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DEPARTMENT OF CHEMISTRY

M.Sc THESIS

ON

PHYTOCHEMICAL INVESTIGATION OF SEEDS OF *Persicaria lapathifolia* AND EVALUATION OF ITS ANTIMICROBIAL ACTIVITIES

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PHYTOCHEMICAL INVESTIGATION OF SEEDS OF *Persicaria lapathifolia* AND EVALUATION OF ITS ANTIMICROBIAL 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 CHEMISTRY(ORGANIC)

BY

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Dedication

I would like to dedicate this thesis work to my father Mr. Humnesa Chibsa and my mother Kabaye Mirkena.

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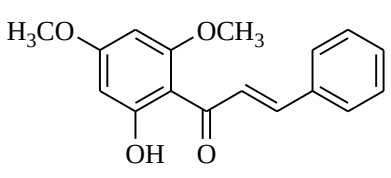
Abstract

Large number of herbal remedies are used to treat microbial diseases; however, the efficacies of most of these plants have not been exhaustively investigated. Thus, the aim of this study was to isolate and characterize compounds from the seeds of *Persicaria lapathifolia* and evaluate their antimicrobial activities. The seeds of *Persicaria lapathafolia* were collected from Jimma University main campus. The air dried plant materials were ground and extracted sequentially with petroleum ether, chloroform and methanol by maceration at room temperature to give crude extract of 4 g, 34 g, and 36 g, respectively. The chloroform extract was selected for further purification following its better antibacterial activity. About 32 g of chloroform extract was subjected to column chromatography that led to the isolation of three pure compounds, named flavokawain B **(1)**, cardamonin **(2)** and pashanone **(3)**. Characterization of these compounds were done using spectroscopic techniques; mainly UV, 1D, 2D NMR, and MS. The crude extracts and isolated compounds were evaluated for their antibacterial and antifungal activity. All extracts and isolated compounds exhibited antifungal activity against *Aspergillus spp* and *Fusarium spp*. The chloroform and methanol extracts had significantly higher inhibitory effect (30 mm) against *Aspergillus spp* and also highest inhibition activity (15 mm) was observed for chloroform extract against *E. coli*. Compound **1** showed marginal activity against *E. coli* (9 mm), but no activity against *S. aureus*, *Trichoderma spp* and *Penicillium spp*. Whereas, compound **2** exhibited better zone of growth inhibition (18 mm) against *S. aureus*, and *Trichoderma spp* strains, which is comparable to that of gentamycin (19 mm) and clotrimazole (20 mm), respectively and no activity against *E. coli*. Compound **3** showed comparable activity against *S. aureus* (12 mm) and *E. coli* (13 mm), and also against *Aspergillus spp* and *Fusarium spp* (20 mm). Chloroform extract and compound **2** demonstrated significant antibacterial and antifungal activities with highest zone of growth inhibition against *S. aureus* and *Trichoderma spp*. which are comparable to that of reference drugs.



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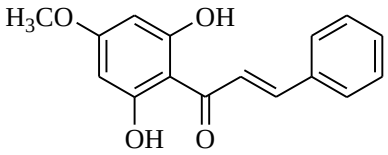

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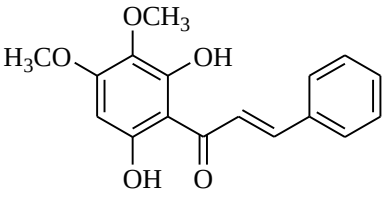

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List of Abbreviations

1D NMR	One Dimensional Nuclear Magnetic Resonance
2D NMR	Two Dimensional Nuclear magnetic Resonance
ATCC	American Type Culture Collection
COSY	Correlation Spectroscopy
DMSO	Dimethyl Sulfoxide
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
MS	Mass Spectrometry
MHz	Mega Hertz
NOESY	Nuclear Overhauser and Exchange Spectroscopy
TLC	Thin Layer Chromatography

Table of Contents

List of Tables

Table Page

List of Figures

Figure Page

1. Introduction

1.1 Background of the study

Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Even today, medicinal plants continue to play a major role in primary health care as therapeutic remedies in many developing countries [1]. The discovery of medicinal plants in different parts of the world is important both to the agriculture and medicine sectors, for the establishment of new directions towards propagation of alternative medicinal crops that offer better economic and social benefits [2].

The control of microbial infection has been remarkably effective since the discovery of antibacterial drugs [2]. However, the emergency of resistance pathogens limits the therapeutic uses of many of the drugs that are in the market and undesirable side effects associated to certain antibiotics have led to the search of new antimicrobial agents from medicinal plants [3]. Higher plants have been shown to be a potential source for new antimicrobial agents. The screening of plant extracts has been of a great interest to scientist for the discovery of new drugs effective in the treatment of several diseases. A number of reports concerning the antibacterial screening of plant extracts of medicinal plants have appeared in the literatures [4].

1.1.2 Natural Products as a Source of Drug Discovery

Most of the currently available drugs for the treatment of different human and animal diseases obtained from natural products, especially medicinal plants [5-8]. Such drugs have been discovered after observing the medicinal use of a particular plant or its parts (leaves, roots, barks, fruits or seed or whole plant) by herbalists and subsequent isolation of bioactive compounds from the plant or part of the plant that had been used traditionally for the treatment of different human illnesses [5-8]. Moreover, some compounds obtained from natural sources have also been used as leads or precursors that can be modified synthetically to improve their therapeutic activities [5, 6].

Introduction and development of several new and highly specific *in vitro* bioassay techniques, chromatographic methods, spectroscopic techniques and other standardized pharmacological methods have also made it much easier to screen, isolate and identify potential drug and precisely from natural sources to alleviate human illnesses [9-12]. There are several reasons that necessitate isolation and characterization of bioactive compounds. Some of the reasons are: distribution of

medicinal plants is not uniform throughout the world to be used by people everywhere; most of the medicinal plants are under threat of extinction due to climate changes and population pressure [13, 14]; isolation and purification of compounds from natural sources is tedious, expensive and time consuming process; and the need to identify the chemical compounds that are responsible for the observed medicinal value of the plant. Once, such compounds are known and their properties and structures determined they can be prepared synthetically without relying on plants.

In addition, the amounts of bioactive compounds obtained from medicinal plants are very small and thereby many plants should be destroyed to harvest significant quantities of such compounds for disease treatment [15, 16]. All these facts necessitated for isolation and characterization of bioactive compounds from medicinal plants and subsequently synthesize them in large scales in laboratories or industries in reasonable costs [11, 16].

It is estimated that today, plant materials have provided the models for 50% modern drugs [17, 18]. The search for plants as a source of potential candidate for drug development is still unsound. From the total of 250,000 to 500,000 species of plants available on earth only 1-10% are being potentially used [19]. Even today, traditional medicine is still the predominant means of health care in developing countries where about 80% of their total population depends on it for their well-being [20], while in Ethiopia about 90% of the populations use traditional medicine for health care needs due to acceptability, availability and affordability [21].

Therefore, traditional medicinal plants are the basis for the development of modern drugs and it has been used for many years in daily life to treat disease all over the world [22] is still wide spread. One of the plant species that is known for its medicinal value is *Persicaria lapathifolia* in folk medicine for its anti-inflammatory, antibacterial, antiviral, antifungal and in the treatment of dysentery. Extracts from aerial part of *P. lapathifolium* have also anthelmintic and anti-emetic properties [23].

The rapidly increasing incidence of antibacterial resistance and the problems presented by antimicrobial agents added in food and public pressure on the food industry to avoid chemical preservatives are the main factors justifying the search and development of new antimicrobial agents, especially from those of natural origin [24]. Therefore, this study was to identify secondary metabolites from the seed of *P. lapathifolia* and evaluate its antimicrobial activities.

1.2 Statement of the problem

Infectious diseases are caused by pathogenic microorganisms, such as bacteria, viruses, or fungi. Infectious diseases have been affecting the global health and economic status and problem in both developed and developing countries accounting approximately for one half of all deaths in tropical countries [25]. However, the emergency of resistance pathogens limits the therapeutic uses of many of the drugs that are in the market and undesirable side effects associated to certain antibiotics have led to the search of new antimicrobial agents from medicinal plants [3].Traditionally, people in Ethiopia have been used medicinal plants to treat different disease and also it has great contribution in primary health care systems [26]. However, most of the existing medicinal plants, mainly those which have documented in traditional uses have not been well investigated for identification of their bioactive principles. *P. lapathifolium* is one of the ethno medicinal plants that have been commonly visited by traditional healers in some parts of Ethiopia. Even though, this plant has been used for the treatment of different alignments including infections during delivery in local medicine, the phytochemical pertaining this plant and its antibiotic activity has not been addressed. Therefore, the present study focused on the isolation and identification of compounds from this plant and evaluation of its antimicrobial activities.

1.3 Objectives of the study

1.3.1 General objective

The main objective of this study was to investigate secondary metabolites from the seed of *P. lapathifolia* and evaluate its antimicrobial activity.

1.3.2 Specific objectives

The specific objectives of this study were:

- To isolate secondary metabolites from the seed extract of *P. lapathifolia* using chromatographic techniques;
- To elucidate the structures of the isolated compounds using spectroscopic techniques, (UV-Vis, 1D, 2D NMR and MS) and
- To evaluate the activities of the crude extracts and isolated compounds against two bacterial strains (*Staphylococccus aureus*, and *Escherichia coli*) and four fungal strains (*Aspergillus spp, Trichoderma spp, Fusarium spp* and *Penicillium spp*).

1.4 Significance of the study

The result of this study would help to

- Identify compounds that are used as antibacterial and antifungal agents in this plant.
- The chemical constituents of this plant provide baseline information for future pharmacological and bio prospecting studies.
- Use this finding for further phytochemical studies on the plant species.
- The discovery of natural products is also important as a means to further refine systems of plant classification.

2. Review of Related Literature

2.1 Botanical Information

2.1.1 The Family *Polygonaceae*

The family *Polygonaceae* is one of the flowering plants that comprises about 50 genera and 1200 species [27, 28]. The largest genera are Eriogonum (240 species), Rumex (200 species), Coccoloba (120 species), *Persicaria* (100 species) and Calligonum (80 species). The family is distributed worldwide, but is most diverse in the North Temperate Zone [29]. The name *Polygonaceae* is believed to be derived from "*Polygonum"* from the Greek poly, "many" and gonu, "knee" referring to the swollen-jointed stem.

Many species are aquatic and naturally grow in the swampy area as floating plants in the rivers or ponds. The leaves vary in shape between lanceolate to oval forms, with the height range from 1 to 30 cm. The stems are usually red, reddish and sometime red-speckled. Generally, flowers are formed in dense clusters from the leaf joints and the youngest are white, pink or greenish [30].

2.1.2 The Genus *Persicaria*

The genus *Persicaria* sometimes called *Polygonum*, found all over the world. They contain diverse pharmacologically active constituents with various properties [31]. The species under the genus are predominantly herbs, found in tropical and temperate regions [30]. Some species are important in traditional medicines. Many of its species and their active principles have been studied for phytochemical screening, biological and pharmacological purposes. Some of them have shown efficacy in the prevention and treatment of diseases such as cancer, malaria, gastric ulcers, bacterial and fungal infections; their toxic effects have also been evaluated [32].

2.1.3 *Persicaria lapathifolium L.*

Persicaria lapathifolium L.(syn. *Persicaria lapathifolia* (L.) S. F. Gray, *Polygonaceae*) is widely distributed from the tropics in the northern hemisphere to the temperate zone and grows wildly on green fields, along rivers and on ditch banks [33].

Figure 1. *Persicaria lapathifolium* (Taken by Ashenafi; 05, June, 2017)

2.4 Ethno medicinal uses of the genus *Persicaria*

The *Persicaria* extract has been used in the folk medicine to cure urinary tract infections in China [34]. *P. cuspidatum* has been used to relieve joint pain, to treat jaundice caused by a bacterial infection or fungal problems and cough with a menorrheal expectoration. *P. cuspidatum* is also used as a decoction to treat liver diseases or as a cream for topical application in burns, wounds and traumatic injuries [35]. Currently it is used in different forms such as powder, decoction or infusion to cure hepatitis and several other diseases [36]. In Korea, the rhizomes of *P. cuspidatum* are commonly used to maintain oral and dental hygiene [37].

In traditional Chinese medicine, *P. multiflorum* has been used to treat mental and physical signs of aging, malaria, constipation and eczema. In Cameroonian traditional medicine, the raffia wine and aqueous extracts of the leaves of *P. limbatum* are respectively used to cure gastrointestinal disorders, venereal diseases and skin infections [38]. In India, preparations of *P. nepalense* are employed for the treatment of colds, influenza, swelling, hemorrhoids, diarrhea and rheumatism [39]. The juice extract of the whole plant is taken orally for fetal mal-position and the decoction is also taken orally for juvenile pregnancy in Cameroon [40]. In Argentina, *P. ferrurgineum* is used to heal infected wounds in local medicine and control several bacteria and fungi [41]. In Bangladesh *P. lapathifolium* is used as an insecticide [42]. In Brazil, *P. spectabile* is used by

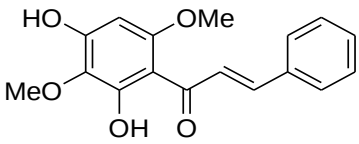
indigenous people for the treatment of diarrhea, ulcers, gingivitis, skin infections and rheumatism [43]. *P. hydropiper* is used as spice to flavor foods by Chinese and Malaysian indigenous because of its strong peppery taste [44]. In Turkey, the roots of *P. amphibium* are very important in folk medicine and are used as an astringent and cleanser for skin [45, 46]; they are also eaten raw, or sometimes they are dried, pounded and the infusion is used in the treatment of chest colds [47]. These data show that the genus is widely distributed around the world and is being used on all continents to control numerous illnesses.

2.5 Compounds from the Genus *Persicaria*

Several investigations were carried out to determine the possible chemical components from *Persicaria* plants. This genus is also well known for producing a wide variety of metabolites including flavonoids [44], terpenoids [48], anthraquinones [49], coumarins [50], phenylpropanoids [51], lignans [52], sesquiterpenoids [53], stilbenoids [54], tannins [55], proteins, amino acids and carbohydrates [56], and sucrose phenyl propanoid esters [50]. Among them, flavonoids are the most common components found in *polygonum* spp. and have been used as chemotaxonomic markers of the genus [53].

Some of the bioactive compounds obtained from the *Persicaria* genus were presented in Fig 2 and Table 1 displays the pharmacological effects of some extracts and compounds isolated from some *Persicaria* plants. The compounds include 2',4'-dihydroxy-3',6'-dimethoxychalcone (**1**) [57, 67]; 2'-hydroxy-4',6'-dimethoxychalcone (**2**) [57]; cardamomin (**3**) [64]; 2',6'-dihydroxy-3',4'-dimethoxychalcone (**4**) [72]; (S)-Pinostrobin (**5**) [59]; (2S)-5-hydroxy-6,7-dimethoxyflavanone (**6**) [59]; (2S)-5,7-dimethoxyflavanone (**7**) [59]; 5-hydroxy-7-methoxyflavanone (pinostrobin) (**8**) [41, 57]; pinocernbrin (**9**) [60]; luteolin (**10**) [61]; apigenin (**11**) [62]; quercetin (**12**) [63]; isorhamnetin (**13**) [63]; physcion (**14**) [65]; emodin (**15**) [66]; chrysophanol (**16**) [59].

Chalcone



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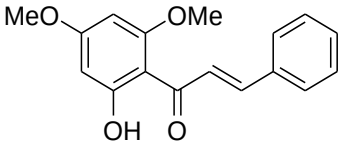

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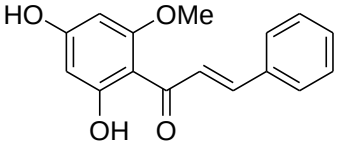

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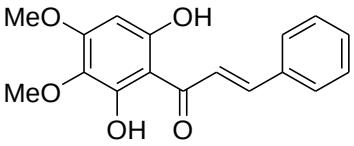

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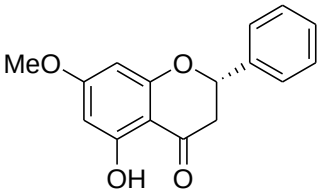
Flavanone



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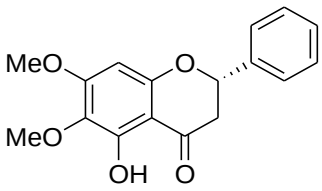

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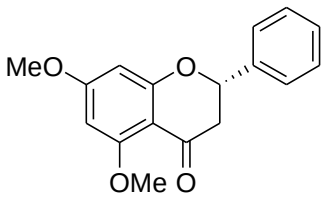

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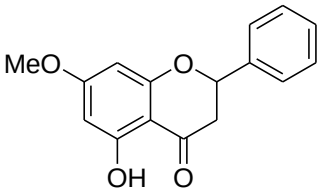

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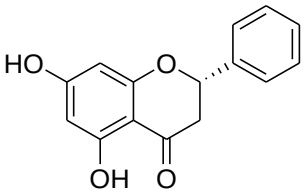

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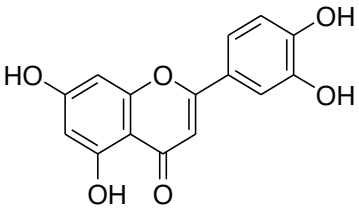
Flavone



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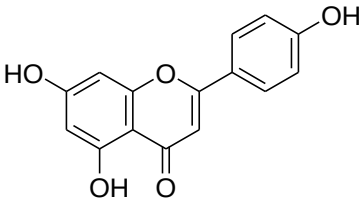

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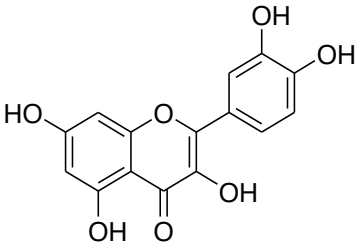

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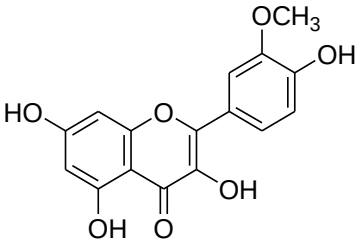

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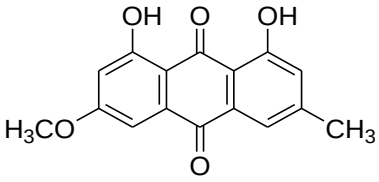
Anthraquinones



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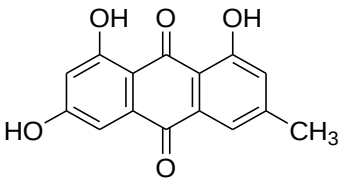

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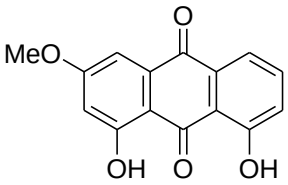

16

Figure 2. Compounds from the genus *Persicaria*

2.6 Pharmacological Effects

Table 1. Pharmacological effects of some extracts and compounds isolated from some *Persicaria*.

