



**Phytochemical and Biological Studies on *Ravenia spectabilis*  
and *Erythrina variegata***

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## ***Declaration***

*I do hereby declare that the materials embodied in this thesis entitled 'Phytochemical and Biological Studies on *Ravenia spectabilis* and *Erythrina variegata*' prepared for the submission to the University of Dhaka, Dhaka, Bangladesh for the degree of Doctor of Philosophy in Pharmaceutical Chemistry are the original research works of mine and have not been previously submitted for the award of any Degree or Diploma.*

(Fatema Tabassum)

Signature of the candidate

## *Certificate*

*This is to certify that the materials included in this thesis entitled “Phytochemical and Biological Studies on *Ravenia spectabilis* and *Erythrina variegata*”, are the original research work submitted by Fatema Tabassum, Registration no. 34, 2014-15, Department of Pharmaceutical chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka, Bangladesh. The thesis contains no material formerly published or written by another person except when due reference is made in the text of the thesis.*

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## List of Abbreviations

$\mu\text{M}$	Micrometre
1D	One-dimensional
2D	Two-dimensional
BHT	Butylated hydroxytoluene
Br s	Broad singlet
$\text{C}_5\text{D}_5\text{N}$	Deuterated pyridine
$\text{CDCl}_3$	Deuterated chloroform
COSY	Correlation spectroscopy
d	Doublet
DCM	Dichloromethane
DPPH	1,1-diphenyl-2-picrylhydrazyl
EA	Ethyl acetate
et al	And Others
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
$\text{IC}_{50}$	50% Inhibition concentration
IR	Infrared
<i>J</i>	Coupling constant
m	multiplet
<i>m/z</i>	Mass-to-charge ratio
mg	Milligram
ME	Methanol
MHz	Megahertz

MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear Magnetic Resonance
NOSEY	Nuclear overhauser enhancement spectroscopy
PE	Pet ether
R <sub>f</sub>	Retention factor
s	Singlet
t	Triplet
TMS	Tetramethylsilane
UV	Ultra Violet
µg/ml	Microgram per milliliter

## Abstract

The methanolic extract of the leaves of *Ravenia spectabilis* Lindl. (Family: Rutaceae) and stem bark of *Erythrina variegata* L. (Family: Fabaceae) were investigated for the isolation of secondary metabolites and evaluation of bioactivities. A total of twenty one compounds were isolated from these two plants, among them six appeared to be new. The structures of the compounds were elucidated mainly by spectroscopic studies including  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, HSQC, HMBC,  $^1\text{H}$ - $^1\text{H}$  COSY and NOSEY experiments and the molecular weights were determined by ESI mass spectrometry. Among the new compounds, four were alkaloids and two were very unusual  $\text{C}_{34}$  terpenoids. These are 3,5-diprenylindole, 3-prenyl-5-(2-keto-but-3-enyl)indole, 3-prenyl-indole-5-carbaldehyde, iso-oligophyline, ravespanol and ravespanone, all of which were isolated from *Ravenia spectabilis*. The known compounds isolated from this plant include ravenoline,  $\gamma$ -fagarine, arborinine, atanine, oligophyline, ravenine, methyl linoleate and  $\beta$ -sitosterol. Phytochemical investigation of *Erythrina variegata* afforded seven known compounds namely scandenone, alpinumisoflavone, lupeol, stigmast-4-en-3-one, stigmasta-4,22-dien-3-one, stigmasterol and  $3\beta,28$ -dihydroxyolean-12-ene. Different fractions of the crude methanolic extract of the investigated plants and some pure compounds, isolated in this study, were screened for their cytotoxic, antimicrobial, thrombolytic and antioxidant activities by standard methods. The new compounds 3,5-diprenylindole, 3-prenyl-5-(2-keto-but-3-enyl)indole and 3-prenyl-indole-5-carbaldehyde were investigated for cytotoxicity using the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] colorimetric assay method. Among the three compounds, 3,5-diprenylindole was found to be most cytotoxic to human pancreatic adenocarcinoma cell lines with  $\text{IC}_{50}$  value of  $9.5 \pm 2.2 \mu\text{M}$ , moderately cytotoxic to human cervical and lung cancer cell lines with  $\text{IC}_{50}$  values of  $11.3 \pm 1.3 \mu\text{M}$  and  $13.5 \pm 1.66 \mu\text{M}$  respectively and weakly cytotoxic to non-tumour cell line (WI-38) with  $\text{IC}_{50}$  value of  $68.5 \pm 3.5 \mu\text{M}$  as compared to the standard ( $0.19 \pm 0.12$  to  $6.3 \pm 0.3 \mu\text{M}$ ). The rest two compounds showed very poor cytotoxicity ( $\text{IC}_{50} > 50 \mu\text{M}$ ) against the four cell lines tested. *In vitro* antimicrobial activity was measured by disc diffusion method against ten gram positive and gram negative bacterial strains using kanamycin as the standard. Among the samples tested, the pet ether fraction of *Ravenia spectabilis* and the

carbontetrachloride fraction of *Erythrina variegata* demonstrated the highest antimicrobial activity against *Bacillus subtilis* and *Bacillus cereus* respectively with zone of inhibition of  $20.5 \pm 0.74$  mm and  $19.5 \pm 1.18$  mm as compared to the standard ( $34.0 \pm 0.50$  mm and  $24.30 \pm 0.44$  mm). Ravenoline isolated from *R. spectabilis* showed moderate inhibition against *Vibrio cholerae* ( $17.2 \pm 0.41$  mm). Mild to moderate thrombolytic activities were observed by arborinine and different fractions of the crude extract with clot lysis ranging from  $30.43 \pm 1.03$  to  $57.78 \pm 0.24$  % as compared to the standard streptokinase with clot lysis of  $74.34 \pm 0.73$  % for Ravenia extract and  $76.54 \pm 0.90\%$  for Erythrina extract. The antioxidant activity was evaluated by DPPH radical scavenging method using butylated hydroxytoluene as the standard. Among the crude extract tested, the chloroform and aqueous extract of *E. variegata* exhibited moderate antioxidant activities with  $IC_{50}$  values of  $67.59 \pm 1.87$   $\mu\text{g/ml}$  and  $75.02 \pm 2.62$   $\mu\text{g/ml}$  respectively as compared to the standard  $23.09 \pm 1.37$   $\mu\text{g/ml}$ . The pure compounds arborinine and ravenoline showed very poor antioxidant activity.



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## 1.1 Rationale and objective of the research work

Since time immemorial natural products and their derivatives have been recognized as a source of therapeutic agents. The world is ornamented with a huge variety of plants, many of which are proved to have significant medicinal properties. These medicinal plants are extremely useful as natural drugs due to their disease-inhibiting capabilities and provide basic bioactive compounds that are less toxic and more effective (Koparde et al., 2019). The plant based systems continue to play an essential role in health care, and according to the estimation by the World Health Organization (WHO), approximately 80 % of the world's inhabitants rely basically on traditional medicines for their primary health care (Mahomoodally et al., 2013).

As per the oral survey made in different areas of the world, it has been recognized that traditional medicines have their own reputation and basic philosophy. So investigation of the chemical constituents of the plants and their pharmacological screening may afford us the basis for developing a lead molecule through drug discovery process. However, the potential use of higher plants as a source of new drugs is still poorly explored. Among the estimated 4-lakh plant species, only 6% have been studied for their activity and very less not more than of 20% have been investigated phytochemically (Koparde, et al., 2019). Thus, there is a need of investigating the various bioactive fractions and the phytoanalysis and phytopharmacological evaluation of medicinal plants for drug discovery.

Plants are the important source of a diverse range of chemical compounds, whereas some of these compounds possessing a wide range of pharmacological activities are either impossible or troublesome to synthesize in the laboratory. About a third of FDA-approved drugs over the past 20 years are based on natural products or their derivatives (Thomford et al., 2018). The past few decades have seen an increase in the use of medicinal plants for health promotion and treatment of diseases in many countries including developed countries. Scientists have found in plants the remedy of diverse diseases ranging from simple skin diseases to complicated cancer. Based on new scientific developments in isolation, identification and testing technology, the most promising recent contribution of medicinal plants research led to the isolation of anticancer agent vinblastine ( from *Catharanthus roseus* ), hypericin (from *Hypericum* species), taxol (from *Taxus braviifolia*), antimalarial drugs such as artemisinin

(*Artemisia annua*) and quinine (*Cinchona* spp.) and anti-AIDS glycyrrhizin (from *Glycyrrhiza* species) were all discovered from natural products and are effective in treating these diseases (Thomford et al., 2018). Ricin, a toxin produced by the beans of *Ricinus communis*, has been found to be a very potent antitumor drug (Spalding, 1991). Further, promising HIV integrase inhibitory activity have been reported from galloyl glucose isolated from *Terminalia chebula* (Singh et al., 2006).

The development of new technologies has revolutionized the screening of natural products in discovering new drugs. Utilizing these technologies gives us an opportunity to perform research in screening new molecules to establish natural products as a major source for drug discovery. The advent of novel technologies including quantum computing, profiling techniques, computational biology techniques, big data, micro fluidics and artificial intelligence will enable scientists to use a combinatorial approach to harness the therapeutic properties of plant-based natural products and simultaneously study their molecular effects in physiological conditions and finally leads to lead structure discovery (Thomford et al., 2018). Plants containing important secondary metabolites and potential biological activities can be genetically engineered to increase the transcription of enzyme responsible for the production of that particular compound. Thus, knowing the potential resources it is possible to increase the content of important active compounds (Owen et al., 1992) and genes are to be encoded in the host organisms to produce lead compounds from plants in industrial scale.

With growing interest in herbal drug development with minimum side effects, there are better opportunities to explore the medicinal and other biological properties of previously inaccessible natural products. To establish its usefulness, it is natural products that have played, and will continue to play, a vital role in drug discovery and are therefore traditionally claimed as the foundations of drug discovery and development (Koparde et al., 2019).

The objectives of the research work are to isolate new compounds and to evaluate the possible biological activities of two plants *Ravenia spectabilis* Lindl. and *Erythrina variegata* L. The following steps were carried out:

- a. Isolation of secondary metabolites from the crude extracts of the selected plants.

- b. Characterization of the isolated compounds by IR, NMR and Mass spectroscopy.
- c. Investigation of the biological activity of different solvent extracts and pure compounds using the following assay techniques
  - Cytotoxicity study by the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay method (Mosmann, 1983).
  - Antimicrobial screening using disc diffusion method (Bauer *et al.*, 1966).
  - Antioxidant activity using DPPH assay method (Brand-Williams *et al.*, 1995).
  - Thrombolytic activity using clot lysis method (Prasad *et al.*, 2006).

## 1.2 The family Rutaceae

Rutaceae, the citrus family composed of 161 genera, with about 1650 species. It includes woody shrubs, trees and a few herbs, which are distributed throughout the world, especially in warm temperate and tropical regions. The largest numbers are found in Africa and Australia, often in semiarid woodlands. Members of the family often feature aromatic leaves with oil glands on the surfaces. Generally the flowers are perfect containing both male and female reproductive organs in the same flower or sometimes unisexual. They are conspicuous for their colour, fragrance and nectar. The fruits are various, consisting, for example, of capsules (genus *Ruta*), follicles (*Zanthoxylum*), drupes (*Amyris*), berries (*Triphasia* and *Citrus*), samaras (hop tree), and schizocarps (*Helietta*) (Encyclopaedia Britannica, 2019; Encyclopedia, 2019).

In Bangladesh, Rutaceae family is represented by 16 genera and 28 species. The genera are *Acronychia*, *Aegle*, *Atalantia*, *Citrus*, *Clausena*, *Glycosmis*, *Luvunga*, *Merope*, *Micromelum*, *Murraya*, *Paramignia*, *Toddalia*, *Triphasia*, *Zanthoxylum* *etc.* (Flora of Bangladesh, n.d.).

### 1.2.1 Economic and medicinal value of Rutaceae

The family Rutaceae, is of great economic importance for its numerous edible fruits of the genus *Citrus*, such as the orange, lime, lemon, mandarin, calamansi, , kumquat, and grapefruit. Non-citrus fruits include the orange berry (*Glycosmis pentaphylla*), colourless sapote (*Casimiroa edulis*), lymenia (*Clymenia polyandra*) and the bael

(*Aegle marmelos*). From a number of species in the genus *Zanthoxylum* (notably Sichuan pepper) spices are made. So rutaceous plants such as *Zanthoxylum* and *Casimiroa* have been used in medicine. A large Australian genus *Boronia* contains some members of which are plants with highly fragrant flowers and are used by the perfume industry (Wikipedia, 2019).

From the very early days, herbal drugs have been used in the treatment of various ailments, though their use has become concentrated in developing countries. The medicine pilocarpine which is used to treat glaucoma as well as for the stimulation of sweat and lachrymal glands is obtained from the genus pilocarpus (Sawaya et al, 2011). The Rutaceae also produces several anticancer agents. The juice extract of *Citrus aurantifolia* showed potential activity against colon cancer, pancreatic cancer, breast cancer and several other cancers (Narang and Jiraungkoorskul, 2016). Fruit extracts of *Limonia acidissima* Linn. showed anticancer activity on selected human breast cancer cell lines (Pradhan et al., 2012). Bioassay of the extract of *Limonia acidissima* Linn. showed that a fraction of the ethanol extract had anticancer activity against SKBR3 and MDA-MB435 human breast cancer cells. The carbazole alkaloids and coumarins from *Clausena* plants exhibit anticancer activity (Huang et al, 2017).

Antibacterial and antifungal activity have been reported for a number of secondary metabolites isolated from Rutaceous plants. The terpinoid isolated from the stem bark of *Teclea afzelii* showed activity against gram-positive and negative bacteria, fungi and *Mycobacterium smegmatis* ( Kuete, et al., 2008). Fungicidal activity has been reported for two prenylated and geranylated acetophenones isolated from *Melicopelunu ankenda* (Kumar et al., 1990). *Toddalia asiatica* is used in the management of malaria and stomach problems.

Currently research has focused on the biological activity of compounds found in citrus species, including compounds called flavanoids, carotenoids and limonoids, especially in terms of their effects on citrus palatability and anti-cancer activity. Citrus flavonoids have potential antioxidant, anti-cancer, antiviral, anti-inflammatory activities, effects on capillarity and cholesterol-lowering ability. The principal carotenoids in pink grapefruit are lycopene and beta-carotene. Fruits and vegetables containing lycopene have been shown to contribute to a significant reduction in



prostate and mammary cancer risk. Recent studies have further shown that limonoids isolated from citrus species inhibit the development of cancer in laboratory animals and in human breast cancer cells as well as reducing cholesterol (Ferguson and Spann 2002).

### 1.2.2 Phytochemicals from Rutaceae family

The family is well known for producing a wide range of phytochemicals, such as phenanthridine, acridone and furo- and pyranoquinoline alkaloids, complex furo- and pyranocoumarins, flavonoids and various types of terpenoids, including the limonoids (Waterman, 1983).

### 1.2.3 The genus *Ravenia*

*Ravenia* is a genus of flowering plants in the citrus family which includes the following species

- ❖ *Ravenia baracoensis* Borhidi & O. Muñiz
- ❖ *Ravenia biramosa* Ducke
- ❖ *Ravenia carabiai* Vict.
- ❖ *Ravenia hiramosa* Ducke
- ❖ *Ravenia infelix* Vell.
- ❖ *Ravenia polygalaecalyx* Ducke
- ❖ *Ravenia pseudalterna* Ducke
- ❖ *Ravenia rosea* Standl.
- ❖ *Ravenia shaferi* P.Wilson
- ❖ *Ravenia simplicifolia* C.Wright ex P.Wilson
- ❖ *Ravenia spectabilis* Engl.
- ❖ *Ravenia urbaniis* Engl. Ex Urban

(The plant list, n.d.)

### 1.2.4 The plant *Ravenia spectabilis*

In the study, one plant species *Ravenia spectabilis* belonging to the family Rutaceae was investigated.

**Synonyms** *Lemonia spectabilis* Lindl.

**Common Name** Lemonia, Limonia, Pink Ravenia (E-Flora of Gandhinagar, n.d.)

#### 1.2.4.1 Taxonomic hierarchy of the plant *Ravenia spectabilis*

---

Kingdom	Plantae
Subkingdom	Viridaeplantae
Phylum	Tracheophyta
Class	Magnoliopsida
Sub Class	Rosidae
Order	Rutales
Family	Rutaceae
Genus	<i>Ravenia</i>
Species	<i>R. spectabilis</i>

---

(Keralaplants.in, n.d.)

#### 1.2.4.2 Morphology of *Ravenia spectabilis*

The plant *Ravenia spectabilis* belonging to the family Rutaceae is a resourceful shrub and broadly spread through the South America and some Asian countries such as



**Figure 1. 1 Whole plant (1) and flower with leaves (2) of *Ravenia spectabilis***

Pakistan, Bangladesh and India (Haque et al., 2013). *Ravenia spectabilis* bearing purplish-red flowers found almost throughout the year. The shrub can be grown in the sun as well as in light shade.

**Growth Form:** Large, woody shrub which is able to grow up to 3 - 5 m tall.

**Foliage:** Elliptic dark green foliage separated into 3 leaflets and measuring about 3 - 5 cm long.

**Flowers:** Bright dark pink flowers, flattened in shape, measuring about 2 - 6 cm wide, 5 sepals, corolla tube pink in colour and corolla tube measuring about 1 - 3 cm long. (Flora & Fauna web, n.d).

#### 1.2.4.3 Reported biological activities of *Ravenia spectabilis*

Previously antimicrobial, antioxidant, cytotoxic and acetylcholinesterase inhibition activity have been reported (Sohrab, et al., 2004; Haque et al., 2013; Viana et al., 2018) from this plant. The plant contains alkaloids, triterpines and other secondary metabolites which may be responsible for its biological activities.

##### **Antimicrobial activity**

The crude methanolic extract showed good activity against *Bacillus cereus* (18 mm), *Bacillus megateriumi* (16 mm) and *Shigella dysenteriae* (15 mm). The growth of *Bacillus cereus* (14 mm) and *Vibrio parahemolyticus* (12 mm) of the carbon tetrachloride partitionate of the methanolic extract possessed significant zone of inhibition. The chloroform partitionate of the methanolic extract possessed moderate activity against *B. cereus* (15 mm) and *S. dysenteriae* (14 mm) (Haque et al., 2011).

##### **Cytotoxic activity**

The n-hexane partitionate of the methanolic extract showed the maximum activity with LC<sub>50</sub> value of 4.26 µg/mL. The carbon tetrachloride as well as chloroform partitionate of the methanolic extract exhibited significant brine shrimp lethality with LC<sub>50</sub> values of 12.15 µg/mL and 22.19 µg/mL, respectively (Haque et al., 2011).

##### **Antioxidant activity**

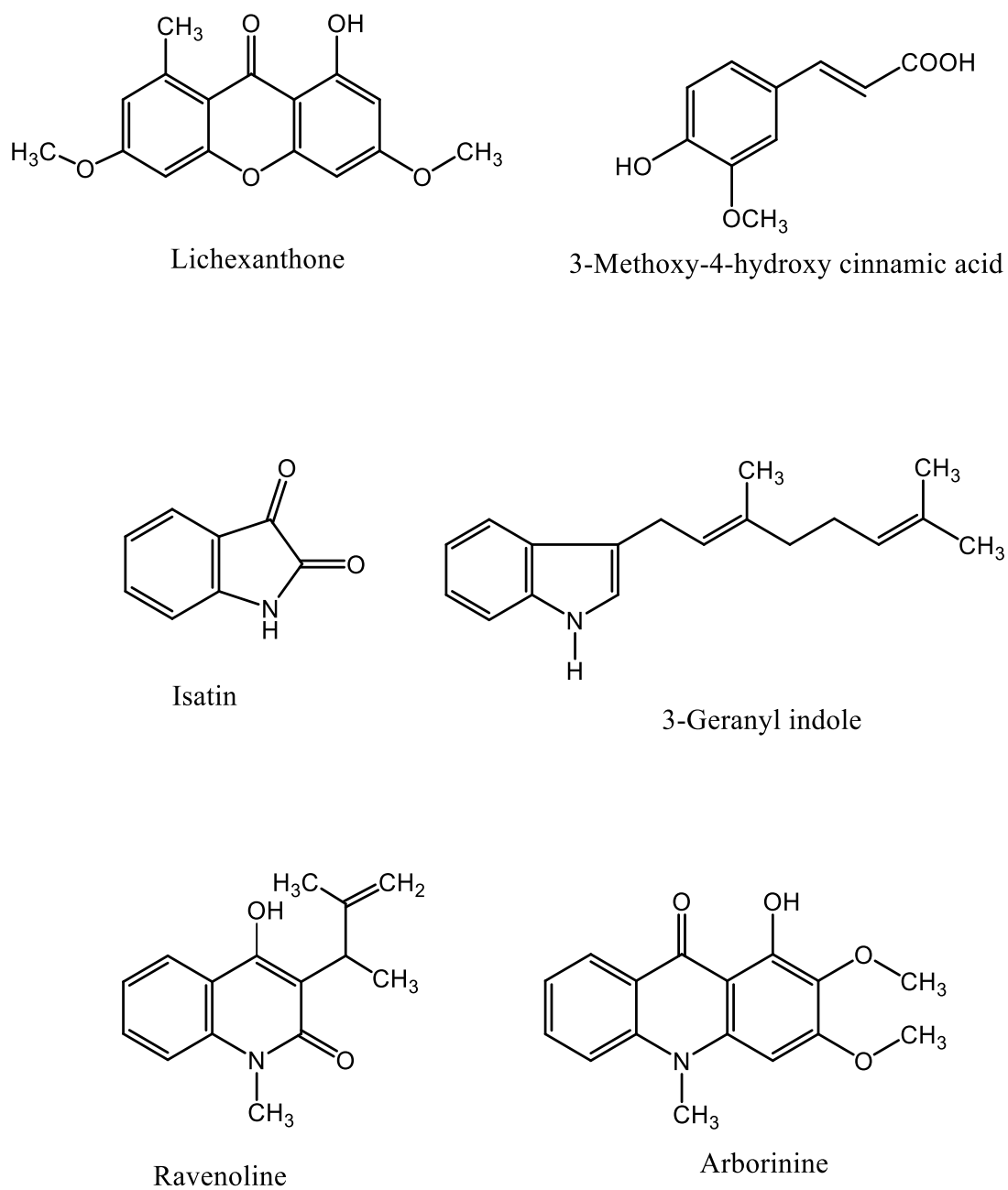
In free radical scavenging assay, the crude methanol extract showed moderate antioxidant activity with IC<sub>50</sub> value 78.25 µg/mL (Haque et al., 2011).

##### **Acetylcholinesterase inhibition**

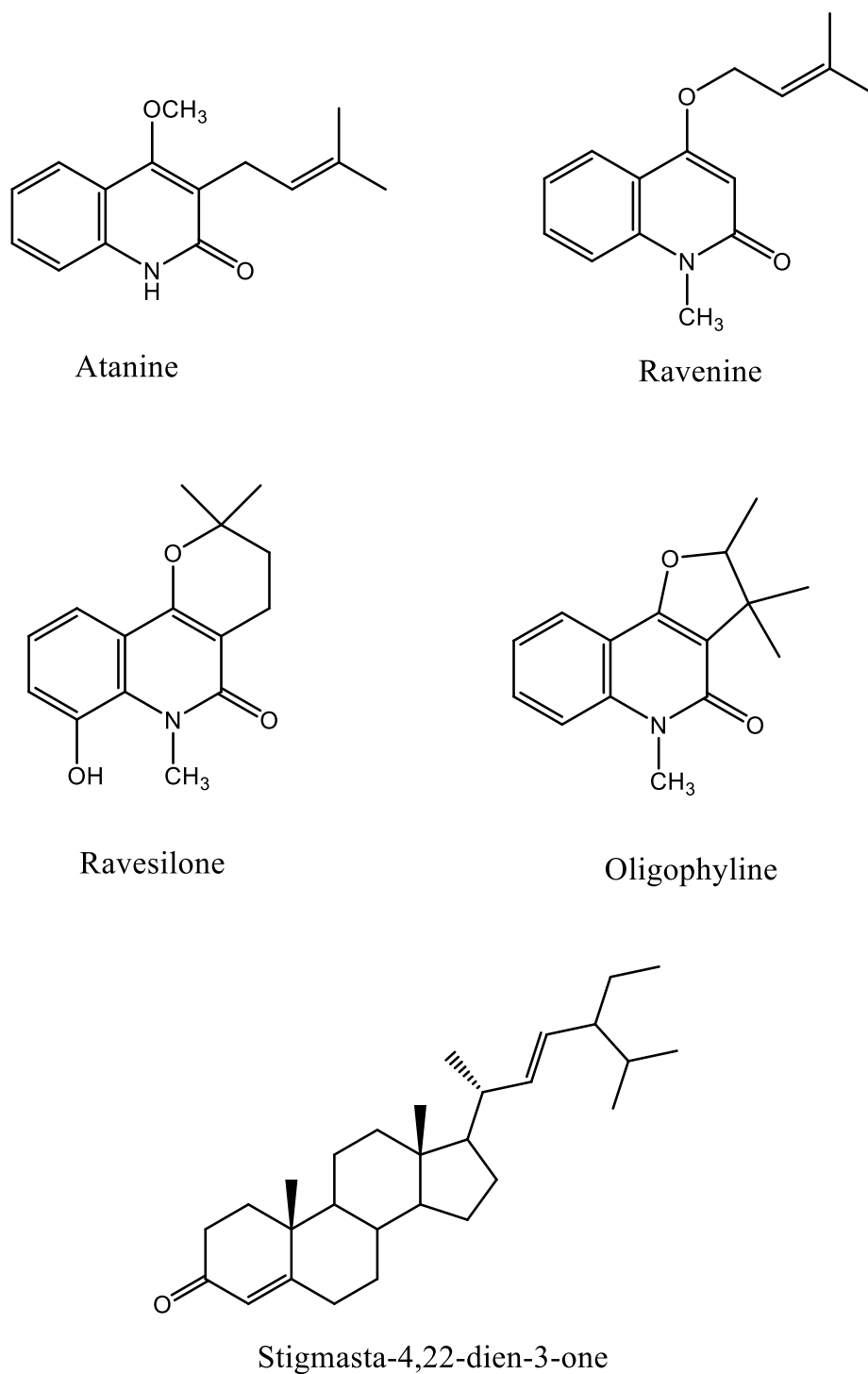
Acetylcholinesterase inhibition of dichloromethane leaves extract of *R. spectabilis* possessed promising AChE inhibition activity (54.4%) and this inhibition is comparable to other plant extracts from Rutaceae such as for fruits of *Aegle marmelos* (44.6%) and leaves of *Esenbeckia leioacarpa* (91.1%) (Viana et al., 2018).

**Table 1.1** Previous phytochemical investigations on *R. spectabilis*

Plant Part studied	Compound Isolated	References
Leaves	Isatin	Viana et al., 2018
	Lichexanthone	
	$\alpha$ -Cadinol	
	$\alpha$ -Spinasterone	
	Lupeol	
Stem bark	Arborinine	Alam et al., 2011
Stem bark	$\gamma$ -Fagarine	Sohrab et al., 2004
	Arborinine	
	Stigmasta-22-dien-3one	
Stem	Ravenoline	Haque et al., 2013
	$\gamma$ -Fagarine	
	Arborinine	
	Atanine	
	2, 3, 3,5-tetramethyl-2, 3, 4, 5-tetrahydrofurano [3,2- c] quinolin-4-one	
	3-Geranyl indole	
	3-Methoxy-4-hydroxy cinnamic acid	
	Stigmasterol	
	Sitosta-4-en-3-one	
	Leaves	
Leaves	Ravesilone	Bhattacharyya & Chowdhury, 1984
Leaves	Spectabiline	Talapatra et al., 1969
	Gamma-fagarine	
	Atanine	
	Ravenine	
Leaves	Ravenine	Paul et al., 1969
	Ravenoline	
	Atanine	
	Arborinine	
	$\gamma$ -Fagarine	



**Figure 1.2** Structures of some previously reported phytochemicals from *R. spectabilis*



**Figure 1.2 (cont.) Structures of some previously reported phytochemicals from *R. spectabilis***

### 1.3 The family Fabaceae

Fabaceae or pea family is a big family comprising of more than 751 genera and about 19,000 species of trees, shrubs, vines, and perennial or annual herbaceous plants. The plant family has immense medicinal importance (Christenhusz & Byng, 2016). Plant species belonging to this family are distributed throughout temperate and tropical regions of the world (Rundel, 1989).

#### 1.3.1 Ecological and economic importance of Fabaceae

Fabaceae is economically and ecologically important plant family due to extraordinary diversity and abundance, the wide variety of edible vegetables they represent and due to the variety of uses in horticulture and agriculture, as a food for the compounds they contain that have medicinal uses and for the oil and fats they contain (Wikipedia, 2019).

The unique ecological role of Fabaceae is in nitrogen fixation. Nitrogen is an element of all proteins and is an essential component in both plant and animal metabolism. Although elemental nitrogen makes up about 80 percent of the atmosphere, it is not directly available to living organisms; nitrogen that can be metabolized by living organisms must be in the form of nitrates or ammonia compounds. Through a mutual benefit arrangement (symbiosis) between legumes and *Rhizobium* bacteria, nitrogen gas (N<sub>2</sub>) is fixed into a compound and then becomes available to the biotic world (Encyclopaedia Britannica, n.d.).

The vital roles of legume seeds are that of supplying most of the protein in regions of high population density and in balancing the deficiencies of cereal protein (Poaceae). Except for the soybean and peanut, the order is not noted for the oil content of the seeds since most seeds have only about 10 percent oil content by weight. The legume seeds generally are highest in carbohydrate compounds, followed by protein and fat. Legumes are thus considered to be energy foods. Nearly all legumes that are used for foods are multipurpose plants, serving for animal forage and soil improvement as well. Fabaceae contains the more important crop plants, such as soybeans, beans, cowpeas (*Vigna*), pigeon peas (*Cajanus cajan*), chick-peas (*Cicer arietinum*), lentils (*Lens culinaris*), peas (*Pisum sativum*) and peanuts (Encyclopaedia Britannica, 2019.).

In addition to their uses as food, legumes are still used as tools in agriculture and forestry. Legumes help to increase soil nitrogen and provide rich sources of vegetable protein for humans, livestock, and wild animals. The plants themselves or plant products like leaves and pods can be tilled into the soil as a nitrogen source or legume crops can be rotated with others for soil improvement. These techniques save farmers billions of dollars in the cost of nitrogen fertilizers (Graham and Vance, 2003).

Industrial farmed legumes include *Acacia*, cultivated for gum arabic, *Indigofera* for the production of indigo, and *Derris*, for the insecticide action of rotenone, a compound it produces. Various legume species are farmed for timber production worldwide, including numerous *Dalbergia* species, *Acacia* species, and *Castanospermum australe*. Some legume species such as alfalfa, sweet clover, colourless clover and various *Prosopis* species are good nectar providers. Many centuries throughout the world legumes have been used as ornamental plants. (Wikipedia, 2019). Their vast diversity of heights, shapes, foliage and flower colour means that this family is commonly used in the design and planting of everything from small gardens to large parks.

In folk medicines, legumes are extensively employed for the treatment of diverse diseases. Isoflavones commonly found in legumes are thought to reduce the risk of cancer and lower cholesterol and soybean phytoestrogens are being studied for use in postmenopausal hormone replacement therapy (Graham and Vance, 2003). Legumes are extensively used in Bangladesh for the treatment of various diseases.



**Table 1.2 Medicinal uses of some Fabaceous species growing in Bangladesh (Ahmed *et al.*, 2009)**

Species	Plant part	Use
<i>Abrus precatorius</i> L.	Bark, leaf	Aphrodisiac, arthritis, rheumatism.
<i>Acacia Arabica</i> (Lam.) Willd. Var. <i>indica</i> Benth.	Leaf, root, bark, flower, seed	Diarrhoea.
<i>Acacia catechu</i> (L.f.) Willd.	Leaf, stem	Diarrhoea.
<i>Acacia farnesiana</i> (L.) Willd.	Roots	Urinary tract infection, lower abdominal pain.
<i>Adenantha pavonina</i> L.	Base of trunk	Diarrhoea.
<i>Caesalpinia nuga</i> (L.) Aiton.	Plant juice, roots, fruit	Burning sensation in urinary tract.
<i>Cassia alata</i> L.	Leaf, stem	Ringworm, diarrhea.
<i>Cassia fistula</i> L.	Root	Diarrhoea.
<i>Cassia sophera</i> L.	Whole plant	Diarrhoea.
<i>Cassia tora</i> L.	Leaf	Diarrhoea.
<i>Clitoria ternatea</i> L.	Leaf, root	Sexual problems, urinary tract infections.
<i>Desmodium gangeticum</i> (L.)	Leaf, root	Chest pain, sexual problems.
<i>Erythrina variegata</i> L.	Leaf	Stomach ache in children.
<i>Mimosa pudica</i> L.	Leaf, bark, root	Frequent urination, sexual problems, burning sensations in the vaginal area, leucorrhoea.
<i>Moghaniam acrophylla</i> (Willd.) Kuntze	Root	Urinary tract infections, frequent urination, lack of or infrequent urination.
<i>Pterocarpus santalinus</i> L.	Stem	Conjunctivitis.
<i>Senna alata</i> (L.) Roxb.	Leaf	Eczema.
<i>Tamarindus indica</i> L.	Leaf, flower	Eye diseases, cataract, rheumatism, dysentery.

### 1.3.2 Phytochemicals from Fabaceae

A high diversity of secondary metabolites are produced by the plants of Fabaceae family which serve as not only defense compounds against herbivores and microbes, but also as incitation compounds to attract pollinating and fruit-dispersing animals. Legumes can produce nitrogen containing secondary metabolites than other plant families as they are nitrogen-fixing plants. Nitrogen include compounds like alkaloids (indolizidine, pyrrolizidine, piperidine, simple indole, pyridine, pyrrolidine, simple isoquinoline, and imidazole alkaloids) cyanogenicglucosides, non-protein amino acids (NPAA) and peptides (lectins,cyclotides ,trypsin inhibitors) and the secondary metabolites without nitrogen are phenolics (phenylpropanoids, flavonoids, isoflavones, catechins, anthocyanins, tannins, lignans, coumarins and furanocoumarins), polyketides (anthraquinones) and terpenoids (especially triterpenoid, steroidal saponins, tetraterpenes) (Wink, 2013).

### 1.3.3 The genus *Erythrina*

*Erythrina* is one of the several genera of Fabaceae family that contains about 130 species. These plants are collectively known as coral tree in horticulture, widely studied and distributed in tropical and subtropical regions around the globe. The generic name is derived from the Greekword (*erythros*), denoting "red", alluding to the bright red flowers of the trees of the genus (Gledhill, 2008). The coral tree or *Erythrina* genus is indigenous to the Old World tropics, particularly from India to Malaysia, but they are native of eastward to eastern Polynesia (the Marquesas) as well as ancient west ward to Zanzibar. Usually they are found in littoral forest on sand based soil, and occasionally in coastal forest. They can grow up to 250m (800ft) in height (Kumar et al., 2010)

Some available species of *Erythrina* genus includes the following

- |                                    |                                       |
|------------------------------------|---------------------------------------|
| ❖ <i>Erythrina abyssinica</i> Lam. | ❖ <i>Erythrina megistophylla</i>      |
| ❖ <i>Erythrina americana</i> Mill. | ❖ <i>Erythrina mulungu</i> Diels      |
| ❖ <i>Erythrina ankaranensis</i>    | ❖ <i>Erythrina perrieri</i>           |
| ❖ <i>Erythrina atitlanensis</i>    | ❖ <i>Erythrina polychaeta</i> Harms   |
| ❖ <i>Erythrina berteroana</i> Urb. | ❖ <i>Erythrina sacleuxii</i>          |
| ❖ <i>Erythrina burana</i> Chiov.   | ❖ <i>Erythrina sandwicensis</i>       |
| ❖ <i>Erythrina crista-galli</i> L. | ❖ <i>Erythrina schimppfii</i> Diels   |
| ❖ <i>Erythrina edulis</i>          | ❖ <i>Erythrina speciosa</i>           |
| ❖ <i>Erythrina flabelliformis</i>  | ❖ <i>Erythrina tahitensis</i>         |
| ❖ <i>Erythrina haerdii</i> Verdc.  | ❖ <i>Erythrina variegata</i> L.       |
| ❖ <i>Erythrina hazomboay</i>       | ❖ <i>Erythrina vespertilio</i> Benth. |

(Wikipedia, 2019)

#### 1.3.4 The plant *Erythrina variegata*

In the study, one plant species belonging to family Fabaceae was investigated.

##### Synonyms

- *Erythrina corallodendrum* var. *orientalis* L.
- *Erythrina indica* Lam.
- *Erythrina orientalis* (L.) Merrill (Kumar et al., 2010).

##### Common names

- Coral tree, Indian coral tree, tiger's-claw (English)
- Gatae (Samoa, Horne Islands, 'Uvea, Cook Islands)
- Dadapaykam (Java, Indonesia (Kumar et al., 2010)

#### 1.3.4.1 Taxonomic hierarchy of the investigated plant *E. variegata* (Kumar *et al.*, 2010)

Kingdom	Plantae – Plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Family	Fabaceae (Legume family)
Subfamily	Papilionoideae
Genus	<i>Erythrina</i> L. – Coral Tree
Species	<i>E. variegata</i> L.

#### 1.3.4.2 Morphology of *Erythrina variegata* L.

*Erythrina variegata* Linn. commonly known as a coral tree, is a fast growing tropical tree usually found in Indonesia, Malaysia, Taiwan, Southern China, Philippines, Africa, Southeast Asia and India (Vanlalremkimi *et al.*, 2016).



**Figure 1. 3 Whole plant (1) and flower (2) of *E. variegata***

**Size:** *Erythrina variegata* is a deciduous tree, 9-88 feet tall tree with fluted bole and much branched crown; their stem and branches are thick and sappy, generally equipped with big, spread prickles (Orwa *et al.*, 2009).

**Leaves:** Leaves are alternate, trifoliolate; stipules lanceolate, 1-1.5 cm long, caducous; petiole 2-28 cm long, unarmed; rachis 10-12 cm long; petiolule up to 1.5cm long, at base with globose glandular stipels; leaflets ovate to broadlyrhomboid, usually wider than long, 4-25 cm x 5-30 cm (Orwa *et al.*, 2009).

**Flowers:** Inflorescence of many-flowered fascicles occurs in terminal or axillary racemes up to 20 cm (8 in) or more long (Preeti K, 2017). *E. variegata* has the typical 'bird flowers' of *Erythrina* spp. scentless, strong and elastic to withstand birds hopping about and poking into the flowers (Orwa et al., 2009).

**Fruit:** Fruit covering dry or hard; Fruit colour brown; Fruits are compressed (Preeti K, 2017).

**Seeds:** Seeds are kidney-shaped, dark purple to red, and 1–1.5 cm (0.4–0.6 in) in length. (Orwa et al., 2009).

**Pod:** Pods are sausage-shaped or long cylindrical, 10-45 cm x 2-3 cm, 1-13-seeded, slightly constricted between the seeds, glabrescent, distinctly veined and exocarp bursting irregularly, indehiscent (Orwa et al., 2009).

#### 1.3.4.3 Economic and medicinal value of *Erythrina variegata*

*Erythrina variegata* is cultivated to fix the soil nitrogen. The large size of the plant makes it suited for planting in golf courses, parks and in other large-scale landscape.

Traditionally various sections of the plant *E. variegata* have been used in the popular system of medicine like Ayurveda, Siddha, Unani and Homeopathy systems for healing of some diseases like fever, bacterial infection, convulsion, inflammation, insomnia, wounds, cuts, cough and helminthiasis (Warrier and Nambiar, 1993). In addition it is also used as anti-asthmatic, febrifuge, antiepileptic and nervine sedative (Anwar, 2006). The juice of the leaves used as an anodyne in toothache and is poured in to the ear to relieve earache. Mixed with honey the leaf juice is ingested to kill different types of worm like tape worm, round worm and thread worm. It is also used to stimulate lactation and menstruation and is used as diuretic, laxative and expectorant (Warrier and Nambiar, 1993).

The plant leaves has found to have powerful effects towards the treatment of various other diseases due to its stomachic, laxative, diuretic, galactagogue and emmenagogue properties; sometimes it is externally used for dispersing venereal buboes, relieve pain of the joints (Suryawanshi, and Patel, 2011). The bark has astringent and antibilious effects; beneficial as a collyrium in ophthalmia and in dysentery. The roots are emmenagogue (Suryawanshi and Patel, 2011).

#### 1.3.4.4 Reported biological activities of *Erythrina variegata*

*Erythrina variegata* has been ethnomedicinally used as a therapeutic agent for a variety of diseases. Alkaloids, flavonoids and other secondary metabolites which are present in this plant might be responsible for its pharmacological activities.

##### **Antimicrobial activity**

New isoflavone named Eryvarins W exhibited a potent antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Tanaka et al., 2011). Isoflavonoids erycristagallin and orientanol B showed the highest anti-MRSA activity (3.13–6.25  $\mu\text{g ml}^{-1}$ ) (Tanaka et al., 2002).

##### **Antioxidant activity**

Isolated compounds 4',5,7-trihydroxy-8-prenyl isoflavone alpinum isoflavone and 6-hydroxygenistein, exhibited high antioxidant activity having  $\text{IC}_{50}$  of 6.42, 8.30 and 8.78  $\mu\text{g/ml}$ , respectively (Rahman et al., 2010).

##### **Anti osteoporotic activity**

Histomorphometric analysis of the proximal end of the tibia showed that the *E. variegata* extract prevented the estrogen deficiency-induced decrease in trabecular thickness and trabecular area (Zhang et al., 2007).

##### **Analgesic and anti-inflammatory activity**

The methanolic extract of the leaf of *E. variegata* at a dose of 500 mg/kg showed significant antinociceptive activity with 49.03% inhibition of writhing response in acetic acid induced writhing model and in radiant heat tail-flick model, the extract also showed significant increase in the tail flick latency at a dose of 500mg/kg body weight with 36.02% elongation of tail flick time (Haque et al., 2006).

##### **CNS effects**

The total alkaloid fraction from the bark showed several characteristic pharmacological effects: neuromuscular blocking, CNS depressant, and anticonvulsant effects (Ghosal et al., 1972). *E. variegata* also causes passivity and decreases spontaneous activity with positive grip strength which indicates CNS relaxant activity (anxiolytic) of this plant (Anwar et al., 2006).

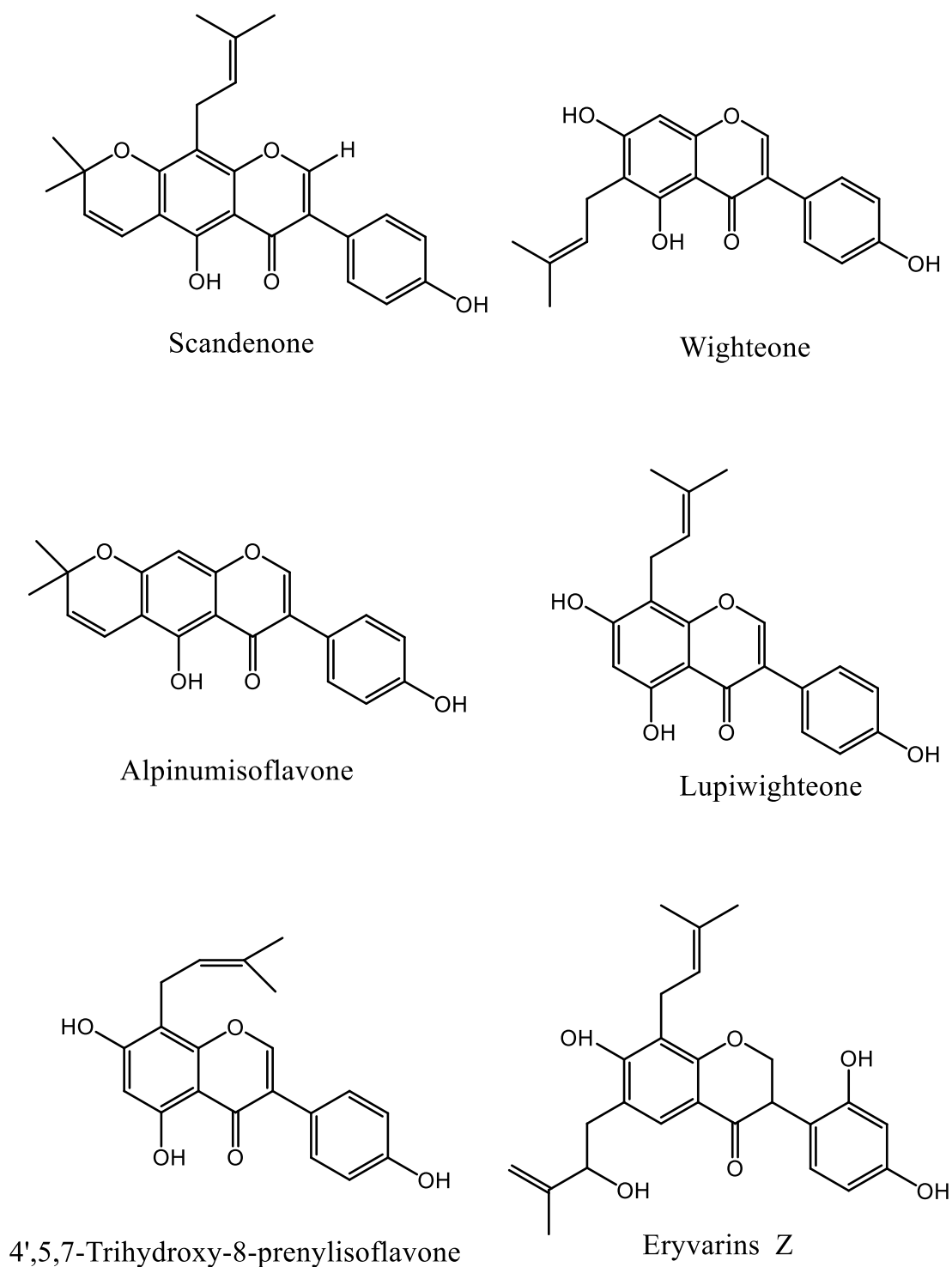
**Table 1.3** Previous phytochemical investigations on *E. variegata*

<b>Plant Part studied</b>	<b>Compound Isolated</b>	<b>References</b>
Flower	Erythrivarines C–G	Zhang et al., 2016
Flower	Erythrivarine A and B	Zhang , 2014
Stem bark	4',5,7-Trihydroxy-8-methylisoflavone 4',5,7-Trihydroxy-8-prenylisoflavone Scandenone	Rahman et al., 2010
Stem bark	Alpinum isoflavone Epilupeol 6-Hydroxygenistein 3 $\beta$ , 28-Dihydroxyolean-12-ene Stigmasterol	Rahman et al., 2007
Stem bark	5,4'-Dihydroxy-8-(3,3-dimethylallyl)-2''-methoxyisopropylfurano[4,5,6,7]isoflavone 5,7,4'-Trihydroxy-6-(3,3-dimethylallyloxiranylmethyl) isoflavone 5,4'-Dihydroxy-8-(3,3-dimethylallyl)-2''-hydroxymethyl-2''-methylpyrano[5,6,6',7]isoflavone 5,4'-Dihydroxy-2'-methoxy-8-(3,3-dimethylallyl)-2'',2''-dimethylpyrano[5,6,6',7], Isoflavanoneeuchrenone b10 Isoerysenegalensein E Wighteone Laburnetin Lupiwighteone Erythrodiol Oleanolic acid	Xiaoli et al., 2006.
Bark	Erysotine, Erythratidine Epi-ery-thratidine 11-Hydroxy-epi-erythratidine	Chawla et al, 1988
Wood	Eryvarin A and B	Tanaka et al., 2000.
Seed	Isolectins (EVLI, EVLII and EVLIII)	Yamasaki et al., 1992

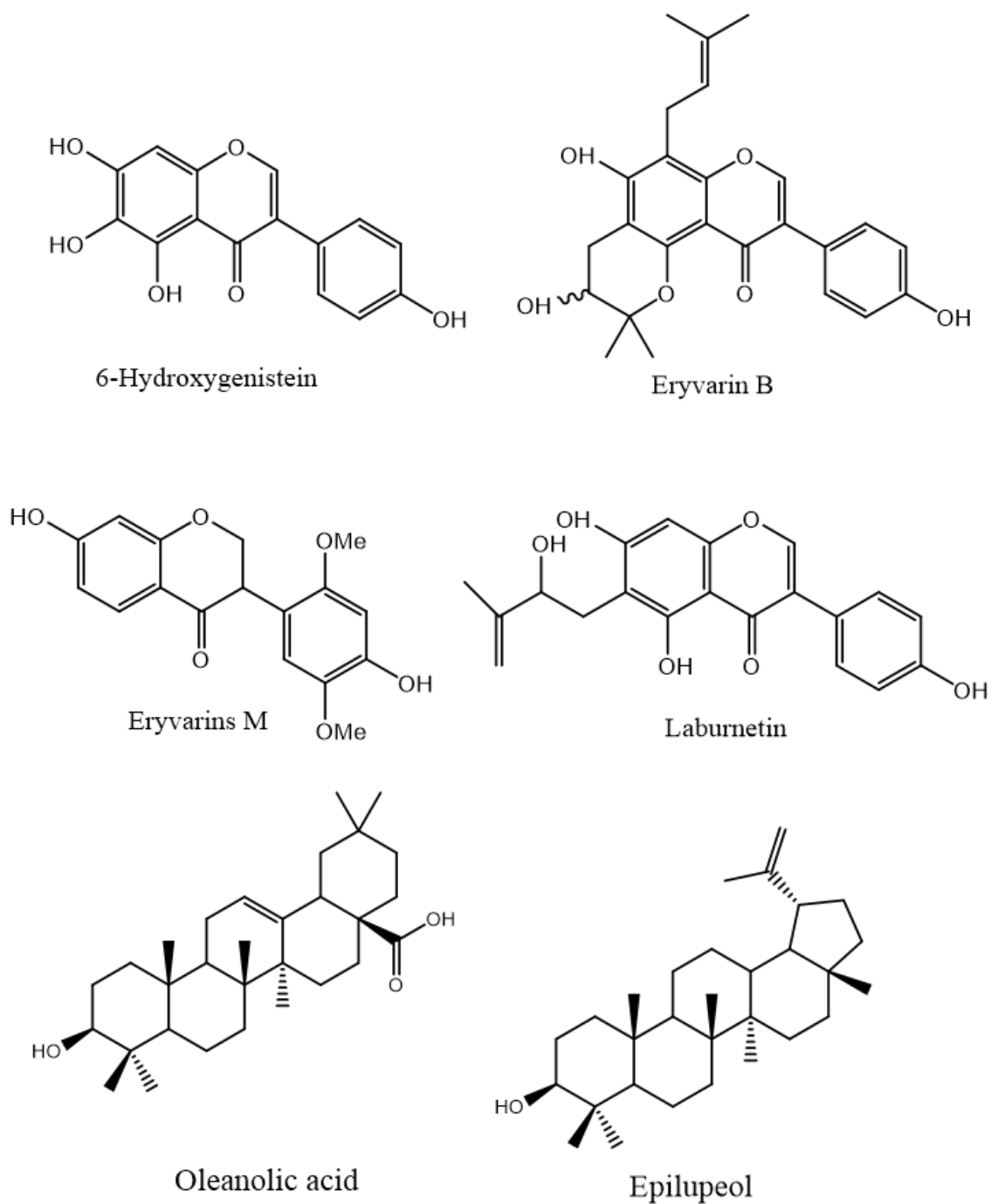
**Table 1.3 (cont.) Previous phytochemical investigations on *E. variegata***

<b>Plant studied</b>	<b>Part</b>	<b>Compound Isolated</b>	<b>References</b>
Root		Eryvarins Y and Z	Tanaka et al., 2015
Root		Eryvarins V, W and X	Tanaka et al., 2011
Root		Biseryvarin A	Tanaka et al., 2010
Root		Eryvarins M, N and O Eryvarins P, Q and R	Tanaka et al., 2004
Root		Eryvarins F and G	Tanaka et al., 2003
Root		Scandenone 5,7,4'-Trihydroxy-6,8-diprenylisoflavone Erycristagallin Erythrabys-sin-II Phaseollin Phaseollidin Isobavachin Cinnamyl phenol	Telikepalli et al., 1990
Various parts		N,N-Dimethyltryptophan Spiroamine alkaloids Carboxylated indole-3-alkylamines	Ghosal et.al,1972
Various parts		Erysotrine	Ghosal, 1970





**Figure 1.4** Structures of some previously reported phytochemicals from *E. variegata*

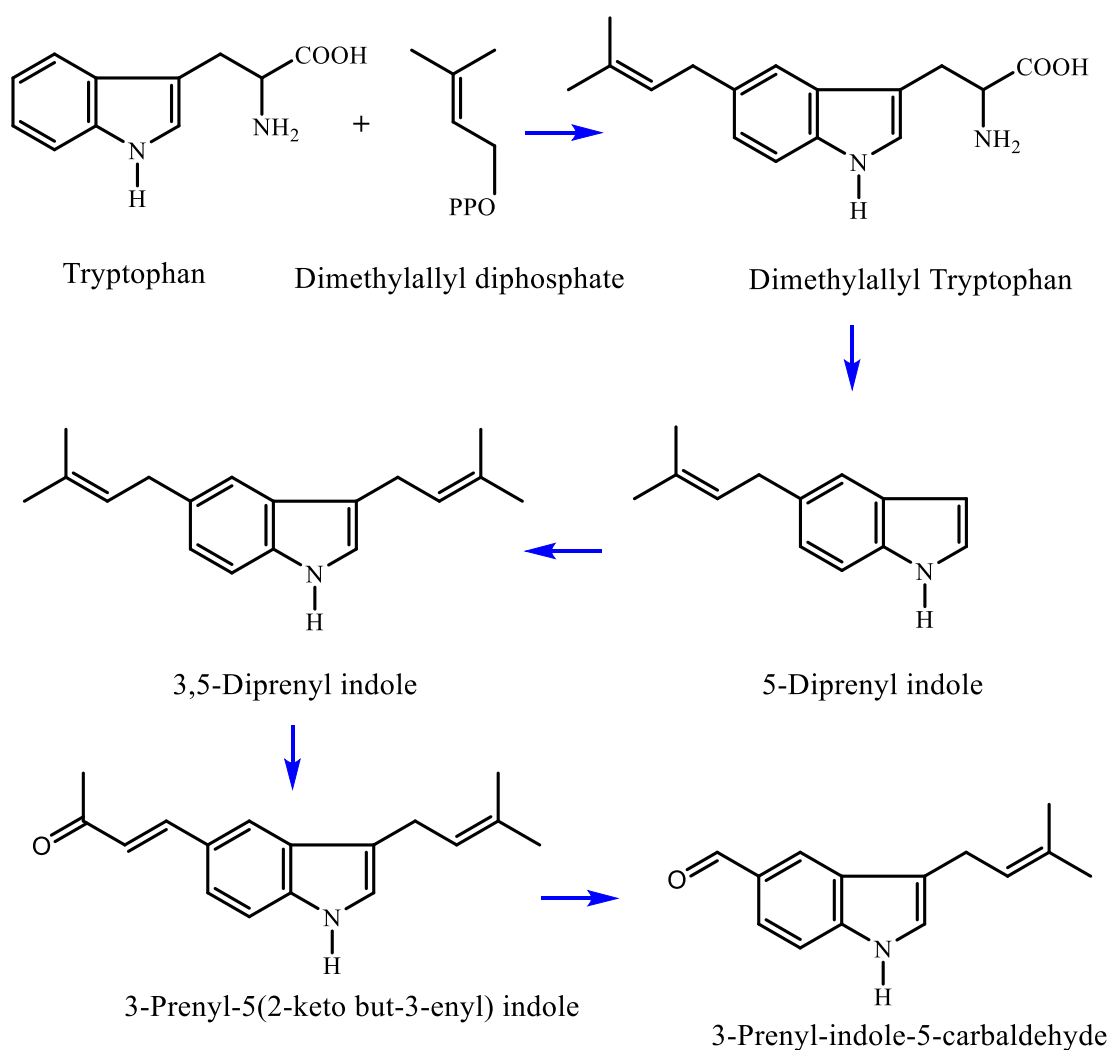


**Figure 1.4 (cont.)** Structures of some previously reported phytochemicals from *E. variegata*

## 1.4 Biosynthesis of secondary metabolites of Rutaceae

### 1.4.1 Biosynthesis of prenylated indole alkaloids

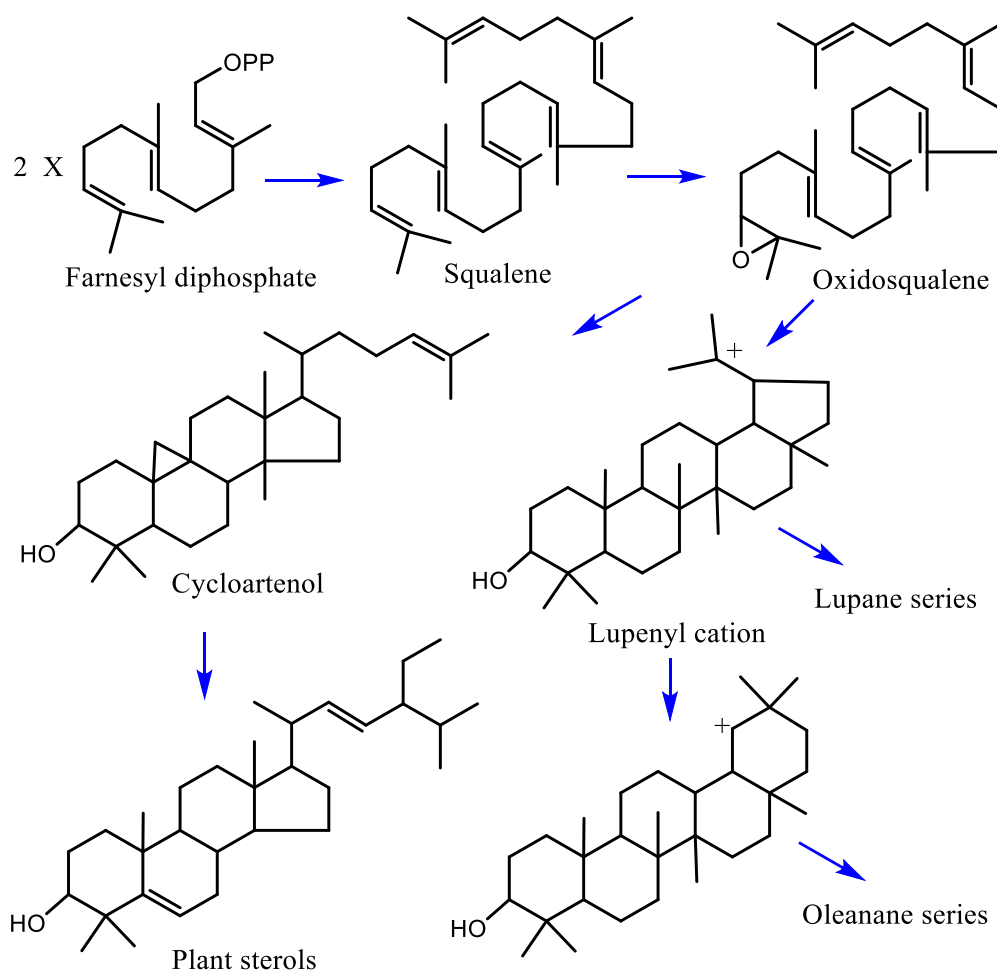
Biosynthesis of indole alkaloid have been studied extensively in fungi and bacteria. The amino acid tryptophan is the precursor of all indole alkaloids. Prenyl transfer reactions catalysed by aromatic prenyltransferases represent key steps in the biosynthesis of these compounds (Steffan et al., 2009, Ozaki et al., 2013). A possible biosynthetic route to prenylated indole alkaloids of ravenia from tryptophan is shown in Scheme 1.1.



