JIMMA UNIVERSITY

COLLEGE OF NATURAL SCIENCES

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DEPARTMENT OF CHEMISTRY

M.Sc THESIS

ON

PHYTOCHEMICAL INVESTIGATIONON OF THE LEAVES OF *Millettia ferruginea ssp. darasana* FABACEAE FAMILY AND EVALUATION FOR ANTIBACTERIAL ACTIVITIES

BY

MESERET CHEMEDA

OCTOBER, 2017

JIMMA, ETHIOPIA

PHYTOCHEMICAL INVESTIGATIONON OF THE LEAVES OF *Millettia ferruginea ssp. darasana* FABACEAE FAMILY AND EVALUATION FOR ANTIBACTERIAL ACTIVITIES

A THESIS SUBMITTED TO SCHOOL OF GRADUTE STUDIES JIMMA UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ORGANIC CHEMISTRY

BY

MESERET CHEMEDA BEZA

ADVISORS

TSEGAYE DEYOU (PhD)

MELKAMU FEYERA (M.Sc)

OCTOBER, 2017

JIMMA, ETHIOPIA

Declaration

I declare that this is my original work, except where the reference is made and has never been submitted anywhere for award of any degree in any university.

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Meseret Chemeda

This M.Sc Thesis has been submitted for examination with our approval as supervisors

_____

Tsegaye Deyou (PhD)

Department of Chemistry

Jimma University

_____

Melkamu Feyera (M.Sc)

Department of Chemistry

Jimma University

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Abbreviations/Acronyms

ATCC	American Type Culture Collection
CC	Column chromatography
COSY	Correlation Spectroscopy
DMSO	Dimethyl Sulfoxide
PE	Petroleum Ether
NMR	Nuclear magnetic resonance
1D NMR	One Dimensional Magnetic Resonance (NMR- ^{1}H)
2D NMR	Two Dimensional Magnetic Resonance (NMR-^{1}H, ^{13}C)
^{13}C-NMR	Isotope Carbon 13 Nuclear Magnetic Resonance
HSQC	Heteronuclear Single Quantum Correlation
HMBC	Heteronuclear Multiple-Bond Correlation
TLC	Thin-layer chromatography
UV	Ultra Violet
WHO	World Health Organization
AMR	Antimicrobial Resistance

Abstract

Bacterial infectious diseases are highly widespread and contribute to the global disease burden and kill many millions of people in developing countries including Ethiopia. The major problem of this health care is due to drug resistance. Therefore, in this study, phytochemical investigation and antibacterial evaluation of the leaf of *Millettia ferrugenia* sub species *darassana* was carried out. Accordingly, the leaf of *Millettia ferrugenia* ssp. *darasana* was exhaustively extracted with methanol/chloroform (1:1, v/v) by maceration at room temperature. The obtained crude extract was evaluated for their antibacterial activities against four bacterial strains (*Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa and Escherichia coli).*The crude extract had shown inhibition zone (11-12.5 mm) on selected bacterial strains. The obtained result has better on *Enterococcus faecalis* than other bacteria species compared to positive control (gentamycin). Based on these antibacterial activities, the resulting crude extract was subjected to chromatographic separation to afford three compounds (**1a-3a**). The identity of the isolated compounds were established as 2'', 2''-dimethylchromene,3',4'-methylenedioxy isoflavone (*Calopogonium isoflavone B)*, 2'', 2''-dimethylchromene, 5'-methoxy, 3',4'-methylenedioxy isoflavone and 7,2'-dimethoxy-4',5'-methylenedioxy isoflavone**,** based on their, 1D (^{1}H, ^{13}C) and 2D, (COSY, HSQC and HMBC) NMR data. All identified compounds were evaluated for antibacterial activity against four bacteria strains. Of these, compound **3a** has demonstrated to have better activity on the *Pseudomonas aeruginosa* bacteria which shows 16 mm inhibition zone compared to gentamycin. Thus, comparing to the standard gentamycin, the crude extract and isolated compounds showed lower zone of inhibition.

1. INTRODUCTION

1.1 Background of the study

Plants have a long history of use as traditional medicines and have become an outstanding and reliable source for an essential role in health care which are considered fewer side effects [1]. Over centuries of research, several medicinal plants have been proved to be effective in the treatment of wide range of simple to complex diseases [2]. A large proportion of the population of developing countries, use traditional medicine alone or in combination with Western drugs to treat a wide variety of health ailments [3]. In Africa, it is estimated that 75% of the rural population rely on herbal medicine for their primary healthcare due to their advantage of being readily available, cheaper, and the process of their preparation is often easier than formulating and producing synthetic drugs [4]. With the renewed interest from Western countries in herbal remedies, and the increasingly urgent need to develop new effective drugs, traditionally used medicinal plants have recently given the attention of the pharmaceutical and scientific communities [5].

Infectious diseases are diseases that cause mortality and morbidity in all regions of the world. The increasing emergence of antimicrobial resistance worsens the impact and shown the risk of negative clinical consequences [6]. Increased prevalence of resistant bacteria, together with lack and high cost of new generation drugs has escalated infection-related morbidity and mortality particularly in developing countries like Ethiopia [6, 7].

Paclitaxel (**1**) from *taxus brevifolia* plant and camptothecin (**2**) from *camptotheca acuminata* tree which used to treat both for different anticancer agents through bioactivity guided isolation methods [8].

Various researchers have been discovering new biologically active compounds from natural products for the most successful source of potential drug leads [9]. The antimicrobials drugs: *pittoviridagenin* **(3)** to treat different diseases such as Dandruff, wound, skin burn, and scabies were obtained from *Pittosporum viridiflorum* Sis [Pittosporaceae] plants and *Ethyl iso-allocholate* **(4)** used for TB and pneumonia, from *Bersama abyssinica* Fresen (Melianthaceae) [10].

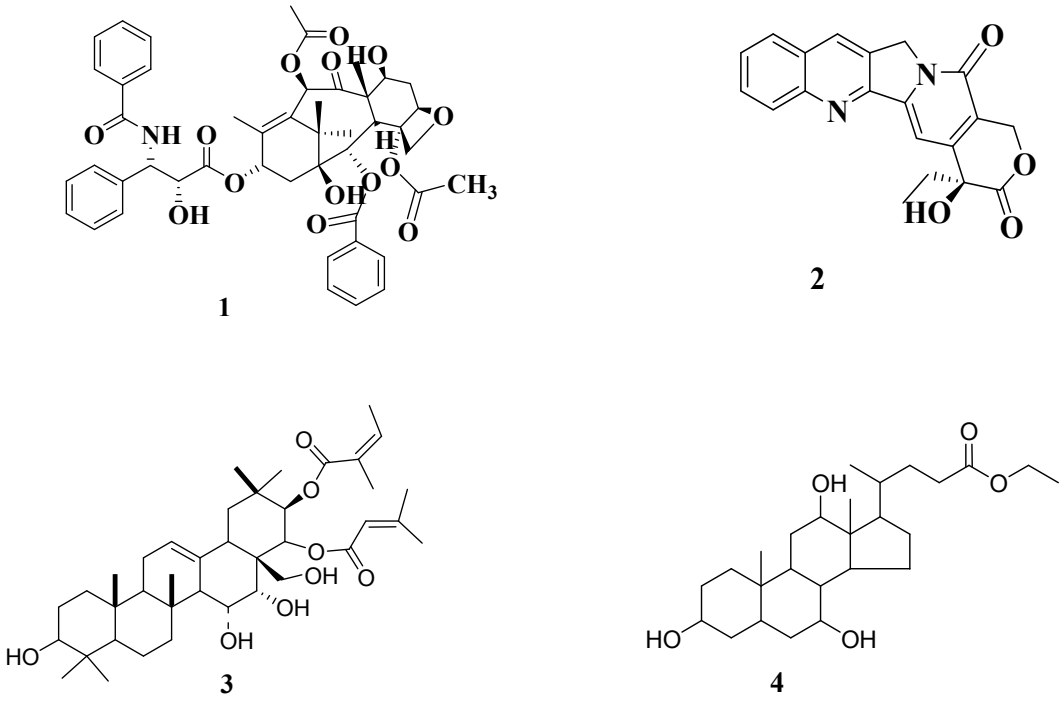
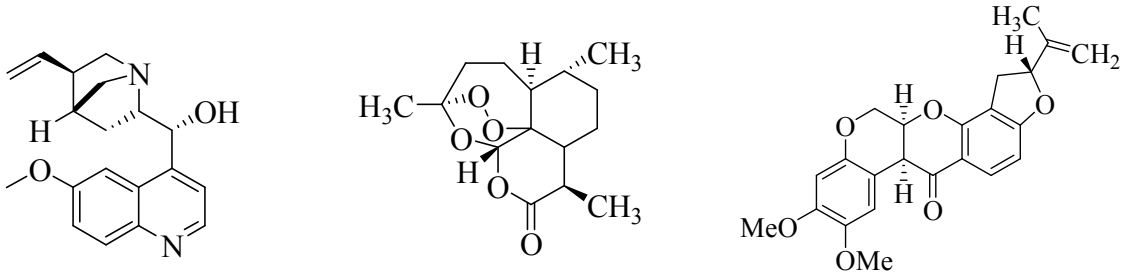


Figure 1 : New biologically active compounds from natural products

Most of the antimalarials were also derived from plants used by indigenous societies in different parts of the world [11]. For example, quinine **(5)** was identified as the active anti-malarial constituent of the *Cinchona* bark [12]. Whereas the newest anti-malarial, artemisinin **(6)**, was isolated from a Chinese traditional plant, Artemisia *annua* [13]. These active plant constituents have served as molecular templates for the development of related synthetic derivatives of quinine such as chloroquine, amodiaquine and primaquine are the most successful antimalarials that safe and more effective than the other molecules[14]. There is still a great potential for plants in the development of new drugs [15].

In developing countries, low-income people such as farmers, people of small isolated villages and native communities use folk medicine for the treatment of common infections [16].
The genus *Millettia* with the major metabolites being flavonoids and isoflavonoids including rotenone **(7)** has an important place in pharmacopoeias with wide ranges of therapeutic effects as antitumoral [17], anti-inflammatory [18], cytotoxicity [19], antiviral [20], insecticidal and trypanocidal [21] and antiplasmodial [22, 23].



5 **6** **7**

Figure 2: Antimalarials drug derived from plants

Moreover, in the search for biodegradable compounds with pesticides activities from plants, the larvicidal activities of rotenoids isolated from *Millettia usaramensis* ssp *usaramensis* have been also reported [22]. A number of investigations had been conducted on different parts of *Millettia ferruginea* sub species *darasana*, but no phytochemical investigation and/or evaluation for biological activities had yet done on the leaves of this plant. Thus, in this work, phytochemical investigation of the leaves of *Millettia ferruginea* ssp. *darasana* was undertaken. The isolated compounds and crude extracts were also evaluated for antibacterial activity against four pathogenic bacteria strain.

1.2 Statement of the problems

Bacterial infectious diseases are highly prevalent and contribute largely to the global disease burden and kill many millions of people in which this burden is serious in developing countries including Ethiopia [6]. The major contributor is the drug resistance, which has become a major clinical and public health problem in the world today. It has led to higher treatment costs, increased morbidity and mortality, and in some cases, permanent loss of specific drug therapies [7]. Moreover, due to less availability, accessibility and affordability of modern drugs coupled with its high cost and adverse effects have worsen the situation; the investigation of safe, more effective, affordable and readily available anti-bacterial agent is necessary to combat or minimize the current situation. Therefore, a continued investigation of medicinal plants and screening against their biological activities is still needed today specifically on those plants which have recognized traditional uses.

Millettia ferrugenia is one of an endemic *Millettia* species which only found in Ethiopia and known to synthesize prenylated flavonoids and isoflavonoids [32]. This plant used traditionally for the treatments of various infection diseases. For instance, it has been commonly used for gonorrhea, fishing or hunting poison and treatment of insects bit [60]. A various study has conducted by different researchers on this plant part for its health aliment. However, there was no much scientific information pertaining to the phytochemical isolation of leaf of *Millettia ferrugenia* and its pharmacological activities. Thus, the present work has focused on isolation, characterization and the evaluation of the bioactive molecules for their antibacterial activity from the leaf of the *Millettia ferrugenia* ssp. *darasana*.

1.3 Objectives of the study

1.3.1 General objective

The main objective of the study was to identify secondary metabolites from the leaf extracts of *Millettia ferruginea* ssp. *darasana* and evaluate their antibacterial activity.

1.3.2 Specific Objectives

The specific objectives of this study were:

- To isolate secondary metabolites from the leaf of *Millettia ferruginea* ssp. *darasana* using chromatographic techniques,
- To elucidate the structures of the isolated compounds using spectroscopic techniques,(1D and 2D NMR),
- To evaluate antibacterial activities of the crude extracts and isolated compounds against four bacterial strains: *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Enterococcus faecalis*

1.4 Significance of the study

Millettia species have been used in traditional medicinal practices to alleviate several aliments, with about 60% of the species are known for their medicinal uses [20]. In Africa particularly in Ethiopia, the use of indigenous plant has played an important role for the treatment of various infection diseases. As several study revealed that numerous medicinal plant has used herbal drug as remedies to treat several health ailments [60]. The family of Leguminosae, *Millettia ferrugenia* is traditionally used as an herbal fish poison and skin infection which caused by insect bites in Ethiopia.

The bioactive phytochemical constituents present in the plant plays a significant role in the development of medicines and drug discovery. However, still there is complex challenge to overcome the health problem due to their drug resistance, less availability, accessibility and affordability of modern drugs coupled with its high cost and adverse effects have worsen the situation. Taking into the account, the plant has a potential to synthesize prenylated flavonoids and isoflavonoids and may combat such complex health problems.

Therefore, the findings of the work would:

- ✓ Help to isolate the promising compound from leaf of *Millettia ferrugenia* which can be used as guide compound in the discovery of antibacterial agents.
- ✓ Provide important information on the chemical profile of the plant that easily available, accessible and affordable for further investigation of new herbal drugs.
- ✓ It also used as guide line for further isolation and purification of bioactive phytochemical constituents.

