzanian medicinal plants used traditionally for the treatment of malaria; *In vitro* antimalarial and *in vitro* cytotoxic activities. *Phytotherapy Research;* 9(7): 504-508.

Gregson, A., and Plowe, C.V. (2005). Mechanisms of Resistance of Malaria Parasites to Antifolates. *Pharmacological Reviews*; 57(1): 117-145.

Guidance For Industry: Single Dose Acute Toxicity Testing for Pharmaceuticals.(1996). Center For Drug Evaluation and Research (CDER). August.

Gurib-Fakim, A. (2006). Medicinal Plants: Traditions of yesterday and drugs of tomorrow; *Molecular Aspects of Medicine,* 27: 1-93.

Hägerstrand, H., and B. Isomaa.(1994). Lipid and protein composition of exovesicles released from human erythrocytes following treatment with amphiphiles Biochim. Biophys. Acta 1190**:**409-415.

Hägerstrand, H., and B. Isomaa. (1989). Vesiculation induced by amphiphiles in erythrocytes. Biochim. *Biophys. Acta* 982**:**179-186.

Haile, Yineger, Delenasaw Yewhalaw and Demel Teketay (2008), Ethnomedical Plants Knowledge and Practice of Oromo Ethnic Group in Southern Ethiopia. *Journal of Ethnobiology and Ethnomedicine.* 4:11.

Harborne J.B., (1998). Phytochemical methods. A guide to modern techniques of plant analysis; 3rd Ed. Chapman and Hall, London.

Hodgkin, D . (1991). Seeking cures in the Jungle. *Insight*, 7: 30.

Huang, P.L., P.L. Huang, H.I. Huang, and S. Lee-Huang, (1992). Developing drugs from traditional medicinal plants, *Chemistry and Industry*, 8 290 – 293.

Ill-Min Chung, Bimal Kumar, Eun-Young Kang and Hyung-in Moon (2009). Antiplasmodial and Cytotoxic Activity of Khellactone Derivertives from Angelica Purpuraefolia Chung. *Phototherapy Research Journal.* 24(3), 469-71.

Iwu, M.M.(1994). African Medicinal Plants in the Search for New Drugs Based on Ethnobotanical Leads; *Ciba Found. Symp;* 185: 116-125.

Kantamreddi V.S.S. and Wright C.W. (2007). Investigation of Indian *Diospyros* Species for Antiplasmodial Properties. Evidence-based Compl and Alternative Medicine 5(2) 187-190.

Kimbi H.K. and Fagbenro-Beyioku A.F. (1996). Efficacy of *Cymbopogon giganteus* and *Enantia chrantha* against chloroquine resistant *Plasmodium yoelii nigeriensis; East African Medical Journal.* 73(10):636-7.

Kofi-Tsekpo, W.M. (1993a). Pharmaceutical Research. *International Journal of BioChemiPhysics,* 2 155-157.

Kofi-Tsekpo, W.M. (1993b). The essential requirements for the incorporation of Traditional Medicines in the Health Care System., No. 65/93. Progress of KEMRI/KETRI 14th Annual Medical Scientific Conference: Recent Advances in Medical Research with a Symposium on Management and Control of Vector Borne Diseases.

Kokwaro, J.O. (1976). Medicinal plants of East Africa. E.A. Literature Bureau, Nairobi.

Kokwaro, J.O.(2009). Medicinal Plants of East Africa. University of Nairobi Press, Nairobi.

Kuria, K. A., De Coster, S., Muriuki, G., Masengo, W., Kibwage, I., Hoogmartens J., Laekeman, G.M. (2001). Antimalarial acitivy of *Ajuga remota* Benth (Labiatae) and *Caesalpinia volkensii* Harms (Caesalpiniaceae) : *In vitro* confirmation of ethnopharmacological use. *Journal of Ethnopharmacology*; 4(2): 141-148.

Kushwahaa S., Sonia V.S., Singha P.K., Banoa N, Kumarb A., Sangwanb R.S., Bhattacharyaa S.M., (2011). *Withania somnifera* chemotypes NMITLI 101R, NMITLI 118R, NMITLI 128R and Withaferin A protect *Mastomys coucha* from *Brugia malayi* infection. Blackwell Publishing Ltd.

Le bras, J. and Deloron, P. (1983). *In-vitro* study of drug sensitivity of *Plasmodium falciparum*: an evaluation of a new semi-microtest. *American Journal of Tropical Medicine and Hygiene,* 32 447 – 451.

Leonardo, K.B., O. Ramiliarisoa, and J. Le Bras, (1995); *In vitro* activity of Atovaquone against the African isolates and clones of *Plasmodium falciparum*; *American Journal of Tropical Medicine and Hygiene,* 53 (4) 388-391.

Leonardo, K.B.,S. Mitaku, A. Skaltsounis. N. Ravelomanantsoa, F. Tillequin, M. Koch and J. LeBras, (1994). *In-vitro* activities of Furoquinoline and Acridone alkaloids against *Plasmodium falciparum*. *Antimicrobial Agents and Chemotherapy*, 1169 - 1171.

Liu, G.Z. (1981). Study of Gossypol as a Male Contraceptive. *Reproduccion;* 5(3): 189-193.

Mahyar UW. Burley J.S., Gyllenhaal C., Soejarto D.D., (1991). Medicinal plants of Seberida Riau Province, Sumatra, Indonesia. *Journal of Ethnopharmacology*. 31(2):217-37, Feb.

Malik, S., Cusido R.M., Mirjalili, M.H., Moyano, E., Palazon, J., Bonfill M.(2011). Production of the anticancer drug Taxol in *Taxus baccata* suspension cultures, A Review. *Process Biochemistry*; 46(1): 23-34.

Matheus S. S., Jose F. O. C., Antoniana U. Mariano G. Z., Gabriela L. A., Ivan M. F. Celso A. C., Jose M. F., Ana M. G., Richard R.S. and Milena B. P.,,(2009) Antimalarial Activity of Betulinic Acid and Derivertives in Vitro Againsst Plasmodium Falciparum and Invivo in P. Berghei – infected mice. *Parasitology Research.* 105(1), 275-279.

Miert S., Pieters L., Totte J., Vlietinck, A.J. (2004). In Vitro Antiplasmodial Activity of Extract ans Fractions From Seven Medicinal Plants Used in Democratic Replublic of Congo. *Journal of Ethnopharmacology*, 93(1), 27-32.

Miguel, M.G. (2010). Antioxidant and Anti-inflammatory Activities of Essential Oils: A Short Review. Molecules; 15: 9252-9287.

Milhouse, W.K., N.F. Weatherly, J.H. Bowdre, and R.E. Desjardins, (1985). *In-vitro* activities of and mechanism of resistance to antifol antimalarial drugs; *Antimicrobial Agents and Chemotherapy*, 27 (4) 525-530.

Mitaku, S., A.L. Skaltsounis, F. Tillequin, M. Koch, J. Pusset and G. Chauviere, (1985). Plantes de Nouvelle-Caledonie, XCVI. Alcaloides de Geijera balansae. *Journal of Natural Products*, 48 772-777.

Mitaku, S., A.L. Skaltsounis, F. Tillequin, M. Koch, J. Pusset and G. Chauviere, (1987). Plantes de Nouvelle-Caledonie, XCVI; Four new alkaloids from *Sarcomelicope dogniensis. Heterocycles*, 26 2057 – 2063.

Mitaku, S., A.L. Skaltsounis, F. Tillequin, M. Koch, J. Pusset, and G. Chauviere (1986). Plantes de Nouvelle-Caledonie, CVI; Alkaloids from *Sarcomelicope glauca*. *Journal of Natural Products,* 49 1091 – 1095.

Mohammed, T., Erko, B., Giday, M. (2014). Evaluation of the antimalarial activity of leaves of *Acokanthera schimperi* and *Croton macrostachyus* against *Plasmodium berghei* in Swiss albino mice. *BMC Complementary and Alternative Medicine*; 14:374.

Muthaura, C.N., G. M. Rukunga, S. C. Chhabra, S. A. Omar, A. N. Guantai, J. W. Gathirwa, F. M. Tolo, P. G. Mwitari,L. K. Keter, P. G. Kirira, C. W. Kimani,G. M. Mungai and E. N. M. Njagi (2007). Antimalarial activity of some plants traditionally used in Meru district of Kenya. *Phytotherapy Research*;21(9): 860-867.

Mwambo Z.H., Foubert K., Chacha M., Kapingu M.C., Magadula J.J., Moshi M.M., Lemiere F., Go J., Peschar R., Vlietinck A., Apers S., Pieters L., (2009). New furanoditerpenoids From *Croton jatrophoides*. *Planta*, 75(3): 262-7.

Norman R.F., A. Olayiwola, S.B. Audrey, D.S. Djaj and g. Zhengang (1985). Medicinal plants in therapy. *Bulletin of the WHO*, 63 (6) 965 – 981.

Peters, W., J.H. Portus, B.L. Robinson, (1975). The chemotherapy of rodent malaria XXII, the valve of drug-resistant strains of *Plasmodium berghei* in screening for

blood schizontocidal activity. *Annals of Tropical Medicine and Parasitology*, 69 (2) 155 –171.

Pillai, P.G., Suresha, P., Mishrab, G., Annapurnaa, M. (2011). Evaluation of the Acute and Sub-acute Toxicity of the Methanolic Leaf Extract of *Plectranthus amoboinicus* (Lour) Spreng in BALBc Mice. *European Journal of Experimental Biolology*; 1: 236-245.

Rathee, D., Rathee, S., Rathee, P., Deep, A., Anandjiwala. S., Rathee D., (2015). HPTLC Densitometric Quantification of Stigmasterol and Lupeol from *Ficus religiosa.* *Arabian Journal of Chemistry*; 8(3): 366-371.

Reynolds, W.F., McLean, S., Poplawski, J., Enriquez, R.G., Escobar, L.I., Leon, I., (1986). Total Assignment of ^{13}C and ^{1}H Spectra of Three Isomeric Triterpene Derivatives by 2D NMR, Tetrahedron; 42: 3419-3428.

Salatino, A. (2007). Traditional Uses, Chemistry and Pharmacology of Croton Species. *Journal of Brazillian Chemistry Society;* 18(1).

Saxena S., Pant N., Jain D.C., Bhakuni R.S., (2003). Antimalarial Agents from Plants. *Current Science*; 85(9): 1314-1329.

Schift, Jr., P.L., (1991). Bisbenzylisoquinoline alkaloids; *Journal of Natural Products*, 54 645 – 749.

Sheetal Anandjiwala, Honnegowda Srinivasa and Mandapati Rajani (2007). Isolation and TLC Densitometric Quantification of Gallicin, Gallic Acid, Lupeol and β-Sitosterol from Bergia suffruticosa, a Hitherto Unexplored Plant. *Chromatographia Journal.* 66(9-10) 725-734.

Sheng, L.H. (1982). Traditional Chinese medicine and immunity; Guangdong Scientific Publishing House, Guangzhou, People's Republic of China 41 – 49.

Steele, J.C., Warhurst, D.C., Kirby, G.C., Simmonds, M.S. (1999) *In Vitro* and *InVivo* Evaluation of Betulinic Acid as an Antimalarial. *Phytotherapy Research;* 13(2), 115-9.

Suksamrarn A., Tanachatchairatana T., and Kanokmedhkakul, S. (2003), Antiplasmodial Triterpenes from Twigs of *Gardenia saxatilis. Journal of Ethnopharmacology*.88(3) 275-2277.

Tane, P., Tatsimo, S., Connolly, J.D. (2004). Cromatocrine, A New Clerodane Diterpene From the Fruits of *Croton macrostachyus*. Tetrahedron Letters; 45(38): 6997-6998.

Tene M., Ndontsa B.L., Tane P., Tamokou J., Kuiate J.R., (2009). Antimicrobial diterpenoids and triterpenoids from the stem bark of *Croton macrostachys*; *International Journal of Biological and Chemical Sciences;* 3(3): 538-544.

Tengwa, M.C., Sonfack, D. C. R., Fokom R., Penlap Beng, V., Amvam Zollo, P.H. (2013). Antifungal and antioxidant activity of crude extracts of three medicinal plants from Cameroon pharmacopeia. *Journal of Medicinal Plant Research*; 7(21): 1537-1542.

Thitima R., Amporn S., Chatchana T., Sudarut W, and Apichart S. (2007). Biological Activities of the Chemical Constituents on *Erythrina stricta* and *Arythrine subumbrans*. *Pharmaca Research Journal*, 30(11), 1398-1403.

United Nations Development Program/world Bank/World Health Organization, (1989). The Leishmaniasis, WHO special programme for research and training in

diseases ninth programme report. Tropical diseases: progress in international research, 1987 – 1988, WHO, Geneva.

United Nations Development Program/World Bank/World Health Organization, (1990). Special programme for research and training in tropical diseases (TDR). Antimonials: Large-scale failure in Leishmaniasis 'alarming' TDR New. 34 1–7.

Wansi J.D., Hussain H., Tcho A.T., Kouam S.F., Specht S., Sarite S.R., Hoerauf A., Krohn K. (2009) Antiplasmodial Activities of Furiquinoline Alkaloids From *Teclea afzelii. Phytotherapy Research*, 24(5): 775-777.

Warrel, D.A., (1993). Treatment and prevention of Malaria. Bruce-Chwatt's Essential Malariology, edited by Gilles H.H. and D.A. Warrel 164-195. Edward Arnold, London.

Wernsdorfer, W.H., and D. Payne, (1991). The dynamics of drug resistance in *Plasmodium falciparum*. *Pharmacology Therapy.* 50 95-121.

World Health Organization (2000). Report of the WHO Expert Committee on Malaria. *WHO Tech. Rep. Ser.;* 1-74.

World Health Organization (2009). WHO Monograph on Selected Medicinal Plants. – Volume 4. Essential Medicine and Health Information Portal. A World Health Organization Portal; 4:1.

Wright, C.W. and Phillipson, J.D., (1990). Natural products and the development of selective antiprotozoal drugs. *Phytotherapy Research*, 4 127 – 139.

Yi Bi, J.X., Xiaoming, W.W,Y.; Shengtao Y.; Luyong Z. (2006). Synthesis and cytotoxic activity of 17-carboxylic acid modified 23-hydroxy betulinic acid ester derivative. *Science Direct*., 17(6):1475-1478.

Yibralgm Z. (2007). Phytochemical Investigation on the stem bark of *Croton macrostachyus* (BISANA). Addis Ababa University School of Graduate Studies. Masters thesis dissertation

Yieneger H., Yewhalaw D., Teketay D., (2008). Ethnomedicinal Plant Knowledge and Practice of the Oromo Ethnic Group in Southwestern Ethiopia. Journal of Ethnobiology and Ethnomedicine. 4(11): 1746.

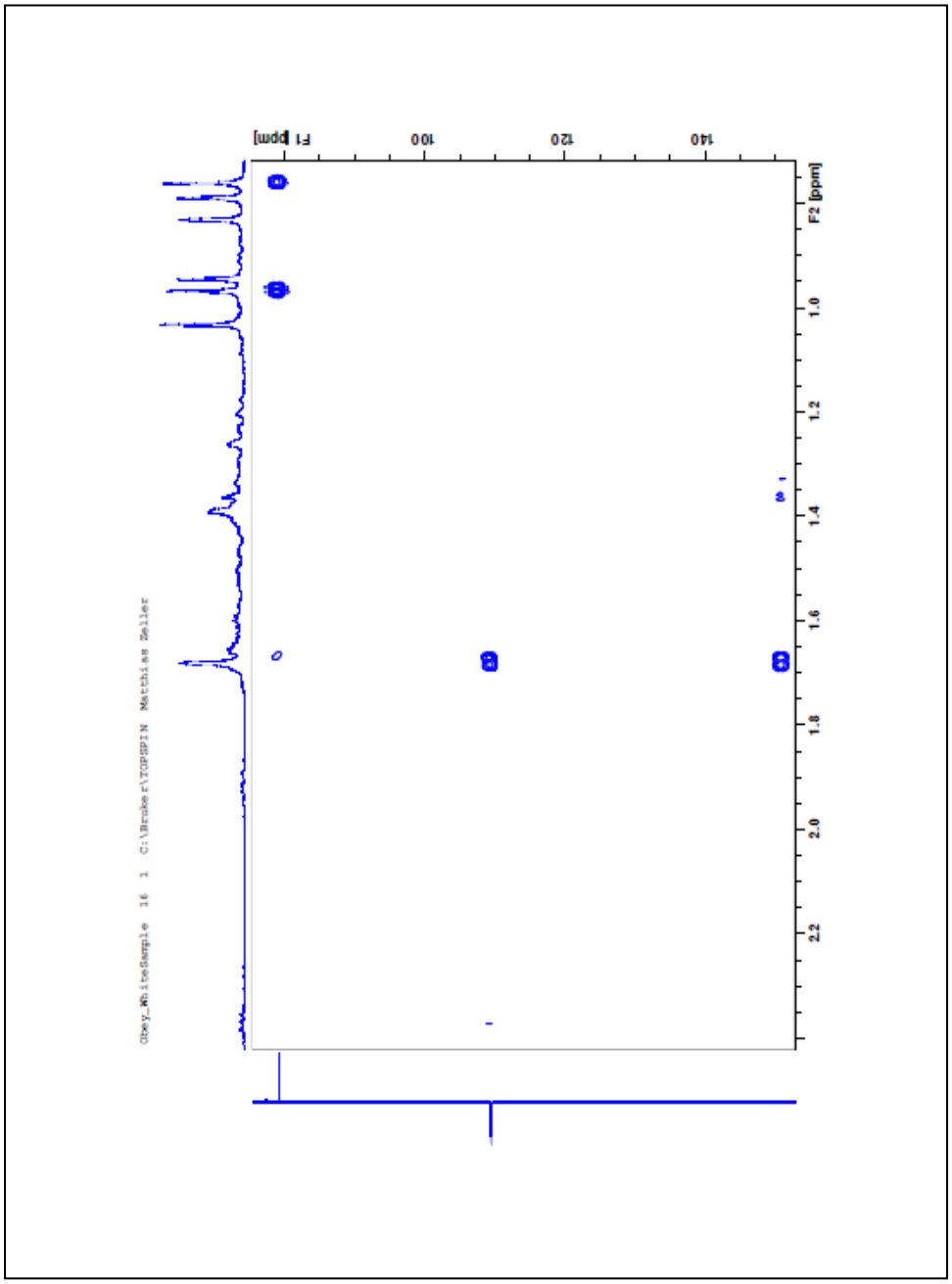
Ziegler H., (2002). *In Vitro Plasmodium falciparum* Drug Sensitivity Assay: Inhibition of Parasite Growth by Incorporation of Stomatocytogenic Amphililes in Erythrocyte Membrane. Antimicrobial Agents and Chemotherapy, 46(5), 1441-1446.

Ziegler H., Hansen, S.S., Stoerk, D., Soren, B.C., Hagerstrand, H., Jaroszewski (2004). The Antiparasitic Compound Licochalcone A Is a Potent Echinocytogenic Agent That Modifies the Erythrocyte Membrane in the Concenteration Range Where Antiplasmodial Activity Is Observed; *Antimicrobial Agents and Chemotherapy;* 48(10): 4067-4071.

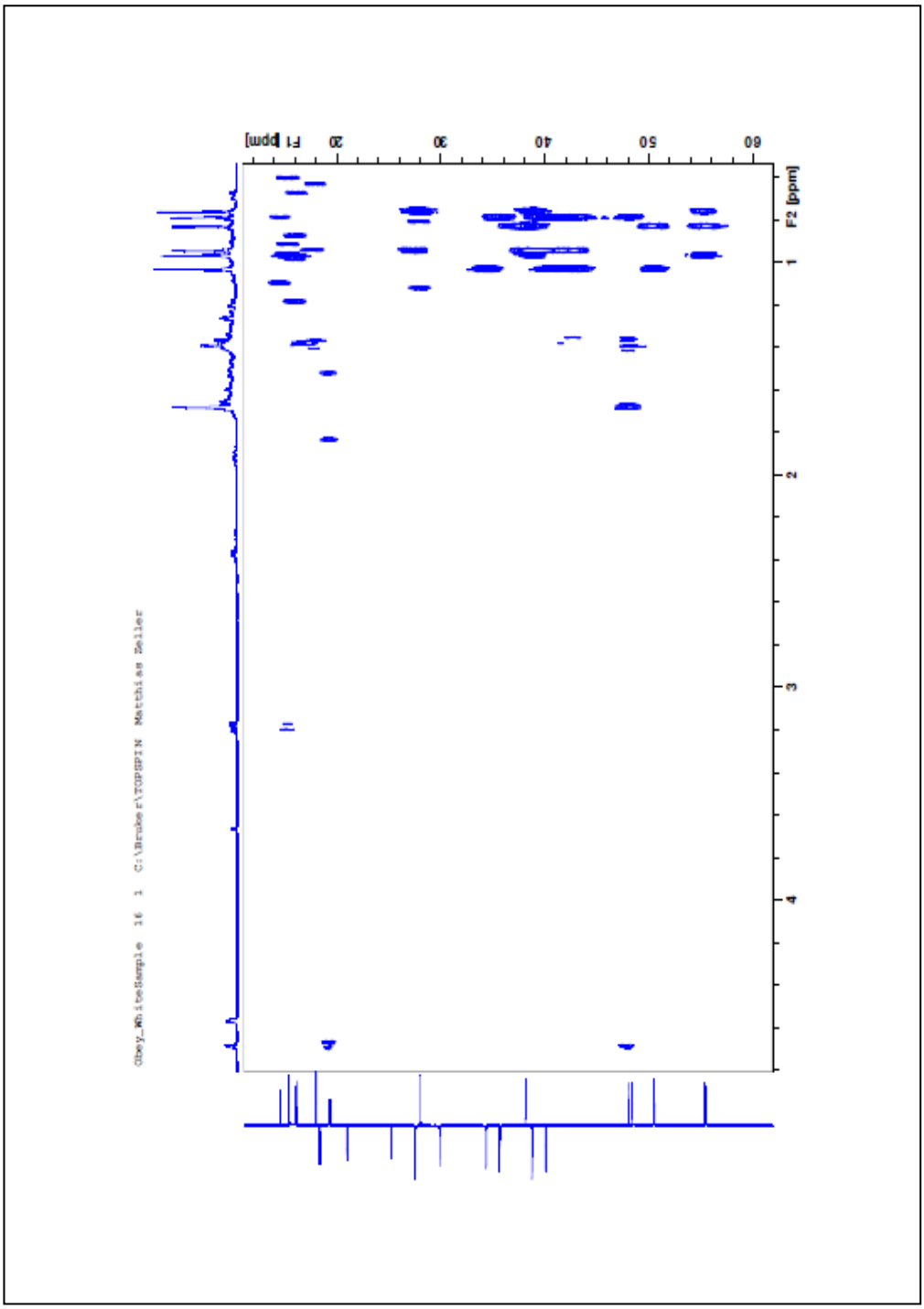
Zore, G.B., Awad, V., Thakre, A.D., Halde, U.K., Meshram, N.S., Surwase, B.S., Karuppayil, S.M. (2007). Activity-directed fractionation and Isolation of Four Antibacterial Compounds From *Abrus precatorius* L. Roots. *Natural Product Research*; 21(10): 933-940.

