ISOLATION OF BIOACTIVE COMPOUNDS FROM SELECTED NIGERIAN MEDICINAL PLANTS FOR MANAGEMENT OF LETROZOLE-INDUCED POLYCYSTIC OVARIAN SYNDROME IN RATS

BY

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ABSTRACT

Polycystic Ovary Syndrome (PCOS) is an endocrine disorder with a global prevalence of 5-10% among women of reproductive age. Women with PCOS have a 2.7-fold increased risk of developing ovarian and cervical cancers. Ethnobotanical survey revealed that plants including *Kigelia africana* (Lam.) Benth. (KA), *Basella alba* L. (BA), *Tetracera potatoria* G. Don (TP) and *Mormodica charantia* L. (MC) are used for treatment of PCOS in southwestern Nigeria. However, there is no reported scientific evidence to validate these claims. This study was, therefore, designed to investigate the effect of the four plants in alleviating polycystic ovary conditions in rats and the associated risk of ovarian and cervical cancers.

Letrozole (1 mg/kg) was used for the induction of PCOS in thirty female albino rats (180-200 g, n=5). Methanol leaf extracts of KA, BA, TP and MC (100 mg/kg *b.w.*) and Clomiphene citrate (1 mg/kg *b.w.*, standard drug) were administered for 15 days. Histopathological evaluation of the ovaries was done microscopically. Luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol were measured using ELISA. Extracts of the most active plants, KA (FHI-111350) and TP (IFE-17794) were successively partitioned into *n*-hexane, dichloromethane and ethyl acetate fractions, and screened for PCOS inhibitory activity. Compounds were isolated from the active fractions using chromatographic techniques. Structures of isolated compounds were elucidated using spectroscopic techniques. Anti-proliferative effect of fractions, isolated compounds and derivatised cinnamic acid analogues were determined on cervix adenocarcinoma (HeLa) and Chinese Hamster ovarian (CHO 1) cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Doxorubicin was used as standard. Data were analysed using one-way ANOVA followed by Student's t-test at $\alpha_{0.05}$.

Animals treated with KA showed normal ovarian stroma with moderate fibroblastic tissues. The levels of FSH in KA, BA, TP and MC treated groups were 0.96±0.08, 1.10±0.23, 0.81±0.04 and 0.69±0.01 mIU/mL, respectively compared to control group (0.93±0.19 mIU/mL). The TP (0.19±0.05 mIU/mL) significantly reduced the level of LH compared with the control group (0.22±0.0l mIU/mL). Hexane and ethyl acetate fractions displayed selective moderate inhibitory effect (IC_{50} = 5.3±1.10 µg/mL) on CHO 1 cell line compared to doxorubicin (IC_{50} = 0.8±0.01 µg/mL). Compounds 3-(3, 4-dimethoxyphenyl) acrylic acid **(1)**, sitosterol **(2)**, methyl 3-(3,4-dihydroxyphenyl) acrylate **(3)**, 2, 3-dihydro-5-(hydroxymethyl) furan-2, 3, 4-triol **(4)**, *p*-coumaric acid **(5)** and caffeic acid **(6)** were isolated from KA, while apigenin **(7)** was isolated from TP.

Compound **1** displayed moderate anti-proliferative activity against HeLa cell line (IC_{50} = 33.5±0.60 µg/mL). Compound **7** inhibited proliferation of HeLa cell line (IC_{50} = 6.2 µg/mL) and had moderate inhibitory effect on CHO l cell line (IC_{50} = 22.2 µg/mL). Derivatised compound N'-(2, 6-dimethoxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (**8**) inhibited both CHO l (IC_{50} = l8.4±4.l µg/mL) and HeLa (IC_{50} = 22.4±0.4 µg/mL) cells.

The extracts of *Kigelia africana* and *Tetracera potatoria* had inhibitory effects on polycystic ovary condition, irregular oestrual cycle and hormonal imbalance in female albino rats. The phenylpropanoids and derivatised analogues exhibited antiproliferative effect on ovarian and cervical cancer cell lines, which could contribute to their use for management of polycystic ovary syndrome.

Keywords: Polycystic ovary syndrome, Leutenizing hormone, Follicle stimulating hormone, Antiproliferative, Bioactive compounds

Word count: 495

DEDICATION

To our LORD JESUS CHRIST, the Saviour of the world

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Thanks to you all!

CERTIFICATION

This is to certify that this project was carried out under my supervision by **Akingbolabo Daniel OGUNLAKIN** in the Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria.

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LIST OF ABBREVIATIONS

PCOS	Polycystic ovary syndrome
CC	Column chromatography
TLC	Thin layer chromatography
ASRM	American Society for Reproductive Medicine
LH	Luteinizing hormone
PCO	Polycystic ovary
NIH	National Institutes of Health
CVD	Cardiovascular disease
FSH	Follicle stimulating hormones
ESHRE	European Society for Human Reproduction and Embryology
AES	Androgen Excess and PCOS Society
T2DM	type II diabetes
GnRH	hypothalamic gonadotrophin releasing hormone
SOD	superoxide dismutase
CAT	catalase
GPx	gluthathione peroxidase
d-ALA-D	d-aminolevulinate dehydratase
CHO cells	Chinese Hamster ovarian cells
HCG	human chorionic gonadotropin
LGA	local government area
FGDs	Focus group discussions
UMi	Use mention index
R_f	Retardation factor
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
DPPH	l, l-diphenyl-2-picryl-hydrazyl-hydrate
DCM	Dichloromethane
prep-TLC	Preparative thin layer chromatography
EIMS	Electron impact mass spectrometry
NMR	Nuclear magnetic resonance
COSY	Correlated spectroscopy
NOESY	Nuclear over Hauser effect spectroscopy
DEPT	Distortionless enhancement by polarisation transfer

HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum correlation
UV	Ultraviolet
IR	Infrared
SEM	Standard error of mean
DHEA	Dehydroepiandrosterone
NIH	National Institutes of Health
CMC	Carboxymethyl cellulose
MDA	Malondialdehyde

APPENDICES

CHAPTER ONE

1.0 INTRODUCTION

l.l Reproductive health problems in premenopausal women

Reproductive health complications increase indisposition and death among reproductive women globally (United Nations, 2012). There are several reports on the effect of menstrual morbidity on women health status. Menstrual complications result in inferiority as these conditions affect education and the health status of women in low-income countries (Tjon, 2007). Affected women, most times, are faced with isolation or neglect from their family members (Tjon, 2007). However, most discussions are focused on male reproductive health issues, such as erectile dysfunction, neglecting the reproductive problems which affect women of reproductive age. The assessment and management of reproductive health conditions in women are often neglected, thus, limiting clinical studies on this subject (Ozcan and Sahin, 2009).

Women, constituting 5l% of Africa's population, still remain the pillars of Africa's economic development (Odii, 2003). Unfortunately, women are plagued by many health challenges including infertility. This is evident in women having difficulty in conceiving after l2 months of continuous sexual intercourse excluding the use of contraceptives. Women with abnormal menstrual cycle are at higher risk of infertility (Wise *et al.,* 2011). About l5% of reproductive age couples are affected by infertility globally (WHO, 2010). In Sub-Saharan Africa, more than 30% of premenopausal women are affected by secondary infertility. Menstrual disorder is an important risk marker for reproductive complications, especially polycystic ovary syndrome (PCOS), which reflects underlying endocrine disorders, and cancer (Grant, 20l0).

l.2 Polycystic ovary syndrome (PCOS)

The first modern medical report on PCOS, presented by Stein and Leventhal (l935), describes amenorrhea, hirsutism and polycystic ovaries as major manifestation of PCOS in seven premenopausal women. Polycystic ovary syndrome, a heterogeneous endocrine disorder in premenopausal women, is connected with several health complications such as chronic menstrual disorder, polycystic ovary morphology (Figure l.2.l) and infertility/sub fertility. Acanthosis nigricans, abdominal obesity, infrequent or absent menses and signs of oversecretion of androgen (hyperandrogenism) manifesting as resistance to insulin action and seborrhea or acne are clinical manifestations of this disorder (Bozdag *et al.*, 20l6). The main manifestations of PCOS include virilisation, obesity, dark patches at the nape of the neck and armpit, alopecia and hirsutism (Figure l.2.2). Women affected by PCOS have great chance of

developing gynaecological cancers, dyslipidemia, type 2 diabetes mellitus, cardiovascular disorders as well as high blood pressure (Shaw *et al.,* 2008).

Globally, about 7% of entire female populace is affected by PCOS. The inclusion of ultrasound as diagnostic tool revealed an enlarged ovary in 22% of women in reproductive years. Some of the accepted diagnostic yardsticks for detecting this disorder in women are National Institutes of Health (NIH) criteria (Zawadzki and Dunaif, l992), 2003 Rotterdam Consensus raised by European Society of Human Reproduction and Embryology (ESHRE), American Society for Reproductive Medicine (ASRM) and Androgen Excess Society (AES) criteria (Rotterdam, 2004). Generally, scientific features such as irregular menstrual phase, weight gain, infertility and confirmation of polycystic ovaries formed the benchmarks for PCOS diagnosis prior incorporation of biochemical markers, for instance luteinizing hormone (LH), follicle stimulating hormone (FSH) and increased testosterone levels in the blood, as tools for PCOS diagnosis. The conventional analytical criteria for PCOS are summarised in Table l.2.l Women affected by PCOS experience abnormal menstrual flow, miscarriages and series of complications during pregnancy (Zawadzki and Dunaif, l992).

Several pathogenesis have been proposed by researchers in the course of investigating and explaining PCOS. Sudden rise in secretion of gonadotropin–releasing hormone (which causes upsurge in secretion of LH), change in insulin secretion and exploit (which causes hyperinsulinemia), associated insulin resistance and reduction of androgen production (which support increase in production of androgen in the ovaries) are the common pathogenesis of PCOS (Tsilchorozidou *et al.,* 2004). However, high level of LH in the body system of affected women still remains the undisputable hallmark of PCOS pathogenesis. Increase of pulse occurrence (frequency) as well as amplitude of LH, evident in affected women, intensify the regularity of gonadotropin releasing hormone (GnRH) in the pituitary gland. Luteinizing hormone's hypersecretion favour the production of androgen by the ovarian thecal cells. Therefore, concentration of androstenedione, testosterone and Dehydroepiandrosterone sulphate (DHEA-S) (the precursor of androgen) in the blood of affected women form the principal endocrine features of PCOS. However, hyperandrogenism have been linked to congenital flaws in ovarian steroidogenesis which is seen as response to hyperinsulinemia and increased stimulation of LH (Erhmann, 2005).

Oxidative stress, arising from excessive production of free radicals coupled with reduced concentration of endogenous antioxidants, could damage the chemical structure and functions of some biomolecules in the body. Oxidative damage contributes to pathogenesis of several inflammation-related diseases. Recent reports confirm the association between oxidative stress and PCOS (Amini *et al.*, 2015; Xu *et al.,* 20l6).

Lifestyle may affect menstrual function via alteration in hormonal levels, which in turn affects ovulation. Access to safe and active cure for infertility is an effective measure in improving reproductive health of women. In the Western countries, where menstrual complications and infertility are often presented as medical condition, treatment includes hormonal therapy (including the use of contraceptives) or non-steroidal anti-inflammatory medications. Some of these medical interventions, aside being beyond the reach of many rural women, come with serious side effects. In low-income countries where medical treatments are sometimes unavailable or unaffordable - especially in the rural areas, the affected women never sought any medical help until the condition becomes severe. Women in these tropical countries, which are blessed with abundant flora, prefer traditional medicines for their health challenges either as cure for menstrual disorders or as family planning agents (Bates and Legro, 20l3; Sonibare and Ayoola, 20l5).

There is association between PCOS and gynaecological malignancies in women. Cancers associated with chronic hormone stimulation among women of reproductive age are endometrial, ovarian and breast cancers. Among PCOS patients, prolonged estrogen stimulation probably result in endometrial malignancy. The connection in-between ovarian cancer and PCOS has been reported (Gottschau *et al.,* 20l5).

l.2.l Treatment and/or management of PCOS

Currently, many therapies are in use to manage PCOS conditions and to induce ovulation. One of the therapies suggests that, the use of oral contraceptive diminishes the prevalence of malignancies in PCOS women. Thus, women with PCOS are commonly treated using oral contraceptives. All these therapies have been reported to have severe effects, which include joint and muscle ache, arthritis and mood swing. Furthermore, due to their potential health hazards, low solubility as well as moderate antioxidant potential, limitations have been placed on their use (Badawy *et al.,* 2011). This necessitates the search for medicinal plants, as natural antioxidants with better solubility, efficacy and safety, which can be used to treat or manage PCOS in reproductive women without limiting their chances of conception.

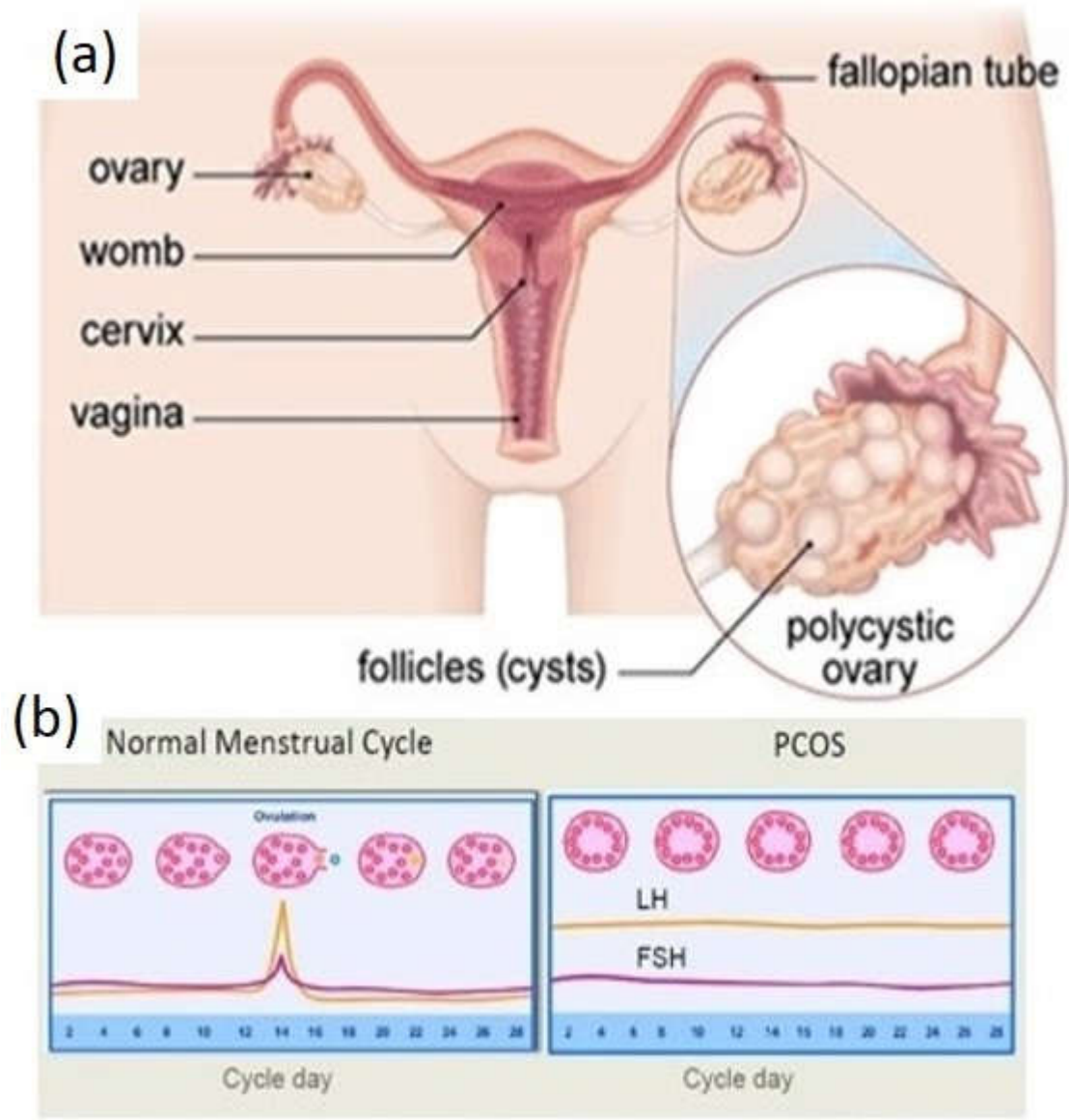


Figure l.2.1: Polycystic ovary with numerous irregular follicles

(a) Morphology of polycystic ovary and (b) level of LH and FSH in normal and polycystic ovary during mestrual cycle

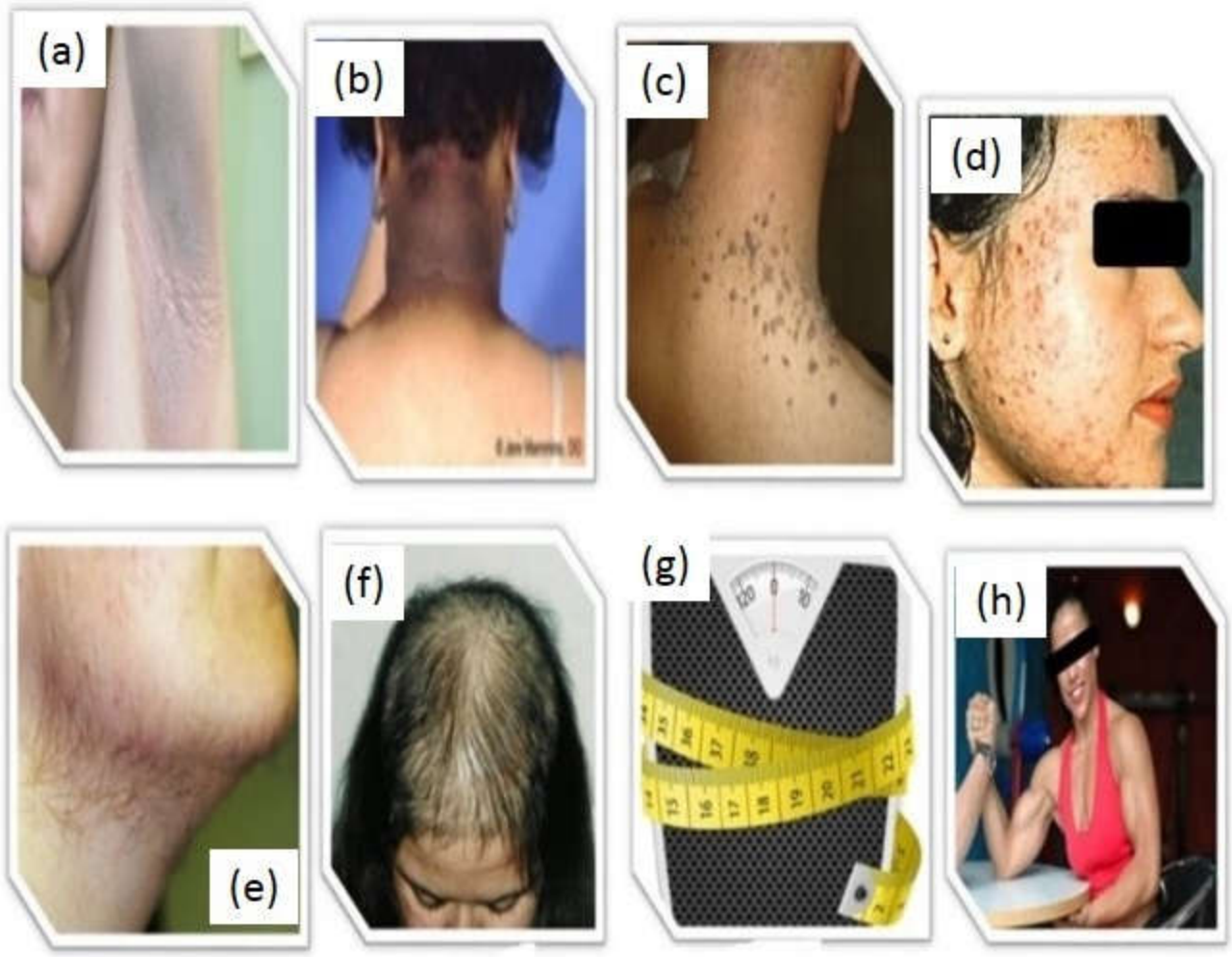


Figure l.2.2: Manifestation of PCOS in women

(a) Dark patches at the armpit, (b) dark patches at the nape of the neck, (c) skin tags, (d) acne, (e) hirsutism, (f) alopecia, (g) obesity and (h) virilisation

Table l.2.l: The old and modern benchmarks for diagnosing PCOS in premenopausal women

NIH Criteria (l990)	Rotterdam Criteria (2003)
i) Oligomenorrhea or amenorrhea ii) Hyperandrogenemia excluding other related disorders	(i) Oligo- and/or anovulation (ii) Clinical and/or biochemical signs of hyperandrogenism (iii) Numerous cysts on the ovaries upon ultrasonography, excluding other related disorders
These two criteria must manifest.	At least two out of these three criteria must manifest.
Other diagnostic markers are total testosterone, free testosterone, androstenedione and DHEA level in the blood sample.	Other diagnostic markers are free androgen index, total testosterone and DHEA level in the blood sample

l.3 Medicinal plants and polycystic ovary syndrome (PCOS)

Fossil records reveal that men, from primordial times through the middle Paleolithic age, have engaged in the use of botanicals and have discovered a lot of medicinal plants in their time. The Neanderthal man's sepulchre, dated 60,000 years, validated the use of medicinal plants among early men. The 4000 years old document of medicinal plants, the Sumerian clay tablet, documents the herbal preparations used for managing and/or treating several illnesses. Papyrus, another document of about 3500 years, contains list of therapeutic plants used all through the period of early civilisation era in Egypt. The use of medicinal plants is also documented by Ancient Chinese. Therefore, plants has always remained the primary source of remedies for several health challenges. Most medicinal plants are processed either by pan-frying, drenched in vinegar or wine before they can be used in order to increase their efficacy. The utilisation of botanicals for treating numerous ailments has greatly contributed to the documentation of folkloric prescriptions for research purpose (Taylor, 2000).

The quantity of higher plant species globally is about 250,000 (lower level is at 2l5, 000 and higher level is as high as 500,000) out of which few are under scientific investigation. The biological activities of about 6% of these medicinal plants have been investigated and compounds have been isolated from about l5%. The availability and advancement of high through put screening methods will definitely encourage scientists to investigate the medicinal importance and phytochemicals of these plants (Arash *et al.,* 20l0).

The quest to understand and document the knowledge of the traditional healing systems has suddenly revived the interest in herbal medicines. Concern in the study of natural products chemistry has recently geared up due to several factors. The numerous therapeutic needs for bioactive molecules with little or no adverse effect can only be met by investigating the pharmacology of secondary metabolites from medicinal plants. Hence, there is urgent need for advancing the procedures used in identifying biologically active natural products and upgrading of methods of isolation, purification and characterisation of these active components (Clark, l996).

l.4. Plants selected for this study

The medicinal plants selected for this study are *Kigelia africana* (Lam.) Benth. (Bignoniaceae) frruit and *Tetracera potatoria* Afzel. ex G. Don (Dilleniaceae) leaf. Other medicinal plants investigated during the course of this research are *Newbouldia laevis* (Beauv.) Seem. ex Bureau (Bignoniaceae), *Basella alba* L. (Basellaceae), *Momordica charantia* L. (Cucurbitaceae) leaves and *Lagenaria breviflora* (Benth.) Roberty (Cucurbitaceae) fruit. The presence of active compounds in *K. africana* justifies its usage as medication for wounds and infections. Extracts of *Kigelia* plant have been reported to have strong mollusicidal as well as antidiabetic activities (Nyarko *et al.,* 2005). This plant is very useful in West African folkloric health system. The leaves are active in the management of stomach discomfort, kidney infections and snakebite, stem and branches are for sores, serpent bite, rheumatism, abdominal and kidney ailments, while fruits are for constipation, gynaecological problems and haemorrhoids (Jackson and Beckette, 20l2). The red and yellow dye produced by boiling fruits and roots, respectively, are used in cloth production. The plant is also a raw material for cosmetics in sun block emulsions and sun ointments. The nutritional composition of *K. africana* leaves is comparable with spinach and other common vegetables. However, little is known of *Tetracera potatoria.* The folkloric uses of *T. potatoria* include treatment of backache, haemorrhoids, diabetes, jaundice, cough and toothache (Betti, 2004). This plant was mentioned in the ethnobotanical survey conducted in the course of this research for treatment of gynaecological disorders, especially menstrual disorders and associated stomach discomfort among premenopausal women in Nigeria.

Newbouldia laevis (Beauv.) Seem. ex Bureau, African border tree, is known in Nigeria as 'Aduruku', Ogirisi" and 'Akoko' among Hausa, Igbo and Yoruba, respectively. In Africa, *N. leavis* is recognised for the management of malaria and numerous illness including stomach pains, septic wounds, coughs, diarrhoea, sexually communicated infections, tooth pain, eye problems, breast malignancy, toothache and difficulty in bowel movement. This plant also has anticonvulsant, antimicrobial and anti-tumour activities (Akerele *et al.,* 2011).

Basella alba L. (Basellaceae) is a perennial vine with various names such as Malabar spinach, creeping spinach and Red vine spinach. It is known as Amunututu among the Yoruba ethnic group of the southwestern Nigeria where it is eaten as vegetable. It is generally found in the humid areas around the world as vigorous soft-stemmed climbing vine, growing up to l0 metre long with characteristic broad heart-shaped and thick semi-succulent green leaves (5-l2 cm wide). This plant is consumed as good source of vitamins A, C, Iron and Calcium. The juice of its leaf, in Nepal, is used to treat flu, its pastes

to treat boils, cooked leaves together with stems are used as laxatives, while fresh flowers are local remedy for toxins (Duke and Ayensu, l985).

Lagenaria breviflora (Benth.) Roberty (Cucurbitaceae) is a perennial climber which forms the jungle coverings in humid part of Africa. The plant has characteristic scabrid and sandpapery leaves, while the fruits (ovoid to 9 cm long) are dark green with creamy blotches. The fruit is highly utilised in West Africa traditionally, for treatment of gastrointestinal problems, measles and microbial infections (Tomori *et al.,* 2007). It is a unique West African fruit, which is known in Nigeria for its characteristic antibacterial and antiviral activities. The phytochemical analysis of *L. breviflora* revealed various chemical constituents such as saponins and phenolics (Elujoba *et al.,* l99l).

Momordica charantia L. (Cucurbitaceae), known as karela or bitter melon is found in humid regions of the East Africa and Asia where it is cultivated for nutritional and medicinal purposes. This perennial climber has characteristic gourd-shaped fruit. The unripe and ripe fruits are green and yellowish, respectively with characteristic bitter taste, which is more prominent in the ripe fruits. *Momordica charantia* acts as antioxidant agent in the management of ailments including abnormal glucose level, inflammation, bacterial infections as well as cancer. There are several clinical records on *M. charantia* extracts' efficacy (primarily from the fruit) against diabetes, dyslipidemia, microbial infections and cancer (Budrat and Shotipruk, 2009).

1.5 Justification for the research

The utilisation of therapeutic flora in the management of challenges facing reproductive wellbeing among womenfolk have been documented worldwide (Zhao, 2011). Oxidative stress, anovulation and polycystic ovary conditions are some of the reproductive health complications affecting reproductive women in Africa (Dumesic and Lobo, 2013). High prevalence of gynaecological cancers among PCOS women has been reported recently. Several ethnobotanical studies conducted in Nigeria have documented numerous medicinal plants for treatment of reproductive health problems. The herbal treatment, mostly administered in form of powder, tea, tonic or tincture, has efficiently supported the body to readjust the menstrual cycle (Grant, 2010; Oyelami *et al*., 2012; Fasola, 2015; Nduche *et al*., 2015; Gadducci *et al.*, 20l6).

Although medicinal plants are effective in restoring the menstrual cycle and associated metabolic disorders among women with PCOS, thorough analysis and study on the effect of these plants on hormonal imbalance, polycystic ovary conditions and accompanying threat of gynaecological malignancies among PCOS patients have not been extensively investigated. The overall target of this

study is to examine the effects of some Nigerian therapeutic plants on polycystic ovary syndrome. Systematic isolation and documentation of bioactive compounds exerting beneficial effects in the cure of PCOS from selected medicinal plants will fast track drug discovery efforts. These drug candidates are open to synthetic optimisation in the future and may as well be suitable for clinical development. At the very least, this work has within it the potential to reveal novel insight into derivatisation of the active compounds originated from medicinal plants for PCOS management. This approach is hinged on decreasing the toxicity and improving other essential features of the compounds such as yield, stability and bioavailability.

1.6 Research hypotheses

Some therapeutic plants mostly utilised in traditional medicine practices in Nigeria for treating PCOS are safe for human consumption and can normalise hormonal imbalance as well as polycystic ovary conditions in reproductive PCOS women. The secondary metabolites in these medicinal plants and derivatised analogues can prevent polycystic ovary conditions and gynaecological cancers among PCOS patients.

l.7 Aim and specific objectives of the study

The aim of this research is to isolate bioactive compound(s) from selected Nigerian medicinal plants (obtained from ethnobotanical survey) commonly used to manage PCOS and derivatised active compound analogues for the management of PCOS conditions.

The specific objectives of the study are to:

1. conduct ethnobotanical survey on therapeutic plants used by Traditional Medical Practitioners for the management of menstrual disorder and infertility
2. evaluate cytotoxicity and antioxidant activities of the selected plants
3. determine the efficacy of selected plants' extracts and fractions on PCOS conditions;
4. isolate and characterise bioactive compounds from the selected plants
5. determine the efficacy of isolated compounds
6. derivatise active compound analogues and determine their activities

CHAPTER TWO

2.0 LITERATURE REVIEW

2.l Female reproductive system

The female reproductive structure consisting of fallopian tubes, a pair of ovaries, uterus and vagina (Figure 2.l.1) is vital for the continuance of mammals' existence. This structure favours conception, establishment of embryo in the uterus, subsequent development of the embryo and delivery of the foetus. Reproductive challenges arise from attacks on the vital organs of the reproductive system. Fallopian tubes, uterus, ovary, cervix and vagina are sites for several diseases. Reproductive health complications increase morbidity and mortality rate among women of reproductive age worldwide (LaRosa *et al.*, 20l6). Even with the relevance of this structure in fertility and wellbeing of females, quite a little is documented of the molecular and cellular machineries that control its growth and functions. Most scientific discussions are focused on male reproductive health issues, neglecting the reproductive problems in women (Ozcan and Sahin, 2009).

Ovaries, approximately the size of almonds, are attached to sides of the uterus by a membrane in the abdominal cavity. The ovaries are attached to the tissue connecting ovaries to fallopian tube (fimbria). A longitudinal section of a healthy ovary reveals peripheral cortex and central medullary regions (Figure 2.1.2). The medulla, which is rich in blood vessels and nerves, form the stroma while the cortex, containing follicles, form the parenchyma. Cortex is the most crucial part of the reproductive system with the oocyte-containing follicles (Heffner and Schust, 20l0).

The stage of maturation of ovarian follicles is a function of their size and cellular composition of follicles as shown in Figure 2.1.3. Practically l–2.5 million follicles for each ovary are produced underneath the tunica albuginea in the course of histogenesis by the conclusion of the fifth intrauterine month while just 250,000–500,000 persist for each ovary after delivery. About 260 follicles in each ovary complete the cycle of maturation and are released as ovum in a normal cycling female. This estimation is for a healthy woman within l0–50 years of age, having normal ovulation (l3 times a year) without pregnancies. There is no ovulation during pregnancy and it has also been found to fluctuate in nursing mothers depending on genetics and regularity of estrous phase. The remaining follicles undergo degeneration (atresia) as shown in Figure 2.1.2. Several ova are lost to atresia during the sixth month of intrauterine period. Ovary also functions as endocrine organ. Estrogen with progesterone secretions occur in the ovary as a response to gonadotropin and paracrine signalling (Coutsoukis, 2007).

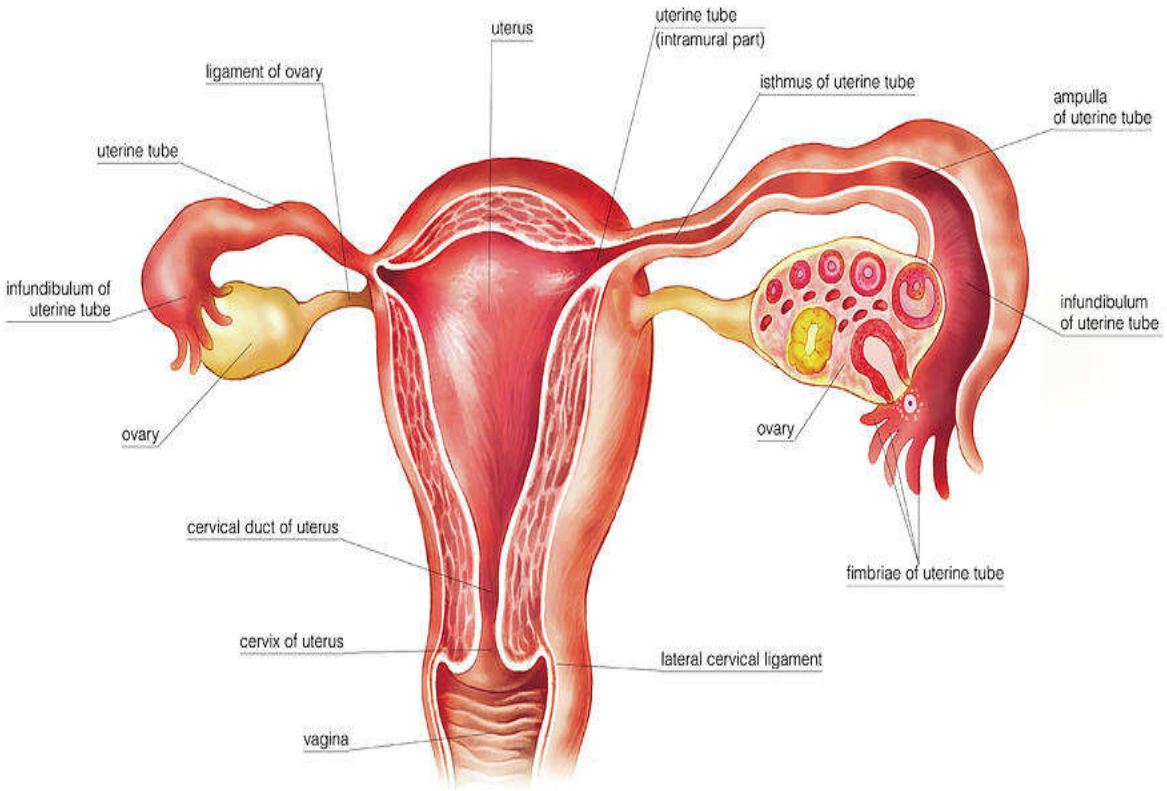


Figure 2.l.1: The female reproductive system
(https://pixels.com/featured/l4-female-genital-system-asklepios-medical-atlas.html)

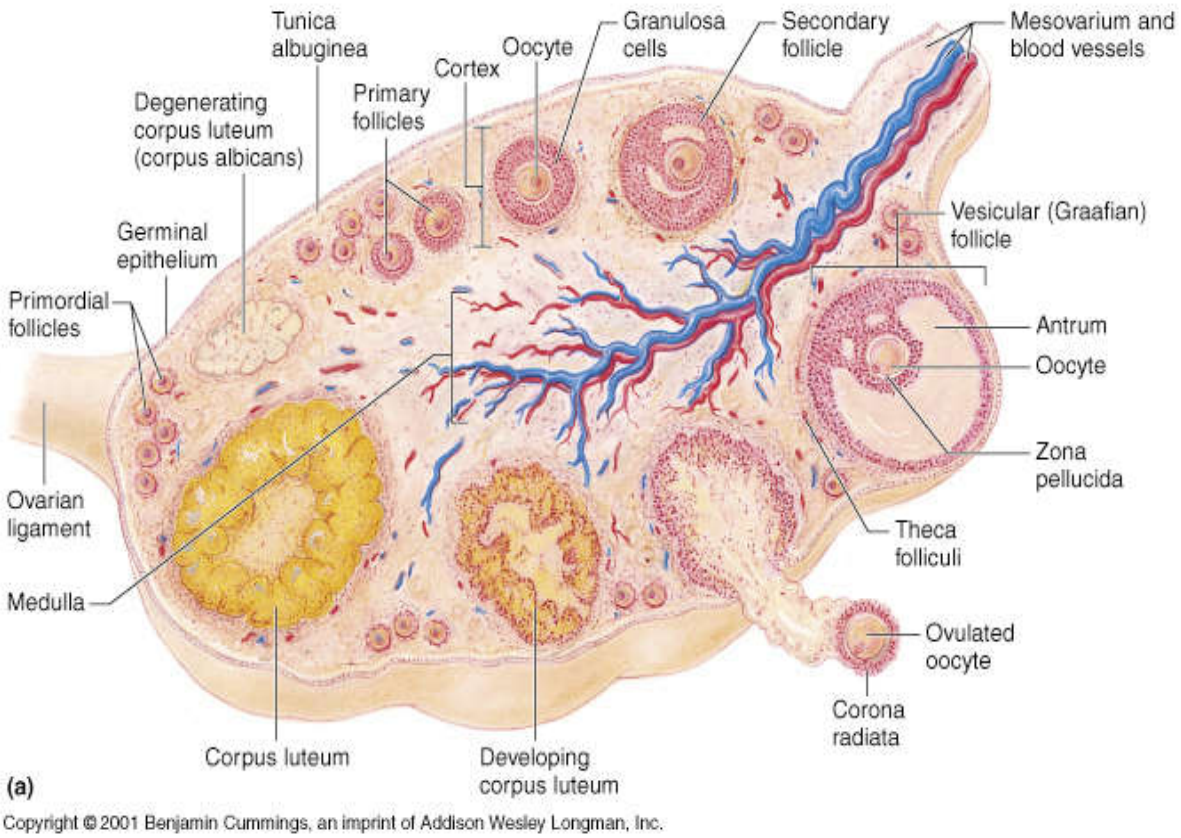


Figure 2.1.2: Schematic picture of the ovary showing emerging follicle as well as oocyte in the ovarian cortex (Donnez and Dolmans, 20l7)

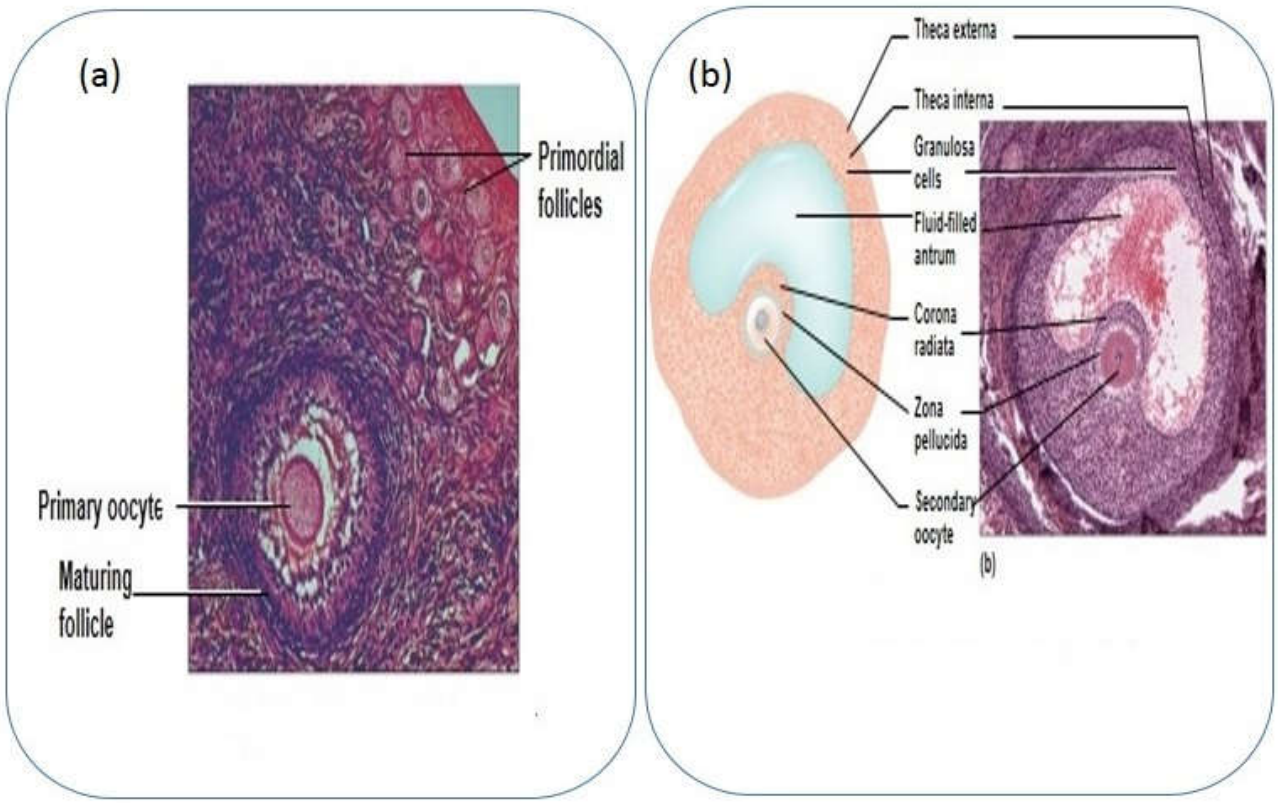


Figure 2.1.3: Features of (a) primary oocyte and (b) matured follicle (Butler and Santoro, 20ll)

2.2. Ovarian-hypothalamic-pituitary feedback loops and ovarian function

Gonadotropins cause the ovaries to induce morphological processes such as folliculogenesis (that is, the cyclic enlistment of follicles to generate a developed follicle meant for ovulation), corpus luteum development and ovarian steroid secretion. These processes occur monthly in rhythmic sequence, resulting in reproductive ovarian phases. Luteinizing hormone (LH) excites thecal and granulosa cells inside the follicle as well as cells of the luteal, which in turn regulate the synthesis and expression of estradiol l7β and progesterone in the ovary. However, the concentration and quality of hormone released is a function of follicle as well as the corpus luteum reputation in the ovaries. Feedback message concerning the ovaries and the hypothalamo-pituitary cycle is vital in regulating the secretion of hormones in the brain and pituitary gland, which are crucial for right functioning of the ovaries. The level of estradiol and progesterone play crucial role in determining the concentration of hormones secreted by ovary. This process is called feedback communications (Figure 2.2.1). In a negative feedback circle, secretion of steroid in the ovary normalises the hypothalamic-hypophyseal unit, which correct the secretion of GnRH and gonadotropin (Yamaji *et al.,* l972).

The inhibitory effect of estradiol-l7β on GnRH and gonadotropin has been reported (Yamaji *et al.,* l972). Little increase in the level of estradiol-l7β inhibits gonadotropins secretion. Therefore, FSH and LH concentrations occurring during follicles development strongly depend on changes in estradiol concentration associated with ripening of the follicle. Increase in circulatory levels of estradiol during follicular phase causes reduction in gonadotropin concentrations (Figure 2.2.2). Women with estradiol deficiency experience increase in LH and FSH circulatory level. This condition can be curtailed by physiologic amounts of estradiol. This results in express and constant downregulation of LH and FSH to the normal levels observed throughout menstrual cycle (Yen *et al.,* l972).

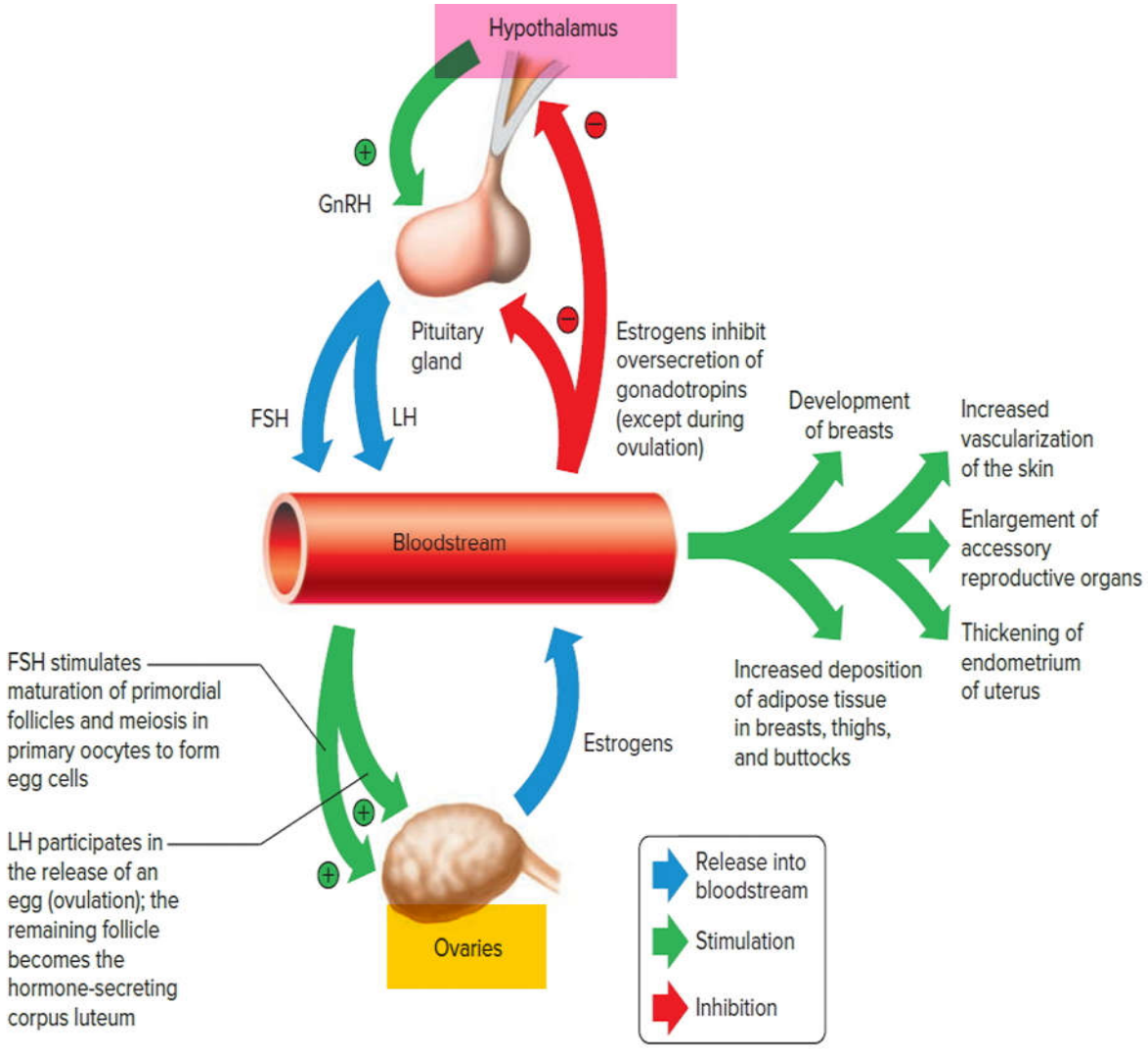


Figure 2.2.1: Schematic diagram highlighting importance of hypothalamic-pituitary-gonadal (HPG) axis (Butler and Santoro, 2011)

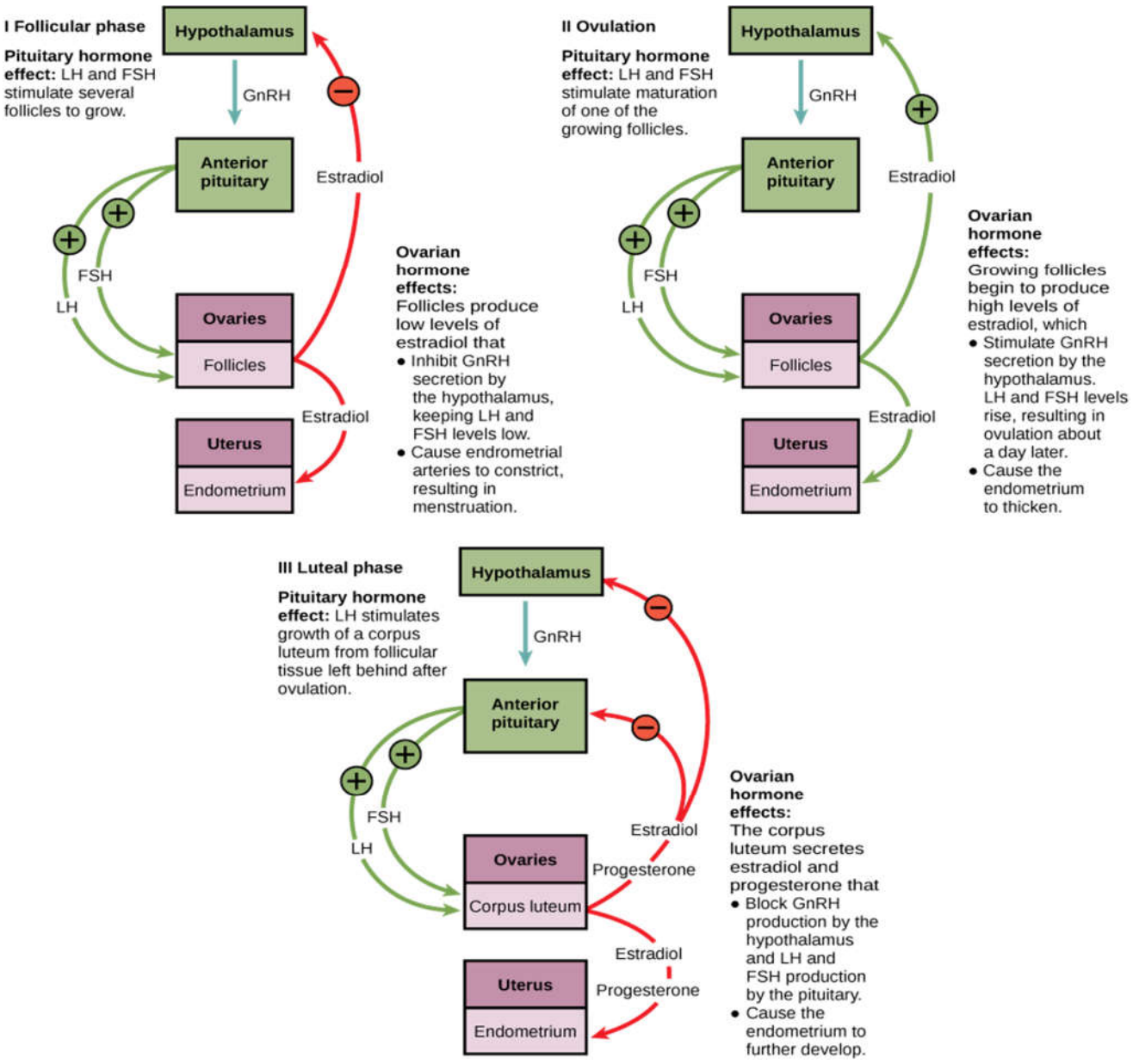


Figure 2.2.2: The ovarian cycle and pituitary hormones (Molnar and Gair, 2015)

2.2.l Causes of irregularities in ovarian-hypothalamic-pituitary feedback loops

2.2.1.1 Excessive extraglandular production of estrogen

In premenopausal women, most of the estrogens in the blood are derived from estradiol secretion in the ovarian follicle or corpus luteum and in adipose tissue as well as skin where peripheral conversion of androstenedione to estrone takes place. Inherited adrenal enlargement, Cushing's syndrome as well as androgen-enhanced malignancies originating from adrenal glands and ovaries among other pathological conditions favour increase in availability of estrogen precursor. This in turn stimulates excessive extraglandular production of estrogen (France and Jackson, l980), which results in abnormal feedback conditions.

2.2.l.2 Aging and obesity

Aging and obesity increase the conversion rate of androgen to estrogen. Excessive production of estrogen, which is not regulated by gonadotrophins (that is, acyclic), distort the original feedback route resulting into cyclic irregularities. This enhances overproduction of endogenous androgen, increase LH pulse incidence (occurrence) or LH amplitude (Apter *et al.,* l994).

2.2.l.3 Irregular secretion of gonadotropin

Quick response of hypothalamic GnRH pulse generator to negative feedback cycle of estradiol has been observed as manifestation of low secretion of gonadotropin in some PCOS affected women. The seasonal change in melatonin concentration regulates the response of the GnRH pulse producer (generator) towards the estradiol negative feedback, which affects the LH pulse pattern in menstrual cycle (Karsch *et al.,* l984). Furthermore, hindered estradiol production or inability of the hypothalamic-pituitary axis to respond to positive estrogen (which may happen all through the first pubertal phase) may cause interruption of LH level beyond the ovulation period. Early upsurge in estradiol or improvement in the sensitivity of hypothalamic-pituitary to estrogen stimulus possibly will encourage untimely LH surge, which can hinder at the same time terminate maturation process of the follicles (Ferin, l999).

2.2.l.4 Pituitary lesions

Pituitary lesions affect menstrual cycle. Trophic hormone deficiency and/or pituitary tumours could result in pituitary lesions. A characteristic pituitary injury takes place in Sheehan's syndrome. Symptoms

of defect in secretion of gonadotropin, thyroid, or adrenal hormone depend on the degree of trophic hormone shortage. The furthermost category of pituitary tumour is the prolactin-secreting adenoma triggering amenorrhea-galactorrhea syndrome in the menstrual phase (Molitch, l996). Hyperprolactinemia subdues the GnRH pulse generator as well as pulsatile discharge of sex hormones (LH and FSH) through unidentified mechanisms. Hypothalamic regulation of prolactin act as inhibitor while hypothalamic element called dopamine, a neurotransmitter, is accountable for regulating prolactin. Therefore, the irregularities in prolactin and associated pulsatile secretion of LH and FSH can be corrected through the use of dopamine agonists (Molitch, l996).

2.3 Polycystic ovary syndrome (PCOS)

The polycystic ovary syndrome (PCOS) remains the leading endocrine malady affecting womenfolk globally. It is associated with characteristic ovulatory dysfunction, over secretion of hyperandrogen and associated polycystic ovaries morphology. In l935, Irving Stein and Michael Leventhal reported the clinical, macroscopic and histological manifestations of PCOS. The nature of PCOS became obvious after the first report of the features of this syndrome by Stein and Leventhal (Stein and Leventhal, l935). These features could manifest as systemic as well as organ-specific abnormalities (Figure 2.3.1). Ovulatory and menstrual dysfunction, hyperandrogenemia and polycystic ovaries are the main features of PCOS. Menstrual dysfunctions, such as oligo-amenorrhea and/or abnormal uterine bleeding are classified as clinical manifestations of PCOS in women **(**Azziz *et al.,* 2006).

There is a relationship between PCOS, excessive weight gain and cardiovascular diseases (CVD). These life threatening factors are more predominant in women nowadays. The high prevalence of PCOS has recently attracted attention of the public. Identification and management of PCOS cost the USA healthcare system about $4 billion every year (Azziz *et al.,* 2005). Therefore, research into pathophysiology as well as lifelong effects of PCOS is of paramount significance in averting the complications which may affect the wellbeing of many PCOS patients in the nearest future.

2.3.l Prevalence of Polycystic ovary syndrome (PCOS)

In several scientific researches, the occurrence of PCOS among reproductive women is assessed to be 5- 20%. The variation in the prevalence obtained across the globe could be linked to the criteria employed and ethnicity of population used for each study (Azziz *et al.,* 2004). The prevalence rate of each polycystic ovary syndrome's manifestation is presented in Figure 2.3.1. In a report on PCOS prevalence in 20l7, ethnicity and diagnostic criteria were identified as variants in determination of PCOS

prevalence. The prevalence of PCOS in Chinese women (using 2003 Rotterdam criterion) is 5.6% while it is 5.5% among Caucasians (using l990 NIH criterion). The effect of the criteria employed in diagnosing PCOS on prevalence became evident in the Middle Eastern region of the world where various prevalence rates were reported using different diagnostic criteria. With l990 NIH criteria, the prevalence of PCOS in this region is 6.l%, 2003 Rotterdam criteria revealed prevalence rate of l6.0% while prevalence of l2.6% was reported using 2006 AES criteria. Among black reproductive women, prevalence of 6.l% (using l990 NIH criteria) has been reported (Ding *et al.,* 20l7). Similar study conducted in Northern Finland estimated 3.4% prevalence of PCOS based on the response of women (having l966 as year of birth) to existence of hirsutism as well as oligo/amenorrhea. In Southeastern Nigeria, presence of numerous ovarian cysts detected using ultrasonography revealed prevalence rate of l8.l% (Ugwu *et al.,* 20l3).

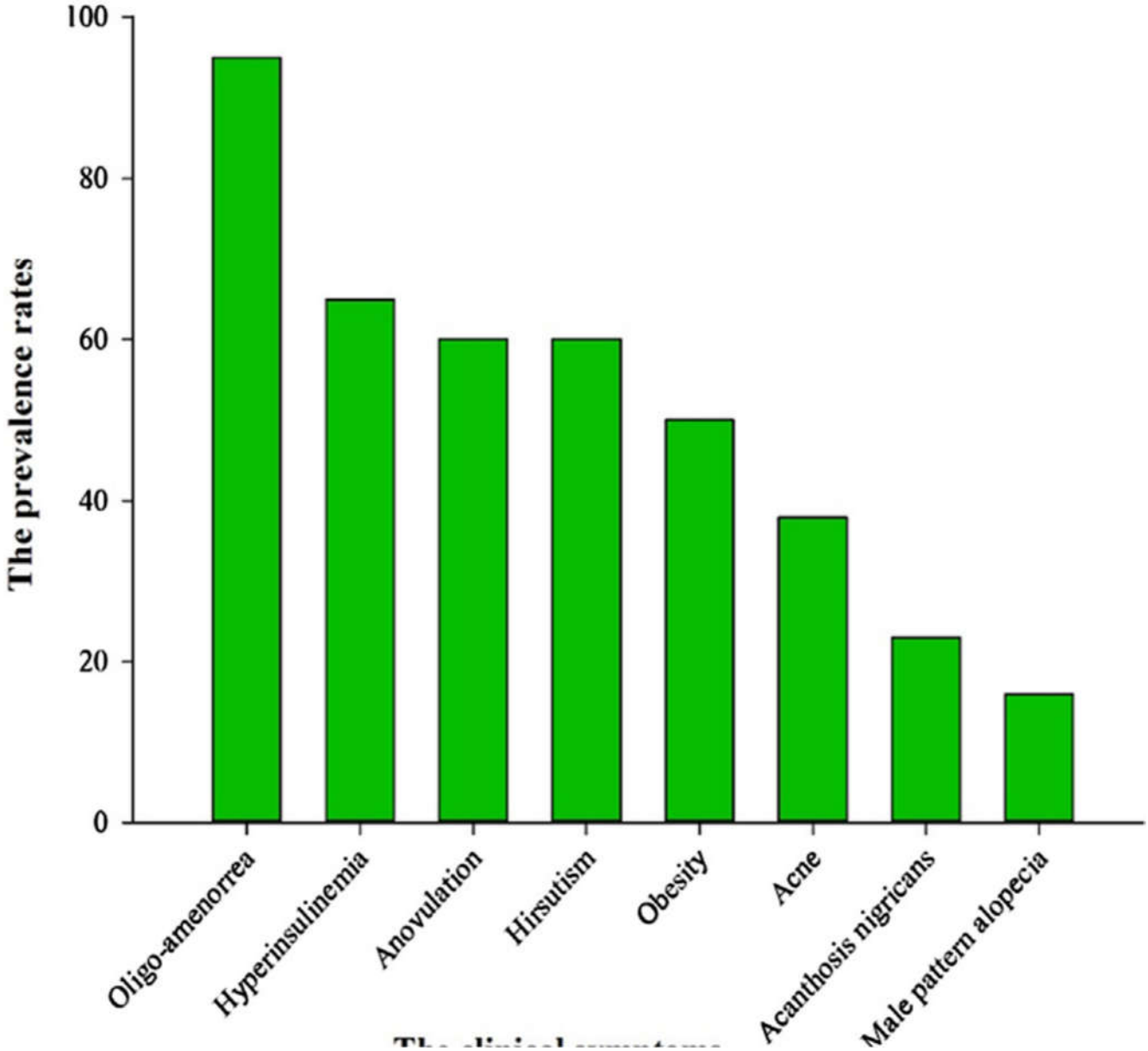


Figure 2.3.1: The prevalence rates of polycystic ovary syndrome (PCOS) manifestations among affected women (Abasian *et al.,* 2018)

2.3.2. Diagnosis of polycystic ovary syndrome (PCOS)

It is a commonly shared perception among medical specialists that the forename PCOS creates a commotion which hinders progress in its management as well as treatment. The name PCOS gives no detailed information about multifaceted interactions that characterise this syndrome. In response to this, several benchmarks have been proposed for establishing this syndrome in women. The NIH criteria, recognised in l990 through the National Institutes of Health, identifies ovulatory dysfunction (irregular menstruation) and over secretion of androgen, while Rotterdam criteria, recognised in 2003 and reviewed in 2004 by the European Society for Human Reproduction and Embryology (ESHRE) in co-operation with the American Society for Reproductive Medicine (ASRM), perceive this disorder in the occurrence of any two out of these three benchmarks: ovulatory dysfunction, over secretion of androgen or PCO which can be detected by ultrasound. Furthermore, Androgen Excess and PCOS Society (AES) measures became well-known in 2006 because it postulated PCO (which can be detected by ultrasound) and over secretion of androgen as benchmarks for detecting PCOS (Azziz *et al.,* 2006; Wijeyaratne *et al*., 20l3).

In all these criteria, hyperandrogenism manifests either as clinical and/or biochemical hyperandrogenism. The application of these criteria excludes other circumstances that imitate PCOS. They are hyperprolactinemia, non-classical inborn excessive growth of adrenal glands, Cushing's syndrome as well as androgen dependent tumours. Nowadays, Rotterdam benchmarks remain the commonest standards for PCOS diagnosis. The PCOS description employed defines the characteristics (phenotypes) of the PCOS in affected women. This separation into characteristics (phenotypes) is a function of the physiognomies of PCOS with over secretion of androgen, PCO and oligo/amenorrhea. The understanding of definite phenotypes of a specific populace is indispensable, as shown through the information that there is a great threat of metabolic disorder in PCOS patients whose phenotype comprises of over secretion of androgen (hyperandrogenism) (Azziz *et al.,* 2006). Nevertheless, the possibility of metabolic disorder among women with PCO and/or without oligo/anovulation has not been reported (Azziz *et al.,* 2006).

2.3.3 Morphological features of polycystic ovaries

Numerous cysts, on the odd occasion bigger than l5 mm, enveloped by a thick layer of theca cells were the first histological parameters reported for PCOS. Other parameters later reported include wider tunica albuginea (the stroma riched in collagen below the exterior of the ovarian epithelium) and ovaries devoid of corpora lutea (Stein and Leventhal, l935). The introduction of ultrasound technology as a tool for

determining morphology of ovary in the l970s has improved the PCO morphology diagnosis instead of ocular inspection or histology. The occurrence of about 12 immature follicles diameter ranging from 2 to 9 mm accompanied with existence of more than one bloated ovary of volume >l0 cm^3 is among the satisfactory measures of PCOS diagnosis (Rotterdam, ESHRE/ASRM, 2004) excluding women taking oral contraceptives. High testosterone, insulin and androstenedione levels in ovulatory PCOS patients have been reported compared with non-PCOS women with normal ovarian morphology, although this might not be important (Falsetti *et al.*, 2002).

2.4 Clinical manifestations of polycystic ovary syndrome

2.4.1 Manifestations of hyperandrogenism

Indicators of over secretion of androgen (hyperandrogenism) comprise seborrhea, acne, hirsutism, alopecia and virilisation (clitoromegaly, which exist occasionally). Excessive weight gain (obesity) and presence of dark patches around the neck make up the scientific features associated with PCOS. Women affected are mostly diagnosed with impaired glucose tolerance. Epidemiological statistics with several controlled studies reveal high occurrence of insulin resistance, undiagnosed type 2 diabetes mellitus and IGT (impaired glucose tolerance) among PCOS patients. Increased threat of impaired lipid metabolism, cardiovascular disease, and endometrial cancer were detected among PCOS patients. Although the relationship between hyperandrogenemia and antioxidant status in PCOS patients still remains unclear, insulin resistance (IR) and hyperglycemia have been reported to increase level of free radicals in PCOS patients. The prevalence of impaired glucose tolerance among PCOS patents is 20-40%. Thus, PCOS patients are more vulnerable to diabetes than normal women (l5% against 2-3% in non-PCOS controls). Acanthosis nigricans, a skin decolouration among PCOS patient, is connected to insulin resistance and over secretion of insulin (hyperinsulinemia). It is dark and silky hyperpigmented skin with papillomatosis in the hollow of the armpit, joints and on the neck (Balen *et al.,* l995). Only 3% of PCOS women are affected by this skin disorder (Balen *et al.,* l995).

2.4.2 Oligomenorrhea, amenorrhea and infertility

It has been reported that among women with oligoanovulation, 65-87% had PCOS. After examining the ovaries of l73 women via pelvic ultrasound scan, 87% of women with polycystic ovaries are down with oligomenorrhea. The symptoms of hyperandrogenism include acne, alopecia and hirsutism. About 90% of women with hirsutism are PCOS patients (Bates and Legro, 20l3) while acne and alopecia affect 35% and 5% of PCOS women, respectively. Amenorrhea and infertility (primary/secondary) affects 30-40%

and more than 60% of PCOS women, respectively (Balen *et al.,* l995). The PCOS patients may be affected by pregnancy hypertension, preeclampsia, gestational diabetes and preterm labour leading to miscarriage during pregnancy (Norman, 2000). Elevated level of LH in obese PCOS patients is an evidence of upset in production pattern of gonadotrophin-releasing hormone which results in abnormal follicular development (Norman, 2000). Therefore, obesity in PCOS favours irregular menses, infertility and miscarriage (Norman, 2000).

2.5 Polycystic ovary syndrome and gynaecological cancers

Cancers which have been linked to chronic stimulation of hormones among women are endometrial, ovarian and breast cancers. Women affected by PCOS are at 2.7-fold increased danger of gynaecological cancers. However, the connection between PCOS and existence of breast cancer has not been established (Dumesic and Lobo, 20l3). Although the defined association between androgen excesses and breast, uterine cancers and melanoma has been validated, there is no significant relationship between PCOS and prevalence of breast cancer (Shobeiri and Jenabi, 20l6).

In women with PCOS, chronic stimulation of estrogen sometimes leads to uncontrollable enlargement of endometrium and endometrial cancer (Gottschau *et al.,* 20l5). Most PCOS patients appear to have 3-fold probability of developing cancer of the endometrium but unaffected by breast cancer. However, PCOS patients have a 2.5-fold greater risk of ovarian cancer than normal control (non-PCOS control). Recently, prevalence of ovarian cancer among PCOS affected women was studied by Ding *et al.* (20l8). In his findings, six PCOS patients (6/8l55) among the PCOS group and l5 non-PCOS patients (l5/32,620) in the control group had ovarian cancer during six months follow-up period. The prevalence of ovarian cancer stood at l2.3 and 7.3 per l00, 000 persons per year among PCOS patients and non-PCOS groups, respectively. No substantial change in the prevalence of ovarian cancer was detected among the groups once breast and endometrial cancer were set as contending risk factors (Ding *et al.,* 20l8).

Oxidative stress has been identified, amidst other factors, as the major element contributing to causes of gynaecological cancers among reproductive women (Krstic *et al.*, 2015). Reactive oxygen species triggers genetic aberrations by damaging DNA, leading to DNA strand breakage, point transfigurations (mutations) and unusual cross-linking of DNA and DNA-protein. The alterations in proto-oncogenes as well as tumour suppressor genes pedal uncontrollable cell multiplication. This uncontrollable cell proliferation is favoured by disrupted DNA repair mechanism. Whenever cells encounter serious DNA damage, *p53* displays its pro-apoptotic effect by eliminating damaged cells, preventing its multiplication into daughter cells. This *p53* gene plays vital role in preserving genomic identity. More than fifty percent

of human cancers arises from destruction of pro-apoptotic purpose of *p53* gene. This includes human cervical carcinoma. This finding surmises that *p53* could be a Knudson-type tumour suppressing agent. Cancers with mutation of *p53* mutations exhibits chemo-resistance, showing that *p53* is responsible for regulating DNA impairment. Chinese Hamster Ovarian (CHO) cell is tumorigenic with characteristic abnormal *p53* function (Hu *et al.*, 1999). Recently, abnormal *p53* tumour suppressor gene was detected in the endometrium of PCOS patients (Shafiee *et al.*, 2015; Gadducci *et al.*, 2016). This justifies the high occurrence of gynaecological cancers among PCOS women (Dumesic and Lobo, 2013; Gottschau *et al.,* 2015).

2.6 Treatment of polycystic ovary syndrome

2.6.l Allopathic medicines and polycystic ovary syndrome

The best acknowledged intervention for PCOS recently is by administration of synthetic medicine which includes tamoxifene, clomiphene citrate, troglitazone and metformin (Figures 2.6.1 and 2.6.2). Clomiphene citrate (Clomid, Serophene) is often the earliest fertility medication of excellence for females with irregular periods, elongated menstrual phases and polycystic ovarian syndrome (PCOS). Clomiphene blocks estrogen, thus, plays the pituitary into production of LH and FSH. This results in increase in follicle growth rate and discharge of follicles. Unfortunately, Clomiphene may cause thinning of the uterine lining and it often lessens the quantity and value of cervical mucus. Whenever this occurs, to restore thickness of uterine lining, combine administration with other hormonal drugs are inevitable. Other adverse effects accompanying the use of clomiphene are ovarian cysts, weight gain, hot flashes, headaches, vomiting and weariness with a faintly increased peril for miscarriage (Gaware *et al.,* 2009).

From time immemorial, studies have proven that insulin resistance is one of the significant causes of PCOS, thus, insulin-sensitising medications are beneficial in PCOS management. Thiazolidinediones and metformin are used to reduce the related insensitivity to insulin in PCOS women**.** Metformin, the insulin-sensitising drug, amend resistance to insulin, reduce serum free testosterone and elevate serum sex hormone binding globulin as well as ensuring regularity of ovulation in PCOS patients. Metformin effectively reduces hyperinsulinemia among non-obese and obese PCOS patients, hinders production of androstenedione with no influence on progesterone in ovarian theca cells. Metformin therapy significantly decreases the total serum testosterone. Moreover, metformin adjusts over secretion of androgen in the ovary and functional hyperandrogenism in the adrenal glands among premenopausal PCOS patients **(**Heibashy *et al.,* 20l3). Administration of metformin is limited by the associated

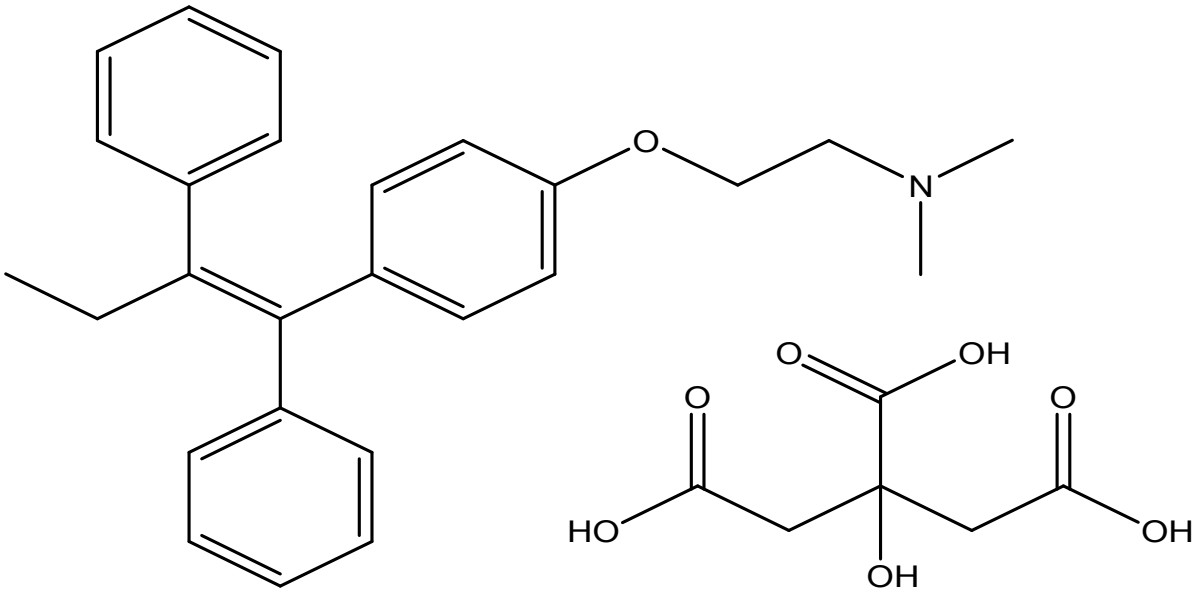
gastrointestinal disturbances such as bloating, diarrhoea, anorexia, flatulence, abdominal pain and its metallic taste (Lashen, 20l0).

2.6.2 History of medicinal plants in polycystic ovary syndrome management

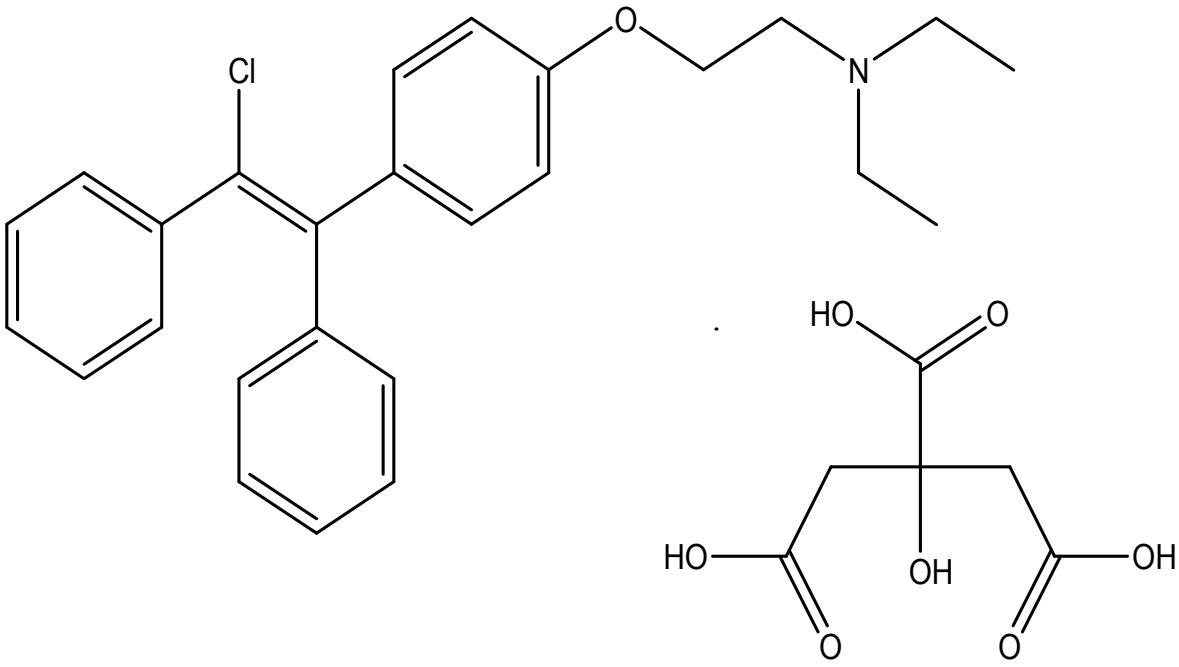
The use of botanicals among PCOS affected women has been found to be beneficial in treating infertility and enhancing the chance of conception significantly among womenfolk (Zhao, 20ll). Several plants have been documented through ethnobotanical studies as effective for reproductive health. The herbal treatment, mostly administered in form of powder, tea, tonic or tincture, has effectively enabled the body to readjust the menstrual cycle in Nigeria (Fasola, 20l5; Nduche *et al.,* 20l5). In line with this, interest has been generated towards the scientific confirmation of the medicinal assertions of some of these botanicals. The qualitative assessment of bioactive constituents of some of common medicinal plants showed that they possess constituents that could elicit pharmacological effects such as cytotoxicity, antioxidant activity and correction of hormonal imbalance, thereby justifying their folkloric uses.

Vitex agnus-castus under trade names Strontan®, Mastodynon®, Phyto-hypophyson® and Agnacaston® have been studied for its effect on cultured brain receptor tissues such as DA2, histamine and 5HT transporters in calf, guinea pig and rats and β-receptors of oestrogen, cloned human μ-opoid receptor cells professionally inserted into ovarian cells of hamster. This medicinal plant reduces prolactin (via dopaminergic effects) and LH levels, binds to β-estrogen receptors, increase serum estradiol level and enhance pregnancy rates among women in productive age (Jarry *et al.,* 2009).

The beneficial result of *Cimicifuga racemosa* on conception in PCOS women has also been published. This medicinal plant binds with receptors of α-estrogen in the pituitary, reduces LH production and also favours high luteal progesterone concentration (Shahin *et al.,* 20l4). In women with PCOS, it enhances endometrial thickness (Shahin *et al.,* 20l4), mends FSH:LH ratio and restricts the action of anti-estrogen when administered together with Clomiphene citrate in the process of treating PCOS (Shahin *et al.,* 20l4). Several scientific findings support the general belief that soy consumption, an isoflavone-rich diet, prevents cardiovascular ailments and post-menopausal effects such as osteoporosis (Malińska and Kiersztan, 2004).

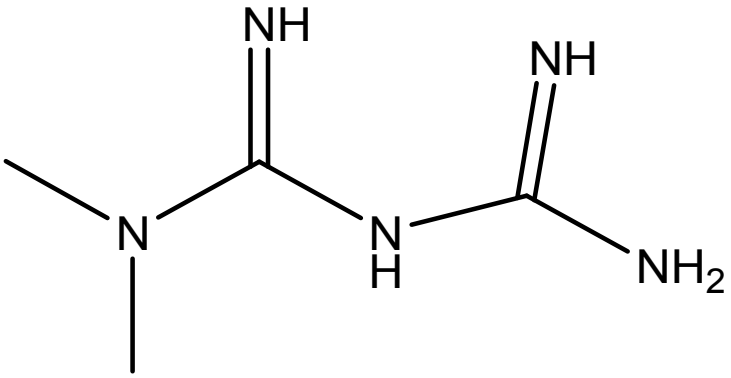


(A) Tamoxifen citrate

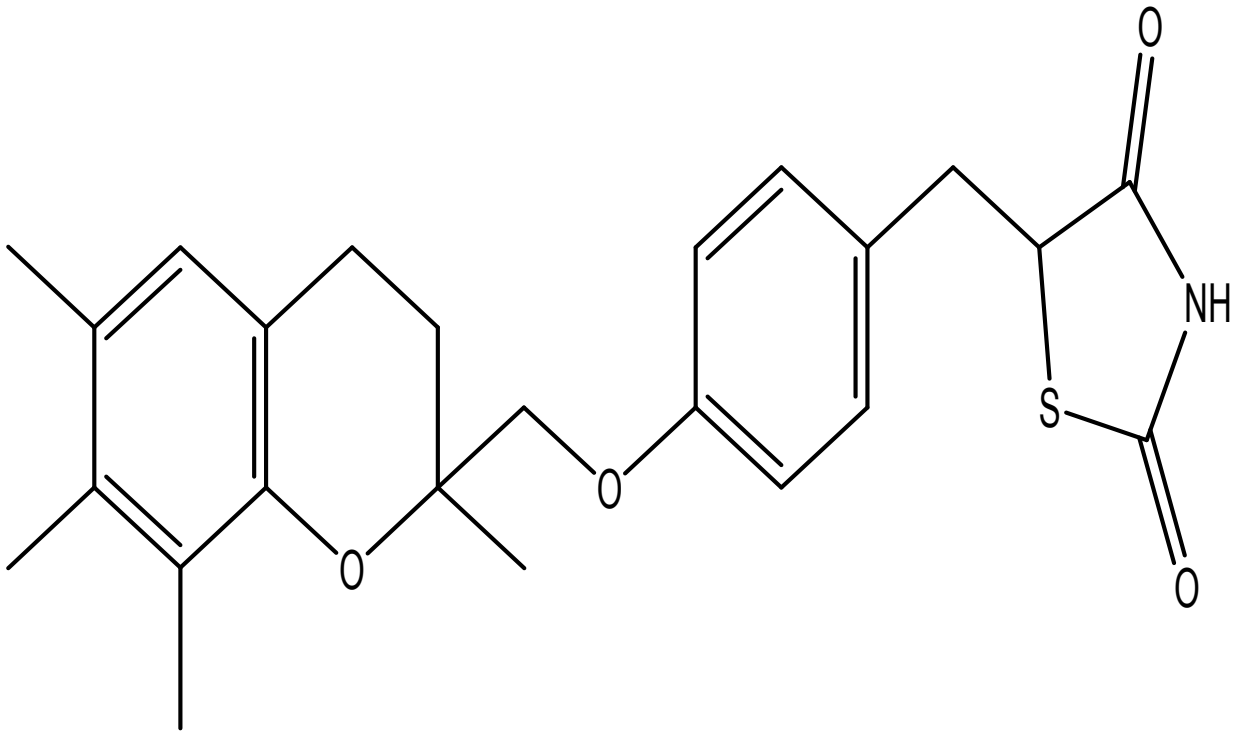


(B) Clomiphene citrate

Figure 2.6.1: Chemical structures of tamoxifen citrate and clomiphene citrate



(C) Metformin



(D) Triglitazone

Figure 2.6.2: Chemical structures of metformin and triglitazone

2.6.2.1. Phyto-antioxidants and PCOS

Reactive oxygen species plays a substantial function in origination of numerous human ailments including inflammation, arthritis, atherosclerosis, cancer and diabetes (Krstic *et al.*, 20l5; Ding *et al.*, 20l8). The recent scientific investigation into roles of free radicals in parthenogenesis of PCOS have revealed that some characteristics in PCOS patients favour overboard generation of free radicals. These characteristics are excessive weight gain (obesity), resistance to insulin, androgen excess and accumulation of fat on the abdomen (abdominal adiposity). The reduction in the level of vitamins and other endogenous antioxidants predispose PCOS patients to oxidative stress. Depletion in antioxidant status in women with PCOS could cause central obesity, dyslipidaemia, resistance to insulin, hypertension and cardiovascular disease (Murri *et al.*, 2013).

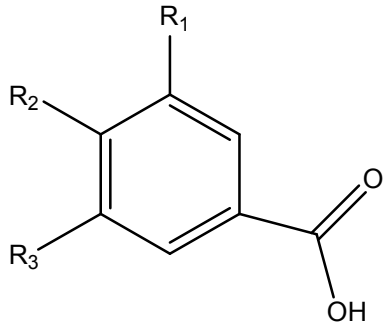
Food supplements rich in antioxidants have been discovered. They significantly improve sensitivity to insulin and additional health threating circumstances in PCOS women (Murri *et al.*, 2013). Antioxidants from natural sources are multidimensional in their enormous activities, and offer the possibility of rectifying hormonal imbalance through ordered consumption of diets containing antioxidants. Hence, it is proposed that antioxidant rich foods and supplements are beneficial in averting oxidative stress in human. Phyto-antioxidants, usually available and less toxic, have been recommended to lessen the menace of ROS. Among these are genistein and diadzein identified to exert positive effect in managing PCOS. Recently, many herbs have been studied in the quest of discovering unique antioxidants, however, there is pressing call to discover additional active antioxidants from plants with different therapeutic uses. The radical scavenging technique using 1,1-diphenyl-2-picrylhydrazyl (DPPH) is generally accepted in the evaluation of antioxidant potential of botanicals because of its high sensitivity and ability to quantify antioxidant potential at small concentrations within a short period (Singh *et al.,* 20l6).

Phenolics and flavonoids still remain the major antioxidant of medicinal plants origin (Cook and Samman, l996; Aggarwal *et al.,* 20l8). Phenolics contribute considerably to the antioxidant potential of numerous plants. Flavonoids comprise maximum number of plant polyphenols. Their collective structural functionality is the diphenylpropane moiety, consisting of two characteristic aromatic rings connected via three carbon atoms which generally form an oxygenated heterocycle. The type of heterocycle involved has resulted in classification of flavonoids into flavanones, flavones, flavonols, anthocyanidins, isoflavones and flavanols (or catechins) (Li *et al.*, 2016). Figures 2.6.3 and 2.6.4 present the structure of each classes of phenolics and flavonoids. Flavonols mostly exist in foods (as either quercetin or kaempferol). Examples of phenolic acids are analogues of benzoic acid and cinnamic acid.

Isoflavone, phenolics, can be grouped as isoflavones, coumestans and lignans. Isoflavones, the most widely considered phytoestrogens, were isolated from soybeans and soy product-containing foods. The main isoflavones existing are genistein and daidzein and their analogues covers more than 65% of the total known isoflavones. Isoflavones, a class of phytoestrogen of which over sixty are known, are isomeric with the flavones but of much fewer existence. They are present in almost one sub-family (the Lotoideae) of the leguminasae, such as soybeans, chickpeas, fava beans, pistachios and peanuts (Harbone, l973). Diadzein (7, 4'-dihydroxyisoflavone) and genistein (5, 7, 4-trihydroxyisoflavone) are potent natural insect repellent (Harbone, l973). This class of flavonoid are problematic to characterise, since they do not react precisely to some colour tests. Few isoflavones (for instance, daizein) give a brilliant light blue colour with ammonia in UV light, but other isoflavones (for example, genistein) look like dull purple spots, transforming to dull brown with ammonia (Mohammed, l996).

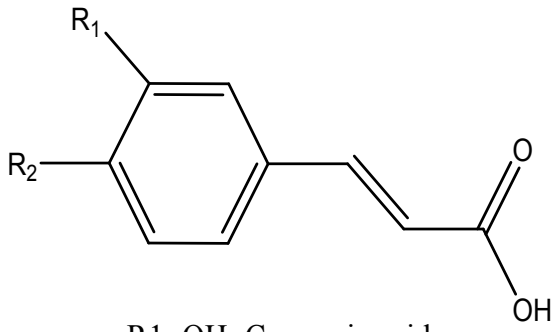
Regardless of the positive functions of antioxidants in PCOS management, limited reports are available on application of antioxidants in the management of PCOS patients. Some available studies in this field presented controversial outcomes (Amini *et al.*, 2015; Borzoei *et al*., 2018; Jazani *et al*., 2019; Darabi *et al*., 2020**)**. To date, evaluating the effect of antioxidants among Nigerian PCOS women has not been investigated. This necessitates critical assessment of Nigerian plants with significant antioxidant activity in the treatment and/or management of PCOS among reproductive women.

Benzoic acid derivatives



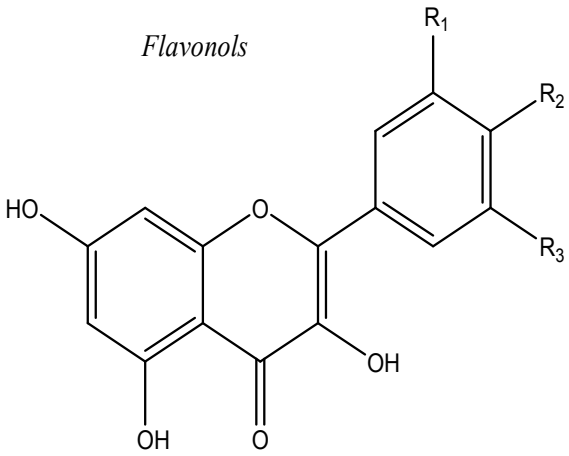
R1=R2=R3=OH; Gallic acid
R1=R2=OH; R3=H; Protocatechuic acid

Cinnamic acid derivatives



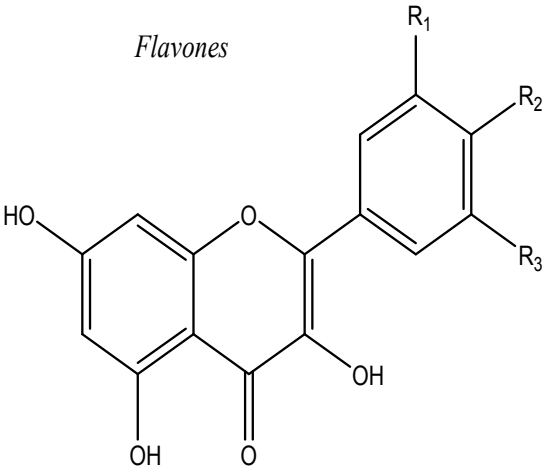
R1=OH; Coumaric acid
R1=R2=OH; Caffeic acid

Flavonols



R1=R2=OH; R3=H; Quercetin

Flavones



R1=R2=H; R3=OH; Apigenin

Figures 2.6.3: Structures of some phenolics and flavonoids (Li *et al.*, 2016)

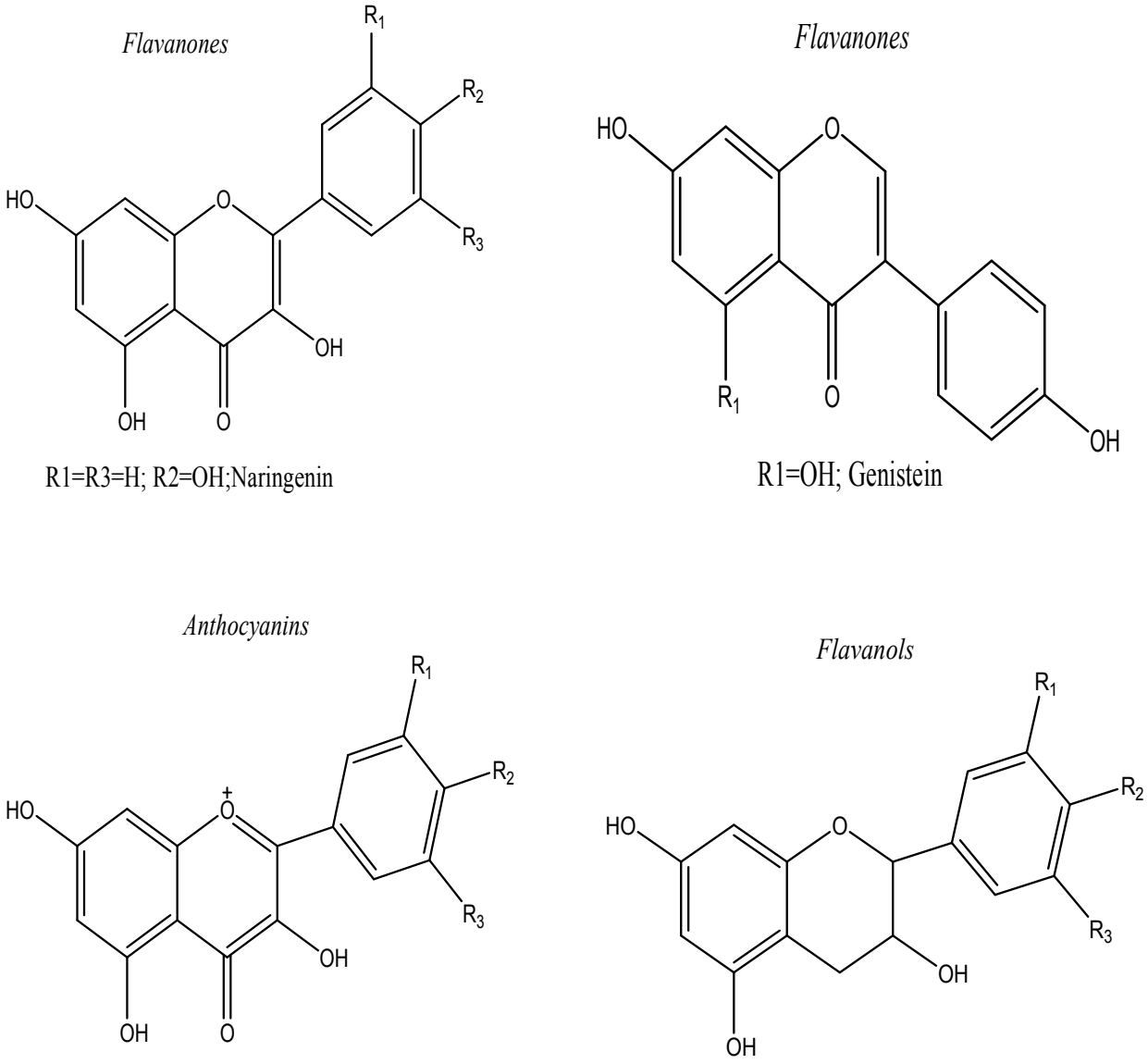


Figure 2.6.4: Structures of some flavonoids (Li *et al.*, 2016)

2.6.2.2 Toxicity of medicinal plants

The utilisation of therapeutic plants for treatment of countless ailments has gained importance in global health care schemes. Generally, it is believed that the safety of these plants is due to the fact that they are from natural source. However, limited scientific facts have been reported on side effects connected with the usage of herbs. Adverse effects, which occasionally manifest when herbs are used, are usually mild, affecting minimal population of patients. The major setback in the use of medicinal plants in clinical settings is that medical practitioners in modern health sector have little or no idea about the use of herbs. Synthetic drugs mostly recommend by this medical practitioners generally consist of a number of chemicals while medicinal plants are good blends of over 400 active principles. The broad physiological actions of medicinal plants are due to the complementary or synergistic interactions between these metabolites which are often without any adverse effect (Philomena *et al.*, 2009).

Several medicinal plants possess adequately diverse compounds with pronounced complexity. Herbal materials such as polysaccharides, mucilages and tannins improve the properties displayed by most active ingredients. Researches reveal that the efficacy of plants extracts cannot be achieved via administration of purified compounds of plants origin. Plants containing a single documented cancer-causing chemical usually possess several other compounds which might be anticancerous, thus nullifying the carcinogenicity of the plant. Furthermore, the availability of other constituents in this plant might even provide significant anticancer effect (Kazemipoor *et al.*, 2012).

The use of brine shrimp is the easiest and generally accepted method of evaluating medicinal plants toxicity. This test is one of the preliminary tools of assessing plants' toxicity. This technique, designed 30 years ago, require small amount of samples for the analysis. Numerous researches have established the correlation in the results of lethal concentration that kills 50% (LC_{50}) achieved by means of Brine Shrimp Lethality Assay and that obtained from Acute Oral Toxicity Assay using mice. Therefore, apart from its accuracy, this technique is economical compared with *in vivo* toxicity assays (Arlsanyolu and Erdemgil, 2006).

2.7 Cinnamic acid derivatives

Cinnamic acid, present in several medicinal plants, displayed numerous biological actions. Many derivatives of cinnamic acid with diverse functional groups exhibited anticancer, antioxidant, antifungal, antimicrobial and antituberculosis activities (De *et al.*, 2011). The α,β-unsaturated amides of cinnamic acid are significant intermediate in the production of anticancer drugs. Cinnamic acid possesses an α,β-unsaturated carbonyl parts, which act as a Michael acceptor, a dynamic part needed in the design of new anticancer agents (Zhu *et al.*, 2011).

2.8 Botanical description of researched plants

2.8.1 *Kigelia africana*

2.8.1.1 Taxonomic description

Kigelia africana, as shown in Figure 2.8.1, exists across humid region of Africa. Common names include Sausage tree (English), Rahaina (Hausa), Pandoro (Yoruba) and Ishi (Igbo) (Abere *et al.*, 20l5). Kigelia is mostly referred to as monospecies genus belonging to family Bignoniaceae. The Bignoniaceae family comprises 82 genus with 827 species, which are are naturally temperate species existing in Southern region of America as trees or herbs. Recently, seven out of eight known clades were highlighted: Bignonieae, Coleeae, Crescentieae, Eccremocarpeae, Oroxyleae, Tecomeae and Tourrettieae (Fischer *et al.*, 2004). Two clades, Bignonieae and Crescentiinae, denote about 80% of the species of this family (Olmstead *et al.*, 2009).

Kingdom: Plantae
Phylum: Tracheophyta
Class: Spermatophytina
Order: Lamiales
Family: Bignoniaceae
Genus: *Kigelia*
Species: *Kigelia africana* (Lam.) Benth. (Abere *et al.*, 20l5)

2.8.1.2 Origin and geographical distribution

Kigelia africana is found mostly in riverbanks, open forests and valleys of Cameroon, Nigeria, Guinea, Kenya, Senegal, South Africa, Tanzania, Namibia and Chad. Sausage trees are cultivated for ornamental purposes in most parts of south Florida. In the open grassland, the thick rounded crown of *K. africana* trees creates shade in gardens and parks. The local names of *K. africana* in Africa are: pandoro (Yoruba, Nigeria) while it is called balam kheera, hathi bailan in India and yago in Luo (Bello *et al.*, 20l6).

2.8.1.3 Morphological description of *Kigelia africana*

Kigelia africana is average to big semi-deciduous tree of around 25 m in height. The leaves arrangement is opposite and crowded in pairs, 3-5 pairs with terminal leaflets, close to the edges of the twigs. The subordinate leaflets are with little petiolate, while the terminal pair have no petioles. The dark-red, cup-like shape flowers, which blossoms at night are suspended on long, rope-like stalks that dangle on the branches of the tree but get detached before daybreak. The characteristic reeking as well as nectar-rich flowers attracts bats, insects and birds as natural pollinating agents. Woody, sausage-like fruits are also

suspended on the twigs by cordlike stalks. This big fruit is able to develop up to about l.l8 m weighing up to l2 kg. The ripe fruit appearing as greyish-brown round sausage contain a tough inedible pulp embedded with numerous seeds (Jackson and Beckett, 20l2).

