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M. Sc THESIS

ON

PHYTOCHEMICAL INVESTIGATION OF LEAVES EXTRACTS OF *Dodonaea angustifolia* AND EVALUATION OF ITS ANTIBACTERIAL ACTIVITIES

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PHYTO-CHEMICAL INVESTIGATION OF LEAVES EXTRACTS OF *Dodonaea angustifolia* AND EVALUATION OF ITS ANTIBACTERIAL ACTIVITIES.

A THESIS SUBMITTED TO SCHOOL OF GRADUATE STUDIES JIMMA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN ORGANIC CHEMISTRY

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Phytochemical Investigation of Leaves extracts of *Dodonaea angustifolia* and Evaluation of its Antibacterial Activities

By

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A Thesis Submitted to School of Graduate Studies Jimma University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Organic Chemistry

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Declaration

I declare that this thesis is my original work and has not been presented anywhere for award of any degree or diploma in any University.

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List of Abbreviations

ATCC	American Type Culture Collection
CC	Column Chromatography
COSY	Correlation Spectroscopy
HSQC	Heteronuclear Single Quantum Correlation
HMBC	Heteronuclear Multiple-Bond Correlation
MHB	Mueller-Hinton Broth
NMR	Nuclear Magnetic Resonance
STDs	Sexually Transmitted Diseases
TLC	Thin-layer Chromatography
TMS	Tetramethylsilane
UV	Ultraviolet
WHO	World Health Organization
HIV	Human Immino Deficiency Virus
AIDS	Aquared Immino Deficiency Syndrom

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Abstract

Natural products from plants and other natural sources have been providing noble and clinically active drugs. The plant-based traditional medicinal system continues to play significant role in health care, in which approximately 80% of the world's population still rely mainly on traditional medicines for their primary health-care. Therefore, this study was undertaken to identify bioactive secondary metabolites from the leaves of *Dodonaea angustifolia* for antibacterial activiities. With this regard, the air dried leaves of *Dodonaea angustifolia* was extracted exhostvely with chloroform/methanol (1:1) by maceration. The chloroform/methanol extract was then partioned between ethyl acetate and H_2O to give ethyl acetate extract. Both $CHCl_3$/CH_3OH and ethyl acetate crude extracts were evaluated for their antibacterial activity against four bacterial strains, namely; *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Enterococcus faecalis* (ATCC 29212). The ethyl acetate portion crude extracts showed antibacterial activities with zone of inhibition ranging from 7-22 mm. The observed activity against *S. aureus* (zone of inhibition, 22 mm) for ethyl acetate soluble extract was comparable with that of the referance drug, gentamycin, which desplayed 23 mm zone of inhibition on the same strain. Based on the superior activity of the ethyl acetate extract, it was subjected to fractionation using column chromatography (CC) over silica gel eluted with petroleum ether containing increasing amounts of ethyl acetate, which resulted with the identification of 5,7-dihydroxy-3,4'6-trimethoxyflavone (**1A**) which showed moderate antibacterial actvity against the tested strains (*E.faecali, E.coli, S.aureus,* and *P. aeruginosa*). The structure of the isolated compound was established using 1D (^1H,^{13}C) and 2D NMR spectroscopic techniques. Devising alternative method of extraction as well as carrying the antioxidant, antifungal and antiplasmodium activities of this plant are recommended for further researchers

1. Introduction

1.1. Background of the Study

The importance of medicinal plants in traditional health care system have been started long time ago and still continue to serve the 80% world populations for their primary healthcare [1, 2]. The traditional usage of medicinal plants is more common in developing countries including Africa, Asia and South America. It is also true that the use of traditional medicine is not completely omitted in developed countries as public interest in natural therapies has increased greatly in industrialized countries. For example, in the United States, about 38% of adults and 12% of children were using some form of traditional medicine in the year, 2007 [3]. In addition to the traditional uses, plants have also been serving as a source of modern drugs in the treatment of malaria by quinine (**1**) from *Cinchona* bark and artemisinin (**2**)[4] from *Artemisia annua*, cancer by the vinca alkaloids, vinblastine (**3**) and vincristine (**4**) from the Madagascan periwinkle, *Catharantus roseus[5]* and taxol (**5)** from some Yew species, and for other infectious diseases

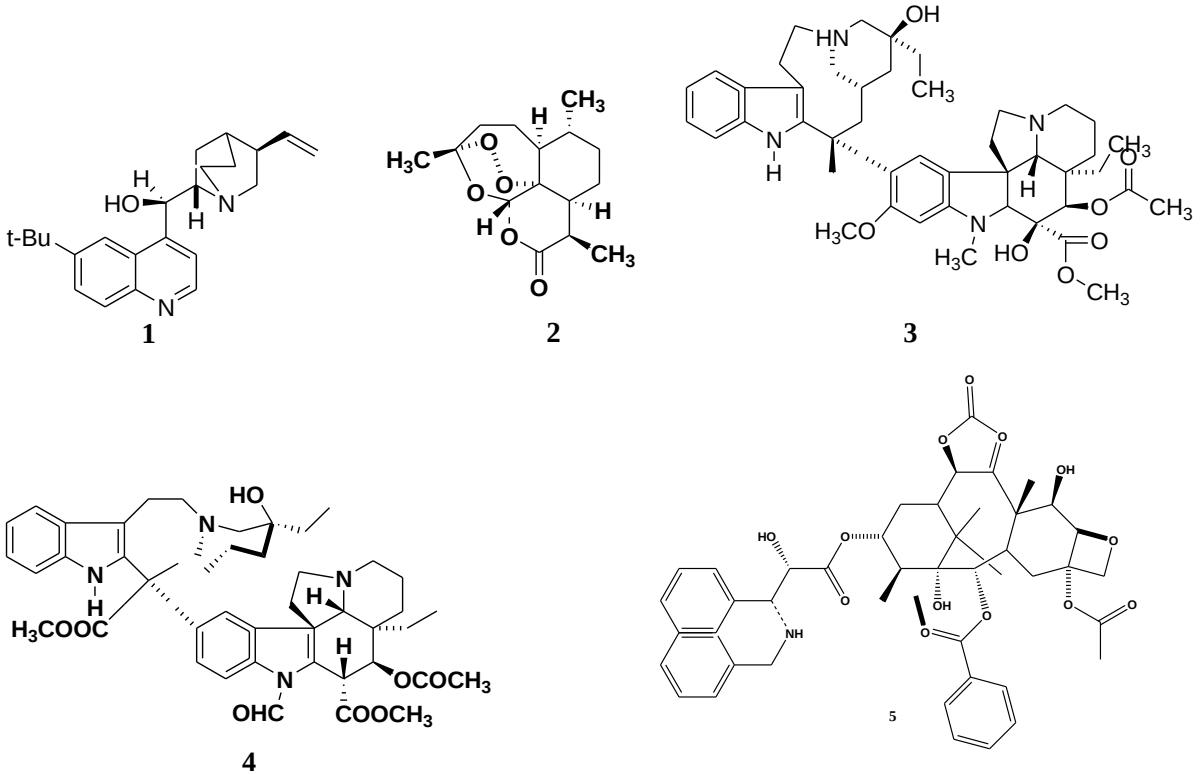


Figure 1 Structure of some drugs isolated from plant.

In general, there is a trend in the world today to shift to natural substances due to various side effects associated to some synthetic drugs with about 200 plant-derived chemical compounds of known structures are being used as drugs or as agents that lead to improvement of human health [6]. During the last two decades, the development of drug resistance as well as the appearance of undesirable side effects of certain antibiotics has lead to the search for new antimicrobial agents mainly from plant extracts with the goal to discover new chemical structures [7, 8]. There have been also increased attentions towards finding natural occurring antioxidants to replace synthetic antioxidants which are being restricted due to their carcinogenicity [9].

Like the rest part of the world, the uses of plants as a medicine is also common in Ethiopia, with close to 80% of the population is still dependent on traditional medicine [10]. However, the work that has been done to investigate Ethiopian traditional medicinal plants both phytochemically and biologically is too scanty. Therefore, the current study is focused on phytochemical investigation of one of the Ethiopian medicinal plants, *Dodonaea angustifolia* for antibacterial principles that could replace the current drugs that are becoming ineffective.

1.2 Statement of the Problem

Infectious diseases are one of the major causes of several numbers of death**s** of children, young and adults in developing countries [11]. It is the second leading cause of death worldwide, and the third leading cause of death in developed countries with a heavy load on people in developing countries by causing morbidity and mortality [12]. The emergence of various drug-resistant organisms limited therapeutic efficacy of many of currently available drugs which is partly attributable to the limitation in both number and structural variety of anti-bacterial drugs in clinical use. Thus, there should be a focus to be given to search bioactive molecules from plants, which could serve as hit or lead in antimicrobial drug discovery. *Dodonaea angustifolia* is one of the *Dodonaea* species that exist in Ethiopia, which is traditionally used for the treatment of various infectious diseases. It has been commonly used for the treatment of various ailments. The leave juice used as wound healing by the traditional healer. The fruit is crushed and mixed with honey and taken for a remedy against malaria and the stem bark is used for teeth brushing. However, there is no scientific

data pertaining to the phytochemical isolation of the natural product and pharmacological activities of the plant. Thus, the present work has focused on isolation, characterization and the evaluation of antibacterial activity of compounds from the leaves of the *Dodonaea angustifolia*

1.3 Objectives of the Study

1.3.1 General Objective

The main objective of this study was to identify secondary metabolites from the leaves of *Dodonaea angustifolia* and evaluate for antibacterial activities.

1.3.2. Specific Objectives

1. To isolate secondary metabolites from the leaves extract of *Dodonaea angustifolia* using chromatographic techniques;
2. To elucidate the structures of the isolated compounds using spectroscopic techniques, 1D and 2D NMR and,
3. To evaluate the antibacterial activities of the crude extracts and isolated compounds against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Enterococcus faecalis* bacterial strains.

1.4 Significance of the Study

The emergence and potential spread of resistance to currently available antibiotics, drugs, including penicillin and that all attempts to use high-throughput screening and combinatorial chemistry, genomics, and vaccine development have so far not delivered a single drug despite the enormous resources these fields received during the past 16 years. At present, there are no promising candidates in the pipeline, signifying the importance of searching for new lead structures in antimicrobial drug development.