2. REVIEW OF RELATED LITERATURE

2.1 Infections Diseases

Infectious diseases caused by bacteria, viruses, fungi or parasites are one of the most challenges in human health due to their high incidence and outbreak rate [33]. It is one of the leading causes of morbidity and mortality worldwide, especially in developing countries [34]. The diseases are catastrophic to all persons, regardless of age, sex, lifestyle, ethnic background, and socioeconomic status. They cause suffering and death which impose a financial burden on society [35, 36]. The main reason is the pathogenic microorganisms are resistant to almost all antibiotics [37]. The diseases can be controlled and prevented by the modern medicines. However, bacteria in general, possess the genetic ability to acquire and transmit resistance to therapeutic agents [38]

Following the massive use of antibiotics in human therapy, bacteria have developed several resistance mechanisms including the efflux of antibiotics [39]. In addition to this problem, antibiotics sometimes associated with adverse effects on host, which include hypersensitivity, depletion of beneficial gut and mucosal micro-organism, immune suppression and allergic reactions [40]. This has created immense clinical problem in the treatment of infectious diseases and impact of death worldwide, causing over 13 million deaths each year [41]. As a result, there is a need to develop alternative antimicrobial drugs, which contribute to drug resistance in AMR bacteria [42]. AMR is the development of resistance in micro-organisms: bacteria, viruses, fungi and parasites to an antimicrobial medicine to which it was previously sensitive. AMR in wide range of infectious agents is a growing public health threat of huge concern to countries and too many sectors [43]. One approach is to screen local medicinal plants for possible antimicrobial properties. Plant materials are remaining an important resource to combat serious diseases in the world.

2.2. Botanical Information

2.2.1 The family Fabaceae

The family Fabaceae, also referred to as the Leguminosae, is the third largest family of flowering plants with 730 genera and over 19,000 species, most of which are shrubs and trees found in both temperate and tropical regions. It is also commonly known as the legume, pea or bean family referring to their typical fruit of these plants. Legumes include a large number of domesticated species harvested as crops for human and animal consumption as well as for oils, fiber, fuel, timber and medicinal production [44]. Leguminous plants are known for their ability to fix atmospheric nitrogen thus replenishing nitrogen deficient soils [45]. The Fabaceae family is subdivided into three sub-families, namely Papilinoideae, Caesalpinoideae, and Mimosoideae [46].

2.2.1.1 The Subfamily Papilionoideae

Papilionoideae is the largest of the three sub-families with over 500 genera and 14,000 species of tree, shrubs and herbs. Its root nodules are containing nitrogen-fixing bacteria and frequently with non-protein amino-acids in the seeds [47]. This sub-family is distinguished from the two other subfamilies by the presence of Papilionoideae flowers, a hilar valve of seeds, fruiting nature including floral development. The Papilionoideae sub-family is divided into 32 tribes. The tribes Tephroseae and Phaseoleae are known to produce prenylated flavonoids and isoflavonoids, some of which possess important biological activities [48]. The genus *Millettia* belongs to this subfamily.

2.2.1.2 The genus Millettia

The genus *Millettia* belongs to the tribe Tephroseae that is known to synthesize prenylated flavonoids and isoflavonoids. The genus has over 200 species worldwide and found widely distributed in tropical Africa, Asia and Australia. *Millettia* plants are trees, shrubs or lianas, or rarely semi-herbaceous plants with woody root stocks [26, 49]. For instance, in Kenya, the genus *Millettia* is represented with the number of species; such as *M. dura, M. lasiantha, M. leucantha, M. oblata, M. tanaensis* and *M. usaramensis* [50]. But *M. ferruginea* is well known agro forestry species only found in Ethiopia

2.2.1.2.1 Millettia Ferrugenia

Millettia ferruginea (Hochst) Baker (*M. ferruginea*) plants, with two distinct subspecies, namely, *ferruginea* and *darasana* belonging to Fabaceae (Leguminosae) family, are widely distributed within the Moist and Wet Weyna Dega agro-climatic zones [51]. The tree is commonly known as 'Berebera' (in Amharic), Sotallo, Kotalu, Sari, Yego (in Afan Oromo), Enghediksho (in Sidama), Zaghia (in Wolaita), Dhadhato (in Gedoffa) languages [52]. Flowering period usually extends from the end of February to the end of March. Its fruits are normally abundant from early June to end of December. Mostly from January to February, seeds are usually released by mechanical or explosive mechanisms from the pod to the ground [53].

Most tree lengths ranged from 25 to 30 meter and leaves are compound with the number of leaflets (A)**.** In most trees, the flower is large with mostly violent on the stalks and golden-brown in colour. The mature pod (B) is flat and pale brown in colour. The shape of the seeds (C) is semi round and nearly golden-brown in colour [53].

(A) (B) (C)

Figure 3: Plant parts of millettia ferrugenia: leaf (A), pod (B) and seed (C)

Millettia ferruginea usually grows in altitude ranging from 1000 to 2500 m above sea level [54]. The natural habitat of *Millettia ferruginea* is generally diverse as it has advanced mechanism of minimizing water loss which enabled it to utilize water in a very conservative manner [55]. It maintained higher tissue water status under mild, moderate and severe water stresses than the other species due to its ability to re-orient its leaves and leaflets during midday, thus avoiding direct solar irradiance. Different regions of Ethiopia including Tigray,

Gondar, Gojam, Shewa, Wollega, Hararghe, Bale, Jimma, Ilubabor, Kefa and Sidamo are commonly potential growing areas of *Millettia ferruginea* [56].

2.3 Ethno medicinal uses of the genus Millettia

Millettia species are used traditionally in different cultures globally. *Millettia* has important place in the pharmacopoeias of Sub-Saharan Africa with numerous therapeutic indications for those ailments. The multiplicity of these activities, well known in traditional medicine is being confirmed by pharmacological [20] studies. Tables 1 summarize few examples of the traditional medicinal uses of some *Millettia* species.

Table 1: Selected Ethno-botanical uses of the *Millettia* species

Species	Plant Part	Uses	Reference
M. auriculata	Leaves	Male infertility	[57]
	Roots	Fish poison, Pesticide and Vermicide	[50]
M. caerulea	Leaf and Stem	Reduce infection in cuts and burns	[51]
M. dura	Entire plant	Fish poison	[58]
M. extensa	Roots	Treat stomach pain	[59]
M. ferruginea	Roots	Treat gonorrhea	[60]
M. pachycarpa	Roots	Treat swelling	[61]
	Seeds	Fish poison	[62]
M. griffoniana	root and stem bark	Treatment of insects bits	[63]
M. pulchra	Roots	Eliminate inflammation, alleviate pain	[64]
M. aboensis	Leaf	used for ulcer healing and as laxatives	[65]

2.4 Phytochemical from the Genus *Millettia*

The genus *Millettia* is a rich source of different classes of flavonoids including isoflavonoids and also consists of minor compound such as coumarins, terpenoids and alkaloids [66].

Isoflavones constitute the largest group of natural isoflavonoids. It has been mostly found in the subfamily Papilionoideae of the Fabaceae family. However, the common emphasis of the fact that isoflavonoids are characteristic metabolites of leguminous plants sometimes leads to overlooking that the presence of isoflavonoids in other families. Previous studies of extracts

of *Millettia* species have led to the isolation of flavones, flavanones, chalcones, rotenoids and isoflavones [67]. .

i) Isoflavones

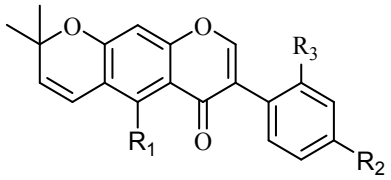
Isoflavones form the largest group of natural isoflavonoids. Among the isoflavonoids of this genus, isoflavones are the most predominant secondary metabolites [67]. Several isoflavonoids have been reported from different *Millettia* species (table 2). The majority of these isoflavonoids contain one or two prenyl group(s) as a side chain, either in acyclic or in cyclic forms, or as a combination of the two forms [68]. There are common structural features which form hydroxylation, geranylation, methoxylation, and methylenedioxy [69].

Table 2: Selected isoflavones isolated from *Millettia* species

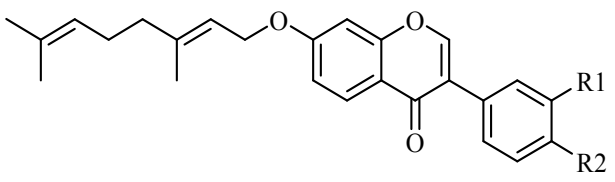
Compounds	Plant Species	Plant Parts	Reference
Alpinumisoflavone **(8)**	*M. thonningii*	Root, seeds and pods	[64]
2'-Methoxyalpinumisoflavone **(9)**			
5-methoxy-4'-O-(3-methyl-2-butenyl), Alpinumisoflavone **(10)**		Root bark, root wood	
7-O-Geranylpseudobaptigenin **(11)**	*M. dura* *M. grifoniana*	Stem bark Root bark	[68,74]
7-O-Geranylformononetin **(12)**			
Calopogonium isoflavone B **(13)**			
7,6'-Dimethoxy-3',4'-Methylenedioxyisoflavone **(14)**	*M. grifoniana*, *M. dura*	Root bark, Stem bark	[68,69]
3',4',6'-Trimethoxy-2",2"-dimethylpyrano[5",6"]isoflavone **(15)**	*M. ichthyochtona*	Leaves	[70]
(2',3',5'-Trimethoxyisoflavone) ;Barbigerone **(16)**	*M. usaramensis* ssp. *usaramensis*	Stem bark	[22]
Millewanin G **(17)**	*M. pachycarpa*	Leaves	[71]
5,6-Dimethoxydurlmillone **(18)**	*M. conraui*	Stem bark	[72,73]
Conrauinones A **(19)**			
6-Methoxydurlmillone **(20)**	*M. griffoniana*	Root bark	[74]

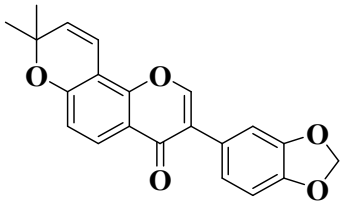
Jamaicin **(21)**			
Calopogoniumisoflavone A **(22)**	*M. dura*	Stem bark	[75]
6-methoxy calopogoniumisoflavone A **(23)**		Seed Pod	[76, 75]
6-Hydroxyldurallone **(24)**			
6-methoxydurallone **(25)**			
Durlettone **(26)**	*M. dura*	Seed,	[77]
Isojamaicin **(27)**	*M. ferruginea*	Stem bark	[53,81]
Milldurone **(28)**	*M. dura*		
Ferugone **(29)**	*M. ferruginea* ssp.	root bark,	[71]
Nordurlettone**(30)**	*darasana*	seeds stem	[32]
	M. grifoniana	bark	
Durlmillone**(31)**		Stem bark	[53]
7-O-Geranylformononetin **(32)**		Root bark	[32,74]
Ichthynone **(33)**		Stem bark	[53;78]

	R_1	**R_2**	**R_3**
8	OH	OH	H
9	OH	OCH_3	H
10	OCH_3	O-Prenyl	H



	R_1	R_2
11	$-OCH_2O-$	
12	H	$-OCH_3$





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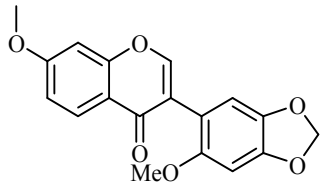

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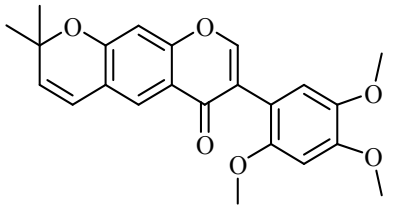

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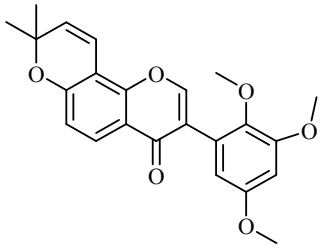

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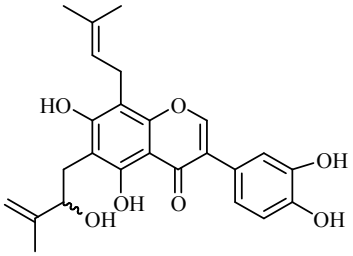

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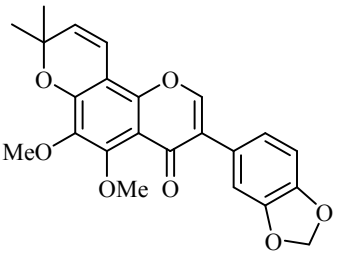

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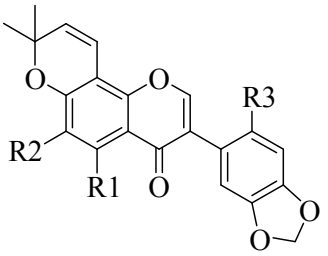
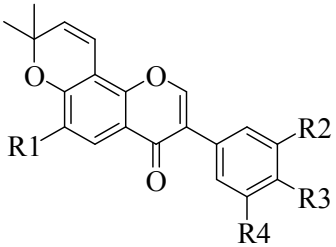
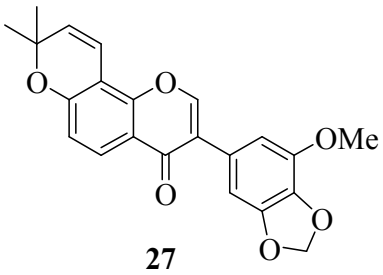
	R1	R2	R3
19	OCH_3	OH	OCH_3
20	H	OCH_3	H
21	H	H	OCH_3



	R1	R2	R3	R4
22	H	H	OCH_3	H
23	OCH_3	H	OCH_3	H
24	OH	OCH_3	OCH_3	H
25	OCH_3	OCH_3	OCH_3	H
26	OH	H	OCH_3	OCH_3





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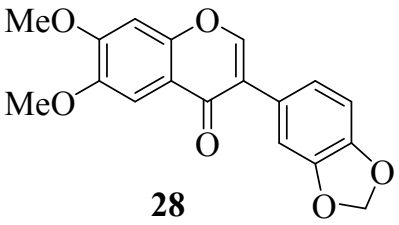

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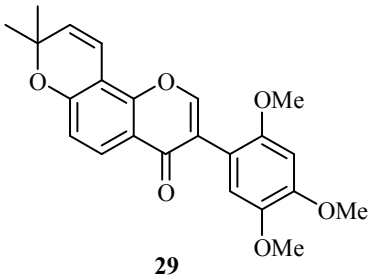

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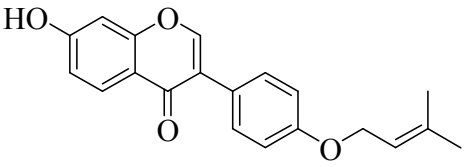

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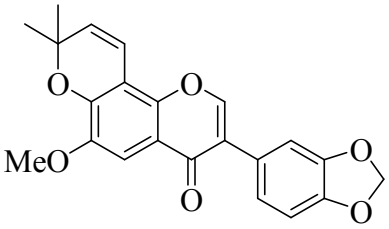

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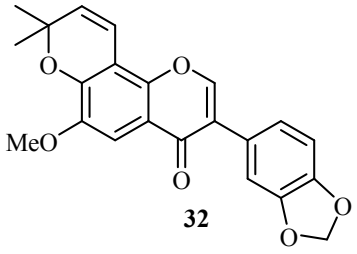

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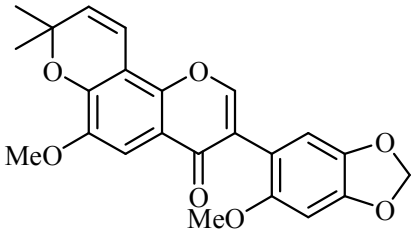

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Figure 4: Structure of isoflavone compounds

ii) Rotenoids

Rotenoids mainly occur in the seeds of *Millettia* plants. These compounds (table 3), especially rotenone (**7**), are considered to be responsible for insecticidal and piscicidal activities observed in the *Millettia* species [22, 76]. Most of the rotenoids previously characterized from these plants have cis-B/C junctions as in rotenone. However, rotenoids of reported from the stem bark of *Millettia usaramensis* have a novel *trans*-B/C ring junction with a (6a*R,* 12a*S*) configuration [77].