2.8.1.4 Ethnobotany of *Kigelia africana*

Kigelia africana has a broad application in African traditional medicine system. Kigelia's different parts are used in Africa for treatment of articular rheumatism, fungal infestations, gonorrhoea, toothache, rheumatism, cough, dysentery, gynaecological and obstetric conditions, sexually transmitted disease, stomach ulcer, pre-birth and post-birth ailments, fibroid, pneumonia, eczema, waist pain, jaundice, skin cancer, breast metastasis and leprosy as shown in Table 2.8.1 (Bello *et al.*, 2016). It is use in the treatment of wound and bacterial infections. It has strong mollusicidal and anti-diabetic activities. The leaves are used in the management of stomach discomfort, kidney disease and snakebite), stem and twigs for sores, serpent bite, rheumatism, stomach and kidney infections, while fruit are used in the treatment of constipation, gynaecological problems and haemorrhoids (Jackson and Beckette, 20l2). The red and yellow dye produced by boiling fruits and roots respectively are used in cloth dying. Nutritional composition of *K. africana* leaves is as good as other green and abundant vegetables including spinach (Glew *et al.,* 20l0) although its edibility has not be documented.

2.8.1.5 Chemical constituents of *Kigelia africana*

About l50 compounds have been isolated from *K. africana.* Some of these compounds are shown in Figures 2.8.2, 2.8.3, 2.8.4, 2.8.5, 2.8.6, 2.8.7, 2.8.8, 2.8.9, 2.8.10 and 2.8.11. These compounds are mainly iridoids, naphthoquinones and flavonoids. Some of the compounds isolated are isovitexin, kojic acid (Sidjui and Zeuko'o, 20l4), ethylgallic acid, chlorogenic acid, kigelianolide, khayanolide B, diacetylkhayanolide E, l-O-deacetyl- 2α-hydroxykhayanolide E, l-O-deacetyl-2α methoxykhayanolide (Jabeen and Riaz, 20l3), sesamin (Sidjui *et al.*, 20l5), β-sitosterol, β-sitosterol glycoside, γ-sitosterol together with Stigmasterol (Sidjui *et al.*, 20l5). In addition, phenolic and flavonoid, with significant antioxidant potentials have been isolated from the plant. Also, berbascoside isolated from *K. africana* displayed extraordinary antioxidant pontential *in vitro* (Alipieva *et al.*, 20l4). Uinone, isopinnatal, kigelinol, isokigelinol, 3-(2′-hydroxy-ethyl)5-(2-hydroxypropyl) dihydro-furan-2(3H)-one, 2-acethylnaphtho [2,3-b]furan-4,9-quinone, 2-(l-hydroxyethyl)-naphtho[2,3-b]furan-4,9-dione, sesamin, 4-hydroxy cinnamic acid, ferulic acid and iridoids isolated from *K. africana* exhibited significant antimicrobial activity (Bello *et al.*, 20l6).

Cytotoxic compounds such as hydroxyethyl-naphtho (2, 3-b) furan-4, 9-dione, ferulic acid, atranorin and 2beta, 3beta and l9 alpha trihydroxy-urs-l2-en-28-oic acid are isolated from *K. africana*. Lapachol (a

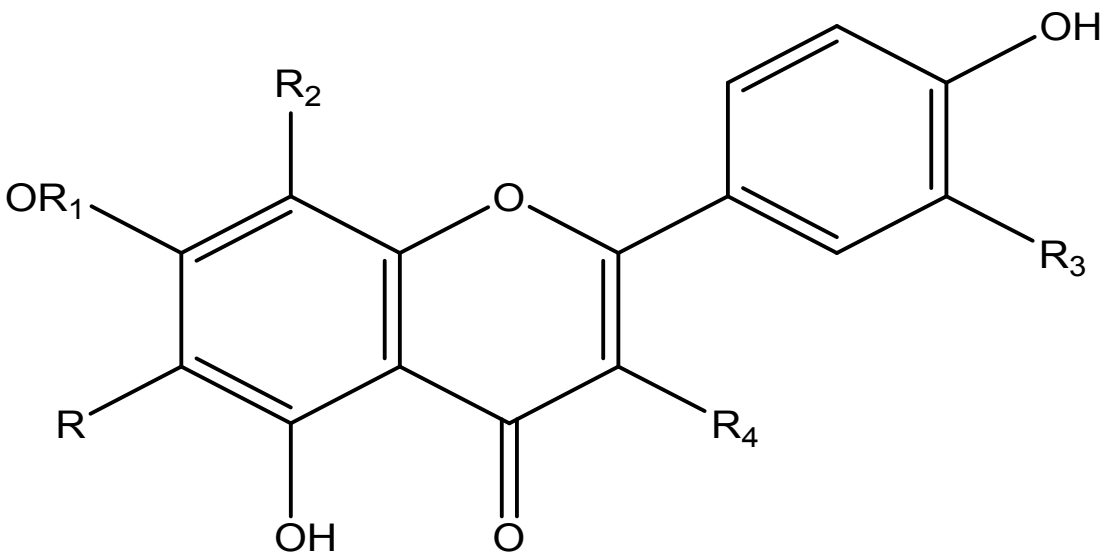
latent anticancer medicine), norviburtinal and isopinnatal have been found to be active on melanoma cell lines (Bello *et al.*, 20l6). Atranorin, linalool and 2beta, 3beta, l9 alphatrihydroxy-urs-l2-en-28-oic acid, isolated from hexane fraction of *K. africana* stem bark displayed significant cytotoxic effect at high concentrations. Yet, the comparative influence of the diverse compounds obtained from *K. africana* to the experimental observations on the cytotoxicity displayed by the extracts remains indescribable, due to the fact that these cytotoxic compounds are exist in this plant in diminutive concentration. The anticancer activity of γ-sitosterol, one of the compounds isolated from this medicinal plant, has continued to be a subject of deliberation (Bello *et al.*, 20l6).

Figure 2.8.1: *Kigelia africana* in Oluponna, Osun State, Nigeria.

Insert: Fruit pods of *K. africana*

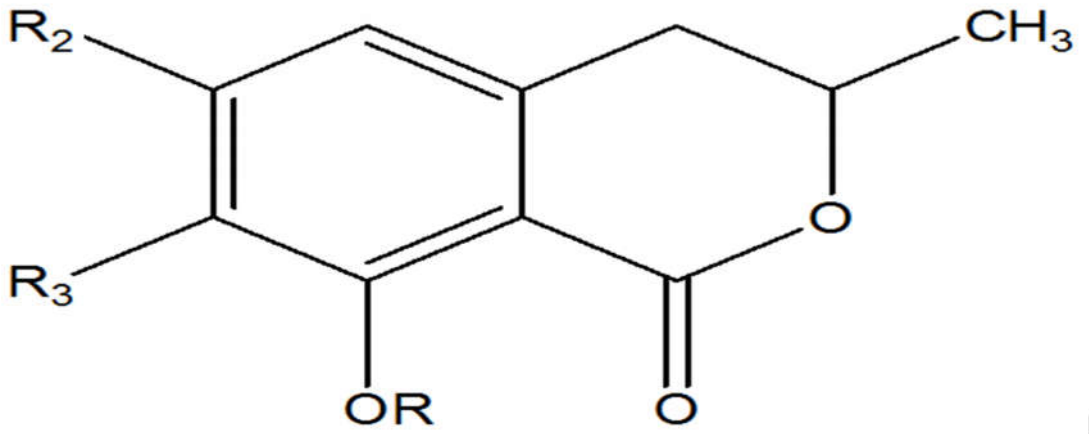
Table 2.8.1: Some ethnobotanical uses of *K. africana* (adapted from Bello *et al.*, 2016)

Country	Plant part	Disease	Method of preparation and administration
Nigeria and Ghana	Bark	Dysentery	The bark is pounded and added to pap made from corn powder.
West and central Africa	Bark	Venereal diseases	Mostly use on children. Treated simultaneously with a drink and wash prepared from decocted bark. Palm wine, in which dried and ground bark is macerated for 2–3 days. About 100 ml is taken daily for 8 days or more.
South Africa	Bark and fruit	Gynecological and obstetric conditions	Chopped bark and fruit boiled in 2 L of water for 1 h, cool before straining and taken orally, half a cup thrice a day for blood cleansing and pelvic pains during pregnancy, or alternatively, an enema with three Size-6 syringes once a day.
Nigeria and Ghana	Bark and fruit	Rheumatism, wounds and malignant tumors	Oily pastes made from the mixture are used to rub on rheumatic parts and on malignant tumors.
Nigeria	leaves	Stomach ulcer	Hot-infusion preparations from it leaves are popularly used to treat stomach ulcer

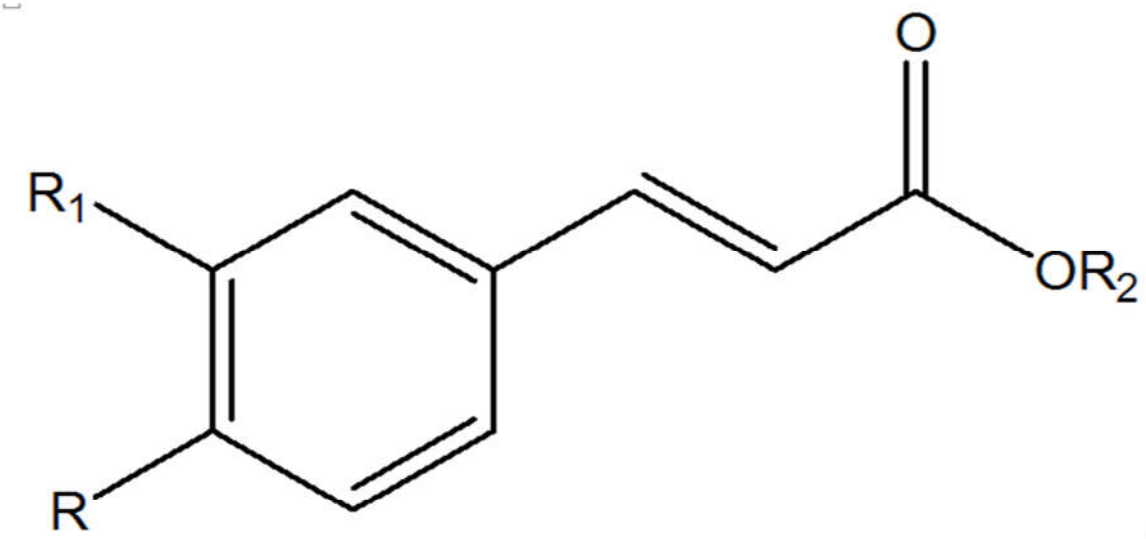


$R=R_1=R_2=H$, $R_3=R_4=OH$	Quercetin
$R=R_1=R_2=R_4=H$, $R_3=OH$	Luteolin
$R=R_1=R_2=R_4=H$, $R=R_3=OH$	6-hydroxyluteolin
$R=R_2=R_4=H$, $R_3=OH$, $R_1=Glu$	Luteolin-7-O-glycoside
$R_2=R_4=H$, $R=R_3=OH$, $R_1=Glu$	6-hydroxyluteolin-7-O-glycoside
$R_1=OH$, $R_2=R_3=H$, $R=Glu$	Isovitexin
$R_1=R_3=R_4=H$, $R=Arab$, $R_2=Glu$	Isoschaftoside

Figure 2.8.2: Some flavonoids isolated from *K. africana* (El-Sayyad, 1981)

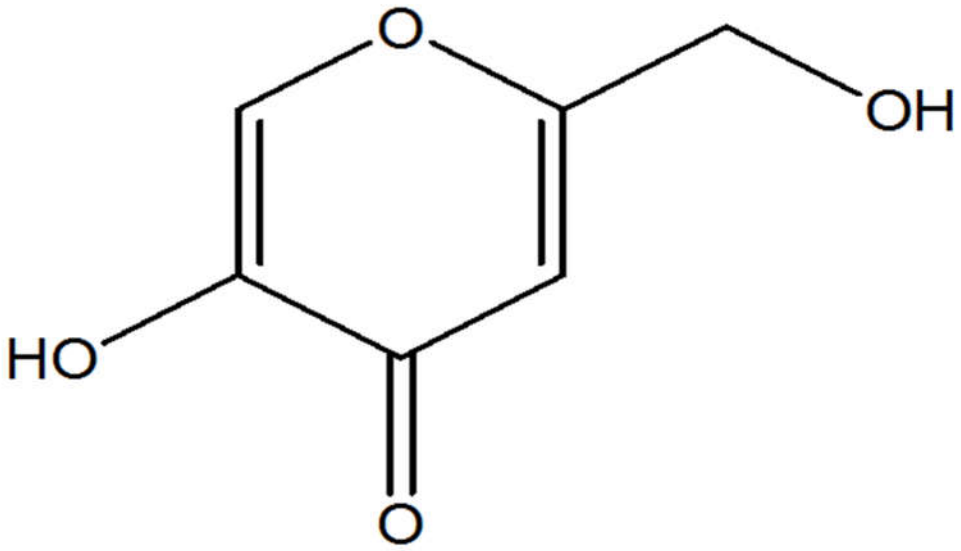


R_1=R_3=H, R_2=OCH_3	6-methoxymellein
R_1=H, R_2=R_3=OCH_3	kigelin
R_1=H, R_2=OCH_3, R_3=OH	3-demethylkigelin
R_1=H, R_2=OH, R_3=OCH_3	6-demethylkigelin

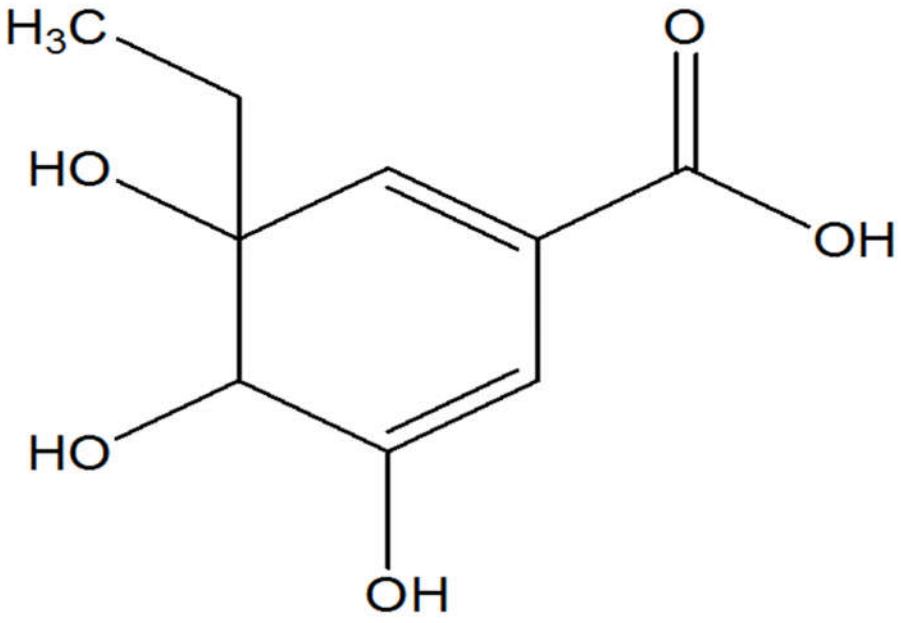


R_1=R_2=H, R=OH	4-hydroxy cinnamic acid
R_2=H, R=R_1=OH	Caffeic acid
R=R_1=OH, R_1=OCH_3	Caffeic acid methyl ester
R_2=H, R=OH, R_1=OCH_3	Ferulic acid

Figure 2.8.3: Structure of methoxymellein, kigelin and cinnamic acid derivatives isolated from *K. africana* (Higgins *et al.*, 2010; Khan *et al.*, 2012)

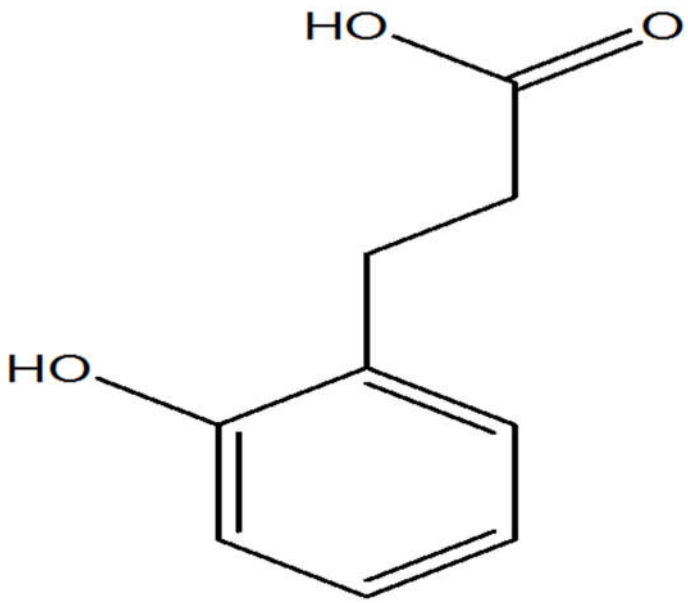


Kojic acid

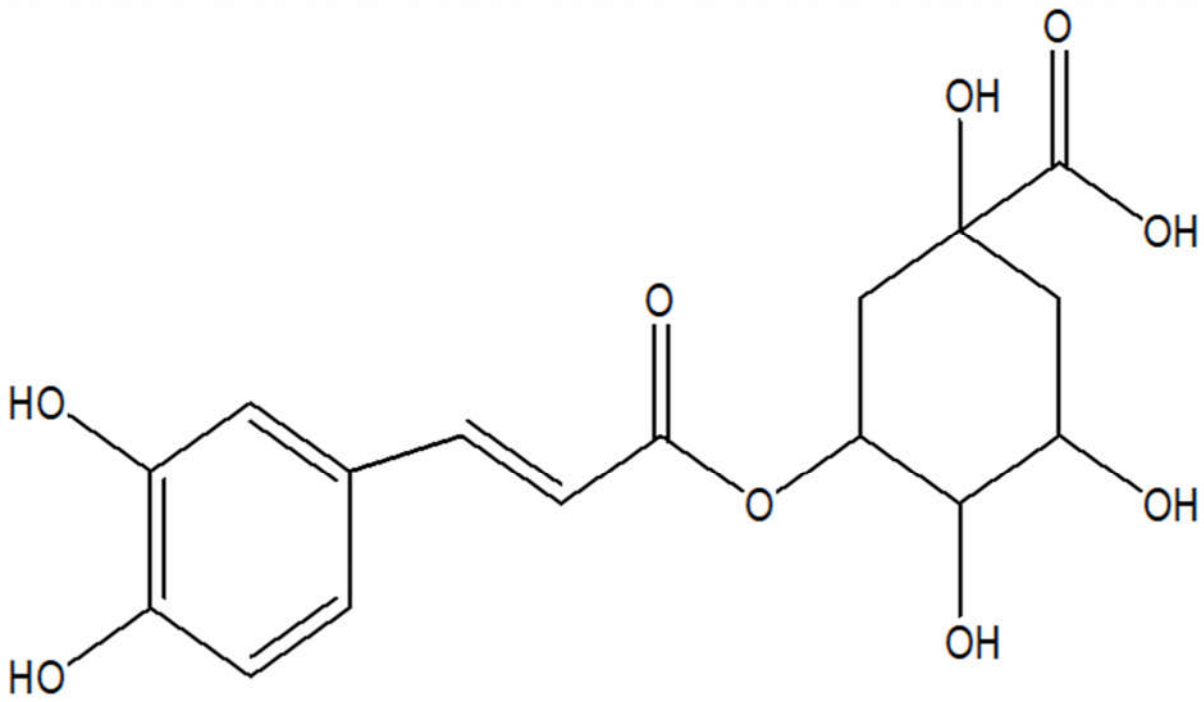


3-ethylgallic acid

Figure 2.8.4: Structure of Kojic acid and 3-ethylgallic acid isolated from *K. africana* (Arkhipov *et al.*, 2014; Bello *et al.*, 20l6)

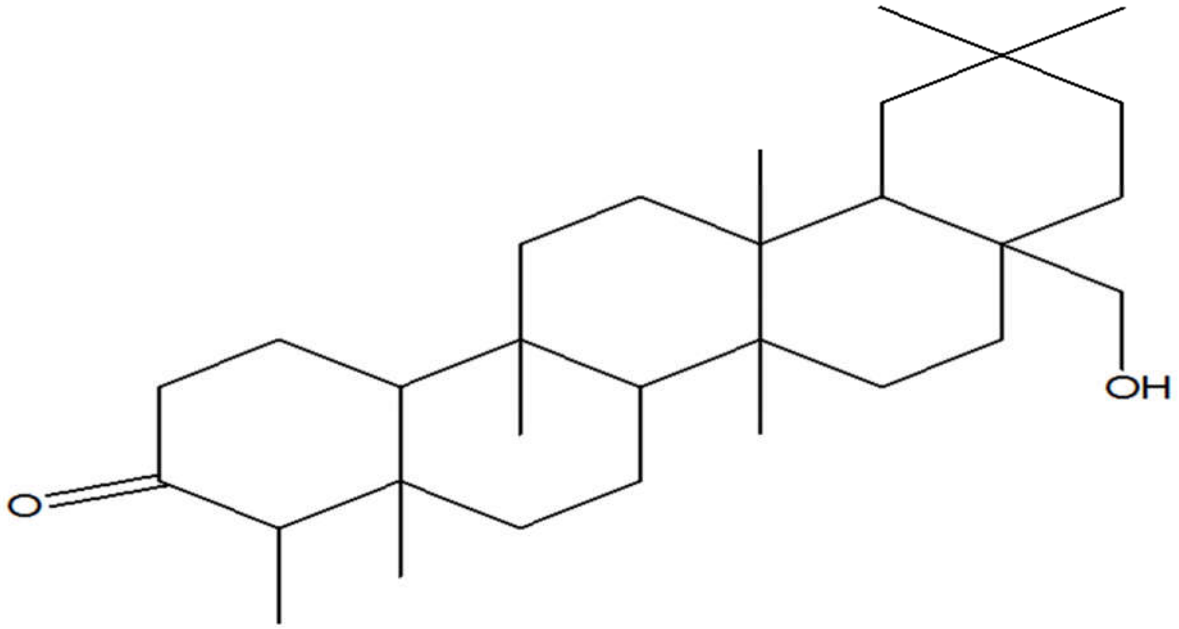


Melilotic acid

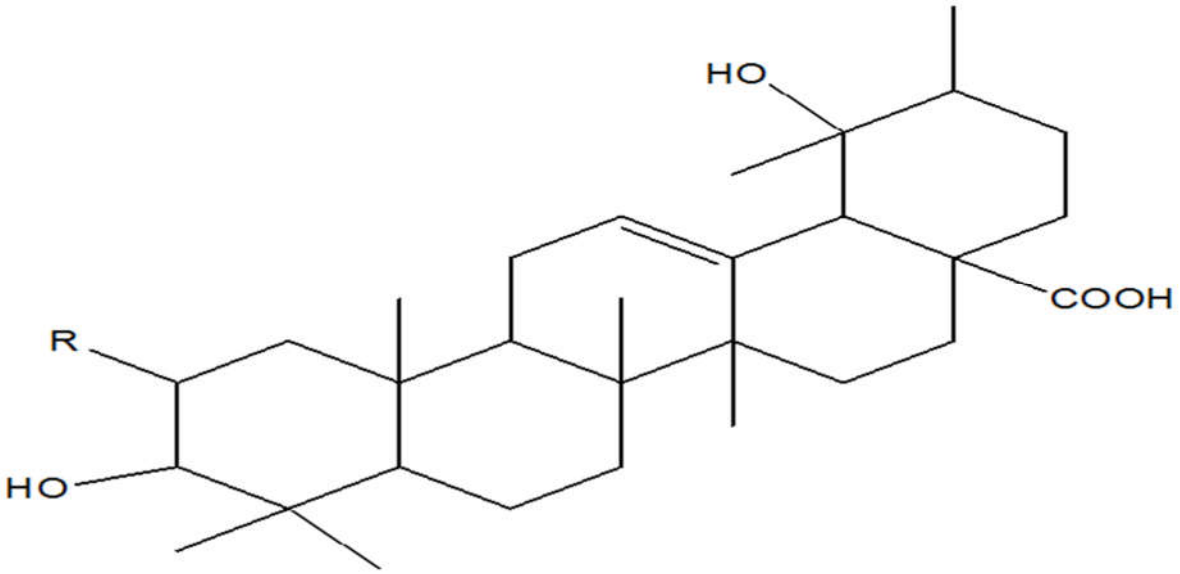


Chlorogenic acid

Figure 2.8.5: Chemical structure of melilotic acid and chlorogenic acid isolated from *K. africana* (Arkhipov *et al.*, 2014; Bello *et al.*, 20l6)



Canophyllol



R=H	Pomolic acid
R=OH	28-hydroxy-pomolic acid

Figure 2.8.6: Chemical structure of canophyllol, pomolic and 28-hydroxy-pomolic acid (Bello *et al.*, 2016)

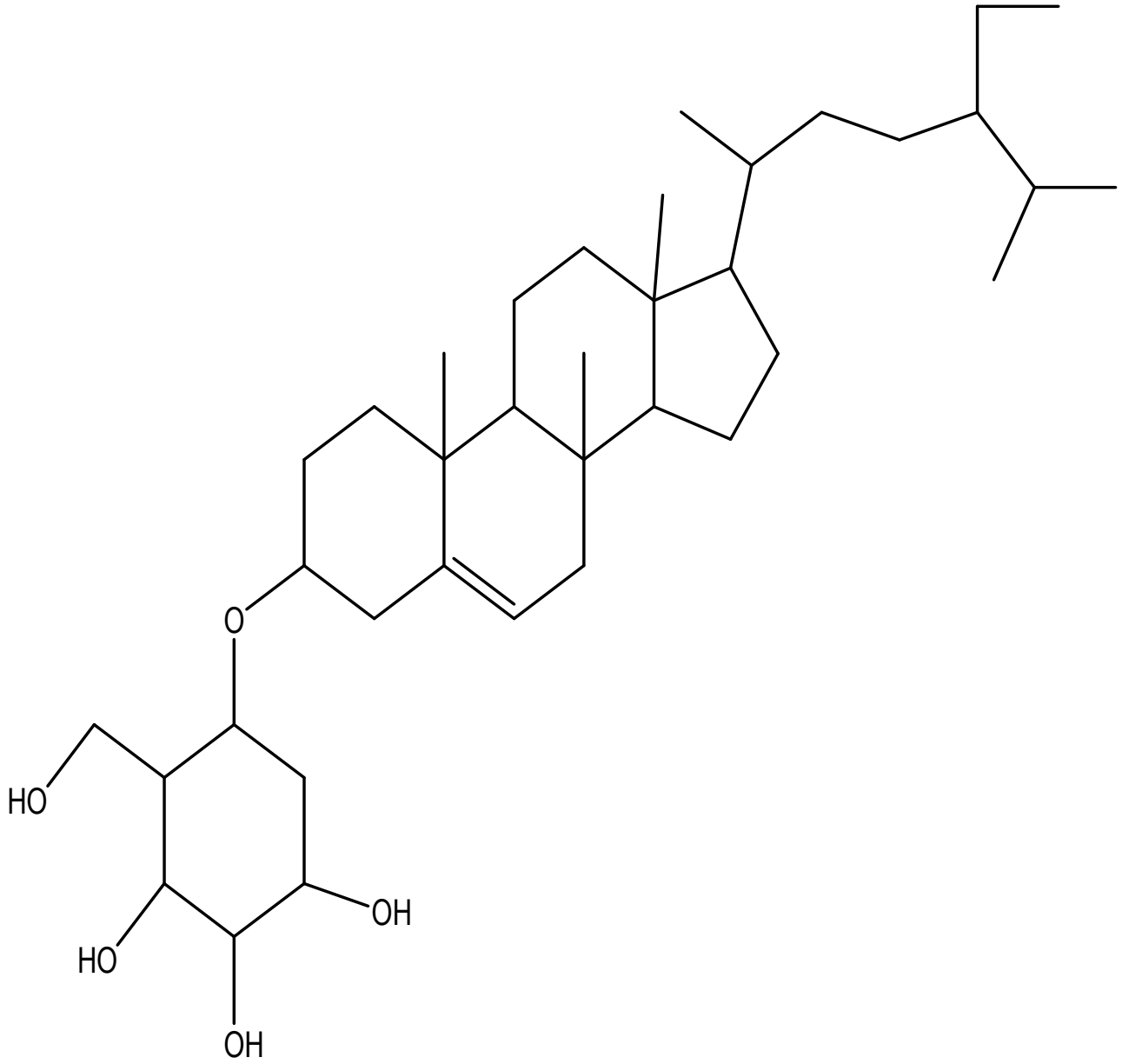


Figure 2.8.7: Chemical structure of sitosterol-D-glucose isolated from *K. africana* (Sidjui *et al.*, 2015)

2.8.2. *Tetracera potatoria*

2.8.2.1 Taxonomic description of *Tetracera potatoria*

Tetracera potatoria (Figure 2.8.12), a scandent shrub or climber that could grow to the heights of 5 m (Burkill, l985), is called ahara-igbo among Yorubas in Nigeria.

Kingdom: Plantae
Phylum: Tracheophyta
Class: Magnoliopsida
Order: Dilleniales
Family: Dilleniaceae
Genus: *Tetracera*
Species: *Tetracera potatoria* Afzel. ex G. Don (Hassler, 20l9).

2.8.2.2 Origin and geographical distribution

Dilleniaceae family are generally shrubs, trees or occasionally lianas or herbs, with a distribution mainly distributed across pantropical, subtropical and temperate regions. This family are mostly found in Australia but very rare in pantropical region (for instance, Africa). The few species available in Africa are in genus *Tetracera*. They consists of ten to fourteen genera with 500 species, and are categorised into four subfamilies. Subfamily Delimoideae contain *Tetracera potatoria* (Fraga *et al.,* 20l0).

2.8.2.3 Morphological description of *Tetracera potatoria*

Tetracera potatoria is a shrub, occasionally sprawling, or lianas, with flexous twigs. Leaves of the plant are spirally organised, simple, with short petiolate deprived of stipules. The base of the leaf is decurrent with margin obviously dentate. The leaves are penninerved while both sides are often scrabid. Fruits are coriaceous capsules, opening with longitudinal slits along ventral and dorsal suture into two valves, with short beak, containing one- to few-seeds. Seeds are glossy, dark-brown to black in colour, with abundant endosperm and microscopically small embryo. The arils are fleshy, fimbriate or laciniate at margin for l/3 to nearly the whole length (Hoogland, l953).

2.8.2.4 Ethnobotanical uses

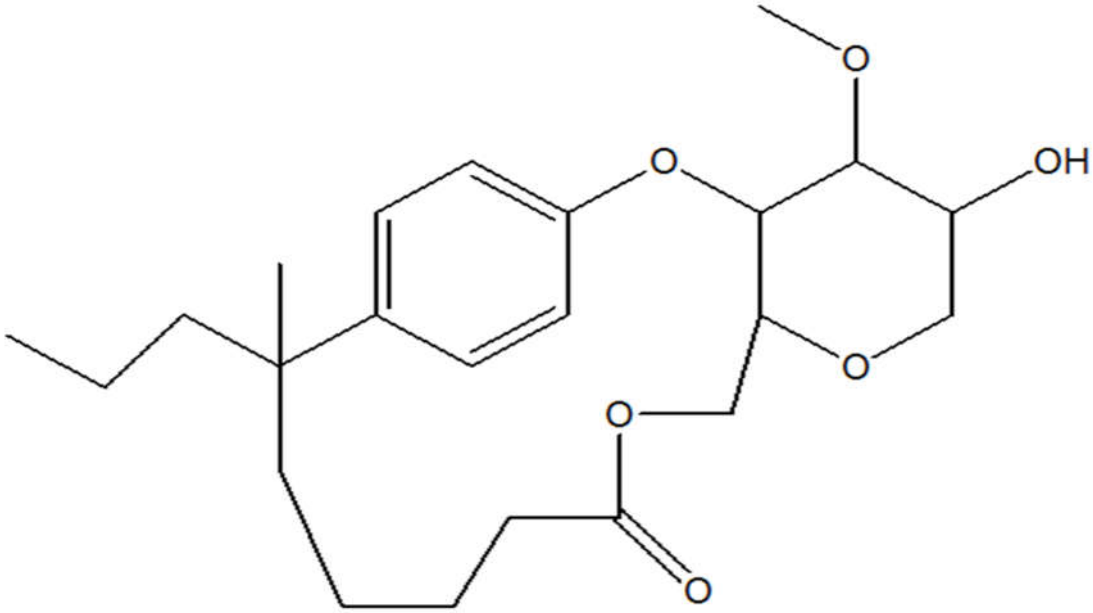
Species is utilised in folk remedies as cure for various illnesses and infections. *Tetracera potatoria*, existing in the forest of Senegal, southern part of Nigeria, Central and Eastern Africa, is effective in the management of back pain, haemorrhoids, diabetes mellitus, jaundice and scurvy. When the leaves and small part of *T. potatoria* stem are boiled in its sap, it can be used as purgative, vermifugal, powerful

diuretic and as cure for gastrointestinal discomfort (Burkill, l985). The sap is active in the management of cough and tooth pain. In Cameroun, its sap is a medication for toothache and cough, TB (tuberculosis) and associated illnesses such as lung infections (Fomogne-Fodjo *et al.*, 20l4). The aqueous extract of the root act as relieve for abdominal discomfort in south-western Nigeria (Adesanwo *et al.*, 2003). *Tetracera potatoria* has also been reported as inflammatory agent and effective remedy against skin infection and sore (Adesanwo *et al.*, 20l3).

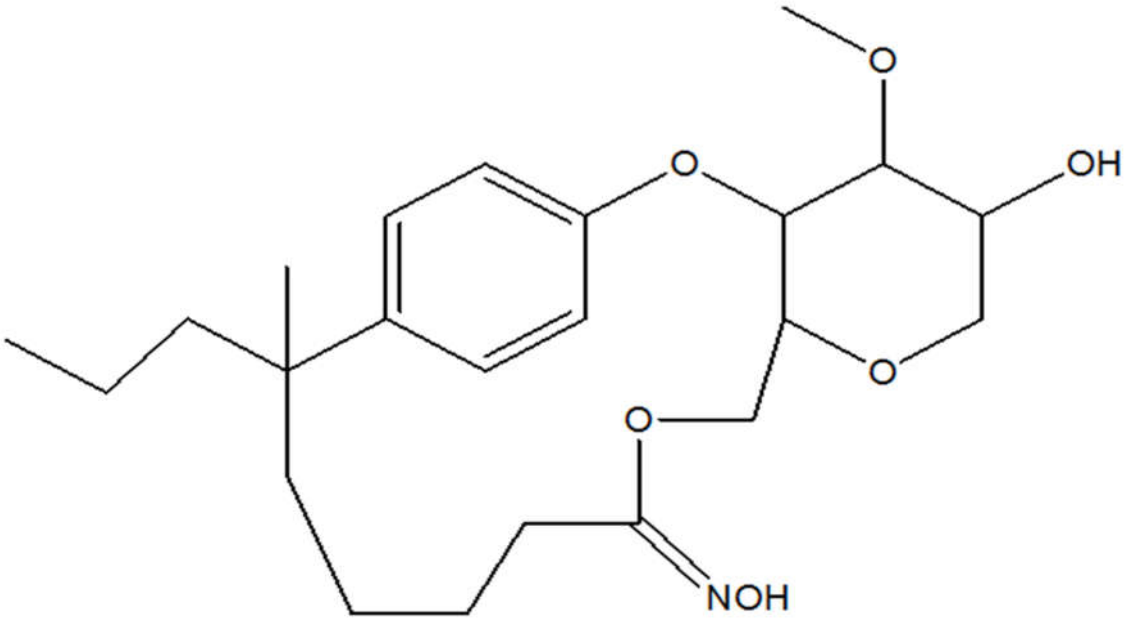
2.8.2.5 Chemical constituents of *Tetracera potatoria*

The phytochemicals reportedly present in the root of *T. potatoria* are tannins, alkaloids, cardiac glycosides, flavonoids and saponins (Adesanwo *et al.*, 20l3). However, few of these compounds have been isolated (Figures 2.8.13, 2.8.14, 2.8.15, 2.8.16). Betulinic acid (Adesanwo *et al.*, 20l3), N hydroxy imidate-tetracerane, stigmast-5-en-3β-yl acetate, tetraceranoate, β-stigmasterol, lupeol and botulin (Fomogne-Fodjo *et al.*, 20l6) have been isolated from various parts of *T. potatoria*. Tetraceranoate, a new compound recently isolated from *T. potatoria* exhibited inhibitory effect against *M. smegmatis* (MIC=7.8 µg/mL), whereas β-stigmasterol, betulinic acid and betulin exhibited significant anti-mycobacterial effect against several strains (Fomogne-Fodjo *et al.*, 20l6).

Figure 2.8.8: *Tetracera potatoria* in Oluponna, Osun State, Nigeria

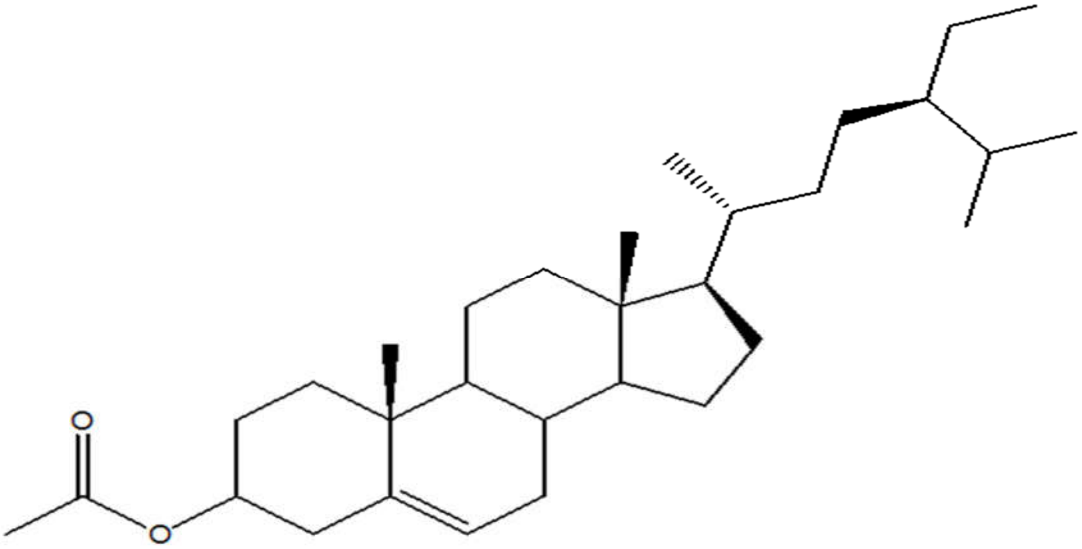


Tetraceranoate

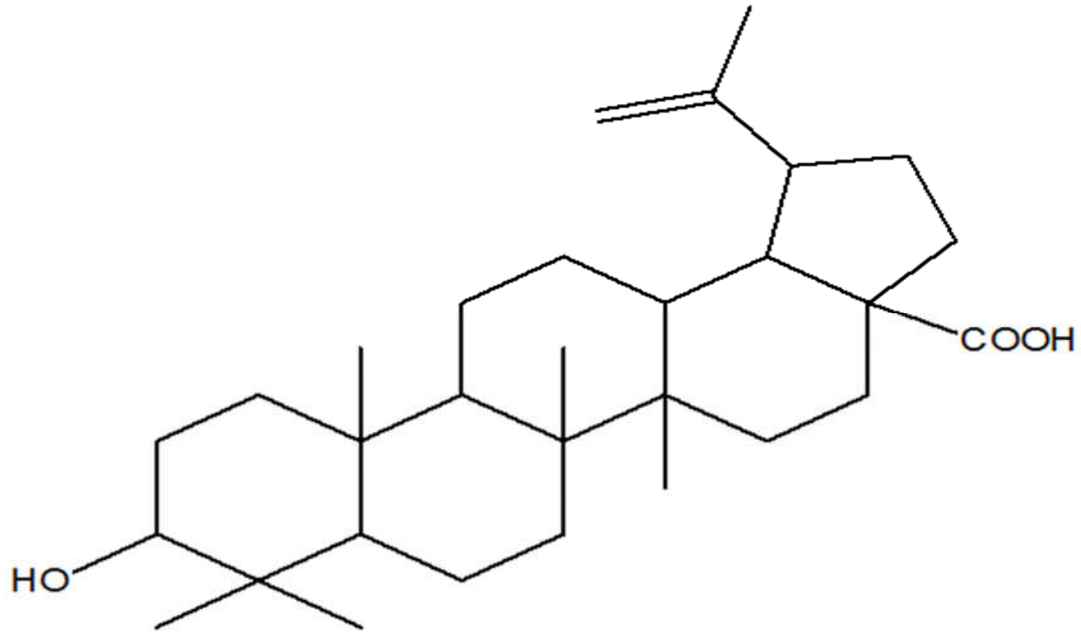


N hydroxy imidate-tetracerane

Figure 2.8.9: Structure of tetraceranoate and N hydroxy imidate-tetracerane isolated from *T. potatoria* (Fomogne-Fodjo *et al.*, 20l6).



Betulin



Betulinic acid

Figure 2.8.10: Structure of betulin and betulinic acid isolated from *T. potatoria* (Adesanwo *et al.*, 2013)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.l Chemicals, materials and reagents

All sovents used for the study, which includes *n*-hexane , chloroform, dichloromethane, ethyl acetate and methanol, were obtained from BDH Ltd, England.The reagents, such as Folin–Ciocaltaeu reagent, Sodium bicarbonate, $FeCl_3$, CH_3COOK, $AlCl_3$, Vitamin C (ascorbic acid), Gallic acid, Quercetin, PCA, Rutin, Genistein, Diadzein and l, l-diphenyl-2-picryl-hydrazyl (DPPH) were supplied by Merck, Germany. Brine Shrimp Egg was bought as Artemia salina cysts, from a pet shop in United State of America. Human cervical carcinoma (HeLa) and Chinese Hamster Ovarian (CHO l) cell lines were obtained from ATCC, Manassas, USA.

3.1.1 Preparation of reagents used

Ferric chloride ($FeCl_3$) solution: 2 g of FeCL3 dissolved in 83 mL *n*-butanol and l5 mL concentrated sulphuric acid.

Aluminium trichloride solution: l g of $AlCl_3$ dissolved l00 mL 95% ethanol.

Formal saline: Mixture of 100 mL of 40% of formaldehyde, 9 g of NaCl and 900 mL of distilled H_2O.

3.2 Ethnobotanical study

The ethnobotanical survey was conducted in Iwo metropolis of Osun State, southwestern Nigeria. Osun State with a landmass of approximately l4,875 Km^2 is located between latitude 7.0° N to 8.0° N and longitude 04°.l0'E to 05°.05'E. The annual rainfall of the southern part differs from that of the Northern part with values of ll25 mm and l475 mm per annum respectively (Abe, l995). The vegetation lies in the lowland rain forest zone of South-Western Nigeria and the derived savannah covers Iwo and Osogbo (Abe, l995). Three local government areas (LGAs) in Iwo metropolis were selected as study area. Figure l shows the study areas namely: Aiyedire LGA (Oluponna, Railway station); Iwo LGA (Iwo town) and OlaOluwa LGA (Obamoro, Ikonifin). Identification of some villages without access to modern health facilities in the study area necessitated the advancement of traditional health care system, which justifies the vast understanding of usage medicinal plant application in this metropolis. The indigenes of this community are Yorubas hosting nomadic Fulanis, Hausas, Ibos and farmers from other countries such as Togo and Benin Republic. The women and men in this region are traders, farmers, civil servants and craftsmen.

Among manifestations of PCOS are menstrual disorder and infertility. Therefore, data on traditional use of medicinal plants known for managing menstrual disorders and female infertility were documented through interviews and Focus group discussions (FGDs) with herb sellers (38.8%), herbalists (l2.7%), community chiefs (4.8%), hunters (l9.0%) and traditional religious leaders (24.6%) in Iwo metropolis. Collection of data was done between June 4 and September l6, 20l6. One hundred and twenty six people fully participated in the study. Interviews and FGDs were conducted with prior permission of the potential participants, aged between 2l and 68 years, in Yoruba language. The list of plants known to be efficacious in the management of menstrual disorders and female infertility in the community and their methods of preparation were documented. The associations of herb sellers, herbalists and hunters in each LGA were visited differently during their meetings where the intention of the survey was made known to all members. All the members were met after the meetings for interviews to collect names of medicinal plants with their modes of preparation and administration. Community chiefs and traditional religious leaders were visited in their various houses to seek further clarifications on some of the plants mentioned at FDGs. Some of these plants were planted in their courtyard. Other uses of the plants mentioned in this survey were also documented. The local and scientific names of medicinal plants mentioned during the survey were validated with research journals. Literatures were also consulted to document the phytochemical constituents of the medicinal plants mentioned.

3.3 Selection of plant for the study

Plants were selected from four UMi categories for this study after literature validation on the extent of work reported on the use of the plants as remedies for female infertility and menstrual disorder. The focus was targeted at plants that have not been previously or extensively reported for the activity. The six plants selected from the ones mentioned in the survey included *Momordica charantia* from the second UMi category (UMi 0.0397), *Basella alba* and *Newbouldia laevis* from the third UMi category (0.03l7), *Kigelia africana* and *Lagenaria breviflora* from the fourth UMi category (UMi 0.0238) and *Tetracera potatoria* from the sixth UMi category (UMi 0.0079).

3.4 Plants collection and extraction

Six plants, *Newbouldia laevis* (Beauv.) Seem. ex Bureau (leaf), *Tetracera potatoria* Afzel. ex G. Don (leaf), *Momordica charantia* L. (leaf), *Kigelia africana* (Lam.) Benth. (fruit), *Basella alba* L. (leaf) and *Lagenaria breviflora* (Benth.) Roberty (fruit) selected from the plants mentioned in the survey based on their UMi categories were collected in Oluponna, Osun state; they were dried and pulverised into coarse powder using a grinding machine. The powdered samples were marcerated in methanol for 72 h at room temperature with occasional stirring and shaking followed by filtration through a fresh cotton plug and

Whatman (Number l) filter paper. Filtrates collected were concentrated *in vacuo* using rotary evaporator (Buchi-Germany) at 40°C. The weights of the crude extracts were determined and the percentage yield was calculated.

3.5 Preliminary thin layer chromatographic analysis of extracts

Small quantities of methanol extracts, l mg/mL, and standards dissolved in methanol were spotted on TLC plates. Thin layer chromatography was performed according to standard method (Harborne, l998) using pre-coated TLC plates (Silica gel G_{60} F_{254} sheets 20 × 20 cm, 0.5 mm thickness, Merck). Thin strips (6.5 x l0 cm) of TLC Silica plates were cut out and impregnated with the fine drop of samples. The mobile phase used for this analysis was ethyl acetate, methanol and water (3.5: l: 0.5). All plates were visualised directly after drying and with the help of UV lamp (Gallenkamp- Germany) at 254 nm and 366 nm. Visualising/locating agents were employed which include aluminium trichloride solution for detection of flavonoids, ferric chloride ($FeCl_3$) solution for detection of phenolics and Dragendorf reagents to identify the existence of alkaloids in the extracts on the plates (Wall, 2000). Furthermore, TLC was also carried out to identify isoflavone (genistein and diadzein) in all methanol extracts. The spots were marked for calculation of retardation factor (R_f) values. The R_f value of each spot was compared with the R_f value of standard genistein and diadzein. The developing solvent system was Chloroform/Methanol (5: 0.5, v/v).

3.6 Brine shrimp lethality assay

The dried methanol extracts and cyclophosphamide (standard), 30 mg each, were re-constituted in MeOH (3 mL) to achieve a stock solution of l0 mg/mL (that is, 10,000 µg/mL) for each extract to be investigated for toxicity. Serial dilution of part of the stock solution was done affording concentrations of l000, 500, l00, l0 and l µg/mL (McLaughlin *et al.*, l998). After 48 h of hatching, ten nauplii were picked and conveyed into each vial different concentrations of test samples using a 23 cm disposable Pasteur pipette. This set up was placed under illumination for 24 h. Sea water was used as control. Nauplii were considered dead when they were immobile and stayed at the bottom of the test tubes. The lethal concentrations of plant extracts, LD_{50} resulting in 50% death of the shrimp larva, LD_{50}, were calculated using the number of surviving naplii after 24 h by using Finney's Probit analysis at 95 confidence intervals.

3.7 Solvent-solvent partitioning

Newbouldia leavis (leaf), *Tetracera potatoria* (leaf), *Mormodica charantia* (leaf), *Kigelia africana* (fruit), *Basella alba* (leaf) and *Lagenaria breviflora* (fruit) crude extracts (5.02, 8.90, 6.06, 5.00, 10.02

mg, respectively) were re-dissolved in methanol-water (3:l) and poured into different separating funnels. Each of these extracts was mixed with aliquots (50 mL) of *n*-hexane, dichloromethane (DCM) and ethyl acetate successively. Each aliquot of *n*-hexane, DCM and ethyl acetate fractions were individually pooled and evaporated *in vacuo* to give residues which were kept in air tight containers for subsequent preliminary assays. The weights of the partitioned extracts were recorded and used to calculate the percentage yield for each extracts

3.8 Diphenyl picryl hydrazyl (DPPH) assay

The free radical scavenging effect of the methanol extract and the fractions was assessed according to the procedures described by Bursal and Gülçin (20ll) with slight modifications. For DPPH assay, 2 mL methanol solution of *Kigelia africana* crude extract, solvents fractions and standards (ascorbic acid and rutin) at varying concentrations (200, l00, 50, 25, l2.5, 6.25 and 3.l25 µg/mL) were added separately to 3 mL (0.004%) of l, l-diphenyl-2-picryl-hydrazyl-hydrate (DPPH). Two mL of methanol was added as test sample in the control. The reaction mixtures were shaken vigorously and kept warm at 27 °C for 30 min in the dark. Absorbance of each vial was measured at 5l7 nm using Spectrumlab 752S UV–VIS spectrophotometer and expressed in percentage inhibition using [l-absorbance of solution with sample and DPPH/absorbance of solution with DPPH] × l00. Percentage inhibition concentration (IC_{50}) value was calculated using the plotted graph of scavenging activity against the concentration of the test samples (using linear regression analysis).

3.9 Measurement of total phenolic content (TPC)

Total phenolic content of the methanol extracts and fractions were measured using Folin-Ciocalteu spectrophotometric method (Miliauskas and Venskutonis, 2004). Five mL of Folin–Ciocalteu's phenol reagent (diluted tenfold) was added into l mL aliquots of each test samples (l00 µg/mL). Afterward, 4 mL of Na_2CO_3 solution in distilled water (7.5 g/l00 mL) was added to mixture in each vials and thereafter incubated at 27 °C for 30 min in the dark. Blank was set up with l mL methanol. Each sample was analysed in triplicate. Absorbance of mixture after 30 min of incubation was read at 765 nm with UV–VIS spectrophotometer (Spectrumlab 752S). Total phenolic content was calculated using linear dose response regression curve (Figure l) generated from the absorbance of Gallic acid. Result of TPC was expressed as mg Gallic acid equivalent/ g of dry weight of extracts.

3.10 Measurement of total flavonoid content (TFC)

The aluminium chloride colorimetry method used in this study was modified procedure reported by Woisky and Salatino (l998). Quercetin was used as standard to plot calibration curve (Figure 2) prepared

by diluting quercetin in ethanol (l00–6.25 µg/mL). Test samples were also prepared by re-constituting 30 mg of all test materials in 30 mL methanol. One mL of each diluted quercetin solutions or test samples (l.0 mL) were mixed separately with l.0 mL of methanol, 0.l mL of l% aluminium chloride, 0.l mL of l M potassium acetate and 2.8 mL. The amount of l% aluminium chloride was substituted by the same amount of distilled water in the blank. Each sample was analysed in triplicate. Incubation was done at 27 °C for 30 min and the absorbance of the reaction was measured at 4l5 nm using UV–VIS spectrophotometer (Spectrumlab 752S). Total flavonoids content for each test samples were deduced from the equation obtained from quercetin calibration curve and expressed in terms of quercetin equivalent (mg Quercetin equivalent/g of extract).

3.11 Large scale collection and extraction of plant materials

The presence of constituents corresponding with the R_f of the standards, genistein and diadzein coupled with strong antioxidant activity of *Tetracera potatoria* (leaf), *Mormodica charantia* (leaf), *Kigelia africana* (fruit) and *Basella alba* (leaf) suggest their beneficial effect in the management of PCOS. These selected plants were collected at Oluponna, Osun state. They were identified at Forest Herbarium, Ibadan (FHI). The voucher specimens were deposited in FHI and Department of Pharmacognosy Herbarium, University of Ibadan (DPHUI), Ibadan. The dried pulverised *Tetracera potatoria* (1 kg), *Mormodica charantia* (1 kg), *Kigelia africana* (3 kg) and *Basella alba* (0.7 kg) powder samples were extracted with methanol at room temperature individually for 72 h with random stirring. The extracts were filtered separately and concentrated *in vacuo* using rotary evaporator (Buchi-Germany). The extracts obtained were kept in the refrigerator (Haier Thermocool Hoomex refrigerator) for subsequent use.

3.12 *In vivo* PCOS study

3.12.1 Dosage for plant extracts

The reported human therapeutic doses of selected medicinal plants mentioned in the ethnobotanical survey conducted for managing irregular menstrual disorder and associated gynaecological disorders are between l to 9 g (Annon., 2017). The dose for rat was calculated considering human to albino rat conversion factor (conversion factor = 0.0l8) according to body surface area. This was done by dividing it by adult human weight of 60 kg and multiplying it by factor to accommodate the body surface area of the animal (Nair and Jacob, 20l6). The calculated dose obtained fall within the range of approximately l00 mg/kg to 900 mg/kg *b.w* rat. Therefore, dose level of l00 mg/kg *b.w.* was used in this research work.

3.12.2 Selection and grouping of animals for assay

Thirty five non-pregnant, female albino rats (l50 - 200 g) with normal estrous cycle were randomly grouped into seven made of five animals per group. Six groups of these albino rats were treated with l mg/kg letrozole orally for a period of 2l consecutive days with 0.5% w/v carboxymethyl cellulose (CMC) as vehicle for the induction of PCOS. The groups were treated with extracts of selected medicinal plants as follows:

Groups one, two, three and four received 100 mg of *K. africana***,** *B. alba*, *Tetracera potatoria* and *M. charantia* extracts, respectively per kilogramme body weight while Group five received l mg of clomiphene citrate (Colid, Pfizer pharamceuticals, USA) per kilogramme body weight for 15 days. Group six (untreated disease control) and seven received 2 mL of 5% w/v CMC in distilled water (Kafali, *et al.,* 2004).

3.12.3 Determination of oestrous cycle pattern

The phases of the oestrous cycle were detected by examining vaginal cytology (Marcondes *et al.,* 2002). Vaginal lavage was obtained with a Pasteur pipette filled with 0.l mL of normal saline (0.9% NaCl), gently inserted into the rat's vagina. The withdrawn vaginal fluid was dropped on a glass slide and immediately viewed under the microscope using x l0 objective to examine the distribution of cells. This was done between 7am and 9am daily throughout the period of the study.

3.12.4 Animal sacrifice

The animals were sacrificed after 15 days of treatment (24 h after last treatment) with 2% sodium pentobarbital (30 mg/kg) for laparotomy. The tissue architecture of the ovaries of the animals euthanized between 0900-ll00 h to minimise diurnal variation were analysed. The blood collected (about 8 mL) were used for determination of the level of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and estradiol in each rat.

3.12.5 Ovarian histology

The ovaries were examined according to standard method (Avwioro, 20l0). Haematoxylin and Eosin stain technique was used. The tissues were observed and dissected into small pieces of not more than 4mm thick into pre-labelled cassettes. These small pieces of ovary tissues were immersed in l0% formal saline for 24 h for fixing. Tissue processing was done automatically using automatic tissue processor (Leica TP l020). The tissues were dehydrated by passing them through various dehydrating reagents such as l0% formal saline and alcohol (70%, 80%, 90% and 95%). The tissues were immersed in the

molten paraffin wax, dispensed into a metal mould, and was transferred to a cold plate to solidify. The tissue block formed was separated from the mould and were trimmed to expose the tissue surface using a rotary microtome at 6 µm. The surfaces were placed on ice and sectioned at 4 µm (ribbon section). The sections floated on water bath (Raymond lamb) set at 55^0C were picked using clean labelled slides, dehydrated on a hotplate (Raymond lamb) set at 60 ^{0}C for l h and viewed under the light microscope using x l00 and x 400 objective.

3.12.6. Hormone analysis

Estradiol was assayed in the serum samples using the ELISA (Fortress Diagnostics limited, Unit 2C Antrim Technology Park, Antrim, BR4l lQS, United Kingdom). Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) were assessed in the serum samples using the ELISA manufactured by Dialab. The samples and the test reagents were equilibrated at room temperature prior the test. Then 0.05 mL of calibrators and rat's samples were pipetted inside the wells followed by addition 0.l mL of dilute enzyme conjugate to each well excluding the blank well. These mixtures were incubated for 60 minutes at room temperature. The mixtures in the microwells were thrown out and the wells were cleaned with 0.2 mL of distilled water. This was done twice in order to remove water in the well. Solution of the substrate (0.l mL) was pipetted into each microwell in the same order and interval as for the enzyme conjugate, blank well was included and incubated for 20 min at room temperature in the dark. Stop solution (0.l mL) was added into each microwell using the same order and timing as for the reaction of the substrate solution. Absorbance of each microwell was read at 450 nm against blank using a microplate reader. The developed colour was stabled for at least 30 minutes and the optical densities were read during this time. All unused specimens and reagent were returned to the refrigerator immediately after use. Required micro well strips were removed carefully from the pouch and the pouch was resealed to prevent condensation in the unused wells. The pouch was returned immediately to refrigerator.

3.13 Anti-proliferative activity

Antiproliferative effect of crude and solvent fractions of *K. africana* and *Tetracera potatoria* on HeLa and CHO cell lines (ATCC, Manassas, USA) were evaluated by standard MTT colorimetric assay (Mosmann, l983). One hundred microliter (l00 µL) of $5 \times l0^4$ cells/mL of HeLa (Human cervical cancer cell lines) and $6 \times l0^4$ cells/mL of CHO l (Chinese Hamster Ovarian cancer cells) proliferating cells in DMEM (Dubecco's modified Eagle's medium) supplemented with l0% FBS were seeded into 96-wells flat bottom plate and incubated overnight at 37 ºC in 5% CO_2. Three different concentrations of crude extracts and their respective solvent fractions (l, l0 and l00 µg/mL) were added to the plate in triplicates

and incubated for 2 days. Fifty microliter (50 µL) of 0.5 mg/mL MTT was added to each well and plate was then further incubated for 4 hours. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was aspirated and l00 µL of DMSO was then added to all test and control wells. The magnitude of enzymatic MTT reduction to its formazan (Figure 3) by NADH of the living cells (Tachon *et al.*, 2009) was calculated by measuring the absorbance at 540 nm, using spectrophotometer (Spectra Max plus, Molecular Devices, CA, USA). The antiproliferative activity was recorded as concentration causing 50% growth inhibition (IC_{50}) for solvent fractions. This assay was repeated for isolated and derivatised compounds.

3.14 Isolation and characterisation of compounds

3.141 Isolation from hexane fraction of *Kigelia africana*

Green coloured n-hexane fraction (0.39 g) was subjected to CC (length of the column = 88 cm; Height of silica in the column = 46 cm; Mesh size = 60-120; Diameter = 2.5 cm) and eluted with Hexane-DCM (50:50) and l00% DCM (Volume collected = 20 mL). Two hundred and twenty fractions were collected. These 220 fractions were pooled to l5 fractions (A-O). Fraction B, C and D (based on similar TLC chromatogram) were pooled to give A^1 (60 mg). Fraction A^1 was rechromatographed (length of the column = 42 cm; Height of silica in the column = l7 cm; Diameter = l cm) using Hex: DCM (50:50) and l00% DCM (Volume collected = 20 mL) which yielded 24 fractions. Sub-fractions l4 to l8 were pooled to give $A^{1}{}_{(l4\text{-}l8)}$ of weight l4.l9 mg. This was subjected to preparative TLC and developed with Hexane-DCM-ethyl acetate (l0:9:l) to yield **l** (8.85 mg, yellow amorphous solid) while sub-fraction $A^{1}{}_{l3}$ crystalized to give **2** (22.43 mg, white amorphous solid).

3.l4.2 Isolation from DCM fraction of *K. africana*

Yellow DCM fraction (3.03 g) was subjected to CC (length of the column = 70 cm; Height of silica in the column = 30 cm; Mesh size = 60-120; Diameter = 4 cm) and eluted with Hexane, Hexane-DCM, DCM-Ethyl acetate and Ethyl acetate-methanol with increasing polarity (volume collected = 20 mL) yielding 572 fractions. These fractions were pooled to 20 fractions (A-T). Fraction K_{II} (fractions 208-247, brown coloured, 630 mg) was subjected to prep-TLC and developed with DCM-ethyl acetate (50:50) to yield **3** (2l.40 mg, light brown amorphous solid) and **4** (8.72 mg, greenish-yellow amorphous solid).

3.14.3 Isolation from ethyl acetate fraction of *K. africana*

Brownish-yellow ethyl acetate fraction (7.48 g) was subjected to CC (length of the column = 56 cm; Height of silica in the column = 34 cm; Mesh size = 60-120; Diameter = 4 cm) and eluted with hexane, hexane-DCM, DCM-Ethyl acetate and Ethyl acetate-methanol with increasing polarity (volume collected = 200 mL) to give l5l fractions. These fractions were pooled to l4 fractions (A-N). Pooled fractions C (fractions 20-25, greenish, 60.02 mg) and D (fractions 26-36, light green, 50.03 mg) were pooled and **2** (27.24 mg, white amorphous solid) crystalized out of this pooled fraction. Pooled fraction I (86-l06, light brownish, l50.60 mg) vial prep-TLC technique (developed with DCM-ethyl acetate (70:30) with few drops of acetic acid) yielded **5** (24.23 mg, pink coloured needle-like crystal**)** was isolated from p. Furthermore, J (fractions l07-ll6, light brownish-yellow, 200.23 mg) was also subjected to prep-TLC (DCM-ethyl acetate (70:30) with few drops of acetic acid) and **6** (44.76 mg, yellow amorphous solid**)** was isolated.

3.l4.4 Isolation from DCM fraction of *Tetracera potatoria*

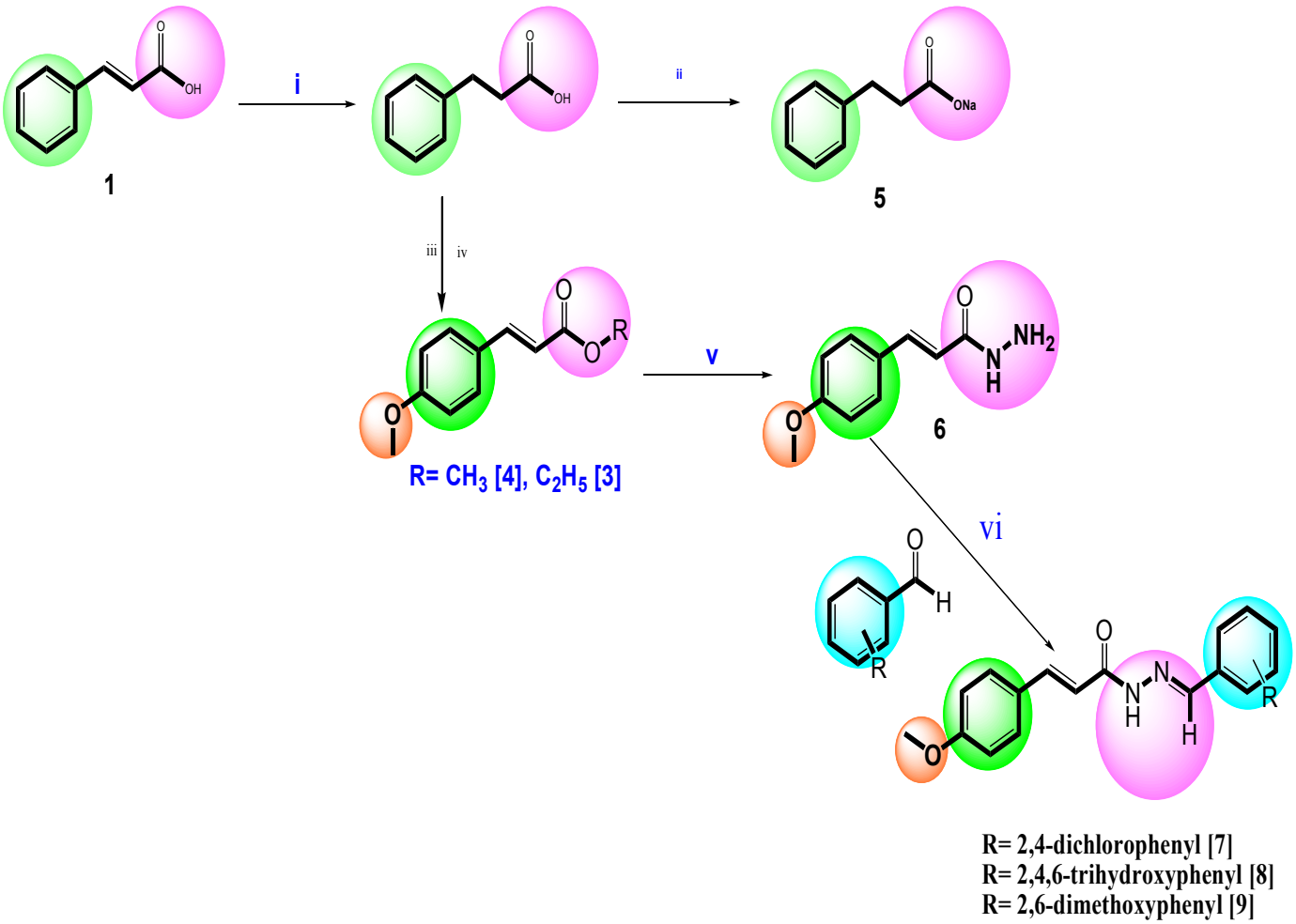
Light-green DCM fraction (2.62 g) was subjected to CC (length of the column = 88 cm; Height of silica in the column = 57 cm; Mesh size = 120-160; Diameter = 2.5 cm) and eluted with hexane, hexane-DCM, DCM-Ethyl acetate and Ethyl acetate-methanol with increasing polarity (volume collected = 200 mL) to give 80 fractions. Fraction 40 (greenish yellow, 200 mg) vial prep-TLC technique (developed with DCM-ethyl acetate - 80:20, with few drops of acetic acid) yielded **7** (6.28 mg, yellow powder**)**.

3.l5. Derivatisation of cinnamic acid

As shown in Scheme 3.l5.1, cinnamic acid (0.l48g, l mmol) was mixed with sodium metal (l mmole) in l5 mL of ethanol at room temperature for 4 hours to give sodium cinnamate (KAD l). The methyl -3-(4-methoxyphenyl) acrylate (KAD 3) (0.l92g, 0.l mmole) was added to 20 mL ethanol and hydrazine hydrate 98% (about 0.04 mL, 0.l2 mmol) and the product, compound (KAD 6), was formed. The intermediate compound, KAD 6 (l mmole, 0.l92 g), was dissolved in 20 mL of ethanol (solvent). To the mixture, l mmole of respective aromatic aldehydes were added, the reaction was catalysed with 0.l5 mL of acetic acid. Large precipitation was observed and the precipitate was filtered, dried and weighed to give compounds KAD 7, 9 and l2 according to the functional groups on aromatic aldehyde used (Scheme 3.15.1).

3.16. Statistical analysis

Values were presented as Mean ± Standard Error of Mean (SEM). Data were analysed using one-way analysis of variance (ANOVA) and group means were compared using Dunnett's Multiple Comparison test, Bonferroni tests using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA. The P values < 0.05 were considered significant.



Scheme 3.l5.1: Synthesis of cinnamic acid derivatives

Reagents and reaction conditions: (i) Pt/H_2, MeOH, reflux at 80°C, 5 h. (ii) Sodium metal, Ethanol, reflux at rt, 4 h. (iii) Methanol at 80°C, reflux 12 h. (iv) Ethanol at 80°C, reflux 9 h. (v) Hydrazine monohydrate 99% (NH_2NH_2) in Ethanol, at 80°C, reflux 8 h. (vi) ArCHO, MeOH, Glacial acetic acid (catalytic amount), reflux at 100°C, 3 h.

CHAPTER FOUR

4.0 RESULTS

4.l Ethnobotanical survey

Table 4.l.1 is the summary of demographic characteristics of all the respondents interviewed during the ethnobotanical survey conducted in three selected local government areas (Figure 4.l.1). Out of the l26 respondents contacted during the period of ethnobotanical survey, 39.7% were males and 60.3% females. Almost 49.0% were between 3l and 40 years old, while l9.0% and 70.6% had primary education and secondary school education, respectively. None of the participants had tertiary education. The hunters and herb sellers consulted were registered members of their various associations at the local government level. About 6l.0%, 3l.0% and l4.0% were practicing Islam, traditional and Christian religions, respectively. The largest percentage of the respondents were herb sellers (38.8%), others were traditional religious leaders (24.6%), hunters (l9.0%), herbalists (l2.7%) and chiefs (4.8%).

The medicinal plants mentioned by these respondents as treatment for female infertility and associated menstrual disorders are listed in Table 4.1.2 and the methods of preparation and enumeration of recipes for herbal preparations were also documented and presented in Table 4.1.3. The characteristics (family, common names and plant parts used) of therapeutic plants mentioned for menstrual disorder and female infertility management are presented in Table 4.1.2. Twenty nine medicinal plants in 20 families were documented. Based on UMi the twenty nine plants were in six categories. One plant each was found in the first (UMi 0.0556) and second (UMi 0.0397) categories, while six plants were found in the third (UMi 0.03l7) category. The fourth (UMi 0.0238), fifth (UMi 0.0l59) and sixth (UMi 0.0079) categories had seven plants each. The plant families mostly used were Euphorbiaceae (20%), Cucurbitaceae (l5%), Bignoniaceae (l0%), Apocynaceae (l0%), Arecaceae (l0%) and Solanaceae (l0%).

Fidelity level (Table 4.1.4), family, species of each medicinal plant mentioned (Figure 4.1.2) and plant parts mentioned in the ethnobotanical survey (Figure 4.1.2) are presented in tables and charts. For the plant species mentioned for management of menstrual disorders and female infertility, the leaves were the morphological part persistently used (40%) in most herbal preparations, then fruit (25%). Other parts of plant, such as the bark (8.6%), seed (5.7%) and whole plant (2.9%), were also used but to a lesser extent (Figure 4.1.3). Among all the plant mentioned, *Picralima nitida, Elaeis quineensis, Cocos nucifera, Tetracera potatoria*, *Euphorbia lateriflora, Musa paradisiaca and Physalis angulata* had fidelity level of l00% while other plants fidelity level varied per medicinal use (Table 4.1.4).

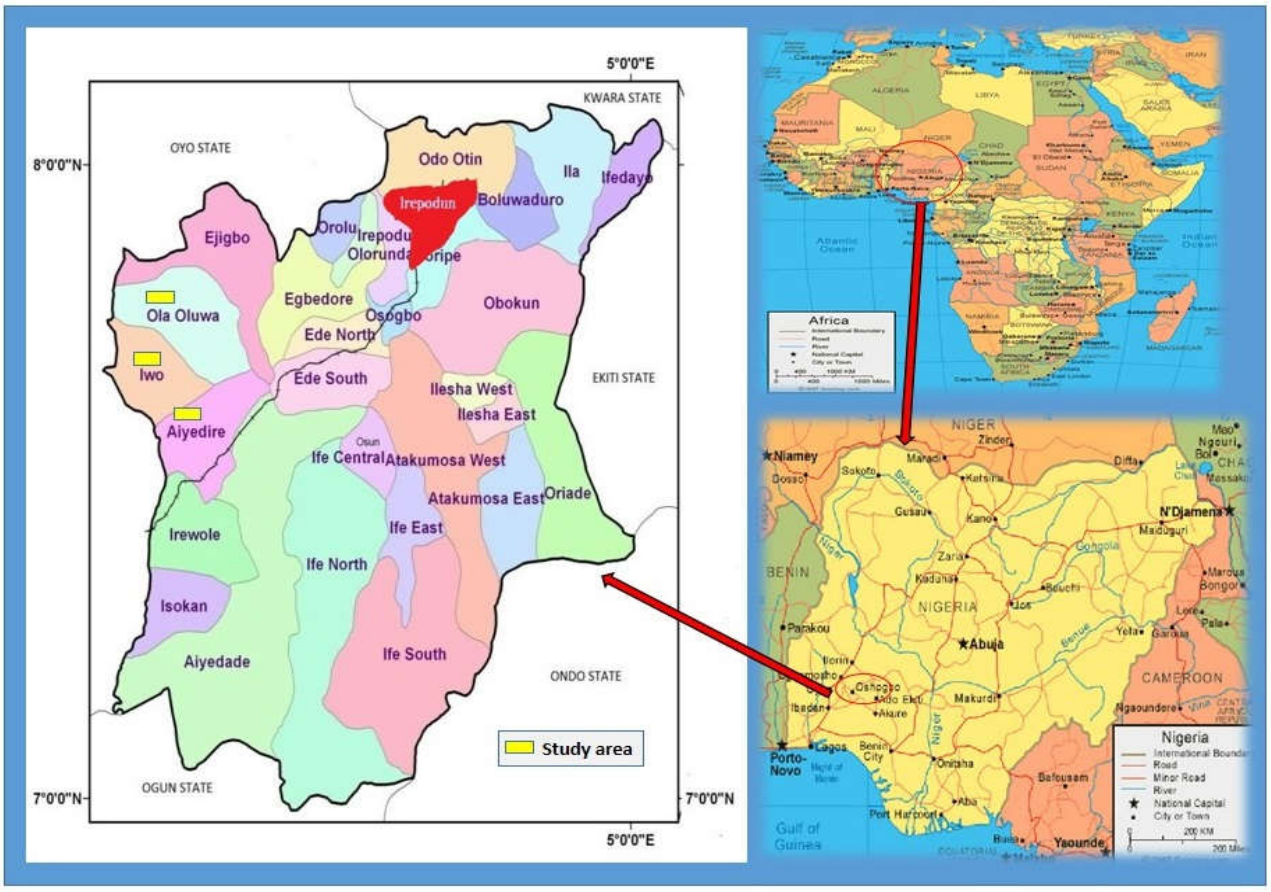


Figure 4.l.1: Map showing the study areas in southwestern Nigeria

Table 4.l.1: Demographic characteristics of the respondents

Characteristics	Specification	Frequency	Percentage (%)
1. Sex	Male	50	39.7
	Female	76	60.3
2. Practice specification	Herb sellers	49	38.8
	Herbalist	l6	l2.7
	Chiefs	6	4.8
	Hunters	24	l9.0
	Traditional religious leaders	3l	24.6
3. Age (year)	2l-3l	30	23.8
	3l-40	40	3l.7
	4l-50	27	2l.4
	5l-60	l2	9.5
	>60	l7	l3.5
4. Religion	Islam	78	6l.9
	Christianity	l8	l4.3
	Traditional	40	3l.7
5. Marital status	Divorce	12	9.5
	Widow(er)	22	17.5
	Single	30	23.8
	Married	64	50.8
6. Educational status	Tertiary institution	0	0.0
	Secondary school	89	70.6
	Primary school	24	l9.0
	No formal education	l3	l0.3
7. Nationality (Tribe)	Nigerian (Yoruba tribe)	l26	l00
	Non-Nigerian	0	0.0

Table 4.1.2: Plants species mentioned as remedy for menstrual disorders and female infertility in Iwo metropolis (Osun State)

Family/ Species	Local name(s)	Part used	Medicinal uses	Growth form	Times stated (n_{is})	Use mention index (UMi)	Number of uses (by respondent, U_{is})	Use value (UV_{is})
Anacardiaceae								
1. Spondia mombin L.	Iyeye	Leaf and seed	Treatment of Female infertility, vaginal infections, malaria and to induce labour	Tree	30	0.0317[l]	4	0.l333
Annonaceae								
2. *Xylopia aethiopica* (Dunal) A. Rich	Eeru alamo	Fruit	Treatment of menstrual disorder, stomach, joint pains and infertility	Tree	26	0.0317[l]	4	0.l539

Table 4.1.2: Plants species mentioned as remedy for menstrual disorders and female infertility in Iwo metropolis (Osun State) contd.

Family/ Species	Local name(s)	Part used	Medicinal uses	Growth form	Times stated (n_{is})	Use mention index (UMi)	Number of uses (by respondent, U_{is})	Use value (UV_{is})
Apocynaceae								
3. *Alstonia boonei* De Wild.	Ahun	Leaf	Treatment of Female Infertility, malaria, and impotence	Tree	l0	0.0238^{m}	3	0.3000
4. *Picralima nitida* Stapf Th. & H. Dur.	Erin	Fruit	Treatment of menstrual disorder	Tree	2	0.0079^{o}	1	0.5000
Arecaceae								
5. *Elaeis quineensis* Jacq.	Eyin (abon)	Fruit (Unripe)	Treatment of Female Infertility	Tree	l0	0.0079^{o}	1	0.l000
6. *Cocos nucifera* L.	Agbon	Fruit water	Treatment of Infertility.	Tree	1	0.0079^{o}	1	l.0000

Table 4.1.2: Plants species mentioned as remedy for menstrual disorders and female infertility in Iwo metropolis (Osun State) contd.

Family/ Species	Local name(s)	Part used	Medicinal uses	Growth form	Times stated (n_{is})	Use mention index (UMi)	Number of uses (by respondent, U_{is})	Use value (UV_{is})
Asteraceae								
7. Vernonia amygdalina Del.	Ewuro	Leaf	Treatment of menstrual disorder, fibroid, stomach ache, ringworm, typhoid fever, headache and diabetes	Tree/ Shrub	45	0.0556[j]	7	0.l556
Basellaceae			Treatment of Female infertility, irregular periods, acne and sterility	Climber	23	0.0317[l]	4	0.l739
8. Basella alba L.	Amunu-tutu, gbowo-le-ganna	Complete aerial parts						

Table 4.1.2: Plants species mentioned as remedy for menstrual disorders and female infertility in Iwo metropolis (Osun State) contd.

Family/ Species	Local name(s)	Part used	Medicinal uses	Growth form	Times stated (n_{is})	Use mention index (UMi)	Number of uses (by respondent, U_{is})	Use value (UV_{is})
Bignoniaceae								
9. *Kigelia africana* (Lam.) Benth	Pandoro	Fruit	Treatment of female Infertility, skin infections and vaginal infections	Tree	l7	0.0238^{m}	3	0.l765
10. Newbouldia laevis (Beauv.) Seem. ex Bureau	Akoko	Bark, root and leaf	Treatment of menstrual disorder, fibroid, impotence and infertility	Tree/ Shrub	25	0.0317^{l}	4	0.l600
Bixaceae								
11. Bixa orellaina L.	Osun-buke	Leaves	Treatment of Female Infertility, stomach ache and diabetes	Shrub	33	0.0238^{m}	3	0.0909

Table 4.1.2: Plants species mentioned as remedy for menstrual disorders and female infertility in Iwo metropolis (Osun State) contd.

Family/ Species	Local name(s)	Part used	Medicinal uses	Growth form	Times stated (n_{is})	Use mention index (UMi)	Number of uses (by respondent, U_{is})	Use value (UV_{is})
Cucurbitaceae								
12. Lagenaria breviflora (Benth.) Roberty	Tangiri	Fruit	Treatment of Irregular menstrual flow, skin infections and diarrhoea	Climber	l0	0.0238^{m}	3	0.3000
13. *Tetracera potatoria*	Ahara	Leaf	Treatment of unhealthy menstruation	Climber	6	0.0079^{o}	1	0.l667
14. Momordica charantia L.	Ejinrin	Leaf, complete aerial parts	Treatment of Female infertility, malaria, diabetes, painful menstruation and to regulate menses	Climber	2l	0.0397^{k}	5	0.238l

Table 4.1.2: Plants species mentioned as remedy for menstrual disorders and female infertility in Iwo metropolis (Osun State) contd.

Family/ Species	Local name(s)	Part used	Medicinal uses	Growth form	Times stated (n_{is})	Use mention index (UMi)	Number of uses (by respondent, U_{is})	Use value (UV_{is})
Euphorbiaceae								
15. *Euphorbia lateriflora* Schum. & Thonn.	Enu opiri	Leaf	Treatment of Irregular menstrual flow	Shrub	l	0.0079^{o}	l	l.0000
16. Bridelia micrantha (Hochst.) Baill.	Aasa, araasa	Leaf and root	Treatment of menstrual irregularity and diabetics	Tree	l0	0.0l59^{n}	2	0.2000
17. Jatropha gossypifolia L.	Lapalapa pupa	Fruit	Treatment of Irregular menstruation, skin infections and excessive bleeding from the vagina	Shrub	l3	0.0238^{m}	3	0.2308

Table 4.1.2: Plants species mentioned as remedy for menstrual disorders and female infertility in Iwo metropolis (Osun State) contd.

Family/ Species	Local name(s)	Part used	Medicinal uses	Growth form	Times stated (n_{is})	Use mention index (UMi)	Number of uses (by respondent, U_{is})	Use value (UV_{is})
18. *Mallotus oppositifolius* (Geisel.) Müll	Ipa, Ija,	Leaf	Treatment of Female Infertility and headache	Shrub	27	0.0l59^{n}	2	0.074l
Fabaceae								
19. Pterocarpus osun Craib	Osun	Seed, leaf	Treatment of Irregular menstrual flow, unhealthy vaginal secretion and skin infections	Tree	4l	0.0238^{m}	3	0.0732
Hypericaceae								
20. *Harungana madagascariensis* Lam. ex Poir	Amuje	Stem bark	Treatment of Irregular menstrual flow and stomach ache	Tree	7	0.0l59^{n}	2	0.2857

Table 4.1.2: Plants species mentioned as remedy for menstrual disorders and female infertility in Iwo metropolis (Osun State) contd.

Family/ Species	Local name(s)	Part used	Medicinal uses	Growth form	Times stated (n_{is})	Use mention index (UMi)	Number of uses (by respondent, U_{is})	Use value (UV_{is})
Menispermaceae								
21. *Cissampelos owariensis* P. Beauv.	Jenjoko	Leaf	Treatment of Stomach disorder during menstruation, excessive bleeding during menstruation, diabetes and infertility	Climber	20	0.0317[l]	4	0.2000
Moraceae								
22. *Erythrophleum suaveolens* Guill and Perr Brenan	Igi obo	Bark and leaf	Treatment of menstrual disorder and skin infections	Tree	3	0.0159[n]	2	0.6667

Table 4.1.2: Plants species mentioned as remedy for menstrual disorders and female infertility in Iwo metropolis (Osun State) contd.

Family/ Species	Local name(s)	Part used	Medicinal uses	Growth form	Times stated (n_{is})	Use mention index (UMi)	Number of uses (by respondent, U_{is})	Use value (UV_{is})
Musaceae								
23. Musa paradisiaca L.	Ogede agbagba	Fruit peel	Treatment of Stomach disorder during menstruation	Tree	5	0.0079^{o}	1	0.2000
Portulacaceae								
24. Talinum triangulare (Jacq.) Willd.	Gbure	Leaf	Treatment of Stomach disorder during menstruation and diabetes	Herb	l5	0.0l59^{n}	2	0.l333

Table 4.1.2: Plants species mentioned as remedy for menstrual disorders and female infertility in Iwo metropolis (Osun State) contd.

Family/ Species	Local name(s)	Part used	Medicinal uses	Growth form	Times stated (n_{is})	Use mention index (UMi)	Number of uses (by respondent, U_{is})	Use value (UV_{is})
Rubiaceae								
25. Morinda lucida Benth.	Oruwo	Root, bark	Treatment of Irregular menstrual flow, malaria, diabetes, stomach disorder during menstruation	Tree	l7	0.0238^{m}	3	0.l765
Santalaceae								
26. Viscum album L.	Afomo-obi	Whole plant	Treatment of Irregular menstrual flow and diabetes	Herb (parasite)	l0	0.0l59^{n}	2	0.2000
Solanaceae								
27. Capsicum frutescens L.	Ata-eye	Fruit	Treatment of Irregular menstrual flow and ulcer	Herb	9	0.0l59^{n}	2	0.2222

Table 4.1.2: Plants species mentioned as remedy for menstrual disorders and female infertility in Iwo metropolis (Osun State) contd.

Family/ Species	Local name(s)	Part used	Medicinal uses	Growth form	Times stated (n_{is})	Use mention index (UMi)	Number of uses (by respondent, U_{is})	Use value (UV_{is})
1. *Physalis angulata* L.	Koropo	Leaf, complete aerial parts	Treatment of Female infertility	Herb	3	0.0079[o]	1	0.3333
Tiliaceae								
2. *Glyphaea brevis* (Spreng.) Monachino	Atori	Leaf and root	Treatment of menstrual disorder, ulcer, diabetes and as abortifacient	Tree/ Shrub	5	0.0317[l]	4	0.8000

[j] = UMi category l, [k] = UMi category 2, [l] = UMi category 3, [m] = UMi category 4, [n] = UMi category 5, [o] = UMi category 6

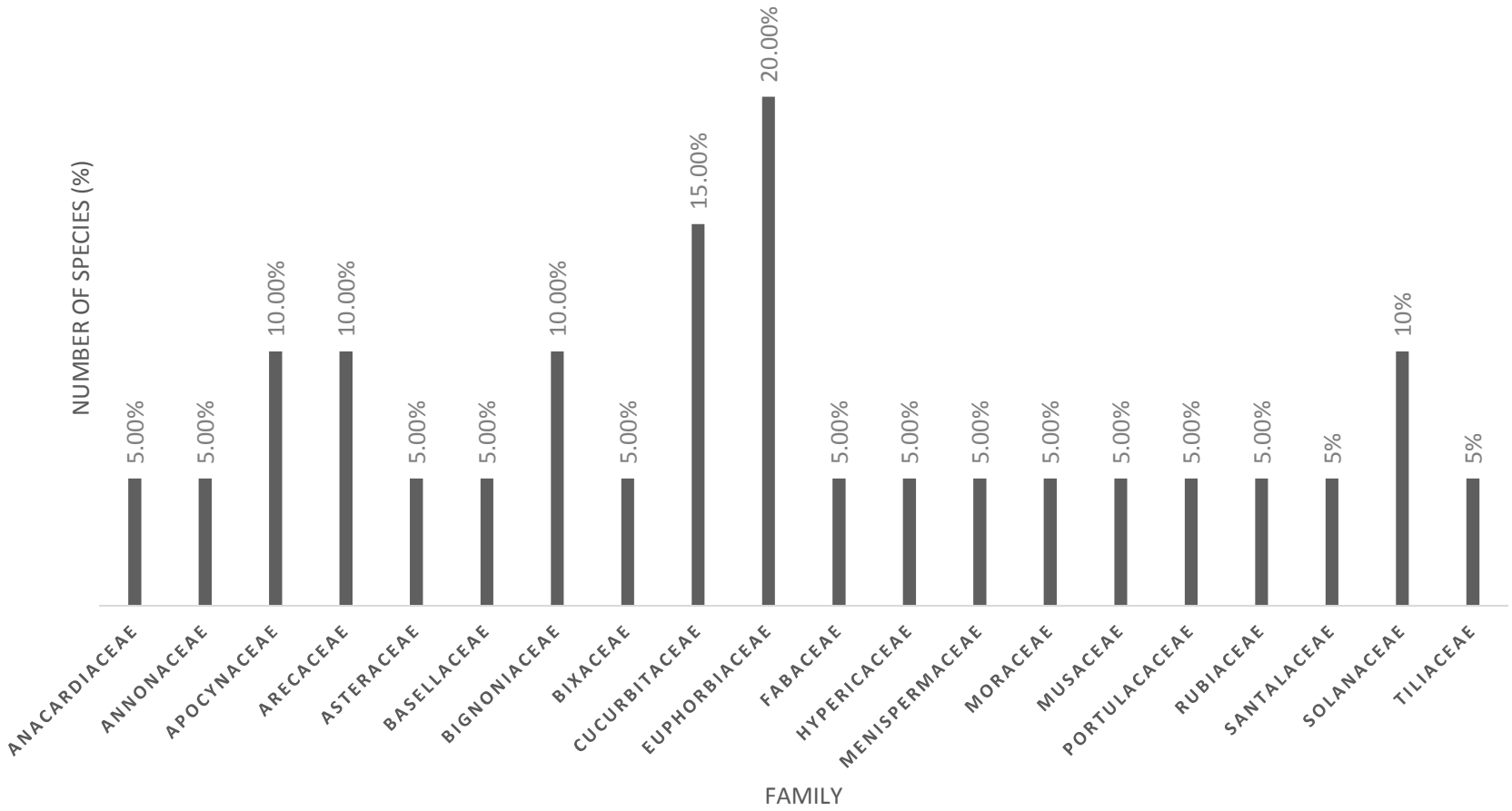


Figure 4.1.2: Family and number of Species of medicinal plants mentioned by respondents

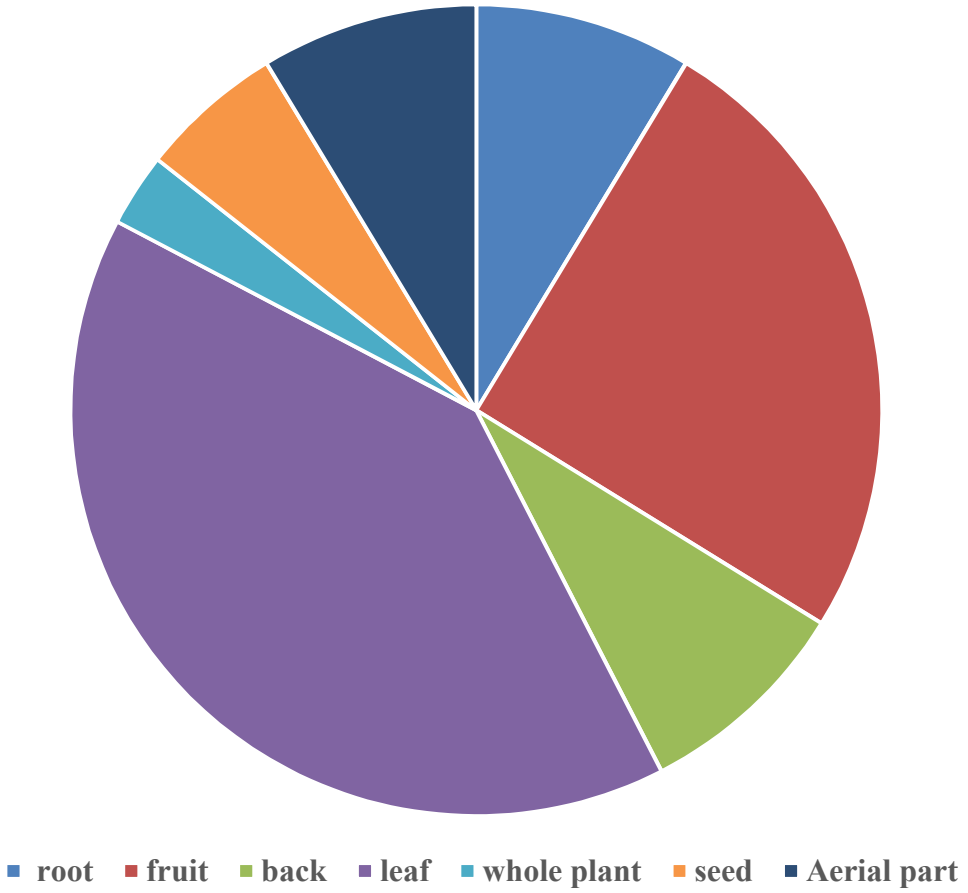


Figure 4.1.3: Plant parts mentioned for the treatment of menstrual disorder and female infertility

Table 4.1.3: Enumeration of recipes used as remedy for menstrual disorder and female infertility

Condition(s)	Method(s) of preparation	Administration
1. Infertility	a). The dried fruit of *Picralima nitida* will be grounded into powder and macerated with *Cocos nucifera* water.	A cup of infusion is to be taken every night
	b). Squeeze *Momordica charantia* leaves	This is to be taken everyday
	c). *Basella alba* plant is macerated with water	The infusion should be taken twice daily
2. Infrequent menstruation (oligomenorrhea)	a). Fruit of *Jatropha gossypifolia*, leaf of *Euphorbia lateriflora* and *Lagenaria breviflora* fruit were burned together	A spoonful of the powder is then mixed with a cup of water and taken as the day of menstruation approaches.
	b). Fruit of *Jatropha gossypifolia*, leaf of *Euphorbia lateriflora* and *Lagenaria breviflora* fruit were burned together and mixed with black soap and potash	The soap is to be bath with as the day of menstruation approaches.
3. Ceased menstruation (amenorrhea)	a). Dried leaves and root of *Bridelia micrantha* and *Xylopia aethiopica* are mixed in a bottle and water is added.	A cup of infusion is to be taken twice a day, morning and night, for duration of 6 months to 1 year

Table 4.1.3: Enumeration of recipes used as remedy for menstrual disorder and female infertility contd.

Condition(s)	Method(s) of preparation	Administration
	b). Bark and leaves of *Erythrophleum suaveolens* are boiled with water and potash. The infusion is then mixed with alcohol.	A cup of the alcoholic part (of the infusion) is to be taken twice a day, morning and night
	c). Squeeze *Physalis angulata* leaves with water.	A glass cup should be taken each day of menstruation
	d). *Bixa orellana* leaves, *Harungana madagascariensis* stem bark and *Morinda lucida* root, are grounded together	This mixture is to be taken with hot pap every morning
1. Unhealthy menses (discoloured or malodourous)	a). Leaves juice of *Tetracera potatoria* . and small potash	This mixture is to be taken every day till the menstruation is over
2. Unhealthy vaginal secretion	a). Squeeze leaf juice of *Pterocarpus osun*	Bathe with water mixed with the juice on the day this vaginal secretion is observed
3. Painful menstruation (dysmenorrhea)	a). Leaves juice of *Cissampelos owariensis* and small potash	This mixture is to be taken every day till the menstruation is over
	b). Leaves juice of *Talinum triangulare* and small potash	This mixture is to be taken every day till the menstruation is over
	c). Fruit peel of *Musa paradisiaca* and sulphur burned together.	The residue is administered with hot pap and must be taken frequently

Table 4.1.4: Fidelity level (FL) for each medicinal plant mentioned per use

Species	Medicinal uses	I_p	I_u	FL (%)
1. *Spondia mombin*	Female infertility	l7	30	56.7
	Vaginal infections	5		l6.7
	Malaria	5		l6.7
	To induce labour	3		l0
2. *Xylopia aethiopica*	Menstrual disorder	7	26	23.3
	Stomach	3		ll.5
	Joint pains	l3		50.0
	Infertility	3		ll.5
3. *Alstonia boonei*	Female Infertility	2	l0	20.0
	Malaria	7		70.0
	Impotence	l		l0.0
4. *Picralima nitida*	Menstrual disorder	2	2	l00
5. *Elaeis quineensis*	Female Infertility	l0	l0	l00
6. *Cocos nucifera*	Infertility.	l	l	l00
7. *Vernonia amygdalina*	Menstrual disorder	3	45	6.7
	Fibroid	5		ll.l
	Stomach ache	l0		22.2
	Ringworm	3		6.7
	Typhoid fever	4		8.9
	Headache	2		4.4
	Diabetes	l8		40.0
8. *Basella alba*	Female infertility	3	23	l3.0
	Irregular periods	l4		60.9
	Acne	4		l7.4
	Sterility	2		8.7

Table 4.1.4: Fidelity level (FL) for each medicinal plant mentioned per use continued

Species	Medicinal uses	I_p	I_u	FL (%)
1. *Kigelia africana*	Female Infertility	7	l7	4l.2
	Skin infections	7		4l.2
	Vaginal infections	3		l7.6
2. *Newbouldia laevis*	Menstrual disorder	6	25	24.0
	Fibroid	l0		40.0
	Impotence	6		24.0
	Infertility	3		l2.0
3. *Bixa orellaina*	Female Infertility	l0	33	30.3
	Stomach ache	6		l8.2
	Diabetes	l7		5l.5
4. *Lagenaria breviflora*	Irregular menstrual flow	3	l0	30
	Skin infections	2		20
	Diarrhoea	5		50
5. *Tetracera potatoria* .	Treatment of unhealthy menstruation	6	6	l00
6. *Momordica charantia*	Female infertility	l	2l	4.8
	Malaria	3		l4.3
	Diabetes	8		38.l
	Painful menstruation	7		33.3
	Regulation of menses	2		9.5
7. *Euphorbia lateriflora*	Irregular menstrual flow	l	l	l00

Table 4.1.4: Fidelity level (FL) for each medicinal plant mentioned per use contd.

Species	Medicinal uses	I_p	I_u	FL (%)
1. *Bridelia micrantha*	Menstrual disorder	4	l0	40
	Diabetics	6		60
2. *Jatropha gossypifolia*	Irregular menstrual flow	3	l3	23.l
	Skin infections	5		38.5
	Excessive bleeding from the vagina	5		38.5
3. *Mallotus oppositifolius*	Female Infertility	l5	27	55.6
	Headache	l2		44.4
4. *Pterocarpus osun*	Irregular menstrual flow	l9	4l	42.2
	Unhealthy vaginal secretion	l7		4l.5
	Skin infections	5		l2.2
5. *Harungana madagascariensis*	Irregular menstrual flow	5	7	7l.4
	Stomach ache	2		28.6
6. *Cissampelos owariensis*	Stomach disorder during menstruation	4	20	20.0
	Excessive bleeding during menstruation	3		l5.0
	Diabetes	7		35.0
	Infertility	6		30.0
7. *Erythrophleum suaveolens*	Menstrual disorder	l	3	33.3
	Skin infections	2		66.7

Table 4.1.4: Fidelity level (FL) for each medicinal plant mentioned per use contd.