**Scheme 1.1 Biosynthesis of prenylated indole alkaloids**

### 1.4.2 Biosynthesis of triterpenes and sterols

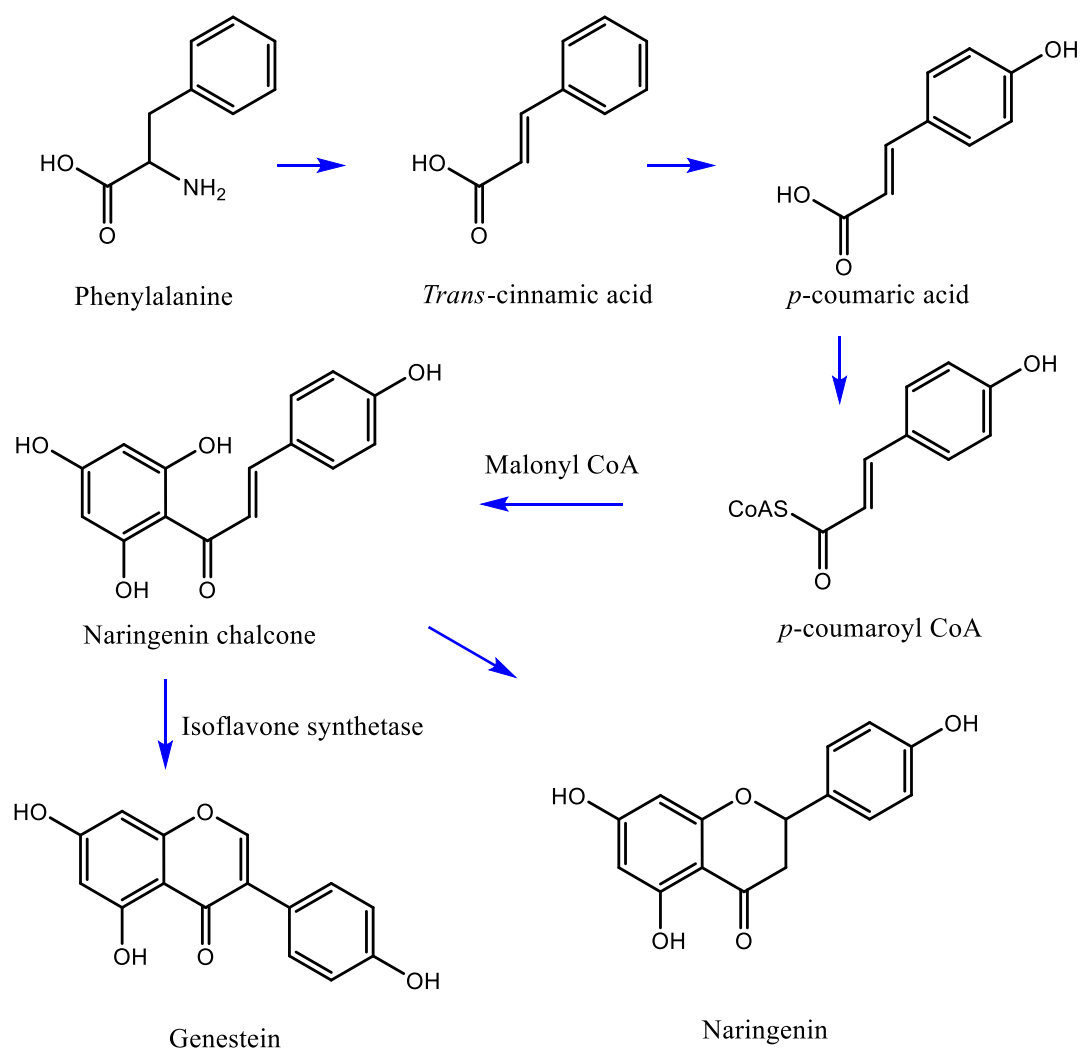
Two molecules of farnesyl diphosphate condensed to form squalene which is the C<sub>30</sub> precursor of triterpenes (Scheme 1.2). The enzyme squalene epoxidase convert squalene into oxidosqualene. 2, 3-oxidosqualene is cyclized into cyclic triterpenes by the enzyme oxidosqualene cyclase. Because of the presence of an epoxide in oxidosqualene, all of the cyclic triterpenes derived from this precursor possess oxygen functionality at the C-3 position. These cyclic triterpenes are further converted into various plant sterols (Kushiro and Ebizuka, 2010; Jäpelt and Jakobsen; 2013, Iturbe-Ormaetxe, 2003).



**Scheme 1.2** Biosynthesis of triterpenes and sterols

### 1.4.3 Biosynthesis of Isoflavonoids and flavonoids

Isoflavones and other related compounds are produced by phenylpropanoid pathway that begins from the amino acid phenylalanine. Phenylalanine is first converted to *trans*-cinnamic acid and then to *p*-coumaric acid. The enzyme *p*-coumarate-CoA converts the latter to *p*-coumaroyl-CoA. Malonyl-CoA then condenses with *p*-coumaroyl-CoA to form naringenin chalcone which is the precursor of all types of flavonoids, isoflavonoids and chalcones (Saito et al., 2013, Gupta et al., 2017)



**Scheme 1.3 Biosynthesis of Isoflavonoids and flavonoids**

## 2.1 Plant materials

At first with the help of a comprehensive literature review two plants namely *Ravenia spectabilis* Lindl. and *Erythrina variegata* L. were selected for the phytochemical and biological investigations.

### 2.1.1 Collection of the plant materials

Leaves of *Ravenia spectabilis* were collected at its fully form from the campus of University of Dhaka in the month of February, 2015 and was identified by the taxonomist Dr. Mahbuba Sultana of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. A voucher specimen (Accession no. 46423) of the plant has been deposited in the same herbarium for future reference.

The stem bark of the second plant *Erythrina variegata* was collected from the area of South Fular road, University of Dhaka in the month of December, 2015 which was identified by another taxonomist Shah Mohammad Ahsan Habib of the same herbarium. A voucher specimen (Accession number DACB No. 46874) of the plant was also deposited in national herbarium for future reference.

The plant parts were cleaned or sorted out properly from dust and other plant materials and were cut into small pieces and subjected to shade drying for one week for the leaf of *Ravenia spectabilis* and two weeks for the stem bark of *Erythrina variegata*. The dried plant parts were then crushed into coarse powder by a high capacity grinding machine with proper care.

### 2.1.2 Extraction of the plant materials

About 1 Kg air dried powdered plant material of *Ravenia spectabilis* (leaf) was soaked in methanol (3L) for 10-15 days and filtered through a cotton plug. The extract (34.5 g) was then concentrated under reduced pressure using a Buchii rotary evaporator.

Dried powder of the second plant *Erythrina variegata* (900 gm) was soaked in 2.5 L methanol for 20 days. The methanol extract was then filtered and concentrated by the same manner and 25.6 g gm of concentrated methanol extract was obtained.

## 2.2 Isolation techniques

Using various chromatographic and other techniques, pure compounds were isolated from the crude and fractionated extracts of *R. spectabilis* and *E. variegata*. A general description of isolation of compounds is discussed below:

### 2.2.1 Vacuum Liquid Chromatography (VLC)

Vacuum liquid chromatography (VLC) is most efficient chromatographic technique for the rapid fractionation of the crude as well complex synthetic and natural products mixture. In the last decade VLC has been progressively applied in the field of natural products as well as in synthetic chemistry because of its simplicity of operation (Maurya et al., 2018). The technique was used for fractionation of both the extracts of *R. spectabilis* and *E. variagata*. The VLC method and apparatus explicated by Professor Pelletier (Pelletieret al., 1986) was pursued here. The column was packed under vacuum with fine VLC grade silica (Kiesel gel 60H) up to a height of 6 cm and subsequently washed with pet-ether to ensure compact packing. The sample was prepared by dissolving it in small amount of methanol and mixing with column grade silica followed by evaporation of the solvent. The dried sample was applied to the top of the column and the elution was commenced with pet-ether, polarity of which was gradually increased by adding more polar solvents like dichloromethane, ethyl acetate and methanol respectively.

### 2.2.2 Thin Layer Chromatography (TLC)

Aluminum or plastic sheet of Precoated silica gel (Keisel gel 60 PF 254) plates (20×20) were used for screening different fractions of the crude extracts, for checking the purity of the isolated compounds and also for identifying a known compound by co-TLC with an authentic sample. The  $R_f$  value may also be used in rough identification of a compound since it is characteristic for each compound in a particular solvent system. Since slight variation in solvent system may affect the  $R_f$  value, co-TLC in different solvent system is often used for identification.

$R_f$  value of a compound can be calculated by the following formula

$$R_f \text{ value} = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent system}}$$

**Table 2.1** Different solvent systems used for VLC of Methanol extract of *R. spectabilis*

<b>Fraction no.</b>	<b>Solvent systems (%)</b>	<b>Volume collected (ml)</b>
<b>1 &amp; 2</b>	100% PE	100
<b>3</b>	2% DCM in PE	100
<b>4</b>	5% DCM in PE	100
<b>5</b>	10% DCM in PE	100
<b>6</b>	15% DCM in PE	100
<b>7</b>	25% DCM in PE	100
<b>8</b>	40% DCM in PE	100
<b>9</b>	50% DCM in PE	100
<b>10</b>	70% DCM in PE	100
<b>11</b>	80% DCM in PE	100
<b>12</b>	100% DCM	100
<b>13</b>	2% EA in DCM	100
<b>14 to 17</b>	5% EA in DCM	100
<b>18</b>	8% EA in DCM	100
<b>19</b>	10% EA in DCM	100
<b>20</b>	15% EA in DCM	100
<b>21</b>	20% EA in DCM	100
<b>22</b>	25% EA in DCM	100
<b>23 to 26</b>	30% EA in DCM	100
<b>27 to 29</b>	50 % EA in DCM	100
<b>30</b>	80 % EA in DCM	100
<b>31 to 34</b>	100 % EA in DCM	100
<b>35 &amp; 36</b>	1% MeOH in EA	100
<b>37</b>	5% MeOH in EA	100
<b>38</b>	25 % MeOH in EA	100
<b>39</b>	50 % MeOH in EA	100
<b>40 to 42</b>	80 % MeOH in EA	100
<b>43</b>	100 % MeOH in EA	100

PE = Petroleum Ether; DCM= Dichloromethane; EA = Ethyl Acetate; MeOH = Methanol



**Table 2.2 Different solvent systems used for VLC of Methanol extract of *E. variegata***

<b>Fraction no.</b>	<b>Solvent systems (%)</b>	<b>Volume collected (ml)</b>
<b>1 &amp; 2</b>	100 % PE	100
<b>3</b>	5 % DCM in PE	100
<b>4</b>	15 % DCM in PE	100
<b>5</b>	30 % DCM in PE	100
<b>6</b>	40 % DCM in PE	100
<b>7</b>	80 % DCM in PE	100
<b>8 to 11</b>	100% DCM	100
<b>12</b>	5 % EA in DCM	100
<b>13</b>	8 % EA in DCM	100
<b>14</b>	10 % EA in DCM	100
<b>15</b>	15 % EA in DCM	100
<b>16</b>	20 % EA in DCM	100
<b>17</b>	30 % EA in DCM	100
<b>18</b>	40 % EA in DCM	100
<b>19</b>	50 % EA in DCM	100
<b>20</b>	60 % EA in DCM	100
<b>21</b>	70 % EA in DCM	100
<b>22</b>	80 % EA in DCM	100
<b>23</b>	90 % EA in DCM	100
<b>24 to 27</b>	100 % EA	100
<b>28</b>	1% MeOH in EA	100
<b>29</b>	5% MeOH in EA	100
<b>30 to 33</b>	10% MeOH in EA	100
<b>34</b>	20% MeOH in EA	100
<b>35</b>	30% MeOH in EA	100
<b>36 to 38</b>	50% MeOH in EA	100
<b>39 &amp; 40</b>	80% MeOH in EA	100
<b>41</b>	90% MeOH in EA	100
<b>42</b>	100 % MeOH	100

PE = Petroleum Ether; DCM= Dichloromethane; EA = Ethyl Acetate; MeOH = Methanol

Each of the fractions of VLC was spotted on TLC plates and using different suitable solvent systems chromatograms were developed. Under the UV light, the TLC plates were examined and then sprayed with spray reagents like Vanillin-sulphuric acid and Dragendorff's reagent. The fractions showing similar type of mixture of chemical compounds were mixed together. Most of the fractions showed mixture of several compounds, suggesting for further fractionation.

### 2.2.3 Gel permeation chromatography

In gel permeation chromatography compounds are separated according to their molecular size. Here it was used for the successful separation of different pigments specially chlorophyll from VLC fractions.

Some VLC fractions of the extracts were selected by observing the TLC plates, for Gel permeation chromatography. A chromatographic column is packed with sephadex (LH-20). A glass column of approximately 30 cm in height and 2.5 cm in diameter was packed with a slurry of sephadex LH-20. A small quantity of chloroform was used to dissolve the sample and added to the top of the column. 20% petrol in methanol was used for the elution. At first chlorophyll and other pigments were eluted and subsequently 1 ml fractions were collected in each test tube. The polarity of the solvent was changed to 10% petrol in chloroform followed by 100% chloroform. To collect more polar compounds still remaining on the column, a solution of 5% methanol in chloroform was used. The column was finally washed with methanol to make it clean and use for the analysis of the next fractions. The solvent systems used as mobile phases in the gel permeation chromatography were listed in the table 2.3

**Table 2.3 The solvent systems used as mobile phases in GPC**

Serial No.	Solvent Systems
1	20% PC (Petroleum Ether : Chloroform=20: 80)
2	10% PC (Petroleum Ether :Chloroform= 10:90)
3	5% PC (Petroleum Ether :Chloroform= 5:95)
4	100% Chloroform
5	5% MC (Methanol: Chloroform=5:95)
6	100% Methanol

#### 2.2.4 Preparative thin layer chromatography (PTLC)

Preparative thin layer chromatography or PTLC is a routinely employed method for the separation and final purification of compounds. The plates were prepared by making a slurry using 35 gm of silica gel (Kieselgel 60 PF 254) with 75 ml of water and spreading it on 5 plates (20 x 20 cm) to produce a layer of 0.5 mm thickness. The plates were dried in the air and then activated by heating in an oven at 105<sup>0</sup>C for 1 hour. The sample was dissolved in a suitable solvent and applied onto the plate as a band by a Pasteur pipette. The chromatography was carried out in a glass tank of 22 x 22 cm with 100 ml of a suitable solvent system. After the development was complete, the plate was dried and the bands of compounds were detected by UV at 254 and 366 nm or by spray reagent (sprayed on one side of the plate). The bands were then scraped off with a spatula and the compound was washed out of the adsorbent by a suitable solvent.

#### 2.2.5 Solvent treatment

By solvent treatment, a compound consisting of the major portion of a mixture of compounds can be purified utilizing selective solvent washing. Initially, a solvent or a solvent mixture in which the desired compound is practically insoluble and other components are soluble is chosen. The undesired components are separated with repeated washing with this solvent or solvent mixture. Other solvent or solvent mixture can be also be utilized until a pure compound is obtained.

### 2.3 Detection of compounds

To analyze the extractives to isolate pure compounds, detection of compounds in TLC plate is very important. The following techniques are used for detecting the compounds in TLC/PTLC plates.

- i. At first the developed chromatogram was examined visually to detect the presence of colored compounds.
- ii. The developed and dried plates were also observed under UV light of both long and short wavelength (254 nm and 366 nm) to detect the spot/band of any compound. Some of the compounds appear as fluorescent spots while the others as dark spots under UV light.

- iii. In this investigation two types of spray reagents were used depending upon the nature of compounds expected to be present in the fractions or the crude extracts.

#### **Vanillin-H<sub>2</sub>SO<sub>4</sub>**

1% vanillin in concentrated sulfuric acid was used as a general spray reagent followed by heating the plates to 100 °C for 2-5 minutes (Stahl, 1966).

#### **Modified Dragendorff's reagent**

Modified Dragendorff's reagent was used to detect alkaloids. The reagent is prepared by mixing equal parts (v/v) of 1.7% bismuth subnitrate dissolved in 20% acetic acid in water and a 40% aqueous solution of potassium iodide (Touchstone and Dobbins, 1977).

### **2.4 Process flow diagram for the isolation and identification compounds**

1 Kg Powdered leaf of *Ravenia spectabilis* Lindl. and 900 gm bark of *Erythrina variegata* L. were soaked in methanol separately for 20 days.



The conc. methanol extracts were fractionated by VLC over Silica gel 60H using different solvents of increasing polarity.



The VLC fractions were then screened by TLC and important VLC fractions were subjected to Gel Permeation Chromatography on Sephadex LH-20.



Twenty one compounds were obtained and purified by Preparative Thin Layer Chromatography (PTLC) or crystallization.



Isolated compounds were characterized by extensive spectroscopic studies like- <sup>1</sup>H NMR, <sup>13</sup>C NMR, HSQC, HMBC, COSY and NOSEY experiments and molecular weight was determined by ESI mass spectrometry.

## 2.5 Instrumentation

### 2.5.1 IR spectroscopy (IR)

Infrared spectroscopy (IR spectroscopy) is the spectroscopy that deals with the infrared region of the electromagnetic spectrum, that is light with a longer wavelength and lower frequency than visible light. In this experiment, IR spectra were recorded as KBr discs or film using a Shimadzu Fourier Transform Infrared Spectrophotometer model no, FTIR-8400.

### 2.5.2 Mass spectrometry (MS)

A substance can be transformed into gas phase ions by various ionization methods. These ions are then accelerated by an electromagnetic field, separated by their mass to charge ( $m/z$ ) ratio and counted by a detector. The signal is recorded and output as a graph of the number of ions detected *versus* their  $m/z$  ratio, called a mass spectrum. (Pavia et al., 2009). In the present work, high-resolution mass spectra were obtained on a Thermo Navigator mass spectrometer coupled to LC using electrospray ionisation (ES) and time-of-flight (TOF) mass spectrometry.

### 2.5.3 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) has been shown to be a powerful spectroscopic method for the structural determination of natural products, especially novel compounds. In the present work, NMR spectra were measured at 400 MHz for  $^1\text{H}$  NMR spectra and 100 MHz for  $^{13}\text{C}$  on a Bruker 400<sup>TM</sup> ASCEND spectrometers in  $\text{CDCl}_3$ .

### 2.5.4 One dimensional (1D) NMR spectra

$^1\text{D}$  NMR spectra are typically displayed as an absorption spectra, the axes of which are the frequency (chemical shift) and the intensity. Many functional groups or types of hydrogens or carbons contained in a molecule can be identified by the characteristic chemical shift values in  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra.

### 2.5.5 $^1\text{H}$ NMR spectra

Protons in a molecule have resonances at various frequencies because of their different chemical environment.  $^1\text{H}$  NMR spectra reveal information about types of hydrogens and the number of each in a molecule based on their chemical shifts, integration values and coupling constants (Pavia et al., 2009).

### 2.5.6 Proton-decoupled $^{13}\text{C}$ NMR spectra

Spin-spin coupling between  $^{13}\text{C}$  atoms are rarely observed but the spin-spin interaction of protons bonded directly to  $^{13}\text{C}$  atoms can split the carbon signal responding to the  $n + 1$  rule. In proton-decoupled  $^{13}\text{C}$  NMR spectra, overlapping multiplets are transformed into singlets and therefore, the spectra are easier to interpret.  $^{13}\text{C}$  NMR spectra show information about the number and types of carbons and functional groups (Pavia et al., 2009).

### 2.5.7 Two dimensional (2D) NMR spectra

2D NMR spectra are obtained by recording resonance signals as a function of two time variables and carrying out two Fourier transformations on a matrix of data. Therefore both of the horizontal and vertical axes in 2D NMR spectra are two chemical shift (frequency) axes. The 2D spectra give cross peaks showing correlations between the two axes and the data are displayed as a series of contours (Mitchell and Costisella, 2007).

### 2.5.8 $^1\text{H}$ - $^1\text{H}$ Correlation Spectroscopy (COSY)

A COSY spectrum shows homonuclear correlations between coupling protons in a molecule. The spectrum provides information on which proton couples with which one. It also indicates  $H-H$  connectivities, *geminal*, *vicinal* or long range couplings (Breitmaier, 2002).

### 2.5.9 Heteronuclear Single Quantum Correlation (HSQC) spectra

A HSQC spectrum displays the heteronuclear correlations of protons with  $^{13}\text{C}$  atoms to which they are directly attached. All C-H single bonds of the molecule can be determined by the HSQC spectrum (Breitmaier, 2002).

### 2.5.10 Heteronuclear Multiple Bond Coherence (HMBC) spectra

The HSQC spectrum shows the  $^1\text{H}$ - $^{13}\text{C}$  correlations through a single bond ( $^1J_{\text{CH}}$ ) and thereby is only applied to  $^{13}\text{C}$  atoms which are attached by protons (Breitmaier, 2002).

### 2.5.11 Nuclear Overhauser Effect Spectroscopy (NOESY)

NOESY spectra display the correlations of protons that are close to each other in space with the common distance of 4.5 Å or less. NOESY spectra are extremely helpful for the determination of relative stereochemistry in molecular structures (Silverstein et al., 2005).

### 2.6.1 Isolation and purification of compounds

Using different chromatographic techniques *Ravenia spectabilis* afforded a total of fourteen pure compounds and *Erythrina variegata* afforded seven compounds. Table 2.4 & Table 2.5 represent the summary of the compounds isolated.

**Table 2.4 Isolation of compounds from crude methanol extracts of *R. spectabilis***

Code	Physical appearance	VLC fractn no.	Sepha fractn no.	Further purification steps	Rf value	Under UV light (254 nm)	Color with vanillin /H <sub>2</sub> SO <sub>4</sub>
TRS-71	Colorless square shaped crystals	7	8-12	Crystallization by n-Hexane	0.54 (50% HT)	Dark quenching	Brown
TRS-157	Brown powder	15	44-46	PTLC using 0.1% Ethyl Acetate in Toluene	0.50 (0.1% ET)	Light brown	Pink
TRS-159	Brown powder	15	32 & 33	PTLC using 0.1% Ethyl acetate in Toluene	0.52 (0.1% ET)	Light brown	Pink
TRS-146	Colorless crystals	14	15	PTLC using 0.1% Ethyl acetate in Toluene	0.54 (0.1% ET)	Blue fluorescent band	Brown
TRS-153	Yellow noncrystalline mass	15	17-20	Crystallization by n-Hexane (with few drops of ethyl acetate)	0.52 (5% ET)	Dark quenching	Brown
TRS-206	Yellowish gummy mass	20	22-25	Crystallization by n-Hexane (with few drops of ethyl acetate)	0.53 (10% ET)	Blue fluorescent band	Brown
TRS-221	Greenish yellowish crystals	19-22	-	Crystallization by n-Hexane (with few drops of ethyl acetate)	0.50 (10% ET)	Blue fluorescent band	Brown
RSD-140	Yellowish gum	14	12-15	PTLC using 0.1% Ethyl acetate in Toluene	0.50 (5% ET)	Blue fluorescent band	Brown
RSD-164	Yellow gum	16	17	PTLC using 5% ethyl acetate in toluene	0.53 (10% ET)	Dark quenching	Brown

**Table 2.4 (cont.) Isolation of compounds from crude methanol extracts of *R. spectabilis***

Code	Physical appearance	VLC fractn no.	Sepha fractn no.	Further purification steps	Rf value	Under UV light (254 nm)	Color with vanillin /H <sub>2</sub> SO <sub>4</sub>
RSD-167	Yellow gum	16	35	PTLC using 5% ethyl acetate in toluene	0.50 (10% ET)	Dark quenching	Brown
RSD-180	Yellowish mass	14	45	PTLC using 0.3% ethyl acetate in toluene	0.50 (0.3% ET)	Light brown	Light brown
RSD-137	Colourless crystals	15	30-35	Crystallization by n-Hexane(with few drops of ethyl acetate)	0.55 (15% ET)	No color	Purple
TRS-121	Colorless fine needle	12	9 & 10	PTLC using 5% hexane in toluene.	0.51 (5% HT)	No color	Purple
TRS-101	White powder	10	4 & 5	PTLC using 5% hexane in toluene.	0.56 (5% HT)	No color	Purple

Here, E=Ethyl acetate, T=Toluene, H=*n*-Hexane



**Table 2.5 Isolation of compounds from crude methanol extracts of *E. variegata***

Code	Physical appearance	VLC fractn no.	Sepha fractn no.	Further purification steps	Rf value	Under UV light (254 nm)	Color with vanillin /H <sub>2</sub> SO <sub>4</sub>
TEV-171	Yellow needle	17	12-13	Crystallization by n-Hexane(with few drops of ethyl acetate)	0.53 (10% ET)	Dark quenching	Yellow
TEV-176	Yellow needle	17	23-26	PTLC using 10% ethyl acetate in toluene	0.53 (10% ET)	Dark quenching	Yellow
TEV-121	Colourless crystals	12	20-25	Crystallization by n-Hexane	0.6 (15% ET)	No color	Purple
TEV-131	Colourless crystals	13	30-33	Crystallization by n-Hexane(with few drops of ethyl acetate)	0.50 (10% ET)	No color	Purple
TEV-131	Colourless crystals	13	30-33	Crystallization by n-Hexane(with few drops of ethyl acetate)	0.50 (10% ET)	No color	Purple
TEV-161	Colourless crystals	17	35-37	Crystallization by n-Hexane(with few drops of ethyl acetate and chloroform)	0.52 (10% ET)	No color	Purple
TEV-1711	Colourless crystals	16	27-32	Crystallization by n-Hexane(with few drops of ethyl acetate)	0.55 (15% ET)	No color	Purple

### 3.1 Characterization of compounds isolated from *Ravenia spectabilis* and *Erythrina variegata*

A total of 21 compounds were isolated from the crude methanol extract of the leaves of *Ravenia spectabilis* and the bark of *Erythrina variegata*. The structures of the compounds were elucidated by extensive NMR studies like  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, HSQC, HMBC, COSY and NOSEY experiments and mass spectrometry. Name of the compounds, their code no. and their chemical nature are listed in Table 3.1 and Table 3.2.

**Table 3.1 Compounds isolated from *Ravenia spectabilis***

Serial number	Code no.	Type of compound	Name of the compound
Compound 1	TRS-71	Indole alkaloid	3,5-Diprenylindole
Compound 2	TRS-157	Indole alkaloid	3-Prenyl-5-(2-keto-but-3-enyl)indole
Compound 3	TRS-159	Indole alkaloid	3-Prenyl-indole-5-carbaldehyde
Compound 4	TRS-146	2-quinolone alkaloid	Iso-oligophyline
Compound 5	TRS-153	2-quinolone alkaloid	Ravenoline
Compound 6	TRS-206	Furoquinoline alkaloid	$\gamma$ -Fagarine
Compound 7	TRS-221	Acridone alkaloid	Arborinine
Compound 8	RSD-140	2-quinolone alkaloid	Atanine
Compound 9	RSD-164	2-quinolone alkaloid	Oligophyline
Compound 10	RSD-167	2-quinolone alkaloid	Ravenine
Compound 11	RSD-180	Ester of fatty acid	Methyl linoleate
Compound 12	RSD-137	Sterol	$\beta$ -Sitosterol
Compound 13	TRS-121	Terpenoid	Ravespanol
Compound 14	TRS-101	Terpenoid	Ravespanone

**Table 3.2** Compounds isolated from *Erythrina variegata*

Serial number	Code no.	Type of compound	Name of the compound
Compound <b>15</b>	TEV-171	Isoflavone	Scandenone
Compound <b>16</b>	TEV-176	Isoflavone	Alpinumisoflavone
Compound <b>17</b>	TEV-121	Triterpinoid	Lupeol
Compound <b>18</b>	TEV-131	Steroid	Stigmast-4-en-3-one
Compound <b>19</b>	TEV-131	Steroid	Stigmasta-4, 22-dien-3-one
Compound <b>20</b>	TEV-161	Steroid	Stigmasterol
Compound <b>21</b>	TEV-1711	Triterpinoid	3 $\beta$ , 28-Dihydroxyolean-12-ene

Altogether ten alkaloids were isolated of which compounds **1-4** were found to be new natural compounds. Compounds **13** and **14** were also new but very unusual C<sub>34</sub> terpenoids. Compound **11** is reported for the first time from *Ravenia spectabilis*. The structures of the compounds are shown in Figure 3.1.

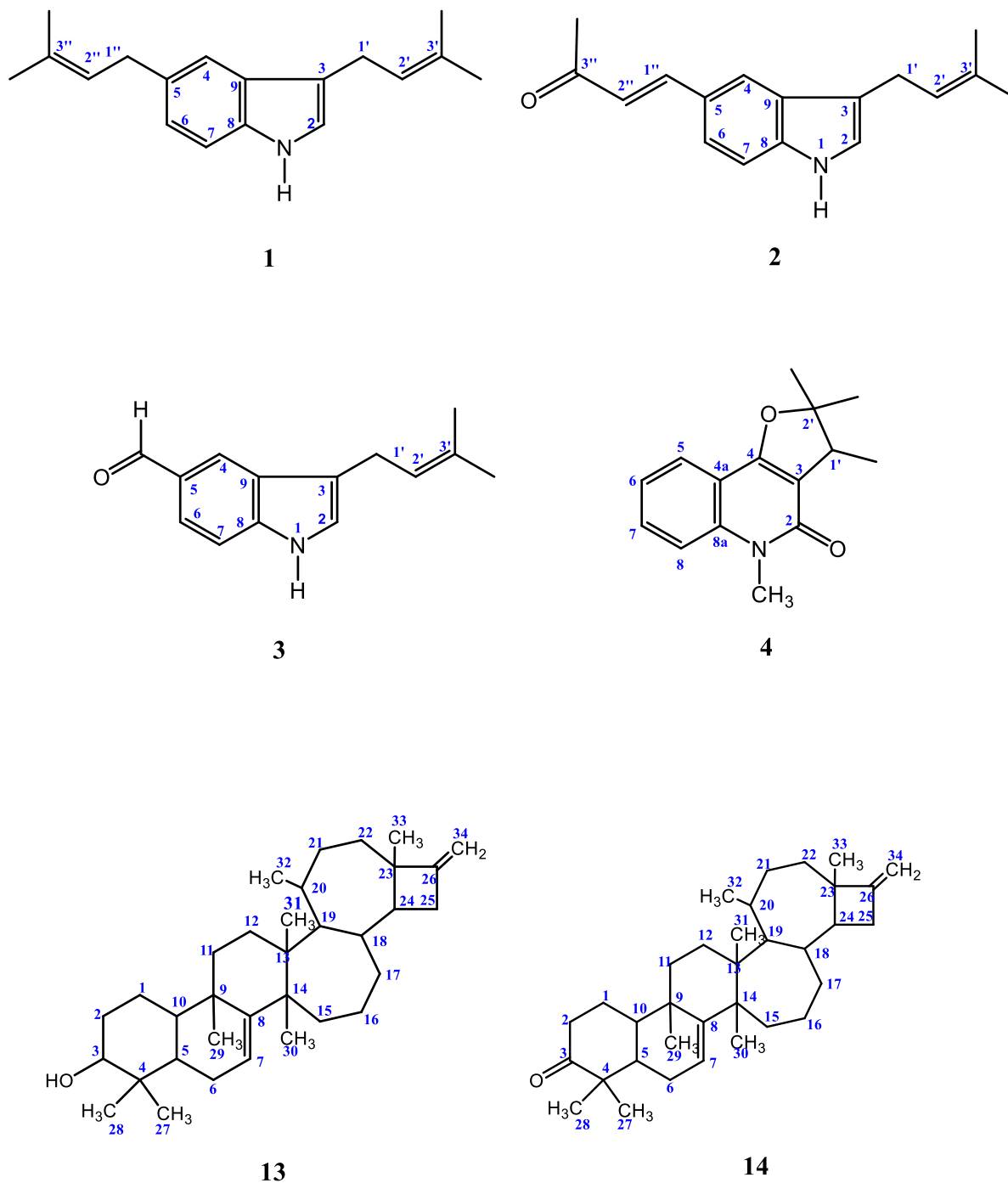


Figure 3.1 Structures of the compounds isolated from *Ravenia spectabilis*

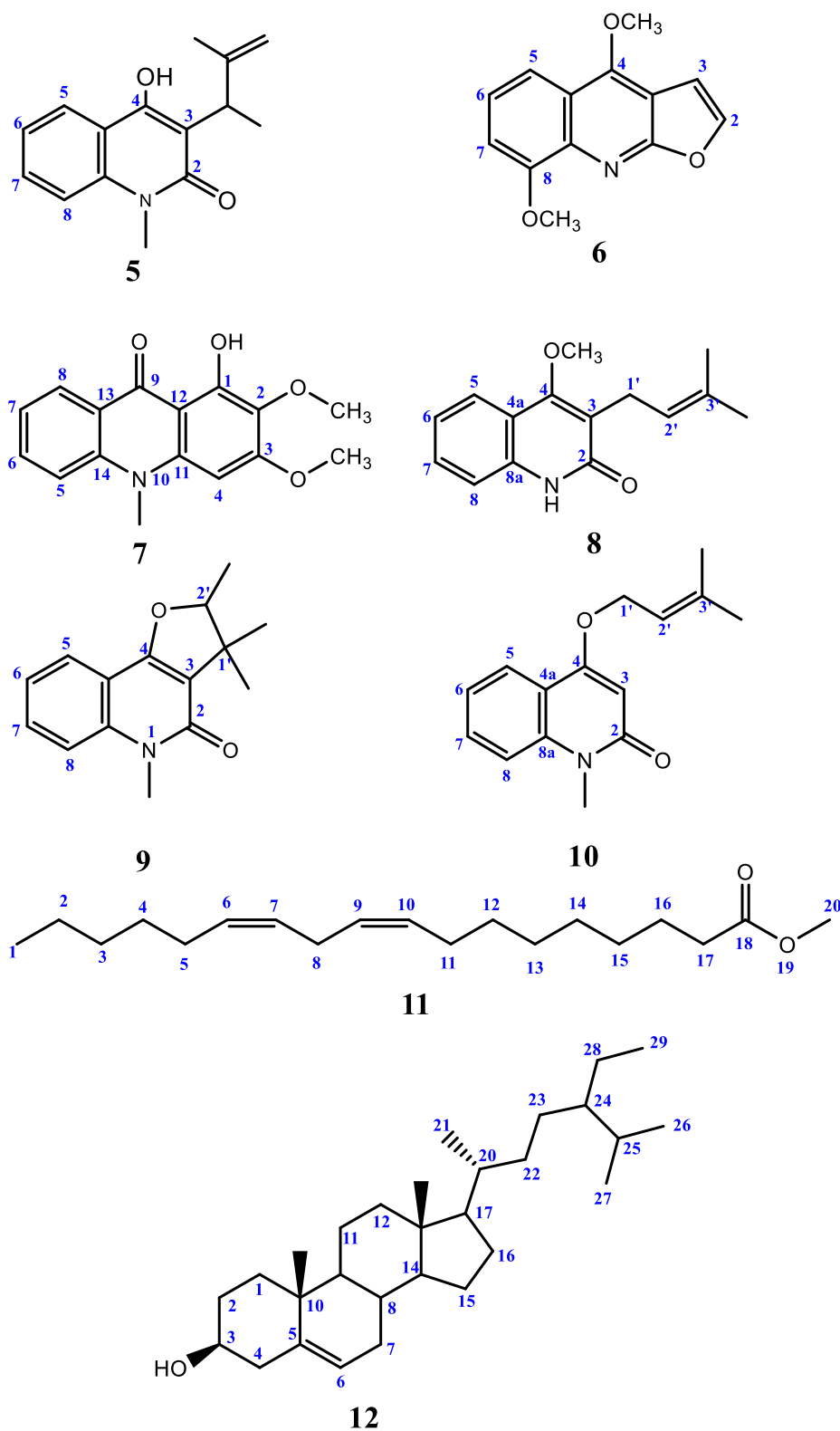


Figure 3.1(cont.) Structures of the compounds isolated from *Ravenia spectabilis*

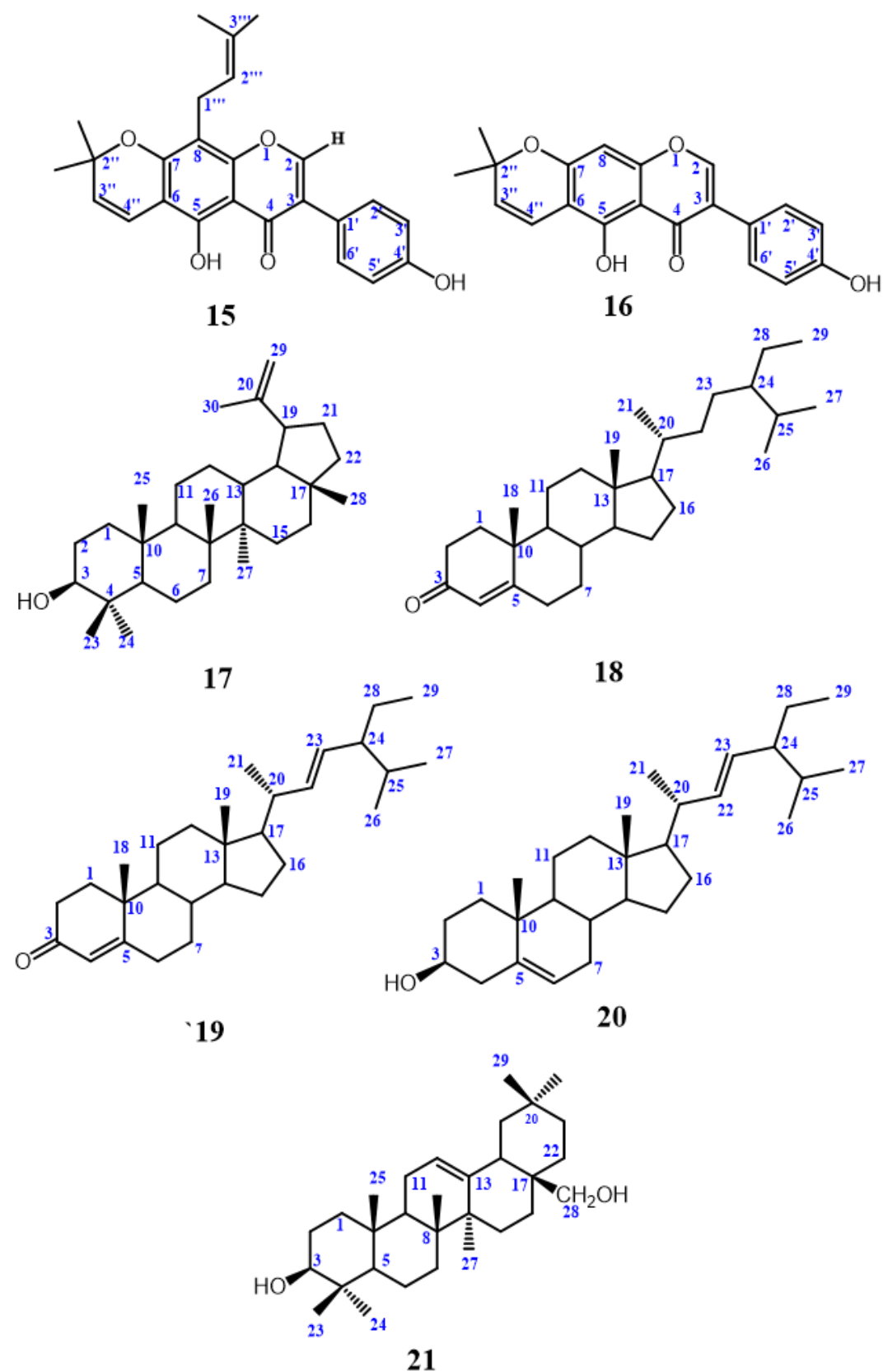


Figure 3.2 Structures of the compounds isolated from *Erythrina variegata*

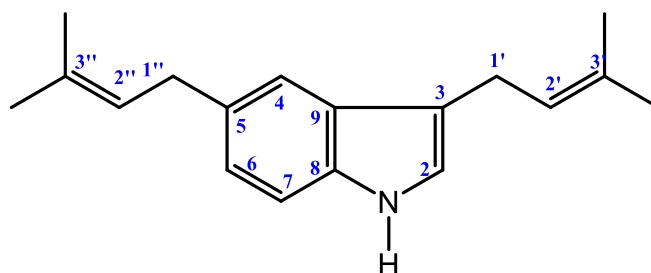
### 3.1.1 Characterization of compound 1 (TRS-71) as 3,5-diprenylindole

Compound **1**, isolated as square shaped crystals, gave deep quenching spot when examined under UV light on a TLC plate and produced brown color when sprayed with vanillin in sulphuric acid reagent followed by heated for 5 minutes. It gave reddish brown color when sprayed with Dragendorff's reagent. The FTIR spectrum of compound **1** showed absorption band at 3398 indicating N-H stretching vibration of an indole ring (Mellich and Becker, 1958). The molecular formula was determined as C<sub>18</sub>H<sub>23</sub>N by HRESIMS (Figure 3.11) measured in the positive ion mode ( $m/z$  254.18, MH<sup>+</sup>).

The <sup>1</sup>H NMR spectrum (Table 3.3, Figure 3.3) displayed three aromatic protons with ABX coupling at  $\delta$  7.43 s, 7.08 dd ( $J = 8.4, 1.0$  Hz) and 7.29 d ( $J = 8.4$  Hz) assignable to H-4, H-6 and H-7 of indole ring respectively, commonly observed in Rutaceae. The proton at position 2 was appeared as a singlet at  $\delta$  6.95. A broad singlet at  $\delta$  7.79 could be assigned to NH proton. The spectrum further revealed the presence of four equivalent methyl groups at  $\delta$  1.82 (12H s), two methine protons at  $\delta$  5.48 & 5.49 (1H br t,  $J = 8.0$  Hz, each) and two methylene protons at  $\delta$  3.49 & 3.51 (2H d,  $J = 8.0$  Hz, each) suggesting the presence of two prenyl groups. The <sup>13</sup>C NMR spectrum displayed all 18 carbons and confirmed the presence of the 3, 5-disubstituted indole nucleus as the methine C signals which normally resonate at  $\delta$  102.10 for C-3 and at  $\delta$  121.7 for C-5 in a typical unsubstituted indole alkaloid (Achenbach & Lowel, 1995), now appeared as quaternary C signals at  $\delta$  115.9 and  $\delta$  132.5, respectively. Other indole C signals appeared in the anticipated chemical shifts typical for the indole nucleus. The position of the two prenyl groups was further confirmed at C-3 and C-5 by an HMBC experiment as the methylene doublets at position C-1' and C-1'' showed <sup>2</sup>J correlation to C-3 and C-5 respectively.

The COSY (Figure 3.9) and NOSEY (Figure 3.10) spectra revealed coupling of H-2 proton to the NH proton. All the carbons and protons were assigned using an HSQC, HMBC, COSY and NOSEY experiments. On the basis of above spectral data discussed, compound **1** was identified as 3, 5-diprenyl indole, which is a new compound. Oxygenated diprenylated indole alkaloids were isolated from *Hexalobus monopetalus* of family Annonaceae (Malebo *et al.*, 2014). This is the first report of

isolation of non-oxygenated diprenylated indole (3,5-diprenylindole) alkaloid from a natural source.



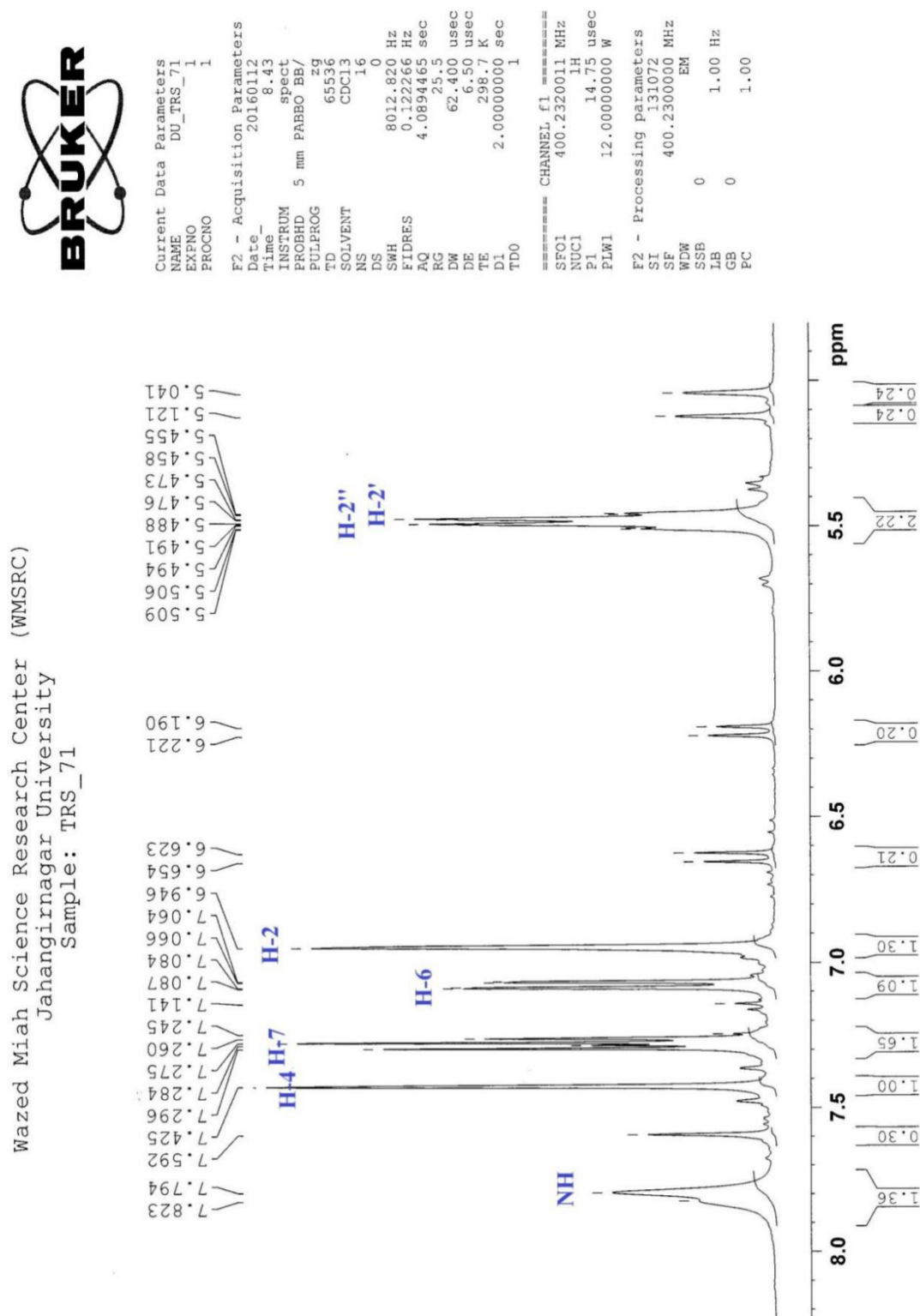
3,5-Diprenyl indole

**Table 3.3 NMR spectroscopic data (400 MHz, CDCl<sub>3</sub>) for compound 1**

Position	$\delta_C$	$\delta_H$	HMBC
2	121.4	6.95 s	135.1 (C-8), 127.8 (C-9), 115.9 (C-3)
3	115.9	---	---
4	118.0	7.43 s	135.1 (C-8), 122.9 (C-6), 34.6 (1'')
5	132.5	---	---
6	122.9	7.08 dd ( $J = 8.4, 1.0$ Hz)	135.1 (C-8), 118.0 (C-4), 34.6 (1'')
7	110.9	7.29 d ( $J = 8.4$ Hz)	132.5 (C-5), 127.8 (C-9)
8	135.1	---	---
9	127.8	---	----
NH		7.79 br s	
1'	24.2	3.49 2H d ( $J = 8.0$ Hz)	115.9 (C-3)
2'	123.2	5.48 br t ( $J = 8.0$ Hz)	115.9 (C-3), 17.8 (C-3'-CH <sub>3</sub> <i>trans</i> )
3'	131.6	---	---
Me-3' <i>cis</i>	25.8	1.82 3H s	123.2(C-2')
Me-3' <i>trans</i>	17.8	1.82 3H s	131.6 (C-3')
1''	34.6	3.51 2H d ( $J = 8.0$ Hz)	132.5 (C-5)
2''	124.6	5.49 br t ( $J = 8.4$ Hz)	25.6 (C-3''-CH <sub>3</sub> <i>cis</i> )
3''	131.8	---	---
Me-3'' <i>cis</i>	25.9	1.82 3H s	124.6(C-2'')
Me-3'' <i>trans</i>	17.9	1.82 3H s	131.8 (C-3'')







Wazed Miah Science Research Center (WMSRC)  
 Jahangirnagar University  
 Sample: TRS\_71



```

Current Data Parameters
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EXPNO     2
PROCNO    1

F2 - Acquisition Parameters
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TD         524288
SOLVENT   CDCl3
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SWH        25252.525 Hz
FIDRES     0.048165 Hz
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RG         2081.5
DW         19.800 usec
DE         6.50 usec
TE         300.1 K
D1         1.00000000 sec
D11        0.03000000 sec
TDO        1

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NUC1       13C
P1         10.00 usec
PLW1      49.00000000 W

===== CHANNEL f2 =====
SFO2      400.2320011 MHz
NUC2       1H
CPDPRG2   waltz16
PCPD2     90.00 usec
PLW2      12.00000000 W
PLW12     0.32231000 W
PLW13     0.26107001 W

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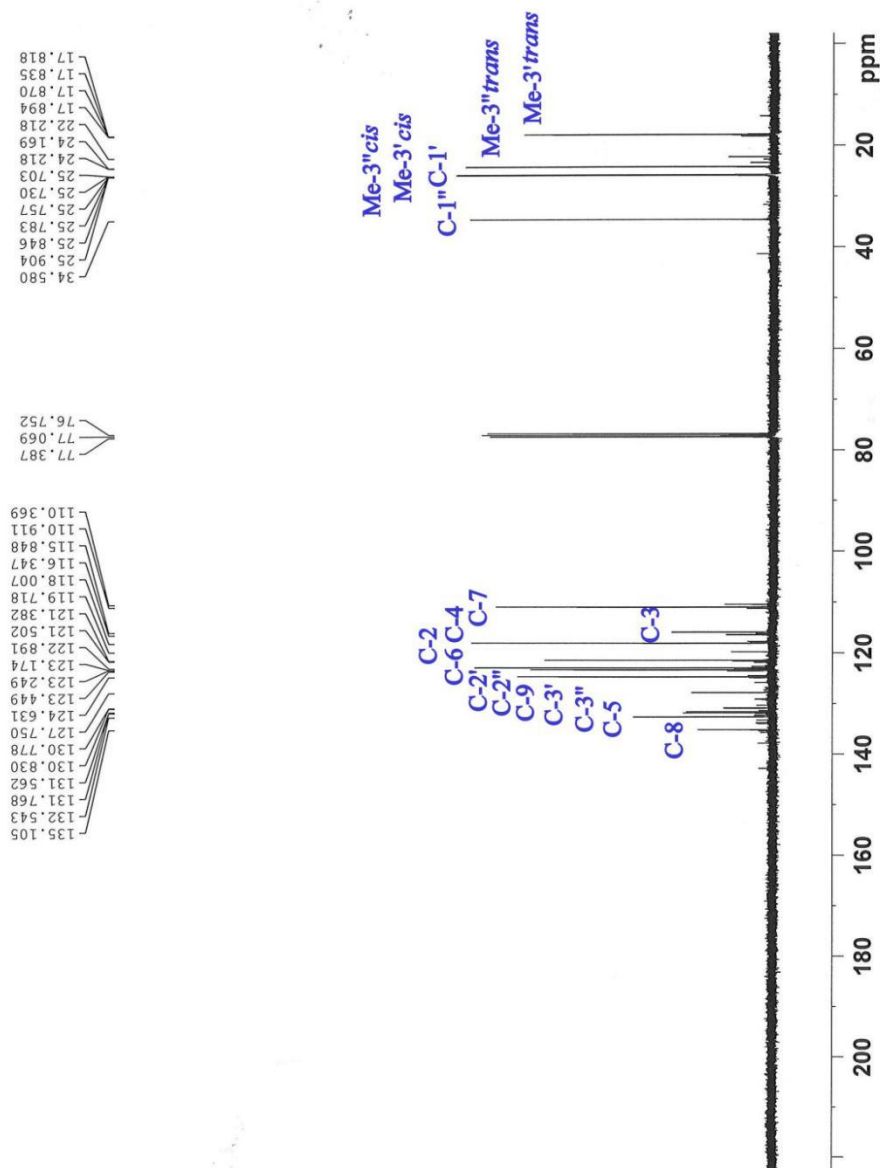


Figure 3.5  $^{13}\text{C}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 1 (TRS-71)



Wazed Miah Science Research Center (WMSRC)  
 Jahangirnagar University  
 Sample: TRS\_71, dept-135

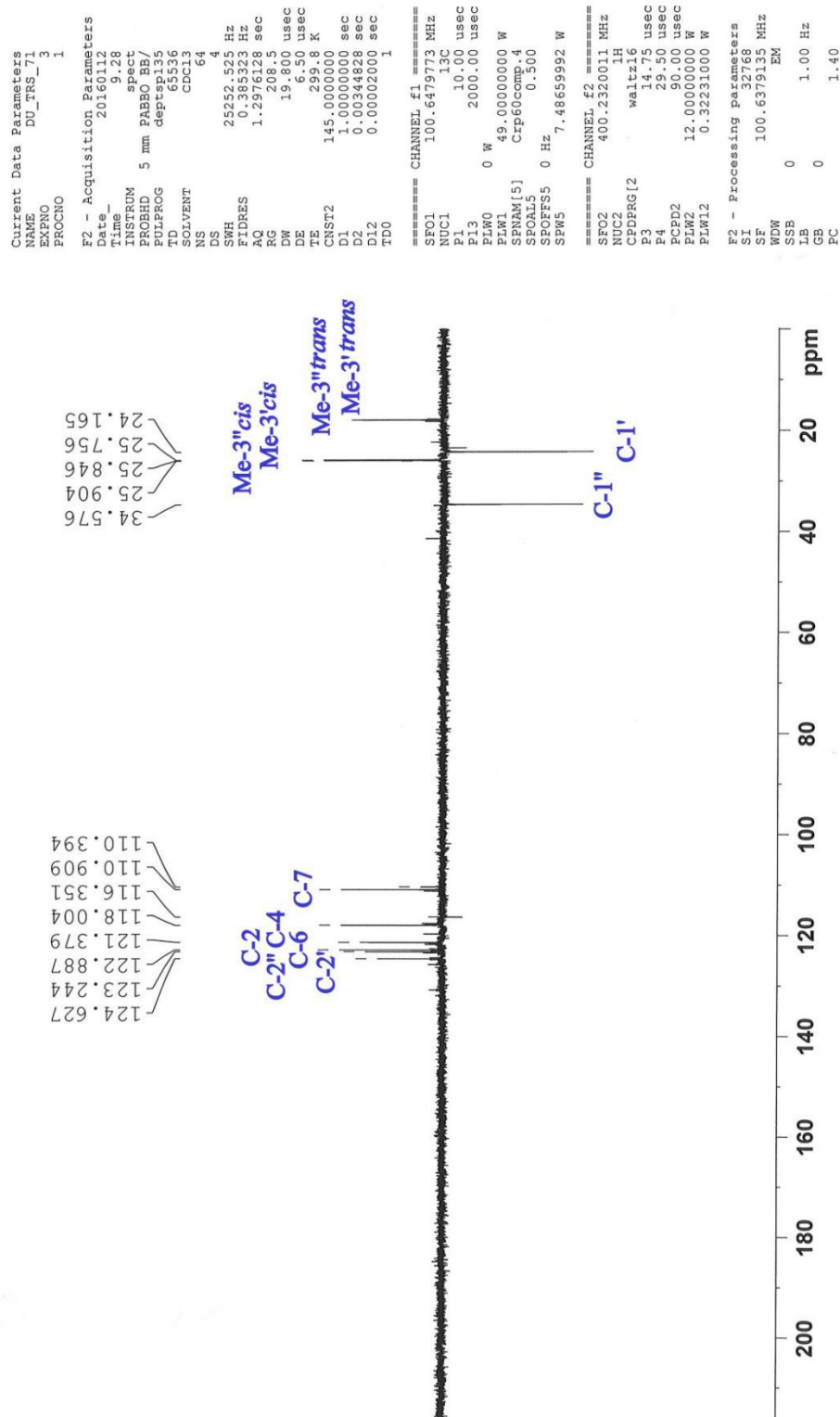


Figure 3.6 DEPT-135 NMR spectrum (400 MHz, CDCl<sub>3</sub>) of compound 1 ( TRS-71)



Wazed Miah Science Research Center (WMSRC)  
 Jahangirnagar University  
 Sample: TRS\_71, hsqc

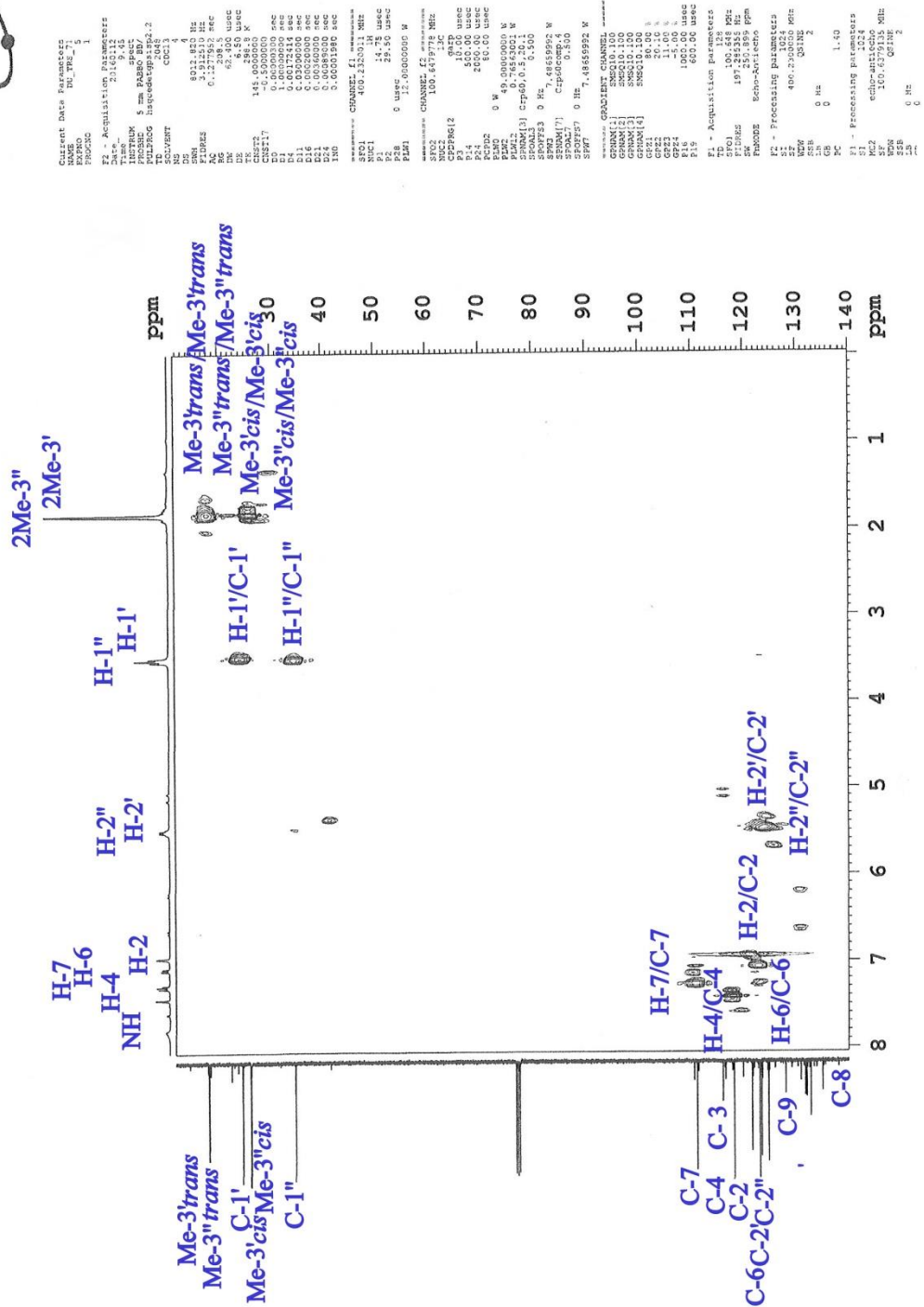


Figure 3.7 HSQC spectrum (400 MHz, CDCl<sub>3</sub>) of compound 1 ( TRS-71)



Wazed Miah Science Research Center (WMSRC)  
 Jahangirnagar University  
 Sample: TRS\_71, hmbc

```

Current Data Parameters
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PROCNO 1
Date_ 20160112
Time 12.11
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DSH 8012.800 Hz
FIDRES 3.912410 Hz
AQ 0.127252 sec
RG 62.400 usec
DE 6.50 usec
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CNS13 10.000000 sec
CNS14 0.000000 sec
D0 0.000000 sec
D1 0.000000 sec
D2 0.00344828 sec
D3 0.000000 sec
D4 0.0500000 sec
D5 0.000000 sec
D6 0.0000000 sec
D7 0.0000000 sec
D8 0.0000000 sec
D9 0.0000000 sec
D10 0.0000000 sec
D11 0.0000000 sec
D12 0.0000000 sec
D13 0.0000000 sec
D14 0.0000000 sec
D15 0.0000000 sec
D16 0.0000000 sec
D17 0.0000000 sec
D18 0.0000000 sec
D19 0.0000000 sec
D20 0.0000000 sec
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NUC2 13C
P1 14.75 usec
P2 0.00 usec
P3 0.00 usec
P4 12.00000000 W
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NUC2 1H
P1 19.00 usec
P2 0.00 usec
P3 0.00 usec
P4 49.00000000 W
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GPNAM[2] SMSQ10.100
GPNAM[3] SMSQ10.100
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GPF4 30.00 %
GPF5 30.00 %
GPF6 30.00 %
GPF7 30.00 %
GPF8 30.00 %
GPF9 30.00 %
GPF10 30.00 %
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GPF14 30.00 %
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CNS14 0.000000 sec
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D2 0.00344828 sec
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D5 0.000000 sec
D6 0.0000000 sec
D7 0.0000000 sec
D8 0.0000000 sec
D9 0.0000000 sec
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SSB 0 Hz
LB 0 Hz
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===== Processing parameters =====
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WDW EM
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LB 0 Hz
GB 0
    