Table 3: Selected rotenoids isolated from *Millettia* species

Compounds	Plants Species	Plants Parts	References
12-Deoxo-12α- methoxylelliptone **(34)**	*M. duchenseni*	Twigs	[79]
6-Methoxy-6α, 12α- dehydrodeguelin **(35)**			
12α-Hydroxyelliptone **(36)**			
Elliptone **(37)**			
6α, 12α-Dehydrodeguelin **(38)**			
6-Hydroxy-6α, 12α-dehydro- α-toxicarol **(39)**			
Griffonianone A **(40)**	*M. griffoniana*	Root bark	[80]
Usararotenoid A **(41)**	*M. usaramensis*	Stem bark	[25]
Rotenone **(42)**	*M. ferruginea*	Seeds	[77,81]
Deguelin **(43)**	*M. dura,* *M. ferrugenia*		

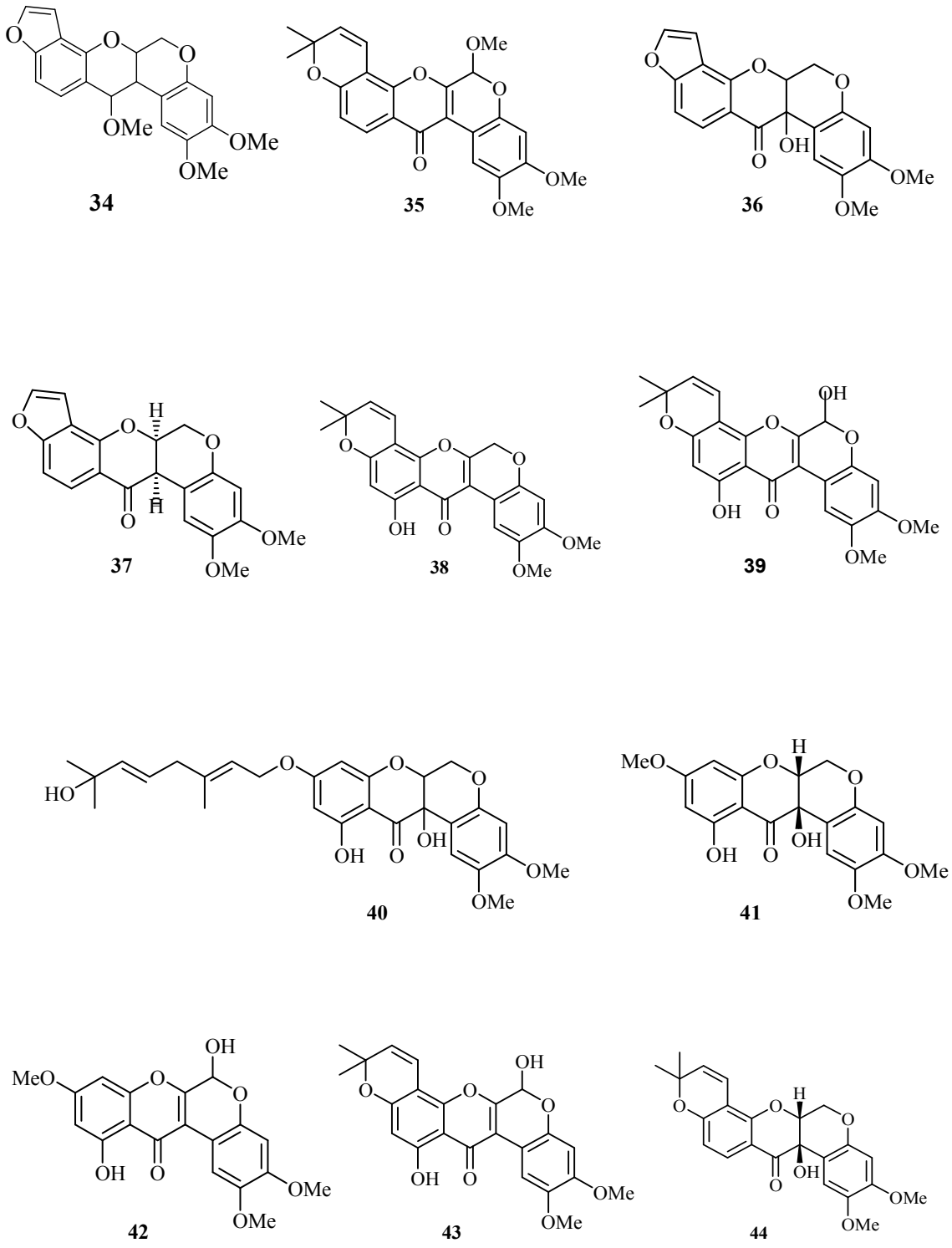


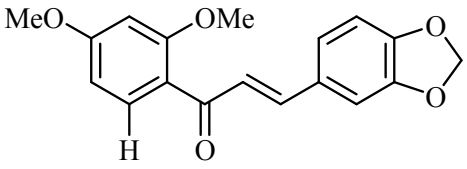
Figure 5: Structure of rotenoids compounds

iii) Chalconoids

Most identified chalcones have prenyl, geranyl or methylenedioxy substituents incorporated in their structures (table 4).

Table 4: Selected chalconoids isolated from *Millettia* species

Compounds	Plant Species	Plant Parts	References
3,4- methylenedioxy- 2',4'-Dimethoxychalcone **(45)**	M *leucantha*	stem bark	[82]
4'-O-Geranylisoliquiritegenin **(46)**	*M. ferruginea* ssp *darassana,* *M. usaramensis* ssp *usaramensis*	root bark, stem bark	[25, 32]
4'-Hydroxylonchocarpin **(47)**	*M. dura* *M. pachycarpa*	Stem bark, seeds	[77, 83]
2'- hydroxy-3, *4*-dimethoxy-[2", 3": 4', 3']- furanochalcone **(48)**	*M.erythrocalyx*	Seed pods	[84]
Lonchocarpin **(49)**	*M.pulchra*	Root Bark	[64,82]
2',4',6'-trimethoxy-3,4-methylenedioxydihydrochalcone **(50)**	*M. leucantha*	stem bark	



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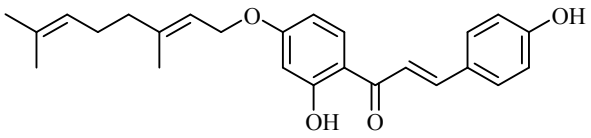

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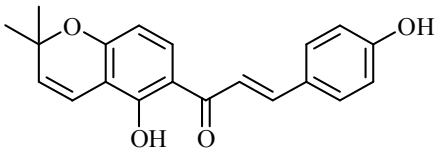

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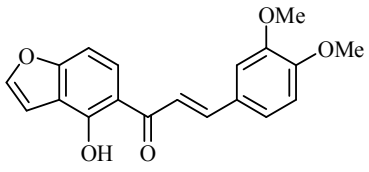

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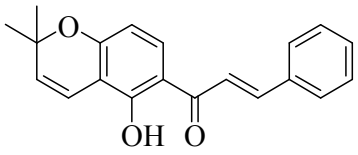

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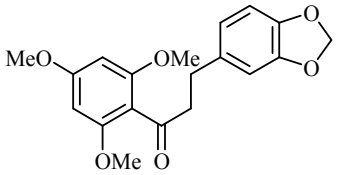

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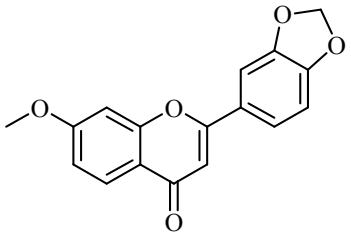
Figure 6: Structure of chalconoids compounds

iv) Flavones

In all cases the furan-ring is on ring A and mostly at 7, 8-position. The only exception to this is pongamol **(60)** from *M. penguensis* which has the furan-ring at 6, 7-position. Some of the flavones of *Millettia* species have listed in table 5.

Table 5: Selected flavones isolated from *Millettia* species

Compounds	Plants Species	Plant Parts	References
7- methoxy-3', 4'- Methylenedioxy flavone **(51)**	*M. leucantha*	stem bark	[82]
Lanceolatin **(52)**	M *sanagana*	root bark	[85]
Kanjone **(53)**			
5-Methoxyfurano[7,8:4",5"]flavone **(54)**			
Sanaganone **(55)**			
Desmethoxykanugin **(56)**	M *leucantha*	stem bark	[82, 85]
Karanjin **(57)**			
6-Hydroxy-2,2-dimethylpyrano-[5",6":8,7]flavone **(58)**	*M.pulchra*	Root bark	[64]
Millettocalyxin B **(59)**	*M. erythrocalyx*	Stem bark	[86]
Pongamol **(60)**	*M.penguensis*	Leaf	[87]



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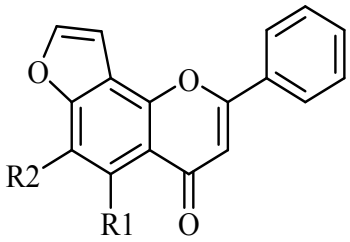
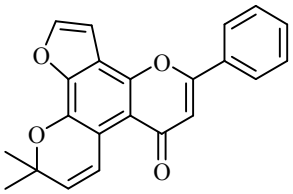


	R1	R2
52	H	H
53	H	OCH_3
54	OCH_3	H



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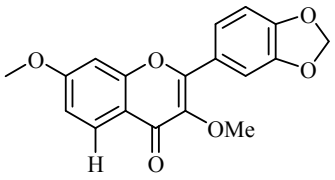

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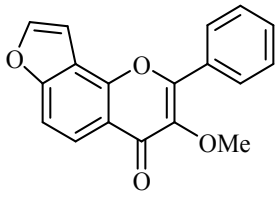

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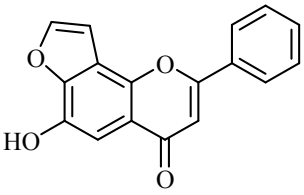

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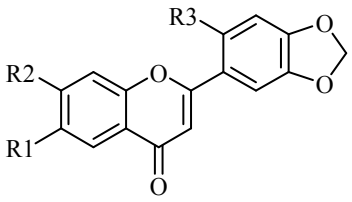

	R1	R2	R3
59	H	OCH_3	OCH_3
60	O-Prenyl	OCH_3	H

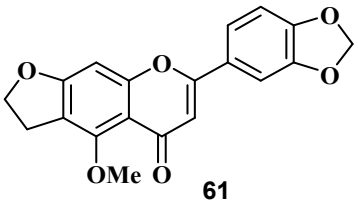

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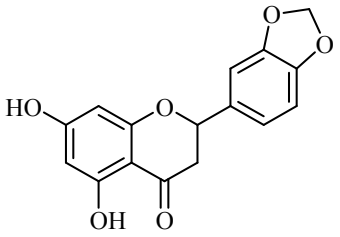
Figure 7: Structure of flavone compounds

v) Flavanones

All the flavanones were characterized from the genus *Millettia* and which are prenylated with lack of oxygenation at C-5 position which lists some of the flavanones reported from *Millettia* (table 6). This is considered to be typical of the flavonoids of the family Fabaceae [52].

Table 6: Selected flavanones isolated from *Millettia* species.

Compounds	Plants Species	Plant Part	References
7-Hydroxy-3',4'-Methylenedioxyflavanone **(62)**	*M. ovalifolia*	Seed	[88]
4'-Hydroxyisolonchocarpin **(63)**	*M. ferruginea*	Stem bark	[53]
Ponganone **(64)**	*M. erythrocalyx*	Root bark	[86]
(−)-(2*S*)-6,3′,4′-trimethoxy-[2″,3″:7,8]-furanoflavanone **(65)**		Seed pods	[85]
Eriodictyol **(66)**	*M. duchesnei*	Ariel part	[79]



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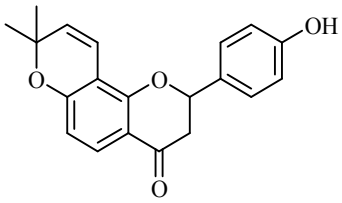

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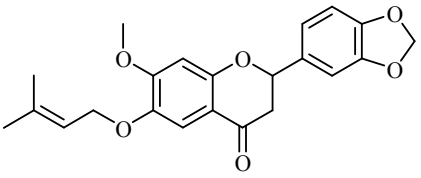

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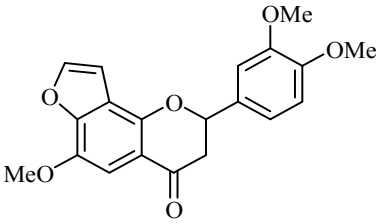

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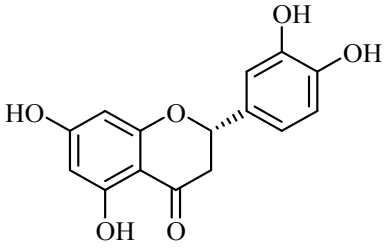

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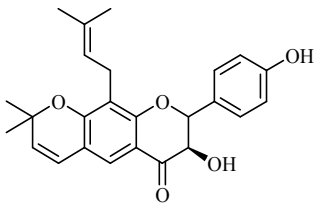
Figure 8: Structure of flavanones compounds

vi) Flavanonols

Two flavanonols namely Lupinifolol **(67)** from *M. pachycarpa* and 7, 4'-Dihydroxy-8, 2', 5'-triprenyldihydroflavanol **(68)** from *M. pulchra* **[**89**].**and 7, 4'-Dihydroxy 8, 3', 5'-triprenyldihydroflavanol from *M. pulchra* ***(*****69**)have been isolated from the *Millettia* genus.



