ANTIHYPERTENSIVE ACTIVITY OF AERIAL PARTS OF *SATUREJA PUNCTATA* (BENTH.) BRIQ. (LAMIACEAE)

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List of Abbreviations and Acronyms

ACE Angiotensin Converting Enzyme

ANOVA Analysis of variance

BP Blood pressure

brs broadened singlet

COSY Correlation Spectroscopy

CVD Cardiovascular disease

DBP Diastolic Blood Pressure

dd doublet of doublets

DEPT Distortional Enhancement Polarization Transfer

DMSO Dimethyl sulfoxide

EFMOH Ethiopian Federal Ministry of Health

HMBC Hetero nuclear Multiple Bond Correlation

HMQC Hetero nuclear Multiple bond Quantum Correlation

HPLC High performance Liquid Chromatography

Hz Hertz

IP Intraperitonial

IV Intravenous

MABP Mean Arterial Blood Pressure

NMR Nuclear Magnetic Resonance

PP Pulse Pressure

SBP Systolic Blood Pressure

SEM Standard Error of Mean

SPSS Statistical package for social science

WHO World Health Organization

Abstract

Antihypertensive activity of aerial parts of *S. puctata* (Benth.) Briq.

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Satureja punctata Benth. Briq. (Lamiaceae) locally known as ‘‘*Lomishet*’’ is among the plants used in Ethiopian traditional medicine for the treatment of various diseases including hypertension. The current study aimed at evaluating the antihypertensive activity of the decoction of the aerial parts of this medicinal plant in guinea model of hypertension and its vasorelaxant effect on isolated aorta. Acute hypertension was induced by surgical procedures involving clamping of the left renal artery and blood pressure was recorded invasively by direct cannulation method from the right common carotid artery. Intravenous administration of the decoction at doses of 10, 20 and 30 mg/kg caused 13.66 ± 0.27, 34.35 ± 0.33, 45.78 ± 0.23% (n=6) fall in ($p < 0.01$) MABP, respectively, in normotensive guinea pig and a respective fall of 16.02 ± 0.28, 38.52 ± 0.50, 52.07 ± 0.42 % (n=6) ($p < 0.01$) MABP in renovascular hypertensive guinea pig. The decoction also caused a dose-dependent relaxation of aorta precontracted with KCl at a concentration of 2.5- 40 mg/ml, with a maximum relaxation of 98.19 % achieved at 40 mg/ml. Preparative reversed-phase HPLC analyses of the aerial part decoction of *S. punctata* resulted in isolation of two phenolic compounds, rosmarinic acid and linarin. The structures of these compounds were elucidated by utilization of spectroscopic techniques, i.e., MS, UV, IR, 1D, and 2D NMR. Further, rosmarinic acid showed significant ($p<0.01$) reduction of MABP by 8.14 ± 0.27, 15.79 ± 0.33 and 31.78 ± 0.64 % (n = 6) at doses of 0.75, 1.5 and 3 mg/kg, respectively, in normotensive guinea pig. The findings of this study suggest that the aerial parts of *S. punctata* have genuine antihypertensive activity.

1. INTRODUCTION

1.1 Background

Hypertension is one of the most common cardiovascular diseases. It is defined as a sustained elevation of arterial blood pressure at or above 140/90 mm Hg (Chobanian *et al*., 2003). The normal level for blood pressure (BP) is below 120/80, where 120 represent the systolic measurement and 80 represent the diastolic measurement. BP between 120/80 and 139/89 is called pre-hypertension to denote increased risk of hypertension (Munir and Karim, 2013). Based on Joint National Committee on prevention, detection, evaluation, and treatment of high BP (JNC -7) report, hypertension is also categorized as; stage 1 (systolic blood pressure (SBP) 140 to 159 or diastolic blood pressure (DBP) 90 to 99 mm Hg), stage 2 (SBP 160 to 179 or DBP 100 to 109 mm Hg) and stage 3 (SBP >180 or DBP >110 mm Hg) (Chobanian *et al*., 2003).

Hypertension is a multi factorial disease involving a complex interplay of genetic and environmental factors and can be classified as primary or essential and secondary (Joshi *et al*., 2012). About 90 to 95% of cases are termed primary hypertension, referring to high BP for which no medical cause can be found. The remaining 5 to 10% of cases, called secondary hypertension, are caused by conditions that affect the kidneys, arteries, heart or endocrine system and also due to glucose tolerance and obesity (Carretero and Oparil, 2000; Tabassum *et al*., 2011).

The pathogenesis of essential hypertension is complex. It can be caused by increase in sympathetic nervous system activity, increase in production of sodium-retaining hormones and vasoconstrictors, deficiencies of vasodilators such as prostacycline and nitric oxide, inappropriate or increased renin secretion and genetic predisposition. Pathogenesis of secondary hypertension include chronic kidney disease, renovascular disease, Cushing's syndrome, drugs such as non-steroidal anti-inflammatory drugs and oral contraceptives (Joshi *et al*., 2012).

Hypertension is one of the major cardiovascular risk factors contributing to myocardial infarction, heart failure, stroke, peripheral vascular insufficiency, kidney failure and premature mortality (Sun *et al*., 2011). Worldwide 7.6 million premature deaths, which is 13.5% of all deaths globally and 92 million disability-adjusted life year annually were attributed to high BP

(Lawes *et al*., 2008). In addition, about 51% of stroke and 45% of ischaemic heart disease deaths are due to elevated BP (Alwan, 2010).

Symptoms associated with hypertension include, shortness of breath, fatigue, dizziness, chest pain, bluish color to lips and skin (cyanosis), heart palpitations, blurred vision, headache and nosebleeds (Rout *et al*., 2010). Hypertension is often called a 'silent killer' because people with this pathological condition can be asymptomatic for years and then have a fatal heart attack or stroke. It is the most common chronic illness. Hypertension is asymptomatic and readily detectable. However, it should be treated in its earliest stages to avoid lethal complication (Jao *et al*., 2012).

1.2 Epidemiology of hypertension

Hypertension is an important growing public health issue in developed as well as developing countries. It is one of the leading causes of disability, mortality and morbidity among the population (Tahla *et al*., 2011). The prevalence of elevated arterial BP increases with age. The global burden of hypertension is that about 26.4% of the world's adult population had hypertension in 2000, and the proportion is expected to increase to 29% by 2025. In the year 2000, an estimated 639 million individuals had hypertension in economically developing countries and this number is expected to rise to 1.15 billion by 2025 (Kearney *et al.*, 2005). Hypertension was thought to be rare in Africa, but it is now recognized as one of the most important causes of cerebrovascular diseases contributing to about 40% of the diseases in the continent. It is one of the major factors for high mortality of adults in sub-Saharan Africa (Opie and Seedat, 2005; Lekoubou *et al*., 2010). According to the health and health-related indicators of Ethiopian Ministry of Health (2008-2009), hypertension was the seventh leading cause of death in Ethiopia in 2008 (EFMOH, 2010). Although there is shortage of extensive data, 22% of men and 14.9% women of Ethiopian adult population has been estimated to have hypertension (Nshisso *et al*., 2012). A community-based study conducted among adult residents in Addis Ababa reported a 31.5% and 28.9% prevalence of hypertension among males and females, respectively (Tesfaye *et al*., 2009).

1.3 Prevention and treatment of hypertension

The level of awareness, treatment and control of hypertension varies considerably among countries. The prevention and control of hypertension has not received due attention by many developing nations (Tirapelli *et al*., 2010). Hypertension is one of the most modifiable risk factors for cardiovascular diseases; however, awareness about its treatment and control is extremely low among developing countries including Ethiopia (Awoke et *al*., 2012). The incidence of hypertension is increasing partly because of the increase in risk factors such as smoking, obesity, change in diet habit, excessive alcohol consumption and physical inactivity (Lawes *et al*., 2008).

Hypertension can best be prevented by adjusting one's lifestyle: salt restriction, moderation of alcohol consumption, high consumption of vegetables and fruits, low-fat diet, body weight reduction, maintenance of healthy weight, regular physical exercise and reducing stress are recommended lifestyle measures that have been shown to be helpful in reducing BP. In addition cessation of smoking is mandatory in order to improve cardiovascular risks (Chobanian *et al*., 2003; Mancia *et al*., 2013). Such appropriate lifestyle changes may safely and effectively delay or prevent hypertension in non hypertensive subjects and contribute to BP reduction in hypertensive individuals already on medical therapy, allowing reduction of the number and doses of antihypertensive agents. Lifestyle changes can also decrease BP, enhance antihypertensive drug efficacy and reduce the risk for cardiovascular diseases (Mancia *et al*., 2013).

As a medical option various classes of antihypertensive drugs such as diuretics, beta-blockers, calcium channel blockers, angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists, alpha-receptor blockers, centrally acting agents and peripheral vasodilators have been used to treat hypertension (Tousoulis *et al*., 2012). These medications are used alone or in combination, while some are only used in combination. For instance, thiazide diuretics such as hydrochlorothiazide can be used alone for the management of hypertension. In addition, some of these drugs are preferred to others depending on the condition of the patient (Chobanian *et al*., 2003). The initial pharmacological treatment of hypertension is antihypertensive monotherapy, which is then followed by two options: either the addition of a second drug, if required, or the switch to another drug, in an attempt to find the appropriate monotherapy for each individual

patient (Mancia *et al*., 2013). Administration of a combination of at least two drugs from the start of treatment is recommended in the guidelines, particularly in patients in whom actual BP and target BP differ by >20 mmHg. This measure facilitates and accelerates the early control of BP and contributes to the maintenance of adequate long term BP control. The most widely used combinations are those containing renin-angiotensin-aldosterone system (RAAS) suppressors such as an angiotensin converting enzyme (ACE) inhibitors or an angiotensin-receptor blocker and a diuretic. Triple combination is needed in ≥ 30% of patients in order to reach the target BP. The most frequently used fixed trials consist of a renin angiotensin- aldostrone system suppressor, a Calcium channel blocker and a thiazide diuretic. The combined effects of the three drugs at optimal doses control BP in a high percentage (>50%) of patients who require triple therapy (Mancia *et al*., 2013; Ruilope, 2013).

In order to prevent damage to critical organs and conditions such as stroke, heart attack, and kidney failure that are caused by high BP, it is important to screen, diagnose, treat, and control hypertension in its earliest stages. This can also be accomplished by increasing public awareness and increasing the frequency of screenings for the condition (Ruilope, 2013). Despite all the effort and the availability of several types of antihypertensive drugs, a successful treatment for hypertension is difficult to achieve. Thus, the development of new pharmacological tools is required to improve the clinical management of this pathology (Laurent *et al*., 2012). The research on naturally occurring BP lowering agents is rapidly expanding due to the high potential of such molecules as new antihypertensive drugs. Recently, a lot of intensive efforts have been channeled into researching plants with hypotensive and antihypertensive therapeutic values (Tabassum *et al*., 2011).

1.4 Medicinal plants used in hypertension treatment

Plants have been a very good source of drugs and many of the currently available medicines have been directly or indirectly derived from them. For this reason, use of natural products as therapeutic agents, especially those derived from plants have been increasing in recent years (Tirapelli *et al*., 2010). A large number of plant derived substances have been evaluated as possible antihypertensive agents. Although several classes of natural products are responsible for the antihypertensive activity of many plant species, the most important activity has been

observed in alkaloids, terpenoids , and flavonoids (Maione *et al.*, 2013). For instance, reserpine (**1**), the purified alkaloid of *Rauwolfia serpentina* (Apocynaceae) was the first potent drug widely used in the long-term treatment of hypertension (Vakil, 1955). Faizi *et al*. (1998) reported that the crude extract of the leaves of *Moringa oleifera* (Moringaeae) showed strong antihypertensive activity and it was indicated that thiocarbamate and isothiocyanate fractions of the crude extract were responsible for the antihypertensive activity. Similarly antihypertensive activities of phenolic constituents of *Zingiber officinalis* (Zingiberaceae) such as 6-gingerol (**2)**, 8-gingerol (**3**) and 10-gingerol (**4**) were reported. It was also reported that the crude extract of ginger lowers BP by mechanism mediated through blockade of Ca^{2+} channel (Ghayur and Gilani, 2005).

The crude extract of *Ocimum basilicum* (Lamiaceae) also causes a fall in systolic, diastolic, and mean BP in a dose-dependent manner; the effect of the extract has been attributed to eugenol (**5**) (Azhar *et al*., 1995). The crude extract of *Marrubium vulgare* (Lamiaceae) is widely used as an antihypertensive in traditional medicine and it has been shown to induce vascular relaxation and decrease SBP in spontaneously hypertensive rats. The diterpene marrubenol (**6**) with vasorelaxant activity was isolated and characterized from the water extract of this plant (El Bardai *et al*., 2001). In addition the extracts prepared from the stem bark of *Croton cajucara* Benth. (Euphorbiaceae) are used in folk medicine for the treatment of hypertension. Chemical investigations on the bark led to the isolation of several diterpenes including trans-dehydrocrotonin (**7**) a diterpene that displays vasorelaxant activity (Guerrero *et al*., 2004). Ahmed *et al*. (2006) reported four flavonoids namely, naringenin (**8**), isoaromadendrin (**9**), taxifolin (**10**) and isosinensin (**11**) which were isolated from *Salvia verbenaca* (Lamiaceae) and three flavonoids namely 5-hydroxy-3,4′,7-trimethoxyflavone (**12**), retusin (**13**) and verbenacoside (**14**) from *Euphorbia cuneata* (Euphorbiaceae) that exhibited significant antihypertensive activity. In addition, several plant species that are rich in phenolic compounds have been reported to cause fall in arterial BP by inhibiting ACE, which plays a key physiological role in BP control (Braga *et al*., 2007; Jimenez-Ferrer *et al*., 2010; De Souza *et al*., 2011). Furthermore, a number of compounds such as peptides, phenylpropanoid glycosides, iridoids, fatty acids and tannins were found to be effective plant derived antihypertensive compounds by showing inhibition of ACE activity (Nyman *et al*., 1998; Loizzo *et al*., 2007; Li *et*

al., 2008). Structural formulae of antihypertensive compounds listed above are depicted in Figure 1.

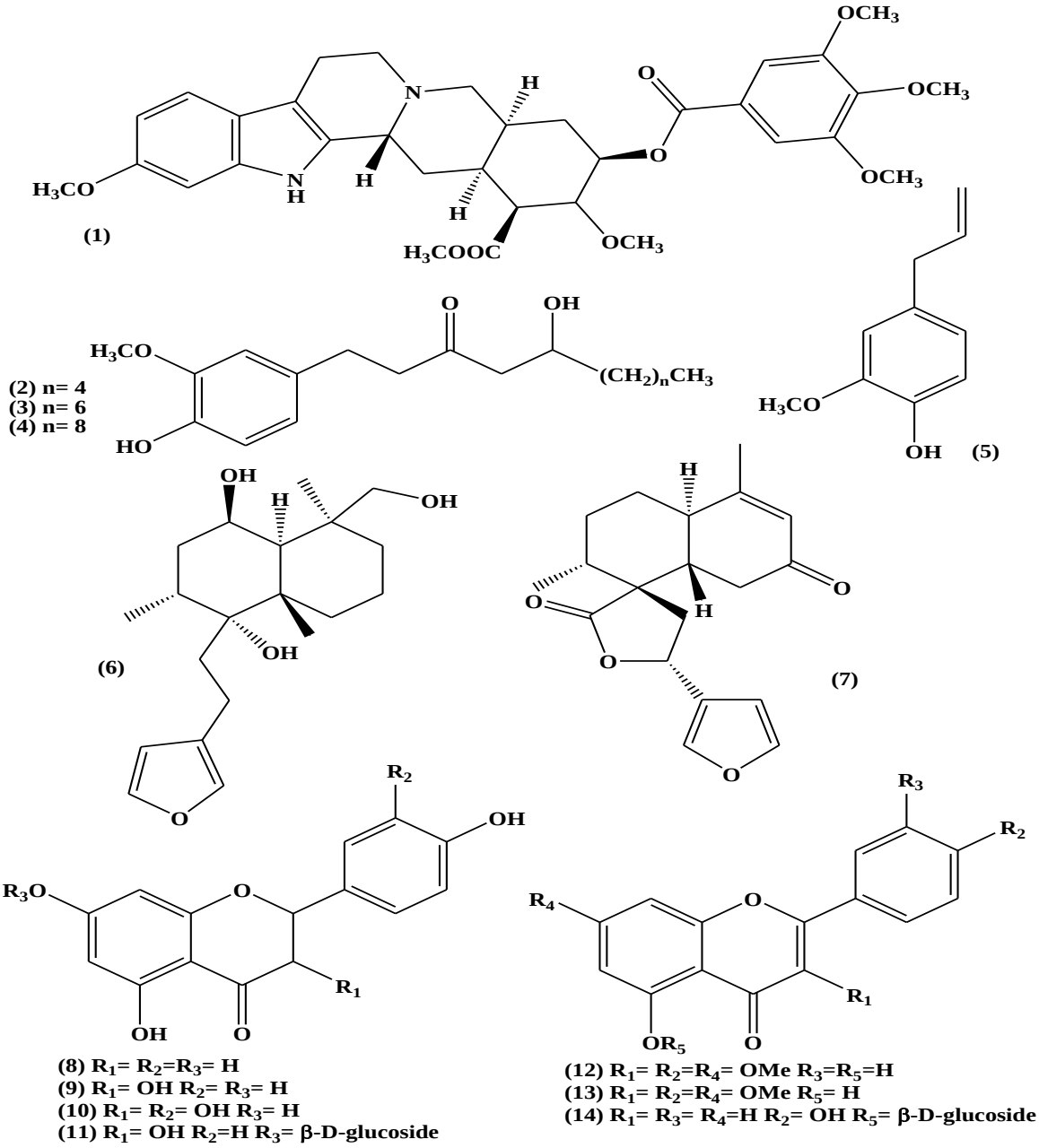


Figure 1: Structural formulae of plant derived antihypertensive compounds

1.4.1 Ethiopian medicinal plants used for hypertension treatment

Ethiopia is a country characterized by a wide range of climatic and ecological conditions possessing enormous diversity of flora, including wide range of potentially useful medicinal plants. Traditional medicinal practices are common in Ethiopia. Because indigenous remedies are culturally better acceptable, accessible and affordable, about 80% of the population in the country uses plant based traditional medicine as their major primary healthcare system (Seid and Tsegaye, 2011).

