ANTI-CANCER AND CYTOTOXIC CONSTITUENTS FROM SOME BANGLADESHI MEDICINAL PLANTS

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ABSTRACT

This thesis describes the isolation and structure elucidation of secondary metabolites from three plants namely *Polyalthia simiarum* Hook. F. and Thom. (Family- Annonaceae), *Glochidion multiloculare* (Roxb. *ex* Willd.) Muell.-Arg. and *Glochidion lanceolarium* (Roxb.) Voigt (Family- Euphorbiaceae) as well as biological studies (anti-cancer, antioxidant, cytotoxic, antimicrobial, analgesic and antiinflamatory) of the extractives from these plants. A total of twelve compounds were isolated of which one (PSB-001, **162**) has been reported as a new compound by us (Kabir *et al*., 2010). The structures of the isolated compounds were elucidated mainly by high field NMR and mass spectroscopic techniques.

The petroleum ether extract of the stem bark of *P. simiarum* afforded four compounds *viz*. PSB-001 (2-Oxo-14,15-bisnor-3,11*E*-kolavadien-13-one, **162**), PSB-007 (Kolavenic acid, **163**), PSB-004 (16β-Hydroxycleroda-3,13(14)*Z*-dien-15,16-olide, **164**) and PSB-008 (16-Oxocleroda-3,13(14)*E*-dien-15-oic acid, **165**). These four compounds are the first report of their occurance from this respective plant. Among these compounds PSB-001 (2-Oxo-14,15-bisnor-3,11*E*-kolavadien-13-one, **162**) represents the second report of the isolation of any 18-carbon containing bisnor-clerodane diterpenoid from nature and it appears to be new. The petroleum ether soluble fraction of the methanol extract of the stem bark of *Glochidion multiloculare* provided five comounds namely, GM-022 (3-*Epi*-lupeol, **166**), GM-023 (Lupeol, **167**), GM-029 (Glochidone, **168**), GM-032 (Glochidonol, **169**) and GM-035 (Glochidiol, **170**). On the other hand, chemical investigation of carbon tetrachloride soluble fraction of the methanol extract of the stem bark of *Glochidion lanceolarium* yielded three compounds GL-022 (Epilupeol, **171**), GL-023 (Glochidonol, **172**) and GL-035 (Glochidone, **173**).

The extractives of *P. simiarum, G. multiloculare* and *G. lanceolarium* were subjected to assay for the anti-cancer activity against EAC tumor bearing mice. The antitumor activity of the ethyl acetate (EA) extract of *P. simiarum* was determined against Ehrlich ascites carcinoma (EAC) in mice at 25 mg/kg and 50 mg/kg body weight intraperitoneally. Significant (p<0.001) increase of survival time by (25 ± 0.57 and 27 ± 0.40 days) by the EA extract treated tumor bearing mice was confirmed with respect to the control group (22 ± 0.12 days). The antitumor activity of the extract/fractions (MEGM, PEFGM, CTFGM, CHFGM)

of stem bark of *G. multiloculare* and also the extracts (MEGL, PEFGL, CTFGL) of *G. lanceolarium* was determined against Ehrlich ascites carcinoma (EAC) in mice at 20 mg/kg body weight intraperitoneally. Significant (p<0.001) increase of survival time by 24 ± 0.12, 26 ± 0.40, 21 ± 0.12 and 27±0.42 days was observed by the extracts MEGM, PEFGM, CTFGM and CHFGM treated tumor bearing mice with respect to the control group (20 ± 0.12 days), whereas the increase of survival time by (25 ± 0.32, 26 ± 0.10 and 23 ± 0.19 days) by the extracts MEGL, PEFGL and CTFGL treated tumor bearing mice was also observed with respect to the control group (20 ± 0.12 days). Treatment with different extracts of the above mentioned plants decreased the intraperitonial tumor burden, thereby reducing the tumor volume, tumor weight, viable tumor cell count and increased the life span of the tumor bearing mice. Hematological studies revealed that the heamoglobin (Hb) content was decreased in EAC treated mice whereas restoration to close to normal levels was observed in extract/fractions treated animals. There was a significant (p<0.001) decrease in RBC and increase in WBC counts in extract/fractions treated animals when compared to EAC affected animals.

All the extractives and purified compounds of *P. simiarum, G. multiloculare,* and *G. lanceolarium* were studied for free radical scavenging activity where *tert*-butyl-1-hydroxytoluene (TBHT) was used as reference standard. In this study, the extracts MEGM, MEGL, PEFGM, PEFGL and PEFPSB of the above mentioned plants showed highest free radical scavenging activity with IC50 values 16.40, 18.32, 19.85, 20.29 and
21.5 µg/ml, respectively. The carbon tetrachloride soluble material (CTFGL) of *G. lanceolarium* exhibited promising antioxidant activity having IC50 value of 22.24 µg/ml. Compound PSB-004 (**164**) and ethyl acetate fraction (EAFPSB) of *P. simiarum* revealed potential antioxidant capacity with IC50 values of 23.5 and 24.5 µg/ml, respectively**.** In this investigation, the CTFGM, CFFGM and AQFGM of *P. simiarum* showed moderate free radical scavenging activity with IC50 values of 27.41, 32.30, and 38.17 µg/ml, respectively.

The crude extracts/fractions of the plants *P. simiarum, G. multiloculare* and *G. lanceolarium* and two pure compounds (PSB-004 and PSB-007) were subjected to brine shrimp lethality bioassay for probable cytotoxic activity. The PEFPSB of *P. simiarum* exhibited highest cytotoxicity with LC50 value of 1.91 µg/ml whereas EAFPSB of *P. simiarum* showed promising activity with LC50 value of 3.65 µg/ml. The degree of lethality was directly proportional to the concentration of extract ranging from the lowest concentration (0.78125

μg/ml) to the highest concentration (400 μg/ml). The positive control drug, vincristine sulfate showed LC50 at 0.32 μg/ml. The LC50 values of PEFGM, CTFGM, CFFGM, MeEGM and AQFGM of *G. multiloculare* were found to be 3.11, 4.96, 7.56, 9.23 and 16.32 μg/ml), respectively. The PEFGM exhibited significant mortality whereas CTFGM and CFFGM showed moderate activity. Besides these, the LC50 values PEFGL, CTFGL, CFFGL, MeEGL and AQFGL of *G. lanceolarium* were found to be 3.09, 6.70, 9.18, 14.24 and 17.68 μg/ml, respectively. In this experiment, compound PSB-004 (**164**) isolated from of *P. simiarum* demonstrated strong cytotoxic activity with LC50 value of 2.29 μg/ml, whereas compound PSB-007 (**163**) of the same plant showed moderate activity with LC50 value of 8.84 μg/ml.

The extractives of the experimental three plants and two purified compounds (PSB-004 and PSB-007 isolated from *P. simiarum*) were screened for their antimicrobial activity against a number of test organism (both Gram-positive, Gram-negative and fungi) by the standardized disc diffusion method. The results obtained were compared with that produced by the standard antibiotic, Kanamycin. The petroleum ether (PEFPSB) and ethyl acetate (EAFPSB) soluble fractions of *P. simiarum* showed promising antibacterial activity with the average zone of inhibition of 20-28 mm and 21-28 mm, respectively at 400 μg/disc. The pet-ether soluble fraction revealed the highest activity against the growth of *B. megaterium* having the zone of inhibition of 28 mm. In this study, the zones of inhibition produced by the extracts CFFGM, PEFGM and CTFGM of *G. multiloculare* ranged from 9-12 mm, 8-10 mm and 7-9 mm, respectively. The carbon tetrachloride fraction (CTFGL) of *G. lanceolarium* was screened against 12 test bacteria and 3 fungi. The zone of inhibition was 07-10 mm. The fraction exhibited moderate activity against the test bacteria *Salmonella typhi* and *Vibrio parahemolyticus* and *Candida albicans, Aspergillus niger* whereas, the petroleum ether soluble fraction (PEFGL) showed poor activity against the test organism. In case of pure compounds, PSB-004 (**164**) obtained from *P. simiarum* demonstrated moderate antimicrobial activity against 12 test bacteria and 3 fungi, having the zone of inhibition 10-14 mm whereas, PSB-007 (**163**) showed poor activity against test bacteria and fungi.

The extracts/fractions of investigated plants were screened for probable analgesic activity using Swiss albino mice as experimental animal. The EA and PE extracts of *P. simiarum* showed analgesic activity in both tail flick and acetic acid induced methods at 50 and 100 mg/kg body weight. Among the extracts, the EA extract revealed a dose dependent analgesic activity in the methods. Both the extractives of *G. multiloculare* (MEGM, PEFGM, CTFGM

and CHFGM) and *G. lanceolarium* (MEGL, PEFGL and CTFGL) at 100 mg/kg b.w., produced significant increase in pain threshold in tail flick method whereas significantly reduced the writhing caused by acetic acid. All the tested extracts/fractions of investigated plants were capable to exhibit moderate analgesic activity.

The extractives of *P. simiarum, G. multiloculare* and *G. lanceolarium* were subjected to assay for the anti-inflamatory activity using Male Wister rats and Swiss albino mice as experimental animal. The EA and PE extracts of *P. simiarum,* showed anti-inflammatory activities at 50- and 100 mg/kg body weight. The EA extract reduced the paw edema considerably (27.5% and 39.10% inhibition after 4h). In addition the extractives of *G. multiloculare* (MEGM, PEFGM, CTFGM and CHFGM) and *G. lanceolarium* (MEGL, PEFGL and CTFGL) at the dose 100 mg/kg body weight showed anti-inflammatory activity. The CTFGL of *G. lanceolarium* reduced the paw edema (0.85± 0.07, after 4h), when compared to carrageenan induced control mice. Therefore, the extractives of the above mentioned plants were found to exhibit moderate anti-inflammatory activity.

For the statistical validity of the results, all the tests were performed in triplicate or more and the data have been presented as mean ± standard deviation.

ACKNOWLEDGEMENTS

All praises to the Almighty Allah who deserves all the credits for successful completion of this thesis.

I wish to convey my heartiest gratitude to my supervisor professor Dr. A. M. Sarwaruddin Chowdhury, Professor of the Department of Applied Chemistry and Chemical Engineering, Faculty of Engineering and Technology, University of Dhaka, for his valuable suggestions, enthusiastic and outstanding guidance and competent supervision during the tenure of research.

I wish to acknowledge with enormous gratitude to my reverend Co-supervisor Professor Dr. Mohammad Abdur Rashid, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka for his day-to-day supervision, constructive suggestions, valuable criticisms and keen interests to complete my research.

I am profoundly indebted to Professor Dr. Choudhury Mahmood Hasan, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, for his extended co-operation and enthusiastic guidance.

I like to convey my respect and gratitude to Professor Dr. Sayed Md. Samsuddin, Chairman, Department of Applied Chemistry and Chemical Engineering, and all the teachers, especially Prof. Dr. Monoronjan Saha, Prof. Dr Abdul Quaiyyum, Prof. Dr. Md. Nurul Amin, Dr. Md. Nurnabi, and also all staffs of the Department of Applied Chemistry and Chemical Engineering, University of Dhaka for their support and cooperation.

It gives me immense pleasure to express sincere appreciation and gratefulness to Dr. Rasheduzzaman Chowdhury, Dr. Rashedul Haque, Dr. Md. Sharifur Rahman, Mr. Md. Abul Kaisar, Assistant Professor, Department of Pharmaceutical Chemistry and Mr. Md. Al Amin Sikder, Lecturer, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka for providing all possible supports during the course of this research work.

I would also like to thank all the staffs of Biomedical Research Center and Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka for their help to carry out this research work.

I am heartly thankful to Ronok Jahan, Lecturer, Department of Pharmacy University of Rajshahi for her for her help and cooperation during the course of this research work.

I acknowledge the valuable co-operation of Dr. Nasim Sultana S.S.O, Dr. Md. Sohrab Hossain S.S.O, Mr. Shahidul Islam, S.S.O, Analytical Research Division, BCSIR Dhaka, for acquiring spectral data and to Dr. Sarder Nasir Uddin, S.S.O, Bangladesh National Herbarium for identifying the plants.

I am also thankful to Dr. Rokeya Begum, Dept. of Agricultural Chemistry, Sher-e-Bangla Agricultural University, Dhaka and Dr. Alomgir Hossain, Assistant Professor Rajendra College, Faridpur for their help and cooperation during the course of this research work.

I express heartiest gratitude to my mother, brothers and sisters for their enthusiasm and prayerful concern towards completion of the research especially to my eldest brother Dr. Jaglul Kabir and Sister-in-law Nurun Nahar Begum, who helped me to carry out the thesis in various ways.

I express my deepest affection, appreciation and thanks to my husband Dr. M. Asaduzzaman and my beloved daughter Sadia Farhin who encouraged me with their love and understanding during difficult moments and also shared my joy and success. Both of them have made immense contribution to my study and life by their support, inspiration, understanding and sacrifices.

Finally, I am grateful to the Bose Centre for Advanced Study and Research in Natural Sciences, University of Dhaka and Bangladesh Science Foundation for financial and Technical /academic support to carry out the research work.

March, 2014 Selina Kabir

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CHAPTER 1

Introduction

1.1 Rationale of the work

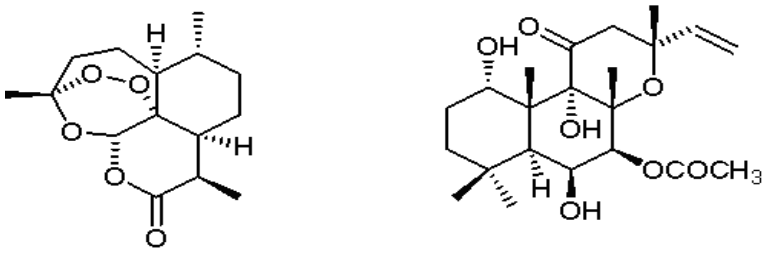
Mankind has been using plants as therapeutic agents for thousands of years and continues to rely on them for health care. This fact seen from the use of natural herbs to cure disease and relieve physical sufferings. The importance of herbs dates back to ancient times for their food value as well as invaluable medicinal properties. Disease emerged with the emergency of life on the earth and the drugs came with the same peace. Though, now a days, most of the medicines, cosmetics, chemicals etc. are synthetically prepared, however, in many cases their origin is natural and more specifically of plants sources (World of Science, 1980).

Plants have formed the basis for traditional systems of medicine which have been used for thousands of years in countries such as China (Chang, 1986) and India (Kapoor, 1990). The use of plants in the traditional medicine of many other cultures has been 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 WHO that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care. Plant products also play an important role in the health care systems of the remaining 20% of the population, mainly residing in the 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 (Arvigo *et al*., 1993). 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 (Fansworth *et al.*, 1985).

Plants are the important source of a diverse range of chemical compounds. Some of these compounds possessing a wide range of pharmacological activity are either impossible or troublesome to synthesize in the laboratory. A phytochemist uncovering these resources

is producing useful materials for screening programs for drug discovery. Emergences of newer diseases also lead the scientists to go back to nature for newer and more effective molecules.

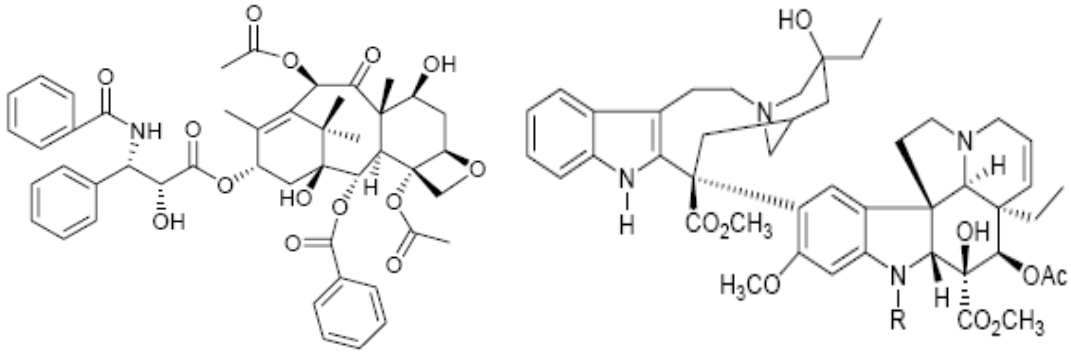
Examples of traditional medicine providing leads to bioactive natural products abound. Artemisinin (qinghaosu) (**1**) is the antimalarial sesquiterpene from a Chinese medicinal herb *Artemisia annua* (Wormwood, Fam. Asteraceae) used in herbal remedies since ancient times. Forskolin (**2**) is the antihypertensive agent from *Coleus forskohlii* Briq (Fam. Laminaceae), a plant whose use was described in ancient Hindu Ayurvedic texts (Bhatt *et al.*, 1988).



1 2

Figure 1.1: Artemisinin (**1**) and forskolin (**2**)

Paclitaxel (Taxol) (**3**) is a natural product derived from the *Taxus baccata* (yew tree, Fam. Taxaceae). It is an important natural product that has made an enormous impact on medical history. It interacts with tubulin during the mitotic phase of the cell cycle, and thus prevents the disassembly of the microtubules and thereby interrupts the cell division (Wani *et al.*, 1991). The original target disease for the compound was ovarian and breast cancers, but now it is used to treat a number of other human tissue proliferating diseases as well (Strobel *et al.*, 2004). A case of serendipity is the discovery of the so-called vinca alkaloids, vincristine (**4**) and vinblastine (**5**), in *Catharanthus roseus*. A random screening programme (conducted at Eli Lilly and Company) of plants with antineoplastic activity found these anticancer agents in the 40th of 200 plants examined. Ethnomedicinal information attributed an anorexigenic effect (i.e. causing anorexia) to an infusion from the plant (Tyler, 1986).



3 **4** R=CHO

5 R=CH_3

Figure 1.2: Paclitaxal (**3**), vincristine (**4**) and vinblastine (**5**)

Many higher plants contain novel metabolities with antimicrobial and antiviral properties. However, in the developed world almost all clinically used chemotherapeutics have been produced by *in vitro* chemical synthesis.

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

There are several familiar approaches to find out lead compounds from plants (Fig 1.3) 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.