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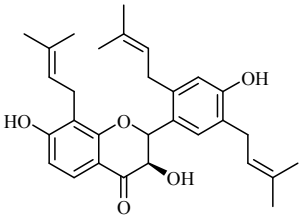

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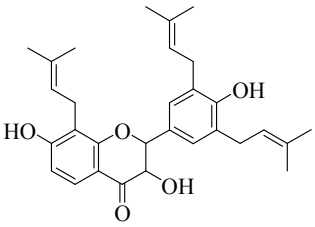

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Figure 9: Structure of flavanonols compounds

vii) Pterocarpans

Pterocarpans are derivatives of isoflavonoids described as benzo-pyrano-benzenes which can be formed by coupling of the B ring to the 4-one position [48]. Although, it contains two chiral centers **[**49**],** only the configurations of (6a*R,* 11a*R)* and (6a*S,* 11a*S)* are sterically (**70**) possible. They are generally bear structural resemblance to isoflavonoids that possess antibiotic activity and are produced by plant tissues in response to infection in (table 7) .

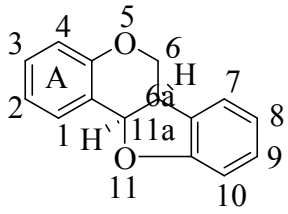

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Figure 10: Skeleton of Pterocarpans

Table 7: Selected Pterocarpans isolated from *Millettia* species

Compounds	Plant Species	Plant Part	Reference
(-)-Maackiain **(71)**	*M. pulchra*	Root Bark	[89]
(-)-Pterocarpin **(72)**			
6α-Methoxypterocarpin **(73)**			
Brandisianin F **(74)**	*M. brandisiana*	leaves	[90]
Medicarpin **(75)**	*M. leucantha*	wood	[19]
4-Hydroxy-3-methoxy-8,9-methylenedioxypterocarpan **(76)**			
2-benzopyrano [4,3-b]furo[2,3h][1]benzopyran- 6(8H)-one**(77)**	*M. pulchra*	root bark	[64]

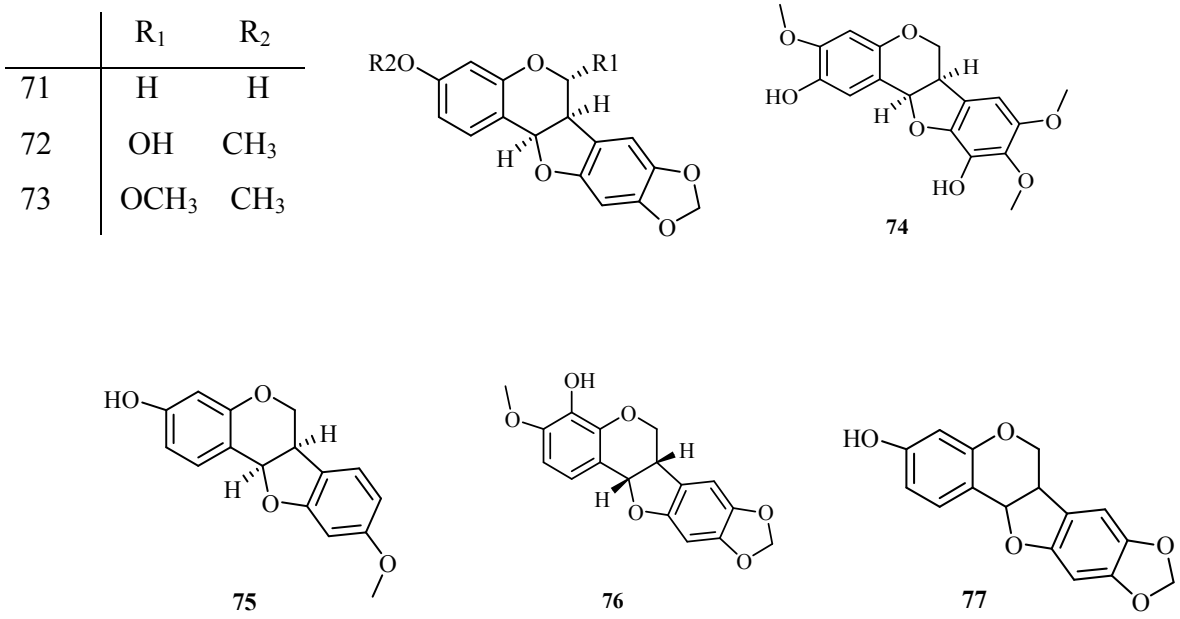


Figure 11: Structure of Pterocarpans compounds

viii) Minor Compounds of the Genus *Millettia*

Some minor compounds including Alkaloids, Coumarins and Terpenoids have been also reported from the genus *Millettia* (table 8).

Table 8: Selected minor compounds from *Millettia* species

Compounds	Plants Species	Plants Part	Reference
Alkaloids			
Millaurine **(78)**	*M. laurentii*	seeds	[91]
O-Acetylmillaurine **(79)**			
Millettonine **(80)**		stem bark	[92]
Coumarins			
Robustic acid **(81)**	*M. thonningii*	Seeds, root bark	[66,93]
Thonningine B **(82)**			
Pervilleanine **(83)**	*M. pervilleana*	root bark	[94]
Terpenoids			
Stigmasterol **(84)**	*M. versicolor* *M. mannii*	leaves; stem bark	[95,96]
Lupeol **(85)**	*M. mannii*	stem bark	[96]
Lupenone **(86)**			

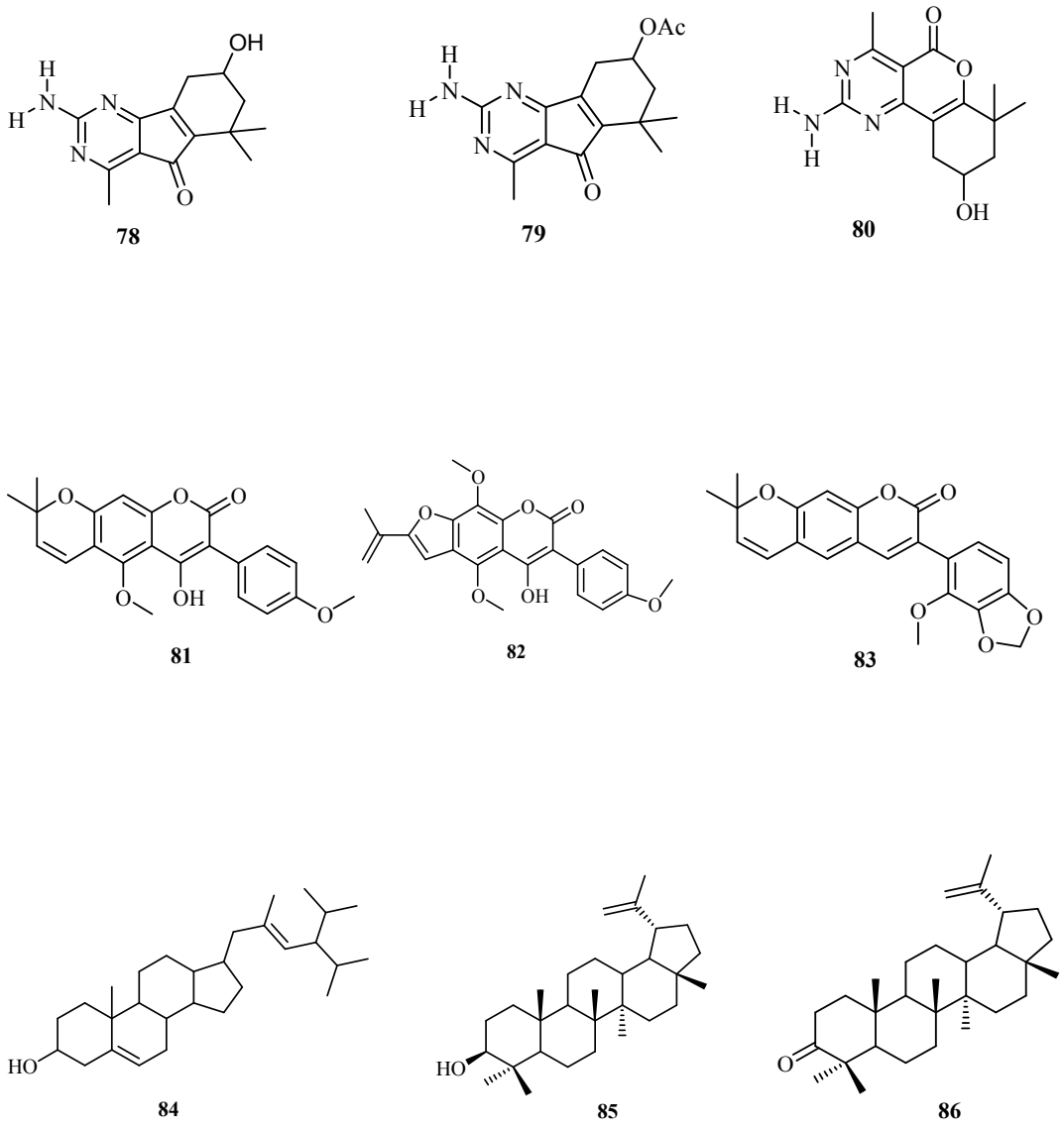


Figure 12: Structure of minor compounds from *Millettia* species

3. MATERIALS AND METHODS

3.1 Chemicals

All chemicals and reagents used for this study were analytical grade and are *n*-hexane petroleum ether, chloroform, methanol and ethyl acetate. They were used for extraction and chromatographic separation with silica gel 60-120 mesh size, distilled water, TLC plate coated with silica gel, iodine for detection of spots on TLC and dimethyl sulfoxide (DMSO) for dissolving sample were also used. Mueller Hinton agar and nutrient broth as culture media were used for antibacterial activity test.

3.2 Apparatus and equipment

NMR spectra were obtained on Bruker Avance 400 MHz (Kensington, NSW, 2052) spectrometer and the spectra were processed with the MestReNova 11.0 software, using residual solvent signal chemical shift as a reference. Rotary evaporator (Laborota, 4000, Heidolph, USA), UV-254 and 365 nm chamber (UV-Tec), column chromatography (300 mm (B-14/23, B-19/26) and 500 mm, B-34/35) were also used.

3.3 Collection of Plant Materials

The leaves of *M. ferruginea* ssp. *darasana* were collected in May 2016 from Limmu Kossa district, Genet, in Jimma Zone. The collected plant materials were air-dried under shade and powdered to suitable size to improve the subsequent extraction.

3.4 Extraction and isolation of plant material

3.4.1 Extraction

The powdered plant materials (500 gm) were soaked in $CHCl_3$ /MeOH (1:1, v/v) and exhaustively extracted three times (3x24 hrs), and followed by filtration in each case. The filtrate was concentrated using rotary evaporator at 50 ºc under reduced pressure to yield 72 gm of green brown crude extract.

3.4.2 Isolation of compounds

The 30 gm of crude extract was subjected to column chromatography (500 mm) filled with silica gel (150 gm) then eluted with petroleum ether in increasing amounts of ethyl acetate gradiently to give fractions. Based on TLC profile, fractions that have the same R_f value were

combined together. The purification of compound **1a** (40 mg) elueted with 3% EtOAc), was achieved by crystallization in methanol as colorless amorphous. Fractions collected with 5 % EtOAc were combined and gave compound **2a** (60 mg) by crystallization in methanol. Similarly the isolated **3a** (30 mg) was obtained from 9% EtOAc after these fractions were subjected to crystallization.

3.5 Test strain

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* A TCC 27853 and *Enterococcus faecalis* (ATCC 29212) were used for antibacterial evaluation of both crude extract and isolated compounds. The standard strains were obtained from Biology Department, Microbiology Research Laboratory, Jimma University. The bacterial strains were reactivated by sub culturing in nutrient broth at 37 ºc and maintained on nutrient agar slant at 4 ºc for further activity.

3.6 Antibacterial activity test

The antibacterial evaluation was undertaken following already established agar disk diffusion methods [97]. Briefly the bacteria stock cultures were maintained on the nutrient agar slants which were stored at 4 ºc. Agar cultures of the test microorganisms were prepared according to manufacturer's instruction. The test solutions were prepared by dissolving 15mg ratio of plant extracts dissolve 2 ml in DMSO. Freshly, grown liquid culture of the test pathogens solution of having similar turbidity with 0.5 McFarland were seeded over the Müeller-Hinton Agar medium with sterile swab. Sterile Whitman filter paper discs (~6 mm) were soaked in the prepared stock solution concentration of the extract and air dried to evaporate the solvent and then applied over the plates that contain both bacteria and Müeller-Hinton Agarat equidistance. Then, the plates take in to incubate at 37 ^{0}c for 24 hr. After the incubation period, the plates were takeout and measured the inhibition zone by transparent ruler to observe the antibacterial activity of the extracts. The inhibition zones formed on disk were measured in millimeter. Each experiment was carried out three times. The average of the inhibition zone of test sample was taken for evaluating the antibacterial activity of the extract.

4. RESULTS AND DISCUSSIONS

4.1 Compounds from leaf of *Millettia ferruginea* sub species darasana

The air dried powder (500 gm) of leaves of *Millettia ferruginea* ssp. *darasana* were exhaustively extracted with methanol/chloroform (1:1, v/v) by maceration at room temperature and yield 72 gm brown crude. The 30 gm crude extract was subjected to chromatographic separation to afford three compounds which are 2'',2''-dimethylchromene, 3',4'-methylenedioxy isoflavone (3 % of EtOAc, 40 mg), 5'-methoxy calopogonium isoflavone (5 % of EtOAc, 60 mg) and 7,2'-dimethoxy-4',5'-methylenedioxy isoflavone (9 % of EtOAc, 30 mg). All are amorphous colorless solid with R_f 0.63, 0.36 and 0.19 respectively. The structures of these compounds were determined by 1D and 2D (COSY, HSQC, HMBC) NMR and compared with reported literature.

4.2 Characterization of the isolated compounds

4.2.1 Compound 1a

Compound **1a** was obtained as a colorless solid with R_f value of 0.63 from PE/EtOAc (97:3 v/v). In ^{1}H NMR spectrum (table 9), the singlet peak at δ_H 7.92 coupled with carbon signals at δ_C 152.0 (C-2), at δ_C 125.8 (C-3) and 175.8 (C-4) in ^{13}C NMR is consistent for isoflavone skeleton for **1a**. The NMR spectra also revealed the presence of pyrano C-3'' (δ_H, 5.72, 6.81) for olefin with two methyl groups C-5'' (δ_H 1.50, *s*, 3*H*) and C-6'' (δ_H 1.25, *s*, 3H) and methylenedioxy δ_C 101.3 with δ_H 5.99(*s*, 2H) group as substituent. This singlet proton showed a ^3J HMBC correlation with carbon C-8a (152.5) and C-4 (175.8).