In Africa, the use of medicinal plants still plays an important role in microbial treatment. Taking into account that plants were potential sources of the existing first line antimicrobial drugs, there is still great potential of identifying antimicrobial drugs from plant-based sources. Thus, screening and identifying the bioactive constituents of traditional medicinal

plants empirically used to treat infectious diseases will have a great contribution in addressing this global problem. flavonoids could exhibited promising activity against different types of ailment.

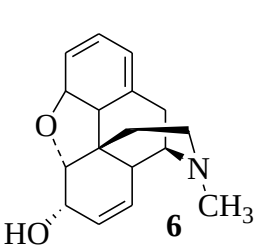
Members of the subfamily sapindaceae including the genus *Dodonaea* produce a broad variety of flavoniods and isoflavones, with wide range of bioactivities. Thus, the study of *Dodonaea angustifolia* may yield promising antibacterial agents. Furthermore, the findings of this research could be useful to provide information of the chemical profile of the plant and can be used as a database and guideline for further isolation and purification of the active principles

2. **Review of Related Literature**

2.1. The Microbial Burden

Infectious diseases are the second leading cause of death worldwide, and the third leading cause of death in developed countries in both children and adults. The burden of infectious diseases falls most heavily on people in developing countries by causing morbidity and mortality [12]. The challenge associated with fighting these diseases has become an increasingly complex one, because of the fast development of resistance to the classic antibiotics; the emergency of multiple drug resistant human pathogenic microorganisms and the changing nature of the infections observed in the elderly and other immune-compromised patients. Antimicrobial resistance is natural biological phenomenon recognized first as a scientific curiosity and then as a threat to the effective treatment of most microbial infections [13]. This demands the search for new sources of antimicrobial substances mainly from medicinal plants, because plants constitute a potentially useful resource for new and safe drugs for the treatment of microbial infections and other diseases [14].

So, to prevent and control infectious diseases it is a vital interest and concern in both developed and developing countries to come up with new remedies for such diseases [13].
To overcome this, new antimicrobial agents with different modes of action against bacterial, fungal, parasite and viral diseases are urgently needed. Thus, screening of medicinal plants for biological activity has resulted in the isolation of active compounds from the early 19th century when morphine (**6**) was isolated from *Papaver somniferum*. The success of the isolation of morphine led to the identification of many other drugs such as quinine (**1**) from *Cinchona spp*, atropine (**7**) from *Atropa belladonna*, reserpine (**8**) from *Rauvolfi serpentina* and digoxin (**9**) from *Digitalis* species [12].



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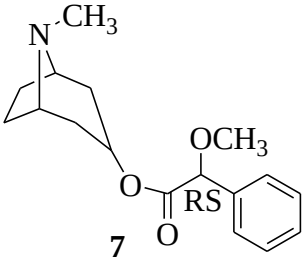

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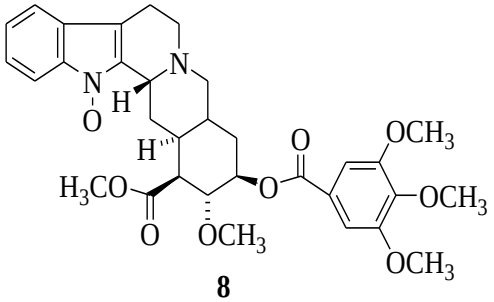

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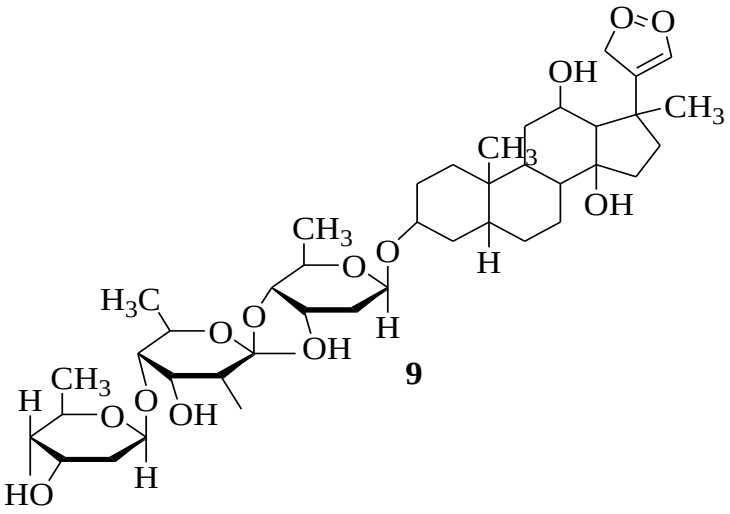

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Figure 2. Some plant based compounds for antibacterial activies

2.1.1 Economical Burden of Infection Disease

Even though the burden of infection diseases has been steadily decreasing in past decades in developed countries, it is still considerable worldwide. Lower respiratory infections, diarrhoeal and HIV/acquired immunodeficiency syndrome (AIDS) diseases are still among the top major killers in 2011 and infection diseases in general are responsible for considerable morbidity in all parts of the world [15].

World wide, an estimated 499 million new cases of curable sectual transimmited infection (as gonorrhoea, chlamydia and syphilis) occurred in 2008; these findings suggested no improvement compared to the 448 million cases occured in 2005. However, wide variations in the incidence of sectual transimmited infections are reported among different regions; the burden of STIs mainly occurs in low-income countries [16]. According to the crucial burden of chlamydia infection in Europe, the economic impact of chlamydia infection has been deeply investigated; in the UK, the cost of chlamydia complications has been estimated to a minimum of € 110 million, annually [17]. Each year, in the USA, direct costs of chlamydia and its complications range between € 1 and 3 billion.

With regard to costs of influenza, results of a 2007 study, referring to 2003 data, highlighted the huge economic brunt of the burden of influenza in the USA, accounting for US$ 87.1 billion across all age groups [18].

Diarrhoeal diseases cause for 4.1 % of the total disability-adjusted life years global burden of disease, and are responsible for 1.8 million deaths every year. An estimated 88 % of that burden is attributable to unsafe supply of water, sanitation and hygiene [19]. Children in the developing world are the most affected by diarrhoeal disease: It is estimated that diarrhoeal diseases account for one in nine child deaths worldwide, making diarrhoea the second leading cause of death among children under the age of 5 after pneumonia [20].

2.1.2 Bacterial Infections and Antibacterial Agent

Bacteria are widely distributed and the most abundant group of unicellular organisms on earth, capable of adapting in a diverse range of environments such as in soil, water and air. They are both useful and harmful to humans. Many parasitic bacteria do not harm their hosts. Some cause disease through different mechanisms including production of toxins and or hydrolytic enzymes. Bacteria have a prokaryotic cell type with a rigid wall which protects the cell against osmotic damage. The structure of the cell wall differs in Gram-positive and Gram-negative bacteria [21].

Gram-negative bacteria differ from Gram-positive by the presence of an outer membrane composed of lipopolysaccharides. It also contains specific proteins for transporting hydrophilic molecules. Other proteins are receptor sites for phages and bacteriocins. The outer membrane in Gram-negative bacteria covers the peptidoglycan layer, which is attached to the outer membrane by lipoproteins. The layer is separated by periplasm from the cytoplasmic membrane. Gram-positive bacteria have thicker peptidoglycan layers with no periplasm[22]. *Staphylococcus aureusis*a spherical Gram-positive parasitic bacterium that causes illnesses ranging from minor skin infections and abscesses, to life-threatening diseases such as pneumonia, meningitis and septicem[23].

Escherichia coli is a Gram-negative bacterium normally present in the intestinal tract of humans and other animals. *Escherichia coli* can sometimes be pathogenic, thus posing a threat to food safety, causing diarrhea, wound and urinary infections [21].

The other problem associated with the emergence of various resistant organisms which limited therapeutic efficacy of many of the available drugs. In addition, it has a serious global economic impact that affects the productivity of individuals, families and the society at large, since it causes energy loss, debilitation and loss of work capacity. Consequently, there is an urgent need for new, affordable and accessible antibacterial agents from nature with novel mechanism of action. The emergence and potential spread of strains of bacteria which are resistant to currently available drugs, has actually prompted the search for new drugs through the use of high-through put screening and combinatorial chemistry, genomics, and vaccine development. However, this effort has yet to deliver a single drug despite the enormous resources expended during the past many years. Natural products will continue to serve as lead structures for the development of antibacterial drugs. This is in fact a pointer that new antibacterial leads may emerge from plants, especially from those with recognized traditional uses.

2.2. Botanical Information

The family *Sapindaceae*, also called soapberry family is a flowering plants consisting of about 2000 species in *ca* 150 genera that are mainly distributed in temperate and tropical regions of the world [24]. The majority of these plants are either shrubs or trees, which are widely used for the treatment of various ailments [25] including its uses as medication to reduce fever, stimulating metabolism uses as antioxidant, analgesic, antiviral, anti-inflammatory, antiulcer and antibacterial activities [26, 27]. It is described that around 10% of the genera including *Dodonaea* and 1% of the species under the Sapindaceae family were registered in Ethiopian flora [24].

2.2.1 Genus Dodonaea

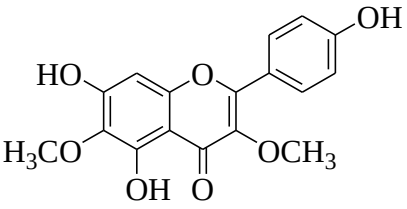
Dodonaea is considered to be largest genus with 70 species most of which are limited to Australia, one in New Guinea *Dodonaea polyandra*, *Dodonaea madagascariensis* endemic to Madagascar. *Dodonaea viscose* and *Dodonaea angustifolia* is widely distributed in almost all continents [28, 29].

2.2.2 Compounds from Genus *Dodonaea*

Dodonaea has been reported to produce diverse groups of secondary metabolites including flavonoids, terpenoids, saponins, tannins, cardiac glycosides, steroids, etc [30]. The major secondary metabolites so far reported are flavonoids and terpenoids [31] which are summarized in Table 1.