Species	Medicinal uses	I_p	I_u	FL (%)
1. *Musa paradisiaca*	Treatment of Stomach disorder during menstruation	5	5	l00
2. *Talinum triangulare*	Stomach disorder during menstruation	4	l5	26.7
	Diabetes	ll		73.3
3. *Morinda lucida*	Irregular menstrual flow	l	l7	5.9
	Malaria	9		60
	Diabetes	6		35.3
	Stomach disorder during menstruation	l		5.9
4. *Viscum album*	Irregular menstrual flow	2	l0	20
	Diabetes	8		80
5. *Capsicum frutescens*	Irregular menstrual flow	3	9	33.3
	Ulcer	6		66.7
6. *Physalis angulata*	Female infertility	3	3	l00
7. *Glyphaea brevis*	Menstrual disorder	l	5	20
	Ulcer	l		20
	Diabetes	2		40
	Abortifacient	l		20

Where I_p is the number of respondents that cited a plant species for a particular ailment and I_u is the total number of respondents who cited the similar plant for managing of any ailments

4.2. Plants extraction and solvent partitioning

The yields of the crude and solvents fractions of the selected medicinal plants were computed and expressed in percentage (Table 4.2.1).

4.3. Thin layer chromatographic (TLC) analysis

The chromatograms of the crude extract of the selected plants are presented in Figures 4.3.1, 4.3.2 and 4.3.3. Flavonoids, phenolics and alkaloids are the classes of compounds detected and the R_f of these classes of compounds are presented in Table 4.3.1. Retardation factor of components in these medicinal plants were calculated and compared with the R_f of known isoflavones - genistein and diadzein (Table 4.3.2).

4.4. Brine shrimp lethality activity of extracts

Toxicity of these selected medicinal plants methanol extracts on brine shrimps is presented in Table 4.4.1. The LC_{50} value of *K. africana* is l442.40 ± l.05 µg/mL, while *T. potatoria* and *M. charantia* had LC_{50} values of 5l7.56 ± 0.l4 µg/mL and l8l8.79 ± 0.22 µg/mL, respectively. Furthermore, *B. alba* and *N. leavis* leaf extracts had LC_{50} values of 29.4l ± 0.l2 µg/mL and l7.29 ± 0.l5 µg/mL, respectively. The LC_{50} value calculated for cyclophosphamide (standard) is 224.74 ± 0.35 µg/mL.

4.5. Antioxidant assay, total phenolic (TPC) and total flavonoid (TFC) content

Antioxidant activity (IC_{50}) and values of TPC and TFC for the extracts and fractions of these medicinal plants are presented in Table 4.5.1. The crude extract, and hexane, DCM and ethyl acetate fractions of *K. africana* had IC_{50} values of 63.34 ± 3.47, ll2.95 ± 0.47, l7.23 ± 0.34 and l2.72 ± 0.04 µg/mL, respectively. The crude extract, DCM and ethyl acetate fractions of *T. potatoria* had IC_{50} values of 220.89 ± 6.99, 89.l5 ± 0.50 and 9.52 ± 0.35 µg/mL, respectively. The IC_{50} values for standards - ascorbic acid and rutin, are 2.76 ± 0.0l and 20.6 ± 9.26 µg/mL, respectively. The ethyl acetate fraction of *T. potatoria* had total phenolics of 7l50.l8 ± ll0.00 µg GAE/g, while crude extract of *B. alba* had total flavonoids of 304.07 ± l.09 mg QE/g.

Table 4.2.1: The percentage yield of the plants crude and solvents fractions

Plants	% yield (w/w) MeOH	% yield (w/w) *n*-hexane fraction	% yield (w/w) DCM fraction	% yield (w/w) ethyl acetate fraction
Newbouldia leavis	6.67	32.27	23.37	7.37
Tetracera potatoria	13.49	6.40	4.49	13.71
Mormodica charantia	8.80	7.14	57.68	25.18
Kigelia africana	4.67	3.96	5.94	11.88
Basella alba	13.91	11.80	8.20	15.20
Lagenaria breviflora	1.90	16.67	1.40	87.43

The percentage yields of the solvent fractions were calculated and expressed as the percentages of the ratio of solvent fractions to the crude extract.

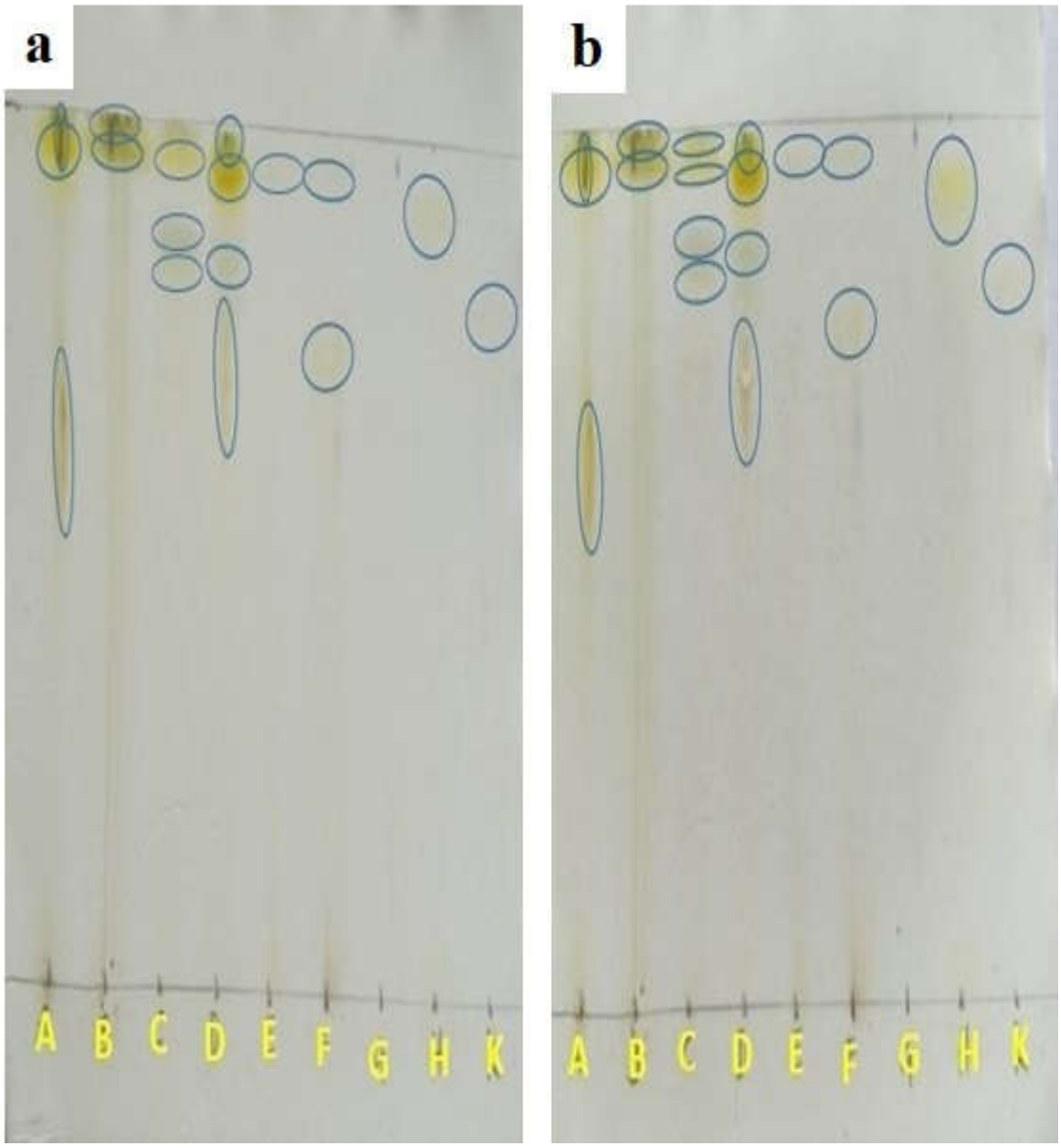


Figure 4.3.1: Chromatogram of (a.) methanol extracts of the selected plants; (b.) yellow spots after spraying with aluminium chloride solution, developed with ethyl acetate, methanol and water (3.5:l:0.5). A - *Momordica charantia*; B - *Tetracera potatoria;* C - *Basella alba*; D - *Newbouldia laevis;* E - *Lagenaria breviflora;* F - *Kigelia africana;* G- Gallic acid; H- Quercetin; K- Protocatechuic acid.

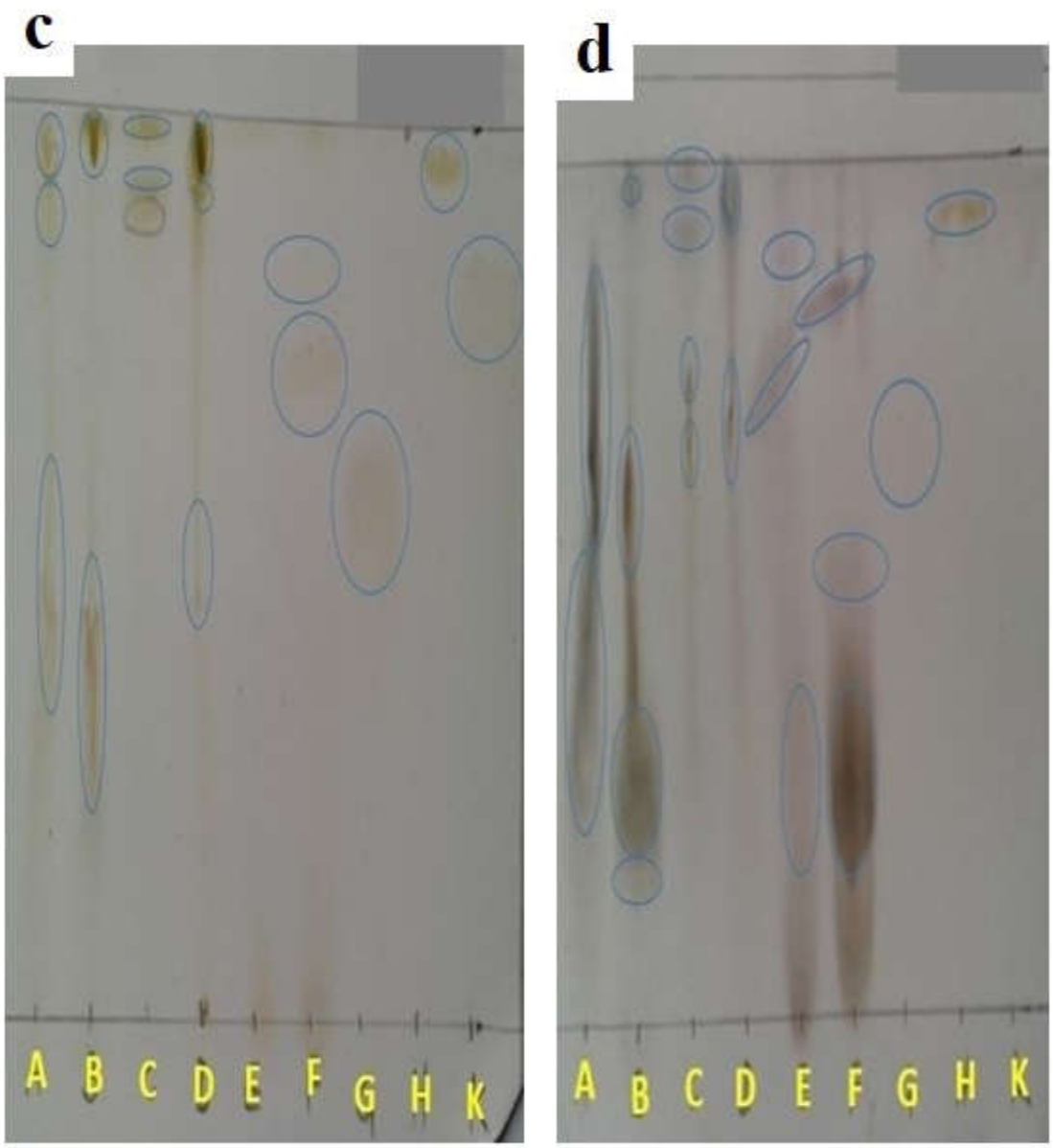


Figure 4.3.2: Chromatogram of (c) orange spots with Drangedorf; (d) Brown, dark and grey spots revealed by ferric chloride solution (heat to ll0 °C) developed with ethyl acetate, methanol and water (3.5:l:0.5). A - *Momordica charantia*; B - *Tetracera potatoria;* C - *Basella alba*; D - *Newbouldia laevis;* E - *Lagenaria breviflora;* F - *Kigelia africana;* G- Gallic acid; H- Quercetin; K- Protocatechuic acid.

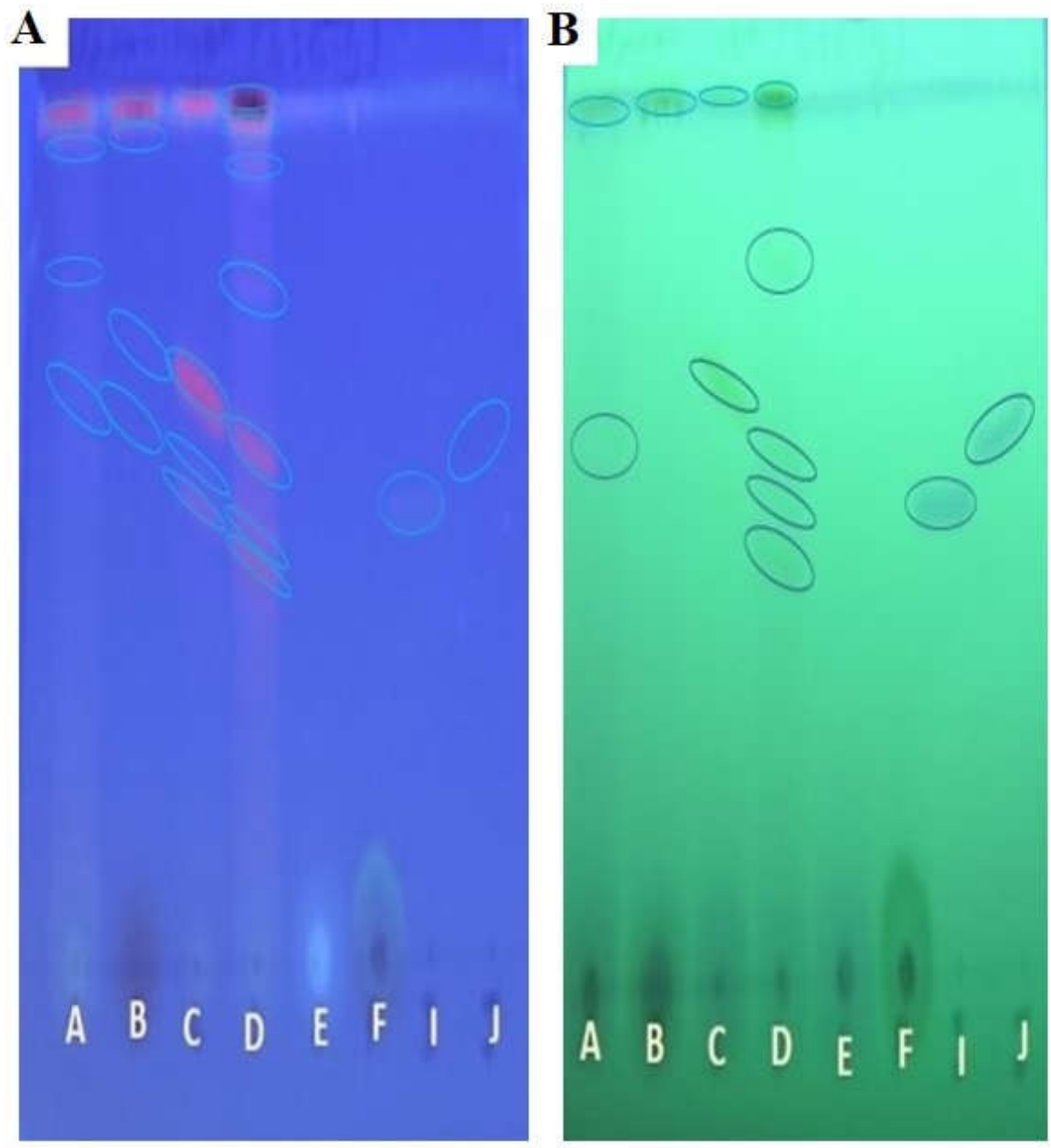


Figure 4.3.3: Chromatogram of methanol extracts of the selected plants with diadzein and genistein as reference at (A) 365 nm and (B) 254 nm developed with Chloroform: methanol (l0: l). A - *Momordica charantia*; B - *Tetracera potatoria;* C - *Basella alba*; D - *Newbouldia laevis;* E - *Lagenaria breviflora;* F - *Kigelia africana*; H- Quercetin; I – Diadzein; J- Genistein

Table 4.3.1: Retardation factors of constituents in samples analysed

VR	Rf								
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**K**
$AlCl_3$	0.59^{O}, 0.94^{Y}, 0.98^{G}	0.94^{Y}, 0.98^{G}	0.74^{G}, 0.78^{Y}, 0.95^{LY}, 0.98^{G}	0.69^{Br}, 0.83^{Y}, 0.86^{G}, 0.95^{DY}, 0.98^{G}	0.95^{LY}	0.76^{O}, 0.98^{LG}	-	0.93^{Y}	0.83^{LBr}
Dragendorf	0.47^{LBr}, 0.89^{Y}, 0.97^{Y}	0.47^{Br}, 0.97^{G}	0.89^{Y}, 0.92^{G}, 0.98^{Y}	0.47^{Br}, 0.89^{G}, 0.89^{Y},	0.98^{LG}	0.70^{O}, 0.83^{LO},	0.56^{O}	0.94^{Br}	0.84^{LBr}
$FeCl_3$	0.64^{Bl}	0.20^{Br}, 0.95^{A}	0.66^{Bl}, 0.76^{Bl}, 0.95^{A}, 0.98^{A}	0.66^{Bl}, 0.95^{A}	0.70^{M}, 0.72^{M}, 0.88^{M}, 0.95^{A}	0.12^{DBr}, 0.52^{M}, 0.81^{M}, 0.98^{A}	-	0.93^{Br}	-

VR= Visualizing reagent, *Rf*= retardation factor, *A* =ash, *Bl* =black, *Br* =brown, *LBr* =light brown, *DBr* =deep brown, *G* =green, *LG* =light green, *LY* =light yellow, *DY* =deep yellow, *O* =orange, *LO* =light orange, *Y* =yellow, *M* =mauve. A - *Momordica charantia*; B - *Tetracera potatoria;* C - *Basella alba*; D - *Newbouldia laevis,* E - *Lagenaria breviflora;* F - *Kigelia africana;* G- Gallic acid; H- Quercetin; K- Protocatechuic acid

Table 4.3.2: Retardation factors of samples analysed with genistein and diadzein as standard

Samples	Rf (at 254 nm)	Rf (at 365 nm)
M. charantia	0.50^{LG}, 0.86^{G}	0.60^{F}, 0.70^{F}, 0.86^{F}, 0.90^{P}
T. potatoria	0.86^{G}	0.60^{LP}, 0.76^{LP}
B. alba	0.48^{LG}, 0.50^{LG}, 0.64^{G}	0.50^{P}, 0.54^{F}, 0.64^{P}
N. leavis	0.42^{G}, 0.44^{LG}, 0.48^{P}, 0.54^{G}	0.42^{P}, 0.48^{P}, 0.78^{P}, 0.80^{F}, 0.86^{F}
L. brevifolia	0.40^{P}	0.30^{F}
K. africana	*NV*	*NV*
Diadzein	0.48^{P}	0.48^{F}
Genistein	0.58^{P}	0.58^{BB}

NV not visible, *G* green, *LG* light green, *F* fluorescence, *P* pink, *LP* light pink, *BB* blue-black.

Table 4.4.1: The LC_{50} (µg/mL) for selected Nigerian medicinal plants mentioned for treatment and management of PCOS using BSLA

Extracts	LC_{50} (µg/mL)
Newbouldia leavis	17.29 ± 0.15***
Tetracera potatoria	517.56 ± 0.14***
Mormodica charantia	1818.79 ± 0.22***
Kigelia africana	1442.40 ± 1.05***
Basella alba	29.41 ± 0.12***
Lagenaria breviflora	257.71 ± 0.12***
Cyclophosphamide	224.74 ± 0.35

Data are presented as means ± (SEM) (n = 3). One-way ANOVA followed by Dunnett's Multiple Comparison Test at $P<0.05$. Each extract was compared with standard (cyclophosphamide) and level of significant difference presented with ***.

Table 4.5.1: The DPPH (IC_{50}), TPC and TFC values of the extracts and their fractions

Extracts	Solvents	DPPH (IC_{50}) (µg/mL)	TPC (µg GAE/g)	TFC (mg QE/g)
Newbouldia leavis	Crude	137.94 ± 4.73***/***	447.84 ± 4.83***	143.23 ± 1.09***
	Hexane	1532.16 ± 17.44***/***	3208.34 ± 27.34**	224.96 ± 0.14^{H}
	DCM	986.85 ± 15.50***/***	4992.50 ± 9.07**	224.83 ± 0.14^{H}
	Ethyl acetate	638.18 ± 5.72***/***	4636.67 ± 7.07***	196.63 ± 0.23***
Tetracera potatoria.	Crude	220.89 ± 6.99***/***	3404.67 ± 6.13^{H}	190.28 ± 12.30***
	Hexane	-	1963.17 ±110.93***	12.85 ± 0.18***
	DCM	89.15 ± 0.50***/***	2518.33 ± 96.17***	3.68 ± 0.16***
	Ethyl acetate	9.52 ± 0.35$^{NS/NS}$	7150.18 ± 110.00^{H}	79. 64 ± 0.15***
Momordica charantia	Crude	314.43 ± 5.57***/***	70.5 ± 5.54***	96.70 ± 13.33***
	Hexane	37.06 ± 0.11**/^{NS}	247.50 ± 80.03***	1.69 ± 0.01***
	DCM	323.55 ± 4.76***/***	4700.33 ± 21.5***	0.54 ± 0.09***
	Ethyl acetate	290.62 ± 2.88***/***	4912.17 ± 10.6***	34. 90 ± 0.01***
Kigelia africana	Crude	63.34 ± 3.47***/***	465.00 ± 0.94***	272.12 ± 12.36***

Table 4.5.1: The DPPH (IC_{50}), TPC and TFC values of the extracts and their fractions contd.

Extracts	Solvents	DPPH (IC_{50}) (µg/mL)	TPC (µg GAE/g)	TFC (mg QE/g)
	Hexane	112.95 ± 0.47***/***	1643.33 ± 7.07***	220.79 ± 0.18***
	DCM	17.23 ± 0.34$^{NS/NS}$	5125.00 ± 1.18^{NS}	176.18 ± 0.99***
	Ethyl acetate	12.72 ± 0.04$^{NS/NS}$	5138.34 ± 8.25***	182.40 ± 0.59***
Basella alba	Crude	156.71 ± 11.03***/***	277.17 ± 3.89***	304.07 ± 1.09^{H}
	Hexane	172.40 ± 2.22***/***	3581.67 ± 20.04^{H}	224.07 ± 0.01***
	DCM	7.17 ± 1.50$^{NS/NS}$	5123.34 ± 4.71^{NS}	223.81 ± 0.05^{NS}
	Ethyl acetate	4.23 ± 0.41$^{NS/NS}$	5016.67 ± 4.71***	205.47 ± 0.14^{H}
Lagenaria breviflora	Crude	242.57 ± 8.90***/***	-	87.15 ± 9.01***
	Hexane	262.91 ± 6.49***/***	2615.67 ± 13.20***	224.32 ±0.05*
	DCM	96.17 ± 2.74***/***	5218.83 ± 3.18^{H}	224.06 ± 0.14^{NS}
	Ethyl acetate	221.45 ± 0.83***/***	4553.00 ± 24.27***	32.00 ± 0.94***
Ascorbic acid		2.76 ± 0.01		
Rutin		20.6 ± 9.26		

Data represented as mean ± (SEM) (n = 3). One-way ANOVA followed by Dunnett's Multiple Comparison Test at $P < 0.05$. Extracts from each solvent with highest TPC and TFC denoted by 'H' were compared with other extracts from the same solvent. Degree of significance is represented by * (significance), ** (moderately significance), *** (highly significance) at $P < 0.05$. *NS* indicates no significant difference with 'H' or same

solvent extracts and fractions. IC_{50} DPPH of each extract was compared with standards (Ascorbic acid and rutin). The asterisk separated by (/) differentiate order of significance from Ascorbic acid and rutin, respectively. *NS* symbolised no significant difference from the standards.

4.6. Effect of the selected medicinal plant extracts of letrozole-induced PCOS

Administration of letrozole for 21 days changed the morphology of the ovary in all treated animals (Figures 4.6.1 and 4.6.2). Histopathology of sections of the ovaries of all the animals before and during the study is presented in Figures 4.6.3 and 4.6.4. Ovary of group I animal showed several Graffian follicles, while ovary of rat in group II revealed follicles with degenerated thecal cells. The morphology of the ovary of animal treated with 100 mg/Kg *b.w.* of *T. potatoria* (Group III) revealed normal antral follicle and stroma with hyperplastic luteinisation. The ovary of group IV animal showed normal thecal cells with mild fibrotic stroma and granular cell hyperplasia. Group V revealed presence of several normal follicles while attretic follicles, degenerated granulosa cells with delineated thecal cells were observed on the ovary of Group VI animal. The ovary of Group VII animal revealed follicle with mild fibrotic stroma. The phase index calculated for each oestrous phase during the period of treatment is presented in Figure 4.6.5

Table 4.6.1 shows the effect of selected medicinal plants extracts and clomiphene citrate (standard drug) on the level of LH, FSH and estradiol in experimental animals. The level of FSH in *K. africana*, *B. alba, T. potatoria* and *M. charantia* treated groups were 0.96±0.08, 1.10±0.23, 0.81±0.04 and 0.69±0.01 mIU/mL, respectively compared to control group (0.93±0.19 mIU/mL). The level of LH in all groups ranged from 0.19±0.05 to 0.23±0.03 mIU/mL with animals treated with *T. potatoria* having the lowest value (0.19±0.05 mIU/mL).

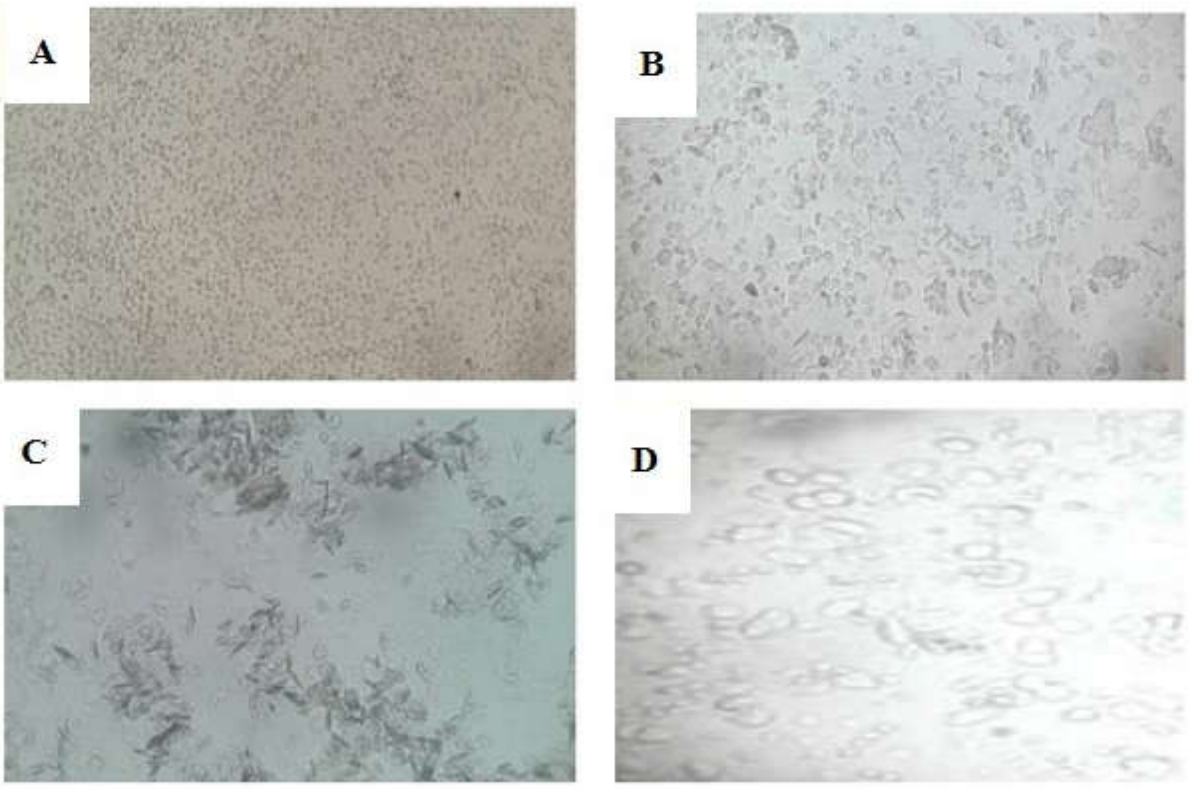


Figure 4.6.1: Photomicrograph of (A) diestrous, (B) proestrous, (C) estrous and (D) metestrous phases of estrous cycle in normal rats (l0x).

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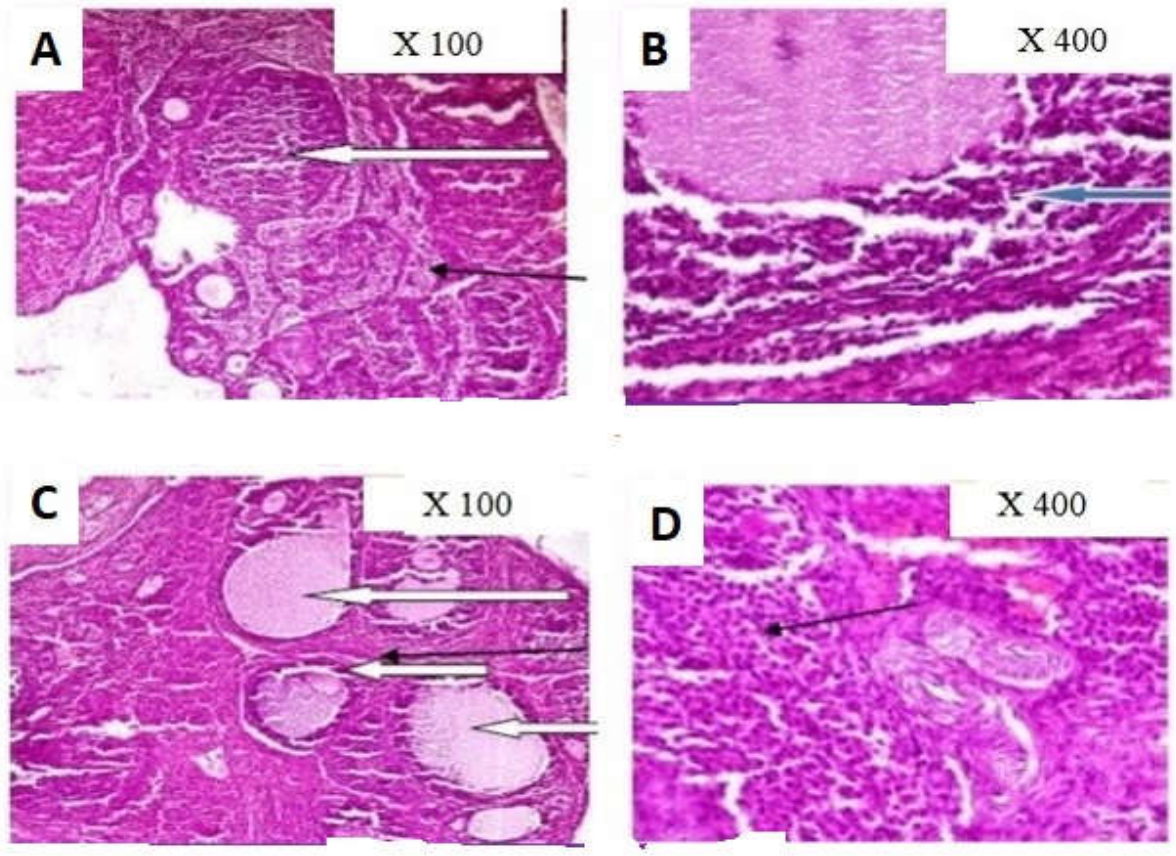


Figure 4.6.2: Section of non-PCOS rat's ovary (A and B) showing normal ovarian stroma with leutinization within the granular cells and PCOS rat ovary (C and D) showing increased number of follicular cysts in the ovaries with fewer corpus lutea as well as thickened theca cell layers after 2l days of distilled water and letrozole administration, respectively. Follicle = white arrow; Thecal layer = black slender arrow.

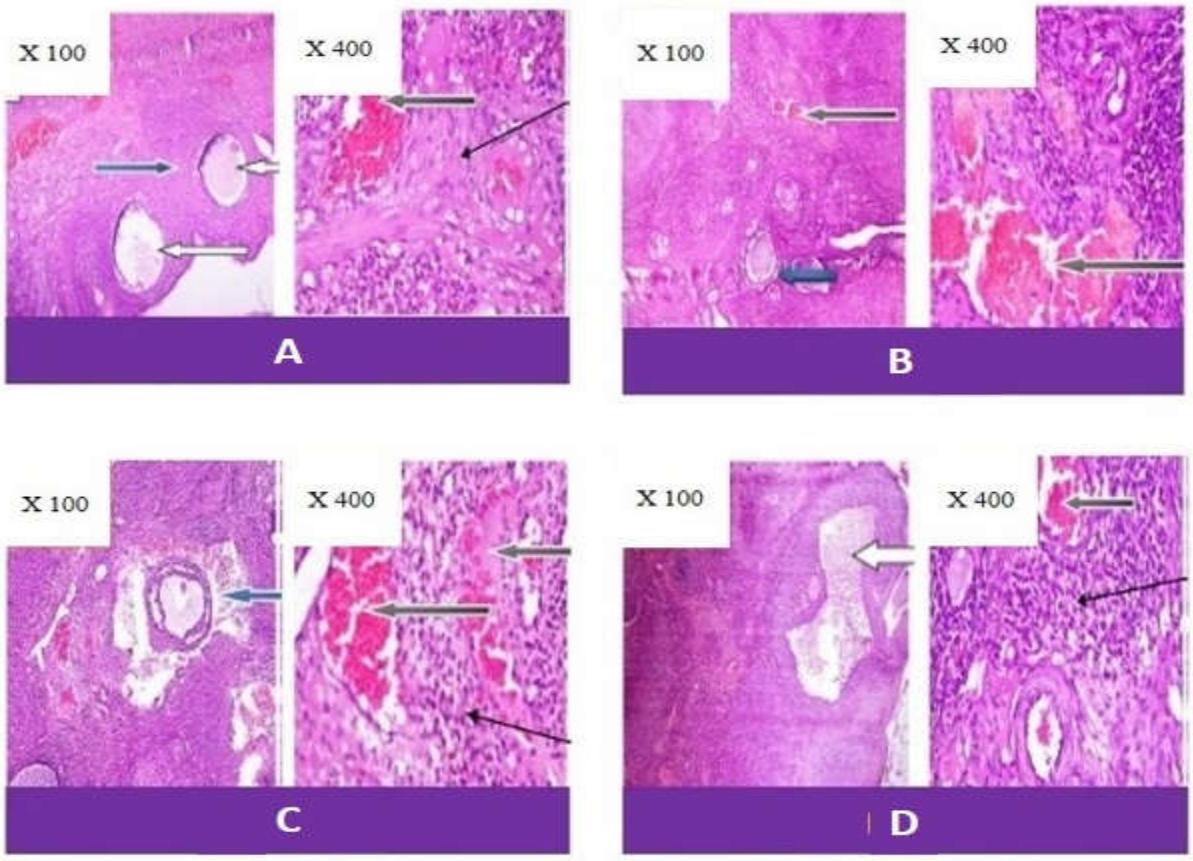


Figure 4.6.3: Photomicrographs of (A) ovary of animal treated with 100 mg/Kg *b.w.* of *K. africana* fruit extract showing several Graffian follicles, (B) ovary of rat treated with 100 mg/Kg *b.w.* of *Basella alba* showing follicles with degenerated thecal cells, (C) ovary of animal treated with 100 mg/Kg *b.w.* of *T. potatoria* showing normal antral follicle and stroma with hyperplastic luteinisation and (D) ovary of animal treated with 100 mg/Kg *b.w.* of *Mormodica charantia* showing normal thecal cells with mild fibrotic stroma and granular cell hyperplasia. Follicle = white arrow; Thecal layer = blue arrow; inflammatory cell = black slender arrow.

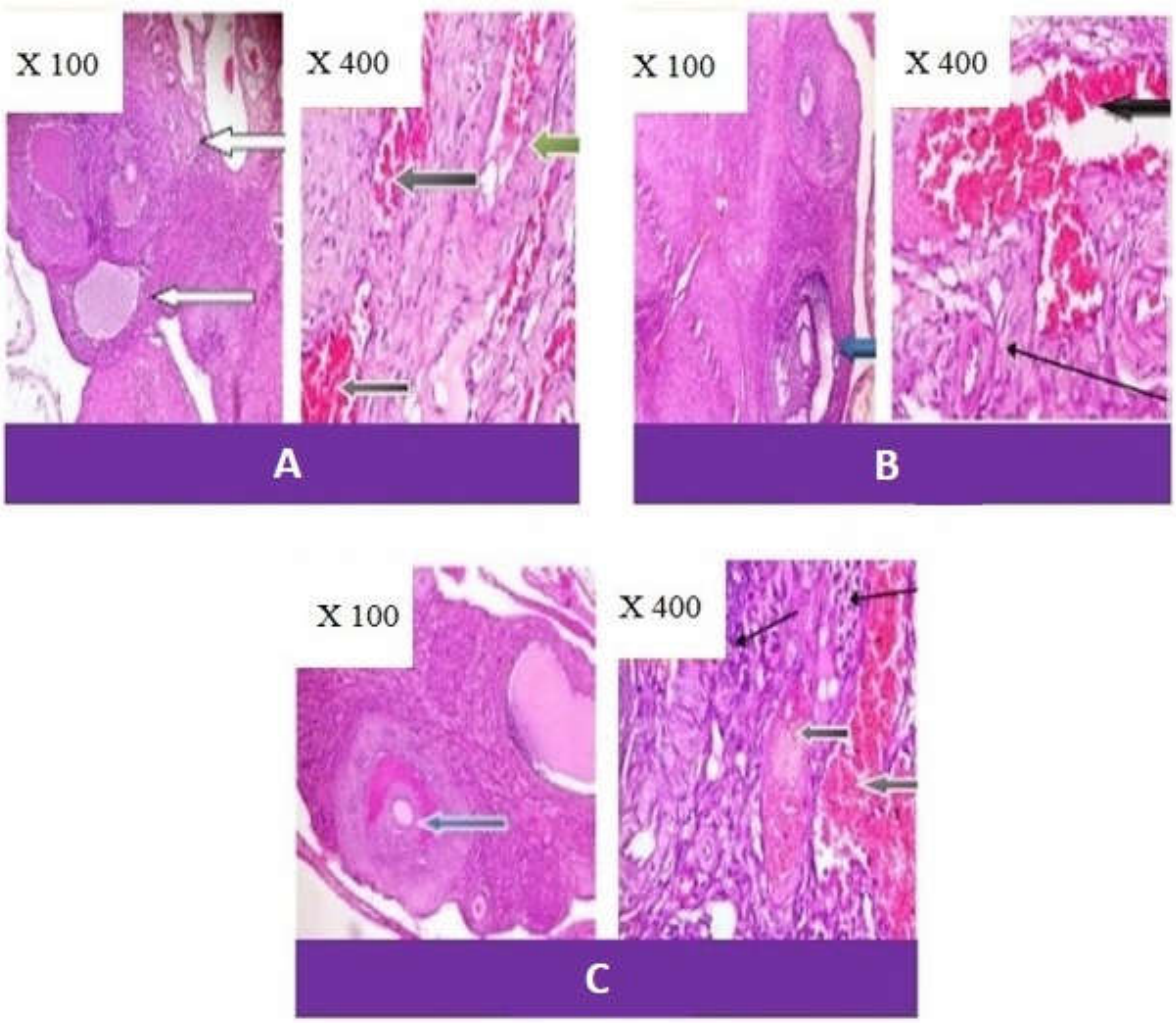


Figure 4.6.4: Photomicrographs of (A) ovary of animal treated with l mg/kg bw of Clomiphene citrate showing presence of several normal follicles, (B) ovary of rat in disease control group showing degenerated granulosa cells as well as delineated thecal cells and (C) ovary of normal control rat showing follicle with mild fibrotic stroma. Follicle = white arrow; Thecal layer = blue arrow; inflammatory cell = black slender arrow.

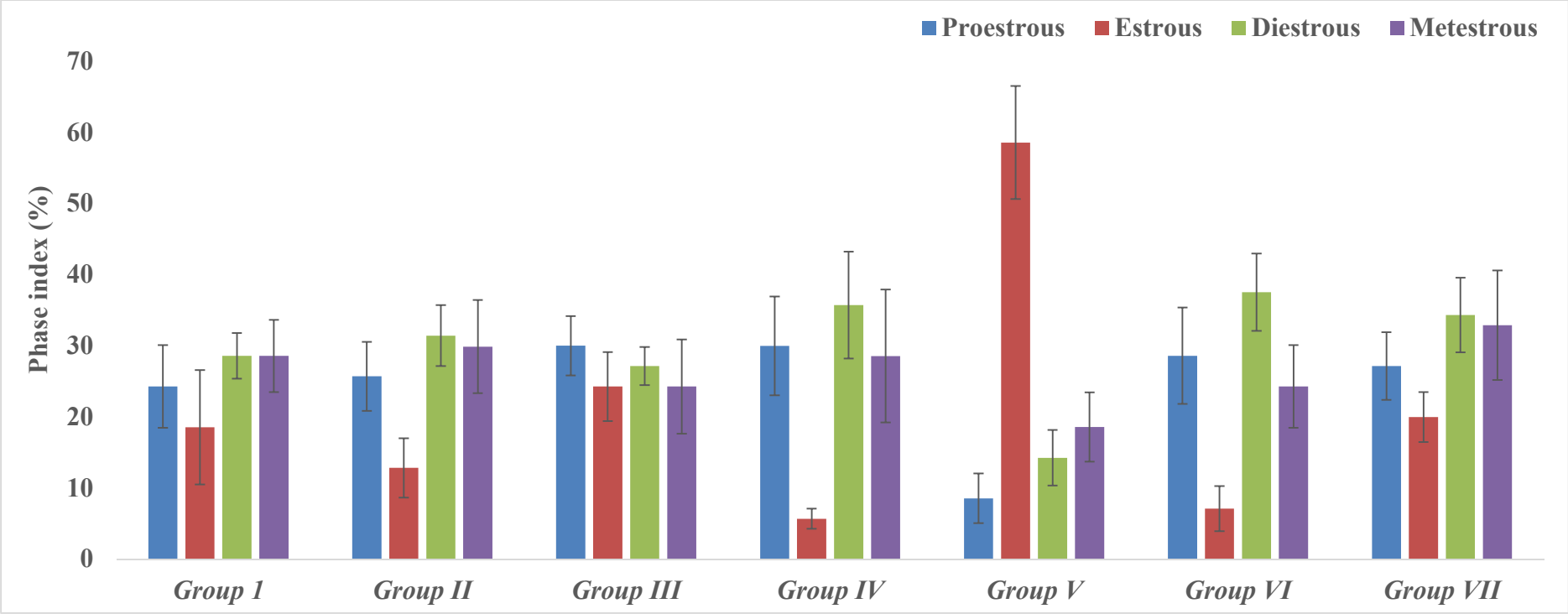


Figure 4.6.5: Estrous cycle phase index of albino rats during l5 days of treatment.

Estrous phase index (%) = Number of days with clear phase smear × l00/total duration of treatment (l5 days).The data represent the mean ± SEM animals, n = 5. Data represented as mean ± (SEM) (n = 5). Group I - l00 mg/kg body weight of *Kigelia africana,* Group II - l00 mg/kg body weight of *Basella alba,* Group III - l00 mg/kg body weight of *Tetracera potatoria*, Group IV - l00 mg/kg body weight of *Mormodica charantia,* Group V - Clomiphene citrate (l mg/kg bw, p.o.), Group VI - disease control group, Group VII - normal control group.

Table 4.6.1: Effect of test samples on LH, FSH and estradiol

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
LH (mIU/mL)	0.2l±0.0l*	0.23±0.03*/#	0.l9±0.05*	0.22±0.0l*	0.23±0.0l*/#	0.23±0.0l*/#	0.22±0.0l
FSH (mIU/mL)	0.96±0.08*	l.l0±0.23*	0.8l±0.04*	0.69±0.0l*	0.75±0.05*	0.73±0.03*	0.93±0.l9
Estradiol (pg/mL)	8.l4±0.45*	7.l8±0.49*	9.36±2.06*	9.26±0.46*	7.63±0.89*	5.70±0.77*	9.84±l.44

Data represented as mean ± (SEM) (n = 5). Evaluated by ANOVA followed by Bonferroni tests. ***** Indicate *P < 0.00l* vs normal control, # indicate *P* < 0.05 vs group III. Group I - l00 mg/kg body weight of *Kigelia africana,* Group II - l00 mg/kg body weight of *Basella alba,* Group III - l00 mg/kg body weight of *Tetracera potatoria*, Group IV - l00 mg/kg body weight of *Mormodica charantia,* Group V - Clomiphene citrate (l mg/kg bw, p.o.), Group VI - disease control group, Group VII - normal control group.

4.7. Antiproliferative effect of crude extracts and fractions of *K. africana* and *T. potatoria*

The antiproliferative effects of extracts and solvent fractions of *K. africana* and *T. potatoria* extracts on Chinese Hamster Ovarian (CHO l) cell line and HeLa cell line is presented in Table 4.7.l. The *K. africana* crude, hexane, DCM and ethyl acetate fractions had IC_{50} values of l8.2 ± 8.2, 5.3 ± 0.l, l3.2 ± 2.8 and 5.3 ± l.l µg/mL, respectively while *T. potatoria* crude, hexane, DCM and ethyl acetate fractions had IC_{50} of >l00, 34.8 ± 0.3, 4l.3 ± 0.8 and >l00 µg/mL, respectively against CHO 1 cell line without inhibiting the proliferation of HeLa cell line.

Table 4.7.l: The IC_{50} (µg/mL) of fractions of *K. africana* and *T. potatoria* extracts on Chinese Hamster Ovarian (CHO l) cell line and HeLa cell line.

Sample (s)	IC_{50} (µg/mL)	
	CHO l cell line	**HeLa cell line.**
K. africana crude	l8.2 ± 8.2	>l00
K. africana hexane fraction	5.3 ± 0.l	>l00
K. africana DCM fraction	l3.2 ± 2.8	79.0 ± 8.7
K. africana ethyl acetate fraction	5.3 ± l.l	>l00
Tetracera potatoria crude	>l00	>l00
Tetracera potatoria hexane fraction	34.8 ± 0.3	>l00
Tetracera potatoria DCM fraction	4l.3 ± 0.8	>l00
Tetracera potatoria ethyl acetate fraction	>l00	>l00
Standard (Doxorubicin)	0.8 ± 0.0l	3.l ± 0.2

4.8. Isolation and purification of isolated compounds

Figures 4.8.1 and 4.8.2 show the flowcharts for the isolated compounds from *K. africana* and *T. potatoria*, respectively. Table 4.8.1 shows the fractions collected from column chromatography of hexane fraction of *K. africana*. Sub-fraction A^1, of hexane fraction, column chromatographic fractionation is presented in Table 4.8.2. Tables 4.8.3 and 4.8.4 shows the column chromatographic fractionation and pooling of DCM column chromatography fractions of *K. africana*, respectively. Column chromatographic fractionation and pooling of ethyl acetate column chromatography fractions of *K. africana* are summarised as shown in Tables 4.8.5 and 4.8.6, while Table 4.8.7 shows column chromatographic fractionation of DCM fraction of *Tetracera potatoria*.

4.9. Structure elucidation of isolated compounds

Nuclear magnetic resonance (NMR) spectra were documented with *Bruker AV (Avance)* spectrometer using deuterated solvents. Proton NMR was measured at 500 MHz while ^{13}C NMR was reordered at 400 and 800 MHz. The chemical shifts were expressed in δ (ppm), referenced to the residual solvent signal and coupling constants (*J*) are in Hz. Splitting patterns of ^{1}H NMR speaks are designated as "s", "d", "dd", "m" and "br s". These symbols indicate "singlet", "doublet", "doublet of doublet", "multiplet" and "broad singlet". The heteronuclear 2D-^{1}H-^{13}C correlations of chemical shift experiment were recorded at 400, 500 and 800 MHz. The low resolution EI-MS (electron impact mass spectra) were carried out with finnigan *MAT 3l2* and *MAT 3l2* spectrometer, attached to *PDP ll/34* computer as display. High resolution Mass measurements were recorded on *Jeol JMS HX ll0* mass spectrometer. The IR absorbance was measured with *FTIR Bruker Vector 22.*

4.10. Characterisation of isolated compounds

Compound **1** was isolated as yellow amorphous solid with molecular formula of $C_{11}H_{12}O_4$ and molecular ion peak of 208 (M^+, 100) in LREIMS. The theoretical mass and observed mass obtained from HREIMS are 208.0736 and 208.0728, respectively. The ^{1}H NMR spectroscopic data displayed signals for allylic protons at 7.61 (IH, d, J = 16Hz, H-2^1) and 6.28 (IH, d, J= 16Hz, H-3^1), aromatic protons (three methane groups) at 7.06 (IH, dd, J = 8.0Hz, J = 1.5Hz, H-6), 7.01 (1-H, d, J = 2.0Hz, H-2) and 6.90 (IH, d, J = 8.5Hz, H-5), and methoxyl group at 3.91(3H, -OCH_3) and 3.77(s, 3H, -OCH_3) as singlet. The ^{13}C-NMR spectra showed that the compound has nine carbon atoms excluding the two methoxyl groups. The two methoxyl groups are attached to para, C-3 (δ_C 150.2), and ortho, C-4 (δ_C 127.4), positions while the functional

group of the compound (carboxylic acid functional group) is at positon C-1' (δ_C 170.8) as shown in Table 4.10.1. This information shows that compound **1** is a cinnamic acid derivative with two substituted methoxyl groups on the aromatic ring system. Compound **1** was elucidated as 3-(3, 4-dimethoxyphenyl) acrylic acid (Figure 4.10.1)**.**

Compound **3** was isolated as light brown amorphous solid with molecular ion peak of 194 (M^+, 100) and molecular formula of $C_{10}H_{10}O_4$ while theoretical mass and observed mass obtained from HREIMS are 194.0579 and 194.0572, respectively. This compound is a cinnamic acid derivative with characteristic olefinic protons at 7.57 (IH, d, *J*= 15.5 Hz, H-2) and 6.26 (IH, d, *J* = 16Hz, H-3) and three aromatic methine protons at 7.05 (1H, d, *J*= 2.0Hz, H-2'), 7.00 (IH, dd, *J* = 8.0Hz, *J*= 1.5Hz, H-6') and 6.85 (IH, d, *J*= 8.0 Hz, H-5') as shown in Table 4.10.3.The ^{13}C-NMR data showed carbon of ester at C-1 (δ_C 167.7) and carbons with hydroxyl groups at C-3' (δ_C 143.7) and C-4' (δ_C 144.6). Compound **3** was elucidated as methyl 3-(3, 4-dihydroxyphenyl) acrylate (Figure 4.10.3). The LREIMS spectrum of compound **4** showed molecular ion peak of 149 (M^+, 100) suggesting a molecular formula of $C_5H_8O_5$. It was isolated as greenish-yellow amorphous solid. The hydroxyl group at position C-2 was observed as singlet at 9.59 ppm while proton attaching to C-2 (δ_C 121.2) and C-3 (δ_C 109.9) were observed as doublet at 7.20 and 6.50 ppm, respectively (Table 4.10.4). Methylene protons which showed at 4.71 ppm as singlet are attached to C-1' (δ_C 57.7). Compound **4** was elucidated as 2, 3-dihydro-5-(hydroxymethyl) furan-2, 3, 4-triol (Figure 4.10.4). The data obtained for compounds **2**, **5** and **6,** shown in Figures 4.10.2, 4.10.5 and 4.10.6, respectiviely were similar with those reported in literatures, as presented in Tables 4.10.2, 4.10.5 and 4.10.6, respectively. The data obtained for compound **7** (isolated from DCM fraction of *T. potatoria*), shown in Figure 4.10.7, were similar with those reported in literatures (Table 4.10.7).

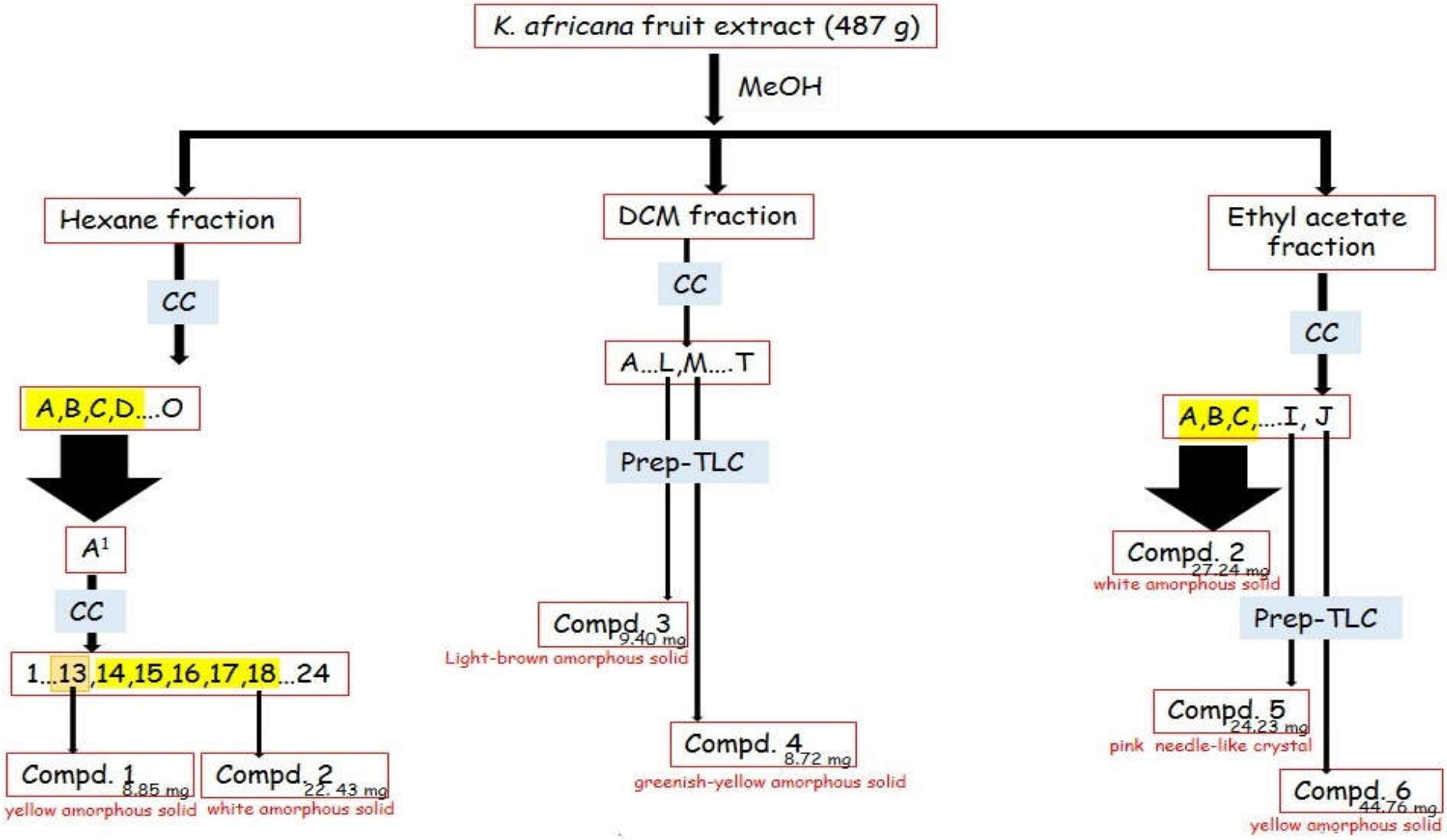


Figure 4.8.1: Flowchat for the compounds isolated from *K. africana* fruit

Table 4.8.l: Pooled column chromatographic fractions of hexane fraction of *K. africana*

S/n	Fractions	Solvent system (Hex: DCM)	Pooled fraction codes	Weight (mg)
l	l-37	50:50	A	l3.28
2	38-48	50:50	B	4.99
3	49-50	50:50	C	25.00
4	5l-6l	50:50	D	30.0l
5	62-72	50:50	E	2.46
6	73-92	50:50	F	6.95
7	93-ll5	0:l00	G	3.44
8	ll6-l25	0:l00	H	6.32
9	l26-l34	0:l00	I	5.88
l0	l35-l40	0:l00	J	l.35
ll	l4l-l66	0:l00	K	7.64
l2	l67-l75	0:l00	L	5.07
l3	l76-l89	0:l00	M	4.53
l4	l90-203	0:l00	N	4.53
l5	204-220	0:l00	O	3.24

Table 4.8.2: Column chromatographic fractionation of A[1]

S/n	Fractions	Solvent system (Hex: DCM: Ethyl acetate)
1	l-2	l00:0:0
2	3-l0	80:20:0
3	ll-l5	60:40:0
4	l6-l8	40:60:0
5	l9-20	20:80:0
6	2l-24	0:80:20

Table 4.8.3: Pooled column chromatographic fractions of DCM fraction of *K. africana*

S/n	Fractions	Solvent system (Hex: DCM: Ethyl acetate: MeOH)
1	l-4	l00:0:0:0
2	4-l0	90:l0:0:0
3	ll-87	50:50:0:0
4	88-l60	0:l00:0:0
5	l6l-223	0:90:l0:0
6	234-282	0:80:20:0
7	283-342	0:60:40:0
8	343-373	0:50:50:0
9	374-435	0:30:70:0
l0	436-498	0:20:80:0
11	499-5l6	0:0:l00:0
12	5l7-572	0:0:90:l0

Table 4.8.4: Pooling of DCM Column chromatography fractions of *K. africana*

S/n	Pooled fractions	Fractions	Weight (mg)
1	A	l-l8	l0.00
2	B	l9-49	2.00
3	C	50-8l	2.50
4	D	82-l03	4.02
5	E	l04-l30	l0.23
6	F	l3l-l67	22.00
7	G	l68-l77	l2.00
8	H	l78-l90	5.23
9	I	l9l-l99	5.23
l0	J	200-204	2.34
11	K	205-207	20.00
l2	L	208-247	630.00
l3	M	248-266	730. 00
l4	N	267-276	l0.02
l5	O	277-309	2.34
l6	P	3l0-326	3.57
l7	Q	327-372	3.56
l8	R	373-380	4.73
l9	S	38l-385	5.33
20	T	386-432	3.54
2l	U	433-530	3.45
22	V	53l-572	2.67

Table 4.8.5: Pooled column chromatographic fractions of ethyl acetate fraction of *K. africana*

S/n	Fractions	Solvent system (Hex: DCM: Ethyl acetate: MeOH)
1	l-2	l00:0:0:0
2	3-27	40:60:0:0
3	28-49	20:80:0:0
4	50-56	0:l00:0:0
5	57-68	0:80:20:0
6	69-83	0:60:40:0
7	84-l08	0:40:60:0
8	l09-l2l	0:20:80:0
9	l22-l36	0:0:l00:0
l0	l37-l47	0:0:80:20
11	l48-l5l	0:0:60:40

Table 4.8.6: Pooling of ethyl acetate Column chromatography fractions of *K. africana*

S/n	Pooled fractions	Fractions	Weight (mg)
1	A	1-10	100.23
2	B	11-19	70.67
3	C	20-25	250.78
4	D	26-36	340.33
5	E	37-59	200.00
6	F	60-66	190.05
7	G	67-74	500.00
8	H	75-78	200.00
9	I	79-85	340.54
10	J	86-106	340.54
11	K	107-116	670.34
12	L	117-132	150.00
13	M	133-139	133.33
14	N	140-142	111.12
15	O	143-151	123.67

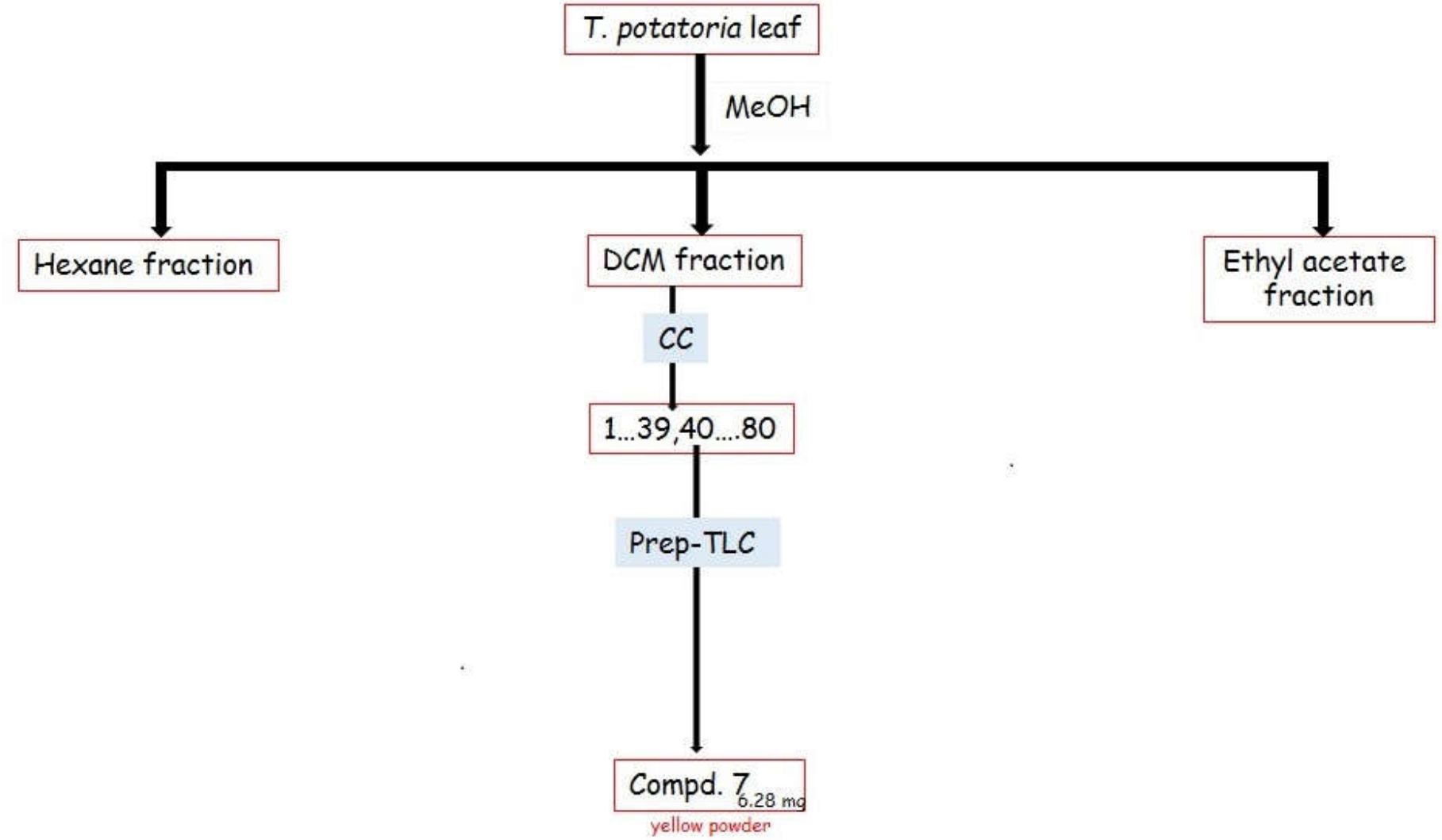


Figure 4.8.2: Flowchat for the compound isolated from *T. potatoria* leaf

Table 4.8.7: Column chromatographic fractionation of DCM fraction of *Tetracera potatoria*.

S/n	Fractions	Solvent system (Hex: DCM: Ethyl acetate: MeOH)
1	l-4	l00:0:0:0
2	5-8	95:05:0:0
3	9-l3	90:l0:0:0
4	l4-20	80:20:0:0
5	2l-23	70:30:20:0
6	24-25	60:40:40:0
7	26-28	40:60:60:0
8	29-39	0:l00:0:0
9	40-50	0:80:20:0
l0	5l-66	0:60:40:20
11	67-80	0:40:60:0

4.10.1. Elucidation of 3-(3, 4-dimethoxyphenyl) acrylic acid (l)

IR (KBr) ν_{max}: 3403, 2950, 2645, l702, l5l5, l033, 848 cm^{-l}

^{l}HNMR (500 MHz, $CDCl_3$): δ(ppm) 7.6l (d, *J*= l6Hz, IH, H-2^{l}), 7.06 (dd, *J* = 8.0Hz, *J* = l.5Hz, IH, H-6), 7.0l (d, *J* = 2.0Hz,l-H, H-2), 6.90 (d, *J* = 8.5Hz, IH, H-5), 6.28 (d, *J* = l6Hz, IH, H-3^{l}), 3.9l(s, 3H, -OCH_3), 3.77(s, 3H, -OCH_3).

^{l3}C-NMR (800 MHz, $CDCl_3$): δ(ppm) l70 (C-l^{l}, -COOH), l50 (C-3), ll2 (C-2), l49 (C-l), l27 (C-4), l23 (C-6), ll4 (C-5), ll5 (C-3'), ll2 (C-3), 55 (-OCH_3), 5l (-OCH_3).

EI-MS *m/z* (% rel. abund.): 209 (M^++l, l0), 208 (M^+, l00), l77 (66), l50 (l2), l49 (l2), l45 (34), l34 (l2), ll7(l3), 82(l2). EI-HRMS: Theoretical mass (208.0736), Observed mass (208.0728).

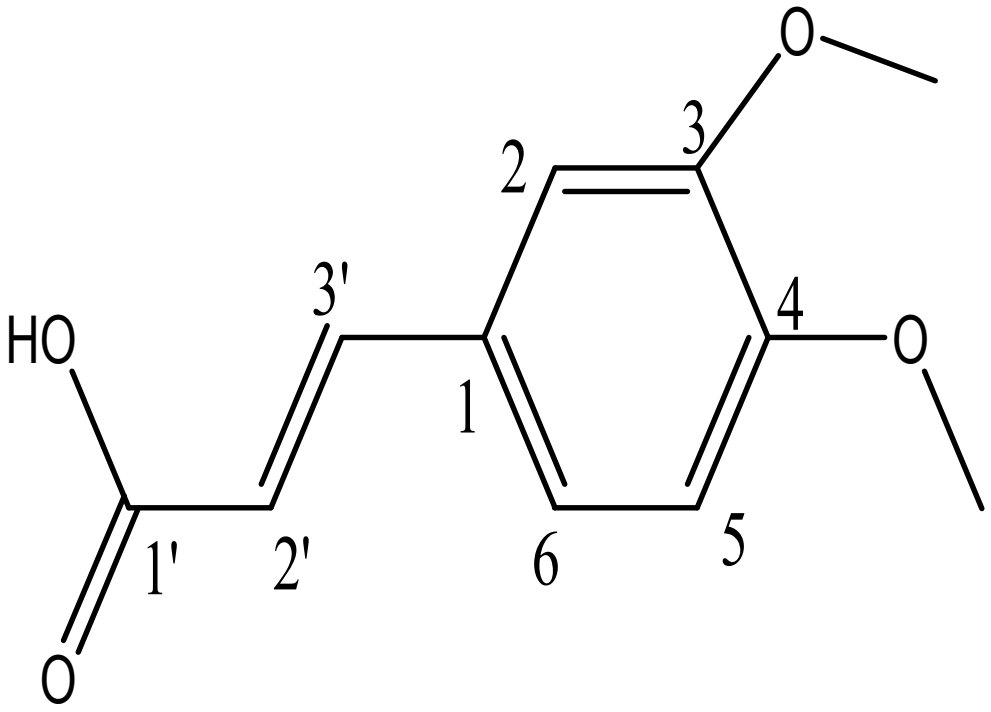


Figure 4.10.l: Structure of 3-(3, 4-dimethoxyphenyl) acrylic acid (l)

Table 4.10.1: Comparison of observed and reported NMR data for compound l

Position	Observed		Reported (Khan *et al.*, 2008)
	$ð_H$	$ð_C$	$ð_H$
	(500 MHz, $CDCl_3$)	(800 MHz, $CDCl_3$)	(400 MHz, CDCl3)
1		l49	
2	7.0l (lH, d, J=2 Hz)	l50	7.02 (lH, d, J=2 Hz)
3		ll2	
4		l27	
5	7.06 (lH, dd, J= l.5, 8 Hz)	l23	7.08 (lH, br.d, J= 8 Hz)
6	6.90 (lH, d, J= 8.5 Hz)	ll4	6.90 (lH, d, J= 8 Hz)
l^l		l70	
2^l	7.6l (IH, d, J=l6 Hz)	l44	7.60 (IH, d, J=l6 Hz)
3^l	6.28 (lH, d, J=l6 Hz)	ll5	6.28 (lH, d, J=l6 Hz
-OCH_3	3.9l (3H, s)	55	3.92 (3H, s)
-OCH_3	3.77 (3H, s)	5l	3.77 (3H, s)

4.10.2. Elucidation of *β*-Sitosterol (2)

IR (KBr) ν_{max}: 3433, 2935, l46l, l375, l056, 956, 836 cm^{-l}

^{l}HNMR (500 MHz, $CDCl_3$): δ (ppm) 5.33 (lH, t, H-6), 3.53 (lH, m, H-3), 0.83 (9H, m, H-27), 0.82 (9H, m, H-29), 0.8l (9H, m, H-26).

^{l3}C-NMR (800 MHz; $CDCl_3$): δ (ppm) 37 (CH_2, C-l), 3l (CH_2, C-2), 7l (CH, C-3), 42 (CH_2, C-4), l40 (C=C, C-5), l2l (C=CH, C-6), 3l (CH_2, C-7), 3l (CH, C-8), 50 (CH, C-9), 36 (C, C-l0), 2l (CH_2, C-ll), 39 (CH_2, C-l2), 42 (C, C-l3), 56 (CH, C-l4), 24 (CH_2, C-l5), 28 (CH_2, C-l6), 56 (CH, C-l7), ll (CH_3, C-l8), l9 (CH_3, C-l9), 36 (CH, C-20), l8 (CH_3, C-2l), 33 (CH_2, C-22), 26 (CH_2, C-23), 45 (CH, C-24), 29 (CH. C-25), l9 (CH_3, C-26), l9 (CH_3, C-27), 23 (CH_2, C-28), l2 (CH_3, C-29).

EI-MS *m/z* (% rel. abund.): 4l5 (M^++l, 32), 4l4 (M^+, l00), 4l2 (20), 400 (20), 399 (43), 397 (l6), 396 (58), 382 (l8), 38l (49), 330 (ll), 329 (44), 304 (l3), 303 (55, 302 (l2), 275 (l3), 273 (29), 27l (l2), 256 (l0), 255 (50), 23l (30), 228 (l0), 2l5 (l3), 2l4 (l2), 2l3 (46), 2l0 (l0), l99 (l8), l87 (l4), l85 (l3), l78 (ll), l77 (l2), l75 (l0), l73 (l7), l7l (l0), l63 (3l), l6l (32), l60 (l7), l59 (37), l58 (l4), l57 (l2), l49 (l7), l47 (26), l46 (l0), l45 (42), l43 (l3), l37 (l2), l35 (27), l33 (29), l3l (l4), l25 (l0), l23 (l3), l2l (25), l20 (l6), ll9 (23), lll (l3), l09 (l9), l07 (37), l05 (28), 97 (20), 95 (35), 93 (23), 9l (20), 85 (l7), 83 (23), 8l (35), 79 (l8), 7l (l9), 69 (20), 67 (l5), 57 (25), 55 (33), 43 (33), 4l (23).

EI-HRMS: Theoretical mass (4l4.3862), Observed mass (4l4.3884).

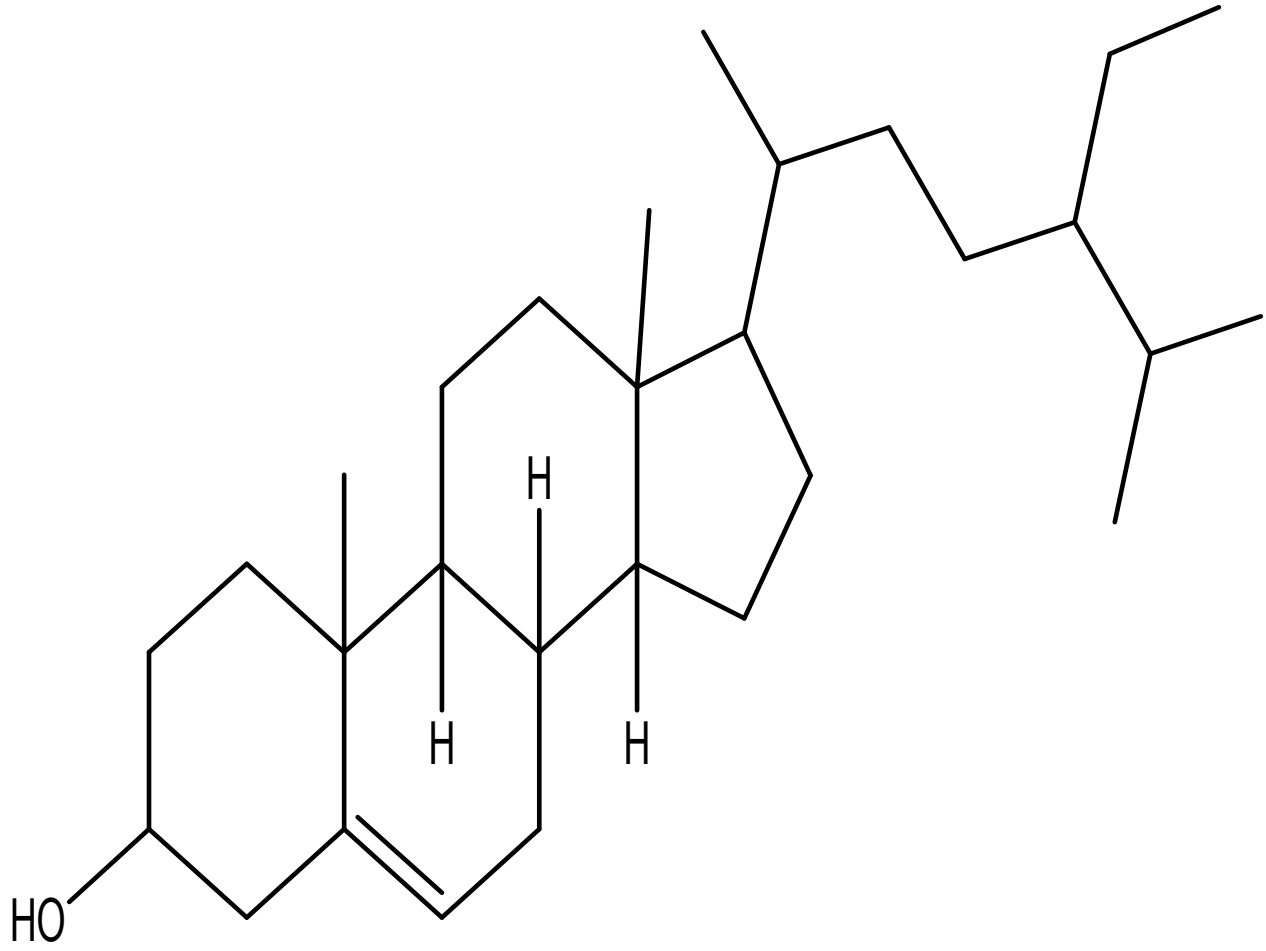


Figure 4.10.2: Structure of l7-(5-ethyl-6-methylheptan-2-yl) 2,3,4,7,8,9,l0,ll,l2,l3,l4,l5,l6,l7-tetradecahydro-l0,l3-dimethyl-lH-cyclopenta[a]phenanthren-3-ol (Sitosterol) (2)

Table 4.10.2: Comparison of observed and reported NMR data for compound 2

Position	δ_H (d, $CDCl_3$, 400 MHz) Reported[a]	δ_H (d, $CDCl_3$, 500 MHz) Observed	δ_C (d, $CDCl_3$, l00 MHz) Reported[a]	δ_C d, $CDCl_3$, 400 MHz) Observed
l			37.25	37.2
2			3l.89	3l.9
3	3.50 (lH, m)	3.53 (lH, m)	7l.79	7l.8
4			42.29	42.2
5			l40.74	l40.7
6	5.35 (lH, m)	5.33 (lH, m)	l2l.70	l2l.7
7			3l.65	3l.6
8			3l.65	3l.6
9			50.l2	50.l
l0			36.l3	36.5
ll			2l.07	2l.0
l2			39.77	39.7
l3			42.29	42.3
l4			56.75	56.7
l5			26.07	26.0
l6			28.23	28.2
l7			56.05	56.0
l8	0.68 (3H, s)	0.66 (3H, s)	ll.84	ll.9
l9	l.02 (3H, s)	0.98 (3H, s)	l9.38	l9.4
20			36.49	36.l
2l			l9.02	l8.7
22			33.93	33.9
23			26.07	26.0
24			45.82	45.8
25			29.l4	29.l
26	0.82 (3H, d, J=6.5 Hz)	0.77-0.84 (9H, m)	l8.77	l9.2
27	0.84 (3H, d, J= 6.5 Hz)	0.77-0.84 (9H, m)	l9.8l	l9.8
28			23.05	23.0
29			l2.l3	l2.0

[a] (Balamurugan *et al.,* 20l2)

4.10.3. Elucidation of methyl 3-(3, 4-dihydroxyphenyl) acrylate (3)

IR (KBr) ν_{max}: 3330, 2927, l842, l5l5, l277, ll70, 773 cm^{-l}

^{l}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 7.57 (d, J = l5.5 Hz, IH, H-2), 7.05 (d, J = 2.0Hz,l-H, H-2'), 7.00 (dd, J = 8.0Hz, J = l.5Hz, IH, H-6'), 6.85 (d, J = 8.0 Hz, IH, H-5'), 6.26 (d, J = l6Hz, IH, H-3), 3.77 (s, 3H, -OCH_3-), 5.63 (s, broad, -OH), 5.49 (s, broad, OH).

^{l3}C-NMR (800 MHz, $CDCl_3$): δ (ppm) l67 (C-3, -$COOCH_3$), ll4 (C-2'), l44 (C–4', -OH), l43 (C-l'), ll5 (C-5'), l22 (C-6'), l43 (C-3'), ll5 (C-5'), 5l (-OCH_3).

EI-MS *m/z* (% rel. abund.): l95 (M^++l, 9.3), l94 (M^+, l00), l63 (83), l45 (ll), l36 (l0), l35 (l7), l34 (20), ll6 (l4), 89 (l3), 77 (l0).

EI-HRMS: Theoretical mass (l94.0579), Observed mass (l94.0572).

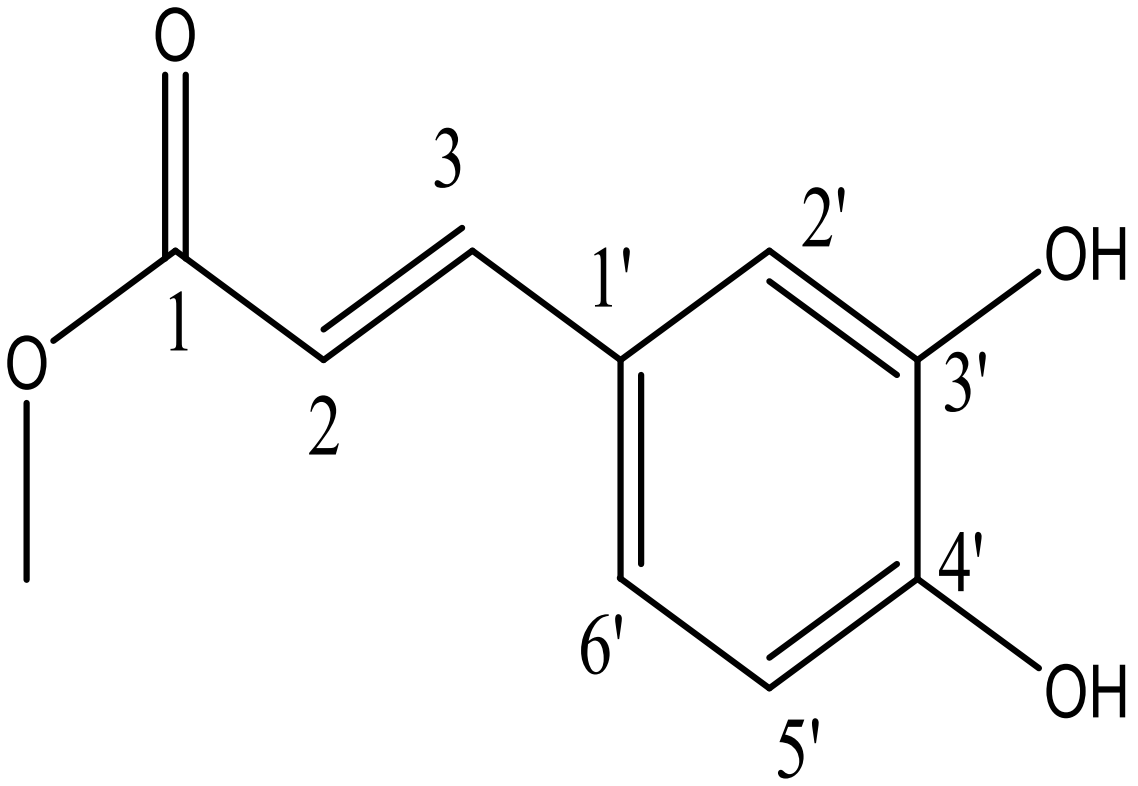


Figure 4.10.3: Structure of methyl 3-(3, 4-dihydroxyphenyl) acrylate (3)

Table 4.10.3: Comparison of observed and reported NMR data for compound 3

Position	Observed		Reported (Konradova *et al.*, 2017)	
	δ_H (500 MHz, $CDCl_3$)	δ_C (800 MHz, $CDCl_3$)	δ_H (500 MHz, $CDCl_3$)	δ_C (126 MHz, $CDCl_3$)
l[l]		l43		-
2[l]	7.05 (lH, d, J= 2Hz)	ll4	7.07 (lH, d, J= 2Hz)	114
3[l]		l43		-
4[l]		l46		147
5[l]	7.00 (lH, dd, J= l.5, 8 Hz)	ll5	6.99 (lH, dd, J= 2.1, 8.2 Hz)	115
6[l]	6.85 (lH, d, J= 2Hz)	l22	6.87 (lH, d, J= 8.7 Hz)	121
1		l67		168
2	7.57 (lH, d, J= l5.5 Hz)	l44	7.57 (lH, d, J= l5.9 Hz)	145
3	6.26 (lH, d, J=l6 Hz)	l33	6.25 (lH, d, J=l5.9 Hz)	-
-OCH_3	3.77 (3H, s)	5l		51
-OH	5.63 (lH, br s)			
-OH	5.48 (lH, br s)			

4.10.4. Elucidation of 2, 3-dihydro-5-(hydroxymethyl) furan-2, 3, 4-triol (4)

IR (KBr) ν_{max}: 3375, 2924, l67l, l52l, ll92, l022, 773 cm^{-l}

^{l}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 9.59 (lH, s), 7.20 (d, J = 3.5 Hz, IH, H-2), 6.50 (d, J = 3.5 Hz, IH, H-3), 4.7l (s, 2H, H-l').

^{l3}C-NMR (800 MHz, $CDCl_3$): δ (ppm) l60 (C-4, -OH), l52 (C-5), l2l (C-2, -OH), l09 (C-3, -OH), 57 (C-l', -OH).

EI-MS *m/z* (% rel. abund.): l49 (M^+ + l, l3), l48 (M^+, l00), ll3 (ll), 7l (l7), 69 (ll), 57 (23), 55 (l6), 53 (l0), 42 (2l), 4l (2l).

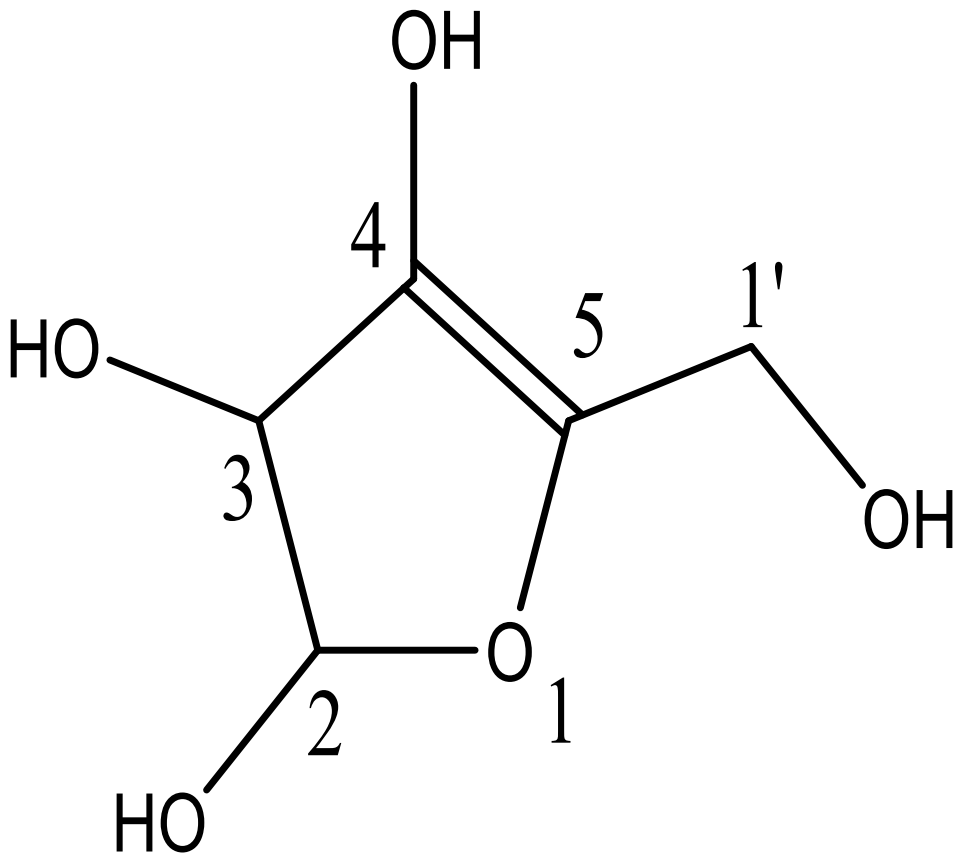


Figure 4.10.4: Structure of 2, 3-dihydro-5-(hydroxymethyl) furan-2, 3, 4-triol (4)

Table 4.10.4: Complete NMR data for compound 4

Position	$ð_H$	$ð_C$	HMBC	^1H-^1H COSY
1				
2	7.20 (1H, d, *J*=3.5 Hz)	121		H-3
3	6.50 (1H, d, *J*= 3.5 Hz)	109	C-4	H-2
4		160		
5		152		
1^1	4.71 (2H, s)	57.8	C-3, C-4	
-OH	9.59 (1H, s)			

4.10.5. Elucidation of 3-(4-hydroxyphenyl) acrylic acid (*p*-Coumaric acid) (5)

IR (MeOH) ν_{max}: 3952, 2928, 1732, 1514, 1218, 1033, 772 cm^{-1}

^{1}H-NMR (500 MHz, CD_3OD): δ 7.59 (1H, d, J = 16 Hz, H-2'), 7.45 (2H, t, J = 2.5 Hz, 4.5 Hz, H-2, H-6), 6.80 (2H, t, J = 2.5 Hz, 4.5 Hz, H-3, H-5), 6.28 (1H, d, J = 16 Hz, H-3').

^{13}C-NMR (800 MHz, CD_3OD): δ 171.2 (C-1'), 161.1 (C-5), 146.4 (C-2'), 131.0 (C-1), 129.8 (C-2), 127.3 (C-6), 116.8 (C-3), 116.0 (C-4), 115.9 (C-3').

EI-MS *m/z* (% rel. abund.): 165 (M^++1, 11), 164 (M^+, 100), 163 (36), 147 (44), 119 (24), 118 (18), 107 (11), 91 (18), 107 (11), 91 (18), 65 (10).

EI-HRMS: Theoretical mass (164.0473), Observed mass (164.0476).

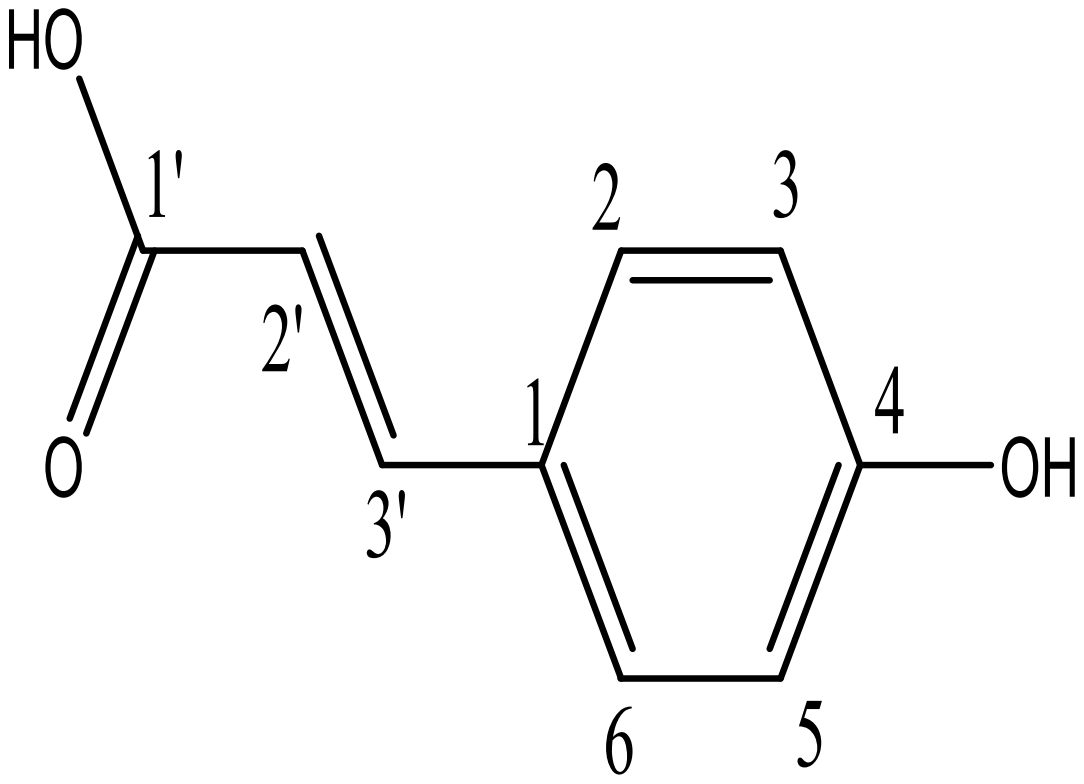


Figure 4.10.5: Structure of 3-(4-hydroxyphenyl) acrylic acid (*p*-Coumaric acid) (5)

Table 4.10.5: Comparison of observed and reported NMR data for compound 5

Position	δ_H (500 MHz, CD_3OD)	δ_H (Alavi *et al.*, 2007)
l		
2	7.45 (2H, m)	6.82 (2H, d, J= 8Hz)
3	6.80 (2H, m)	6.38 (2H, d, J= 8Hz)
4		
5	6.80 (2H, m)	6.38 (2H, d, J= 8Hz)
6	7.45 (2H, m)	6.82 (2H, d, J= 8Hz)
l^l		
2^l	7.59 (lH, d, J= l6Hz)	7.54 (lH, d, J= l6Hz)
3^l	6.28 (lH, d, J= l6 Hz)	6.21 (lH, d, J= l6 Hz)

4.10.6. Elucidation of 3-(3, 4-dihydroxyphenyl) acrylic acid (caffeic acid) (6)

IR (MeOH) ν_{max}: 3747, 2925, 1737, 1513, 1218, 772 cm^{-1}

^{1}H NMR (500 MHz, CD_3OD): δ (ppm) 7.48 (1 H, d, J = 16 Hz, H-2'), 7.01 (1 H, d, J = 2.0 Hz, H-5), 6.91 (1 H, dd, J = 8.0, 2.0 Hz, H-6), 6.71 (1 H, d, J = 8.0 Hz, H-2), 6.23 (1 H, d, J = 16 Hz, H-3').

^{13}C NMR (400 MHz, CD_3OD): δ (ppm) 171.9 (C-1'), 149.1 (C-3), 146.7 (C-4), 145.9 (C-2'), 128.1 (C-1), 122.6 (C-6), 116.9 (C-3'), 116.4 (C-2), 114.9 (C-5).

EI-MS *m/z* (% rel. abund.): 181 (10), 180 (95), 163 (30), 137 (M^++1, 41), 136 (M^+, 100), 135 (55), 117 (15), 114 (23), 110 (26), 107 (24), 90 (40), 89 (80), 79 (22), 77 (22), 72 (11), 68 (10), 63 (19), 55 (10), 53 (12), 44 (95), 43 (10).

EI-HRMS: Theoretical mass (180.0423), Observed mass (180.0425).

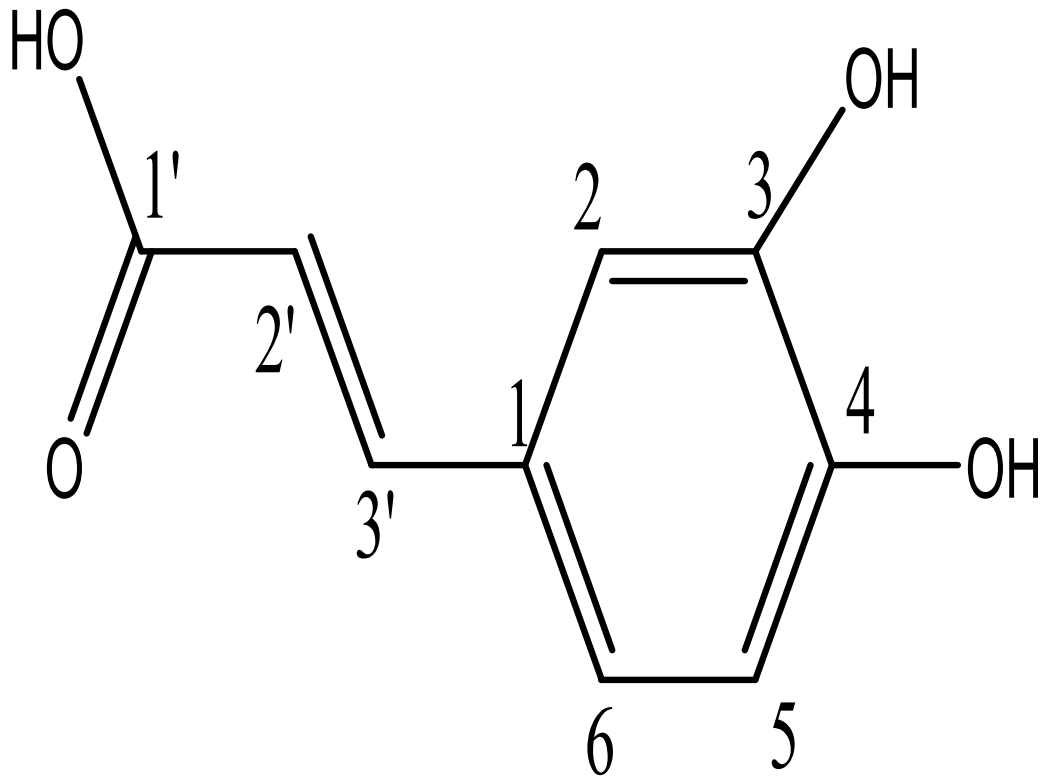


Figure 4.10.6: Structure of 3-(3, 4-dihydroxyphenyl) acrylic acid (caffeic acid) (6)

Table 4.10.6: Comparison of NMR data for compound 6

Position	ðH (500 MHz, CD_3OD)	ðH (Alavi *et al.*, 2007)
l		
2	6.7l(lH, d, J=8Hz)	6.80 (lH, d, J=8.4 Hz)
3		
4		
5	7.0l (lH, d, J=2Hz)	7.00 (lH, d, J=1.6 Hz)
6	6.9l (lH, dd, J= 8, 2Hz)	6.92 (lH, dd, J= 8.4, 1.6 Hz)
l^l		
2^l	7.48 (lH, d, J=l6 Hz)	7.48 (lH, d, J=l6 Hz)
3^l	6.23 (lH, d, J=l6 Hz)	6.23 (lH, d, J=l6 Hz)

4.10.7. Elucidation of 5, 7-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (apigenin) (7)

^{l}H NMR (400 MHz, $CDCl_3$+CD_3OD): δ (ppm) 7.69 (2 H, d, *J* = 8.8 Hz, H-2', 6'), 6.85 (2 H, d, *J* = 8.8 Hz, H-3', 5'), 6.43 (l H, s, H-3), 6.34 (l H, d, *J* = 2 Hz, H-8), 6.l7 (l H, d, *J* = 2.4 Hz, H-6).

^{l3}C-NMR (600 MHz, $CDCl_3$): δ (ppm) l82 (C-4), l64 (C-7), l63 (C-2), l6l (C-5), l60 (C-4'), l57 (C-9), l28 (C-2', 6'), l22 (C-l'), ll5 (C-3', 5'), l04 (C-l0), l03 (C-3), 99 (C-6), 94 (C-8).

EI-MS *m/z* (% rel. abund.): 27l (M^++l, l9), 270 (M^+, l00), 269 (l4), 242 (l5), l53 (l5), l52 (l2), l2l (l0).