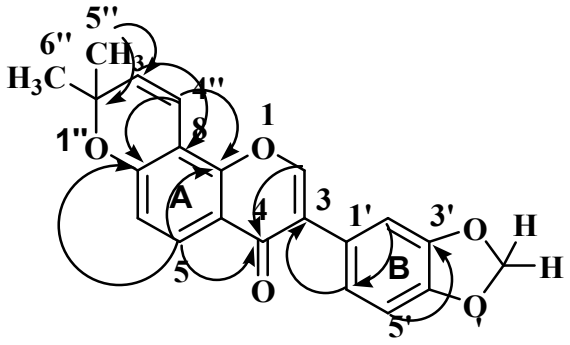
The ^{1}H NMR and COSY spectra showed, two *ortho*-coupled aromatic doublets at δ_H 8.06 (*d*, *J*=8)and δ_H 6.85 (*d*, *J*=12)are , respectively assigned to H-5 and H-6 of the ring A. . Furthermore three mutually coupled aromatic AMX-type protons were observed at δ_H 6.85 (1H, *d*, *J* =12), 6.87 (1H, *d*, *J* =8), 6.98 (1H, *d*, *J* =8), 7.92 (1H, *s*) and assigned to H-5', H-6' and H-2' of the ring B, respectively.

The attachment of a 2'', 2''-dimethylchromene was fixed at C-7/C-8 of the ring A based on the HMBC coupling of the two olefinic doublets (H-3" and H-4") with ring A carbon atoms, and the coupling nature of the H-5/H-6 protons of ring A. Whereas, the methylenedioxy

group was placed at ring B (C-3'/C-4') due to HMBC associations of its protons to the ring B carbon atoms (C-3'/C-4'). Based on these spectroscopic data, compound **1a** was identified as 2'', 2''-dimethylchromene, 3', 4'-methylenedioxy isoflavone (calopogonium isoflavone B). This compound was previously reported from the root bark of *M. grifoniana* [98].

Table 9: NMR spectroscopic data for compound **1a** in ($CDCl_3$ 400 MHz)

	Compound 1a observed		**Reported**		
Position	δ_H (*m*, *J* in Hz)	δ_C	δ_H (*m*, *J* in Hz)	δ_C	HMBC
1					
2	7.92 (*s*)	152.0	7.91(*s*)	151.9	C-4, C-8a
3		125.8		125.7	
4		175.8		175.7	
4a		118.4		118.3	
5	8.06 (*d, J=8*)	126.9	8.05(*d*, *J*=9)	126.7	C-4,C-7,C-8a
6	6.85 (*d*)	115.4	6.84(*d*)	115.2	C-5, C-8, C-4a
7		157.5		157.3	
8		109.3		109.2	
8a		152.5		152.3	
1'		125.0		124.8	
5'	6.87 (*d, J=8*)	108.5	6.86(*d*,*J*=7.8)	108.4	C-3',C-4'
6'	6.98 (*d*,*J=8*)	122.5	6.96(*d*,*J*=7.8)	122.4	C-3,C-2'
3'		147.8		147.6	
4'		147.8		147.6	
2'	7.09	109.5	7.08(d, *J*= 1.8)	108.4	C-6'
OCH_2O	5.99 (*s*)	101.3	5.98(*s*)	101.2	C-3',C-4'
1''					
2''		77.8		77.7	
3''	5.72 (*d, J=8*)	130.4	5.51(*d*, *J*=10.2)	130.3	C-8
4''	6.81 (*d, J=12*)	115.1	6.79(*d*, *J*=10.2)	114.9	
5''-CH_3	1.50(*s*)	28.3	1.49(*s*)	28.2	C-2'',C-3''
6''-CH_3	1.25(*s*)	28.3		28.2	



1a

Figure 13: HMBC correlation of calopogonium isoflavone B

4.2.2 Compound 2a

Compound **2a** was obtained as an amorphous solid with R_f 0.36 value from PE/EtOAc (95:5 v/v). From ^{1}H and ^{13}C NMR data (table 10) similarly, compound **2a** is an isoflavone derivative, having a methoxy (δ_H3.93 and δ_C 56.8), a methylenedioxy (δ_H 6.01 and δ_C 101.5) and a 2'', 2''-dimethylchromene groups having C-5''(δ_H 1.50, *s*, 3H) and C-6''(δ_H 1.25, *s*, 3H) with C-5'' (δ_C 28.3) and C-6''(δ_C 29.8) as substituents in table 10.

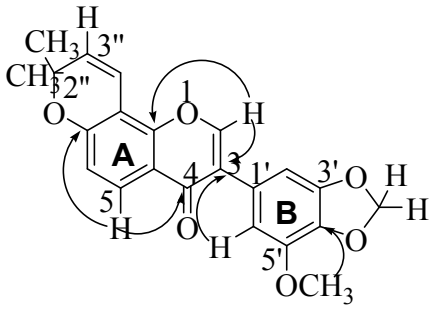
In ^{1}H NMR, the aromatic doublet at δ_H 8.06 was assigned to H- 5 owing to its HMBC correlations with δ_C 157.5 (C-7), 152.5 (C-8a) and 175. 8 (C-4) and deshielding effect of the carbonyl, consequently the doublet at δ_H 6.88 (J = 8 Hz), and H-5 coupling partner (from COSY spectrum) was assigned to H-6 of the ring A, which is substituted at C-7 and C-8. The proton at δ_H 6.80 of 2, 2-dimethylchromene system showed HMBC correlations with C-7 (δ_C 157.5) and C-8 (δ_C 109.0) on ring A suggesting that 2'', 2''-dimethylchromene group is located at C-7/C-8 on ring A. Furthermore, the ^{1}H NMR spectrum revealed two mutually coupled aromatic proton signals at δ_H 6.84 and 6.72, assigned to H-2' (δ_C 109.3) and H-6' (δ_C 103.4), respectively. This proton (δ_H 6.84) showed HMBC correlation with its neighboring carbon atoms, C-2' (δ_C 103.4), C-3(δ_C 125.0) and C-4' (δ_C 135.5), while the one at δ_H 6.72 showed HMBC correlation with C-4'/C-6' to support their placement on ring B.

The methoxy group (δ_H 3.93; δ_C 56.8) was assigned to C-5' (δ_C 148.9) based on HMBC correlation with this carbon. The methylenedioxy group was therefore placed at C-3' (δ_C 135.5) and/C-4' (δ_C 143.7). The NMR data of **2a** (table 10) is in close resemblance with

those of **1a** except the occurrence of a methoxy group in the former in ring B at C-5'. Therefore, the compound **2a** was named as is 2'', 2''-dimethylchromene, 5'-methoxy-3', 4'-methylenedioxyisoflavone.

Table 10: NMR spectroscopic data for **2a** in ($CDCl_3$ 400 MHz)

Compound 2a			
Position	δ_H (*m*, *J* in Hz)	δ_C	HMBC
1			
2	7.94 (1H,*s*)	152.2	C-3, C-4 , C-8a
3		124.9	
4		175.9	
4a		118.4	
5	8.06 (1H,*d, J=8*)	126.9	C-4,C-7,C-8a
6	6.88 (1H,*d, J=8*)	115.5	C-8, C-4a,
7		157.5	
8		109.0	
8a		152.4	
1'		126.5	
2'	6.72 (1H, *s*)	103.4	C-4',C-6'
3'		135.5	
4'		143.7	
5'		149.0	
6'	6.84 (1H, *s*)	109.3	C-3,C-2', C-4'
1			
2	6.01 (2H, *s*)	101.5	C-4',C-5'
1''			
2''		77.9	
3''	5.73 (1H, *d, J=12*)	130.5	C-8,C-2''
4''	6.81 (1H, *d,J=8*)	115.0	
5''	1.50 (3H, *s*)	28.3	C-2'',C-3''
6''	1.25 (3H, *s*)	29.8	
$-OCH_3$	3.93 (3H, *s*)	56.8	C-4'



2a

Figure 14: HMBC correlation of 5'-methoxy calopogonium isoflavone

4.2.3 Compound 3a

Compound **3a** is solid amorphous with R_f value 0.19. From NMR data (table 11), it consists of two benzene ring, two methoxy group, one olefinic group, one methylene dioxy and carbonyl group, which confirmed that the compound is isoflavone derivative.

The NMR spectra (Appendix 3a and 3b) shown that **3a** accommodating two methoxyl (δ_H 3.91; δ_C 56.9 and δ_H 3.66; δ_C 56.6) and methylenedioxy (δ_H 6.01; δ_C 101.2) groups as substituents.

In ^{1}H NMR (Appendix 3a) further indicated that the presence of an aromatic protons AMX spin system at δ_H 7.98*(d, J*=8), 7.08*(dd, J*= 4) and 7.15*(d, J* = 4) were observed on ring A and assigned to H-5, H-6 and H-8, respectively, with C-7 (δ_C 163.6) being substituted with methoxy group δ_H 3.91, δ_C 56.1) as it was established from HMBC correlation of the methoxy protons with C-7. These two aromatic doublet protons have HMBC correlation with C-4, C-7and C-8a, and C-4a and C-8 respectively. Those assigned protons were also confirmed by HSQC correlation.

One of the protons in the AMX spin system is highly de shielded which assigned to H-5 due to more electron withdrawing group i.e., carbonyl group. This implies the AMX aromatic protons are in ring A and two singlet aromatic protons (δ_H = 6.88, H, *s* and 6.83, H, *s*) are attached at ring B. On the ring A, one methoxy group at C-7 which also agrees with the biogenetic oxygenation of this position. The second methoxy and methylenedioxy groups were consequently placed in ring B. In order to allow for the presence of two singlets and the biogenetic oxygenation of C-4', the methoxy and methylenedioxy groups were placed at C-2' and C-4'/C-5', respectively. This placement is also further supported by ^{13}C NMR data (δ_C

152.8, 147. 9, 140.3) respectively. The singlet proton at ring B which assigned at C-3' has HMBC correlation with C-1'but no correlation with proton on C-6' as shown from NMR data. From NMR spectra, the protons which assigned to C-5 and C-6 on ring A are *ortho*-coupled aromatic doublets at δ_H (7.98 and 7, 08) and shown in their COSY spectra.
Therefore, Compound **3a** was identified as 7, 2'-dimethoxy-4', 5'-methylenedioxyisoflavone, previously isolated from stem bark of *Millettia dura* [26, 98].

Table 11: NMR spectroscopic data for **3a** in ($CDCl_3$, 400 MHz)

	Observed NMR data		**Reported NMR data**		
Position	**δ_H (*m*, *J* in Hz)**	**δ_C**	**δ_H (*m*, *J* in Hz)**	**δ_C**	**HMBC**
1					
2	8.22 (1H, *s*)	154.6	8.19 (1H, *s)*	154.0	C-4,C-8a,C-1',C-3
3		121.8		122.1	
4		174.3		175.7	
4a		117.5		118.4	
5	7.98 (1H, *d, J=8)*	126.9	7.89 (1H, *d*)	127.8	C-4,C-7,C-8a
6	7.08 (1H, *d, J=4)*	114.7	6.98 (1H, *dd*)	114.3	C-4a,C-8
7		163.6		163.8	
8	7.15 (1H, *s)*	100.6	6.98 (1H, *d*)	100.1	C-4a,C-6
8a		157.5		157.9	
1'		112.7		112.2	
2'		152.8		152.9	
3'	6.88 (1H, *s*)	95.5		95.4	C-1'
4'		147.9		148.3	
5'		140.3		141.4	
6'	6.83 (1H, *s*)	111.0		111.1	
1''					
2''	6.01 (2H, *s*)	101.2		101.4	C-4',C-5'
-OCH_3(7)	3.91 (3H, *s*)	56.1		55.7	C-7
OCH_3(2')	3.66 (3H, *s*)	56.6		56.9	C-2'

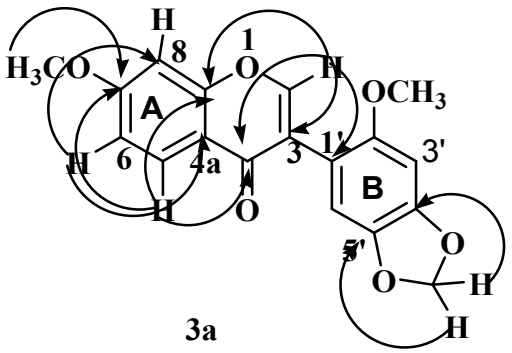


Figure 15: HMBC correlation of 7, 2'-dimethoxy-4', 5'-methylenedioxy isoflavone

4.3 Summery of Physical Properties and Spectroscopic Data for Isolated Compounds

(1) Calopogonium Isoflavone B

Amorphous with Rf. 0.63 values from PE/EtOAc (97:3 v/v). ^{1}H NMR δ (400 MHz, $CDCl_3$) 1.50 (3H, *s*,) , 5.72 (1H, *d*, 3'''-H, *J*=8), 5.99 (2H, *s*), 6.81 (1H, *d*, 4''-H, *J*=12), 6.85 (1H, *d*, 6-H), 6.87 (1H, *d*, 5'-H), 6.98 (1H, *dd*, 6'-H, *J*=8),7.92 (1H, *s*, 2-H) and 8.06 (1H, *d*, 5-H,*J*=8). ^{13}C NMR δc (MHz, $CDCL_3$) is 28.28 (C-2'''), 101.3 (C-2''), 108.5 (C-5'), 122.5 (C-6'), 109.5 (C-2'), 115.4(C-6), 115.1(C-4''), 130.4 (C-3''), 118.4 (C-4a), 125.8 (C-3), 125.0 (C-1'), 147.8 (C-3'), 147.8 (C-4'), 152.0 (C-2), 152.5 (C-8a), 157.5 (C-7) and 175.9 (C-4).