APPENDICES

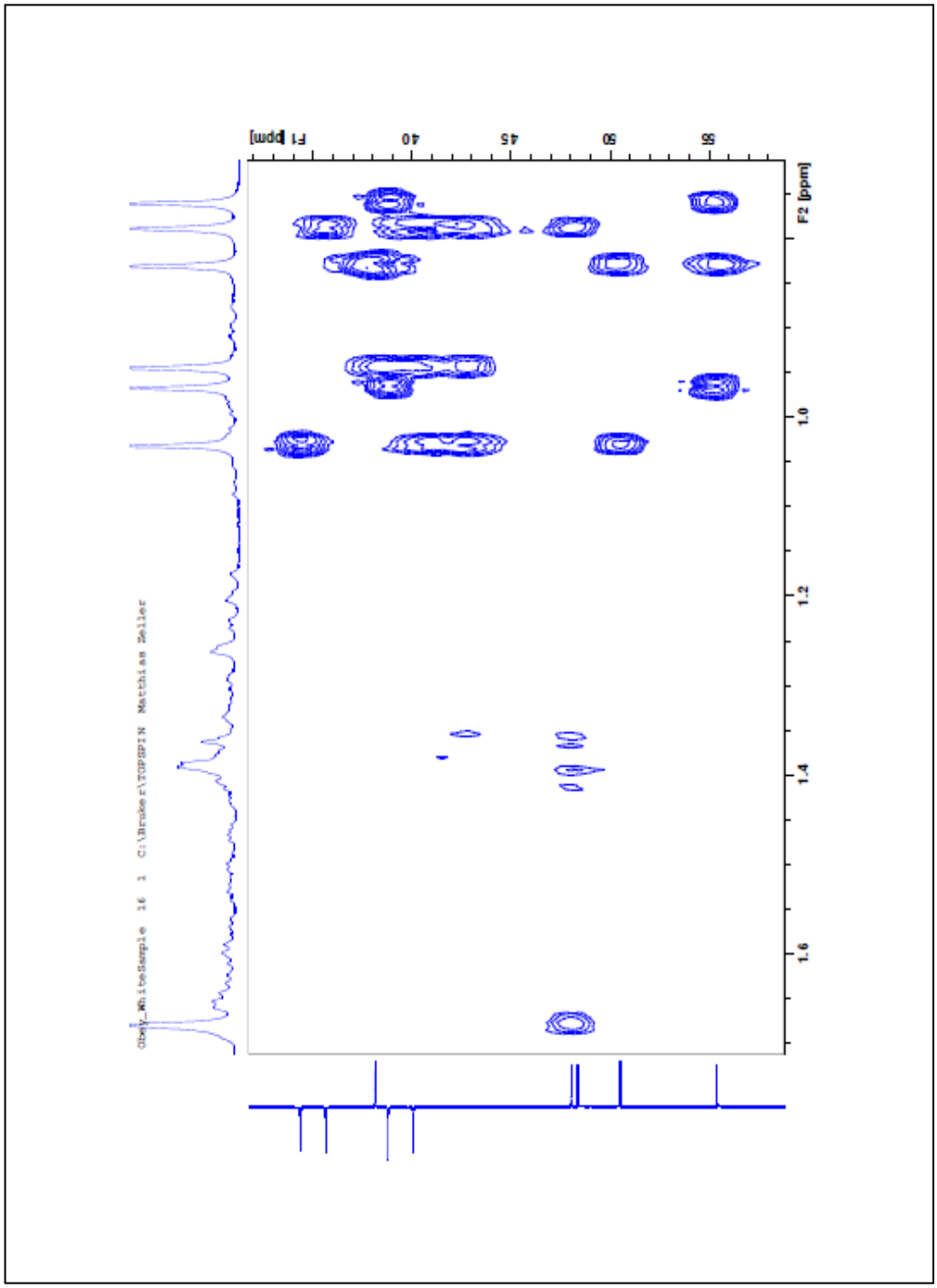
Appendix I: Heternuclear Multibond Correlation Spectroscopy for fraction F5 film 1



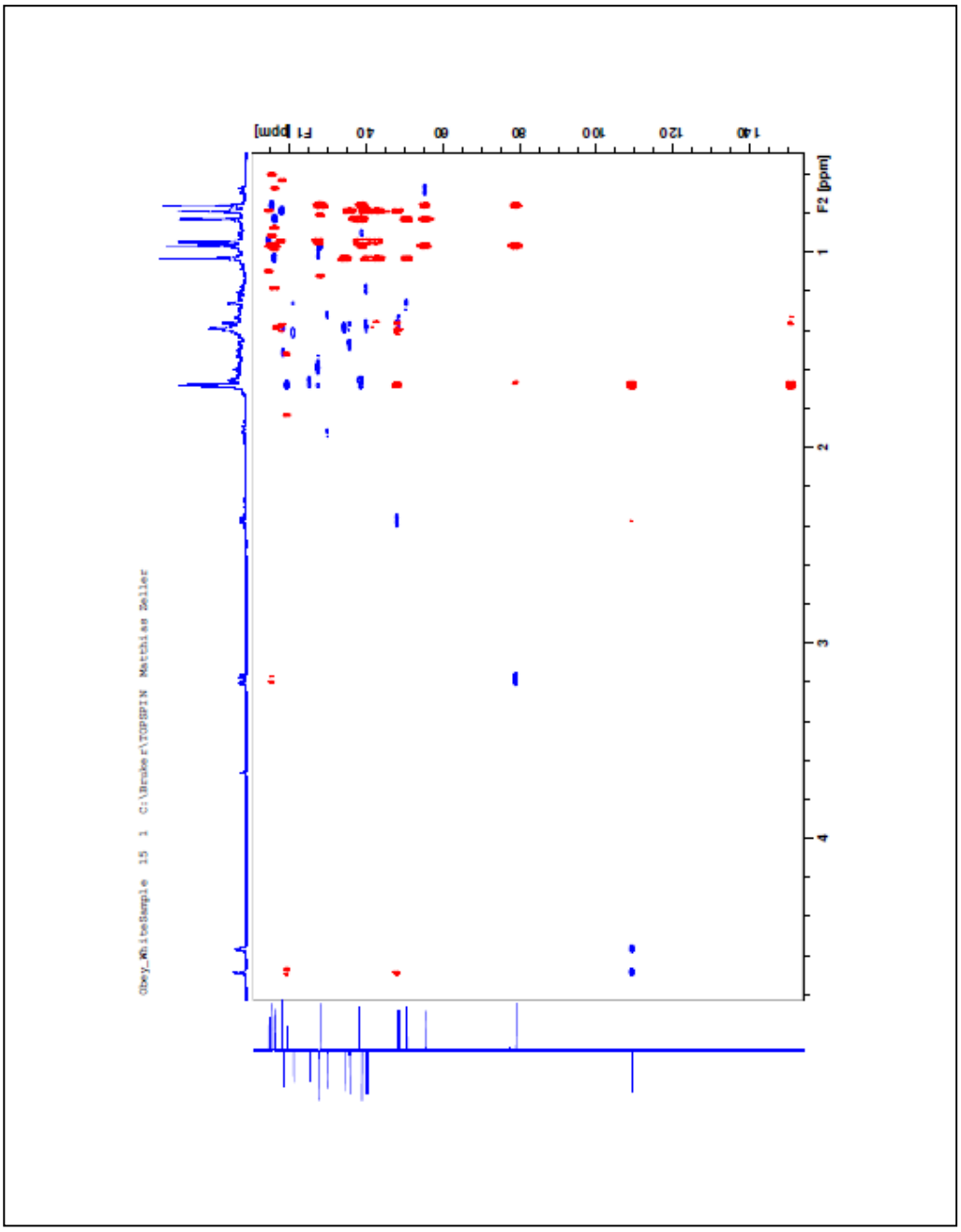
Appendix II: Heternuclear Multibond Correlation Spectroscopy for fraction F5 film 2



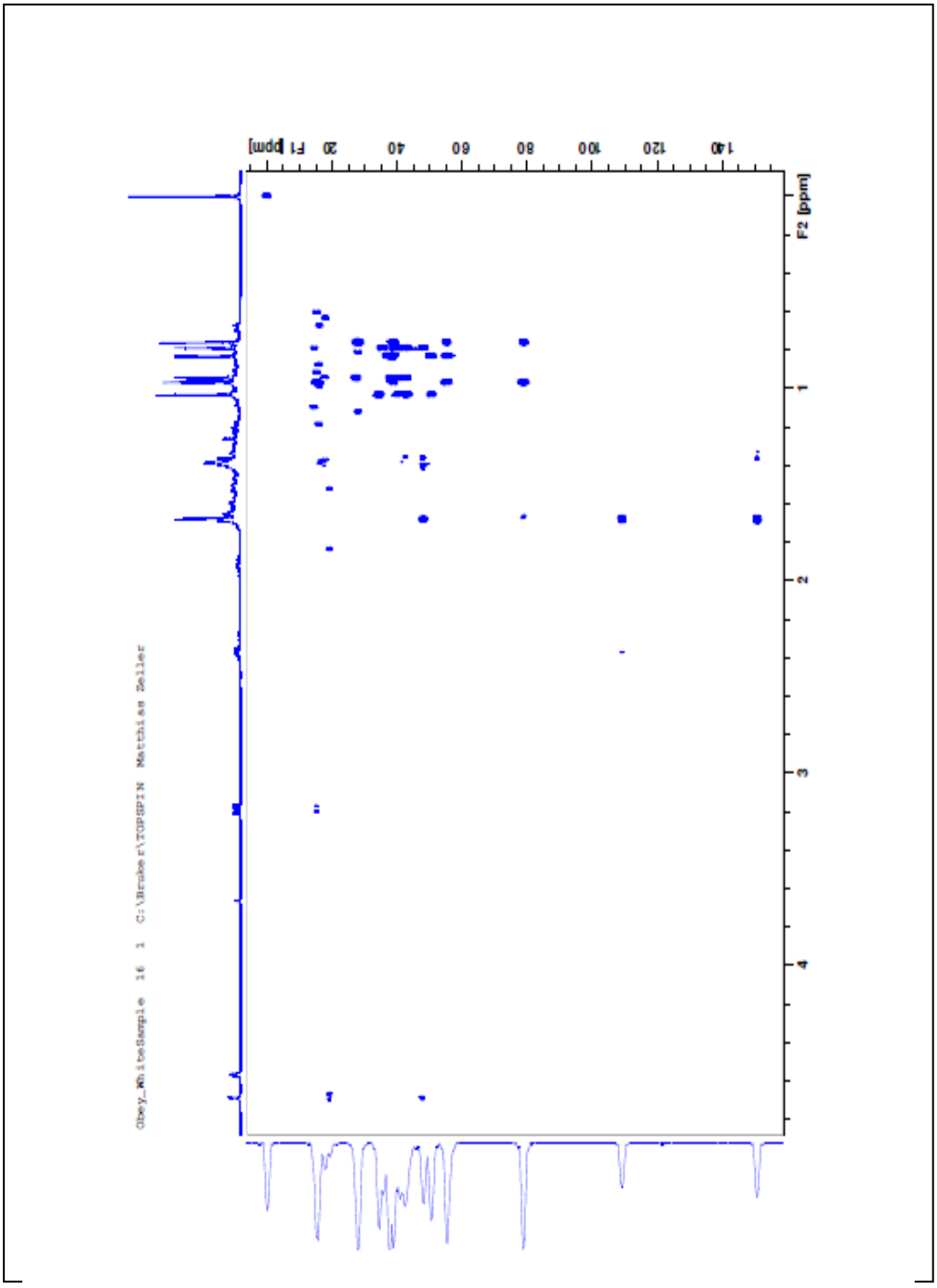
Appendix III: Heternuclear Multibond Correlation Spectroscopy for fraction F5 film 3



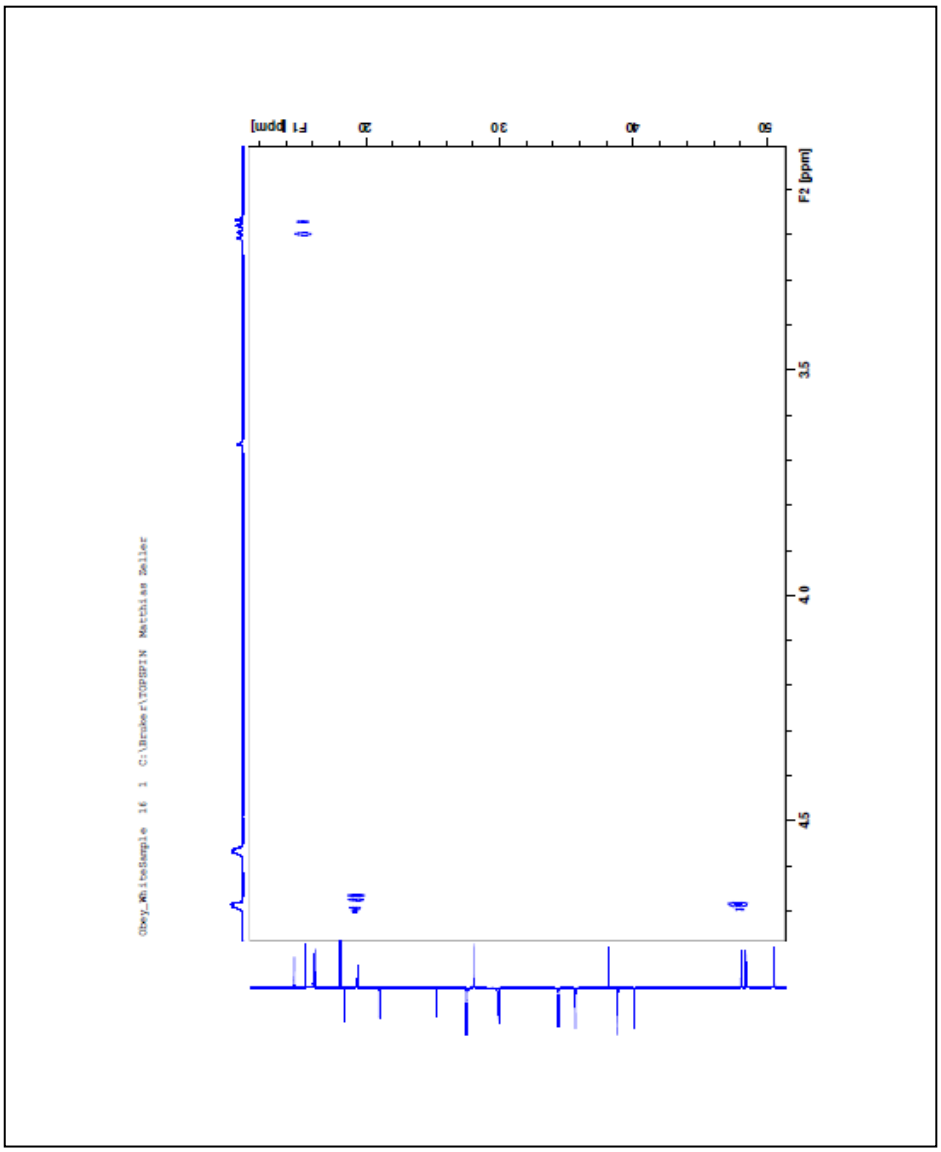
Appendix IV: Heternuclear Multibond Correlation Spectroscopy for fraction F5 film 4



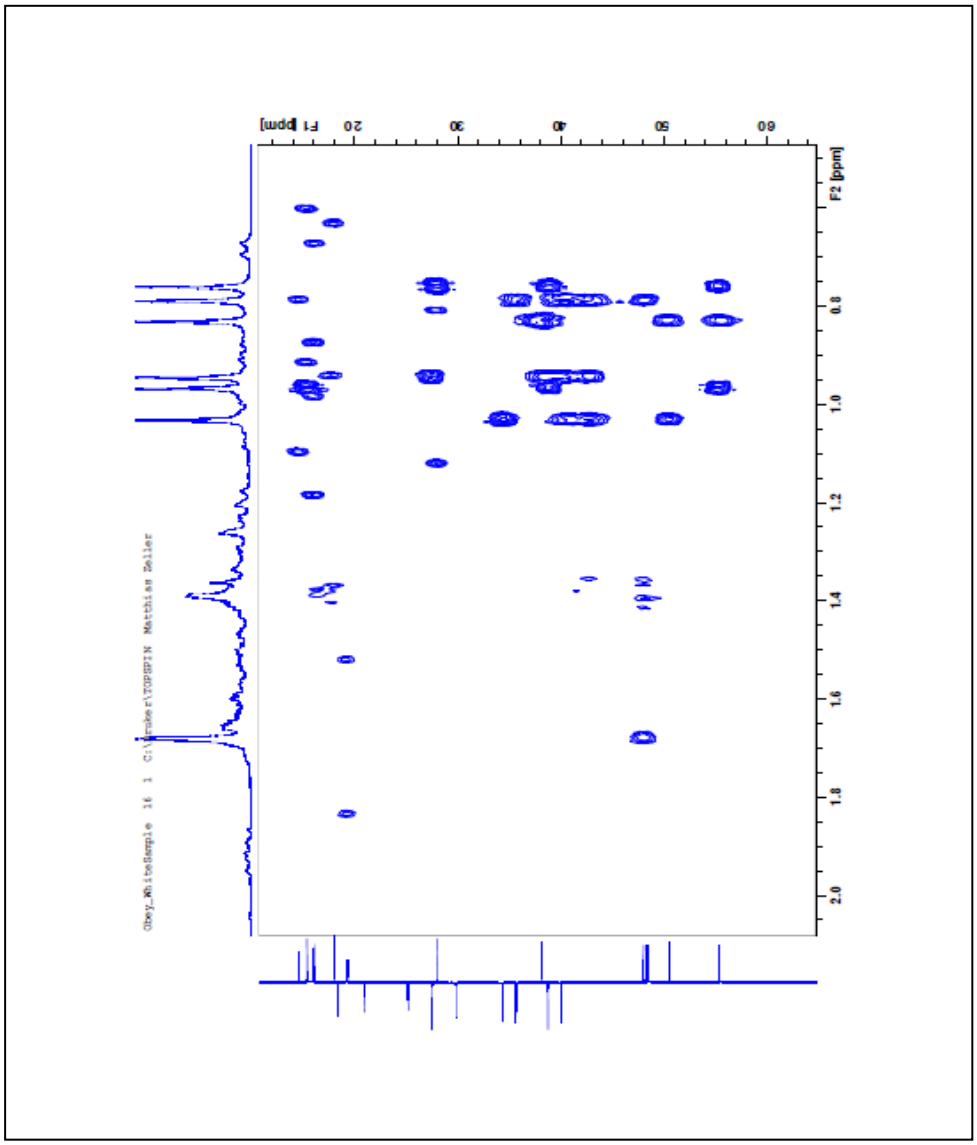
Appendix V: Heternuclear Multibond Correlation Spectroscopy for fraction F5 film 5



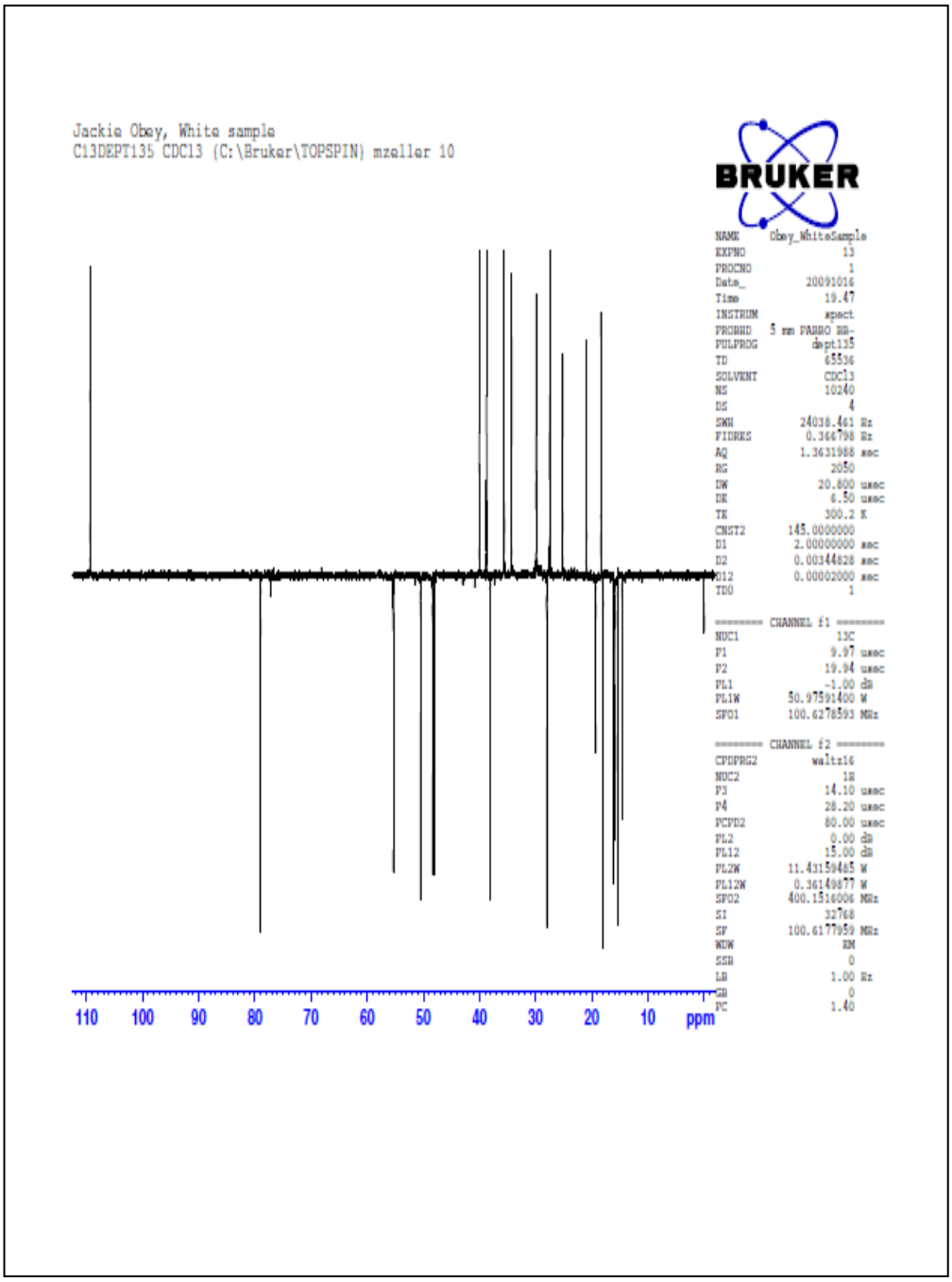
Appendix VI: Heternuclear Multibond Correlation Spectroscopy for fraction F5 film 6



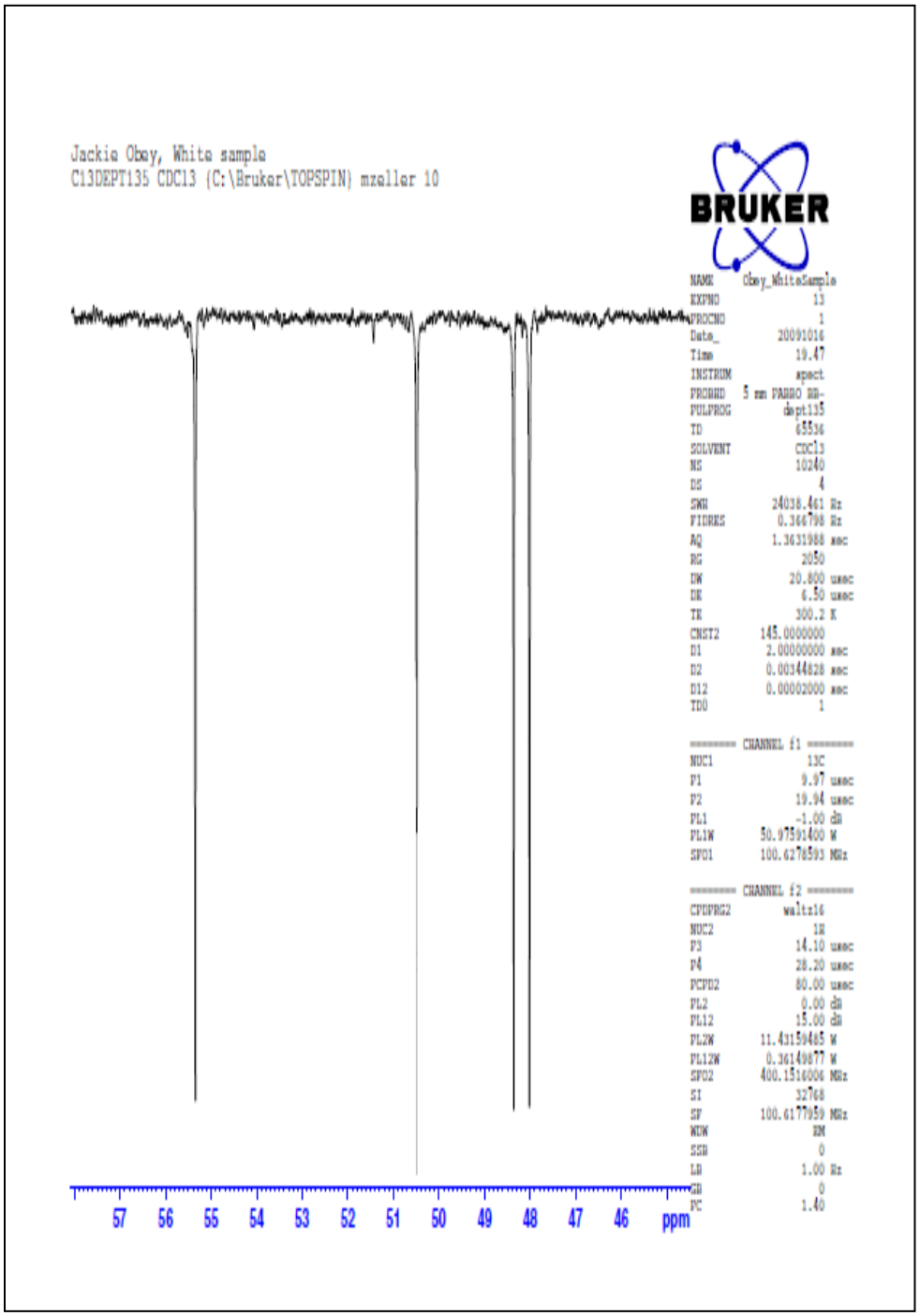
Appendix VII: Heternuclear Multibond Correlation Spectroscopy for fraction F5 film 7



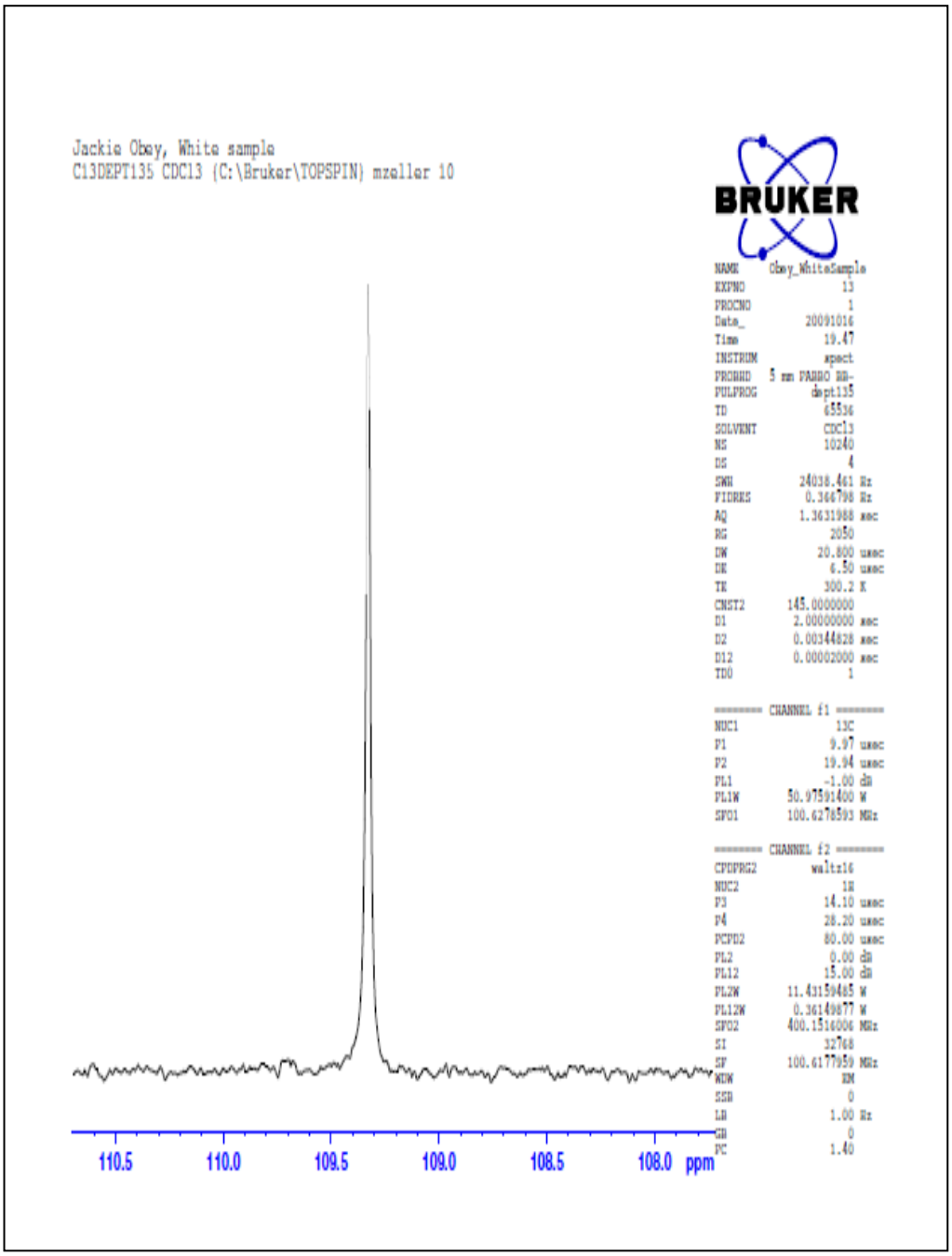
Appendix VIII: Distortionless Enhancement by Polarization Transfer (DEPT) ^{13}C Spectrum for fraction F5, film 1



