



**Chemical and pharmacological profiling of *Jatropha*
pandurifolia and *Syzygium reticulatum***

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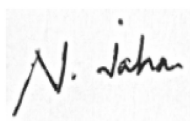
"Indeed your Lord is Allah Who created the heavens and the earth in six Days,¹ then established Himself on the Throne. He makes the day and night overlap in rapid succession. He created the sun, the moon, and the stars—all subjected by His command. The creation and the command belong to Him 'alone'. Blessed is Allah—Lord of all worlds!"

Surah Al-A'raf - 7:54

**Dedicated to My Respected Parents and
Beloved Family**

Declaration

I hereby declare that the thesis entitled “**Chemical and pharmacological profiling of *Jatropha pandurifolia* and *Syzygium reticulatum***” has been prepared for submission to the University of Dhaka, Dhaka, Bangladesh, for the degree of Doctor of Philosophy in Pharmaceutical Chemistry. I confirm that I am the sole author of this thesis. To the best of my knowledge, this thesis contains no material previously published by any other person, except where due acknowledgement has been made. Furthermore, this thesis contains no material which has been accepted as part of the requirements for any other academic degree or non-degree program, in English or any other language.



Signature of the candidate

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Certificate

This is to certify that the research work presented in this thesis, entitled “**Chemical and pharmacological profiling of *Jatropha pandurifolia* and *Syzygium reticulatum***” is the original research work carried out by Nisrat Jahan (Re-registration No. 36, Session 2023-24) under our supervision. The thesis contains no material previously published or written by another person, except where proper reference is made in the text.



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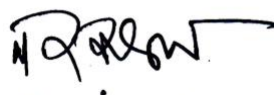
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List of Abbreviations

IC₅₀	50% inhibitory concentration	MHz	Megahertz
LC₅₀	50% lethal concentration	µg/mL	Microgram per milliliter
LD₅₀	50% lethal dose	µm	Micrometre
DPPH	2,2-diphenyl-1-picrylhydrazyl	mg	Milligram
BHT	Butylated hydroxytoluene	R_f	Retardation factor
STK	Streptokinase	UV	Ultraviolet
et al	And others	GABA	Gamma-aminobutyric acid
s	Singlet	OF	Open field
br s	Broad singlet	HC	Hole cross
dd	Doublet of doublet	DMSO	Dimethylsulfoxide
t	Triplet	CDCl₃	Deuterated chloroform
m	Multiplet	Jp-ML	Methanolic leaf extract of <i>J. pandurifolia</i>
TMS	Tetramethylsilane	Jp-EAL	Ethyl acetate leaf extract of <i>J. pandurifolia</i>
NMR	Nuclear magnetic resonance	Jp-CL	Chloroform leaf extract of <i>J. pandurifolia</i>
2D	Two-dimensional	Jp-HL	n-Hexane leaf extract of <i>J. pandurifolia</i>
COSY	Correlation spectroscopy	Sr-ML	Methanolic leaf extract of <i>S. reticulatum</i>
J	Coupling constant	Sr-EAL	Ethyl acetate leaf extract of <i>S. reticulatum</i>
Hz	Hertz	Sr-CL	Chloroform leaf extract of <i>S. reticulatum</i>
HMBC	Heteronuclear multiple bond correlation	Sr-HL	n-Hexane leaf extract of <i>S. reticulatum</i>
MS	Mass spectrometry	Sr-ML	Methanolic leaf extract of <i>S. reticulatum</i>
m/z	Mass-to-charge ratio		

Publications

1. **Nisrat Jahan**, khozirah Shaari, Sk Nazrul Islam, A.T.M. Zafrul Azam, Fatema-Tuz-Zohora* and Monira Ahsan, **Chemical and biological profiling of three ferulic acids alkyl esters isolated from *Jatropha pandurifolia* (family: Euphorbiaceae) stem bark**, *Biomedical and pharmacology journal*. 2023, 16(2): 817-826.
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Conference

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Abstract

Jatropha pandurifolia Andrews (family: Euphorbiaceae) and *Syzygium reticulatum* (Wight) Walp. (family: Myrtaceae) were studied for their secondary metabolites and their biological activities were evaluated both *in vitro* and *in vivo*. Through extensive NMR experiments—including ^1H NMR, ^{13}C NMR, and ^1H - ^1H COSY, HSQC and mass spectrometry, a total of fifteen compounds were identified from the plant extracts. Thirteen of these chemical compounds were isolated specifically from the stem bark of *J. pandurifolia* including 2-epi-jatrogrossidione, integerrimene, jatrophatrione, citlaltitrone, dotriacontyl *trans*-ferulate, triacontyl *trans*-ferulate, octacosyl *trans*-ferulate, hexacosyl *trans*-ferulate, octacosyl *cis*-ferulate, β -sitosterol, stigmasterol, n-dotriacontanol, and 1,2-dioleoyl-3-palmitoyl-glycerol. Dotriacontyl *trans*-ferulate was isolated and characterized as a previously unreported molecule. Bis(2-ethylhexyl) phthalate and stigmast-4-en-3-one were isolated from the leaves of *S. reticulatum*, marking the first report of these compounds from this plant species. *In vivo* investigation was carried out to assess CNS depressant, antidiarrheal, and analgesic activities, while *in vitro* study was assessed for antioxidant, cytotoxic, and thrombolytic properties. Furthermore, *in silico* investigation of selected pure compounds were conducted using molecular docking and ADMET (absorption, distribution, metabolism, excretion, and toxicity) profiling. CNS depressant activity was carried out by open field and hole cross methods at 100 mg/kg, 200 mg/kg, and 300 mg/kg, where diazepam was used as standard. In open field and hole cross method, highest inhibition of movements were 87.37% (**P<0.001) and 84.78% (*P<0.05), respectively for methanolic extract of *J. pandurifolia* leaf at 300 mg/kg. Furthermore, at the same dose highest inhibition of locomotor activity were 73.68% (**P<0.001) and 80.43% (**P<0.01) for ethyl acetate extract of *S. reticulatum* leaf in aforementioned methods. Diazepam exhibited 91.58% and 95.65% movement inhibition, respectively. The antidiarrheal effect was performed using the castor oil induced diarrhea model at 200, 400, and 600 mg/kg, using loperamide as the standard. The frequency of diarrhea was reduced by 85.95% at 600 mg/kg of Jp-ML, where loperamide showed 98.57% gastric inhibition. At the same dose, Sr-EAL exhibited a delay in diarrhea onset of 87.53% where loperamide showed 94.84% gastric inhibition. The analgesic activity was assessed by acetic acid induced writhing

method and formalin induced nociception test. Jp-ML showed the highest dose-dependent inhibition of writhing by 66.67% (** $P < 0.01$) at 300 mg/kg, whereas indomethacin showed 78.43% pain reduction. In the formalin test, pain inhibition were 63.92% (*** $P < 0.001$) in the early phase and 70.35% (*** $P < 0.001$) in the late phase at the same dose. On the other hand, Sr-EAL showed the highest inhibition of writhing by 70.59% (*** $P < 0.001$) at 300 mg/kg. Inhibition of paw licking were recorded by 54.55% (*** $P < 0.001$) and 70.52% (*** $P < 0.001$) in the early phase and late phase, respectively. Antioxidant activity was estimated using the DPPH free radical scavenging assay, with BHT as the standard. The lowest IC_{50} value were 8.11 $\mu\text{g/mL}$ and 10.34 $\mu\text{g/mL}$ for JP-ML and Sr-EAL, respectively. Pure compounds (JP-10, JP-13, JP-25, and JP-460) exhibited promising antioxidant activity, with highest antioxidant activity of JP-25 ($IC_{50} = 13.10 \mu\text{g/mL}$). Cytotoxicity was assessed using the brine shrimp lethality assay, with vincristine sulphate as the standard. The LC_{50} values were 1.60 $\mu\text{g/mL}$ for Jp-EAL and 1.70 $\mu\text{g/mL}$ for Sr-ML. Among the four pure compounds tested, JP-460 exhibited the strongest cytotoxicity, with LC_{50} value of 1.27 $\mu\text{g/mL}$. Cytotoxicity test was also carried out on the HeLa cell line both qualitatively and quantitatively. In qualitative test JP-13 and JP-460 showed a cell survival rate of over 95%, whereas JP-200 resulted in a cell survival rate of only 5%, indicating strong cytotoxicity. In quantitative MTT assay, JP-200 exhibited LD_{50} value of 272.45 $\mu\text{g/mL}$. Thrombolytic activity was evaluated using streptokinase as standard. The highest clot lysis activity were 63.32% and 67.58% for the Jp-ML and Sr-EAL, respectively. JP-460 demonstrated significant clot lysis activity, with 71.04% clot rupture. Finally, molecular docking studies revealed that compound JP-460 exhibited strong binding affinity (-9.4 kcal/mol) and binding efficacy (0.41 (kcal/mol per non-hydrogen atom) to the human GABA-A receptor. The ADMET assessment confirmed that the isolated compounds have preferable pharmacokinetic and safety profiles, supporting their potential for therapeutic application.

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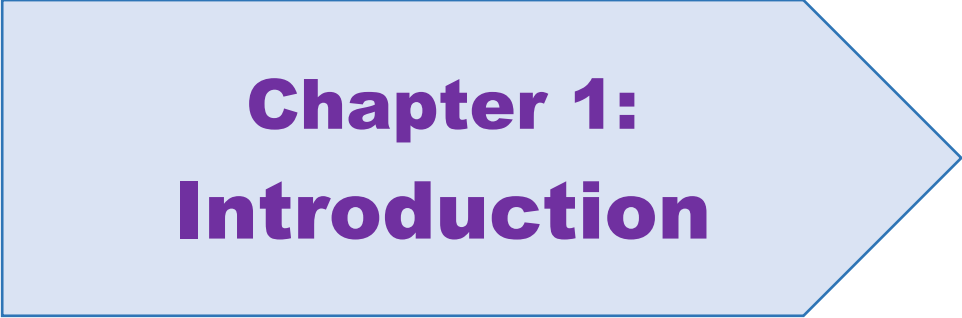
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Chapter 1: Introduction

1.1 Background of the study

Medicinal plants are crucial not only in promoting health and well-being, but also in advancing medical science. Over the past decade, it has been shown that both industrialized and developing countries exhibit a strong preference and willingness to embrace natural remedies (Ekor, 2014). Our Earth is home to approximately 420,000 plant species, of which around 35,000 are used by indigenous communities for various purposes. Approximately 20% of these plants have been evaluated through pharmaceutical or biological testing, although only about 10% progress beyond the initial biological screening stage. Medicinal plants have been used for healing since ancient times, establishing a tradition that has persisted for thousands of years across various cultures around the world (Šantić et al., 2017).

The WHO emphasizes that about 70% to 95% of the population in developing countries relies on traditional herbal medicines as their primary form of treatment for various ailments (Mohamed et al., 2012). With the advent of advanced tools and instrumentation in recent decades, more detailed investigations into phytochemicals—such as carbohydrates, amino acids, lipids, and other secondary metabolites—have become possible, leading to significant advancements in the health sector. These phytochemicals offer remarkable potential for the discovery of new, novel, and therapeutically beneficial compounds that could contribute to a modern therapeutic revolution. However, the use of plant materials is not without risk; toxic effects may occur if plants are misidentified, improperly purified, or if they interact adversely with standard medications. Furthermore, the safety and efficacy of plant-based treatments can be influenced by environmental conditions, storage methods, preparation techniques, and the complex interactions between multiple bioactive constituents (Zhang, 2011). To improve therapeutic effectiveness and minimize side effects, scientists frequently develop synthetic analogues of natural compounds and test them in clinical trials. Nevertheless, synthetic drugs can still pose serious health risks, including tissue, and organ damage. As public interest in traditional medicine continues to rise, this growing demand has motivated researchers to explore and identify new plant-derived compounds with promising therapeutic potential (Jamshidi-Kia et al., 2017).

Researchers are increasingly investigating medicinal plants to isolate bioactive compounds, understand their biological activities, and scientifically validate

traditional uses through detailed pharmacological and toxicological evaluations. Due to the natural origin and chemical diversity, medicinal plants are recognized as important assets in modern pharmaceutical and biotech innovation. Ongoing studies are uncovering auspicious leads for disease management. At long last, research on medicinal plant serves as a captious bridge between traditional healing practices and contemporary biomedical science.

1.2 Rationality and objectives of the study

Chemical investigations focus on the identification and characterization of active compounds within plant extracts for understanding the mechanisms of action of the compounds, and their suitability for drug development. In parallel, biological investigations examine the pharmacological effects of these compounds to assess their efficacy in addressing specific health conditions, and their overall safety. Molecular docking and ADMET analysis aid rational drug design and optimization the safety of lead molecules. By integrating both chemical and biological approaches along with computational investigation, researchers can gain a holistic understanding of the composition and therapeutic value of natural substances, ultimately identifying promising candidates for pharmaceutical research and clinical trials. Accordingly, the objectives of this research are to isolate pure compounds, and evaluate the selective biological activities of two medicinal plants: *Jatropha pandurifolia* and *Syzygium reticulatum*. The following investigations have been conducted:

1. Isolation of secondary metabolites from the crude extracts of the chosen plants.
2. Characterization of the isolated compounds using ^1H NMR, ^{13}C NMR, 2D NMR, mass spectrometry etc.
3. Examination of the biological activity of various solvent extracts and pure compounds through the following assay techniques.
 - CNS depressant activity (Gupta et al., 1971; Takagi et al., 1971)
 - Antidiarrheal Activity (Degu et al., 2020; Sairam et al., 2003)
 - Analgesic Activity (Tjølsen et al., 1992; Couto et al., 2011)
 - Antioxidant activity (Brand Williams et al., 1995)
 - Cytotoxicity in brine shrimp (Waghulde et al., 2019)

- Cytotoxicity in HeLa cell line (Khatibi et al., 2017; Ala et al., 2018)
- Thrombolytic Activity (Prasad et al., 2007)

4. Molecular docking (Trott & Olson, 2010) and ADMET (absorption, distribution, metabolism and excretion) profile of pure compounds. (Fu et al., 2024).

1.3 Plant review

1.3.1 The plant family-Euphorbiaceae

The family, also called the “spurge family,” comprises a wide range of succulent plants, from lawn weeds to cactus-like species, and includes 283 genera and approximately 7,500 species. Flowers are typically unisexual and clustered in structures known as cyathia. The milky latex has strong skin-irritant activity, and chronic exposure can result in carcinogenic effects. Despite its toxicity, it can also be a source of oil or wax. Plants in this family are known for their anti-inflammatory, antimicrobial, and antiviral properties, which have applications in cosmetics and pharmaceuticals. The toxic components of Euphorbiaceae species are specific diterpenes, commonly known as phorboids, including tiglane, ingenane, and daphnane derivatives (Hohmann et al., 2000; Morris Kupchan et al., 1976).

Ornamental Plants

- ✓ *Codiaeum*
- ✓ Copperleaf (*Acalypha*),
- ✓ Sandbox Tree (*Hura*)
- ✓ *Phyllanthus*

Economically important plants

- ✓ Castor-Oil Plant (*Ricinus Communis*)
- ✓ Omphalea, Cassava (*Manihot Esculenta*)
- ✓ Rubber (*Hevea*)
- ✓ Croton (*Croton Tiglium*)

1.3.1.1 The plant genus- *Jatropha*

The genus name *Jatropha* is derived from the Greek words *jatrós* (doctor) and *trophé* (food), implying its traditional medicinal use. *Jatropha* (Family: Euphorbiaceae), an ornamental plant genus, comprises approximately 170 species of woody trees, shrubs, subshrubs, and herbs, found in seasonally dry tropical regions. Traditionally, it has been used in Africa, Asia, and Latin America to treat various ailments, including swelling, inflammation, stomachache, toothache, leprosy, vertigo, dysentery, dyscrasia, anemia, diabetes, skin diseases, bronchitis, and asthma. Several bioactive compounds have been reported from this genus, such as alkaloids, polypeptides,

flavonoids, diterpenes (e.g. jatrophine, jatropham, jatrophone), curcin, and fatty acids (Malhotra et al., 2020). These constituents are associated with a range of pharmacological activities, including antimicrobial, antiprotozoal, anti-inflammatory, antioxidant, protoscolicidal, insecticidal, and molluscicidal effects (Sabandar et al., 2013).

1.3.1.2 The plant- *Jatropha pandurifolia* Andrew

In the study, *Jatropha pandurifolia* Andrews belonging to Euphorbiaceae family was investigated.

- ❖ **Synonym:** *J. integerrima* Jacq, *J. hastate* Jacq, and *J. coccinea*.
- ❖ **Common name:** Peregrina, fire-Cracker, spicy jatropha.
- ❖ **Bangla name:** Jatropha ful.
- ❖ **Plant part:** Leaf and stem bark.

1.3.1.3 Taxonomical hierarchy of *J. pandurifolia*

Rank	Scientific Name with Common Name
Kingdom:	Plantae - Plants
Sub kingdom:	Tracheobionta - Vascular plants
Super division:	Spermatophyta - Seed plants
Division:	Magnoliophyta - Flowering plants
Class:	Magnoliopsida - Dicotyledons
Subclass:	Rosidae
Order:	Euphorbiales
Family:	Euphorbiaceae - Spurge family
Genus:	<i>Jatropha</i> - Nettlepurge
Species:	<i>Jatropha pandurifolia</i> - Peregrina

1.3.1.4 Morphology of *J. pandurifolia*

J. pandurifolia is an upright, ornamental, evergreen shrub that grows between 3 to 10 feet tall, reaching almost tree-like proportions as it ages in frost-free climates. The plant can be staked and trained to grow with a single trunk for two to three feet, though it does not provide shade (Gilman & Watson, 1993).

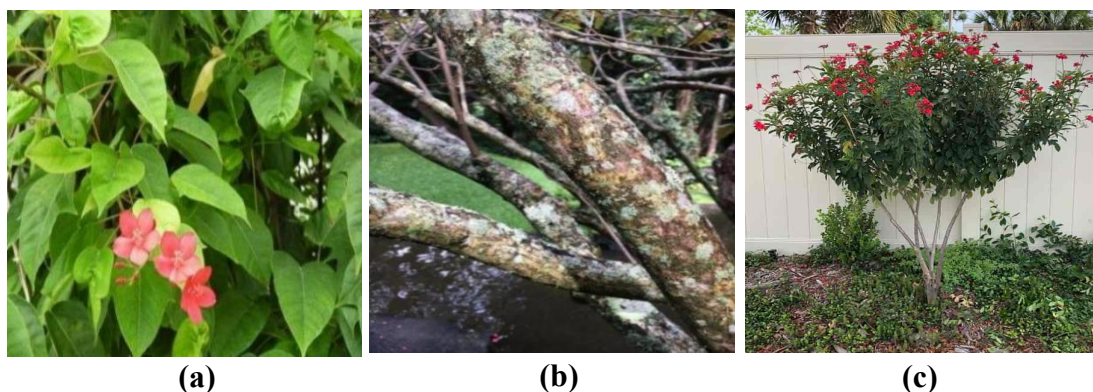


Figure 1.1 : Leaves (a), bark (b) and full plant (c) of *J. pandurifolia*

Leaves: Green and velvety, flecked with purple beneath, reaching up to 7 inches in length with 3, 5, or 7 lobes, or divided into up to 11 segments (Abdul Rahman et al., 2014)

Flower: 1 inch wide with five deep red petals and yellow stamens, arranged in branched clusters on 4 inch-long stalks at the tips of branches (Ghani, 2003).

Fruit: Rounded trigonous and about 1 × 1 cm with splitting opening.

Seed: Ovoid-ellipsoid, 8-9 × 5 mm, buff colored, mottled blackish brown (Krishnan & Paramathma, 2009)

Latex/ Sap: May cause skin irritation and rash while contamination (Kolawole et al., 2016).

Stem: Distinct periderm with wide cortex and xylem with void cylinder xylem, phloem and pith (Dehgan, 1982).

1.3.1.5 Distribution of *J. pandurifolia*

This plant is cultivated worldwide but is native to the Caribbean Islands, including Cuba, the Dominican Republic, Haiti, Java, Celebes, the Philippines, Indo-Malayan region, tropical America, tropical Africa, and some subtropical regions. *J. pandurifolia* is traditionally used for its health benefits in Asia, Latin America, and Africa (Quinlan et al., 2002).

1.3.1.6 Review of traditional use of *J. pandurifolia*

J. pandurifolia has traditionally been used to treat wounds, skin infections, and inflammation. Its leaves and latex are applied topically in folk medicine for their healing potential. A review of its traditional uses is provided below:

Table 1.1: Reported traditional uses of *J. pandurifolia*

Part	Traditional Use	Reference
Leaf	Warts, tooth ache, ring worm, gastric emptying, styptic, emetic agent	Pertino et al., 2007
	For veterinary purposes: Skin diseases, piles, sores	Nayak et al., 2010
Leaf decoction	Antitussive, antiseptic	Thomas et al., 2008
Oil	Purgative, rheumatic pain, skin disease	Nayak et al., 2010
Root	Antidote, gum bleeding	Van den Berg et al., 1995

1.3.1.7 Review of phytochemicals in *J. pandurifolia*

Previous studies confirmed different phytoconstituents, such as alkaloids, glycosides, diterpenes, lignins, tannins, saponins, steroids, peptides, fatty acids, carotenoids, flavonoids, phenolic compounds, coumarin, curcin, etc. from this species (Dietrich et al., 2004). A detailed phytochemical review is provided below:

Table 1.2: Reported phytochemicals present in *J. pandurifolia*

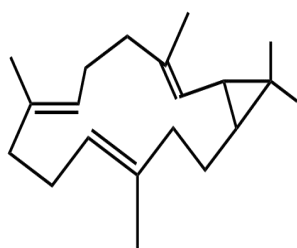
Parts	Phytochemicals	Reference
Whole plant	Jatrophone, Jatrophatrione, Jatropholone A–B, Rioloatrione, Lathyrane, Cleistanthane, Jatrophadioxan, Casbene	Sutthivaiyakit et al., 2003 Sackett et al., 1996 Kinghorn & Evans, 1975
Latex	Jatrophine, Jatropham, Curcin, Integerrimides A, Integerrimides B	Mongkolvisut et al., 2006 Devappa et al., 2010
Leaves, root, seed, oil	Stigmasterol, beta sitosterol, Anthocyanin, Integerrimene, Citlaltirione, Caniojane, 1,11- bisepicaniojane, 2-epicaniojane, 2 α -hydroxyjatropholone, 2 β -hydroxyjatropholone	Bhagat & Kulkarni, 2010 Das et al., 2008
Seed	Curcin, Palmitic acid, Oleic acid, Linoleic acid	Luo et al., 2006

1.3.1.8 Review of pharmacological activities of *J. pandurifolia*

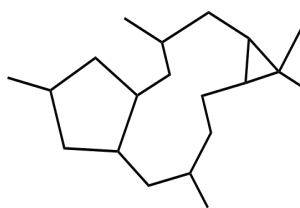
The aforementioned phytochemicals exhibit various promising biological activities, including antimicrobial, antiinflammatory, antioxidant, thrombolytic, and cytotoxic properties, highlighting the therapeutic potential of *J. pandurifolia* in pharmaceutical applications. A detailed pharmacological review is given below:

Table 1.3: Reported pharmacological activities of *J. pandurifolia*

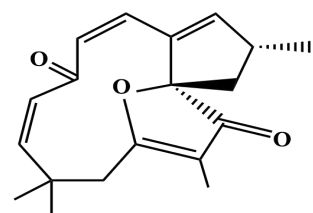
Plant Part	Pharmacological activity	Reference
Antimicrobial effect		
Leaf, stem, root, latex, and oil	<i>Erwinia carotovora</i>	Rajesh et al., 2012
	<i>Pseudomonas aeruginosa</i>	Rampadarath et al., 2014
	<i>Xanthomonas citri</i>	
	<i>Xanthomoas campestris</i>	
Bark	<i>Staphylococcus aureus</i> ,	Akhter et al., 2008
	<i>S. typhi</i> , <i>S. paratyphi</i> ,	Gaikwad et al., 2012
	<i>S. dysenteriae</i> ,	
	<i>Bacillus subtilis</i> , <i>B. cereus</i> , <i>B. megaterium</i> ,	
	<i>Escherichia coli</i> ,	
	<i>Pseudomonas aeruginosa</i> ,	
	<i>Vibrio mimicus</i> ,	
	<i>Sarcina lutea</i> , <i>Shigella boydii</i> .	
Antifungal effect		
Leaf, stem, root, and bark	<i>Alternaria alternata</i>	Rajesh et al., 2012
	<i>Aspergillus flavus</i>	Akhter et al., 2008
	<i>Aspergillus niger</i>	
	<i>Fusarium oxysporum</i>	
	<i>Rhizoctonia solani</i>	
	<i>Trichoderma viride</i>	
	<i>S. cerevaceae</i>	
	<i>Candida albicans</i>	
Miscellaneous		
Root, and bark	Antiinflammatory effect	Sutthivaiyakit et al., 2009
	Antituberculosis effect	
	Antiplasmodial activity	
	Cytotoxicity activity	
Leaf, and latex	Anticancer activity	Sharma & Singh, 2013
	Cardiovascular action	Mursu et al., 2014
	Cholinesterase activity	
Flower	Antioxidant activity	Kuspradini et al., 2016



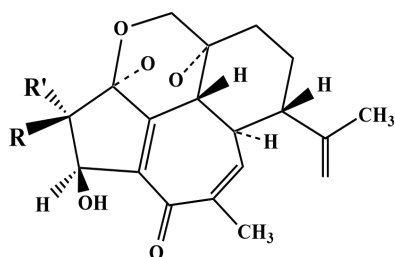
Casbene



Lathyrane



Jatrophone



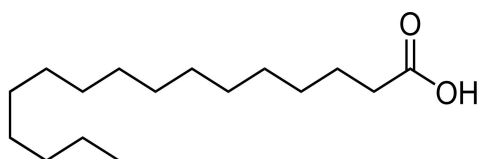
2-epicaniojane:

R = CH₃, R' = H

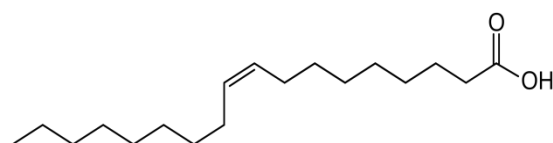
Caniojane :

R = H, R' = CH₃

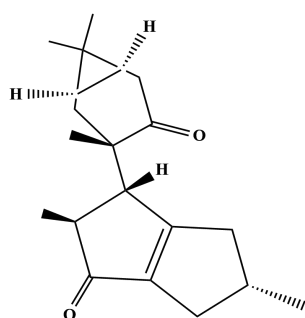
1,11- bisepicaniojane:

R = H, R' = CH₃(with 1,11 bis *epi*)

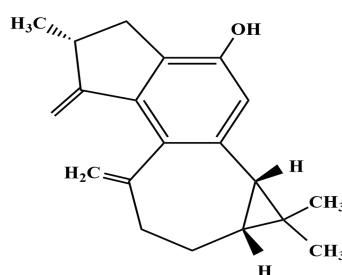
Palmitic acid



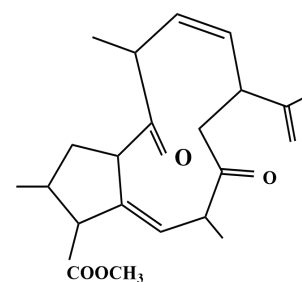
Oleic acid



Riolozatrione



Jatropholone A



Integerrimene

Figure 1.2 : Reported phytochemicals isolated from *J. pandurifolia*

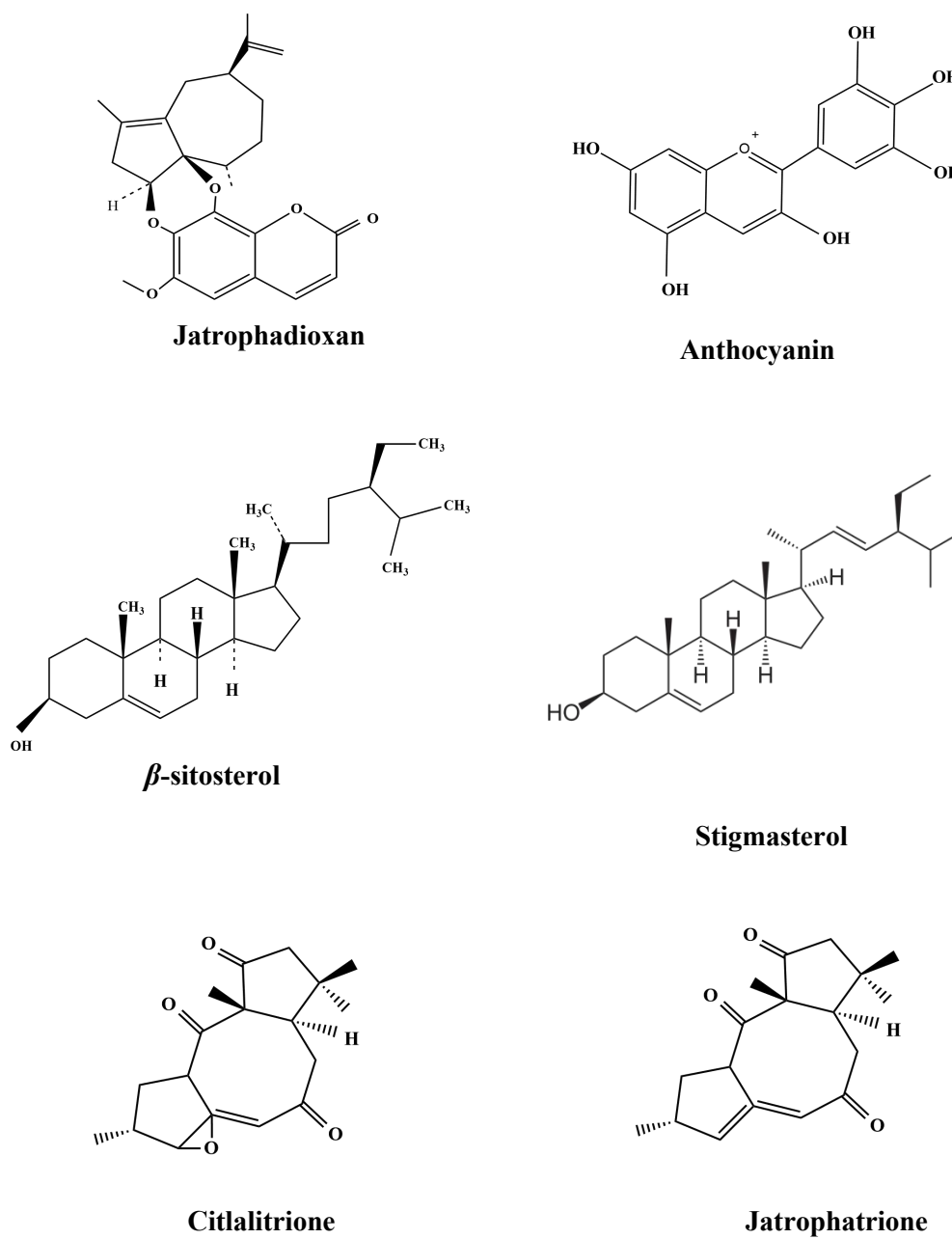


Figure 1.2 (cont.): Reported phytochemicals isolated from *J. pandurifolia*

1.3.2 The plant family-Myrtaceae

The Myrtaceae family includes about 140 genera and 3,400 species, primarily found in subtropical and tropical regions worldwide. It is especially diverse in Australia, which is home to some of the tallest flowering plants, such as Eucalyptus trees. Extensive information has been gathered on the traditional uses of this family, which serve as remedies for a wide range of conditions, including asthma, parasitic infections, bronchitis, colic, cholera, eye sores, cough, diabetes, diarrhea, dysentery, jaundice, stomach pain, toothache, ulcers, wounds, and more. They are also known for their astringent, carminative, diuretic, anthelmintic, and laxative properties (Rahman & Zaman, 2015).

1.3.2.1 The plant genus- *Syzygium*

Syzygium, a flowering plant genus within the Myrtaceae family, comprises approximately 1,200–1,800 species. Its native range extends from Africa and Madagascar to Southern East Asia and the Pacific, with Southeast Asia, including Indonesia, Malaysia, East India, Myanmar, the Philippines, and Thailand (Mudiana, 2016). *Syzygium* species predominantly inhabit tropical and subtropical environments, ranging from lowland and montane rainforests to swamps, ultramafic forests, savannahs, and limestone forests. Morphologically, they are characterized by narrow leaves, short petioles, and flexible twigs, with leaves densely clustered at branch tips (Uddin et al., 2022). In addition to their ecological significance—supporting wildlife with their fruits—many species are cultivated as ornamental plants. Their versatility underscores their importance in medicine, agriculture, and horticulture.

1.3.2.2 The plant-*Syzygium reticulatum* (Wight) Walp.

The plant *Syzygium reticulatum* (Wight) Walp. has undergone little investigation till date.

- ❖ **Synonym:** *Eugenia mangifolia* Wall, *Eugenia mangifolia* Wall. ex Duthie, *Eugenia reticulata* Wight, *Jambosa reticulata* Blum
- ❖ **Bangla name:** Jali jamrul
- ❖ **Plant part:** Leaf



Figure 1.3 : Leaves (a) and full plant (b) of *S. reticulatum*

Syzygium reticulatum (Wight) Walp. is commonly found in Bangladesh (Sylhet), India (Assam, Meghalaya), and Myanmar. It is a medium-sized evergreen tree with ovate-lanceolate leaves that have thick, leathery veins on both sides. The tree produces drupe-like fruits containing covered seeds. The fruiting and flowering period extends from March to June (Jahan et al., 2023). Limited ethnopharmacological data are available specifically for this species. Recently, *S. reticulatum* has been reported with antioxidant, cytotoxic, analgesic, and anti-diarrheal activity (Das et al., 2024).

1.3.2.3 Taxonomical hierarchy of *S. reticulatum*

Rank	Scientific Name with Common Name
Kingdom:	Plantae - Plants
Phylum:	Tracheophyta
Class:	Magnoliopsida
Order:	Myrtales
Family:	Myrtaceae
Genus:	<i>Syzygium</i>
Species:	<i>Syzygium reticulatum</i> (Wight) Walp

1.3.2.4 Review of traditional use of *Syzygium* genus

S. reticulatum is not widely documented for traditional use. As this plant shares same phytochemicals with other *Syzygium* species, it may be locally used to treat digestive disorders, diabetes, inflammation, and various infections like other species of this genus. A detailed review is given below:

Table 1.4: Reported traditional use of *Syzygium* genus

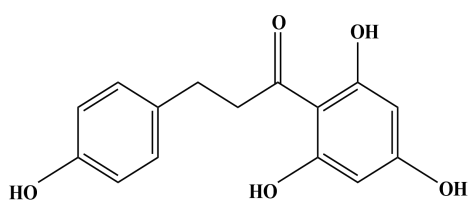
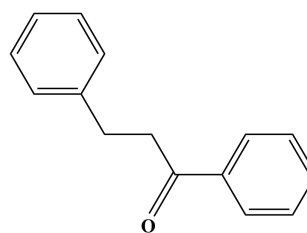
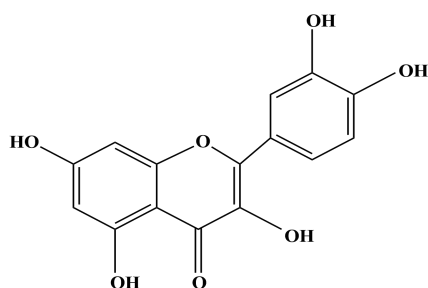
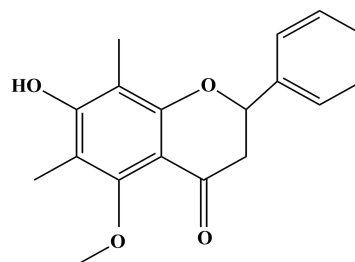
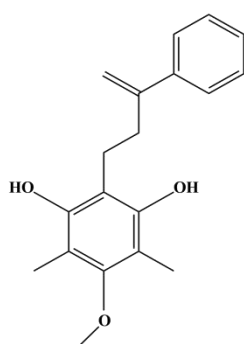
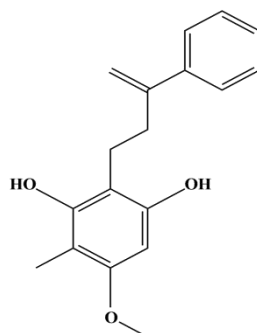
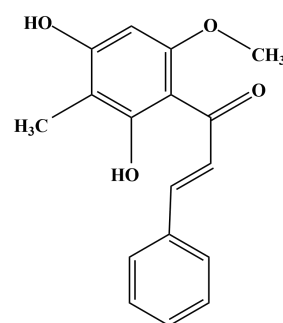
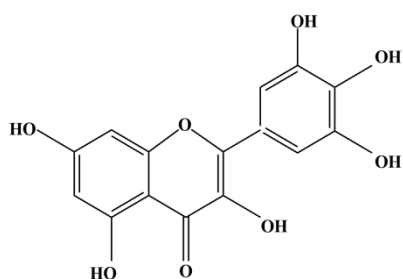
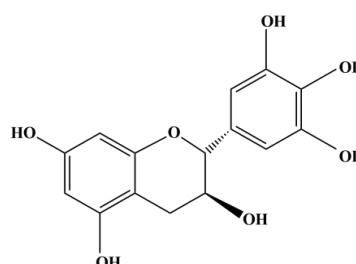
Plant Name	Indications	References
<i>S. anisatum</i>	Antiseptic	Bryant & Cock, 2016
<i>S. jambos</i>	Hemorrhages, leprosy, wounds, ulcers, syphilis and lung diseases	Reis et al., 2021
<i>S. australe</i>	Skin infection	Noé et al., 2019
<i>S. aqueum</i>	Antibiotic, labour pain	Manaharan et al., 2013
<i>S. cumini</i>	Cough, diarrhea, inflammation, ringworm, leucorrhea, diabetes, dysentery and gastrointestinal issues.	Dharani, 2016
<i>S. formosum</i>	Allergic reaction	Duyen Vu et al., 2019
<i>S. polyanthum</i>	Diabetes, high blood pressure, gastritis, ulcers, diarrhea, and skin conditions.	Ismail & Ahmad, 2019
<i>S. nervosum</i>	Diabetes, diarrhea, wounds, breast inflammation, Itching and scabies	Pham et al., 2020

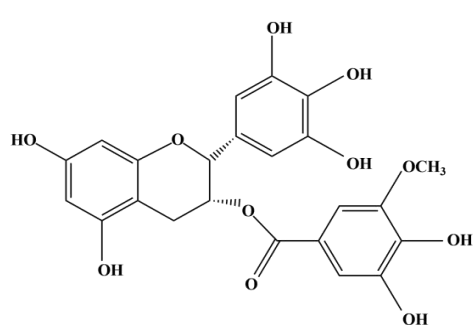
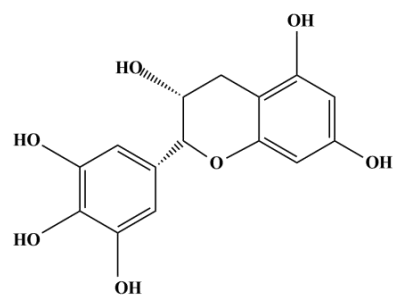
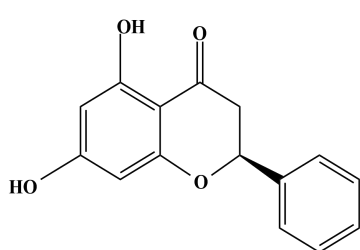
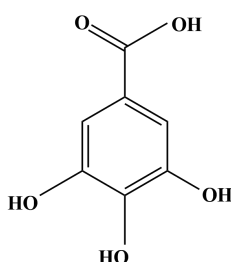
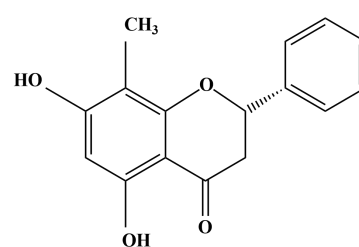
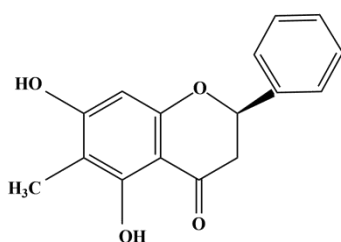
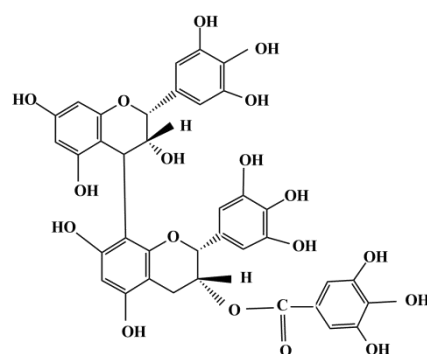
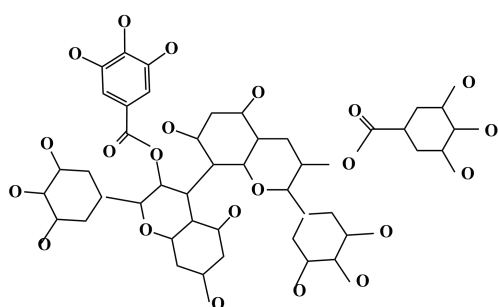
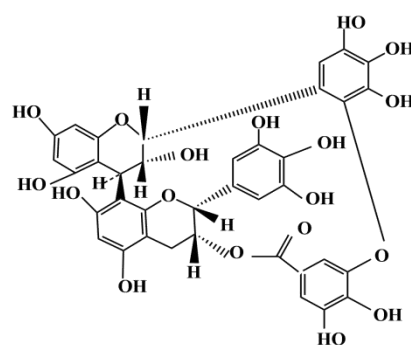
1.3.2.5 Review on the phytochemical and pharmacological activity of *Syzygium* genus

The *Syzygium* genus is abundant in bioactive compounds such as flavonoids, phenolic acids, tannins, and essential oils, which are associated with its medicinal benefits. Scientific studies have demonstrated that species within this genus exhibit antidiabetic, antioxidant, antimicrobial, anti-inflammatory, and anticancer properties. These findings support their traditional uses and highlight their potential in the development of new therapeutic agents. A detailed review on phytochemicals and pharmacological activities is provided below:

Table 1.5: Phytochemicals and pharmacological activity of *Syzygium* genus

Species	Compounds	Properties	Reference
<i>S. aqueum</i>	Europetin-3-O-rhamnoside, Myrigalone-B and G, Phloretin, Gallocatechin, (-)-epigallocatechin 3-O-gallate	Antihyperglycemic, Antiinflammatory activity	Sobeh et al., 2018
<i>S. corticosum</i>	Fouquierol, Ursolic acid, Melaleucic acid, 2,3-dihydrosideroxylin	Cytotoxicity	Ren et al., 2018
<i>S. cumini</i>	Lupeol, Quercetin, β sitosterol, Stigmasterol, gallic acid, (-)-epigallocatechin, Prodelphinidin B-2 3''-O-gallate, Prodelphinidin B-2 3,3''-O-gallate	Antidiabetic, Hypolipidemic, Cardioprotective, Thrombolytic, Anticancer, Membrane Stabilizing Activity	Nahid et al., 2017 Barbhuiya & Godiya, 2019
<i>S. aromaticum</i>	Oleanolic acid lactone. 3- β -hydroxy-11-oxo-olean-12-en-28-oic acid	Anticancer activity	Jimoh et al, 2017 Kasai et al., 2016
<i>S. szemaoense</i>	Syzygium ursanolides	Antimicrobial	Xu et al., 2020
<i>S. kusukusense</i>	Ursolic acid, 2 α -hydroxybetulinic acid, Platanic acid, Betulinic acid, Hyptatic acid A	Cytotoxic	Bai et al., 2014
<i>S. samarangense</i>	Myricitrin, 2',4'-dihydroxy-6'-methoxy-3'-methyldihydrochalcone, 7-hydroxy-5-methoxy-6,8-dimethylfoavanone, Stercurensin, Pinocembrin, 8-methylpinocembrin, (-)-strobopinin, Samarangenin A	Antioxidant	Hu et al., 2018 Sobeh et al., 2019

**Phloretin****2',4'-dihydroxy-6'-methoxy-3'-methyldihydrochalcone****Quercetin****7-hydroxy-5-methoxy-6,8-dimethylfoavanone****Myrigalone-B****Myrigalone-G****Stercurensin****Myricetin****Galocatechin****Figure 1.4: Reported phytochemicals isolated from *Syzygium* genus**

**(-)-epigallocatechin 3-O-gallate****(-)-epigallocatechin****Pinocembrin****Gallic acid****8-methylpinocembrin****(-)-strobopinin****Prodelphinidin B-2 3''-O-gallate****Prodelphinidin B-2 3,3''-O-gallate****Samarangenin A****Figure 1.6 (cont.): Reported phytochemicals isolated from *Syzygium* genus**

1.4 Biosynthesis of secondary metabolites

1.4.1 Biosynthesis of alkyl ferulates

The biosynthesis of alkyl ferulates take place within the cytosol, and/or the endoplasmic reticulum, and involved in three major steps:

- Production of ferulic acid.
- Reduction of a fatty acyl chain to fatty alcohol or derivatives.
- Finally coupling of ferulic acid and alkyl moieties to alkyl ferulate.

The amino acid phenylalanine, formed by the shikimic acid pathway, serves as the precursor of ferulic acid. In the biosynthesis of ferulic acid, phenylalanine is transformed into cinnamic acid by the enzyme phenylalanine ammonia-lyase. Hydroxylation at the C4 position on the phenyl ring produces p-coumaric acid by the enzyme cinnamate 4-hydroxylase. Further hydroxylation at C3 by p-coumarate 3-hydroxylase leads to the formation of caffeoyl-CoA. Finally, caffeoyl-CoA O-methyltransferase causes methylation of the hydroxyl group at C3 to produce feruloyl-CoA.

The alkyl chain of alkyl ferulates is usually composed of long-chain saturated fatty alcohols, formed from corresponding fatty acid homologs. C16 and C18 fatty acids are produced by the fatty acid synthetase complex. In plants, long-chain fatty acid elongation occurs via the fatty acid elongase complex, catalyzing the sequential addition of C2 moieties from malonyl-CoA to C16 and C18 fatty acid precursors. Once elongated to a specific chain length, the fatty acyl-CoAs are reduced by fatty acyl reductases to the corresponding fatty alcohols. The highly specific acyl transferases finally transfer feruloyl-CoA to alkyl moieties to form alkyl ferulates (Domergue and Kosma, 2017; de Oliveira et al., 2015).

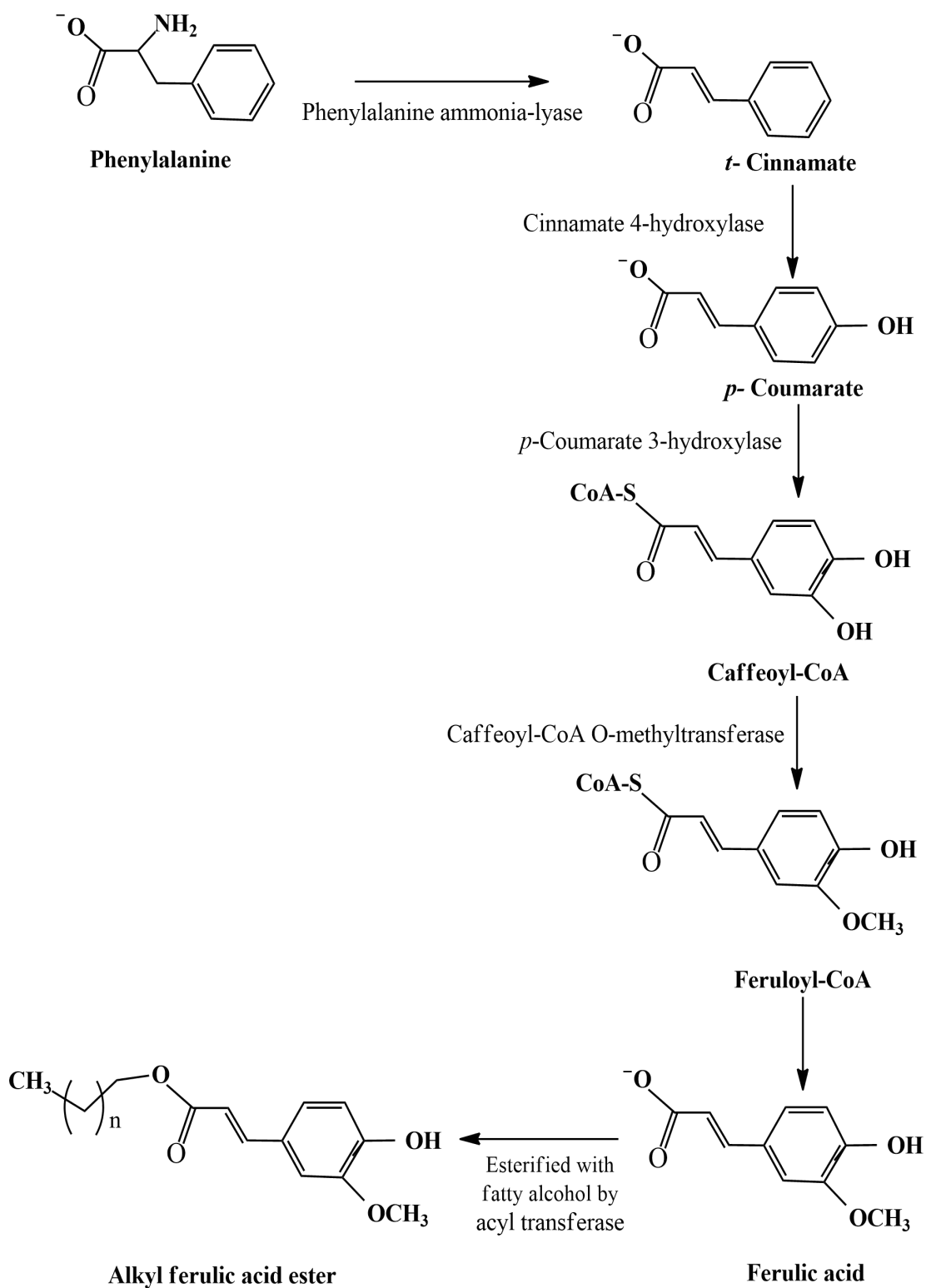


Figure 1.5: Biosynthesis of alkyl ferulic acid esters

1.4.2 Biosynthesis of diterpenes

Synthases that produce isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) interact with prenyltransferases to form oligoprenyl diphosphates (OPP). Farnesyl diphosphate (FPP, C15) and geranylgeranyl diphosphate (GGPP, C20) are produced following the interaction of DMAPP and IPP (GPP, C10) (Li & Dickschat, 2022). Terpene synthases (TPSs) aid in the cyclization of terpenes, whereas cytochrome P450s (CYPs) further modify the terpene skeleton, adding bioactive functional groups and making the molecule more polar (Souza et al., 2024). Casbene is recognized as a precursor for various polycyclic and macrocyclic diterpenes, such as those of the types daphnane, ingenane, tiglane, lathyrane, jatrophone, and casbane (Fattahian et al., 2020). Casbene-derived diterpenes are biosynthesized starting with GGPP, where the diphosphate group is removed, generating a delocalized cation. This cation interacts with the terminal double bond of the C(14, 15) bond, converting it into a cembrene intermediate. The C15 tertiary carbocation then undergoes cyclization and rearrangement, resulting in a range of carbon skeletons.

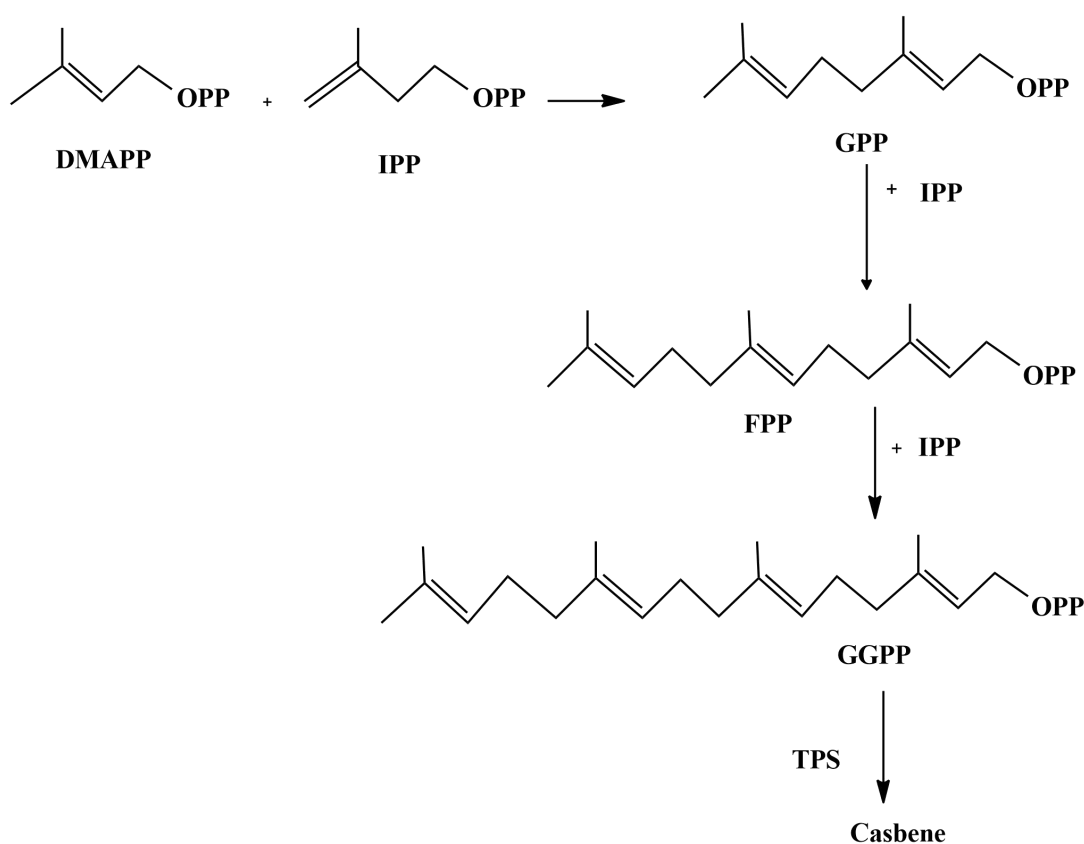


Figure 1.6: Biosynthesis of casbene from primary precursors

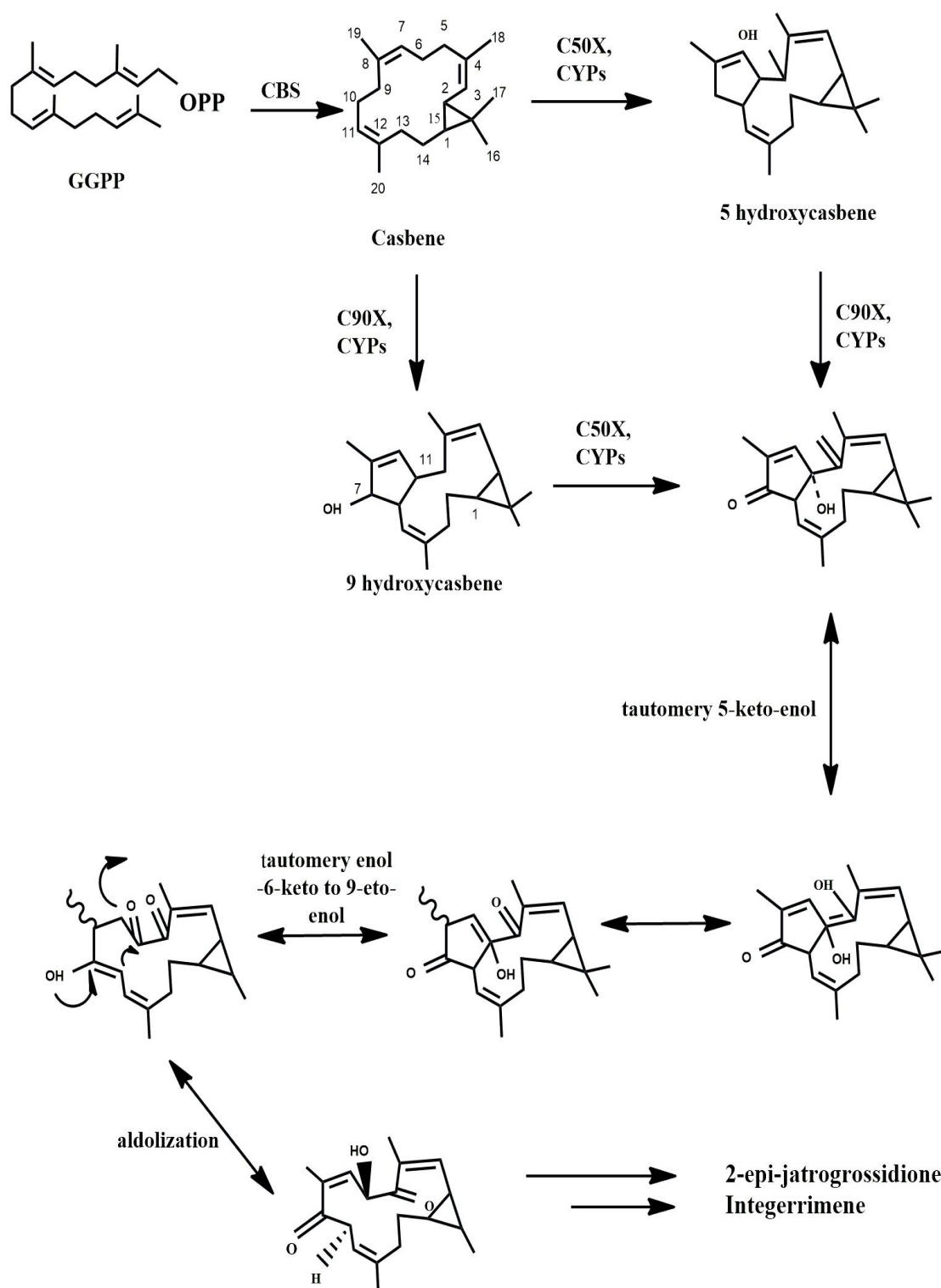
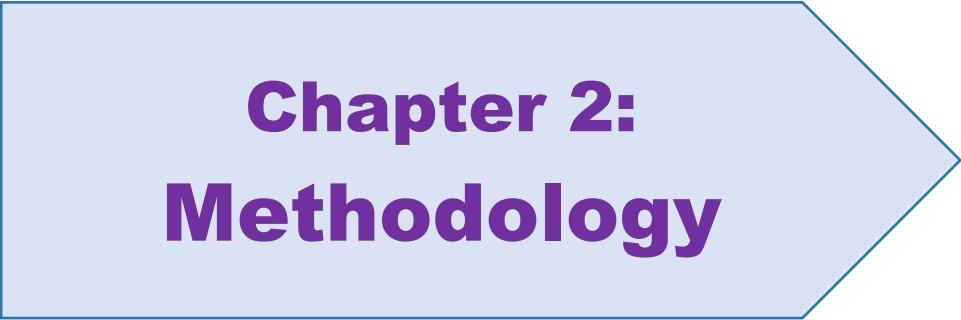


Figure 1.7: Biosynthesis of diterpenes (Integerrimene and 2-epi-jatrogrossidione)



Chapter 2: Methodology

2.1 Plant materials

Jatropha pandurifolia and *Syzygium reticulatum* were chosen for the phytochemical and biological investigations following an intensive literature review.

2.1.1 Collection and identification of the plant

J. pandurifolia was obtained in August 2019 from the botanical garden of the University of Dhaka, Bangladesh, while *S. reticulatum* was collected in 2022 from Lawachara, Sylhet. A taxonomist at the Bangladesh National Herbarium, Ms. Nasrin Aktar, identified the plant specimens and deposited a voucher specimen for future documentation (DACB Accession No. JP: 54445, SR: 65585).

2.1.2 Preparation of plant extracts

Both plant parts were thoroughly cleaned to remove dirt, air-dried, and ground into a coarse powder. *J. pandurifolia* stem bark (3.0 kg) and leaves (2.2 kg) were soaked in ethyl acetate and methanol, respectively. The leaves of *S. reticulatum* (2.0 kg) were soaked in methanol. After being soaked in solvents for 15 days, the plant materials were filtered through cotton plug, and finally concentrated using a Buchi rotary evaporator under low pressure.

2.1.3 Solvent partitioning

The methanolic leaves of *J. pandurifolia* and *S. reticulatum* were fractionated sequentially with solvents of different polarities using the Kupchan method (Emran et al., 2015). For *J. pandurifolia*, n-hexane (Jp-HL: 15 g), ethyl acetate (Jp-EAL: 17 g), chloroform (Jp-CL: 10 g), and aqueous (Jp-Aq: 1.5 g) fractions, as well as for *S. reticulatum*, n-hexane (Sr-HL: 12.76 g), ethyl acetate (Sr-EAL: 17.83 g), chloroform (Sr-CL: 2.83 g), and aqueous fractions (Sr-Aq: 2.0 g) were collected.

2.1.4 Solvents and reagents

In this experiment, analytical/laboratory grade solvents and reagents were used. Distilled methanol, toluene, n-hexane, chloroform, ethyl acetate, dimethyl sulfoxide (DMSO), and ether were organic solvents used in the study. Additionally, the study required the following supplies: saline water, tween 80, diazepam, loperamide, indomethacin, butylated hydroxytoluene (BHT), vincristine sulfate, and streptokinase.

2.2 Isolation techniques

Pure compounds were isolated from the crude and different fractionated portions of *J. pandurifolia* and *S. reticulatum* using various chromatographic techniques. A general description of the isolation of compounds is discussed below:

2.2.1 Chromatographic techniques

Chromatographic methods proved highly effective for separating and purifying compounds derived from plant extracts. Column chromatography and thin layer chromatography were consistently and effectively employed in our current investigation. These techniques facilitated the fractionation of extracts from both plants.

2.2.1.1 Thin Layer Chromatography (TLC)

TLC is an analytical technique used to separate and identify compounds in a mixture, where the sample moves along a plate with a thin layer of material, such as silica gel, carried by a solvent. As the compounds move at varying speeds, they form distinct spots (Santiago & Strobel, 2013). The R_f value (retention factor) is a numerical indicator used in TLC to identify compounds based on how far they travel on the TLC plate. The R_f value is always between 0 and 1. It is calculated using the formula:

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

2.2.1.2 Vacuum Liquid Chromatography (VLC)

VLC is a chromatographic technique used to separate and purify compounds from complex mixtures based on their polarity using high vacuum (Hostettmann et al., 1998). Here, VLC was used to quickly fractionate the crude ethyl acetate (Jp-EA: 42.4 grams) fraction of the stem bark. The vacuum-activated flow of the column allows for the most effective separation of different compounds. Fine VLC-grade silica (Kiesel gel 60H) was packed into the column up to 6 cm height. To ensure compact packing, n-hexane was used as a washing solvent. Chloroform was used to dissolve the material, which was then mixed with silica and dried. The sample was applied to the top of the column. Non-polar n-hexane was used to elute the column. Then polarity of

the eluting solvent was increased by increasing amount of polar solvents like ethyl acetate and methanol (Maurya et al., 2018) (Table 2.1)

Table 2.1 : Different solvent systems used in VLC of Jp-ML

Beaker no.	Solvent system	Collected volume (ml)
1	100% n-hexane	150 ml
2	EA : n-hexane (1:99)	150 ml
3	EA : n-hexane (2:98)	150 ml
4	EA : n-hexane (4:96)	150 ml
5	EA : n-hexane (6:94)	150 ml
6	EA : n-hexane (8:92)	150 ml
7,8	EA : n-hexane (10:90)	150 ml
9, 10	EA : n-hexane (12:88)	150 ml
11	EA : n-hexane (15:85)	150 ml
12	EA : n-hexane (18:82)	150 ml
13, 14	EA : n-hexane (20:80)	150 ml
15	EA : n-hexane (25:75)	150 ml
16	EA : n-hexane (30:70)	150 ml
17-20	EA : n-hexane (35:65)	150 ml
21-23	EA : n-hexane (50:50)	150 ml
24-27	EA : n-hexane (80:20)	150 ml
28-30	100% EA	150 ml
31-35	MeOH : EA (30:70)	150 ml

[EA: Ethyl acetate; MeOH: Methanol]

For *J. pandurifolia*, VLC (9-12) and VLC (17-20) fractions were subjected to gel permeation chromatography in different solvent systems. After performing TLC screening, prominent GPC fractions were further subjected to column chromatography.