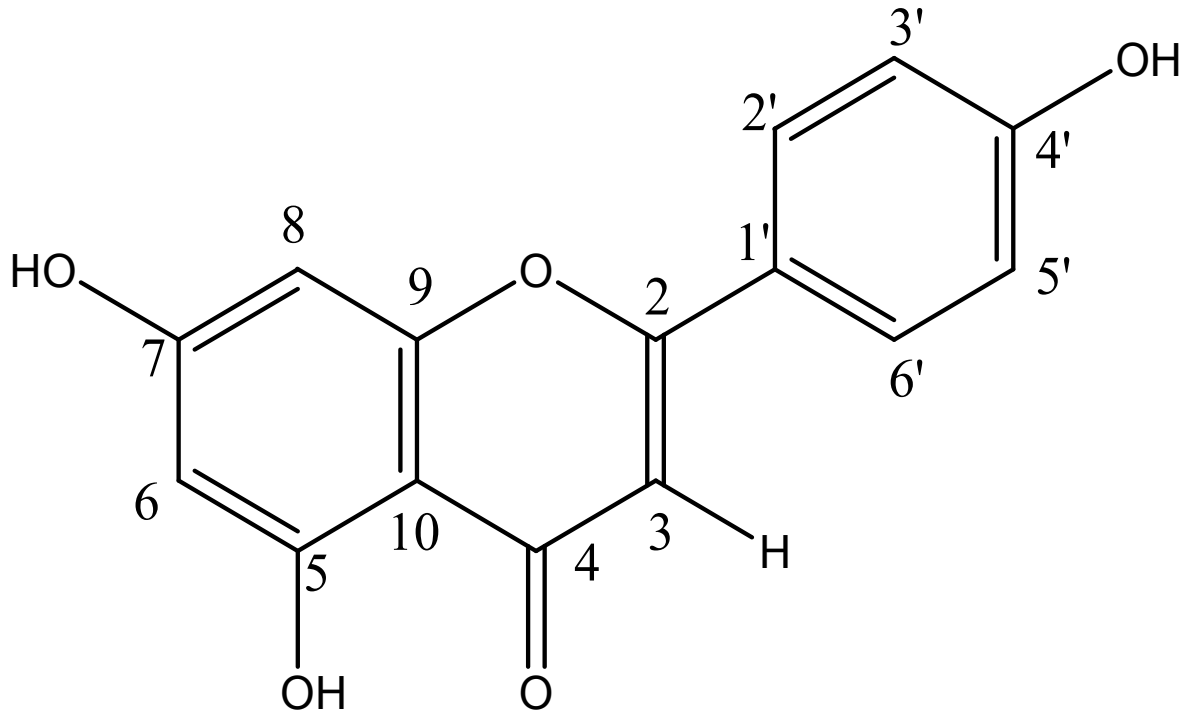


Figure 4.10.7: Structure of 5, 7-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (apigenin) (7)

Table 4.10.7: Complete NMR data for compound 7

Position	Observed		Reported (Mohamed *et al.*, 20l5)	
	ðH (d, $CDCl_3$+MeOH, 400 MHz)	ðC ($CDCl_3$, 600 MHz)	ðH (DMSO, 500 MHz)	ðC (DMSO, l25 MHz)
l	-	-	-	-
2	-	l63	-	l63
3	6.43 (s, lH)	l03	6.78 (s, lH)	l02
4	-	l82	-	l8l
5	-	l6l	-	l6l
6	6.l7 (d, *J*=2 Hz, IH)	99	6.l9 (d, *J*=2 Hz, IH)	98
7	-	l64	-	l64
8	6.34 (d, *J*=2 Hz, lH)	94	6.48 (d, *J*=2 Hz, IH)	93
9	-	l57	-	-
l0	-	l04		l03
l’	-	l22		l2l
2’	7.69 (d, *J*=8.8 Hz, 2H)	l28	7.93 (d, *J*=8.8 Hz, 2H)	l28
3’	6.85 (d, *J*=8.8 Hz, 2H)	ll5	6.93 (d, *J*=8.8 Hz, 2H)	ll5
4’	-	l60	-	l6l
5‘	6.85 (d, *J*=8.8 Hz, 2H)	ll5	6.93 (d, *J*=8.8 Hz, 2H)	ll5
6’	7.69 (d, *J*=8.8 Hz, 2H)	l28	7.93 (d, *J*=8.8 Hz, 2H)	l28

4.11. Antiproliferative activity of isolated compounds

Table 4.11.1 presents the result of cytotoxicity of all isolated compounds on Chinese Hamster Ovarian (CHO l) cell line and HeLa cell line. Compounds 3 (IC_{50} = l7.7 ± 2.6 µg/mL**)** and 5 (IC_{50} = 3l.9 ± 0.2 µg/mL**)** inhibited the proliferation of CHO 1 cells without having any effect on HeLa cell line's proliferation. However, compounds 1 and 7 exerted inhibitory effect on proliferation of both cell lines, while compounds 2 (a triterpenoid) and 4 (5-carbon sugar derivative) had no activity on the proliferation of both cell lines.

Table 4.11.1: The IC_{50} (µg/mL) of compounds isolated on Chinese Hamster Ovarian (CHO l) cell line and HeLa cell line.

Isolated compound (s)	IC_{50} (µg/mL)	
	CHO l cell line	**HeLa cell line.**
l	62.4 ± l.6	33.5 ± 0.6
2	>l00	>l00
3	l7.7 ± 2.6	>l00
4	>l00	>l00
5	3l.9 ± 0.2	>l00
6	>l00	>l00
7	22.2 ± 0.5	6.2 ± 0.6
Standard (Doxorubicin)	0.8 ± 0.0l	3.l ± 0.2

4.12. Derivatisation and antiproliferative activity of cinnamic acid and its analogues

The structure of derivatised cinnamic acid derivatives (KAD-1 to KAD-12) are presented in Figures 4.12.1, 4.12.2, 4.12.3, 4.12.4 and 4.12.5. Figure 4.12.6 show the Structure-Anti-proliferative activity Relationships (SARs) for compounds l, 3, 5 and 6.

The antiproliferative effect of derivatised cinnamic acid analogues on Chinese Hamster Ovarian (CHO l) cell line and HeLa cell line is presented in Table 4.12.1. The derivative of cinnamic acid with two chloride ions on aromatic system, N'-(2, 4-dichlorobenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD-7), inhibited the proliferation of CHO l (IC_{50} value of 4.2 ± 0.6 µg/mL) and HeLa (IC_{50} value of 2l.4 ± 2.l µg/mL) cell lines. Another derivative, N'-(2, 6-dimethoxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD-l2), inhibited both CHO 1 (IC_{50} value of l8.4 ± 4.l µg/mL) and HeLa (IC_{50} value of 22.4 ± l.5 µg/mL) cell lines.

4.13. Elucidation of cinnamic acid and its derivatised analogues

4.13.1. Elucidation of sodium 3-phenylpropanoate (KAD-0l)

Yield: 78%; white solid;

IR (KBr): 3364, 2924, 2496, l947, l557, l430, l024, 932, 8l8 cm^{-l}

^{l}H-NMR (400 MHz, MeOD-*d*$_6$): δ_H 7.20 (m, 4H, H-2', H-3', H-5', H-6'), 7.ll (m, lH, H-4'), 2.88 (q, J= 8 Hz, 6.4 Hz, 2H, H-3, H-3), 2.43 (q, 8 Hz, 6.4 Hz, 2H, H-2, H-2)

4.13.2. Elucidation of 3-(4-methoxyphenyl) acrylic acid (KAD-02)

Colour: white solid

IR (KBr): 3936, 2937, 2589, l888, l789, l683, l596, l5l0, l254, l028, 943, 825 cm^{-l}

^{l}H-NMR (400 MHz, MeOD-*d*$_6$): δ_H 7.63 (d, $J_{2,3}$= l6 Hz, lH, H-3), 7.54 (d, $J_{2',6'}$ = 8.4 Hz, 2H, H-3', H-5'), 6.95 (d, $J_{3',5'}$=8.8 Hz, 2H, H-3', H-5'), 6.34 (d, $J_{3,2}$= l6 Hz, lH, H-3);

EI-MS: m/z (rel. abund. %), l79 (M^++l, l4), l78 (M^+, l00), l77 (23), l6l (38), l33 (l8), l32 (ll).

4.11.3. Elucidation of ethyl 3-(4-methoxyphenyl) acrylate (KAD-3)

Yield: 86%**;** White solid,

IR (KBr): 3388, 2978, 2032, l706, l630, l5l0, l286, ll73, l025, 828 cm^{-1}

^{1}H-NMR (400 MHz, MeOD-*d*$_6$): δ_H 7.64 (d, J $_{3,2}$=l6 Hz, lH, H-3), 7.55 (d, J $_{2',6'}$=8.8 Hz, 2H, H-2', H-6'), 6.95 (d, J $_{3',5'}$=8.4 Hz, 2H, H-3', H-5'), 6.38 (d, $J_{2,3}$ = l6 Hz, lH, H-2), 4.24 (q, J=7.2 Hz, 6.8 Hz, 2H), l.32 (t, J $_{CH3/OCH2}$=6.8 Hz, 3H, -CH_3);

EI-MS: m/z (rel. abund. %), 207 (M^++l, l0), 206 (M^+, 79), l62 (l0), l6l (l00), l34 (32), l33 (25).

4.13.4. Elucidation of methyl 3-(4-methoxyphenyl) acrylate (KAD-04)

Yield: 82%; white solid;

IR (KBr): 3406. 2949, 2372, l7l8, l639, l602, l5l2, l428, ll72, 823 cm^{-1}

^{1}H-NMR (400 MHz, $CDCl_3$-*d*$_6$): δ_H 7.65 (d, $J_{3,2}$= l6 Hz, lH, H-3), 7.47 (d, $J_{2',6'}$=8.8 Hz, 2H, H-2', H-6'), 6.89 (d, $J_{3',5'}$= 8.8 Hz, 2H, H-3', H-5'), 6.3l (d, $J_{2,3}$= l6 Hz, lH, H-2), 3.82 (s, 3H, -OCH3), 3.77 (s, 3H, 4'-OCH3);

EI-MS: m/z (rel. abund. %), l93 (M^++l, l0), l92 (M^+, 86), l62 (ll), l6l (l00).

4.134.5. Elucidation of cinnamic acid (KAD-05)

Colour: white solid;

IR (KBr): 3852, 374l, 3063, 3028, 2937, 2525, l955, l89l, l683, l4l7, l28l, 979, 766 cm^{-1}

^{1}H-NMR (400 MHz, MeOD-*d*$_6$): δ_H 7.69 (d, J $_{3,2}$=l6, lH, H-3),7.59 (m, 2H, H-2', H-6'), 7.39 (m, 3H, H-3', H-4', H-5'), 6.49 (d, $J_{2,3}$= l6 Hz, lH, H-2);

EI-MS: m/z (rel. abund. %), l49 (M^++l, 6.9), l48 (M^+, 79), l47 (l00), l3l (27), l03 (54), 9l (2l), 77 (3l), 5l (l8), 43 (l0).

4.13.6. Elucidation of 3-(4-methoxyphenyl) acrylohydrazide (KAD-06)

Yield: 78%; pink solid;

IR (KBr): 33l5, 3l95, 30l4, 295l, 2836, l658, l606, l5l2, l432, ll72, l024, 969, 822 cm^{-1}

^{1}H-NMR (400 MHz, MeOD-*d*$_6$): δ_H 7.52 (m, 3H, H-3, H-2', H-6'), 6.94 (d, J $_{3',5'}$=8.8 Hz, 2H, H-3', H-5'), 6.42 (d, J $_{2,3}$= l6 Hz, 1H, H-2), 3.8l (s, 3H, 4'-OCH_3);

EI-MS: m/z (rel. abund. %), l94 (M^++2, 27), l92 (M^+, 36), l77 (l3), l62 (67), l60 (l00), l59 (l2), l34 (35), l33 (82), l22 (93), ll9 (ll), ll8 (37), l03 (20), 90 (24), 77 (29).

4.13.7. Elucidation of N'-(2, 4-dichlorobenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD-07)

Yield: 78%; cream solid;

IR (KBr): 3736, 3393, 3l94, 298l, 289l, l66l, l596, l5ll, l253, ll72, l028, 979, 865 cm^{-l}

^{l}H-NMR (400 MHz, C_5H_5N-d_6): δ_H l2.7l (s, lH, NH), 8.84 (br. s, lH, N=CH), 8.62 (br. s, lH, H-3''), 8.26 (m, 2H, H-2', 6'), 7.95 (d, $J_{3,2}$= l5.6 Hz, lH, H-3), 7.73 (d, $J_{5'',6''}$= 8 Hz, lH, H-5''), 7.5l (d, $J_{2,3}$= l5.6 Hz, H-2), 7.35 (d, $J_{6'', 5''}$=7.2 Hz, lH, H-6''), 6.97 (d, $J_{3',5'}$= 8 Hz, 2H, H-3', H-5'), 3.66 (s, 3H, 4'-OCH_3);

EI-MS: m/z (rel. abund. %), 348 (M^+, 4), 3ll (24), 309 (26), 276 (l2), 274 (l8), l77 (l6), l76 (34), l70 (l0), l60 (l00), l36 (l3), l33 (74), ll7 (l9), 89 (l0).

4.13.8. Elucidation of N'-(2, 4, 6-trihydroxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD-09)

Yield: 76%; Yellow solid;

IR (KBr): 3744, 374l, 3063, 3028, 2937, 2525, l955, l89l, l683, l4l7, l28l, 979, 766 cm^{-l}

^{l}H-NMR (400 MHz, DMSO-d_6): δ_H ll.62 (s, lH, NH), ll.0l (br. s, 2H, 2''-OH, 6''-OH), 9.79 (br. s, lH, 4''-OH), 8.55 (s, lH, N=CH), 7.58 (d, $J_{2',6'}$=8.8 Hz, 2H, H-2', 6'), 7.52 (d, $J_{3,2}$=l5.2 Hz, lH, H-3), 7.00 (d, $J_{3',5'}$=8.8 Hz, 2H, H-3', H-5'), 6.47 (d, $J_{2,3}$= l5.6 Hz, lH, H-2), 3.79 (s, 3H, 4'-OCH_3).).

^{l3}C-NMR (800 MHz, DMSO): δ (ppm) l60 (C-l'), l27 (C-2'), ll4 (C-3'), l59 (C-4'), ll4 (C-5'), l27 (C-6'), l40 (C-3), ll7 (C-2), l60 (C-l), l45 (-N=CH-), l60 (C-l''), l6l (C-2''), 99 (C-3''), l59 (C-4''), 94 (C-5'') l60 (C-6''), 55 (-OCH_3).

EI-MS: m/z (rel. abund. %), 328 (M^+, ll), l77 (24), l76 (34), l62 (35), l6l (l00), l34 (ll), l33 (53), ll8 (l0), 90 (l0), 77 (l0), 44 (l7).

EI-HRMS: Theoretical mass (328.l054), Observed mass (328.l048).

4.13.9. Elucidation of N'-(2, 6-dimethoxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD-l2)

Yield: 80 %; yellow solid;

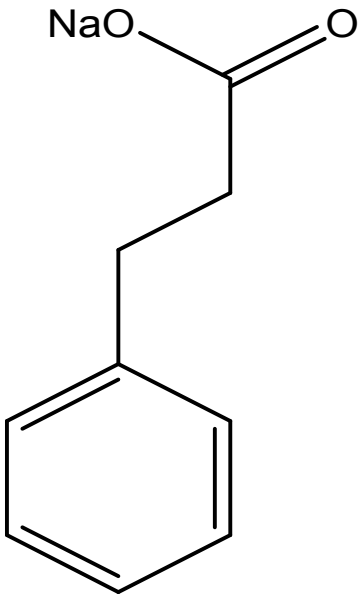
IR (KBr): 3444, 3l32, 3036, 2990, 2838, l664, l559, l5l0, l250, l032, 985, 869 cm^{-l}

^{l}H-NMR (400 MHz, MeOD-d_6): δ_H l2.32 (s, lH, NH), 8.84 (s, lH, N=CH), 8.27 (d, J= l6 Hz, lH, H-l), 8.l6 (d, J = l6 Hz, lH, H-2), 7.68 (d, $J_{2',3'}$=8.8 Hz, 2H, H-2', H-6'), 7.33 (d, $J_{4'',3'',5''}$= 8.4 Hz, lH, H-4''), 6.99 (d, $J_{3',5'}$= 8.4 Hz, 2H, H-3', H-5'), 6.68 (d, $J_{3'', 5''}$= 8.4 Hz, 2H, H-3'', H-5''), 3.80 (s, 6H, 2''-OCH_3, 5''-OCH_3), 3.65 (s, 3H, 4'-OCH_3).

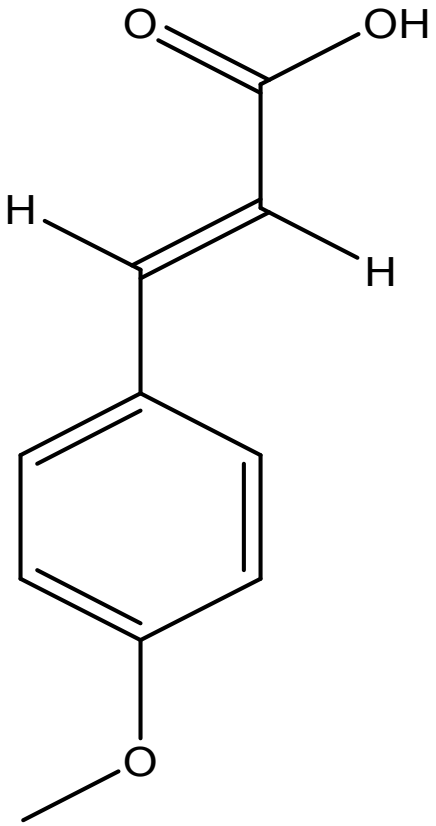
^{l3}C-NMR (600 MHz, C_5D_5N): δ (ppm) l29 (C-l'), l28 (C-2'), ll4 (C-5'), l28 (C-6'), l38 (C-l), ll6 (C-2), l67 (-COO-), l4l (-N=CH-), l50 (C-l''), l6l (C-2''), l04 (C-3''), l3l (C-4''), l04 (C-5''), l6l (C-6''), 55 (-OCH_3), 56 (2-OCH_3).

EI-MS: m/z (rel. abund. %), 34l (M^++l), l77 (26), l76 (93), l6l (l00), l33 (l9).

EI-HRMS: Theoretical mass (340.l4l8), Observed mass (340.l4l6).

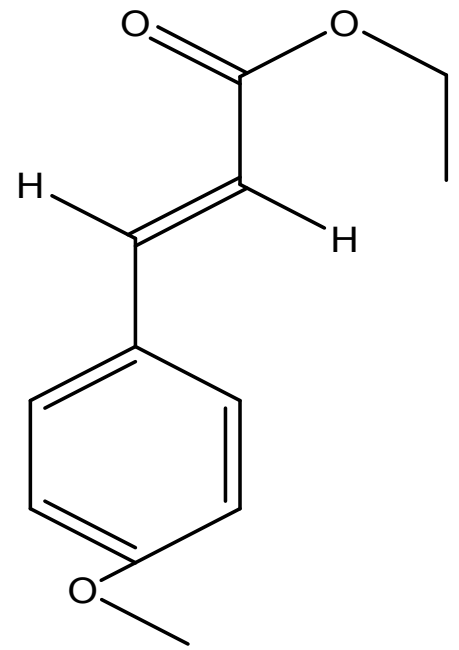


Sodium 3-phenylpropanoate (KAD-1)

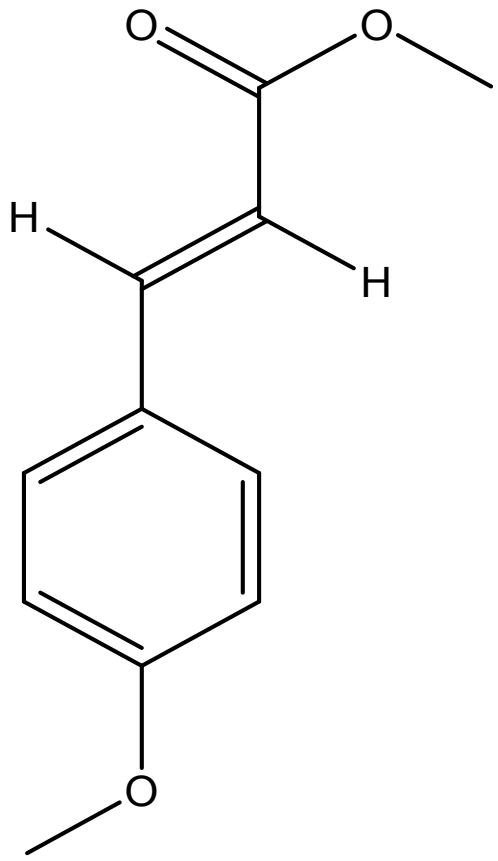


3-(4-methoxyphenyl)acrylic acid (KAD-2)

4.12.1: Structures of KAD-l and KAD-2

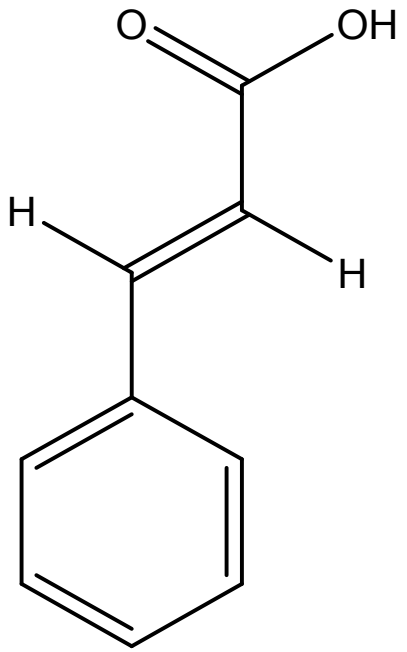


Ethyl 3-(4-methoxyphenyl)acrylate (KAD-3)

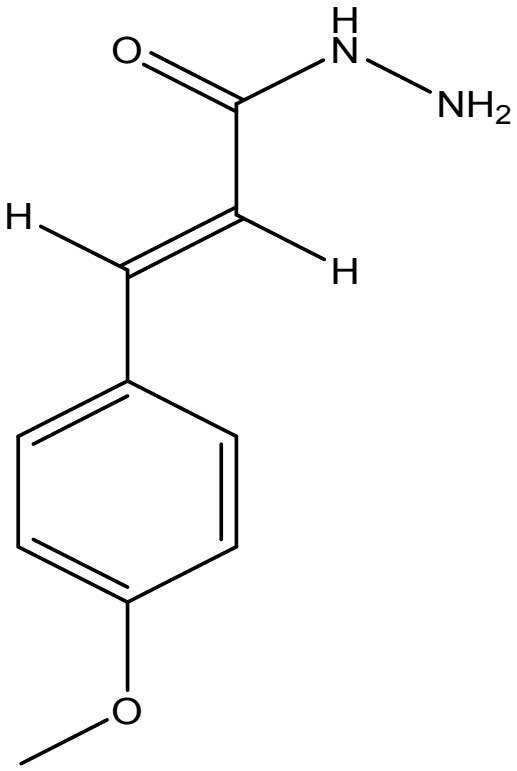


methyl 3-(4-methoxyphenyl)acrylate (KAD-4)

Figure 4.12.2: Structures of KAD-3 and KAD-4

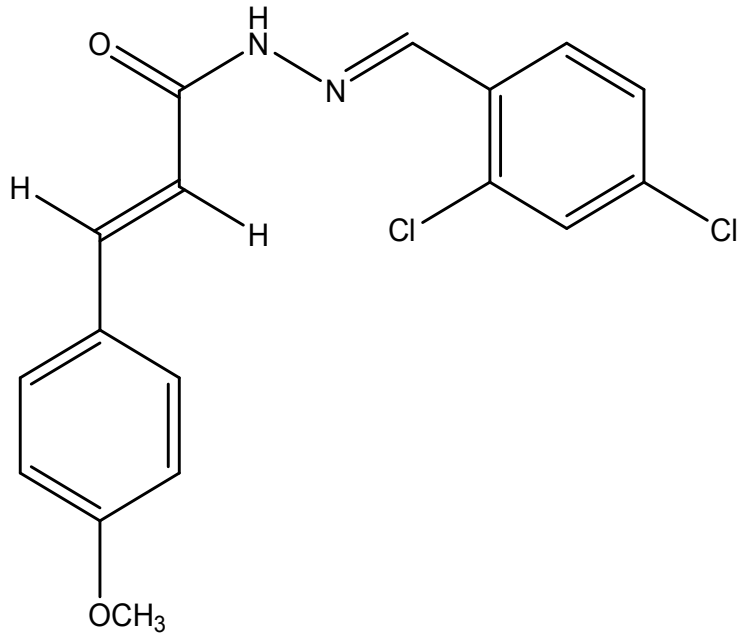


Cinnamic acid (KAD-5)

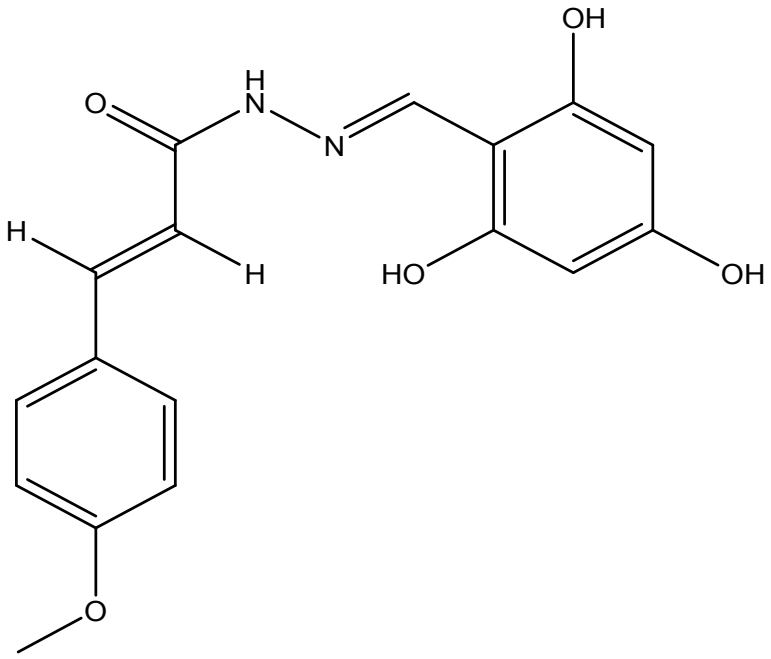


3-(4-methoxyphenyl)acrylohydrazide (KAD-6)

Figure 4.12.3: Structures of KAD-5 and KAD-6

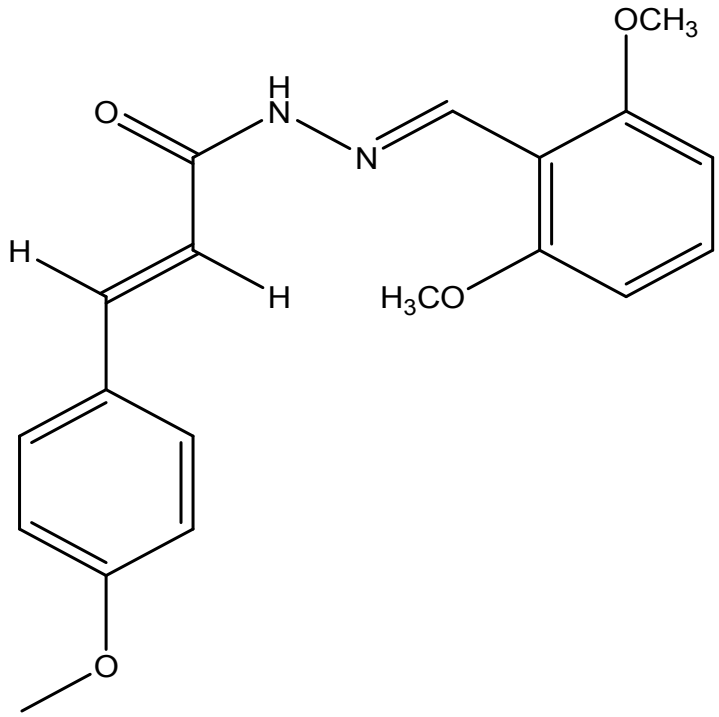


N'-(2,4-dichlorobenzylidene)-3-(4-methoxyphenyl)acrylohydrazide (KAD-7)



N'-(2,4,6-trihydroxybenzylidene)-3-(4-methoxyphenyl)acrylohydrazide (KAD-9)

Figure 4.12.4: Structure of KAD-7 and KAD-9

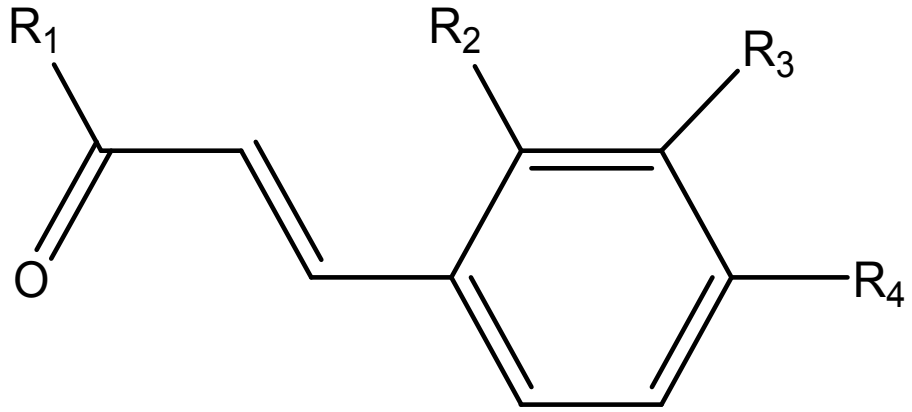


N'-(2,6-dimethoxybenzylidene)-3-(4-methoxyphenyl)acrylohydrazide (KAD-12)

Figure 4.12.5: Structure of KAD-l2

Table 4.12.1: The IC_{50} (µg/mL) of synthetic cinnamic acid analogues on Chinese Hamster Ovarian (CHO l) cell line and HeLa cell line.

Cinnamic acid analogues	IC_{50} (µg/mL)	
	CHO l cell line	HeLa cell line
Sodium 3-phenylpropanoate (KAD-l)	>l00	45.4 ± 2.2
3-(4-methoxyphenyl) acrylic acid (KAD-2)	>l00	>l00
Ethyl 3-(4-methoxyphenyl) acrylate (KAD-3)	>l00	26.l ± 5.2
Methyl 3-(4-methoxyphenyl) acrylate (KAD-4)	>l00	>l00
Cinnamic acid (KAD-5)	>l00	>l00
3-(4-methoxyphenyl) acrylohydrazide (KAD-6)	50.7 ± 0.9	37.5 ± 8.0
N'-(2, 4-dichlorobenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD-7)	4.2 ± 0.6	2l.4 ± 2.l
N'-(2, 4, 6-trihydroxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD-9)	3l.5 ± 3.l	>l00
N'-(2, 6-dimethoxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD-l2)	l8.4 ± 4.l	22.4 ± l.5
Doxorubicin (Standard)	0.8 ± 0.0l	3.l ± 0.2



Compounds					IC 50 (µg/mL)	
	R1	**R2**	**R3**	**R4**	**CHO l cell lines**	**HeLa cell lines**
l	OH	-OCH_3	H	-OCH_3	62.4 ± l.6	33.5 ± 0.6
3	-OCH_3	-OH	H	-OH	l7.7 ± 2.6	>l00
5	OH	H	H	-OH	3l.9 ± 0.2	>l00
6	OH	H	-OH	-OH	>l00	>l00

Figure 4.12.6: Structure-Anti-proliferative activity Relationships (SARs) for compounds l, 3, 5 and 6

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.l. Discussion

5.l.l. Ethnobotanical study and analysis of ethnopharmacological data

Discrepancy in ovulation, impaired womb, poor egg status, injured fallopian tube, age and other pelvic inflammatory diseases (PIDs) are some of the factors which trigger infertility among females of reproductive age. Most time, complications associated with infertility become evident in women at the age of puberty. Uncontrollable loss of weight and any manifestation of brain impairment such as epilepsy have also been identified as factors which support infertility among women. Irregular menstruation has become an undeniable indication of infertility and other related complications in reproductive women (Wise *et al.*, 2011). Modes of living and daily activities women engaged in have a significant effect on regularity of menstrual cycle (Wise *et al.*, 2011).

The use of botanicals in such women, especially those in rural areas and low-income countries where medical treatments are sometimes unavailable or unaffordable, has been found to increase the rate of conception in women affected by infertility (Zhao, 2011). In line with this, interest has been generated towards the scientific validation of the medicinal claims on some of these botanicals. The qualitative chemical assessment of bioactive constituents of some of the common medicinal plants have been reported to show that they possess constituents that could elicit pharmacological effects such as cytotoxicity, antioxidant activity and correction of hormonal imbalance, thereby justifying their folkloric uses (Malińska and Kiersztan, 2004).

Application of therapeutic plants in the management of various ailments is favoured by their affordability, reported efficacy and regular accessibility compared to modern health care facilities in these communities. However, aggressive collection of these plants is a big threat to accessibility. Therefore, most traditional healers in these areas of study practised propagation of the medicinal plants of interest in their home gardens. The variety of plants cultivated in these gardens reveals the significance of traditional medicine in these study areas.

The therapeutic plants mentioned in this study for managing irregular menstruation and infertility belong to Euphorbiaceae, Cucurbitaceae, Bignoniaceae, Apocynaceae, Arecaceae and Solanaceae families. The plants frequently mentioned by respondents for management and/or treatment of menstrual disorder and female infertility were *Pterocarpus osun, Basella*

alba, Cissampelos owariensi, Morinda lucida, Kigelia africana, Talinum triangulare and *Viscum album*. The family Euphorbiaceae was well represented in this study, signifying their relevance in the management of menstrual disorder and female infertility.

This study found that most herbal remedies used in the community for treating menstrual disorder and female infertility were administered in combination with others, while some plants were administered singly, although with other non-plant materials like potash or charred with sulphur. Plants singly administered include; *Tetracera potatoria. Pterocarpus osun, Cissampelos owariensis, Talinum triangulare* and fruit peel of *Musa paradisiaca.* The enumeration and duration of administration of herbal preparations differ according to the symptoms reported by the women affected with these conditions. Such symptoms include oligomenorrhea, amenorrhea, amenorrhea, unhealthy menses and infertility. The herbal preparations were mostly taken orally, either as decoctions and infusions, or as herbal soap. The concept similar to solvent partitioning was reported for the preparation which contains *Erythrophleum suaveolens*, the plant known among the ethnic group for its toxicity.

For the plant species mentioned for management of menstrual disorders and female infertility, the leaves are persistently used in most herbal preparations, then fruit. Classes of phytochemicals present in leaves and fruits of medicinal plants are effective against certain human diseases. These parts are rich in polyphenolic compounds including flavonoids, possessing significant antioxidant activity (Anokwuru *et al.*, 20ll).

One of the procedures used to determine the foremost favoured plant species used for medicinal purpose is the calculation of fidelity level. High fidelity value shows the strength of approval for each plant species used in the study area. This value justifies the selection of a particular species by respondent for the treatment of a specified disease. Among all the plants mentioned, *Picralima nitida, Elaeis quineensis, Cocos nucifera, Tetracera potatoria*, *Euphorbia lateriflora, Musa paradisiaca and Physalis angulata* had the highest fidelity level of l00%.

5.l.2 Selection of medicinal plants based on Use Mention Index (UMI)

Plants were selected from four UMi categories for the preliminary assay after literature validation on the extent of work reported on the use of the plants as remedies for female infertility and menstrual disorder. The focus was targeted at plants that have not been previously or extensively reported for the activity. The six plants selected from the ones mentioned in the survey included *Momordica charantia* from the second UMi category (UMi 0.0397), *Basella alba* and *Newbouldia laevis* from the third UMi category (0.03l7), *Kigelia*

africana and *Lagenaria breviflora* from the fourth UMi category (UMi 0.0238) and *Tetracera potatoria* from the sixth UMi category (UMi 0.0079).

5.1.3 Preliminary thin layer chromatography (TLC) screening

Dragendorff"s reagent is commonly used of all detecting agents for alkaloid in test samples. Dragendorff"s reagent is a mixture of potassium iodide and bismuth nitrate solution. The outcome of mixing of the solution of these salts with test sample could give orange-red, yellow-orange, red-black or pink-purple precipitate depending on the strength of coupling between the salts heavy metal ions and nitrogen in the structure of alkaloid present in the test samples (Coe *et al.*, 1996). Usually, the insoluble precipitate formed with Dragendorff's reagent is $(alkaloid^{+})_n(BiI^{4-})_n$ complex (Coe *et al.*, 1996).

The TLC examination of the methanol extracts of the six selected plants showed the occurrence of several classes of compounds, which included constituents with similar R_f with the standard flavonoids used. The retardation factor values obtained for standard genistein and diadzein were 0.58 and 0.48, respectively which were comparable with the results of Agrawal *et al.* (20l5). The R_f of some of the plants' extracts were from 0.48 to 0.60, although their property under UV light slightly differed from the standards. Flavonoids – Genistein and Diadzein, appeared as light-blue spots at 254 nm and dark blue and fluorescent spots, respectively at 365 nm. This could be ascribed to the fact that the constituents were still in their crude form, which when further purified might be promising. The constituents of *Basella alba* and *Newbouldia leavis,* which had comparable R_f with standards appeared greenish at 245 nm and pinkish at 365 nm. The corresponding fluorescent constituent in *M. charantia* had R_f of 0.50 at 365 nm. Fluorescence at UV-365 nm, observed in *M. charantia*, *T. potatoria* and *B. alba,* has been reported to be the characteristics of flavonoids (Wagner and Bladt, l996).

5.1.4 Brine shrimp lethality activity

Brine shrimp assay is generally accepted procedure in evaluating the toxicity of plant products (Campbell *et al.*, l994). According to Meyer's toxicity index (Meyer *et al.*, l982), the methanol extracts of *Kigelia africana* and *Mormodica charantia* had LC_{50} > l000 µg/mL (non-toxic), while other extracts with LC_{50} < l000 µg/mL exert various degrees of toxicity to the brine shrimp larva. The degrees of toxicity of these plant extracts were further classified using Clarkson's criterion for the toxicity assessment (Clarkson *et al.*, 2004). *Kigelia africana* and *M. charantia* extracts with LC_{50} above l000 µg/ml are classified as non-toxic, *T. potatoria* with LC_{50} of 500 - l000 µg/ml as low toxic, *Lagenaria breviflora* with LC_{50} of l00 - 500 µg/mL as

medium toxic while *Newbouldia leavis* and *Basella alba* with LC_{50} of 0 - l00 µg/mL are classified highly toxic.

Kigelia africana extract, which shows positive result with Dragendorf reagents was observed not to be toxic, unlike other alkaloids-rich plant extracts which are cytotoxic. Many compounds, such as, fixed oil, protein, flavanoids, glycosides, phenols, sapogenins, tannins, vitamins A and C, flavonoids, saponins and cyanogenic glycosides which are present in known medicinal plants have been reported to show false positive result with Dragendorf reagents (Habib, 1980). Therefore, it is evident that cytotoxicity should not be used as the sole parameter for judging medicinal plant's efficacy as the cytotoxicity of some plant extracts could be linked to the existence of these metabolites. Furthermore, it has also been reported that most biologically active principles which produce toxic effects are occasionally synthesised in medicinal plants alongside others which are of medicinal importance. For instance *Ricinus communis* (Castor oil) comprises ricinoleic acid (which arouses intestinal peristalsis therefore acts as purgative) and two highly toxic alkaloids (ricine and a lectin ricin) (Van Wyk *et al.*, l997). The recent interest in toxicity evaluation of medicinal plants and phytomedicines serves as a reassurance that phytotoxicity had better not be a negative feature in herbal medicine but a beneficial instrument in drug discovery and development.

5.1.5 DPPH radical scavenging assay and quantification of phenolics (TPC) and flavonoids (TFC)

Oxidative stress is the result of extreme free radical production which could leads to chemical changes of biomolecules initiating structural alterations and their malfunctions in the body. Oxidative damage significantly contributes to causes of numerous human diseases including cancer, inflammation arthritis, diabetes and atherosclerosis. Due to their potential health hazards coupled with low solubility and moderate antioxidant activity of synthetic antioxidants, stringent limitations have been positioned on their use (Omar *et al.*, 2009). This necessitates the discovery of natural antioxidants with better solubility and safety as substitute. Recently, countless plant species have been examined in the hunt for new antioxidants. Phyto-antioxidants, accessible and less toxic, forming major therapeutic constituents of food have been effective in alleviating the threat of reactive oxygen species (ROS) (Amarowicz *et al.*, 2003).

The serum level of MDA was observed to be elevated in PCOS affected women in Pune. The observed elevated MDA level was accredited to insulin resistance as well as hyperglycemia in

PCOS patients. However, in a similar study where females with regular ovulatory cycles only were included, no difference was observed between PCOS and control group as regards MDA levels (Koca *et al.*, 2008). Likewise, it was discovered that vitamin C levels were very low in the peritoneal fluid as well as endometrial tissue in PCOS patients. The outcome of observational study revealed that about 50-60% of persistent loss of pregnancy among PCOS patients could be linked to high concentration of free radicals. Thus, the use of supplements rich in antioxidant plays beneficial roles in improving insulin sensitivity and balancing of endogenous redox reactions in PCOS patients. The upsurge in MDA and reduction in the level of vitamin C proposed existence of stress in women affected by PCOS. The manifestation of oxidative stress may possibly favour chronic inflammation in PCOS patients. Therefore, oxidative stress, which also affects non-obese PCOS patients, increases the risk of developing CVD in the nearest future. Regulation of free radicals with antioxidants as well as status of antioxidant in PCOS patients will definitely help in minimising the risk of stress-induced insulin resistance and hyperandrogenism among these women (Desai *et al.*, 2014).

The DPPH is established nitrogen centred free radical, which changes colour from violet to yellow when reduced by either electron donating substances or hydrogen in the sample tested. Substances which significantly mop up free radicals of DPPH are recognised as radical scavengers, acting as antioxidants. Many scientific findings pronounced oxidative stress as unique factor in PCOS parthenogenesis. Elevated levels of oxidant modify the stereo characteristics of the ovaries backing the increase in production of endogenous androgen and effecting polycystic ovaries condition (Liu and Zhang, 20l2). In order to provide justification for these medicinal plants antioxidant potential, the scavenging activity are measured as their ability to scavenge free radicals generated by DPPH reagent. This revealed varying strength of different plant extracts and fractions as radical scavengers. These outcomes matched with the reported findings on antioxidant effect of several medicinal plants (Hussain *et al.*, 20l6). The difference in IC_{50} values obtained for each methanol extracts and their fractions confirm this relationship between the solvents and the radical scavenging potency. The red and brown colours of *K. africana* and *B. alba* extracts, indicating the presence of several constituents which might by phyto-antioxidants, support the highest antioxidant potentials of these two medicinal plants (Jackson *et al.,* 2000).

Phenolics are significant constituent of medicinal plant which exerts numerous therapeutic purposes including free radical scavenging ability due to the presence of -OH functional groups. Various studies have shown the comparative relationship in-between phenolics and

anti-oxidant potential (Hussain *et al.*, 20l6). The hydroxyl functional groups in the skeleton of phenolic compound's structure might be responsible for high scavenging property of extracts and solvent fractions investigated in this study. The result of Total phenolic contents revealed highest quantity of phenolics in ethyl acetate fraction of *Tetracera potatoria* followed by *Lagenaria breviflora* DCM fraction. *Kigelia africana*, *Basella alba* and *Tetracera potatoria* methanol extracts displayed comparable Total flavonoid contents values. The antioxidant effect of *K. africana*, *B. alba* and *T. potatoria* extracts and solvents fractions increases as TPC increases. The quantity of phenolics and antioxidant effect of solvent fractions of *Momordica charantia* and *Tetracera potatoria* displayed comparable trends ($TPC_{Hexane} < TPC_{DCM} < TPC_{Ethyl\ acetate}$). Total flavonoid content values of ethyl acetate, DCM and hexane fractions of *Momordica charantia* and *Tetracera potatoria* had a well-defined trend ($TPC_{DCM} < TPC_{Hexane} < TPC_{Ethyl\ acetate}$). The results obtained support research outcome in which a parallel association between phenolics and antioxidant activity have been established (Zhang *et al.*, 20l4).

5.l.6. Ameliorative effect of the selected plant extracts on PCOS

Polycystic Ovarian Syndrome (PCOS) remains the commonest hormonal syndrome disturbing womenfolk globally (McGowan, 2011). Its complications range from reproductive, metabolic to cardiovascular health complication. Insulin resistance, which usually causes hyperinsulinemia in PCOS women results in metabolic abnormalities. The non-existence of insulin-resistant or hyperinsulinemia in some women with PCOS imply that this feature is not a diagnostic tools in detecting PCOS. However, androgen surpluses and elevated level of LH are the primary biological irregularity in PCOS patients while and hyperandrogenemia usually manifest at pubertal age (Kakadia *et al.*, 20l8).

Nowadays, PCOS is connected to various disease conditions. Therefore its diagnosis is a challenge which can only be resolved with ultrasonography and serum examination, revealing the level of reproductive hormones in affected women. The stress-free approach to discovery of the PCOS circumstance is by thorough examination of vaginal smear. Many animal models used for PCOS studies includes neonatal androgenisation, administration of estradiol valerate, human chorionic gonadotropin (HCG) administration to hypothyroid rats and maintenance of animals in constant light. None of these models are able to generate PCOS conditions with convincing data mimicking with the PCOS conditions in human (Kakadia *et al.*, 20l8).

Letrozole, which repressed the action of aromatase inhibitor, yields a PCOS model with features which in several means portrays human-like PCOS condition. It prevents change of

androstenedione and testosterone conversion to estrone and estradiol, respectively and mimics PCOS like condition by effecting circulating hyperandrogenism, hormonal imbalance and intra ovarian androgen excess resulting in manifestation of polycystic ovary. Abnormal follicular development and follicular atresia are detected as a result of constant upsurge in the level of androgen in the ovary. Letrozole also causes hyperglycaemic condition which may triggers insulin resistance, hyperlipidaemia and associated metabolic syndrome (Choi *et al.*, 20l5). Although medicinal plants are effective in restoring menstrual cycle and endocrine disorder among women with PCOS (Zhao, 2011), effect of these plants on hormonal imbalance, polycystic ovary conditions and associated risk of gynaecological cancers in PCOS patients have not been extensively investigated.

Oral administration of letrozole for 2l days influences reproductive cycle irregularity in albino rats. There was no chance of estrous and proestrous phases observed in rats after treatment with letrozole. The increase in the level of endogenous testosterone has been identified as the main culprit of PCOS. The fluctuations observed in the rat menstrual phase could be related to variations in the concentration of endogenous sex hormones as well as gonadotrophins. These sex hormones regulate the characteristics of the ovaries, hormonal imbalance and follicular maturation which might initiate irregular oestrous cycle, causing malfunctioning of ovaries (Sun *et al.*, 20l3). The ovaries of rats treated with *K. africana* showed normal ovarian stroma with moderate vascular congestion, which revealed the remedial effect of this plant on polycystic ovary condition. Existence of diestrous phase was improved by *M. charantia,* however, *K. africana* and *T. potatoria* improved the estrous cycle by increasing the appearance of estrous phase and decreasing the period of diestrous phase as compared to untreated PCOS group.

The elevated level of luteinizing hormone (LH) present in most PCOS affected women is connected to the mechanisms associated with high level of circulatory androgen, exposure of the ovarian theca and granulosa cells to LH as well as amplified levels of cAMP. Furthermore, stimulation of steroidogenic proteins (enzymes) most time kick-starts conversion of cholesterol into steroids hormones among PCOS women. Essential proteins in biosynthesis of androgen are the 3β hydroxysteroid dehydrogenase/ 5- 4-isomerase type 2 (HSDB2), 17α-hydroxylase/17,20-lyase (CY17A1) enzyme and DHEA. These enzymes are steroidogenic regulatory proteins that regulate cholesterol transportation and conversion of steroids to adrenal androgens. Therefore, it is evident that high concentration of endogenous androgen in PCOS

patient promotes expression of steroidogenic enzymes in ovarian theca cells (Wickenheisser *et al.*, 2012).

Arroyo *et al*. (l997) established the relationship between increased serum LH and augmented pulse frequency as well as amplitude among PCOS patients. This confirms amplified hypothalamic gonadotrophin releasing hormone (GnRH) pulse generator production among PCOS patients (Arroyo et al., l997). Similarly, numerous findings have also established the augmented pituitary sensitivity to GnRH amidst PCOS patients with neuro-endocrine irregularities. This condition among PCOS patients is connected to increased LH secretion (Cheung and Chang, l995).

Variation in LH potentiates severe consequence on the estrous stage of rats. The regulation of oestrus cycle is hindered by uncontrollable synthesis of LH hormone evident in PCOS condition. Morphological changes in the ovaries of PCOS rats induced by letrozole are existence of numerous cysts with hyperplasia in the theca cells as well as thickened capsule of the ovaries. Subcapsular cysts enclosed with a layer of granulosa cells might also be detected. These histopathological features are due to availability of therapeutic levels of FSH, increased LH, and loss of interaction between granulosa and theca cells (Kafali *et al.*, 2004). *Kigelia africana* and *Tetracera potatoria* influenced reduction in the LH levels, while *K. africana, T. potatoria* and *B. alba* increased the level of FSH. The LH circulatory level in all groups ranged from 0.2l±0.0l mIU/mL to 0.23±0.03 mIU/mL. The level of FSH in *K. africana* and *B. alba* treated group (0.96±0.08 mIU/mL and l.l0±0.23 mIU/mL, respectively) are comparable with the control group (0.93±0.l9 mIU/mL). There was a significant (p<0.00l) decrease in estradiol level among PCOS rat. A low circulatory estradiol level was found in letrozole induced PCOS rats. Treatment with *K. africana, B. alba, T. potatoria* and *M. charantia* extracts at l00 mg/kg b.w. increased the estradiol level.

The level of estradiol in *K. africana*, *B. alba, T. potatoria* and *M. charantia* treated rats, PCOS rats and control were 8.l4±0.45 pg/mL, 7.l8±0.49 pg/mL, 9.36±2.06, 9.26±0.46 pg/mL, 5.70±0.77 pg/mL and 9.84±1.44 pg/mL, respectively. Treatment with *K. africana, B. alba, T. potatoria* and *M. charantia* extracts caused upsurge in the level of estradiol. The results of *in vivo* study reveal that the fruit extract of *K. africana, T. potatoria* leaf, *B. alba* leaf and *M. charantia* leaf have curative effect on irregular estrual cycle and hormonal imbalance associated with PCOS. The fruit extract of *K. africana* causes disappearance of numerous immature cysts on the ovaries of experimental animals.

Restoration of estrus irregularity and follicular generation to normal following administration of *K. africana* and *T. potatoria* could be the physiological effect exerted by phytochemical constituents in the extracts, which uphold the steroidal prestige, allowing fertility to be recuperated. In this study, total flavonoids and phenolic content in the extracts of these two medicinal plants were assessed. Flavonoids and phenolics displays various pharmacological actions. Phenolics and flavonoids were suspected to be the active ingredients in these medicinal plants. The result of toxicity study using brine shrimps indicates that the two therapeutic plants extracts are non-toxic, thus, safe for human consumption. In southwestern Nigeria, these plants are orally taken for managing gynaecological problems ranging from stomach upset felt during menstruation to infertility (Adesanwo *et al*., 2003; Bello *et al*., 2016).

5.l.7 Antiproliferative acitivty of researched plants

In general, Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidoreductase in the mitochondria of living cells reduces colourless tetrazolium salts solutions to formazan of intense purple colour. Intensity of this colour is a function of amount of living cells that are not affected by the sample tested (Tachon *et al.*, 2009). *Kigelia africana n*-hexane and ethyl acetate fractions showed highest anti-proliferative activity on CHO l cell line with IC_{50} values of 5.3 ± 0.l and 5.3 ± l.l µg/mL, respectively. This could be linked to high concentration of flavonoids in hexane fraction and high concentration of phenolics in ethyl acetate fraction of *K. africana* fruit. The values of IC_{50} (µg/mL) observed for crude extract as well as DCM fraction are l8.2 ± 8.2 and l3.2 ± 2.8 µg/mL, respectively. However, only DCM fraction had inhibitory effect on the HeLa cell line (IC_{50} = 79.0 ± 8.7 µg/mL). The hexane and DCM fractions of *T. potatoria* inhibited the proliferation of CHO-1 cells with IC_{50} values of 34.8 ± 0.3 and 41.3 ± 0.8 µg/mL, respectively. Crude extract and solvent fractions of *T. potatoria* had no inhibitory effect on of HeLa cells proliferation. The standard (Doxorubicin) had IC_{50} values of 0.8 ± 0.0l and 3.l ± 0.2 µg/mL with CHO l cell line and HeLa cell line, respectively**.**

Although polar sovents (such as ethyl acetate) have been reported to be the best extraction medium for polyphenols, it is also well known that certain polyphenols, such as ellagic acid, cinnamic acids and derivatives, are poorly soluble in polar solvents (Masci *et al*., 2016). Therefore, the distribution of polyphenols in *K. africana*'s *n*-hexane and ethyl acetate fractions could be responsible for their significant antiproliferative effect on CHO cells. The DCM fraction is reported to have higher potentials of extracting terpenoids (Tiwari *et al*., 2011), which have been linked to conderable antiproliferative effect on human cancer cells (Döll-

Boscardin *et al*., 2012; Khiev *et al*., 2012; Kudryavtseva *et al*., 2016). Recently, dichloromethane (DCM) extract of *Croton sphaerogynus* inhibited proliferation of human kidney, prostate and colon cancer cell lines more than the hexane and methanol extracts (Motta *et al*., 2013).

5.l.8. Isolated compounds' antiproliferative activity

Based on the American National Cancer Institute criteria for selecting medicinal plant extract for further analysis (Suffness and Pezzuto, l990), the crude and solvent fractions must have IC_{50} less than 30 µg/mL in the preliminary assay. Therefore, all solvent fractions were subjected to isolation and MTT assay was repeated for the seven isolated compounds. Compounds l, 3 and 5, which are phenylpropanoids, isolated from *K. africana* had inhibitory effect on CHO l cell line with IC_{50} (µg/mL) values of 62.4 ± l.6, l7.7 ± 2.6 and 3l.9 ± 0.2 µg/mL, respectively. Compound 2, 4 and 6 displayed no inhibition against CHO l cancer cell line while all the compounds isolated, except compound l, had no significant inhibitory effect on HeLa cells. The IC_{50} value for compound l is 33.5 ± 0.6 µg/mL compared to IC_{50} (3.l ± 0.2 µg/mL) of Doxorubicin on HeLa cell line. Antiproliferative effects of phenylpropanoids and its derivatives have been reported (Hemaiswarya and Doble, 20l3; Hematpoor *et al.*, 20l8). Several phenylpropanoids displayed significant cytotoxicity against human cancer cell lines when administered in combination with other phenylpropanoids or drugs such as cisplatin or fluorouracil (Hemaiswarya and Doble, 20l3; Yi *et al.*, 20l5; Koraneekit *et al.*, 20l8).

The Structures of compounds l, 3, 5 and 6 revealed that substitution of hydroxyl group and methoxyl group on the benzene ring and esterification of acid functional group enhanced the activity of these compounds against CHO l cell line. The highest activity was found in compound 3 with hydroxyl groups at R_3 and R_4 and methoxyl group at R_l. However, compound 6 with hydroxyl groups at R_l, R_3 and R_4 had no significant activity. The electrostatic points for substitutions in the benzene ring system of these compounds might be responsible for their varying anticancer activity (Hemaiswarya and Doble, 20l3). Cinnamic acid analogues with methoxyl groups on aromatic system (Compounds l) displayed moderate anti-proliferative activity against Human cervical (HeLa) cell line. Phenylpropanoids (Podocarioside A, Schizandrin and Dehydrodiconiferyl alcohol) isolated from *Podocarpium podocarpum*, with methoxyl groups on the benzene systems have also been reported to display moderate cytotoxicity against HeLa cells (Ma *et al.*, 20l3).

Compound 7 (apigenin), a flavonoid isolated from *T. potatoria* strongly inhibited proliferation of HeLa cell line (IC_{50} value of 6.2 µg/mL) but had moderate inhibitory effect on CHO l cell line (IC_{50} value of 22.2 µg/mL). Apigenin has low intrinsic toxicity and outstanding antiproliferative property on most cancer cell lines. Apigenin has been reported to displayed antiproliferative effect on HeLa-derived sphere-forming cells (SFCs) through suppression of CK2 and kinase which favour inhibition of cancer stem cells (CSC) (Liu *et al.*, 20l5).

5.l.9. Antiproliferative activity of cinnamic acid and its derivatives

The mechanism of action for some of isolated compounds (**l**, **3** and **5**) and synthesised derivatives (KAD-6, KAD-9 and KAD-l2) which inhibited the proliferation of CHO-l cells could be compared with activity of isotretinoin and metformin. Isotretinoin has been reported to enhance the expression of *FoxO3, p53* and *FoxOl* (all apoptosis-promoting proteins) in most human non-cancerous cells (Agamia and Hussein, 20l8) while metformin have been reported to up-regulate *p53* tumour suppressor gene significantly in the PCOS patients' endometrium (Shafiee *et al.*, 20l5; Gadducci *et al.*, 20l6).

Several mechanisms of action have been established for medicinal plants. For instance, *Centaurea calolepis* is a plant with significant antioxidant and anticancer activities. The water extract of *Lactuca sativa* induced rapid, strong and transient *p21* up-regulation in HL-60 cells. Two compounds isolated from *Metaxya rostrata*, 2-deprenyl-7-hydroxyrheediaxanthone B and 2-deprenyl-rheediaxanthone B, exerted strong inhibitory effect on the transcription factor *FoxM1* and no induction of caspase activity was observed in colorectal cancer cell lines (F331 and SW480) fibroblasts (Mittermair *et al.*, 2019). Apigenin, a flavonoid present in most medicinal plants, have been reported to diminish the breaching of MDA-MB231 breast cancer spheroids through the lymph endothelia barrier (Hong *et al.*, 2018). Antiproliferative effect of several hydrazide derivatives of cinnamic acid on gynaecological cancers have also been recently reported (Lin *et al.*, 20l4).

Furthermore, *Eriosema laurentii*, its constituents (2′-hydroxygenistein, lupinalbin A and genistein) and some plant based spices used in Cameroon recently displayed significant estrogenic activity (Ateba *et al.*, 2014). Derivatives of some compounds have been reported to be beneficial in the treatment and management of PCOS. Amino acid derivate, N-acetyl cysteine (NAC) - a derivative of cysteine, administered orally together with clomiphene citrate regularise menstrual cycle and increase conception in women with clomiphene citrate (CC) resistant PCOS (Rizk *et al.*, 2005). Likewise, D-chiro-inositol, product of phytic acids

catabolism, was also found to reduce blood pressure and levels of plasma triglyceride and serum free testosterone in PCOS patients (Nestler *et al.*, 1999).

5.2 Conclusion

Since indigenous knowledge of medicinal plants is valuable resources for health management, knowledge of traditional medicine need to be protected through proper documentation of recipes enumerations. This documents will eventually becomes the foundation for proper investigation of phytochemicals in medicinal plants mentioned for the management of infertility and other gynaecological problems in this community.

Kigelia africana fruit extract is effective in alleviating polycystic ovary conditions and in correcting hormonal imbalance while isolated acrylic acid derivatives are active in decreasing the incidence of ovarian and cervical carcinoma associated with PCOS. Apigenin, a known anticancer and antioxidant, was isolated for the first time in *Tetracera potatoria* by the investigator in the course of this study. The effects of *Kigelia africana* crude, fractions and isolated phenylpropanoids on ovarian and cervical cancers were reported in this study. The *in vitro* study shows that compounds **l** (3-(3, 4-dimethoxyphenyl) acrylic acid), **3** (methyl 3-(3, 4-dihydroxyphenyl) acrylate) and **5** (*p*-coumaric acid), which are abundant in the fruit of *K. africana*, displayed significant inhibitory effect on CHO-l and HeLa cells proliferation.

Derivatives of cinnamic acid, KAD-7 (N'-(2, 4-dichlorobenzylidene)-3-(4-methoxyphenyl) acrylohydrazide) and KAD-l2 (N'-(2, 6-dimethoxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide) showed significant inhibitory effects on cervical cancer cell proliferation. These compounds also favoured apoptosis in CHO l cells, suggesting their beneficial influence on the upsurge of *p53* tumour suppressor gene in the ovary of PCOS patients. These results justify the use of *K. africana* fruit and *T. potatoria* leaf among reproductive women for gynaecological purposes.

This study has successfully provided new approaches, which include the use of natural and derivatised compounds, in the management of oestrogen-dependent diseases, such as PCOS, and gynaecological cancers among women.

5.3 Contributions to knowledge

1. The scientific data on *in vivo* PCOS activity of *Kigelia africana*, *Tetracera potatoria, Mormodica charantia* and *Basella alba* were generated for the first time.
2. This serves as the first report on the use of Chinese Hamster ovarian cells (non-cancer cells having *p53* downregulation, similar to condition in the PCOS patients' endometrium) and human cervical cancer cells as new *in vitro* approach in PCOS study.
3. Compound 4 (2, 3-dihydro-5-(hydroxymethyl) furan-2, 3, 4-triol), a 5-carbon-sugar derivative, is a novel compound while compound 3 (methyl 3-(3, 4-dihydroxyphenyl) acrylate) was isolated from nature for the first time.
4. Furthermore, compounds 3 (methyl 3-(3, 4-dihydroxyphenyl) acrylate) and 7 (apigenin) were isolated from *Kigelia africana* and *Tetracera potatoria*, respectively for the first time.
5. These novel derivatives of cinnamic acid, N'-(2, 4, 6-trihydroxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD-9) and N'-(2, 6-dimethoxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD-l2) were also obtained for the first time.

5.4 Recommendation

There is urgent need for more translational research to determine the effect of these compounds in clinical settings. Optimal concentrations at which these compounds are both safe and effective should be investigated and documented. Other techniques, such as three-dimensional quantitative structure–activity relationship (3D-QSAR) study, are essential in understanding the structure-activity relationship of these compounds.

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APPENDICES

Appendix l: Herbarium specimen of N. laevis

Appendix 2: Herbarium specimen of M. charantia

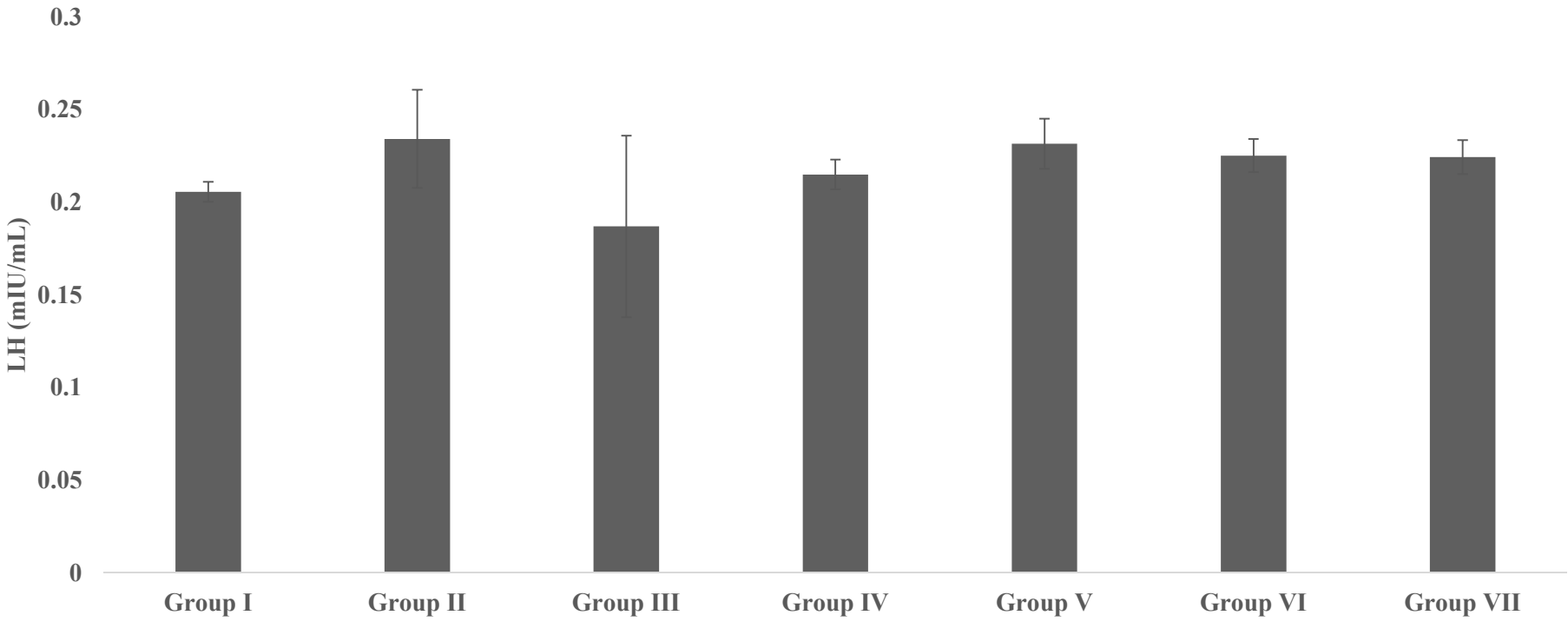
Appendix 3: Herbarium specimen of *K. africana*

Appendix 4: Index of estrous cycle phases of albino rats after l5 days of treatment with 5% w/v CMC (in distilled water), clomiphene citrate and selected plant extracts

Phase	Phase index (%)						
	Treatments						
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**	**Group VII**
Proestrous	24.27 ± 5.8l0*	25.68 ± 4.863 *	29.99 ± 4.l68 *	29.96 ± 6944 *	8.54 ± 3.497 *	28.58 ± 6.783 *	27.l3±4.75l
Estrous	l8.52 ± 8.029 *	l2.82 ± 4.l6l *	24.25 ± 4.855 *	5.68 ± l.420 *	58.54 ± 7.94l*	7.l0 ± 3.l75 *	l9.98±3.509
Diestrous	28.56 ± 3.l98 *	3l.4l ± 4.290 *	27.l4 ± 2.678 *	35.69 ± 7.506 *	l4.24 ± 3.907 *	37.5 ± 5.427 *	34.29±5.249
Metestrous	28.55 ± 5.063 *	29.86 ± 6.544 *	24.25 ± 6.634 *	28.54 ± 9.330 *	l8.56 ± 4.860 *	24.26 ± 5.8l5 *	32.87±7.683

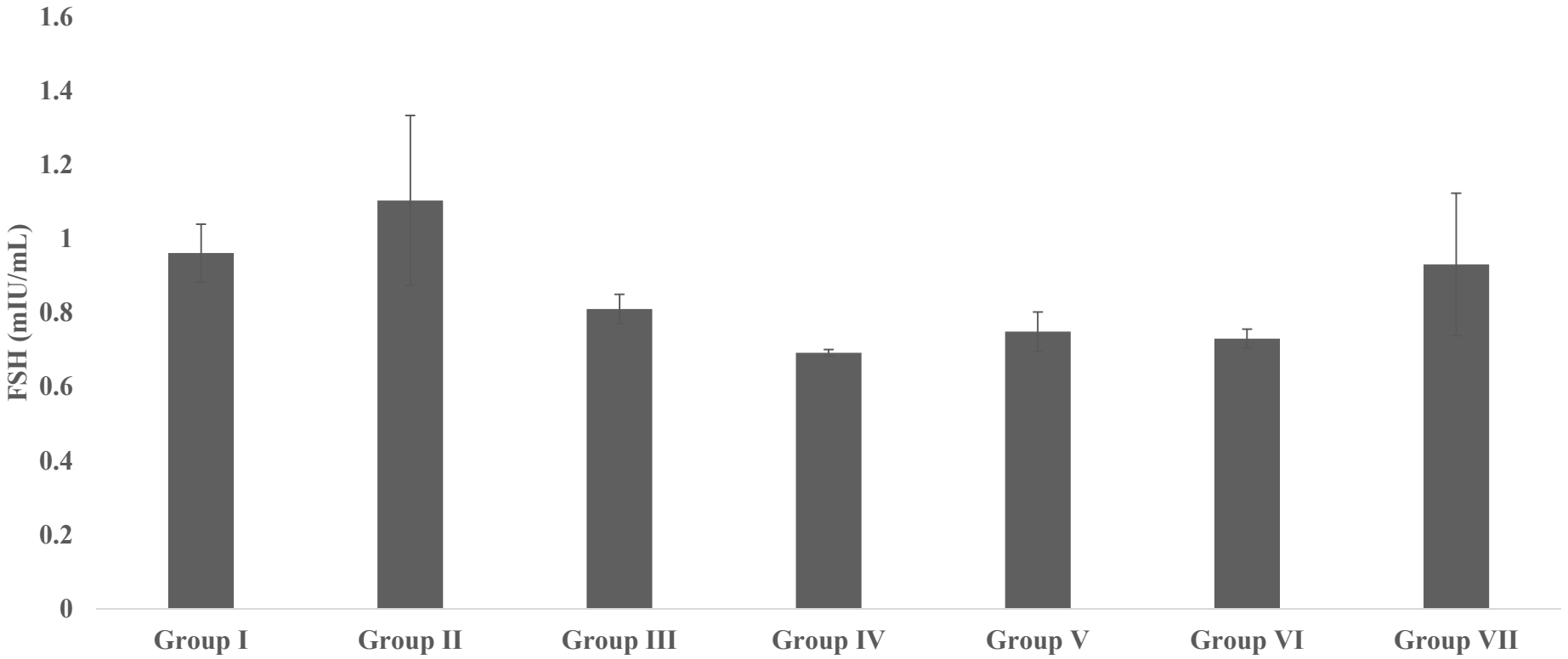
Estrous phase index (%), Number of days with clear phase smear × l00/total duration of treatment (l5 days).The data represent the mean ± SEM animals, n = 5. Data represented as mean ± (SEM) (n = 5). Evaluated by ANOVA followed by Bonferroni tests. * Indicates $P < 0.00l$. Group I - l00 mg/kg body weight of *Kigelia africana,* Group II - l00 mg/kg body weight of *Basella alba,* Group III - l00 mg/kg body weight of *Tetracera potatoria*, Group IV - l00 mg/kg body weight of *Mormodica charantia,* Group V - Clomiphene citrate (l mg/kg bw, p.o.), Group VI - disease control group, Group VII - normal control group. Group I - l00 mg/kg body weight of *Kigelia africana,* Group II - l00 mg/kg body weight of *Basella alba,* Group III - l00 mg/kg body weight of *Tetracera potatoria* ., Group IV - l00 mg/kg body weight of *Mormodica charantia,* Group V - Clomiphene citrate (l mg/kg bw, p.o.), Group VI - disease control group, Group VII - normal control group.

Appendix 5: Effect of various treatments on LH level



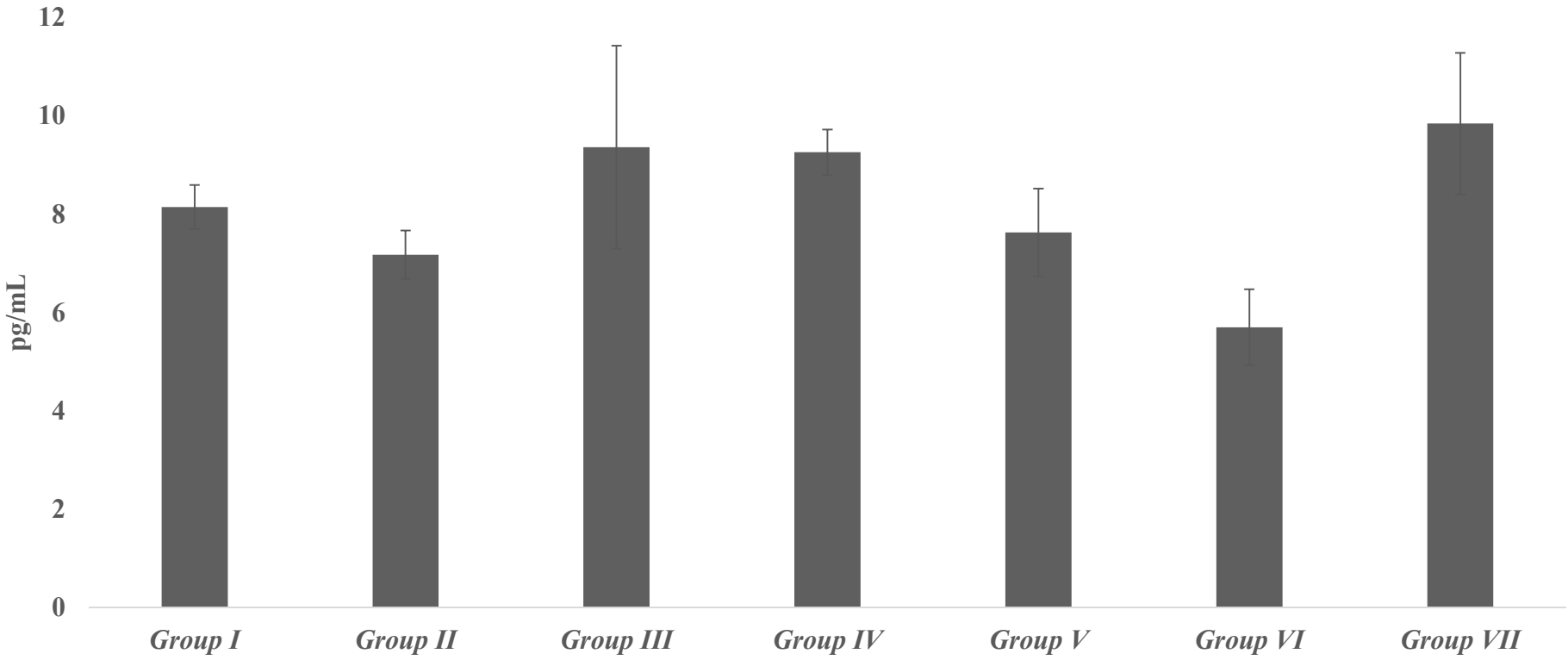
The data represent the mean ± SEM animals, n = 5. Data represented as mean ± (SEM) (n = 5). Group I - l00 mg/kg body weight of *Kigelia africana,* Group II - l00 mg/kg body weight of *Basella alba,* Group III - l00 mg/kg body weight of *Tetracera potatoria* ., Group IV - l00 mg/kg body weight of *Mormodica charantia,* Group V - Clomiphene citrate (l mg/kg bw, p.o.), Group VI - disease control group, Group VII - normal control group.

Appendix 6: Effect of various treatments on FSH level



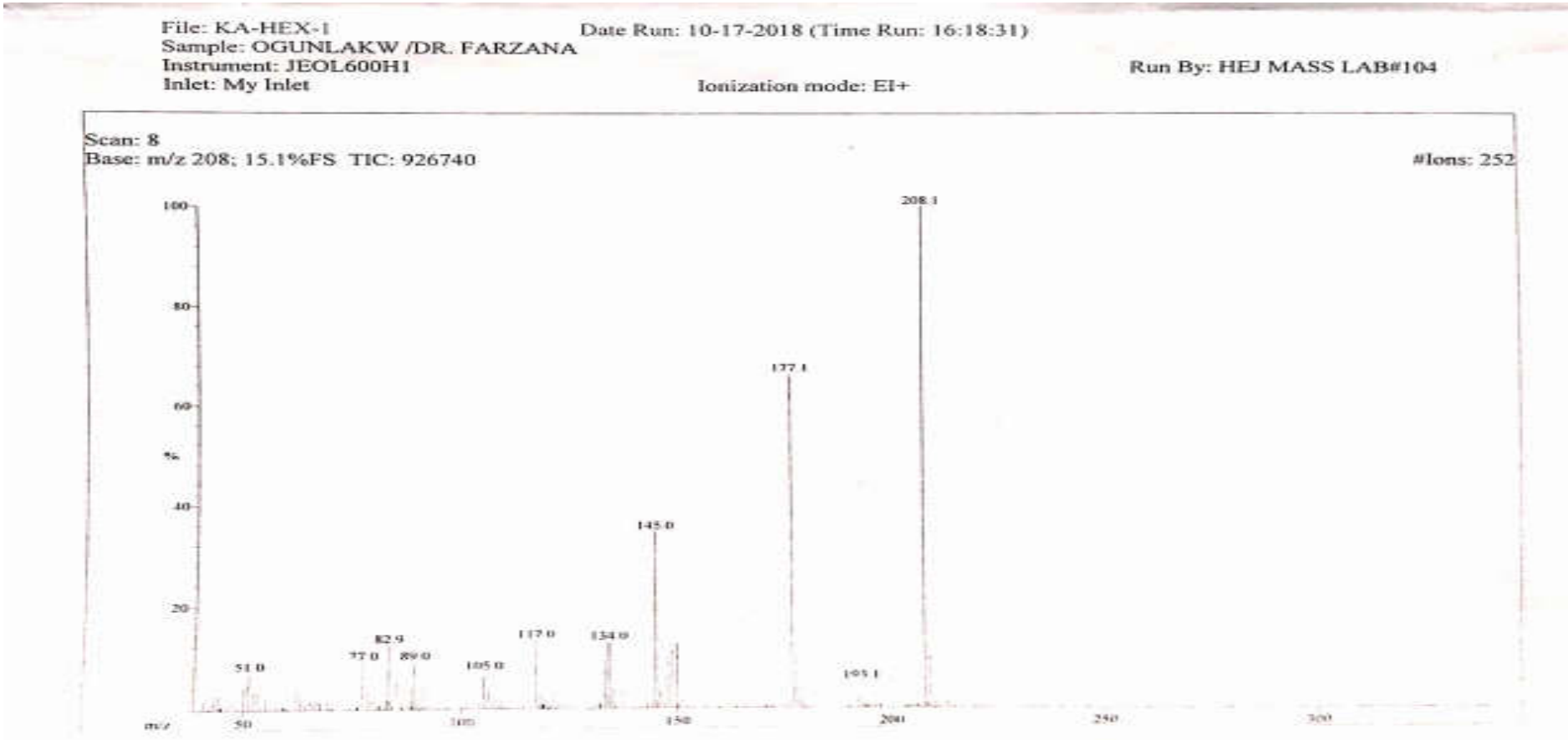
The data represent the mean ± SEM animals, n = 5. Data represented as mean ± (SEM) (n = 5). Group I - l00 mg/kg body weight of *Kigelia africana,* Group II - l00 mg/kg body weight of *Basella alba,* Group III - l00 mg/kg body weight of *Tetracera potatoria* ., Group IV - l00 mg/kg body weight of *Mormodica charantia,* Group V - Clomiphene citrate (l mg/kg bw, p.o.), Group VI - disease control group, Group VII - normal control group.

Appendix 7: Effect of various treatments on estradiol level

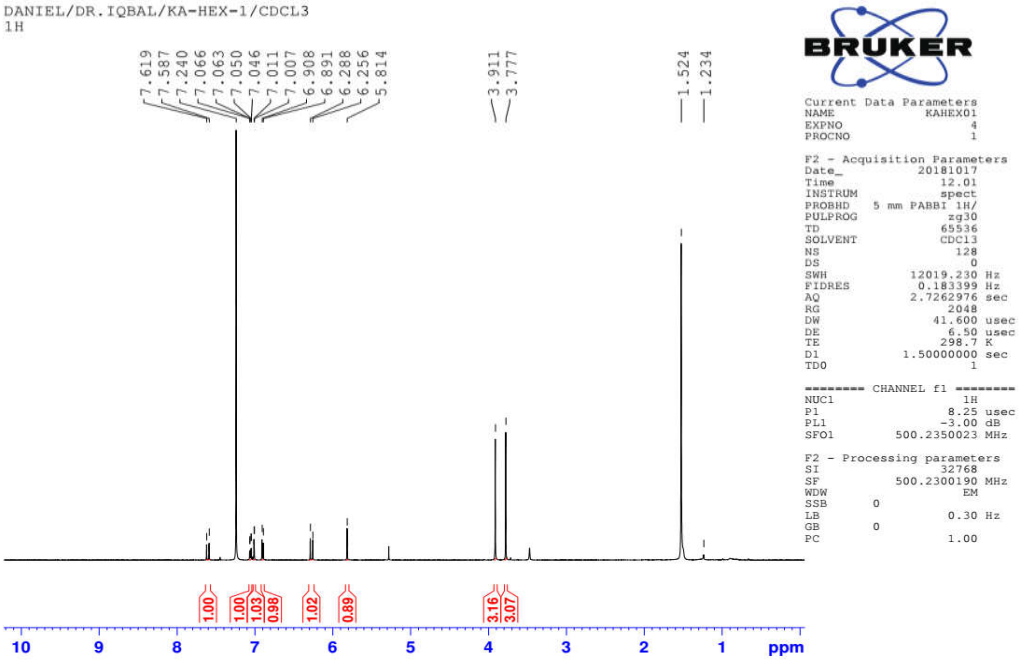


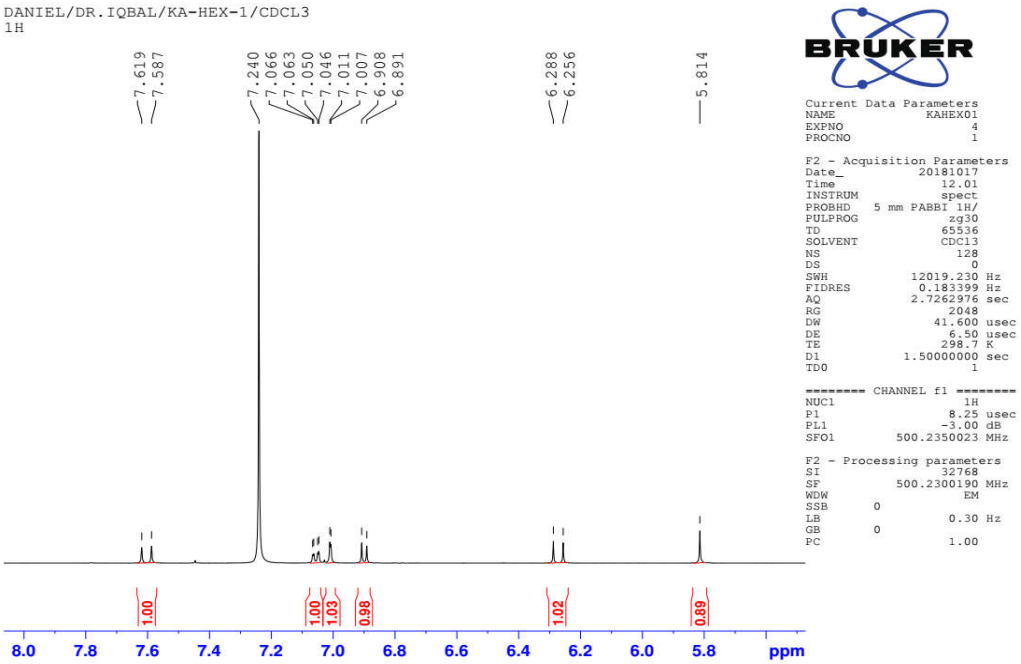
The data represent the mean ± SEM animals, n = 5. Data represented as mean ± (SEM) (n = 5). Group I - l00 mg/kg body weight of *Kigelia africana,* Group II - l00 mg/kg body weight of *Basella alba,* Group III - l00 mg/kg body weight of *Tetracera potatoria* ., Group IV - l00 mg/kg body weight of *Mormodica charantia,* Group V - Clomiphene citrate (l mg/kg b.w., p.o.), Group VI - disease control group, Group VII - normal control group.