Large numbers of plants are used for the treatment of hypertension in the traditional medical practice of Ethiopia. Some *in vivo* and *in vitro* studies have been conducted to evaluate antihypertensive activities of these plants. For instance, Ayele *et al*. (2010) evaluated 80% methanol extract of the leaves of *Syzygium guineense* (Willd) D.C. (Myrtaceae) and showed that it causes significant reduction of BP in a 1-kidney-1-clip rat model and dose-dependent relaxation of guinea pig aorta precontracted with KCl. Similarly Ambaye *et al*. (2002) reported that "Gebto Arekei", the traditional medicinal spirit prepared from a fermented brew containing *Lupinus albus* seeds causes reduction in BP in anaesthetized renovascular hypertensive guinea-pigs. Mengistu *et al*. (2012) also reported that crude aqueous leaf extract of *Moringa stenopetala* Baker f. (Moringaceae) causes significant fall in BP in normotensive anaesthetized guinea pigs and dose and time dependent inhibition of KCl induced contraction on guinea pig aorta.

1.5. The genus *Satureja*

The genus *Satureja* consists of about 100-200 species distributed in Europe, Africa, Asia, and America. The species are regularly found in sunny, dry and rocky habitats. The genus *Satureja* belongs to the family Lamiaceae (Labiatae). Members of the genus are small shrubs or sub-shrubs and perennial herbs. In Ethiopia, the genus is represented by eight species: *S. abyssinica*, *S. imbricate*, *S. kilimandschari*, *S. paradoxa*, *S. punctata*, *S. pseudosimensis*, *S. simensis* and *S. unguentaria* (Ryding *et al*., 2006).

Plants belonging to the genus *Satureja* have long been used as spices and natural food preservatives, culinary flavors and in aromatherapy (Mihajilov-Krstev *et al*., 2012). The aerial parts of some *Satureja* species are used as herbal tea in traditional medicine to treat various disorders including cramps, muscle pain, nausea, indigestion, diarrhea, diabetes, hypertension

and infectious diseases (Abate, 1989; Hajhashemi *et al*., 2012). *Satureja* species are economically and medicinally important because of their high content of essential oils (Momtaz and Abdollahi, 2010).

1.5.1 Pharmacological activities

Among the aromatic plant species, the essential oils and extracts of different species belonging to the genus *Satureja* showed significant antimicrobial activity against various species of bacteria and fungi. In previous studies, *S. hortensis* (Gulluce *et al*., 2003; Sahin *et al*., 2003), S. *biflora*, *S. pseudosimensis* and *S. masukensis* (Vagionas *et al*., 2007), *S. thymbra* (Giweli *et al*., 2012), *S. punctata* (Belay *et al*., 2011), *S. abyssinica* and *S. paradoxa* (Tolossa *et al*., 2007) have demonstrated a good antibacterial and antifungal activity. In addition, *Satureja* species are famous for their potential pharmacologic activities such as antioxidant (Radonic and Milos, 2003; Ozkan *et al*., 2007; Bagheri *et al*., 2013), antiviral (Yamasaki *et al*., 1998; Loizzo *et al*., 2008), vasodilatory (Sanchez de Rojas *et al*., 1996b; Sanchez de Rojas *et al*., 1999), anti-inflammatory and antinociceptive (Hajhashemi *et al*., 2002; Amanlou *et al*., 2005; Hajhashemi *et al*., 2012), antispasmodic and antidiarrheal (Hajhashemi *et al*., 2000), antiprotozoal (Tariku *et al*., 2010), antimycobacterial (Askun *et al*., 2012), anticholinesterase (Ozturk, 2012), hepatoprotective (Wolde *et al*., 2010; Ahmadvand *et al*., 2012), antidiabetic, antihyperlipidemic and stimulation of reproduction (Abdollahi *et al*., 2003) and cytotoxic (Sadeghi *et al*., 2013). These pharmacologic effects are mostly correlated to the presence of essential oils, phenolic compounds and terpenoids (Momtaz and Abdollahi, 2010).

1.5.2 Other uses

Due to the presence of high amount of thymol and carvacrol in *Satureja* species essential oils obtained from the leaves and flowers of *Satureja* species are commonly used as preservative and flavoring material in food, pharmaceutical, cosmetic and perfume industries (Ozturk, 2012). In addition, insecticidal activity of essential oil of *Satureja* was also reported (Ayvaz *et al*., 2010).

1.5.3 Phytochemistry

Volatile oils, phenolic compounds, sterols, acids, gums and mucilages have been reported as the main components of *Satureja* species (Momtaz and Abdollahi, 2010; Askun *et al*., 2012).

Analysis of the essential oil composition of several *Satureja* species indicated the presence of γ -terpinene (**15**) (Hassanpouraghdam *et al*., 2009; Ghotbabadi *et al*., 2012), *p*-cymene (**16**) (Oke *et al*., 2009; Sadeghi *et al*., 2013), carvacrol (**17**) (Hassanpouraghdam *et al*., 2009)**,** thymol (**18**) (Radonic and Milos, 2003; Sadeghi *et al*., 2013) and linalool (**19**) (Vagionas *et al*., 2007; Ghotbabadi *et al*., 2012) as major components. Similarly oleanolic acid (**20**), ursolic acid (**21**), β-sitosterol (**22**) and β-sitosterol-β-D-glucoside (**23**) (Escudero *et al*., 1985) and flavonoids such as apigenin (**24**), luteolin (**25**), genkwanin (**26**), chrysoeriol (**27**), diosmetin (**28**), quercetin (**29**), luteolin 7-O-β-D-glucoside (**30**), luteolin 7-O-β-D-rutinoside (**31**), luteolin 3'-O-β-D-glucoside (**32**), cirsimaritin (**33**), xanthomicrol (**34**) and vicenin-2 (**35**) have been isolated and identified from solvent extract of the aerial parts of *Satureja* species (Ferreres *et al*., 1987; Saeidnia *et al*., 2011).

In addition, naringenin (**36**), eriodictyol (**37**) and luteolin were reported from hot water extract of *S. obovata* (Sanchez de Rojas *et al*., 1996b; Sanchez de Rojas *et al*., 1999). Analysis of solvent extracts of *Satureja* species such as *S. icarica, S. coerulea, S. montana* and *S. cilicica* revealed that carvacrol, hesperidin (**38**), rosmarinic acid (**39**) and caffeic acid (**40**) are the main components of the aerial parts (Askun *et al*., 2012; Hassanein *et al*., 2014). Figure 2 depicts the structural formulae of compounds isolated from *Satureja* species.

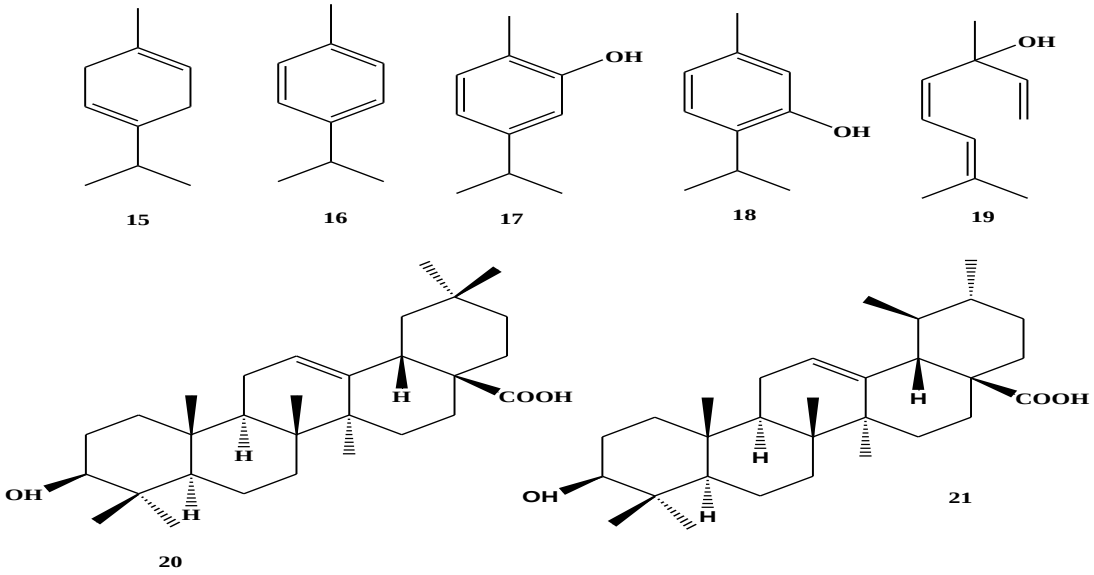
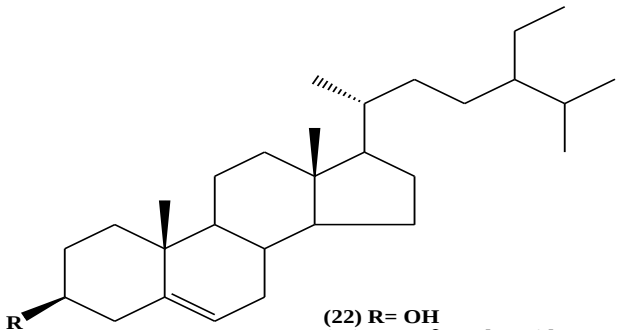
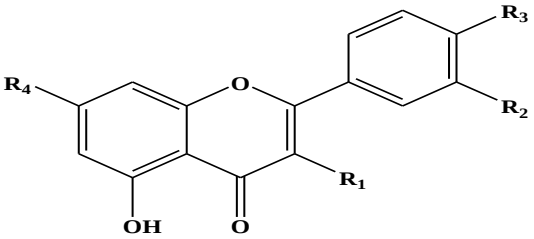


Figure 2: Structural formulae of compounds isolated from the genus *Satureja*

Figure 2 Continued

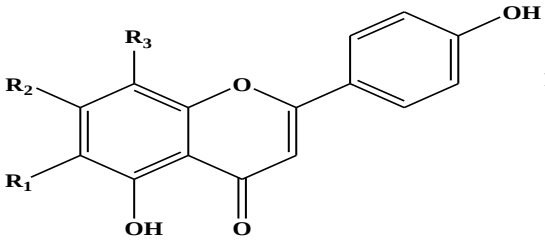


(22) R= OH
(23) R= O-β-D-glucoside

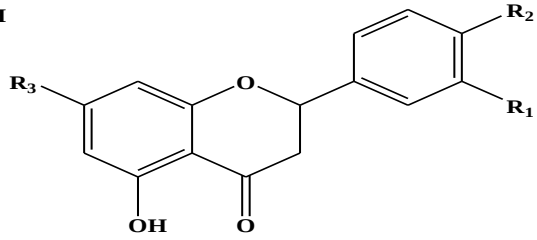


(24) R_1= R_2= H R_3= R_4= OH
(25) R_1= H R_2 = R_3 = R_4 = OH
(26) R_1 = R_2 = H R_3= OH R_4 = OCH_3
(27) R_1 = H R_2 = OCH_3 R_3 = R_4= OH
(28) R_1= H R_2 = R_4 = OH R_3 = OCH_3

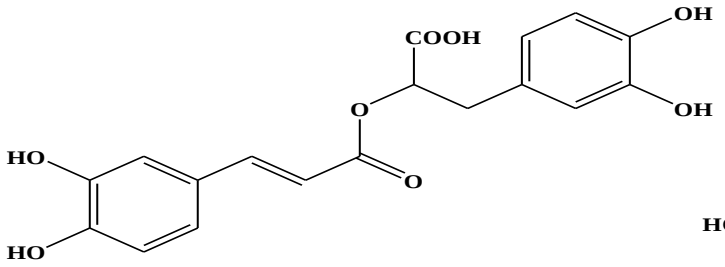
(29) R_1= R_2 = R_3 = R_4 = OH
(30) R_1 = H R_2 = R_3 = OH R_4 = O-β-D-glucoside
(31) R_1= H R_2 = R_3 = OH R_4 = O-β-D-rutinoside
(32) R_1= H R_3 = R_4 = OH R_2 = O-β-D-glucoside



(33) R_1 = R_2 = OCH_3 R_3 = H
(34) R_1 = R_2 = R_3 = OCH_3
(35) R_1= R_3= β-D-glucoside R_2 = OH



(36) R_1 = H R_2 = R_3 = OH
(37) R_1= R_2 = R_3 = OH
(38) R_1= OH R_2 = OCH_3 R_3 = O-β-D-rutinoside



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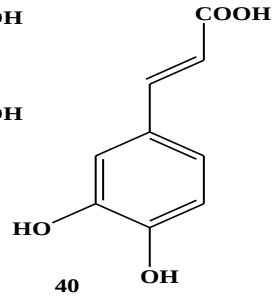

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1.6 *Satureja punctata* (Benth.) Briq.

S. punctata is a sub-shrub or small shrub which grows up to 10-100 cm high (Figure 3), often with long branches with distinctive purple/violet flowers. It is known to grow on stony slopes mainly on limestone (Rydring *et al*., 2006).

Figure 3**:** Aerial parts of *Satureja punctata* (Benth.) Briq.

1.6.1 Ethnobotonical uses

S. punctata is known by the vernacular name *"Lomishet"* (Amharic) in Ethiopia. The Ethiopian traditional healers make preparations from the aerial parts of *S. punctata* for the treatment of various diseases including hypertension, diabetes, liver disease, headache and febrile illness (Abate, 1989; Yineger *et al*., 2008). For the treatment of hypertension, the decoction of the dried aerial parts of the plant is taken orally as a tea (Abate, 1989).

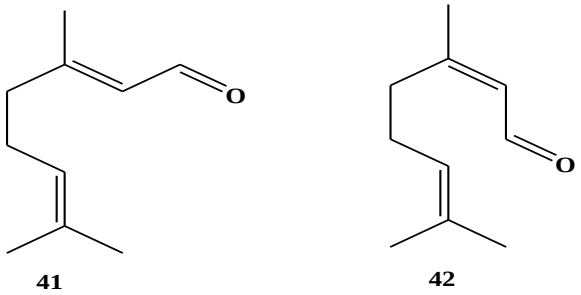
1.6.2 Pharmacological activities

The essential oil of *S. punctata* showed significant antiprotozoal activity (Tariku *et al*., 2010) and antibacterial activity (Belay *et al*., 2011). In addition, the total hydroalcoholic extract of *S. punctata* and its methanolic and aqueous fractions were reported to possess antidiabetic activity

(Tsegaye *et al*., 2010). Similarly, the crude aqueous extract of *S. punctata* and its methanol and chloroform fractions possess hepatoprotective and antioxidant activity (Wolde *et al*., 2010).

1.6.3 Phytochemistry

S. punctata contains essential oil, which is composed of 67 compounds; i.e, oxygenated monoterpenes, oxygenated sesquiterpenes, sesquiterpene hydrocarbons, monoterpene hydrocarbons and non-terpenic compounds. The main constituents of the oil are geranial **(41)** and neral **(42)** (Chagonda and Chalchat, 2005; Tariku *et al*., 2010). Furthermore, preliminary phytochemical screening of the methanolic fraction of *S. punctata* revealed the presence of alkaloids, flavonoids, tannins and phenolic compounds (Wolde *et al*., 2010).



41 42

Figure 4: Structural formulae of compounds isolated from essential oil of *Satureja puctata* (Benth.)

1.7 Rationale for the study

Hypertension is an important worldwide public health challenge because of its high frequency and concomitant risks of cardiovascular and kidney diseases (Munir and Karim, 2013). Three quarters of people with hypertension live in developing countries with limited health resources and where people have a very low awareness of hypertension and poor BP control (WHO, 2005).

In addition, though various antihypertensive drugs have been used to manage hypertension, the efficacy of these drugs is only 40% - 60%, and usually two or more antihypertensive drugs from different categories need to be combined to achieve optimal results and thus increasing the cost of treatment and side effects (Sun *et al*., 2011). Furthermore, numerous patients remain unresponsive to available pharmacological interventions and left with severely high BP (Kloet *et*

al., 2013). Successful and effective way for managing hypertension has long been a challenge for medical researchers. Therefore, new, more effective, safe and economical antihypertensive agents from plant sources are needed as substitutes for synthetic drugs.

In Ethiopian traditional medicine *S. punctata* (Benth.) has been widely used for long time in treatment of hypertension, however, evaluation of its antihypertensive activity is not reported.

2. OBJECTIVES

2.1 General objective

The general objective of this study is to evaluate the potential antihypertensive activity of decoction and compounds isolated from the aerial parts of *S. punctata* (Benth.) Briq.

2.2 Specific objectives

- To evaluate the effect of the decoction in animal model of hypertension
- To evaluate the hypotensive effect of the decoction in normotensive guinea pigs
- To study the possible mechanism of action of the decoction *in vivo*
- To evaluate the vasorelaxant effect of the decoction on isolated aorta *ex vivo*
- To isolate the active compound(s)
- To evaluate the *in vivo* blood pressure lowering effect the compound isolated from the decoction.

3. MATERIAL AND METHODS

3.1 Materials

3.1.1 Plant material

The aerial parts of *S. punctata* (Benth.) Briq. were collected in February 2013 from Suba-State Forest, Menagesha, West of Addis Ababa, Ethiopia. The plant material was authenticated by Professor Ensermu Kelbessa, the National Herbarium, Department of Plant Biology and Biodiversity Management, Addis Ababa University, where a voucher specimen (collection number DH 001) was deposited.

3.1.2. Chemicals and drugs

The following chemicals and solvents were used for the experiments. Acetylcholine chloride and sodium chloride (Sigma Aldrich, UK), atropine sulfate injection (Renaudin lab, France), sodium pentobarbital injection (Apoteksbolaget, Umeå, Sweden), heparin injection (Rotex Medica, Germany), normal saline (0.9% NaCl) (Ethiopian Pharmaceutical Manufacturing, Addis Ababa, Ethiopia), potassium chloride, magnesium sulphate, calcium chloride, potassium dihydrogen phosphate and sodium bicarbonate (BDH Chemicals Ltd., England), glucose (Epharmecor, Madrid, Spain), methanol (Carlo Erba reagent SPA, France) and acetic acid (Pharmacos Ltd., Germany). All the chemicals were of analytical grade; they were either purchased from local chemical suppliers or obtained from laboratories of the Department of Physiology and the Department of Pharmaceutical Chemistry and Pharmacognosy, Addis Ababa University.

3.1.3 Experimental animals

Healthy adult guinea pigs of either sex weighing 400-450 g were used for the experiments. The animals were purchased from Ethiopian Health and Nutrition Research Institute (EHNRI) and placed in the animal house of Department of Pharmacology, College of Health Sciences, Addis Ababa University. The animals were kept at room temperature, exposed to a 12 h light/dark cycle. They were allowed free access to standard commercial diet and water *ad libitum* and acclimatized for one week prior to the study. All experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline (ILAR, 1996).