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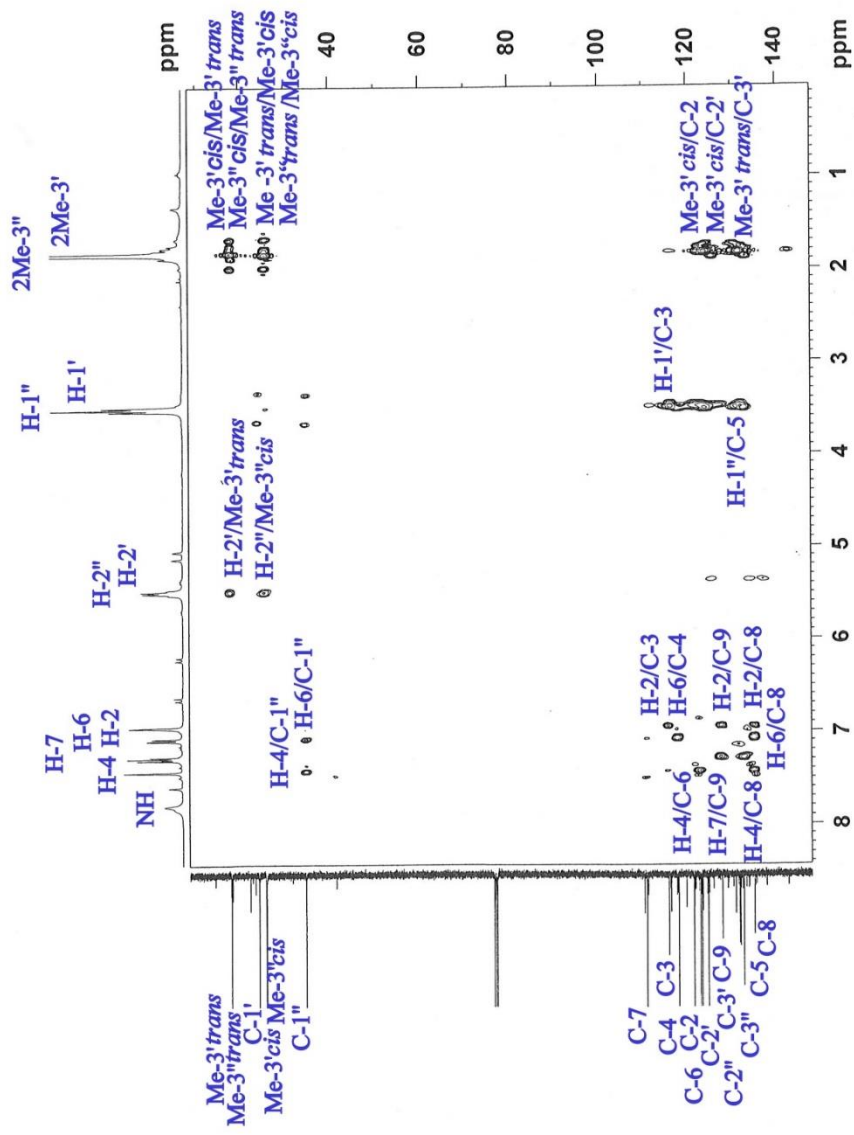
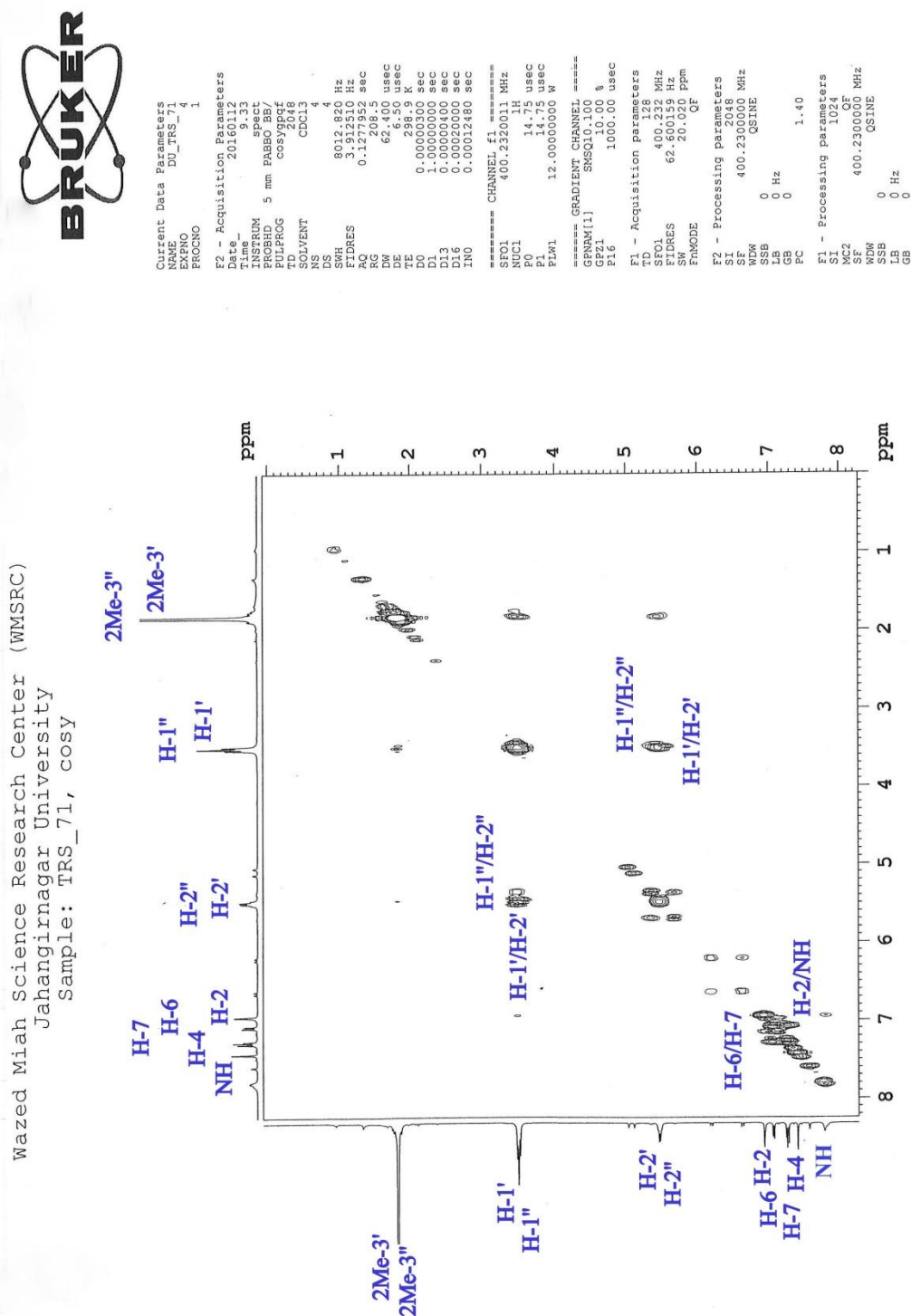
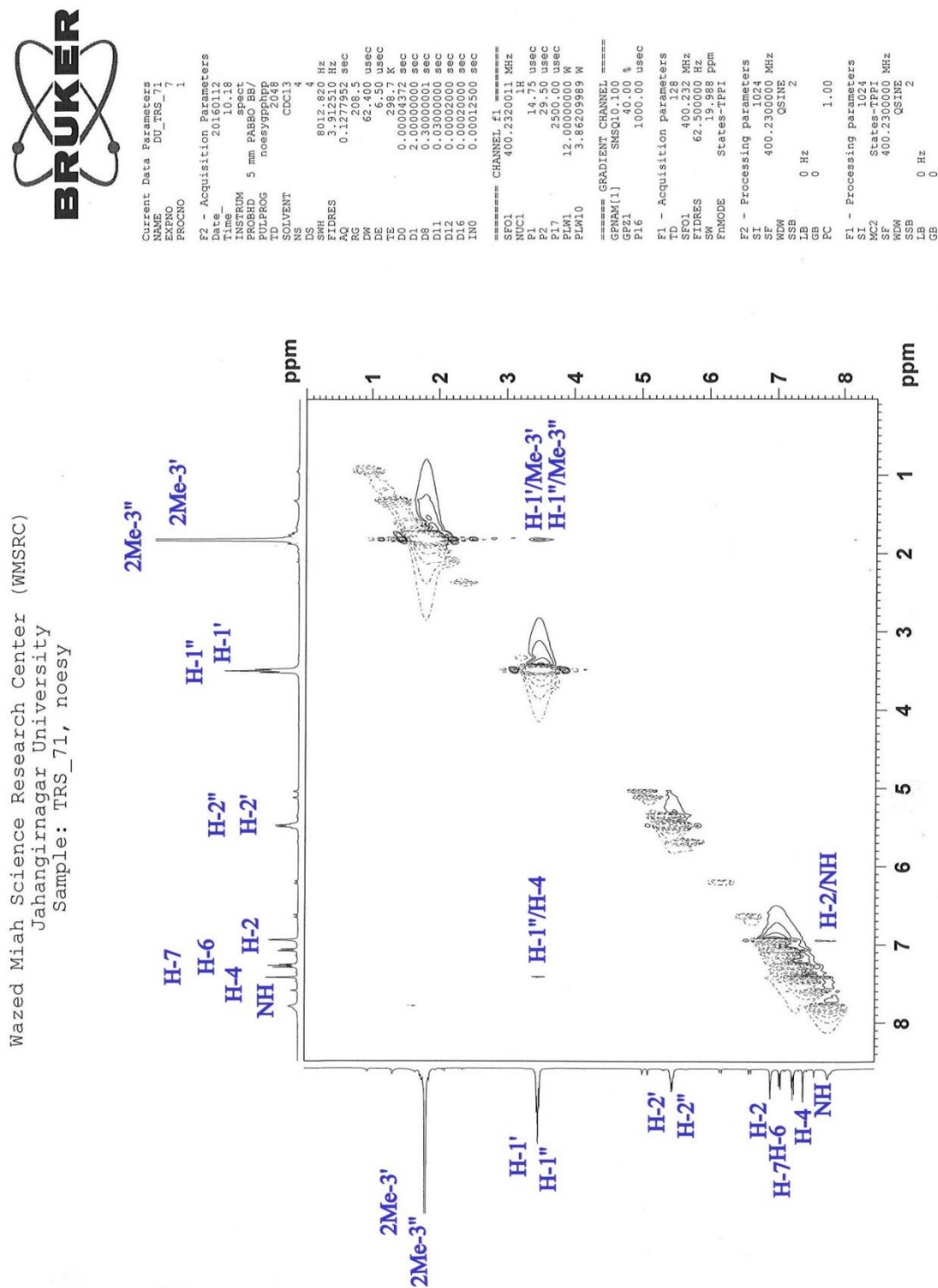


Figure 3.8 HMBC spectrum (400 MHz, CDCl<sub>3</sub>) of compound 1 (TRS-71)

Figure 3.9 COSY spectrum (400 MHz, CDCl<sub>3</sub>) of compound 1 ( TRS-71)

Figure 3.10 NOSEY spectrum (400 MHz, CDCl<sub>3</sub>) of compound 1 ( TRS-71)



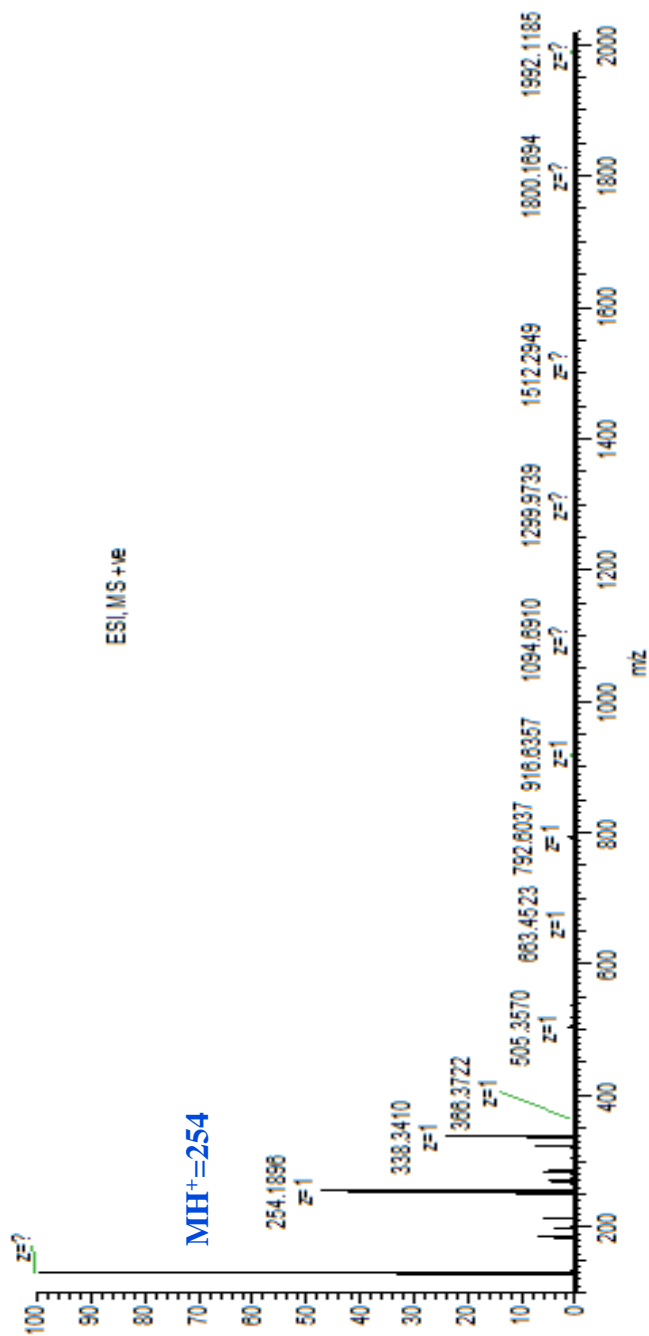


Figure 3.11 Mass spectrum of compound 1 ( TRS-71)

### 3.1.2 Characterization of compound 2 (TRS-157) as 3-prenyl-5-(2-keto-but-3-enyl)indole

Compound **2**, isolated as brown mass gave quenching spot when examined under UV light at 254 nm on a TLC plate. The HRESIMS measured in the positive ion mode (Figure 3.17) exhibited a base peak at  $m/z$  254.153 ( $MH^+$ ) corresponding to the molecular formula  $C_{17}H_{19}NO$ .

The  $^1H$ NMR spectrum (Table 3.4, Figure 3.12) indicated the presence of three aromatic protons with ABX coupling, appeared at  $\delta$  7.79 d ( $J = 0.8$  Hz), 7.45 dd ( $J = 8.4, 1.2$  Hz) and 7.37 d ( $J = 8.4$  Hz) assignable to H-4, H-6 and H-7 respectively.

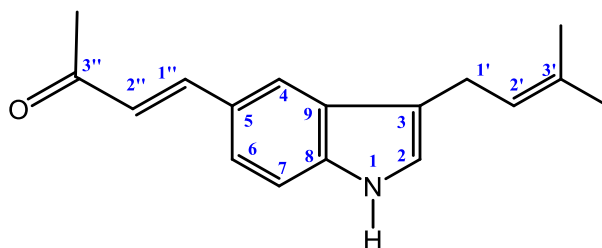
The NH proton of indole ring was observed as a broad peak at  $\delta$  8.12. The spectrum further revealed the presence of two methyl groups at  $\delta$  1.80 (3H, s) and  $\delta$  1.79 (3H, s), one methine proton at  $\delta$  5.44 (1H br t,  $J = 7.0$  Hz) and two methylene protons at  $\delta$  3.48 (2H, d,  $J = 7.0$  Hz) suggesting the presence of a prenyl group.

The spectrum also revealed the presence of two olefinic protons resonating at  $\delta$  7.70 (d,  $J=16$ Hz) and 6.75 (d,  $J=16$ Hz) and an acetyl methyl singlet at 2.42 (3H, s) suggesting the presence of a 2-keto-3-butenyl chain.

The  $^{13}C$  NMR spectrum revealed the presence of seventeen carbons including three methyl carbons at  $\delta$  at 17.8, 25.7 & 27.3, a methylene carbon at  $\delta$  23.9 (C-1') and a carbonyl carbon at  $\delta$  198.8.

The position of the prenyl group was confirmed at C-3 by the HMBC (Figure 3.16) experiment as the methylene group at position C-1' showed  $^2J$  correlation to C-3. The position 2-keto-3-butenyl group was confirmed at C-5 as C-1'' showed  $^3J$  correlation with H-4 and H-6.

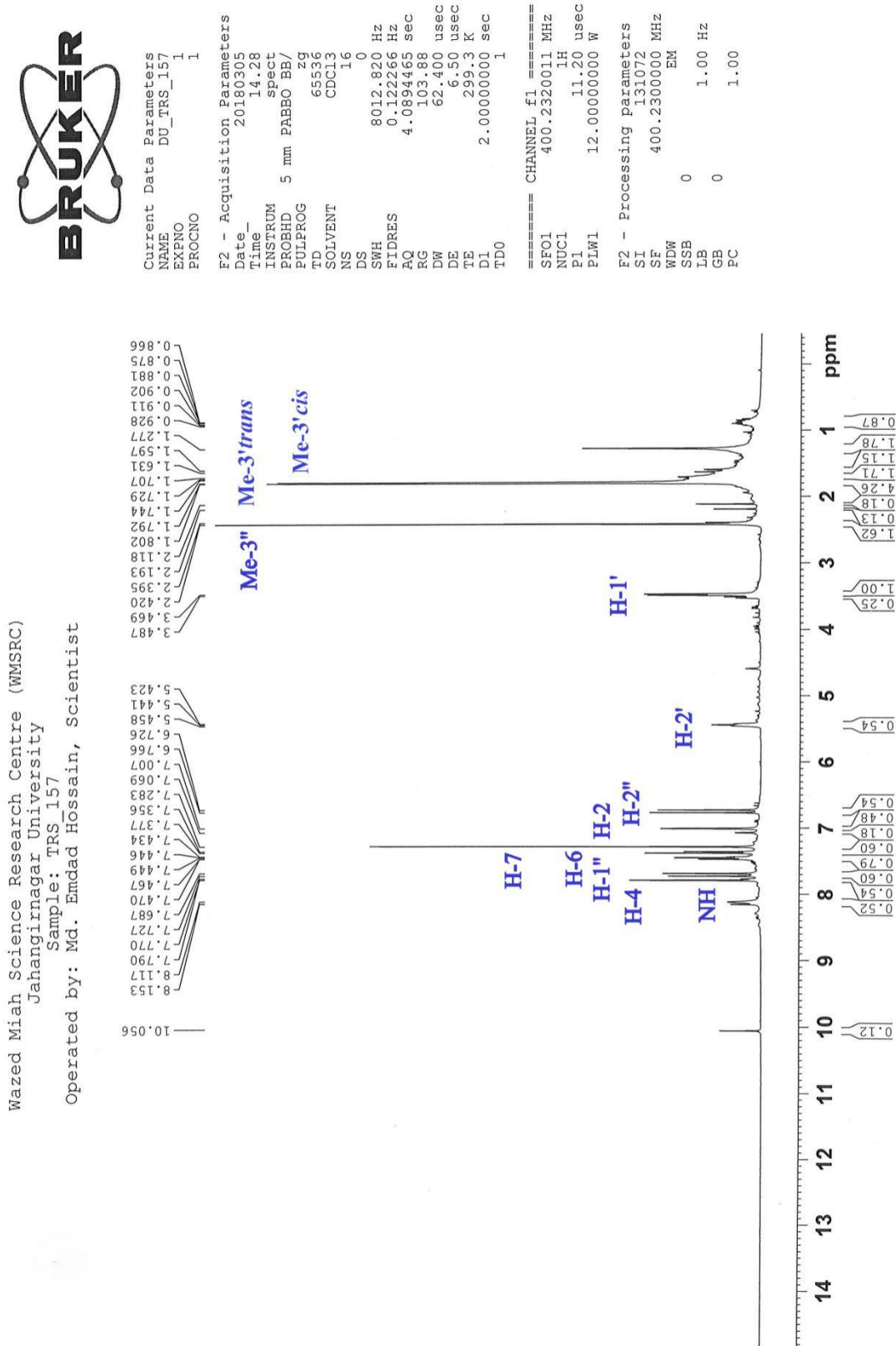
The HSQC (Figure 3.15) spectrum also showed all the expected couplings between carbon and hydrogen. On the basis of above spectral data discussed, compound **2** was identified as 3-prenyl-5-(2-keto-but-3-enyl)indole which is a new compound.

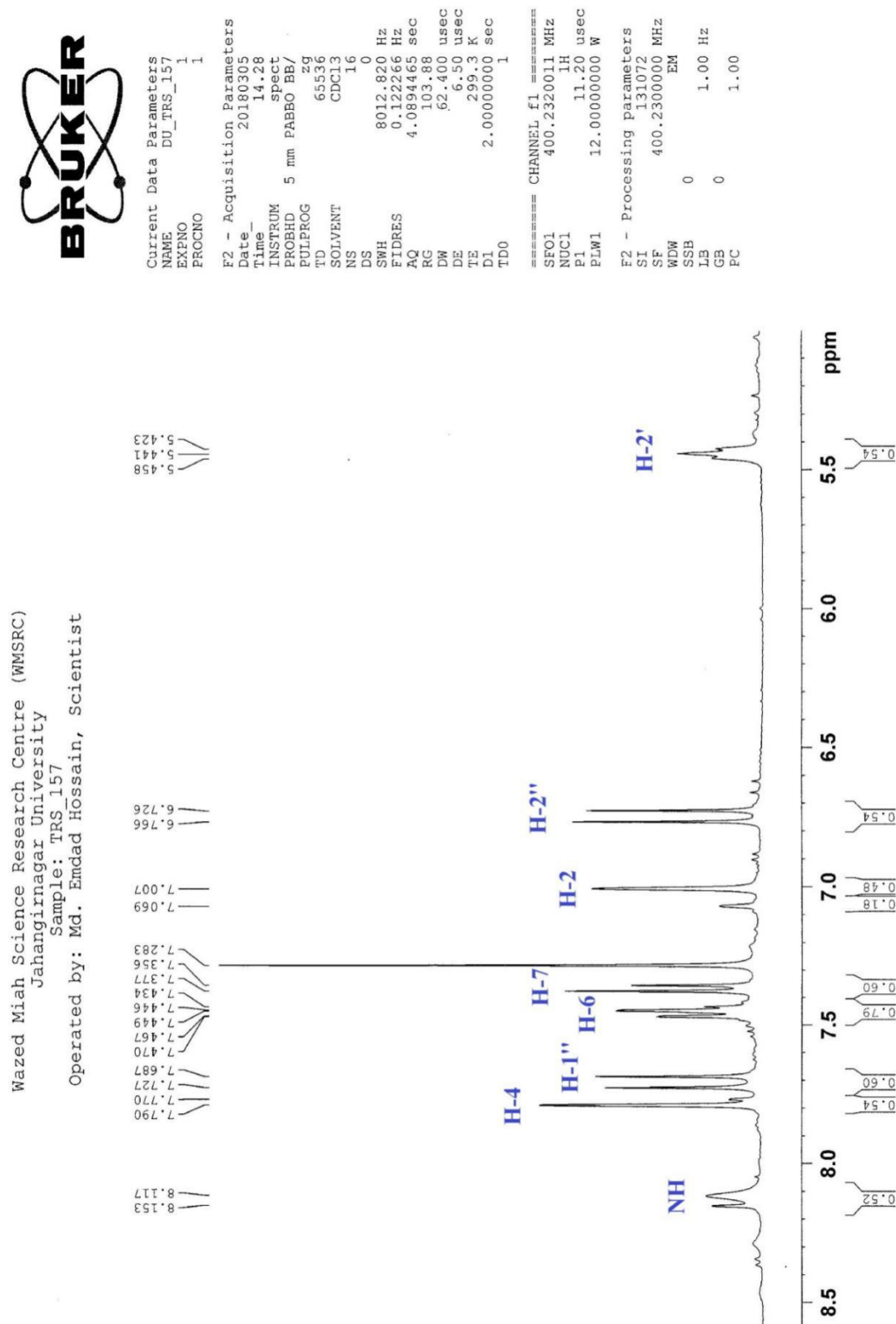


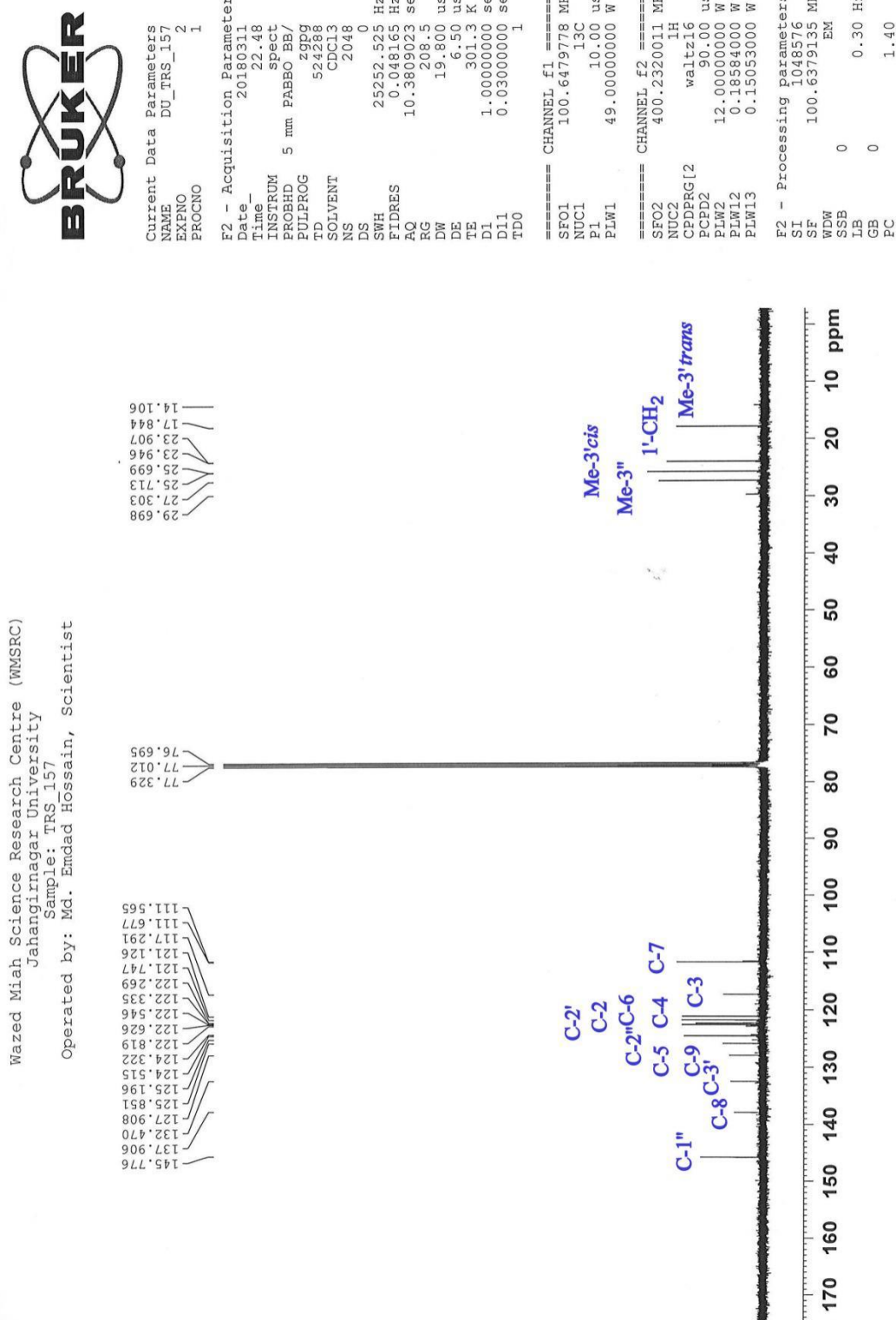
3-Prenyl-5(2-keto-but-3-enyl) indole

**Table 3. 4 NMR spectroscopic data (400 MHz, CDCl<sub>3</sub>) for compound 2**

Position	$\delta_c$	$\delta_H$	HSQC	HMBC
2	122.3	7.01 s	122.3	137.9 (C-8), 127.9 (C-9), 117.3 (C-3)
3	117.3	---	---	---
4	121.1	7.79 d ( $J = 0.8\text{Hz}$ )	121.1	145.8 (1'') 137.9 (C-8), 121.7 (C-6)
5	125.9	---	---	---
6	121.7	7.45 dd ( $J = 8.4, 1.2\text{ Hz}$ )	121.7	145.8 (1''), 137.9 (C-8), 121.1 (C-4),
7	111.7	7.37 d ( $J = 8.4\text{ Hz}$ )	111.7	125.9 (C-5), 127.9 (C-9)
8	137.9	---	---	---
9	127.9	---	---	----
NH		8.12 br s		
1'	23.9	3.48 d ( $J = 7.0\text{ Hz}$ )	23.9	132.5 (C-3'), 127.9 (C-9), 122.5 (C-2'), 117.3 (C-3)
2'	122.5	5.44 br t ( $J = 7.0\text{ Hz}$ )	122.5	----
3'	132.5	---	---	---
Me-3' <i>cis</i>	25.7	1.79 3H s	25.7	132.5 (C-3'), 122.5 (C-2'), 17.8 (Me-3' <i>trans</i> )
Me-3' <i>trans</i>	17.8	1.80 3H s	17.8	132.5 (C-3'), 122.5 (C-2'), 25.7 (Me-3' <i>cis</i> )
1''	145.8	7.70 d ( $J = 16\text{ Hz}$ )	145.8	198.8 (C=O), 121.1 (C-4), 121.7 (C-6)
2''	124.5	6.75 ( $J = 16\text{ Hz}$ )	124.5	125.9 (C-5)
3'' (C=O)	198.8	---	---	---
Me-3''	27.3	2.42 3H s	27.3	198.8 (C=O)





Figure 3.14 <sup>13</sup>C NMR spectrum (400 MHz, CDCl<sub>3</sub>) of compound 2 (TRS-157)

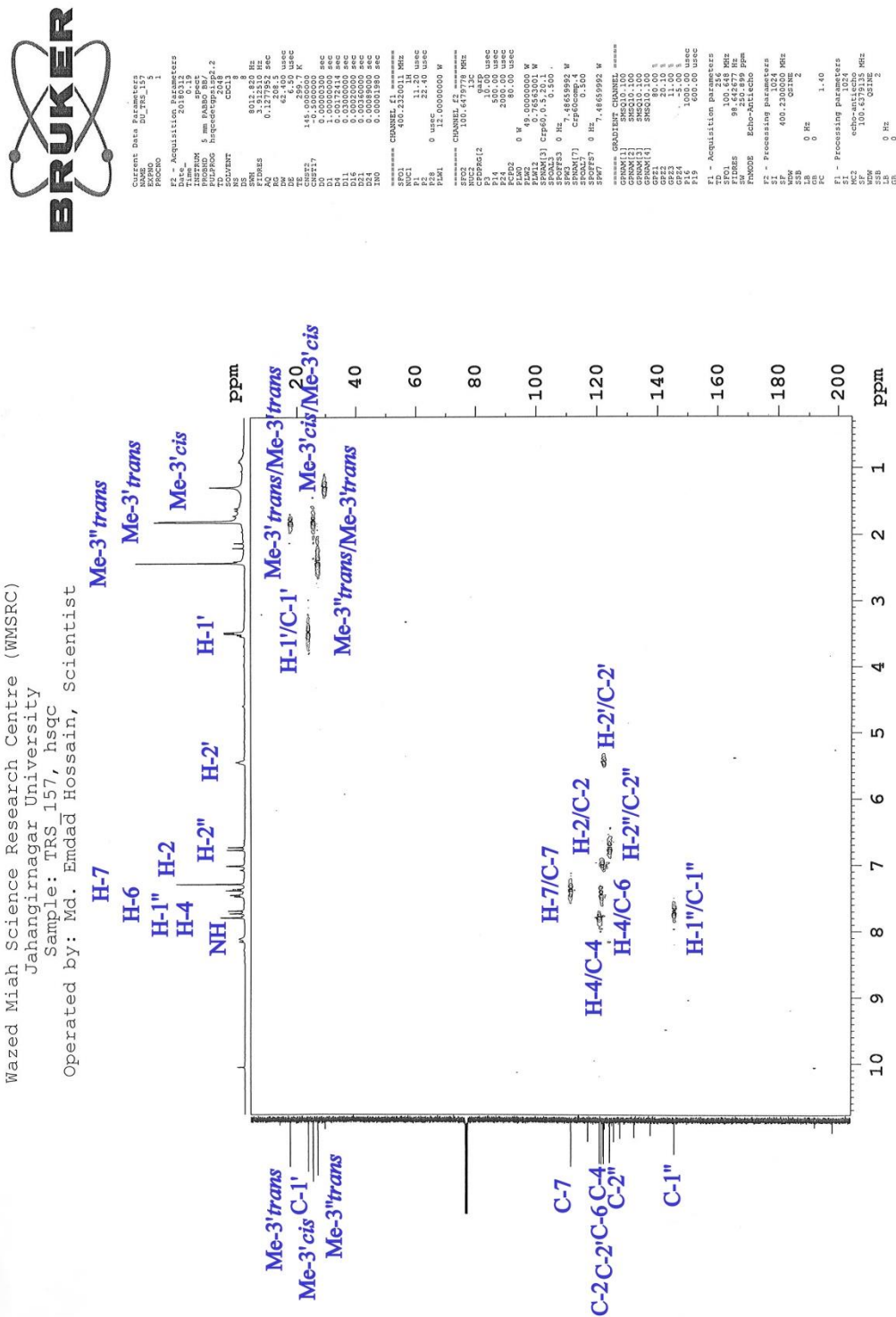


Figure 3. 15 HSQC spectrum (400 MHz, CDCl<sub>3</sub>) of compound 2 (TRS-157)



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 Jahangirnagar University  
 Sample: TRS\_157, hmbc  
 Operated by: Md. Emdad Hossain, Scientist

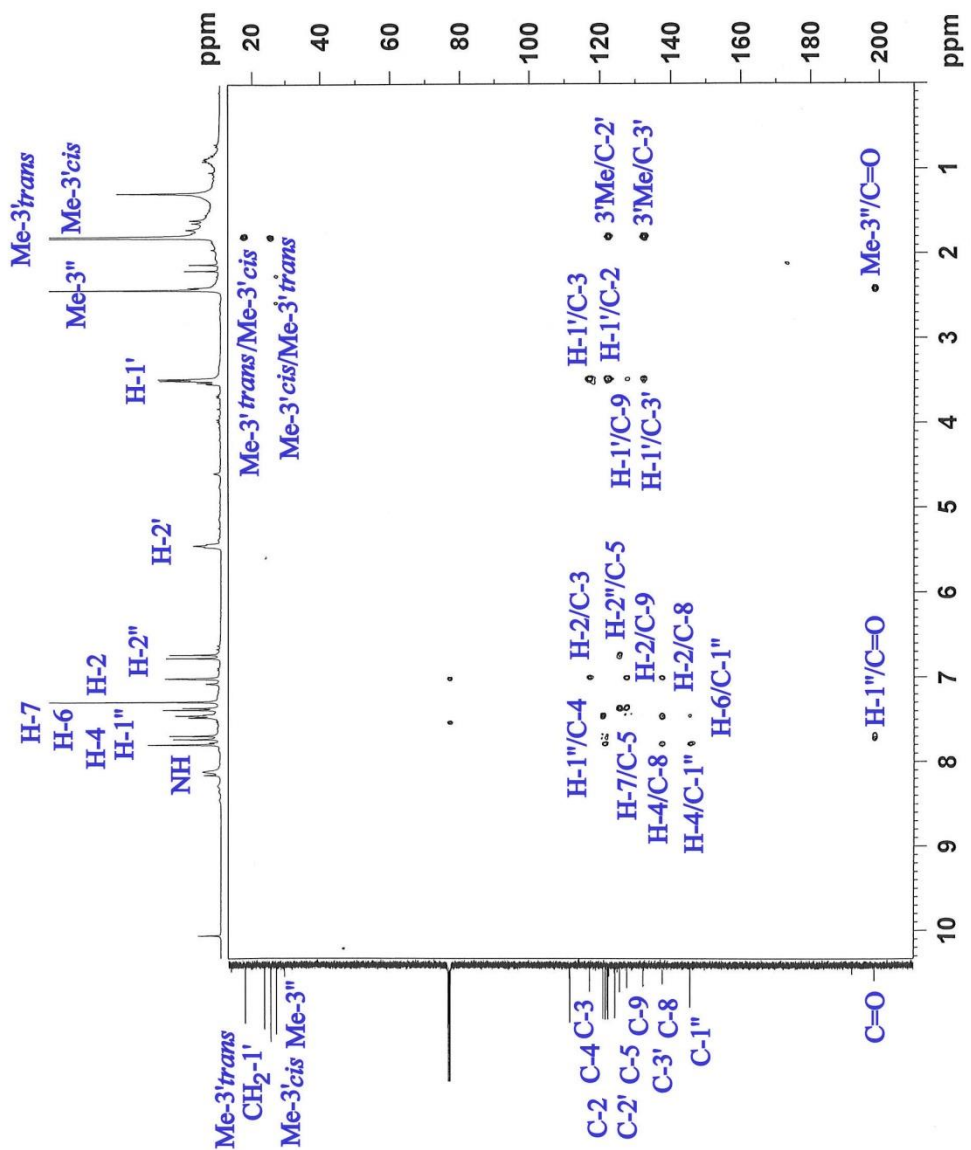


Figure 3. 16 HMBC spectrum (400 MHz, CDCl<sub>3</sub>) of compound 2 (TRS-157)



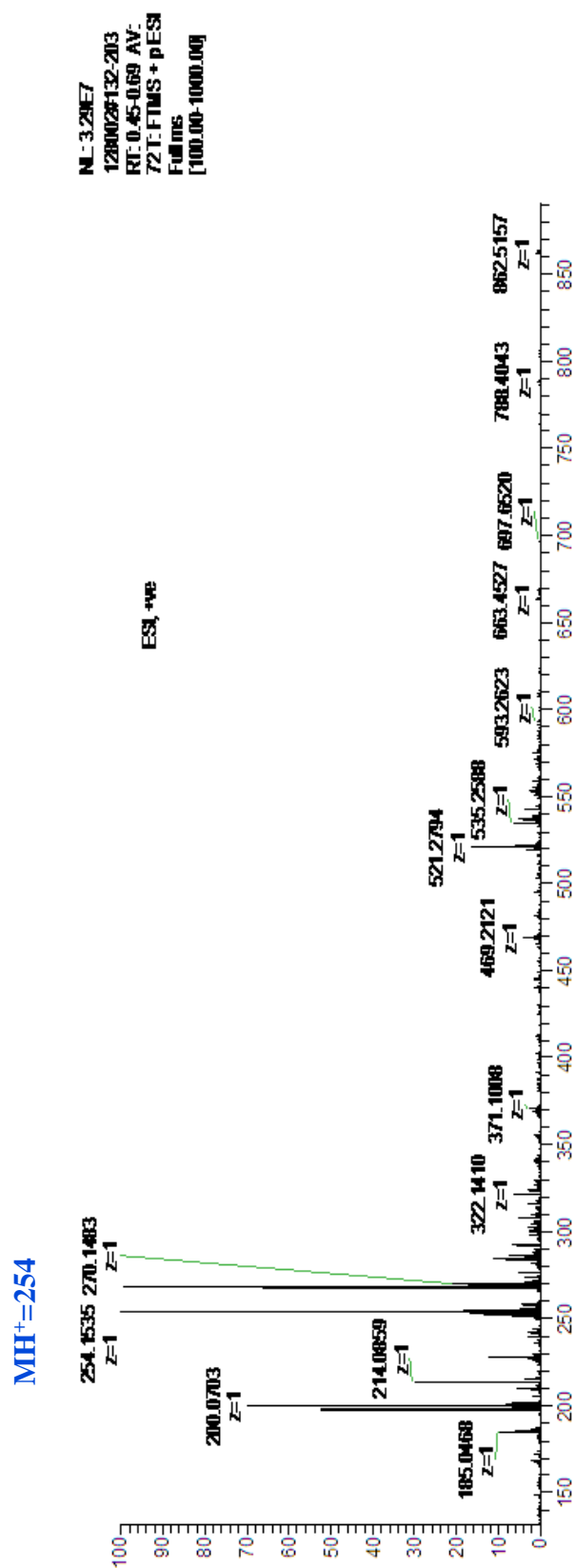


Figure 3. 17 Mass spectrum of compound 2 (TRS-157)

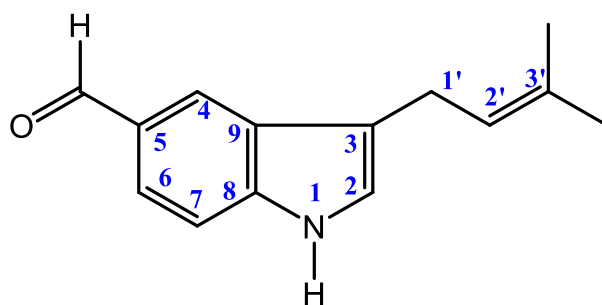
### 3.1.3 Characterization of compound 3 (TRS-159) as 3-prenyl-indole-5-carbaldehyde

Compound **3** was isolated as brown gummy mass, showed deep quenching spot when examined under UV light on a TLC plate and produced brown color when sprayed with vanillin in sulphuric acid reagent followed by heating for 5 minutes.

The HRESIMS of compound **3** (Figure 3.23) measured in the positive ion mode showed a  $MH^+$  ion peak at  $m/z$  214.122 which is in agreement with the molecular formula  $C_{14}H_{15}NO$ .

The  $^1H$  NMR spectrum (Table 3.5, Figure 3.18) indicated the presence of three aromatic protons with ABX coupling at  $\delta$  8.06 s, 7.69 br d ( $J = 8.0$  Hz) and 7.35 d ( $J = 8.0$  Hz) assignable to H-4, H-6 and H-7 respectively. The H-2 proton of the indole was appeared as a singlet at  $\delta$  6.98. A broad singlet at  $\delta$  8.16 and a sharp singlet at 9.97 suggested the presence of an NH and an aldehydic protons respectively. The spectrum further revealed the presence of two equivalent methyl groups at  $\delta$  1.71 (6H s), one methine proton at  $\delta$  5.36 (1H br t,  $J = 8.0$  Hz) and one methylene proton at  $\delta$  3.49 (2H d,  $J = 8.0$  Hz) indicating a prenyl group in the molecule. The  $^{13}C$  NMR spectrum (Figure 3.19) displayed fourteen carbons including two methyl carbons at  $\delta_c$  17.8 (Me-3'*trans*) and 25.54 (Me-3'*cis*), a methylene carbon at  $\delta$  24.13 (C-1') and a carbonyl carbon at  $\delta$  192.5. A DEPT-135 experiment (Figure 3.20) confirmed the aldehydic and methylene carbon at  $\delta$  192.57 and 24.13 respectively. From the HMBC experiment, the position of the prenyl group was observed at C-3 as the methylene doublets at position C-1' showed  $^2J$  correlation to C-3, whereas the aldehyde group displayed  $^3J$  correlation to C-4 and C-6, thus confirming its attachment to C-5. The HSQC spectrum (Figure 3.21) also showed all the expected  $^1J$  correlations between carbon and hydrogen.

Thus compound **2** was identified as a new indole alkaloid and named 3-prenyl-indole-5-carbaldehyde.



3-Prenyl-indole-5-carbaldehyde

**Table 3.5** NMR spectroscopic data (400 MHz, CDCl<sub>3</sub>) for compound **3**

Position	$\delta_C$	$\delta_H$	HSQC	HMBC
2	122.4	6.98	122.5	140.0 (C-8), 127.8 (C-9), 118.2 (C-3)
3	118.2	---	---	---
4	124.4	8.06 s	124.5	192.5 (C=O), 140.0 (C-8), 122.6(C-6)
5	129.6	---	---	---
6	122.6	7.69 br d ( $J = 8.0$ Hz)	122.7	192.5 (C=O), 140.0 (C-8), 124.4 (C-4),
7	111.3	7.35 d ( $J = 8.0$ Hz)	111.3	129.6 (C-5), 127.8 (C-9)
8	140.0	---	---	---
9	127.8	---	---	---
NH		8.16br s		
1'	24.13	3.49 2H d ( $J = 8.0$ Hz)	24.03	132.6(C-3'), 127.8(C-9), 122.2(C-2'), 118.2 (C-3)
2'	122.2	5.36 br t ( $J = 8.0$ Hz)	122.3	----
3'	132.6	---	---	---
Me-3' <i>cis</i>	25.54	1.71 3H s	25.8	132.6 (C-3'), 122.2 (C-2'), 17.8 (3'-Me <i>trans</i> )
Me-3' <i>trans</i>	17.8	1.71 3H s	17.8	132.6(C-3'), 122.2 (C-2') 25.8 (3'-Me <i>cis</i> )
HC=O	192.5	9.97 1H s	192.5	124.4(C-4), 122.6 (C-6)

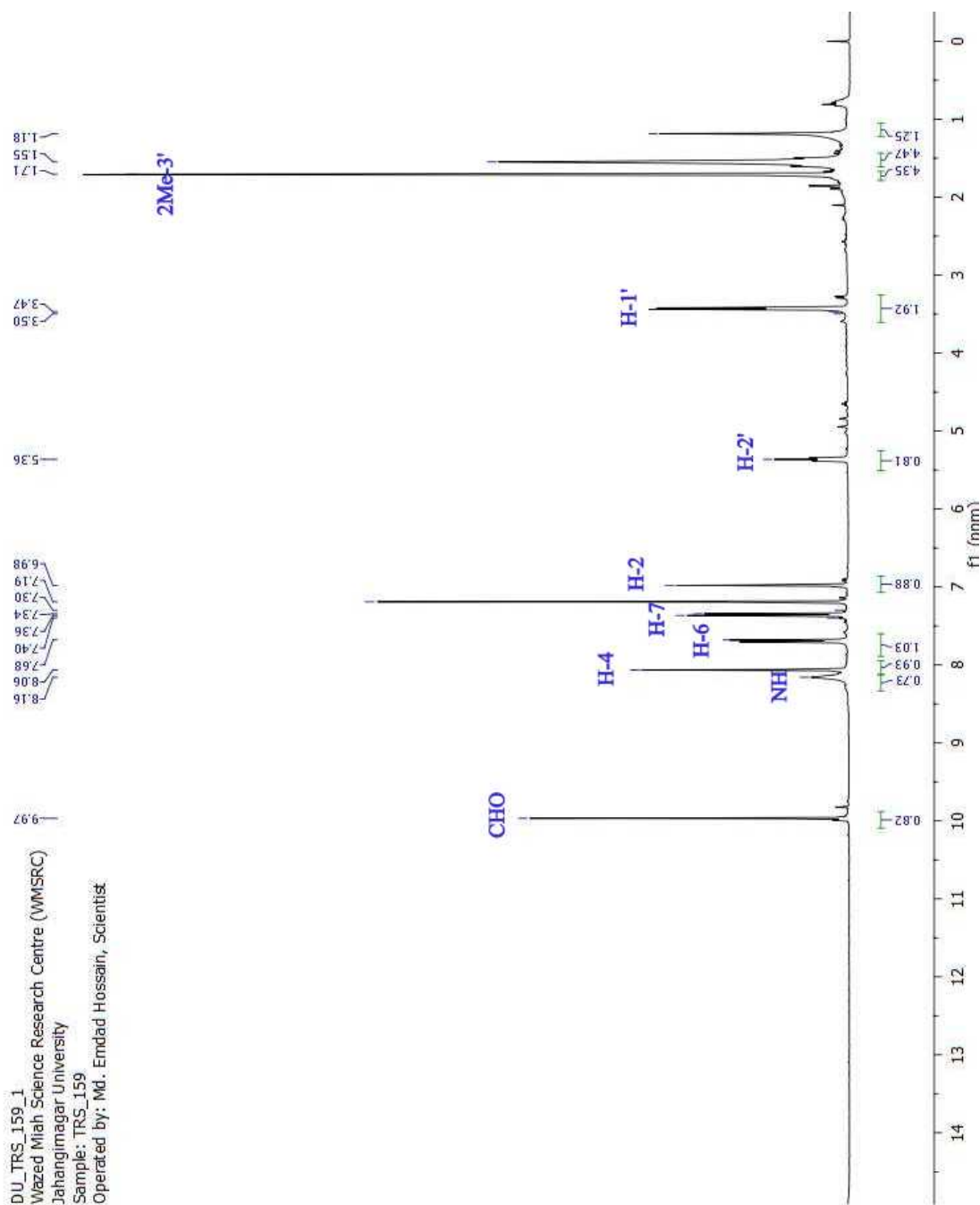
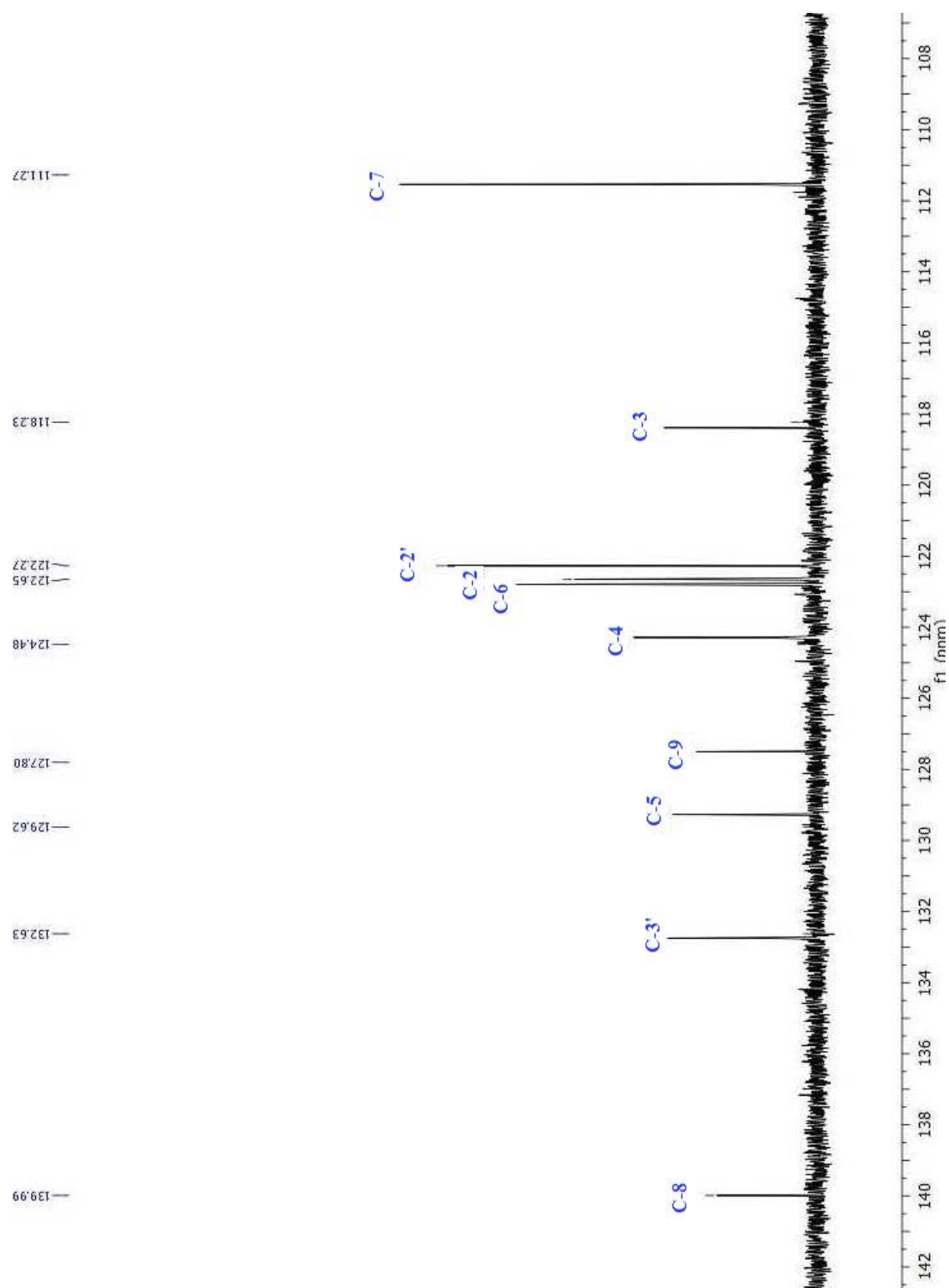


Figure 3. 18  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 3 (TRS-159)



**Figure 3.19** Partially expanded <sup>13</sup>C NMR spectrum (400 MHz, CDCl<sub>3</sub>) of compound 3 (TRS-159)

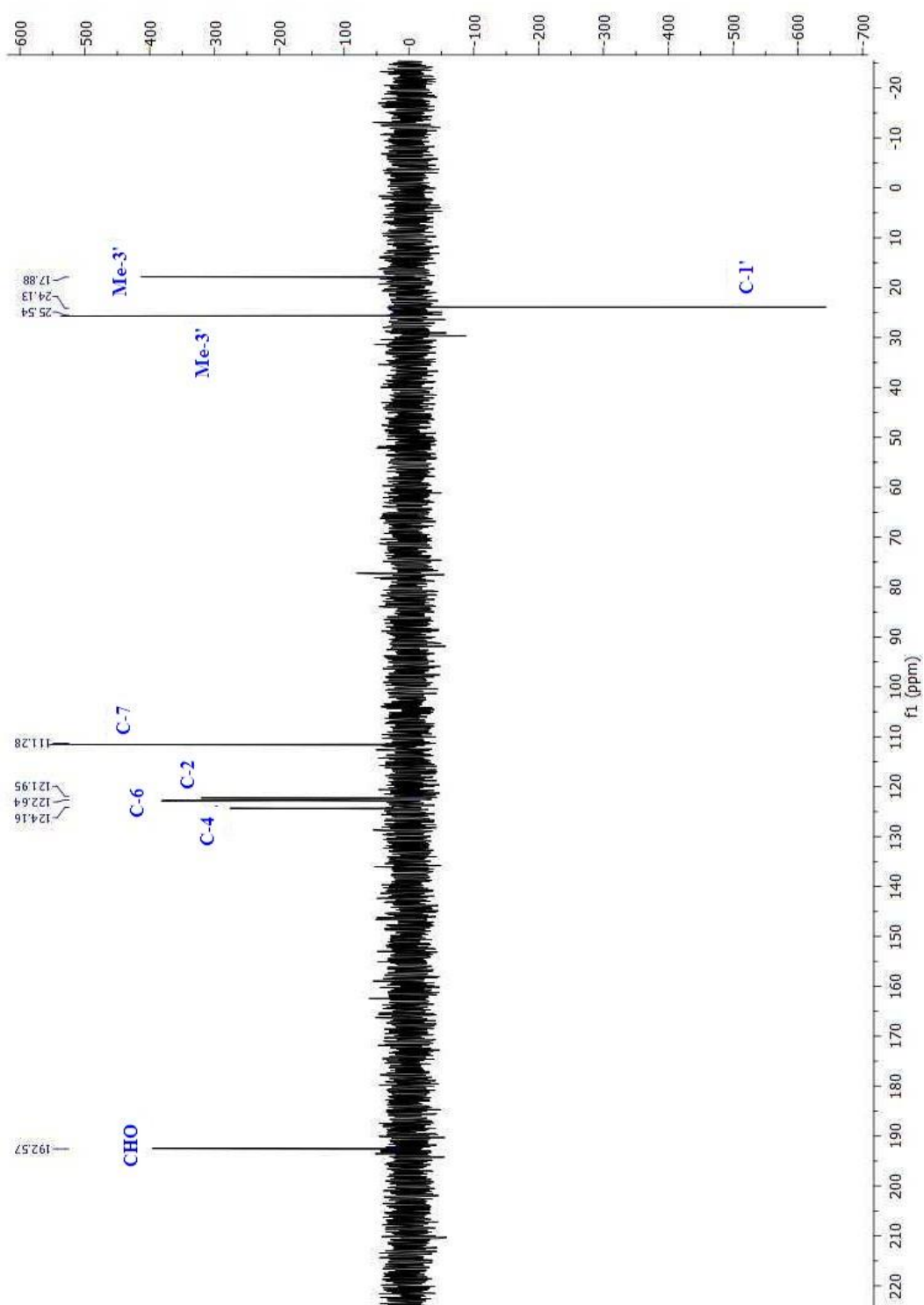


Figure 3. 20 DEPT-135 spectrum (400 MHz, CDCl<sub>3</sub>) of compound 3 (TRS-159)



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 Jahangirnagar University  
 Sample: TRS\_159, hsqc  
 Operated by: Md. Emdad Hossain, Scientist

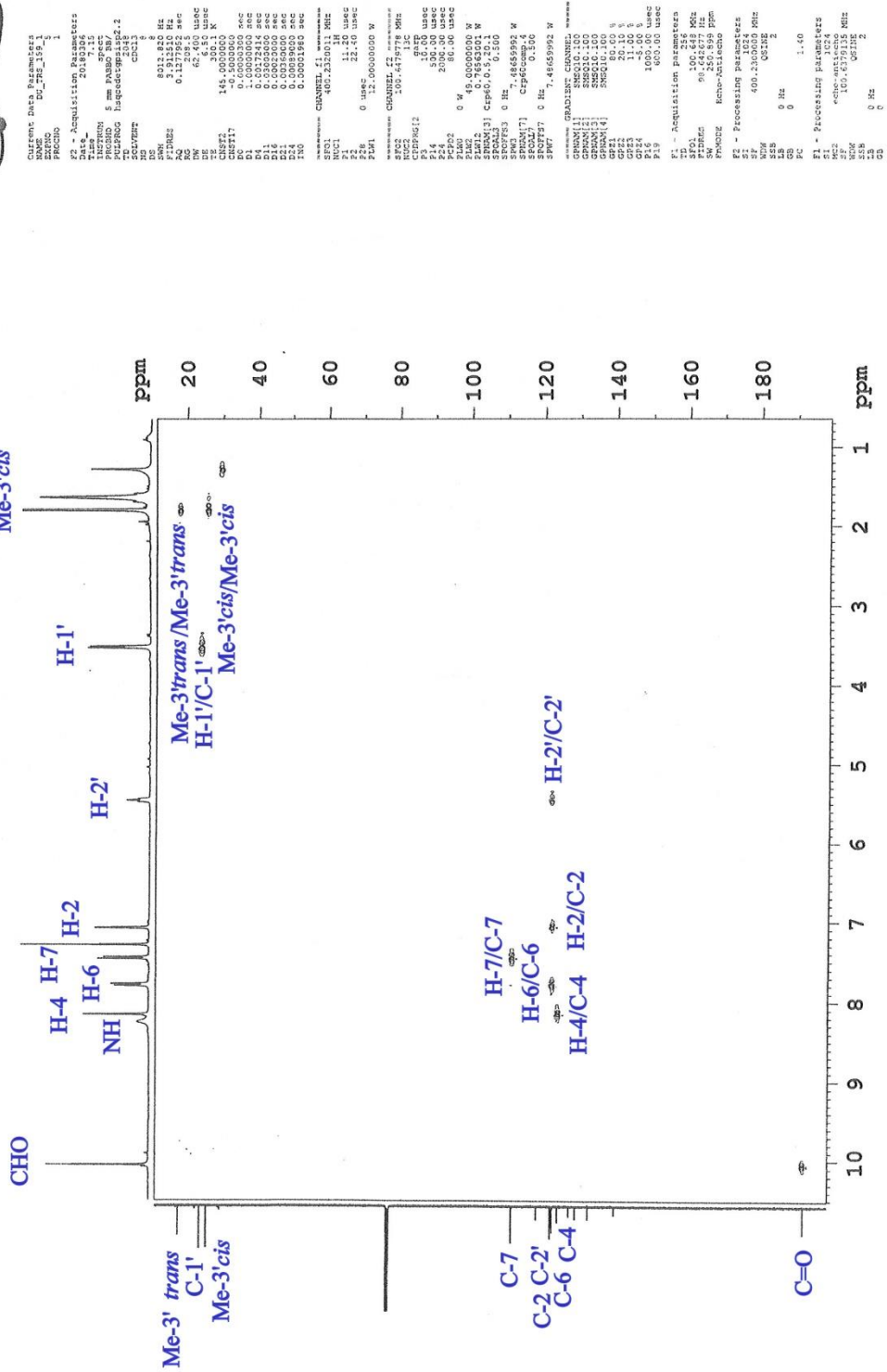


Figure 3. 21 HSQC spectrum (400 MHz, CDCl<sub>3</sub>) of compound 3 (TRS-159)