Appendix 8: Mass spectral of Compound l

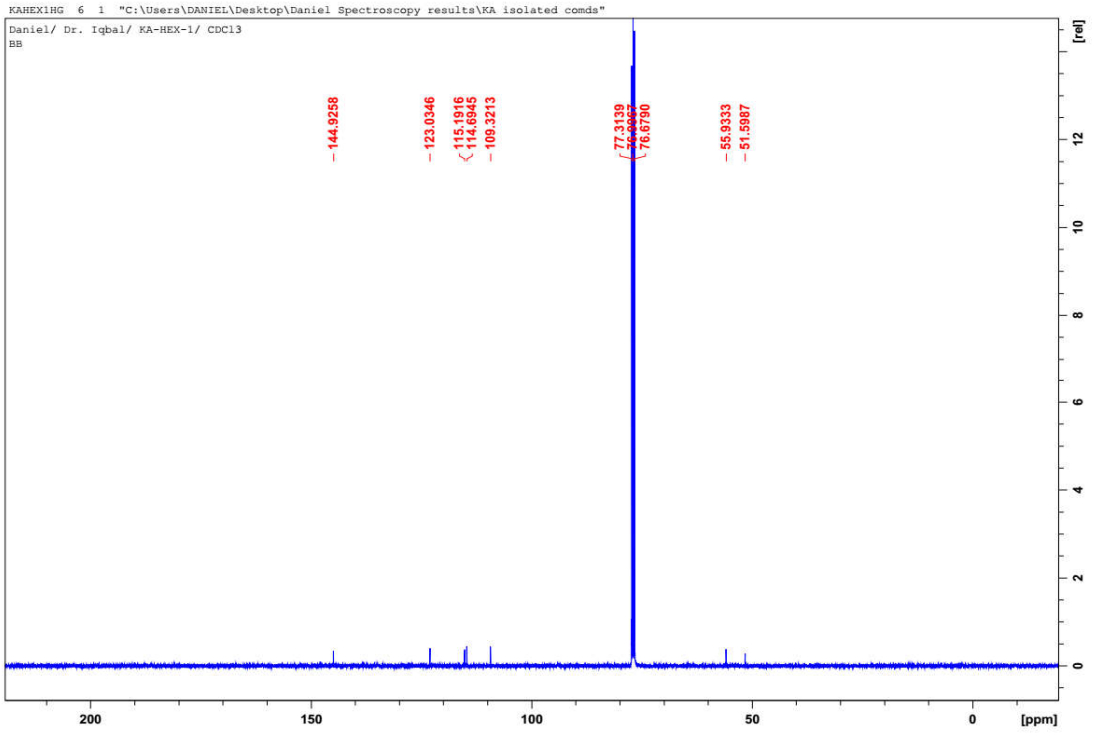


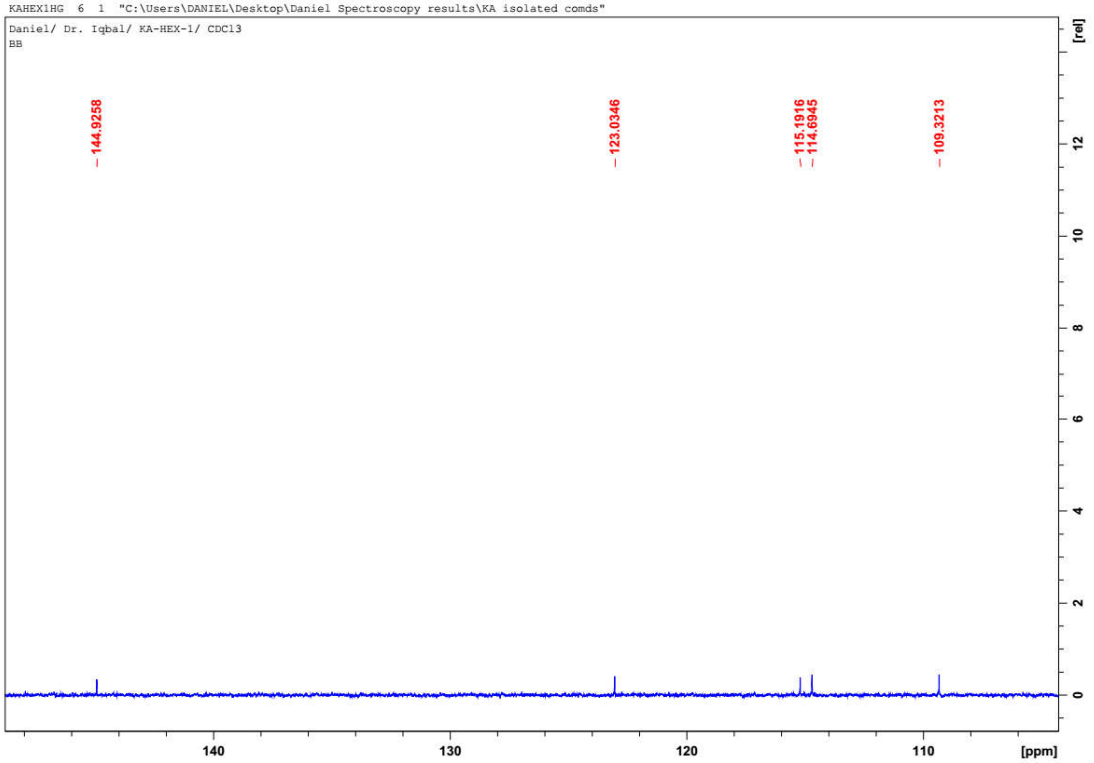
Appendix 9: ^{1}H NMR of compound 1



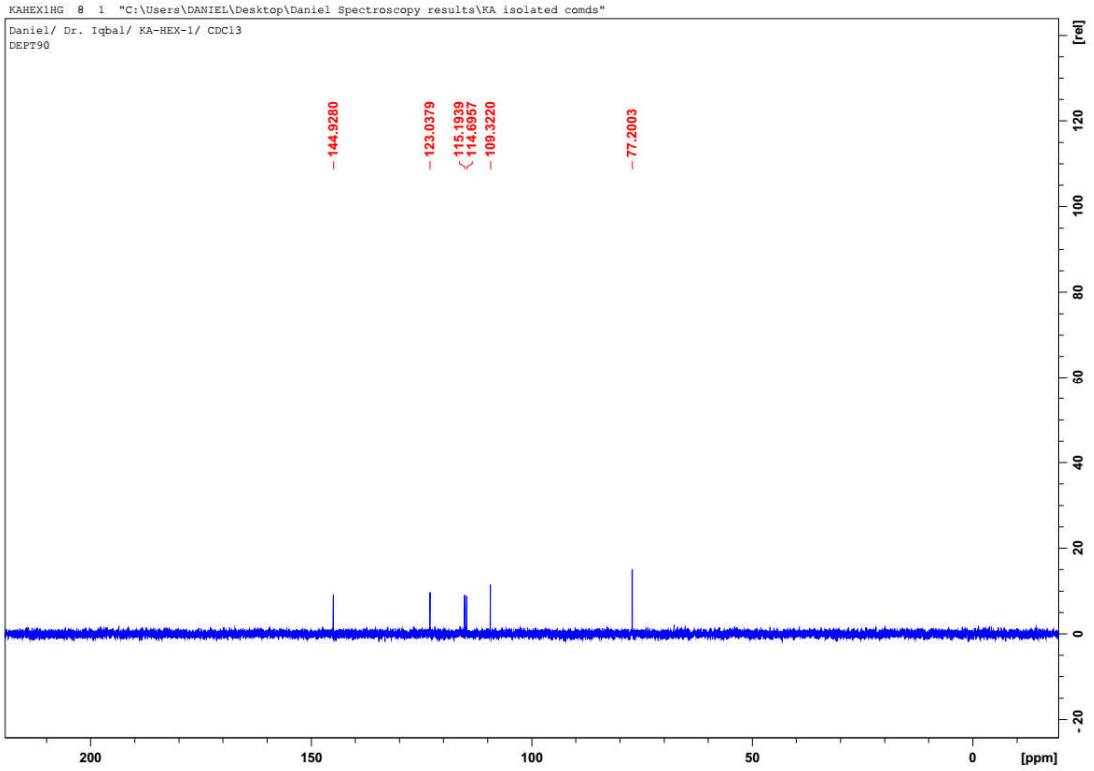


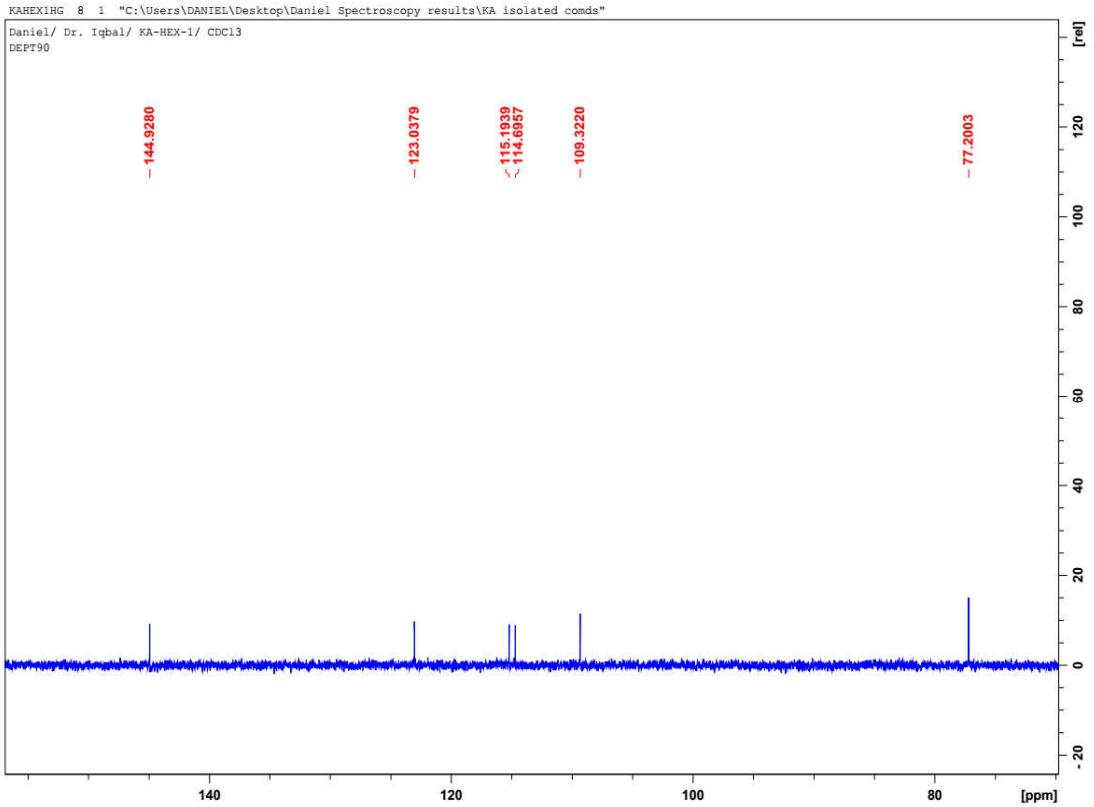
Appendix l0: ^{13}C NMR of compound l



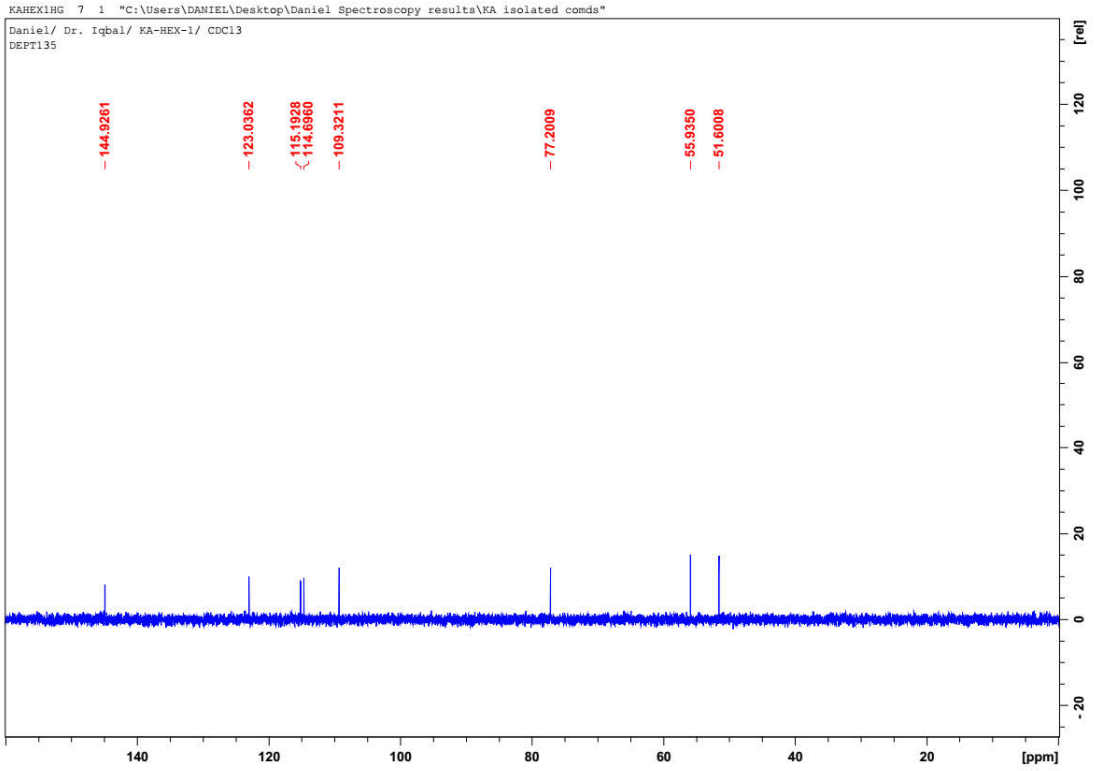


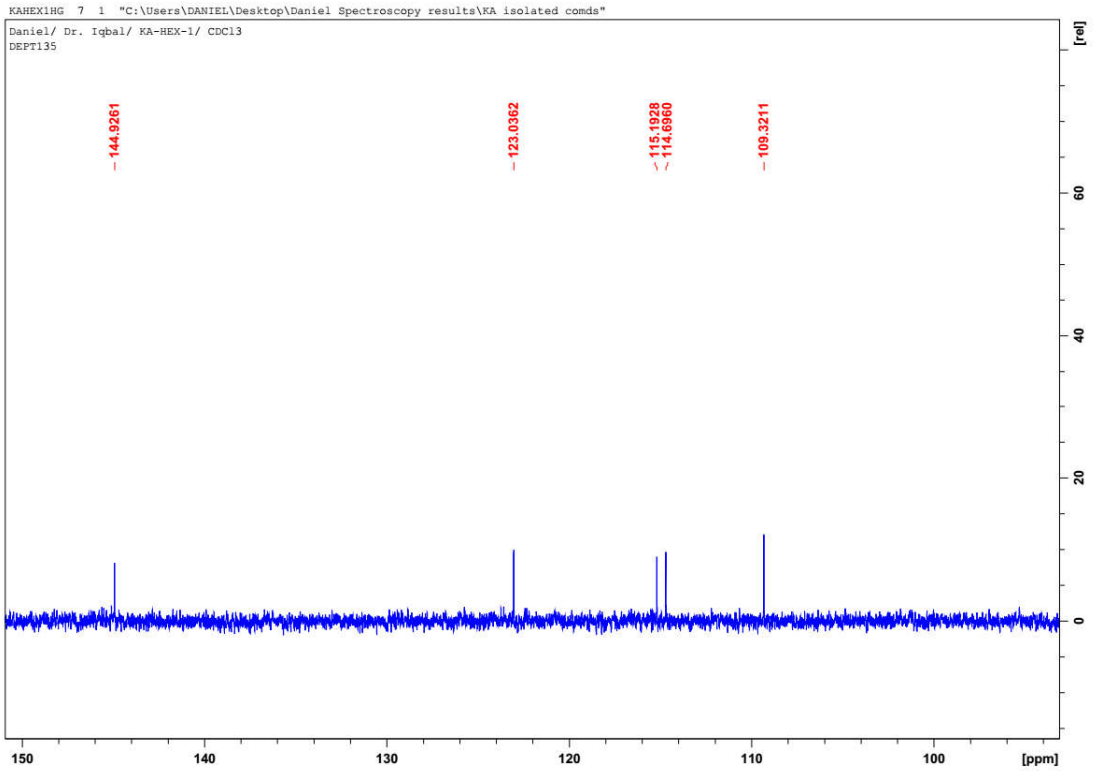
Appendix ll: DEPT 90 of compound l



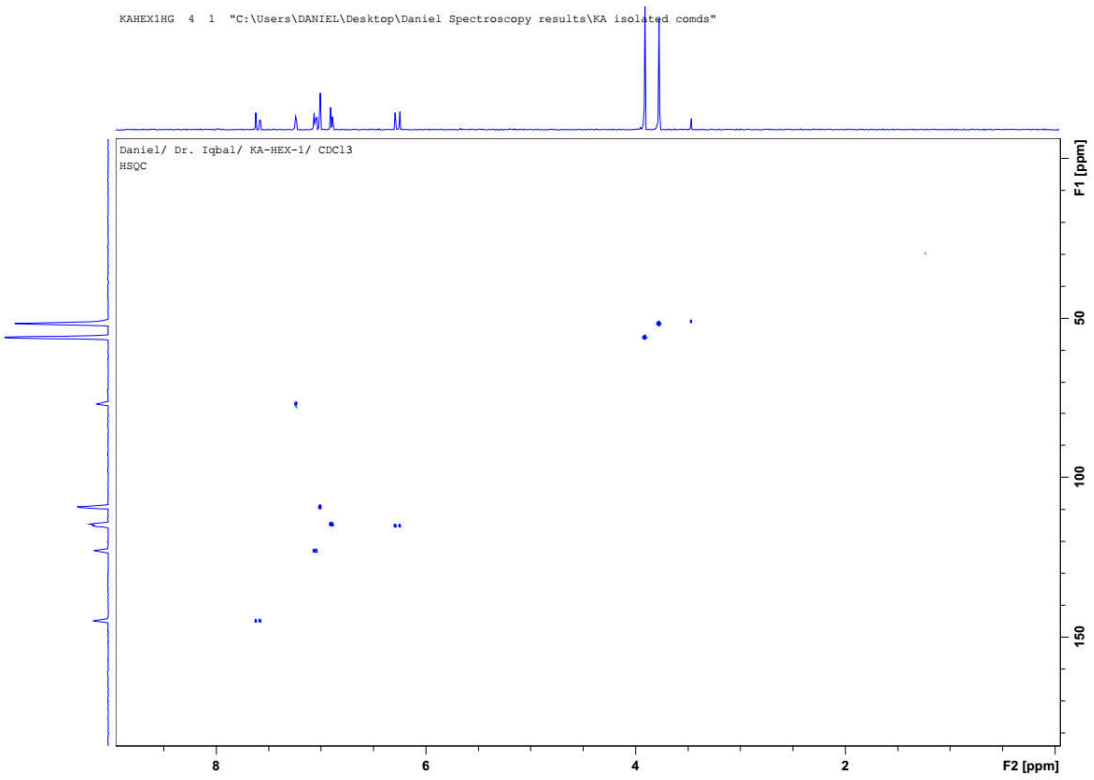


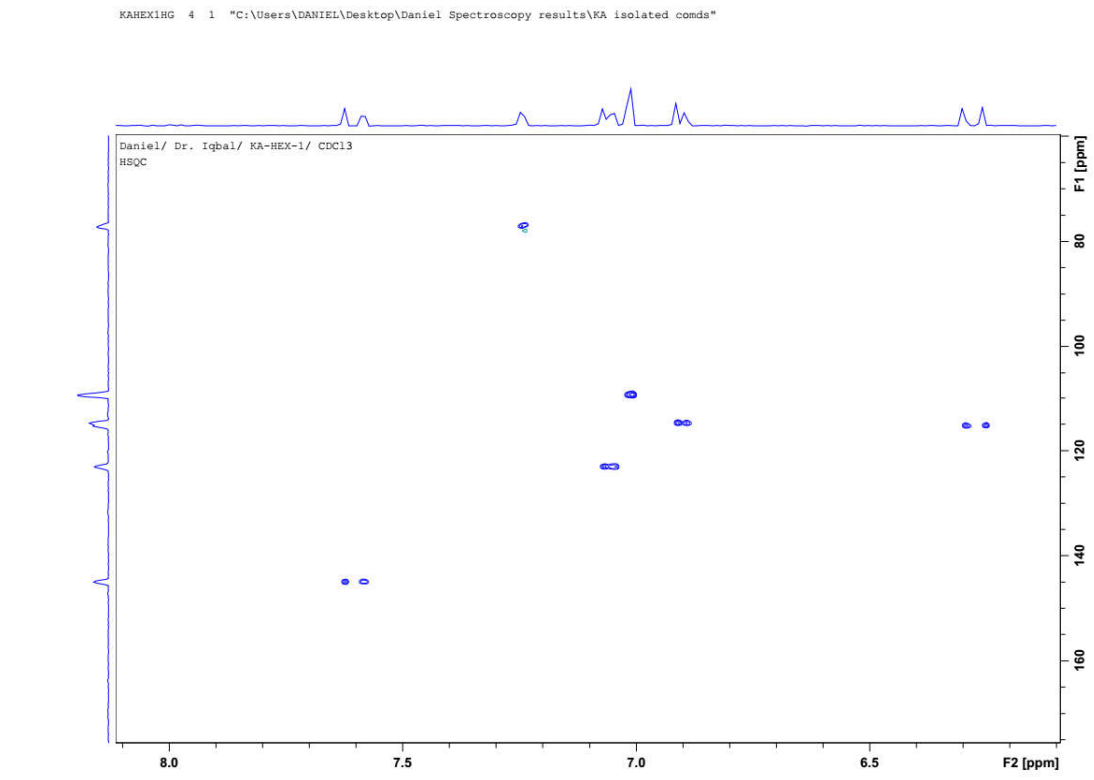
Appendix l2: DEPT l35 of compound l



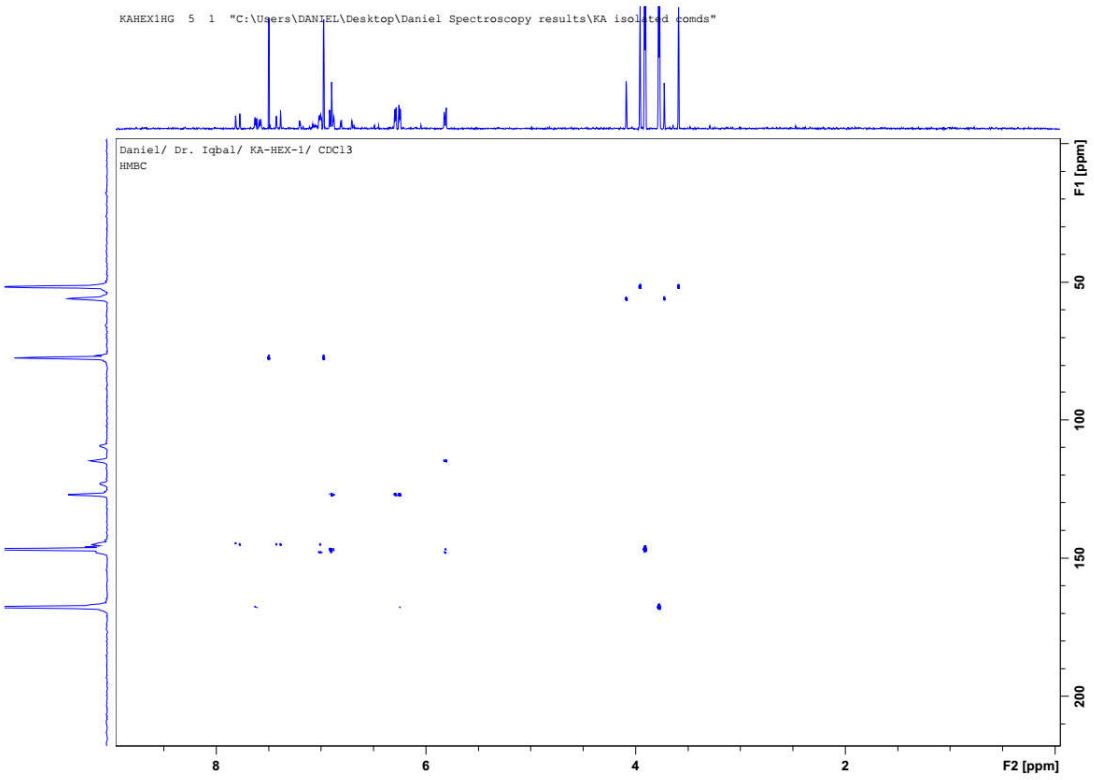


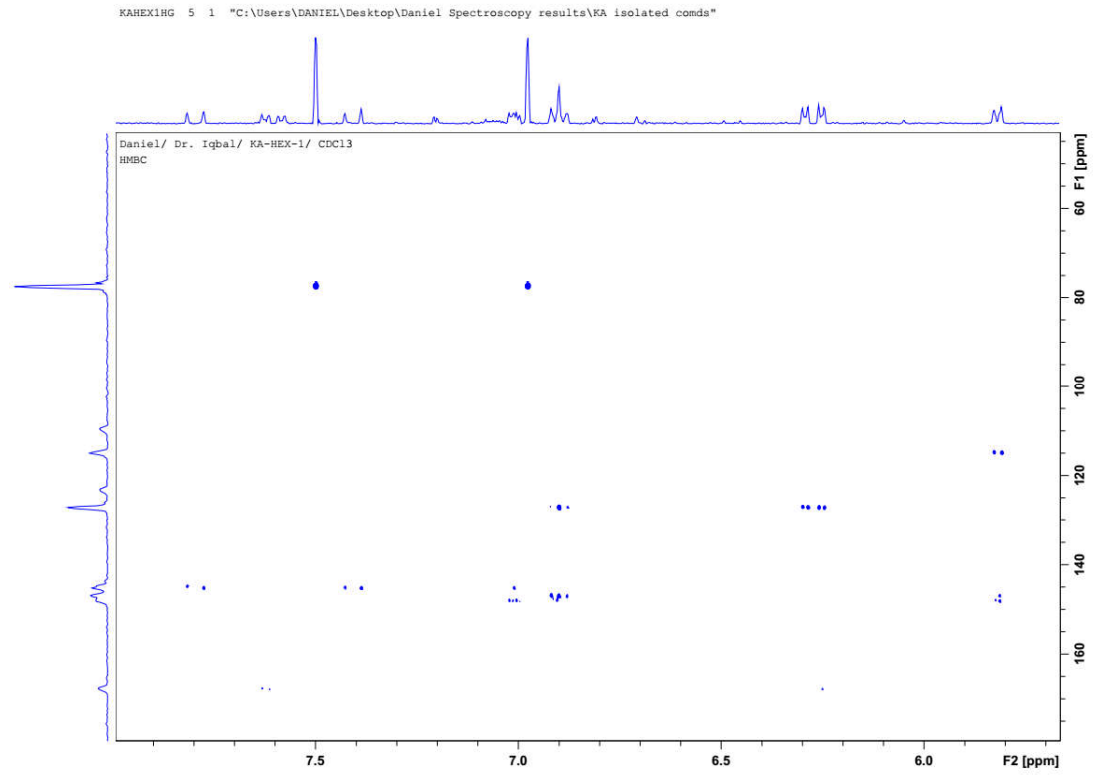
Appendix l3: HSQC of compound l



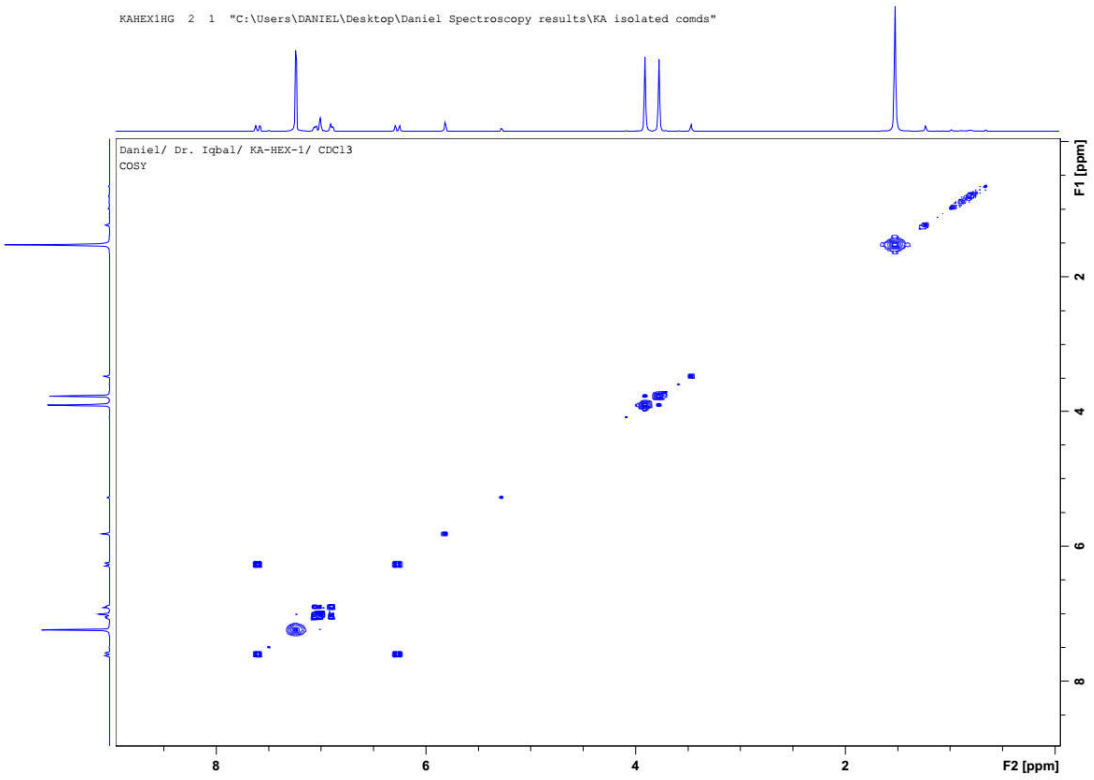


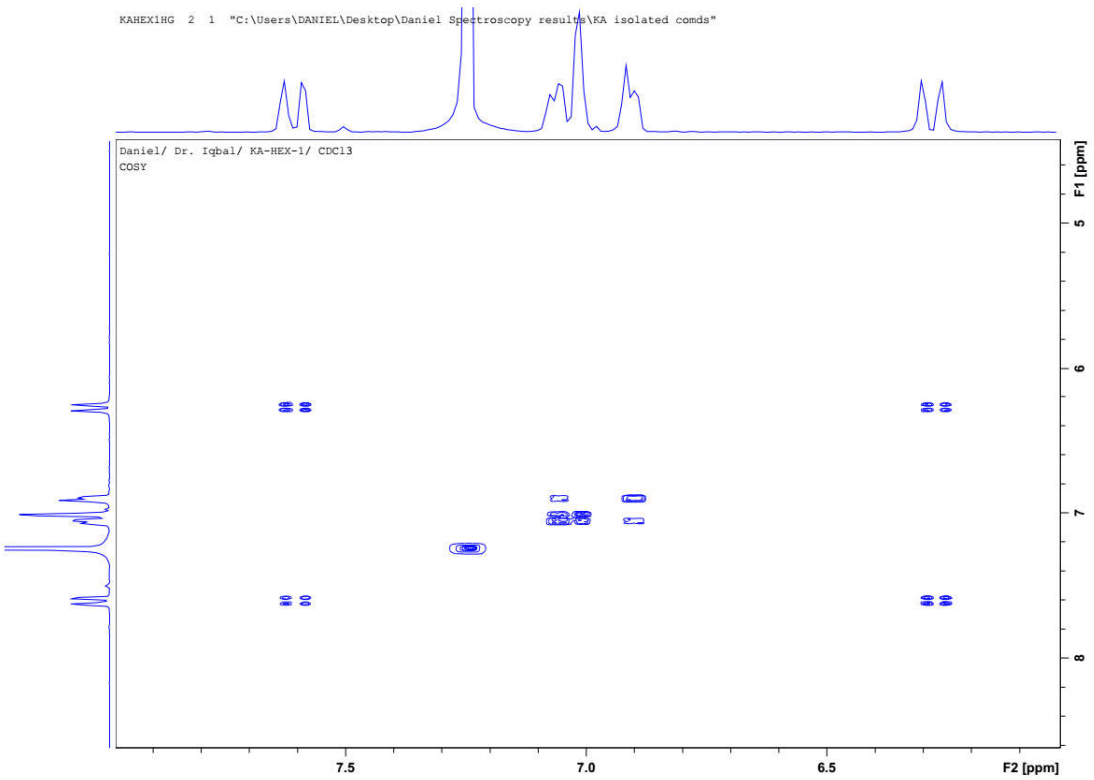
Appendix l4: HMBC of compound l



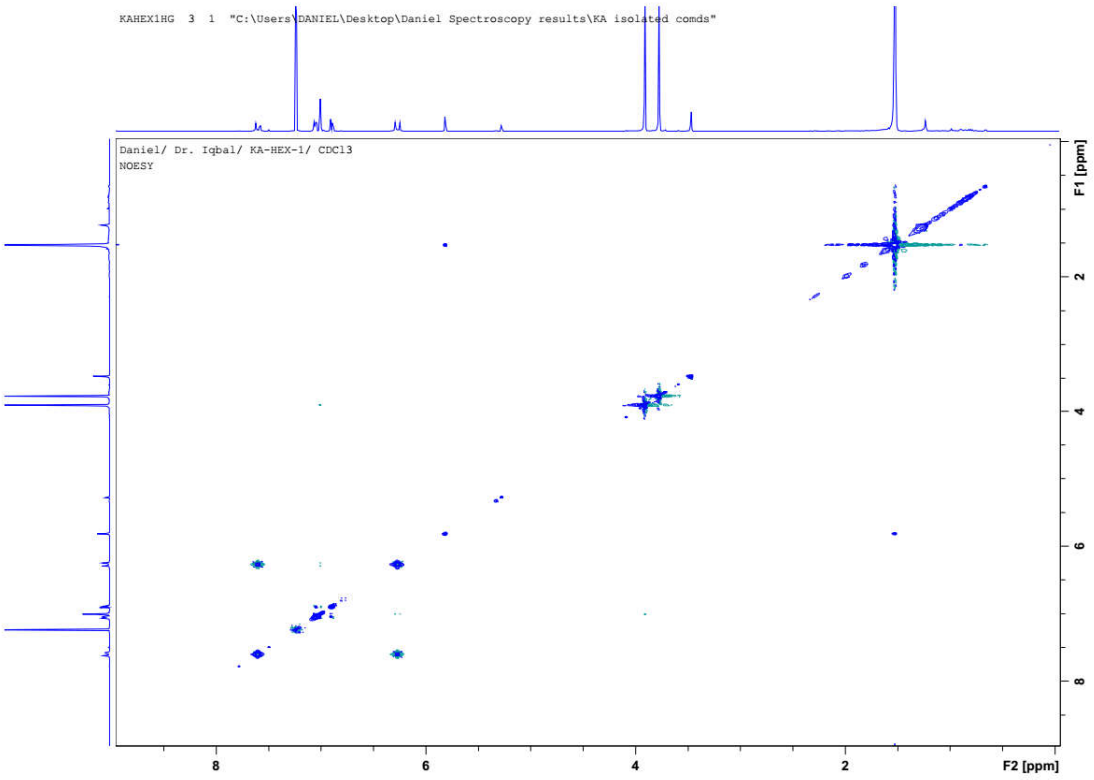


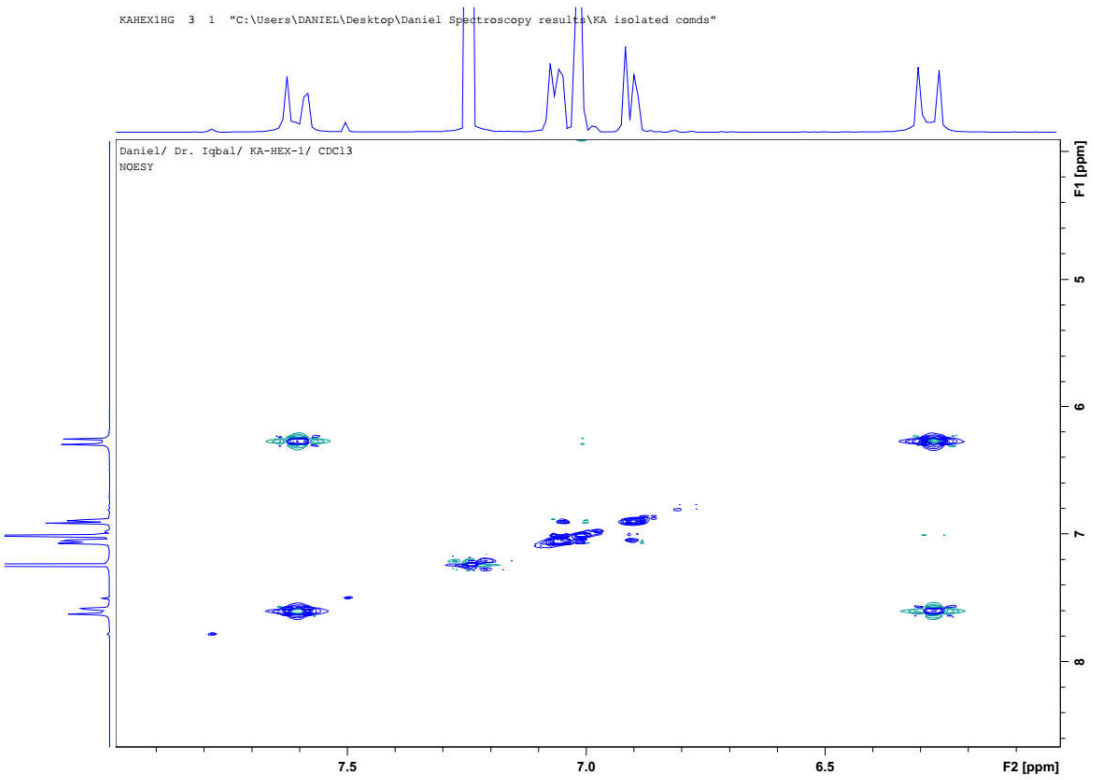
Appendix l5: COSY of compound l



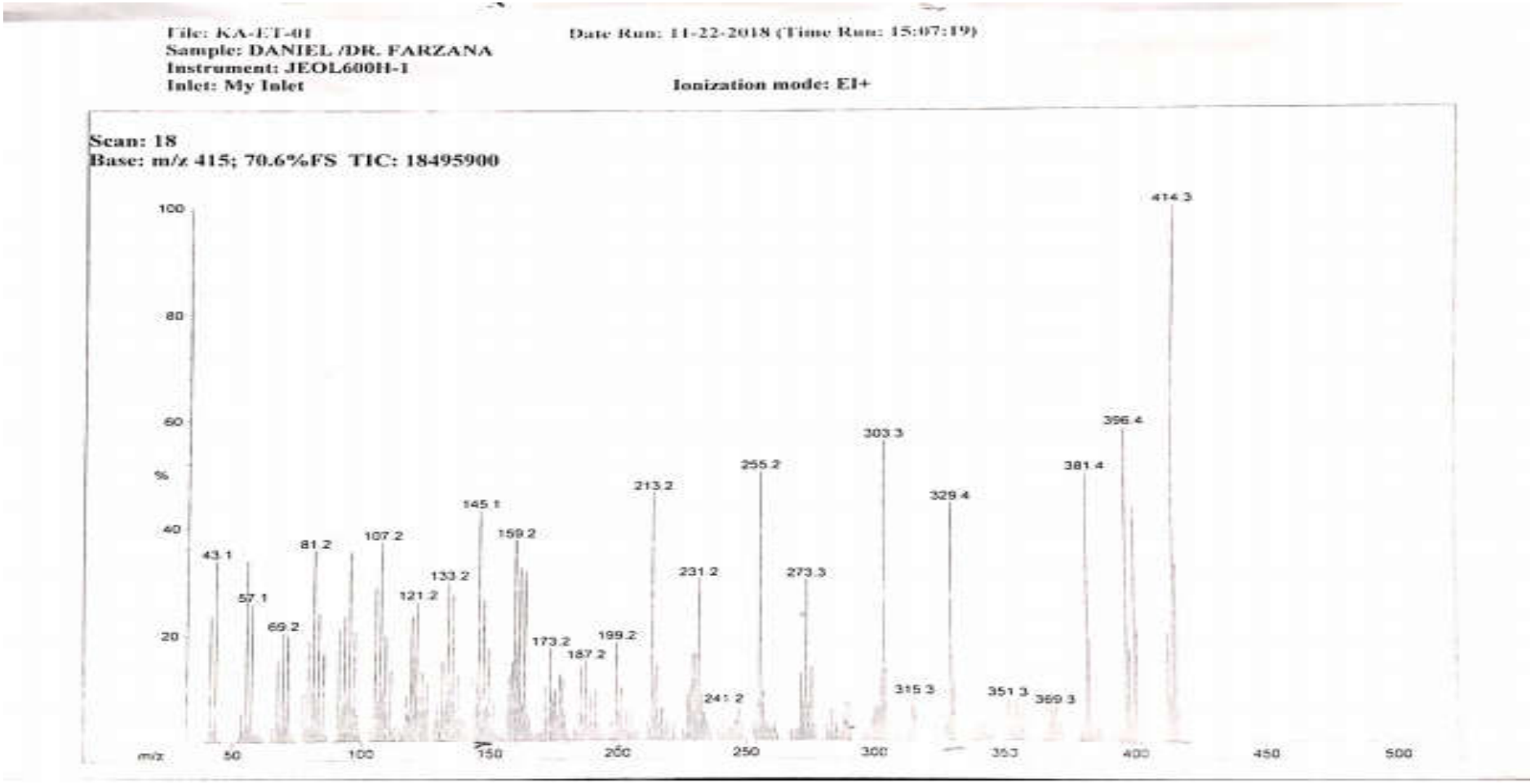


Appendix l6: NOESY of compound l

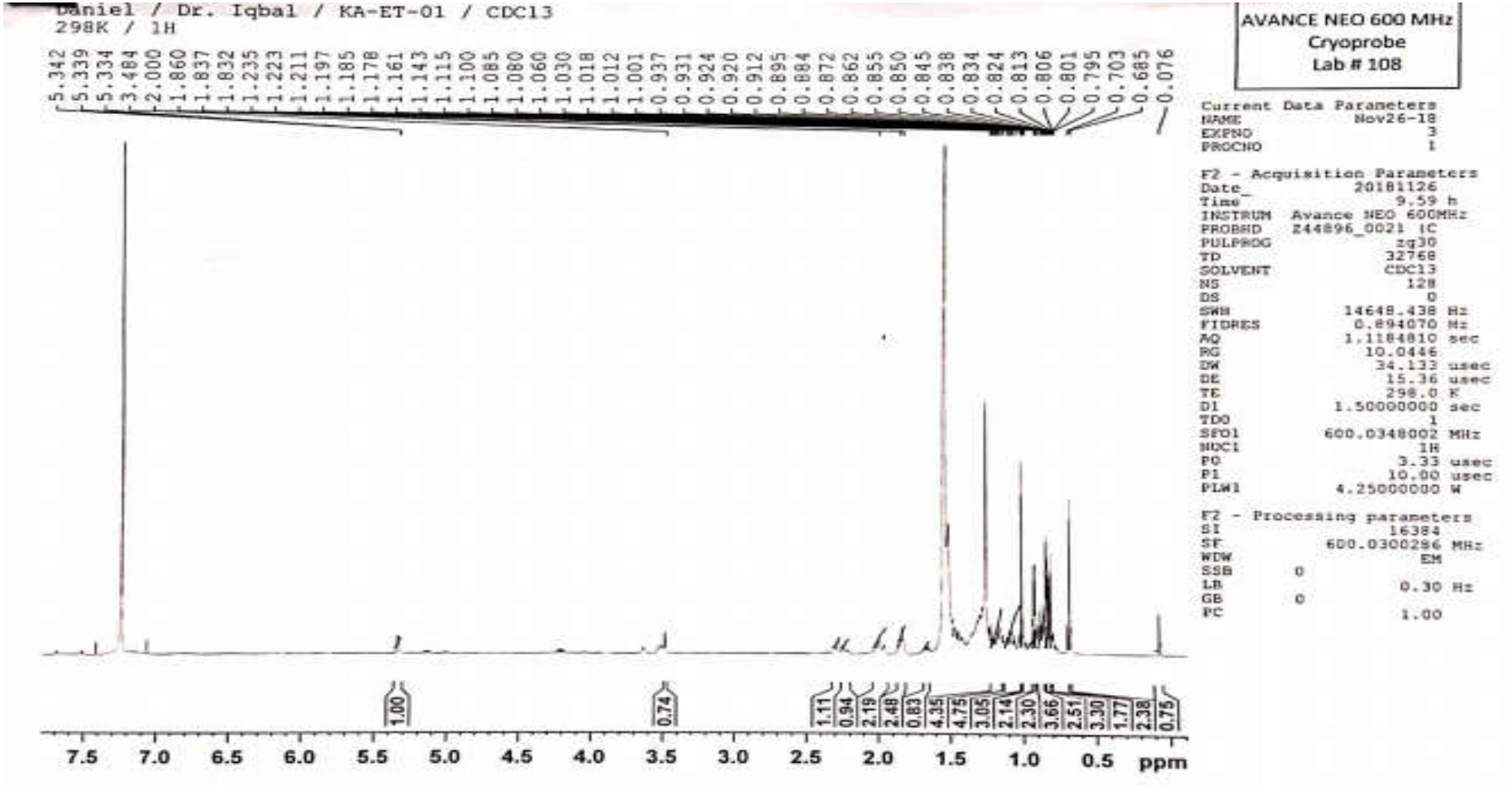




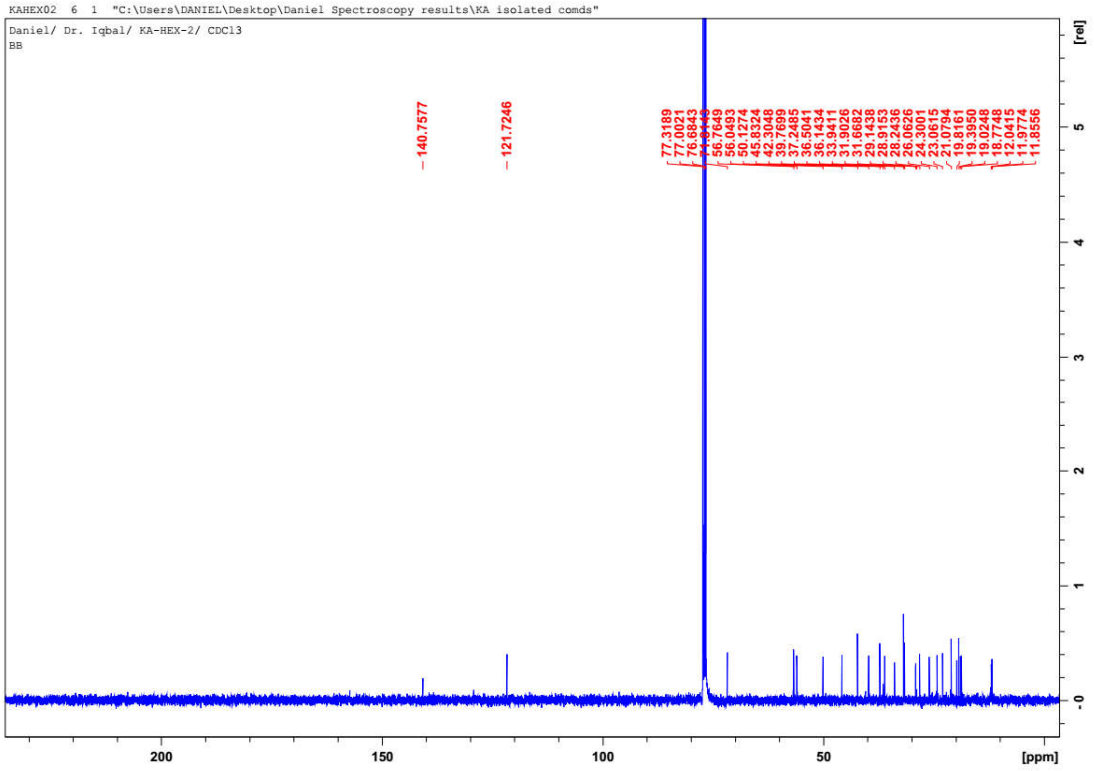
Appendix l7: Mass spectral of Compound 2

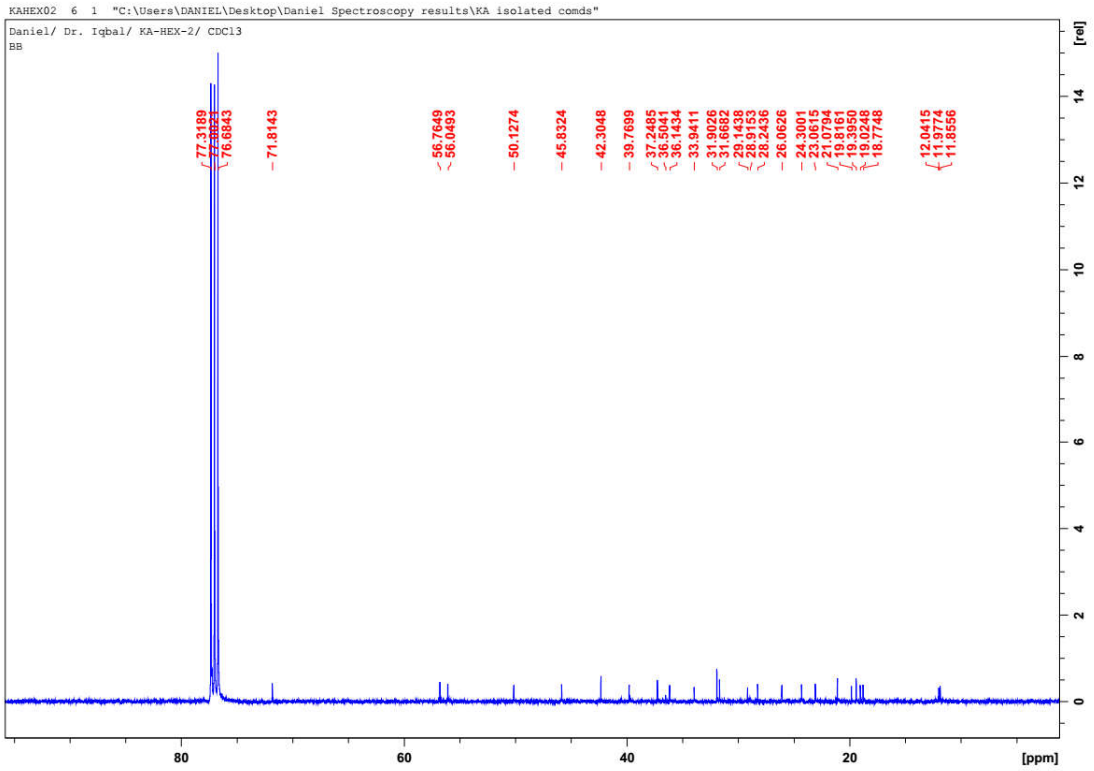


Appendix l8: ^{1}H NMR of compound 2

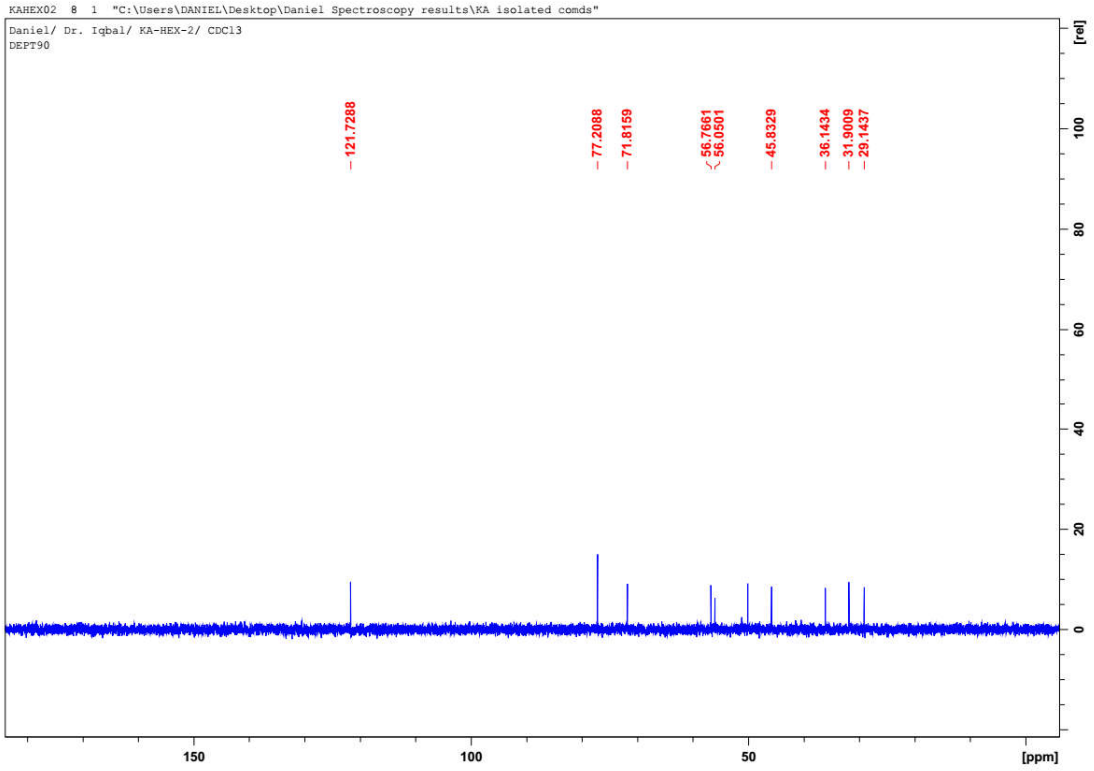


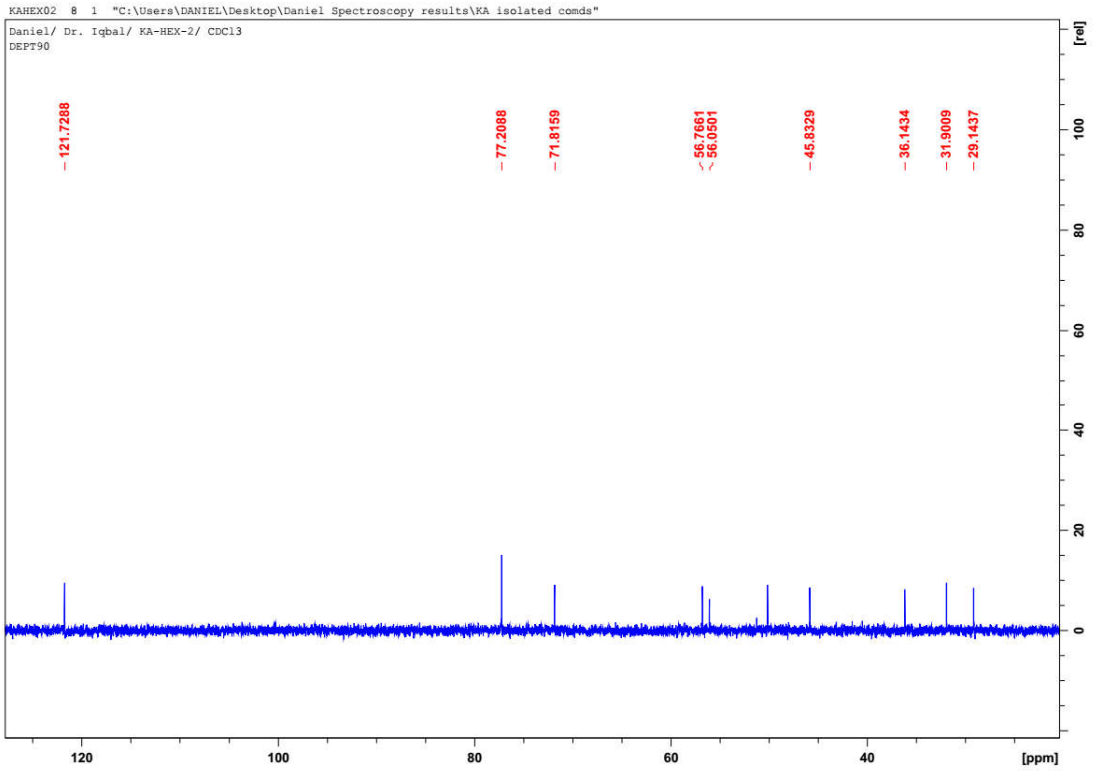
Appendix l9: ^{13}C NMR of compound 2



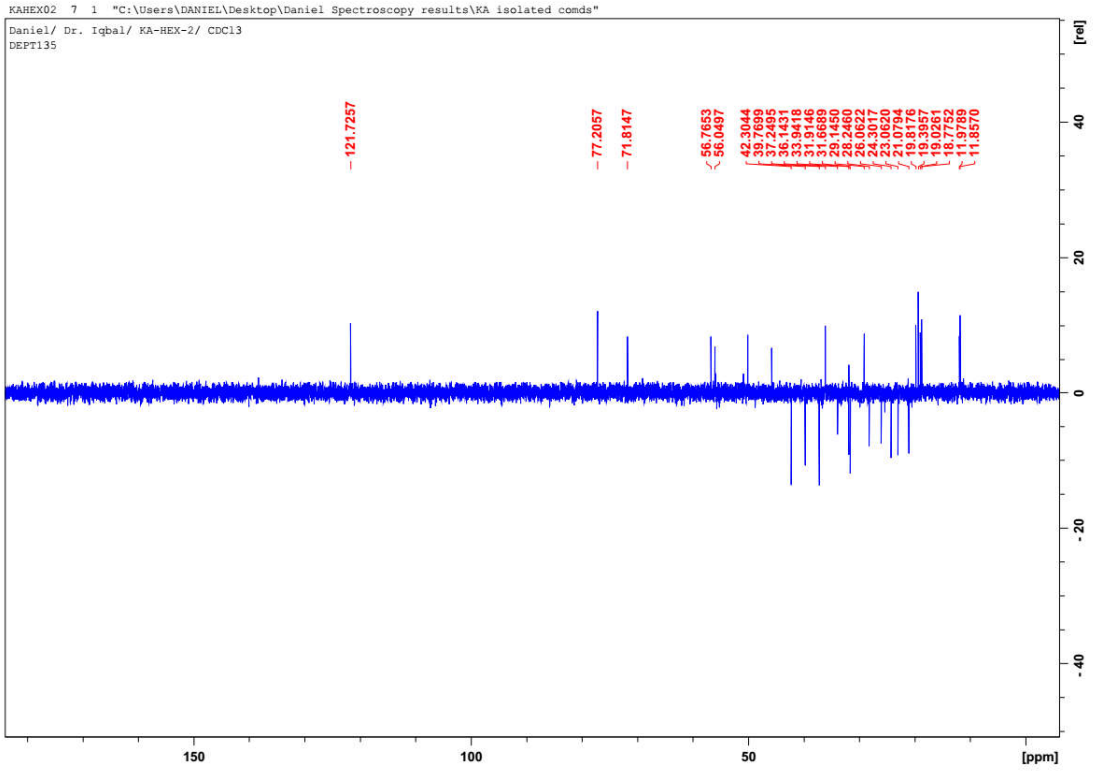


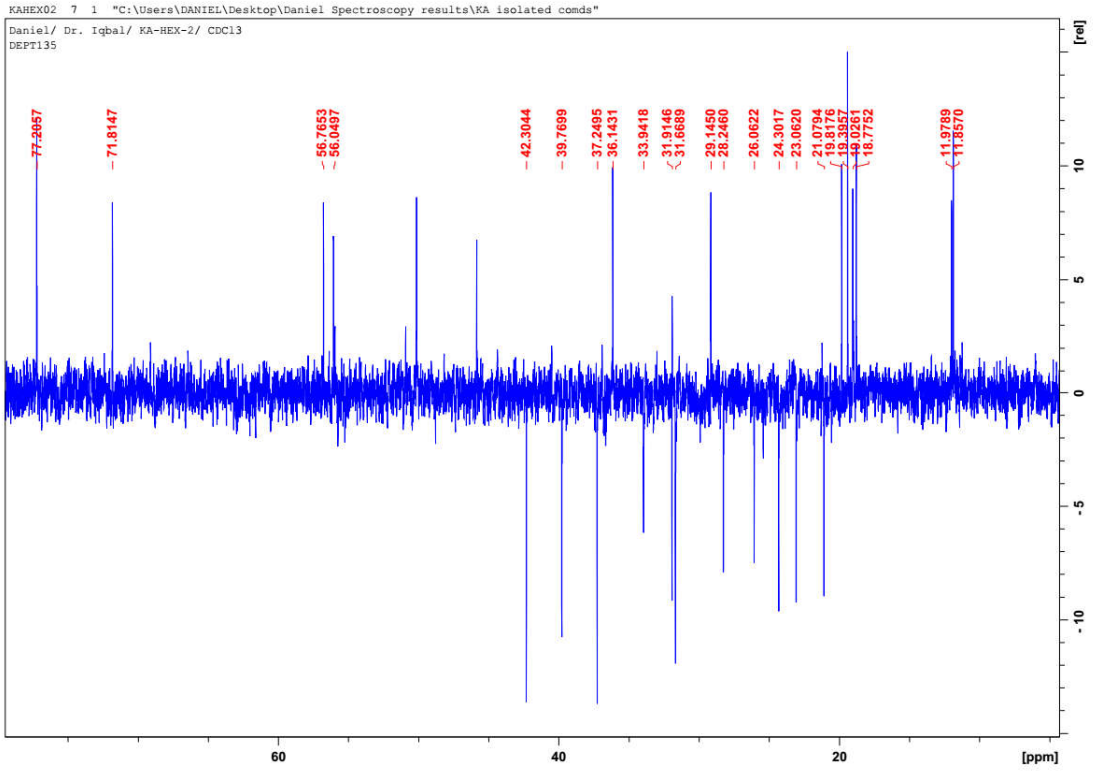
Appendix 20: DEPT 90 of compound 2



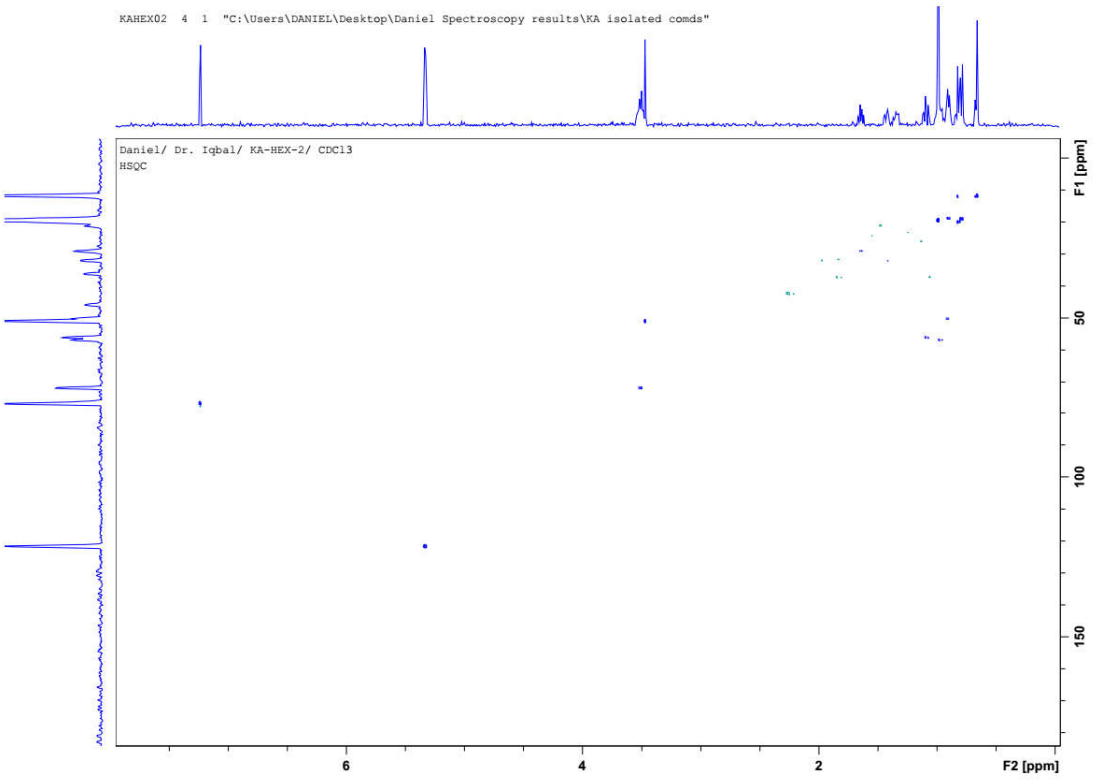


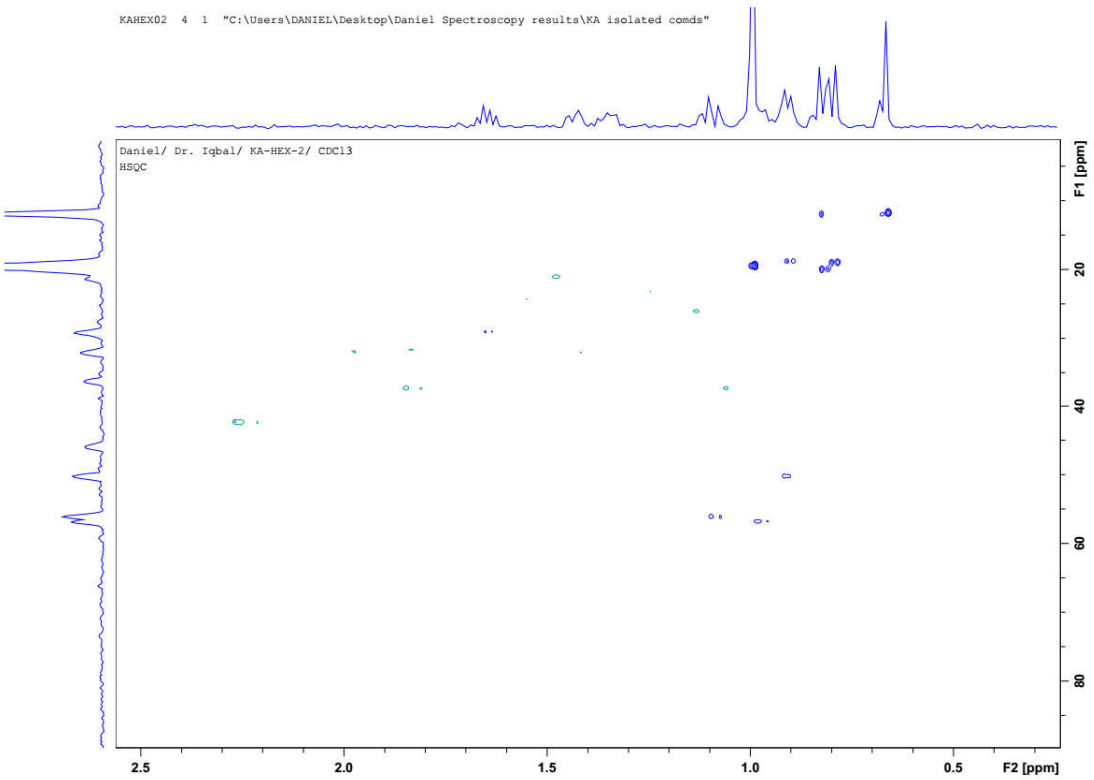
Appendix 2l: DEPT l35 of compound 2



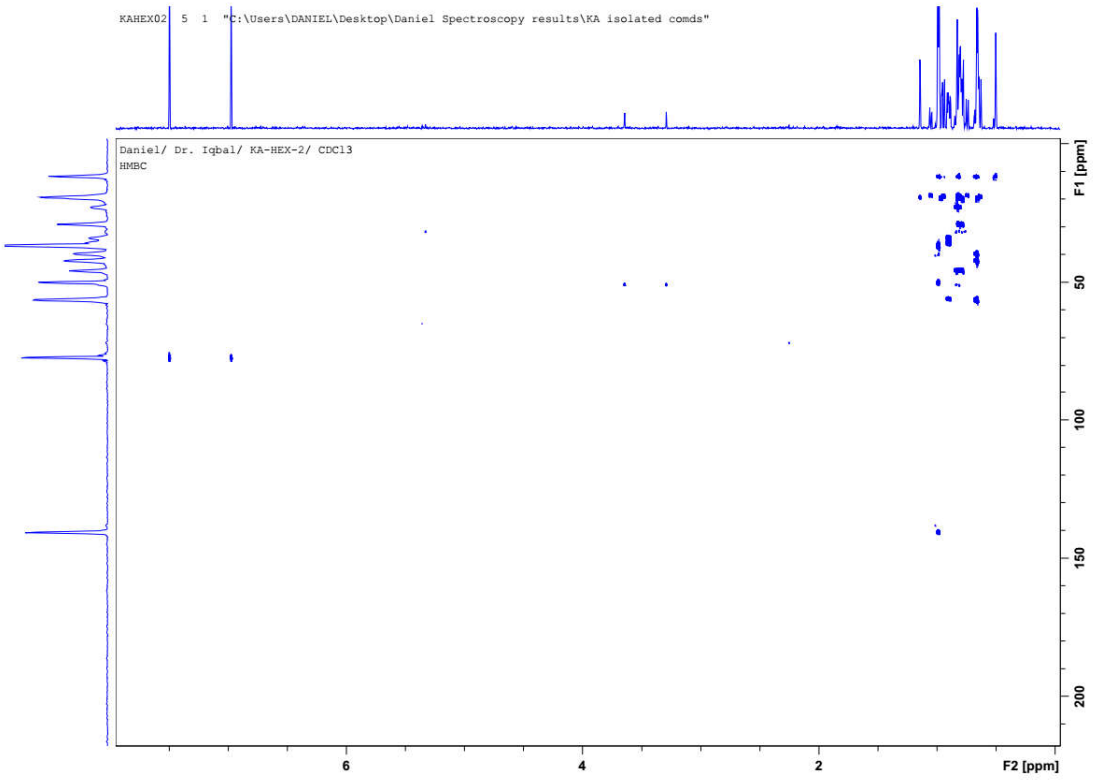


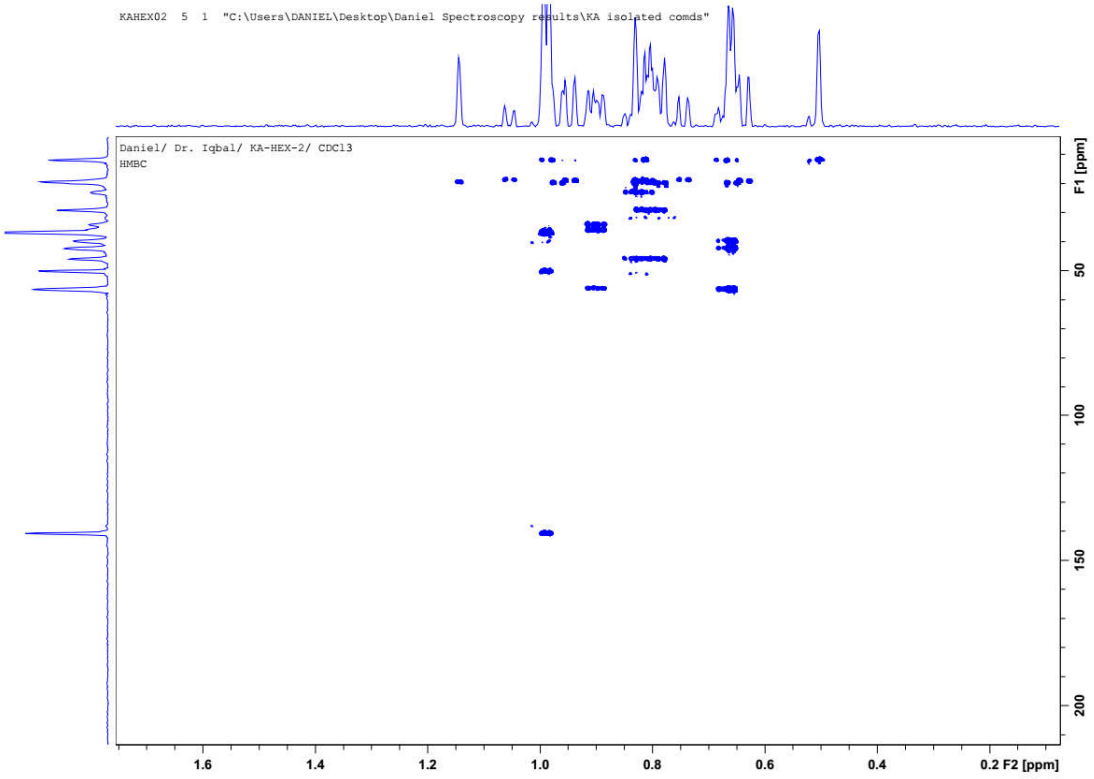
Appendix 22: HSQC of compound 2



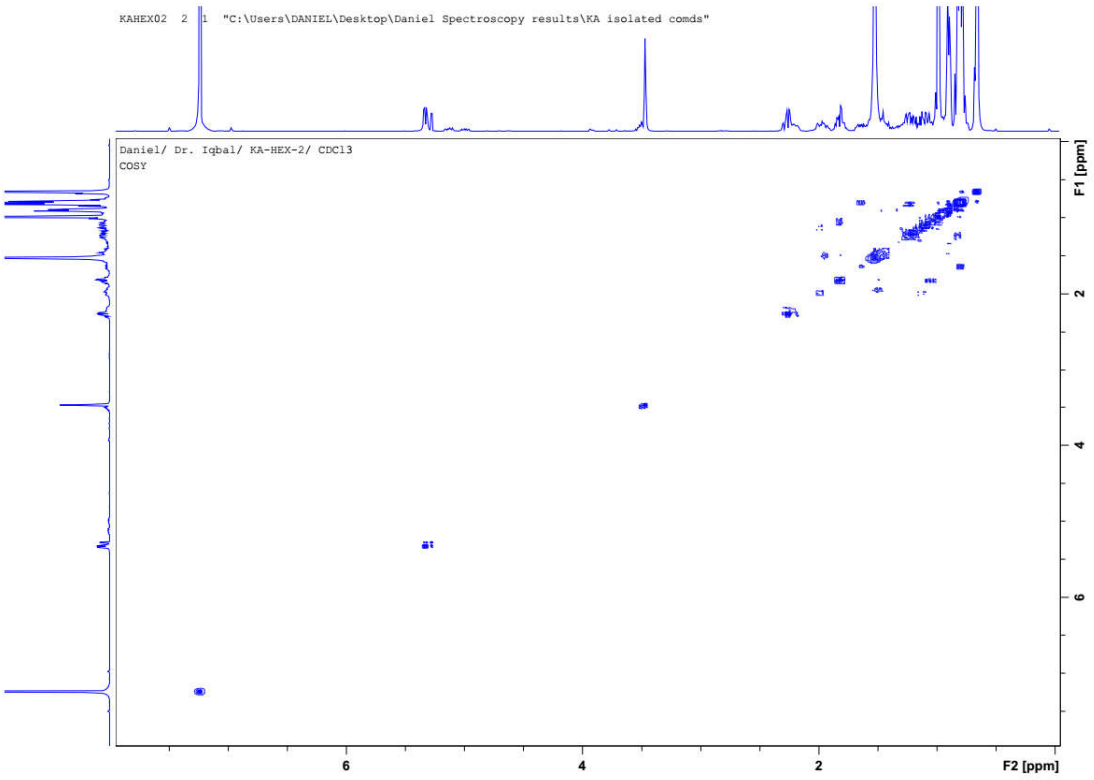


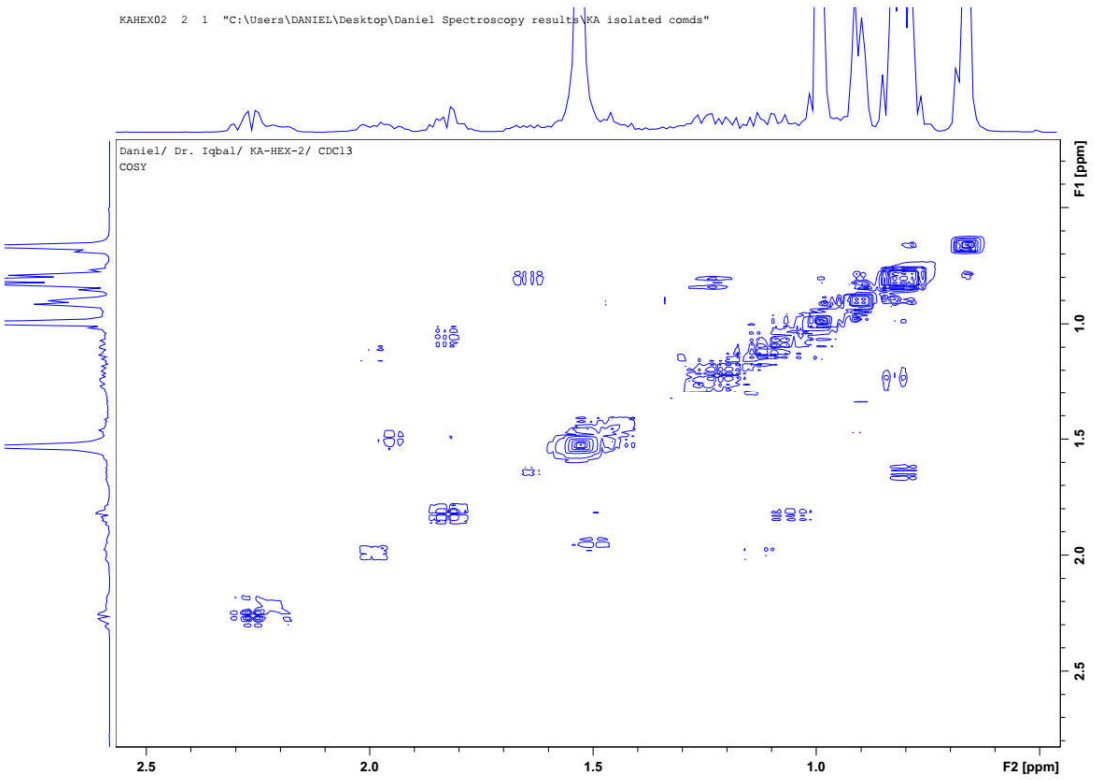
Appendix 23: HMBC of compound 2



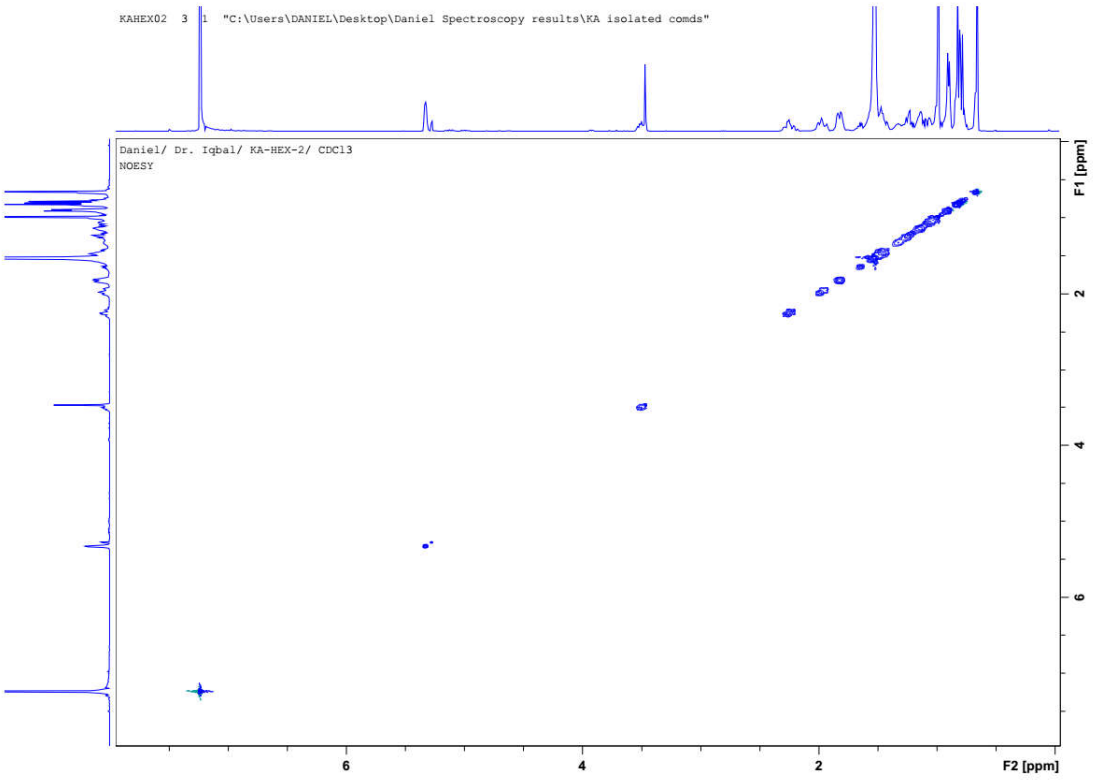


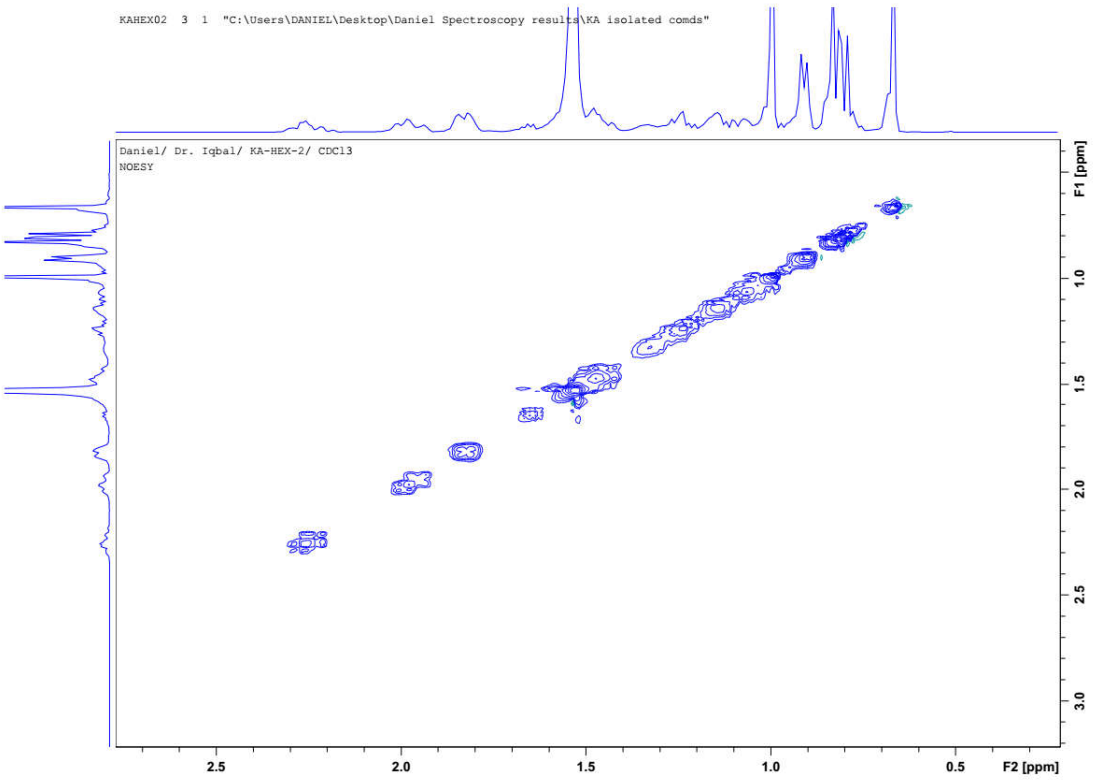
Appendix 24: COSY of compound 2





Appendix 25: NOESY of compound 2



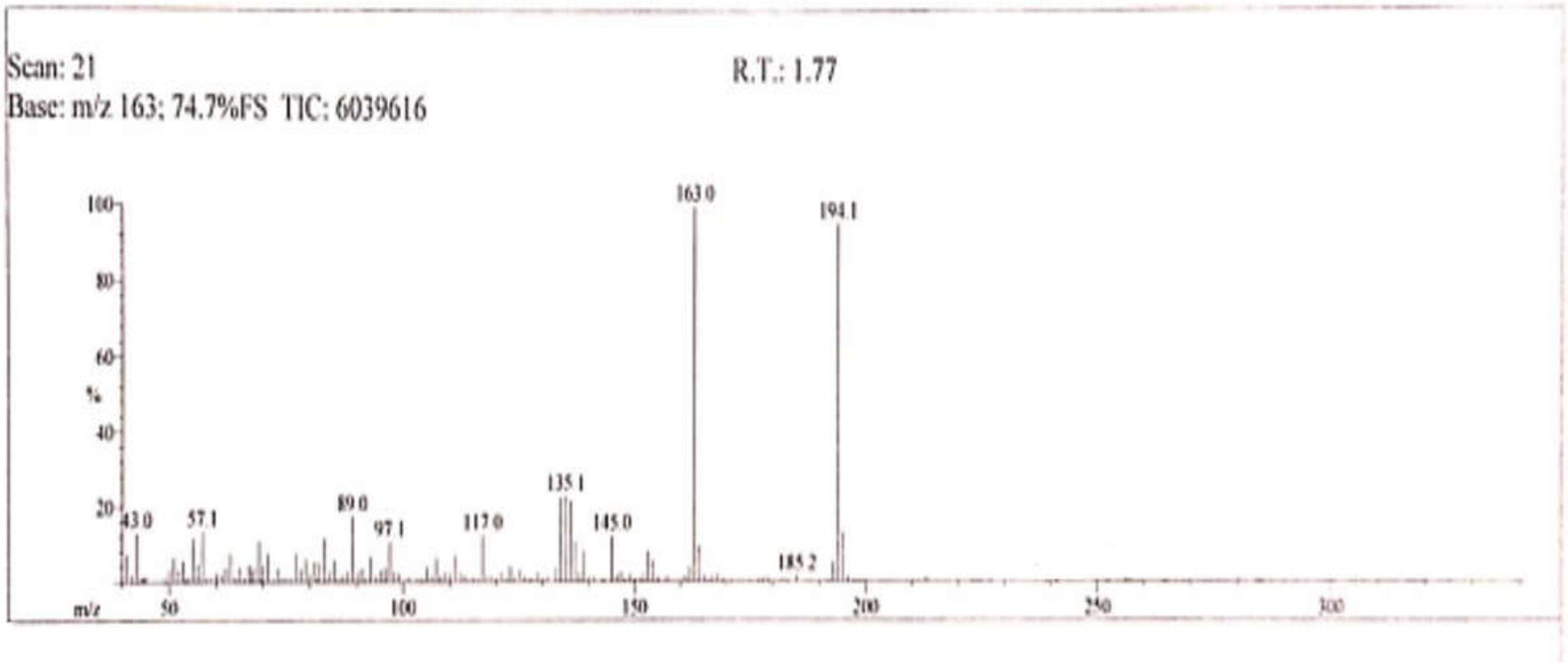


Appendix 26: Mass spectral of compound 3

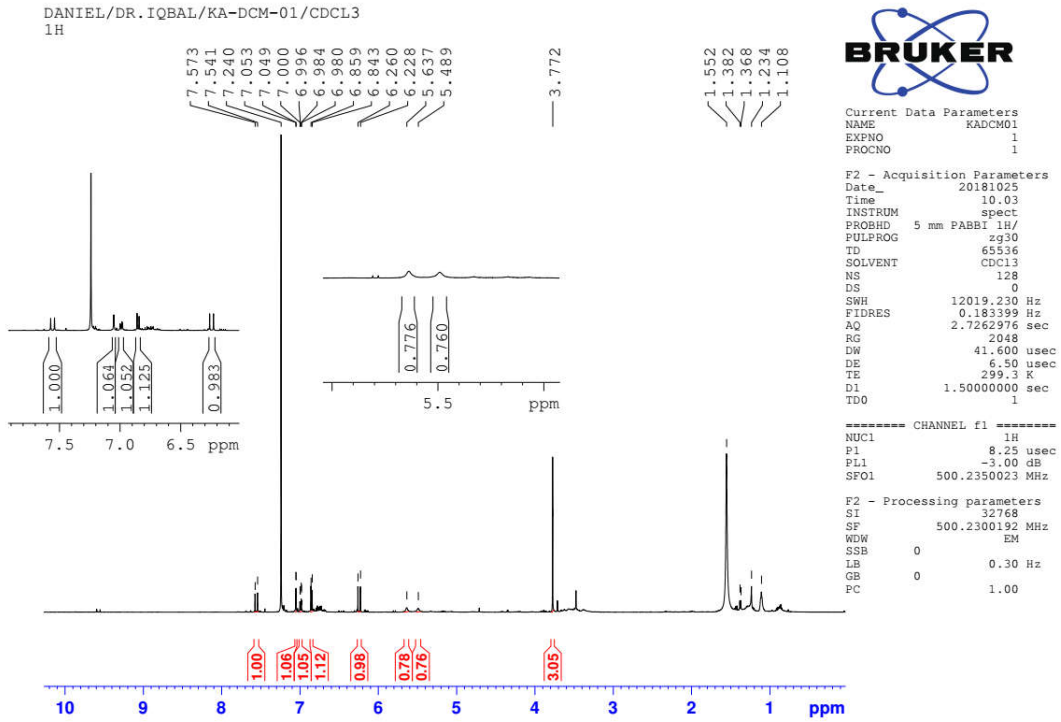
File: KE-21
Sample: DANIEL /DR. FARZANA
Instrument: JEOL JMS600H-1

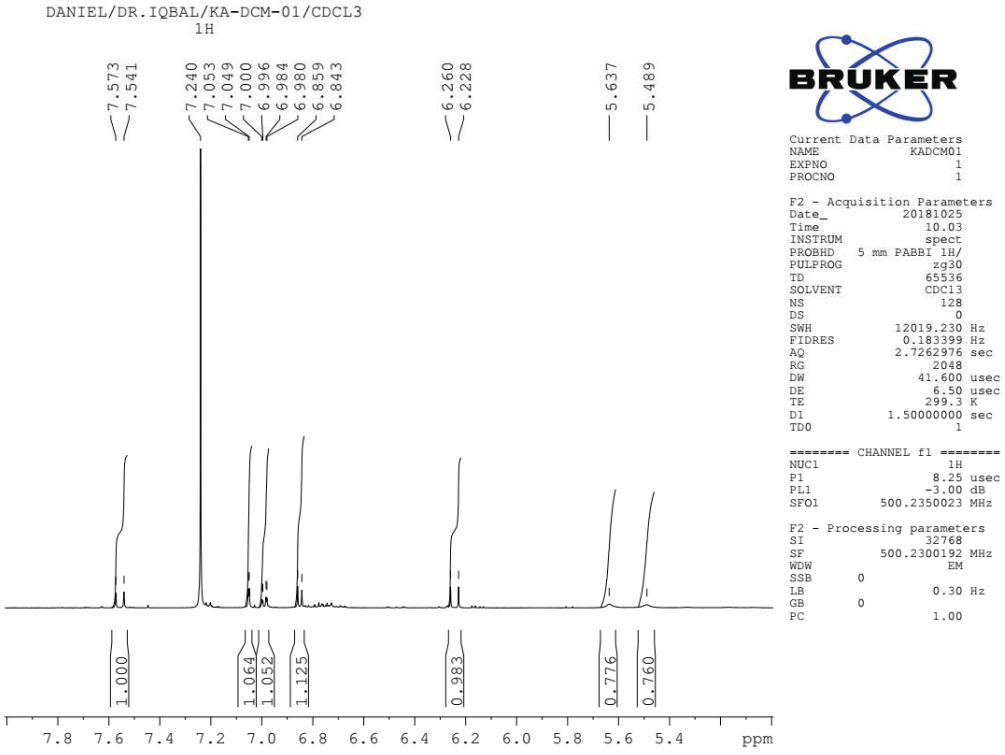
Date Run: 01-17-2019 (Time Run: 10:57:25)

