CYTOTOXIC AND ANTI-MICROBIAL CONSTITUENTS FROM SOME BANGLADESHI *BRIDELIA* AND *ERYTHRINA* SPECIES

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Declaration

I do hereby declare that the materials embodied in this thesis entitled "Cytotoxic and antimicrobial constituents from some Bangladeshi Bridelia and Erythrina species" prepared for submission to the University of Dhaka, Dhaka, Bangladesh for the Degree of Doctor of Philosophy in Pharmaceutical Chemistry are the original research works of mine and have not been previously submitted for the award of any Degree or Diploma.

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ABSTRACT

This thesis describes the isolation and structure elucidation of secondary metabolites from four plants *Bridelia verrucosa, B. stipularis, B. tomentosa* (Family: Phyllanthaceae) and *Erythrina fusca* (Family: Fabaceae) as well as biological studies of the extractives from these plants along with *E. variegata* (Family: Fabaceae). A total of nineteen compounds were isolated of which three appear to be new. The structures of the isolated compounds were elucidated mainly by spectroscopic studies including high field NMR.

The stem bark of *B. verrucosa* afforded eight compounds *viz.* glochidonol (BVSP-1, **46**), brassicasterol (BVSP-2, **47**), friedelin (BVSP-4, **48**); 5β-24S-ethylcholestan-3β-ol (BVSP-6, **49**); stigmasterol (BVSP-34, **50**); 5,5'-dihydroxysesamine (BVBC-2, **51**), 3-ketoleanane (**53**) and pinoresinol (BVS-65, **54**). Except stigmasterol (**50**) and pinoresinol (**54**) this is the first report of the isolation of compounds **46-49**, **53** and **54** from the genus *Bridelia* and **51** has been identified as a new natural product. Phytochemical investigations of methanolic extract of the stem bark of *B. stipularis* provided two triterpenes namely, glut-5(6)-en-3-one (BS-01, **55**), glut-5(6)-en-3α-ol (BS-06, **56**) and a cholestane type compound (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one (BS-11, **57**). All these are the first report of their occurrence from *B. stipularis* while (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one is a new compound. On the other hand, phytochemical investigation of methanol extract of the stem bark of *B. tomentosa* yielded friedoolean-5(6),14(15)-dien-3-one (BT-1, **58**), β-taraxerol (BT-6, **59**), D_4-stigmasterone (BT-8, **60**) and lupeol (BT-14, **61**). This is the first report of their occurrence from this plant and friedoolean-5(6),14(15)-dien-3-one (**58**) is a novel compound. The carbon tetrachloride soluble fractions of a methanol extract of the stem bark of *E. fusca* provided three flavonoids namely, shinpterocarpin (EF-7, **62**), lupinifolin (EF-8, **63**) and 3,9-dihydroxy-4-(3,3-dimethylallyl)[6a*R*,11a*R*]-pterocarpan (**64**) and a steroid, β-sitosterol (EF-38, **65**). All these are the first report except **65** from *E. fusca*.

The crude extracts and organic soluble material of the investigated plants and some purified compounds were screened for their antimicrobial activity against a wide range of Gram-positive and Gram-negative microorganisms and fungi by the standardized disc diffusion method. Kanamycin and Griseofulvin were used as reference drugs for the test.

R=

BVSP-2: Brassicasterol (**47**)

BVSP-34: Stigmasterol (**50**)

EF-38: β-Sitosterol (**65**)

R= OH, R_1= O BVSP-1: Glochidonol (**46**)

R= H, R_1 < OH, H BT-14: Lupeol (**61**)

BVBC-2: 5,5'-dihydroxysesamine (**51**)

R= OH, R_1= CH_3, R_2=

BS-11: (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one (**57**)

R= H, R_1= H, R_2=

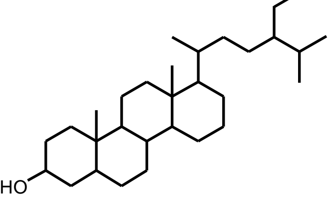
BT-8: D_4-Stigmasterone (**60**)

Pinoresinol, BVS-65, **54**

BVSP-4: Friedelin (**48**)

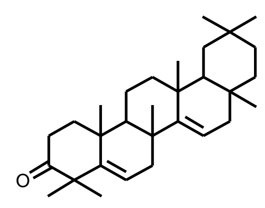
R= O, BS-01: Glut-5(6)-en-3-one (**55**)

R < H, OH BS-06: Glut-5(6)-en-3α ol (**56**)



BVSP-6: 5β -24S-ethylcholestan-3α-ol (**49**)

BVSC-8: 3-Ketooleanane, (**53**)



BT-1: Friedoolean-5(6),14(15)-dien-3-one (**58**)

BT-6: β -Taraxerol **(59)**

EF-7: Shinpterocarpin (**62**)

EF-8: Lupinifolin (**63**)

EF-12: 3,9-dihydroxy-4-(3,3-dimethylallyl) [6aR,11aR]-pterocarpan (**64**)

All the samples showed mild to moderate antimicrobial activity against thirteen Gram positive and Gram negative bacterial strains and three fungi, where the stem bark extract of *B. tomentosa* demonstrated the highest inhibition of growth against *B. cereus* (23.2 mm) and *C. albicans* (17.5 mm). The purified compounds isolated from the investigated plants showed mild to moderate antibacterial activity. BS-01 (**55**) and BS-06 (5**6**) revealed strong inhibition of growth of *E. coli* (22.7 mm) and *C. albicans* (20.8 mm), respectively.

The plants were subjected to brine shrimp lethality bioassay for probable cytotoxicity. The aqueous soluble fraction of the leaf of *B. stipularis* revealed the highest cytotoxic activity with LC_{50} value of 12.59 μg/ml. On the other hand, the *n*-hexane soluble fraction of the stem bark of *E. variegata* revealed the strongest LC_{50} value of 14.66 μg/ml. The isolated pure compound EF-8 (**62**) displayed the strongest cytotoxic activity with LC_{50} value of 3.17 μg/ml, as compared to standard vincristine sulfate (LC_{50} 0.45 μg/ml).

Free radical scavenging activity, total antioxidant capacity and total phenolic content of the extractives were also investigated. Free radical scavenging assay of the methanol extract of the leaf of *B. verrucosa* revealed highest activity having IC_{50} of 6.35 μg/ml while the chloroform soluble fraction of the leaf of *E. fusca* showed IC_{50} at 5.50 μg/ml. The IC_{50} (7.3 μg/ml) of the isolated pure compound BVBC-2 (**51**) indicated strong free radical scavenging activity.

The aqueous and chloroform soluble materials of the leaf of *B.stipularis* demonstrated promising total pehonolic content (98.76 and 94.52 mg of GAE/100 g of dried extract). On the other hand, the highest total antioxidant capacity of methanolic extract of the leaf of *B. tomentosa* and *E. fusca* were found as 612.54 and 587.66 mg of ascorbic acid/g of dried extract, respectively.

All the extractives of five plants were also studied for their thrombolytic potential. The carbon tetrachloride soluble fraction and methanol extract of leaf and aqueous soluble material of bark of *B. tomentosa,* methanol extract of bark of *B. stipularis* and carbon tetrachloride soluble partitionate of leaf of *B. verrucosa* exhibited highest thrombolytic activity with clot lysis value of 41.46%, 34.85%, 37.04%, 36.45% and 33.72%, respectively. Standard streptokinase was used as positive control which exhibited 61.50% lysis of clot while the negative control water revealed 2.56% clot lysis.

In conclusion, a total 19 compounds including three new molecules (**51**, **57** and **58**) have been isolated from *B. verrucosa*, *B. stipularis* and *B. tomentosa*, respectively. Also, the methanolic extract of the investigated plants and their Kupchan fractions showed significant antibacterial, cytotoxic, antioxidant and thrombolytic activities. These plants can be further studied in order to find out their unexplored efficacy against other diseases as well as to isolate more chemically interesting and biologically important drug candidates.

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1.1 Rational of the work

Today standing in the twenty-first century, in the age of massive development of modern science, mankind still depends on nature for its survival. This fact is seen from the use of natural herbs to cure diseases and relieve physical sufferings. The importance of herbs dates back to ancient times for their food value as well as invaluable medicinal properties. It is not certain when and how man learned to use plant appropriately but it is thought that; animal was the first guide to show them the use of plants. Because animals naturally know or feel how to get relief from unusual internal conditions and during the long span of time men gradually developed a knowledge of naturally occurring drugs which was transmitted at one time orally, later in written form as papyri, baked clay tablets, parchments, manuscript herbals, printed herbals, pharmacopoeias and other works, and most recently by computerized information-retrieval system (Evans, 1989).

The art of medicine is very ancient. There are countless herbal remedies and cures, which have been passed on by generations. The list of such herbal medications are endless, each unique in their value and origin, some of which we are still unaware of but some of the drugs which have been used in the days of antiquity are still now used, but in a modified form. As far as records from history of pharmacy, it appears that the Babylonians (about 3000 years BC) made clay models of the human body and early writings indicate that, they were aware of medicinal effects of a number of plants (Tyler and Brady, 1985).

Probably the oldest pharmacopoeia of the Emperor Shen Nung (Burger, 1970) of China written between 2730 BC and 3000 BC contains the uses and recipes of hundreds and thousands of Chinese traditional medicines. As evident from the Papyrus Ebers (written in about 1500 BC found in the tomb of a mummy and now kept in the University of Leipzig); the ancient Egyptians possessed a good knowledge of the medicinal properties of hundreds of plants. Many of the plant drugs which are used in present day, like henbane (*Hyoscymus spp.*); mandrake (*Mandragora officinarum*); opium (*Papaver somniferum* fruit); castor oil (oil of *Ricinus communis* seed) and many others were in common use in Egypt about 4500 years ago (Ghani, 2003).

The Materia Medica of the great Greek physician Hippocrates (406 ~ 370 BC) consists of some 300 to 400 medicinal plants which included opium, mint, rosemary, sage and verbena. The far-ranging scientific work of Aristotle (384 ~ 322 BC); a Greek philosopher, included an effort to catalogue the properties of the various medicinal herbs known at that time. Theophrastus (Core, 1962) (370 ~ 285 BC); a student of Pleto, in his "*Historia plantarum*" described the use of nearly 500 medicinal plants. The encyclopedic work of Dioscorides (1st Century AD)-De Materia Medica was the forerunner of all modern pharmacopoeias and an authoritative text on botanical medicine. The work featured about 600 medicinal plants. Two of the 37 volumes of books written by Pliny De Elder (23 ~ 70 AD) were devoted to medical botany and these included a large number of medicinal plants.

Galen (131 ~ 200 AD); a Greek pharmacist-physician who lived in Rome, described the method of preparing formulas containing plant and animal drugs. He devoted considerable time in compiling this knowledge, which was distributed through out 20 books (Tyler and Brady, 1985).

When the Roman glory began to decline in the Middle Ages, the Arabs contributed much to the vast field of medicine. A canon of medicine by Ibn Sina bears a testimony to this. With the advent of the Muslim conquerors, the Mohammedan system of medicine was introduced in the sub-continent and the system was prevalent during the region of Mughal and Pathan. The Arabian system was superior standard and was fairly advanced for the period. An excellent account of the Indian sub-continent can be seen in the book written by Abu Mansur in that period (Core, 1962).

The discovery of the medicinal plants of the Indian sub-continent was made hundred of years ago (Chopra *et al.,* 1933). Rigveda (4500 ~ 1600 BC); Atharvaveda, Ayurveda, Susruta and Charaka are amongst the oldest treatises on Indian medicinal plants (Chopra *et al.,* 1958).

One of the earliest book containing the description of therapeutic agents were known as the "Herbal" and the oldest herbals were the "Ortus sanitatis" meaning the garden of Health, written in Latin and published at Mainz in 1491 (Wallis, 1967).

Ancient manuscripts, pictures painted on the walls of tombs, plant remains found in caves and burial sites and craving in stones preserved through the ages have given modern scientists at least fragmentary insight into the knowledge of those people about the use of plants. Allopathic and homeopathic system of medicines today are based on the doctrines expatiated by Galen (Sofowra, 1982).

Today, a lot of modern medicines have been discovered as a result of the scientific follow-up of the traditional herbal preparations. A classic example is the latest development of the anti-malarial drug "Quinhasu" from the herb "*Artemisia annua*" which has been used in China against malaria for 2000 years. Withering (1741-1799) introduced digitalis (purple foxglove) as a therapeutic agent for dropsy the active principles from the same material are used in the treatment of heart failure today.

With centuries of efforts scientists have found in plants the remedy of diverse diseases ranging from simple skin diseases to complicated cancer. Based on new scientific developments in isolation, identification and testing technology, the most promising recent contribution of medicinal plants research led to the isolation of anticancer agent taxol (from *Taxus bravifolia*); hypercin (from *Hypericum* species) and anti-AIDS glycyrrhizin (from *Glycyrrhiza* species).

Plants have formed the basis for traditional systems of medicine, which have been used for thousands of years in many countries (Chang, 1986; Kapoor, 1990) and extensively documented (Schultes *et al.*, 1990). These plant-based systems continue to play an essential role in health care and it has been estimated by the World Health Organization (WHO) that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care (Arvigo *et al.,* 1993). Plant products also play an important role in the health care systems of the remaining 20% of the population, mainly residing in developed countries. In a study it has been shown that at least 119 chemical substances, derived from 90 plant species, can be considered as important drugs that are in use in one or more countries. Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine (Farnsworth *et al.,* 1985).

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During the last two decades, the renewed interest in investigating natural products has lead to the advent of several important drugs. For example, recently ricin, a toxin produced by the beans of *Ricinus communis*, has been found to be a very potent antitumor drug (Spalding, 1991; Gupta, 1992). Further, HIV inhibitory activity has been discovered in some novel complex angular coumarins isolated from *Calophyllum lanigerum* (Kashman *et al.,* 1992) and taxol is another example of effective antitumor agent found from *Taxus bravifolia*. Plants containing important secondary metabolites and potential biological activities can be genetically engineered to increase the transcription of enzyme responsible for the production of that particular compound. Thus, knowing the potential resources it is possible to increase the content of important active compounds (Owen *et al.,* 1992) and genes are to be encoded in the host organisms to produce lead compounds from plants in industrial scale.

In fact, plants are the important source of a diverse range of chemical compounds. Some of these compounds possessing a wide range of pharmacological activities are either impossible or trouble some to synthesize in the laboratory. A phytochemist uncovering these resources is producing useful materials for screening programs for drug discovery.

Recently developed genetic engineering technology in plants has further increased their importance in the field of medicine, for example, in the production of antibodies by expression of an appropriate gene in the plant. By using this technique it is possible to modify the activity or regulate the properties of the key enzymes responsible for the production of secondary metabolites. Thus, by knowing the potential resources it is possible to increase the content of the important active compounds (Owen *et al.,* 1992) and in the future genes responsible for very specific biosynthetic processes may be encoded into host organism to facilitate synthetic transformation that is produce lead compounds from plants in industrial scale.

Thus, plant is a biosynthetic laboratory and the curative phyto-elements produced inside a plant through a flow of biochemical reactions significantly contribute to the traditional and modern medicines. These interesting active ingredients are nothing but the chemical defense against diseases which can hold back numerous pathological discrepancies and can reset physiological harmony.

The goals of using plants as sources of therapeutic agent are (Daniel *et al.,* 2001)

a) To isolate bioactive compounds for direct use as drugs, e.g. digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine;
b) To produce bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon (and other narcotic analgesics); taxotere, teniposide, verapamil, and miodarone, which are based, respectively, on galegin, Δ9- tetrahydrocannabinol, morphine, taxol, podophyllotoxin, khellin, and khellin;
c) To use agents as pharmacological tools, e. g., lysergic acid diethylamide, mescaline, yohimbin; and
d) To use the whole plant or part of it as a herbal remedy, e.g., cranberry, echinacea, feverfew, garlic, etc.

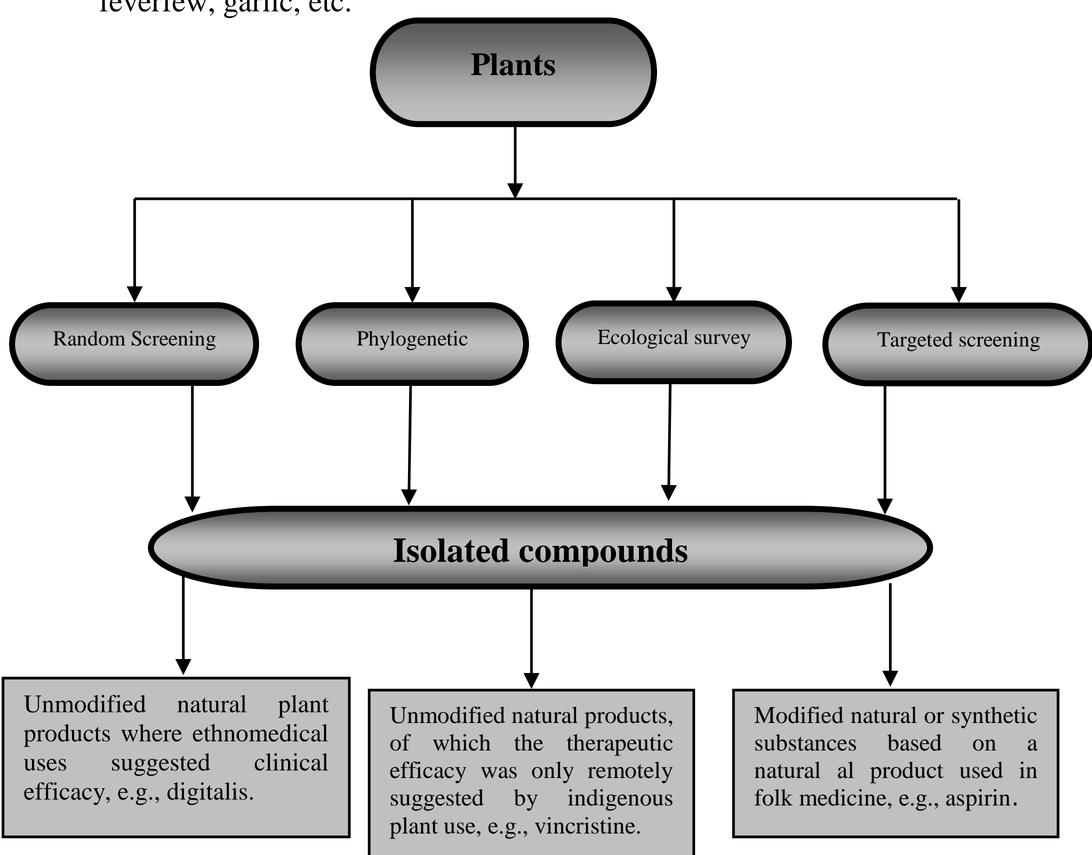

Figure 1.1: Lead compound search and utilization from plants.

There are several familiar approaches to find out lead compounds from plants (Fig 1.1) and the isolated bioactive compounds are utilized in three basic ways (Cox, 1994):

a) Unmodified natural plant products where ethnomedical uses suggested clinical efficacy, e.g., digitalis.
b) Unmodified natural products of which the therapeutic efficacy was only remotely suggested by indigenous plant use, e.g., vincristine.
c) Modified natural or synthetic substances based on a natural product used in folk medicine, e.g., aspirin.

1.2 Objectives of the work

Nature has been a source of several medicines for treating various types of diseases in humans and animals for many years (Dev, 1997). Natural products are naturally derived metabolites and/or by-products from plants, microorganisms or animals (Baker *et al.,* 2000). In the synthesis or design of new drugs the structure of the chemical substances still play a major role in treatment of diseases either as the drug or as a predecessor. The worlds best known and most universally used medicinal agent is aspirin, which is related to salicin and was originated from the plant genera, *Salix* and *Populus* (Strobel *et al.,* 2004). Examples abound of natural product use, especially in small native populations in a myriad of remote locations on earth. For instance, certain tribal groups in the Amazon basin, the highland people of Papua New Guinea and the Aborigines of Australia, each have identified certain plants to provide relief of symptoms, varying from colds to massive wounds and intestinal ailments (Isaacs, 2002).

Nature has endowed Bangladesh with a lavish gift of medicinal plants most of which grow in jungles, forests and gardens without care. They can also be cultivated in large scale, if desired. Many of these plants have been used in the indigenous system of medicine for centuries without having any knowledge of active principles responsible for therapeutic activities. Researches on some of these plants in the recent years have been much rewarding. As already mentioned, Bangladesh is blessed with numerous medicinal plants belonging to various families including Phyllanthaceae and Fabaceae. Although a large number of plants are included in the above two families and some of the plants have been investigated all over the world, very little is known regarding the chemistry and

pharmacology of *Bridelia* and *Erythrina* species growing in Bangladesh. So, attempts have been taken to study the chemical constituents along with their pharmacological aspects especially cytotoxic and antibacterial profiles of some *Bridelia* and *Erythrina* species available in Bangladesh. The plants selected for investigation include

***Bridelia* species:**	***Erythrina* species:**
1. *Bridelia verrucosa* Haines.	1. *Erythrina fusca* Lour.
2. *Bridelia stipularis* (L) Blume.	2. *Erythrina variegata* L.
3. *Bridelia tomentosa* Blume.	

These investigations may provide some remarkable compounds, with promising pharmacological activities. If considerable results are obtained, these can be used as remedy for the treatment of some diseases. Since these plants are available in Bangladesh, these may produce a cost-effective treatment. Thus, the objective was to explore the possibility of developing new drug candidates from these plants for the treatment of various diseases.

1.3 The plant family Phyllanthaceae

Phyllanthaceae is the family of flowering plants in the eudicot order Malpighiales. It is most closely related to the family Picrodendraceae. The Phyllanthaceae are most numerous in the tropics, with many in the South Temperate Zone, and a few ranging as far north as the middle of the North Temperate Zone (Hoffman *et al.,* 2006).

The name "Phyllanthaceae" was first validly published by Ivan Ivanovich Martynov in 1820 in a Russian book entitled *Tekhno-botanico Slovar.* A proposal to conserve this name was published in 2007 (Reveal *et al.,* 2007). Martynov's name was rarely used in the 180 years after he published it. During that time, the plants that are now in Phyllanthaceae were placed in the large and heterogeneous family Euphorbiaceae. The monophyly of Euphorbiaceae had long been held in doubt by some, but the first strong evidence of its polyphyly came in 1993 with the first maximum parsimony analysis of DNA sequences of the gene rbcL from a large number of seed plants (Chase *et al.,* 1993). Since the 1993 study, all subsequent phylogenetic analyses have shown that the old concept of Euphorbiaceae consisted of several lineages that did not together form a clade in the order Malpighiales. Euphorbiaceae is now defined as a

much smaller family than it had been in the twentieth century (Charles *et al.,* Toru, 2007). Pandaceae, Phyllanthaceae, Picrodendraceae, Putranjivaceae, Peraceae and Centroplacaceae have been removed from it (Stevens, 2001).

However, the obsolete, older concept of Euphorbiaceae, known as Euphorbia*ceae sensu lato,* is sometimes still used for continuity and convenience (Hoffmann *et al.,* 2006). It was the subject of a book and two papers which stood as the standard works on Phyllanthaceae until that family was revised by Hoffmann and co-authors in 2006.

1.4 Taxonomy of the plant family Phyllanthaceae

Taxonomically, the family Phyllanthaceae is divided into two subfamilies: **Antidesmatoideae** and **Phyllanthoideae**. Antidesmatoideae is divided into six tribes and Phyllanthoideae is divided into four. The tribe Antidesmateae of Antidesmatoideae and the tribes Bridelieae and Wielandieae of Phyllanthoideae are further divided into subtribes (Wikipedia).

Phyllanthaceae comprises about 2000 species which are grouped into 54 to 60 genera. Some of the genera are poorly defined, and the number of genera in the family is likely to change as the classification is further refined. The genus Phyllanthus, one of the largest genera of flowering plants, with over 1200 species, has more than half of the species in the family (Kathriarachchi *et al.,* 2006). Some of the genera have recently been "absorbed" by others, while other genera have recently been divided. The largest genera and the approximate number of species in each are *Phyllanthus* (1270); *Cleistanthus* (140); *Antidesma* (100); *Aporosa* (90); *Uapaca* (60); *Baccaurea* (50) and *Bridelia* (50) (Stevens, 2001).

Table 1.1: List of 54 genera from the 2006 revision of Phyllanthaceae (Hoffman *et al.*, 2006).

Genera		
Actephila	*Didymocistus*	*Nothobaccaurea*
Amanoa	*Discocarpus*	*Oreoporanthera*
Andrachne	*Distichirrhops*	*Pentabrachion*
Antidesma	*Flueggea*	*Phyllanthus*
Apodiscus	*Gonatogyne*	*Plagiocladus*
Aporosa	*Heywoodia*	*Poranthera*
Ashtonia	*Hieronyma*	*Protomegabaria*
Astrocasia	*Hymenocardia*	*Pseudolachnostylis*
Baccaurea	*Jablonskia*	*Richeria*
Bischofia	*Keayodendron*	*Savia*
Bridelia	*Lachnostylis*	*Securinega*
Celianella	*Leptonema*	*Spondianthus*
Chascotheca	*Leptopus*	*Tacarcuna*
Chonocentrum	*Lingelsheimia*	*Thecacoris*
Chorisandrachne	*Maesobotrya*	*Uapaca*
Cleistanthus	*Margaritaria*	*Wielandia*
Croizatia	*Martretia*	*Zimmermannia*
Dicoelia	*Meineckia*	*Zimmermanniopsis*

1.5 Botanical features of plant family Phyllanthaceae (Hoffman *et al.*, 2006)

Phyllanthaceae is an unusually diverse family for its moderate size. It can be recognized only by a combination of characters because there are a few exceptions to almost everything that is generally true of the family. It is most notable for having two ovules in each locule of the ovary, a trait that clearly distinguishes it from Euphorbiaceae.

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1.5.1 Habit and leaf form

The Phyllanthaceae are nearly all trees, shrubs or herbs. A few are climbers or succulents, and one species, *Phyllanthus fluitans* is aquatic. Unlike many of the Euphorbiaceae, none have latex, and only a very few produce a resinous exudates. Any hairs, if present, are almost always simple. Rarely are they branched or scale-like. Thorns and other armament are rare.

Stipules are produced with each leaf, but in some, these fall before the leaf is fully mature. Leaves are present, except for a few species of *Phyllanthus* that have flattened, leaf like stems called cladodes that bear flowers along their edges. The leaves are compound in Bischofia, but otherwise simple and usually alternate. Rarely are they opposite, in fascicles, or in whorls around the stem. The leaf margin is almost always entire, rarely toothed. A petiole is nearly always present, often with a pulvinus at its base.

1.5.2 General anatomy

Plants with laticifers (commonly, articulated or non-articulated)

1.5.3 Stem anatomy

Cork cambium present; initially deep-seated (rarely); or superficial. Nodes tri-lacunar, or unilacunar. Primary vascular tissue bicollateral, or centrifugal. Cortical bundles present (occasionally); or absent. Medullary bundles present (occasionally); or absent. Internal phloem present (occasionally); or absent. Secondary thickening developing from a conventional cambial ring (mostly, even in lianes); or anomalous; from a single cambial ring. The secondary phloem stratified into hard (fibrous) and soft (parenchymatous) zones (very rarely. e.g. in *Bridelia*); or not stratified. 'Included' phloem present (occasionally, e.g. Dalechampia); or absent. Xylem with libriform fibres. Vessel end-walls scalariform, or simple (usually). Vessels with vestured pits (rarely); or without vestured pits. Wood parenchyma apotracheal, or paratracheal (or very sparse, or absent).

1.5.4 Reproductive type, pollination

Plants monoecious, or dioecious, or hermaphrodite. Pollination entomophilous.

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1.5.5 Inflorescences, floral, food and seed morphology

The inflorescences are usually in the axils of leaves, rarely below the leaves or at the ends of stems. In Uapaca, the flowers are in a pseudanthium, a tight bundle of flowers that resembles a single flower. Except for four species of Aporosa, the flowers are unisexual, the plants being either monoecious or dioecious. The flowers are actinomorphic in form. The sepals are three to eight in number, usually free from each other. Petals may be absent or present. If present, there are usually four to six, and their color is yellow to green, or rarely, pink or maroon. A nectary disk is often present. It may be in the form of a ring, or divided into segments. The stamens are three to ten in number, or rarely more, free or variously fused. The fruit is a schizocarp, drupe, or berry. In some, the schizocarp breaks up explosively. The ovary is superior. The number of locules in the ovary is highly variable, usually from two to five, but sometimes as many as fifteen. The placentation is apical, with a pair of ovules hanging by their funicles from the top of each locule. Often, only one of the ovules will develop into a seed. A single, massive obturator may cover the micropyles of both ovules, or each ovule may have its own thin obturator. The mega gametophyte is of the Polygonum type. The style is usually 2-lobed or bifid, sometimes entire, or rarely multifid.

1.5.6 Physiology and biochemistry

Cyanogenic or not cyanogenic. Cynogenic constituents tyrosine derived or phenylalanine derived or of Hegnauer's 'Group C'. Alkaloids present (commonly); or absent. Iridoids not detected. Proanthocyanidins present (rarely); or absent; when present, cyanidin and delphinidin (in one Phyllanthus species). Flavonols present, or absent; kaempferol, or kaempferol and quercetin. Ellagic acid present or absent. Arbutin absent. Saponins/sapogenins present (rarely); or absent. Aluminium accumulation demonstrated (but in relatively few genera); or not found. Sugars transported as sucrose or as sugar alcohols + oligosaccharides + sucrose (e.g. *Phyllanthus*).

1.6 Medicinal uses of Phyllanthaceae species in Bangladesh

Traditional use of some ethno medicinal plants of Phyllanthaceae species are described in Table 1.2 (Ahmed *et al.*, 2009).

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

Species	Plant part	Use
***Antidesma ghaesembilla* Gaertn.**	• Bark	Astringent and tonic, jaundice, fever Rheumatoid arthritis, etc.
	• Leaf	Wound healing, severe headache, skin disease, abdominal swelling.
***Antidesma acidum* Retz.**	• Leaf paste	Diabetes, insanity, lumps in throat, remitting fever, lymphoma.
***Antidesma roxburghii* Wall.**	• Whole plant	Hysteria, insanity, epilepsy, dysentery, facial paralysis, febrile convulsion, gout, post natal indigestion, remitting fever, stomach disorders.
***Antidesma bunius* Spreng.**	• Leaf	Snakebite, syphilitic catexia.
***Antidesma montanum* Spreng.**	• Leaf	Ulcer and lumber pain.
	• Root	Stomachache.
***Phyllanthus acidus* L. Skeels.**	• Fruit	Astringent, liver tonic, constipation, piles, biliousness.
	• Root & seed	Cathartic.
***Phyllanthus emblica* L.**	• Seed	Asthma, bronchitis and biliousness
	• Fruit	Laxative, diuretic in heart disease, liver complaint, piles, dysentery and diarrhea.
	• Flower	Cooling agent and as snuff in nasal hemorrhage.
***Phyllanthus maderaspatensis* L.**	• Leaf	Expectorant and diaphoretic.
	• Seed	Carminative, laxative, liver tonic and ophthalmic. Bronchitis, pain in ear.
***Phyllanthus niruri* L.**	• Whole plant	Diuretic in gonorrhea and other genitourinary disease.
	• Root	Jaundice.
	• Leaf, bark	Bruises, wounds, scabby infection and purgative.
***Phyllanthus reticulates* Poir.**	• Whole plant	Smallpox and syphilis.
	• Leaf juice	Dysentery.
	• Fruit	Astringent, inflammation and blood disease.

1.7 The plant genus *Bridelia* Willd.

Bridelia is a plant genus of the family Phyllanthaceae. This genus comprises approximately between 60 and 70 species and found throughout tropical and subtropical regions of the world, mainly in Africa, Australia and Asia. About 50 species are distributed in Tropical Africa, Madagascar, Yemen and in Asia ranging from India and South China throughout Indochina, Malaysia to North Australia and the Solomons and Vanuatu Islands. The species in Southeast Asia are usually a part of the primary and secondary forest vegetation either as large trees or as shrubs/smaller trees in the under storey. Some species are scrambling and one is reported to be a climber. Some different species seem to be restricted to a certain type of habitat, e.g. *Bridelia parvifolia* sand dunes, *Bridelia oligantha* dry savannahs. The species occur from the sea level up to 1800 m. (Ngueyem *et al.,* 2009)

The genus *Bridelia* was named in honor of Samuel Elisée Bridel-Brideri by the German botanist Carl Ludwig Willdenow. *Bridelia* species are used as food plants by the larvae of some *Lepidoptera* species including *Endoclita malabaricus*.

The genus is also known as:

1. *Candelabria* Hochst.
2. *Gentilia* A.Chev. & Beille.
3. *Pentameria* Klotzsch ex Baill.
4. *Tzellemtinia* Chiov.

1.7.1 Taxonomic hierarchy of the genus *Bridelia*

- **Domain:** Eukaryota
- **Kingdom:** Plantae
  - **Subkingdom:** Viridaeplantae
    - **Phylum:** Tracheophyta
      - **Division:** Magnoliophyta
    - **Class:** Magnoliopsida
      - **Subclass:** Dilleniidae
        - **Order:** Malpighiales
          - **Family:** Phyllanthaceae
            - **Tribe:** Bridelieae
              - **Genus:** *Bridelia*

1.7.2 *Bridelia* species available in Bangladesh

Some available species of *Bridelia* are listed in the table below:

Table 1.3: Some *Bridelia* species available in Bangladesh.

1. *B. atroviridis* Mull. Arg.	8. *B. grandis* Pierre.	15. *B. scleuroneuroide* Pax.
2. *B. balansae* Tutcher.	9. *B. ndellensis* Beille.	16. *B. scleuroneura* Mull. Arg
3. *B. brideliifolia* (Pax) Fedde.	10. *B. ovata* Decne.	17. *B. siamensis* Craib.
4. *B. cathartica* Bertol.	11. *B. pervilleana* Baill.	18. *B. stipularis* (L) Blume.
5. *B. crenulata* Roxb.	12. *B. retusa* (L) Spreng.	19. *B. tomentosa* Blume.
6. *B. ferruginea* Benth.	13. *B. ripicola* Leonard.	20. *B. verrucosa* Haines.
7. *B. glauca* f. *balansae* Blume.	14. *B. scandens* (Roxb). Willd.	

1.7.3 Medicinal uses

Bark, roots and leaves of at least ten *Bridelia* species are used in African and Asian traditional and local medicines for treating several ailments including sexual diseases (*Bridelia atroviridis*); bronchitis (*Bridelia balansae*); anemia (*Bridelia cathartica*); intestine disorders and painful joints (*Bridelia michranta*); dental caries (*Bridelia grandis*); fever, diabetes and diarrhoea (*Bridelia ndellensis*); rheumatism (*Bridelia retusa*); rheumatism, abdominal pain and arthritis (*Bridelia sclereoneura*); fever (*Bridelia tomentosa*). The best studied species *Bridelia ferruginea* is used for bladder troubles, diabetes, dysentery, rheumatism pain and for its antimicrobial activity (Ngueyem *et al.,* 2009).

1.7.4 Ethnomedicinal properties and phytoconstituents of some *Bridelia* species

Ethnomedicinal properties and phytoconstituents of some *Bridelia* species are listed below in Table 1.4.

Table 1.4: Ethnomedicinal properties and phytoconstituents of some *Bridelia* species.

Species and plant part used	Identified phytoconstituents and pharmacological activities	References
B. atroviridis Mull. Arg. Bark, leaves	Saponins, tannins, antimicrobial activity, cardiovascular activity	Abbiw, 1990; Agyare *et al.,* 2006; Corallo *et al.,* 1997 and Neuwinger, 2000.
B. balansae Tut. Leaves	Balansenate I (**9**); II (**10**); bridelone (**11**); bridelonine (**12**)	Tsai *et al.,* 2003.
B. brideliifolia Fed. Twig bark, root, leaves	-	Neuwinger, 2000.
B. cathartica Bert. Roots, leaves, stem bark	Anthocyanins, flavonoids, tannins, anti-anemia for its high amount of iron	Watt and Breyer Brandwijk 1962; Hedberg *et al.,* 1983; Chhabra *et al.,* 1990; Ouma *et al.,* 1997 and Neuwinger 2000.
B. crenulata Roxb. Bark	Luteoforol (3,4,4,5,7-pentahydroxyl droxyflavon) (**13**)	Ramesh *et al.,* (2001a, b).
B. ferruginea Benth. Fruit, leaves, stem-bark, roots	Tannins, gallocatechin-(4-O-7) epi-gallocatechin (**1**); flavonoids and biflavonoids, caffeoyl esters, anti-complement, anti-oxidant, anti-viral, diabetes, anti-inflammatory, anti-microbial activity, effects on rat bladder smooth muscle, quercetin (**2**); quercetin-3-neohesperidoside (**3**); rutin (**4**); myricetin-3-glucoside (**5**); myricetin-3-rhamnosi- de (**6**); 5-demethoxypelta-tin-5-O-d-glucopyra-noside (**7**); peltatin-5-O-d-glucopy-ranoside (**8**)	Cimanga *et al.,* 1999, 2001; Tona *et al.,* 1998; Adebayo and Ishola 2009; Abubakar *et al.,* 2007; Pieters and Vlietinck 2005; Talla *et al.,* 2002; Ampofo 1979; Iwu 1980, 1983; Akinpelu *et al.,* 2000; Olajide *et al.,* 1999, 2003; Pedersen *et al.,* 2009; Magassouba *et al.,* 2007; Onoruvwe *et al.,* 2001; Akinpelu *et al.,* 2000; Olajide & Makinde Modupe 2000; Rashid *et al.,* 2000; Neuwinger 2000 and Oliver-Bever 1986.

Table-1.4: Ethnomedicinal properties and phytoconstituents of some *Bridelia* species. cont'd

Species and plant part used	Identified phytoconstituents and pharmacological activities	References
B. glauca f. *balansae* Blume. Leaves	Bridelionoside, bridelioside, (*7R*, *8S*)-5-methoxydihydrodehydrodico niferyl alcohol-4-O-gluco pyranosi deglochi- dioboside	Sueyoshi *et al.,* 2006, 2007.
B. grandis Pierre Bark, leaves	Antimicrobial activity against oral streptococci, anti-trypanosomal and anti plasmodial activity	Ngueyem *et al.,* 2008; Atindehou *et al.,* 2004 and Neuwinger 2000.
B. micrantha Bark, leaves, root	Taraxerol (**14**); gallic and ellagic acid, friedelin (**15**); delphinidin (**16**); methyl salicylate, anti-diarrhoeal activity, anti-plasmodial activity, weak cytotoxic activity	Watt and Breyer-Brand wijk 1962; Lin *et al.,* 2002; Steenkamp (2003); Ajaiyeoba *et al.,* 2006; Gradé *et al.,* 2009; Gbolade 2009; Abo *et al.,* 2008; Ssegawa and Kasenene 2007; Clarkson *et al.,* 2004; Hamill *et al.,* 2003 and Neuwinger, 2000.
B. mollis Hutch. Root	-	Neuwinger, 2000.
B. moonii Thwaites. Bark	Glochidone (**17**)	Carpenter *et al.,* 1980.
B. monoica Merr. Leaves, root, stem	Stigmasterol, sitosterol, friedelan-3-ol (**18**); glutin-5-en-3-ol, friedelin (**15**)	Hui and Fung, 1968 and Roosita *et al.,* 2008.
B. ndellensis Beille. Stem bark	Hypoglycemic effect	Sokeng *et al.,* 2005 and Neuwinger, 2000.
B. ovata Decne. Leaves, stem bark	24-methyl-lanosta-9(11);25-dien-3-one (**19**); 24,24-di methyl-lanosta-9-(11)-25-dien-3-one (**20**); campeste-rol, stigmasterol, sitosterol, friedelin (**15**); friedelan-3-ol (**18**); *trans*tria-contyl-4-hydroxy-3-methoxycinna-mate	Boonyaratavej *et al.,* 1992.
B. pervilleana Baill. Leaves	-	Neuwinger, 2000.

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Table-1.4: Ethnomedicinal properties and phytoconstituents of some *Bridelia* species. cont'd

Species and plant part used	Chemical compounds identified & pharmacological activities	References
B. retusa (L) Spreng Leaves, root, bark	Isoflavone, 4-[(*E*)-6-methyl-4oxohept-2-en-2-yl)] benzoic acid (**21**); 4-[(*E*)-6-methyl-4-oxohept-2,5-dien-2-yl)] benzoic acid, 4-[(*R*)-6-methyl-4-oxohept-5-en-2-yl)] benzoic acid (**22**); 4-[(*R*)-6-methyl-4-oxoheptan-2-yl)] benzoic acid (**23**); (−)-isochaminic acid (**24**); 5-allyll 1,2,3-trimetoxy-benzene (elemicin) (**25**); (+)-sesamin (**26**); 4-isopropyl benzoic acid, cyanogenic glycol-sides, triterpenes, ketone and tannins	Jayasinghe *et al.,* 2003; Jain *et al.,* 2004; Ayyanar & Ignacimuthu 2005 and Kshirsagar & Singh, 2001.
B. rippicola Lenard Leaves, bark, plant powder	-	Neuwinger, 2000.
B. scleuroneura Mull. Arg. Bark, leaves, root, twig bark	Antinociceptive and anti-inflamma-tory activity	Dimo *et al.,* 2006; Tabuti *et al.,* 2003 and Gradé *et al.,* 2009.
B.scleuroneuroides Pax. Root	-	Watt and Breyer-Brandwijk, 1962.
B. siamensis Craib. Leaves	(−)-Ovatolide (**27**)	Delgado and Clardy, 1993.
B. stipularis Blume Leaves, roots	Bridelyl alcohol, phlobatannin, taraxenone	Sengupta and Ghosh, 1963 and Desai *et al.,* 1976.
B. tomentosa Blume Leaves, root, bark	24-methyl-lanosta-9(11)-25-dien-3-one (**19**); 24,24-dimethyl-lanosta-9(11)-25-dien-3-one (**20**)	Boonyaratavej, 1990.

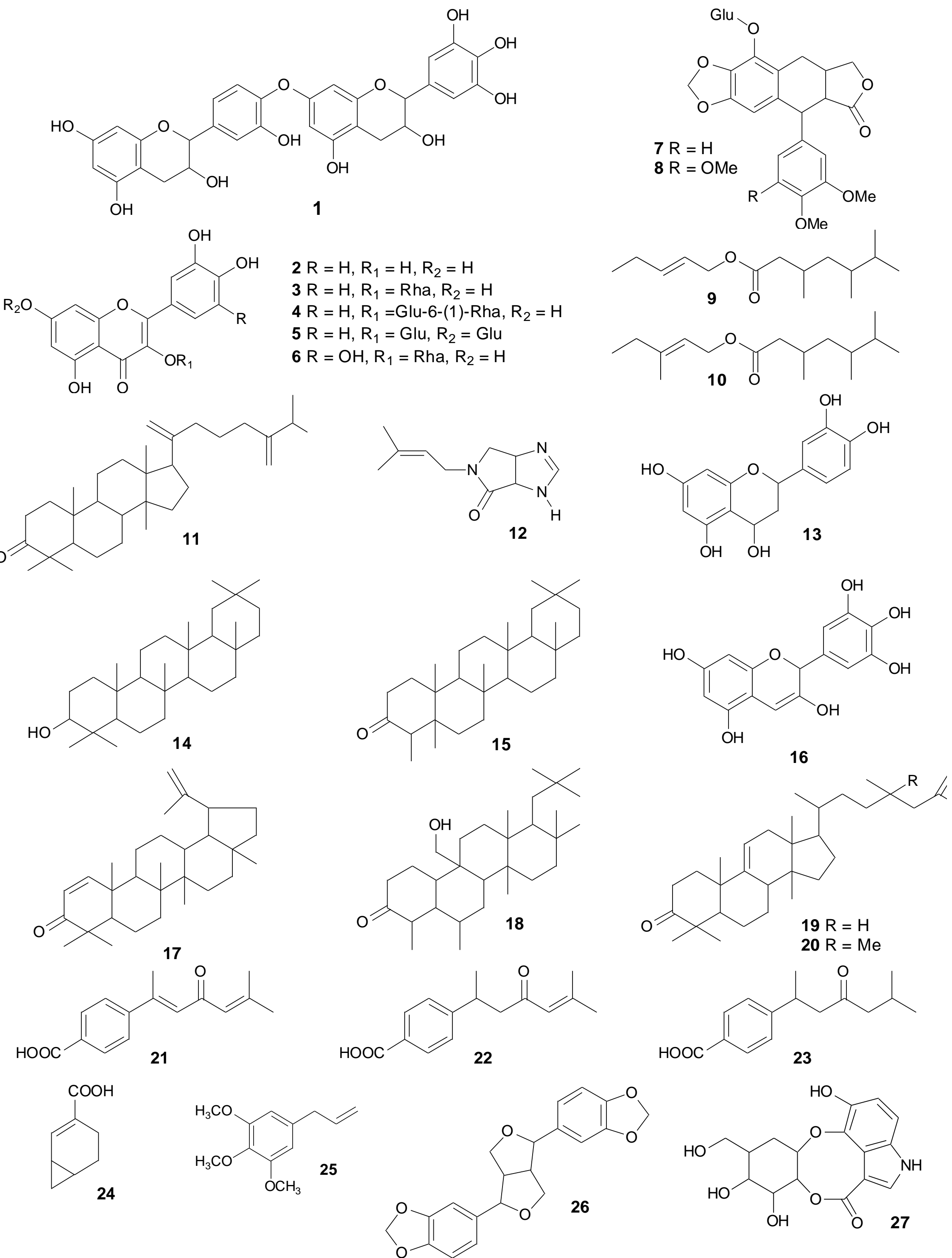

Figure 1.2: Structures of some compounds found in the genus *Bridelia*.

1.8 Description of the investigated plants of the genus *Bridelia*

1.8.1 Taxonomic hierarchy of *Bridelia verrucosa* Haines

- **Domain:** Eukaryota
- **Kingdom:** Plantae
  - **Subkingdom:** Viridaeplantae
    - **Phylum:** Tracheophyta
      - **Division:** Magnoliophyta
    - **Class:** Magnoliopsida
      - **Subclass:** Dilleniidae
    - **Order:** Malpighiales
      - **Family:** Phyllanthaceae
        - **Tribe:** Bridelieae
          - **Genus:** *Bridelia*
            - **Species***:* *B. verrucosa*

1.8.2 Description of the investigated plant *B. verrucosa* (Kirtikar and Basu, 1994)

Taxonomic name	:	*Bridelia verrucosa* Haines		
Synonyms	:	*Bridelia montana* Willd		
		Bridelia sikkimensis Gerhmann		
Common names	:	Ghiyai, Gondii, Gayo		
Vernacular names	:	**Assam**	:	Kaiso
		Bombay	:	Asano
		Nepal	:	Geio
Habit and habitat	:	Large shrub or small 3 m strangling tree without thorns, twigs are glabrous and verrucose; occasional in the forest.		
Leaf	:	Leaf-blade elliptic-obovate or broadly elliptic.		
Perulae	:	Pubescent		
Petioles	:	5 mm long		

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Stipules	:	Lanceolate, 2 mm long, acute, glabrous, not persistent.
Flowers	:	Arranged only in fascicles, which are commonly unisexual, although sometimes bisexual; flowers shortly pedicellate or sessile.
Male flowers	:	Pedicels 1 mm long, glabrous; sepals oblong-lanceolate, 2 mm long, acute, glabrous; petals spathulate-flabelliform, 1 mm long; disc pentagonal, 1 mm diameter; stamina column 1 mm high; anthers 0.5 mm long; pistillode conical, bifid, 0.5 mm long.
Female flowers	:	Sessile; sepals triangular-ovate, 1.5 mm long, sparingly pubescent outside at the base, otherwise glabrous; petals elliptic-oblong, 1 mm long; outer disc roundly pentagonal, inner disc completely enveloping the ovary, only the styles exserted; ovary 1 mm diam., glabrous; styles 2, bifid, 0.5 mm long.
Fruits	:	Ovoid, 5-6 mm long, 4 mm diam. when dried, black; pyrene 1, furrowed, 4 x 3 mm, black.
Life span	:	Perennial.
Flowering time	:	September to November.
Fruiting time	:	October to December.
Distribution	:	Sub-Himalayan Tract, Khasia hills, Northern India from Punjab & Himachal Pradesh to Bihar & Bengal, Nepal, Sikkim, Orissa and Upper Burma.
Distribution in Bangladesh	:	Chittagong hill tracts, especially in Khagrachhori and Panchouri district.
Medicinal uses	:	Anthelmintic and astringent.

1

2

Figure 1.3: *Bridelia verrucosa* Haines flower (1) and leaves with fruits (2).

1.8.3 Taxonomic hierarchy of *Bridelia stipularis* (L) Blume

- **Domain:** Eukaryota
- **Kingdom:** Plantae
  - **Subkingdom:** Viridaeplantae
    - **Phylum:** Tracheophyta
      - **Division:** Magnoliophyta
    - **Class:** Magnoliopsida
      - **Subclass:** Dilleniidae
    - **Order:** Malpighiales
      - **Family:** Phyllanthaceae
        - **Tribe:** Bridelieae
          - **Genus:** *Bridelia*
            - **Species:** *B. stipularis*

1.8.4 Description of the investigated plant *B. stipularis* (Nasir, 2006; Prain, 1963)

Taxonomic name	:	*Bridelia stipularis* (L) Blume.
Synonyms	:	*Bridelia scandens* (Roxb.) Willd. *Bridelia dasycalyx* Kurz. *Bridelia zollingeri* Miq. *Clutia stipularis* Linn.
Common names	:	Harinhara
Vernacular names	:	**Bengali** : Patkhowi **Marma** : Moo gach, So mui **Tripura** : Khaipak
Habit and habitat	:	Large evergreen scandent shrub attaining 1.5 feet to 2 feet and sometimes more or less climbing shrub; grows in shady, moist forest floors.
Leaf	:	Leaves 5-20 cm, subcoriaceous, elliptic-obovate or orbicular-oblong.
Stipules	:	Stipules ovate-triangular, 3-9 × 2-4 mm.
Flowers	:	Small, in small axillary clusters or long spikes, often subtended by long stipular bracts. Usually 2-6-flowered, sometimes grouped into many-flowered spikes or on terminal small-leaved branches.
Male flowers	:	Male flowers 0.6-1 cm in diameter; pedicels very short, up to 1 mm; receptacle cup-shaped.
Female flowers	:	Female flowers up to 12 mm in diam.; pedicels short and stout; receptacle nearly funnel-shaped.
Fruits	:	Drupes ovoid, 0.7-1.3 × 0.6-1.1 cm, bilocular. Seeds semiovoid, 6-8 × 3-6 mm, yellow to brown, smooth, adaxial surface flattened or slightly concave.
Life span	:	Perennial.
Flowering time	:	November to March.

Fruiting time	**:**	November to March.
Distribution	**:**	Nepal, India, Sri-Lanka, Southern China, Indo-China, Malaysia, Indonesia and the Philippines.
Distribution in Bangladesh	**:**	Forests of Chittagong, Chittagong Hill Tracts, Sylhet, Gazipur, Cox's Bazar, Sayadpur (Rangpur).
Medicinal use	**:**	Amoebic dysentery, chest pain, constipation, astringent diarrhea, leucoderma and strangury (Nasir, 2006). Decoction of bark is used for cough, fever and asthma. Also hypotensive and hypoglycaemic on animals. Leaves are used for jaundice (Krishnan, 1992). The roots are used as medicine for reducing inflammation. Fruits are used to induce vomiting and as an antitoxic.

1

2

Figure 1.4: *Bridelia stipularis* Blume leaves with fruits (1) and flowers (2).

1.8.5 Taxonomic hierarchy of *Bridelia tomentosa*

- **Domain:** Eukaryota
- **Kingdom:** Plantae
    - **Subkingdom:** Viridaeplantae
        - **Phylum:** Tracheophyta
            - **Division:** Magnoliophyta
        - **Class:** Magnoliopsida
            - **Subclass:** Dilleniidae
        - **Order:** Malpighiales
            - **Family:** Phyllanthaceae
                - **Tribe:** Bridelieae
                    - **Genus:** *Bridelia*
                        - **Species:** *B. tomentosa*

1.8.6 Description of *B. tomentosa* (Cowan *et al.*, 1929; Krishnan, 1992)

Taxonomic name	:	*Bridelia tomentosa* (Blume)
Synonyms	:	*Bridelia lanceaefolia* Roxb. *Bridelia monoica* Lour. *Bridelia phyllanthoides* W. Fitzg
Common names	:	Pop-gun seeds
Local names	:	Khy
Vernacular names	:	**Bengali** : Mindri, Sirai
Habit and habitat	:	Shrubs or small evergreen trees, usually 2 - 5 m tall, rarely up to 12 m tall; bark dark-grey; branches slender and elongate, pubescent when young, glabrous with age; latex absent. Grows in open forest, vine thickets, monsoon forest and the drier types of rain forest.
Leaf	:	Stipules linear-lanceolate, ca. 7 mm long, usually caducous, pubescent; petioles 3 - 5 mm long, pubescent.

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Flowers	**:**	Numerous flowers in each fascicle. Calyx lobes about 1.5-2 x 0.5-0.9 mm. Petals small and inconspicuous, about 0.5-0.6 x 0.4-0.5 mm.
Fruits	**:**	Fruits globular, about 6-7 x 4-6 mm, calyx persistent at the base of the fruit.
Seeds	**:**	Brown-red, oblong-ovate, 3.5 - 4 mm long and 3 mm wide.
Seedlings	**:**	Cotyledons obreniform, wider than long about 7 x 10 mm, apex excavated, base truncate, petiole hairy.
Life span	**:**	Perennial.
Flowering time	**:**	Almost throughout the year.
Fruiting time	**:**	Almost throughout the year.
Distribution	**:**	Widespread in shrub lands and wood lands; Malay islands, South China, Taiwan, Philippines, south east Asia, Australia.
Distribution in Bangladesh	**:**	Srimangal, Sylhet, Chittagong District and Singhara forest, Dinajpur.
Medicinal uses	**:**	Leaves used as herbal medicine for traumatic injury; roots used in epidemic influenza, neurasthenia; barks containing 8% of tannin and used as astringent and colic.

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Figure 1.5: *Bridelia tomentosa* Blume leaves with fruits (1) and leaves (2).

1.9 The plant family: Fabaceae or Leguminosae

Systematic position

Class: Dicotyledonae

Sub-class: Polypetalae

Series: Calyciflorae

Order: Rosales

Family: Fabaceae (From ITIS report)

The Fabaceae or Leguminosae, commonly known as the legume, pea, or bean family, is a large and economically important family of flowering plants. The group is the third largest land plant family with 730 genera and over 19,400 species, behind only the Orchidaceae (orchids) and Asteraceae (daisies, sunflowers) and second only to Poaceae (grasses) in terms of agricultural and economic importance.. It is surpassed in size only by the orchid family (Orchidaceae) with about 20,000 species and the sunflower family (Asteraceae) with about 24,000 species. The largest genera are *Astragalus* (over 2,400 species); *Acacia* (over 950 species); *Indigofera* (around 700 species); *Crotalaria* (around 700 species); and *Mimosa* (around 500 species).

Legumes includes a large number of domesticated species harvested as crops for human and animal consumption as well as for oils, fiber, fuel, fertilizers, timber, medicinals, chemicals, and horticultural varieties (Lewis *et al.,* 2005). In addition, the family includes several species studied as genetic and genomic model systems (e.g., pea, *Pisum sativum*, barrel medic, *Medicago truncatula* and trefoil, *Lotus corniculatus*).

Legumes vary in habit from annual and perennial herbs to shrubs, trees, vines and even a few aquatics. Ranging in size from some of the smallest plants of deserts and arctic or alpine regions to the tallest of rain forest trees, legumes are a conspicuous, and often dominant, component of most of the vegetation types distributed throughout temperate and tropical regions of the world (Rundel, 1989). Legumes are particularly diverse in tropical forests and temperate shrub lands with a seasonally dry or arid climate. This preference for semi-arid to arid habitats is related to a nitrogen demanding metabolism. While many species have the ability to colonize barren and marginal lands because of their capacity to "fix" atmospheric nitrogen via a symbiotic association with root-nodulating bacteria, this is just one of several ways in which legumes obtain high levels of nitrogen to meet the demands of their metabolism (McKey, 1994 and Sprent, 2001).

Over the past 30 years, the study of legume classification and biology has benefited from major advances in our understanding of the morphology, evolution and systematics and ecology of the family (Polhill, 1994; Lewis *et al.,* 2005). Much of this knowledge is recorded in the venerable Advances in Legume Systematics series, first published in 1981 by the Royal Botanic Gardens at Kew, United Kingdom. Coming 25 years after publication of the first volumes of Advances in Legume Systematics (Polhill and Raven, 1981, parts 1 & 2); the most recent authoritative (and only illustrated) guide to the family, Legumes of the World, by Lewis, Schrire, Mackinder, and Lock (2005, Royal Botanic Garden, Kew) continues that tradition.

A number of important agricultural plants, including: *Glycine max* (soybean); *Pisum sativum* (pea); *Cicer arietinum* (chickpeas); *Medicago sativa* (alfalfa); *Arachis hypogaea* (peanut) and *Glycyrrhiza glabra* (licorice) are among the best known members of Fabaceae. A number of species are also weedy pests in different parts of the world,

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including: *Cytisus scoparius* (broom); *Ulex europaeus* (gorse); *Pueraria lobata* (kudzu) and a number of *Lupinus* species.

1.10 Taxonomy of the plant family Fabaceae

Taxonomically, Fabaceae has been traditionally divided into three subfamilies, the Caesalpinioideae, Mimosoideae, and Papilionoideae (although sometimes these have been ranked as separate families, as in Caesalpiniaceae, Mimosaceae, and Papilionaceae); and considered most closely related to the Connaraceae and Sapindaceae on the basis of anatomy, morphology, and biogeographic distributions (reviewed in Polhill and Raven, 1981). The recognition of three subfamilies is based on characteristics particularly of the flower, including size, symmetry, aestivation of petals, sepals (united or free); stamen number and heteromorphy, pollen (single or polyads); but also presence of a pleurogram, embryo radicle shape, leaf complexity, and presence of root nodules (reviewed in Lewis *et al.,* 2005). Differences in these characteristics led to the view that the Mimosoideae and Papilionoideae are unique and distinct lineages in the family which arose independently within a paraphyletic "basal" caesalpinioid assemblage (Polhill, 1994).

As Lewis *et al.,* 2005 pointed out, while there has been some disagreement as to whether Fabaceae should be treated as one family (composed of three subfamilies) or three, there is a growing body of evidence from morphology and molecules to support the legumes being one monophyletic family. This view has been reinforced not only by the degree of inter relatedness of taxonomic groups within the legumes compared to that between legumes and its relatives, but also by recent molecular phylogenetic studies (Doyle *et al.,* 2000; Kajita *et al.,* 2001; Wojciechowski, 2003; Wojciechowski *et al.,* 2004) showing strong support for a monophyletic family that is more closely related to Polygalaceae, Surianaceae, and Quillajaceae, which together form the order Fabales (Angiosperm Phylogeny Group, 2003).

So, taxonomically the three recognized subfamilies of Fabaceae are

1. **Papilionoideae**
2. **Caesalpinioideae**
3. **Mimosoideae**

The recognition of three subfamilies is based on characteristics particularly of the flower, including size, symmetry, aestivation of petals, sepals (united or free); stamen number and heteromorphy, pollen (single or polyads); but also presence of a pleurogram, embryo radicle shape, leaf complexity, and presence of root nodules (Lewis *et al.,* 2005).

1.10.1 Papilionoideae:

Members of the subfamily Papilionoideae have true papilionaceous flowers in which the upper petal is outside the lateral petals in the bud. This subfamily includes 476 genera and 14,000 species, most of the members of the Fabaceae family with typical pea-like flowers are *Dalea* (smoke tree); *Lupinus* (lupine); *Lathyrus* (sweet pea); *Erythrina* (coral tree); *Robinia* (black locust) and *Astragalus* (locoweed) etc. One acceptable alternative name for the subfamily Papilionoideae is Faboideae.

1.10.2 Caesalpinioideae:

Members of the subfamily Caesalpinioideae have flowers that are bilateral, typically with five distinct petals, upper petal (banner) enveloped in the bud by the lateral wings. This subfamily includes 153 genera *Cassia* (senna); *Cercis* (redbud); *Bauhinia* (orchid tree); *Cercidium* (palo verde); *Parkinsonia* (Jerusalem thorn); *Caesalpinia* (Brazil wood); *Haematoxylum* (logwood); *Ceratonia* (carob); *Tamarindus* (tamarind) and *Delonix* (royal poinciana) etc.

1.10.3 Mimosoideae:

Members of the subfamily Mimosoideae have flowers with radial symmetry, small, inconspicuous corollas and numerous, showy stamens. The flowers are typically in many flowered heads or spikes. This subfamily includes about 80 genera of which *Acacia* (wattle); *Albizia* (silk tree); *Samanea* (monkey pod); *Prosopis* (mesquite) and *Calliandra* (powder puff) etc are well known.

Table 1.5: A short synoptic list of genera distributed in various sub families under Fabaceae family (Free Dictionary by Farlex).

Sub family	Some well known genera of the sub family	Image
Papilionoideae	*Stizolobium*, *Ononis*, *Myroxylon*, *Onobrychis*, *Ormosia*, *Oxytropis*, *Pachyrhizus*, *Dalea*, *Parochetus*, *Phaseolus*, *Pickeringia*, *Piscidia*, *Dalbergia*, *Glycine*, *Desmodium*, *Desmanthus*, *Crotalaria*, *Daviesia*, *Dipogon*, *Coronilla*, *Genista*, *Castanospermum*, etc.	Figure 1.6: *Castanospermum austral*
Caesalpinioideae	*Acrocarpus*, *Bauhinia*, *Senna*, *Petteria*, *Parkinsonia*, *Gledtissia*, *Delonix*, *Chamaecrista*, *Ceretonia*, *Cassia*, etc.	Figure 1.7: *Bauhinia galpinii*
Mimosoideae	*Mimosa*, *Acacia*, *Adinathera*, *Albizia*, *Anadenanthera*, *Albizzia*, *Leukaena*, *Lysiloma*, *Calliandra*, *Enterolobium*, *Pithecolibium*, etc.	Figure 1.8: *Albizia distachya*

1.11 Botanical features of Fabaceae

1.11.1 Habit

The plants are trees, shrubs, herbs or lianas; resinous, or not resinous. The family includes herbs, shrubs, trees and vines distributed throughout the world, especially the tropical rain forest and even a few aquatics.

Ranging in size from some of the smallest plants of deserts and arctic/alpine regions to the tallest of rain forest trees, legumes are a conspicuous, and often dominant, component of most of the vegetation types distributed throughout temperate and tropical regions of the world. Legumes are particularly diverse in tropical forests and temperate shrub lands with a seasonally dry or arid climate. This preference for semi-arid to arid habitats is related to a nitrogen demanding metabolism. While many species have the ability to colonize barren and marginal lands because of their capacity to "fix" atmospheric nitrogen via a symbiotic association with root-nodulating bacteria, this is just one of several ways in which legumes obtain high levels of nitrogen to meet the demands of their metabolism (Lewis *et al.,* 2005).

1.11.2 Leaf form

Morphologically, Fabaceae is characterized by leaves simple to compound (pinnate, rarely palmate, or bipinnate); unifoliate, trifoliate (*Medicago*, *Trifolium*); sometimes phyllodic (many species of *Acacia*); or reduced to a tendril (as in *Lathyrus*); spirally arranged, with stipules present that are sometimes large and leaf-like (*Pisum*) or developed into spines (*Prosopis*, *Robinia*).

1.11.3 Flowers

Flowers are usually regular or irregular (i.e., actinomorphic to zygomorphic in symmetry, respectively); bisexual, with a single superior carpel (hypogynous to perigynous); pentamerous, arranged singly or in racemes, spikes, or heads.

1.11.4 Fruits

The principal unifying feature of this family is the fruit, the legume (Polhill, 1994). The fruit is technically called a legume or pod. It is composed of a single seed-bearing carpel that splits open along two seams. Legume fruits come in an enormous variety of shapes and sizes, including indehiscent pods that do not split open. With a few exceptions, legumes are typically one-chambered pods (one locule); with parietal placentation along the adaxial suture, ovules two to many, in two alternating rows on a single placenta, typically dry and dehiscent along one or both sutures (legume); occasionally constricted into one-seeded sections (loments) or indehiscent (samara, drupe).

1.11.5 Fossil record

The Fabaceae contains over 19,000 extant species widely distributed throughout the world in many ecological settings, from deserts of high latitudes to seasonally dry and wet tropical forests of equatorial regions (Lewis *et al.,* 2005). Legumes appear to be diversified during the Early Tertiary (Herendeen *et al.,* 1992) to become a ubiquitous feature of modern terrestrial biotas, similar to the timing of diversification of several other modern families of angiosperms (e.g., Fagaceae; Manos and Standford, 2001). Although legumes are now highly diverse in tropical to subtropical Africa and South America, the fossil data alone argue against a moist, warm tropical origin.

The fossil record of the Fabaceae is abundant and diverse, particularly in the Tertiary, with fossil flowers, fruits, leaflets, wood, and pollen known from numerous localities; some examples are shown in the figures below (Crepet and Taylor, 1985, 1986; Crepet and Herendeen, 1992; Herendeen, 1992; Herendeen *et al.,* 1992). Although there are several reports of earlier fossils, Sindora-like pollen (subf. Caesalpinioideae) from the Maastrichtian of Canada, Columbia, and Siberia (Raven and Polhill, 1981) and woods similar to Cassia s. l. and Mimosoideae from the same time period (e.g., Müller-Stoll and Mädel, 1967); they cannot be assigned unequivocally to legumes. The first definitive legumes appear during the Late Paleocene (ca. 56 Mya; Herendeen, 2001; Herendeen and Wing, 2001; Wing *et al.,* 2004). Representatives of all three traditionally recognized subfamilies, the caesalpinioids, mimosoids, and papilionoids (Polhill *et al.,* 1981); as well as other taxonomically large clades within these subfamilies (e.g., “genistoids”); are recorded from the fossil record soon afterward, beginning around 50 to 55 Mya (Herendeen *et al.,* 1992). Indeed, the occurrence of diverse assemblages of taxa representing all three subfamilies at multiple localities dating from the middle to upper Eocene, especially the Mississippi Embayment of southeastern North America, suggests that most major lineages of woody legumes (except for the tribe Cercideae) were present and extensive diversification had taken place by this time (Herendeen *et al.,* 1992).

Fig 1.9: *Caesalpinia claibornensis* (Caesalpinioideae) fossil legume (fruit) described from the Middle Eocene (40-50 Mya) Claiborne Formation in western Tennessee.

Left: Holotype (IU 15826-5853) showing outlines of seed chambers. Right: Single-seeded fruit (IU 15826-5848) (Herendeen & Dilcher, 1991).

Fig1.10: *Acacia eocaribbeanensis* (Mimosoideae). Specimen showing numerous stamens with long filaments (USNM 458372, holotype) (Herendeen, Dilcher and Crepet, 1992).

Fig 1.11: *Cladrastis* subgenus *Platycarpa* (Papilionoideae) fossil fruit (Herendeen and Dilcher, 1990).

Fig 1.12: *Diplotropis claibornensis* fossil fruit (IU 15826-7235) from the Middle Eocene Claiborne Formation of the southeastern North America (Herendeen and Dilcher, 1990).

Attempts to estimate the age of legumes and diversification in the family, based on molecular sequence data, have been published in recent years. Wikström *et al.,* 2001 used a nonmolecular clock based analysis of the three gene data set (plastid atpB & rbcL, and nuclear 18S rDNA genes; Soltis *et al.,* 2000) with a minimum age of 84 Ma for the split between Fagales and Cucurbitales as an internal calibration point, and estimated an age for Fabaceae of 74-79 Ma. A comprehensive analysis of rates of molecular evolution and estimated ages for crown groups within the legume family has been presented by Lavin *et al.,* 2005. In this study, Tertiary macrofossils that showed distinctive combinations of apomorphic characters or features were used to constrain the minimum age of 12 specific internal nodes to estimate ages of a number of the clades identified in recent family-wide phylogenetic analyses of plastid matK (Wojciechowski *et al.,* 2004) and rbcL (Kajita *et al.,* 2001) gene sequence data.

Their findings indicate the age of the legume crown clade differs by only 1.0 to 2.5 Ma from the age of the stem clade and the oldest caesalpinioid, mimosoid, and papilionoid crown clades show approximately the same age range of 40 to 59 Ma, findings consistent with a rapid diversification of the family soon after its origin during the Late Paleocene. Remarkably, three large clades that include papilionoids traditionally considered derived (Polhill *et al.,* 1981; Polhill, 1994); the "dalbergioids" (Lavin *et al.,* 2001), "Hologalegina" (Wojciechowski *et al.,* 2000) and "mirbelioids" (Crisp *et al.,* 2000), all

have ages estimates in the 50-Ma time frame or older. One of these, Hologalegina, contains many of the well-known temperate, herbaceous species of legumes grown as food and forage crops (e.g., alfalfa, clovers, peas, and lentils).

1.11.6 Physiology and biochemistry

Nitrogen-fixing root nodules present (seemingly the norm in Papilionoideae and Mimosoideae); or absent (seemingly, from many Caesalpinioideae). Cyanogenic (rarely); or not cyanogenic (mostly). Cynogenic constituents tyrosine-derived, or phenylalanine-derived, or of Hegnauer's 'Group C', or leucine-derived. Alkaloids present (commonly); or absent. Iridoids not detected. Proanthocyanidins present, or absent; when present, cyanidin, or delphinidin, or cyanidin and delphinidin. Flavonols present (mostly); or absent; kaempferol and quercetin, or quercetin and myricetin, or kaempferol, quercetin, and myricetin. Ellagic acid consistently absent (from 54 species and 41 genera, representing all three subfamilies). Arbutin present, or absent. Aluminium accumulation not found. Sugars transported as sucrose (in numerous species and genera from all three subfamilies). C_3 physiology recorded directly in *Acacia*, *Alysicarpus*, *Amorpha*, *Arachis*, *Astragalus*, *Caragana*, *Cassia*, *Cercidium*, *Crotalaria*, *Dalea*, *Dolichos*, *Genista*, *Gleditsea*, *Glycine*, *Hoffmanseggia*, *Indigofera*, *Lespedeza*, *Lotus*, *Lupinus*, *Medicago*, *Mimosa*, *Olneya*, *Phaseolus*, *Pisum*, *Prosopis*, *Pueraria*, *Robinia*, *Sesbania*, *Spartium*, *Stylosanthes*, *Tephrosia*, *Trifolium*, *Vicia* and *Vigna*. Anatomy non-C_4 type (recorded from numerous genera representing all three subfamilies).

1.11.7 Agricultural and economic importance of legumes

Legumes have demonstrated agricultural importance for thousands of years, beginning with the domestication of lentils (*Lens esculenta*) in Iran dating to 9,500 to 8,000 years ago, their use as a food source during the prehistory of North and South America (beans, more than 3,000 years ago); and their use by the Roman Empire as a food source and for soil improvement (Graham and Vance, 2003). Today legumes are an increasingly invaluable food source not just for humans, accounting for 27% of the world's primary crop production, but also for farm animals (Graham and Vance, 2003). Legumes were grown on more than 13% of the total arable land under cultivation in the world in 2004 (Gepts *et al.,* 2005). Grain legumes alone contribute 33% of the dietary protein nitrogen

needs of humans, while soybeans (*Glycine max*) and peanut (*Arachis hypogeae*) provide more than 35% of the world's processed vegetable oil and a rich source of dietary protein for the poultry and pork industries (Graham and Vance, 2003).

While they produce nitrogen-containing protein in abundance, legumes are deficient in sulfur containing amino acids and other nutrients needed by people and animals. For this reason, legumes and cereal crops are often raised together to account for the amino acids and other elements they are each deficient in (Gepts *et al.,* 2005). The primary dietary legumes grown, such as bean (*Phaseolus vulgaris*); pea (*Pisum sativum*); chickpea (*Cicer arietinum*); broad bean (*Vicia faba*); pigeon pea (*Cajanus cajan*); cowpea (*Vigna unguiculata*); and lentils (Graham and Vance, 2003); include representatives of each of the four clades within papilionoids, the genistoids, dalbergioids, hologalegina, and phaseoloid/millettioids. Many legumes form root nodules to fix atmospheric nitrogen in a symbiotic relationship with the soil bacteria 'rhizobia'.

Legumes are extremely diverse in their abilities to nodulate, not all species can and there is a wide variety of nodules that form legumes, depending on the species in symbiosis. In addition to their uses as food, legumes are still used as tools in agriculture and forestry as the Romans did. The plants themselves or plant products like leaves and pods can be tilled into the soil as a nitrogen source or legume crops can be rotated with others for soil improvement. These techniques save farmers billions of dollars in the cost of nitrogen fertilizers (Graham and Vance, 2003). Industrially, legumes have many uses in making biodegradable plastics, oils, dyes, and biodiesel fuel.

Legumes are used traditionally in folk medicines, but also demonstrate importance in modern medicine. Isoflavones commonly found in legumes are thought to reduce the risk of cancer and lower cholesterol and soybean phytoestrogens are being studied for use in postmenopausal hormone replacement therapy (Graham and Vance, 2003). Legumes also produce a hypoglycemic effect when eaten, making them a recommended food for diabetics (Gepts *et al.,* 2005).

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

Medicinal uses of some plants of Fabaceous species growing in Bangladesh are described in Table 1.6.

Table 1.6: Medicinal uses of some Fabaceous species in Bangladesh.

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

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1.13 The plant genus *Erythrina* L.

Erythrina is a genus of flowering plants in the pea family, Fabaceae. The generic name is derived from the Greek word (*erythros*); meaning "red," referring to the flower color of certain species (Gledhill, D., 2008). It contains about 130 species, which are distributed in tropical and subtropical regions worldwide. Unlike many other genera of forage tree legumes, *Erythrina* is pantropical, consisting of some 112 species, 70 neotropical, 31 African and 12 Asian. Only one species, *E. fusca*, occurs in both the New and Old Worlds. They are trees, growing up to 30 m (98 ft) in height.

Particularly in horticulture, the name coral tree is used as a collective term for these plants. Flame trees is another vernacular name, but may refer to a number of unrelated plants as well. Many species of *Erythrina* have bright red flowers, and this may be the origin of the common name. However, the growth of the branches can resemble the shape of sea coral. Not all species of *Erythrina* have bright red flowers; the Wiliwili (*E. sandwicensis*) has extraordinary variation in its flower colour, with orange, yellow, salmon, green and white all being found within natural populations. This striking colour polymorphism is likely unique in the genus. Other popular names, usually local and particular to distinct species, liken the flowers' red hues to those of a male chicken's wattles, and/or the flower shape to its leg spurs. Commonly seen Spanish names for any local species are *bucaré*, *frejolillo* or *porotillo*, and in Africans some are called *kaffirboom. Mullumurikku* is a widespread name in Kerala.

The genus is probably of South American origin but the ability of the seeds to float and retain viability after prolonged immersion in salt water and the probable riverine, coastal or estuarine environments inhabited by the ancestral species have resulted in worldwide distribution. Pollination by birds and a marked ability to hybridise have resulted in a tremendous amount of ecological and morphological diversity, both within and between species, but within rather close cytological and phytochemical relationships. The alkaloids of *Erythrina* are distinct from those of other legumes and they all possess an unusual high activity, low affinity nitrate reductase system distinct from known nitrate reduction patterns in other angiosperms (Neill 1988).

Phytochemical analysis has demonstrated the presence of terpenes in plants from the *Erythrina* genus (Nkengfack *et al.,* 1997); that are also recognized as bioactive alkaloid-rich plants (Ghosal *et al.,* 1971; Barakat *et al.,* 1977) and flavonoids, especially, isoflavones, pterocarpanes, flavanones and isoflavanones (Chacha *et al.,* 2005). Some of these flavonoids have demonstrated a wide variety of biological activities (Table 2). Studies have demonstrated the presence of analgesic and anti-inflammatory effects in extracts obtained from *E. senegalensis*, *E. velutina* and *E. mulungu* (Vasconcelos *et al.,* 2003). In folk medicine, various species are utilized as a tranquilizer, against insomnia and to treat inflammation (Garcia-Mateos *et al.,* 2001).

1.13.1 Taxonomic hierarchy of the genus *Erythrina*

- **Domain:** Eukaryota
- **Kingdom:** Plantae
    - **Subkingdom:** Viridaeplantae
        - **Phylum:** Tracheophyta
            - **Subphyllum:** Euphyllophytina
        - **Class:** Dicotyledone
            - **Subclass:** Polypetaleae
                - **Order:** Fabales
                - **Family:** Fabaceae
            - **Subfamily:** Faboideae
                - **Tribe:** Phaseoleae
                    - **Genus:** *Erythrina*

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1.13.2 Some available species of *Erythrina*

Some available species of *Erythrina* are listed in Table 1.7.

Table 1.7: Some available species of *Erythrina*.

1. *E. abyssinica* Lam.	8. *E. humeana* Spreng.	15. *E. speciosa* Andrews.
2. *E. americana* Mill.	9. *E. lanceolata* Standl.	16. *E. stricta* Roxb.
3. *E. coralloides* D. C.	10. *E. lysistemon* Hutch.	17. *E. suberosa* Roxb.
4. *E. decora* Harms.	11. *E. mulungu* Diels Mart.	18. *E. tuxtlana* Krukoff.
5. *E. falcata* Benth.	12. *E. polychaeta* Harms.	19. *E. velutina* Willd.
6. *E. fusca* Lour.	13. *E. sandwicensis* O. Deg.	20. *E. zeyheri* Harv.
7. *E. herbacea* L.	14. *E. schimpffii* Diels.	

1.13.3 Medicinal uses

Erythrina has been used in folk medicine for treatment of insomnia, malaria fever, venereal disease, asthma and toothache. *Erythrina* species are widely prescribed in traditional medicine against frequent diseases from microbial and parasitic origin. The ailments treated are bacterial, fungal, parasitic and viral diseases, gastrointestinal disorders, liver disorders, sexual asthenia, nervous disorders, sterility, eye diseases and kidney pain.

South American Indians used *Erythrina* as a fish poison. In addition, there are reports of its use as a narcotic and anthelmintic. The first compounds isolated from *Erythrina* were alkaloids. β-erythroidine was used for a brief time as a muscle relaxant in surgery and in treatment of schizophrenia. Tea from *Erythrina* flowers is regularly used as a relaxant in Mesoamerica. Subsequently, homoerythrina alkaloids were investigated for their anti-cancer activity. Recently, research involving *Erythrina* has focused on other chemical effects, primarily the antimicrobial action of *Erythrina* lectins and the enzymology of proteinase inhibitors isolated from *Erythrina* (Payne, 1991).

E. abyssinica and *E. senegalensis* are prescribed in ethno veterinary medicine practices against brucellosis, oedema, hygroma, dropsy, bacterial infections, skin diseases (Byavu *et al.,* 2000; Ejobi *et al.,* 2007). *E. vogelii* is used in Cameroon (Ali *et al.,* 2010) against microbial infections. *E. sigmoidea* is prescribed against inflammations (Njamen *et al.,* 2004; Kouam *et al.,* 2007; Udem *et al.,* 2010) and cancer (Watjen *et al.,* 2007).

E. milbraedii is used to prepare remedies against prostate (Tchokouaha *et al.,* 2010). *E. poeppigiana* and *E. fusca* are used against microbial infections (Sato *et al.,* 2003); fever and inflammations (Innok *et al.,* 2009).

All plant parts (leaves, stem barks, roots and flowers) are used for the preparation of remedies in the form of decoction, maceration, infusion, powders or calcinates to treat diseases. The treatments are administered by oral routes and baths.

1.13.4 Phytochemical data analysis of the genus *Erythrina* L.

The phytochemical data analysis showed predominance of alkaloids and flavonoids in the *Erythrina* genus. It is important to note that alkaloids are recognized as markers for plants of this genus in addition to showing a wide array of biological activities, and being important candidates in the development of new drugs.

Some important alkaloids that are distributed within plants from the *Erythrina* genus are erytharbine, erythartine, erysotramidine, erysodine and erysotrine, shown in Figure 1.13. It is noteworthy that a characteristic feature of these alkaloids is the spiro structure in the rings bearing the nitrogen atom.

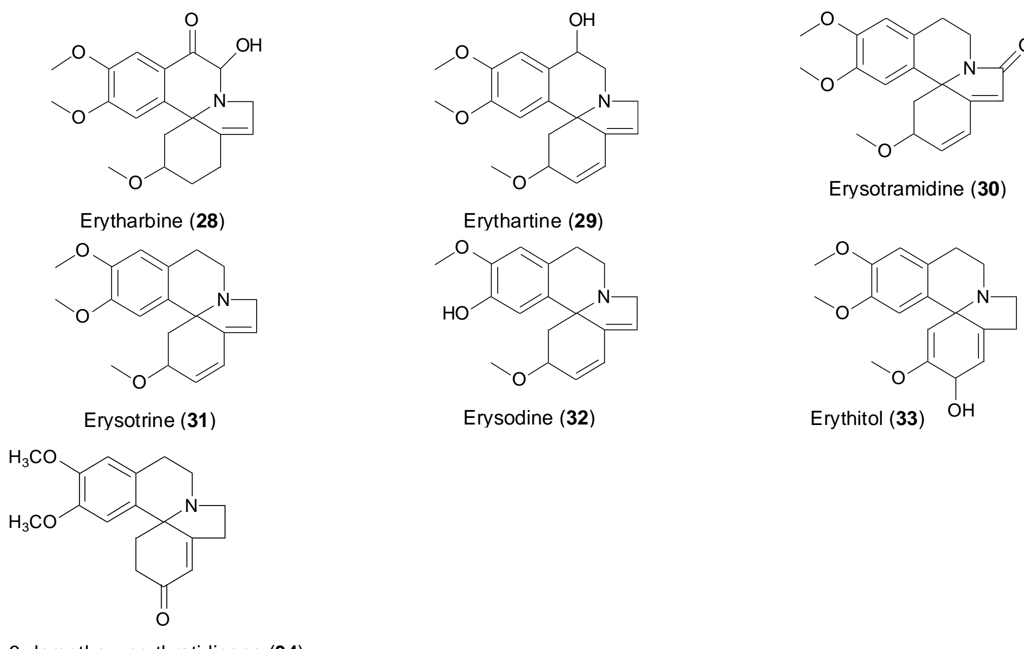

Figure 1.13: Structures of some alkaloids reported from *Erythrina* species.

Lupinifolin (**35**)

Lonchocarpal A (**36**)

Figure 1.14: Structures of some flavonoids reported from *Erythrina* species.

Wighteone (**37**)

Erythrinine A (**38**)

Alpinum isoflavone (**39**)

Eryvarine D (**40**)

Eryvarine E (**41**)

Eryvarine C (**42**)

Figure 1.15: Structures of some isoflavonoids reported from *Erythrina* species.

Sandwicensin (**43**)

Phaseollidin (**44**)

Erythrabissin I (**45**)

Figure 1.16: Structures of some pterocarpans from *Erythrina* species.

1.13.5 Biological activities of some *Erythrina* species

Analysis of the biological activity data (Table 1.8) shows the wide variety of biological activity of plants from the *Erythrina* genus, and shows too that most of this corroborates with popular knowledge and uses. It is noteworthy to point out that most of these activities, mainly the antibacterial and analgesic properties, confirm the different popular applications of extracts obtained from plants of this genus. Despite the wide array of

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available data related to plants of this genus, there is still a need for more research about some of them. The *Erythrina* genus, have the potential to provide new compounds for the development of drugs for the treatment of diseases such as cancer, diabetes and hypertension.

Table-1.8: Biological activity of some *Erythrina* species.

Species	Plant Part	Biological Activities	Reference
E. abyssinica Lam.	Bark, leaf, root, root bark	Mitogenic activity, cell culture, molluscicidal, (*Biomphalaria pfeifferi*); antibacterial (Gram positive species, *Escherichia coli*); Anti-yeast (*Saccharomyces cerevisiae*); antimalárico.	Tachibana *et al.,* 1993; Kloos, *et al.,* 1987; Kamat, *et al.,* 1981; Taniguchi *et al.,* 1978; Yenesew, *et al.,* 2004.
E. abyssinica Lam.	Bark	Antibacterial (*Pseudomonas aeruginosa, Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*).	Omer *et al.,* 1998.
E. abyssinica Lam.	Leaf, trunk	Uterine relaxing and stimulant, muscle relaxing and stimulant, peripheric muscle relaxing and stimulant, antidiarrheal, antibacterial (*Salmonella typhi*, *Shigella flexneri*, *Shigella dysenteriae*, *Shigella boyd*ii, *Shigella sonnei*); antiviral, toxic effect in rats.	Chagnon, 1984; Maikere-faniyo *et al.,* 1989; Vlietinck, *et al.,* 1995.
E. addisoniae Hutch. & Dalziel	Stem bark	Anti-inflammatory.	Talla *et al.,* 2003.
E. americana Mill.	Bark	Plant germination inhibition, molluscicidal.	Dominguez & Alcorn, 1985.
E. americana Mill.	Seed	Central nervous system depressor.	Garin-Aguilar *et al.,* 2000.
E.arborescens Roxb.	Leaf, stem	Hypotensive, cytotoxic antispasmodic, uterine stimulant.	Dhar *et al.,* 1968.
E. berteroana Urb.	Leaf	Anti-yeast, antibacterial.	Caceres *et al.,* 1987.
E. breviflora DC.	Leaf, stem	Cancer induction.	Caldwell & Brewer, 1983.
E. caffra Thunb.	Leaf, bark	Antibacterial, COX_1 inhibitor,	Pillay *et al.,* 2001.
E. cristagalli Linn.	Aerial parts, bark	Anti-inflammatory, analgesic, antibacterial, antifungal.	Mino *et al.,* 2002; Perez & Anesini, 1994.