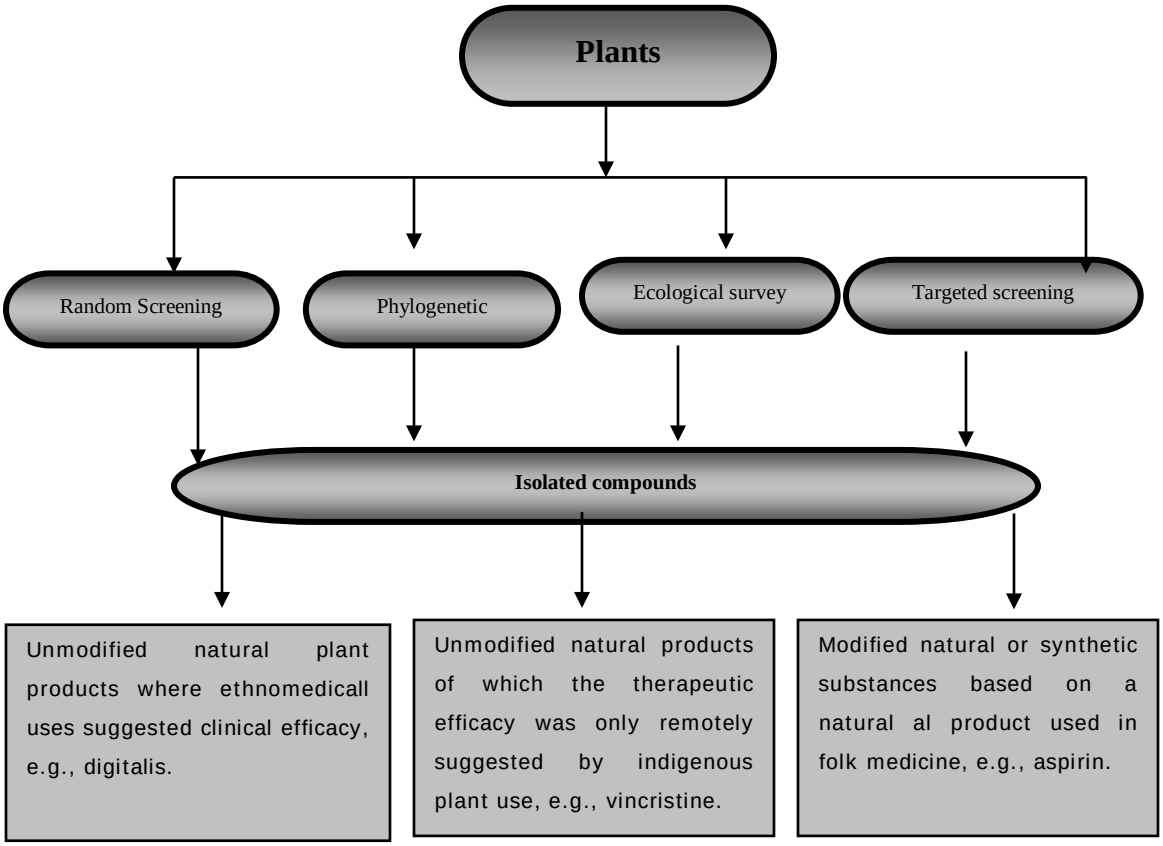


Figure 1.3: Lead compound search and utilization from plants

1.2 Anti-cancer activity

Cancer, the second leading cause of death worldwide next to cardiovascular diseases, is a group of more than 100 different diseases, characterized by uncontrolled cellular growth, local tissue invasion, and distant metastases (Dashora *et al*., 2010). Cancer is caused by internal factors (tobacco, chemicals, radiations and infectious organisms) and external factors (mutation, hormones and immune conditions) (Kuper *et al*., 2002) and can be treated with surgery, radiation, chemotherapy, hormone therapy and biological therapy. Chemotherapy is still a major challenge to the cancer patients because such highly potent drug can be toxic and less than 1% of injected drug molecules can reach their target cells, whereas the rest may damage healthy cells and tissue especially bone marrow, epithelial tissues, reticulo-endothelial system and gonads (Kathiriya *et al*., 2010). Multidisciplinary scientific investigations are making best efforts to combat this disease, but the sure-short, perfect cure is yet to be brought into world medicine. Moreover, the rate of increase of

cancer incidence and lack of anticancer drugs has forced scientists to pharmacological and chemical investigation of anticancer agents from medicinal plants (Koduru *et al*., 2006). The worldwide upsurge use of the herbal preparation and medicinal plants with its isolated active compounds has provided one of the most importance sources for pharmaceutical industry for lead compound. Furthermore, over a 100 new products are in clinical development, particularly as anticancer agents and anti-infectives (Hafidh *et al*., 2009). Emerging evidence suggests that a number of plants are known to be the source of useful drugs in modern medicine (Sadiq *et al*., 2009) and have been accepted currently as one of the main source of cancer chemoprevention drug discovery and development (Gonzales and Valerio, 2006) due to their diverse pharmacological properties including cytotixic and cancer chemopreventive effects (Dahiru and Obidoa, 2007). Over 60% of currently used anti-cancer agents are derived in one-way or another from natural sources, including plants, marine organisms and microorganisms (Newman *et al*., 2003). It is estimated that more than 50% of all the patients diagnosed with cancer explore complementary and alternative medicine – especially herbal medicine (Nelson et al., 2004). Although, the mechanism of interaction between phytochemicals and cancer cells has been studied extensively and augmented the interest of pharmalogical evaluation of various plants used in Bangladeshi traditional systems of medicine (Kumar *et al*., 2007). Hence, the natural products now have been contemplated of exceptional value in the development of effective anticancer agents with minimum host cell toxicity.

Different types of antibiotics such as *Actinomycin D* (**6**) and *Bleomycin* (**7**) are used as anticancer drugs. Antibiotic is a chemical substance produced by a microorganism which is also sensitive to other microorganism. These compounds produce their antitumor effects by forming relatively stable complexes with DNA, thereby inhibiting DNA or RNA synthesis or both (Henry, 1974; Montgomery and Struck, 1973; Brockmann *et al*., 1956; Cobb and Walkler, 1958).

Structure of some well known chemotherapeutic agents (antibiotic)

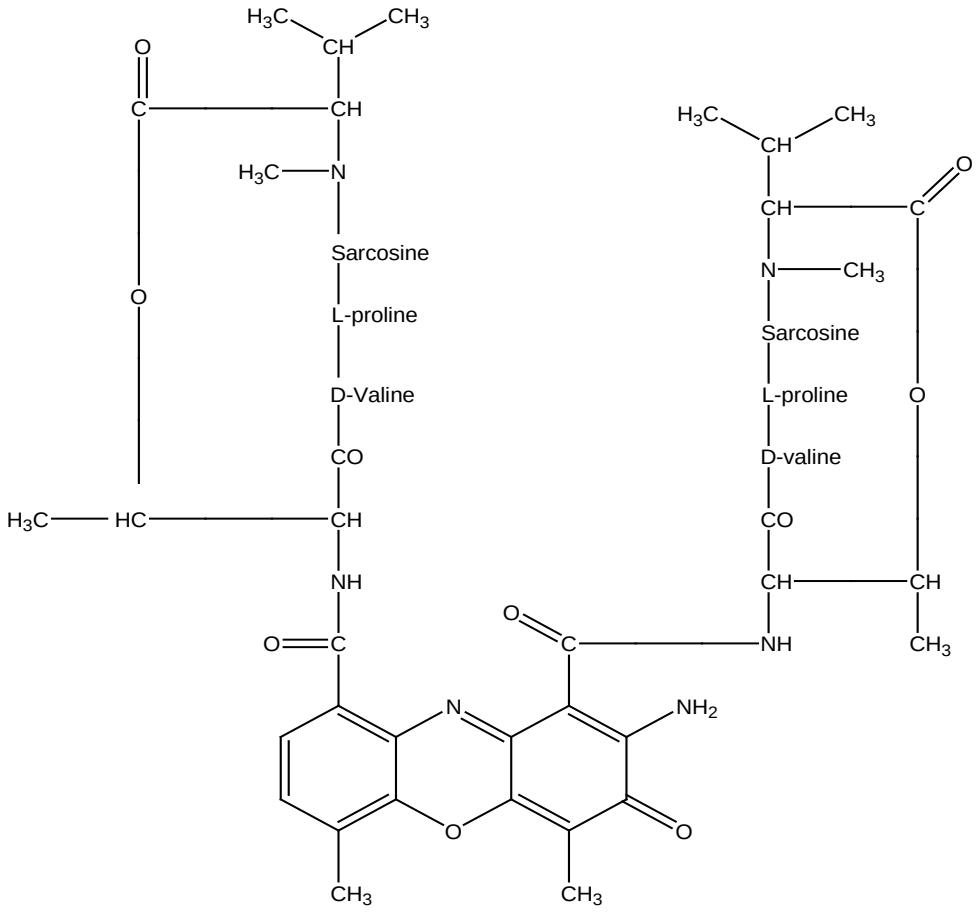


Figure 1.4: *Actinomycin D* (**6**)

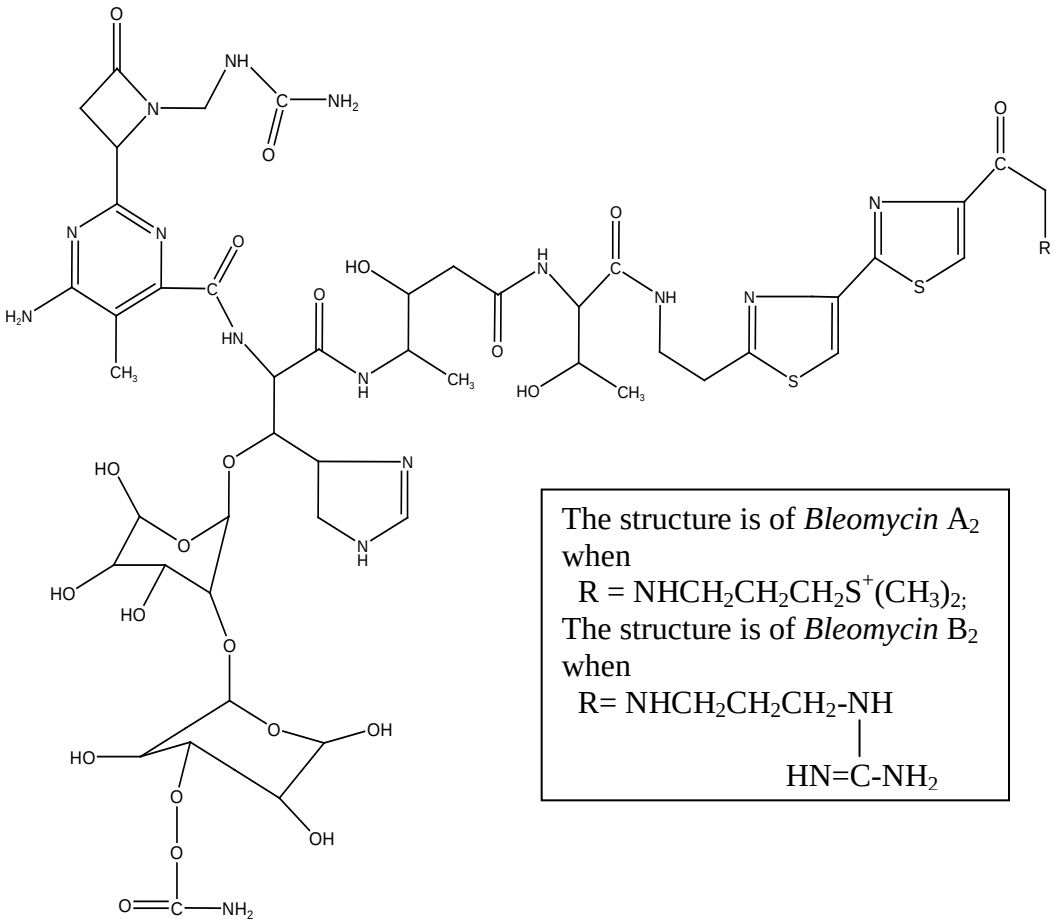


Figure 1.5: *Bleomycin* (**7**)

1.3 Objective of the work

For more than 50 years, natural products have served us well in combating infectious bacteria and fungi. During the 20th century, microbial and plant secondary metabolites helped to double our life span, reduced pain and suffering, and revolutionized medicine. Historically, nature has provided the source for the majority of the drugs in use today. This owes in large part to their structural complexity and clinical specificity. A renaissance of natural products-based drug discovery is coming because of the trend of combining the power of diversified but low-redundancy natural products with systems biology and novel assays.

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). These chemical substances still play a major role in drug treatment of diseases either as the drug or as a 'forebear' in the synthesis or design of new drugs. The worlds best known and most universally used medicinal agent is aspirin, which is related to salicin, which was originated from the plant genera, *Salix* sp. and *Populus* sp. (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).

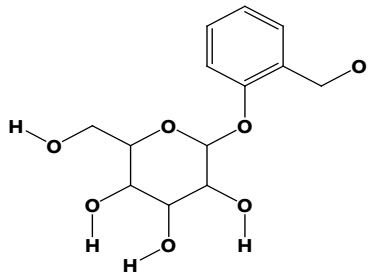


Figure 1.6: Slicing (**8**)

Even with untold centuries of human experience behind us, and a movement into a modern era of chemistry and automation, it is still evident that natural product-based compounds have had an immense impact on modern medicine. For instance, about 40% of prescription drugs are based on them. Furthermore, well over 50% of the new chemical products registered by the FDA as anticancer, antimigraine, and antihypertensive agents were natural products or derivatives thereof in the time-frame of 1981-2002 (Newman *et al.*, 2003).

Bangladesh is blessed with numerous medicinal plants belonging to various families including, Annonaceae and Euphorbiaceae. Although a large number of plants included in the above families have been investigated all over the world, very little is known regarding the chemistry and pharmacology of the plant species growing in Bangladesh. Thus, with this concern, we selected some Bangladeshi plants for chemical investigations (Table 1.1) and evaluate their pharmacological (mainly anti-cancer, antioxidant, antibacterial, cytotoxic, analgesic and antiinflamatory) profiles.

These investigations may provide some interesting compounds, which may be pharmacologically active. If significant results are obtained, these can be used as remedies for the treatment of some diseases. Since these plants are available in Bangladesh and as they are used in the folkloric medicines by rural patients, these may be also cost-effective treatment. These prompted us to embark upon their investigation. So, the objective is to explore the possibility of developing new drug candidates from these plants for the treatment of various diseases as with especial emphasis on anticancer. The selected plants are shown in table 1.1.

Table -1.1: List of studied plants and plant parts

Family name	Botanical name	Local name	Plant parts	Bangladesh National Harbarium voucher/ access. no.
Annonaceae	*Polyalthia simiarum* (Hook. F. and Thom.)	Arjan	Stem bark	DACB -34201
Euphorbiaceae	*Glochidion multiloculare* (Rottler *ex* Willd.) Voigt	Aniatori/ Paniatori	Stem bark	DACB -34200
Euphorbiaceae	*Glochidion lanceolarium* (Roxb.) Voigt	Anguti/ Bhauri	Stem bark	DACB -34199

1.4. The plant family: Annonaceae

Annonaceae is a large plant family including trees, shrubs and climbers with aromatic wood and leaves. The family belongs to the order annonales. The family comprises of 120 genera and more than 2100 species over the world (Trease & Evans, 1993). The plants of this family are widely distributed in the tropics of both hemispheres, mainly in evergreen rain forest region. About 51 genera (950 species) exist in Asia and Australia. In Africa and Madagascar, there are about 40 genera (450 species) and in America about 38 genera (740 species) (Takhtajan, 1969).

1.4.1. Annonaceous species available in Bangladesh

Annonaceous plants grow well in Bangladesh. They are found in plain areas as well as in hilly areas like Sylhet and Chittagong. According to the recent reports of Bangladesh National Harberium, the following Annonaceous plants are available in Bangladesh as shown in table- 1. 2.

Table- 1.2: Annonaceaeous species available in Bangladesh

Genus	Species
1. *Alphonsea*	*Alphonsea lutea* *Alphonsea ventricosa*
2. *Annona*	*Annona reticulata* *Annona roxburghiana* *Annona squamosa*
3. *Artabotrys*	*Artabotrys odorotissimus* *Artabotrys suaveolenes* *Artabotrys uncinatus*
4. *Cananga*	*Cananga odorata*
5. *Desmos*	*Desmos longiflorus* *Desmos chinensis*
6. *Goniothalamus*	*Goniothalamus sesquipedalis*
7. *Melodorum*	*Melodorum polyalthum* *Melodorum rubiginosum* *Melodorum rufinereae* *Melodorum wallichii*
8. *Miliusa*	*Miliusa velutina* *Miliusa roxburghiana*
9. *Mitrephora*	*Mitrephora tomentosa*
10. *Polyalthia*	*Polyalthia cerasoides* *Polyalthia longifolia* *Polyalthia longifolia* var. *pendulla* *Polyalthia simiarum* *Polyalthia suberosa*
11. *Sageraea*	*Sageraea listeri*
12. *Unona*	*Unona discolor* *Unona dunolii*
13. *Uvaria*	*Uvaria hamiltonii* *Uvaria furrugianea* *Uvaria longifolia* *Uvaria macrophylla*

1.4.2. Medicinal Importance and other uses of Annonaceous plants

The medicinal importance and other uses of Annonaceous plants are listed in table-1.3.

Table- 1.3 The medicinal importance and other uses of Annonaceous plants

Genus/Species	Plant parts /isolated compound	Medicinal or other uses
1. *Annona* (a) *Annona bullata*	Bullatacin & Bullatacinone (acetogenins)	Selective cytotoxic agent in human tumor cell line. Bullatacin is a pesticidal at a concentration of 1 ppm (Hui *et al*., 1989)
(b) *Annona glabra*	Liriodenine (alkaloid)	Antibacterial, antifungal & antitiumor agent. (Warthen *et a*l., 1969; Hufford *et al*., 1980)
(c) *Annona muricata*	Flowers, fruits, seeds & roots	Effective in cough & chronic dysentry, emetic, astringent, antispasmodic & parasiticidal (Hossain *et al*., 1991)
(d) *Annona reticulata*	Fruits	Effective against biliousness & thirst (Ayurveda) & also used as anthelmentic (Kirtikar & Basu, 1980)
(e) *Annona sonegalensis*	Extracts of stem bark	Showed good antibacterial activity (Hasan *et al*., 1988a)
	Root bark	Antineoplastic activity against sarcoma 180 ascites tumor cells (Adesogan & Durodola, 1976)
(f) *Annona squamosa*	Leaves & Fruits	In boils & ulcer. Tonic effect on the body which increases blood, muscular strength, relieve vomiting, lessen burning sensation & biliouness (Ayurveda)
	Seeds	Fatal to insect & worm (Kirtikar & Basu, 1980)

Genus/Species	Plant parts/ Isolated Products	Medicinal or other uses
2. *Artabotrys* (a) *Artabotrys odorotissimus*	Leafs extract Essential oils from flower Alkaloidal mixtures	In treatment of cholera (Ayurveda) In perfumery (Chopra *et al*., 1953) Showed antibacterial action (Haider, 1988)
(b) *Artabotrys suaveolens*	Leaves	Used against cholera (Kirtikar & Basu 1980)
3. *Cananga* (a) *Cananga odorata*	Oils from flowers	In treatment of gout, opthalmia & cephalalgia (Kirtikar & Basu, 1980) important raw materials for perfumery.
4. *Desmos* (a) *Desmos longiflorus* (b) *Desmos chinensis*	Alkaloids from stem bark Chloroform extract	Good antibacterial agent & antifungal agent (Hossain, 1991) Strong inhibitor of tyrosine kinase enzyme.
5. *Goniothalamus* (a) *Goniothalamus giganteus*	Acetogenins Styryl lactones	Selectively & significantly cytotoxic to human tumor cell. Some of them active against murine leukemia. One of them was insecticidal & inhibited formation of crown gall tumor on potato discs. Antimitotic acetogenin was also isolated. (Alkofahi *et al*., 1988; Fang *et al*., 1990,1991) Cytotoxic to human tumor cell (Fang *et al*., 1990)
(b) *Goniothalamus macrophyllus*	Plant constituents	An embryotoxic and teratogenic compound was isolated (Sam *et al*., 1987)

Contd.

Genus/species	Plant parts/Isolated Products	Medicinal and other uses
(c) *G. Grifithi* and *G. sesquipedalis*	Powdered leaves Smoke from burning leaves Crude extract and pure compounds	Relieves labor pain. Mosquito repellant (Talapatra *et al*., 1985) Antibacterial agent (Hasan *et al*., 1994b, 1994c)
(d) *G. malayanus* *G. montanus* *Gi tapis*	Different parts of these plants	Used for procurement of abortion & undefined post natal treatment (Burkhill, 1966).
6. *Miliusa* (b) *Miliusa tomentosa*	Essential oils from this plant	Used as analgesic & posseses antibacterial activity (Menon & Kar 1970, Kar & Jain, 1971)
(b) *Miliusa cf. banacea*	Oxoaporphine alkaloids from root	Has been reported as good bioactive and cytotoxic compounds, (Harrigan *et al*., 1994)
(c) *Miliusa velutina*	Sesquiterpenes (Spathulenol) and aromatic ester (Benzyl benzoate) from stem bark	Enamul *et al*., (1998)
7. *Polyalthia* (a) *P. longifolia*	Volatile oils from this plant Alkaloids from rnethanol extract of stern bark Crude chloroform extract	Anti bacterial agent (Kar & Jain, 1971) Good antibacterial and antifungal agent (Hasan *et al*., 1988b) Good antibacterial agent (Shahnaz, 1986)
(b) *P. longifolia var pendulla*	Different plant parts and pure compound (lanuginosine)	Antimicrobial (Ferdous *et al*., 1992 & Hasan *et al*., 1994, 1994a)

Contd.

Genus/species	Plant parts/Isolated Products	Medicinal and other uses
(c) *P. suaveolens*	Extract of bark	In black water fever (leishmaniasis) & stomach disorder (Keay *et al*., 1964)
8. *Uvaria* (a) *Uvaria afzelli*	Plant parts	Good activity against Bacillus subtilis, Microbacterium semagmatis & Staph. aureus (Hufford *et al*., 1981)
(b) *Uvaria chamae*	C-benzylated flavonoids	Cytotoxic against human carcinoma of the nasopharynx (in vitro) (Laswell &Hufford 1977a, Hufford & Oguntimein, 1980)
(c) *Uvaria duclis*	Root bark	Astringent, stimulant & alternative properties (Kirtikar & Basu, 1980)
9. *Xylopia* (a) *Xylopia aethiopica*	Plant parts and isolated diterpenes	Anticaugh, antifungal & antibacterial agent (Boakye, 1977)
(b) *Xylopia danguyella*	Plant parts	CNS depressant & hypotensive (Cordell, 1981)

1.4.3. Taxonomy of Annonaceae

On the basis of morphology and habitat, the Annonaceae is a very homogeneous plant family. They are trees or shrubs, sometimes climbing, usually evergreen, with resin canals and septate pith in the stems.

The leaves are alternate, entire and exstipulate.

The fragrant flowers frequently open before all the parts are fully developed. They are terminal, leaf-opposed or crowded, hermaphrodite or rarely unisexual, regular, mostly trimerous. The stamens are usually numerous, hypogenous, spirally arranged. The carpels

are generally numerous and free, very rarely united in a one celled ovary with pariental placentas.