Ionization mode: EI+

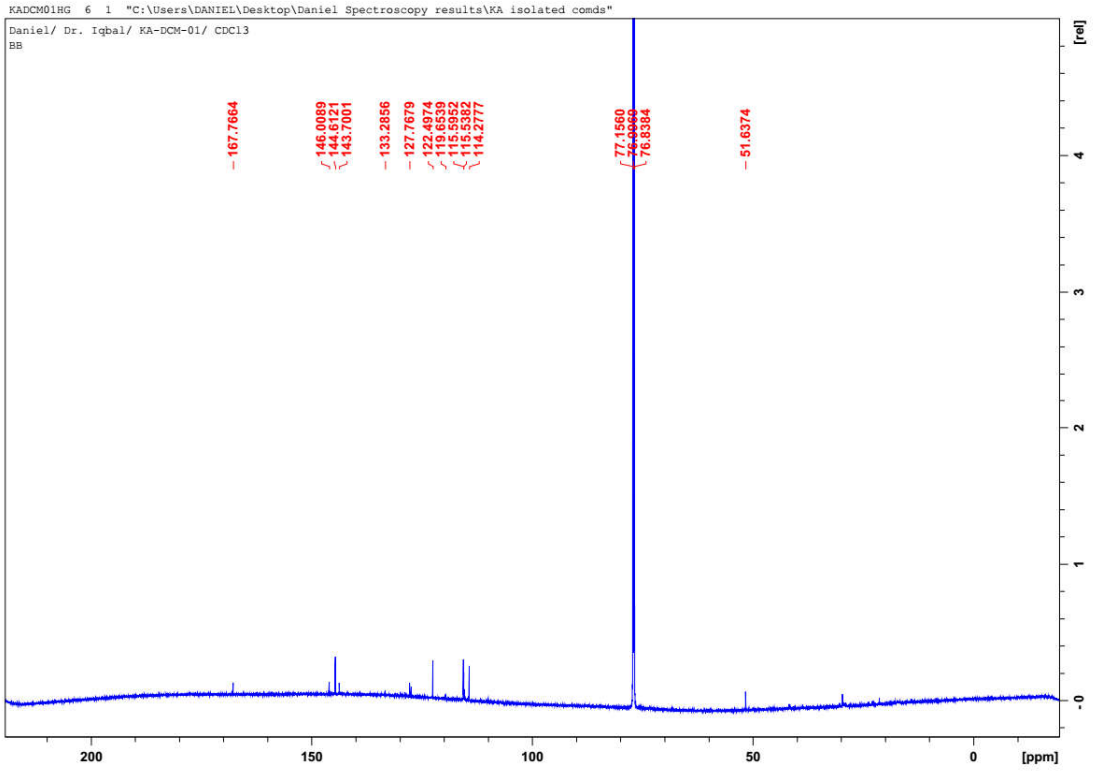


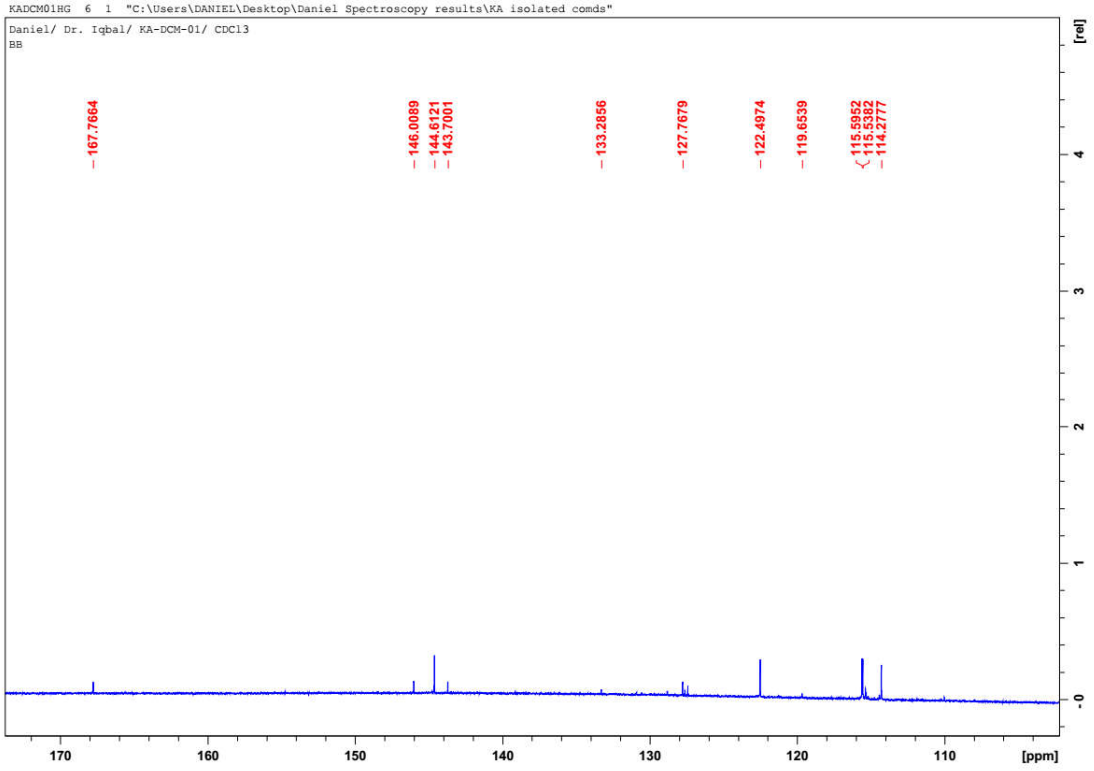
Appendix 27: ^{1}H NMR of compound 3



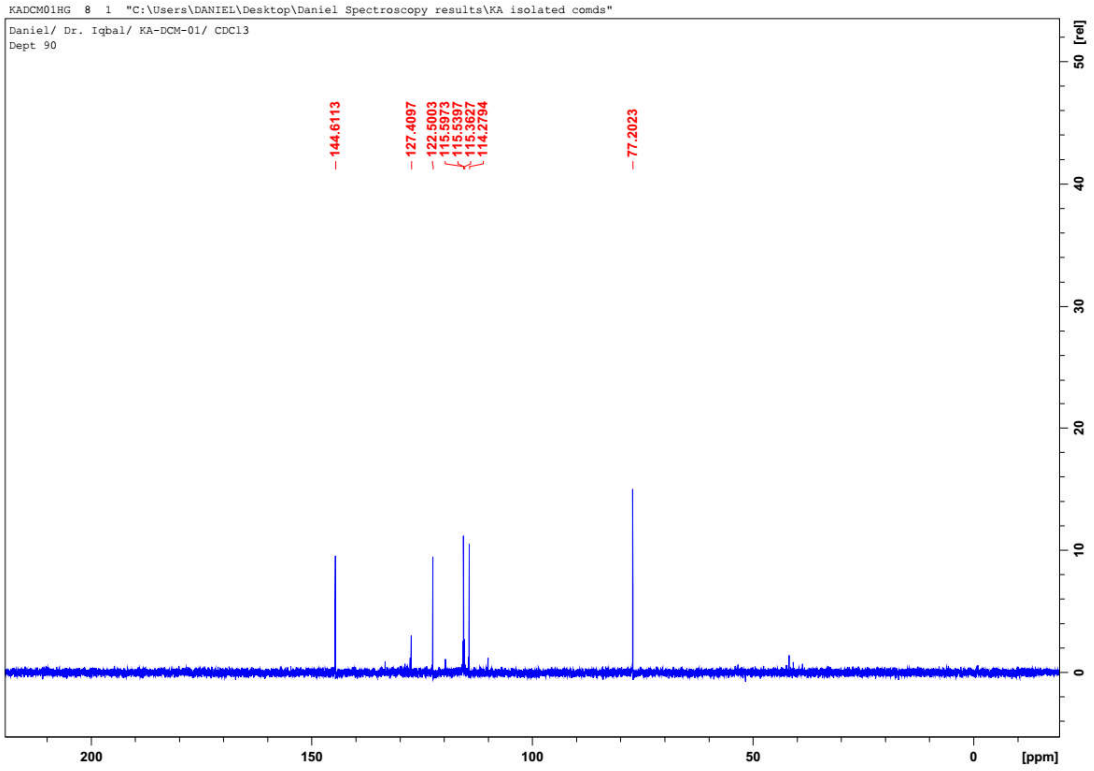


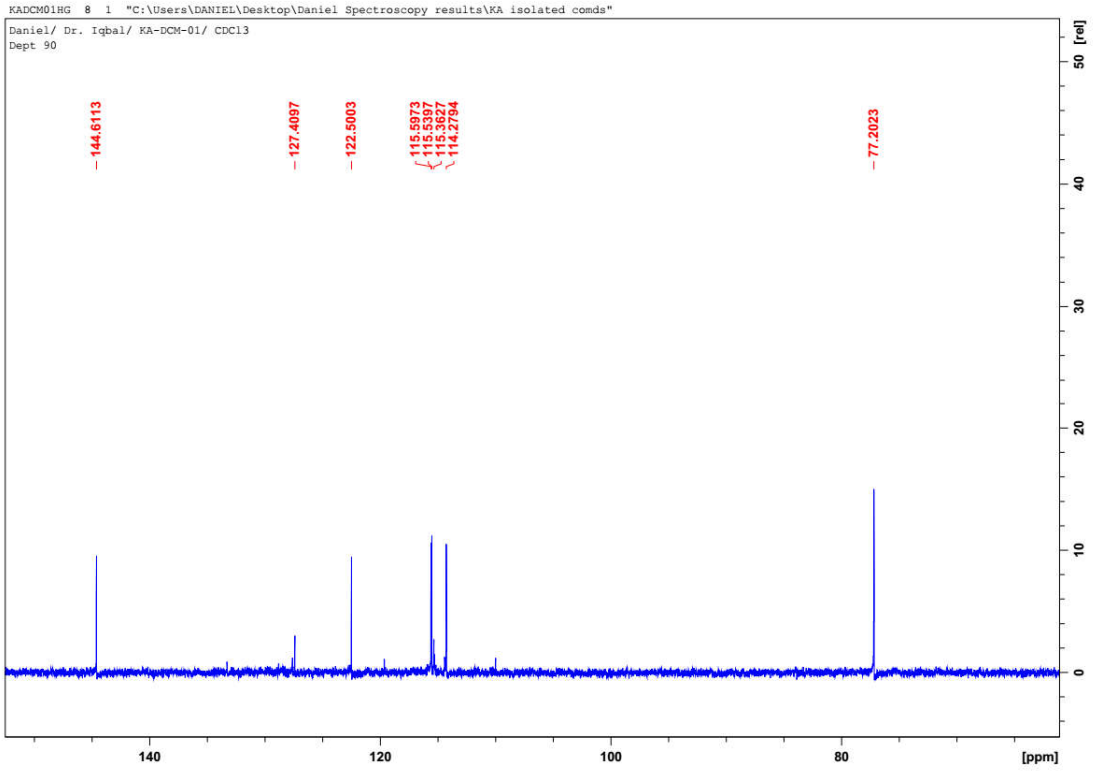
Appendix 28: ^{13}C NMR of compound 3



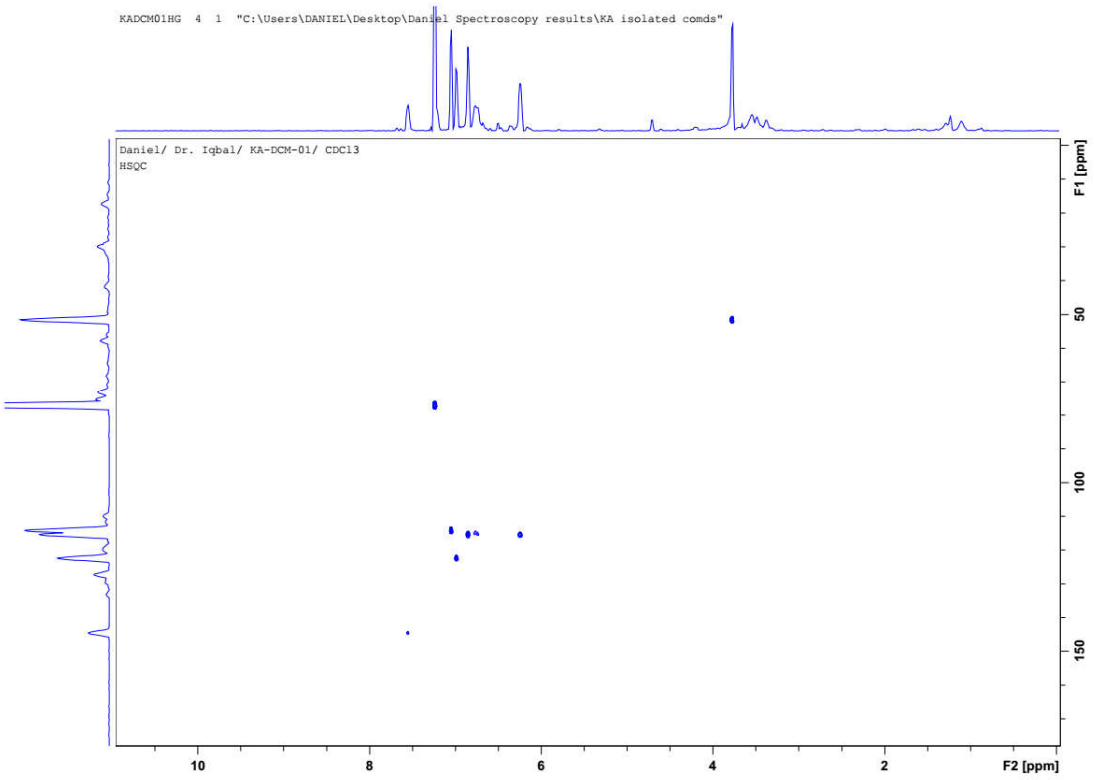


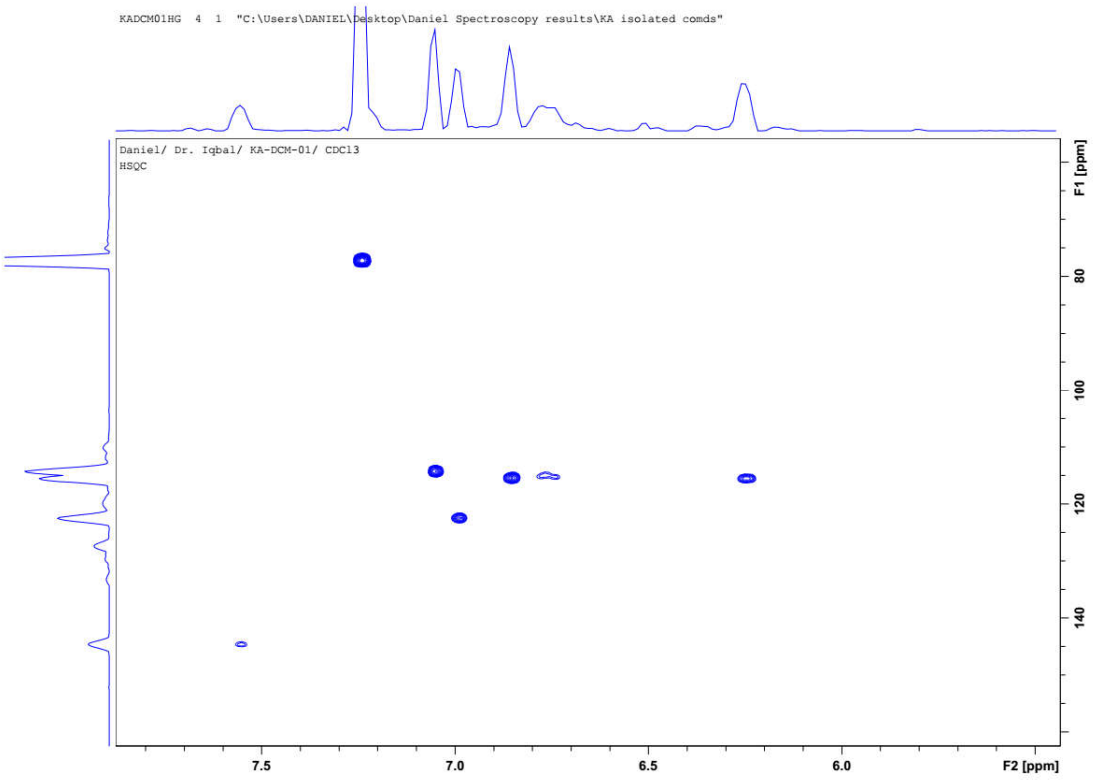
Appendix 29: DEPT 90 of compound 3



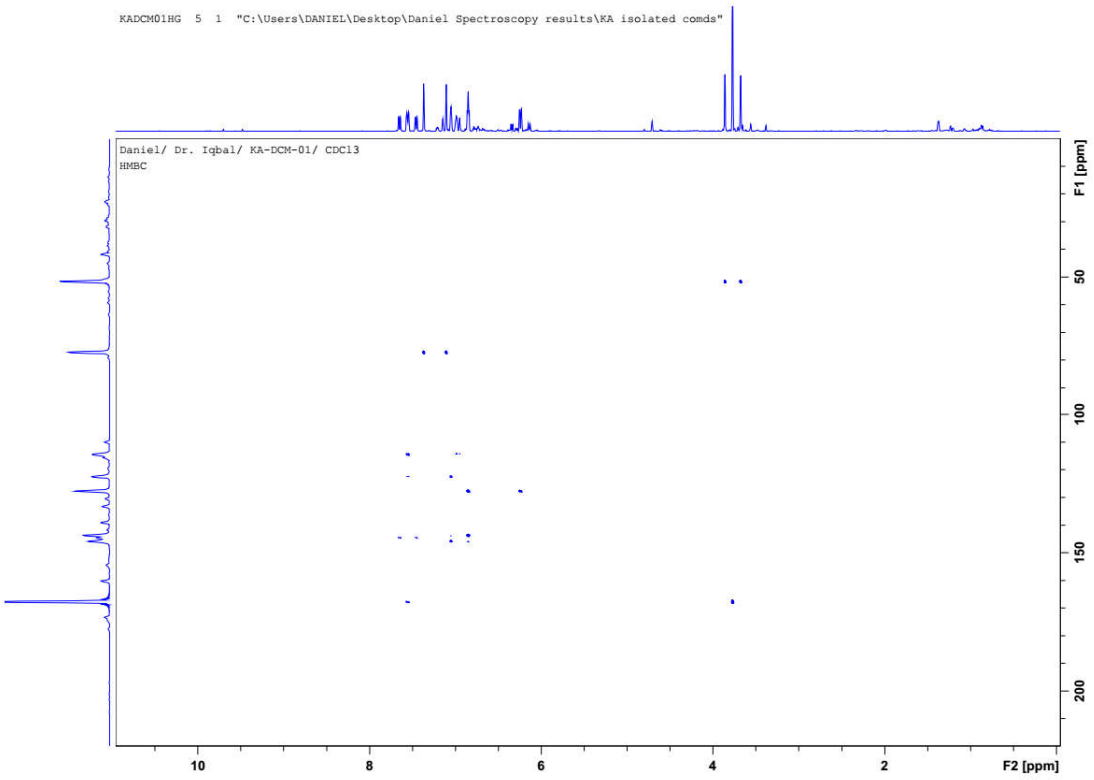


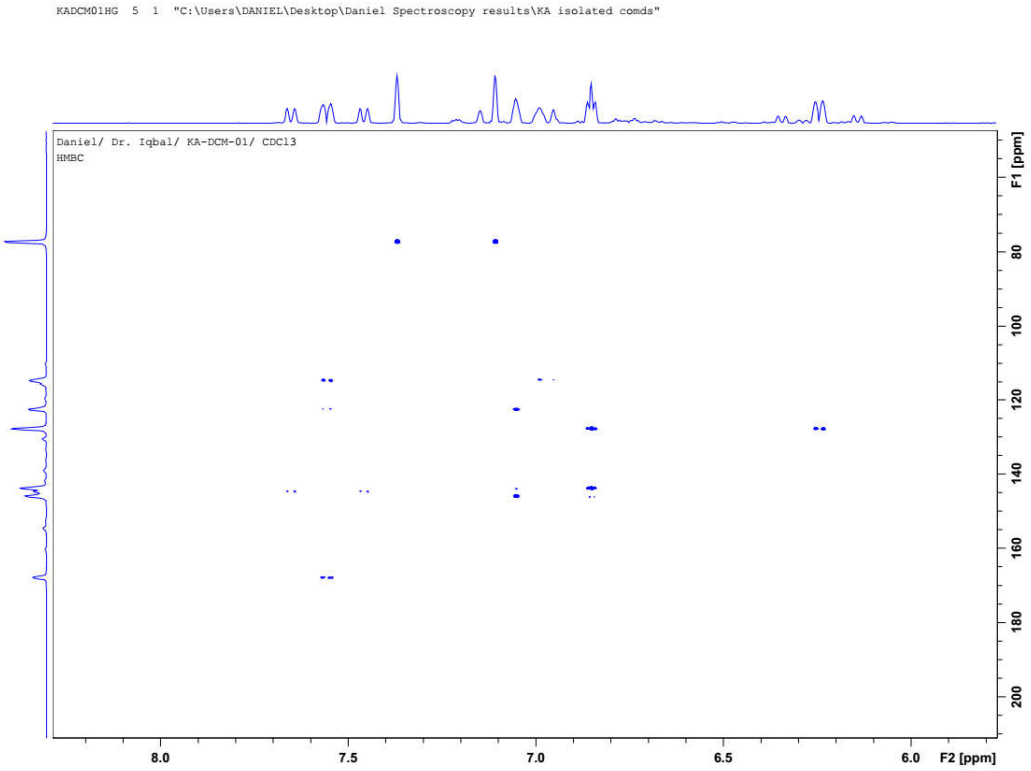
Appendix 30: HSQC of compound 3



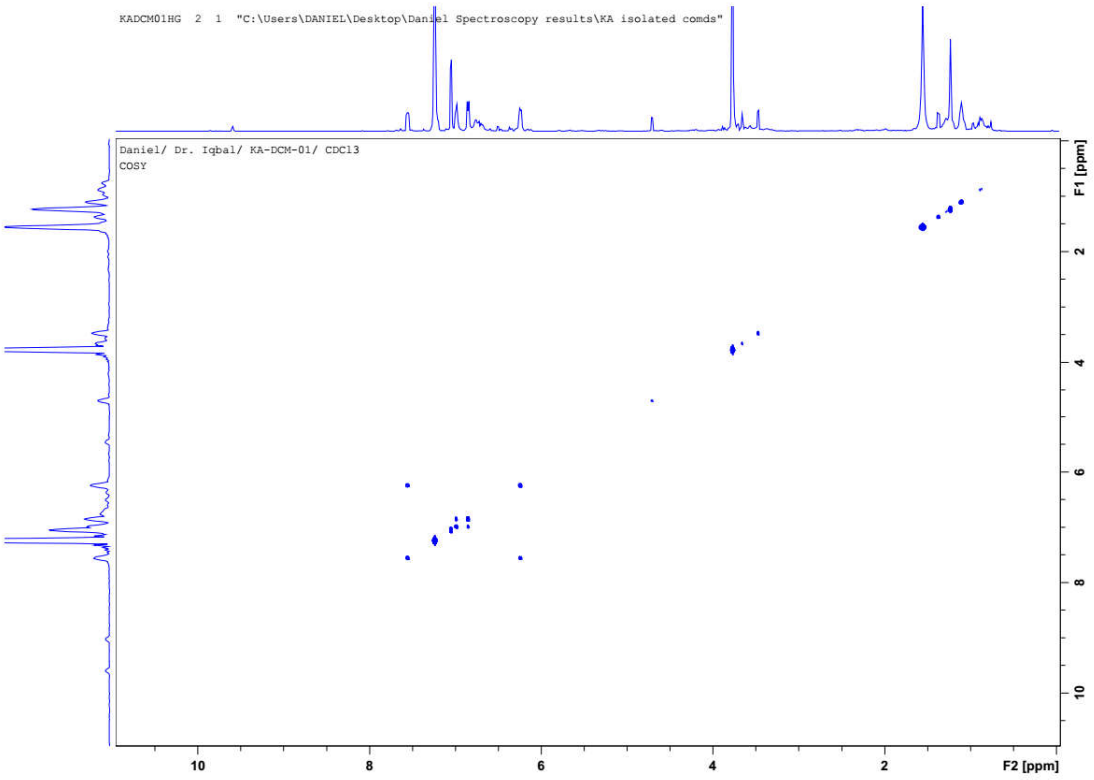


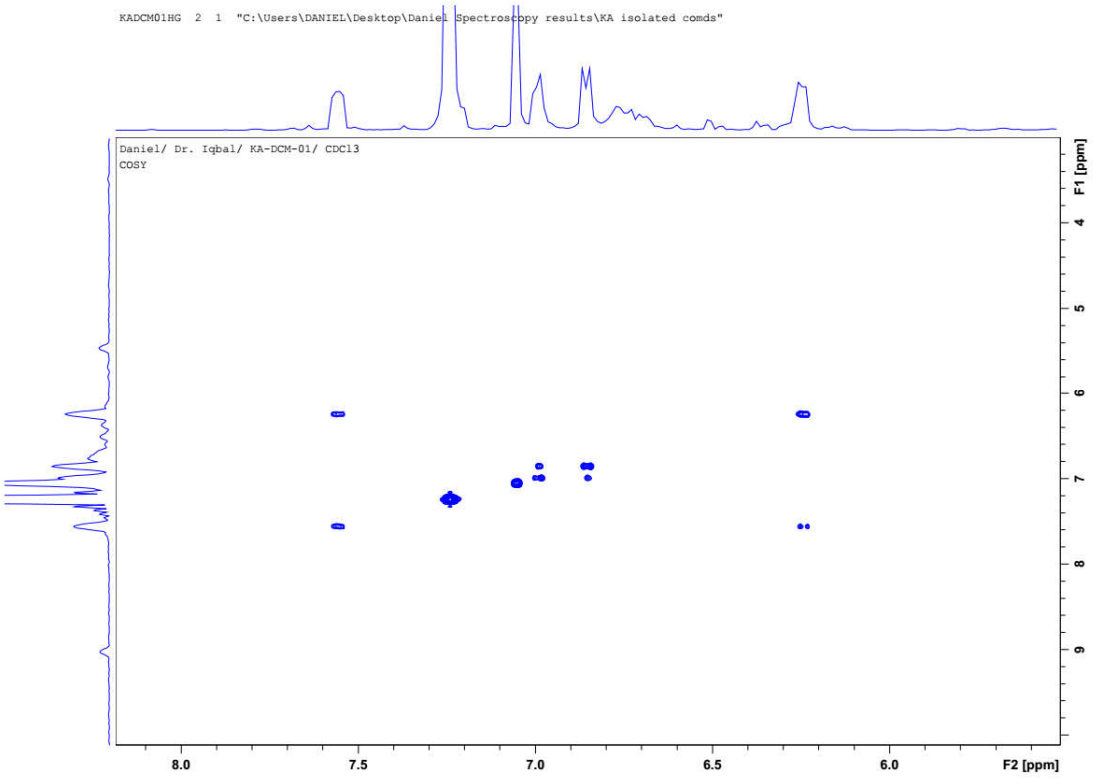
Appendix 3l: HMBC of compound 3



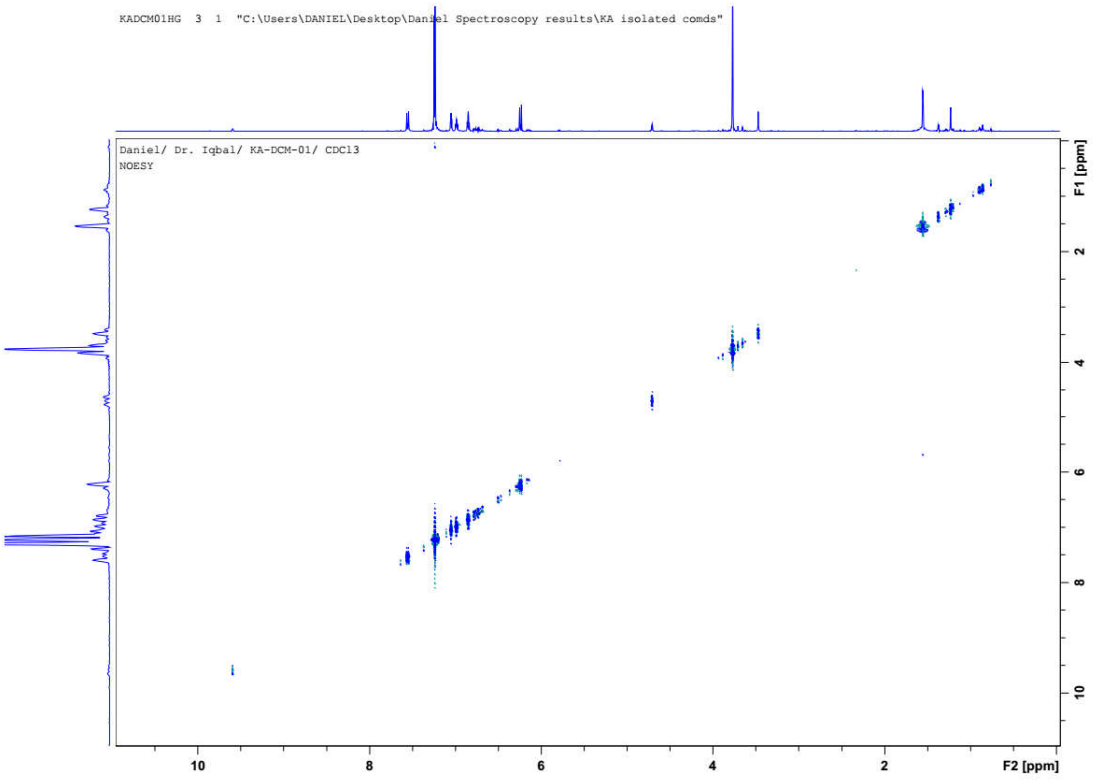


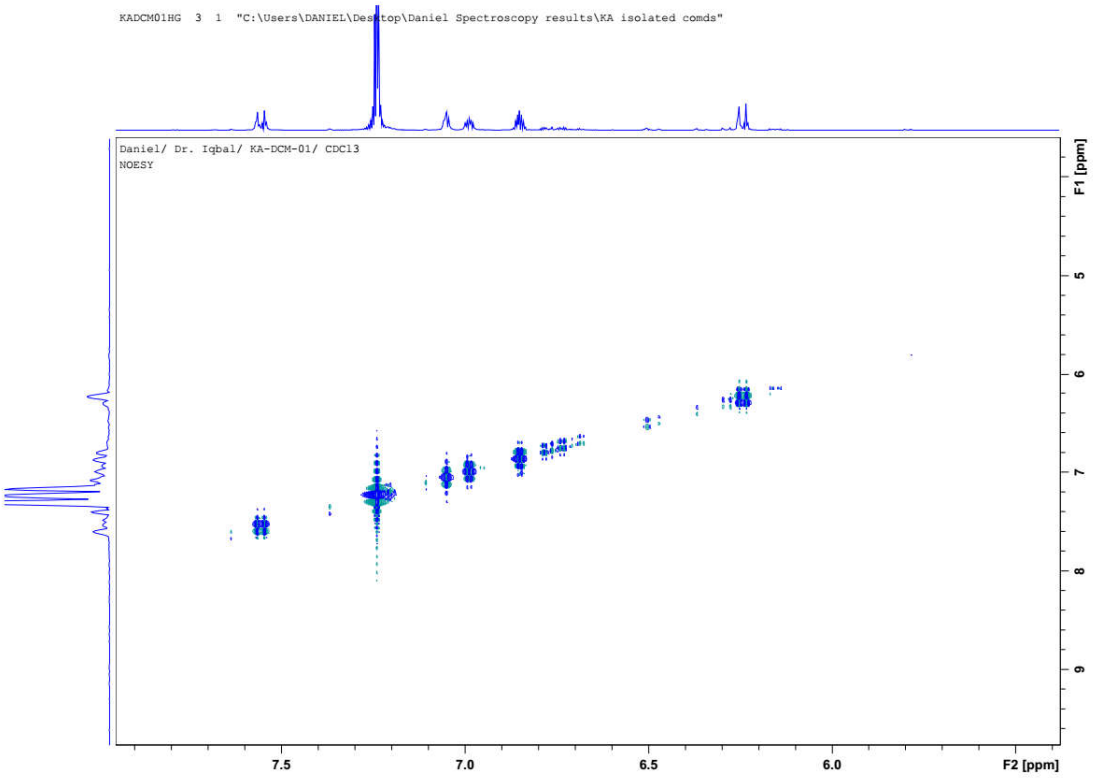
Appendix 32: COSY of compound 3



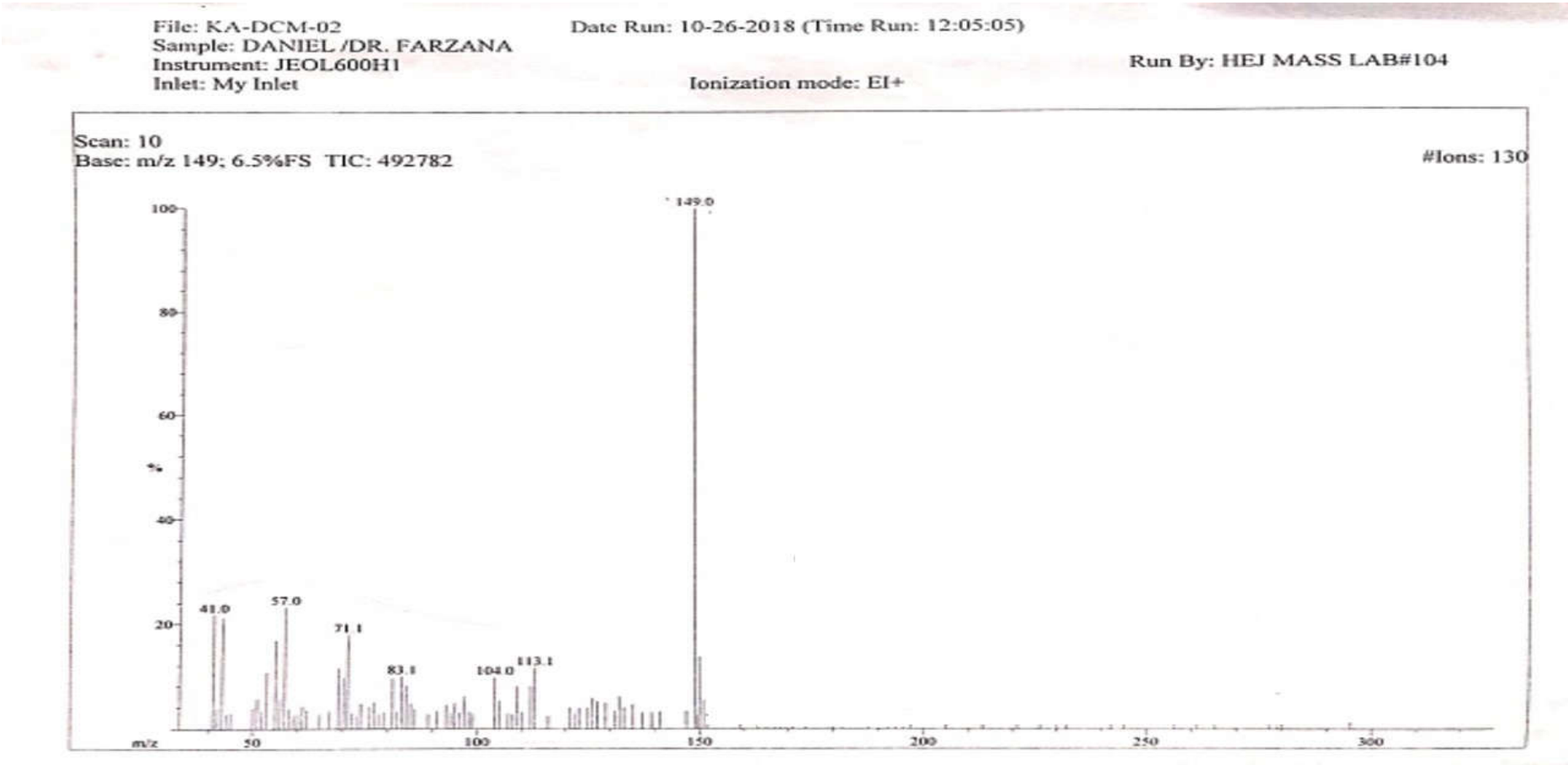


Appendix 33: NOESY of compound 3

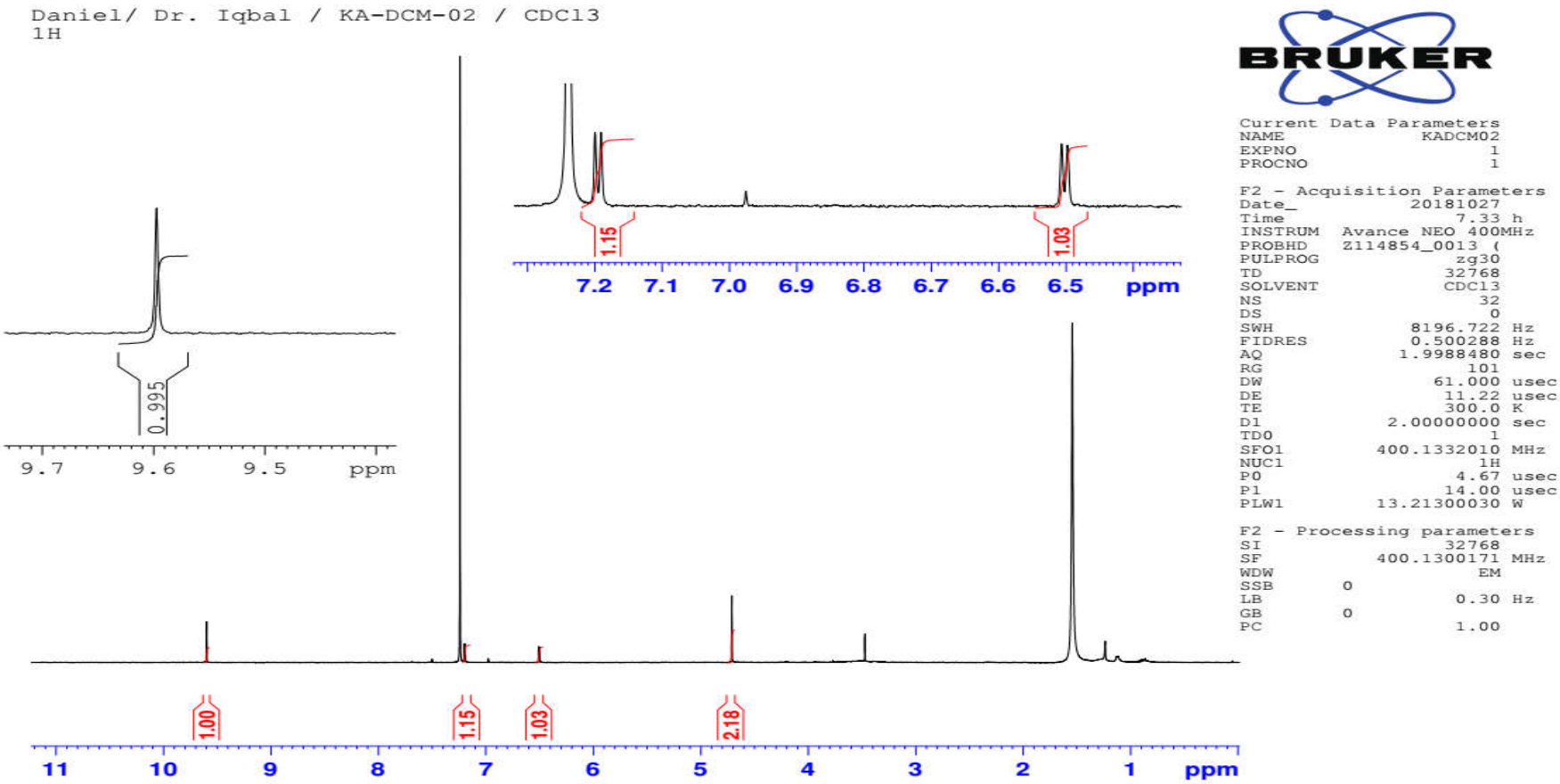




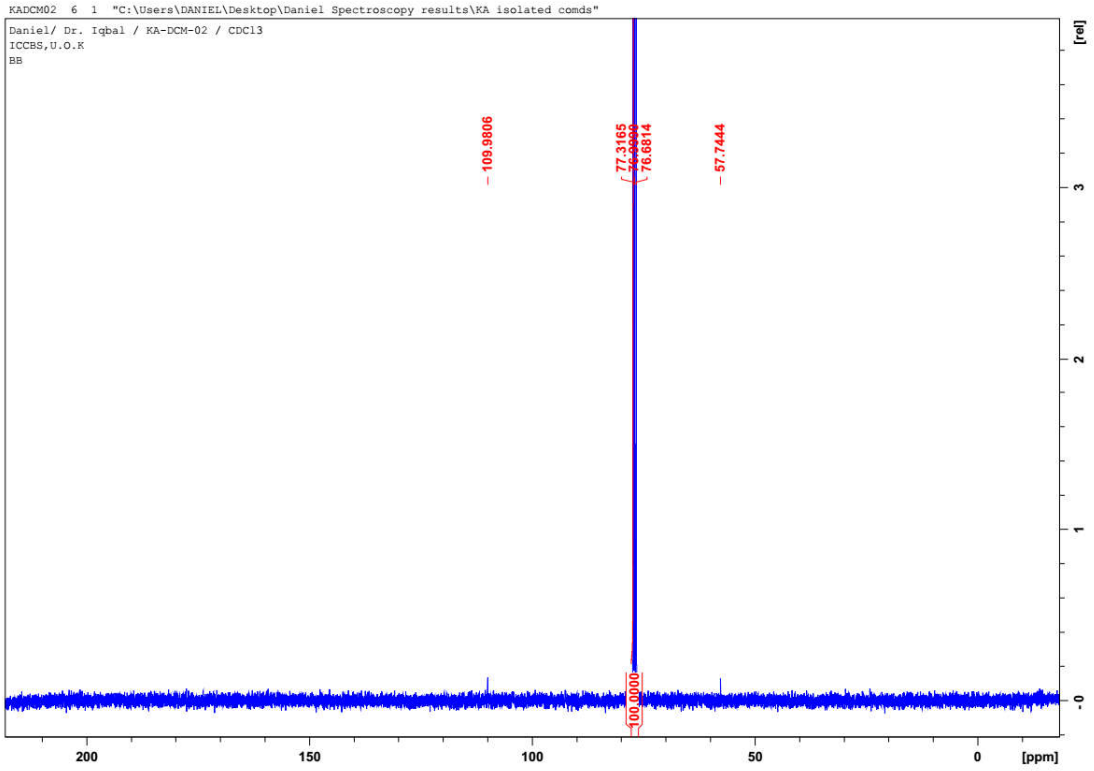
Appendix 34: Mass spectral of compound 4