Table 1: List of compounds isolated from *Dodonaea* species**.**

Compound class	Compound name	Plant source	Ref
Flavonoids	3,5,7-Trihydroxy-4'-methoxyflavone **(10)**	*Dodonaea viscose*	[32]
	5,7,4'-Trihydroxy-3,6-dimethoxyflavone(**11**)	"	[33, 34]
	5,7-Dihydroxy-3,6,4'-trimethoxyflavone/santin(**12**)	"	[33, 35]
	5-Hydroxy-3,7,4'-trimethoxyflavone (**13**)	"	[27]
	5-Hydroxy-3,6,7,4'-tetramethoxyflavone(**14**)	"	
	3,5-Dihydroxy-7,4',-dimethoxyflavone (**15**)	"	
	3,4',5,7-Tetrahydroxyflavone(**16**)	"	[33, 34]
	Pinocembrin (**17**)	"	[34]
	Methylenebissantin (**18**)	"	[36]
	Kaempferol3-methylether (**19**)	"	[36]
	5,7,4'-Trihydroxy-3'(3-methylbut-2-enyl)-3-methoxyflavones(**20**)	*Dodonaea polyandra*	[37]
	7-Dihydroxy-3'(3-methylbut-2-enyl)-3,4'-dimethoxyflavone(**21**)	"	[37]
	5,7,4'-Trihydroxy-3',5'(3-methylbuyt-2-enyl)-3-methoxyflavone (**22**)	"	[37]
	5,7,4'-Trihydroxy-3',5'(3-methylbuyt-2-enyl)-3-methoxy-6metoxyflavone(**23**)	"	[37]
	5,7,4'-Trihydroxy-3'-(3-methylbuyt-2-enyl)-3-methoxy-5'methoxyflavone (**24**)	"	[37]
Phenolics	Vanillicacid (**25**)	*Dodonaea.viscosa*	[36]
	NebrodensideA (**26**)	"	[36]
	Docosylcaffeate (**27**)	"	[36]
Terpenoids	5-(2-Furan-3-yl-ethyl)-8α-hydroxymethyl-5,6-dimethyl-3,4,4α,5,6,7,8,8α-octahydro-naphthalene-1-carboxylicacid (**28**)	hautriwaiclactone	[38]



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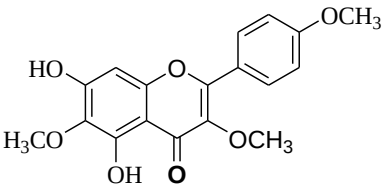

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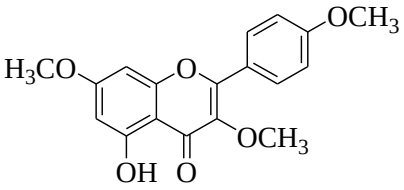

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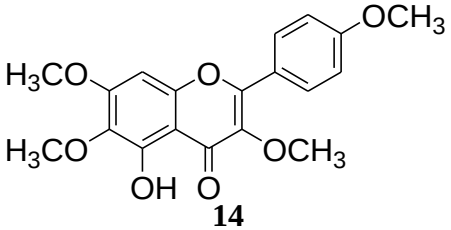

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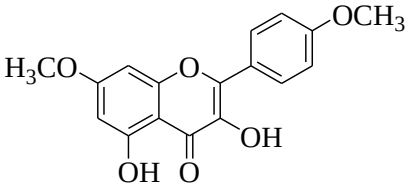

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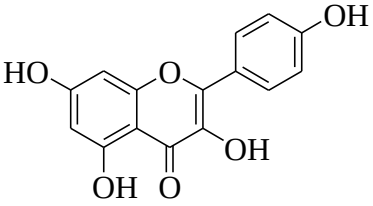

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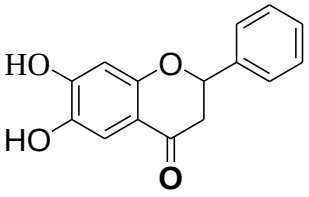

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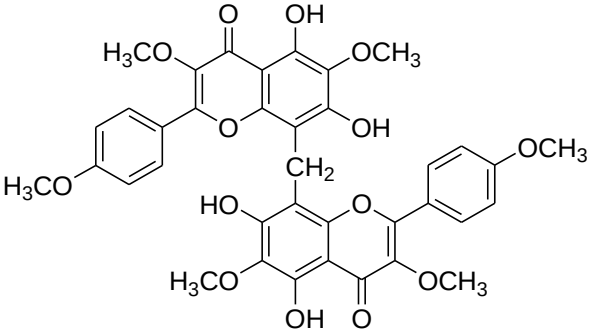

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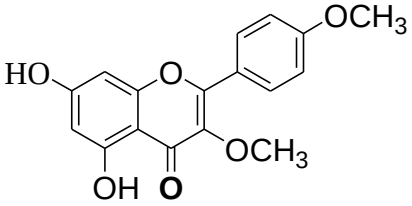

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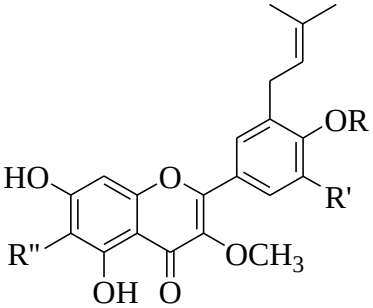
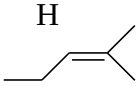
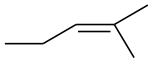


	R	R'	R"
20	H	H	H
21	CH3	H	H
22	H		H
23	H		OCH_3
24	H	H	OCH3

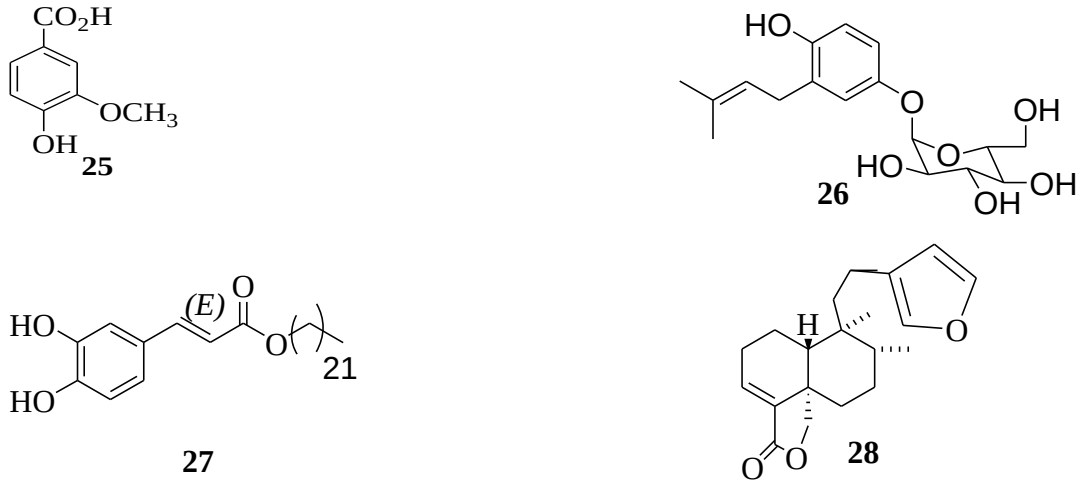


Figure 4 Structures of compounds reported from *Dodonaea* species

2.2.3 Biological Activities of the Genus *Dodonaea*

The alcohol and aqueous extracts of the root of genus *Dodonaea* significantly reduced diarrhea in mice with reduction in weight of stools showing its anti-diarrheal property [39]. The leaves extract of genus *Dodonaea* exhibited anti-diabetic [52, 32.40.41], anti-inflammatory [42, 43], antioxidant [28, 33, 35], hypotensive [27], antiviral [44], analgesic [27], antipyretic [45] and wound healing activities [46]. Furthermore, the crude extract of the seeds of *Dodonaea* viscosa showed anti-malarial activity [47]. Literature report showed that different solvent extracts of the various parts of *D. angustifolia* were capable of suppressing the germination and growth of various plants demonstrating its allelopathic potential [48].

2.2.4 *Dodonaea angustifolia*

Dodonaea angustifolia, hop-bush in English "*Kitkita"* in Amharic and "*Ittacha"* in Afaan Oromo is mainly found in Australia, Africa, Asia and South America [49, 50] with its greatest center of diversity appears to be the Southeast Asian region. *Dodonaea angustifolia* is a multi stemmed shrub or single-stemmed small tree up to 7 m tall. It occurs in different African countries like Ethiopia, Kenya, Somalia, Senegal, Nigeria, Mozambique, Madagascar, Democratic Republic of Congo, and South Africa in natural habitat and home cultivated in Ghana, Nigeria and Cameroon. It is an evading plant in East Africa which

reproduces itself from seed very freely and grows on dry rocky slopes between 1500 and 2100 m. [51]

Figure. 3 Picture of *D. angustifolia* on uncultivated area taken from Estern Wollega ,Gobu Sayo woreda, Anno district in 2016 (taken by Getachew Nemera).

2.2.5 Traditional Uses of *Dodonaea angustifolia*

Some plants under the genus *Dodonaea* are known to be used for the treatment of a wide range of diseases and illness. The aerial parts of *Dodonaea angustifolia* are used in folk medicine against fevers, swellings and colds in Latin America, China, Africa, and India [52, 53] while the juice made from the powdered leave is used for the treatment of trachoma and to expel roundworms as anti-helmentic [27]. There is also a report indicating that various parts of this plant are used in the traditional systems of medicine for contraceptive effect [54]. In the Tigray region of Ethiopia, the crushed fruit mixed with honey and taken for a remedy against malaria [55]. The stems are also used as fumigants to treat rheumatism [27].

3 Materials and Methods

3.1 Chemicals, Apparatus and Instruments

Rotary evaporator (Heidolph, USA) for solvent evaporation, Uv chamber for detection of spots on TLC plate, glass column chromatography (300 mm (B-14/23,B-19/26) and 500 mm, B-34/35); Smith Scientific Eden bridge; U.K) for separation and purification techniques, mortar and pestle for grinding, and round bottom flask of size 250, 500, and 1000 mL, measuring cylinder, filter papers (cotton swab), weighting balances, oven for drying purpose, TLC analyses were carried out on Merck pre-coated silica gel 60 F_{254} plates. Column chromatography was run on silica gel 60 Å (70-230 mesh)**.** Chloroform (99%), methanol (99.99%), from Blurex Laboratories Ltd, petroleum ether and ethyl acetate (99%), from Fisher Scientific, UK are chemicals used for extraction and gradient column elution. Mueller Hinton agar and nutrient broth as culture media were used for antibacterial activity test. Deuterated chloroform ($CDCl_3$, 99.8%) and dimethylsulfoxide (DMSO, 99.96%) solvents were used for obtaining NMR spectra, Vertical Laminar Flow Cabinet hood CLB-201-14 and Genlab incubator were used in the study.