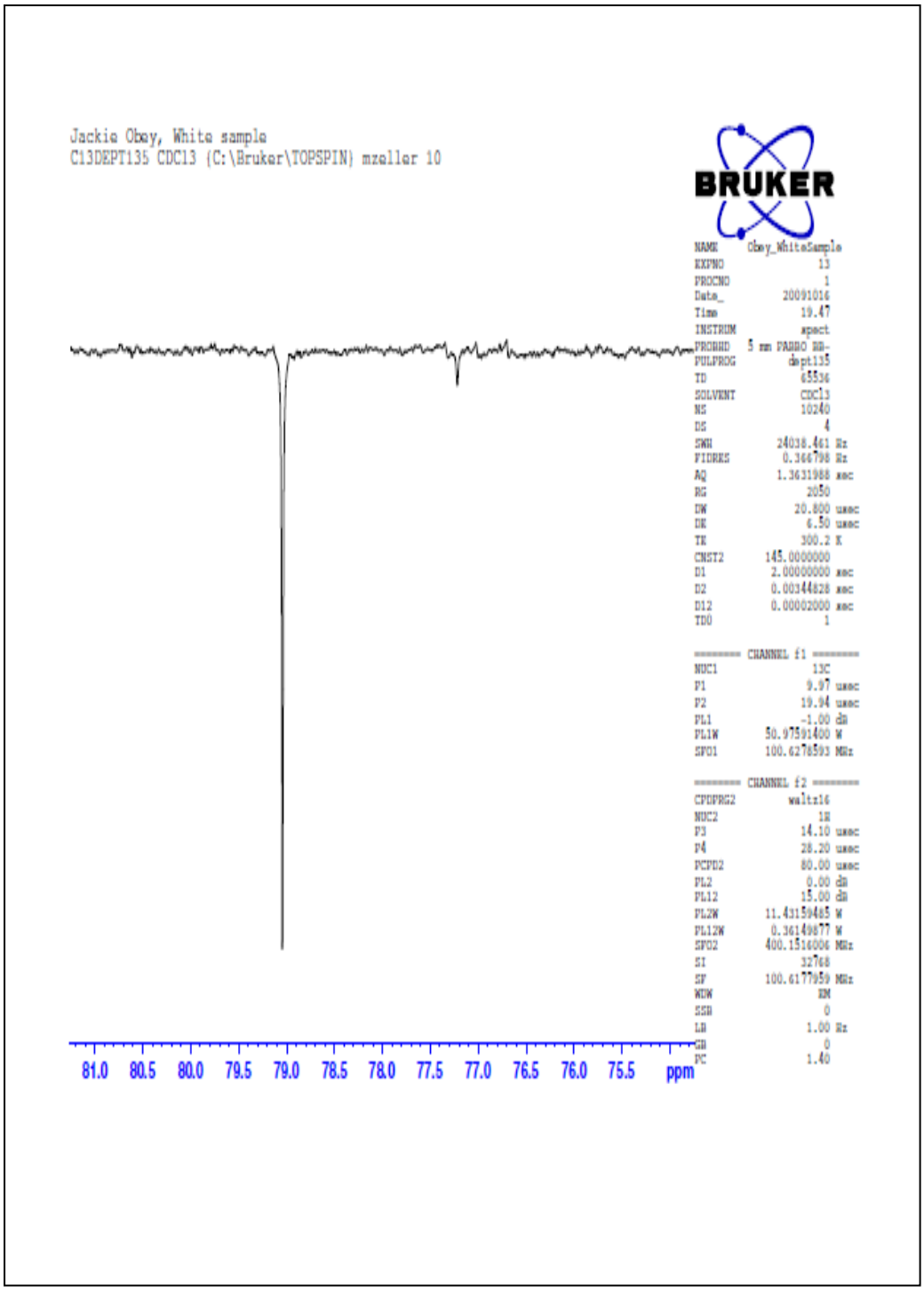
Appendix IX: Distortionless Enhancement by Polarization Transfer (DEPT) ^{13}C Spectrum for fraction F5, film 2



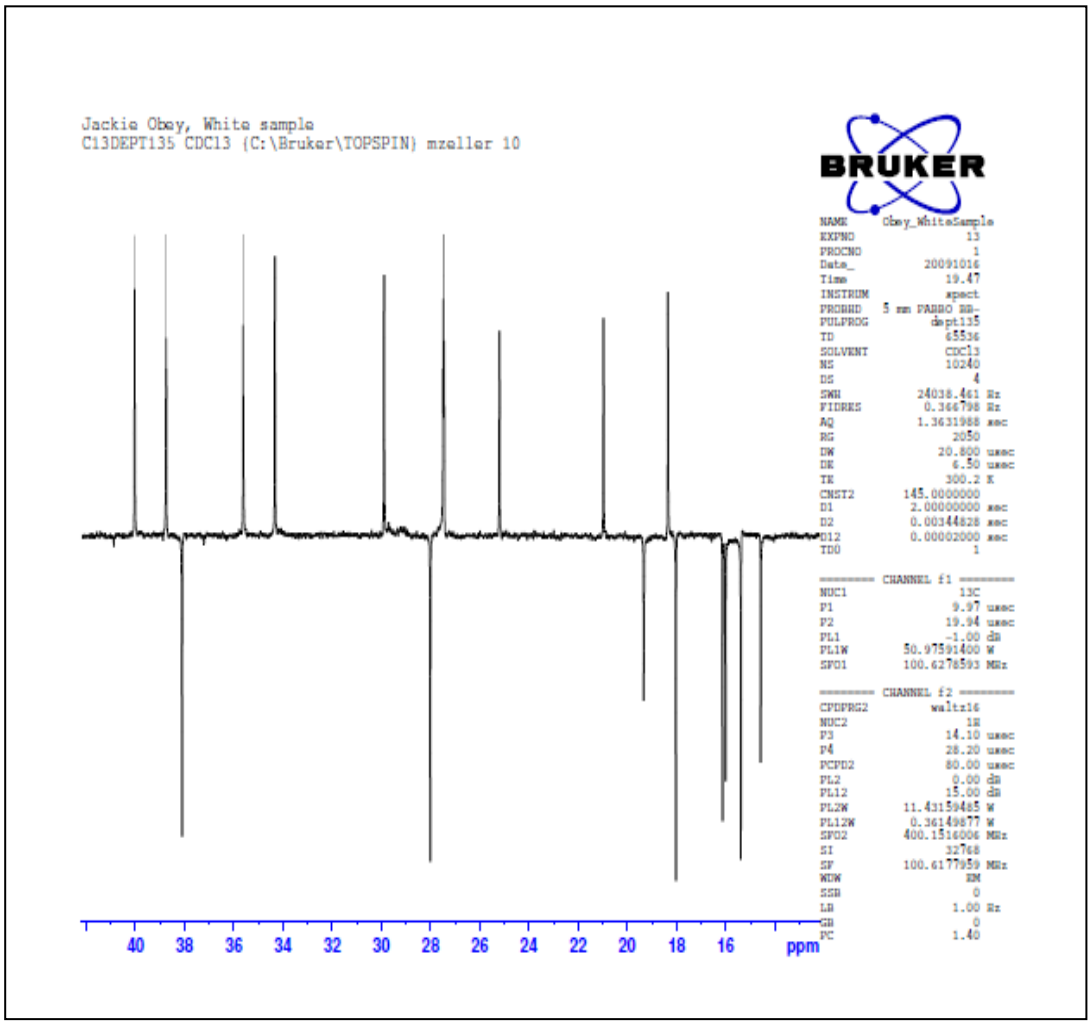
Appendix X: Distortionless Enhancement by Polarization Transfer (DEPT) ^{13}C Spectrum for Compound F5, film 3



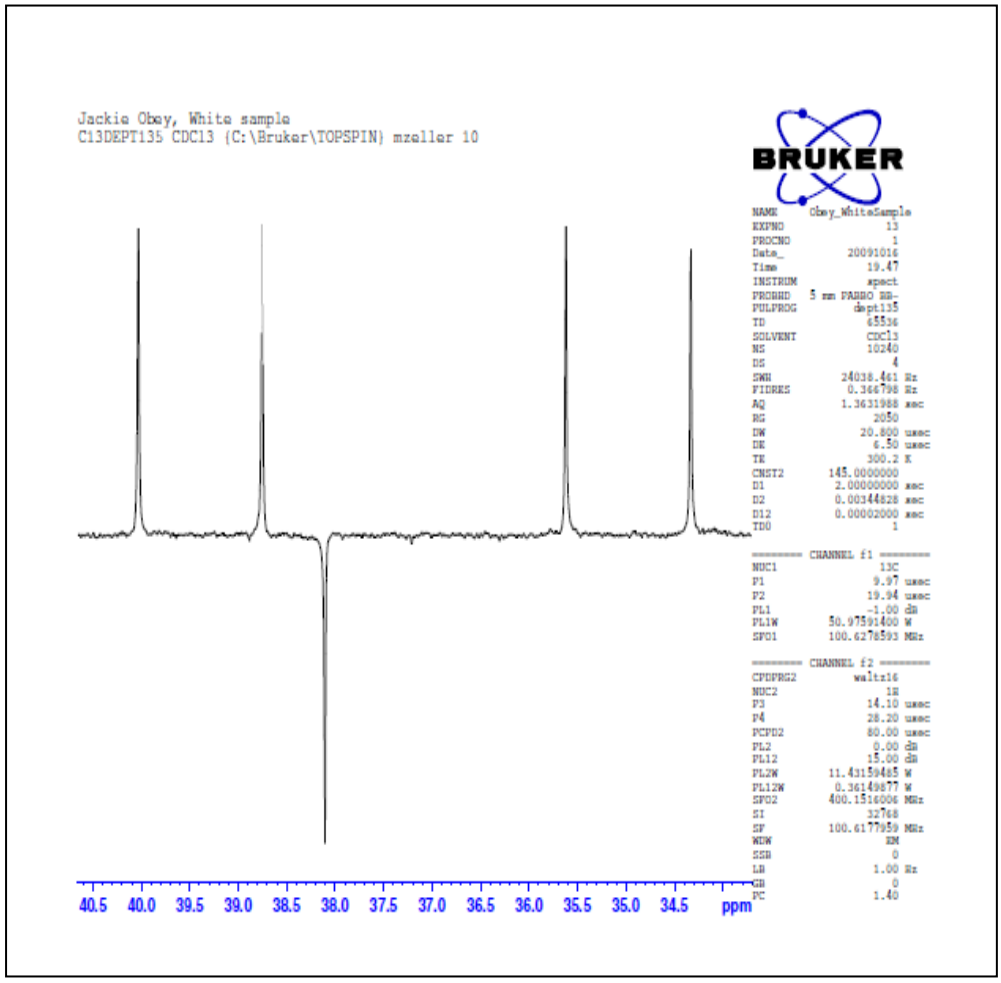
Appendix XI: Distortionless Enhancement by Polarization Transfer (DEPT) ^{13}C Spectrum for Compound F5, film 4



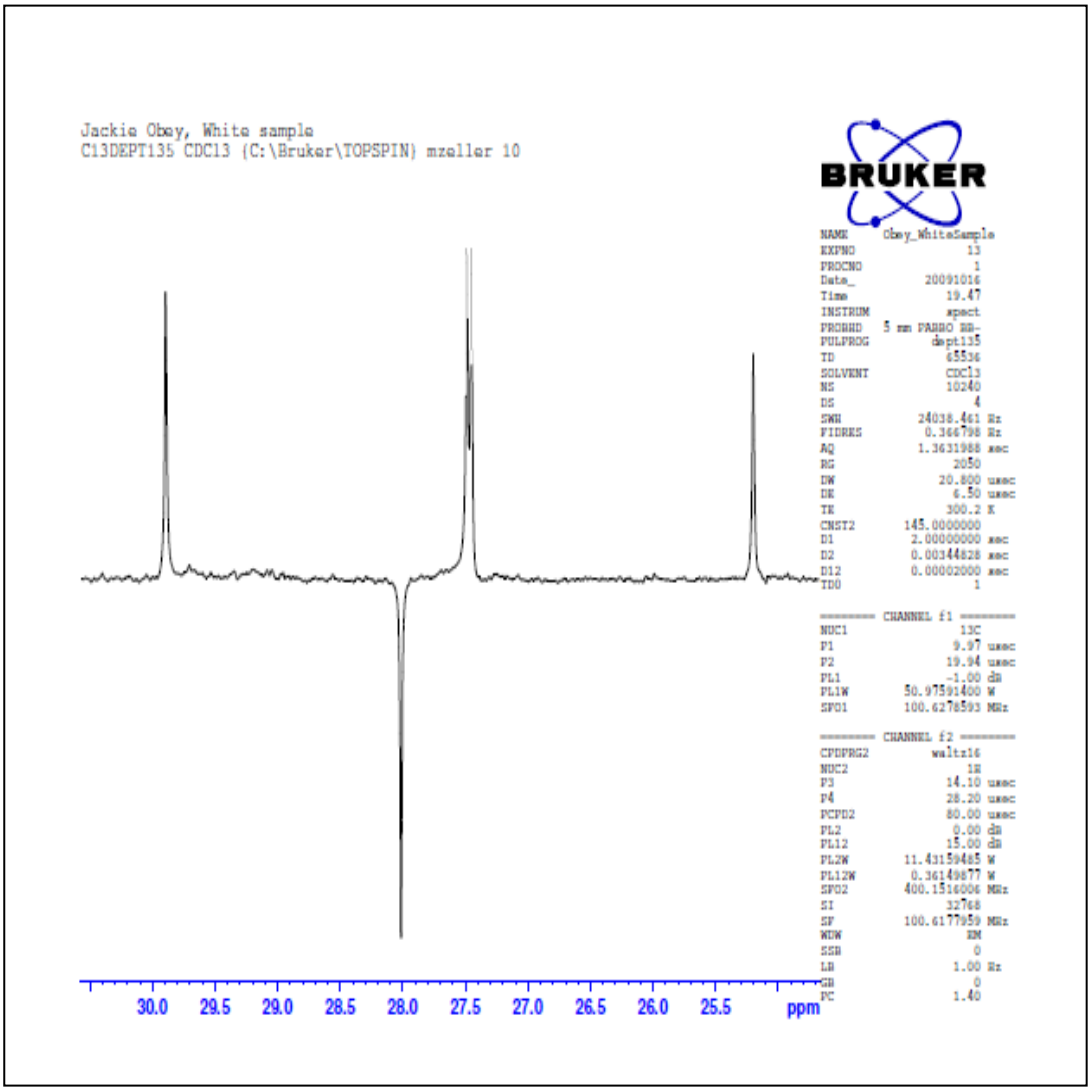
Appendix XII: Distortionless Enhancement by Polarization Transfer (DEPT) ^{13}C Spectrum for Fraction F5, film 5



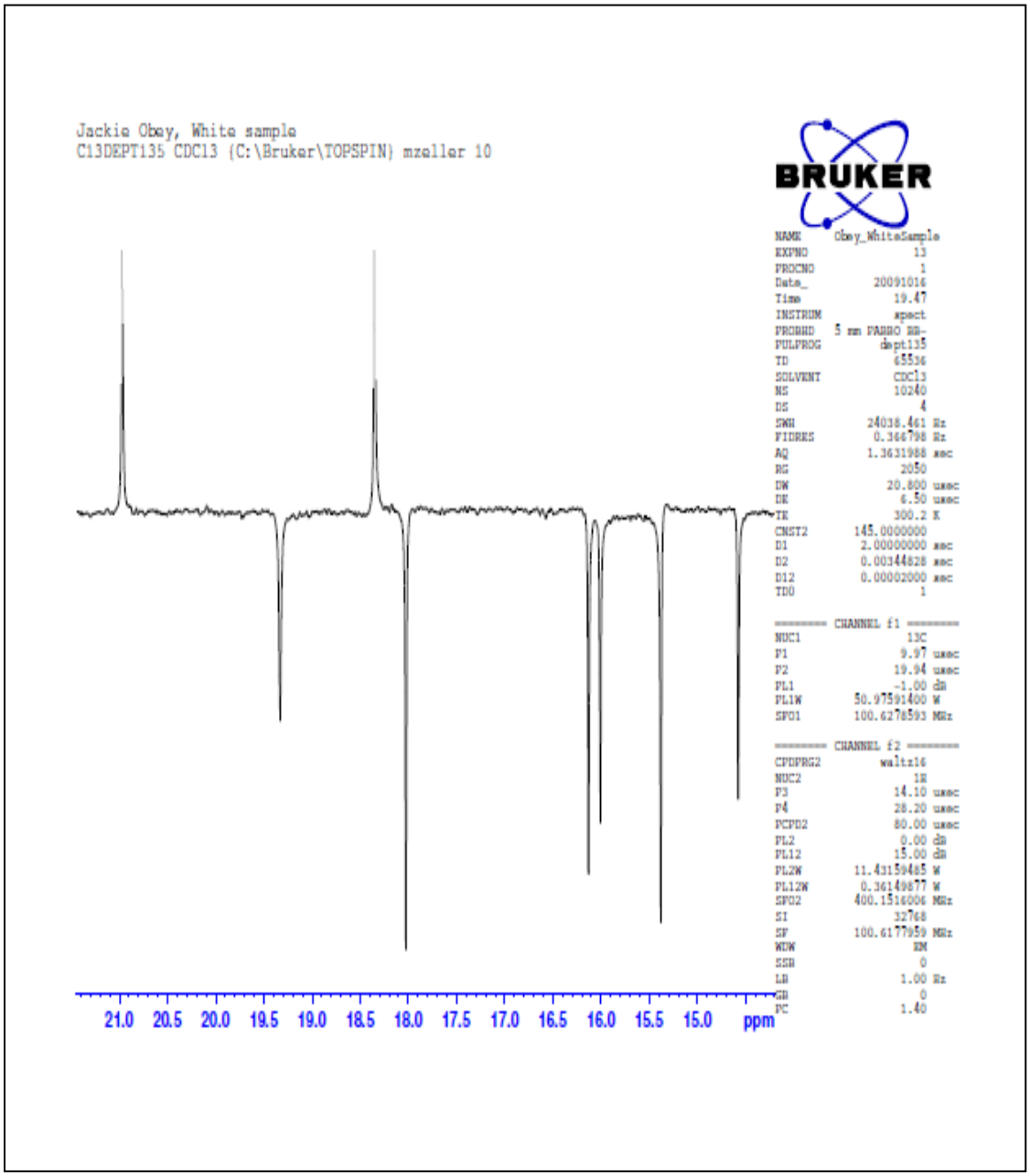
Appendix XIII: Distortionless Enhancement by Polarization Transfer (DEPT) ^{13}C Spectrum for Fraction F5, film 6



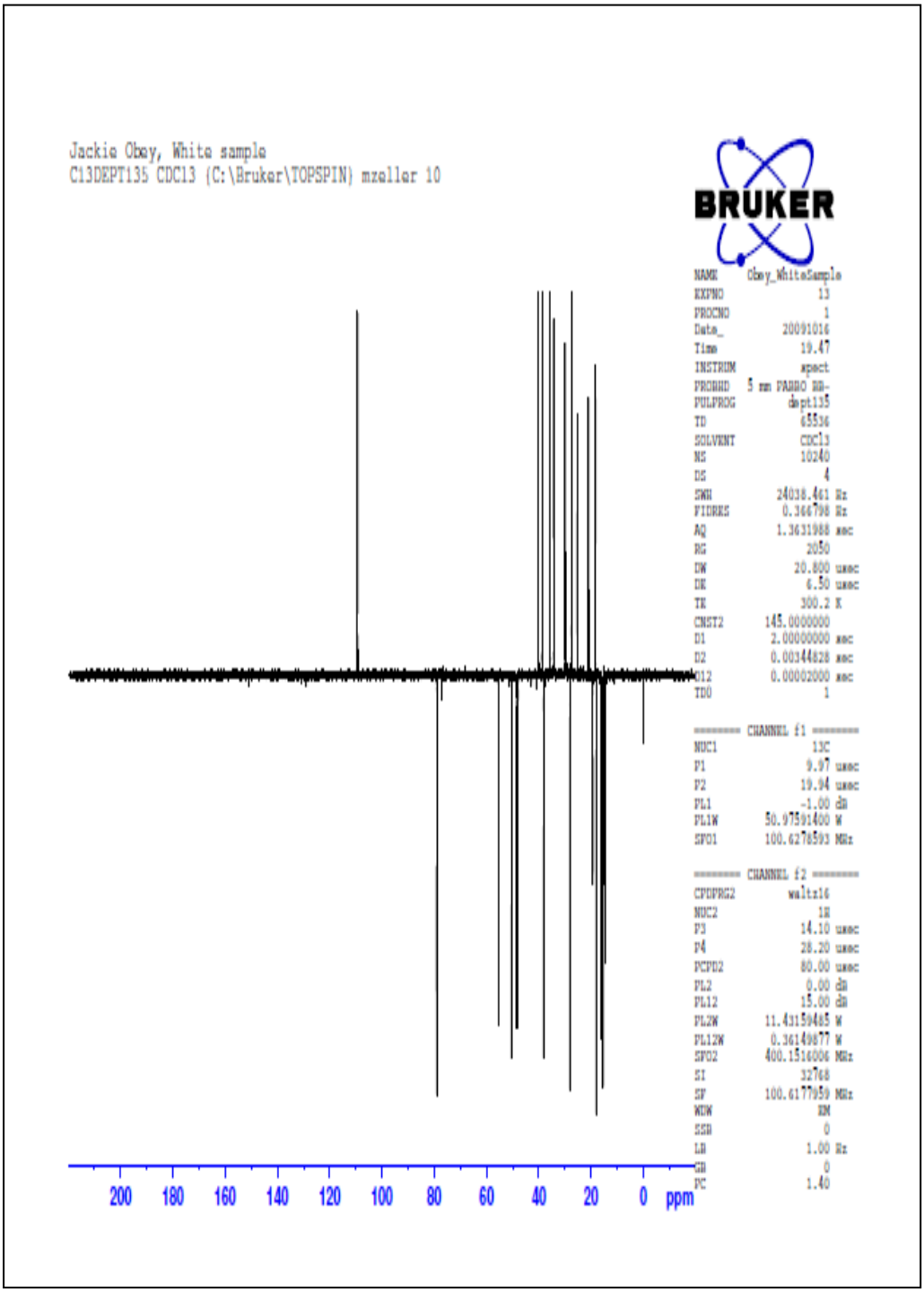
Appendix XIV: Distortionless Enhancement by Polarization Transfer (DEPT) ^{13}C Spectrum for Fraction F5, film 7



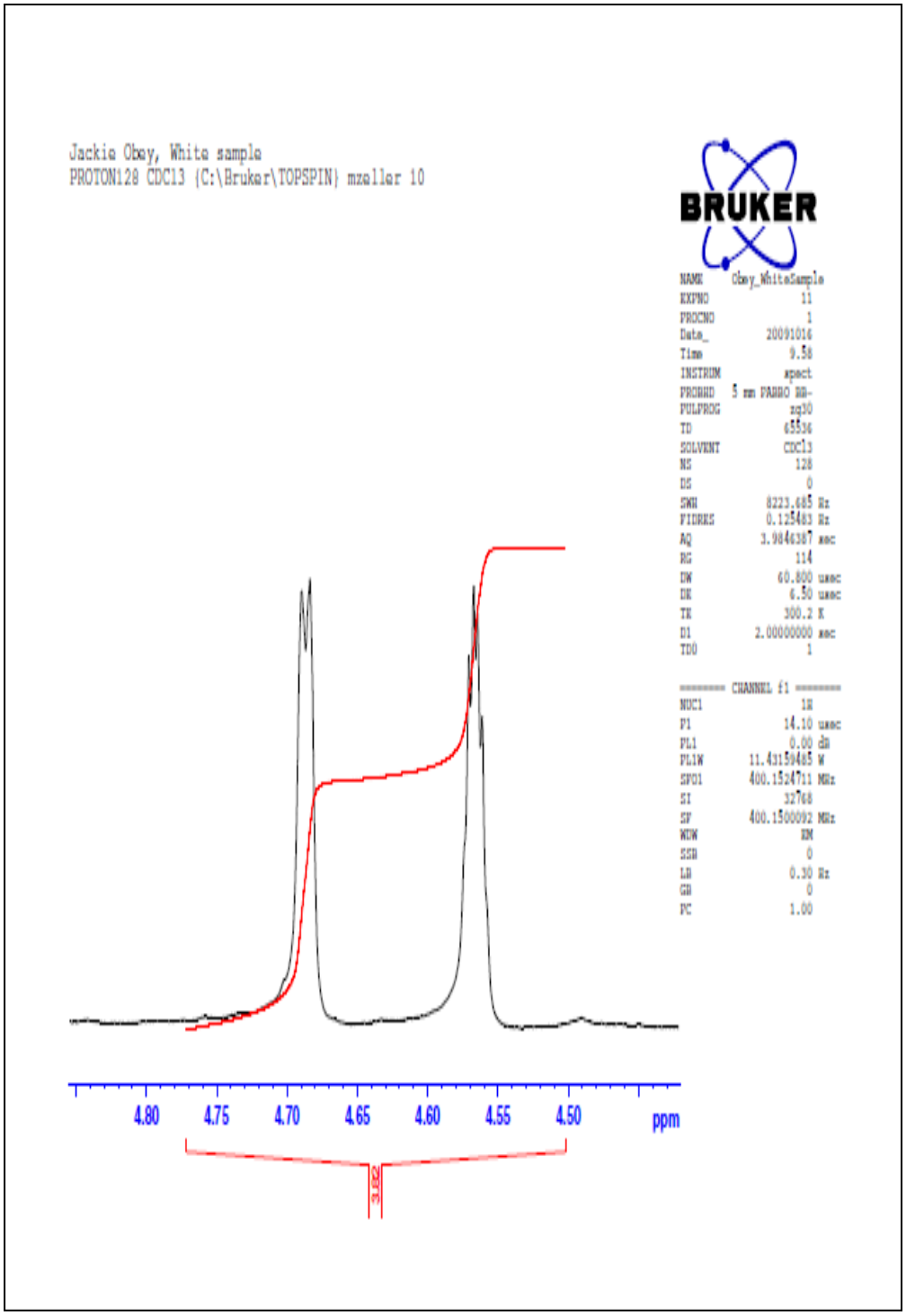
Appendix XV: Distortionless Enhancement by Polarization Transfer (DEPT) ^{13}C Spectrum for Fraction F5, film 8