Plants	Extracts, compounds	Pharmacological effects	*In vitro*	Reference
P. spectabile	Ethyl acetate extract	*Anti-Staphylococcus aureus*	*In vitro*	[58]
	2'-hydroxy-4',6'-dimethoxychalcone (**1**)	*Anti-Staphylococcus aureus*	*In vitro*	
	2',4'-dihydroxy-3',6'-dimethoxychalcone (**2**)	*Anti-Vero cells*	*In vitro*	
P. limbatum	Cardamomin (**3**)	*Leukemia* (anticancer)	*In vitro*	[64]
		Cervix (anticancer)	*In vitro*	
	2',4'-dihydroxy-3',6'-dimethoxychalcone (**2**)	*Leukemia* (anticancer)	*In vitro*	
		Cervix (anticancer)	*In vitro*	
P. cuspidatum	Physcion (**14**)	*Inhibition of melanogenesis effect*	*In vitro*	[65]
	Emodin (**15**)	*Anti-human prostate cancer cells*	*In vitro*	[66]
P. senegalense	2',6'-dihydroxy-4'-methoxydihydrochalcone	*Anti- plasmodium falciparum*	*In vitro*	[67]
	2',4'-dihydroxy-3',6'-dimethoxychalcone (**2**)	*Anti- plasmodium falciparum*	*In vitro*	

2.7 Biosynthetic Pathway of Chalcone

The production of chalcones as natural products represents the convergence of two synthetic pathways; the acetate and the shikimate pathways. Chalcones are synthesized by sequential condensation of 3 molecules malonyl-CoA with one of p-coumaroyl-CoA. This reaction is carried out by the enzyme chalcones synthase (CHS). The final step is believed to be the cyclization of a tetra ketide precursor.

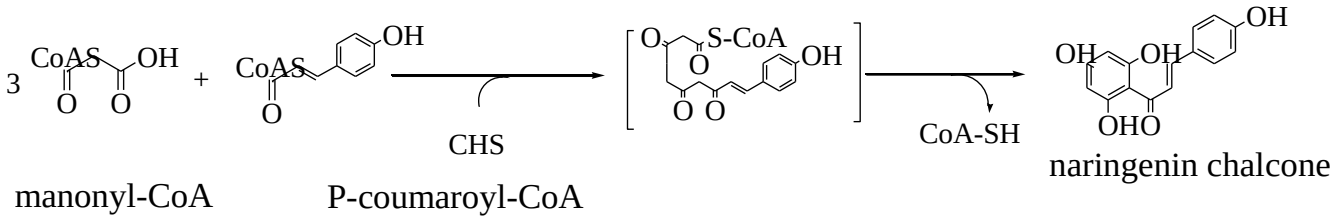

Figure 3. Scheme for biosynthesis of chalcone

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals

All chemicals used were analytical grade. Solvents used for extraction of plant materials were petroleum ether, chloroform and methanol. Silica gel 60-120 mm size, silica gel coated TLC, iodine for detection of spots on TLC. $CDCl_3$ and acetone-d_6 solvent, DMSO, gentamicin, Mueller Hinton agar, nutrient agar and saline solution were used as a culture medium during antimicrobial test.

3.1.2 Apparatus and Equipment

Rotary evaporator (Heidolph, USA) for solvent evaporation, UV chamber (LF-206.LS, EEC) for detection of spots on TLC plate, Melting point apparatus (MFB 590010T, Griftin, Britain); UV-Visible photo-spectroscopy (JENWAY 6705, UK), column chromatography (300 mm (B-14/23, B-19/26) and 500 mm, (B-34/35); Smith Scientific Eden bridge; UK) 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), weighing balances, oven, Bunsen burner, Vertical Laminar Flow Cabinet CLB-201-14, incubator, were used for this study.

3.2 Methods

3.2.1 Collection and Preparation of the Plant Specimens

The fresh plant material was collected from Oromia region, Jimma University main campus on October 2016. The collected plant material was chopped into smaller pieces and shade dried in chemistry laboratory department at room temperature. The air dried plant material was ground to small size to facilitate easy solvent penetration. The powdered plant material was stored in suitable container until used for extraction.

3.3 Extraction

The powdered plant material (865 g) was sequentially extracted with petroleum ether (2.5 L), chloroform (2.0 L), and methanol (2.0 L) based on increasing polarity for 72 hr with occasional shaking by maceration at room temperature. The filtrate was then separated from the mark using fresh cotton plug. The filtrate was concentrated using Rotary evaporator at 50 ℃ under reduced pressure then stored at room temperature.

3.4 Isolation

The chloroform extract that showed better antibacterial activity was selected for further isolation of the bioactive compounds. The chloroform crude extract (32 g) was adsorbed on 50 g silica gel and applied on column chromatography already packed with silica gel.

The column was first eluted using 100% petroleum ether, and then with increasing gradient of ethyl acetate in petroleum ether (1, 2, 4, 6, 8, 10, 15, 30, 50, 70, 80 up to 100%) during chromatographic separation. The fractions were concentrated under reduced pressure using a Rotary evaporator at 50 °C. The fractions were examined by TLC and the spots developed were visualized under UV light at 254 and 365 nm and then by exposure to iodine vapor. The fractions that showed the same TLC profiles (Rf) were combined and concentrated to dryness under reduced pressure using Rotary evaporator.

3.5 Identification of compounds

The isolated compounds were characterized by the various spectral techniques namely; ^{1}H-NMR, ^{13}C-NMR, HMBC, HMQC, NOESY, UV-Vis and MS.

3.6 Antibacterial and Antifungal Activities Test

3.6.1 Test strains

The crude extracts and isolated compounds were evaluated for *in vitro* antibacterial activities against two bacterial strains (*Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923) and the antifungal activities against four fungus strains (*Aspergillus spp, Trichoderma spp*, *Fusarium spp* and *Penicillium spp*) by disc diffusion method. All strains obtained from Jimma University department of biology in microbiology laboratory culture collection.

3.6.2 Bioassay (Agar Disk Diffusion Method)

Agar disk diffusion method was used to evaluate the antibacterial and antifungal activities of both crude extract and isolated compounds on nutrient agar. Briefly, the bacteria stock cultures were maintained on the nutrient agar slants which were stored at 4 ℃. Agar cultures of the test microorganisms were prepared according to manufacture instruction. The test solutions were prepared by dissolving 0.01 g ratio of plant extracts to achieve final stock concentrations of 100 mg/mL in DMSO. Freshly, grown liquid culture of the test pathogens solution of having similar turbidity with 0.5 McFarland were seeded over the Mueller-Hinton Agar medium with sterile swab.

Sterile Whatman filter paper discs (6 mm) were soaked with stock solution of the extract then placed over the seeded plates at equidistance. The plates were then inverted and incubated at 37 ℃ for 24 hr. After the incubated period, the plates were for observed a clearance zone around the disks which indicates a positive antibacterial activities of the respective plant extracts. The clear zones formed around each disk were measured in millimeter.

Bioassay for antifungal activities was the same method described for bacteria will be adopted. The inoculated medium will be incubated for three days. For each bacterial and fungal strain, the standard gentamicin and clotrimazole were taken as positive control respectively and pure solvent DMSO as the negative control.

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4. Results and Discussion

4.1 Result for extraction and isolated of secondary metabolites from seed of *P. lapathifolia*

The air dried seed of *Persicaria lapathifolia* was sequentially extracted using petroleum ether, chloroform and methanol by maceration at room temperature to yield 4 g, 34 g and 36 g, respectively. The crude extract of chloroform (32 g) was subjected to column chromatography for further purifications. Chromatographic purification of the extract resulted in the isolation of three compounds; compounds **1**, **2** and **3** from 1%, 2% and 8% petroleum ether to ethyl acetate, respectively. The extraction, isolation, and purification of compounds process are summarized as follow.

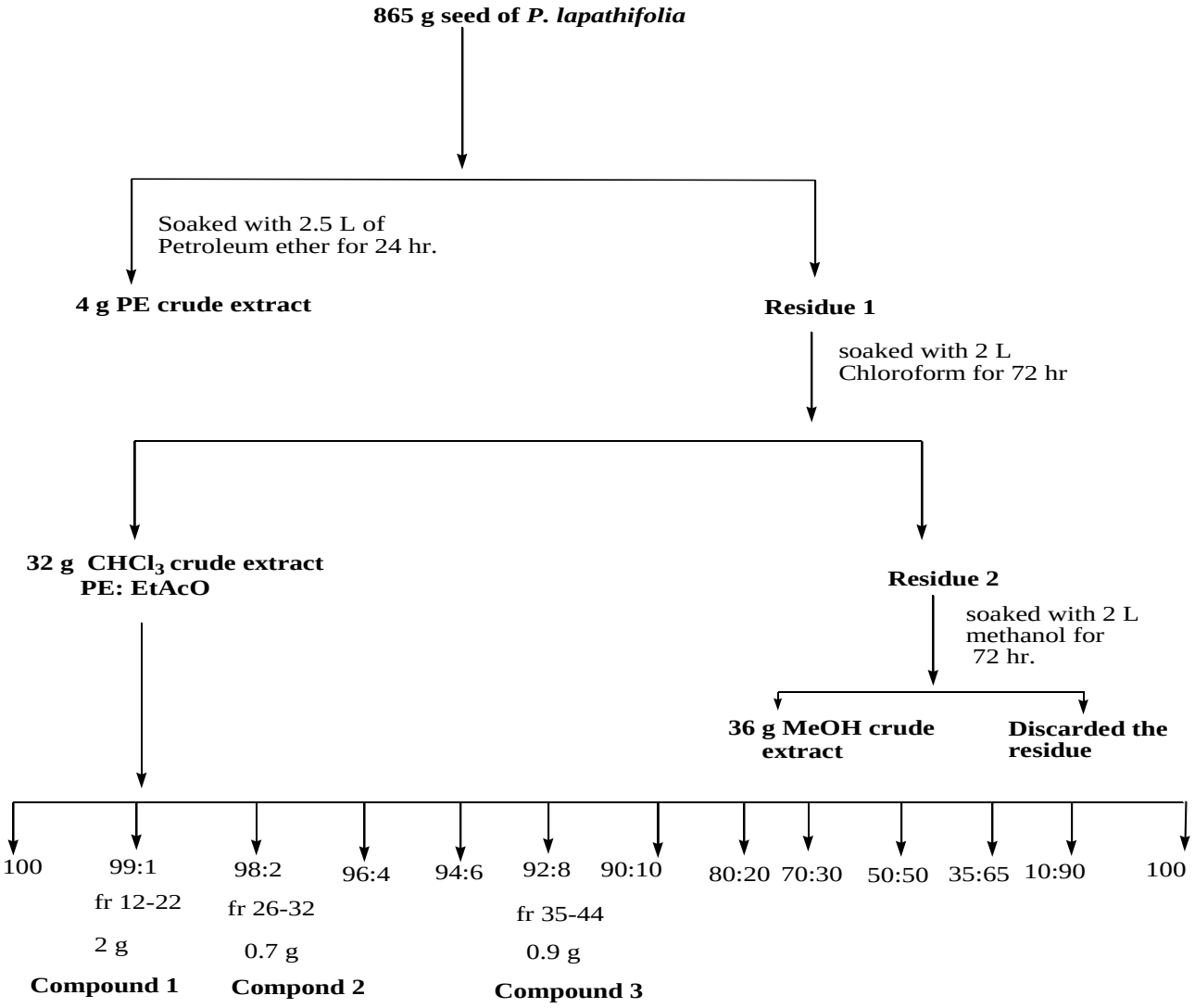

Figure 4. Scheme for extraction, isolation and purification of *Persicaria lapathifolia* seed

4.2 Characterization of the isolated compounds

4.2.1 Characterization of compound 1

Compound **1** was isolated as yellow amorphous solid from fraction eluted with 1% ethyl acetate in petroleum ether. It showed melting point of 87-89 ^{o}C and UV spectrum maximum at (MeOH, $\lambda_{max, nm}$) were observed at 219 and 342 nm. The positive mode ESI-MS spectrum showed molecular ion peak of *m/z* 307 $[M + Na]^+$ and 591 $[2M + Na]^+$, both corresponding to the molecular formula $C_{17}H_{16}O_4$, which indicated ten degrees of unsaturations.

The ^{1}HNMR spectrum showed signals for ten protons (Table **2**) including highly downfield shifted signal at δ_H 14.32 for phenolic hydroxyl group involved in hydrogen bonding. The presence of a typical carbonyl carbon signal at δ_C 192.7 in ^{13}CNMR spectrum and a *trans*-configured double bond (δ_H 7.79, 1H, *d*, *J* = 15.6 Hz, H-α; δ_H 7.91, 1H, *d*, *J* = 15.6 Hz, H-β) confirmed the existence of the chalcone skeleton. The presence of five mutually coupled multiplet aromatic protons at δ_H 7.60-7.39 confirmed by HMBC analysis has indicated mono-substituted aromatic ring A. In ring B, two upfield shifted (due to di-*ortho* oxygenation) *meta* coupled proton signals at δ_H 5.96 (1H, *d*, 2.0) and 6.10 (1H, *d*, 2.0) where assigned to H-3' and H-5', respectively, which otherwise fully substituted with two methoxyl (δ_H 3.90 and 3.82) and one hydroxyl (δ_H 14.32) groups. The NOESY between all protons can be observed simultaneously. NOESY spectrum confirm that H-3' coupled on space with 4'-OC<u>H</u>$_3$, and H-5' coupled with 6'-OC<u>H</u>$_3$ and also H-5' coupled with 4'-OC<u>H</u>$_3$.

The ^{13}C NMR spectrum (Table **2**) showed 17 carbon signals; which accounted for two methoxyl group, nine methine and six quaternary carbon atoms. The position of the methoxyl groups, δ_H 3.90 and 3.82 were established at C-6' (δ_C 166.5) and C-4' (δ_C 168.5), respectively based on their HMBC correlations to vicinal carbons. Whereas the hydroxyl group (δ_H 14.32) was placed at C-2' (δ_C 162.6) *peri* to carbonyl carbon.

These data are consistent with the compound being 2'-hydroxy-4', 6'-dimethoxychalcone; trivial name, flavokawain B, which was previously reported from *P. lapathifolium* and *P. ferrugineum* [41, 68].

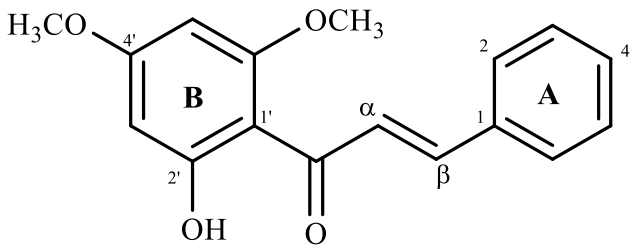

Figure 5. Structure of compound **1**

Table 2. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compound **1** (in $CDCl_3$) and flavokawain B.