Wazed Miah Science Research Centre (WMSRC)  
 Jahangirnagar University  
 Sample: TRS\_159, hmbc  
 Operated by: Md. Emdad Hossain, Scientist

```

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PROCNO   1

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RG         208.5
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PC         301.2 K
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NUC2       13C
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D2         0.0344828 sec
D3         0.0500000 sec
D4         0.0000000 sec
D5         0.0000000 sec
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PL1       0.0000000 W
SFO2     12.0000000 MHz
NUC2     13C
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PL2       0.0000000 W

CHANNEL f2
SFO1     100.6197173 MHz
NUC1     13C
P1        10.00 usec
PL1       0.0000000 W

CHANNEL f3
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GPRM[24] 50.00 usec
GPRM[25] 1000.00 usec

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GB         0
PC         301.2 K
PC2       1.40

F1 - Processing parameters
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LB         62.400 Hz
GB         0
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PC2       1.40
    
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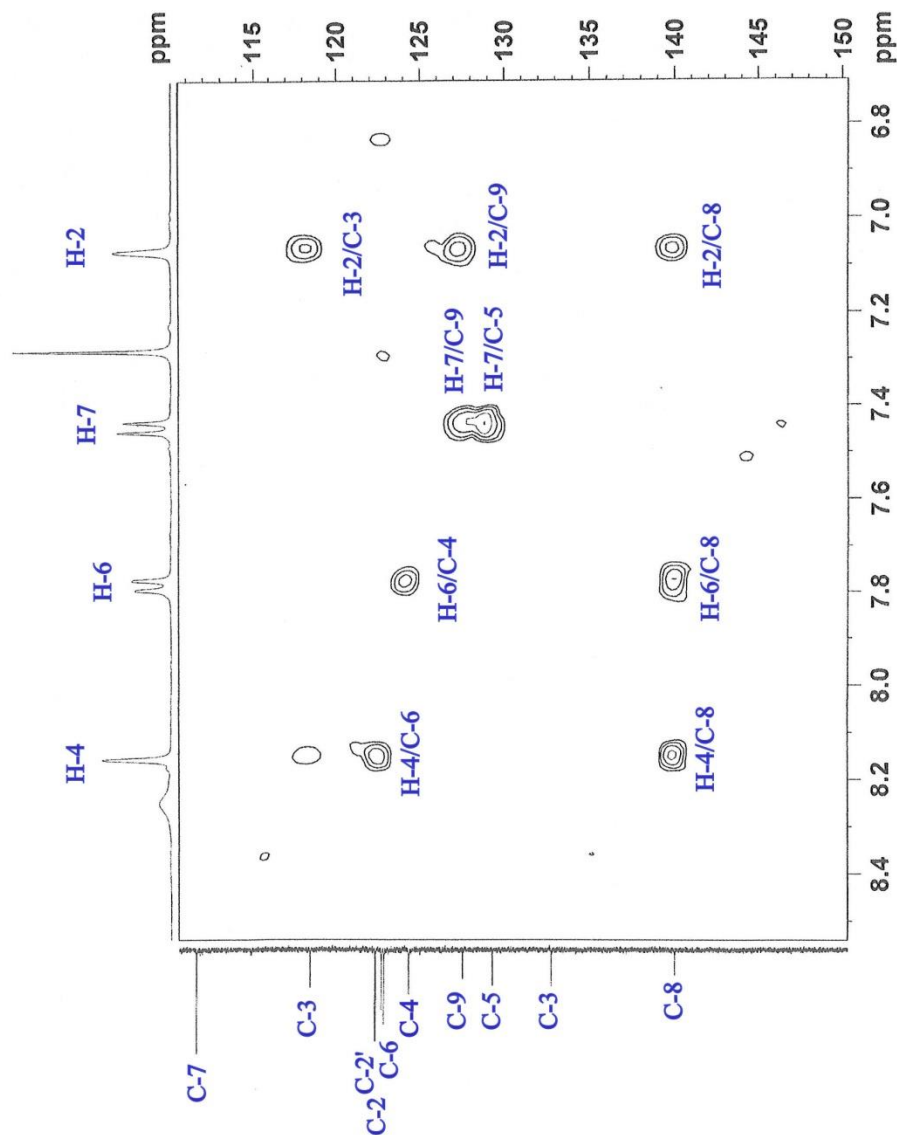


Figure 3.22 Partially expanded HMBC spectrum (400 MHz, CDCl<sub>3</sub>) of compound 3 (TRS-159)



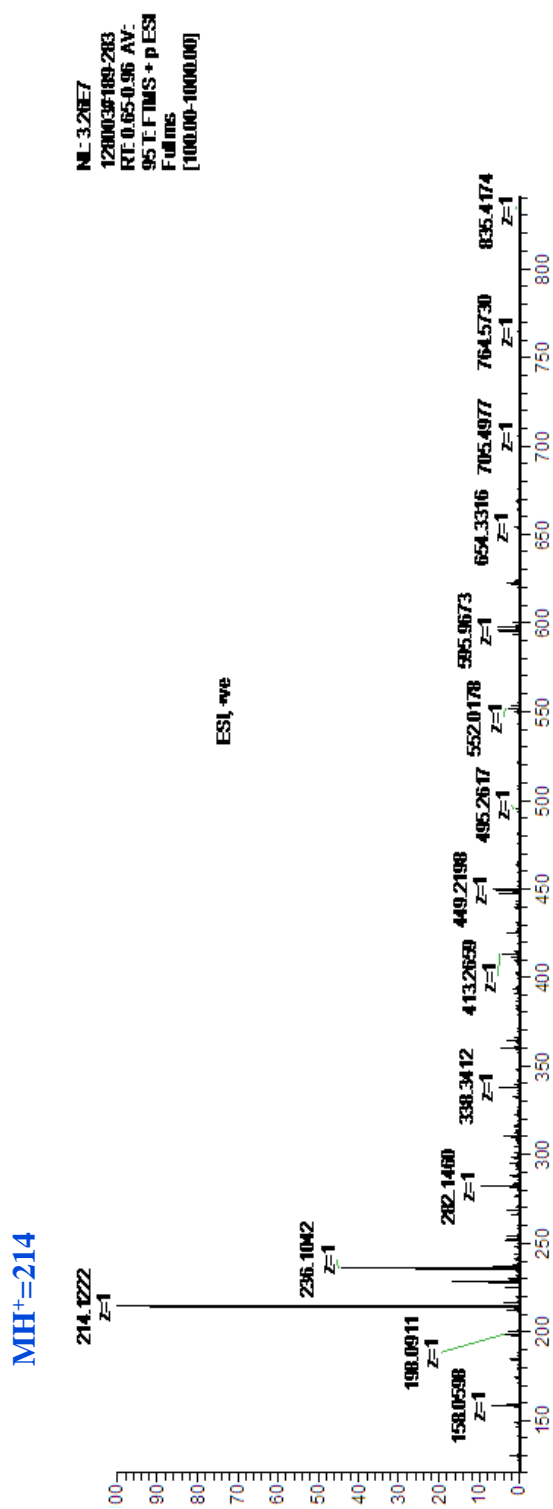


Figure 3. 23 Mass spectrum of compound 3 (TRS-159)

### 3.1.4 Characterization of compound 4 (TRS-146) as iso-oligophyline

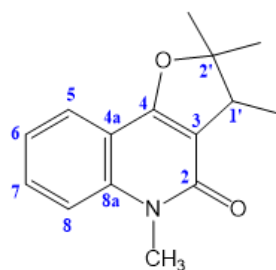
Compound 4, isolated as yellowish mass, was appeared as a pinkish spot under 254 nm UV light on a TLC plate, gave dark brown colour after spraying with vanillin-sulphuric acid reagent followed by heating for 5 minutes and produced orange red color when sprayed with Dragendorff's reagent.

The molecular formula of compound 4 was determined as C<sub>15</sub> H<sub>17</sub> NO<sub>2</sub> by HRESIMS (Figure 3.30) measured in the positive ion mode (*m/z* 244.13, MH<sup>+</sup>).

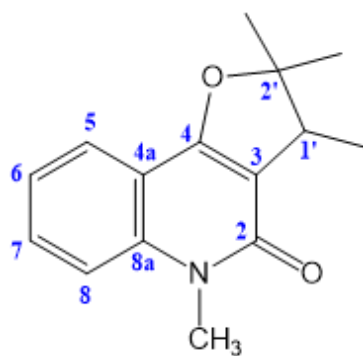
The <sup>1</sup>H NMR spectrum (Table 3.6, Figure 3.24) displayed signals indicating the presence of four aromatic proton multiplets at δ 7.72 d (*J* = 7.2 Hz), 7.17 dd (*J* = 8.0, 7.8 Hz), 7.52 dd (*J* = 8.5, 8.0) and 7.32 d (*J* = 8.5 Hz) assignable to H-5, H-6, H-7 and H-8 respectively suggesting the presence of *ortho* disubstituted aromatic ring of the 2-quinolones.

A three proton singlet resonating at δ 3.67 could be assigned to N-methyl group. In addition the spectrum showed two methyl singlets at δ 1.40 and 1.44, a methyl doublet at δ 1.33 (*J* = 6.2 Hz) and a methine multiplet at δ 3.25. All the <sup>1</sup>H NMR signals of compound 4 were found to be similar to those of oligophyline (isolated previously from *Euxylophora paraensis*) except that the methine signal now appears at a high field placing its position at C-1'. In oligophyline the methine appeared at a low field at δ 4.59 due to presence of oxygen in the same carbon.

The <sup>13</sup>C NMR, the spectrum (Figure 3.26) exhibited three methyl carbons at δ 22.5, 28.9 and 14.2, a methylene carbon at δ 44.7 and an N-methyl carbon at δ 29.0. The COSY spectrum (Figure 3.29) revealed the coupling between H-1' protons to Me-1' protons and also between the protons as expected for the benzene ring. The HSQC and HMBC experiment showed all expected <sup>1</sup>*J*, <sup>2</sup>*J* and <sup>3</sup>*J* coupling among the carbons and protons. Thus compound 4 was identified as new a 2-quinolone alkaloid and was given the trivial name iso-oligophyline.



Iso-oligophyline

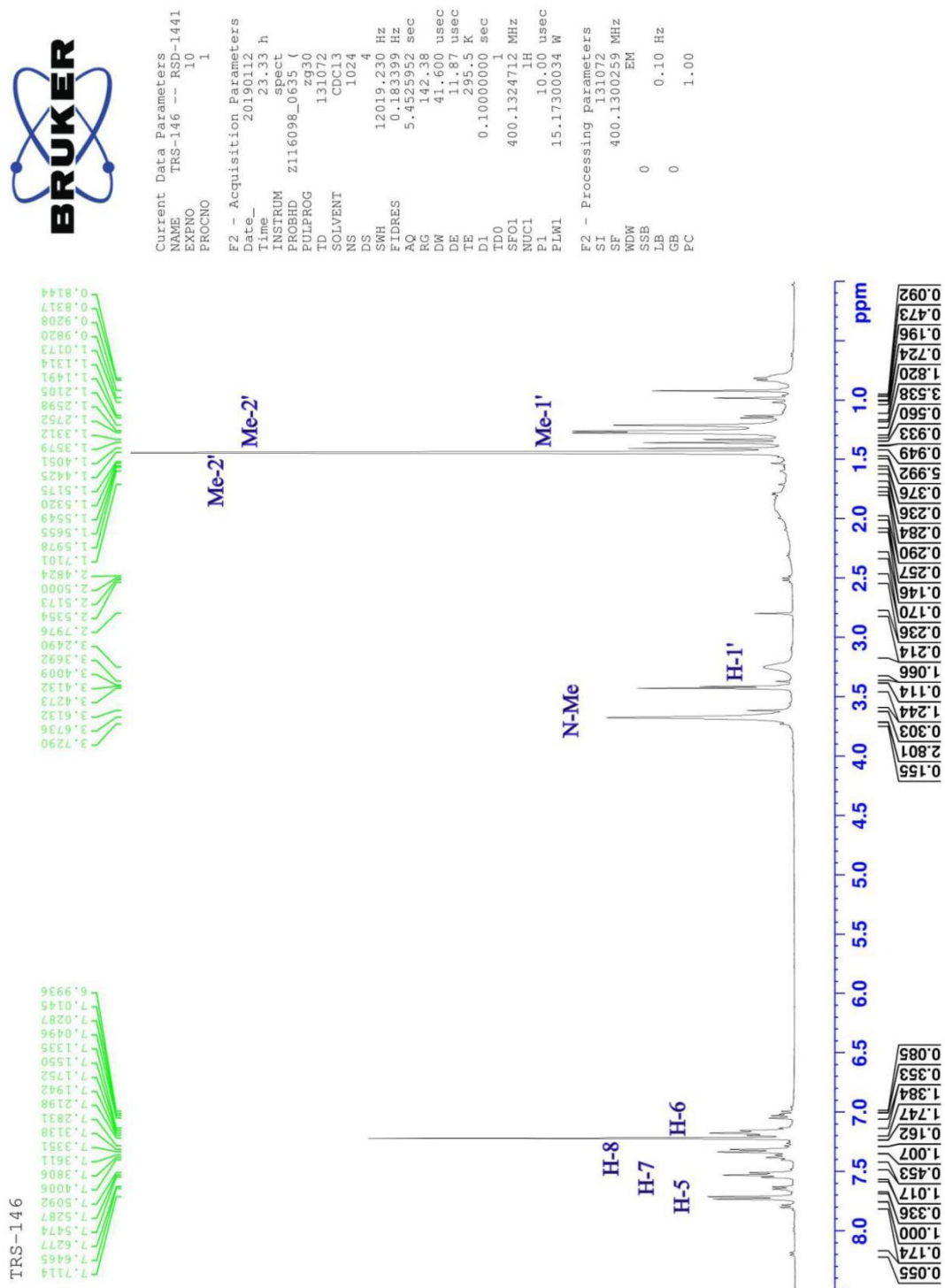


Iso-oligophylline

**Table 3.6 NMR spectroscopic data (400 MHz, CDCl<sub>3</sub>) for compound 4**

Position	$\delta_C$	$\delta_H$	HMBC
2	c	---	---
3	114.5	---	---
4	160.7	---	---
4a	113.2	---	---
5	123.2	7.72 d ( $J = 7.2$ Hz)	140.5 (C-8a), 130.8 (C-7), 160.7 (C-4)
6	121.5	7.17 dd ( $J = 8.0, 7.2$ Hz)	113.2(C-4a)
7	130.8	7.52 dd ( $J = 8.5, 8.0$ Hz)	140.5 (C-8a), 123.2 (C-5)
8	114.5	7.32 d ( $J = 8.5$ Hz)	121.5(C-6), 113.2(C-4a)
8a	140.5	---	---
1'	44.7	3.25 1H m	----
Me-1'	14.2	1.33 3H d ( $J = 6.2$ Hz)	92.7(C-2'), 44.7(C-1')
2'	92.7	-	---
Me-2'	22.5	1.44 3H s	92.7(C-2'), 44.7 (C-1'), 28.9 (Me-2')
Me-2'	28.9	1.40 3H s	92.7(C-2'), 44.7(C-1'), 22.5 (Me-2')
N-Me	29.0	3.67 3H s	--

C= not observed

Figure 3.24  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 4 (TRS-146)

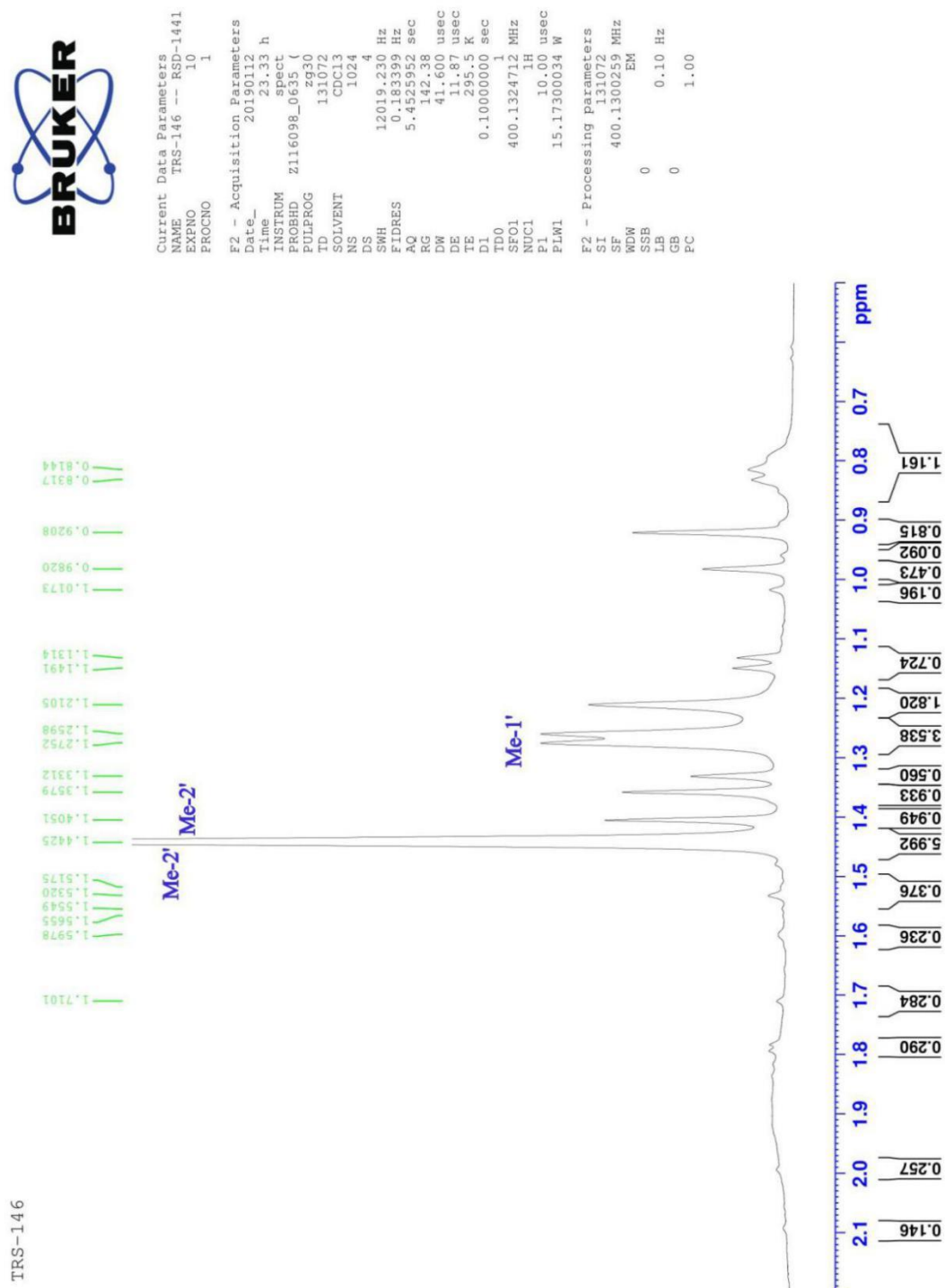


Figure 3. 25 Partially expanded  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of

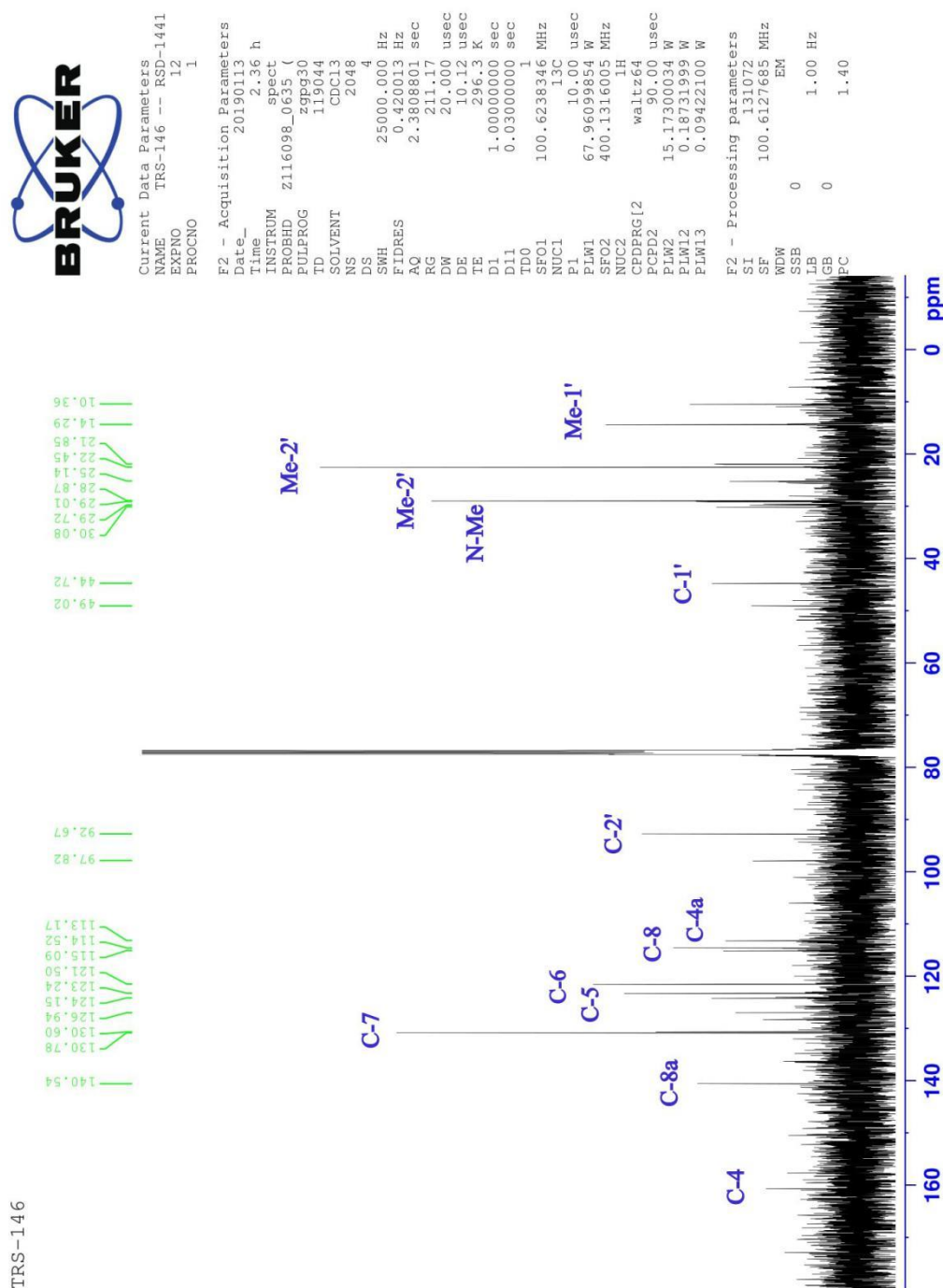


Figure 3. 26  $^{13}\text{C}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 4 (TRS-146)

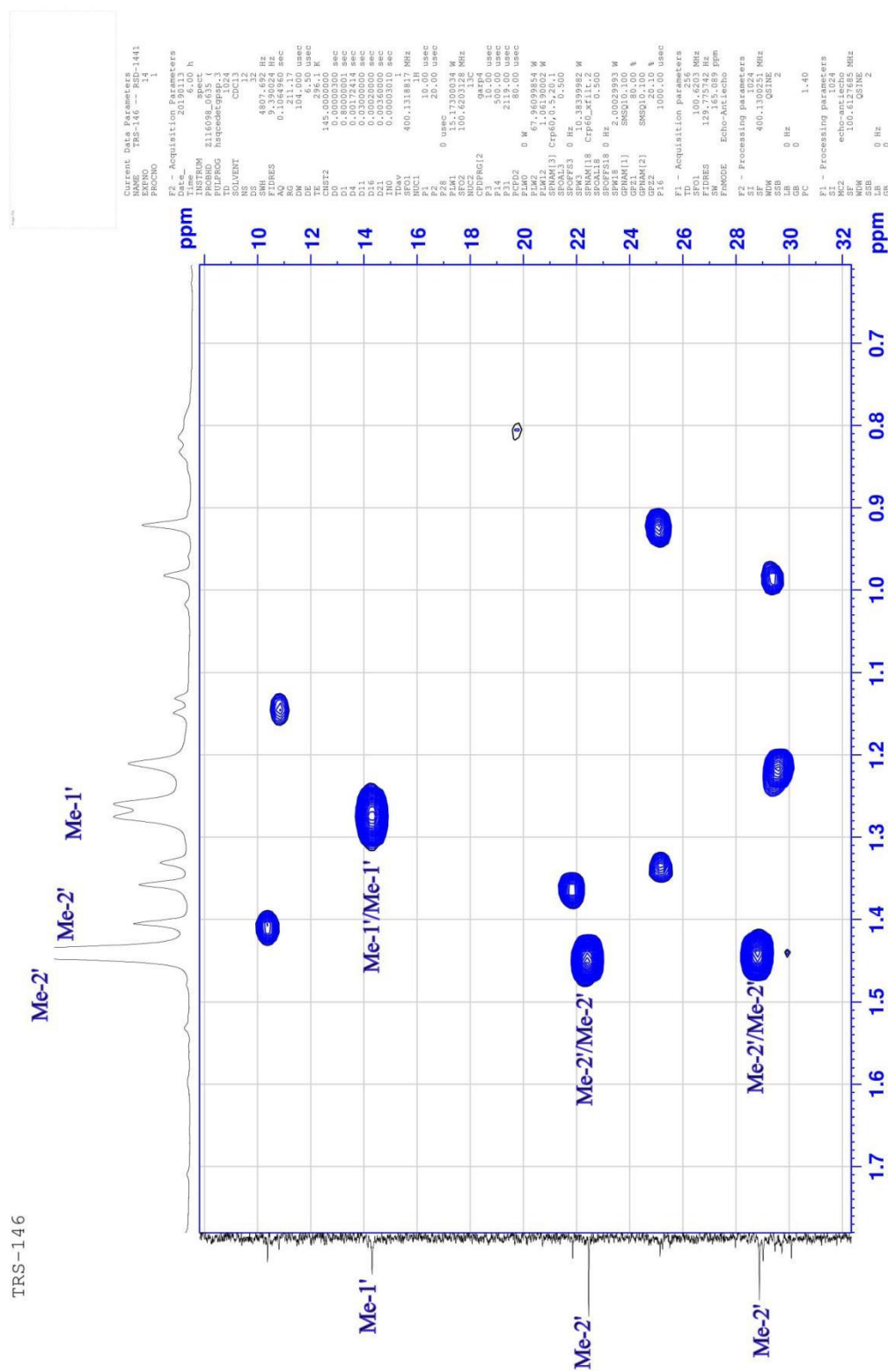


Figure 3.27 Partially expanded HSQC spectrum (400 MHz, CDCl<sub>3</sub>) of compound 4 (TRS-146)







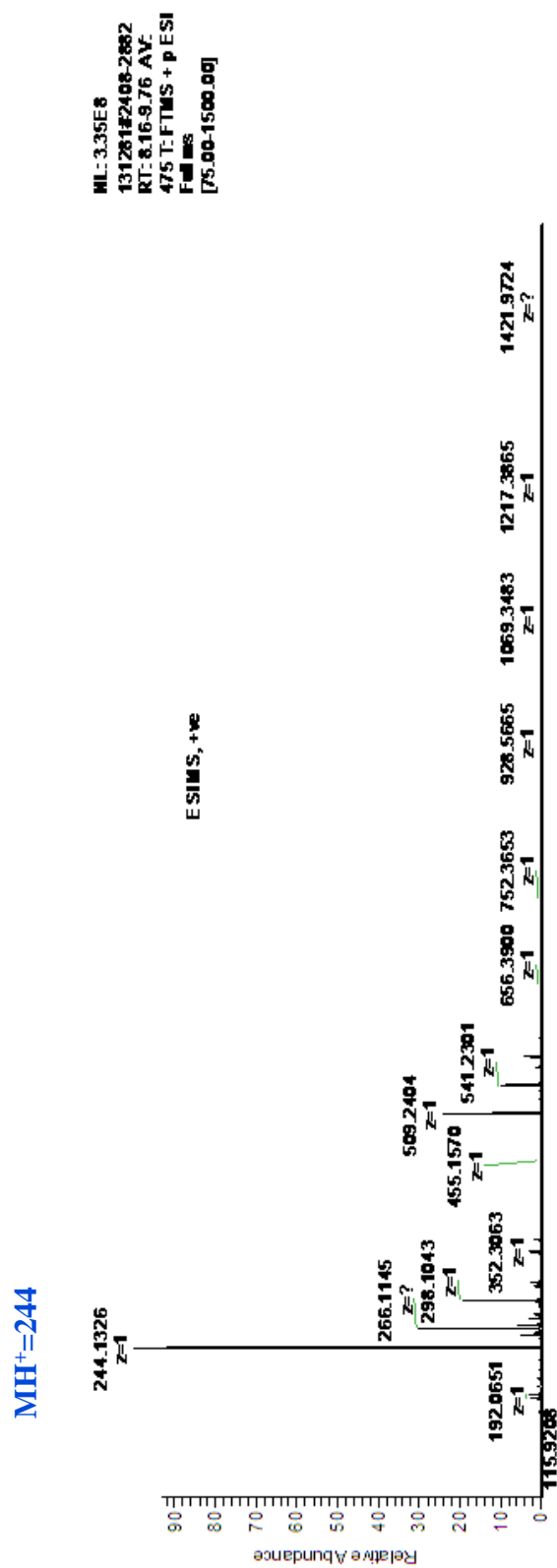


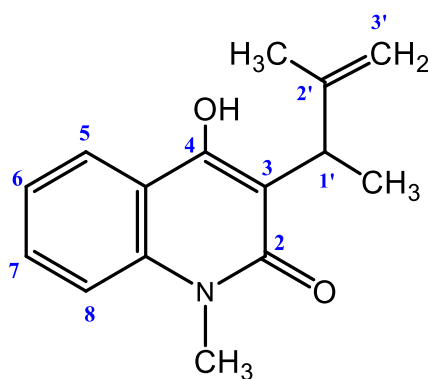
Figure 3.30 Mass spectrum of compound 4 (TRS-146)

### 3.1.5 Characterization of compound **5** (TRS-153) as Ravenoline

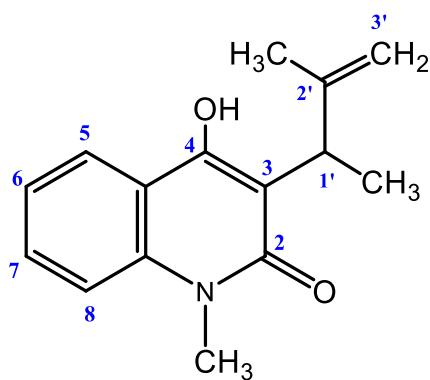
Compound **5** was isolated as colourless crystals, produced brown spot on a TLC plate after spraying with vanillin in sulphuric acid reagent and heated for 5 minutes. It produced reddish brown spot when sprayed with Dragendorff's reagent.

The  $^1\text{H}$  NMR spectral data (Table 3.7, Figure 3.31) of compound **5** demonstrated the presence of four aromatic protons with ABCD coupling at  $\delta$  7.95 dd ( $J = 8.0, 1.6$  Hz), 7.24 ddd ( $J = 8.0, 7.2, 0.8$  Hz), 7.57 ddd ( $J = 8.0, 7.2, 1.6$  Hz) and 7.34 d ( $J = 8.0$  Hz) assignable to H-5, H-6, H-7 and H-8 respectively of a 2-quinolone ring.

The *N*-methyl group (3H s), at position 1 and hydroxyl proton at position 4 appeared as singlets at  $\delta$  3.75 and 7.33. In addition the spectrum revealed the presence of a methyl doublet at  $\delta$  1.42 ( $J = 7.2$  Hz) indicating an adjacent methine proton at  $\delta$  4.18 q ( $J = 7.2$  Hz), an exomethylene group at  $\delta$  5.36 s, 5.28 s and a deshielded tertiary methyl group at  $\delta$  1.85 (3H s). All these NMR data suggested the presence of a 1, 2-dimethyl-1-propenyl chain at C-3 of the quinolone molecule. Thus compound **5** was identified as ravenoline isolated previously from *Ravenia spectabilis* and the structure was further confirmed by comparing its  $^1\text{H}$  NMR data with those published in the literature (Haque et al., 2013).



Ravenoline



Ravenoline

**Table 3.7**  $^1\text{H}$ NMR spectroscopic data (400 MHz,  $\text{CDCl}_3$ ) for compound **5**

Position	Compound 5	Ravenoline
	$\delta_{\text{H}}$	(Haque et al., 2013) $\delta_{\text{H}}$
5	7.95 dd ( $J = 8.0, 1.6$ Hz)	7.92 dd ( $J = 8.0, 1.2$ Hz)
6	7.24 ddd ( $J = 8.0, 7.2, 0.8$ Hz)	7.22 dd ( $J = 8.0, 1.2$ , Hz)
7	7.57 ddd ( $J = 8.0, 7.2, 1.6$ Hz)	7.54 dd ( $J = 7.2, 1.5$ Hz)
8	7.34 d ( $J = 8.0$ Hz)	7.31 d ( $J = 8.2$ Hz)
1'	4.18 q ( $J = 7.2$ Hz)	4.17 m
3'	5.36 s, 5.28 s	5.33 s, 5.26 s
Me-1'	1.42 3H d ( $J = 7.2$ Hz)	1.38 3H d ( $J = 7.2$ Hz)
Me-2'	1.85 3H s	1.82 3H s
OH	7.33 s	7.31 s
N-Me	3.75 s	3.72 s



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 Jahangirnagar University  
 Sample: TRS 153  
 Operated by: Md. Emdad Hossain, Scientist

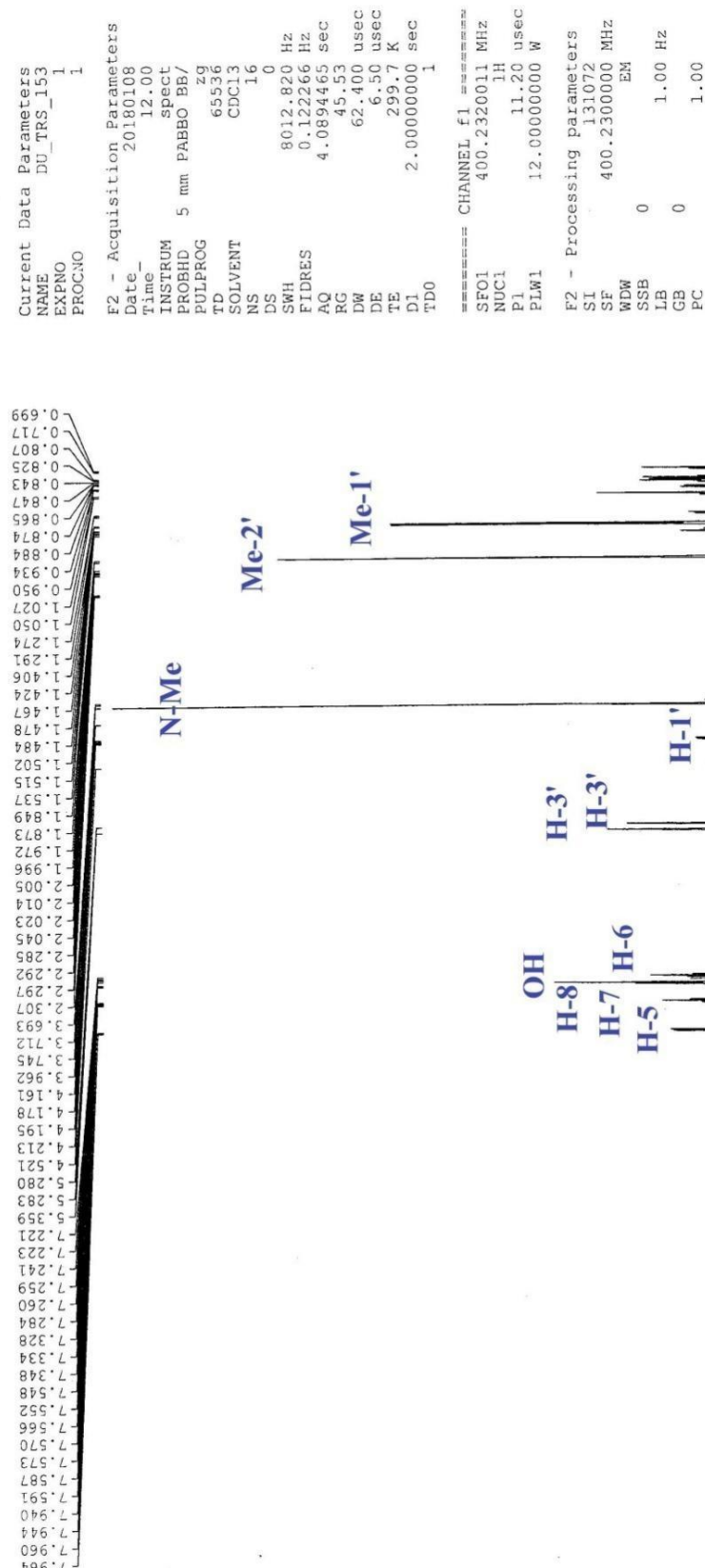


Figure 3.31  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 5 (TRS-153)

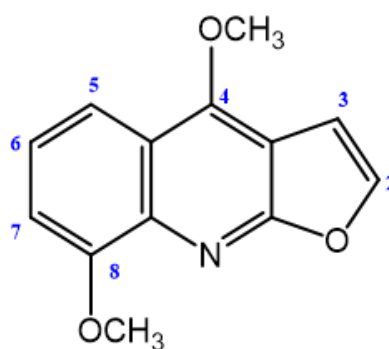
### 3.1.6 Characterization of compound 6 (TRS-206) as $\gamma$ -fagarine

Compound **6** was isolated as yellowish gummy mass, produced brown color when sprayed with vanillin in sulphuric acid reagent and heated for 5 minutes. It produced reddish brown color when sprayed with Dragendorff's reagent.

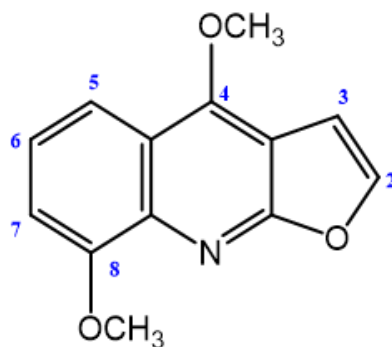
The  $^1\text{H}$  NMR spectrum (Table 3.8, Figure 3.32) showed two doublets at  $\delta$  7.68 and 7.11 with coupling constant of 2.8 Hz which could be assigned to H-2 and H-3 protons of a furan ring.

Three aromatic protons with ABC coupling at  $\delta$  7.87 dd ( $J = 8.6, 1.0$  Hz), 7.39 dd ( $J = 8.6, 7.7$  Hz) and 7.09 dd ( $J = 7.7, 1.0$  Hz) assignable to H-5, H-6 and H-7 of a quinoline ring of the furoquinoline alkaloid.

In addition, the spectrum showed two methoxy groups at  $\delta$  4.49 and 4.11 (3H s, each), assignable to OMe-4 and OMe-8 respectively. Compound **6** was identified as  $\gamma$ -fagarine as all these  $^1\text{H}$  NMR data were found to be in close agreement with those reported for the alkaloid isolated previously from the same plant (Sohrab et al., 2004).



$\gamma$ -fagarine

 $\gamma$ -fagarine**Table 3.8**  $^1\text{H}$ NMR spectroscopic data (400 MHz,  $\text{CDCl}_3$ ) for compound 6

Position	Compound 6 $\delta_{\text{H}}$	$\gamma$ -Fagarine (Sohrab et al., 2004) $\delta_{\text{H}}$
2	7.68 d ( $J = 2.8\text{Hz}$ )	7.62 d ( $J = 2.4\text{ Hz}$ )
3	7.11 d ( $J = 2.8\text{ Hz}$ )	7.05 br s
5	7.87 dd ( $J = 8.6, 1.0\text{ Hz}$ )	7.82 d ( $J = 8.4\text{ Hz}$ )
6	7.39 dd ( $J = 8.6, 7.7\text{ Hz}$ )	7.34 t ( $J = 8.2\text{ Hz}$ )
7	7.09 d ( $J = 7.7, 1.0\text{ Hz}$ )	7.04 d ( $J = 8.2\text{ Hz}$ )
OMe-4	4.49 3H s	4.42 3H s
OMe-8	4.11 3H s	4.06 3H s

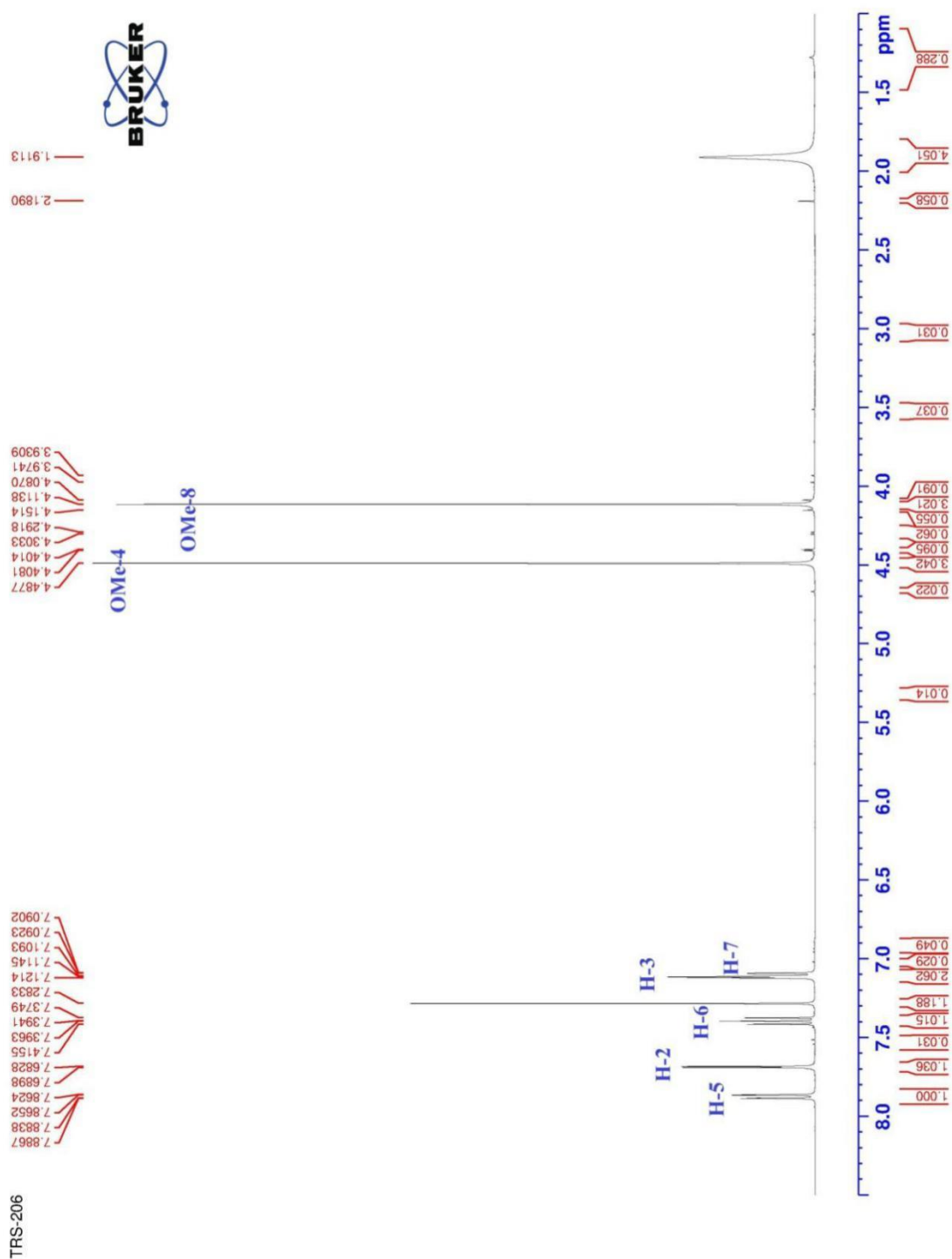


Figure 3.32  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 6 (TRS-206)



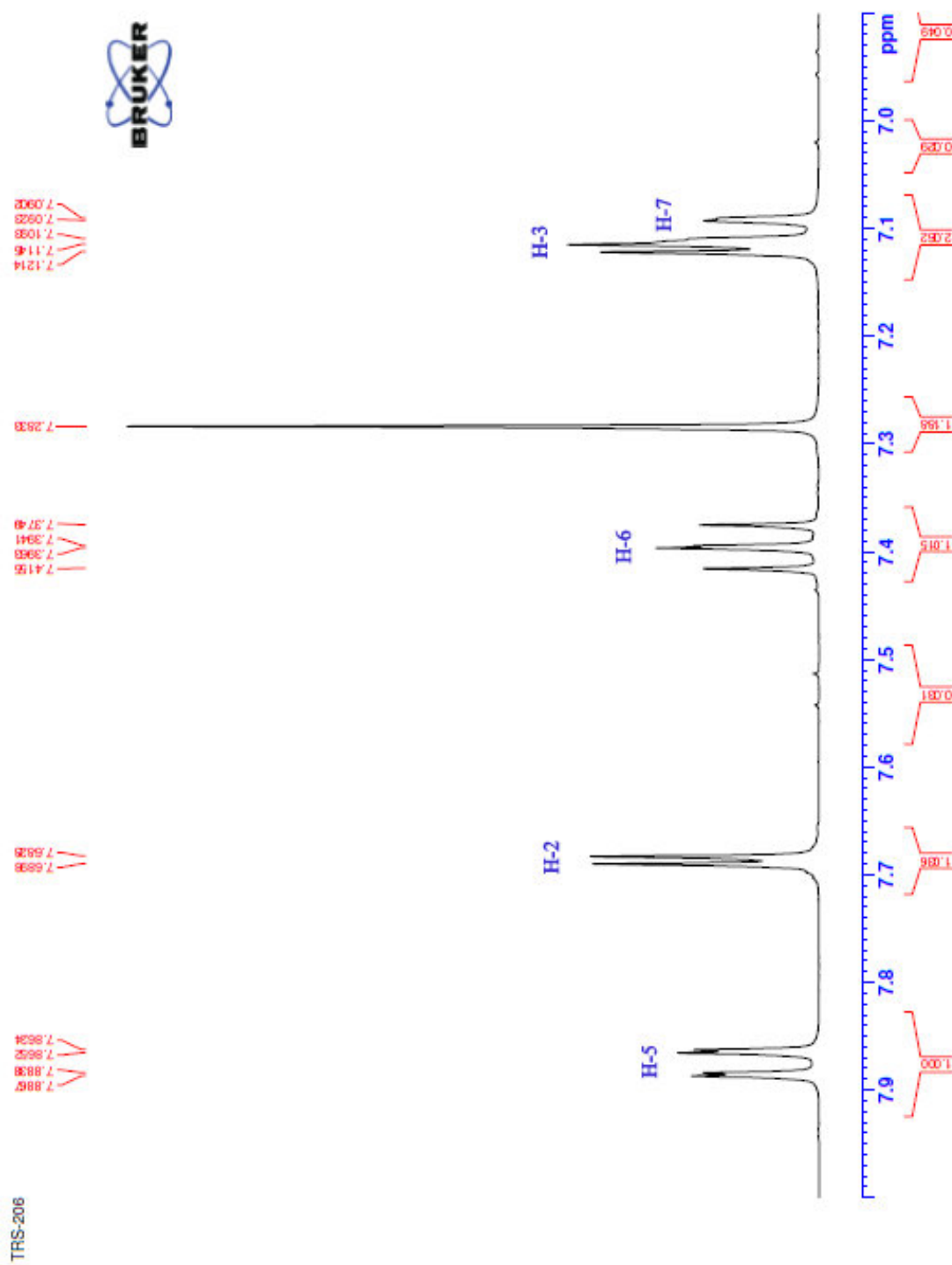


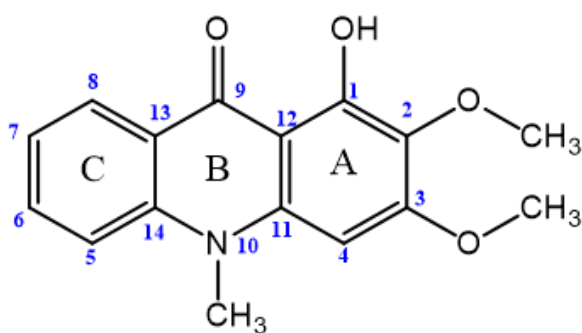
Figure 3.33 Partially expanded <sup>1</sup>H NMR spectrum (400 MHz, CDCl<sub>3</sub>) of compound 6 (TRS-206)

### 3.1.7 Characterization of compound 7 (TRS-221) as arborinine

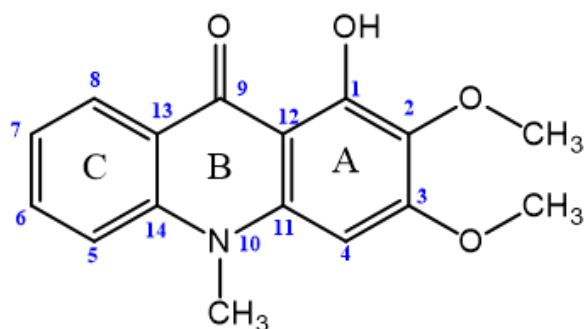
Compound **7**, isolated as greenish yellowish crystals, produced brown colored spot on a TLC plate when sprayed with vanillin in sulphuric acid reagent followed by heating for 5 minutes and reddish brown color when sprayed with Dragendorff's reagent.

The  $^1\text{H}$  NMR spectrum (Table 3.9, Figure 3.34) indicated five aromatic protons, an *N*-methyl and two methoxy groups. The four aromatic protons resonating at  $\delta$  7.50 d ( $J = 6.8$ ), 7.77 ddd ( $J = 8, 6.8, 1.4$  Hz), 7.33 dd ( $J = 8.0, 6.8$  Hz), 8.49 dd ( $J = 8.0, 1.4$  Hz) suggested an *ortho* disubstituted benzene ring and could be assigned to H-5, H-6, H-7 and H-8 respectively. The appearance of H-8 proton at a much lower field ( $\delta$  8.49) is due to the deshielding effect of the carbonyl oxygen at C-9 of the acridone molecule. The remaining aromatic protons, which appeared as a singlet at  $\delta$  6.32 and two methoxy resonating at  $\delta$  3.98 and  $\delta$  4.05, must be placed in ring A.

The  $^1\text{H}$  NMR data of compound **7** was found to be in close agreement with those reported for arborinine and was isolated previously from *Ravenia spectabilis* (Haque et al., 2013).



Arborinine



Arborinine

**Table 3.9**  $^1\text{H}$ NMR spectroscopic data (400 MHz,  $\text{CDCl}_3$ ) for compound 7

Posit <sup>n</sup>	Compound 7	Arborinine
	$\delta_{\text{H}}$	(Haque et al., 2013) $\delta_{\text{H}}$
H-4	6.32 s	6.29 s
H-5	7.50 d ( $J=6.8$ )	7.51 d ( $J=8.8$ Hz)
H-6	7.77 ddd ( $J=8.0, 6.8, 1.4$ Hz)	7.72 ddd ( $J=7.2$ Hz)
H-7	7.33 dd ( $J=8.0, 6.8$ Hz)	7.30 t ( $J=7.4$ Hz)
H-8	8.49 dd ( $J=8.0, 1.4$ Hz)	8.47 dd ( $J=6.4$ Hz)
N-Me	3.88 s	3.85 s
OMe-2	3.98 s	4.02 s
OMe-3	4.05 s	3.93 s

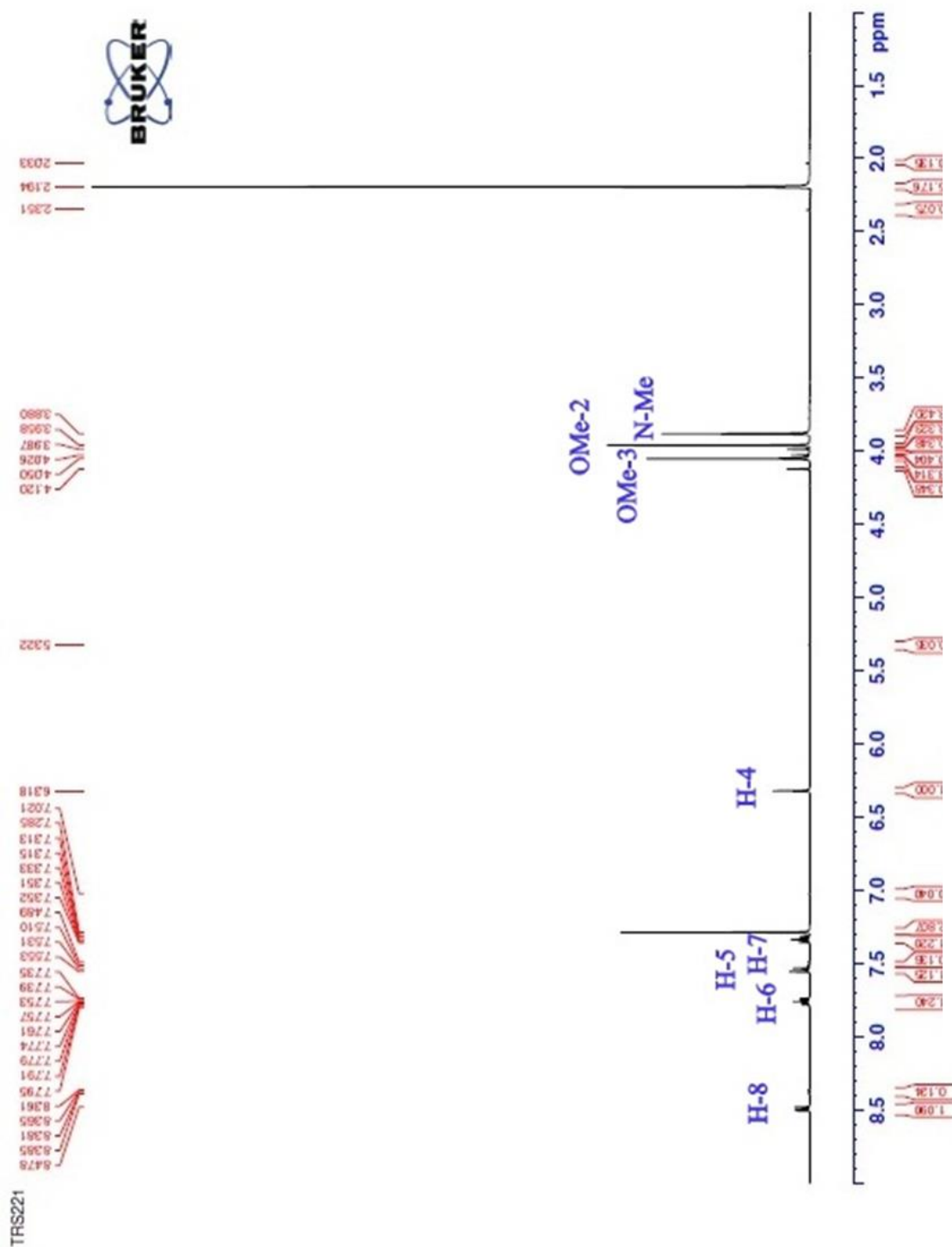


Figure 3.34  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 7 (TRS-221)

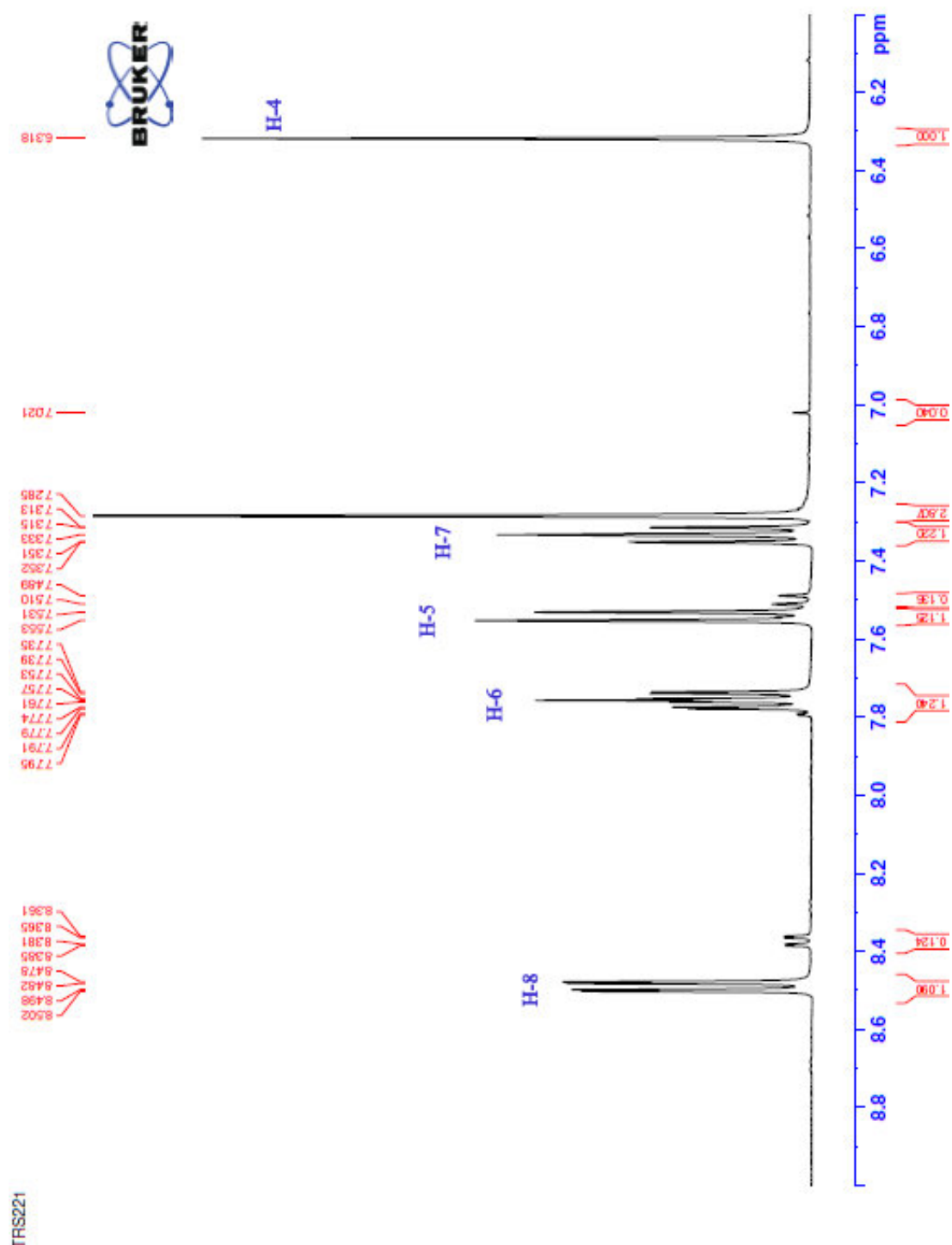


Figure 3.35 Partially expanded  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 7 (TRS-221)

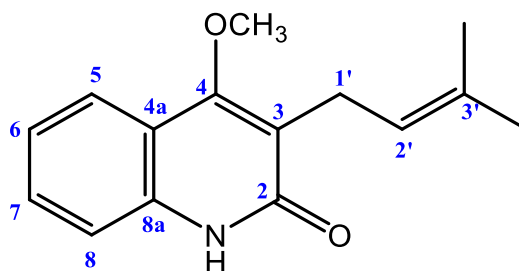
### 3.1.8 Characterization of compound **8** (RSD-140) as atanine

Compound **8**, obtained as yellowish gum, produced brown color on a TLC plate, when sprayed with vanillin in sulphuric acid reagent followed by heated for 5 minutes. It produced reddish brown color when sprayed with Dragendorff's reagent.

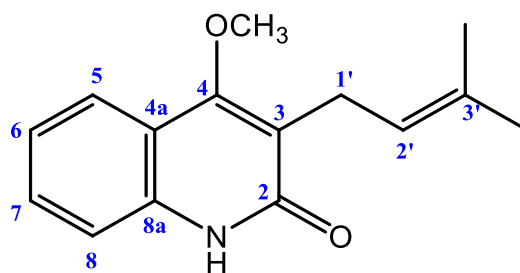
The  $^1\text{H}$  NMR spectrum (Table 3.10, Figure 3.36) revealed the presence of four adjacent aromatic protons comprising an ABCD coupling system, resonating at  $\delta$  7.68 dd ( $J = 8.0, 1.0$  Hz), 7.14 ddd ( $J = 8.0, 7.2, 1.0$  Hz), 7.39 ddd ( $J = 8.0, 7.04, 1.6$  Hz), 7.24 d ( $J = 8.0$  Hz) which could be attributed to H-5, H-6, H-7 and H-8 respectively. The H-5 proton appearing at a higher field at  $\delta$  7.68 is typical for 4-alkoxy-2-quinolones. Therefore, the methoxy group at  $\delta$  3.87 must be placed at C-4.

The spectrum also showed a benzylic methylene group ( $\delta$  3.35, 2H d,  $J = 6.8$  Hz), an olefinic proton ( $\delta$  5.22 br t,  $J = 8.0$ ) and two methyls at  $\delta$  1.75 and  $\delta$  1.63. All these signals together indicated the presence of a prenyl group which must be attached to C-3. The NH proton appeared at  $\delta$  10.85 as a broad singlet.

Thus compound **8** was identified as atanine, previously isolated from this plant (Haque et al., 2013).



Atanine



Atanine

**Table 3. 10**  $^1\text{H}$ NMR spectroscopic data (400 MHz,  $\text{CDCl}_3$ ) for compound 8

Position	Compound 8	Atanine
	$\delta_{\text{H}}$	(Haque et al., 2013) $\delta_{\text{H}}$
H-5	7.68dd ( $J = 8$ Hz, 1.0 Hz)	7.76 dd ( $J = 8.1, 1.1$ Hz)
H-6	7.14 ddd ( $J = 8, 7.2, 1.0$ Hz)	7.20 ddd ( $J = 8.1, 7.2, 1.0$ Hz)
H-7	7.39 ddd ( $J = 8, 7.04, 1.6$ Hz)	7.45 ddd ( $J = 8.1, 7.2, 1.2$ Hz)
H-8	7.24 d ( $J = 8.0$ Hz)	7.27 d ( $J = 8.1$ Hz)
H-1'	3.35 2H, d ( $J = 6.8$ Hz)	3.56d ( $J = 6.9$ Hz)
H-2'	5.22 br t ( $J = 6.8$ Hz)	5.28 br t ( $J = 6.9$ Hz)
NH	10.85 br s	10.82 br s
Me-3'	1.63 s	1.69 s
Me-3'	1.75 s	1.82 s
OMe-4	3.87 s	3.89 s

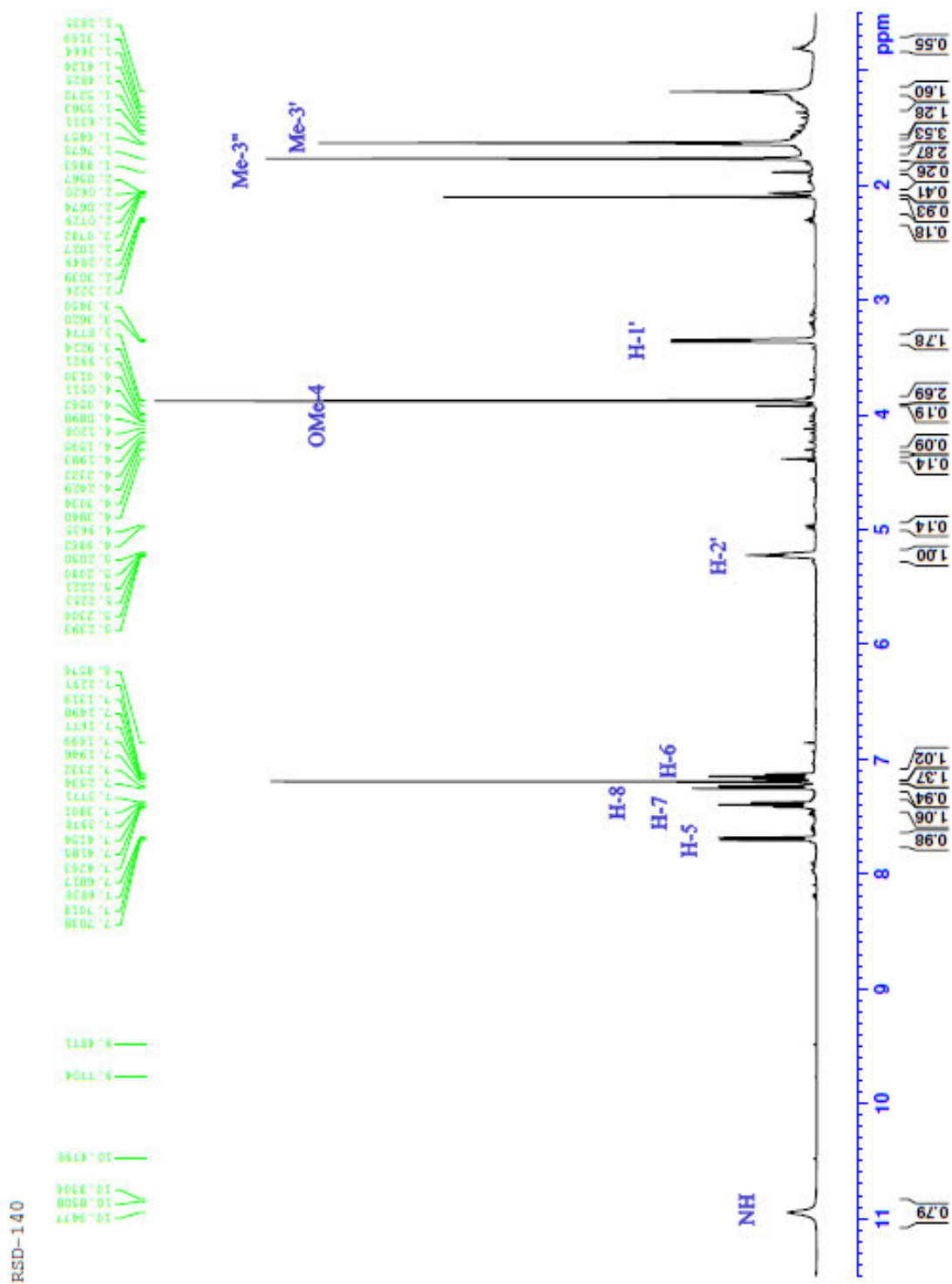


Figure 3.36 <sup>1</sup>H NMR spectrum (400 MHz, CDCl<sub>3</sub>) of compound 8 (RSD-140)



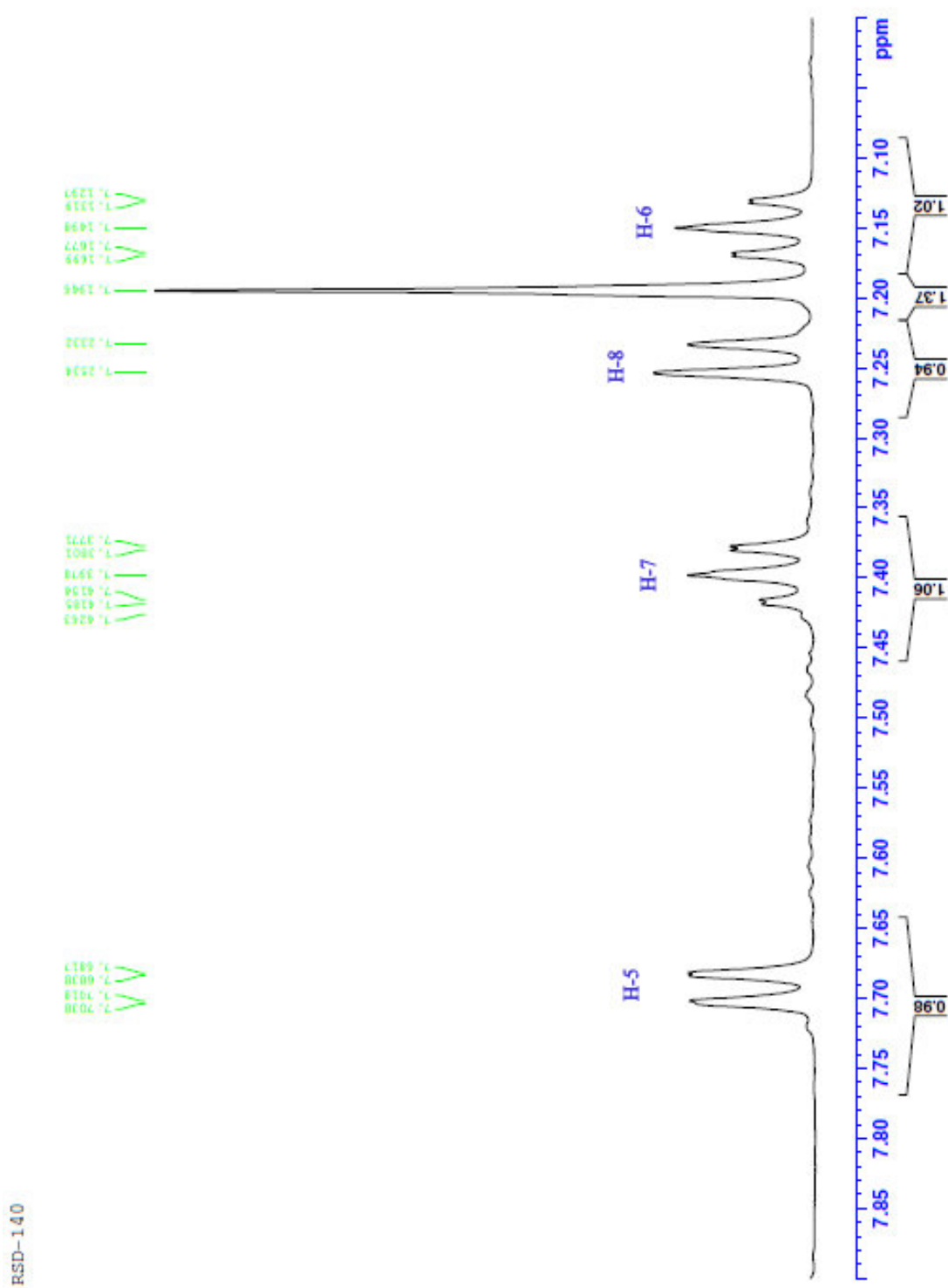


Figure 3. 37 Partially expanded  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 8 (RSD-140)

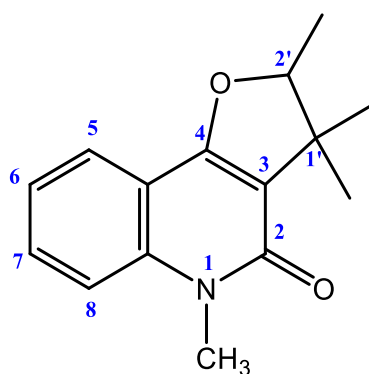
### 3.1.9 Characterization of compound **9** (RSD-164) as oligophyline

Compound **9** was appeared as yellow gum, produced brown color on a TLC plate when sprayed with vanillin in sulphuric acid reagent and heated for 5 minutes. It gave reddish brown color when sprayed with Dragendorff's reagent.

The  $^1\text{H}$ NMR spectral (Table 3.11, Figure 3.38) data of compound **9** demonstrated the presence of four aromatic protons with ABCD coupling resonating at  $\delta$  7.76 dd ( $J = 7.6, 1.2$  Hz), 7.21 dd ( $J = 7.6, 7.6$  Hz), 7.55ddd ( $J = 8.8, 7.2, 1.6$  Hz) and 7.34 d ( $J = 8.4$  Hz), assignable to H-5, H-6, H-7 and H-8 respectively .

The *N*- methyl group at position 1 appeared as a singlet at  $\delta$  3.67(3H, s).

The spectrum revealed the presence of two methyl singlet at  $\delta$  1.26 and 1.48 (3H s, each) could be assignable to C-1'. A methyl doublet appeared at  $\delta$  1.45 (3H d,  $J = 6.4$  Hz), a deshielded quartet at  $\delta$  4.59 q (1H q,  $J = 6.8$  Hz), now appearing at a low field than H-1' proton of compound **4**, could be placed at C-2', to which an oxygen is attached. The  $^1\text{H}$  NMR data of compound **9** was found to be in close agreement with those reported for 2, 3, 3,5-tetramethyl-2, 3, 4, 5-tetrahydrofurano [3,2- c] quinolin-4-one (Haque et al., 2013). The compound also named as oligophyline was previously isolated from *Euxylophora paraensis* (Grundon, 1987).



Oligophyline



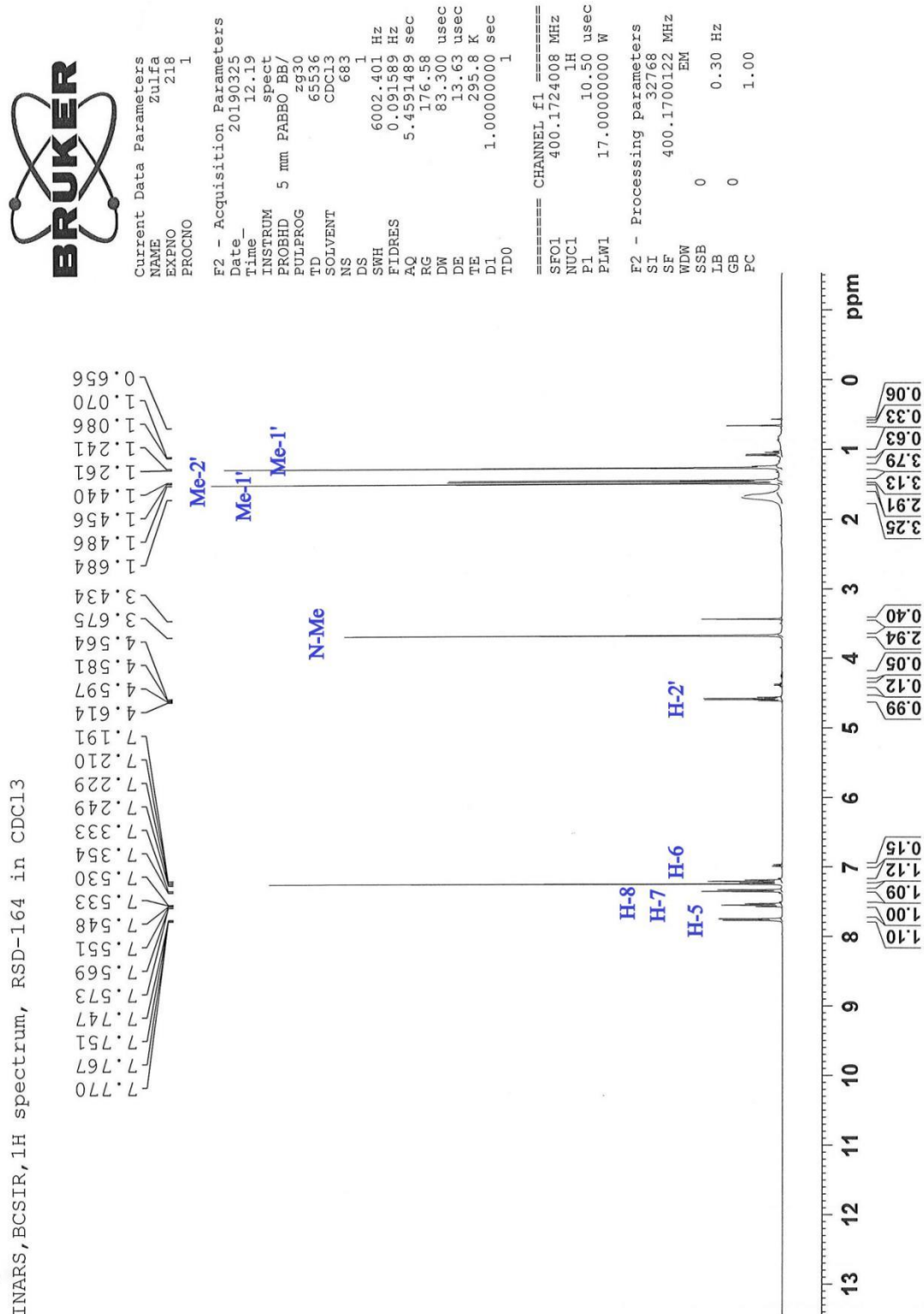


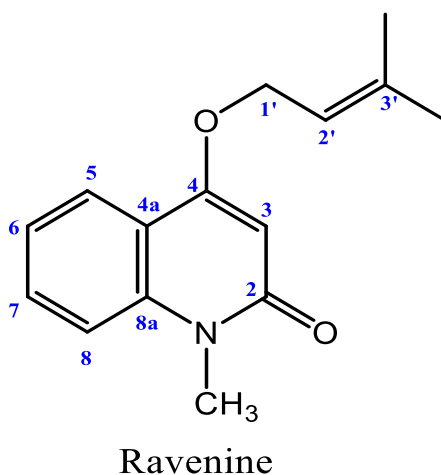
Figure 3. 38 <sup>1</sup>H NMR spectrum (400 MHz, CDCl<sub>3</sub>) of compound 9 (RSD-164)

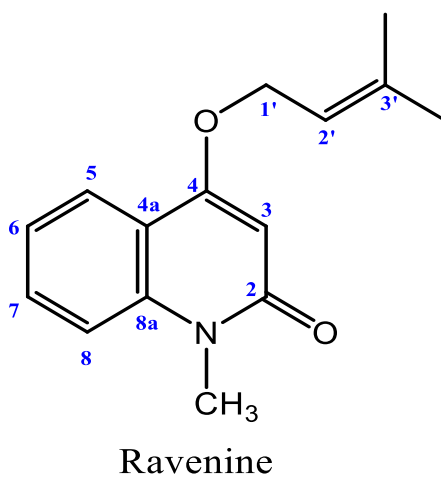
### 3.1.10 Characterization of compound 10 (RSD-167) as ravenine

Compound **10**, isolated as yellow gum, produced brown color on a TLC plate when sprayed with vanillin in sulphuric acid reagent followed by heating for 5 minutes and gave reddish brown color when sprayed with Dragendorff's reagent.

The  $^1\text{H}$  NMR spectrum (Table 3.12, Fig 3.39) demonstrated the presence of four aromatic protons as ABCD coupling resonating at  $\delta$  8.02 dd ( $J = 1.2, 8$  Hz), 7.25 dd ( $J = 7.2, 8$  Hz), 7.59 ddd ( $J = 1.6, 6.5, 8.5$  Hz), 7.35 d ( $J = 8.4$  Hz) assignable to H-5, H-6, H-7 and H-8 respectively. The spectrum also revealed the presence of N-methyl proton appeared as a singlet at  $\delta$  3.69. A singlet resonating at  $\delta$  6.07 assignable for the proton at position 3.

The spectrum also showed two methyl protons of three proton intensity appeared as singlets at  $\delta$  1.78 and  $\delta$  1.90, two oxymethylene protons at  $\delta$  4.6 (2H d  $J = 7.0$  Hz) and one methine proton at  $\delta$  5.56 (1H t  $J = 7.2$  Hz). The  $^1\text{H}$ NMR data of compound **10** was found to be in close agreement with those reported for ravenine (Paul & Bose, 1968).





**Table 3.12**  $^1\text{H}$  NMR spectroscopic data (400 MHz,  $\text{CDCl}_3$ ) for compound 10

Position	Compound 10 $\delta_{\text{H}}$	Ravenine (Paul & Bose, 1968) $\delta_{\text{H}}$
3	6.07 s	6.03 s
5	8.02 dd ( $J=1.2, 8.0$ Hz)	7.98 dd ( $J= 1.2, 8.2$ )
6	7.25 dd ( $J=7.2, 8.0$ Hz)	7.21 dd ( $J= 7, 8.2$ )
7	7.59 ddd ( $J=1.6, 6.5, 8.5$ Hz)	7.54 ddd ( $J= 1.6, 6.6, 8.6$ )
8	7.35 d ( $J= 8.4$ Hz)	7.31 d ( $J= 8.2$ )
1'	4.6 (2H d $J= 7.0$ Hz)	4.64 d ( $J= 6.6$ )
2'	5.56 (1H t $J= 7.0$ Hz)	5.52 d ( $J= 7.2$ )
3'- $\text{CH}_3$	1.78 s	1.76 s
3'- $\text{CH}_3$	1.84 s	1.82 s
N- $\text{CH}_3$	3.69 s	3.67s

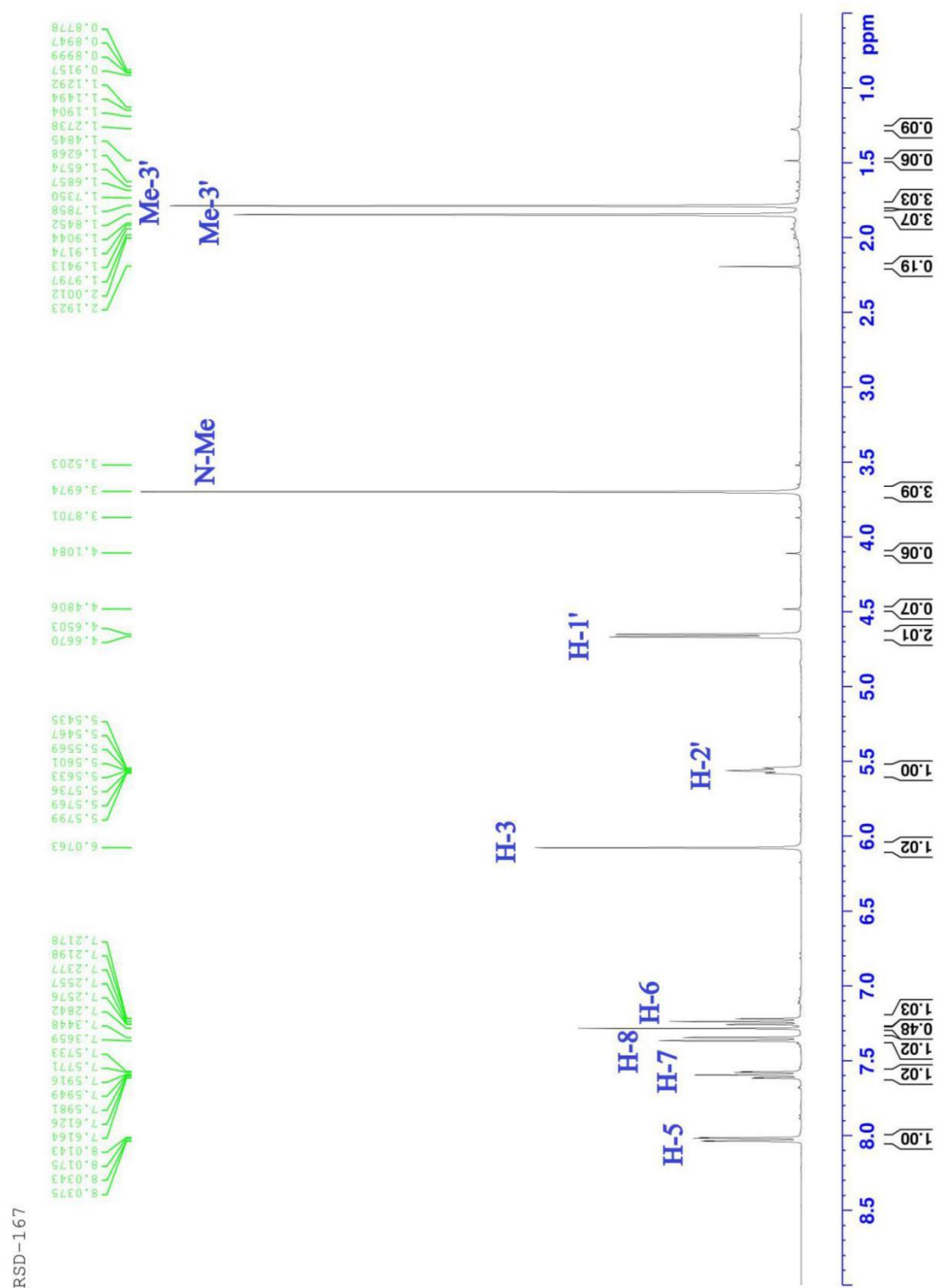


Figure 3. 39  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 10 (RSD-167)

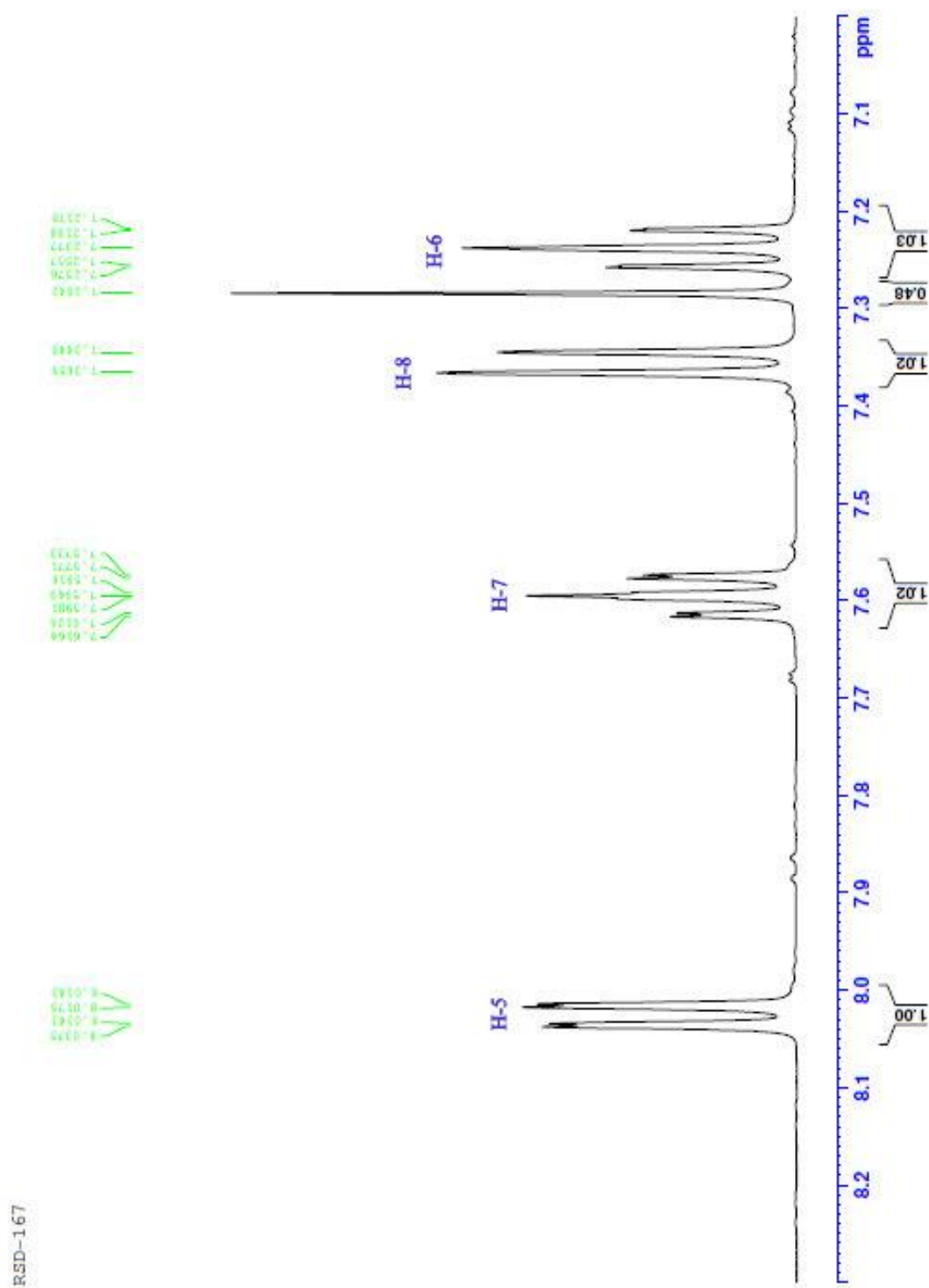


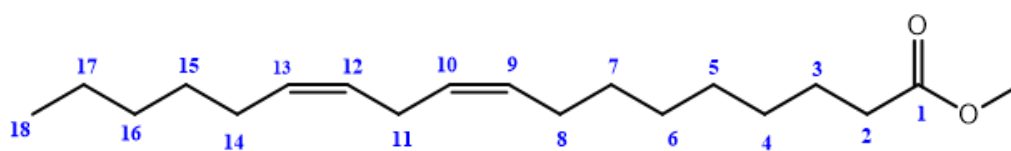
Figure 3.40  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 10 (RSD-167)



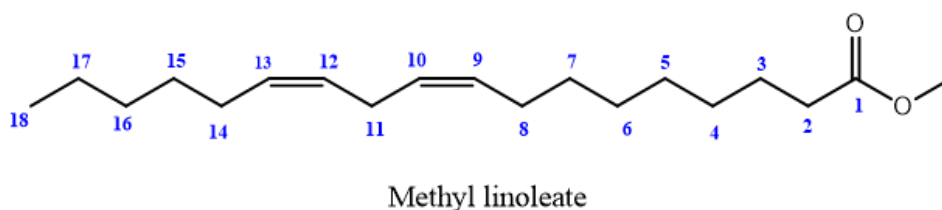
### 3.1.11 Characterization of compound **11** (RSD-180) as methyl linoleate

Compound **11** was obtained as light yellowish mass, produced light brown color on a TLC plate when sprayed with vanillin in sulphuric acid reagent and heated for 5 minutes.

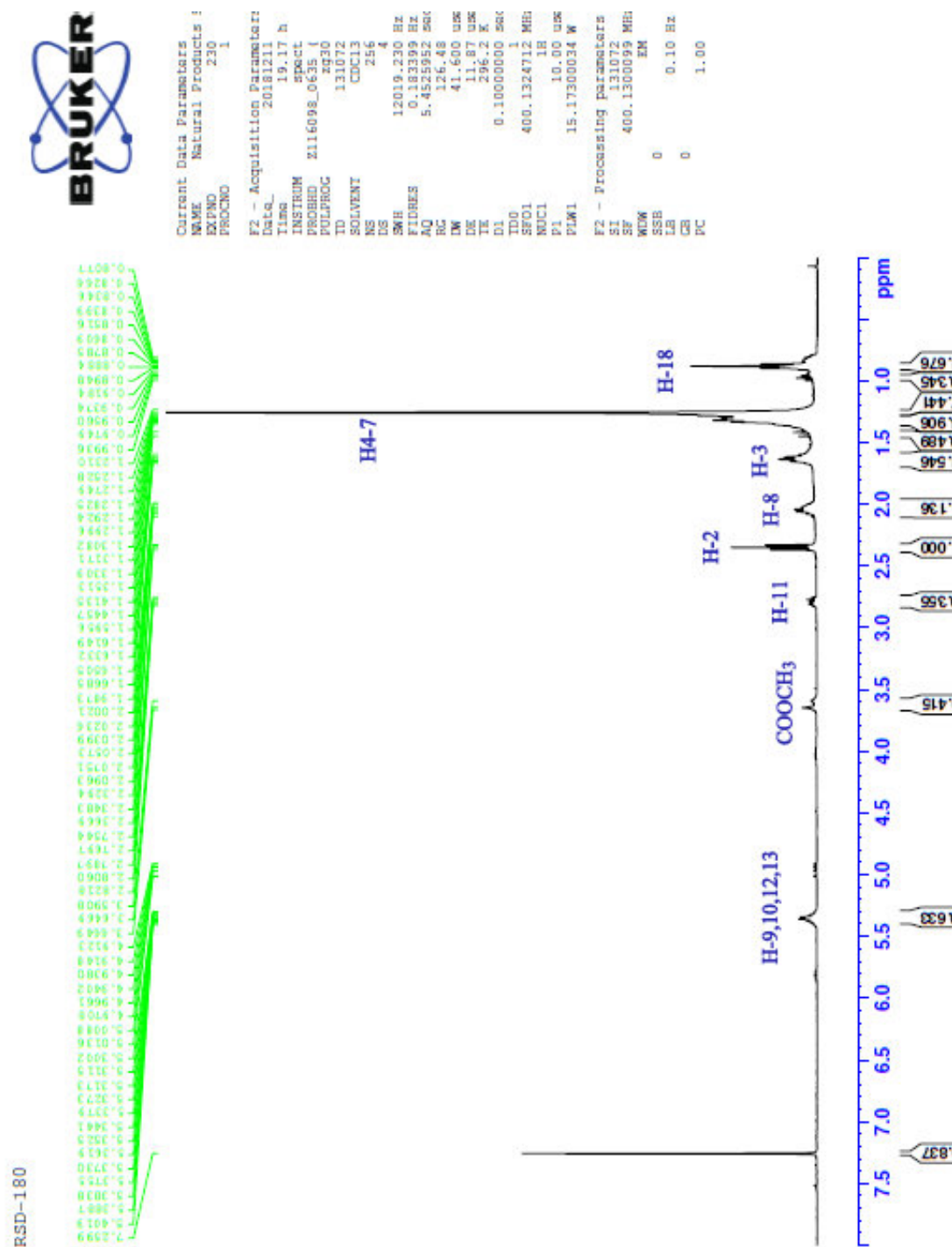
The  $^1\text{H}$  NMR spectral data (Table 3.13, Figure 3.41) of compound **11** demonstrated the presence of four olefinic proton multiplets resonating at  $\delta$  5.36, a methyl triplet at  $\delta$  0.89 and a methoxy group at  $\delta$  3.65, which could be assigned to two conjugated doublets at C-9 & C-12, a terminal methyl group (C-18) and the methyl ester moiety ( $\text{COOCH}_3$ ) of an unsaturated fatty acid respectively. The bis-allylic protons ( $=\text{CH}-\underline{\text{CH}_2}-\text{CH}=\text{}$ ) appeared at  $\delta$  2.79 (2H m H-11) and the protons resonating at  $\delta$  2.07 (4H m) are the allylic protons ( $\underline{\text{CH}_2}-\text{CH}=\text{CH}$ ) of C-8 and C-14. The protons directly adjacent to the carbonyl group resonated at  $\delta$  2.37 (2H t H-2) and the  $\text{OOC}-\text{CH}_2-\underline{\text{CH}_2}$  protons resonated at  $\delta$  1.63 (2H, m H-3). The methylene protons of fatty chain appeared at  $\delta$  1.26-1.32 (14H m). All these data are found to be in close agreement with those reported for methyl linoleate (Diaz and Gavin, 2007).