2.2.1.3 Gel Permeation Chromatography (GPC)

Gel permeation chromatography separates compounds based on their molecular size passing through a porous gel-based stationary phase. Here, larger molecules are unable to enter the pores and therefore elute through the column more quickly, while

smaller molecules diffuse into the gel pores, resulting in a longer elution time. This variation in elution time confirms an effective size based separation of compounds (Ghamartale et al., 2021). Using a pasteur pipette, the sample was dissolved in 20% petroleum ether-chloroform and carefully placed at the top of a Sephadex LH-20 column. Different solvent systems in increasing polarity were used to elute the fractions. Test tubes with numbers were used to collect the eluates. Remaining polar chemicals from the column was eluted with a 5% methanol-chloroform solution, 10% methanol-chloroform solution, and 100% methanol. To prepare the column for subsequent analysis, it was thoroughly cleaned with methanol to ensure the removal of any residual compounds. The used solvent systems used as mobile phases in the GPC are listed below (Table 2.2).

Table 2.2: Different solvent systems used in GPC of Jp-ML

Solvent systems
20% PC (Petroleum Ether: Chloroform= 20: 80)
15% PC (Petroleum Ether: Chloroform= 15:85)
10% PC (Petroleum Ether: Chloroform= 10:90)
5% PC (Petroleum Ether: Chloroform= 5:95)
100% Chloroform
5% MC (Methanol: Chloroform=5:95)
10% MC (Methanol: Chloroform=10:90)
100% Methanol

Table 2.3: Different GPC fractions from VLC fractions of Jp-ML

VLC fraction no.	GPC fraction no.	Collected volume (ml)
VLC (9-12)	C-31 to C-55	120 ml
	C-56 to C-75	100 ml
VLC (17-20)	C-26 to C-40	100 ml
	C-41 to C-60	100 ml
	C-61 to C-90	100 ml

2.2.1.4 Column Chromatography (CC)

A glass column with 92 cm in length and 4.5 cm in diameter was used where powdered silica gel 60 H (Kieselgel 60, mesh 70-230) was used as stationary phase. A slurry of silica gel in n-hexane was poured into the column until two-thirds of column filled. During this process, it is imperative to avoid the production of any bubbles or contamination. The sample was then gently loaded onto the top of the silica gel bed (Srivastava et al., 2021) and a small round piece of cotton was placed on the sample to prevent its disturbance during solvent addition. Then, the solvent was introduced and the eluted fractions were collected in numbered test tubes for further analysis. The polarity of the solvent system was gradually increased by using ethyl acetate and methanol to elute more polar compounds (Table 2.4).

Table 2.4: Different CC fractions from GPC fractions of Jp-ML

VLC fraction no.	GPC fraction no.	CC fraction no.	Solvent system	Collected volume (ml)
VLC (9-12)	GPC (31-55)	CC (100-114)	EA : n-hexane (0.5 : 99.5)	200
	GPC (56-75)	CC (451-491)	EA : n-hexane (5 : 95)	250
VLC (17-20)	GPC (26-40)	CC (136-200)	EA : n-hexane (3 : 97)	200
	GPC (41-60)	CC (265-272)	EA : n-hexane (6: 94)	180
	GPC (61-90)	CC (394-435)	EA : n-hexane (6: 94)	150
		CC (401-491)	EA : n-hexane (3: 97)	100

Methanolic leaf extract of *S. reticulatum* was subjected to column chromatography using different solvent systems. Among different fractions the prominent fractions were focused for compound isolation after performing TLC screening (Table 2.5).

Table 2.5: Different CC fractions Sr-ML extract

Fraction no.	Solvent system	Collected volume (ml)
CC (15-25)	EA : n-hexane (8 : 92)	100
CC (31-60)	EA : n-hexane (7 : 93)	200

2.2.1.5 Preparative Thin Layer Chromatography (PTLC)

PTLC is routinely performed for the final purification of the compound. In PTLC, a relatively large amount of sample is used compared to TLC. Glass plates coated with silica gel (Kieselgel 60 PF 254) are used for PTLC. The sample is dissolved in a suitable solvent and applied as a thin band, positioned 2 cm from the edge of the TLC plate. The plates are then developed using a solvent system previously optimized through TLC analysis. Subsequently, the plates are allowed to dry, and the compounds are visualized under UV light (either short or long wavelength) or using various reagent sprays. Following visualization, the compounds are scraped off the plate and eluted with an appropriate solvent to obtain pure compounds or further purification (Sherma & Fried, 1987).

2.2.2 Solvent treatment/ Recrystallization

At the outset, a solvent or a mixture of solvents is carefully selected, in which the desired compound exhibits practical insolubility while other components dissolve readily. This chosen solvent facilitates the separation of undesired components through repeated washing. Additional solvents or solvent mixtures may be employed until a state of purity is achieved for the desired compound. If the compound is crystalline it was dissolved in minimum amount of solvent and left overnight for recrystallization.

2.3 Detection/ visualization of the compounds

There are different methods for visualization or detection of compound on a TLC plate that are described below-

1. Visual detection: The developed chromatogram was visually inspected to observe and detect any colored compounds present on the plate.

2. UV light: A UV lamp offers the convenience of illuminating TLC plates with either short-waved (254 nm) or long-waved (365 nm) ultraviolet light. Typically, commercially available TLC plates contain a fluorescent substance like zinc sulfide embedded in the silica or alumina. In 254 nm, compound is appeared as dark spot against green background and in 365 nm, compound is appeared as fluorescent spot against dark background. This method is often the first choice due to its simplicity

and speed. It is particularly effective for visualizing aromatic compounds and highly conjugated systems, as these tend to strongly absorb UV light. However, other functional groups that do not absorb UV light at these wavelengths will not appear dark under the UV lamp, despite still being present on the plate.

3. Spray reagents: For more precise detection primarily two different spray reagents were used.

A. Vanillin-Sulfuric acid reagent: A 1% vanillin-sulfuric acid reagent was sprayed onto the TLC plate, followed by heating at 100 °C for 2–5 minutes. This treatment resulted in the appearance of different colors for various compounds present on the plate (Wu et al., 2019).

B. Modified Dragendorff's reagent: 1.7% (v/v) of bismuth subnitrate was prepared by dissolving in 20% of acetic acid. 40% aqueous solution of potassium iodide was also prepared. Modified Dragendorff's reagent was produced by mixing equal parts of these two reagents. When sprayed onto alkaloids and other nitrogen-containing compounds, this reagent produces orange to red spots, indicating their presence (Kiran et al., 2015).

2.4 Isolation and purification of the compounds

The detail of isolated compounds from the both plants are listed below in Table 2.6 and Table 2.7.

Table 2.6: Isolated compounds from ethyl acetate fraction of *J. pandurifolia*

Code	Amount found & physical appearance	VLC no.	Sepha & column fraction no.	Further purification steps	R _f value	UV-light 254 nm 366 nm	Spraying with Vanillin-H ₂ SO ₄
JP-199a	2.5 mg; colorless crystal	17-20	GPC (41-60) CC (265-268)	PTLC using 6.0% ET	0.66	Quenching spot, Blue fluorescent	Dark Purple
JP-200	5.0 mg; white mass	17-20	GPC (41-60) CC (269-272)	PTLC using 6.0% ET	0.40	Yellowish spot, Blue fluorescent	Light brown
JP-460	10 mg; colorless mass	17-20	GPC (61-90) CC (394-435)	PTLC using 6.0% ET	0.55	Yellowish spot, Quenching spot	Dark blue
JP-500	1.5 mg; colorless crystal	17-20	GPC (41-60) CC (265-268)	PTLC using 6.0% ET	0.74	Light Yellowish, Blue fluorescent	Yellowish color
JP-05	Colorless crystal	17-20	GPC (26-40) CC (136-200)	PTLC using 3.0% ET	0.65	Quenching spot, bright yellow fluorescent	Dark blue
JP-25	4.0 mg; white crystal	25	N/A	Rerystallization by n-hexane	0.5	Quenching spot, yellow fluorescent	Dark purple
JP-81	3.0 mg; colorless crystal	17-20	GPC (61-90) CC (401-491)	PTLC using 3.0% ET	0.70	Quenching spot, blue fluorescent	Dark blue
JP-13	5.0 mg; white crystal	13	N/A	Recrystallization by n-hexane	0.72	Quenching spot, Yellow fluorescent	Dark purple
JP-10	5.0 mg; white crystal	10	N/A	Recrystallization by n-hexane	0.65	Quenching spot, Yellow fluorescent	Dark purple

Table 2.6 (cont.): Isolated compounds from ethyl acetate fraction of *J. pandurifolia*

Code	Amount found & physical appearance	VLC no.	Sepha & column fraction no.	Further purification steps	R _f value	UV-light 254 nm, 366 nm	Spraying with Vanillin-H ₂ SO ₄
JP-6	5.0 mg; colorless needle shaped crystal	9-12	GPC (56-75) CC (451-491)	PTLC using 5.0% ET	0.45	Quenching spot, pale yellow fluorescent	Dark purple
JP-66	1.5 mg; colorless crystal	17-20	GPC (61-90) CC (401-491)	PTLC using 3.0% ET	0.25	invisible, brown	Dark purple
JP-93	4.0 mg; White powder	9-12	GPC (31-55) CC (100-114)	PTLC using 0.5% ET	0.80	Quenching spot, light blue fluorescent	Bright deep blue

Table 2.7: Isolated compounds from methanolic fraction of *S. reticulatum*

Code	Amount found & physical appearance	VLC no.	Sepha & column fraction no.	Further purification steps	R _f value	UV-light 254 nm, 366 nm	Spraying with Vanillin-H ₂ SO ₄
SR-60	1.0 mg; colorless powder	N/A	CC (15-25)	PTLC using 8.0% ET	0.71	Quenching spot, yellowish fluorescent	Dark blue
SR-15	2.0 mg; colorless powder	N/A	CC (31-60)	PTLC using 7.0% ET	0.88	Quenching spot, pale yellowish fluorescent	Light purple

2.5 Analytical techniques

2.5.1 Mass Spectrometry (MS)

Mass spectrometry determines molecular mass and molecular formula. It works by ionizing the chemical compounds which are then analyzed according to their mass-to-charge ratios (m/z) (Király et al., 2016). In this study, JEOL JMS-600H, EIMS (Electron Impact Mass Spectrometry) was used.

2.5.2 Nuclear Magnetic Resonance spectroscopy (NMR)

NMR spectroscopy is an analytical technique that uses magnetic fields and radio waves that gives detailed information about organic molecules. In this study, NMR spectra were acquired using a Bruker 400TM ASCEND spectrometer. CDCl₃ used as solvent and tetramethylsilane (TMS) as the internal standard ($\delta_H = 0$). Chemical shifts are denoted in parts per million (ppm) and coupling constants (J) in hertz. This method is widely applied in structure elucidation of compound involved in drug development (Speight, 2017).

2.5.2.1 1D NMR spectroscopy

In one-dimensional NMR spectroscopy the spectrum plots signal intensity (y-axis) against chemical shift (x-axis, in ppm), showing how nuclei resonate at different magnetic field strengths. This shift is dictated by both the electron density distribution surrounding the nucleus and its interactions with adjacent nuclei (Yan et al., 2016).

2.5.2.1.1 ¹H NMR spectroscopy

¹H NMR spectrum helps identify the probable local chemical environments of protons within a compound's structure through their chemical shifts. It also reveals connectivity by showcasing coupling constants and patterns among protons. Additionally, reflects the number of protons corresponding to each signal (Lim and Lewis, 2013).

2.5.2.1.2 ¹³C NMR spectroscopy

¹³C NMR spectroscopy is used to identify and characterize the carbon framework of a molecule. It provides information about the number, type, and environment of carbon atoms (e.g., alkyl, aromatic, carbonyl). This is often used alongside ¹H NMR and 2D NMR techniques for complete structural elucidation of compounds (Koenig, 1999).

2.5.2.2 2D NMR spectroscopy

In 2D NMR, both the x and y axes feature chemical shift scales, and the resulting two-dimensional spectra are visualized as a grid, similar to a map (Lindon, 2005) in contrast to the single axis in 1D NMR spectroscopy.

2.5.2.2.1 Correlated Spectroscopy (COSY)

COSY provides information about the connectivity of atoms within a molecule, specifically focusing on proton-proton interactions. It allows to determine how protons are coupled by correlating signals of protons that are connected through scalar couplings (J -coupling).

2.5.2.2.2 Heteronuclear Single Quantum Coherence (HSQC) spectroscopy

HSQC provides information about the direct interactions between protons and heteronuclei (such as C or N) within a molecule. One axis represents the chemical shifts of protons, while the other axis corresponds to the chemical shifts of the heteronuclei. This technique helps to identify which protons are directly bonded to heteronuclei by correlating the signals of these nuclei and provides a more detailed understanding of the molecular structure (Chen et al., 2015).

2.5.2.2.3 Heteronuclear Multiple Bond Correlation (HMBC) spectroscopy

HMBC is designed to detect correlations between protons and heteronuclei that are separated by 2–4 bonds, unlike HSQC, which detects direct 1-bond interactions. This technique enables to understand the overall connectivity of a molecule, and elucidate the larger structural framework of the molecule (Reynolds, 2017).

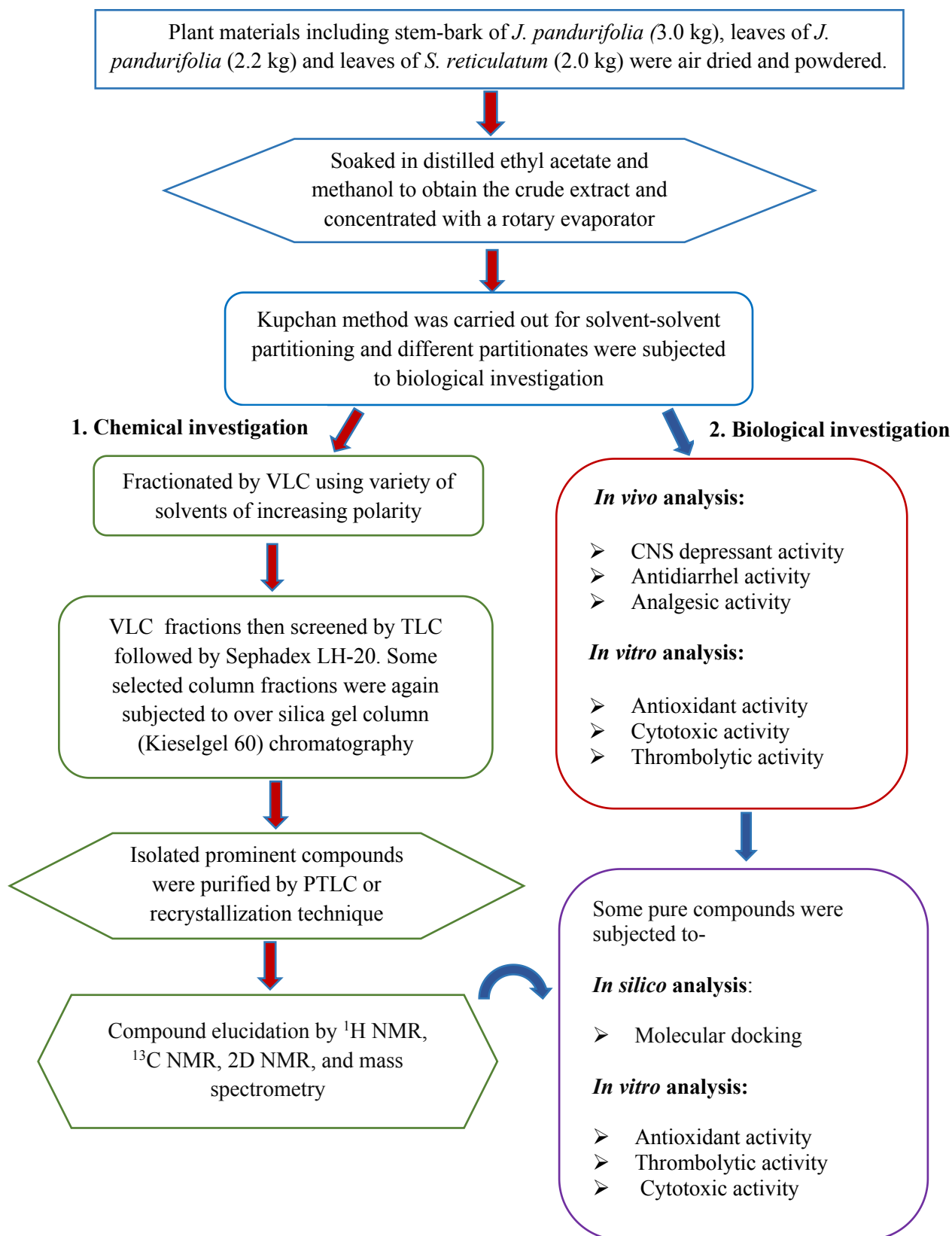


Figure 2.1: Process flow diagram of the current research

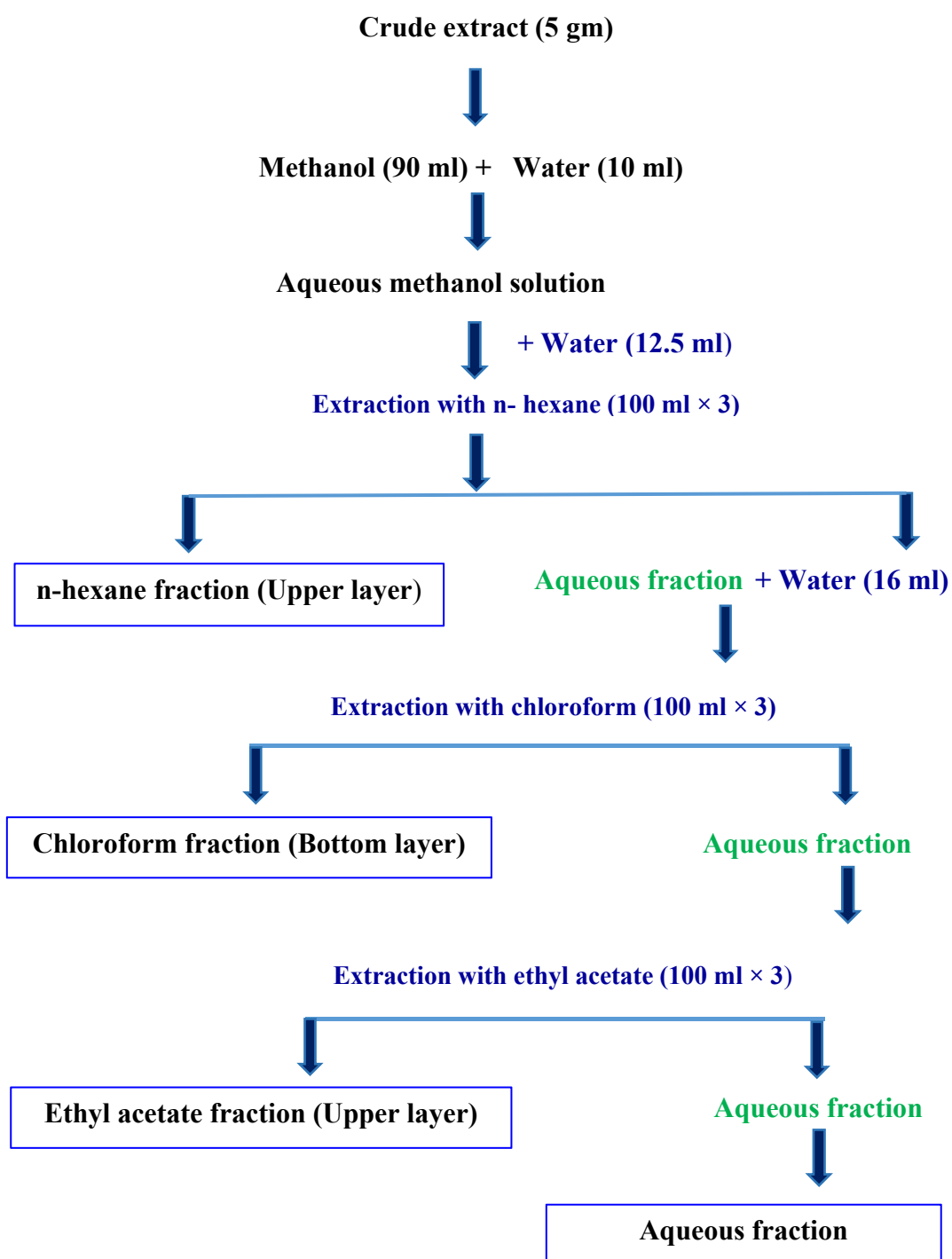


Figure 2.2: Modified Kupchan partitioning protocol

**Chapter 3:
Result & Discussion
(Chemical
Investigation)**

3.1 Characterization of isolated compounds

In this study, *Jatropha pandurifolia* and *Syzygium reticulatum* were examined for their phytochemical constituents and biological potency. A total of fifteen compounds were isolated from both plants, among them thirteen compounds isolated from the crude ethyl acetate extract of *J. pandurifolia* stem bark and two compounds isolated from the crude methanolic extract of *S. reticulatum* leaf (Table 3.1). The structures of the compounds were determined by ^1H NMR, ^{13}C NMR, 2D NMR (COSY and HSQC) and mass spectrometry.

Table 3.1: Compounds isolated from *J. pandurifolia* and *S. reticulatum*

Sl. No.	Code No.	Type of compound	Name of the compound
Compound 1	JP-199a	Diterpene	2-epi-jatrogrossidione
Compound 2	JP-200	Diterpene	Integerrimene
Compound 3	JP-460	Diterpene	Jatrophatrione
Compound 4	JP-500	Diterpene	Citlaltione
Compound 5	JP-05	n-alkyl <i>trans</i> -ferulate	Dotriacontyl <i>trans</i> -ferulate
Compound 6	JP-25	n-alkyl <i>trans</i> -ferulate	Triacetyl <i>trans</i> -ferulate
Compound 7	JP-81	n-alkyl <i>trans</i> -ferulate	Octacosyl <i>trans</i> -ferulate
Compound 8	JP-13	n-alkyl <i>trans</i> -ferulate	Hexacosyl <i>trans</i> -ferulate
Compound 9	JP-10	n-alkyl <i>cis</i> -ferulate	Octacosyl <i>cis</i> -ferulate
Compound 10	JP-06a	Phytosterol	Beta-sitosterol
Compound 11	JP-06b	Phytosterol	Stigmasterol
Compound 12	JP-66	Fatty alcohol	n-Dotriacontanol
Compound 13	JP-93	Triglyceride	1, 2-Dioleoyl-3-palmitoyl-glycerol or 1, 3-dioleoyl-2-palmitoyl-glycerol
Compound 14	SR-60	Phthalate ester	Bis(2-ethylhexyl) phthalate
Compound 15	SR-15	Phytosterol	Stigmast-4-en-3-one

Compound 1, 6, 7, 8, 9, 12 and 13 were isolated for the first time from *J. pandurifolia* stem bark. Additionally, compound 14 and 15 were also reported for the first time from the second plant *S. reticulatum* leaf. Notably, compound 5 is a new compound isolated for the first time from any natural source.

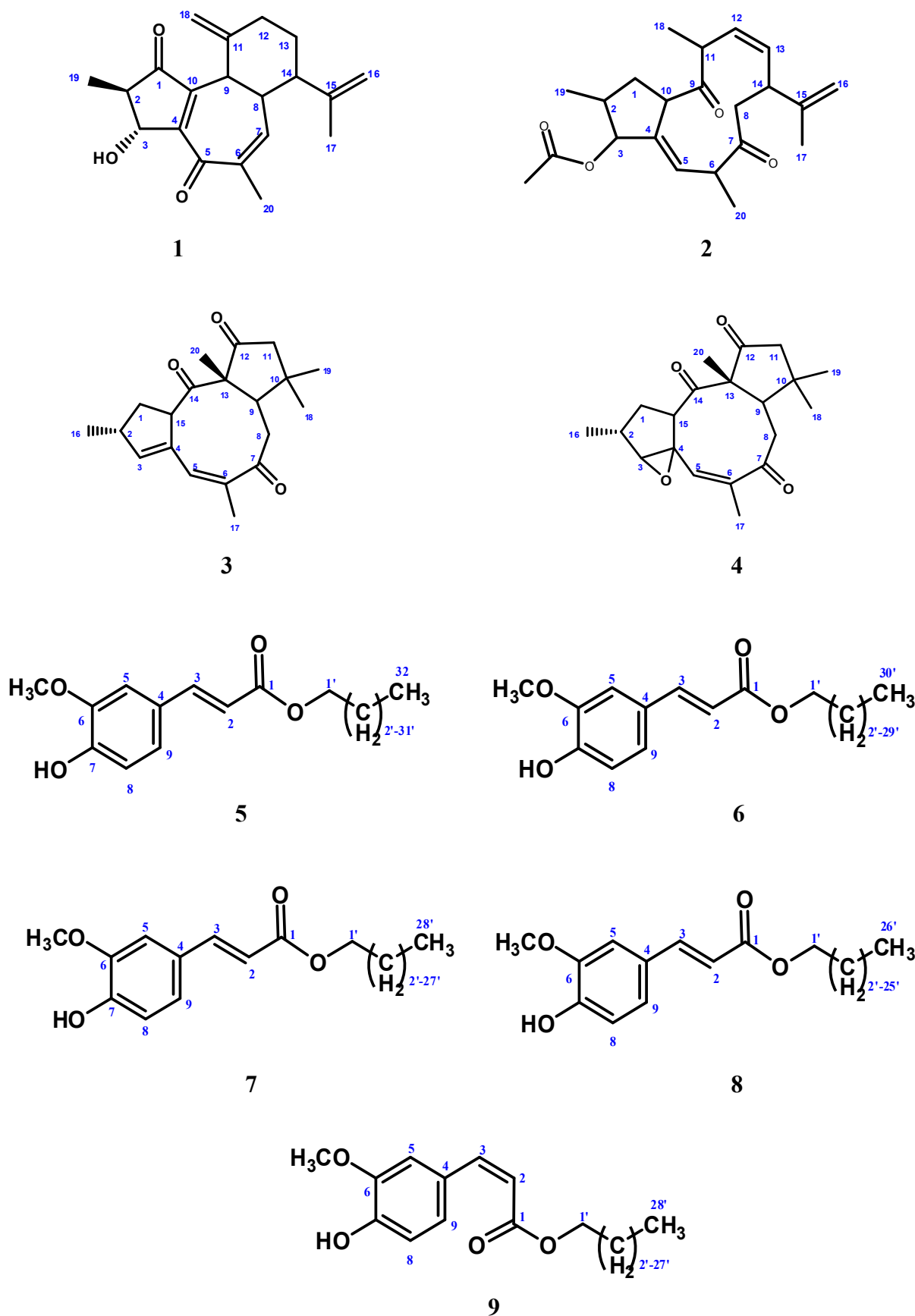


Figure 3.1: Structures of the compounds isolated from *J. pandurifolia*

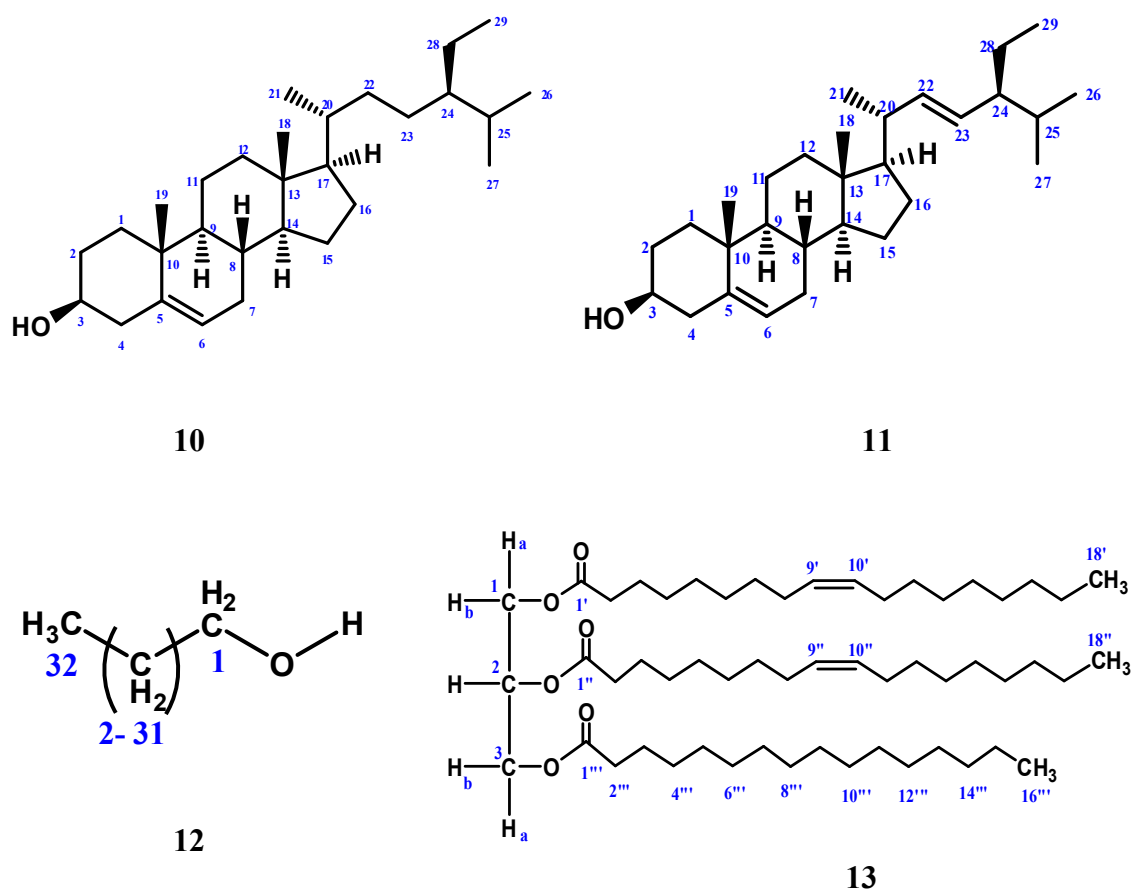


Figure 3.1 (cont.) Structures of the compounds isolated from *J. pandurifolia*

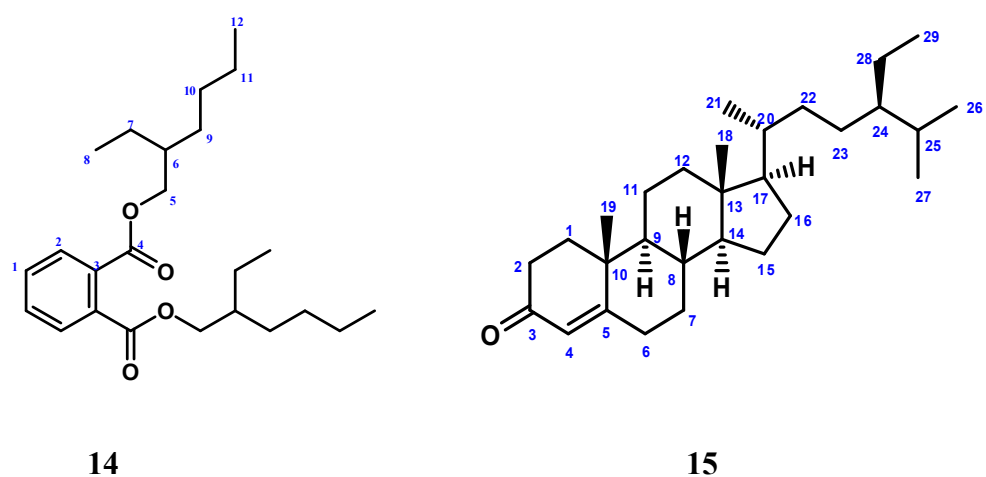


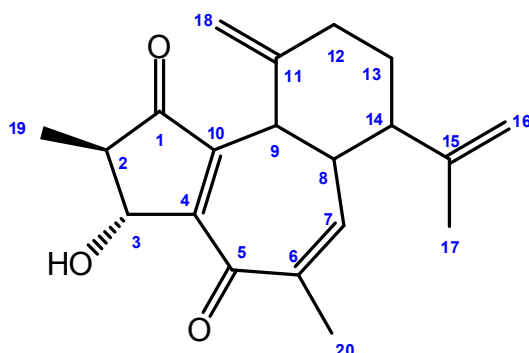
Figure 3.2: Structures of the compounds isolated from *S. reticulatum*

3.1.1 Characterization of compound 1 (JP-199a) as 2-*epi*-Jatrogrossidione

Compound **1** was obtained as colorless fine needles, which appeared as a quenching spot under UV light at 254 nm and a blue fluorescent spot at 366 nm on a thin layer chromatography plate. After spraying with vanillin-sulfuric acid reagent and heating for 3 minutes, it developed a dark purple spot.

The ^1H NMR spectrum (400 MHz, CDCl_3 ; Table: 3.2, Figure: 3.4) displayed an olefinic proton at δ 5.90 ($J = 5.4, 2.4$ Hz), an oxymethine proton at δ 4.87 t ($J = 2$ Hz), and two exocyclic methylene groups at δ 4.84, 4.82, and δ 4.75, 4.10, assignable to H-7, H-3, H-16 and H-18, respectively. The spectrum further exhibited two methyl groups, resonating at a comparatively low field at δ 1.84 ($J = 1.4$ Hz) and δ 1.58, suggesting their attachment to unsaturated carbon atoms, which could be attributed to H-20 and H-17, respectively. In addition, the spectrum showed a secondary methyl resonating at δ 1.30 with a coupling constant of $J = 7.6$ Hz, attributed to H-19. The ^{13}C NMR spectrum (150 MHz, CDCl_3 ; Table: 3.2, Figure: 3.6) further corroborated the presence of two exomethylene groups (δ 113.6/146.8 and δ 108.5/149.0) and a carbinol carbon at δ 76.9 (C-OH, C-3). All these NMR data of compound **1** were found to be identical to those reported for the rhamnofolane diterpenoid, 2-*epi*-jatrogrossidione, previously isolated from *Jatropha grossidentata* (Jakupovic et al., 1988) and *Jatropha gaumeri* (Can-Ake et al., 2004).

The COSY spectrum (600 MHz, CDCl_3 ; Figure: 3.8) showed the expected vicinal correlation between H-2 to H-3, H-7 to H-8, H-8 to H-9, H-2 to H-19, H-8 to H-14 etc. This is the first report on the isolation of 2-*epi*-jatrogrossidione from *J. pandurifolia*.



2-*epi*-jatrogrossidione

Table 3.2: NMR spectral data (400 MHz, CDCl₃) of compound 1 (JP-199a)

Position	Compound 1		2- <i>epi</i> -jatrogrossidione (Can-Ake et al., 2004; Jakupovic et al., 1988)	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1	---	---	---	209.6
2	2.40 dq ($J = 7.6, 3.6$ Hz)	48.5	2.41 dq ($J = 7.6, 2.4$ Hz)	48.3
3	4.87 t ($J = 2$ Hz)	76.9	4.88 br s	76.8
4	---	---	---	148.4
5	---	---	---	198.4
6	---	141.5	---	141.3
7	5.90 dq ($J = 5.4, 2.4$ Hz)	137.4	5.91 dq ($J = 5.1, 1.6$ Hz)	137.2
8	2.60 m	43.9	2.60 dddq ($J = 12, 12, 5, 1.5$ Hz)	43.7
9	3.18 br d ($J = 11.2$ Hz)	45.6	3.18 d ($J = 12.0$ Hz)	45.4
10	---	---	---	160.5
11	---	149.0	---	148.9
12	2.41 m, 2.30 m	36.5	2.44 ddd ($J = 12, 5.6, 2.4$ Hz) 2.29 brd t ($J = 12.6, 11.6, 4.4$ Hz)	36.4
13	1.90 m, 1.45 m	34.5	1.89 m, 1.47 dddd ($J = 12, 12, 12, 5.2$ Hz)	34.3
14	2.34 m	51.6	2.37 ddd ($J = 12, 12, 4.4$ Hz)	51.4
15	---	146.8	---	146.6
16	4.84 br s, 4.82 br s	113.6	4.85 br s, 4.82 br s	113.4
17	1.58 3H, s	18.8	1.59 3H, brs	18.7
18	4.75 br s, 4.10 br s	108.5	4.76 br s, 4.11 br s	108.4
19	1.30 3H, d ($J = 7.6$ Hz)	14.6	1.32 3H, d ($J = 7.6$ Hz)	14.5
20	1.84 3H, t ($J = 1.4$ Hz)	19.0	1.85 3H, t ($J = 1.6$ Hz)	18.9

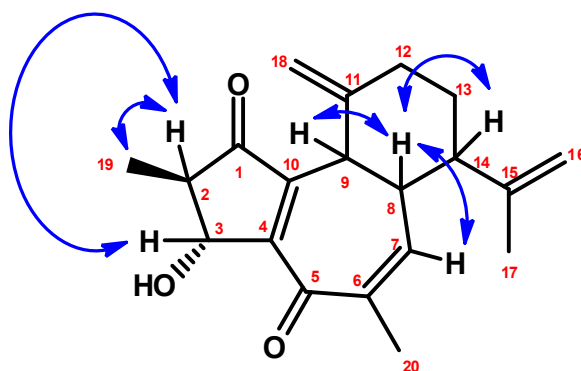
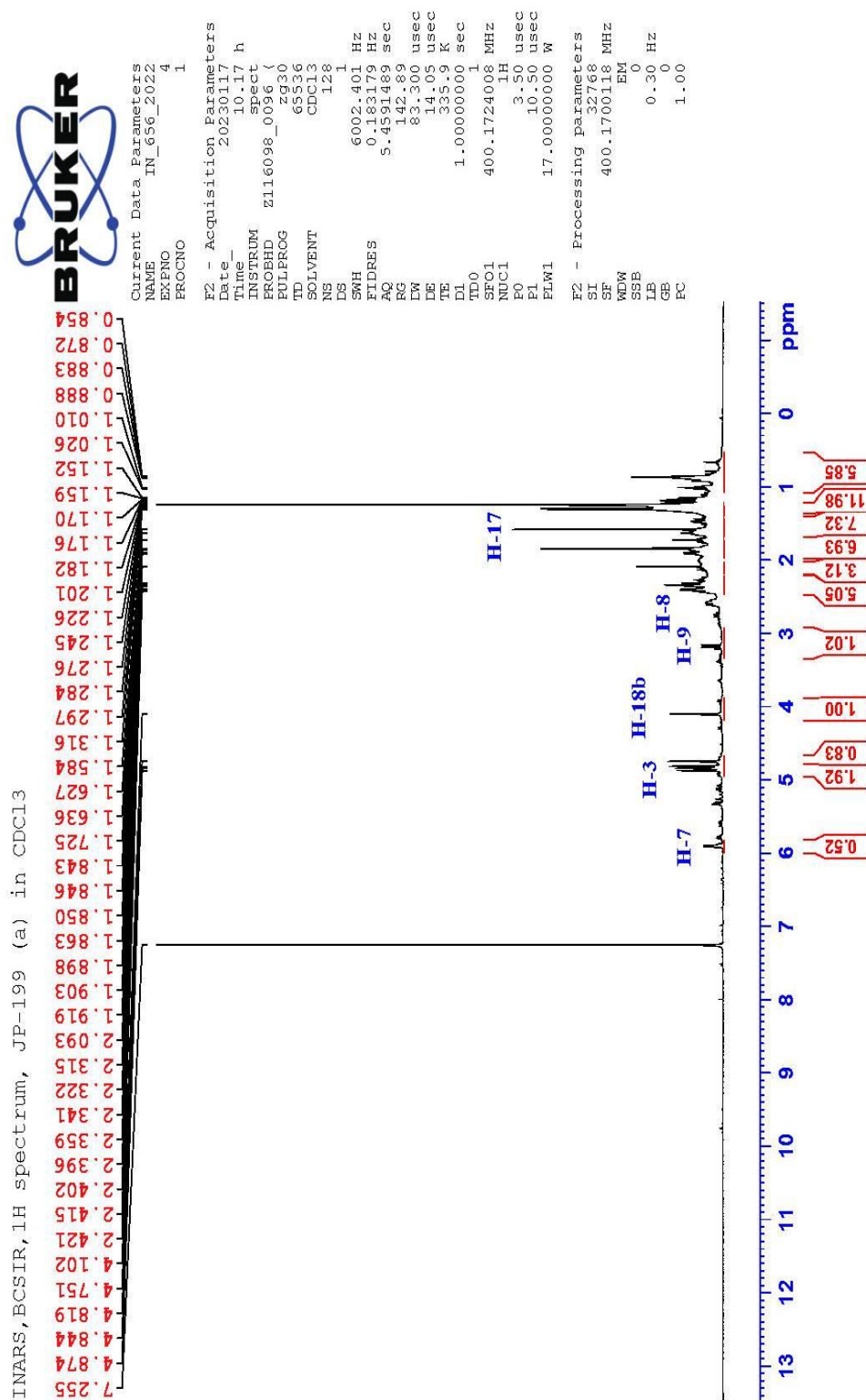


Figure 3.3 Key COSY correlations in compound 1

Figure 3.4: ¹H NMR (400 MHz, CDCl₃) of compound 1 (JP-199a)

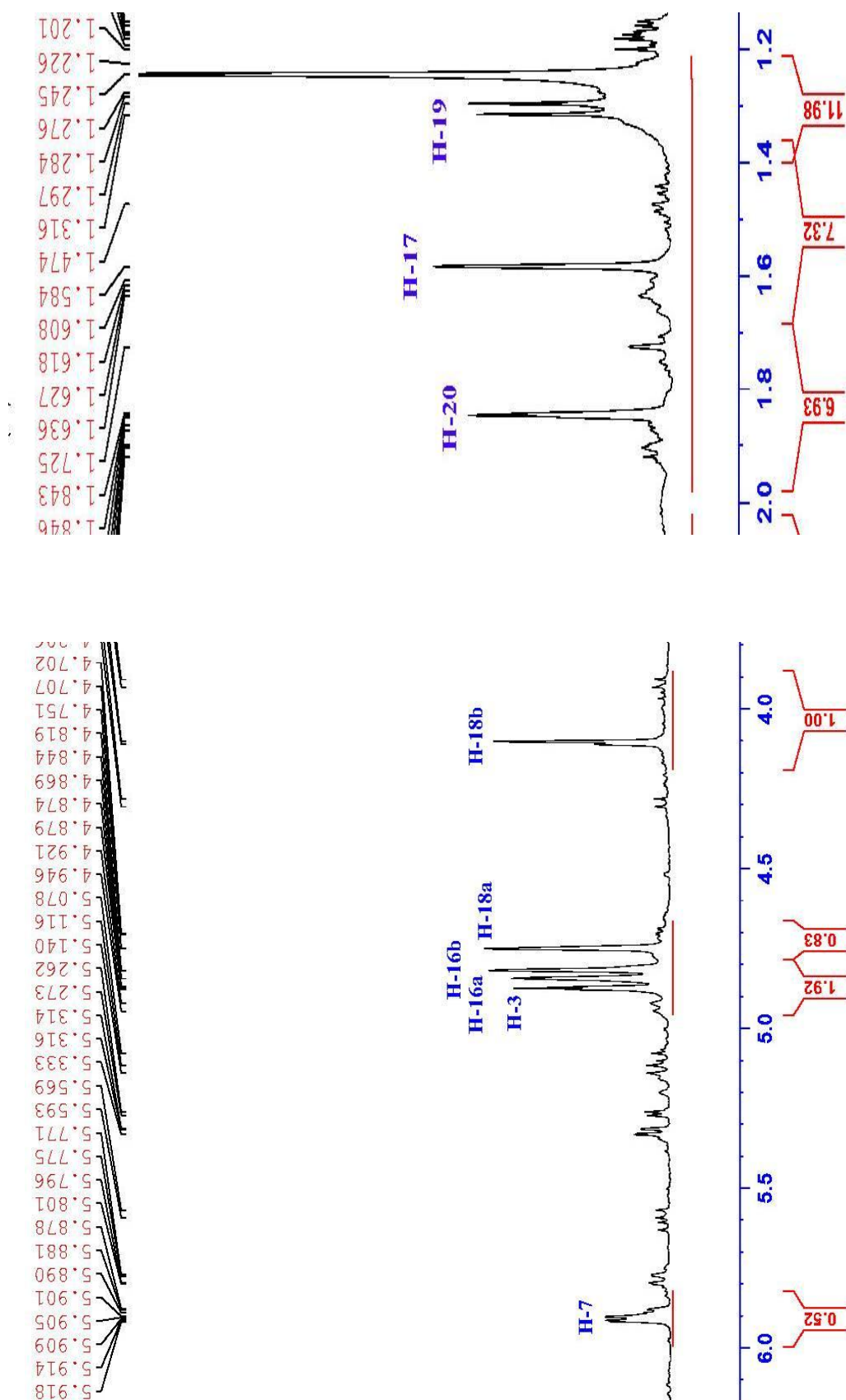
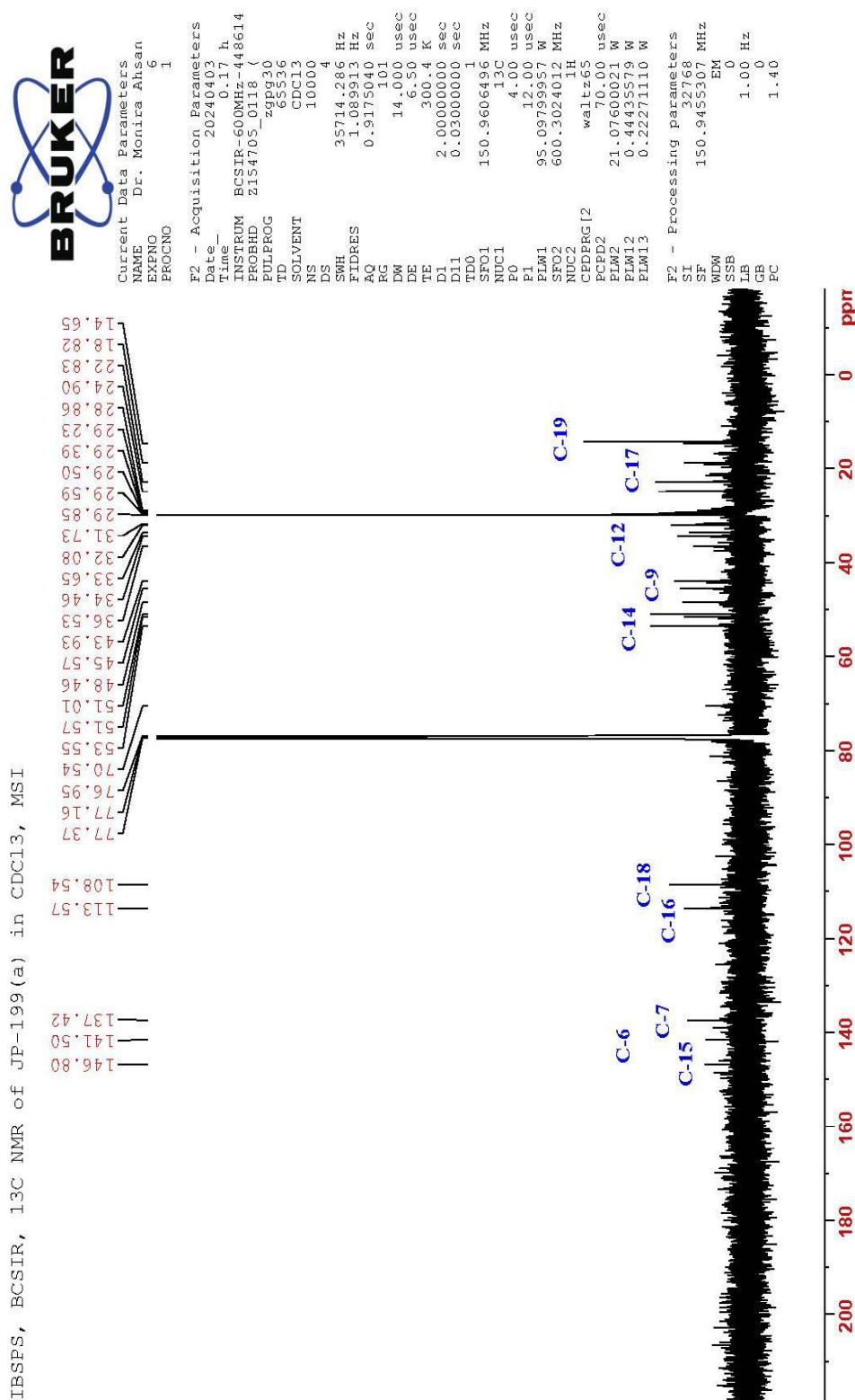


Figure 3.5: Partially expanded ^1H NMR (400 MHz, CDCl_3) of compound 1 (JP-199a)

Figure 3.6: ^{13}C NMR (150 MHz, CDCl_3) of compound 1 (JP-199a)

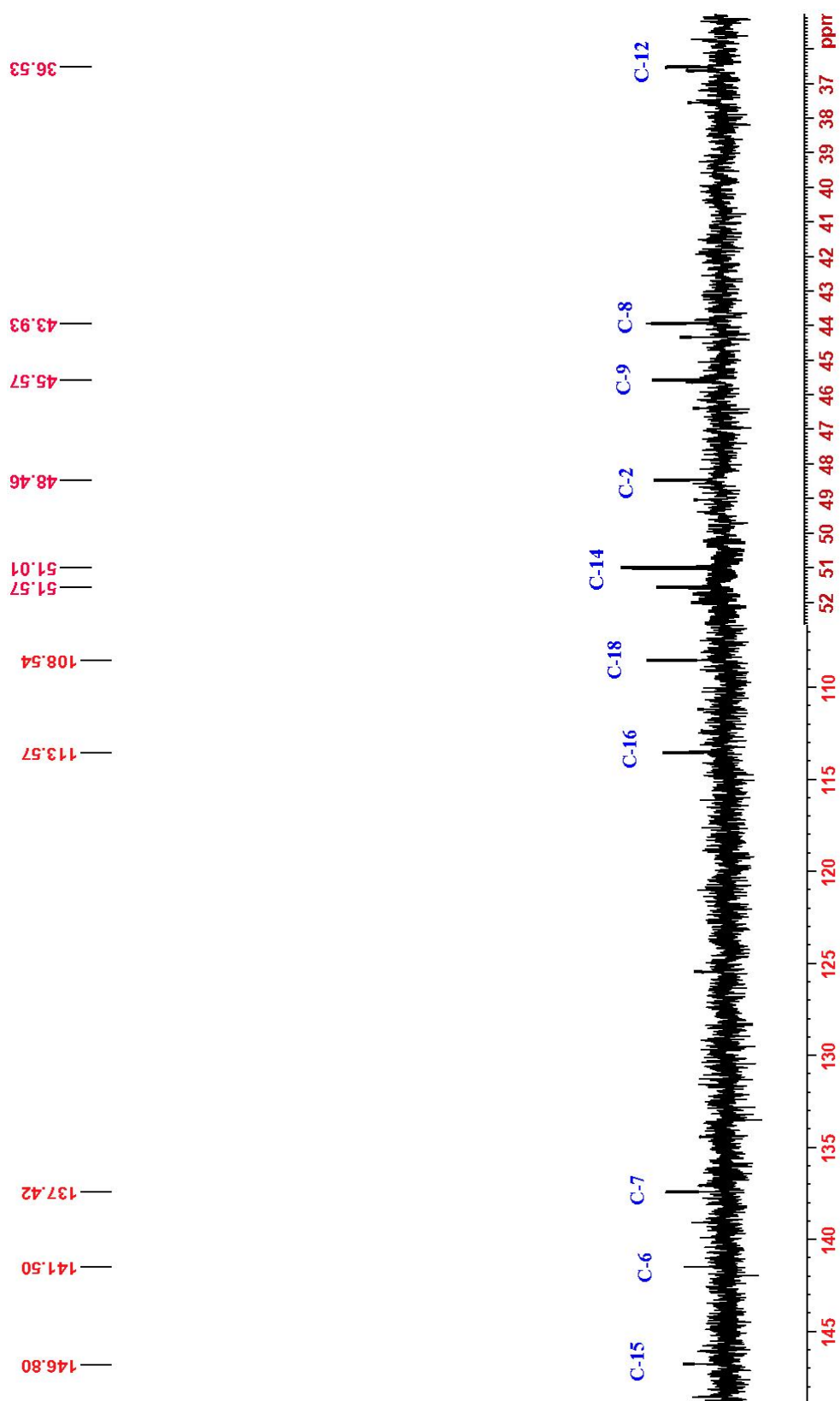
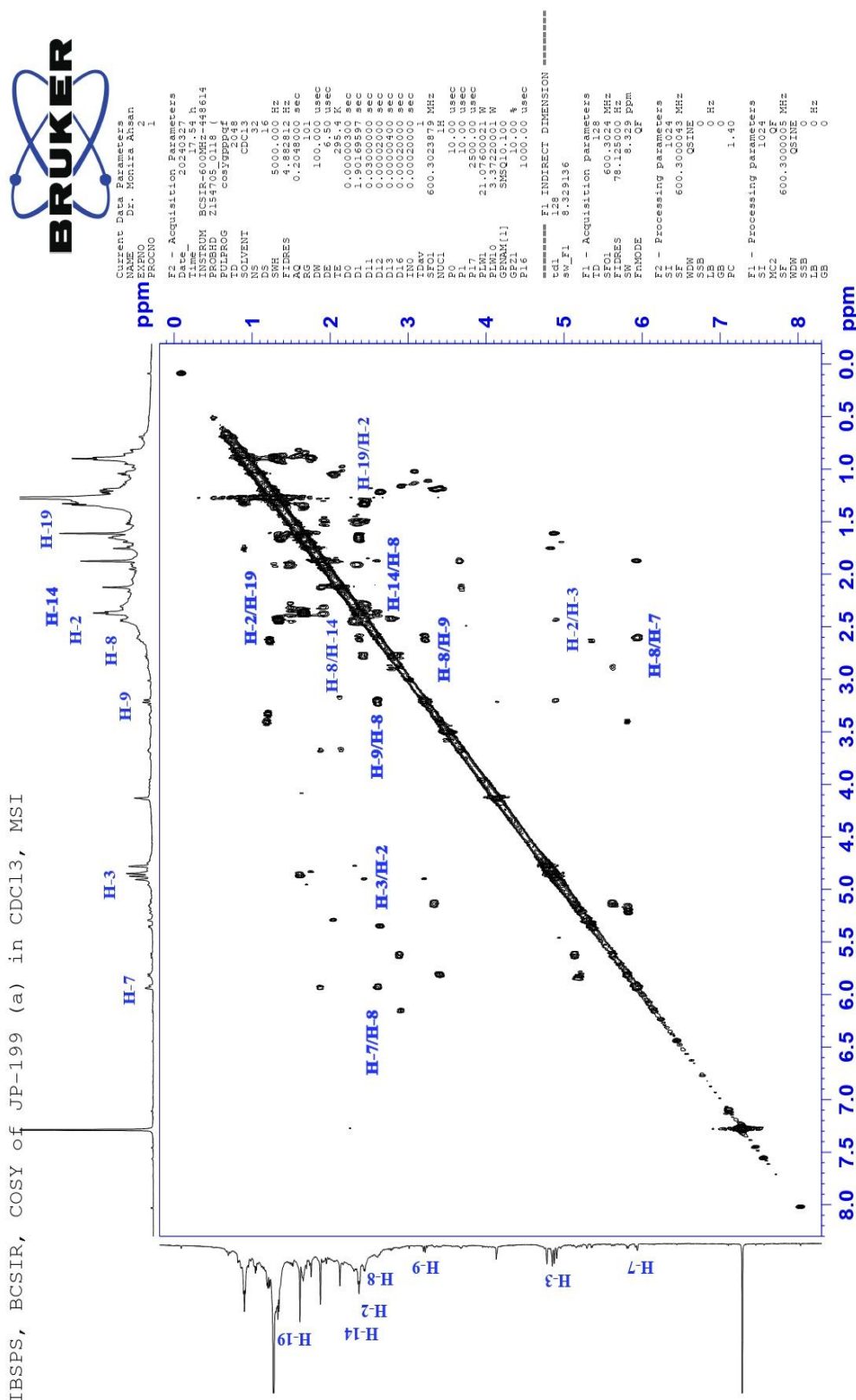


Figure 3.7: Partially expanded ^{13}C NMR (150 MHz, CDCl_3) for compound 1 (JP-199a)

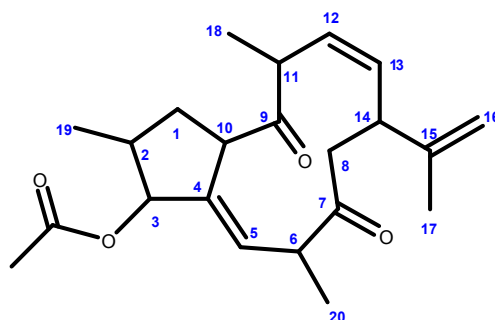
Figure 3.8: COSY spectrum (600 MHz, CDCl₃) of compound 1 (JP-199a)

3.1.2 Characterization of compound 2 (JP-200) as Integerrimene

Compound **2** was obtained as white mass that showed a yellowish spot under UV light at 254 nm and a blue fluorescent spot at 366 nm on a thin layer chromatography plate. When sprayed with vanillin-sulfuric acid reagent and heated for 3 minutes, it turned a light brown color.

The ^1H NMR spectrum (400 MHz, CDCl_3 ; Table: 3.3, Figure: 3.9) displayed three olefinic protons appeared as doublets of doublets at δ 5.78 ($J = 10, 1.6$ Hz), 5.11 ($J = 15.2, 9.2$ Hz) and 5.60 ($J = 15.2, 9.6$ Hz), assignable to H-5, H-12 and H-13, respectively of a diterpenoid. The spectrum also showed three secondary methyl groups at δ 1.17 ($J = 6.8$ Hz), 1.01 ($J = 6.4$ Hz), and 1.15 ($J = 6.8$ Hz) along with a deshielded methyl group at δ 1.72, which could be attributed to H-18, H-19, H-20 and H-17, respectively. An exocyclic methylene group was appeared at δ 4.81 and 4.74 (br s, each), attributed to H-16. The presence of an acetoxy group was revealed by the presence of the methyl singlet at δ 2.09 and an oximethine proton at δ 5.27 d ($J = 4.8$ Hz) assigned to H-3.

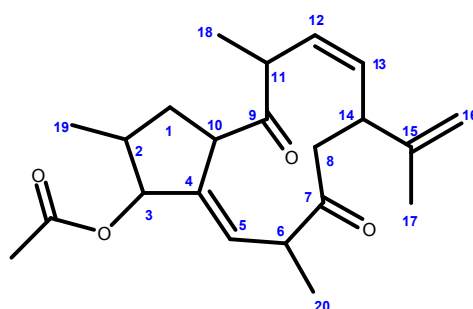
The ^1H NMR data of compound **2** was found identical to those reported for the macrocyclic diterpene integerrimene (Sutthivaiyakit et al., 2003) having 8,9-secorhamnofolate skeleton, previously isolated from the same plant.

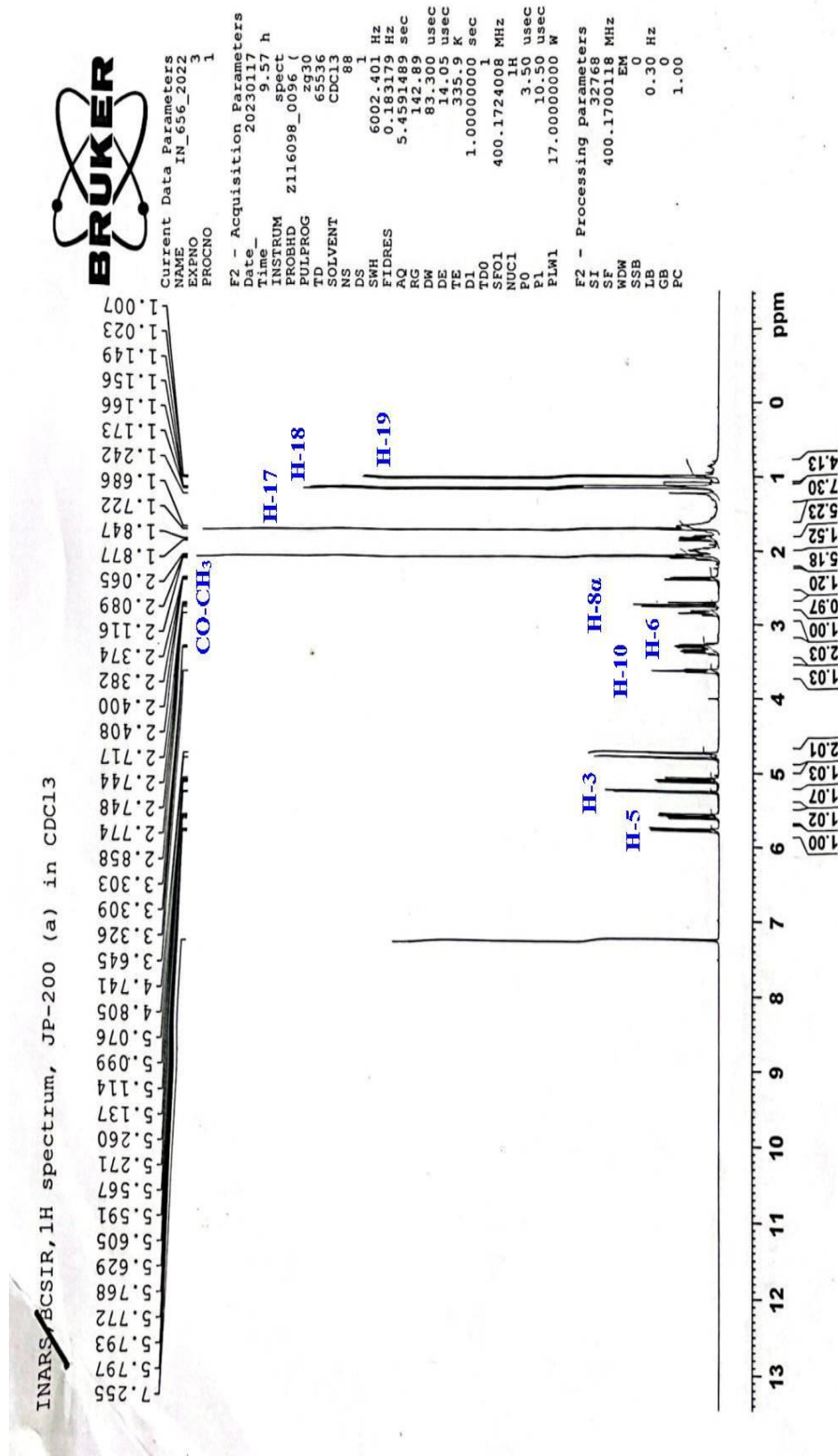


Integerrimene

Table 3.3: NMR spectral data (400 MHz, CDCl₃) of compound 2 (JP-200)

Position	Compound 2	Integerrimene (Sutthivaiyakit et al., 2003)
	δ_{H}	δ_{H}
3	5.27 d ($J = 4.8$ Hz)	5.25 d ($J = 4.6$ Hz)
5	5.78 dd ($J = 10, 1.6$ Hz)	5.77 dd ($J = 10.3, 1.8$ Hz)
6	3.37 dq ($J = 10, 7.2$ Hz)	3.36 dq ($J = 10.3, 7.2$ Hz)
8	α 2.75 dd ($J = 10.4, 12$ Hz) β 2.39 dd ($J = 10.4, 3.2$ Hz)	α 2.73 dd ($J = 12.2, 10.5$ Hz) β 2.38 dd ($J = 10.4, 2.9$ Hz)
10	3.65 dt ($J = 8.4, 1.6$ Hz)	3.63 dt ($J = 6.6, 1.8$ Hz)
11	3.31 dq ($J = 9.2, 6.8$ Hz)	3.29 dq ($J = 9.2, 6.7$ Hz)
12	5.11 dd ($J = 15.2, 9.2$ Hz)	5.10 dd ($J = 15.2, 9.4$ Hz)
13	5.60 dd ($J = 15.2, 9.6$ Hz)	5.58 dd ($J = 15.2, 9.6$ Hz)
14	2.86 dt ($J = 9.5, 3.0$ Hz)	2.85 br dt ($J = 9.6, 2.9$ Hz)
16	4.81 br s, 4.74 br s	4.80 br s, 4.73 br s
17	1.72 3H, s	1.71 3H, s
18	1.17 d ($J = 6.8$ Hz)	1.16 3H, d ($J = 6.7$ Hz)
19	1.01 3H, d ($J = 6.4$ Hz)	1.00 3H, d ($J = 6.6$ Hz)
20	1.15 3H, d ($J = 6.8$ Hz)	1.15 3H, d ($J = 7.2$ Hz)
COCH ₃	2.09 3H, s	2.09 3H s

**Integerrimene**

Figure 3.9: ¹H NMR (400 MHz, CDCl₃) of compound 2 (JP-200)

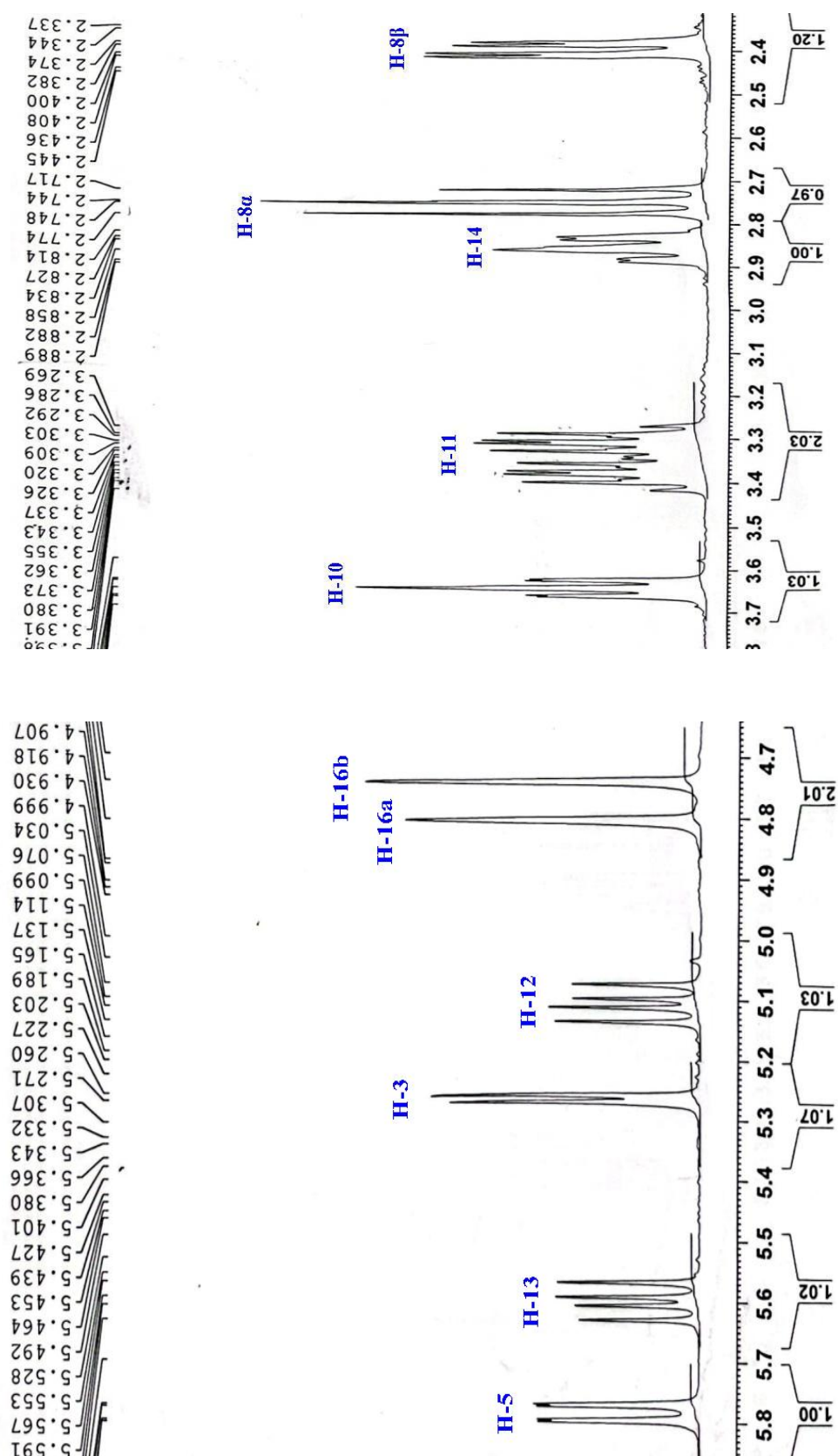


Figure 3.10: Partially expanded ¹H NMR (400 MHz, CDCl₃) (2.4-3.7) and (4.7-5.8) of compound 2 (JP-200)

3.1.3 Characterization of compound 3 (JP-460) as Jatrophatrione

Compound **3** obtained as a white powdery mass, was appeared as a yellowish spot under UV light at 254 nm and a quenching spot at 366 nm on a precoated TLC plate, and when sprayed with vanillin in sulfuric acid reagent followed by heating for 3 minutes, it developed a dark blue color.