NMR spectra were obtained on Bruker Avance III 400 spectrometer, using the residual solvent peaks as reference at South Korea, South Polo University. The spectra were processed using MestReNova 11.0 software.

3.2 Collection and Preparation of Plant specimens

The leaves of *Dodonaea angustifolia* were collected from Eastern Wollega Zone, Gubu Sayo district, Anno town in April 2016. The plant material was air-dried under shade and powdered to suitable size by using mechanical grinder to improve the subsequent extraction by rendering the sample more homogenous, increasing the surface area, and facilitating the penetration of solvent into the cells.

3.3 Extraction and Isolation

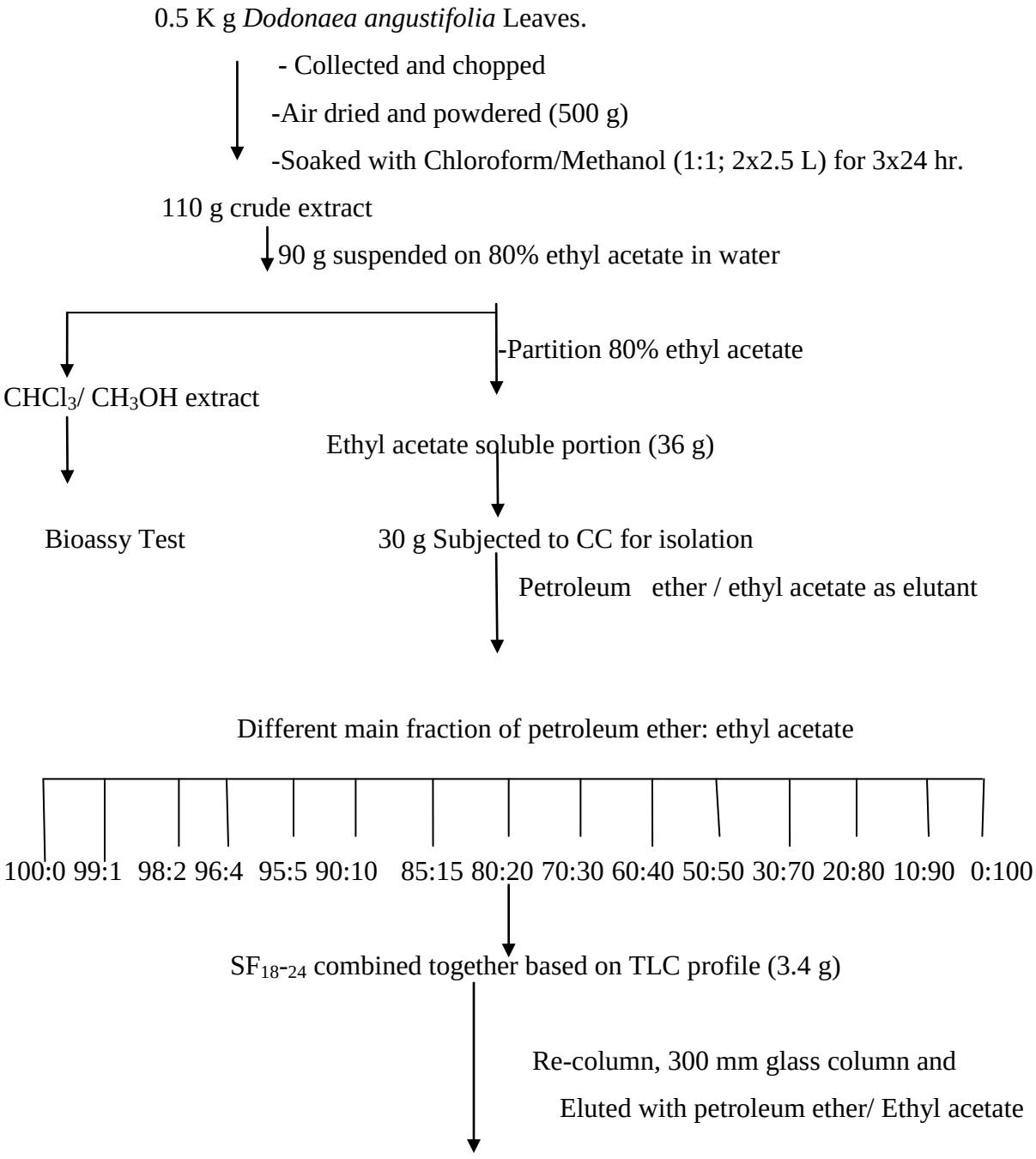
3.3.1 Extraction of Leaves of *Dodonaea angustifolia*

The air-dried and powdered leaves of *Dodonaea angustifolia* 0.5 Kg was soaked in 1:1 ratio of chloroform /methanol 2.5 L each and exhaustively extracted 72 hr (3x24 hr). The filtrate was then separated from the mark using fresh cotton plug and the mark was macerated three times with the same solvent system. The filtrate was concentrated using rotary evaporator at 50 °C under reduced pressure to yield 110 g (22%) black crude extract and stored at room temperature. The portion of the crude extract (90 g) was subjected to differential extraction between ethyl acetate and water in 8:2 ratios. The process (partitioning) was repeated three times using the same ratio of ethyl acetate and water (8:2) followed by concentration of the ethyl acetate layer. The ethyl acetate extracts were then combined and concentrated to afford the dried weight of 36 g from plant material and (40%) from crude extract.

3.3.2 Isolation of compound by column Chromatography.

A 30 g portion of the ethyl acetate extract was adsorbed on 6 g silica gel (60-130 mesh) and subjected to column chromatography (500 mm diameter) on silica gel eluting with petroleum ether with increasing amounts of ethyl acetate gradient. The following ratio of solvent combination were used in the elution process in petroleum ether to ratio of 100:00, 99:1, 98:2, 96:4, 90:10, 85:15, 80:20, 70:30, 50:50, 30:70, 20:80, 00:100 for each ratio fifty fraction with 30 mL was collected.

After TLC analysis, the fractions from SF18-24 (3.4 g) collected with 20% ethyl acetate in petroleum ether was subjected to column chromatography again on silica gel using increasing amounts of ethyl acetate in petroleum ether to give 250 mg of compound (**1A**)



F-6 (250 mg) Yellow powdered solid (**1A)**

3.4 Culture Media

3.4.1. Test Strains

Once the isolate bacterial strains were obtained, the antibacterial susceptibility test was done in the order of selection of the strain, prepare and standardize inoculum suspension, plate inoculated, antibacterial to disks added, incubated the plates and measured the zone of inhibition for interpretation was considered to know the potency of plant extract.

In vitro antibacterial activity was examined for the two crude extracts and for the isolated compound 1A from leave of *D. angustifolia*. Among microorganisms investigated, the two gram-positive and two-gram negative bacterial strains, *Staphylococcus aureus ATCC 25923*, *Enterococcus faecalis ATCC 29212*, *Escherichia coli ATCC 25922* and *Pseudomonas aeruginosa ATCC 27853* respectively.

The standard strains were obtained from, Medical Microbiology Research Laboratory, Jimma hospital, donated previously from Ethiopia Public Health Institute, Addis Ababa, . The bacteria strains were reactivated by sub culturing in nutrient broth at 37 ºC and maintained on nutrient agar slant at 4 ºC for further use in microbiology laboratory, Department of Biology Jimma University.

3.4.2 Standardization of Inoculums

To standardize the inoculums density for a susceptibility test, a Barium sulphate ($BaSO_4$) turbidity standard, equivalent to a 0.5 McFarland standard, should be used. Accordingly, $BaSO_4$ 0.5 McFarland standard was prepared, by adding 0.5 mL of 0.048 $molL^{-1}$ barium chloride ($BaCl_2$) 1.175% w/v dehydrated barium chloride ($BaCl_2.2H_2O$) in to 99.5 mL of 0.18 $molL^{-1}$ Sulphuric acid (H_2SO_4, 1% v/v) with constant stirring to maintain a suspension. This mixture was considered to be equivalent to cell density of $1.5x10^8$ $CFUmL^{-1.}$ The turbidity standard is then aliquot into test tubes identical to those used to prepare the inoculums suspension. McFarland turbidity standard tubes were sealed with para film to prevent evaporation. Barium sulphate turbidity was compared with identical tubes containing inoculums 0.85% NaCl saline solution [56]. For each bacterial strain, gentamycin was taken as positive control and pure solvent (DMSO) as the negative control [57].

3.5 Antibacterial Activity Test

3.5.1 Agar Disc Diffusion Method.

Agar disc diffusion method was used to evaluate the antibacterial activities of the crude extract and isolated compound on nutrient agar. Accordingly, an overnight culture of the test strains whose cell densities were adjusted to 0.5 McFarland standards (equivalent to 1 to $2x10^8$ $CFUmL^{-1}$ bacterial suspensions) were uniformly spread on pre-prepared sterile Muller Hinton Agar plates to form lawn cultures. Then, 0.2 g crude extract of (1:1) chloroform/ methanol was dissolved in DMSO. The stock solution was prepared with concentrations of 200 $mgmL^{-1}$for each solvent extract. The blotting paper discs (6 mm) was soaked in various dilute solvent extracts and dried for 5 min. to avoid flow of extracts in the test media. The discs were placed on a plate with standardized culture suspension at equidistance to one another to avoid overlap of zones of growth inhibitions. Antibacterial activity of potential plant extract against bacterial pathogens was determined after incubation of the plates for 24 hr. at 37 ºC, by measuring the diameter of zone of inhibition of growth. The diameter of zone of growth inhibition surrounding the discs was measured and expressed in millimeter (mm) using transparent ruler [58]. Each experiment was carried out in triplicates. The mean of the inhibition zone of each test sample was taken for evaluating the antibacterial activity of the extract.