Fruiting carpels are sessile or stipitate, mostly indehiscent. The fruit is usually an aggregate of berries, but in free genera, specially *Annona*, the berries coalesce with an edible fleshy receptable and the fruits arc worth eating.

The seeds have a copious ruminate endosperm a minute embryo.

1.4.4 Taxonomical hierarchy of the investigated plant *P. simiarum* (Cronquist, 1981)

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Angiospermae

Order: Magnoliales

Family: Annonaceae

Genus: *Polyalthia*

Species: *Polyalthia simiarum*

Scientific name: *Polyalthia simiarum* (Hook. F. and Thom.)

Synonym: *Guatteria simiarum* Hook. F. and Thom. (1855)

English name: Not known

Local name: Arjan

1.4.5 General botanical feature (Khanam and Rahman, 2002)

Plant structure:

A tree. Stem and branches glabrous.

Height:

Not known

Leaves:

Leaves with petiole 0.5-0.7 cm long; lamina 10-22 × 5-12.5 cm, ovate-oblong or oblong-lanceolate, acuminate, shining above, pale beneath, membranous, pubescent along the nerves and midrib.

Flowers:

Flowers 2.5-3.5 cm, yellowish green, spreading. Pedicles 1.2-3.5 cm, nacked above, slender.

Bracts:

Bracts 1 or 2, scaly, basal.

Sepals:

Sepals 0.2-0.3 cm long, bluntly ovate, recurved, pubescent outside.

Fruits:

Ripe carpels 10, 3-3.5 cm long, obovoid, glabrous, turning orange-red to blue-black in ripening, gradually narrowed to a stalk 2.5-5 cm long.

Seeds:

Seeds 2.5-2.7 × 1.3-1.4 cm, ovoid, grooved and transversely ribbed.

Flowering and fruiting:

May-September.

Habitat and distribution:

P. simiarum Hook f. and Thom., locally known as Arjan, is a very tall tree which grows in Cox's Bazaar hillside of Bangladesh. It is also available in India, Bhutan, Burma, Thailand, Laos and Vietnam (Khanam and Rahman, 2002).

Figure 1.7: Photograph of *P. simiarum*

Figure 1.8: Photograph of flower and leaves of *P. simiarum*

1.4.6. Chemistry of the the genus *Polyalthia*

The genus *Polyalthia* (Annonaceae) comprises about 70 species, out of which only eight are indigenous to the Indian subcontinent. These include *P. longifolia, P. longifolia var. pendulla, P. simiarium, P. suberosa, P. cerasoides, P. fragens, P. coffeoides and P. jentensii* (Choi *et al*., 2000). Biological evaluations of *Polyalthia* species have shown them to exhibit cytotoxic, antimicrobial (Ferdous *et al*., 1992; Rashid *et al*., 1996), anticancer (Sashidhara *et al*., 2009), antimalarial, antimycobacterial (Konokmedhakul *et al*., 2007; and Konokmedhakul *et al*., 2003), HIV-inhibitory (Li *et al*., 1993) activities. **A recent and comprehensive review of the constituents of *Polyalthia* species (Padmaa and Khosa, 2009) has revealed that although *P. simiarum* has never been subjected to chemical studies, previous investigations on *Polyalthia* species resulted in the isolation of various types of secondary metabolites, including alkaloids, acetogenins, clerodane diterpenes, triterpenes, benzopyran derivatives, flavonoids, and polyacetylenes.**

Table-1.4: List of isolated compounds from *Polyalthia* genus.

Species	Isolated compounds **Clerodane Diterpenoids**	References
P. longifolia var. *pendulla*	16-Hydroxycleroda-l3-ene-15,16-olide-3-one (**9**)	Chang *et al*., 2006
P. longifolia	16-Hydroxycleroda-3,13-dien-15,16-olide (**10**)	Hara *et al*., 1995
P. longifolia, P. viridis, P. barnesil	16α-Hydroxycleroda-3,13*Z*-dien-15,16-olide (**11**)	Zhao *et al*., 1991; Kijjoa *et al*., 1993; Ma *et al*., 1994
P. longifolia var. *pendulla, P. viridis*	16β-Hydroxycleroda-3,13*Z*-dien-15,16-olide (**12**)	Hasan *et al*., 1994; Rashid *et al*., 1996; Kijjoa *et al*., 1990
P. longifolia	16α-Methoxycleroda-3,13*Z*-dien-15,16-olide (**13**)	Chakrabarty, and Nath, 1992
P. longifolia, P. longifolia var. *pendulla*	Cleroda-3,13-dien-15,16-olide (**14**)	Hara *et al*., 1995; EI-Razek *et al.,* 2003
P. viridis, P. Longifolia, P. longifolia var. *Pendulla, P. cheliensis*	Cleroda-3,13*E*-dien-l5-oic acid (kolavenic acid) (**15**)	Kijjoa *et al*., (1990 and 1993); Hara *et al*., 1995; Phadnis *et al*., 1988; Chen *et al*., 2000; Hasan *et al*., 1994; Rashid *et al*., 1996; Hao *et al*., 1995
P. longofolia var. *pendulla*	Kolavenolic acid (**16**)	Chang *et al*., 2006
P. longifolia, P. viridis, P. longifolia var. *pendulla*	16-Oxocleroda-3,13*E*-dien-l5-oic acid (**17**)	Hara *et al*., 1995; Phadnis *et al*., 1988; Kijjoa *et al*., 1993; Hasan *et al*., 1994; Rashid *et al*., 1996

Species	**Isolated compounds**	**References**
	Clerodane diterpenoids	
P. longifolia	16-Oxocleroda-3,13*Z*-dien-l5-oic acid (polyalthialdoic acid) (**18**)	Zhao *et al*., 1991
P. longifolia var. *pendulla*	16-Hydroxycleroda-3,13-dien-l5-oic acid (**19**)	Chen *et al*., 2000
P. viridis, P. longifolia var. *pendulla*	3,12 *E*-Kolavdien-l5-oic acid-l6-al (**20**)	Kijjoa *et al*., 1993; Chen *et al*., 2000
P. longifolia var. *pendulla*	4 (18),12*E*-Kolavdien-l5-oic acid-l6-al (**21**)	Saleem *et al*., 2005
P. viridis	14,15 -Bisnor-3,11*E*-kolavadien-l3-one (**22**)	Kijjoa *et al*., 1990
P. longifolia	16-Hydroxycleroda-4 (18),-13-dien-15,16-olide (**23**)	Hara *et al*., 1995
P. cheliensis	16α-Hydroxycleroda-4 (18), 13 (14)*Z*-dien-15,16-olide (**24**)	Hao *et al*., 1995
P. longifolia	Cleroda-4 (18),13*E*-dien-l5-oic acid (**25**)	Hara *et al*., 1995
P. longifolia	16-Oxocleroda-4 (18),13*E*-dien-l5-oic acid (**26**)	Hara *et al*., 1995
P. longifolia	16-Oxocleroda-3,13 (14)*E*-dien-l5-oic acid methyl ester (**27**)	Phadnis *et al*., 1988
P. longifolia	Cleroda-4 (18),13-dien-15,16-olide (**28**)	Hara *et al*., 1995
P. barnesil	3β,16α-Dihydroxycleroda-4 (18),13 (14)*Z*-dien-15,16-olide (**29**)	Ma *et al.*, 1994
P. barnesil	4β,16α-Dihydroxycleroda-13 (14)*Z*-en-15,16-olide (**30**)	Ma *et al.*, 1994
P. longifolia var. *pendulla*	2-Oxokolavenic acid (**31**)	Hasan *et al*., 1995

Contd.

Species	Isolated compounds	References
	Abeo- clerodane diterpenoid	
P. viridis, P. longifolia var. *pendulla*	(4-2)-abeo-16(R and S)-2,13*Z*-clerodadien-15,16-olide-3-al (**32**)	Kijjoa *et al.*, 1993; Chen *et al*., 2000
P. longifolia var. *pendulla*	Solidagonal acid (**33**)	Chen *et al*., 2000
	Halimane diterpenoids	
P. longifolia	16-Hydroxy-*ent*-halima-5 (10),13-dien-15,16-olide (**34**)	Hara *et al*., 1995
P. longifolia	*Ent*-halima-5 (10),13*E*-dien-l5-oic acid (**35**)	Hara *et al*., 1995
P. longifolia	16-Oxo-*ent*-halima-5 (10),13*E*-dien-l5-oic acid (**36**)	Hara *et al*., 1995
P. longifolia	*Ent*-halima-5 (10),13*E*-dien-15,16-olide (**37**)	Hara *et al*., 1995
P. longifolia	*Ent*-halima-1(10),13*E*-dien-l5-oic acid (**38**)	Hara *et al*., 1995
P. longifolia	*Ent*-halima-1(10),13*E*-dien-15,16-olide (**39**)	Hara *et al*., 1995
P. longifolia var. *pendula*	3β,5β,16α-Trihydroxyhalima-13 (14)-en 15,16-olide (**40**)	Chen *et al*., 2000
	Labdane diterpenoids	
P. longifolia var. *pendulla*	Labd-13*E*-en-8-o1-15-oic acid (**41**)	Chen *et al*., 2000
P. macropoda	(4S,9R,l0R)-Methyl-l8-carboxy-labda-8,13*E*-diene-l5-oate (**42**)	Richomme *et al*., 1991
	Triterpenoids	
P. viridis	Polycarpol (**43**)	Hammoniere *et al*., 1976
P. longifolia, P. suberosa	α-Amyrin (**44**)	Goyal and Gupta (1986 and 1985)

Contd.

Species	**Isolated compounds**	**References**
	Triterpenoids	
P. longifolia, P. suberosa	β-Amyrin (**45**)	Goyal and Gupta (1986 and 1985)
P. suberosa	Lupeol (**46**)	Goyal and Gupta, 1986
P. suberosa	Suberosol (**47**)	Li *et al*., 1993
	Benzenoids	
P. longifolia var. *pendulla*	*Trans*-methyl ferulate (**48**)	Chang *et al*., 2006
	Benzosesquiterpenoids	
P. cheliensis	3",3"'-Bispolycerasoidol (**49**)	Zhu *et al*., 2002
P. cheliensis	Polycerasoidol (**50**)	Zhu *et al*., 2002
P. cheliensis	Isopolycerasoidol (**51**)	Zhu *et al*., 2002
P. longifolia var. *pendulla*	Liriodenine (**52**)	Hasan *et al*., 1995
	Oxoaporphines	
P. longifolia var. *pendulla*	Lanuginosine (oxoxylopine) (**53**)	Hasan *et al*., 1995
P. suaveolens	Oxonuciferine (lysicamine) (**54**)	Cave *et al*., 1978
P. suaveolens	Oxostephanine (**55**)	Ferdous *et al*., 1992
P. cauliflora var. *baccarii*	*O*-Methylmoschatoline (**56**)	Guinaudeau *et al*., 1978
P. cauliflora var. *baccarii*	Atherosperrnidine (**57**)	Guinaudeau *et al*., 1978
P. cauliflora var. *baccarii*	Thailandine (**58**)	Guinaudeau *et al*., 1978

Contd.

Species	**Isolated compounds**	**References**
	Aporphines	
P. longifolia var. *pendulla*	(-)-Norboldine (**59**)	Chen *et al*., 2000
P. longifolia var. *pendulla*	(+)-Norboldine (**60**)	Chen *et al*., 2000
P. emarginata	(-)-Anonaine (**61**)	Guinaudeau *et al*., 1978
P. longifolia var. *Pendulla*	(-)-Asimilobine (**62**)	Chen *et al*., 2000
P. debilis	Dehydroanonaine (**63**)	Somdej *et al*., 2003
P. cauliflora var. *baccarii*	(+)-Boldine (**64**)	Jossang *et al*., 1984
P. cauliflora var. *baccarii*	(+)-Predicentrine (**65**)	Jossang *et al*., 1984
P. longifolia	(+)-Norlirioferine (**66**)	Wu, 1989
P. longifolia	(+)-*O*-Methylbulbocapnine-β-*N*-oxide (**67**)	Wu, 1989
P. longifolia	(+)-*O*-Methylbulbocapnine-α-*N*-oxide (**68**)	Wu, 1989
P. longifolia	(+)-*N*-Methylnandigerine-β-*N*-oxide (**69**)	Wu, 1989
P. oligosperma	(+)-Polygospermine (**70**)	Guinaudeau *et al*., 1978
P. cauliflora var. *baccarii*	(+)-Dehydropedicentrine (**71**)	Jossang *et al*., 1984
P. oligosperma	(+)-Norconovine (**72**)	Guinaudeau *et al*., 1978
P. oliveri	(+)-*N*-Methylcorydine (**73**)	Hammonniere *et al*., 1977
P. bullata	7,7'-Bisdehydro-*O*-methylisopiline (**74**)	Connolly *et al*., 1996
P. bullata	7-Dehydronornuciferinyl-7'-dehydro- *O*-methylisopiline (**75**)	Connolly *et al*., 1996
P. bullata	Urabain (**76**)	Connolly *et al*., 1996
P. debilis	Bidebiline A (**77**)	Somdej *et al*., 2003

Contd.

Species	**Isolated compounds**	**References**
	Aporphines	
P. debilis	Bidebiline B (**78**)	Somdej *et al*., 2003
P. debilis	Bidebiline C (**79**)	Somdej *et al*., 2003
P. debilis	Bidebiline D (**80**)	Somdej *et al*., 2003
	Proaporphine	
P. longifolia	(+)-Stepharine (**81**)	Chang *et al*., 2000
	7-Substituted aporphines	
P. oliveri	(-)-Oliveridine (**82**)	Hammonniere *et al*., 1977
P. oliveri	(-)-Oliverine (**83**)	Hammonniere *et al*., 1977
P. suaveolens	(-)-Noroliverine (**84**)	Cave *et al*., 1978
P. longifolia	(-)-Noroliveroline (**85**)	Wu *et al*., 1990
P. suaveolens	(-)-Guatterine (**86**)	Cave *et al*., 1978
P. suaveolens	(-)-Polyalthin (**87**)	Cave *et al*., 1978
P. oliveri	(-)-Oliveroline-β-*N*-oxide (**88**)	Hammonniere *et al*., 1977
P. oliveri	*N*-Methylpachypodanthine- *N*-oxide (**89**)	Hammonniere *et al*., 1977
	Indolosequiterpenes	
P. suaveolens	Polyaveolensin (**90**)	Okarie, 1980 and 1981
P. suaveolens	Polyavolensinol (**91**)	Okarie, 1980 and 1981
P. suaveolens	Polyaveolensinone (**92**)	Okarie, 1980 and 1981
P. suaveolens	Polyaveolinamide (**93**)	Okarie, 1980
	Isoquinolines	
P. baccarii	Beccapoline (**94**)	Goyal and Gupta, 1985
P. baccarii, P. cauliflora var. *baccarii*	Beccapolinium (**95**)	Jossang *et al*., (1982 and 1984)
P. cauliflora var. *baccarii*	Beccapolydione (**96**)	Jossang *et al*., 1984

Contd.

Species	**Isolated compounds**	**References**
	Benzylisoquinolines	
P. nitidissima	*N*, *N*'-Dimethtllindoldhamine (**97**)	Jossang *et al*., 1983
P. nitidissima	Isodaurisoline (**98**)	Jossang *et al*., 1983
P. nitidissima	7-*O*-Methtllindoldhamine (**99**)	Jossang *et al*., 1983
P. nitidissima	7'-*O*-Methtllindoldhamine (**100**)	Jossang *et al*., 1983
	Tetrahydroprotoberberines	
P. oligosrerma	(-)-Kikemanine (**101**)	Guinaudeau *et al*., 1978
P. stenopetala	(-)-Discretamine (**102**)	Lavault *et al*., 1990
P. stenopetala	(-)-Thaipetaline (**103**)	Lavault *et al*., 1990
	8-Oxoprotoberberines	
P. longifolia var. *pendulla*	Pendulamine A (**104**)	Shaheen *et al*., 2003
P. longifolia var. *pendulla*	Pendulamine B (**105**)	Shaheen *et al*., 2003
P. longifolia var. *pendulla*	(-)-8-Oxopolyalthiaine (**106**)	Chen *et al*., 2000
	Morphinanedienones	
P. longifolia var. *Pendulla*	(-)-Norpallidine (**107**)	Chen *et al*., 2000
P. cauliflora var. *baccarii*	(-)-Sebiferine (**108**)	Jossang *et al*., 1984
	Morphinandienone	
P. longifolia	(-)-Pallidine (**109**)	Tamayo-Castillo *et al*., 1989
	Azafluorenes	
P. longifolia var. *Pendulla*	6-Hydroxy-7-methoxyonychine (**110**)	Chen *et al*., 2000
P. longifolia	Onychine (**111**)	Chakrabarty and Patra, 1990
P. longifolia	Polylongine (**112**)	Wu, 1989

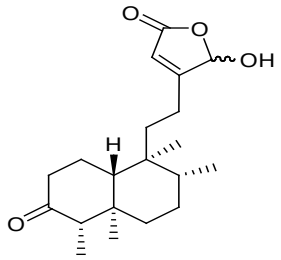
Contd.

Species	**Isolated compounds**	**References**
	Azaanthracenes	
P. suberosa	Kalasinamide (**113**)	Tuchinda *et al.*, 2000
	Imide	
P. longifolia var. *pendulla*	Bisclerodane imide (**114**)	Saleem *et al.*, 2005
P. longifolia var. *pendulla*	4(18)Olefinic isomer of bisclerodane imide (**115**)	Saleem *et al.*, 2005
	Amide	
P. longifolia var. *pendulla*	*N-Trans*-feruloyltyramine (**116**)	EI-Razek *et al.*, 2003
P. longifolia var. *pendulla*	*N-Cis*-feruloyltyramine (**117**)	EI-Razek *et al.*, 2003
	Lignan	
P. longifolia var. *pendulla*	(+)-Syringaresinol (**118**)	EI-Razek *et al.*, 2003
P. longifolia	Eudesmin (**119**)	Kakisawa *et al.*, 1972
	Steroids	
P. longifolia	β-Sitosterol (**120**)	Goyal and Gupta, 1985
P. longifolia	Stigmasterol (**121**)	Goyal and Gupta, 1985
P. suberosa	β-Sitosterol-D-glucoside (**122**)	Dan *et al.*, 1985
P. suberosa	β-Sitosterol-β-D-glucoside (**123**)	Dan *et al.*, 1985
	Furans	
	Evectic acid	
P. evecta	19-(2-Furyl) nonadeca-5,7-diynoic acid (**124**)	Kanokmedhakul *et al.*, 2006
P. evecta	19- (2-Furyl) nonadeca-5-ynoic acid (**125**)	Kanokmedhakul *et al.*, 2006

Contd.