The ^1H NMR spectrum (400 MHz, CDCl_3 ; Table: 3.4, Figure: 3.11) revealed one secondary methyl singlets as in compound **1** at δ 1.12 ($J = 7.2$ Hz), assignable to H-16. In addition, the spectrum displayed three quaternary methyl singlets at δ 1.21, 0.91, and 1.47, attributed to H-18, H-19 and H-20, respectively. The spectrum further showed a deshielded methyl at δ 1.94, indicating its attachment to an unsaturated carbon ($\text{CH}_3\text{-C}=\text{C}$), which could be attributed to H-17. Two olefinic protons singlet at δ 5.87 and 6.13 which are assignable to H-3 and H-5, respectively.

All these ^1H NMR data were found identical to those reported for jatrophatrione (Melchor-Martínez et al., 2017) and thus compound **3** was identified as jatrophatrione, a macrocyclic rhamnofolane diterpene.

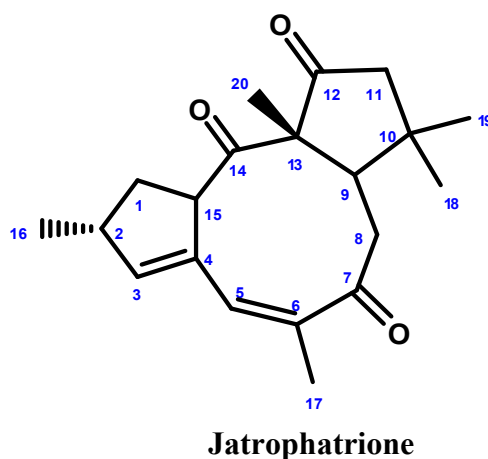
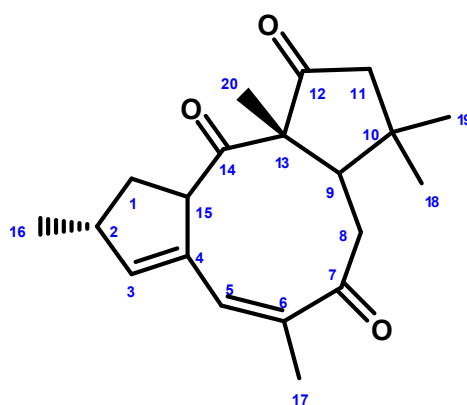
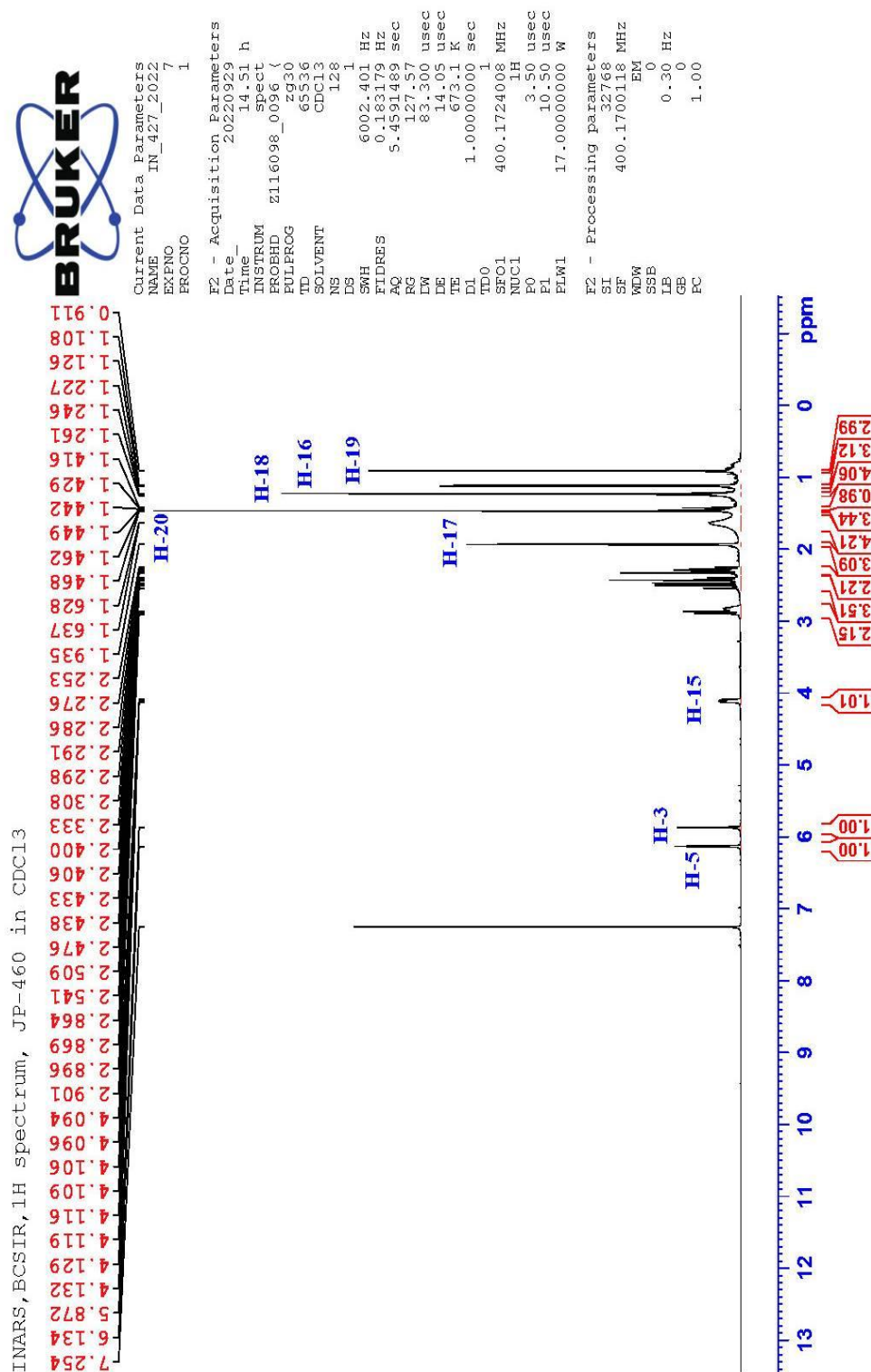


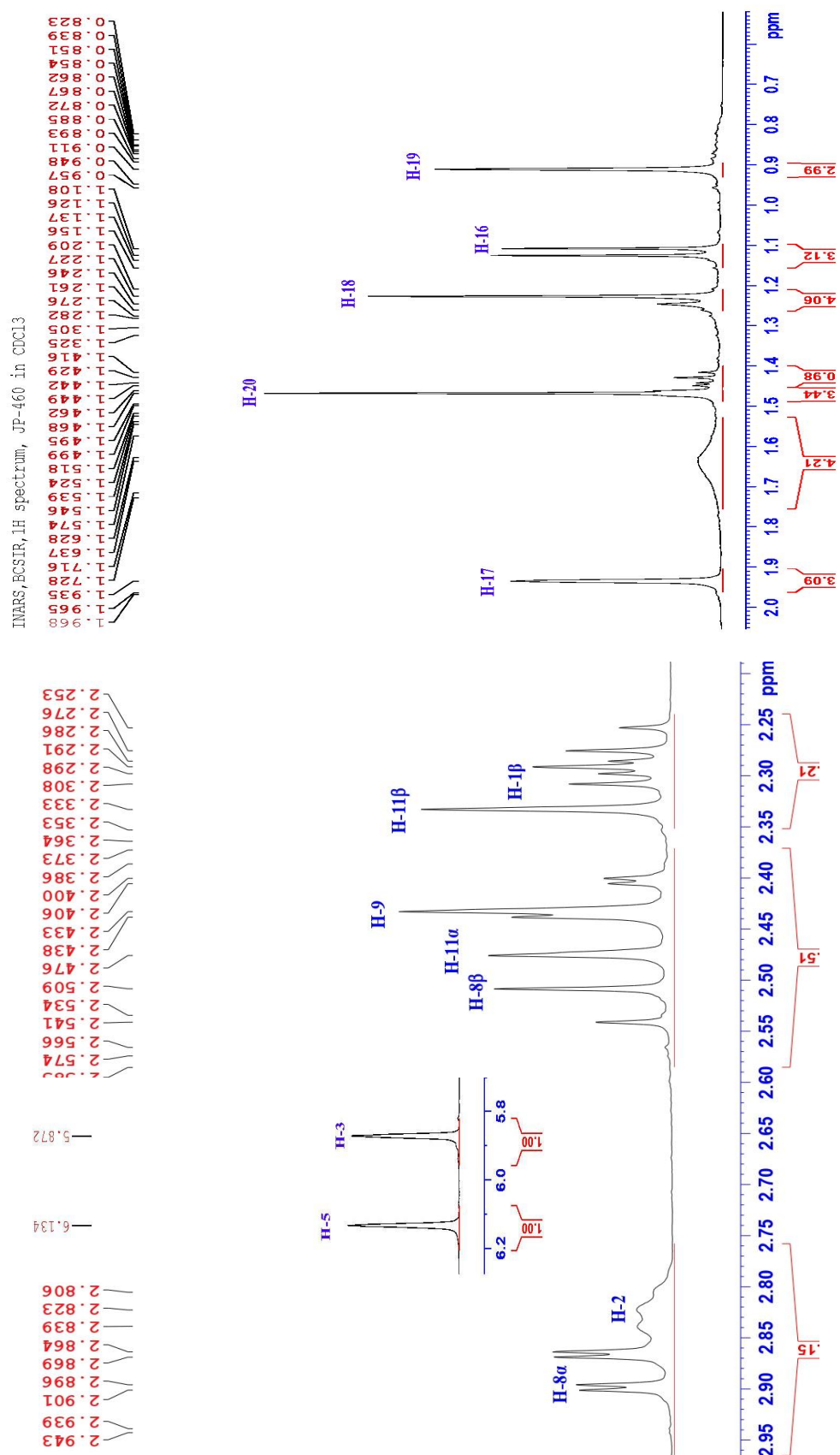
Table 3.4: NMR spectral data (400 MHz, CDCl₃) of compound 3 (JP-460)

Position	Compound 3		Jatrophatrione (Melchor-Martínez et al., 2017)	
		δ_{H}		δ_{H}
1	α	1.45 ddd ($J=13.1, 5.2, 5.1$ Hz)	α	1.45 ddd ($J=13.1, 5.2, 5.1$ Hz)
	β	2.30 ddd ($J=13.1, 9.1, 9.0$ Hz)	β	2.30 ddd ($J=13.1, 9.1, 9.0$ Hz)
2		2.82 m		2.80-2.87 m
3		5.87 br s		5.89 br s
5		6.13 br s		6.15 br s
8	α	2.89 dd ($J = 12.8, 2$ Hz)	α	2.90, dd ($J = 13.0, 2.1$ Hz)
	β	2.51 dd ($J = 13.2, 12.8$ Hz)	β	2.52 dd ($J = 13.1, 13$ Hz)
9		2.43 dd ($J = 13.1, 2.1$ Hz)		2.43 dd ($J=13.1, 2.1$ Hz)
11	α	2.45 d ($J=17$ Hz)	α	2.46 d ($J=16.7$ Hz)
	β	2.32 d ($J=17$ Hz)	β	2.33 d ($J=16.7$ Hz)
15		4.12 dddd ($J=9.1, 5.1, 2.9, 1.5$ Hz)		4.12 dddd ($J=9.1, 5.1, 2.9, 1.5$ Hz)
16		1.12 d ($J=7.2$ Hz)		1.13 d ($J=7.0$ Hz)
17		1.94 s		1.95 b s
18		1.21 s		1.24 s
19		0.91 s		0.92 s
20		1.47 s		1.48 s



Jatrophatrione

Figure 3.11: ¹H NMR (400 MHz, CDCl₃) of compound 3 (JP-460)

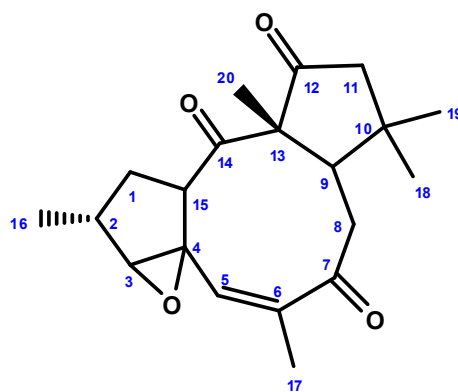
Figure 3.12: Partially expanded ¹H NMR (400 MHz, CDCl₃) of compound 3 (JP-460)

3.1.4 Characterization of compound 4 (JP-500) as Citlaltirione:

Compound **4** was obtained as colorless needle shaped crystals. On a TLC plate, it appeared as light yellow spot at 254 nm and a blue fluorescent spot at 366 nm. Spraying with vanillin-sulfuric acid reagent followed by heating for 3 minutes, it developed a yellowish color.

The ^1H NMR spectrum (400 MHz, CDCl_3 ; Table: 3.5, Figure: 3.13) were found similar to that of compound **3**, except that it displayed only one olefinic proton instead of two, at δ 5.50 ($J = 1.6$ Hz), which is assignable to H-5. As in compound **3**, its spectrum also exhibited five methyl signals including one secondary methyl at δ 1.15 ($J = 7.6$ Hz), three quaternary methyl at δ 1.26, 0.96 and 1.43, assignable to H-16, H-18, H-19, and H-20, respectively, and a deshielded methyl at δ 1.93, assignable to H-17. Instead of the olefinic proton at position 3, an oximethine proton was appeared at δ 3.29 as a singlet, suggesting the formation of an epoxy group.

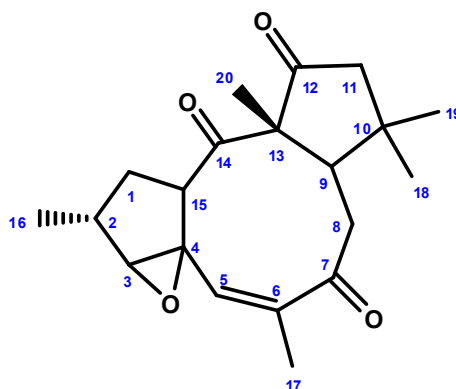
The ^1H NMR spectrum data of compound **4** was found identical to those reported for the macrocyclic diterpenoid citlaltirione (Melchor-Martínez et al., 2017), a known compound isolated from the same plant.



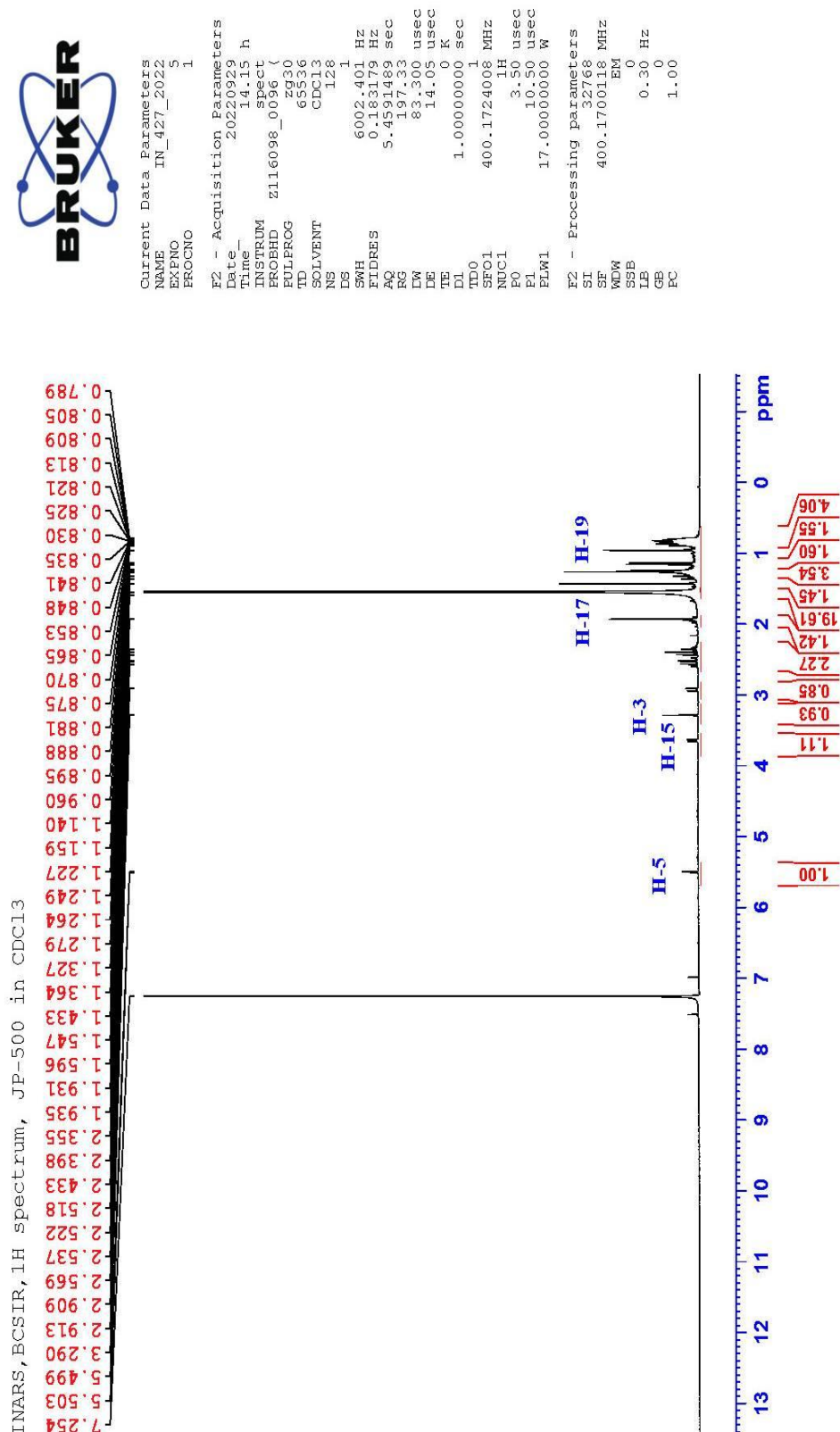
Citlaltirione

Table 3.5: NMR spectral data (400 MHz, CDCl₃) of compound 4 (JP-500)

Position	Compound 4		Citlaltirione (Melchor-Martínez et al., 2017)	
	δ_H		δ_H	
1	α	1.32 ddd ($J=14.8$, Hz)	α	1.35 ddd ($J=13, 1.5, 1.3$ Hz)
	β	1.95 ddd ($J=13.7, 9.5, 8.8$ Hz)	β	1.95 ddd ($J=13.7, 9.5, 8.8$ Hz)
2		2.34 m		2.36 dqd ($J=8.8, 7.5, 1.3$ Hz)
3		3.29 s		3.30 s
5		5.50 q ($J=1.6$ Hz)		5.51 q ($J=1.6$ Hz)
8	α	2.93 dd ($J=12.4, 1.6$ Hz)	α	2.95 dd ($J=12.7, 1.8$ Hz)
	β	2.54 dd ($J=12.8, 7.6$ Hz)	β	2.58dd ($J=13.3, 12.7$ Hz)
9		2.50 dd ($J=12.8, 18.8$ Hz)		2.51 dd ($J=13.3, 1.8$ Hz)
11		2.40 s		2.42 d ($J=12$ Hz)
15		3.64 d ($J=8.8$, Hz)		3.66dd ($J=9.5, 1.5$ Hz)
16		1.15 d ($J=7.6$ Hz)		1.16 d ($J=7.5$ Hz)
17		1.93 d ($J=1.6$ Hz)		1.95 d ($J=1.6$ Hz)
18		1.26 s		1.28 s
19		0.96 s		0.97 s
20		1.43 s		1.44 s



Citlaltirione

Figure 3.13: ¹H NMR (400 MHz, CDCl₃) of compound 4 (JP-500)

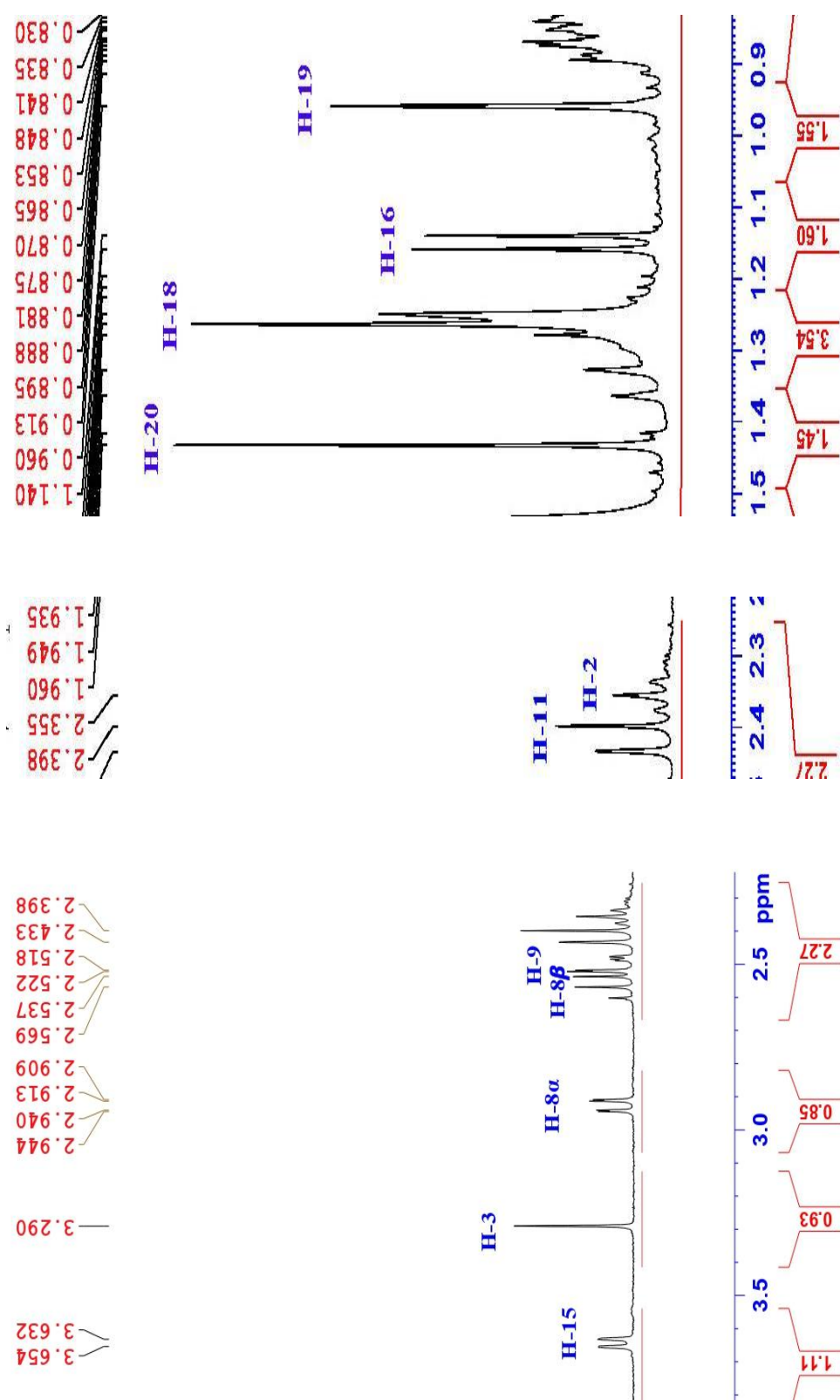
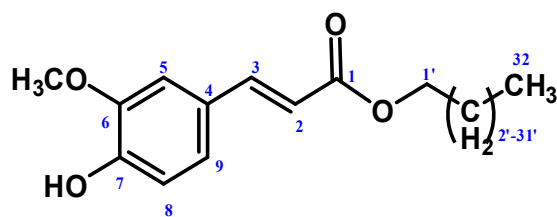


Figure 3.14: Partially expanded ^1H NMR (400 MHz, CDCl_3) of compound 4 (JP-500)

3.1.5 Characterization of compound 5 (JP-05) as Dotriacontyl *trans*-ferulate

Compound 5 was obtained as white amorphous powder. On a TLC plate, it appeared as a quenching spot at 254 nm and bright yellowish fluorescence spot at 366 nm. When sprayed with vanillin-sulfuric acid reagent and heated for 3 minutes, it developed a dark blue spot.

The EIMS (Figure: 3.22) of compound 5 displayed a molecular ion peak at m/z 642.4 (10.8%, M^+) and a base peak at m/z 614.1 (100%) attributed to the loss of $\text{CH}_2\text{-CH}_2$ (28) from the molecular ion. The ^1H NMR spectrum (400 MHz, CDCl_3 ; Table: 3.6 Figure: 3.16) showed three aromatic proton signals at δ 7.03 br s, 6.91 ($J = 8.2$ Hz), and 7.06 ($J = 8.2$ Hz) indicating the presence of a trisubstituted benzene ring and were attributed to H-5, H-8, and H-9, respectively. Further the spectrum displayed two olefinic proton doublets at δ 6.29 and 7.61 with a large coupling constant of 16 Hz (each) indicating their *trans* relationship and a methoxy group singlet at δ 3.92. All these data together indicated the presence of a *trans*-ferulic acid moiety in the molecule. A deshielded methylene group appeared as a triplet at δ 4.18 ($J = 6.8$ Hz) suggesting its attachment to an oxygen atom and to another methylene group. This signal along with a multiplet at δ 1.25-1.38 integrated for 54 protons, three 2H multiplets at δ 1.69, 1.39 & 1.28 and a terminal methyl signal at δ 0.87 ($J = 6.8$ Hz) is characteristic for an aliphatic chain with carbon C-32. The ^{13}C NMR spectrum (150 MHz, CDCl_3 ; Table: 3.6, Figure: 3.18) revealed the presence of one ester carbonyl at δ 167.41 and two olefinic methine carbons at δ 115.69 and 144.64. In the HSQC spectrum the later two carbons showed direct correlation to the *trans* coupled protons at δ 6.29 and 7.60, respectively. In addition, the ^{13}C NMR spectrum exhibited three aromatic methine carbons at δ 109.27, 114.7 & 123.06, two oxygenated carbons at δ 147.88 & 146.78 and a quaternary carbon at δ 127.07. The methoxy carbon and oxymethylene carbon signals were appeared at δ 55.94 and 64.64, respectively. Other aliphatic carbon signals were appeared at δ 28.78 (C-2'), 29.32-31.94 (C-3'- C-29'), 26.01 (C-30'), 22.71(C-31') and 14.14 (terminal methyl, C-32'). The HSQC (600 MHz, CDCl_3 ; Table: 3.6, Figure: 3.20) spectrum showed the expected direct (1J) correlations between protons and carbon signals. On the basis of above observations, compound 5 was identified as dotriacontyl *trans*- ferulate which is a new compound, isolated for the first time from a natural source.

Dotriacontyl *trans*-ferulateTable 3.6: NMR spectral data (400 MHz, CDCl₃) of compound 5 (JP-05)

Position	Compound 5		
	δ_{H}	δ_{C}	HSQC
1	---	167.41	---
2	6.29 d ($J = 16$ Hz)	115.69	115.69
3	7.60 d ($J = 16$ Hz)	144.64	144.64
4	---	127.07	---
5	7.03 br s	109.27	109.27
6	---	147.88	---
7	---	146.78	---
8	6.91 d ($J = 8.2$ Hz)	114.70	114.70
9	7.06 br d ($J = 8.2$ Hz)	123.06	123.06
1'	4.18 2H, t ($J = 6.8$ Hz)	64.64	64.64
2'	1.69 2H, m	28.78	28.78
3'-29'	1.25-1.38 54 H, m	29.32-31.94	29.32-31.94
30'	1.39 m	26.01	26.01
31'	1.28 m	22.71	22.71
32'	0.87 3H, t ($J = 6.8$ Hz)	14.14	14.14
OCH ₃	3.92 3H, s	55.94	55.94
OH-7	5.85 br s	---	----

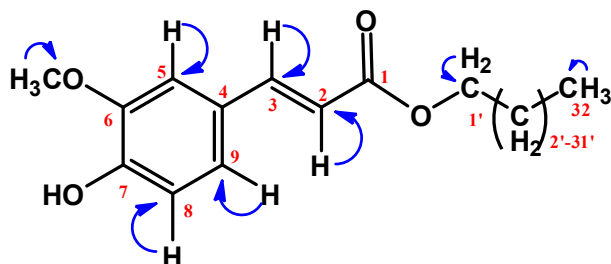
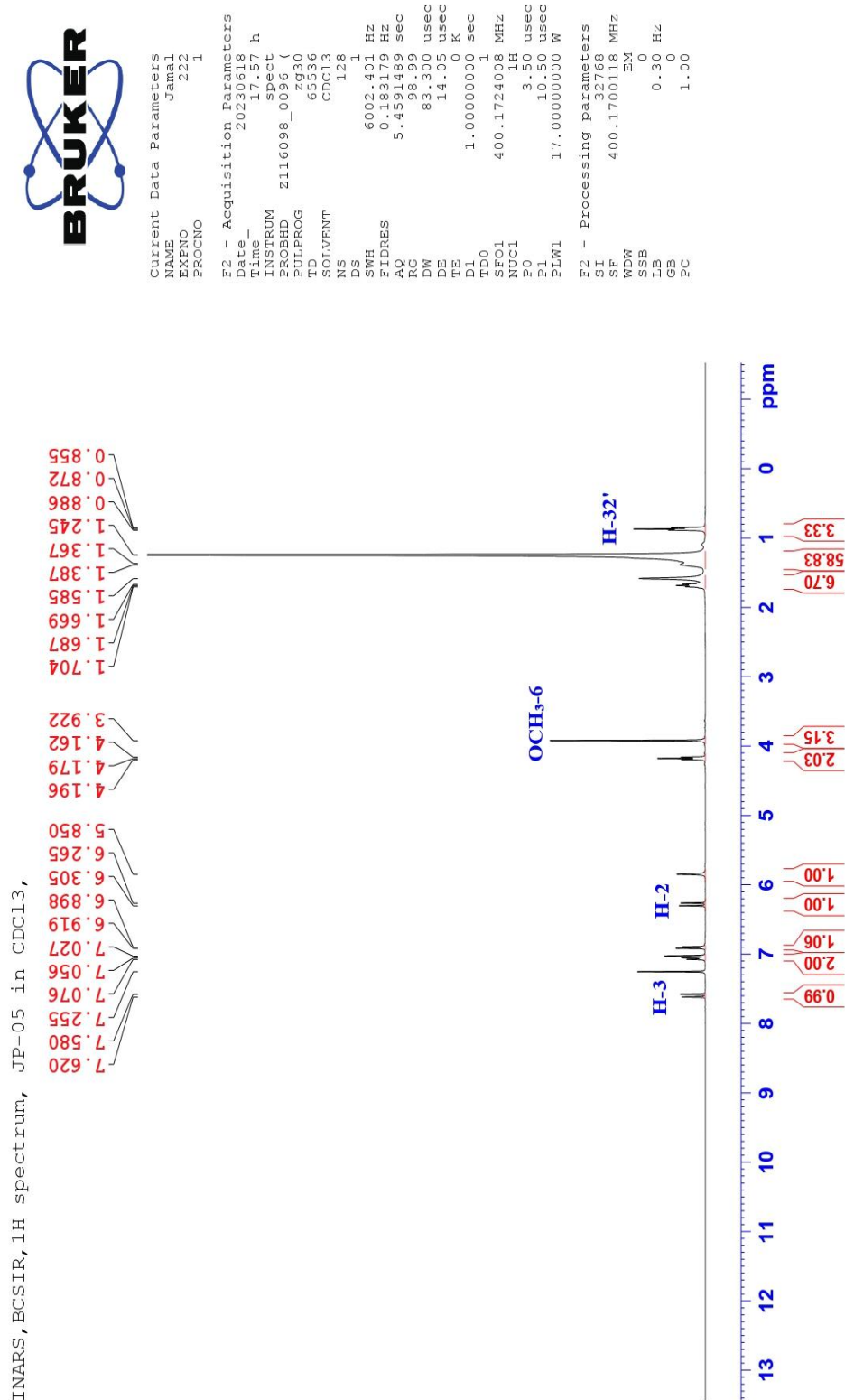


Figure 3.15 Key HSQC correlations in compound 5

Figure 3.16: ¹H NMR (400 MHz, CDCl₃) of compound 5 (JP-05)

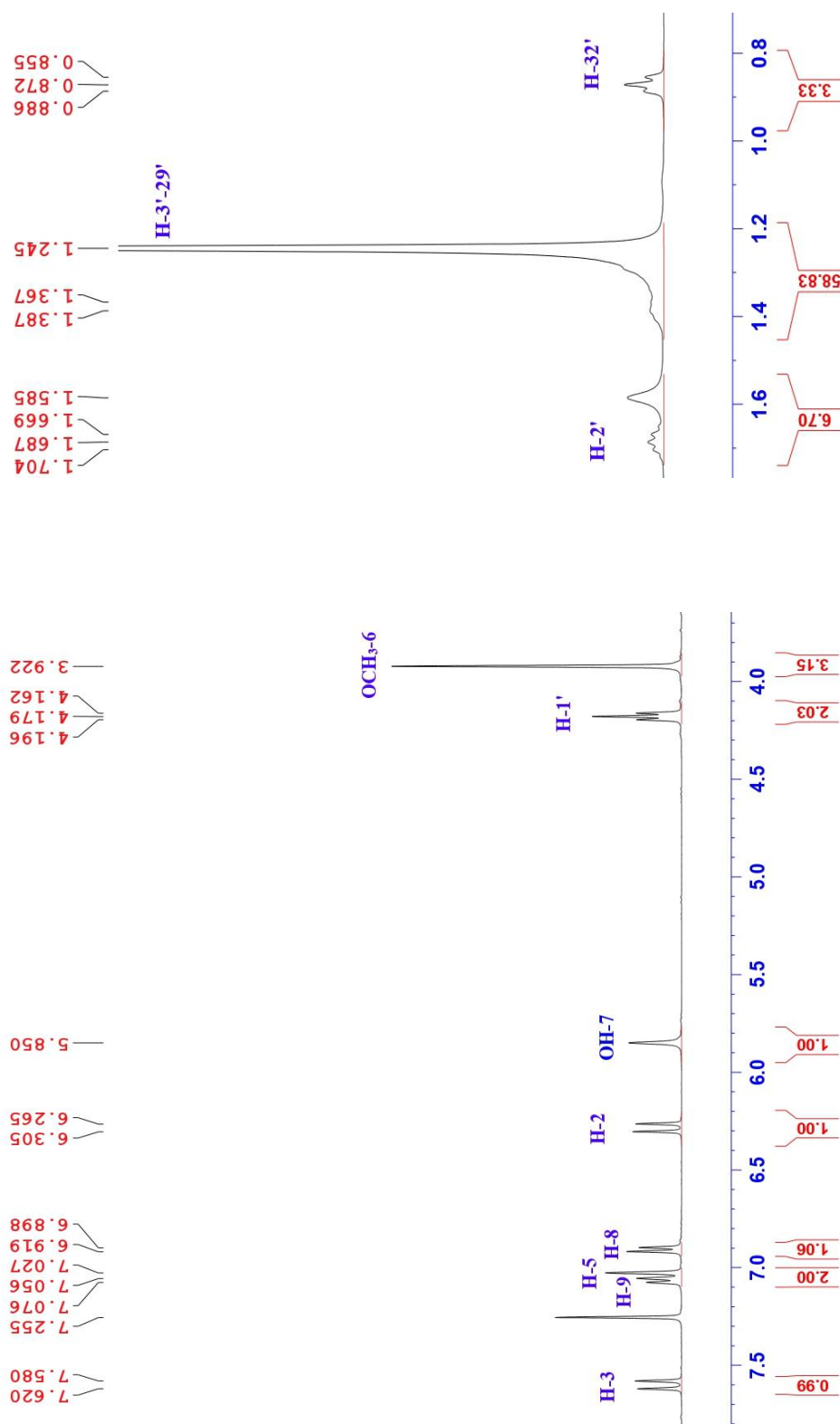
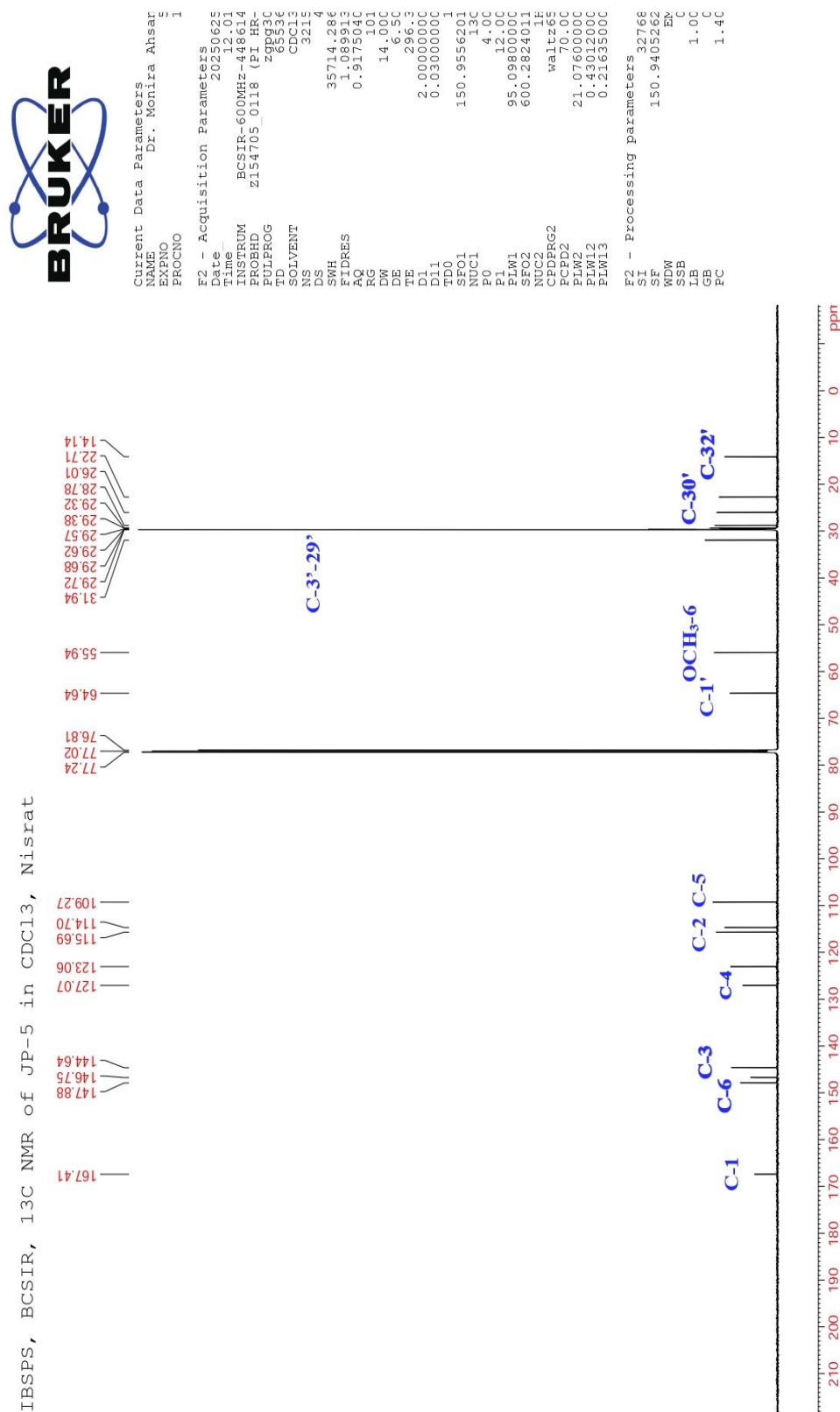


Figure 3.17: Partially expanded ¹H NMR (400 MHz, CDCl₃) of compound 5 (JP-05)

Figure 3.18: ¹³C NMR (150 MHz, CDCl₃) of compound 5 (JP-05)

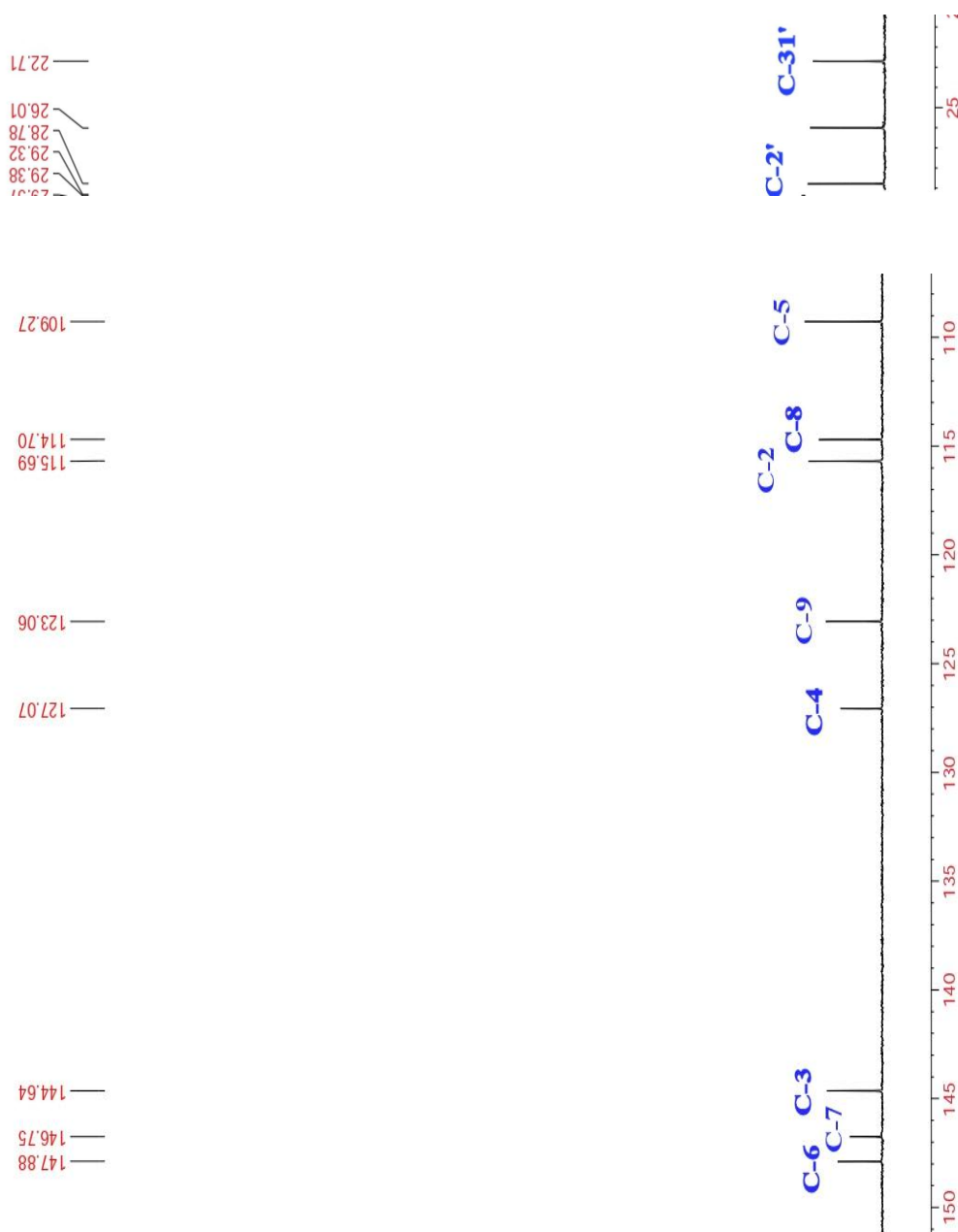
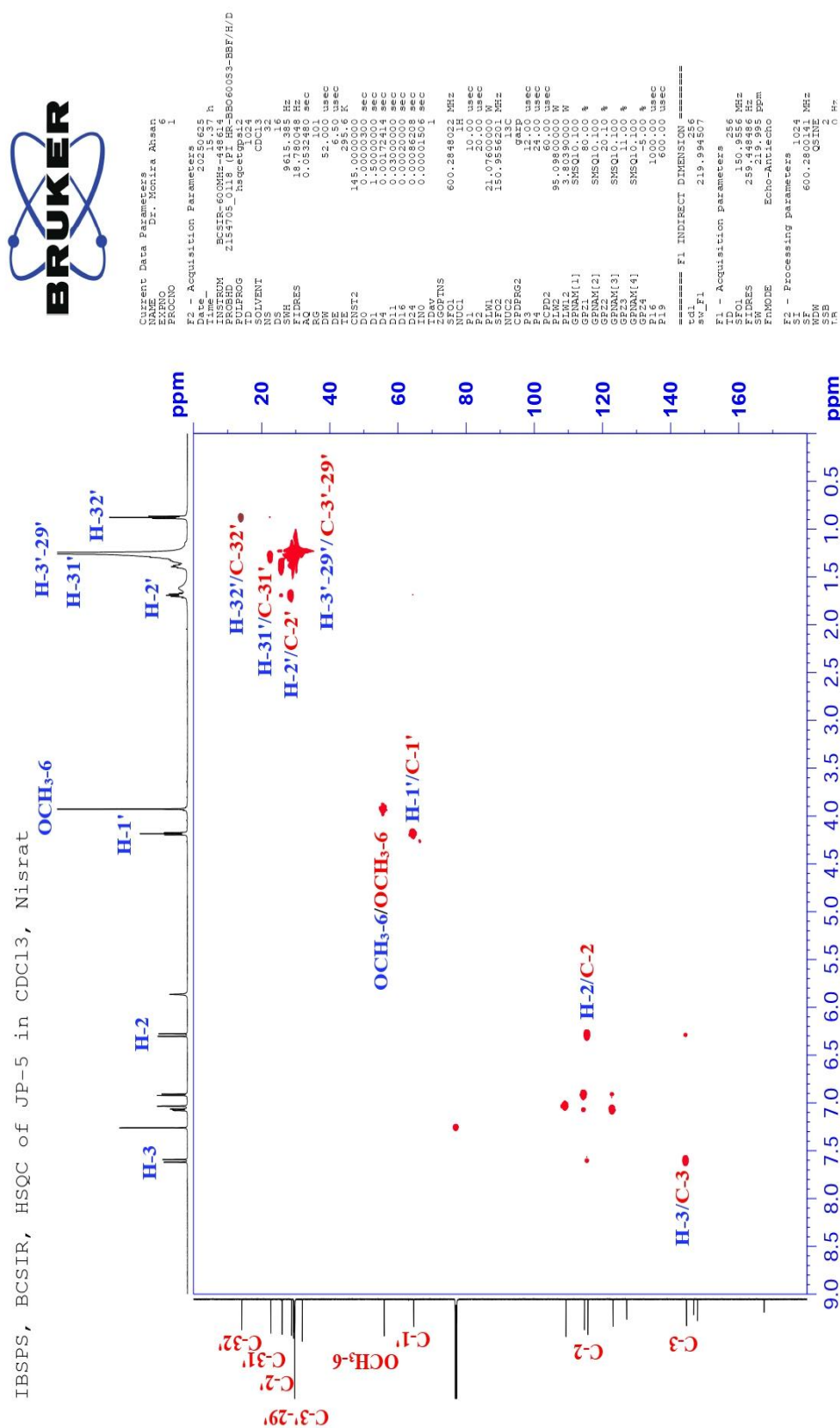
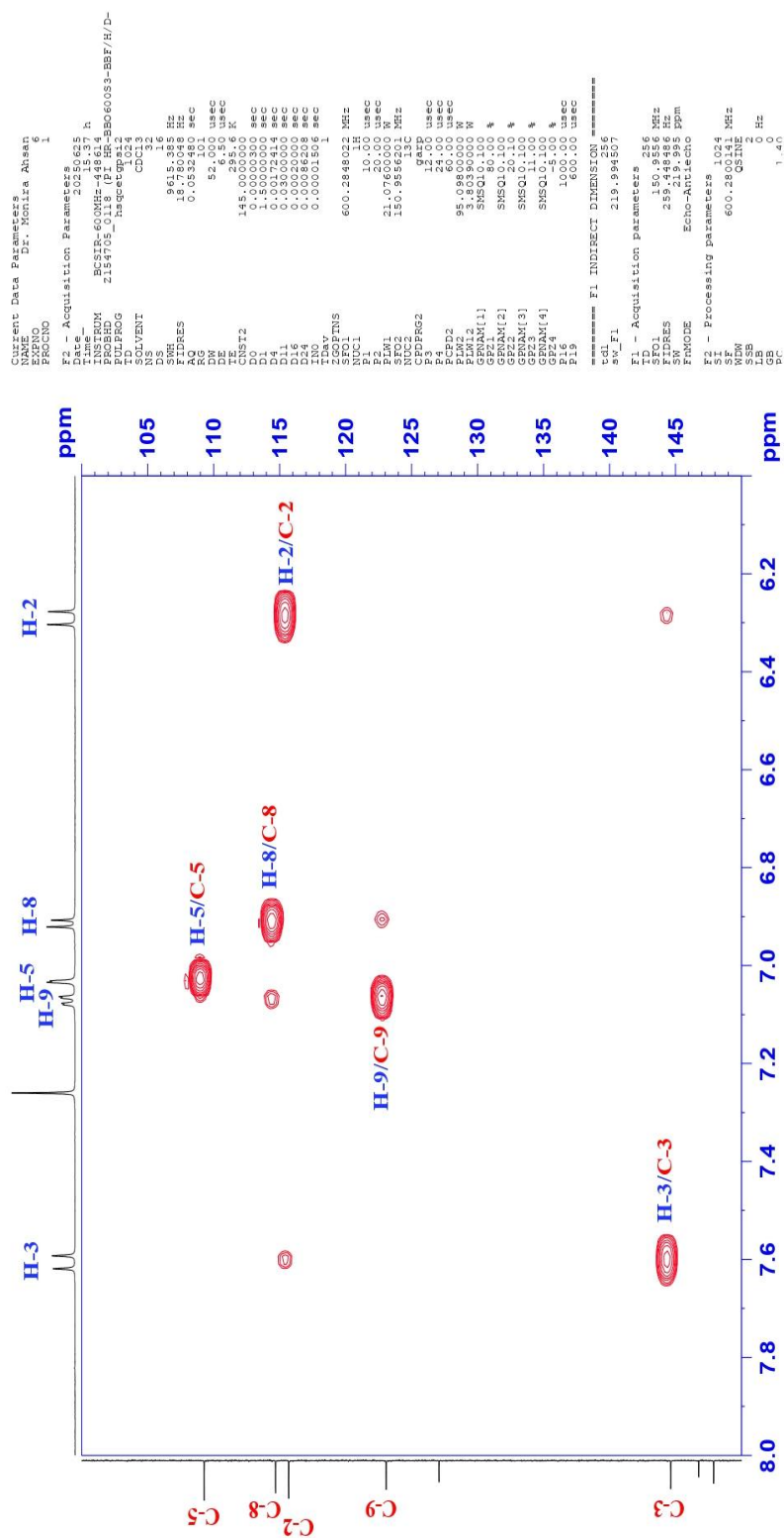


Figure 3.19: Partially expanded ^{13}C NMR (150 MHz, CDCl_3) of compound 5 (JP-05)

Figure 3.20: HSQC (600 MHz, CDCl₃) spectrum of compound 5 (JP-05)

IBSPS, BCSIR, HSQC of JP-5 in CDCl₃, Nisrat



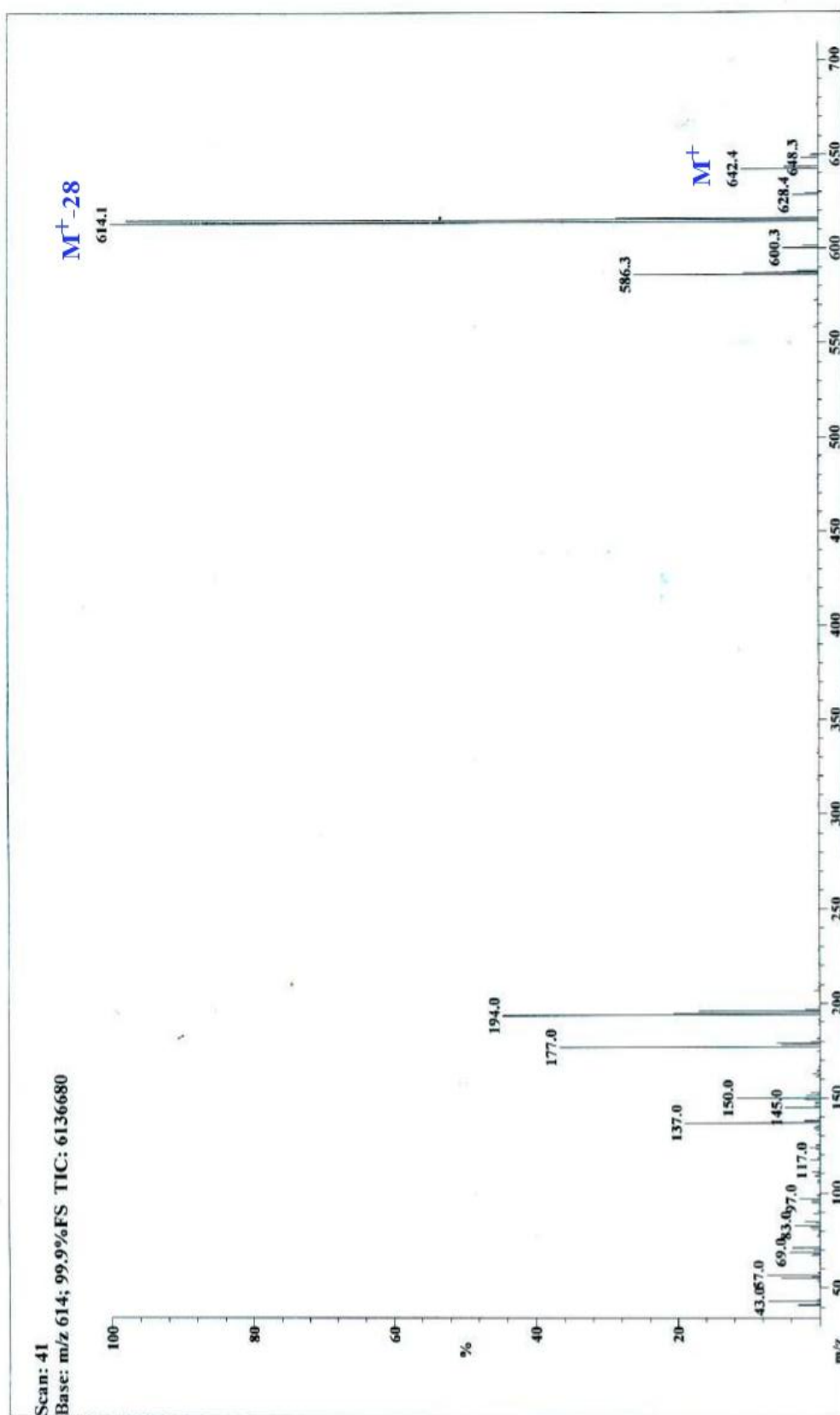


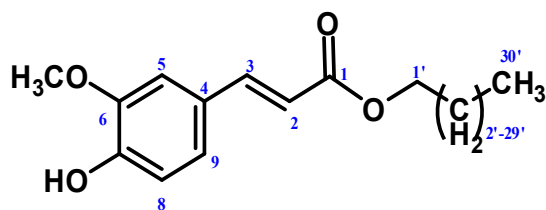
Figure 3.22: Mass spectrum of compound 5 (JP-05)

3.1.6 Characterization of compound 6 (JP-25) as Triacontyl *trans*-ferulate:

Compound **6**, isolated as white crystalline powder, produced a quenching spot at 254 nm and a yellow fluorescent spot at 366 nm on a TLC plate. When sprayed with vanillin in sulfuric acid reagent followed by heating for 2 minutes, it developed a dark purple color.

The EIMS (Figure: 3.29) of compound **6** displayed a molecular ion peak at m/z 614.2 (100%, M^+). The ^1H NMR spectrum (400 MHz, CDCl_3 ; Table: 3.7, Figure: 3.24) was similar to that of compound **5**, except that the multiplet at δ 1.25-1.38 was integrated for 50 protons instead of 54 protons suggesting the n-alkyl part as a triacontyl moiety. In the spectrum, three aromatic proton signals resonated at δ 7.03 br s, 6.91 ($J = 8$ Hz) and 7.07 ($J = 8.1$ Hz) were assigned as H-5, H-8 and H-9, respectively. The methoxy group resonated at δ 3.93 and the *trans*-coupled olefinic protons appeared at δ 6.29 and 7.61 (d, $J = 16$ Hz each). The triacontyl part was confirmed by the oximethylene triplet at δ 4.18 ($J = 8$ Hz), three 2H multiplets at δ 1.68, 1.38 and 1.27, an upfield 50 H multiplet at δ 1.25-1.38 and a terminal methyl signal at δ 0.88 ($J = 6.5$ Hz). The ^{13}C NMR spectrum (150 MHz, CDCl_3 ; Table: 3.7, Figure: 3.25) was similar to that of compound **5** where the ester carbonyl resonated at δ 167.42 and two olefinic methine carbons at δ 115.69 and 144.64. In addition, the spectrum exhibited three aromatic methine carbons at δ 109.28, 114.7 and 123.06, two oxygenated carbons at δ 147.89 & 146.75 and a quaternary carbon at δ 127.07. The methoxy carbon and oxymethylene carbon signals were resonated at δ 55.94 and 64.64, respectively. The aliphatic carbon signals were appeared at δ 28.78 (C-2'), 29.32-31.94 (C-3'- C-27'), 26.01 (C-28'), 22.71 (C-29') and 14.14 (terminal methyl, C-30'). The HSQC (600 MHz, CDCl_3 ; Table: 3.7, Figure: 3.27) spectrum was used for correct assignments of carbons and protons.

All these data allowed identification of compound **6** as triacontyl *trans*-ferulate and were found identical to those reported for the compound (Ochienget al., 2017). This long chain alkyl ferulate was isolated for the first time from *J. pandurifolia*.