4. Results and Discussion

4.1 Characterization of Isolated Compound (1A)

Compound **1A** was isolated as a yellow solid from the ethyl acetate soluble portion of the 1:1 chloroform/methanol extract from leaves of *D. angustifolia*. The ^{1}H NMR spectrum revealed the presence of three proton singlet signals at δ_H 3.84, 3.89, and 4.03, each integrated for three protons for three methoxy groups. It also further showed the presence of five aromatic proton signals at δ_H 6.54 (1H, s), δ 7.02 (2H, dd, J = 8, 4 Hz) and δ 8.07 (2H, dd, J = 8, 4 Hz) and two hydroxyl proton signal at δ_H 12.91 (1H, s) and δ_H 6.66(1H, s).

The ^{13}C-NMR spectrum revealed that ten quaternary carbons which are attributed to five oxygenated aromatic carbons at δ_C 138.5 (C-3), 151.8 (C-5), 130.1 (C-6), 152.2 (C-7), and 161.1 (C-4'); two oxygenated olefinic carbons at δ_C 155.1 (C-9) and 156.2 (C-2); two aromatic quaternary carbons at δ_C 106.2 (C-10) and 122.9 (C-1'); and an α ,β-unsaturated carbonyl carbon at δ_C 179.3 (C-4) and five methine carbon signals are also observed in the aromatic region at δ_C 93.2 (C-8), 114.2 (C-3', 5') and 130.3 (C-2', 6'). Furthermore, the spectrum demonstrated the presence of three methoxy groups at δ_C 55.6, 60.2 and 61.0 as showed in Table 2 (Appendix 3, 4)

Table 2: The ^{1}H and ^{13}C NMR spectral data of compound (1A) and literature for santin [33]

Position	Compound **(1A)**		Santin **(12)**		Remark
	δ_H	δ_C	δ_H	δ_C	
2		156.2		156.2	Quaternary
3		138.5		138.4	Quaternary
4		179.3		179.2	Quaternary
5		151.9		155.1	Quaternary
6		130.1		130.1	Quaternary
7		152.3		151.8	Quaternary
8	6.54 (1H, s)	93.2	5.57 (1H, s)	93.1	CH
9		155.1		152.2	Quaternary
10		106.3		106.1	Quaternary

1'		122.9		122.7	Quaternary
2'and 6'	7.02 (2H,d J=7.01)	130.3	7.02 (2H, d)	130.2	CH
3'and 5'	8.07 (2H,d, J=8.66)	114.2	8.07 (2H, d)	114.2	CH
4'		161.1		161.7	Quaternary
4'-OMe	3.89 (3H, s)	55.6	3.70 (3H, s)	55.5	CH_3
3-OMe	3.84 (3H, s)	60.3	3.80 (3H,)	60.2	CH_3
6-OMe	4.03 (3H, s)	61.0	3.80 (3H, s)	60.9	CH_3

Assessments made on the basis of 1D (^{1}H-^{13}C), COSY, HSQC and HMBC correlations; δ in ppm; Coupling constants are in Hz.

The ^{1}H-^{1}H COSY spectrum (Appendix 5) showed the correlation between two aromatic protons each symmetrically placed on para substituted benzene ring "B" at δ_H 7.01 and δ_H 8.07. The two protons are found coupled to each other but no other ^{1}H-^{1}H correlations are observed from its COSY spectrum as illustrated in Figure 5

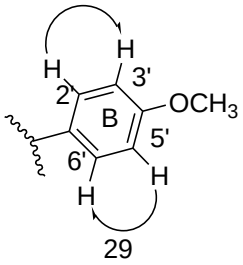


Figure. 5 . Structure of compound (**1A**) based on COSY spectrum

Whereas in ring A, only a single up field proton signal at δ_H 6.54 because of di*ortho* oxygenation is characteristic for proton at C-8 (H-8), otherwise fully substituted with two hydroxyl and a methoxyl groups.

The downfield shifted proton signal at δ_H 12.91 (1H, s) for chelated hydroxyl group involved in hydrogen bonding was placed at C-5, *peri* to carbonyl group.

Table 3 HSQC data for compound 1A.

Carbon (^{13}CNMR)		Proton(^{1}HNMR)		Multiplicity	Remark
No	δ_C (ppm)	No	δ_H (ppm)		
OMe-4'	55.6	4'(OCH_3)	3.89	S	CH_3
OMe-3	60.3	3(OCH_3)	3.84	S	CH_3
OMe-6	61.0	6(OCH_3)	4.03	S	CH_3
8	93.2	8	6.54	S	CH
2'and 6'	130.3	2'and 6'	7.02	dd (J = 8, 4)	CH
3'and 5'	114.2	3'and 5'	8.07	dd (J = 8, 4)	CH

The HSQC spectrum showed correlation such that the methyl proton signals at δ 3.89, 3.84 and 4.03 correlate with the carbons at δ_C 55.6, 60.3 and 61.0, respectively and other correlations observed were between aromatic protons at δ_H 6.54, 7.02 (2H, d) and 8.07(2H, d) with aromatic carbon signals at δ_C 93.3, 114.2 (C-3' & C-5') and 130.3 (C-2' & C-6'), respectively as shown in table 3 (Appendix 6)

Table. 4 Observed correlations in HMBC data of compound 1A

Carbon		HMBC(^{1}H-^{13}C)
No	δ_C (ppm)	
OMe-4'	55.6	H (4'-OMe') ↔,C-4'
OMe-3	60.3	H (3-OMe)↔C-3,
OMe-6	61.0	H (6-OMe)↔C-6,
C-8	93.2	H-8'↔C-7, H-8↔C-9,H-, H-8↔C-6
C-3'	114.2	H-3'↔C-2', H-3'↔C-4', H-3'↔, C-5'
C-2'	130.3	H-2'↔C-3', H-2'↔C-1', H-2'↔C-2, H-2' ↔C-6'
		H (5-OH)↔C-5, H (5-OH)↔C-6, H (5-OH) ↔C-10
OH on C-5		
OH on C-7		H (7-OH)↔C-7, H (7-OH)↔C-6, H(7-OH)↔C-8,

The HMBC spectral analysis revealed that the proton signal at δ_H 6.54 (H-8) showed long range coupling with the carbon signals at δ_C 152.3 (C-7), 151.1 (C-9), 130.1 (C-6) and 106.3 (C-10)**.** The most downfield shifted signal in the ^{1}H NMR at δ_H 12.91 (5-OH) showed HMBC cross coupling with its neighboring carbons, C-5 (δ 151.9), C-6 (δ 130.1) and C-10 (δ 106.3) confirming the position of this hydroxyl group at C-5.as showed in Figure 6 and Appendix 7.

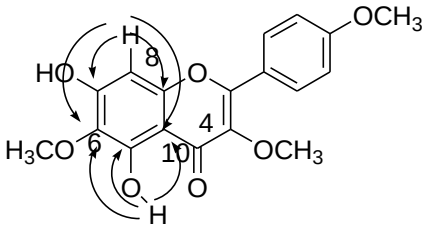


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Figure. 6 Structure of compound 1A based on HMBC spectrum

The signal at δ_H 6.66 in ^{1}H NMR spectrum showed no HSQC correlation indicating that the group is for phenolic hydroxylic group. It also show that HMBC correlations with C-7, C-6 and C-8, supported the placement of the group at C-7 as indicated in Figure 7.

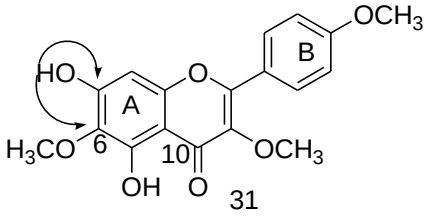

Figure 7 Structure of compound **1A** based on HMBC spectrum

The spectrum also shown significant correlation led to the conclusion that the methoxy groups at δ_H 4.03 and 3.84 are clearly attached to C-6 (δ_C 130.1) and C-3 (δ_C 138.5), respectively as showed in Figure 7. The signal due to the third methoxy group at δ_H 3.89 correlates with the carbon signal at δ_C 161.2 (C-4') establishing the location of the third methoxy group at C-4' as shown in Figure 8.

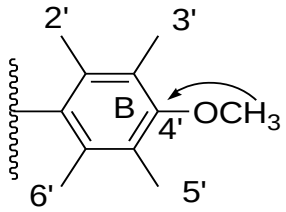

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Figure 8. Structure compound **1A** based on HMBC spectrum

The HMBC Spectrum on ring B also shown correlation of H-2'(δ_H 8.07) with C-3' δ_C (130.3), C-1' (122.9), C-2 (156.2), C-6' (130.3) and H-3' (7.02) with C-4' (161.1),C-2' (130.3). The overall HMBC structure was given as indicated in Table 3 and Figure 9.

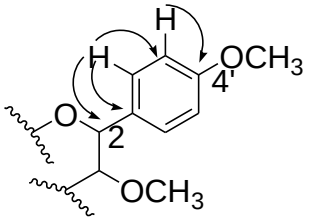

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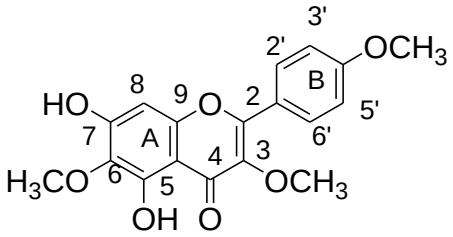
Figure 9. Structure of compound **1A** based on HMBC spectrum.

Finally from the assignments made on the basis of ^{1}H NMR, ^{13}C NMR, COSY, HSQC and HMBC correlations and spectra the structure of the isolated compound was deduced as in Figure 10.



1A

Figure 10 Final Structure of Isolated compound.

The above physical and spectral data of compound **1A** are in close agreement with the data presented in the literature for santin (5,7-dihydroxy 3,6,4'-trimethoxyflavone) [33], which was previously isolated from the leaves of *Dodonaea viscosa* [43], *Parthenium hysterophorus* [59] and the aerial parts of *Tanacetum microphyllum* [60].