Table-1.8 cont'd: Biological activity of some *Erythrina* species

Species	Plant Part	Biological Activities	Reference
E. cristagalli Linn.	Fresh fruit, leaf, stem	Anti-phagocytic.	Yannitsaros, 1996.
E. cristagalli Linn.	Leaf, stem	Cytotoxic and antiviral.	Simoes *et al.,* 1999.
E. eriotricha Harms.	Root bark	Antibacterial.	Nkengfack *et al.,* 1995.
E. excelsa Baker	Root bark	Antibacterial, antifungal.	Taniguchi *et al.,* 1993.
E. fusca Lour.	Seeds	Central nervous system depressor.	Widianto *et al.,* 1980.
E. glauca Willd.	Bark	Anti-viral	Mc Kee *et al.,* 1997.
E. humeana Spreng.	Bark, leaf	Antibacterial, COX_1 inhibitor.	Pillay *et al.,* 2001.
E. indica Lour.	Leaf	Antifungal, antibacterial.	Ross *et al.,* 1980.
E. indica Lour.	Leaf	Central nervous system depressor.	Ratnasooriya & Dharmasiri, 1999.
E. indica Lour.	Root bark	Anti-mycobacterial, antibacterial.	Waffo *et al.,* 2000.
E. indica Lour.	Stem bark	Cytotoxic.	Nkengfack *et al.,* 2001.
E. latissima Mey.	Bark, leaf	COX_1 inhibitor, antibacterial.	Pillay *et al.,* 2001.
E. lysistemon Hutch.	Bark, leaf	COX_1 inhibitor, anti-yeast antibacterial.	Pillay *et al.,* 2001; Rabe & Van Staden 1997; Motsei *et al.,* 2003.
E. lysistemon Hutch.	Stem bark	Estrogenic, rises seric LDL, bone formation stimulant, anti-diabetic.	Njamen *et al.,* 2007.
E. resupinata Roxb.	Roots	Fetal anti-implantation, uterine stimulant, anti-tumoral, abortive, toxicity evaluation.	Aswal *et al.,* 1984.
E. sacleuxii Hua.	Leaf, root bark	Anti-malarial, cytotoxic.	Gessler *et al.,* 1994; Gessler *et al.,* 1995,
E. senegalensis DC.	Bark, root, stem bark, flowers	Anti-malarial, analgesic, anti-inflammatory, anti bacterial, molluscicidal.	Saidu *et al.,* 2000; Etkin 1997; Okunji & Iwu 1988; Hussain & Deeni, 1991; Ajaiyeoba *et al.,* 2004.
E. senegalensis DC.	Raiz	Antiviral.	Silva *et al.,* 1997.

Table-1.8 cont'd: Biological activity of some *Erythrina* species

Species	Plant Part	Biological Activities	Reference
E. sigmoidea Hua.	Bark, root bark, stem bark	Anti-yeast, antibacterial, skeletal muscle relaxing, anti-spasmodic, antifungal.	Biyiti *et al.,* 1988; Benedicta *et al.,* 1993; Nkengfack *et al.,* 1994; Nkeh *et al.,* 1996.
E. stricta Roxb.	Leaf, stem	Spasmolytic, analgesic, diuretic, hypothermic, anti-convulsant, toxicity evaluation, cytotoxic, anti-yeast, anti protozoan, antiviral, antifungal.	Bhakuni *et al.,* 1988; Dhar *et al.,* 1968.
E. suberosa Roxb.	Leaf, stem bark	Anti-spermatogenic, anti-tumoral, anti-androgen, anti-gonadotropin, cytotoxic, hypo glycaemic, toxicity evaluation of spasm, anti- hypotensive.	Dhar *et al.,* 1968.
E. subumbrans (Hassk.) Merr.	Aerial parts	Fetal anti-implantation, uterine stimulant, anti tumor, abortive effect, toxicity evaluation.	Aswal *et al.,* 1984.
E. ulei Harms.	Bark	Anti-crustacean, DNA linker, anti-oxidant.	Desmarcheilier *et al.,* 1996. Desmarcheilier *et al.,* 1997.
E. variegata L.	Bark	Anti gastric ulcer.	Muto *et al.,* 1994.
E. variegata L.	Flowers	Anti-yeast, antibacterial.	Avirutnant *et al.,* 1983.
E. variegata L.	Fresh flowers	Anxiolytic.	Flausino *et al.,* 2007.
E. velutina Willd.	Leaf	Anti-inflammatory, analgesic.	Marchioro *et al.,* 2005.
E. vespertilio Benth.	Bark	Inhibition of platelet aggregation, serotonin release inhibition.	Rogers *et al.,* 2001.
E. vogelii Hook.f.	Root bark	Anti-fungal.	Queiroz *et al.,* 2002.
E. zeyheri Harv.	Leaf	Anti-bacterial, COX_1 inhibitor.	Pillay *et al.,* 2001.

1.14 Description of the investigated plants of the genus *Erythrina*

1.14.1 Taxonomic hierarchy of *Erythrina fusca* Lour

- **Domain:** Eukaryota
- **Kingdom:** Plantae
  - **Subkingdom:** Viridaeplantae
    - **Phylum:** Tracheophyta
      - **Subphyllum:** Euphyllophytina
    - **Class:** Dicotyledone
      - **Subclass:** Polypetaleae
        - **Order:** Fabales
          - **Family:** Fabaceae
            - **Subfamily:** Faboideae
          - **Tribe:** Phaseoleae
            - **Genus:** *Erythrina*
              - **Species:** *E. fusca*

1.14.2 Description of *E. fusca* (Orwa *et al.*, 2009)

Taxonomic name	:	*Erythrina fusca* Lour.		
Synonyms	:	*Erythrina atrosanguinea* Ridl.		
		Erythrina glauca Willd.		
		Erythrina ovalifolia Roxb.		
Common names	:	Pannya mandar, Harikakra, Kanta madar		
Vernacular names	:	**English**	:	Coral bean, purple coral tree
		Philipino	:	Korung-korung
		French	:	Immortelle blanche
		Indonesian	:	Cangkring, kane, rase
		Thai	:	Thong lang nam
Habit and habitat	:	Medium to large spreading tree, 10-15 m tall; crown rounded; trunk short, spiny (spines 1-2 cm long); much		

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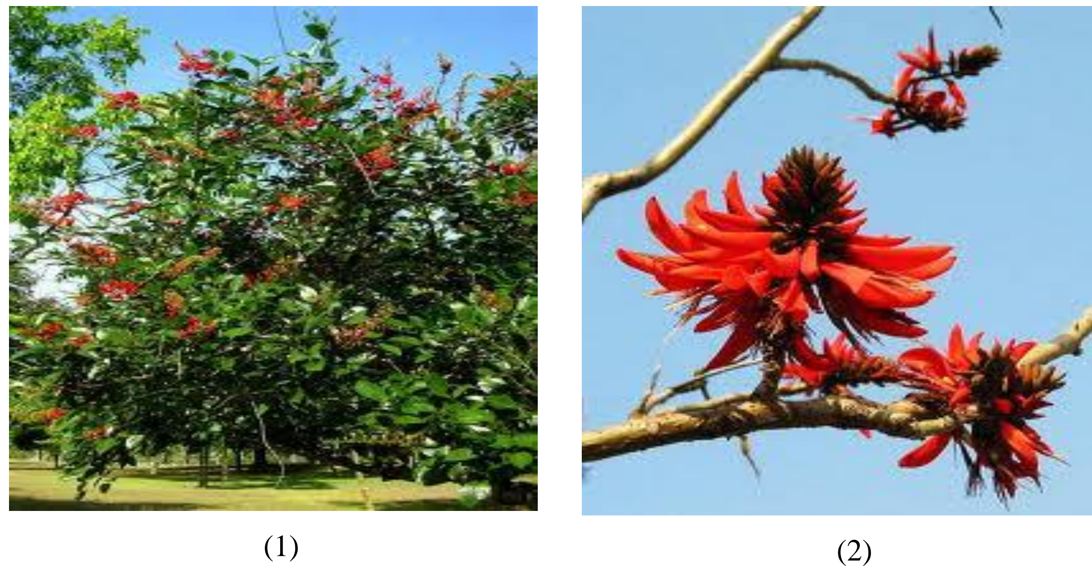
		branched, bark brownish-grey or olive-brown, flaky; branches spreading. Most commonly found on the outer margins of swamps on sites that are not permanently flooded.
Leaf	**:**	Alternate and trifoliate.
Petioles	**:**	Up to 25 cm long and sometimes sparsely prickly.
Stipules	**:**	Orbicular and caduceus.
Flowers	**:**	Pale brick-red or salmon flowers in fascicles scattered along the rachis, covered with deciduous, ferruginous hairs, mostly unarmed peduncle up to 13 cm long.
Fruits	**:**	Fruit a woody, linear compressed pod, dehiscent, slightly constricted between 3-15 seeds; stipe stout, 1.5 cm long; beak 2 cm long, velvety, ferruginously hairy when young, later glabrescent.
Seeds	**:**	Oblong-ellipsoid, 12-18 x 5-8 mm, dark brown or black.
Life span	**:**	Perennial.
Flowering time	**:**	January to March.
Fruiting time	**:**	April to June.
Distribution	**:**	*E. fusca* has the widest distribution of any *Erythrina* species; it grows on coasts and along rivers in tropical Asia (India, Sri Lanka, Myanmar, Indonesia and Bangladesh); Australia, the Mascarene Islands, Madagascar, Africa, and the southern America.
Distribution in Bangladesh	**:**	Every where in Bangladesh.
Medicinal uses	**:**	In Thailand, its root, bark and leaves are used as an antipyretic.



(1) (2)

Figure 1.17: *Erythrina fusca* Lour tree (1) and flowers (2).

1.14.3 Taxonomic hierarchy of *Erythrina variegata* L.

- **Domain:** Eukaryota
- **Kingdom:** Plantae
    - **Subkingdom:** Viridaeplantae
        - **Phylum:** Tracheophyta
            - **Subphyllum:** Euphyllophytina
        - **Class:** Dicotyledone
            - **Subclass:** Polypetaleae
                - **Order:** Fabales
                    - **Family:** Fabaceae
                        - **Subfamily:** Faboideae
                    - **Tribe:** Phaseoleae
                        - **Genus:** *Erythrina*
                            - **Species:** *E. variegata*

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1.14.4 Description of *E. variegata* L. (Website: 1)

Taxonomic name : *Erythrina variegata* L.

Synonyms : *E. indica* Lam.
E. variegata var. *Orientalis* (L.) Merr

Common names : Tiger's Claw, Indian Coral Tree and Sunshine Tree

Vernacular names :
- **Bengali** : Madar, Mandar, Palitamadar
- **Assam** : Modar
- **Fiji** : Drala
- **Thailand** : Thong lang
- **Vietnam** : Vong nem

Habit and Habitat : Thorny deciduous medium to large tree, commonly reaching 15 to 20 m in height in 20 to 25 years; the smooth bark is streaked with vertical lines of green, buff, grey and white; small black prickles cover the stem and branches. It is well adapted to the humid and semi-arid and tropics and subtropics, occurring in zones with annual rainfall of 800 to 1500 mm distributed over a five- to six- month rainy season.

Leaf : The leaves are pinnate with three leaflets. The leaflets are usually variegated, medium to light green, heart shaped, 7 to 12 cm wide and 12 to 18 cm long.

Flowers : Brilliant orange-red flowers emerge in dense, cofiioal inflorescences 5 to 7 cm long and 2 to 3 cm wide, usually after the leaves have dropped. Flowering is normally followed by a lavish production of seed.

Fruits : The pods are thick and black-1.5 to 2 cm wide and 15 to 20 cm long. Each contains 5 to 10 egg-shaped seeds. These are glossy brown, red or purple and are 6 to 10 mm in diameter and 12 to 17 mm long.

Life span : Perennial..

Flowering time	:	February to May.
Fruiting time	:	April to August.
Distribution	:	Native to the tropical and subtropical regions of eastern Africa, southern Asia, northern Australia, and the islands of the Indian Ocean and the western Pacific Ocean east to Fiji.
Distribution in Bangladesh	:	Forests of Chittagong, Chittagong Hill Tracts, Cox's Bazar and Sylhet, al.so planted throughout the country.
Medicinal use	:	Leaves are stomachic, anthelmintic, laxative, diuretic, and emmenagogue; applied externally for dispersing venereal buboes, relieve pain of the joints and inflammations; juice is poured into the ear to relief earache and is used as an anodyne in toothache. The bark is astringent, febrifuge anti-bilious and anthelmintic; useful in dysentery and as a collyrium in ophthalmia. The roots are emmenagogue (Yusuf *et al.,* 2009). Total alkaloid fraction from bark showed neuromuscular blocking, smooth muscle relaxant, CNS depressant and anticonvulsant effects (Rastogi & Mehrotra, 1993).

(1) **(2)** **(3)**

Figure 1.18: ***Erythrina variegata*** **L. tree (1); leaves (2) and flowers (3).**

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2.1 Phytochemical Investigation of the experimental plants

Two plant genus and five plant species belonging to the Phyllanthaceae and Fabaceae family were investigated in this study.

Name of plant	Family	Genus	Plant part
Bridelia verrucosa	Phyllanthaceae	*Bridelia*	Stem bark and leaf
Bridelia stipularis	Phyllanthaceae	*Bridelia*	Stem bark and leaf
Bridelia tomentosa	Phyllanthaceae	*Bridelia*	Stem bark and leaf
Erythrina fusca	Fabaceae	*Erythrina*	Stem bark and leaf
Erythrina variegata	Fabaceae	*Erythrina*	Stem bark and leaf

The chemical investigations of the first four plants will be discussed here in different sections.

2.2 Phytochemical investigation of *Bridelia verrucosa*

2.2.1 Collection and preparation of the plant material

Leaf and stem bark of *B. verrucosa* was collected from the village of Panchouri, Khagrachhori District in February 2007 and identified in Bangladesh National Herbarium where voucher specimens have been maintained representing this collection (Accession No. DACB-31376). After proper washing, the leaf and stem bark (after cutting into small pieces) was sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature (not more than 40 °C for better grinding. The dried plant material was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka, Bangladesh.

2.2.2 Extraction of the plant material

The powdered stem bark (550 g) and leaf of *B. verrucosa* (325 g) were separately soaked in 1.5 L and 750 mL of methanol in a clean, round bottomed flask (5 and 3 L, respectively). The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at low temperature (36-40 ºC) and reduced pressure with a Buchii Rotavapour to have a concentrate of the crude extracts. The concentrated extract was then air dried to get solid residue. The weight of the crude extract obtained from stem bark was 27.5 g and from leaf was 16.25 g.

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2.2.3 Solvent-solvent partitioning of crude extract

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wagenen *et al*. (1993). 5 g of dried methanol extract was triturated with 90 ml of methanol containing 10 ml of distilled water. The crude extract was dissolved completely. This is the mother solution, which was partitioned off successively by three solvents of different polarity; *n*-hexane, carbon tetrachloride and chloroform. The whole partitioning process is schematically shown in Figure 2.1. All the fractions were evaporated to dryness (Table 2.1) and were used for further analysis.

2.2.3.1 Partitioning with *n*-hexane

The mother solution was taken in a separating funnel. 100 ml of the *n*-hexane was added to it and the funnel was shaken and then kept undisturbed. The organic portion was collected. The process was repeated thrice; *n*-hexane fractions were collected together and evaporated in a Rotary evaporator.

2.2.3.2. Partitioning with carbon tetrachloride (CCl_4)

To the mother solution left after washing with *n*-hexane, 12.5 ml of distilled water was added and mixed. It was then taken in a separating funnel and extracted with CCl_4 (100 ml X 3). The CCl_4 fractions were collected together and evaporated. The aqueous fraction was preserved for the next step.

2.2.3.3. Partitioning with chloroform ($CHCl_3$)

To the mother solution that left after washing with *n*-hexane and CCl_4, 16 ml of distilled water was added and mixed uniformly. It was then taken in a separating funnel and extracted with $CHCl_3$ (100 ml X 3). The $CHCl_3$ soluble fractions were collected together and evaporated. The aqueous methanolic fraction was preserved as aqueous soluble fraction.

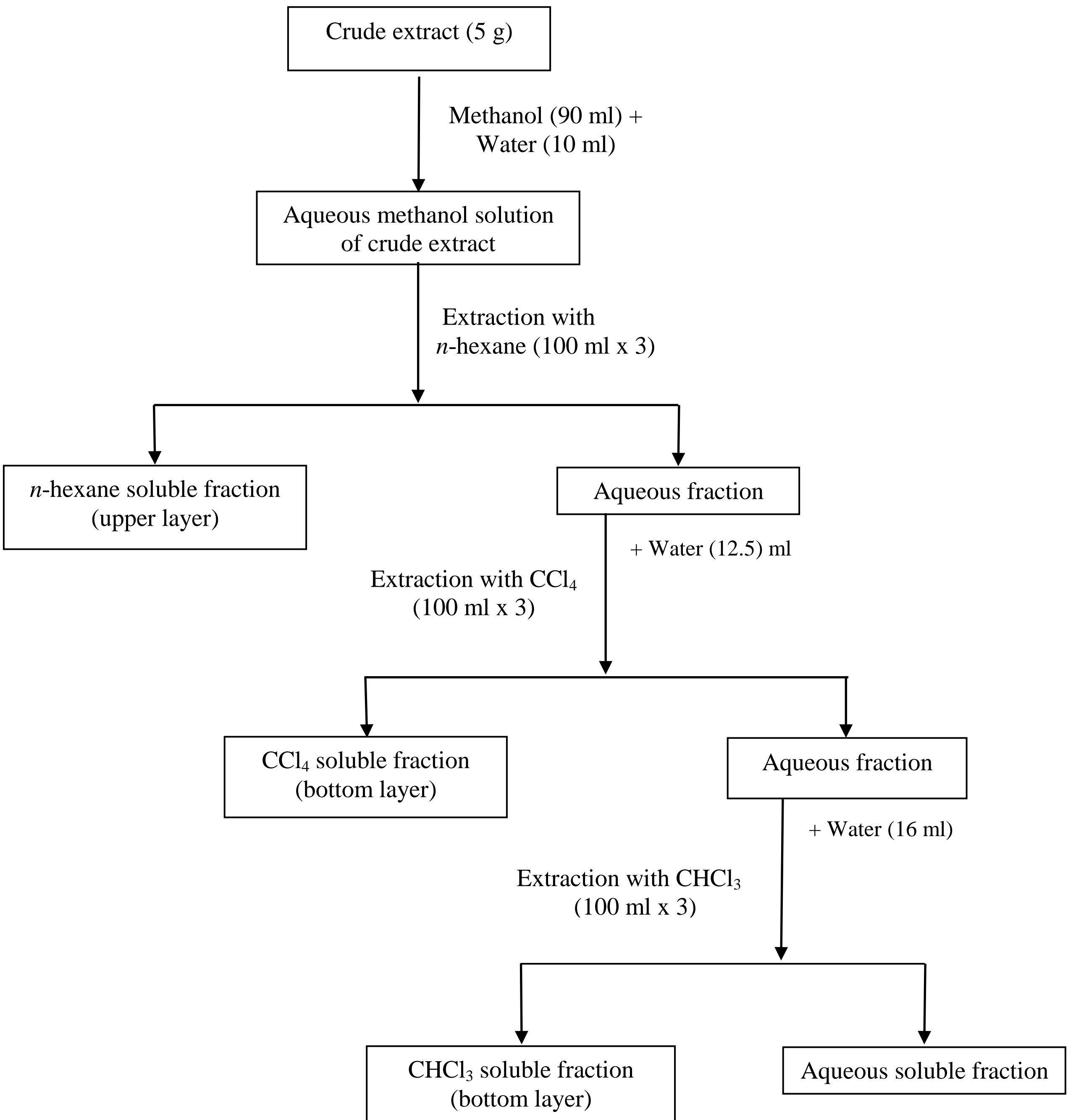

Figure 2.1: Schematic representation of the modified Kupchan partitioning of the stem bark of methanolic extract of *B. verrucosa*.

The process was repeated for several times and after evaporation the weight of the different soluble fractions obtained is mentioned in Table 2.1.

Table 2.1: Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of *B. verrucosa*.

Plant Part	**Weight (g) of methanolic extract for Kupchan partitioning**	**Fraction code**	Weight (g) of soluble fractions
Stem bark	**20**	**HSF**	3.5
		CSF	1.24
		CHSF	1.6
		AQSF	11.4
Leaf	**10**	**HSF**	3.1
		CSF	0.62
		CHSF	0.62
		AQSF	4.0

HSF= *n*-hexane soluble fraction; CSF= carbon tetrachloride soluble fraction; CHSF= chloroform soluble fraction; AQSF= aqueous soluble fraction

2.2.4 Gel permeation chromatography of *n*-hexane soluble fraction of the stem bark of *B. verrucosa*

The column was packed with Lipophilic Sephadex (LH-20). At first dried sephadex was soaked in a mixture of solvents with a ratio of *n*-hexane- dichloromethane- methanol (2:5:1) for at least 12 hours for proper swelling. After that, the slurry of sephadex was made and added into a glass column having the length and diameter of 55 cm and 1.1 cm, respectively. When sufficient height of the stationary phase was obtained, a few hundred milliliter of solvent mixture with the same ratio was run through the column for proper packing of the column. The sample (320 mg) was dissolved in this solvent mixture and subsequently applied on top of the column with the help of a Pasteur pipette. The column was then eluted with the same solvent mixture and finally the column was washed with dichloromethane and methanol mixtures of increasing polarity. Solvent systems used as mobile phases in the analysis of extract were listed in Table 2.2.

Table 2.2: Different solvent systems used for gel permeation (Lipophilic Sephadex LH-20) chromatography of *n*-hexane soluble materials of the stem bark of *B. verrucosa*.

Fraction No.	**Solvent systems**	**Volume collected (mL)**
1-19	*n*-Hexane-dichloromethane-methanol (2:5:1)	60
20-22	Dichloromethane-methanol (9:1)	25
23-24	Dichloromethane-methanol (1:1)	25
25-30	Methanol 100%	120

2.2.5 Analysis of column fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with vanillin sulfuric acid reagent followed by heating at 100-110 ºC for 5-10 minutes as well.

Depending on the TLC behavior a number of similar samples were detected and the column fractions were added, these were then purified by employing various techniques. A list of isolated compounds has been summarized in Table 2.3.

Table 2.3: List of isolated compounds from *n*-hexane soluble fraction of the methanolic extract of the stem bark of *B. verrucosa*.

Column fraction	Mobile Phase	R_f value	Amount (mg)	Code
11-18	Toluene- Ethyl acetate (90:10)	0.71	7.23	BVSP-1
19-23	Toluene- Ethyl acetate (95:5)	0.50	6.78	BVSP-2
Sub column 1-4	Toluene- Ethyl acetate (95:5)	0.63	5.3	BVSP-4
Sub column 5-9	Toluene- Ethyl acetate (95:5)	0.56	7.14	BVSP-6
Sub column 34-43	Toluene- Ethyl acetate (95:5)	0.62	6.5	BVSP-34

2.2.5.1 Isolation of compound BVSP-1

The fractions 11 to 18 showed similar TLC feature. So, they were bulked together. After, evaporation of all the solvents, white crystals appeared. The crystals obtained after evaporation of solvents were washed with *n*-hexane to remove the adhered coloring materials and recrystallized from *n*-hexane and ethyl acetate to afford a pure compound as needles in a sample vial with *n*-hexane and chloroform which was found to be pure by TLC screening. The isolated compound was marked as BVSP-1 (7.23 mg).

2.2.5.2 Isolation of compound BVSP-2

The fractions 19-23 were combined together depending on the identical TLC characterization and subjected to preparative TLC over silica gel F_{254} using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated three times for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a purple colored band was visualized. The band was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent afforded a colorless compound BVSP-2 (6.78 mg).

2.2.5.3 Column chromatography (CC) of fractions 11-18

The column was packed with silica gel (Kieselgel 60H, mesh 70-230). Slurry of silica gel was added into a glass column having the length and diameter of 60 cm and 1.5 cm respectively. When sufficient height of the adsorbent bed was obtained, 100 mL of 100% *n*-hexane and then 1% ethyl acetate in *n*-hexane was run through the column for proper packing of the column. The sample was prepared by adsorbing 100 mg of fraction 11-18 onto silica gel (Kieselgel 60H, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. The column was then eluted with 1% ethyl acetate in *n*-hexane,

followed by mixtures of *n*-hexane and ethyl acetate of increasing polarities. Solvent systems used as mobile phases in the analysis of fraction 11-18 are listed in Table 2.4.

Table 2.4: Different solvent systems used for column chromatography (Kiselgel 60 mesh 70-230) of fractions 11-18 from Sephadex (LH-20) column of *n*-hexane soluble materials of the stem bark of *Bridelia verrucosa*.

Fraction No.	Solvent Systems	Volume collected (mL)/test tube
1-34	*n*-Hexane-Ethyl acetate (99:1)	20
35-46	*n*-Hexane-Ethyl acetate (98:2)	20
47-50	*n*-Hexane-Ethyl acetate (95:5)	20

2.2.5.4 Analysis of Column Chromatography fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with vanillin-sulfuric acid reagent and then heating at 100-110 ºC for 5-10 minutes.

2.2.5.5 Isolation of compound BVSP-4

Fractions 1-4 showing identical spots on TLC were combined in a beaker and was kept at room temperature undisturbed for several days. The crystals obtained after evaporation of solvents were washed with *n*-hexane to remove the adhered coloring materials and recrystallized from *n*-hexane and ethyl acetate to afford a pure compound designated as BVSP-4 (5.3 mg) as needles.

2.2.5.6 Isolation of compound BVSP-6

The sub-fractions 5-9 were combined together depending on the identical TLC characterization and subjected to preparative TLC over silica gel F_{254} using toluene-ethyl acetate (95:5) as the developing solvent. The process was repeated five times for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a pink colored band was visualized. The band was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent afforded a colorless compound BVSP-6 (7.14 mg).

2.2.5.7 Isolation of compound BVSP-34

Column fractions 34-43 gave similar TLC feature and were bulked together. Then it was subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (95:5)]. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating in 110 ºC for 5 minutes and a purple colored band was visualized. The stationary phase was scrapped off from the area having no spray

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reagent and eluted with ethyl acetate. Evaporation of the solvent yielded white needles which melted at 163 ºC. The compound was analyzed over TLC to check for purity and found to be a pure compound which was termed as BVSP-34 (6.5 mg).

2.2.6 Column chromatography of carbon tetrachloride soluble fraction of the stem bark of *B. verrucosa*

A portion of carbon tetrachloride soluble fraction (270 mg) was subjected to column chromatography (CC) for fractionation. The column was packed with column grade silica gel (Kieselgel 60H, mesh 70-230). Slurry of silica gel in petroleum ether (60-80 ºC) was added into a glass column having a length and diameter of 84 and 2 cm, respectively. When the desired height of adsorbent bed was obtained, a few hundred milliliter of solvent was run through the stationary phase for proper packing of the column. After packing, the sample was prepared by adsorbing carbon tetrachloride soluble material onto silica gel (Kieselgel 60H, mesh 70-230) and allowed to dry up to a free flowing powder like mass and subsequently applied on top of the adsorbent layer. Then the column was eluted with petroleum ether, followed by mixtures of petroleum ether and ethyl acetate and then ethyl acetate, finally with ethyl acetate and methanol in order of increasing polarities. The flow was maintained at a constant rate (2 ml/min) for better separation. Solvent system used as mobile phase in CC analysis of carbon tetrachloride soluble material and has been listed in Table 2.5. The eluates were collected in test tubes. A total of 150 fractions were collected each 20 ml.

Table 2.5: Different solvent systems used for the column chromatographic analysis of the carbon tetrachloride soluble fraction of the stem bark of *B. verrucosa*.

Fraction no.	Solvent systems	Volume collected ml/test tube
1 – 4	Petroleum ether - Ethyl acetate = (98 : 2)	20
5 – 8	Petroleum ether - Ethyl acetate = (95 : 5)	20
9 – 22	Petroleum ether - Ethyl acetate = (92.5 :7.5)	20
23 – 48	Petroleum ether - Ethyl acetate = (90: 10)	20
49 – 62	Petroleum ether - Ethyl acetate = (88: 12)	20
63 – 73	Petroleum ether - Ethyl acetate = (85 : 15)	20
74 – 85	Petroleum ether - Ethyl acetate = (80 : 20)	20
86 – 99	Petroleum ether - Ethyl acetate = (75 : 25)	20
100 – 110	Petroleum ether - Ethyl acetate = (70 : 30)	20
111 – 117	Petroleum ether - Ethyl acetate = (60 : 40)	20
118 – 130	Petroleum ether - Ethyl acetate = (50 : 50)	20
131 – 137	Ethyl acetate (100%)	20
138 – 141	Ethyl acetate : Methanol = (99 : 1)	20
142 – 145	Ethyl acetate : Methanol = (98 : 2)	20
146 – 150	Ethyl acetate : Methanol = (95 : 5)	20

2.2.7 Analysis of column fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with vanillin sulfuric acid reagent followed by heating at 100-110 ºC for 5-10 minutes as well. Depending on the TLC behavior a number of similar samples were detected and the column fractions were added, these were then purified by employing various techniques. A list of isolated compounds has been summarized in Table 2.6.

Table 2.6: List of isolated compounds from carbon tetrachloride soluble fraction of the methanolic extract of the stem bark of *B. verrucosa*.

Column fraction	Mobile Phase	R_f value	Amount (mg)	Code
8-12	Toluene - Ethyl acetate 90:10	0.41	7.53	BVSC-8
74-76	Toluene - Ethyl acetate 90:10	0.54	7.10	BVBC-2

2.2.7.1 Isolation of compound BVSC-8

Fractions 8-12 gave identical TLC features, were combined in a beaker and kept undisturbed at room temperature for several days. Crystals were produced upon evaporation of the solvents. The crystals thus obtained were washed with *n*-hexane to remove adhered color materials and recrystallized from *n*-hexane and ethyl acetate to afford a pure compound designated as BVSC-8 (7.53 mg) as needles.

2.2.7.2 Isolation of compound BVBC-2

Fractions 74-76 were combined together. Evaporation of solvents yielded a whitish mass which was purified by preparative TLC over silica gel F_{254} using toluene-ethyl acetate (9:1) as the developing solvent. The process was repeated twice for better separation and isolation of the compound of interest. The desired band was scrapped off and eluted with ethyl acetate to yield BVBC-2 (7.10 mg) as whitish mass.

2.2.8 Column chromatography of chloroform soluble fraction of the stem bark of *B. verrucosa*

A portion of chloroform soluble fraction (500 mg) was subjected to column chromatography (CC) for fractionation. The column was packed with column grade silica gel (Kieselgel 60H, mesh 70-230) and eluted as described in section 2.2.6. Solvent system used as mobile phase in CC analysis of chloroform soluble material has been listed in Table 2.7. The eluates were collected in test tubes. A total of 170 fractions were collected each 20 ml.

Table 2.7: Different solvent systems used for the column chromatographic analysis of the chloroform soluble fraction of the stem bark of *B. verrucosa*.

Fraction no.	Solvent systems	Volume collected ml/test tube
1 – 3	Petroleum ether - Ethyl acetate = (98 : 2)	20
4 – 6	Petroleum ether - Ethyl acetate = (95 : 5)	20
7 – 18	Petroleum ether - Ethyl acetate = (92.5 :7.5)	20
19 – 37	Petroleum ether - Ethyl acetate = (90: 10)	20
38 – 57	Petroleum ether - Ethyl acetate = (88: 12)	20
58 – 75	Petroleum ether - Ethyl acetate = (85 : 15)	20
76 – 85	Petroleum ether - Ethyl acetate = (80 : 20)	20
86 – 100	Petroleum ether - Ethyl acetate = (75 : 25)	20
101 – 115	Petroleum ether - Ethyl acetate = (70 : 30)	20
116 – 125	Petroleum ether - Ethyl acetate = (60 : 40)	20
126 – 137	Petroleum ether - Ethyl acetate = (50 : 50)	20
138 – 145	Ethyl acetate (100%)	20
146 – 152	Ethyl acetate - Methanol = (99 : 1)	20
153 – 160	Ethyl acetate - Methanol = (98 : 2)	20
161 – 170	Ethyl acetate - Methanol = (95 : 5)	20

2.2.9 Analysis of column fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with vanillin sulfuric acid reagent followed by heating at 100-110 ºC for 5-10 minutes as well. Depending on the TLC behavior a number of similar samples were detected and the column fractions were added, these were then purified by employing various techniques. A list of isolated compounds has been summarized in Table 2.8.

Table 2.8: List of isolated compounds from chloroform soluble fraction of the methanolic extract of the stem bark of *B. verrucosa*.

Column fraction	Mobile phase	R_f value	Amount (mg)	Code
59-69	Chloroform - Methanol (97:3)	0.42	3.54 mg	BVS-65

2.2.9.1 Isolation of compound BVS-65

Evaporation of solvents from the fractions 59-69 yielded a yellowish mass which was subjected to preparative TLC over silica gel F_{254} using chloroform-methanol (97:3) as the developing solvent. The desired band was scrapped off on an aluminum foil and eluted initially using a 19:1 mixture of chloroform-methanol (19:1) to yield BVS-65 (3.54 mg).

2.2.10 Properties of the isolated compounds

The physical properties of the isolated compounds and their reactions to vanillin-H_2SO_4 spray reagent are summarized in the Table 2.9.

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Table 2.9 Properties of the isolated compounds from stem bark of *B. verrucosa*.

Isolated compounds	Physical form	Color	Color with vanillin-H_2SO_4
BVSP-1	Crystal	White	Deep purple
BVSP-2	Needles	Colorless	Purple
BVSP-4	Crystal	Transparent	Yellow
BVSP-6	Mass	Whitish	Pink
BVSP-34	Crystal	White	Purple
BVSC-8	Crystal	White	Purple
BVBC-2	Mass	Colorless	Bluish pink which turns into ash
BVS-65	Mass	Yellow	Orange

2.3 Phytochemical investigation of *Bridelia stipularis*

2.3.1 Collection and preparation of the plant material

Leaf and stem bark of *B. stipularis* was collected from the village of Panchouri, Khagrachhori District in February 2007 and identified in Bangladesh National Herbarium where voucher specimens have been maintained representing this collection (Accession No. DACB-31378). After proper washing, the leaf and stem bark (after cutting into small pieces) was sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature (not more than 40 °C) for better grinding. The dried plant material was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka, Bangladesh.

2.3.2 Extraction of the plant material

The powdered stem bark (550 g) and leaf (325 g) of *B. stipularis* were separately soaked in 1.5 L and 750 mL methanol in a clean, round bottomed flask (5 and 3 L, respectively). The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at low temperature (36-40 ºC) and reduced pressure with a Buchii Rotavapour to have a concentrate of the crude extracts. The concentrated extract was then air dried to solid residue. The weight of the crude extract obtained from stem bark was 30 g and from leaf was 15 g.

2.3.3 Solvent-solvent partitioning of crude extract

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wagenen *et al*., (1993) as described in section 2.2.3. The whole partitioning process is schematically shown in Figure 2.1. All the fractions were evaporated to dryness (Table 2.10) and were used for further analysis.

The process was repeated for several times and after evaporation the weight of the different soluble fractions obtained is mentioned in Table 2.10.

Table 2.10: Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of *B. stipularis*.

Plant Part	Weight (g) of methanolic extract for Kupchan partitioning	Fraction code	Weight (g) of soluble fractions
Stem bark	10	HSF	2.75
		CSF	0.65
		CHSF	0.92
		AQSF	4.30
Leaf	10	HSF	3.30
		CSF	0.81
		CHSF	0.71
		AQSF	4.20

HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction

2.3.4 Vacuum Liquid Chromatography (VLC) of the crude extract of stem bark of *B. stipularis*

2.3.4.1. Packing of Column

The column was packed with fine VLC grade silica gel (Kieselgel 60H). A column having 14 cm length and 10 cm in diameter was packed with the silica gel up to a height of 6 cm under reduced pressure. The column was then washed with petroleum ether to expedite compact packing.

2.3.4.2. Sample Input

The sample was prepared by adsorbing 15 g of crude extract onto column grade silica gel (Kieselgel 60H, mesh 70-230) with continuous stirring under air blower in a porcelain basin. Stirring continued until the sample becomes a free flowing powder. The free flowing sample was then placed on the packed silica into the column. Finally a cotton plug was placed on the sample.

2.3.4.3. Development of Chromatogram

After placing the sample, the column was eluted with petroleum ether, followed by mixtures of petroleum ether and ethyl acetate and finally washed by methanol. Solvent

systems used as mobile phases in the VLC analysis of crude extract were listed in Table 2.11.

Table 2.11: Solvent systems used for VLC analysis of the crude extract of stem bark of *B. stipularis*.

Fraction no.	Solvent systems	Volume collected (ml)
1	100% Pet. Ether	50
2	Pet. ether - Ethyl acetate (98 : 2)	100
3	Pet. ether - Ethyl acetate (95 : 5)	100
4	Pet. ether - Ethyl acetate (92.5 : 7.5)	100
5 (A+B)	Pet. ether - Ethyl acetate (90 : 10)	75 + 75
6 (A+B)	Pet. ether - Ethyl acetate (80 : 20)	75 + 75
7 (A+B)	Pet. ether - Ethyl acetate (75 : 25)	75 + 75
8 (A+B)	Pet. ether - Ethyl acetate (65 : 35)	100 + 100
9 (A+B)	Pet. ether - Ethyl acetate (55 : 45)	100 + 100
10 (A+B)	Pet. ether - Ethyl acetate (50 : 50)	100 + 100
11 (A+B)	Pet. ether - Ethyl acetate (45 : 55)	100 + 100
12 (A+B)	Pet. ether - Ethyl acetate (40 : 60)	100 + 100
13 (A+B)	Pet. ether - Ethyl acetate (35 : 65)	100 + 100
14 (A+B)	Pet. ether - Ethyl acetate (30 : 70)	100 + 100
15 (A+B)	Pet. ether - Ethyl acetate (25 : 75)	100 + 100
16 (A+B)	Pet. ether - Ethyl acetate (20 : 80)	100 + 100
17 (A+B)	Pet. ether - Ethyl acetate (15 : 85)	100 + 100
18 (A+B)	Pet. ether - Ethyl acetate (10 : 90)	100 + 100
19 (A+B)	Pet. ether - Ethyl acetate (5 : 95)	100 + 100
20 (A+B)	Ethyl acetate (100 %)	100 + 100
21 (A+B)	Ethyl acetate - Methanol (98:2)	50 + 50
22 (A+B)	Ethyl acetate - Methanol (95:5)	50 + 50
23 (A+B)	Ethyl acetate - Methanol (90:10)	50 + 50
24 (A+B)	Ethyl acetate - Methanol (80:20)	50 + 50
25 (A+B)	Ethyl acetate - Methanol (70:30)	50 + 50

2.3.5 Analysis of VLC fractions by thin layer chromatography (TLC)

All the above VLC fractions were screened by TLC using various solvent systems. The plates were observed under UV light and by spraying with vanillin-sulfuric acid reagent followed by heating at 100-110 ºC for 5-10 minutes as well. Depending on the TLC behavior fractions 4 & 5A and 9A-11B were selected for further investigation.

Table 2.12: List of isolated compounds from VLC fraction of the methanolic extract of the stem bark of *B. stipularis*.

Column fraction	TLC Solvent System	R_f value	Amount (mg)	Code
4 & 5A	*n*-Hexane - Ethyl acetate (99:1)[3t]	0.32	8.23	BS-01
9A - 11B	Toluene - Ethyl acetate (97:3)[2t]	0.47	5.31	BS-6
9A - 11B	Toluene - Ethyl acetate (98:2)[5t]	0.38	7.01	BS-11

2.3.5.1 Isolation and purification of compounds from the selected VLC fractions

Five compounds have been isolated and purified from the different VLC fractions by adopting various techniques.

2.3.5.2 Isolation of BS-01

The VLC fractions 4 & 5A were mixed together due to their identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (99: 1)]. The process was repeated thrice for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a purple colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of solvent yielded white crystals and was designated as **BS-01** (8.23mg).

2.3.5.3 Gel permeation chromatography of VLC fractions 9A-11B of the stem bark of *B. stipularis*

The column was packed as described in section 2.2.4. The sample (450 mg) was dissolved in the solvent mixture (*n*-hexane - dichloromethane - methanol= 2:5:1) and subsequently applied on top of the adsorbent layer with the help of a Pasteur pipette. The column was then eluted with the same solvent mixture and finally the column was washed with dichloromethane and methanol mixtures of increasing polarity. Solvent systems used as mobile phases in the analysis of extract were listed in Table 2.2

Table 2.13: Different solvent systems used for gel permeation (Sephadex LH-20) of VLC fractions 9A-11B of the stem bark of *B. stipularis*.

Fraction No.	Solvent systems	Volume collected (ml)
1-36	*n*-Hexane - Dichloromethane - Methanol (2:5:1)	75
37-47	Dichloromethane – Methanol (9:1)	25
48-59	Dichloromethane – Methanol (1:1)	50
50-55	Methanol 100%	120

2.3.5.3.1 Isolation of BS-06

The LH-50 column fractions 20-26 of the VLC column fraction 9A-11B were mixed together due to their identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (97:3)]. The process was repeated twice for better separation of the desired band. A small portion of

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the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 ºC for 5 minutes and a pink colored band was visualized. The unsprayed protion of the band at stationary phase was scrapped off on aluminium foil and eluted with ethyl acetate. Evaporation of ethyl acetate yielded white needle like crystals which was checked for purity and termed as **BS-06** (5.31 mg).

2.3.5.3.2 Isolation of BS-11

The yellowish color of LH-50 column fractions 20-26 of the VLC column fraction 9A-11B was washed with *n*-hexane and due to their identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (98:2)]. The process was repeated five times for better separation of the bands. From the developed plates two yellow colored bands were visualized after spraying with vanillin-sulfuric acid reagent followed by heating in 110º C for 5 minutes. The bands were separately scrapped on to an aluminum foil and eluted with ethyl acetate. Colorless mass was obtained in both cases which were checked for purity and termed as **BS-11** (7.01 mg).

2.3.6 Properties of the isolated compounds

The physical properties of the isolated compounds and their reactions to vanillin-H_2SO_4 spray reagent are summarized in the Table 2.14.

Table 2.14 Properties of the isolated compounds from stem bark of *B. stipularis*.

Isolated compounds	Physical form	Color	Color with vanillin-H_2SO_4
BS-01	Crystal	White	Purple
BS-06	Needle	White	Pink
BS-11	Solid	White	Yellow

2.4 Phytochemical investigation of *Bridelia tomentosa*

2.4.1 Collection and preparation of the plant material

Leaf and stem bark of *B. tomentosa* was collected from the village of Panchouri, Khagrachhori District in February 2007 and identified in Bangladesh National Herbarium where voucher specimens have been maintained representing this collection (Accession No. DACB-31377). After proper washing, the leaf and stem bark (after cutting into small pieces) was sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature (not more than 40 °C for better grinding. The dried plant material was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka, Bangladesh.

2.4.2 Extraction of the plant material

The powdered stem bark of *B. tomentosa* (575 g) was separately soaked in 1.5 L methanol and 325 g of powdered leaf of was also separately soaked in 750 mL methanol in a clean, round bottomed flask (5 and 3 L, respectively). The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at low temperature (36-40 ºC) and reduced pressure with a Buchii Rotavapour to have a concentrate of the crude extracts. The concentrated extract was then air dried to solid residue. The weight of the crude extract obtained from stem bark was 35 g and from leaf was 15.5 g.

2.4.3 Solvent-solvent partitioning of crude extract

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wagenen *et al*., (1993) as described in section 2.2.3. The whole partitioning process is schematically shown in Figure 2.1. All the fractions were evaporated to dryness and were used for further analysis.

The process was repeated for several times and after evaporation the weight of the different soluble fractions obtained is mentioned in Table 2.15.

Table 2.15: Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of *B. tomentosa*.

Plant Part	Weight (g) of methanolic extract for Kupchan partitioning	Fraction code	Weight (g) of soluble fractions
Stem bark	15	HSF	3.4
		CSF	0.94
		CHSF	1.28
		AQSF	6.5
Leaf	10	HSF	2.7
		CSF	0.83
		CHSF	0.69
		AQSF	4.5

HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction

2.4.4 Vacuum Liquid Chromatography (VLC) of the crude extract of stem bark of *B. tomentosa*

2.4.4.1 Packing of Column

The column was packed with fine VLC grade silica gel (Kieselgel 60H). A column having 14 cm length and 10 cm in diameter was packed with the silica gel up to a height

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of 6 cm under reduced pressure. The column was then washed with *n*-hexane to speed up compact packing.

2.4.4.2 Sample Input

The sample was prepared by adsorbing 20 g of crude extract onto column grade silica gel (Kieselgel 60H, mesh 70-230) with continuous stirring under air blower in a porcelain basin. Stirring continued until the sample becomes a free flowing powder. The free flowing sample was then placed on the packed silica into the column. Finally a cotton plug was placed sample.

2.4.4.3 Development of Chromatogram

After placing the sample, the column was eluted with *n*-hexane, followed by mixtures of *n*-hexane and ethyl acetate and finally washed by methanol. Solvent systems used as mobile phases in the VLC analysis of crude extract were listed in Table 2.16.

Table 2.16: Solvent systems used for VLC analysis of the crude extract of stem bark of *B. tomentosa*.

Fraction no.	Solvent systems	Volume collected (ml)
1	100% *n*-Hexane	50
2 (A+B)	*n*-Hexane - Ethyl acetate (98 : 2)	50 + 50
3 (A+B)	*n*-Hexane - Ethyl acetate (95 : 5)	50 + 50
4 (A+B)	*n*-Hexane - Ethyl acetate (92.5 : 7.5)	75 + 75
5 (A+B)	*n*-Hexane - Ethyl acetate (90 : 10)	75 + 75
6 (A+B)	*n*-Hexane - Ethyl acetate (80 : 20)	75 + 75
7 (A+B)	*n*-Hexane - Ethyl acetate (75 : 25)	75 + 75
8 (A+B)	*n*-Hexane - Ethyl acetate (60 : 40)	100 + 100
9 (A+B)	*n*-Hexane - Ethyl acetate (55 : 45)	100 + 100
10 (A+B)	*n*-Hexane - Ethyl acetate (50 : 50)	100 + 100
11 (A+B)	*n*-Hexane - Ethyl acetate (45 : 55)	100 + 100
12 (A+B)	*n*-Hexane - Ethyl acetate (40 : 60)	100 + 100
13 (A+B)	*n*-Hexane - Ethyl acetate (30 : 70)	100 + 100
14 (A+B)	*n*-Hexane - Ethyl acetate (25 : 75)	100 + 100
15 (A+B)	*n*-Hexane - Ethyl acetate (20 : 80)	100 + 100
16 (A+B)	*n*-Hexane - Ethyl acetate (15 : 85)	100 + 100
17 (A+B)	*n*-Hexane - Ethyl acetate (10 : 90)	100 + 100
18 (A+B)	*n*-Hexane - Ethyl acetate (5 : 95)	100 + 100
19 (A+B)	Ethyl acetate (100 %)	100 + 100
20 (A+B)	Ethyl acetate - Methanol (99:1)	50 + 50
21 (A+B)	Ethyl acetate - Methanol (97:3)	50 + 50
22 (A+B)	Ethyl acetate - Methanol (90:10)	50 + 50
23 (A+B)	Ethyl acetate - Methanol (75:25)	50 + 50

2.4.5 Analysis of VLC fractions by thin layer chromatography (TLC)

All the above VLC fractions were screened by TLC using various solvent systems. The plates were observed under UV light and by spraying with vanillin-sulfuric acid reagent followed by heating at 100-110 ºC for 5-10 minutes as well. Depending on the TLC behavior fractions 4(A+B), 5B & 6A, 7B & 8A and 12(A+B) & 13A were selected for further investigation.

Table 2.17: List of isolated compounds from VLC fraction of the methanolic extract of the stem bark of *B. tomentosa*.

Column fraction	TLC Solvent System	R_f value	Amount (mg)	Code
4(A+B)	Toluene - Ethyl acetate (95:5)	0.345	8.04	BT-1
5B & 6A	Toluene - Ethyl acetate (95:5)	0.455	5.11	BT-6
7B & 8A	Toluene - Ethyl acetate (90:10)	0.683	6.29	BT-8
12(A+B) & 13A	Toluene - Ethyl acetate (90:10)[3t]	0.321	6.31	BT-14

2.4.5.1 Isolation and purification of compounds from the selected VLC fractions

Four compounds was isolated and purified from the different VLC fractions by adopting various techniques.

2.4.5.2 Isolation of BT-1

Evaporation of solvents of the VLC fractions 4(A+B) furnished impure white crystalline mass. Repeated washing of these crystalline mass with *n*-hexane and then recrystallizing with *n*-hexane and ethyl acetate yielded white crystals. After TLC with solvent system toluene: ethyl acetate (95:5) a single spot was observed after spraying with vanillin-sulfuric acid reagent followed by heating in 110º C for 5 minutes. These crystals were designated as compound **BT-1** (8.04 mg).

2.4.5.3 Isolation of BT-6

The VLC fractions 5B & 6A were mixed together due to their identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (95:5)]. A deep purple colored band was visualized after spraying a portion of the developed plate with vanillin-sulfuric acid reagent followed by heating at 110 ºC for 5 minutes. The unsprayed portion of the band at stationary phase was scrapped off and eluted with ethyl acetate. Colorless mass was obtained which was checked for purity and termed as **BT-6** (5.11 mg).

2.4.5.4 Isolation of BT-8

Again, the VLC fractions 7B & 8A were mixed together due to similar TLC characteristics and subjected to preparative TLC [stationary phase - silica gel F_{254}, mobile phase: toluene-ethyl acetate (90:10)], the process was repeated thrice for better separation and isolation of the desired band. From the developed plates a purple colored band was visualized after spraying a small portion of the PTLC plate with vanillin-sulfuric acid reagent followed by heating at 110 ºC for 5 minutes. The unsprayed stationary phase was scrapped off and eluted with ethyl acetate. After evaporation of the solvent fine tree like crystals were obtained which was termed as **BT-8** (6.29 mg).

2.4.5.5 Isolation of BT-14

Again, the VLC fractions 12A & 13B were mixed together due to identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (90:10)]. The process was repeated thrice for better separation and isolation of the desired band. From the developed plates a purple colored band was visualized after spraying a small portion of the PTLC plate with vanillin-sulfuric acid reagent followed by heating at 110 ºC for 5 minutes. The unsprayed portion of the stationary phase was scrapped off and eluted with ethyl acetate. After evaporation of the solvent fine tree like crystals were obtained which after purity test was termed as **BT-14** (6.31 mg).

2.4.6 Properties of the isolated compounds

The physical properties of the isolated compounds and their reactions to vanillin-H_2SO_4 spray reagent are summarized in the Table 2.18.

Table 2.18: Properties of the isolated compounds from the stem bark of *B. tomentosa*.

Isolated compounds	Physical form	Color	Color with vanillin-H_2SO_4
BT-1	Crystal	White	Violet
BT-6	Amorphous	Whitish	Deep purple
BT-8	Crystals	Tree like	Purplish
BT-14	Crystal	Tree like	Purple

2.5 Phytochemical investigation of *Erythrina fusca*

2.5.1 Collection and preparation of the plant material

Leaf and stem bark of *E. fusca* was collected from Nandail, Mymensingh District in September 2011 and identified in Bangladesh National Herbarium where voucher specimens have been maintained representing this collection (Accession No. DACB-

35902). After proper washing, the leaf and stem bark (after cutting into small pieces) was sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature (not more than 40 °C) for better grinding. The dried plant material was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka, Bangladesh.

2.5.2 Extraction of the plant material

The powdered stem bark of *E. fusca* (800 g) was separately soaked in 2.25 L methanol and powdered leaf of (550 g) was also separately soaked in 1.5 mL methanol in a clean, round bottomed flask (7.5 and 5 L, respectively). The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 20 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at low temperature (36-40 ºC) and reduced pressure with a Buchii Rotavapour to have a concentrate of the crude extracts. The concentrated extract was then air dried to solid residue. The weight of the crude extract obtained from stem bark was 40 g and from leaf was 25 g.

2.5.3 Solvent-solvent partitioning of crude extract

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wagenen *et al.*, (1993) as described in section 2.2.3. The whole partitioning process is schematically shown in Figure 2.1. All the fractions were evaporated to dryness and were used for further analysis.

The process was repeated for several times and after evaporation the weight of the different soluble fractions obtained is mentioned in Table 2.19.

Table 2.19: Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of *E. fusca*.

Plant Part	Weight (g) of methanolic extract for Kupchan partitioning	Fraction code	Weight (g) of soluble fractions
Stem bark	30	HSF	8.25
		CSF	2.50
		CHSF	2.00
		AQSF	13.1
Leaf	15	HSF	4.9
		CSF	1.23
		CHSF	0.95
		AQSF	6.7

HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction

2.5.4 Column chromatography of carbon tetrachloride soluble fraction of the stem bark of *E. fusca*

A portion of carbon tetrachloride soluble fraction (1.4 g) was subjected to column chromatography (CC) for fractionation. The column was packed with silica gel (Kieselgel 60H, mesh 70-230). Slurry of silica gel in petroleum ether (60-80 ºC) was added into a glass column having a length and diameter of 84 and 2 cm, respectively. When the desired height of adsorbent bed was obtained, a few hundred milliliter of solvent was run through the stationary phase for proper packing of the column. After packing, the sample was prepared by adsorbing carbon tetrachloride soluble material onto silica gel (Kieselgel 60H, mesh 70-230) and allowed to dry up to a free flowing powder like mass and subsequently applied on top of the adsorbent layer. Then the column was eluted with petroleum ether, followed by mixtures of petroleum ether and ethyl acetate and then ethyl acetate, finally with ethyl acetate and methanol in order of increasing polarities. Solvent system used as mobile phase in CC analysis of carbon tetrachloride soluble material and has been listed in Table 2.20. The eluates were collected in test tubes. A total of 250 fractions were collected each 20 ml.

Table 2.20: Different solvent systems used for the column chromatographic analysis of the carbon tetrachloride soluble fraction of the stem bark of *E. fusca*.

Fraction no.	Solvent systems	Volume collected ml/test tube
1 - 7	Petroleum ether - Ethyl acetate = (99 : 1)	20
8 - 19	Petroleum ether - Ethyl acetate = (98 : 2)	20
20 - 34	Petroleum ether - Ethyl acetate = (97 : 3)	20
35 - 45	Petroleum ether - Ethyl acetate = (95 : 5)	20
46 - 61	Petroleum ether - Ethyl acetate = (92.5 :7.5)	20
62 - 76	Petroleum ether - Ethyl acetate = (90: 10)	20
77 - 86	Petroleum ether - Ethyl acetate = (87.5: 12.5)	20
87 - 110	Petroleum ether - Ethyl acetate = (85 : 15)	20
111 - 142	Petroleum ether - Ethyl acetate = (80 : 20)	20
143 - 158	Petroleum ether - Ethyl acetate = (75 : 25)	20
159 - 173	Petroleum ether - Ethyl acetate = (70 : 30)	20
174 - 182	Petroleum ether - Ethyl acetate = (65 : 35)	20
183 - 190	Petroleum ether - Ethyl acetate = (55 : 45)	20
191 - 207	Petroleum ether - Ethyl acetate = (50 : 50)	20
208 - 211	Petroleum ether - Ethyl acetate = (35 : 65)	20
212 - 220	Petroleum ether : Ethyl acetate = (20 : 80)	20
221 - 229	Ethyl acetate (100%)	20
230 - 237	Ethyl acetate - Methanol = (99 : 1)	20
238 - 242	Ethyl acetate - Methanol = (97 : 3)	20
243 - 250	Ethyl acetate - Methanol = (95 : 5)	20

2.5.5 Analysis of column fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with vanillin sulfuric acid reagent followed by heating at 100-110 ºC for 5-10 minutes as well. Depending on the TLC behavior a number of similar samples were detected and the column fractions were added, these were then purified by employing various techniques. A list of isolated compounds has been summarized in Table 2.21.

Table 2.21: List of isolated compounds from carbon tetrachloride soluble fraction of the methanolic extract of the tem bark of *E. fusca*.

Column fraction	Mobile phase	R_f value	Amount (mg)	Code
90-97	Toluene - Ethyl acetate (97:3)	0.657	6.12	EF-7
90-97	Toluene: Ethyl acetate (97:3)	0.600	8.20	EF-8
117-122	Toluene - Ethyl acetate (90:10)	0.218	4.50	EF-12
119-127	Toluene - Ethyl acetate (85:15)	0.571	6.87	EF-38

2.5.5.1 Isolation of compound EF-7

EF-7 was isolated as yellowish mass by evaporation of solvent from the silica column fractions 90-97 of the carbon tetrachloride soluble portion of methanolic extract of the stem bark of *E. fusca*. The yellowish residue was then subjected to preparative TLC over silica gel F_{254} using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated thrice for better separation of the desired band. After excitation with 254 nm UV light, quenching was observed by the desired band as a uniform layer. Then the band was scrapped off on an aluminum foil and eluted with ethyl acetate. Evaporation of the solvent yielded an oily transparent liquid. It was checked for purity and denoted as EF-7 (6.12 mg).

2.5.5.2 Isolation of compound EF-8

Evaporation of solvents from the CC fractions 90-97 of the carbon tetrachloride soluble fraction provided a yellowish mass. The fraction was then subjected to a preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (97:3)]. The process was repeated thrice for better separation of the desired band. The desired band was identified under UV light, then scrapped off and eluted with ethyl acetate to yield EF-8 (8.20 mg) as yellow needles on standing at room temperature.

2.5.5.3 Isolation of compound EF-12

Sub-fractions 90-97 showing identical spots on TLC were combined in a beaker. After drying the solvent a yellowish mass was obtained which was then subjected to preparative TLC over silica gel F_{254} using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated five times for better separation of the desired band. A small portion of the developed plate was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a pink colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent yielded a compound EF-12 (4.5 mg) as amorphous powder.

2.5.5.4 Isolation of compound EF-38

Sub-fractions 119-227 showing identical spots on TLC were combined in a beaker. The fraction was then subjected to a preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (85:15)]. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating in 110 ºC for 5 minutes and a purple colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent yielded white needles. The compound was analyzed over TLC to check for purity and found to be a pure compound which was termed as EF-38 (6.87 mg).

2.5.5.5 Properties of the isolated compounds

The physical properties of the isolated compounds and their reactions to vanillin-H_2SO_4 spray reagent are summarized in the Table 2.22.

Table 2.22: Properties of the isolated compounds from stem bark of *E. fusca*.

Isolated compounds	Physical form	Color	Color with vanillin-H_2SO_4
EF-7	Oily liquid	Transparent	Magenta
EF-8	Mass	Yellow	Deep orange
EF-12	Powder	White	Pink
EF-38	Needles	White	Scarlet

2.6 Phytochemical investigation of *Erythrina variegata*

2.6.1 Collection and preparation of the plant material

Leaf and stem bark of *E. variegata* was collected from Savar, Dhaka District in September 2011 and identified in Bangladesh National Herbarium where voucher specimens have been maintained representing this collection (Accession No. DACB-35901). After proper washing, the leaf and stem bark (after cutting into small pieces) was sun dried for several days. The plant materials were then oven dried for 24 hours at

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considerably low temperature (not more than 40 °C) for better grinding. The dried plant material was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka, Bangladesh.

2.6.2 Extraction of the plant material

The powdered stem bark of *E. variegata* (650 g) was separately soaked in 1.75 L methanol and 450 g of powdered leaf of was also separately soaked in 1 L methanol in a clean, round bottomed flask (5 and 3 L, respectively). The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 20 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at low temperature (36-40 ℃) and reduced pressure with a Buchii Rotavapour to have a concentrate of the crude extracts. The concentrated extract was then air dried to solid residue. The weight of the crude extract obtained from stem bark was 30 g and from leaf was 15 g.

2.6.3 Solvent-solvent partitioning of crude extract

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wagenen *et al.*, (1993) as described in section 2.2.3. The whole partitioning process is schematically shown in Figure 2.1. All the fractions were evaporated to dryness and were used for further analysis.

The process was repeated for several times and after evaporation the weight of the different soluble fractions obtained is mentioned in Table 2.23.

Table 2.23: Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of *Erythrina variegata*.

Plant Part	Weight (g) of methanolic extract for Kupchan partitioning	Fraction code	Weight (g) of soluble fractions
Stem bark	10	HSF	2.75
		CSF	0.65
		CHSF	0.92
		AQSF	4.30
Leaf	10	HSF	3.30
		CSF	0.81
		CHSF	0.71
		AQSF	4.20

HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction

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3.1 Characterization of BVSP-1 as glochidonol (46)

The *n*-hexane soluble portion of the crude methanolic extract of the stem bark of *B. verrucosa* was subjected to silica gel column chromatography by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities to obtain 43 fractions. Fractions 11-18 showing identical spots on TLC were combined in a beaker and it was kept at room temperature undisturbed for several days. The crystals obtained after evaporation of solvents were washed with *n*-hexane to remove the adhering coloring materials and recrystallization from *n*-hexane and ethyl acetate to afforded needle like crystals designated as BVSP-1 (7.23 mg, **46**). It was found to be soluble in chloroform, dichloromethane and ethyl acetate.

The crystals of BVSP-1 melted at 227-229 ºC which was similar to that reported (228-230 ºC) for glochidonol (Thu *et al.*, 2010). The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$; Figures 3.2-3.3; Table 3.1) exhibited typical signals for 48 protons including seven tertiary methyl, a carbinol and a terminal exomethylene proton. In the ^{1}H-NMR of BVSP-1 seven three proton singlets indicated seven tertiary methyl groups at δ 1.03 (H-23), 1.03 (H-24), 0.80 (H-25), 1.01 (H-26), 0.95 (H-27), 0.78 (H-28) and 1.66 (H-30). The spectrum also demonstrated a proton doublet at δ 3.80 (1H, d, *J*= 2.8 Hz) corresponding to oxymethine proton at C-1. It also showed olefinic protons at δ 4.54 (H_a-29) and δ 4.66 (H_b-29, d, *J*= 1.6 Hz).

The ^{13}C-NMR (125 MHz, $CDCl_3$; Figures 3.4-3.6; Table 3.2) displayed a total of 30 carbon resonances including a carbonyl carbon at δ 215. 4. The ^{13}C-NMR spectrum also showed typical signal at δ 79.0 for the oxymethine carbon at C-1 while two signals at δ 150.8 and δ 109.7 could be assigned to C-20 and C-29, respectively.

All these features are indicative of the presence of a lupane-type triterpenoid type skeleton in BVSP-1.

The melting point and ^{1}H and ^{13}C-NMR spectral data (Tables 3.1 and 3.2, respectively) of BVSP-1 were compared with the published data of glochidonol and found to be identical (Thu *et al.*, 2010). Thus, compound BVSP-1 was characterized as glochidonol (**46**). This is the first report of isolation of glochidonol from *B. verrucosa*.

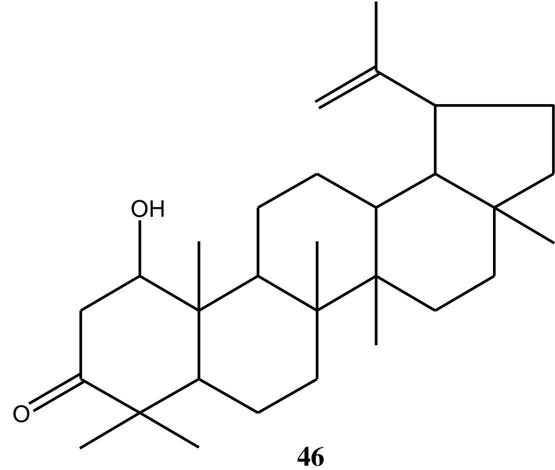

Figure 3.1: Structure of BVSP-1 (glochidonol, **46**).

Table 3.1: ^{1}H-NMR spectral data of BVSP-1 (46) and glochidonol (Thu *et al.*, 2010) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	BVSP-1 (46)	**Glochidonol**
H-1	3.80 d (2.8)	3.89 1H, d (2.8)
H_a-2	2.99 dd (14.3, 8.1)	3.00 dd (14.5, 8.0)
H_b-2	2.38 m	2.38 m
Me-23	1.03 s	1.06 s
Me-24	1.03 s	1.06 s
Me-25	0.80 s	0.84 s
Me-26	1.01 s	1.05 s
Me-27	0.95 s	0.98 s
Me-28	0.78 s	0.80 s
H_a-29	4.54 s	4.56 s
H_b-29	4.66 d (1.6)	4.68 d (1.5)
Me-30	1.66 s	1.68 s

Table 3.2: ^{13}C-NMR spectral data of BVSP-1 (46) and glochidonol (Thu *et al*., 2010) in $CDCl_3$.

Position of carbon	Glochidonol	BVSP-1 (46)
C-1	79.7	79.0
C-2	45.2	45.3
C-3	215.5	215.4
C-4	47.2	47.2
C-5	51.5	53.3
C-6	19.7	18.9
C-7	33.1	33.0
C-8	43.0	43.0
C-9	50.8	49.4
C-10	43.0	43.0
C-11	23.2	22.5
C-12	25.3	25.7
C-13	38.1	38.1
C-14	41.3	41.5
C-15	27.6	27.6
C-16	35.6	35.6
C-17	43.0	43.1
C-18	48.9	48.4
C-19	48.0	48.0
C-20	150.7	150.8
C-21	29.9	28.4
C-22	40.0	39.9
C-23	27.9	27.9
C-24	19.9	20.5
C-25	11.8	11.8
C-26	16.0	16.4
C-27	14.5	14.9
C-28	18.1	18.2
C-29	109.5	109.7
C-30	19.4	20.4

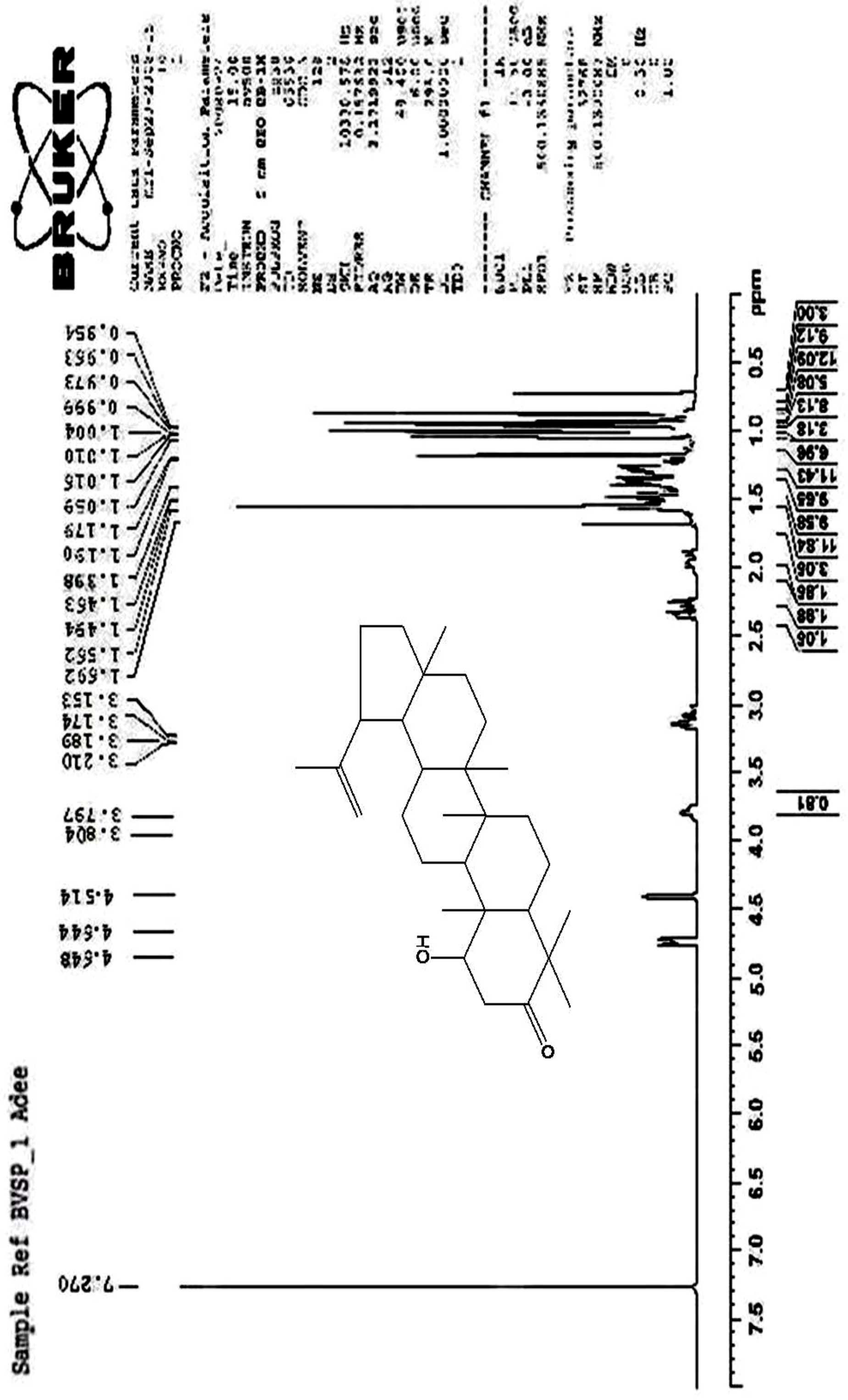

Figure 3.2: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVSP-1 (46).

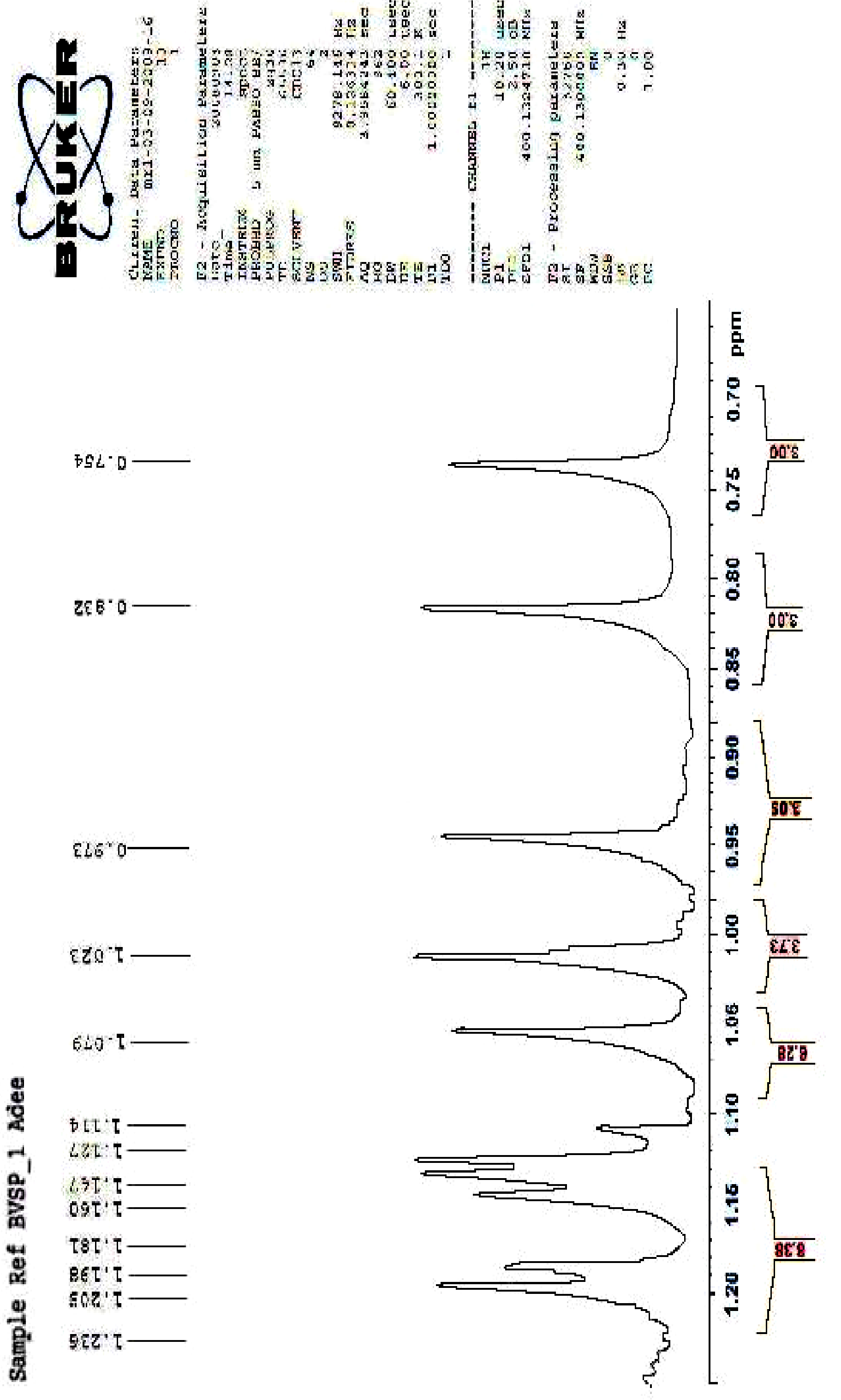

Figure 3.3: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVSP-1 (46).

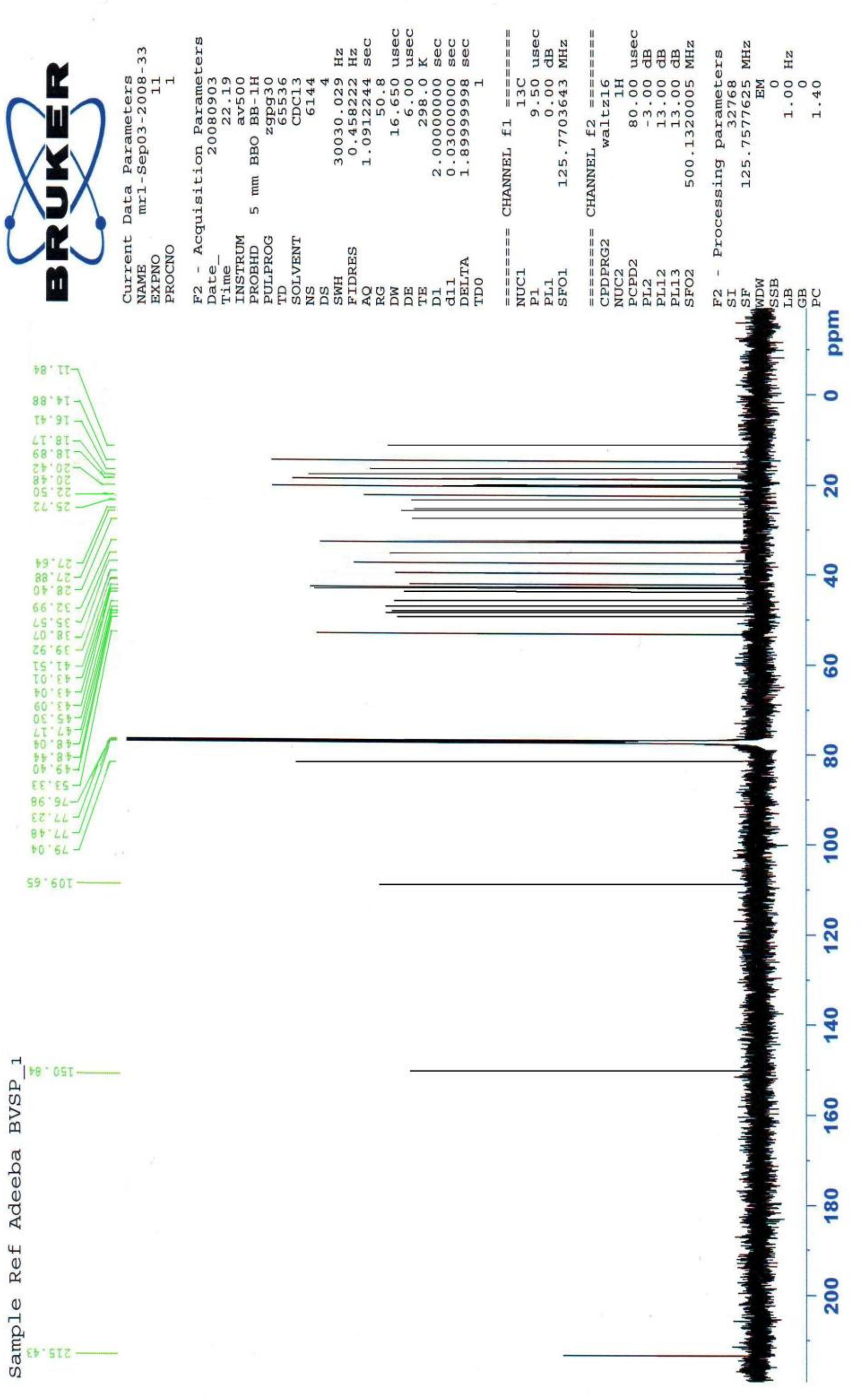

Figure 3.4: ^{13}C-NMR spectrum of BVSP-1 (46) in $CDCl_3$.

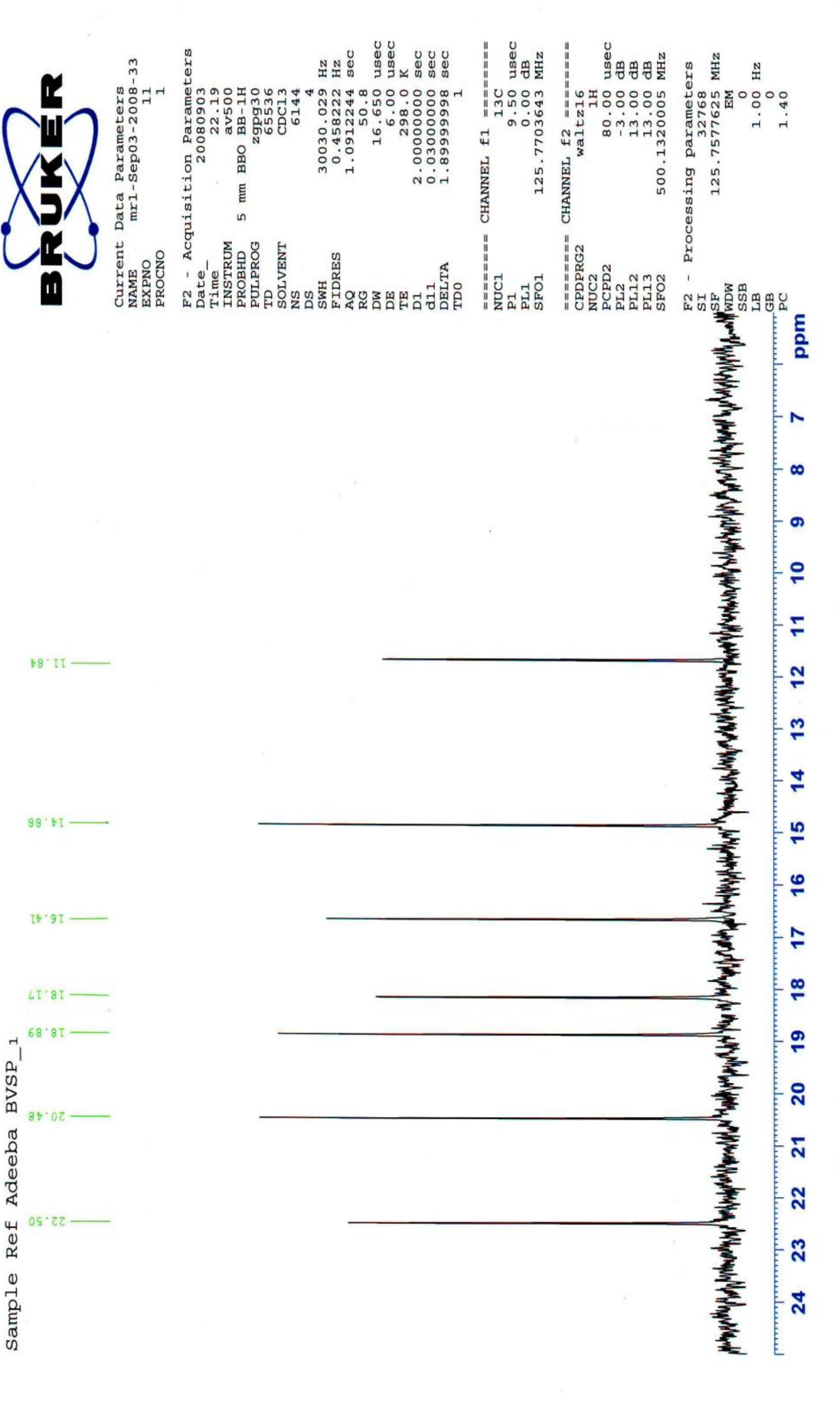

Figure 3.5: Partially expanded ^{13}C-NMR spectrum of BVSP-1 (46) in $CDCl_3$.

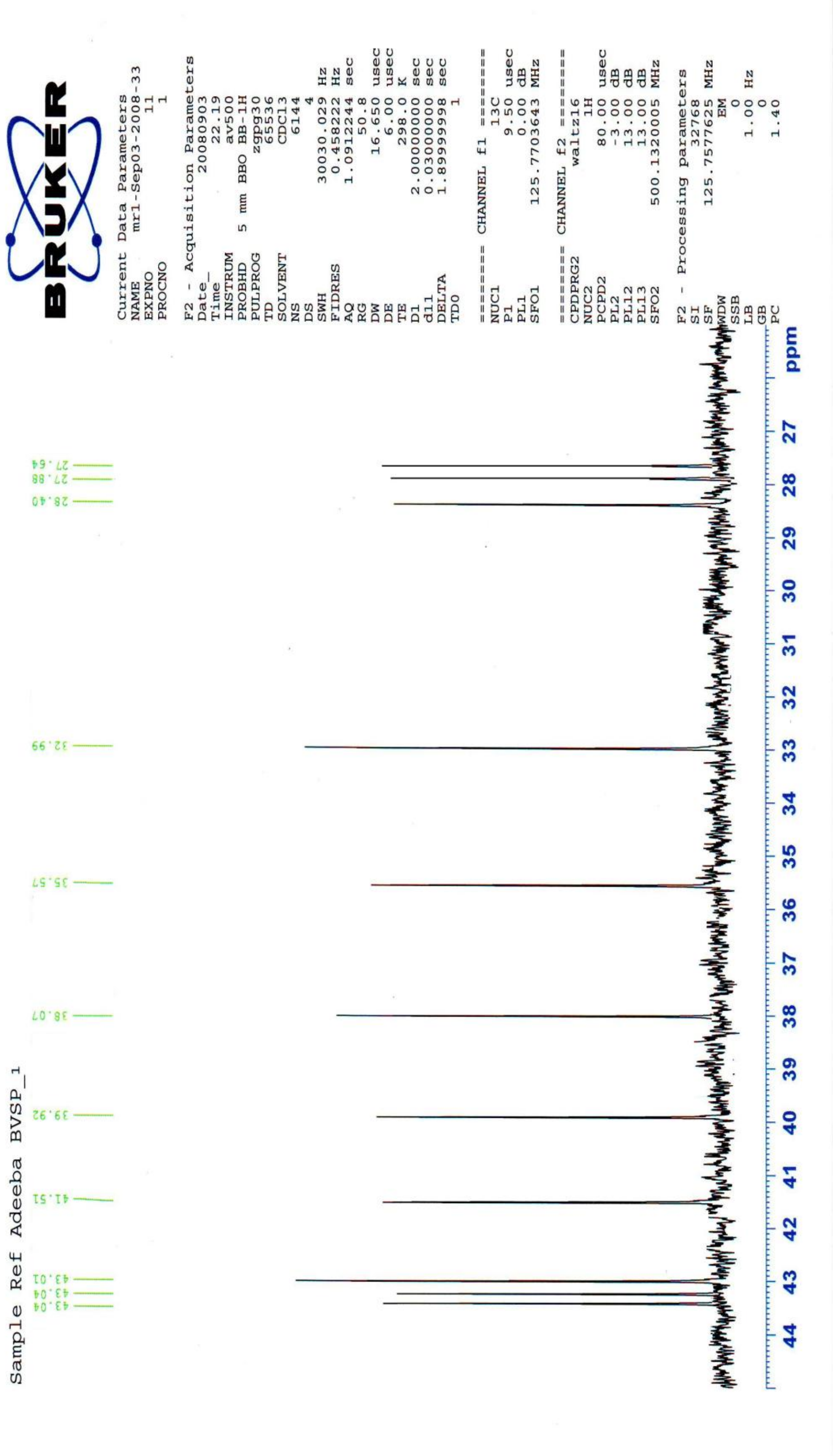

Figure 3.6: Partially expanded ^{13}C-NMR spectrum of BVSP-4 (46) in $CDCl_3$.