	Compound 1			**Flavokawain B [69]**	
Position	**δ_H (*J* in Hz)**	**δ_C**	**HMBC**	**δ_H (*J* in Hz)**	**δ_C**
1		135.7			135.7
2/6	7.60 (2H, *m*)	128.4	C-1, C-3, C-4, C-5, C-6, Cβ	7.61 (*dd*, 7.8, 1.2, 2H)	128.3
3/5	7.42 (2H, *m*)	128.9	C-1, C-2, C-4, C-5, C-6	7.42 (*dd*, 2H)	129.0
4	7.39 (1H, *m*)	127.6	C-2, C-3, C-5, C-6	7.39 (*t*, 1H)	127.7
α	7.79 (1H, *d*, 15.6)	130.1	C-1, CO	7.79 (*d*, 15.6, 1H)	130.2
β	7.91 (1H, *d*, 15.6)	142.3	C-1, C-2, C-6, CO	7.91 (*d*, 15.6, 1H)	142.5
1'		106.4			106.5
2'		162.6			162.6
3'	6.10 (1H, *d*, 2.0)	93.9	C-1', C-4', C-5'	6.10 (*d*, 2.4, 1H)	93.9
4'		168.5			168.5
5'	5.96 (1H, *d*, 2.0)	91.3	C-1', C-3', C-6'	5.96 (*d*, 2.4, 1H)	91.4
6'		166.5			166.4
6'-OCH_3	3.90 (3H, *s*)	55.6	C-6'	3.90 (*s*, 3H)	55.7
4'-OCH_3	3.82 (3H, *s*)	55.9	C-4'	3.84 (*s*, 3H)	56.0
2'-OH	14.32 (1H, *s*)			14.27 (*s*, 1H)	
CO		192.7			192.8

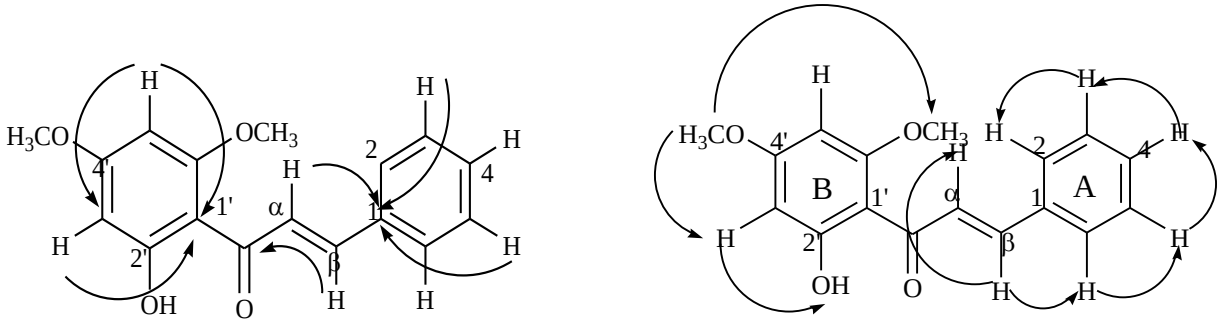

Figure 6. HMBC and NOESY for compound **1**

4.2.2 Characterization of compound 2

The second compound **2** was isolated as red amorphous solid. It showed melting point 144-145 ^{o}C and UV spectrum maximum (MeOH, $\lambda_{max,\ nm}$) were observed at 221, 288 and 335 nm.The ESIMS of this compound showed a sodium adduct ion $[M + Na]^+$ at *m/z* 293 and $[2M + Na]^+$ at *m/z* 563, which is consistent with $C_{16}H_{14}O_4$ molecular formula.

The ^{1}H NMR spectral (Table **3**) features were virtually identical to that of compound **1** with the two downfield shifted proton signal at δ_H 8.26 and 7.80 (*J* = 15.6 Hz) for *trans*-configured α, β-unsaturated chalcone moiety and five mutually coupled aromatic protons (δ_H 7.70-7.43) for mono substituted phenyl ring A. The only notable difference is observed in ring B, where the two upfield shifted *meta* coupled protons (δ_H 5.96 and 6.10 (*d*, 2.0)) observed in compound **1** have been replaced with chemically equivalent two proton singlet (δ_H 6.04) in compound **2**. The chemical equivalency of these protons can only be happen if this ring is symmetric. Furthermore, the presence of only one methoxyl protons at δ_H 3.82 and a chelated hydroxyl proton at δ_H 12.02 allowed the placement of these groups at C-4' (δ_C 167.3) and C-2' (δ_C165.5) respectively, supporting the aforementioned argument. Therefore, the two protons at δ_H 6.04 were equivocally assigned to H-3' and H-5'.

The ^{13}CNMR spectrum (Table **3**) showed 16 carbon signal including one methoyl group, nine methine and six quaternary carbon atoms. Therefore, based on these spectroscopic data, compound **2** was identified as 2', 6'-dihydroxy-4'-methoxychalcone, trivial name cardamonin, previously reported from bark of *Lindera umbellate* [70].

Table 3. ^{1}H (500 MHz), ^{13}C (125 MHz) and HMBC NMR data of compound **2** (in acetone-d_6)

Compound 2			
Position	**δ_H (*J* in Hz)**	**δ_C**	**HMBC**
1		136.5	
2/6	7.44 (2H, *m)*	129.2	C-1, C-3, C-4, C-5, C-6
3/5	7.70 (2H, *m*)	129.8	C-2, C-4, C-5, C-6
4	7.43 (1H, *m)*	128.4	C-2, C-3, C-5, C-6
α	7.80 (1H, *d*, 15.6)	131.0	C-1, CO
β	8.26 (1H, *d*, 15.6)	143.0	C-1, C-2, C-6, CO
1'		106.2	
2'/6'		165.5	
3'	6.04 (1H, *s*)	94.7	C-1', C-2', C-4', C-5'
4'		167.3	
5'	6.04 (1H, *s*)	94.6	C-1', C-3', C-4', C-6'
4'-OCH_3	3.82 (3H, *s*)	55.9	C-4'
2'/6'-OH	12.02 (2H, *s*)		C-1', C-2', C-3', C-5', C-6'
CO		193.5	

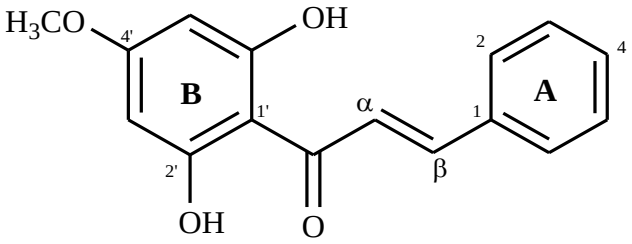

Figure 7. Structure of compound **2**

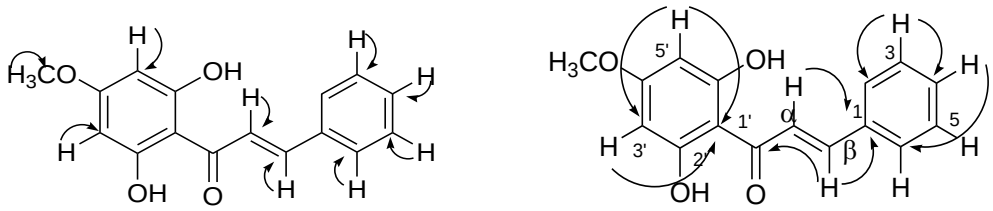

Figure 8. HMQC and HMBC for compound **2**

4.2.3 Characterization compound 3

Compound **3** was isolated as orange amorphous solid with melting points of 134-135 ^{o}C. The UV spectrum maximum (MeOH, $\lambda_{max, nm}$) were observed at 221 and 342 nm. The ^{1}H and ^{13}C NMR (Table **4**) spectral pattern were similar to that of compound **1** except for the absence of one upfield shifted aromatic proton in compound **3**. The presence of five mutually coupled aromatic protons (δ_H 7.61-7.39) for mono substituted phenyl ring, *trans*-configured two olefinic protons (δ_H 7.81 and 7.91), two methoxyl groups (δ_H 3.93 and 3.91), a singlet shielded aromatic proton (δ_H 6.07) and chealeted hydroxyl proton (δ_H 14.36) clearly indicating that compound **3** is a derivative of compound **1**.

The ^{13}CNMR (Table **4**) spectrum showed signals for 17 carbon atoms including signals for two methyl groups, eight methine and seven quaternary carbon atoms. This indicated that it has additional substituent (hydroxyl group) on ring B at C-3′/6'. The position of the two methoxyl groups at δ_H 3.93 (δ_C 56.2) and δ_H 3.91 (δ_C 61.0) were deduced from the downfiled chemical shift value (δ_C 61.0) of the former methoxyl group indicating its devoid of planarity due to the presence of di-*ortho* substitution and hence these methoxyl groups were placed at C-3' (δ_C 128.5) and C-4' (δ_C 155.5), respectively.

Thus, based on the above evidence, compound **3** was found to be 2′, 6′-dihydroxy-3′, 4′-dimethoxychalcone, trivial name pashanone (**3**), a compound previously isolated from *Polygonum ferrugineum* and *Polygonum hydropiper* [71, 72].

Table 4. ^{1}H (500 MHz), ^{13}C (125 MHz) and HMBC NMR data of compound **3** (in $CDCl_3$)

	Compound 3		**Pashanone [72]**
Position	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1		135.6	
2/6	7.61 (2H, *m)*	128.6	7.70 (*m*, 2H)
3/5	7.41 (2H, *m*)	129.0	7.50 (*m*, 2H)
4	7.39 (1H, *m)*	127.6	7.30 (*m*, 1H)
α	7.81 (1H, *d*, 15.6)	130.3	7.80 (*d*, 16, 1H)
β	7.91 (1H, *d*, 15.6)	142.7	7.92 (*d*, 16, 1H)
1'		106.6	
2'		159.0	
3'		128.5	
4'		155.5	
5'	6.07 (1H, *s*)	90.0	6.08 (*s*, 1H)
6'		159.2	
3'-OCH_3	3.91 (3H, s)	61.0	3.92 (*s*, 3H)
4'-OCH_3	3.93 (3H, s)	56.2	3.96 (*s*, 3H)
2'/ 6'-OH	14.36 (2H, *s*)		14.35 (*s*, 2H)
CO		193.4	

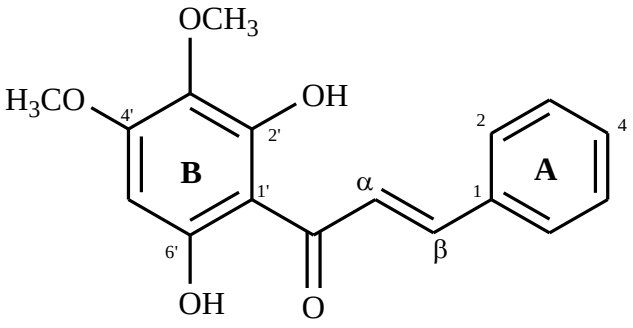

Figure 9. Structure of compound **3**

4.3 Antimicrobial Activities

The crude extracts (chloroform and methanol) and the isolated compounds (**1**, **2** and **3**) were *in vitro* assayed (Table **5**) against two bacterial strains (*Staphylococccus aureus* and *Escherichia coli*). The antibacterial activities of these extracts and the isolated compounds were comparatively assessed by the diameter of zone of inhibition in millimeters and zones of inhibition more than 6 mm were taken into consideration. Both extracts showed marginal inhibitory activities against both tested bacterial strains, with the highest activity is observed for chloroform extract against *Escherichia coli*.

The inhibition displayed on both Gram negative and Gram positive bacteria for the isolated compounds were good with variable degree of potency between the tested compounds. Compound **1** showed marginal activity against Gram-negative bacteria (*Escherichia coli)* while it has little or no inhibitory activity against Gram positive bacteria (*Staphylococccus aureus*). Whereas, compound **2** exhibited highest zone of growth inhibition (18 mm) on Gram-positive bacteria strain (*Staphylococccus aureus*), which is comparable to that of reference drug, gentamycin (19 mm) and has no activity against *Escherichia coli*. This is may be related to the polar/none polar nature of the compounds, as the two compounds differ from one another by presence of methoxy and free phenol at position C-6′.

It is therefore believed that compound **1** having a non-polar methyl ether moiety at C-6′ can pass the outer lipid membrane of the Gram-negative bacteria. Whereas, Gram positive bacteria, which has no such outer membrane were expected to be most susceptible to the more polar compound **2**. Compound **3** showed comparable activity against both strains, as it possesses both polar and none polar groups.

The antifungal activities of crude extracts of chloroform, methanol, and compound **1**, **2**, **3**, against *Aspergillus spp*, *Trichoderma spp*, *Fusarium spp* and *Penicillium spp* were also determine with clotrimazole as reference standard. All extracts and isolated compounds exhibited antifungal activities against *Aspergillus spp* and *Fusarium spp*. The chloroform and methanol extracts revealed significantly higher inhibitory effect (30 mm) against *Aspergillus spp*, which was followed by compound **3** (20 mm) at the same incubation period. In addition to that, methanol and chloroform extracts exhibited equal antifungal activities (30 mm) against *Aspergillus spp* and (20 mm) against *Fusarium spp*. There was no activity exhibited by compound **1** against *Trichoderma*

spp and *Penicillium spp.* Whereas, compound **2** exhibited highest zone of growth inhibition (22 mm) against *Trichoderma spp* strain, which is comparable to that of reference drug, clotrimazole (20 mm).

Table 5. Inhibition zone (mm) of crudes and isolated compounds for bacteria and fungus strains.

Growth inhibition zone (mm)									
		Crude extracts		Isolated compounds			Controls		
Strains	**Gram**	**CE-1**	**CE-2**	**1**	**2**	**3**	**G**	**C**	**DMSO**
S.aureus	*+ve*	12	10	NI	18	12	19	-	NI
E.coli	*-ve*	15	8	9	NI	13	17	-	NI
Aspergillus spp	-	30	30	10	15	20	-	23	NI
Trichoderma spp	-	14	18	NI	22	18	-	15	NI
Fusarium spp	-	20	20	10	19	20	-	22	NI
Penicillium spp	-	18.5	16.5	NI	10	14	-	15	NI

Key: CE-1 = Chloroform extract, CE-2 = Methanol extract, NI = No zone of inhibition, G = gentamycin, C = Clotrimazole, *+ve* = Gram-postive, *-ve* = Gram-negative.

5. Conclusion and Recommendations

5.1 Conclusion

The chromatographic separation of seed extract of *Persicaria lapathilium* led to the isolation of three pure compounds. These compounds were identified to be flavokawain B (**1**); cardamonin (**2**); and pashanone (**3**). The crude extract of chloroform and compound **2** showed considerable zone of inhibition in bacteria strains; *Escherichia coli*, and *Staphylococccus aureus*, respectively. However, compound **2** exhibited comparable growth inhibition for *Staphylococccus aureus* measured as 18 mm to the standard gentamycin 19 mm. The chloroform extract and the isolated compounds demonstrated significant antibacterial and antifungal activities with compound **2** showed highest zone of growth inhibition against *Staphylococccus aureus* and *Trichoderma spp* strains, which are comparable to that of reference drugs, which are almost comparable to that of gentamycin and clotrimazole. The scientific findings of the present study proved that the activities of the crude extract and the isolated compounds support the traditional use of the plant against various infectious diseases as evidenced by the promising in vitro assay result.

5.2 Recommendations

- The present study used only gravity column with the help of ethyl acetate and petroleum ether as an eluent. Further phytochemical investigation on *P. lapathifolia* should be done using HPLC on the polar extracts of the plant. As a result, more phenolic compounds along with their glycosides could be identified.
- Chloroform and methanol solvents gave better extract yield and better antimicrobial activities which suggest that there are still more polar unidentified phenolic compounds present in the seeds of the plant.
- All extracts and isolated compounds exhibited antifungal activities against *Aspergillus spp* and *Fusarium spp*. and also compound **2** showed more activity against Gram positive bacteria rather than Gram negative bacteria; hence, the mechanism of action, the MIC and MBC for compounds should be studied further.
- More biological assay on other strains needs to be conducted on various extracts of the plant so as to establish the traditional use of the plant.

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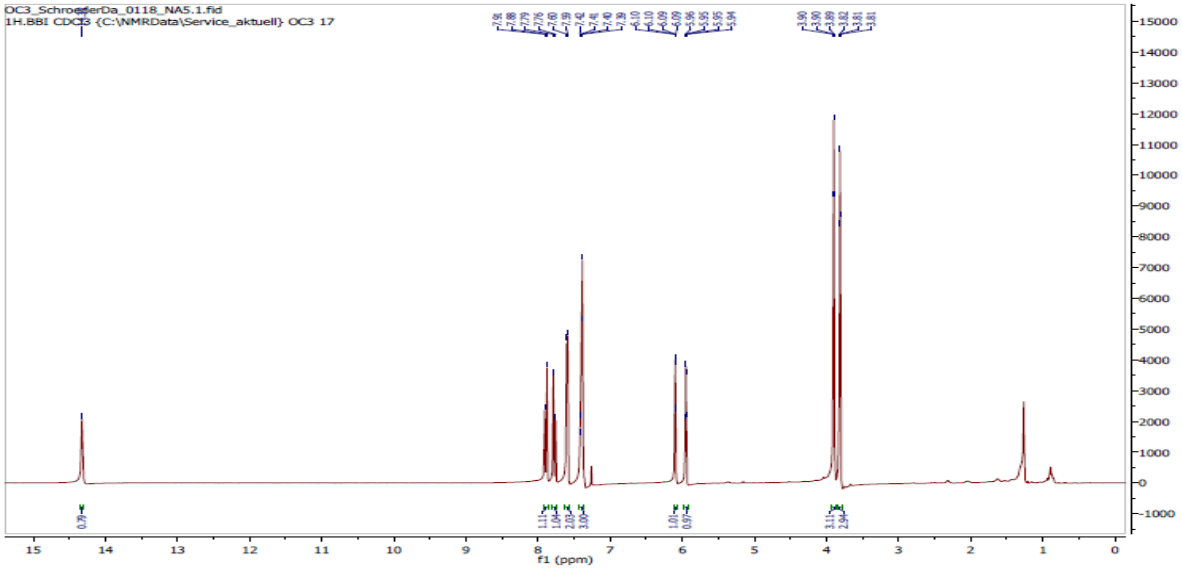
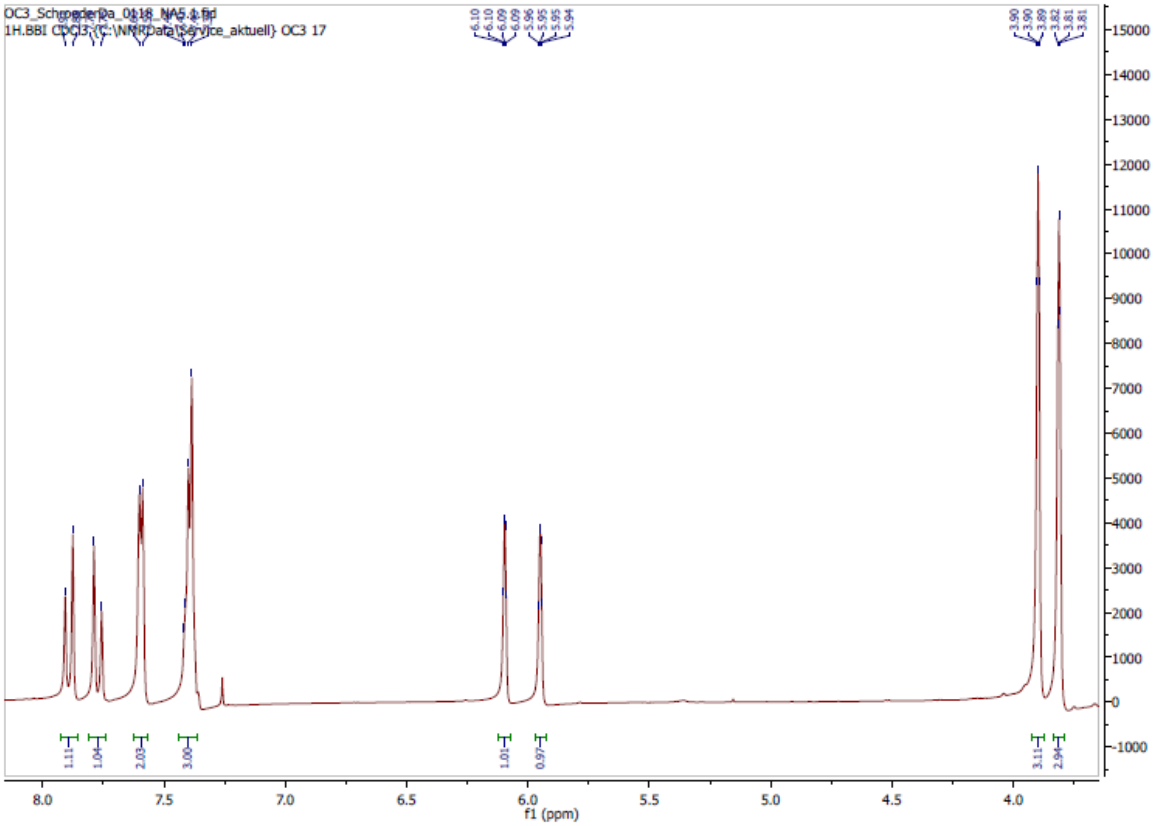
Appendixes