Species	**Isolated compounds**	**References**
	Furans	
P. suberosa	1-(2-Furyl)pentacosa-16,18-diyne (**126**)	Patoomratana *et al.*, 2001
P. suberosa	23-(2-Furyl) tricosa-5,7-diynoic acid (**127**)	Patoomratana *et al.*, 2001
	Prenylated benzopyran derivatives	
P. sclerophylla	Polycerasoidin (**128**)	Gonzalez *et al.*, 1996
P. sclerophylla	Polycerasoidol (**129**)	Gonzalez *et al.*, 1996
P. cerasoides	Polycerasoidin methyl ester (**130**)	Gonzalez *et al.*, 1996
P. sclerophylla	(6*E*, l0*E*)-Isopolycerasoidol (**131**)	Gonzalez *et al.*, 1996
	Phenylpropene derivative	
P. cerasoides, P. sclerophylla	*Trans*-asarone (**132**)	Gonzalez *et al.*, 1996
	Pyrone derivative	
P. longifolia var. *pendulla*	3-Acetyl-4-hydroxy-6-methyl-pyran-2-one (**133**)	EI-Razek *et al.*, 2003
	Styryl-**lactones**	
P. crassa	(+)-Crassalactone A (**134**)	Tuchinda *et al.*, 2006
P. crassa	(+)-Crassalactone B (**135**)	Tuchinda *et al.*, 2006
P. crassa	(+)-Crassalactone C (**136**)	Tuchinda *et al.*, 2006
P. crassa	(+)-Crassalactone D (**137**)	Tuchinda *et al.*, 2006

Previously reported compounds from *Polyalthia* genus



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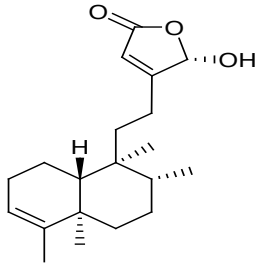
16-Hydroxycleroda-l3-ene-15,16-olide-3-one



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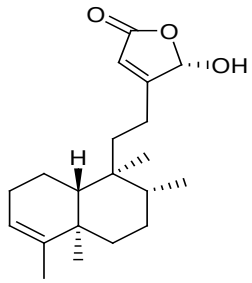
16-Hydroxycleroda-3,13-dien-15,16-olide



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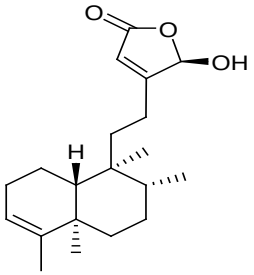
16α-Hydroxycleroda-3,13*Z*-dien-15,16-olide



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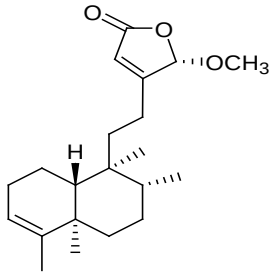

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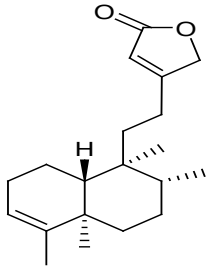

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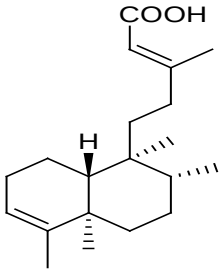
Cleroda-3,13-dien-15,16-olide



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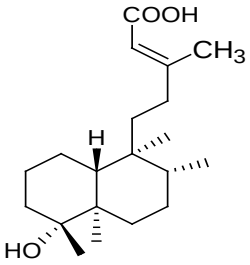
Cleroda-3,13*E*-dien-l5-oic acid (Kolavenic acid)



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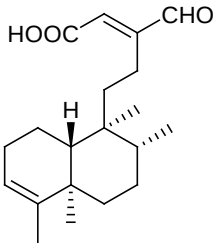
Kolavenolic acid



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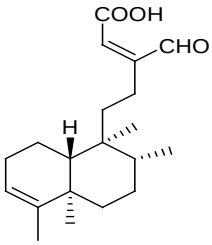
16-Oxocleroda-3,13*E*-dien-l5-oic acid



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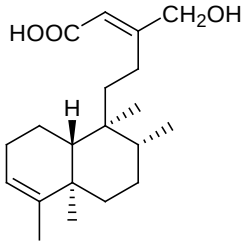
16-Oxocleroda-3,13*Z*-dien-l5-oic acid (Polyalthialdoic acid)



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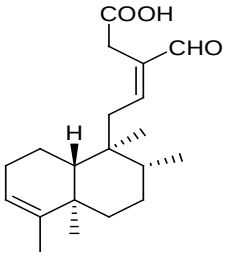
16-Hydroxycleroda-3,13-dien-l5-oic acid



20
3,12 *E*-Kolavdien-l5-oic acid-l6-al

Figure 1.9: Structure of compounds reported from *Polyalthia* genus

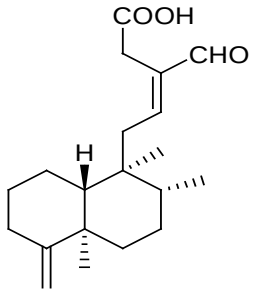
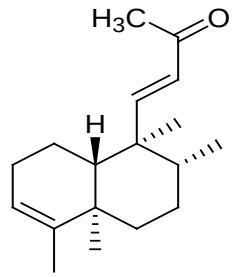
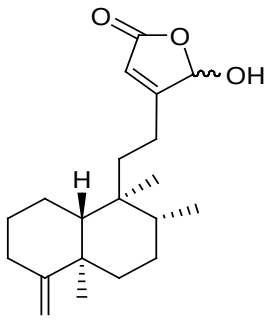
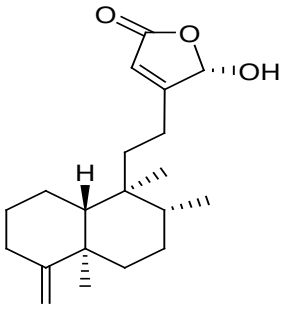
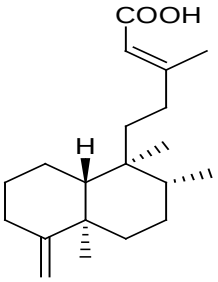
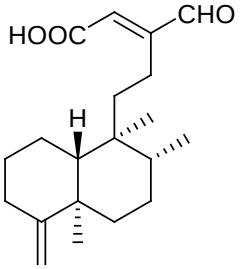
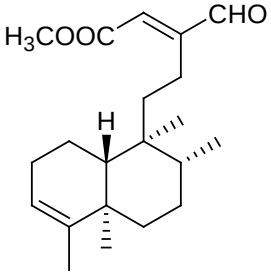
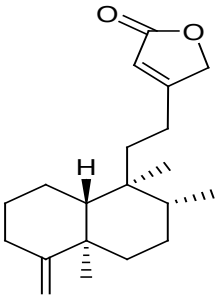
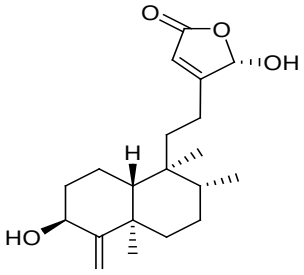
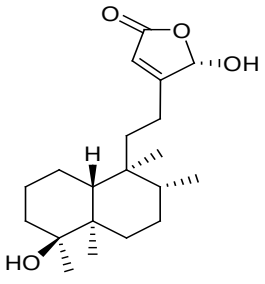
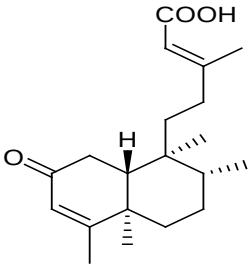
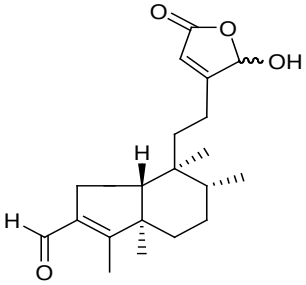
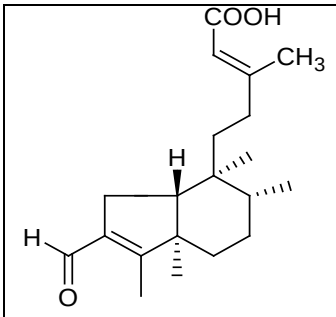
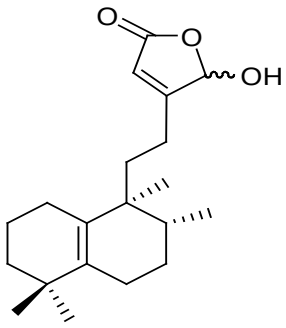
 **21** 4 (18),12*E*-Kolavdien-l5-oic acid-l6-al	 **22** 14,15-Bisnor-3,11*E*-kolavadien-l3-one	 **23** 16-Hydroxycleroda-4(18),-13-dien-15,16-olide
 **24** 16α-Hydroxycleroda-4(18),13 (14)*Z*-dien-15,16-olide	 **25** Cleroda-4(18),13*E*-dien-l5-oic acid	 **26** 16-Oxocleroda-4(18),13*E*-dien-l5-oic acid
 **27** 16-Oxocleroda-3,13(14)*E*-dien-l5-oic acid methyl ester	 **28** Cleroda-4(18),13-dien-15,16-olide	 **29** 3β,16α-Dihydroxycleroda-4(18),13 (14)*Z*-dien-15,16-olide
 **30** 4β,16α-Dihydroxycleroda-13 (14)*Z*-en-15, 16-olide	 **31** 2-Oxokolavenic acid	 **32** (4 -2)-abeo-16 (R and S)-2,13*Z*-clerodadien-15,16-olide-3-al

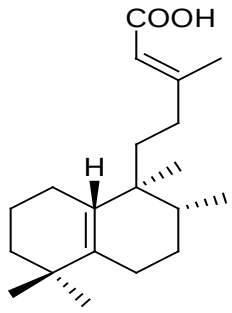
Figure 1.10: Structure of compounds reported from *Polyalthia* genus (contd.)



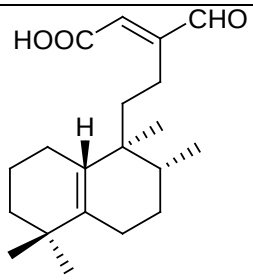
33
Solidagonal acid



34
16-Hydroxy-*ent*-halima-5(10), 13-dien-15,16-olide



35
Ent-halima-5(10),13*E*-dien-l5-oic acid



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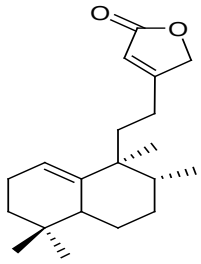
16-Oxo-*ent*-halima-5(10),13*E*-dien-l5-oic acid



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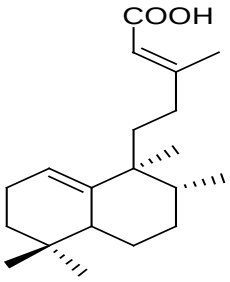
Ent-halima-5 (10),13*E*-dien-15,16-olide



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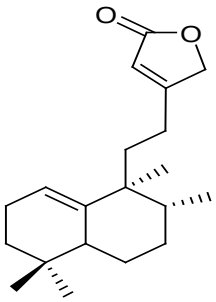
Ent-halima-1(10),13*E*-dien-l5-oic acid



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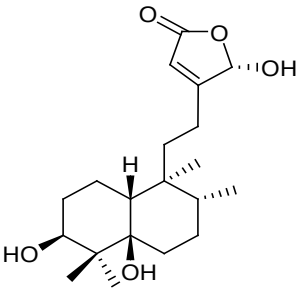
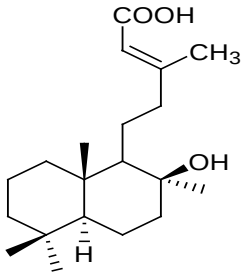
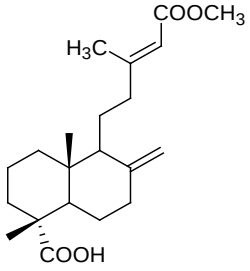
Ent-halima-1(10),13*E*-dien-15,16-olide



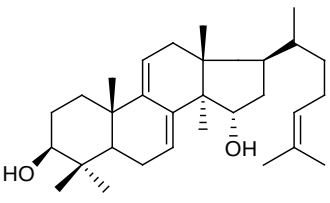
40
Ent-halima-1(10),13*E*-dien-15,16-olide



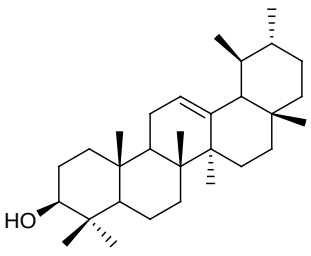
41
Labd-13*E*-en-8-o1-15-oic acid



42
(4S,9R,l0R)-Methyl-l8-carboxy-labda-8,13*E*- diene-l5-oate

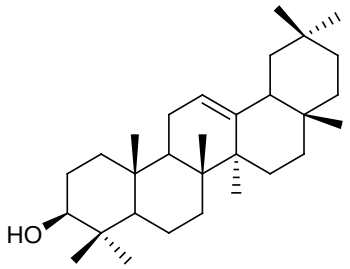


43
Polycarpol

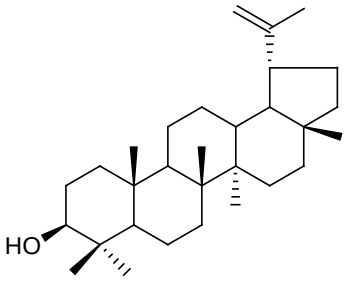


44
α-Amyrin

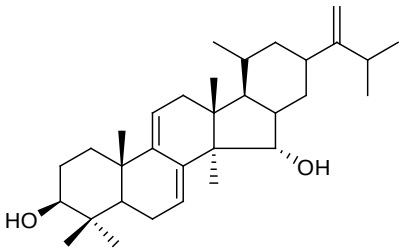
Figure 1.11: Structure of compounds reported from *Polyalthia* genus (contd.)



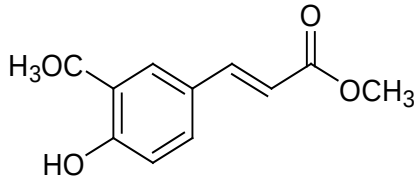
45
β-Amyrin



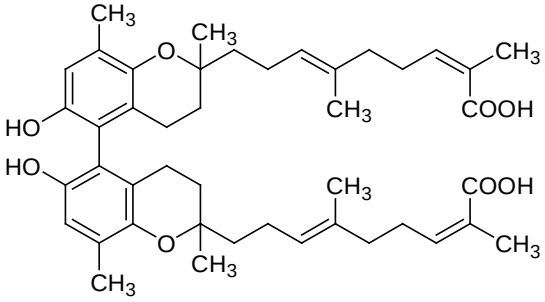
46
Lupeol



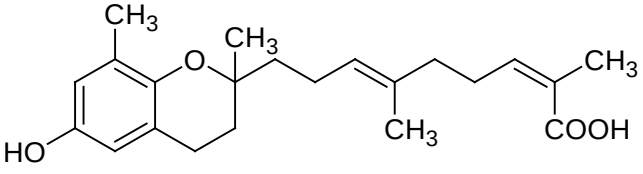
47
Suberosol



48
Trans-methyl ferulate

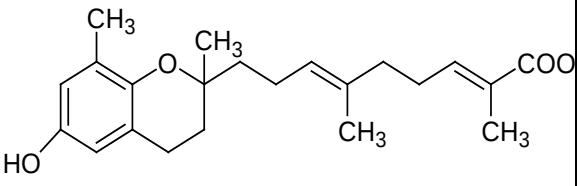


49
3", 3"'-Bispolycerasoidol

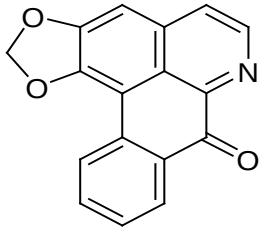


50
Polycerasoidol

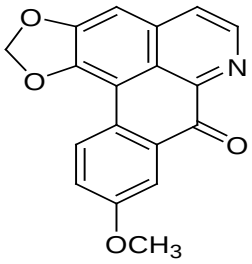
Figure 1.12: Structure of compounds reported from *Polyalthia* genus (contd.)



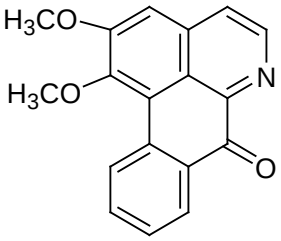
51
Isopolycerasoidol



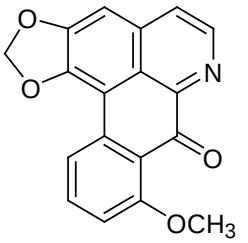
52
Liriodenine



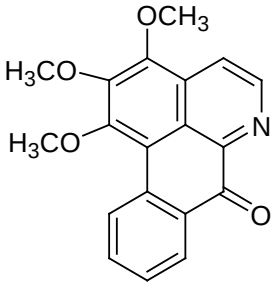
53
Lanuginosine (Oxoxylopine)



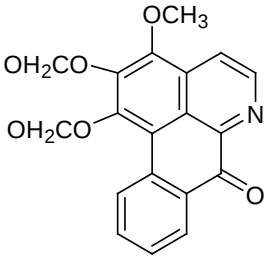
54
Oxonuciferine (Lysicamine)



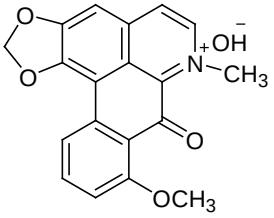
55
Oxostephanine



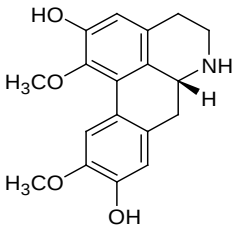
56
O-Methylmoschatoline



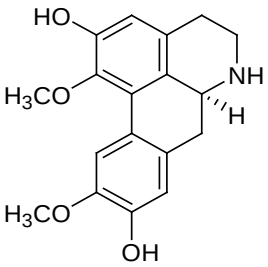
57
Atherosperrnidine



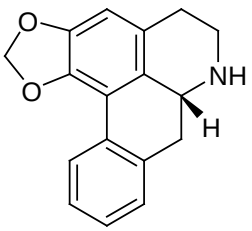
58
Thailandine



59
(-)-Norboldine

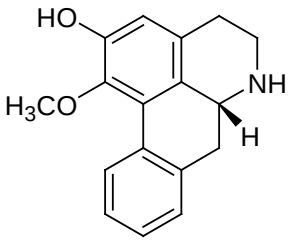


60
(+)-Norboldine

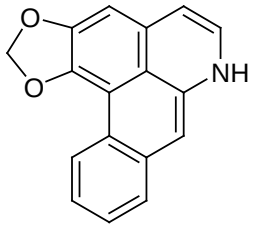


61
(-)-Anonaine

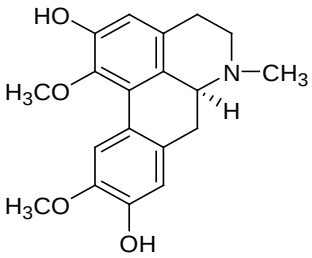
Figure 1.13: Structure of compounds reported from *Polyalthia* genus (contd.)



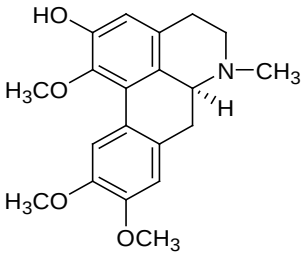
62
(-)-Asimilobine



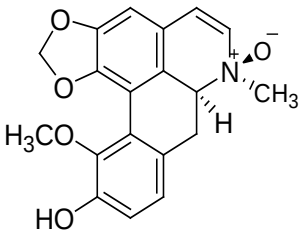
63
Dehydroanonaine



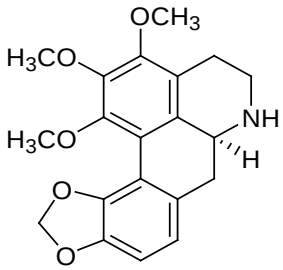
64
(+)-Boldine



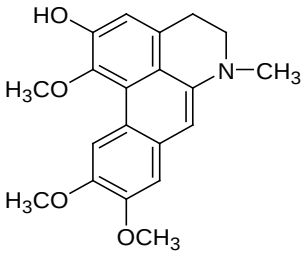
65
(+)-Predicentrine



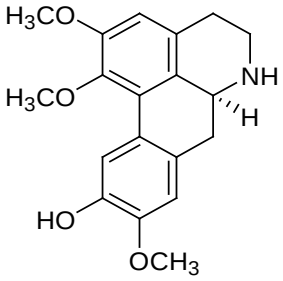
66
(+)-Norlirioferine



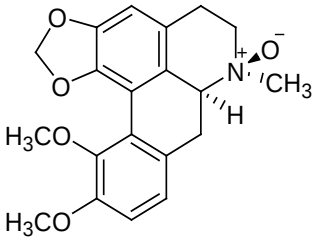
67
(+)-*O*-Methylbulbocapnine-β-*N*-oxide



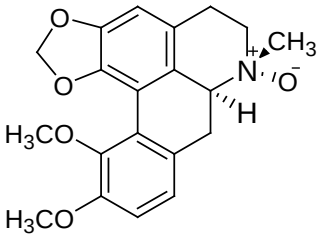
68
(+)-*O*-Methylbulbocapnine-α-*N*-oxide



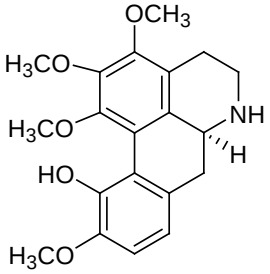
69
(+)-*N*-Methylnandigerine-β-*N*-oxide



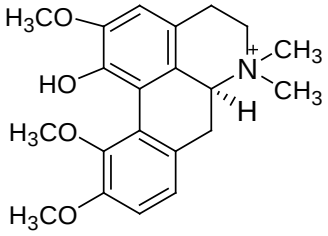
70
(+)-Polygospermine



71
(+)-Dehydropedicentrine

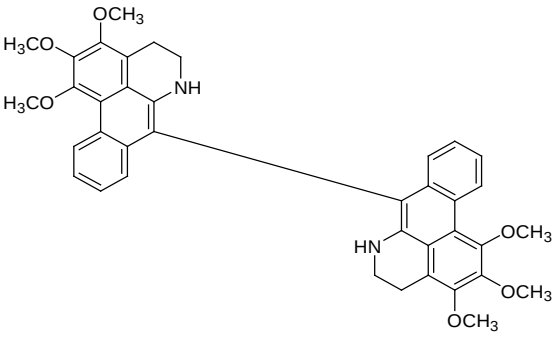


72
(+)-Norconovine

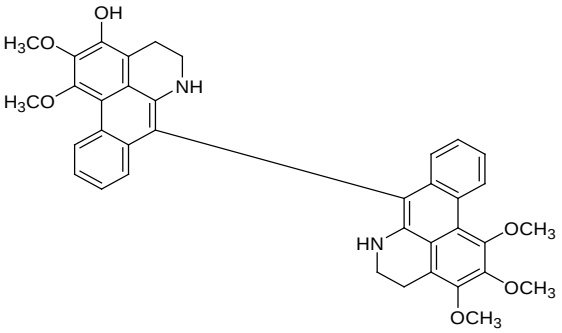


73
(+)-*N*-Methylcorydine

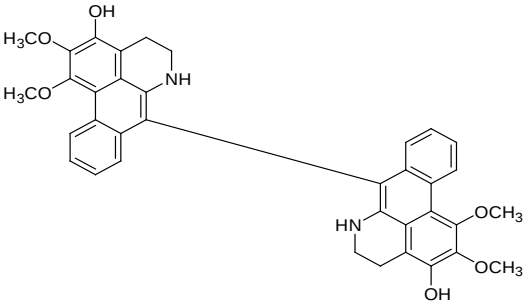
Figure 1.14: Structure of compounds reported from *Polyalthia* genus (contd.)