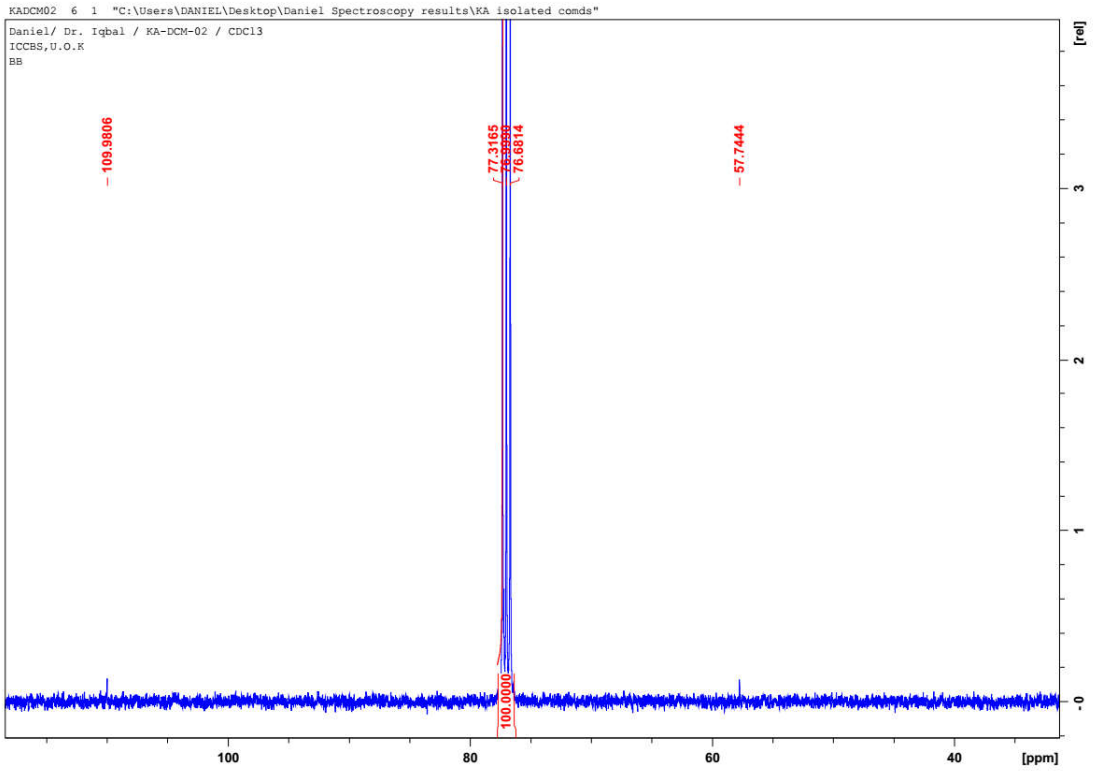


Appendix 35: ^{1}H NMR of compound 4

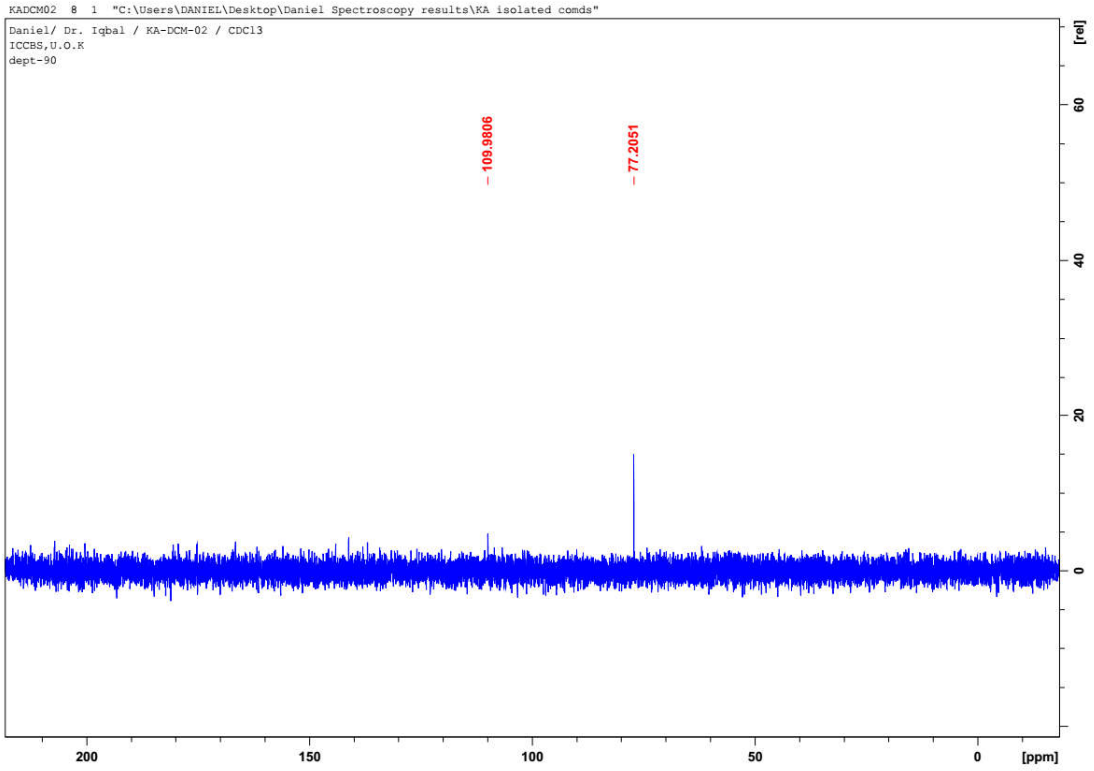


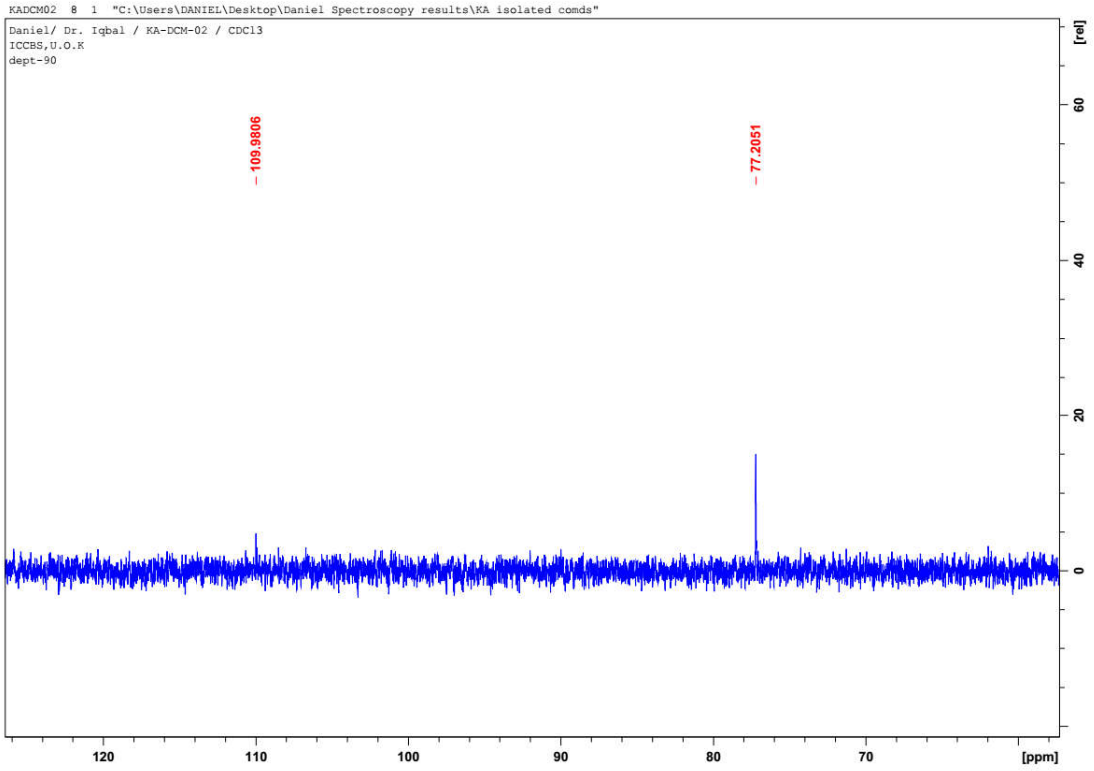
Appendix 36: ^{13}C NMR of compound 4



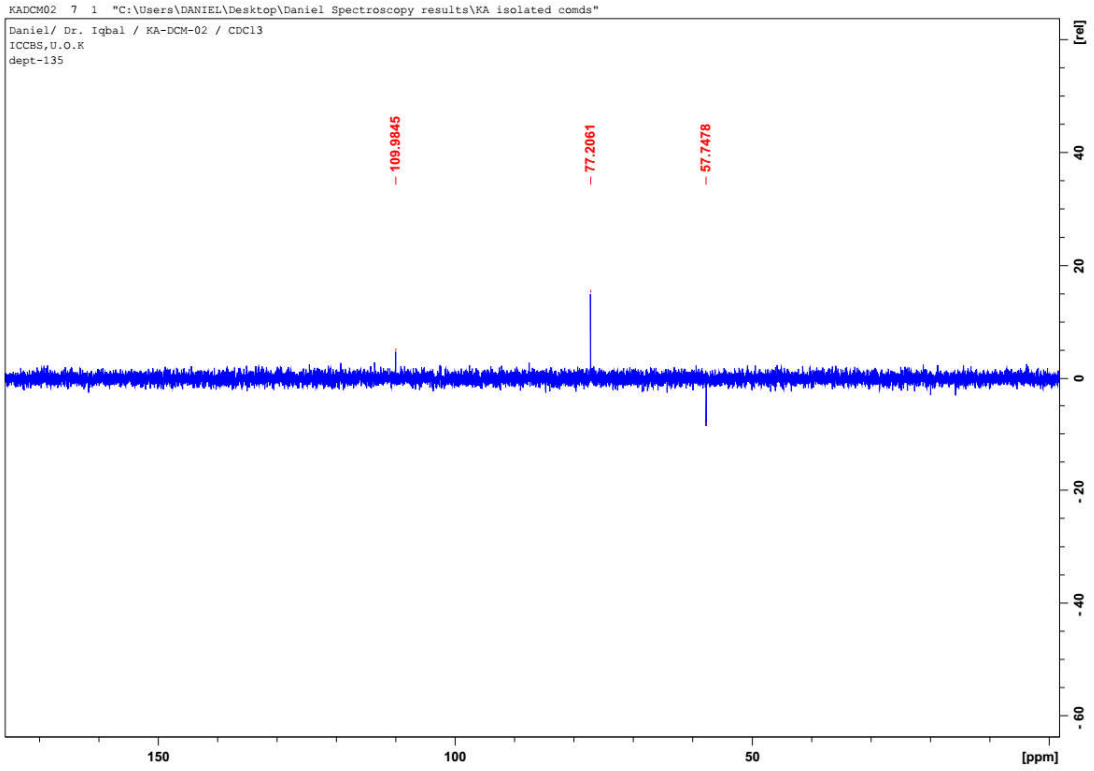


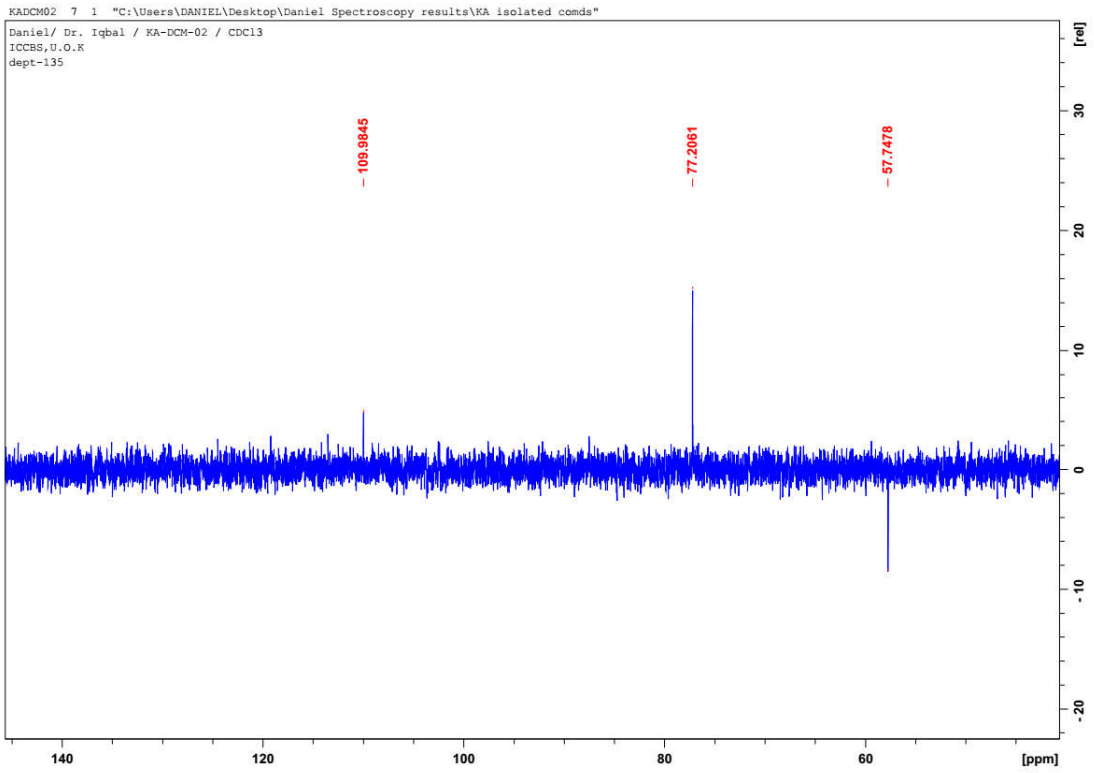
Appendix 37: DEPT 90 of compound 4



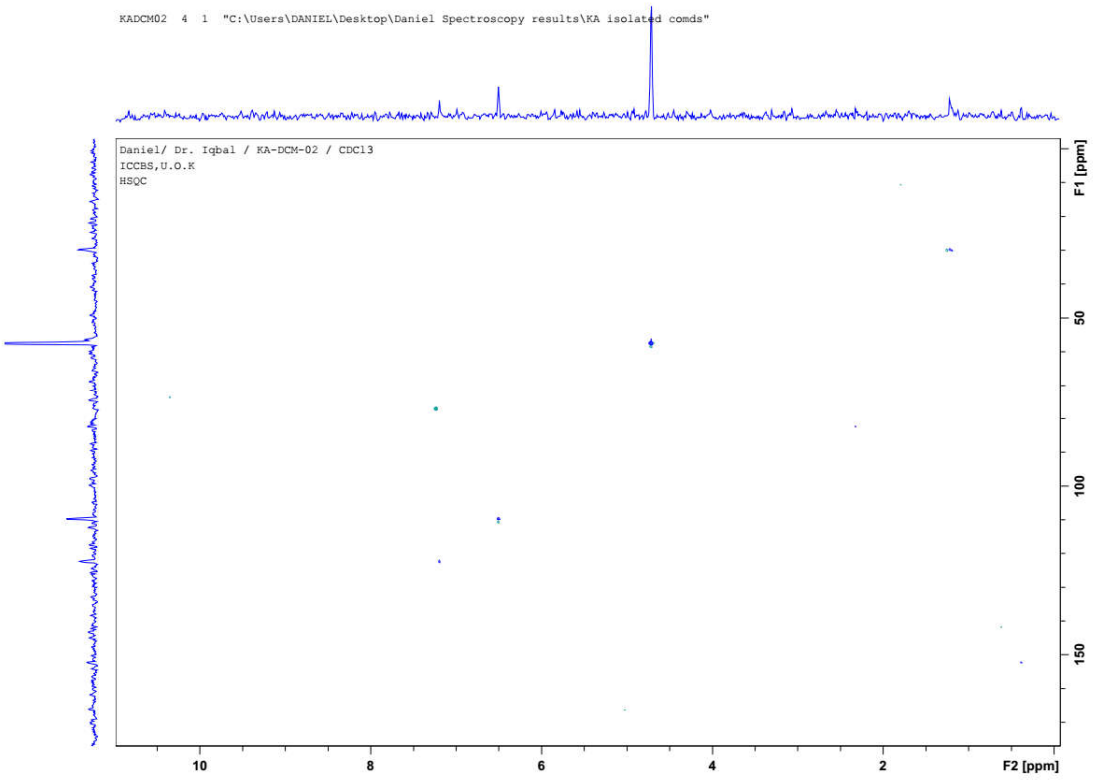


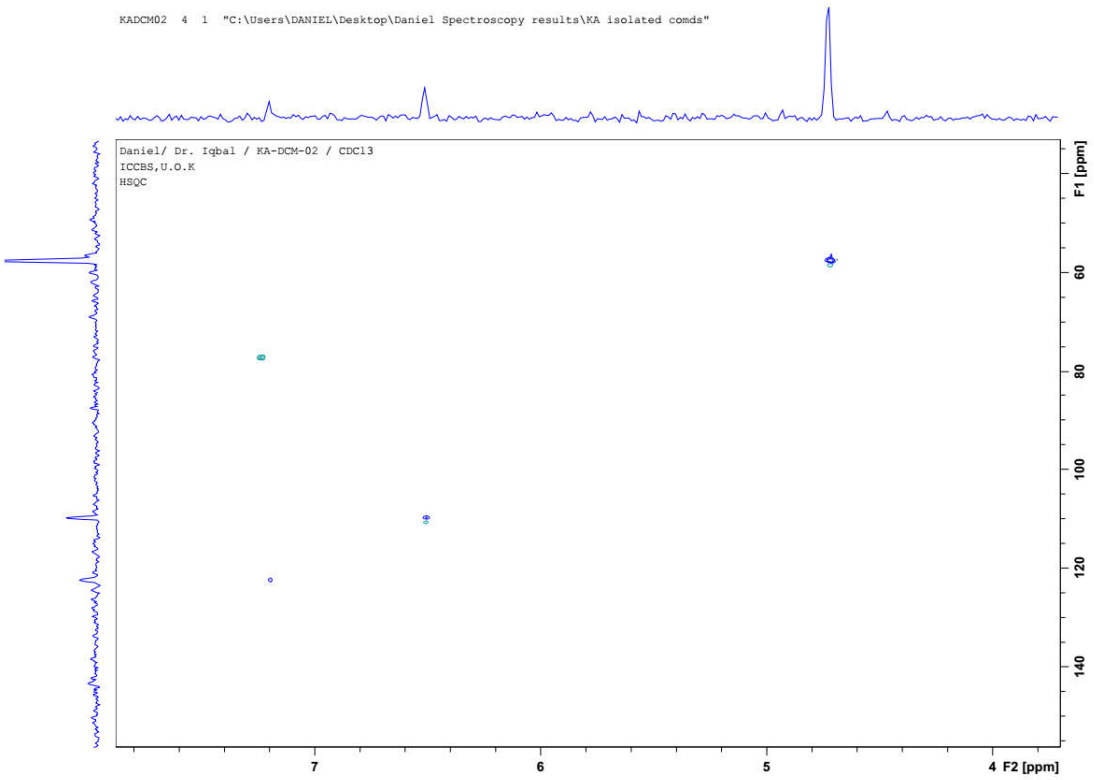
Appendix 38: DEPT l35 of compound 4



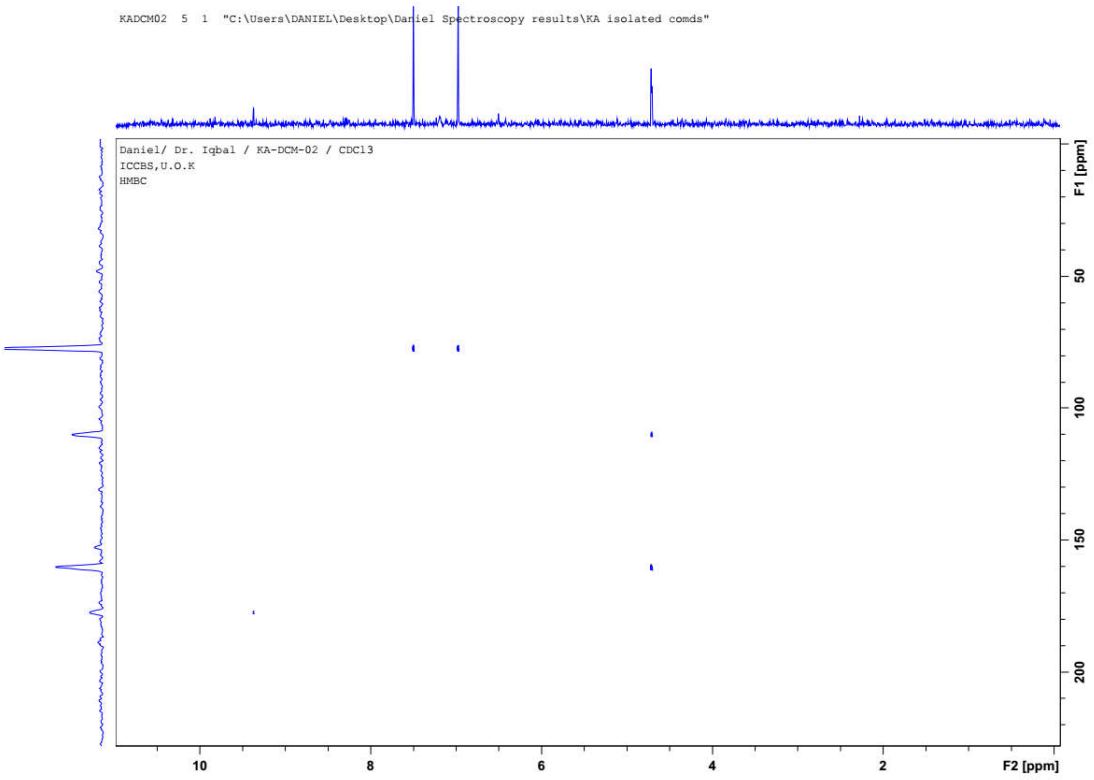


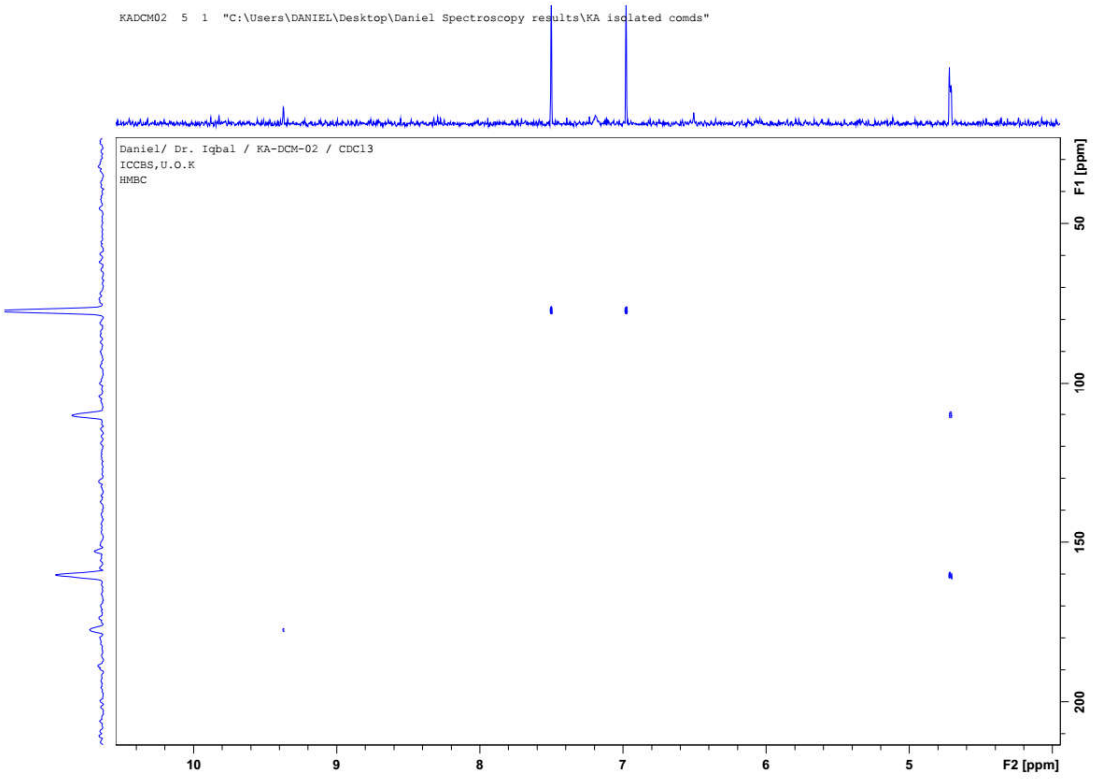
Appendix 39: HSQC of compound 4



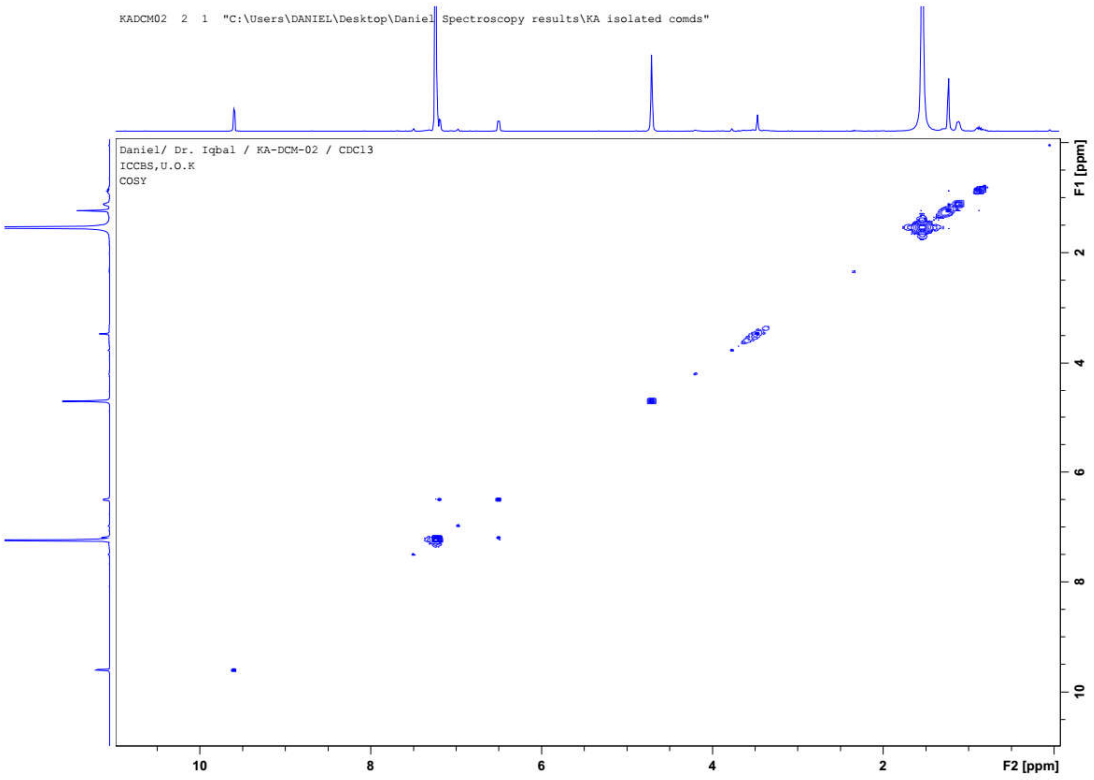


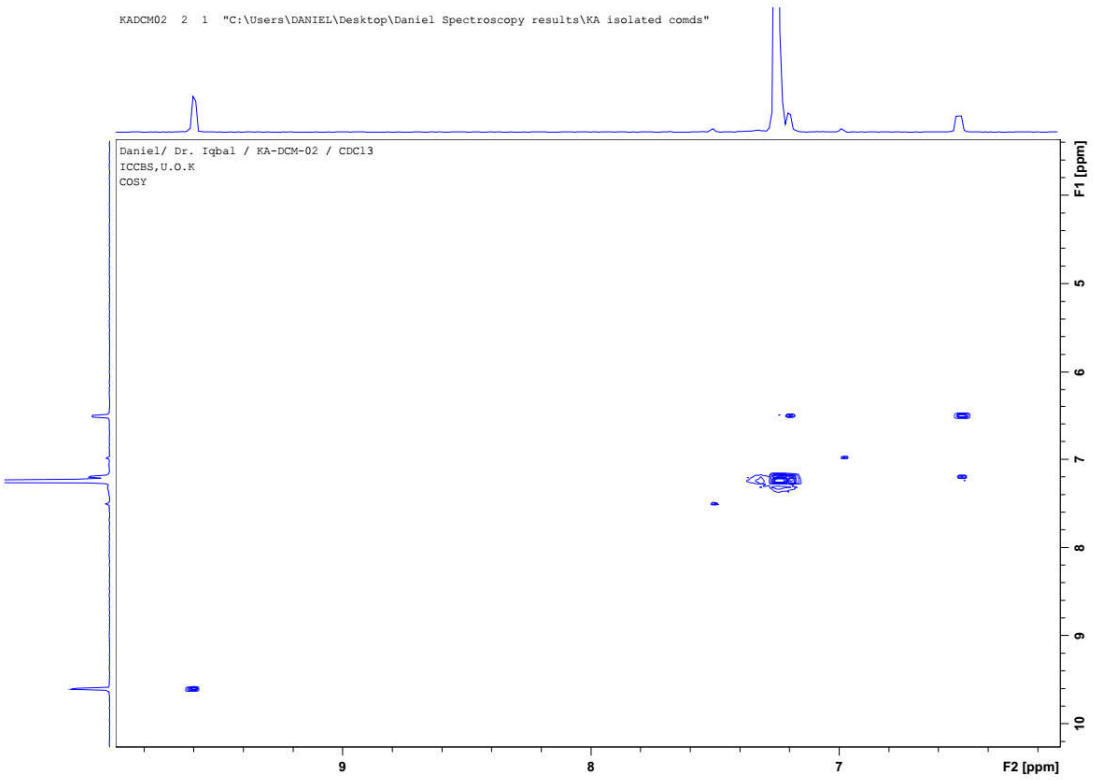
Appendix 40: HMBC of compound 4



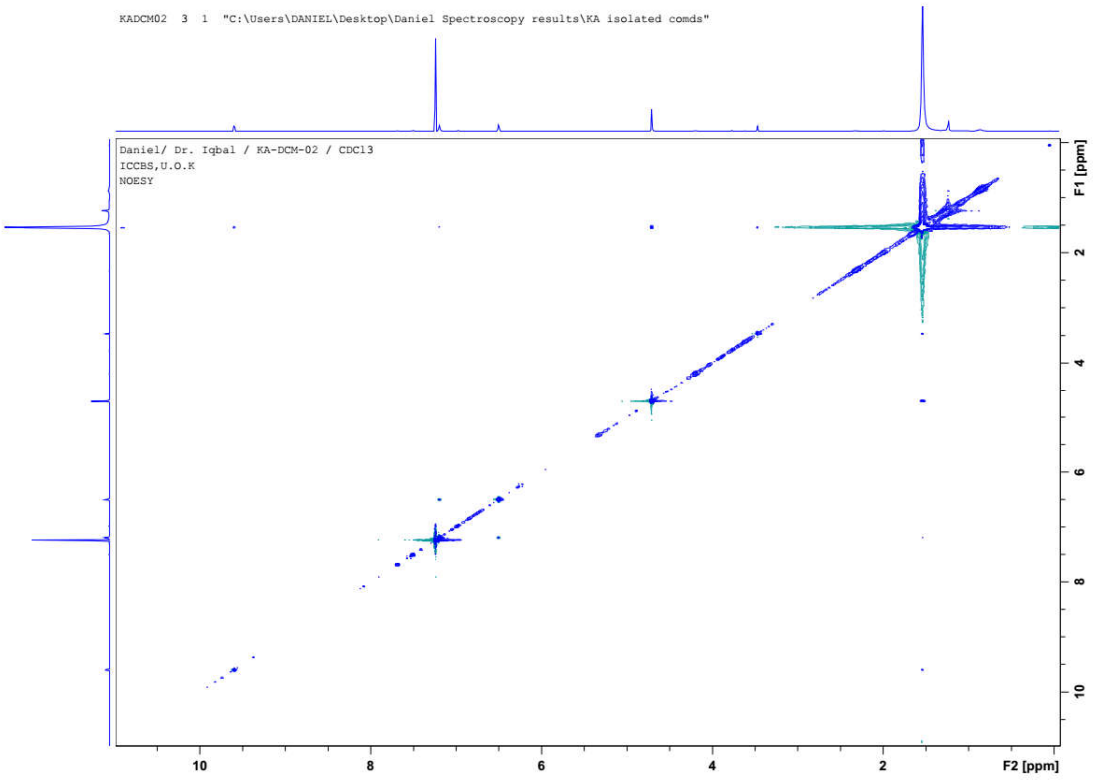


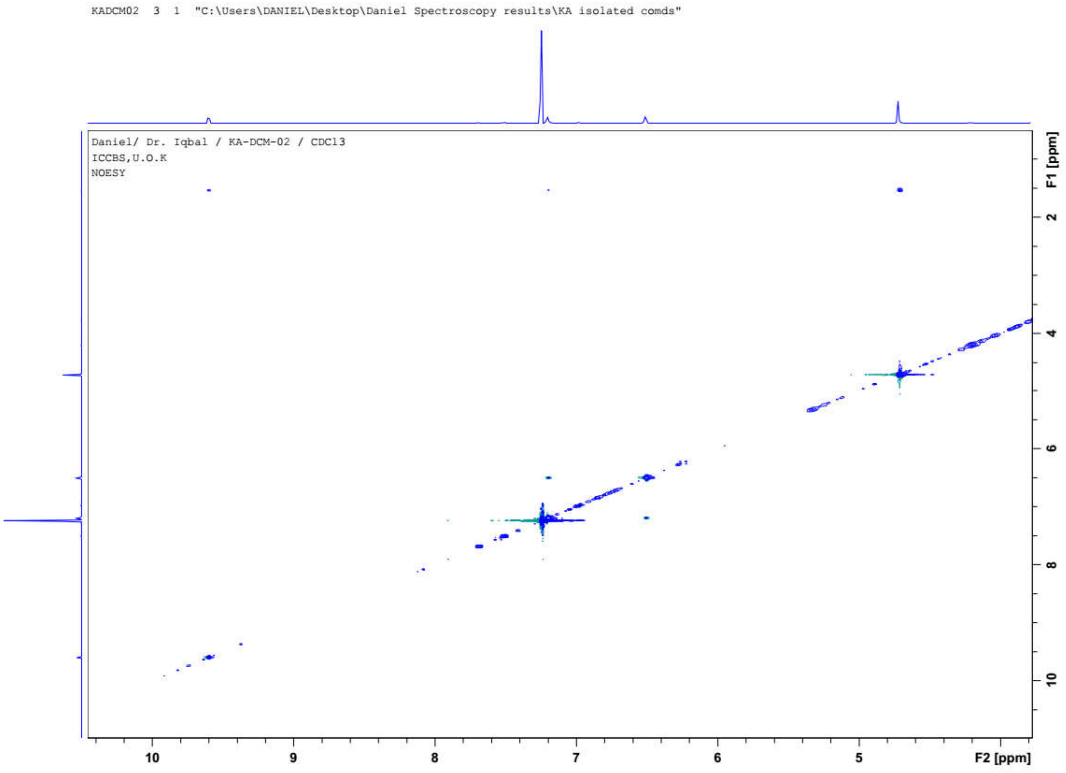
Appendix 4l: COSY of compound 4



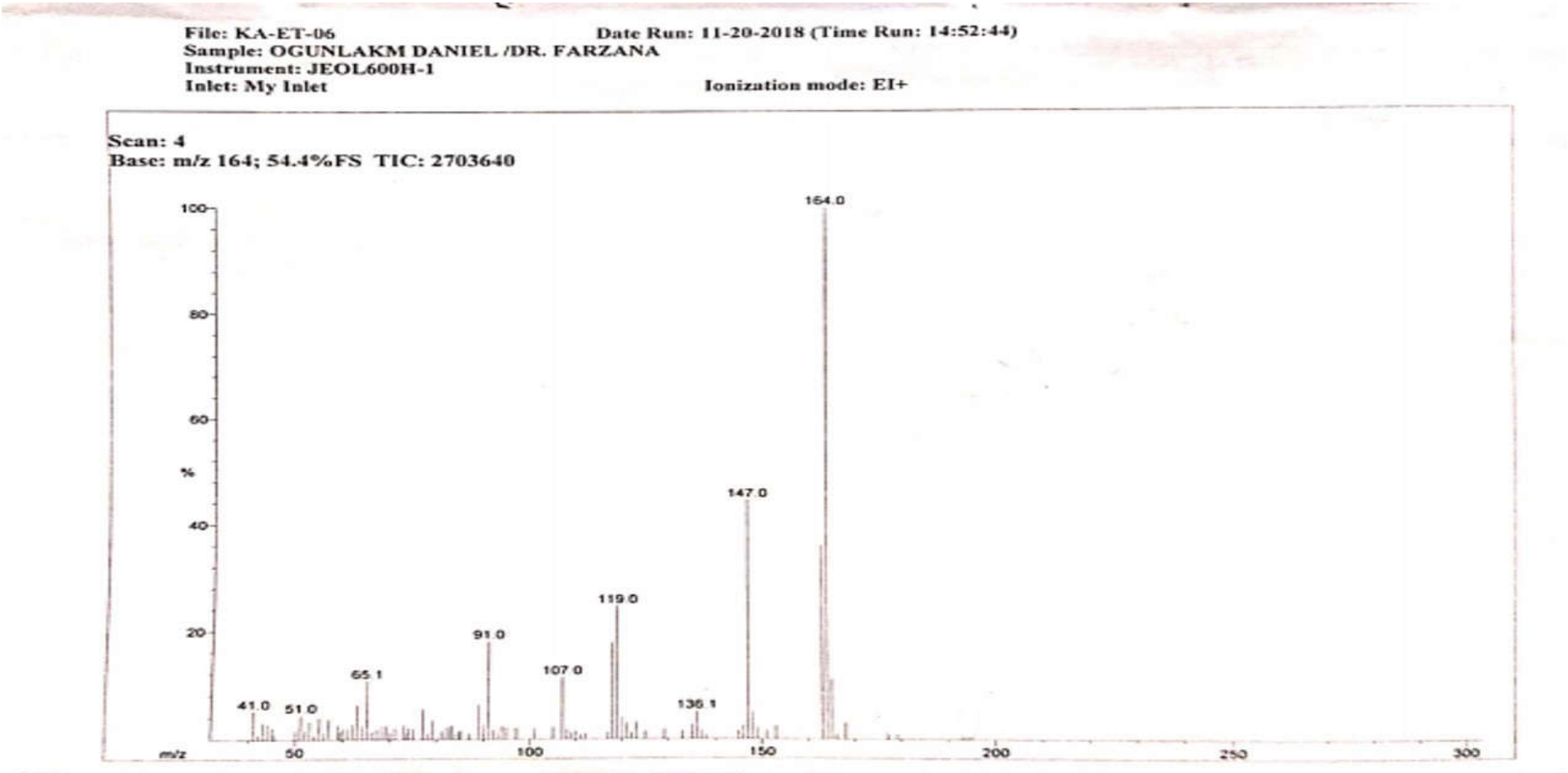


Appendix 42: NOESY of compound 4

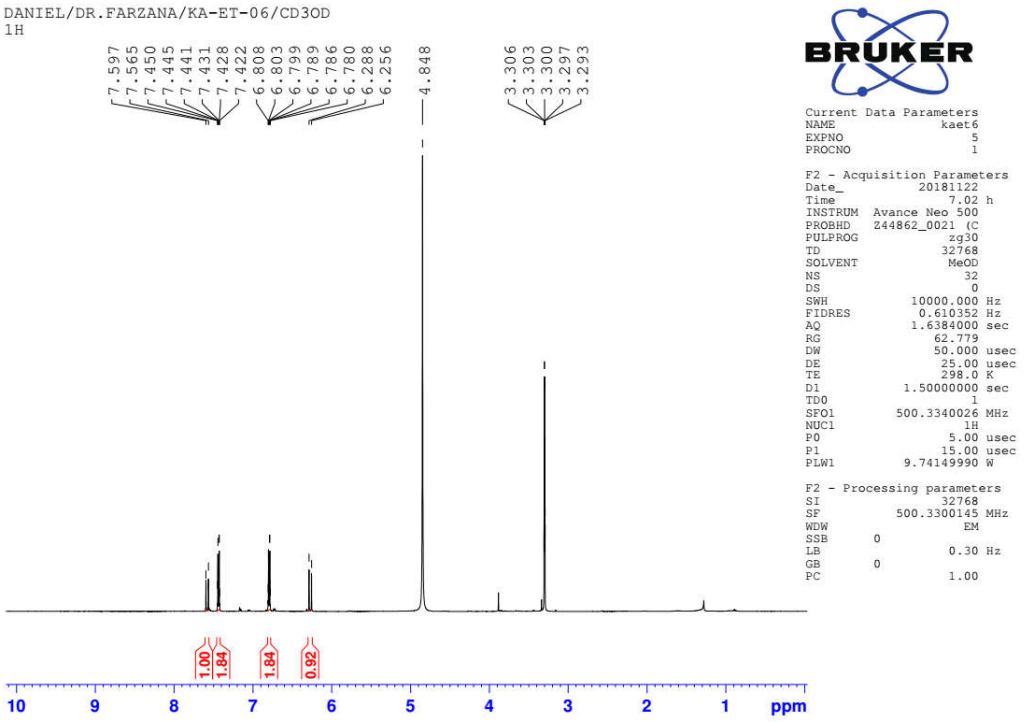


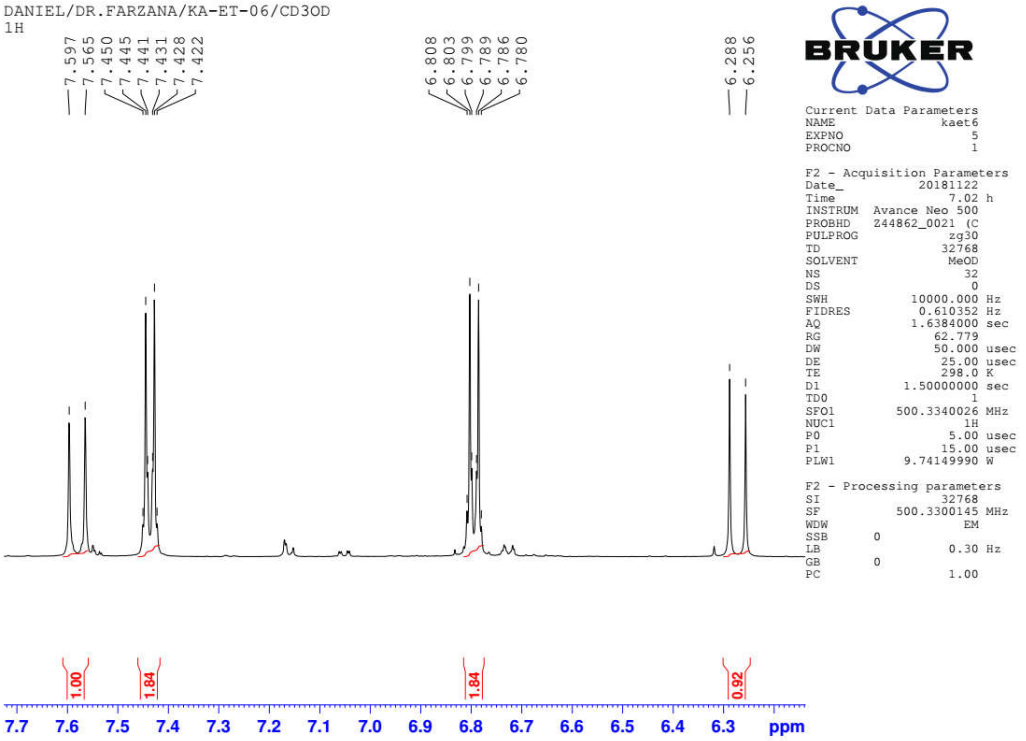


Appendix 43: Mass spectral of compound 5

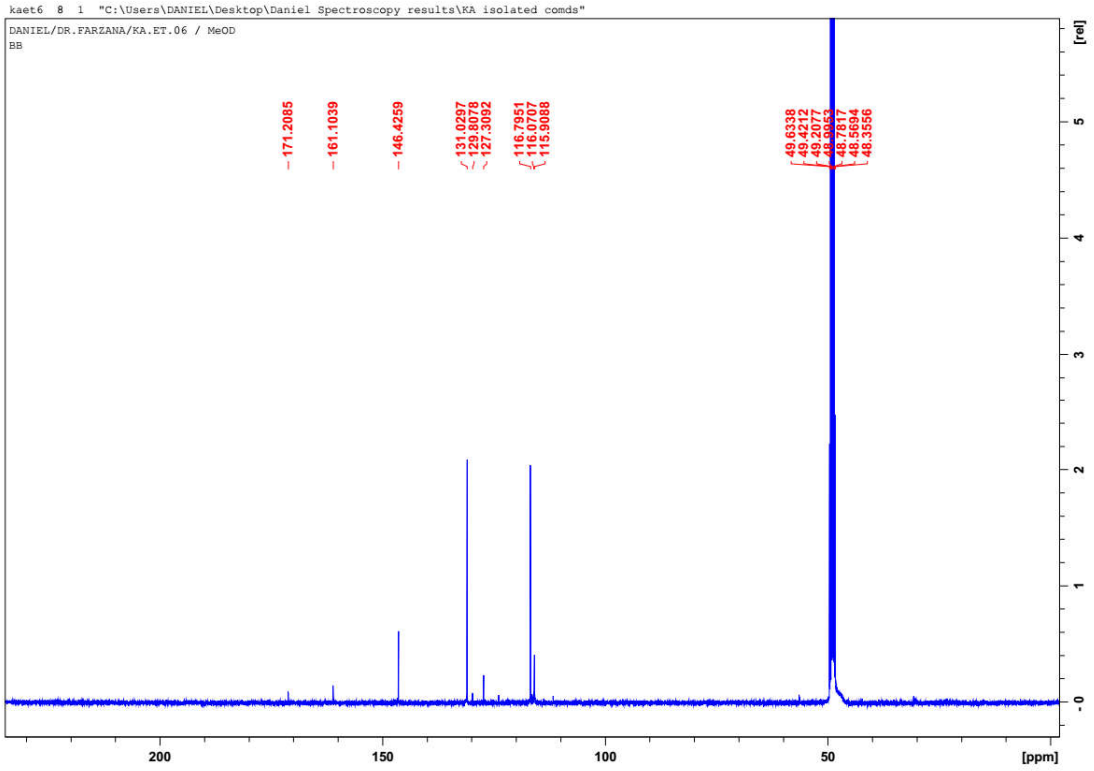


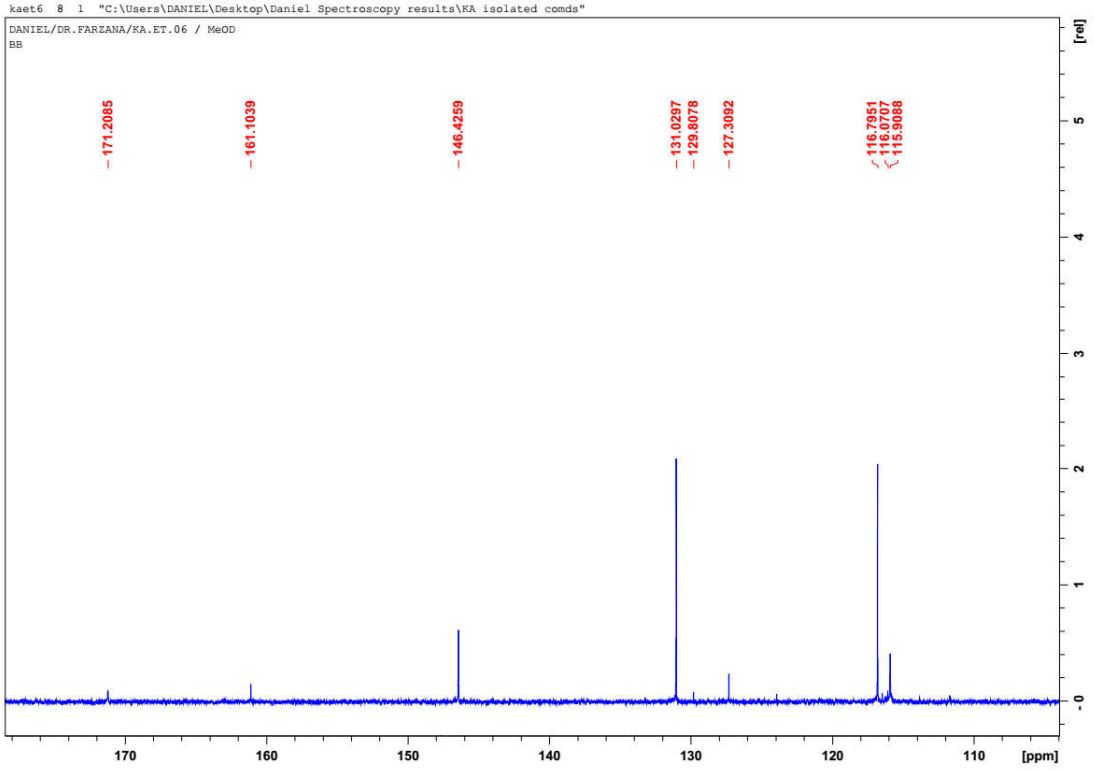
Appendix 44: ^{1}H NMR of compound 5



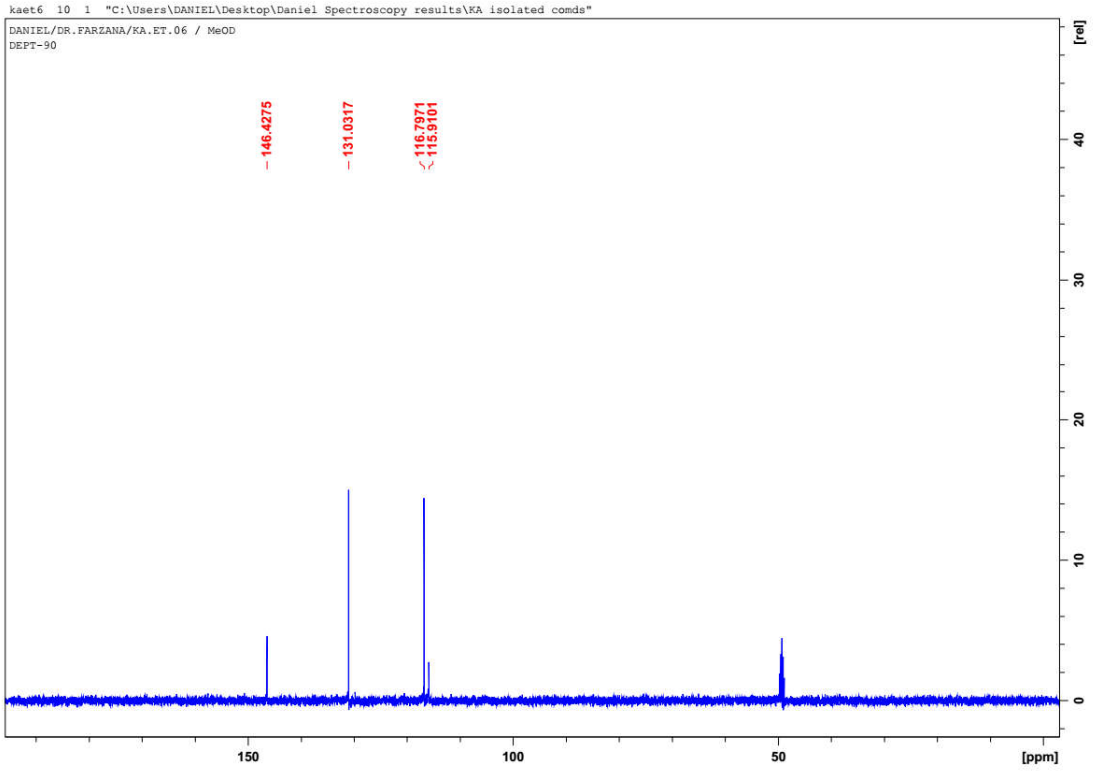


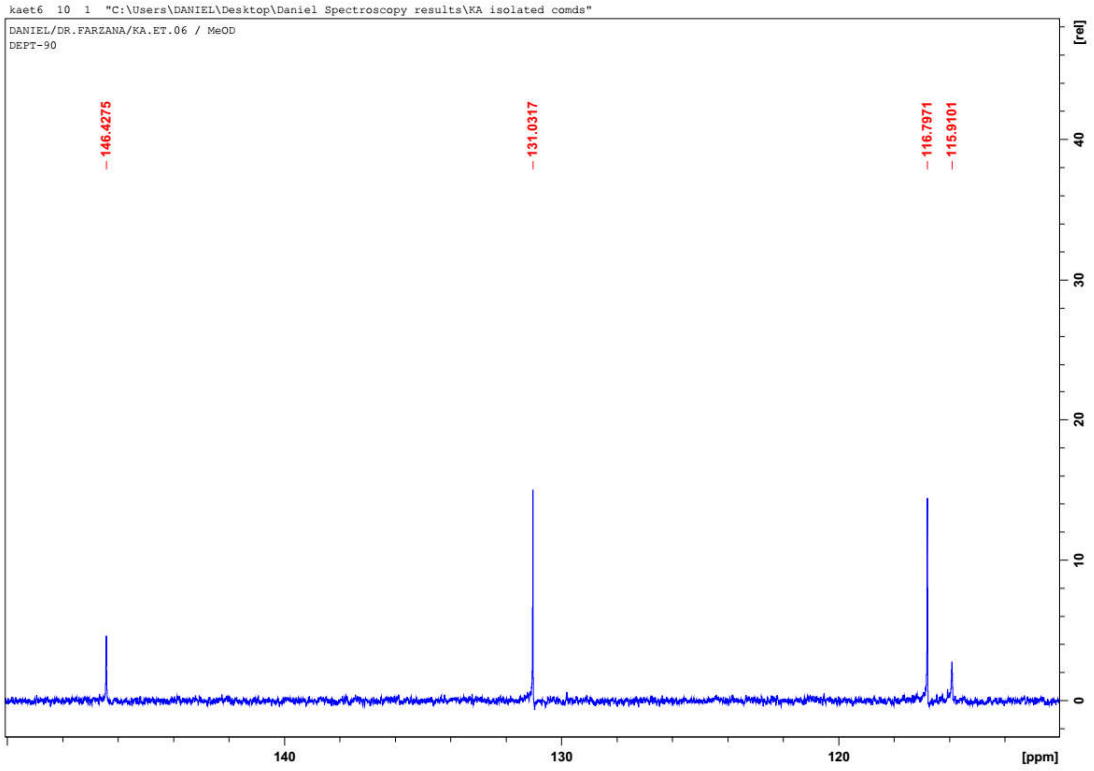
Appendix 45: ^{13}C NMR of compound 5



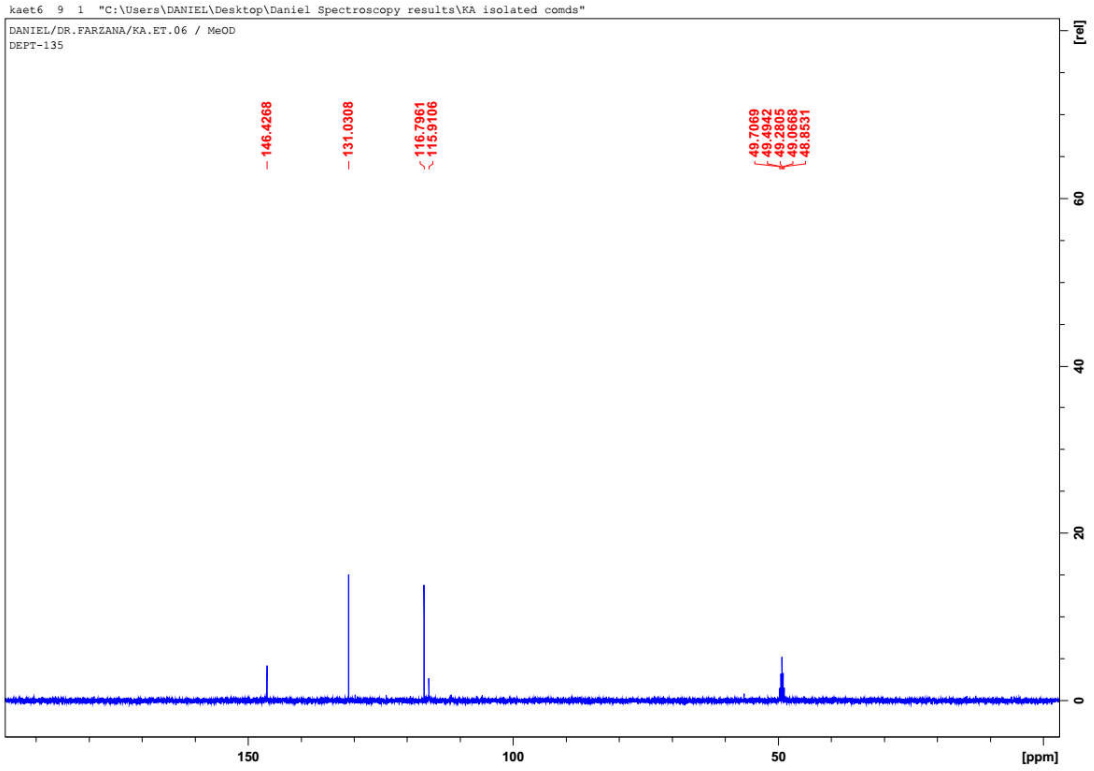


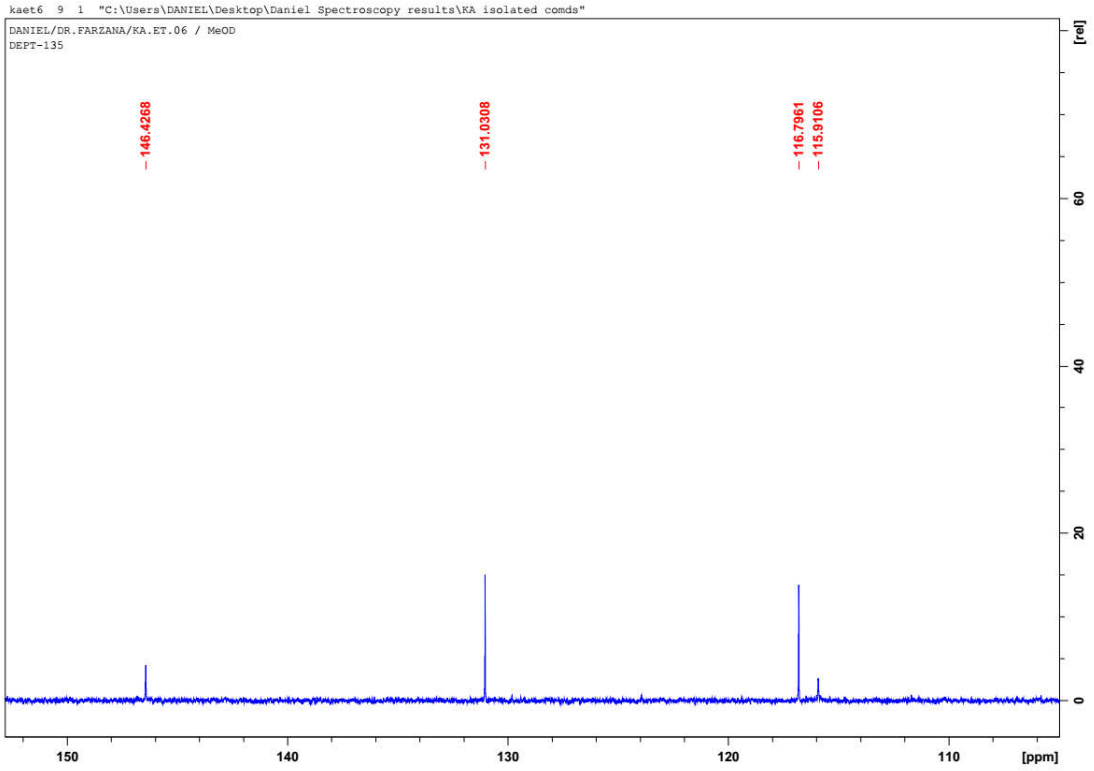
Appendix 46: DEPT 90 of compound 5



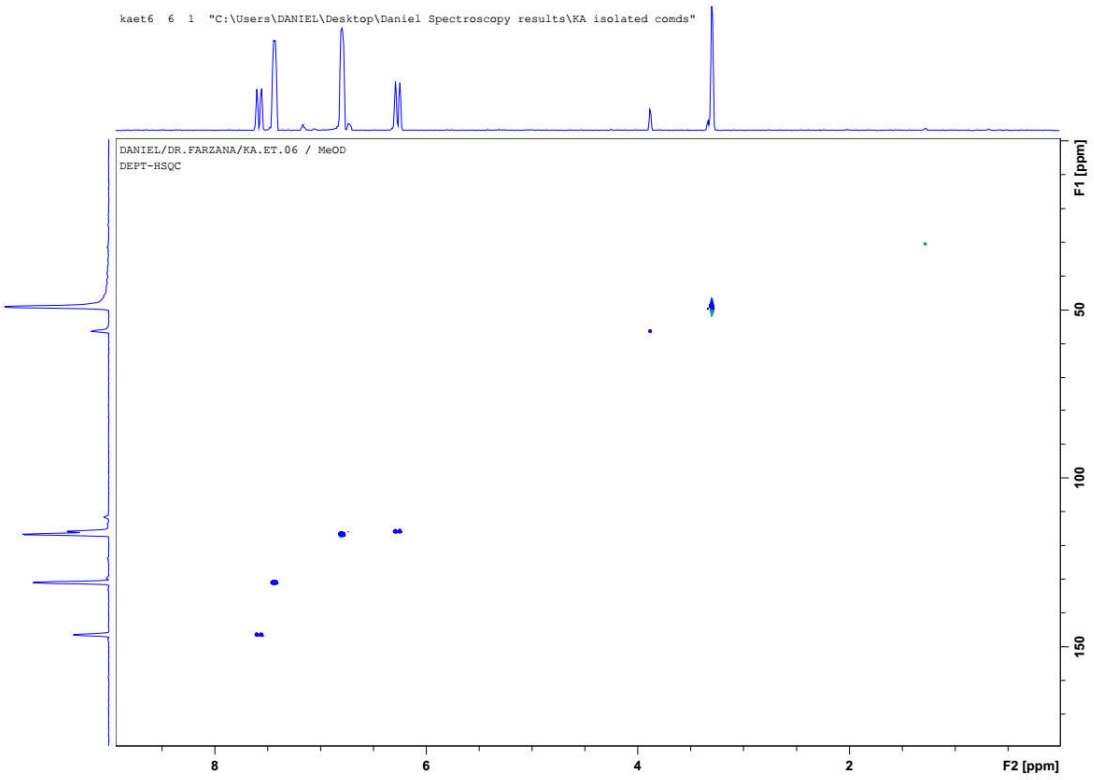


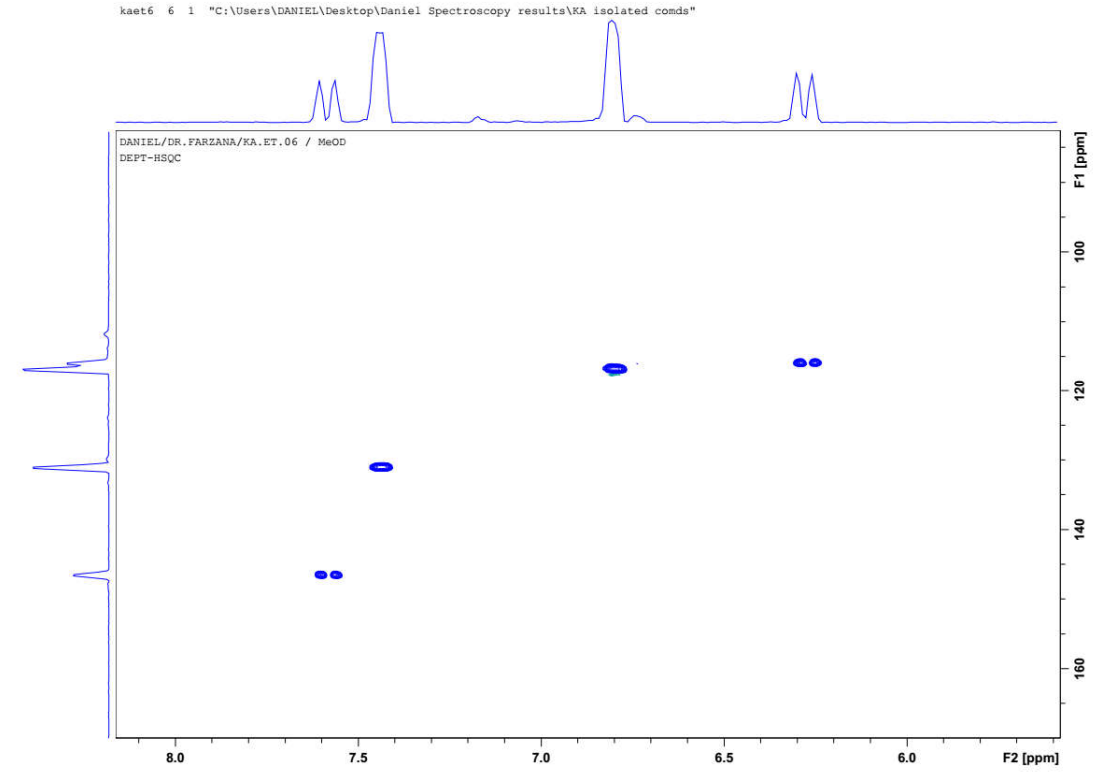
Appendix 47: DEPT l35 of compound 5



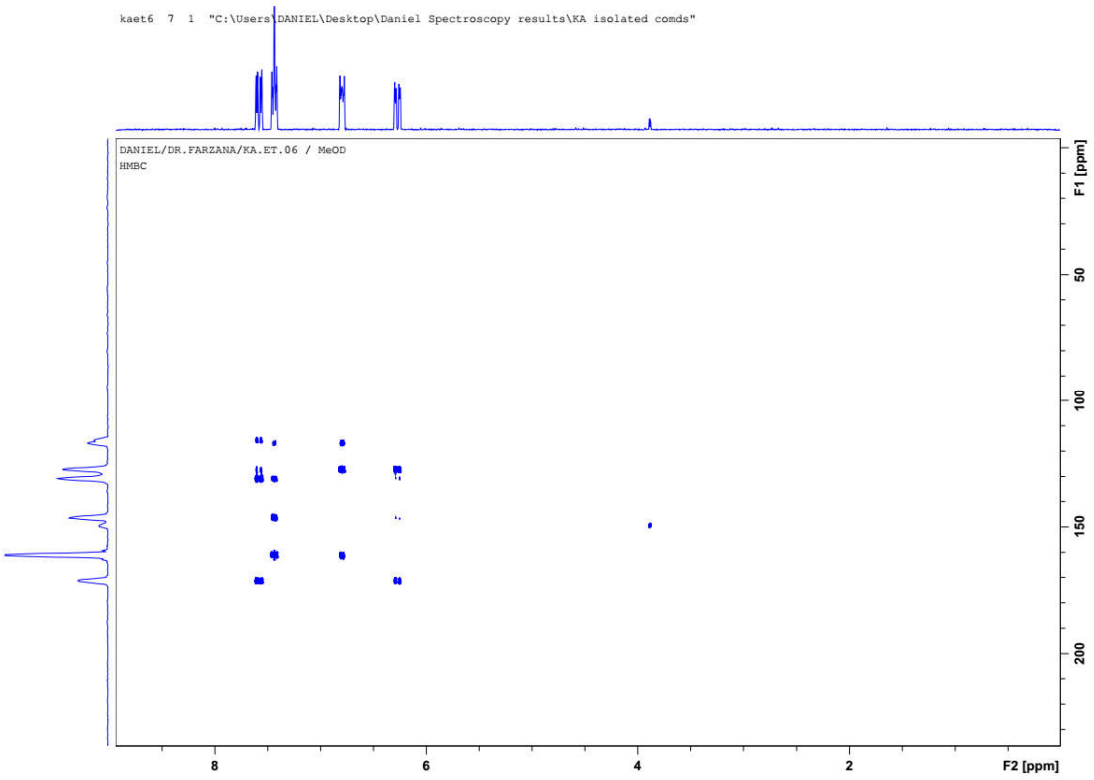


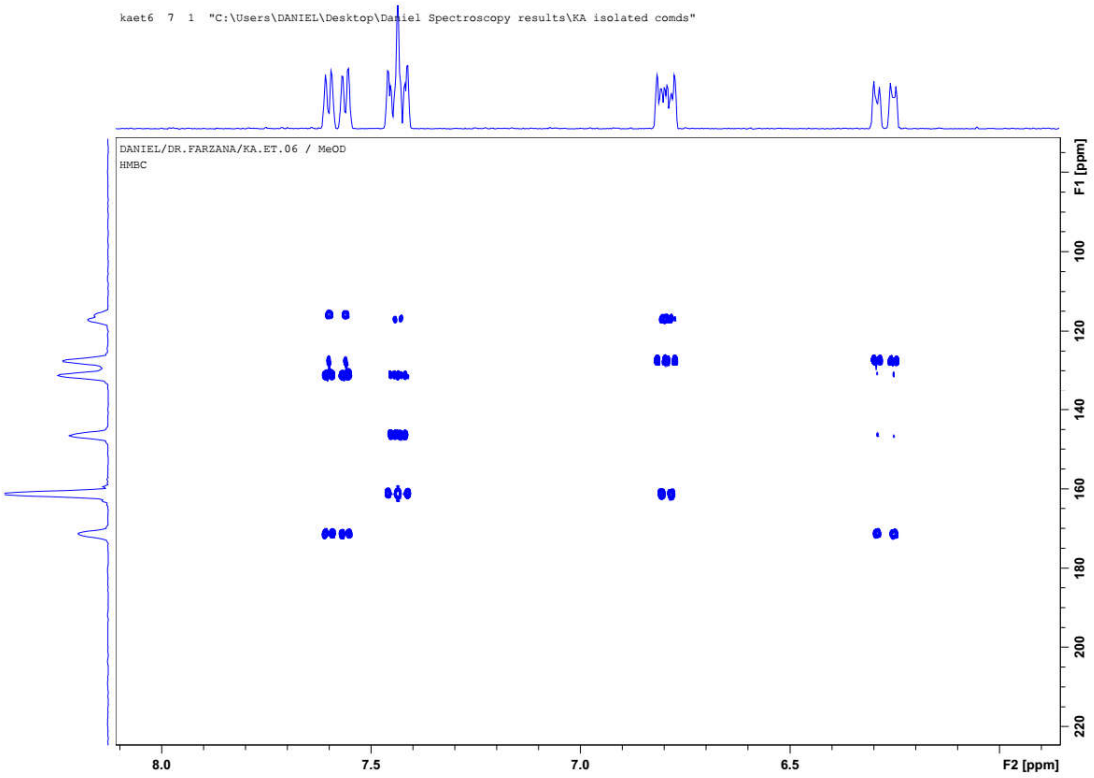
Appendix 48: HSQC of compound 5



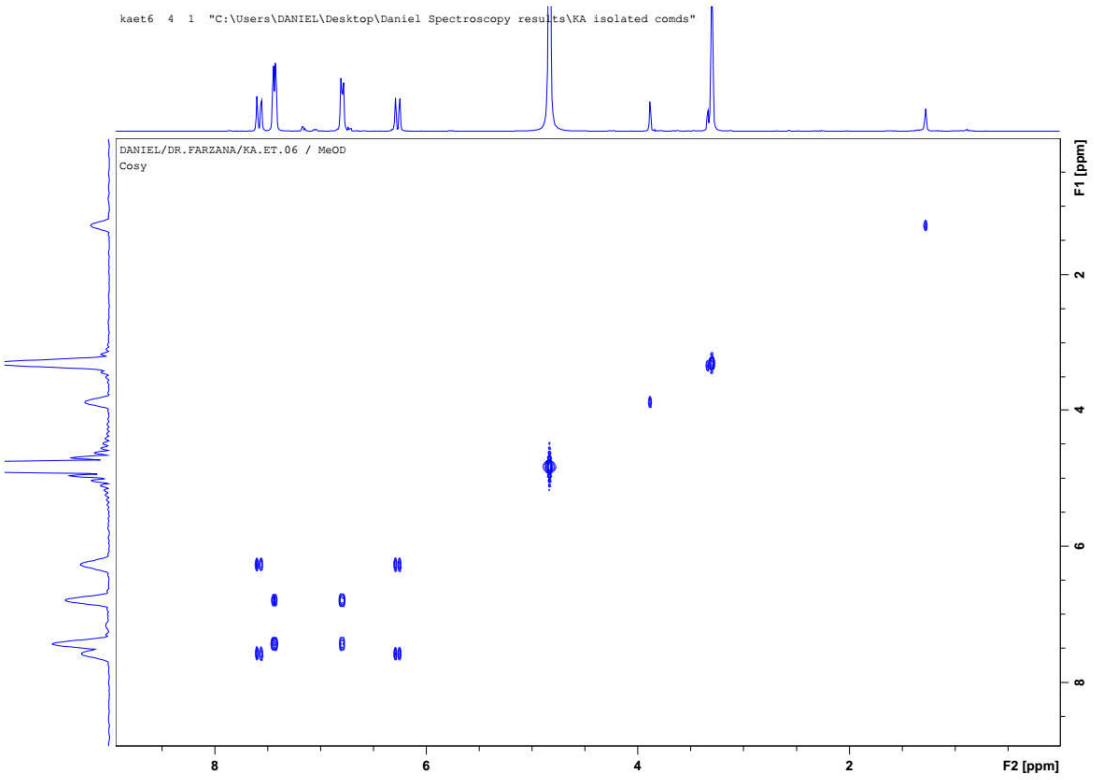


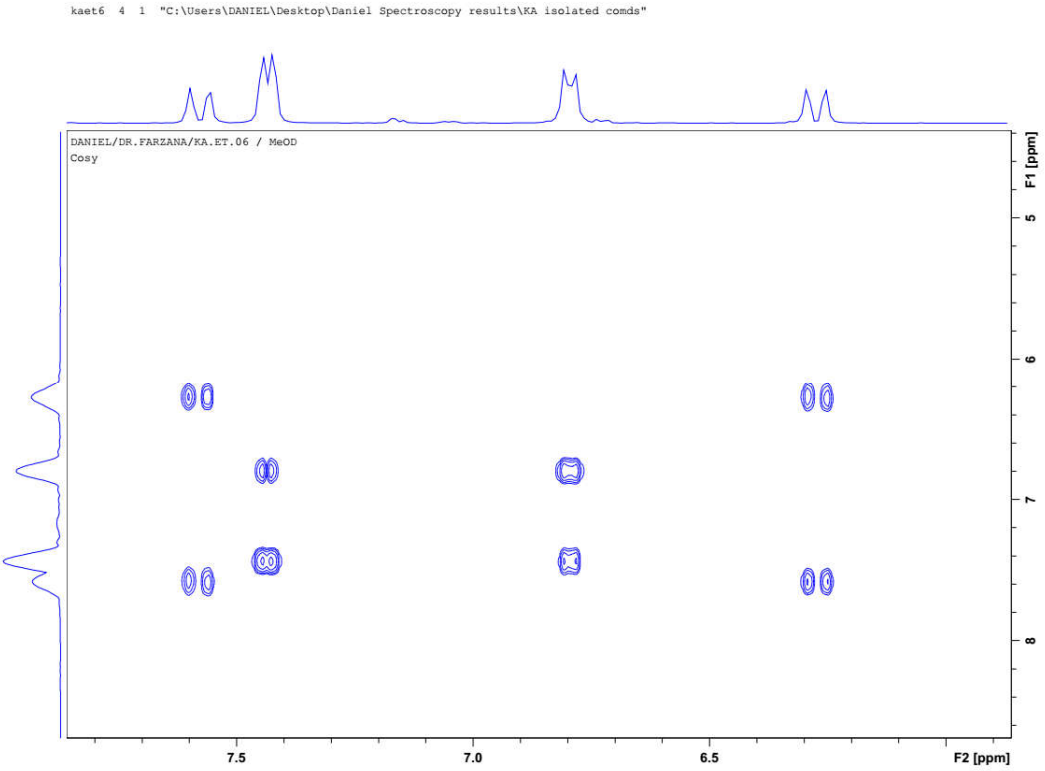
Appendix 49: HMBC of compound 5



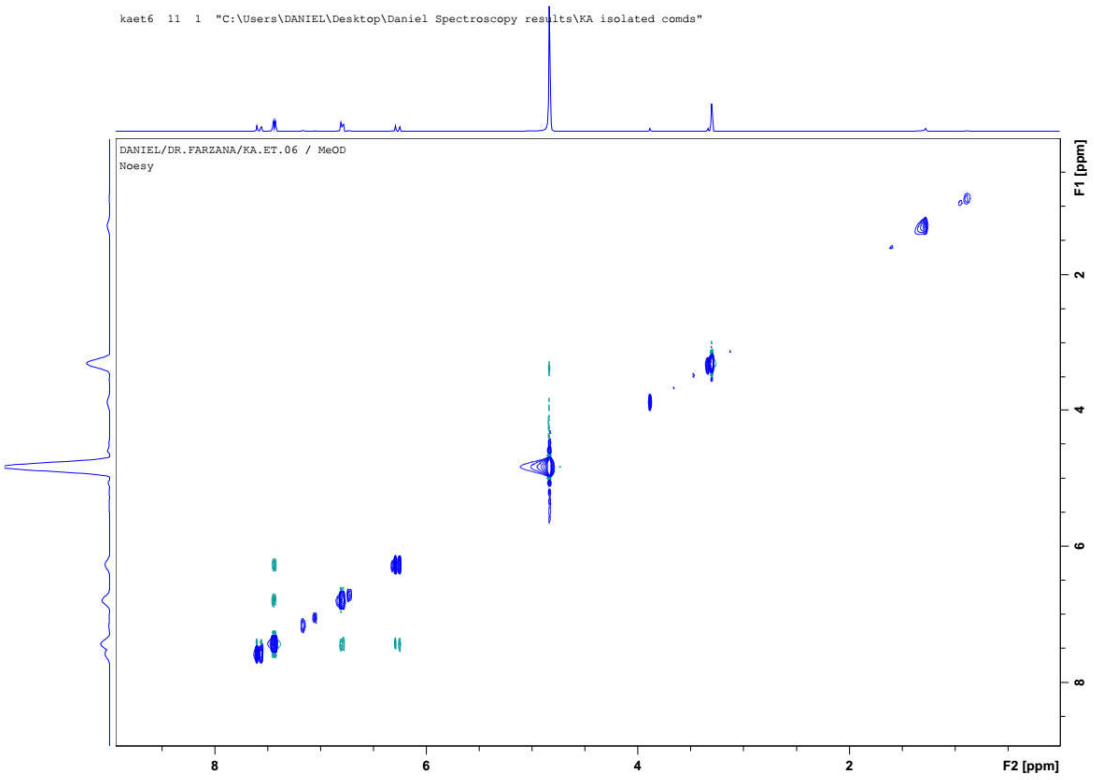


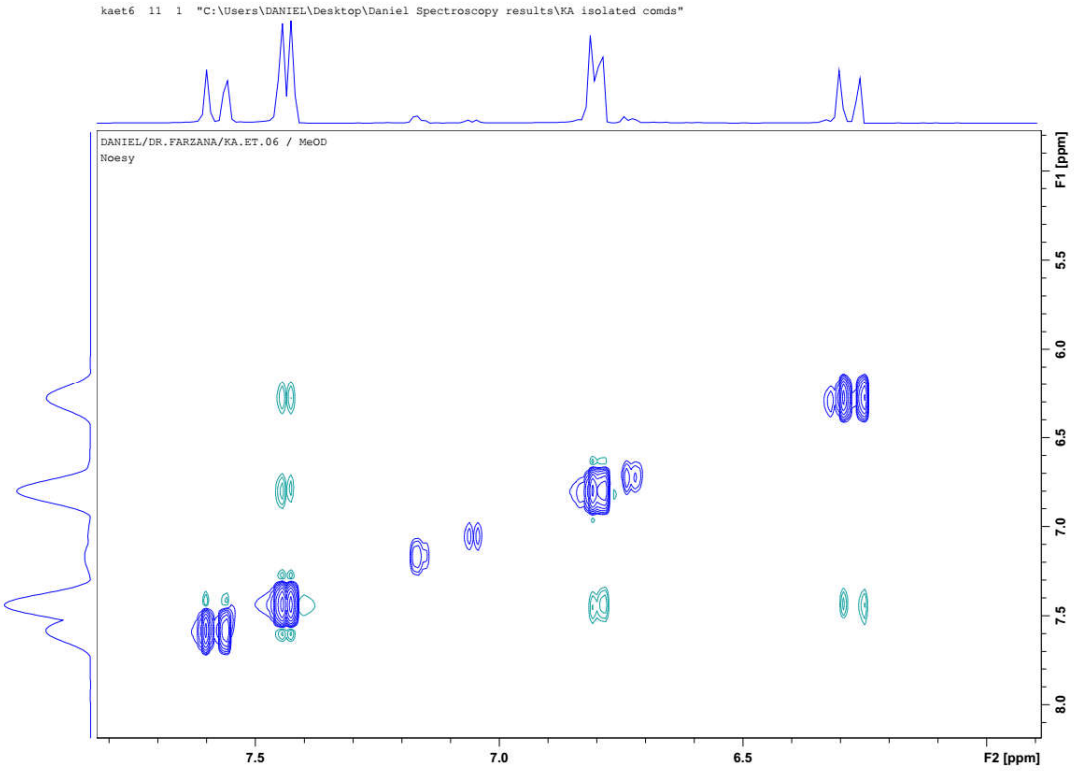
Appendix 50: COSY of compound 5



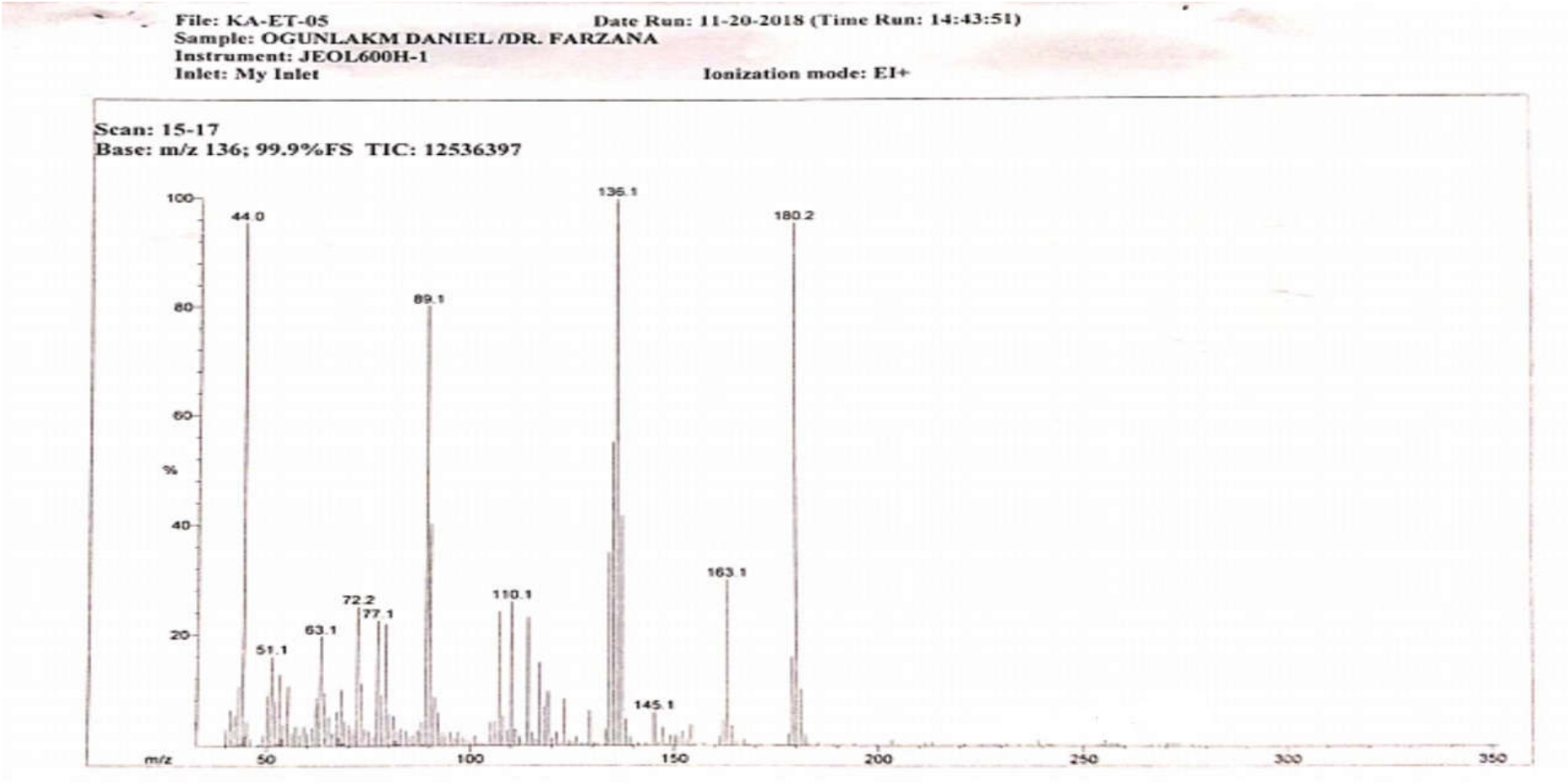


Appendix 5l: NOESY of compound 5

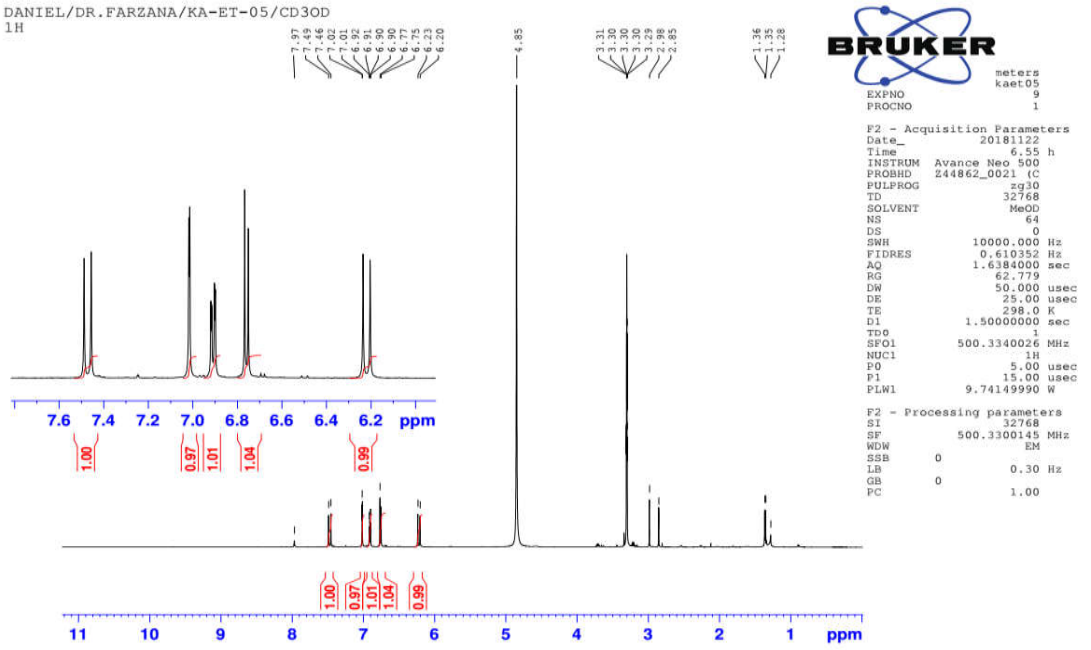


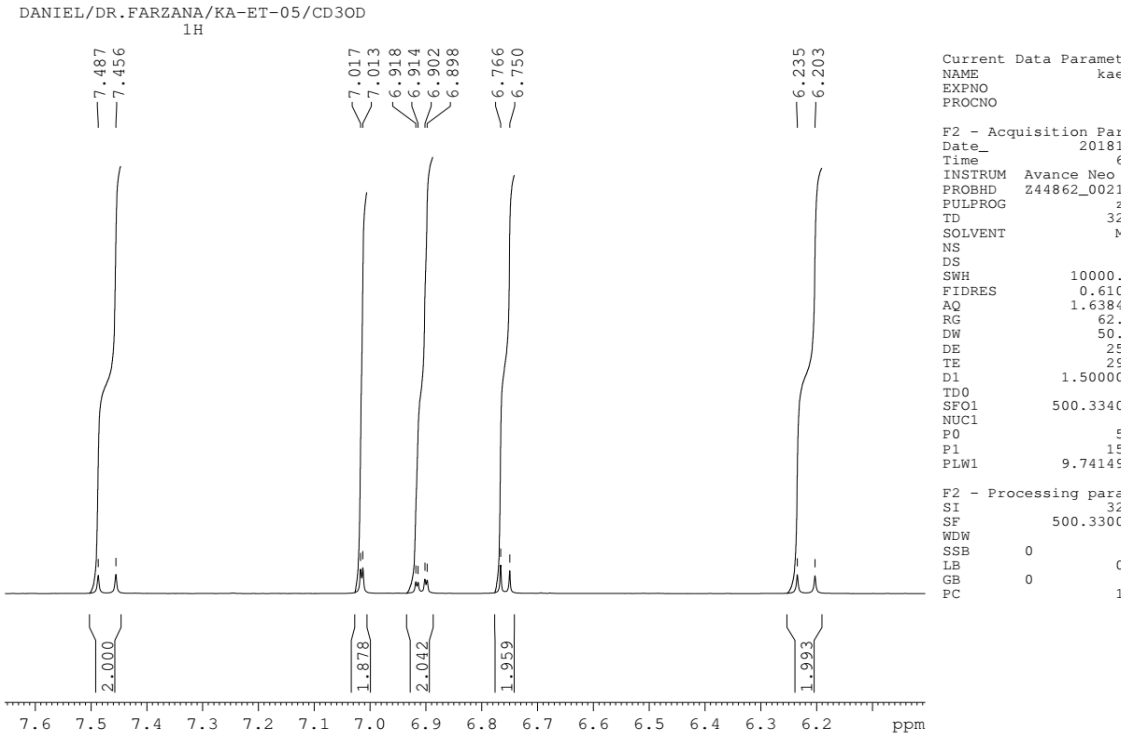


Appendix 52: Mass spectral of compound 6

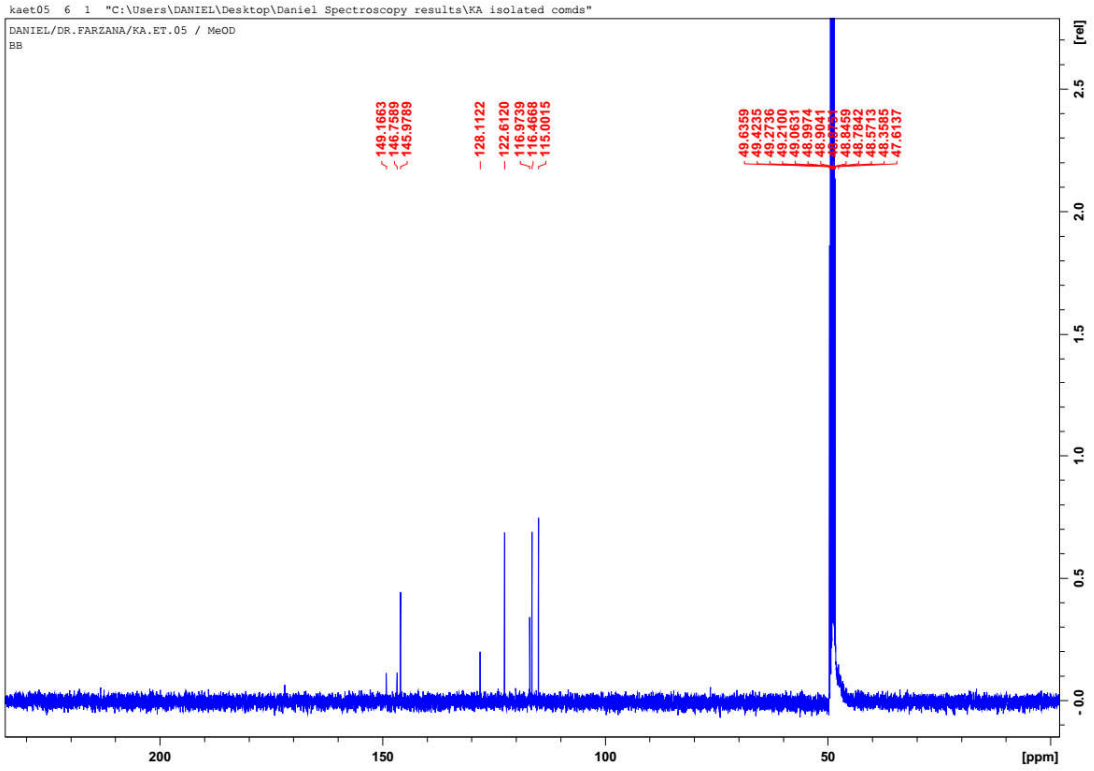


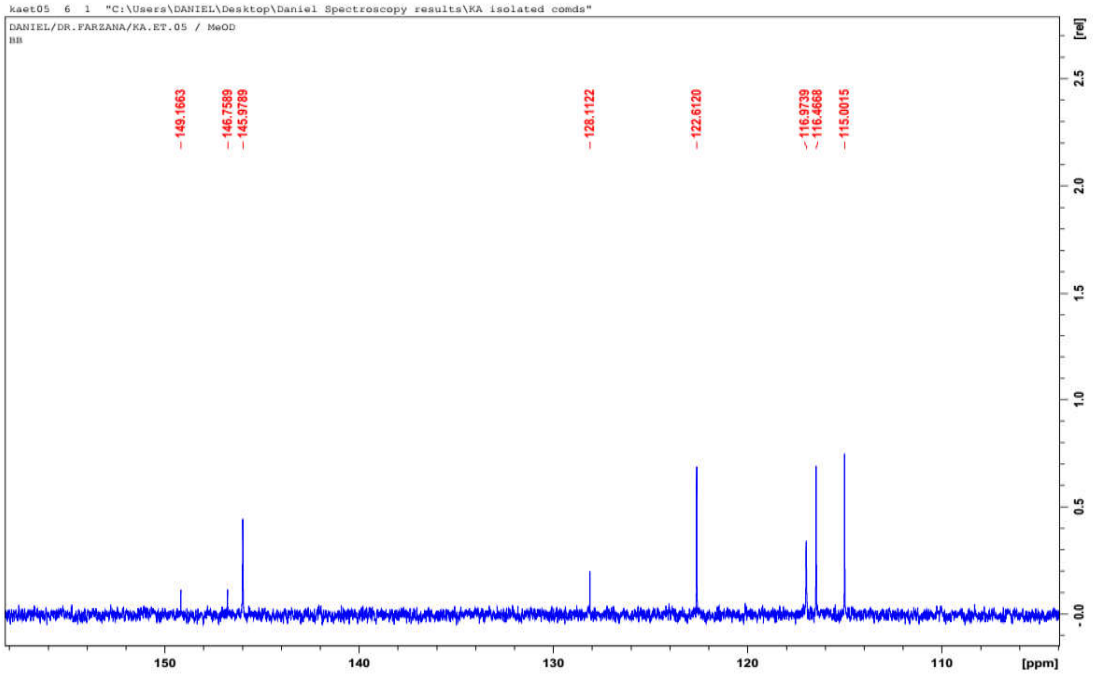
Appendix 53: ^{1}H NMR of compound 6



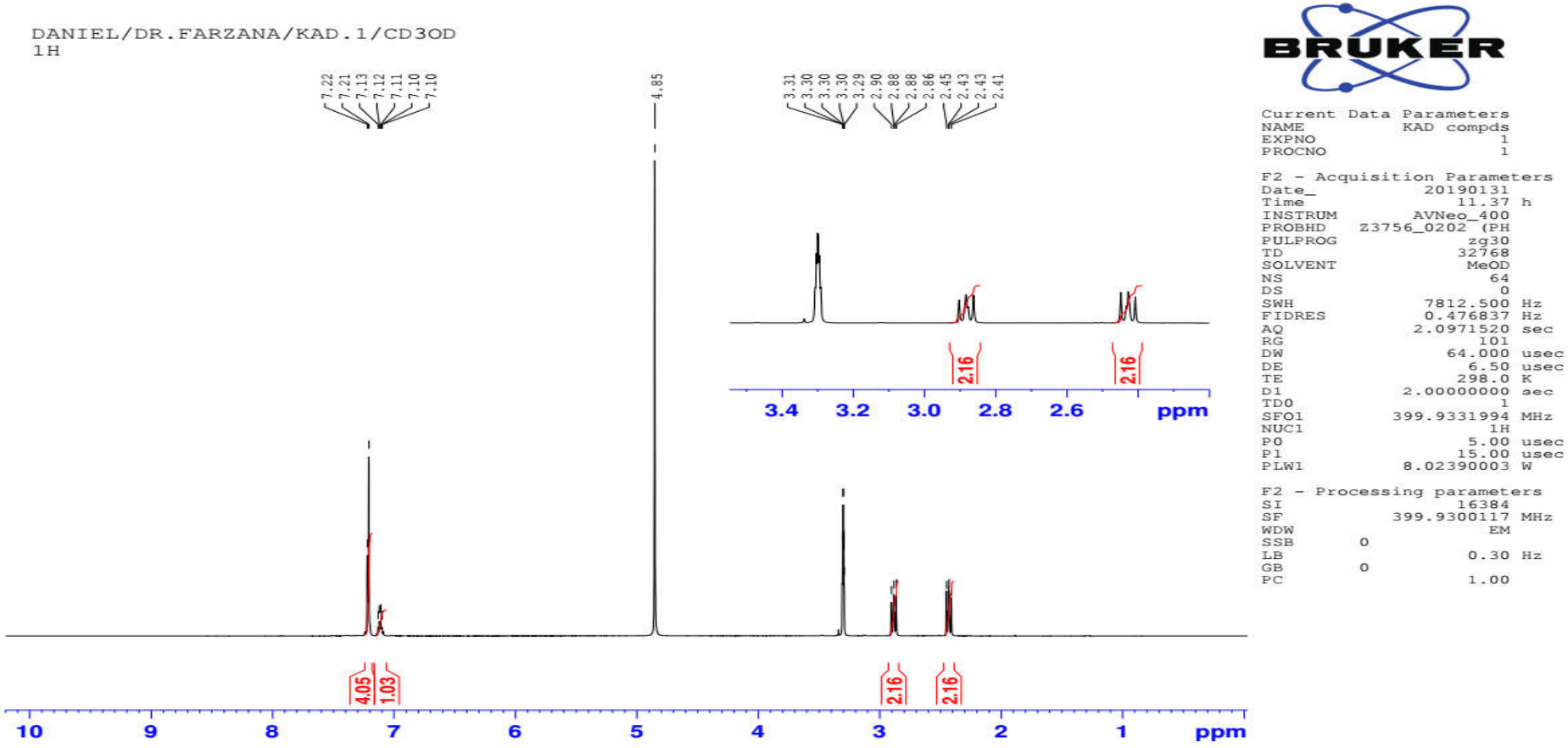


Appendix 54: ^{13}C NMR of compound 6

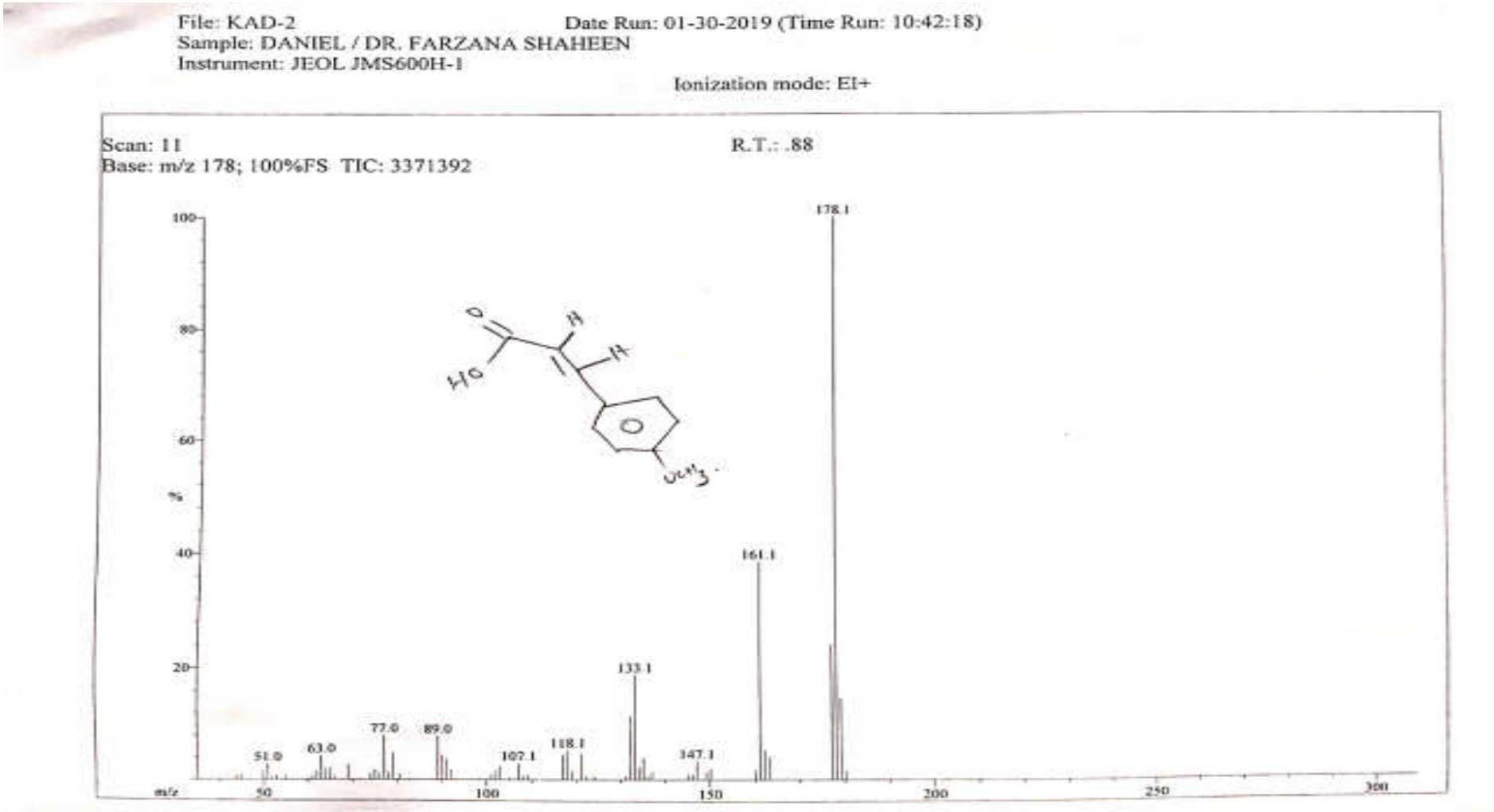




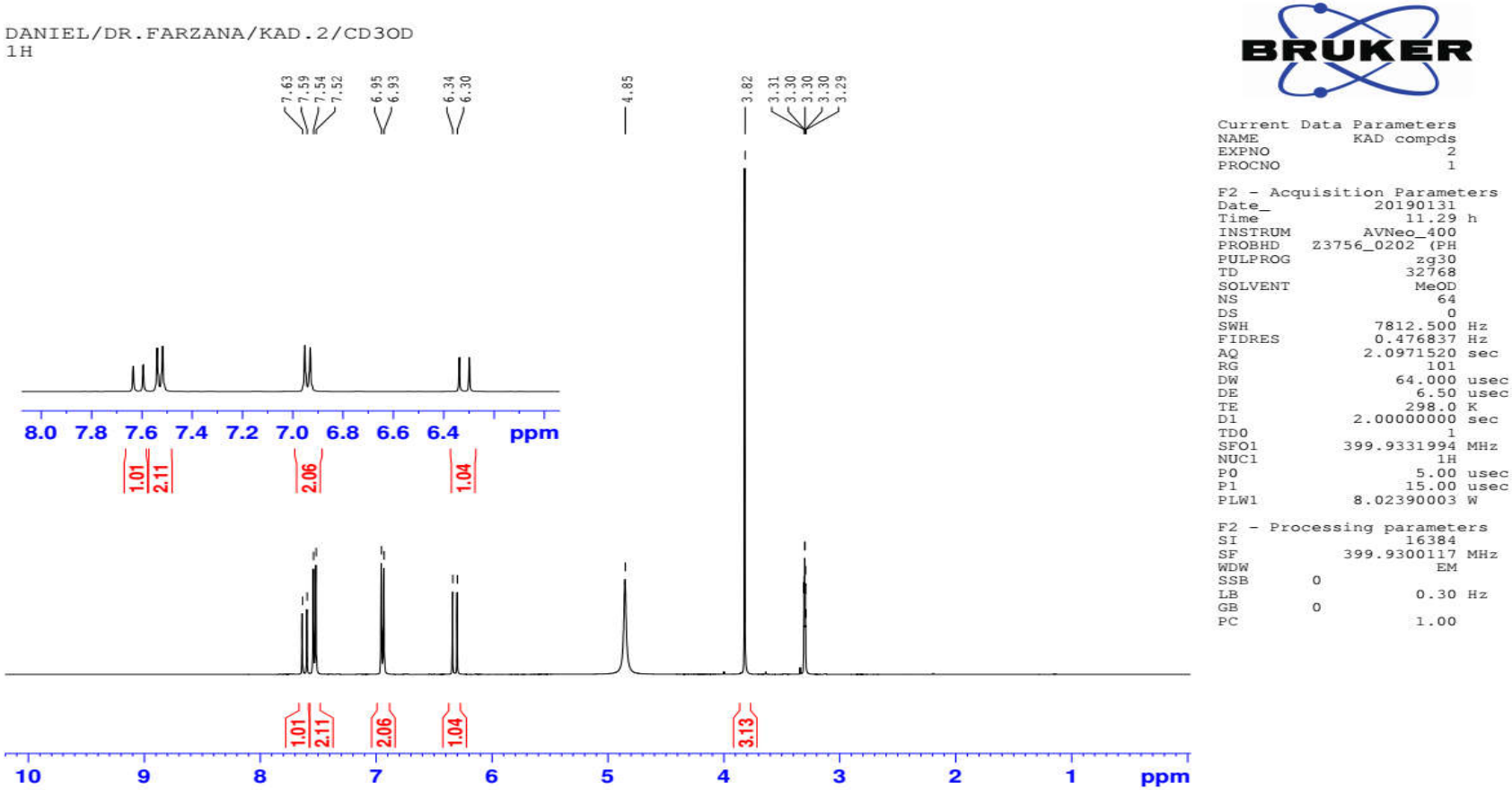
Appendix 55: ^{1}H NMR of KAD-l



Appendix 56: Mass spectral of KAD-2



Appendix 57: ^{1}H NMR of KAD-2

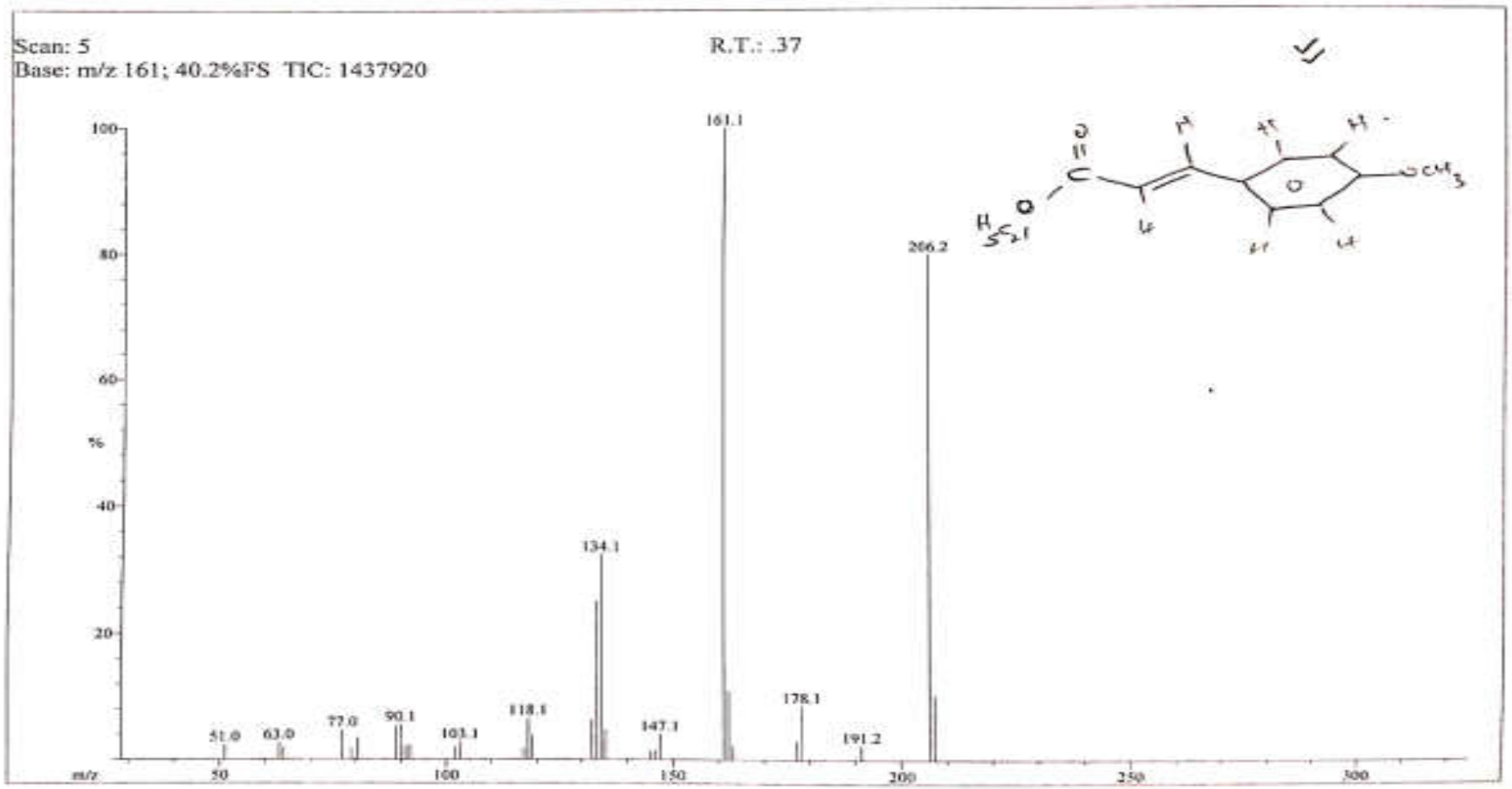


BRUKER

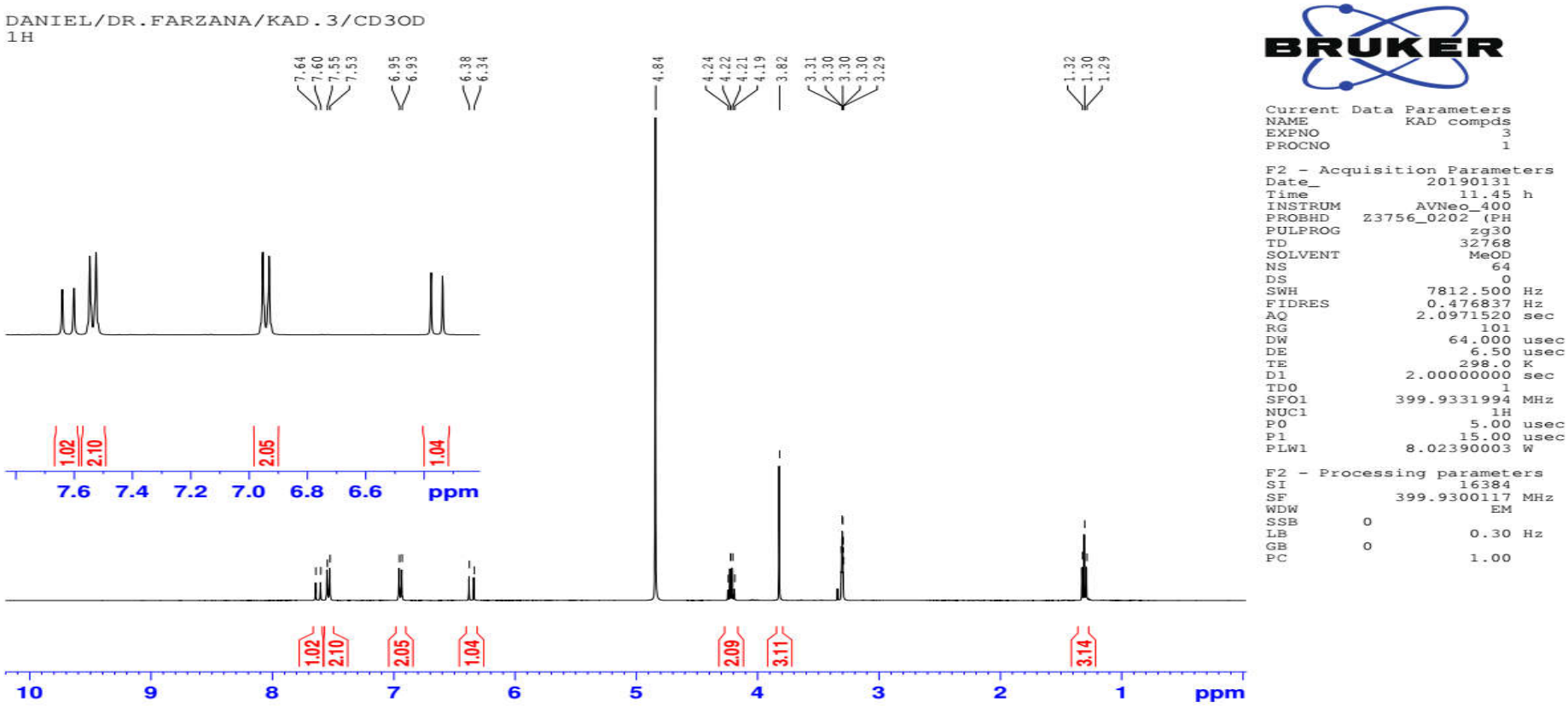
```
Current Data Parameters
NAME            KAD compds
EXPNO                    2
PROCNO                   1

F2 - Acquisition Parameters
Date_             20190131
Time                11.29 h
INSTRUM         AVNeo_400
PROBHD    Z3756_0202 (PH
PULPROG             zg30
TD                 32768
SOLVENT             MeOD
NS                    64
DS                     0
SWH             7812.500 Hz
FIDRES          0.476837 Hz
AQ              2.0971520 sec
RG                   101
DW                64.000 usec
DE                 6.50 usec
TE                298.0 K
D1             2.00000000 sec
TD0                    1
SFO1          399.9331994 MHz
NUC1                  1H
P0                 5.00 usec
P1                15.00 usec
PLW1           8.02390003 W

F2 - Processing parameters
SI                 16384
SF            399.9300117 MHz
WDW                   EM
SSB        0
LB                 0.30 Hz
GB         0
PC                 1.00
```

Appendix 58: Mass spectral of KAD-3

File: KAD-3 Date Run: 01-30-2019 (Time Run: 10:16:46)
Sample: DANIEL / DR. FARZANA SHAHEEN
Instrument: JEOL JMS600H-1
Ionization mode: EI+

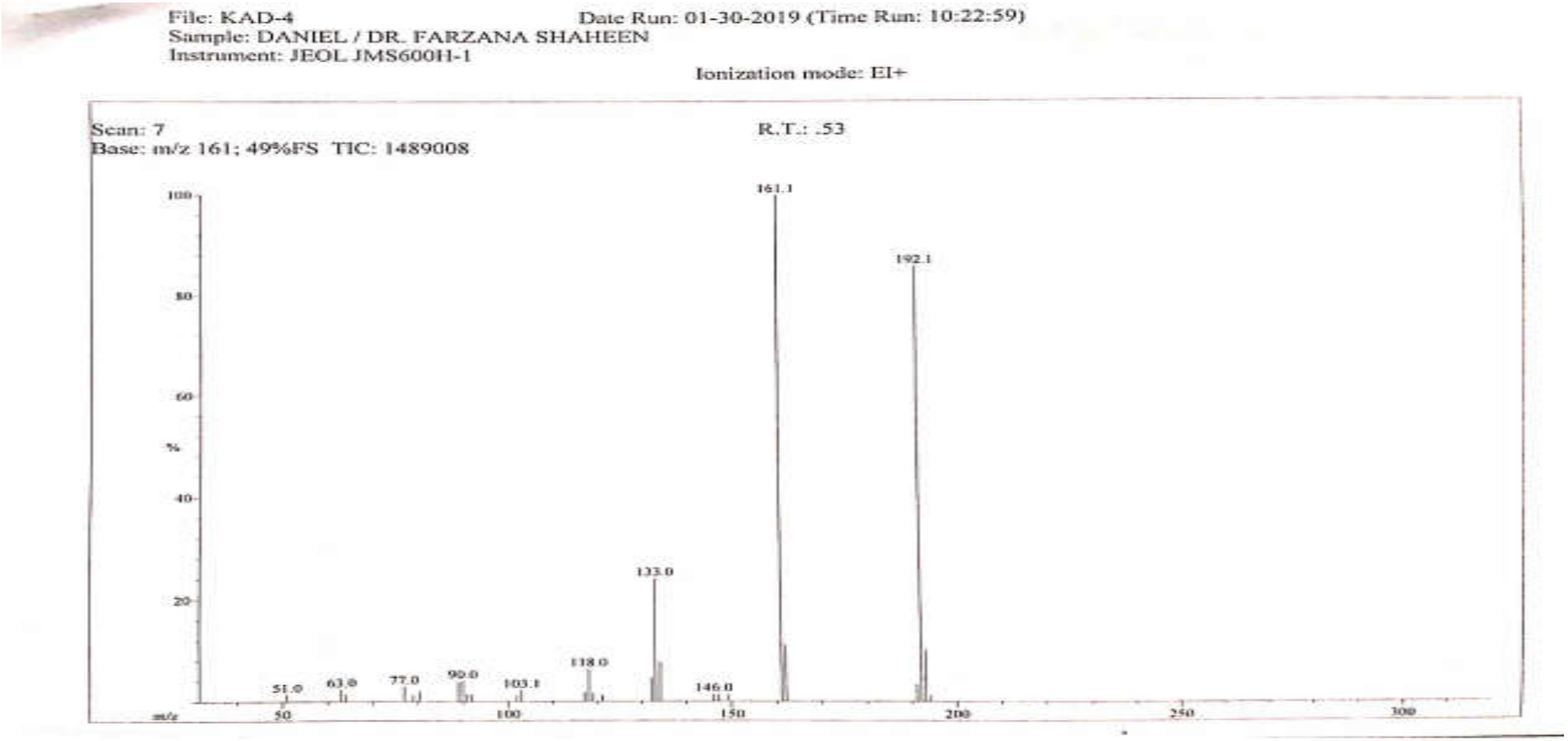


Appendix 59: ^{1}H NMR of KAD-3

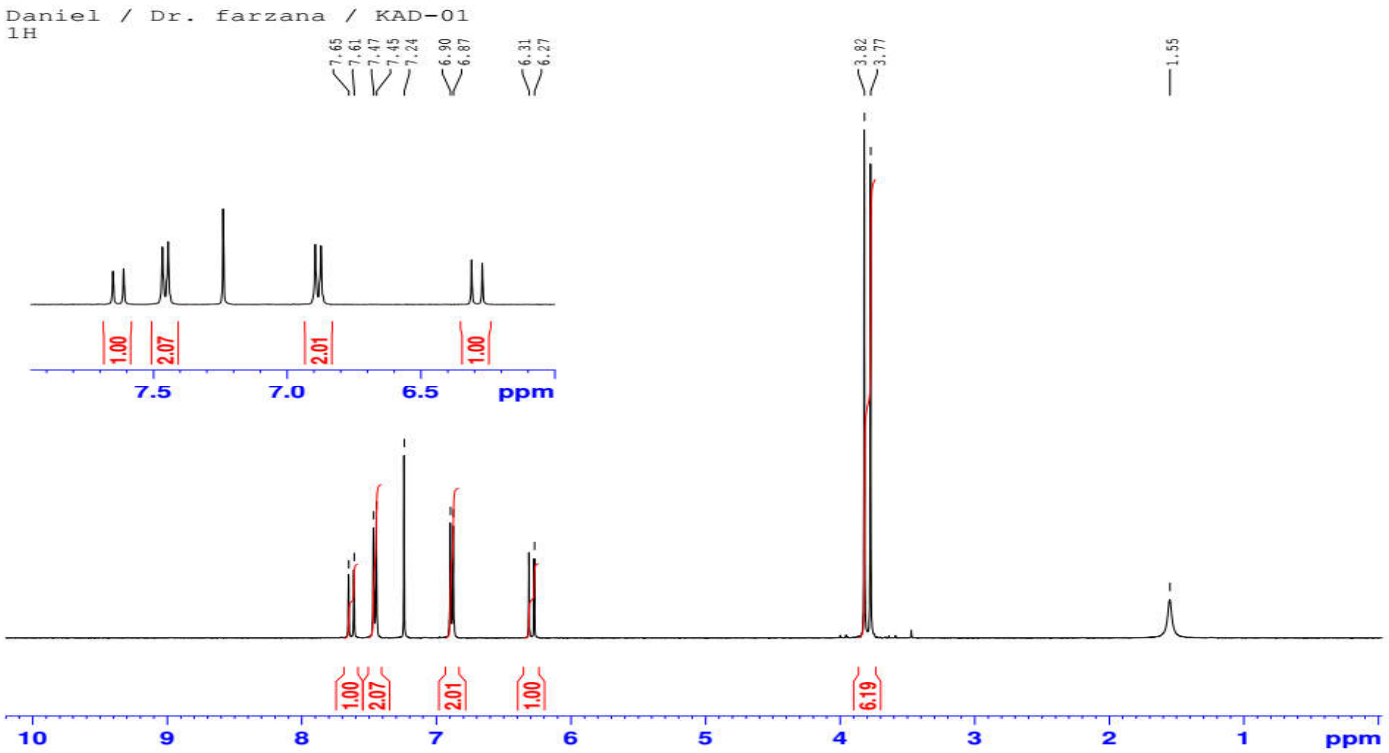



```
Current Data Parameters
NAME                  KAD compds
EXPNO                          3
PROCNO                         1

F2 - Acquisition Parameters
Date_                   20190131
Time                       11.45 h
INSTRUM                AVNeo_400
PROBHD    Z3756_0202 (PH
PULPROG                    zg30
TD                        32768
SOLVENT                    MeOD
NS                           64
DS                            0
SWH                    7812.500 Hz
FIDRES                 0.476837 Hz
AQ                     2.0971520 sec
RG                           101
DW                        64.000 usec
DE                         6.50 usec
TE                        298.0 K
D1                     2.00000000 sec
TD0                            1
SFO1                 399.9331994 MHz
NUC1                          1H
P0                         5.00 usec
P1                        15.00 usec
PLW1                   8.02390003 W

F2 - Processing parameters
SI                         16384
SF                   399.9300117 MHz
WDW                           EM
SSB                  0
LB                         0.30 Hz
GB                   0
PC                         1.00
```

Appendix 60: Mass spectral of KAD-4



Appendix 6l: ^{1}H NMR of KAD-4



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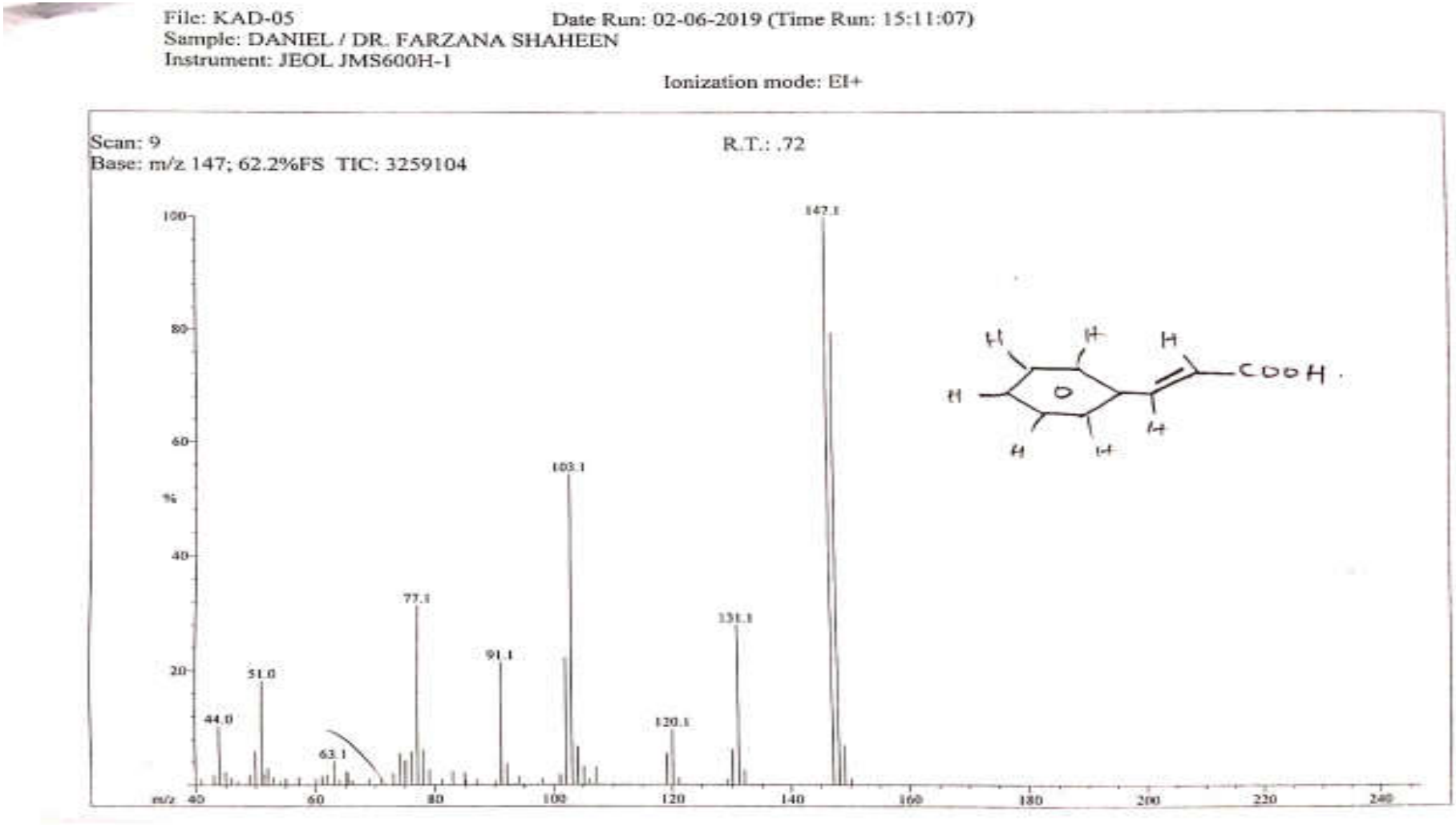
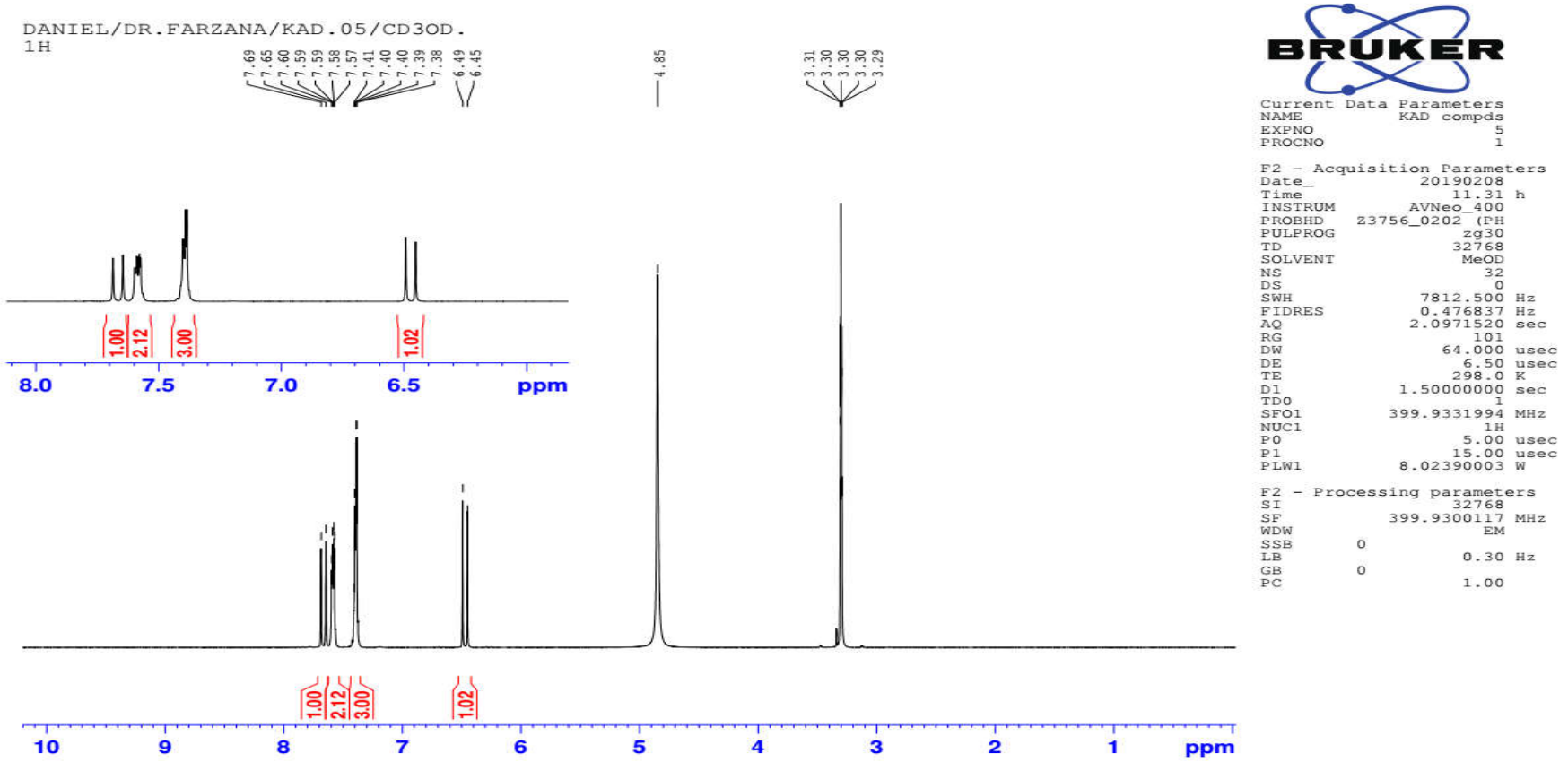
```
Current Data Parameters
NAME                  KAD compds
EXPNO                          4
PROCNO                         1

F2 - Acquisition Parameters
Date_                   20190201
Time                        8.03 h
INSTRUM                AVNeo_400
PROBHD    Z3756_0202 (PH
PULPROG                     zg30
TD                         32768
SOLVENT                    CDCl3
NS                             8
DS                             0
SWH                     7812.500 Hz
FIDRES                  0.476837 Hz
AQ                      2.0971520 sec
RG                           101
DW                        64.000 usec
DE                         6.50 usec
TE                        298.0 K
D1                     1.50000000 sec
TD0                            1
SFO1                  399.9331994 MHz
NUC1                          1H
P0                         5.00 usec
P1                        15.00 usec
PLW1                   8.02390003 W

F2 - Processing parameters
SI                         16384
SF                    399.9300178 MHz
WDW                           EM
SSB                 0
LB                         0.30 Hz
GB                  0
PC                         1.00
```

Appendix 62: Mass spectral of KAD-5



Appendix 63: ^{1}H NMR of KAD-5

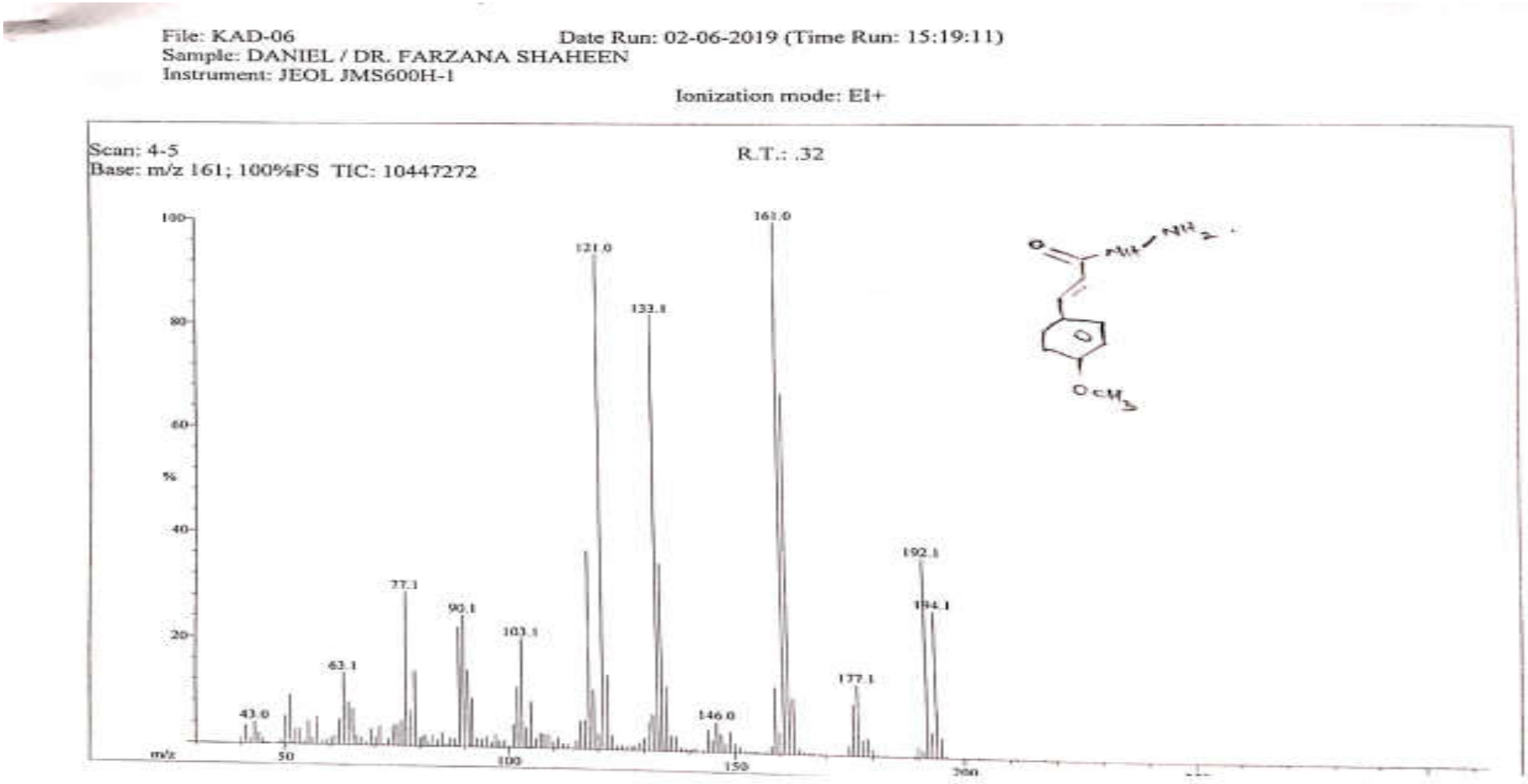


Current Data Parameters
NAME KAD compds
EXPNO 5
PROCNO 1

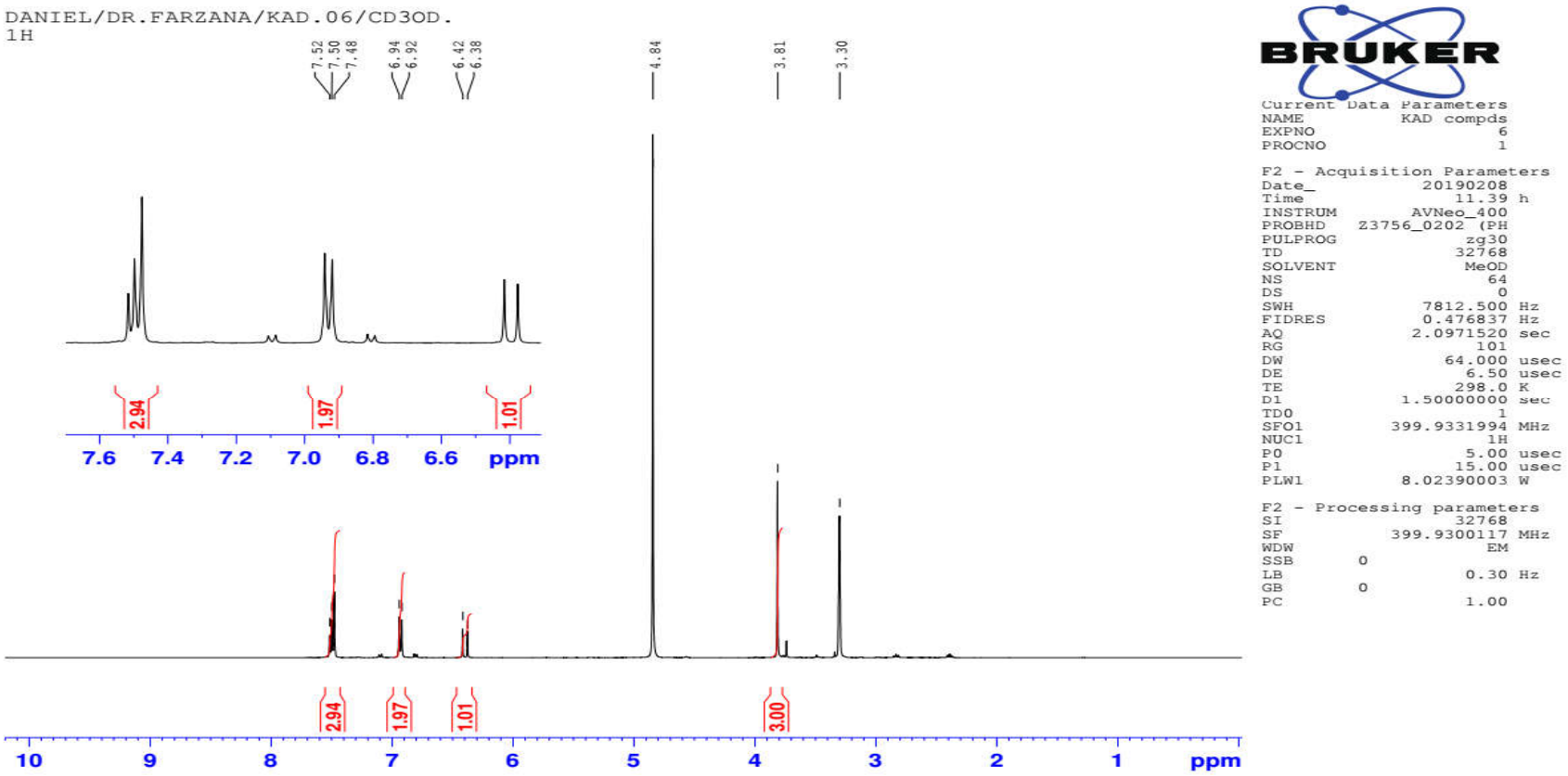
F2 - Acquisition Parameters
Date_ 20190208
Time 11.31 h
INSTRUM AVNeo_400
PROBHD Z3756_0202 (PH
PULPROG zg30
TD 32768
SOLVENT MeOD
NS 32
DS 0
SWH 7812.500 Hz
FIDRES 0.476837 Hz
AQ 2.0971520 sec
RG 101
DW 64.000 usec
DE 6.50 usec
TE 298.0 K
D1 1.50000000 sec
TD0 1
SFO1 399.9331994 MHz
NUC1 1H
P0 5.00 usec
P1 15.00 usec
PLW1 8.02390003 W

F2 - Processing parameters
SI 32768
SF 399.9300117 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

Appendix 64: Mass spectral of KAD-6



Appendix 65: ^{1}H NMR of KAD-6



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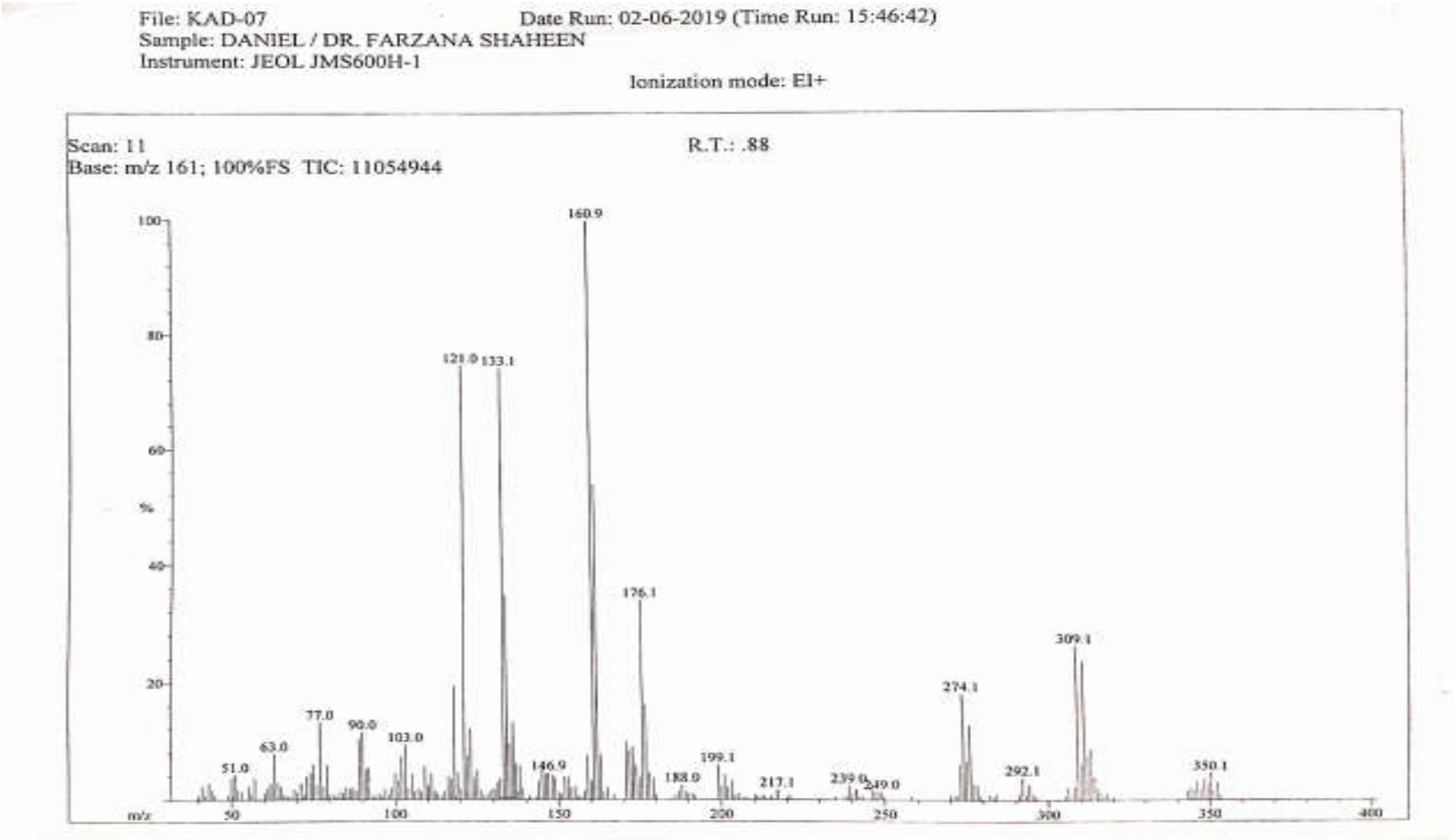
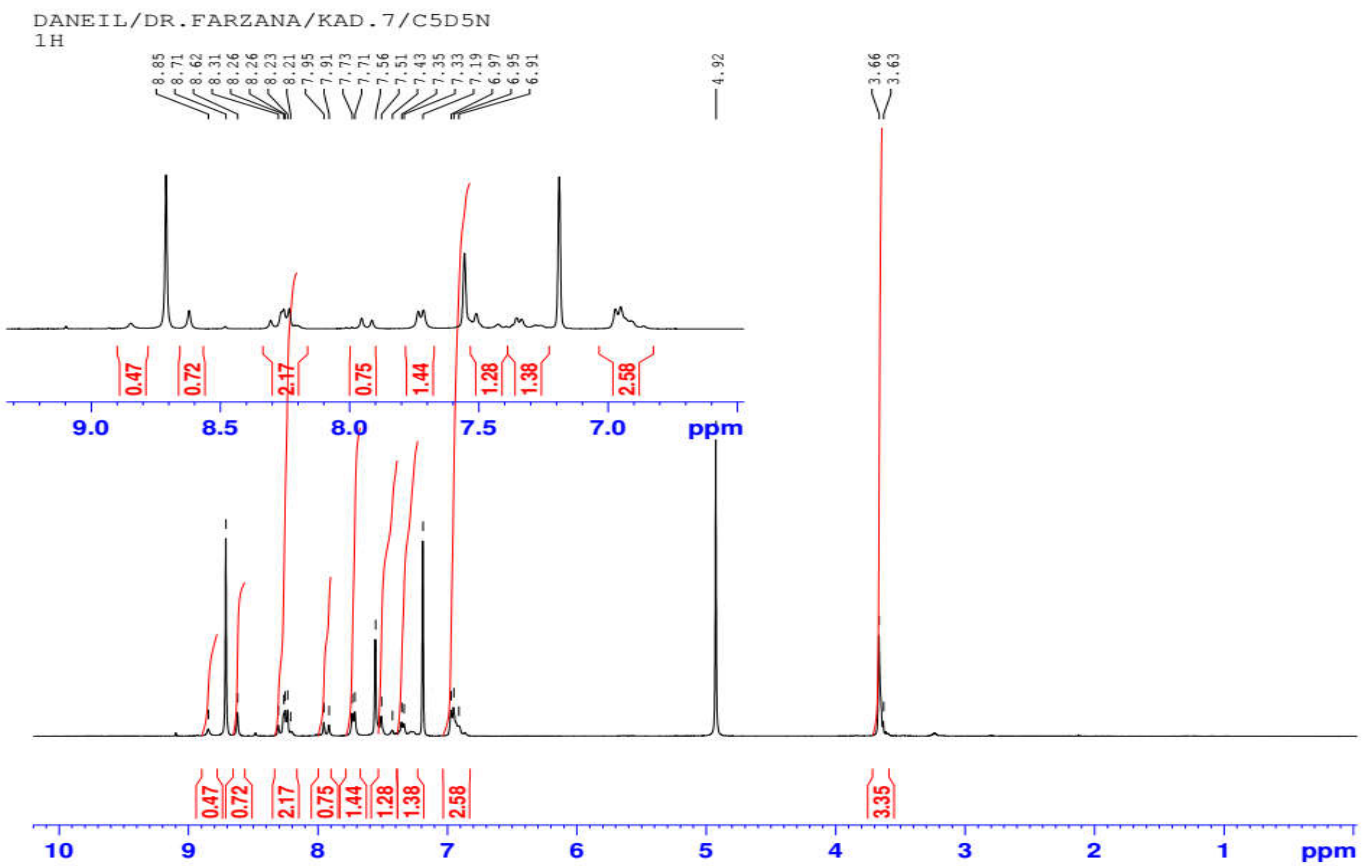
```
Current Data Parameters
NAME                  KAD compds
EXPNO                          6
PROCNO                         1

F2 - Acquisition Parameters
Date_                   20190208
Time                      11.39 h
INSTRUM                AVNeo_400
PROBHD    Z3756_0202 (PH
PULPROG                    zg30
TD                        32768
SOLVENT                    MeOD
NS                           64
DS                            0
SWH                    7812.500 Hz
FIDRES                 0.476837 Hz
AQ                     2.0971520 sec
RG                           101
DW                        64.000 usec
DE                         6.50 usec
TE                        298.0 K
D1                     1.50000000 sec
TD0                           1
SFO1                 399.9331994 MHz
NUC1                         1H
P0                         5.00 usec
P1                        15.00 usec
PLW1                   8.02390003 W

F2 - Processing parameters
SI                        32768
SF                   399.9300117 MHz
WDW                          EM
SSB                   0
LB                         0.30 Hz
GB                    0
PC                         1.00
```

Appendix 66: Mass spectral of KAD-7



Appendix 67: ^{1}H NMR of KAD-7



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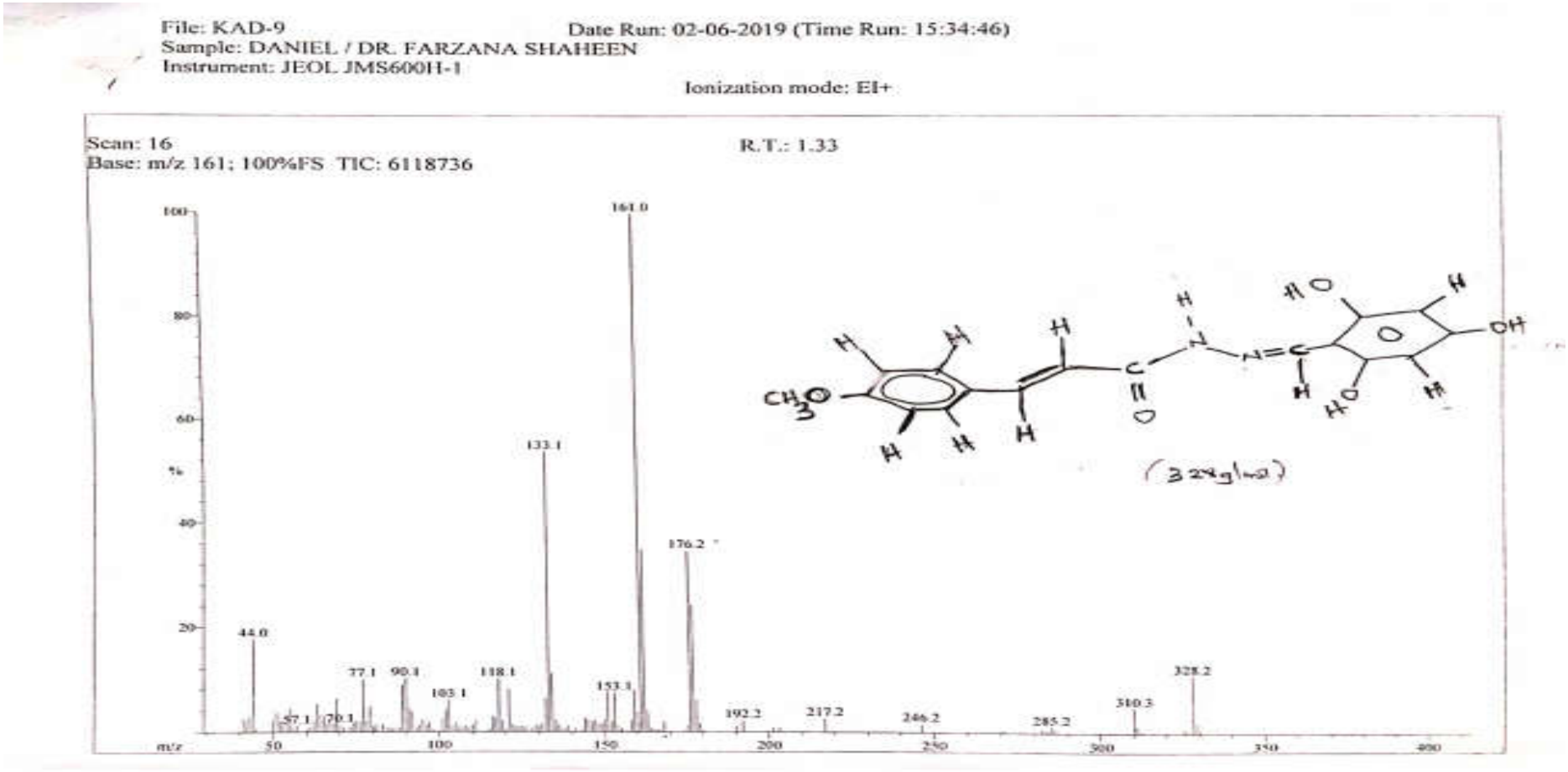
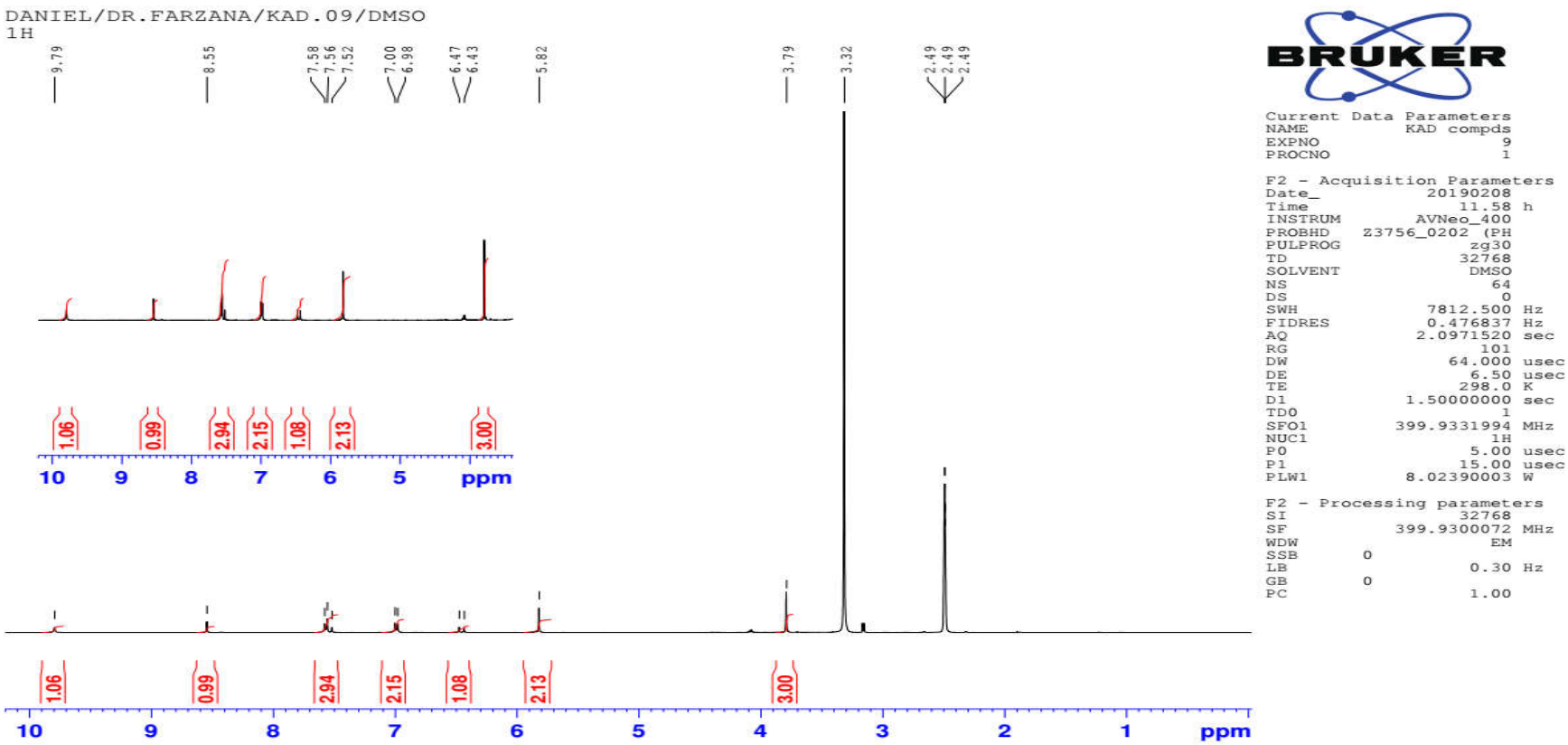
```
Current Data Parameters
NAME                  KAD compds
EXPNO                          7
PROCNO                         1

F2 - Acquisition Parameters
Date_                   20190212
Time                        8.29 h
INSTRUM                AVNeo_400
PROBHD    Z3756_0202 (PH
PULPROG                    zg30
TD                        32768
SOLVENT                     Pyr
NS                          128
DS                            0
SWH                    7812.500 Hz
FIDRES                 0.476837 Hz
AQ                     2.0971520 sec
RG                          101
DW                       64.000 usec
DE                        6.50 usec
TE                       298.0 K
D1                     1.50000000 sec
TD0                            1
SFO1                399.9331994 MHz
NUC1                         1H
P0                        5.00 usec
P1                       15.00 usec
PLW1                8.02390003 W

F2 - Processing parameters
SI                        32768
SF                  399.9306022 MHz
WDW                          EM
SSB                 0
LB                        0.30 Hz
GB                  0
PC                        1.00
```

Appendix 68: Mass spectral of KAD-9



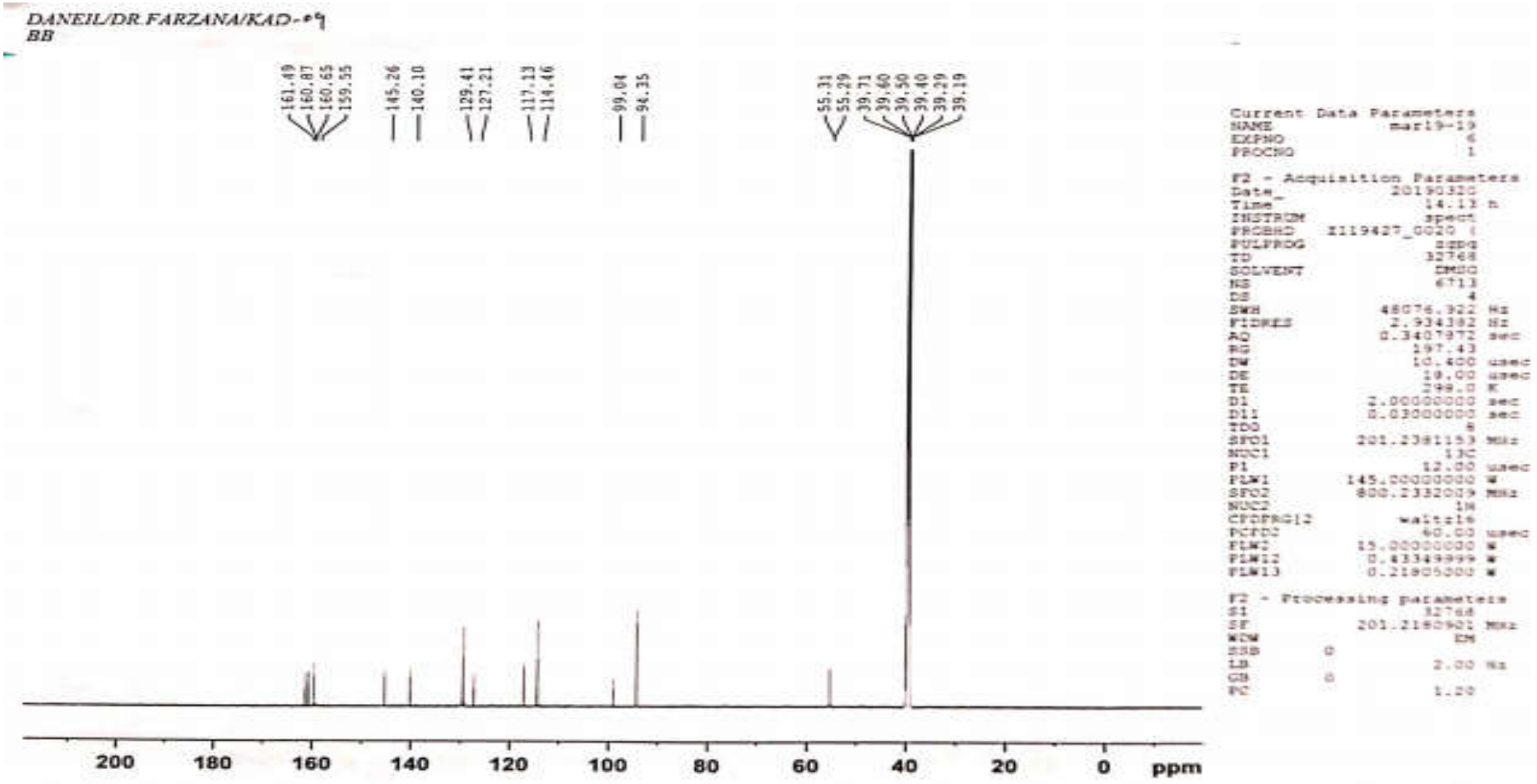
Appendix 69: ^{1}H NMR of KAD-9



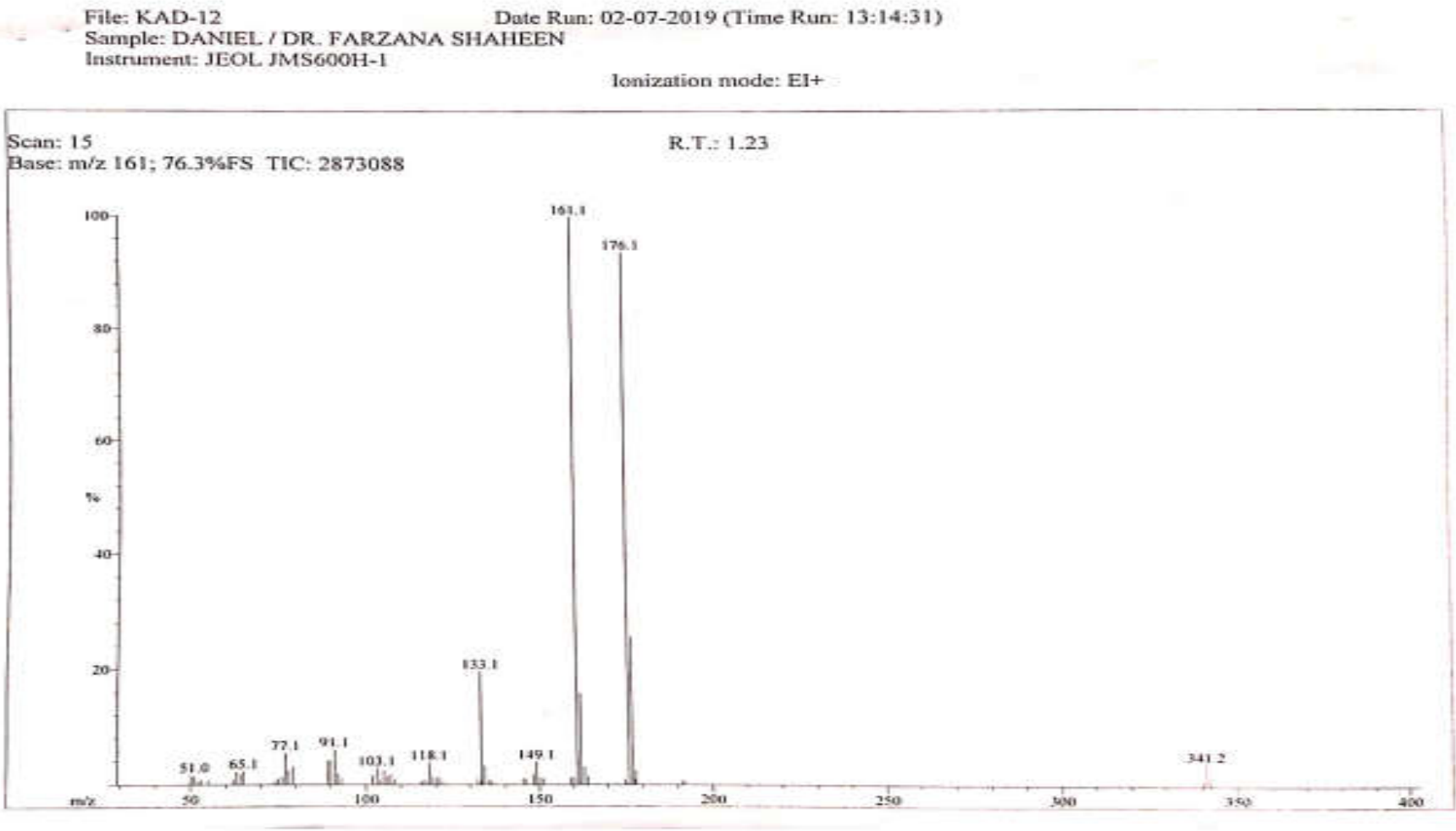
BRUKER

```
Current Data Parameters
NAME                  KAD compds
EXPNO                          9
PROCNO                         1

F2 - Acquisition Parameters
Date_                   20190208
Time                       11.58 h
INSTRUM                AVNeo_400
PROBHD    Z3756_0202 (PH
PULPROG                     zg30
TD                         32768
SOLVENT                     DMSO
NS                             64
DS                              0
SWH                     7812.500 Hz
FIDRES                  0.476837 Hz
AQ                      2.0971520 sec
RG                            101
DW                         64.000 usec
DE                          6.50 usec
TE                         298.0 K
D1                    1.50000000 sec
TD0                             1
SFO1                  399.9331994 MHz
NUC1                           1H
P0                          5.00 usec
P1                         15.00 usec
PLW1                  8.02390003 W

F2 - Processing parameters
SI                         32768
SF                    399.9300072 MHz
WDW                            EM
SSB                 0
LB                          0.30 Hz
GB                  0
PC                          1.00
```

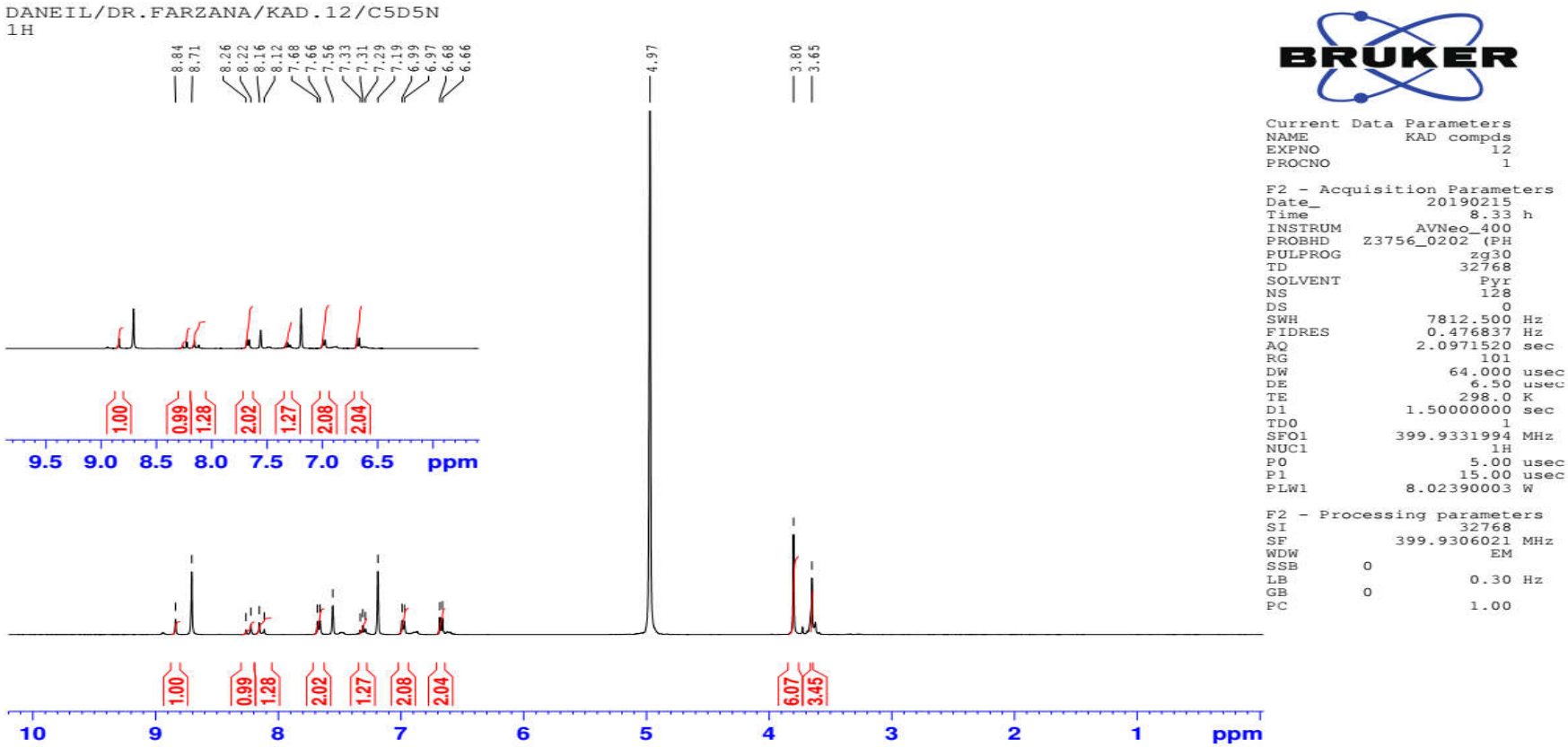
Appendix 70: ^{13}C NMR of KAD-9



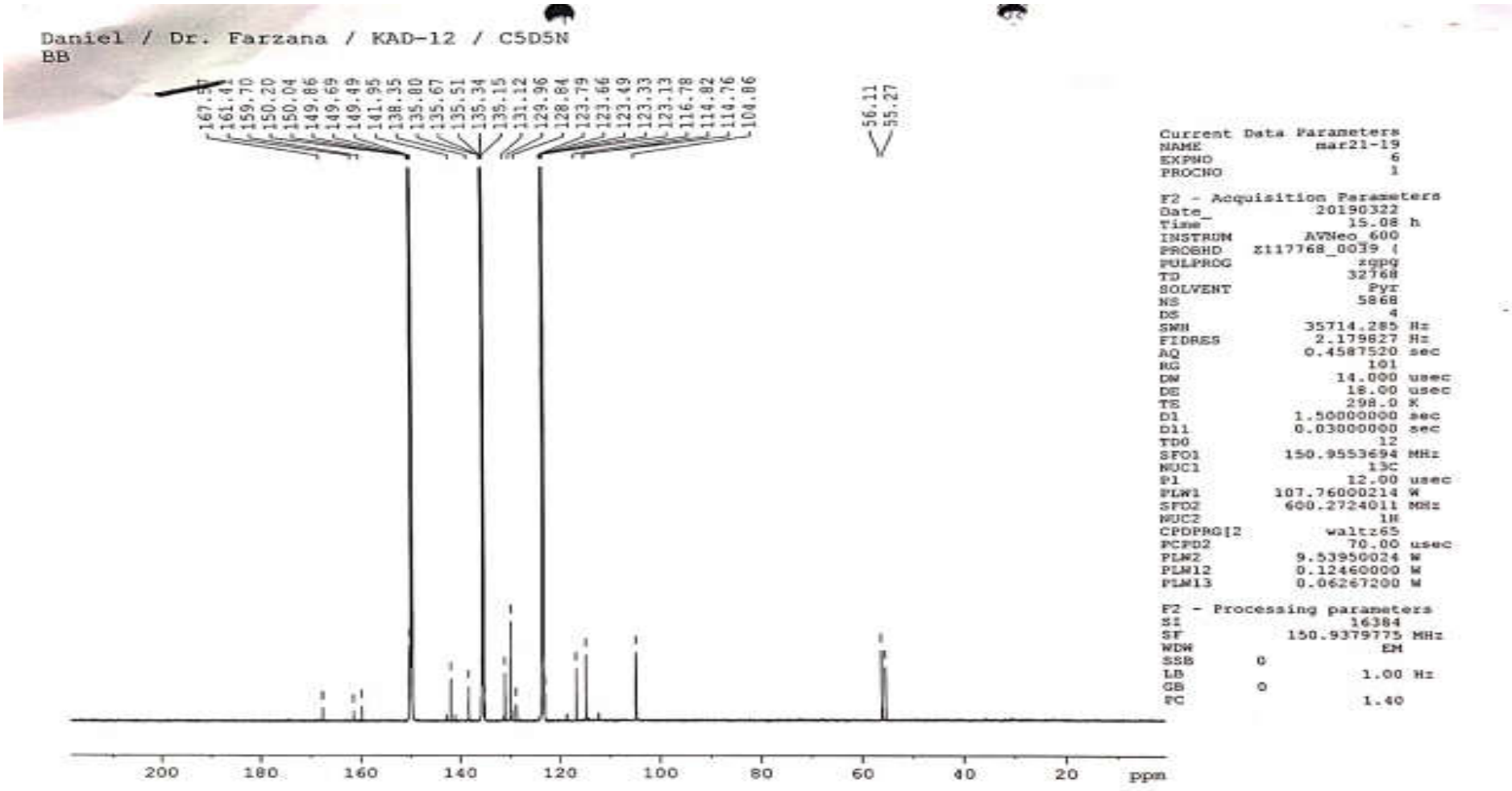
Appendix 7l: Mass spectral of KAD-l2



Appendix 72: ^{1}H NMR of KAD-l2

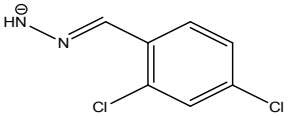
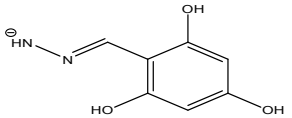
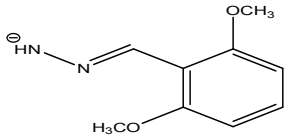