Triacontyl *trans*-ferulateTable 3.7: NMR spectral data (400 MHz, CDCl₃) of compound 6 (JP-25)

Position	Compound 6		
	δ_{H}	δ_{C}	HSQC
1	---	167.42	---
2	6.29 d ($J = 16$ Hz)	115.69	115.69
3	7.61 d ($J = 16$ Hz)	144.64	144.64
4	---	127.07	---
5	7.03 br s	109.28	109.27
6	---	147.89	---
7	---	146.75	---
8	6.91 d ($J = 8.1$ Hz)	114.70	114.70
9	7.07 br d ($J = 8.1$ Hz)	123.06	123.06
1'	4.18 2H, t ($J = 8$ Hz)	64.64	64.64
2'	1.68 2H, m	28.78	28.78
3'-27'	1.25-1.38 50 H, m	29.32-31.94	29.32-31.94
28'	1.38 m	26.01	26.01
29'	1.27 m	22.71	22.71
30'	0.88 3H, t ($J = 6.5$ Hz)	14.14	14.14
OCH ₃	3.93 3H, s	55.94	55.94
OH-7	5.85 br s	---	----

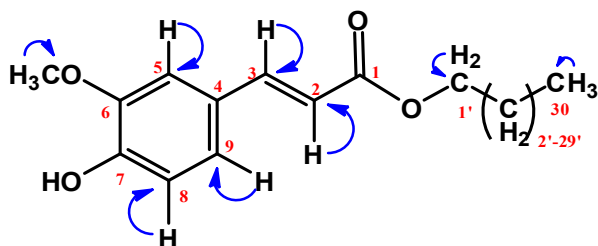
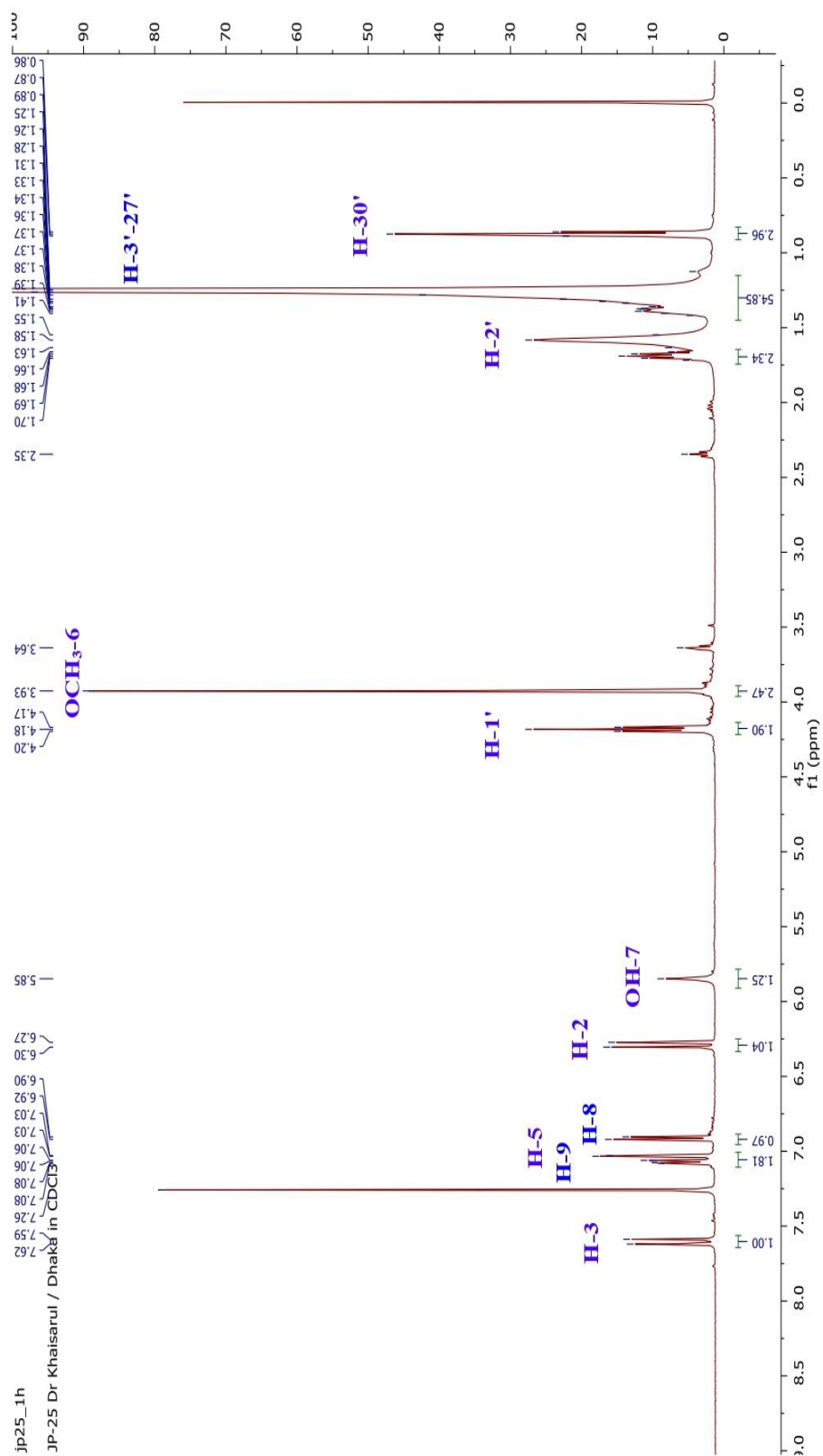
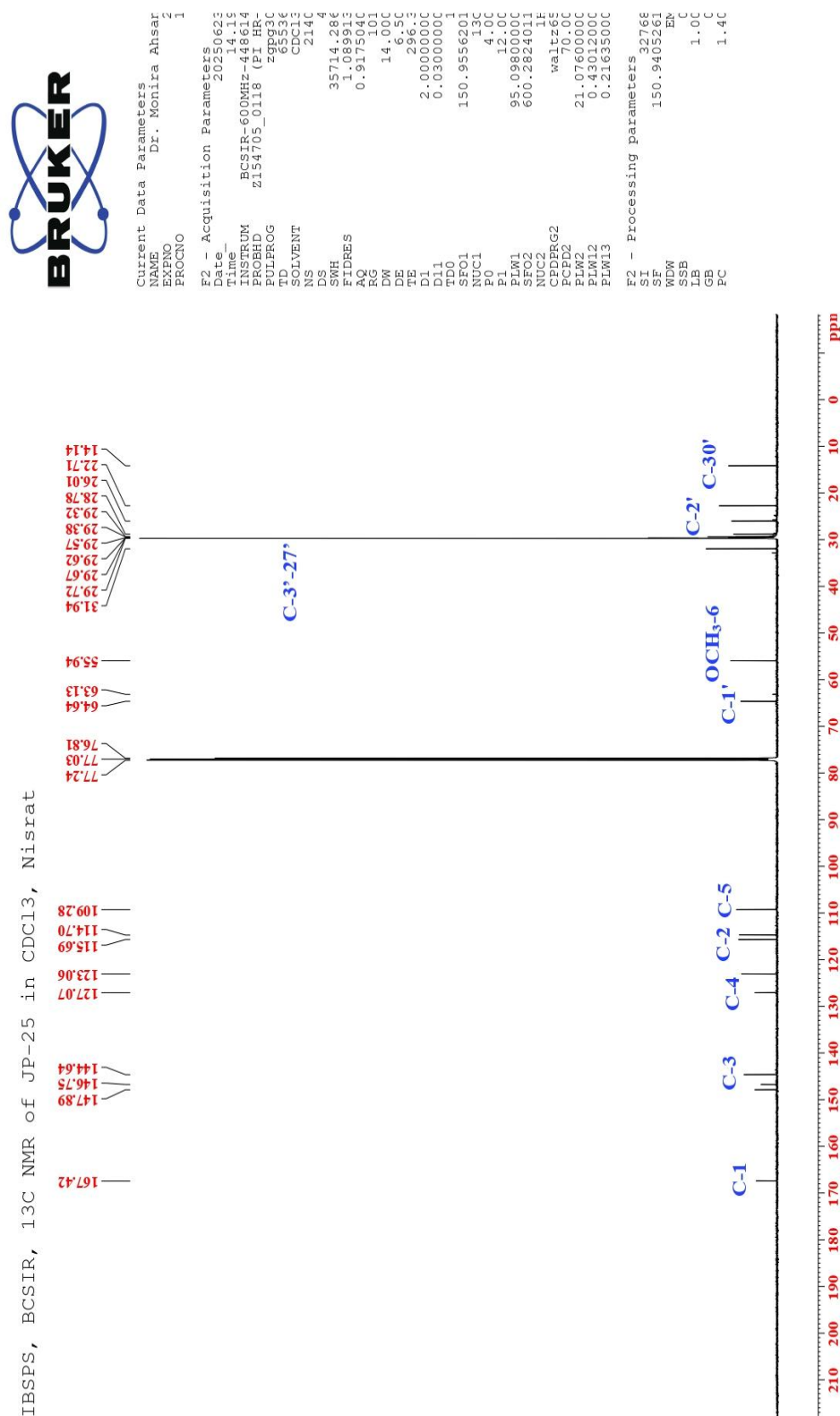


Figure 3.23: Key HSQC correlations in compound 6

Figure 3.24: ¹H NMR (400 MHz, CDCl₃) of compound 6 (JP-25)

Figure 3.25: ¹³C NMR (150 MHz, CDCl₃) of compound 6 (JP-25)

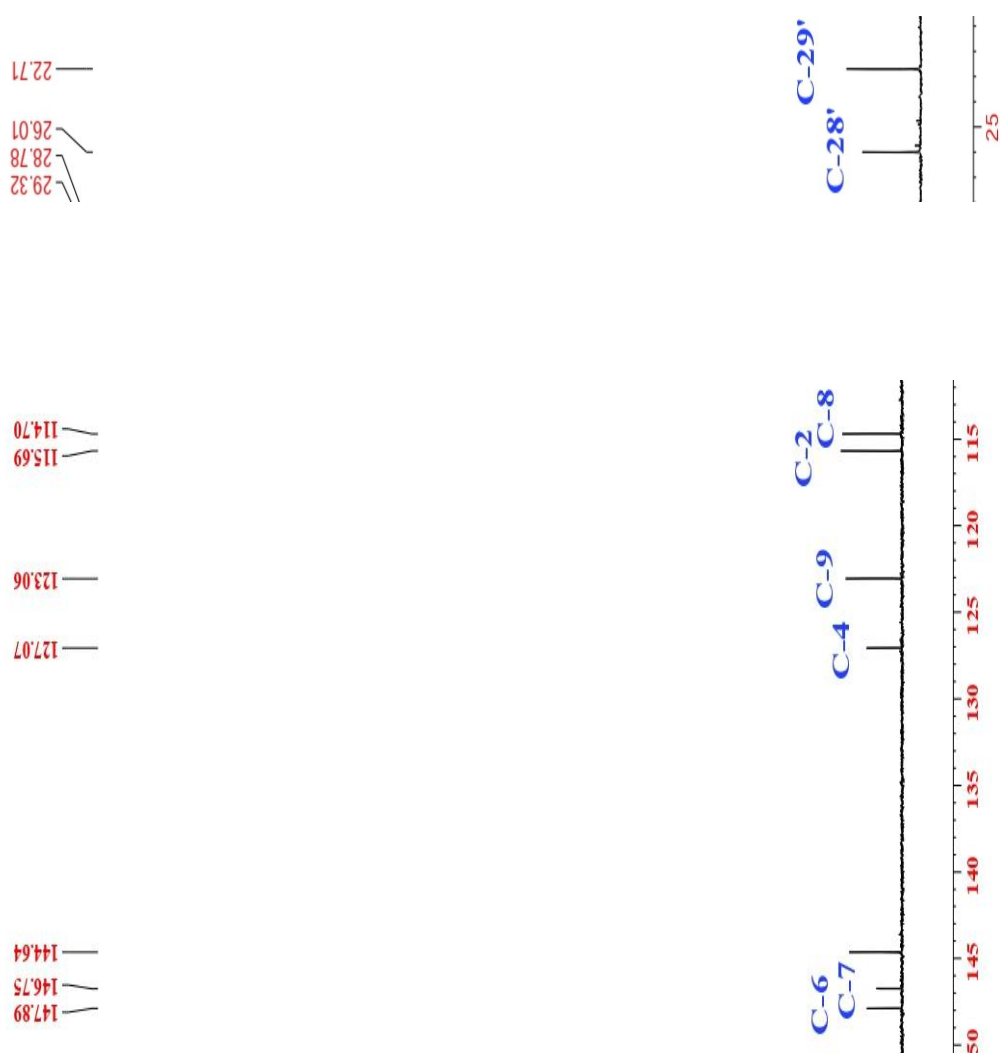


Figure 3.26: Partially expanded ^{13}C NMR (150 MHz, CDCl_3) of compound 6 (JP-25)

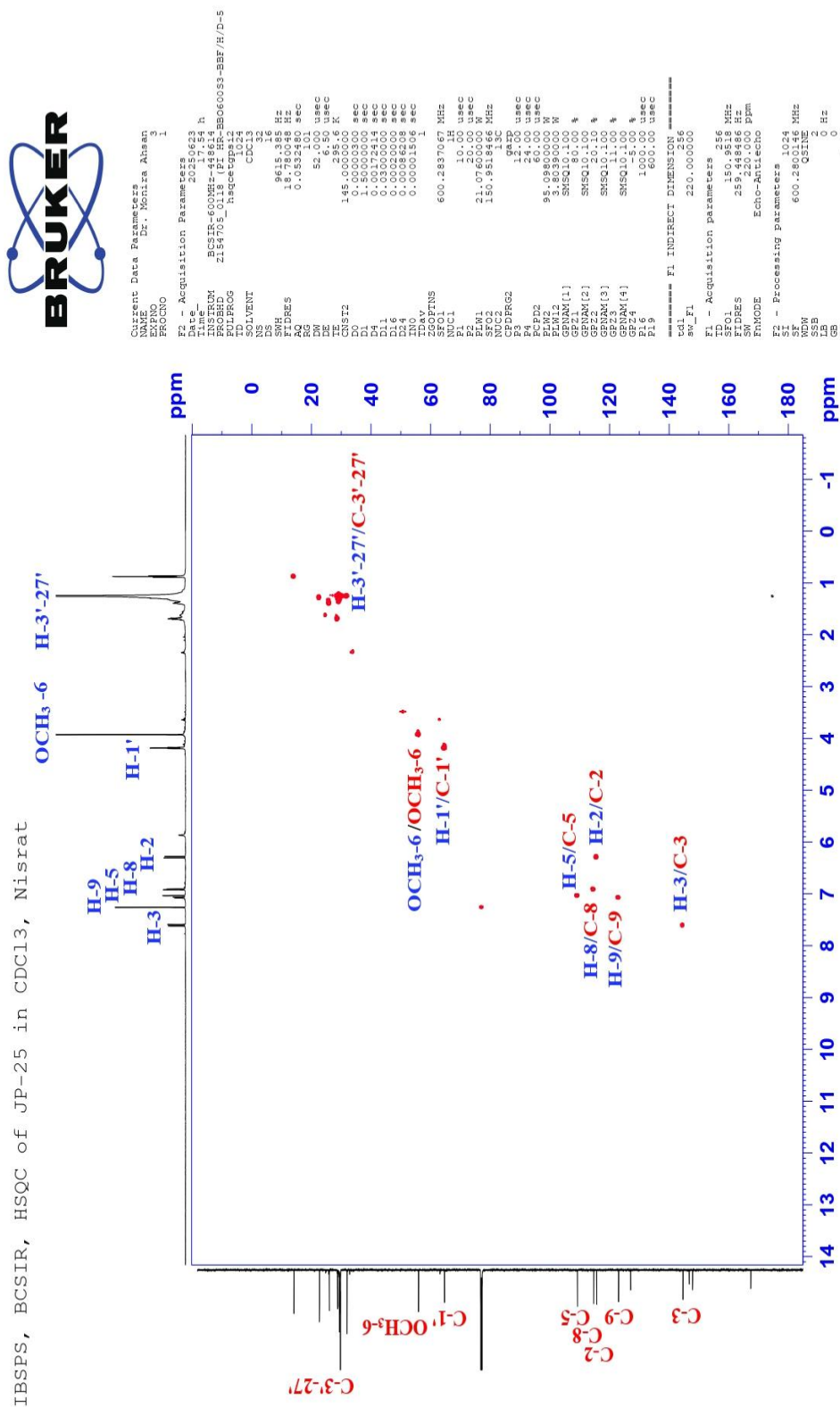


Figure 3.27: HSQC (600 MHz, CDCl₃) spectrum of compound 6 (JP-25)



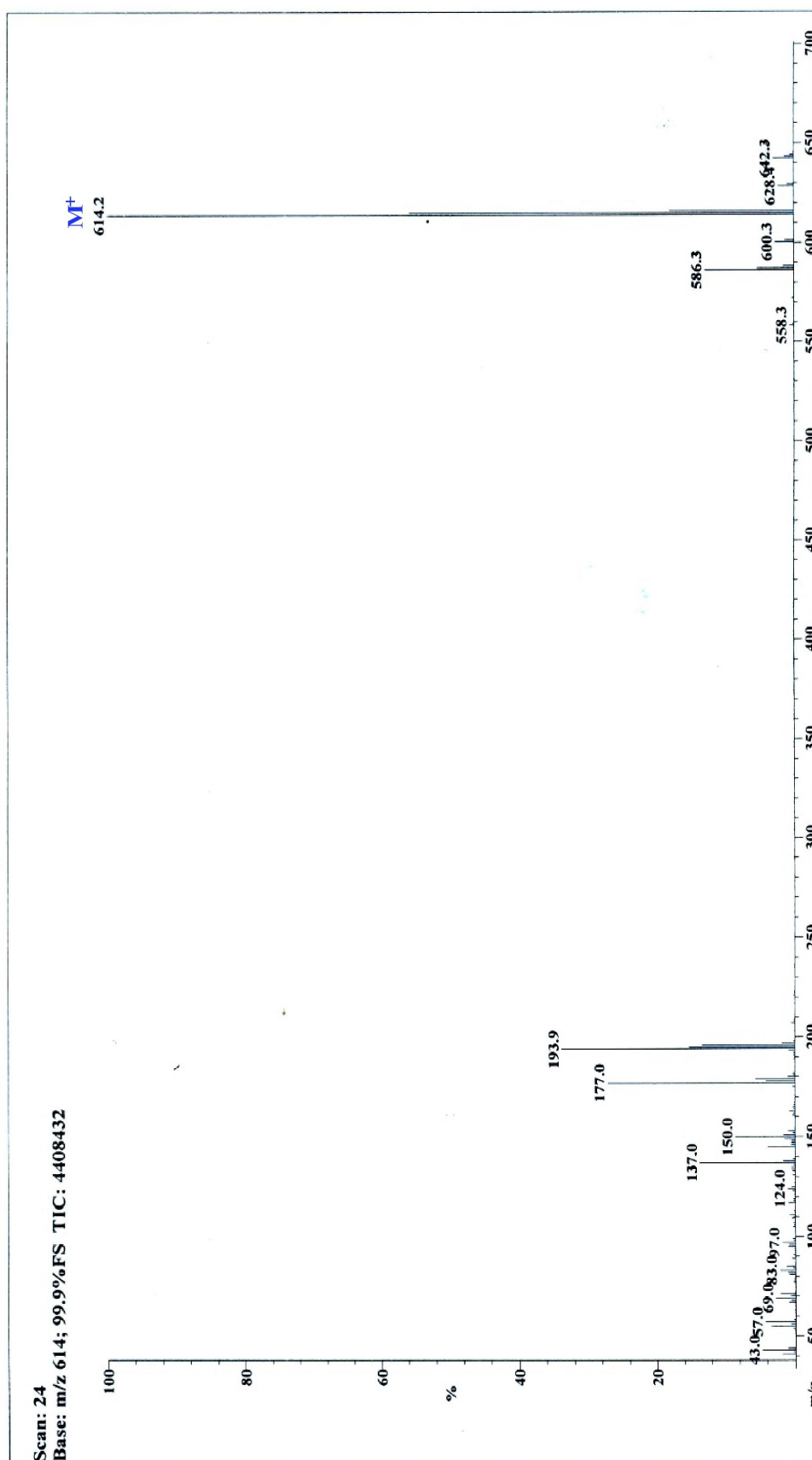


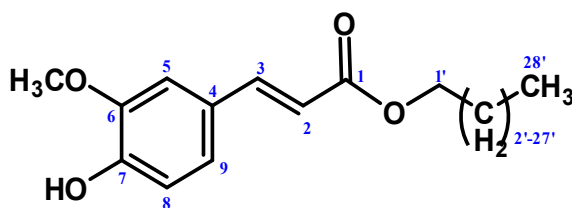
Figure 3.29: Mass spectrum of compound 6 (JP-25)

3.1.7 Characterization of compound 7 (JP-81) as Octacosyl *trans*-ferulate:

Compound 7 was obtained as white crystalline powder. On a TLC plate, it appeared as a quenching spot under UV light at 254 nm and a blue fluorescent spot at 366 nm. When sprayed with vanillin-sulfuric acid reagent and heated for 2 minutes, it produced a dark blue spot.

The ^1H NMR spectrum (400 MHz, CDCl_3 ; Table: 3.8, Figure: 3.30) presented three aromatic proton multiplets at δ 7.03 ($J = 1.6$ Hz), δ 6.91 ($J = 8.2$ Hz) and δ 7.07 ($J = 8.2, 1.6$ Hz), indicating the presence of a trisubstituted benzene ring and attributed to H-5, H-8, and H-9, respectively. Further the spectrum exhibited two olefinic proton doublets at δ 6.28 and δ 7.60 with a coupling constant of 16 Hz each, attributed to H-2 and H-3, respectively and a methoxy group singlet at δ 3.92. These data indicated the presence of a *trans* ferulic acid moiety in the molecule. An oximethylene group resonated as a triplet at δ 4.18 ($J = 6.6$ Hz), assignable to H-1'. A multiplet at δ 1.25-1.39 integrated for 50 protons assignable to H-3'-27'. Another multiplet at δ 1.68 is attributed to H-2'. The terminal methyl signal was appeared at δ 0.87 ($J = 6.8$ Hz) and assignable to H-28'. These data supported the presence of an octacosane moiety in the structure.

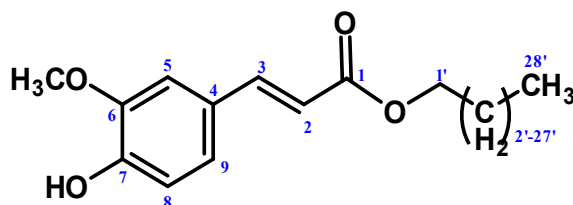
The ^1H NMR data were consistent to those reported for octacosyl *trans*-ferulate (Gutiérrez-Tlahque et al., 2019). On the basis of above observation compound 7 was identified as octacosyl *trans*-ferulate. This is the first report on the isolation of this compound from *J. pandurifolia*.

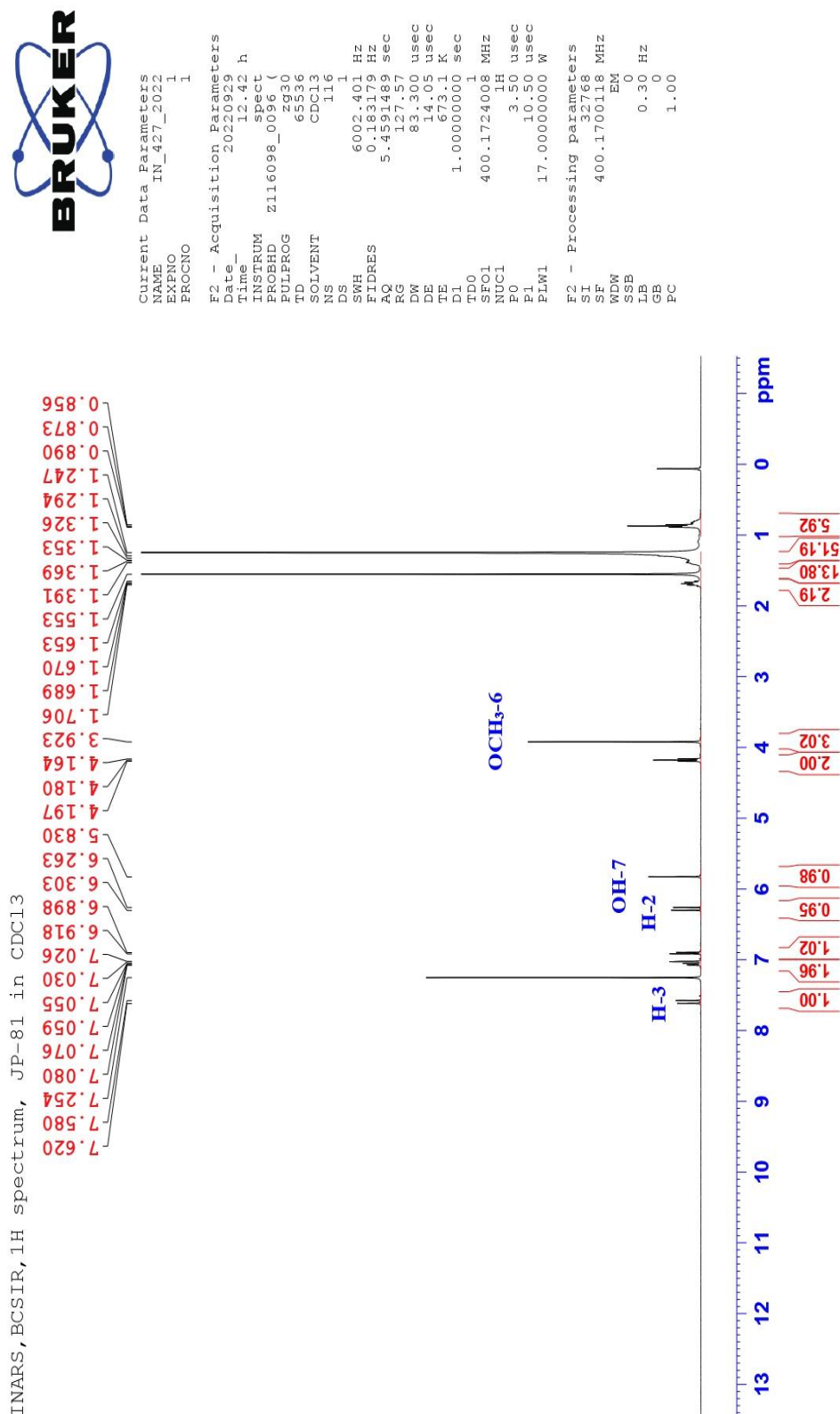


Octacosyl *trans*-ferulate

Table 3.8: NMR spectral data (400 MHz, CDCl₃) of compound 7 (JP-81)

Position	Compound 7	Octacosyl <i>trans</i> -ferulate (Gutiérrez-Tlahque et al., 2019)
	δ_{H}	δ_{H}
2	6.28 d ($J = 16$ Hz)	5.81 d ($J = 13$ Hz)
3	7.60 d ($J = 16$ Hz)	6.79 d ($J = 13$ Hz)
5	7.03 d ($J = 1.6$ Hz)	7.77 d ($J = 2$ Hz)
8	6.91 d ($J = 8.2$ Hz)	6.87 d ($J = 8.5$ Hz)
9	7.07 dd ($J = 8.2, 1.6$ Hz)	7.10 d ($J = 8.5$ Hz)
1'	4.18 2H, t ($J = 6.6$ Hz)	4.11 2H, t ($J = 6$ Hz)
2'	1.68 2H m	1.68 2H m
3'-27'	1.25-1.39 50 H, m	1.27 m
28'	0.87 3H, t ($J = 6.8$ Hz)	0.90 3H, t ($J = 6.8$ Hz)
OCH ₃	3.92 3H, s	3.95 3H, s
OH-7	5.83	5.87

Octacosyl *trans*-ferulate

Figure 3.30: ¹H NMR (400 MHz, CDCl₃) of compound 7 (JP-81)

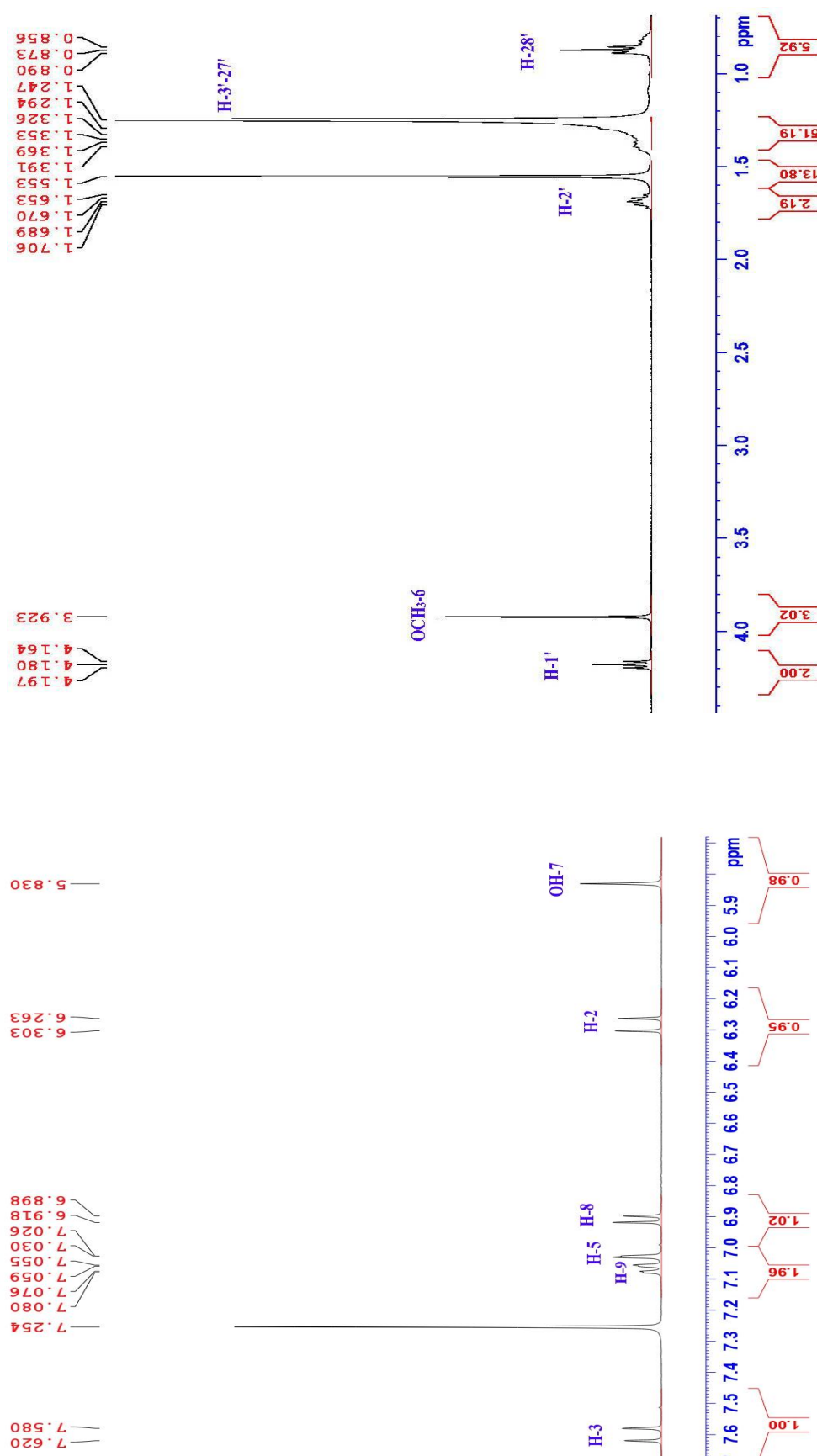


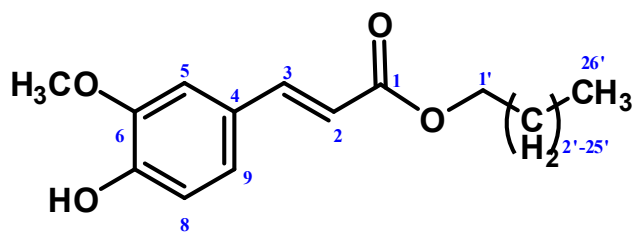
Figure 3.31: Partially expanded ^1H NMR (400 MHz, CDCl_3) (0.8-4.50) and (5.9-7.6) of compound 7 (JP-81)

3.1.8 Characterization of compound 8 (JP-13) as Hexacosyl *trans*-ferulate:

Compound **8** obtained as white crystalline powder, gave quenching spot at 254 nm and a yellow fluorescent spot at 366 nm on a TLC plate. After spraying with vanillin in sulfuric acid reagent and heating for 3 minutes, it developed a dark purple color.

The ^1H NMR spectrum (400 MHz, CDCl_3 ; Table: 3.9, Figure: 3.32) displayed three aromatic proton signals at δ 7.03 br s, 6.91 ($J = 8$ Hz), and 7.07 ($J = 8.1$ Hz) indicating the presence of a trisubstituted benzene ring as in compound **5** and were attributed to H-5, H-8, and H-9, respectively. The spectrum also exhibited a methoxy group at δ 3.92 and two olefinic proton doublets at δ 6.29 and 7.61 with a large coupling constant of 16 Hz (each) indicating their *trans* relationship instead of *cis*, unlike compound **5**. These data suggested the presence of a *trans* ferulic acid moiety in the molecule. An oximethylene group appeared as triplet at δ 4.18 ($J = 6.2$ Hz) could be attributed to H-1'. A multiplet signal at δ 1.64 that is assignable to H-2'. Another multiplet at δ 1.25-1.39 integrated for 46 protons assignable to H-3'-25'. The terminal methyl signal was appeared at δ 0.88 ($J = 6.5$ Hz) and assigned as H-26'. These data supported the presence hexacosyl moiety in the molecule. The ^1H NMR data were found identical to those reported for hexacosyl *trans*-ferulate (Fraga et al., 2017).

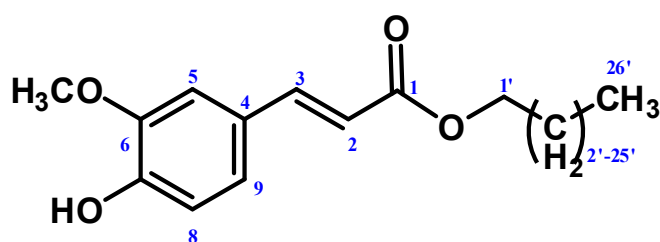
All these data permitted the identification of compound **6** as hexacosyl *trans*-ferulate. This n-alkyl *trans*-ferulate is isolated for the first time from *J. pandurifolia*.



Hexacosyl *trans*-ferulate

Table 3.9: NMR spectral data (400 MHz, CDCl₃) of compound 8 (JP-13)

Position	Compound 8	Hexacosyl <i>trans</i> -ferulate (Fraga et al., 2017)
	δ_H	δ_H
2	6.29 d ($J = 16$ Hz)	6.30 d ($J = 15.9$ Hz)
3	7.61 d ($J = 16$ Hz)	7.62 7.57 d ($J = 15.9$ Hz)
5	7.03 br s	7.04 d ($J = 1.8$ Hz)
8	6.91 d ($J = 8$ Hz)	6.92 ($J = 8.1$ Hz)
9	7.07 dd ($J = 8.1$ Hz)	7.08 dd ($J = 8.1, 1.8$ Hz)
1'	4.18 2H, t ($J = 6.2$ Hz)	4.20 2H t ($J = 6.7$ Hz)
2'	1.64 2H m	1.70 2H, m
3'-25'	1.25-1.39 46 H, m	1.22 46 H m
26'	0.88 3H, t ($J = 6.5$ Hz)	0.89 3H, t ($J = 6.7$ Hz)
OCH ₃	3.92 3H, s	3.94 3H, s
OH-7	5.93 br s	5.85 br s

Hexacosyl *trans*-ferulate

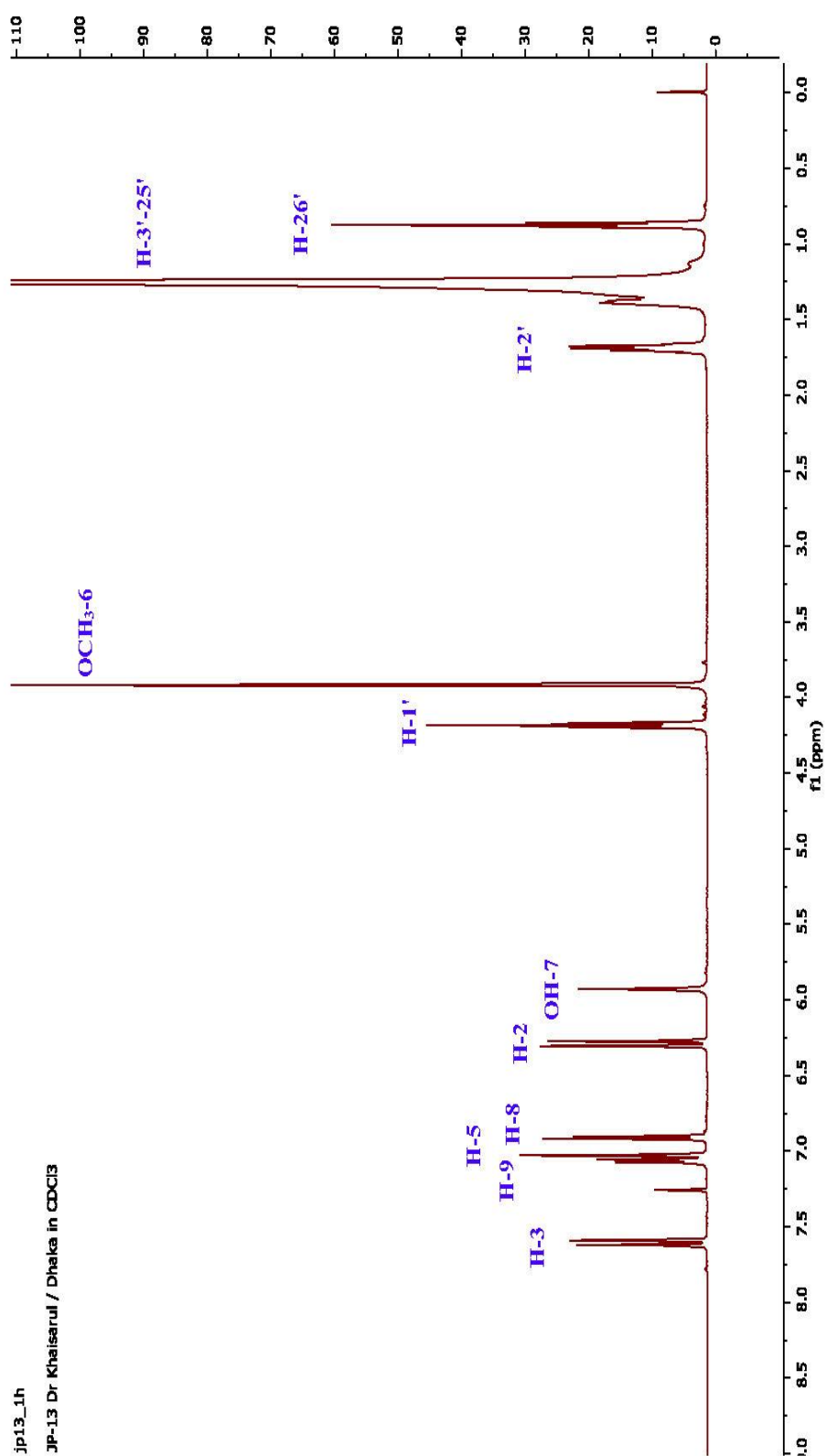


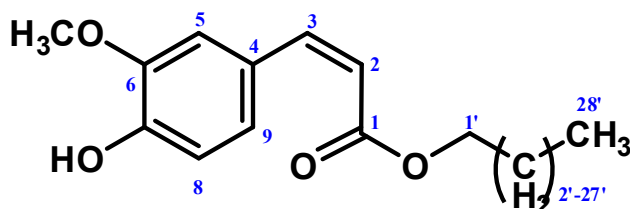
Figure 3.32: Partially expanded ¹H NMR (400 MHz, CDCl₃) of compound 8 (JP-13)

3.1.9 Characterization of compound 9 (JP-10) as Octacosyl *cis*-ferulate:

Compound 9 was obtained as white crystalline powder. On a TLC plate, it appeared as a quenching spot under UV light at 254 nm and a yellow fluorescent spot at 366 nm. When sprayed with vanillin-sulfuric acid reagent and heated for 3 minutes, it turned deep purple color.

The ^1H NMR spectrum (400 MHz, CDCl_3 ; Table: 3.10, Figure: 3.33) exhibited three aromatic proton signals at δ 7.77 br s, 6.88 ($J = 8$ Hz), and 7.10 ($J = 8$ Hz), indicating the presence of a trisubstituted benzene ring and attributed to H-5, H-8 and H-9, respectively. Further the spectrum displayed two olefinic proton doublets at δ 5.82 and 6.79 ($J = 12$ Hz, each) and a methoxy group singlet at δ 3.93. All these data together indicated the presence of a *cis*-ferulic acid moiety in the molecule. A deshielded methylene group appeared as a triplet at δ 4.11 ($J = 6$ Hz) suggesting it's attachment to an oxygen atom and to another methylene group. This signal along with a multiplet at δ 1.25-1.34 integrated for 50 protons, a 2H multiplet at δ 1.64, and a terminal methyl signal at δ 0.88 ($J = 6.5$ Hz) is characteristic for an aliphatic chain with carbon C-28.

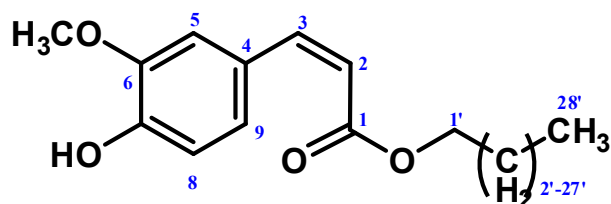
The ^1H NMR data matched to those reported for n-alkyl ferulate, octacosyl *cis*-ferulate (Gutiérrez-Tlahque et al., 2019). On the basis of the above observation, compound 9 was identified as octacosyl *cis*-ferulate, which is the first report on the isolation of this n-alkyl *cis*-ferulate from *J. pandurifolia*.



Octacosyl *cis*-ferulate

Table 3.10: NMR spectral data (400 MHz, CDCl₃) of compound 9 (JP-10)

Position	Compound 9	Octacosyl <i>cis</i> -ferulate (Gutiérrez-Tlahque et al., 2019)
	δ_H	δ_H
2	5.82 d ($J = 12$ Hz)	5.81 d ($J = 13$ Hz)
3	6.79 d ($J = 12$ Hz)	6.79 d ($J = 13$ Hz)
5	7.77 br s	7.77 d ($J = 2$ Hz)
8	6.88 d ($J = 8$ Hz)	6.87 d ($J = 8.5$ Hz)
9	7.10 d ($J = 8$ Hz)	7.10 d ($J = 8.5$ Hz)
1'	4.11 2H, t ($J = 6$ Hz)	4.11 2H, t ($J = 6$ Hz)
2'	1.64 2H, m	1.68 2H, m
3'-27'	1.25-1.34 50H, m	1.27 m
28'	0.88 3H, t ($J = 6.5$ Hz)	0.90 3H, t ($J = 6.8$ Hz)
OCH ₃	3.93 3H, s	3.95 3H, s
OH-7	5.83 br s	5.87 br s

Octacosyl *cis*-ferulate

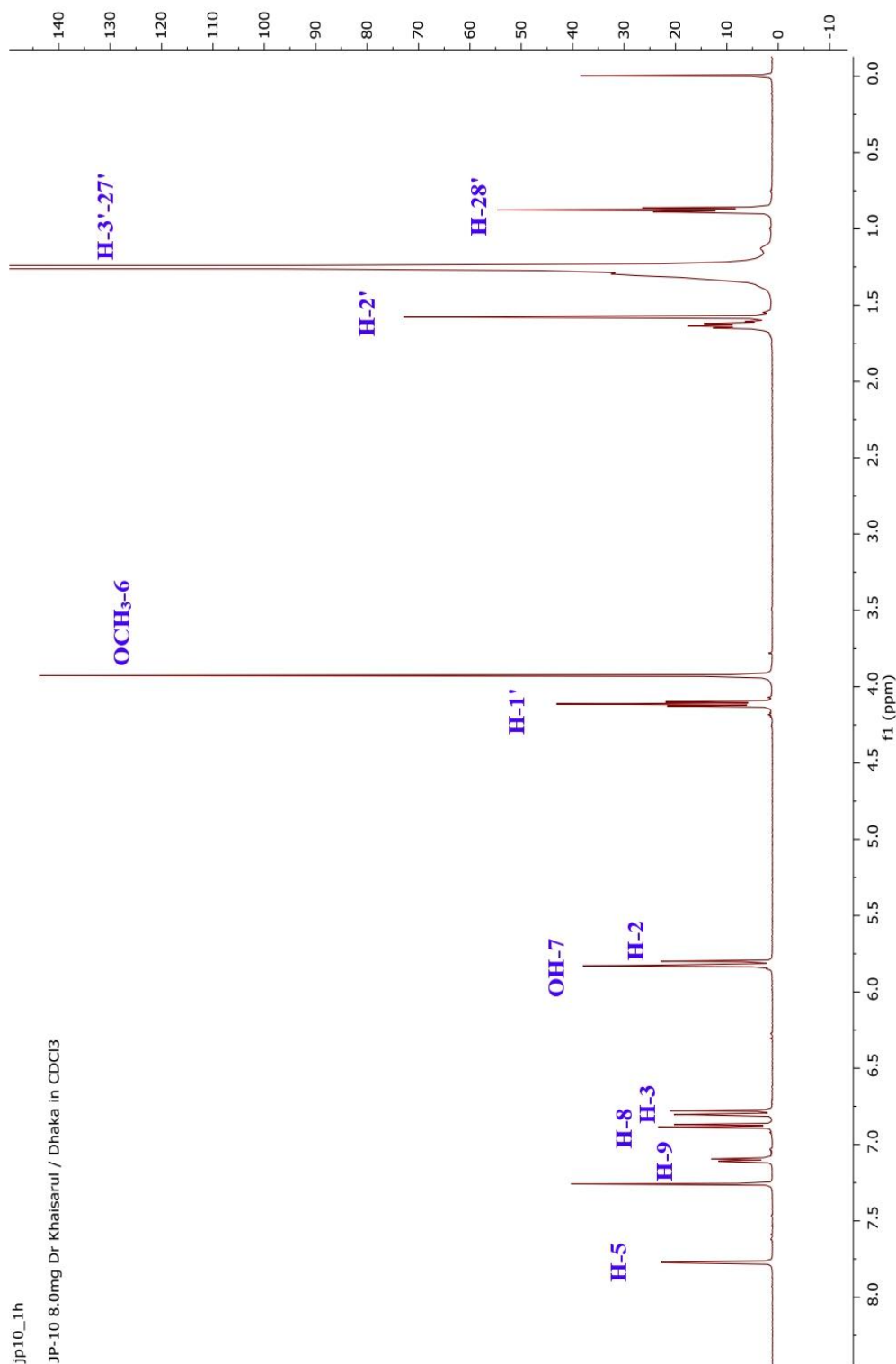


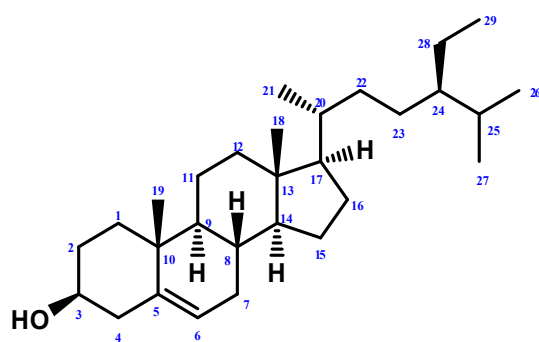
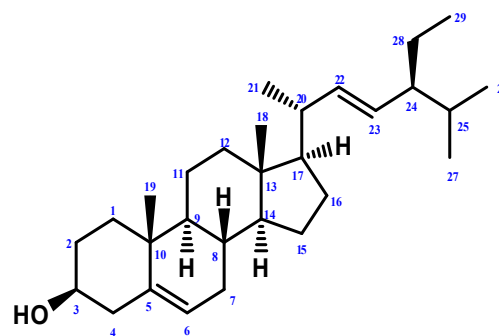
Figure 3.33: Partially expanded ¹H NMR (400 MHz, CDCl₃) of compound 9 (JP-10)

3.1.10 Characterization of compound **10** and compound **11** (JP-06) as a mixture of β -sitosterol and stigmasterol

Compounds **10**, and **11** seemed as a single spot on the TLC plate and could not be separated into individual compounds. The mixture was obtained as colorless needle-shaped crystals. On a TLC plate, JP-06 was appeared as quenching spot at 254 nm and ale yellow fluorescent at 366 nm. When sprayed with vanillin-sulfuric acid reagent followed by heating for 3 minutes, it immediately developed a purple color and then turned greyish green color after 60 minutes.

The ^1H NMR spectrum (400 MHz, CDCl_3 ; Table: 3.11, Figure: 3.34) exhibited two tertiary methyl groups at δ 0.67, and δ 1.00 assignable to H-18 and H-19, respectively. The spectrum also showed three secondary methyl doublets at δ 0.91 ($J = 6.8$ Hz), δ 0.81 ($J = 6.8$ Hz), and δ 0.83 ($J = 7.2$ Hz) assignable to H-21, H-26, and H-27, respectively. The 6th methyl group was appeared as a triplet δ 0.84 ($J = 7.2$ Hz) which could be attributed to H-29. The spectrum further revealed a signal at δ 5.35 indicating an olefinic proton, which is assignable to H-6 and a multiplet at δ 3.55 indicating a oxymethine proton assignable to H-3. Thus, compound **10** was identified as β -sitosterol. The structure was further confirmed by comparing its ^1H NMR data with those published by Pateh et al., 2009.

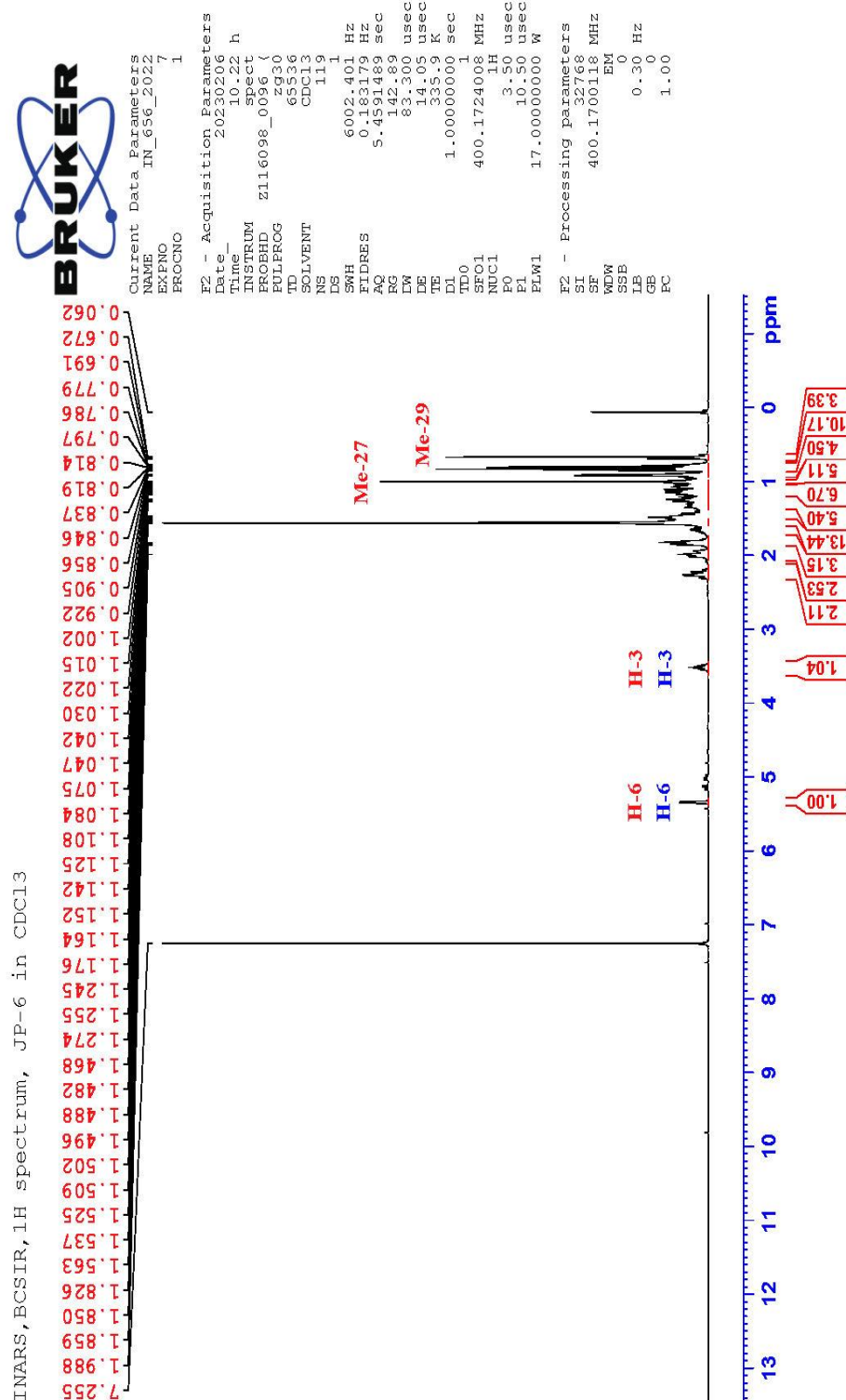
In addition to the signals mentioned for compound **10**, the ^1H NMR spectrum (Table: 3.11, Figure: 3.34) also showed another set of two tertiary methyl groups at δ 0.69 and 1.00, a secondary methyl at δ 1.04 m was assignable to H-21. The methyl signals at position 27 and 29 were not observed due to overlapping by methyl peaks of compound **10**. The spectrum also revealed an olefinic proton at δ 5.35 and oxymethine proton at δ 3.55, which were ascribed as H-6 and H-3, respectively. Additionally, the spectrum showed two characteristic olefinic protons as doublet of doublet at δ 5.14 ($J = 15.2, 8.4$ Hz) and δ 5.00 ($J = 15.2, 8.8$ Hz), attributable to H-22 and H-23, respectively. These ^1H NMR data closely matched to those reported for stigmasterol (Marliyana et al. 2021). Thus, compound **11** was identified as stigmasterol.

 β -Sitosterol

Stigmasterol

Table 3.11: NMR spectral data (400 MHz, CDCl₃) of compound 10 and 11 (JP-06)

Position	Compound 10	β -sitosterol (Pateh et al., 2009)	Compound 11	Stigmasterol (Marliyana et al., 2021)
	δ_H	δ_H	δ_H	δ_H
H-3	3.55 m	3.53 m	3.55 m	3.51 m
H-6	5.35 br s	5.37 br s	5.35 br s	5.34 m
Me-18	0.67 3H s	0.68 3H s	0.69 s	0.70 s
Me -19	1.00 3H s	1.01 3H s	1.00 s	1.01 s
H-21	0.91 d ($J = 6.8$ Hz)	0.92 d ($J = 6.4$ Hz)	1.04 d m	1.03 m
H-22	-	-	5.14 1H, dd ($J = 15.2, 8.4$ Hz)	5.16 m
H-23	-	-	5.00 1H, dd ($J = 15.2, 8.8$ Hz)	5.03 m
Me -26	0.81 d ($J = 6.8$ Hz)	0.81 d ($J = 6.4$ Hz)	0.79 d ($J = 7.2$ Hz)	0.80 m
Me -27	0.83 d ($J = 7.2$ Hz)	0.83 d ($J = 6.4$ Hz)	Peak overlapped	0.85 m
Me -29	0.84 t ($J = 7.2$ Hz)	0.85 t ($J = 7.5$ Hz)	Peak overlapped	0.81 t

Figure 3.34: ¹H NMR (400 MHz, CDCl₃) of compound 10 and 11 (JP-06)

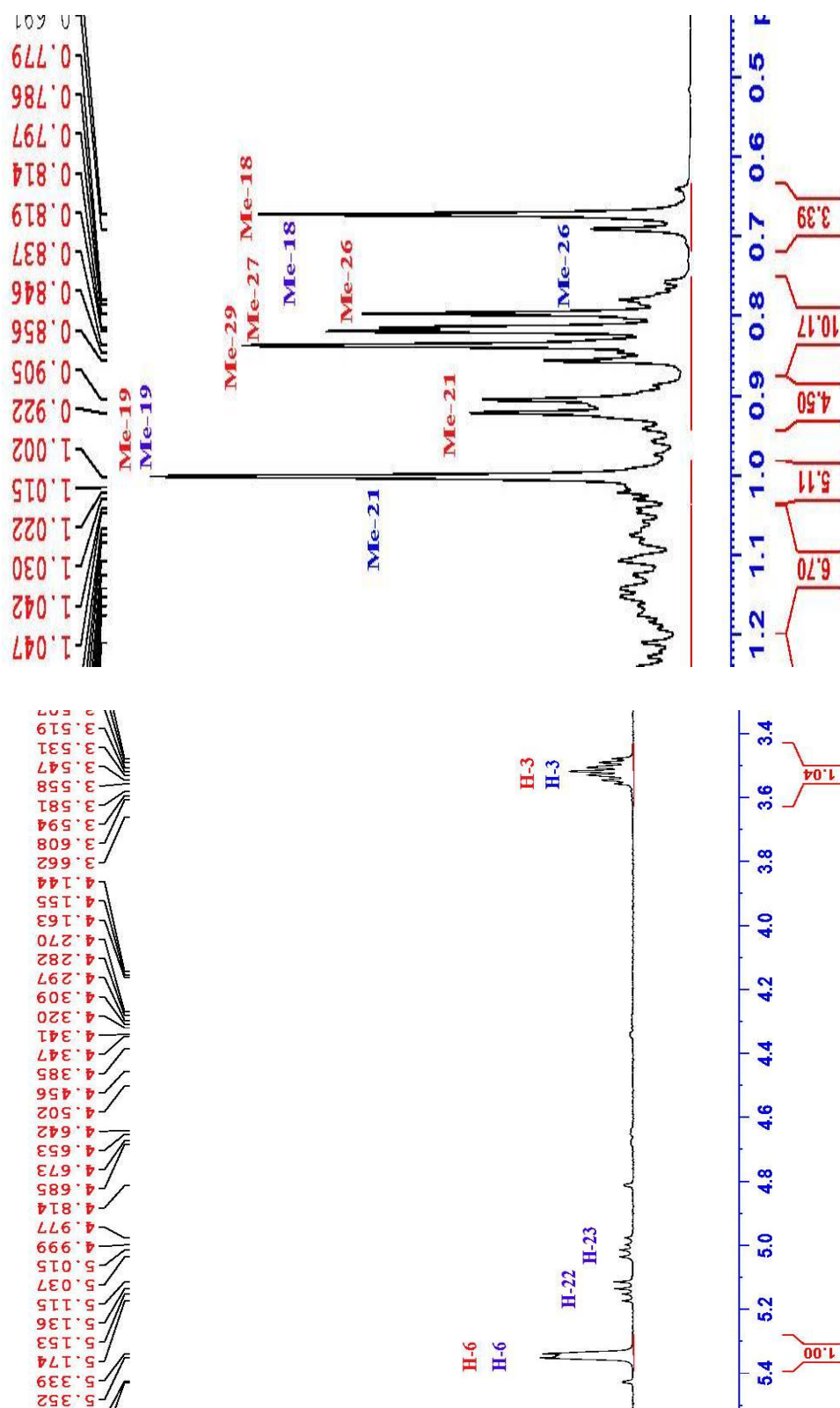


Figure 3.35: Partially expanded ^1H NMR (400 MHz, CDCl_3) of compound 10 (red color) and compound 11 (blue color) (JP-06)

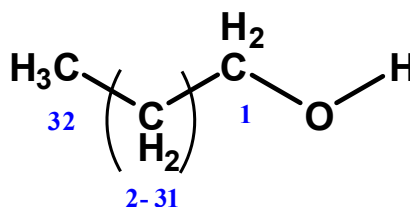
3.1.11 Characterization of compound 12 (JP 66) as n-dotriacontanol

Compound **12** appeared as a colorless crystal, was invisible at 254 nm and brown color at 366 nm. After spraying with vanillin in sulfuric acid reagent followed by heating for 3 minutes, it developed a deep purple spot.

The ^1H NMR spectrum (400 MHz, CDCl_3 ; Table: 3.12, Figure: 3.36) exhibited a triplet at δ 3.63, integrated for 2H with a coupling constant of $J=6.6$ Hz, characteristic of an oxymethylene group at C-1 of an aliphatic alcohol. The spectrum further displayed a 2H multiplet at δ 1.55, 58 H multiplet at δ 1.25 and a 3H triplet at δ 0.89 ($J = 6.8$ Hz), assignable to a methyl group at C-2, twenty nine methylene groups at C-3 to C-31 and a terminal methyl group at C-32, respectively.

These data suggested compound **12** to be a long chain aliphatic alcohol with 32 carbons and thus it was identified as n-dotriacontanol (Thi Vie Huong et al., 2019).

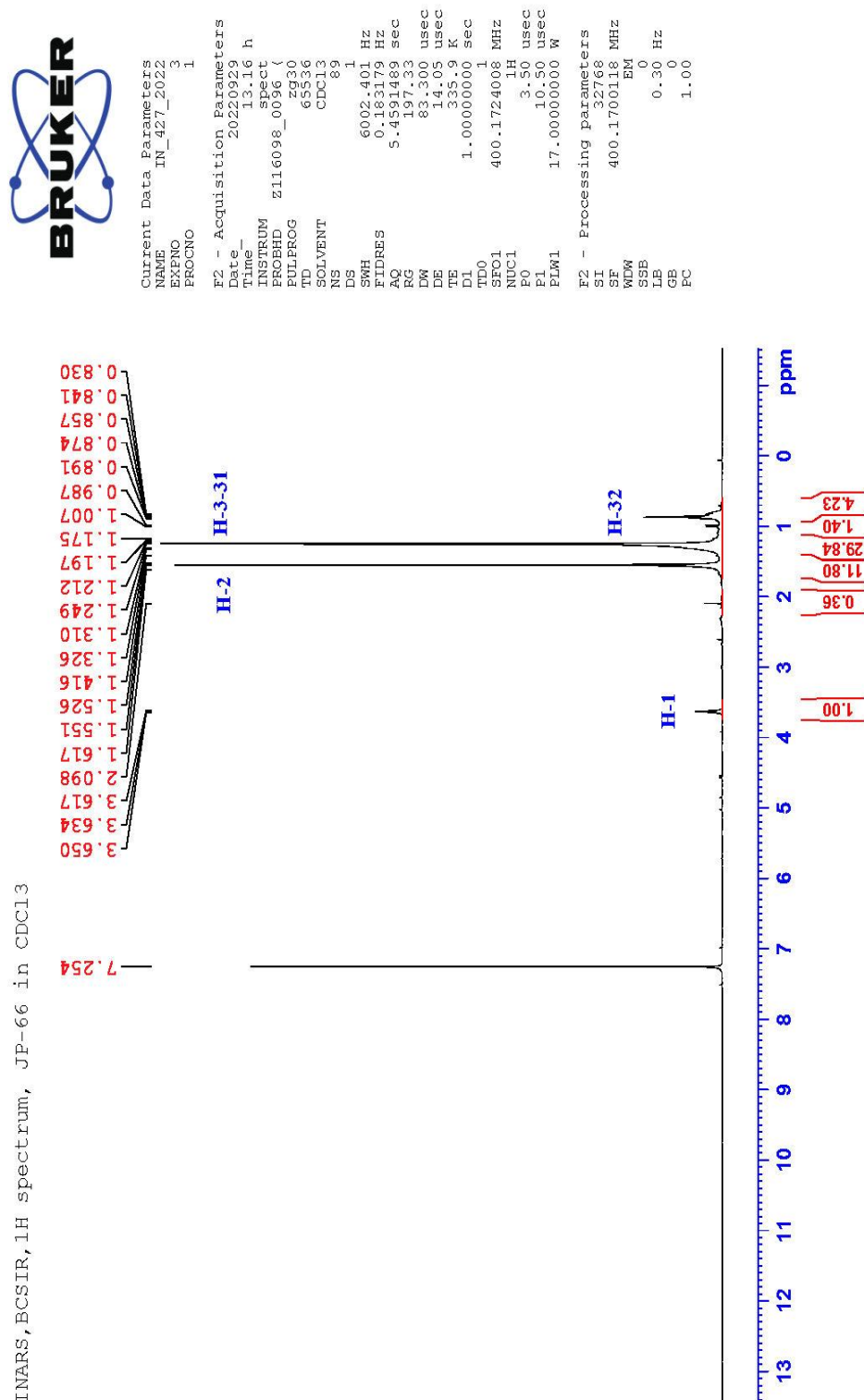
This long chain aliphatic alcohol was isolated for the first time from *J. pandurifolia*.



n-dotriacontanol

Table 3.12: NMR spectral data (400 MHz, CDCl_3) of compound 12 (JP-66)

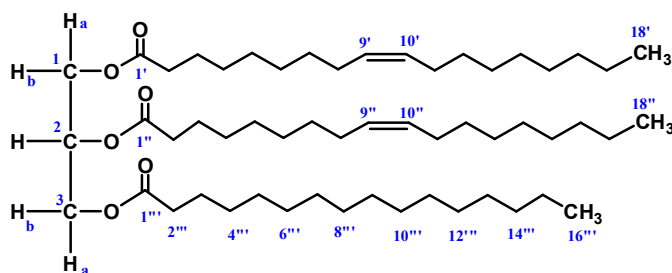
Position	Compound 12	n-dotriacontanol (Thi Vie Huong et al., 2019)
	δ_{H}	δ_{H}
H-1	3.63 2H, t ($J = 6.6$ Hz)	3.64 2H, t ($J = 6.5$ Hz)
H-2	1.55 2H, m	1.56 2H, q ($J = 7.5$ Hz)
H-3 to H-31	1.25 58 H, m	1.25 58H, br s
H-32	0.89 3H, t ($J = 6.8$ Hz)	0.87 3H, t ($J = 6.5$ Hz)

Figure 3.36: ¹H NMR (400 MHz, CDCl₃) of compound 12 (JP-66)

3.1.12 Characterization of compound 13 (JP- 93) as 1, 2-dioleoyl-3-palmitoyl-glycerol or 1, 3-dioleoyl-2-palmitoyl-glycerol

Compound **13** was obtained as white powder which appeared as a quenching spot under UV light at 254 nm and a light blue fluorescent spot at 366 nm on a thin layer chromatography plate. After spraying with vanillin in sulfuric acid reagent and heating for 3 minutes, it developed a bright deep blue spot.