3.2 Methods

3.2.1 Preparation of plant material

The aerial parts of *S. punctata* were air-dried under shade at room temperature and were grind into powder. The decoction of the plant material was prepared by boiling the powdered dried plant material (100 g) in distilled water (1.5 L) for 30 min. The extract was then filtered with new nylon cloth and with Whatman No. 1 filter paper. The filtrate was freeze dried in a lyophilizer (Gperon, Korea) and the resulting dry extract was kept in a desiccator until use.

3.2.2. *In vivo* blood pressure lowering activity test

3.2.2.1 In normotensive guinea pigs

Six normotensive male guinea pigs per group were used for the experiment and arterial BP was recorded according to the method described by Gilani (1991). Experimental animals, in the current case guinea pigs, were anaesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (40 mg/kg, i.p.). The temperature of the animals was maintained at 37 °C by the use of a heated table and an overhead lamp. The anaesthetized guinea pigs were fixed on a supine position on a dissecting table. At the stage of light anesthesia, as determined by the absence of corneal or pedal withdrawal reflexes and spontaneous movements, a longitudinal mid-tracheal incision was made through the anterior of the neck to expose the trachea, the right common carotid artery and the left jugular vein. The trachea was exposed and the animals were allowed to breathe spontaneously using artificial ventilator (Bioscience, 815-51190-1, Sheemess, Kent, UK) by inserting tracheal cannula after tracheotomy. For arterial BP recording the left carotid artery was cannulated *via* an arterial cannula filled with heparinized saline, which was connected to a pressure transducer (BBC, Goez Metrawatt, Model SE 120). The cannulation of the right jugular vein was performed in the same manner as the cannulation of carotid artery with similar cannula to facilitate intravenous injections of normal saline and the test samples. The exposed surface for the cannulation was covered with cotton wool moistened with warm normal saline (0.9% NaCl). The animals were allowed to equilibrate before commencing the experiment and BP was monitored until a steady baseline level was obtained. After stabilization of BP, the animals were injected with 0.2 ml normal saline intravenously (i.v.). The experiments were started with a control recording of basal BP with stable systolic and diastolic phases.

Administration was through jugular vein slowly over a duration of 15 sec and then flushed with 0.2 ml normal saline. Arterial BP was allowed to return to the resting level between injections. The values for the systolic and diastolic BP was calculated from the calibration scale made at the beginning of each experiment. Pulse pressure (PP) was obtained by subtracting diastolic blood pressure (DBP) from systolic blood pressure (SBP) and mean arterial blood pressure (MABP) was calculated according to the formula:

$$MABP = DBP + 1/3 (SBP - DBP)$$

Changes in BP were recognized as the difference between the steady state values before injection and the changed readings seen after test sample injection and it was calculated by the following formula:

$$\text{Change BP} = \text{Baseline BP-BP after injection of sample}$$

Pilot experiments were carried out to estimate the doses to be used for the study. Accordingly, the samples were freshly dissolved in normal saline as vehicle and the decoction was tested at the doses of 10, 20 and 30 mg/kg of body weight. Whereas the isolated compound was tested at the doses of 0.75, 1.5 and 3 mg/kg of body weight. To study the mechanism of action of the test extract, response to i.v. injections of acetylcholine (1 μg/kg) and the extract was compared in presence of atropine (1 mg/kg) in another set of experiments.

3.2.2.2 In renovascular hypertensive guinea pigs

The ability of the decoction to lower BP in hypertensive animals was investigated according to the method given by Goldblatt *et.al* (1934). Six male guinea pigs were used for the induction of acute renal hypertension; guinea pigs were anaesthetized by i.p. injection of 40 mg/kg sodium pentobarbital and cannulation was performed in similar manner as in normotensive guinea pigs as stated under section 3.2.2.1. After allowing BP to stabilize for a period of one hour, the animals were subjected to a second surgical procedure to ligate renal artery. In the procedure, a midline incision was made through the abdominal wall. The intestine was carefully extirpated and then the left renal artery was clamped. The renal artery was occluded for 4 h following the

surgery. Then the arterial clip was removed and BP was monitored continuously until a renewed increase to the starting level was obtained. Within 10-15 min a stable hypertension was achieved and the animals were injected with 0.2 ml normal saline i.v. as control; whereas for the test the `animals were injected with the same volume test solutions. Test solutions were prepared by dissolving the decoction in normal saline at the doses of 10, 20 and 30 mg/kg of body weight in similar manner as performed in normotensive animals. Increase in BP after reopening of the renal artery and reduction in BP after administration of the test sample was determined in mmHg (Vogel and Vogel, 2002). Percent inhibition of hypertensive BP values under sample treatment compared to pretreatment hypertension values was calculated by the following formula:

$$\% \text{ Inhibition of hypertensive BP} = \frac{\text{Baseline hypertensive BP- BP after injection of sample}}{\text{Baseline hypertensive BP}} \times 100$$

3.2.3 *In vitro* vasorelaxant activity

The *in vitro* vasorelaxant property of the decoction was studied on isolated thoracic aorta of guinea pigs of either sex. A method described by Bohn and Schonafinger (1989) was employed to carry out the test. Accordingly, the animals were killed by stunning on the head. The descending thoracic aorta was immediately removed, excess fat and connective tissues were trimmed off and the whole length of aorta was cut spirally resulting in a short strip (about 2 cm long) that was prepared to be used for the experiment. The strips were mounted in tissue bath containing 2.5 ml Kreb-Henseleit solution (composition in mM: 118.2 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 1.3 KH_2PO_4, 25 $NaHCO_3$ and 11.7 glucose) at 37°C and aerated with oxygen. A resting tension of 1 g was applied to the tissue and allowed to stabilize for about 1 h before application of any drug, during which period it was washed every 15 min to prevent the accumulation of metabolic products. After stabilization, the aorta was contracted by addition of KCl (bath concentration of 80 mM) to the bath. Effect of the extract was first determined on the resting baseline of the tissue to see if it had any vasoconstrictor effect. Once a contraction plateau was achieved, increasing concentrations of the decoction dissolved in water (1.25 - 40 mg/ml) were cumulatively added every 2 min and tension change of the tissue was recorded. The effect of extract on resting tension was tested with isometric sensors and traced using a

polygraph (BBC, Goez Metrawatt, Model SE 120) and percentage relaxation value was calculated by the following formula:

% Relaxation = % (Maximum contraction induced by KCl - Change in contraction)

3.2.4 Chromatographic techniques

3.2.4.1 High performance liquid chromatography (HPLC) analysis

3.2.4.1.1 Analytical HPLC

HPLC analysis of the decoction of *S. punctata* was performed using Agilent 1260 infinity series HPLC system equipped with a G-1311B quaternary pump and G1314F VW (Variable wave length) UV detector (Agilent, Germany) according to published techniques for the separation of phenolic constituents (Caponio *et al*., 1999). The column used for analytical purpose was Agilent Eclipse plus C_{18} (4.6 x 100 mm, with a particle size, 3.5 µm, USA). The mobile phase used was 0.5% acetic acid in water (A) and in methanol (B). A gradient elution was performed as follows: 10-60% B (0-18 min) and 60-10% B (in 15 min) with a solvent flow rate of 0.3 ml/min. The eluates were detected at 365 nm. The column temperature was set at 25 ^{o}C and injection volume was 10 µl.

3.2.4.1.2 Preparative HPLC

A Waters 600 controller HPLC (Waters Corporation, USA) equipped with quaternary pump was used to carry out preparative HPLC. The column used was Reprosil-Pur C_{18}-AQ (8 x 250 mm, with a particle size, 5 µm, Germany). The sample was dissolved in distilled water and loaded to the preparative column and then eluted with the solvents. The flow rate was 3 ml/min and a linear gradient from 0% to 60% B in 120 min was applied. The fractions containing the major compounds were collected and their purity was checked in the analytical HPLC. Solvent was removed in rotary evaporator (Laborota 4000, Germany) and remaining residue was subjected to spectroscopic analyses.

3.2.5 Structural elucidation of active compounds

NMR spectra were recorded on Bruker Avance DMX400 FT-NMR spectrometer instrument operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C, at room temperature using deutrated water and DMSO-d_6. A region from 0 to 20 ppm for ^{1}H and 0 to 205 ppm for ^{13}C was employed for scanning. Chemical shifts (δ) are reported in ppm units relative to an internal standard tetramethyl silane (TMS) and coupling constants (J) are given in Hz. Multiplicities of ^{1}H NMR signals are indicated as *s* (singlet), *d* (doublet), *dd* (doublet of doublets), *t* (triplet) and *m* (multiplate). FT-IR spectra were recorded on a Perkin-Elmer 65 IR spectrometer (in KBr). Ultraviolet (UV) spectra of the compounds (in methanol) were determined using a Shimadzu UV 1800 spectrometer (200-400 nm) at room temperature. The negative ion high-resolution ESI (Electron spray ionization) mass spectra were obtained from a Orbitrap Elite mass spectrometer (Thermofisher Scientific, Germany) equipped with a HESI electrospray ion source (positive spray voltage 3.5 kV; negative spray voltage 4.0 kV; capillary temperature 275 °C, source heater temperature 200 °C; FTMS resolution 30.000). Nitrogen was used as sheath gas. The collision induced dissociation (CID) mass spectra obtained in the ion trap (buffer gas: helium) were recorded with normalized collision energies of 45-65 %. The instrument was externally calibrated by the Pierce LTQ Velos ESI positive ion calibration solution (product number 88323) and Pierce ESI negative ion calibration solution (product number 88324) from Thermofisher Scientific, USA. The data were evaluated using the software Xcalibur 2.7 SP1.

3.2.6 Data analysis

Results of the study were expressed as mean ± standard error of mean (SEM). Data were analyzed using Windows SPSS Version 19 and Graph Pad-Prism version 2 (Graph Pad Software, San Diego, CA, USA). Comparison among groups and statistical significance was determined by one-way ANOVA followed by Tukey's HSD *post-hoc* multiple comparison tests for the *in vitro* study. Comparison of blood pressure parameters among groups and statistical significance was determined by one-way ANOVA and independent student t-test at a 95% confidence interval ($\alpha = 0.05$). Paired *t-test* was used to compare between initial or control blood pressure and blood pressure after treatment with the samples. The results were considered significant when $P<0.05$.

4. RESULTS AND DISCUSSION

4.1 *In vivo* blood pressure lowering activity of the decoction

4.1.1 In normotensive guinea pigs

The result of this study showed that decoction of *S. punctata* (Benth.) Briq. possesses BP lowering activity in anesthetized normotensive guinea pigs. BP was recorded invasively by direct cannulation using surgical procedures involving common carotid artery. The i.v. administration of the extract showed significant dose dependent reduction of SBP, DBP and MABP compared with vehicle (0.9% NaCl; 0.2 ml), as shown in a typical tracing of the experiment (Figure 5) and Table 1. At doses of 10, 20 and 30 mg/kg, the extract caused 13.66 ± 0.27, 34.35 ± 0.33 and 45.78 ± 0.23% (n=6) fall in MABP respectively. On the other hand, the decline in pulse pressure was significant at the dose of 30 mg/kg. In various epidemiological studies, PP has been found to be a stronger predictor for future risk of cardiovascular events such as stroke, coronary, heart disease and heart failure than SBP, DBP or MABP particularly in older individuals (Vaccarino *et.al*, 2000). Hence, the extract may help in reductions of cardiovascular events.

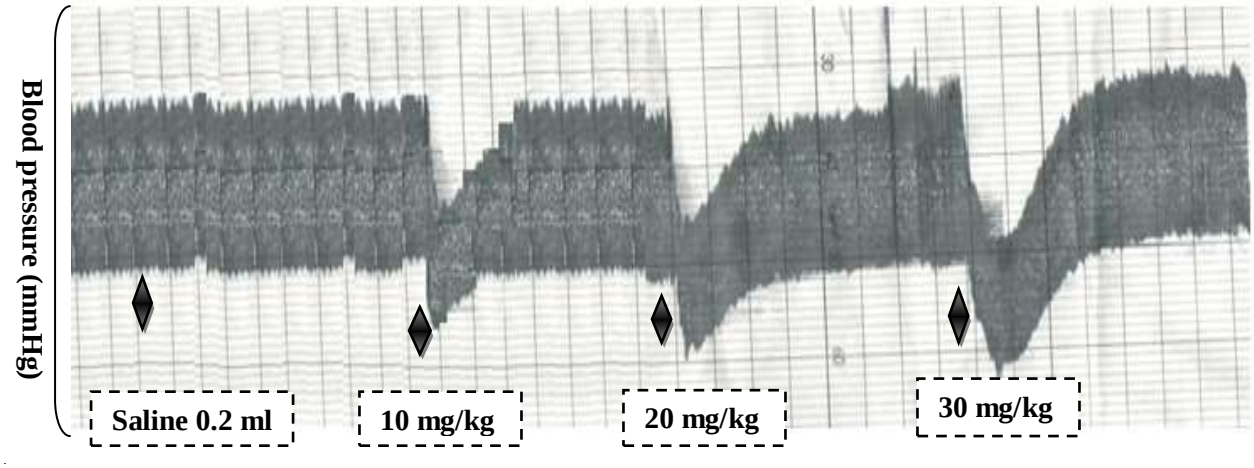


Figure 5: Tracing showing the blood pressure-lowering effect of the aerial part decoction of *Satureja punctata* in anaesthetized normotensive guinea pigs. ♦ Shows the point at which the test sample was administered.

The BP-lowering effect of the extract was not altered by pretreatment of the animals with atropine (1 mg/kg) (Figure 6). Acetylcholine (Ach) at a dose of 1 μg/kg produced a considerable drop in BP however pretreatment of animals with atropine (1 mg/kg) completely abolished its effect on BP. Unlike the effects of acetylcholine, the BP lowering action of the extract was not blocked by atropine; a competitive blocker of acetylcholine at a muscarinic receptor (Gilani *et al*., 2005b). This suggests that the BP lowering effect of the plant extract is mediated through mechanism(s) independent of muscarinic receptor activation.

Table 1: The effect of the aerial part decoction of *Satureja punctata* on blood pressure of anaesthetized normotensive guinea pigs.

Dose (mg/kg)	Parameters (mmHg)			
	SBP	DBP	MABP	PP
Control	81.93 ± 0.67	53.51± 0.47	62.98 ± 0.54	28.42 ± 0.21
10	73.22 ± 0.43*[a]	44.96 ± 0.38*[a]	54.38 ± 0.39*[a]	28.26 ± 0.12
20	58.83 ± 0.26*[a]	32.61 ± 0.27*[a]	41.35± 0.26*[a]	26.23 ± 0.07
30	49.78 ± 0.25*[a]	26.34 ± 0.31*[a]	34.15 ± 0.27*[a]	23. 44 ± 0.39*[a]

Values are presented as mean ± SEM; n = 6; *(P < 0.01); significant; a= in comparison with control. Route of administration, i.v., SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, MABP = Mean Arterial Blood pressure and PP = Pulse Pressure

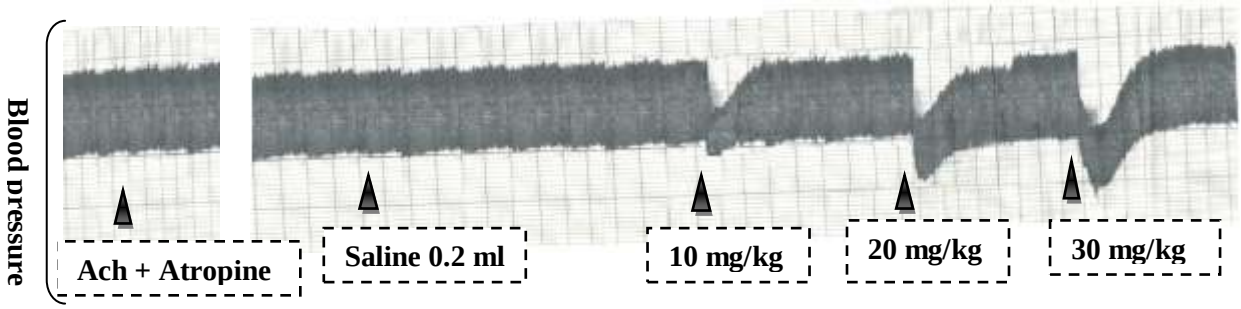


Figure 6: Tracing showing the blood pressure-lowering effect of the aerial part decoction of *Satureja punctata*, as compared to that of acetylcholine, in the presence of atropine in anaesthetized normotensive guinea pigs. ▲ Shows the point at which the test sample was administered.

4.1.2 In renovascular hypertensive guinea pigs

The anaesthetized guinea pigs showed a gradual increment in SBP, DBP and MBP after clamping of the left renal artery. Acute renal hypertension was induced by clamping the left renal artery for 4 h in anaesthetized guinea pigs. After reopening of the vessel SBP, DBP and MABP raised from the BP measurements before clamping of renal artery, as shown in Table 2. The clamping of the left renal artery reduces blood flow to the left kidney and causes kidney ischemia. Ischemia of the kidney consequently results in elevation of blood pressure by activation of the renin-angiotensin system. The ischemic kidney secrets renin, after reopening of the vessel, accumulated renin is released into circulation. Renin catalyzes the first and rate limiting step in the formation of angiotensin II. It acts on angiotensinogen to release angiotensin I, which is cleaved by angiotensin converting enzyme to yield angiotensin II. Increased formation of angiotensin II, a potent vasoconstrictor, causes elevation of BP leading to acute hypertension (Vogel and Vogel, 2002).

The results of this study showed that i.v. administration of the decoction of *S. punctata* to anaesthetized renovascular hypertensive guinea pigs caused significant fall in SBP, DBP and MABP in a dose-dependent manner compared with the pretreatment hypertensive BP (Table 2). At doses of 10, 20 and 30 mg/kg the extract caused a respective fall of 16.02 ± 0.28, 38.52 ± 0.50, 52.07 ± 0.42 % (n=6) in MABP. The extract lowered both SBP and DBP in the present study; however, DBP appeared to be affected more than SBP. This result indicates that the extract has more effect on preload than after load and the pattern is the same in normotensive animal model. Diastolic hypertension predominates before age 50, either alone or in combination with SBP elevation. DBP is a more potent cardiovascular risk factor than SBP until age 50 thus the extract could be preferentially helpful in such cases (Chobanian *et al*., 2003).

The BP lowering effect of decoction of *S. punctata* could be the result of single or combined action of different phytochemicals present in the extract. Previous phytochemical reports indicated the presence of phenolic compounds as major component in *Satureja* species (Momtaz and Abdollahi, 2010). In addition it has been described that herbs rich in phenolic phytochemicals present beneficial effect in management of cardiovascular diseases such as hypertension (Gorzalczany *et al*., 2013; Mihailovic-Stanojevic *et al*., 2013) and other chronic

oxidation linked diseases including diabetes and cancer (Ahmadvand *et al*., 2013; Sadeghi *et al*., 2013). The effect of the extract in normotensive and hypertensive animals followed similar pattern. This indicates that there was no major difference in the models used in terms of blood pressure lowering efficacy and therapeutic index of the extract.