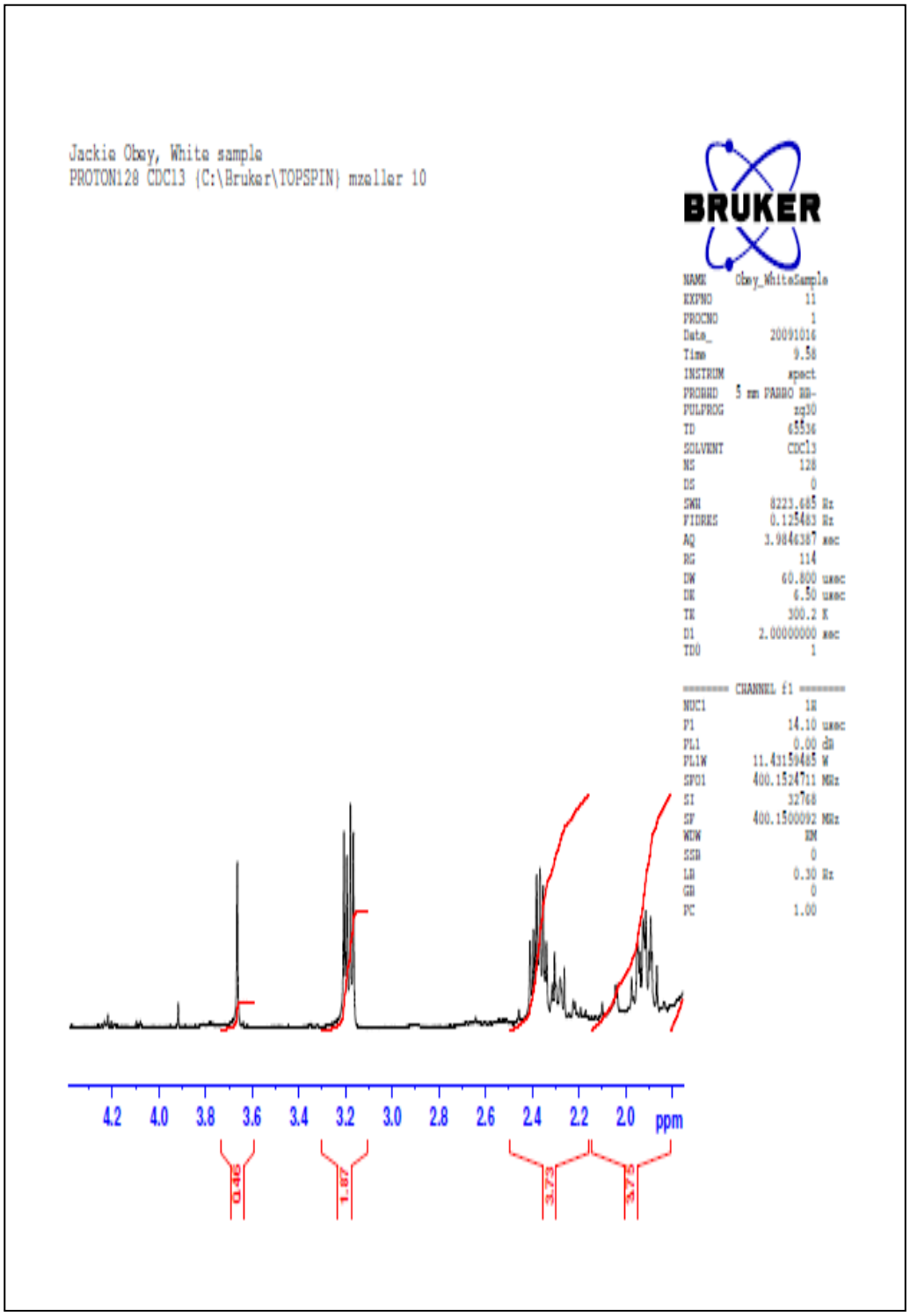
Appendix XVI: Distortionless Enhancement by Polarization Transfer (DEPT) ^{13}C Spectrum for Fraction F5, film 9



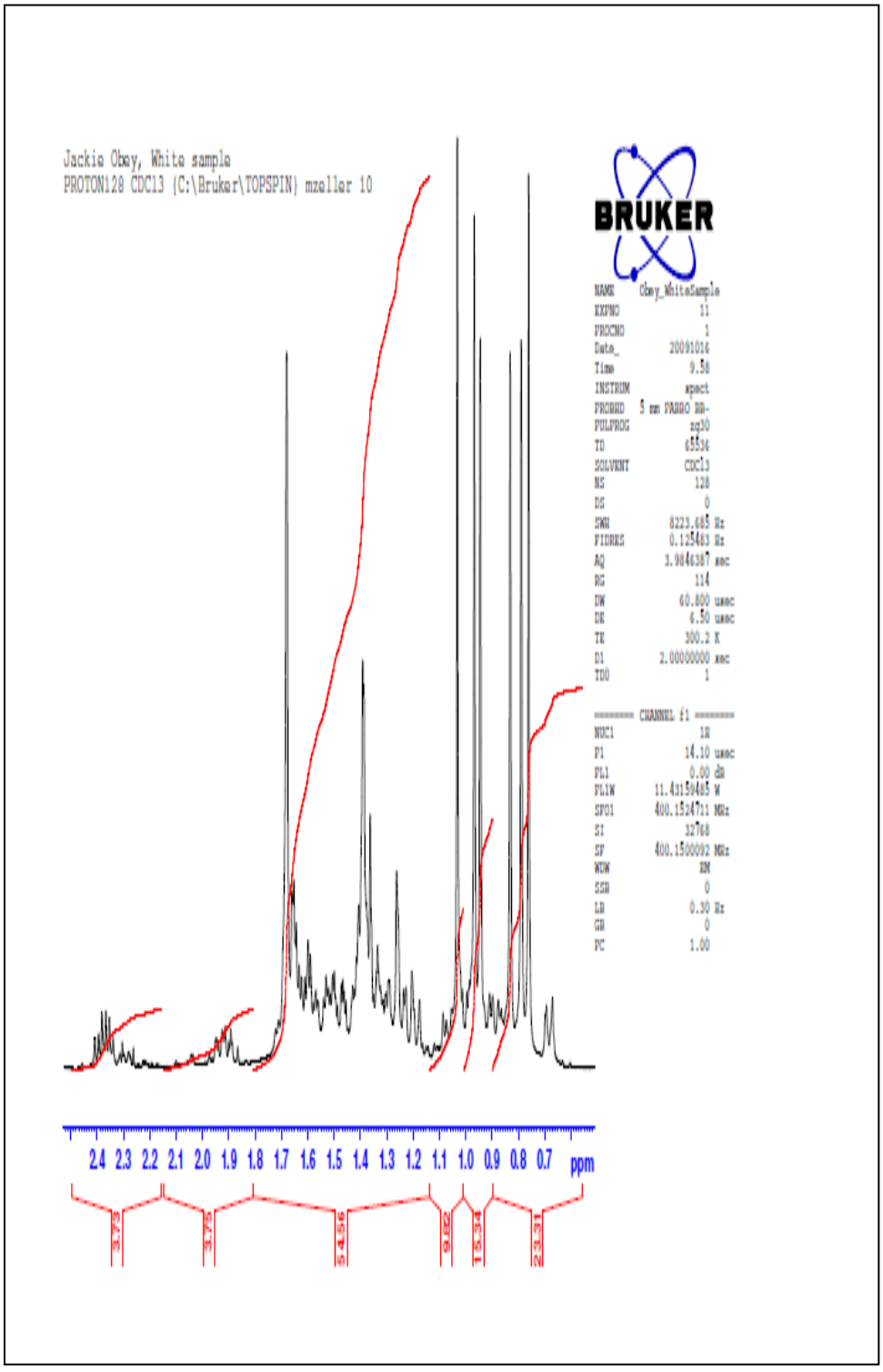
Appendix XVII: Proton ^{1}H Nuclear Magnetic Resonance (NMR) for F5, film 1



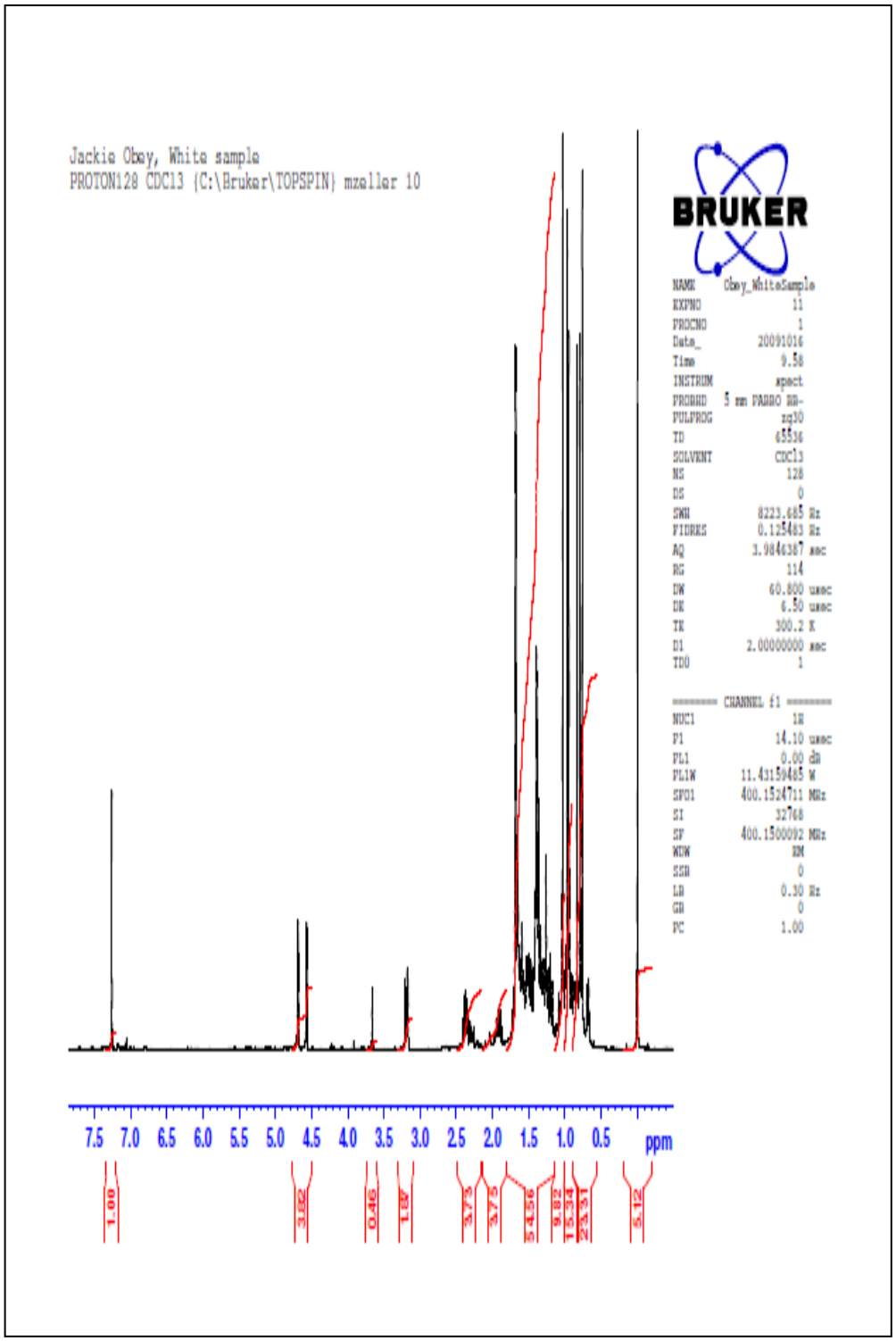
Appendix XVIII: Proton ^{1}H Nuclear Magnetic Resonance (NMR) for F5, film 2



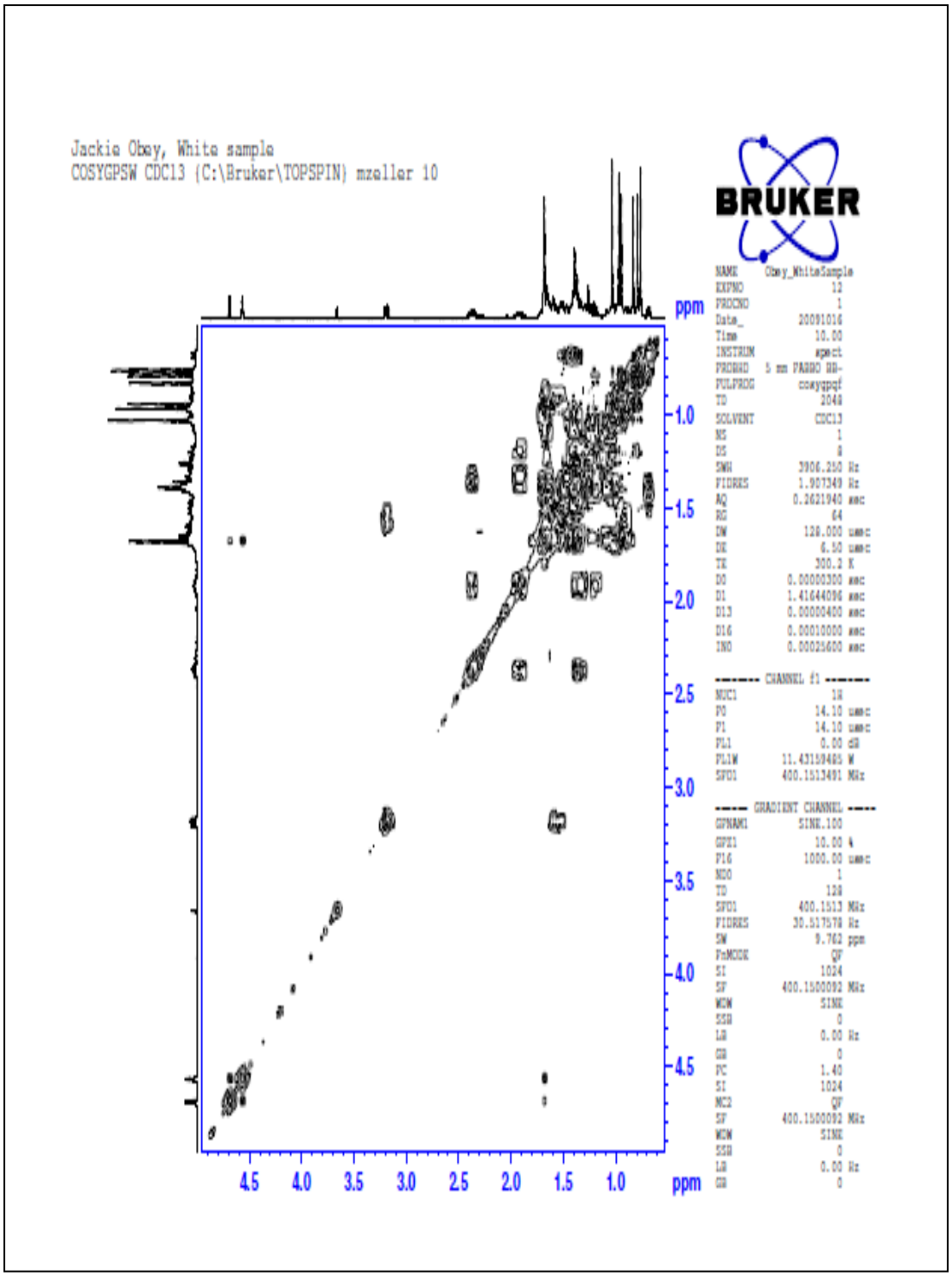
Appendix XIX: Proton ^{1}H Nuclear Magnetic Resonance (NMR) for F5, film 3



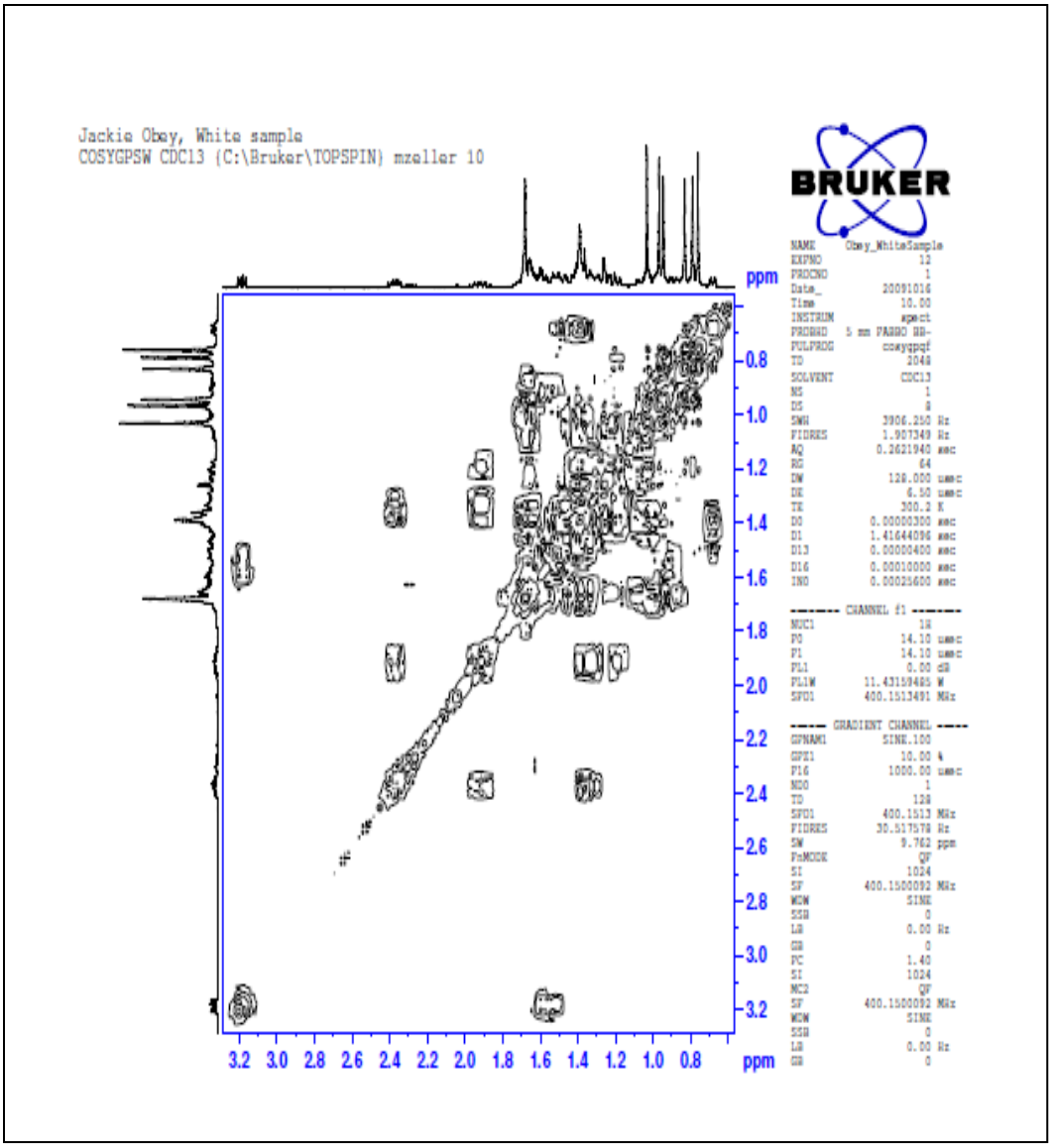
Appendix XX: Proton ^{1}H Nuclear Magnetic Resonance (NMR) for F5, film 4



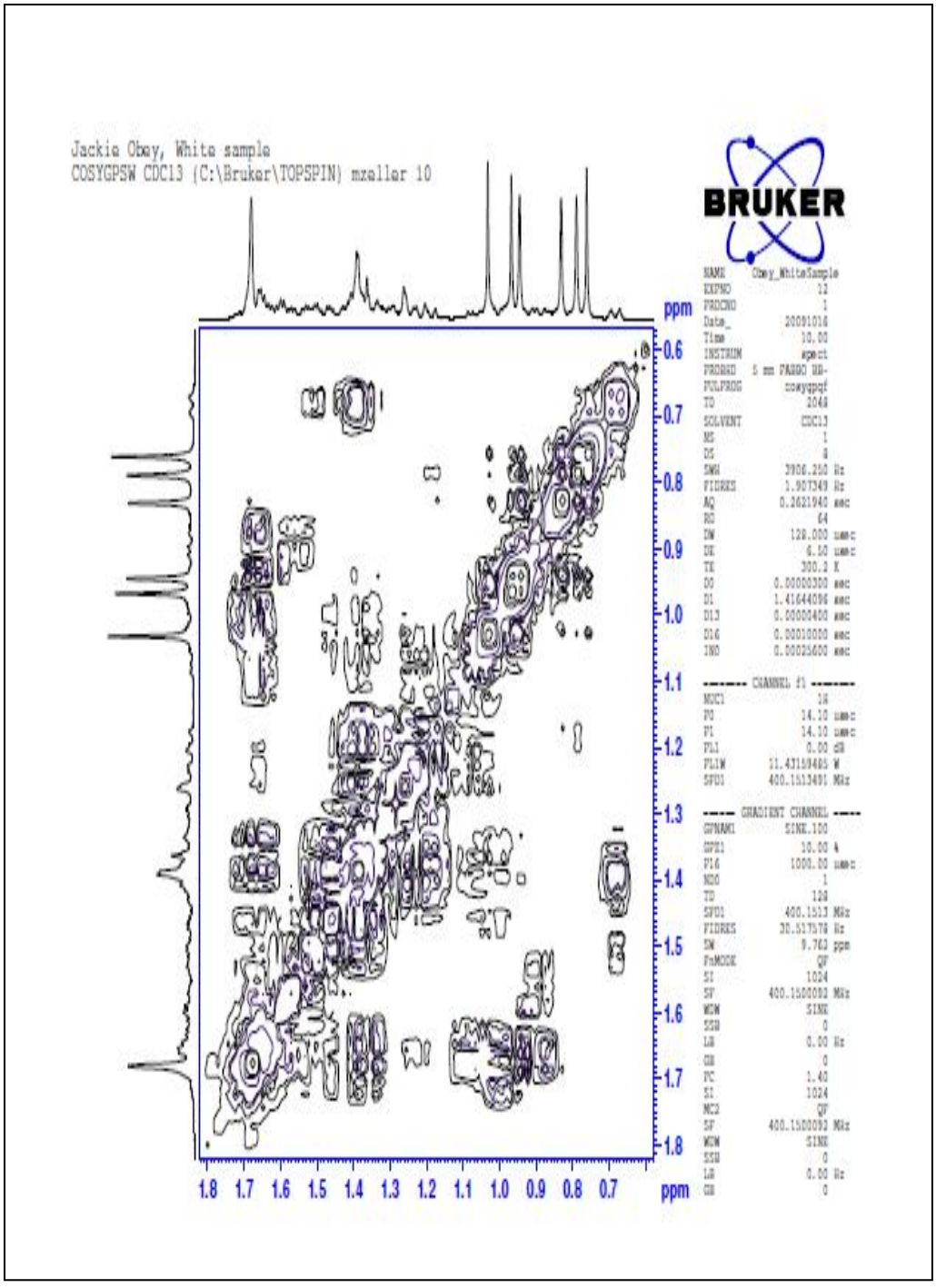
Appendix XXI: Correlation Spectroscopy (COSY) for fraction F5: film 1