3.2 Characterization of BVSP-2 as brassicasterol (47)

The *n*-hexane soluble portion of the crude methanol extract of the stem bark of *B. verrucosa* was subjected to silica gel column chromatography by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities to obtain forty three fractions.
Fractions 19-23 were combined together depending on identical TLC behaviour and subjected to preparative TLC over silica gel F_{254} using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated three times for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a purple colored band was visualized. The band was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent afforded colorless needles of BVSP-2 (6.78 mg, **50**). The melting point of the crystals of BVSP-2 was found to be at 153-156 ºC which was similar to that reported (154-156 ºC) for brassicasterol (Jinming *et al.*, 2001). It was soluble in chloroform, dichloromethane and ethyl acetate.
The ^{1}H-NMR spectrum (500 MHz, $CDCl_3$; Figures 3.8-3.9; Table 3.3) showed a multiplet of one proton intensity at δ 3.62 for a oxymethine proton at H-3. The typical signal for the olefinic C-6 proton of the steroidal skeleton was evident from a doublet at δ 5.36 (*J*= 6.0 Hz) that integrated for one proton. The *trans*-olefinic protons H-22 and H-23 appeared as characteristic downfield signals at δ 5.17 (2H, m) and δ 5.20 (2H, m) due to couplings with the neighbouring olefinic and methine protons. The spectrum further revealed signals at δ 0.69 and 0.83 (3H each) assignable to two tertiary methyl groups at C-13 (H_3-18) and C-10 (H_3-19), respectively. The ^{1}H-NMR spectrum also showed four doublets of three proton intensity each at δ 1.00 (3H, d, *J*= 6.5 Hz), 0.84 (3H, d, *J*= 6.5 Hz), 0.93 (3H, d, *J*= 6.5 Hz) and1.21 (3H, d, *J*= 6.5 Hz) assignable to the methyl groups at Me-21, Me-26, Me-27, Me-28, respectively. All these features are indicative of the presence of a steroidal nucleus.
Furthermore, the melting point and ^{1}H-NMR spectral data (Table 3.3) of BVSP-2 were compared with the reported values of brassicasterol and found to be identical (Jinming *et al.*, 2001). Thus, BVSP-2 was identified as brassicasterol (**50**). This is the first report of isolation of brassicasterol from *B. verrucosa*.

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Figure 3.7: Structure of BVSP-2 (brassicasterol, **47**).

Table 3.3: ^{1}H- NMR spectral data of BVSP-2 (47) and brassicasterol (Jinming *et al*., 2001) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	BVSP-2 (47)	**Brassicasterol**
H-3	3.62 m	3.53 m
H-6	5.36 d (6.0)	5.35 d (5.2)
H-18	0.69 s	0.69 s
H-19	0.83 s	0.83 s
Me-21	1.00 d (6.5)	1.00 d (6.5)
H-22	5.17 m	5.17-5.20 m
H-23	5.22 m	5.17-5.20 m
Me-26	0.84 d (6.5)	0.84 d (6.5)
Me-27	0.93 d (6.5)	0.91 d (6.8)
Me-28	1.21 d (6.5)	1.17 d (6.5)

Figure 3.8: ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of BVSP-2 (47).

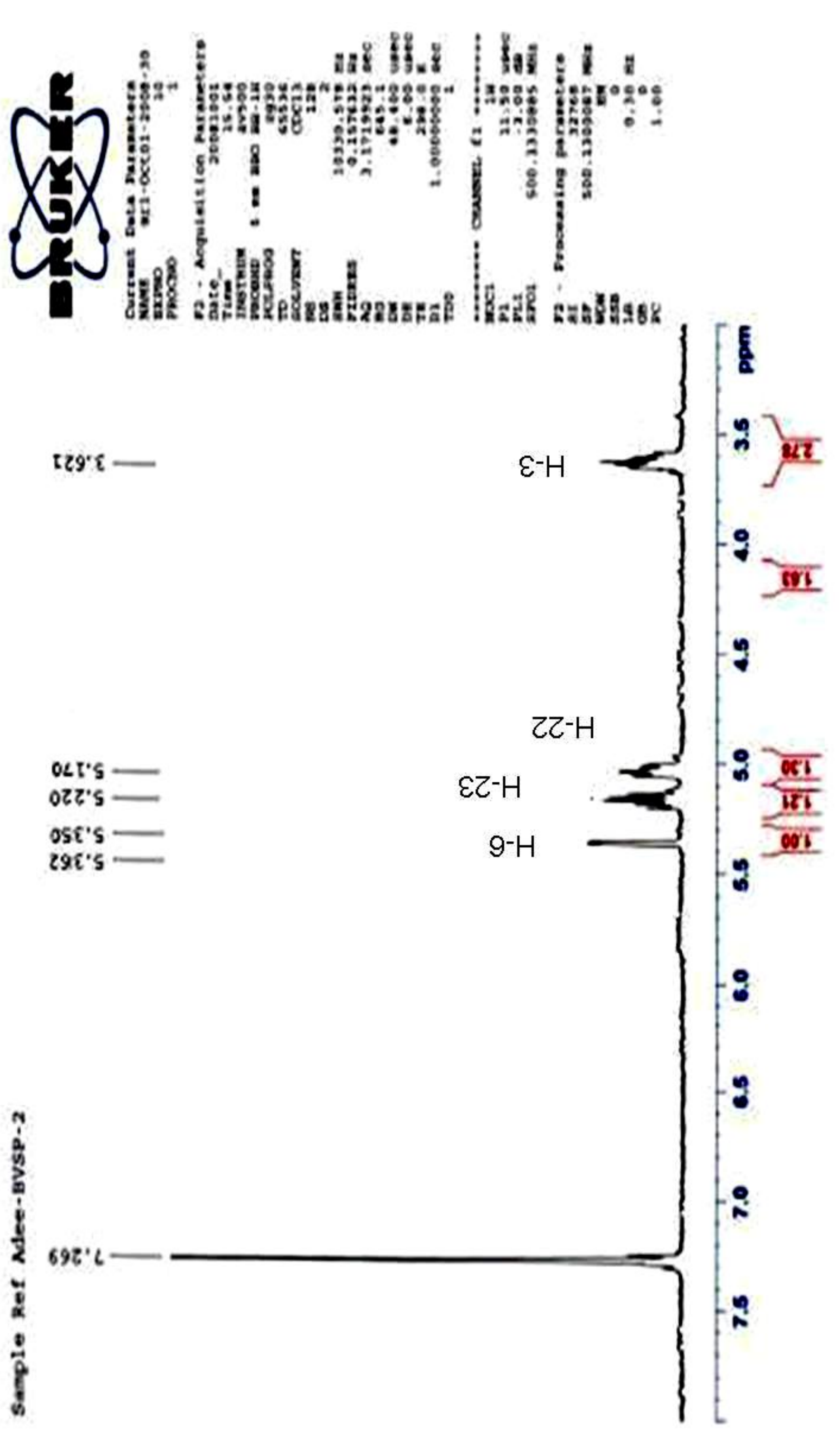

Figure 3.9: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of BVSP-2 (47).

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3.3 Characterization of BVSP-4 as friedelin (48)

The *n*-hexane soluble portion of the crude methanolic extract of the stem bark of *B. verrucosa* was subjected to silica gel column chromatography by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities to obtain forty three fractions. Fractions 1-4 showing identical spots on TLC were combined in a beaker and it was kept at room temperature undisturbed for several days. The crystals obtained after evaporation of solvents were washed with *n*-hexane to remove the adhered coloring materials and recrystallized from *n*-hexane and ethyl acetate to afford a pure compound as needles designated as BVSP-4 (5.3 mg, **48**). It was found to be soluble in chloroform, dichloromethane and ethyl acetate.

The crystals of BVSP-4 melted at 268 ºC which was identical to that reported (267 ºC) for friedelin (Akihisa *et al.*, 1992). The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$; Figures 3.11-3.12; Table 3.4) showed seven singlets between δ 0.74-1.19 corresponding to seven methyl groups and a doublet of three proton intensity at δ 0.90 (3H, J= 6.4 Hz). These confirmed the presence of eight methyl groups in a pentacyclic type triterpenoid skeleton. However, the lack of any olefinic proton resonance around δ 5.00-5.50 and oxymethine proton signal around δ 3.00 demonstrated the absence of double bond between C-12 and C-13 and the oxymethine proton at C-3.

The ^{13}C-NMR (125 MHz, $CDCl_3$; Figures 3.13-3.16; Table 3.5) displayed 30 carbon resonances including a carbonyl carbon at δ 213.54 ppm. The DEPT spectrum (Figures 3.17-3.19) indicated that twenty three out of the thirty carbons in BVSP-4 had attached protons. The DEPT experiment further revealed the presence of eight methyl, eleven methylene, four methine and seven quarternary carbon atoms. All these features are indicative of the presence of a triterpenoid type carbon skeleton in BVSP-4 having no double bond between C-12 and C-13, and no oxymethine proton at C-3.

The melting point, ^{1}H and ^{13}C-NMR spectral data (Tables 3.4 and 3.5, respectively) of BVSP-4 were compared with the published data of friedelin and found to be identical (Akihisa *et al.*, 1992). Thus, compound BVSP-4 was characterized as friedelin (**48**). This is the first report of isolation of friedelin from *B. verrucosa*.

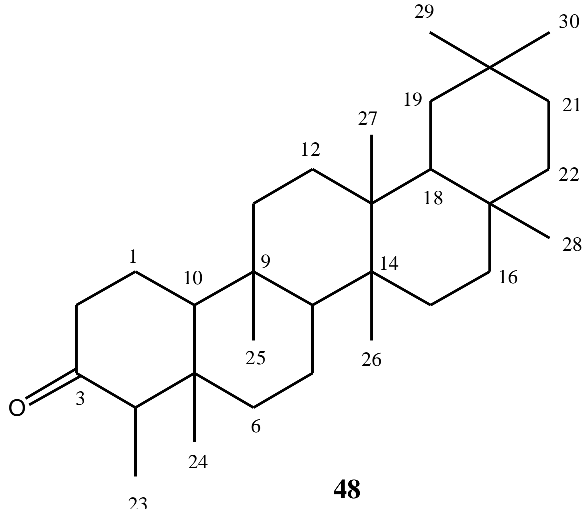

Figure 3.10: Structure of BVSP-4 (friedelin, **48**).

Table 3.4: ^{1}H- NMR spectral data of BVSP-4 (48) and friedelin (Akihisa *et al.*, 1992) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	BVSP-4 (48)	**Friedelin**
Me-23	0.90 d (6.4)	0.88 d (7.0)
Me-24	0.74 s	0.73 s
Me-25	0.88 s	0.87 s
Me-26	1.02 s	1.01 s
Me-27	1.06 s	1.05 s
Me-28	1.19 s	1.18 s
Me-29	0.97 s	0.95 s
Me-30	1.01 s	1.00 s

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Table 3.5: ^{13}C-NMR spectral data of BVSP-4 (48) and friedelin (Akihisa *et al*., 1992) in $CDCl_3$.

Position of carbon	Type of carbon	Friedelin	BVSP-4 (48)
C-1	CH_2	22.3	22.5
C-2	CH_2	41.5	41.5
C-3	>C=O	213.2	213.5
C-4	CH	58.2	58.5
C-5	C	42.1	42.4
C-6	CH_2	41.3	41.8
C-7	CH_2	18.2	18.5
C-8	CH	53.1	53.3
C-9	C	37.4	37.7
C-10	CH	59.4	59.7
C-11	CH_2	35.6	35.9
C-12	CH_2	30.5	30.1
C-13	C	39.7	39.2
C-14	C	38.4	38.5
C-15	CH_2	32.4	32.9
C-16	CH_2	36.0	36.0
C-17	C	30.0	30.2
C-18	CH	42.8	43.0
C-19	CH_2	35.3	35.4
C-20	C	28.1	28.4
C-21	CH_2	32.7	32.6
C-22	CH_2	39.2	39.5
C-23	CH_3	6.8	7.0
C-24	CH_3	14.6	14.8
C-25	CH_3	17.9	18.1
C-26	CH_3	20.2	20.4
C-27	CH_3	18.6	18.9
C-28	CH_3	32.1	32.0
C-29	CH_3	35.0	35.0
C-30	CH_3	31.8	31.3

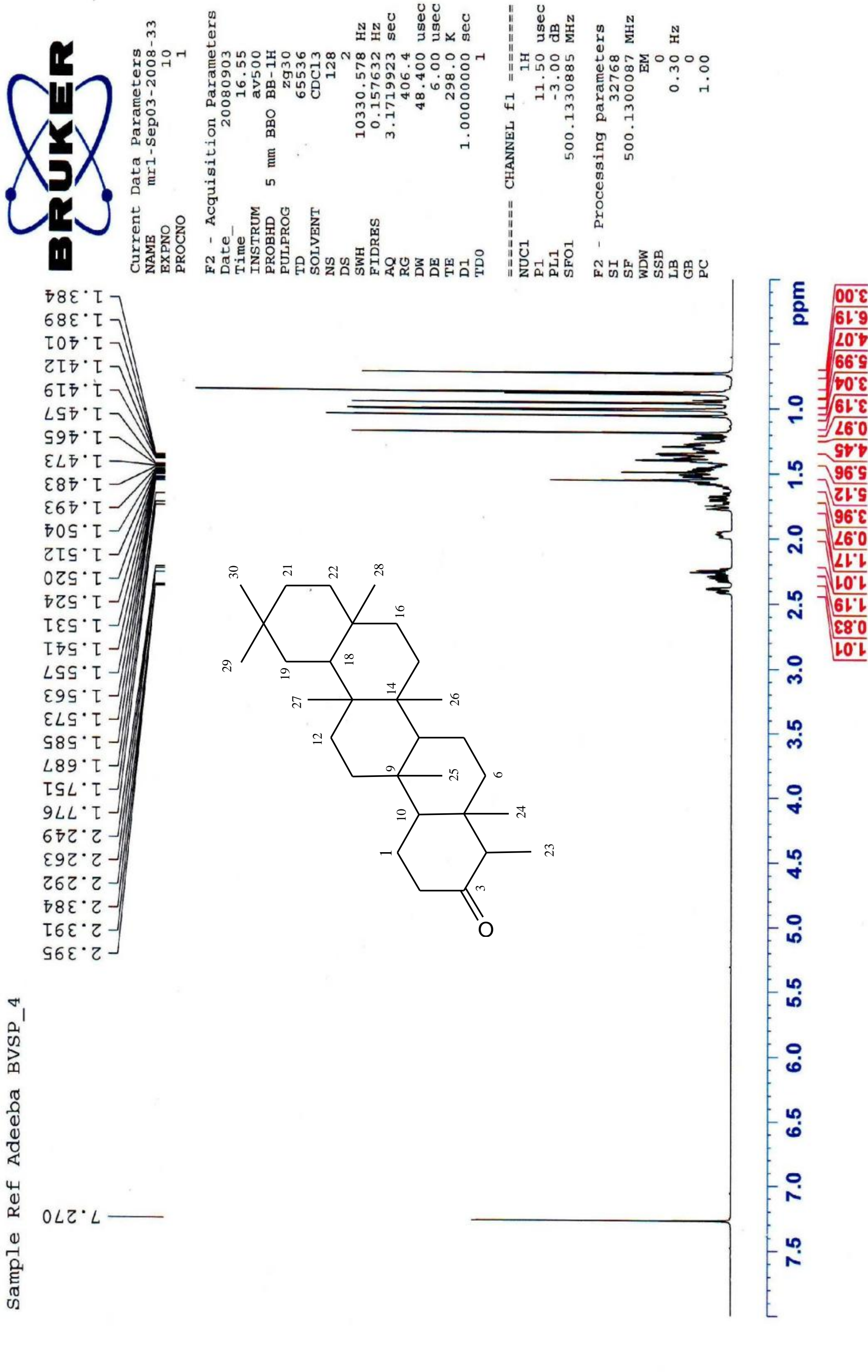

Figure 3.11: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVSP-4 (48).

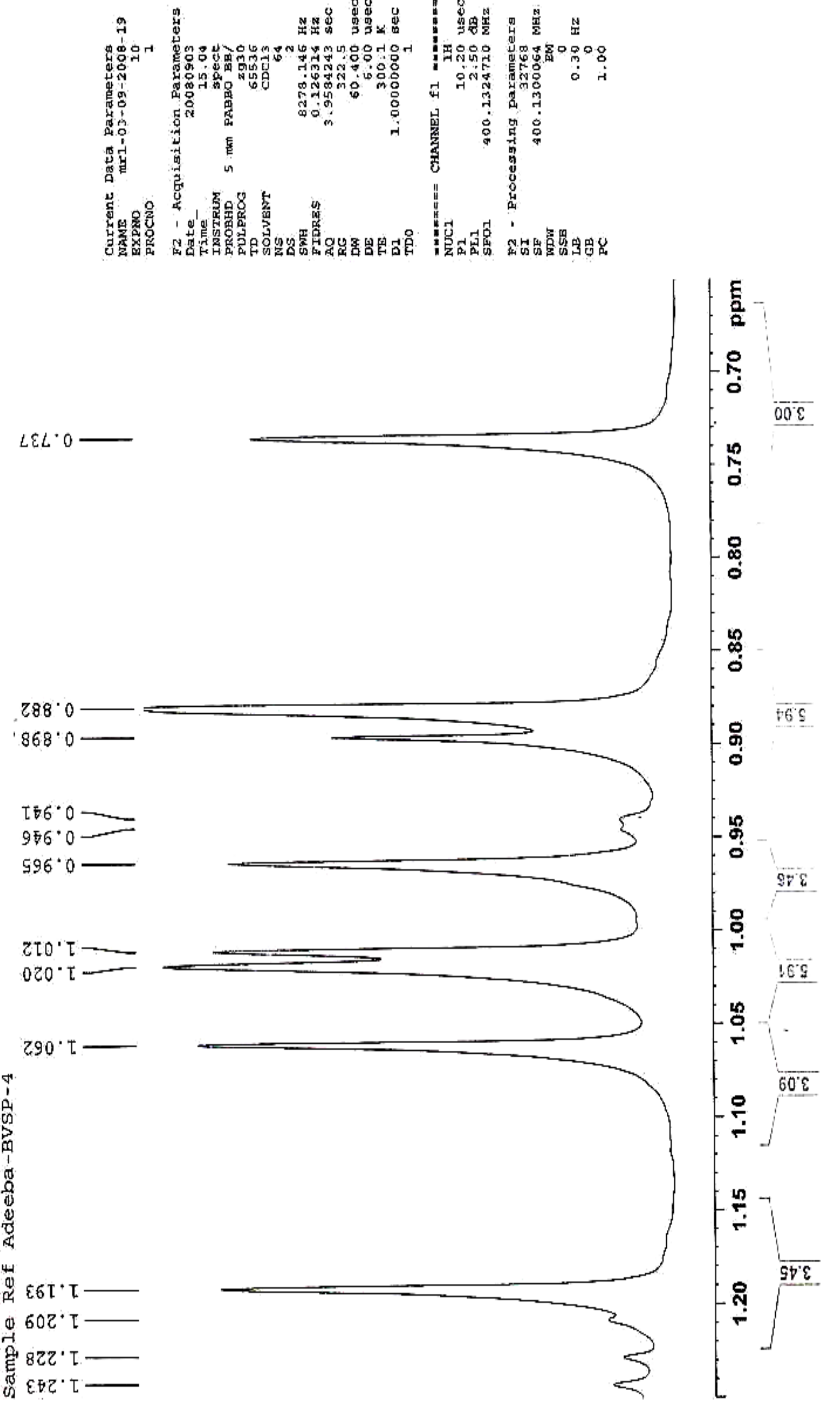

Figure 3.12: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVSP-4 (48).

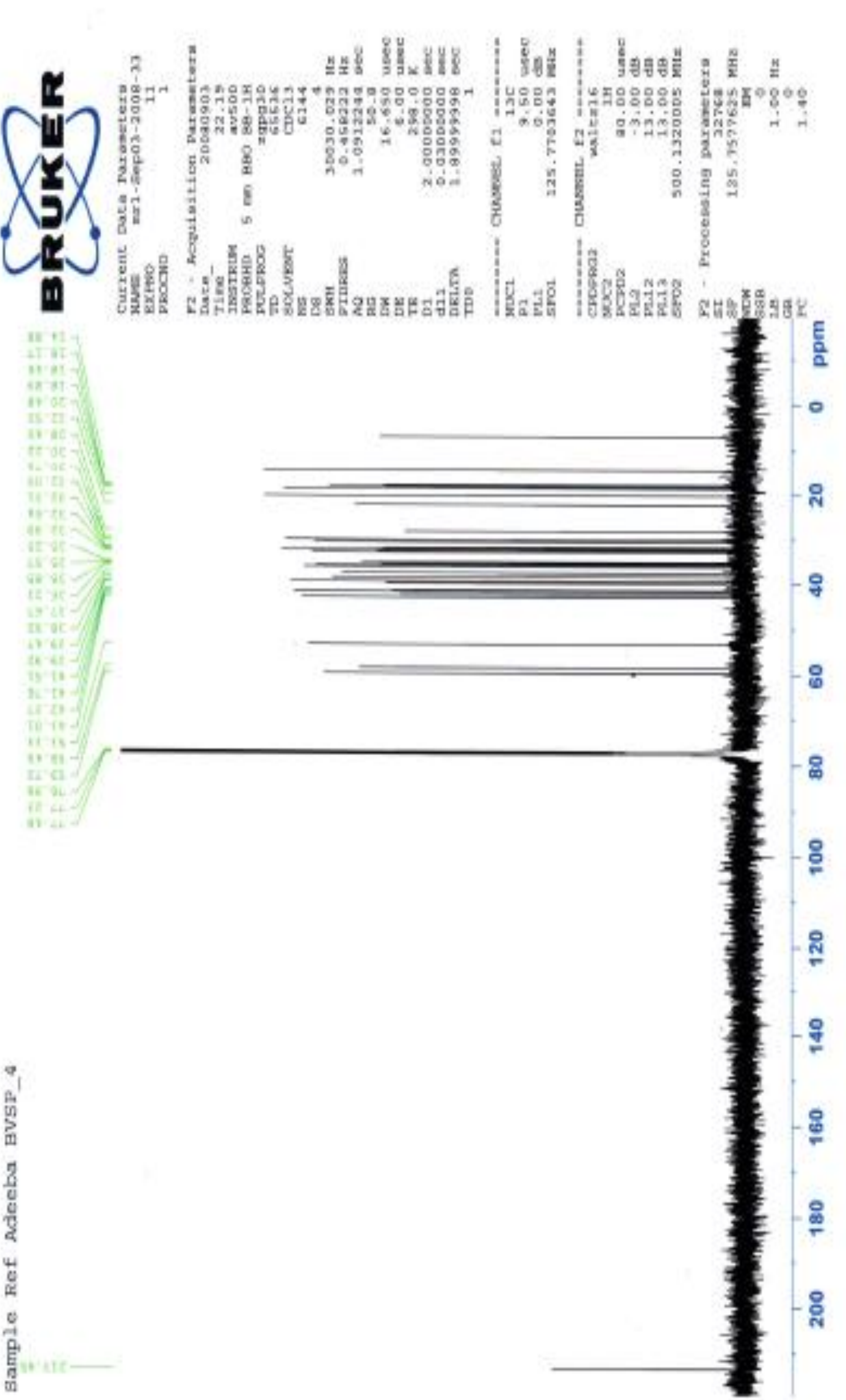

Figure 3.13: ^{13}C-NMR spectrum of BVSP-4 (48) in $CDCl_3$.

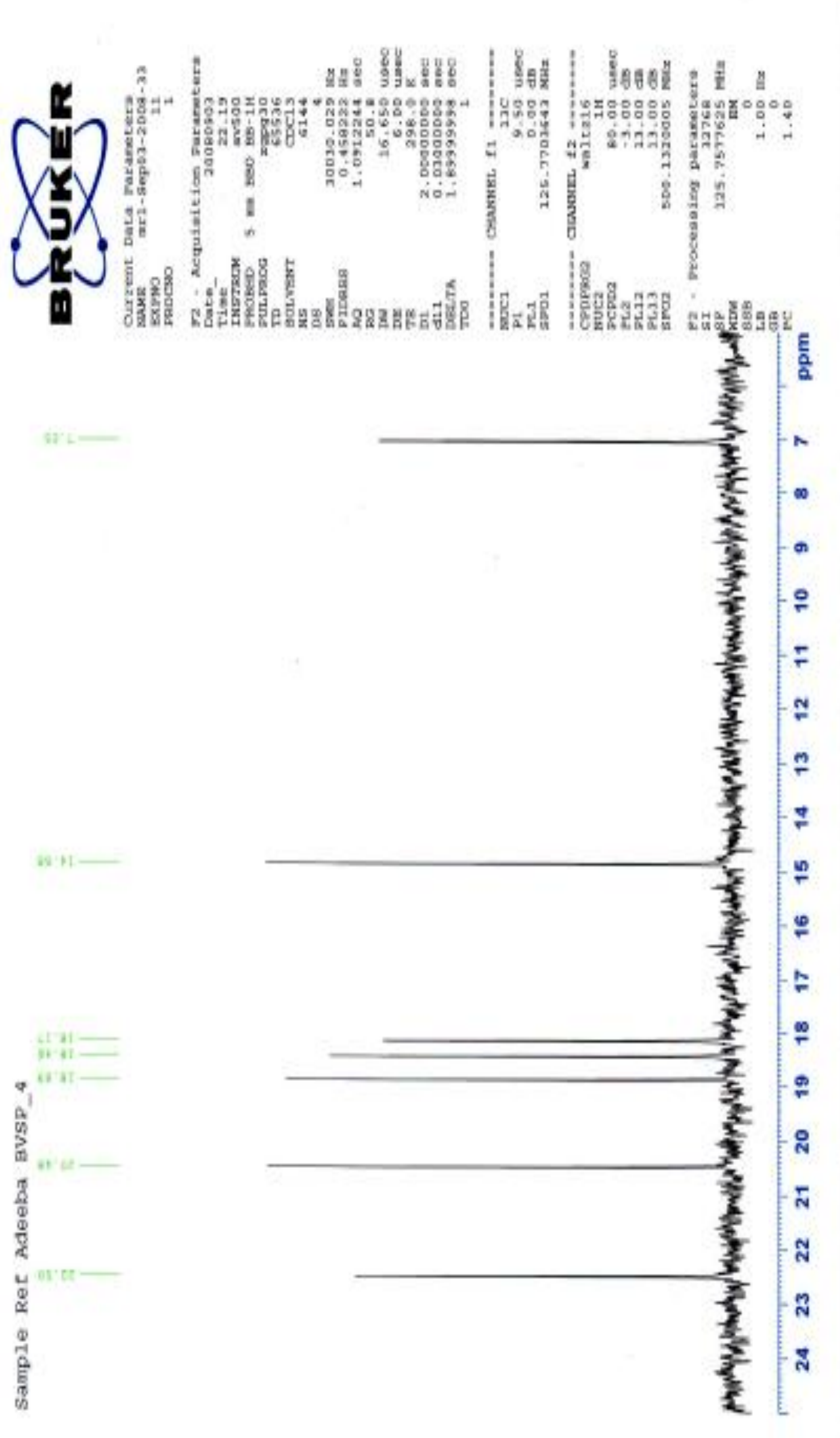

Figure 3.14: Partially expanded ^{13}C-NMR spectrum of BVSP-4 (48) in $CDCl_3$.

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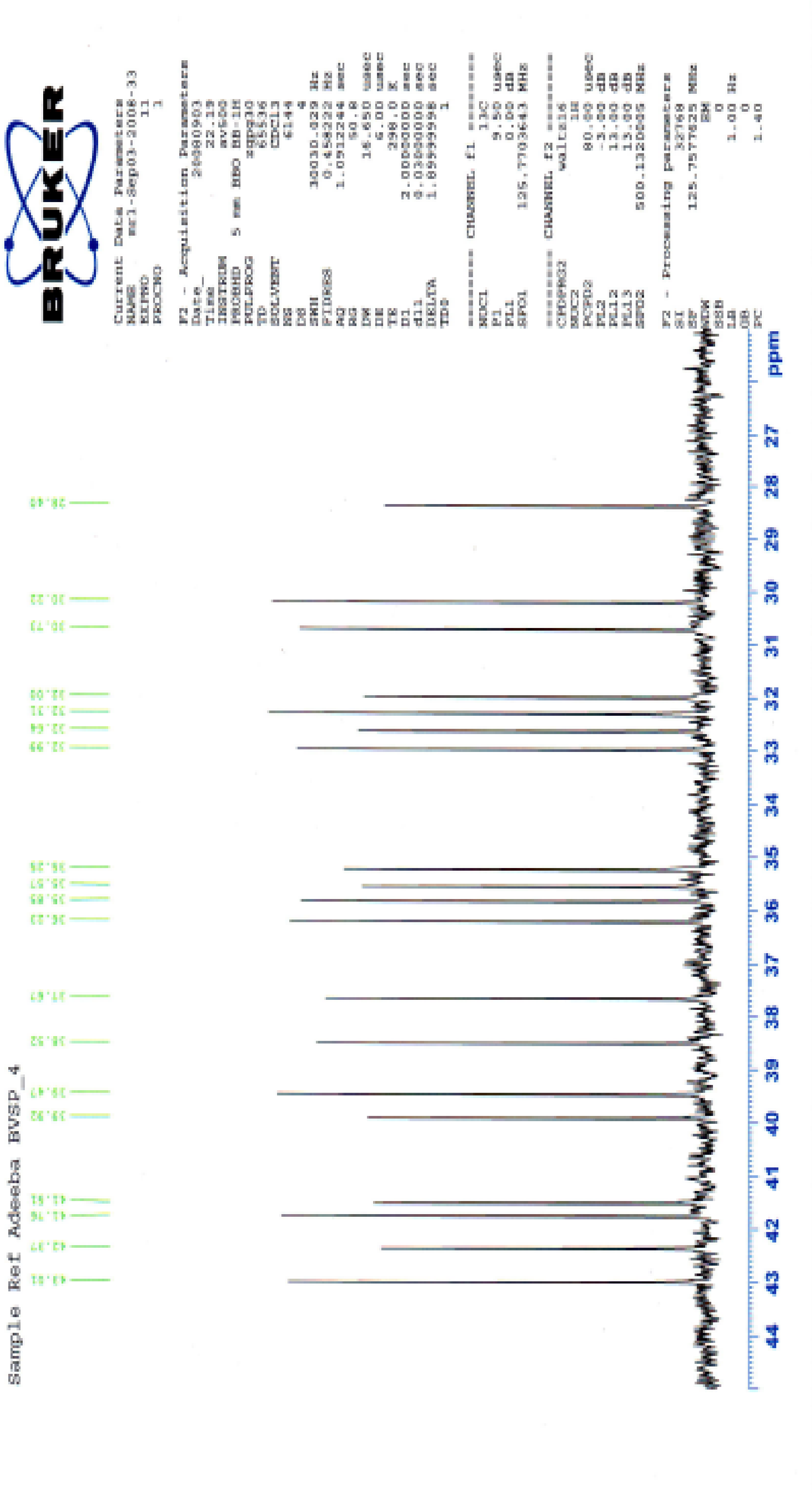

Figure 3.15: Partially expanded ^{13}C-NMR spectrum of BVSP-4 (48) in $CDCl_3$.

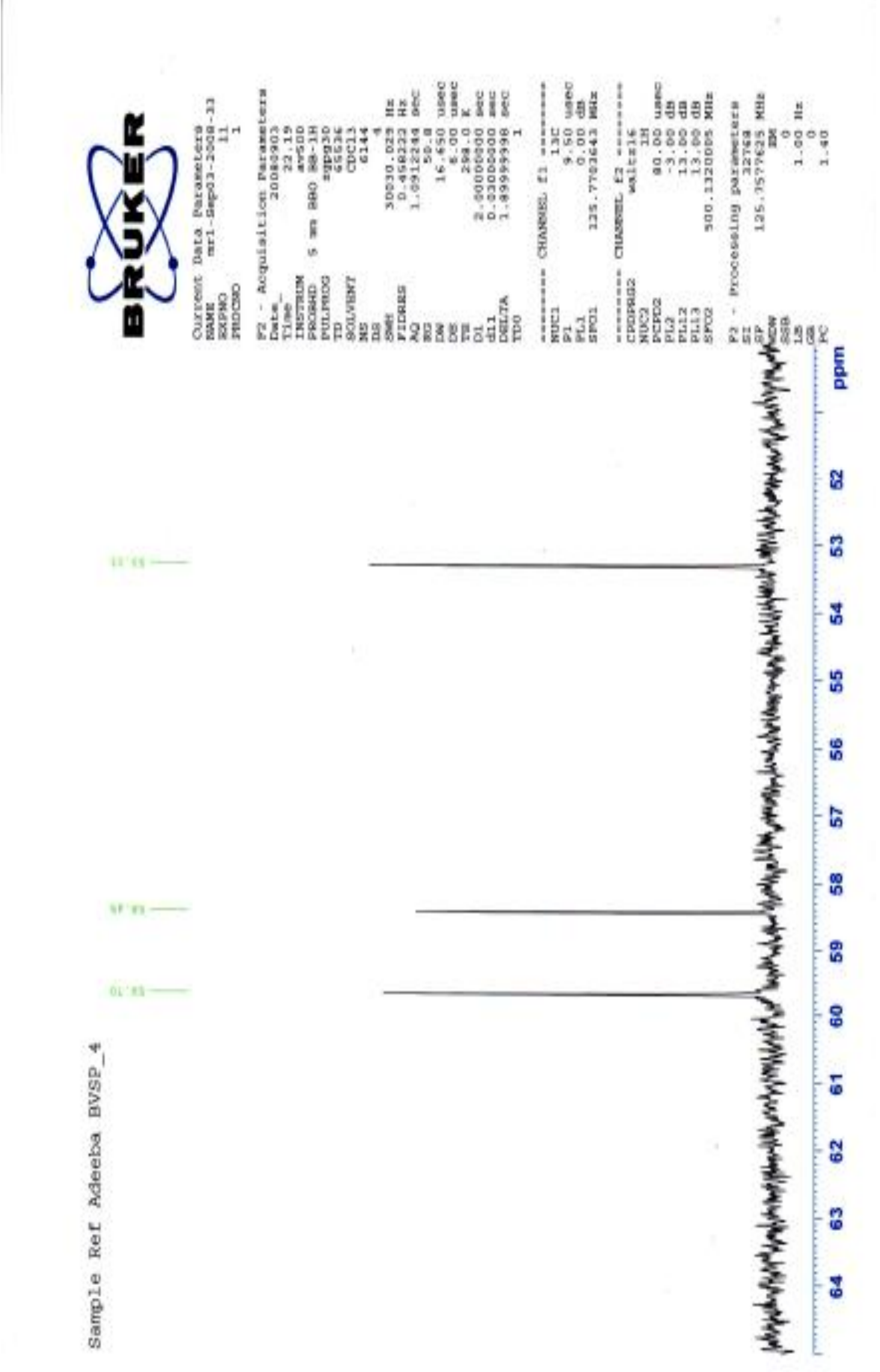

Figure 3.16: Partially expanded ^{13}C-NMR spectrum of BVSP-4 (48) in $CDCl_3$.

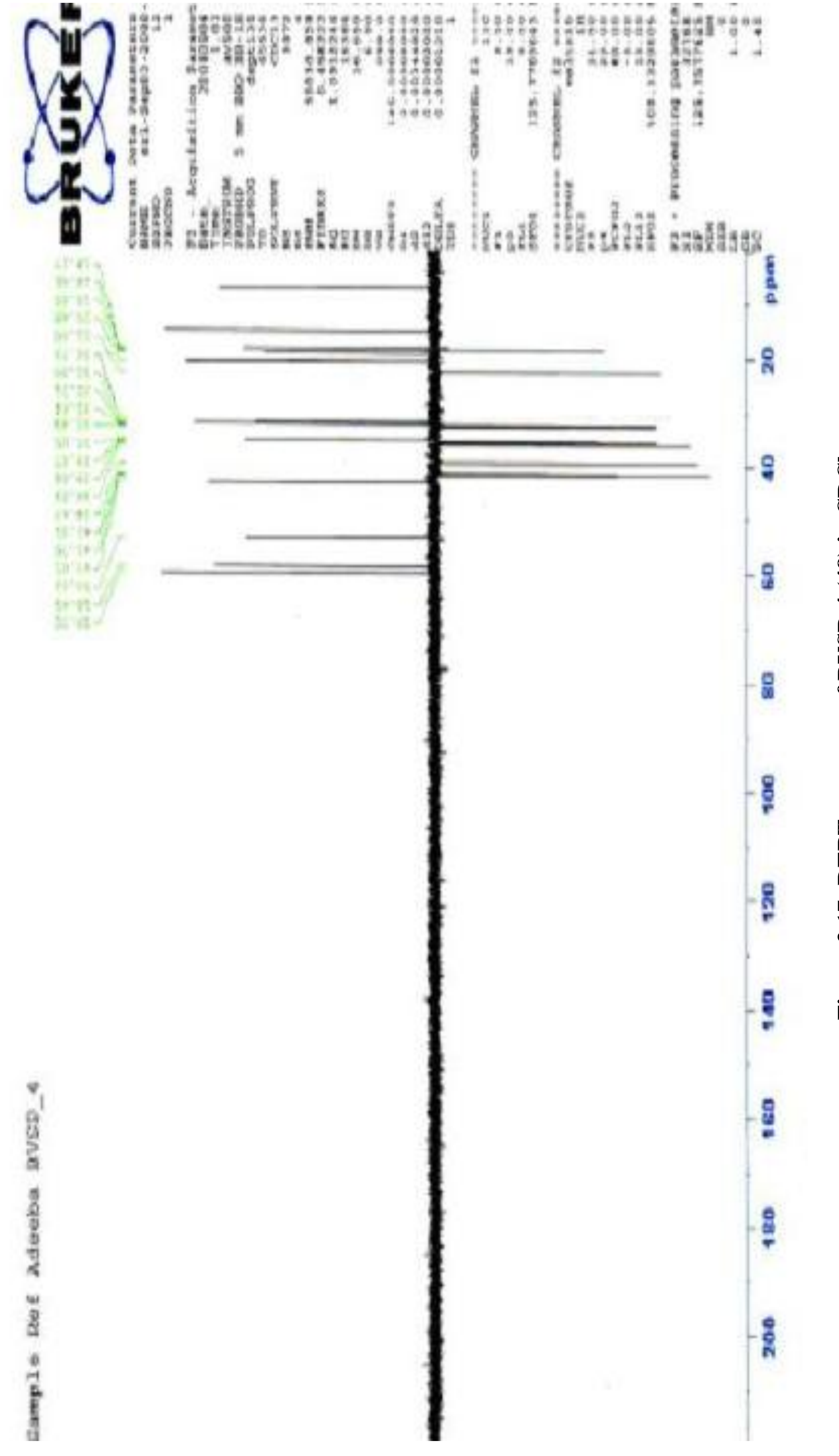

Figure 3.17: DEPT spectrum of BVSP-4 (48) in $CDCl_3$.

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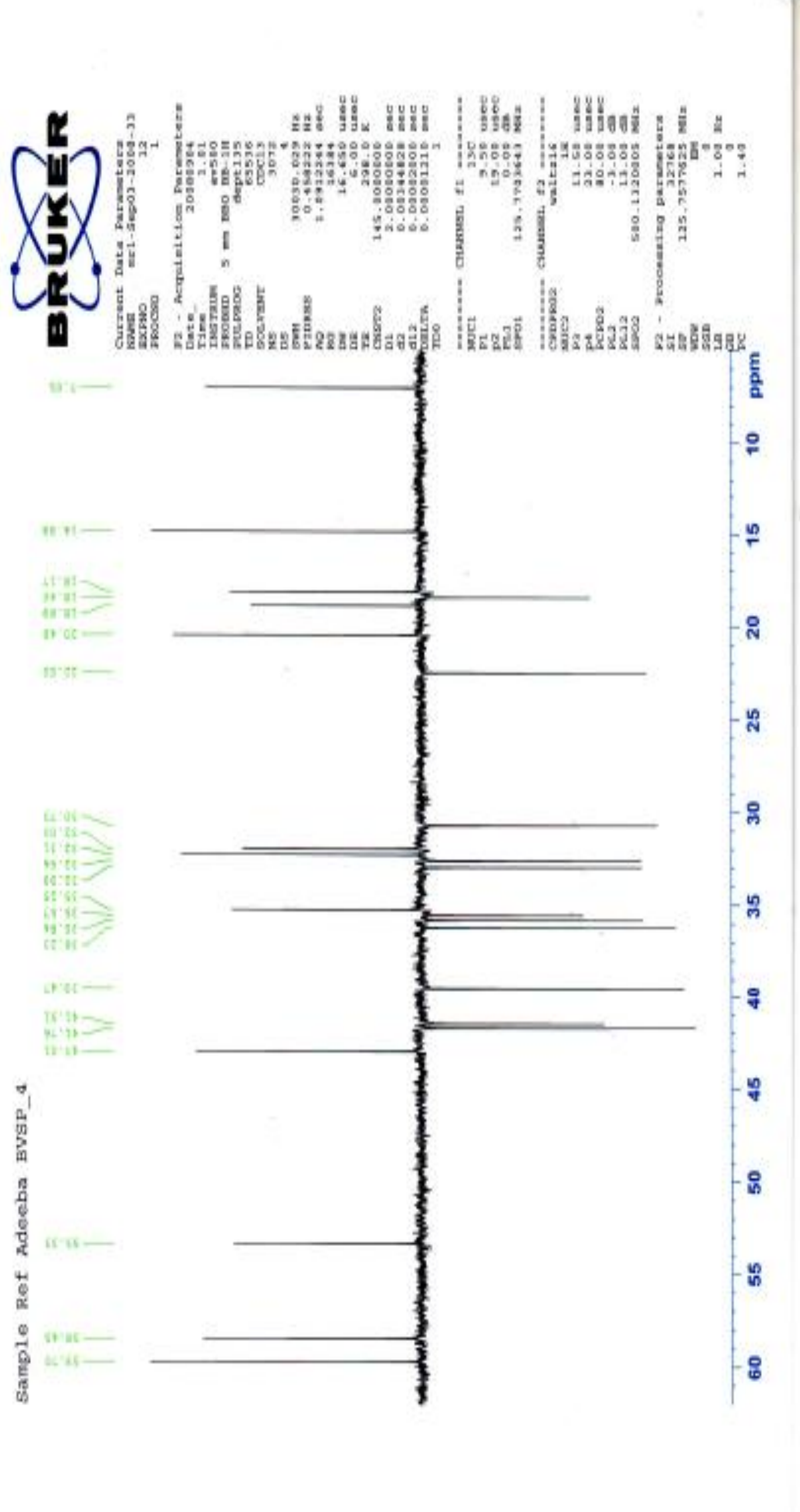

Figure 3.18: Partially expanded DEPT spectrum of BVSP-4 (48) in $CDCl_3$.

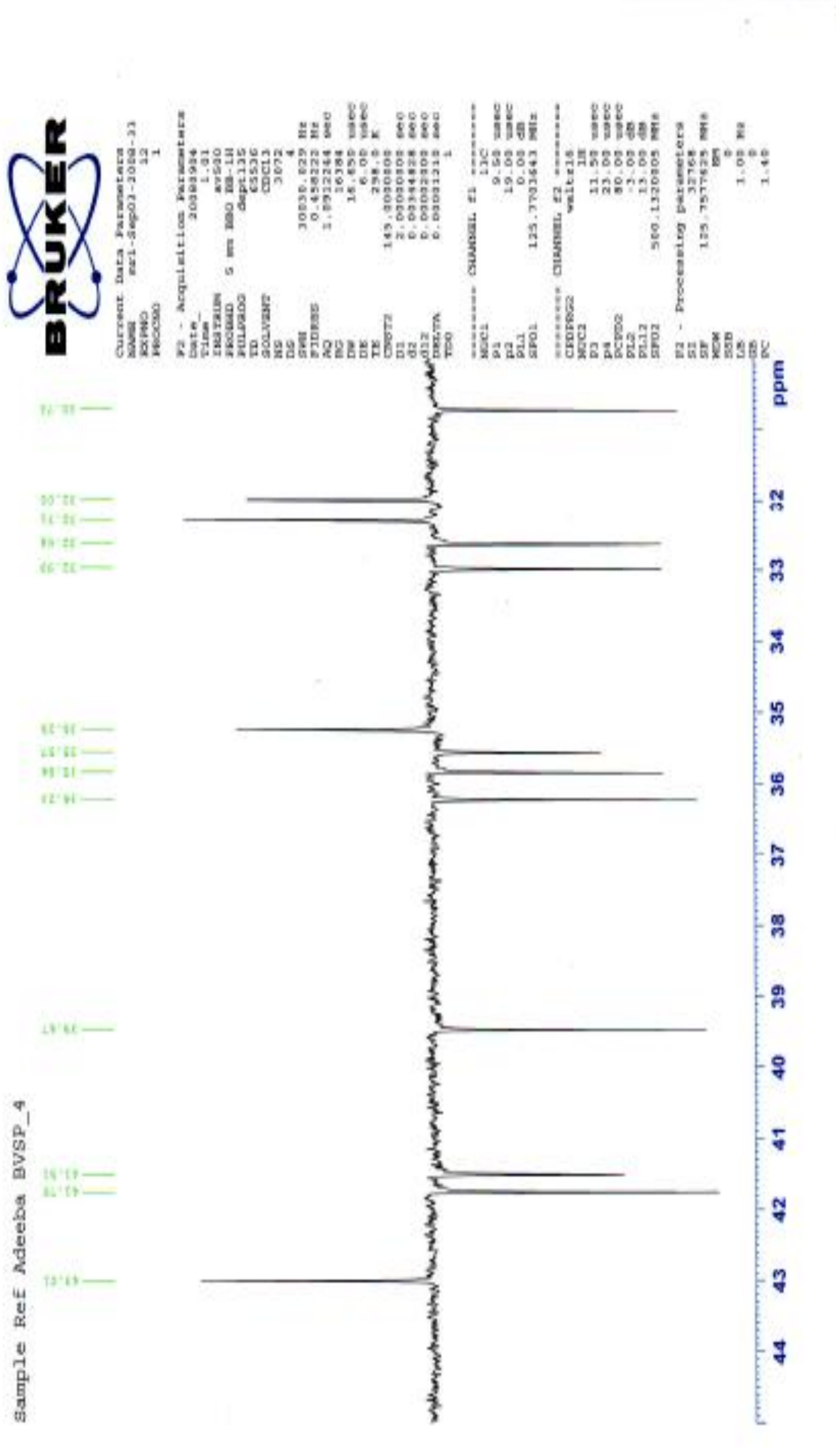

Figure 3.19: Partially expanded DEPT spectrum of BVSP-4 (48) in $CDCl_3$.

3.4 Characterization of BVSP-6 as 5β-24S-ethylcholestan-3β-ol (49)

The *n*-hexane soluble fraction of the crude methanolic extract of the stem bark of *B. verrucosa* was subjected to silica gel column chromatography by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities to obtain 50 sub-fractions. The sub-fractions 5-9 were combined together depending on the identical TLC characterization and subjected to preparative TLC over silica gel F_{254} using toluene-ethyl acetate (95:5) as the developing solvent. The process was repeated five times for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a pink colored band was visualized. The band was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent afforded a colorless compound BVSP-6 (7.14 mg) (**49**). The compound was analyzed over TLC to check its purity and found as a pure compound which was soluble in *n*-hexane, dichloromethane, ethyl acetate and acetone.

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$; Figures 3.21-3.22; Table 3.6) revealed a one proton multiplet at δ 3.75, the position and multiplicity of which was indicative of H-3 in a steroidal nucleus. It also displayed signals of three proton intensity at δ 0.93 and 0.70 indicating two tertiary methyl groups at C-10 and C-13. Again doublets (each 3H intensity) at δ 0.87 (d, *J*= 8.0 Hz) and 0.83 (d, *J*= 7.2 Hz) were observed due to secondary methyl groups at C-25. Another doublet at δ 0.92 (d, *J*= 6.4 Hz) was assigned to a methyl group at C-20. A triplet of three proton intensity at δ 0.89 (*J*= 7.5 Hz) due to primary methyl group to the primary methyl group at C-28. The absence of any signal between δ 5.00 and 6.00 suggested the absence of olefinic proton between C-5 and C-6 as observed in sitosterol and stigmasterol type of steroidal compounds.

These spectral features (Table 3.6) were found to be in close agreement with those of 5β-24S-ethylcholestan-3β-ol (**49**) (Luo *et al.*, 2009). This is the first report of isolation of 5β-24S-ethylcholestan-3β-ol from *B. verrucosa*.

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Figure 3.20: Structure of BVSP-6 (5β-24S-ethylcholestan-3β-ol**, 49**).

Table 3.6: ^{1}H-NMR spectral data of BVSP-6 (49) and 5β-24S-ethylcholestan-3β-ol (Luo *et al.*, 2009) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (J in Hz).	
	BVSP-6 (49)	**5β-24S-Ethylcholestan-3β-ol**
H-3	3.99 m	4.10 m
Me-18	0.70 s	0.64 s
Me-19	0.93 s	0.96, s
Me-21	0.92 d (6.4)	0.90 d (6.7)
Me-26	0.87 d (8.0)	0.82 d (7.7)
Me-27	0.83 d (7.2)	0.80 d (7.7)
Me-29	0.89 t (7.5)	0.85 t (7.5)

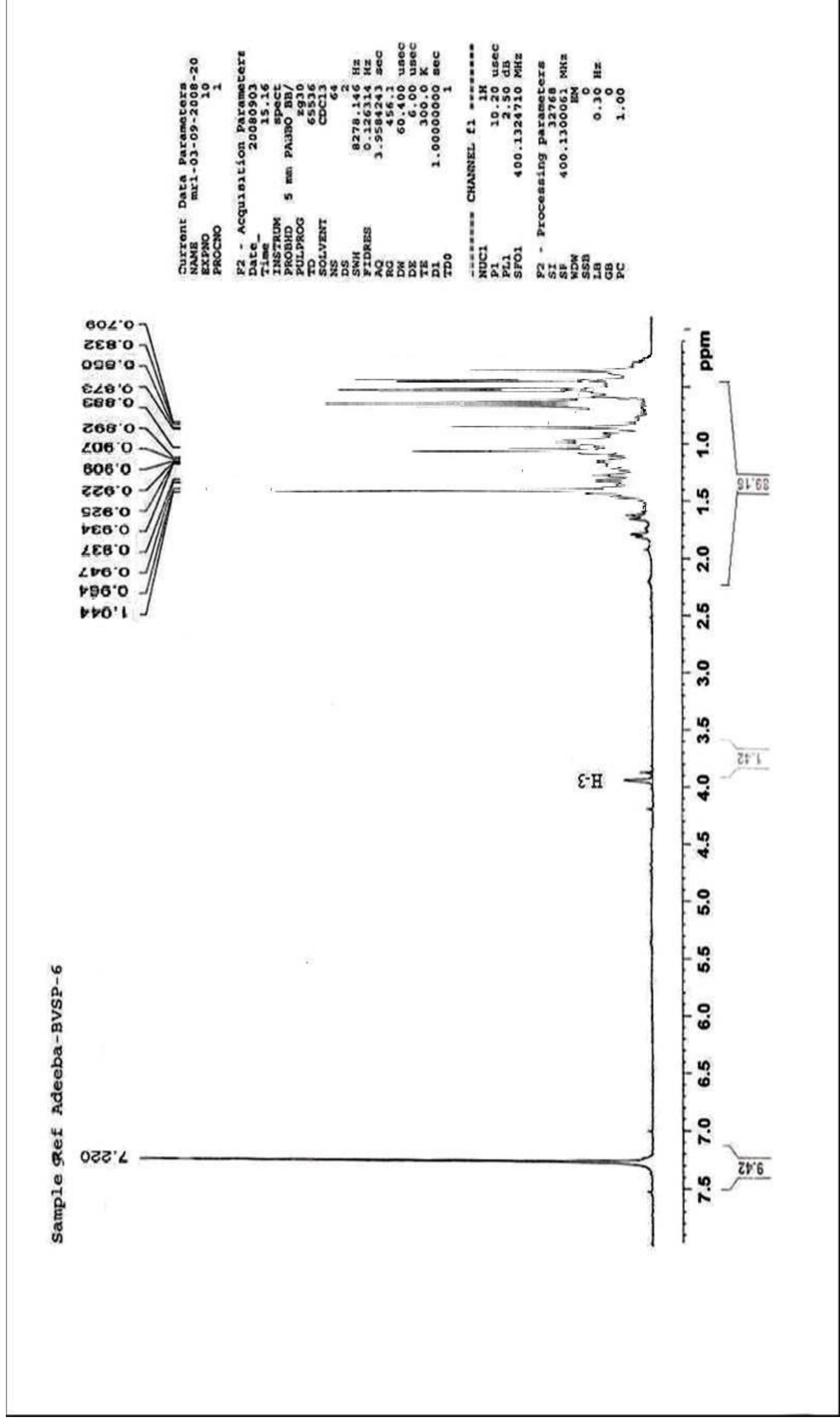

Figure: 3.21: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVSP-6 (49).

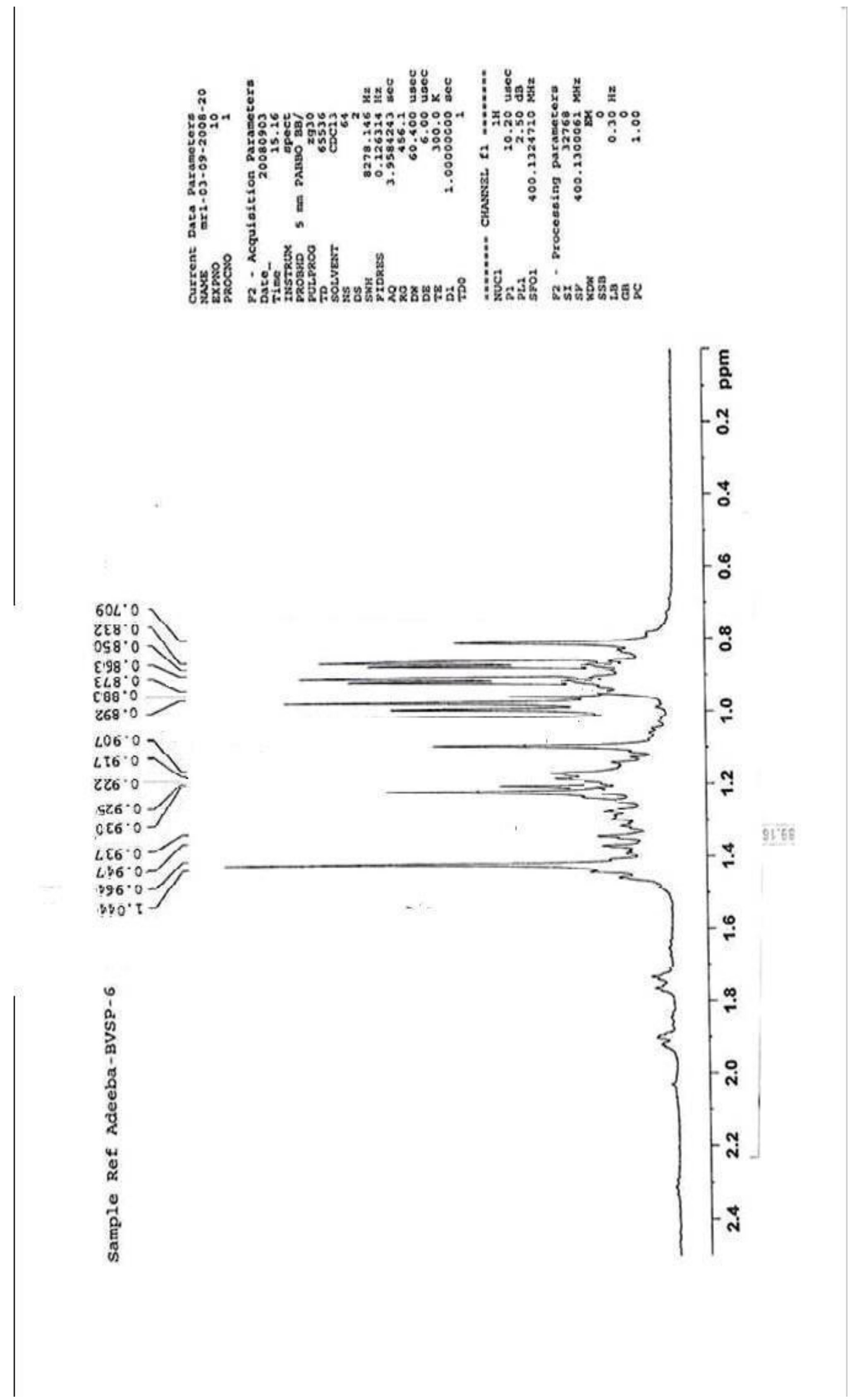

Figure 3.2 : Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVSP-6 (49).

3.5 Characterization of BVSP-34 as stigmasterol (50)

Fractions 34-43 from silica gel column chromatography of the *n*-hexane soluble portion of the methanol extract of the stem bark of *B. verrucosa* gave similar TLC feature and were combined together. Then it was subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (95:5)]. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating in 110 ºC for 5 minutes and a purple colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent yielded white needles which melted at 163 ºC which was identical to that published (163 ºC) for stigmasterol (Khan and Mandal, 1991). The compound was analyzed over TLC to check for purity and found to be a pure compound which was termed as BVSP-34 (6.5 mg, **50**). It was also found to be soluble in *n*-hexane, dichloromethane, ethyl acetate and acetone.

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$; Figure 3.24; Table 3.7) revealed a one proton multiplet at δ 3.49, the position and multiplicity of which was indicative of H-3 in a steroidal nucleus. The typical C-6 proton was evident as a broad singlet at δ 5.40 that integrated for one proton.

The olefinic protons H-22 and H-23 appeared as characteristic downfield signals at δ 5.14 (dd, *J*= 15.0, 6.5 Hz) and δ 5.04 (dd, *J*= 15.0, 6.5 Hz) which were comparable to the ^{1}H-NMR data of authentic stigmasterol. It further displayed signals of three proton intensity at δ 0.67 and 1.00 indicating two tertiary methyl protons at C-10 and C-13. Again doublets (each 3H) at δ 0.80 and 0.84 (*J*= 6.0 Hz) were observed due to secondary methyl groups at C-25 and a triplet at δ 0.82 (*J*= 6.5 Hz) was due to primary methyl group. Another doublet near δ 0.90 (*J*= 6.5 Hz) was assigned to a methyl group at C-20. These spectral features (Table 3.4) as well as melting point of BVSP-34 were found to be in close agreement with those of stigmasterol (Khan and Mandal, 1991). Moreover, the spectrum of BVSP-34 was super imposable to the ^{1}H-NMR spectrum acquired for an authentic stigmasterol previously isolated in our laboratory. So, BVSP-34 was identified as stigmasterol (**50**). This is the first report of isolation of stigmasterol from *B. verrucosa*.

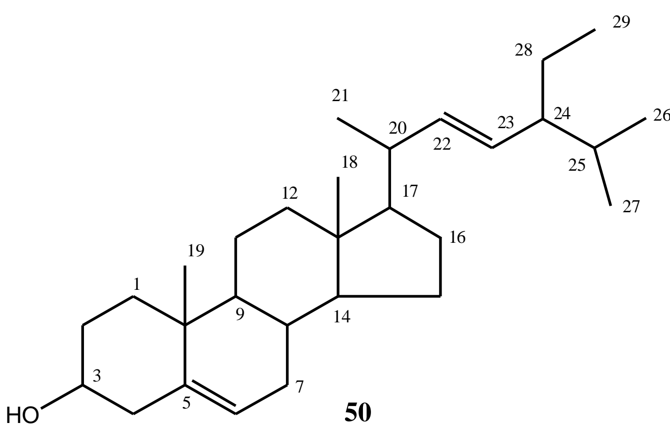

Figure 3.23: Structure of BVSP-34 (stigmasterol, **50**).

Table 3.7: ^{1}H-NMR spectral data of BVSP-34 (50) and stigmasterol (Khan and Mandal, 1991) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	BVSP-34 (50)	Stigmasterol
H-3	3.49 m	3.52 m
H-6	5.35 m	5.32 m
Me-18	0.67 s	0.65 s
Me-19	1.00 s	1.00 s
Me-20	0.90 d (6.5)	0.90 d (6.7)
Me-21	0.91 d (6.2)	0.93 d (6.6)
H-22	5.14 dd (15.0 and 6.5)	5.17 dd (14.7 and 6.3)
H-23	5.04 dd (15.0 and 6.5)	5.04 dd (15.0 and 6.5)
Me-25	0.84 d (6.0)	0.84 d (6.0)
	0.80 d (6.0)	0.80 d (6.0)
Me-28	0.82 t (6.5)	0.81 t (6.6)

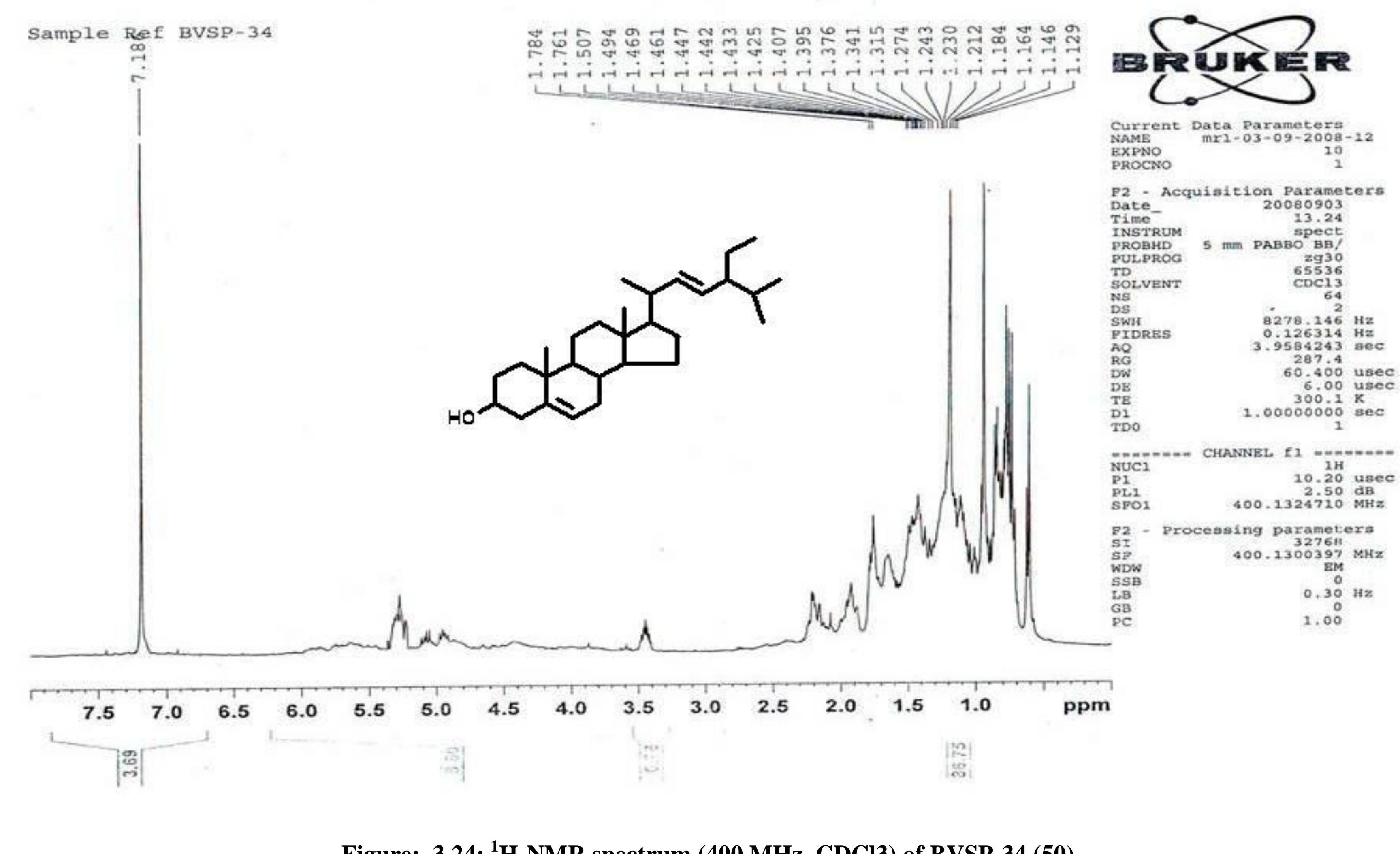

Figure: 3.24: ^{1}H-NMR spectrum (400 MHz, CDCl3) of BVSP-34 (50).

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3.6 Characterization of BVBC-2 as 5, 5'-dihydroxysesamine (51)

The carbon tetrachloride soluble partitionate (270 mg) of the crude methanolic extract of stem bark of *B. verrucosa* was subjected to silica gel column chromatography [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (9:1)] and 150 fractions were collected. Fractions 74-76 were combined together. Evaporation of solvents yielded a whitish mass which was purified by preparative TLC over silica gel F_{254} using toluene-ethyl acetate (9:1) as the developing solvent. The process was repeated twice for better separation and isolation of the compound of interest. The desired band was scrapped off and eluted with ethyl acetate to yield BVBC-2 (7.1 mg, **51**) as whitish mass. It was found to be soluble in chloroform, ethyl acetate and methanol.

The ^{1}H-NMR (400 MHz, $CDCl_3$; Figures 3.26-3.28; Table 3.8) spectral data of BVBC-2 could be assigned by careful comparison of the data reported for structurally related compound, sesamine (Pelter *et al*., 1976; Jayasinghe *et al*., 2003) as well as with the help of ^{1}H-^{1}H COSY spectrum (Figure 3.29). It showed a one proton multiplet at δ 3.05 which could be assigned to the methine proton, H-1. In the ^{1}H-^{1}H COSY spectrum it was found to couple with a vicinal proton at δ 4.84 (d, J= 5.2 Hz, H-2) and also with a pair of methylene protons at δ 3.85 (dd, J= 9.0 and 6.0 Hz, H-8eq) and δ 4.52 (dd, J= 9.0 and 6.0 Hz, H-8ax). Another pair of methylene protons were observed at δ 4.05 (d, J= 9.6 Hz, H-4eq) and δ 3.92 (d, J= 9.6 Hz, H-4ax) which were geminally coupled to each other only indicating the absence of a vicinal proton at H-5 where a hydroxyl group may be present in compound BVBC-2 as compared to a hydrogen at C-5 in sesamine.

Jayasinghe *et al*., 2003 reported the presence of an aromatic proton at H-5' in sesamine (**52**) which coupled with the vicinal aromatic proton H-6' to give a doublet with a J value of 7.90 Hz, a typical ortho coupling but H-6' proton in BVBC-2 proton appeared as a doublet (J= 1.6 Hz) at δ 6.90 Hz due to the presence of H-2' aromatic proton indicating the absence of H-5' proton. So, there may be a OH group in compound BVBC-2. From the above spectral evidences it can be assumed that the isolated compound BVBC-2 could be an asymmetrically substituted 2,6-diaryl *cis*-3,7-dioxabicyclo [3.3.0] octane derivative (Pelter *et al*., 1976).

On the basis of the above spectral data as well as by comparison with that of sesamine (**52**), the proton chemical shifts of BVBC-2 have been assigned (Table-3.8). The remaining spectral data were similar to that of the reported compound sesamine. So BVBC-2 was identified as 5,5'-dihydroxysesamine (**51**). This is the first report of occurrence of 5,5'-dihydroxysesamine (**51**) from any nature and synthetic source. However, additional spectral data is required to confirm its structure.

Figure 3.14: Structures of BVBC-2 (5,5'-dihydroxysesamine, **51**) and sesamine (**52**).

Table 3.8: ^{1}H-NMR spectral data of BVBC-2 (51) and sesamine (52) (Jayasinghe *et al*., 2003) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	BVBC-2 (51)	Sesamine (52)
H-1	3.05 m	3.05 m
H-2β	4.84 d (5.2)	4.71, d (4.4)
H-4eq/ H-4α	4.05 d (9.6)	4.23, dd (9.3 and 6.8)
H-4ax/ H-4β	3.92 d (9.6)	3.87, dd (9.3 and 3.7)
H-5	-	3.05, m
H-6β	4.84 d (5.2)	4.71, d (4.4)
H-8eq/ H-8β	3.85 dd (9.0 and 6.0)	3.87, dd (9.3 and 3.7)
H-8ax/ H-4α	4.52 dd (9.2 and 8.0)	4.23, dd (9.3 and 6.8)
H-2'	6.92 d (1.6)	6.84 d (1.2)
H-5'	-	6.77 d (7.9)
H-6'	6.95 d (1.6)	6.80 d (1.5)
H-2''	6.86 bs	6.84 d (1.2)
H-5''	6.80 d (7.6)	6.77 d (7.9)
H-6''	6.88 dd (1.6, 0.8)	6.80 d (1.5)
$2 \times$ -OCH_2O-	5.99 s	5.95, s
	5.96 s	5.95, s

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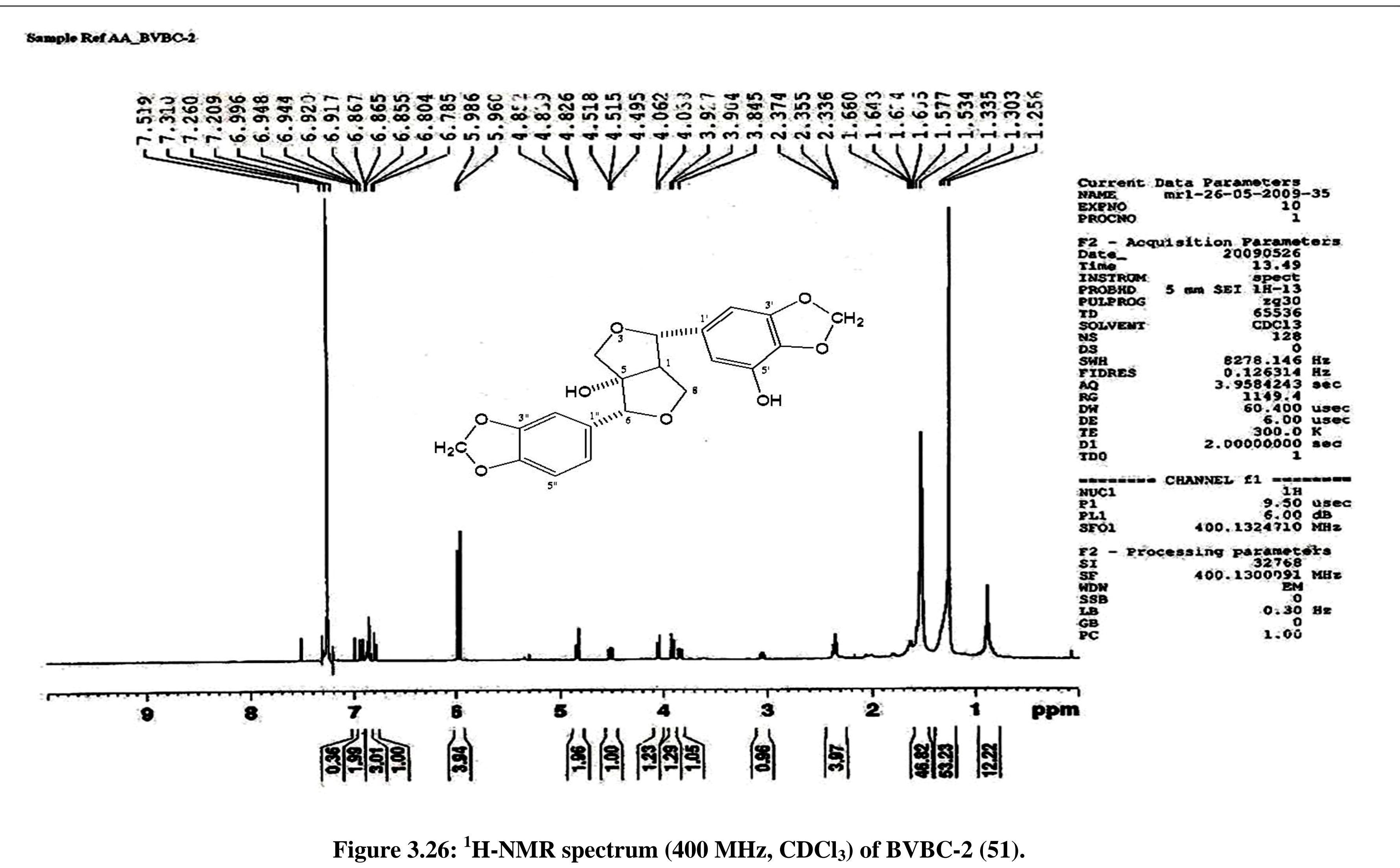

Figure 3.26: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVBC-2 (51).

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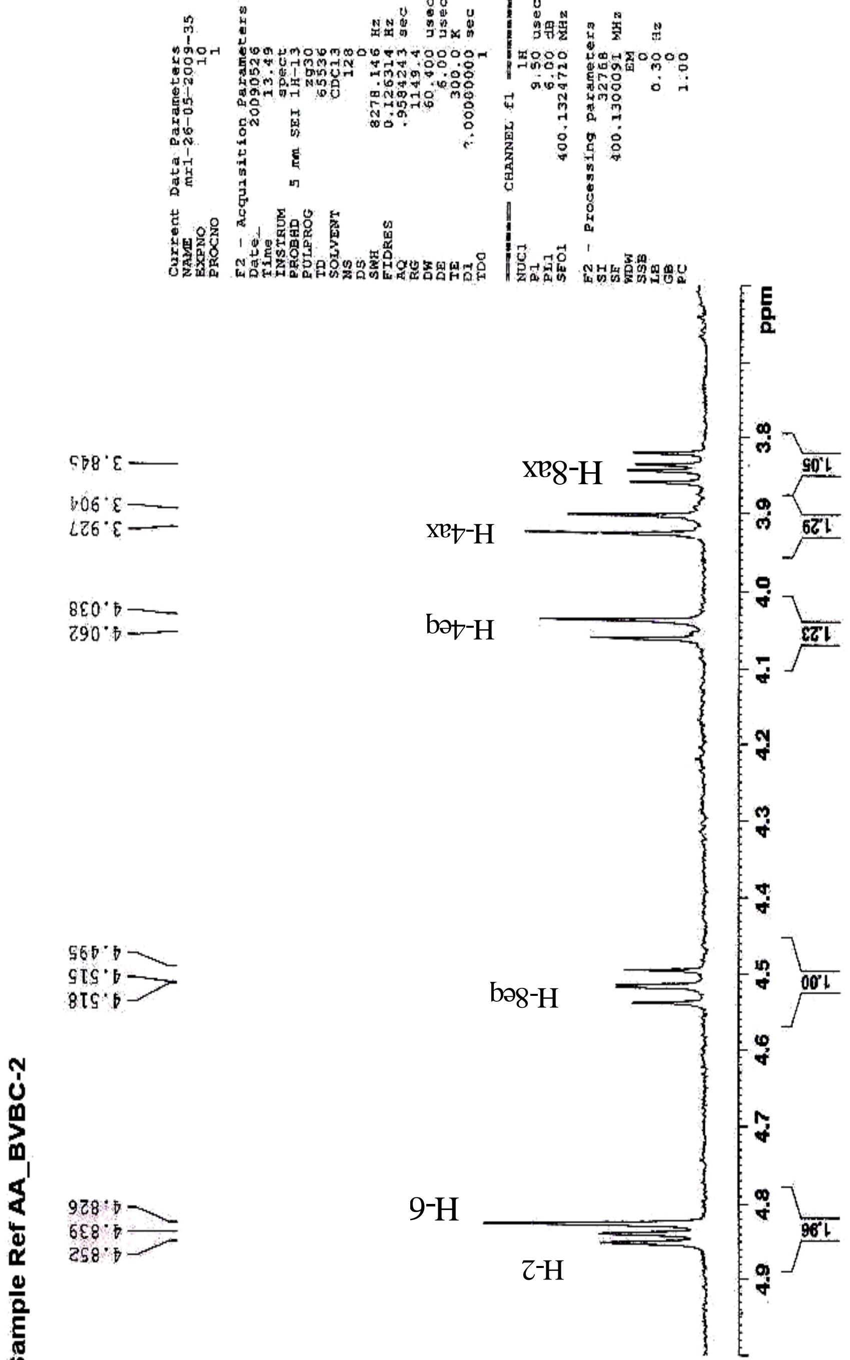

Figure 3.27: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVBC-2 (51).

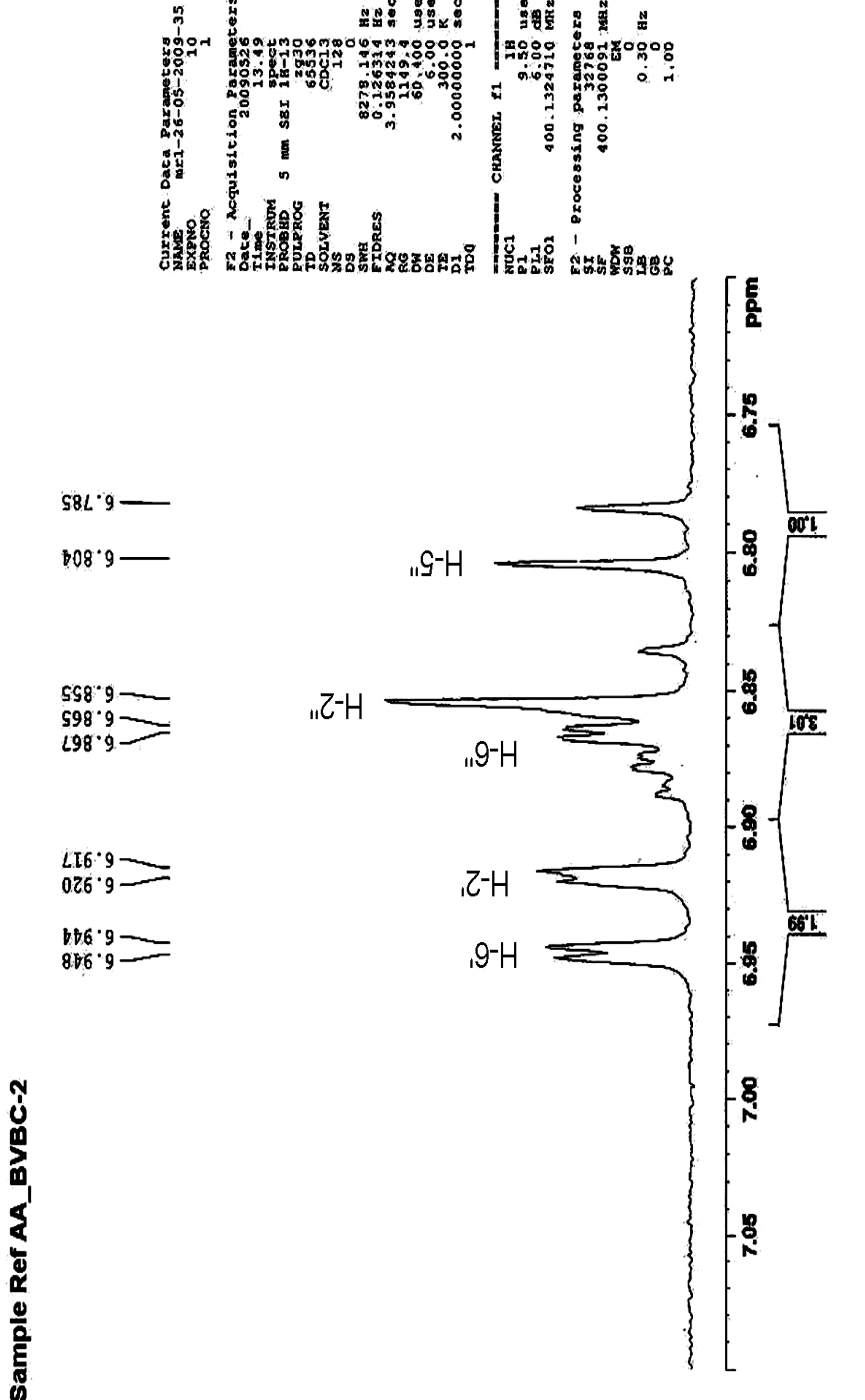

Figure 3.28: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVBC-2 (51).

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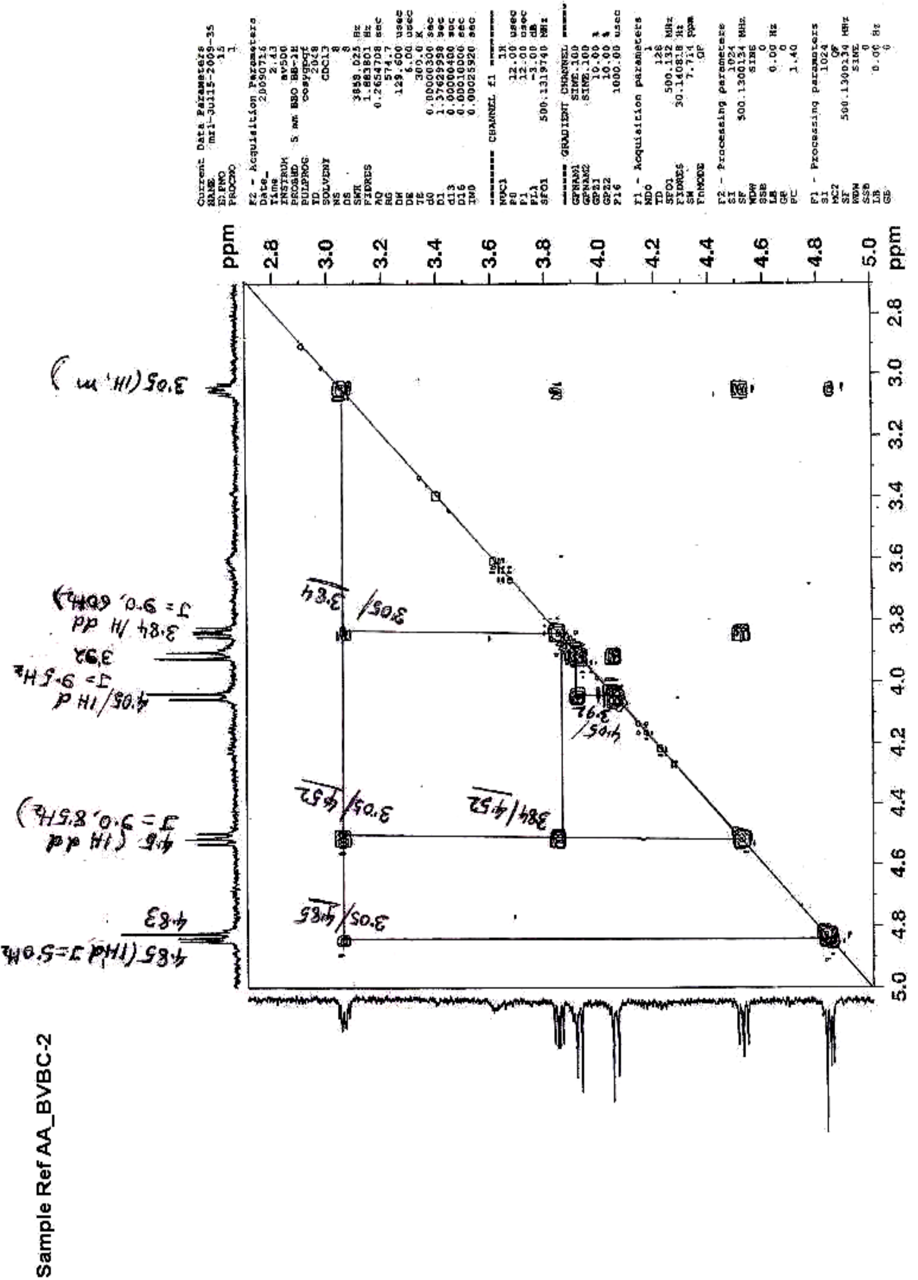

Figure 3.29: ^{1}H-^{1}H COSY spectrum of BVBC-2 (51) in $CDCl_3$.

3.7 Characterization of BVSC-8 as 3-ketooleanane (53)

The carbon tetrachloride soluble portion of the crude methanolic extract of the stem bark of *B. verrucosa* was subjected to silica gel column chromatography by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities to obtain one hundred fifty fractions.

Fractions 8-12 showing identical spots on TLC were combined in a beaker and it was kept undisturbed at room temperature for several days. The crystals obtained after evaporation of solvents were washed *n*-hexane to remove adhered coloring materials and recrystallized from *n*-hexane and ethyl acetate to afford a pure compound designated as needles designated BVSC-8 (7.53 mg, **53**) as needles. It was found to be soluble in chloroform, dichloromethane and ethyl acetate.

The crystals of BVSC-8 melted at 235-236 ^{o}C which was identical to that reported (233-235 ºC) for 3-ketooleanane (Krishnaveni *et al.*, 2000). The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$; Figures 3.31 -3.32; Table 3.9) showed eight singlets corresponding to eight methyl groups between δ 0.72-1.25. However, the absence of any olefinic proton resonance near δ 5.00 and oxymethine proton signal around δ 3.00 suggested the absence of double bond between C-12 and C-13, and the oxymethine proton at C-3. All these are indicative of the presence of a pentacyclic triterpenoid type skeleton in BVSC-8 having no double bond between C-12 and C-13.

The melting point and ^{1}H-NMR spectral data (Tables 3.9) of BVSC-8 were compared with the published data of 3-ketooleanane and found to be identical (Krishnaveni *et al.*, 2000). Thus, compound BVSC-8 was characterized as 3-ketooleanane (**53**). This is the first report of isolation of 3-ketooleanane from *B. verrucosa*.