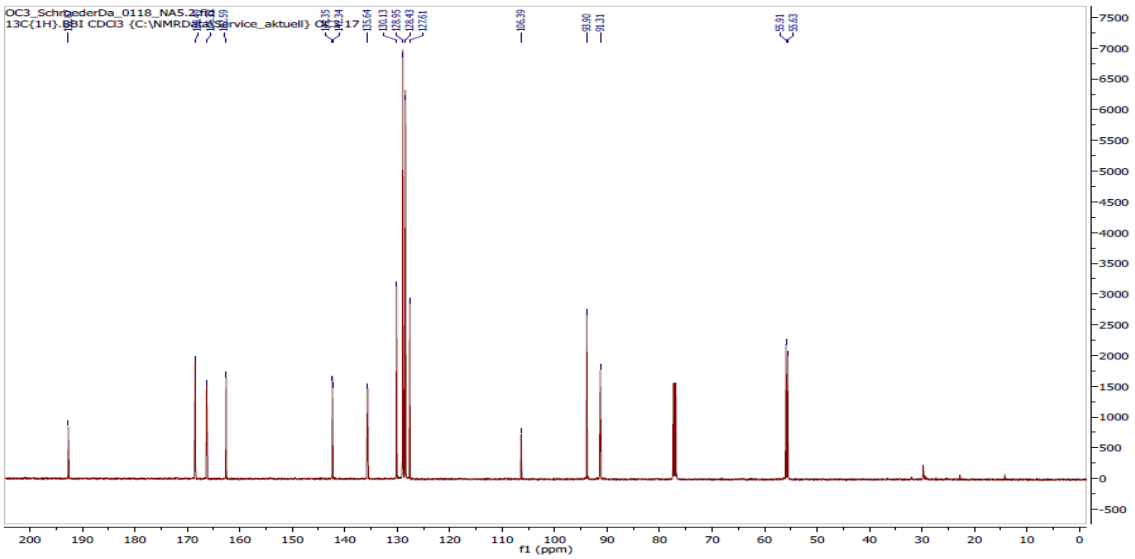
Appendix 1A: ^{1}H NMR spectrum of Flavokawain B (**1**), $CDCl_3$, 500 MHz



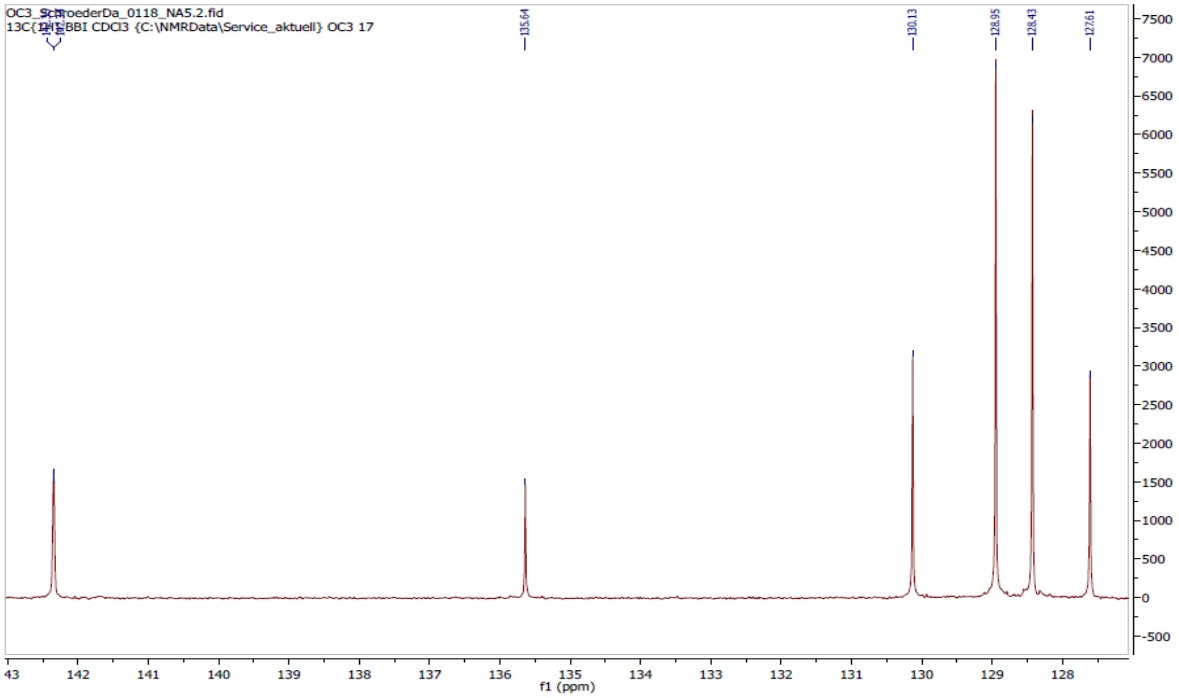
Appendix 1A: Expand ^{1}H NMR spectrum of Flavokawain B (**1**), $CDCl_3$, 500 MHz



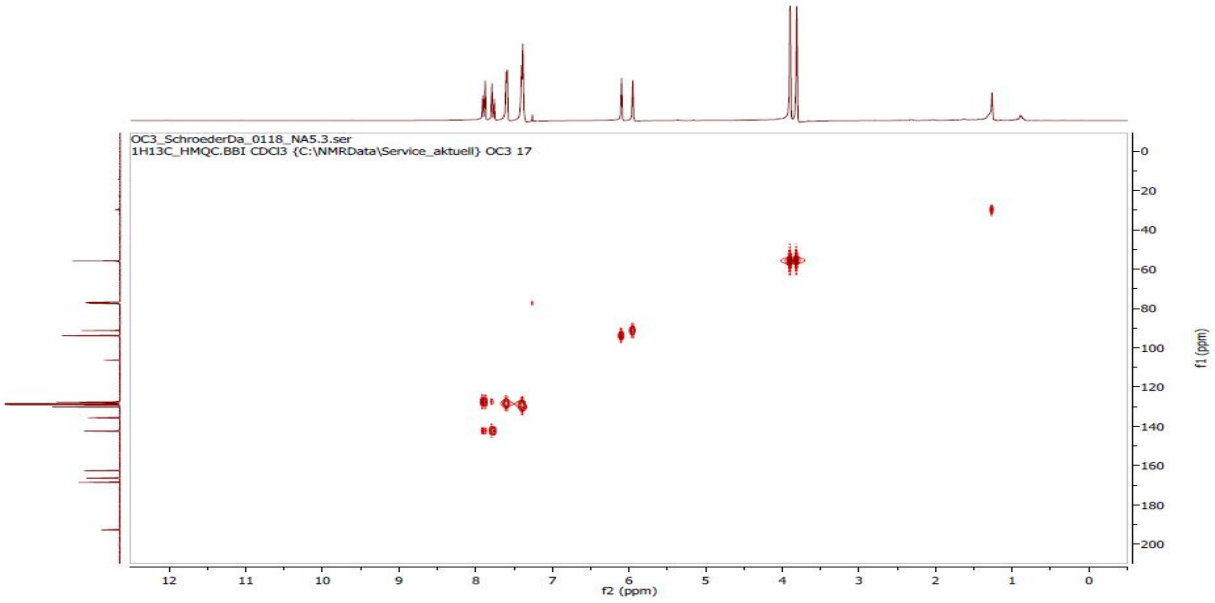
Appendix 1B: ^{13}C NMR spectrum of Flavokawain B (**1**), $CDCl_3$, 125 MHz



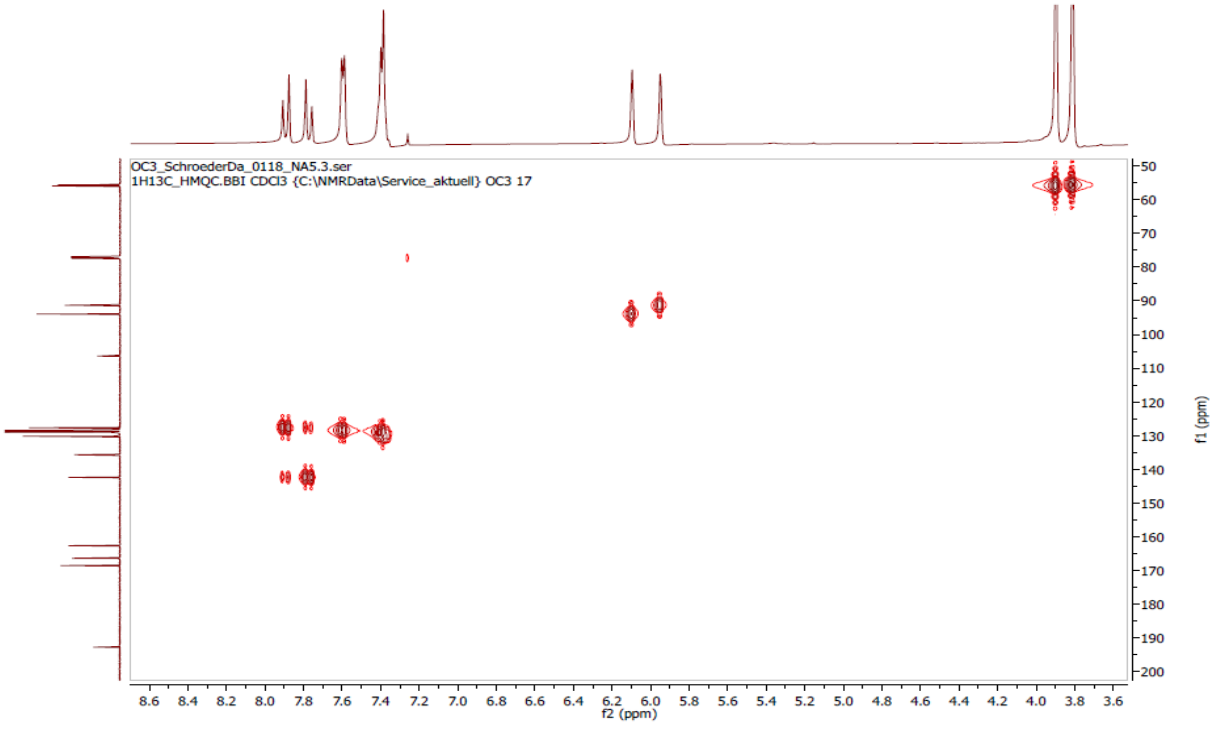
Appendix 1B: Expand ^{13}C NMR spectrum of Flavokawain B (**1**), $CDCl_3$, 125 MHz



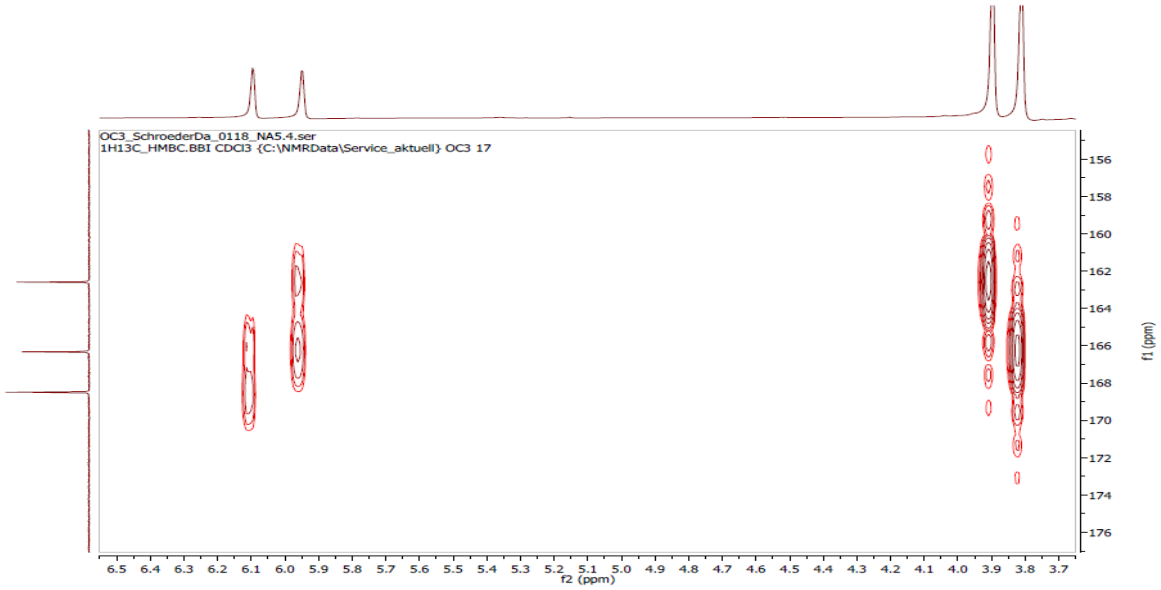
Appendix 1C: HMQC spectrum of Flavokawain B **(1),** $CDCl_3$, 500 MHz



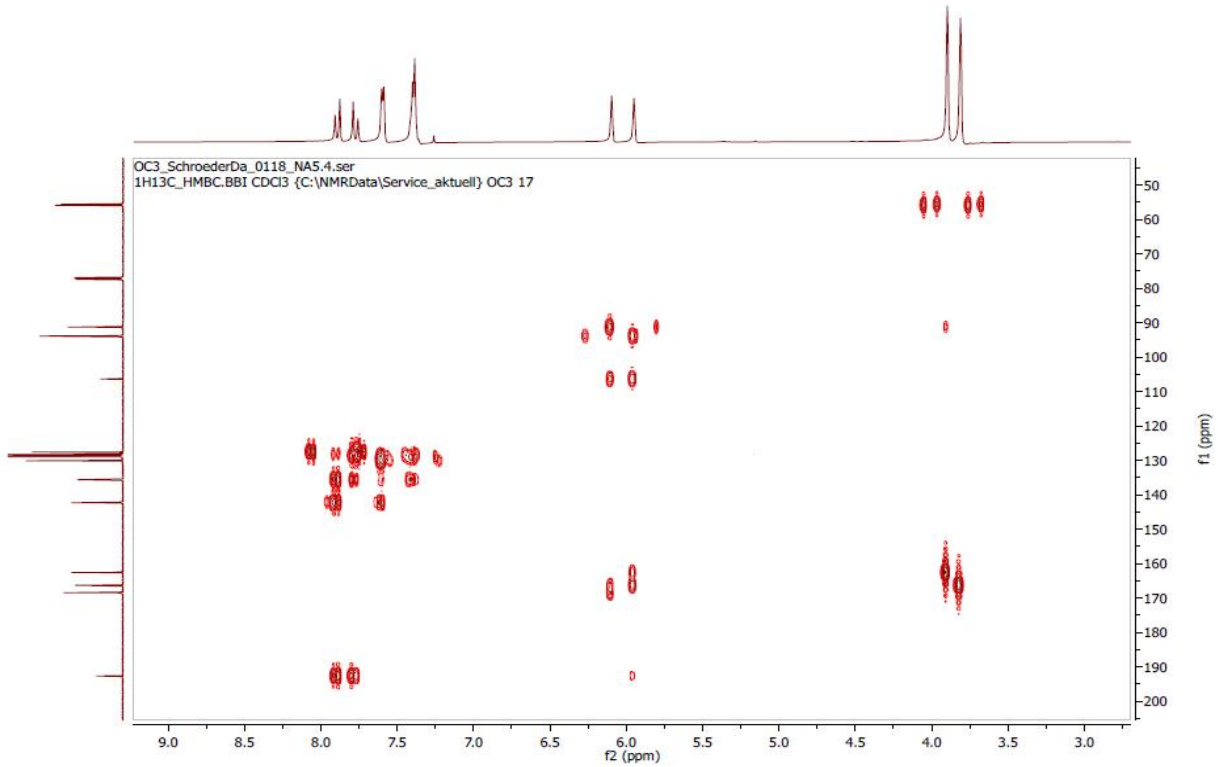
Appendix 1D: Expand HMQC spectrum of Flavokawain B **(1),** $CDCl_3$, 500 MHz