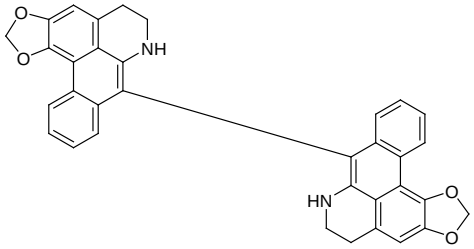
74
7, 7'-Bisdehydro-*O*-methylisopiline



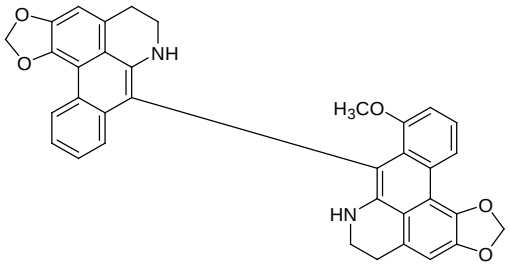
75
7-Dehydronornuciferinyl-7'-dehydro- *O*-methylisopiline



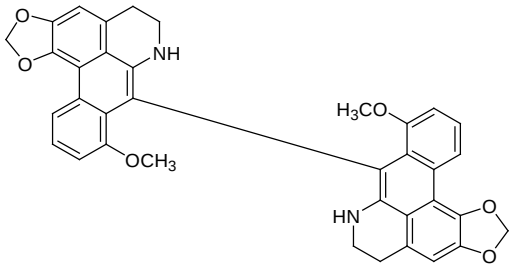
76
Urabain



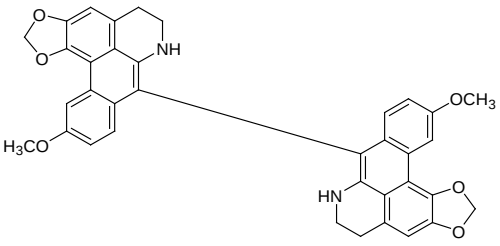
77
Bidebiline A



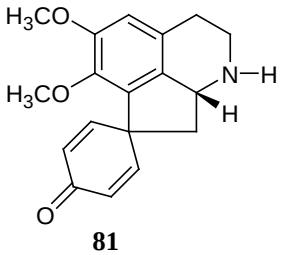
78
Bidebiline B



79
Bidebiline C

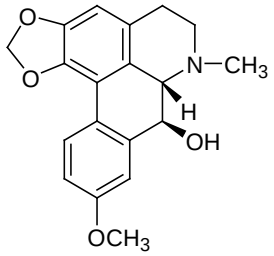


80
Bidebiline D

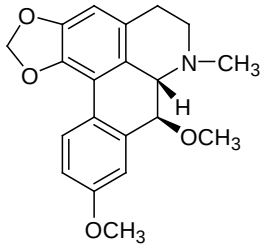


81
(+)-Stepharine

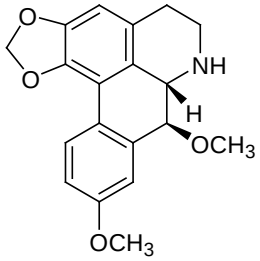
Figure 1.15: Structure of compounds reported from *Polyalthia* genus (contd.)



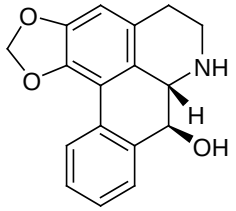
82
(-)-Oliveridine



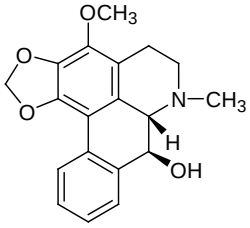
83
(-)-Oliverine



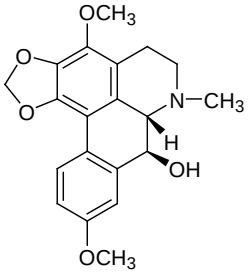
84
(-)-Noroliverine



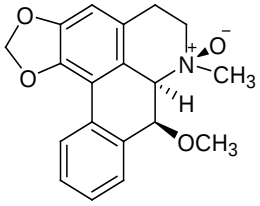
85
(-)-Noroliveroline



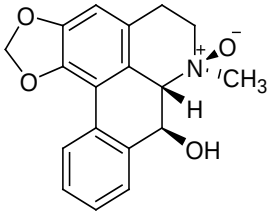
86
(-)-Guatterine



87
(-)-Polyalthin

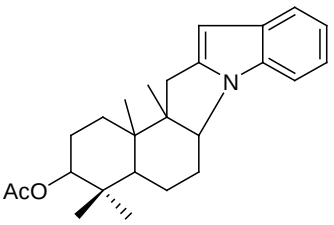


88
(-)-Oliveroline-β-*N*-oxide

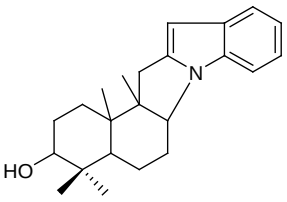


89
N-Methylpachypodanthine- *N*-oxide

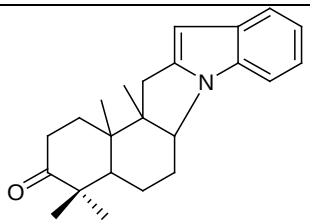
Figure 1.16: Structure of compounds reported from *Polyalthia* genus (contd.)



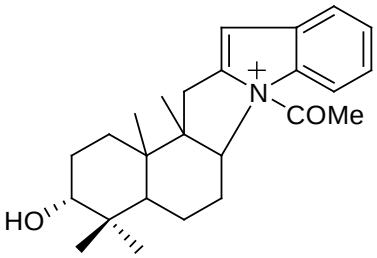
90
Polyaveolensin



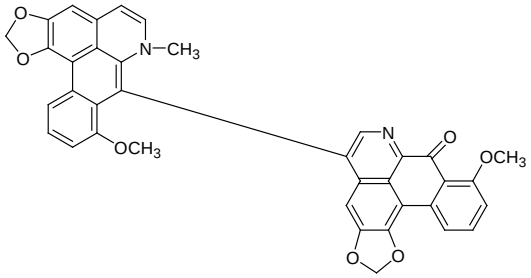
91
Polyavolensinol



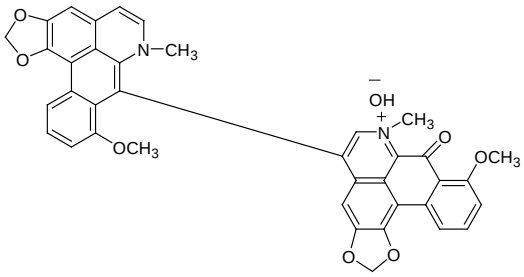
92
Polyaveolensinone



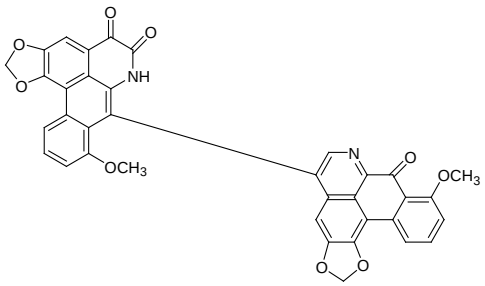
93
Polyaveolinamide



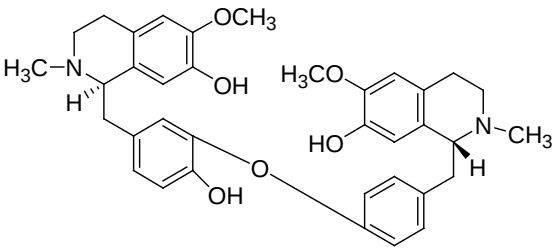
94
Beccapoline



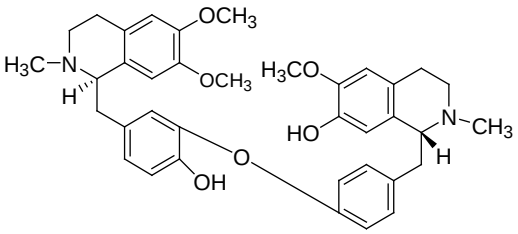
95
Beccapolinium



96
Beccapolydione

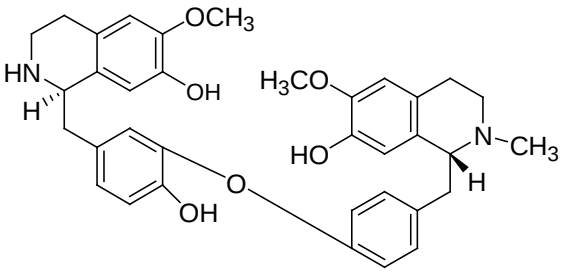


97
N, *N*'-Dimethtllindoldhamine

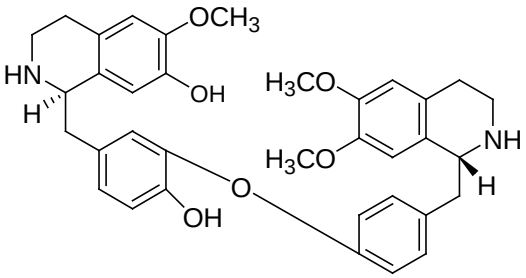


98
Isodaurisoline

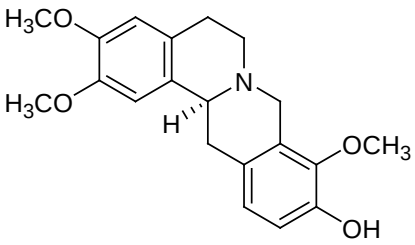
Figure 1.17: Structure of compounds reported from *Polyalthia* genus (contd.)



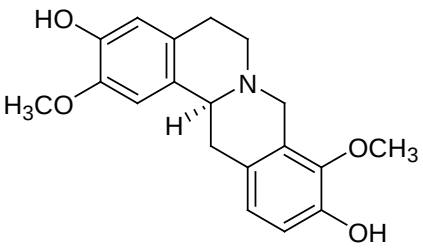
99
7-*O*-Methtllindoldhamine



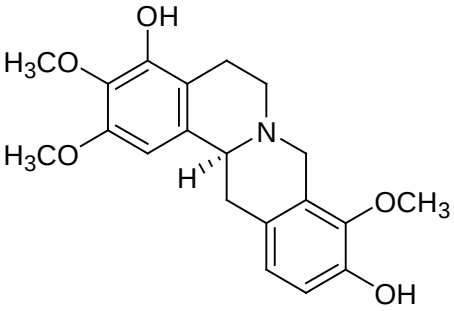
100
7'-*O*-Methtllindoldhamine



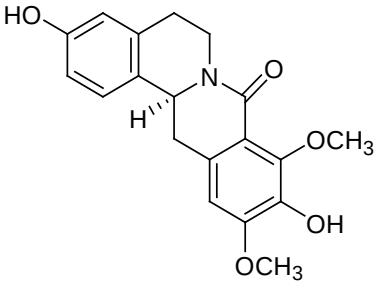
101
(-)-Kikemanine



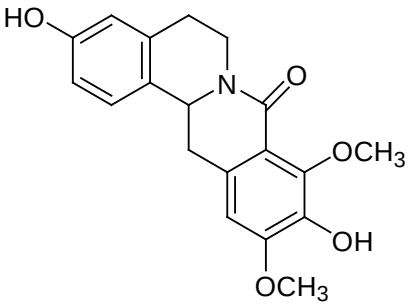
102
(-)-Discretamine



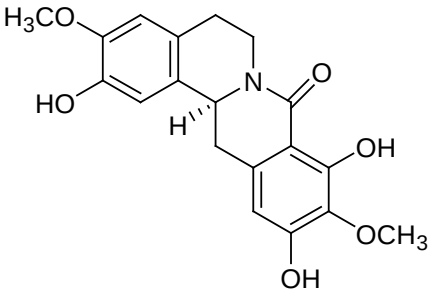
103
(-)-Thaipetaline



104
Pendulamine A

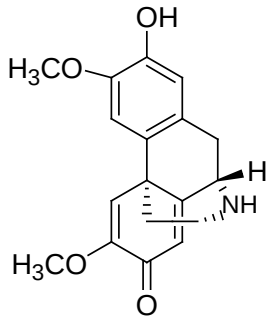


105
Pendulamine B

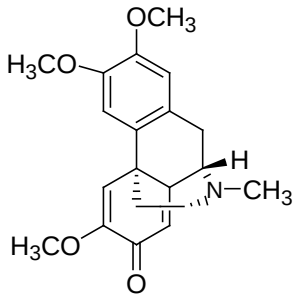


106
(-)-8-Oxopolyalthiaine

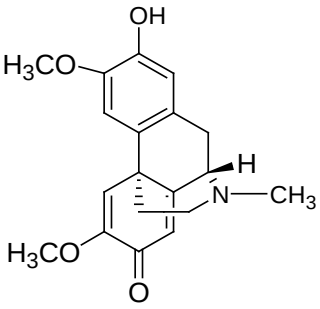
Figure 1.18: Structure of compounds reported from *Polyalthia* genus (contd.)



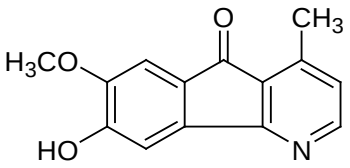
107
(-)-Norpallidine



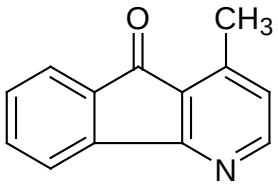
108
(-)-Sebiferine



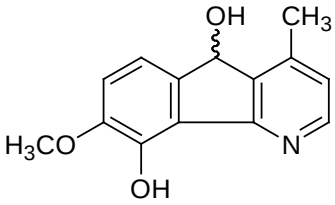
109
(-)-Pallidine



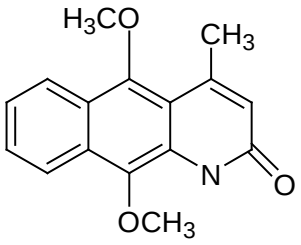
110
6-Hydroxy-7-methoxyonychine



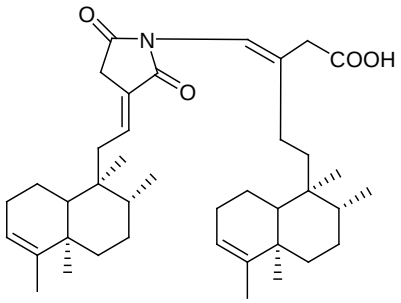
111
Onychine



112
Polylongine

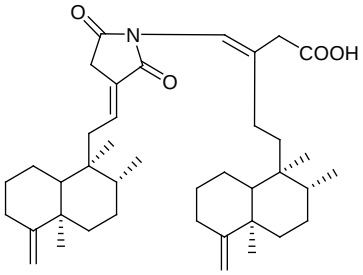


113
Kalasinamide

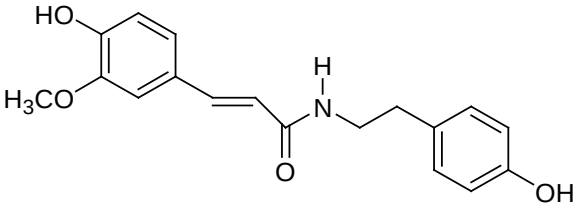


114
Bisclerodane imide

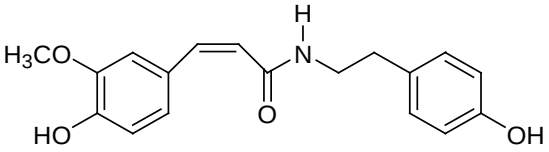
Figure 1.19: Structure of compounds reported from *Polyalthia* genus (contd.)



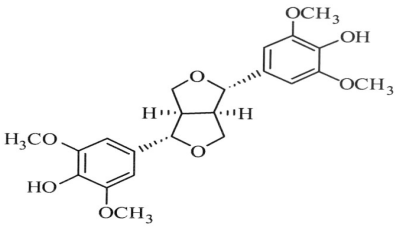
115
4 (18)Olefinic isomer of bisclerodane imide



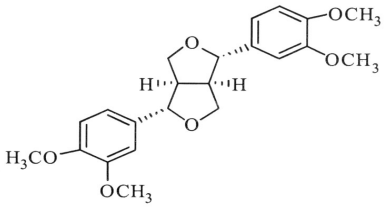
116
N-Trans-feruloyltyramine



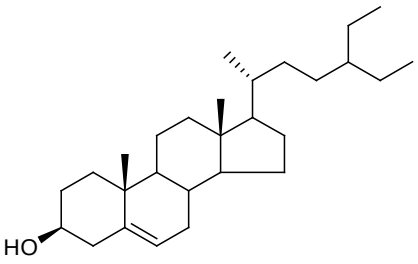
117
N-Cis-feruloyltyramine



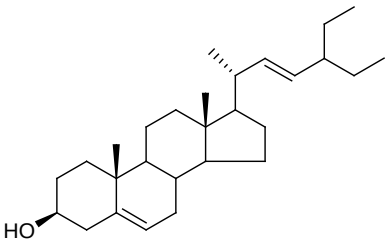
118
(+)-Syringaresinol



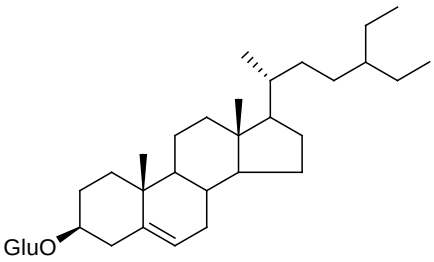
119
Eudesmin



120
β-Sitosterol

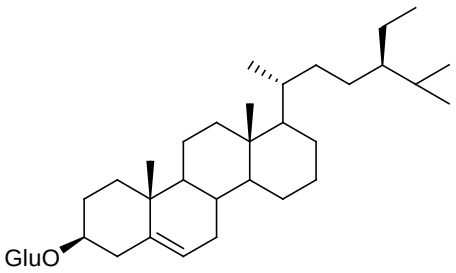


121
Stigmasterol

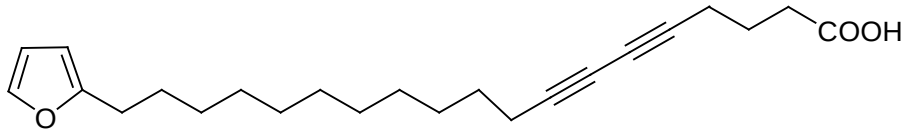


122
β-Sitosterol-D-glucoside

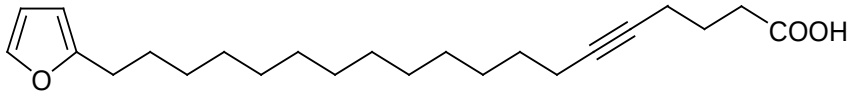
Figure 1.20: Structure of compounds reported from *Polyalthia* genus (contd.)