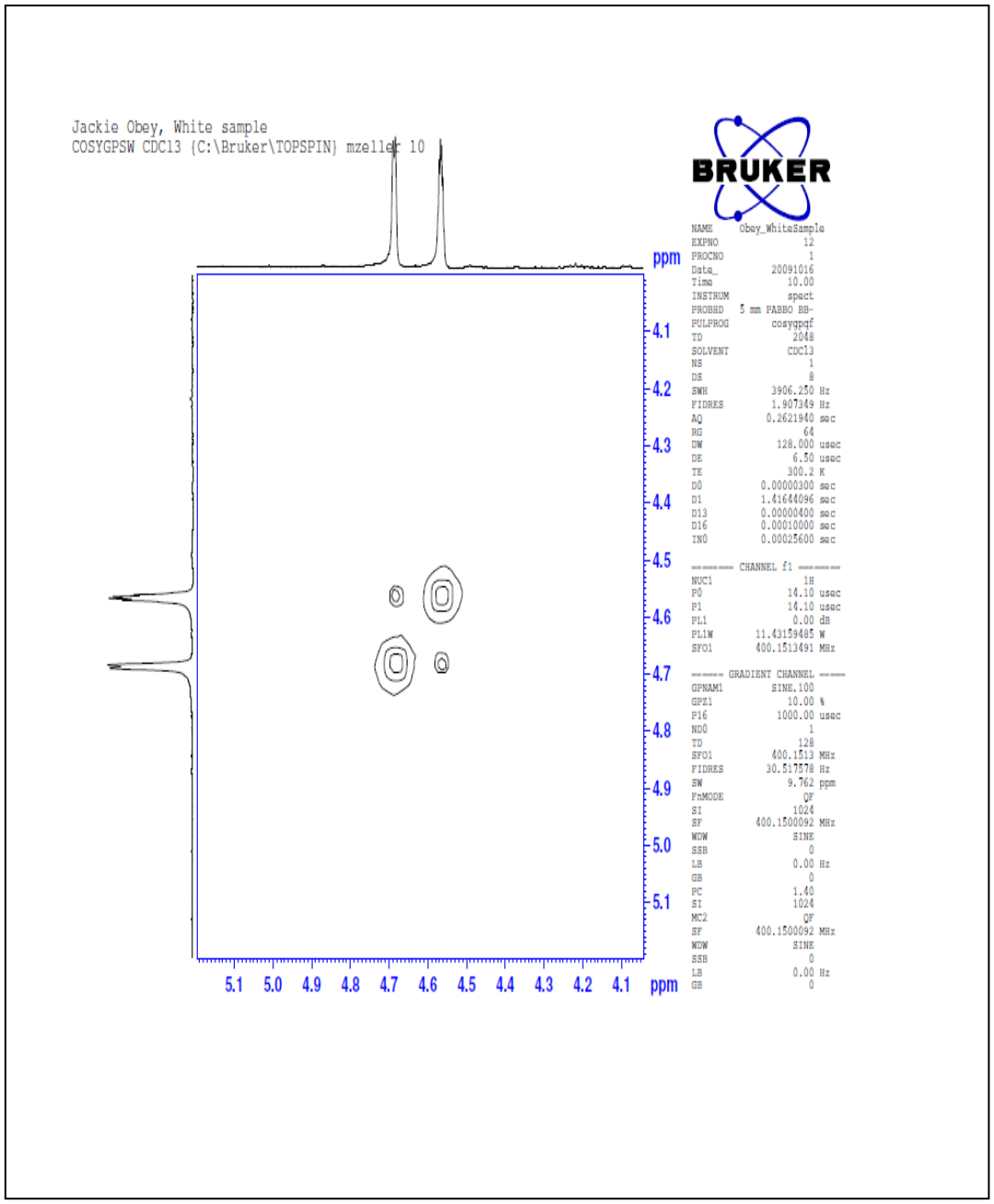
Appendix XXII: Correlation Spectroscopy (COSY) for fraction F5, film 2



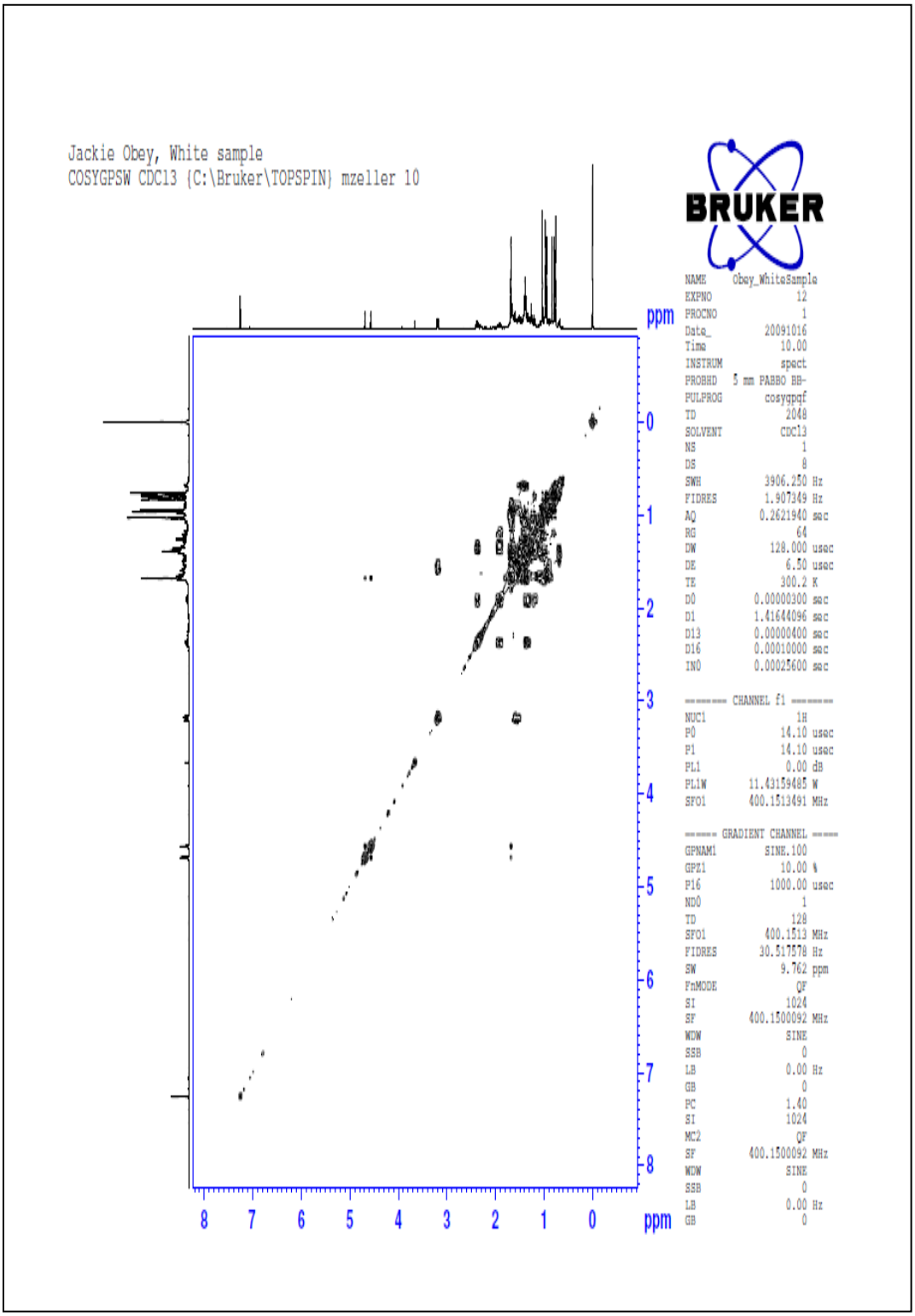
Appendix XXIII: Correlation Spectroscopy (COSY) for fraction F5, film 3



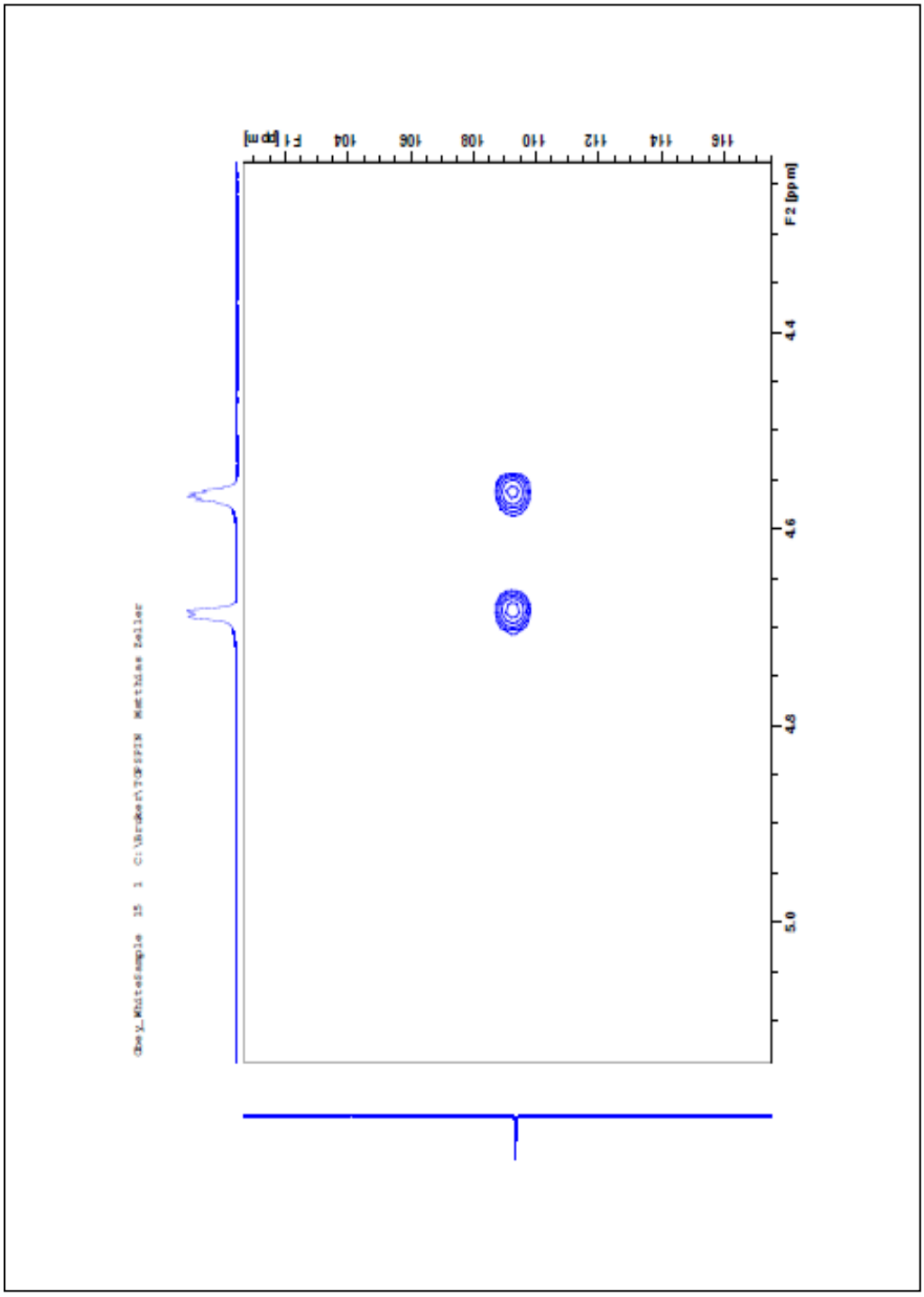
Appendix XXIV: Correlation Spectroscopy (COSY) for fraction F5, film 4



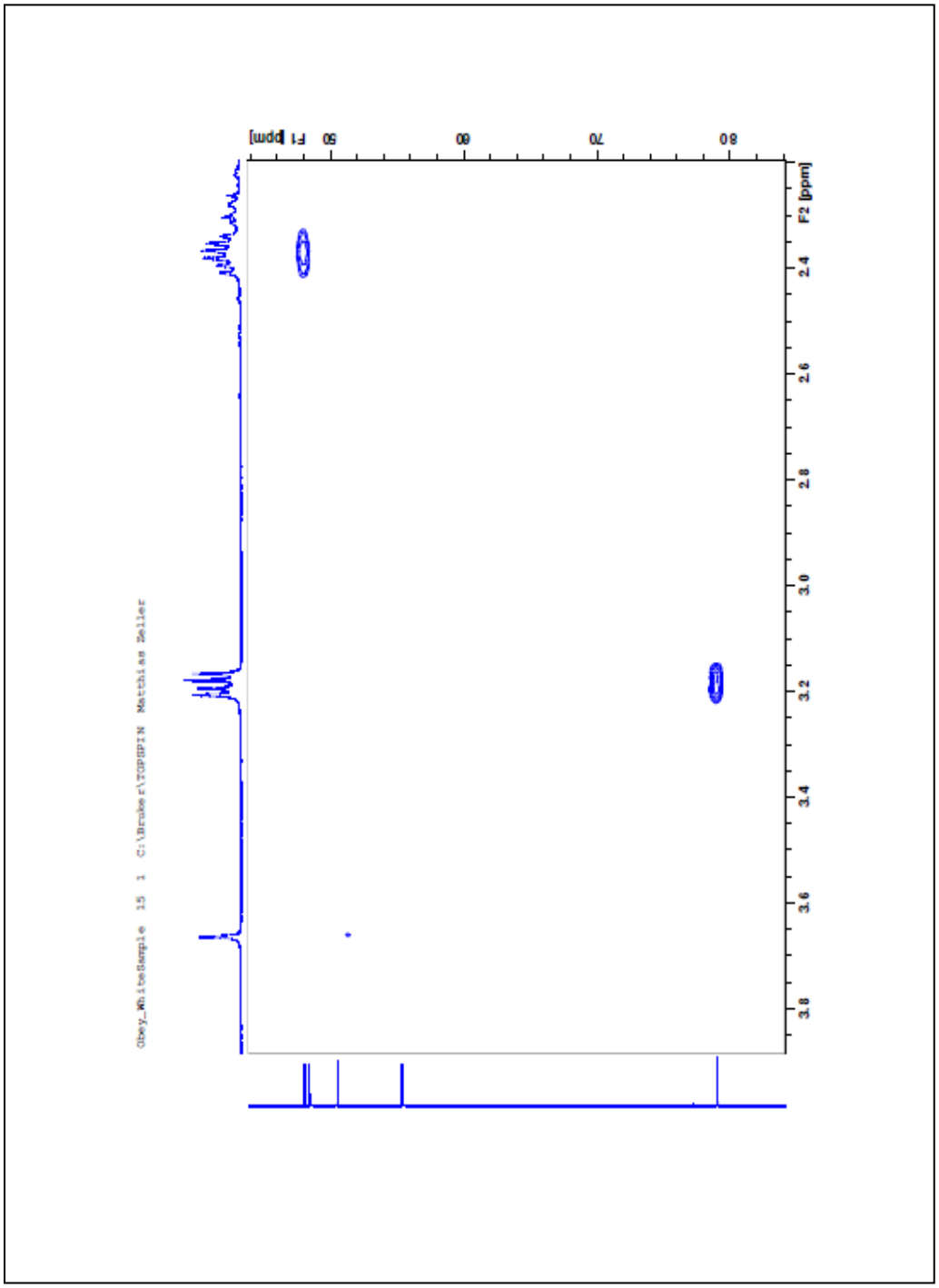
Appendix XXV: Correlation Spectroscopy (COSY) for fraction F5, film 5



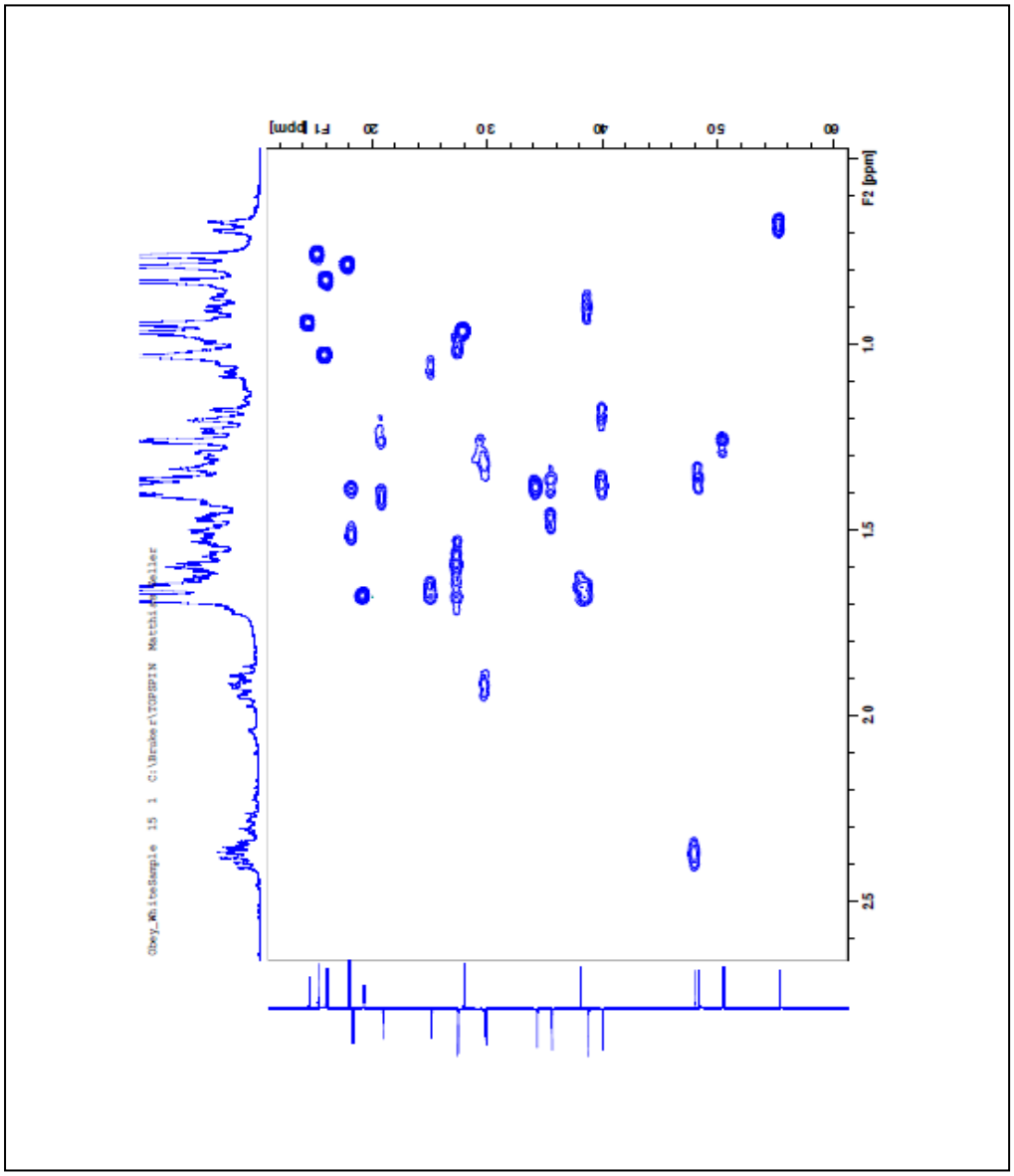
Appendix XXVI: Heteronuclear Single Quantum Coherence (HSQC) Spectroscopy for fraction F5 film 1



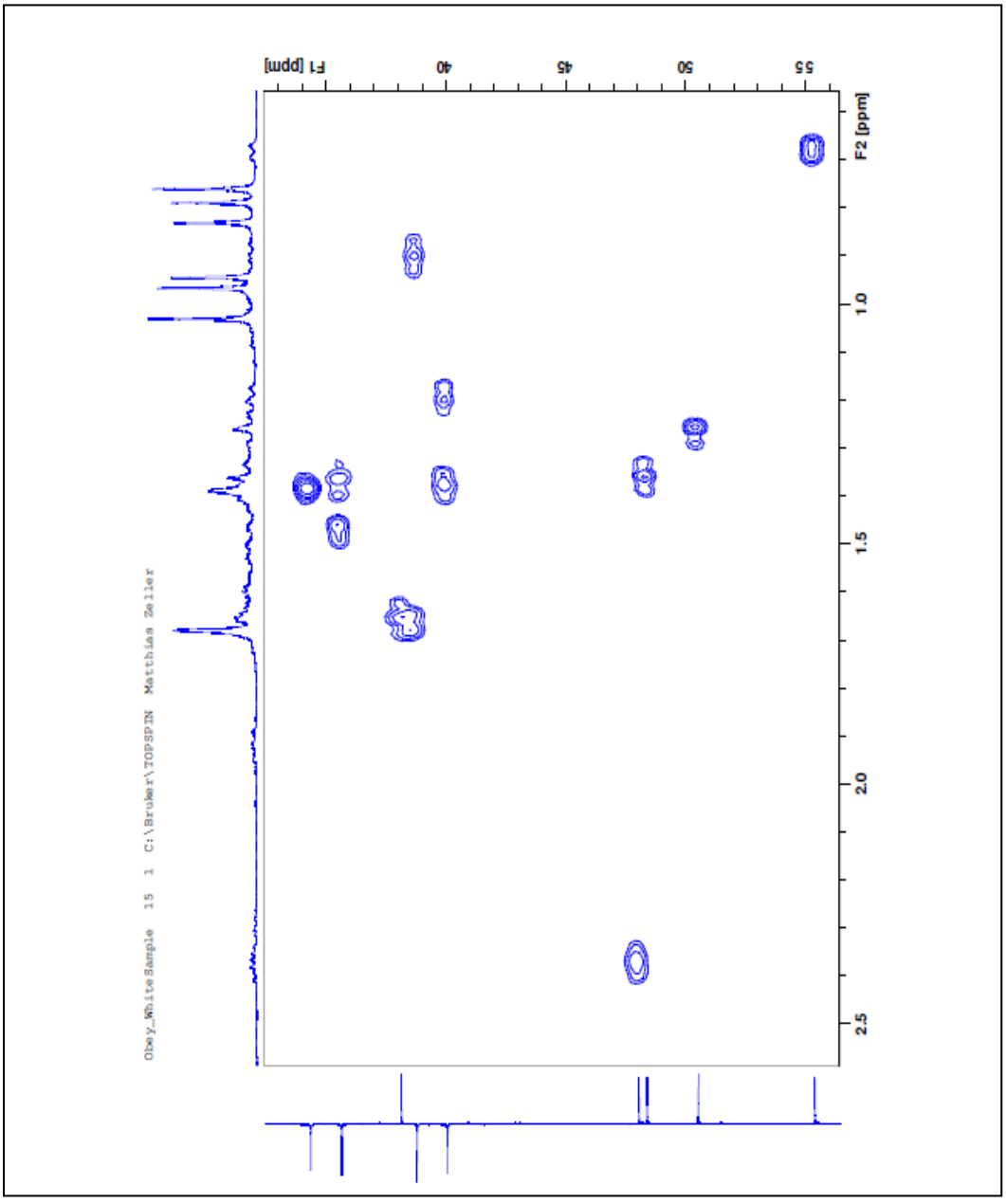
Appendix XXVII: Heteronuclear Single Quantum Coherence (HSQC) Spectroscopy for fraction F5 film 2



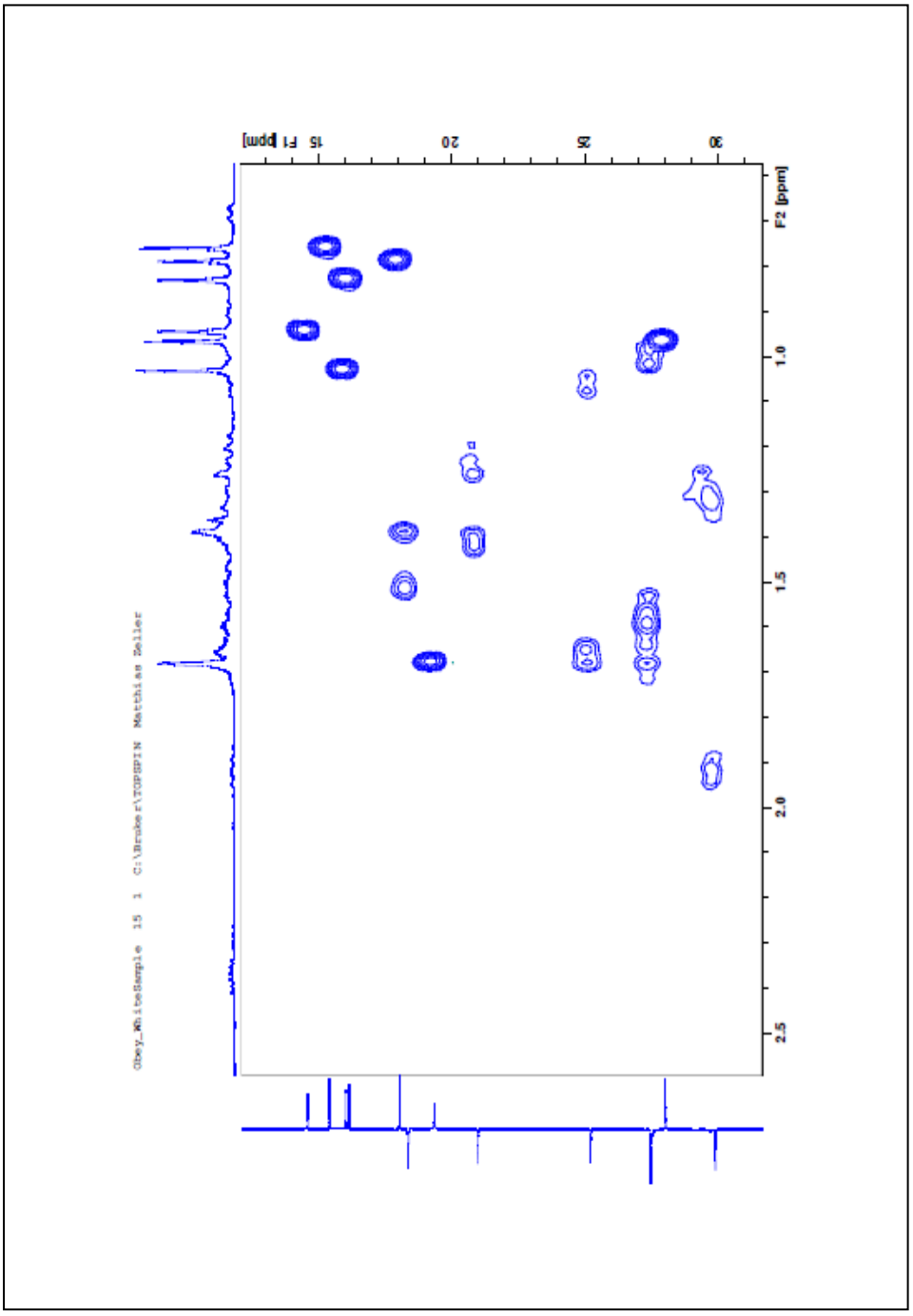
Appendix XXVIII: Heteronuclear Single Quantum Coherence (HSQC) Spectroscopy for fraction F5 film 3