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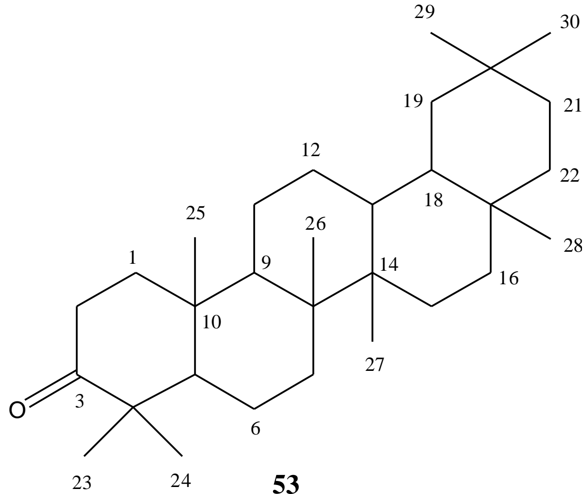

Figure 3.30: Structure of BVSC-8 as 3-ketooleanane (**53**).

Table 3.9: ^{1}H- NMR spectral data of BVSC-8 (53) and 3-ketooleanane (Krishnaveni *et al.*, 2000) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$.	
	BVSC-8 (53)	3-ketooleanane
H-3	-	-
H-6	-	-
Me-23	1.25 s	1.25 s
Me-24	1.18 s	1.18 s
Me-25	0.87 s	0.88 s
Me-26	0.72 s	0.75 s
Me-27	1.05 s	1,07 s
Me-28	0.96 s	0.96 s
Me-29	1.01 s	1.02 s
Me-30	1.00 s	0.98 s

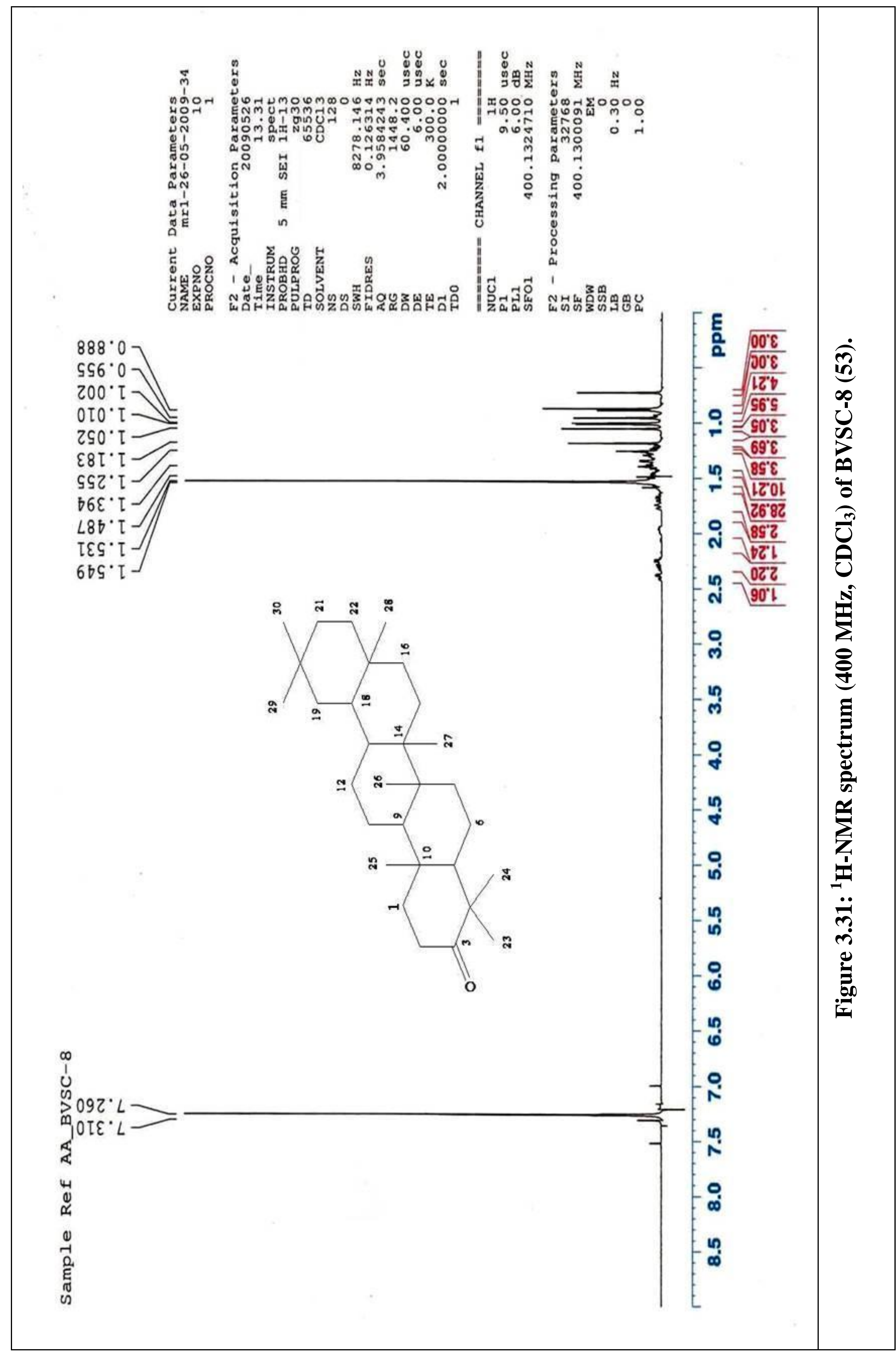

Figure 3.31: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVSC-8 (53).

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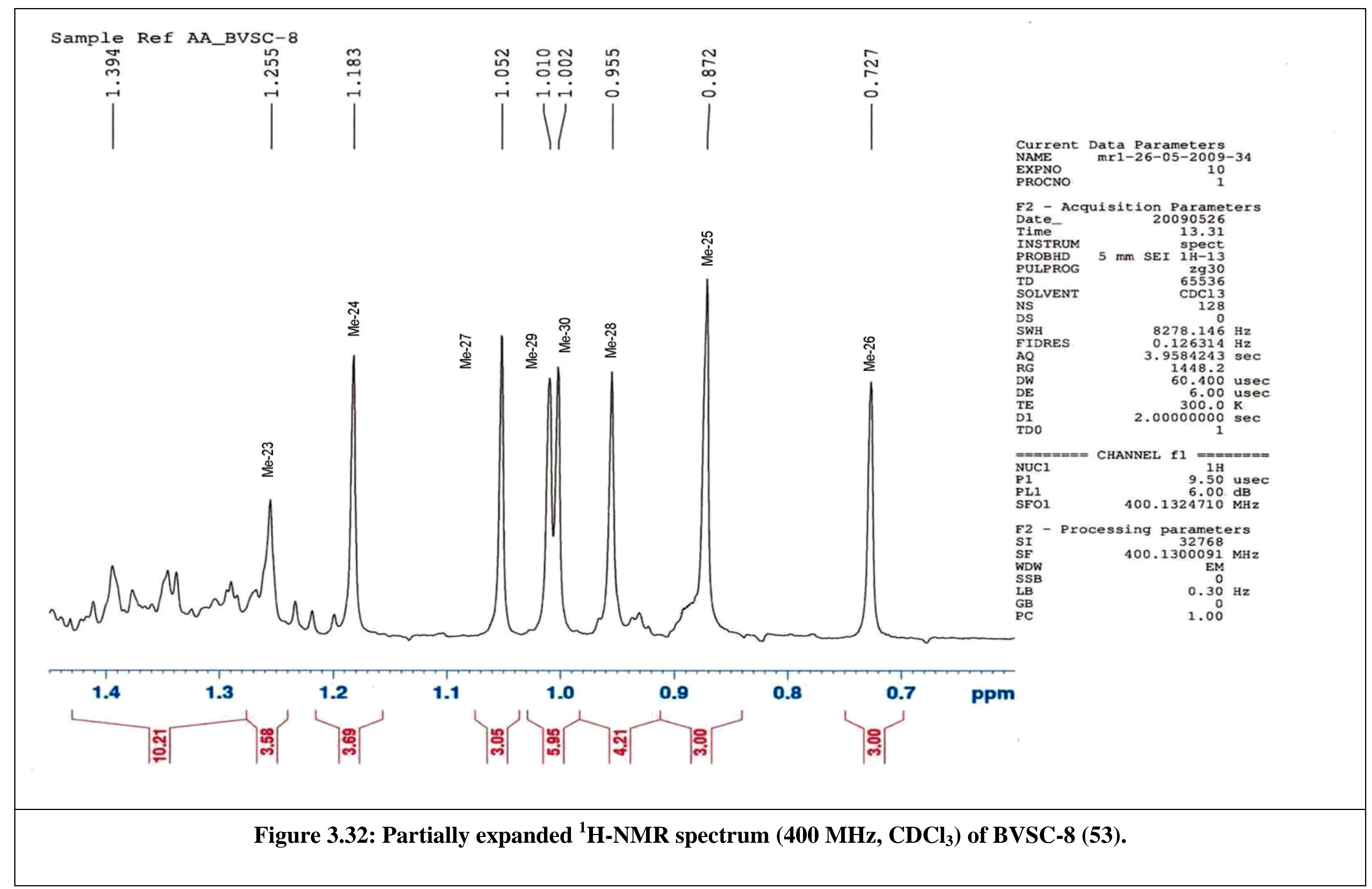

Figure 3.32: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVSC-8 (53).

3.8 Characterization of BVS-65 as pinoresinol (54)

Compound BVS-65 was isolated as yellowish mass from the column chromatographic fractions 59-69 of the chloroform soluble materials of the methanolic extract of the stem bark of *B. verrucosa*. The concentrated fraction was subjected to preparative TLC over silica gel F_{254} using chloroform-methanol (97:3) as the developing solvent. The desired band was scrapped off on an aluminum foil and eluted initially using a mixture of chloroform-methanol (19:1) to yield BVS-65 (3.54 mg, **54**). The compound was found to be soluble in dichloromethane, ethyl acetate, acetone and methanol.

The ^{1}H-NMR (400 MHz, $CDCl_3$; Figures 3.34-3.36; Table 3.10) spectrum of BVS-65 indicative of the typical pattern of two 1,3,4-trisubstituted benzene rings displayed signals at δ 6.88 (2H, d, *J*= 8.0 Hz, H-5', H-5''), δ 6.81 (2H, dd, *J*= 8.0, 1.6, H-6', H-6'') and δ 6.86 (2H, d, *J*= 1.6 Hz, H-2', H-2'') and two methoxy groups at δ 3.84 (6H). It also revealed a multiplet of two proton intensity at δ 3.09 (Perez *et al.*, 1995) for H-1 and H-5 and a doublet for H-2 and H-6 at δ 4.72 with *J* value of 4.00 Hz and another double doublet at δ 4.17 (*J*= 9.2, 7.2 Hz) that integrated for two protons, which could be attributed to $H\text{-}4_{eq}$ and $H\text{-}8_{eq}$. The ^{1}H-NMR spectrum further showed a double doublet at δ 3.81 (*J*= 8.8 and 3.6) for two protons assignable to $H\text{-}4_{ax}$ and $H\text{-}8_{ax}$.

These ^{1}H-NMR spectral data indicated that BVS-65 was a lignan of the 2,6-diaryl-3,7-dioxobicyclo [3.3.0] octane type (Pelter *et al.*, 1976). Moreover, all the other proton shifts were similar to that published for pinoresinol (Maria *et al.*, 1991) as shown in Table 3.7. So, the compound BVS-65 was identified as pinoresinol (**54**). This is the first report of isolation of pinoresinol from *B. verrucosa*.

Figure 3.33: Structure of BVS-65 (pinoresinol, **54**).

Table 3.10: ^{1}H-NMR spectral data of BVS-65 (54) and pinoresinol (Maria *et al.*, 1991) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	BVS-65 (54)	**Pinoresinol**
H-1, 5	3.09 m	3.1 m
H-2, 6	4.72 d (4.0)	4.73 d (4.0)
H_{eq}-4, 8	4.17 dd (9.2 and 7.2)	4.25 dd (9.0 and 6.5)
H_{ax}-4, 8	3.81 dd (8.8 and 3.6)	3.87 dd (9.0 and 4.0)
H-2', 2''	6.86 d (1.6)	6.89 d (2.0)
H-4', 4''	5.56 s	5.59 s
H-5', 5''	6.88 d (8.0)	6.88 d (8.0)
H-6', 6''	6.81 dd (8.0 and 1.6)	6.82 dd (8.0 and 2.0)
OMe-3', 3''	3.84 s	3.87 s

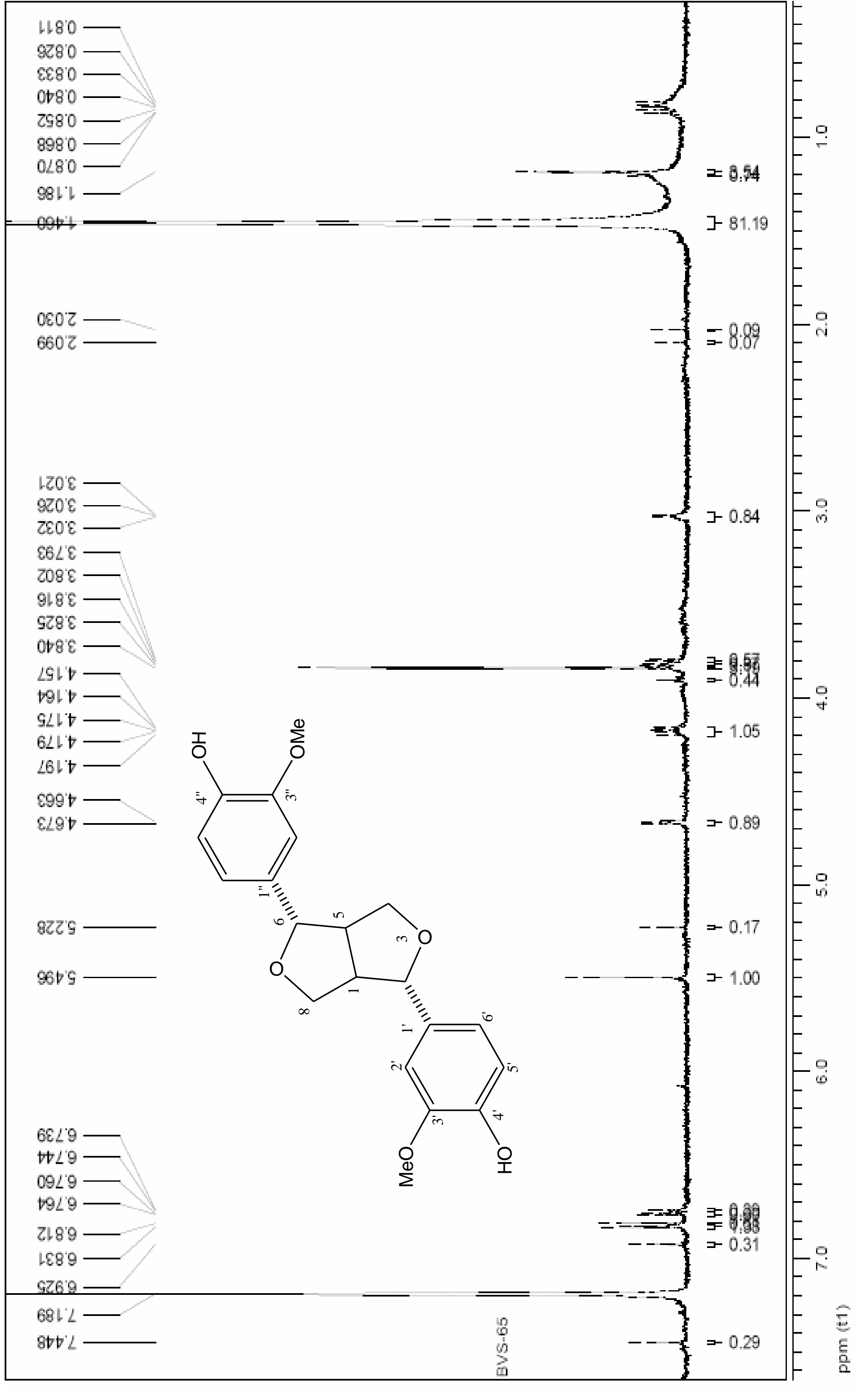

Figure 3.34: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVS-65 (54).

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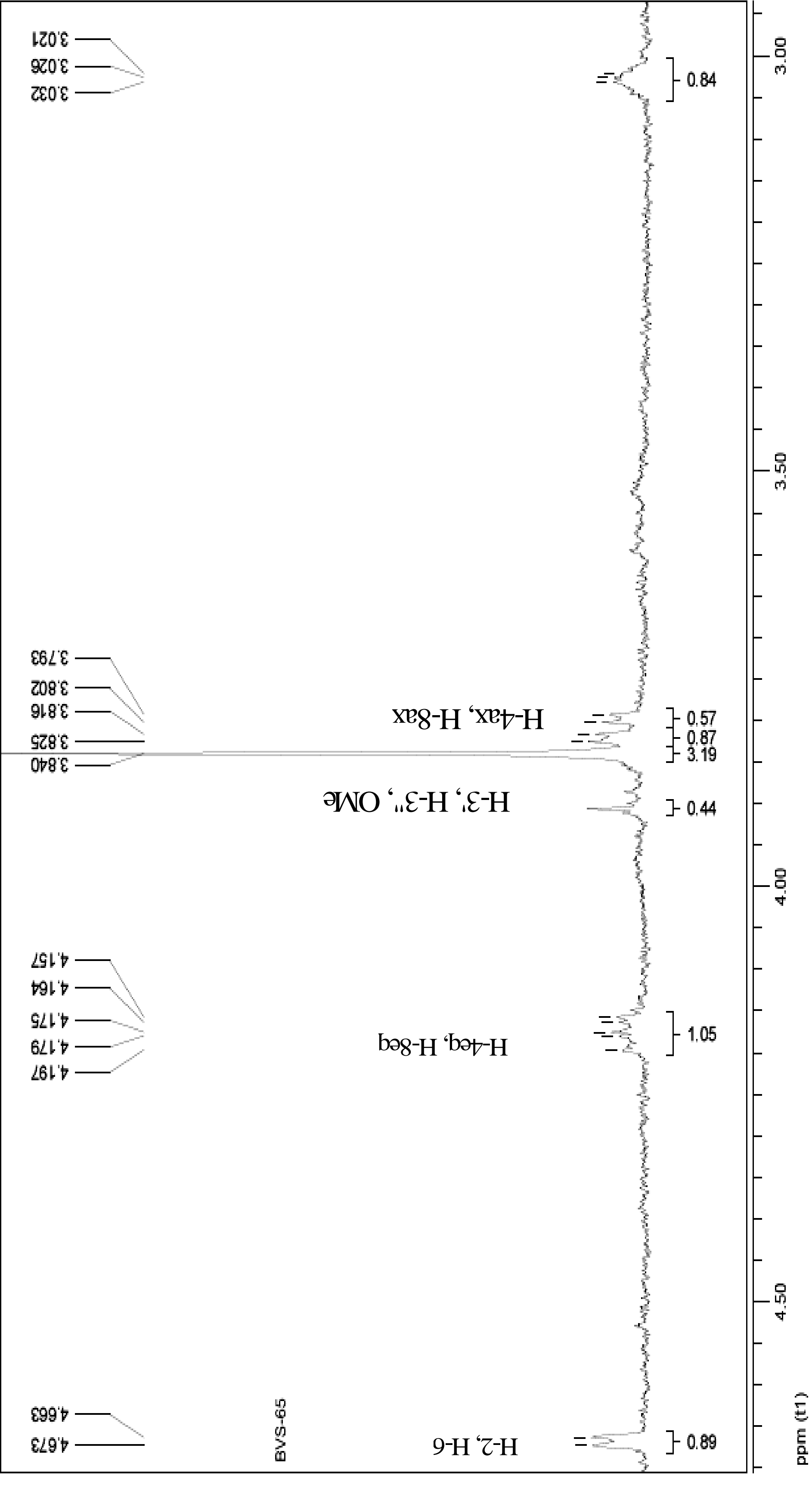

Figure 3.35: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVS-65 (54).

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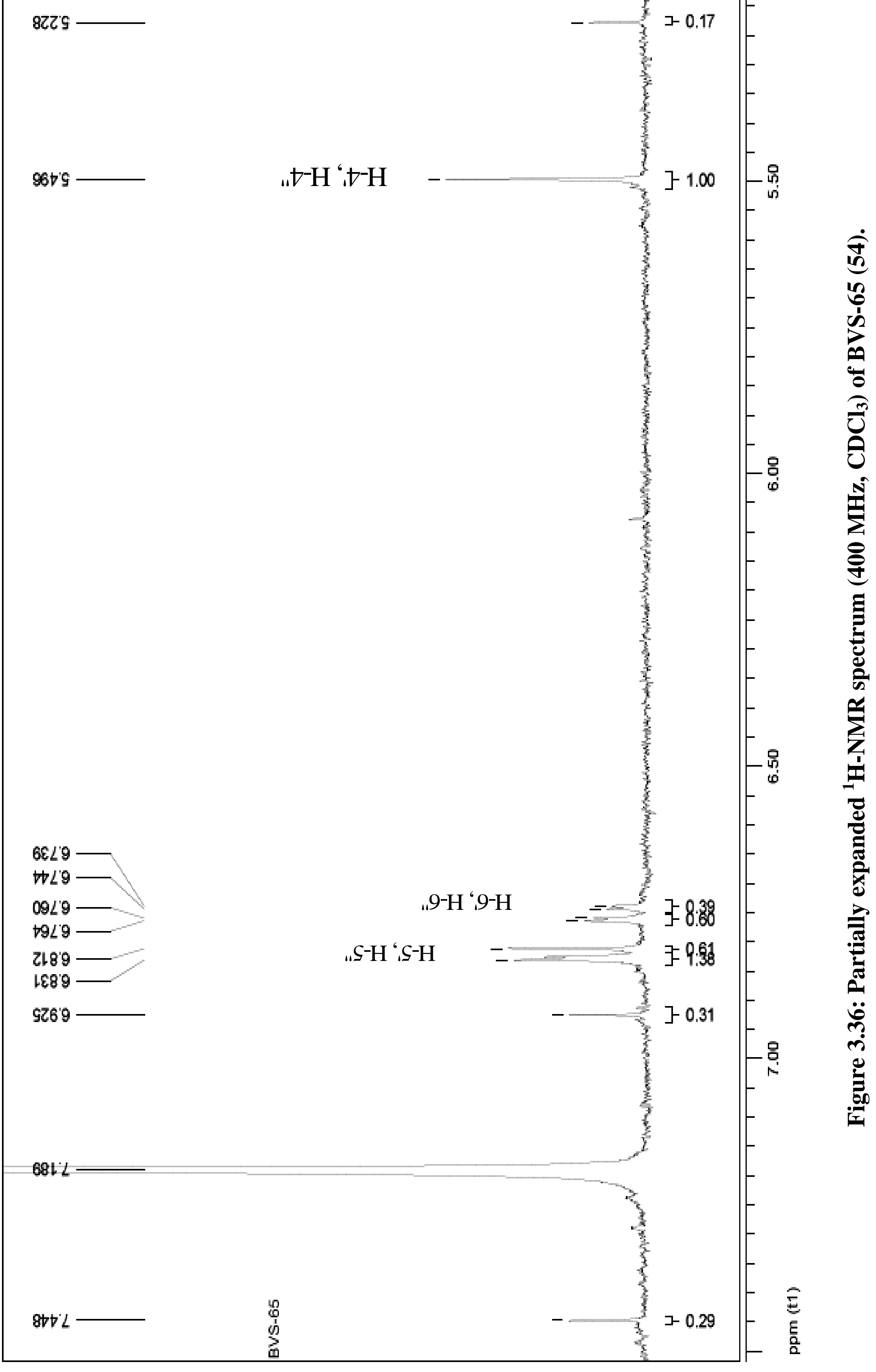

Figure 3.36: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BVS-65 (54).

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3.9 Characterization of BS-01 as glut-5(6)-en-3-one (55)

BS-01 was isolated as white crystals from the VLC fractions 4 and 5A of the crude methanolic extract of the stem bark of *B. stipularis*. These fractions were mixed together due to their identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (99: 1)]. The process was repeated thrice for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 ºC for 5 minutes and a purple colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of solvent yielded white crystals and was designated as BS-01 (8.23mg, **55**). The crystals melted at 207-210 ºC which was identical to that reported (208-210 ºC) for glut-5(6)-en-3-one (Shazid *et al*., 2012). The compound was found to be soluble in dichloromethane, chloroform, ethyl acetate, acetone and methanol.

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$; Figures 3.38-3.40; Table 3.11) of BS-01 displayed an olefinic proton signal at δ 5.61 (1H, m) which could be assigned to H-6 of a pentacyclic triterpenoid-type carbon skeleton. The spectrum also showed eight three proton singlets at δ 0.75, 0.89, 0.93, 0.96, 1.03, 1.10, 1.16 and 1.17 attributable to eight methyl groups present in the molecule.

Comparison of the ^{1}H-NMR spectral data with reported values of glut-5(6)-en-3-one (Shazid *et al*., 2012) demonstrated that all the proton shifts were similar as showed in Table 3.11. On this basis BS-01 was characterized as glut-5(6)-en-3-one, which was further approved by co-TLC with an authentic glut-5(6)-en-3-one previously isolated in our laboratory. Therefore, the compound BS-01 was characterized as glut-5(6)-en-3-one (**55**). This is the first report of isolation of glut-5(6)-en-3-one from *B. stipularis*.

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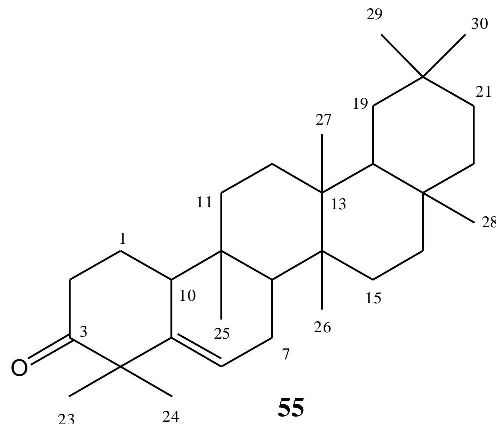

Figure 3.37: Structure of BS-01 (glut-5(6)-en-3-one, **55**).

Table 3.11: ^{1}H-NMR spectral data of BS-01 (55) and glut-5(6)-en-3-one (Shazid *et al*., 2012) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$.	
	BS-01 (55)	Glut-5(6)-en-3-one
H-6	5.61 m	5.69 m
Me-23	1.16 s	1.23 s
Me-24	1.17 s	1.24 s
Me-25	0.75 s	0.82 s
Me-26	1.03 s	1.10 s
Me-27	0.96 s	1.03 s
Me-28	1.10 s	1.17 s
Me-29	0.89 s	0.96 s
Me-30	0.93 s	0.99 s

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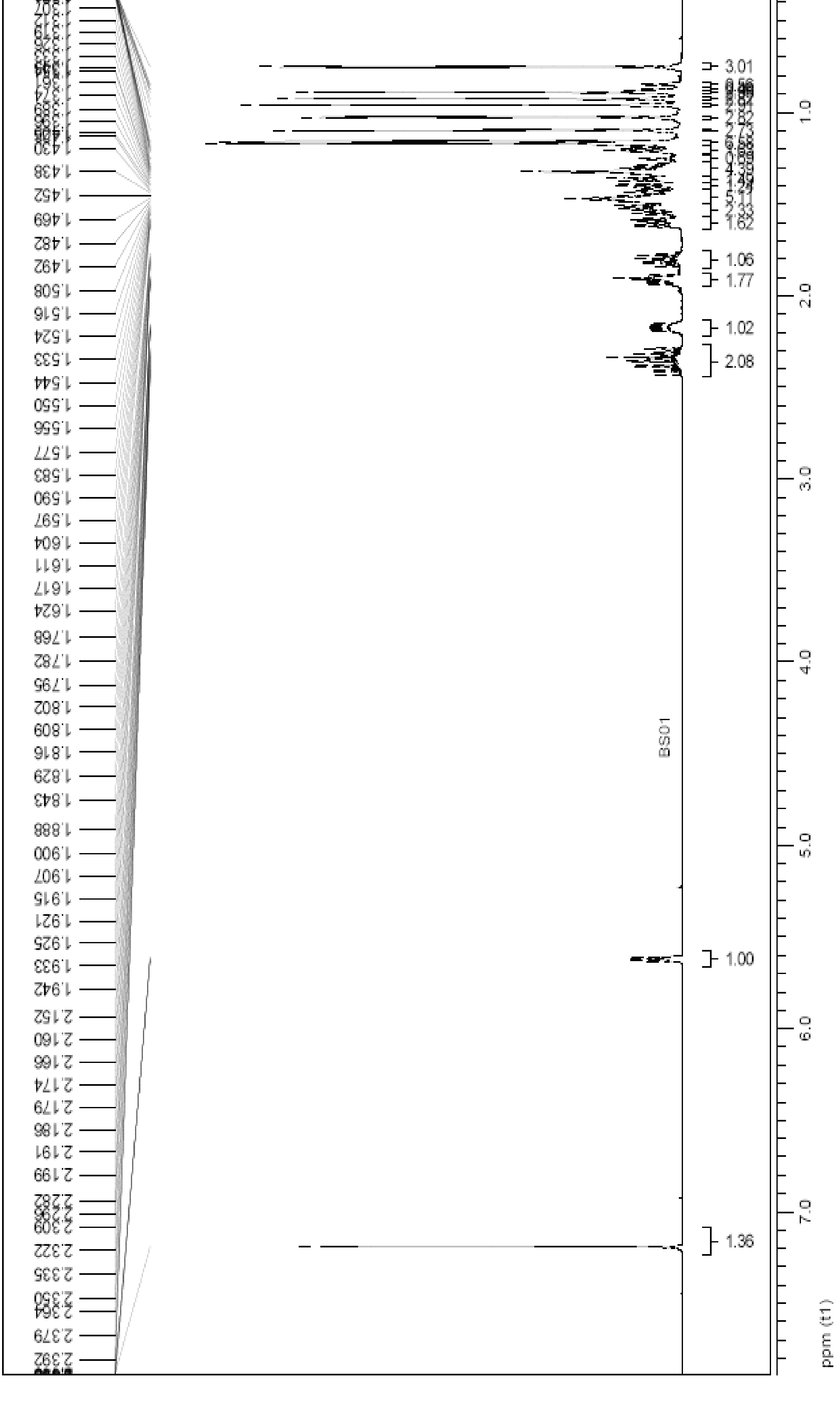

Figure 3.38: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BS-01 (55).

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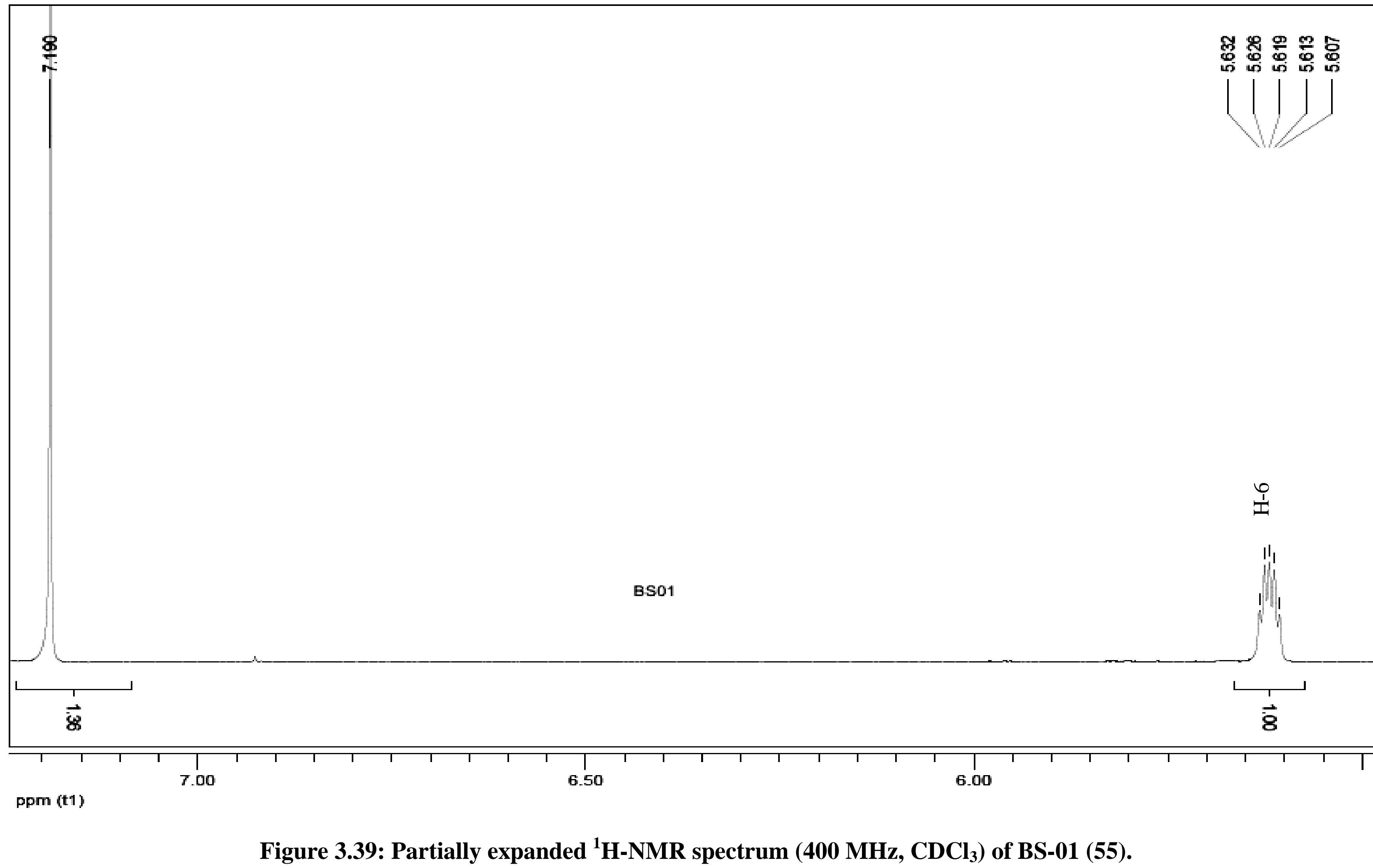

Figure 3.39: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BS-01 (55).

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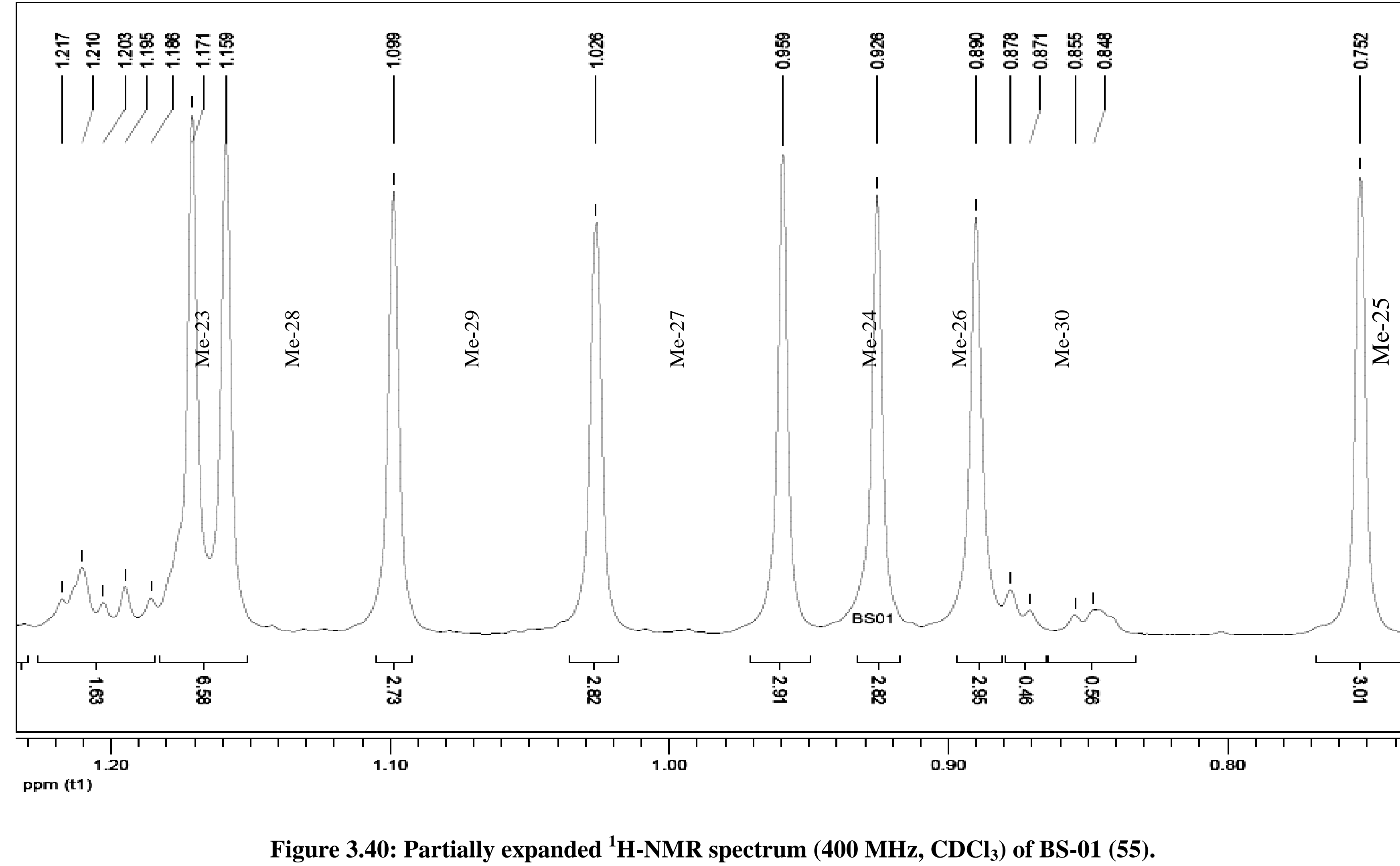

Figure 3.40: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BS-01 (55).

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3.10 Characterization of BS-06 as glut-5(6)-en-3α-ol (56)

Fractions 20-26 of the LH-50 column chromatography obtained from VLC fractions 9A-11B of the crude methanolic extract of the stem bark of *B. stipularis* were mixed together due to their identical TLC spots and subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (97:3)]. The process was repeated twice for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 ºC for 5 minutes and a pink colored band was visualized. The unsprayed portion of the band at stationary phase was scrapped off on aluminium foil and eluted with ethyl acetate. Evaporation of ethyl acetate yielded white needle like crystals which melted at 203 ºC. This was identical to the melting point (205 ºC) of glut-5(6)-en-3α-ol (Xue-jing *et al.*, 2012). The compound was checked for purity and termed as BS-06 (5.31 mg, **56**). It was found to be soluble in dichloromethane, chloroform, ethyl acetate and acetone.

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$; Figures 3.42-3.44; Table 3.12) of BS-06 displayed an olefinic proton signal at δ 5.64 (1H, d, *J*=6.4 Hz) and an oxymethine proton at δ 3.47 (1H, t, *J*= 2.8 Hz) which were assigned to H-6 and H-3, respectively. The oxymethine proton at δ 3.47 was axial in configuration as confirmed by a triplet with *J* value 2.8 Hz (Xue-jing *et al.*, 2012). The ^{1}H-NMR spectrum also showed eight methyl groups resonances as singlets at δ 0.85, 0.95, 0.99, 1.01, 1.05, 1.10, 1.14 and 1.17.

On the basis of the above spectral data, compound BS-06 was characterized as glut-5(6)-en-3α-ol (**57**) which was confirmed by comparison of its spectral data (Table 3.12) and melting point with reported values (Xue-jing *et al.*, 2012). This is the first report of isolation of glut-5(6)-en-3α-ol (**56**) from *B. stipularis*.

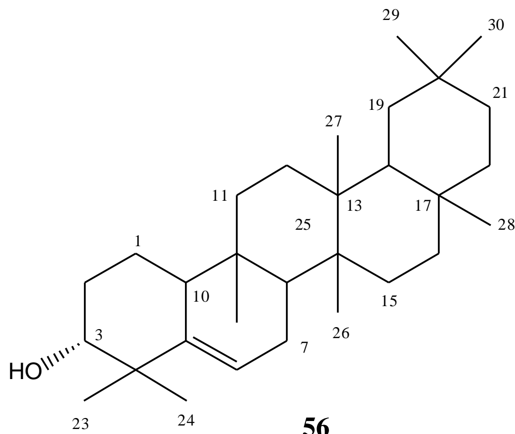

Figure 3.41: Structure of BS-06 (glut-5(6)-en-3α-ol, **56**).

Table 3.12: ^{1}H-NMR spectral data of BS-06 (56) and glut-5(6)-en-3α-ol (Xue-jing *et al.*, 2012) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	BS-06 (56)	**Glut-5(6)-en-3α-ol**
H-3	3.47 t (2.8)	3.51 t (2.9)
H-6	5.64 d (6.4)	5.63 d (6.41)
Me-23	1.17 s	1.16 s
Me-24	1.01 s	1.00 s
Me-25	0.85 s	0.85 s
Me-26	0.99 s	0.99 s
Me-27	1.05 s	1.05 s
Me-28	1.14 s	1.14 s
Me-29	1.10 s	1.08 s
Me-30	0.95 s	0.95 s

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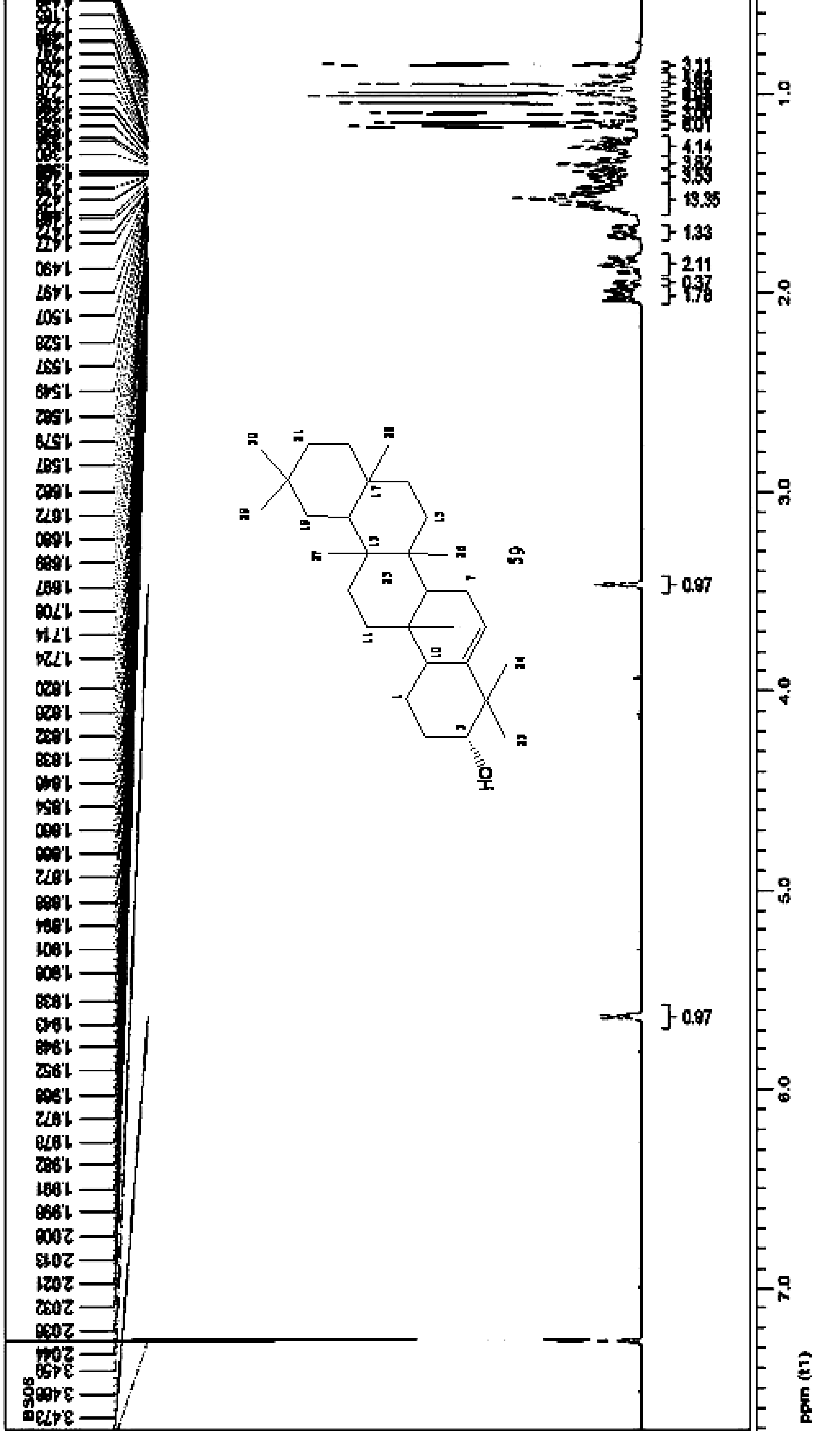

Figure 3.42: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BS-06 (56).

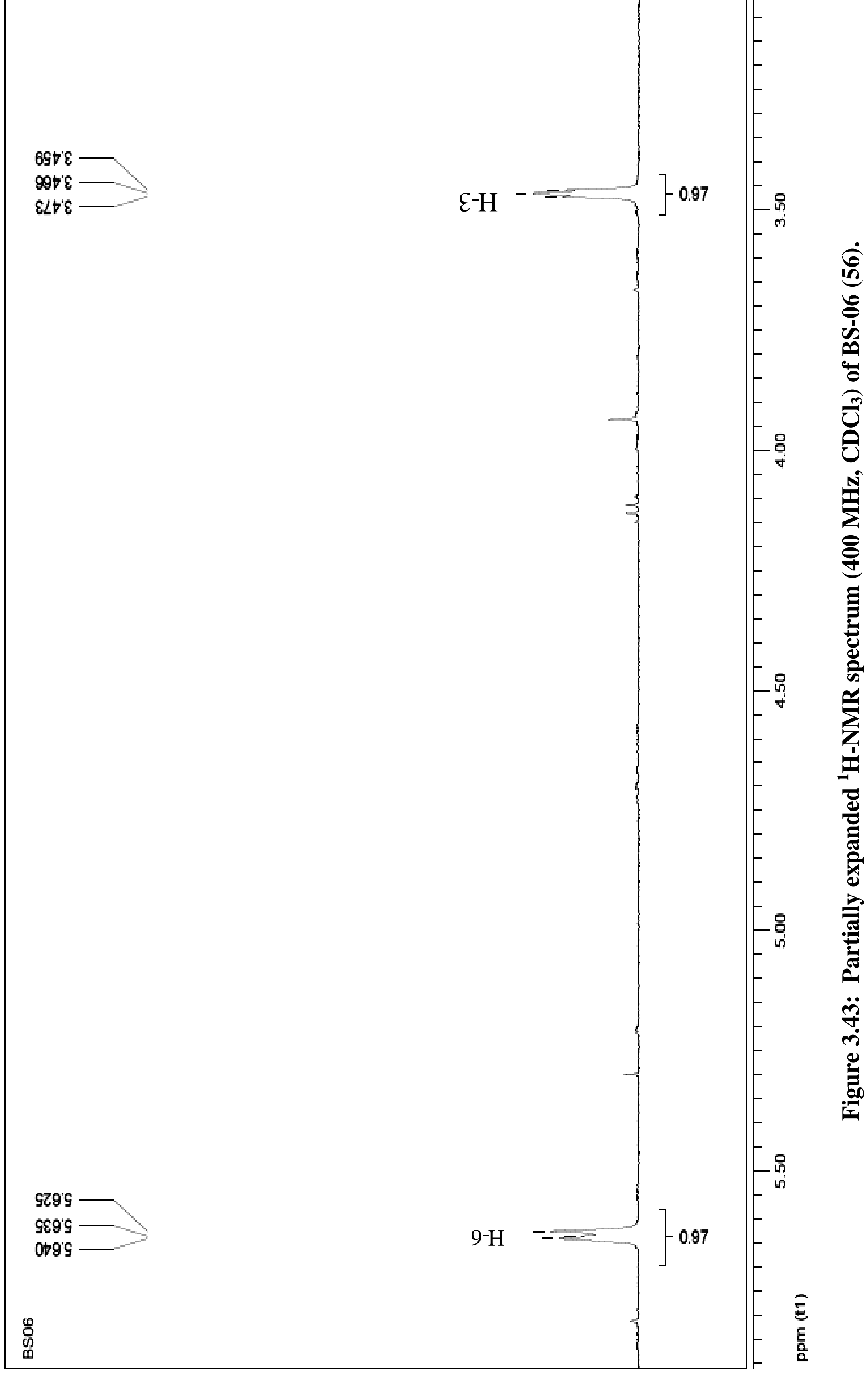

Figure 3.43: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BS-06 (56).

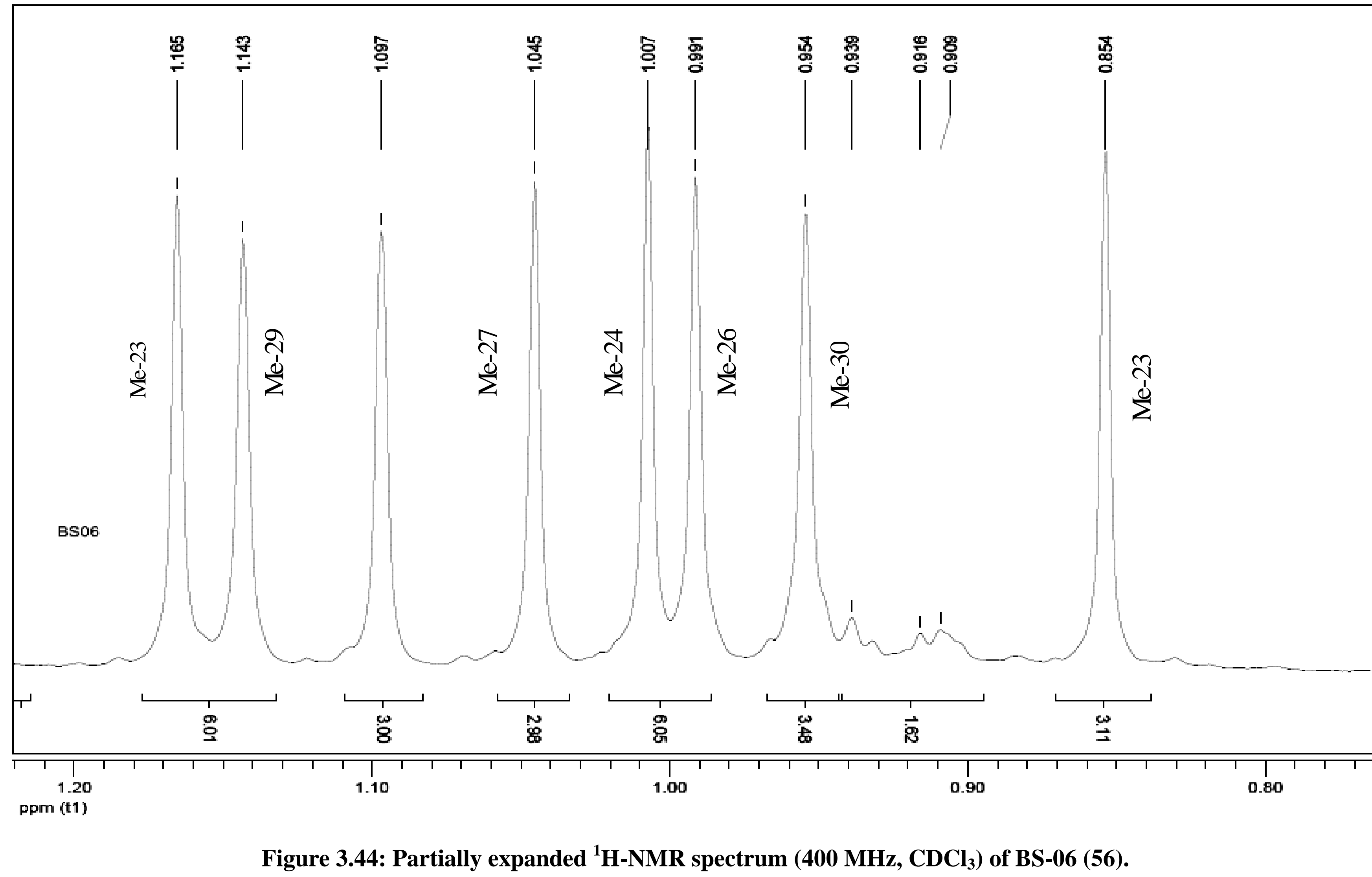

Figure 3.44: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BS-06 (56).

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3.11 Characterization of BS-11 (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one (57)

Vacuum liquid column chromatography of the methanolic extract of the stem bark of *B. stipularis* by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities provided 46 fractions. Fractions 9A-11B showing identical spots on TLC were bulked together and subjected to gel permeation chromatography over lipophilic LH-20 to give 55 fractions. Based on the TLC fractions 20-26 of LH-20 were again combined and evaporation of solvents afforded yellowish mass. This was subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (98:2)]. The process was repeated five times for better separation of the desired band, which was scrapped off and eluted with ethyl acetate. Evaporation of solvent yielded a pure white amorphous compound BS-11 (7.01 mg, **57**). The compound was soluble in *n*-hexane, dichloromethane, ethyl acetate and methanol.

The ^{1}H-NMR spectrum of the compound BS-11 (500 MHz, $CDCl_3$; Figures 3.46-3.48; Table 3.13) displayed signals at δ 0.70 (s, 3H), 0.85 (d, 6H, *J*= 6.8 Hz, overlapped), 1.27 (d, 3H, *J*= 6.7 Hz) and 1.30 (s, 3H) attributable to the methyl groups at C-18, C-26/27, C-21 and C-19, respectively in a cholestane type carbon skeleton. In the ^{1}H-NMR spectrum, the olefinic protons at H-22 and H-23 appeared as characteristic downfield signals at δ 5.20 (m, 1H) and δ 5.06 (m, 1H) (Aiello *et al.*, 1988). Another olefinic proton signal at δ 5.70 (bs, 1H) was attributed to the trisubstituted double bond conjugated with the ketone group located at C-3, and therefore, the trisubstituted double bond could be assigned at C-4 and C-5 (Mellado *et al.*, 2004). The ^{1}H-NMR spectrum further displayed signals of a oxymethine proton at δ 3.67 (m, 1H) and a tertiary methyl proton at δ 0.84 indicating a hydroxyl group at C-7 and a methyl group at C-28, respectively (Notaro *et al.*, 1992). In the literature, it was revealed that if the hydroxyl group was situated at C-24, the proton chemical shifts of C-24, C-22 and C-23 moved upfield to δ 3.77, 5.49 and 5.38 which do not match with the resonances present at the ^{1}H-NMR spectrum of BS-11.

On this basis as well as by comparison of two ^{1}H-NMR data of with that of chemical shifts of protons of steroidal ketone compounds reported in the literature (Aiello *et al.*, 1988; Mellado *et al.*, 2004), it can be assumed that the isolated compound BS-11 could be a (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one (**57**). This is the first report of occurrence of the compound from any nature and synthetic source. However, additional spectral data is required to confirm its structure.

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Figure 3.45: Structure of BS-11 (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one, **57**).

Table 3.13: ^{1}H-NMR spectral data of BS-11 (57) in $CDCl_3$.

Proton(s)	**δ_H in ppm in $CDCl_3$, (*J* in Hz).**
	BS-11 (57)
H-4	5.70 bs
H-7	3.67 m
Me-18	0.70 s
Me-19	1.33 s
Me-21	1.27 d (6.7)
H-22	5.06 (15.5 and 9.0)
H-23	5.20 (15.0 and 8.5)
Me-26	0.85 d (6.8)
Me-27	0.85 d (6.8)
Me-28	0.84 s

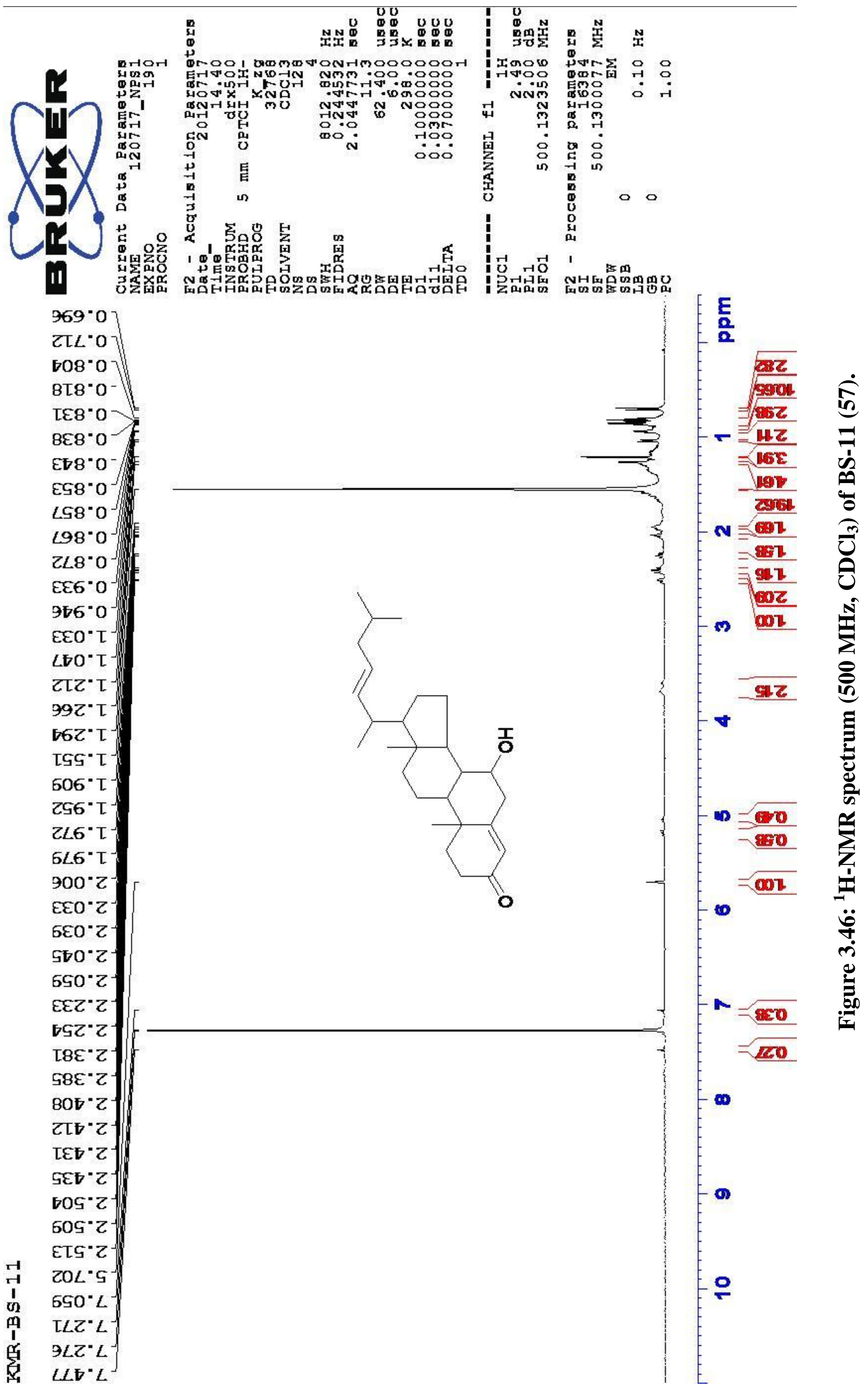

Figure 3.46: ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of BS-11 (57).

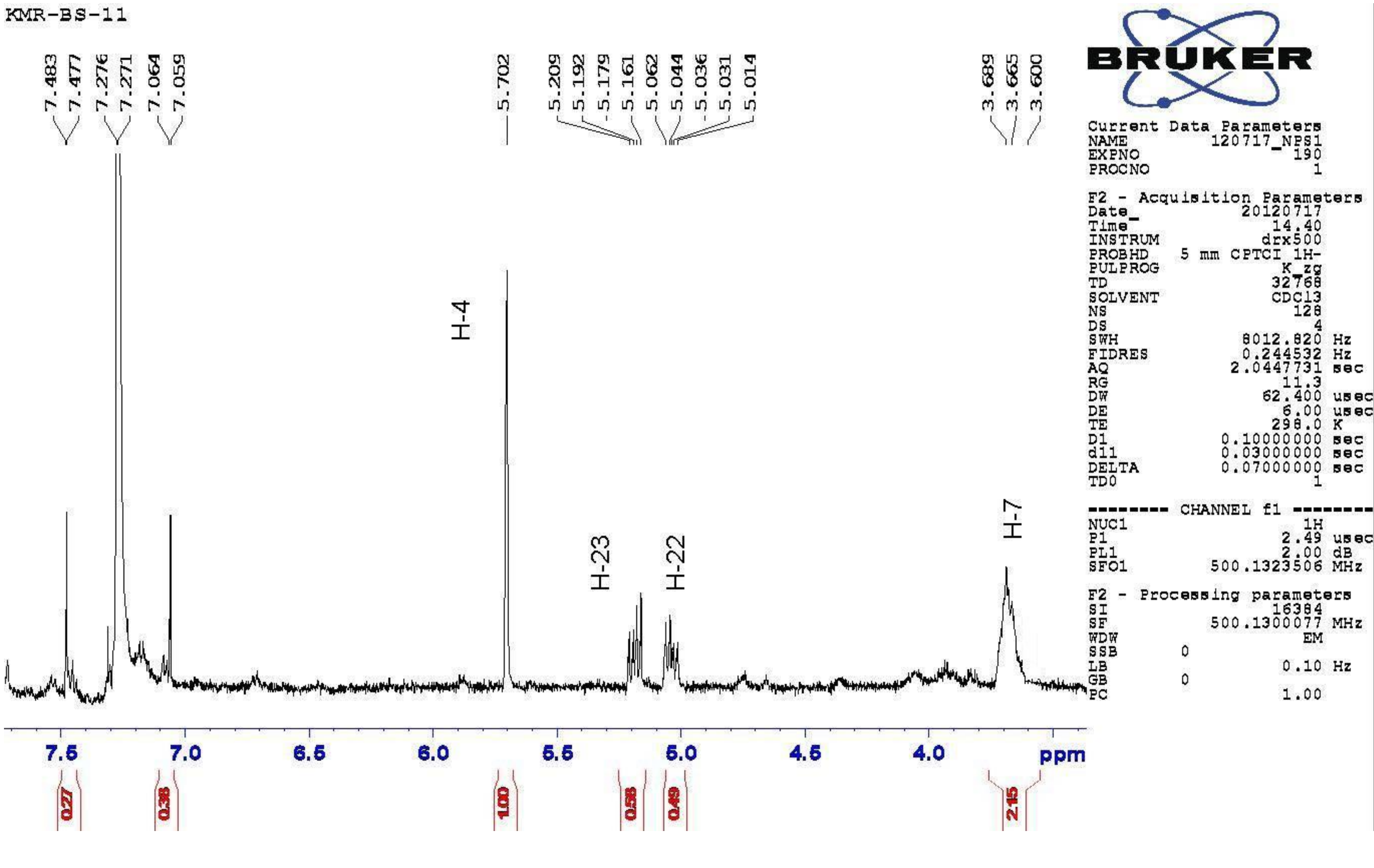

Figure 3.47: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of BS-11 (57).

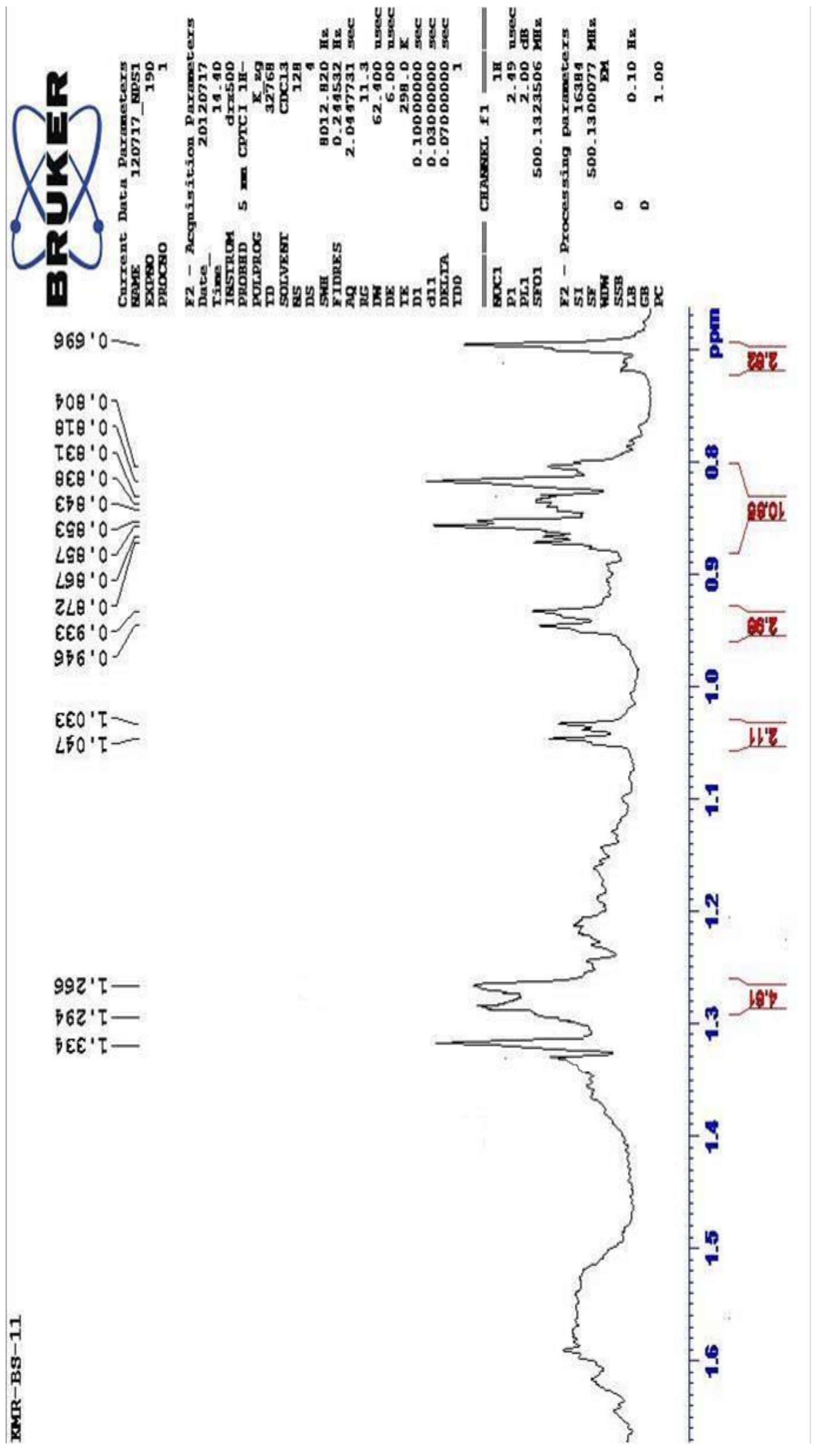

Figure 3.48: Partially epanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of BS-11 (57).

3.12 Characterization of BT-1 as friedoolean-5(6),14(15)-dien-3-one (58)

VLC fractions 4(A+B) of the methanolic extract of the stem bark of *B. tomentosa* were mixed together and subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (95:5)]. A deep purple colored band was visualized after spraying a portion of the developed plate with vanillin-sulfuric acid reagent followed by heating at 110 ºC for 5 minutes. The unsprayed portion of the band was scrapped off and eluted with ethyl acetate to provide a colorless mass which was checked for purity and termed as BT-1 (8.04 mg, **58**). It was found to be soluble in petroleum ether, chloroform and ethyl acetate.

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$; Figures 3.50-3.52; Table 3.14) of BT-1 showed eight methyl group resonances as singlets at δ 0.81, 0.95, 0.98, 1.02, 1.08, 1.16, 1.22 and 1.23 attributable to eight methyl groups present in the molecule (Xue-jing *et al.*, 2012). The ^{1}H-NMR spectrum displayed two olefinic proton signals at δ 5.68 (1H, m) and δ 5.56 (1H, dd, J= 6.4 and 2.1 Hz) which could be assigned to H-6 and H-15 of a pentacyclic triterpenoid skeleton (Shazid *et al.*, 2012; Fakir *et al.*, 2009, Moulisha *et al.*, 2009).

Comparison of the ^{1}H-NMR partial spectral data with reported values it was suggested the presence of a typical taraxaren-type pentacyclic triterpene skeleton (Xue-jing *et al.*, 2012; Shazid *et al.*, 2012; Fakir *et al.*, 2009, Moulisha *et al.*, 2009).

On the basis of the above spectral data as well as by comparison with that of chemical shifts of protons of pentacyclic triterpenes reported in the literatures (Xue-jing *et al.*, 2012; Shazid *et al.*, 2012; Fakir *et al.*, 2009 and Moulisha *et al.*, 2009), it can be assumed that the isolated compound BT-1 could be a friedoolean-5(6),14(15)-dien-3-one (**58**). This is the first report of occurrence of the compound from any nature and synthetic source. However, additional spectral data is required to confirm its structure.

Figure 3.49: Structures of BT-1 (friedoolean-5(6),14(15)-dien-3-one, **58**), glut-5(6)-en-3-one (**55**), glut-5(6)-en-3α-ol (**56**), β-taraxerol (**59**) and friedoolean-14-en,3-one [**58(i)**].

Table 3.14: ^{1}H-NMR spectral data of BT-1 (58) and other references (Xue-jing *et al.*, 2012; Shazid *et al.*, 2012; Fakir *et al.*, 2009 and Moulisha *et al.*, 2009) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).				
	BT-1 (58)	glut-5(6)-en-3-one (**55**)	glut-5(6)-en-3α-ol (**56**)	β-taraxerol (**59**)	friedoolean-14-en,3-one [**58(i)**]
H-6	5.68 m	5.69 m	-	-	-
H-15	5.56 dd (8.2, 3.6)	-	-	5.57 dd (8.0, 3.2)	5.56 dd (7.8, 2.7)
Me-23	1.23 s	-	1.16 s	-	-
Me-24	1.00 s	-	1.00 s	-	-
Me-25	0.81 s	-	0.85 s	-	-
Me-26	0.98 s	-	0.99 s	-	-
Me-27	1.08 s	-	1.05 s	-	-
Me-28	1.22 s	-	1.14 s	-	-
Me -29	1.16 s	-	1.08 s	-	-
Me-30	0.95 s	-	0.95 s	-	-

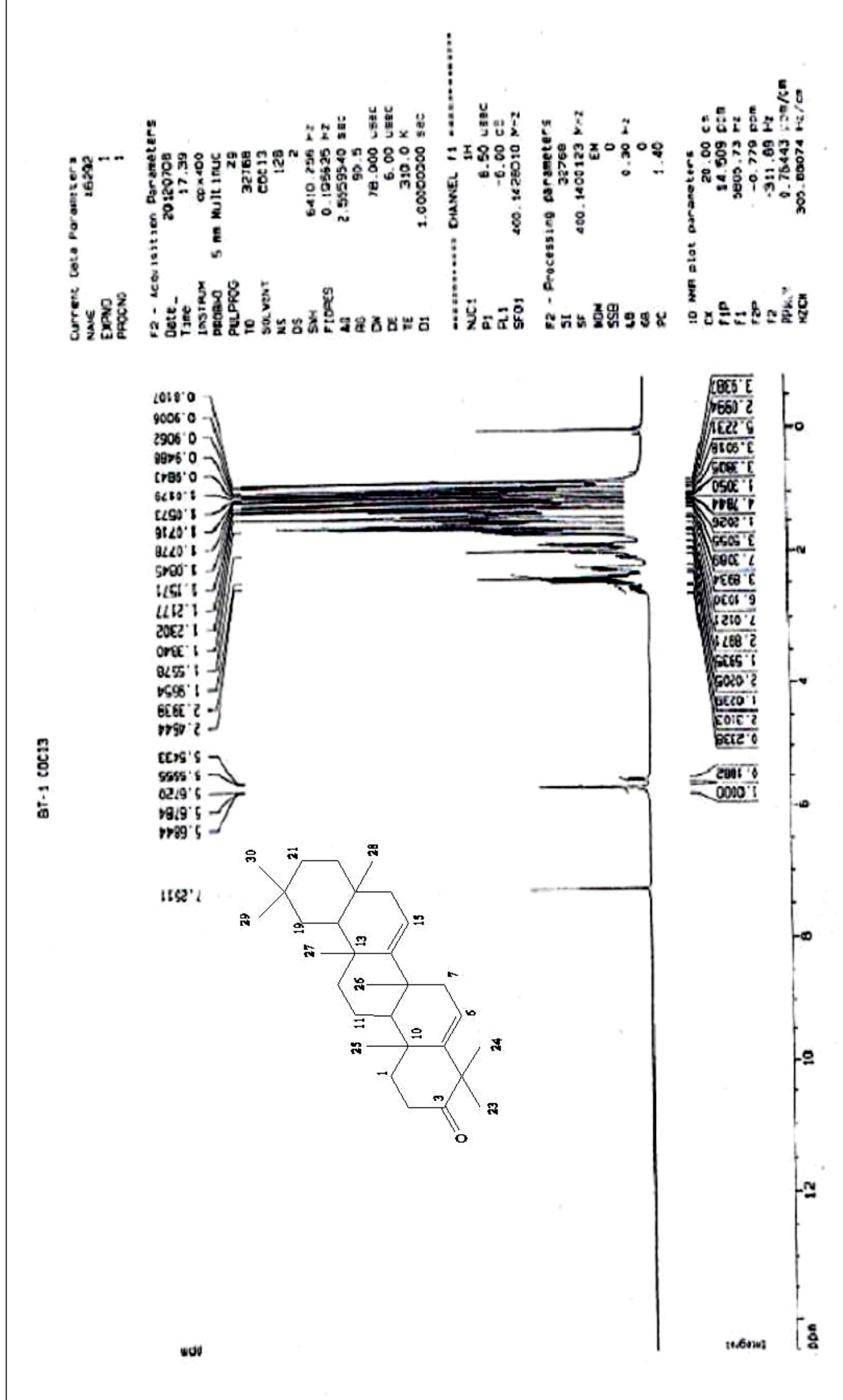

Figure 3.50: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BT-1 (58).

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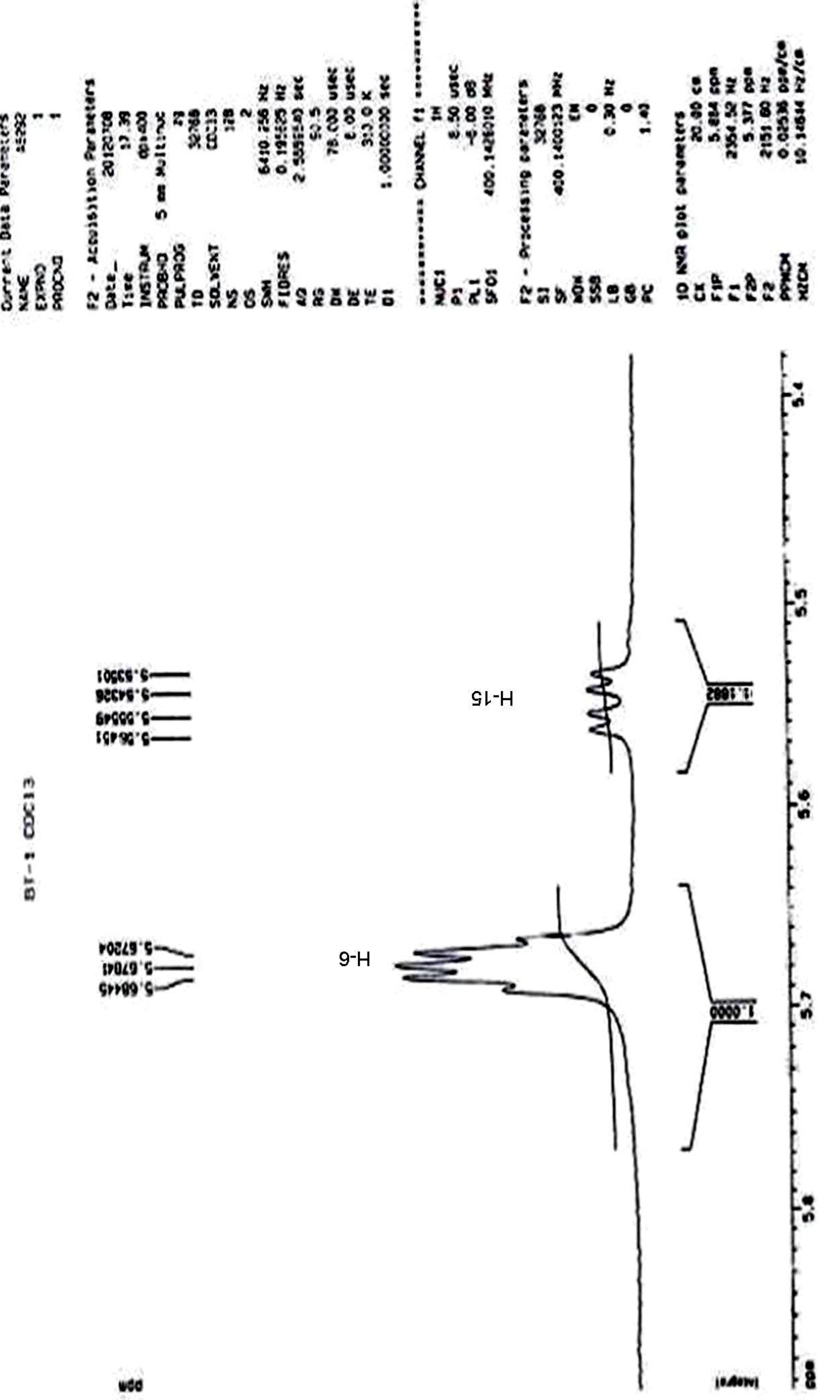

Figure 3.51: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BT-1 (58).

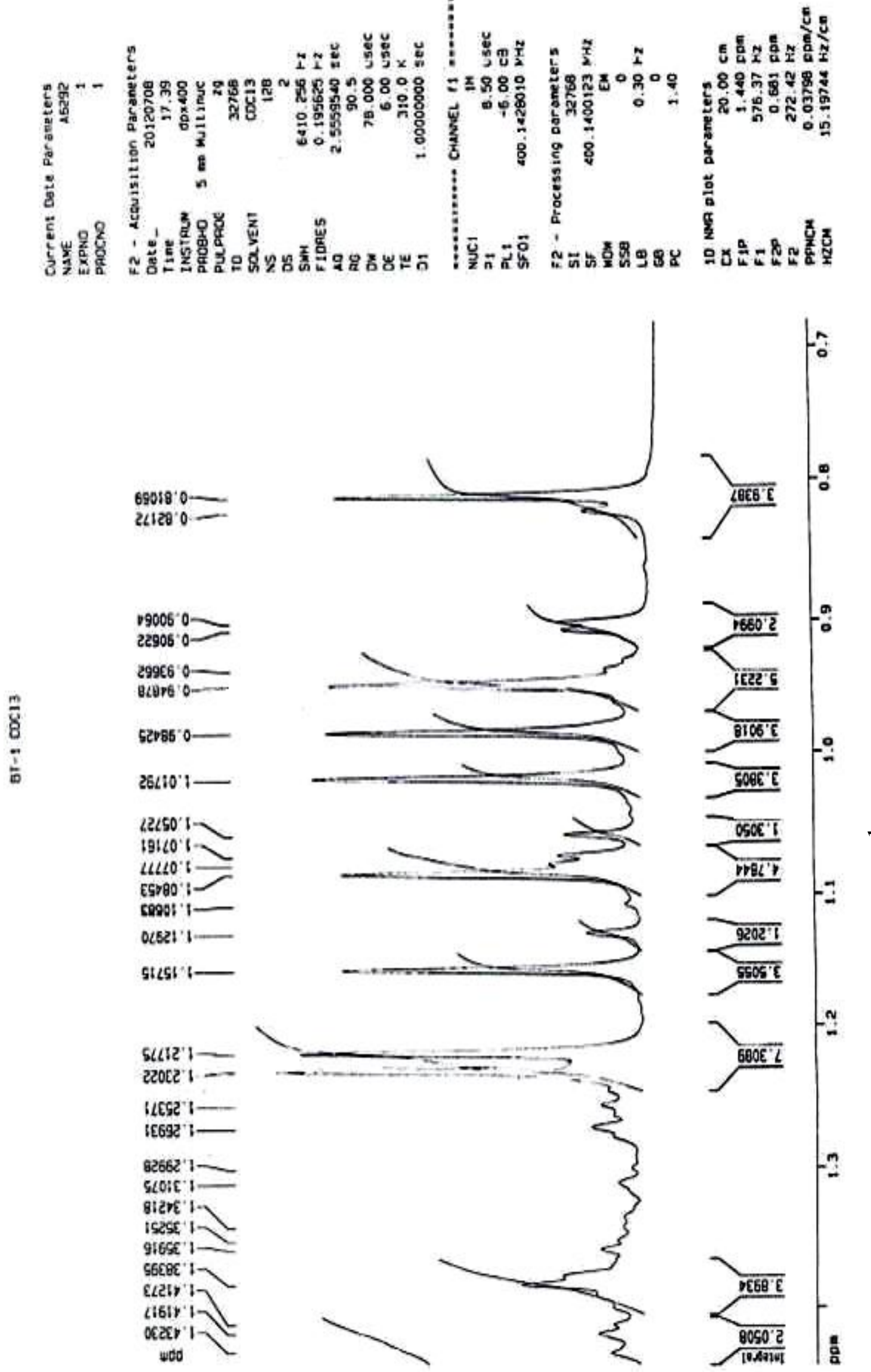

Figure 3.52: Partially expanded ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of BT-1 (58).

3.13 Characterization of BT-6 as β-taraxerol (59)

BT-6 was isolated as colorless mass from the VLC fractions 5B and 6A of the methanolic extract of the stem bark of *B. tomentosa*. Fractions 5B and 6A were mixed together due to their identical TLC spots and subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (95:5)]. A deep purple colored band was visualized after spraying a portion of the developed plate with vanillin-sulfuric acid reagent followed by heating at 110 ºC for 5 minutes. The unsprayed portion of the band on the stationary phase was scrapped off and eluted with ethyl acetate. Colorless mass was obtained which was checked for purity and termed as BT-6 (5.11 mg, **59**). It was found to be soluble in petroleum ether, chloroform and ethyl acetate.

The electrospray ionization mass spectrum (ESI-MS) of BT-6 (Figure 3.54) exhibited a pseudomolecular ion $(M+H)^+$ peak at *m/z* 427 which was consistent with molecular ion at 426 and molecular formula of $C_{30}H_{50}O$. The ^{1}H-NMR spectrum (500 MHz, $CDCl_3$; Figures 3.55-3.57; Table 3.15) of BT-6 showed eight three-proton singlets at δ 0.78, 0.80, 0.89, 0.89, 0.91, 0.93, 0.96 and 1.07, which were attributed to the methyl group protons at Me-28, Me-27, Me-23, Me-24, Me-29, Me-30, Me-25 and Me-27, respectively. The double doublet (*J*= 8.0, 3.5 Hz) of one proton intensity centered at δ 5.52 was assigned to the olefinic proton at C-15. Another double doublet (*J*= 10.5, 3.5 Hz) centered at δ 3.17 could be assigned to the oxymethine proton at C-3. The large couplings (*J*= 10.5, 3.5 Hz) of this proton (H-3) with the vicinal methylene protons (H_2-2) suggested a β orientation of the hydroxyl group at C-3. All the above ^{1}H-NMR signals suggested the presence of a typical taraxaren-type pentacyclic triterpene skeleton.

These spectral features (Table 3.15) were in close agreement to those published for β-taraxerol (Fakir *et al*., 2009). On this basis, the identity of BT-6 was confirmed as β-taraxerol (**59**). This is the first report of isolation of β-taraxerol form *B. tomentosa*.

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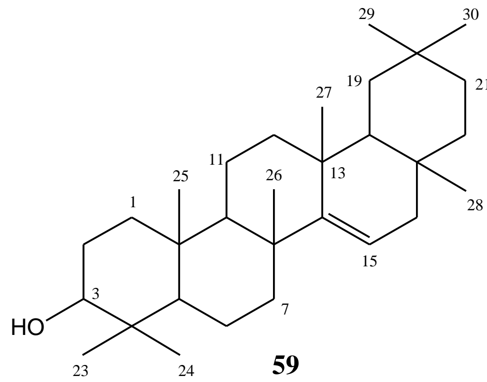

Figure 3.53: Structure of BT-6 (β-taraxerol, **59**).