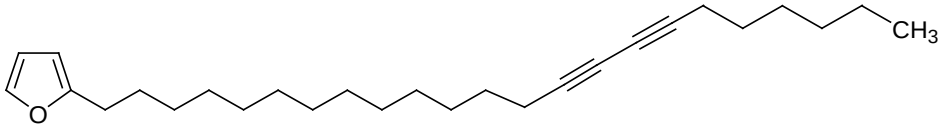
123
β-Sitosterol-β-D-glucoside



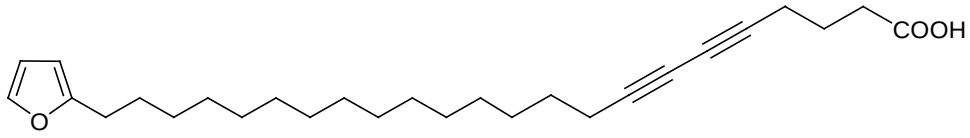
124
19- (2-Furyl) nonadeca-5,7-diynoic acid



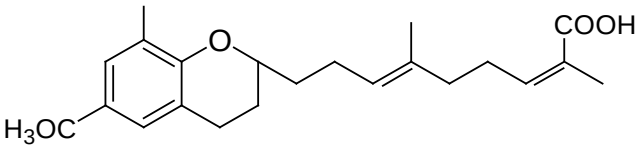
125
19- (2-Furyl) nonadeca-5-ynoic acid



126
1- (2-Furyl) pentacosa-16,18-diyne

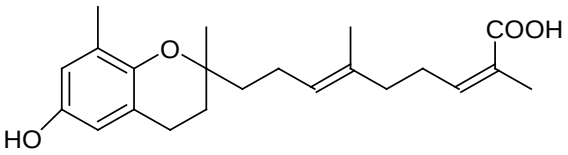


127
23- (2-Furyl) tricosa-5,7-diynoic acid

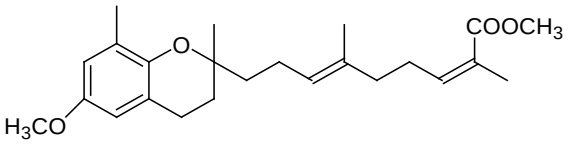


128
Polycerasoidin

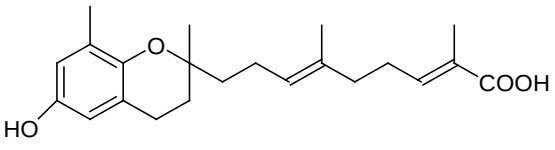
Figure 1.21: Structure of compounds reported from *Polyalthia* genus



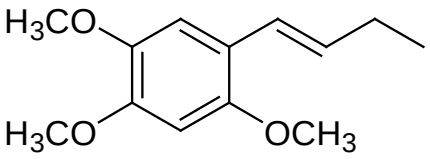
129
Polycerasoidol



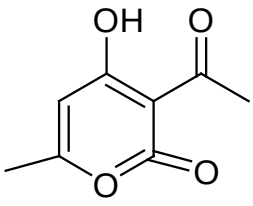
130
Polycerasoidin methyl ester



131
(6*E*, l0*E*)-Isopolycerasoidol

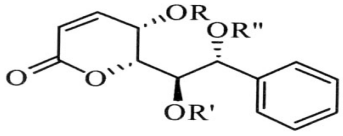


132
Trans-asarone



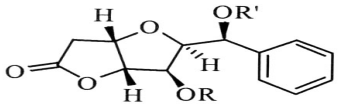
133

3-Acetyl-4-hydroxy-6-methyl-pyran-2-one



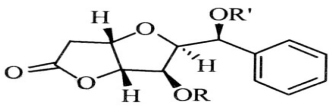
R=cinnamoyl,R'=R"=H

134
(+)-Crassalactone A



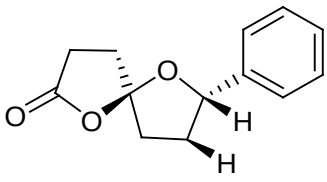
R=cinnamoyl,R'=H

135
(+)-Crassalactone B



R'=cinnamoyl,R=H

136
(+)-Crassalactone C



137
(+)-Crassalactone D

Figure 1.22: Structure of compounds reported from *Polyalthia* genus

1.4.7 Medicinal uses of *P. simiarum*

In the traditional system of medicine, the plants are used as a bitter tonic, abortifacient, febrifuge, a cure for scorpion strings, hypertension and as a respiratory stimulant (Kirtikar and Basu, 1980)

1.5 The plant family: Euphorbiaceae (Rahman, 2008)

The plant under investigation- *Glochidion multiloculare* (Roxb. ex Willd.) Muell.-Arg. and *Glochidion lanceolarium* (Roxb.), Vogit belongs to the family Euphorbiaceae. The Euphorbiaceae family are a family of dioecious or monoecious, often poisonous, prostrate, erect or scandent annual herbs to trees, often with milky juice, spiny or unarmed. Indumentum absent or of simple, branched or stellate hairs or peltate scales, the hairs sometimes urticating. Leaves simple or compound, alternate, rarely opposite, usually petiolate or sessile, stipulate or exstipulate, entire or toothed, peltate or not, eglandular or glandular, stipules free or connate, sometimes spathaceous, membranous, capilliform, sub-persistent or readily caducous. Inflorescence terminal, axillary, lateral or leaf-opposed, cymes, paniculate, racemose, spicate, cyathial or with the flowers fasciculated or solitary. Flowers small or minute, unisexual, perianth sepaloid, rarely petaloid, sometimes distinguishable as calyx and corolla, occasionally wanting in one or both the sexes. Calyx in both sexes usually of 3-6, imbricate, valvate, or open equal or unequal lobes or free sepals. Corolla in one or both sexes of 3-6, free, rarely united, sun-valvate or imbricate, petals sometimes united. Male flowers with stamens usually as many or twice, as many as petals or reduced to one, sometimes very many, distinct or monadelphous, anthers 2-celled, dehiscing longitudinally, transversly or by an apical pore, intrastaminal disc usually present in multistaminate flowers, pistillode sometimes present. Female flowers with or without staminodes, usually pedicelled. Ovary superior, 3-locular with 1 or 2 pendulous ovules in each lobule, in axile placentas, styles 3, distinct or basally connate, each with 2 lobes. Fruit a capsule or drupe. Seeds often with a caruncle and copious fleshy endosperm.

A very large family with about 300 genera and 7500 species, cosmopolitan but with the strongest representation in the humid tropics of both hemispheres. In Bangladesh, this family is represented by 47 genera and 141 species.

1.6 The plant genus: *Glochidion*

Glochidion are a taxon of plants in the family Euphorbiaceae. It comprises about 300 species, distributed from Madagascar to the Pacific Islands ("Glochidion," 2010).

1.6.1 Occurrence overview

Glochidion are distributed from Madagascar to the Pacific Islands. They are predominantly found in Southeast Asia.

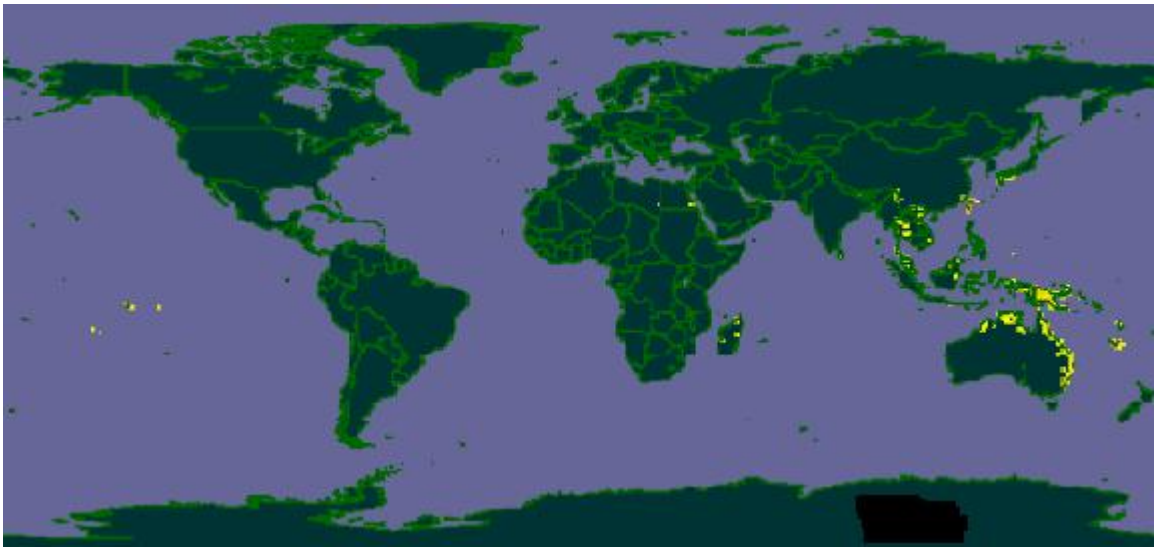


Figure 1.23: This Map depicts density of data for Glochidion, registered within the GBIF network index and not necessarily true species occurrence density gradients. The data in the GBIF network index may not represent the full distribution of *Glochidion* (GBIF, 2010).

1.6.2 Taxon description

These are trees or shrubs, monoecious, rarely dioecious. Leaves simple, alternate, petiolate, stipulate, distichous or spirally arranged on the branchlets, entire, penninerved. Flowers in axillary clusters or short cymes. Male flowers pedicellate or subsessile, slender, sepals 5-6 in 2 series, imbricate, stamens 3-8, connate into oblong or ellipsoid column, shorter than the sepals, anthers 2-locular, extrorse, linear, longitudinally dehiscent, disc lacking. Female flowers pedicellate or subsessile, stout and short, sepals as in male flowers but slightly thicker, ovary globose, 3-15 locular, ovules 2 per locule,

styles connate into a short thick cylindrical column, lobed or teethed at the apex, rarely free. Fruit a capsule, globose or depressed-globose, with 3-15 bivalvate cocci when dry, rarely unlobed, each coccus 2-seeded. Seeds hemispherical or laterally compressed (Ahmed *et al*., 2008).

1.6.3 Taxon range (Catalogue of Life, 2007)

- *Glochidion annamense*
- *Glochidion apodogynum*
- *Glochidion arborescens*
- *Glochidion arboreum*
- *Glochidion arnottianum*
- *Glochidion butonicum*
- *Glochidion cacuminum*
- *Glochidion cagayanense*
- *Glochidion calciphilum*
- *Glochidion caledonicum*
- *Glochidion calocarpum*
- *Glochidion caloneurum*
- *Glochidion calospermum*
- *Glochidion camiguinense*
- *Glochidion canarum*
- *Glochidion discogyne*
- *Glochidion disparilaterum*
- *Glochidion dumicola*
- *Glochidion atrovirens*
- *Glochidion auii*
- *Glochidion azaleon*
- *Glochidion bachmaense*
- *Glochidion chamaecerasus*
- *Glochidion chevalieri*
- *Glochidion chlamydogyne*
- *Glochidion chodoense*
- *Glochidion chondrocarpum*
- *Glochidion christopheraenii*
- *Glochidion cinerascens*
- *Glochidion compressicaule*
- *Glochidion concolor*
- *Glochidion conostylum*
- *Glochidion cordatum*
- *Glochidion coriaceum*
- *Glochidion ferdinandi*
- *Glochidion ferdinandii*

- *Glochidion gamblei*
- *Glochidion gardneri*
- *Glochidion gaudichaudii*
- *Glochidion geoffirayi*
- *Glochidion gigantifolium*
- *Glochidion gillespiei*
- *Glochidion gimi*
- *Glochidion glaberrimum*
- *Glochidion glabrum*
- *Glochidion glaucescens*
- *Glochidion glaucifolium*
- *Glochidion glaucogynum*
- *Glochidion glaucops*
- *Glochidion glaucum*
- *Glochidion lanceolarium*
- *Glochidion lanceolatum*
- *Glochidion lancifolium*
- *Glochidion lancilimbum*
- *Glochidion lancisepalum*
- *Glochidion latistylum*
- *Glochidion laurifolium*
- *Glochidion leiostylum*
- *Glochidion lenormandi*
- *Glochidion malabaricum*
- *Glochidion flavidum*
- *Glochidion flavum*
- *Glochidion flexuosum*
- *Glochidion foliosum*
- *Glochidion formanii*
- *Glochidion formosanum*
- *Glochidion fortuni*
- *Glochidion frodinii*
- *Glochidion frondosum*
- *Glochidion fulvirameum*
- *Glochidion fuscum*
- *Glochidion galorii*
- *Glochidion heterocalyx*
- *Glochidion heterodoxum*
- *Glochidion lichenisilvae*
- *Glochidion ligulatum*
- *Glochidion littorale*
- *Glochidion liukiuense*
- *Glochidion llanosi*
- *Glochidion lobocarpum*
- *Glochidion loerzingii*
- *Glochidion longfieldiae*
- *Glochidion longipedicellatum*
- *Glochidion lucidum*

- *Glochidion marchionicum*
- *Glochidion marianum*
- *Glochidion marojejiense*
- *Glochidion marquesanum*
- *Glochidion martii*
- *Glochidion medogense*
- *Glochidion mehipitense*
- *Glochidion meijeri*
- *Glochidion melvilliorum*
- *Glochidion merrillii*
- *Glochidion metanubigenum*
- *Glochidion microbotrys*
- *Glochidion microphyllum*
- *Glochidion mishmiense*
- *Glochidion mitrastylum*
- *Glochidion molle*
- *Glochidion moluccanum*
- *Glochidion monostylum*
- *Glochidion montanum*
- *Glochidion montiberica*
- *Glochidion moonii*
- *Glochidion moorei*
- *Glochidion mop*
- *Glochidion ornatum*
- *Glochidion lutescens*
- *Glochidion luzonense*
- *Glochidion macphersonii*
- *Glochidion macrocarpum*
- *Glochidion macrochorion*
- *Glochidion macrophyllum*
- *Glochidion macrosepalum*
- *Glochidion macrostigma*
- *Glochidion magnificum*
- *Glochidion maingayi*
- *Glochidion majus*
- *Glochidion muelleri*
- *Glochidion multilobum*
- *Glochidion myrianthum*
- *Glochidion myrtifolium*
- *Glochidion nadeaudii*
- *Glochidion namilo*
- *Glochidion nanogynum*
- *Glochidion neilgherrense*
- *Glochidion nemorale*
- *Glochidion nepalense*
- *Glochidion nervosum*
- *Glochidion nesophilum*
- *Glochidion nitidum*

- *Glochidion salomonis*
- *Glochidion sambiranense*
- *Glochidion santisukii*
- *Glochidion taitense*
- *Glochidion talboti*
- *Glochidion pleiosepalum*
- *Glochidion podocarpum*
- *Glochidion poeppigianum*
- *Glochidion quercinum*
- *Glochidion quinquestylum*

1.6.4: Some reported species found in Bangladesh (Ahmed *et al*., 2008)

- *Glochidion arborescence*
- *Glochidion assamicum*
- *Glochidion fagifolium*
- *Glochidion heyneanum*
- *Glochidion hirsutum*
- *Glochidion khasicum*
- *Glochidion lanceolarium*
- *Glochidion multiloculare*
- *Glochidion oblatum*
- *Glochidion sphaerogynum*
- *Glochidion thomsonii*
- *Glochidion velutinum*
- *Glochidion zeylanicum*

1.7: Description of *Glochidion multiloculare* (Roxb. *ex* Willd.) Muell.-Arg.

1.7.1: Taxonomic hierarchy of the investigated plant

(Catalogue of Life, 2007)

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Euphorbiales

Family: Euphorbiaceae

Genus: *Glochidion*

Species: *Glochidion multiloculare*

Scientific name: *Glochidion multiloculare* (Roxb. *ex* Willd.) Muell.-Arg.

Synonym: *Agyneia multilocularis* Roxb. *ex* Willd. (1803), *Bradleia multilocularis* (Roxb. *ex* Willd.) Spreng. (1826), *Phyllanthus multilocularis* (Roxb. *ex* Willd.) Muell.-Arg. (1866).

1.8: Description of *Glochidion lanceolarium* (Roxb.) Voigt

1.8.1: Taxonomic hierarchy of the investigated plant

(Cronquist, 1981)

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Euphorbiales

Family: Euphorbiaceae

Genus: *Glochidion*

Species: *Glochidion lanceolarium*

Scientific name: *Glochidion lanceolarium* (Roxb.) Voigt

English name: Not known

Local name: Kechchua, Bhauri, Kakra, Anguti (Syihet).

1.8.2: General botanical features (Rahman, 2008)

Plant structure:

A small to medium-sized evergreen tree with spreading crown.

Height:

Usually 1-3m tall, rarely 7-12 m tall

Steam: Greyish or greyish-brown.

Leaves:

Leaves green 6.3-15cm long, elliptic-lanceolate, acutely or obtusely acuminate.

Flowers:

Flowers small, monoecious or dioecious, in axillary clusters. Male flowers greenish yellow with capillary predicels; female flowers few, sessile, deeper yellow than the male.

Fruits:

Capsule, depressed-globose.

Flowering and fruiting:

April-December

Distribution:

Glochidion lanceolarium (Roxb.) Voigt grows in Chittagong, Cox's Bazaar and Sylhet of Bangladesh. It is also available in Bhutan, India, Myanmar and Nepal.

Figure 1.24: Photograph of leaves of *G. lanceolarium*

Figure 1.25: Photograph of fruits of *G. lanceolarium*

1.9: Molecular phylogenetics of Euphorbiaceae *sensu lato*

Following the break-up of Euphorbiaceae sensu lato (Malpighiales) into five major lineages recognised at family level by the Angiosperm Phylogeny Group, molecular phylogenetic studies have been carried out in the last five years with the objective of clarifying circumscription and internal relationships. The studies are a collaboration between the Smithsonian Institution, Vienna University, and RBG Kew. Multi-gene analyses of the two largest segregate families, Euphorbiaceae sensu stricto and Phyllanthaceae, are now published, with Picrodendraceae and a revised classification of Euphorbiaceae s.s. to follow (Royal, 2010).

Molecular phylogenetic studies have shown that Phyllanthus is paraphyletic over Reverchonia, Glochidion, Sauropus, and Breynia. A recent revision of the family Phyllanthaceae has subsumed all four of these genera into Phyllanthus (Hoffmann *et al*., 2006). This enlarged version of Phyllanthus might eventually be divided into smaller genera, but much more research will be needed before anyone knows how to do this. Progress continues to be made in this area (Kathriarachchi *et al*., 2006; Pruesapan *et al*., 2008)

1.9.1: The plant family: Phyllanthaceae (Hoffmann, 2007; Hoffmann *et al*., 2006)

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 exudate. 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, leaflike 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.

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 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 (Hutchinson, 1973). 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 megagametophyte is of the Polygonum type (Webster, 1994). The style is usually 2-lobed or bifid, sometimes entire, or rarely multifid. The fruit is a schizocarp, drupe, or berry. In some, the schizocarp breaks up explosively.

Phyllanthaceae comprises about 2000 species (Kathriarachchi *et al*., 2005; Samuel, *et al*., 2005). Depending on the author, these 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).

1.9.2: Taxonomic hierarchy of the investigated plant

(Encyclopedia of Life, 2010)

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Malpighiales

Family: Phyllanthaceae

Genus: *Glochidion*

Species: *Glochidion multiloculare*

Scientific name: *Glochidion multiloculare* (Rottler *ex* Willd.) Voigt

Synonyms: *Agyneia multilocularis* Roxb. *ex* Willd. (1803), *Bradleia multilocularis* (Roxb. *ex* Willd.) Spreng. (1826), *Phyllanthus multilocularis* (Roxb. *ex* Willd.) Muell.-Arg. (1866).

English name: Not known

Local name: Aniatori, Keotomi, Keoura, Paniatori, Pannyaturi.

1.9.3: General botanical features (Rahman, 2008)

Glochidion multiloculare is an evergreen shrub or small tree.

Plant structure:

An evergreen shrub or small tree, branchlets glabrous or puberulous, sometimes prickly with thickened stipules, twigs angular.

Trunk & bark:

Bark brownish, peeling off in thin, papery transparent flakes, tuberculated or watery, blaze reddish.

Leaves:

Leaves stipulate, stipules 2-4 mm long, acuminate, petiolate, petioles 1-4 mm long, leaf blade elliptic, elliptic-oblong or bluntly apiculate, base cuneate, shining, entire, coriaceous, pale and glabrous or rarely pubescent beneath, often yellow-green above when dry, lateral veins 5-8 pairs.

Flowers:

Flowers small, greenish-yellow, on short, stout pedicles, both male and females clustered in the leaf axils. Male flowers pedicellate, pedicels 5-8 mm long, slender, sepals 6, biseriate, stamens extrorse, longitudinally dehiscent, anthers usually 6. Female flowers with pedicels 3-5 mm long, stout, sepals 6 or more, glabrous, ovary 10-15 celled, glabrous, style a hollow grooved cone on top of the ovary.

Fruits:

Fruit a capsule, strongly depressed-globose, 10-15 lobed, 1.5-2.4 cm in diameter, base and apex intruded.

Seeds: Seeds are red.

Flowering and fruiting: July-January.

Distribution: *Glochidion multiloculare* (Roxb. *ex* Willd.) Muell.-Arg. grows in Modhupur, Tanghail of Bangladesh. It is also available in Bhutan, India, Myanmar and Nepal.

Figure 1.26: Photograph of the whole plant of *G. multiloculare*

Figure 1.27: Photograph of flowers and fruits of *G. multiloculare*

1.10: Medicinal uses of *Phyllanthus* taxon of Bangladesh (Ghani, 1998)

Medicinal uses of *Phyllanthus* taxon of Bacgladesh are listed below (Table-1.5).