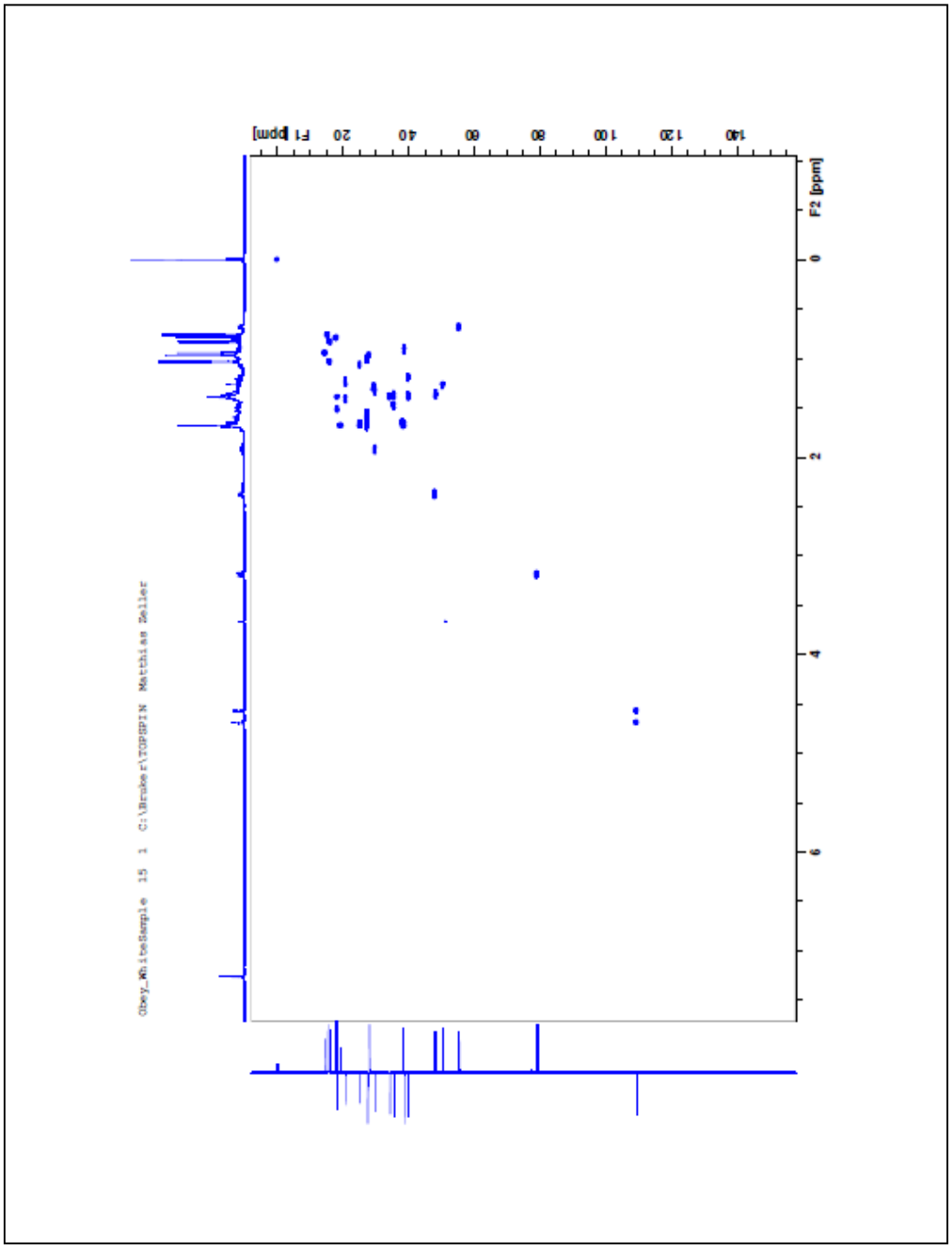
Appendix XXIX: Heteronuclear Single Quantum Coherence (HSQC) Spectroscopy for fraction F5 film 4



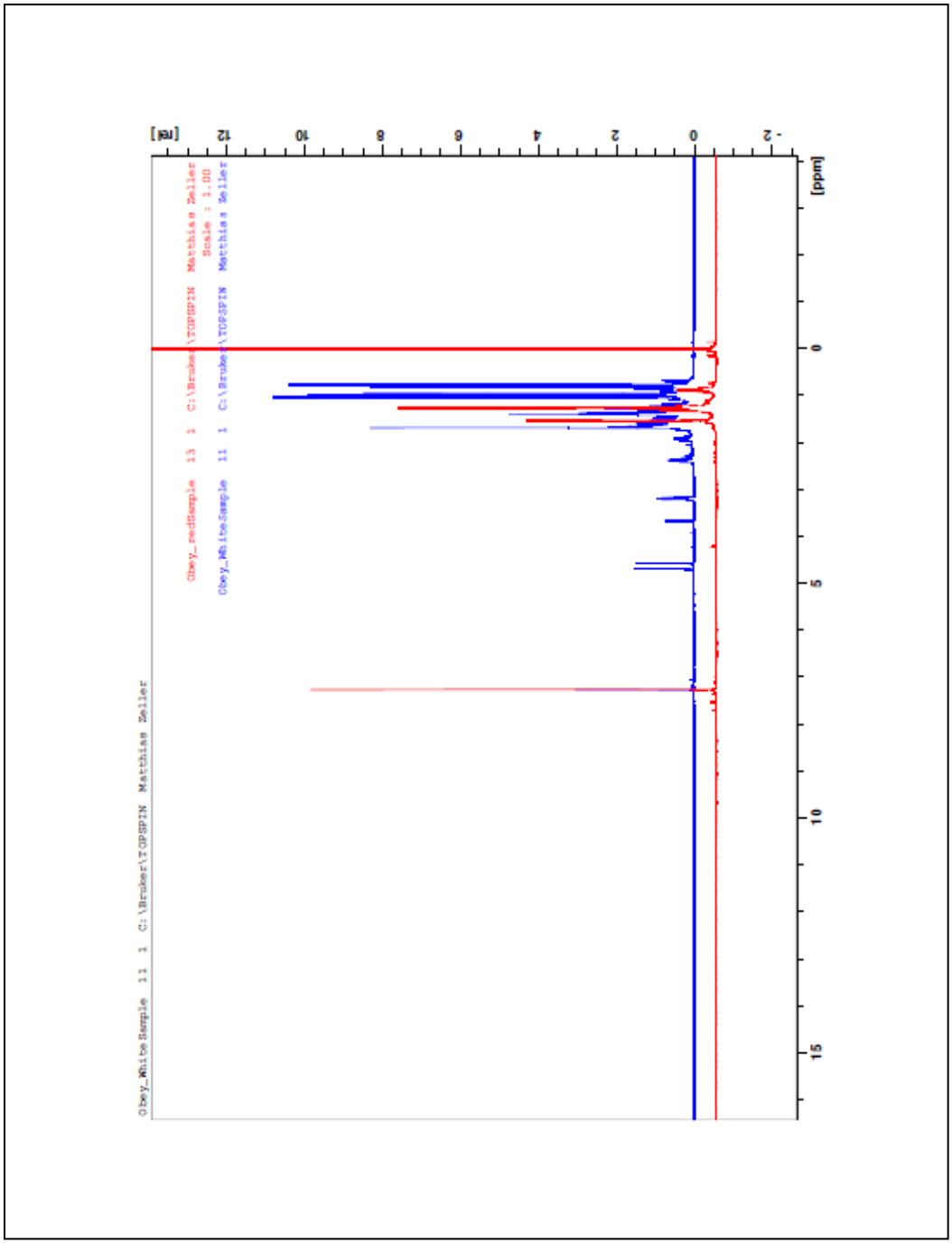
Appendix XXX: Heteronuclear Single Quantum Coherence (HSQC) Spectroscopy for fraction F5 film 5



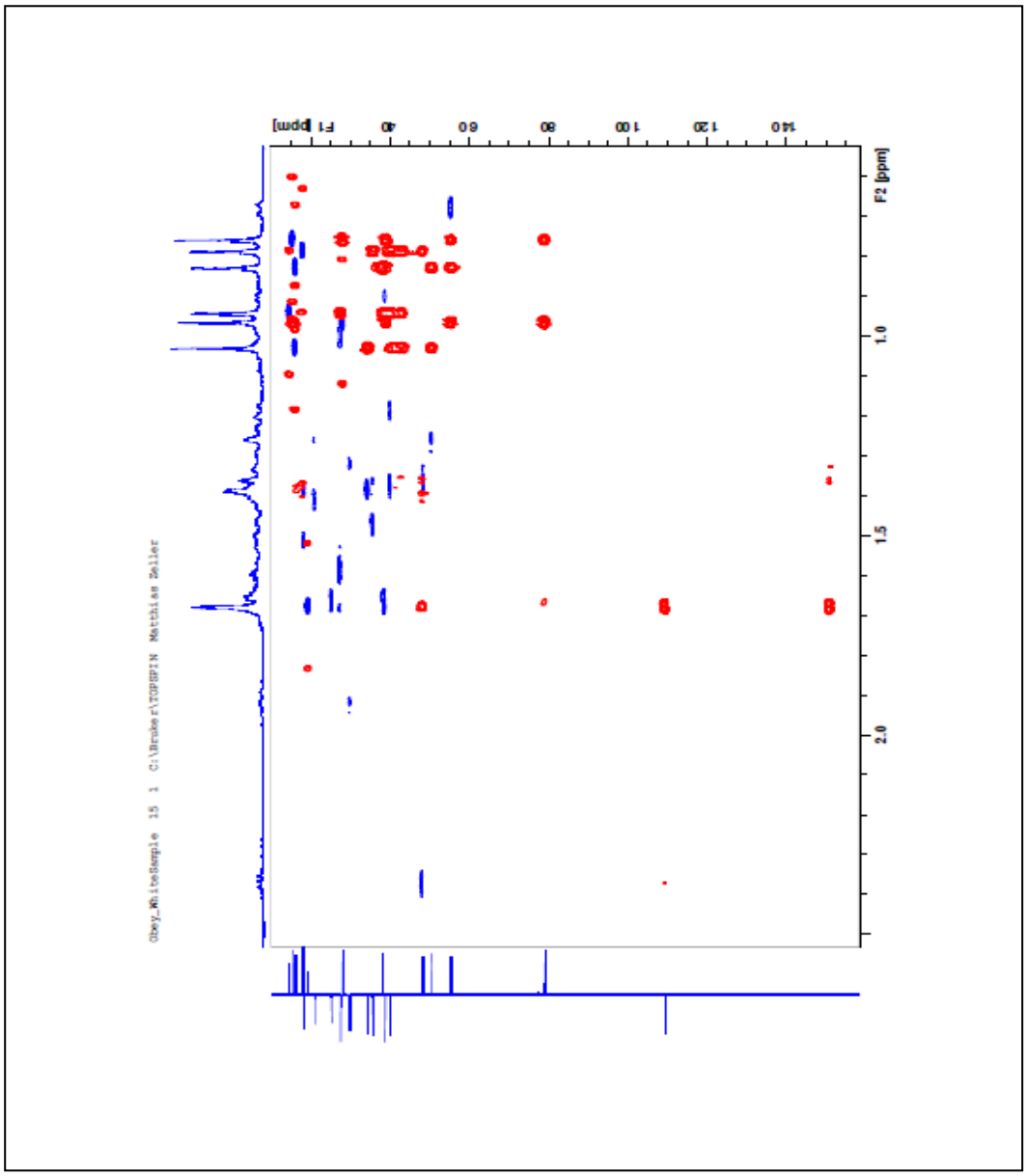
Appendix XXXI: Heteronuclear Single Quantum Coherence (HSQC) Spectroscopy for fraction F5 film 6



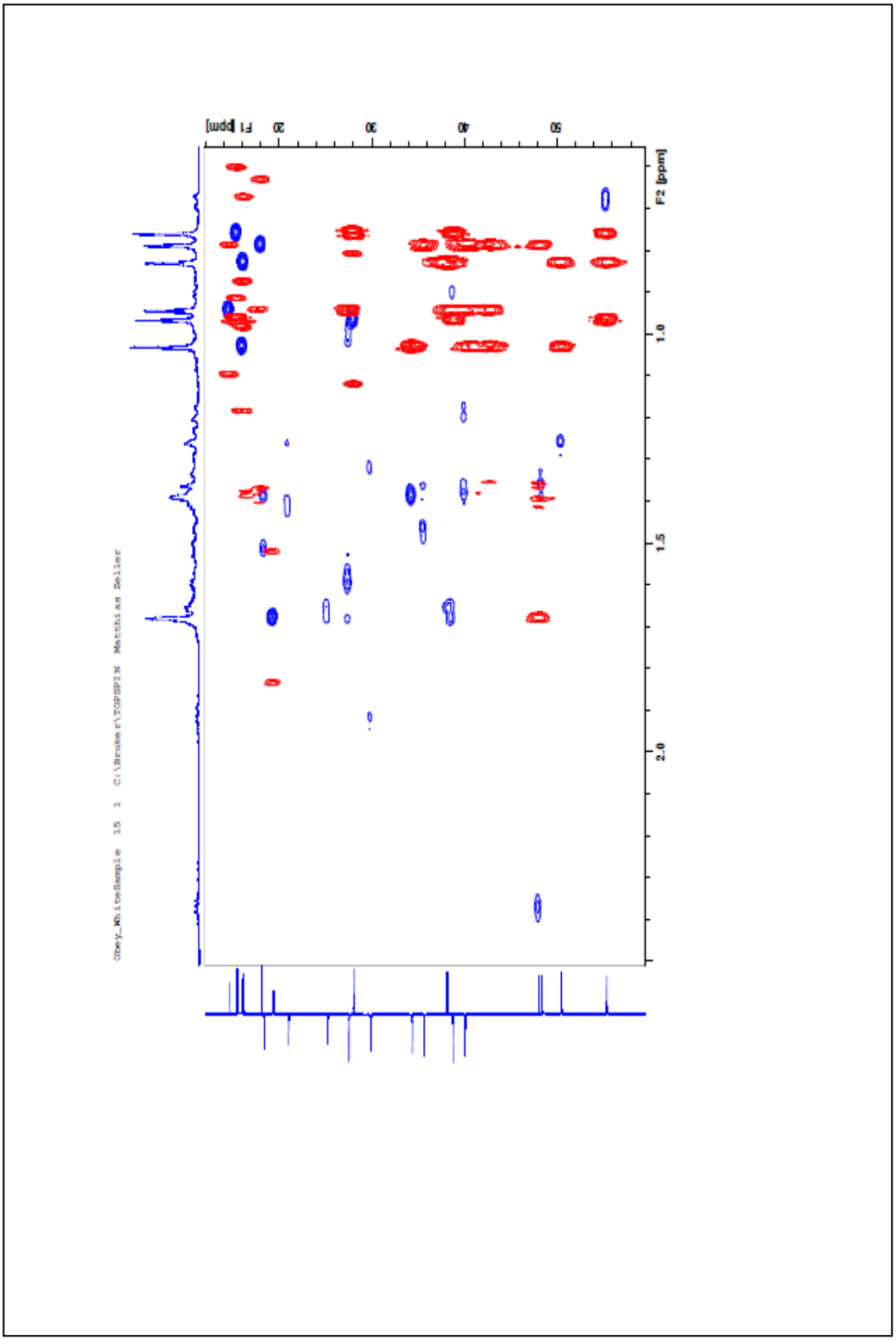
Appendix XXXII: Overlay of red/white samplesF5 and $F5_2$



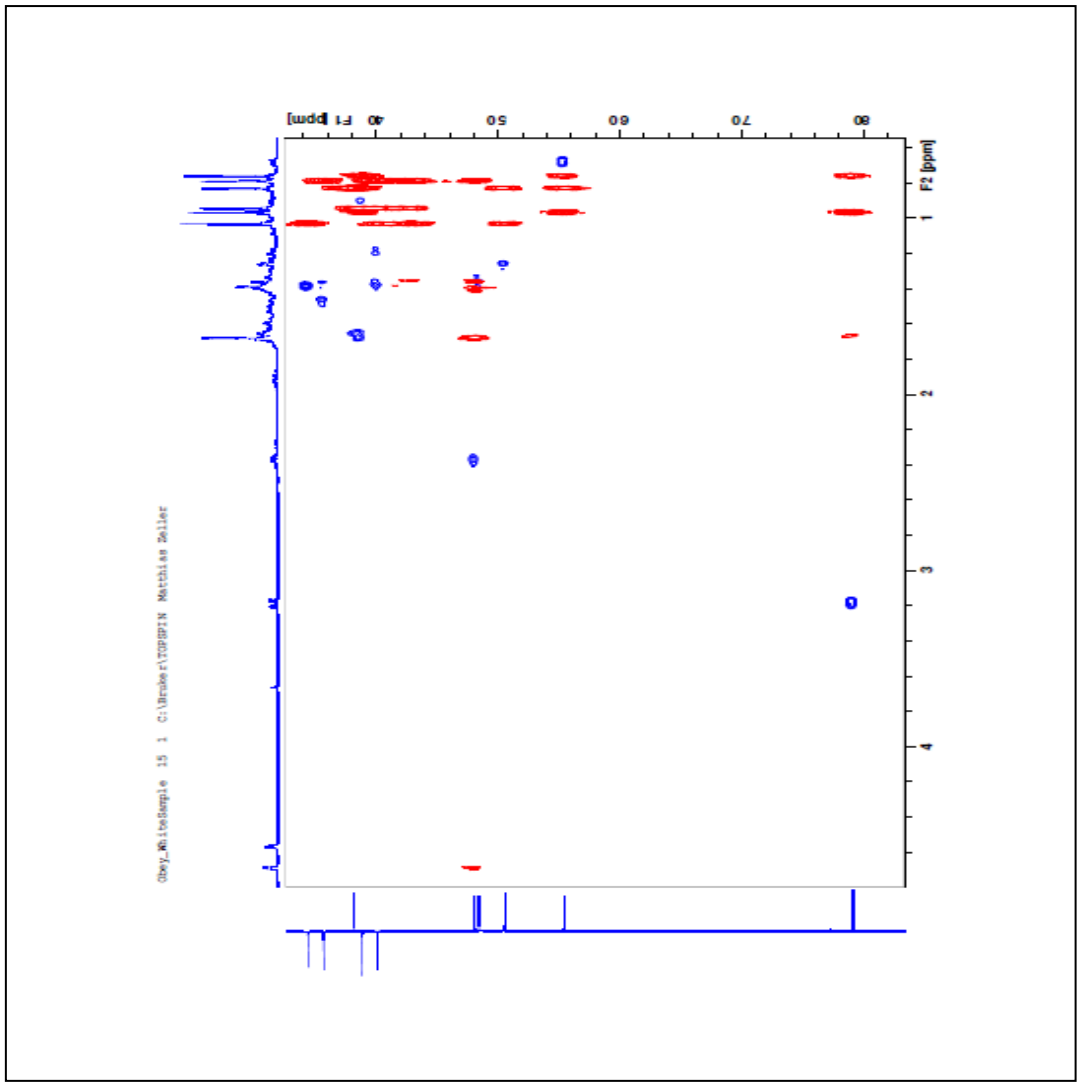
Appendix XXXIII: Overlay of HMBC Spectra for fraction F5, film 1



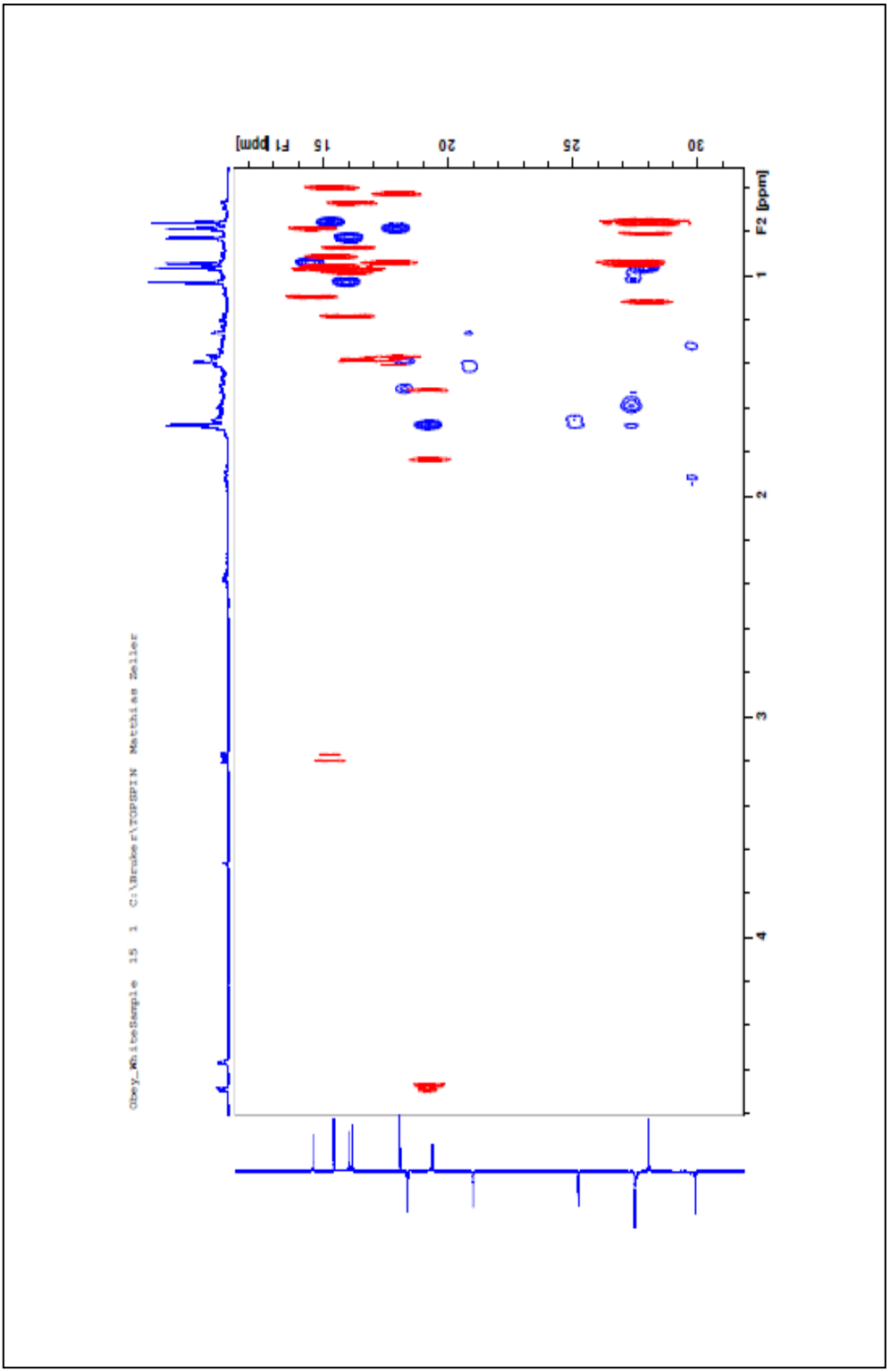
Appendix XXXIV: Overlay of HMBC Spectra film 2



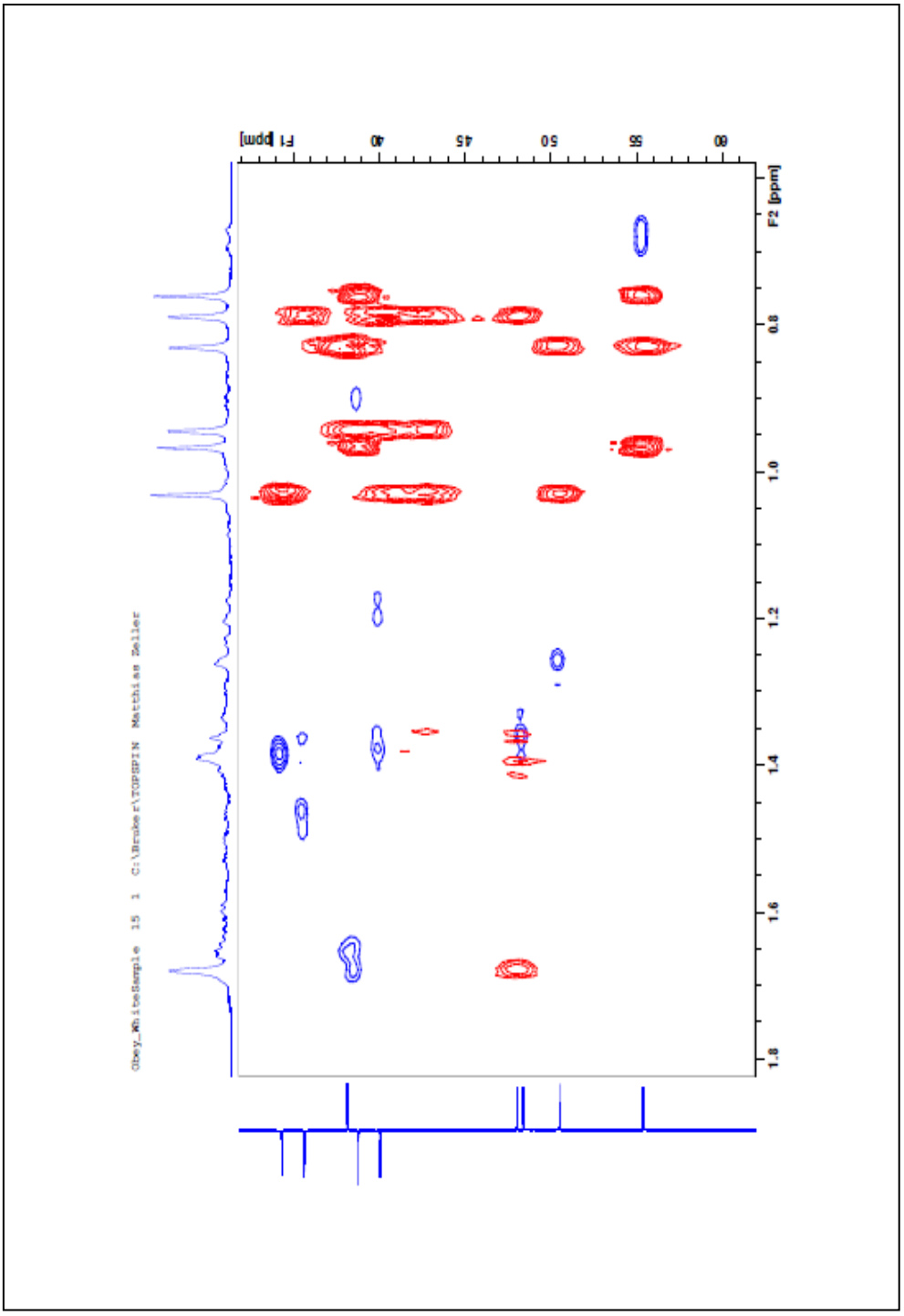
Appendix XXXV: Overlay of HMBC Spectra for fraction F5, film 3