Table 3.15: ^{1}H-NMR spectral data of BT-6 (59) and β-taraxerol in $CDCl_3$ (Fakir *et al.*, 2009) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	BT-6 (59)	**β-taraxerol**
H-3	3.17 dd (10.5 and 3.5)	3.19 d (9.2)
H-15	5.52 dd (8.0 and 3.5)	5.57 dd (8.0 and 3.2)
Me-23	0.89 s	0.90 s
Me-24	0.89 s	0.90 s
Me-25	0.96 s	0.97 s
Me-26	1.07 s	1.08 s
Me-27	0.80 s	0.82 s
Me-28	0.78 s	0.80 s
Me-29	0.91 s	0.92 s
Me-30	0.93 s	0.94 s

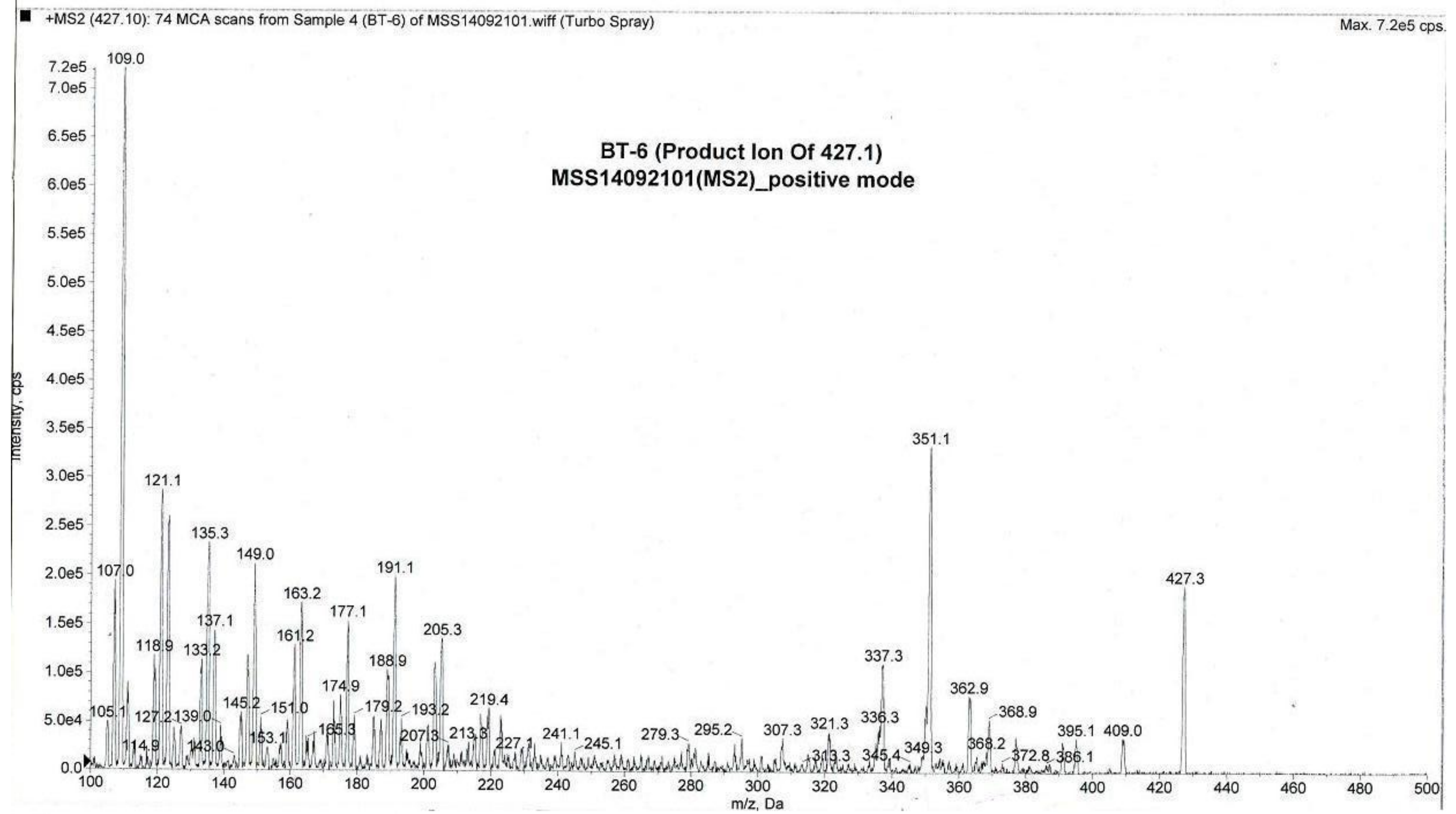

Figure 3.54: ESI-Mass spectrum of BT-6 (59).

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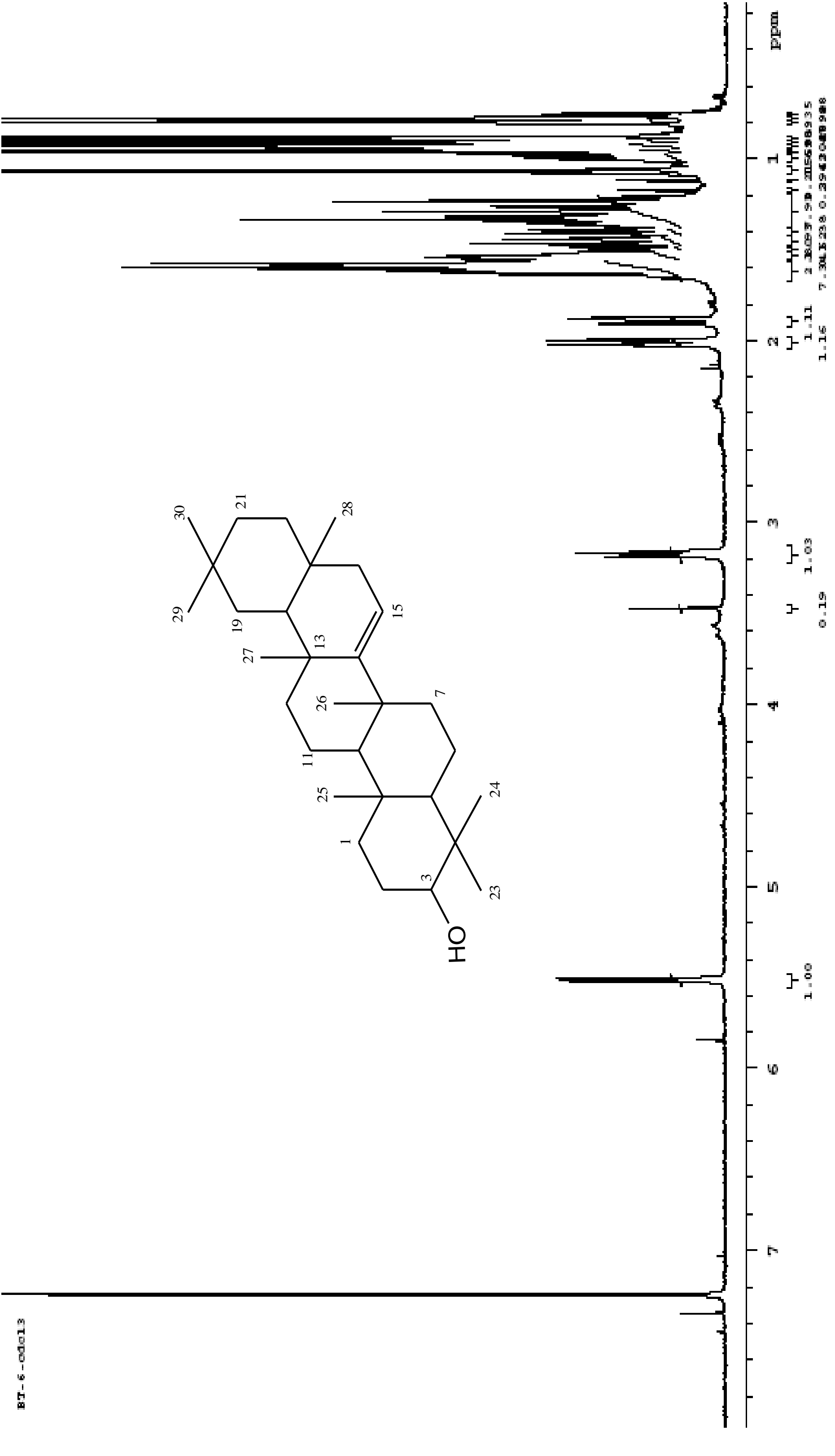

Figure 3.55: ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of BT-6 (59).

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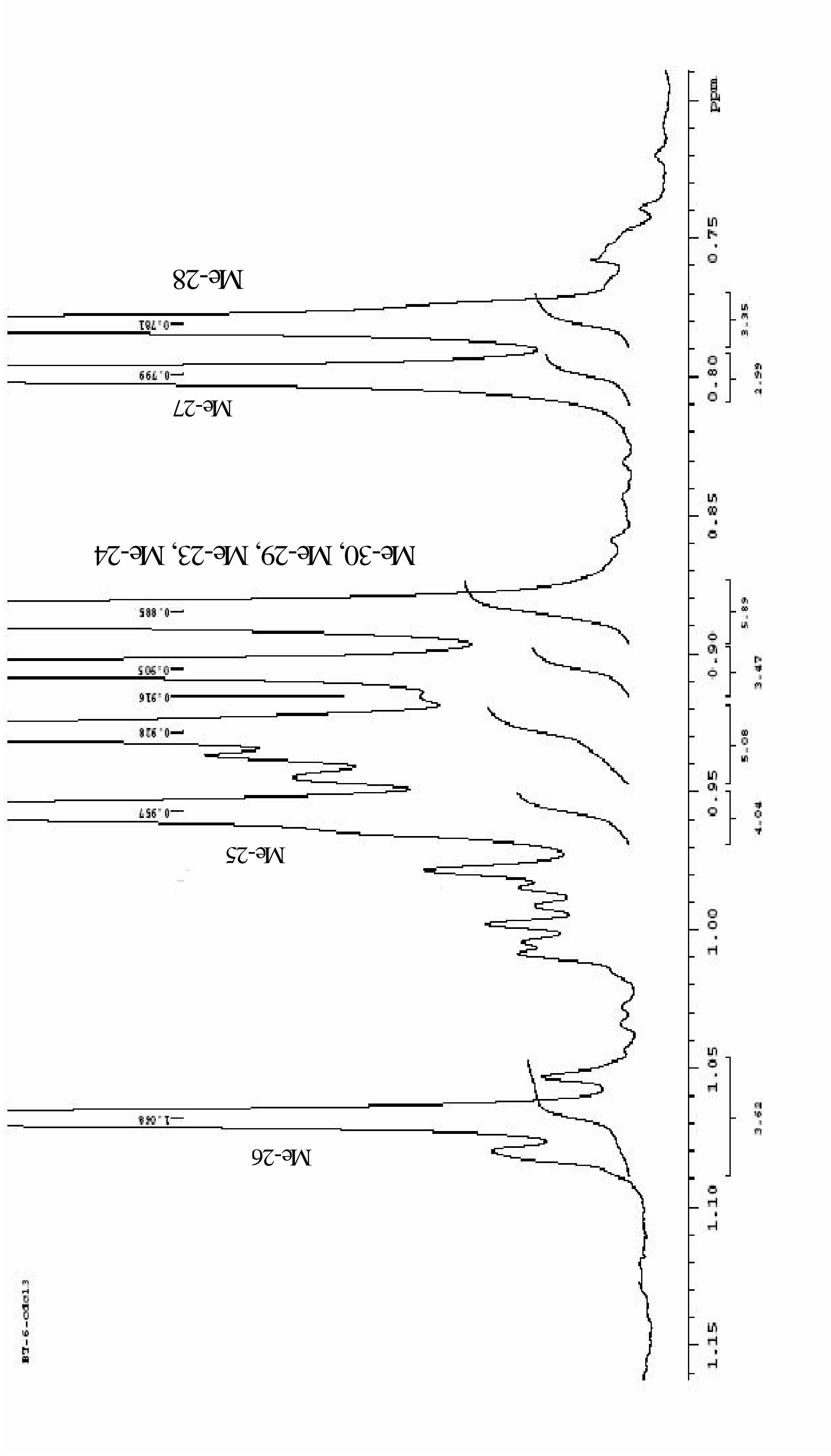

Figure 3.56: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of BT-6 (59).

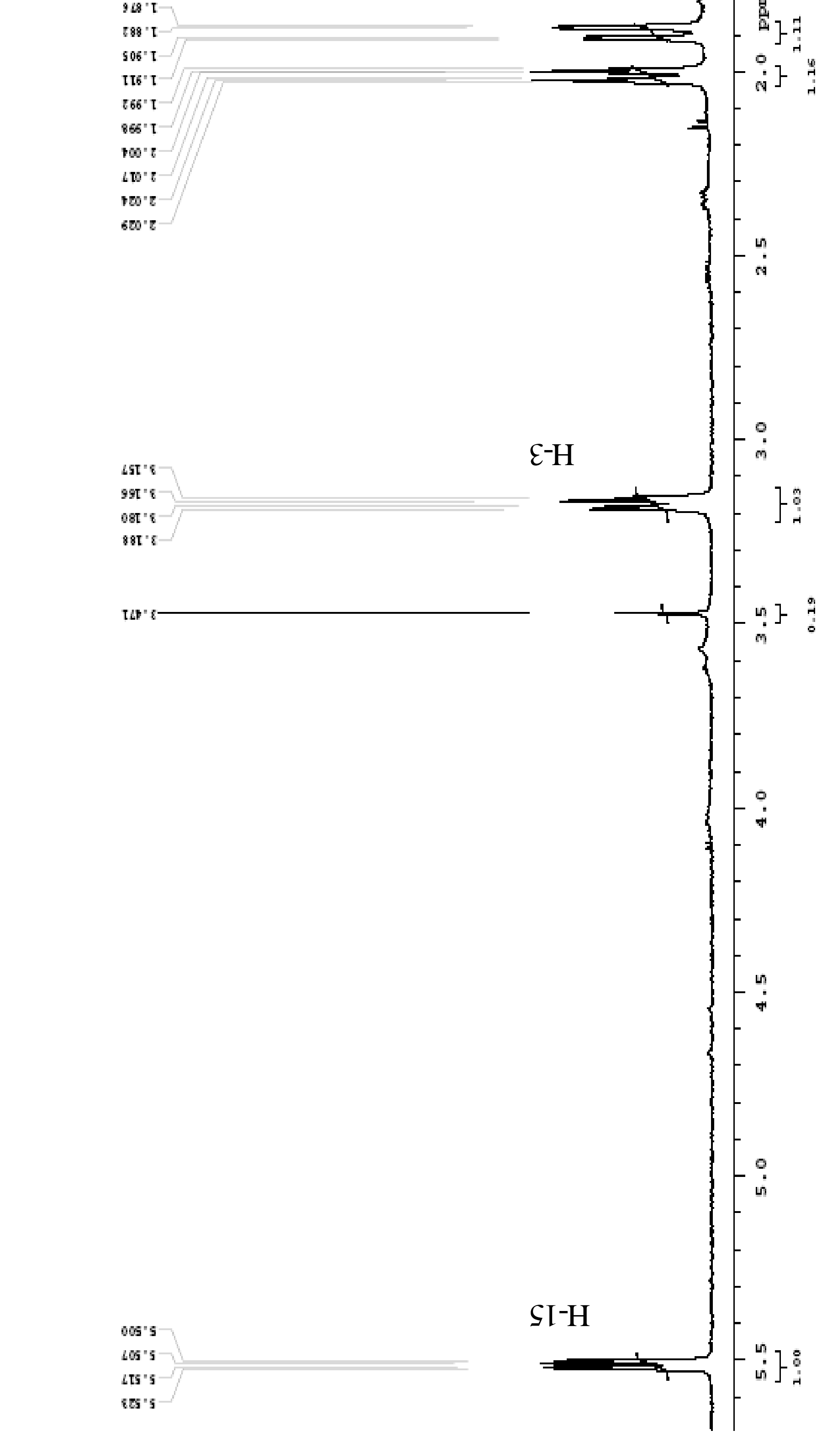

Figure 3.57: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of BT-6 (59).

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3.14 Characterization of BT-8 as D_4-stigmasterone (60)

BT-8 was isolated as crystals from the VLC fractions 12(A+B) and 13A of the methanolic extract of stem bark of *B. tomentosa*. VLC fractions 12(A+B) and 13A were mixed together due to their identical TLC characteristics and subjected to preparative TLC (Stationary phase - Silica gel F_{254}, Mobile phase - toluene: ethyl acetate = 90: 10), the process was repeated thrice for better separation and isolation of the desired band. From the developed plates a purple colored band was visualized after spraying a small portion of the PTLC plate with vanillin-sulfuric acid reagent followed by heating at 110 ºC for 5 minutes. The unsprayed stationary phase was scrapped off and eluted with ethyl acetate. After evaporation of the solvent fine tree like crystals were obtained which was termed as **BT-8** (6.29 mg, **60**). It was found to be soluble in petroleum ether, *n*-hexane, ethyl acetate, chloroform and methanol.

The electrospray ionization mass spectrum (ESI-MS) of BT-8 (Figure 3.59) exhibited a pseudomolecular ion $(M+H)^+$ peak at *m/z* 411 which was consistent with molecular ion at 410 that suggested it to be a steroid with molecular formula of $C_{29}H_{46}O$. The ^{1}H-NMR spectrum (500 MHz, $CDCl_3$; Figure 3.60; Table 3.16) showed resonances of six methyl groups, two of which were doublets at δ 0.80 d (*J*= 4.6 Hz) and 0.89 d (*J*= 4.6 Hz), associated to an isopropyl group substituent. Three double bond resonances of methine protons were observed at δ 5.76 as a singlet, 5.13 as a double doublet (*J*= 8.3, 15.1 Hz) and 5.01 also as a double doublet (*J*= 8.5, 15.0 Hz). This is characteristic of H-4, H-22 and H-23 in D_4-stigmasterone. Other spectral features are in close agreement with those of D_4-stigmasterone (Georges *et al.*, 2006), as well as the ^{1}H-NMR spectrum of BT-8 was super imposable to that of the spectrum acquired for an authentic sample previously isolated in our laboratory. So, the compound BT-8 was identified as D_4-stigmasterone (**60**). This is first report of isolation of D_4-stigmasterone (**59**) from *B. tomentosa*.

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Figure 3.58: Structure of BT-8 (D$_4$-stigmasterone, **60**).

Table 3.16: ^{1}H-NMR spectral data of BT-8 (60) and D$_4$-stigmasterone (Georges *et al.*, 2006) in $CDCl_3$.

Proton(s)	**δ_H in ppm in $CDCl_3$, (*J* in Hz).**	
	BT-8 (60)	**D$_4$-stigmasterone**
H-4	5.76 s	5.72 s
Me-18	0.71 s	0.73 s
Me-19	0.85 s	0.83 s
Me-21	1.01 s	1.04 s
Me-22	5.13 dd (15.1 and 8.3)	5.15 dd (15.2 and 8.4)
Me-23	5.01 dd (15.0 and 8.5)	5.02 dd (15.2 and 8.4)
Me-26	0.80 d (4.6)	0.81 d (4.5)
Me-27	0.89 d (4.6 Hz)	0.88 d (4.5)
Me-29	0.81 t (6.6 Hz)	0.82 t (4.5)

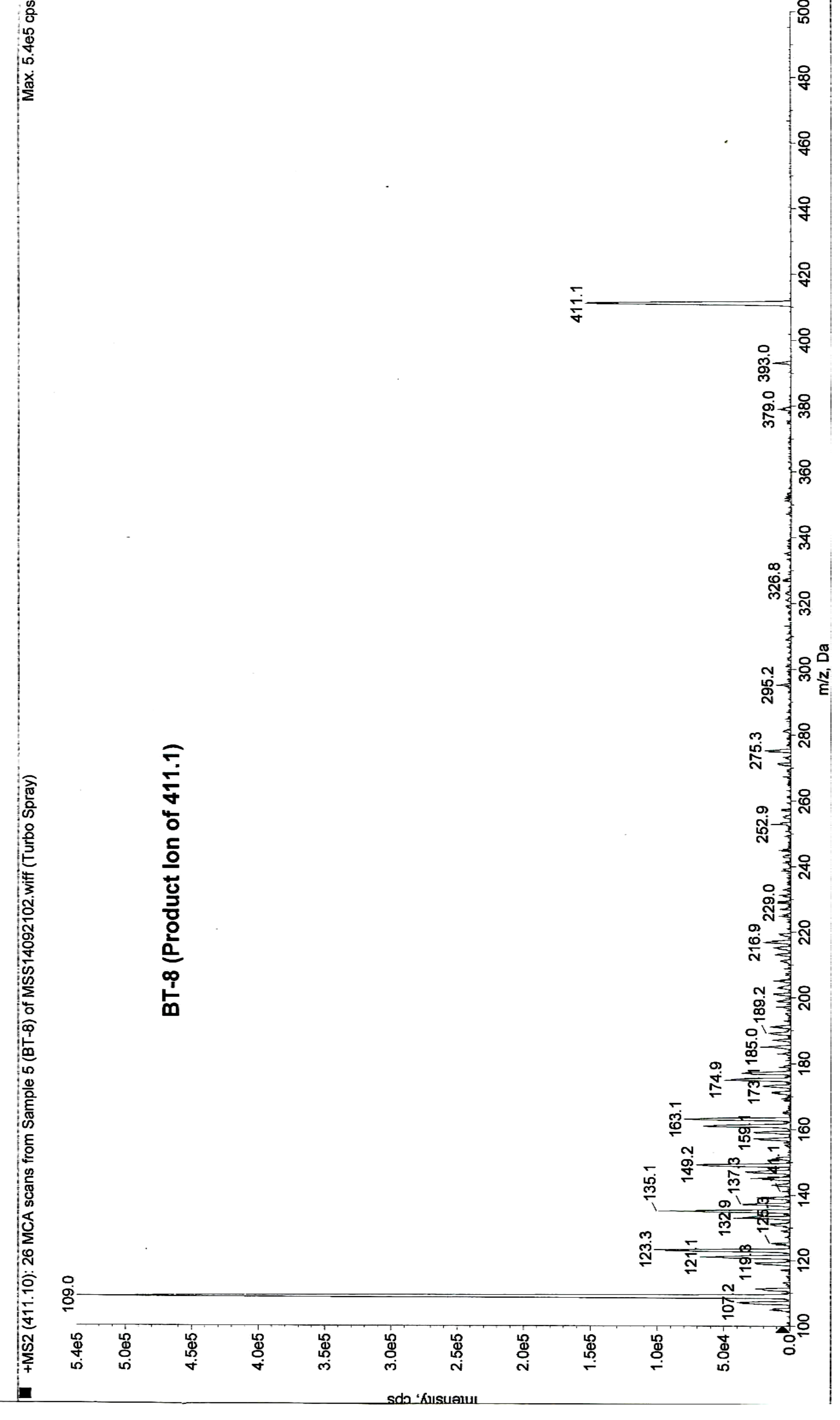

Figure 3.59: ESI-Mass spectrum of BT-8 (60).

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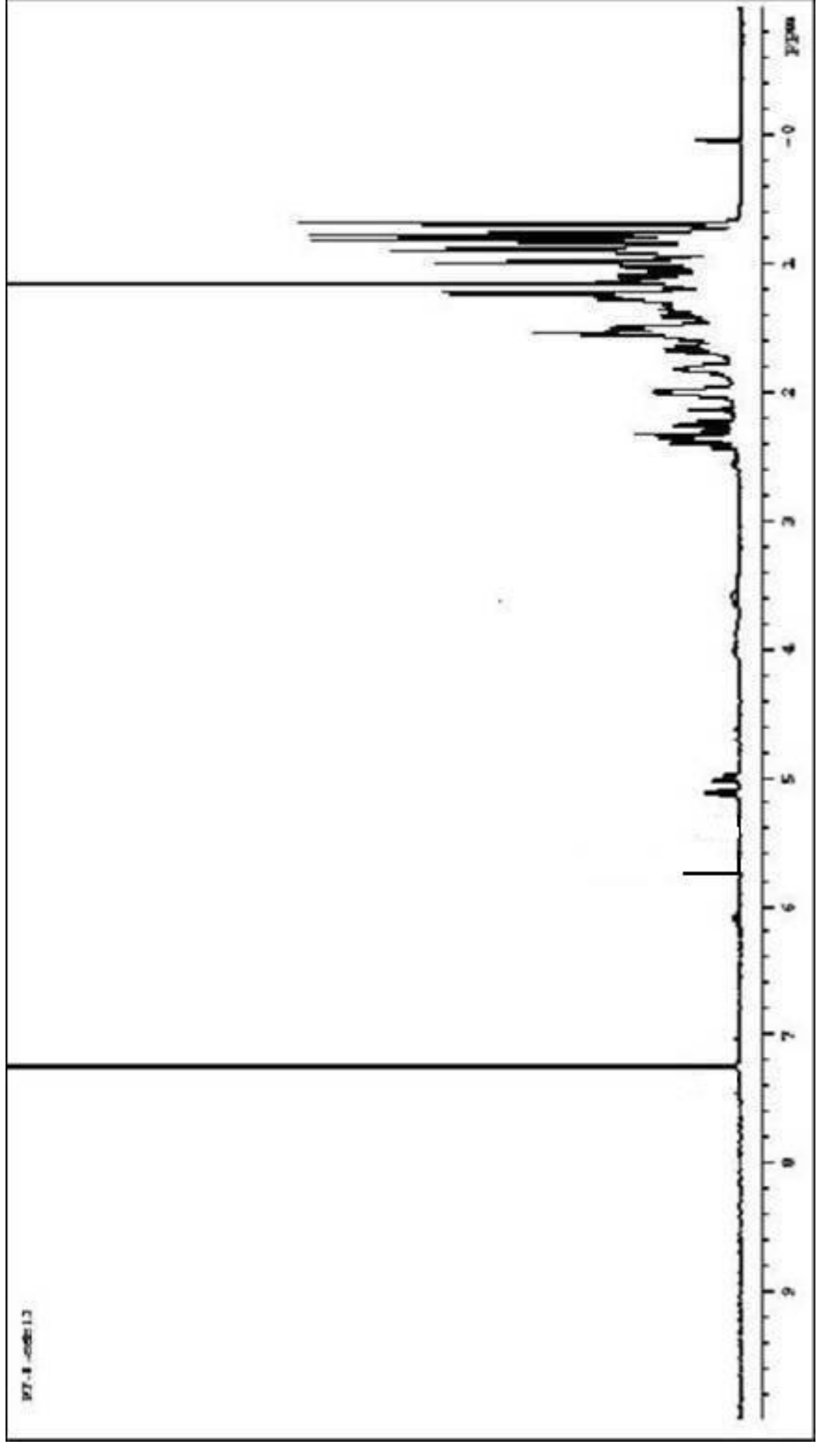

Figure 3.60: ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of BT-8 (60).

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3.15 Characterization of BT-14 as lupeol (61)

BT-14 was isolated as crystals from the VLC fractions 12(A+B) and 13A of the methanolic extract of stem bark of *B. tomentosa*. VLC fractions 12(A+B) and 13A were mixed together due to their identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (90:10)]. The process was repeated thrice for better separation and isolation of the desired band. From the developed plates a purple colored band was visualized after spraying a small portion of the PTLC plate with vanillin-sulfuric acid reagent followed by heating at 110 ºC for 5 minutes. The unsprayed portion of the band on the stationary phase was scrapped off and eluted with ethyl acetate. After evaporation of the solvent fine tree like crystals were obtained which after purity test was termed as BT-14 (6.31 mg, **61**).

The crystals were found to be soluble in petroleum ether, *n*-hexane, ethyl acetate, chloroform and sparingly soluble in methanol.

The electrospray ionization mass spectrum (ESI-MS) of BT-14 (Figure 3.62) exhibited a pseudomolecular ion $(M+H)^+$ peak at *m/z* 427 which was consistent with molecular ion at 426 that suggested it to be a triterpenoid with molecular formula of $C_{30}H_{50}O$. The ^{1}H-NMR spectrum (500 MHz, $CDCl_3$; Figure 3.63; Table 3.17) of BT-14 showed a double doublet (J= 11.2, 4.8 Hz) of one proton intensity at δ 3.20, typical for an oxymethine proton at C-3 in a triterpene. The splitting pattern of this proton revealed the β orientation of the C-3 oxygenated substituent. The spectrum also displayed two broad one proton singlet at δ 4.68 and 4.56 (1H each) assignable to the vinylic protons at C-29. The ^{1}H-NMR spectrum further showed a characteristic multiplet of one proton intensity at δ 2.36 which could be ascribed to the proton at C-19 and seven singlets at δ 0.96, 0.78, 0.83, 1.02, 0.93, 0.82 and 1.67 (3H each) assignable to the methyl protons at C-4 (H_3-23, H_3-24), C-10 (H_3-25), C-8 (H_3-26), C-14 (H_3-27), C-17 (H_3-28) and C-20 (H_3-30), respectively. The downfield methyl group resonance at δ 1.67 was assigned to the vinylic methyl group at C-20 (H_3-30).

By comparing the ^{1}H-NMR spectral data (Table 3.17) with previously published values (Jahan *et al.*, 2010) BT-14 was identified as lupeol (**61**). The identity of BT-14 was further substantiated by co-TLC with an authentic lupeol previously isolated in our laboratory. This is the first report of isolation of lupeol from *B. tomentosa*.

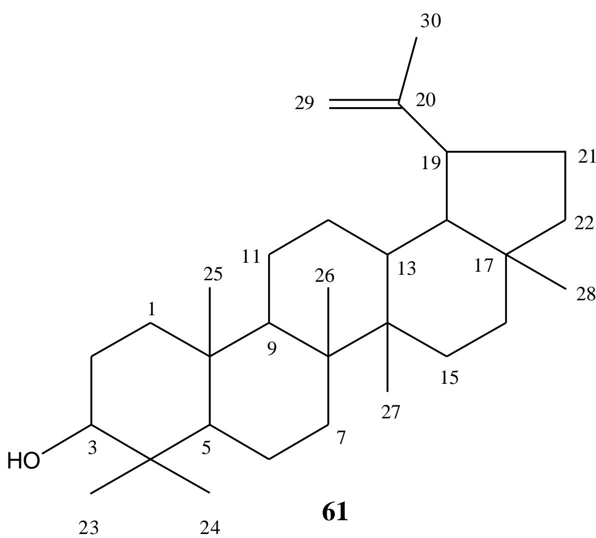

Figure 3.61: Structure of BT-14 (lupeol, **61**).

Table 3.17: ^{1}H-NMR spectral data of BT-14 (61) and lupeol (Jahan *et al.*, 2010) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	BT-14 (61)	**Lupeol**
H-3	3.20 dd (11.2 and 4.8)	3.20 dd (11.5 and 5.0)
H-19	2.30 m	2.28 m
Me-23	0.96 s	0.95 s
Me-24	0.78 s	0.78 s
Me-25	0.83 s	0.84 s
Me-26	1.02 s	1.02 s
Me-27	0.94 s	0.93 s
Me-28	0.75 s	0.82 s
Me_a -29	4.56 s	4.55 s
Me_b -29	4.68 s	4.67 s
Me-30	1.67 s	1.68 s

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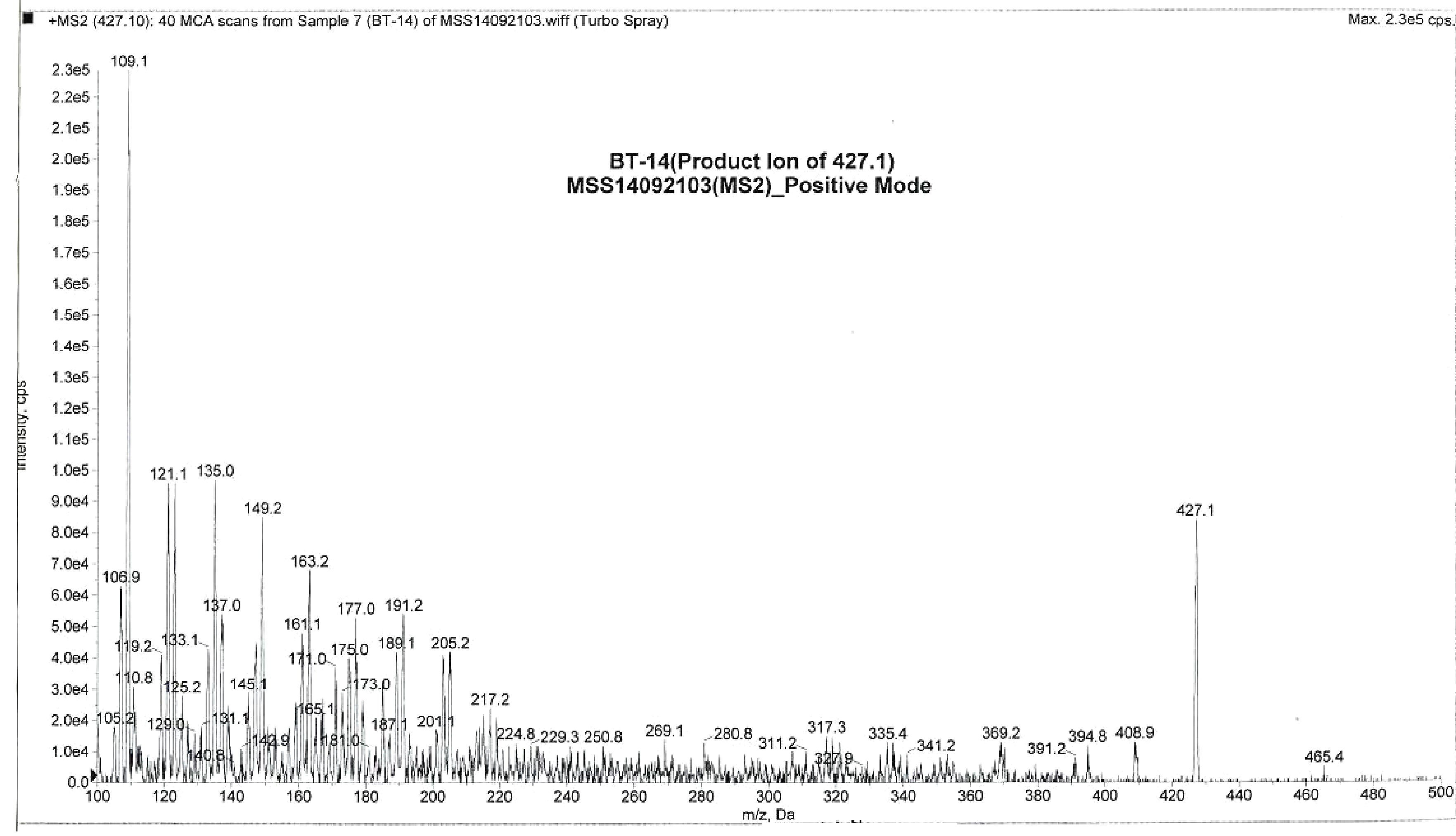

Figure 3.62: Mass spectrum of BT-6 (61).

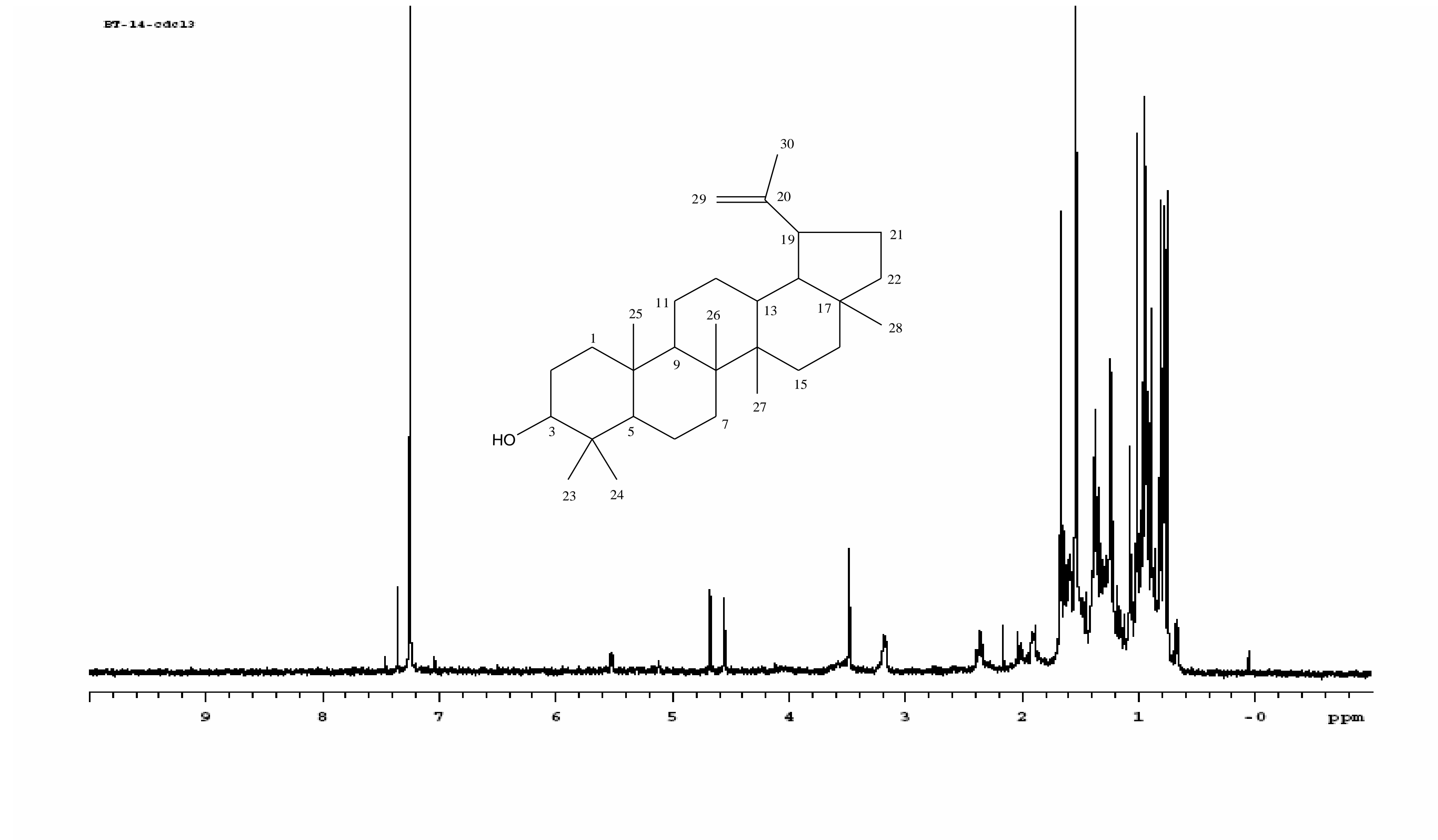

Figure 3.63: ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of BT-14 (61).

3.16 Characterization of EF-7 as shinpterocarpin (62)

EF-7 was isolated as yellowish mass by evaporation of solvent from the silica column fractions 90-97 of the carbon tetrachloride soluble portion of methanolic extract of the stem bark of *E. fusca*. The yellowish residue was then subjected to preparative TLC over silica gel F_{254} using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated thrice for better separation of the desired band. After excitation with 254 nm UV light, quenching was observed by the desired band as a uniform layer. Then the band was scrapped off on an aluminum foil and eluted with ethyl acetate. Evaporation of the solvent yielded an oily transparent liquid. It was checked for purity and denoted as EF-7 (6.12 mg, **62**). The compound was soluble in *n*-hexane, ethyl acetate, chloroform and methanol.

The ^{1}H-NMR spectrum of the compound EF-7 (500 MHz, $CDCl_3$; Figures 3.65-3.70; Table 3.18) showed a one proton multiplet at δ 3.50 (m) due to 6a-H, at δ 5.52 (d, *J*= 6.5 Hz) for 11a-H, two proton signals at δ 3.63 (dd, *J*=11.0, 5.0 Hz) and 4.25 (dd, *J*=11.0, 11.0 Hz) due to 6-H_2 which indicated a pterocarpan skeleton. In the ring A, the presence of two doublets aromatic protons at δ 7.43 (1H, d, *J*= 8.0 Hz) for H-1 and 6.36 (1H, d, *J*= 8.0 Hz) for H-2 indicated that C-3 and C-4 of the ring were substituted. In the D ring, the presence of two doublets with one aromatic proton intensity each at δ 6.97 (1H, d, *J*= 8.0 Hz) for H-7 and 6.44 (1H, d, *J*= 2.0 Hz) for H-8 indicated that the C-9 was substituted with hydroxyl group. The ^{1}H-NMR spectrum further showed two singlets with three proton intensity at δ 1.45 and 1.44 assignable to the methyl protons at C-6'.

Comparison of ^{1}H-NMR data for EF-7 were found to be similar with those for shinpterocarpin (Table 3.18) (Kitagawa *et al.*, 1994). Therefore, the compound EF-7 was identified as shinpterocarpin (**62**). This is the first report of isolation of shinpterocarpin (**62**) from *E. fusca*.

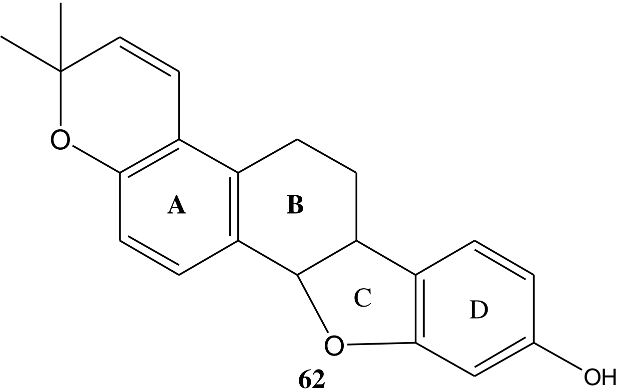

Figure 3.64: Structure of EF-7 (shinpterocarpin, **62**).

Table 3.18: ^{1}H-NMR spectral data of EF-7 (62) and shinpterocarpin (Kitagawa *et al.*, 1994) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	EF-7 (62)	**Shinpterocarpin**
H-1	7.43 d (8.0)	7.25 d (8.8)
H-2	6.36 d (8.0)	6.48 d (8.8)
H-6	3.63 dd (11.0 and 5.0)	3.65 dd (10.5 and 9.4)
	4.25 dd (11.0 and 11.0)	4.33 dd (10.5 and 4.4)
H-6a	3.5 m	3.61 m
H-7	6.97 d (8.0)	7.13 d (8.3)
H-8	6.57 dd (8.0 and 2.0)	6.38 dd (8.3 and 2.2)
H-10	6.44 d (2.0)	6.31 d (2.2)
H-11a	5.52 d (6.5)	5.48 d (6.6)
H-4'	6.52 d (10.0)	6.60 d (9.9)
H-5'	5.52 d (10.0)	5.67 d (9.9)
6' methyl	1.45 s	1.39 s (6'-CH_3 × 2)
	1.41 s	

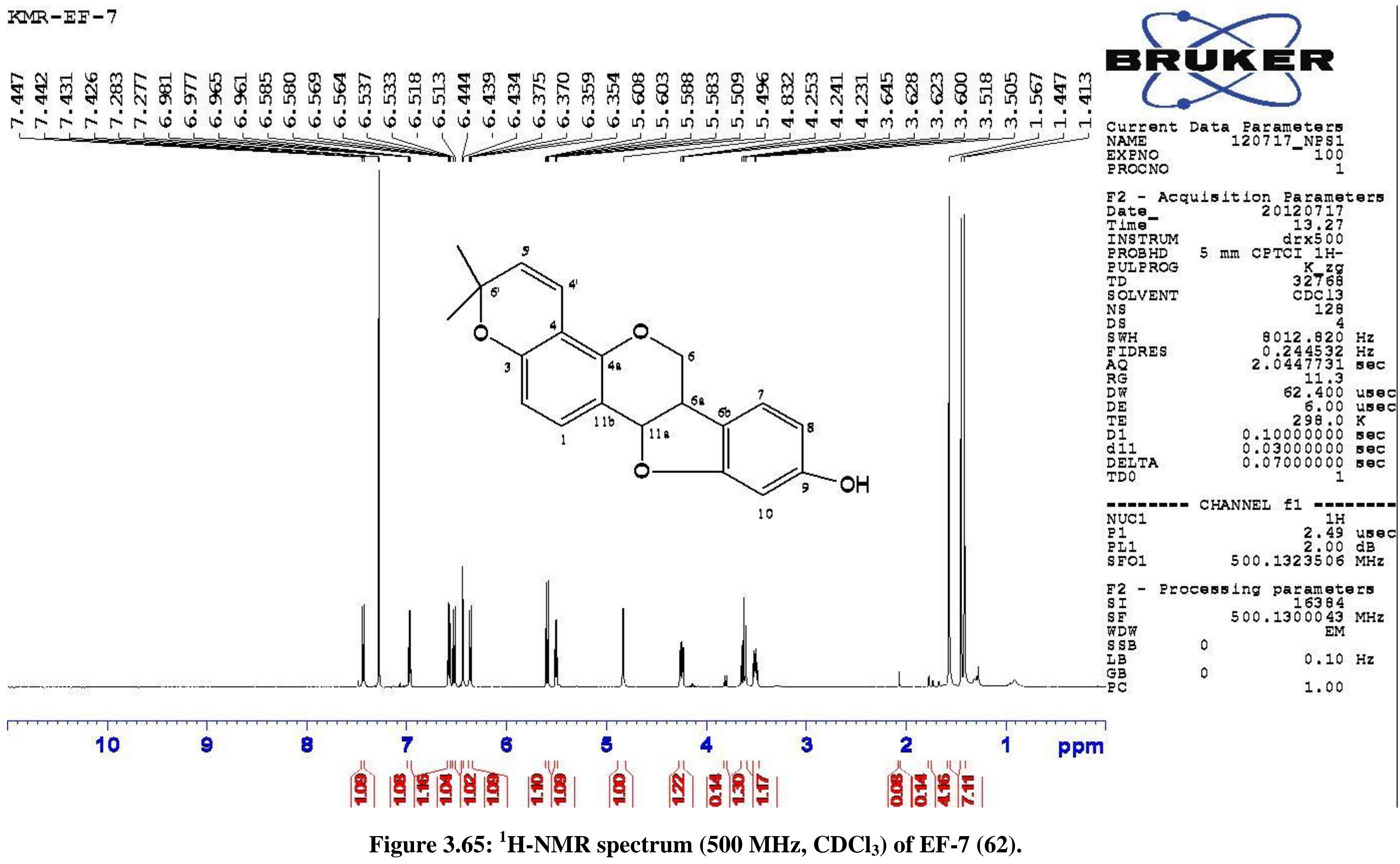

Figure 3.65: ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-7 (62).

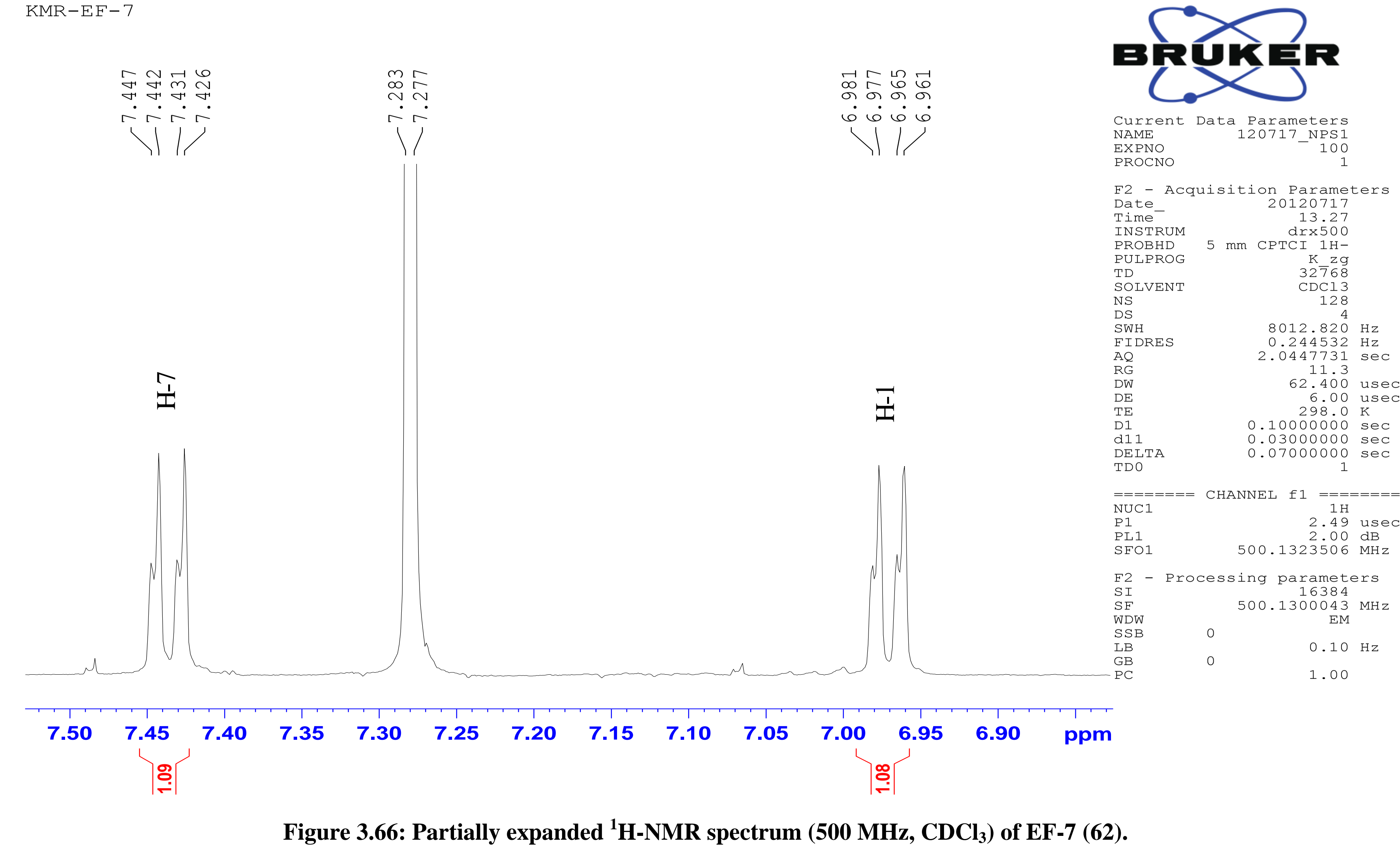

Figure 3.66: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-7 (62).

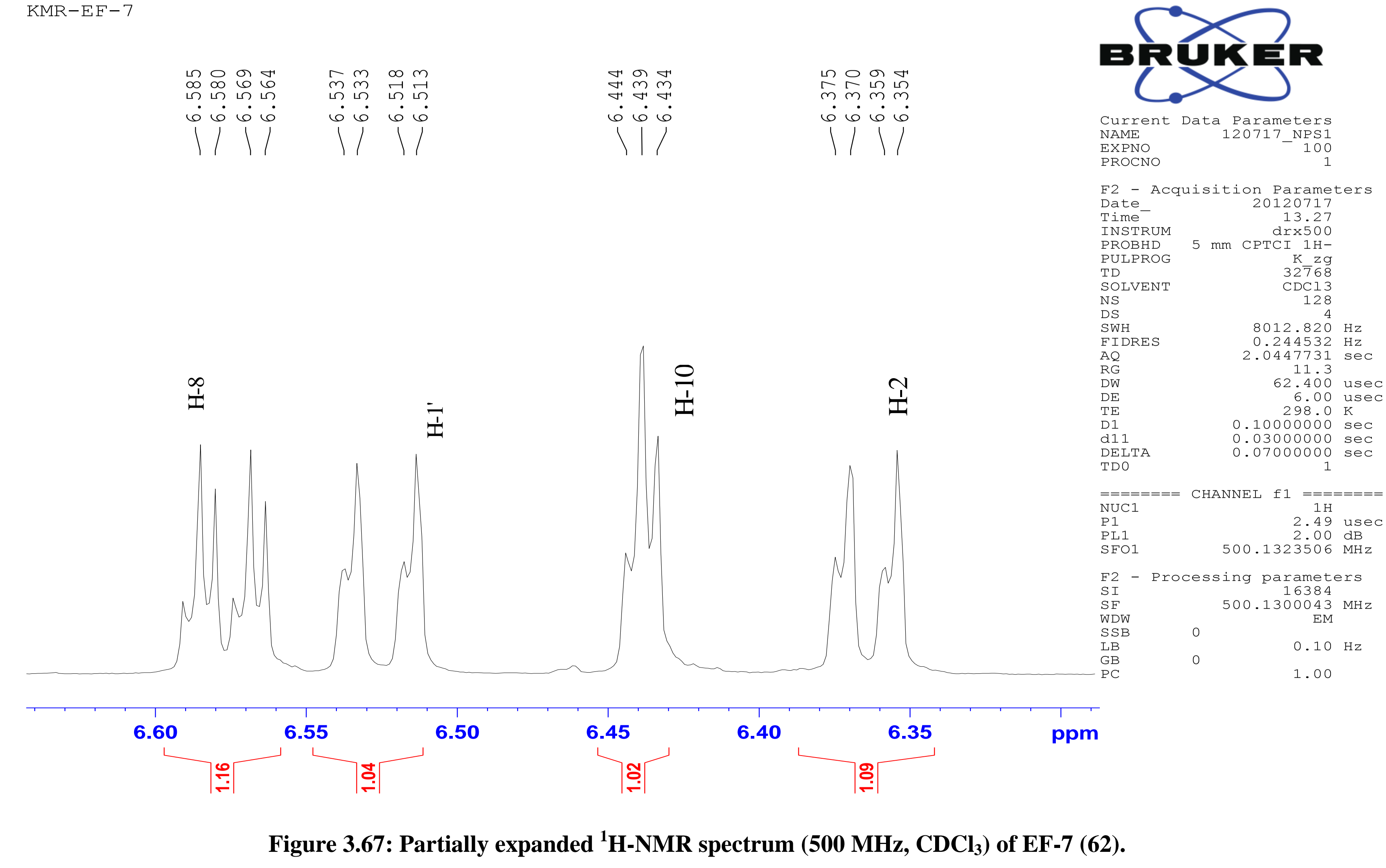

Figure 3.67: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-7 (62).

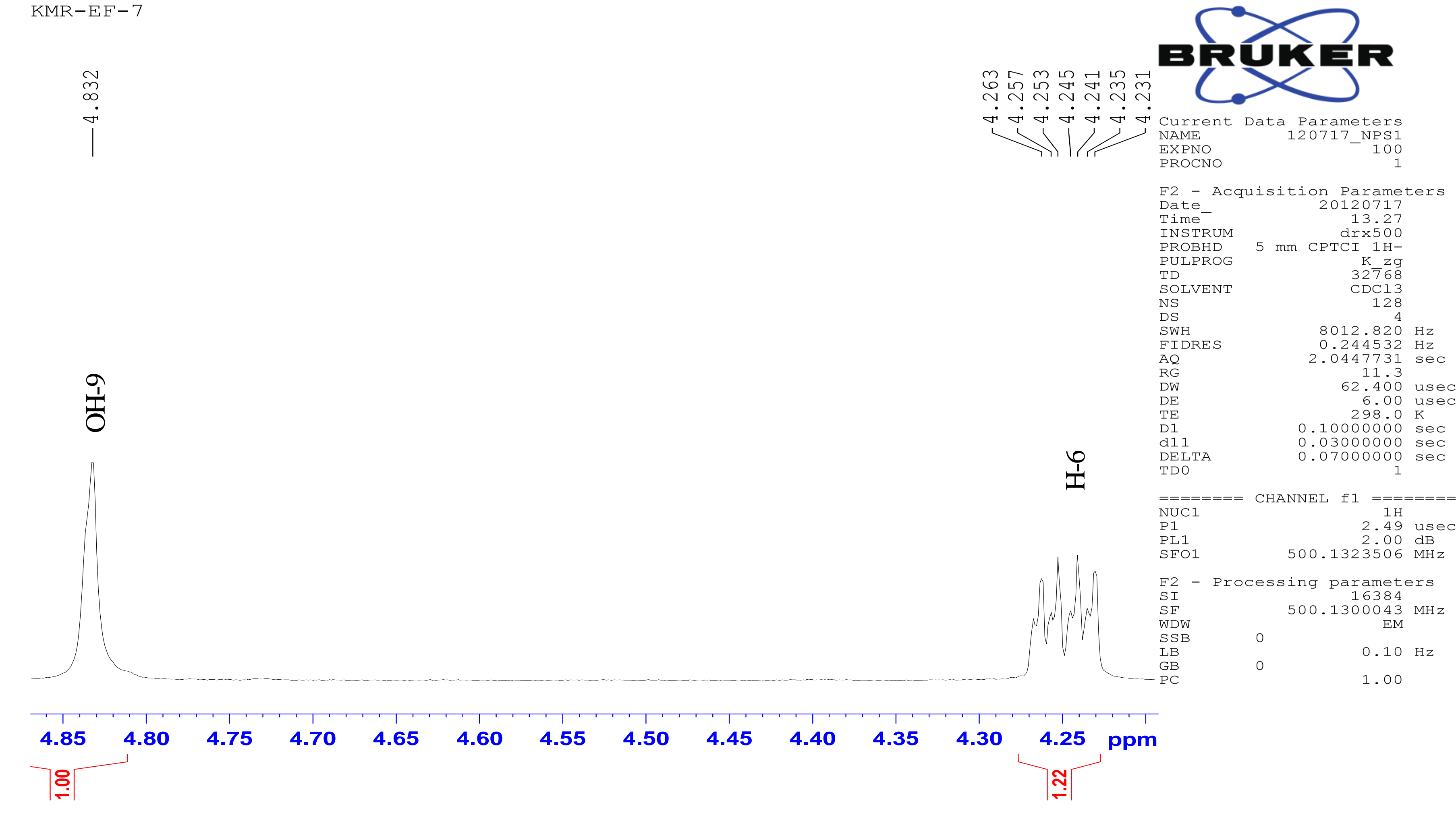

Figure 3.68: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-7 (62).

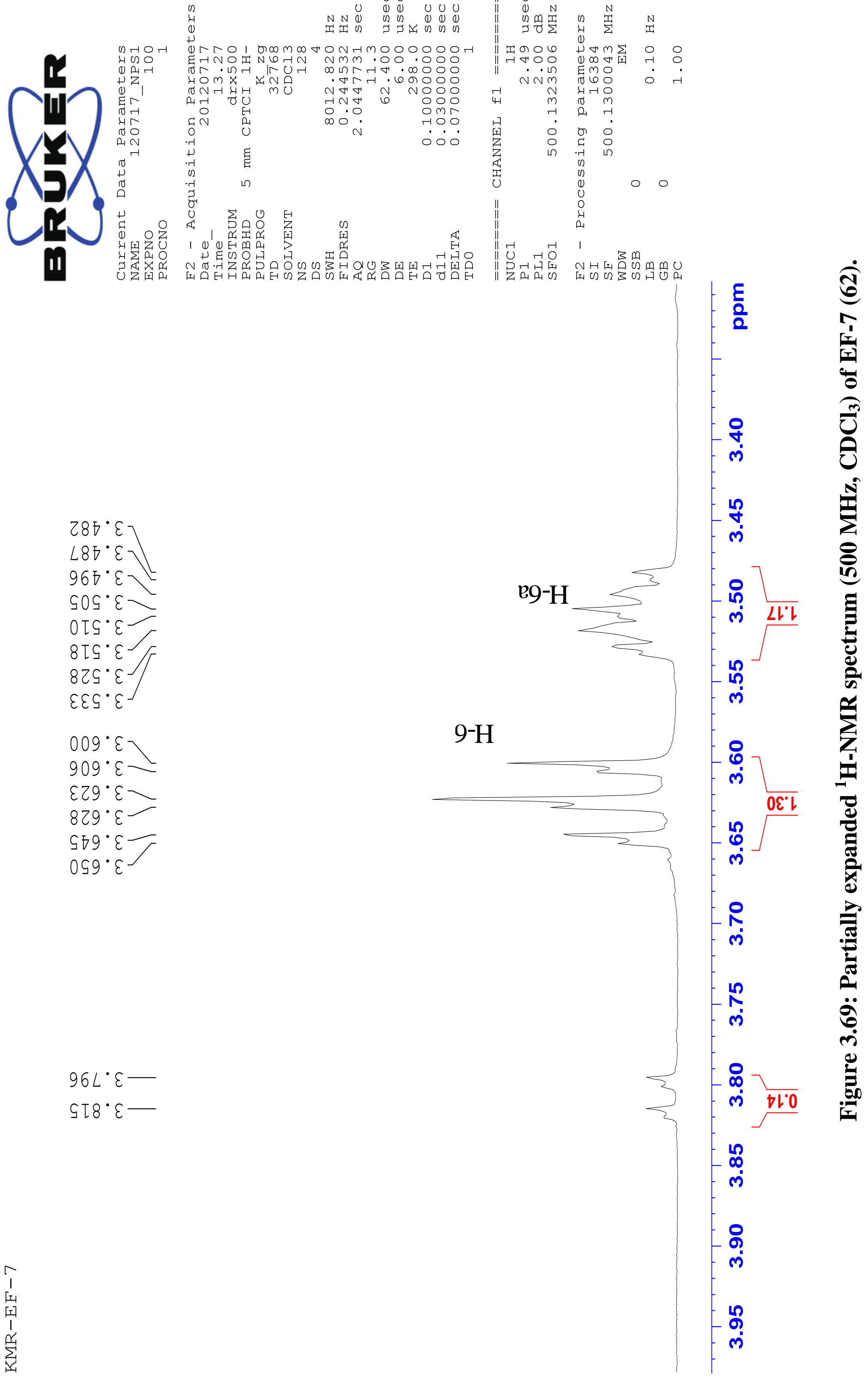

Figure 3.69: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-7 (62).

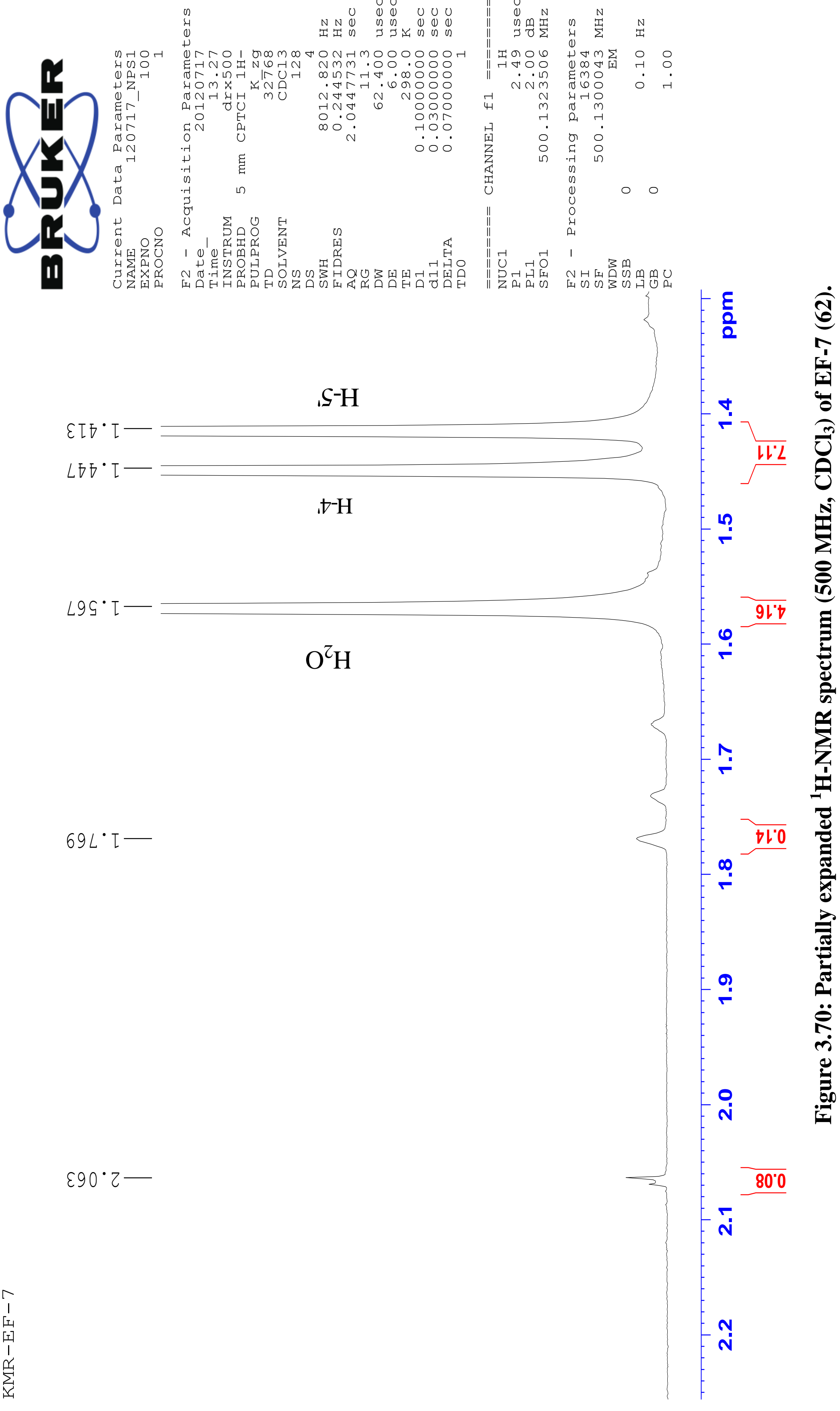

Figure 3.70: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-7 (62).

3.17 Characterization of EF-8 as lupinifolin (63)

EF-8 was obtained as a yellowish mass by evaporation of solvent from the CC fractions 90-97 of the carbon tetrachloride soluble fraction of stem bark of *E. fusca*. The fraction was then subjected to a preparative TLC over silica gel F_{254} using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated thrice for better separation of the desired band. The desired band was identified under UV light, then scrapped off and eluted with ethyl acetate to yield EF-8 (8.20 mg, **63**) as yellow needles on standing at room temperature. The compound was soluble *n*-hexane, ethyl acetate, chloroform and methanol.

TLC examination of the compound EF-8 showed a bright yellow colored spot which turned into orange spot when sprayed with vanillin-sulfuric acid reagent indicated the presence of a flavonone type compound.

The ^{1}H-NMR spectrum (500 MHz, $CDCl_3$; Figures 3.72-3.75; Table 3.19) of EF-8 revealed a sharp singlet characteristic for the chelated hydroxyl group at C-5 in a pyranoflavonone skeleton at δ 12.25 (1H, s, OH-5). The diagnostic features for a flavonone nucleus were evident δ 3.04 (1H, dd, *J*= 14.5, 7.3 Hz), 2.80 (1H, dd, *J*= 13.5, 2.0 Hz) and δ 5.34 (1H, dd, *J*= 12.0, 2.0 Hz) which could be assigned to H-3α, H-3β and H-2 of the flavanone nucleus, respectively. The doublet at δ 5.50 (1H, d, *J*= 9.5 Hz, H-3") and δ 6.63 (1H, d, *J*= 10.0 Hz, H-4"), each equivalent to one proton and the two singlets at δ 1.44 (3H, s) and δ 1.46 (3H, s) were characteristic for the *cis* double bond and *gem*-dimethyl group of a 2,2-dimethyl-chromene moiety, respectively (Rao and Srimannarayana, 1984). Two *ortho*-coupled doublets (*J*= 8.5 Hz) centered at δ 7.33 (2H) and 6.87 (2H) were assigned to the protons H-2' & H-6' and H-3' & H-5' of the *para*-disubstituted benzene ring (C ring). The presence of an isoprenyl group was inferred from the singlets at δ 1.67 (3H, s, CH_3-4''') and δ 1.65 (3H, s, CH_3-5'''), the doublet at δ 3.22 (1H, d, *J*= 7.0 Hz, H-1''') and the triplet at δ 5.10 (1H, t, *J*= 7.1 Hz, H-2''').

These ^{1}H-NMR data (Table 3.19) of compound EF-8 showed similarity with that of lupinifolin (Mahihol *et al.*, 1997). Thus, compound EF-8 was identified as lupinifolin (**63**). It was previously isolated from the bark of the same plant (Innok *et al.*, 2009).

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Figure 3.71: Structure of EF-8 (lupinifolin, **63**).

Table 3.19: ^{1}H-NMR spectral data of EF-8 (63) and lupinifolin (Mahidol *et al*., 1997) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	EF-8 (63)	**Lupinifolin**
2	5.34 dd (12.0 and 2.0)	5.30 dd (12.7 and 3.3)
3α	3.04 dd (14.5 and 7.3)	3.04 dd (17.3 and 12.7)
3β	2.80 dd (13.5 and 2.0)	2.78 dd (17.3 and 3.3)
2', 6'	7.33 d (8.5)	7.28 d (8.5)
3', 5'	6.88 d (8.5)	6.84 d (8.5)
3''	5.50 d (9.5)	5.48 d (10.0)
4''	6.63 d (10.0)	6.63 d (10.0)
5''	1.44 s	1.44 s
6''	1.46 s	1.44 s
1'''	3.22 d (7.0)	3.20 br d (7.0)
2'''	5.15 t (7.1)	5.14 t (7.0)
4'''	1.66 s	1.64 br s
5'''	1.67 s	1.64 br s
5-OH	12.25 s	12.20 s

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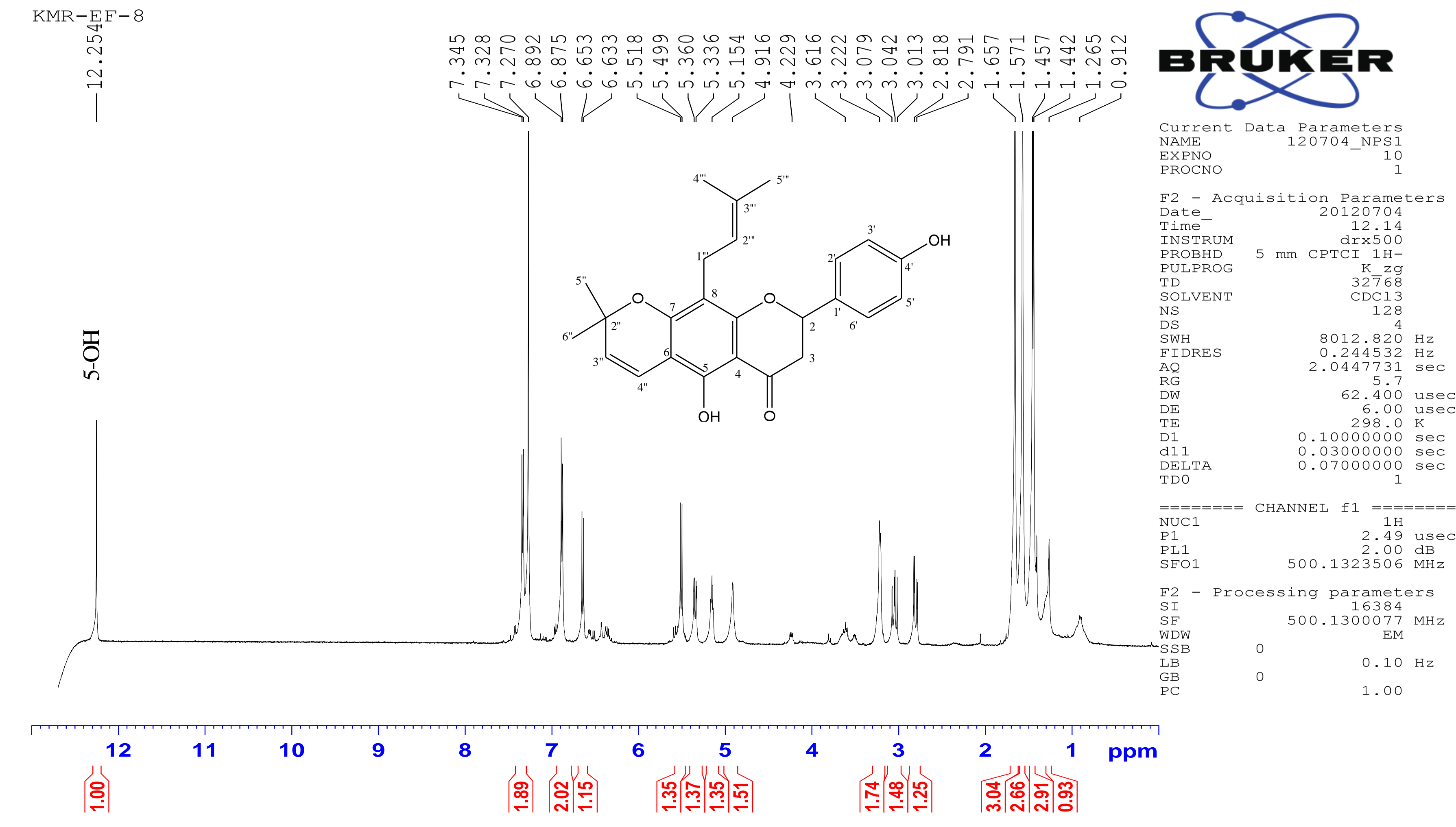

Figure 3.72: ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-8 (63).

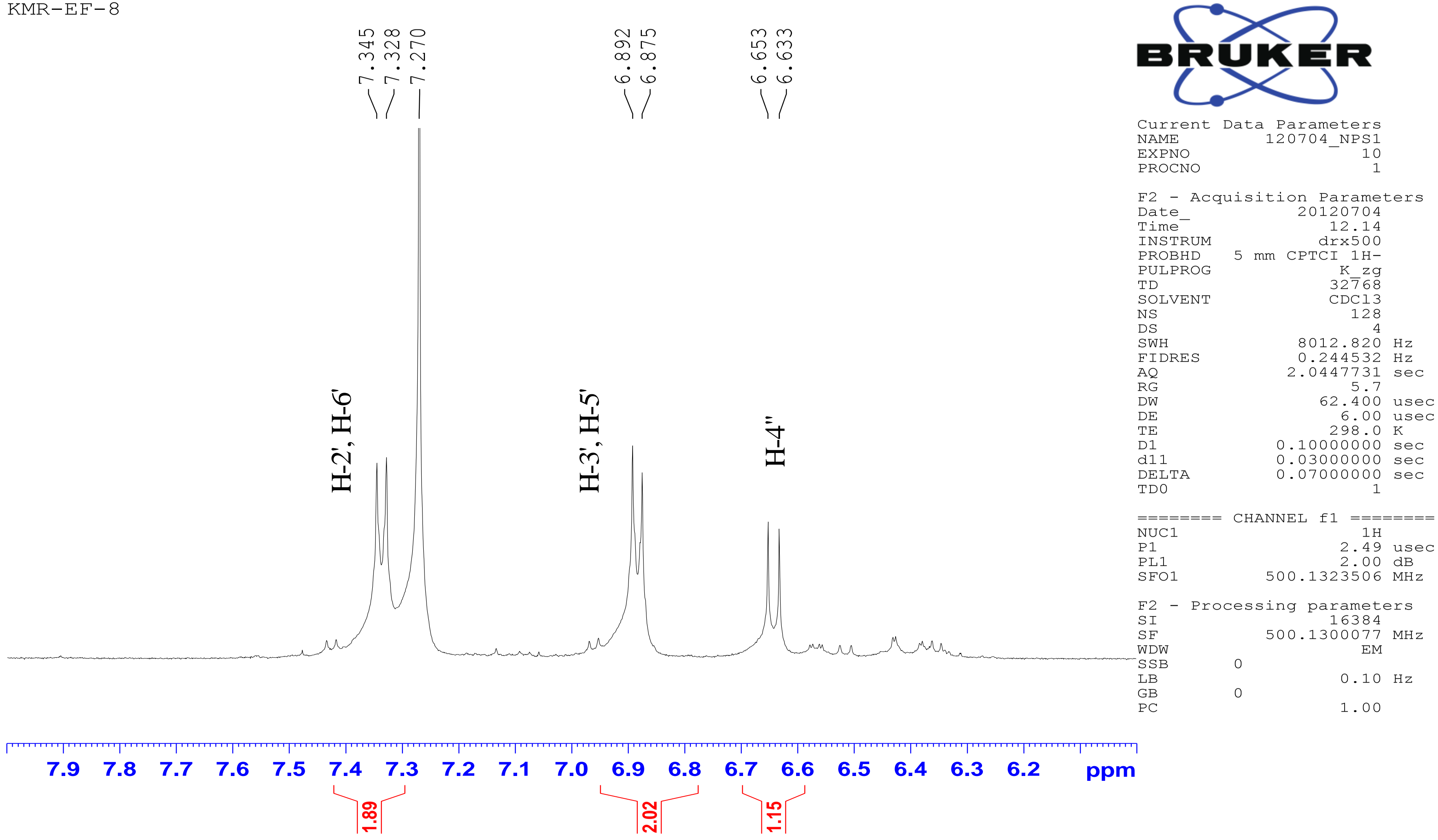

Figure 3.73: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-8 (63).

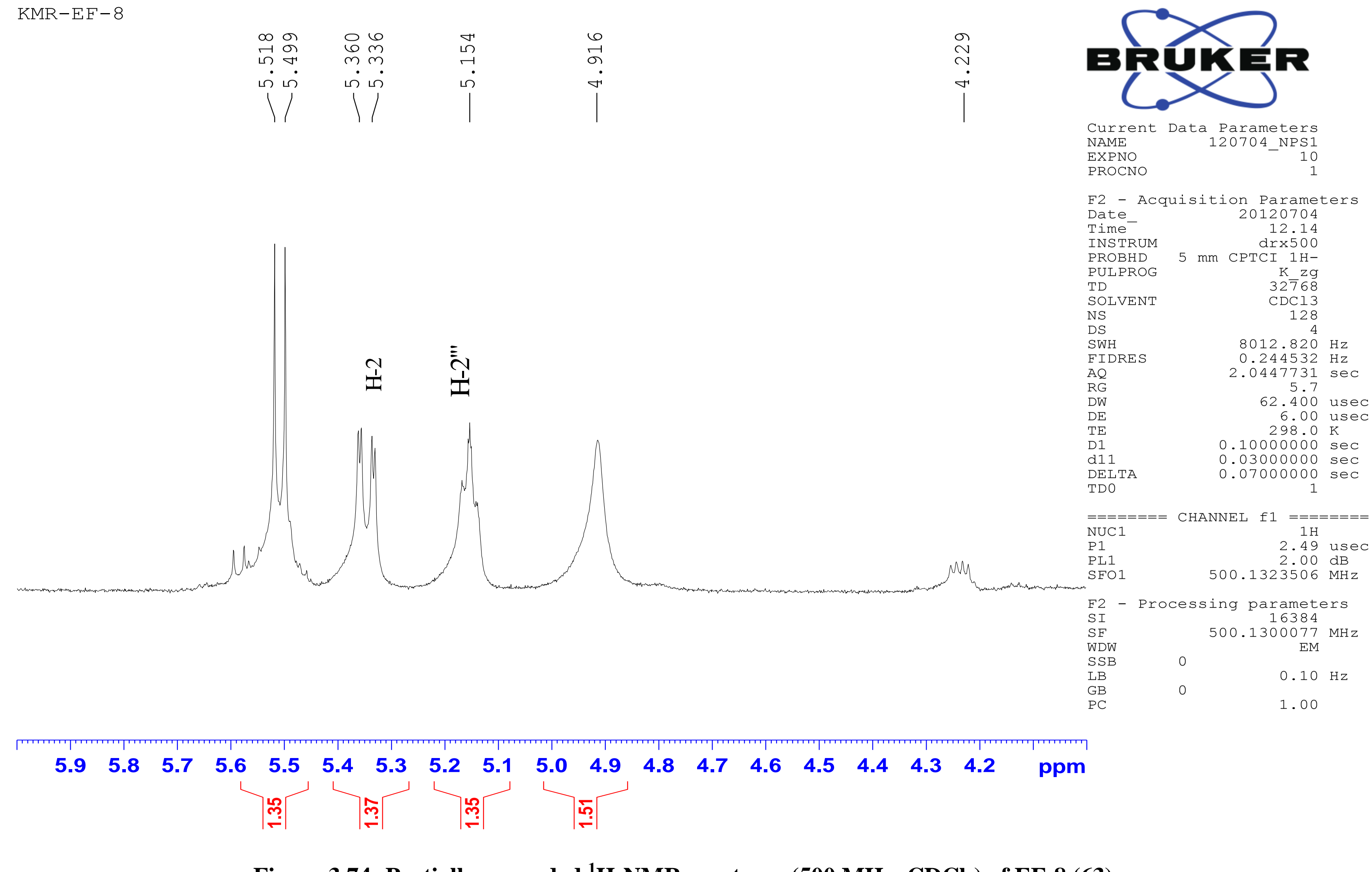

Figure 3.74: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-8 (63).

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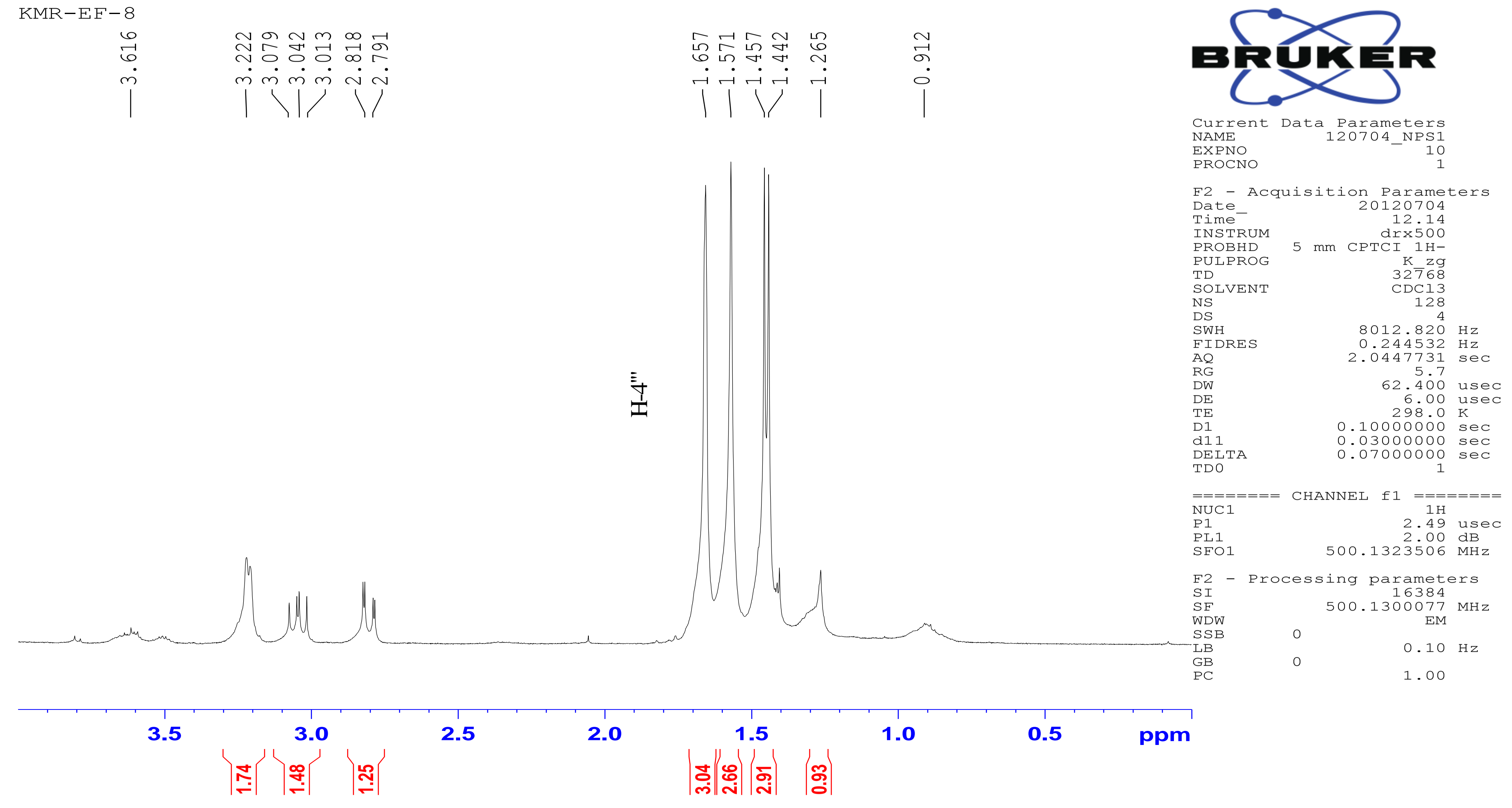

Figure 3.75: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-8 (63).

3.18 Characterization of EF-12 as 3,9-dihydroxy-4-(3,3 dimethylallyl) [6aR,11aR]-pterocarpan (64)

The carbon tetrachloride soluble fraction of the crude methanolic extract of the stem bark of *E. fusca* was subjected to silica gel column chromatography by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities to obtain two hundred and fifty sub-fractions. Sub-fractions 90-97 showing identical spots on TLC were combined in a beaker. After drying the solvent a yellowish mass was obtained which was then subjected to preparative TLC over silica gel F_{254} using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated five times for better separation of the desired band. A small portion of the developed plate was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a pink colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent yielded a compound EF-12 (4.5 mg, **64**) as amorphous powder. The compound was analyzed over TLC to check its purity and found a pure compound which was also found to be soluble in *n*-hexane, ethyl acetate, chloroform and methanol.

The ^{1}H-NMR spectrum of the compound EF-12 (500 MHz, $CDCl_3$; Figures 3.77-3.81; Table 3.20) showed a set of proton resonances at δ 3.63 (dd, *J*=11.0, 5.0 Hz) and 4.25 (dd, *J*=11.0, 5.0 Hz) due to 6-H_2, at δ 3.51 (m) due to 6a-H and at δ 5.47 (d, *J*= 6.5 Hz) for 11a-H suggested the presence of an -O-CH_2-CH-CH-O- skeleton of B and C rings of a pterocarpan nucleus.