Methyl linoleate

**Table 3.13**  $^1\text{H}$  NMR spectroscopic data (400 MHz,  $\text{CDCl}_3$ ) for compound 11

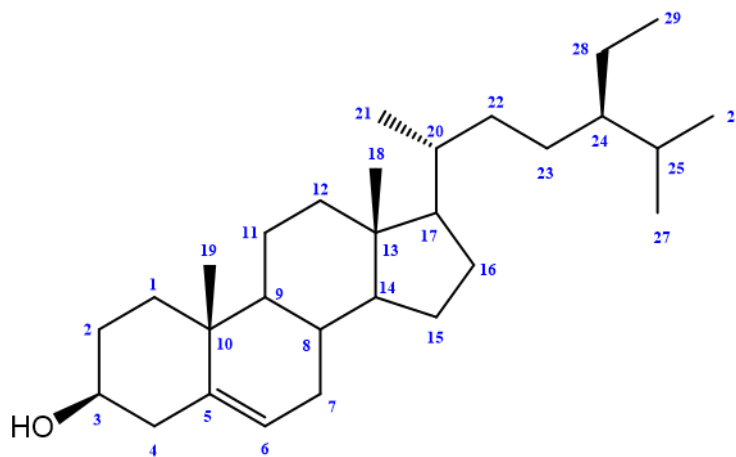
Position	Compound 11 $\delta_{\text{H}}$	Me-linoleate (Diaz and Gavin, 2007) $\delta_{\text{H}}$
2	2.37 2H t (1H t $J = 7.2$ Hz)	2.3 2H t
3	1.63 2H m	1.6 2H m
4-7	1.26-1.32, 8H m	1.3, 8H m
8	2.07 2H m	2.0 2H m
9,10, 12, 13	5.36 m	5.3 m
11	2.79 2H m	2.7 2H m
14	2.07 2H m	2.0 2H m
15-17	1.26-1.32, 6H m	1.3, 6H m
18	0.89, 3H t (1H t $J = 6.8$ Hz)	0.9, 3H t
$\text{COOCH}_3$	3.65 3H s	3.60 3H s

Figure 3.41 <sup>1</sup>H NMR spectrum (400 MHz, CDCl<sub>3</sub>) of compound 11 (RSD-180)

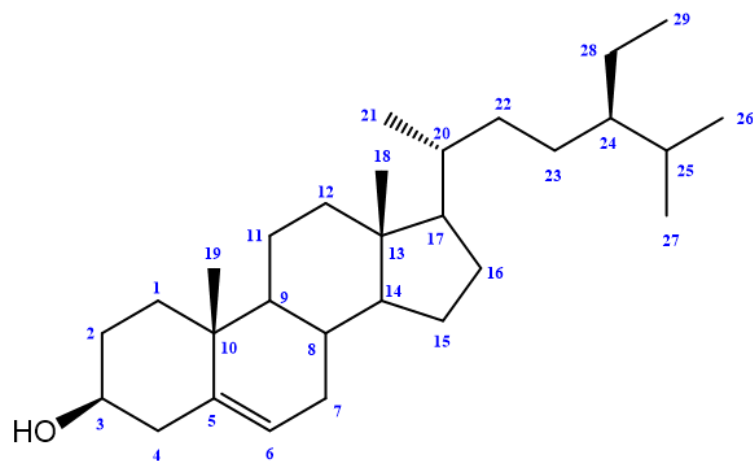
### 3.1.12 Characterization of compound **12** (RSD-137) as $\beta$ -sitosterol

Compound **12**, isolated as colorless crystal produced dark purple color on a TLC plate when sprayed with vanillin in sulphuric acid reagent followed by heating for 5 minutes

The  $^1\text{H}$  NMR spectrum (Table 3.14, Figure 3.42) of compound **12** showed the presence of six high intense peaks indicating the presence of six methyl groups resonating at  $\delta$  0.75, 0.82, 0.84, 0.85, 0.92 and 0. 1.00 ppm which are assignable for H-18, H-26, H-27, H-29, H-21 and H-19 respectively. The proton corresponding to the H-3 of a sterol moiety was appeared as a multiplet at  $\delta$  3.53 ppm, H-6 olefinic proton appeared at  $\delta$  5.35 ppm. Compound **12** was identified as  $\beta$ -sitosterol by comparing its  $^1\text{H}$  NMR data with those published in the literature (Pateh et al, 2009).



$\beta$ -Sitosterol



$\beta$ -Sitosterol

**Table 3.14**  $^1\text{H}$  NMR spectroscopic data (400 MHz,  $\text{CDCl}_3$ ) for compound 12 (RSD-137) as  $\beta$ -Sitosterol

Position	RSD-137 $\delta_{\text{H}}$	$\beta$ - Sitosterol (Patch et al, 2009) $\delta_{\text{H}}$
H-3	3.53 1H m	3.53 1H m
H-6	5.35 1H d ( $J = 5.0$ Hz)	5.37 1H br s
H-18	0.75 3H s	0.68, 3H s
H-19	1.00 3H s	1.01, 3H s
H-21	0.92 d ( $J = 6.5$ Hz)	0.92 d ( $J = 6.4$ Hz)
H-26	0.82 d ( $J = 7.2$ Hz)	0.81 d ( $J = 6.4$ Hz)
H-27	0.84 d ( $J = 7.2$ Hz)	0.83 d ( $J = 6.4$ Hz)
H-29	0.85 t ( $J = 7.2$ Hz)	0.85 t ( $J = 7.5$ Hz)

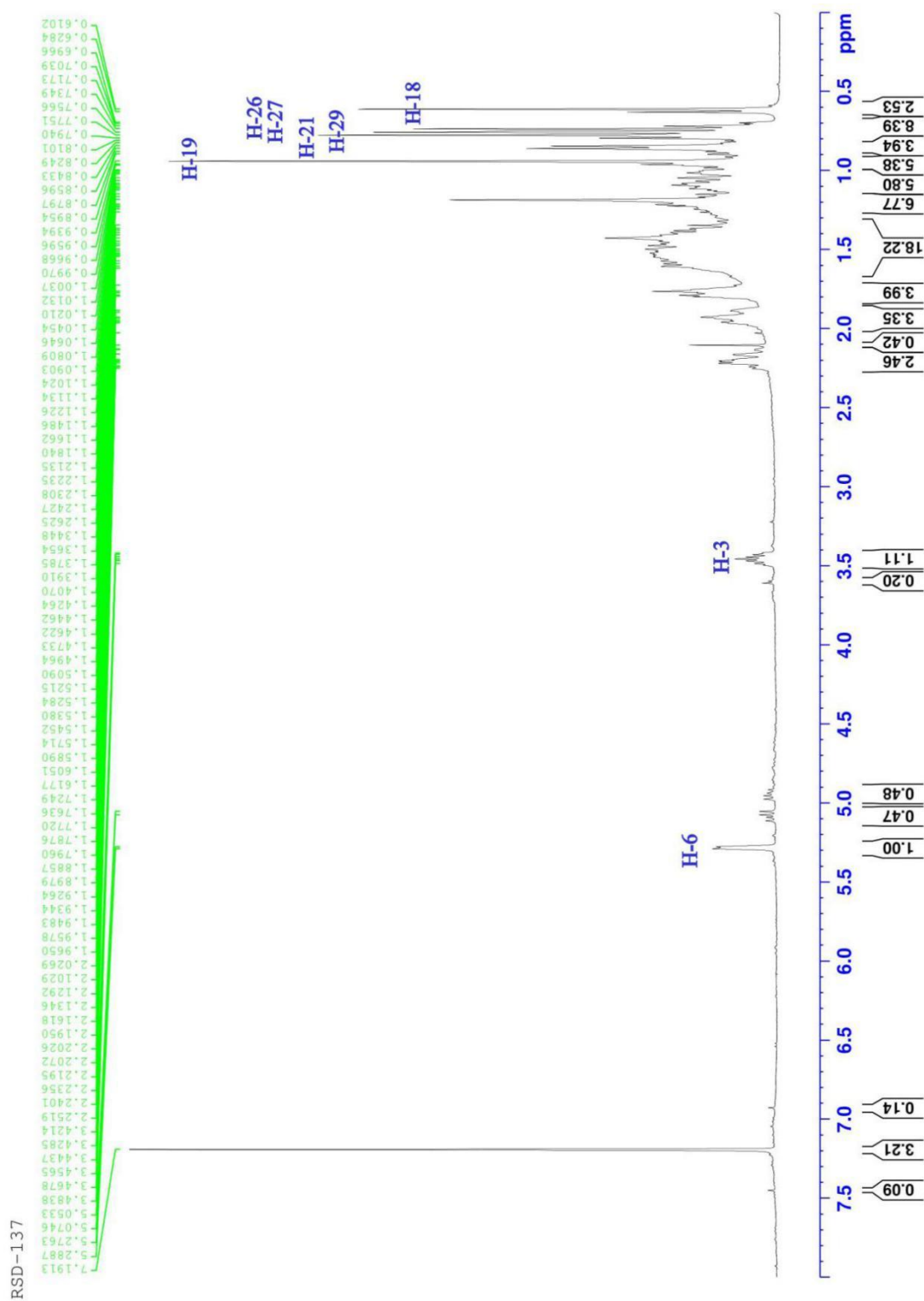


Figure 3.42  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of Compound 12 (RSD-137)

### 3.1.13 Characterization of compound **13** (RSD-1441 or TRS-121) as ravespanol

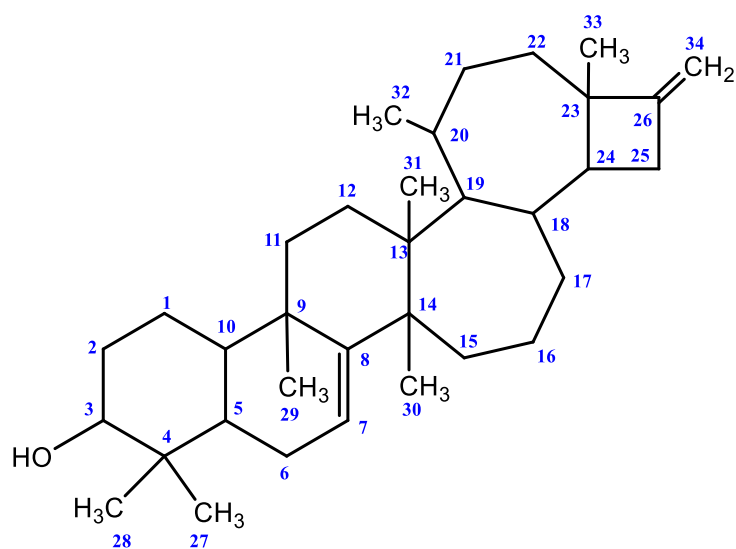
Compound **13**, isolated as fine needles, was invisible when examined under UV light on a TLC plate and produced brown color when sprayed with vanillin in sulphuric acid reagent followed by heating for 5 minutes.

Compound **13** and compound **14** were found to be very unusual and closely related C-34 terpenoids which differ only in position 3. Compound **13** contain a hydroxyl group and the other contain a keto group at C-3 as confirmed by the 1D and 2D spectroscopic data.

The  $^1\text{H}$ ,  $^{13}\text{C}$  NMR,  $^1\text{H}$ - $^1\text{H}$  COSY, HSQC and HMBC spectra in both  $\text{CDCl}_3$  and  $\text{C}_5\text{D}_5\text{N}$  were available for RSD-1441. The  $^1\text{H}$  NMR spectrum (Table 3.15, Figure 3.43) showed an olefinic proton at  $\delta$  5.33 d ( $J = 5.1$  Hz), two exomethylene protons  $\delta$  4.98, 4.94 (br s, each), an oxymethine proton at  $\delta$  3.70, six methyl singlets at  $\delta$ , 0.90, 0.95, 1.11, 1.12, 1.21, 1.25 and a methyl doublet  $\delta$  0.96 d ( $J = 6.6$  Hz). The  $^{13}\text{C}$  NMR spectrum displayed 34 carbons including two unsaturated quaternary carbons at  $\delta$  156.8, 158.0, an unsaturated methine carbon at  $\delta$  118.8 and an unsaturated methylene carbon at  $\delta$  107.4, indicating the presence of a tri-substituted olefinic group and an exomethylene group. Further the spectrum revealed a carbinol carbon at  $\delta$  75.8 and seven methyl carbons at  $\delta$  13.4 - 26.5. The HSQC spectrum (Figure 3.46 & Figure 3.51) showed  $^1J$  connectivities of twelve methylene protons to the methylene carbons at  $\delta$  20.9, 30.0, 25.1, 32.8, 34.4, 34.3, 30.3, 28.8, 31.4, 38.4, 24.0 and 107.4. The spectrum also showed  $^1J$  connectivities of eight methine protons to methine carbons at  $\delta$  75.8, 35.8, 118.8, 47.8, 28.7, 54.6, 37.7 and 28.0. The methyl carbons were also assigned from the HSQC. In the HMBC spectrum (Figure 3.52) the methyls at  $\delta$  1.25 and 0.90 showed common correlations to the carbinol carbon at  $\delta$  75.8 (C-3), a quaternary carbon at  $\delta$  39.6 (C-4) and methine carbon at  $\delta$  35.8 (C-5). These two methyls also correlated to each other, thus indicating them as germinal methyls at position 27 and 28 respectively. The olefinic proton at  $\delta$  5.33 revealed  $^3J$  correlations to  $\delta$  35.8 (C-5), 37.0 (C-9) and 53.4 (C-14), and therefore could be assigned to H-7. The methyl at  $\delta$  1.21, which could be assigned to H-29, indicated  $^{2/3}J$  correlations to  $\delta$  32.8 (C-11), revealed  $^3J$  connectivities to C-8 and C-15 and the later to C-12, therefore confirming their position at 30 and 31. The methyl doublet at  $\delta$  0.96 showed

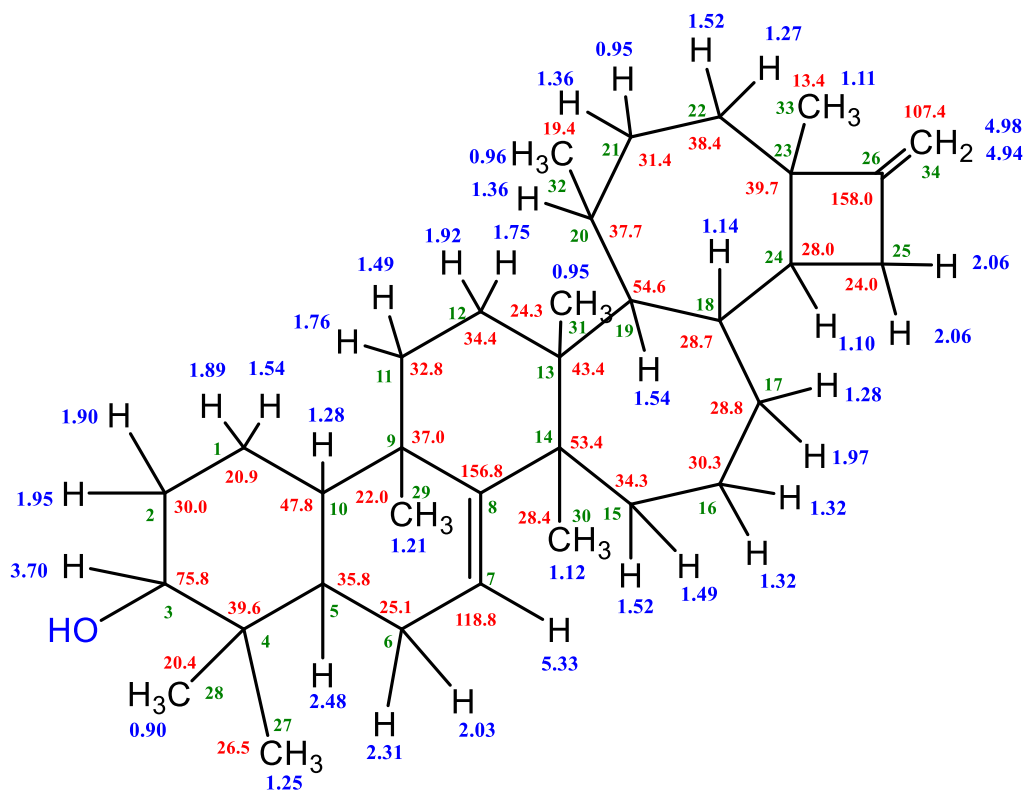
$^2J$  correlation to  $\delta$  37.0 (C-9), 47.8 (C-10) and 156.8 (C-8). Two methyl groups resonating at  $\delta$  1.12 and 0.95 showed common connectivities to C-13 and C-14, in addition, the former 37.7 and  $^3J$  correlation to  $\delta$  31.4 to which a shielded proton resonating at  $\delta$  0.95 is attached. The later revealed  $^3J$  correlation to  $\delta$  54.6 (H-19), and thus could be assigned to H-21 and the methyl doublet could be assigned to H-32. In the COSY spectrum (Figure 3.40), the H-21 proton showed correlations to protons at  $\delta$  1.52 and 1.27 (thus placing them at 22), which showed direct connectivities to the carbon at  $\delta$  38.4. The remaining methyl at  $\delta$  1.11 showed  $^3J$  correlation to the methylene carbon at  $\delta$  38.4 (C-22), quaternary carbon at  $\delta$  39.7 (C-23), a methine carbon at  $\delta$  28.0 C-24) and the unsaturated carbon at  $\delta$  158.0 (C-26). The exomethylene protons revealed  $^3J$  correlation to C-23 and C-26. In the COSY spectrum the H-15 proton showed coupling to H-16 and the latter to H-17 protons. In the HMBC spectrum H-17 proton showed  $^3J$  correlation to C-24 and H-18 proton to C-26.

Thus the structure of compound 13 was tentatively determined and given a trivial name ravespanol.



Ravespanol

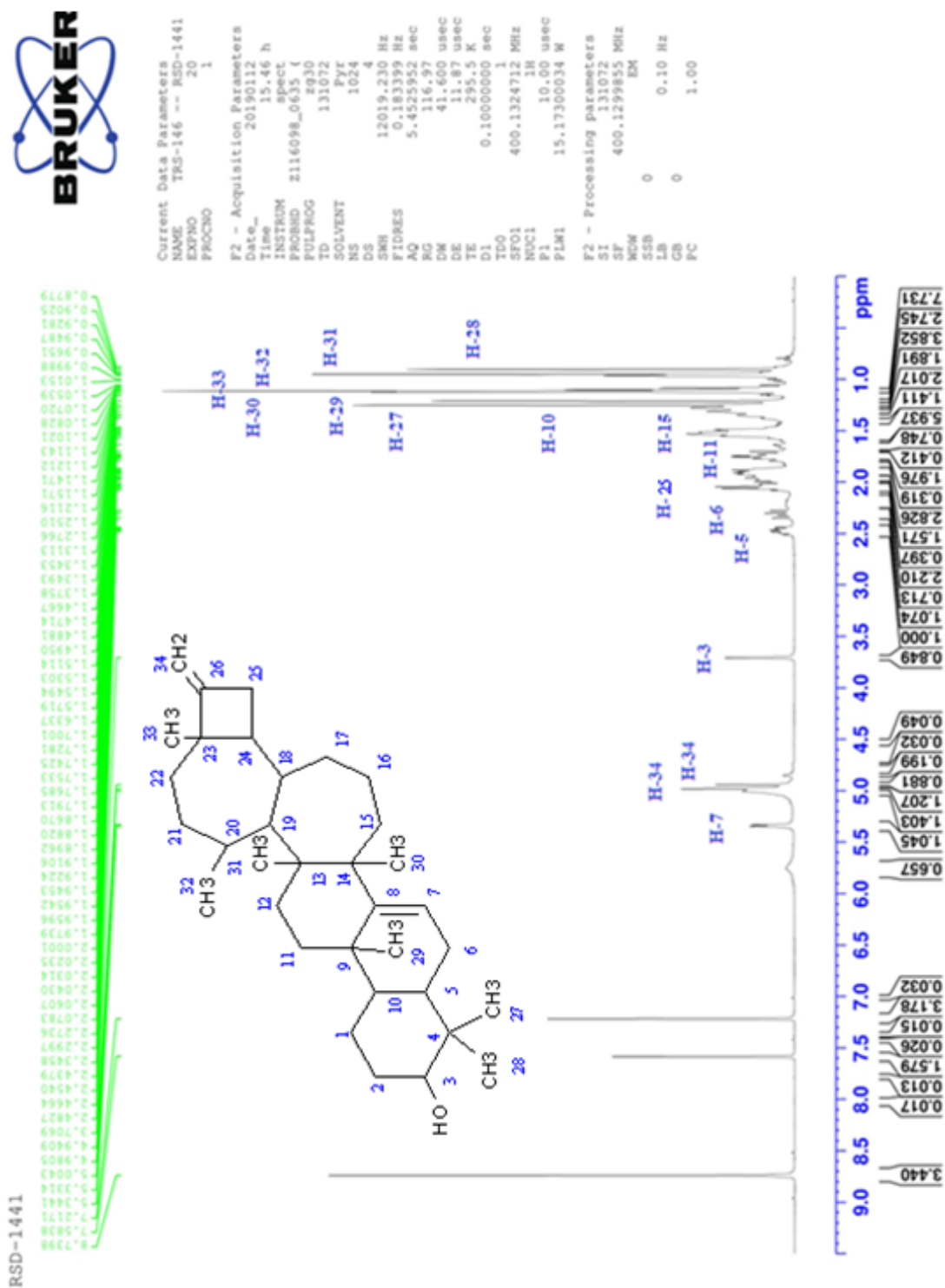




Ravespanol

**Table 3.15 NMR spectroscopic data (400 MHz, C<sub>5</sub>D<sub>5</sub>N) for compound 13 (TRS 1441)**

Positn	$\delta_C$	$\delta_H$	HMBC
1	20.9	1.89, 1.54	---
2	30.0	1.90, 1.95	---
3	75.8	3.70 br s	20.9 (C-1)
4	39.6	---	---
5	35.8	2.48	20.4 (C-28), 39.6 (C-4), 47.8 (C-10),
6	25.1	2.31, 2.03	39.6 (C-4), 156.8 (C-8),
7	118.8	5.33 d ( $J = 5.1$ Hz)	25.1 (C-6), 35.8 (C-5), 37.0 (C-9), 53.4 (C-14)
8	156.8	---	---
9	37.0	---	---
10	47.8	1.28	---
11	32.8	1.76m, 1.49 m	22.0 (C-29), 43.4 (C-13), 53.4 (C-14), 47.8 (C-10) 34.4 (C-12), 156.8 C-8)
12	34.4	1.92 m, 1.75 m	24.3 (C-31), 32.8 (C-11), 37.0 (C-9), 37.0 (C-9), 47.8 (C-10)
13	43.4	---	---
14	53.4	---	---
15	34.3	1.52m, 1.49 m	53.4 (C-14), 28.7 (C-18)
16	30.3	1.32 m, 1.32 m	---
17	28.8	1.97, 1.28	28.0 (C-24)
18	28.7	1.14 m	24.0 (C-25),
19	54.6	1.54 m	----
20	37.7	1.36 m	---
21	31.4	0.95 m, 1.36 m	---
22	38.4	1.27 m, 1.53 m	28.0 (C-24) 158.0 (C-26)
23	39.7	---	---
24	28.0	1.10 m	24.0 (C-25),
25	24.0	2.06, 2Hm	13.4 (C-33), 107.4 (C-34), 158.0 (C-26)
26	158.0	---	---
27	26.5	1.25 3H s	20.4 (C-28), 35.8 (C-5), 39.6 (C-4), 75.8 (C-3)
28	20.4	0.90 3H s	26.5 (C-27), 35.8 (C-5), 39.6 (C-4), 75.8 (C-3)
29	22.0	1.21 3H s	32.8 (C-11), 37.0 (C-9), 47.8 (C-10), 156.8 (C-8)
30	28.4	1.12 3H s	34.3 (C-15), 43.4 (C-13), 53.4 (C-14), 156.8 (C-8)
31	24.3	0.95 3H s	34.4 (C-12), 43.4 (C-13), 53.4 (C-14)
32	19.4	0.96 3H d ( $J = 6.6$ Hz)	31.4 (C-21), 37.7 (C-20)
33	13.4	1.11 3H s	28.0 (C-24), 38.4 (C-22), 39.7 (C-23), 158.0 (C-26)
34	107.4	4.98, 4.94 br s, each	24.0 (C-25), 39.7 (C-23)

Figure 3. 43 <sup>1</sup>H NMR spectrum (400 MHz, C<sub>5</sub>D<sub>5</sub>N) of compound 13 (TRS-1441)

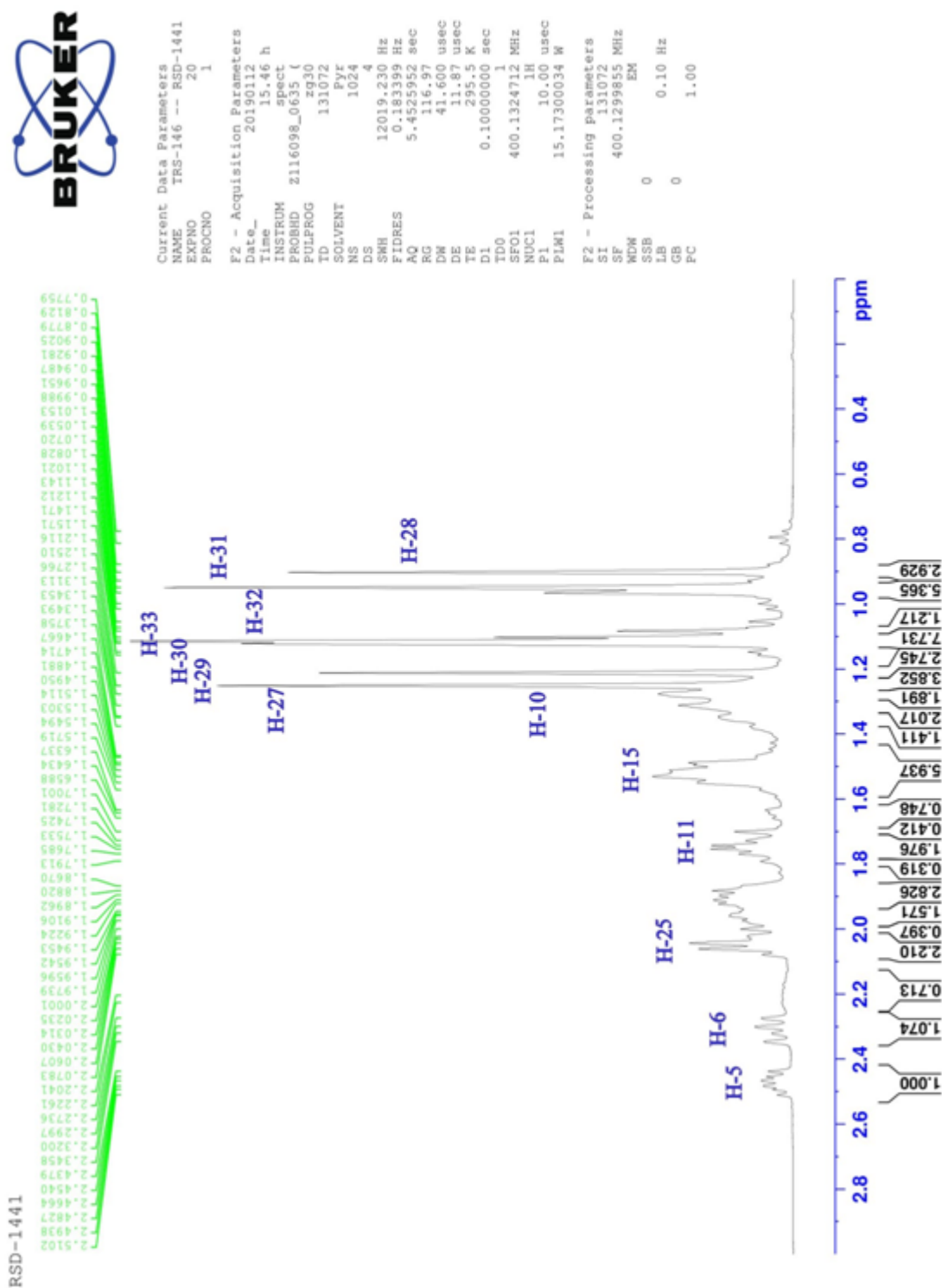


Figure 3.44 Partially expanded  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{C}_5\text{D}_5\text{N}$ ) of compound 13 (RSD-1441)

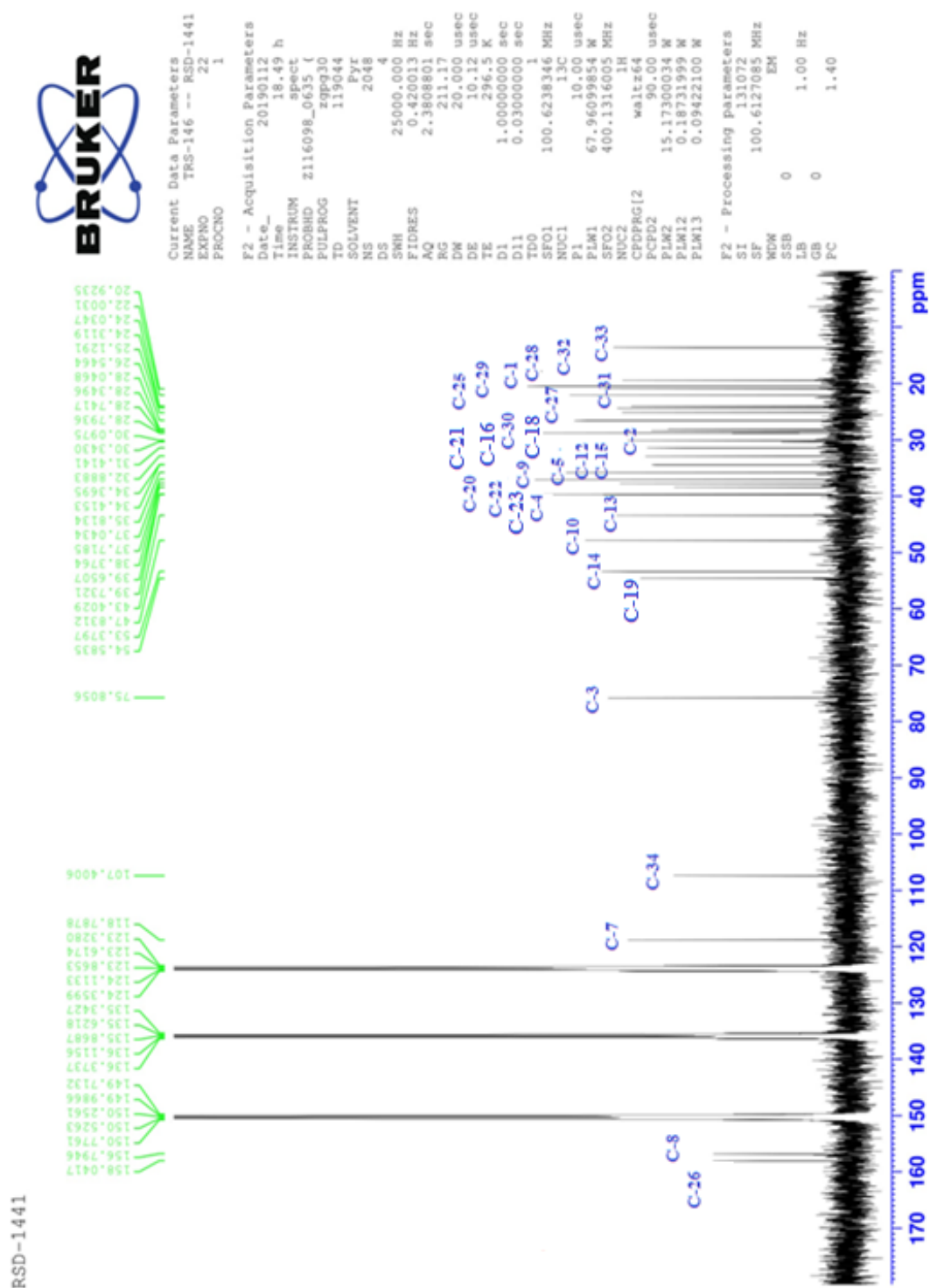
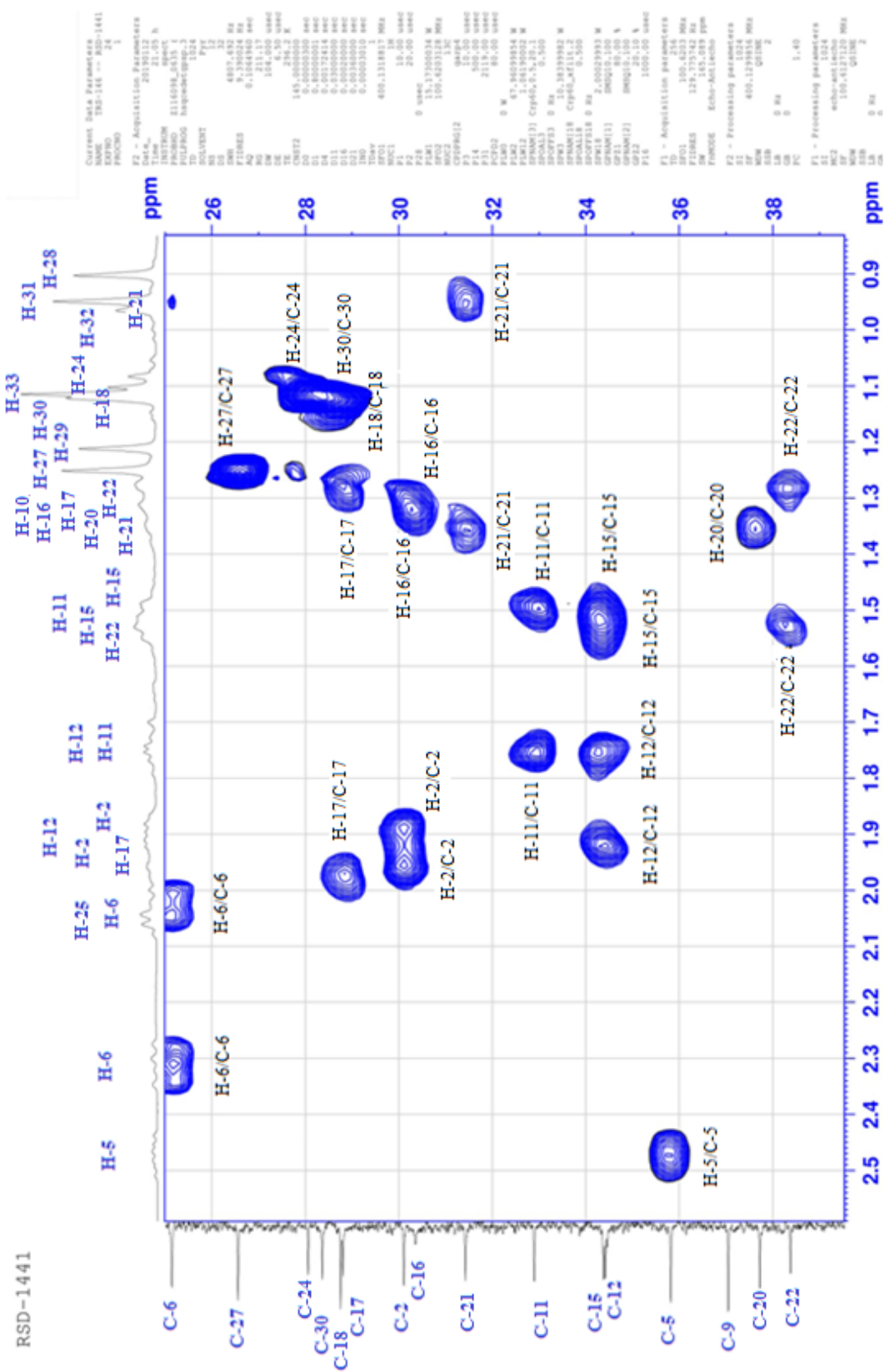


Figure 3.45  $^{13}\text{C}$  NMR spectrum (400 MHz,  $\text{C}_5\text{D}_5\text{N}$ ) of compound 13 (RSD-1441)

Figure 3.46 Partial HSQC spectrum (400 MHz, C<sub>5</sub>D<sub>5</sub>N) of compound 13 (RSD-1441)

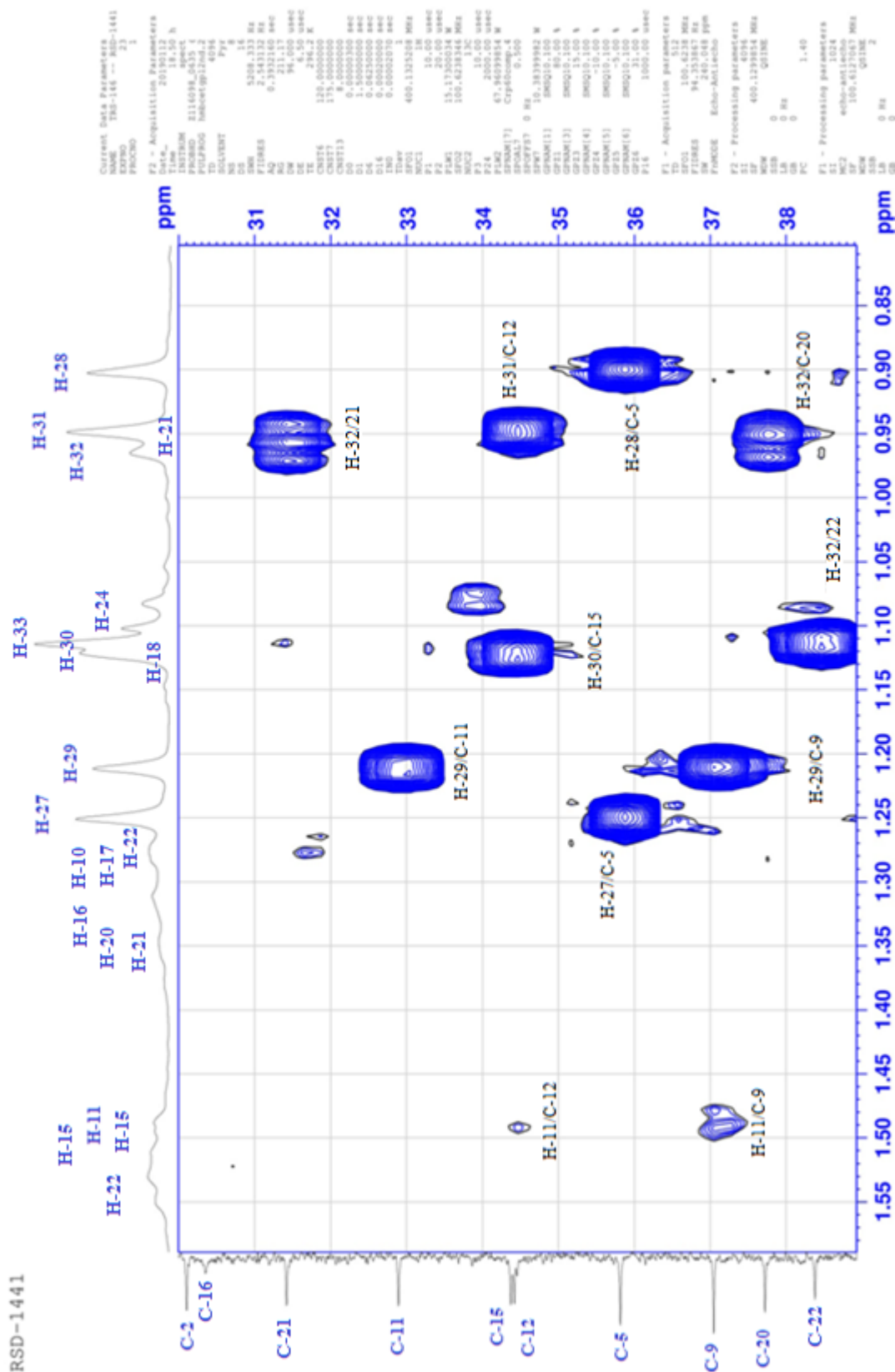


Figure 3. 47 Partial HMBC spectrum (400 MHz, C<sub>5</sub>D<sub>5</sub>N) of compound 13 (RSD-1441)

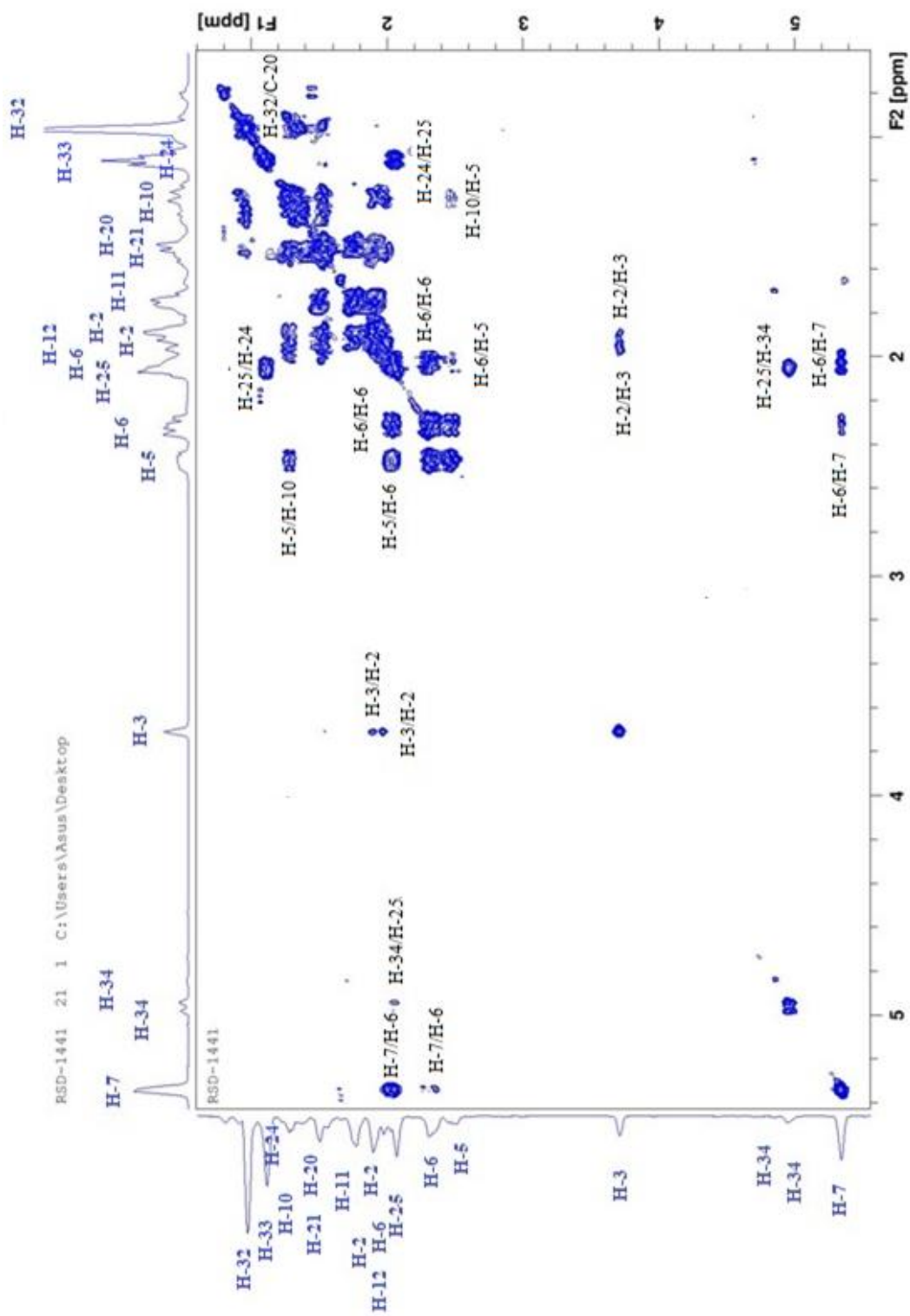


Figure 3. 48 COSY spectrum (400 MHz, C<sub>5</sub>D<sub>5</sub>N) of compound 13 (RSD-1441)



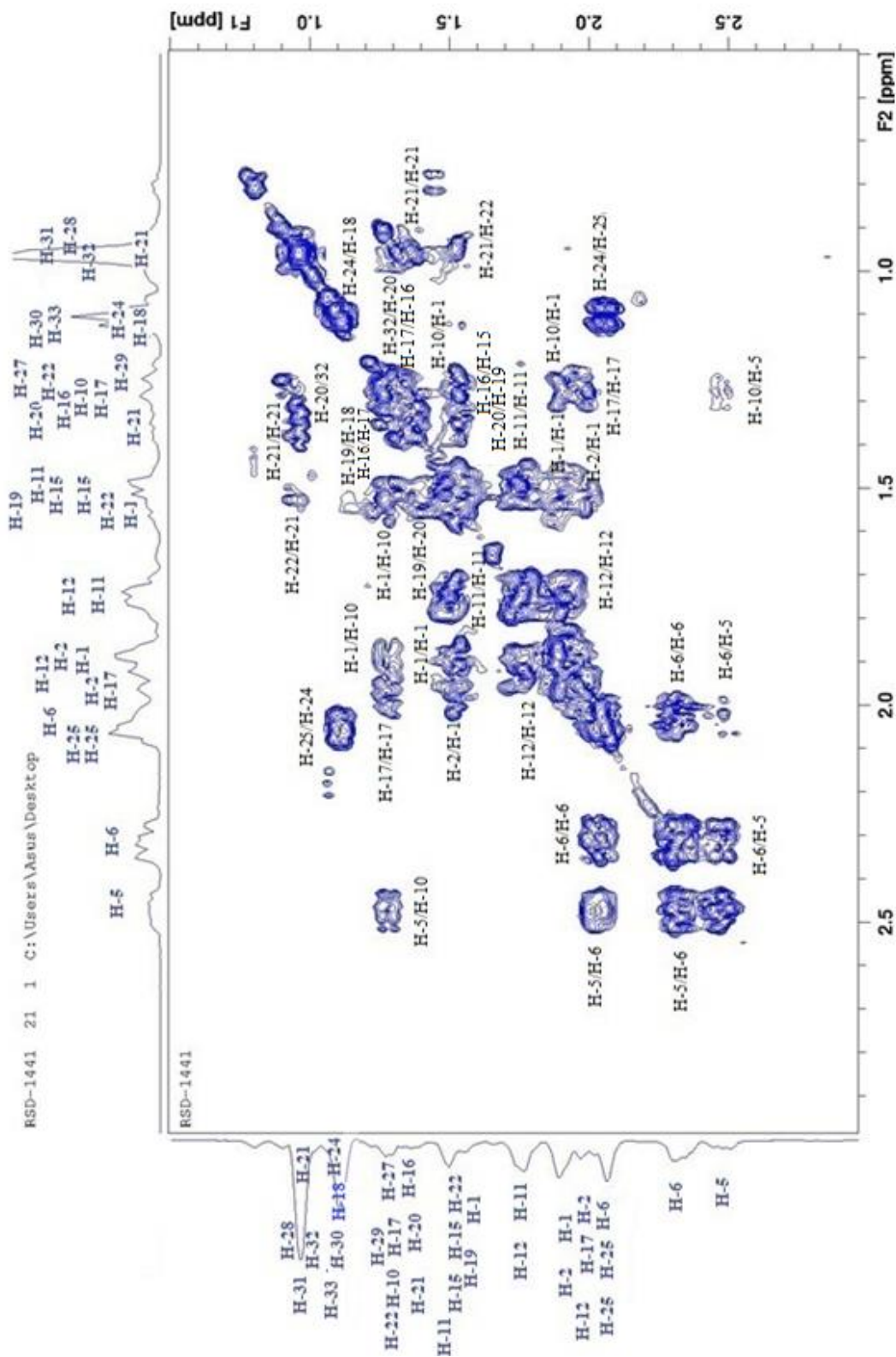
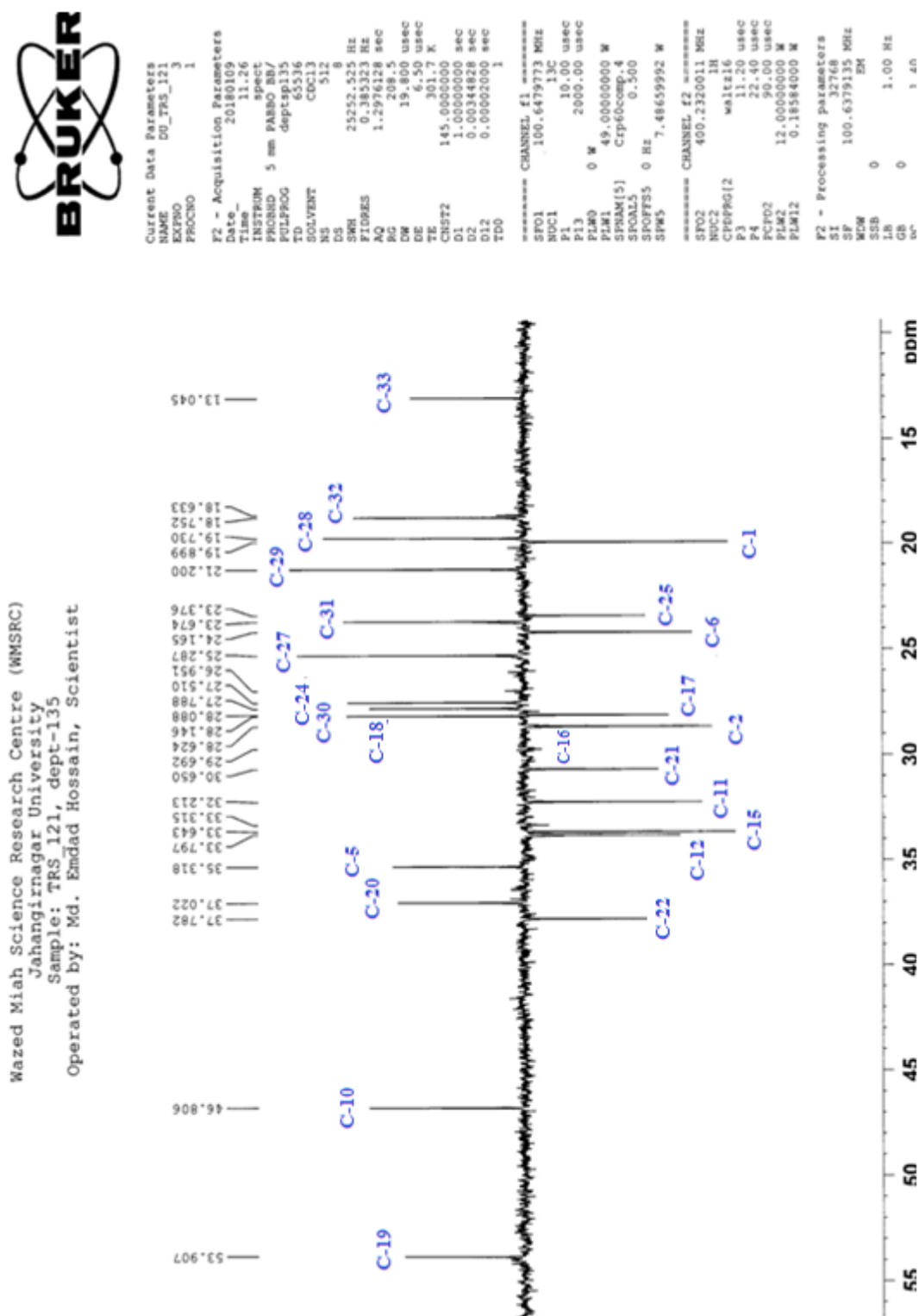


Figure 3. 49 Partial COSY spectrum (400 MHz,  $C_5D_5N$ ) of compound 13 (TRS-1441)

**Table 3.16** NMR spectroscopic data (400 MHz, CDCl<sub>3</sub>) for compound 13 (TRS-121)

Posit <sup>n</sup>	δ <sub>C</sub>	δ <sub>H</sub>	HMBC
1	19.9	1.85,1.40	35.3 (C-5)
2	28.6	1.84, 1.65	38.7 (C-4)
3	76.5	3.46 t ( <i>J</i> = 2.4 Hz)	19.9 (C-1), 35.3 (C-5)
4	38.7	---	---
5	35.3	1.93	---
6	24.2	2.18 ddd (18.2,10, 1.8), 1.86 m	117.8 (C-7), 156.3 (C-8)
7	117.8	5.20 dd ( <i>J</i> = 6.4, 2 Hz)	24.2 (C-6), 35.3 (C-5), 52.7 (C-14)
8	156.2	----	----
9	36.3	---	----
10	46.8	1.08 m	---
11	32.2	1.87 m, 1.47 m	42.8 (C-13), 156.2 (C-8), 36.3 (C-9)
12	33.8	1.92m	42.8 (C-13), 53.9 (C-18),
13	42.8	---	---
14	52.7	---	---
15	33.6	---	---
16	29.7	---	---
17	28.1	---	---
18	28.2	---	---
19	53.9	1.48 m	---
20	37.0	---	---
21	30.7	0.85 m, 1.30 m	---
22	37.8	---	---
23	39.2	---	---
24	27.8	1.08 m	23.4 (C-25)
25	23.4	2.01, 2H m	13.0 (C-33), 106.4 (C-34), 157.8 C-26)
26	157.8	---	---
27	25.3	0.99 3H s	19.7 (C-28),35.3 (C-5), 38.7 (C-4), 76.5 (C-3)
28	19.7	0.78 3H s	25.3 (C-27),35.3 (C-5), 38.7 (C-4), 76.5 (C-3)
29	21.2	1.09 3H s	32.2 (C-11), 36.3 (C-9), 46.8 (C-10), 156.2 (C-8)
30	27.8	1.05 3H s	33.6 (C-15), 42.8 (C-13), 52.7 (C-14), 156.2 (C-8)
31	23.7	0.88 3H s	33.8 (C-12), 42.8 (C-13), 52.7 (C-14)
32	18.8	0.89 3H d ( <i>J</i> = Hz)	30.7 (C-21), 37.0 (C-20)
33	13.1	1.04 3H s	27.8 (C-24), 37.8 (C-22), 39.2 (C-23), 157.8 (C-26)
34	106.4	4.81 , 4.79 br s, each	23.4 (C-25), 39.2 (C-23)

Figure 3. 50 DEPT-135 NMR spectrum (400 MHz, CDCl<sub>3</sub>) of compound 13 ( TRS-121)

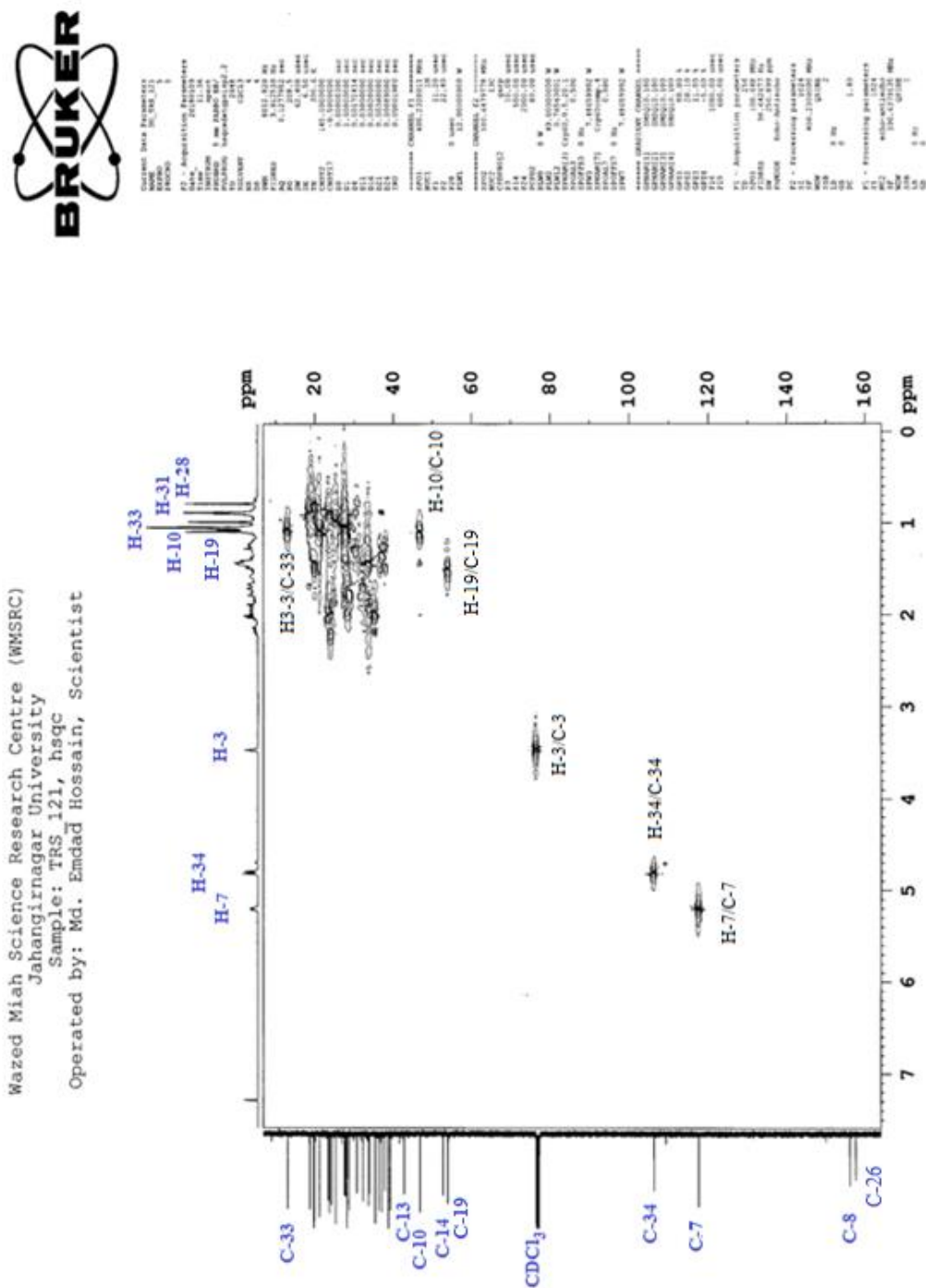
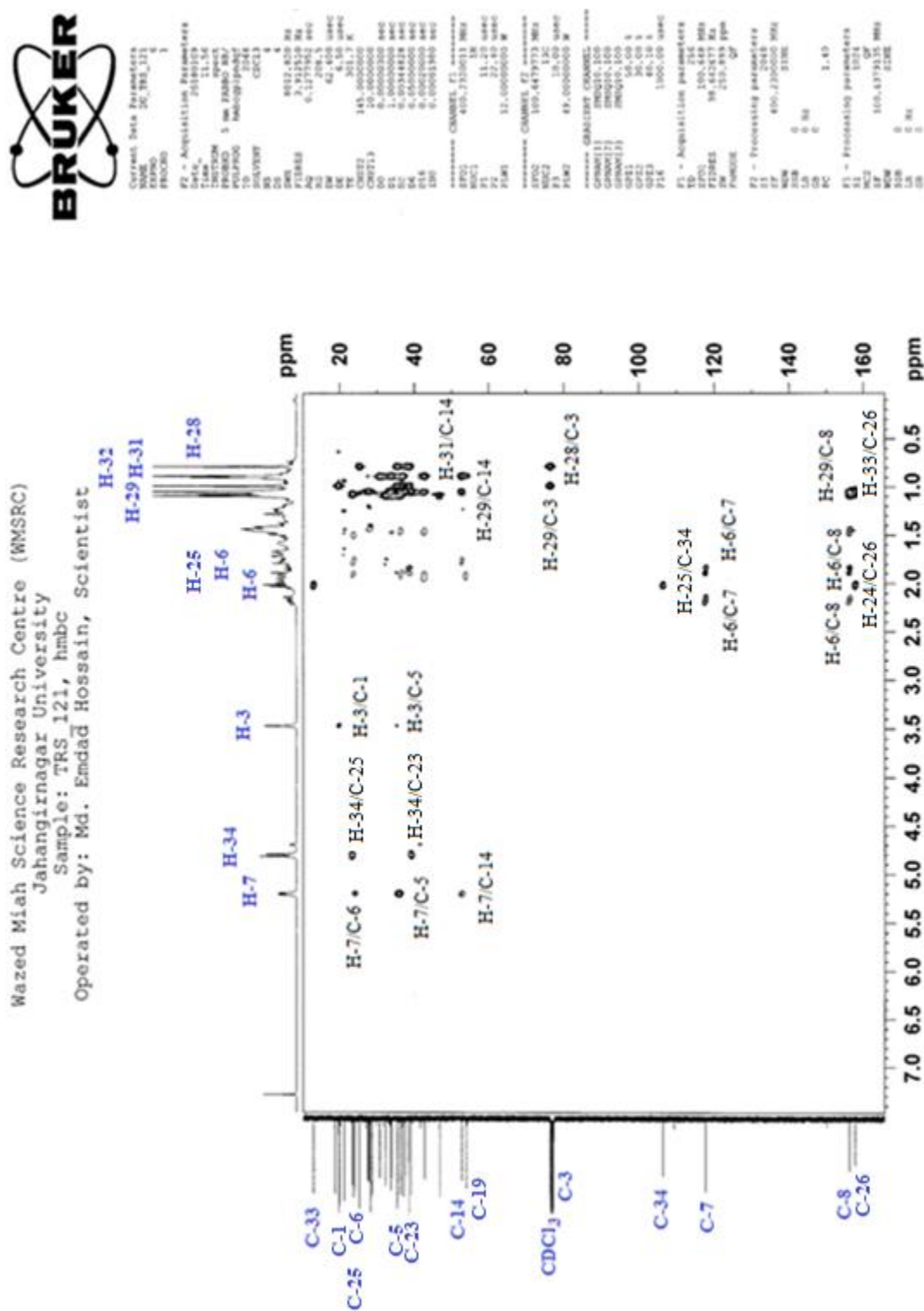


Figure 3.51 HSQC spectrum (400 MHz, CDCl<sub>3</sub>) of compound 13 ( TRS-121)

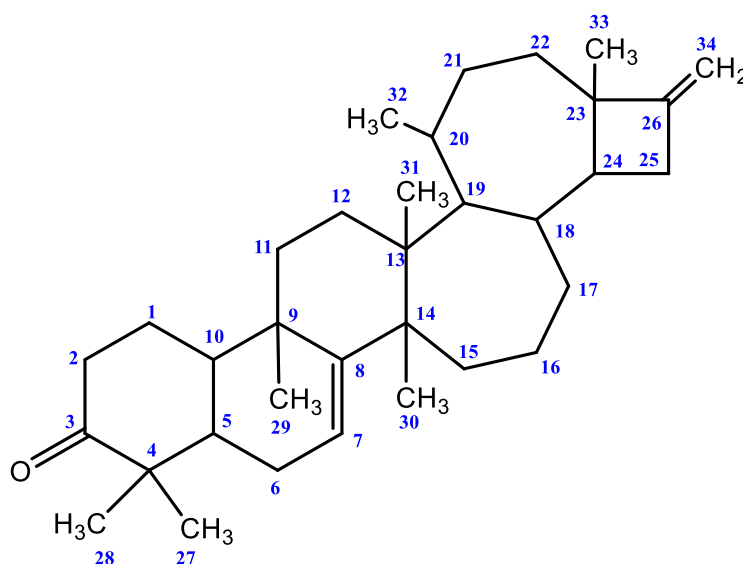
Figure 3.52 HMBC spectrum (400 MHz, CDCl<sub>3</sub>) of compound 13 ( TRS-121)

### 3.1.14 Characterization of compound 14 (TRS-101) as ravespanone

Compound 14, isolated as colorless powder, was invisible when examined under UV light on a TLC plate and produced brown color when sprayed with vanillin in sulphuric acid reagent and heated for 5 minutes.

The  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR,  $^1\text{H}$ - $^1\text{H}$  COSY, HSQC and HMBC spectral data of compound 14 were very similar to those of compound 13, except that in the  $^1\text{H}$  NMR spectrum, the oxymethine proton and in the  $^{13}\text{C}$  NMR spectrum, the carbinol carbon disappeared indicating the absence of the hydroxyl group at position 3. Instead of the carbinol carbon at  $\delta$  75.8 a carbonyl carbon resonating at 216.5 was appeared, suggesting the replacement of the hydroxyl group by a keto group.

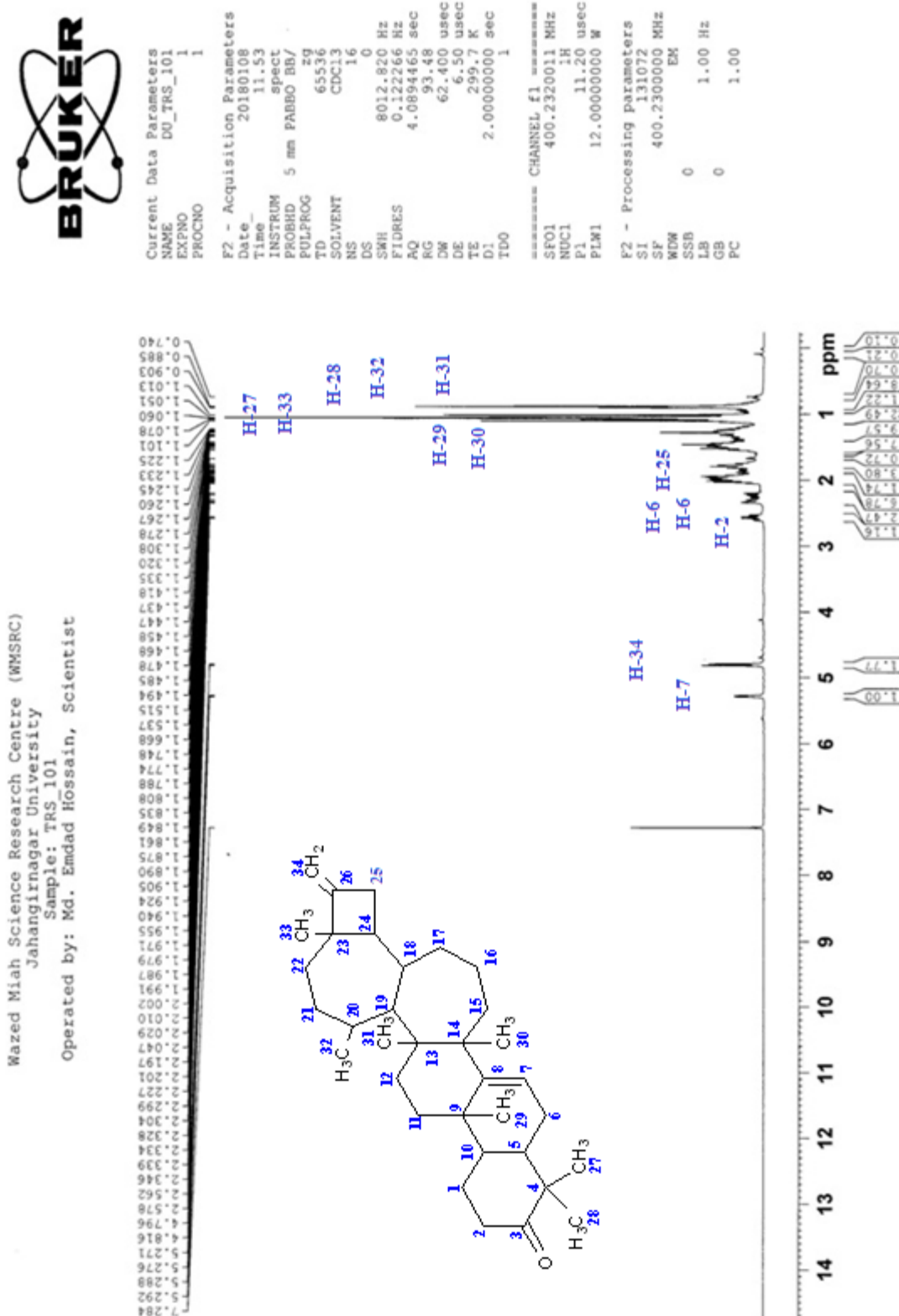
The  $^1\text{H}$ NMR spectrum (Table 3.17, Figure 3.53) displayed an olefinic proton at  $\delta$  5.28 dd ( $J = 6.6, 1.8$  Hz), two exomethylene protons  $\delta$  4.82, 4.80 (br s, each), six methyl singlets at  $\delta$ , 0.89, 1.01, 1.04, 1.05, 1.06, 1.10 and a methyl doublet  $\delta$  0.90 d ( $J = 6.9$  Hz). The  $^{13}\text{C}$  NMR spectrum revealed all 34 carbons including seven methyls, twelve methylenes, seven methines and eight quaternary carbons. The HMBC and HSQC spectra revealed all the expected correlations. Thus the tentative structure of ravespanone is shown in figure.



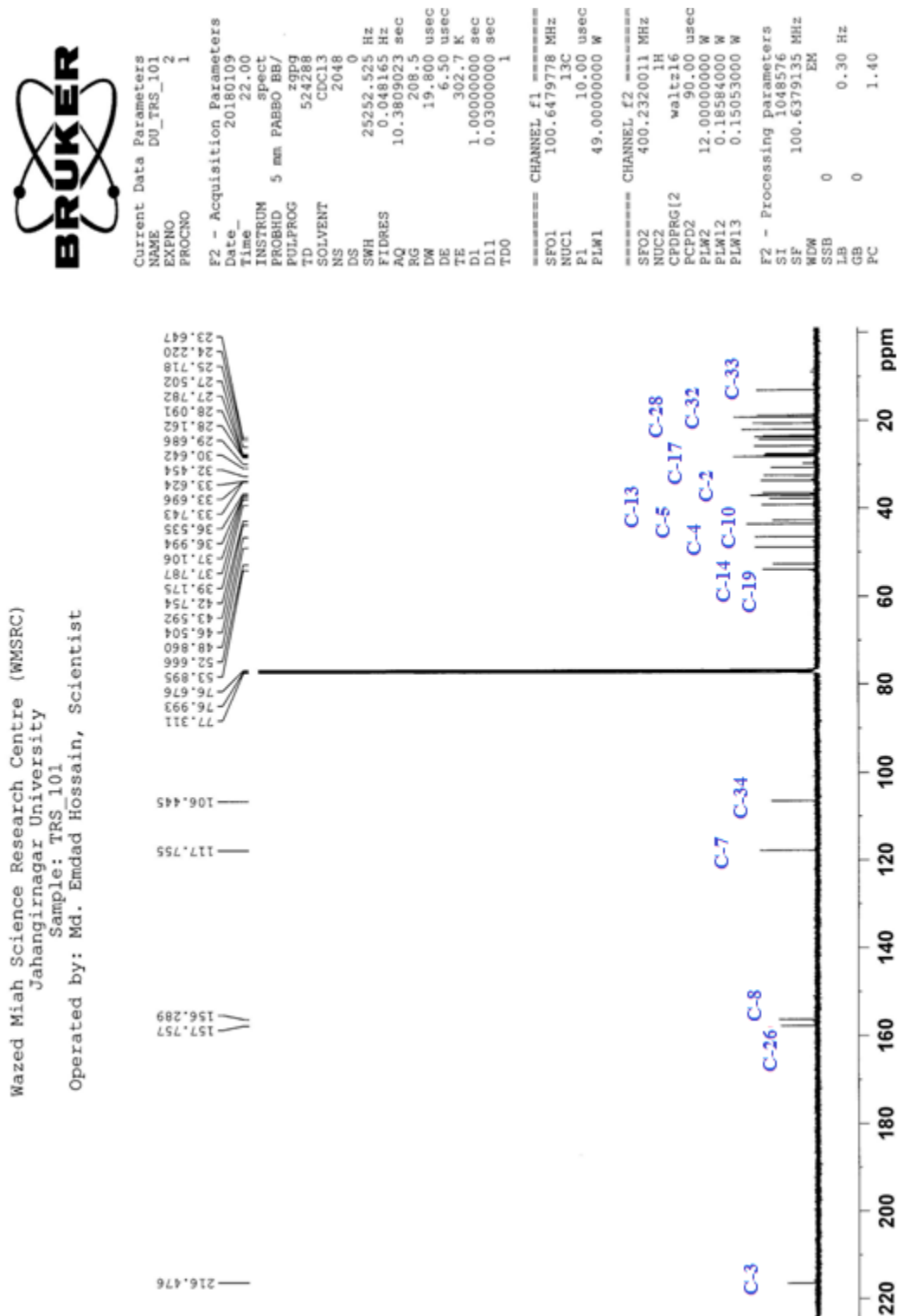
Ravespanone

**Table 3.17 NMR spectroscopic data (400 MHz, CDCl<sub>3</sub>) for TRS-101(Compound 14)**

Posit <sup>n</sup>	δ <sub>C</sub>	δ <sub>H</sub>	HMBC
1	25.7	1.85 m, 1.93 m	---
2	37.1	2.57 m, 2.32 m	25.7 (C-1), 216.5 (C-3)
3	216.5	---	---
4	48.9	---	---
5	43.6	1.85m	---
6	24.2	2.23 m, 1.96 m	43.6 (C-5), 117.8 (C-7), 156.3 (C-8)
7	117.8	5.28 dd ( <i>J</i> = 6.6, 1.8 Hz)	24.2 (C-6), 36.5 (C-9), 43.6 (C-5), 52.7 (C-14)
8	156.3	---	---
9	36.5	---	---
10	46.5	1.50 m	---
11	32.4	---	---
12	33.7	1.76 m	20.6 (C-29), 32.4 (C-11), 52.7 (C-14)
13	42.8	---	---
14	52.7	---	---
15	33.6	---	---
16	29.7	---	---
17	28.1	---	---
18	28.2	---	---
19	53.9	1.52 m	---
20	37.0	---	---
21	30.6	---	---
22	37.8	---	---
23	39.2	---	---
24	27.8	1.08	23.4 (C-25)
25	23.4	2.01, 2H m	13.1 (C-33), 106.4 (C-34), 157.8 C-26)
26	157.8	---	---
27	22.0	1.05 3H s	19.2 (C-28), 43.6 (C-5), 48.9 (C-4), 216.5 (C-3)
28	19.2	1.01 3H s	22.0 (C-27), 43.6 (C-5), 48.9 (C-4), 216.5 (C-3)
29	21.2	1.06 3H s	32.4 (C-11), 36.5 (C-9), 46.5 (C-10), 156.3 (C-8)
30	27.5	1.103H s	33.6 (C-15), 42.8 (C-13), 52.7 (C-14), 156.3 (C-8)
31	23.7	0.89 3H s	33.7 (C-12), 42.8 (C-13), 52.7 (C-14)
32	18.8	0.90 3H d ( <i>J</i> = 6.9 Hz)	30.6 (C-21), 37.0 (C-20)
33	13.1	1.04 3H s	27.8 (C-24), 37.8 (C-22), 39.2 (C-23), 157.8 (C-26)
34	106.4	4.82 , 4.80 br s, each	23.4 (C-25), 39.2 (C-23),

Figure 3.53 <sup>1</sup>H NMR spectrum (400 MHz, CDCl<sub>3</sub>) of compound 14 ( TRS-101)



Figure 3.54  $^{13}\text{C}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 14 ( TRS-101)

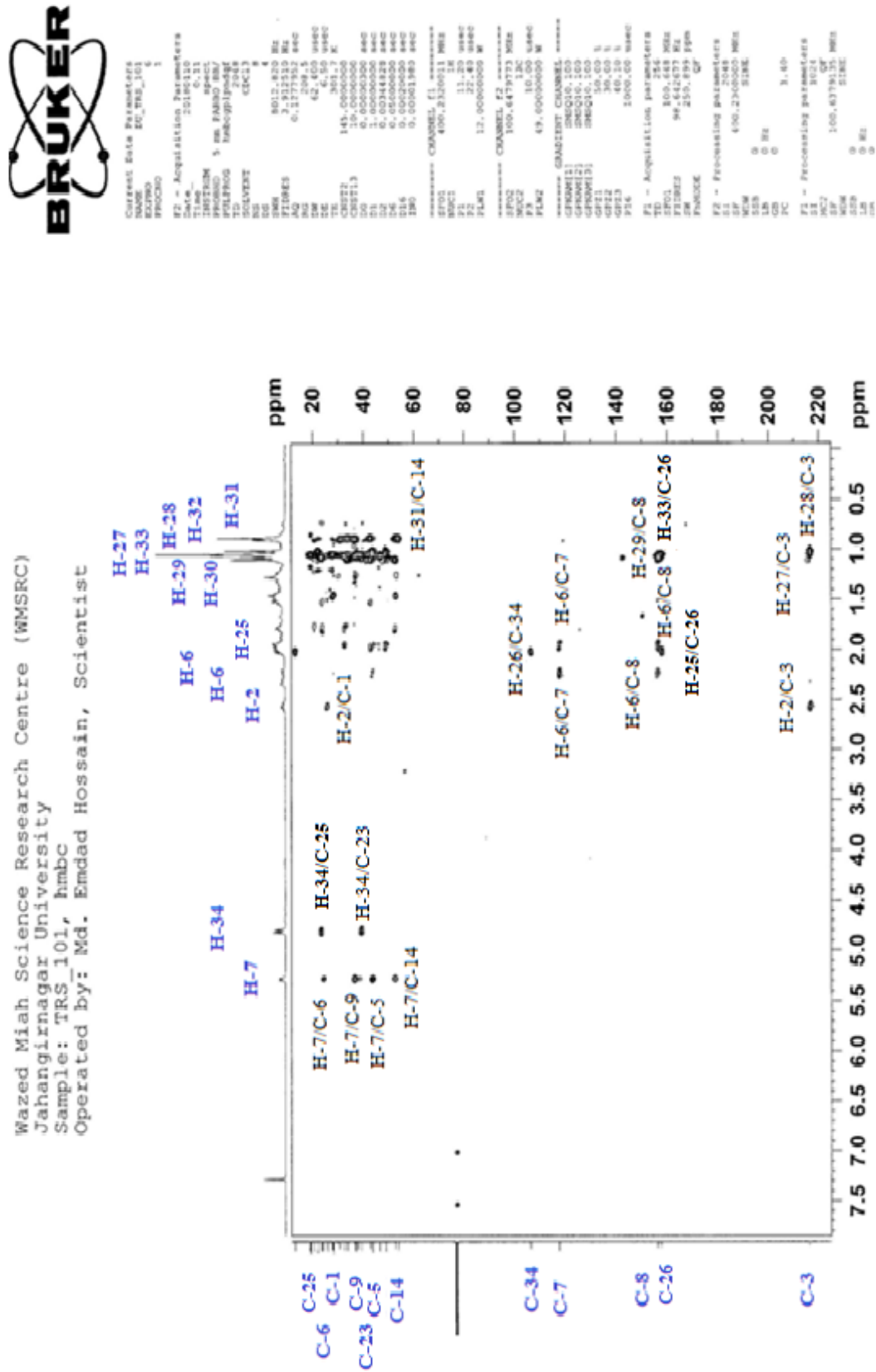
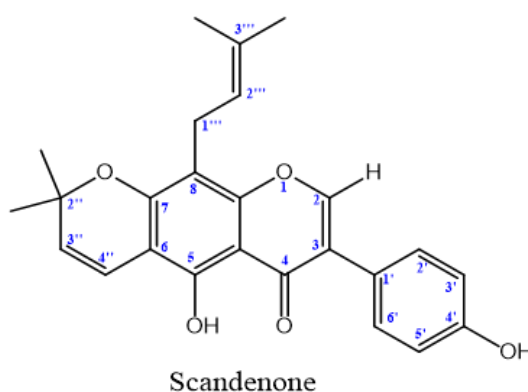


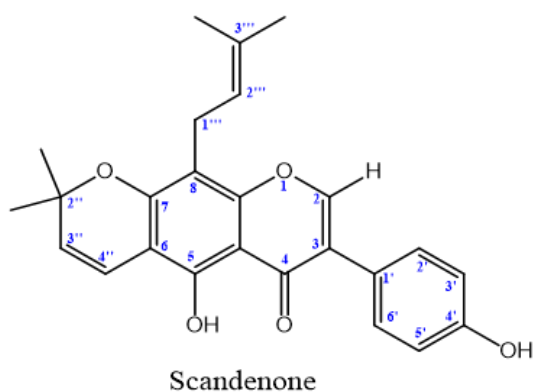
Figure 3. 55 HMBC spectrum (400 MHz, CDCl<sub>3</sub>) of compound 14 ( TRS-101)

### 3.1.15 Characterization of compound **15** (TEV-171) as scandenone

Compound **15** was isolated as yellow needle shaped crystals, produced yellow colored spot on a TLC plate when sprayed with vanillin in sulphuric acid reagent and heated for 5 minutes.

$^1\text{H}$  NMR spectrum (Table 3.18, Figure 3.56) showed a pair of doublets at  $\delta$  5.65 and 6.75 and two equivalent methyl groups resonating at  $\delta$  1.49 (6H, s), indicating the presence of a 2,2-dimethylchromene ring. A singlet integrating for one proton at  $\delta$  7.90 is of characteristic for C-2 proton of the isoflavone skeleton. The  $^1\text{H}$ NMR spectrum also displayed a pair of doublets each of which were integrating for two protons centered at  $\delta$  6.84 and 7.35 (2H d, each,  $J = 8.8$  Hz), typical of a *para* di-substituted aromatic ring nucleus. The relatively upfield resonance at  $\delta$  6.84 of H-3' and H-5' suggested the presence of an oxygenated substituent at C-4' as a hydroxyl group. The  $^1\text{H}$  NMR spectrum of the compound showed two methyl groups resonating at  $\delta$  1.70 and 1.83 (3H s, each), a triplet at  $\delta$  5.20 ( $J = 7.3$  Hz) and a methylene group at  $\delta$  3.42 (2H d,  $J = 7.3$  Hz). These signals suggested the presence of a prenyl group side chain attached to the C-8 of the isoflavone nucleus. A singlet at  $\delta$  13.15 could be assignable to the chelated hydroxyl group at C-5. These data enabled the identification of the compound as scandenone. The  $^1\text{H}$  NMR data of compound **15** were found to be very similar to those reported for scandenone previously isolated from the stem bark of this plant (Rahman et al., 2010).





**Table 3.18**  $^1\text{H}$  NMR spectroscopic data (400 MHz,  $\text{CDCl}_3$ ) for compound 15

Position	Compound 15 $\delta_{\text{H}}$	Scandenone (Rahman et al., 2010) $\delta_{\text{H}}$
2	7.90 s	7.88 s
2'/6'	7.35 2H d ( $J = 8.8$ Hz)	7.39 2H d ( $J = 8.0$ Hz)
3'/5'	6.84 2H d ( $J = 8.4$ Hz)	6.90 2H d ( $J = 8.0$ Hz)
3''	5.65 H d ( $J = 12$ Hz)	5.60 H d ( $J = 10$ ) Hz
4''	6.75, H d ( $J = 12$ Hz)	6.72 H d ( $J = 10$ Hz)
2 Me-2''	1.49 6H, s	1.46 6Hs
1'''	3.42 d ( $J = 7.6$ Hz)	3.39, d ( $J = 7.2$ Hz)
2'''	5.20 t ( $J = 7.3$ Hz)	5.17 t ( $J = 7.2$ Hz)
Me-3'''	1.83 3Hs	1.80 3Hs
Me-3'''	1.70 3H s	1.68 3H s
OH-4'	5.36 s	4.32 s
OH-5	13.15 s	13.17 s

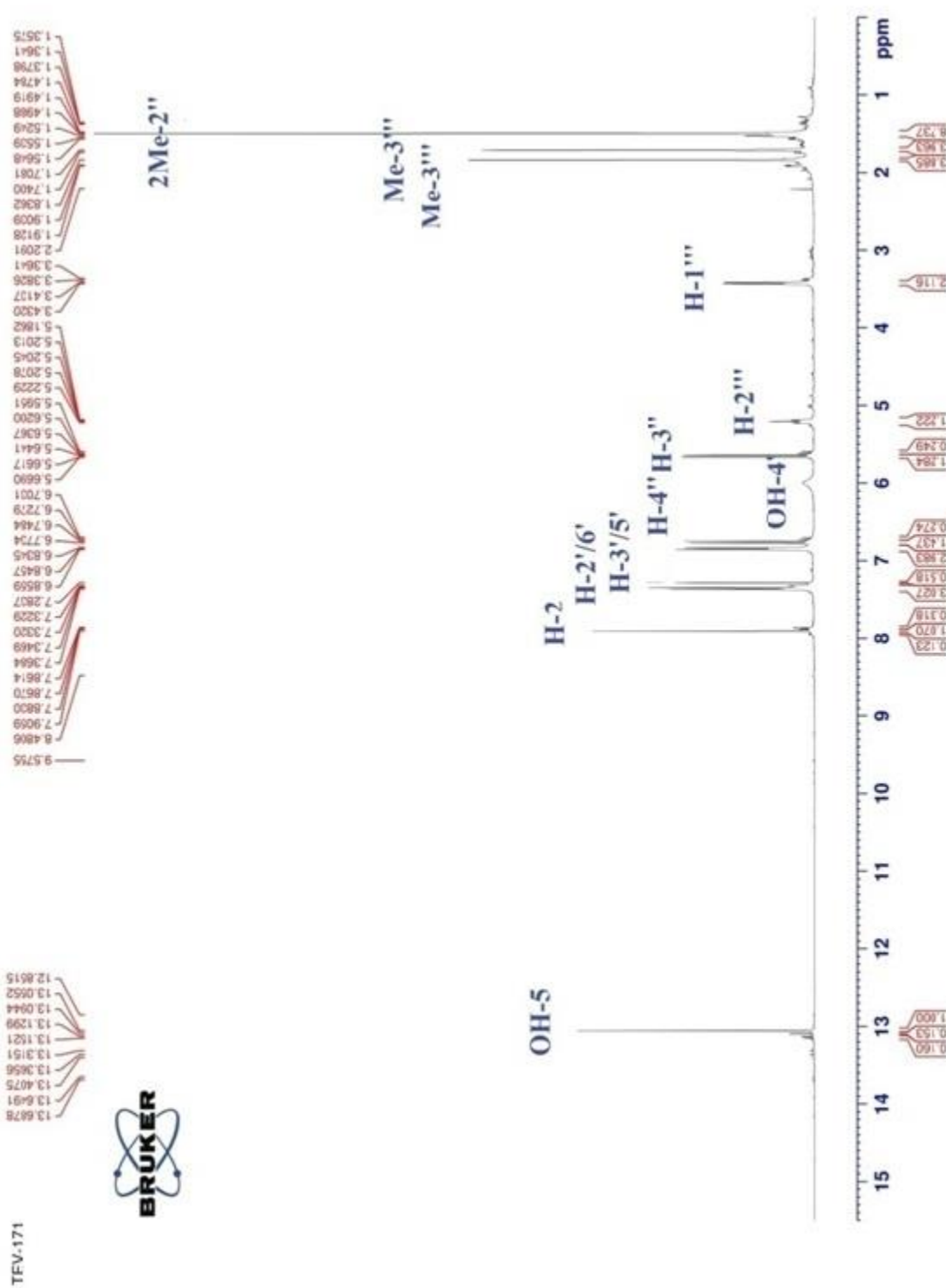


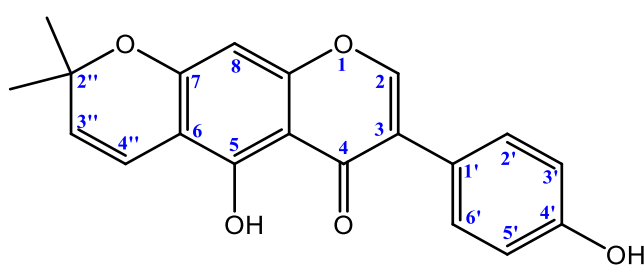
Figure 3.56 <sup>1</sup>H NMR spectrum (400 MHz, CDCl<sub>3</sub>) of compound 15 (TEV-171)

### 3.1.16 Characterization of compound **16** (TEV-176) as alpinumisoflavone

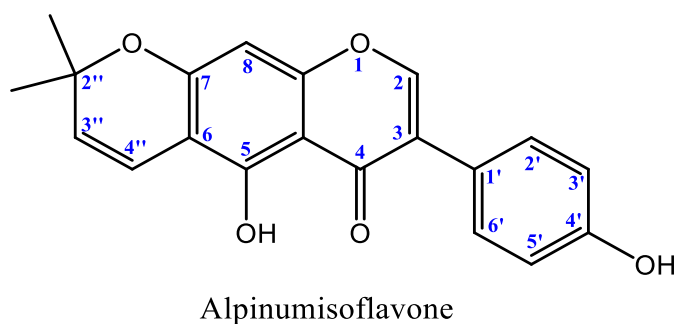
Compound **16** was isolated as yellow needle shaped crystals produced yellow spot on a TLC plate when sprayed with vanillin in sulphuric acid reagent and heated for 5 minutes.

The  $^1\text{H}$  NMR spectrum (Table 3.19, Figure 3.57) of compound **16** was found very similar to that of compound **15** except that a sharp singlet integrated for one proton appeared at  $\delta$  6.35 in place of the prenyl group signals at position C-8. The presence of 2, 2-dimethylchromene ring was indicated by a pair of doublets at  $\delta$  5.67 and 6.74 ( $J=10$  Hz, each) and two equivalent methyl group at  $\delta$  1.49 (6H s).

The characteristic C-2 proton of the isoflavone skeleton was evident as a singlet at  $\delta$  7.84 of one proton intensity. The H-2/ 6' and H-3'/5' protons of the *para* disubstituted benzene ring appeared at  $\delta$  7.41 and 6.91 (2H d,  $J = 8.4$  Hz, each) respectively. The chelated hydroxyl group at C-5 resonated at  $\delta$  13.15. These data permitted the identification of compound **16** as alpinumisoflavone. The structure was further confirmed by comparison of the  $^1\text{H}$  NMR data with those published (Hussain et al., 2011).



Alpinumisoflavone



**Table 3.19**  $^1\text{H}$  NMR spectroscopic data (400 MHz,  $\text{CDCl}_3$ ) for compound 16

Position	Compound 16 $\delta_{\text{H}}$	Alpinumisoflavone (Hussain et al., 2011) $\delta_{\text{H}}$
2	7.84 1H s	7.83 1H s
8	6.35 1H s	6.34 1H s
H-2'/6'	7.41 2H d ( $J=8.4$ Hz)	7.27 2H d ( $J=8.5$ Hz)
H-3'/5'	6.91 2H d ( $J=8.4$ Hz)	6.96 2H d ( $J=8.5$ Hz)
H-3''	5.67 1H d ( $J=10$ Hz)	5.53 1H d ( $J=10.6$ Hz)
H-4''	6.74 1H d ( $J=10$ Hz)	6.60 1H d ( $J=10.6$ Hz)
2Me-2''	1.49 6H s	1.48 6H s
OH-5	13.15 1H s	13.14 1H s

Wazed Miah Science Research Centre (WMSRC)  
 Jahangirnagar University  
 Sample: TEV\_176  
 Operated by: Md. Emdad Hossain, Scientist