(2) 5' methoxy Calopogonium Isoflavone

White gray solid amorphous with R_f 0.36 value. 1H NMR δ_H (400 MHz, $CDCL_3$) 1.5 (3H, *s*, 5'''-H, 1,25 (3H, *s*, 6''-H), 3.93 (3H, *s*, 5'-OCH_3), 5.73 (1H, *d*, 3''-H, *J*=12), 6.0 (2H, *s*, 2'-H), 6.72 (1H, *s*, 2'-H), 6.80 (1H, *d*, 4''-H,*J*=4), 6.84 (1H, *s*, 6'-H), 6.88 (1H, *d*, 6-H),7.94 (1H, *s*, 2-H), 8.06 (1H, *d*, 5-H, *J*=8). For ^{13}C NMR δc (MHz, $CDCL_3$); 28.3 (C-5'''), 29.85 (C-6'''), 56.8 (C-5'), 77.9 (C-2'''), 101.5 (C-2''), 103.4 (C-2'), 109.0 (C-8), 109.3 (C-6'), 115.0 (C-4'''), 115.5 (C-6), 118.4 (C-4a), 124.9 (C-3), 126.5 (C-1'), 126.9 (C-5), 130.5 (C-3'''), 135.5 (C-4'), 143.7 (C-3'), 149.0 (C-5'), 152.2 (C-2), 152.4 (C-8a), 157.5 (C-7) and 175.9 (C-4).

(3) **7,2'-dimethoxy-4',5'-methylenedioxy Isoflavone**

Colorless (white) solid amorphous with R_f 0.19 value.^{1}H NMR δ (400 MHz, $CDCL_3$): 3.66 (3H, *s*, 2'-OCH_3), 3.91 (3H, *s*, 7-OCH_3), 6.01 (2H, *s*, 2''-H), 6.83 (1H, *s*, 6'-H), 6.88 (1H, *s*, 3'-H),7.08 (1H, *d*, 6-H, *J*=4), 7.15 (1H, *d*, 8-H),7.98 (1H, *d*, 5-H, *J*=8). 8.22 (1H, *s*, 2-H).^{13}C NMR δc (MHz, $CDCL_3$) is 56.1(C-7-OCH3), 56.9 (C-2'-OCH3), 95.5 (C-3'), 100.6 (C-8), 101.2(C-2''),111.0 (C-6'), 121.8 (C-3),114.7(C-6),117.5 (C-4a), 112.7 (C-1'), 126.9 (C-5), 140.3 (C-5'), 147.9 (C-4'), 152.8 (C-2'),154.6 (C-2),157.5 (C-8a), 163.6 (C-7) and 174.3 (C-4).

4.4 Antibacterial Activity Test

The methanol/chloroform (1:1 v/v) extracts and three isolated compounds; **1a, 2a and 3a** were evaluated for antibacterial activity on four bacterial strains; *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli (*ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) employing the disk diffusion method. The zone of inhibition measured in millimeter was presented in table 12 for 0.15 mg/ml concentration. The inhibition zone for crude extract and isolated compounds is analyzed against standard positive control.

Table 12: Antibacterial activity of crude extract and isolated compounds from *Millettia ferrugenia* leaf

Bacterial strain	**Gram Strain**	**Crude Extract**	**Inhibition zone of isolated Compounds (mm)**			**Controls**	
			C-1a	C-2a	C-3a	G	DMSO
S. aureus	+ve	12	15	10	14	21	NI
E. faecalis	+ve	12.5	10	8	8	21	NI
P. aeruginosa	-ve	11	7	12	16	26	NI
E. coli	-ve	11	7	7	7	25	NI

Key: NI; Not inhibited, G; Gentamycin, C=compound, DMSO= Dimethyl Sulfoxide

The zone of growth inhibitions in the above table (**12**) are different based on the bacterial strains and test samples. The crude extract and all isolated compounds (**1a, 2a and 3a**) were showed considerable bacterial inhibitions zone comparing to the positive control. The crude extract shows good inhibition zone on gram positive bacteria (*Enterococcus faecalis)* when compared with the positive control. The compound **1a** show better effect on positive bacteria (*S.aureus)* than the other compared to positive control. Regarding gram strain negative; *Pseudomonas aeruginosa* on compound **3a** has shown good inhibition zone when compared to gentamycin.

5. Conclusion and Recommendations

5.1 Conclusion

In the present study, three compounds were isolated from leaf of *Millettia ferrugenia, darasana* sub species. These compounds are **1a, 2a and 3a** were chosen for characterization based on their TLC plate. It was fractionated and purified by column chromatographic separation with increasing gradient of ethyl acetate in petroleum ether. Among these, compound (**1a**) [68, 74] and (**3a)** [26, 98] were isolated from the root bark of *M. grifoniana* and stem bark of *M. dura* respectively. The isolated compounds from leaf extraction of darasana species were extracted earlier from other sub species of *Millettia* family indicate that the same compound was found in different sub species of the same genus.

The antibacterial activities of both the extract and isolated compounds were tested against four bacterial strains *Enterococcus faecalis, Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* using Agar disc diffusion method; meanwhile, the inhibition zone was measured. Besides, the calopogonium isoflavone compound is comparable effect on *Staphylococcus aureus*(15 mm) compared to positive control (21 mm) *and* 7, 2'-dimethoxy-4', 5'-methylenedioxy Isoflavone better potent(Appendixes 1) on *Pseudomonas aeruginosa* bacteria strain having 16 mm which compared to again positive control (26 mm).Whereas 5'-methoxy Calopogonium isoflavone **(2a)** compound has less effect on four bacteria strains.

Therefore, the *in vitro* antibacterial activity tests has shown that antibacterial activity of the isolated compounds from the leaf of *Millettia ferrugenia darasana* sub species to be the promising source of bioactive compounds that could be used as lead compounds in search of new clinically effective antibacterial compounds.

The characterized compounds were identified as calopogonium isoflavone (**1a**), 5'-Methoxylcalopogonium isoflavone **(2a)** and 7, 2'-dimethoxy-4', 5'-methylenedioxy Isoflavone (**3a)** was confirmed from the biosynthetic pathway. However, these three compounds were not reported so far from the *darasana leaf*.

5.2 Recommendation

Based on the current finding, further studies on the same plant are recommended as:

- ✓ The plant used as guide line for further isolation and purification of bioactive phytochemical constituents.
- ✓ Since the isolated compounds are class of isoflavone, developing the choice of extraction method; it is further carrying out on the antioxidant, antiplasmodial and antifungal activities for further study.
- ✓ Since the isolated compounds are class of isoflavone, developing the choice of extraction method and further studies out the antioxidant, antiplasmodial and antifungal activities of this plant are recommended for further study

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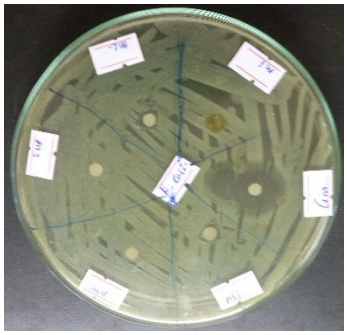
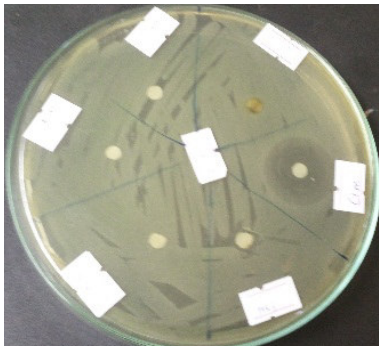
Appendices

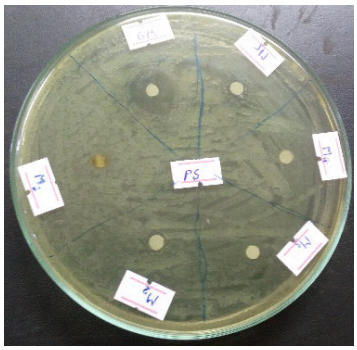
Appendices 1: Bioassays test of crude extract and isolated compounds' inhibition zone



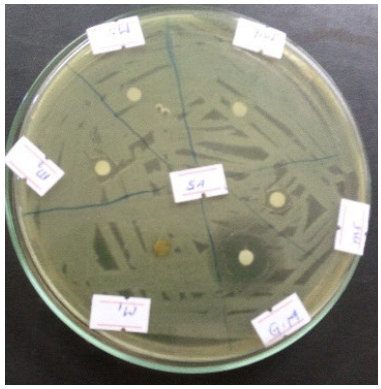
E.coli



E. Faecalis

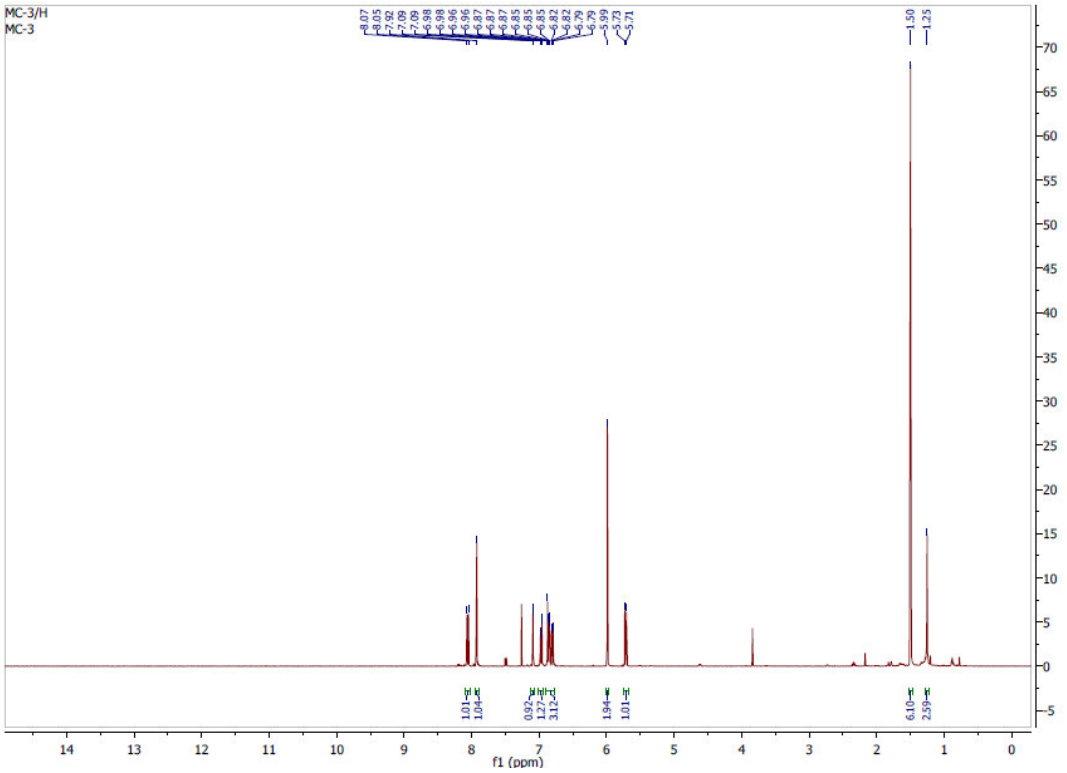


P. aeruginosa

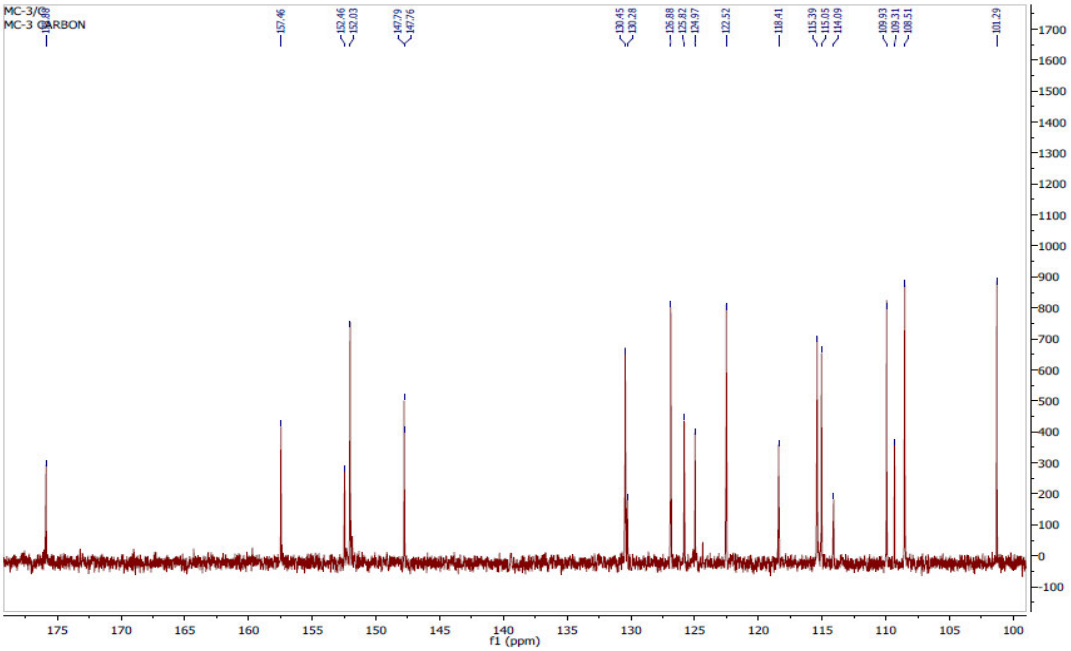


S.aureus

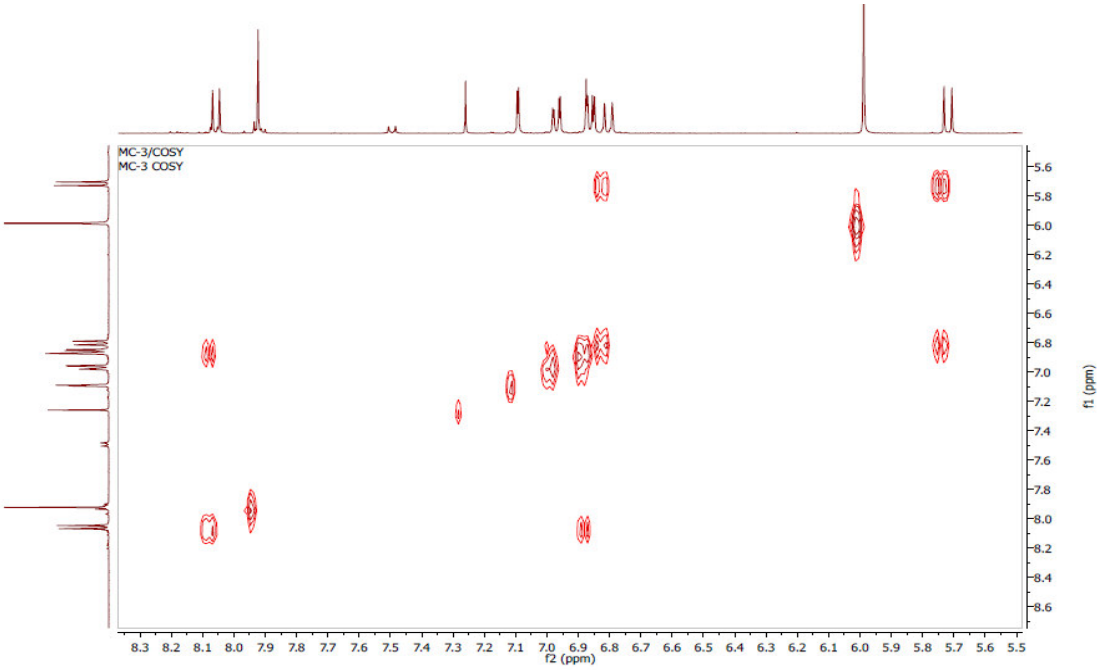
Appendices 2: 2a ^{1}H NMR Spectra of compound **1a** in $CDCl_3$