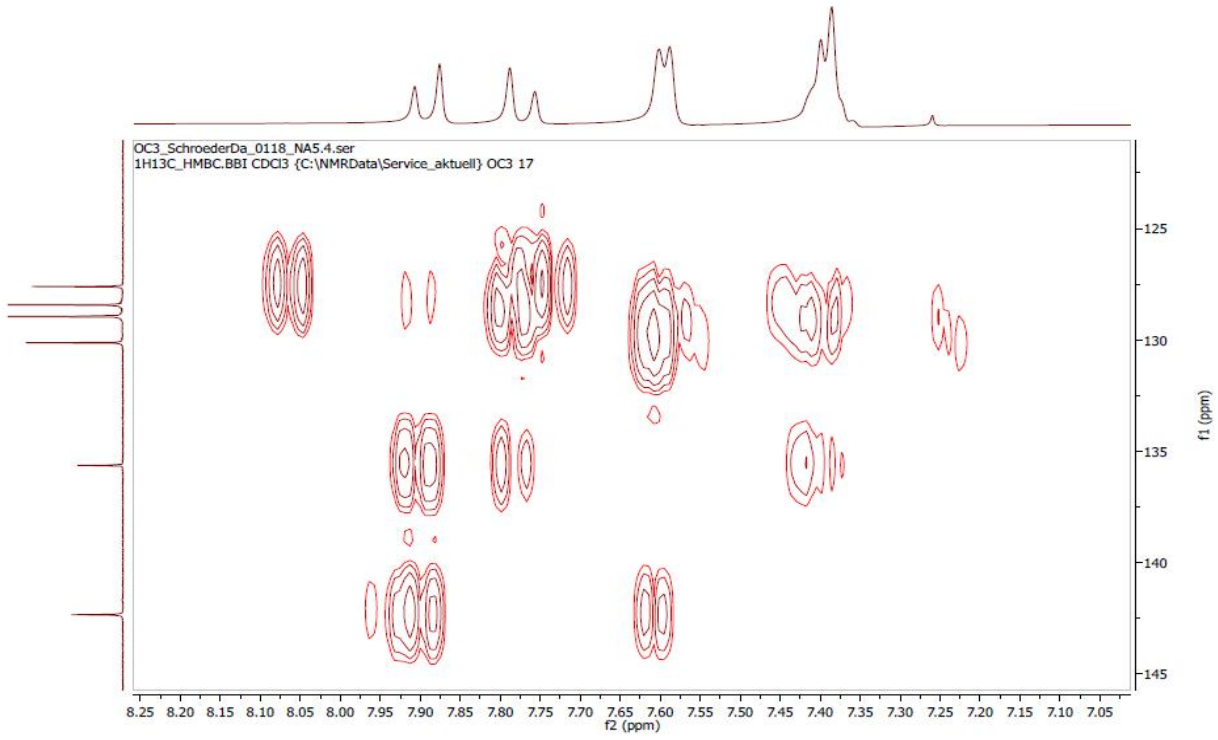
Appendix 1E: Expand HMBC spectrum of Flavokawain B **(1),** $CDCl_3$, 500 MHz



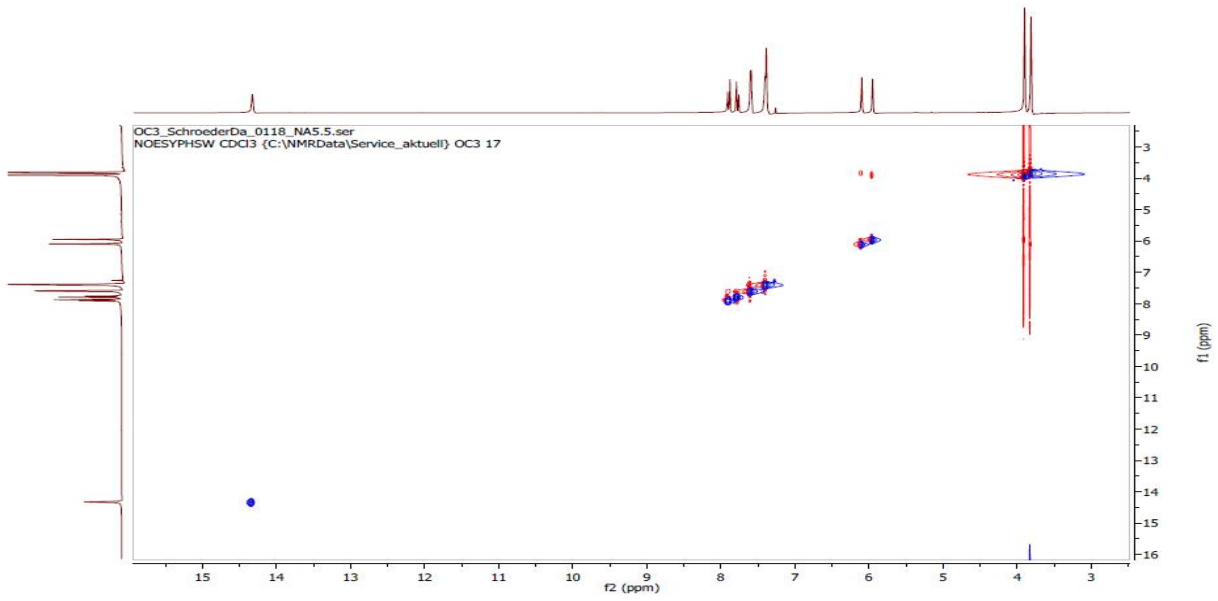
Appendix 1F: HMBC spectrum of Flavokawain B **(1),** $CDCl_3$, 500 MHz



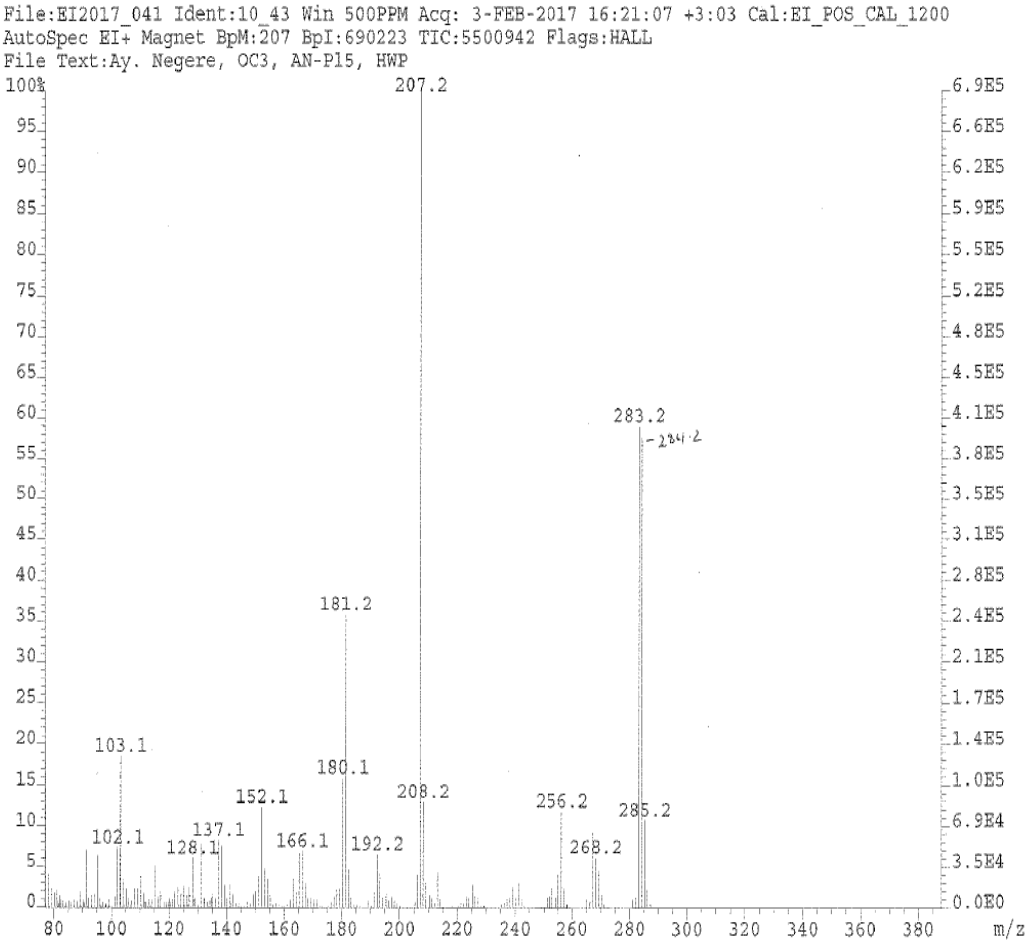
Appendix 1G: Expand HMBC spectrum of Flavokawain B **(1),** $CDCl_3$, 500 MHz



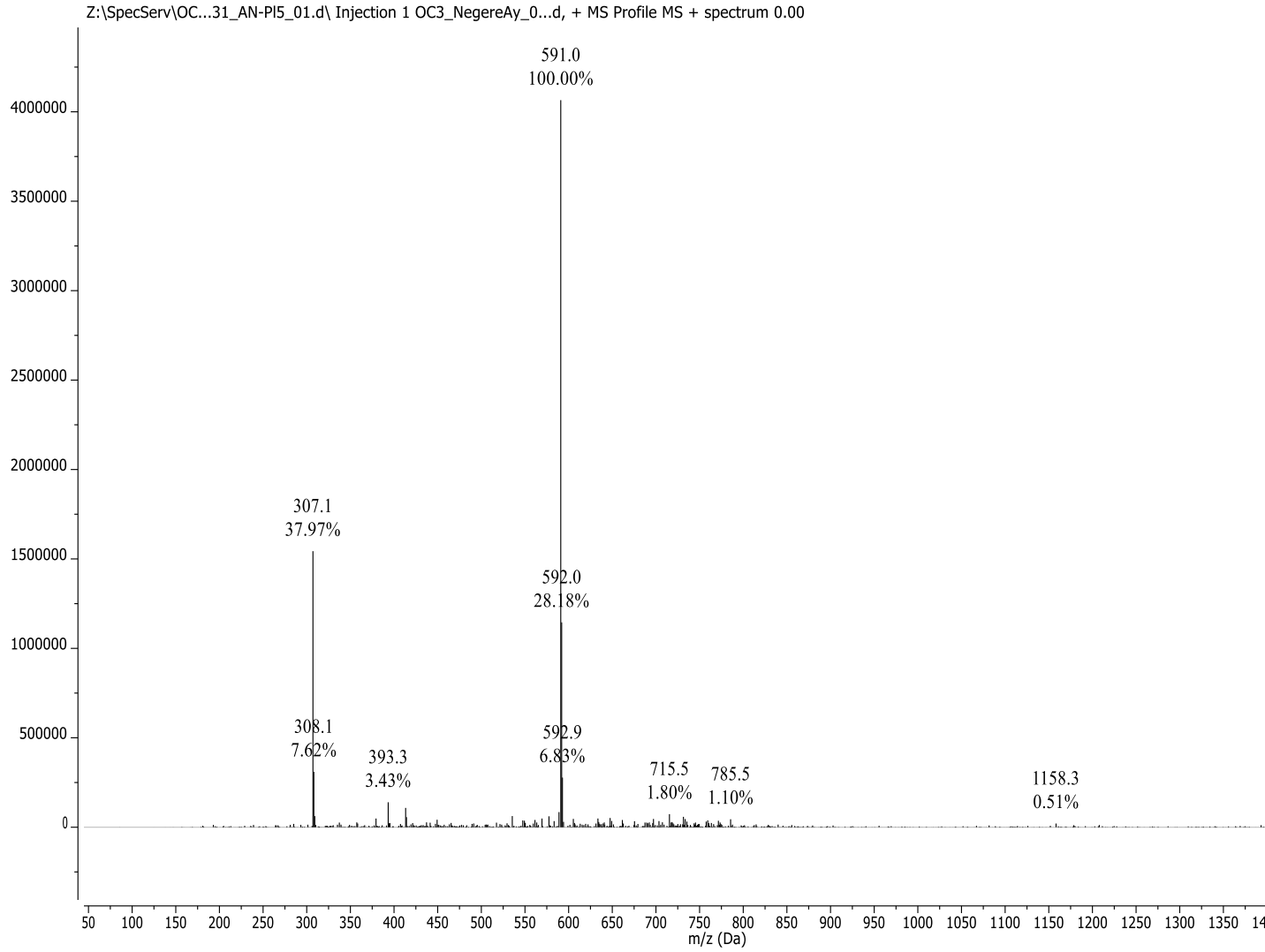
Appendix 1H: NOESY spectrum of Flavokawain B **(1),** $CDCl_3$, 500 MHz



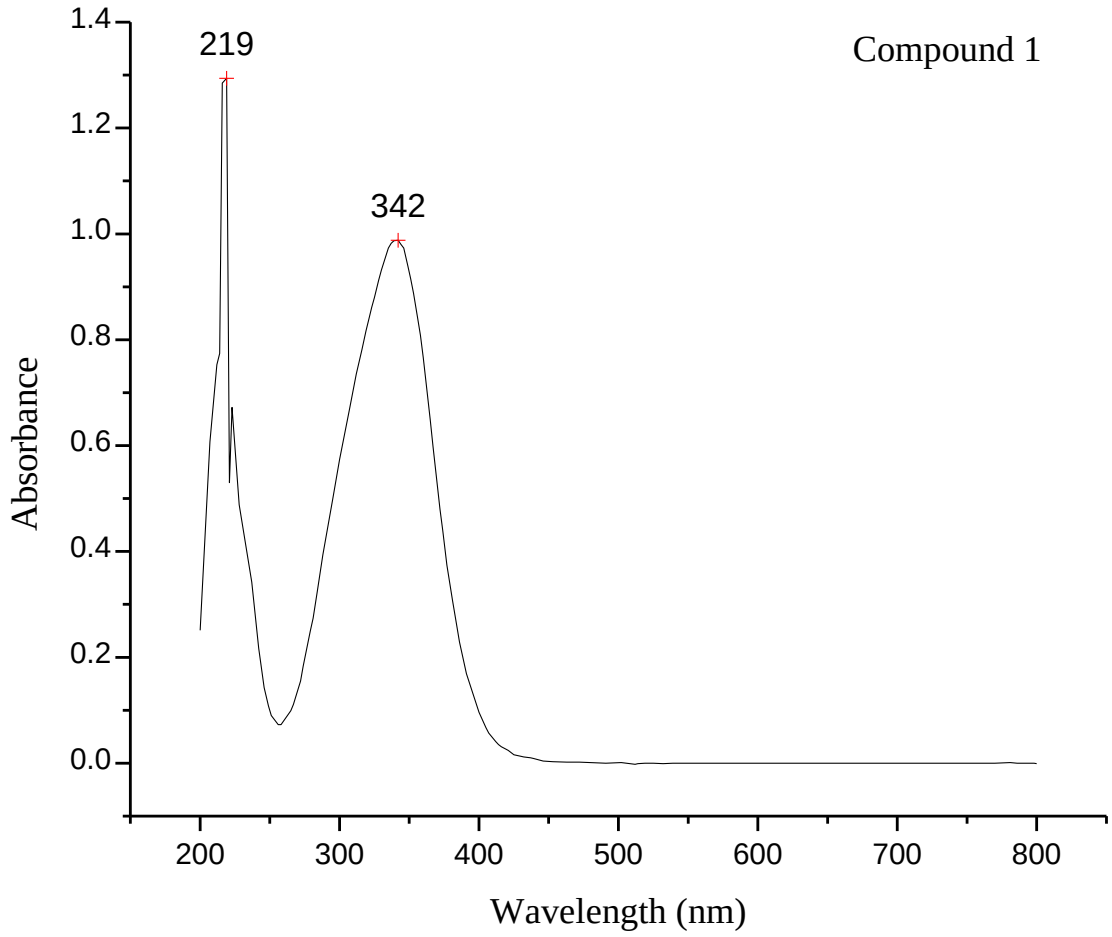
Appendix 1I: ESI-MS spectrum of Compound **1**



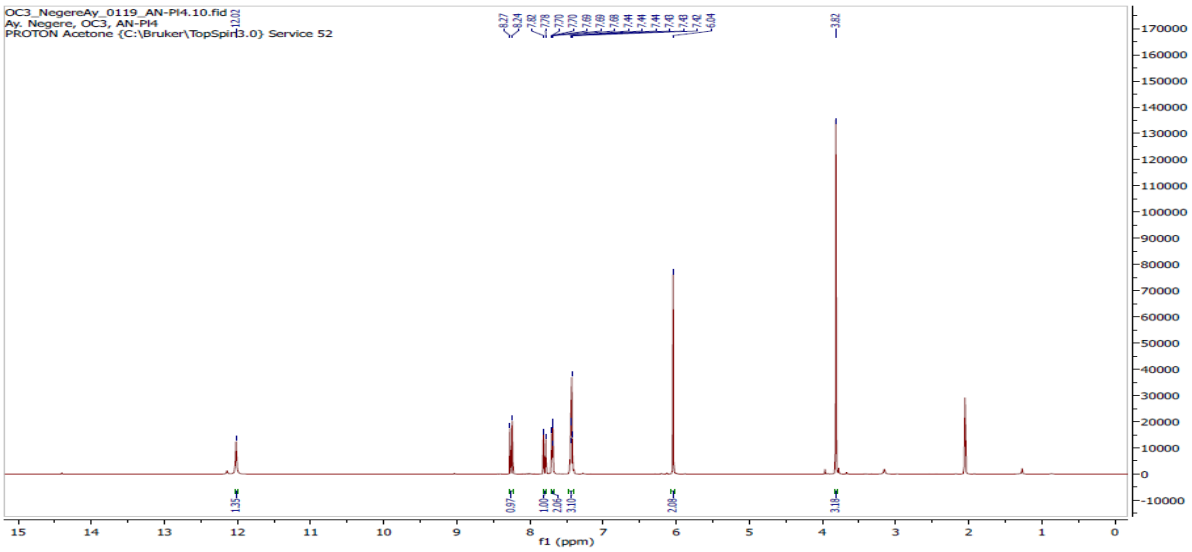
Appendix 1J: ESI-MS spectrum of Compound **1**