```

Current Data Parameters
NAME      DU_TEV_176
EXPNO    1
PROCNO   1

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Time     13.01
INSTRUM spect
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PULPROG zg
TD       65536
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DS       0
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FIDRES   0.122266 Hz
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RG       118.34
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DE       6.50 use
TE       299.3 K
D1       2.00000000 sec
TD0      1

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NUC1    1H
P1      11.20 use
PLW1    12.00000000 W

F2 - Processing Parameters
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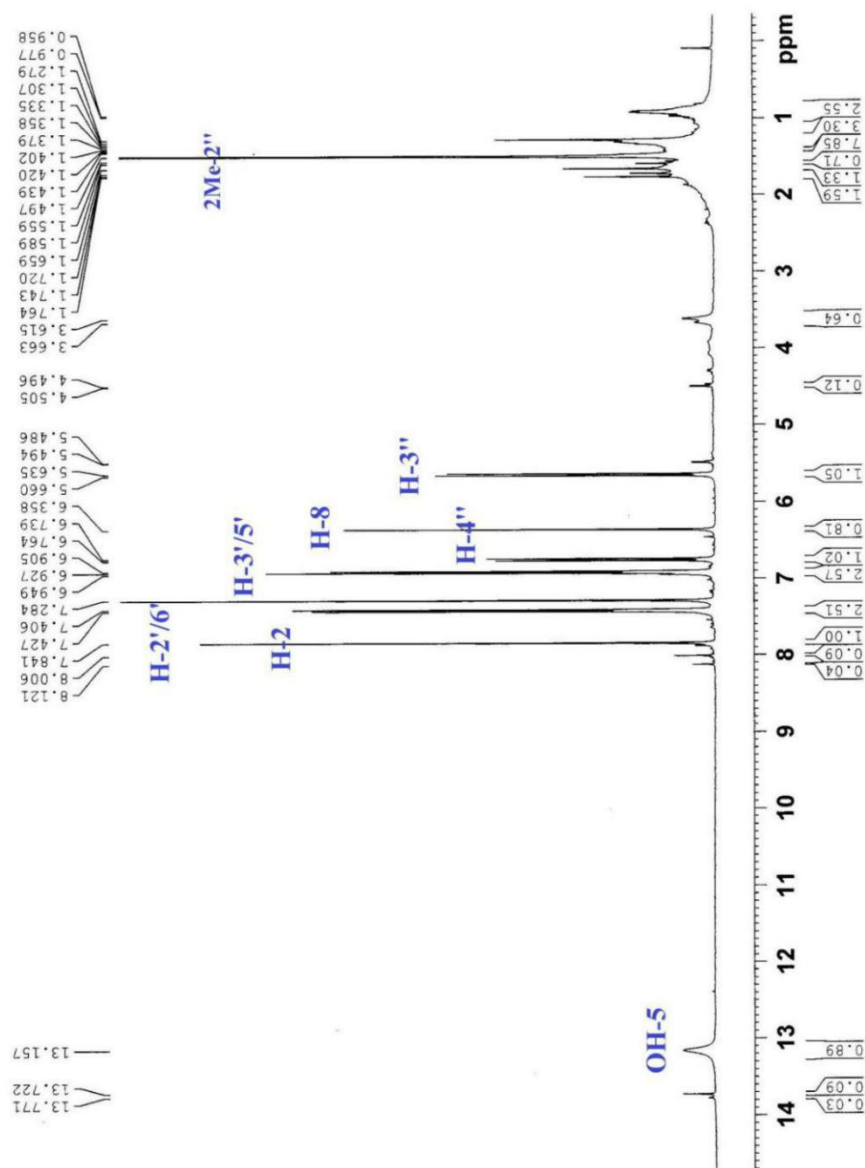


Figure 3.57  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 16 (TEV-176)

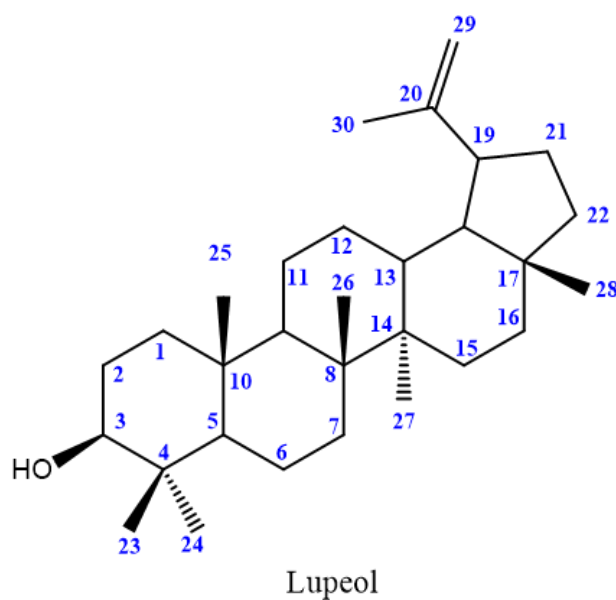


### 3.1.17 Characterization of compound 17 (TEV-121) as lupeol

Compound **17** isolated from VLC fraction **12** as colourless crystals, was invisible when examined under UV light. The compound produced bright purple colour when sprayed with vanillin in sulphuric acid reagent followed by heating for two minutes.