The ^{1}H-NMR spectrum further revealed the presence of a pair of *ortho*-coupled doublets with one proton intensity each at δ 7.42 (1H, d, *J*=8.5 Hz) (for H-1) and δ 6.39 (1H, d, *J*= 8.0 Hz) (for H-2) in the ring-A showed that C-3 and C-4 of the ring were substituted. The presence of three aromatic protons at δ 6.97 (1H, d, *J*= 8.0 Hz) (H-7), 6.57 (dd, *J*= 8.0, 2.0 Hz) (H-8) and 6.43 (1H, d, *J*= 2.0 Hz) (H-10) indicated ABX spin system and protons of two hydroxyl groups at δ 4.79 and 4.80. Comparison of ^{1}H-NMR data for EF-12 with that of the published in the literature (Cottiglia *et al.*, 2005) the structure of EF-12 was established as 3,9-dihydroxy-4-(3,3-dimethylallyl)[6a*R*,11a*R*]-pterocarpan (**64**). This is the first report of isolation of 3,9-dihydroxy-4-(3,3-dimethylallyl)[6a*R*,11a*R*]-pterocarpan (**64**) from *E. fusca*.

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Figure 3.76: Structure of EF-12 (3,9-dihydroxy-4-(3,3-dimethylallyl)[6aR,11aR]-pterocarpan, **64**).

Table 3.20: ^{1}H-NMR spectral data of EF-12 (64) and 3,9-dihydroxy-4-(3,3-dimethylallyl)[6aR,11aR]-pterocarpan (Cottiglia *et al.*, 2005) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	EF-12 (64)	**3,9-Dihydroxy-4-(3,3-dimethylallyl) [6aR, 11aR]-pterocarpan**
1	7.42 d (8.5)	7.29 d (8.4)
2	6.39 d (8.0)	6.31 d (8.4)
6	3.63 dd (11.0 and 5.0)	3.45 t-like (11.2)
	4.25 dd (11.0 and 5.0)	4.10 dd (11.2 and 5.2)
6a	3.51 m	3.02 m
7	6.97 d (8.0)	6.68 d (8.0)
8	6.57 dd (8.0 and 2.0)	6.21 dd (8.0 and 2.4)
10	6.43 d (2.0)	6.24 d (2.4)
11a	5.47 d (6.5)	5.25 d (6.8)
1'	3.36 m	3.58 d (8.0)
2'	5.27 d (4.5)	5.41 t (8.0)
4'	1.81 s	1.61 s
5'	1.75 s	1.71 s
3-OH	4.79 s	4.71 s
9-OH	4.80 s	4.81 s

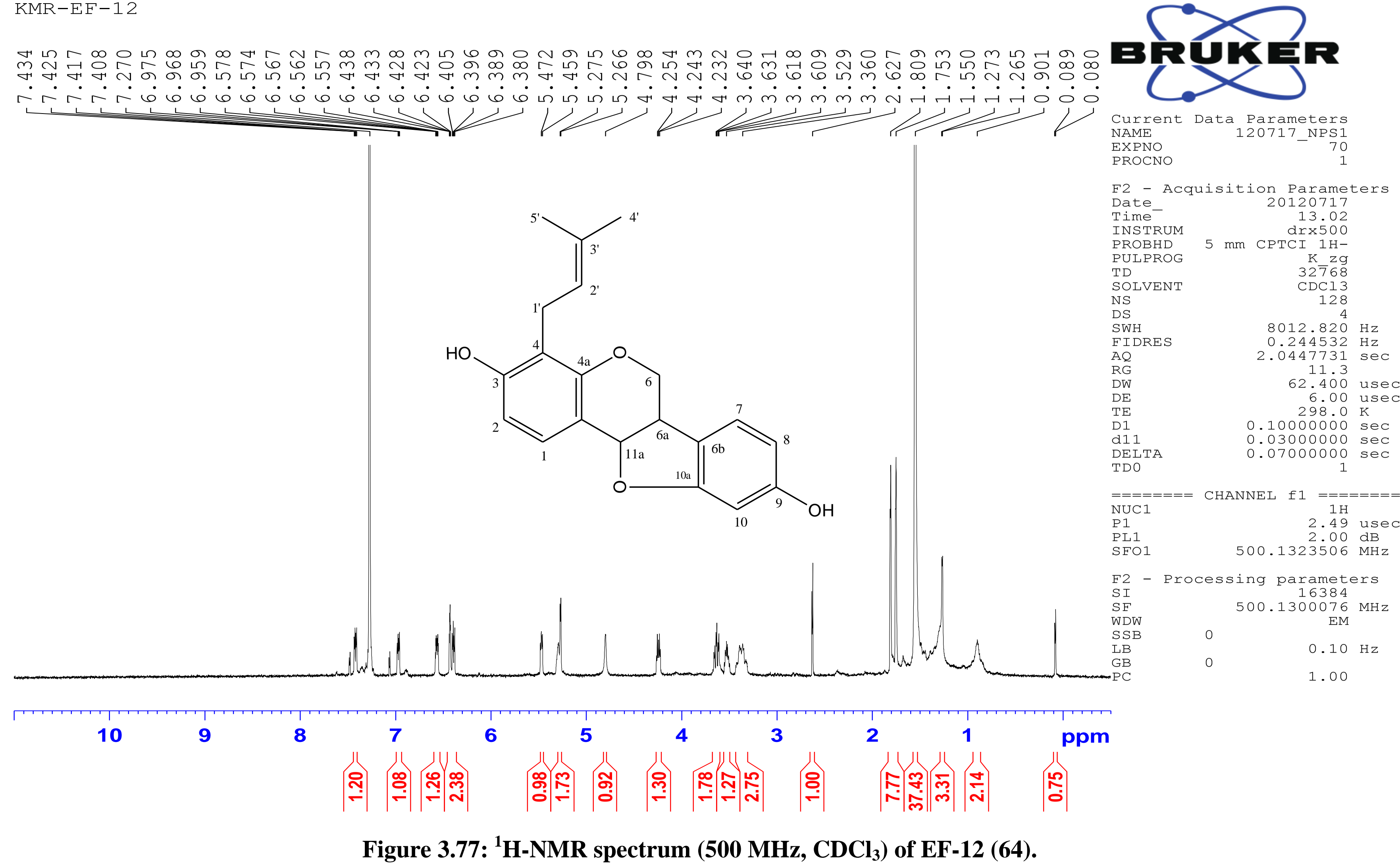

Figure 3.77: ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-12 (64).

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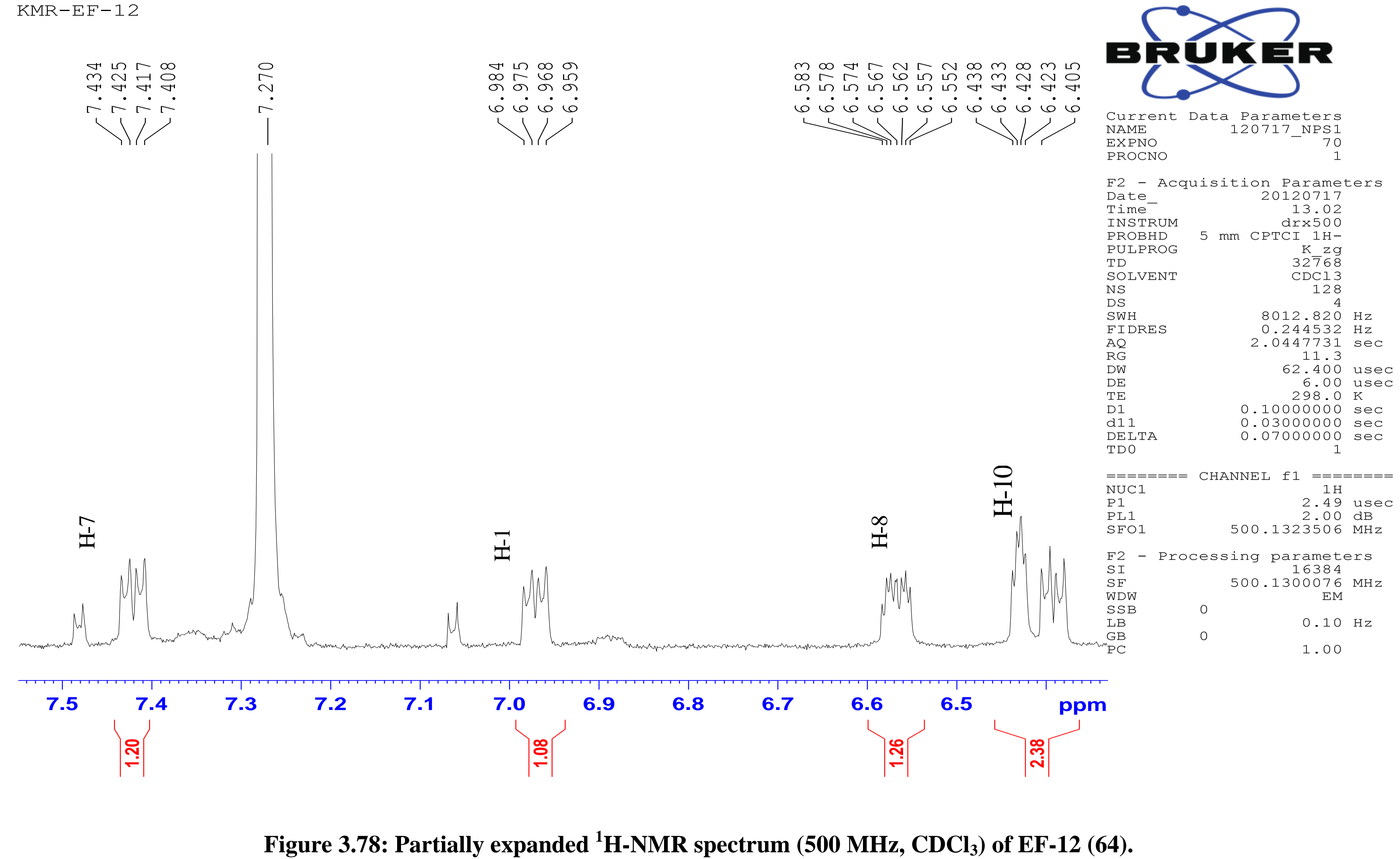

Figure 3.78: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-12 (64).

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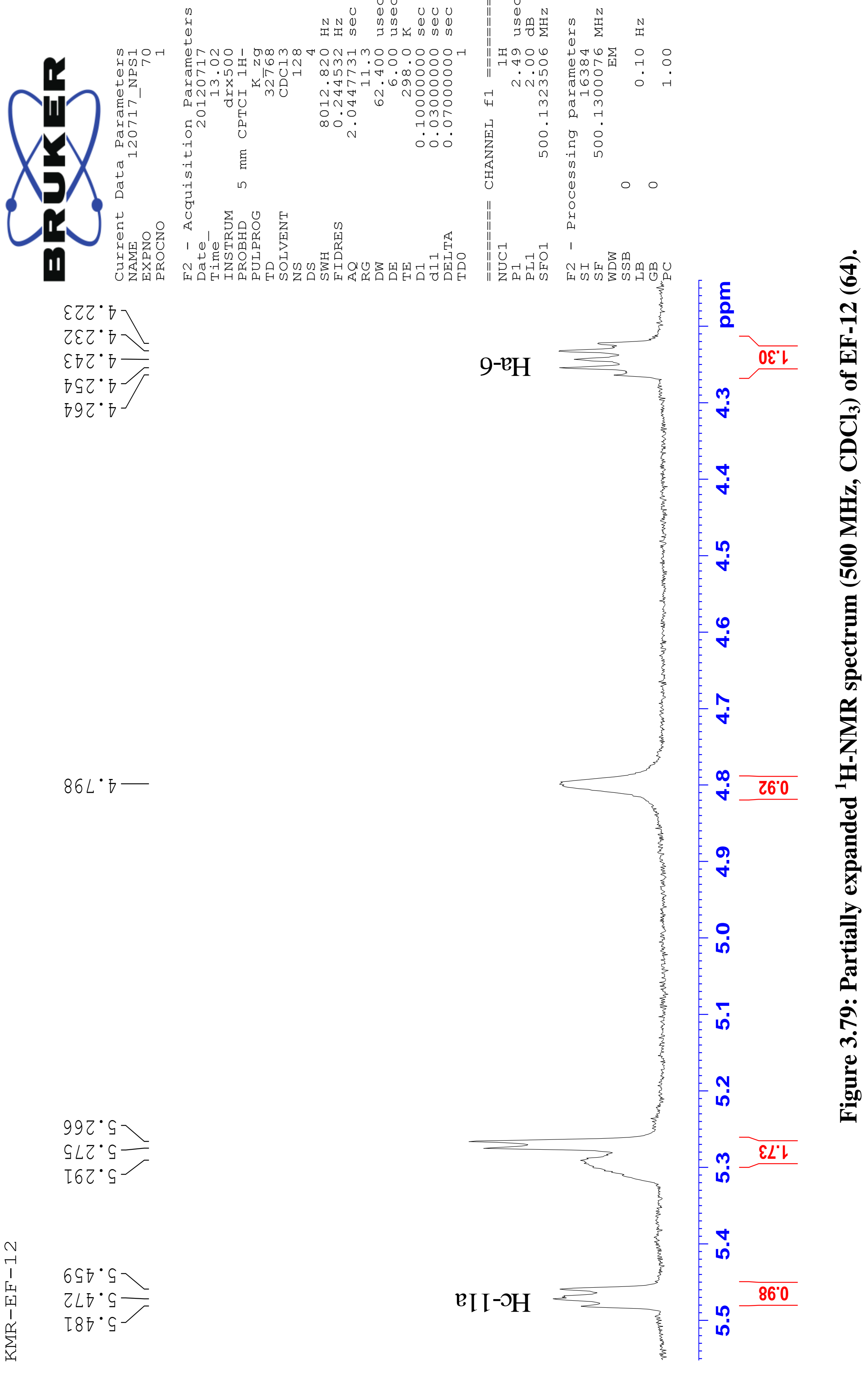

Figure 3.79: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-12 (64).

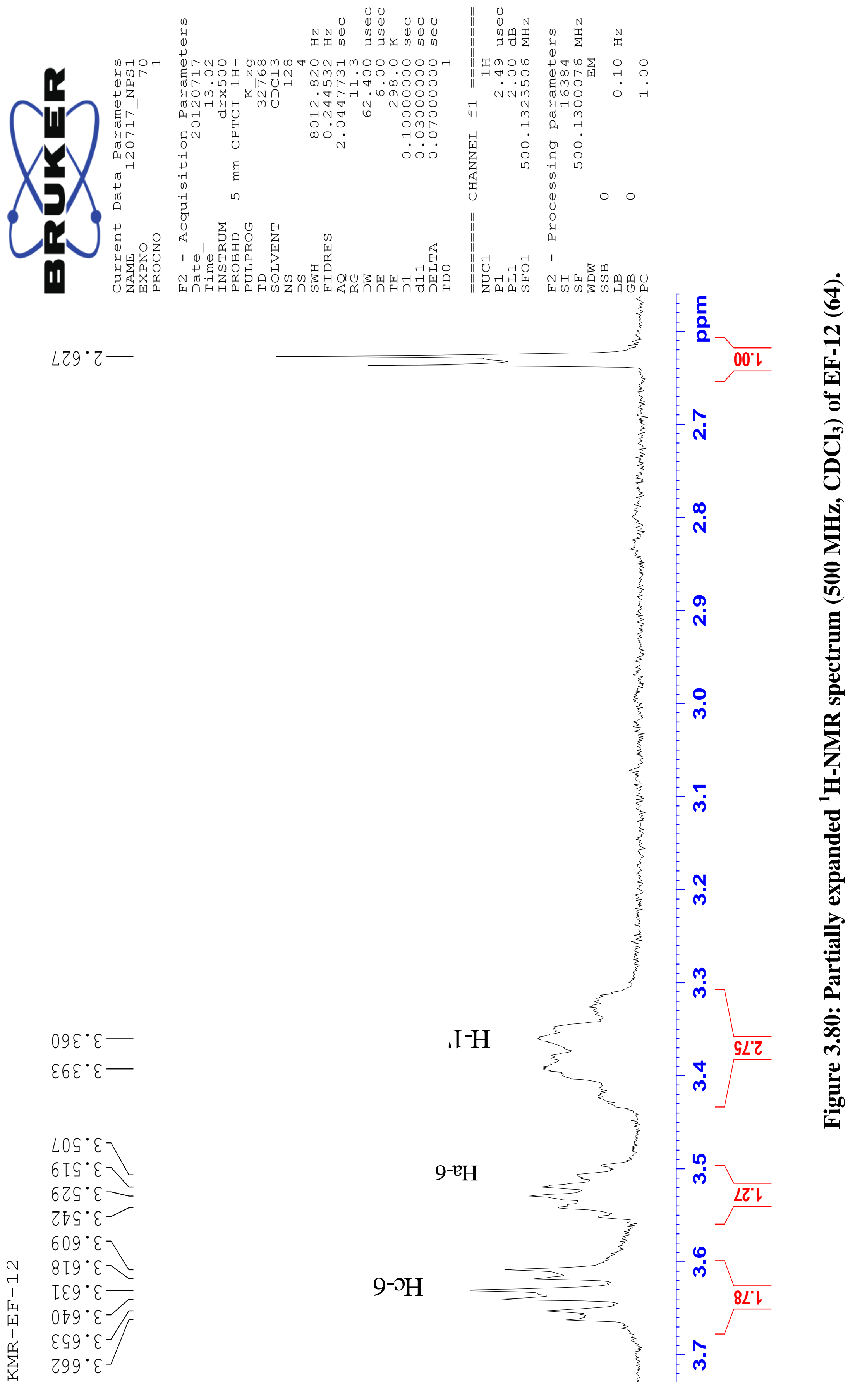

Figure 3.80: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-12 (64).

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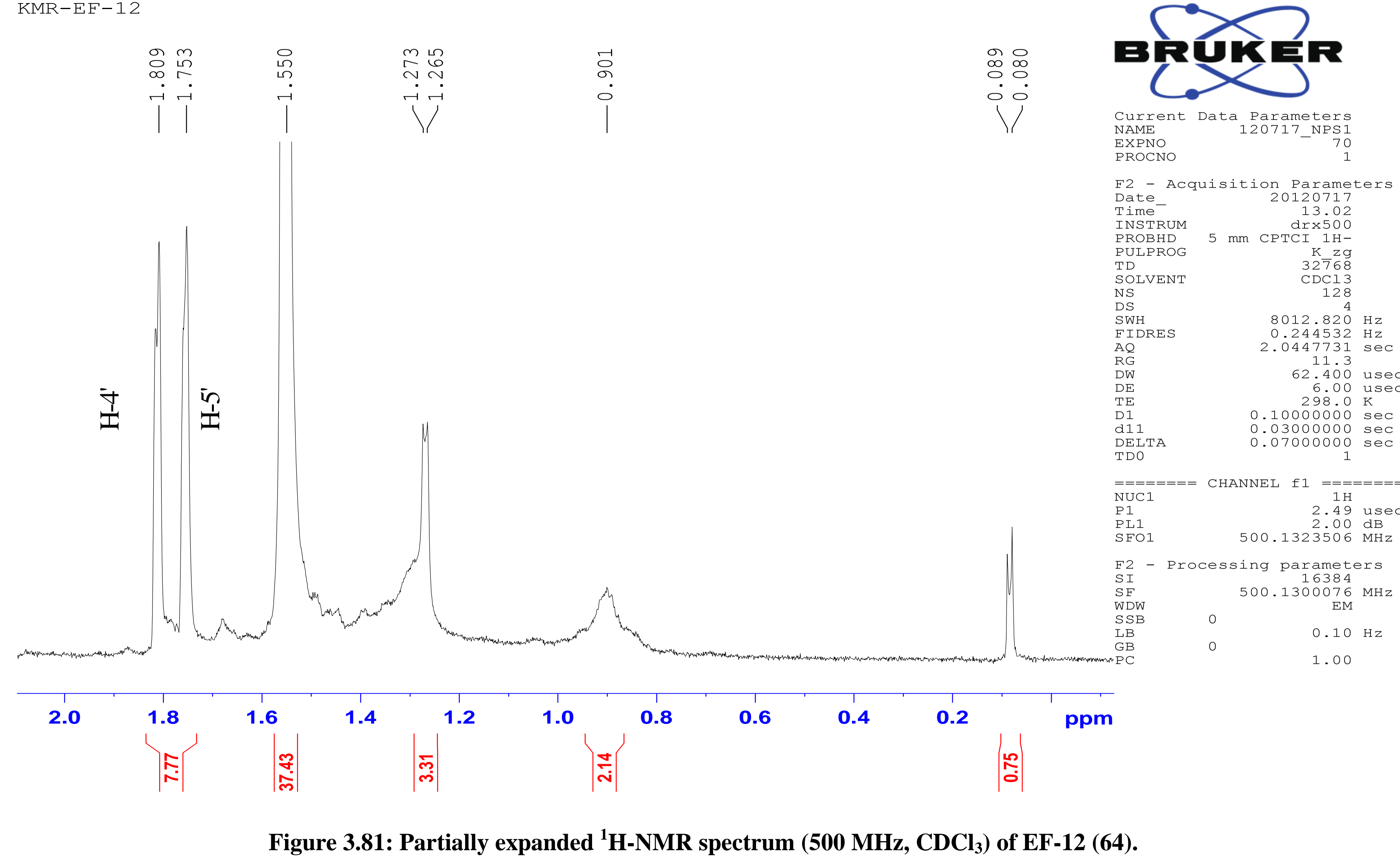

Figure 3.81: Partially expanded ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of EF-12 (64).

3.19 Characterization of EF-38 as β-sitosterol (65)

EF-38 was obtained as crystals by evaporation of solvent from the CC fractions 119-127 of the carbon tetrachloride soluble fraction of stem bark of *E. fusca*. The fraction was then subjected to a preparative TLC [stationary phase: silica gel F_{254}, mobile phase: toluene-ethyl acetate (85:15)]. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating in 110 ºC for 5 minutes and a purple colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent yielded white needles which melted at 137 ºC which was identical to that published for β-sitosterol (Morales *et al.,* 2003). The compound was analyzed over TLC to check for purity and found to be a pure compound which was termed as EF-38 (6.87 mg, **65**). It was also found to be soluble in *n*-hexane, dichloromethane, ethyl acetate and acetone.

The ^{1}H-NMR spectrum of EF-38 (400 MHz, $CDCl_3$; Figure 3.83; Table 3.21) revealed a one proton multiplet at δ 3.58, the position and multiplicity of which was indicative of H-3 of the steroid nucleus. A triplet at δ 5.35 (*J*= 7.0 Hz) was evident for the typical olefinic proton at H-6 of the steroidal skeleton. The spectrum further revealed signals at δ 0.73 and δ 1.04 (3H each) assignable to two tertiary methyl groups at Me-18 and Me-19, respectively. The ^{1}H-NMR spectrum also showed two doublets centered at δ 0.82 (*J*= 7.0 Hz) and 0.80 (*J*= 6.9 Hz) which could be attributed to two methyl groups at Me-26 and Me-27, respectively. The doublet at δ 0.93 (*J*= 6.5 Hz) demonstrated a methyl group at Me-21. On the other hand, the triplet of three-proton intensity at δ 0.86 (*J*= 7.3) could be assigned to the primary methyl group for Me-29. The above spectral features (Table 3.21) are in close agreement to those observed for β-sitosterol **(65)** (Morales *et al.,* 2003). The identity of EF-38 was further confirmed by co-TLC with an authentic β-sitosterol previously isolated in our laboratory.

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Figure 3.82: Structure of EF-38 (β-sitosterol, **65**).

Table 3.21: ^{1}H-NMR spectral data of EF-38 (65) and β-sitosterol (Morales *et al.*, 2003) in $CDCl_3$.

Proton(s)	δ_H in ppm in $CDCl_3$, (*J* in Hz).	
	EF-38 (65)	**β-sitosterol**
H-3	3.58 m	3.52 m
H-6	5.35 t (7.0)	5.36 br d (5.3)
Me-18	0.73 s	0.70 s
Me-19	1.04 s	1.01 s
Me-21	0.93 d (6.5)	0.92 d (6.6)
Me-26	0.82 d (7.0)	0.84 d (7.0)
Me-27	0.80 d (6.90)	0.81 d (7.0)
Me-29	0.86 t (7.3)	0.85 t (7.0)

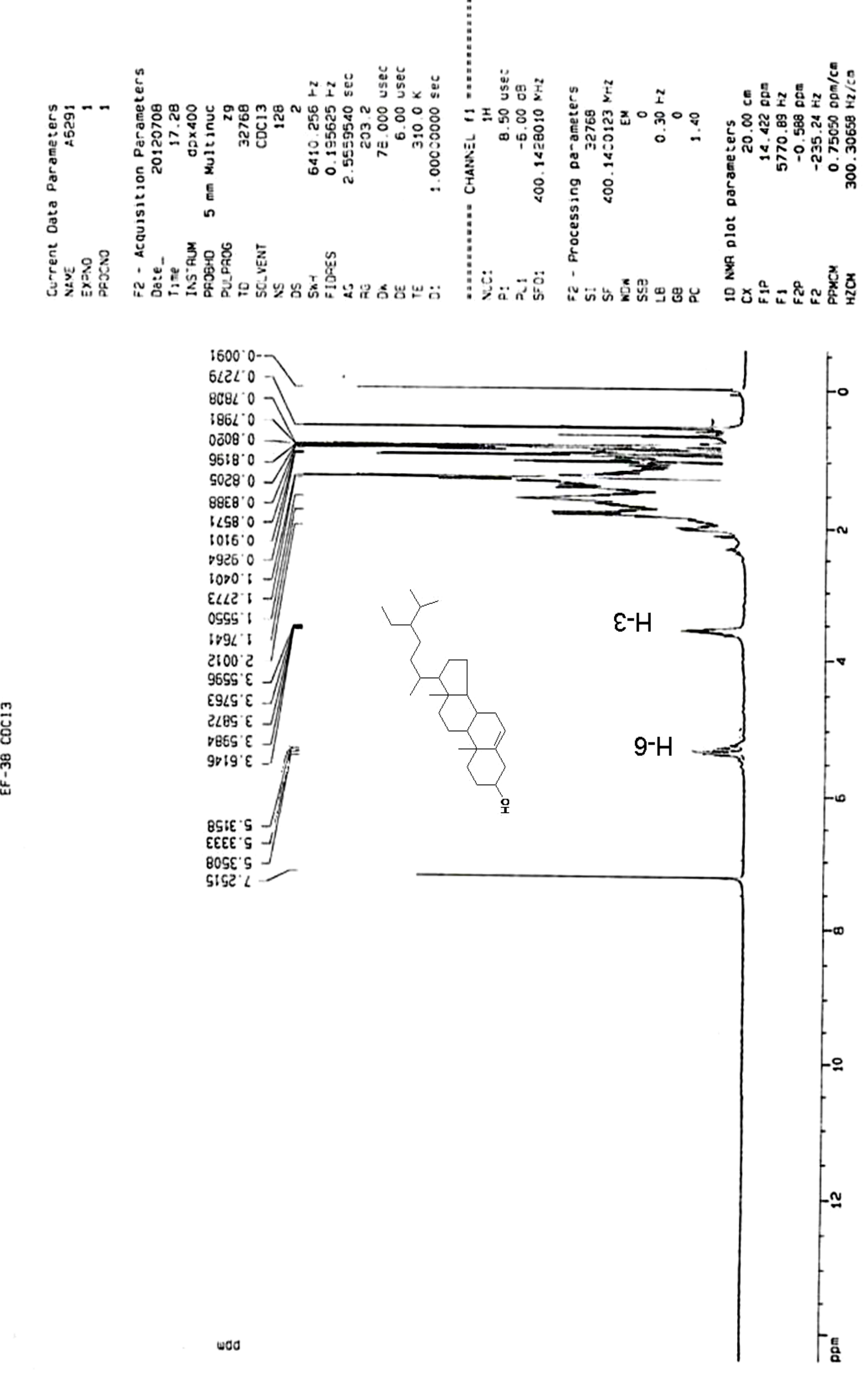

Figure 3.83: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of EF-38 (65).

4.1 Principle

Antimicrobial screening was done by disc diffusion method following the process illustrated by Bauer et al., 1966.

4.2 Test samples

Test samples and preparation of the sample discs of various extracts are listed in Table 4.1.

Table 4.1: List of test samples and preparation of sample discs for various extracts.

Sample	Extract	Dose (µg/disc)	Amount for 20 discs (mg)
B. verrucosa (Stem bark and leaf)	Me	400	8
	HSF	400	8
	CSF	400	8
	CHSF	400	8
	AQSF	400	8
	BVSP-4 (**48**)	50	1
	BVBC-2 (**51**)	50	1
	BVS-65 (**54**)	50	1
B. stipularis (Stem bark and leaf)	Me	400	8
	HSF	400	8
	CSF	400	8
	CHSF	400	8
	AQSF	400	8
	BS-01(**55**)	50	1
	BS-06 (**56**)	50	1
B. tomentosa (Stem bark and leaf)	Me	400	8
	HSF	400	8
	CSF	400	8
	CHSF	400	8
	AQSF	400	8
	BT-1 (**58**)	50	1
	BT-6 (**59**)	50	1
E. fusca (Stem bark and leaf)	Me	400	8
	HSF	400	8
	CSF	400	8
	CHSF	400	8
	AQSF	400	8
	EF-7 (**62**)	50	1
	EF-8 (**63**)	50	1
	EF-12 (**64**)	50	1
E. variegata (Stem bark and leaf)	Me	400	8
	HSF	400	8
	CSF	400	8
	CHSF	400	8
	AQSF	400	8

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction

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4.3 Test organisms

The test organisms used for antimicrobial screening are listed in Table 4.2.

Table 4.2: List of test organisms used for antimicrobial screening.

Gram positive bacteria	Gram negative bacteria	Fungi
Bacillius cereus *B. megaterium* *B. subtilis* *Staphylococcus aereus* *Sarcina lutea*	*Escherichia coli* *Pseudomonas aeruginosa* *Salmonella paratyphi* *S. typhi* *Shigella boydii* *S. dysenteriae* *Vibrio mimicus* *V. parahemolyticus*	*Aspergillus niger* *Candida albicans* *Saccharomyces cerevisiae*

4.4 Results and discussion

4.4.1 Antimicrobial activity of the stem bark *B. verrucosa*

Antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the stem bark of *B. verrucosa* showed low to moderate activity with zones of inhibition being between the ranges of 9 to 15 mm against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the stem bark of *B. verrucosa* were ranged from 10-15, 9-14, 11-15, 10-14 and 10-14 mm, respectively (Table 4.3) at a concentration of 400 μg/disc. The carbon tetrachloride soluble materials of the methanol extract showed the highest inhibitory activity against *S. paratyphi* having the zone of inhibition 15 mm. The *n*-hexane soluble materials gave moderate zone size of 14.0, 13.9, 13.5 and 13.6 mm against *S. paratyphi*, *S. typhi*, *V. mimicus* and *V. parahemolyticus*, respectively. At the same time, the carbon tetrachloride partitionate of methanol extract showed mild to moderate activity having zone size of 14.2, 15.0 and 13.6 mm, respectively against *E. coli, S. typhi* and *S. dysenteriae*. Again, the chloroform soluble materials showed zone of inhibition of 13.8, 14.0, 14.3 13.5 and 14.1 mm against *B. cereus*, *S. aureus*, *S. lutea*, *V. mimicus* and *V. hemolyticus*, respectively indicating moderate activity. The aqueous soluble materials showed low activity against all the strains ranging from 10-15 mm. The antibacterial activity of test samples of the stem bark of *B. verrucosa* is shown in Table 4.3.

Antifungal screening of the partitionates gave the zone of inhibition was between 10-15 mm indicating mild to moderate activity in contrast to griseofulvin as standard. The carbon tetrachloride soluble materials gave highest activity against *C. albicans* with zone size of 14.2 mm.

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Table 4.3: Antimicrobial screening of the stem bark of *B. verrucosa* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

Test bacteria and fungi	Diameter of zone of inhibition (mm)					
	Me	HSF	CSF	CHSF	AQSF	KAN
Gram positive bacteria						
B. cereus	14.3 ± 1.53	9.9 ± 1.15	11.6 ± 1.52	13.8 ± 2.01	10.7 ± 1.37	35
B. megaterium	10.9± 1.81	12.0± 1.72	14.9 ± 1.15	12.4± 1.40	11.4 ± 1.36	35
B. subtilis	11.3 ± 2.08	9.6 ± 1.53	12.3± 2.52	10.6± 2.08	11.1 ± 1.60	36
S. aereus	12.3 ± 2.31	12.4 ± 1.70	11.6 ± 1.26	14.3 ± 1.53	12.6 ± 1.60	32
S. lutea	10.6 ± 2.52	11.7 ± 2.46	12.6 ± 2.08	14.0 ± 1.70	13.6 ± 2.52	27
Gram negative bacteria						
Escherichia coli	13.7 ± 0.76	13.1 ± 2.43	14.2 ± 1.67	12.6 ± 0.80	12.8 ± 1.73	25
P. aeruginosa	14.7± 0.50	12.8± 1.29	13.1± 0.87	13.3 ± 0.78	12.9 ± 0.31	20
S. paratyphi	13.1 ± 0.76	14.0 ± 1.59	14.2 ± 1.67	12.6 ± 0.80	11.6 ± 0.36	27
S. typhi	13.4 ± 0.79	13.9 ± 0.97	15.0 ± 1.10	13.4 ± 1.12	11.3 ± 0.80	22
S. boydii	12.9 ± 1.22	13.1 ± 1.11	12.8 ± 0.76	14.5 ± 1.40	11.5 ± 0.35	27
S. dysenteriae	12.8± 2.02	13.3 ± 0.58	13.6 ± 1.26	11.1 ± 1.76	14.3 ± 0.80	25
V. mimicus	13.1 ± 1.04	13.5 ± 2.18	12.6 ± 1.15	13.5 ± 1.29	10.1 ± 0.76	25
V. parahemolyticus	14.8 ± 2.02	13.6 ± 1.53	12.9 ± 0.31	14.1 ± 1.53	11.1 ± 1.04	20
Fungi						**GRI**
A. niger	12.1 ± 0.76	13.1 ± 0.58	11.3 ± 0.76	12.3 ± 1.04	12.4 ± 0.97	20
C. albicans	12.2 ± 0.75	12.6 ± 1.53	14.2 ± 0.44	13.3 ± 2.08	13.1± 0.76	18
S. cerevisiae	13.6 ± 1.53	12.8 ± 1.31	10.6 ± 1.22	13.4 ± 0.85	13.1 ± 0.76	19

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

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4.4.2 Antimicrobial activity of the leaf of *B. verrucosa*

On the other hand, the leaf of the methanol extract and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the plant *B. verrucosa* showed mild to moderate activity with zones of inhibition ranging from 12 to 16 mm against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the leaf of *B. verrucosa* were ranged from 12-16, 12-16, 12-15, 11-16 and 11-13 mm, respectively (Table 4.4) at a concentration of 400 µg/disc. The chloroform soluble materials of the methanol extract showed the highest inhibitory activity against *P. aeruginosa* having the zone of inhibition 15.6 mm. In case of the methanol extract the growth of *B. megaterium* (13.8 mm), *S. lutea* (14.6 mm), *P. aeruginosa* (14.1 mm), *S. paratyphi* (15.1 mm), *S. typhi* (14.1 mm), *S. boydii* (14.3 mm) and *V. parahemolyticus* (13.7 mm) was moderately inhibited. The *n*-hexane soluble materials also gave moderate zone size of 14.7, 14.8, 14.2, 15.1, 15.3, 15.0 and 14.3 mm against *B. cereus*, *B. subtilis*, *S. aereus*, *E. coli*, *P. aeruginosa*, *S. typhi* and *S. dysenteria*, respectively. Also, the carbon tetrachloride partitionate of methanol extract showed mild to moderate activity against *B. cereus, S. aereus*, *S. lutea*, *P. aeruginosa*, *S. paratyphi* and *V. mimicus* having zone of inhibition 13.7, 14.1, 14.5, 14.5 and 13.8 mm, respectively. Again, moderate activity was shown by the chloroform soluble materials with zone of inhibition of 14.2, 14.5, 15.6, 14.2 and 14.7 mm against *B. cereus*, *E. coli*, *P. aeruginosa*, *S. typhi* and *V. mimicus*, respectively. The aqueous soluble materials showed low activity against all the strains ranging from 11-13 mm. Out of all samples, the aqueous soluble materials showed lowest zone of inhibition of 11 mm against *S. aereus* indicating low activity. Table 4.4 shows the antibacterial activity of test samples of the leaf of *B. verrucosa*.

The zone of inhibition was between 11-16 mm indicating moderate to strong activity in contrast to griseofulvin as standard In case of antifungal screening. The *n*-hexane soluble materials gave highest activity against *C. albicans* with zone size of 15.4 mm.

Table 4.4: Antimicrobial screening of the leaf of *B. verrucosa* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

Test bacteria and fungi	Diameter of zone of inhibition (mm)					
	Me	HSF	CSF	CHSF	AQSF	KAN
Gram positive bacteria						
B. cereus	12.1 ± 0.76	14.7 ± 1.12	13.7 ± 0.22	14.2 ± 0.85	11.7 ± 0.93	35
B. megaterium	13.8± 1.42	12.7± 1.40	12.6 ± 0.91	11.7± 0.67	12.5 ± 0.98	35
B. subtilis	13.2 ± 0.87	14.8 ± 1.21	12.9± 1.16	13.5± 1.33	12.4 ± 1.16	36
S. lutea	14.6 ± 1.11	12.9 ± 1.25	14.1 ± 1.61	13.9 ± 1.61	12.7 ± 0.36	32
S. aereus	12.9 ± 1.93	14.2 ± 1.23	13.6 ± 0.75	12.4 ± 0.83	11.0 ± 0.70	27
Gram negative bacteria						
E. coli	12.6 ± 1.16	15.1 ± 0.45	13.0 ± 1.68	14.5 ± 1.20	11.2 ± 0.60	25
P. aeruginosa	14.1± 0.60	15.3± 0.61	14.5± 0.15	15.6 ± 0.50	12.2 ± 0.70	20
S. paratyphi	15.1 ± 1.49	14.0 ± 0.61	14.5 ± 0.68	13.1 ± 0.70	12.7 ± 0.95	27
S. typhi	14.1 ± 0.87	15.0 ± 1.44	13.2 ± 1.00	14.2 ± 0.45	11.5± 0.96	22
S. boydii	14.3 ± 0.95	14.0 ± 0.70	13.6 ± 1.11	12.3 ± 0.68	11.3 ± 0.70	27
S. dysenteriae	13.4± 0.75	14.3 ± 1.00	13.5 ± 0.79	13.9 ± 0.36	11.3 ± 0.72	25
V. mimicus	13.5 ± 0.81	13.4 ± 0.62	13.8 ± 0.38	14.7 ± 0.40	11.6 ± 0.71	25
V. parahemolyticus	13.7 ± 0.70	13.5 ± 0.81	13.4 ± 0.78	13.5 ± 0.60	11.8 ± 0.40	20
Fungi						**GRI**
A. niger	13.3 ± 0.56	13.7 ± 0.91	13.9 ± 0.85	14.2 ± 0.61	12.5 ± 0.60	20
C. albicans	14.3 ± 0.50	15.3 ± 0.51	11.2 ± 1.46	13.5 ± 0.79	11.8± 0.85	18
S. cerevisiae	13.6 ± 0.46	15.3 ± 0.61	13.2 ± 0.56	14.2 ± 0.56	11.8 ± 0.40	19

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.3 Antimicrobial activity of the stem bark of *B. stipularis*

Antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the stem bark of *B. stipularis* showed moderate to strong activity against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs with zones of inhibition between the ranges of 11.5 to 23 mm. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the stem bark of *B. stipularis* were ranged from 14-23, 12-19, 13-21, 13-23 and 11-18 mm, respectively (Table 4.5) at a concentration of 400 μg/disc. The methanol extract showed the highest inhibitory activity against *S. aereus* having the zone of inhibition 23 mm. In case of the methanol extract the growth of *B. subtilis* (21.1 mm), *S. aereus* (23 mm), *S. paratyphi* (21.9 mm), *S. typhi* (20.7 mm), *S. boydii* (20.3 mm) and *V. mimicus* (20.3 mm) was strongly inhibited. The *n*-hexane soluble materials gave moderate zone size of 18.7, 18.9, 18.8 and 18.7 mm against *B. megatrium*, *S. paratyphi*, *S. typhi* and *S. boydii*, respectively. At the same time, the carbon tetrachloride partitionate of methanol extract showed strong activity against *B. megatrium* and *E. coli* with zone of inhibition of 21 and 20.8 mm, respectively. It also gave moderate activity against *S. lutea,* *S. paratyphi* and *S. typhi* with zone of inhibition 18.7, 18.1 and 18.8 mm, respectively. Again, the chloroform soluble materials showed zone of inhibition of 20.4, 19.7, 20 and 22.3 mm against *S. aureus*, *S. paratyphi*, *S. dysenteriae* and *V. mimicus*, respectively indicating moderate activity. The aqueous soluble materials showed low to moderate activity against all the strains ranging from 11-18 mm. Out of all samples, the aqueous soluble materials showed lowest zone of inhibition of 11.9 mm against *V. parahemolyticus* indicating lowest activity. The antibacterial activity of test samples of the stem bark of *B. stipularis* is shown in Table 4.5.

Antifungal screening In case of various extracts of the stem bark of *B. stipularis*, the zone of inhibition was between 9-16 mm indicating mild to strong activity in contrast to griseofulvin as standard. The chloroform soluble materials gave highest activity against *A. niger* with zone size of 15.1 mm.

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Table 4.5: Antimicrobial screening of the stem bark of *B. stipularis* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

Test bacteria and fungi	Diameter of zone of inhibition (mm)					
	Me	HSF	CSF	CHSF	AQSF	KAN
Gram positive bacteria						
B. cereus	18.5 ± 0.82	14.8 ± 0.95	13.8 ± 0.46	16.7 ± 1.15	17.2 ± 1.32	35
B. megaterium	17.5 ± 1.32	18.7 ± 0.85	21.0 ± 0.66	17.1 ± 1.36	17.4 ± 0.59	35
B. subtilis	21.1 ± 2.05	15.7 ± 0.35	17.7 ± 1.11	19.4 ± 0.50	16.9 ± 0.42	36
S. lutea	21.0 ± 1.68	17.9 ± 0.74	18.7 ± 0.76	17.6 ± 0.32	16.8 ± 0.42	27
S. aereus	23.0 ± 1.68	17.3 ± 0.45	15.6 ± 0.76	20.4 ± 0.85	17.5 ± 1.06	32
Gram negative bacteria						
E. coli	19.1 ± 0.63	17.7 ± 0.25	20.8 ± 0.60	18.5 ± 0.77	17.6 ± 0.40	25
P. aeruginosa	14.7 ± 0.50	12.8 ± 1.29	13.1 ± 0.87	13.3 ± 0.78	13.0 ± 0.31	20
S. paratyphi	21.9 ± 0.86	18.9 ± 0.42	18.1 ± 0.67	19.7 ± 0.74	17.6 ± 1.26	27
S. typhi	20.7 ± 2.09	18.8 ± 0.17	18.8 ± 0.46	16.3 ± 1.40	17.3 ± 0.74	22
S. boydii	20.3 ± 1.29	18.7 ± 0.21	17.4 ± 0.42	18.7 ± 0.72	16.6 ± 0.46	27
S. dysenteriae	20.1 ± 1.12	17.5 ± 0.12	15.6 ± 0.47	20.0 ± 0.81	16.9 ± 0.55	25
V. mimicus	20.3 ± 0.91	17.9 ± 0.55	17.5 ± 0.51	22.3 ± 0.55	14.9 ± 1.11	25
V. parahemolyticus	17.4 ± 1.17	16.4 ± 0.31	16.9 ± 0.38	17.8 ± 0.42	11.9 ± 0.78	20
Fungi						**GRI**
A. niger	13.6 ± 2.07	11.9 ± 0.40	16.4 ± 0.64	15.1± 1.02	9.4 ± 0.46	20
C. albicans	14.7 ± 0.4 0	13.0 ± 0.42	12.4 ± 1.45	11.1 ± 0.90	13.4 ± 0.66	18
S. cerevisiae	11.7 ± 1.45	14.1 ± 1.02	12.3 ± 1.62	12.7 ± 1.37	10.9 ± 0.70	19

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

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4.4.4 Antimicrobial activity of the leaf of *B. stipularis*

In comparison to standard Kanamycin discs antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the leaf of *B. stipularis* also showed moderate to strong activity against various Gram positive and Gram negative bacterial strains with zones of inhibition between the ranges of 12 to 22 mm. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the leaf of *B. stipularis* were ranged from 14-22, 15-22, 14-20, 15-22 and 12-18 mm, respectively (Table 4.6) at a concentration of 400 µg/disc. The methanol extract showed the strongest inhibitory activity against *S. lutea* having the zone of inhibition 21.6 mm and the growth of *B. subtilis* (20.5 mm), *S. lutea* (21.6 mm) and *E. coli* (20 mm) was strongly inhibited. Again, the growth of *S. paratyphi*, *S. boydii* and *V. mimicus* was moderately inhibited with a zone of inhibition of 19.4, 19.4 and 19.6 mm, respectively. The *n*-hexane soluble materials gave strong zone size of 21.2 mm against *S. aereus* and 20 mm against *S. dysenteriae*. The *n*-hexane soluble extract also showed moderate activity with zone size of 19.7, 19.1 and 18.7 mm against *B. subtilis*, *S. paratyphi* and *V. parahemolyticus*, respectively. Simultaneously, the carbon tetrachloride partitionate of the methanol extract showed moderate activity against *S. aereus*, *S. lutea, S. typhi* and *V. mimicus* with zone of inhibition 19.4, 19.7, 19.1 and 19.8 mm, respectively. Again, the chloroform soluble materials showed zone of inhibition of 21.3 mm against *B. subtilis* indicating strong activity but it also gave zone size of 18.5, 18.1, 19.4 and 18 mm against *S. aureus*, *E. coli, S. paratyphi* and *S. dysenteriae*, respectively indicating moderate activity. The aqueous soluble materials showed low to moderate activity against all the strains ranging from 12-18 mm. The zone of inhibition of *B. subtilis*, *S. aereus* and *E. coli* was 17.9, 17.0 and 17.7 mm, respectively indicating mild activity. Out of all samples, the aqueous soluble materials showed lowest zone of inhibition of 12.2 mm against *P. aeruginosa* indicating lowest activity. For test samples of the leaf of *B. stipularis* the antibacterial activity is shown in Table 4.6.

In case of antifungal screening, the zone of inhibition was between 10.5-15.5 mm indicating moderate to strong activity in contrast to griseofulvin as standard. The *n*-hexane soluble materials gave highest activity against *C. albicans* with zone size of 15.5 mm.

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Table 4.6: Antimicrobial screening of the leaf of *B. stipularis* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

Test bacteria and fungi	Diameter of zone of inhibition (mm)					
	Me	HSF	CSF	CHSF	AQSF	KAN
Gram positive bacteria						
B. cereus	15.6 ± 0.89	18.2 ± 0.50	15.6 ± 0.61	16.7 ± 0.35	14.9 ± 0.78	35
B. megaterium	15.2 ± 0.70	17.5 ± 0.87	16.1 ± 0.87	15.6 ± 0.25	15.3 ± 0.76	35
B. subtilis	20.5 ± 1.12	19.7 ± 0.85	17.6 ± 0.61	21.3 ± 1.91	17.9 ± 0.65	36
S. lutea	21.6 ± 0.68	18.1 ± 0.67	19.7 ± 0.57	17.5 ± 0.85	15.3 ± 0.40	27
S. aereus	19.1 ± 0.80	21.2 ± 1.50	19.4 ± 0.75	18.5 ± 0.80	17.0 ± 0.74	32
Gram negative bacteria						
E. coli	20.0 ± 1.52	17.8 ± 0.61	17.2 ± 0.47	18.1 ± 0.38	17.7 ± 0.55	25
P. aeruginosa	14.1 ± 0.60	15.3 ± 0.61	14.5 ± 0.15	15.6 ± 0.50	12.2 ± 0.70	20
S. paratyphi	19.4 ± 0.35	19.1 ± 0.73	17.9 ± 1.15	19.4 ± 0.70	15.9 ± 0.40	27
S. typhi	19.1 ± 0.38	17.6 ± 1.25	19.1 ± 0.29	16.4 ± 1.03	15.7 ± 0.56	22
S. boydii	19.4 ± 1.03	17.8 ± 0.23	18.8 ± 0.31	17.6 ± 0.27	15.8 ± 0.95	27
S. dysenteriae	19.3 ± 0.87	20.0 ± 0.21	18.3 ± 0.35	18.0 ± 0.61	15.9 ± 0.15	25
V. mimicus	19.6 ± 0.78	16.2 ± 1.80	19.8 ± 2.15	15.6 ± 0.70	14.8 ± 0.81	25
V. parahemolyticus	17.4 ± 0.42	18.7 ± 0.86	18.4 ± 1.03	16.5 ± 0.49	15.3 ± 1.01	20
Fungi						**GRI**
A. niger	11.3 ± 0.49	11.8 ± 0.40	15.6 ± 0.63	11.6 ± 1.02	11.9 ± 0.78	20
C. albicans	15.4 ± 0.42	15.5 ± 0.65	11.2 ± 1.46	10.5 ± 0.79	11.8 ± 0.85	18
S. cerevisiae	14.0 ± 0.61	15.4 ± 0.67	13.1 ± 0.65	14.4 ± 0.46	13.1 ± 0.71	19

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

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4.4.5 Antimicrobial activity of the stem bark of *B. tomentosa*

Antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the stem bark of *B. tomentosa* also showed moderate to strong activity against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs with zones of inhibition between the ranges of 14 to 23.5 mm. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the stem bark of *B. tomentosa* were ranged from 19-24, 15-20, 15-21, 17-21 and 14-17 mm, respectively (Table 4.7) at a concentration of 400 μg/disc. The methanol extract showed the strongest inhibitory activity against *B. cereus* having the zone of inhibition 23.2 mm and the growth of *B. subtilis* (20.7 mm), *S. aereus* (21 mm), *S. lutea* (21.4 mm), *E. coli* (22.4 mm), *S. paratyphi* (21.1 mm), *S. typhi* (22.3 mm), *S. boydii* (21.3 mm), *S. dysenteriae* (20.9 mm), *V. mimicus* (21.2 mm) and *V. parahemolyticus* (21.3 mm) was strongly inhibited. The *n*-hexane soluble materials gave moderate zone size of 18.1 mm against *S. aereus* and *S. lutea*. The *n*-hexane soluble extract also showed moderate activity with zone size of 18.8 mm against *S. paratyphi*, 19.9 mm against *V. miniscus* and 18.7 mm against *V. parahemolyticus*. At the same time, the carbon tetrachloride partitionate of the methanol extract showed strong activity against *S. typhi* with zone size of 20.5 mm. It also gave moderate activity against *S. aereus*, *E. coli* and *V. mimicus* with zone of inhibition 19.6, 18.9 and 18.2 mm, respectively. Again, the chloroform soluble materials showed zone of inhibition of 20.3 mm against *S. paratyphi* and 20.1 mm against *S. boydii* indicating very strong activity but it also gave zone size of 19, 19.4, 18.9 and 19.5 mm against *B. cereus*, *B. subtilis, S. boydii* and *S. dysenteriae*, respectively indicating strong activity. The aqueous soluble materials showed low to moderate activity against all the strains ranging from 12-17 mm. Out of all samples, the aqueous soluble materials showed lowest zone of inhibition of 12.7 mm against *P. aeruginosa* indicating lowest activity. The antibacterial activity of test samples of the stem bark of *B. tomentosa* is shown in Table 4.7.

For antifungal screening, the zone of inhibition was between 10-16 mm indicating moderate to strong activity in contrast to griseofulvin as standard. The chloroform soluble materials gave highest activity against *S. cerevisiae* with zone size of 15.9 mm.

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Table 4.7: Antimicrobial screening of the stem bark of *B. tomentosa* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

Test bacteria and fungi	Diameter of zone of inhibition (mm)					
	Me	HSF	CSF	CHSF	AQSF	KAN
Gram positive bacteria						
B. cereus	23.2 ± 1.47	17.8 ± 0.66	16.9 ± 1.10	19.0 ± 0.31	15.0 ± 0.49	35
B. megaterium	19.1 ± 0.60	17.5 ± 0.40	16.4 ± 0.50	17.3 ± 0.47	14.4 ± 0.74	35
B. subtilis	20.7 ± 0.55	16.8 ± 0.50	18.6 ± 0.61	19.4 ± 0.31	14.9 ± 0.17	36
S. lutea	21.4 ± 0.50	18.1 ± 0.57	16.8 ± 0.61	18.9 ± 0.78	14.8 ± 0.31	27
S. aereus	21.0 ± 0.95	18.1 ± 0.35	19.6 ± 0.59	17.6 ± 0.42	15.8 ± 0.46	32
Gram negative bacteria						
E. coli	22.4 ± 0.35	17.9 ± 0.21	18.9 ± 0.57	17.8 ± 0.27	15.2 ± 0.51	25
P. aeruginosa	16.6 ± 0.21	14.8 ± 0.50	13.8 ± 1.01	13.8 ± 0.65	12.7 ± 0.85	20
S. paratyphi	21.2 ± 0.82	18.8 ± 0.50	17.5 ± 0.32	20.3 ± 0.53	15.2 ± 0.51	27
S. typhi	22.3 ± 0.75	17.6 ± 0.31	20.5 ± 0.38	17.3 ± 0.60	15.0 ± 0.31	22
S. boydii	21.3 ± 1.40	17.7 ± 0.66	16.9 ± 0.55	20.1 ± 0.45	15.4 ± 0.31	27
S. dysenteriae	20.9 ± 0.72	17.7 ± 0.56	15.9 ± 0.59	19.5 ± 1.05	16.8 ± 0.36	25
V. mimicus	21.2 ± 1.10	19.9 ± 0.47	18.2 ± 0.50	17.3 ± 0.40	14.4 ± 0.50	25
V. parahemolyticus	21.3 ± 0.59	18.7 ± 0.55	17.1 ± 0.67	18.1 ± 0.68	13.8 ± 1.29	20
Fungi						**GRI**
A. niger	13.6 ± 0.31	11.4 ± 0.83	10.2 ± 0.42	10.8 ± 0.50	13.2 ± 0.31	20
C. albicans	13.4 ± 0.91	14.2 ± 0.61	15.6 ± 0.32	13.6 ± 1.14	10.2 ± 0.40	18
S. cerevisiae	11.4 ± 0.66	14.6 ± 0.31	12.6 ± 1.10	15.9 ± 0.17	12.9 ± 0.72	19

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.6 Antimicrobial activity of the leaf of *B. tomentosa*

Again, antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the leaf of *B. tomentosa* also showed mild to strong activity against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs with zones of inhibition between the ranges of 13 to 23 mm. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the leaf of *B. tomentosa* were ranged from 16-23, 14-20, 13-21, 14-20 and 13-17 mm, respectively (Table 4.8) at a concentration of 400 µg/disc. The methanol extract showed the strongest inhibitory activity against *V. parahemolyticus* having the zone of inhibition 22.1 mm and the growth of *B. megaterium* (21.6 mm), B. *subtilis* (20 mm), *E. coli* (20 mm), *S. paratyphi* (20.3 mm), *S. typhi* (20.9 mm), *V. mimicus* (21.3 mm) and *V. parahemolyticus* (22.1 mm) was strongly inhibited. The *n*-hexane soluble materials gave moderate zone size of 18.5, 18.8, 19.6, 18.1 and 18.3 mm against *B. megaterium*, *B. subtilis*, *S. aereus, S. paratyphi* and *S. typhi*, respectively. The carbon tetrachloride partitionate of the methanol extract showed strong activity against *B. megaterium* with zone size of 20.1 mm. It also gave moderate activity against *B. cereus*, *B. subtilis*, *S. aereus*, S. *lutea, S. boydii* and *S. dysenteriae* with zone of inhibition 19.1, 18.3, 18.8, 18.1, 18.8 and 18.1 mm, respectively. Again, the chloroform soluble materials gave zone size of 18.1, 19.1, 18.3 and 18.1 mm against *B. subtilis*, E. *coli, V. mimicus* and *V. parahemolyticus*, respectively indicating moderate activity. The aqueous soluble materials showed low to moderate activity against all the strains ranging from 13-17 mm. The zone of inhibition of *B. megaterium*, *S. aereus*, *E. coli*, *P. aeruginosa*, *S. paratyphi* and *S. dysenteriae* was 15.6, 16.7, 16.3, 15.6, 15.6 and 15.7 mm, respectively indicating mild activity. Further of all the samples, the aqueous soluble materials showed lowest zone of inhibition of 13.2 mm against *B. subtilis* indicating lowest activity. Table 4.8 shows the antibacterial activity of test samples of the leaf of *B. tomentosa*.

Using griseofulvin as standard the zone of inhibition was between 10-18 mm indicating moderate to strong activity in case of antifungal screening. The methanol extract gave highest activity against *C. albicans* with zone size of 17.5 mm.

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Table 4.8: Antimicrobial screening of the leaf of *B. tomentosa* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

Test bacteria and fungi	Diameter of zone of inhibition (mm)					
	Me	HSF	CSF	CHSF	AQSF	KAN
Gram positive bacteria						
B. cereus	18.0 ± 1.71	16.9 ± 0.75	19.1 ± 0.15	17.8 ± 0.56	15.1 ± 0.70	35
B. megaterium	21.6 ± 0.97	18.5 ± 0.87	20.1 ± 0.25	17.4 ± 0.91	15.6 ± 0.57	35
B. subtilis	22.0 ± 0.70	18.8 ± 0.35	18.3 ± 0.67	18.1 ± 0.68	13.2 ± 0.42	36
S. lutea	19.4 ± 0.35	17.6 ± 0.27	18.1 ± 0.40	16.0 ± 0.42	14.6 ± 0.50	27
S. aereus	19.9 ± 1.12	19.6 ± 0.50	18.8 ± 0.56	16.9 ± 0.68	16.7 ± 0.60	32
Gram negative bacteria						
E. coli	20.9 ± 0.71	17.4 ± 0.42	16.6 ± 0.31	19.1 ± 0.25	16.3 ± 0.55	25
P. aeruginosa	15.9 ± 0.40	14.0 ± 0.60	13.5 ± 0.15	14.8 ± 0.82	15.6 ± 0.63	20
S. paratyphi	20.3 ± 0.96	18.1 ± 0.72	17.8 ± 0.78	17.0 ± 0.56	15.6 ± 0.31	27
S. typhi	20.9 ± 0.70	18.3 ± 0.85	16.9 ± 0.75	16.8 ± 0.42	14.8 ± 0.50	22
S. boydii	19.8 ± 0.42	16.8 ± 0.50	18.8 ± 0.42	16.8 ± 0.42	15.3 ± 0.45	27
S. dysenteriae	20.8 ± 0.76	17.5 ± 1.04	18.1 ± 0.92	17.3 ± 0.59	15.7 ± 0.32	25
V. mimicus	21.3 ± 0.72	17.2 ± 0.31	16.5 ± 0.32	18.3 ± 0.78	14.4 ± 0.23	25
V. parahemolyticus	22.1 ± 0.72	17.9 ± 0.45	16.9 ± 0.15	18.1 ± 0.55	14.2 ± 0.70	20
Fungi						**GRI**
A. niger	13.9 ± 0.30	11.9 ± 0.32	15.8 ± 0.31	14.7 ± 0.60	13.8 ± 0.71	20
C. albicans	17.5 ± 0.17	15.1 ± 0.35	11.5 ± 1.60	12.2 ± 0.87	10.7 ± 1.65	18
S. cerevisiae	13.1 ± 0.72	14.2 ± 0.60	11.8 ± 0.56	13.5 ± 0.38	12.8 ± 0.46	19

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

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4.4.7 Antimicrobial activity of the stem bark *E. fusca*

Antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the stem bark of *E. fusca* showed low to strong activity with zones of inhibition being between the ranges of 12-19 mm against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the stem bark of *E. fusca* were ranged from 14-20, 13-19, 13-18, 12-20 and 13-19 mm, respectively (Table 4.9) at a concentration of 400 µg/disc. The chloroform soluble fractions of the methanol extract showed the highest inhibitory activity against *B. cereus* having the zone of inhibition 19.3 mm. In case of the methanol extract the growth of *B. megaterium* (18.1 mm), *B. subtilis* (18.4 mm), *S. aereus* (19.1 mm), *S. lutea* (18.3 mm), *S. paratyphi* (19.2 mm) and *V. mimicus* (14.8 mm) was strongly inhibited. The *n*-hexane soluble materials gave strong zone size of 18.6, 18.3 and 18.1 mm against *B. megaterium*, *B. subtilis* and *S. dysenteriae*. On the other hand, the carbon tetrachloride partitionate of methanol extract showed mild to moderate activity against *E. coli, S. paraytyphi* and *V. mimicus* having zone of inhibition 17.3, 17.6 and 16.9 mm respectively. Again, the chloroform soluble materials showed maximum zone of 19.3 mm against *B. cereus* and zone of 17.6, 17.4, 17.7 16.8 and 16.5 mm against *B. subtilis*, *S. aureus*, *E. coli*, *S. paratyphi* and *S. boydii*, respectively indicating strong to moderate activity. The aqueous soluble materials also showed low to strong activity against all the strains ranging from 12-19 mm. Of all the samples, the chloroform soluble materials showed lowest zone of inhibition of 12.1 mm against *B. cereus* indicating low activity. The antibacterial activity of test samples of the stem bark of *E. fusca* is shown in Table 4.9.

In case of antifungal screening, the zone of inhibition was between 11-17 mm indicating mild to moderate activity in contrast to griseofulvin as standard. The carbon tetrachloride soluble materials gave highest activity against *C. albicans* with zone size of 16.1 mm.

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Table 4.9: Antimicrobial screening of the stem bark of *E. fusca* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

Test bacteria and fungi	Diameter of zone of inhibition (mm)					
	Me	**HSF**	**CSF**	**CHSF**	**AQSF**	**KAN**
Gram positive bacteria						
B. cereus	17.5± 0.40	16.9± 0.75	15.2± 0.55	19.3± 0.21	14.2± 0.51	35
B. megaterium	18.1± 0.68	18.6± 0.70	14.9± 0.17	12.4± 0.70	14.4± 0.74	35
B. subtilis	18.4± 0.75	18.3± 0.40	16.8± 0.36	17.6± 0.42	14.8± 0.31	36
S. aereus	19.1± 0.74	18.1± 0.55	16.7± 0.45	17.4± 0.42	15.8± 0.56	32
S. lutea	18.3± 0.85	17.5± 0.32	16.6± 0.31	15.6± 0.31	17.6± 0.31	27
Gram negative bacteria						
E. coli	17.8± 0.78	16.9± 0.75	17.3± 0.60	17.7± 0.66	18.3± 0.85	25
P. aeruginosa	14.4± 0.35	13.4± 0.36	12.5± 0.69	12.1 ± 0.87	12.7± 0.85	20
S. paratyphi	19.2± 0.61	17.7± 0.56	17.6± 0.49	16.8± 0.36	18.1± 0.93	27
S. typhi	16.2± 0.87	17.1± 0.21	16.8± 0.36	16.2± 0.81	15.4± 0.31	22
S. boydii	16.8± 0.36	17.8± 0.56	17.5± 0.31	16.5± 0.35	15.4± 0.31	27
S. dysenteriae	16.9± 0.45	18.1± 0.55	16.7± 0.35	15.8± 0.56	17.2± 0.31	25
V. mimicus	18.5± 0.53	17.6± 0.31	16.9± 0.15	15.8± 0.31	16.1± 0.53	25
V. parahemolyticus	15.8± 0.31	15.1± 0.35	15.5± 0.74	16.1± 0.85	15.6± 0.46	20
Fungi						**GRI**
A. niger	14.4± 0.42	12.2± 0.87	13.7± 0.72	13.9± 0.57	13.5± 0.35	20
C. albicans	15.1± 0.91	15.6± 0.31	16.1± 0.85	14.6± 0.64	12.5± 0.32	18
S. cerevisiae	15.5± 0.38	12.5± 0.32	11.9± 0.72	13.2± 0.51	12.1± 0.74	19

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.8 Antimicrobial activity of the leaf of *E. fusca*

Again, the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the leaf of *E. fusca* also showed low to mild activity against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs with zones of inhibition between the ranges of 11-20 mm. At a concentration of 400 μg/disc the zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the leaf of *E. fusca* were ranged from 13-19, 12-20, 11-18 mm, 12-18 and 13-18 mm, respectively (Table 4.10) The *n*-hexane soluble fraction showed the highest inhibitory activity against *S. lutea* having the zone of inhibition 19.2 mm. In case of the methanol extract the growth of *B. cereus* (18.2 mm), *S. lutea* (18.4 mm), *E. coli* (18.1 mm) and *S. typhi* (18.2 mm) was moderately inhibited. The *n*-hexane soluble materials gave highest zone size of 17.9 mm *B. cereus,* 18.1 mm against *B. megaterium*, 18.1 mm against *V. mimicus* and 18.1 mm against *V. parahemolyticus*. At the same time, the carbon tetrachloride partitionate of methanol extract showed relatively mild activity against *B. cereus, E. coli* and *S. typhi* having zone of inhibition 17.4, 16.7 and 16.9 mm respectively. Again, the chloroform soluble materials showed maximum zone of 17.3 mm against *S. dysenteriae* and inhibition zone of 17.2, 16.5, 16.8, and 17.2 mm against *B. megaterium*, *S. partyphi*, *S. typhi* and *S. boydii*, respectively indicating relatively mild to moderate activity. The aqueous soluble materials also showed low to relatively strong activity against all the strains ranging from 13-18 mm. Out of all samples, the carbon tetrachloride soluble materials showed lowest zone size of 11.5 mm against *P. aeruginosa* indicating low activity. The antibacterial activity of test samples of the leaf of *E. fusca* is shown in Table 4.10.

Yet again, the zone of inhibition for antifungal screening was between 10-16 mm indicating low to strong activity in contrast to griseofulvin as standard. The crude methanol extract gave highest activity against *A. niger* with zone size of 15.5 mm.

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Table 4.10: Antimicrobial screening of the leaf of *E. fusca* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

Test bacteria and fungi	Diameter of zone of inhibition (mm)					
	Me	HSF	CSF	CHSF	AQSF	KAN
Gram positive bacteria						
B. cereus	18.2± 0.83	17.9± 0.36	17.4± 0.91	15.8± 0.40	17.1± 0.72	35
B. megaterium	17.1± 0.32	18.1± 0.72	15.7± 0.65	17.2± 0.72	15.4± 0.92	35
B. subtilis	17.5± 0.53	16.9± 0.35	14.7± 0.35	16.1± 0.71	13.5± 0.32	36
S. aereus	17.9± 0.44	17.6± 0.31	16.1± 0.55	15.1± 0.32	15.3± 0.55	32
S. lutea	18.4± 0.97	19.2± 0.15	16.2 ± 0.61	15.2± 0.51	15.6± 0.31	27
Gram negative bacteria						
E. coli	18.1± 0.72	17.8± 0.78	16.7± 0.40	15.1± 0.32	16.8± 0.64	25
P. aeruginosa	13.5± 0.25	12.1± 0.47	11.5± 0.15	12.8± 0.75	13.8± 0.25	20
S. paratyphi	17.9± 0.74	16.9± 0.75	14.7± 0.35	16.5± 0.35	17.3± 0.59	27
S. typhi	18.2± 0.42	17.4± 0.36	16.9± 0.55	16.8± 0.42	16.5± 0.35	22
S. boydii	17.3± 0.59	17.7± 0.56	15.9± 0.32	17.2± 0.47	16.9± 0.15	27
S. dysenteriae	16.5± 0.35	17.5± 0.29	16.1± 0.53	17.3± 0.59	13.8± 0.71	25
V. mimicus	17.8± 0.36	18.1± 0.55	16.2± 0.36	14.5± 0.40	13.8± 0.71	25
V. parahemolyticus	16.9± 0.15	17.9± 0.45	14.2± 0.15	15.1± 0.45	12.2± 0.70	20
Fungi						**GRI**
A. niger	15.5± 0.51	14.5± 0.36	15.4± 0.31	12.3± 0.25	13.8± 0.71	20
C. albicans	14.4± 0.35	13.8± 0.35	10.2± 0.46	12.2± 0.61	11.9± 0.61	18
S. cerevisiae	14.1± 0.76	13.5± 0.15	13.5± 0.15	14.2± 0.30	12.8± 0.46	19

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

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4.4.9 Antimicrobial activity of the stem bark *E. variegata*

Antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the stem bark of *E. variegata* showed low to strong activity with zones of inhibition being between the ranges of 11-19 mm against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs. The crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the stem bark of *E. variegata* zones of inhibition ranged from 14-19, 12-18, 11-19, 13-17 and 11-15 mm, respectively (Table 4.11) at a concentration of 400 µg/disc. The crude methanol extract showed the maximum inhibitory activity against *B. cereus* having the zone of inhibition 18.2 mm. In case of the methanol extract the growth of *S. typhi* (17.9 mm), *S. boydii* (17.3 mm), *S. dysenteriae* (17.1 mm) and *V. mimicus* (17.9 mm) was moderately inhibited. The *n*-hexane soluble materials gave moderate zone size for of 17.7 mm against *E. coli*, 17.1 mm against *S. paratyphi* and 17.6 mm against *S. typhi*. The carbon tetrachloride partitionate of methanol extract gave maximum zone of inhibition of 18.1 mm. At the same time, it showed mild to moderate activity against *S. lutea, S. paraytyphi*, *S. typhi* and *V. mimicus* having zone of inhibition 16.7, 17.4, 16.7 and 17.2, mm respectively. Again, the chloroform soluble materials showed maximum zone of 16.8 mm against *S. typhi* indicating weak activity. The aqueous soluble materials also showed low to moderate activity against all the strains between 11-16 mm. Out of all samples, the chloroform soluble materials showed lowest zone of inhibition of 11.7 mm against *P. aeruginosa* indicating low activity. Table 4.11 shows the antibacterial activity of test samples of the stem bark of *E. variegata.*

For the antifungal screening, the zone of inhibition was between 9-15 mm indicating low to moderate activity in contrast to griseofulvin as standard. The *n*-hexane soluble materials gave highest activity against *C. albicans* with zone size of 14.4 mm.

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Table 4.11: Antimicrobial screening of the stem bark of *E. variegata* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

Test bacteria and fungi	Diameter of zone of inhibition (mm)					
	Me	HSF	CSF	CHSF	AQSF	KAN
Gram positive bacteria						
B. cereus	18.2± 0.68	16.8± 0.42	15.3± 0.84	14.4± 0.74	14.9± 0.17	35
B. megaterium	15.2± 0.84	16.1± 0.75	16.2 ± 0.23	15.3± 0.47	13.1± 0.65	35
B. subtilis	14.2± 0.45	13.3± 0.45	12.1± 0.65	13.9± 0.55	12.7± 0.45	36
S. aereus	14.9± 0.55	12.1± 0.35	11.9± 0.55	13.9± 0.47	11.8± 0.56	32
S. lutea	15.1± 0.32	16.8± 0.61	16.7± 0.57	14.5± 0.38	14.3± 0.53	27
Gram negative bacteria						
E. coli	16.6± 0.44	17.7± 0.45	18.1± 0.72	16.5± 0.35	15.3± 0.45	25
P. aeruginosa	14.4± 0.35	12.2± 0.72	12.3± 0.55	13.1± 0.35	11.7± 0.42	20
S. paratyphi	16.8± 0.42	17.1± 0.76	17.4± 0.42	15.4± 0.61	15.2± 0.55	27
S. typhi	17.9± 0.68	17.6± 0.42	16.7± 0.53	16.8± 0.51	14.9± 0.20	22
S. boydii	17.3± 0.59	16.8± 0.36	16.4± 0.42	14.4± 0.35	15.1± 0.49	27
S. dysenteriae	17.1± 0.67	14.7± 0.56	16.1± 0.78	15.9± 0.55	15.1± 0.75	25
V. mimicus	17.9± 0.45	15.6± 0.59	17.2± 0.56	16.3± 0.87	13.8± 0.23	25
V. parahemolyticus	15.1± 0.65	16.1± 0.32	14.2± 0.45	14.7± 0.55	12.9± 0.78	20
Fungi						**GRI**
A. niger	12.9± 0.55	13.7± 0.51	13.6± 0.42	11.8± 0.78	9.8± 0.55	20
C. albicans	13.5± 0.49	14.4± 0.42	12.6± 0.56	11.1± 0.35	12.6± 0.44	18
S. cerevisiae	13.3± 0.32	12.2± 0.56	11.4± 0.30	12.9± 0.74	12.7± 0.85	19

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

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4.4.10 Antimicrobial activity of the leaf of *E. variegata*

On the other hand, the leaf of the methanol extract and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the plant *E. variegata* showed low to moderate activity with zones of inhibition ranging from 11-18 mm against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the leaf of *E. variegata* were ranged from 11-18, 12-17, 11-17, 12-17 and 11-17 mm, respectively (Table 4.12) at a concentration of 400 µg/disc. The crude methanol extract showed the highest inhibitory activity against *E. coli* having the zone of inhibition 17.8 mm. In case of the methanol extract the growth of *B. cereus* (16.5 mm), *S. typhi* (16.6 mm), *S. dysenteriae* (17.2 mm) and *V. parahemolyticus* (16.5 mm) was strongly inhibited. The *n*-hexane soluble materials also gave strong zone size of 16.9, 16.9, 17.3 and 17.1 mm against *S. lutea*, *E. coli*, *S. typhi* and *S. dysenteriae*. Also, the carbon tetrachloride partitionate of methanol extract showed mild to moderate activity against *E. coli*, *S. paratyphi*, *S. typhi* and *S. dysenteriae* having zone of inhibition of 16.4, 16.5, 16.5, 14.5 and 16.7 mm, respectively. Again, the chloroform soluble materials showed strong zone of inhibition of 17.3 and 16.9 mm against *S. paratyphi* and *S. dysenteriae*, respectively indicating strong activity. But *S. lutea* and *E. coli* had a zone of 15.5 mm each indicating mild inhibition. The aqueous soluble materials showed low to moderate activity against all the strains ranging from 11-17 mm. Out of all samples, the aqueous soluble materials showed lowest zone of inhibition of 11.2 mm against *B. subtilis* indicating low activity and strongest against *S. paratyphi* with a zone of 16.9 mm. The antibacterial activity of test samples of the leaf of *E. variegata* is shown in Table 4.12.

The zone of inhibition was between 11-16 mm indicating moderate to strong activity in contrast to griseofulvin as standard in case of antifungal screening. The *n*-hexane soluble materials gave highest activity against *C. albicans* with zone size of 15.4 mm.

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Table 4.12: Antimicrobial screening of the leaf of *E. variegata* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

Test bacteria and fungi	Diameter of zone of inhibition (mm)					
	Me	HSF	CSF	CHSF	AQSF	KAN
Gram positive bacteria						
B. cereus	16.5± 0.35	15.9± 0.75	15.7± 0.57	14.8± 0.46	14.3± 0.45	35
B. megaterium	13.1± 0.31	13.5± 0.25	14.4± 0.42	15.1± 0.42	12.2± 0.96	35
B. subtilis	11.1± 0.74	15.8± 0.46	12.3± 0.38	13.5± 0.26	11.2± 0.61	36
S. aereus	12.7± 0.40	12.2± 0.42	11.8± 0.78	14.6± 0.50	15.2± 0.36	32
S. lutea	15.3± 0.55	16.6± 0.35	15.8± 0.66	15.5± 0.40	14.7± 0.45	27
Gram negative bacteria						
E. coli	17.8± 0.78	16.9± 0.61	16.4± 0.42	15.5± 0.51	14.7± 0.45	25
P. aeruginosa	13.1± 0.62	13.5± 0.31	11.2± 0.68	12.2± 0.50	12.1± 0.35	20
S. paratyphi	15.4± 0.31	14.7± 0.51	16.5± 0.25	17.3± 0.59	16.9± 0.65	27
S. typhi	16.6± 0.31	17.3± 0.59	16.5± 0.46	13.7± 0.56	13.3± 0.91	22
S. boydii	15.2± 0.76	16.4± 0.36	14.6± 0.46	14.8± 0.76	15.1± 0.76	27
S. dysenteriae	17.2± 0.31	17.1± 0.76	16.7± 0.35	16.9± 0.49	14.3± 0.55	25
V. mimicus	15.4± 0.78	14.2± 0.31	15.5± 0.35	13.9± 0.74	12.7± 0.45	25
V. parahemolyticus	16.5± 0.55	15.8± 0.31	13.1± 0.66	13.8± 0.71	12.9± 0.86	20
Fungi						**GRI**
A. niger	13.7± 0.51	14.4± 0.36	15.3± 0.31	12.9± 0.25	11.2± 0.71	20
C. albicans	14.3± 0.31	13.7± 0.45	10.4± 0.55	11.8± 0.66	9.9± 0.26	18
S. cerevisiae	14.7± 0.47	13.7± 0.45	13.1± 0.67	13.4± 0.44	12.4± 0.42	19

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

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4.4.11 Antimicrobial activity of the pure compounds

Some of the isolated pure compounds from the four investigated plants were also subjected to antimicrobial screening which showed low to strong activity with zones of inhibition being between the ranges of 11 to 23 mm against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs. The pure compounds BVSP-4 (**48**), BVBC-2 (**51**), BVS-65 (**54**), BS-01 (**55**), BS-06 (**56**), BT-1 (**58**), BT-6 (**59**), EF-7 (**62**), EF-8 (63) and EF-12 (64) isolated from the stem bark of *B. verrucosa*, *B. stipularis*, *B. tomentosa* and *E. fusca* produced zone of inhibition ranging from 11-16, 12-16, 11-17, 17-23, 16-20, 16-21, 14-21, 16-18, 14-19 and 15-19 mm, respectively (Table 4.13 and 4.14) at a concentration of 50 µg/disc. The highest zone of inhibition (22.7 mm) was produced by BS-01 (**55**) against *E. coli*.

In case of antifungal screening, the zone of inhibition was between 10-21 mm indicating mild to strong activity in contrast to griseofulvin as standard. BS-06 (**56**) gave highest activity against *C. albicans* with zone size of 20.8 mm.

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Table 4.13: Antimicrobial screening of the some of the pure compounds from *B. verrucosa* and *B. stipularis* (50 µg/disc) and Kanamycin (30 µg/disc).

Test bacteria and fungi	Diameter of zone of inhibition (mm)					
	BVSP-4	BVBC-2	BVS-65	BS-01	BS-06	KAN
Gram positive bacteria						
B. cereus	11.9± 0.33	13.9± 0.75	13.5± 0.71	19.0± 0.60	16.1± 0.66	35
B. megaterium	13.1± 0.31	14.5± 0.66	14.6± 0.41	18.4± 0.50	16.9± 0.91	35
B. subtilis	12.1± 0.54	12.8± 0.46	12.3± 0.38	17.5± 0.87	16.3± 1.05	36
S. aereus	12.9± 0.91	12.2± 0.29	13.0± 0.78	17.8± 0.40	18.2± 0.89	32
S. lutea	14.6± 0.55	14.5± 0.57	11.8± 0.66	21.6± 0.87	20.7± 1.22	27
Gram negative bacteria						
E. coli	13.2± 0.58	12.1± 0.49	12.9± 0.71	22.7± 0.65	18.8± 0.42	25
P. aeruginosa	13.1± 0.62	13.5± 0.31	14.6± 0.41	17.9± 0.76	18.4± 0.76	20
S. paratyphi	14.8± 0.31	13.1± 0.74	14.3± 0.55	20.1± 0.65	18.4± 0.76	27
S. typhi	12.8± 0.31	15.1± 0.76	16.5± 0.46	22.1± 0.65	20.6± 0.86	22
S. boydii	15.2± 0.76	16.4± 0.36	14.6± 0.46	20.2± 1.29	18.1± 0.86	27
S. dysenteriae	14.3± 0.55	14.8± 0.76	16.7± 0.35	21.2± 0.36	19.2± 0.66	25
V. mimicus	13.3± 0.91	14.2± 0.31	13.5± 0.35	21.3± 0.91	16.9± 0.61	25
V. parahemolyticus	13.1± 0.66	13.9± 0.74	14.6± 0.22	20.7± 0.60	18.9± 0.59	20
Fungi						**GRI**
A. niger	13.7± 0.51	14.4± 0.36	15.3± 0.31	19.1± 0.35	18.2± 0.59	20
C. albicans	14.3± 0.31	13.7± 0.45	10.4± 0.55	18.5± 0.60	20.8± 0.45	18
S. cerevisiae	14.7± 0.47	13.7± 0.45	13.1± 0.67	18.2± 0.31	20.2± 0.83	19

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

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Table 4.14: Antimicrobial screening of the some of the pure compounds from *B. tomentosa* and *E. fusca* (50 µg/disc) and Kanamycin (30 µg/disc).

Test bacteria and fungi	Diameter of zone of inhibition (mm)					
	BT-1	BT-6	EF-7	EF-8	EF-12	KAN
Gram positive bacteria						
B. cereus	17.7± 0.57	19.1± 0.45	17.8± 0.66	16.9± 1.10	19.0± 0.31	35
B. megaterium	16.1± 0.59	16.7± 0.55	18.5± 0.87	19.1± 0.60	15.6± 0.57	35
B. subtilis	18.3± 0.85	14.9± 0.96	19.4± 0.31	18.8± 0.35	14.9± 0.17	36
S. aereus	16.9± 0.87	16.4± 1.08	17.6± 0.27	18.1± 0.40	61.0± 0.42	32
S. lutea	19.6± 0.83	20.8± 0.50	16.9± 0.68	16.7± 0.60	17.6± 0.42	27
Gram negative bacteria						
E. coli	19.5± 0.21	19.1± 0.57	17.9± 0.21	17.8± 0.27	17.4± 0.42	25
P. aeruginosa	15.8± 0.98	16.9± 0.68	14.8± 0.82	14.8± 0.50	16.6± 0.21	20
S. paratyphi	21.3± 0.46	19.7± 0.70	17.5± 0.32	17.8± 0.78	15.2± 0.51	27
S. typhi	18.9± 0.55	20.1± 0.90	18.3± 0.85	18.8± 0.42	17.3± 0.60	22
S. boydii	20.1± 0.92	19.8± 0.25	16.9± 0.55	16.8± 0.42	17.7± 0.66	27
S. dysenteriae	20.3± 0.85	19.6± 0.70	16.8± 0.36	17.3± 0.59	15.9± 0.59	25
V. mimicus	19.1± 0.87	17.2± 0.56	17.2± 0.31	17.3± 0.40	16.5± 0.32	25
V. parahemolyticus	17.2± 0.36	18.4± 1.08	18.1± 0.55	16.9± 0.15	17.9± 0.15	20
Fungi						**GRI**
A. niger	13.6± 0.55	15.8± 0.66	13.6± 0.31	11.9± 0.32	13.2± 0.31	20
C. albicans	12.8± 0.99	14.2± 1.23	14.2± 0.61	13.6± 1.14	15.1± 0.35	18
S. cerevisiae	14.2± 0.36	12.7± 0.45	12.6± 1.10	14.2± 0.60	14.2± 0.60	19

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

5.1 Principle

Brine shrimp lethality bioassay was done by following the method illustrated by Meyer *et al.*, 1982.

Table 5.1: Test samples of experimental plants.

Name of Plant	Extract	Amount of extract (mg)
Bridelia verrucosa (Stem bark and leaf)	Crude MeOH extract	4.0
	n-Hexane soluble fraction	4.0
	CCl_4 soluble fraction	4.0
	Chloroform soluble fraction	4.0
	Aqueous soluble fraction	4.0
	BVSP-4 (**48**)	1.0
	BVBC-2 (**51**)	1.0
	BVS-65 (**54**)	1.0
Bridelia stipularis (Stem bark and leaf)	Crude MeOH extract	4.0
	n-Hexane soluble fraction	4.0
	CCl_4 soluble fraction	4.0
	Chloroform soluble fraction	4.0
	Aqueous soluble fraction	4.0
	BS-01(**55**)	1.0
	BS-06 (**56**)	1.0
	BS-11 (**57**)	1.0
Bridelia tomentosa (Stem bark and leaf)	Crude MeOH extract	4.0
	n-Hexane soluble fraction	4.0
	CCl_4 soluble fraction	4.0
	Chloroform soluble fraction	4.0
	Aqueous soluble fraction	4.0
	BT-6 (**60**)	1.0
Erythrina fusca (Stem bark and leaf)	Crude MeOH extract	4.0
	n-Hexane soluble fraction	4.0
	CCl_4 soluble fraction	4.0
	Chloroform soluble fraction	4.0
	Aqueous soluble fraction	4.0
	EF-7 (**62**)	1.0
	EF-8 (**63**)	1.0
	EF-12 (**64**)	1.0
Erythrina variegata (Stem bark and leaf)	Crude MeOH extract	4.0
	n-Hexane soluble fraction	4.0
	CCl_4 soluble fraction	4.0
	Chloroform soluble fraction	4.0
	Aqueous soluble fraction	4.0

Table 5.2: Effect of vincristine sulfate (positive control) on shrimp nauplii.