Table- 1.5: Medicinal uses of Phyllanthus taxon of Bangladesh

Species	Plant part	Use
P. acidus	Fruit	Fruit is edible, astringent, appetiser and tonic to liver, and used in bronchitis, biliousness, piles and urinary concretions. It purifies and enriches the blood and is useful in thirst, vomiting and constipation.
	Root	Used as cathartic.
	Seed	Used as cathartic.
P. emblica	Fruit	Fruit is used as a source of vitamin C, as a diuretic, refrigerant, carminative, stomachic, laxative, antacid and tonic; promotes children's resistance to cold and cough. It is also used in vomiting and burning urination, and as a hair tonic. Dried fruit is astringent and used in haemorrhoids, diarrhoea, dysentery, anaemia, jaundice dyspepsia. It is also useful in insomnia, skin problems, gall pain, leucorrhoea, tympanitis and as an anti-emetic.
	Flower	Flowers are cooling and aperient
	Bark	Bark is astringent
P. freternus	Whole plant	Plant is used as a diuretic in dropsical affections and in gonorrhoea, leucorrhoea, dyspepsia, colic, diarrhoea and dysentery. It si also regarded as deobstruent, stomachic, febrifuge and antiseptic. The plant is useful in diabetes.
	Leaves	Leaves are used as poultice on swellings and ulcers.
	Shoots	Tender shoots are used in curing chronic dysentery.
	Root	Fresh roots are beneficially used in jaundice.
P. reticulatus	Leaves	Leaves are used as astringent, diuretic and alterative. Juice of leaf is used for curing diarrhoea in children and, mixed with camphor and cubebs, used as a remedy for spongy and bleeding gums.
	Bark	Bark is used as astringent, diuretic and alterative.

1.11: Some reported biological works of *Glochidion* and *Phyllanthus* taxon

Some reported biological works of *Glochidion* and *Phyllanthus* taxon are listed in Table-1.6

Table-1.6: Some reported biological works of *Glochidion* and *Phyllanthus* taxon

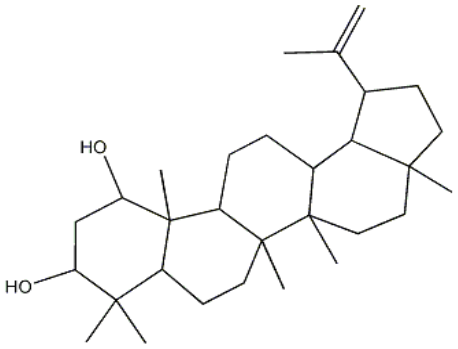
Species	Investigation and Result	References
G. eriocarpum	Cytotoxic activity was observed. Glochieriosides A and B exhibited significant cytotoxic activity against HL-60, HT-29, MCF-7 and SK-OV-3 human cancer cell lines with the IC_{50} values of 5.5, 6.8, 29.1, and 22.7 microM for glochierioside A, respectively, and 6.6, 18.6, 36.1, and 16.0 microM for glochierioside B. Glochidone was less active with IC_{50} values greater than 100 microM while lup-20(29)-en-1beta,3beta-diol was moderately active with IC_{50} values of 43.3, 67.0, 66.1, and 48.0 microM, respectively.	Kiem *et al*., 2009
G. sphaerogynum	Cytotoxic activity of lupane-type triterpenes from *Glochidion sphaerogynum*. Induction of apoptosis was observed.	Puapairoj *et al*., 2005
G. zeylanicum	Potential anti-tumor promoting activity of lupane-type triterpenoids from the stem bark of *Glochidion zeylanicum* was observed.	Tanaka *et al*., 2004
Phyllanthus niruri	An aqueous extract of the plant *Phyllanthus niruri* inhibits endogenous DNA polymerase of hepatitis B virus and binds to the surface antigen of hepatitis B virus in vitro. The extract also inhibits woodchuck hepatitis virus (WHV) DNA polymerase and binds to the surface antigen of WHV in vitro.	Venkateswaran *et al.*, 1987
P. amarus	Extract of *Phyllanthus amarus* (*P. amarus*) significantly inhibited hepatocarcinogenesis induced by N-nitrosodiethylamine (NDEA) in a dose dependent manner.	Jeena *et al*., 1999
P. amarus	The *Phyllanthus amarus* plant suppresses HBV mRNA transcription in vitro and exhibits therapeutic potential in chronic HBV carriers, although further work is necessary to define its mechanism of action.	Ott *et al*., 1997

Phyllanthus niruri	An aqueous extract of *Phyllanthus niruri* (Euphorbiaceae) inhibited human immunodeficiency virus type-1 reverse transcriptase (HIV-1-RT).	Ogata *et al*., 1992
P. amarus	Aqueous extract of *Phyllanthus amarus* (*P. amarus*) treatment exhibited potent anticarcinogenic activity against 20-methylcholanthrene (20-MC) induced sarcoma development and increased the survival of tumour harboring mice.	Rajeshkumar et al., 2002

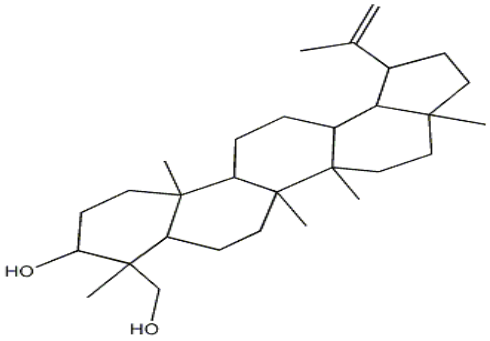
1.12: Chemistry of the genus *Glochidion*

Previous phytochemical investigations of Glochidion genus led to the isolation (1-beta,3-beta)-form of 20 (29)-Lupene-1,3-diol (**138**) (Kiem *et al*., 2009), 3-alpha form of 20 (29)- Lupene 3,23-diol (**139**) (Puapairoj *et al*., 2005), Acuminaminoside (**140**); Glochidacuminoside A (**141**); Glochidacuminoside B (**142**); Glochidacuminoside C (**143**); Glochidacuminoside D (**144**) and Glochidiolide (**152**) (Otsuka *et al*., 2004), Epilupeol (**144**); 5,7,3',4'-Tetra-*O*-methyl-*ent*-epicatechin 3-*O*-β-D-glucopyranoside (**149**); 5,7,3',4'-Tetra-*O*-methylepicatechin (**150**) (Thu *et al*., 2010), Glochidine (**145**) (Johns *et al*., 1967), Glochiflavanosides (A-D) (**146**) (Ostuka *et al*., 2001), Glochidioboside (**147**) (Evans, 2007), Glocheriosides (A and B) (**148**) (Yang *et al*., 2007), Glochidiol (**151**) and Glochidol (**159**) (Hui and Li, 1976), Glochidionolactone (A-F) (**153, 154, 155, 156, 157, 158)** (Ostuka *et al*., 2000), Glochidone (**160**) (Kiem, *et al*., 2009; Hui and Li, 1976), Glochidonol (**161**) (Thu *et al*., 2010; Hui and Li, 1976).

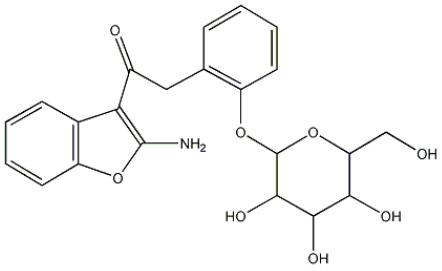
Previously reported compounds from *Glochidion* genus



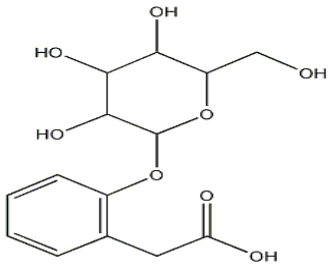
138
(1-beta,3-beta)-form of 20 (29)-Lupene-1, 3-diol



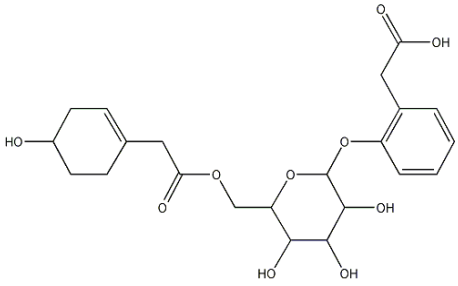
139
3-alpha form of 20 (29)- Lupene 3,23-diol



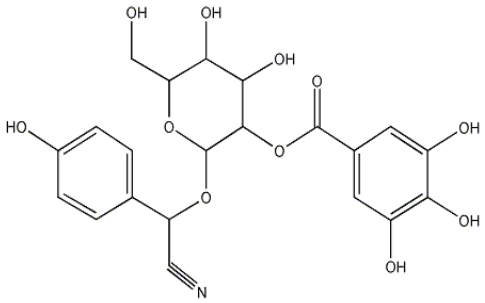
140
Acuminaminoside



141
Glochidacuminoside A

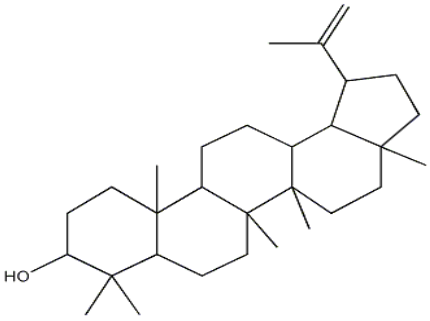


142
Glochidacuminoside B

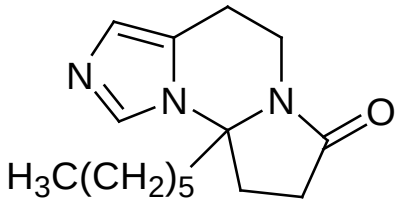


143
Glochidacuminoside D

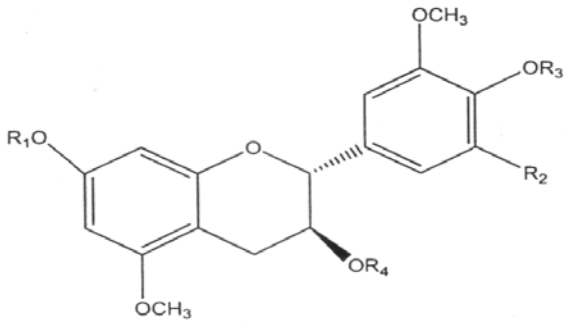
Figure-1.28: Structure of compounds reported from *Glochidion* genus.



144
Epilupeol



145
Glochidine

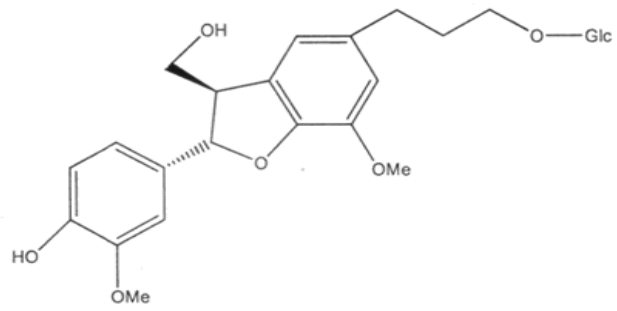


146

Glochiflavanosides A- D

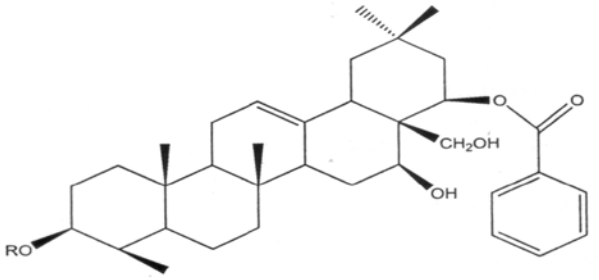
Glochiflavanosides	R_1	R_2	R_3	R_4
A	GLC	H	H	H
B	GLC	OCH_3	H	H
C	H	OCH_3	H	H
D	H	H	H	GLC

Figure-1.29: Structure of compounds reported from *Glochidion* genus (contd.)



147

Glochidioboside

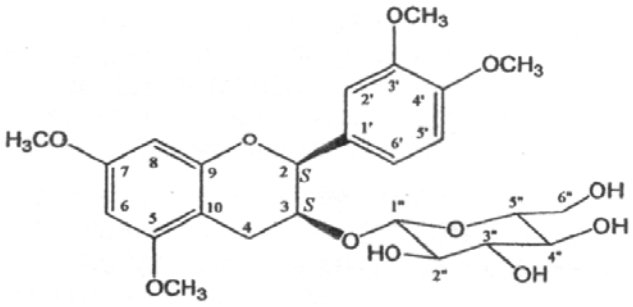


148

Glocheriosides A and B

Glocheriosides A:R = β –D- glucopyranosyll - (1 - 3)- α- L- arabinopyranoside

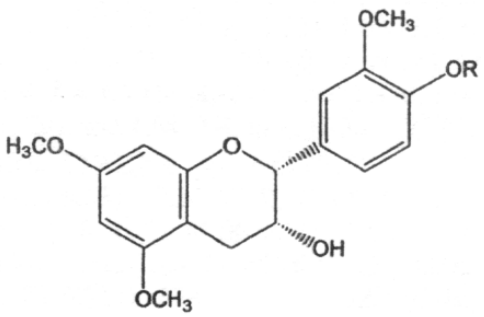
Glocheriosides B:R = β –D- glucopyranosyll - (1- 3)- β - d- xylopyranoside



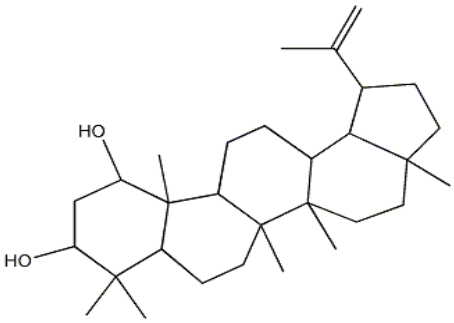
149

5,7,3',4'-Tetra-*O*-methyl-*ent*-epicatechin 3-*O*-β-D-glucopyranoside

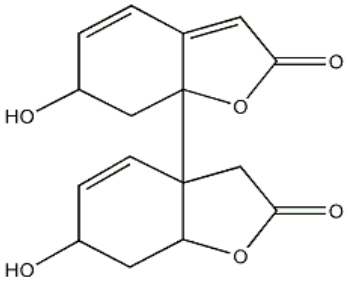
Figure-1.30: Structure of compounds reported from *Glochidion* genus (contd.)



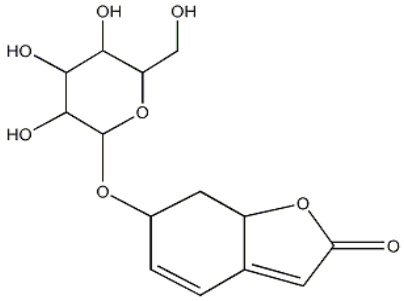
150
5,7,3',4'-Tetra-*O*-methylepicatechin



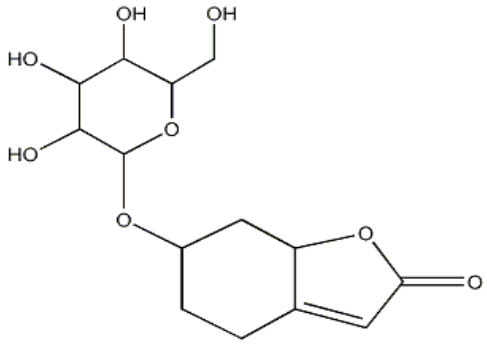
151
Glochidiol



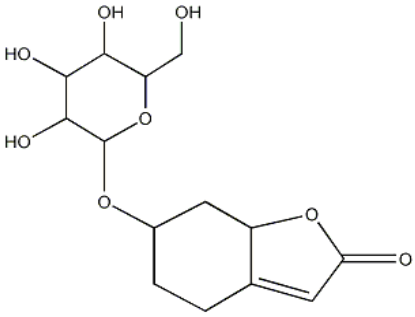
152
Glochidiolide



153
Glochidionolactone A

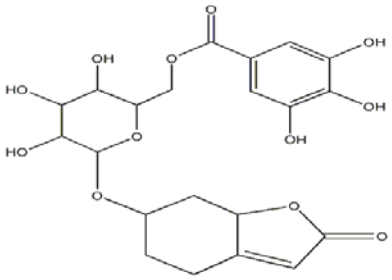


154
Glochidionolactone B

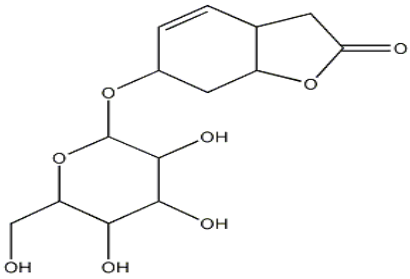


155
Glochidionolactone C

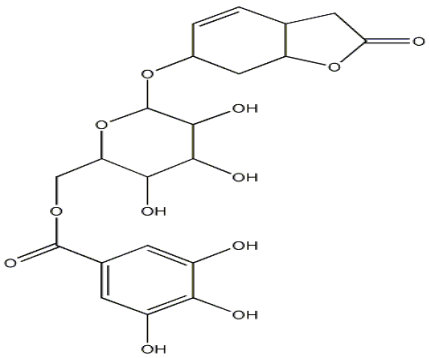
Figure-1.31: Structure of compounds reported from *Glochidion* genus (contd.)



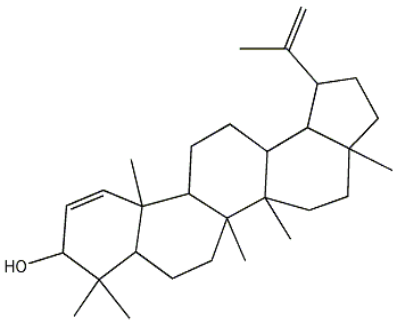
156
Glochidionolactone D



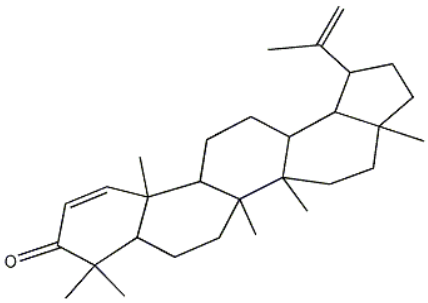
157
Glochidionolactone E



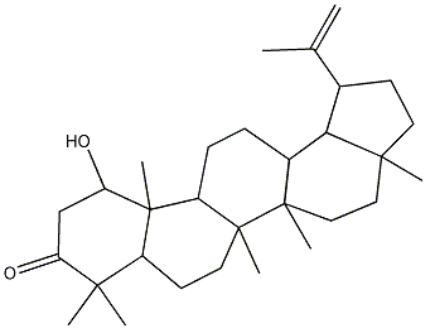
158
Glochidionolactone F



159
Glochidol



160
Glochidone



161
Glochidonol

Figure-1.32: Structure of compounds reported from *Glochidion* genus.

1.13: Chemistry of *G. multiloculare*

Phytochemical investigation for this plant was found four compounds (eg, Glochidiol, glochilocudiol, glochidone and dimedone) (Talapatra *et al.*, 1973).

1.14: Chemistry of *G. lanceolarium*

Previous phytochemical investigations of G. lanceolarium led to the isolation of triterpenes 3-epilupeol, glochidone and glochidiol from the bark and roots (Asolkar *et al*., 1992).