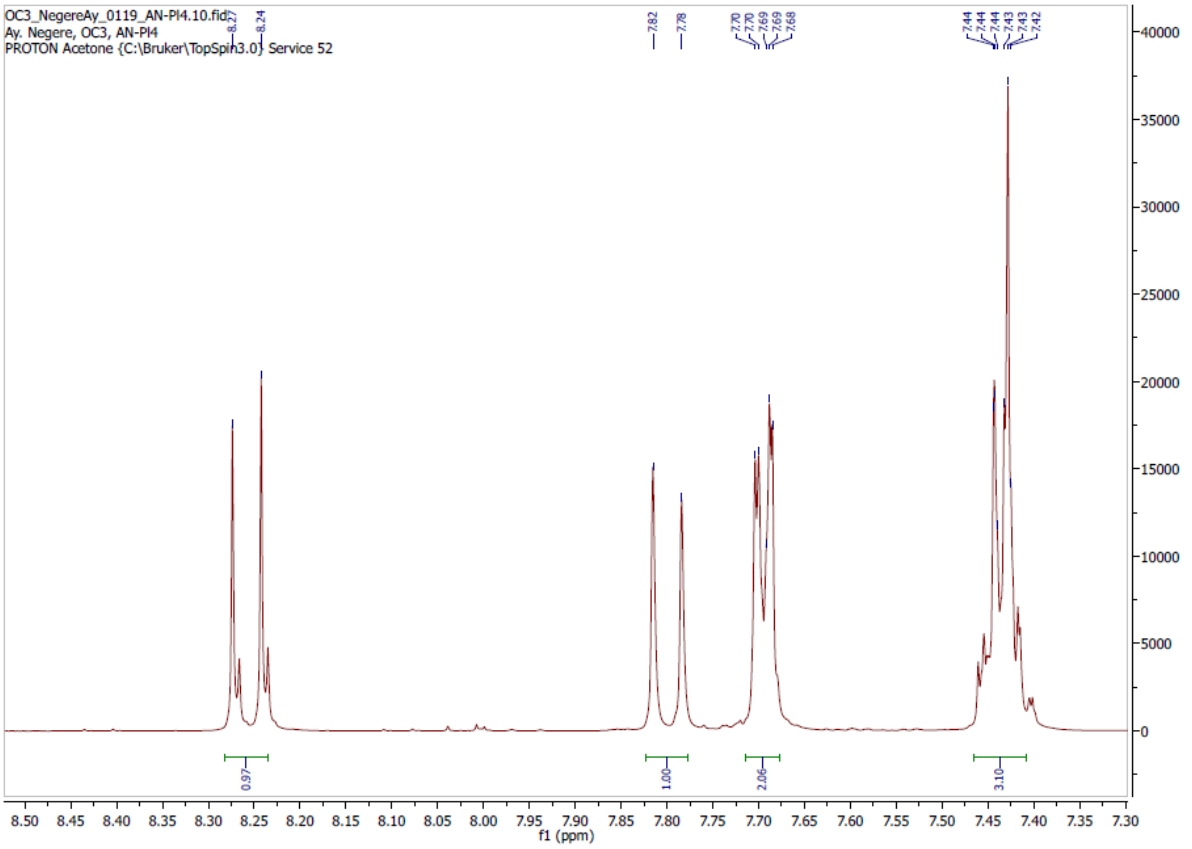
Appendix 1K: UV- VIS Spectroscopy for compound **1.**



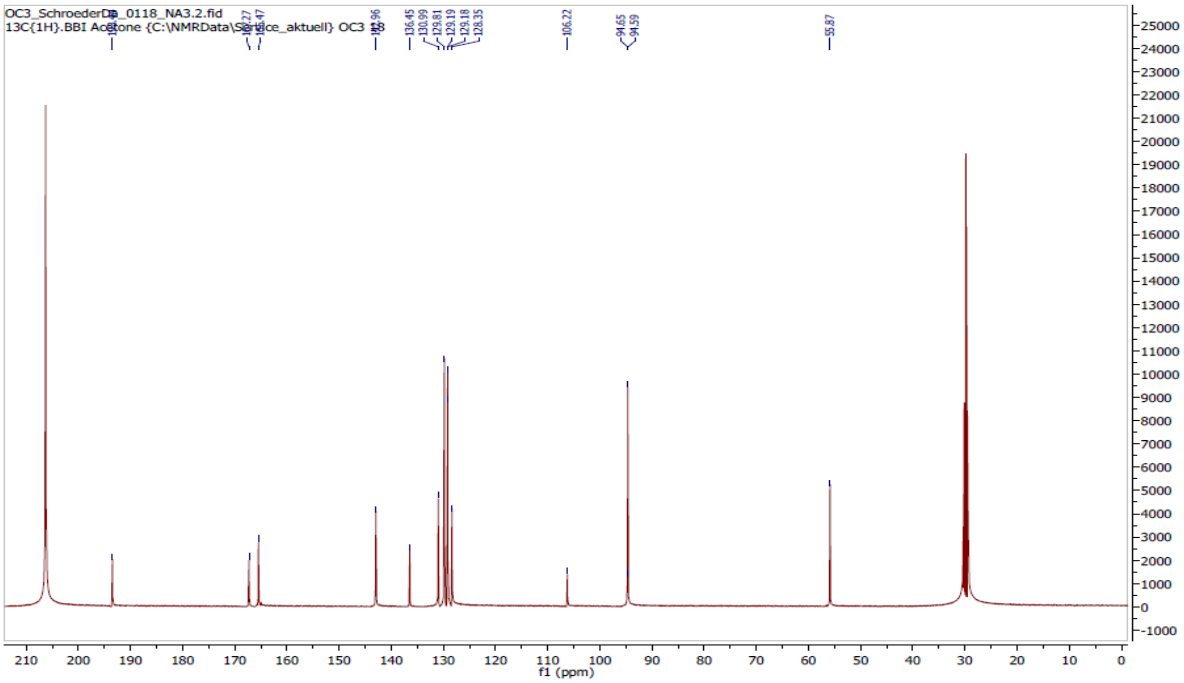
Appendix 2A: ^{1}H NMR spectrum of cardamonin **(2),** Acetone, 500 MHz



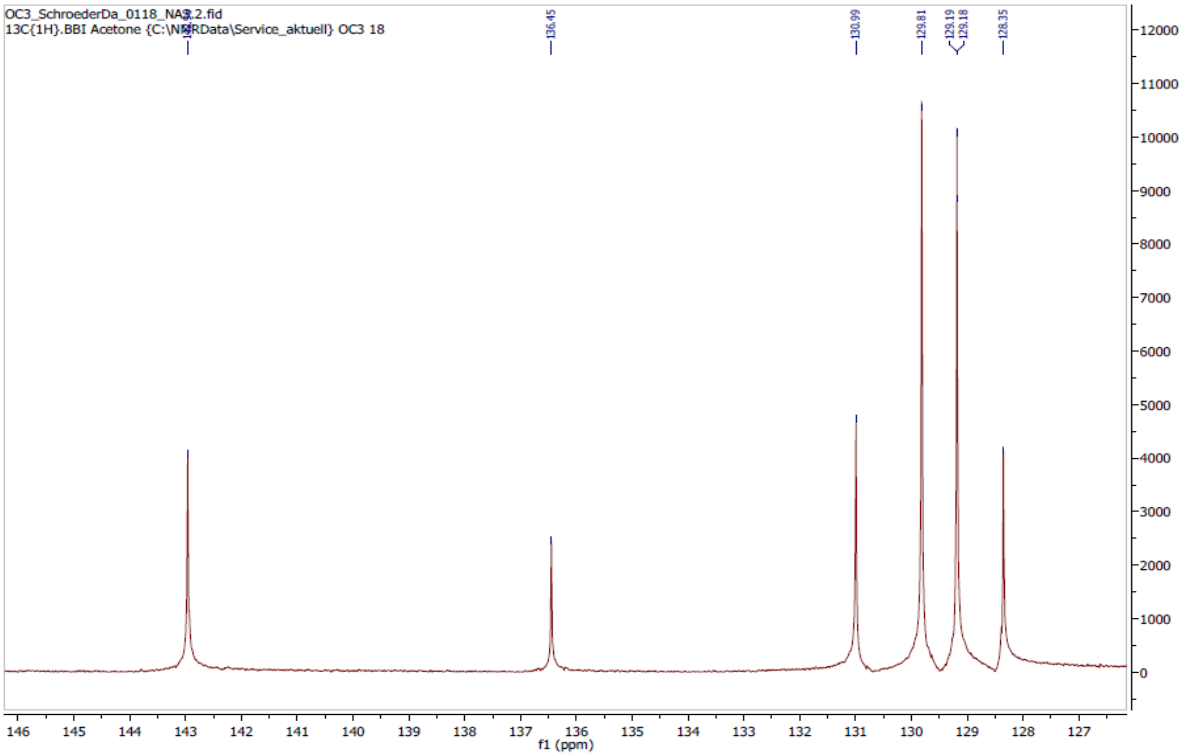
Appendix 2B: ^{1}H NMR spectrum of cardamonin **(2),** Acetone, 500 MHz



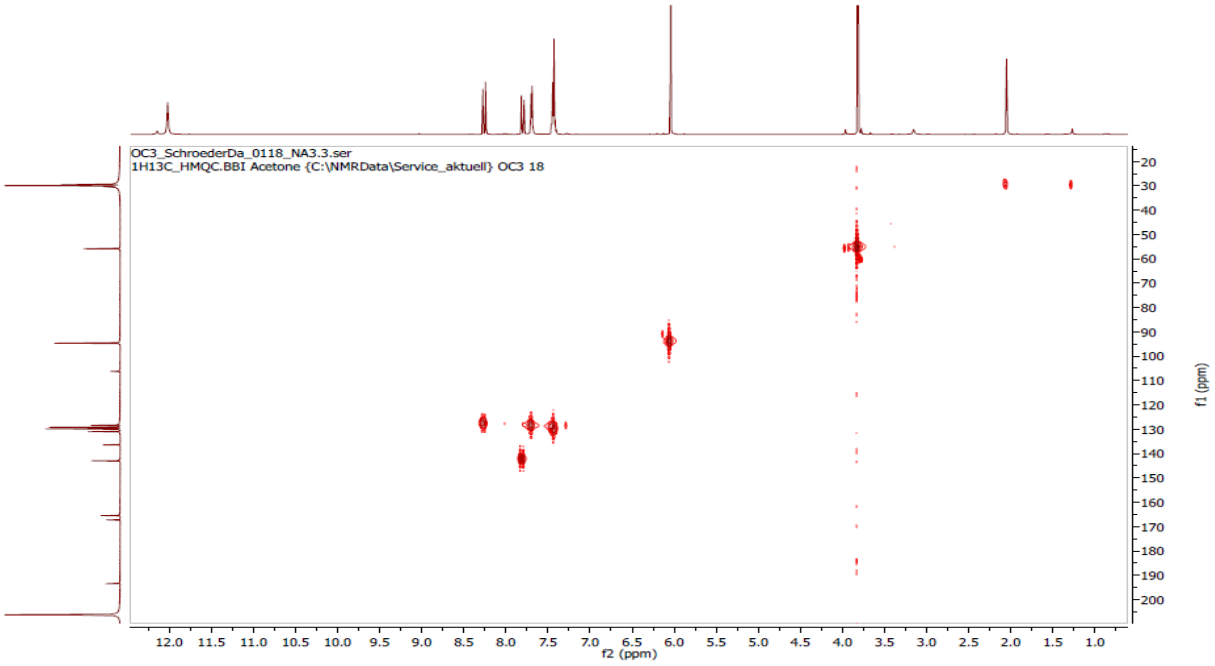
Appendix 2C:[13]C NMR spectrum of cardamonin **(2),** Acetone, 125 MHz



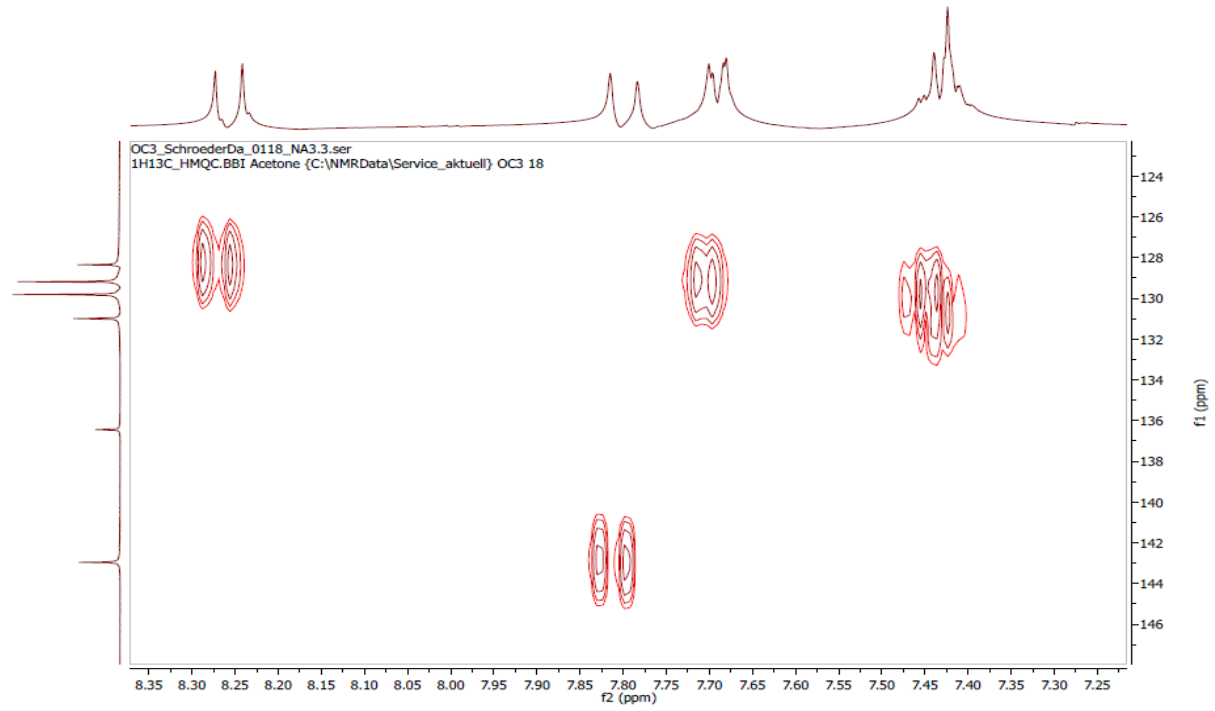
Appendix 2D: [13]C NMR spectrum of cardamonin **(2),** Acetone, 125 MHz



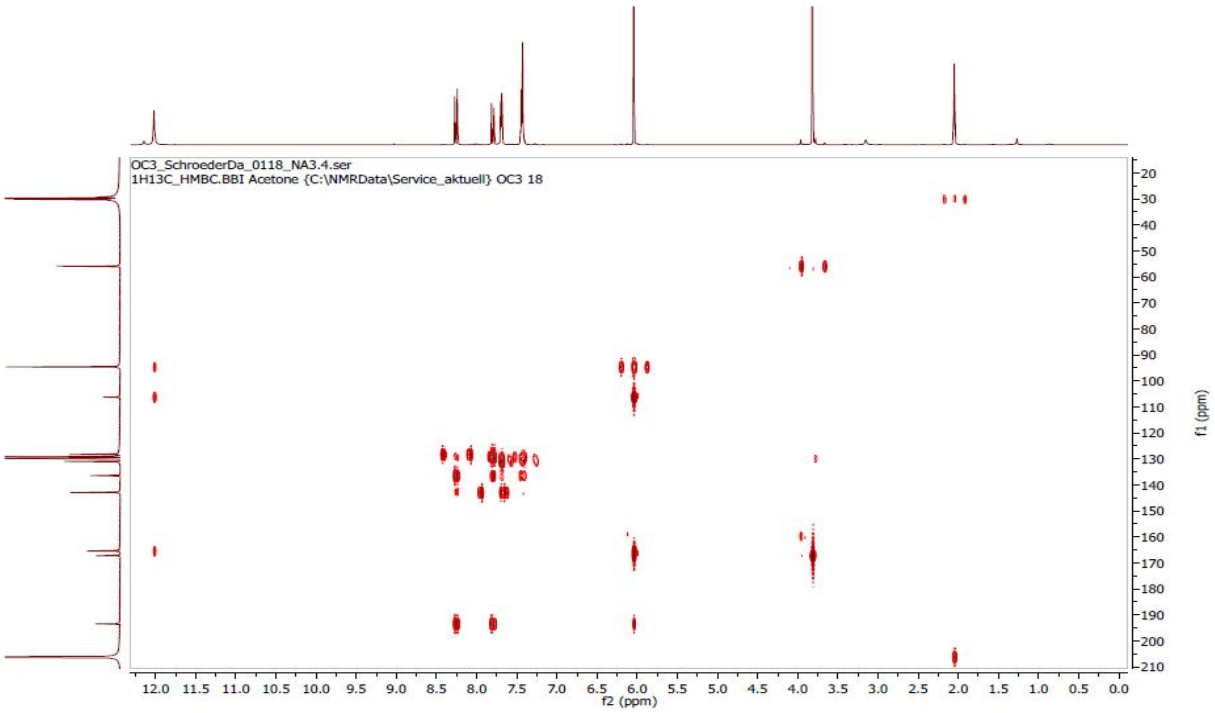
Appendix 2E: HMQC spectrum of cardamonin **(2),** Acetone, 500 MHz