Conc. (C) (µg/ml)	Log C	% Mortality	LC_{50} (µg/ml)	LC_{90} (µg/ml)
0.0390	-1.40894	20	0.45	10.0
0.078125	-1.10721	30		
0.15625	-0.80618	30		
0.3125	-0.50515	40		
0.625	-0.20142	50		
1.25	0.09691	70		
2.5	0.39794	80		
5	0.69897	80		
10	1	90		
20	1.30103	100		

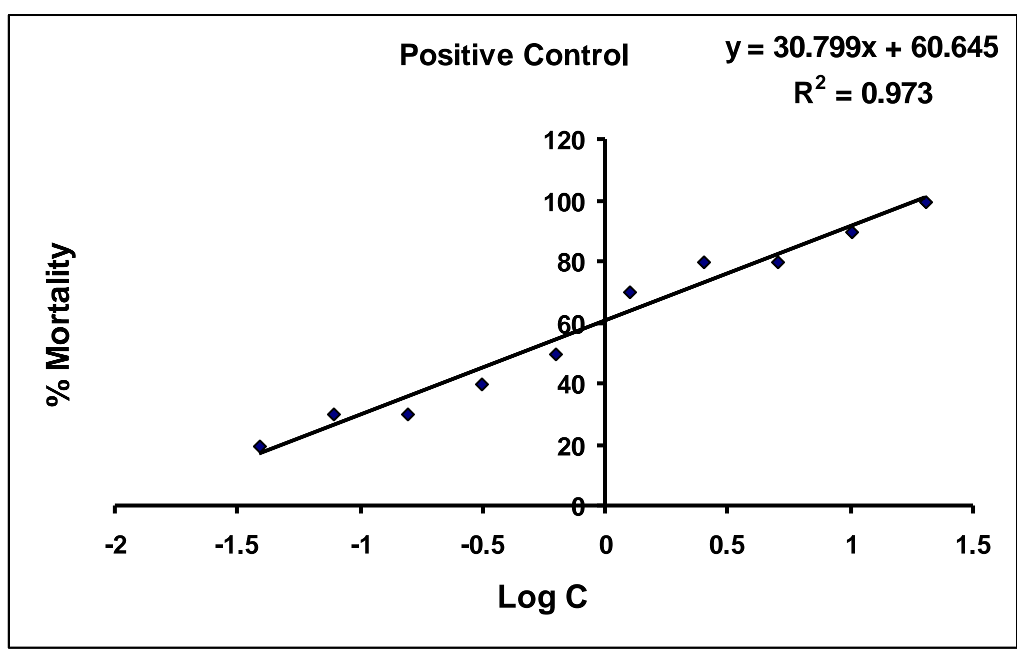

Figure 5.1: Effect of vincristine sulfate on brine shrimp nauplii.

5.2 Results and discussion of the test samples

The crude extracts of the stem bark and leaf of the plants of the genus *Bridelia*; *B. verrucosa*, *B. stipularis* and *B. tomentosa* and *Erythrina*; *E. fusca* and *E. variegata* were screened by brine shrimp lethality bioassay for probable cytotoxic activity. In this bioassay, the crude extract and fraction showed varying degrees of lethality indicating that the extractives and the compounds are biologically active. Each of the test samples showed different mortality rate at different concentrations. The mortality rate of brine shrimp was found to be increased with increase in concentration of the test samples and a plot of percent mortality versus log concentration on the graph paper produced an approximate linear correlation between them. It is evident that all the test samples were lethal to brine shrimp nauplii.

Table 5.2 gives the results of the brine shrimp lethality after 24 hour exposure to the positive control, vincristine sulfate. The positive control, compared with the negative control (sea water) was lethal, showing significant mortality to the shrimp.

The LC_{50} and LC_{90} values of *n*-hexane, carbon tetrachloride, chloroform, soluble fraction and methanolic extract of both the stem bark and leaf of *B. verrucosa*, *B. stipularis*, *B. tomentosa*, *E. fusca* and *E. variegata* along with the pure compounds isolated from the investigated plants together with the positive control are shown in Table 5.3-5.15 and graphs are in Figures 5.1-5.60. However, varying degree of lethality to *Artemia salina* was observed with exposure to different dose levels of the test samples. The degree of lethality was directly proportional to the concentration of the extracts ranging from the lowest concentration (0.78125 µg/ml) to the highest concentration (400 µg/ml). Maximum mortalities took place at a concentration of 400 µg/ml, whereas least mortalities were at 0.78125 µg/ml concentration. In other words, mortality increased gradually with the increase in concentration of the test samples.

From the results of the brine shrimp lethality bioassay it can be well predicted that the crude extract and partitionate fraction possess bioactive principles and have considerable cytotoxic potency. Comparison with positive control vincristine sulfate signifies that cytotoxicity exhibited by the crude extracts and further bioactivity guided investigation can be done to find out potent antitumor and pesticidal compounds.

Table 5.3: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the stem bark of *B. verrucosa* on brine shrimp nauplii.

***Bridelia verrucosa* (Stem bark)**											
Conc. (C) (µg/ml)	**Log C**	**% Mortality**					**LC_{50}, LC_{90} (µg/ml)**				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
400	2.602	100	100	100	100	100	6.3 ± 0.25, 170.0± 1.33	5.1 ± 0.95, 72.4± 0.97	3.1 ± 0.62, 204.2± 0.75	0.71± 0.14, 43.7± 0.95	7.08± 0.44, 97.7± 1.59
200	2.301	90	100	90	100	100					
100	2.000	90	100	80	100	90					
50	1.699	70	90	70	100	80					
25	1.398	70	80	70	90	80					
12.5	1.097	50	70	60	80	70					
6.25	0.797	40	50	60	70	40					
3.125	0.495	40	40	50	70	40					
1.563	0.194	30	30	50	50	20					
0.781	-0.107	30	20	40	40	10					

In case of *B. verrucosa*, the crude methanol extract of the stem bark as well as its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fraction were subjected to brine shrimp lethality bioassay by using the method of Meyer *et al*., 1982. The LC_{50} values were found to be 6.3, 5.1, 3.1, 0.71 and 7.08 µg/ml and LC_{90} values were found to

be 170.0, 72.4, 204.2, 43.7 and 97.7 µg/ml (Table 5.3), respectively. The positive control drug, vincristine sulfate showed LC_{50} at 0.45 µg/ml and LC_{90} at 10.0 µg/ml (Table 5.2). The chloroform soluble fraction of methanol extract of the stem bark demonstrated strong cytotoxic activity, while the other three partitionates along with the methanolic extract showed low to moderate cytotoxic activity.

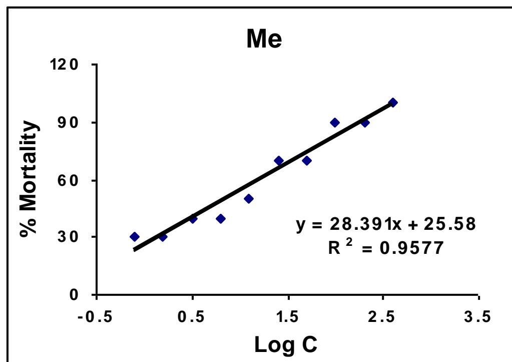

Figure 5.2: Effect of crude methanol extract of the stem bark of *B. verrucosa* on brine shrimp nauplii.

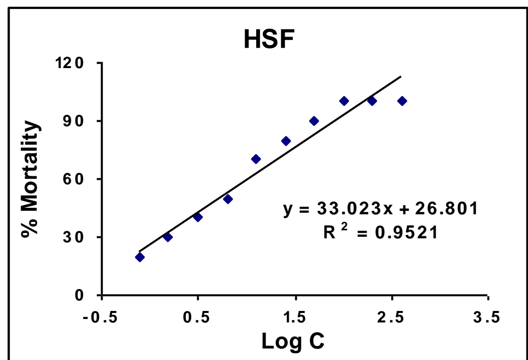

Figure 5.3: Effect of n-hexane soluble fraction of the stem bark of *B. verrucosa* on brine shrimp nauplii.

CSF

% Mortality

Log C

y = 20.338x + 41.627

R 2 = 0.963

Figure 5.4: Effect of CCl_4 soluble fraction of the stem bark of *B. verrucosa* on brine shrimp nauplii.

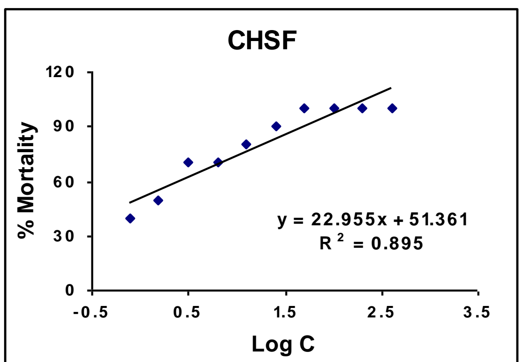

Figure 5.5: Effect of chloroform soluble fraction of the stem bark of *B. verrucosa* on brine shrimp nauplii.

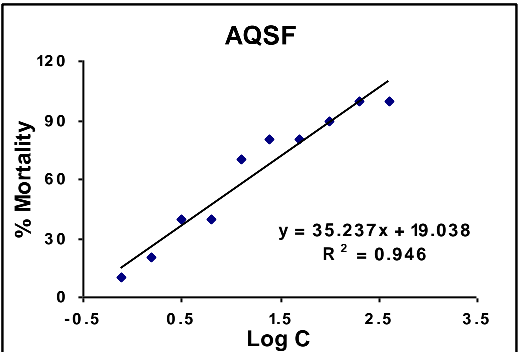

Figure 5.6: Effect of aqueous soluble fraction of the stem bark of *B. verrucosa* on brine shrimp nauplii.

Table 5.4: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the leaf of *B. verrucosa* on brine shrimp nauplii.

***Bridelia verrucosa* (Leaf)**											
Conc. (C) (µg/ml)	**Log C**	**% Mortality**					**LC_{50}, LC_{90} (µg/ml)**				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
400	2.602	100	100	100	100	100	2.51± 0.14 , 70.8± 2.35	8.13± 0.56, 134.9± 1.31	1.2± 0.40, 77.6± 1.18	3.13± 0.36, 58.9± 0.40	7.94± 0.36, 128.8± 0.52
200	2.301	100	100	100	100	90					
100	2.000	100	90	90	100	80					
50	1.699	100	80	80	100	80					
25	1.398	80	60	80	90	70					
12.5	1.097	60	50	70	70	70					
6.25	0.797	50	40	70	60	50					
3.125	0.495	50	30	60	50	40					
1.563	0.194	40	30	50	40	30					
0.781	-0.107	40	20	40	20	10					

Again, for brine shrimp lethality bioassay with *B. verrucosa* leaf, the chloroform soluble fraction gave strong cytotoxic activity with LC_{50} value of 1.2 µg/ml and LC_{90} value of 77.6 µg/ml. The crude methanolic extract and its carbon tetrachloride soluble fraction gave LC_{50} values of 2.51and 3.13 µg/ml and LC_{90} values of 70.8 and 58.9 µg/ml, respectively (Table 5.4) which exhibited moderate cytotoxic activity with comparison to the standard vincristine sulfate (LC_{50} value 0.45 µg/ml). The *n*-hexane and aqueous soluble fraction showed low cytotoxic activity with LC_{50} values of 8.13 and 7.94 µg/ml and LC_{90} values of 134.9 and 128.8 µg/ml, respectively.

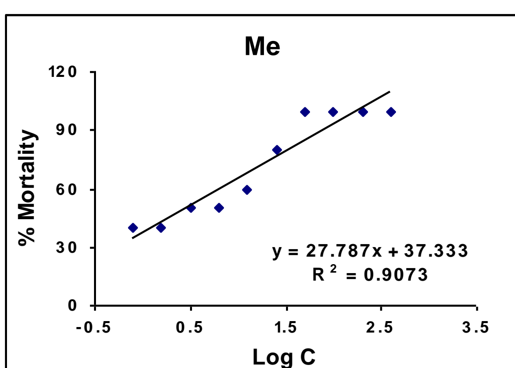

Figure 5.7: Effect of crude methanol extract of the leaf of *B. verrucosa* on brine shrimp nauplii.

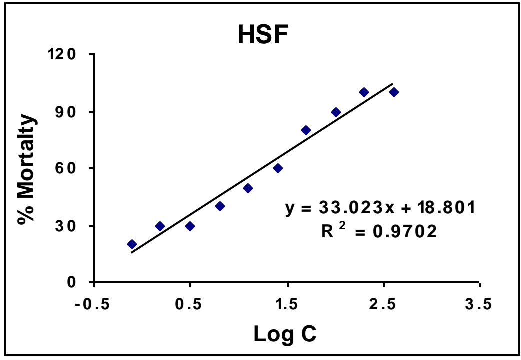

Figure 5.8: Effect of *n*-hexane soluble fraction of the leaf of *B. verrucosa* on brine shrimp nauplii.

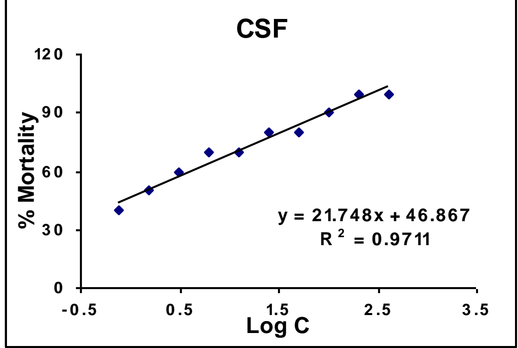

Figure 5.9: Effect of CCl_4 soluble fraction of the leaf of *B. verrucosa* on brine shrimp nauplii.

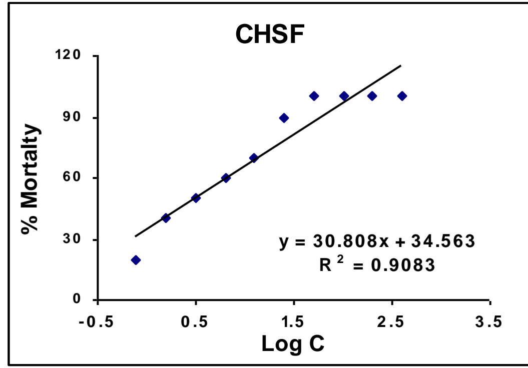

Figure 5.10: Effect of chloroform soluble fraction of the leaf of *B. verrucosa* on brine shrimp nauplii.

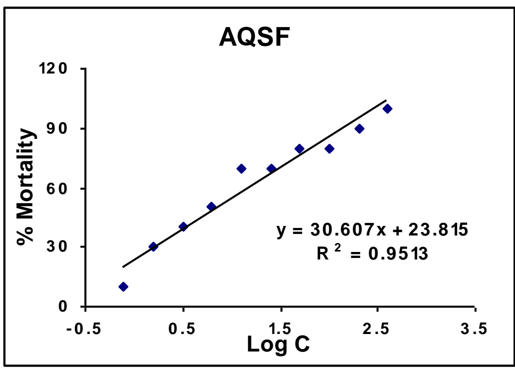

Figure 5.11: Effect of aqueous soluble fraction of the leaf of *B. verrucosa* on brine shrimp nauplii.

Table 5.5: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the stem bark of *B. stipularis* on brine shrimp nauplii.

***Bridelia stipularis* (Stem bark)**												
Conc. (C) (µg/ml)	**Log C**	**% Mortality**					**LC_{50} (µg/ml)**				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
400	2.602	100	100	100	100	100	8.51± 0.19, 199.5± 1.31	7.94 ± 0.43, 138.0± 1.49	4.47± 0.65, 131.8± 0.45	1.20± 0.51, 112.2± 2.2	4.70± 1.08, 70.8± 0.9
200	2.301	90	90	90	90	100					
100	2.000	90	90	90	90	80					
50	1.699	70	80	80	80	70					
25	1.398	70	60	70	70	70					
12.5	1.097	50	60	60	80	60					
6.25	0.797	40	50	50	70	50					
3.125	0.495	40	30	50	60	40					
1.563	0.194	30	30	40	50	40					
0.781	-0.107	30	10	30	40	20					

The lethality of the crude methanolic extract and its *n*-hexane, carbon tetrachloride and aqueous soluble fraction of the stem bark of *B. stipularis* gave LC_{50} values of 8.51, 7.94, 4.47 and 4.7 µg/ml and LC_{90} values of 199.5, 138.0, 131.8 and 70.8 µg/ml respectively (Table 5.5) which exhibited low cytotoxic activity with comparison to the standard vincristine sulfate (LC_{50} value 0.45 µg/ml). But the chloroform soluble fraction showed high cytotoxic activity with LC_{50} value of 1.2 µg/ml.

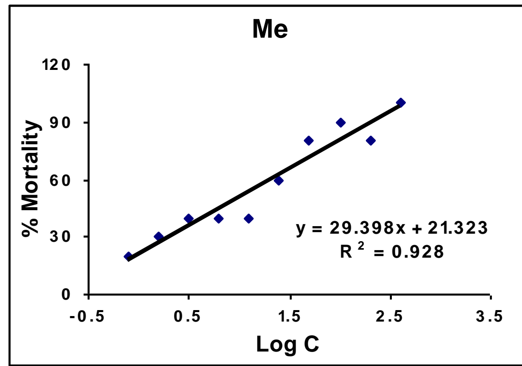

Figure 5.12: Effect of crude methanol extract of the stem bark of *B. stipularis* on brine shrimp nauplii.

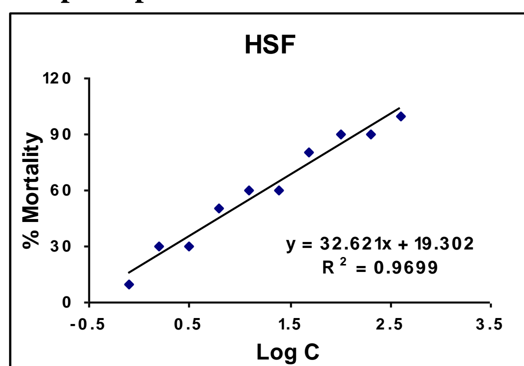

Figure 5.13: Effect of *n*-hexane soluble fraction of the stem bark of *B. stipularis* on brine shrimp nauplii.

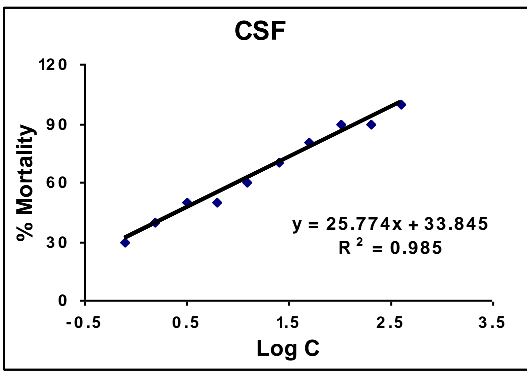

Figure 5.14: Effect of CCl_4 soluble fraction of the stem bark of *B. stipularis* on brine shrimp nauplii.

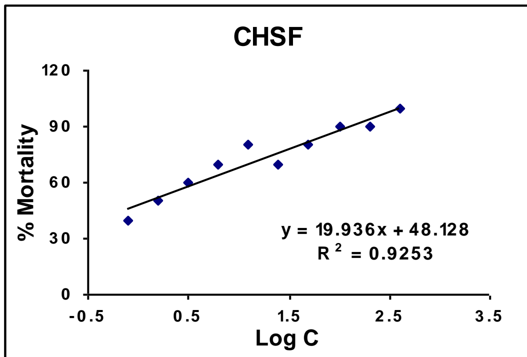

Figure 5.15: Effect of chloroform soluble fraction of the stem bark of *B. stipularis* on brine shrimp nauplii.

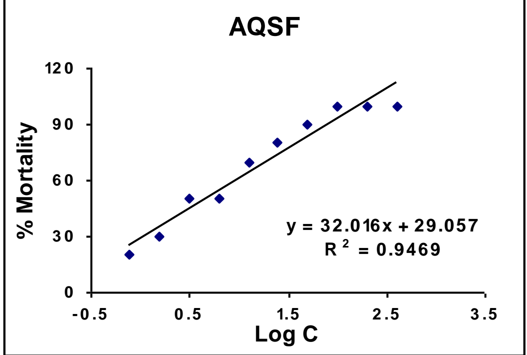

Figure 5.16: Effect of aqueous soluble fraction of the stem bark of *B. stipularis* on brine shrimp nauplii.

Table 5.6: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the leaf of *B. stipularis* on brine shrimp nauplii.

***Bridelia stipularis* (Leaf)**											
Conc. (C) (µg/ml)	**Log C**	**% Mortality**					**LC_{50}, LC_{90} (µg/ml)**				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
400	2.602	100	100	100	100	90	3.16± 0.18, 91.2± 1.36	6.31 ± 0.18, 100.0 ± 1.71	1.99± 0.25, 95.5± 1.36	3.98± 0.98, 102.3± 0.76	12.59 ± 1.3, -
200	2.301	100	100	90	90	70					
100	2.000	90	100	90	90	70					
50	1.699	80	90	80	90	60					
25	1.398	80	70	80	80	60					
12.5	1.097	70	50	70	70	50					
6.25	0.797	60	50	70	60	40					
3.125	0.495	60	50	70	50	30					
1.563	0.194	40	30	40	30	30					
0.781	-0.107	30	10	30	20	10					

On the other hand, in case of brine shrimp lethality bioassay with *B. stipularis* leaf, the crude methanolic extract and its *n*-hexane, carbon tetrachloride and chloroform soluble fraction gave LC_{50} values of 3.16, 6.31, 1.99 and 3.98 µg/ml and LC_{90} values of 91.2, 100.0, 95.5 and 102.3 µg/ml respectively (Table 5.6) which exhibited moderate cytotoxic activity with comparison to the standard vincristine sulfate (LC_{50} value 0.45 µg/ml). The aqueous soluble fraction showed low cytotoxic activity with LC_{50} value of 12.59 µg/ml.

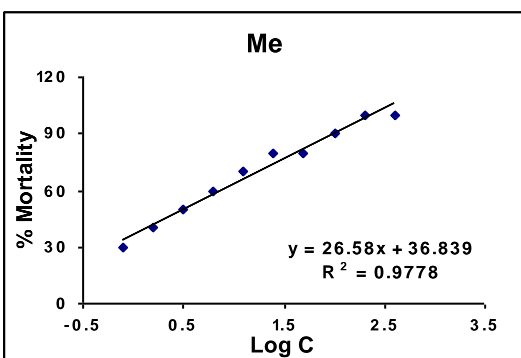

Figure 5.17: Effect of crude methanol extract of the leaf of *B. stipularis* on brine shrimp nauplii.

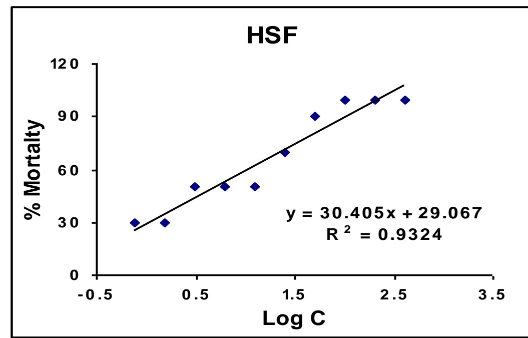

Figure 5.18: Effect of *n*-hexane soluble fraction of the leaf of *B. stipularis* on brine shrimp nauplii.

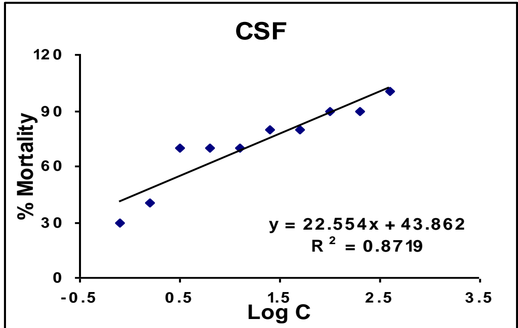

Figure 5.19: Effect of CCl_4 soluble fraction of the leaf of *B. stipularis* on brine shrimp nauplii.

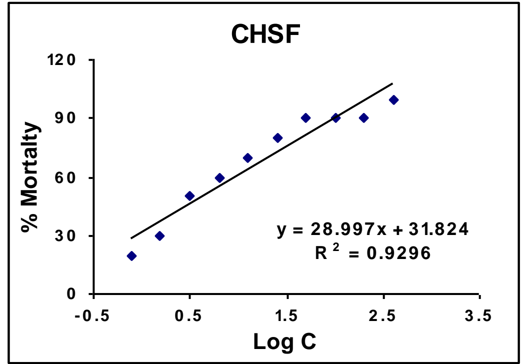

Figure 5.20: Effect of chloroform soluble fraction of the leaf of *B. stipularis* on brine shrimp nauplii.

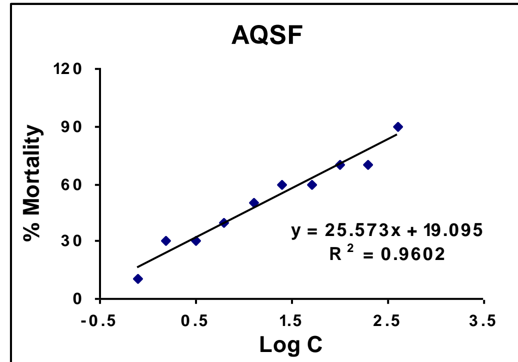

Figure 5.21: Effect of aqueous soluble fraction of the leaf of *B. stipularis* on brine shrimp nauplii.

Table 5.7: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the stem bark of *B. tomentosa* on brine shrimp nauplii.

***Bridelia tomentosa* (Stem bark)**											
Conc. (C) (µg/ml)	**Log C**	**% Mortality**					**LC_{50}, LC_{90} (µg/ml)**				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
400	2.602	100	100	100	100	100	12.02± 0.38, 87.1± 1.85	8.13± 0.36, 141.2± 0.65	7.08± 1.00, 117.5± 1.31	1.59± 0.22, 56.2± 0.23	4.47± 0.73, 112.2± 0.4
200	2.301	100	100	90	100	100					
100	2.000	80	100	90	100	90					
50	1.699	80	90	90	80	80					
25	1.398	50	80	70	90	70					
12.5	1.097	40	60	70	90	60					
6.25	0.797	40	60	30	70	50					
3.125	0.495	30	30	30	60	40					
1.563	0.194	20	20	30	50	30					
0.781	-0.107	10	20	20	30	20					

The brine shrimp lethality bioassay with *B. tomentosa* stem bark, the chloroform soluble fraction showed prominent cytotoxic activity with LC_{50} and LC_{90} values of 1.59 and 56.2 µg/ml, respectively. The crude methanolic extract and its *n*-hexane and carbon tetrachloride soluble fraction gave LC_{50} values of 12.02, 8.13 and 7.08 µg/ml and LC_{90} values of 87.1, 141.2, and 117.5 µg/ml, respectively (Table 5.7) which exhibited moderate cytotoxic activity with comparison to the standard vincristine sulfate (LC_{50} value 0.45 µg/ml). The aqueous soluble fraction showed reasonable cytotoxic activity with LC_{50} value of 4.47 µg/ml and LC_{90} value of 112.2 µg/ml.

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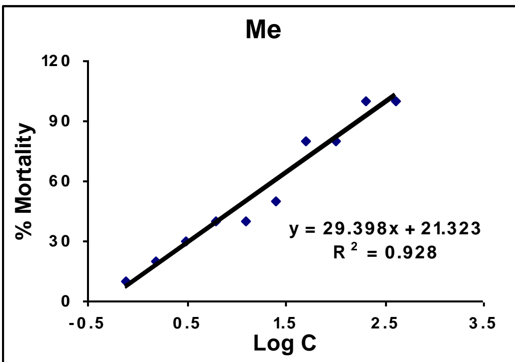

Figure 5.22: Effect of crude methanol extract of the stem bark of *B. tomentosa* on brine shrimp nauplii.

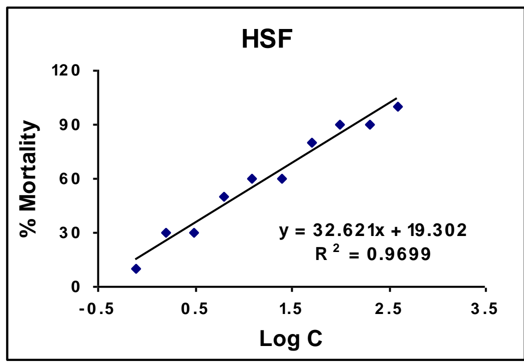

Figure 5.23: Effect of *n*-hexane soluble fraction of the stem bark of *B. tomentosa* on brine shrimp nauplii.

Figure 5.24: Effect of CCl_4 soluble fraction of the stem bark of *B. tomentosa* on brine shrimp nauplii.

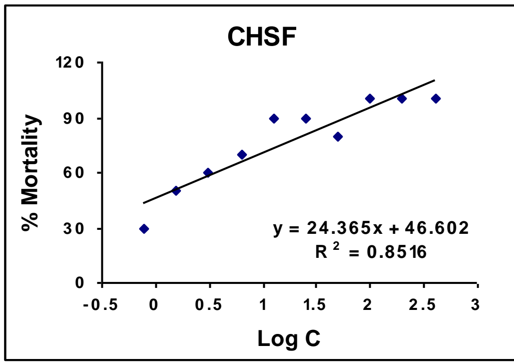

Figure 5.25: Effect of chloroform soluble fraction of the stem bark of *B. tomentosa* on brine shrimp nauplii.

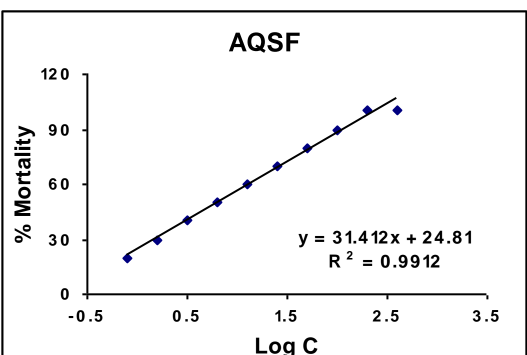

Figure 5.26: Effect of aqueous soluble fraction of the stem bark of *B. tomentosa* on brine shrimp nauplii.

Table 5.8: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the leaf of *B. tomentosa* on brine shrimp nauplii.

***Bridelia tomentosa* (Leaf)**												
Conc. (C) (µg/ml)	**Log C**	**% Mortality**					**LC_{50}, LC_{90} (µg/ml)**				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
400	2.602	100	100	100	100	100	5.75± 0.42, 102.3± 0.75	3.55± 0.58, 75.9± 1.93	11.22± 0.4, 128.8± 1.12	4.37± 0.78, 69.2± 1.24	8.51± 1.21, 107.2± 0.41
200	2.301	100	100	90	100	100					
100	2.000	80	100	90	100	90					
50	1.699	80	90	90	100	80					
25	1.398	80	80	80	80	70					
12.5	1.097	70	80	50	70	60					
6.25	0.797	60	70	30	50	50					
3.125	0.495	50	50	20	40	30					
1.563	0.194	30	30	10	30	30					
0.781	-0.107	10	10	10	20	10					

Yet again, brine shrimp lethality bioassay with *B. tomentosa* leaf, the crude methanolic extract and its *n*-hexane, and chloroform soluble fraction gave LC_{50} values of 5.75, 3.55 and 4.37 µg/ml and LC_{90} values of 102.3, 75.9 and 69.2 µg/ml, respectively (Table 5.8) which exhibited moderate cytotoxic activity with comparison to the standard vincristine sulfate (LC_{50} value 0.45 µg/ml). The carbon tetrachloride and aqueous soluble fraction showed low cytotoxic activity with LC_{50} value of 11.22 and 8.51 µg/ml and LC_{90} values of 128.8 and 107.2 µg/ml, respectively.

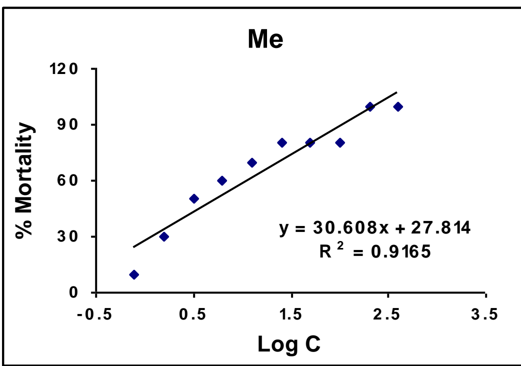

Figure 5.27: Effect of crude methanol extract of the leaf of *B. tomentosa* on brine shrimp nauplii.

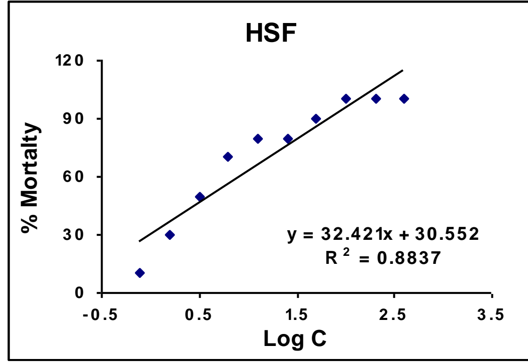

Figure 5.28: Effect of *n*-hexane soluble fraction of the leaf of *B. tomentosa* on brine shrimp nauplii.

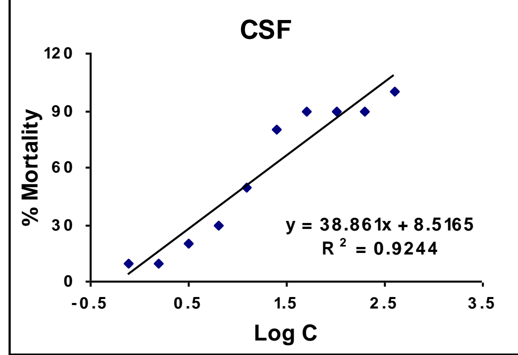

Figure 5.29: Effect of CCl_4 soluble fraction of the leaf of *B. tomentosa* on brine shrimp nauplii.

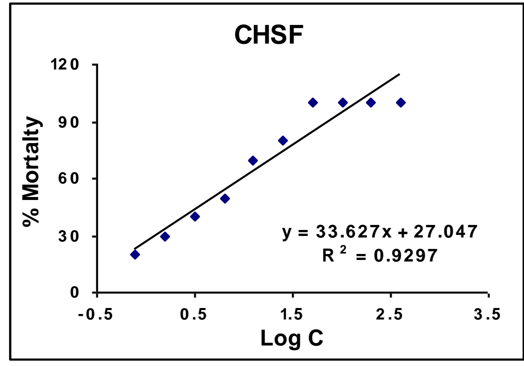

Figure 5.30: Effect of chloroform soluble fraction of the leaf of *B. tomentosa* on brine shrimp nauplii.

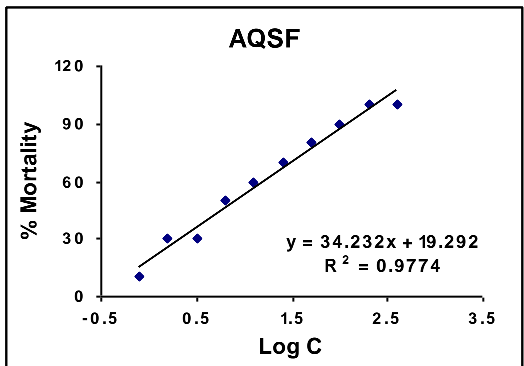

Figure 5.31: Effect of aqueous soluble fraction of the leaf of *B. tomentosa* on brine shrimp nauplii.

Table 5.9: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the stem bark of *E. fusca* on brine shrimp nauplii.

***Erythrina fusca* (Stem bark)**											
Conc. (C) (µg/ml)	**Log C**	**% Mortality**					**LC_{50}, LC_{90} (µg/ml)**				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
400	2.602	100	100	100	100	100	9.45± 0.45, 216.83 ± 0.43	4.96± 0.59, 98.66 ± 0.3	1.55± 0.3, 65.68 ± 0.23	4.79± 0.65, 103.31 ± 0.49	8.95± 0.70, 136.28 ± 0.47
200	2.301	70	100	100	90	100					
100	2.000	90	100	90	90	90					
50	1.699	80	80	90	80	70					
25	1.398	80	70	80	80	70					
12.5	1.097	70	70	90	70	50					
6.25	0.797	40	50	70	70	50					
3.125	0.49	40	40	70	60	30					

	5										
1.563	0.194	40	40	50	20	30					
0.781	-0.107	30	20	20	10	10					

The brine shrimp lethality bioassay with *E. fusca* stem bark, the carbon tetrachloride soluble fraction showed prominent cytotoxic activity with LC_{50} and LC_{90} values of 1.55 and 65.68 µg/ml. The *n*-hexane and chloroform soluble fraction gave LC_{50} values of 4.96 and 4.79 µg/ml and LC_{90} values of 98.66, and 103.31 µg/ml, respectively (Table 5.9) which exhibited moderate cytotoxic activity with comparison to the standard vincristine sulfate (LC_{50} value 0.45 µg/ml). The crude methanolic extract and aqueous soluble fraction showed reasonable cytotoxic activity with LC_{50} value of 9.45 and 8.95 µg/ml, respectively and LC_{90} value of 216.83 and 136.28 µg/ml, respectively.

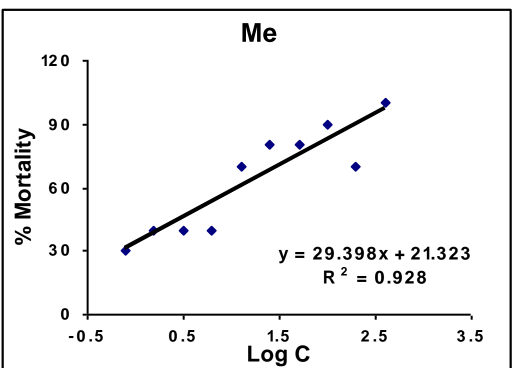

Figure 5.32: Effect of crude methanol extract of the stem bark of *E. fusca* on brine shrimp nauplii.

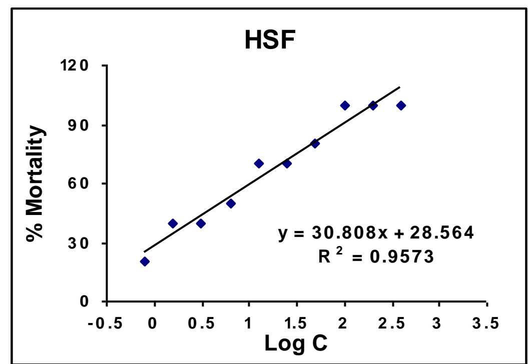

Figure 5.33: Effect of *n*-hexane soluble fraction of the stem bark of *E. fusca* on brine shrimp nauplii.

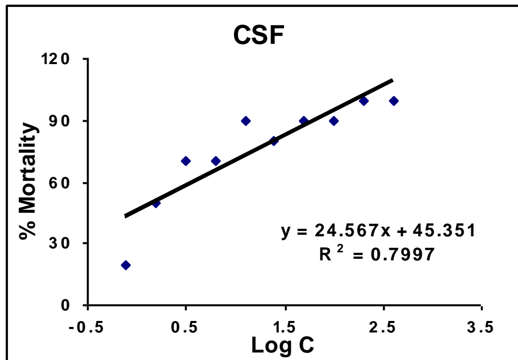

Figure 5.34: Effect of CCl_4 soluble fraction of the stem bark of *E. fusca* on brine shrimp nauplii.

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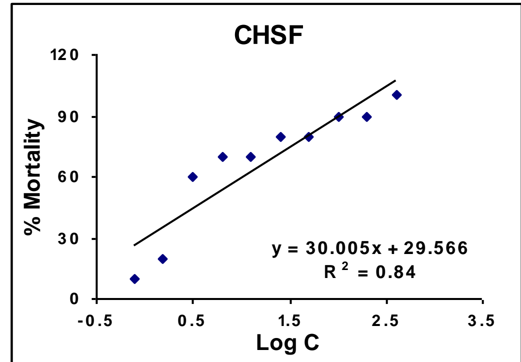

Figure 5.35: Effect of chloroform soluble fraction of the stem bark of *E. fusca* on brine shrimp nauplii.

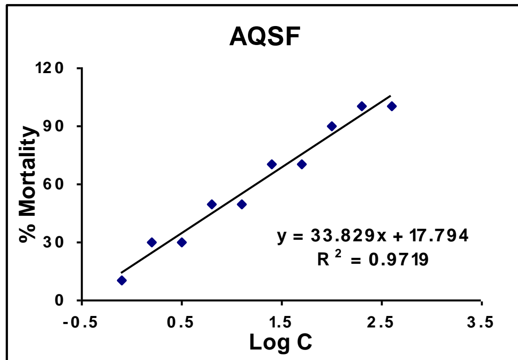

Figure 5.36: Effect of aqueous soluble fraction of the stem bark of *E. fusca* on brine shrimp nauplii.

Table 5.10: Effect of methanol extract (Me), n-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the leaf of *E. fusca* on brine shrimp nauplii.

<table>
<tr><th colspan="12">Erythrina fusca (Leaf)</th></tr>
<tr><th rowspan="2">Conc. (C) (µg/ml)</th><th rowspan="2">Log C</th><th colspan="5">% Mortality</th><th colspan="5">LC_{50}, LC_{90} (µg/ml)</th></tr>
<tr><th>Me</th><th>HSF</th><th>CSF</th><th>CHSF</th><th>AQSF</th><th>Me</th><th>HSF</th><th>CSF</th><th>CHSF</th><th>AQSF</th></tr>
<tr><td>400</td><td>2.602</td><td>100</td><td>100</td><td>100</td><td>100</td><td>100</td><td rowspan="10">7.34± 0.40, 137.7± 0.08</td><td rowspan="10">7.96± 0.19, 113.7± 0.38</td><td rowspan="10">6.82± 0.67, 163.4± 0.22</td><td rowspan="10">7.63± 0.16, 220.3± 0.33</td><td rowspan="10">7.89± 0.52, 116.2± 0.69</td></tr>
<tr><td>200</td><td>2.301</td><td>100</td><td>100</td><td>90</td><td>80</td><td>100</td></tr>
<tr><td>100</td><td>2.000</td><td>80</td><td>90</td><td>80</td><td>80</td><td>90</td></tr>
<tr><td>50</td><td>1.699</td><td>80</td><td>70</td><td>80</td><td>70</td><td>80</td></tr>
<tr><td>25</td><td>1.398</td><td>70</td><td>80</td><td>70</td><td>70</td><td>70</td></tr>
<tr><td>12.5</td><td>1.097</td><td>50</td><td>60</td><td>60</td><td>50</td><td>60</td></tr>
<tr><td>6.25</td><td>0.797</td><td>50</td><td>60</td><td>50</td><td>50</td><td>50</td></tr>
<tr><td>3.125</td><td>0.495</td><td>50</td><td>30</td><td>40</td><td>60</td><td>30</td></tr>
<tr><td>1.563</td><td>0.194</td><td>30</td><td>20</td><td>30</td><td>40</td><td>30</td></tr>
<tr><td>0.781</td><td>-0.107</td><td>10</td><td>10</td><td>20</td><td>0</td><td>10</td></tr>
</table>

Again, brine shrimp lethality bioassay with *E. fusca* leaf, the crude methanolic extract and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fraction gave LC_{50} values of 7.34, 7.96, 6.82, 7.63 and 7.89 µg/ml and LC_{90} values of 137.70, 113.77, 163.41, 220.35 and 116.29, respectively (Table 5.10) which exhibited low cytotoxic activity with comparison to the standard vincristine sulfate (LC_{50} value 0.45 µg/ml).

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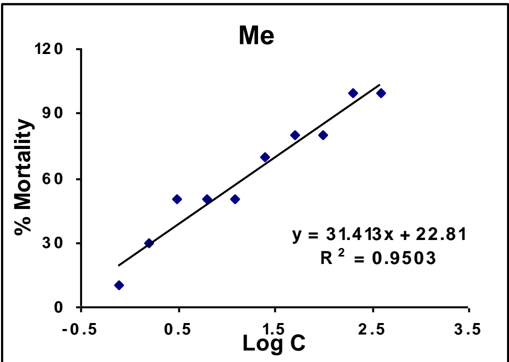

Figure 5.37: Effect of crude methanol extract of the leaf of *E. fusca* on brine shrimp nauplii.

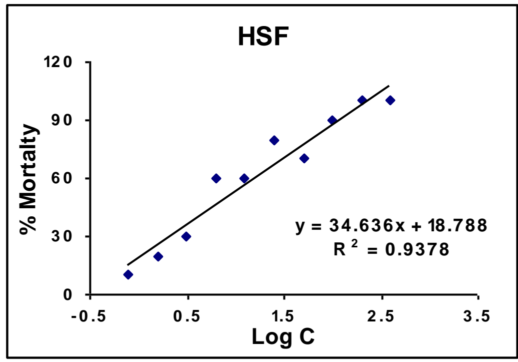

Figure 5.38: Effect of *n*-hexane soluble fraction of the leaf of *E. fusca* on brine shrimp nauplii.

Figure 5.39: Effect of CCl_4 soluble fraction of the leaf of *E. fusca* on brine shrimp nauplii.

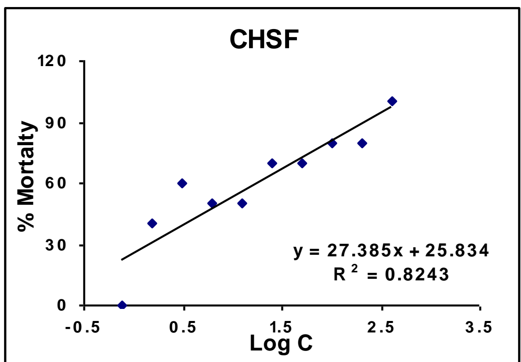

Figure 5.40: Effect of chloroform soluble fraction of the leaf of *E. fusca* on brine shrimp nauplii.

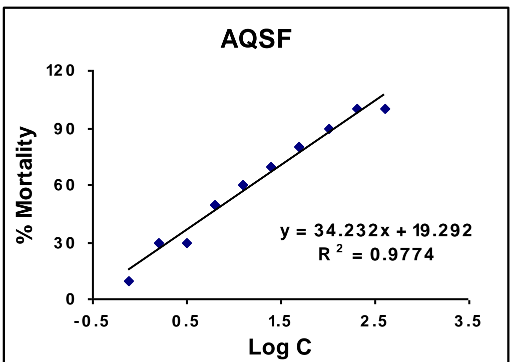

Figure 5.41: Effect of aqueous soluble fraction of the leaf of *E. fusca* on brine shrimp nauplii.

Table 5.11: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the stem bark of *E. variegata* on brine shrimp naupli.

Erythrina variegata **(Stem bark)**											
Conc. (C) (µg/ml)	**Log C**	**% Mortality**					**LC_{50}, LC_{90} (µg/ml)**				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
400	2.602	100	100	100	90	100	9.98± 0.14, 174.1± 0.86	14.66± 0.75, 178.5± 0.7	7.86± 0.45, 150.2± 1.19	12.87± 0.99, 308.4± 0.46	5.13± 0.21, 94.42± 0.63
200	2.301	90	90	100	80	100					
100	2.000	80	90	80	80	90					
50	1.699	70	70	70	70	80					
25	1.398	80	60	70	50	80					
12.5	1.097	50	50	60	60	70					
6.25	0.797	40	30	40	40	60					
3.125	0.495	30	10	50	40	50					
1.563	0.194	30	20	30	30	30					
0.781	-0.107	10	10	10	0	10					

The lethality of the crude methanolic extract and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fraction of the stem bark of *E. variegata* gave LC_{50} values of 9.98, 14.66, 7.86, 12.87 and 5.13 µg/ml and LC_{90} values of 174.13, 178.56, 150.25, 308.42 and 94.42 µg/ml respectively (Table 5.11) which exhibited low cytotoxic activity with comparison to the standard vincristine sulfate (LC_{50} value 0.45 µg/ml).

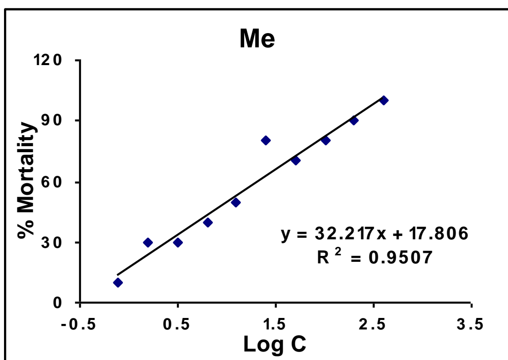

Figure 5.42: Effect of crude methanol extract of the stem bark of *E. variegata* on brine shrimp nauplii.

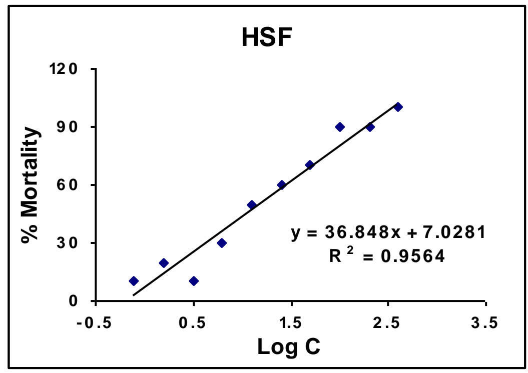

Figure 5.43: Effect of *n*-hexane soluble fraction of the stem bark of *E. variegata* on brine shrimp nauplii.

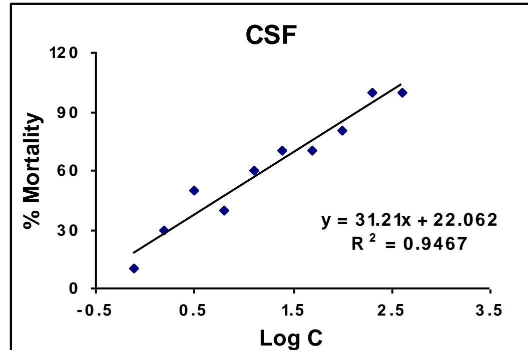

Figure 5.44: Effect of CCl_4 soluble fraction of the stem bark of *E. variegata* on brine shrimp nauplii.

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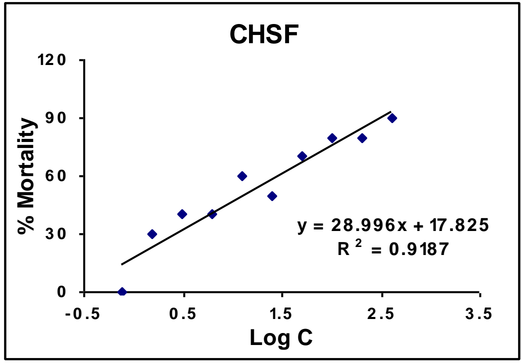

Figure 5.45: Effect of chloroform soluble fraction of the stem bark of *E. variegata* on brine shrimp nauplii.

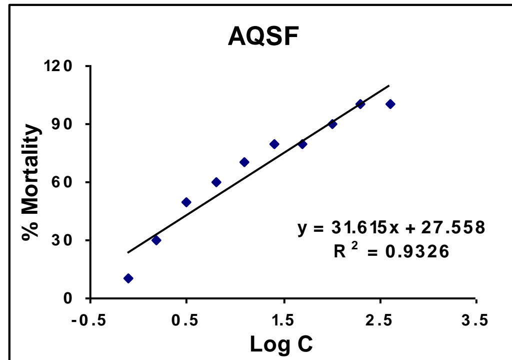

Figure 5.46: Effect of aqueous soluble fraction of the stem bark of *E. variegata* on brine shrimp nauplii.

Table 5.12: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the leaf of *E. variegata* on brine shrimp nauplii.

Erythrina variegata **(Leaf)**												
Conc. (C) (µg/ml)	**Log C**	**% Mortality**					**LC_{50}, LC_{90} (µg/ml)**				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
400	2.602	100	100	100	100	100	6.61± 0.41, 136.6± 0.52	4.89± 0.52, 85.19± 0.50	10.81± 0.92, 126.4± 0.74	4.40± 0.30, 96.79 ± 1.23	7.66± 0.41, 124.5± 0.32
200	2.301	90	100	90	100	90					
100	2.000	80	90	90	90	90					
50	1.699	80	90	80	90	80					
25	1.398	80	80	80	80	70					
12.5	1.097	70	70	60	60	70					
6.25	0.797	50	60	40	50	50					
3.125	0.495	40	50	20	50	40					
1.563	0.194	20	30	10	40	20					
0.781	-0.107	20	10	10	20	10					

On the other hand, in case of brine shrimp lethality bioassay with *E. variegata* leaf, the crude methanolic extract and its *n*-hexane, chloroform and aqueous soluble fraction gave LC_{50} values of 6.61, 4.89, 4.40 and 7.66 µg/ml and LC_{90} values of 136.65, 85.19, 96.79 and 124.58 µg/ml respectively (Table 5.12) which exhibited low cytotoxic activity with comparison to the standard vincristine sulfate (LC_{50} value 0.45 µg/ml). The carbon tetrachloride soluble fraction showed very low cytotoxic activity with LC_{50} and LC_{90} value of 10.81 and 126.47 µg/ml.

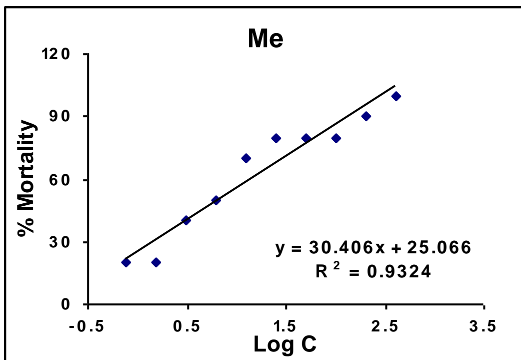

Figure 5.47: Effect of crude methanol extract of the leaf of *E. variegata* on brine shrimp nauplii.

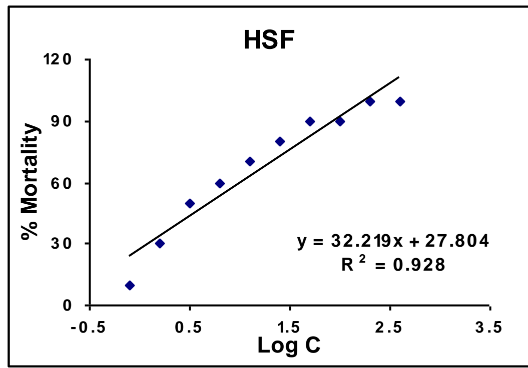

Figure 5.48: Effect of *n*-hexane soluble fraction of the leaf of *E. variegata* on brine shrimp nauplii.

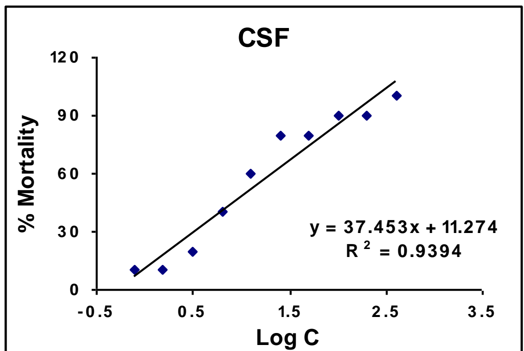

Figure 5.49: Effect of CCl_4 soluble fraction of the leaf of *E. variegata* on brine shrimp nauplii.

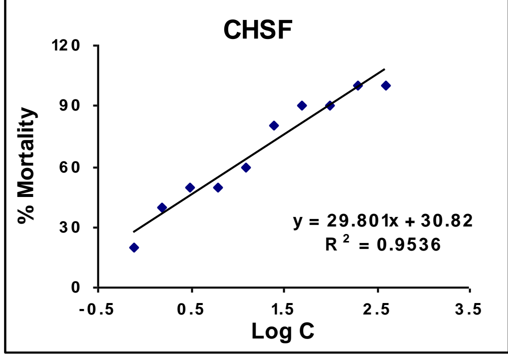

Figure 5.50: Effect of chloroform soluble fraction of the leaf of *E. variegata* on brine shrimp nauplii.

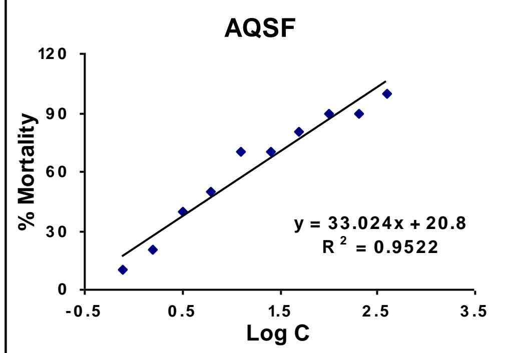

Figure 5.51: Effect of aqueous soluble fraction of the leaf of *E. variegata* on brine shrimp nauplii.

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Table 5.13: Effect of pure compounds BVSP-4 (48), BVBC-2 (51) and BVS-65 (54) isolated from the stem bark of *B. verrucosa* on brine shrimp nauplii.

***Bridelia verrucosa* (Stem bark)**							
Conc. (C) (µg/ml)	**Log C**	**% Mortality**			**LC_{50}, LC_{90} (µg/ml)**		
		BVSP-4 **(48)**	BVBC-2 **(51)**	BVS-65 **(54)**	BVSP-4 **(48)**	BVBC-2 **(51)**	BVS-65 **(54)**
400	2.602	100	100	100	13.80, 213.79	8.11, 109.14	10.12, 164.82
200	2.301	90	100	100			
100	2.000	80	90	80			
50	1.699	70	80	70			
25	1.398	60	80	60			
12.5	1.097	40	60	60			
6.25	0.797	30	40	40			
3.125	0.495	30	40	30			
1.563	0.194	20	20	30			
0.781	-0.107	10	10	10			

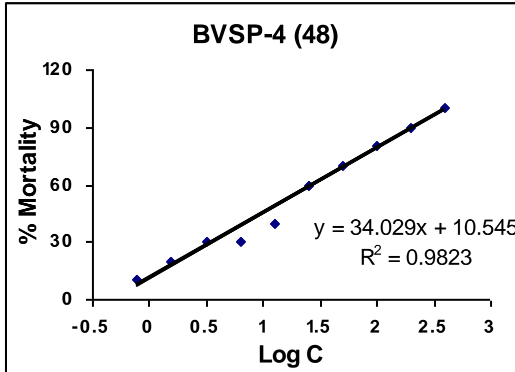

Figure 5.52: Effect of BVSP-4 (48) from the stem bark of *B. verrucosa* on brine shrimp nauplii.

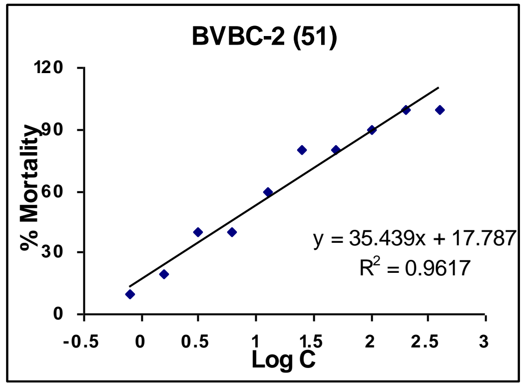

Figure 5.53: Effect of BVBC-2 (51) from the stem bark of *B. verrucosa* on brine shrimp nauplii.

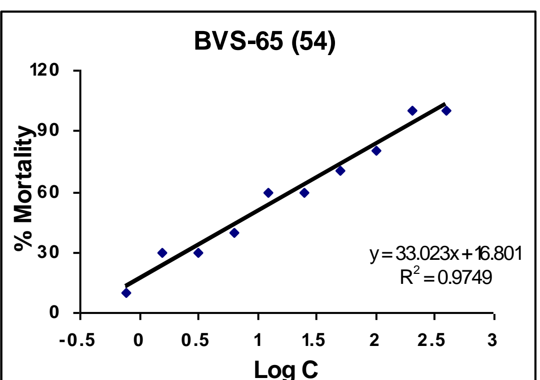

Figure 5.54: Effect of BVS-65 (54) from the stem bark of *B. verrucosa* on brine shrimp nauplii.

Table 5.14: Effect of pure compounds BS-01 (55), BS-06 (56) and BT-6 (59) isolated from the stem bark of *B. stipularis* and *B. tomentosa* on brine shrimp nauplii.

Bridelia stipularis and *B. tomentosa* (Stem bark)							
Conc. (C) (μg/ml)	Log C	% Mortality			LC_{50}, LC_{90} (μg/ml)		
		BS-01 (**55**)	BS-06 (**56**)	BT-6 (**59**)	BS-01 (**55**)	BS-06 (**56**)	BT-6 (**59**)
400	2.602	100	90	100	7.64, 101.16	8.53, 218.27	7.71, 99.31
200	2.301	100	90	100			
100	2.000	90	80	90			
50	1.699	80	70	90			
25	1.398	80	70	80			
12.5	1.097	70	60	60			
6.25	0.797	50	50	50			
3.125	0.495	30	40	20			
1.563	0.194	20	30	30			
0.781	-0.107	10	10	10			

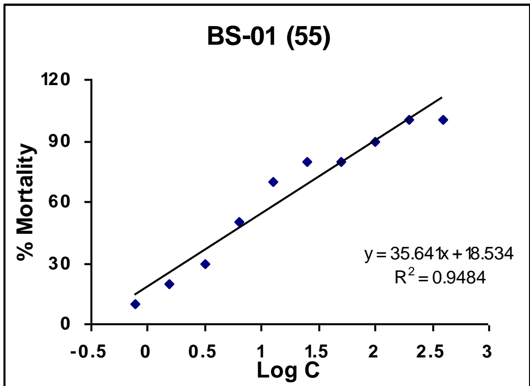

Figure 5.55: Effect of BS-01 (55) from the stem bark of *B. stipularis* on brine shrimp nauplii.

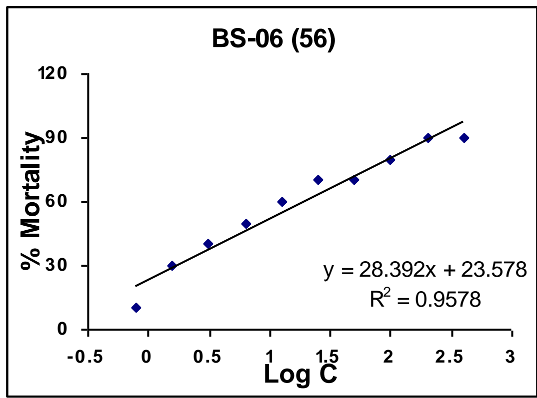

Figure 5.56: Effect of BS-06 (56) from the stem bark of *B. verrucosa* on brine shrimp nauplii.

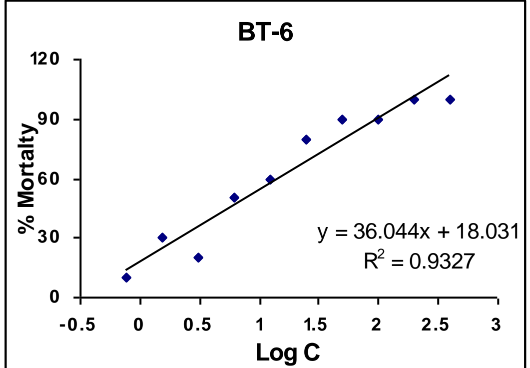

Figure 5.57: Effect of BT-6 (59) from the stem bark of *B. verrucosa* on brine shrimp nauplii.

Table 5.15: Effect of pure compounds EF-7 (62), EF-8 (63) and EF-12 (64) isolated from the stem bark of *E. fusca* on brine shrimp nauplii.

Erythrina fusca (Stem bark)							
Conc. (C) (µg/ml)	**Log C**	**% Mortality**			**LC_{50}, LC_{90} (µg/ml)**		
		EF-7 (**62**)	EF-8 (**63**)	EF-12 (**64**)	EF-7 (**62**)	EF-8 (**63**)	EF-12 (**64**)
400	2.602	100	100	100	4.70, 129.12	3.17, 62.95	4.81, 86.90
200	2.301	90	90	100			
100	2.000	90	90	90			
50	1.699	70	90	90			
25	1.398	80	80	80			
12.5	1.097	60	70	70			
6.25	0.797	70	40	60			
3.125	0.495	50	40	50			
1.563	0.194	40	30	20			
0.781	-0.107	10	10	20			

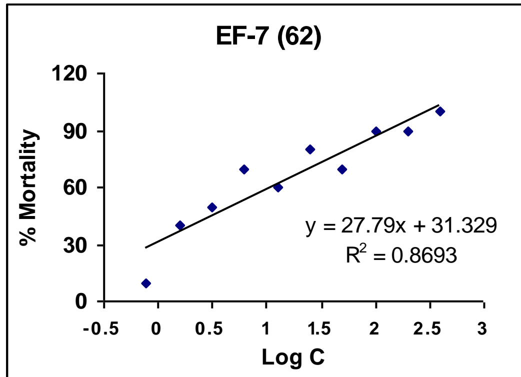

Figure 5.58: Effect of EF-7 (62) from the stem bark of *E. fusca* on brine shrimp nauplii.

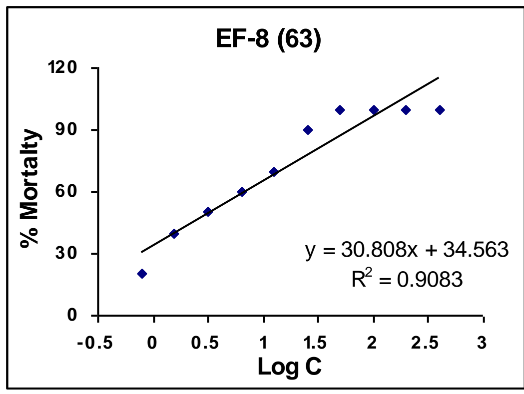

Figure 5.59: Effect of EF-8 (63) from the stem bark of *E. fusca* on brine shrimp nauplii.

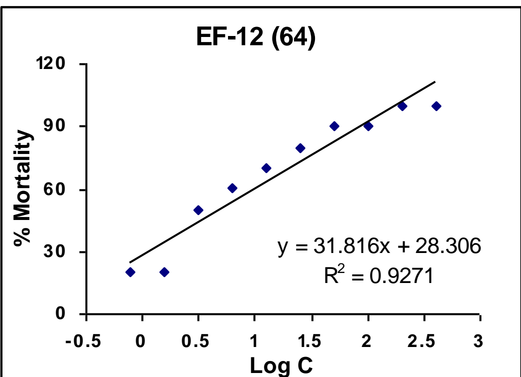

Figure 5.60: Effect of EF-12 (64) from the stem bark of *E. fusca* on brine shrimp nauplii.

The brine shrimp lethality bioassay with pure compounds showed moderate to mild cytotoxic activity. The LC_{50} and LC_{90} values of the pure compound EF-8 (**62**) isolated from the stem bark of *Erythrina fusca* gave promising LC_{50} and LC_{90} values of 3.17 and 62.95 μg/ml, respectively. The other isolated pure compounds BVSP-4 (**48**), BVBC-2 (**51**), BVS-65 (**54**), BS-01 (**55**), BS-06 (**56**) and BT-6 (**59**) gave LC_{50} values of 13.8, 8.11, 10.12, 7.64, 8.53 and 7.71 μg/ml and LC_{90} values of 213.79, 109.14, 164.82, 101.16, 218.27 and 99.31 μg/ml, respectively (Table 5.13 and 5.14) which exhibited moderate cytotoxic activity with comparison to the standard vincristine sulfate (LC_{50} value 0.45 μg/ml). The other two pure compounds EF-7 (**61**) and EF-12 (**63**) showed reasonable cytotoxic activity with LC_{50} values of 4.70 and 4.81 μg/ml and LC_{90} value of 129.12 and 86.90 μg/ml, respectively (Table 5.15).

5.2.1 Statistical Analysis

Three replicates of each sample were used for statistical analysis and the values are reported as mean ± SD.

5.2.2 Conclusion

The crude extracts of the plants, *B. verrucosa*, *B. stipularis, B. tomentosa*, *E. fusca* and *E. variegata* and their Kupchan fractions and some purified compounds were screened by brine shrimp lethality bioassay for probable cytotoxic activity. In this bioassay, the crude extract and compounds showed lethality indicating that the compounds are biologically active. Each of the test samples showed different mortality rate at different concentrations. The mortality rate of brine shrimp was found to be increased with increase in concentration of the test samples and a plot of percent mortality versus log concentration on the graph paper produced an approximate linear correlation between them. It is evident that all the test samples were lethal to brine shrimp nauplii.

From the results of the brine shrimp lethality bioassay and comparison with positive control vincristine sulfate signifies that cy./totoxicity exhibited by the crude extracts and further bioactivity guided investigation can be done to find out potent antitumor and pesticidal compound. However, this can not be confirmed without further studies and specific tests.

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6.1 Assays for total phenolic contents

6.1.1 Principle

Total phenolic content of *B. verrucosa*, *B. stipularis*, *B. tomentosa*, *E. fusca* and *E. variegata* extractives were measured employing the method as described by Skerget *et al*., 2005 involving Folin-Ciocalteu reagent as oxidizing agent and gallic acid as standard (Majhenic *et al*., 2007).

6.1.2 Standard curve preparation

Gallic acid (standard) solution of different concentration ranging from 100 µg/ ml to 0 µg / ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of Na_2CO_3 (7.5% w/v) solution was added to 0.5 ml of gallic acid solution. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 760 nm. A graph was constructed by plotting the absorbance in ordinate against the concentration in abscissa where a linear relationship was obtained which was used as a standard curve for determination of the total phenolic content of the samples.

6.1.3 Sample preparation

1 mg of each of the extractives was taken and dissolved in the distilled water to get a sample concentration of 250 µg/ml in every case.

6.2 Antioxidant activity evaluation by DPPH method

6.2.1 Principle

The free radical scavenging activity (antioxidant capacity) of the plant extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was estimated by the method developed by Brand-Williams *et al.,* 1995.

6.3 Total antioxidant assay by phosphomolybdenum method

6.3.1 Principle

The phosphobolybdenum method was based on the reduction of molybdenum, Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate-Mo (V) complex with a maximal absorption at 695 nm. As it being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extracts (Prieto *et al.*, 1999).

6.4 Results and discussions

6.4.1 Total phenolic content (TPC)

The methanolic extract of the stem bark and the leaf of the three *Bridelia* species, *B. verrucosa*, *B. stipularis*, *B. tomentosa* and also two *Erythrina* species, *E. fusca* and *E. variegata* along with their different partitionates i.e. *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials were subjected to total phenolic content determination. Based on the absorbance values of the various test solutions, reacted with Folin-Ciocalteu reagent and compared with the standard solutions of gallic acid (Table 6.1), results of the colorimetric analysis of the total phenolics are given in Table 6.2 and 6.3 Total phenolic content of the samples are expressed as mg of GAE (gallic acid equivalent)/100 g of extractives.

Table 6.1: Standard curve preparation by using gallic acid.

SL. No.	Conc. Of the standard (µg/ml)	Absorbance	Regression line	R^2
1	100	1.620	y = 0.0162x + 0.0215	0.9985
2	50	0.866		
3	25	0.450		
4	12.5	0.253		
5	6.25	0.120		
6	3.125	0.059		
7	1.5625	0.034		
8	0.78125	0.022		
9	0.3906	0.020		
10	0	0.011		

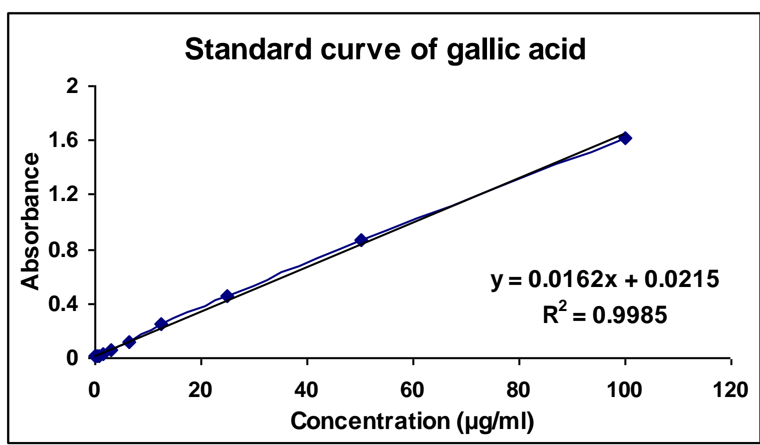

Figure 6.1: Standard curve of gallic acid for total phenolic content determination.

Table 6.2: Results of the test samples for total phenolic content determination of *Bridelia* species.

Sample	Conc. (µg/ml)	Mean Absorbance	Gallic acid equivalent (mg of GAE/100 g of dried extract)
B. verrucosa (Stem bark)			
Me	250	0.069±0.004	2.95±0.22
HSF	250	0.98±0.008	59.15±0.46
CSF	250	1.043±0.011	63.03±0.65
CHSF	250	1.125±0.098	68.14±6.02
AQSF	250	1.174±0.010	89.14±0.62
B. verrucosa (Leaf)			
Me	250	0.144±0.013	7.54±0.77
HSF	250	1.045±0.006	63.18±0.37
CSF	250	0.763±0.003	45.79±0.16
CHSF	250	0.074±0.011	3.26±0.65
AQSF	250	1.328±0.012	80.63±0.74
B. stipularis (Stem bark)			
Me	250	0.324±0.030	18.67±1.84
HSF	250	1.059±0.032	63.90±1.99
CSF	250	0.282±0.020	16.08±1.22
CHSF	250	0.349±0.018	20.22±1.11
AQSF	250	1.424±0.009	86.59±0.58
B. stipularis (Leaf)			
Me	250	0.562±0.022	33.38±1.33
HSF	250	0.764±0.013	45.85±0.77
CSF	250	0.843±0.018	50.71±1.13
CHSF	250	1.432±0.204	87.31±0.59
AQSF	250	1.621±0.021	98.76±1.27
B. tomentosa (Stem bark)			
Me	250	0.517±0.011	30.59±0.68
HSF	250	0.813±0.013	48.86±0.81
CSF	250	1.259±0.068	76.39±4.20
CHSF	250	1.233±0.265	74.78±1.33
AQSF	250	1.574±0.041	95.81±1.54
B. tomentosa (Leaf)			
Me	250	0.437±0.016	25.65±0.99
HSF	250	0.752±0.012	45.09 ±0.71
CSF	250	0.637±0.004	38.01±0.23
CHSF	250	0.426±0.012	24.97±0.74
AQSF	250	1.432±0.020	87.05±1.24

Values are expressed as average±SD (n=3); Me = Methanolic extract; HSF = *n*-hexane soluble fraction; CSF = carbon tetrachloride soluble fraction; CHSF = chloroform soluble fraction and AQSF = aqueous soluble fraction.

The amount of total phenolic content was found differ in different extractives compared with gallic acid equivalent. The amount of total phenolic content in case of the three *Bridelia* species varied for different partitionates ranging from 2.95 mg to 98.76 mg of GAE/100 g of dried extract. The highest total phenolic content was found from aqueous soluble fraction of the leaf of *B. stipularis* (98.76 mg of GAE/100 g of dried extract) and

the lowest from the stem bark of the methanolic extract of *B. verrucosa* (2.95 mg of GAE/100 g of dried extract).

Table 6.3: Results of the test samples for total phenolic content determination *Erythrina* species.

Sample	Conc. (µg/ml)	Mean Absorbance	Gallic acid equivalent (mg of GAE / 100 g of dried extract)
E. fusca (Stem bark)			
Me	250	0.187±0.008	10.22±0.505
HSF	250	0.768±0.008	46.11±0.463
CSF	250	0.164±0.006	8.82±0.351
CHSF	250	0.458±0.013	26.92±0.829
AQSF	250	1.158±0.008	70.17±0.463
E. fusca (Leaf)			
Me	250	0.380±0.010	22.13±0.617
HSF	250	0.207±0.010	11.47±0.600
CSF	250	0.232±0.003	12.97±0.189
CHSF	250	0.522±0.003	30.90±0.223
AQSF	250	1.545±0.016	94.02±1.004
E. variegata (Stem bark)			
Me	250	0.172±0.014	9.31±0.840
HSF	250	0.564±0.015	33.51±0.916
CSF	250	0.087±0.012	4.02±0.747
CHSF	250	1.247±0.005	75.65±0.327
AQSF	250	1.553±0.048	94.52±2.981
E. variegata (Leaf)			
Me	250	1.157±0.023	70.11±1.448
HSF	250	0.504±0.007	29.78±0.432
CSF	250	0.842±0.018	50.65±1.123
CHSF	250	0.065±0.006	2.66±0.372
AQSF	250	1.33±0.020	80.77±1.235

Values are expressed as average±SD (n=3); Me = Methanolic extract; HSF = *n*-hexane soluble fraction; CSF = carbon tetrachloride soluble fraction; CHSF = chloroform soluble fraction and AQSF = aqueous soluble fraction.

Similarly, for the two *Erythrina* species the total phenolic content varied from 2.66 g to 94.52 g of GAE/100 g dried extract). The highest total phenolic content was found from aqueous soluble fraction of the stem bark of *E. variegata* (94.52 g of GAE/100 g of dried extract) and the lowest from the leaf of chloroform soluble fraction of *E. variegata* (2.66 g of GAE/100 g of dried extract).

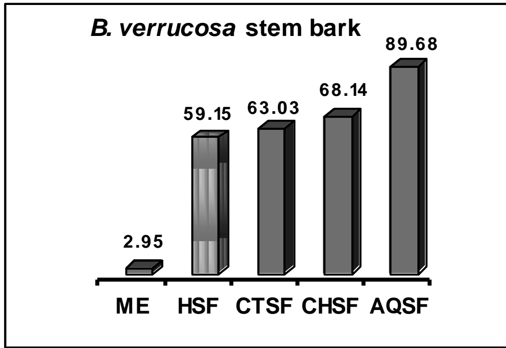

Figure 6.2: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the stem bark of *B. verrucosa*.

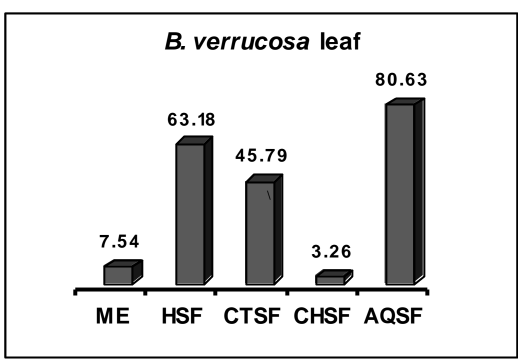

Figure 6.3: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the leaf of *B. verrucosa*.

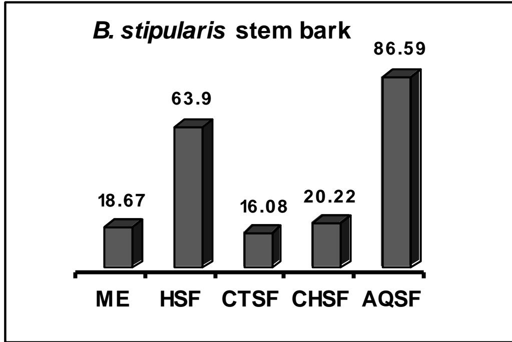

Figure 6.4: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the stem bark of *B. stipularis*.

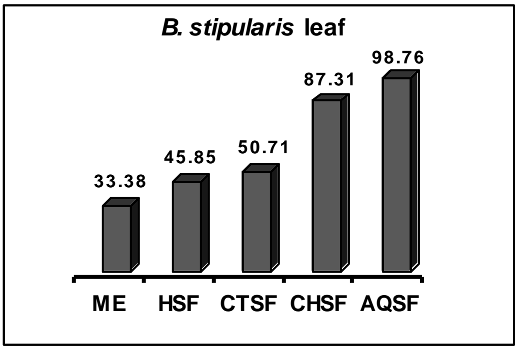

Figure 6.5: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the leaf of *B. stipularis*.

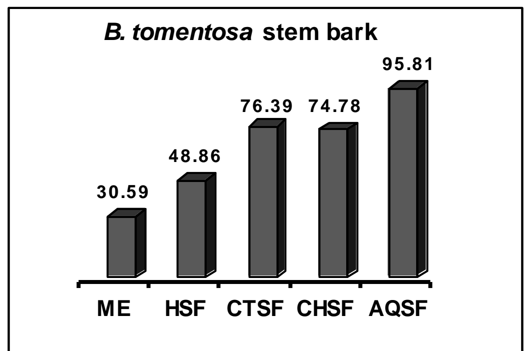

Figure 6.6: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the stem bark of *B. tomentosa*.

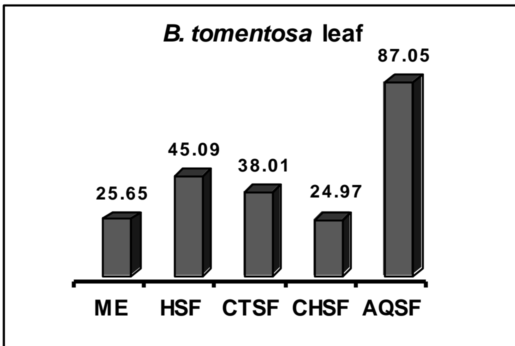

Figure 6.7: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the leaf of *B. tomentosa.*

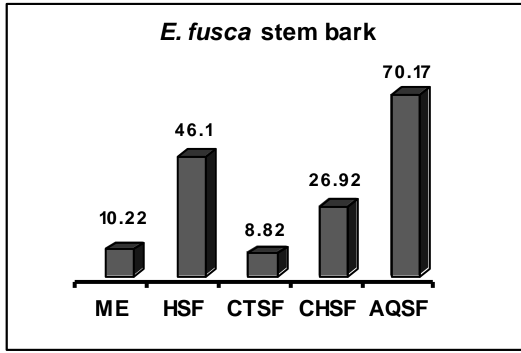

Figure 6.8: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the stem bark of *E. fusca*.

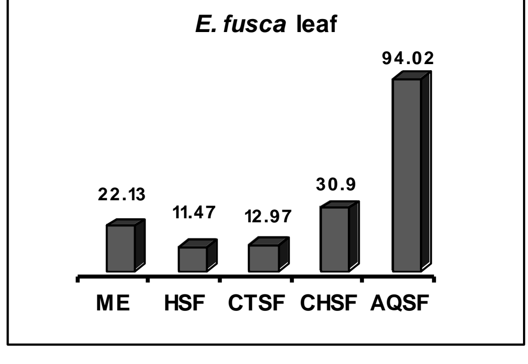

Figure 6.9: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the leaf of *E. fusca*.

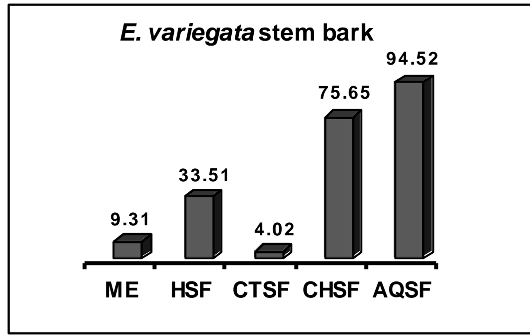

Figure 6.10: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the stem bark of *E. variegata*.

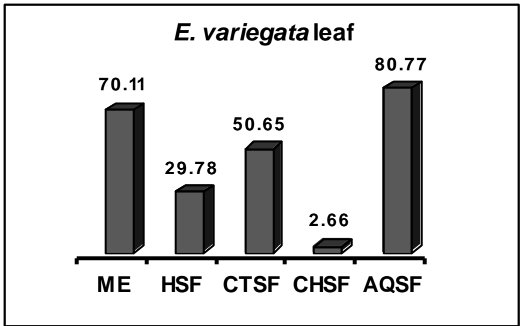

Figure 6.11: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the leaf of *E. variegata*.

6.4.2 Antioxidant activity evaluation by DPPH method

The methanolic extract of the three *Bridelia* species, *B. verrucosa*, *B. stipularis* and *B. tomentosa* and two species of *Erythrina* species, *E. fusca* and *E. variegata* available in Bangladesh (Me) and different partitionates i.e. *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous (AQSF) soluble fractions were subjected to free radical scavenging activity by the method of Brand-Williams *et al,*. 1995. Smaller IC_{50} value corresponds to a higher activity of the plant extracts (Maisuthisakul *et al.*, 2007). Here, *tert*-butyl-1-hydroxytoluene (BHT) and ascorbic acid (ASA) were used as reference standard.

In this investigation, the free radical scavenging activity of the methanolic extract of the leaf of *B. verrucosa* was found to be highest having IC_{50} value of 6.35 µg/ml and lowest activity was shown by the aqueous soluble fraction of the leaf of *B. tomentosa* with IC_{50} being 26.4 µg/ml. The other soluble fractions of the three plants demonstrated high to moderate free radical scavenging activity with the IC_{50} value ranging from 7.34 µg/ml to 21.3 µg/ml, as compared to the standards, i.e. *tert*-butyl-1-hydroxytoluene (BHT),

(IC_{50} = 24.35 µg/ml) and ascorbic acid, ASA (IC_{50} = 5.80 µg/ml). Again, for the *Erythrina* species free radical scavenging activity of the chloroform soluble fraction of the leaf of *E. fusca* was highest with IC_{50} value of 5.5 µg/ml and lowest activity was shown by the chloroform soluble fraction of the leaf of *E. variegata* with IC_{50} being 21.3 µg/ml. Other soluble fractions of the two plants demonstrated high to moderate free radical scavenging activity with the IC_{50} value ranging from 5.5 µg/ml to 21.3 µg/ml, as compared to the standards. The IC_{50} (7.3 µg/ml) of the isolated pure compound BVBC-2 (**51**) indicated strong free radical scavenging activity. All the results are shown in Tables 6.4-6.15 and Figures 6.12-6.65.

Table 6.4: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of *B. verrucosa*.