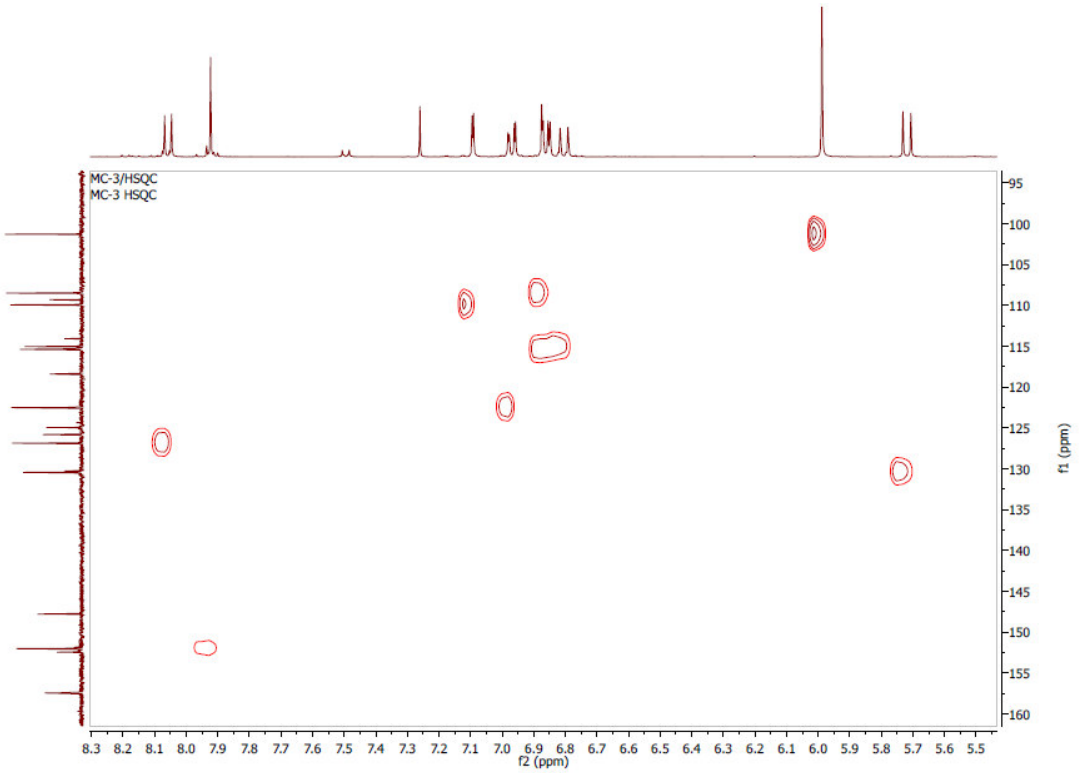
Appendices 3: 2b ^{13}C NMR Spectra of compound **1a** in $CDCl_3$



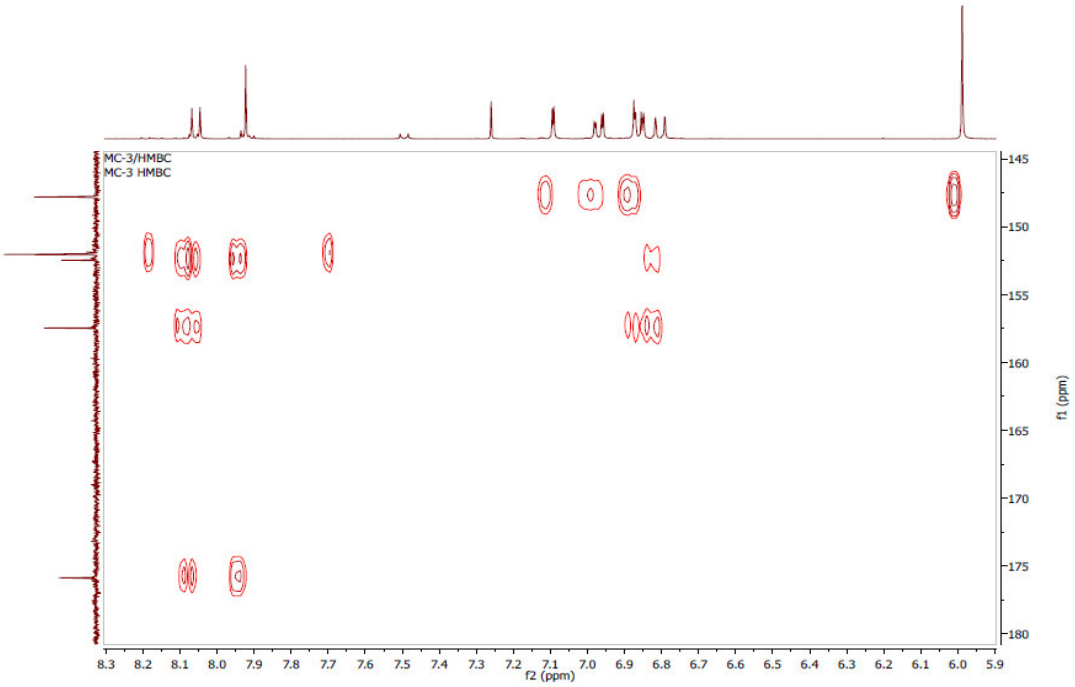
Appendices 4: 2c COSY Spectrum of compounds **1a** in $CDCl_3$



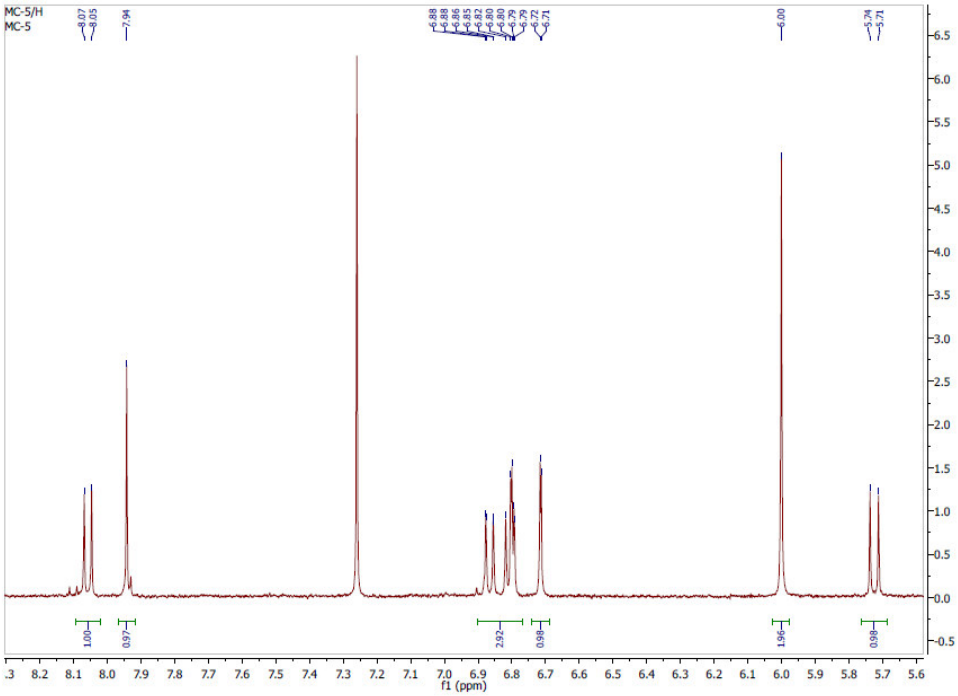
Appendices 5: 2d HSQC Spectrum of compound **1a** in $CDCl_3$



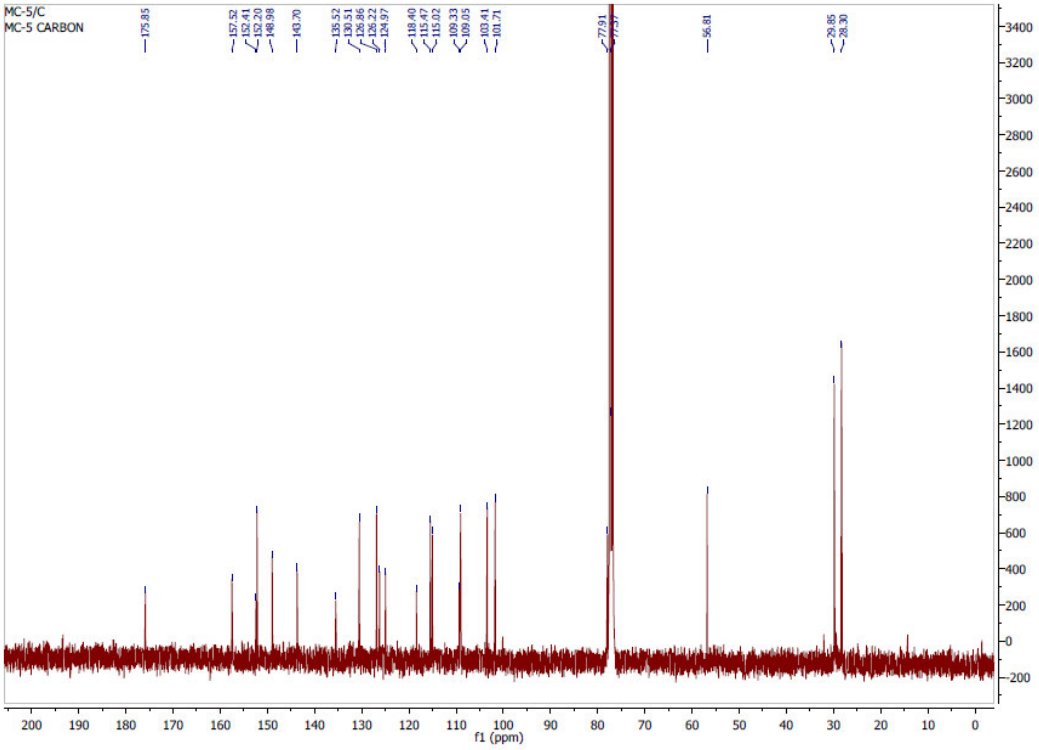
Appendices 6: 2e HMBC Spectrum of compound **1a** in $CDCl_3$



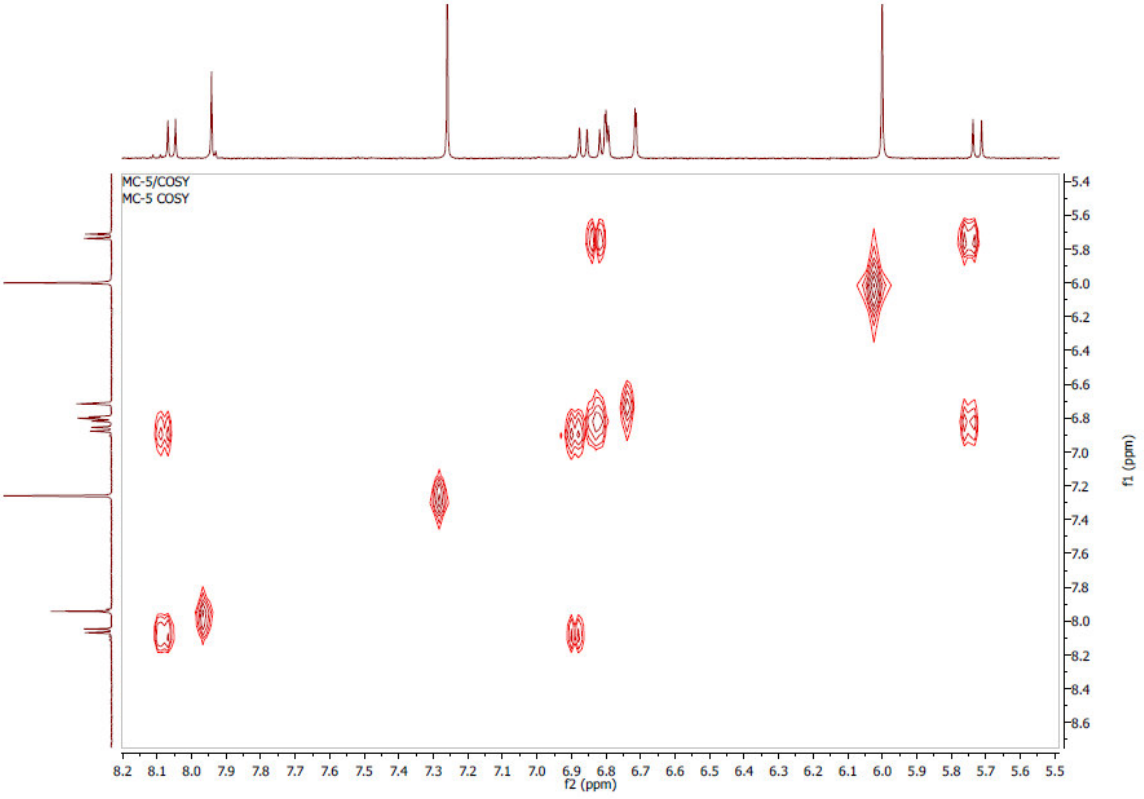
Appendices 7: 3a ^{1}H NMR Spectra of compound **2a** in $CDCl_3$