The ^1H NMR spectrum (400 MHz, CDCl_3 ; Table: 3.13, Figure: 3.37) displayed an oxymethylene proton at δ 5.26, which could be attributed to sn-2 of glycerol part of a triglyceride. The sn-1a and sn-3a resonate at the same field at δ 4.13 (2H, each) with a coupling constant of 12 and 6 Hz. sn-1b and sn-3b also appeared as a doublet at δ 4.28 (2H, each, $J = 12, 4.2$ Hz). In addition, the spectrum revealed signals at δ 0.87 (9H m) which assigned to the terminal methyl group of three fatty acid chain assignable to H-8', H-18'' and H-16'''. The spectrum also showed four olefinic proton multiplet at δ 5.33 assignable to H-9', H-9'', H-10' and H-10'' of two oleic acids. Therefore the third acyl group was from a saturated fatty acid. Other significant resonances were found at δ 2.30, and δ 1.60 (6 H m, each), assignable to H-2', H-2'', H-2''', H-3', H-3'' and H-3''' of three acyl group. The H-8', H-8'', H-11' and H-11'' methylene group of oleic acid slightly deshielded due to it's close attachment of the double bond and appeared at δ 2.03 (8 H m). The fatty chain resonated at δ 1.24-1.29, integrated for 64 protons of which 16 H assigned as H-4'-7', H-4''-7'' and 24 H as H-12'-17' and H-12''-17''. The rest 24 H indicated 12 methylene group of the saturated fatty acyl group which were attributed to H-4''' to H-15'''. On the basis of above data compound **13** is a triacylglycerol consisting of two oleic and the palmitic acid moiety (Ruksilp, 2020). Thus compound **13** was identified as 1,2-dioleoyl-3-palmitoyl-glycerol or 1,3-dioleoyl-2-palmitoyl-glycerol and isolated for the first time from *J. pandurifolia*.



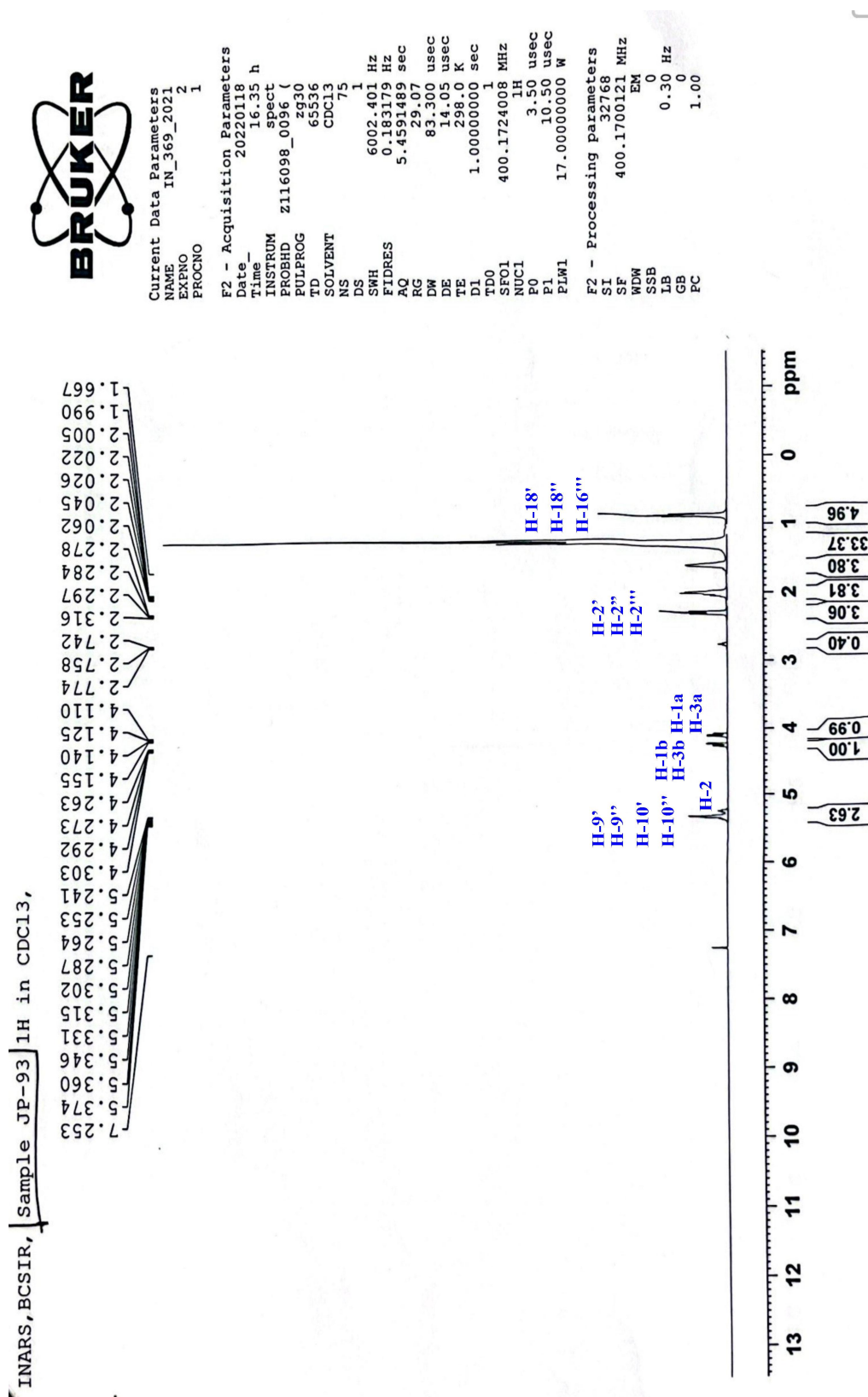
1, 2-dioleoyl-3-palmitoyl-glycerol or 1,3-dioleoyl-2-palmitoyl-glycerol

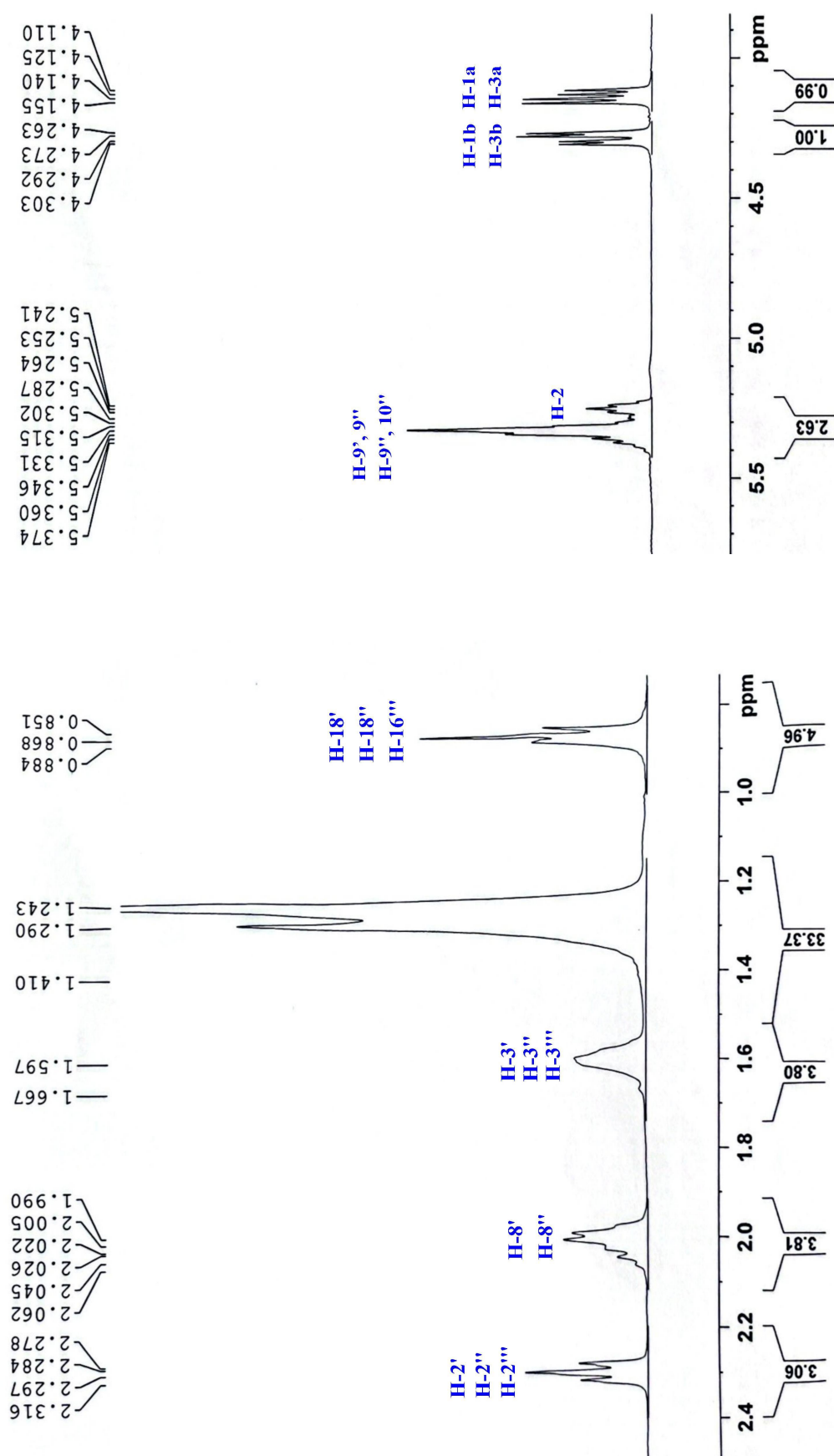
Table 3.13: NMR spectral data (400 MHz, CDCl₃) of compound 13 (JP-93)

Position	Compound 13 (Glycerol chain)	Glycerol (Wang et al., 2010)
	δ_H	δ_H
1a	4.13 dd ($J = 12, 6.0$ Hz)	4.13 m
1b	4.28 dd ($J = 12, 4.2$ Hz)	4.28 m
2	5.26 m	5.24 m
3a	4.13 dd ($J = 12, 6.0$ Hz)	4.13 m
3b	4.28 br d ($J = 12, 4.2$ Hz)	4.28 m

Position	Compound 13 (Oleic chain*)	Oleic acid (Ruksilp, 2020)	Position	Compound 13 (Palmitic chain*)	Palmitic acid (Ruksilp, 2020)
	δ_H	δ_H		δ_H	δ_H
2', 2''	2.30 4H m	2.35 ($J = 7.5$ Hz)	2'''	2.30 2H, m	2.30
3', 3''	1.60 4H m	1.60 m	3'''	1.60 2H, m	
4'-7'	1.24-1.29		4'''-15'''	1.24-1.29	
4''-7''	16H, m			24H, m	
8', 8''	2.03 4H, m	2.01 m	---	---	
9', 10'	5.33 4H, m	5.34 m	---	---	
9'', 10''					
11', 11''	2.03 4H, m	2.01 m	---	---	
12'-17'	1.24-1.29		---	---	
12''-17''	24H, m				
18', 18''	0.87 6H, m	0.88 t ($J = 7.0$ Hz)	16'''	0.87 3H, m	0.84 3H, m

* = Position interchangeable

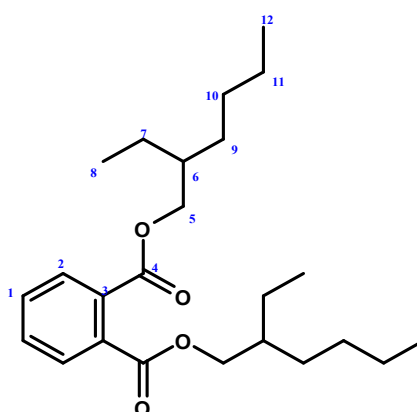
Figure 3.37: ¹H NMR (400 MHz, CDCl₃) compound 13 (JP-93)

Figure 3.38: Partially expanded ^1H NMR (400 MHz, CDCl_3) of compound 13 (JP-93)

3.1.13 Characterization of compound 14 (SR- 60) as bis(2-ethylhexyl) phthalate

Compound **14** was appeared as a colorless oily mass. On a TLC plate, it showed a quenching spot at 254 nm and a yellowish spot at 366 nm. After spraying with vanillin in sulfuric acid reagent followed by heating for 2 minutes, it developed a blue colored spot.

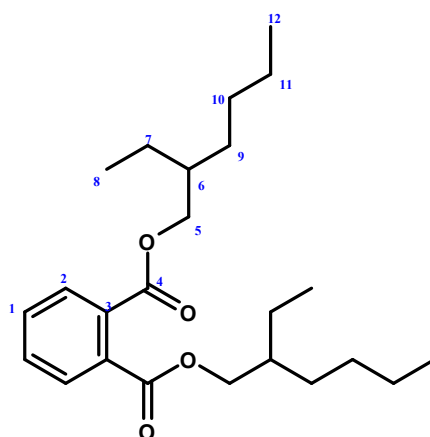
The ^1H NMR spectrum (600 MHz, CDCl_3 ; Table: 3.14, Figure: 3.39) showed the resonances of two multiplets in the aromatic region at δ 7.71 and δ 7.53 (2H m, each) which are assignable to H-1 and H-2, respectively, the coupling pattern of which suggested an orthodisubstituted benzene ring, characteristic of a phthalate ester. The presence of two oxymethylene group at δ 4.21 (4H m), two methine groups at 1.68 (2H m), two slightly deshielded methylene groups at δ 1.44 (4H m), six typical methylene groups at δ 1.25-1.36 and four methyl groups at δ 0.91 indicated the presence of two 2-ethylhexyl moiety esterified with two carboxyl group of phthalic acid. The ^{13}C NMR (150 MHz, CDCl_3 ; Table: 3.14, Figure: 3.41) spectrum suggested the symmetry of the molecule with three aromatic carbons at δ 132.6, 131.0 and 129.0, one carbonyl group at δ 167.9, and one oxymethylene carbon at δ 68.3. On the basis of these above data compound **14** was identified as bis (2-ethylhexyl) phthalate. The structure was further corroborated by comparing it's ^1H NMR and ^{13}C NMR data to those reported by Kdimy et al., 2022 and Dharmayani et al., 2023. This compound was isolated for the first time from *Syzygium reticulatum*.



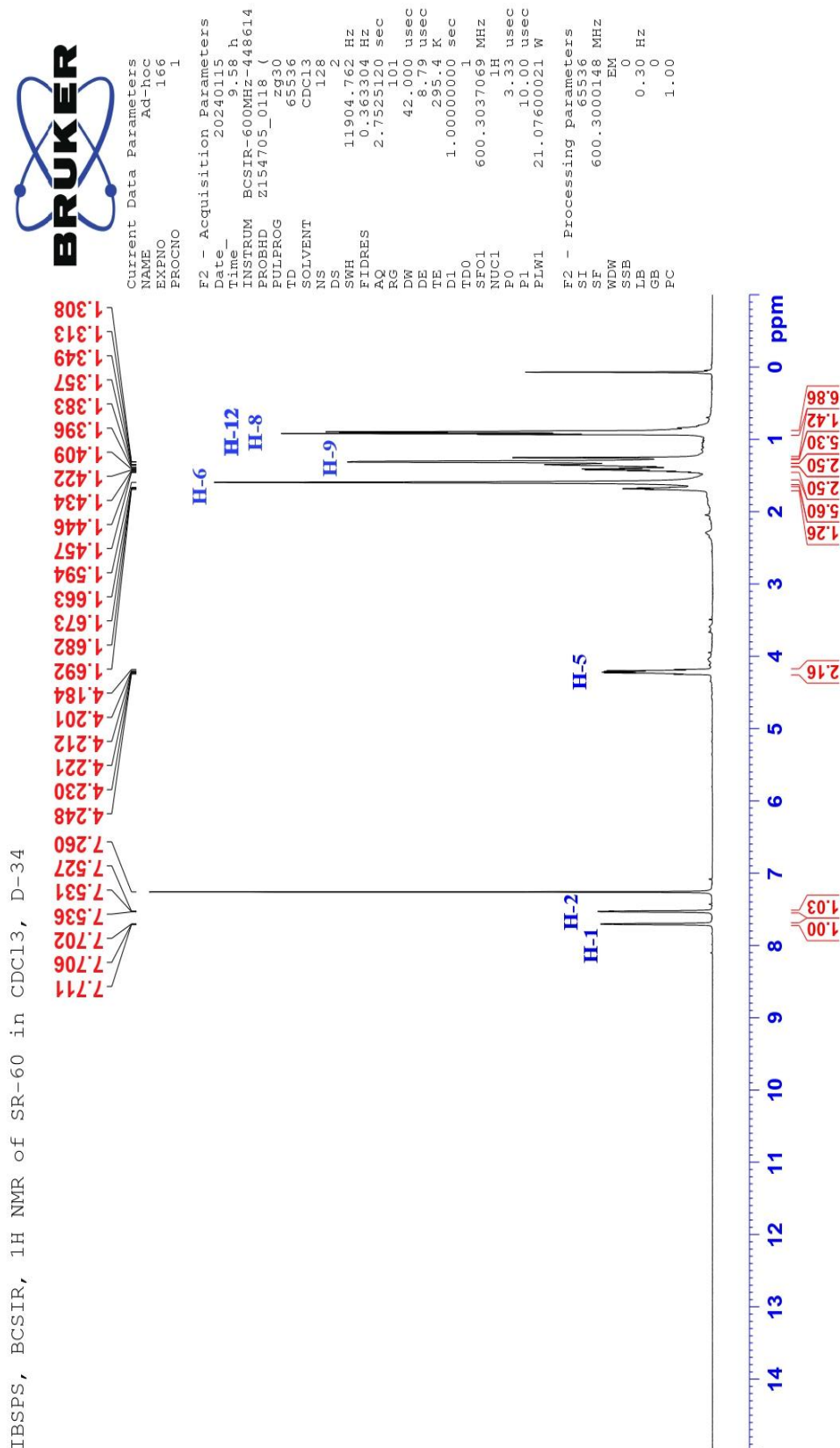
Bis(2-ethylhexyl) phthalate

Table 3.14: ^1NMR spectral data (600 MHz, CDCl_3) of compound 14 (SR-60)

Position	Compound 14		Bis (2-ethylhexyl) phthalate	
	δ_{H}	δ_{C}	(Kdimy et al., 2022)	(Dharmayani et al., 2023)
1	7.71 2H, m	132.6	7.58-7.68 m	133.4
2	7.53 2H, m	131.0	7.70-7.78 m	132.0
3	---	129.0	---	129.6
4	---	167.3	---	168.0
5	4.21 4H, m	68.3	4.19-4.29 m	68.2
6	1.68 2H, m	38.9	1.71 hepta ($J = 6.0$ Hz)	39.6
7	1.25-1.36 4H, m	30.5	1.46 tdd ($J = 7.2, 6.4, 3.3$ Hz)	31.6
8	0.91 6H, m	29.1	0.91-1.00 m	29.6
9	1.44 4H, m	23.9	1.30-1.43 m	24.4
10	1.25-1.36 4H, m	23.1	1.30-1.43 m	23.6
11	1.25-1.36 4H, m	14.2	1.30-1.43 m	14.3
12	0.91 6H, m	11.1	0.91-1.00 m	11.3



Bis(2-ethylhexyl) phthalate

Figure 3.39: ¹H NMR (600 MHz, CDCl₃) of compound 14 (SR-60)

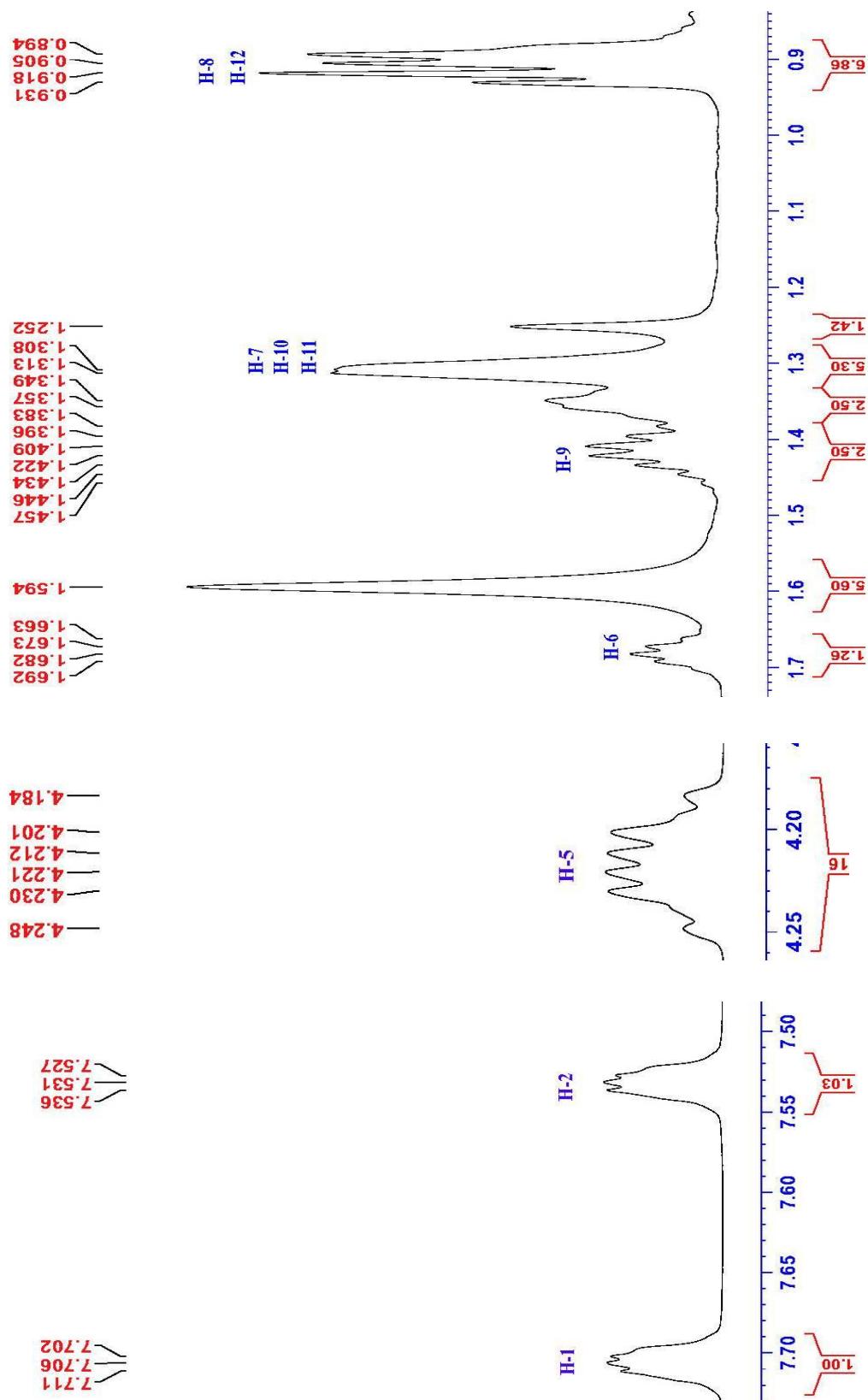
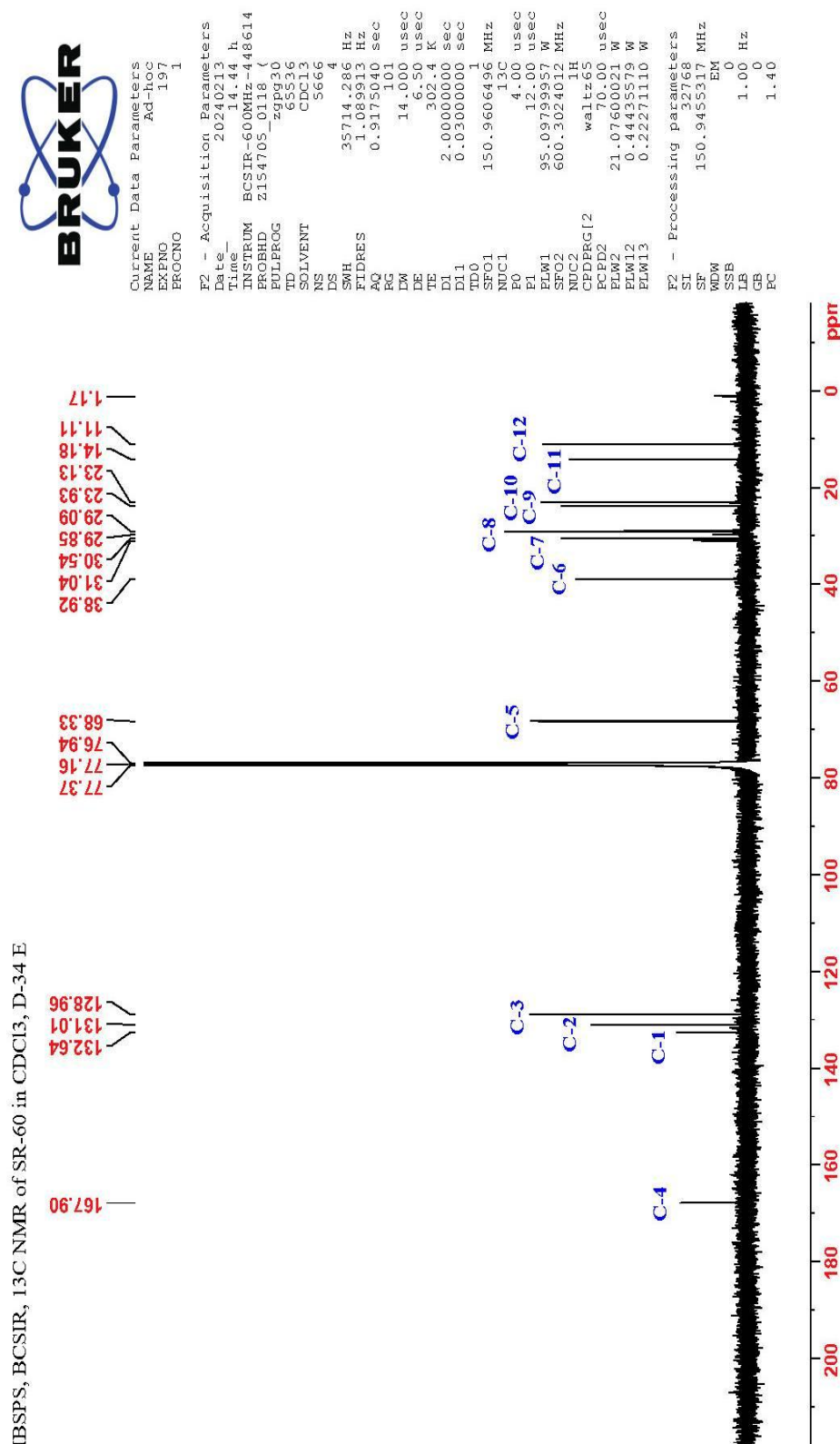


Figure 3.40: Partially expanded ^1H NMR (600 MHz, CDCl_3) of compound 14 (SR-60)

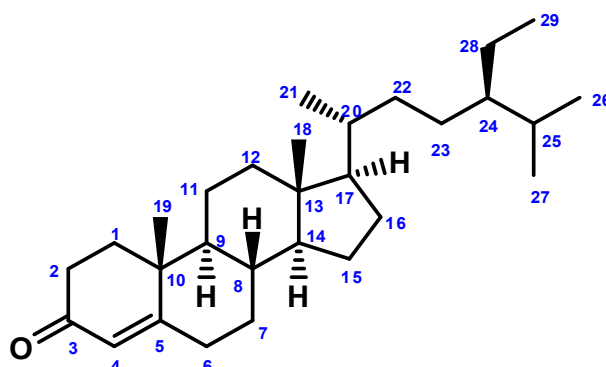
Figure 3.41: ^{13}C NMR (150 MHz, CDCl_3) of compound 14 (SR-60)

3.1.14 Characterization of compound **15** (SR-15) as Stigmast-4-en-3-one:

Compound **15** was obtained as white amorphous powder. On a TLC plate, it appeared as a quenching spot under UV light at 254 nm and a pale yellowish fluorescent spot at 366 nm. After spraying with vanillin-sulfuric acid reagent and followed by heating for 3 minutes, it developed a light purple colored spot.

The ^1H NMR revealed (600 MHz, CDCl_3 ; Table: 3.15, Figure: 3.42) the resonances of two tertiary methyl groups at δ 0.71 and 1.18, corresponding to H-18, and H-19, respectively. Additionally, three secondary methyl group signals appeared at δ 0.92 ($J = 6.6$ Hz), 0.84 ($J = 7.8$ Hz), and 0.81 (3H, $J = 6.6$ Hz) could be assigned to H-21, H-26, and H-27, respectively. Furthermore, a methyl triplet was detected at δ 0.85 ($J = 7.8$ Hz) that is attributed to H-29. The spectrum also revealed a highly deshielded olefinic proton at δ 5.72, indicating it's close proximity to a carbonyl group and could be assigned to H-4 of a 3-keto-4-ene steroid. The ^{13}C NMR data (150 MHz, CDCl_3 ; Table: 3.15, Figure: 3.44) showed the presence of 29 carbon signals including two olefinic carbons at δ 123.7 and 171.8, a carbonyl carbon at δ 199.7, six methyl carbons at δ 19.8, 19.0, 18.7, 17.4, 12.0 and 12.0.

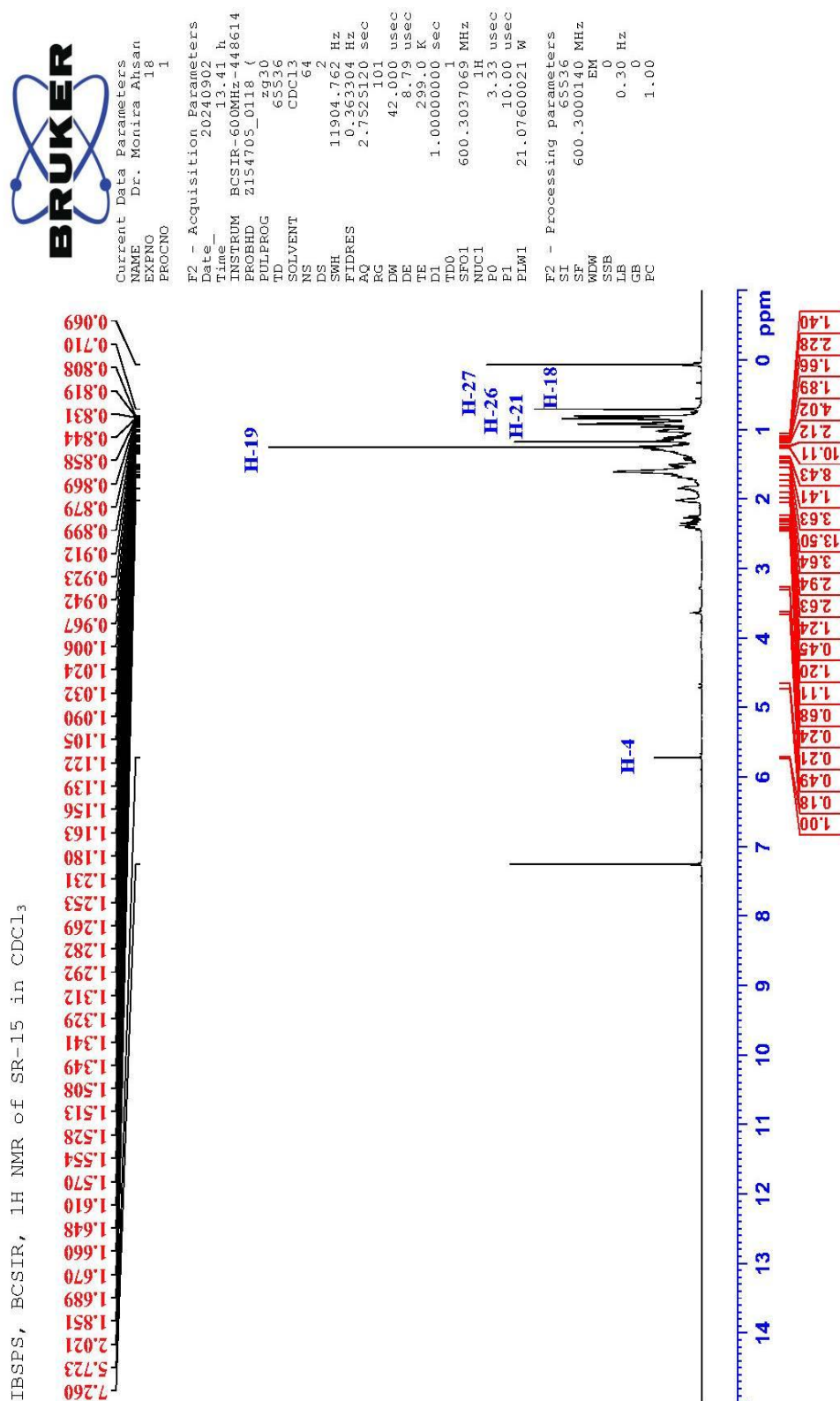
The ^1H NMR and ^{13}C NMR data of compound **15** were matched to those reported for Stigmast-4-en-3-one (Jibril et al., 2019). It is a known natural compound isolated for the first time from *Syzygium reticulatum*.

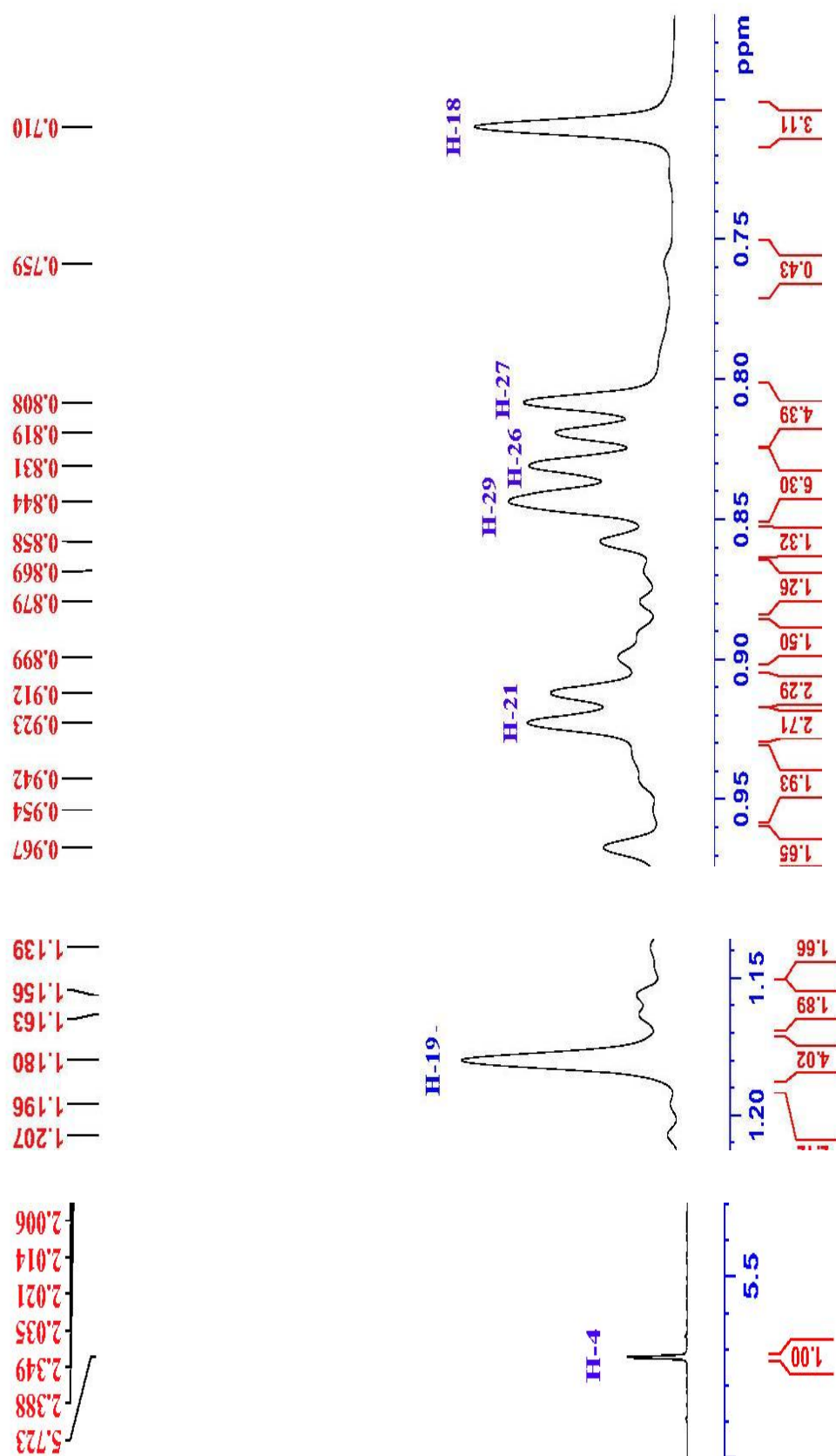


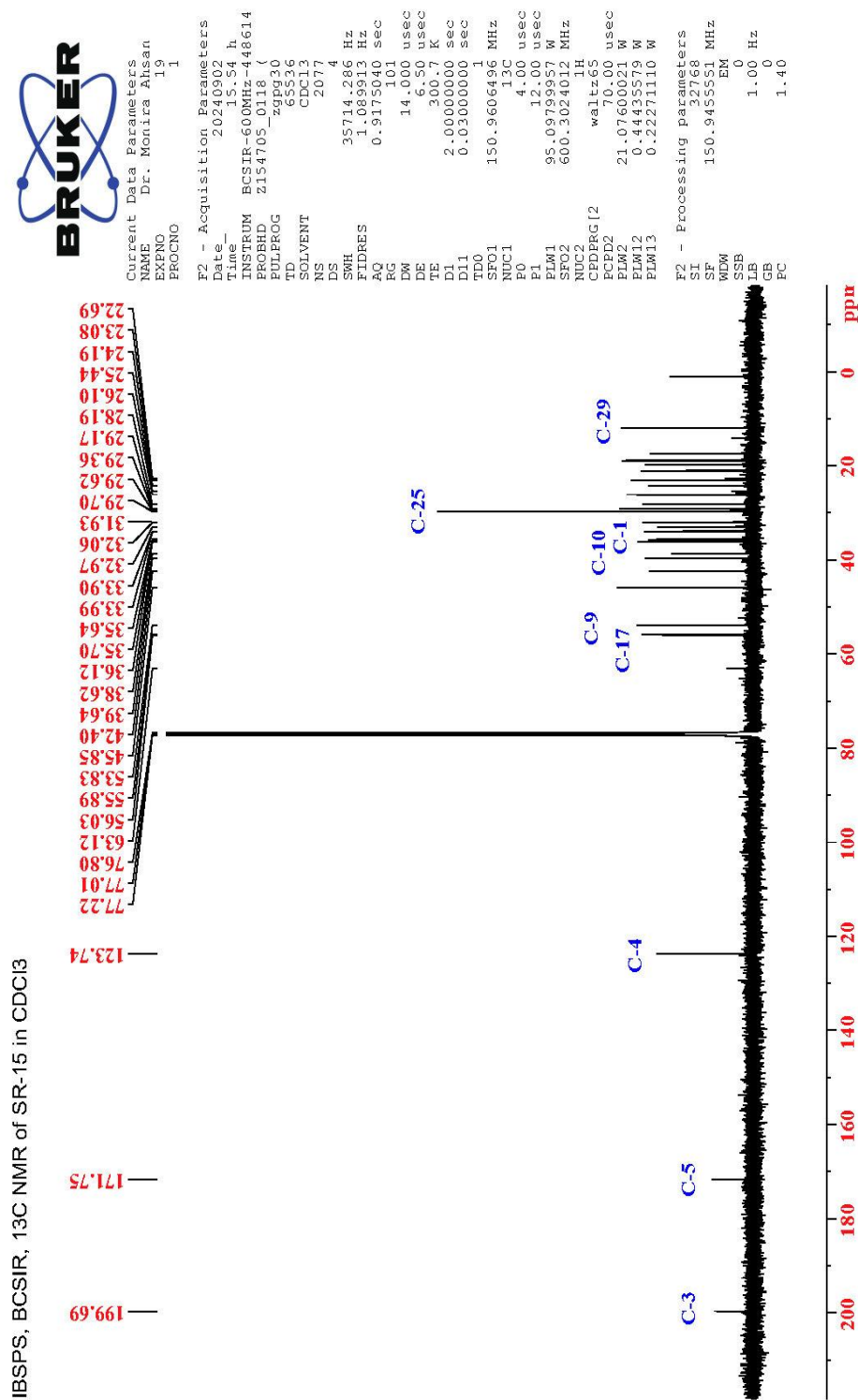
Stigmast-4-en-3-one

Table 3.15: NMR spectral data(600 MHz, CDCl₃) of compound 15 (SR-15)

Position	Compound 15		Stigmast-4-en-3-one (Jibril et al., 2019)	
	δ_H	δ_C	δ_H	δ_C
1	-	35.6	-	35.6
2	-	34.0	-	33.9
3	-	199.7	-	199.7
4	5.72 1H, s	123.7	5.72 1H, s	123.8
5	-	171.8	-	171.8
6	-	33.9	-	33.8
7	-	33.0	-	32.9
8	-	35.7	-	35.6
9	-	53.8	-	53.8
10	-	38.6	-	38.6
11	-	21.0	-	21.1
12	-	39.6	-	39.6
13	-	42.4	-	42.3
14	-	55.9	-	55.8
15	-	24.2	-	24.1
16	-	28.2	-	28.2
17	-	56.0	-	55.9
18	0.71 3H, s	12.0	0.71 3H, s	12.2
19	1.18 3H, s	17.4	1.18 3H, s	17.3
20	-	36.1	-	36.1
21	0.92 3H, d ($J = 6.6$ Hz)	18.7	0.92 3H, d ($J = 6.5$ Hz)	18.7
22	-	32.1	-	32.0
23	-	26.1	-	26.1
24	-	45.9	-	45.8
25	-	29.2	-	29.1
26	0.84 ($J = 7.8$ Hz)	19.8	0.84 3H, d ($J = 6.8$ Hz)	19.8
27	0.81 3H, d ($J = 6.6$ Hz)	19.0	0.82 3H, d ($J = 6.8$ Hz)	19.0
28	-	23.1	-	23.0
29	0.85 3H, d ($J = 7.8$ Hz)	12.0	0.85 3H, m	11.9

Figure 3.42: ¹H NMR (600 MHz, CDCl₃) of compound 15 (SR-15)

Figure 3.43: Partially expanded ¹H NMR (600 MHz, CDCl₃) of compound 15 (SR-15)

Figure 3.44: ^{13}C NMR (150 MHz, CDCl_3) of compound 15 (SR-15)

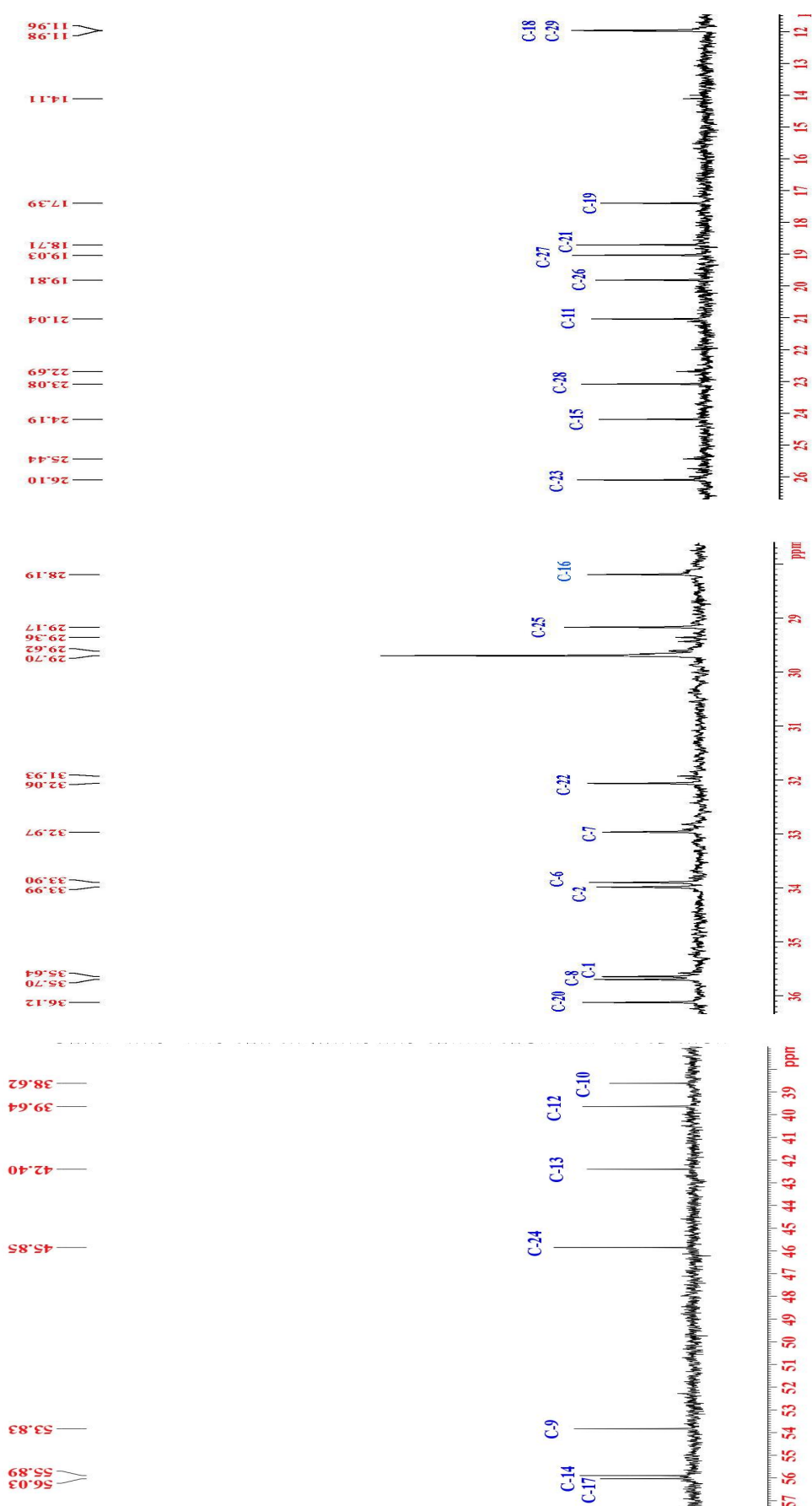


Figure 3.45: Partially expanded ^{13}C NMR (150 MHz, CDCl_3) of compound 15 (SR-15)

**Chapter 4:
Result & Discussion
(Biological
Investigation)**

4.1 Introduction to biological investigation

In this research, biological investigation was performed on different fractionates of methanolic *J. pandurifolia* stem bark and *S. reticulatum* leaf extract. *In vivo* experiment was evaluated according to CNS depressant, antidiarrheal and analgesic activities. *In vivo* experiment was carried out according to antioxidant, cytotoxicity, and thrombolytic activities.

In vivo Analysis

4.1.1 CNS depressant activity

CNS depressants exert their effects on the CNS by enhancement of gamma-aminobutyric acid (GABA) (Cutler et al., 2023) that permits chloride ions to enter in CNS. It makes the neuron less responsive to stimulant neurotransmitters, causing neuronal hyperpolarization leading to CNS depression. Common CNS depressants include benzodiazepines, barbiturates, opioids, alcohol, and several plant derived compounds such as flavonoids and alkaloids.

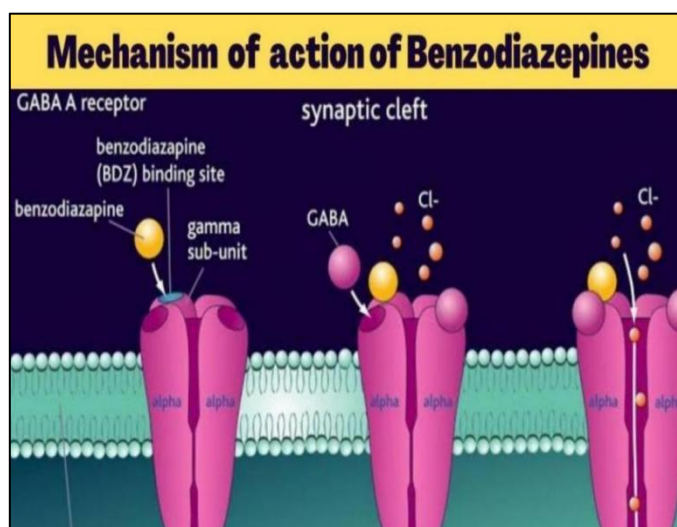


Figure 4.1: Mechanism of action of benzodiazepine

4.1.1.1 Principle of CNS depressant activity test

The open field (OF) and hole cross (HC) tests are commonly used to assess the effects of drugs on animal activity and underlying psychological feature. In these paradigms, the frequency of movement reflects the level of exploration, arousal, and emotional state of animal over a period of time (Choleris et al., 2001). Benzodiazepines make CNS more resistant to excitation, which leads to CNS depression (Molin et al., 2012).

4.1.1.2 Materials and experimental procedure

Equipment and reagents

- | | |
|-----------------------|------------------------|
| ✧ Saline water | ✧ Feeding needle |
| ✧ Diazepam | ✧ Tween 80 |
| ✧ Syringe & stopwatch | ✧ DMSO |
| ✧ Beaker | ✧ Open field apparatus |
| ✧ Marker & gloves | ✧ Hole cross apparatus |

Animals

Thirty Swiss-albino male mice (29–34 g) were obtained from the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B), Mohakhali, Dhaka. The animals were maintained under standard environmental conditions, including 55–65% relative humidity, a 12-hour light/dark cycle, and a temperature of 24°C. Food and water were provided *ad libitum*. The mice were fed standard rodent chow formulated by ICDDR, Bangladesh. All experimental procedures were conducted in accordance with the ethical guidelines and approval of ICDDR, Bangladesh.

Animal grouping and dosing

Rats were randomly assigned into the following six groups:

Group-1: Negative control: 0.9% NaCl (10 ml/kg, orally)

Group-2: Positive control: Standard (Diazepam) (1 mg/kg b.w, intra peritoneal)

Group-3: Methanolic extract of *J. pandurifolia* leaf (100 mg/kg b.w, orally)

Group-4: Methanolic extract of *J. pandurifolia* leaf (200 mg/kg of b.w, orally)

Group-5: Methanolic extract of *J. pandurifolia* leaf (300 mg/kg of b.w, orally)

Group-6: Ethyl acetate extract of *S. reticulatum* leaf (100 mg/kg of b.w, orally)

Group-7: Ethyl acetate extract of *S. reticulatum* leaf (200 mg/kg of b.w, orally)

Group-8: Ethyl acetate extract of *S. reticulatum* leaf (300 mg/kg of b.w, orally)

The solutions were prepared fresh on the experiment day.

Statistical analysis

Mean±SD was used to display the findings of each experiment. One-way analysis of variance (ANOVA) was employed to compare test groups to negative control group

using SPSS 20 software. Significance level were considered as $P \leq 0.05$ (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$).

Preparation of negative control (0.9% of NaCl)

0.9 g of sodium chloride was weighed and added to distilled water, then mixed thoroughly to make a final volume of 100 mL.

Preparation of positive control (Diazepam)

The required amount of diazepam (for four mice) was weighed and dissolved in 4 mL of saline containing two drops of Tween-80 and 1 mL of DMSO. Each animal received 1 mL of the solution via intraperitoneal injection.

Preparation of test sample

The required amount of extract (for four mice) was calculated and measured. It was then dissolved in 4 mL of saline containing two drops of Tween-80 and 1 mL of DMSO. Each mouse received 1 mL of the solution orally.

A. Locomotor activity test by open field method

The test was performed to assess spontaneous locomotor activity in mice, following the method described by Gupta et al. (1971). The open field apparatus used in this experiment was a white box measuring 30 cm × 50 cm, with walls 27 cm high. The floor was divided into a grid of square blocks, alternately colored black and white. The mice were grouped as previously described. The number of squares visited by each animal was recorded over a 3 minute period at 0, 30, 60, 90, and 120 minutes. The percentage of movement inhibition was calculated using the following formula:

% of CNS depressant activity:

$$\frac{\text{Mean movement in control group} - \text{Mean movement in test group}}{\text{Mean movement in control group}} \times 100$$

(MMC= Mean Movements of Control Group; MMT= Mean Movements of Test Group)

B. Locomotor activity test by hole test method

The hole cross test was conducted according to the method of Takagi et al. (1971), with minor modifications. The apparatus consisted of a wooden box (30 × 20 × 14 cm³) with a partition containing a 3 cm diameter hole, positioned at a height of 7.5 cm in the center of the box. Treated mice were placed at the center of one side of the chamber and the number of times each mouse passed through the hole to the opposite chamber was recorded over a 3 minute period at 0, 30, 60, 90, and 120 minutes. The percentage of movement inhibition was calculated using the following formula:

% of CNS depressant activity:

$$\frac{\text{Mean movement in control group} - \text{Mean movement in test group}}{\text{Mean movement in control group}} \times 100$$

(MMC= Mean Movements of Control Group; MMT= Mean Movements of Test Group)



Figure 4.2: Experiment with open field and hole cross method

4.1.1.3 Result and discussion

4.1.1.3.1 *Jatropha pandurifolia*

A. Open field method

Group-1: Negative control (0.9% NaCl solution)

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins
M1	80	63	55	33	53
M2	89	58	58	47	58
M3	94	72	69	95	98
M4	76	62	60	55	76
Mean	84.75	63.75	60.5	57.5	71.25
STD	8.22	5.91	6.028	26.60	20.39

Group-2: Positive control (Diazepam 1 mg/kg)

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	23	22	15	10	6	$= \frac{MMC - MMT}{MMC} \times 100$ $= \{(71.25 - 6) / 71.25\} \times 100$ $= 91.58\%$
M2	32	34	27	17	4	
M3	30	24	20	14	9	
M4	26	23	15	10	5	
Mean	27.75	25.75	19.25	12.75	6	
STD	4.03	5.56	5.68	3.40	2.16	

Group-3: Jp-ML 100 mg/kg

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	34	23	62	54	45	$= \frac{MMC - MMT}{MMC} \times 100$ $= \{(71.25 - 28) / 71.25\} \times 100$ $= 60.70\%$
M2	76	10	14	46	21	
M3	68	59	40	55	9	
M4	54	64	36	45	37	
Mean	58	39	38	50	28	
STD	18.40	26.60	19.66	5.23	16.12	

Group-4: Jp-ML 200 mg/kg

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	13	3	10	9	8	$= \frac{MMC - MMT}{MMC} \times 100$ $= \{(71.25 - 11.25) / 71.25\} \times 100$ $= 84.21\%$
M2	23	22	22	19	21	
M3	33	25	22	15	6	
M4	56	43	36	22	10	
Mean	31.25	23.25	22.5	16.25	11.25	
STD	18.41	16.38	10.63	5.62	6.70	

Group-5: Jp-ML 300 mg/kg

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	55	30	23	22	17	$= \frac{MMC - MMT}{MMC} \times 100$ $= \{(71.25 - 9) / 71.25\} \times 100$ $= 87.37\%$
M2	27	30	11	10	2	
M3	57	45	36	12	11	
M4	43	38	21	17	6	
Mean	45.5	35.75	22.75	15.25	9	
STD	13.80	7.23	10.28	5.38	6.48	

Table 4.1: CNS depressant activity of Jp-ML (Open field)

Group	Treatment	Movements (Mean± STD)	% of inhibition of movements
Group 1	Negative control (0.9% NaCl solution; 10 mL/kg)	71.25±20.39	----
Group 2	Positive control (Diazepam; 1 mg/kg)	6± 2.16	91.58%
Group 3	Jp-ML (100 mg/kg)	28± 16.12	60.70%***
Group 4	Jp-ML (200 mg/kg)	11.25± 6.7	84.21%***
Group 5	Jp-ML (300 mg/kg)	9± 6.48	87.37%***

Values are expressed as mean ± SD (n = 4); Significance level at $P \leq 0.05$ (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) in One way ANOVA; Test groups were compared to negative control group

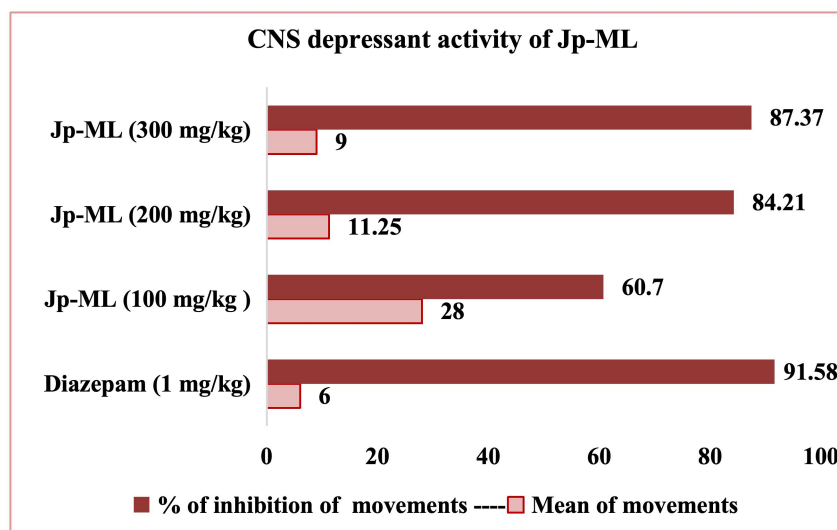


Figure 4.3: CNS depressant activity of Jp-ML (Open field)

In open field test, at 100, 200, and 300 mg/kg, the number of movements dropped to 28 ± 16.12 , 11.25 ± 6.7 , and 9 ± 6.48 , showing significant movement inhibition by 60.70% ($***P < 0.001$), 84.21% ($***P < 0.001$), and 87.37% ($***P < 0.001$), respectively (Table 4.1; Figure 4.3). These values were much lower than the control group (71.25 ± 20.39). Diazepam showed 91.58% locomotor inhibition. The results indicated that Jp-ML may exert strong central nervous system depressant effects, potentially through GABA-A receptor modulation.

C. Hole cross method

Group-1: Negative control (0.9% NaCl solution)

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins
M1	18	14	13	8	16
M2	12	16	13	11	10
M3	11	13	12	8	9
M4	8	5	9	10	11
Mean	12.25	12	11.75	9.25	11.5
STD	4.19	4.83	1.89	1.50	3.11

Group-2: Positive control (Diazepam 1 mg/kg)

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	5	5	4	3	0	$= \frac{\text{MMC} - \text{MMT}}{\text{MMC}} \times 100$ $= \{(11.5 - 0.5)/11.5\} \times 100$ $= 95.65\%$
M2	14	9	7	2	1	
M3	8	5	4	1	1	
M4	1	1	2	0	0	
Mean	7	5	4.25	1.5	0.5	
STD	5.48	3.27	2.06	1.29	0.58	

Group-3: Jp-ML 100 mg/kg

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	18	16	14	12	6	$= \frac{\text{MMC} - \text{MMT}}{\text{MMC}} \times 100$ $= \{(11.5 - 6.5)/11.5\} \times 100$ $= 43.48\%$
M2	20	14	18	12	10	
M3	13	14	10	8	3	
M4	18	12	11	9	7	
Mean	17.25	14	13.25	10.25	6.5	
STD	2.99	1.63	3.59	2.06	2.89	

Group-4: Jp-ML 200 mg/kg

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	7	6	2	2	1	$= \frac{\text{MMC} - \text{MMT}}{\text{MMC}} \times 100$ $= \{(11.5 - 4.25)/11.5\} \times 100$ $= 63.04\%$
M2	11	13	12	10	12	
M3	8	8	8	4	2	
M4	9	4	6	2	2	
Mean	8.75	7.75	7	4.5	4.25	
STD	1.71	3.86	4.16	3.79	5.19	

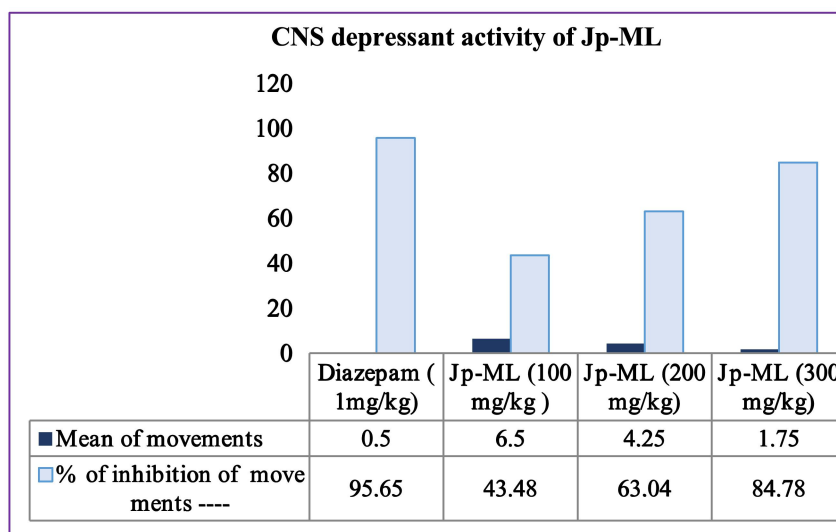
Group-5: Jp-ML 300 mg/kg

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	8	7	7	3	1	$= \frac{MMC - MMT}{MMC} \times 100$
M2	10	9	4	4	2	
M3	16	12	10	4	3	$= \{(11.5 - 1.75) / 11.5\} \times 100$
M4	12	9	8	3	1	
Mean	11.5	9.25	7.25	3.5	1.75	$= 84.78\%$
STD	3.42	2.06	2.50	0.58	0.96	

Table 4.2: CNS depressant activity of Jp-ML (Hole cross)

Group	Treatment	Movements (Mean± STD)	% of inhibition of movements
Group 1	Negative control (0.9% NaCl solution; 10 mL/kg)	11.5±3.11	----
Group 2	Positive control (Diazepam; 1 mg/kg)	0.5± 0.58	95.65%
Group 3	Jp-ML (100 mg/kg)	6.5± 2.89	43.48%
Group 4	Jp-ML (200 mg/kg)	4.25± 5.19	63.04%*
Group 5	Jp-ML (300 mg/kg)	1.75± 0.96	84.78%*

Values are expressed as mean ± SD (n = 4); Significance level at $P \leq 0.05$ (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$) in One way ANOVA; Test groups were compared to negative control group

**Figure 4.4: CNS depressant activity of Jp-ML (Hole cross)**

In the hole cross test, the mean number of movement were recorded as 6.5 ± 2.89 at 100 mg/kg, 4.25 ± 5.19 at 200 mg/kg, and 1.75 ± 0.96 at 300 mg/kg when compared to negative control group (11.5 ± 3.11) (Table 4.2; Figure 4.4). Inhibition of movements were counted as 43.48%, 63.04% (* $P < 0.05$), and 84.78% (* $P < 0.05$) for aforementioned doses. Diazepam treated group showed 95.65% inhibition with lowest movements. It was projected that Jp-ML showed strong CNS depressant property in hole cross method too.