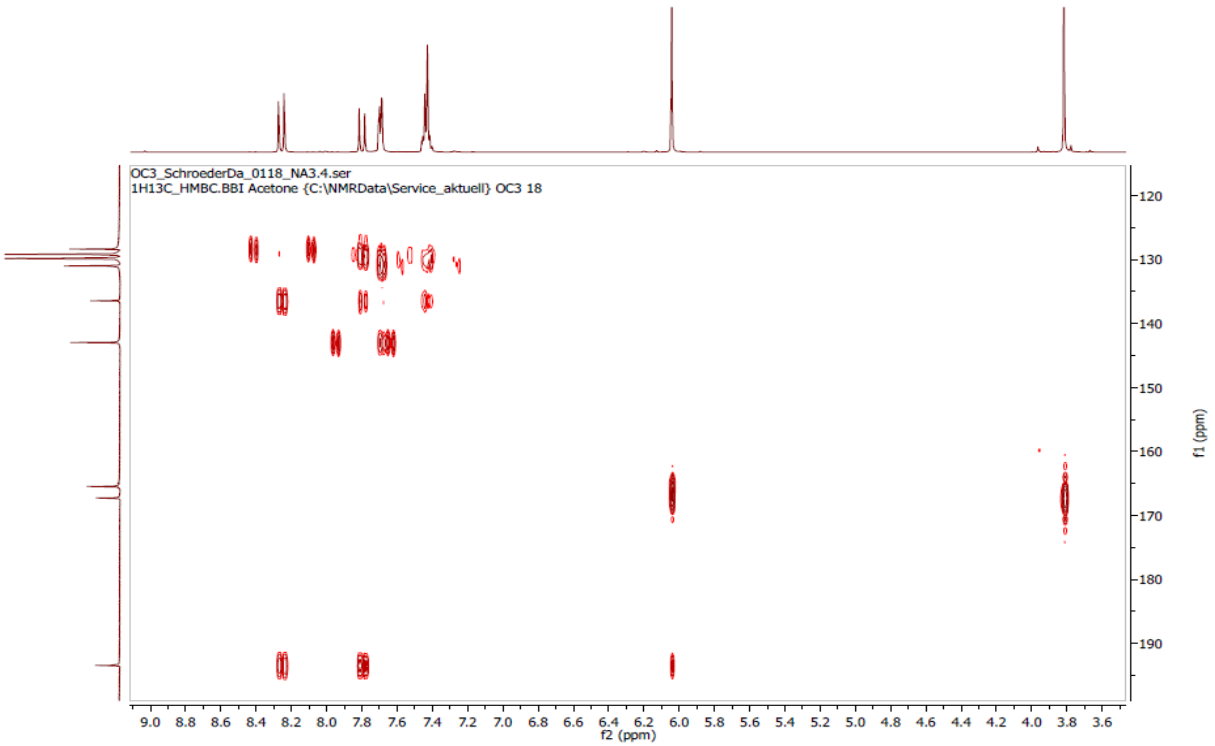
Appendix 2F: Expand HMQC spectrum of cardamonin **(2),** Acetone, 500 MHz



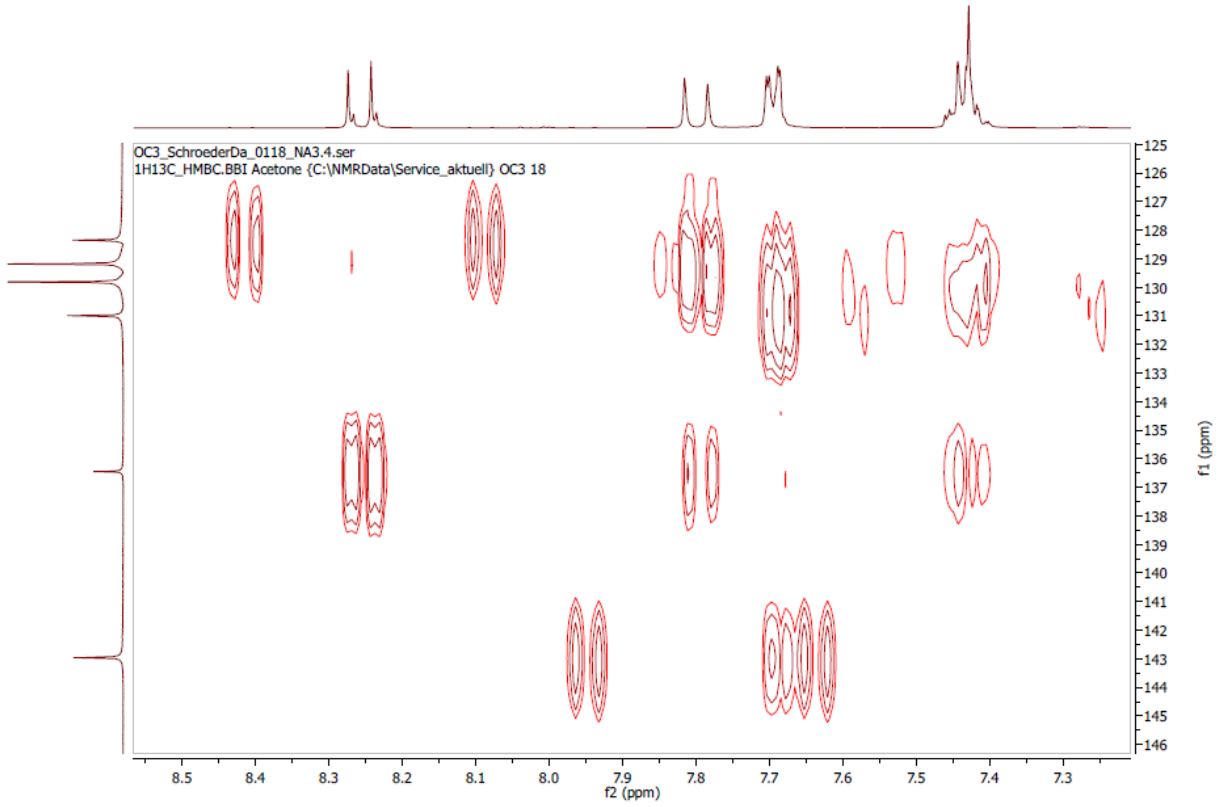
Appendix 2G: HMBC spectrum of cardamonin **(2),** Acetone, 500 MHz



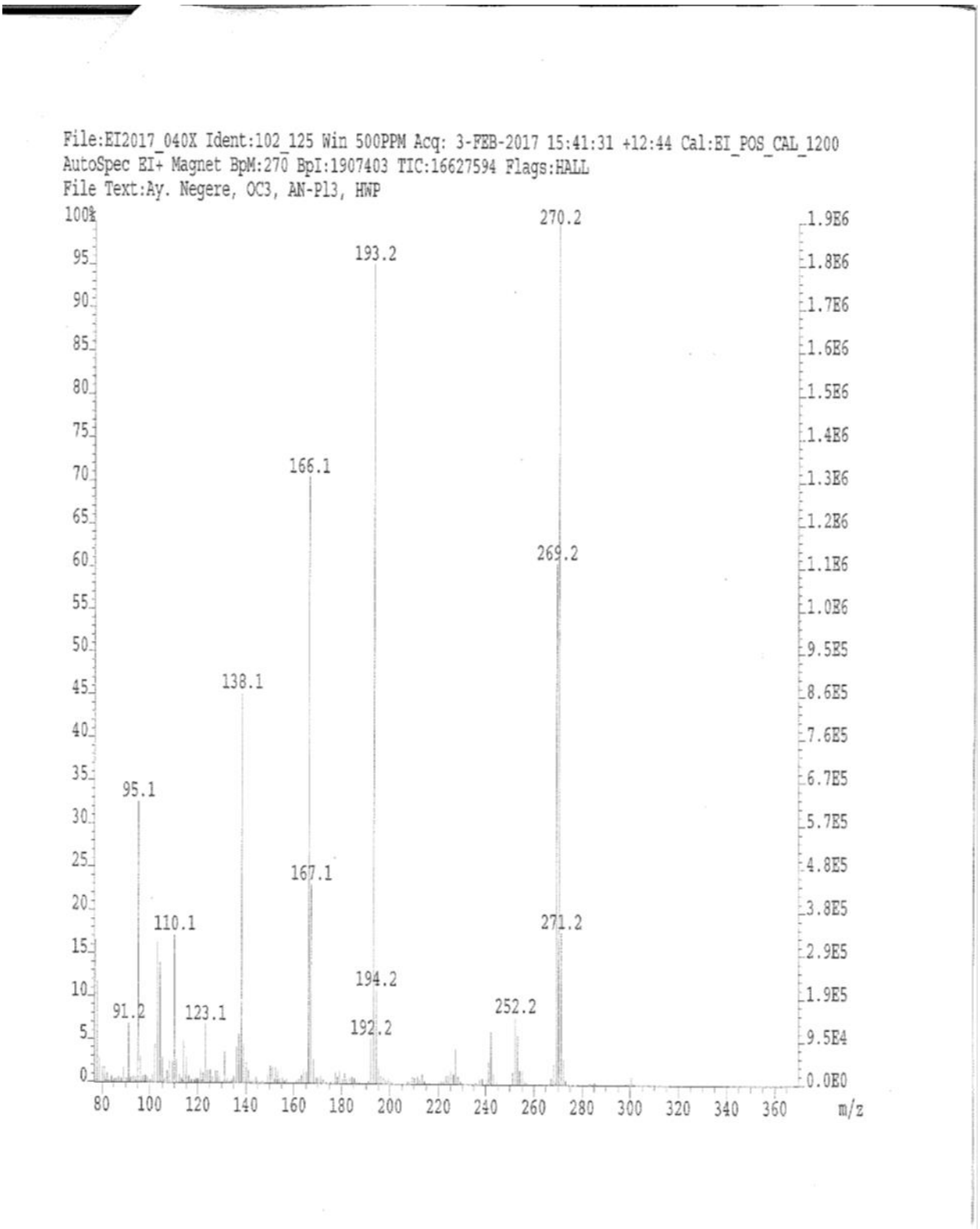
Appendix 2H: HMBC spectrum of cardamonin **(2),** Acetone, 500 MHz



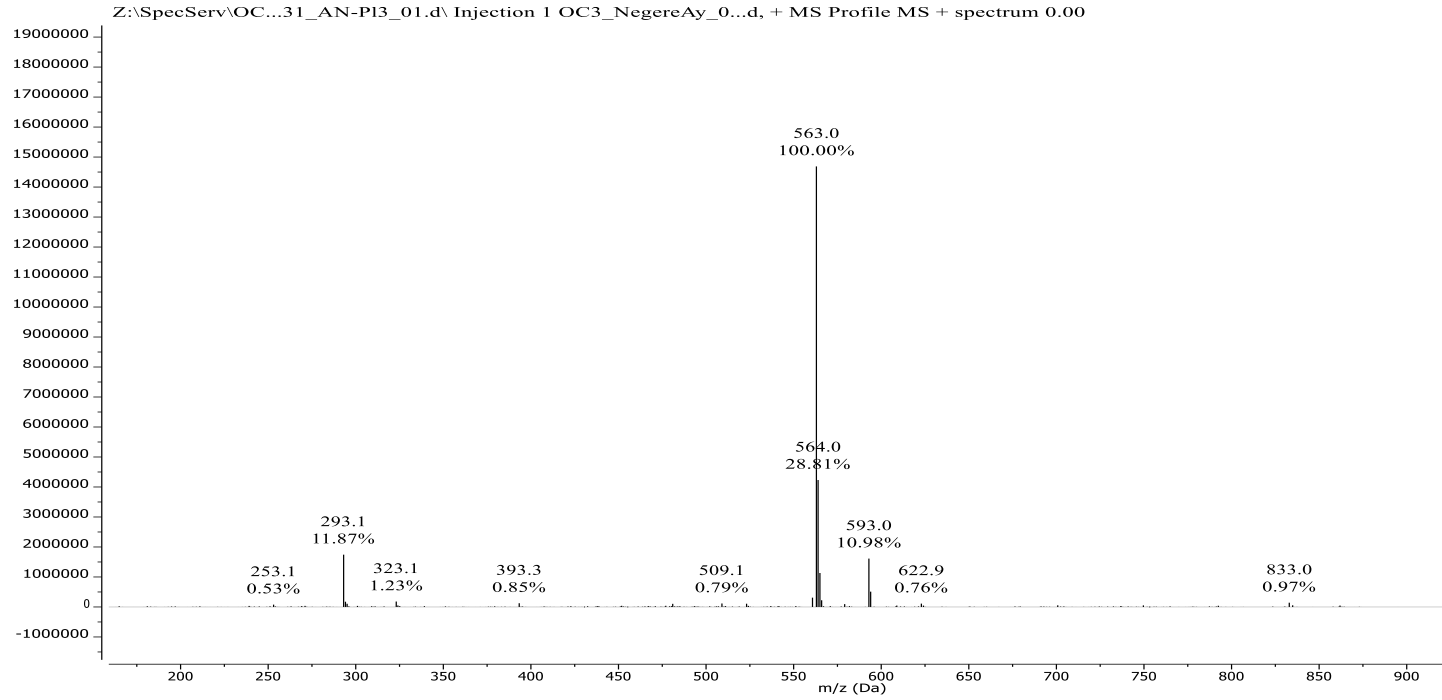
Appendix 2I: Expand HMBC spectrum of cardamonin **(2),** Acetone, 500 MHz



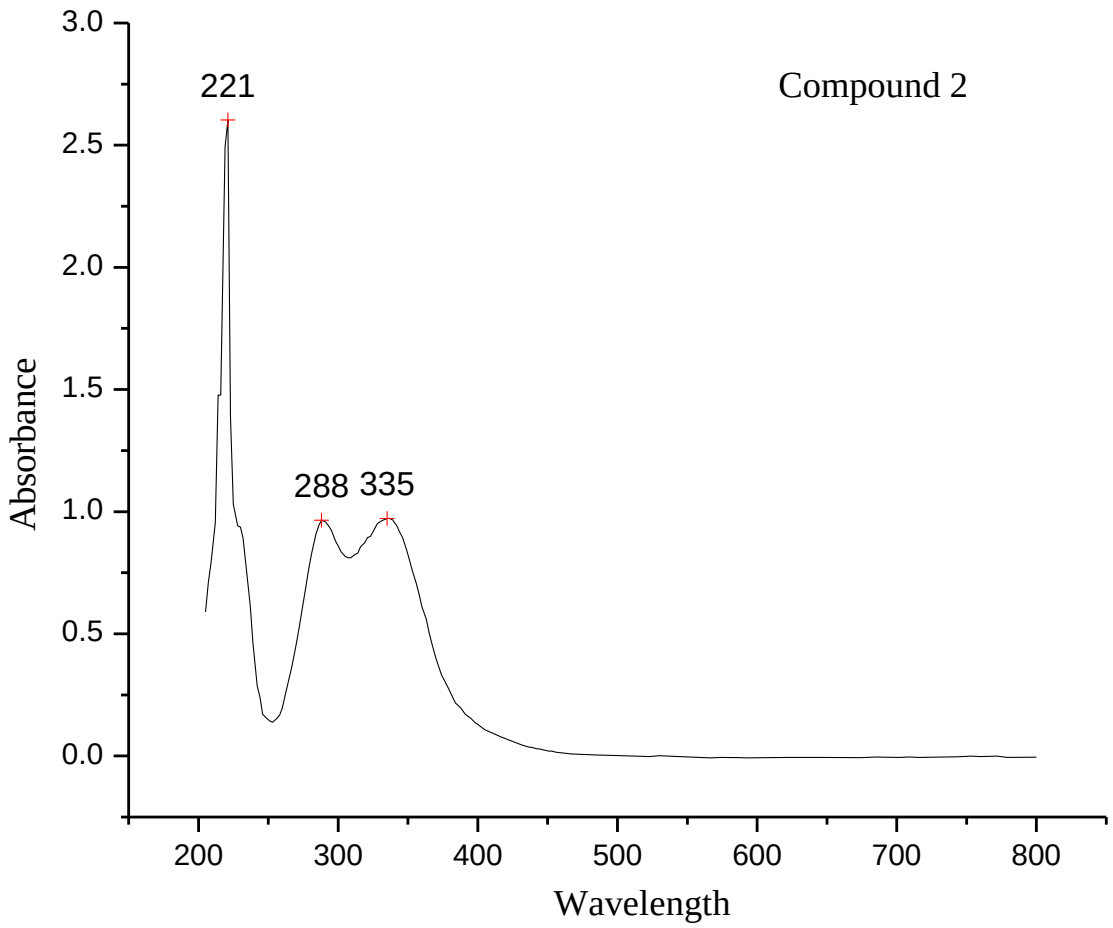
Appendix 2J: ESI-MS spectrum of Compound **2**