Table 2: The effect of the aerial part decoction of *Satureja punctata* on blood pressure of anaesthetized renovascular hypertensive guinea pigs.

Treatment	Parameters (mmHg)			
	SBP	DBP	MABP	PP
Normotensive	80.46 ± 0.57	53.12 ± 0.38	62.23 ± 0.43	27.33 ± 0.34
Induction of HPN	106.88 ± 0.48[a]	74.11 ± 0.34[a]	85.04 ± 0.38[a]	32.77 ± 0.17*[a]
10 mg/kg	92.79 ± 0.59*[b]	60.75 ± 0.46*[b]	71.43 ± 0.49*[b]	32.04 ± 0.25
20 mg/kg	72.25 ± 0.47*[b]	42.29 ± 0.35*[b]	52.28 ± 0.38*[b]	29.96 ± 0.26
30 mg/kg	58.89 ± 0.54*[b]	31.7 ± 0.37*[b]	40.76 ± 0.42*[b]	27.19 ± 0.21*b

Values are presented as mean ± SEM (n = 6), *($P < 0.01$); significant; a = in comparison with normotensive control; b = in comparison with renal hypertensive control. Route of administration, i.v., SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, MABP = Mean Arterial Blood pressure and PP = Pulse Pressure

4.2 *In vitro* vasorelaxant activity of decoction

The present study also demonstrated that decoction of *S. punctata* had a vasorelaxant effect on a KCl precontracted, isolated guinea pig aorta. The extract caused a dose-dependent relaxation as shown in Figure 7 and Table 3. The extract did not exhibit any vasoconstrictor activity on the resting baseline of guinea pig aorta. The results (Table 3) are expressed as the percentage contraction, taking the control KCl-induced contraction before the application of the test extract as 100%, and no significant effect was observed at a concentration of 1.25 mg/ml. However significant dose dependent relaxation was seen at concentrations ranging from 2.5 to 40.0 mg/ml ($p < 0.01$). Potassium induces vascular smooth muscle contraction through activation of voltage dependent calcium channels (Soncini *et al.*, 2011). The ability of the extract to cause relaxation of the potassium-induced contraction on aorta strips could be associated with blockade of calcium influx. On the other hand, the vascular endothelium plays an essential role in the control

of smooth muscle relaxation *via* synthesis or release of vasodilator substances such as nitric oxide (NO) and prostacyclin (PGI_2) (Stankevicius *et al*., 2003). Since the evaluation was performed in endothelium intact aorta strips the mechanism of action of the extract may in part involve endothelium derived vasorelaxing factors, however these effect of the extract needs to be demonstrated in endothelium denuded preparation and in the presence of nitric oxide synthase inhibitors such as *N*-nitro-L-arginine methyl ester (L-NAME) and PGI_2 production inhibitors.

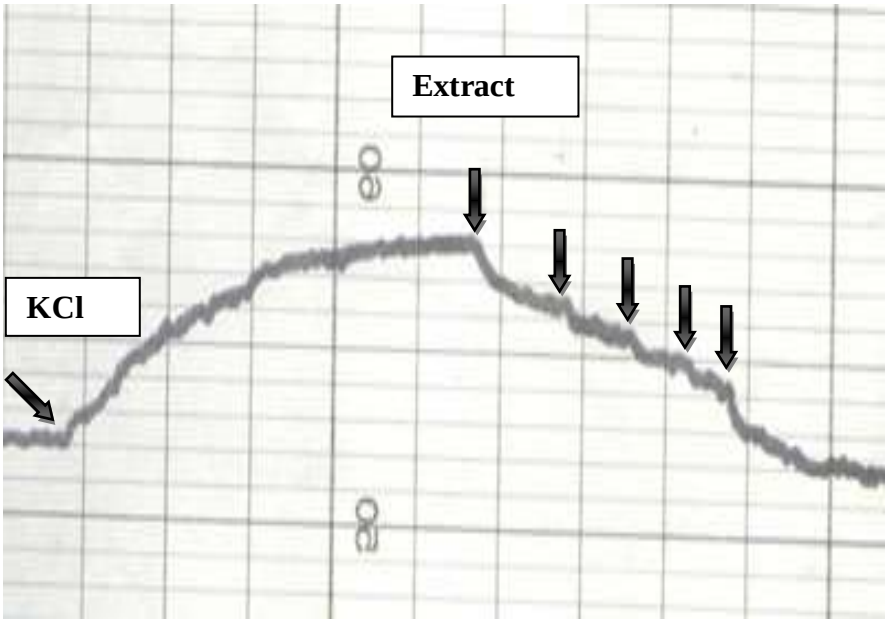


Figure 7: Tracing of relaxation of precontracted aorta caused by cumulative addition of the aerial part decoction of *Satureja punctata*

Vasorelaxant activity of the decoction of *S. punctata* could be associated with the presence of phenolic compounds such as phenolic acids and flavonoids, which have been implicated in vasodilator activities of several plants (Rosalia *et al*., 2010; Soncini *et al.*, 2011; Gorzalczany *et al*., 2013). Another *Satureja* sp., namely, *S. obovata,* has been previously reported to exhibit significant vasorelaxant activity in endothelium-intact aorta pre-contracted by KCl (Sanchez de Rojas *et al*., 1996b).

Table 3: Vasorelaxant effect of the aerial part decoction of *Satureja punctata* on guinea-pig thoracic aorta precontracted with 80 mM KCl

Concentration (mg/ml)	% Contraction induced by KCl	% Relaxation of KCl precontracted aorta
0.0	100 ± 0.0	0.00 ± 0.00
1.25	97.85 ± 0.13	2.15 ± 0.13
2.5	93.02 ± 0.38	6.98 ± 0.38*
5	71.13 ± 0.71	28.87 ± 0.71*
10	37.15 ± 0.58	62.85 ± 0.58*
20	23.93 ± 0.51	76.23 ± 0.51*
30	12.73 ± 0.13	87.27 ± 0.13*
40	1.81 ± 0.15	98.19 ± 0.15*

Values are presented as mean ± SEM (n = 6), *($P < 0.01$) significant

Interestingly, the results from *in vitro* evaluation of vasorelaxant activity strongly supported the *in vivo* BP reduction effect of the extract. From these results it could be suggested that the BP lowering effect of the extract involves relaxation of the vascular system. The decoction of *S. punctata* was able to exhibit significant dose dependent relaxant effect on isolated guinea pig thoracic aorta contracted with KCl, and a dose-dependent arterial BP lowering in anaesthetized normotensive and renovascular hypertensive guinea pigs, representing the first attempt to describe the pharmacological evidences of vasodilatory and antihypertensive effect of this plant.

4.3 Isolation of compounds

The decoction of the aerial parts of *S. punctata* gave brown powder with a calculated yield of 13.45% w/w of dried plant material. Analysis of the decoction of *S. punctata* by analytical and preparative RP C_{18} column HPLC in solvent system 0.5% acetic acid in water (A) and in methanol (B) led to the isolation of two compounds. The compounds were coded as SP-1(detected at Rt = 7.2 min) and SP-2 (detected at Rt = 12.3 min). The purity of these compounds

was checked by analytical HPLC. Figure 8 depicts the chromatogram of aerial part decoction of *Satureja punctata* using the mobile phase 0.5% acetic acid in water (A) and in methanol (B) with gradient elution 10-60% B (0-18 min), detected at 365 nm.

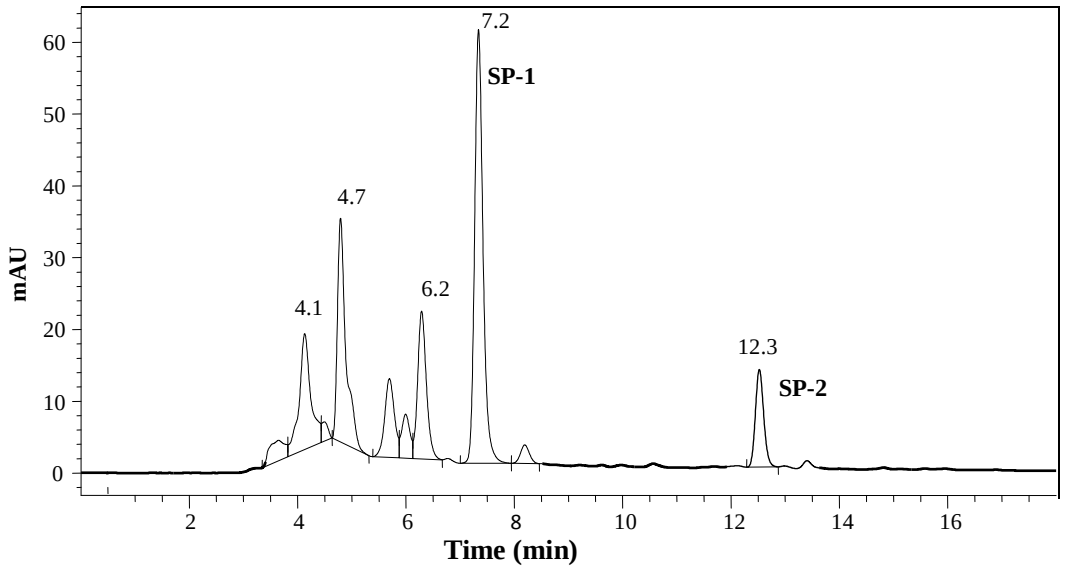


Figure 8: HPLC chromatogram of aerial part decoction of *Satureja punctata*, using solvent system 0.5% acetic acid in water (A) and in methanol (B) with gradient elution of 10-60% B (0-18 min), detected at 365 nm.

4.4 Structural elucidation of isolated compounds

4.4.1 SP-1

SP-1 was isolated as a yellowish red amorphous powder. The UV spectrum of SP-1 exhibited absorption maxima at 330 nm and a shoulder at 290 nm (Appendix I). This could be due to a phenolic acid with two aromatic rings (Wettasinghe *et al*., 2001). The IR spectrum showed that the compound has OH groups (3413 cm^{-1}), sp^3 carbons (2930), carbonyl groups (around 1699 cm^{-1}) and aromatic rings (1605, 1518, 1446 cm^{-1}). The band due to stretching of carboxylic acid O-H (3300-2500) is very broad and it overlapped with the band of stretching of free OH groups (3650-32003 cm^{-1}). In addition the peak due to carbonyl group seems boarder and this could be due to the presence of two carbonyl groups (acidic and esteric) (Appendix I).

The negative-ion ESI mass spectrum of SP-1 showed pseudomolecular ion at *m/z* 359.0757 ([M-H]$^-$), indicating a relative molecular weight (M_r) of 360 (Figure 9). The molecular formula of SP-1 was determined to be $C_{18}H_{16}O_8$, using the MS data along with UV, IR, ^{1}H, ^{13}C NMR and DEPT spectral data.

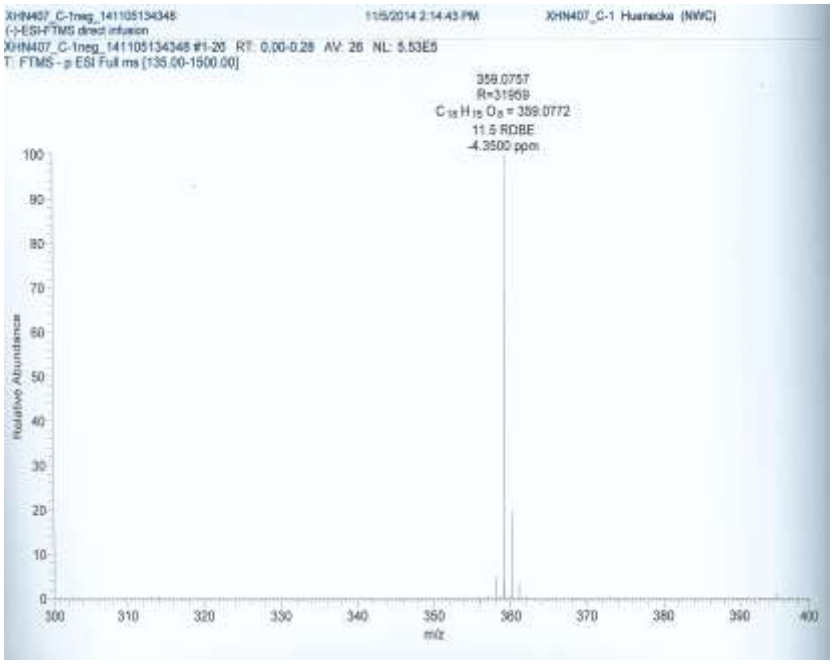
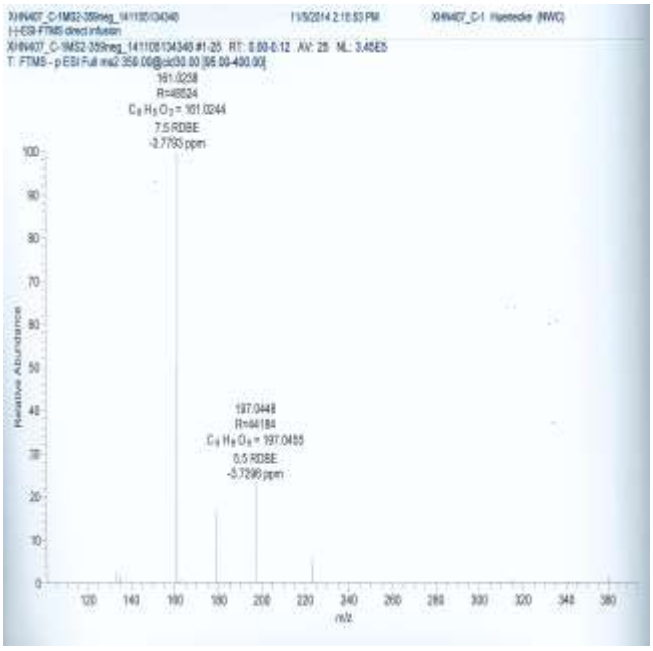
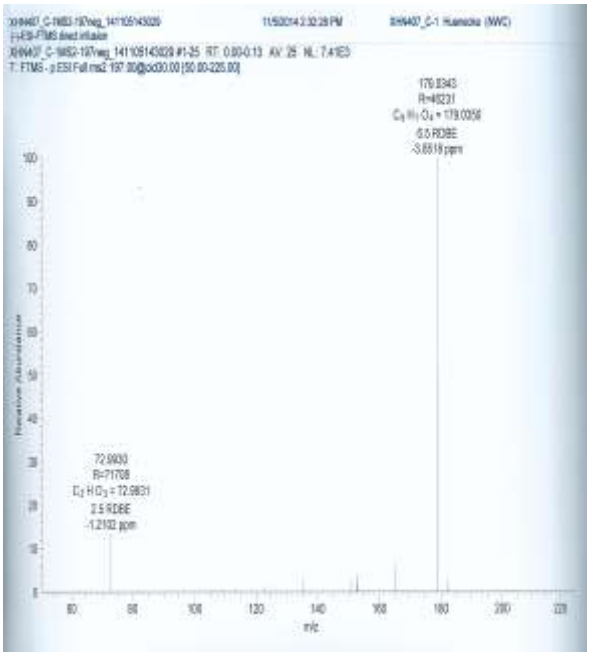
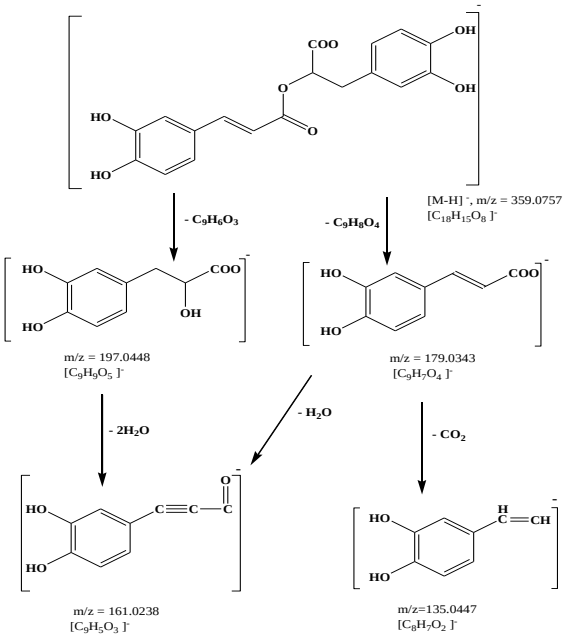


Figure 9: MS Spectrum of SP-1

The [M-H]$^-$ ion was selected for collision induced dissociation (CID) to produce MS/MS spectra. The prominent MS/MS ion was then selected for further MS^3 analysis. The fragmentation behavior of the [M-H]$^-$ ion is characterized by losses of $C_9H_6O_3$, $C_9H_8O_4$ and two H_2O followed by a CO_2 unit (Figure 10). Fragment ions at *m/z* 197.0448, 179.0343, 161.0238 and 135.0447 were detected. The fragment ion at m/z 179.0343 shows the presence of a caffeic acid moiety. The *m/z* at 197.0448 indicates a 3,4 dihydroxyphenyl lactic acid group. The dehydrated ion fragments of these two molecular parts constituents at *m/z* 161.0338 were observed in MS^2 spectrum. Further fragmentation was characterized by loss of CO_2 unit, *m/z* 135.0447, as observed in MS^3 spectrum.