4.1.1.3.2 *Syzygium reticulatum*

A. Open field method

Group-1: Negative control (0.9% NaCl solution)

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins
M1	80	63	55	33	53
M2	89	58	58	47	58
M3	94	72	69	95	98
M4	76	62	60	55	76
Mean	84.75	63.75	60.5	57.5	71.25
STD	8.22	5.91	6.028	26.60	20.39

Group-2: Positive control (Diazepam 1 mg/kg)

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	23	22	15	10	6	$= \frac{MMC - MMT}{MMC} \times 100$ $= \{(71.25 - 6) / 71.25\} \times 100$ $= 91.58\%$
M2	32	34	27	17	4	
M3	30	24	20	14	9	
M4	26	23	15	10	5	
Mean	27.75	25.75	19.25	12.75	6	
STD	4.03	5.56	5.68	3.40	2.16	

Group-6: Sr-EAL 100 mg/kg

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	65	60	55	45	40	$= \frac{MMC - MMT}{MMC} \times 100$ $= \{(71.25 - 43.25)/71.25\} \times 100$ $= 39.30\%$
M2	112	75	65	55	43	
M3	55	54	40	43	40	
M4	76	65	51	60	50	
Mean	77	63.5	52.75	50.75	43.25	
STD	24.86	8.89	10.34	8.10	4.72	

Group-7: Sr-EAL 200 mg/kg

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	58	13	19	27	18	$= \frac{MMC - MMT}{MMC} \times 100$ $= \{(71.25 - 30.75)/71.25\} \times 100$ $= 56.84\%$
M2	95	46	38	40	31	
M3	63	20	53	37	39	
M4	79	27	22	20	35	
Mean	73.75	26.5	33	31	30.75	
STD	16.76	14.20	15.73	9.20	9.11	

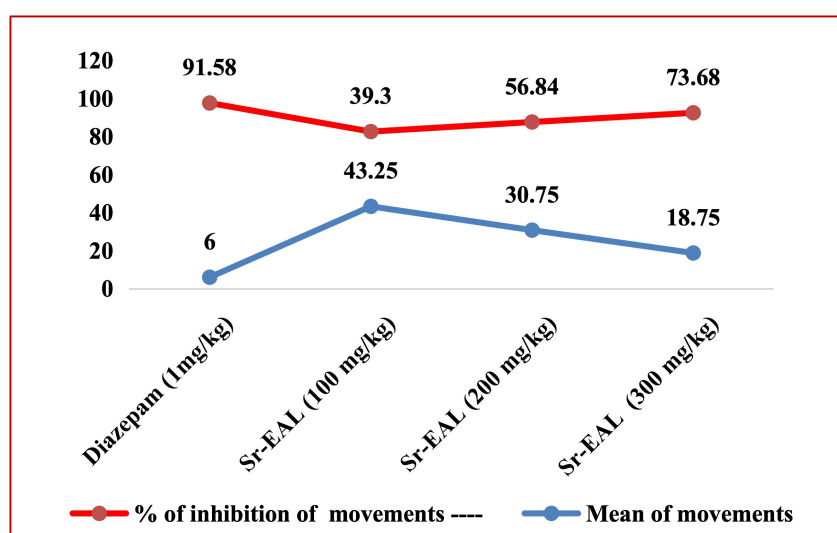
Group-8: Sr-EAL 300 mg/kg

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	95	65	30	20	14	$= \frac{MMC - MMT}{MMC} \times 100$ $= \{(71.25 - 18.75)/71.25\} \times 100$ $= 73.68\%$
M2	66	47	37	16	10	
M3	76	55	51	47	41	
M4	77	43	28	12	10	
Mean	78.5	52.5	36.5	23.75	18.75	
STD	12.07	9.71	10.41	15.84	14.95	

Table 4.3: CNS depressant activity of Sr-EAL (Open field)

Group	Treatment	Movements (Mean± STD)	% of inhibition of movements
Group 1	Negative control (0.9% NaCl solution; 10 mL/kg)	71.25± 20.39	---
Group 2	Positive control (Diazepam; 1 mg/kg)	6± 2.16	91.58%
Group 6	Sr-EAL (100 mg/kg)	43.25±4.72	39.30%**
Group 7	Sr-EAL (200 mg/kg)	30.75±9.11	56.84%***
Group 8	Sr-EAL (300 mg/kg)	18.75±14.95	73.68%***

Values are expressed as mean ± SD (n = 4); Significance level at $P \leq 0.05$ (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$) in One way ANOVA; Test groups were compared to negative control group

**Figure 4.5: CNS depressant activity of Sr-EAL (Open field)**

For Sr-EAL, the mean number of movements were 43.25 ± 4.72 with 39.30% inhibition (** $P < 0.01$), 30.75 ± 9.11 with 56.84% inhibition (*** $P < 0.001$), and 18.75 ± 14.95 with 73.68% inhibition (*** $P < 0.001$) for 100, 200 and 300 mg/kg, respectively in comparison to the negative control (71.25 ± 20.39), indicating a strong depressive effect (Table 4.3; Figure 4.5).

B. Hole cross method**Group-1: Negative control (0.9% NaCl solution)**

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins
M1	18	14	13	8	16
M2	12	16	13	11	10
M3	11	13	12	8	9
M4	8	5	9	10	11
Mean	12.25	12	11.75	9.25	11.5
STD	4.19	4.83	1.89	1.50	3.11

Group-2: Positive control (Diazepam 1 mg/kg)

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	5	5	4	3	0	$= \frac{\text{MMC} - \text{MMT}}{\text{MMC}} \times 100$ $= \{(11.5 - 0.5)/11.5\} \times 100$ $= 95.65\%$
M2	14	9	7	2	1	
M3	8	5	4	1	1	
M4	1	1	2	0	0	
Mean	7	5	4.25	1.5	0.5	
STD	5.48	3.27	2.06	1.29	0.58	

Group-6: Sr-EAL 100 mg/kg

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	14	9	15	16	7	$= \frac{\text{MMC} - \text{MMT}}{\text{MMC}} \times 100$ $= \{(11.5 - 8.75)/11.5\} \times 100$ $= 23.91\%$
M2	35	25	22	17	16	
M3	6	5	6	2	3	
M4	10	8	5	8	9	
Mean	18.33	11.75	12	10.75	8.75	
STD	12.92	9.00	8.04	7.09	5.44	

Group-7: Sr-EAL 200 mg/kg

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	15	5	5	3	1	$= \frac{MMC - MMT}{MMC} \times 100$ $= \{(11.5 - 4.00)/11.5\} \times 100$ $= 65.22\%$
M2	17	15	14	9	8	
M3	14	10	8	5	4	
M4	11	5	6	4	3	
Mean	14.25	8.75	8.25	5.25	4	
STD	2.50	4.79	4.03	2.63	2.94	

Group-8: Sr-EAL 300 mg/kg

Mice ID	0 th mins	30 th mins	60 th mins	90 th mins	120 th mins	% inhibition of movement (after 120 mins)
M1	14	16	13	12	7	$= \frac{MMC - MMT}{MMC} \times 100$ $= \{(11.5 - 2.25)/11.5\} \times 100$ $= 80.43\%$
M2	9	7	6	9	2	
M3	9	8	2	3	0	
M4	10	8	8	2	0	
Mean	10.5	9.75	7.25	6.5	2.25	
STD	2.38	4.19	4.57	4.80	3.30	

Table 4.4: CNS depressant activity of Sr-EAL (Hole cross)

Group	Treatment	Movements (Mean± STD)	% of inhibition of movements
Group 1	Negative control (0.9% NaCl solution; 10 mL/kg)	11.5± 3.11	---
Group 2	Positive control (Diazepam; 1 mg/kg)	0.5± 0.58	95.65%
Group 6	Sr-EAL (100 mg/kg)	8.75±5.44	23.91%
Group 7	Sr-EAL (200 mg/kg)	4±2.94	65.22%*
Group 8	Sr-EAL (300 mg/kg)	2.25±3.30	80.43%**

Values are expressed as mean ± SD (n = 4); Significance level at $P \leq 0.05$ (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) in One way ANOVA; Test groups were compared to negative control group

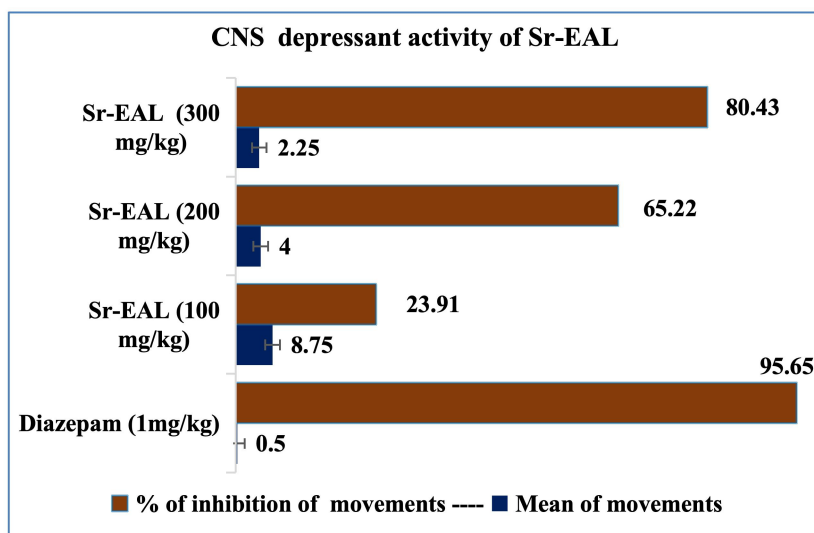


Figure 4.6: CNS depressant activity of Sr-EAL (Hole cross)

Moderate to mild CNS depressant activity were recorded as 23.91%, 65.22% (* $P < 0.05$), and 80.43% (** $P < 0.01$) for the 100 mg/kg, 200 mg/kg, and 300 mg/kg doses, respectively, whereas diazepam exhibited 95.65% CNS depressant activity (Table 4.4; Figure 4.6). The inhibition of mice passage through the chamber hole showed a decreasing trend with 8.75 ± 5.44 , 4 ± 2.94 , and 2.25 ± 3.30 movements, respectively.

Locomotor activity refers to changes in alertness, with a reduction in locomotor activity being indicative of a depressive effect. In this study, locomotor activity was measured using hole cross and open field tests, and it was found that a significant reduction in head dips during exploratory behavior has been reported as a measure of CNS depressant activity for both plants extracts (Couto et al., 2011). Notably, both plants exhibited a strong to mild CNS depressive effect in a dose-dependent manner that could be an indicator of presence of numerous flavonoids and neuroactive steroids that mimic benzodiazepine in the CNS (Sofidiya et al., 2022).

4.1.2 Antidiarrheal activity

Antidiarrheal treatment primarily aims to restore hydration and manage electrolyte loss to prevent serious complications (Sisay et al., 2019). Interference of NaCl and water reabsorption leads to a hypersecretory state, causing diarrheal symptom. Opiates, anticholinergics, and adsorbents are commonly used to treat diarrhea while natural compounds like flavonoids, tannins, saponins, alkaloids have also demonstrated prominent antidiarrheal activity (Mekuriaw et al., 2021).

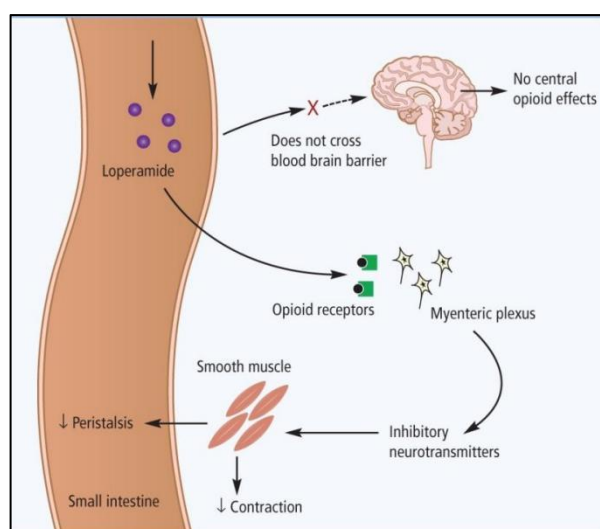


Figure 4.7: Mechanism of action of loperamide

4.1.2.1 Principle of antidiarrheal activity test

The intestinal hydrolysis of castor oil produces ricinoleic acid and promotes the formation of nitric oxide (NO), which elevates cyclic AMP (cAMP) and cyclic GMP (cGMP). These consequences alter mucosal permeability, electrolyte transport, and increase gastric motility. Loperamide slows down gut motility, which helps reduce diarrhea by allowing more time for water and electrolytes to be absorbed from the intestine.

4.1.2.2 Materials and experimental procedure

Equipment and reagents

- | | |
|-----------------------|-----------------------|
| ✧ Saline water | ✧ Feeding needle |
| ✧ Loperamide | ✧ Tween 80 |
| ✧ Castor oil | ✧ DMSO |
| ✧ Syringe & stopwatch | ✧ Observation chamber |
| ✧ Beaker | ✧ Marker & gloves |

Animal grouping and dosing

Rats were randomly assigned into following six groups:

Group-1: Negative control: 0.9 % NaCl solution (10 mL/kg, orally)

Group-2: Positive control: Standard (Loperamide) (5 mg/kg, intra peritoneally)

Group-3: Methanolic extract of *J. pandurifolia* leaf (200 mg/kg, orally)

Group-4: Methanolic extract of *J. pandurifolia* leaf (400 mg/kg, orally)

Group-5: Methanolic extract of *J. pandurifolia* leaf (600 mg/kg, orally)

Group-6: Ethyl acetate extract of *S. reticulatum* leaf (200 mg/kg, orally)

Group-7: Ethyl acetate extract of *S. reticulatum* leaf (400 mg/kg, orally)

Group-8: Ethyl acetate extract of *S. reticulatum* leaf (600 mg/kg, orally)

The solutions were prepared fresh on the experiment day.

Preparation of negative control (0.9% of NaCl)

0.9 g of sodium chloride was weighed and added to distilled water, then mixed thoroughly to make a final volume of 100 mL.

Preparation of positive control (Loperamide)

The required amount of loperamide was weighed and dissolved in 4 mL of saline water with two drops of Tween-80 and 1 mL of DMSO. The solution was administered intraperitoneally at a dose of 1 mL per mouse.

Preparation of test sample

The required amount of extract was calculated and measured (for 4 mice). The extract was dissolved in 4 mL of saline water with two drops of Tween-80 and 1 mL of DMSO. Each mouse was given 1 mL orally.

Castor oil induced diarrheal test (Sairam et al., 2003, Degu et al., 2020)

After 18 hours of fasting, all mice were allowed to take treatments. Following the treatments, 0.5 mL of castor oil was given orally to induce diarrhea. Mice were individually placed in cages and the floor was covered with absorbent paper. Animals were carefully observed over a period of 4 hours to record the onset of diarrhea, the total number of defecations, and the average weight of wet feces. Additionally, the

number of mice with diarrheal droppings within the 4 hour period was counted. The calculation was done using following formula:

$$\% \text{ of diarrheal inhibition: } \frac{WFC - WFT}{WFC} \times 100$$

$$\% \text{ fecal output: } \frac{\text{Mean of defecation in test/standard group}}{\text{Mean of defecation in negative control group}} \times 100$$

(WFC = Mean Weight of Wet Feces in Control Group; WFT = Mean Weight of Wet Feces in Test Group)



Figure 4.8: Experiments on mice to collect stool and defecation timing

4.1.2.3 Result and discussion

4.1.2.3.1 *Jatropha pandurifolia*

Castor Oil induced method

Group-1: Negative control (0.9% NaCl solution)

Mice ID	1 hr	2 hr	3 hr	4 hr
M1	0.43	0.51	0.36	0.29
M2	0.26	0.79	0.21	0.14
M3	0.65	0.45	0.29	0.03
M4	0.1	0.88	0.55	1.22
Mean	0.36	0.6575	0.3525	0.42
STD	0.24	0.21	0.15	0.54

Group-2: Positive control (Loperamide 5 mg/ kg)

Mice ID	1 hr	2 hr	3 hr	4 hr
M1	0.059	0	0	0.013
M2	0	0	0	0
M3	0.0118	0	0	0.0110
M4	0	0	0	0
Mean	0.018	0	0	0.006
STD	0.03	0.00	0.00	0.01

$$\begin{aligned}
 \% \text{ of diarrheal inhibition} &= \frac{WFC - WFT}{WFC} \times 100 \\
 &= \{(0.42 - 0.006) / 0.42\} \times 100 \\
 &= 98.57\%
 \end{aligned}$$

$$\begin{aligned}
 \% \text{ fecal output} &= \frac{\text{Mean defecation in test group}}{\text{Mean defecation in negative control group}} \times 100 \\
 &= \frac{3.25}{7} \times 100 \\
 &= 46.42\%
 \end{aligned}$$

Group-3: Jp-ML 200 mg/kg

Mice ID	1 hr	2 hr	3 hr	4 hr
M1	0.016	0.39	1	0.49
M2	0.118	0.027	0.05	0.01
M3	0.17	0.095	0.355	0.311
M4	0.426	0.33	0.238	0.224
Mean	0.183	0.211	0.411	0.259
STD	0.17	0.18	0.41	0.20

$$\begin{aligned}
 \% \text{ of diarrheal inhibition} &= \frac{WFC - WFT}{WFC} \times 100 \\
 &= \{(0.42 - 0.259) / 0.42\} \times 100 \\
 &= 38.33\%
 \end{aligned}$$

$$\begin{aligned}
 \% \text{ of fecal output} &= \frac{\text{Mean defecation in test group}}{\text{Mean defecation in negative control group}} \times 100 \\
 &= \frac{6.5}{7} \times 100 \\
 &= 92.86\%
 \end{aligned}$$

Group-4: Jp-ML 400 mg/kg

Mice ID	1 hr	2 hr	3 hr	4 hr
M1	0.33	0.53	0.15	0.09
M2	0.95	0.62	0.5	0
M3	0.99	0.25	0.23	0.22
M4	0.1	0.45	0.29	0.27
Mean	0.5925	0.4625	0.2925	0.145
STD	0.45	0.16	0.15	0.12

$$\begin{aligned}
 \% \text{ of diarrheal inhibition} &= \frac{WFC - WFT}{WFC} \times 100 \\
 &= (0.42 - 0.145) / 0.42 \times 100 \\
 &= 65.48\%
 \end{aligned}$$

$$\begin{aligned}
 \% \text{ of fecal output} &= \frac{\text{Mean defecation in test group}}{\text{Mean defecation in negative control group}} \times 100 \\
 &= \frac{6}{7} \times 100 \\
 &= 85.71 \%
 \end{aligned}$$

Group-5: Jp-ML 600 mg/kg

Mice ID	1 hr	2 hr	3 hr	4 hr
M1	0	0.32	0.07	0.02
M2	0	0.21	0.12	0.02
M3	0	0.66	0.22	0.106
M4	0	0.21	0.14	0.09
Mean	0	0.35	0.1375	0.059
STD	0	0.21	0.06	0.05

$$\begin{aligned}
 \text{\% of diarrheal inhibition} &= \frac{WFC - WFT}{WFC} \times 100 \\
 &= (0.42 - 0.059) / 0.42 \times 100 \\
 &= 85.95\%
 \end{aligned}$$

$$\begin{aligned}
 \text{\% of fecal output} &= \frac{\text{Mean of defecation in test group}}{\text{Mean of defecation in negative control group}} \times 100 \\
 &= \frac{4.5}{7} \times 100 \\
 &= 64.29\%
 \end{aligned}$$

Table 4.5: Antidiarrheal activity of Jp-ML (Castor oil induced diarrhea)

Group	Treatment	Weight of wet feces (Mean±STD)	% of diarrheal inhibition	Number of defecation (Mean±STD)	% fecal output
Group 1	Negative control (0.9% NaCl solution; 10 mL/kg)	0.42 ±0.5	----	7±0.71	---
Group 2	Positive control (Loperamide; 5 mg/kg)	0.006±0.01	98.57%	3.25±0.29	46.42%
Group 3	Jp-ML (200 mg/kg)	0.259±0.2	38.33%	6.5±0.58	92.86 %***
Group 4	Jp-ML (400 mg/kg)	0.145±0.12	65.48%	6±0.82	85.71%***
Group 5	Jp-ML (600 mg/kg)	0.059±0.05	85.95%	4.5±0.41	64.29%***

Values are expressed as mean ± SD (n = 4); Significance level at $P \leq 0.05$ (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$) in One way ANOVA; Test groups were compared to negative control group

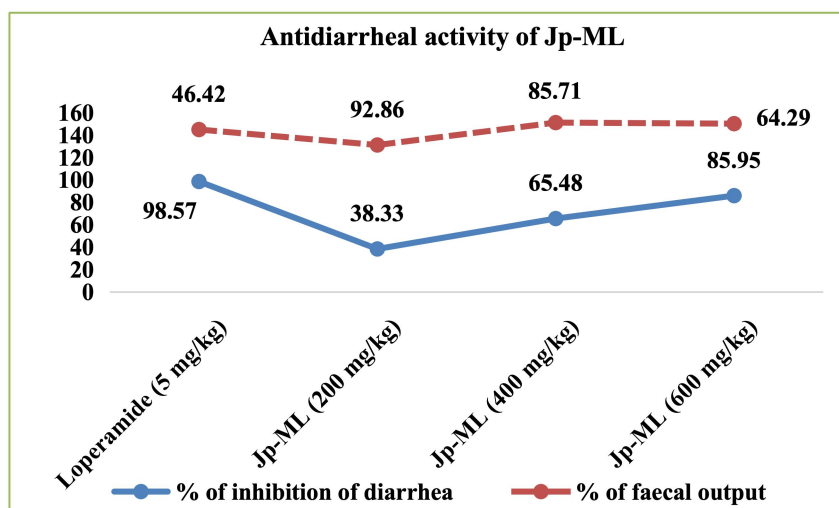


Figure 4.9: Antidiarrheal activity of Jp-ML (Castor oil induced diarrhea)

Jp-ML at doses of 200, 400, and 600 mg/kg showed notable inhibition in diarrheal frequency by 38.33%, 65.48%, and 85.95%, respectively, while loperamide recorded a 98.57% inhibition of gastric motility (Table 4.5; Figure 4.9). Total number of defecations were 6.5 ± 0.58 , 6 ± 0.82 , and 4.5 ± 0.41 for the respective doses. A significant dose-dependent reduction in fecal output was observed of 92.86% (** $P < 0.001$), 85.71% (** $P < 0.001$) and 64.29% (** $P < 0.001$) for same doses respectively. The onset of diarrhea were 60, 80, and 110 minutes for 200, 400, and 600 mg/kg doses, respectively, indicating a delay in diarrheal onset. Additionally, the maximum reduction in wet stool weight was observed at the 600 mg/kg dose (0.059 g), compared to the negative control group (0.42 g).

4.1.2.3.2 *Syzygium reticulatum*

Castor oil induced method

Group-1: Negative control (0.9% NaCl solution)

Mice ID	1 hr	2 hr	3 hr	4 hr
M1	0	0.319	0.187	0
M2	0	0.412	0.276	0.231
M3	0	0.055	0	0
M4	0	0.423	0.321	0.234
Mean	0	0.30225	0.196	0.1163
STD	0.00	0.17	0.14	0.13

Group-2: Positive control (Loperamide 5 mg/kg)

Mice ID	1 hr	2 hr	3 hr	4 hr
M1	0.059	0	0	0.013
M2	0	0	0	0
M3	0.0118	0	0	0.0110
M4	0	0	0	0
Mean	0.018	0	0	0.006
STD	0.03	0.00	0.00	0.01

$$\begin{aligned}
 \% \text{ of diarrheal inhibition} &= \frac{WFC - WFT}{WFC} \times 100 \\
 &= \{(0.1163 - 0.006) / 0.1163\} \times 100 \\
 &= 94.84\%
 \end{aligned}$$

$$\begin{aligned}
 \% \text{ of fecal output} &= \frac{\text{Mean defecation in test group}}{\text{Mean defecation in negative control group}} \times 100 \\
 &= \frac{3.25}{6.13} \times 100 \\
 &= 53.02\%
 \end{aligned}$$

Group-6: Sr-EAL 200 mg/kg

Mice ID	1 hr	2 hr	3 hr	4 hr
M1	0	0.126	0.111	0.152
M2	0	0.177	0	0
M3	0	0.12	0.064	0.054
M4	0	0	0	0.122
Mean	0	0.10575	0.04375	0.082
STD	0.00	0.07	0.05	0.07

$$\begin{aligned}
 \% \text{ of diarrheal inhibition} &= \frac{WFC - WFT}{WFC} \times 100 \\
 &= \{(0.1163 - 0.082) / 0.1163\} \times 100 \\
 &= 29.49\%
 \end{aligned}$$

$$\% \text{ of fecal output} = \frac{\text{Mean defecation in test group}}{\text{Mean defecation in negative control group}} \times 100$$

$$= \frac{4.8}{6.13} \times 100$$

$$= 78.03\%$$

Group-7: Sr-EAL 400 mg/kg

Mice ID	1 hr	2 hr	3 hr	4 hr
M1	0.233	0	0	0
M2	0	0	0	0
M3	0.157	0	0	0.253
M4	0	0	0	0
Mean	0.098	0	0	0.063
STD	0.12	0.00	0.00	0.13

$$\% \text{ of diarrheal inhibition} = \frac{WFC - WFT}{WFC} \times 100$$

$$= \{(0.1163 - 0.063) / 0.1163\} \times 100$$

$$= 45.83\%$$

$$\% \text{ of fecal output} = \frac{\text{Mean defecation in test group}}{\text{Mean defecation in negative control group}} \times 100$$

$$= \frac{4.13}{6.13} \times 100$$

$$= 67.37\%$$

Group-8: Sr-EAL 600 mg/kg

Mice ID	1 hr	2 hr	3 hr	4 hr
M1	0	0.143	0	0
M2	0	0	0	0
M3	0	0	0	0.058
M4	0	0.112	0	0
Mean	0	0.064	0	0.0145
STD	0.00	0.07	0.00	0.03

$$\% \text{ of diarrheal inhibition} = \frac{WFC - WFT}{WFC} \times 100$$

$$= \{(0.1163-0.0145)/0.1163\} \times 100$$

$$= 87.53\%$$

$$\% \text{ of fecal output} = \frac{\text{Mean defecation in test group}}{\text{Mean defecation in negative control group}} \times 100$$

$$= \frac{3.63}{6.13} \times 100$$

$$= 59.22\%$$

Table 4.6 : Antidiarrheal activity of Sr-EAL (Castor oil induced diarrhea)

Group	Treatment	Weight of wet feces (Mean±STD)	% of diarrheal inhibition	Number of defecation (Mean±STD)	% fecal output
Group 1	Negative control (0.9% NaCl solution; 10 mL/kg)	0.1163	----	6.13± 0.25	---
Group 2	Positive control (Loperamide; 5 mg/kg)	0.006± 0.01	94.84%	3.25 ± 0.5	53.02%
Group 6	Sr-EAL (200 mg/kg)	0.082± 0.07	29.49%	4.8± 0.29	78.03%***
Group 7	Sr-EAL (400 mg/kg)	0.063± 0.13	45.83%	4.13± 0.25	67.37%***
Group 8	Sr-EAL (600 mg/kg)	0.0145± 0.03	87.53%	3.63± 0.63	59.22%***

Values are expressed as mean ± SD (n = 4); Significance level at $P \leq 0.05$ (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$) in One way ANOVA; Test groups were compared to negative control group

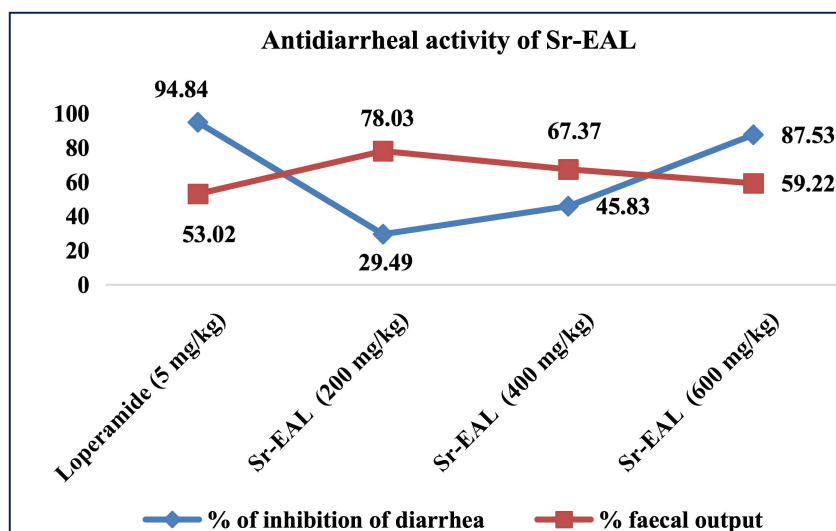


Figure 4.10: Antidiarrheal activity of Sr-EAL (Castor oil induced diarrhea)

Significant delays in diarrheal rates were recorded by 29.49%, 45.83%, and 87.53% for 200, 400, and 600 mg/kg doses of Sr-EAL, respectively, whereas loperamide showed a 94.84% inhibition of gastric motility (Table 4.6; Figure 4.10). The total number of defecations over 4 hours were 4.8 ± 0.29 , 4.13 ± 0.25 , and 3.63 ± 0.63 for the respective doses. Fecal output was reduced significantly by 78.03% (** $P < 0.001$), 67.37% (** $P < 0.001$), and 59.22% (** $P < 0.001$), respectively. The onset of diarrhea was delayed to 60, 100, and 120 minutes for the aforementioned doses. 400 mg/kg dose showed the greatest reduction in wet stool weight (0.0145 g), compared to 0.1163 g recorded in the negative control group.

The crude leaf extract of both plants significantly prolonged the onset of diarrhea and reduced total defecations. This supports the notion that the extracts possess strong antidiarrheal activity likely attributable to their rich phytochemical content, including phenols, tannins, and alkaloids (Jahan et al., 2022). Therefore, it could be presumed that both plants might act as antidiarrheal agents by slowing down gut motility, helping more electrolyte reabsorption and regulating frequency of bowel movements.

4.1.3 Analgesic activity

Pain involves in inflammation and tissue damage by synthesis of prostacyclin and prostaglandin E₂ (Calati et al., 2015). Opioids and NSAIDs are effective but they cause many serious health risks (Farshchi et al., 2009). Various plant-derived substances like flavonoids, alkaloids, terpenoids, and phenolic compounds offer safer alternatives to synthetic painkillers with fewer side effects.

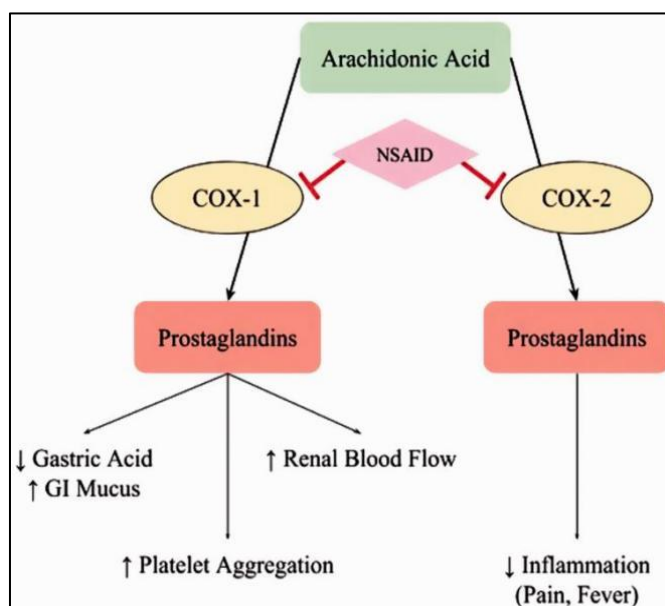


Figure 4.11: Mechanism of action of analgesic drugs

4.1.3.1 Principle of analgesic activity test

Acetic acid and formalin solution induce local inflammation resulting in the perception of pain (nociception). Formalin induced model was carried out in early phase and late phase. The biological response to this pain is typically observed by some indicators like writhing and licking behavior in mice. Being a potent NSAID indomethacin reduces the synthesis of prostaglandins, which are key mediators of pain.

4.1.3.2 Materials and experimental procedure

Equipment and reagents

- | | |
|---------------------|-----------------------|
| ✧ Saline Water | ✧ Syringe & stopwatch |
| ✧ Indomethacin | ✧ Beaker |
| ✧ Acetic acid | ✧ Marker & gloves |
| ✧ Formalin solution | ✧ Feeding needle |

Animal grouping and dosing

Rats were randomly assigned into following six groups:

Group-1: Negative control: 0.9 % NaCl (10 mL/kg, orally)

Group-2: Positive control: Standard (Indomethacin) (10 mg/ kg, intra peritoneal)

Group-3: Methanolic extract of *J. pandurifolia* leaf (100 mg/kg of b.w, orally)

Group-4: Methanolic extract of *J. pandurifolia* leaf (200 mg/kg of b.w, orally)

Group-5: Methanolic extract of *J. pandurifolia* leaf (300 mg/kg of b.w, orally)

Group-6: Ethyl acetate extract of *S. reticulatum* leaf (100 mg/kg of b.w, orally)

Group-7: Ethyl acetate extract of *S. reticulatum* leaf (200 mg/kg of b.w, orally)

Group-8: Ethyl acetate extract of *S. reticulatum* leaf (300 mg/kg of b.w, orally)

The solutions were prepared fresh on the experiment day.

Preparation of negative control (0.9% of NaCl)

0.9 g of sodium chloride was weighed and added to distilled water, then mixed thoroughly to make a final volume of 100 mL.

Preparation of standard drug (Indomethacin)

The required amount of indomethacin was weighed and dissolved in the appropriate amount of saline water, along with two drops of Tween-80 and 1 ml of DMSO. Each mouse received 1 ml of the solution via intraperitoneal injection.

Preparation of test sample

The required amount of extract was calculated and measured. The extract was dissolved in 4 ml of saline water, along with two drops of Tween-80 and 1 ml of DMSO. Each mouse received 1 ml of the solution orally.

A. Acetic-Acid induced writhing test (Couto et al., 2011)

Mice were injected with acetic acid (0.6%, 10 mL/kg, i.p.) after 30 minutes of aforementioned treatments. Analgesic activity was assessed by counting the number of writhes after the administration of acetic acid. Each animal was placed in a glass jar. Following a five minute latency period, the total number of abdominal muscle

contractions and hind limb stretches was recorded over a 30 minute period. The following formula was used to assess analgesic activity:

$$\% \text{ of Analgesic activity} = \frac{\text{MWC} - \text{MWT}}{\text{MWC}} \times 100$$

(MWC= Mean Writhing in Control Group; MWT= Mean Writhing in Test Group)



Figure 4.12: Observation of writhing in mice

B. Formalin induced nociception test (Tjølsen et al., 1992)

Mice were injected with formalin (5%, 20 mL/kg, i.p.) after 30 minutes of aforementioned treatments. The time (in seconds) taken for the injected paw to be licked or bitten was considered a measure of the pain response. Paw licking time was recorded during the early phase (0 to 5 minutes) and late phase (20 to 30 minutes) after formalin injection. The following formula was used to quantify analgesic activity:

$$\% \text{ of Analgesic activity} = \frac{\text{MLC} - \text{MLT}}{\text{MLC}} \times 100$$

(MLC= Mean Licking in Control Group; MLT= Mean Licking in Test Group)

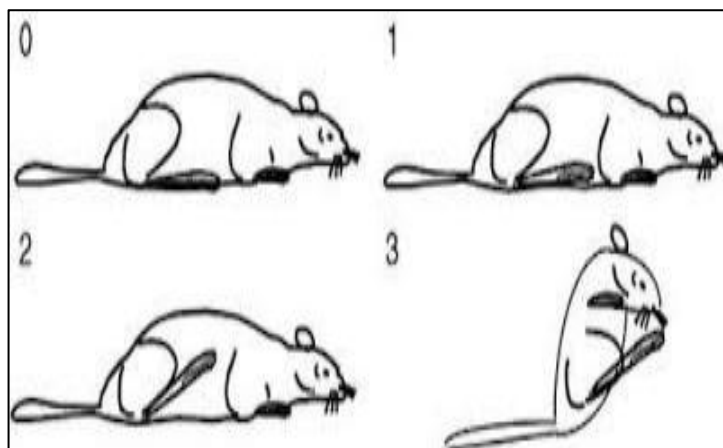


Figure 4.13: Observation of paw licking in mice

4.1.3.3 Result and discussion

4.1.3.3.1 *Jatropha pandurifolia*

A. Acetic-acid induced writhing test

Group-1: Negative control group: 0.9 % NaCl solution

Mice ID	Number of writhing
M1	10
M2	12.5
M3	15
M4	13.5
Mean	12.75
STD	2.94

Group-2: Positive control: Indomethacin (10 mg/kg)

Mice ID	Number of writhing	% of analgesic activity
M1	3	$= \frac{\text{MWC} - \text{MWT}}{\text{MWC}} \times 100$ $= \{12.75 - 2.75 / 12.75\} \times 100$ $= 78.43\%$
M2	3	
M3	2	
M4	3	
Mean	2.75	
STD	0.5	

Group-3: Jp-ML 100 mg/kg

Mice ID	Number of writhing	% of analgesic activity
M1	10	$= \frac{MWC - MWT}{MWC} \times 100$
M2	11	
M3	12	$= \{12.75 - 9.5 / 12.75\} \times 100$
M4	5	$= 25.49\%$
Mean	9.5	
STD	3.11	

Group-4: Jp-ML 200 mg/kg

Mice ID	Number of writhing	% of analgesic activity
M1	13	$= \frac{MWC - MWT}{MWC} \times 100$
M2	3.5	
M3	5.5	$= \{12.75 - 6.875 / 12.75\} \times 100$
M4	5.5	$= 46.08\%$
Mean	6.875	
STD	4.19	

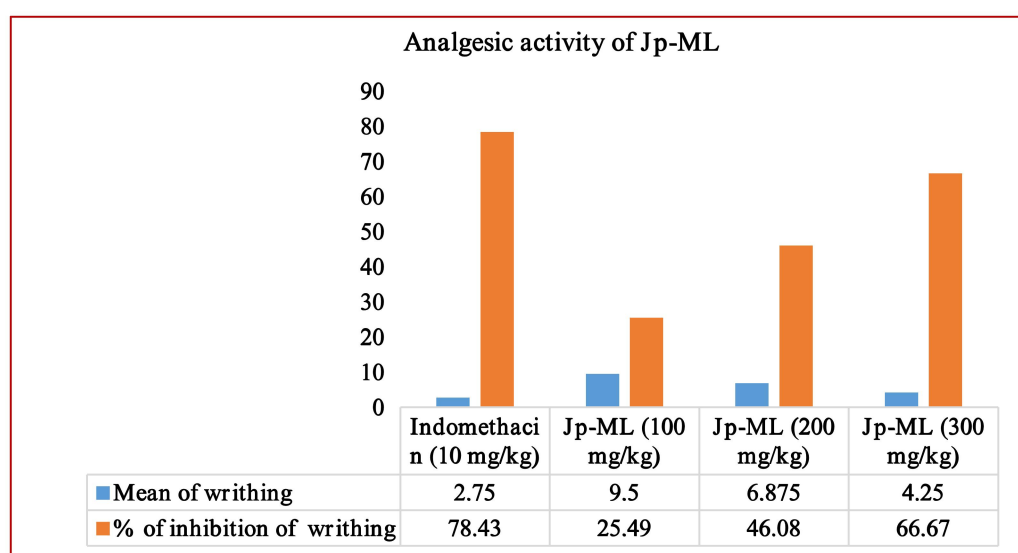
Group-5: Jp-ML 300 mg/kg

Mice ID	Number of writhing	% of analgesic activity
M1	8.5	$= \frac{MWC - MWT}{MWC} \times 100$
M2	3	
M3	3.5	$= \{12.75 - 4.25 / 12.75\} \times 100$
M4	2	$= 66.67\%$
Mean	4.25	
STD	2.90	

Table 4.7: Analgesic activity of Jp-ML (Acetic acid induced writhing)

Group	Treatment	Writhing (Mean± STD)	% of inhibition of writhing
Group 1	Negative Control (0.9% NaCl solution; 10 mL/kg)	12.75± 2.10	-----
Group 2	Positive control (Indomethacin; 10 mg/kg)	2.75± 0.5	78.43%
Group 3	Jp-ML (100 mg/kg)	9.5± 3.11	25.49%
Group 4	Jp-ML (200 mg/kg)	6.875 ± 4.19	46.08%**
Group 5	Jp-ML (300 mg/kg)	4.25± 2.90	66.67% **

Values are expressed as mean ± SD (n = 4); Significance level at $P \leq 0.05$ (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$) in One way ANOVA; Test groups were compared to negative control group

**Figure 4.14: Analgesic activity of Jp-ML (Acetic acid induced writhing)**

The extract significantly reduced the number of writhes to 9.5 ± 3.11 , 6.875 ± 4.19 , and 4.25 ± 2.90 , corresponding to 25.49%, 46.08% (** $P < 0.01$), and 66.67% (** $P < 0.01$) pain inhibition for 100, 200, and 300 mg/kg, respectively, compared to the negative control group (12.75 ± 2.10) (Table 4.7; Figure 4.14). Indomethacin (10 mg/kg) showed 78.43% (2.75 ± 0.5 writhes). These results suggest that Jp-ML possesses significant peripheral analgesic activity significantly lowering the quantity of acetic acid induced abdominal spasms.

B. Formalin induced nociception test**Group-1: Negative control group: 0.9 % NaCl Solution**

Early Phase		Late Phase	
Mice ID	Number of licking	Mice ID	Number of licking
M1	85	M1	120
M2	88	M2	153
M3	92	M3	153
M4	87	M4	144
Mean	88	Mean	142.5
STD	2.94	STD	15.59

Group-2: Positive Control group: Indomethacin (10 mg/kg)**Early Phase**

Mice ID	Number of licking
M1	30
M2	42
M3	42
M4	35
Mean	37.25
STD	5.85

% of analgesic activity

$$= \frac{MLC - MLT}{MLC} \times 100$$

$$= \{88 - 37.25 / 88\} \times 100$$

$$= 57.67\%$$

Late Phase

Mice ID	Number of licking
M1	35
M2	52
M3	49
M4	46
Mean	45.5
STD	7.42

% of analgesic activity

$$= \frac{MLC - MLT}{MLC} \times 100$$

$$= \{142.5 - 45.5 / 142.5\} \times 100$$

$$= 68.07\%$$

Group-3: Jp-ML 100 mg/kg**Early Phase**

Mice ID	Number of licking	% of analgesic activity
M1	75	$= \frac{MLC - MLT}{MLC} \times 100$
M2	68	$= \{88 - 71.5/88\} \times 100$
M3	73	$= 18.75\%$
M4	70	
Mean	71.5	
STD	3.11	

Late Phase

Mice ID	Number of licking	% of analgesic activity
M1	90	$= \frac{MLC - MLT}{MLC} \times 100$
M2	86	$= \{142.5 - 95.25/142.5\} \times 100$
M3	93	$= 33.16\%$
M4	112	
Mean	95.25	
STD	11.53	

Group-4: Jp-ML 200 mg/kg**Early Phase**

Mice ID	Number of licking	% of analgesic activity
M1	55	$= \frac{MLC - MLT}{MLC} \times 100$
M2	49	$= \{88 - 50.5/88\} \times 100$
M3	53	$= 42.61\%$
M4	45	
Mean	50.5	
STD	4.43	

Late Phase

Mice ID	Number of licking	% of analgesic activity
M1	95	$= \frac{MLC - MLT}{MLC} \times 100$
M2	65	$= \{142.5 - 72/142.5\} \times 100$
M3	75	$= 49.47\%$
M4	53	
Mean	72	
STD	17.78	

Group-5: Jp-ML 300 mg/kg**Early Phase**

Mice ID	Number of licking	% of analgesic activity
M1	23	$= \frac{MLC - MLT}{MLC} \times 100$
M2	35	
M3	38	$= \{88 - 31.75 / 88\} \times 100$
M4	31	$= 63.92$
Mean	31.75	
STD	6.5	

Late Phase

Mice ID	Number of licking	% of analgesic activity
M1	44	$= \frac{MLC - MLT}{MLC} \times 100$
M2	40	
M3	42	$= \{142.5 - 42.25 / 142.5\} \times 100$
M4	43	$= 70.35\%$
Mean	42.25	
STD	1.71	

Table 4.8: Analgesic activity of Jp-ML (Formalin induced pain)

Group	Treatment	Early Phase (0-5 mins)		Late phase (15-30 mins)	
		Licking time (Mean± STD)	Inhibition of pain (%)	Licking time (Mean ± STD)	Inhibition of pain (%)
Group 1	Negative control (0.9% NaCl solution; 10 mL/kg)	88±2.94	---	142.5± 15.5	---
Group 2	Positive control (Indomethacin; 10 mg/kg)	37.25±5.85	57.67	45.5 ± 7.42	68.07
Group 3	Jp-ML (100 mg/kg)	71.5±3.11	18.75***	95.25±11.53	33.16***
Group 4	Jp-ML (200 mg/kg)	50.5±4.43	42.61***	72 ± 17.78	49.47***
Group 5	Jp-ML (300 mg/kg)	31.75±6.5	63.92***	42.25± 1.71	70.35***

Values are expressed as mean ± SD (n = 4); Significance level at $P \leq 0.05$ (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) in One way ANOVA; Test groups were compared to negative control group

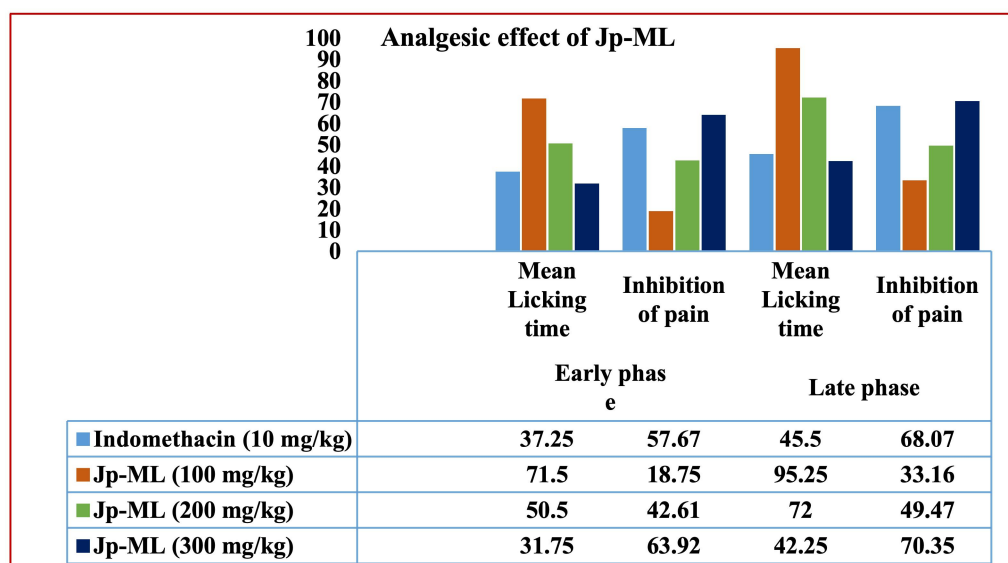


Figure 4.15: Analgesic activity of Jp-ML (Formalin induced pain)

The formalin-induced nociception test is commonly used as a model for evaluating tonic and inflammatory pain (Lee and Jeong, 2002). In the early phase, significant pain reduction were recorded as 18.75% (**P<0.001), 42.61% (**P<0.001), and 63.92% (**P<0.001), respectively, where paw licking counts were 71.5±3.11, 50.5±4.43, and 31.75±6.5. In the late phase, pain reduction was noted as 33.16% (**P<0.001), 49.47% (**P<0.001), and 70.35% (**P<0.001) with paw licking numbers were 95.25±11.53, 72± 17.78, and 42.25± 1.71 for 100 mg/kg, 200 mg/kg, and 300 mg/kg, respectively (Table 4.8; Figure 4.15). Indomethacin inhibited the paw licking episode by 57.67 and 68.07 % in early and late phase, respectively. The significant effects observed in both phases of the formalin-induced nociception test suggest that the extract exhibits both peripheral and central analgesic activity.

4.1.3.3.2 *Syzygium reticulatum*

A. Acetic-acid induced writhing test

Group-1: Negative control group: 0.9 % NaCl solution

Mice ID	Number of writhing
M1	10
M2	12.5
M3	15
M4	13.5
Mean	12.75
STD	2.10

Group-2: Positive Control group: Indomethacin (10 mg/kg)

Mice ID	Number of writhing	% of analgesic activity
M1	3	$= \frac{MWC - MWT}{MWC} \times 100$
M2	3	
M3	2	$= \{12.75 - 2.75 / 12.75\} \times 100$
M4	3	
Mean	2.75	$= 78.43\%$
STD	0.5	

Group-6: Sr-EAL 100 mg/kg

Mice ID	Number of writhing	% of analgesic activity
M1	8	$= \frac{MWC - MWT}{MWC} \times 100$
M2	0	
M3	9	$= \{12.75 - 6.125 / 12.75\} \times 100$
M4	7.5	
Mean	6.125	$= 51.96\%$
STD	4.13	

Group-7: Sr-EAL 200 mg/kg

Mice ID	Number of writhing	% of analgesic activity
M1	6	$= \frac{MWC - MWT}{MWC} \times 100$ $= \{12.75 - 4.625 / 12.75\} \times 100$ $= 63.73\%$
M2	3	
M3	4	
M4	5.5	
Mean	4.625	
STD	1.38	

Group-8: Sr-EAL 300 mg/kg

Mice ID	Number of writhing	% of analgesic activity
M1	5.5	$= \frac{MWC - MWT}{MWC} \times 100$ $= \{12.75 - 3.75 / 12.75\} \times 100$ $= 70.59\%$
M2	4.5	
M3	2.5	
M4	2.5	
Mean	3.75	
STD	1.5	

Table 4.9: Analgesic activity of Sr-EAL (Acetic acid induced writhing)

Group	Treatment	Writhing (Mean± STD)	% of inhibition of writhing
Group 1	Negative control (0.9% NaCl solution, 10 mL/kg)	12.75± 2.10	-----
Group 2	Positive control (Indomethacin; 10 mg/kg)	2.75± 0.5	78.43%
Group 6	Sr-EAL (100 mg/kg)	6.125± 4.13	51.96%***
Group 7	Sr-EAL (200 mg/kg)	4.625± 1.38	63.73%***
Group 8	Sr-EAL (300 mg/kg)	3.75± 1.5	70.59%***

Values are expressed as mean ± SD (n = 4); Significance level at $P \leq 0.05$ (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$) in One way ANOVA; Test groups were compared to negative control group

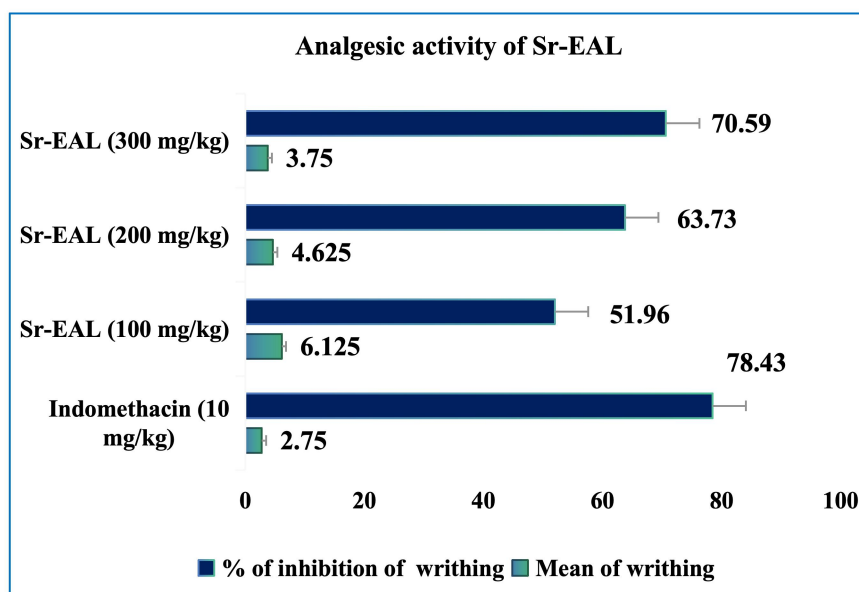


Figure 4.16: Analgesic activity of Sr-EAL (Acetic acid induced writhing)

Mice treated with the Sr-EAL extract showed fewer cramps compared to the control group, with writhing counts of 6.125 ± 4.13 , 4.625 ± 1.38 , and 3.75 ± 1.5 at 100 mg/kg, 200 mg/kg, and 300 mg/kg, respectively. Significant dose-dependent inhibition of writhing were recorded as 51.96% (** $P < 0.001$), 63.73% (** $P < 0.001$), and 70.59% (** $P < 0.001$) for the respective doses, whereas indomethacin inhibited writhing by 78.43% (Table 4.9; Figure 4.16).

B. Formalin induced nociception test

Group-1: Negative control group: 0.9 % NaCl Solution

Early Phase		Late Phase	
Mice ID	Number of licking	Mice ID	Number of licking
M1	85	M1	120
M2	88	M2	153
M3	92	M3	153
M4	87	M4	144
Mean	88	Mean	142.5
STD	2.94	STD	15.59

Group-2: Positive Control group: Indomethacin (10 mg/kg)**Early Phase**

Mice ID	Number of licking	% of analgesic activity
M1	30	$= \frac{MLC - MLT}{MLC} \times 100$
M2	42	$= \{88 - 37.25/88\} \times 100$
M3	42	$= 57.67\%$
M4	35	
Mean	37.25	
STD	5.85	

Late Phase

Mice ID	Number of licking	% of analgesic activity
M1	35	$= \frac{MLC - MLT}{MLC} \times 100$
M2	52	$= \{142.5 - 45.5/142.5\} \times 100$
M3	49	$= 68.07\%$
M4	46	
Mean	45.5	
STD	7.42	

Group-6: Sr-EAL 100 mg/kg**Early Phase**

Mice ID	Number of licking	% of analgesic activity
M1	72	$= \frac{MLC - MLT}{MLC} \times 100$
M2	63	$= \{88 - 68/88\} \times 100$
M3	71	$= 22.73\%$
M4	66	
Mean	68	
STD	4.24	

Late Phase

Mice ID	Number of licking	% of analgesic activity
M1	82	$= \frac{MLC - MLT}{MLC} \times 100$
M2	80	$= \{142.5 - 74.75/142.5\} \times 100$
M3	71	$= 47.54\%$
M4	66	
Mean	74.75	
STD	7.54	

Group-7: Sr-EAL 200 mg/kg**Early Phase**

Mice ID	Number of licking	% of analgesic activity
M1	50	$= \frac{MLC - MLT}{MLC} \times 100$
M2	70	$= \{88 - 50.25/88\} \times 100$
M3	41	
M4	40	$= 42.90\%$
Mean	50.25	
STD	13.91	

Late Phase

Mice ID	Number of licking	% of analgesic activity
M1	55	$= \frac{MLC - MLT}{MLC} \times 100$
M2	69	$= \{142.5 - 57/142.5\} \times 100$
M3	54	
M4	50	$= 60\%$
Mean	57	
STD	8.29	

Group-8: Sr-EAL 300 mg/kg**Early Phase**

Mice ID	Number of licking	% of analgesic activity
M1	28	$= \frac{MLC - MLT}{MLC} \times 100$
M2	44	$= \{88 - 40/88\} \times 100$
M3	50	
M4	38	$= 54.55 \%$
Mean	40	
STD	9.38	

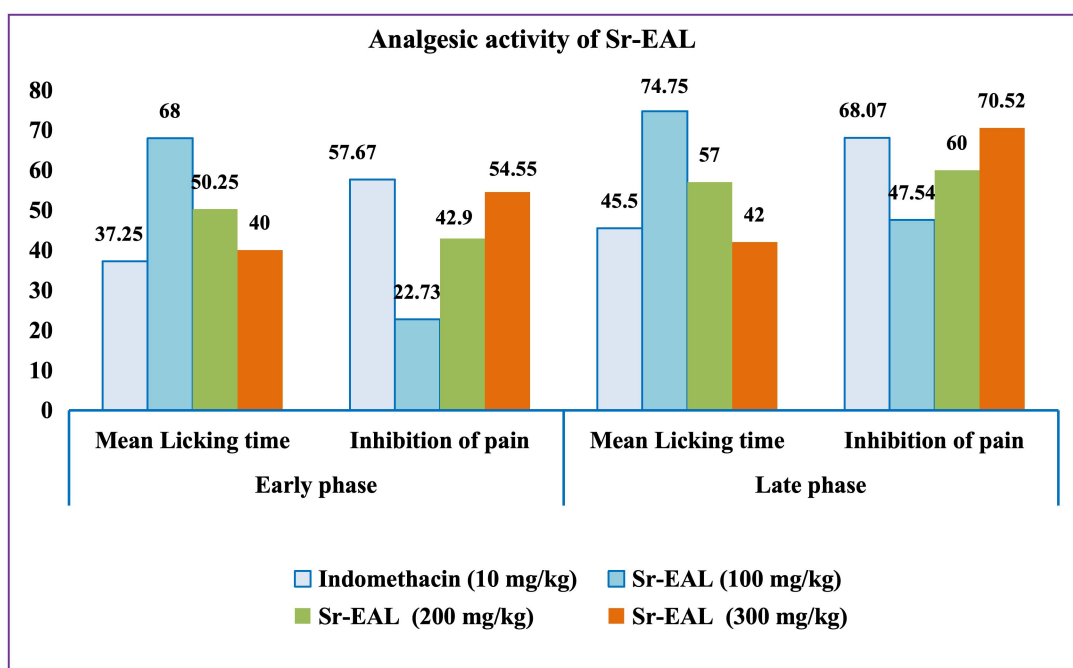
Late Phase

Mice ID	Number of licking	% of analgesic activity
M1	32	$= \frac{MLC - MLT}{MLC} \times 100$
M2	50	$= \{142.5 - 42/142.5\} \times 100$
M3	45	
M4	41	$= 70.52\%$
Mean	42	
STD	7.62	

Table 4.10: Analgesic activity Sr-EAL (Formalin induced pain)

Group	Treatment	Early Phase (0-5 mins)		Late phase (15-30 mins)	
		Licking time (Mean± STD)	Inhibition of pain (%)	Licking time (Mean ± STD)	Inhibition of pain (%)
Group 1	Negative Control (0.9% NaCl solution, 10 mL/kg)	88±2.94	---	142.5±15.5	---
Group 2	Positive control (Indomethacin; 10 mg/kg)	37.25±5.85	57.67	45.5±7.42	68.07
Group 6	Sr-EAL (100 mg/kg)	68±4.24	22.73**	74.75±7.54	47.54***
Group 7	Sr-EAL (200 mg/kg)	50.25±13.91	42.9***	57±8.29	60***
Group 8	Sr-EAL (300 mg/kg)	40±9.38	54.55***	42.00±7.62	70.52***

Values are expressed as mean ± SD (n = 4); Significance level at P ≤ 0.05 (*P<0.05; **P<0.01, ***P<0.001) in One way ANOVA; Test groups were compared to negative control group

**Figure 4.17: Analgesic activity of Sr-EAL (Formalin induced pain)**

In the early phase, strong to moderately significant pain reduction were recorded by 22.73% (**P<0.01), 42.9% (***P<0.001), and 54.55% (***P<0.001) with paw licking counts of 68 ± 4.24 , 50.25 ± 13.91 , and 40 ± 9.38 at 100 mg/kg, 200 mg/kg, and 300 mg/kg doses of Sr-EAL, respectively. In the late phase, percentage of pain reductions were 47.54% (***P<0.001), 60% (***P<0.001), and 70.52% (***P<0.001) with decreased paw licking frequency by 74.75 ± 7.54 , 57 ± 8.29 , and 42 ± 7.62 , respectively (Table 4.10; Figure 4.17). Indomethacin exhibited paw licking reduction by 57.67% and 68.07% in the early and late phases, respectively.

Centrally acting analgesics have been shown to inhibit acetic acid induced pain (Trongsakul et al., 2003). In formalin induced nociception model, the early phase caused by direct activation of nociceptors and a late phase resulting from inflammatory responses (Shibata et al., 1979). Peripheral analgesics predominantly affect the second phase, whereas central analgesics are effective in both phases (Yamamoto et al., 2002). Significant inhibition in both phases, Jp-ML and Sr-EAL extracts exhibit both central and peripheral analgesic activities, assuring a strong assumption for their prospective development as efficient analgesic agents.

In vitro Analysis

4.1.4 Antioxidant activity

Oxidative stress is induced by formation of free radicals and associated with numerous degenerative conditions, including cancer, inflammation, cardiovascular and neurological disorders (Christodoulou et al., 2022). Widely used antioxidants are vit-C, vit-E, glutathione, melatonin etc. while phytochemicals such as curcumin, resveratrol, quercetin, flavonoid and phenol are also well studied for preventing oxidation.

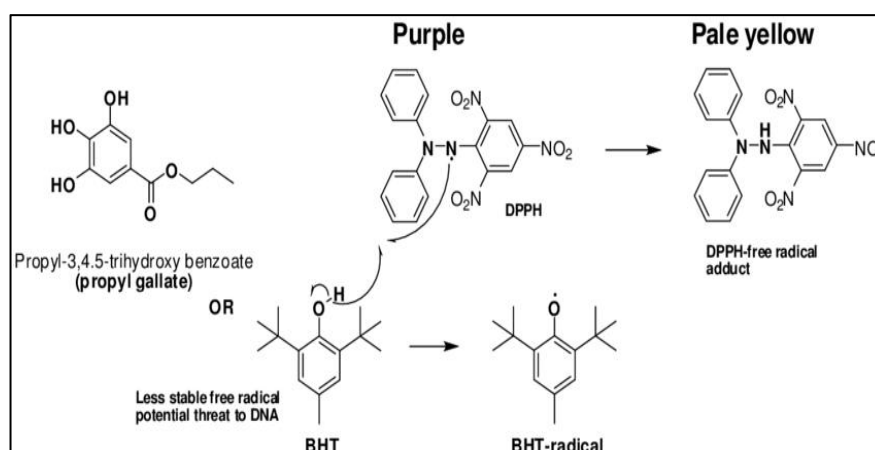


Figure 4.18: Mechanism of action of BHT

4.1.4.1 Principle of antioxidant activity (DPPH test)

When a DPPH (2,2-diphenyl-1-picrylhydrazyl) solution comes into contact with an antioxidant, it accepts hydrogen from the antioxidant and reduced into DPPH-H (Yapıcı et al., 2021). After reduction the solution changes from violet to a light yellow color. Butylated hydroxytoluene is widely used to prevent oxidation by neutralizing different radicals.

4.1.4.2 Materials and experimental procedure

Equipment and reagents

- | | |
|--|-------------------|
| ✧ 1,1-diphenyl-2-picrylhydrazyl (DPPH) | ✧ Eppendorf tube |
| ✧ Methanol | ✧ Distilled water |
| ✧ UV spectrophotometer | ✧ Light proof box |
| ✧ Butylated Hydroxy Anisole (BHA) | ✧ Micropipette |

Preparation of negative control (DPPH solution)

A DPPH solution with a concentration of 20 µg/mL was prepared in methanol. The resulting solution was stored in an amber glass container, protected from light.

Preparation of positive control (BHT)

BHT was used as the positive control and was prepared by dissolving butylated hydroxytoluene (BHT) in methanol at a concentration of 8000 µg/mL. This mother solution was then serially diluted to obtain a range of concentrations from 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, 1.5625 µg/mL, and 0.7813 µg/mL.

Preparation of test sample

After dissolving various extractives in methanol, a mother solution was prepared with a concentration of 4000 µg/mL. The stock solution was serially diluted to obtain a range of concentrations from 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.63 µg/mL, 7.83 µg/mL, 3.91 µg/mL, 1.96 µg/mL, and 0.98 µg/mL.

DPPH free radical scavenging assay (Brand-Williams et al., 1995)

2 mL of DPPH solution (20 µg/mL in methanol) was mixed with 2 mL of various concentrations of the test samples. The mixture was then incubated for 30 minutes at 25 °C in a dark environment, after which the UV absorbance was measured at 517 nm. IC₅₀ values were determined by plotting concentrations against the percentage of free radical scavenging and applying the regression equation. BHT and methanolic DPPH solution were used as the positive and negative controls, respectively. DPPH radical scavenging activity was calculated using the following equation:

$$I\% = 1 - \{A_1 / A_0\} \times 100$$

A₀= absorbance of the control and A₁= absorbance of test samples or standard.