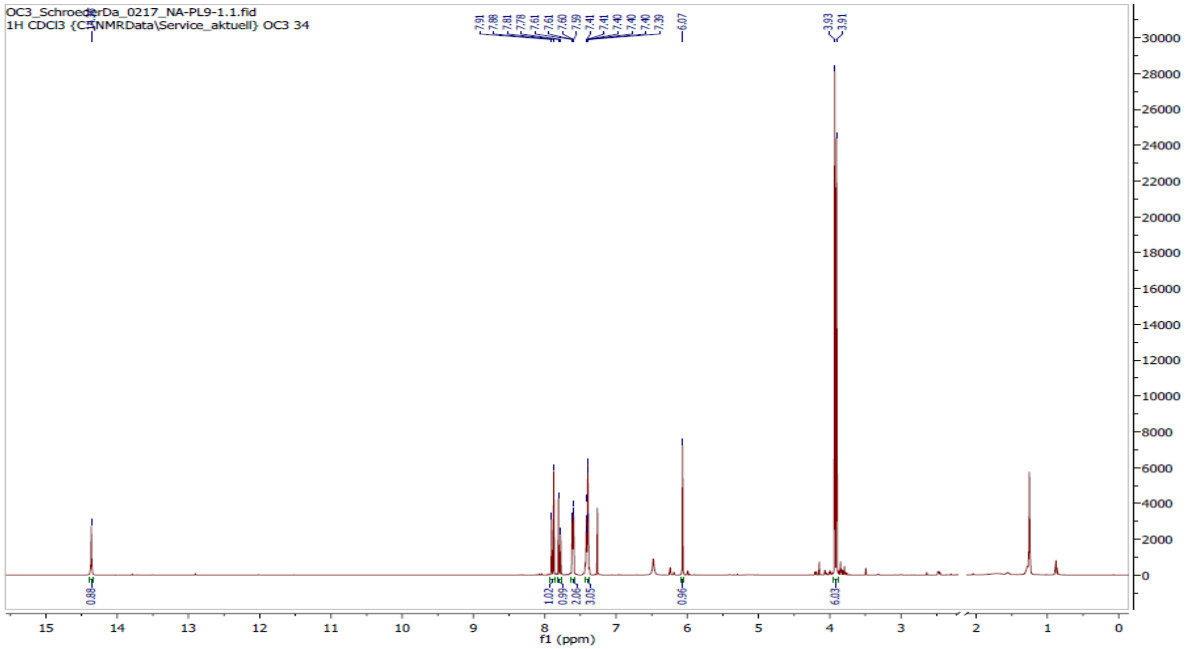
Appendix 2K: ESI-MS spectrum of Compound **2**



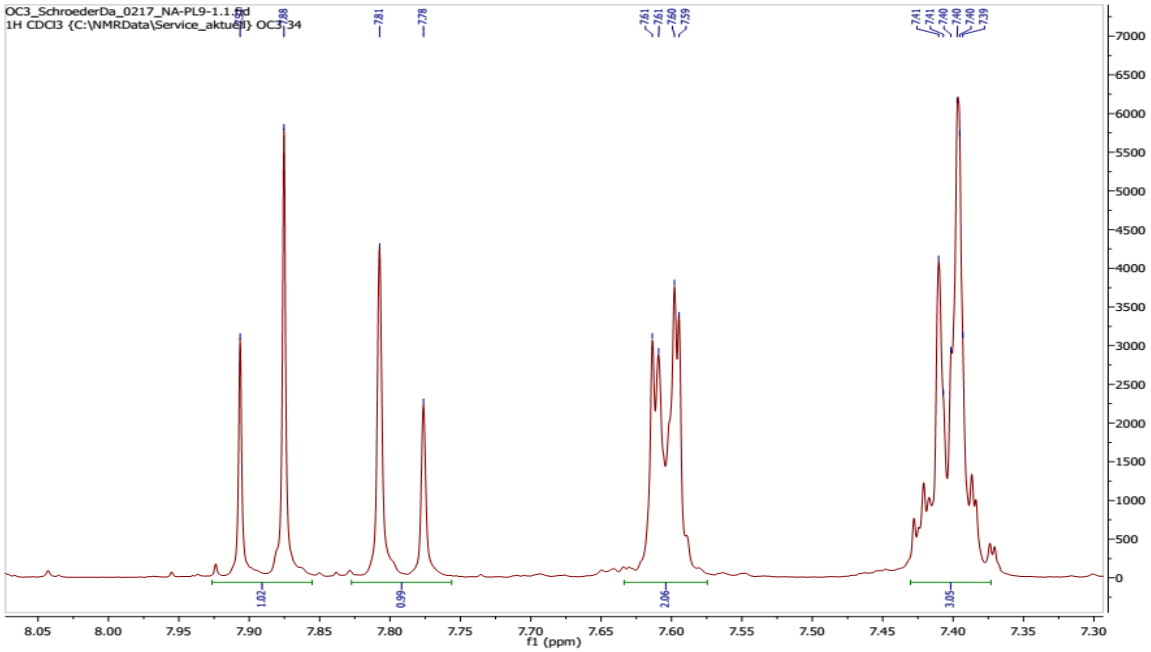
Appendix 2L: UV- VIS Spectroscopy for compound **2**.



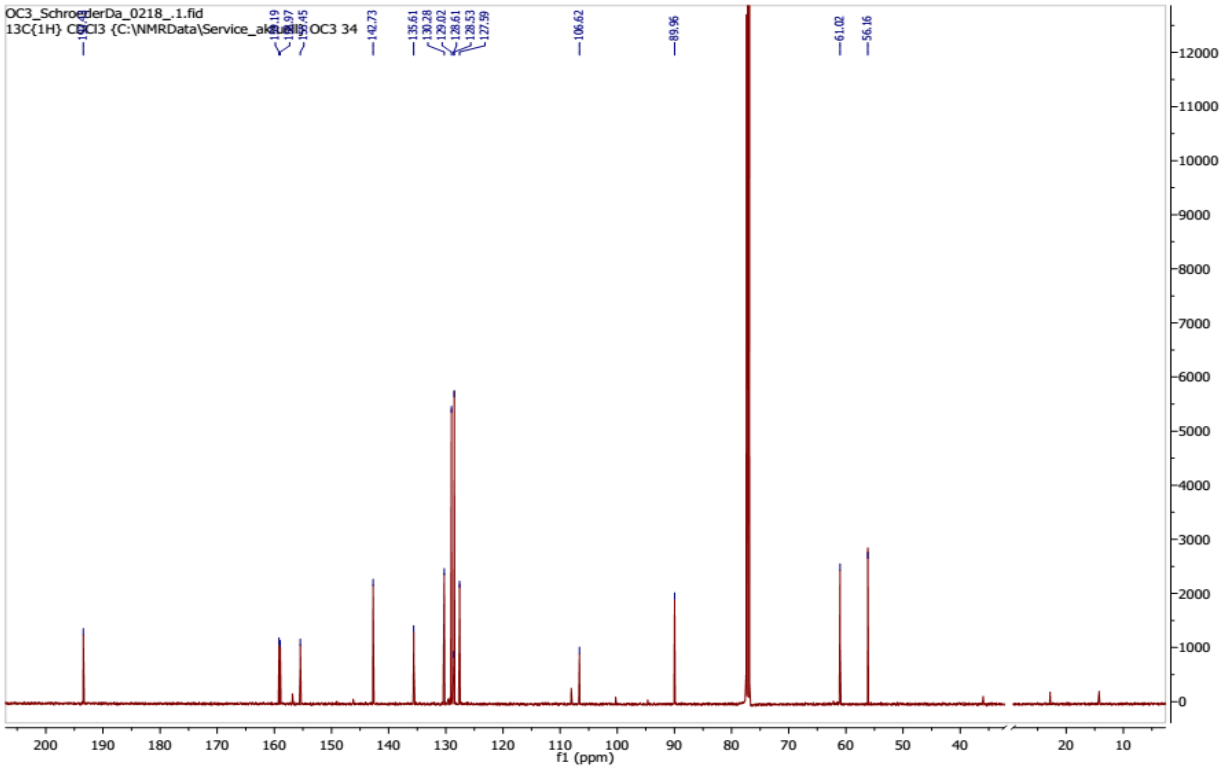
Appendix 3A: ^{1}H NMR spectrum of pashanone **(3),** $CDCl_3$, 500 MHz



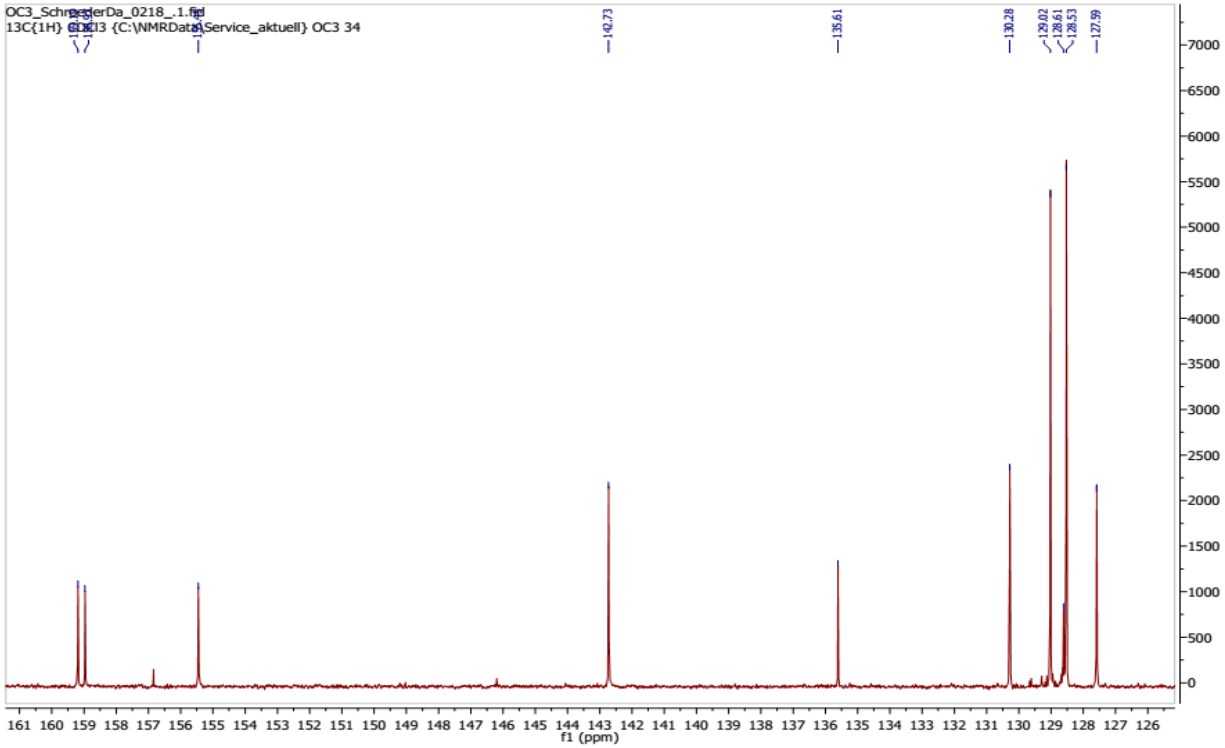
Appendix 3B: ^{1}H NMR spectrum of pashanone **(3),** $CDCl_3$, 500 MHz



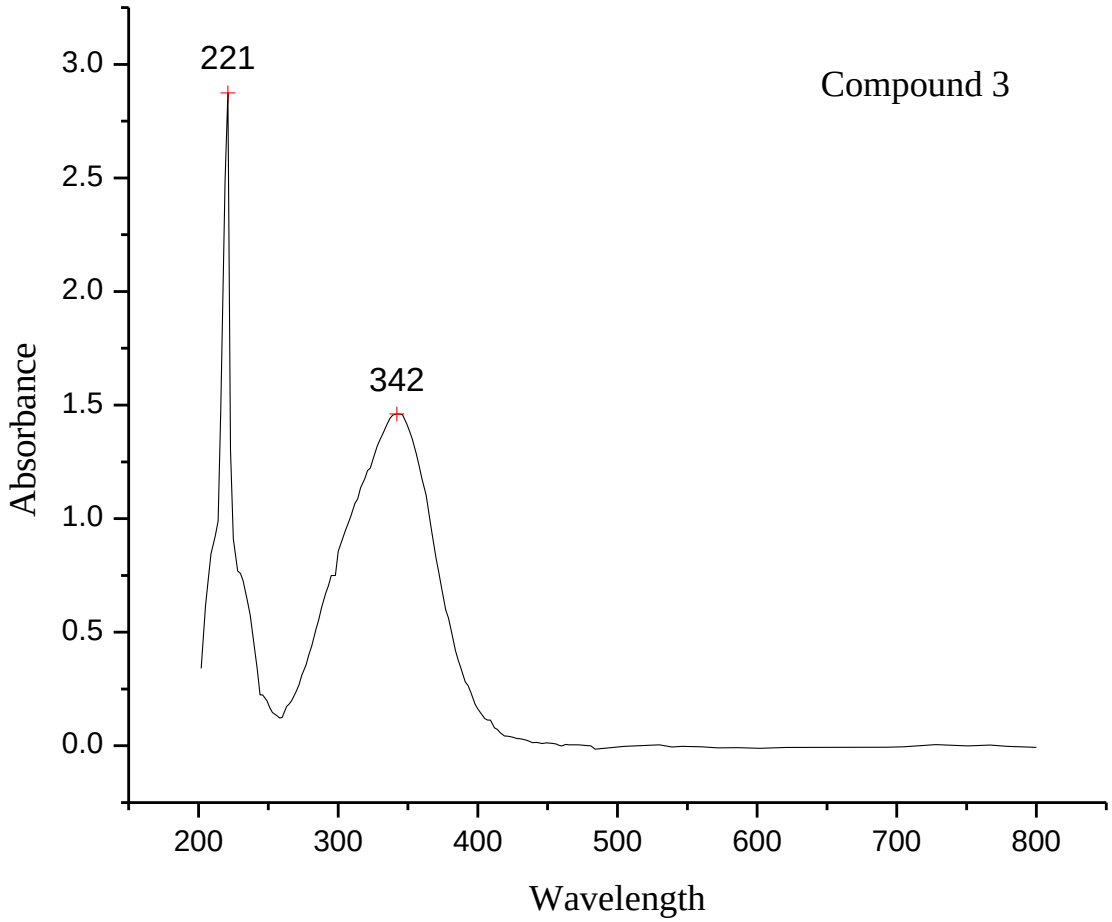
Appendix 3C: ^{13}C NMR spectrum of pashanone **(3),** $CDCl_3$, 125 MHz



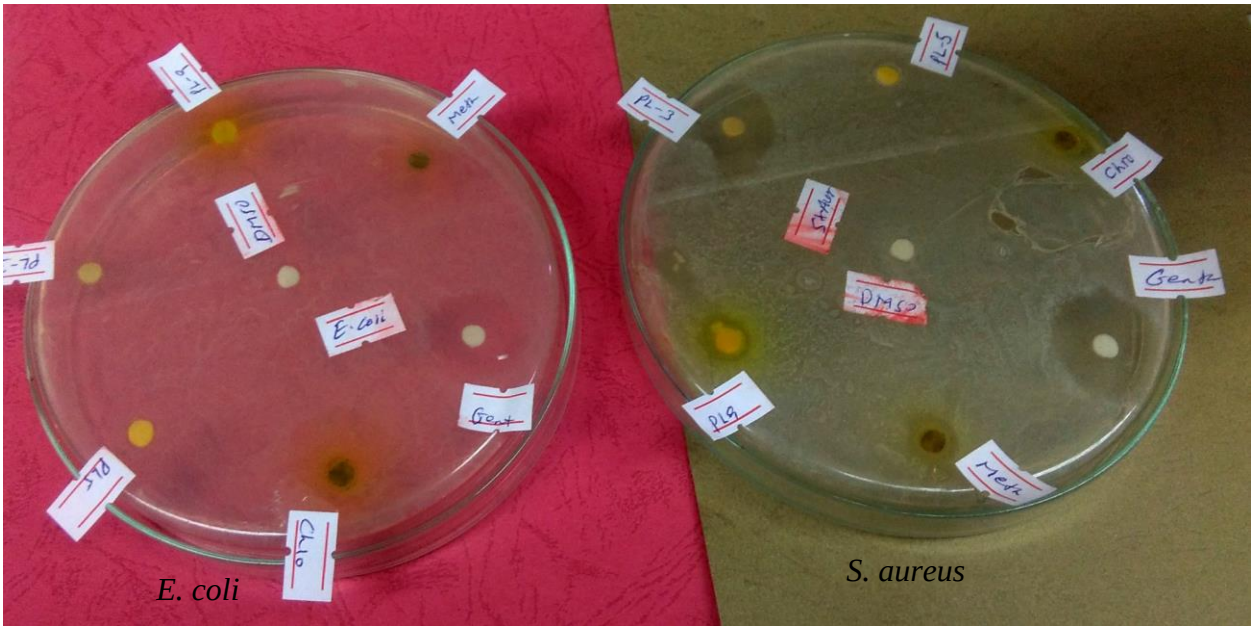
Appendix 3D: ^{13}C NMR spectrum of pashanone **(3),** $CDCl_3$, 125 MHz

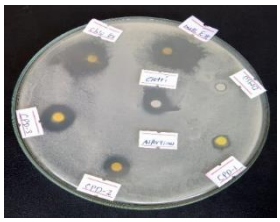


Appendix 3E: UV- VIS Spectroscopy for compound **3**.

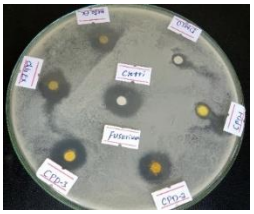


Appendix 4A: Zone of growth inhibition for crude and isolated compounds of bacteria and fungi

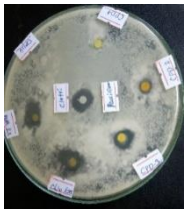




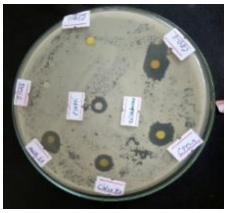
Aspergillus spp.



Fusarium spp.



Penicillium spp.



Trichoderma spp.