4.1.1. Summary of Spectral and Physical data of the Isolated Compound 1A.

Compound **1A**: Yellow solid; soluble in methanol; TLC: Rf 0.64 (mobile phase EtOAc :petrol, (2:8); have molecular formula $C_{18}H_{16}O_7$; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.84 (3H, *s*, OCH_3), 3.89 (3H, *s*, OCH_3), 4.03 (3H, *s*, OCH_3), 6.54 (1H, *s*, H-8), 7.02 (2H, *d*, *J* = 8.80, H-2' and 6'), 8.07 (2H, *d*, *J* = 8.80, H-3'and 5') and 12.91(1H, *s*, 5-OH) and 6.66(1H,s,7-OH) and ^{13}C NMR (400 MHz, $CDCl_3$) δ in ppm: C-2(δ 156.2), C-3(138.5), C-4 (179.3), C-5(151.9), C-6(130.1), C-7(152.3) C-8 (93.2), C-9 (155.1), C-10 (106.3), C-1'(122.9), C-2', 6'), (130.3), (C-3', 5') (114.2) , C-4'(161.1), OCH_3 (61.0), OCH_3 (60.3) and OCH_3 (55.6).

4.2 Antibacterial activity Evaluation

The methanol/chloroform (1:1 v/v) extract, the ethyl acetate fraction and the isolated compounds; 1A were evaluated for antibacterial activity on four bacterial strains; *Staphylococccus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). The disk diffusion method was used and the zone of growth inhibition of the isolates was measured in millimeter (mm) compared to the standard positive control, gentamycin and negative control, DMSO. The zone of inhibition measured was presented in Table 5 for 200 mg/ml concentration. The zone of growth inhibition for crude extract and isolated compounds was analyzed against standard positive control gentamycin

Table 5 Antibacterial activity of crude extracts and isolated compound from *D. angustifolia*

Bacterial strains		**Crude extract (mm).**		**isolated compound and control zone of inhibition**		
		$CHCl_3/CH_3OH$ extract	**EtOA-soluble partition.**	**Isolated compoud**	**G**	**DMSO**
E.faecalis	+ve	NI	7	12	22	NI
S.aureus	+ve	NI	22	10	23	NI
E.coli	-ve	11	12	15	23	NI
P.aeruginosa	-Ve	9	NI	13	29	NI

Key: NI = No Inhibition, EtOA = ethyl acetate, G = gentamycin, DMSO= dimethyl sulfoxide, +ve = positive, -ve = Negative

The zone of growth inhibitions (Table 5) are different based on the types of bacterial strains and the test samples. The two crude extracts and the isolated compound showed considerable bacterial growth inhibition comparing to the positive control, gentamycin. The crude extract designated as $CHCl_3/CH_3OH$ exhibited higher zone of growth inhibition (11mm) on gram negative bacteria, Escherichia coli while the ethyl acetate extract showed highest growth inhibition (22 mm) on gram-positive bacteria, *Staphylococccus aureus*. The isolated compound showed good activities against the tested bacterial strains. The compound from the genus; 5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone showed zone of growth inhibition 12, 10, 15, 13 mm in diameter against, *E. faecalis, S. aureus E. coli* and *P. aeruginosa* respectively. The diameter of growth inhibition zone displayed on both gram negative and gram positive were good with variable degree of potency the tested compound. However, compound (1A) has shown significant anti-bacterial activity on gram negative bacterial strains (*E. coli* and *P. aeruginosa)* than gram positive bacterial strains *(E. faecalis and S. aureus).* Comparable the isolated compound display good activity on *E. coli* and less activity on *S. aeureus* .

5. Conclusion and Recommendation

5.1 Conclusion

It has been documented that *Dodonaea angustifolia* is widely used around the world as analgesic, anti-inflammatory, antiviral, antifungal, anti-ulcerogenic, pasmolytic, laxative, antimicrobial, hypotensive, rheumatism, gout, hemorrhoids, fractures and snake bites. In the course of this project work, one compound, **1A** was identified from the leaves of *Dodonaea angustifolia.* It was isolated from (8:2) petroleum ether/ ethyl acetate extract. The antibacterial activities of the compound have an effect on tested bacteria as compared to gentamycin hence accounting for the traditional use of this plant against bacteria. Moreover, the structural elucidation of this compound was done by using spectroscopic methods NMR. The characterized compounds was identified as 5, 7-dihydroxy-3, 4', 6-trimethylflavone, trivial name santin, their occurrence in the leaves of *Dodonaea* was confirmed from the biosynthetic pathway

Therefore, the *in vitro* antibacterial activity tests revealed that antibacterial activity of the isolated compound from the leaves of *Dodonaea angustifolia* to be the promising source of bioactive compounds that could be used as lead compounds in search for new clinically effective antimicrobial compounds.

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5.2 Recommendation

I. The phytochemical investigation of *D. angustifolia* yielded for the isolation of compounds from the genus further phytochemical investigation on *D. angustifolia* should be done using HPLC so as to check the presence of phenyllanthraquinones, which serves as a marker compound in the genus *Dodonaea*.

II. The crude extracts and isolated compounds showed considerable clear zone inhibition on four bacteria strains *Staphylococccus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa*, the other phytochemicals from the genus should be done on another type of bacteria strain for better activity.

III. The clear zone of inhibition measured for isolated compound on *E.coli* was 15 mm as compared to the standard genetamycin was about 23 mm in diameter, for this compound the minimum inhibition concentration should be done further.

IV. The isolated compound showed more potency for gram-negative bacteria; in contrast to the ethyl acetate soluble extract was more active for gram positive bacteria rather than gram negative bacteria. thus, the mechanism of action of these compound should be studied further

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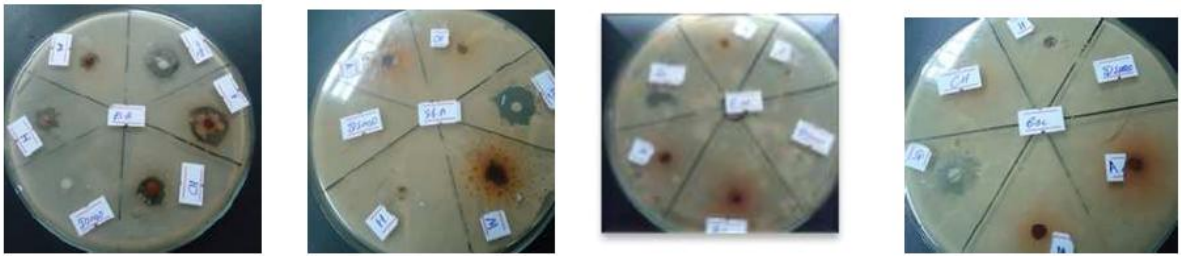
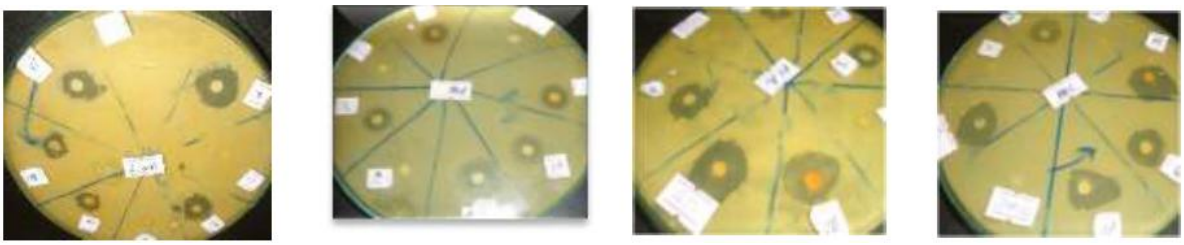
7. Appendices

Appendix 1 Bioassays Tests of Crude Extracts Zone of Growth Inhibition in mm



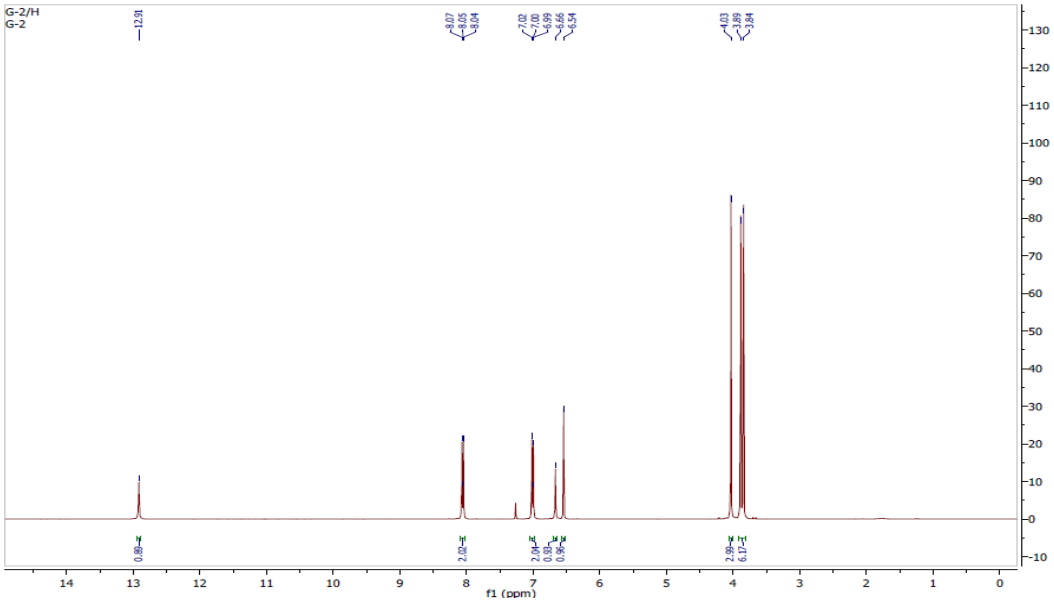
E. Faecalis *E. coli* *S. aureus* *P. aeruginosa*