The  $R_f$  value of the compound was found to be 0.6 in 15% of ethyl acetate in toluene. Lupeol is a pencyclic triterpenoid containing six tertiary methyls, a vinylic methyl and an exomethylene group. It is a common triterpenoid of plants and also previously isolated from the leaves of *Erythrina variegata*.

Compound **17** was identified as lupeol by co-TLC with authentic sample using different solvent systems.

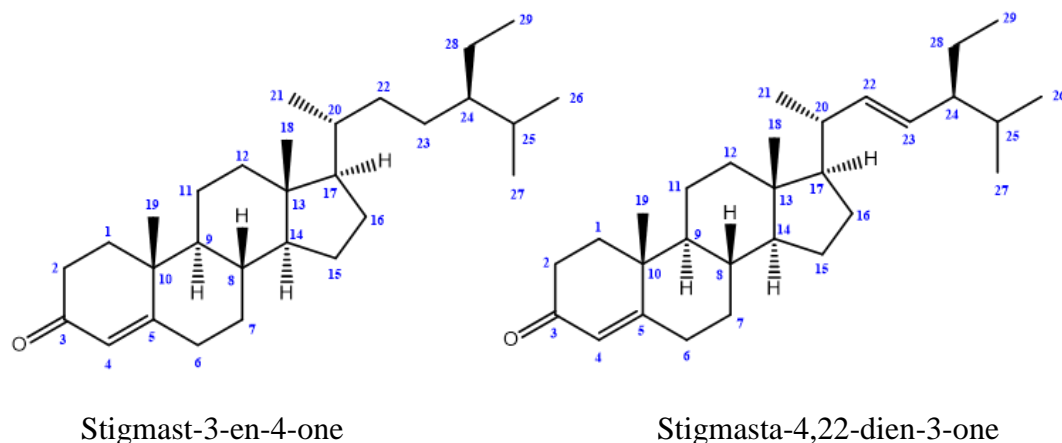


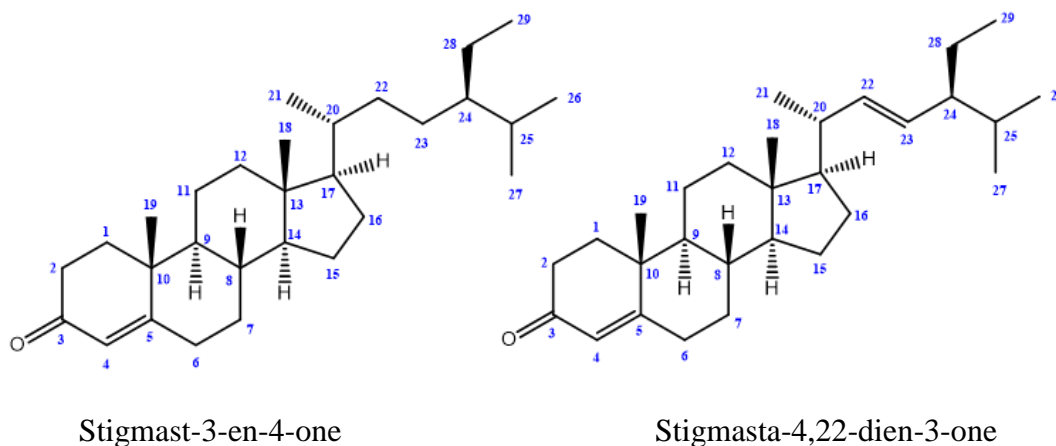
### 3.1.18 Characterization of compound 18 & compound 19 (TEV-131) as a mixture of stigmast-4-en-3-one and stigmasta-4, 22-dien-3-one

Compound **18** and compound **19** (TEV-131) were isolated as mixtures from VLC fraction 13. The compounds were found as colourless crystals and produced purple color when sprayed with vanillin in sulphuric acid reagent, followed by heating for 5 minutes. The compounds were appeared as a single spot on a TLC plate and therefore could not be separated from each other.

The  $^1\text{H}$  NMR spectrum of compound **18** (Table 3.20, Figure 3.58) showed resonances for six methyl groups at  $\delta$  0.73s, 0.83 *d* ( $J = 7.2$  Hz), 0.86 *d* ( $J = 7.2$  Hz), 0.87 *t* ( $J = 7.2$  Hz), 0.94 *d* ( $J = 6.6$  Hz) and 1.20 s assignable to H-18, H-27, H-26, H-29, H-21 and H-19 respectively. An olefinic proton appeared as a sharp singlet at  $\delta$  5.74 assignable to H-4. The  $^1\text{H}$  NMR spectrum were found similar to those reported for sitosta-4-en-3-one (Jibril *et al*, 2019). Thus compound 18 was identified as stigmast-4-en-3-one.

The remaining signals of the  $^1\text{H}$  NMR spectrum (Figure 3.59) include two methyl singlets at 0.75 and 1.20, three methyl doublets, 0.83 ( $J = 7.2$  Hz), 0.86 ( $J = 7.2\text{Hz}$ ) and 1.04 ( $J = 6.8$  Hz), a methyl triplet at 0.87 ( $J = 7.2$  Hz) and an olefinic proton singlet at  $\delta$  5.74. In addition, the spectrum displayed two *trans* olefinic protons, as indicated by the large coupling constant of 15.2 Hz, resonated at 5.04 dd and 5.17 dd ( $J = 15.2, 8.4$  Hz, each). On this basis, compound **19** was identified as stigmasta-4-22-dien-3-one. All these  $^1\text{H}$ NMR data were found to be in close agreement with those reported for stigmasta-4, 22-dien-3-one (Jibril *et al*, 2019).



**Table 3.20** NMR spectroscopic data (400 MHz, CDCl<sub>3</sub>) for compounds **18** and **19**

Position	Compound <b>18</b>	Stigmast-4-en-3-one (Jibril <i>et al</i> , 2019)	Compound <b>19</b>	Stigmasta-4,22-dien-3-one (Jibril <i>et al</i> , 2019)
	$\delta_H$	$\delta_H$	$\delta_H$	$\delta_H$
H-4	5.74 1H s	5.72 1H s	5.74 1H s	5.72 1H s
H-18	0.73 3H s	0.71 3H s	0.75 3H s	0.73 3H s
H-19	1.20 3H s	1.18 3H s	1.20 3H s	1.18 3H s
H-21	0.94 3H d ( $J = 6.4$ Hz)	0.92 3H d ( $J = 6.5$ Hz)	1.04 3H d ( $J = 6.8$ Hz)	1.02 3H d ( $J = 7.5$ Hz)
H-22	---	---	5.17 1H dd ( $J = 15.2, 8.4$ Hz)	5.15 1H dd ( $J = 15.5, 9.0$ Hz)
H-23	---	---	5.04 1H dd ( $J = 15.2, 8.4$ Hz)	5.03 1H dd ( $J = 15.5, 9.0$ Hz)
H-26	0.86 3H d ( $J = 7.2$ Hz)	0.84 3H d ( $J = 6.8$ Hz)	0.83 3H d ( $J = 7.2$ Hz)	0.80 3H d ( $J = 6.0$ Hz)
H-27	0.83 3H d ( $J = 7.2$ Hz)	0.82 3H d ( $J = 6.8$ Hz)	0.86 3H d ( $J = 7.2$ Hz)	0.85 3H d ( $J = 6.0$ Hz)
H-29	0.87 3H t ( $J = 7.2$ Hz)	0.85 3H m	0.83 3H t ( $J = 6.4$ Hz)	0.81 3H m