***B. verrucosa* (Stem bark)**											
Blank	**Conc.** (µg/ml)	**% Inhibition**					**IC_{50} (µg/ml)**				
		Me	**HSF**	**CSF**	**CHSF**	**AQSF**	**Me**	**HSF**	**CSF**	**CHSF**	**AQSF**
0.395	500	85.06	90.63	92.66	90.63	92.91	11.6± 0.39	10.51± 0.43	11.65± 0.80	15.8± 0.15	15.5± 0.36
0.395	250	75.95	90.38	88.61	85.57	87.59					
0.395	125	57.22	85.06	82.53	82.53	74.94					
0.395	62.5	58.48	82.03	76.46	81.01	70.89					
0.395	31.25	65.57	83.54	73.16	65.82	60.51					
0.395	15.625	52.91	72.91	55.19	49.62	49.87					
0.395	7.813	46.08	31.39	38.48	32.15	44.05					
0.395	3.906	36.46	23.8	11.65	30.13	25.06					
0.395	1.953	23.8	12.41	8.61	7.85	20.51					
0.395	0.0977	12.41	4.05	4.56	3.54	15.95					

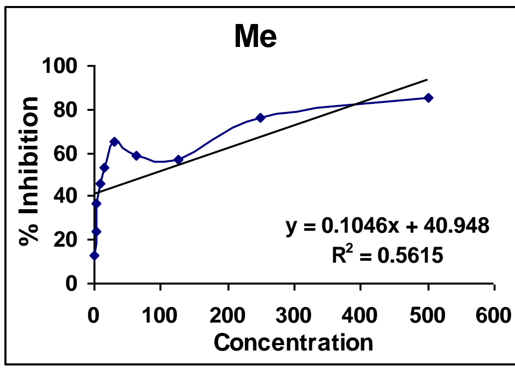

Figure 6.12: Free radical scavenging activity of the methanol fraction (Me) of the stem bark of *B. verrucosa*.

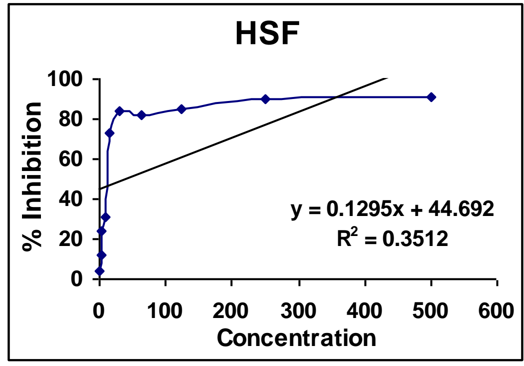

Figure 6.13: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the stem bark of *B. verrucosa*.

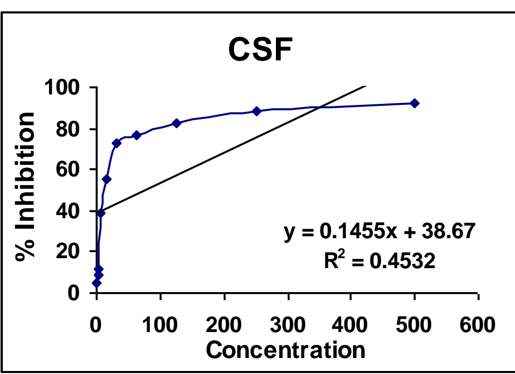

Figure 6.14: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the stem bark of *B. verrucosa*.

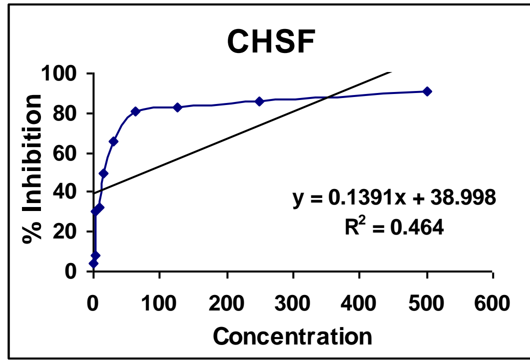

Figure 6.15: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of *B. verrucosa*.

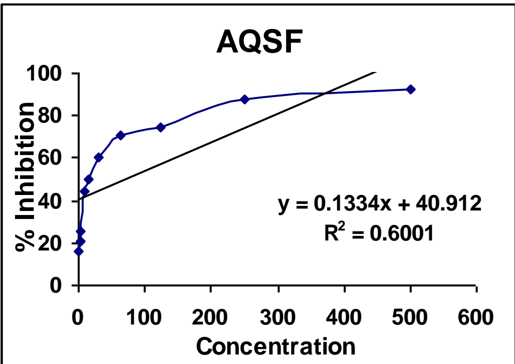

Figure 6.16: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of *B. verrucosa*.

Table 6.5: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of *B. verrucosa*.

***B. verrucosa* (leaf)**											
Blank	**Conc.** (µg/ml)	**% Inhibition**					**IC_{50} (µg/ml)**				
		Me	**HSF**	**CSF**	**CHSF**	**AQSF**	**Me**	**HSF**	**CSF**	**CHSF**	**AQSF**
0.395	500	89.11	90.38	90.63	93.67	76.46	6.35± 0.80	25.8± 0.21	5.9± 0.85	5.25± 0.10	15.8± 0.40
0.395	250	85.57	87.09	91.9	90.63	75.44					
0.395	125	85.57	76.2	85.82	86.84	70.38					
0.395	62.5	84.3	65.32	75.7	80	69.37					
0.395	31.25	81.27	52.15	67.59	77.97	57.22					
0.395	15.625	69.87	42.28	65.82	67.09	50.13					
0.395	7.813	51.65	26.84	64.05	65.32	43.54					
0.395	3.906	42.28	23.8	25.06	37.72	24.81					
0.395	1.953	33.16	17.22	23.54	30.13	20.51					
0.395	0.0977	28.86	4.3	9.87	16.71	12.15					

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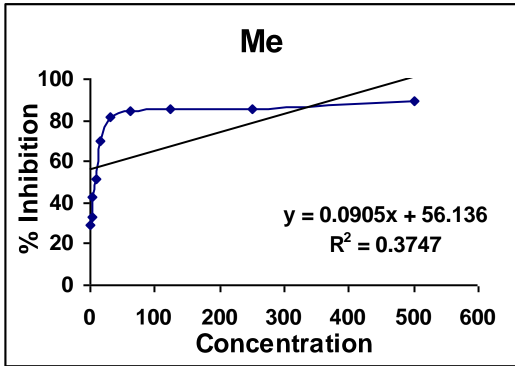

Figure 6.17: Free radical scavenging activity of the methanol fraction (Me) of the leaf of *B. verrucosa*

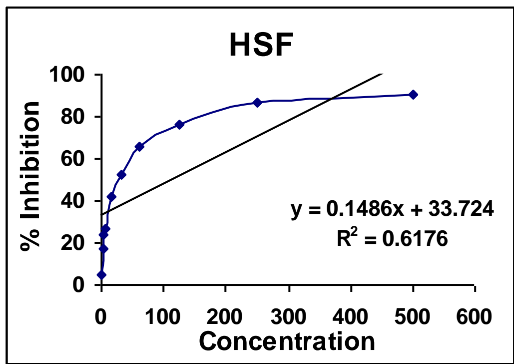

Figure 6.18: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the leaf of *B. verrucosa*

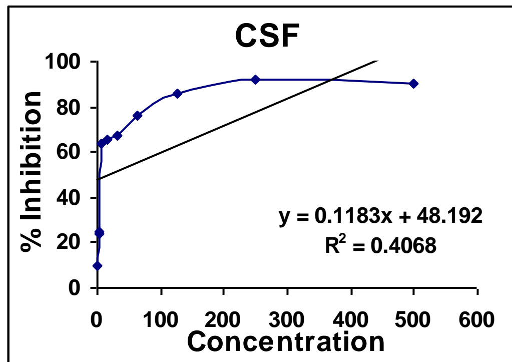

Figure 6.19: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the leaf of *B. verrucosa*

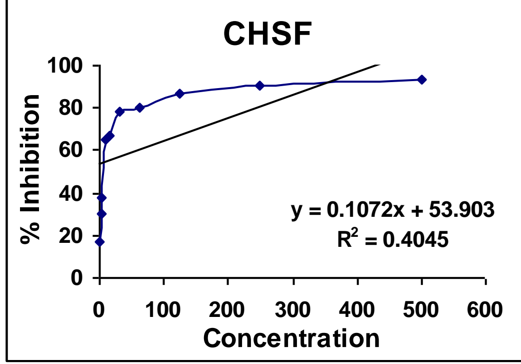

Figure 6.20: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of *B. verrucosa*.

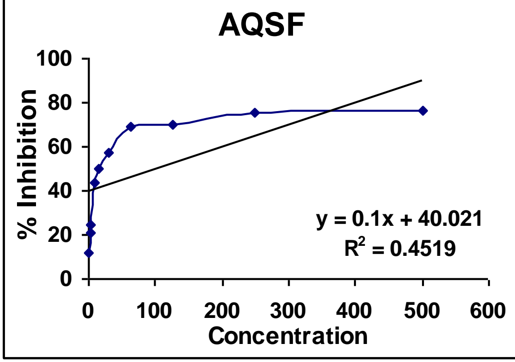

Figure 6.21: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of *B. verrucosa*.

Table 6.6: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of *B. stipularis*.

B. stipularis (stem bark)												
Blank	**Conc.** (µg/ml)	**% Inhibition**					**IC_{50} (µg/ml)**					
		Me	**HSF**	**CSF**	**CHSF**	**AQSF**	**Me**	**HSF**	**CSF**	**CHSF**	**AQSF**
0.395	500	94.68	90.38	86.58	94.68	87.59	9.10± 0.40	10.20± 0.55	15.60± 0.50	13.2± 0.10	21.3± 0.40
0.395	250	92.15	85.06	78.23	92.15	80.25					
0.395	125	86.58	80.25	65.06	86.84	70.63					
0.395	62.5	77.97	71.39	61.01	82.53	56.2					
0.395	31.25	75.7	70.13	54.94	76.46	49.87					
0.395	15.625	71.9	68.61	49.62	52.91	45.32					
0.395	7.813	43.29	37.47	23.54	42.03	39.49					
0.395	3.906	25.82	23.54	17.47	30.13	24.81					
0.395	1.953	14.43	10.63	11.65	21.27	13.16					
0.395	0.0977	6.58	1.52	2.03	13.67	9.87					

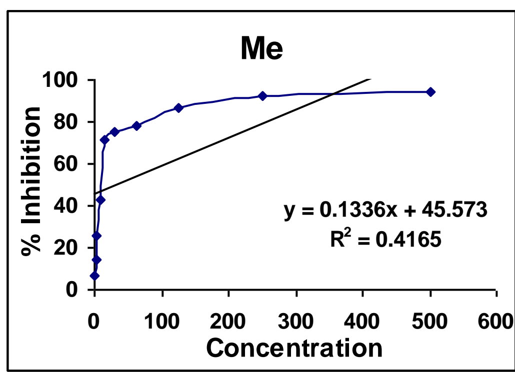

Figure 6.22: Free radical scavenging activity of the methanol fraction (Me) of the stem bark of *B. stipularis*.

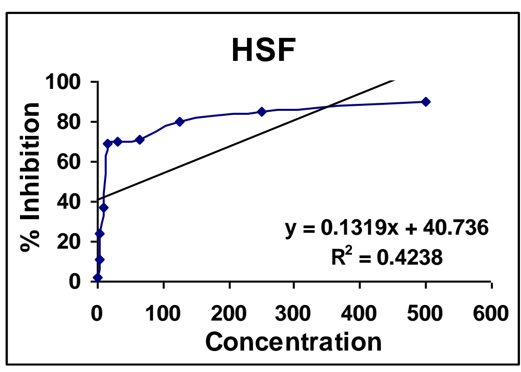

Figure 6.23: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the stem bark of *B. stipularis*.

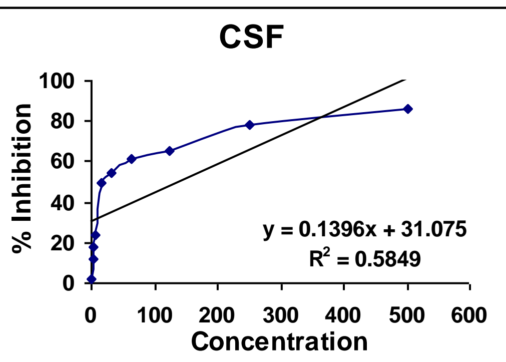

Figure 6.24: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the stem bark of *B. stipularis*.

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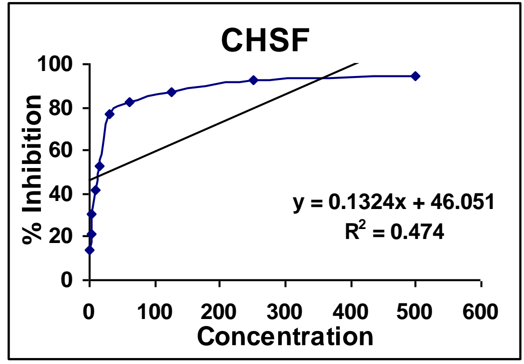

Figure 6.25: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of *B. stipularis*.

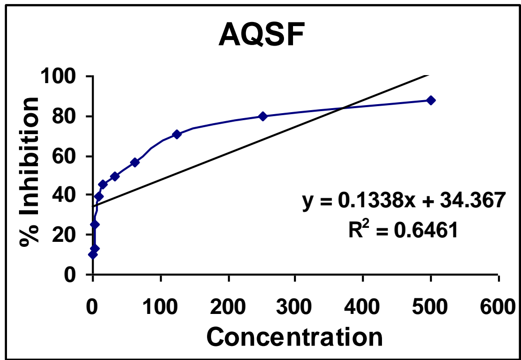

Figure 6.26: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of *B. stipularis*.

Table 6.7: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of *B. stipularis*.

***B. stipularis* (leaf)**											
Blank	**Conc.** (µg/ml)	**% Inhibition**					**IC_{50} (µg/ml)**				
		Me	**HSF**	**CSF**	**CHSF**	**AQSF**	**Me**	**HSF**	**CSF**	**CHSF**	**AQSF**
0.395	500	87.59	79.24	90.13	93.42	85.82	13.9± 0.80	11.8± 0.55	9.75± 0.74	7.34± 0.46	21.3± 0.60
0.395	250	84.05	76.71	84.05	90.63	77.97					
0.395	125	75.19	73.67	75.19	87.09	76.46					
0.395	62.5	70.13	71.65	69.87	80.76	69.87					
0.395	31.25	63.04	65.06	66.58	70.63	52.91					
0.395	15.625	51.14	52.15	60.25	60.00	46.33					
0.395	7.813	42.03	46.33	42.78	50.63	34.68					
0.395	3.906	30.13	26.84	26.84	31.9	28.35					
0.395	1.953	21.77	19.24	17.72	17.72	10.89					
0.395	0.0977	16.71	5.82	7.09	10.38	6.08					

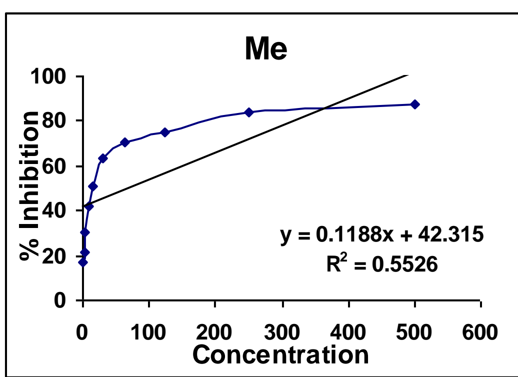

Figure 6.27: Free radical scavenging activity of the methanol fraction (Me) of the leaf of *B. stipularis*.

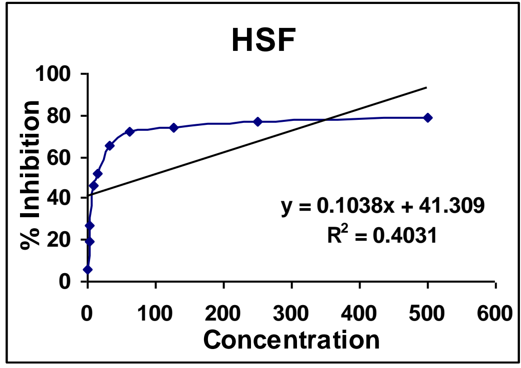

Figure 6.28: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the leaf of *B. stipularis*.

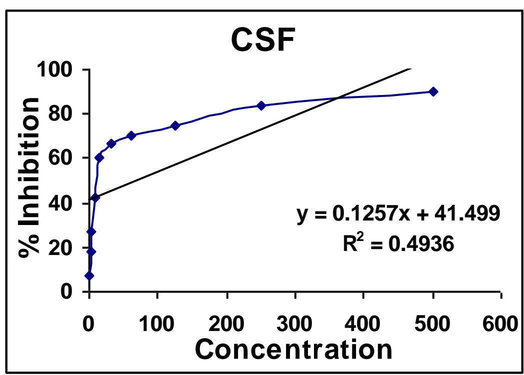

Figure 6.29: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the leaf of *B. stipularis*.

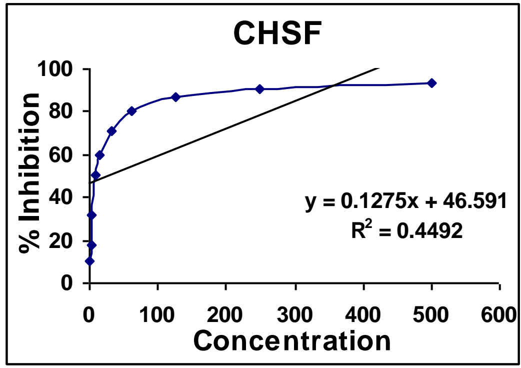

Figure 6.30: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of *B. stipularis*.

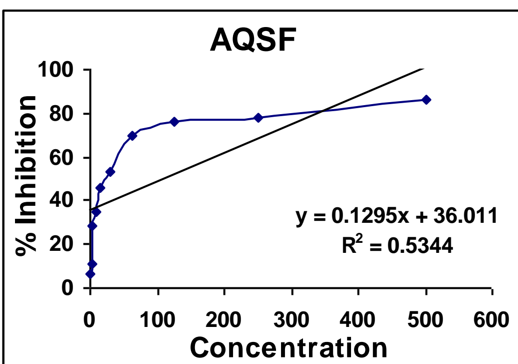

Figure 6.31: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of *B. stipularis*.

Table 6.8: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of *B. tomentosa*.

B. tomentosa (stem bark)											
Blank	Conc. (µg/ml)	% Inhibition					IC_{50} (µg/ml)				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
0.395	500	90.63	93.16	90.63	92.15	91.39	7.55± 0.27	10.35± 0.48	10.50± 0.44	7.35± 0.40	17.60± 0.33
0.395	250	89.37	88.1	89.62	86.33	87.09					
0.395	125	86.33	89.62	81.77	84.56	75.44					
0.395	62.5	75.44	84.05	75.70	79.49	68.86					
0.395	31.25	67.85	78.99	76.46	72.91	56.2					
0.395	15.625	61.27	69.37	58.73	61.52	47.85					
0.395	7.813	49.62	35.95	41.52	50.38	32.41					
0.395	3.906	32.41	20.76	19.75	28.86	17.22					
0.395	1.953	21.27	15.44	11.14	22.28	6.08					
0.395	0.0977	7.59	2.03	1.27	10.38	2.53					

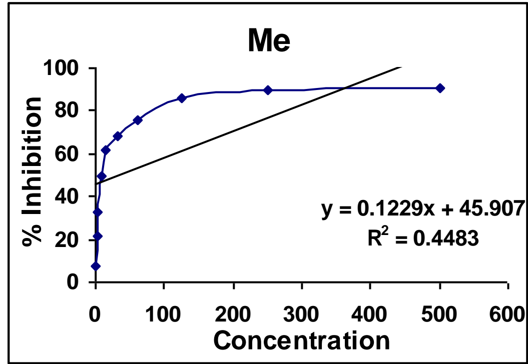

Figure 6.32: Free radical scavenging activity of the methanol fraction (Me) of the stem bark of *B. tomentosa*.

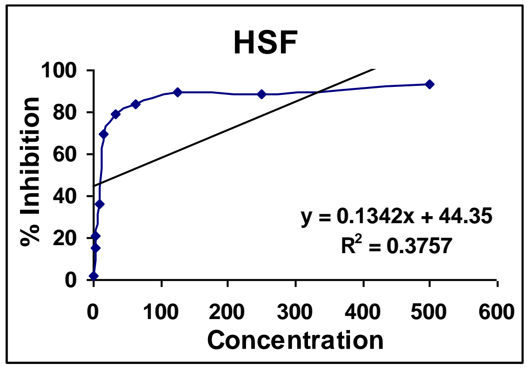

Figure 6.33: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the stem bark of *B. tomentosa*.

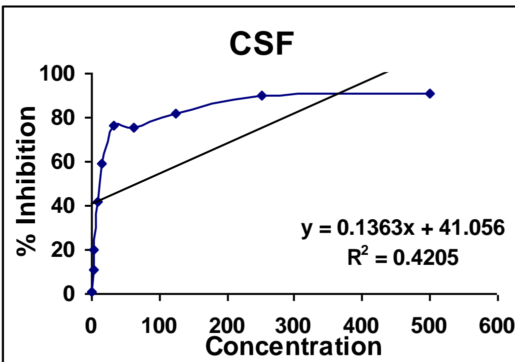

Figure 6.34: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the stem bark of *B. tomentosa*.

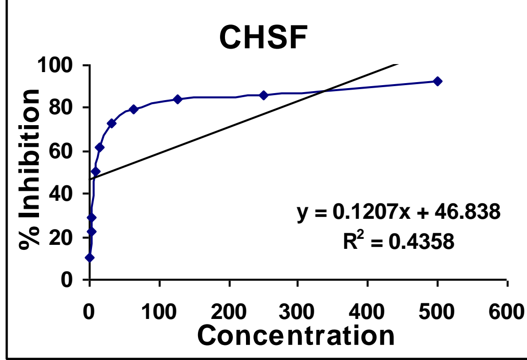

Figure 6.35: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of *B. tomentosa*.

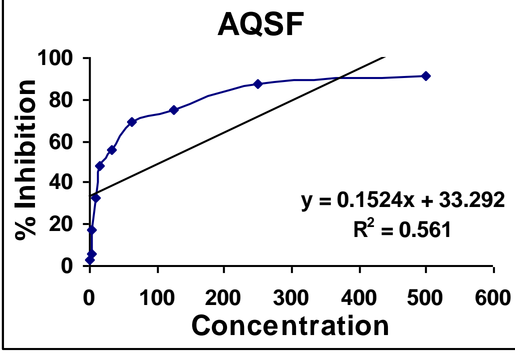

Figure 6.36: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of *B. tomentosa*.

Table 6.9: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of *B. tomentosa*.

B. tomentosa (leaf)												
Blank	Conc. (µg/ml)	% Inhibition					IC_{50} (µg/ml)				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
0.395	500	93.16	92.91	94.18	91.39	85.06	9.70± 0.25	11.20± 0.50	8.8± 0.10	3.9± 0.80	26.4± 0.45
0.395	250	90.13	88.1	90.38	87.09	77.97					
0.395	125	87.09	84.56	87.59	90.38	67.85					
0.395	62.5	82.53	76.96	81.52	87.09	61.52					
0.395	31.25	75.44	63.8	78.48	83.04	53.67					
0.395	15.625	61.01	58.73	69.37	76.46	32.41					
0.395	7.813	43.54	36.46	45.06	69.37	23.04					
0.395	3.906	30.63	24.3	23.54	50.38	19.75					
0.395	1.953	20.76	16.2	13.67	28.35	11.14					
0.395	0.0977	1.52	7.09	1.52	18.73	4.3					

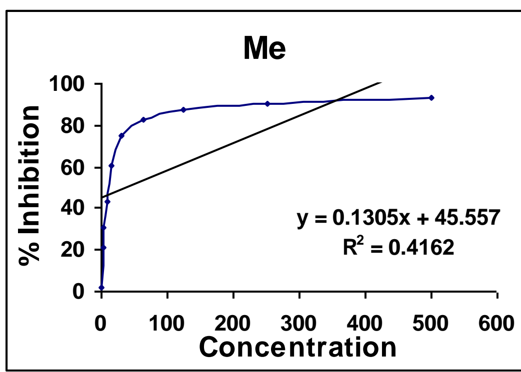

Figure 6.37: Free radical scavenging activity of the methanol fraction (Me) of the leaf of *B. tomentosa*.

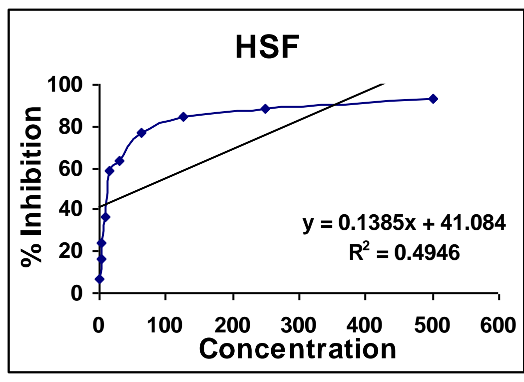

Figure 6.38: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the leaf of *B. tomentosa*.

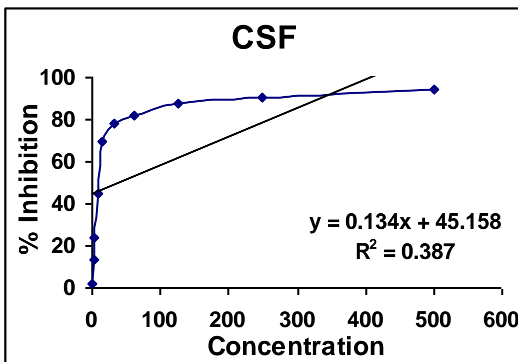

Figure 6.39: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the leaf of *B. tomentosa*.

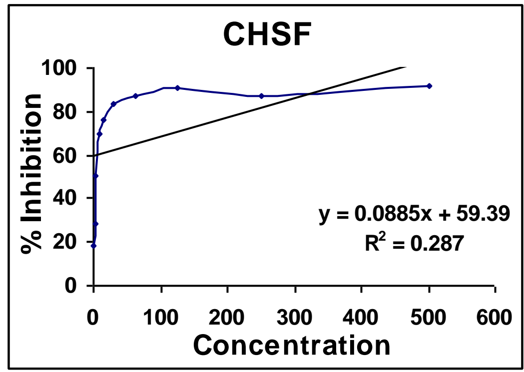

Figure 6.40: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of *B. tomentosa*.

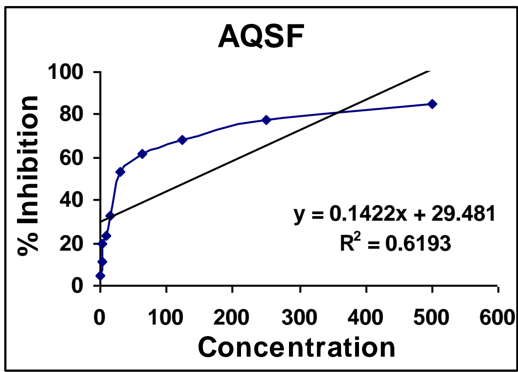

Figure 6.41: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of *B. tomentosa*.

Table 6.10: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of *E. fusca*.

***E. fusca* (stem bark)**											
Blank	**Conc.** (μg/ml)	**% Inhibition**					***IC_{50} (μg/ml)***				
		Me	**HSF**	**CSF**	**CHSF**	**AQSF**	**Me**	**HSF**	**CSF**	**CHSF**	**AQSF**
0.395	500	94.68	90.38	91.39	86.58	89.62	11.8± 0.35	7.7± 0.95	13.1± 0.75	8.8± 0.95	12.1± 0.45
0.395	250	91.90	85.32	85.82	80.76	81.77					
0.395	125	83.29	81.52	76.46	73.67	74.43					
0.395	62.5	75.44	75.95	69.37	68.86	70.13					
0.395	31.25	65.06	66.84	57.22	62.78	59.75					
0.395	15.625	53.67	61.27	50.13	54.68	52.15					
0.395	7.813	42.78	50.89	43.54	47.85	45.06					
0.395	3.906	27.85	24.81	24.81	34.94	35.19					
0.395	1.953	24.30	16.71	20.51	19.24	19.24					
0.395	0.0977	18.73	6.08	12.15	5.82	13.67					

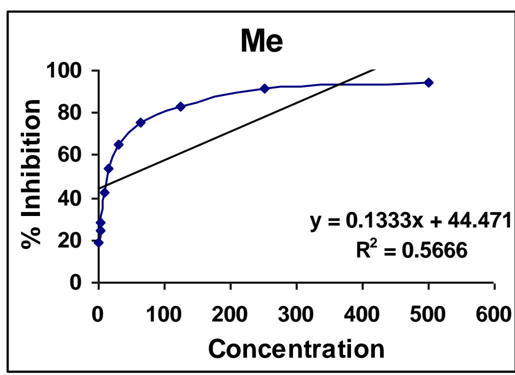

Figure 6.42: Free radical scavenging activity of the methanol fraction (Me) of the stem bark of *E. fusca*.

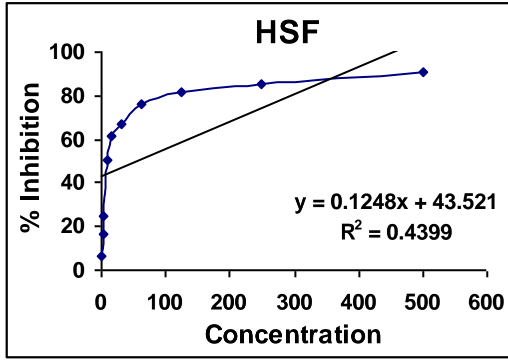

Figure 6.43: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the stem bark of *E. fusca*.

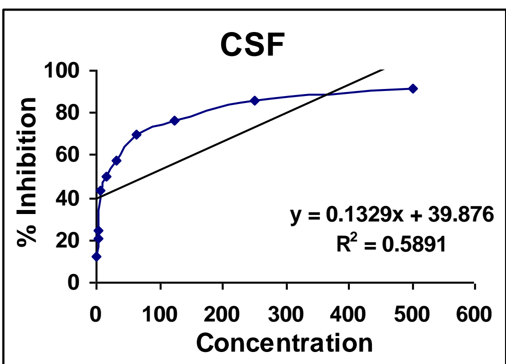

Figure 6.44: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the stem bark of *E. fusca*.

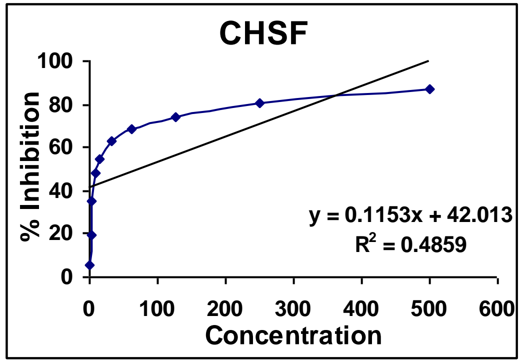

Figure 6.45: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of *E. fusca*.

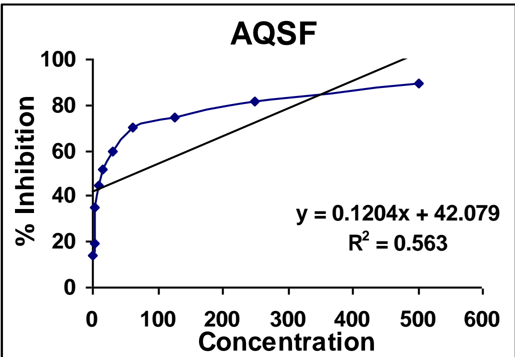

Figure 6.46: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of *E. fusca*.

Table 6.11: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of *E. fusca*.

E. fusca (leaf)											
Blank	Conc. (µg/ml)	% Inhibition					IC_{50} (µg/ml)				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
0.395	500	93.16	91.9	88.61	94.18	95.7	9.9± 0.95	7.9± 0.70	19.9± 0.95	5.5± 0.60	12.6± 0.20
0.395	250	89.37	88.1	81.52	88.10	92.15					
0.395	125	81.01	87.09	73.67	82.53	85.57					
0.395	62.5	76.46	78.99	62.78	75.44	81.52					
0.395	31.25	67.85	70.63	52.91	71.65	75.95					
0.395	15.625	53.42	62.28	47.34	66.08	52.66					
0.395	7.813	46.08	49.11	39.49	52.66	44.81					
0.395	3.906	29.62	37.22	29.87	46.08	26.84					
0.395	1.953	14.94	14.18	16.96	34.94	16.71					
0.395	0.0977	9.62	8.61	7.34	13.42	10.38					

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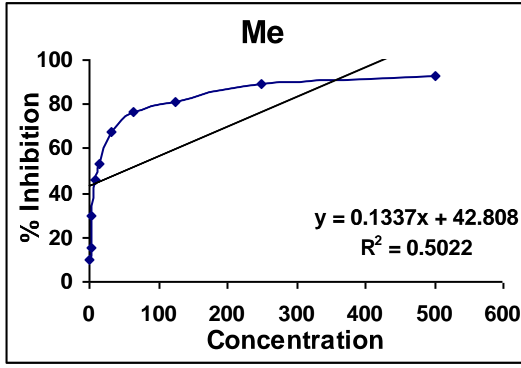

Figure 6.47: Free radical scavenging activity of the methanol fraction (Me) of the leaf of *E. fusca*.

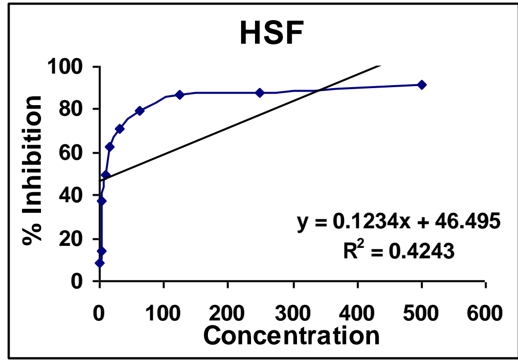

Figure 6.48: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the leaf of *E. fusca*.

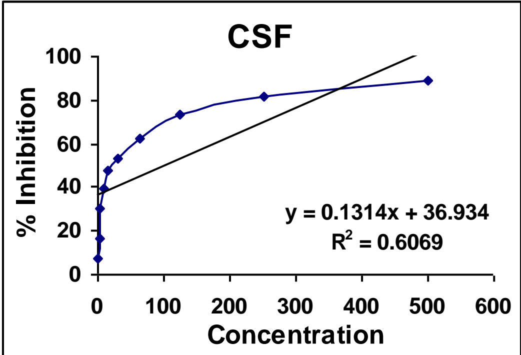

Figure 6.49: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the leaf of *E. fusca*.

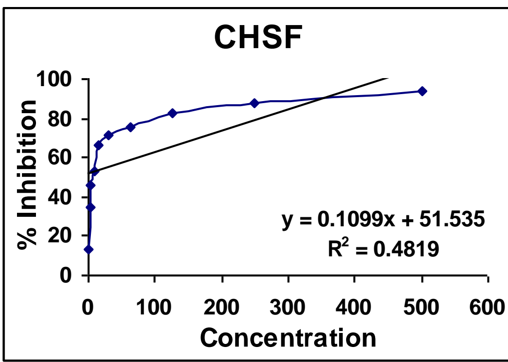

Figure 6.50: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of *E. fusca*.

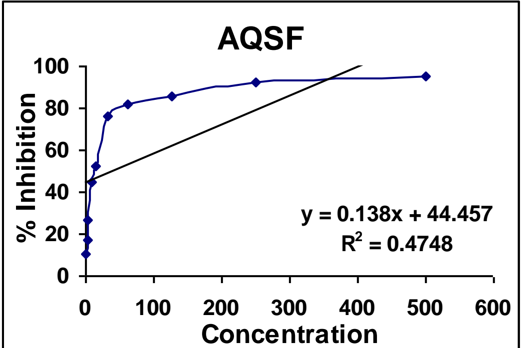

Figure 6.51: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of *E. fusca*.

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Table 6.12: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of *E. variegata*.

E. variegata (stem bark)											
Blank	Conc. (µg/ml)	% Inhibition					IC_{50} (µg/ml)				
		Me	HSF	CSF	CHSF	AQSF	Me	HSF	CSF	CHSF	AQSF
0.395	500	91.90	91.39	92.91	95.19	92.66	6.6± 0.25	13.2± 0.61	16.5± 0.40	7.9± 0.80	7.3± 0.50
0.395	250	83.80	87.09	88.1	91.39	88.35					
0.395	125	75.19	77.72	76.96	85.57	90.13					
0.395	62.5	72.41	70.38	64.81	77.47	81.52					
0.395	31.25	69.11	62.53	53.42	73.92	75.95					
0.395	15.625	61.01	50.63	48.61	65.57	67.59					
0.395	7.813	53.42	44.81	43.54	48.86	51.39					
0.395	3.906	48.86	35.95	23.8	29.37	32.66					
0.395	1.953	34.43	16.96	18.73	19.75	6.58					
0.395	0.0977	21.01	8.35	12.15	6.08	1.77					

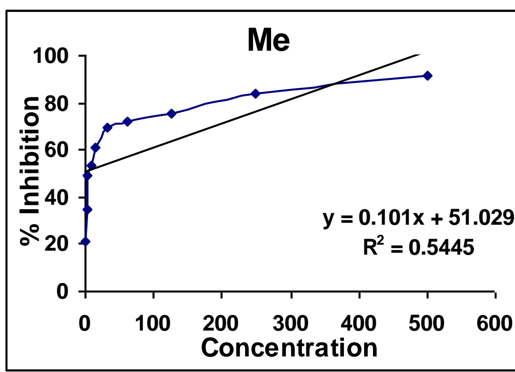

Figure 6.52: Free radical scavenging activity of the methanol fraction (Me) of the stem bark of *E. variegata*.

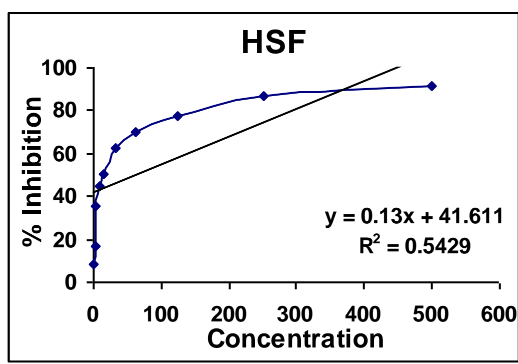

Figure 6.53: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the stem bark of *E. variegata*.

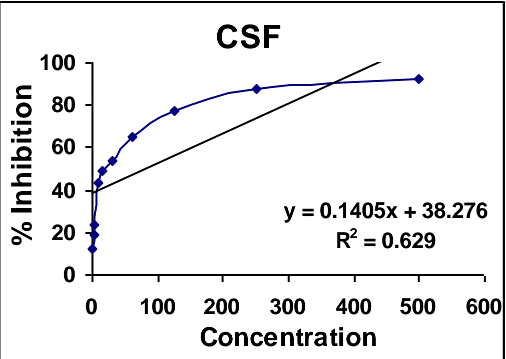

Figure 6.54: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the stem bark of *E. variegata*.

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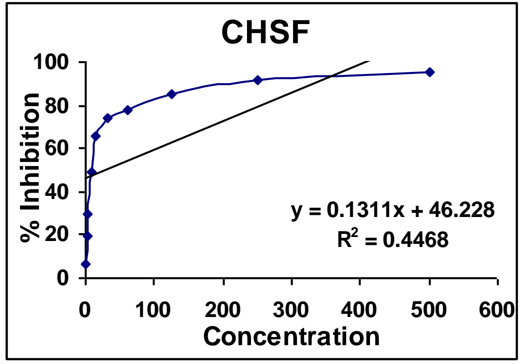

Figure 6.55: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of *E. variegata*.

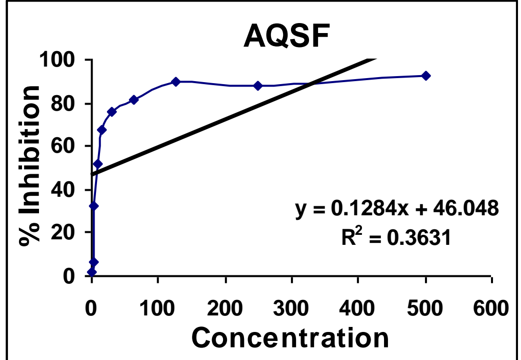

Figure 6.56: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of *E. variegata*.

Table 6.13: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of *E. variegata*.

***Erythrina variegata* (leaf)**											
Blank	**Conc.** (μg/ml)	**% Inhibition**					**IC_{50} (μg/ml)**				
		Me	**HSF**	**CSF**	**CHSF**	**AQSF**	**Me**	**HSF**	**CSF**	**CHSF**	**AQSF**
0.395	500	90.13	90.89	93.92	85.82	93.42	14.7± 0.85	10.45± 0.65	14.3± 0.25	21.3± 0.50	7.34± 0.45
0.395	250	87.59	84.56	91.14	77.97	90.63					
0.395	125	77.72	79.49	86.61	76.46	87.09					
0.395	62.5	83.04	70.89	81.52	69.87	80.76					
0.395	31.25	65.06	69.37	75.44	52.91	70.63					
0.395	15.625	50.38	67.85	51.39	46.33	59.75					
0.395	7.813	46.08	36.46	40.76	34.68	50.63					
0.395	3.906	16.96	22.53	28.86	28.35	31.9					
0.395	1.953	11.65	9.62	20.25	10.89	17.72					
0.395	0.0977	1.01	2.03	12.41	6.08	10.38					

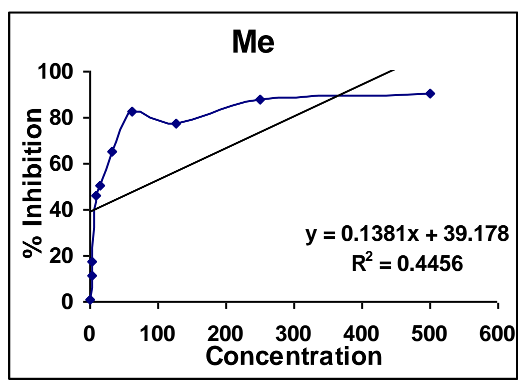

Figure 6.57: Free radical scavenging activity of the methanol fraction (Me) of the leaf of *E. variegata.*

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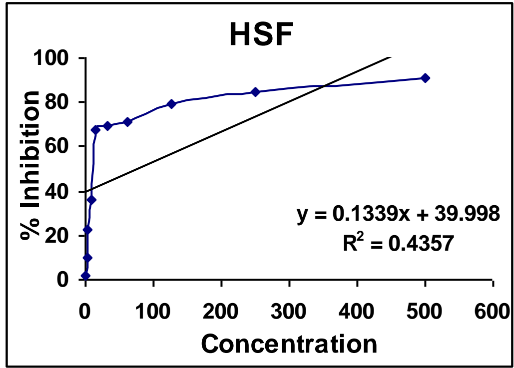

Figure 6.58: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the leaf of *E. variegata*.

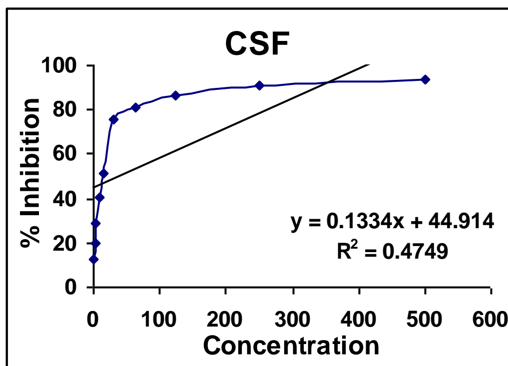

Figure 6.59: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the leaf of *E. variegata*.

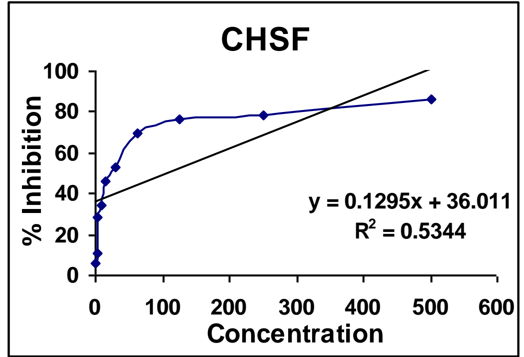

Figure 6.60: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of *E. variegata*.

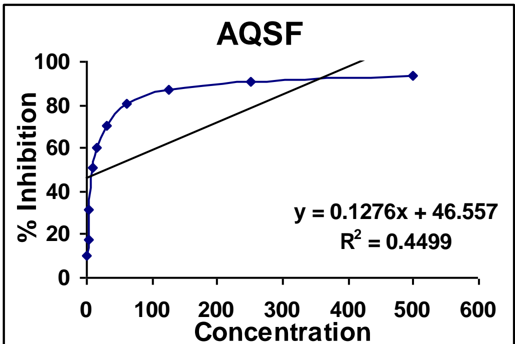

Figure 6.61: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of *E. variegata*.

Table 6.14: Free radical scavenging activity of the isolated pure compounds BVSP-4, BVSP-6, BVBC-2 and BVS-65 isolated from the stem bark of *B. verrusosa*.

Blank	Conc. (μg/ml)	% Inhibition				IC_{50} (μg/ml)			
		BVSP-4	BVSP-6	BVBC-2	BVS-65	BVSP-4	BVSP-6	BVBC-2	BVS-65
0.395	500	85.82	90.13	92.66	97.72	17.9	14.7	7.3	8.4
0.395	250	80.25	87.59	88.35	94.68				
0.395	125	76.46	77.72	90.13	90.13				
0.395	62.5	69.87	83.04	81.52	82.78				
0.395	31.25	53.67	65.06	75.95	75.95				
0.395	15.625	47.09	50.38	67.59	56.20				
0.395	7.813	32.15	46.08	51.39	49.62				
0.395	3.906	19.75	16.96	32.66	35.95				
0.395	1.953	9.62	11.65	6.58	21.27				
0.395	0.0977	4.56	1.01	1.77	8.35				

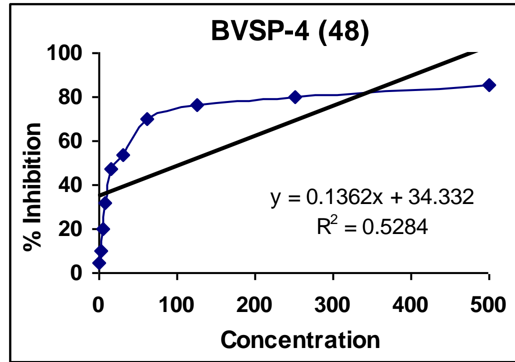

Figure 6.62: Free radical scavenging activity BVSP-4 (**48**) isolated from the stem bark of *B. verrucosa.*

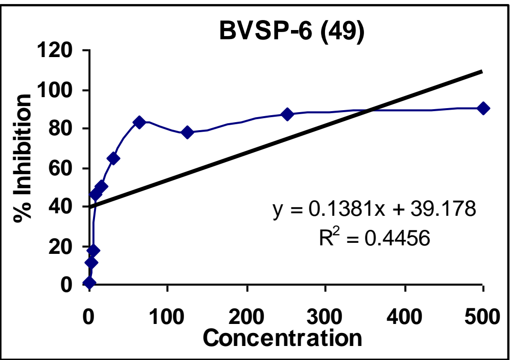

Figure 6.63: Free radical scavenging activity BVSP-6 (**49**) isolated from the stem bark of *B. verrucosa.*

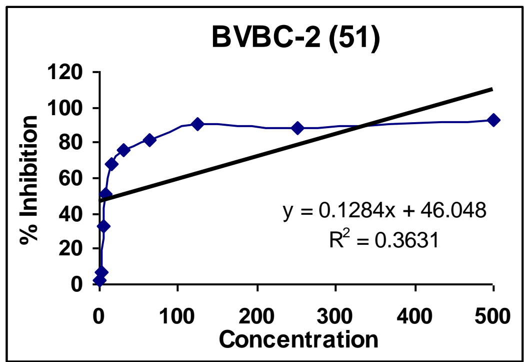

Figure 6.64: Free radical scavenging activity BVBC-2 (**51**) isolated from the stem bark of *B. verrucosa.*

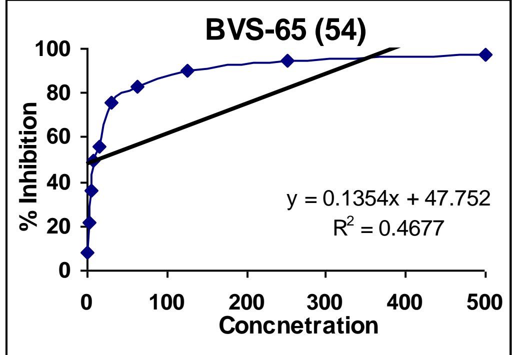

Figure 6.65: Free radical scavenging activity BVS-65 (**54**) isolated from the stem bark of *B. verrucosa.*

Table 6.15: Free radical scavenging activity of the isolated pure compounds BS-11, EF-7, EF-8 and EF-12 isolated from the stem bark of *B. stipularis* and *E. fusca*.

Pure compounds									
Blank	**Conc.** (μg/ml)	**% Inhibition**				**IC_{50} (μg/ml)**			
		BS-11	**EF-7**	**EF-8**	**EF-12**	**BS-11**	**EF-7**	**EF-8**	**EF-12**
0.395	500	95.7	90.38	87.59	95.19	12.6	8.8	7.7	7.9
0.395	250	92.15	87.09	91.14	91.39				
0.395	125	85.57	80.51	75.19	85.57				
0.395	62.5	81.52	75.44	80.51	77.47				
0.395	31.25	75.95	67.85	65.57	73.92				
0.395	15.625	52.66	53.67	49.87	65.57				
0.395	7.813	44.81	47.34	49.11	48.86				
0.395	3.906	26.84	24.56	20.76	29.37				
0.395	1.953	16.71	12.41	9.62	19.75				
0.395	0.0977	4.3	0.76	1.77	6.08				

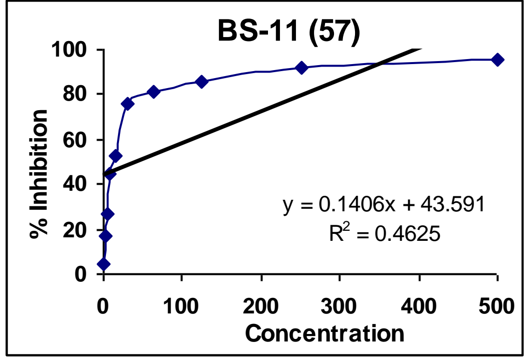

Figure 6.66: Free radical scavenging activity BS-11 (**57**) isolated from the stem bark of *B. verrucosa.*

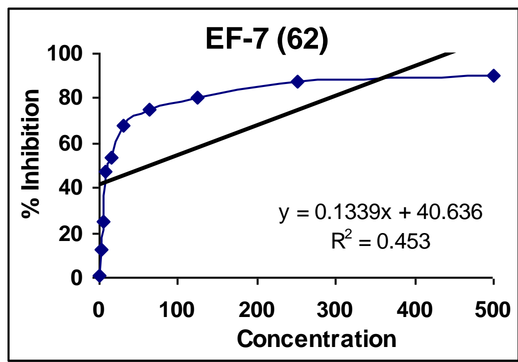

Figure 6.67: Free radical scavenging activity EF-7 (**62**) isolated from the stem bark of *E. fusca.*

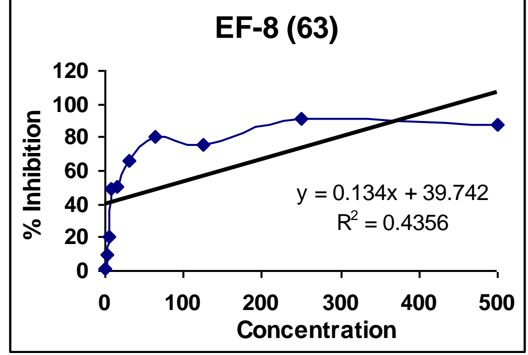

Figure 6.68: Free radical scavenging activity EF-8 (**63**) isolated from the stem bark of *E. fusca.*

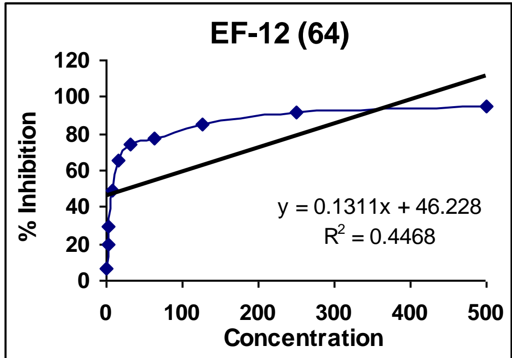

Figure 6.69: Free radical scavenging activity EF-12 (**64**) isolated from the stem bark of *E. fusca.*

6.4.3 Total antioxidant capacity assay by the phosphomolybdenum method

Total antioxidant capacity in terms of absorbance values of the methanol extract (Me) of *B. verrucosa*, *B. stipularis* and *B. tomentosa*, *E. fusca* and *E. variegata* available in Bangladesh and fractions generate from the crude extract i.e. *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous (AQSF) soluble partitionates were determined by the phosphomolybdenum method and were expressed as equivalents of ascorbic acid as standard (Table 6.15 and 6.16). In case of *Bridelia* species, the total antioxidant capacity of the methanolic extract of the leaf of *B. tomentosa* was highest (612.54 mg of ascorbic acid/g of dried extract) and *n*-hexane soluble fraction of the stem bark of *B. stipularis* being the lowest (142.66 mg of ascorbic acid/g of dried extract). The fractions of the three plants demonstrated varying degrees of antioxidant capacity as compared to the standard ascorbic acid. Again, for the *Erythrina* species antioxidant capacity of the methanol extract of the leaf of *E. fusca* was highest being 587.66 mg of ascorbic acid/g of dried extract and lowest capacity was shown by the chloroform soluble fraction of the leaf of *E. variegata* being 96.66 mg of ascorbic acid/g of dried extract. The

other remaining fractions of the two plants demonstrated high to moderate antioxidant capacity as compared to the standard.

Table 6.16: Standard curve preparation by using ascorbic acid.

SL. No.	Conc. Of the standard (μg/ml)	Absorbance	Regression line	R^2
1	100	0.298	y = 0.003x + 0.1057	0.9845
2	200	0.641		
3	400	1.465		
4	600	2.1		
5	800	2.54		
6	1000	3		

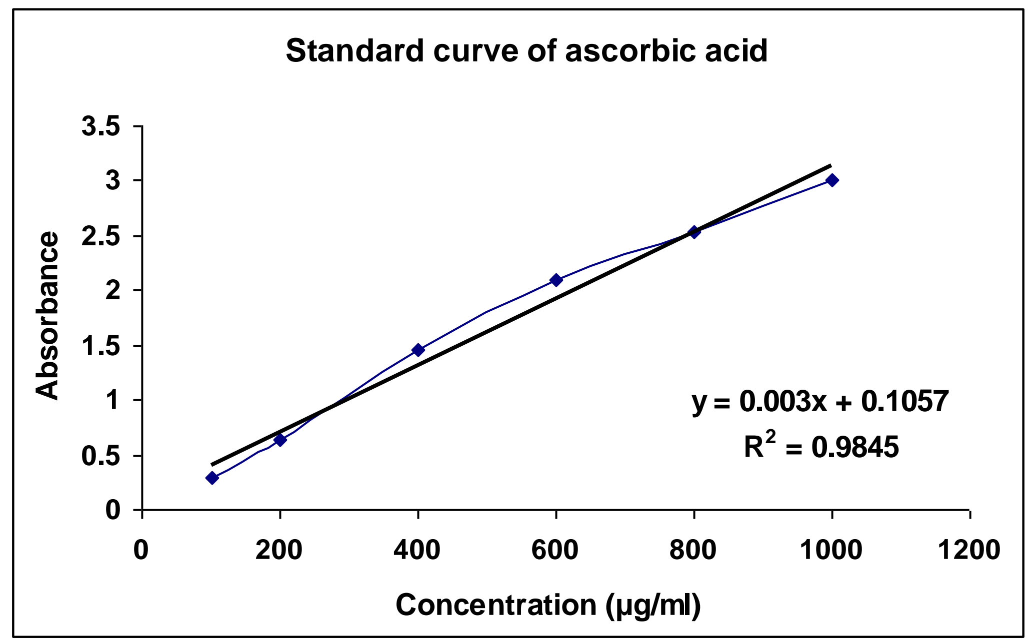

Figure 6.70: Standard curve of ascorbic acid for total antioxidant capacity determination.

Table 6.17: Total antioxidant capacity of the methanolic extracts and its different soluble fractions of stem bark and leaf of *Bridelia* species.

Soluble fractions	*B. verrucosa*		*B. stipularis*		*B. tomentosa*	
	Stem bark (mg of ascorbic acid/g of dried extract)	Leaf (mg of ascorbic acid/g of dried extract)	Stem bark (mg of ascorbic acid/g of dried extract)	Leaf (mg of ascorbic acid/g of dried extract)	Stem bark (mg of ascorbic acid/g of dried extract)	Leaf (mg of ascorbic acid/g of dried extract)
Me	542.88±1.61	484.43±1.11	248.99±0.75	392.54±2.32	442.77±3.93	612.54±1.79
HSF	217.32±0.91	161.88±1.02	142.66±0.63	243.54±0.73	254.32±0.83	210.99±1.38
CSF	257.1±1.36	208.43±0.77	226.32±0.4	192.77±1.32	211.66±0.98	185.77±0.61
CHSF	151.88±0.76	148.43±0.85	181.21±1.08	226.32±0.95	153.77±0.65	152.21±1.24
AQSF	462.88±2.65	279.99±1.83	285.66±0.30	293.32±2.40	254.32±0.81	287.10±0.54

Values are expressed as average±SD (n=3); Me = Methanolic extract; HSF = *n*-hexane soluble fraction; CSF = carbon tetrachloride soluble fraction; CHSF = chloroform soluble fraction and AQSF = aqueous soluble fraction.

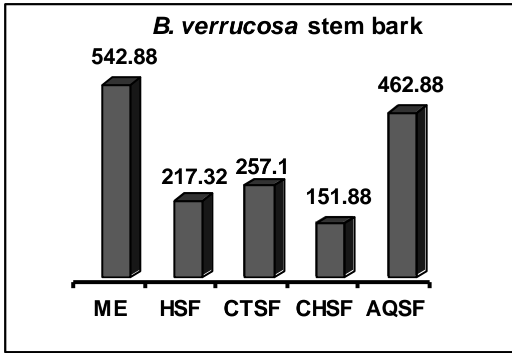

Figure 6.71: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the stem bark of *B. verrucosa*.

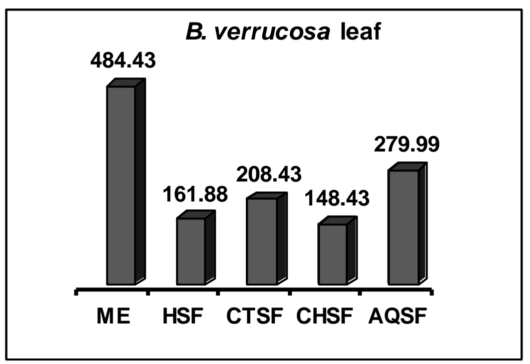

Figure 6.72: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the leaf of *B. verrucosa*.

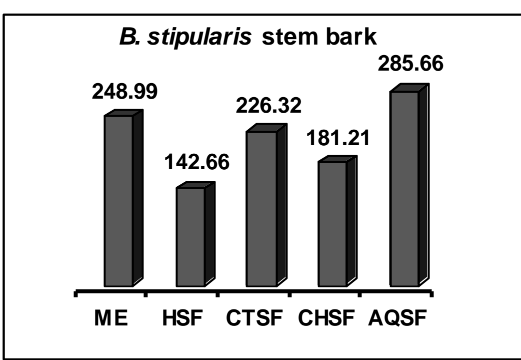

Figure 6.73: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the stem bark of *B. stipularis*.

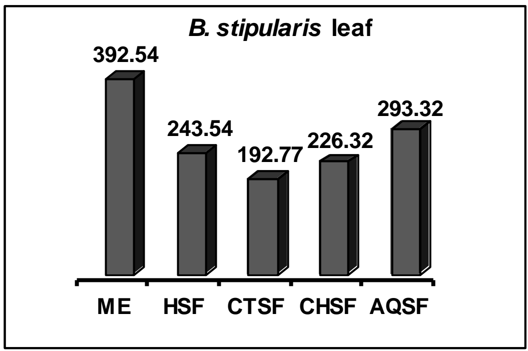

Figure 6.74: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the leaf of *B. stipularis*.

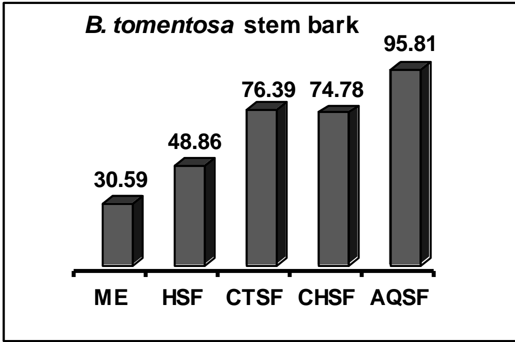

Figure 6.75: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the stem bark of *B. tomentosa*.

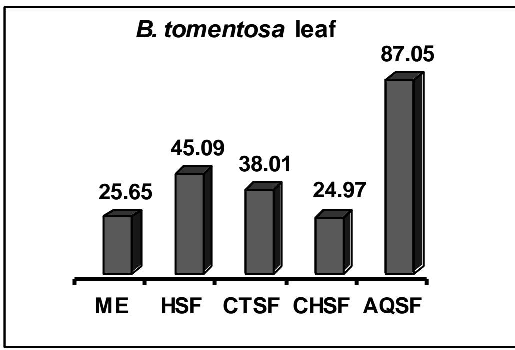

Figure 6.76: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the leaf of *B. tomentosa*.

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Table 6.18: Total antioxidant capacity of the methanolic extracts and its different soluble fractions of *Erythrina* species.

Soluble fractions	*E. fusca*		*E. variegata*	
	Stem bark (mg of ascorbic acid/g of dried extract)	Leaf (mg of ascorbic acid/g of dried extract)	Stem bark (mg of ascorbic acid/g of dried extract)	Leaf (mg of ascorbic acid/g of dried extract)
Me	472.21±2.78	587.66±0.85	454.43±0.42	289.21±1.06
HSF	175.66±0.63	142.21±0.52	175.99±0.73	120.66±0.58
CSF	125.99±0.29	106.10±0.89	221.99±0.46	256.10±0.60
CHSF	216.54±0.47	260.77±0.19	146.54±0.77	96.66±0.92
AQSF	415.10±2.12	153.77±0.63	191.10±1.35	251.77±0.74

Values are expressed as average±SD (n=3); Me = Methanolic extract; HSF = *n*-hexane soluble fraction; CSF = carbon tetrachloride soluble fraction; CHSF = chloroform soluble fraction and AQSF = aqueous soluble fraction.

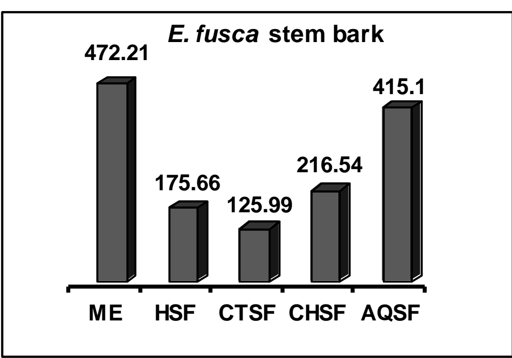

Figure 6.77: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the stem bark of *E. fusca*.

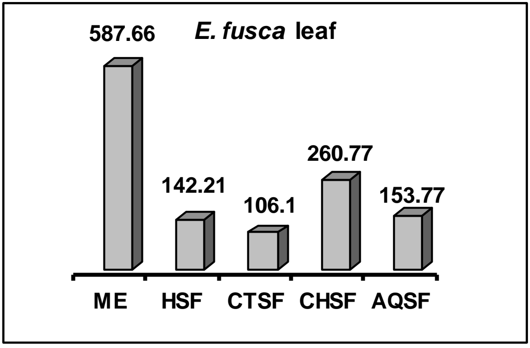

Figure 6.78: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the leaf of *E. fusca*.

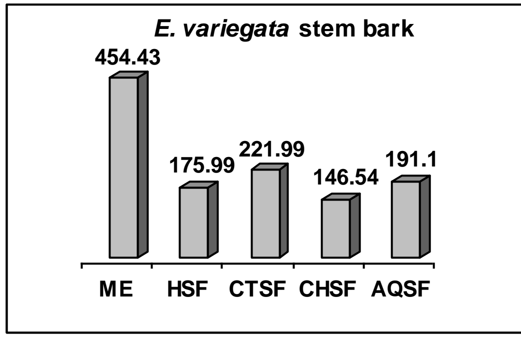

Figure 6.79: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the stem bark of *E. variegata*.

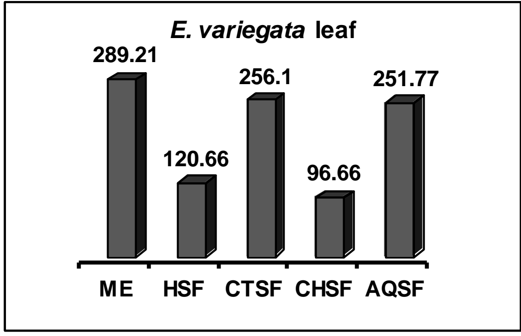

Figure 6.80: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the leaf of *E. variegata*.

7.1 Introduction

One of the major causes of cardiovascular disease is lack of proper blood circulation due to formation of clots. Thrombi or emboli can lodge in a blood vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. This can result in damage, destruction (infarction), or even death of the tissues (necrosis) in that area. A blood clot (thrombus) is formed from fibrinogen by thrombin and is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator (tPA). Fribrinolytic drugs have been used to dissolve thrombi in acutely occluded coronary arteries thereby to restore blood supply to ischaemic myocardium, to limit necrosis and to improve prognosis (Laurence, 1992).

Streptokinase is an antigenic thrombolytic agent used for the treatment of acute myocardial infarction. It reduces mortality as effectively as the nonantigenic altreplase in most infarct patients while having the advantages of being much less expensive. Tissue-type Plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators. All available thrombolytic agents still have significant shortcomings, including the need for large doses to be maximally effective, limited fibrin specificity and a significant associated bleeding tendency. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs (Nicolini *et al*., 1992; Adams *et al*., 1991; Lijnen *et al*., 1991; Marder, 1993; Wu *et al*., 2001). Heparin and Aspirin are only moderately efficient for acceleration of lysis and prevention of reocclusion, but are safe. More selective thrombin inhibitors and antiplatelet agents are more potent, but their safety remains to be confirmed. Continued investigation in this area will provide new insights and promote progress toward the development of the ideal thrombolytic therapy, characterized by maximized stable coronary arterial thrombolysis with minimal bleeding (Collen, 1990).

For the treatment of several diseases herbal preparations have been used since ages and are often perceived as safe because they are "natural" (Gesler, 1992). In India, in recent years, there is increased research on traditional ayurvedic herbal medicines on the basis of their known effectiveness in the treatment of ailments for which they have been traditionally applied. Considerable efforts have been directed towards the discovery and development of natural products from various plant and animal sources which have antiplatelet (Briggs *et al*., 2000; Demrow *et al*., 1995), anticoagulant (Leta *et al*., 2002; Zhguang *et al*., 2000), antithrombotic (Rajapakse, 2005) and thrombolytic activity. Epidemiologic studies have provided evidence that foods with experimentally proved

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antithrombotic effect could reduce risk of thrombosis. Herbs showing thrombolytic activity have been studied and some significant observations have been reported (Yamamoto *et al.*, 2005).

The aim of present study was to investigate the thrombolytic activity of methanol extracts and its different soluble fractions of stem bark and leaf of three *Bridelia* and two *Erythrina* species available in Bangladesh.

7.2 Experimentals

7.2.1 Preparation of sample

The thrombolytic activity of all extractives was evaluated by a method using streptokinase (SK) as standard substance. 10 mg of each plant extract (crude methanol extract and its kupchan fractions of the stem bark of *B. verrucosa*, *B. stipularis*, *B. tomentosa*, *E. fusca* and *E. variegata*) was suspended in 1 ml distilled water and the suspension was shaken vigorously on a vortex mixer. The suspension was kept over night and decanted to remove the soluble supernatant, which was filtered through a 0.22 micron syringe filter. Then this solution is ready for the study to check *in vitro* thrombolytic activity (Prasad, *et al.*, 2007).

7.2.2 Streptokinase (SK)

Commercially available lyophilized Altepase (Streptokinase) vial (Beacon Pharmaceuticals Ltd) of 15,00,000 I.U., was collected and mixed well with 5 ml sterile distilled water. This suspension was used as a stock from which 100µl (30,000 I.U) was used for *in vitro* thrombolysis (Prasad, *et al.*, 2007).

7.2.3 Blood Sample

Whole blood (n=10) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1 ml of blood was transferred to the previously weighed sterile vials and was allowed to form clots.

7.3 Assay for Thrombolytic activity

Aliquots (5 ml) of venous blood were drawn from healthy volunteers which were distributed in ten different pre weighed sterile micro centrifuge tube/Eppendorf tube (1 ml/tube) and incubated at 37 °C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each vial having clot was again weighed to determine the clot weigh (clot weight = weight of clot containing tube – weight of tube alone).

To each vial containing pre-weighed clot, 100 µl aqueous solution of different partitionates along with the crude extracts was added separately. As a positive control,

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100 µl of streptokinase (SK) and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the control vials. All the vials were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, the released fluid was removed and vials were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

% of clot lysis = (wt of released clot /clot wt) × 100

7.4 Results and Discussion

As a part of discovery of cardio protective drugs from natural sources, the extractives of stem bark and leaf of the three *Bridelia* and two *Erythrina* species available in Bangladesh were subjected to assays for thrombolytic activity and the results are presented in Table 7.1 and 7.2. Addition of 100µl SK, a positive control (30,000 I.U.), to the clots and subsequent incubation for 90 minutes at 37 °C, showed 66.7% lysis of clot. On the other hand, distilled water was treated as negative control which exhibited a negligible percentage of lysis of clot (8.12%). The mean difference in percentage of clot lysis between positive and negative control was found to be significant.

In this study, the methanol extract of the stem bark and leaf of *B. verrucosa*, *B. stipularis* and *B. tomentosa* exhibited thrombolytic activity ranging from 20.94% to 36.45%. The *n*-hexane soluble fraction (HSF) from the stem bark and leaf of three *Bridelia* species showed 10.43% to 28.21%. The carbon tetrachloride soluble fraction (CSF) exhibited clot lysis activity from 22.06% to 41.46%, the chloroform soluble fraction (CHSF) from 8.94% to 23.07% and the aqueous soluble fraction (AQSF) revealed thrombolytic activity between 9.24% to 37.04%.

Again, in this study, the methanol extract of the stem bark and leaf of *E. fusca* and *E. variegata* demonstrated thrombolytic activity ranging from 12.45% to 15.65% and 12.28% to 24.48%. The *n*-hexane soluble fraction (HSF), carbon tetrachloride soluble fraction (CSF), the chloroform soluble fraction (CHSF) and the aqueous soluble fraction (AQSF) of the methanolextract of the stem bark and leaf of *E. fusca* and *E. variegata* exhibited clot lysis ranging from 14.27% to 15.33% & 12.65% to 23.54%, 8.02% to 22.75% & 5.62% to 15.78%, 6.34% to 12.19% & 9.46% to 46.73, respectively.

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Table 7.1: Thrombolytic activity (in terms of % clot lysis) of *B. verrucosa*, *B. stipularis* and *B. tomentosa*.

***B. verrucosa* (stem bark)**				
Fractions	W_1	W_2	W_3	% of lysis
Me	4.8956 ± 0.15	5.3520 ± 0.06	5.2165 ± 0.02	29.69 ± 0.97
HSF	4.8682 ± 0.00	5.4435 ± 0.10	5.3428 ± 0.09	17.50 ± 0.54
CSF	4.8954 ± 0.17	5.2189 ± 0.04	5.1467 ± 0.02	22.32 ± 0.95
CHSF	4.8154 ± 0.05	5.2185 ± 0.08	5.1255 ± 0.01	23.07 ± 0.86
AQSF	4.7817 ± 0.18	5.3107 ± 0.08	5.2130 ± 0.13	18.47 ± 2.24
***B. verrucosa* (leaf)**				
	W_1	W_2	W_3	% of lysis
Me	4.8756 ± 0.08	5.3386 ± 0.03	5.2271 ± 0.01	24.08 ± 0.68
HSF	4.8682 ± 0.06	5.4435 ± 0.00	5.3428 ± 0.04	17.50 ± 0.41
CSF	4.9762 ± 0.09	5.2781 ± 0.10	5.1763 ± 0.02	33.72 ± 1.05
CHSF	4.8813 ± 0.10	5.3892 ± 0.03	5.3438 ± 0.09	8.94 ± 0.22
AQSF	4.6689 ± 0.11	5.2056 ± 0.07	5.1560 ± 0.13	9.24 ± 1.86
***B. stipularis* (stem bark)**				
	W_1	W_2	W_3	% of lysis
Me	4.7870 ± 0.10	5.1582 ± 0.04	5.0229 ± 0.08	36.45 ± 0.66
HSF	4.7747 ± 0.09	5.2446 ± 0.10	5.1151 ± 0.04	27.56 ± 0.73
CSF	4.8069 ± 0.01	5.2529 ± 0.06	5.1196 ± 0.01	30.06 ± 0.55
CHSF	4.6709 ± 0.10	5.0970 ± 0.09	5.0039 ± 0.02	21.85 ± 0.47
AQSF	4.7420 ± 0.08	5.3244 ± 0.10	5.1721 ± 0.01	28.73 ± 0.86
***B. stipularis (*leaf)**				
Fractions	W_1	W_2	W_3	% of lysis
Me	4.9863 ± 0.12	5.4876 ± 0.05	5.3486 ± 0.04	27.73 ± 0.92
HSF	4.8854 ± 0.11	5.3218 ± 0.06	5.1987 ± 0.01	28.21 ± 0.82
CSF	4.8639 ± 0.11	5.3465 ± 0.07	5.2188 ± 0.03	26.46 ± 0.73
CHSF	4.9769 ± 0.07	5.4712 ± 0.05	5.3982 ± 0.02	14.77 ± 0.51
AQSF	4.8792 ± 0.11	5.3478 ± 0.03	5.2168 ± 0.03	27.96 ± 0.79
***B. tomentosa* (stem bark)**				
Fractions	W_1	W_2	W_3	% of lysis
Me	4.8708 ± 0.11	5.4598 ± 0.05	5.3391 ± 0.01	20.49 ± 0.72
HSF	4.7375 ± 0.10	5.2563 ± 0.09	5.2020 ± 0.02	10.43 ± 0.48
CSF	4.7678 ± 0.10	5.1586 ± 0.06	5.0724 ± 0.01	22.06 ± 0.32
CHSF	4.7813 ± 0.09	5.2239 ± 0.03	5.1609 ± 0.03	16.13 ± 0.43
AQSF	4.8321 ± 0.03	5.2587 ± 0.05	5.1007 ± 0.01	37.04 ± 0.78
***B. tomentosa* (leaf)**				
Fractions	W_1	W_2	W_3	% of lysis
Me	4.7583 ± 0.09	5.2786 ± 0.03	5.0973 ± 0.01	34.85 ± 1.00
HSF	4.8953 ± 0.17	5.3865 ± 0.11	5.2804 ± 0.08	21.60 ± 1.37
CSF	4.8537 ± 0.00	5.2895 ± 0.09	5.1087 ± 0.06	41.46 ± 0.98
CHSF	4.9765 ± 0.07	5.4487 ± 0.15	5.3786 ± 0.02	14.85 ± 1.11
AQSF	4.8569 ± 0.04	5.2786 ± 0.19	5.1874 ± 0.01	21.63 ± 2.33

W_1 = Weight of vial alone; W_2 = Weight of clot containing vial; W_3 = Weight of clot containing vial after clot disruption; Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction

Table 7.2: Thrombolytic activity (in terms of % clot lysis) of *E. fusca* and *E. variegata*.

E. fusca **(stem bark)**				
Fractions	W_1	W_2	W_3	% of lysis
Me	4.7355 ± 0.12	5.1508 ± 0.01	5.0991 ± 0.09	12.45 ± 1.31
HSF	4.8124 ± 0.18	5.2631 ± 0.10	5.1988 ± 0.03	14.27 ± 0.98
CSF	4.8242 ± 0.34	5.2458 ± 0.07	5.1499 ± 0.06	22.75 ± 0.59
CHSF	4.7377 ± 0.25	5.1211 ± 0.02	5.0968 ± 0.12	6.34 ± 0.47
AQSF	4.8599 ± 0.09	5.2391 ± 0.01	5.1905 ± 0.01	12.82 ± 0.35
E. fusca **(leaf)**				
Fractions	W_1	W_2	W_3	% of lysis
Me	4.8122 ± 0.16	5.2743 ± 0.01	5.2035 ± 0.02	15.65 ± 0.94
HSF	5.2202 ± 0.21	5.7155 ± 0.01	5.6368 ± 0.10	15.33 ± 0.32
CSF	5.1043 ± 0.13	5.6606 ± 0.06	5.6160 ± 0.14	8.02 ± 1.78
CHSF	4.8928 ± 0.23	5.2941 ± 0.02	5.2452 ± 0.04	12.19 ± 1.07
AQSF	4.9631 ± 0.17	5.2375 ± 0.18	5.1954 ± 0.08	15.35 ± 0.35
E. variegata **(stem bark)**				
Fractions	W_1	W_2	W_3	% of lysis
Me	4.8759 ± 0.09	5.4632 ± 0.22	5.3194 ± 0.03	24.48 ± 1.97
HSF	4.7659 ± 0.20	5.3174 ± 0.02	5.1876 ± 0.02	23.54 ± 0.60
CSF	4.8216 ± 0.17	5.3267 ± 0.13	5.2885 ± 0.06	5.62 ± 0.90
CHSF	4.7953 ± 0.09	5.1876 ± 0.03	5.0278 ± 0.05	40.73 ± 1.67
AQSF	4.8712 ± 0.09	5.3718 ± 0.07	5.2368 ± 0.03	26.97 ± 0.94
E. variegata **(leaf)**				
Fractions	W_1	W_2	W_3	% of lysis
Me	4.7761 ± 0.15	5.2738 ± 0.07	5.2127 ± 0.10	12.28 ± 0.18
HSF	4.8766 ± 0.18	5.3470 ± 0.06	5.2875 ± 0.07	12.65 ± 0.54
CSF	4.7760 ± 0.07	5.2246 ± 0.05	5.1538 ± 0.04	15.78 ± 0.40
CHSF	4.9860 ± 0.11	5.5124 ± 0.20	5.4626 ± 0.19	9.46 ± 1.44
AQSF	4.8460 ± 0.19	5.5023 ± 0.01	5.2213 ± 0.01	42.82 ± 1.43

W_1 = Weight of vial alone; W_2 = Weight of clot containing vial; W_3 = Weight of clot containing vial after clot disruption; Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction

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SUMMARY

This dissertation describes the isolation and structure elucidation of secondary metabolites from four plants *Bridelia verrucosa, B. stipularis, B. tomentosa* (Family: Phyllanthaceae) and *Erythrina fusca* (Family: Fabaceae) as well as biological studies of the extractives from these plants along with *E. variegata* (Family: Fabaceae). A total of nineteen compounds were isolated of which three appear to be new. The structures of the isolated compounds were elucidated mainly by spectroscopic studies including high field NMR.

Kupchan partitioning method was used for partitioning the crude methanolic extract and the various chromatographic techniques were used to isolate the pure compounds from the investigated plants. For the *n*-hexane soluble materials of the stem bark of *B. verrucosa*, gel permeation (Lipophilic Sephadex LH-20) chromatography was used followed by column and preparative thin layer chromatography (PTLC). The stem bark of *B. verrucosa* afforded eight compounds *viz.* glochidonol (BVSP-1, **46**), brassicasterol (BVSP-2, **47**), friedelin (BVSP-4, **48**); 5β-24S-ethylcholestan-3β-ol (BVSP-6, **49**); stigmasterol (BVSP-34, **50**); 5,5'-dihydroxysesamine (BVBC-2, **51**), 3-ketoleanane (**53**) and pinoresinol (BVS-65, **54**). Except stigmasterol (**50**) and pinoresinol (**54**) this is the first report of the isolation of compounds **46-49**, **53** and **54** from the genus *Bridelia* and **51** has been identified as a new natural product.

In case of the stem bark of *B. stipularis* and *B. tomentosa* vacuum liquid chromatography was used for fractioning the methanolic extract followed by gel permeation chromatography and then PTLC was done to isolate two triterpenes namely, glut-5(6)-en-3-one (BS-01, **55**), glut-5(6)-en-3α-ol (BS-06, **56**) and a cholestane type compound (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one (BS-11, **57**). All these are the first report of their occurrence from *B. stipularis* while **57** is a new compound. On the other hand, phytochemical investigation of methanol extract of the stem bark of *B. tomentosa* yielded friedoolean-5(6),14(15)-dien-3-one (BT-1, **58**), β-taraxerol (BT-6, **59**), D_4-stigmasterone (BT-8, **60**) and lupeol (BT-14, **61**). This is the first report of their occurrence from this plant and **58** is a novel compound.

Again, for the stem bark of *E. fusca* column chromatography of the carbon tetrachloride soluble materials was carried out. The fractions were then subjected to PTLC to afford three flavonoids namely, shinpterocarpin (EF-7, **62**), lupinifolin (EF-8, **63**) and 3,9-

dihydroxy-4-(3,3-dimethylallyl)[6a*R*,11a*R*]-pterocarpan (**64**) and a steroid, β-sitosterol (EF-38, **65**). All these are the first report from *E. fusca*.

The crude extracts and organic soluble material of the investigated plants and some purified compounds were screened for their antimicrobial activity against a wide range of Gram-positive and Gram-negative microorganisms and fungi by the standardized disc diffusion method. Kanamycin and Griseofulvin were used as reference drugs for the test.

All the samples showed mild to moderate antimicrobial activity against thirteen Gram positive and Gram negative bacterial strains and three fungi, where the stem bark extract of *B. tomentosa* demonstrated the highest inhibition of growth against *B. cereus* (23.2 mm) and *C. albicans* (17.5 mm). The purified compounds isolated from the investigated plants showed mild to moderate antibacterial activity. BS-01 (**55**) and BS-06 (5**6**) revealed strong inhibition of growth of *E. coli* (22.7 mm) and *C. albicans* (20.8 mm), respectively.

The plants were subjected to brine shrimp lethality bioassay for probable cytotoxicity. The aqueous soluble fraction of the leaf of *B. stipularis* revealed the highest cytotoxic activity with LC_{50} value of 12.59 µg/ml. On the other hand, the *n*-hexane soluble fraction of the stem bark of *E. variegata* revealed the strongest LC_{50} value of 14.66 µg/ml. The isolated pure compound EF-8 (**62**) displayed the strongest cytotoxic activity with LC_{50} and LC_{90} values of 3.17 and 62.95 µg/ml, respectively as compared to standard vincristine sulfate (LC_{50} 0.45 µg/ml and LC_{90} 10 µg/ml).

Free radical scavenging activity, total antioxidant capacity and total phenolic content of the extractives were also investigated. Free radical scavenging assay of the methanol extract of the leaf of *B. verrucosa* revealed highest activity having IC_{50} of 6.35 µg/ml while the chloroform soluble fraction of the leaf of *E. fusca* showed IC_{50} at 5.50 µg/ml. The IC_{50} (7.3 µg/ml) of the isolated pure compound BVBC-2 (**51**) indicated strong free radical scavenging activity.

The aqueous and chloroform soluble materials of the leaf of *B.stipularis* demonstrated promising total pehonolic content (98.76 and 94.52 mg of GAE/100 g of dried extract). On the other hand, the highest total antioxidant capacity of methanolic extract of the leaf of *B. tomentosa* and *E. fusca* were found as 612.54 and 587.66 mg of ascorbic acid/g of dried extract, respectively.

All the extractives of five plants were also studied for their thrombolytic potential. The carbon tetrachloride soluble fraction and methanol extract of leaf and aqueous soluble material of bark of *B. tomentosa,* methanol extract of bark of *B. stipularis* and carbon tetrachloride soluble partitionate of leaf of *B. verrucosa* exhibited highest thrombolytic

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activity with clot lysis value of 41.46%, 34.85%, 37.04%, 36.45% and 33.72%, respectively. Standard streptokinase was used as positive control which exhibited 61.50% lysis of clot while the negative control water revealed 2.56% clot lysis.

In conclusion, a total 19 compounds including three new molecules (**51**, **57** and **58**) have been isolated from *B. verrucosa*, *B. stipularis* and *B. tomentosa*, respectively. In case of biological investigation, the methanolic extract of the investigated plants and their Kupchan fractions showed significant antibacterial, cytotoxic, antioxidant and thrombolytic activities. These plants can be further studied in order to find out their unexplored efficacy against other diseases as well as to isolate more chemically interesting and biologically important drug candidates.

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LIST OF PUBLICATIONS

1. **Adeeba Anjum**, Mohammad R. Haque, Mohammad S. Rahman, Choudhury M. Hasan, Md. Ekramul Haque, Mohammad A. Rashid. 2011. *In vitro* antibacterial, antifungal and cytotoxic activity of three Bangladeshi *Bridelia* species. *International Research of Pharmacy and Pharmacology*. **1(7)**, 149-154. ISSN 2251-0176.
2. **Adeeba Anjum**, M. A. Sikder, Mohammad R. Haque, Choudhury M. Hasan, Mohammad A. Rashid. 2013. *In vitro* antioxidant and thrombolytic activities of *Bridelia s*pecies growing in Bangladesh. *J. Sci. Res*. **5(2)**, 343-351.