Appendix 2 Bioassays Tests of Isolated Compound Zone of Growth Inhibition in mm

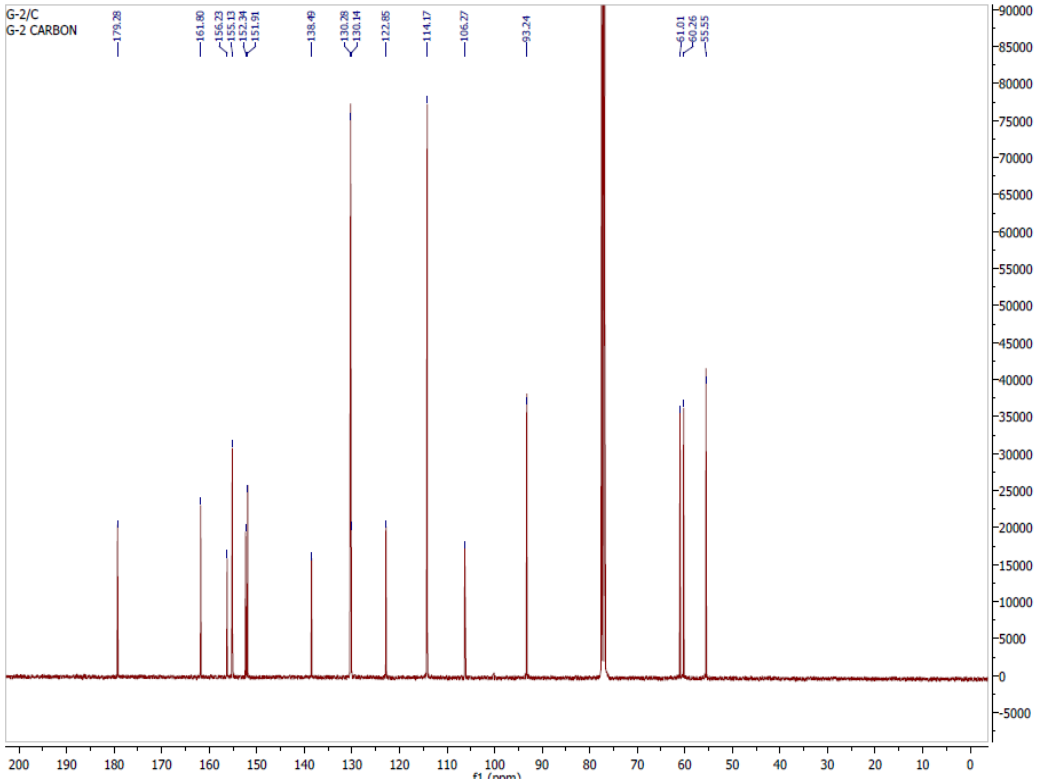


E. faecalis *E. coli* *S. aureus* *P. aeruginosa*

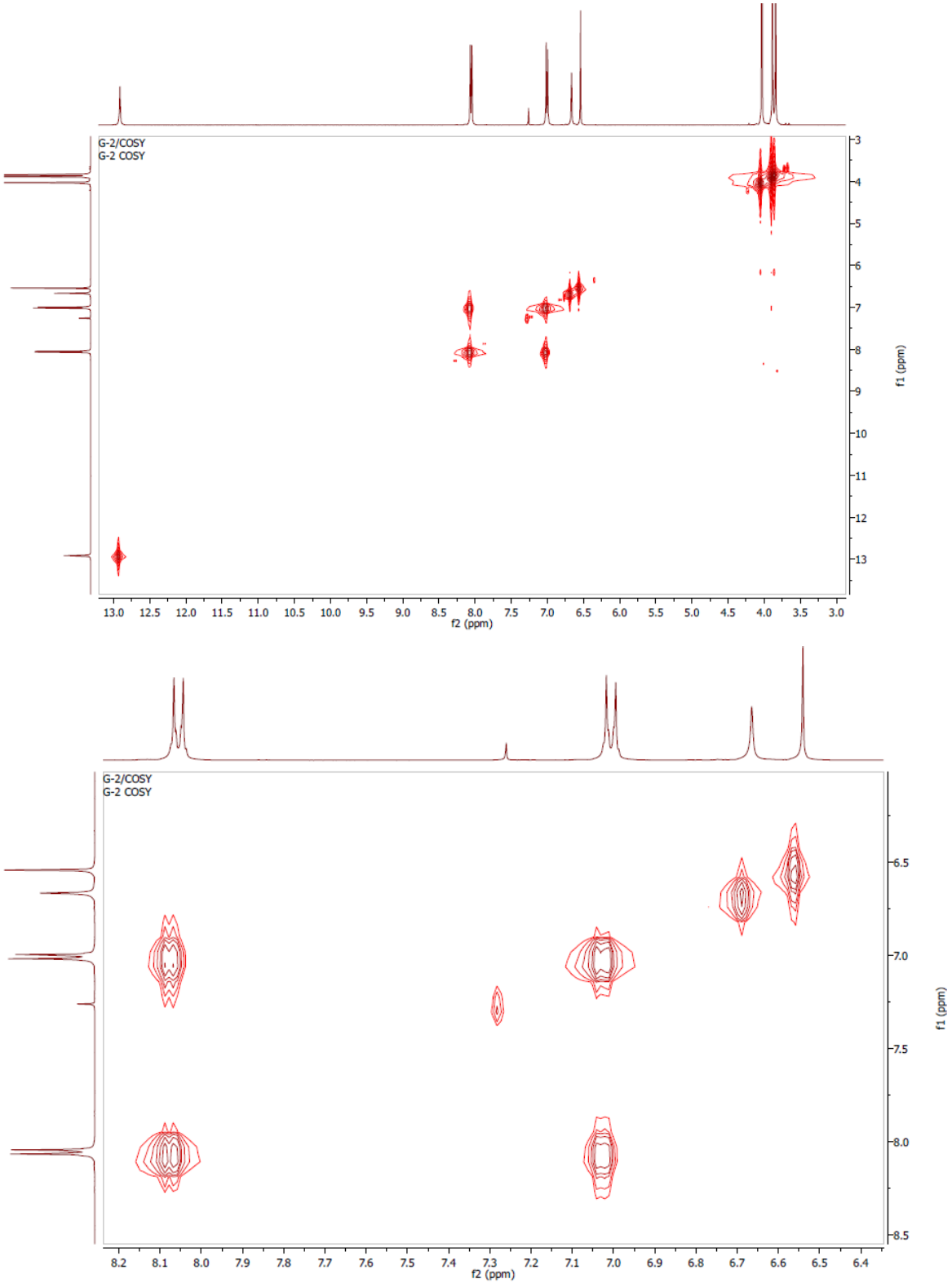
Appendix 3 ^{1}H-NMR Spectrum of compound **(1A)** in $CDCl_3$



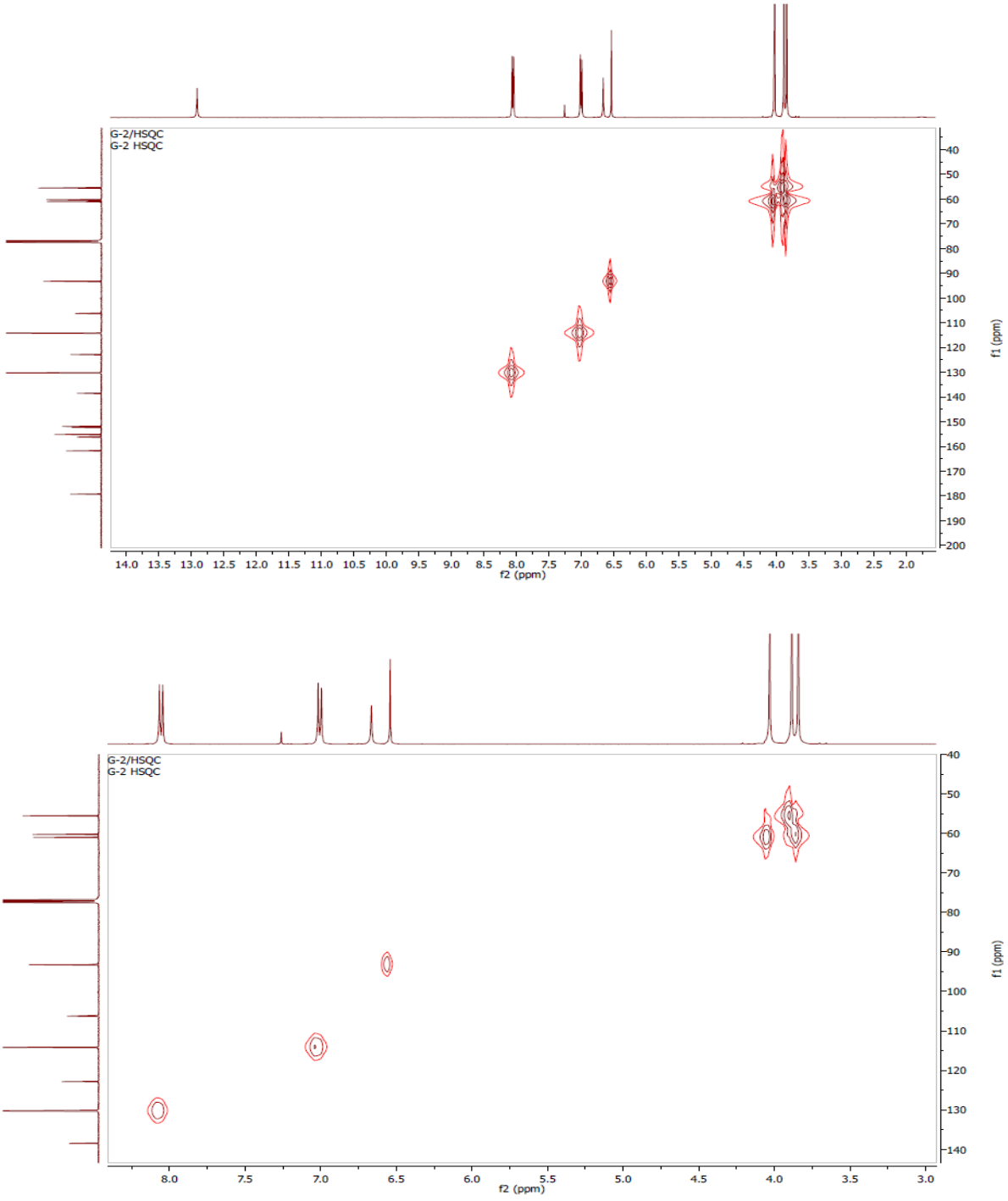
Appendix 4. 13C-NMR Spectrum of Compound **(1A)** in CDCl