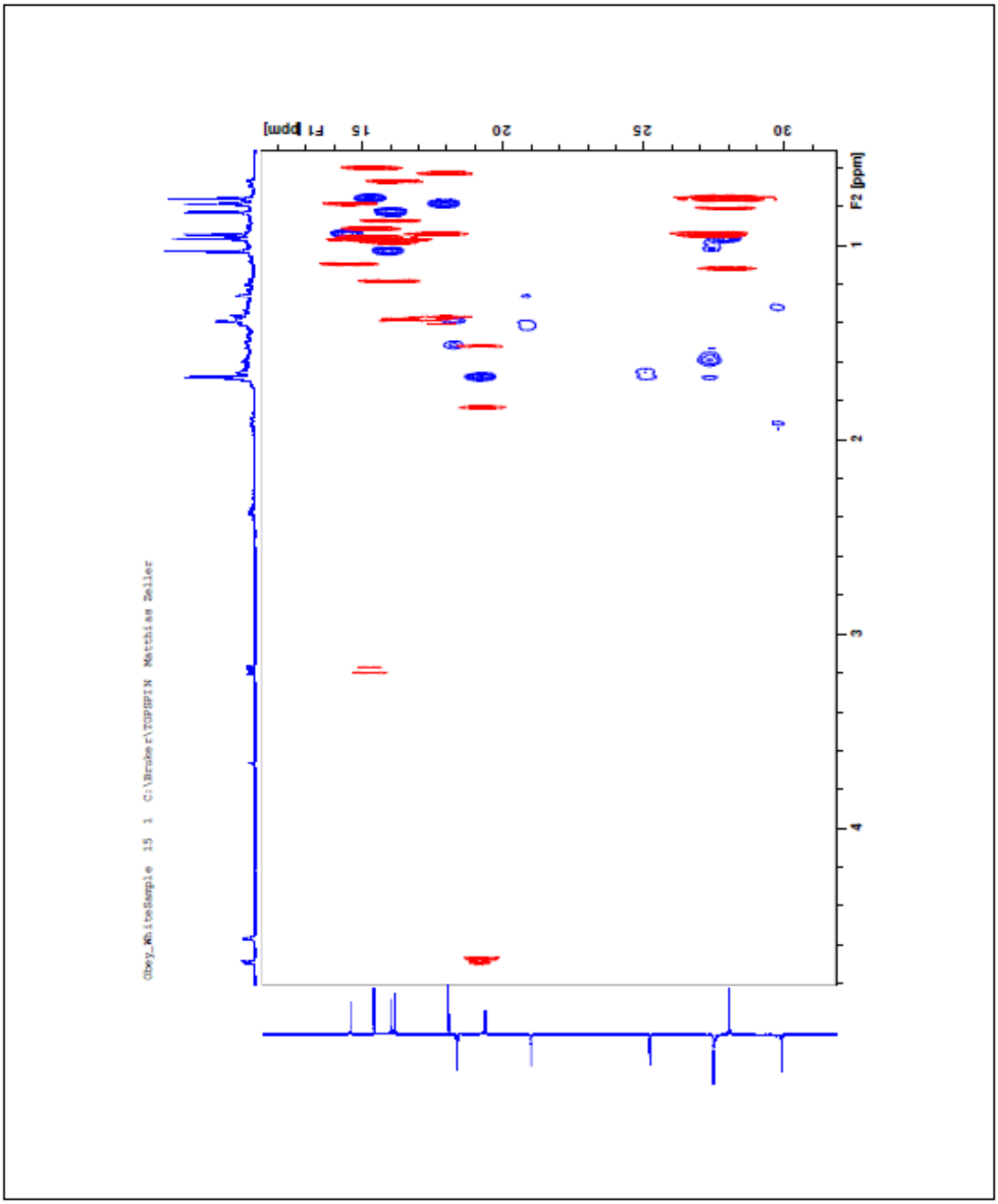
Appendix XXXVI: Overlay of HMBC Spectra film 4



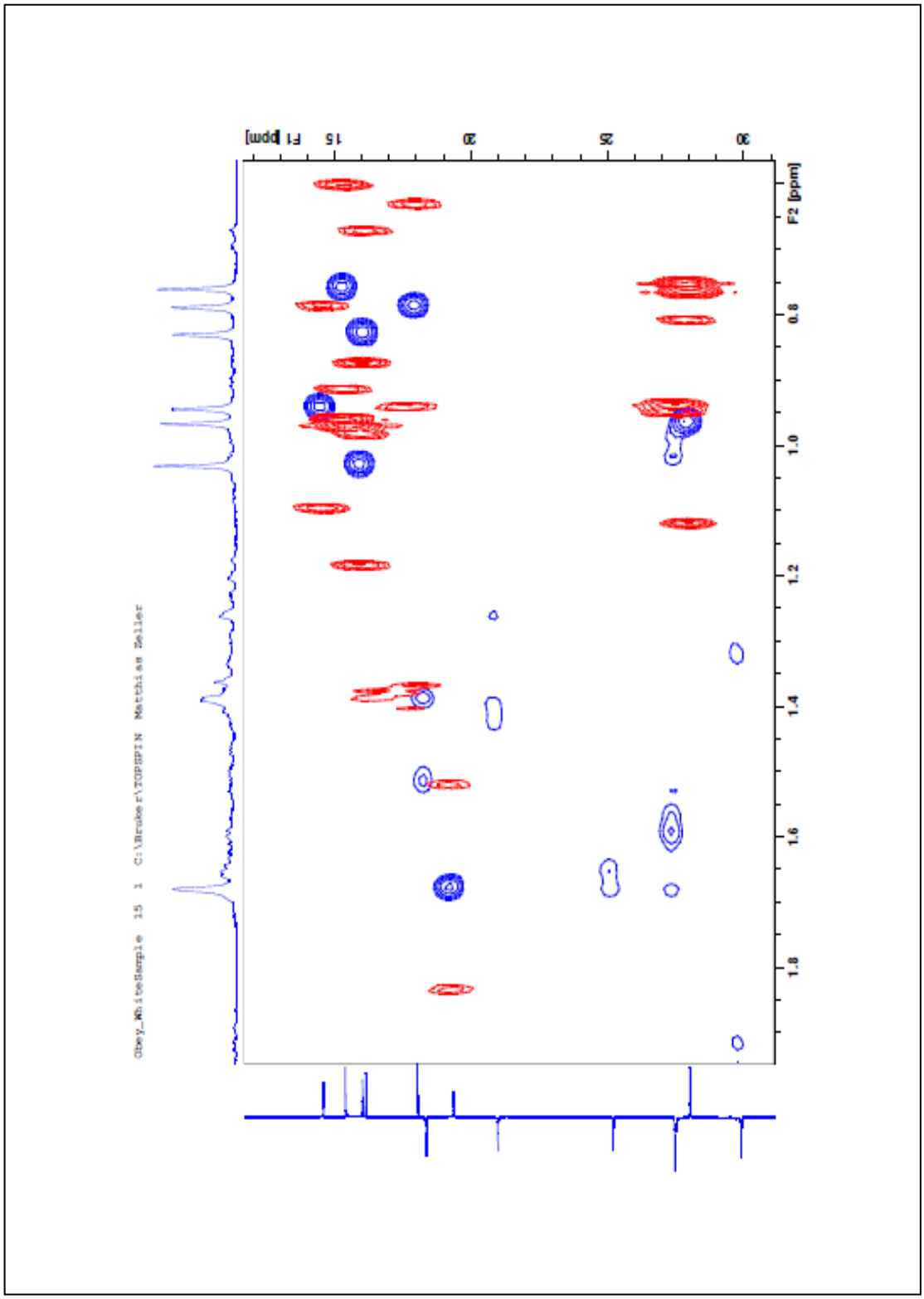
Appendix XXXVII: Overlay of HMBC Spectra film 5



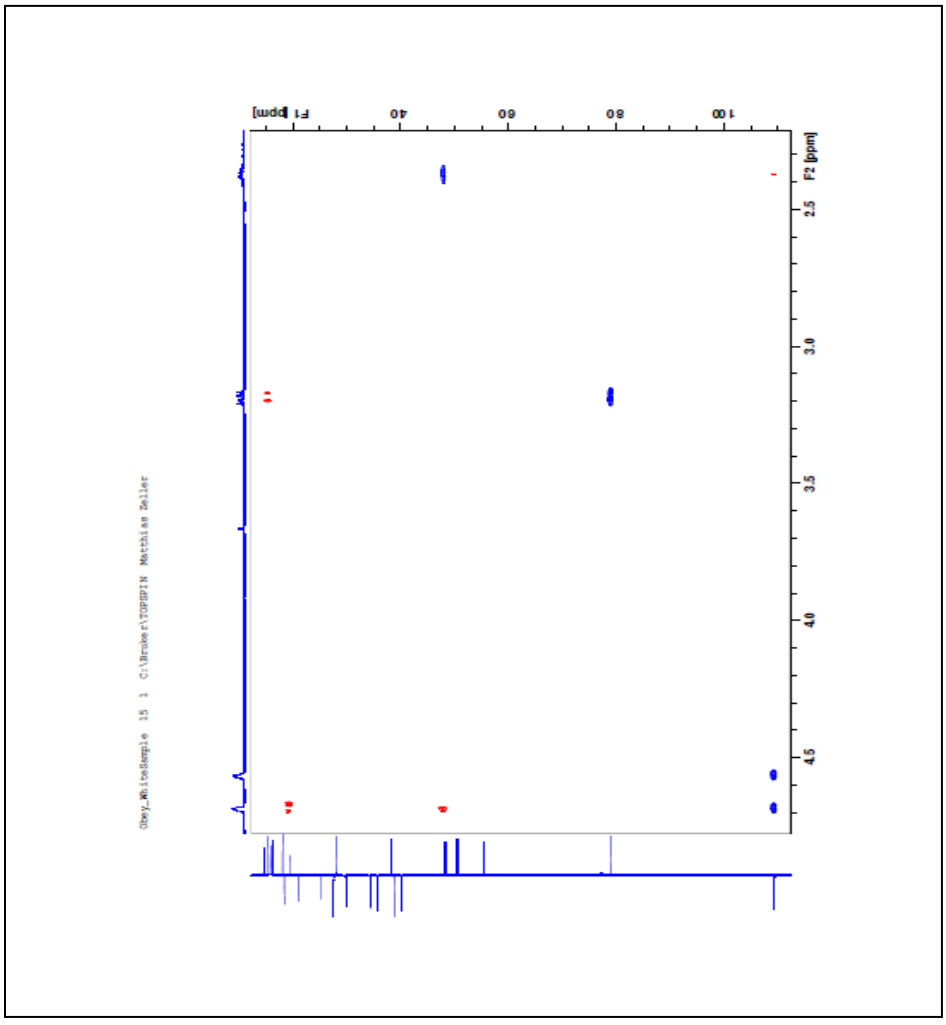
Appendix XXXVIII: Overlay of HMBC Spectra film 6



Appendix XXXIX: Overlay of HMBC Spectra film 7



Appendix XL: Overlay of HMBC Spectra for fraction f5, film 8



Appendix XLI: Parasite Density After Treatment with Artemether Control, Butanol and Methanol Extracts

	art 10ng/ml		L4a10ug/ml		BuOH1000ug/ml		MeOH	
1	100	86	52	66	40	754	854	766
2	112	136	72	54	38	186	870	1223
3	216	552	52	116	92	148	98	84
4	466	740	66	102	266	124	32	24
5	440	414	140	146	206	402	52	30
6	512	614	470	418	564	196	98	72
7	490	424	590	834	816	152	126	80
8	430	764	334	946	926	260	250	106
9	386	490	638	1089	868	534	246	162
10	250	366	686	744	866	798	304	200
11	238	602	738	948	822	604	382	326
12	26	238	584	724	678	580	314	160

Appendix XLII: Parasite Density After Treatment with Chloroquine Control, F5 Pure Compound, and Ethyl Acetate Extract.

	CQ 1000ng/ml		F5 100ug/ml		EtOAc1000ug/ml		L4a25ug/ml	
1	42	88	38	1387	442	198	1417	162
2	66	72	56	252	808	764	876	88
3	60	66	54	78	62	44	70	28
4	42	52	88	136	90	36	20	26
5	44	42	176	176	100	86	30	14
6	54	76	206	226	66	58	44	46
7	58	60	104	280	164	146	38	92
8	64	36	248	246	78	122	66	42
9	74	152	292	316	240	138	166	66
10	240	458	440	394	210	196	170	50
11	360	338	478	392	332	226	246	68
12	84	468	690	384	382	188	144	76

Appendix XLIII: Parasite Density After Treatment with Chloroquine Control, Ethyl acetate, Butanol and Methanol Extracts.

	CQ		EtOAc		BuOH		MeOH	
1	158	164	30	30	108	38	60	82
2	180	138	48	72	44	104	144	106
3	22455	292	172	270	382	404	176	118
4	66	8	32	32	1245	1209	588	150
5	284	14596	14213	10567	6428	4880	1499	1253
6	192	842	11709	5989	8898	8367	7806	6478
7	212	324	10569	9060	18514	17570	16233	15154
8	2754	238	10813	10475	13814	10126	11320	9863
9	6546	292	10348	10256	11204	9829	9671	9988
10	13884	408	12401	10343	13495	11326	11190	8745
11	14128	10950	12567	12052	13329	10396	11124	8487
12	952	1569	1028	968	862	826	390	386

Appendix XLIV: Parasite Density After Well Treatment with Artemether Control, $F5_2$ Pure Compound and F5 Pure Compound.

	art		$f5_2$		f5		$F5_2$	
1	568	808	292	300	2980	416	216	172
2	4013	4081	976	1543	11826	2499	400	242
3	13866	13905	4978	3967	12198	11593	2123	1839
4	20030	15899	13802	14195	15306	14925	8323	10986
5	16367	14311	15549	14275	14845	14203	14833	16551
6	15703	15232	15922	15296	14169	13604	14863	14163
7	15968	16146	13912	16024	14650	14336	14891	14580
8	13186	12026	9992	10559	9976	9473	9397	13056
9	14937	12042	12842	10887	11378	8842	11312	11376
10	13094	12445	14420	12810	11743	11463	10652	12020
11	12493	11784	10928	11429	13183	13802	10986	10170
12	694	892	688	916	1012	962	478	418

APPENDIX XLV: Maintaining the *In Vitro* Cultures

LABORATORY CULTURE PROCEDURES

All procedures (except centrifugation) were performed in a laminar flow biological safety cabinet. The cabinet was wiped with 70% industrial methylated spirit (IMS) and paper tissues at the beginning and end of each day. The vacuum tubing and trap were cleaned by flushing several times with tap water then with 70% IMS at the end of each day.

At the end of each day the gas regulator was checked that the needle valve was off and the main stop dock fully closed. The incubators were checked to ensure that they were at 37^{o}C. The pipette-aids were checked that they were off. The gas supply to the gas-o-matic was checked to ensure that it was off at the cylinder. The front of the hood was checked to see if it is covered with the roll-down plastic sheet.

PREPARATION OF MEDIUM (1640/HEPES)

The hands were washed and used only glass ware which is specifically designated for preparing media and is used for no other purpose. To 1000 ml graduated cylinder 2 drops of liquid detergent was added.

200 ml tap water was added, agitated vigorously by swirling and emptied into sink, the cylinder was rotated so that the detergent solution comes into contact with all parts of the inner surface of the cylinder. While holding the cylinder was inverted and allowed to drain for 10 sec. The procedure was repeated multiple times until no visible bubbles of detergent were formed with water and drained. To cylinder 100 ml autoclaved distilled water was added and rinsed 6 times.

To cylinder 100 ml autoclaved distilled water. 5.94g powdered HEPES acid was weighed out into a clean weigh boat (a spatula or other instrument was not used to add powder or to enter stock container; another weigh boat was used to transfer powder. The chemicals were not returned to the stock bottle, no re-entry; small amounts were transferred at a time so that waste is minimized). The powder was poured into the cylinder containing 100ml water.

The weigh boat was washed with autoclaved distilled water from a wash bottle to dissolve remaining powder and added to cylinder. The weigh boat was washed until no more powder was visible.

A one-liter packet of powdered RPMI medium 1640 was removed (Preferably no more no more than 6 months old, but powdered RPMI may be used until indicated expiry date) from refrigerator. Carefully the packet was opened so that no powder is spilled. The contents were added to cylinder containing HEPES acid. The packet was washed carefully with autoclaved distilled water from a wash bottle to dissolve the remaining powder and added to cylinder. If container is a drum the lid was washed also. The packet was washed until no powder was visible and no pink color was imparted when water was added. The packet was washed two more times.

The neck of the cylinder was washed with autoclaved distilled water from the washing bottle. To the cylinder approx. 200ml autoclaved tissue culture distilled water was added and swirled the contents until all the powder was wetted. More water was added to 960 ml mark on cylinder. Gentamycin was added to a final concentration of 10 µg/ml.

The cylinder neck was covered with parafilm and mixed until the powder was dissolved and the gentamycin was well mixed into the medium. Approx. 200ml medium was

poured into a 0.2 µm filter unit and filtered with the aid of a vacuum line. In a laminar flow hood, using a full aseptic technique, filtered medium was poured into a sterile 500ml plastic flask. The procedure was repeated until all medium was filtered and transferred to 2 flasks.

The flasks were labeled with medium names and date prepared. The medium was left overnight in the incubator at 37^{o}C and observed the following morning to see if there are any traces of contamination. If there is no contamination, the medium was stored at 4^{o}C until needed. The maximum life span is 4 weeks.

PREPARATION OF SODIUM BICARBONATE

The hands were washed. 2.5g tissue culture sodium bicarbonate was weighed into a weigh boat. The chemical was carefully poured into a 50 ml sterile plastic centrifuge tube. The weigh boat was washed with autoclaved distilled water as before until no powder remains. The weigh boat was washed two more times as before.

Autoclaved tissue culture water was added to 50 ml mark on the tube. Tube cap was replaced tightly and mixed until all powder was dissolved. A 20 ml syringe and Millipore 'millex-GS' 0.22 µm filter unit to sterilize directly into a 25 cm^2 flask. It was stored at 4^{o}C for no more than 2 weeks.

PREPARATION OF WASH MEDIUM (WM)

The volume of 1640/HEPES and 5% sodium bicarbonate needed was calculated from the formula:

1640/HEPES =0.96 x final volume

5% bicarb. =0.040 x final volume

After handwashing, appropriate number of 25 cm^2 flasks (one for each 25ml) was placed under hood and caps were loosened. The stock 1640/HEPES and 5% sodium bicarbonate were removed from refrigerator and placed under hood.

Using aseptic technique bicarbonate was added to one flask containing 1640/HEPES. The pipette was discarded (do not enter bottle of bicarbonate). The procedure was repeated for other flasks. The flasks were flushed with 5% O_2, 3% CO_2, 92% N_2 and sealed.

To wash 5ml blood, 22.5 ml WM (21.6 ml 1640/HEPEs and 0.9 ml bicarbonate) were prepared. To wash 50 ml blood, about 90ml WM was prepared.

PREPARATION OF MEDIUM WITH 10% SERUM (cms)

The amounts of 1640/HEPES, 5% bicarbonate and serum were calculated from the formula;

1640/HEPES =0.8640 x final volume

5% bicarb. = 0.036 x final volume

Serum = 0.1 x final volume

(For 50ml final volume, these amounts are 43.20, 1.80 and 5.00 ml. The hands were washed; the required volume of serum was thawed in 37^oC incubator and removed to room temperature as soon as thawed. Appropriate number of 25 cm^2 flasks (one each 50 ml CMS) were selected and placed under hood and caps loosened. The 1640/HEPES and 5% bicarbonate were removed from the refrigerator and placed under hood with thawed serum. Using aseptic technique, 1640/HEPES was added to flasks. The 5% bicarbonate was added to one flask containing 1640/HEPES and mixed until color is uniform. If one

flask was being prepared, 5% bicarbonate was taken up in the same pipette for all flasks and pipette discarded. Do not re-enter bottle of bicarbonate.

The serum was mixed by aspirating and expelling from pipette 5 times. A desired volume of serum was aspirated into pipette and transferred to flask containing 1640/HEPES and bicarbonate and mixed by aspirating and expelling 10 times. The procedure was repeated until all flasks contain serum, (use same pipette).The flasks were flushed with 5% O_2, 3% CO_2, and 92% N_2.

FLUSHING TUBES AND FLASKS WITH 5% O_2, 3% CO_2, 92% N_2.

The main stopcock of gas cylinder and a can of plugged Pasteur pipettes were opened. The needle valve was opened slowly so that gas flows at a rate of 2.5 I/min, and allowed the gas to flush the tubing for 5 sec. The tubing was kinked to stop the gas flow. A pipette was removed by touching with the fingers only and only the pipette was removed, and without allowing the distal half of the pipette to touch the sides of the can or any other pipette. The pipette was attached to gas line tubing. The pipette/ tubing were held in the right hand.

The distal half of the pipette was moved back and forth in full flame for 4 sec. The cap was removed from culture flask/ tube using the little finger of the right hand and inserted pipette into flask, the tubing was unlinked and the gas was allowed to flush the flask (5 seconds for 15 ml tube, 10 sec. for 25 cm^2 flask holding it in almost horizontal. The tubing was re-kinked, the pipette was removed quickly, the cap replaced and sealed tightly. While holding kinked tube/pipette, the needle valve was closed and the pipette removed from tubing and discarded.

COLLECTION OF SERUM

Consent for one unit of blood was obtained from at least six adults. A convenient time for blood collect was arranged and ensured that a clinician is available to draw blood. Every prospective donor was ensured that they read and completed the "blood donation for *in vitro* culture" questionnaire (questionnaires are stored in the culture laboratory). On the basis of the completed form, it was decided whether the donor was acceptable or not.

For suitable donors, one unit of blood was collected into a bag with no anticoagulant. The blood in the tubing was not allowed to drain back into the bag; this will suck in unsterile air and may contaminate the entire serum batch. The taking tube containing blood was tied in an air tight knot close to the pack. The surplus tubing was cut off, in the process, some of the blood was drained (about 2 ml or so) in the tubing into a 7 ml bijou bottle. This aliquot of blood will be used for screening. The bag and the bijou bottle were labeled with reference number of the donor.

The blood in the bag was allowed to clot at room temperature for four hours, then in the refrigerator at 4^{o}C overnight. While the blood is clotting at 4^{o}C, the aliquot in the bijou bottle was taken to be tested at KEMRI microbiology for hiv and hepatitis antigens. Any donations which were HIV +ve or which are positive for hepatitis antigen were incinerated.

The hands were washed; the clotted blood was centrifuged at 4500 rpm for 5 min. The serum was expressed into transfer bag. If RBCs have been transferred along with serum, the serum was centrifuged and expressed into transfer bag again.

Using aseptic technique, appropriate numbers of 650 ml sterile plastic bottles were selected to accommodate the serum batch. The plastic sterile bottles were placed in a water bath at 56^{o}C for 40 minutes to heat inactive. The water level was ensured that it is 1 cm below bottom of the plastic bottle's top (or there is a danger that the serum will be contaminated by water).

Using each bag of serum separately, a small amount (50 ml) of complete medium was prepared with serum (CMS) and maintained a culture for one week to test whether each particular batch of serum supports growth. If it does, the separate serum donations were pooled in the ratio of their volume so that each 650 ml bottle finally contains the same volume of pooled serum, and the same proportions of individual donations.

5 ml or 10 ml aliquot of pooled serum was added to pre-labeled polypropylene 5 or 10 ml snap-top tubes. The tops of tubes were snapped tightly; the tubes were placed upright in -20^{o}C freezer overnight then transferred to -80^{o}C freezer. Note: If tubes are to be placed at -80^{o}C ensure that appropriate tubes are used otherwise the tubes will crack resulting in contamination of the serum.

COLLECTION OF FRESH ERYTHROCYTES

a**)Collection of erythrocytes from donor**

The hands were washed, in the laminar flow hood 3ml ACD was added to 50 ml sterile plastic centrifuge tube. 70% of ethanol was squited onto sterile cotton gauze and

cleansed donor's arm. Using 19 or 20 gauge needle and 20 ml -30 ml syringe, draw 20 ml blood.

In a laminar flow hood, using aseptic technique the needle was removed from syringe and blood added to tube, the cap was replaced firmly and the contents mixed by inverting tube slowly several times. The tubes were labeled with initial of donor, blood group, the date collected and stored at 4^{o}C until used. The tube contents were mixed at least once a week by several inversions.

i)**Formula of acid citrate dextrose (ACD)**

Tri-sodium citrate 22.00g

Citric acid 8.0g

Dextrose 24.5g

Autoclaved distilled water to 1000 ml

Sterilize by filtration, store at 4^{o}C.

NOTE:

When large numbers of culture flasks are being maintained, it is difficult to collect sufficient amounts of blood in the laminar flow hood as described above.

Therefore two pints of blood (O positive blood group), and 6-7 days old should be collected from National Blood Bank (Next to the malaria control program, on the left side of WTRL, Nairobi unit).

The blood collected at no cost since it for research purposes. Blood pints that are not completely full, that is, the donor was unable to completely fill the pint, is more easily available and may be used.

b) Collection of erythrocytes from blood collected from the blood bank:

The hands were washed and the tubes labeled with blood group and the date collected. The blood bag was shaken gently. Using aseptic technique the delivery tube was snipped to blood bag and aliquot content into 50 ml tubes and store at 4^{o}C until used. The tube contents were mixed at least once a week by several inversions.

WASHING ERYTHROCYTES

The 50 ml centrifuged tube of blood/ACD was placed in laminar flow hood. A sterile 10 ml pipette was attached to pipette –aid. The blood was mixed gently drawing up and expelling from pipette 5 times. The desired amount of blood was removed and transferred to a 15 ml conical centrifuge tube and centrifuged at 1500 rpm for 5 min. The blood/ACD tube was returned to 4^{o}C refrigerator. 22.5 ml wash medium was prepared for each 5 ml volume of blood being washed. The flask was tightened without gassing and set aside in hood. The pilot flame was ignited on touch-o-matic . The tubes were transferred from centrifuge to laminar flow hood and the caps loosened on tubes. The vacuum tube was removed from holder and remove a sterile unplugged Pasteur pipette from the can touching with fingers only the one to be removed and without allowing the distal half of the pipette to touch the sides of the ca n or any other pipette. The pipette was attached to vacuum line and moved the distal half back and forth in full flame for 4 sec.

The cap of centrifuge tube was removed using the little finger of the right hand. The foot switch was operated to turn on vacuum pump. The supernatant fluid and buffy coat was aspirated; suction was sufficient to cause a steady flow of fluid into pipette, but not so strong as to produce air bubbles or turbulence. The cap was replaced on the tube and the pipette discarded. If suction was not correct the vacuum was adjusted.