Wazed Miah Science Research Centre (WMSRC)  
 Jahangirnagar University  
 Sample: TEV\_131  
 Operated by: Md. Emdad Hossain, Scientist

```

Current Data Parameters
NAME DU_TEV_131
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PROCNO 1

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SOLVENT CDCl3
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FIDRES 0.122266 Hz
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RG 165.43
DW 62.400 usec
DE 6.50 usec
TE 288.0 K
D1 2.00000000 sec
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NUC1 1H
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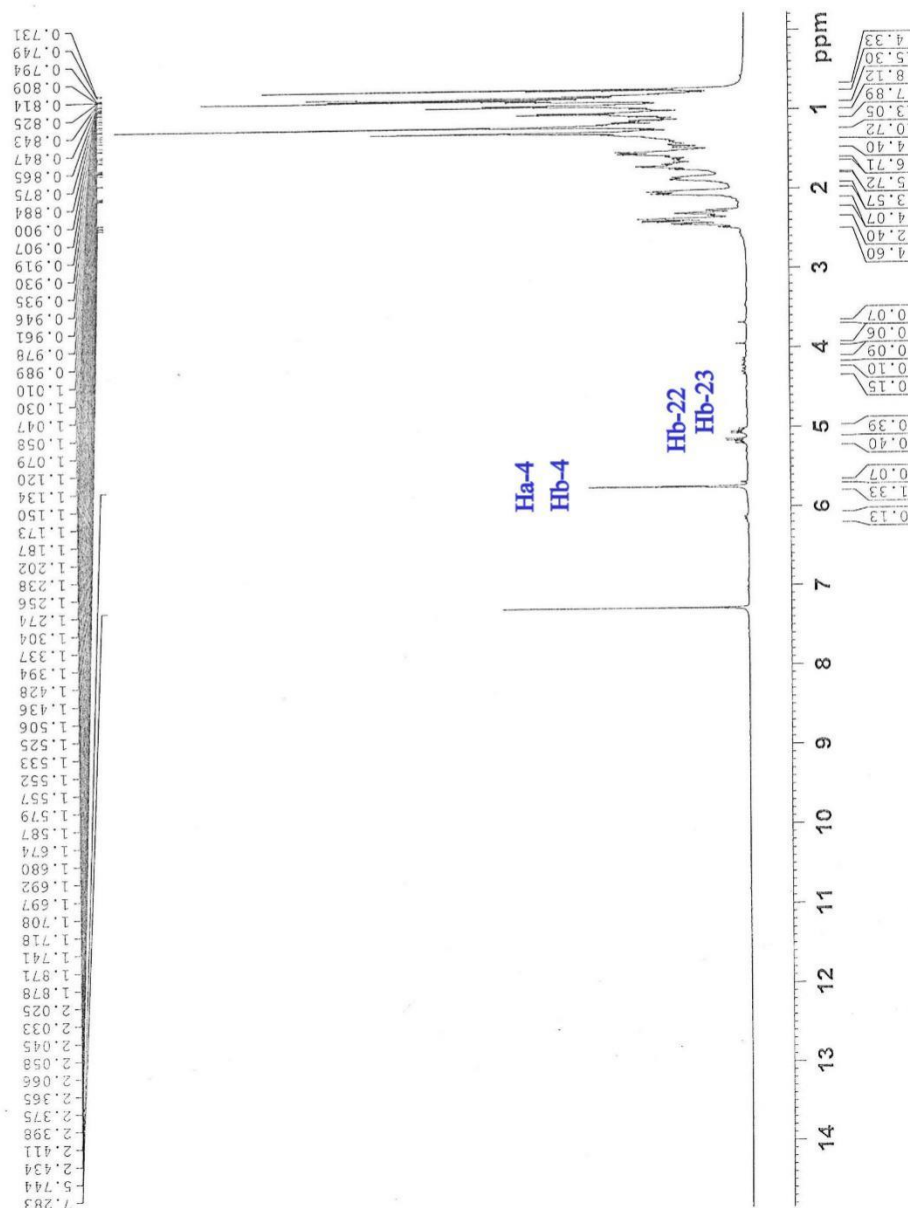


Figure 3.58  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 18 (TEV-131) (Ha for compound 18 & Hb for compound 19)



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 Jahangirnagar University  
 Sample: TEV 131  
 Operated by: Md. Emdad Hossain, Scientist

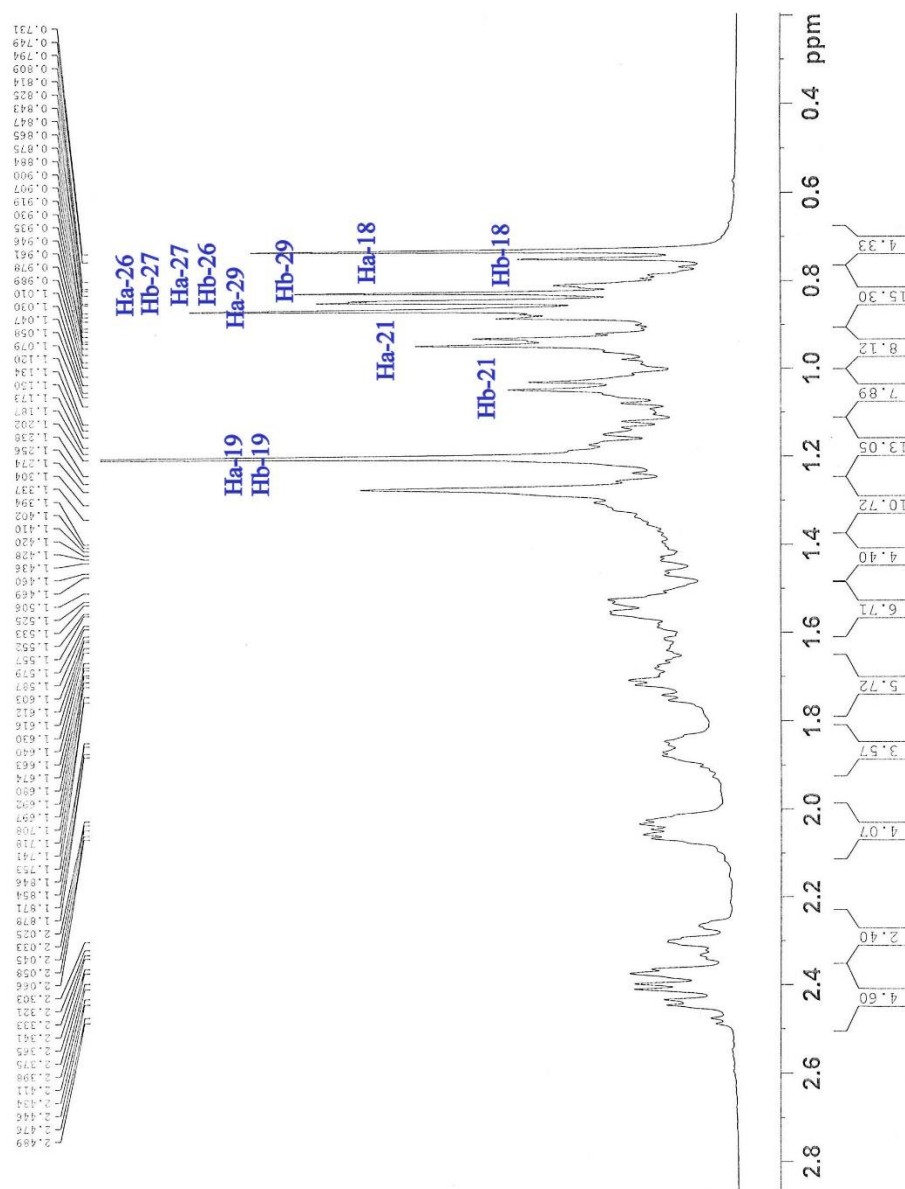
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SOLVENT CDCl3
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DS 0
SWH 8012.820 Hz
FIDRES 0.122266 Hz
AQ 4.0894465 sec
RG 165.43
DQ 62.400 usec
DE 6.50 usec
TE 298.0 K
D1 2.0000000 sec
TD0 1

===== CHANNEL f1 =====
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NUC1 1H
P1 11.20 usec
PLW1 12.0000000 W

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PC 1.00
  
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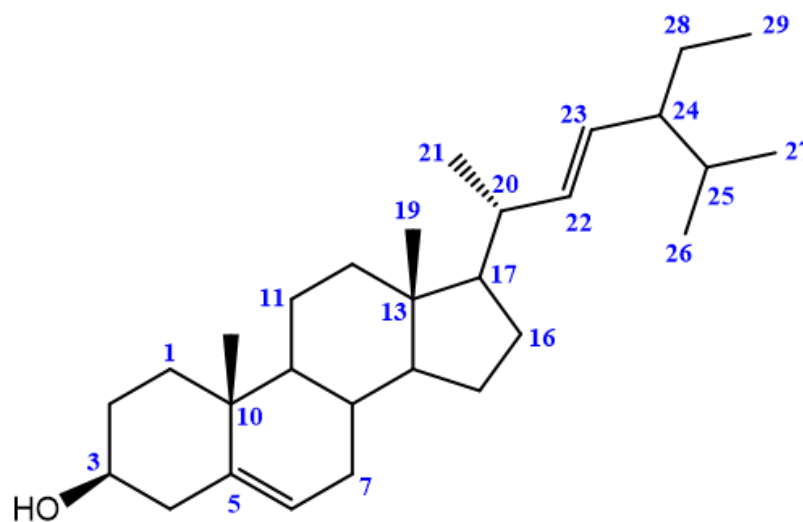


**Figure 3.59** Partially expanded  $^1\text{H}$  NMR spectrum of compound 18 & compound 19 (Ha for compound 18 & Hb for compound 19)

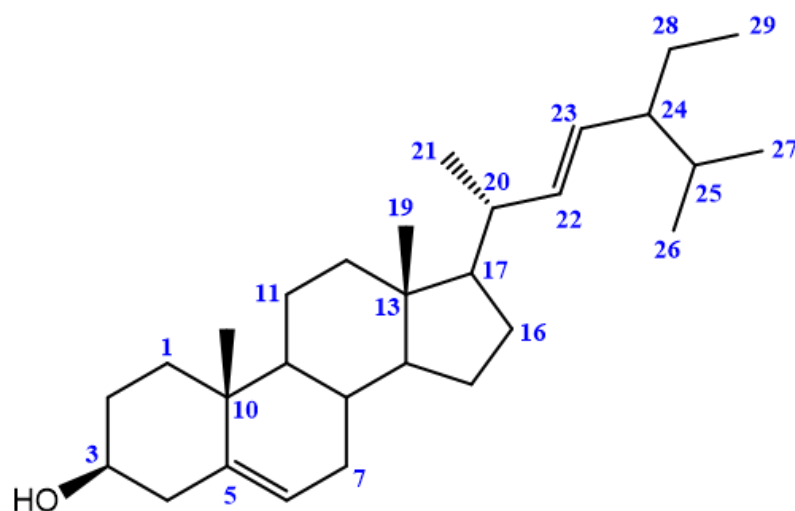
### 3.1.19 Characterization of compound **20** (TEV-161) as stigmasterol

Compound **20** was obtained as colorless crystals, gave no colour when examined under UV light and produced purple color when sprayed with vanillin in sulphuric acid reagent and heated for 5 minutes.

The  $^1\text{H}$  NMR spectrum (Table 3.21, Figure 3.61) of compound **20** showed the presence of six methyl groups resonating at  $\delta$  0.72, 0.82, 0.83, 0.87, 1.03 and 1.04 ppm which could be assignable to H-18, H-26, H-29, H-27, H-19 and H-21 respectively. The proton corresponding to the H-3 of a sterol moiety was appeared as a multiplet at  $\delta$  3.54 ppm. Two olefinic protons appeared downfield at  $\delta$  5.04 ppm and  $\delta$  5.16 ppm which are assignable for H-22 and H-23. Thus the compound was identified as stigmasterol. The structure was further confirmed by comparison of the  $^1\text{H}$  NMR data with those published (Pateh et al, 2009) and by co-TLC with an authentic sample.



Stigmasterol



Stigmasterol

Table 3.21  $^1\text{H}$  NMR spectroscopic data (400 MHz,  $\text{CDCl}_3$ ) for Compound 20

Position	Compound 20	Stigmasterol (Patch et al., 2009)
	$\delta_{\text{H}}$	$\delta_{\text{H}}$
H-3	3.54, 1H m	3.52 1H m
H-6	5.57, 3H d ( $J = 5.1\text{ Hz}$ )	5.36 1H br,s
H-18	0.72, 3H, s	0.70, 3H s
H-19	1.03 3H, s	1.01, 3H s
H-21	1.04, 3H d ( $J = 7.8\text{ Hz}$ )	1.02, 3H d ( $J = 7.5\text{ Hz}$ )
H-22	5.16 1H dd ( $J = 15.0, 8.6\text{ Hz}$ )	5.14, 1H m
H-23	5.04 1H dd ( $J = 15.0, 8.6\text{ Hz}$ )	4.98, 1H m
H-26	0.82, 3H d ( $J = 7.0\text{ Hz}$ )	0.80, 3H d ( $J = 6.5\text{ Hz}$ )
H-27	0.87, 3H d ( $J = 6.3\text{ Hz}$ )	0.85, 3H d ( $J = 6.5\text{ Hz}$ )
H-29	0.83, 3H t ( $J = 7.0\text{ Hz}$ )	0.80 3H t ( $J = 7.5\text{ Hz}$ )

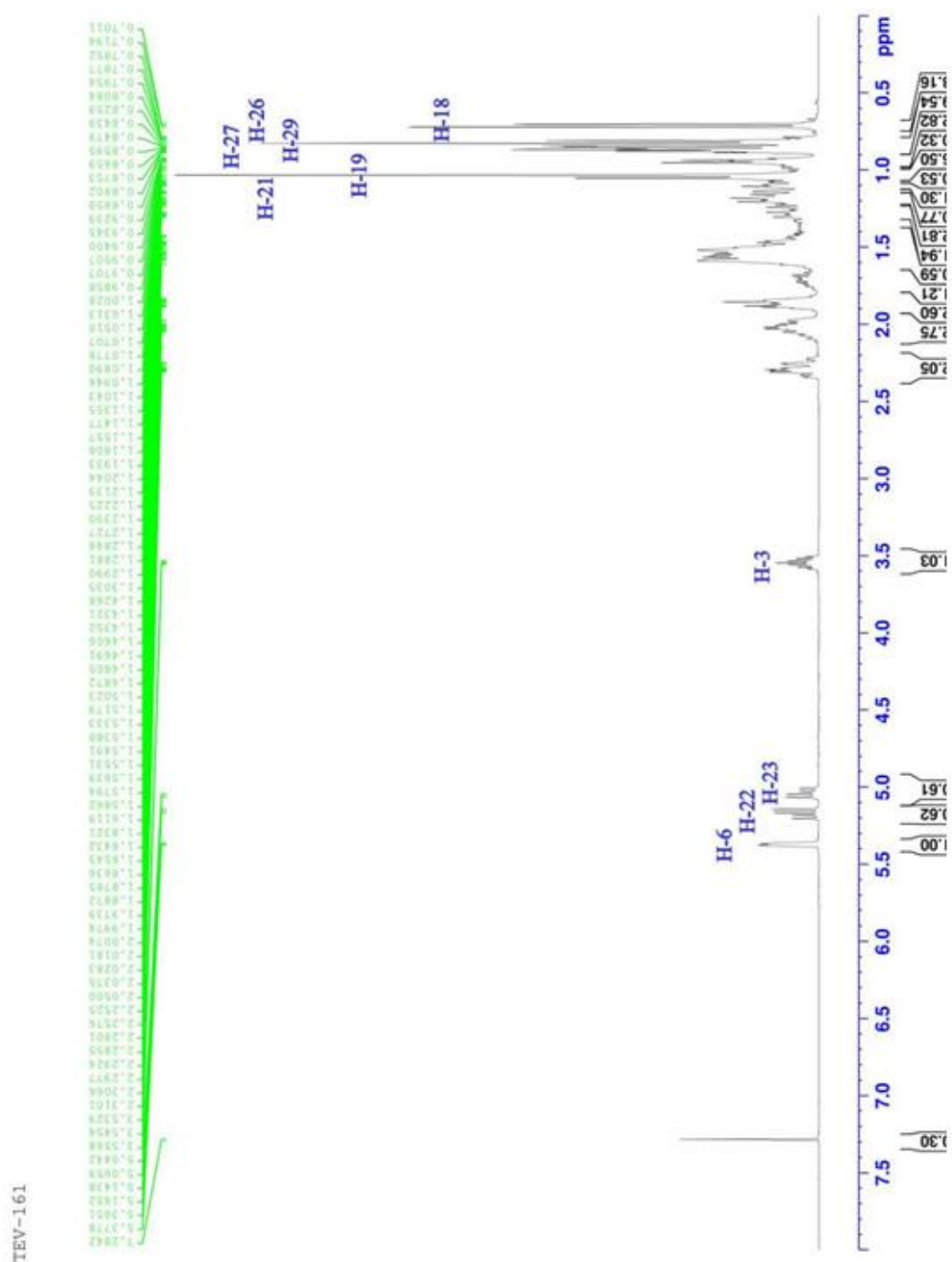


Figure 3. 60 <sup>1</sup>H NMR spectrum (400 MHz, CDCl<sub>3</sub>) of compound 20 (TEV-161)

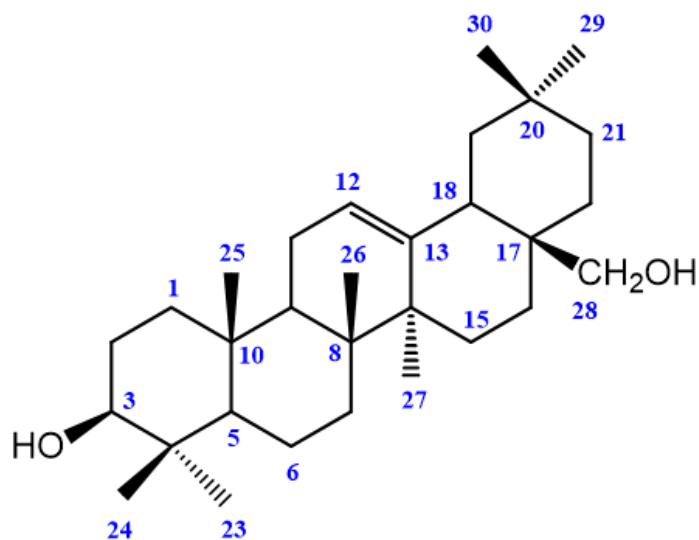


### 3.1.20 Characterization of compound **21**(TEV-1711) as 3 $\beta$ ,28-dihydroxyolean-12-ene

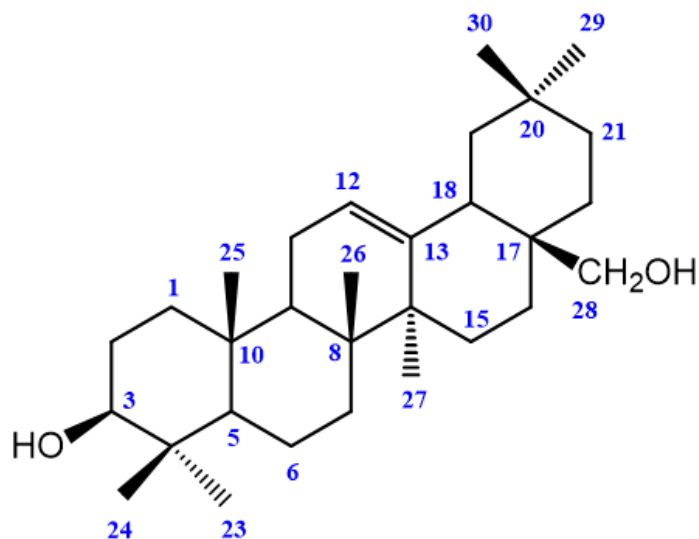
Compound **21**, isolated as colorless crystals, was invisible when examined under UV light on a TLC plate and produced purple color when sprayed with vanillin in sulphuric acid reagent followed by heating for 5 minutes.

The  $^1\text{H}$  NMR spectrum showed seven methyl groups resonating at  $\delta$  0.75, 0.90, 0.91, 0.95, 0.96, 1.01 and 1.18 (3H s, each) which are assignable for H-24, H-25, H-30, H-29, H-23, H-26 and H-27 respectively and an olefinic proton at  $\delta$  5.24 d ( $J=10.8$  Hz).

The typical oxymethylene ( $-\text{CH}_2\text{OH}$ ) protons were seen as a pair of doublets centered at  $\delta$  3.23 and 3.57 ( $J = 11$  Hz) and an oxymethine proton at  $\delta$  3.28 m (H-3). These  $^1\text{H}$  NMR data revealed the presence of a triterpenoid structure with a hydroxymethyl group at C-28 and a hydroxyl function at C-3. The  $^1\text{H}$  NMR spectrum of compound **21** were found similar to those reported for 3 $\beta$ ,28-dihydroxyolean-12-ene (Ragasa et al., 2014). Thus, compound **21** was identified as 3 $\beta$ ,28-dihydroxyolean-12-ene previously isolated from *Erythrina variegata*.



3 $\beta$ ,28-dihydroxyolean-12-ene



3 $\beta$ ,28-dihydroxyolean-12-ene

Tables 3.22  $^1\text{H}$  NMR spectroscopic data (400 MHz,  $\text{CDCl}_3$ ) for compound 21

Protons	TEV-1711	3 $\beta$ ,28-Dihydroxyolean-12-ene
	$\delta_{\text{H}}$	(Ragasa et al., 2014) $\delta_{\text{H}}$
H-3	3.28 m	3.18 m
H-12	5.24 d ( $J = 10.8$ Hz)	5.18 d
H-23	0.96 3H s	0.96 3H s
H-24	0.75 3H s	0.74 3H s
H-25	0.90 3H s	0.87 3H s
H-26	1.01 3H s	0.98 3H s
H-27	1.18 3H s	1.15 3H s
H-29	0.95 3H s	0.953H s
H-30	0.91 3H s	0.88 3H s
H-28a	3.23 d ( $J = 11.0$ Hz)	3.20 d
H-28b	3.57 d ( $J = 11.0$ Hz)	3.52 d

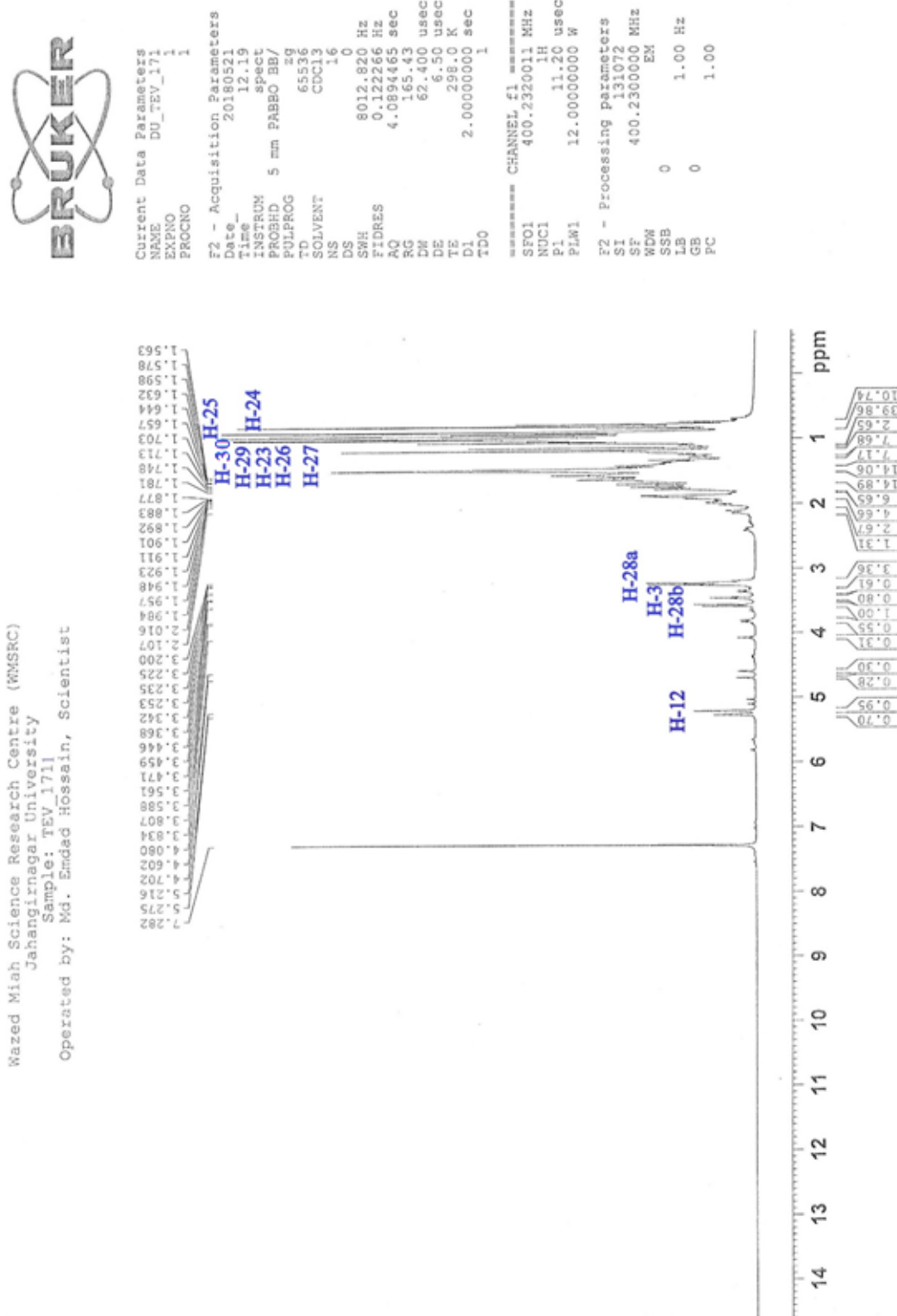


Figure 3. 61  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound 21 (TEV-1711)

## 4.1 Introduction to Biological Investigation

Plants are one of the most important and well known source for the treatment of various kinds of diseases of both human beings and animals (Spinella, 2001). Due to the immense necessity, today the investigation of medicinal plants with modern technology has become the leading areas of research in plant sciences. Biological Investigations involves the introduction of scientific method through practical application where pharmacologically active biomolecules and natural chemicals are investigated from medicinal plants. It also concentrated on the separation of active pharmacological compounds from natural resources (Williams & Ahmad Z, 1999). Currently, structure-activity relationships (SAR) studies due to their influence on the drug design particularly of novel drugs have introduced them as one of the major part of phytochemistry, which is an advanced areas of pharmaceutical sciences (Aslam, 2016).

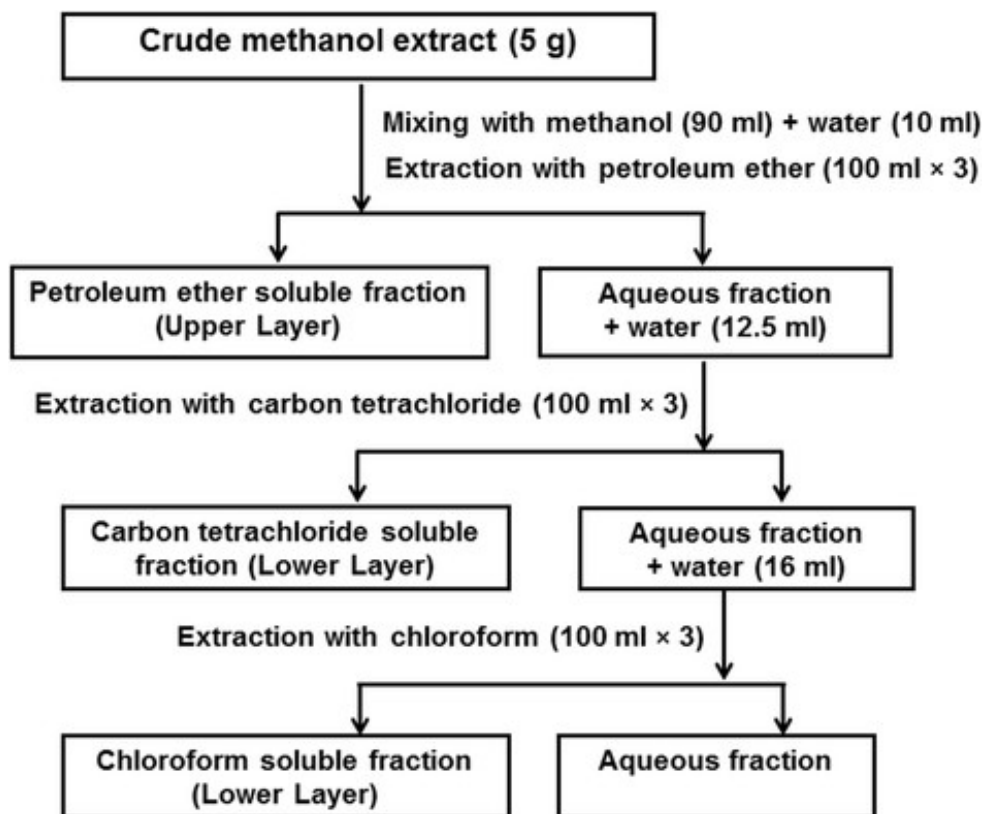
In spite of vigorous competition from other drug discovery methods, natural products are still providing their fair share of new clinical candidates and drugs (Butler, 2004). These compounds are still a significant source of new drugs, especially in the areas like anticancer, anti-hypertensive, anti-infective, immunosuppression and neurological diseases (Butler, 2004).

The objective of this research work was to investigate different biological activities i.e., cytotoxic, antimicrobial, antioxidant and thrombolytic activities of different solvent fractions of crude methanolic extracts and isolated pure compounds of *Ravenia spectabilis* and *Erythrina variegata*.

## 4.2 Experimental Design of investigated plant's extracts

### 4.2.1 Solvent-Solvent partition of crude extracts by Modified Kupchan Partition

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by (Van Wagenen et al. 1993). The crude extract (5 gm) was dissolved in 10% aqueous methanol. It was extracted with pet-ether, then with carbon tetrachloride and finally with chloroform. All the four fractions were evaporated to dryness and were used for further analysis (Van Wagenen et al., 1993). The whole partitioning process is schematically shown in Figure 4.1



**Figure 4.1** Schematic representation of the modified Kupchan Partitioning of methanolic crude extract of *R. spectabilis* and *E. variegata*

### 4.3 Evaluation of biological activities

#### 4.3.1 Cytotoxic activity

Cancer is the second reason of death worldwide. In recent years, many researchers have focused on the anticancer effect of medicinal plants and their isolated components due to the side effect of chemotherapeutic agents which is the main treatment of cancer. Plant natural product chemistry has played an active role in generating a significant number of drug candidate compounds in a drug discovery program. It is significant that over 60% of presently used anticancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms (Kaur et al., 2011). Therefore, natural protection against cancer has been recently receiving a great deal of attention not only from cancer patients but, surprisingly, from physicians as well. Many of the medicinal plants maintain the health and vitality of individuals and also cure diseases, including cancer without causing toxicity.

##### 4.3.1.1 Principle

Enzyme-based methods using MTT, rely on a reductive coloring reagent and dehydrogenase in a viable cell to determine cell viability with a colorimetric method. This method is easy-to-use, safe, has a high reproducibility, and is widely used in both cell viability and cytotoxicity tests. In the method, MTT is reduced to a purple formazan by NADH. However, MTT formazan is insoluble in water, and it forms purple needle shaped crystals in the cells. Therefore prior to measuring the absorbance, an organic solvent is required to solubilize the crystals. Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture media from the plate wells due to floating cells with MTT formazan needles, giving significant well-to-well error.

In this experiment, we evaluated the cytotoxic activity of three new alkaloids 3,5-iprenyl indole, 3-prenyl-5(2-keto-but-3-enyl) indole and 3-prenyl-indole-5-carbaldehyde isolated from *Ravenia spectabilis*. The experiment was done in School of Cancer and Pharmaceutical Science, King's College London as a collaboration research work.

#### 4.3.1.2 Cell line and cell culture

A panel of three immortalised human tumour cell lines and one non-tumour cell line WI-38 were used for the cytotoxicity screening of the three isolated new alkaloids i.e., 3,5-diprenylindole (compound 1), 3-prenyl-5-(2-keto-but-3-enyl)indole (compound 2) and 3-prenyl-indole-5-carbaldehyde (compound 3). HeLa (human cervical cancer), MIA-PaCa-2 (human pancreatic adenocarcinoma), and A549 (lung cancer) cell lines were obtained from the American Type Culture Collection and LGC. All cell-lines were maintained in monolayer culture in 75 cm<sup>2</sup> flasks (TPP, Switzerland) under a humidified 5% CO<sub>2</sub> atmosphere at 37°C. The HeLa cell line was maintained in Dulbecco's Modified Eagles Media (DMEM; Invitrogen) supplemented with foetal bovine serum (10% v/v; Invitrogen), L-glutamine (2mM; Invitrogen), non-essential amino acids (1x; Invitrogen) and Penicillin-Streptomycin (1% v/v, Invitrogen). For MIA PaCa2, Dulbecco's MEM, supplemented with L-glutamine (2mM; Invitrogen) and foetal calf serum (10%, Biosera UK) was used. For A549, F12-K medium (Sigmaaldrich), foetal bovine serum (10%, Biosera UK), non-essential amino acids (1x; Invitrogen) and Penicillin-Streptomycin (1% v/v, Invitrogen) was used for sub-culturing. The WI38 line was maintained in antibiotic free Dulbecco's Modified Eagles Media (DMEM; Invitrogen) supplemented with foetal bovine serum (10% v/v; Invitrogen), L-glutamine (2mM; Invitrogen) and non-essential amino acids (1x; Invitrogen). For passaging, cells were washed with PBS (GIBCO 14040, Invitrogen, UK), incubated with trypsin (GIBCO 25300, Invitrogen, UK), and re-seeded into fresh medium. For seeding, cells were counted using a Neubauer haemocytometer (Assistant, Germany) by microscopy (Nikon, USA) on a non-adherent suspension of cells that were washed in PBS, trypsinised, centrifuged at 8°C at 8000 rpm for 5 min and re-suspended in fresh medium.

#### 4.3.1.3 MTT Assay

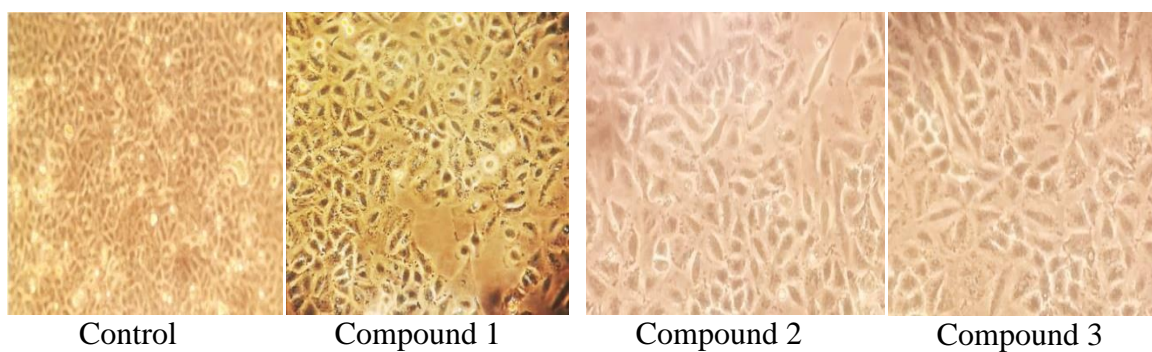
The cells were grown in normal cell culture conditions at 37°C under a 5% CO<sub>2</sub> humidified atmosphere using appropriate medium. The cell count was adjusted to 10<sup>5</sup> cells/ml/ and 5,000-15,000 cells were added per well depending on the cell line. The cells were incubated for 24 hours and 1 µl of the appropriate inhibitor concentrations

were to the wells in triplicates. After 96 h of continuous exposure to each compound, the cytotoxicity was determined using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Lancaster Synthesis Ltd, UK) colorimetric assay.<sup>19</sup> Absorbance was quantified by spectrophotometry at  $\lambda = 570$  nm (Envision Plate Reader, PerkinElmer, USA). IC<sub>50</sub> values were calculated by a dose-response analysis using the Prism Graphpad Prism® software.

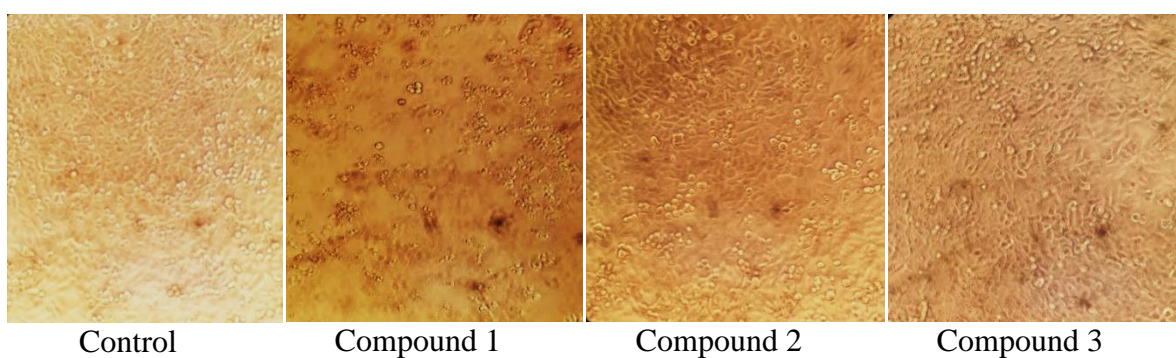
#### 4.3.1.4 Results and discussion

Among the isolated new compounds, 3,5-diprenylindole (compound 1) possessed highest cytotoxicity (Table 4.1, Figure 4.2 to Figure 4.4) to human pancreatic adenocarcinoma cell lines with IC<sub>50</sub> value of  $9.5 \pm 2.2$   $\mu$ M, moderately cytotoxic to human cervical and lung cancer cell lines with IC<sub>50</sub> values of  $11.3 \pm 1.3$   $\mu$ M and  $13.5 \pm 1.66$   $\mu$ M respectively and weakly cytotoxic to non-tumour cell line (WI-38) with IC<sub>50</sub> value of  $68.5 \pm 3.5$   $\mu$ M as compared to the standard ( $0.19 \pm 0.12$  to  $6.3 \pm 0.3$   $\mu$ M). The other two compounds 3-prenyl-5-(2-keto-but-3-enyl)indole (compound 2) and 3-prenyl-indole-5-carbaldehyde (compound 3) showed poor cytotoxicity (here, IC<sub>50</sub> values >50) against the four cell lines tested.

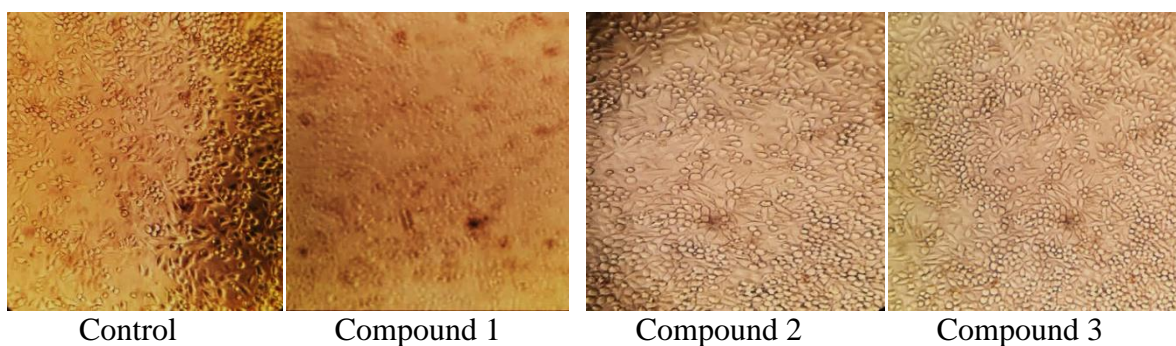




**Figure 4.2 Cytotoxicity study of three new alkaloids and control (Gemcitabine) against A549 Cells**



**Figure 4.3 Cytotoxicity study of three new alkaloids and control (Gemcitabine) against Hela cells**



**Figure 4.4 Cytotoxicity study of three new alkaloids and control (Gemcitabine) against Mia PaCa 2 Cells**

**Table 4. 1 Cytotoxic activity of the isolated pure compounds against different tumour cell lines**

<b>Compounds</b>	<b>HeLa (Cervical) IC<sub>50</sub> (μM)</b>	<b>A549 (Lung) IC<sub>50</sub> (μM)</b>	<b>MIA PaCa2 (Pancreatic) IC<sub>50</sub> (μM)</b>	<b>WI-38 (Non-tumour lung fibroblast) IC<sub>50</sub> (μM)</b>
Compound 1	11.3 ± 1.3	13.5 ± 1.66	9.5 ± 2.2	68.5 ± 3.5
Compound 2	>50	>50	>50	>100
Compound 3	>50	>50	>50	>100
Gemcitabine	3.3 ± 0.8	0.19 ± 0.12	0.6 ± 0.4	6.3 ± 0.3

N.B. Concentration range used – 100 μM to 1 μM, experiment performed in triplicate using MTT assay

### 4.3.2 Antimicrobial Assay

Among the general population, infectious diseases are a common cause of morbidity and mortality, particularly in the developing countries (Silva and Fernandes 2010). A wide range of medicinal plant extracts and isolated phytochemicals are used to treat several infections as they have potential antimicrobial activity. Pharmacological industries have introduced a number of new antibiotics in the last three decades, but resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to the antibacterial drugs. Such a fact is cause for concern, because of the number of patients in hospitals who have suppressed immunity and due to new multidrug resistant bacterial strains. As a result, new infections can occur in hospitals resulting in high mortality (Nascimento et al., 2000). Hence, more studies pertaining to the use of plants as antimicrobial agents should be emphasized, especially those related to the control of antibiotic resistant microbes.

#### 4.3.2.1 Principle of disc diffusion method

Disc diffusion method is one of the widely used and popular method for susceptibility testing of bacteria. In this method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Test samples containing paper discs (6 mm diameter) are dried and sterilized and are placed on nutrient agar medium uniformly seeded with the test microorganisms. The plates are kept at low temperature (4°C) for 16 to 24 hours to allow maximum diffusion of the test materials to surrounding media (Barry, 1976). For optimum growth of the organisms the plates are then inverted and incubated at 37°C for 24 hours.

The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition and the diameter of zone of inhibition (expressed in millimeter) is then measured to evaluate the antimicrobial activity of the test agent (Bauer et al., 1966).

Blank discs and standard antibiotic (Kanamycin) discs are used as negative and positive control. The experiment was done in triplicate and the inhibitory activity of the samples were determined by comparing the average sizes of inhibition zones (mm).

In the present study, different organic fractions of the crude methanol extract of *Ravenia spectabilis* and *Erythrina variegata* and the pure compounds named arborinine, ravenoline, scandenone and alpinumisoflavone isolated from these two plants were tested for antimicrobial activity by disc diffusion method. The experiment was carried out thrice and the mean of the readings is recorded (Bauer et al., 1966).

#### 4.3.2.2 Materials and method

##### Bacterial strains

The bacterial strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. Both gram positive and gram-negative organisms were taken for the test and they are listed in the following table:

**Table 4.2 List of bacteria used in antibacterial screening**

Types of Strains	
Bacteria	
Gram positive	<i>Staphylococcus aureus</i> <i>Bacillus subtilis</i> <i>Bacillus cereus</i>
Gram negative	<i>Shigella dyenteriae</i> <i>Salmonella typhi</i> <i>Salmonella paratyphi</i> <i>Escherichia coli</i> <i>Pseudomonas aeruginosa</i> <i>Vibrio cholerae</i> <i>Klebsiella pneumonia</i>

##### Equipments and reagents

All procedures were performed using the equipment available in the Phytochemical Research Laboratory, University of Dhaka. The equipments and apparatus are autoclave, laminar air flow hood, incubator, spirit burner, sterile forceps, ethanol, incubating loop, sterile forceps, Whatman no. 3 filter paper discs, nutrient agar media,

screw cap vials, screw cap test tubes, micro pipette ,nose mask and hand gloves, petri-dishes ,sterile cotton etc.

### **Culture Medium**

The nutrient agar media was used to demonstrate the antimicrobial activity and to make subculture of the test organisms. It is composed of bacto peptone (0.5 gm), sodium chloride (0.5 gm), bacto yeast extract (1.0 gm), bacto agar (2.0 gm), distilled water q.s. 100ml.

#### **4.3.2.3 Experimental**

##### **Preparation of the medium**

Each of the constituents of the medium were properly measured and taken in a conical flask and distilled water was added to it to make the required volume. The contents were then heated in a water bath to make a clear solution. The medium was then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15-lbs. pressure at 121°C for 20 minutes. The slants were used for making fresh culture of bacteria that were in turn used for sensitivity study,

##### **Preparation of Test sample**

Test samples were prepared from crude methanol extracts of *Ravenia spectabilis* and *Erythrina variegata* and pure compounds were isolated from the two mentioned plants.

##### **Sterilization Procedure**

In order to avoid contamination and cross contamination by the test organisms the anti microbial screening was done in Laminar hood and all types of precautions were highly maintained UV light was switched on an hour before working in Laminar Hood.

Different glasswares such as petri dishes were sterilized by autoclaving at a temperature of 121°C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs and swabs were also sterilized.

### **Preparation of Subculture**

The test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The aseptic condition was maintained under laminar air cabinet. The inoculated strains were then incubated for 24 hours at 37°C for their optimum growth. For the sensitivity test these fresh cultures were used.

### **Preparation of the Test Plate**

With the help of a sterilized transfer loop, the test organisms were transferred from the subculture to the test tubes containing about 10 ml of melted and sterilized agar medium. To get a uniform suspension of the organisms, the test tubes were shaken by rotation. The bacterial suspension was instantly transferred to the sterilized petri dishes. The petri dishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media. All these procedures were done in an aseptic area.

### **Disc preparation**

#### **Preparation of Blank Discs**

Blank discs were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

#### **Standard Discs**

Kenamycin standard disc was used as the reference here. Standard discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample.

#### **Preparation of Sample Discs with Test Sample**

Metrical (BBL, Cocksville, USA) filter paper discs were made carefully and taken in a blank wide mouth screw cap vial. These discs were then sterilized properly. Measured amounts of each test sample were dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. Then the discs were soaked with solutions of test samples and dried.

### **Diffusion and Incubation**

All the discs (sample discs, standard antibiotic discs and control discs) were placed gently on the previously marked spots in the agar plates pre-inoculated with test bacteria. The plates were then kept in a refrigerator at 4°C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37°C for 24 hours.

### **Determination of antimicrobial activity**

The antimicrobial potency of the test agents were measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. The antimicrobial activities of the test materials were determined after incubation, by measuring the diameter of the zones of inhibition in millimeter with a slide calipers.

#### **4.3.2.4 Results and Discussion**

Antimicrobial screening of the petroleum ether, carbon tetrachloride, chloroform and aqueous soluble fractions of the crude methanol extract of the leaf of *Ravenia spectabilis* showed mild to moderate activity (Table 4.3) against various gram positive and gram negative bacterial strains in comparison to standard Kanamycin discs.

Among the eight gram (+)ve and (-)ve bacteria, antimicrobial activity of all fractions of *R. spectabilis* and the isolated pure compounds (ravenoline and arborinine) showed highest antibacterial activity against *Vibrio cholerae* and the pet-ether fraction of the plant showed the highest activity ( $20.5 \pm 0.74\text{mm}$ ) against *Bacillus subtilis*. This fraction showed very small zone of inhibition against all other bacteria (except *Vibrio cholerae*). Carbon tetrachloride fraction of the plant showed very little activity or no activity against most of the bacteria. The chloroform fraction of the plant showed moderate antimicrobial activity against *Staphylococcus aureus*, *Shigella dysenteriae* and *Vibrio cholera* ( $11.6 \pm 0.71\text{ mm}$ ,  $15.5 \pm 0.66\text{ mm}$  &  $18.1 \pm 0.33\text{ mm}$  respectively). The aqueous fraction and the pure compound ravenoline (isolated from *R. spectabilis*) have shown moderate activity against *Bacillus subtilis* and good

activity against *Vibrio cholerae*. The pure compound arborinine showed very little activity or no activity against most of the bacteria.

Different partitionates of methanol extract of the stem bark of *Erythrina variegata* were tested for antimicrobial activities (Table 4.4) against two gram (+)ve and four gram(-)ve bacteria. All the partitionates and the isolated pure compounds (scandenone and alpinumisoflavone) showed mild to moderate antimicrobial activity against most of the microorganisms. The carbontetrachloride fraction showed highest antibacterial activity against *Bacillus cereus* ( $19.5 \pm 1.18$  mm). The petroleum ether, carbon tetrachloride and chloroform extracts exhibited good antimicrobial activity against *Bacillus cereus*. The carbontetrachloride fraction showed prominent antibacterial activity against *Bacillus subtilis* ( $18.9 \pm 0.39$  mm) and also the aqueous fraction of the methanol extract of *E. variegata* showed good activity against *Salmonella paratyphi* ( $17.8 \pm 0.72$  mm). The pet-ether and carbon tetrachloride fraction also exhibited pretty good activity ( $18.3 \pm 0.77$  mm &  $15.7 \pm 0.88$  mm) against *Vibrio cholerae*. The isolated pure compounds scandenone and alpinumisoflavone (isolated from *E. variegata*) showed mild activity (some cases no activity) against the test microorganisms.



**Table 4.3 Antimicrobial activity of the extracts and pure compounds of *R. spectabilis***

Sample	Diameter of Zone of Inhibition (mm)							
	Gram Positive Bacteria			Gram Negative Bacteria				
	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>	<i>Salmonella typhi</i>	<i>Shigella dysenteriae</i>	<i>Escherichia coli</i>	<i>Vibrio cholerae</i>	<i>Klebsiella pneumoniae</i>
<b>Pet Ether fraction</b> (400 µgm/disc)	20.5±0.74	8.2±0.49	7.2±0.51	Absent	7.2±0.41	7.2±0.24	17.7±0.68	7.3±0.37
<b>Carbon Tetra Chloride fraction</b> (400 µgm/disc)	10.2±0.36	Absent	7.3±0.37	Absent	7.6±0.56	9.2±0.2	18.1±0.33	Absent
<b>Chloroform fraction</b> (400 µgm/disc)	16.1±0.6	11.6±0.71	7.2±0.25	Absent	15.5±0.66	8.1±0.33	18.1±0.33	7.0±0.58
<b>Aquaous fraction</b> (400 µgm/disc)	10.8±0.47	7.2±0.36	Absent	Absent	7.3±0.51	8.1±0.31	16.3±0.51	Absent
<b>Arborinine</b> (100 µgm/disc)	7.3±0.61	7.3±0.38	Absent	Absent	7.2±0.55	7.7±0.68	16.2±0.24	Absent
<b>Ravenoline</b> (100 µgm/disc)	10.1±0.44	8.1±0.29	7.6±0.40	Absent	Absent	7.1±0.17	17.2±0.41	Absent
<b>Kanamycin</b> (30 µgm/disc)	34.0±0.5	30.3±0.62	42.8±0.72	35±0.6	27.6±0.29	37.6±0.29	30.3±0.62	40.3±0.62

The diameter of zone of inhibition was expressed as mean± SD

**Table 4.4** Antimicrobial activity of the extracts and pure compounds of *E. variegata*

Sample	Diameter of Zone of Inhibition (mm)					
	Gram Positive Bacteria			Gram Negative Bacteria		
	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>	<i>Pseudomonas aeruginosa</i>	<i>Salmonella paratyphi</i>	<i>Shigella dysenteriae</i>	<i>Vibrio cholerae</i>
<b>Pet Ether fraction</b> (500 µgm/disc)	10.7±0.63	18.5±89	Absent	9.2±0.72	10.6±0.71	18.3±0.77
<b>Carbon Tetra Chloride Fraction</b> 500 µgm/disc)	18.9±0.39	19.5±1.18	Absent	13.1±0.33	11.8±0.68	15.7±0.88
<b>Chloroform fraction</b> (500 µgm/disc)	12.7±0.78	17.8±0.69	12.7±0.44	9.0±0.75	10.8±0.70	13.6±0.55
<b>Aquaous fraction</b> (500 µgm/disc)	13.5±0.94	Absent	14.3±0.57	17.8±0.72	10.4±0.92	9.7±0.60
<b>Scandenone</b> (100 µgm/disc)	Absent	10.7±0.82	10.4±0.73	9.4±0.76	Absent	Absent
<b>Alpinum isoflavone</b> (100 µgm/disc)	12.1±0.54	9.0±0.72	Absent	11.0±1.06	Absent	7.6±0.55
<b>Kanamycin</b> (30 µgm/disc)	25.8±0.59	24.3±0.44	29.4±0.92	24.3±0.45	27.4±0.73	30.1±1.36

The diameter of zone of inhibition was expressed as mean± SD

### 4.3.3 Thrombolytic activity assay

Blood clot formation has been considered as a severe problem of blood circulation (Ramjan et al., 2014). Thrombous formation within the blood vessels obstructs blood flow through the circulatory system leading hypertension, stroke to the heart, anoxia and so on. The complete deprivation of oxygen and infarction is a mode of cell death (Sultana et al., 2012). Thrombolytic drugs are used to dissolve blood clots in a procedure termed thrombolysis. Medicinal plants are considered as an important source of new chemical substances with potential therapeutic effects. Several plants such as *Ocimum sanctum*, *Curcuma longa*, *Azadirachta indica*, *Tulbaghia violaceae*, *Anacardium occidentale* etc. have been proved to possess thrombolytic activity and many such plants are yet to be scientifically studied (Fathima et al., 2015).

#### 4.3.3.1 Principle

In this investigation, thrombolytic activity was determined by the method developed by Prasad et al., which is a simple and easy method.

According to this method a pre-weighted clot formed from collected human blood has been applied with the sample to be tested and the amount of clot lysis was measured and results were expressed as percentage of clot lysis with reference to that of standard streptokinase sample (Prasad et al., 2006).

#### 4.3.3.2 Materials and Methods

##### Equipments and Reagents

Eppendorf tubes, distilled water, blood from human volunteers, streptokinase, incubator, test tube, vortex mixture

##### Preparation of sample

The dry crude extracts (10 mg) were suspended in 10 ml of distilled water and it was kept overnight. Then the soluble supernatant was decanted and filtered.

##### Preparation of Standard

Commercially available lyophilized alteplase (streptokinase) vial (Beacon Pharmaceutical Ltd.) of 1,500,000 I.U. was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 µl (30,000 I.U) was used for *in vitro* thrombolysis.

### **Blood Sample Collection**

Whole blood (20 ml) was drawn from two healthy human volunteer without a history of oral contraceptive or anticoagulant therapy.

### **Thrombolytic Activity**

Aliquots (20 ml) of venous blood were drawn from healthy volunteers which were distributed in forty different pre-weighed sterile eppendorf tube (0.5 ml/tube) and incubated at 37° C for 45 minutes. The serum was completely removed after clot formation, without disturbing the clot. Each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone).

As a negative non-thrombolytic control, 100 µl of distilled water and as a positive control, 100 µL of streptokinase (SK) were separately added to the control tubes. All the tubes were then incubated at 37° C for 90 minutes and observed for clot lysis. After incubation, the released of fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption.

Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

$$\% \text{ of clot lysis} = (\text{Weight of the clot after lysis} / \text{Weight of clot before lysis}) \times 100$$

#### **4.3.3.3 Results and Discussion**

In search of cardioprotective properties of the extracts obtained from *Ravenia spectabilis* and *Erythrina variegata* were assessed for thrombolytic activity and the results are presented in Table 4.5 to Table 4.12.

The extractives and the pure compounds of leaf of *Ravenia spectabilis* showed moderate thrombolytic activity. Among all the fractions and pure compounds, the pet ether fraction showed highest clot lysis activity ( $48.85 \pm 2.17 \%$ ), whereas standard streptokinase at 37 °C showed  $74.34 \pm 0.73 \%$  lysis of the clot as compared to distilled water showing a negligible lysis of clot ( $3.93 \pm 0.70 \%$ )

The extractives of the stem bark of *Erythrina variegata* showed moderate thrombolytic activity. The pet ether and aqueous fraction showed highest clot lysis

activity ( $56.78 \pm 0.55$  % and  $57.78 \pm 0.24$  % respectively), whereas standard streptokinase at  $37$  °C showed  $76.54 \pm 0.9$  % lysis of the clot as compared to distilled water showing a negligible lysis of clot ( $3.49 \pm 0.28$  %)

**Table 4.5 Thrombolytic Activity (% of clot lysis) of the extractives of *R. spectabilis* for Experiment 1**

Extractives	W1 g	W2 g	W3 g	clot before lysis, W4=W2-W1g	Weight of lysis clot, W5= W2-W3 g	% of lysis
PE	0.796	1.416	1.101	0.619	0.314	50.790
CTC	0.801	1.214	1.092	0.413	0.122	29.539
CL	0.787	1.406	1.141	0.618	0.264	42.797
AQ	0.791	1.398	1.182	0.606	0.216	35.613
Arborinine	0.794	1.315	1.142	0.520	0.172	33.205
Blank	0.766	1.114	1.101	0.347	0.013	3.738
Streptokinase	0.721	1.934	1.022	1.213	0.912	75.185

Here, PE = pet ether soluble fraction, CTC = carbontetrachloride soluble fraction, CL= chloroform soluble fraction, AQ = aqueous soluble fraction, Blank = distilled water, W1 = Weight of vial, W2 = Weight of clot containing vial, W3 = Weight of clot containing vial after clot disruption

**Table 4.6 Thrombolytic Activity (% of clot lysis) of the extractives of *R. spectabilis* for Experiment 2**

Extractives	W1 g	W2 g	W3 g	clot before lysis, W4=W2-W1g	Weight of lysis clot, W5= W2-W3 g	% of lysis
PE	0.766	1.430	1.121	0.664	0.308	46.500
CTC	0.811	1.214	1.087	0.403	0.127	31.563
CL	0.776	1.409	1.143	0.633	0.266	42.015
AQ	0.782	1.342	1.162	0.560	0.179	32.071
Arborinine	0.794	1.291	1.132	0.497	0.159	32.034
Blank	0.756	1.124	1.112	0.368	0.012	3.342
Streptokinase	0.771	1.773	1.032	1.001	0.740	73.944

Here, PE = pet ether soluble fraction, CTC = carbontetrachloride soluble fraction, CL= chloroform soluble fraction, AQ = aqueous soluble fraction, Blank = distilled water, W1 = Weight of vial, W2 = Weight of clot containing vial, W3 = Weight of clot containing vial after clot disruption

**Table 4.7 Thrombolytic Activity (% of clot lysis) of the extractives of *R. spectabilis* for Experiment 3**

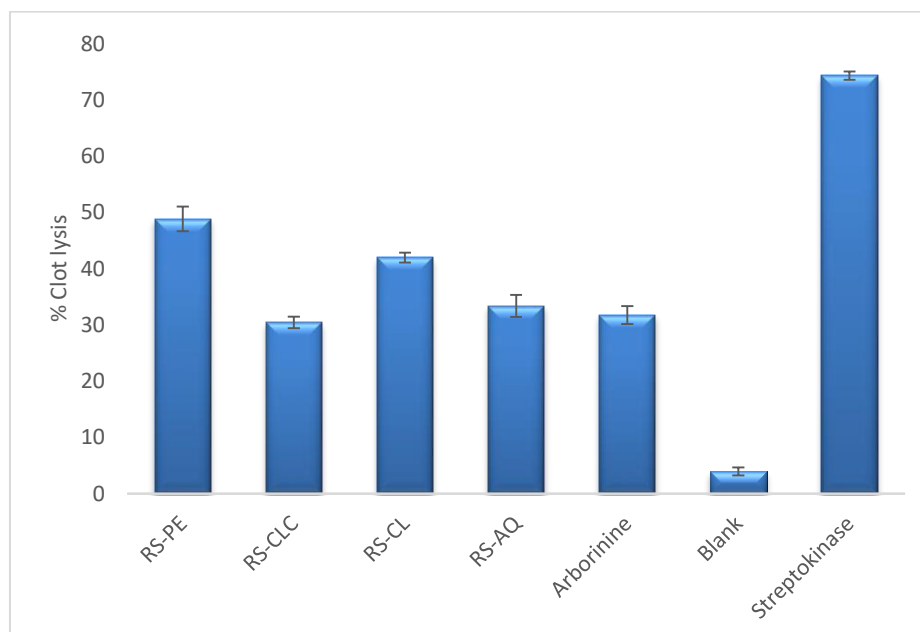
Extractives	W1 g	W2 g	W3 g	clot before lysis, W4=W2-W1g	Weight of lysis clot, W5=W2-W3 g	% of lysis
PE	0.777	1.489	1.1385	0.711	0.350	49.255
CTC	0.815	1.652	1.3993	0.836	0.252	30.201
CL	0.786	1.375	1.1332	0.588	0.241	41.080
AQ	0.772	1.364	1.1724	0.591	0.191	32.375
Arborinine	0.775	1.301	1.1432	0.525	0.157	30.005
Blank	0.746	1.339	1.311	0.592	0.028	4.721
Streptokinase	0.723	1.820	1.009	1.096	0.810	73.885

Here, PE = pet ether soluble fraction, CTC = carbontetrachloride soluble fraction, CL= chloroform soluble fraction, AQ = aqueous soluble fraction, Blank = distilled water, W1 = Weight of vial, W2 = Weight of clot containing vial, W3 = Weight of clot containing vial after clot disruption

**Table 4.8 Mean value of the Thrombolytic Activity (% of clot lysis) of the extractives of *R. spectabilis***

Extractives	Experiment 1	Experiment 2	Experiment 3	Mean± Stdev
RS-PE	50.790	46.500	49.255	48.85±2.17
RS-CCL4	29.539	31.563	30.201	30.43±1.03
RS-CHCL3	42.797	42.015	41.080	41.96±0.859
RS-AQUA	35.613	32.071	32.375	33.35±1.96
Arborinine	33.205	32.034	30.005	31.74±1.61
Blank	3.738	3.342	4.721	3.93±0.70
Streptokinase	75.185	73.944	73.885	74.34±0.73

Here, RS= *R. spectabilis*, PE=pet ether soluble fraction, CTC=carbontetrachloride soluble fraction, CL= chloroform soluble fraction, AQ= aqueous soluble fraction , Blank=distilled water

**Figure 4.5 Thrombolytic Activity (% of clot lysis) of different extractives of *Ravenia spectabilis***

**Table 4.9 Thrombolytic Activity (% of clot lysis) of the extractives of *E. variegata* for Experiment 1**

Extractives	W1 g	W2 g	W3 g	clot before lysis, W4=W2-W1g	Weight of lysis clot, W5= W2-W3 g	% of lysis
PE	0.830	1.705	1.206	0.874	0.499	56.143
CTC	0.847	1.631	1.382	0.783	0.248	30.823
CL	0.821	1.865	1.412	1.043	0.452	44.770
AQ	0.856	1.292	1.039	0.435	0.253	57.647
Blank	0.851	1.994	1.951	1.143	0.042	3.182
Streptokinase	0.834	1.726	1.051	0.891	0.675	77.503

Here, PE = pet ether soluble fraction, CTC = carbontetrachloride soluble fraction, CL= chloroform soluble fraction, AQ = aqueous soluble fraction, Blank = distilled water, W1 = Weight of vial, W2 = Weight of clot containing vial, W3 = Weight of clot containing vial after clot disruption

**Table 4.10 Thrombolytic Activity (% of clot lysis) of the extractives of *E. variegata* for Experiment 2**

Extractives	W1 g	W2 g	W3 g	clot before lysis, W4=W2-W1g	Weight of lysis clot, W5= W2-W3 g	% of lysis
PE	0.841	1.915	1.301	1.073	0.613	57.053
CTC	0.863	1.611	1.378	0.748	0.233	31.737
CL	0.827	1.795	1.374	0.967	0.421	43.379
AQ	0.877	1.730	1.238	0.852	0.491	58.067
Blank	0.831	1.754	1.721	0.923	0.032	3.750
Streptokinase	0.842	1.986	1.112	1.143	0.874	75.715

Here, PE = pet ether soluble fraction, CTC = carbontetrachloride soluble fraction, CL= chloroform soluble fraction, AQ = aqueous soluble fraction, Blank = distilled water, W1 = Weight of vial, W2 = Weight of clot containing vial, W3 = Weight of clot containing vial after clot disruption



**Table 4.11 Thrombolytic Activity (% of clot lysis) of the extractives of *E. variegata* for Experiment 3**

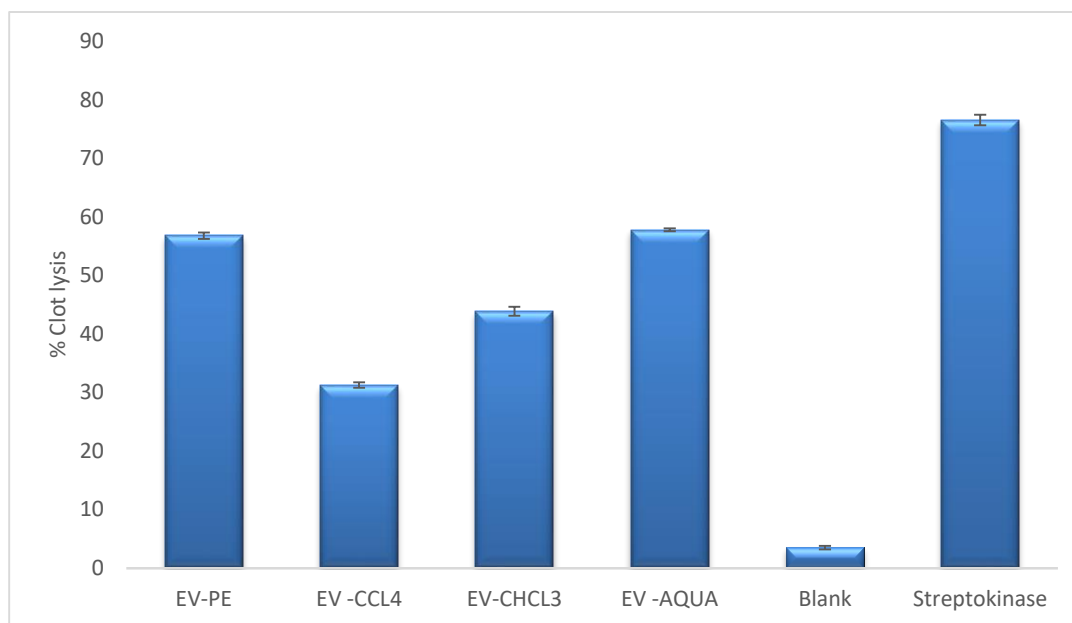
Extractives	W1 g	W2 g	W3 g	clot before lysis, W4=W2-W1g	Weight of lysis clot, W5= W2-W3 g	% of lysis
PE	0.841	1.915	1.301	1.073	0.613	57.158
CTC	0.863	1.611	1.378	0.748	0.233	31.191
CL	0.827	1.795	1.374	0.967	0.421	43.536
AQ	0.877	1.730	1.238	0.852	0.491	57.647
Blank	0.831	1.754	1.721	0.923	0.032	3.561
Streptokinase	0.842	1.986	1.112	1.143	0.874	76.418

Here, PE = pet ether soluble fraction, CTC = carbontetrachloride soluble fraction, CL= chloroform soluble fraction, AQ = aqueous soluble fraction, Blank = distilled water, W1 = Weight of vial, W2 = Weight of clot containing vial, W3 = Weight of clot containing vial after clot disruption

**Table 4.12 Mean value of the Thrombolytic Activity (% of clot lysis) of the extractives of *E. variegata***

Extractives	Experiment 1	Experiment 2	Experiment 3	Mean± Stdev
EV-PE	56.143	57.053	57.158	56.78±0.55
EV -CTC	30.823	31.737	31.191	31.25±0.45
EV-CL	44.770	43.379	43.536	43.89±0.76
EV -AQ	57.647	58.067	57.647	57.78±0.24
Blank	3.182	3.750	3.561	3.49±0.28
Streptokinase	77.503	75.715	76.418	76.54±0.9

Here, EV = *E. variegata*, PE=pet ether soluble fraction, CTC=carbontetrachloride soluble fraction, CL= chloroform soluble fraction, AQ= aqueous soluble fraction, Blank=distilled water



**Figure 4.6** Thrombolytic Activity (% of clot lysis) of different extractives of *Erythrina variegata*

#### 4.3.4 Antioxidant activity

Antioxidants obtained from the natural resources can boost up the capability of antioxidant activity of plasma thereby diminishing the possibility for particular diseases namely stroke, malignancy and cardio vascular diseases (Prior and Cao, 2000). Plants generate phenolics, flavonoids as secondary metabolites almost in all parts particularly in the leaves, fruits, seeds, roots and bark (Mathew and Abraham, 2006) , which are well known for their prominent scavenging actions against free radicals. Although many synthetic antioxidants are also available but they contain various unwanted side effects (Ito et al., 1983), including the liver damage and production of cancer in laboratory animals (Gao et al., 1999; Williams et al., 1999). Thus there exists the need of highly potent, relatively safer in terms of toxicity and side effects as well as cost minimizing antioxidants. In this regard the medicinal plants seem to be the best choice for meeting the necessity of required antioxidants from natural sources.

##### 4.3.4.1 Principle

The DPPH method is the most frequently used assay for the evaluation of the free radical-scavenging capacity of plant extracts. The free radical scavenging activities (antioxidant capacity) of the plant extracts on the stable radical 1,1- diphenyl- 2-picrylhydrazyl (DPPH) were estimated by mixing 2.0 ml of methanol solution of the extract at different concentration with 2.0 ml of a DPPH methanol solution (20 µg/ml). The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extract as compared to that of butylated hydroxytoluene (BHT) by UV spectrophotometer (Brand-Williams, 1995).

##### 4.3.4.2 Materials and Methods

###### Equipments and reagents

The apparatus, reagents and other components used in this experiment are 1,1-diphenyl-2-picrylhydrazyl (DPPH) , butylated hydroxytoluene (BHT) , distilled water, methanol , UV spectrophotometer (UV-1650PC,SHIMADZU), micropipette , eppendorf tube , Light proof box .

### **Preparation of positive control**

In this study, butylated hydroxytoluene (BHT) was used as positive control. Calculated amount of BHT was dissolved in methanol to get a mother solution having a concentration 400 µg/ml. Serial dilution was made using the mother solution to get different concentrations from 200.0 to 0.78125 µg/ml.

### **Test sample preparation**

Necessary amount of different extractives (pet-ether, carbon tetrachloride, chloroform and aqueous extracts) were measured and dissolved in methanol to get a mother solution having a concentration 400 µg/ml. Serial dilution was made using the mother solution to get different concentrations from 200.0 to 0.78125 µg/ml.

### **Preparation of DPPH solution**

For the preparation of total required amount of DPPH solution, 20 mg of DPPH was weighed and dissolved in 1 liter methanol to get a DPPH solution having a concentration of 20 µg/ml. As the DPPH solution is oxygen and light sensitive, it was prepared in an amber glass bottle and kept in light-proof box.

### **Evaluation of free radical scavenging activity**

2.0 ml of a methanol solution of the sample (Control or extractives) at different concentration from 200.0 to 0.78125 µg/ml were mixed with 2.0 ml of a DPPH methanol solution (20 µg/ml). After 30 minutes reaction period at room temperature in dark place, the absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer.

Inhibition of free radical DPPH in percent (I%) was calculated as follows

$$I\% = 1 - \{A_{\text{sample}} / A_{\text{blank}}\} \times 100$$

Where A blank is the absorbance of control reaction (containing all reagents except the test material).

Extract concentration providing 50% inhibition (IC<sub>50</sub>) was calculated from the graph plotted inhibition percentage against extract concentration.

#### 4.3.4.3 Results and Discussion

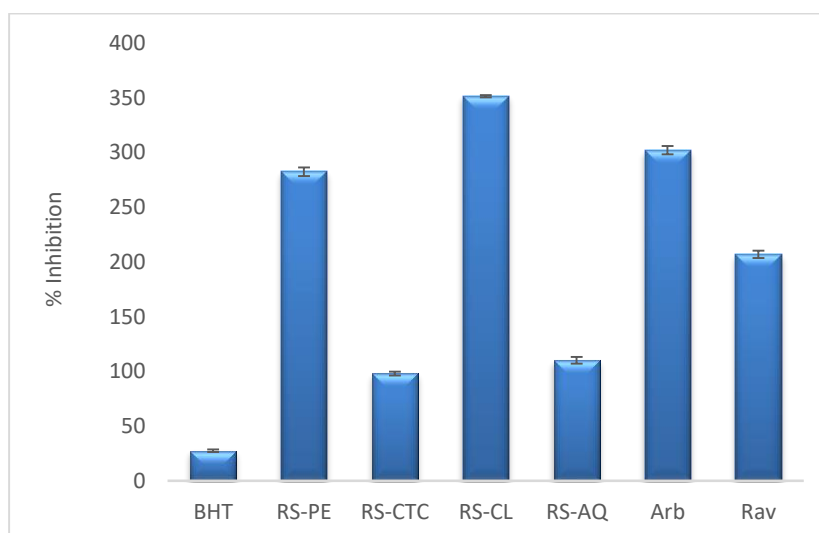
Four different partitionates of the methanolic extract of *Ravenia spectabilis* and *Erythrina variegata* and two isolated pure compounds (arborinine & ravenoline) from *Ravenia spectabilis* were subjected to free radical scavenging activity by the method developed by Brand-Williams *et al.*, 1995. Here butylatedhydroxytoluene (BHT) was used as standard.

Among all the fractions, carbon tetrachloride and aqueous fraction of *Ravenia spectabilis* showed moderate inhibitory activity ( $IC_{50}$  value were  $97.88 \pm 1.73 \mu\text{g/ml}$  and  $110.08 \pm 3.10 \mu\text{g/ml}$  respectively). Here the  $IC_{50}$  value of the reference butylated hydroxy toluene (BHT) was  $27.54 \pm 1.29 \mu\text{g/ml}$  (Table 4.13).

Carbon tetrachloride, chloroform and aqueous fraction of *E.variegata* showed moderate inhibitory activity with  $IC_{50}$  value of  $93.85 \pm 1.04 \mu\text{g/mL}$ ,  $67.59 \pm 1.87 \mu\text{g/mL}$  and  $75.02 \pm 2.62 \mu\text{g/mL}$ , respectively (Table 4.21) as compared to standard ( $23.09 \pm 1.37 \mu\text{g/mL}$ ).

**Table 4.13** IC<sub>50</sub> values of the standard and partitionates of *Ravenia spectabilis*

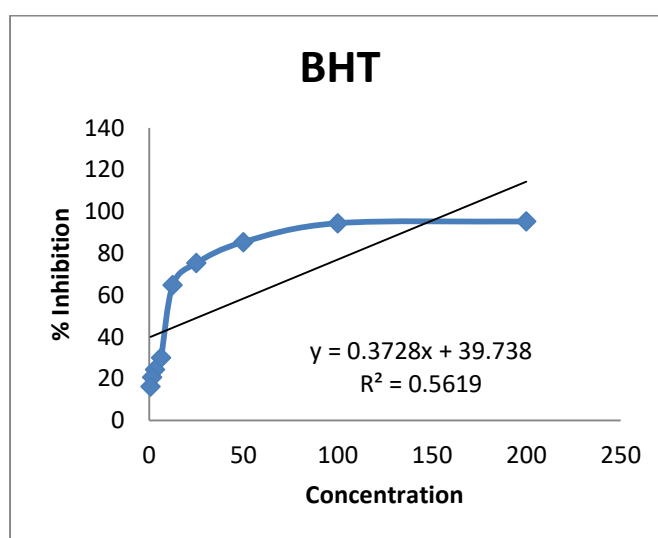
Sample code	Test sample	IC <sub>50</sub> value (µg/ml)
BHT	Butylated hydroxytoluene	27.54 ± 1.29
RS-PE	Pet-ether soluble fraction of the methanolic extract of the plant	282.22 ± 3.83
RS-CTC	Carbontetrachloride soluble fraction of the methanolic extract of the plant	97.88 ± 1.73
RS-CL	Chloroform soluble fraction of the methanolic extract of the plant	351.28 ± 0.963
RS-AQ	Aqueous soluble fraction of the methanolic extract of the plant	110.08 ± 3.10
Rav	Ravenoline	206.903 ± 3.28
Arb	Arborinine	301.92 ± 3.75

**Figure 4.7** Free radical scavenging activity of BHT, different fractions, arborinine and ravenoline of the leaf extract of *R. spectabilis*

**Table 4.14 Free radical scavenging activity of Butylated hydroxytoluene (BHT)**

Conc. (µgm/ml)	Absorbance			% Inhibition			IC <sub>50</sub> value			IC <sub>50</sub> (µg/ml) Mean± Stdev
	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	
200	0.012	0.015	0.013	95.68	94.60	95.32				
100	0.014	0.016	0.017	94.96	94.24	93.88				
50	0.038	0.041	0.043	86.33	85.25	84.53				
25	0.067	0.069	0.07	75.89	75.17	74.82				
12.5	0.097	0.099	0.098	65.10	64.38	64.74	26.05	28.38	28.20	27.54 ±1.29
6.25	0.192	0.195	0.196	30.93	29.85	29.49				
3.125	0.209	0.212	0.21	24.82	23.74	24.46				
1.5625	0.22	0.222	0.219	20.86	20.14	21.22				
0.78125	0.231	0.233	0.234	16.90	16.18	15.82				

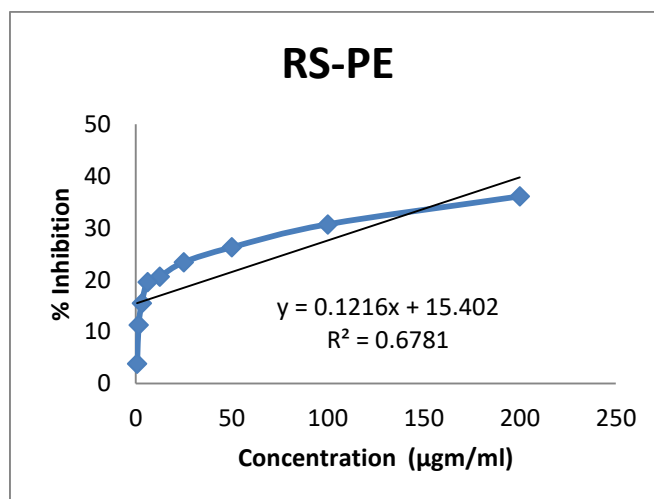
Here, Ex 1 = Experiment 1, Ex 2 = Experiment 2, Ex 3= Experiment 3, Absorbance of the Blank = 0.278

**Figure 4.8 Free radical scavenging activity of BHT (Butylated hydroxytoluene)**

**Table 4.15** Free radical scavenging activity of pet ether soluble fraction (RS-PE) of the leaf extract of *Ravenia spectabilis*

Conc. (µgm/ml)	Absorbance			% Inhibition			IC <sub>50</sub> value			IC <sub>50</sub> (µg/ml) Mean± Stdev
	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	
200	0.176	0.174	0.183	36.69	37.41	34.17				
100	0.192	0.191	0.195	30.93	31.29	29.85				
50	0.205	0.201	0.209	26.25	27.69	24.82				
25	0.213	0.21	0.216	23.38	24.46	22.30	281.88	278.57	286.22	282.22 ±3.83
12.5	0.22	0.217	0.225	20.86	21.94	19.06				
6.25	0.223	0.22	0.228	19.78	20.86	17.98				
3.125	0.235	0.232	0.238	15.46	16.54	14.38				
1.5625	0.244	0.239	0.257	12.23	14.02	7.553				
0.78125	0.267	0.264	0.271	3.956	5.035	2.517				

Here, Ex 1 = Experiment 1, Ex 2 = Experiment 2, Ex 3= Experiment 3, Absorbance of the Blank = 0.278

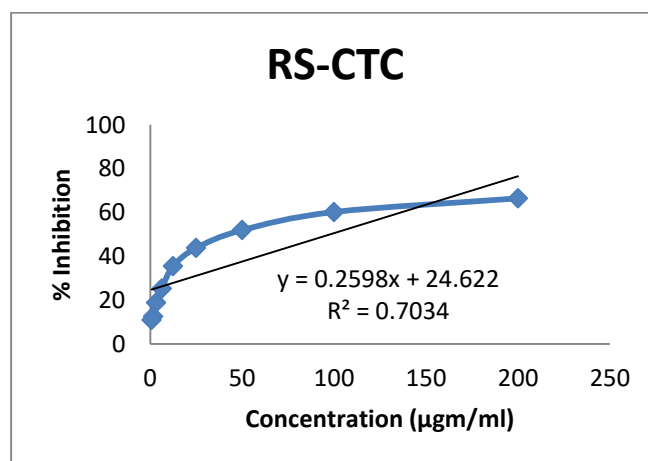
**Figure 4.9** Free radical scavenging activity of pet ether fraction of *R. spectabilis*



**Table 4.16 Free radical scavenging activity of carbontetrachloride soluble fraction (RS-CTC) of the leaf extract of *Ravenia spectabilis***

Conc. ( $\mu\text{g}/\text{ml}$ )	Absorbance			% Inhibition			IC <sub>50</sub> value			IC50 ( $\mu\text{g}/\text{ml}$ ) Mean $\pm$ Stdev
	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	
200	0.09	0.102	0.088	67.62	63.30	68.34				
100	0.112	0.112	0.108	59.71	59.71	61.15				
50	0.138	0.128	0.135	50.35	53.95	51.43				
25	0.16	0.151	0.158	42.44	45.68	43.16	97.92	99.60	96.12	97.88 $\pm$ 1.73
12.5	0.182	0.176	0.18	34.53	36.69	35.25				
6.25	0.213	0.2	0.21	23.38	28.05	24.46				
3.125	0.237	0.206	0.234	14.74	25.89	15.82				
1.5625	0.247	0.238	0.245	11.15	14.38	11.87				
0.78125	0.253	0.24	0.25	8.992	13.66	10.07				

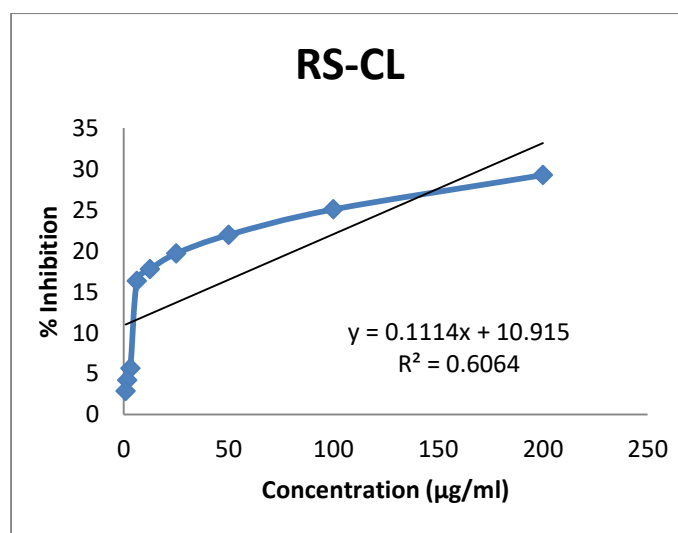
Here, Ex 1 = Experiment 1, Ex 2 = Experiment 2, Ex 3= Experiment 3, Absorbance of the Blank = 0.278

**Figure 4.10 Free radical scavenging activity of carbontetrachloride fraction of *R. spectabilis***

**Table 4.17 Free radical scavenging activity of chloroform soluble fraction (RS-CL) of the leaf extract of *Ravenia spectabilis***

Conc. ( $\mu\text{g}/\text{ml}$ )	Absorbance			% Inhibition			IC <sub>50</sub> value			IC <sub>50</sub> ( $\mu\text{g}/\text{ml}$ ) Mean $\pm$ Stdev
	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	
200	0.198	0.197	0.195	28.77	29.13	29.85				
100	0.208	0.208	0.209	25.17	25.17	24.82				
50	0.218	0.217	0.216	21.58	21.94	22.30				
25	0.225	0.224	0.221	19.06	19.42	20.50				
12.5	0.233	0.228	0.225	16.18	17.98	19.06	351.06	352.34	350.45	351.28 $\pm$ 0.963
6.25	0.234	0.233	0.231	15.82	16.18	16.90				
3.125	0.264	0.262	0.261	5.03	5.75	6.11				
1.5625	0.267	0.266	0.266	3.95	4.31	4.31				
0.78125	0.271	0.27	0.269	2.51	2.87	3.23				

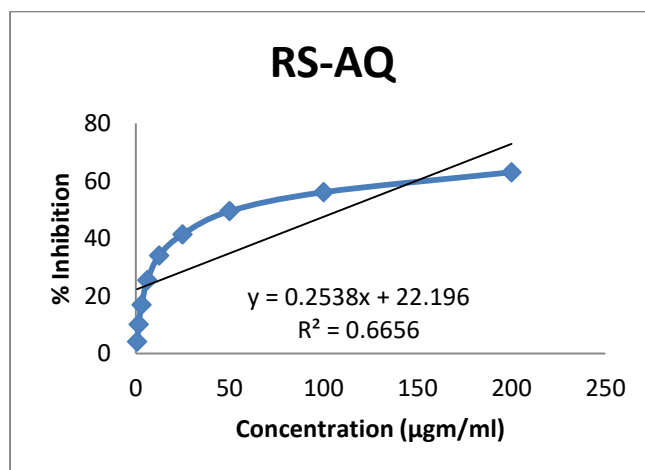
Here, Ex 1 = Experiment 1, Ex 2 = Experiment 2, Ex 3= Experiment 3, Absorbance of the Blank = 0.278

**Figure 4.11 Free radical scavenging activity of chloroform fraction of *R. spectabilis***

**Table 4.18** Free radical scavenging activity of aqueous soluble fraction (RS-AQ) of the leaf extract of *Ravenia spectabilis*

Conc. (µgm/ml)	Absorbance			% Inhibition			IC <sub>50</sub> value			IC <sub>50</sub> (µg/ml) Mean± Stdev
	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	
200	0.111	0.099	0.098	60.07	64.38	64.74				
100	0.122	0.121	0.123	56.11	56.47	55.75				
50	0.138	0.141	0.142	50.35	49.28	48.92				
25	0.16	0.163	0.165	42.44	41.36	40.64				
12.5	0.182	0.185	0.183	34.53	33.45	34.17	113.57	107.61	109.06	110.08 ±3.10
6.25	0.203	0.205	0.213	26.97	26.25	23.38				
3.125	0.231	0.225	0.237	16.90	19.06	14.74				
1.5625	0.247	0.25	0.252	11.15	10.07	9.35				
0.78125	0.263	0.265	0.271	5.39	4.67	2.51				

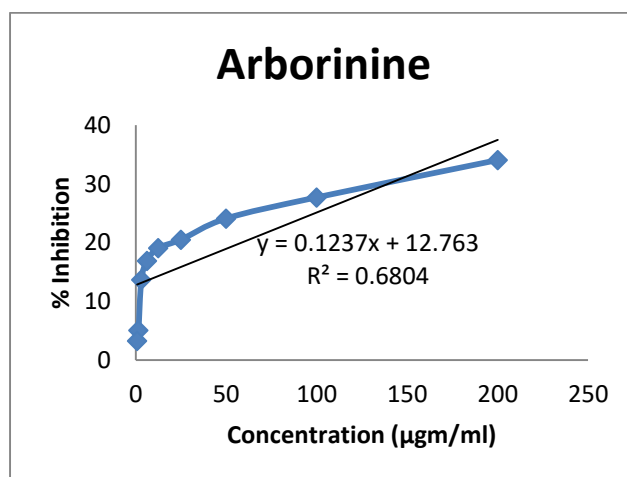
Here, Ex 1 = Experiment 1, Ex 2 = Experiment 2, Ex 3= Experiment 3, Absorbance of the Blank = 0.278

**Figure 4.12** Free radical scavenging activity of aqueous fraction of *R. spectabilis*

**Table 4.19 Free radical scavenging activity of arborinine**

Conc. (µgm/ml)	Absorbance			% Inhibition			IC <sub>50</sub> value			IC <sub>50</sub> (µg/ml) Mean± Stdev
	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	
200	0.183	0.185	0.182	34.17	33.45	34.53				
100	0.201	0.203	0.199	27.69	26.97	28.41				
50	0.211	0.213	0.209	24.10	23.38	24.82				
25	0.221	0.223	0.219	20.50	19.78	21.22				
12.5	0.225	0.227	0.223	19.06	18.34	19.78	300.40	306.21	299.18	301.92 ±3.75
6.25	0.231	0.233	0.229	16.90	16.18	17.62				
3.125	0.24	0.242	0.238	13.66	12.94	14.38				
1.5625	0.264	0.266	0.262	5.035	4.31	5.75				
0.78125	0.269	0.271	0.267	3.23	2.51	3.95				

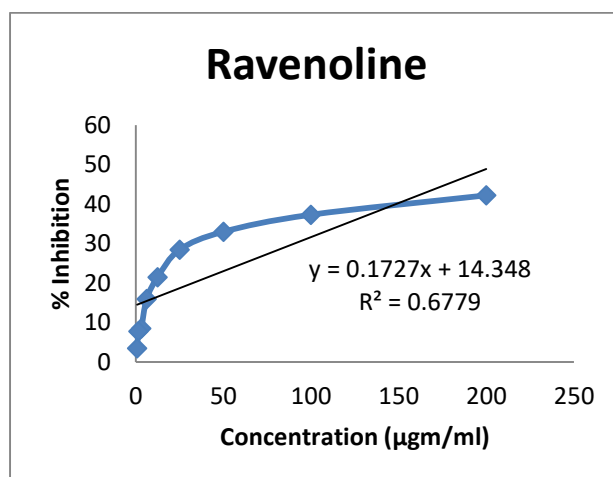
Here, Ex 1 = Experiment 1, Ex 2 = Experiment 2, Ex 3= Experiment 3, Absorbance of the Blank = 0.278

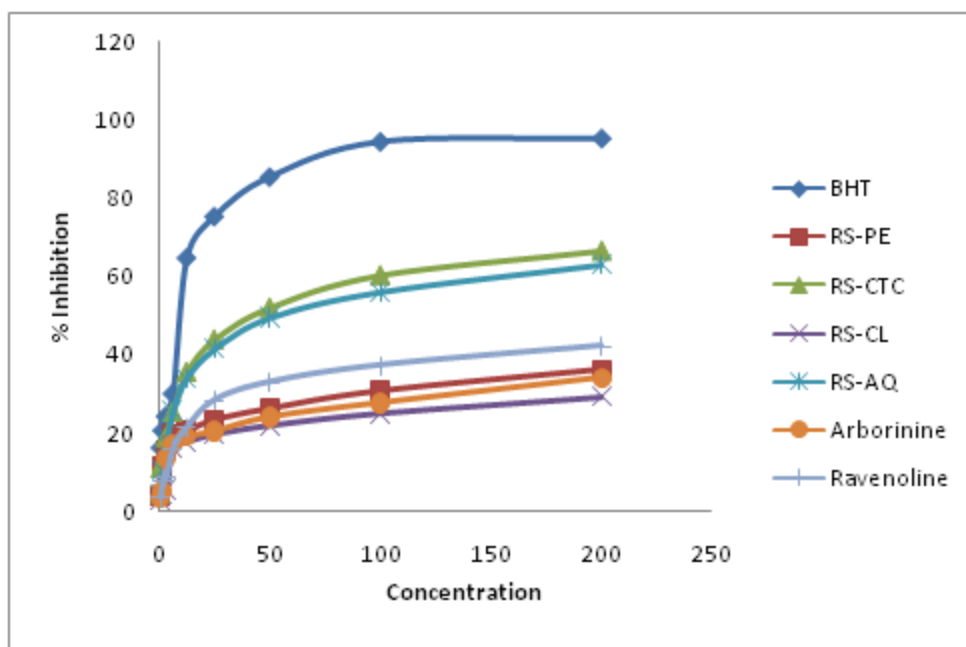
**Figure 4.13 Free radical scavenging activity of arborinine**

**Table 4.20 Free radical scavenging activity of ravenoline**

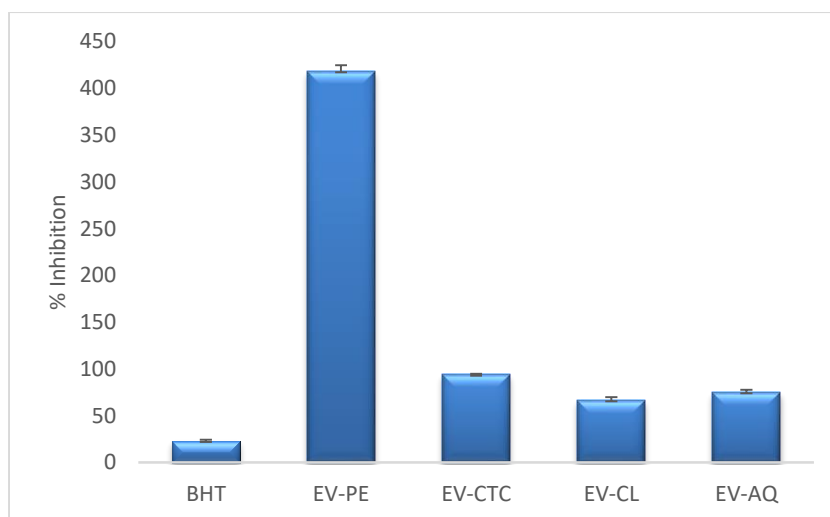
Conc. ( $\mu\text{gm}$ /ml)	Absorbance			% Inhibition			IC <sub>50</sub> value			IC <sub>50</sub> ( $\mu\text{g/ml}$ ) Mean $\pm$ Stdev
	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	
200	0.161	0.159	0.162	42.08	42.80	41.72				
100	0.176	0.173	0.174	36.69	37.76	37.41				
50	0.188	0.185	0.186	32.37	33.45	33.09				
25	0.195	0.193	0.209	29.85	30.57	24.82				
12.5	0.219	0.217	0.219	21.22	21.94	21.22	209.18	203.13	208.39	206.90 3 $\pm$ 3.28
6.25	0.235	0.232	0.234	15.46	16.54	15.82				
3.125	0.255	0.253	0.255	8.273	8.99	8.27				
1.5625	0.257	0.255	0.257	7.55	8.27	7.55				
0.78125	0.269	0.267	0.269	3.23	3.95	3.23				

Here, Ex 1 = Experiment 1, Ex 2 = Experiment 2, Ex 3= Experiment 3, Absorbance of the Blank = 0.278

**Figure 4.14 Free radical scavenging activity of ravenoline**



**Figure 4.15** Free radical scavenging activity of BHT, different fractions, arborinine and ravenoline of the leaf extract of *R. spectabilis*



**Figure 4.16** Free radical scavenging activity of BHT and different fractions of the bark extract of *E. variegata*

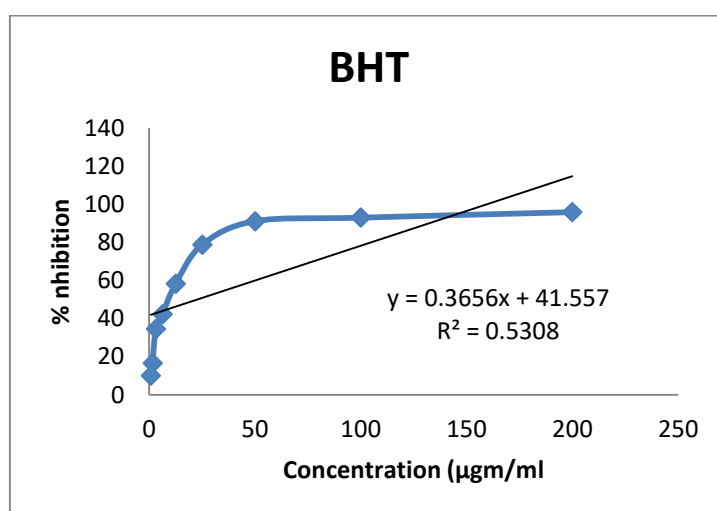
**Table 4.21** IC<sub>50</sub> values of the standard and partitionates *Erythrina variegata*

Sample code	Test sample	IC <sub>50</sub> value (µg/ml)
BHT	Butylated hydroxytoluene	23.09 ± 1.37
EV-PE	Pet-ether soluble fraction of the methanolic extract of the plant	418.21 ± 6.40
EV -CTC	Carbontetrachloride soluble fraction of the methanolic extract of the plant	93.85 ± 1.04
EV-CL	Chloroform soluble fraction of the methanolic extract of the plant	67.59 ± 1.87
EV -AQ	Aqueous soluble fraction of the methanolic extract of the plant	75.02 ± 2.62

**Table 4.22 Free radical scavenging activity of BHT**

Conc. (µgm/ml)	Absorbance			% Inhibition			IC <sub>50</sub> value			IC <sub>50</sub> (µg/ml) Mean± Stdev
	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	
200	0.010	0.013	0.011	96.25	95.13	95.88				
100	0.018	0.02	0.019	93.25	92.50	92.88				
50	0.023	0.025	0.024	91.38	90.63	91.01				
25	0.056	0.058	0.057	79.02	78.27	78.65				
12.5	0.110	0.114	0.111	58.80	57.30	58.42	21.73	24.49	23.04	23.09± 1.37
6.25	0.153	0.155	0.154	42.69	41.94	42.32				
3.125	0.173	0.176	0.175	35.20	34.08	34.45				
1.5625	0.221	0.224	0.223	17.22	16.10	16.47				
0.78125	0.239	0.242	0.24	10.48	9.36	10.11				

Here, Ex 1 = Experiment 1, Ex 2 = Experiment 2, Ex 3= Experiment 3, Absorbance of the Blank = 0.267



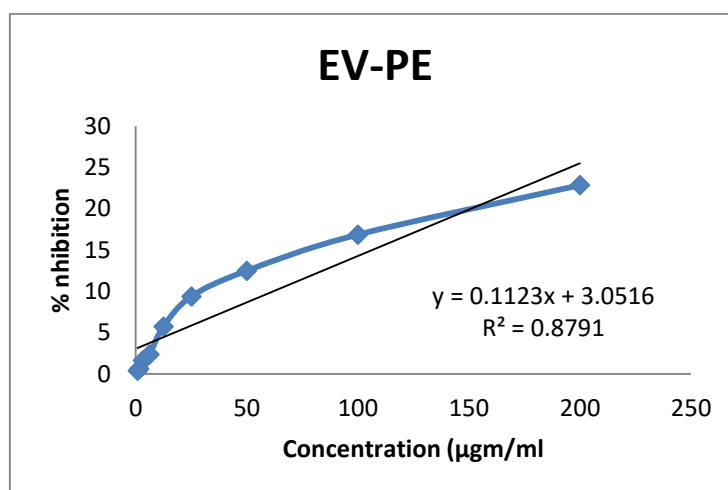
**Figure 4.17 Free radical scavenging activity of BHT (Butylated hydroxytoluene)**



**Table 4.23** Free radical scavenging activity of pet ether soluble fraction (EV-PE) of the bark extract of *E. variegata*

Conc. ( $\mu\text{gm}$ /ml)	Absorbance			% Inhibition			IC <sub>50</sub> value			IC50 ( $\mu\text{g/ml}$ ) Mean $\pm$ Stdev
	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	
200	0.207	0.205	0.206	22.47	23.22	22.84				
100	0.224	0.22	0.222	16.10	17.60	16.85				
50	0.236	0.231	0.234	11.61	13.48	12.35				
25	0.244	0.24	0.242	8.61	10.11	9.36				
12.5	0.251	0.252	0.252	5.99	5.61	5.61	425.19	412.59	416.84	418.21 $\pm$ 6.40
6.25	0.262	0.259	0.261	1.87	2.99	2.24				
3.125	0.264	0.261	0.263	1.12	2.24	1.49				
1.5625	0.266	0.264	0.266	0.37	1.12	0.37				
0.78125	0.267	0.265	0.266	0	0.749	0.37				

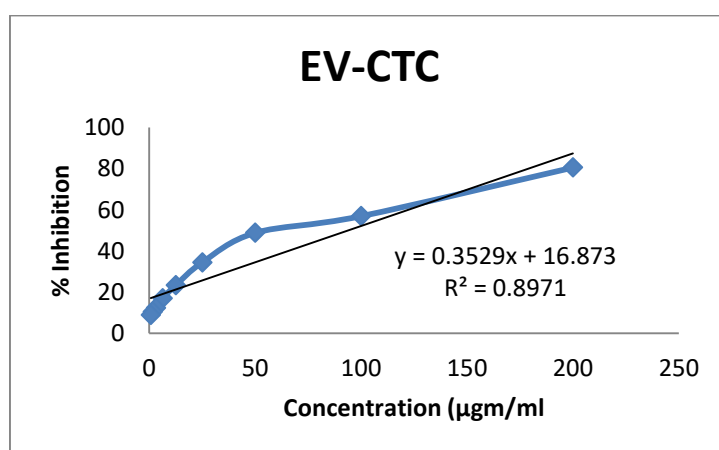
Here, Ex 1 = Experiment 1, Ex 2 = Experiment 2, Ex 3= Experiment 3, Absorbance of the Blank = 0.267

**Figure 4.18** Free radical scavenging activity of pet ether fraction of *E. variegata*

**Table 4.24** Free radical scavenging activity of carbontetrachloride fraction (EV-CTC) of the bark extract of *Erythrina variegata*

Conc. (µgm/ml)	Absorbance			% Inhibition			IC <sub>50</sub> value			IC50 (µg/ml) Mean± Stdev
	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	
200	0.052	0.054	0.05	80.52	79.77	81.27				
100	0.115	0.116	0.114	56.92	56.55	57.30				
50	0.138	0.135	0.137	48.31	49.43	48.68				
25	0.175	0.173	0.177	34.45	35.20	33.70				
12.5	0.205	0.206	0.203	23.22	22.84	23.97	93.93	94.85	92.77	93.85± 1.04
6.25	0.222	0.223	0.22	16.85	16.47	17.60				
3.125	0.234	0.235	0.233	12.35	11.98	12.73				
1.5625	0.238	0.242	0.237	10.86	9.36	11.23				
0.78125	0.242	0.245	0.243	9.36	8.25	8.988				

Here, Ex 1 = Experiment 1, Ex 2 = Experiment 2, Ex 3= Experiment 3, Absorbance of the Blank = 0.267

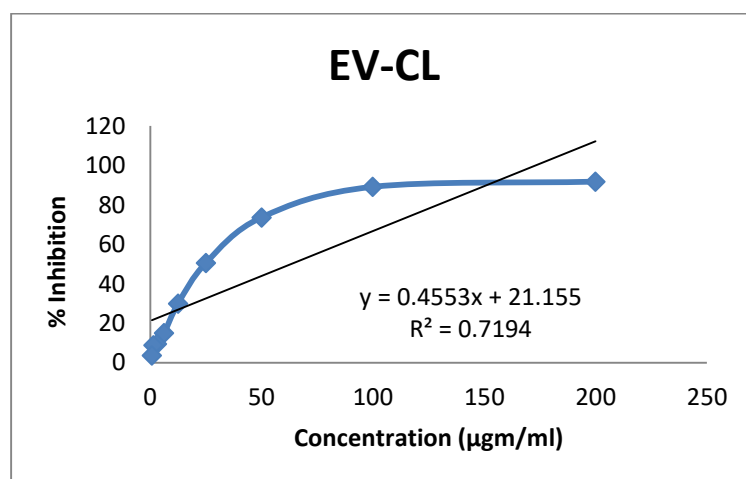


**Figure 4.19** Free radical scavenging activity of carbontertachloride fraction of *E. variegata*

**Table 4.25** Free radical scavenging activity of chloroform fraction (EV-CL) of the bark extract of *Erythrina variegata*

Conc. (µgm/ml)	Absorbance			% Inhibition			IC <sub>50</sub> value			IC <sub>50</sub> (µg/ml) Mean± Stdev
	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	
200	0.022	0.021	0.023	91.76	92.13	91.38				
100	0.031	0.026	0.03	88.38	90.26	88.76				
50	0.091	0.04	0.08	65.91	85.01	70.03				
25	0.14	0.113	0.143	47.56	57.67	46.44				
12.5	0.186	0.177	0.198	30.33	33.70	25.84	66.535	66.484	69.762	67.59± 1.87
6.25	0.229	0.224	0.228	14.23	16.10	14.60				
3.125	0.245	0.238	0.241	8.239	10.86	9.737				
1.5625	0.243	0.242	0.245	8.988	9.363	8.239				
0.78125	0.257	0.259	0.256	3.745	2.996	4.119				

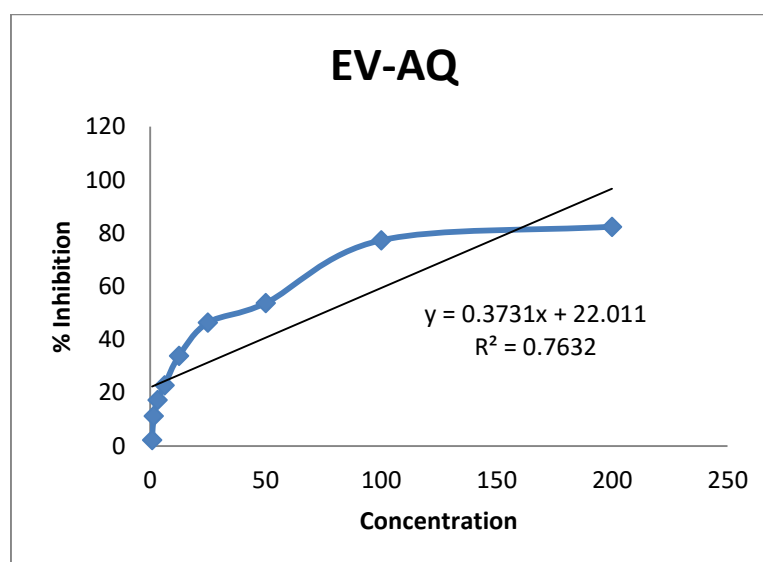
Here, Ex 1 = Experiment 1, Ex 2 = Experiment 2, Ex 3= Experiment 3, Absorbance of the Blank = 0.267

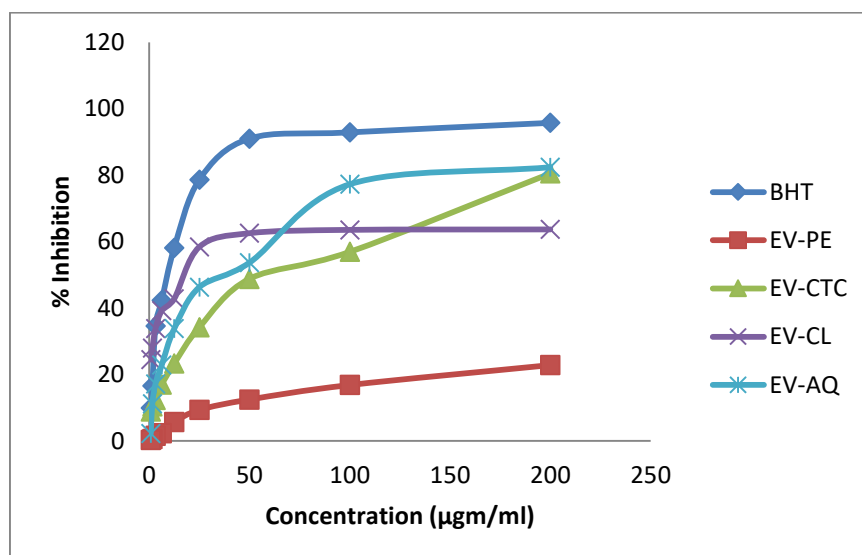
**Figure 4.20** Free radical scavenging activity of chloroform fraction of *E. variegata*

**Table 4.26 Free radical scavenging activity of aqueous fraction (EV-AQ) of the bark extract of *Erythrina variegata***

Conc. (µgm/ml)	Absorbance			% Inhibition			IC <sub>50</sub> value			IC <sub>50</sub> (µg/ml) Mean± Stdev
	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	Ex1	Ex2	Ex3	
200	0.046	0.052	0.043	82.77	80.52	83.89				
100	0.06	0.063	0.059	77.52	76.40	77.90				
50	0.122	0.128	0.121	54.30	52.05	54.68				
25	0.147	0.143	0.14	44.94	46.44	47.56	74.90	77.71	72.46	75.02± 2.62
12.5	0.177	0.178	0.175	33.70	33.33	34.45				
6.25	0.206	0.211	0.201	22.84	20.97	24.71				
3.125	0.222	0.22	0.221	16.85	17.60	17.22				
1.5625	0.237	0.237	0.237	11.23	11.23	11.23				

Here, Ex 1 = Experiment 1, Ex 2 = Experiment 2, Ex 3= Experiment 3, Absorbance of the Blank = 0.267

**Figure 4.21 Free radical scavenging activity of aqueous fraction of *E. variegata***



**Figure 4.22** Free radical scavenging activity of BHT and different fractions of the bark extract of *E. variegata*.

## Summary

Rutaceae and fabaceae are two important plant families which produce diverse chemical compounds possessing a wide range of biological activities. In Bangladesh many species of these two families are found growing wildly or cultivated throughout the country. In order to obtain new drug molecules with promising pharmacological activities, two plants from these two families, *Ravenia spectabilis* and *Erythrina variegata*, were chosen for the present study.

In this work, the air-dried and powdered leaf of *Ravenia spectabilis* (1kg) and stem bark of *Erythrina variegata* (900 gm) were extracted with methanol. The extracts were fractionated by VLC (vacuum liquid chromatography) and some of the selected fractions were subjected to column chromatography over lipophilic sephadex (LH-20). A total of twenty one compounds were isolated and purified by preparative thin layer chromatography (PTLC) and crystallization technique. The structure elucidation was carried out based on  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, HSQC, HMBC,  $^1\text{H}$ - $^1\text{H}$  COSY and NOSEY spectral data and the molecular weights were determined by ESI mass spectrometry.

From the methanolic extract of the leaf of *Ravenia spectabilis*, fourteen compounds were isolated which are 3,5-diprenylindole (**1**), 3-prenyl-5-(2-keto-but-3-enyl)indole (**2**), 3-prenyl-indole-5-carbaldehyde (**3**), iso-oligophyline (**4**), ravenoline (**5**),  $\gamma$ -fagarine (**6**), arborinine (**7**), atanine (**8**), oligophyline (**9**), ravenine (**10**), methyl linoleate (**11**),  $\beta$ -sitosterol (**12**), ravespanol (**13**) and ravespanone (**14**). The methanolic extract of the stem bark of *Erythrina variegata* provided seven known compounds namely scandenone (**15**), alpinumisoflavone (**16**), lupeol (**17**), stigmast-4-en-3-one (**18**), stigmasta-4,22-dien-3-one (**19**), stigmasterol (**20**) and 3 $\beta$ ,28-dihydroxyolean-12-ene (**21**). Among the isolated compounds, compounds **1-4** are new alkaloids, whereas compounds **13** and **14** are unusual novel C<sub>34</sub> terpenoids, all of which were isolated from *Ravenia spectabilis*.

Three new indole alkaloids (**1-3**) were investigated for cytotoxicity assay by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay method. A panel of three immortalized human tumour cell lines i.e., HeLa (human cervical cancer), MIA-PaCa-2 (human pancreatic adenocarcinoma) and A549 (lung cancer) cell lines and a non-tumour cell line WI-38 were employed for cytotoxicity

screening using gemcitabine as the standard. Compound **1** (3,5-diprenylindole) was found to be most cytotoxic to human pancreatic adenocarcinoma cell lines with IC<sub>50</sub> value of  $9.5 \pm 2.2 \mu\text{M}$ , moderately cytotoxic to human cervical and lung cancer cell lines with IC<sub>50</sub> values of  $11.3 \pm 1.3 \mu\text{M}$  and  $13.5 \pm 1.66 \mu\text{M}$  respectively and weakly cytotoxic to non-tumour cell line (WI-38) with IC<sub>50</sub> value of  $68.5 \pm 3.5 \mu\text{M}$  as compared to the standard ( $0.19 \pm 0.12 \mu\text{M}$  to  $6.3 \pm 0.3 \mu\text{M}$ ). Compounds 2 and 3 showed very poor cytotoxicity (IC<sub>50</sub> >50  $\mu\text{M}$ ) against the four cell lines tested.

By using the protocol designed by Kupchan and modified by VanWagenen et al. the crude methanolic extracts of *Ravenia spectabilis* and *Erythrina variegata* were subjected to solvent-solvent partitioning. Different organic soluble material of the investigated plants and some pure compounds were screened for their antimicrobial activity against three gram-positive and seven gram-negative bacteria by the standardized disc diffusion method. Kanamycin was used as reference drug for the test. All the samples showed mild to moderate antimicrobial activity against different bacterial strains, where the pet ether fraction of *Ravenia spectabilis* and the carbontetrachloride fraction of *Erythrina variegata* demonstrated the highest antimicrobial activity against *Bacillus subtilis* and *Bacillus cereus* respectively with zone of inhibition of  $20.5 \pm 0.74 \text{ mm}$  and  $19.5 \pm 1.18 \text{ mm}$  as compared to the standard ( $34.0 \pm 0.5 \text{ mm}$  and  $24.3 \pm 0.44 \text{ mm}$ ). Ravenoline isolated from *R. spectabilis* promising inhibition against *Vibrio cholerae* ( $17.2 \pm 0.41 \text{ mm}$ ).

*In vitro* thrombolytic activity of different extracts of the investigated plants were carried out according to the method of Prasad et al. (2006) using streptokinase (100 $\mu\text{l}$ ) as the standard. Mild to moderate thrombolytic activities were observed by arborinine and different fractions of the crude extract with clot lysis ranging from  $30.43 \pm 1.03$  to  $57.78 \pm 0.24 \%$  as compared to the standard streptokinase with clot lysis of  $74.34 \pm 0.73 \%$  for *Ravenia* extract and  $76.54 \pm 0.9 \%$  for *Erythrina* extract.

*In vitro* antioxidant activity was evaluated by DPPH radical scavenging method using butylated hydroxytoluene as the standard. Among the crude extracts tested, the chloroform and aqueous extract of *E. variegata* exhibited moderate antioxidant activities with IC<sub>50</sub> values of  $67.59 \pm 1.87$  and  $75.02 \pm 2.62 \mu\text{g/ml}$  respectively as compared to the standard  $23.09 \pm 1.57 \mu\text{g/ml}$ . The pure compounds arborinine and ravenoline showed very poor antioxidant activity

## Publications/ Communications

1. Fatema Tabassum, Choudhury Mahmood Hasan, Mohammad Mehedi Masud, Sheikh Nazrul Islam and Monira Ahsan. Phytochemical and Biological Investigations of *Ravenia spectabilis*; Asian Journal of Chemistry, Vol. 31, No. 1 (2019), 139-142.
2. Fatema Tabassum, Choudhury Mahmood Hasan, Mohammad Mehedi Masud, Md. Imran Nur Manik and Monira Ahsan. Isolation and Characterization of Secondary Metabolites and Evaluation of Antimicrobial, Antioxidant and Thrombolytic Potentials of *Erythrina variegata* L. Bark; Asian Journal of Chemistry, 31(8):1842-1846.
3. Fatema Tabassum, Choudhury Mahmood Hasan, Mohammad Mehedi Masud and Monira Ahsan. A poster titled '3,5-Diprenyl indole from *Ravenia spectabilis*' was presented in 18<sup>th</sup> International Congress of International Society for Ethnopharmacology & the 5<sup>th</sup> International Congress of the Society for Ethnopharmacology, India (ISE-SFEC 2018) at University of Dhaka, Bangladesh, 13-15 January 2018 (abstract published) and achieved the 'Best Poster Presentation Award'.



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