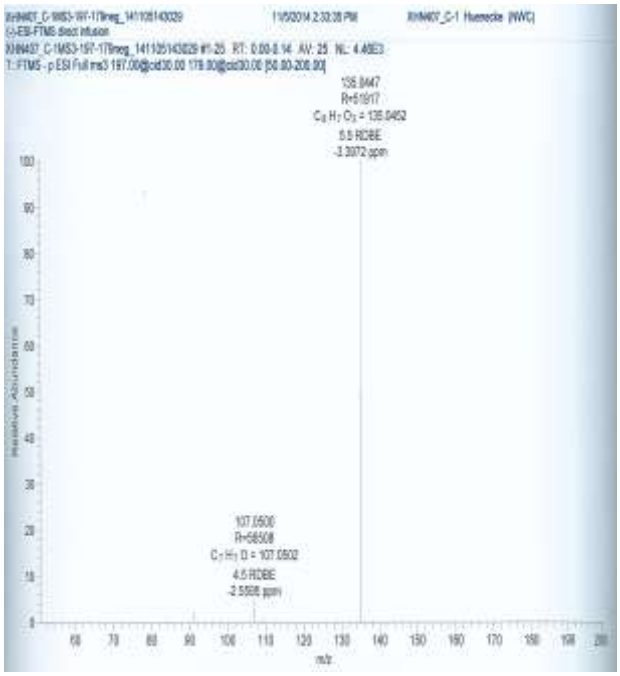


Figure 10: MS fragmentation pattern of SP-1

The ^{1}H NMR spectrum of SP-1 (Appendix I) showed the presence of two doublets at δ 7.29 and δ 6.05 which were assigned to a pair of trans-olefinic protons on the bases of the observed large proton-proton coupling (*J* = 16). In addition, there were six protons observed in aromatic region, which were assignable to two discrete sets of protons of the 3, 4-dihydroxy phenyl units. The assignments were established based on their splitting patterns and *J*-coupling constants, i.e, H-2 (δ 6.88, *d*, *J* = 1.6 Hz), H-6 (δ 6.78, *dd*, *J* = 8.4, 1.6 Hz), H-5' (δ 6.69, *d* , *J* = 8.0 Hz), H-2' (δ 6.69, *d*, *J* = 1.6 Hz), H-5 (δ 6.68, *d*, *J* = 8.4 Hz), H-6' (δ 6.58, *dd*, *J* = 8.0, 1.6 Hz). Further three sets of multiplets at δ 2.91(*dd*, *J* = 14.4, 7.2 Hz), δ 2.96 (*dd*, *J* = 14.4, 4.8 Hz), and δ 5.02 (*dd*, *J* = 7.2, 4.8 Hz) were consistent with the presence of –CH-O-CH_2- unit based on their coupling pattern. The complete assignments of the ^{1}H chemical shifts of SP-1 are listed in Table 4.

^{13}C NMR spectrum along with DEPT experiment revealed the presence of 18 carbon atoms (Appendix I), which showed one CH_2 carbon, nine CH carbons and eight quaternary carbons. The presence of two carbonyl signals was evident from the ^{13}C NMR data, of which one was identified as carboxylic acid carbonyl (δ 174.35) and the other corresponds to ester carbonyl (δ 168.48).

The appearance of twelve aromatic carbons consisting of six tertiary carbons (δ 122.80, 121.89, 117.28, 116.14, 116.09 and 115.18) and six quaternary carbons, of which four were phenoxyl carbons (δ 147.18, 144.16, 143.79 and 142.90) and the remaining two were non hydroxylated carbons (δ 128.73 and 126.74) confirmed the presence of two sets of 3, 4-dihydroxyphenyl groups. In addition, the presence of two olefinic tertiary carbons (δ 146.84 and 113.48), one oxygenated methine carbon (δ 73.96) and one methylene carbon (δ 36.09) were supported by the ^{13}C NMR, DEPT and HMQC spectra.

The ^{1}H-NMR and ^{13}C-NMR spectra (Table 4) assigned with the aid of DEPT, ^{1}H-^{1}H shift COSY, HMQC and HMBC experiment (Appendix I), revealed the presence of a caffeoyl group and a 3,4 dihydroxyphenyl lactic acid moiety in SP-1.

By combining the information from all spectral data and by comparing data reported in the literature (Lu and Foo, 1999; Satake *et al*., 1999), the structure of SP-1 was determined to be rosmarinic acid (α-O-caffeoyl-3, 4-dihydroxyphenyl lactic acid) (Figure 11).

Table 4: Comparison of the ^{1}H and ^{13}C NMR spectral data of SP-1 and 'rosmarinic acid'

Assignment	^{1}H NMR (ppm)		^{1}H-^{1}H COSY	^{13}C NMR (ppm)		HMBC
	Rosmarinic acid[a]	SP-1*	SP-1*	Rosmarinic acid[a]	SP-1*	SP-1*
1	-	-	-	125.66	126.74	-
2	7.06 (*d*, 2.0)	6.88 (*d*, 1.6)	H-6	115.18	115.18	C-6, 7
3- OH	-	-	-	145.18	144.16	-
4- OH	-	-	-	148.81	147.18	-
5	6.77 (*d*, 8.2)	6.68 (*d*, 8.4)	H-6	116.13	116.09	C-1, 3, 4
6	7.01 (*dd*, 8.2, 2.0)	6.78 (*dd*, 8.4, 1.6)	H-5, H-2	121.49	122.80	C-2, 5, 7
7	7.46 (*d*, 15.9)	7.29 (*d*, 16)	H-8	145.35	146.84	C-2, 6, 8, 9
8	6.24 (*d*, 15.9)	6.05 (d, 16)	H-7	116.13	113.48	C-1, 9
9	-	-	-	166.29	168.48	-
1'	-	-	-	128.71	128.73	-
2'	6.68 (*d*, 2.0)	6.69 (*d*, 1.6)	H-6'	116.90	117.28	C-4', 6', 7'
3'-OH	-	-	-	144.13	143.79	-
4'-OH	-	-	-	144.00	142.90	-
5'	6.64 (*d*, 8.0)	6.69 (*d*, 8.0)	H-6'	115.63	116.14	C-1', 3'
6'	6.52 (*dd*, 8.0, 2.0)	6.58 (*dd*, 8.0, 1.6)	H-2', H-5'	120.02	121.89	C-2', 4', 7'
7' H-a	2.89 (*dd*, 14.3, 8.6)	2.91 (*dd*, 14.4, 7.2)	H-8', H-7b	36.85	36.09	C-2', 6', 8'
H-b	2.99 (*dd*, 14.3, 4.1)	2.96 (*dd*, 14.4, 4.8)	H-8', H-7a	-	-	C-6', 8'
8'	5.02 (*dd*, 8.6, 4.1)	5.02 (*dd*, 7.2, 4.8)	H-7'	75.54	73.96	C-7', 9'
9'	-	-		172.05	174.35	-

[a]Measured in DMSO-d_6 , Satake *et al*. (1999) ; *Measured in D_2O

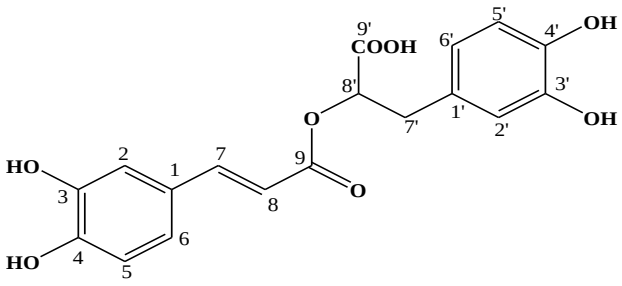


Figure 11: The structural formula of SP-1 (Rosmarinic acid)

4.4.2 SP-2

SP-2 was obtained as a yellow amorphous solid and showed absorption λ max at 283 and 319 nm, which is characteristic for a substituted flavone skeleton (Mabry *et al*., 1970). The IR spectrum showed that the compound has OH group (3432 cm^{-1}), sp^3 carbons (2926), a carbonyl group (1651cm^{-1}) and aromatic rings (1611, 1513, 1446 cm^{-1}) (Appendix II).

The identity of SP-2 was further confirmed by a series of 1D and 2D NMR experments, including ^1H and ^{13}C NMR, DEPT, ^1H-^1H COSY, HMQC and HMBC. The ^1H-NMR spectrum (Appendix II) of SP-2 showed one hydroxyl group at δ 12.92 (*s*) assigned to OH at position-5, which suggested the presence of a carbonyl group adjacent to the hydroxyl group. In addition the ^1H NMR spectrum (Table 5) displayed signals of *ortho*-coupled protons at δ = 8.06 and 7.16 (each 2H, *d*, *J* = 8.8 Hz) assignable to H-2', 6' and H-3', 5', respectively, and *meta*-coupled protons at δ = 6.80 and 6.46 (each 1H, *d*, *J* = 2.4 Hz) assigned to H-8 and H-6, respectively. The spectrum further revealed the presence of a methoxyl group at δ = 3.87, an isolated proton at δ = 6.96 (1H, *s*) assigned to H-3, and two signals assignable to anomeric sugar protons at δ = 5.07 (*d*, *J* = 7.2) for glucose and at δ = 4.55 (*brs*) for rhamnose proton. The remaining sugar protons were displayed at δ 3.75 - 3.29. In addition protons of CH_3 for rhamnose moiety appeared at δ 1.08 (*d*, *J* = 6).

The ^{13}C NMR spectrum of SP-2 (Appendix II) displayed a total of 28 carbon atoms which along with DEPT experiment, revealed the presence of one CH_2 carbon, seventeen CH carbons, eight

non-protonated carbons and one methoxyl carbon and one methyl carbons. The existence of seven methines of the flavone skeleton (δ 94.78, 100.5, 103.80, 114.709 (2C) and 128.45 (2C)) and one methoxyl carbon at δ 55.55 were supported by the ^{13}C NMR, DEPT and HMQC spectra. The presence of two sugar units was confirmed by the existence of 12 carbons in the ^{13}C NMR and DEPT spectra, of which two were anomeric carbon signals at δ = 99.95 and 99.66 for rhamnose and glucose unit respectively, eight methine carbon signals (four signals at δ 70.74, 73.06, 75.66, 76.25 were accounted for O-glucose moiety and four signals at δ 68.3, 69.6, 70.34 and 72.06 belong to *O*-rahmnose moiety), one oxymethylene carbon at δ = 66.08 for glucose and one methyl carbon at δ =17.77 for rhamnose were supported by ^{13}C NMR, DEPT and HMQC spectra. Eight quaternary carbons were identified in SP-2 from ^{13}C-NMR and DEPT-135 spectra, including one signal due to carbonyl carbon (δ = 182.01; ketone carbonyl). The complete assignments of all carbon atoms are listed in Table 5.

In the HMBC spectrum of SP-2 a long range coupling between the anomeric H-1''' at δ = 4.55 and C-6" at δ = 66.08 demonstrated that the rhamnose moiety was connected to C-6" of the glucose unit which, in turn, was linked to the acacetin moiety. It was also proved by the HMBC spectrum that the anomeric proton H-1" at δ = 5.07 of glucose exhibited correlation with C-7 at δ 162.95 of the aglycone unit indicating the site of glucosylation at position-7. Further the coupling constant (*J* = 7.2) of the anomeric proton of glucose (H-1" at δ = 5.07) indicated β-configuration of the glucose unit (Markham and Mabry, 1975). The position of the methoxyl group was assigned to C-4' on the basis of HMBC correlations between C-4' at δ = 162.42 and the proton of the methoxyl group at δ = 3.87. Although the signals of some of the sugar protons overlapped in the ^{1}H NMR spectrum, the complete assignment of all protons and carbon resonances was achieved with the help of ^{1}H- ^{1}H COSY, HMQC and HMBC experiments (Appendix II).

Thus by combining the information from the spectral data and by comparing with data reported in the literature (Ina and Lida 1981; Nazauk and Gudej, 2003) the structure of SP-2 was determined to be acacetin-7-O-β-D - rutinoside (linarin) (Figure 12).

Table 5: Comparison of the ^{1}H and ^{13}C NMR spectral data of SP-2 and 'Linarin'

Assignment	^{1}H NMR (ppm)		^{1}H-^{1}H COSY	^{13}C NMR (ppm)		HMBC
	Linarin*	SP-2	SP-2	Linarin*	SP-2	SP-2
1	-	-	-	-	-	-
2	-	-	-	162.94	163.96	-
3	6.98 (*s*)	6.96 (*s*)	-	103.80	103.80	C-2, 4, 10, 1'
4	-	-	-	182.10	182.01	-
5-OH	12.90 (*s*)	12.92 (*s*)	-	161.13	161.13	C-5, 6, 10
6	6.45 (*d*, 2.1)	6.46 (*d*, 2.4)	H-8	100.51	100.50	C-5, 7, 8, 10
7	-	-	-	163.93	162.95	-
8	6.79 (*d*, 2.1)	6.80 (*d*, 2.4)	H-6	94.77	94.78	C-6, 7, 9, 10
9	-	-	-	156.96	156.97	-
10	-	-	-	105.45	105.46	-
1'	-	-	-	122.66	122.67	-
2'	8.05 (*d*, 8.9)	8.06 (*d*, 8.8)	H-3'/H-5'	128.45	128.45	C-1', 3'/5', 4'
3'	7.15 (*d*, 8.9)	7.16 (*d*, 8.8)	H-2'/H-6'	144.69	114.70	C-1', 2'/6', 4'
4'-OCH_3	-	-	-	162.42	162.43	-
5'	7.16 (*d*, 2.4)	7.16 (*d*, 2.4)	H-2'/H-6'	144.69	114.70	C-1', 2'/6', 4'
6'	8.06 (*d*, 8.8)	8.06 (*d*, 8.8)	H-3'/H-5'	128.45	128.45	C-1', 3'/5', 4'
O-CH_3	3.86 (*s*)	3.87 (*s*)	-	55.56	55.55	C-4'
1"	5.06 (*d*, 7.1)	5.07 (*d*, 7.2)	H-2"	99.92	99.66	C-7'
2"	-	3.44b	-	73.06	73.06	-
3"	-	3.42b	-	76.24	76.25	-
4"	-	3.35b	-	69.58	70.74	-
5"	-	3.67b	-	76.24	75.66	-
6"	-	3.41b	-	66.08	66.08	-
1'"	4.55(*s*)	4.55(*brs*)	-	99.92	99.95	C-6", C-2'"
2'"	-	3.74b	-	70.73	70.34	-
3'"	-	3.29b	-	70.33	69.60	-
4'"	-	3.75b	-	72.04	72.06	-
5'"	-	3.40b	-	68.31	68.3	-
6'"	1.08 (*d*, 6.1)	1.08 (*d*, 6.0)	H-5'"	17.79	17.77	C-4'", C-5'"

* Nazauk and Gudej, 2003, b signal patterns are unclear due to overlap

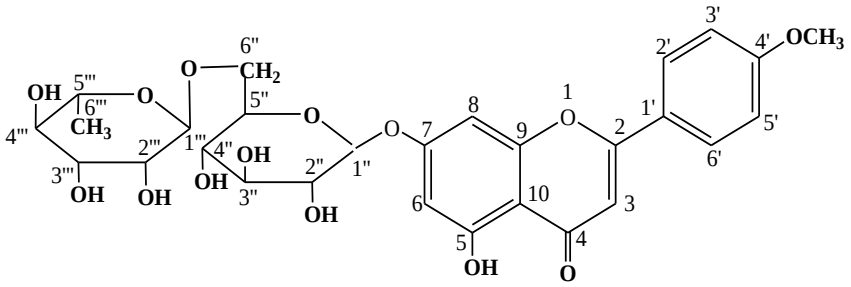


Figure 12: Structure of SP-2 (Linarin)

4.5 Blood pressure lowering activity of isolated compound

4.5.1 Rosmarinic acid

The i.v. administration of rosmarinic acid induced significant dose-dependent fall in SBP, DBP and MABP in anesthetized normotensive guinea pigs as depicted in Table 6 and Figure 13, tracing from a typical experiment.

At doses of 0.75, 1.5 and 3 mg/kg the compound caused 8.14 ± 0.27, 15.79 ± 0.33 and 31.78 ± 0.64% (n = 6) fall in MABP respectively. The result of the study showed that the isolated compound has also more effect on DBP than SBP like the decoction. The effect was observed at relatively low doses. A single dose of rosmarinic acid, as low as 0.75 mg/kg, caused fall in the MABP of normotensive guinea pigs in a range of 5 mm Hg. It has been estimated that, in humans suffering from hypertension, a reduction of 5 mm Hg in MABP can decrease mortality due to stroke and coronary events by 14% and 9% respectively (Chobanian *et al*., 2003), reinforcing the importance of this findings.

The result of this study demonstrated that the BP reducing effect of decoction of *S. punctata* could be associated with presence of rosmarinic acid and linarin. Linarin (SP-2) has been reported to have anti-hypertension effect on spontaneously hypertensive rats (Lv *et al*., 2013).Thus the antihypertensive effect of the decoction could be due to combined effect of rosmarinic acid and linarin. In line with the present result, the hot water extract of *Thymus serpyllum* L. (Lamiaceae) which contains high level of rosmarinic and caffeic acids and trace amounts of quercetin and luteolin has been reported to induce a significant decrease of SBP and DBP in spontaneously hypertensive rats (Mihailovic-Stanojevic *et al*., 2013).

Table 6: The effect of rosmarinic acid on blood pressure of anaesthetized normotensive guinea pigs.

Dose (mg/kg)	Parameters (mmHg)			
	SBP	DBP	MABP	PP
Control	82.58 ± 0.40	54.36 ± 0.29	63.77 ± 0.31	28.22 ± 0.24
0.75	77.12 ± 0.34*[a]	49.20 ± 0.24*[a]	58.58 ± 28*[a]	28.07 ± 0.25
1.5	70.40 ± 0.52*[a]	45.35 ± 0.28*[a]	53.70 ± 0.36*[a]	25.05 ± 0.12
3	59.74 ± 0.45*[a]	35.43 ± 0.42*[a]	43.54 ± 0.23*[a]	24. 31 ± 0.11*[a]

Values are presented as mean ± SEM; n = 6; *(P < 0.01); significant; a= in comparison with control. Route of administration, i.v., SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, MABP = Mean Arterial Blood pressure and PP = Pulse Pressure

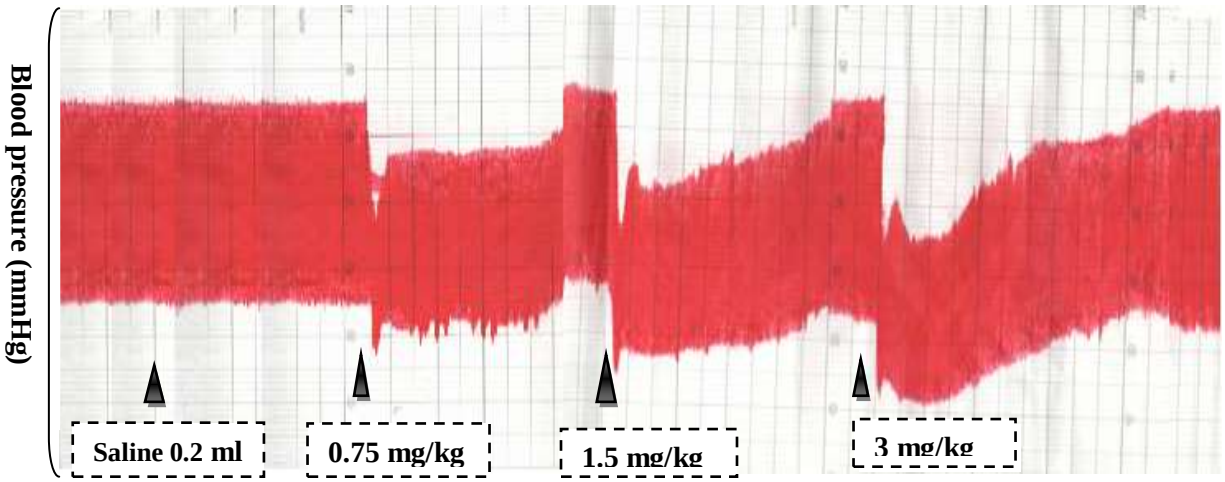


Figure 13: Tracing showing the blood pressure lowering effect of rosmarinic acid in anaesthetized normotensive guinea pigs. ▲ Shows the point at which the test solution was administered.