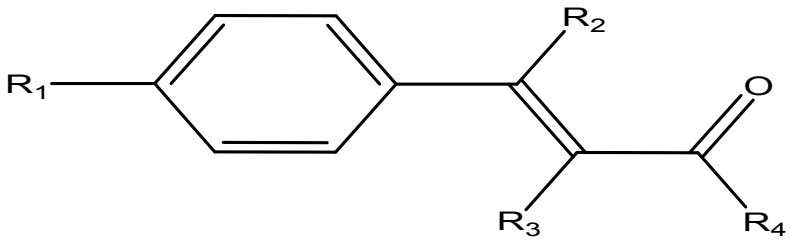
Appendix 73: ^{13}C NMR of KAD-l2



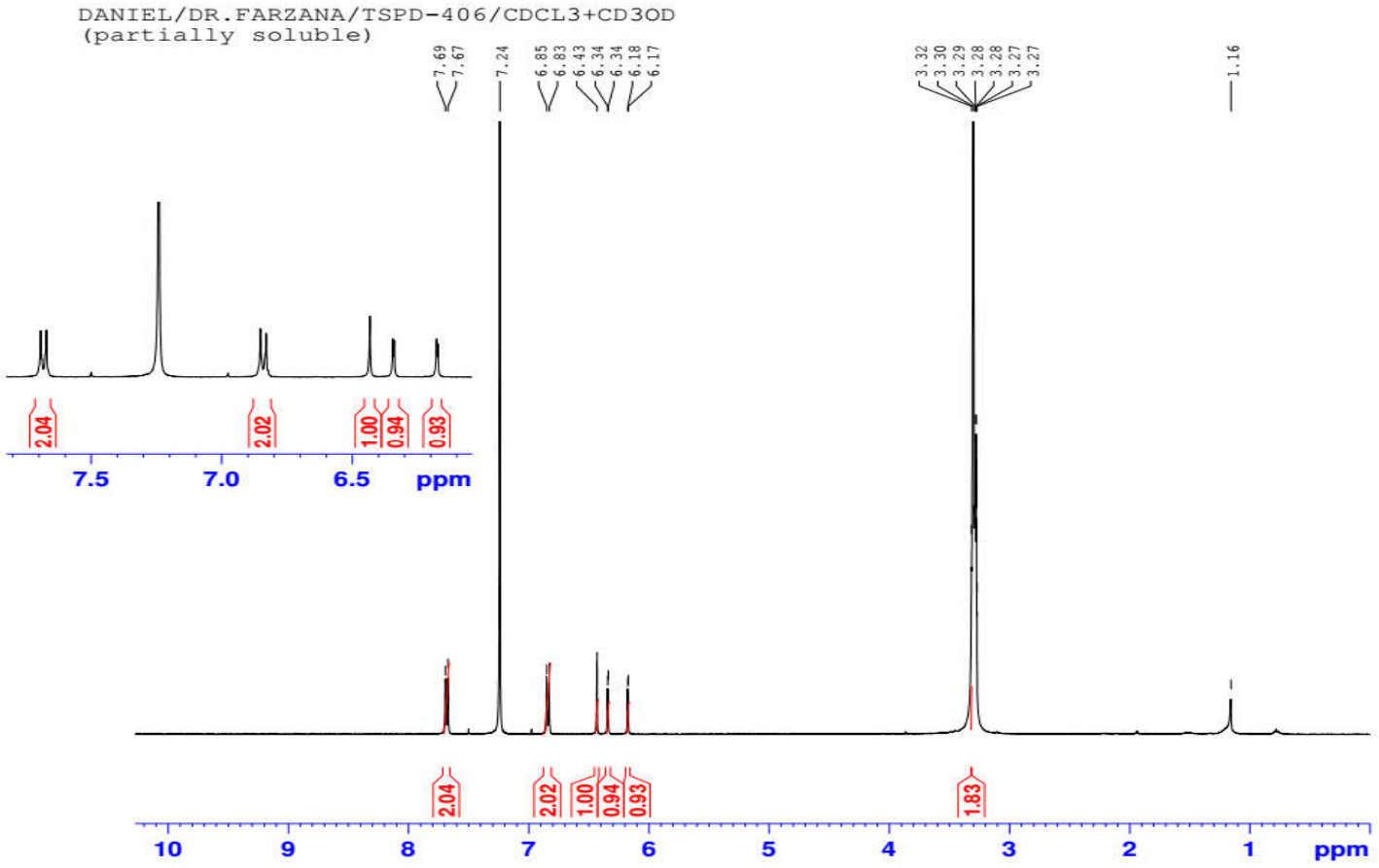

```
Current Data Parameters
NAME                  mar21-19
EXPNO                        6
PROCNO                       1

F2 - Acquisition Parameters
Date_                 20190322
Time                     15.08 h
INSTRUM                AVNeo_600
PROBHD    Z117768_0039 (
PULPROG                   zgpg
TD                       32768
SOLVENT                    Pyr
NS                          5868
DS                             4
SWH                   35714.285 Hz
FIDRES                 2.179827 Hz
AQ                     0.4587520 sec
RG                           101
DW                        14.000 usec
DE                        18.00 usec
TE                        298.0 K
D1                    1.50000000 sec
D11                   0.03000000 sec
TD0                            12
SFO1                150.9553694 MHz
NUC1                         13C
P1                        12.00 usec
PLW1                107.76000214 W
SFO2                600.2724011 MHz
NUC2                         1H
CPDPRG[2                waltz65
PCPD2                     70.00 usec
PLW2                 9.53950024 W
PLW12                0.12460000 W
PLW13                0.06267200 W

F2 - Processing parameters
SI                         16384
SF                  150.9379775 MHz
WDW                           EM
SSB                  0
LB                         1.00 Hz
GB                   0
PC                         1.40
```

Appendix 74: Structure-Anti-proliferative activity Relationships (SARs) for cinnamic acid and its analogues

Compounds					IC_{50} (µg/mL)	
	R_1	R_2	R_3	R_4	CHO l cell line	HeLa cell line
KAD-l	-H	$-CH_2$	$-CH_2$	-ONa	>l00	45.4 ± 2.2
KAD-2	$-OCH_3$	-H	-H	-OH	>l00	>l00
KAD-3	$-OCH_3$	-H	-H	$-OCH_2CH_3$	>l00	26.l ± 5.2
KAD-4	$-OCH_3$	-H	-H	$-OCH_3$	>l00	>l00
KAD-5	-H	-H	-H	-OH	>l00	>l00
KAD-6	$-OCH_3$	-H	-H	$-NHNH_2$	50.7 ± 0.9	37.5 ± 8.0
KAD-7	$-OCH_3$	-H	-H		4.2 ± 0.6	2l.4 ± 2.l
KAD-9	$-OCH_3$	-H	-H		3l.5 ± 3.l	>l00
KAD-l2	$-OCH_3$	-H	-H		l8.4 ± 4.l	22.4 ± l.5
Doxorubicin (Standard)					0.8 ± 0.0l	3.l ± 0.2



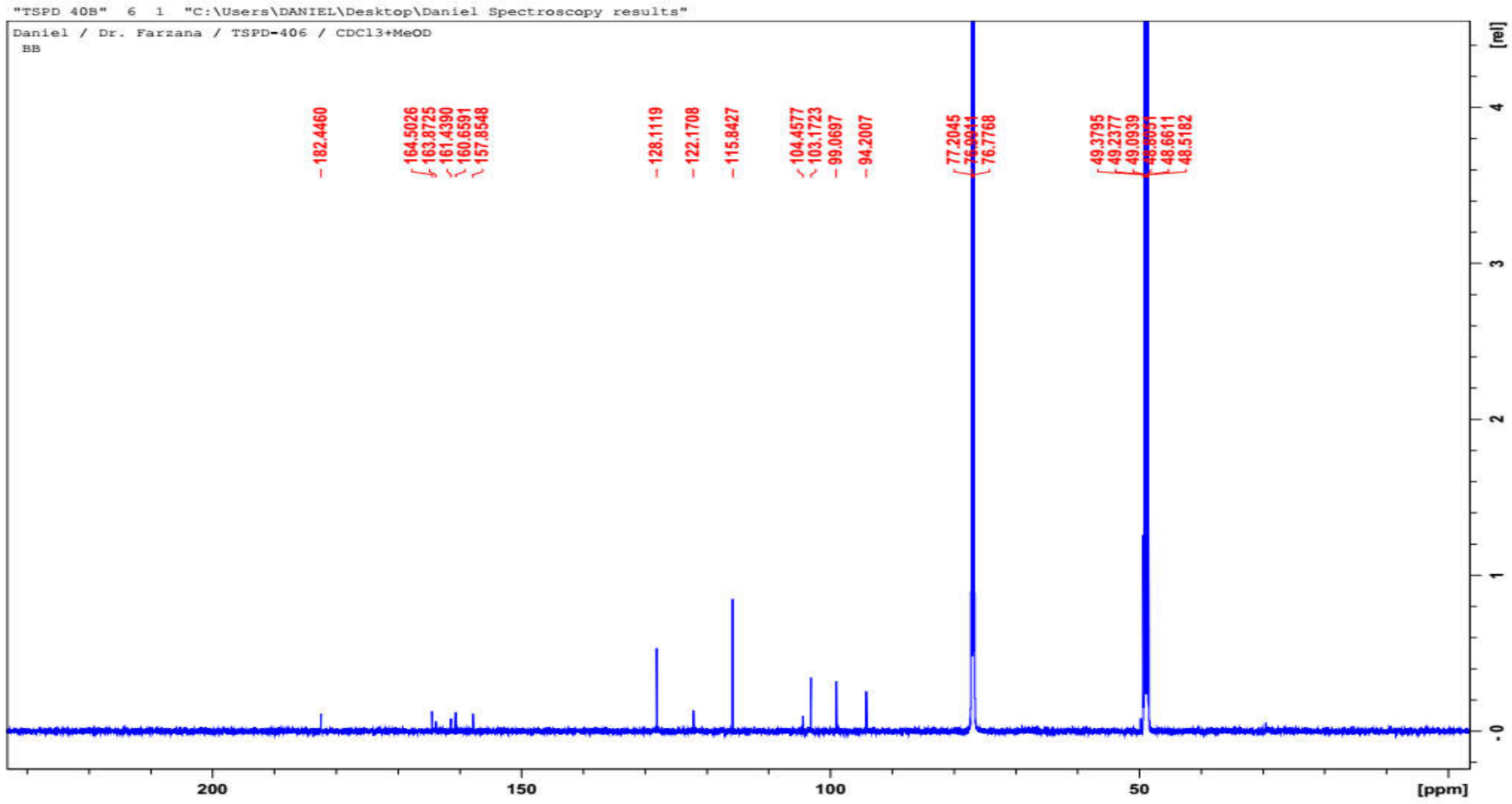
Appendix 75: ^{1}H NMR of compound 7



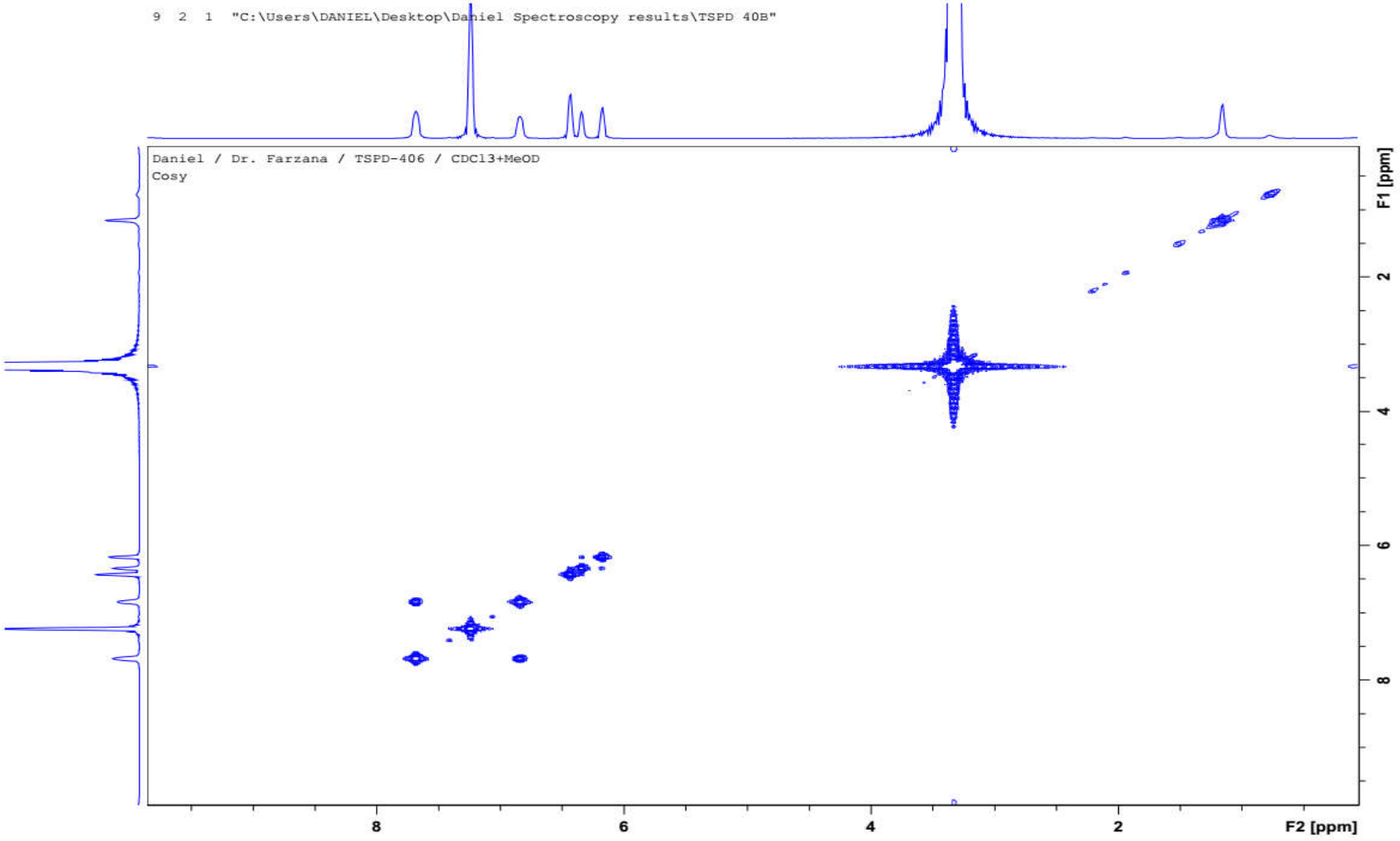
BRUKER

```
Current Data Parameters
NAME                  TSPD 40B
EXPNO                       2
PROCNO                      1

F2 - Acquisition Parameters
Date_               20190108
Time                    7.45 h
INSTRUM   Avance NEO 400MHz
PROBHD    Z114854_0013 (
PULPROG                 zg30
TD                     32768
SOLVENT                CDCl3
NS                       128
DS                         0
SWH                 8196.722 Hz
FIDRES              0.500288 Hz
AQ                  1.9988480 sec
RG                       101
DW                    61.000 usec
DE                    12.86 usec
TE                    300.0 K
D1                  1.50000000 sec
TD0                        1
SFO1             400.1332010 MHz
NUC1                      1H
P0                     4.67 usec
P1                    14.00 usec
PLW1            13.21300030 W

F2 - Processing parameters
SI                     32768
SF               400.1300170 MHz
WDW                       EM
SSB          0
LB                     0.30 Hz
GB           0
PC                     1.00
```

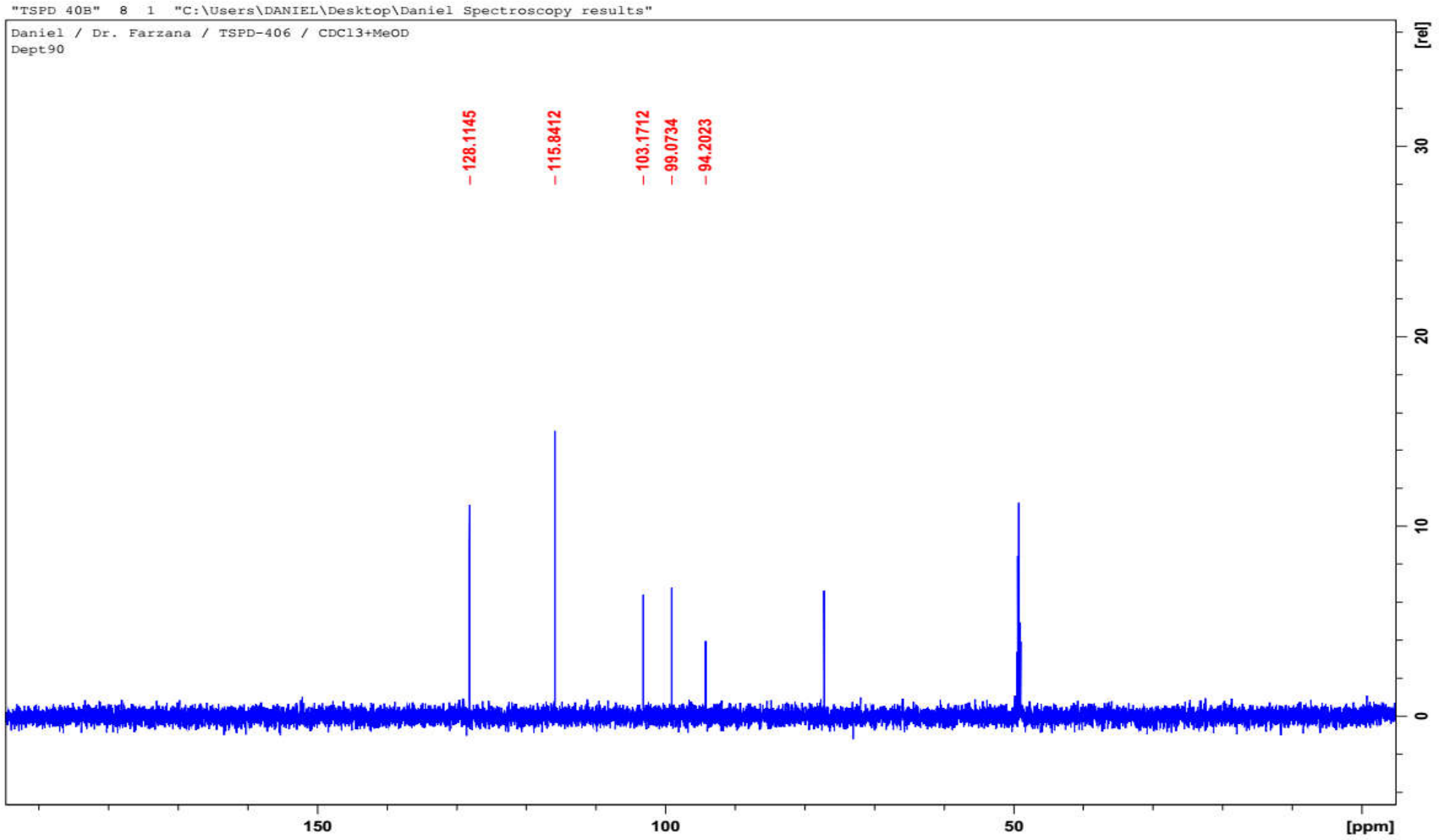
Appendix 76: ^{13}C NMR of compound 7



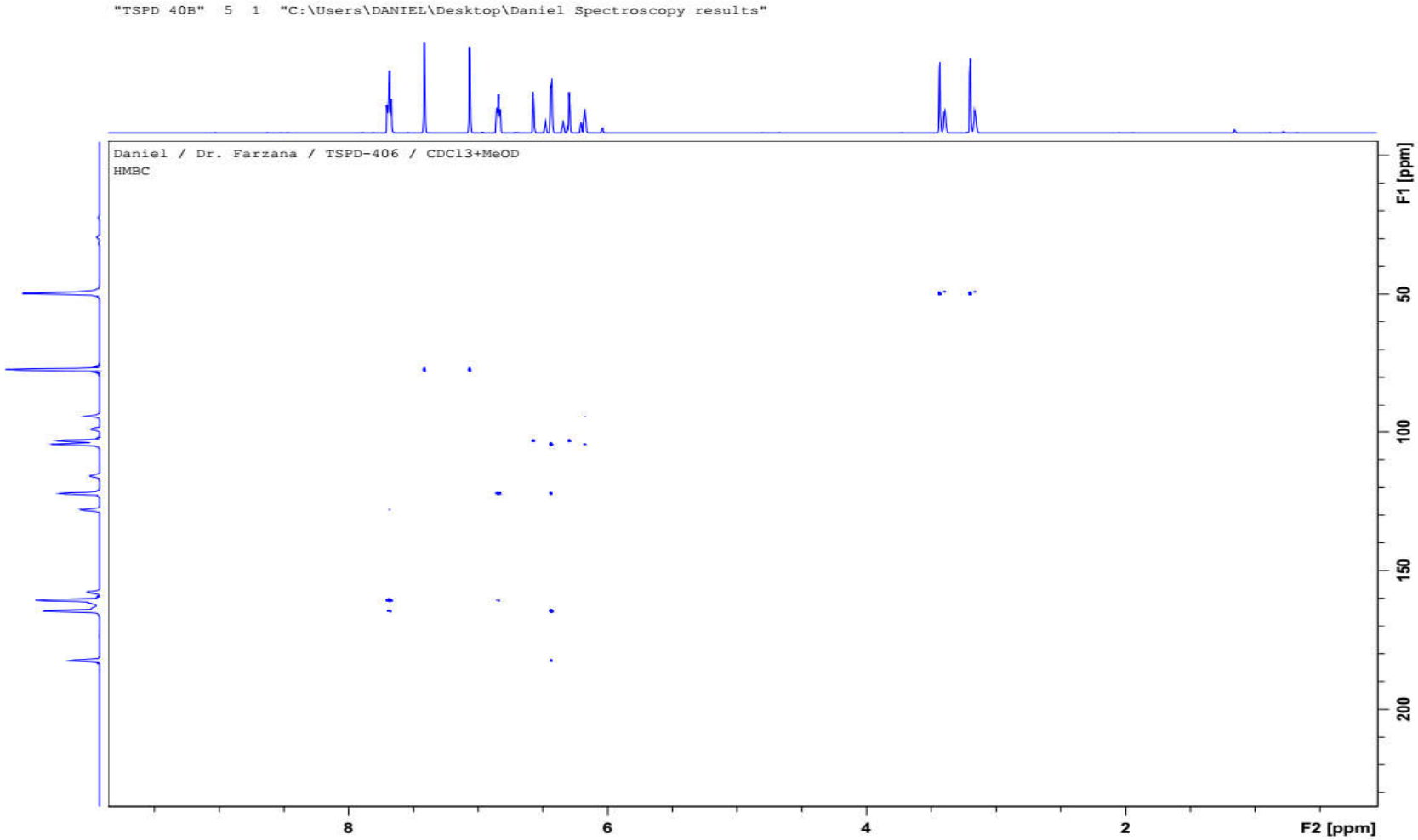
Appendix 77: COSY of compound 7



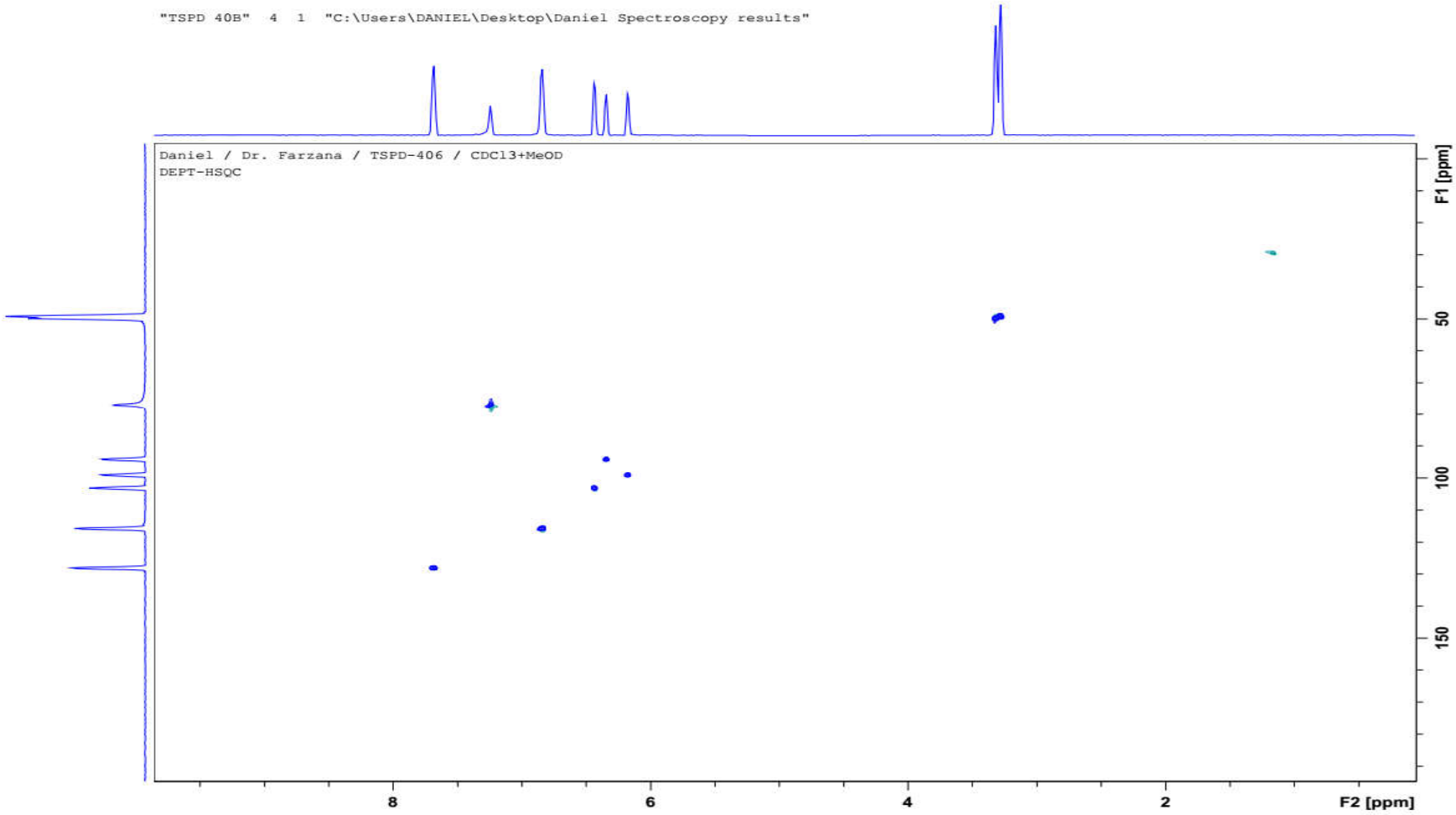
Appendix 78: DEPT 90 of compound 7



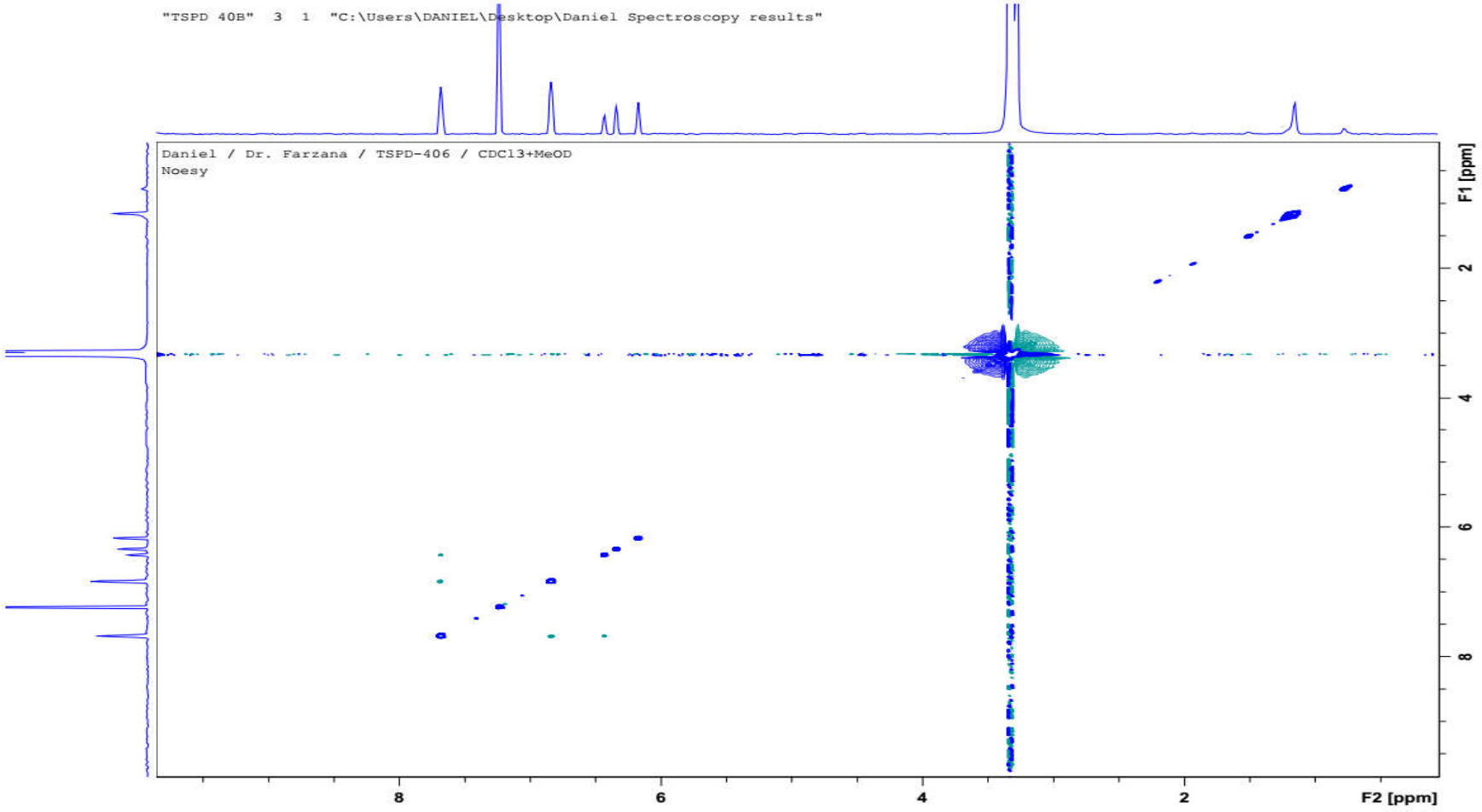
Appendix 79: HMBC of compound 7



Appendix 80: HSQC of compound 7



Appendix 81: NOESY of compound 7



Appendix 82: Ethical approval certificate obtained for *in vivo* PCOS study

UNIVERSITY OF IBADAN
ANIMAL CARE AND USE RESEARCH ETHICS COMMITTEE
(UI-ACUREC)
2nd Floor, Room 311, Faculty of Veterinary Medicine Building,
University of Ibadan, Nigeria. 0817 691 7269
E-mail: animaluseresearch@yahoo.com/animaluseresearch@gmail.com

Our Ref:..... Date: 19-12-2019

Assigned Number: UI-ACUREC/19/0051

NOTICE OF FULL APPROVAL AFTER FULL COMMITTEE REVIEW

Re: Isolation of Bioactive Compounds from Nigerian Medical Plants for the Remedial Treatment of Letrozole-Induced Polycystic Ovarian Syndrome

Name and Address of Principal Investigator: Ogunlakin Akingbolabo Daniel
Department of Pharmacognosy
Faculty of Pharmacy
University of Ibadan, Ibadan

Date of receipt of valid application: 3/5/2019

Date of meeting when final determination on ethical approval was made: 21/11/2019

This is to inform you that the research described in the submitted protocol, have been received and *given full approval by the* UI-ACUREC. **Please, note that the Committee has approved only the animal component of the study.**

This approval dates from **21/11/2019 to 20/11/2020.** If there is delay in starting the research, please inform UI-ACUREC so that the dates of approval can be adjusted accordingly.

Note that no activity related to this research may be conducted outside of these dates. It is expected that you submit your annual report as well as an annual request for the project renewal to the UI-ACUREC at least four weeks before the expiration of this approval in order to avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenet of the code including ensuring that all adverse events are reported promptly to the UI-ACUREC. No changes are permitted in the research without prior approval by the UI-ACUREC except in circumstances outlined in the code.

You are to note that UI-ACUREC reserves the right to monitor and conduct compliance visit to your research site without previous notification.

.....
Prof. S. I. B. Cadmus
Chairman, UI-ACUREC

Appendix 83: Publication 1

ORIGINAL PAPER

Nig. J. Pharm. Res. 2019, 15 (1) pp
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Ethnobotanical survey of medicinal plants used as remedy for female infertility and menstrual disorder in Southwestern Nigeria

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background*:* In Sub-Saharan Africa, more than 30% of premenopausal women are affected with secondary infertility. Nigerian (Yoruba) women show a higher tendency towards exaggerated ovarian (PCOS) response to stimulation for assisted conception associated with a higher prevalence of Polycystic Ovary Syndrome.

Objective: Identify and document medicinal plants traditionally used to treat female infertility and menstrual disorders among Yoruba ethnic group.

Materials and Methods: Data on medicinal plants traditionally used to manage menstrual disorders and female infertility were collected through interviews and Focus group discussions (FGDs).

Results: Twenty nine plant species belonging to 20 families were mentioned for management of infertility and menstrual disorder. Plant families mostly used were Euphorbiaceae (20%), Cucurbitaceae (15%), Bignoniaceae (10%), Apocynaceae (10%), Arecaceae (10%) and Solanaceae (10%).

Conclusion: This study provides documentation of medicinal plants used in the management and treatment of infertility and menstrual disorder in Southwestern Nigeria.

Keywords: Female infertility, Menstrual disorder, Medicinal plants, Bioactive constituents.

INTRODUCTION

Reproductive health problem is the prominent cause of global morbidity and mortality among premenopausal women (United Nations, 2012). Several researchers have reported that menstrual morbidity influenced health status, quality of life, social integration and educational status of women in developing countries (Tjon, 2007). However, most discussions are focused on male reproductive health issues, such as erectile dysfunction, neglecting the reproductive problems, which affect women of reproductive age. The assessment and management of reproductive health conditions in women are often neglected thus limiting clinical studies on this subject (Ozcan and Sahin, 2009). Women, constituting 51% of Africa's population, still remain the pillars of Africa's economic development (Rogombe, 1985). Unfortunately, the women folk is plagued by many health challenges, some of which include infertility. Zegers--Hochschild *et al*. (2009) defined infertility as failure to achieve a clinical pregnancy after 12 months (or more) of regular unprotected sexual intercourse. Women with irregular menstrual cycles have been reported to be victims of infertility (Wise *et al*., 2011). Menstrual disorder is an important risk marker for other reproductive complications such as oligomenorrhea, amenorrhea, polycystic ovary syndrome and recently, gynaecological cancers (Engman *et al.,* 2005).

About 15% of reproductive aged couples are affected by infertility globally (WHO, 2010). In Sub-Saharan Africa, not less than 30% of premenopausal women are affected with secondary infertility. Wada *et al.* (1994) reported the high prevalence of infertility among Nigerian (Yoruba) women. Nigerian (Yoruba) women show higher propensity towards exaggerated ovarian response to stimulate assisted conception which has been linked with a higher occurrence of Polycystic Ovary Syndrome (Wada *et al.,* 1994). Reproductive health can be improved by either medications or by fertility control (Siedlecky, 2001). In Western countries, where menstrual irregularity and infertility and/ or subfertility are repeatedly presented as a medical condition, treatment includes hormonal therapy (including contraception) or non-steroidal anti-inflammatory drugs. Some of these medical interventions aside being beyond the reach of many rural women come with serious side effects. In low-income countries where medical treatments are sometimes unavailable or unaffordable, especially in the rural areas, affected women never sought any medical help until the condition becomes severe. Women in these tropical countries, which are blessed with abundant flora prefer traditional medicine for their health challenges including menstrual disorders or family planning (Levin, 2001; Castle, 2003; Bearinger *et al*., 2007; Williamson *et al*., 2009; Sonibare and Ayoola, 2015).

The use of botanicals in such women has been found to increase the rate of conception in women affected by infertility (Zhao, 2011). Several Nigerian plants have been documented through ethnobotanical studies as effective for reproductive health (Soladoye *et al.*, 2014; Nduche *et al*., 2015; Fasola, 2015). The herbal treatment, mostly administered in form of powder, tea, tonic or tincture, has effectively enabled the body to readjust the menstrual cycle (Nduche *et al*., 2015; Fasola, 2015). In line with this, interest has been generated towards the scientific validation of the medicinal claims on some of these botanicals. The qualitative chemical assessment of bioactive constituents of some of the common medicinal plants have been reported to show that they possess constituents that could elicit pharmacological effects such as cytotoxicity, antioxidant activity and correction of hormonal imbalance, thereby justifying their folkloric uses. Several beneficial effects of Isoflavones in human have been documented. Several data support the general belief that soy consumption, an isoflavone-rich diet, prevents cardiovascular ailments and post-menopausal effects such as osteoporosis (Rice-Evans and Packer, 2003; Malińska and Kiersztan, 2004). Therefore, this study was designed to identify, document and to assess (qualitatively) the isoflavone constituents of a number of botanicals conventionally used to treat menstrual disorders and female infertility among Yoruba ethnic group in Iwo metropolis of Osun State, Nigeria.

METHODOLOGY

Study area

The study was conducted in Iwo metropolis of Osun State, South-western Nigeria. Osun State with a landmass of 14,875 Km^2 can be located between latitude 7.0° N to 8.0° N and longitude 04°.10'E to 05°.05'E. The annual rainfall of the southern part differs from that of the Northern part with values of 1125 mm and 1475 mm per annum respectively (Abe, 1995). The forest reserve vegetation lies in the lowland rain forest zone of South-Western Nigeria and the derived savannah covering Iwo and Osogbo (Abe, 1995). Three local government areas (LGAs) in Iwo metropolis were selected as study area. Figure 1 shows the study areas namely: Aiyedire LGA (Oluponna, Railway station); Iwo LGA (Iwo town) and OlaOluwa LGA (Obamoro, Ikonifin). Identification of some villages without access to modern health facilities in the study area necessitated the advancement of

traditional health care system, which justifies the vast understanding of medicinal plant's application in this metropolis. The indigenes of this community are Yorubas hosting nomadic Fulanis, Hausas, Ibos and farmers from other countries such as Togo and Benin Republic. The women and men in this region are traders, farmers, civil servants and craftsmen.

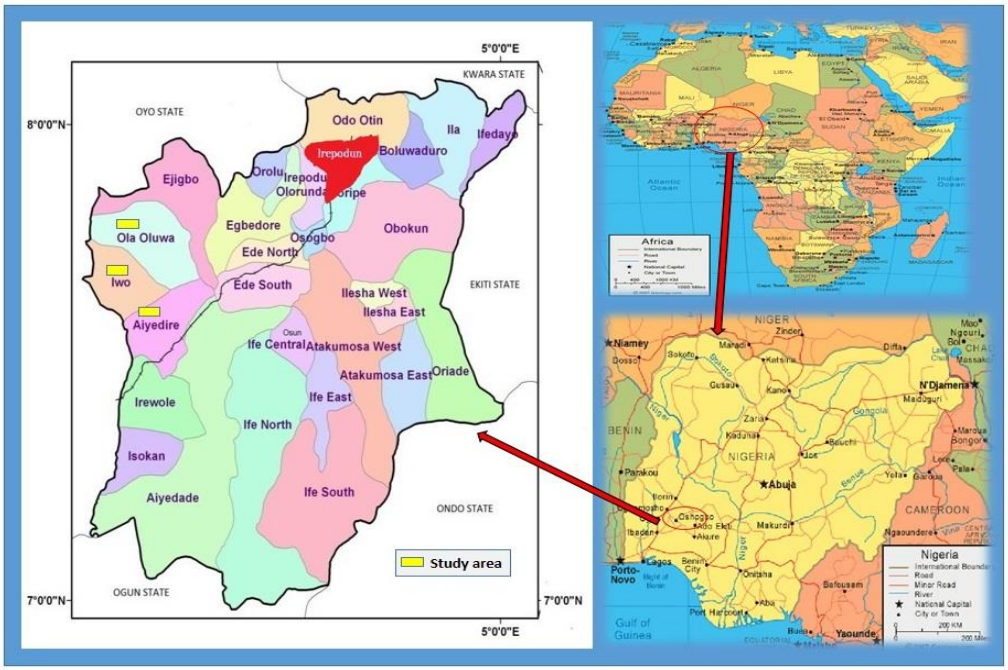

Figure 1. Map showing the study areas in Southwestern Nigeria

Data Collection

Data on traditional use of medicinal plants known for managing menstrual disorders and female infertility were documented through interviews and Focus group discussions (FGDs) with herb sellers (38.8%), herbalists (12.7%), community chiefs (4.8%), hunters (19.0%) and traditional religious leaders (24.6%) in Iwo metropolis. Collection of data was done between June 4 and September 16, 2016. One hundred and twenty six people fully participated in the study. Interviews and Focus Group Discussions were conducted with prior permission of the potential participants, aged between 21 and 68 years, in Yoruba language. The list of plants known to be efficacious in the management of menstrual disorders and female infertility in the community and their methods of preparation were documented. The associations of herb sellers, herbalists and hunters in each LGA were visited at separate times during their meeting where the intention of the survey was made known to all members. All the members were met after the meeting for interviews to collect names of medicinal plants with their modes of preparation and administration. Community chiefs and traditional religious leaders were visited in their various houses to seek further clarifications on some of the plants mentioned at FDGs. Some of these plants were planted in their courtyard. Other uses of the plants mentioned in this survey were also documented. The local and scientific names of medicinal plants mentioned during the survey were validated with research journals.

Data Analysis

All data obtained were analysed and presented in proportion, percentages and frequencies. The significance level of each species of the medicinal plants mentioned was evaluated and ranked with use value, UV (Phillips and Gentry, 1993) and use mentions index, UMI (Andrade-Cetto, 2009). Use value is the ratio of the number of uses mentioned by a respondent for a particular species (U_{is}) and number of interviews by the informant (n_{is}), while use mention index (UMI) is the ratio of number of use mentioned for a particular plant and the number of entire population interviewed. Fidelity level (expressed in percentage) was calculated as the ratio of the number of respondents that mentioned a plant species for a particular ailment (I_p) and the total number of respondents who knows the same plant for management of any ailment (I_u) (Friedman *et al.*, 1986).

RESULTS AND DISCUSSION

Of the 126 respondents, 39.7% were males and 60.3% females. Almost 49.0% were between 31 and 40 years old, while 19.0% and 70.6% had primary education and secondary school education, respectively. None of the participants had tertiary education. The hunters and herb sellers consulted were registered members of their various associations at the local government level. About 61.0%, 31.0% and 14.0% were practicing Islam, traditional and Christian religions, respectively. Table 1 shows the demographic characteristics of all the participants interviewed via semi-structured questionnaires and FGDs. Largest percentage of the respondents were herb sellers (38.8%), others were traditional religious leaders (24.6%), hunters (19.0%), herbalists (12.7%) and chiefs (4.8%).

Table 1: Demographic Characteristics of the Respondents

Characteristics	Specification	Total	Percentage (%)
1. Sex	Male	50	39.7
	Female	76	60.3
2. Practice specification	Herb sellers	49	38.8
	Herbalist	16	12.7
	Chiefs	6	4.8
	Hunters	24	19.0
	Traditional religious leaders	31	24.6
3. Age	21-31	30	23.8
	31-40	40	31.7
	41-50	27	21.4
	51-60	12	9.5
	>60	17	13.5
4. Religion	Islam	78	61.9
	Christianity	18	14.3
	Traditional	40	31.7
5. Marital status	Married	64	50.8
	Divorce	12	9.5
	Single	30	23.8
	Widow(er)	22	17.5
6. Educational status	Tertiary institution	0	0
	Secondary school	89	70.6
	Primary school	24	19.0
	No formal education	13	10.3
7. Nationality (Tribe)	Nigerian (Yoruba tribe)	126	100
	Non-Nigerian	0	0

The characteristics (family, common names and plant parts used) of medicinal plants used in the management of menstrual disorder and female infertility are presented in Table 2.

224 **Table 2: Plants species mentioned as remedy for menstrual disorders and female infertility in Iwo metropolis**
225 **(Osun State)**

Family/ Species	Local name(s)	Part used	Medicinal uses	Growth form	Times stated (n_{is})	Use mention index (UMI)	Number of uses (by respondent, U_{is})	Use value (UV)
Anacardiaceae								
1. Spondia mombin L.	Iyeye	Leaf and seed	Treatment of Female infertility, vaginal infections, malaria and to induce labour	Tree	30	0.032[l]	4	0.133
Annonaceae								
2. *Xylopia aethiopica* (Dunal) A. Rich	Eeru alamo	Fruit	Treatment of menstrual disorder, stomach, joint pains and infertility	Tree	26	0.032[l]	4	0.154
Apocynaceae								
3. Alstonia boonei De Wild.	Ahun	Leaf	Treatment of Female Infertility, malaria, and impotence	Tree	10	0.024[m]	3	0.300
4. *Picralima nitida* Stapf Th. & H. Dur.	Abere	Fruit	Treatment of menstrual disorder	Tree	2	0.008[o]	1	0.500
Arecaceae								
5. Elaeis quineensis Jacq.	Eyin (abon)	Fruit (Unripe)	Treatment of Female Infertility	Tree	10	0.008[o]	1	0.100
6. *Cocos nucifera* L.	Agbon	Fruit water	Treatment of Infertility.	Tree	1	0.008[o]	1	1.000
Asteraceae								
7. Vernonia amygdalina Del.	Ewuro	Leaf	Treatment of menstrual disorder, fibroid, stomach ache, ringworm, typhoid fever, headache and diabetes	Tree/ Shrub	4 5	0.056[j]	7	0.156
Basellaceae								
8. Basella alba L.	Amunu-tutu, gbowo-le-ganna	Complete aerial parts	Treatment of Female infertility, irregular periods, acne and sterility	Climber	23	0.032[l]	4	0.174
Bignoniaceae								
9. *Kigelia africana* (Lam.) Benth	Pandoro	Fruit	Treatment of female Infertility, skin infections and vaginal infections	Tree	17	0.024[m]	3	0.177
10. Newbouldia laevis (Beauv.) Seem. ex Bureau	Akoko	Bark, root and leaf	Treatment of menstrual disorder, fibroid, impotence and infertility	Shrub	25	0.032[l]	4	0.160
Bixaceae								
11. Bixa orellaina L.	Osun-buke	Leaves	Treatment of Female Infertility, stomach ache and diabetes	Shrub	33	0.024[m]	3	0.091
Cucurbitaceae								
12. Lagenaria breviflora (Benth.) Roberty	Tangiri	Fruit	Treatment of Irregular menstrual flow, skin infections and diarrhoea	Climber	10	0.024[m]	3	0.300
13. *Momordica cabraei*	Ahara	Leaf	Treatment of unhealthy menstruation	Climber	6	0.008[o]	1	0.167
14. Momordica charantia L.	Ejinrin	Leaf, complete aerial parts	Treatment of Female infertility, malaria, diabetes, painful menstruation and to regulate menses	Climber	21	0.040[k]	5	0.238
Euphorbiaceae								
15. Euphorbia lateriflora Schum. & Thonn.	Enu opiri	Leaf	Treatment of Irregular menstrual flow	Shrub	1	0.008[o]	1	1.000

16.	*Bridelia micrantha* (Hochst.) Baill.	Aasa, araasa	Leaf and root	Treatment of menstrual disorder and diabetics	Tree	10	0.016[n]	2	0.200
17.	*Jatropha gossypifolia* L.	Lapalapa pupa	Fruit	Treatment of Irregular menstrual flow, skin infections and excessive bleeding from the vagina	Shrub	13	0.024[m]	3	0.231
18.	*Mallotus oppositifolius* (Geisel.) Müll	Ipa, Ija,	Leaf	Treatment of Female Infertility and headache	Shrub	27	0.016[n]	2	0.074
Fabaceae									
19.	*Pterocarpus osun* Craib	Osun	Seed, leaf	Treatment of Irregular menstrual flow, unhealthy vaginal secretion and skin infections	Tree	41	0.024[m]	3	0.073
Hypericaceae									
20.	*Harungana madagascariens is* Lam. ex Poir	Amuje	Stem bark	Treatment of Irregular menstrual flow and stomach ache	Tree	7	0.016[n]	2	0.286
Menispermaceae									
21.	*Cissampelos owariensis* P. Beauv.	Jenjoko	Leaf	Treatment of Stomach disorder during menstruation, excessive bleeding during menstruation, diabetes and infertility	Climber	20	0.032[l]	4	0.200
Moraceae									
22.	*Erythrophleum suaveolens* Guill and Perr Brenan	Igi obo	Bark and leaf	Treatment of menstrual disorder and skin infections	Tree	3	0.016[n]	2	0.667
Musaceae									
23.	*Musa paradisiaca* L.	Ogede agbagba	Fruit peel	Treatment of Stomach disorder during menstruation,	Tree	5	0.008[o]	1	0.200
Portulacaceae									
24.	*Talinum triangulare* (Jacq.) Willd.	Gbure	Leaf	Treatment of Stomach disorder during menstruation and diabetes	Herb	15	0.016[n]	2	0.133
Rubiaceae									
25.	*Morinda lucida* Benth.	Oruwo	Root, bark	Treatment of Irregular menstrual flow, malaria, diabetes, stomach disorder during menstruation	Tree	17	0.024[m]	3	0.177
Santalaceae									
26.	*Viscum album* L.	Afomo-obi	Whole plant	Treatment of Irregular menstrual flow and diabetes	Herb (parasite)	10	0.016[n]	2	0.200
Solanaceae									
27.	*Capsicum frutescens* L.	Ata-eye	Fruit	Treatment of Irregular menstrual flow and ulcer	Herb	9	0.016[n]	2	0.222
28.	*Physalis angulata* L.	Koropo	Leaf, complete aerial parts	Treatment of Female infertility	Herb	3	0.008[o]	1	0.333
Tiliaceae									
29.	*Glyphaea brevis* (Spreng.) Monachino	Atori	Leaf and root	Treatment of menstrual disorder, ulcer, diabetes and as abortifacient	Shrub	5	0.032[l]	4	0.800

Twenty-nine medicinal plants in 20 families were documented. Based on UMI the twenty-nine plants were in six categories. One plant each was found in the first (UMI 0.056) and second (UMI 0.040) categories, while six plants were found in the third (UMI 0.032) category. The fourth (UMI 0.024), fifth (UMI 0.016) and sixth (UMI 0.008) categories had seven plants each. The plant families mostly used were Euphorbiaceae (20%), Cucurbitaceae (15%), Bignoniaceae (10%), Apocynaceae (10%), Arecaceae (10%) and Solanaceae (10%) as shown in Figure 2. The plants frequently mentioned by respondents for management and/or treatment of menstrual disorder and female infertility were *Pterocarpus osun, Basella alba, Cissampelos owariensi, Morinda lucida, Kigelia africana, Talinum triangulare* and *Viscum album*. In a study similar to this one, Nduche *et al*. (2015) reported that 62 plant species belonging to 41 families were being used as remedy for fertility conditions in Ebonyi State of Nigeria. Another study that surveyed medicinal plants used in the management and treatment of various female reproductive health challenges in Southwestern part of Nigeria reported 61 plant species belonging to 32 families (Fasola, 2015). The family Euphorbiaceae was well represented in this study, signifying their relevance in the management of menstrual disorder and female infertility.

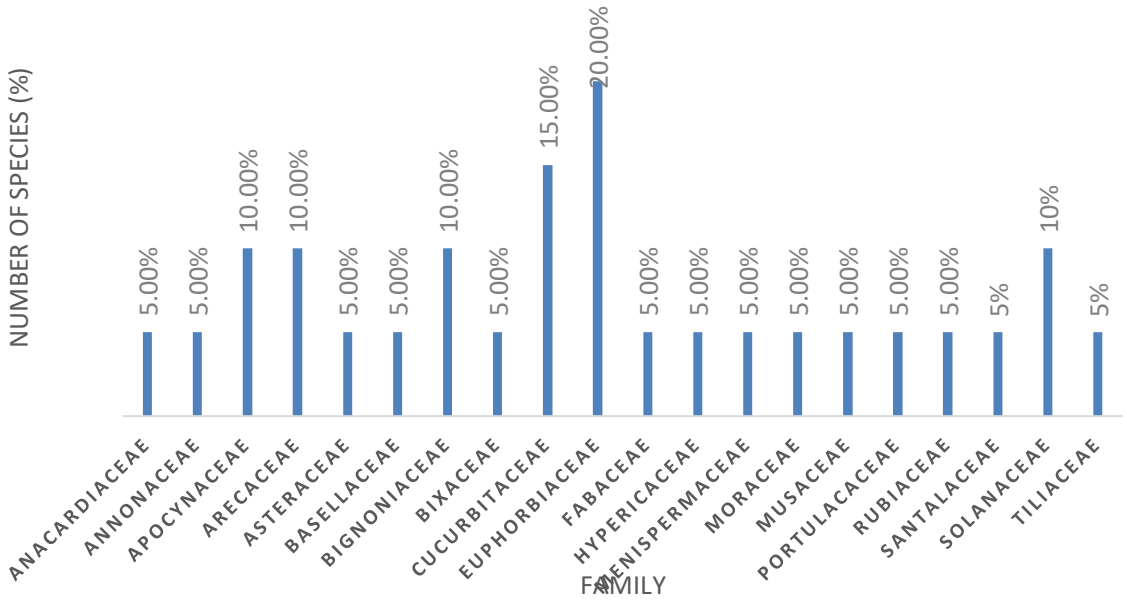

Figure 2: Family and number of Species of medicinal plants mentioned for the treatment of menstrual disorder and female infertility

The study found that most herbal remedies used by the community for treating menstrual disorder and female infertility were administered in combination, while some plants, such as *Tetracera* sp., *Pterocarpus osun, Cissampelos owariensis, Talinum triangulare* and fruit peel of *Musa paradisiaca* were administered singly although with other non-plant materials like potash or charred with sulphur. The enumeration and duration of administration of herbal preparations differ according to the symptoms reported by the women affected with these conditions. Such symptoms include oligomenorrhea, amenorrhea, amenorrhea, unhealthy menses and infertility. The herbal preparations were mostly taken orally, either as decoctions and infusions, or as herbal soap. The concept similar to solvent partitioning was reported for the preparation which contains *Erythrophleum suaveolens*, the plant known among the ethnic group for its toxicity (Table 3).

Table 3: Enumeration of recipes used as remedy for menstrual disorder and female infertility

Conditions	Method(s) of preparation	Administration
1. Infertility	a). The dried fruit of *Picralima nitida* will be grounded into powder and macerated with *Cocos nucifera* water.	A cup of infusion is to be taken every night
	b). Squeeze *Momordica charantia* leaves	This is to be taken everyday
	c). *Basella alba* plant is macerated with water	The infusion should be taken twice daily
2. Infrequent menstruation (oligomenorrhea)	a). Fruit of *Jatropha gossypifolia*, leaf of *Euphorbia lateriflora* and *Lagenaria breviflora* fruit were burned together	A spoonful of the powder is then mixed with a cup of water and taken as the day of menstruation approaches.
	b). Fruit of *Jatropha gossypifolia*, leaf of *Euphorbia lateriflora* and *Lagenaria breviflora* fruit were burned together and mixed with black soap and potash	The soap is to be bath with as the day of menstruation approaches.
3. Ceased menstruation (amenorrhea)	a). Dried leaves and root of *Bridelia micrantha* and *Xylopia aethiopica* are mixed in a bottle and then water is added.	A cup of infusion is to be taken twice a day, morning and night, for duration of 6 months to 1 year
	b). Bark and leaves of *Erythrophleum suaveolens* are boiled with water and potash. The infusion is then mixed with alcohol.	A cup of the alcoholic part (of the infusion) is to be taken twice a day, morning and night
	c). Squeeze *Physalis angulata* leaves with water.	A glass cup should be taken each day of menstruation
	d). *Bixa orellana* leaves, *Harungana madagascariensis* stem bark and *Morinda lucida* root, are grounded together	This mixture is to be taken with hot pap every morning
4. Unhealthy menses (discoloured or malodourous)	a). Leaves juice of *Momordica cabraei* and small potash	This mixture is to be taken every day till the menstruation is over
5. Unhealthy vaginal secretion	a). Squeeze leaf juice of *Pterocarpus osun*	Bathe with water mixed with the juice on the day this vaginal secretion is observed
6. Painful menstruation (dysmenorrhea)	a). Leaves juice of *Cissampelos owariensis* and small potash	This mixture is to be taken every day till the menstruation is over
	b). Leaves juice of *Talinum triangulare* and small potash	This mixture is to be taken every day till the menstruation is over
	c). Fruit peel of *Musa paradisiaca* and sulphur burned together.	The residue is mixed with pap and should be taken regularly

For the plant species mentioned for management of menstrual disorders and female infertility, the leaves (40%) were the morphological part persistently used in most herbal preparations, then fruit (25%). Other parts of plant used are bark (8.6%), seed (5.7%) and whole plant (2.9%) (Figure 3).

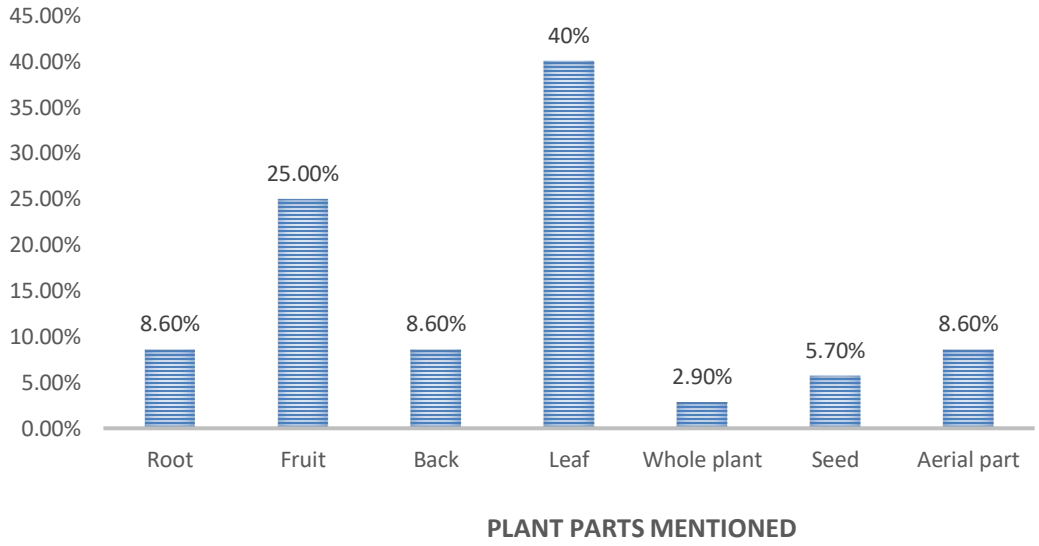

Figure 3: Plant parts mentioned for the treatment of menstrual disorder and female infertility

One of the procedures used to determine the foremost used plant species used for medicinal purpose is the calculation of fidelity level. High fidelity value shows the strength of approval for each plant species used in the study area. This value justifies the selection of a particular species by respondent for the treatment of a specified disease. Among all the plant mentioned, *Picralima nitida, Elaeis quineensis, Cocos nucifera, Tetracera* sp., *Euphorbia lateriflora, Musa paradisiaca and Physalis angulata* had fidelity level of 100% (Table 4[m1]).

CONCLUSION

Medicinal plants were commonly used in the study area because of their better affordability, reported efficacy and regular accessibility compared to modern health care facilities. However, aggressive collection of these plants with medicinal importance is a big threat to availability. Therefore, most traditional healers practiced propagation of the medicinal plants of interest in their home gardens. The indigenous knowledge of medicinal plants is valuable resources for health management. Knowledge of traditional medicine use need to be protected through proper documentation of recipes enumerations. This documentation becomes the foundation for proper investigation of phytochemicals and validation of pharmacological claims of medicinal plants use for management of infertility and other gynaecological problems in this community and for future.

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RESEARCH ARTICLE

Antiproliferative and ameliorative effects of *Tetracera potatoria* and its constituent

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Abstract

The effect of *Tetracera potatoria* leaves and its isolated constituent on polycystic ovarian syndrome (PCOS) and associated gynaecological cancers was investigated. Crude extract showed the lowest level of luteinizing hormone and the highest level of estradiol and follicle stimulating hormone, comparable to the normal control group. The hexane ($IC_{50} = 34.8 \pm 0.3$ µg/mL), and dichloromethane ($IC_{50} = 41.3 \pm 0.8$ µg/mL) fractions inhibited the proliferation of Chinese Hamster ovarian (CHO) cells. The dichloromethane fraction was subjected to purification using column chromatography, which led to the isolation of apigenin. The structure of the isolated compound was confirmed by the reported spectroscopic data. Apigenin inhibited the proliferation of both CHO and HeLa cells with an IC_{50} values of 22.2 ± 0.5 and 6.2 ± 0.6 µg/mL, respectively. The apigenin was isolated and reported for the first time in *T. potatoria*. The leaves extract of *T. potatoria* showed curative effect on irregular estrus cycle and hormonal imbalance. The isolated constituent showed anticancer potential, hence it could reduce the risk of gynaecological cancers among PCOS patients.

Keywords *Tetracera potatoria* · Antiproliferative · Flavonoids · Polycystic ovary syndrome · Luteinizing hormone · Follicle stimulating hormone

Introduction

Polycystic ovary syndrome (PCOS) is the leading endocrine disorder affecting women worldwide. It is connected with ovulatory dysfunction, polycystic ovarian morphology and hyperandrogenism (Legro et al. 2013). In most studies, the incidence of PCOS among women is assessed to be between 5 and 20%. The criteria used and ethnicity of the studied population have been identified as factors responsible for the variation in the prevalence of PCOS (Azziz et al. 2004; Ehrmann 2005). In South-eastern Nigeria, prevalence rate of 18.1% was reported when presence of numerous ovarian cysts was used as diagnostic criteria (Ugwu et al. 2013). Women affected with PCOS are at 2.7-fold increased risk of developing gynaecological cancers such as ovarian and cervical cancers. However, there is no established relationship between PCOS and breast cancer (Dumesic and Lobo 2013; Ding et al. 2017). Abnormal *p53* tumour suppressor gene was recently discovered in the endometrium of PCOS patients (Shafiee et al. 2015; Gadducci et al. 2016).

Tetracera potatoria is a medicinal plant found in wooded areas of Senegal, southern part of Nigeria, Central and Eastern Africa (Dalzel 1937). The leaves or a portion of the stem are boiled in its own sap and used as a powerful diuretic, vermifugal and purgative, as well as for the treatment of gastrointestinal and other stomach complaints (Burkill 1985; Betti 2004). The sap is also used for the treatment of cough and toothache (Oliver-Bever 1960). In south-western Nigeria, the aqueous extract from the root is an active remedy for intestinal disorders (Adesanwo et al. 2003). It is used in traditional treatment of inflammation, skin infection and ulcer (Adesanwo et al. 2013). Several classes of phytochemicals such as tannins, alkaloids, cardiac glycosides, flavonoids

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and saponins have been reported to be present in *Tetracera potatoria* (Adesanwo et al. 2013), however, betulinic acid, N hydroxy imidate-tetracerane, β-stigmasterol, stigmast-5-en-3β-yl acetate, tetraceranoate, lupeol and botulin are the few compounds isolated from various parts of *T. potatoria* so far (Adesanwo et al. 2013; Fomogne-Fodjo et al. 2017). In an ethnobotanical survey conducted in the course of this study, *T. potatoria* leaf juice was mentioned for management of menstrual disorder, stomach upset and infertility among premenopausal women in South-western Nigeria (Ogunlakin and Sonibare 2020). This study therefore reported the effect of *Tetracera potatoria* leaves and its constituent on polycystic ovarian syndrome and associated gynaecological cancer.

Materials and methods

Plant material

Fresh leaves of *Tetracera potatoria* were collected in Oluponna (7° 35′ 34.7″ N 4° 11′ 27.5″ E), Ayedire Local Governmet Area, Osun state of Nigeria on 19th of February, 2017. The plant was identified and authenticated by Mr. G. Ibhanesebhor, a taxonomist-in-charge of the Herbarium of Obafemi Awolowo University, Ile-Ife, Nigeria where voucher specimen (IFE Herbarium-17794) was also deposited.

Extraction and solvent partitioning

The air-dried plant material was pulverised. Pulverised plant material (1300 g) was extracted with methanol for 72 h at room temperature. The extract was filtered and the filtrate was concentrated *in vacuo* yielding dark-green sticky extract. The extract was stored in refrigerator. Solvent–solvent partitioning was done in accordance with standard procedure (Van Wagenen et al. 1993). The *n*-hexane, dichloromethane (DCM) and ethyl acetate fractions obtained were concentrated *in vacuo* to give residues, which were kept in air tight containers for subsequent bioassay. The percentage yields of *n*-hexane, DCM and ethyl acetate fractions were calculated.

In vitro antioxidant assay and quantification of phenolics and flavonoids

The antioxidant potential of the methanol extract and the fractions of *T. potatoria* was evaluated using 1, 1-diphenyl-2-picryl-hydrazyl-hydrate (Mensor et al. 2001; Bursal and Gülçin 2011). Total phenolic content (TPC) of the methanol extract and fractions were measured using Folin-Ciocalteu spectrophotometric method (Miliauskas et al. 2004). The aluminium chloride colorimetry method was used for quantification of flavonoids according to the standard procedure (Woisky and Salatino 1998).

Spectroscopic analysis

Nuclear magnetic resonance (NMR) spectra were recorded on *Bruker AV (Avance)* spectrometer (^{1}H NMR at 400 MHz; ^{13}C NMR at 600 MHz) using the nondeuterated solvent peaks as internal standard. Low resolution electron impact mass spectra were recorded on a finnigan *MAT 312* and *MAT 312* spectrometer. TLC was carried out using silica gel 60 GF_{254} pre-coated aluminium sheets by Sigma Aldrich, Germany. Melting point of the compound was determined on Buchi® M-560 melting point apparatus.

Isolation and characterisation of apigenin from DCM fraction

Light-green coloured DCM fraction (2.62 g) was subjected to column chromatography and eluted with hexane, DCM, ethyl acetate and methanol in different ratios to give 80 fractions of 200 mL each. The fractions were analysed by TLC using pre-coated TLC plates (Silica gel G_{60} F_{254} sheets 20 × 20 cm, 0.5 mm thickness) using appropriate solvent systems as mobile phases. Twelve different pooled fractions, D_1 (fractions 1–4), D_2 (fractions 5–8), D_3 (fractions 9–13), D_4 (fractions 14–20), D_5 (fractions 21–23), D_6 (fractions 24–25), D_7 (fractions 26–28), D_8 (fractions 29–39), D_9 (fraction 40), D_{10} (fractions 41–50), D_{11} (fractions 51–66), D_{12} (fractions 67–80) were obtained. Fraction D_9 (greenish yellow, 200 mg) vial prep-TLC technique (developed with DCM-ethyl acetate—80:20 and few drops of acetic acid) yielded compound **1** (6.28 mg, yellow coloured powder).

Antiproliferative studies

Antiproliferative effect of crude and solvent fractions of *Tetracera potatoria* on HeLa and CHO cell lines (ATCC, Manassas, USA) was evaluated by standard MTT colorimetric assay (Mosmann 1983). One hundred microliter (100 µL) of viable cells (5×10^4 cells/mL of HeLa and 6×10^4 cells/mL of CHO cells) cultured in DMEM (Dubecco's modified Eagle's medium; Sigma chemical Co., St. Louis, MO, USA) supplemented with 10% FBS were seeded into 96-wells microliter plate and incubated overnight at 37 °C in 5% CO_2. The extracts, solvent fractions and Doxorubicin at three different concentrations (1, 10 and 100 µg/mL) prepared in triplicate were added to the plate and incubated for 48 h. Fifty microliter (50 µL) of 0.5 mg/mL MTT was added to each well after 4 h incubation of the mixture. The same volume of DMSO was then added to all test and control wells and the absorbance was measured at 540 nm using spectrophotometer (Spectra

Max plus, Molecular Devices, CA, USA). The antiproliferative effect of all the test samples was reported as IC_{50} value (µg/mL). This assay was repeated for the isolated compound **1**.

In vivo studies

Experimental animals

Laboratory-bred virgin female Wistar rats, weighing between 150 and 200 g with regular estrous cycles were used. All animals were housed in cages under 12 h to 12 h light–dark cycle at a temperature of 20–25 °C. The cages were lined with soft wood shavings, used as beddings to absorb waste products from the animals, and changed regularly. They had free access to constant food pellets (Ladokun feeds Nig. Ltd.) and water ad libitum during the course of this study. Animals were acclimatized for minimum of 1 week before the start of experimental procedures. The experiment was conducted in accordance with the directions of Guide for the Care and Use of Laboratory Animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. In addition, the experiment was approved by University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC/19/0051).

Induction of PCOS and administration of test samples

The in vivo study was based on the crude extract of *T. potatoria*. Twenty non-pregnant, female albino rats (150–200 g) with normal estrous cycle were randomly grouped into four made of five albino rats per group. Groups I-III were treated with l mg/kg letrozole orally for a period of 2 l consecutive days using 0.5% w/v carboxymethyl cellulose (CMC) as vehicle for the induction of PCOS. Group I received 100 mg of *T. potatoria* methanol extract per kilogramme body weight, while Group II received l mg of clomiphene citrate (Colid, Pfizer pharamceuticals, USA) per kilogramme body weight. Groups III (untreated disease control) and IV (normal control) received 2 mL of 5% w/v CMC in distilled water (Kafali et al. 2004). The test samples were administered daily for 15 days via oral route. The dose level of 100 mg/kg *b.w.* was used in this study based on human therapeutic dose mentioned in the preliminary ethnobotanical survey conducted for managing irregular menstrual disorder and associated gynaecological disorders. The dose for rat was calculated considering human to albino rat conversion factor (conversion factor = 0.018) according to body surface area (Nair and Jacob 2016).

Determination of oestrous cycle pattern and hormonal analysis

The phases of the oestrous cycle were detected by examining vaginal cytology (Marcondes et al. 2002). Vaginal lavage was obtained with a Pasteur pipette filled with 0.1 mL of normal saline (0.9% NaCl), gently inserted into the rat's vagina. The withdrawn vaginal fluid was dropped on a glass slide and immediately evaluated microscopically. The oestrus cycle was monitored and the proportion of leukocytes, epithelial and cornified cells was expressed as phase index (%). The levels of Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH) and estradiol in the serum of the rats were measured using the Enzyme Linked Immuno Sorbent Assay (ELISA). Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) were assessed in the serum samples using the microwell kits manufactured by Fortress Diagnostics limited, United Kingdom, while estradiol level was measured by microwell kits manufactured (Dialab, Austria). The samples and the test reagents were equilibrated at room temperature prior the test. Then 0.05 mL of calibrators and rat's samples were pipetted inside the wells followed by addition 0.1 mL of dilute enzyme conjugate to each well excluding the blank well. These mixtures were incubated for 60 min at room temperature. The mixtures in the microwells were thrown out and the wells were cleaned with 0.2 mL of distilled water. This was done twice in order to remove water in the well. Solution of the substrate (0.1 mL) was pipetted into each microwell in the same order and interval as for the enzyme conjugate, blank well was included and incubated for 20 min at room temperature in the dark. Stop solution (0.1 mL) was added into each microwell using the same order and timing as for the reaction of the substrate solution. Absorbance of each microwell was read at 450 nm against blank using a microplate reader. The developed colour was stabled for at least 30 min and the optical densities were read during this time.

Ovarian histology

The animals were euthanized 24 h after last treatment, between 0900 and 1100 h to minimise diurnal variation, with 2% sodium pentobarbital (30 mg/kg) for laparotomy to collect abdominal aorta blood and ovaries of the overnight fasted rats. The tissue architecture of the dissected ovaries was examined according to standard method (Avwioro 2010). Haematoxylin and Eosin stain technique was used. The tissues were observed and dissected into small pieces of not more than 4 mm thick into pre-labelled cassettes. These small pieces of ovary tissues were immersed in 10% formal saline for 24 h for fixing. Tissue processing was done automatically using automatic tissue processor (Leica TP 1020). The tissues were dehydrated by passing them through

various dehydrating reagents such as 10% formal saline and alcohol (70%, 80%, 90% and 95%). Formal saline is the mixture of 100 mL of 40% of formaldehyde, 9 g of NaCl and 900 mL of distilled H_2O. The tissues were immersed in the molten paraffin wax, dispensed into a metal mould, and was transferred to a cold plate to solidify. The tissue block formed was separated from the mould and were trimmed to expose the tissue surface using a rotary microtome at 6 µm. The surfaces were placed on ice and sectioned at 4 µm (ribbon section). The sections floated on water bath (Raymond lamb) set at 55 °C were picked using clean labelled slides, dehydrated on a hotplate (Raymond lamb) set at 60 °C for l h and viewed under the light microscope using ×100 and ×400 objective.

Statistical analysis

The results of all assays were expressed as mean ± standard error of mean. The experiments were carried out in triplicate. The data were analysed with GraphPad (Version 5, GraphPad Prism Software Inc., San Diego, CA,). One-way ANOVA followed by Dunnett's Multiple Comparison Test were employed to test for the statistical differences between the groups at $p < 0.05$.

Results

Plant extraction

The percentage yields of the crude methanol extract, *n*-hexane, DCM and ethyl acetate fractions were 13.49% (175.37 g), 6.40% (11.23 g), 4.49% (7.87 g) and 13.71% (24.11 g), respectively.

In vitro antioxidant assay and quantification of phenolics and flavonoids

The dichloromethane (DCM) and ethyl acetate fractions of *T. potatoria* displayed free radical scavenging activity with IC_{50} values of 89.15 ± 0.50 and 9.52 ± 0.35 µg/mL, respectively, among which ethyl acetate fraction was found to be more potent, compared with the standards, ascorbic acid and rutin (IC_{50} values of 2.76 ± 0.01 and 20.6 ± 9.26 µg/mL, respectively) (Table 1). The result of total phenolic content (TPC) showed that ethyl acetate fraction of *T. potatoria* had the highest TPC (7150.18 ± 110.00 µg GAE/g). The crude extract had the highest Total flavonoid content (190.28 ± 12.30 mg QE/g).

Spectroscopic analysis

Isolation and characterisation of apigenin from DCM fraction

Compound **1,** isolated as a yellow powder (MP = 345.2–347.1 °C) from the DCM fraction of *T. potatoria* had the following spectroscopic data; ^{1}H NMR spectrum (400 MHz, $CDCl_3 + CD_3OD$): δ (ppm) 7.69 (2H, d, J = 8.8 Hz, H-2′), 6.85 (2H, d, J = 8.8 Hz, H-3′), 6.43 (1H, s, H-3), 6.34 (1H, d, J = 2 Hz, H-8), 6.17 (1H, d, J = 2.4 Hz, H-6). ^{13}C NMR (600 MHz, $CDCl_3$): δ (ppm) 182.4 (C=O, C-4), 164.5 (C, C-7), 163.8 (C, C-2), 161.4 (C, C-5), 160.6 (C, C-4′), 157.8 (C, C-9), 128.1 (CH, C-2′), 128.1 (CH, C-6′), 122.1 (C, C-1′), 115.8 (CH, C-3′), 115.8 (CH, C-5′), 104.4 (C, C-10), 103.1 (CH, C-3), 99.0 (CH, C-6), 94.2 (CH, C-8). EI-MS *m/z* (% rel. abun.): 271 (M^++1, 19), 270 (M^+, 100), 269 [M–(H)], 253 [M–(H)–(OH)]. The NMR spectroscopic data of the compound (Fig. 1) is presented in Table 2.

Antiproliferative studies

The hexane and DCM fractions of *T. potatoria* inhibited the proliferation of CHO cells with IC_{50} values of 34.8 ± 0.3 and 41.3 ± 0.8 µg/mL, respectively, while the crude and ethyl acetate fraction had insignificant effect on CHO cells proliferation. Crude extract and solvent fractions of *T. potatoria* exerted no inhibitory effect on HeLa cells proliferation (Table 3). The compound, apigenin, inhibited proliferation of HeLa (IC_{50} value of 6.2 ± 0.6 µg/mL) and CHO cell lines

Table 1 The DPPH (IC_{50}), TPC and TFC values of the extract and solvent fractions of *T. potatoria*

Extracts	Solvents	DPPH (IC_{50}) (µg/mL)	TPC (µg GAE/g)	TFC (mg QE/g)
T. potatoria.	Crude	220.89 ± 6.99***/***	3404.67 ± 6.13	190.28 ± 12.30
	Hexane	–	1963.17 ± 110.93	12.85 ± 0.18
	DCM	89.15 ± 0.50***/***	2518.33 ± 96.17	3.68 ± 0.16
	Ethyl acetate	$9.52 \pm 0.35^{NS/NS}$	7150.18 ± 110.00	79. 64 ± 0.15
Ascorbic acid		2.76 ± 0.01		
Rutin		20.6 ± 9.26		

Data represented as mean ± (SEM) (n = 3). One-way ANOVA followed by Dunnett's Multiple Comparison Test at $p = 0.05$. IC_{50} DPPH of each extract was compared with standards (Ascorbic acid and rutin) with level of significant difference represented by *** or asterisk separated by (/) indicate order of significance from Ascorbic acid and rutin respectively, *NS* no significant difference from the standards

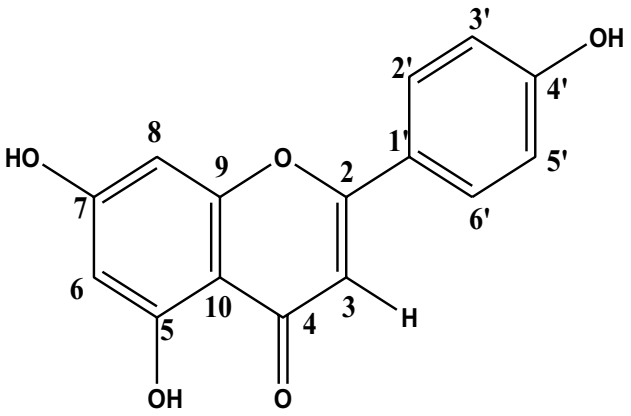

Fig. 1 Structure of 5, 7-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (apigenin) (1)

(IC_{50} value of 22.2 ± 0.5 µg/mL). The standard, Doxorubicin, had IC_{50} values of 0.8 ± 0.0 l µg/mL and 3.1 ± 0.2 µg/mL on CHO and HeLa, respectively.

In vivo studies

Effect of plant extract on oestrous cycle pattern and hormonal analysis

Oral administration of letrozole for 2 l days influenced reproductive cycle irregularity in female albino rats (Fig. 2). For the period of letrozole administration the estrous and proestrous phases were absent in the letrozole treated rats. As treatment commenced all treated rats exhibited acyclic conditions, however, *T. potatoria* extract improved the estrous cycle (Fig. 2) by increasing the appearance of estrous phase (index = 24.25%) and decreasing the duration of diestrous phase (index = 24.25%) when compared with disease control group (estrous index = 7.1%; diestrous index = 24.26%). *Tetracera potatoria* influenced reduction in the LH level and increased the level of FSH as shown in Table 4. The LH circulatory level in all groups ranged from 0.19 ± 0.05 mIU/mL to 0.23 ± 0.03 mIU/mL. Group I, having rats treated with *T. potatoria* methanol extract, had the least value (0.19 ± 0.05 mIU/mL). The level of FSH in *T. potatoria* treated group (0.81 ± 0.04 mIU/mL) was comparable with that of the control group (0.93 ± 0.19 mIU/mL). There was a significant ($p < 0.00$ l) reduction in the level of estradiol (5.70 ± 0.77 pg/

Table 3 The IC_{50} (µg/mL) of crude extract, fractions and isolated compound of *Tetracera potatoria* on Chinese Hamster Ovarian (CHO 1) cell line and HeLa cell line

Sample (s)	IC_{50} (µg/mL)	
	CHO 1 cell line	HeLa cell line.
Tetracera potatoria crude	> 100	> 100
Tetracera potatoria hexane fraction	34.8 ± 0.3	> 100
Tetracera potatoria DCM fraction	41.3 ± 0.8	> 100
Tetracera potatoria ethyl acetate fraction	> 100	> 100
Compound **1**	22.2 ± 0.5	6.2 ± 0.6
Standard (Doxorubicin)	0.8 ± 0.01	3.1 ± 0.2

Table 2 1H and 13C-NMR data of compound **1** compared with reported literature

Position	Observed		Reported (Nabavi et al. 2015)	
	δ_H (d, $CDCl_3$ + MeOH, 400 MHz)	δ_C ($CDCl_3$, 600 MHz)	δ_H (DMSO, 500 MHz)	δ_C (DMSO, 125 MHz)
l	–	–	–	–
2	–	163	–	163
3	6.43 (s, lH)	103	6.78 (s, lH)	102
4	–	182	–	181
5	–	161	–	161
6	6.17 (d, *J* = 2 Hz, IH)	99	6.19 (d, *J* = 2 Hz, IH)	98
7	–	164	–	164
8	6.34 (d, *J* = 2 Hz, lH)	94	6.48 (d, *J* = 2 Hz, IH)	93
9	–	157	–	–
10	–	104		103
l′	–	122		121
2′	7.69 (d, *J* = 8.8 Hz, 2H)	128	7.93 (d, *J* = 8.8 Hz, 2H)	128
3′	6.85 (d, *J* = 8.8 Hz, 2H)	115	6.93 (d, *J* = 8.8 Hz, 2H)	115
4′	–	160	–	161
5′	6.85 (d, *J* = 8.8 Hz, 2H)	115	6.93 (d, *J* = 8.8 Hz, 2H)	115
6′	7.69 (d, *J* = 8.8 Hz, 2H)	128	7.93 (d, *J* = 8.8 Hz, 2H)	128

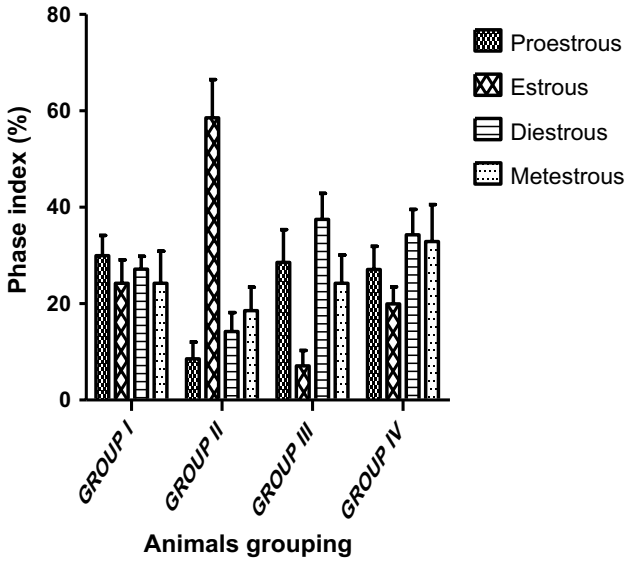

Fig. 2 Index of estrous cycle phases of albino rats after 15 days of treatment with 5% w/v CMC (in distilled water), clomiphene citrate and selected plant extracts. The data represent the mean ± SEM animals, n = 5. Data represented as mean ± (SEM) (n = 5). Group I—100 mg/kg body weight of *Tetracera potatoria*, Group II—Clomiphene citrate (1 mg/kg bw, p.o.), Group III—disease control group, Group IV—normal control group

Table 4 Effect of various treatments on LH, FSH and estradiol

Parameters	Group I	Group II	Group III	Group IV
LH (mIU/mL)	0.19 ± 0.05*	0.23 ± 0.01*	0.23 ± 0.01*	0.22 ± 0.01
FSH (mIU/mL)	0.81 ± 0.04*	0.75 ± 0.05*	0.73 ± 0.03*	0.93 ± 0.19
Estradiol (pg/mL)	9.36 ± 2.06*	7.63 ± 0.89*	5.70 ± 0.77*	9.84 ± 1.44

Data represented as mean ± (SEM) (n = 5). Evaluated by ANOVA followed by Bonferroni tests. *Indicate $p < 0.001$ versus normal control

Group I—100 mg/kg body weight of *Tetracera potatoria*, Group II—Clomiphene citrate (1 mg/kg bw, p.o.), Group III—disease control group, Group IV—normal control group

mL) in PCOS rats. However, treatment with *T. potatoria* caused an upsurge in the level of estradiol (9.36 ± 2.06 pg/mL) in the rats compared to the normal control group which had 9.84 ± 1.44 pg/mL.

Ovarian histology

Administration of letrozole for 21 days changed the morphology of the ovary in all treated animals (Fig. 3). The morphology of the ovary of animal treated with 100 mg/kg *b.w.* of *T. potatoria* (Group I) revealed presence of normal antral follicle with clear antrum, while oocytes are surrounded by granulosa cells. The stroma revealed hyperplastic luteinisation as shown in Fig. 3.

Discussion

Oxidative stress has been identified as one of the major factors contributing to PCOS and gynaecological cancers pathogenesis among reproductive women (Murri et al. 2013; Krstic et al. 2015). Among women with PCOS, oxidative stress favours the pathogenesis of gynaecological cancers (Ding et al. 2017). Reactive oxygen species (ROS) cause mutation of protooncogenes and tumour suppressor genes in the DNA (Ziech et al. 2011), resulting in uncontrollable cell proliferation. For instance, abnormal *p53* tumour suppressor gene was recently observed in the endometrium of PCOS patients (Shafiee et al. 2015; Gadducci et al. 2016). Therefore, to provide justification for the antioxidant potential of the plant, which might play a significant role in reducing the risk of PCOS, the DPPH radical scavenging activity was measured. The result revealed varying strength of antioxidant potential in the crude extract and fractions of the plant. This outcome matched with the reported findings on antioxidant effect of several medicinal plants (Hussain et al. 2016). Phenolics are significant constituents of medicinal plants, which exert numerous therapeutic activities including free radical scavenging ability due to the presence of OH functional groups. Various studies have shown the comparative relationship between phenolics and anti-oxidant potential (Hussain et al. 2016). The hydroxyl functional groups in the skeleton of phenolic compound's structure might be responsible for high scavenging property of extracts and solvent fractions of *T. potatoria* investigated in this study. The total phenolic content was highest in ethyl acetate fraction. The antioxidant power of *T. potatoria* extract and solvents fractions increased as TPC increased. The quantity of phenolics and antioxidant effect of solvent fractions of *Tetracera potatoria* displayed comparable trends ($TPC_{Hexane} < TPC_{DCM} < TPC_{Ethyl\ acetate}$). Total flavonoid content values of ethyl acetate, DCM and hexane fractions of *Tetracera potatoria* had a well-defined trend ($TPC_{DCM} < TPC_{Hexane} < TPC_{Ethyl\ acetate}$). The results obtained support other research outcomes in which a parallel association between phenolics and antioxidant activity have been established (Zhang et al. 2014).

The data of the isolated compound corresponds with the reported data for 4′,5,7-trihydroxyflavone, also known as apigenin (Nabavi et al. 2015). The compound apigenin is reported for the first time in *T. potatoria*.

Cancer is one of the leading causes of death globally. In women with PCOS, chronic stimulation of estrogen sometimes leads to uncontrollable enlargement of endometrium and endometrial cancer (Gottschau et al. 2015). It has been

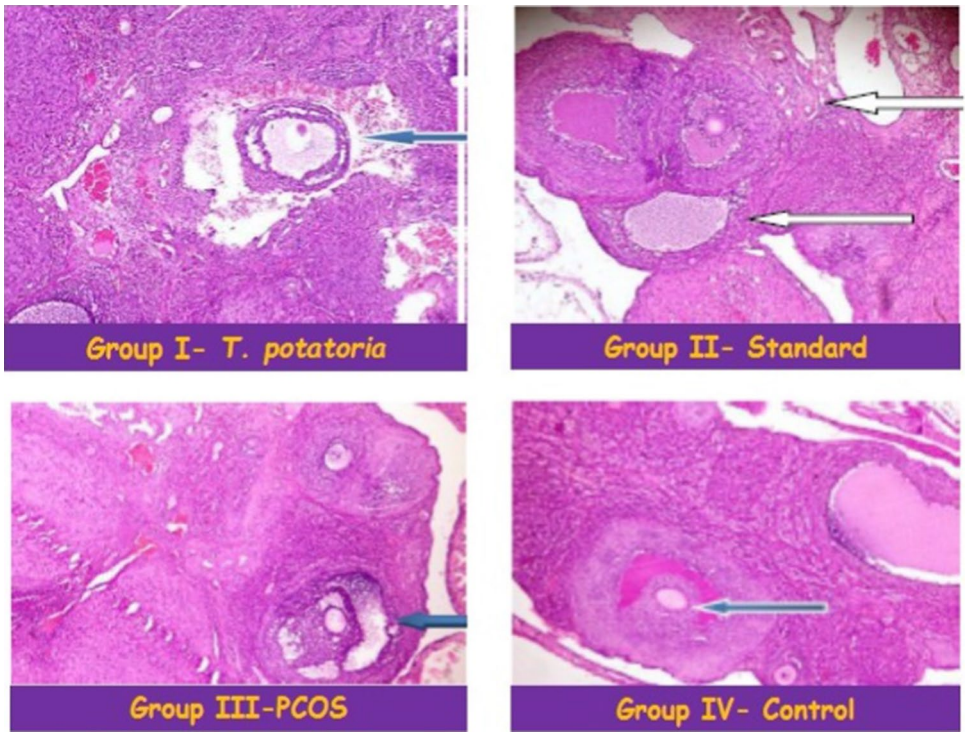

Fig. 3 Section of ovaries of normal rats, controls and rats treated with medicinal plant extract (x 100). Group I received 100 mg of *T. potatoria* extract per kilogramme body weight, Group II received l mg of clomiphene citrate (Colid, Pfizer pharamceuticals, USA) per kilogramme body weight. Groups III is untreated disease control. Group IV (normal control) received 2 mL of 5% w/v CMC in distilled water. Follicle = white arrow; thecal layer = blue arrow

reported that PCOS patients appear to have threefold probability of developing cancer of the endometrium but unaffected by breast cancer. However, PCOS patients have a 2.5-fold greater risk of ovarian cancer than normal control (non-PCOS control). Since abnormal *p53* tumour suppressor gene has been observed in the PCOS patients' endometrium (Shafiee et al. 2015), Chinese Hamster Ovarian (CHO l) cell, tumorigenic cell with characteristic abnormal *p*53 function (Hu et al. 1999) was selected for this study. Also, the human cervical cancer (HeLa) cell was selected for this study based on high global prevalence of gynaecological cancers among reproductive women, especially among women with PCOS (Sankaranarayanan and Ferlay 2006).

The hexane and DCM fractions of *T. potatoria* inhibited the proliferation of CHO-1 cells with 34.8 ± 0.3 and 41.3 ± 0.8 µg/mL IC_{50} values, respectively. The crude extract and solvent fractions had no inhibitory effect on HeLa cells proliferation. The standard (Doxorubicin) had IC_{50} values of 0.8 ± 0.0 l and 3.1 ± 0.2 µg/mL, on CHO l cell line and HeLa cell line, respectively.

Phenolic compounds have been widely investigated for their anti-cancer activities and low toxicity. One particular tumour suppressor gene codes for the protein p53 can induce cell cycle arrest in a DNA damage cell. Then, it can induce transcription of genes involved in DNA repair or, if the damage cannot be repaired, the p53 protein eventually initiates cell suicide, thereby preventing the DNA damaged cell becoming a mutated cell. Interestingly, some flavonoids have shown ability to induce cell cycle arrest at G1/S phase (Yoshida et al. 1992), at G2/M (Lian et al. 1998) or at both G1/S and G2/M phases (Traganos et al. 1992). Apigenin (**1**) was reported to suppress various human cancers in vitro and in vivo by multiple biological effects, such as triggering cell apoptosis and autophagy, inducing cell cycle arrest, suppressing cell migration and invasion, and stimulating an immune response (Cardenas et al. 2016). Recently, apigenin was reported to show anti-cancer activities by stimulating an immune response (Cardenas et al. 2016). The inhibitory effect of apigenin on the proliferation of HeLa and CHO cells was reported in earlier studies (Lepley et al. 1996; Souza et al. 2017). Furthermore, apigenin downregulated CK2α expression and inhibited the self-renewal capacity of sphere-forming cells in HeLa cells (Liu et al. 2015). These support our postulation that any bioactive compounds used for management and/or treatment of PCOS should be able to inhibit proliferation of CHO cells, a non-cancerous cell which possesses one of the main characteristics found recently in PCOS patients' endometrium i.e. abnormal *p53* function (Hu et al. 1999; Shafiee et al. 2015).

Polycystic ovary syndrome remains the commonest hormonal syndrome disturbing womenfolk globally. Variation in LH potentiates severe consequence on the estrous stage of rats. The regulation of estrus cycle is hindered by uncontrollable synthesis of LH hormone evident in PCOS condition (Zangeneh et al. 2012). Androgen surpluses and elevated level of LH are the primary biological irregularity in PCOS patients, while hyperandrogenemia usually manifest at pubertal age (Kakadia et al. 2018). Animal models used for

PCOS studies include neonatal androgenization, administration of estradiol valerate, human chorionic gonadotropin (HCG) administration to hypothyroid rats and maintenance of animals in constant light. None of these models are able to generate PCOS conditions with convincing data mimicking the PCOS conditions in human (Kakadia et al. 2018). Letrozole, which inhibits the action of aromatase inhibitor, yields a PCOS model with features which in several ways portray human-like PCOS condition. It blocks change of androstenedione and testosterone conversion to estrone and estradiol, respectively and mimics PCOS-like condition by effecting circulating hyperandrogenism, hormonal imbalance and intra ovarian androgen excess resulting in manifestation of polycystic ovary. Abnormal follicular development and follicular atresia are detected as a result of constant upsurge in the level of androgen in the ovary. Letrozole also causes hyperglycaemic condition, which may trigger insulin resistance, hyperlipidaemia and associated metabolic syndrome (Choi et al. 2015). Although medicinal plants are effective in restoring menstrual cycle and endocrine disorder among women with PCOS (Zhao 2011), effects of plants such as *T. potatoria* on hormonal imbalance, polycystic ovary conditions and associated risk of gynaecological cancers in PCOS patients have not been extensively investigated.

Oral administration of Letrozole for 21 days influences reproductive cycle irregularity in albino rats as observed in the present study. There was no chance of estrous and proestrous phases observed in rats after treatment with letrozole. The increase in the level of endogenous testosterone has been identified as the main culprit of PCOS. The fluctuations observed in the rat menstrual phase could be related to variations in the concentration of endogenous sex hormones as well as gonadotrophins. These sex hormones regulate the characteristics of the ovaries, hormonal imbalance and follicular maturation, which might initiate irregular oestrous cycle, causing malfunctioning of ovaries (Sun et al. 2013). *Tetracera potatoria* improved the estrous cycle by increasing the appearance of estrous phase and decreasing the period of diestrous phase as compared to untreated PCOS group.

The elevated level of luteinizing hormone (LH) present in most PCOS affected women is connected to the mechanisms associated with high level of circulatory androgen, exposure of the ovarian theca and granulosa cells to LH as well as amplified levels of cAMP. Variation in LH potentiates severe consequence on the estrous stage of rats. The regulation of oestrus cycle is hindered by uncontrollable synthesis of LH hormone evident in PCOS condition. Morphological changes in the ovaries of PCOS rats induced by letrozole are shown by the existence of numerous cysts with hyperplasia in the theca cells as well as thickened capsule of the ovaries. Subcapsular cysts enclosed with a layer of granulosa cells might also be detected. These histopathological features are due to availability of therapeutic levels of FSH, increased LH, and loss of interaction between granulosa and theca cells (Kafali et al. 2004). In our study, *Tetracera potatoria* influenced reduction in the level of LH and increased FSH. Also, a low circulatory estradiol level was found in letrozole-induced PCOS rats. However, treatment with *T. potatoria* extract at 100 mg/kg b.w. increased the estradiol level.

The level of estradiol in *Tetracera potatoria* was 9.36 ± 2.06 pg/mL compared to and PCOS rats, which had 5.70 ± 0.77 pg/mL. Treatment with *T. potatoria* leaf had curative effect on irregular estrual cycle and hormonal imbalance associated with PCOS. Restoration of estrus irregularity and follicular generation to normal following administration of *T. potatoria* could be the physiological effect exerted by phytochemical constituents in the extracts, which uphold the steroidal prestige, allowing fertility to be recuperated.

Conclusion

The antipoliferative effect of the hexane and DCM fractions of *Tetracera potatoria* and its isolated compound **1** (apigenin) on Chinese Hamster Ovarian (CHO) cells is noteworthy. This isolated compound also inhibited human cervical cancer HeLa cells. The plant and its isolated constituent could provide basis for the development of newer antiproliferative agents

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Authors contribution ADO and MAS contributed to the study conception and design. Material preparation, data collection and analysis were performed by all authors. MAS supervised the complete project. AJ, FS and SFS hosted part of the study. The first draft of the manuscript was written by ADO and all authors wrote the manuscript. All authors read and approved the final manuscript. All data were generated in-house and no paper mill was used.

Compliance with ethical standards

Ethical statement Ethical clearance for animal experimental work was obtained from Animal Care and Research Ethics Committee of the University of Ibadan, Nigeria prior to the commencement of experiments (UI-ACUREC/19/0051).

Conflict of interest Akingbolabo Daniel Ogunlakin has no conflict of interest. Mubo Adeola Sonibare has no conflict of interest. Almas Jabeen has no conflict of interest. Farzana Shaheen has no conflict of interest. Syeda Farah Shah has no conflict of interest.

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