4.1.4.3 Result and discussion

4.1.4.3.1 Antioxidant activity of plant extracts

Table 4.11: IC₅₀ of *J. pandurifolia* and *S. reticulatum*

Test sample	Sample code	IC ₅₀ value (µg/mL)
<i>J. pandurifolia</i>	Jp-ML (Methanolic extract)	8.11
	Jp-EAL (Ethyl acetate extract)	30.55
	Jp-CL (Chloroform extract)	439.10
	Jp-HL (n-Hexane extract)	667.38
<i>S. reticulatum</i>	Sr-ML (Methanolic extract)	14.33
	Sr-EAL (Ethyl acetate extract)	10.34
	Sr-CL (Chloroform extract)	230.40
	Sr-HL (n-Hexane extract)	628.99
Positive control	Butylated hydroxytoluene (BHT)	2.41
Negative control	Methanolic DPPH solution	93.22

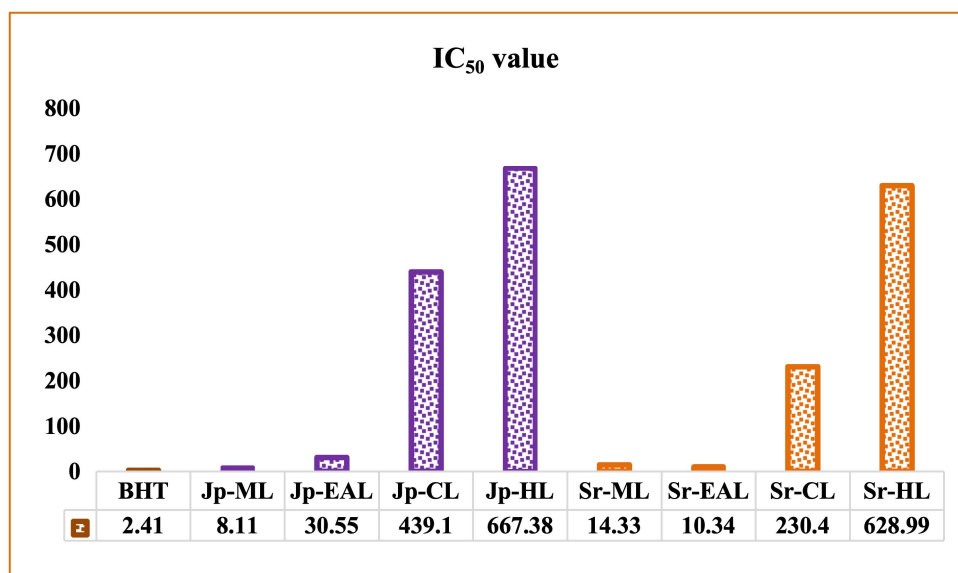
Figure 4.19: IC₅₀ of *J. pandurifolia* and *S. reticulatum*

Table 4.12: IC₅₀ value of butylated hydroxytoluene (BHT)

Conc. ($\mu\text{g/ mL}$)	Abs. 1	Abs. 2	Abs. 3	Mean Absorbance	% of Scavanging	IC ₅₀ ($\mu\text{g/ mL}$)
200	0.05	0.071	0.031	0.03	95.52	
100	0.052	0.072	0.083	0.069	89.7	
50	0.075	0.082	0.08	0.079	88.21	
25	0.142	0.128	0.129	0.133	80.15	2.41
12.5	0.147	0.163	0.167	0.159	76.23	
6.25	0.255	0.266	0.265	0.262	60.90	
3.125	0.359	0.371	0.35	0.360	46.27	
1.563	0.522	0.546	0.525	0.531	20.75	
0.7813	0.612	0.620	0.619	0.617	7.93	

Absorbance of the Blank = 0.67

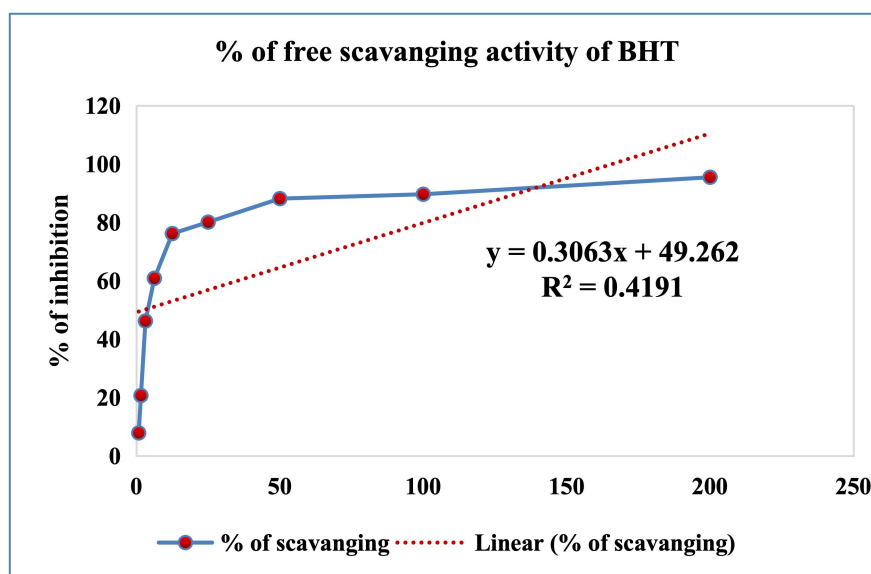
**Figure 4.20: IC₅₀ value of butylated hydroxytoluene (BHT)**

Table 4.13: IC₅₀ value of Jp-ML and Jp-EAL

Jp-ML					Jp-EAL				
Conc. ($\mu\text{g}/\text{mL}$)	Abs. 1	Abs. 2	Mean Absorbance $\pm$ STD	% of Scavenging	Abs. 1	Abs. 2	Abs. 3	Mean Absorbance $\pm$ STD	% of Scavenging
1000	0.156	0.17	0.163 $\pm$ 0.01	75.67	0.181	0.184	0.183	0.184 $\pm$ 0.002	72.54
500	0.188	0.198	0.193 $\pm$ 0.01	71.18	0.185	0.193	0.187	0.193 $\pm$ 0.004	71.2
250	0.219	0.201	0.21 $\pm$ 0.01	68.66	0.23	0.21	0.219	0.228 $\pm$ 0.01	65.97
125	0.231	0.225	0.228 $\pm$ 0.00	65.97	0.26	0.23	0.22	0.239 $\pm$ 0.02	64.32
62.5	0.249	0.269	0.259 $\pm$ 0.01	61.34	0.292	0.273	0.251	0.273 $\pm$ 0.02	59.25
31.25	0.268	0.286	0.277 $\pm$ 0.01	58.66	0.315	0.318	0.301	0.310 $\pm$ 0.01	53.73
15.63	0.301	0.291	0.296 $\pm$ 0.01	55.82	0.322	0.326	0.329	0.326 $\pm$ 0.004	51.34
7.813	0.341	0.285	0.313 $\pm$ 0.04	53.28	0.33	0.343	0.322	0.333 $\pm$ 0.01	50.30
3.91	0.331	0.347	0.339 $\pm$ 0.01	49.4	0.37	0.384	0.363	0.371 $\pm$ 0.01	44.62
1.96	0.472	0.454	0.463 $\pm$ 0.01	39.73	0.38	0.378	0.445	0.401 $\pm$ 0.04	40.13
0.98	0.585	0.567	0.576 $\pm$ 0.01	14.03	0.496	0.459	0.485	0.480 $\pm$ 0.019	28.34
IC ₅₀ ($\mu\text{g}/\text{mL}$) = 8.11					IC ₅₀ ($\mu\text{g}/\text{mL}$) = 30.55				

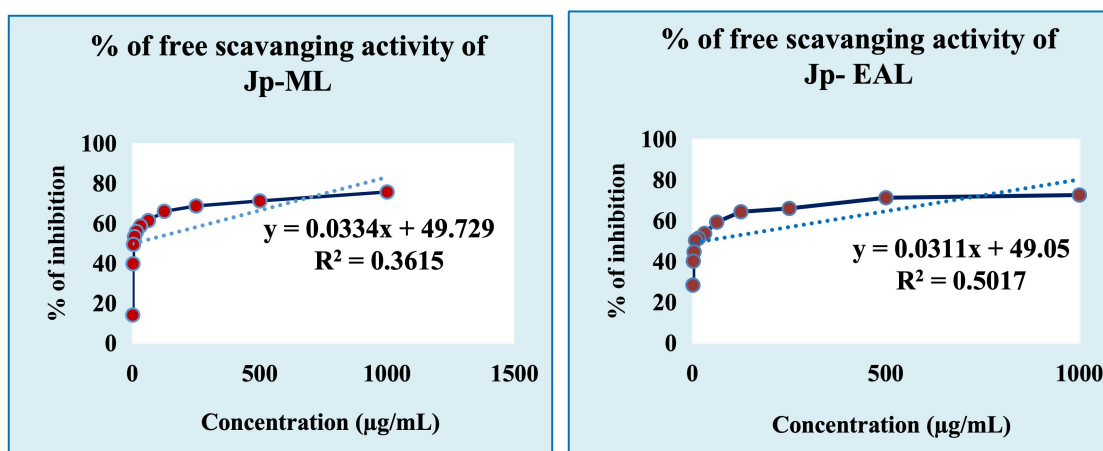
Figure 4.21: IC₅₀ value of Jp-ML and Jp-EAL

Table 4.14: IC₅₀ value of Jp-CL and Jp-HL

Jp-CL						Jp-HL				
Conc. ($\mu\text{g}/\text{mL}$)	Abs. 1	Abs. 2	Abs. 3	Mean Absorbance $\pm$ STD	% of Scavenging	Abs. 1	Abs. 2	Abs. 3	Mean Absorbance $\pm$ STD	% of Scavenging
1000	0.312	0.349	0.335	0.332 $\pm$ 0.02	70.14	0.278	0.269	0.26	0.269 $\pm$ 0.01	59.88
500	0.354	0.324	0.444	0.374 $\pm$ 0.06	59.18	0.378	0.363	0.387	0.376 $\pm$ 0.01	43.98
250	0.367	0.332	0.363	0.354 $\pm$ 0.02	47.16	0.41	0.418	0.402	0.41 $\pm$ 0.01	38.81
125	0.394	0.382	0.379	0.385 $\pm$ 0.01	42.53	0.417	0.425	0.418	0.42 $\pm$ 0.01	37.31
62.5	0.419	0.423	0.403	0.415 $\pm$ 0.01	38.06	0.439	0.455	0.429	0.441 $\pm$ 0.01	34.18
31.25	0.434	0.412	0.417	0.421 $\pm$ 0.01	37.16	0.457	0.486	0.458	0.467 $\pm$ 0.01	30.3
15.63	0.452	0.444	0.436	0.444 $\pm$ 0.01	33.73	0.489	0.506	0.505	0.5 $\pm$ 0.01	25.37
7.813	0.463	0.455	0.432	0.450 $\pm$ 0.01	32.84	0.537	0.533	0.529	0.533 $\pm$ 0.00	20.44
3.91	0.481	0.477	0.452	0.470 $\pm$ 0.01	29.85	0.566	0.581	0.569	0.572 $\pm$ 0.01	14.63
1.96	0.637	0.536	0.555	0.576 $\pm$ 0.05	14.03	0.594	0.611	0.619	0.608 $\pm$ 0.01	9.25
0.98	0.585	0.567	-	0.576 $\pm$ 0.01	14.03	0.599	0.621	0.658	0.626 $\pm$ 0.03	6.57
IC ₅₀ ($\mu\text{g}/\text{mL}$) = 439.10						IC ₅₀ ($\mu\text{g}/\text{mL}$) = 667.38				

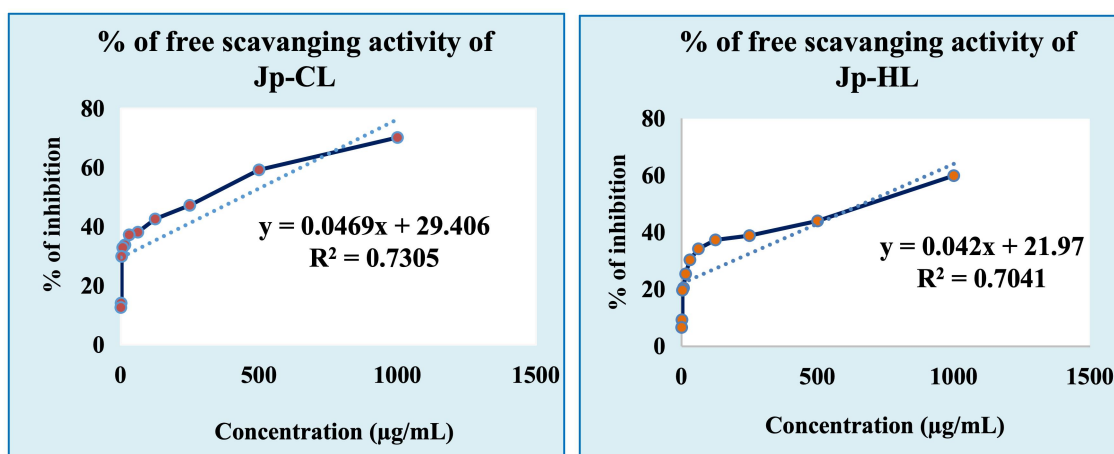
Figure 4.22: IC₅₀ value of Jp-CL and Jp-HL

Table 4.15: IC₅₀ value of Sr-ML and Sr-EAL

Sr-ML						Sr-EAL				
Conc. ($\mu\text{g}/\text{mL}$)	Abs. 1	Abs. 2	Abs. 3	Mean Absor bance $\pm$ STD	% of Scavan ging	Abs. 1	Abs. 2	Abs. 3	Mean Absor bance $\pm$ STD	% of Scavangi ng
1000	0.048	0.024	0.042	0.038 $\pm$ 0.01	94.58	0.119	0.12	0.13	0.123 $\pm$ 0.01	82.45
500	0.149	0.148	0.129	0.142 $\pm$ 0.01	79.78	0.209	0.219	0.208	0.212 $\pm$ 0.01	69.8
250	0.182	0.247	0.174	0.201 $\pm$ 0.04	71.36	0.18	0.197	0.205	0.194 $\pm$ 0.01	72.36
125	0.228	0.234	0.27	0.244 $\pm$ 0.02	65.24	0.261	0.262	0.251	0.258 $\pm$ 0.00	63.25
62.5	0.249	0.263	0.349	0.287 $\pm$ 0.05	59.1	0.281	0.275	0.305	0.287 $\pm$ 0.02	59.12
31.25	0.275	0.322	0.342	0.313 $\pm$ 0.03	55.43	0.321	0.299	0.319	0.313 $\pm$ 0.01	55.41
15.63	0.326	0.341	0.359	0.342 $\pm$ 0.02	51.3	0.353	0.340	0.354	0.349 $\pm$ 0.01	50.28
7.813	0.348	0.347	0.352	0.349 $\pm$ 0.00	50.28	0.378	0.380	0.394	0.384 $\pm$ 0.01	45.3
3.91	0.367	0.404	0.399	0.390 $\pm$ 0.02	44.44	0.362	0.355	0.366	0.361 $\pm$ 0.01	48.58
1.96	0.416	0.426	0.406	0.416 $\pm$ 0.01	40.74	0.411	0.412	0.404	0.409 $\pm$ 0.00	41.74
0.98	0.453	0.499	0.461	0.471 $\pm$ 0.02	32.9	0.47	0.461	0.464	0.465 $\pm$ 0.00	33.9
IC ₅₀ ($\mu\text{g}/\text{mL}$) = 14.33						IC ₅₀ ($\mu\text{g}/\text{mL}$) = 10.34				

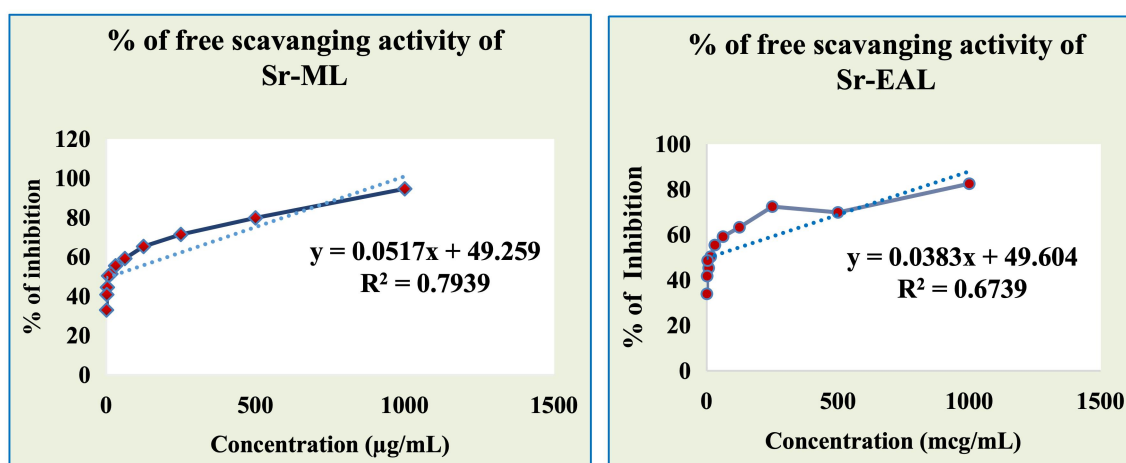
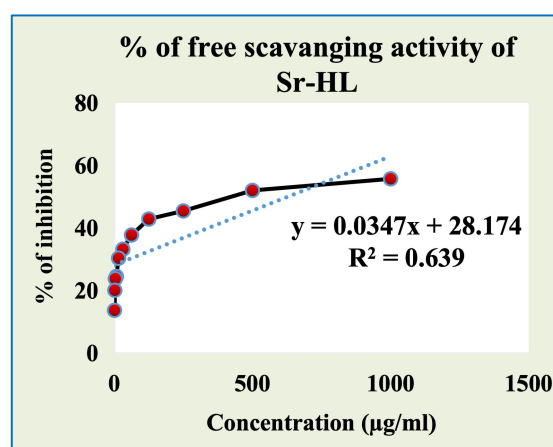
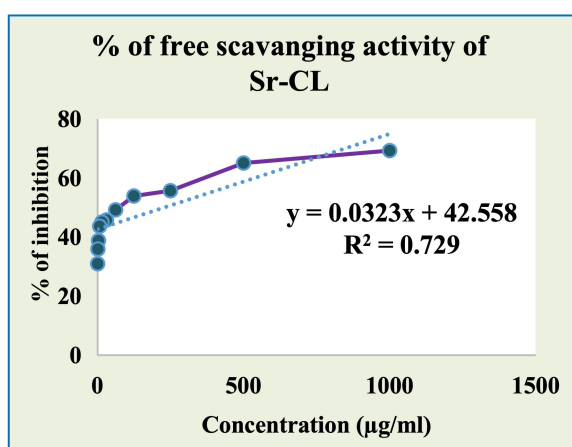
Figure 4.23: IC₅₀ value of Sr-ML and Sr-EAL

Table 4.16: IC₅₀ value of Sr-CL and Sr-HL

Sr-CL						Sr-HL				
Conc. ($\mu\text{g}/\text{mL}$)	Abs. 1	Abs. 2	Abs. 3	Mean Absor bance $\pm\text{STD}$	% of Scavan ging	Abs. 1	Abs. 2	Abs. 3	Mean Absor bance $\pm$ STD	% of Scavangi ng
1000	0.23	0.2	0.218	0.216 $\pm$ 0.02	69.22	0.334	0.329	0.27	0.311 $\pm$ 0.04	55.7
500	0.244	0.25	0.236	0.243 $\pm$ 0.01	65.01	0.357	0.313	0.341	0.337 $\pm$ 0.02	51.99
250	0.325	0.299	0.309	0.311 $\pm$ 0.01	55.64	0.355	0.398	0.396	0.383 $\pm$ 0.02	45.44
125	0.311	0.349	0.313	0.324 $\pm$ 0.02	53.85	0.422	0.394	0.387	0.401 $\pm$ 0.02	42.85
62.5	0.348	0.352	0.369	0.357 $\pm$ 0.01	49.15	0.436	0.452	0.423	0.437 $\pm$ 0.01	37.75
31.25	0.379	0.391	0.372	0.381 $\pm$ 0.01	45.73	0.498	0.452	0.457	0.469 $\pm$ 0.03	33.21
15.63	0.415	0.401	0.342	0.386 $\pm$ 0.04	45.01	0.490	0.472	0.505	0.489 $\pm$ 0.02	30.34
7.813	0.426	0.398	0.364	0.396 $\pm$ 0.03	43.59	0.522	0.57	0.498	0.530 $\pm$ 0.04	24.50
3.91	0.429	0.394	0.467	0.430 $\pm$ 0.04	38.75	0.547	0.522	0.536	0.535 $\pm$ 0.01	23.78
1.96	0.437	0.444	0.469	0.450 $\pm$ 0.02	35.88	0.555	0.574	0.554	0.561 $\pm$ 0.01	20.09
0.98	0.513	0.482	0.460	0.485 $\pm$ 0.03	30.91	0.586	0.634	0.598	0.606 $\pm$ 0.02	13.68
IC ₅₀ ($\mu\text{g}/\text{mL}$) = 230.40						IC ₅₀ ($\mu\text{g}/\text{mL}$) = 628.99				

Figure 4.24: IC₅₀ value of Sr-CL and Sr-HL

The term "IC₅₀" represents the concentration of an antioxidant needed to inhibit 50% of the DPPH radicals, serving as a measure of antioxidant effectiveness. It provides an analytical description of the radical scavenging capacity (Koca et al., 2023). Lower IC₅₀ values indicate higher radical scavenging activity of antioxidants. According to Setha et al. (2013), antioxidant strength is categorized into four levels: very strong (IC₅₀ < 50 µg/mL), strong (IC₅₀: 50–100 µg/mL), moderate (IC₅₀: 101–150 µg/mL), and weak (IC₅₀: 250–500 µg/mL). Jp-ML exhibited strong antioxidant properties with the lowest IC₅₀ value of 8.11 µg/mL, while the non-polar n-hexane fraction (Jp-HL) showed negligible antioxidant activity with an IC₅₀ of 667.38 µg/mL. The IC₅₀ values for other fractions were as follows: Jp-EAL: 30.55 µg/mL (strong activity), Jp-CL: 439.1 µg/mL (weak activity) (Table 4.13-Table 4.14, Figure 4.21-Figure 4.22). In *S. reticulatum*, Sr-EAL: 10.34 µg/mL (strong potency), Sr-ML: 14.33 µg/mL (strong potency), Sr-CL: 230.4 µg/mL (moderate potency), and Sr-HL: 628.99 µg/mL (inactive) (Table 4.15-Table 4.16, Figure 4.23, Figure 4.24). BHT showed IC₅₀ value of 2.41 µg/mL (Table 4.12, Figure 4.20).

Revealing significant scavenging property, the plant extracts confirm the presence of phenolic and flavonoid compounds, which are known as for their role in oxidative stress diminution. These results reinforce their possibility as natural antioxidant.

4.1.4.3.2 Antioxidant activity of pure compounds

Table 4.17: IC₅₀ value of isolated pure compounds

Sample code	IC ₅₀ value (µg/ml)
BHT	2.41
JP-10	16.17
JP-13	14.12
JP-25	13.10
JP-460	43.43

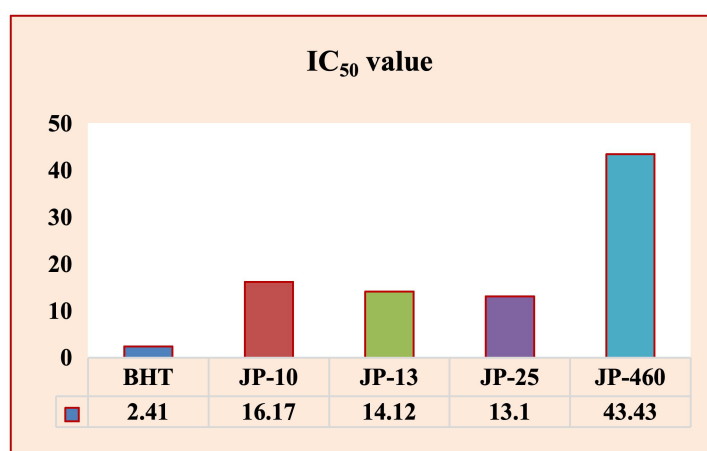


Figure 4.25: IC₅₀ value of isolated pure compounds

Table 4.18: IC₅₀ value of JP-10 and JP-13

JP-10						JP-13				
Conc. (µg/ mL)	Abs. 1	Abs. 2	Abs. 3	Mean Absor bance± STD	% of Scava nging	Abs. 1	Abs. 2	Abs. 3	Mean Absor bance± STD	% of Scavangi ng
1000	0.112	0.113	0.123	0.116± 0.01	81.92	0.106	0.128	0.132	0.122± 0.01	81
500	0.146	0.155	0.167	0.156± 0.01	75.56	0.126	0.133	0.125	0.128± 0.00	79.99
250	0.246	0.237	0.222	0.235± 0.01	63.28	0.211	0.208	0.199	0.206± 0.01	67.88
125	0.283	0.256	0.28	0.273± 0.01	57.41	0.231	0.256	0.245	0.244± 0.01	61.88
62.5	0.311	0.278	0.287	0.292± 0.02	54.33	0.295	0.270	0.260	0.275± 0.02	56.99
31.25	0.286	0.311	0.309	0.302± 0.01	52.88	0.291	0.290	0.277	0.286± 0.01	55.35
15.63	0.319	0.320	0.312	0.317± 0.00	50.44	0.288	0.311	0.319	0.306± 0.02	52.11
7.813	0.315	0.345	0.339	0.333± 0.02	47.97	0.32	0.341	0.329	0.33±0 .01	48.42
3.91	0.345	0.330	0.345	0.34±0 .01	46.98	0.321	0.322	0.329	0.324± 0.00	49.40
1.96	0.317	0.367	0.387	0.357± 0.04	44.21	0.354	0.387	0.399	0.38±0 .02	40.77
0.98	0.364	0.362	0.351	0.359± 0.01	43.98	0.462	0.432	0.441	0.445± 0.02	30.42
IC ₅₀ (µg/ mL) = 16.17						IC ₅₀ (µg/ mL) = 14.12				

Absorbance of blank: 0.64

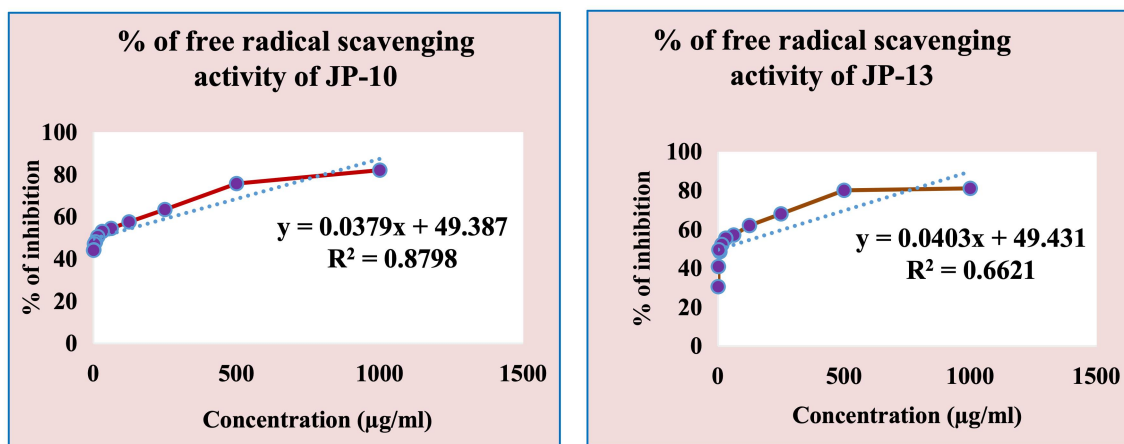
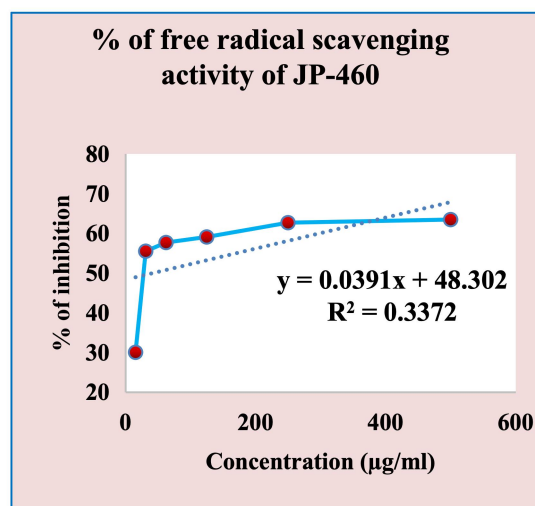
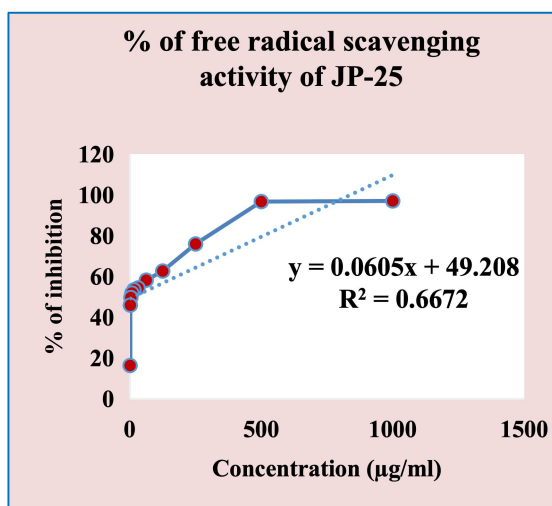
Figure 4.26: IC₅₀ value of JP-10 and JP-13

Table 4.19: IC₅₀ value of JP-25 and JP-460

JP-25					JP-460			
Conc. (µg/ mL)	Abs. 1	Abs. 2	Mean Absorbance ±STD	% of Scavan ging	Abs. 1	Abs. 2	Mean Absorbance± STD	% of Scavan ging
1000	0.015	0.023	0.019±0.01	97.03	0.231	0.237	0.234±0.01	63.44
500	0.021	0.021	0.021±0.00	96.71	0.243	0.235	0.239±0.01	62.66
250	0.163	0.145	0.154±0.01	75.93	0.27	0.254	0.262±0.01	59.06
125	0.224	0.254	0.239±0.02	62.67	0.263	0.279	0.271±0.01	57.66
62.5	0.261	0.273	0.267±0.01	58.21	0.291	0.279	0.285±0.01	55.47
31.25	0.288	0.296	0.292±0.01	54.38	0.461	0.435	0.448±0.02	30
15.63	0.296	0.300	0.298±0.00	53.44				
7.813	0.311	0.305	0.308±0.00	51.88				
3.91	0.329	0.315	0.322±0.01	49.69				
1.96	0.338	0.354	0.346±0.01	45.96				
0.98	0.540	0.53	0.535±0.12	16.40				
IC ₅₀ (µg/ mL) = 13.10					IC ₅₀ (µg/ mL) = 43.43			

Figure 4.27: IC₅₀ value of JP-25 and JP-460

JP-10, JP-13, JP-25, and JP-460 were subjected to DPPH test. Promising IC_{50} values were 16.17, 14.12, 13.10, and 43.43 $\mu\text{g/mL}$ for JP-10, JP-13, JP-25, and JP-460, respectively (Table 4.18-Table 4.19, Figure 4.26-Figure 4.27), which are isolated compounds from *J. pandurifolia*. These findings are noteworthy, suggesting that the isolated pure compounds possess strong antioxidant activity that highlights the potential of these compounds as candidates for the development of new antioxidant agents. This was the first time study of these pure compounds according to antioxidant assay.

4.1.5 Cytotoxic activity

Cytotoxic activity refers to the ability of a compound to kill or retard the growth of cells, particularly multiple malignant cells that is essential in the development of anticancer agents. Alkaloids, flavonoids, terpenoids, and lignans are linked to cytotoxic effects through interfering the pathways of cell cycle.

A. Brine shrimp lethality bioassay

The brine shrimp lethality bioassay is a cost effective and bench-top assay that is utilized to identify active cytotoxic agents (Meyer et al., 1982). The assay needs a few sample requirement (2 to 20 mg or less) (Sarah et al., 2017). Being an anticancer drug, vincristine sulphate binds to tubulin proteins, prevents the polymerization of tubulin dimers and finally triggers apoptosis.

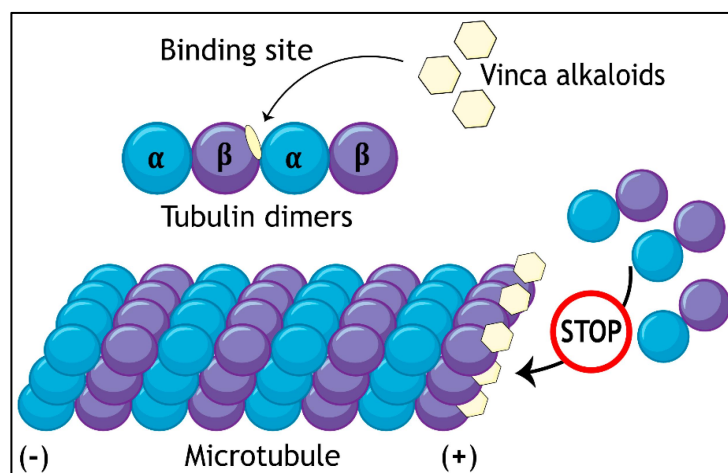


Figure 4.28: Mechanism of action of vincristine sulphate

4.1.5.1 Principle of brine shrimp lethality bioassay

The brine shrimp lethality bioassay is based on a test sample's ability to kill the biological organism known as brine shrimp nauplii (*Artemia salina* Leach). In this assay, a fixed number of nauplii were treated with various concentrations of the sample. After a certain incubation period, the number of surviving nauplii was counted. The percentage of lethality was then calculated for each concentration.

4.1.5.2 Materials and experimental procedure

Equipment and reagents

- *Artemia salina* leach (brine shrimp eggs)
- Small tank with perforated divider
- Dimethylsulphoxides (DMSO)
- Eppendorf tubes
- Sea water or NaCl solution
- Lamp to attract the shrimps
- Magnifying glass
- Spatula
- Test tubes
- Pipette
- Micropipette

Preparation of artificial sea water

An artificial salt solution, also known as brine shrimp salt (35 g/L), was prepared by dissolving 28 grams of salt in 800 milliliters of tap water. The mixture was stirred with a stirring rod until the salt was completely dissolved.

Hatching of brine shrimps

Brine shrimp eggs, namely *Artemia salina* Leach, were obtained from pet shops in the Katabon, Dhaka. Artificial seawater was prepared in a tank, which was divided by a perforated partition. The shrimp eggs were placed in one section of the tank covered to create a dark environment. The opposite section of the tank was exposed to light from a bulb. After 48 hours, the shrimp developed into nauplii, which were then mature enough to be used in experimental assays.

Incorpoarting the shrimp

Using a Pasteur pipette, ten live shrimp were carefully transferred into each test tube containing 5 ml of artificial seawater. A magnifying glass was used to count the nauplii accurately.

Preparation of negative control (seawater+DMSO)

Three pre-labeled test tubes, each containing 5 ml of simulated seawater, were filled with 30 µl of DMSO (Dimethyl sulfoxide) and introduced with 10 brine shrimp nauplii to serve as control groups in the experiment.

Preparation of positive control (VS)

Vincristine sulfate (0.4 mg) was dissolved in DMSO to obtain an initial concentration of 40 µg/ml. Subsequent dilutions were carried out to prepare a range of concentrations, including 40 µg/ml, 20 µg/ml, 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL, 0.3125 µg/mL, 0.15625 µg/mL, 0.078125 µg/mL.

Preparation of test solutions (Meyer et al., 1982)

For the experiment, 4 mg of each extract was dissolved in dimethyl sulfoxide (DMSO), and solutions of varying concentrations (1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.63 µg/mL, 7.8313 µg/mL, 3.91 µg/mL, 1.96 µg/mL, and 0.98 µg/mL) were obtained by the serial dilution technique using simulated seawater.

Procedure of brine shrimp lethality assay (Waghulde et al., 2019)

The solutions with different concentrations were added to pre-marked vials containing 10 live brine shrimp nauplii in 5 ml of simulated seawater. After 24 hours post-experiment initiation, the test tubes under investigation were inspected using a magnifying glass to determine the number of surviving shrimp. The percentage of mortality was then calculated for each dilution. The LC₅₀ value represents the concentration of the test sample that causes the death of 50% of the test subjects after a specified period of exposure. The mortality rate was used by following formula:

$$\% \text{ mortality} = (\text{no. of dead nauplii} / \text{initial total no. of live nauplii}) \times 100$$

4.1.5.3 Result and discussion

4.1.5.3.1 Cytotoxic activity of plant extracts (brine shrimp lethality bioassay)

Table 4.20: LC₅₀ value of *J. pandurifolia* and *S. reticulatum*

Test sample	Sample code	LC ₅₀ value (µg/ml)
<i>J. pandurifolia</i>	Jp-ML (Methanolic extract)	5.81
	Jp-EAL (Ethyl acetate extract)	1.60
	Jp-CL (Chloroform extract)	2.96
	Jp-HL (n-Hexane extract)	2.78
<i>S. reticulatum</i>	Sr-ML (Methanolic extract)	1.70
	Sr-EAL (Ethyl acetate extract)	5.10
	Sr-CL (Chloroform extract)	3.22
	Sr-HL (n-Hexane extract)	1.97
Positive control	Vincristine Sulphate (VS)	0.83
Negaive control	Seawater + DMSO	340.84

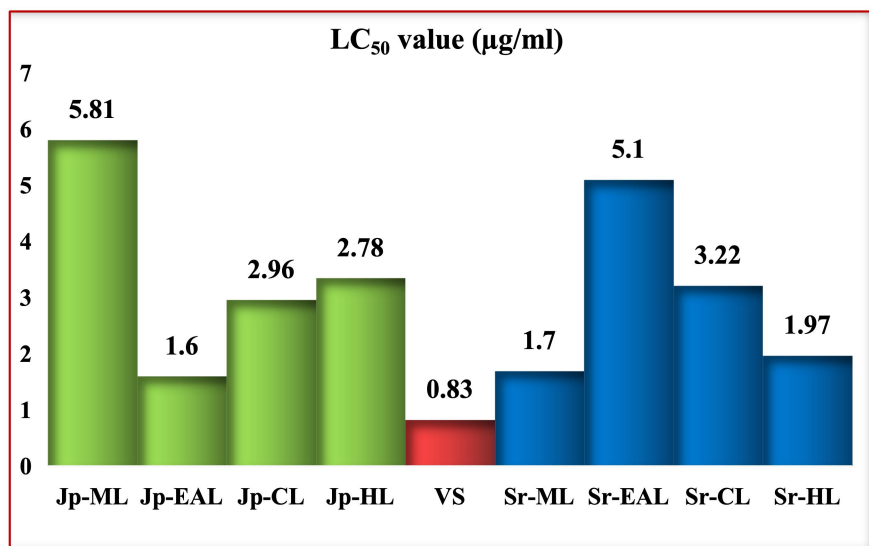
Figure 4.29: LC₅₀ value of *J. pandurifolia* and *S. reticulatum*

Table 4.21: LC₅₀ value of vincristine sulphate

Conc. (µg/ mL)	Log C	1	2	3	Mean % of mortality	LC ₅₀ (µg/ mL)
40	2.699	100	100	100	100±0.00	0.83
20	2.398	100	100	100	100±0.00	
10	2.097	90	90	100	93.33±5.77	
5	1.796	80	90	80	83.33±5.77	
2.5	1.495	80	80	80	80±0.00	
1.25	1.194	60	60	70	63.33±5.77	
0.625	0.893	60	60	50	56.67±5.77	
0.312	0.591	30	40	30	33.33±5.77	
0.15633	0.292	30	30	20	26.67±5.77	
0.0783	-0.01	20	30	30	26.67±5.77	

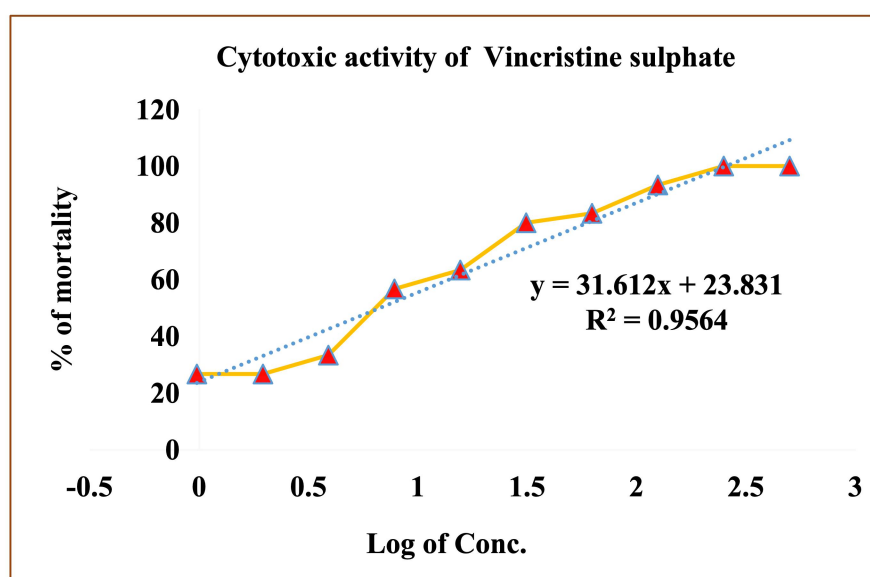
**Figure 4.30: LC₅₀ value of vincristine sulphate**

Table 4.22: LC₅₀ value of Jp-ML and Jp-EAL

Jp-ML						Jp-EAL			
Conc. (µg/ mL)	Log C	1	2	3	Mean % of mortality	1	2	3	Mean % of mortality
1000	3	90	90	80	86.67±5.77	80	80	90	83.33±5.77
500	2.699	90	90	90	90±0.00	70	90	80	80±10
250	2.398	60	60	60	60±0.00	60	60	70	63.33±5.77
125	2.097	60	70	60	63.33±5.77	50	50	60	53.33±5.77
62.5	1.796	30	40	30	33.33±5.77	70	50	50	56.67±11.55
31.25	1.495	30	40	40	36.67±5.77	40	50	50	46.67±5.77
15.63	1.194	50	60	50	53.33±5.77	30	50	50	43.33±5.77
7.813	0.893	40	40	40	40±0.00	30	40	40	36.67±5.77
3.91	0.591	30	40	30	33.33±5.77	50	50	40	46.67±5.77
1.96	0.292	20	20	30	23.33±5.77	30	40	30	33.33±5.77
0.98	-0.01	10	20	20	16.67±5.77	20	10	10	13.33±11.55
LC ₅₀ (µg/ mL) = 5.81						LC ₅₀ (µg/ mL) = 1.60			

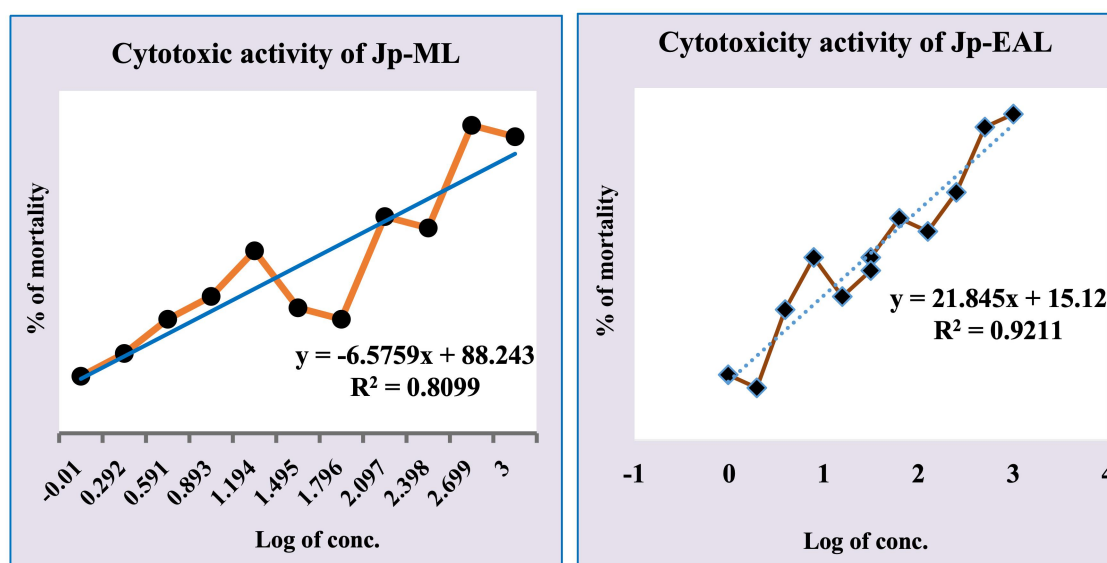
Figure 4.31: LC₅₀ value of Jp-ML and Jp-EAL

Table 4.23: LC₅₀ value of Jp-CL and Jp-HL

Conc. (µg/ mL)	Log C	Jp-CL				Jp-HL			
		1	2	3	Mean % of mortality	1	2	3	Mean % of mortality
1000	3	50	60	50	53.33±5.77	40	40	40	40±0.00
500	2.699	50	50	40	46.67±5.77	40	40	30	36.67±5.77
250	2.398	40	40	40	40±0.00	40	30	30	33.33±5.77
125	2.097	40	40	30	36.67±5.77	40	40	40	40±0.00
62.5	1.796	30	30	40	33.33±5.77	30	30	30	30±0.00
31.25	1.495	40	40	30	36.67±5.77	30	30	20	26.67±5.77
15.63	1.194	50	30	30	36.67±11.55	10	0	0	3.33±5.77
7.813	0.893	30	30	20	26.67±5.77	10	10	0	6.67±5.77
3.91	0.591	20	30	40	30±10.00	0	0	0	0
1.96	0.292	20	30	10	20±10.00	0	0	0	0
0.98	-0.01	10	10	10	10±0.00	0	0	0	0
LC ₅₀ (µg/ mL) = 2.96					LC ₅₀ (µg/ mL) = 2.78				

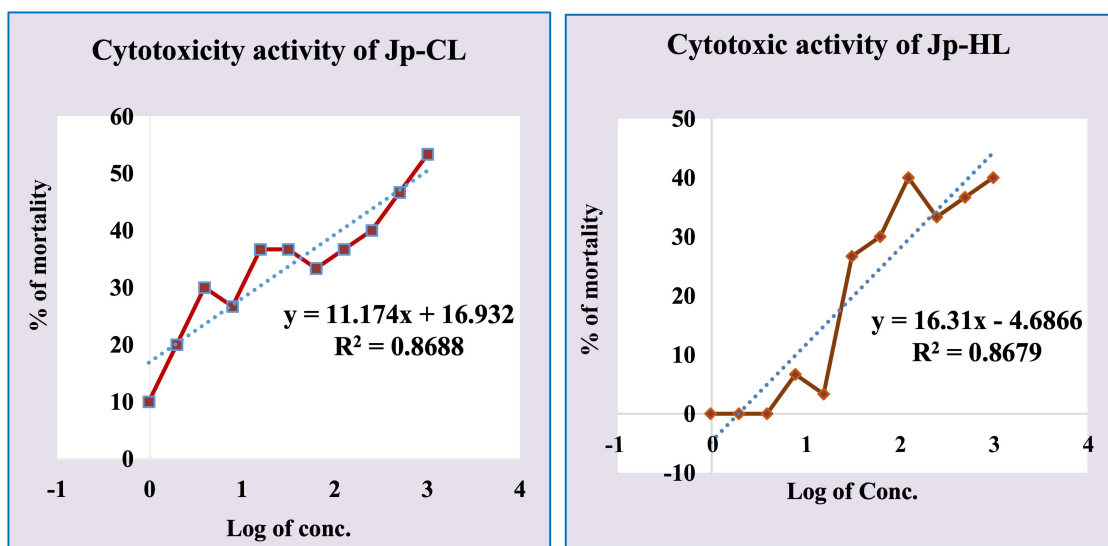
Figure 4.32: LC₅₀ value of Jp-CL and Jp-HL

Table 4.24: LC₅₀ value of Sr-ML and Sr-EAL

Conc. (µg/ mL)	Log C	Sr-ML				Sr-EAL			
		1	2	3	Mean % of mortality	1	2	3	Mean % of mortality
1000	3	70	70	60	66.67±5.77	80	80	80	80±0.00
500	2.699	70	70	70	70±0.00	70	70	70	70±0.00
250	2.398	70	60	70	66.67±5.77	60	60	50	56.67±5.77
125	2.097	80	50	60	63.33±15.28	60	60	60	60±0.00
62.5	1.796	40	30	50	40±10.00	40	40	40	40±0.00
31.25	1.495	40	50	40	43.33±5.77	40	40	40	40±0.00
15.63	1.194	50	40	30	40±10.00	40	40	40	40±0.00
7.813	0.893	60	50	40	50±10.00	40	40	40	40±0.00
3.91	0.591	40	50	20	36.67±15.28	30	30	30	30±0.00
1.96	0.292	20	20	30	23.33±5.77	20	20	20	20±0.00
0.98	-0.01	10	10	10	10±0.00	10	10	10	10±0.00
LC ₅₀ (µg/ mL) = 1.70					LC ₅₀ (µg/ mL) = 5.10				

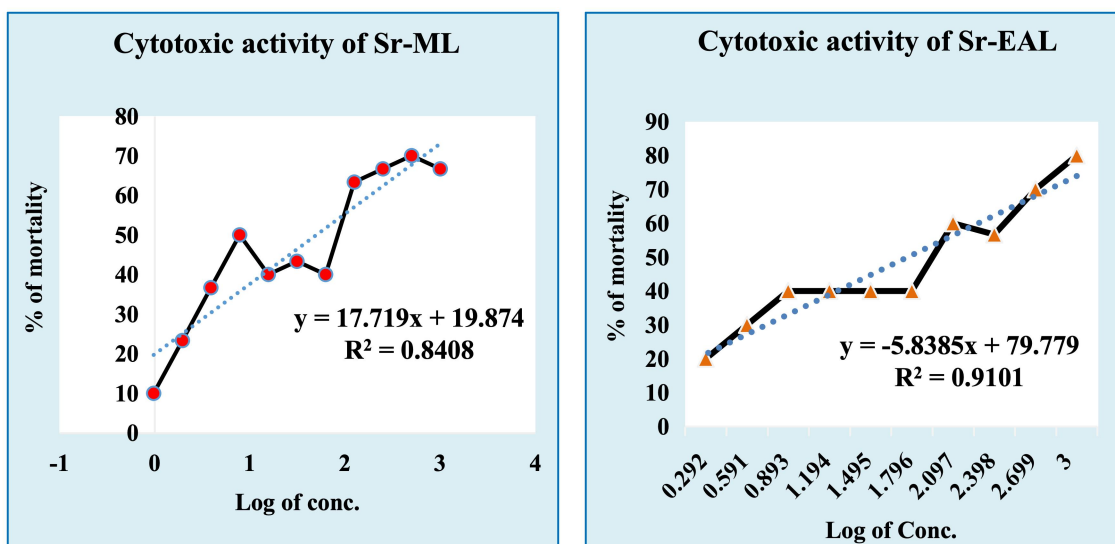
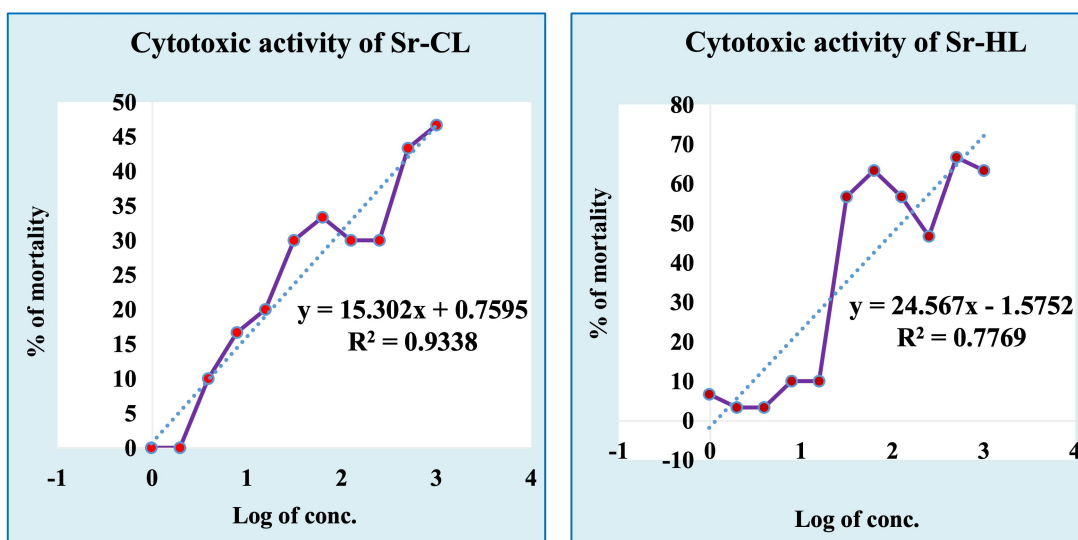
Figure 4.33: LC₅₀ value of Sr-ML and Sr-EAL

Table 4.25: LC₅₀ value of Sr-CL and Sr-HL

Conc. (µg/ mL)	Log C	Sr-CL				Sr-HL			
		1	2	3	Mean % of mortality	1	2	3	Mean % of mortality
1000	3	50	50	40	46.67± 5.77	60	70	60	63.33±5.77
500	2.699	40	50	40	43.33± 5.77	60	70	70	66.67±5.77
250	2.398	30	30	30	30± 0.00	50	50	40	46.67±5.77
125	2.097	30	30	30	30± 0.00	50	50	70	56.67±14.14
62.5	1.796	30	30	40	33.33± 5.77	60	50	80	63.33±15.28
31.25	1.495	20	30	40	30± 10.0	40	60	70	56.67±15.28
15.63	1.194	20	20	20	20± 0.00	10	10	10	10±0.00
7.813	0.893	10	20	20	16.67± 5.77	10	10	10	10±0.00
3.91	0.591	10	10	10	10	0	10	0	3.33±5.77
1.96	0.292	0	0	0	0	0	0	10	3.33±5.77
0.98	-0.01	0	0	0	0	0	10	10	6.67±5.77
LC ₅₀ (µg/ mL) = 3.22					LC ₅₀ (µg/ mL) = 1.97				

Figure 4.34: LC₅₀ value of Sr-CL and Sr-HL

LC₅₀ value above 1000 µg/mL is considered nontoxic, a value between 500-1000 µg/mL is classified as weakly cytotoxic, LC₅₀ value of 100-500 µg/mL indicates moderate toxicity, and LC₅₀ value between 0-100 µg/mL is indicative of strong cytotoxicity (Meyer et al., 1982; Santos Pimenta et al., 2003). The results showed that the Jp-ML, Jp-EAL, Jp-CL, and Jp-HL fractions exhibited significantly strong lethality on brine shrimp, with LC₅₀ values of 5.81, 1.6, 2.96, and 2.78 µg/mL, respectively, which were notably lower than the negative control LC₅₀: 340.84 µg/mL) (Table 4.22-Table 4.23, Figure 4.31-Figure 4.32). The second plant extract also demonstrated significant cytotoxicity against brine shrimp in a dose-dependent manner. Strong cytotoxicity was found for Sr-ML, Sr-EAL, Sr-CL, and Sr-HL with LC₅₀ values of 1.70, 5.10, 3.22, and 1.97 µg/mL, respectively, (Table 4.24-Table 4.25, Figure 4.33-Figure 4.34) compared to the negative control. Standard vincristine sulfate had LC₅₀ value of 0.83 µg/mL (Table 4.21, Figure 4.30).

The results provide a exploratory indication of the toxicological profile of the extracts that could be contributing to the safety profile in pharmacological research. The observed cytotoxicity may be aligned to the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, and phenolics that are well known to disrupt cell membranes and interfere with cellular metabolism.

4.1.5.3.2 Cytotoxicity activity of pure compounds (brine shrimp lethality bioassay)

Table 4.26: LC₅₀ value of pure compounds

Sample code	LC ₅₀ value (µg/ml)
VS	0.83
JP-10	1.56
JP-13	1.35
JP-25	1.29
JP-460	1.27

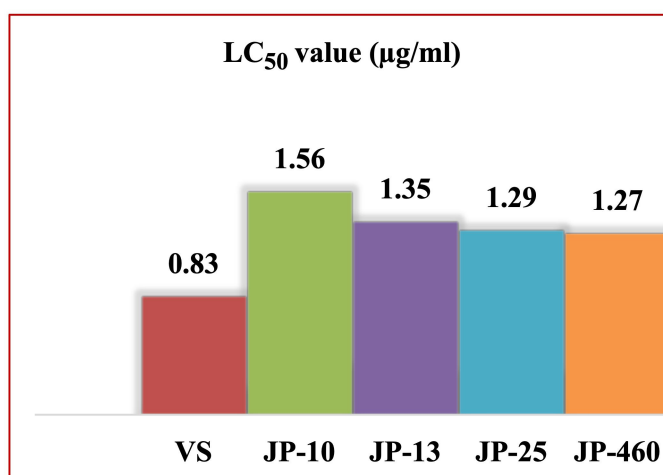


Figure 4.35: LC₅₀ value of pure compounds

Table 4.27: LC₅₀ value of JP-10 and JP-13

Conc. ($\mu\text{g}/\text{mL}$)	Log C	JP-10				JP-13			
		1	2	3	Mean % of mortality	1	2	3	Mean % of mortality
1000	3	100	100	100	100 $\pm$ 0.00	100	100	100	100 $\pm$ 0.00
500	2.699	100	90	90	86.67 $\pm$ 15.28	80	90	80	83.33 $\pm$ 5.77
250	2.398	80	80	80	80 $\pm$ 0.00	70	70	80	73.33 $\pm$ 5.77
125	2.097	60	60	60	80 $\pm$ 0.00	60	70	60	63.33 $\pm$ 5.77
62.5	1.796	40	40	30	36.67 $\pm$ 5.77	60	60	60	60 $\pm$ 0.00
31.25	1.495	30	30	30	30 $\pm$ 0.00	50	40	50	46.6 $\pm$ 5.77
15.63	1.194	30	30	30	30 $\pm$ 0.00	50	50	50	50 $\pm$ 0.00
7.813	0.893	30	30	30	30 $\pm$ 0.00	40	40	40	40 $\pm$ 0.00
3.91	0.591	20	20	20	20 $\pm$ 0.00	40	30	30	33.33 $\pm$ 5.77
1.96	0.292	20	20	10	16.67 $\pm$ 5.77	30	30	30	30 $\pm$ 0.00
0.98	-0.01	10	20	20	16.67 $\pm$ 5.77	10	10	20	10 $\pm$ 0.00
LC ₅₀ ($\mu\text{g}/\text{mL}$) = 1.56					LC ₅₀ ($\mu\text{g}/\text{mL}$) = 1.35				

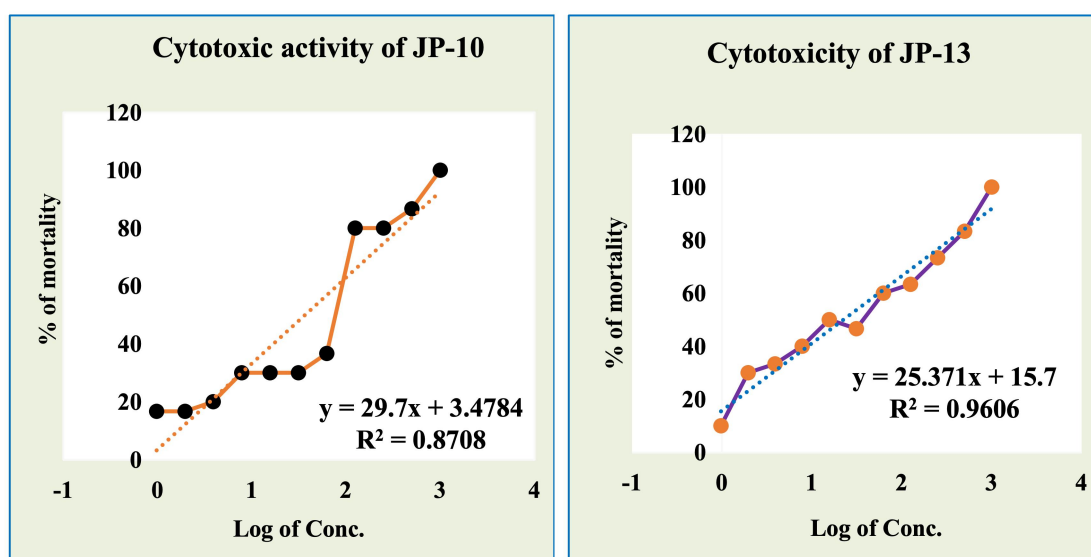
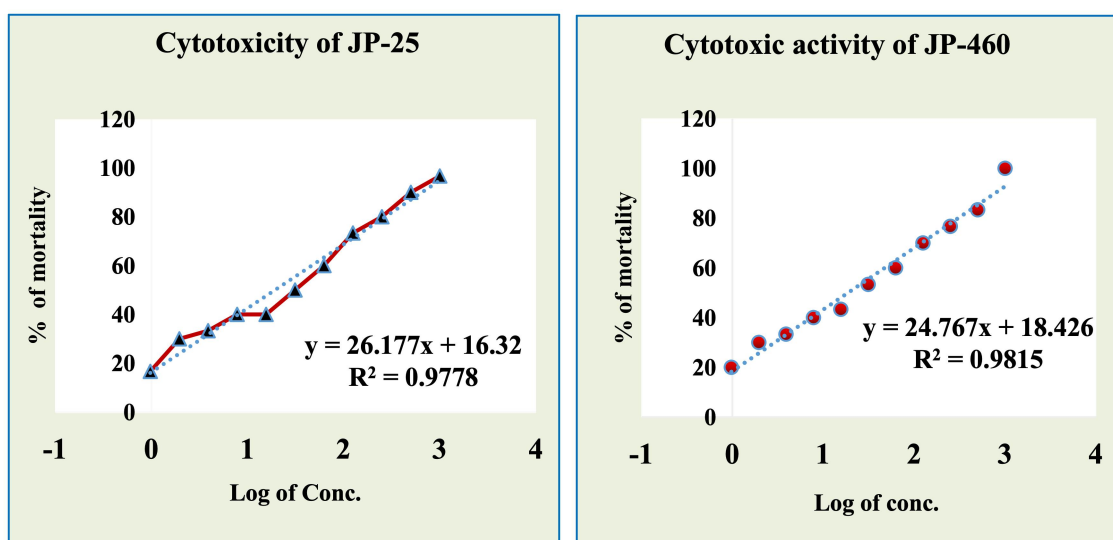
Figure 4.36: LC₅₀ value of JP-10 and JP-13

Table 4.28: LC₅₀ value of JP-25 and JP-460

JP-25						JP-460			
Conc. (µg/ mL)	Log C	1	2	3	Mean % of mortality	1	2	3	Mean % of mortality
1000	3	100	100	90	96.67	100	100	100	100±0.00
500	2.699	90	90	90	90	80	80	90	83.33±5.77
250	2.398	80	80	80	80	80	70	80	76.67±5.77
125	2.097	70	80	70	73.33	70	70	70	70±0.00
62.5	1.796	60	60	60	60	60	70	50	60±10.00
31.25	1.495	50	50	50	50	50	60	50	53.33±5.77
15.63	1.194	40	40	40	40	40	50	40	43.33±5.77
7.813	0.893	40	40	40	40	40	40	40	40±0.00
3.91	0.591	30	40	30	33.33	30	40	30	33.33±5.77
1.96	0.292	30	30	30	30	30	30	30	30±0.00
0.98	-0.01	20	20	10	16.67	20	30	10	20±10.0
LC ₅₀ (µg/ mL) = 1.29						LC ₅₀ (µg/ mL) = 1.27			

Figure 4.37: LC₅₀ value of JP-25 and JP-460

JP-10, JP-13, JP-25, and JP-460 showed LC_{50} values of 1.56, 1.35, 1.29, and 1.27 $\mu\text{g/mL}$, respectively, (Table 4.27-Table 4.28, Figure 4.36-Figure 4.37) indicating strong cytotoxicity. This supports their potential use as anticancer agents or in cancer cell prevention.

B. Cytotoxicity in HeLa cell line (Qualitative test)

HeLa cells are first human cell lines developed from the cervical cancer epithelial cells of Henrietta Lacks (Landry et al., 2013). Researchers use human cell lines as models to study cellular activities in species other than humans. HeLa cells, being an immortal human cell line, are widely used in evaluation of cell mortality, viability, proliferation etc.

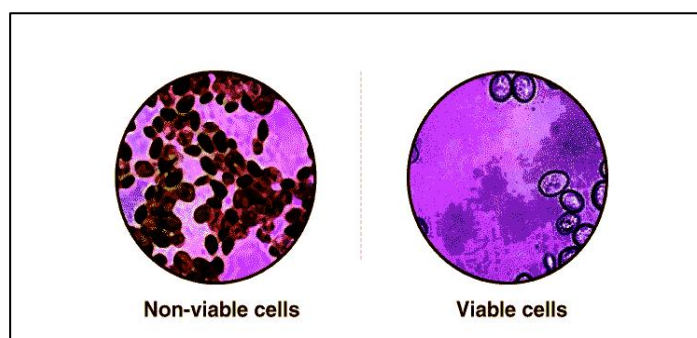


Figure 4.38: Pathogenesis of cell damage

4.1.5.4 Principle of cell viability test

A cell viability assay is performed to determine the proportion of living to dead cells. These assays are designed to evaluate the physical and physiological condition of cells in response to external stimuli or chemical agents. They are commonly employed *in vitro* studies to assess the effects of drugs in cell-mediated cytotoxicity assays, particularly in terms of cell proliferation.