Furthermore, Li *et al*. (2008) reported that rosmarnic acid isolated from the whole plant extract of *Rabdosia coetsa* showed *in vitro* angiotensin-converting enzyme (ACE) inhibitory activity

and this could further support the *in vivo* BP lowering activity of rosmarinic acid observed in the present study. The significant BP lowering effect of the decoction demonstrated in renovascular hypertensive guinea pigs under section 4.1.2, which could be associated with the presence of high level of rosmarinic acid in the extract, may be linked with the ACE inhibitory activity of rosmarinic acid since hypertension was induced *via* activation of the renin-angiotensin system. Therefore, together with the *in vitro* vasodilatory activity showed by the extract, it could be suggested that the BP lowering effect of rosmarinic acid may partly be explained by its effect on the renin-angiotensin system. However, in addition to the suggested mechanisms of action, mode of action of the extract and the isolated compounds needs to be elucidated since various mechanisms of action have been hypothesized for BP lowering activity of natural products.

Rosmarinic acid is a caffeic acid ester that is common in Lamiaceae plants. The biosynthesis of rosmarinic acid starts with the amino acids L-phenylalanine and L-tyrosine. Rosmarinic acid, first isolated from rosemary (*Rosmarinus officinalis*; Lamiaceae), is found in a number of medicinal plants (Petersen *et al*., 2009). It occurs in plants that are thought to have health benefits and it has been reported to possess various pharmacological activities including antioxidant (Del Bano *et al*., 2003; Hossain *et al*., 2009), anti-inflammatory (Swarup *et al*., 2007), inhibition of oxidation of low density lipoprotein (Ahmadvand *et al*., 2012), antiangiogenic (Furtado *et al*., 2010), antitumor (Osakabe *et al*., 2004), antimicrobial (Bernardes *et al*., 2010) and HIV-1-inhibiting properties, among others (Dubois *et al*., 2008). Oxidative stress is known to be one of the factors in cardiovascular problems such as hypertension, thus the above listed pharmacological activities could likely support the BP lowering effect of rosmarinic acid observed in the present study.

5. CONCLUSION AND RECOMMENDATIONS

In the present study, phytochemical analysis of decoction of *S. punctata* and evaluation of the antihypertensive activity of the decoction and an isolated compound were carried out. Two phenolic compounds were isolated from the active extract and identified as rosmarinic acid and linarin. The extract showed significant dose dependent *in vitro* vasorelaxant effect and *in vivo* BP lowering activity in normotensive and renovascular hypertensive anaesthetized guinea pigs. Therefore, it seems that the extract is useful in hypertension associated with renal impairment cases at least in guinea pigs. A single dose of the extract, as low as 10 mg/kg, reduced the MABP of renovascular guinea pigs by about 14 mm Hg. It can therefore be concluded that the antihypertensive activity of the plant is in part due to the presence of rosmarinic acid and linarin. Hence, findings of this study support the traditional use of the aerial parts of this plant for the treatment of hypertension.

Based on the findings the following recommendations are proposed

- As the present study was only focused on *in vitro* vasorelaxant effect in isolated aorta and BP lowering effect in anesthetized normotensive and renovascular hypertensive animal models, investigation of the decoction and the isolated compounds on other antihypertensive models should be necessary.
- Isolation of other minor compounds and evaluation of their antihypertensive activities could be necessary.
- Structure of SP-2 should be further confirmed using MS, ^{1}H NMR and ^{13}C NMR
- Toxicological studies including acute, sub-acute and chronic toxicity studies are mandatory before considering the plant for further studies.

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7. APPENDIX

Appendix I: UV, IR and NMR spectrum of Rosmarinic acid (SP-1)

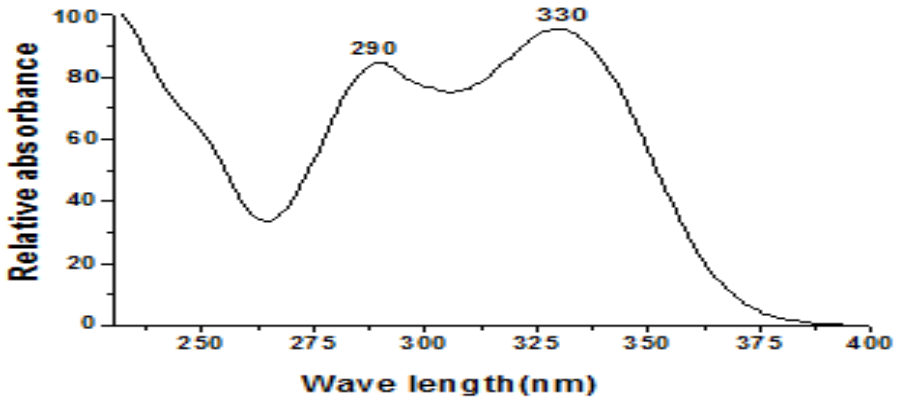


Figure A: UV spectrum of SP-1

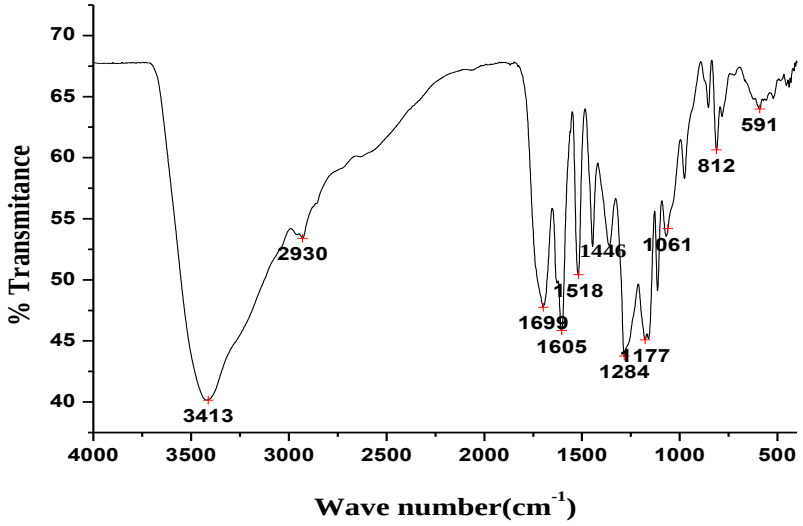


Figure B: IR spectrum of SP-1

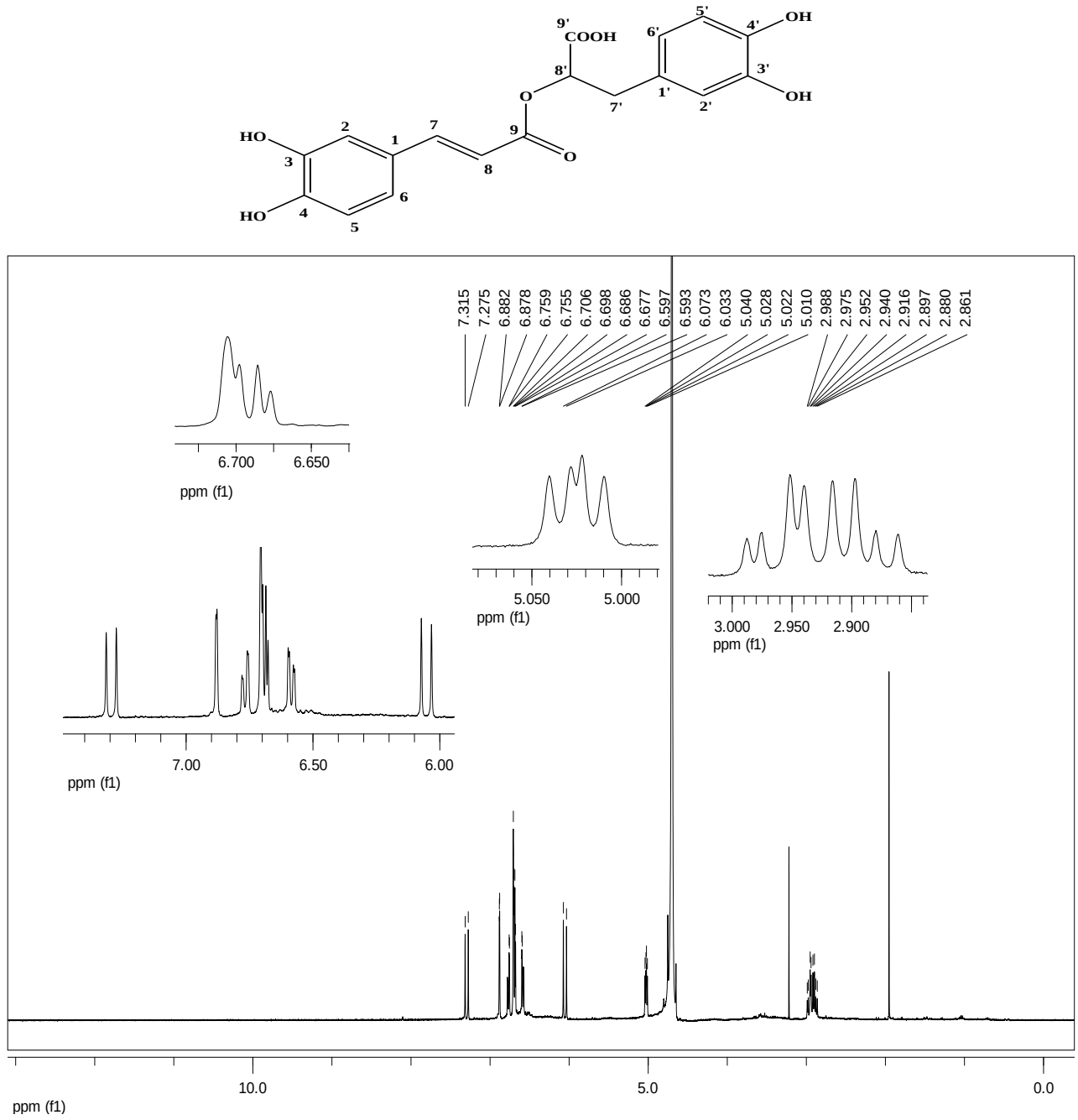


Figure C: ^{1}H NMR spectrum of SP-1 in D_2O

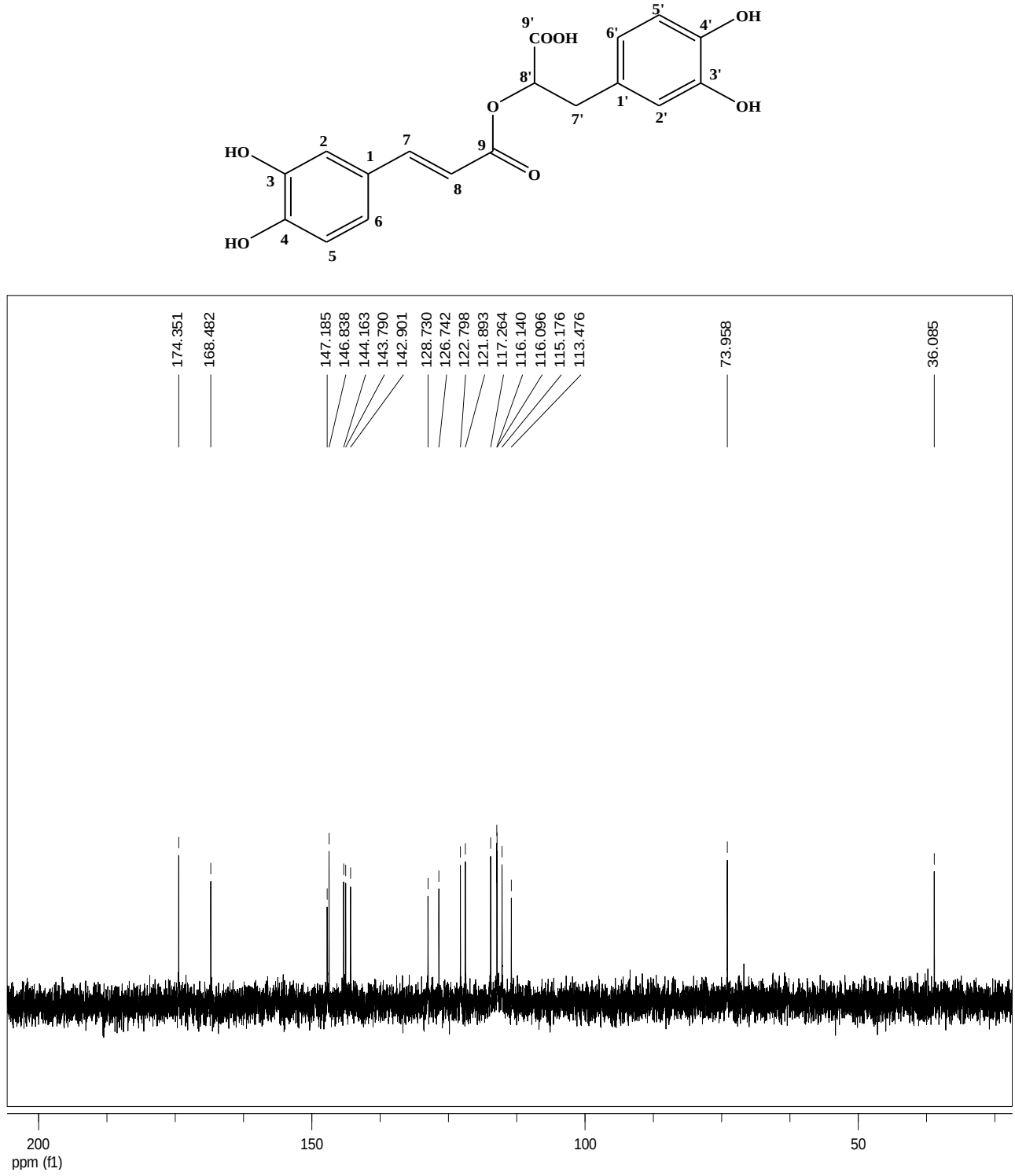
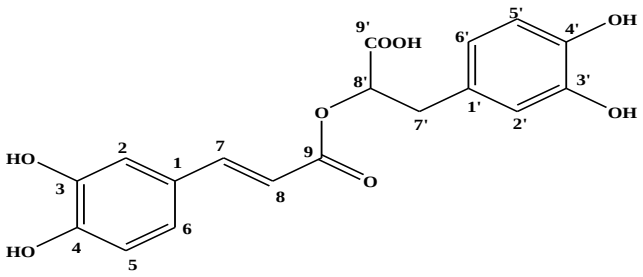