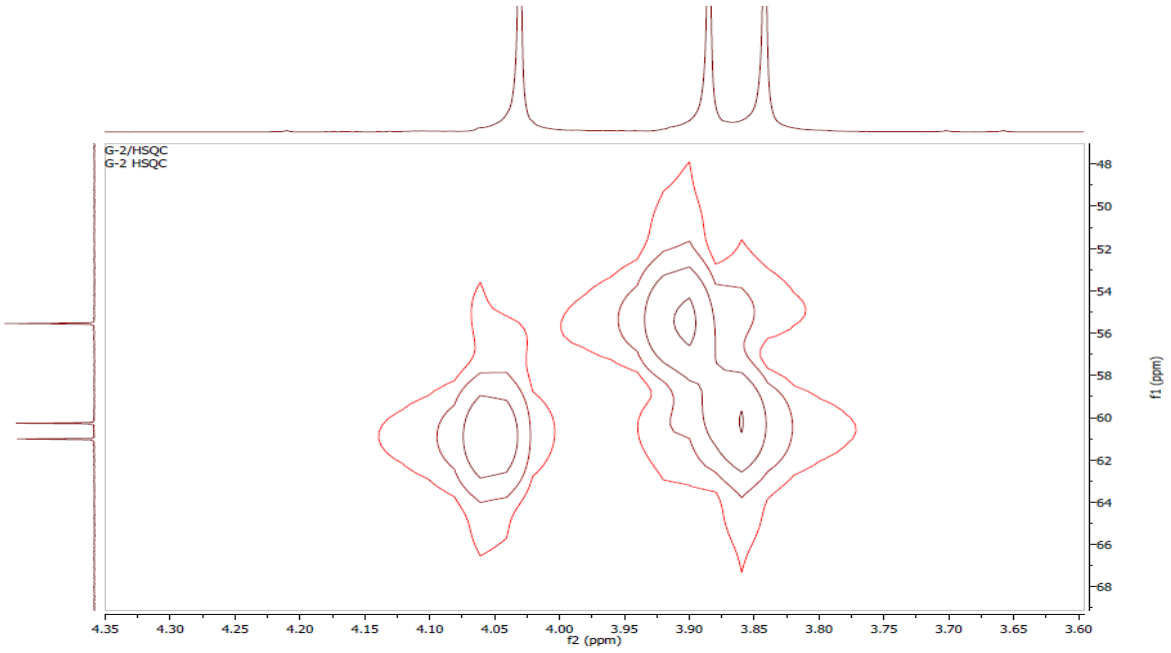


Appendix 5 COSY Spectrum of Compound (**1A**) in $CDCl_3$

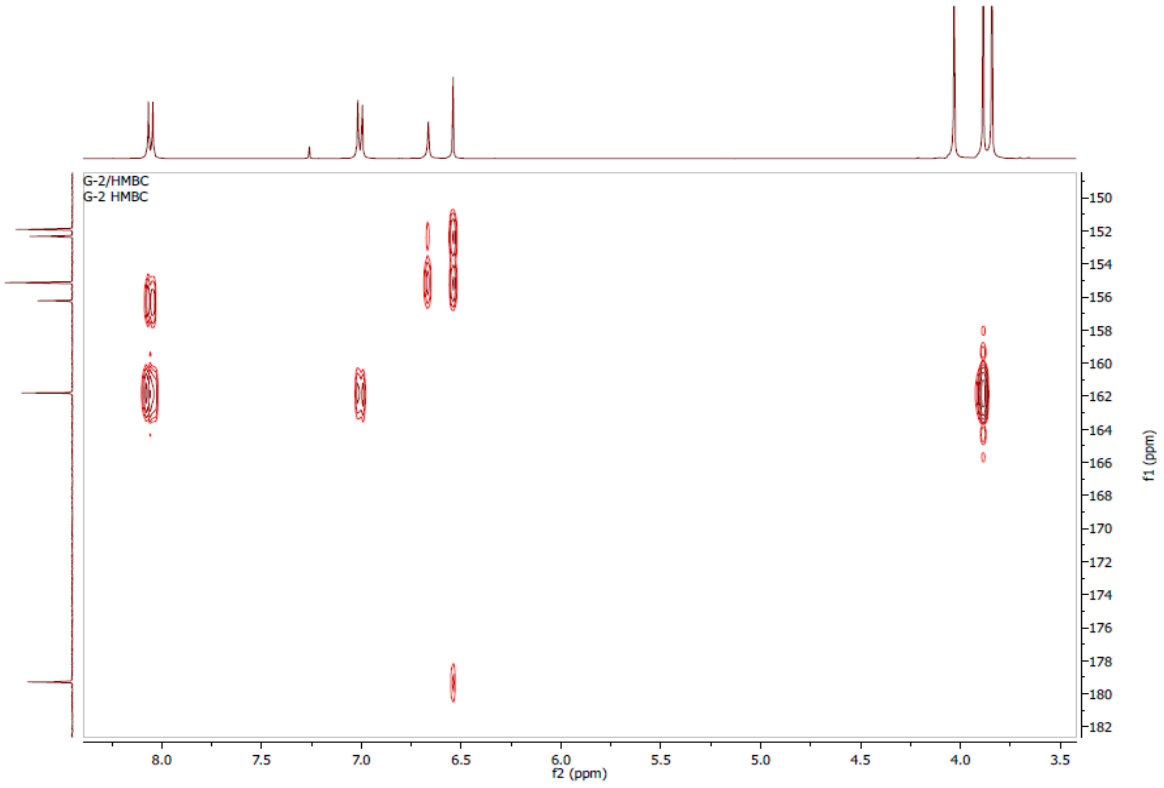


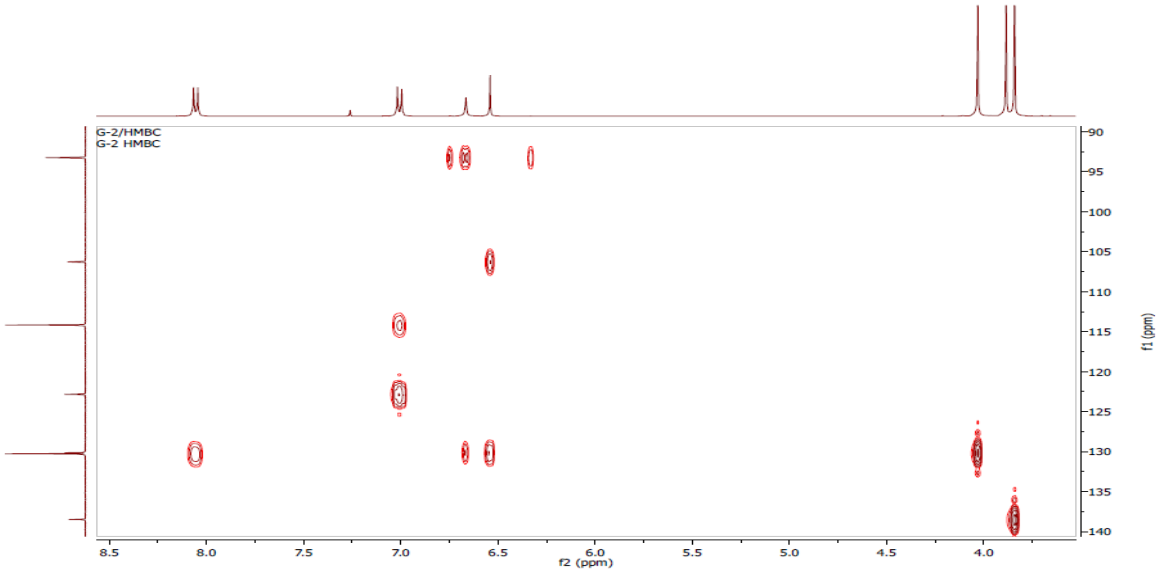
Appendix 6. HSQC Spectrum of compound **(1A)** in $CDCl_3$

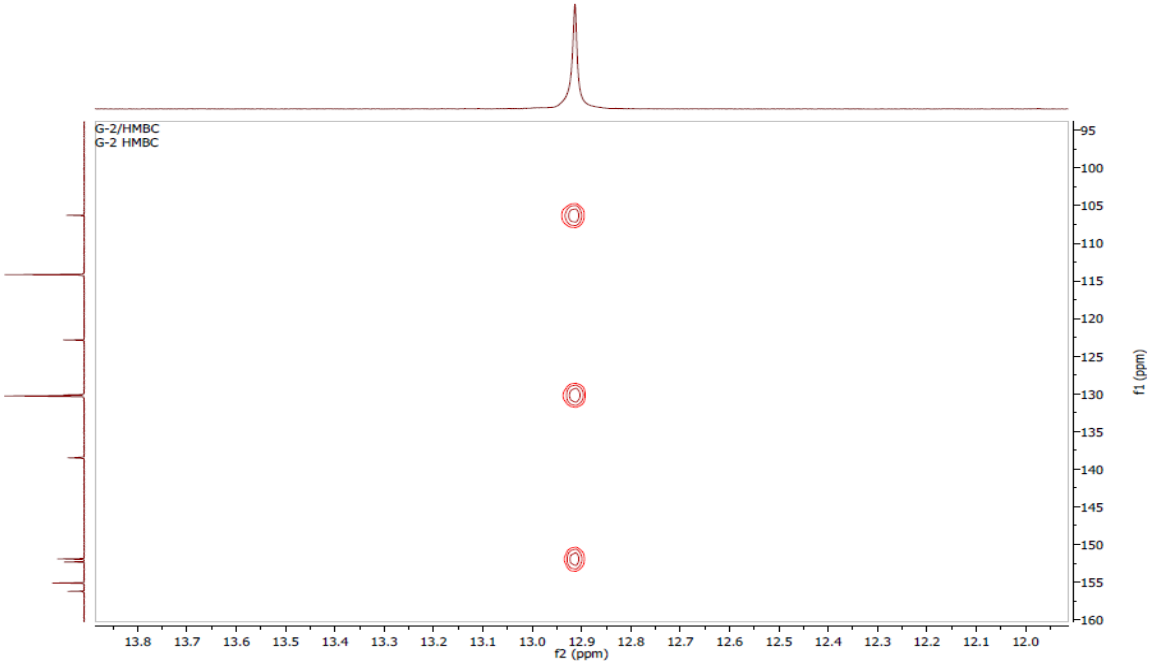




Appendix 7 HMBC Spectrum of compound **(1A)** in $CDCl_3$




G-2/HMBC
G-2 HMBC
f2 (ppm)
f1 (ppm)


G-2/HMBC
G-2 HMBC
f2 (ppm)
f1 (ppm)