4.1.5.5 Materials and experimental procedure

Equipment and reagents

- | | |
|-----------------|-----------------------|
| ✧ 48-well plate | ✧ Cell culture media |
| ✧ 15-ml tubes | ✧ Antibiotics (P+S) |
| ✧ Tips | ✧ Gentamycin |
| ✧ Gloves | ✧ Serological pipette |
| ✧ Culture flask | ✧ Trypsin |

Instruments used

- Trinocular microscope with camera (Optika, Italy)
- Biological Bio Safety Cabinet (Model: NU-400E, Nuaire, USA)
- Hemocytometer
- CO₂ Incubator (Nuaire, USA)

Cell culture

The cytotoxicity test was conducted using a cell viability assay at the Center for Advanced Research in Science, University of Dhaka. HeLa, a human cervical cancer cell line, was cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS), 0.25% gentamicin, and 1% penicillin-streptomycin (1:1).

Procedure of cell viability assay (Khatibi et al., 2017)

JP-13, 460, and 200 were subjected to cell viability assay. Each pure compound (0.5 mg) was dissolved in 1 mL of 4% DMSO solution. Cells (4.0×10^4 /200 μ L) were seeded onto a 48-well plate and incubated at 37°C with 5% CO₂. The following day, 50 μ L of the sample (autoclaved) was added to each well, and cytotoxicity was assessed after 48 hours of incubation under an inverted light microscope. Duplicate wells were used for each sample. Cell viability was calculated by following formula.

$$\text{Cell viability (\%)} = (\text{Live cells}) / (\text{Total number of cells}) \times 100$$

4.1.5.6 Result and discussion**Table 4.29: Cell viability of isolated pure compounds**

Sample ID	Survival of Cells	Remarks
Solvent -	HeLa	Cytotoxicity was observed for sample (JP-200) on HeLa cell line
Solvent +	100%	
JP-13	>95%	
JP-460	>95%	
JP-200	<5%	

In the cancer cell-containing plate with a 4% DMSO solution, a 100% cell survival rate was observed. JP-13 and JP-460 showed a cell survival rate above 95%, indicating minimal cytotoxic effect on the cancer cells. JP-200 exhibited cytotoxic activity, with a cell survival rate below 5%, indicating that the majority of the cells underwent mortality (Table 4.29, Figure 4.39).

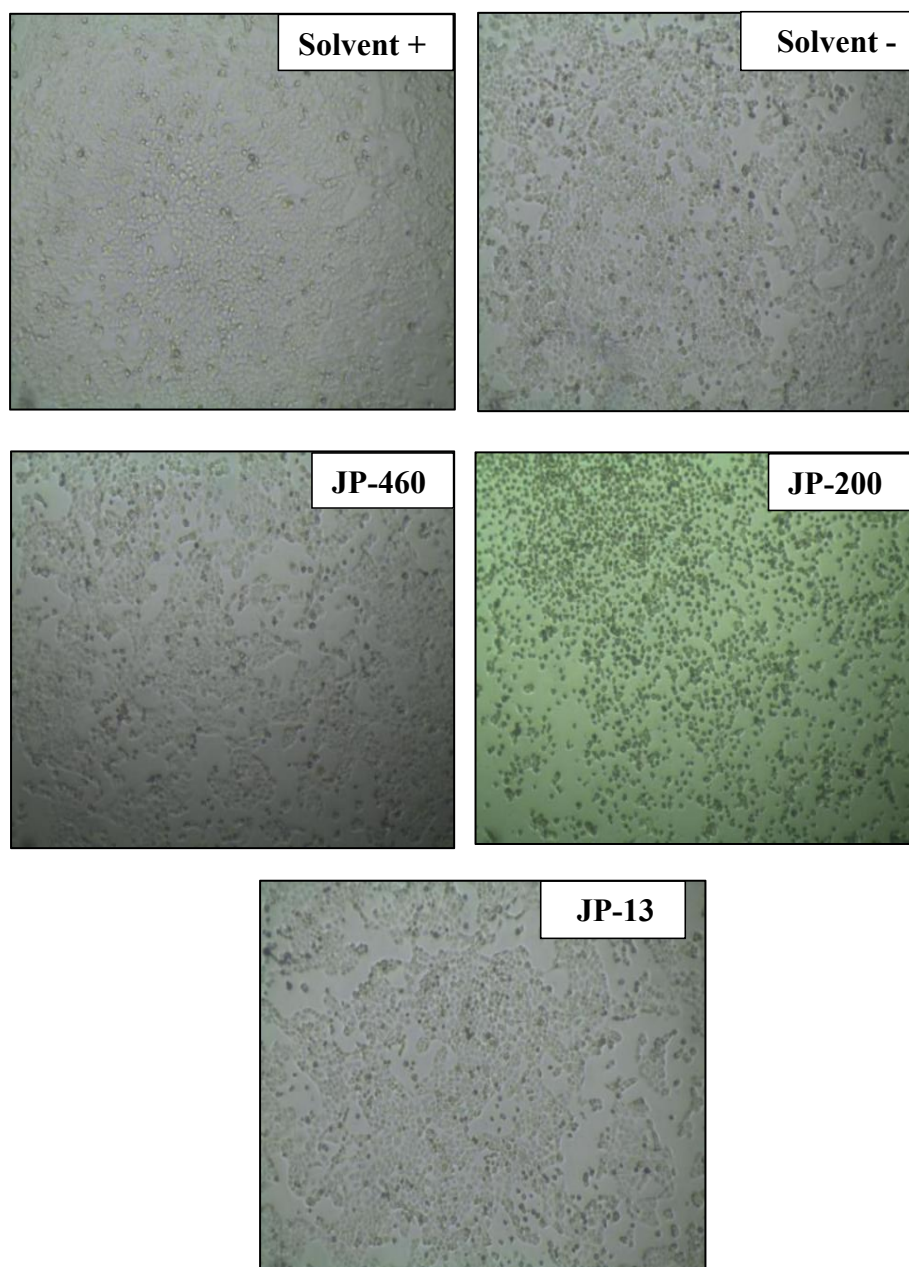


Figure 4.39: Visualization of affected cell area after treatment

C. Cell viability by MTT assay (Quantitative test)

The MTT assay is a colorimetric assay that quantitatively measures cell viability as well as cytotoxicity. This is an efficient method that serves information on cellular activity of cells in response to treatment that is related to development of anticancer drug.

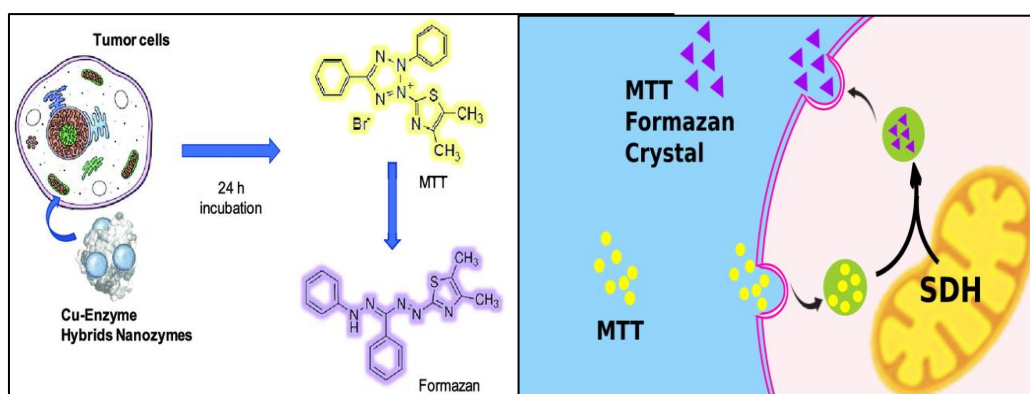


Figure 4.40: Mechanism of action of MTT assay

4.1.5.7 Principle of MTT assay (Riss et al., 2016)

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first cell viability assay. MTT salt (a tetrazolium salt) is reduced by NADPH dependent cellular oxidoreductase enzymes, resulting in the formation of purple formazan crystals that occurs in the mitochondria of viable cells. The amount of purple formazan formed is directly proportional to the number of live cells. The LD₅₀ can be calculated using a linear regression equation that relates concentration to the percentage of cell viability.

4.1.5.8 Materials and experimental procedure

Equipment and reagents

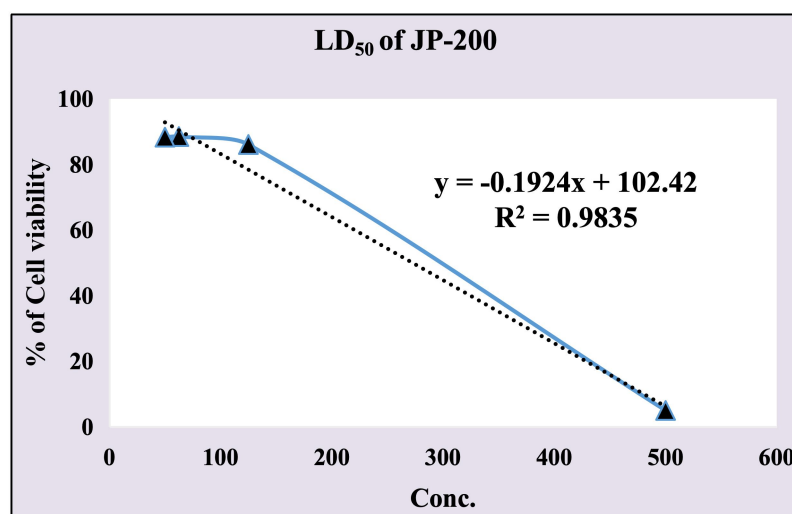
- | | |
|---|--------------------------------|
| ✧ Biological Bio Safety Cabinet (Model: NU-400E, Nuaire, Hemocytometer USA) | ✧ Gloves |
| ✧ CO ₂ Incubator (Nuaire, USA) | ✧ Culture flask |
| ✧ Antibiotics (P+S), T 96-well plate | ✧ Cell culture media and flask |
| ✧ Trinocular microscope with camera | ✧ Trypsin |
| ✧ (Optika, Italy) | ✧ Gentamycin |
| ✧ 15-ml tubes | ✧ Serological pipette |
| | ✧ Tips |

Procedure of MTT assay (Ala et al., 2018)

HeLa cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium) containing 1% penicillin-streptomycin (1:1), 0.2% gentamycin, and 10% fetal bovine serum (FBS). HeLa cells ($2.0 \times 10^4/100 \mu\text{L}$) were seeded onto a 96-well plate and incubated at 37°C with 5% CO_2 . The following day, 25 μL of the sample (autoclaved) was added to each well. Cell viability was examined after 48 hours of incubation using the CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, USA). Duplicate wells were used for each sample. Absorbance was measured at 570 nm (Multiskan FC, Thermo Scientific, DE). Inhibition activity was expressed as percentages of the control with DMSO.

4.1.5.9 Result and discussion**Table 4.30: LD₅₀ of JP-200**

Sample ID	Survival of HeLa Cells (%)	LD ₅₀
Control	100	
500 $\mu\text{g/mL}$	5	272.45 $\mu\text{g/mL}$
125 $\mu\text{g/mL}$	86.04	
62.5 $\mu\text{g/mL}$	88.47	
50 $\mu\text{g/mL}$	88.28	

**Figure 4.41: LD₅₀ of JP-200**

A higher LD₅₀ value indicates lower toxicity or a non-toxic nature of the compound (Indrayudha et al., 2013). The U.S. National Cancer Institute classifies cytotoxic compounds into four categories: very toxic (LD₅₀ ≤ 20 µg/mL), moderately cytotoxic or quite active, (LD₅₀ between 21-200 µg/mL, weakly cytotoxic (LD₅₀ between 201-500 µg/mL, and non-toxic (LD₅₀ ≥ 500 µg/mL) (Abdel-Hameed et al., 2012). An LD₅₀ value of 272.45 µg/mL (Table 4.30, Figure 4.41) indicates a weak cytotoxic potential in inhibiting HeLa cervical cancer cells (Figure 4.42). However, if the cytotoxicity is moderate or weak, the compound may be classified as a chemopreventive agent with the ability to prevent and inhibit the growth of cancer cells (Prayong et al., 2008).

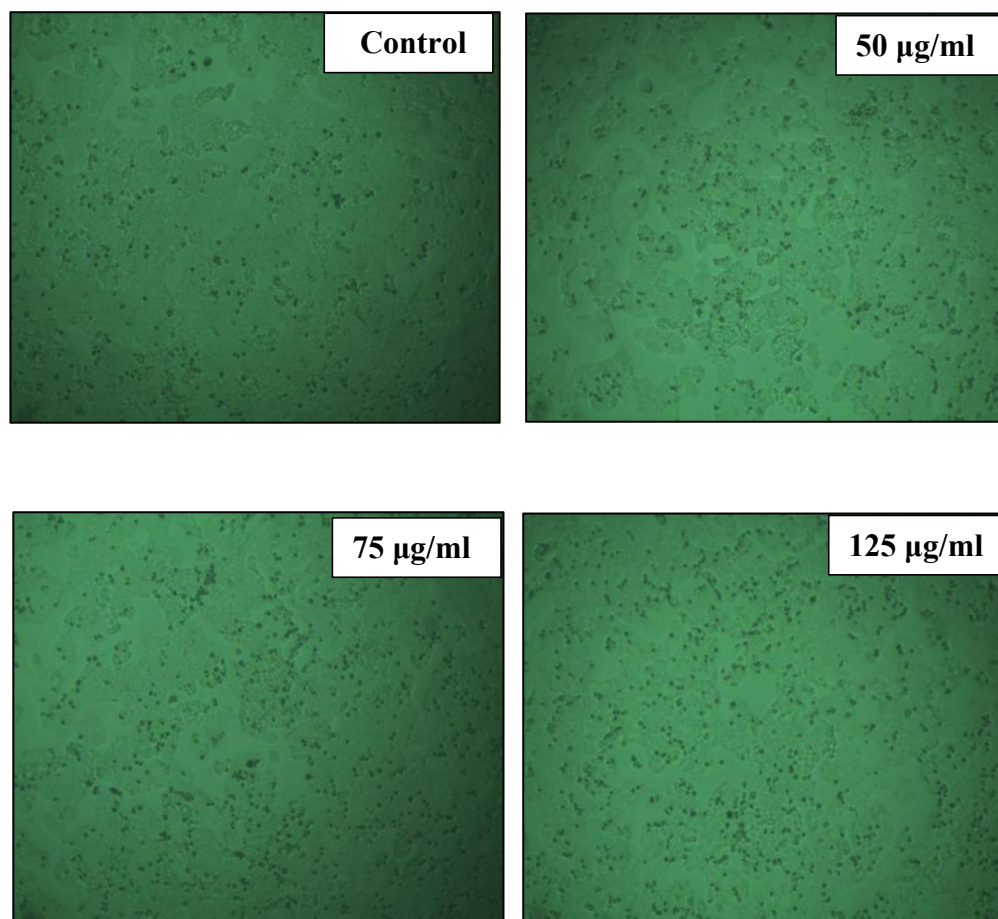


Figure 4.42: Visualization of affected cell area after treatments with JP-200

4.1.6 Thrombolytic activity

Thrombus or blood clot plays a critical role in vascular diseases including myocardial infarction, hypoxia and hypertension (Sultana et al., 2012). Plasminogen activator, urokinase and streptokinase are widely used for treating thrombotic diseases and managing cerebral venous sinus thrombosis patients. Bioactive phytochemicals, such as flavonoids, alkaloids, saponins, and tannins are also responsible for clot management.

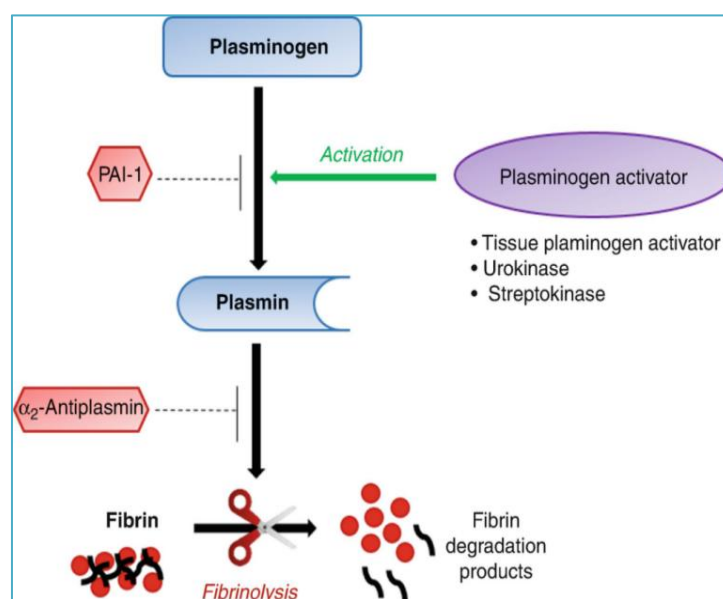


Figure 4.43: Mechanism of action of streptokinase

4.1.6.1 Principle of thrombolytic activity test

Fresh venous blood is allowed to promote thrombus formation under standard conditions. The test sample may produce fibrinolysis and clot breakdown. The clot mass reduction indicated thrombolytic activity. Streptokinase converts plasminogen to plasmin and promotes clot lysis.

4.1.6.2 Materials and experimental procedure

Equipment and reagents

- | | |
|--------------------|-----------------------------|
| ➤ Human volunteers | ➤ Normal saline (0.9% NaCl) |
| ➤ Eppendorf tubes | ➤ Incubator |
| ➤ Streptokinase | ➤ Test tube |

Preparation of negative control (0.9% of NaCl)

0.9 g of sodium chloride was weighed and added to distilled water, then mixed thoroughly to make a final volume of 100 mL.

Preparation of positive control (STK)

Approximately 5 mL of sterile distilled water was added to a commercially obtained lyophilized vial of streptokinase (STK) with a potency of 1,500,000 International Units (I.U.), and the solution was thoroughly mixed. This prepared solution served as a stock from which 100 μ L (30,000 I.U.) was served as positive standard.

Collection of blood

Blood was collected from healthy human volunteers with no history of taking oral contraceptives or anticoagulant medications. 1 ml of this blood was then dispensed into pre-weighed sterile Eppendorf tubes, where it was left undisturbed to form clot.

Procedure of thrombolytic activity (Prasad et al., 2007)

5 ml of venous blood were drawn into individual sterile vials, each pre-weighed and holding 1 ml of blood. After 45 minutes of incubation at 37°C, the tubes were allowed to clot and were weighed again. The clear part was carefully removed after the clot had formed. The clot weight was determined by subtracting the initial weight from the final weight. 100 μ L of test samples (1 mg/100 μ L water) were added in each vial. A positive control of 100 μ L streptokinase (30,000 I.U.) and a negative control of 100 μ L 0.9% NaCl solution were used, respectively. Clot lysis was observed after 90 minutes of incubation at 37°C. After the clear plasma was removed, the vials were weighed again to determine the weight difference. The clot lysis percentage was calculated using the formula below.

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

4.1.6.3 Result & discussion

4.1.6.3.1 Thrombolytic activity of plant extracts

Table 4.31: Thrombolytic activity of *J. pandurifolia* and *S. reticulatum*

Test sample	Sample code	LC ₅₀ value (µg/ml)
<i>J. pandurifolia</i>	Jp-ML (Methanolic extract)	63.32±2.85
	Jp-EAL (Ethyl acetate extract)	46.87±0.23
	Jp-CL (Chloroform extract)	32.37±2.69
	Jp-HL (n-Hexane extract)	43.22±3.40
<i>S. reticulatum</i>	Sr-ML (Methanolic extract)	65.11±4.65
	Sr-EAL (Ethyl acetate extract)	67.58±5.27
	Sr-CL (Chloroform extract)	36.42±8.58
	Sr-HL (n-Hexane extract)	48.56±3.78
Positive control	Streptokinase (STK)	72.47±1.6
Negative control	0.9% NaCl Solution	16.58±2.66

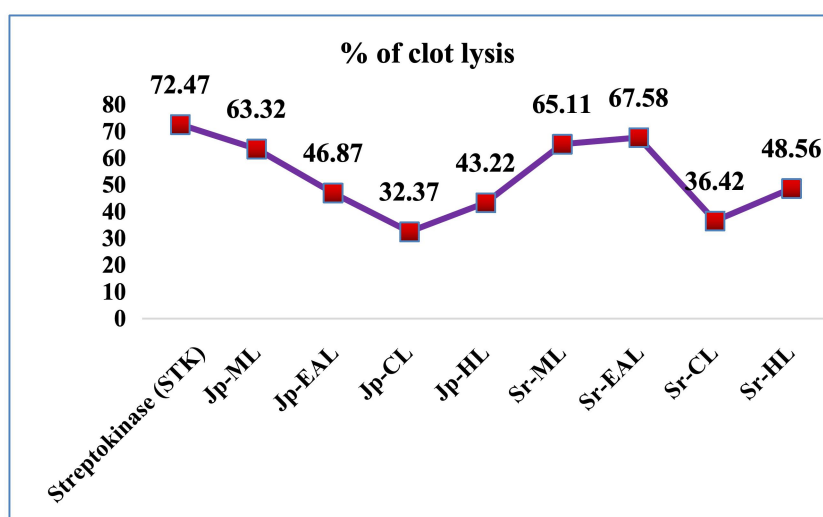
Figure 4.44: Thrombolytic activity of *J. pandurifolia* and *S. reticulatum*

Table 4.32: Mean value of thrombolytic activity of *J. pandurifolia* and *S. reticulatum*

Group	W1 g	W2 g	W3= (W2-W1)	W4 g	W5= (W4-W1)	% of clot lysis	Mean % of clot lysis
Negative control	0.9374	1.1380	0.2006	0.9674	0.030	14.96	16.58±2.66
	0.9865	1.1264	0.1399	1.0127	0.0262	18.72	
Positive control	0.8943	1.2231	0.3288	1.1363	0.2420	73.60	72.47±1.6
	0.9849	1.3474	0.3625	1.2435	0.2586	71.34	
Jp-ML	0.8071	1.2130	0.4059	1.0559	0.2488	61.30	63.32±2.85
	0.9953	1.3296	0.3343	1.2137	0.2184	65.33	
Jp-EAL	0.9134	1.3087	0.3953	1.0993	0.1859	47.03	46.87±0.23
	0.9136	1.3095	0.3959	1.0985	0.1849	46.70	
Jp-CL	0.5412	1.2866	0.7454	0.7630	0.2218	29.76	32.37±2.69
	0.9091	1.3333	0.4242	1.0575	0.1484	34.98	
Jp-HL	0.9523	1.2265	0.2742	1.0642	0.1119	40.81	43.22±3.40
	0.9843	1.2465	0.2622	1.1039	0.1196	45.62	
Sr-ML	0.9117	0.9942	0.0825	0.9627	0.051	61.82	65.11±4.65
	0.9844	1.2758	0.2914	1.1837	0.1993	68.39	
Sr-EAL	0.7904	1.2333	0.4429	1.0730	0.2826	63.85	67.58±5.27
	0.8555	1.3576	0.5021	1.2135	0.3580	71.30	
Sr-CL	0.9200	1.0528	0.1328	0.9603	0.0403	30.35	36.42±8.58
	0.9843	1.2521	0.2678	1.0981	0.1138	42.49	
Sr-HL	0.8259	1.1466	0.3207	0.973	0.1471	45.88	48.56±3.78
	0.9842	1.3295	0.3453	1.1611	0.1769	51.23	

W1= Weight of empty vial; W2= Weight of vial + clot; W3 = (W2-W1)= Weight of clot (before treatment); W4= Weight of vial +clot (after treatment); W5= (W4-W1)=Weight of clot (after treatment); % of clot lysis (W5/W3) ×100

Clot lysis activity was recorded as 63.32±2.85%, 46.87±0.23%, 32.37±2.69%, and 43.22±3.40% for Jp-ML, Jp-EAL, Jp-CL, and Jp-HL, respectively, in comparison to control group (16.58%±2.66). The highest fibrinolytic activity was found for the polar methanolic fraction, and the lowest activity was observed for the less polar chloroform fraction. Streptokinase exhibited a thrombolytic activity of 72.47±1.6%. On the other hand, thrombolytic activity was found to be 65.11±4.65%, 67.58±5.27%,

36.42±8.58%, and 48.56±3.78% for Sr-ML, Sr-EAL, Sr-CL, and Sr-HL, respectively (Table 4.32, Figure 4.44). The selected plant species exhibited significant thrombolytic and/or fibrinolytic activity, as evidenced by a reduction in clot mass ensuring the reservoir of flavonoids, alkaloids, tannins, and saponins (Jahan et al., 2018).

4.1.6.3.2 Thrombolytic activity of pure compounds

Table 4.33: Thrombolytic activity of isolated pure compounds

Sample ID	% of clot lysis
Negative control	16.58±2.66
Positive control	72.47±1.6
JP-460	71.04%±6.22
JP-10	68.92%±5.09
JP-13	66.56%±7.04
JP-25	70.81%±12.22

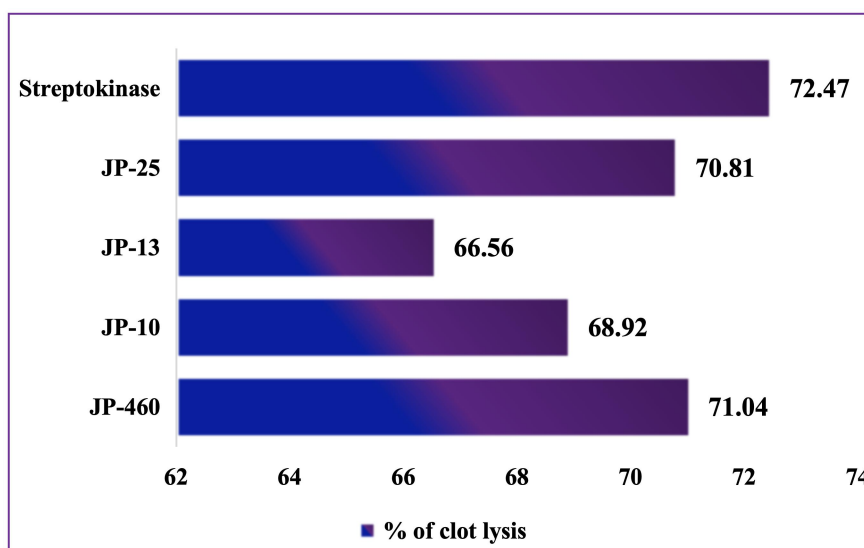


Figure 4.45: Thrombolytic activity of pure compounds

Table 4.34: Mean value of thrombolytic activity of isolated pure compounds

Group	W1 g	W2 g	W3= (W2-W1)	W4 g	W5= (W4-W1)	% of clot lysis	Mean % of clot lysis
JP-460	0.7812	1.2868	0.5056	1.1626	0.3814	75.44	71.04±6.22
	0.7723	1.2764	0.5041	1.1082	0.3359	66.64	
JP-10	0.7785	1.2325	0.4540	1.0751	0.2966	65.32	68.92±5.09
	0.7954	1.3349	0.5395	1.1866	0.3912	72.52	
JP-13	0.7773	1.2633	0.4860	1.125	0.3477	71.54	66.56±7.04
	0.7875	1.3088	0.5213	1.1085	0.3210	61.58	
JP-25	0.7986	1.2784	0.4798	1.0968	0.2982	62.16	70.81±12.22
	0.8041	1.2530	0.4489	1.1608	0.3567	79.46	

JP-460, JP-10, JP-13, and JP-25 demonstrated significant clot rupture activity, with values of 71.04%±6.22, 68.92%±5.09, 66.56%±7.04, and 70.81%±12.22, respectively (Table 4.34, Figure 4.45). This is the first study to report that these pure compounds significantly inhibited clot formation, highlighting their potential as new thrombolytic agents.

**Chapter 5:
Result & Discussion
(Computational
Investigation)**

5.1 Computational investigation

This investigation was carried out to estimate drug binding affinity and pharmacokinetic profile according to molecular docking and ADMET analysis.

In silico Analysis

5.1.1 Molecular Docking

A structure-based computational approach known as molecular docking is used to predict the interactions between ligands and target macromolecules, determining both the binding affinity and efficacy (Muhammed et al., 2022). Molecular docking serves as a pivotal technique, particularly in virtual screening and drug repurposing efforts.

5.1.1.1 Principle of molecular docking test

Versatile docking tools are employed in this process, including AutoDock Vina, AutoDock, GOLD, MOE, ICM, Glide, and FlexX (Chen, 2015). Docking evaluates how effectively a ligand fits into the active site of a biological target protein along with the nature of this interactions. In computational drug discovery, docking typically involves small molecule–macromolecule interactions, such as protein–ligand docking (Prieto-Martínez et al., 2018) (Tilahun Muhammed & Aki-Yalcin, 2024).

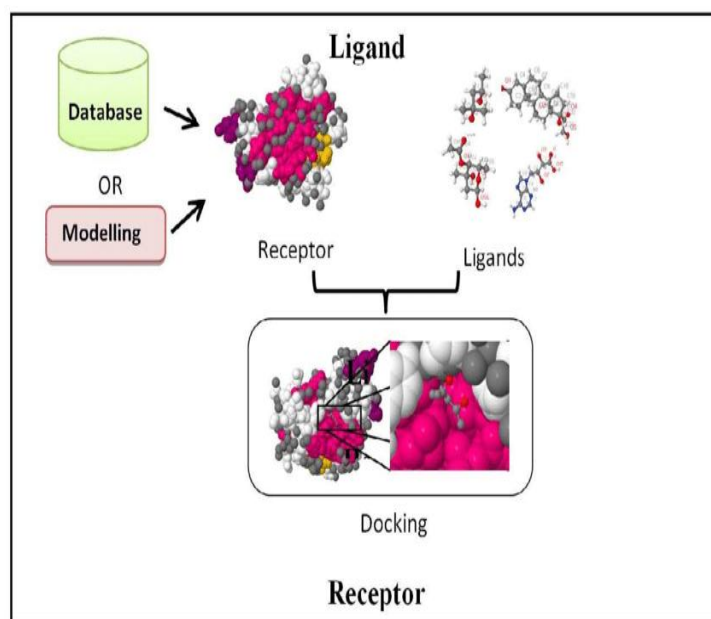


Figure 5.1: Principle of molecular docking

5.1.1.2 Materials and experimental procedure

Preparation of macromolecules

The protein targets were used as docking receptors; they were taken from the RCSB Protein Data Bank (PDB ID: 6X3X) (Kim et al., 2020). Prior to docking, the receptor's active site was cleared of all bound ligands, and water molecules.

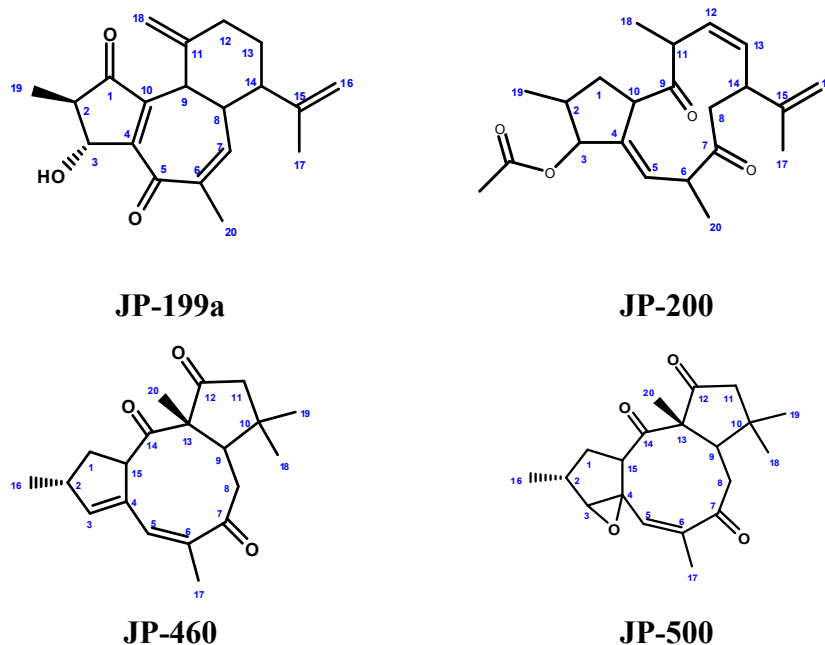


Figure 5.2: Structures of the isolated compounds

Molecular docking study of human GABA-A receptor

The AutoDockTools (ADT), and AutoDock Vina (Forli, et al., 2016) programs were used to conduct the molecular docking research (Trott & Olson, 2010). When docking the compounds against the protein's benzodiazepine binding site (PDB ID: 6X3X), standard protocol was followed. With a grid point spacing of 1.0 Å, the grid box was built using 18, 18, and 20 pointing in the x, y, and z directions, respectively. The grid box in the middle measures 90.391 Å, 125.706 Å, and 106.501 Å. For every ligand, nine distinct conformations were produced, ordered by binding energies, and scored using AutoDock Vina scoring procedures. To examine how ligands, and target receptors interact, the conformations with the most advantageous (lowest) free binding energy were selected. To visualize the data, Discovery Studio Visualizer v24 was utilized (Dassault Systèmes, 2024).

5.1.1.3 Result and discussion

JP-199a, JP-200, JP-460, and JP-500 showed favorable docking scores within the benzodiazepine binding site of human GABA-A receptor.

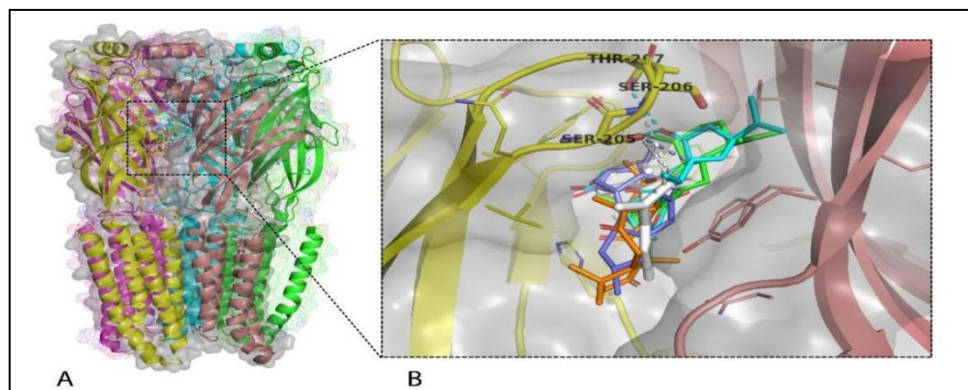


Figure 5.3: A) Human GABA-A receptor; B) Overlay of compound 1 (green stick), compound 2 (cyan stick), compound 3 (slate stick), compound 4 (orange stick) & diazepam (white stick) bound to the benzodiazepine binding site of human GABA-A receptor (PDB ID: 6X3X)

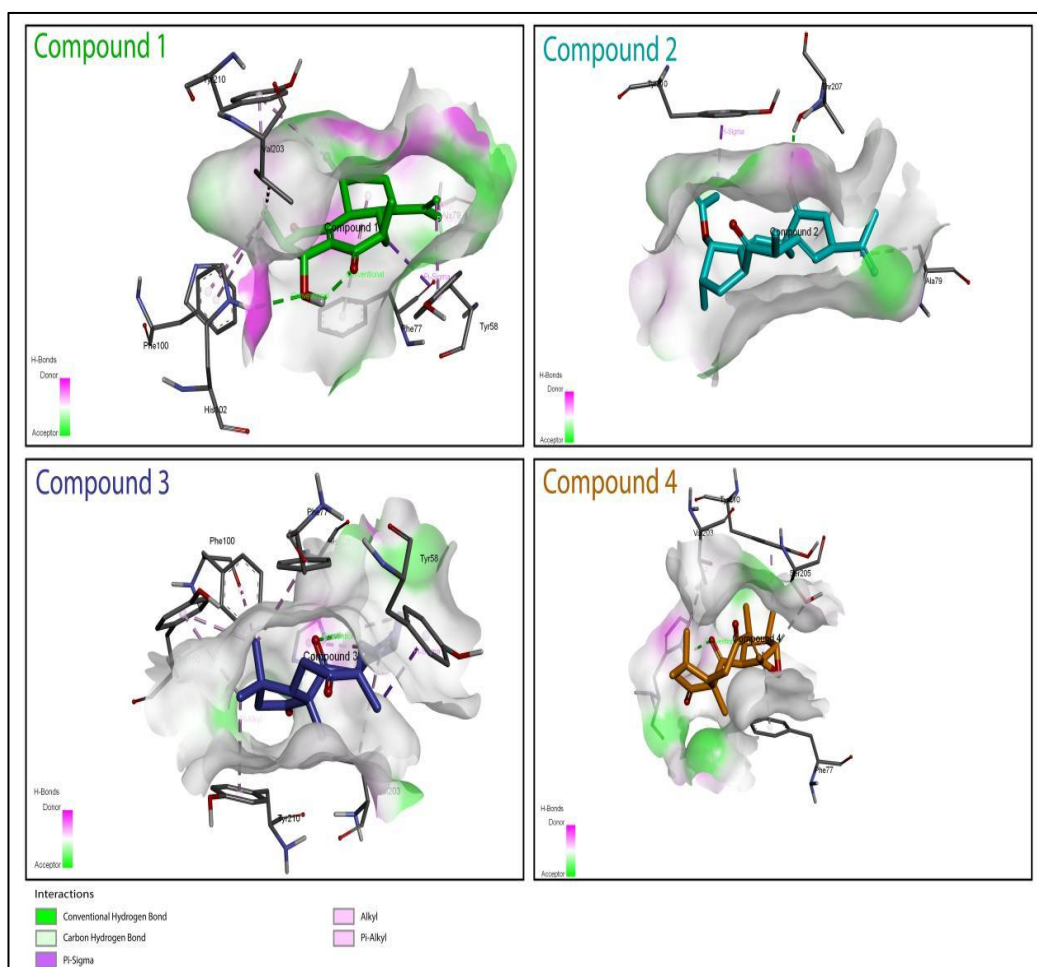


Figure 5.4: Interaction profiles with human GABA-A receptor

Table 5.1: Molecular docking scores of the compounds on GABA-A receptor

Compounds	Binding affinity (kcal/mol)	Ligand efficiency (kcal/mol per non-hydrogen atom)	Ligand-receptor interactions
Compound 1 (JP-199a)	-9.0	0.39	Phe-100 (pi-alkyl), His-102 (pi-alkyl), Val-203 (alkyl) and Tyr-210 (pi-alkyl) of $\alpha 1$ subunit as well as Tyr-58 (pi-alkyl and pi-sigma), Phe-77 (pi-alkyl) and Ala-79 (alkyl) of $\gamma 2$ subunit of human GABA-A receptor.
Compound 2 (JP-200)	-8.8	0.34	Phe-100 (pi-alkyl), His-102 (pi-alkyl), Val-203 (alkyl), Ser-205 (H-bond), Thr-207 (H-bond) and Tyr-210 (pi-sigma) of $\alpha 1$ subunit as well as Tyr-58 (pi-alkyl), Phe-77 (pi-alkyl) and Ala-79 (alkyl) of $\gamma 2$ subunit of human GABA-A receptor.
Compound 3 (JP-460)	-9.4	0.41	Phe-100 (pi-alkyl), His-102 (H-bond and pi-alkyl), Tyr-160 (pi-alkyl), Val-203 (alkyl), Ser-205 (H-bond) and Tyr-210 (pi-alkyl) of $\alpha 1$ subunit as well as Tyr-58 (pi-sigma) and Phe-77 (pi-alkyl) of $\gamma 2$ subunit of human GABA-A receptor.
Compound 4 (JP-500)	-5.7	0.24	Phe-100 (van der Waals), His-102 (van der Waals), Val-203 (alkyl), Ser-205 (H-bond) and Tyr-210 (pi-alkyl) of $\alpha 1$ subunit as well as Phe-77 (pi-alkyl) of $\gamma 2$ subunit of human GABA-A receptor.
Diazepam	-10.3	0.52	(Kim et al., 2020)

Compound **1** interacted with Phe-100 (pi-alkyl), His-102 (pi-alkyl), Val-203 (alkyl), and Tyr-210 (pi-alkyl) of $\alpha 1$ subunit as well as Tyr-58 (pi-alkyl and pi-sigma), Phe-77 (pi-alkyl), and Ala-79 (alkyl) of $\gamma 2$ subunit of human GABA-A receptor. Such interaction may stabilize local and global organization of the human GABA-A receptor.

Compound **2** interacted with Phe-100 (pi-alkyl), His-102 (pi-alkyl), Val-203 (alkyl), Ser-205 (H-bond), Thr-207 (H-bond), and Tyr-210 (pi-sigma) of $\alpha 1$ subunit as well as Tyr-58 (pi-alkyl), Phe-77 (pi-alkyl), and Ala-79 (alkyl) of $\gamma 2$ subunit of human GABA-A receptor.

Compound **3** interacted with Phe-100 (pi-alkyl), His-102 (H-bond and pi-alkyl), Tyr-160 (pi-alkyl), Val-203 (alkyl), Ser-205 (H-bond), and Tyr-210 (pi-alkyl) of $\alpha 1$ subunit as well as Tyr-58 (pi-sigma), and Phe-77 (pi-alkyl) of $\gamma 2$ subunit of human GABA-A receptor.

Compound **4** interacted with Phe-100 (van der Waals), His-102 (van der Waals), Val-203 (alkyl), Ser-205 (H-bond), and Tyr-210 (pi-alkyl) of $\alpha 1$ subunit as well as Phe-77 (pi-alkyl) of $\gamma 2$ subunit of human GABA-A receptor (Figure 5.4).

Compound **3** and compound **1** showed high binding affinities -9.4 , and -9.0 kcal/mol respectively, whereas the binding affinity of diazepam, was scored to be -10.3 kcal/mol with RMSD value of 0.354. These findings suggest that the isolated compound 3 and 1 might be promising positive modulators of human GABA-A receptor with binding affinity presented in Table 5.1, whereas compound **2**, and compound **4** (-8.8 , and -5.7 kcal/mol) might be a moderate modulator of human GABA-A receptor. The Ligand efficiency was observed with 0.41, 0.39, 0.34, and 0.24 kcal/mol, respectively. These isolated compounds are, therefore, responsible for the CNS depressant activity of the plant observed in this study.

5.1.2 ADMET Analysis

ADMET analysis is a fundamental aspect of drug discovery that predicts potential therapeutic or toxicological consequences. It represents a captious component of computational drug design and development (Flores-Holguín et al., 2021).

5.1.2.1 Principle of ADMET analysis

The principle of ADMET analysis is to illustrate the pharmacokinetic profile and behavior of a drug molecule within a biological system in terms of absorption, distribution, metabolism, excretion, and toxicity. ADMET helps in screening and optimizing compounds for efficacy and safety, reducing the risk of failure in later stages.

5.1.2.2 Materials and experimental procedure

Using the ADMETlab 3.0 server, the important ADMET characteristics for the isolated compounds were examined (Fu et al., 2024). Human intestinal absorption (HIA), Caco-2 permeability, cytochrome P450 enzyme inhibition level, P-glycoprotein (P-gp) inhibitory activity, Ames toxicity, carcinogenicity, and other characteristics of the isolated compounds were taken into account for the analysis. Finally, the ADMET-score values were adjusted between 0 and 1 according to the scores of the drugs in DrugBank (<https://go.drugbank.com/>) database in which “1” indicates the best and “0” means the worst.

5.1.2.3 Result and discussion

Absorption, distribution, metabolism, excretion, and toxicity profile are done in significant dimensions. Optimum values for each criteria and values for each compounds are given below:

Table 5.2: Absorption properties of the isolated compounds

Property	JP-199a value	JP-199a Decision	JP-200 Value	JP-200 Decision	JP-460 Value	JP-460 Decision	JP-500 Value	JP-500 Decision
Caco-2 Permeability	-4.836	●	-4.763	●	-4.752	●	-4.707	●
Optimal: higher than -5.15 Log unit								
MDCK Permeability	-4.474	●	-4.593	●	-4.501	●	-4.533	●
< 2 × 10 ⁻⁶ cm/s indicates low permeability, 2-20 × 10 ⁻⁶ cm/s indicates medium permeability, > 20 × 10 ⁻⁶ cm/s indicates highly passive permeability								
PAMPA	0.317	●	0.008	●	0.683	●	0.794	●
The experimental data for Peff was logarithmically transformed (logPeff). Molecules with log Peff values below 2.0 were classified as low-permeability (Criteria 0), while those with log Peff values exceeding 2.5 were classified as high-permeability (Criteria 1).								
Pgp-inhibitor	0.899	●	0.993	●	0.975	●	0.866	●
Criteria 1: Inhibitor; Criteria 0: Non-inhibitor; The output value confirms the probability of being a Pgp-inhibitor								
Pgp-substrate	0.002	●	0.006	●	0.015	●	0.003	●
Criteria 1: Inhibitor; Criteria 0: Non-inhibitor; The output value confirms the probability of being a Pgp-substrate								
HIA	0.008	●	0.002	●	0.001	●	0.007	●
HIA indicates Human Intestinal Absorption, Criteria 1: HIA+(HIA < 30%), Criteria 0: HIA-(HIA ≥ 30%), The output value confirms the probability of being a HIA+								
F_{20%}	0.001	●	0.013	●	0.004	●	0.002	●
F_{20%} indicates 20% Bioavailability , Criteria 1: F _{20%} +, Criteria 2: F _{20%} -								
F_{30%}	0.111	●	0.382	●	0.021	●	0.02	●
F_{30%} indicates 30% Bioavailability , Criteria 1: F _{30%} +, Criteria 2: F _{30%} -								
F_{50%}	0.474	●	0.954	●	0.815	●	0.748	●
F_{50%} indicates 50% Bioavailability , Criteria 1: F _{50%} +, Criteria 2: F _{50%} -								

Table 5.3: Distribution properties of the isolated compounds

Property	JP-199a Value	JP-199a Decision	JP-200 Value	JP-200 Decision	JP-460 Value	JP-460 Decision	JP-500 Value	JP-500 Decision
PPB	94.702	●	95.825	●	87.696	●	64.439	●
Drugs with high protein-bound may have a low therapeutic index. Plasma Protein Binding Optimal: < 90%.								
VDss	0.23	●	-0.137	●	0.088	●	0.236	●
Volume Distribution, Optimal: 0.04-20L/kg								
BBB	0.018	●	0.373	●	0.014	●	0.018	●
Blood-Brain Barrier Penetration, Category 1: BBB+; Category 0: BBB-, The output value is the probability of being BBB+								
Fu	4.978	●	4.747	●	13.164	●	39.798	●
The fraction unbound in plasma, Low: <5%; Middle: 5~20%; High: > 20%								
OATP1B1 inhibitor	0.9	●	0.595	●	0.999	●	0.998	●
Category 0: Non-inhibitor, Category 1: inhibitor, The output value confirms the probability of being OATP1B1 inhibitor within the range of 0 to 1								
OATP1B3 inhibitor	0.999	●	0.997	●	1.0	●	1.0	●
Category 0: Non-inhibitor; Category 1: inhibitor, The output value is the probability of being inhibitor, within the range of 0 to 1								
BCRP inhibitor	0.044	●	0.028	●	0.001	●	0.001	●
Category 0: Non-inhibitor; Category 1: inhibitor, The output value is the probability of being inhibitor, within the range of 0 to 1								
MRP1 inhibitor	0.956	●	0.996	●	0.989	●	0.96	●
Category 0: Non-inhibitor; Category 1: inhibitor, The output value is the probability of being inhibitor, within the range of 0 to 1								

Table 5.4: Metabolism properties of the isolated compounds

Property	JP-199a Value	JP-199a Decision	JP-200 Value	JP-200 Decision	JP-460 Value	JP-460 Decision	JP-500 Value	JP-500 Decision
CYP1A2 inhibitor	0.001	●	0.0	●	0.0	●	0.0	●
Category 1: Inhibitor; Category 0: Non-inhibitor; The output value is the probability of being inhibitor								
CYP1A2 substrate	0.001	●	0.0	●	0.071	●	0.029	●
Category 1: Substrate; Category 0: Non-substrate; The output value is the probability of being substrate								
CYP2C19 inhibitor	0.046	●	0.058	●	0.96	●	0.985	●
Category 1: Inhibitor; Category 0: Non-inhibitor; The output value is the probability of being inhibitor								
CYP2C19 substrate	0.019	●	0.984	●	0.963	●	0.998	●
Category 1: Substrate; Category 0: Non-substrate; The output value is the probability of being substrate								
CYP2C9 inhibitor	0.006	●	0.001	●	0.394	●	0.326	●
Category 1: Inhibitor; Category 0: Non-inhibitor; The output value is the probability of being inhibitor								
CYP2C9 substrate	0.007	●	0.572	●	0.012	●	0.004	●
Category 1: Substrate; Category 0: Non-substrate; The output value is the probability of being substrate								
CYP2D6 inhibitor	0.0	●	0.0	●	0.0	●	0.001	●
Category 1: Inhibitor; Category 0: Non-inhibitor; The output value is the probability of being inhibitor								

Table 5.4 (cont.): Metabolism properties of the isolated compounds

Property	JP-199a Value	JP-199a Decision	JP-200 Value	JP-200 Decision	JP-460 Value	JP-460 Decision	JP-500 Value	JP-500 Decision
CYP2D6 substrate	0.0	●	0.0	●	0.0	●	0.0	●
Category 1: Substrate; Category 0: Non-substrate; The output value is the probability of being substrate								
CYP3A4 inhibitor	0.086	●	0.488	●	0.871	●	0.469	●
Category 1: Inhibitor; Category 0: Non-inhibitor; The output value is the probability of being inhibitor								
CYP3A4 substrate	0.456	●	0.007	●	1.0	●	0.994	●
Category 1: Substrate; Category 0: Non-substrate; The output value is the probability of being substrate								
CYP2B6 inhibitor	0.999	●	0.001	●	0.758	●	0.008	●
Category 1: Inhibitor; Category 0: Non-inhibitor; The output value is the probability of being inhibitor								
CYP2B6 substrate	0.0	●	0.987	●	0.005	●	0.161	●
Category 1: Substrate; Category 0: Non-substrate; The output value is the probability of being substrate								
CYP2C8 inhibitor	0.734	●	0.992	●	0.95	●	0.632	●
Category 1: Inhibitor; Category 0: Non-inhibitor; The output value is the probability of being inhibitor								
HLM Stability	0.903	●	0.957	●	0.996	●	0.967	●
Human liver microsomal (HLM) stability, Category 0: stable+ (HLM > 30 min); Category 1: unstable- (HLM 30 min). The output value is the probability of human liver microsomal instability, where a value closer to 1 indicates a higher likelihood of instability								

Table 5.5: Excretion properties of the isolated compounds

Property	JP-199a Value	JP-199a Decision	JP-200 Value	JP-200 Decision	JP-460 Value	JP-460 Decision	JP-500 Value	JP-500 Decision
CL Plasma	8.844	●	9.713	●	7.874	●	9.197	●
The unit of predicted CL plasma penetration is ml/min/kg. >15 ml/min/kg: high clearance; 5-15 ml/min/kg: moderate clearance; < 5 ml/min/kg: low clearance								
T _{1/2}	1.142	●	0.582	●	0.566	●	0.86	●
The unit of predicted T _{1/2} is hours. ultra-short half-life drugs: T _{1/2} < 1 hour; short half-life drugs: T _{1/2} between 1-4 hours; intermediateshort half-life drugs: T _{1/2} between 4-8 hours; long half-life drugs: T _{1/2} > 8 hours.								

Table 5.6: Toxicity properties of the isolated compounds

Property	JP-199a Value	JP-199a Decision	JP-200 Value	JP-200 Decision	JP-460 Value	JP-460 Decision	JP-500 Value	JP-500 Decision
hERG Blockers	0.044	●	0.016	●	0.144	●	0.146	●
Molecules with $IC_{50} \leq 10\mu M$ or $\geq 50\%$ inhibition at $10\mu M$ were classified as hERG+ (Category 1), while molecules with $IC_{50} > 10\mu M$ or $< 50\%$ inhibition at $10\mu M$ were classified as hERG- (Category 0). The output value is the probability of being hERG+								
hERG Blockres (10μm)	0.445	●	0.296	●	0.562	●	0.524	●
Molecules with $IC_{50} \leq 10\mu M$ are classified as hERG+ (Category 1), and molecules with $IC_{50} > 10\mu M$ are classified as hERG- (Category 0). The output value is the probability of being hERG+								
DILI	0.885	●	0.755	●	0.713	●	0.571	●
Drug Induced Liver Injury. Category 1: drugs with a high risk of DILI; Category 0: drugs with no risk of DILI. The output value is the probability of being toxic								
AMES Mutagenicity	0.513	●	0.097	●	0.63	●	0.81	●
AMES Toxicity, Category 1: Ames positive(+); Category 0: Ames negative(-); The output value is the probability of being toxic								
Rat Oral Acute Toxicity	0.282	●	0.498	●	0.776	●	0.771	●
Rat Oral Acute Toxicity. Category 0: low-toxicity, > 500 mg/kg; Category 1: high-toxicity, < 500 mg/kg. The output value is the probability of being toxic								
FDAMDD	0.609	●	0.324	●	0.899	●	0.714	●
FDA Maximum (Recommended) Daily Dose. Category 1: FDAMDD (+); Category 0: FDAMDD (-); The output value is the probability of being positive								

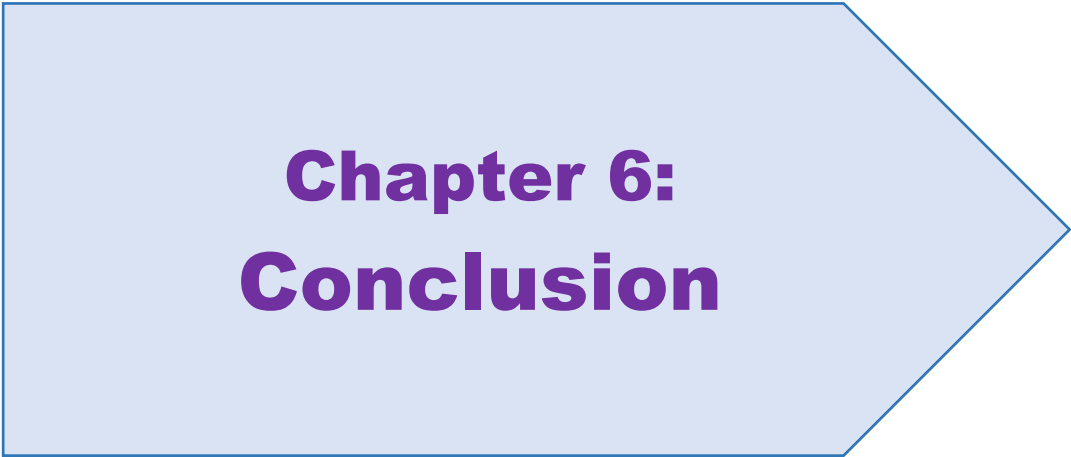
Table 5.6 (cont.): Toxicity properties of the isolated compounds

Property	JP-199a Value	JP-199a Decision	JP-200 Value	JP-200 Decision	JP-460 Value	JP-460 Decision	JP-500 Value	JP-500 Decision
Skin Sensitization	0.804	●	0.208	●	0.839	●	0.798	●
Category 1: Sensitizer; Category 0: Non-sensitizer. The output value is the probability of being toxic								
Carcinogenicity	0.883	●	0.404	●	0.793	●	0.751	●
Category 1: carcinogen; Category 0: non-carcinogen; The output value is the probability of being toxic								
Eye Corrosion	0.007	●	0.007	●	0.019	●	0.0	●
Eye Corrosion, Category 1: corrosive; Category 0: noncorrosive; The output value is the probability of being corrosive								
Eye Irritation	0.545	●	0.116	●	0.422	●	0.065	●
Eye Irritation, Category 1: irritant; Category 0: nonirritant; The output value is the probability of being irritants								
Respiratory	0.699	●	0.503	●	0.845	●	0.685	●
Category 1: respiratory toxicants; Category 0: non-respiratory toxicants. The output value is the probability of being toxic								
Human Hep atotoxicity	0.797	●	0.546	●	0.851	●	0.726	●
Human Hepatotoxicity, Category 1: H-HT positive(+); Category 0: H-HT negative(-); The output value is the probability of being toxic								
Drug-induced Nephrotoxicity	0.332	●	0.186	●	0.805	●	0.326	●
Category 0: non-nephrotoxic (-); Category 1: nephrotoxic (+). The output value is the probability of being nephrotoxic (+)								

Table 5.6 (cont.): Toxicity properties of the isolated compounds

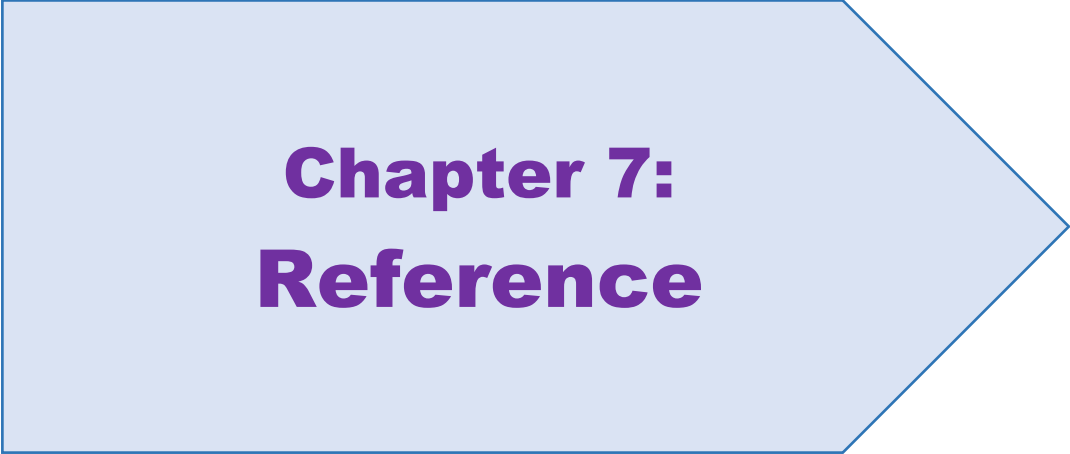
Property	JP-199a Value	JP-199a Decision	JP-200 Value	JP-200 Decision	JP-460 Value	JP-460 Decision	JP-500 Value	JP-500 Decision
Hematotoxicity	0.534	●	0.458	●	0.413	●	0.402	●
Category 0: non-hematotoxicity (-); Category 1: hematotoxicity (+). The output value is the probability of being hematotoxicity (+)								
Genotoxicity	0.499	●	0.762	●	0.984	●	0.981	●
Category 0: non-Genotoxicity (-); Category 1: Genotoxicity (+). The output value is the probability of being ototoxicity (+)								
RPMI-8226 Immunotoxicity	0.103	●	0.036	●	0.072	●	0.17	●
Category 0: non-cytotoxicity (-); Category 1: cytotoxicity (+). The output value is the probability of being ototoxicity (+)								
A549 Cytotoxicity	0.26	●	0.084	●	0.419	●	0.223	●
Category 0: non-cytotoxicity (-); Category 1: cytotoxicity (+). The output value is the probability of being ototoxicity (+)								
Hek293 Cytotoxicity	0.354	●	0.205	●	0.423	●	0.788	●
Category 0: non-cytotoxicity (-); Category 1: cytotoxicity (+). The output value is the probability of being ototoxicity (+), within the range of 0 to 1.								
Drug-induced Neurotoxicity	0.618	●	0.752	●	0.919	●	0.899	●
Category 0: non-neurotoxic (-); Category 1: neurotoxic (+). The output value is the probability of being neurotoxic (+)								
Ototoxicity	0.847	●	0.664	●	0.484	●	0.714	●
Category 0: non-ototoxicity (-); Category 1: ototoxicity (+).The output value is the probability of being ototoxicity (+)								

ADME and toxicity predictions were conducted across 53 biological endpoints. To validate the scoring function, ADMET scores of compounds were evaluated using data from the DrugBank database. For assessing absorption characteristics, various endpoints were considered, including human intestinal absorption (HIA) and Caco-2 permeability models. Molecules that inhibit a greater number of CYP isoforms (1A2, 2C9, 2C19, 2D6, and 3A4) are more likely to be involved in drug–drug interactions (DDIs) with other medications (Cheng et al., 2011). In this study, CYP promiscuity, was assessed. Models predicting P-glycoprotein inhibition or substrate properties were employed as key indicators for evaluating drug candidates from multiple pharmacokinetic perspectives (Broccatelli et al., 2011). Half-life ($T_{1/2}$) and plasma clearance (CL-plasma) were considered to reflect drug accumulation within the kidneys, which may lead to nephrotoxicity (Kido et al., 2011). Consequently, these parameters were included as indicators of excretion in defining the ADMET score. The toxicity assessment covered several important endpoints, including AMES mutagenicity, acute oral toxicity (AO), carcinogenicity (CARC), and hERG channel inhibition. Inhibition of the hERG potassium channel can prolong QT interval, indicating serious cardiac adverse effects. AMES mutagenicity testing was also performed to evaluate the potential for teratogenicity and genotoxicity. Additional toxicological endpoints, such as human hepatotoxicity, drug-induced nephrotoxicity, hematotoxicity, neurotoxicity, and ototoxicity, represent significant concerns for human health (Wang et al., 2012). Based on the parameters described above, compounds JP-199, JP-200, and JP-460 demonstrated favorable *in silico* ADMET profiles, as reflected by their ADMET score values. The compounds demonstrated advantageous properties such as effective absorption in the gastrointestinal tract and blood-brain barrier penetration, suggesting their appropriateness for oral administration and effects on the CNS. Furthermore, the absence of anticipated CYP450 enzyme inhibition suggests a lower risk of drug-drug interactions. The majority of the compounds were also predicted to be non-toxic, exhibiting no risks of mutagenic or liver toxicity. The ADMET scoring method aids an inclusion approach to assessing and ranking potential drug candidates by integrating important safety and toxicity data profile. This study also highlights early identification of safe lead compound in predictive and efficient pattern.



Chapter 6: Conclusion

This study represents a comprehensive investigation into the phytochemical and pharmacological profiles of *J. pandurifolia* and *S. reticulatum*. Spectroscopic techniques, including ^1H NMR, ^{13}C NMR, ^1H - ^1H COSY, HSQC, and mass spectrometry were employed in the structural elucidation of isolated compounds. Among the fifteen compounds, compound 1, 6, 7, 8, 9, 12, and 13 were identified for the first time from *J. pandurifolia* stem bark, while compound 14 and 15 were newly reported from *S. reticulatum* leaf. Notably, compound 5 represents a new natural molecule, previously unreported in any literature. Methanolic extract *J. pandurifolia* (Jp-ML) leaf and ethyl acetate extract of *S. reticulatum* (Sr-EAL) leaf were subjected to *in vivo* evaluation for determining CNS depressant, antidiarrheal, and analgesic properties at various doses. Both Jp-ML and Sr-EAL exhibited mild to strong dose-dependent inhibition of locomotor activity in the open field and hole cross models. A strongly significant reduction in diarrheal frequency and onset of action was recorded for both the extracts at highest dose (600 mg/kg) indicating promising gastrointestinal protective activity. Both the extracts also exhibited significant reductions in writhing and paw licking that ensure effective pain suppression at (300 mg/kg). Different fractionates (Jp-ML, Jp-EAL, Jp-CL, Jp-HL, Sr-ML, Sr-EAL, Sr-CL and Sr-HL) and pure compounds (JP-10, JP-13, JP-25, JP-460) were subjected to *in vitro* evaluation of antioxidant, cytotoxic and thrombolytic activity. JP-ML, Sr-EAL along with triacontyl *trans*-ferulate (JP-25) showed low IC_{50} value, revealing promising antioxidant activity. Low LC_{50} values were recorded for Jp-EAL, Sr-ML and jatrophatrione (JP-460) indicating high cytotoxicity. Integerrimene (JP-200) significantly reduced cell survival rate that confirms strong cytotoxic potential in different concentrations reflecting low LD_{50} value on HeLa cell line. Jp-ML, Sr-EAL and jatrophatrione (JP-460) exhibited significant clot rupture capacity indicating potential thrombolytic activity. *In silico* molecular docking of JP-199a, 200, 460, 500 were performed and the investigation indicates strong interaction of these pure compounds with GABA-A receptor that aligns to CNS depressive potential. ADMET profiling predicted acceptable pharmacokinetic behavior with minimal hepatotoxicity, nephrotoxicity, or genotoxicity for these pure compounds. These results enhance the safety profile of the identified phytoconstituents and reinforce their drug-likeness potential. Overall, this study explores the phytochemical and pharmacological properties of *J. pandurifolia* and *S. reticulatum* that highlights their potential as valuable sources of drug leads in the context of drug discovery.



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