Figure D: ^{13}C NMR spectrum of SP-1 in D_2O



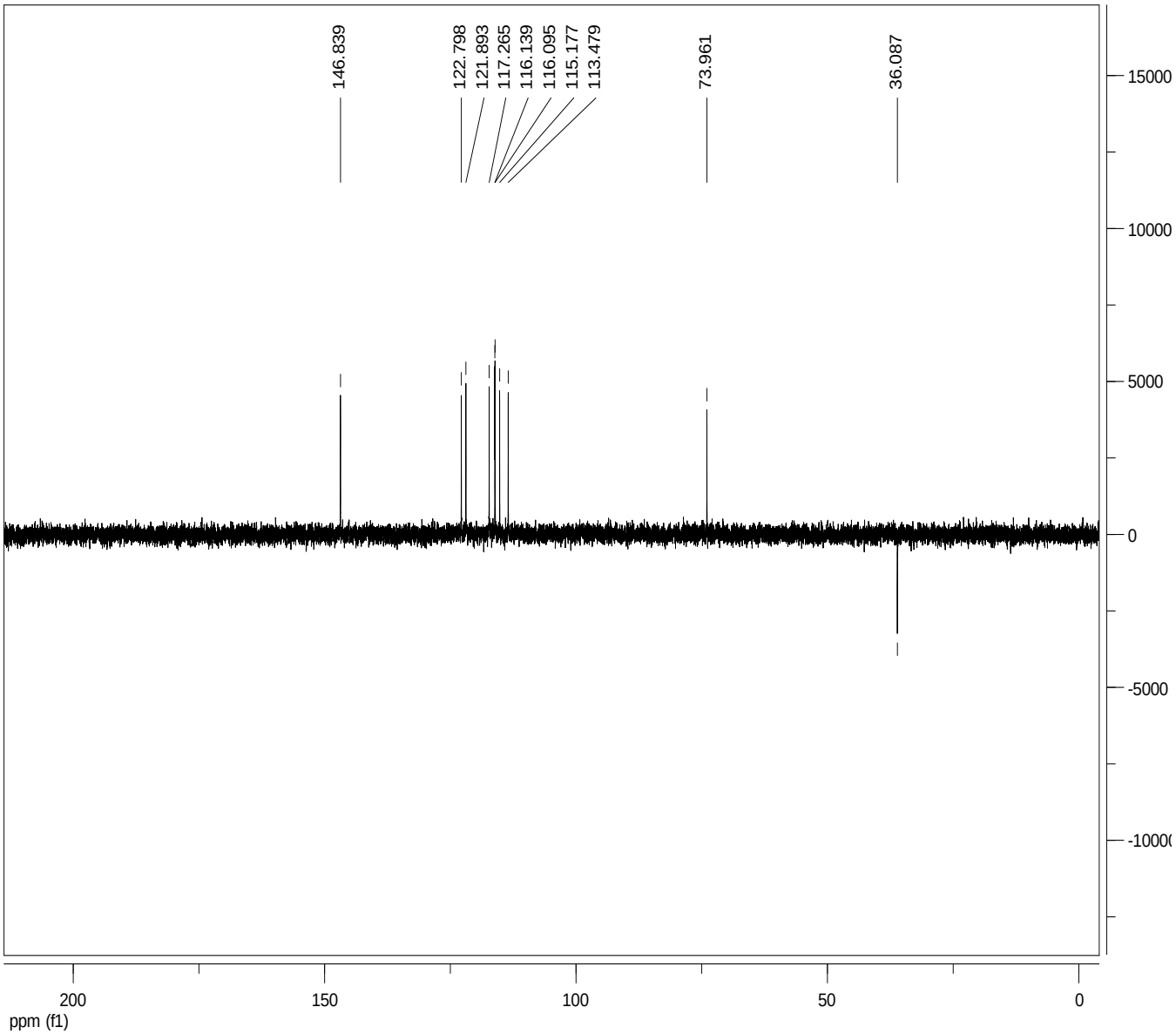


Figure E: DEPT-135 spectrum of SP-1 in D_2O

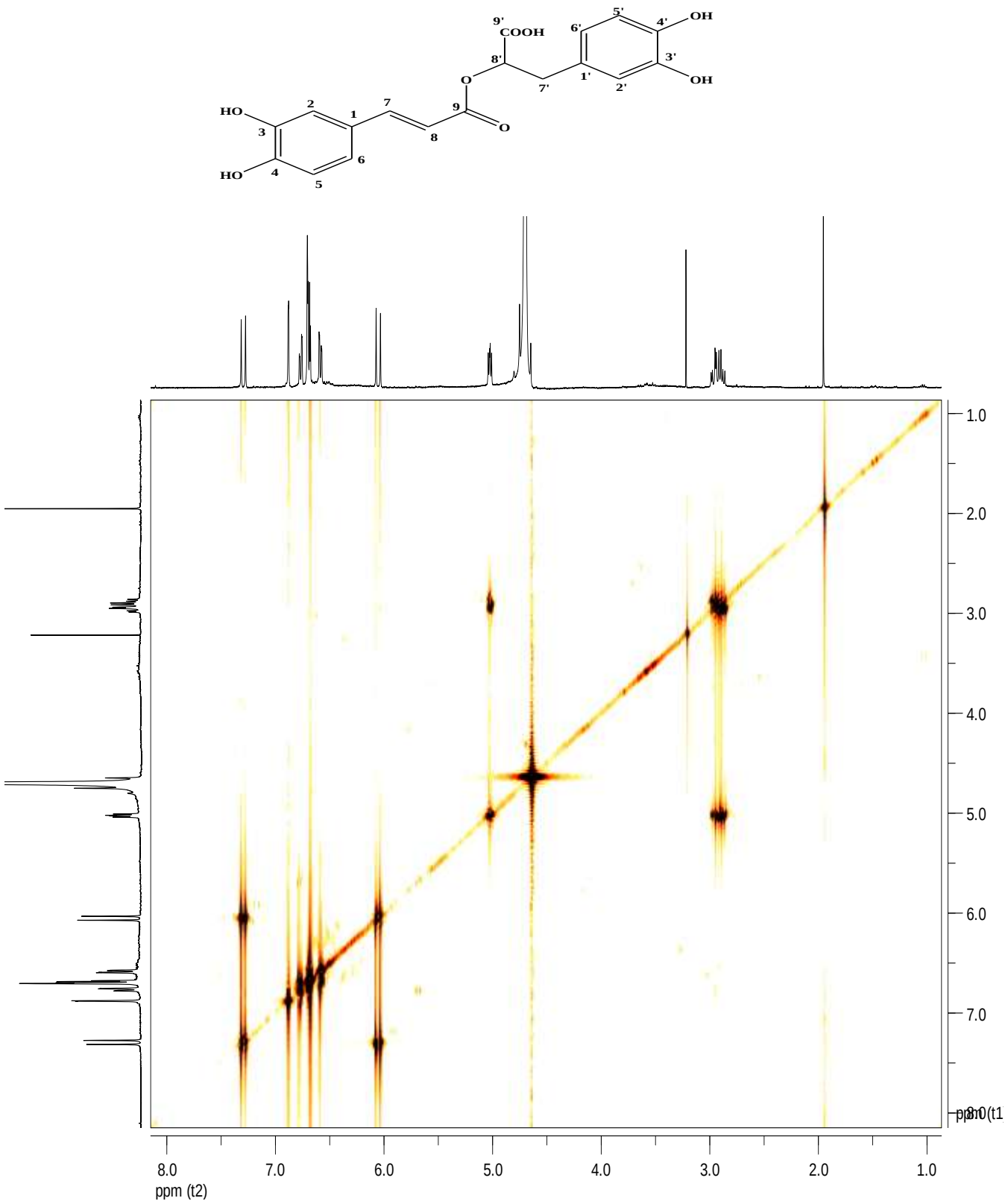


Figure F: ^{1}H-^{1}H COSY of SP-1 in D_2O

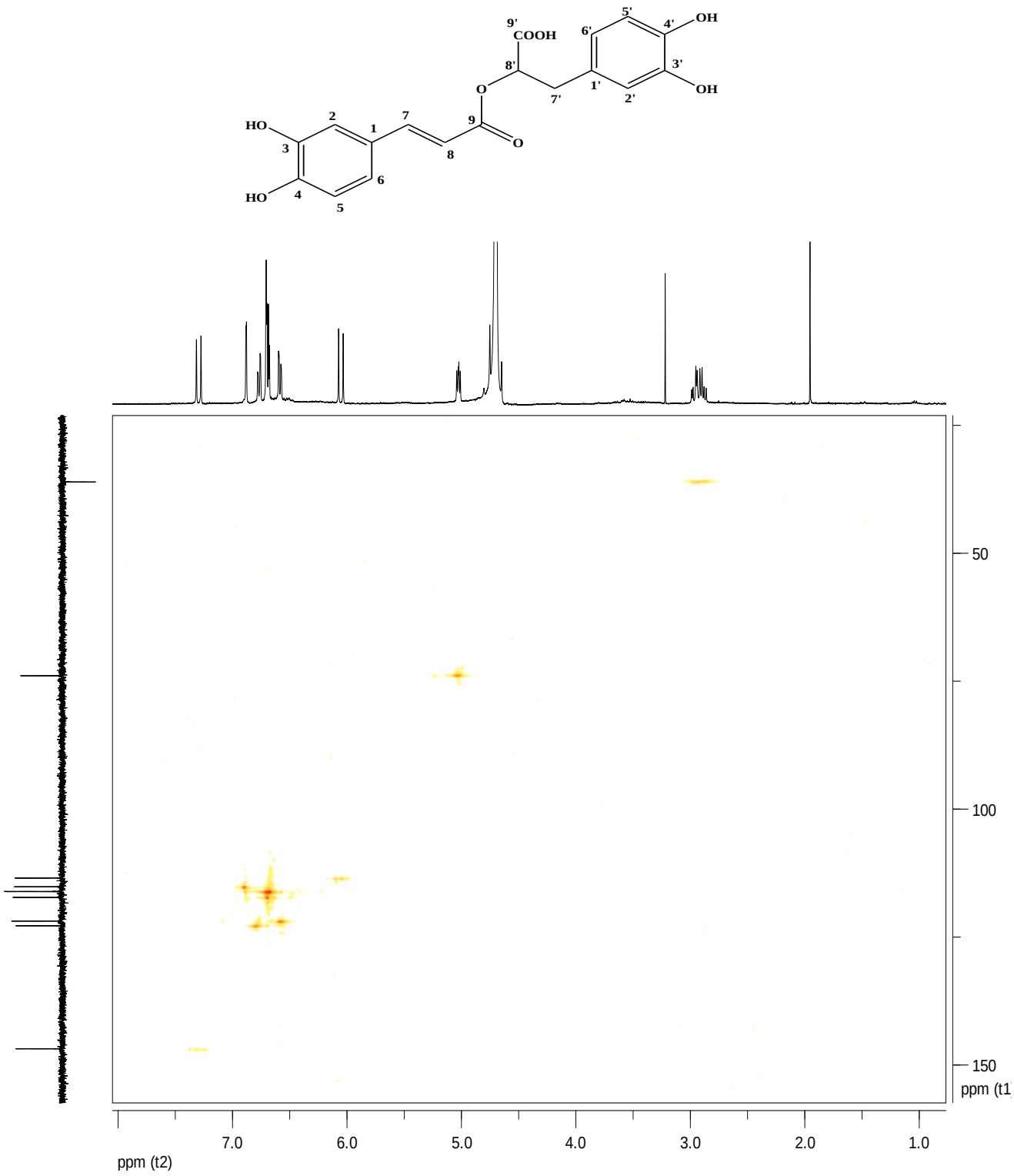


Figure G: HMQC spectrum of SP-1 in D_2O

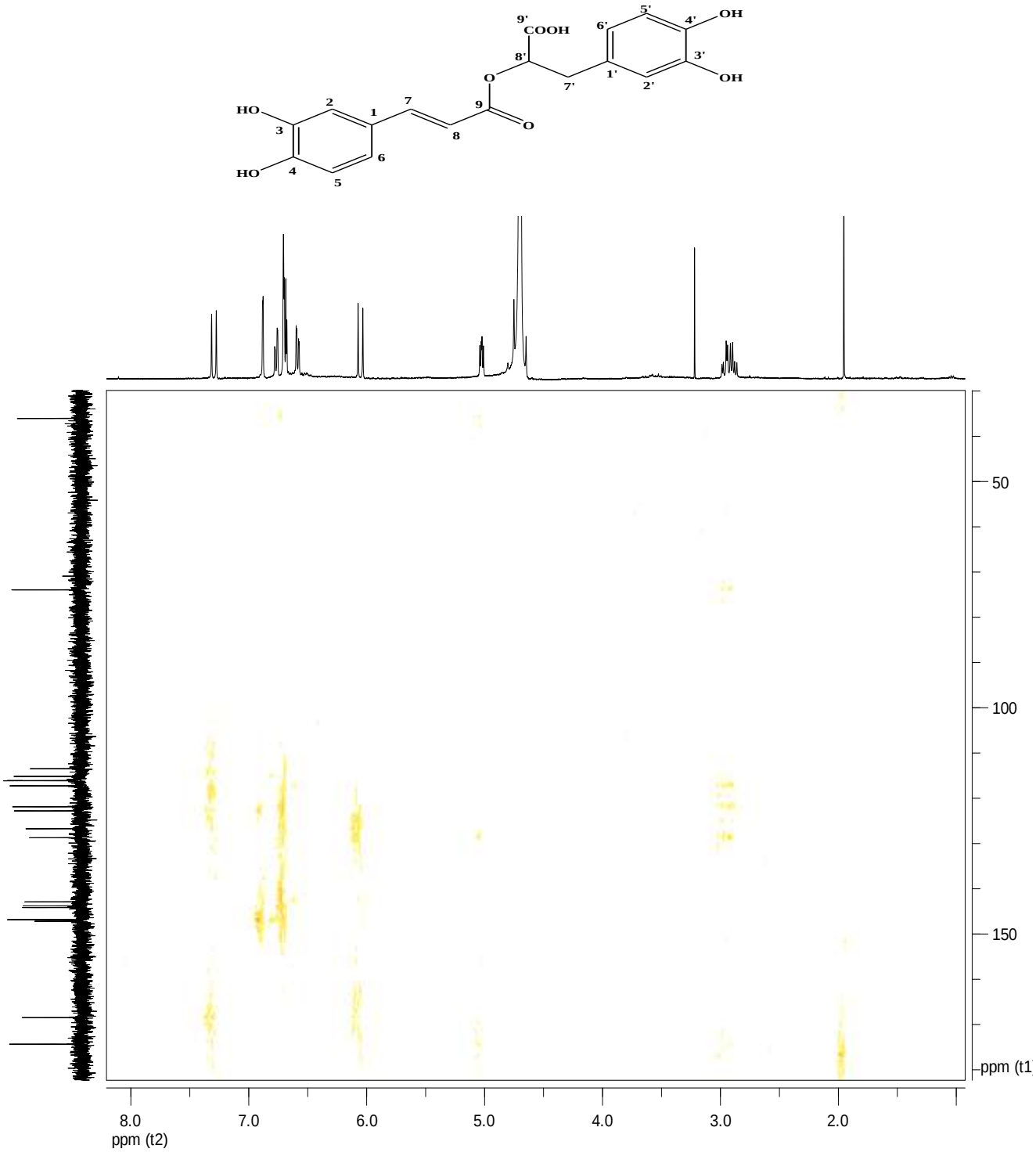


Figure H: HMBC spectrum of SP-1 in D_2O

Appendix II: UV, IR and NMR spectrum of Linarin (SP-2)