The tube cap was removed using the little finger of the right hand. 10 ml wash medium was added to tube and gently resuspended cells 5 times using 10 ml pipette. Repeat procedure until all erythrocytes have been washed.

The packed cell volume of wash medium was estimated and resuspended cells (50% RBC suspension). If cells are not to be used immediately the tube was flushed with 5% O_2, 3% CO_2, 92% N_2 and stored at 4^oC. This preparation was not used until after 2 days.

NOTE: If 50 ml of blood is to be washed the following procedure has been found to be successful at the WTRL, Nairobi unit.

The 90 ml of wash medium was prepared and centrifuged the whole blood in the 50 ml tube at 1500 rpm for 5 min and discarded the supernatant. The resulting blood cells were washed three times, at each wash using 20 ml of wash medium as explained above.

The packed cell volume of the cells was estimated and resuspended cells with wash medium 50% RBC suspension. If cells are not to be used immediately the tube was flushed with 5% O_2, 3% CO_2, 92% N_2 and stored at 4^oC. This preparation was not used until after 2 days.

PREPARING NEW CULTURES FROM OLD CULTURES (5 ML VOLUME, 6% HCT)

The hands were washed, the gas cylinder main stop cock was turned on and the pilot flame was ignited on touch-o-matic . A 50% RBC suspension was prepared (> 12 days < 28 days old). The necessary volumes of old culture, 50% RBC and medium needed for 5 ml, 6% haematocrit (hct) new cultures was determined as follows;

Culture Volume (CV)	=	5/D	500 ml
50% RBC volume	=	6/50 (5-CV)	540 ml
Medium volume	=	5-(CV + RV)	400 ml

Where:D is the reciprocal of the desired dilution factor e.g D = 10 for 1:10 dilution .

The required number of 25 cm^2 flasks were selected and labeled them (initials of scientist, parasite reference number, date). The required amount of CMS, 50% RBC was added to the flask(s), the flasks were flushed with 5% O_2, 3%CO_2, 92% N_2, and placed flasks in the 37^oC incubator for 20 min to equilibrate to 37^oC.

The warmed flasks plus old cultures to be diluted were placed under hood and loosened the caps. The old culture flasks were gently swirled to achieve the homogenous suspension. The desired volume of old culture was removed and added to flasks

containing fresh RBC and medium was mixed by swirling flask with a circular motion and placed horizontally on the work surface. The procedure was repeated for the remaining new cultures.

The flasks were flushed with gas, changing the pipette between each different strain/isolate. The new culture were mixed again by swirling with a circular motion and placed horizontally in the 37^{o}C incubator.

PREPARING NEW CULTURES FROM CRYOPRESERVED INFECTED BLOOD

The hands were washed, the blood was thawed and reconstituted isotonicity as per protocol of July 1985. This should yield approx. 0.25 ml packed cell volume. Under laminar flow hood 0.7 ml 50% RBC , 9.1 ml medium with 10% serum (CMS) was added to thawed packed cells and mixed well using the 10 ml pipette.

The cells suspension was taken up in the 10 ml pipette and transferred 5ml to each of the two 25 cm^2 flasks. The flasks were flushed with 5% O_2, 3%CO_2, 92% N_2, the caps were tightened and placed horizontally in the 37^{o}C incubator.

PREPARING NEW CULTURES FROM FRESH INFECTED BLOOD

NOTE: Parasite viability was prolonged when the infected heparinised blood was cooled to 4^{o}C as soon as possible after collection and kept at this temperature until the culture was started. Successful recovery of parasites from cooled blood 24 hr after collection was possible.

The hands were washed. In laminar flow hood, 4 ml of fresh infected blood was placed in a 15ml centrifuge tube. The blood was washed by mixing 0.6 ml of 50% suspension of fresh infected blood and 4.4 ml medium with 10% serum was added to a 25 cm^2 flask.

The flask was flushed with 5% O_2, 3% CO_2, 92% N_2 and was placed horizontally in 37^{o}C incubator.

CHANGING MEDIUM AND MAKING SLIDES

The hands were washed. The flasks of medium with serum (CMS) were removed from refrigerator and placed in 37^{o}C incubator for 20 min to equilibrate. The gas cylinder main stopcock was opened. The pilot flame was ignited on touch-o-matic and turn on pipet-aid.

The frosted glass slides were labeled. Sterile glass pipettes were prepared; the square form can was held in one hand, the lid was supported with the other hand, and the pipettes were shaken gently down towards the lid end. The can was held horizontally. The lid was removed and placed in a corner of the hood. The mouth of the can and the pipette ends which are exposed were flamed. The can was placed carefully in the horizontal position on top of the lid (Sterile pipettes ends were kept well above the bench surface. Plugged and unplugged pipettes were required for changing medium.

Culture flasks were removed (1 or 2 slides were made, 1-4 if only changing medium) from 37^{o}C incubator, placed in hood and caps loosened. The flasks were removed carefully to avoid disturbing cell sediment.

The sterile unplugged Pasteur pipette was removed from can, touching with fingers only the one to remove and without allowing the distal half of the pipette to touch sides of the can or any other pipette.

The pipette was attached to vacuum tube and moved distal of pipette back and forth in full flame for 4 sec. Pipette/vacuum tube was kept in left hand.

The culture flask was carefully picked up with the left hand, keeping it horizontal. Using the little finger of the right hand the cap was removed from culture flask. The flask was tilted so that medium flows towards the mouth and pipette inserted. The foot switch was operated to apply vacuum and aspirated approx. 80% of the supernatant, continuing to tilt flask. Promptly remove pipette so that no red cells are aspirated. The cap was replaced loosely on flask and laid the flask flat on the work surface. This left about 1ml medium and red cells in the flask. The pipette was discarded.

If no slide was made, directions indicated to move to step (19). A plugged, sterile Pasteur pipette was removed as in step (9), attached rubber teat and flame as in step (10).

The cap was removed from flask as in step (11), the culture flask was tilted slightly so that medium flows away from mouth. Gentle pressure was applied to teat, pipette tip was inserted into pool of medium and aspirated medium approx. 4mm into pipette tip. The angle of flask was adjusted to almost horizontal, a pipette tip was placed in settled red cell layer, expressed medium and re-aspirated repeatedly, 4-6 times until a uniform red cell suspension is present in the tip.

The volume was all expressed but 2mm of the red cell suspension, the pipette removed, the cap replaced loosely and a small drop of the suspension applied to a glass slide and the pipette discarded.

A thin smear of the red cell suspension was made using the end of another glass slide and immediately the slide was placed in the stream of the air at the edge of the hood to hasten drying.

A 5 ml pipette was fitted to the pipette aid. Removed the cap from the flask of warm medium as in step 11, 4 ml medium was aspirated into the pipette and replaced cap on flask of medium.

The cap was removed from culture flask as in step 10, placed the tip pipette in flask, run in slowly, removed pipette, replaced cap loosely. The flask contents were swirled gently to resuspend the cells and replaced the flask upright on the work surface. The flask was flushed with 5%O_2, 3%CO_2, 92%N_2 . The flask was replaced horizontally in a 37^{o}C incubator.

LABELED HYPOXANTHINE UPTAKE TEST

The labeled hypoxanthine uptake test required; good growth rate of original culture (>3), haematocrit of 1.5% with parasitaemia of 0.5%, drug solutions, unparasitised controls (URBC), well mixer(manual titerman 12 channel (25-200µl), gas box in incubator, Eppendorf multipipette plus 4981.

a)<u>Setting up a 96 well plate test culture.</u>

The test media with CMS was made enough for 225µl per well (96 wells = 19.2ml say 20ml) as follows :- the parasitaemia level was set at 0.5% from the old culture ie. If culture has 2% parasitaemia then a ¼ dilution may be done and culture marked up to 5ml with media at 6% haematocrit as per a normal dilution. The haematocrit became 1.5% by making up the 5ml primary dilution to the final volume of 20mls with CMS.

The test solution was put and the rediluted continuing culture solution into the incubator to warm up to 37^{o}C before the addition of the culture.

The test drugs solutions were made up from the frozen standards in the freezer. NB: The quoted figures of ng/ml (1^{st} well concentration) resulting from dilution taking into account the plate dilutions of (i)1/2 and then (ii) 1/9 with addition of the media.

The plate with 25µl plain CMS per well was charged using the Eppendorf multipipette plus 4981; the sterile dispensing tip was put onto connection and adjusted the amount to be dispensed accordingly. NB the tip is the only sterile component. After filling the tip was discharged X 2 to ensure that the ratchet has engaged. 25µl of drug solution was added to row B in pairs, the tip was changed between drugs (Fig 2 page 41). The ½ dilutions were made with 'manual titerman 12 channel' from row B downwards doing 15-20 aspirations per well group. Trying not to touch the sides of the well when removing the mixer. The final 25µl was discarded from row H and the tips. The last 4 columns of row A was charged with 200µl unparasitised controls (URBC) using the Eppendorf multipipette plus 4981 dispenser. URBCs are made up from 30µl of 50% hct washed RBCs and 0.97 ml CMS.

The 'old culture' was added to the large volume of equilibrated CMS plus RBC to make up to the final test media. The drugs under test were divided into Bijou bottles to prevent drug cross contamination. 200µl of the test media was placed into each well starting with the parasitized controls and then moving to row H of the drug pairs and moving upwards. The combitip was refilled as required to maintain sterility.

If only one drug was being tested, the test culture was transferred to a 50 ml centrifuge tube instead of several Bijou bottles.

The gas box was placed into the 37^{o}C incubator and incubated for 48 hours re-gassing at 24 hours.

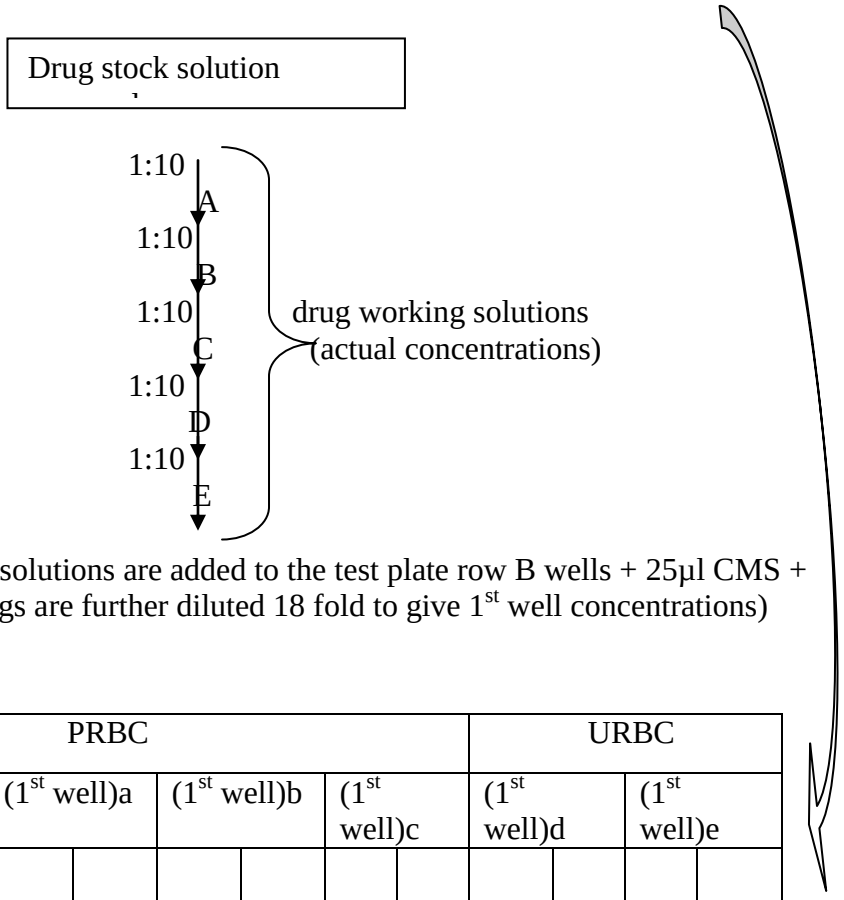
b) PREPARATION OF DRUG STOCKS AND WORKING SOLUTIONS

For new compounds whose 50% inhibitory concentration (IC_{50}) is not known; 10 – 15 mg of the drug powder was weighed into a 7ml Bijou bottle. Ensuring that all the necessary safety precautions are undertaken, that is wearing clean gloves, face-mask and a face shield. In the hood the cap of the Bijou bottle was loosened and the drug compound dissolved in 1 ml solvent. If all the powder is not dissolved, little amounts of solvent (500µl) was added slowly at a time until the powder is completely dissolved. DMSO was used as the standard solvent for dissolving *C. macrostachyus* extract drug compounds, while sterile distilled water was used to dissolve the control drugs chloroquine and artemisinin. The stock was diluted in a series of 1:10 dilutions to fit one complete test plate leaving a space for a control assay (Prepare five 1:10 dilutions). The drug concentration prepared is referred to as the actual concentration**.** On the test plate well the actual drug concentration was further diluted by a factor of 18 due to addition 25µl of CMS and 200µl of test culture. The drug concentration on the wells that drug was added to on the test plate (row B) is therefore referred to as 1st well concentration and is indeed the concentration of interest as shown on the flow diagram.

A test plate was set up as explained earlier using the working solutions prepared. One or two drug concentration ranges will enable you obtain the 50% inhibitory concentration (IC50) of the compound that you are testing. The IC_{50} concentration obtained now acts as

a reference point for any further assays to be carried out and the drug concentration that enabled you to get the IC_{50} is the only one carried forward for further testing.

Preparation of drug solutions:



(When the drug working solutions are added to the test plate row B wells + 25µl CMS + 200µl test culture the drugs are further diluted 18 fold to give 1st well concentrations)

A	PRBC								URBC			
B	Controls		(1st well)a		(1st well)b		(1st well)c		(1st well)d		(1st well)e	
C												
D												
E												
F												
G												
H												

DETERMINATION OF ^{3}H-HYPOXANTHINE UPTAKE

a.USING THE MICROBETA TRILUX LIQUID SCINTILLATION AND LUMINESCENCE COUNTER

Once the harvesting procedure was completed and the sealed filter mats have been placed in the cassettes for counting in the micro beta trilux liquid scintillation counter, the computer was switched on from the mains and on the CPU. From the desk top menu, **My computer → drive (C:) molecular_b → mbw → results** was selected. All the files in results were cleared by highlighting on them and pressing the delete button. Drive C was closed and returned to the desktop. Microbeta windows were selected from the desk top, once open the light on the start button display will be red. At this point the cassettes containing the filter mats were loaded to be read in the microbeta counter. This machine holds a maximum of sixteen cassettes. The last cassettes to be loaded did not contain a filter mat and had an ID support plate clipped to its side –containing the stop protocol bar code. (The bar code with the stop protocol enabled the microbeta counter to stop once all the other plates were read.)

Cassettes were loaded beginning with the shelve at the bottom, marked 1, this was the first cassette to be read. On the first cassette, the code label was clipped which showed the microbeta counter which counting protocol to use. In the case of tritium counting – protocol 1. Once all the cassettes containing the filter mats to be read were loaded, the empty cassette clipped with the stop protocol bar code was then loaded. The door to the microbeta counter was closed and the machine was switched on. The machine was given an ample time (5 minutes) for it to load the protocols and programs (loading parameters was shown at the right hand bottom of the computer monitor screen). Once the microbeta

counter wass ready, the light on the start button display on the computer monitor was indicated green. Select **protocols** ⟶ **general** ⟶ **open** ⟶ **tritium** (the protocol indicated was 1). At this point the plate map was observed to ensure that all the 96 squares was read. By selecting: plate map. If all the squares had a green circle around them and a number 1 to 96 then they were read and clicked okay.

If not all the squares had a green circle around them, then "auto fill" and "okay" were clicked.

Clicking okay returns you to **protocols** ⟶ **general** ⟶ **open** ⟶ **tritium.** Start was clicked and each cassette was read for 48 minutes that is ½ minute per well. Once all the cassettes are read, the beta counter senses the bar code on the stop protocol and counting stops.

a) RETRIEVAL OF RESULTS

The results were observed and saved on a diskette allowing one to analyse them at a different work station. Retrieving the results was done by minimizing the microbeta windows program. From the desktop selecting ⟶**My computer** ⟶ **drive (C:)** ⟶ ⟶**molecular_b** ⟶ **mbw**⟶ **results.** The files in results was double clicked one at a time. Once the file opens go to **file** ⟶ **save as** ⟶ **A:** under file name a desired name was given. Under save as, type select Microsoft excel 97 & 5.0/95 work sheet then click save. The result is saved in excel format.

APPENDIX XLVII: Detailed Procedures for *In Vivo* Experiments

STAINING THIN BLOOD SMEAR SLIDES WITH GIEMSA STAIN SOLUTION

The slide having thin blood smear was placed on the rack near the sink. 100% methanol was poured onto the whole surface of the slide to allow fixation of the thin smear onto the slide. A 4% giemsa staining solution was poured on the slide ensuring the whole surface is covered. The giemsa staining solution was allowed to remain on the slide for a period of 10 minutes. The giemsa staining solution was washed off using running tap water. The slide was dried and observed under the microscope.

PREPARATION OF GIEMSA STAINING SOLUTION

a) Preparing stock buffer solution for giemsa stain:

33g of Na_2HPO_4 and 7g of NaH_2PO_4 were dissolved to final volume of 200 mls distilled water.

b) Preparing working buffer solution for giemsa stain

20 mls of the stock buffer solution which was prepared above and made to 1000mls with distilled water.

c) Preparing giemsa working solution

The number of slides to be stained was counted. 4mls of giemsa working solution for each slide to be stained was prepared. 4% concentration of giemsa in the working buffer solution was prepared.

CALCULATING PARASITAEMIA

Parasitaemia = the number of red blood cells in 10,000 red blood cells. It is expressed as a percentage.

Parasitized cell = red blood cell containing parasite, this could either be a multiple infection (more than one parasite) or a single infection (one parasite). Whether a cell contains a single or multiple infection, it is counted as one parasitized cell.

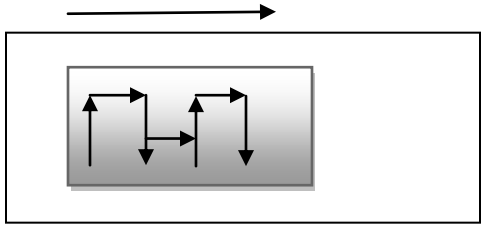
An appropriate area on the blood film was selected. At least three fields counting the number of red blood cells in each fields and the average number of red blood cells observed in a field was determined and labeled as A.

The number of fields needed to cover 10000 cells was determined (10000/A) and labeled as B. The number of parasitized red blood cells in B fields was counted and recorded as C.

- If parasitized cells in 10,000 cells = C

  Then parasitized cells in 100 cells (percentage parasitemia) = $\frac{C \times 100}{10{,}000}$

The parasites on the slide were counted ensuring that the same field is not observed twice, by counting from one end of the selected area then moved the slide to another field as illustrated below.



CALCULATION OF GROWTH RATE (GR)

$$\text{Growth rate (GR)} = \left(\frac{\%\ \text{parasitaemia final}}{\%\ \text{parasitaemia initial}}\ \text{X dilution}\right)\ 2/\text{days}$$

where:

$$\text{dilution (d)} = \frac{\text{Parasitaemia new}}{\text{Parasitaemia old}}\ \text{X}\ \left(\text{GR}\right)^{\text{days}/2}$$

CALCULATION OF PERCENT PARASITAEMIA AND PERCENT SUPPRESSION OF PARASITAEMIA (PSP) FOR BOTH *IN VIVO* AND IMMUNOPROPHYLACTIC STUDIES

a. Calculation of percent suppression of parasitaemia for the ethyl acetate extract against P. berghei infected BALB/c mice.

$$\%\ \text{Suppression of parasitaemia} = \left(\frac{\text{Parasitemia of negative control - Parasitemia of test}}{\text{Parasitemia of negative control}}\right) \times 100$$

$$\%\ \text{Suppression of parasitaemia (PC)} = \frac{(17.39-2.15)}{17.39} \times 100 = 87.64\%$$

$$\%\ \text{Suppression of parasitaemia (500)} = \frac{(17.39-3.18)}{17.39} \times 100 = 81.71\%$$

$$\%\ \text{Suppression of parasitaemia (250)} = \frac{(17.39-7.26)}{17.39} \times 100 = 58.25\%$$

$$\%\ \text{Suppression of parasitaemia (100)} = \frac{(17.39-6.64)}{17.39} \times 100 = 61.82\%$$

b. Calculation of percent suppression of parasitaemia against then methanol extract of *C. macrostachyus*

$$\% \text{ Suppression of parasitaemia} = \frac{(\text{Parasitemia of NC} - \text{Parasitemia of test})}{\text{Parasitemia of NC}} \times 100$$

$$\% \text{ Suppression of parasitaemia (PC)} = \frac{(18.33 - 0.51)}{18.33} \times 100 = 97.22\%$$

$$\% \text{ Suppression of parasitaemia (500)} = \frac{(18.33 - 5.84)}{18.33} \times 100 = 68.14\%$$

$$\% \text{ Suppression of parasitaemia (250)} = \frac{(18.33 - 12.17)}{18.33} \times 100 = 33.61\%$$

$$\% \text{ Suppression of parasitaemia (100)} = \frac{(18.33 - 13.30)}{18.33} \times 100 = 27.44\%$$

Calculation of percent suppression of parasitaemia for the aqueous extract experiment.

$$\% \text{ Suppression of parasitaemia} = \frac{(\text{Parasitemia of NC} - \text{Parasitemia of test})}{\text{Parasitemia of NC}} \times 100$$

$$\% \text{ Suppression of parasitaemia (PC)} = \frac{(18.72 - 0.15)}{18.72} \times 100 = 99.20\%$$

$$\% \text{ Suppression of parasitaemia (500)} = \frac{(18.72 - 5.27)}{18.72} \times 100 = 71.85\%$$

$$\% \text{ Suppression of parasitaemia (250)} = \frac{(18.72 - 10.44)}{18.72} \times 100 = 44.23\%$$

$$\% \text{ Suppression of parasitaemia (100)} = \frac{(18.72 - 14.16)}{18.72} \times 100 = 24.36\%$$

Calculation of percent suppression of parasitaemia of the butanol extract experiment.

$$\% \text{ Suppression of parasitaemia} = \frac{(\text{Parasitemia of NC} - \text{Parasitemia of test})}{\text{Parasitemia of NC}} \times 100$$

% Suppression of parasitaemia (PC) $= \frac{(18.2-0)}{18.2} \times 100 = 100\%$

% Suppression of parasitaemia (500) $= \frac{(18.2-3.56)}{18.2} \times 100 = 80.44\%$

% Suppression of parasitaemia (250) $= \frac{(18.2-7.16)}{18.2} \times 100 = 60.66\%$

% Suppression of parasitaemia (100) $= \frac{(18.2-4.97)}{18.2} \times 100 = 72.69\%$

Calculation of percent suppression of parasitaemia for immunoprophylaxis using the ethyl acetate extract of *C. macrostachyus.*

% Suppression of parasitaemia = $\frac{\text{Parasitemia of NC - Parasitemia of test}}{\text{Parasitemia of NC}} \times 100$

% Suppression of parasitaemia (PC) $= \frac{(17.36-0.2)}{17.36} \times 100 = 98.85\%$

% Suppression of parasitaemia (500) $= \frac{(17.36-0.02)}{17.36} \times 100 = 99.88\%$

% Suppression of parasitaemia (250) $= \frac{(17.36-1.23)}{17.36} \times 100 = 92.91\%$

% Suppression of parasitaemia (100) $= \frac{(17.36-4.51)}{17.36} \times 100 = 74.02\%$

Calculation of standard deviation and standard error for the immunoprophylactic experiment with the ethyl acetate extract.

Calculation of percent suppression of parasitaemia for immunoprophylaxis using the aqueous extract of *C. macrostachyus.*

% Suppression of parasitaemia = $\frac{\text{Parasitemia of NC - Parasitemia of test}}{\text{Parasitemia of NC}} \times 100$

% Suppression of parasitaemia (PC) $= \frac{(17.19-2.54)}{19.19} \times 100 = 85.32\%$

% Suppression of parasitaemia (500) = $\frac{(17.19 - 4.16)}{17.19} \times 100 = 75.78\%$

% Suppression of parasitaemia (250) = $\frac{(17.19 - 11.35)}{17.19} \times 100 = 33.97\%$

% Suppression of parasitaemia (100) = $\frac{(17.19 - 16.41)}{17.19} \times 100 = 4.54\%$

Calculation of percent suppression for the methanol extract in the immunoprophylactic experiment.

% Suppression of parasitaemia = $\frac{\text{(Parasitemia of NC - Parasitemia of test)}}{\text{Parasitemia of NC}} \times 100$

% Suppression of parasitaemia (PC) = $\frac{(16.1 - 0.24)}{16.1} \times 100 = 98.51\%$

% Suppression of parasitaemia (500) = $\frac{(16.1 - 2.73)}{16.1} \times 100 = 83.04\%$

% Suppression of parasitaemia (250) = $\frac{(16.1 - 5.66)}{16.1} \times 100 = 64.84\%$

% Suppression of parasitaemia (100) = $\frac{(16.1 - 6.94)}{16.1} \times 100 = 56.89\%$

Calculation of standard deviation and standard error

Calculation of percent suppression of parasitaemia for the butanol extract in BALB/c mice.

% Suppression of parasitaemia = $\frac{\text{(Parasitemia of NC - Parasitemia of test)}}{\text{Parasitemia of NC}} \times 100$

% Suppression of parasitaemia (PC) = $\frac{(16.77 - 0.1)}{16.77} \times 100 = 99.94\%$

% Suppression of parasitaemia (500) = $\frac{(16.77 - 1.33)}{16.77} \times 100 = 92.07\%$

% Suppression of parasitaemia (250) = $\frac{(16.77 - 2.74)}{16.77} \times 100 = 83.66\%$

% Suppression of parasitaemia (100) = $\frac{(16.77 - 2.41)}{16.77} \times 100 = 85.63\%$

Calculation of standard deviation and standard error for the immunoprophylaxis experiment using the Butanol extract in BALB/c mice.