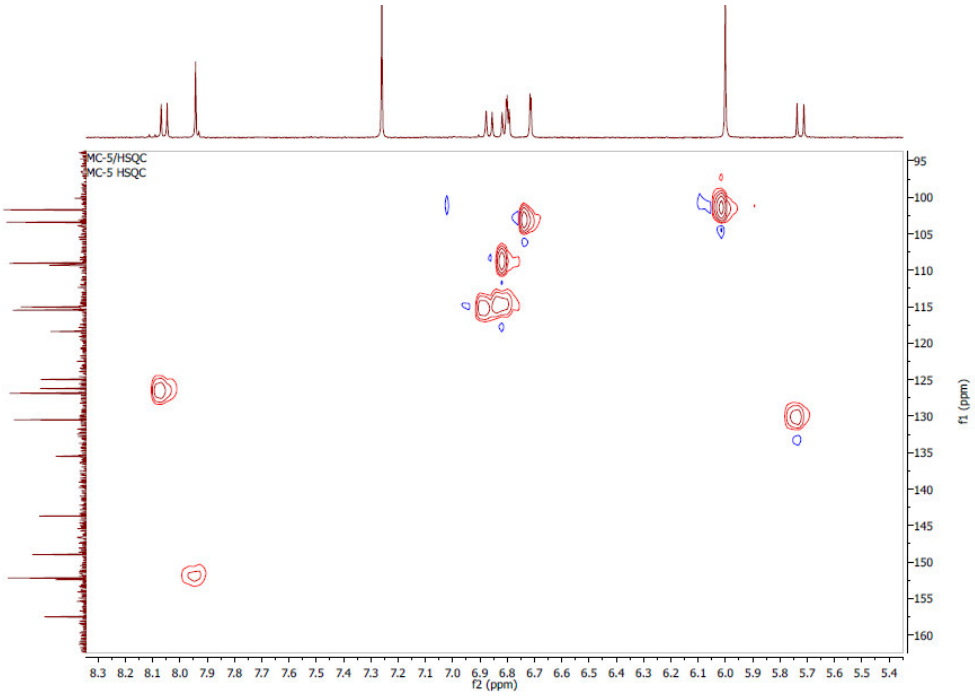
Appendices 8: 3b ^{13}C NMR Spectra of compound **2a** in $CDCl_3$



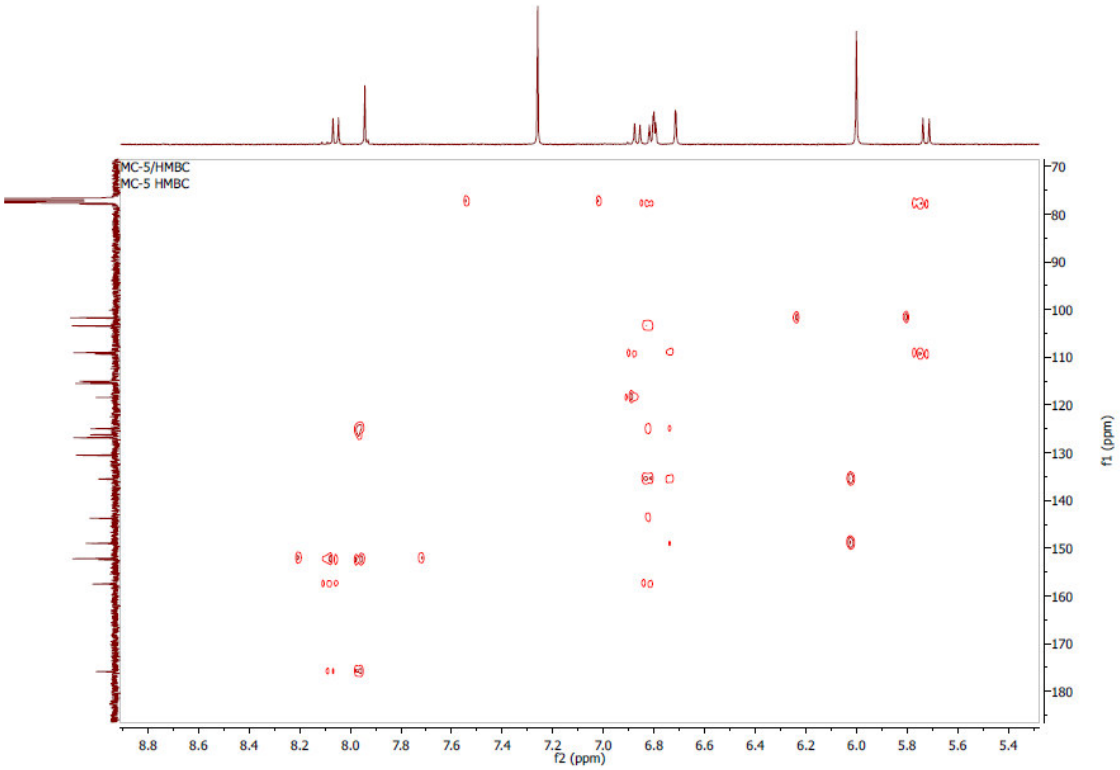
Appendices 9: 3c COSY Spectrum of compound **2a** in $CDCl_3$



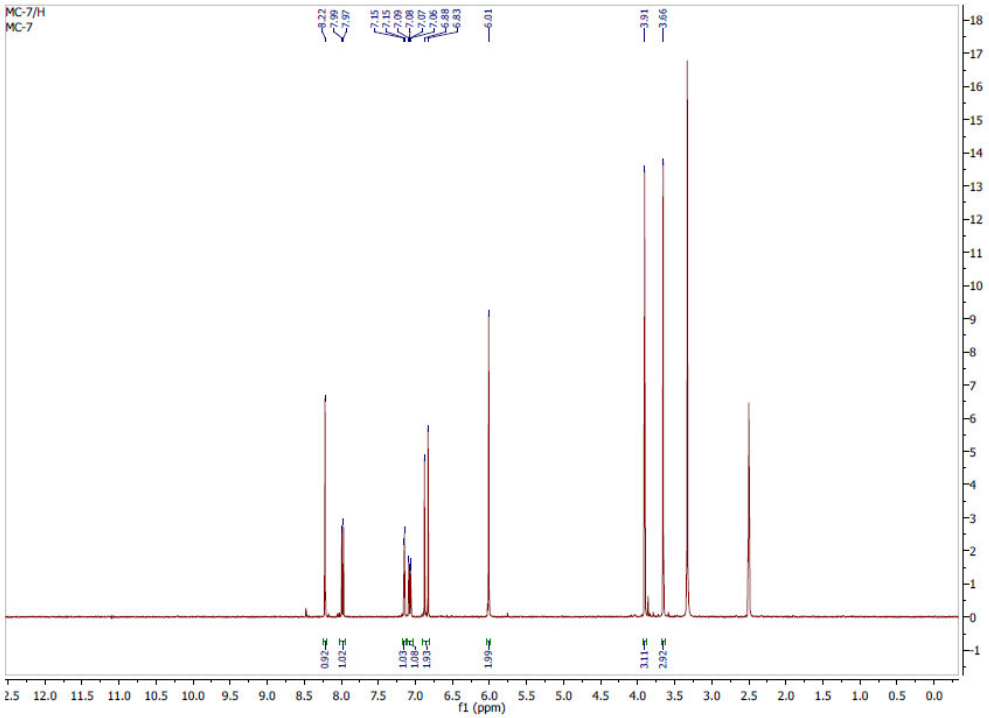
Appendices 10: 3d HSQC Spectrum of compound **2a** in $CDCl_3$



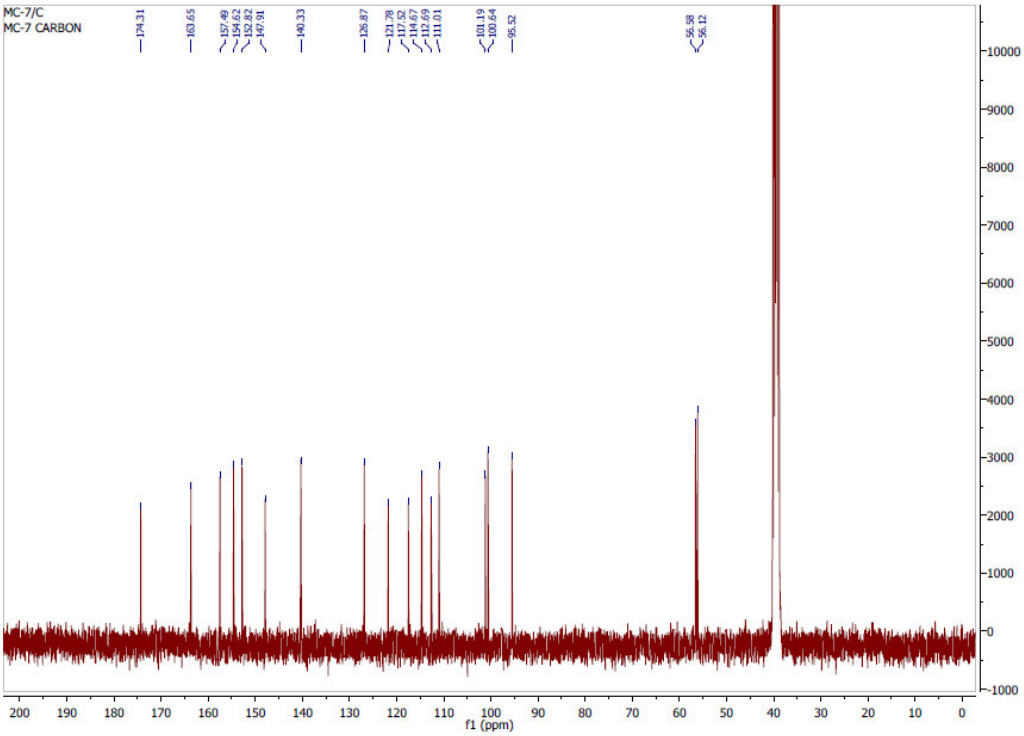
Appendices 11: 3e HMBC Spectrum of compound **2a** in $CDCl_3$



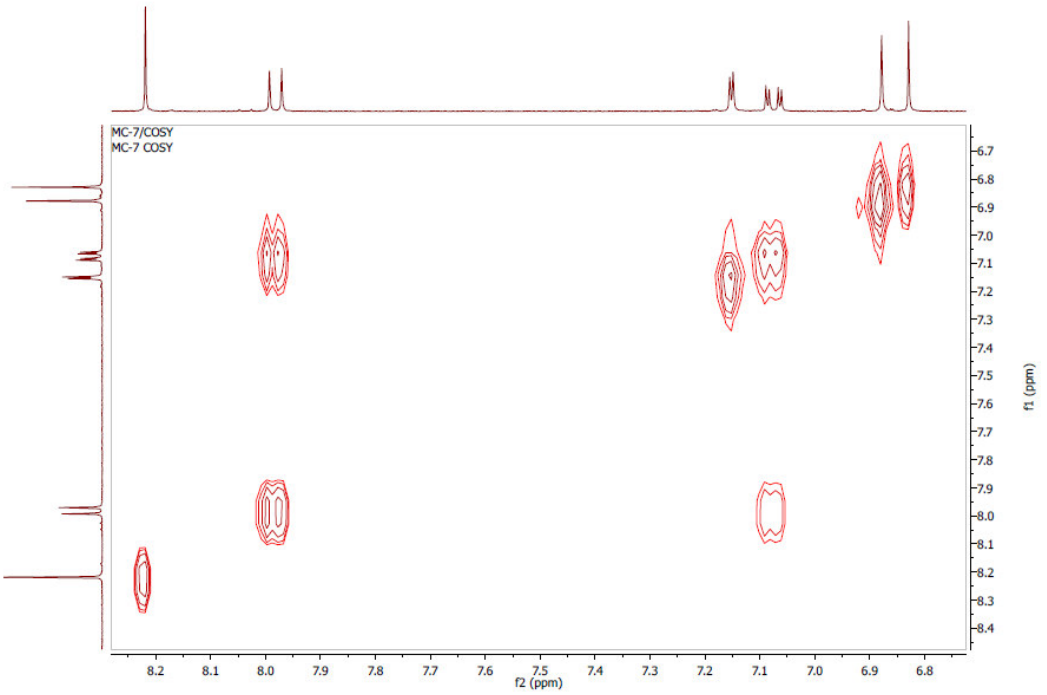
Appendices 12: 4a ^{1}H NMR Spectra of compound **3a** in $CDCl_3$



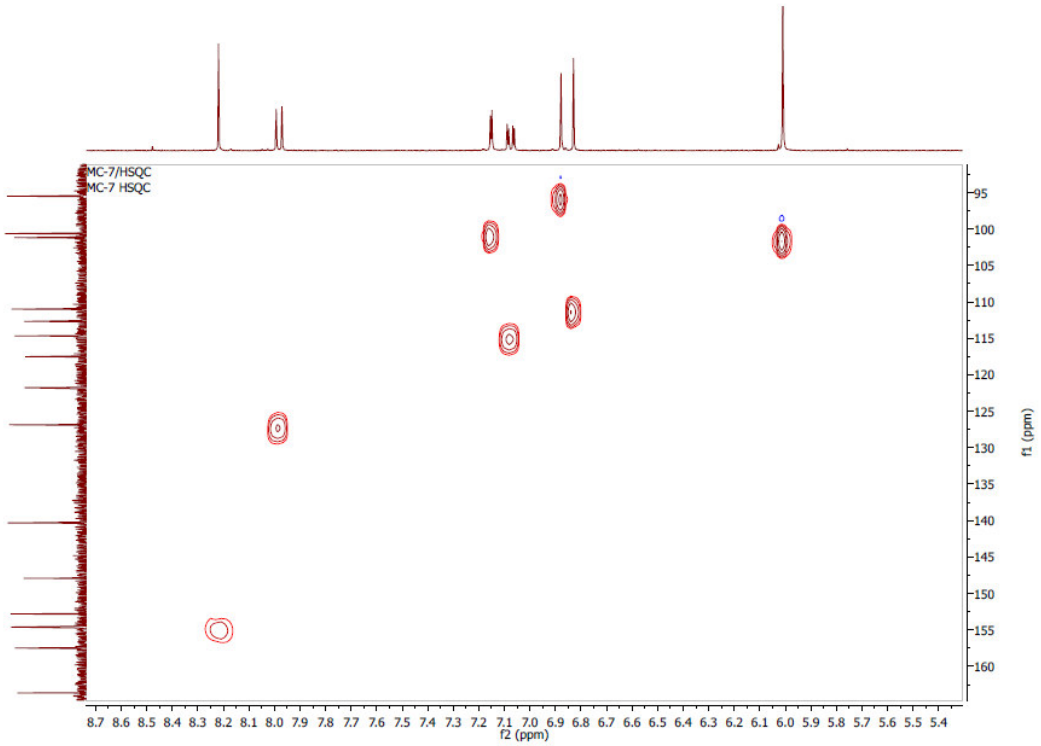
Appendices 13: 4b ^{13}C NMR Spectra of compound **3a** in $CDCl_3$



Appendices 14: 4c COSY Spectrum of compound **3a** in $CDCl_3$



Appendices 15: 4d HSQC Spectrum of compound **3a** in $CDCl_3$



Appendices 16: 4e HMBC Spectra of compound **3a** in $CDCl_3$