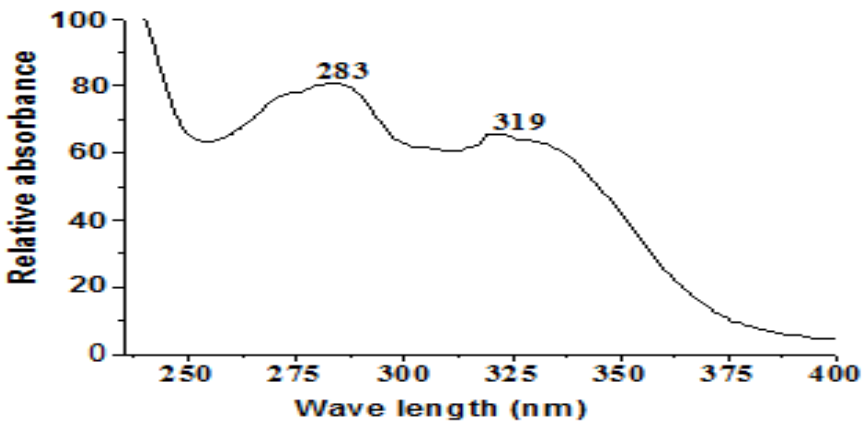


Figure I: UV spectrum of SP-2

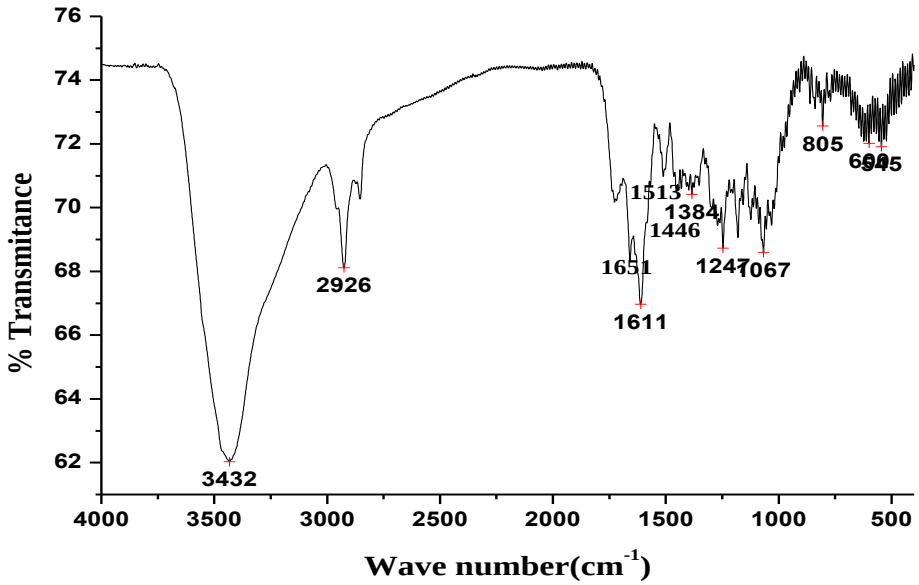


Figure II: IR spectrum of SP-2

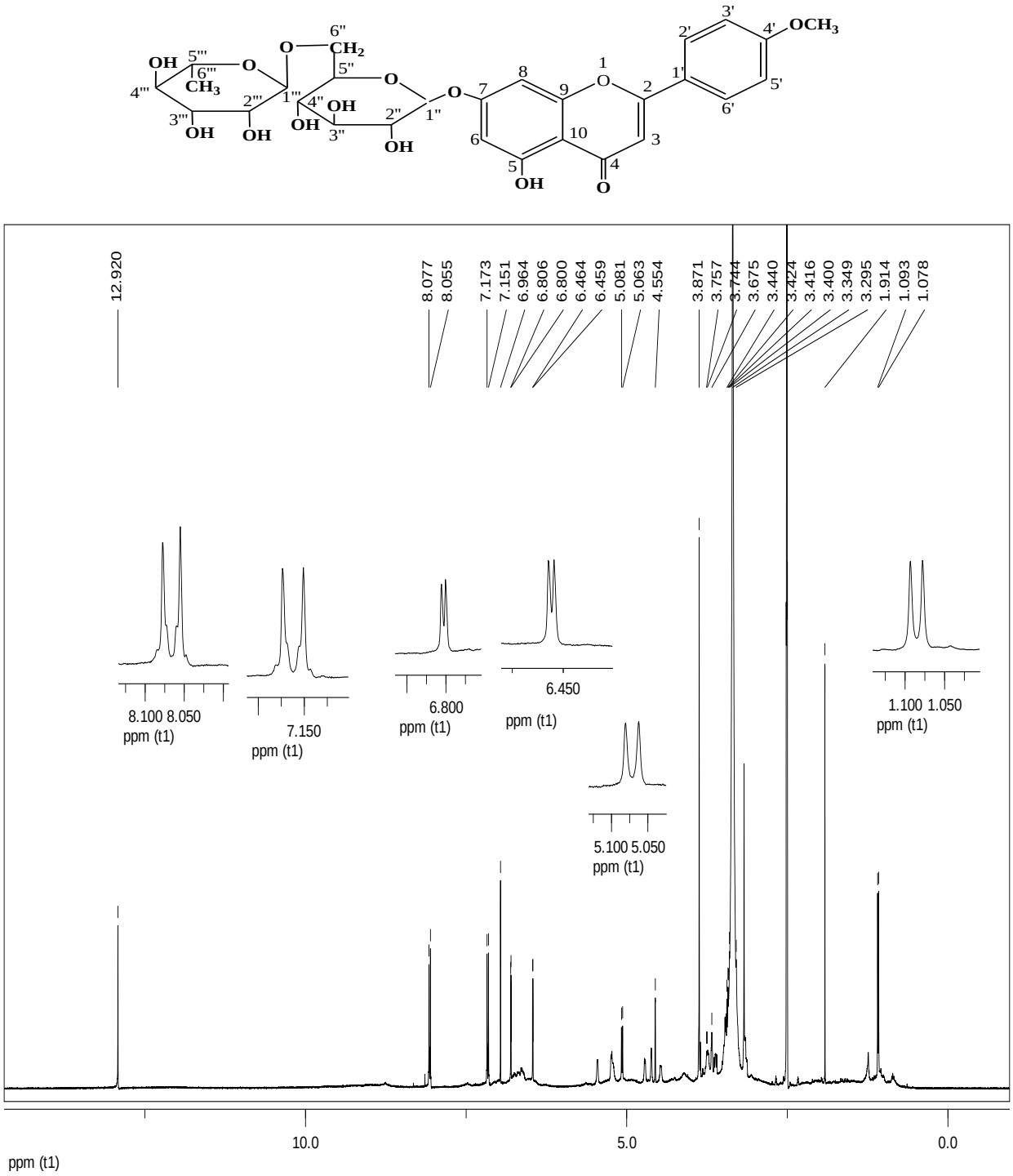


Figure III: ^{1}H NMR spectrum of SP-2 in DMSO-d_6

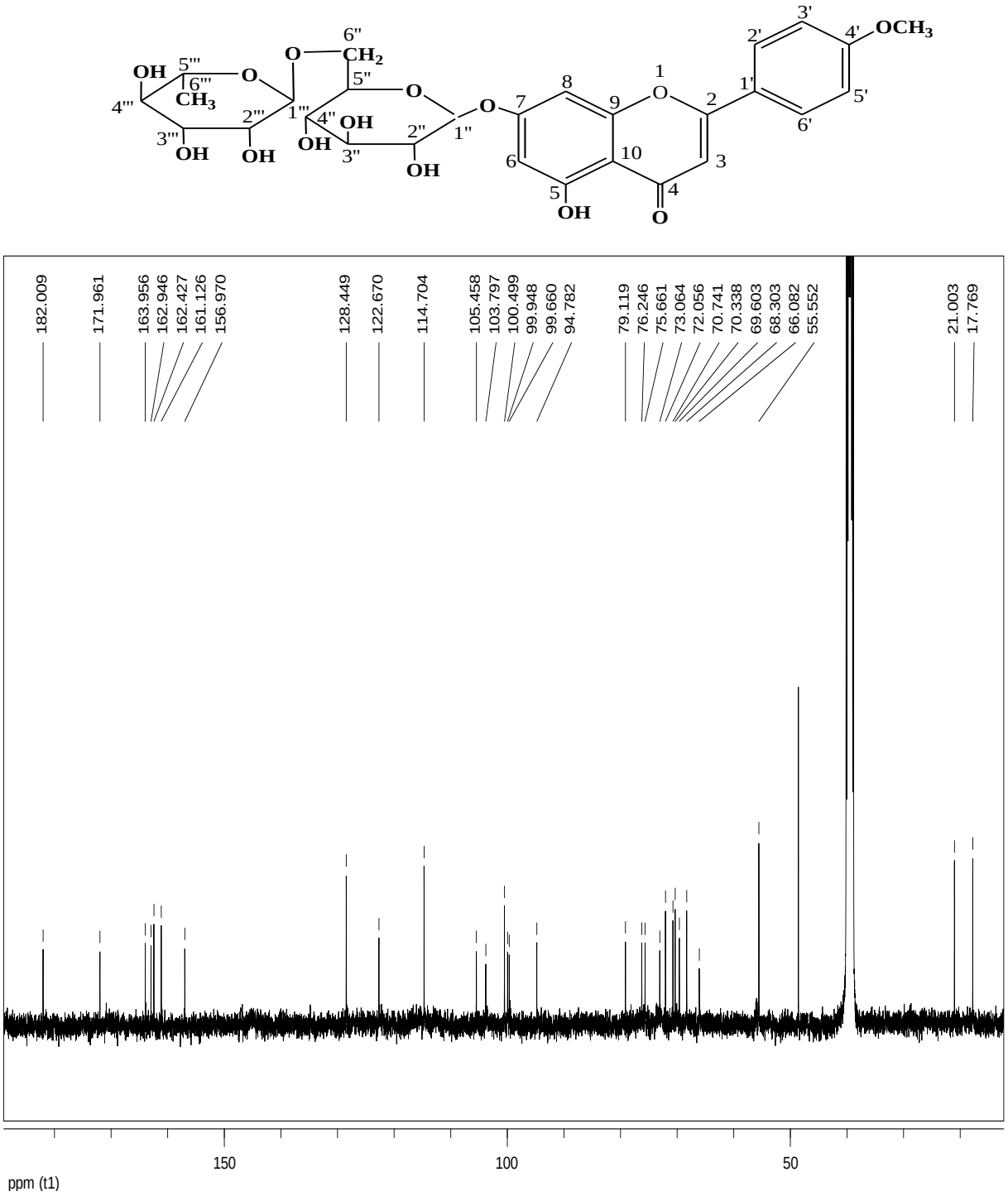


Figure IV: ^{13}C NMR spectrum of SP-2 in DMSO-d_6

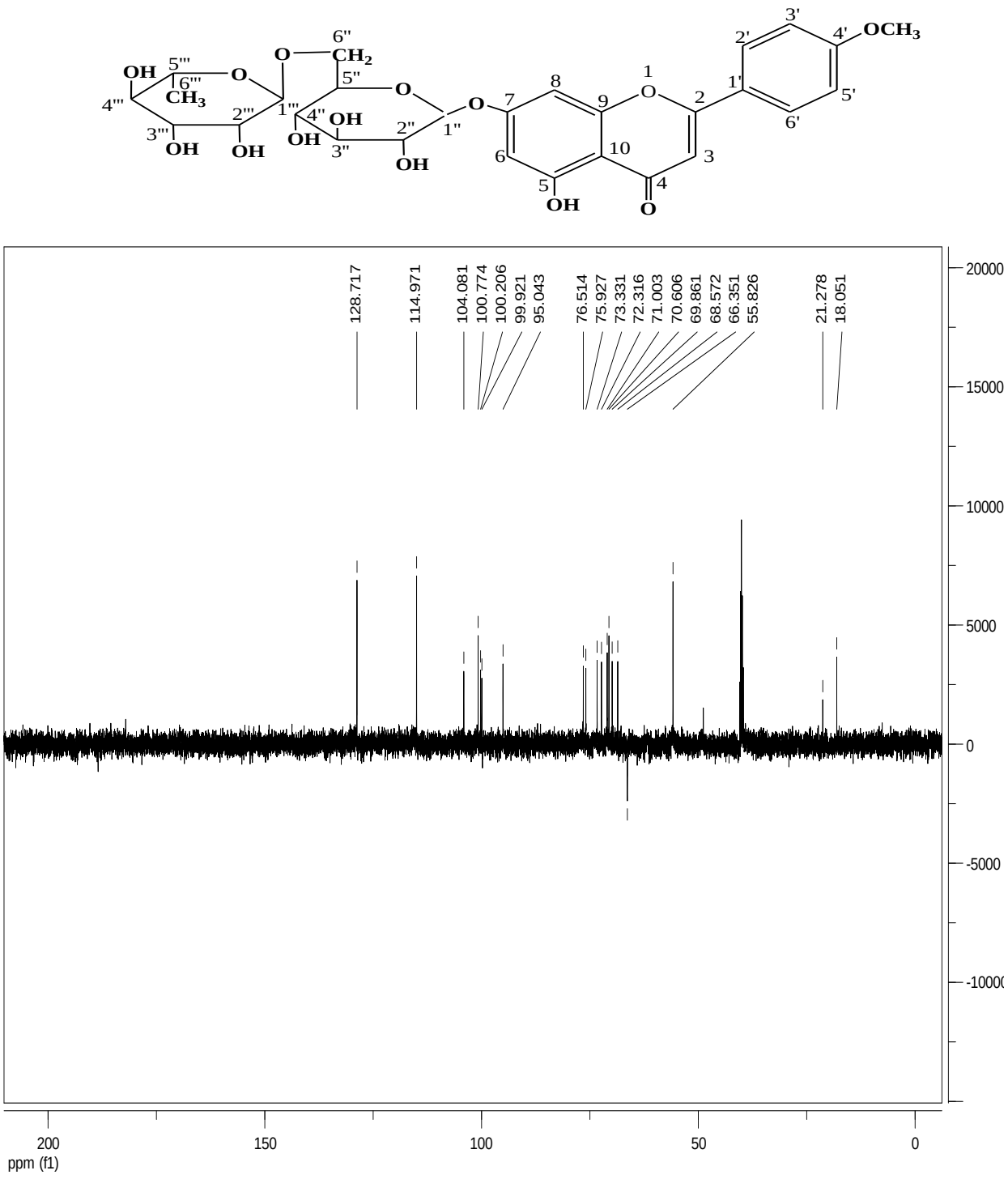


Figure V: DEPT-135 spectrum of SP-2 in DMSO-d_6

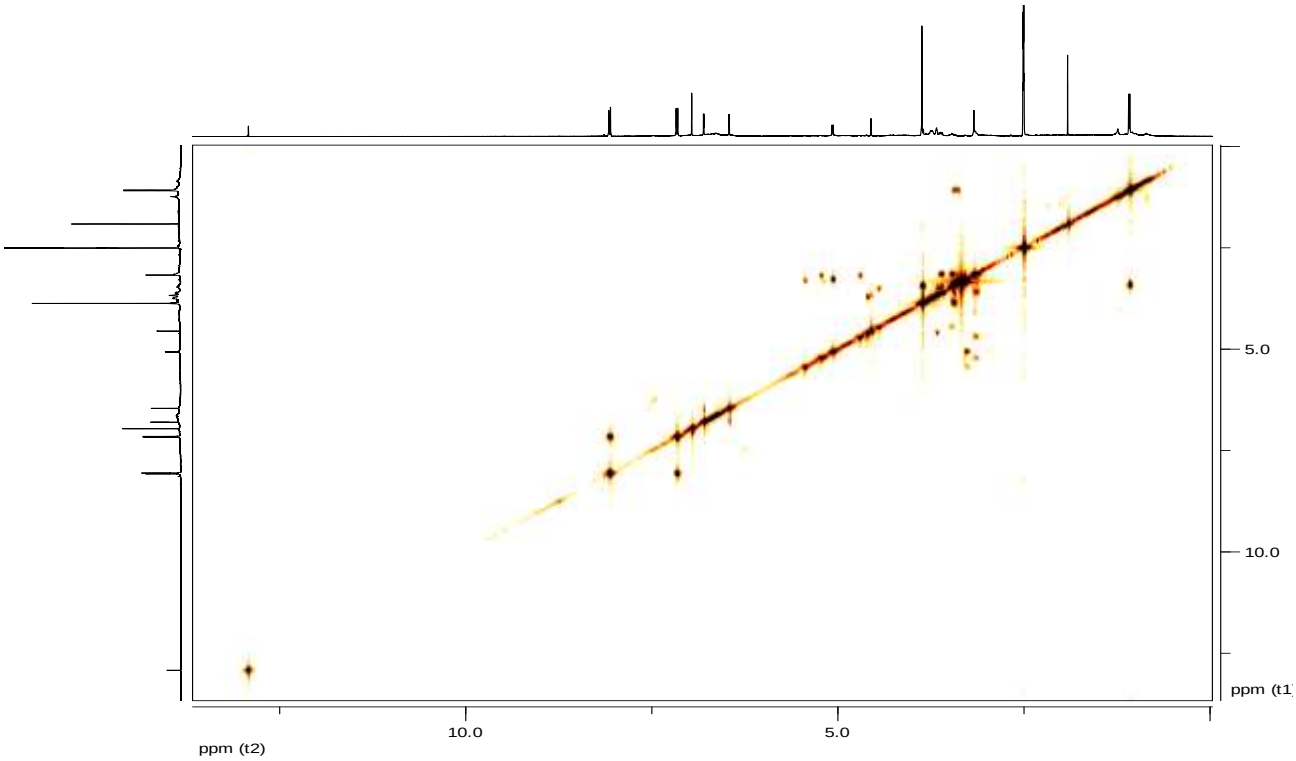


Figure VI: ^{1}H-^{1}H COSY of SP-2 in DMSO-d_6

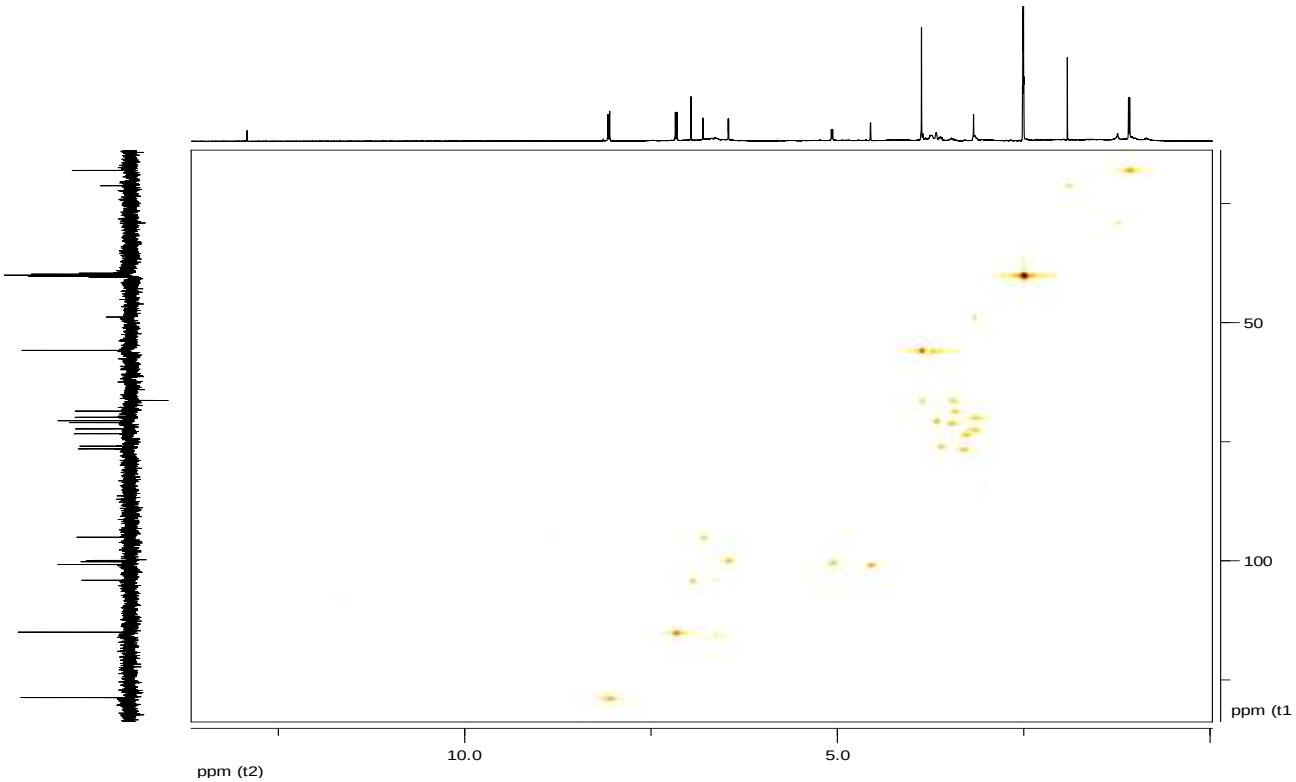


Figure VII: HMQC spectrum of SP-2 in DMSO-d_6

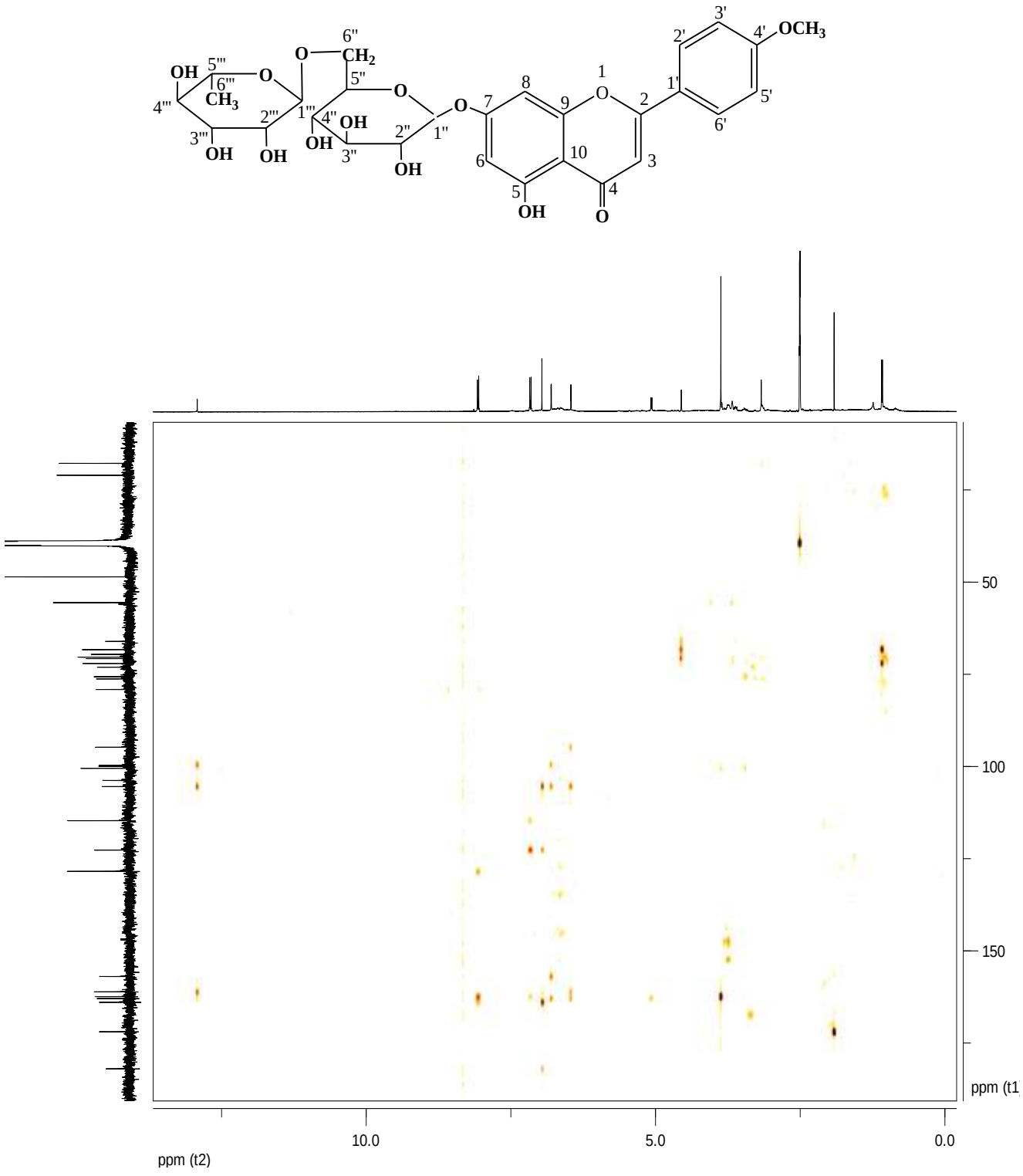


Figure VIII: HMBC spectrum of SP-2 in DMSO-d_6