1.15: Terpenoids

Terpenes consist of five carbon isoprene units, derived from mevalonate and are classified broadly according to the number of isoprene units as follows:

I. Monoterpenes (C_{10})
II. Sesquiterpenes (C_{15})
III. Diterpenes (C_{20})
IV. Triterpenes (C_{30})

1.15.1: Biosynthesis of terpenoids

The terpenoids represent a large diverse class of secondary metabolites. They are constructed from isoprene (2-methyl butadiene) units. The first set of reactions starts with the formation of β-hydroxy-p-methylglutaryl CoA (HMG COA) from acetyl CoA and acetoacetyl CoA. HMG CoA is reduced to mevalonic acid which is then converted into isopentenylpyrophosphate through 5-phosphomevalonate, 5- pyrophosphomevalonate and 3-phospho-5-pyrophosphomevalonate. Isopentenylpyrpohosphate is then isomerized into dimethylallylpyrophosphate. Isopentenylpyrophosphate and dimethyl allyl pyrophosphate are then condensed to form geranyl pyrophosphate. From the geranyl pyrophosphate monoterpenes are formed. Geranyl pyrophosphate is condensed with another molecule of dimethylallylpyrophosphate to form farnesyl pyrophosphate

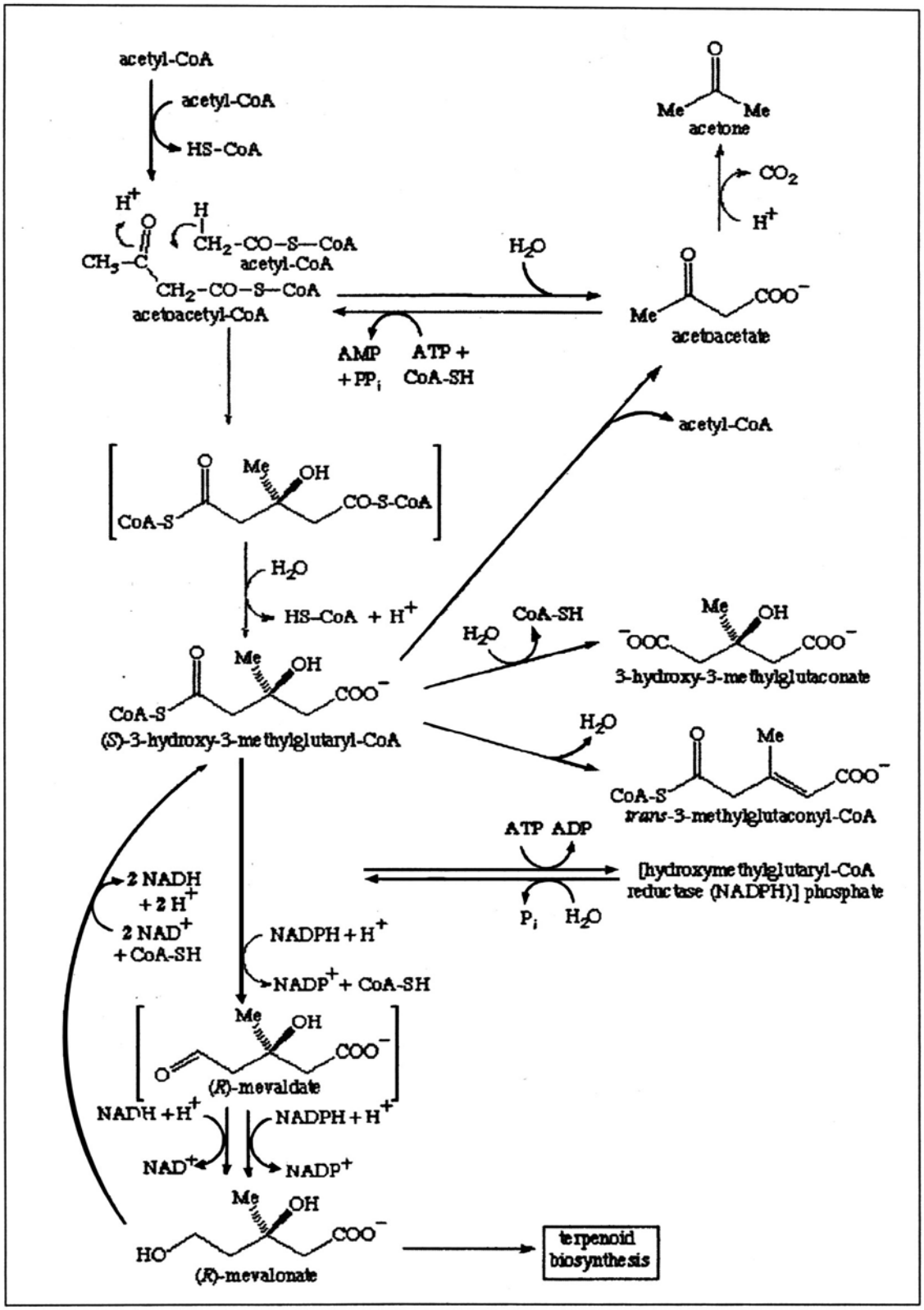


Figure 1.33: Biosynthesis of mevalonate (IUBMB, 2005)

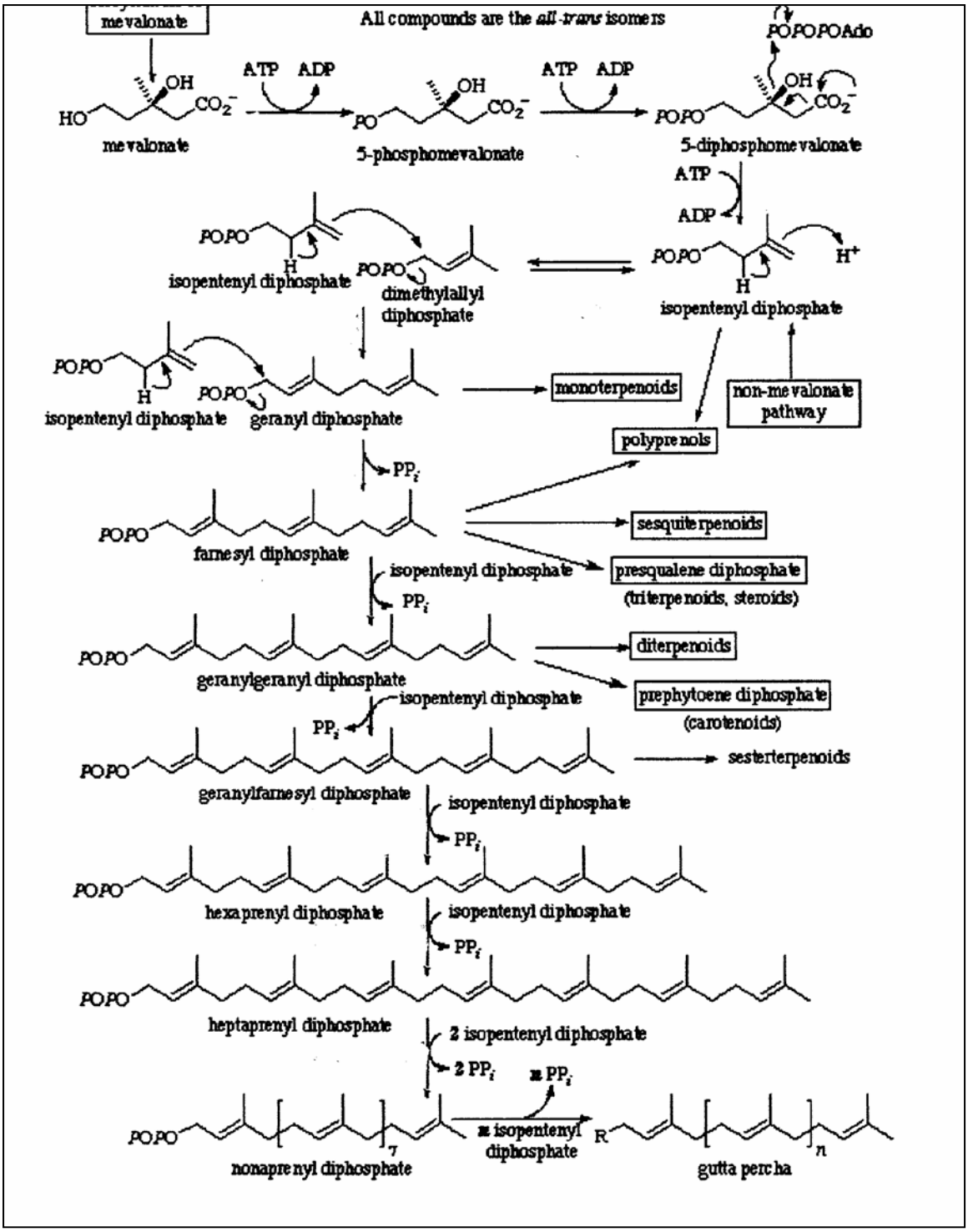


Figure 1.34: Biosynthesis of terpenoids (IUBMB, 2005)

Biosynthesis of triterpenes:

Biosynthetically squalene or the 3S isomer of 2,3-epoxy-2,3-dihydrosqualene is the immediate precursor of all triterpenoids (Newman, A.A. 1972). Triterpenoids are formed by the cyclisation of these two precursors followed by rearrangement. 3(S)-2,3-epoxy-2,3-dihydrosqualene (squalene-2,3-epoxide) undergoes cyclisation to give 3β-hydroxytriterpenoids which by oxidation and reduction can be transformed into 3α-hydroxytriterpenoids.

Cyclisation of squalene-2,3-epoxide in a *chair-boat-chair-boat* conformation and by a subsequent sequence of rearrangements leads to lanosterol, cycloartenol and cucurbitacin I (J.D. Connolly and K.H. Overton, 1972). From cycloartenol, other terpenoids are formed. Desmosterol is formed from lanosterol by a sequence of modification reactions. β-Sitosterol and stigmasterol are formed by the addition of extra carbon atoms to the side chain of desmosterol in plants. Cyclisation of squalene-2,3-epoxide in the *chair-chair-chair-boat* conformation leads to the dammarane ring system. This cyclisation goes through a series of carbonium ion intermediates to a cation from which dammaranes, euphanes and tirucallanes are thought to be derived. According to the scheme suggested by Eschenmoser *et al*, 1955, the transformation of the carbonium ion intermediates into euphol or tirucallol occurs either by a concerted process or via the appropriate ethylenic intermediates.

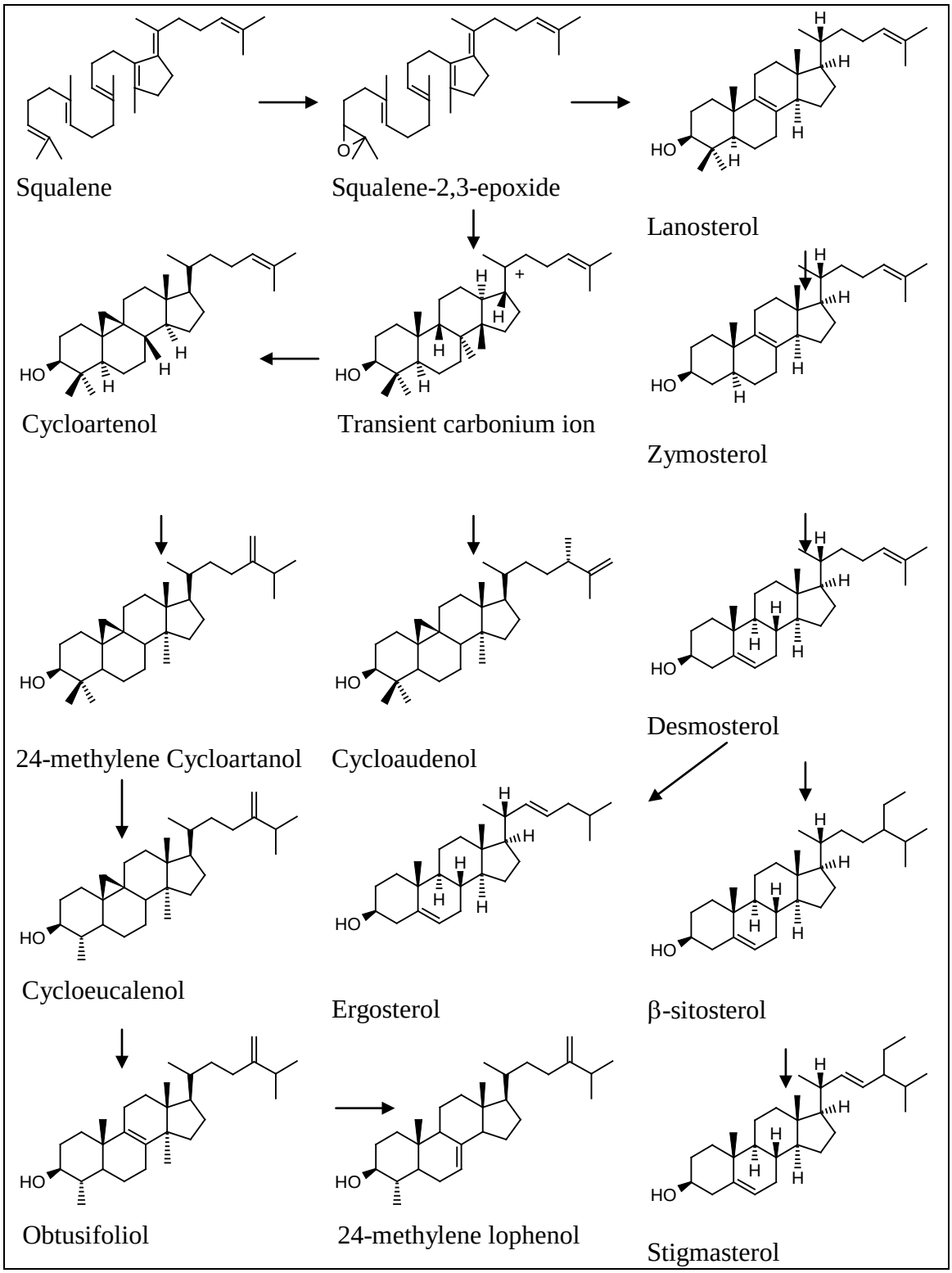


Figure 1.35: Biosynthesis of triterpenoids and phytosterols (Trease and Evans, 1989; Evans, 1996).

CHAPTER 2

Materials and Methods- Chemical

2.1. Methods

The chemical investigation of a plant can be divided roughly into the following major steps:

a) Collection and proper identification of the plant materials

b) Preparation of plant sample

c) Extraction

d) Fractionation and isolation of compounds

e) Structural characterization of purified compounds

The last step will be discussed in Chapter-3. However, other steps will be presented here in connection with concerned plants.

2.2 Selection of plant samples

After surveying and reviewing the literature, the whole plant/plant part (s) were collected from authentic sources and identified by a taxonomist. A voucher specimen was deposited for each sample at Bangladesh National Herbarium (BNH) with an accession number for each sample. The plant species belonging to Annonaceae and Euphorbiaceae families were selected for chemical studies.

2.3 Chemical investigation of *Polyalthia simiarum* (Hook. F. and Thom.).

Plant parts: The fresh stem bark of the plant *Polyalthia simiarum* (Family-Annonaceae) was selected for phytochemical investigation.

2.3.1 Collection and preparation of plant material

The stem bark of *P. simiarum* was collected from Mirpur, Dhaka in the month of June 2008. It was taxonomically identified by Mr. Sarder Nasir Uddin, Scientific Officer, Bangladesh National Herbarium, Ministry of Environment and Forest, Dhaka,

Bangladesh, (accession number DACB-34201). The stem bark of the plant was cut into small pieces, cleaned, dried and pulverized.

2.3.2 Extraction

The air dried powdered plant material (750 g) was sequentially extracted in a Soxhlet apparatus with petroleum ether (60-80^{O}C) followed by ethyl acetate. The extractives were filtered through a fresh cotton plug and followed by Whatman no.1 filter paper. The volume of the filtrate were concentrated with a rotary evaporator at temperature between 40^{o}-50^{o}C and reduced pressure and afforded pet-ether extract (PE, 3.5g), ethyl acetate extract (EA, 2.5g).

2.3.3 Initial screening of the extractive by thin layer chromatography (TLC)

Thin layer chromatographic technique was used for initial screening of the extractives in different solvent systems. As per TLC reports, petroleum ether soluble extract (PE) was selected for further chemical investigation.

2.3.4 Investigation of the petroleum ether soluble extract (PE)

A portion of the PE (700 mg) was subjected to column chromatography (CC) for fractionation.

2.3.5 Investigation of the petroleum ether soluble material

Column Chromatography

The column was packed with silica gel (Kieselgel, mesh 70-230). Slurry of silica gel in *n*-hexane 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 petroleum ether soluble material onto silica gel (Kieselgel 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 *n*-hexane, followed by mixtures of *n*-hexane 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 petroleum ether soluble

material and has been listed in Table 2.1. The elutes were collected in test tubes. A total of 150 fractions were collected each 20 ml.

Table-2.1: Solvent systems used for column chromatography (CC) of petroleum ether (PE) soluble materials

Fraction no.	Solvent systems		Volume collected (ml/test tube)
1-4	Hexane+ Ethyl acetate	(98 : 2)	20
5-8	Hexane + Ethyl acetate	(95 : 5)	20
9-21	Hexane + Ethyl acetate	(92.5 : 7.5)	20
22-38	Hexane + Ethyl acetate	(90 : 10)	20
39-52	Hexane + Ethyl acetate	(85 : 15)	20
53-64	Hexane + Ethyl acetate	(80 : 20)	20
65-78	Hexane + Ethyl acetate	(70 : 30)	20
79-91	Hexane + Ethyl acetate	(50 : 50)	20
92-105	Ethyl acetate	(100%)	20
106-110	Ethyl acetate + Methanol	(90 : 1)	20
111-120	Ethyl acetate + Methanol	(98 : 2)	20
121-128	Ethyl acetate + Methanol	(95 : 5)	20
129-135	Ethyl acetate + Methanol	(90 : 10)	20
136-143	Ethyl acetate + Methanol	(85 : 15)	20
144-150	Ethyl acetate + Methanol	(70 : 30)	20

2.3.6 Analysis of CC fractions by thin layer chromatography (TLC)

All the CC fractions were screened by TLC using various solvent systems. The plates were monitored under UV light and by spraying with vanillin-sulphuric acid reagent followed by heating at 100-110^{o} C for 5-10 minutes. Depending on the TLC behaviour, fractions 16-22, 50-60, 66-68 and 87-102 were selected for further investigation. Four compounds have been isolated and purified from the different CC fractions by adopting various techniques.

2.3.6.1 Isolation of PSB-001 (162)

The column fractions 16-22 were mixed together due to their identical TLC characteristics. This combination of column fractions eluted with 7.5-10% EtOAc in *n*-hexane was subjected to PTLC (Stationary phase:- Silica gel PF_{254}, Mobile phase:- Toluene : Ethyl acetate : Acetic acid = 98 :2: 1, multiple developments, Thickness of plates:- 0.5mm). From the developed plates, a set of identical bands (UV radiation active)

was scrapped separately and eluted with conventional process. This compound was given light orange color spot after spray with vanillin-sulfuric acid solution by heating at 110° C for 5 minutes. The compound, so obtained was designated PSB-001 (9.5 mg).

2.3.6.2 Isolation of PSB-007 (163)

The column fractions 50-60 were bulked together on the basis of TLC reports. This combination of column fractions eluted with 15-20% EtOAc in *n*-hexane was subjected to PTLC using Toluene- EtOAc- AcOH (92:8:1) as the developing solvents afforded compound PSB-007 (8.4 mg). This compound was given violet color spot after spray with vanillin-sulfuric acid solution by heating at 110° C for 5 minutes.

2.3.6.3 Isolation of PSB-004 (164)

The column fractions 66-68 were combined together as they showed similar TLC feature. This combination of column fractions eluted with 20-30% EtOAc in *n*-hexane was subjected to PTLC (Stationary phase:- Silica gel PF_{254}, Mobile phase:- Toluene : Ethyl acetate : Acetic acid = 90 : 10: 1, multiple developments, Thickness of plates:- 0.5mm). From the developed plates a set of identical bands were scrapped and eluted with conventional process.This compound was given violet color spot after spray with vanillin-sulfuric acid solution by heating at 110° C for 5 minutes.The compound, so obtained was designated PSB-004 (10.2 mg).

2.3.6.4 Isolation of PSB-008 (165)

The column fractions 87-102 were mixed together as they showed similar TLC feature. This combination of column fractions eluted with 50-100% EtOAc in *n*-hexane was subjected to PTLC with Toluene- EtOAc-AcOH (70: 30: 1) as the mobile phase yielded compound PSB-008 (1.5). This compound was given greenish color spot after spray with vanillin-sulfuric acid solution by heating at 110° C for 5 minutes.

2.4 Chemical investigation of *Glochidion multiloculare* (Roxb. *ex* Willd.) Muell.-Arg.

Plant parts: The fresh stem bark of the plant *Glochidion multiloculare* (Family-Euphorbiacea)) was selected for phytochemical investigation.

2.4.1 Collection and preparation of plant material

The stem bark of *G. multiloculare* was collected from Modhupur, Tanghail in the month of April, 2009. It was taxonomically identified by Mr. Sarder Nasir Uddin, Scientific Officer, Bangladesh National Herbarium, Ministry of Environment and Forest, Dhaka, Bangladesh, where a voucher specimen (DACB-34200) representing this collection has been deposited. The stem bark of the plant was cut into small pieces, cleaned, dried and pulverized.

2.4.2: Preparation of extracts:

The air dried powdered plant material (1000 g) was successively cold extracted with methanol (7 days) at room temperature with occasional shaking and stiring. The extractives were filtered through fresh cotton plug and followed by whatman no.1 filter paper. The filtrate were then concentrated by a Buchii rotavapor at low temperature and pressure and afforded methanol extract (41.7398g). The cold methanol extract (10 g) was subjected to Solvent-Solvent partitioning using the protocol designed by Kupchan and modified by Wagene (Vanwagenen *et al*., 1993). The extract was portioned successively with petroleum ether, carbon tetrachloride, and chloroform. Evaporation of solvents afforded petroleum ether (PEFGM, 3.8g), carbon tetrachloride (CTFGM, 2.5g), chloroform (CFFGM, 500mg) and aqueous (AQFGM, 1.3g).

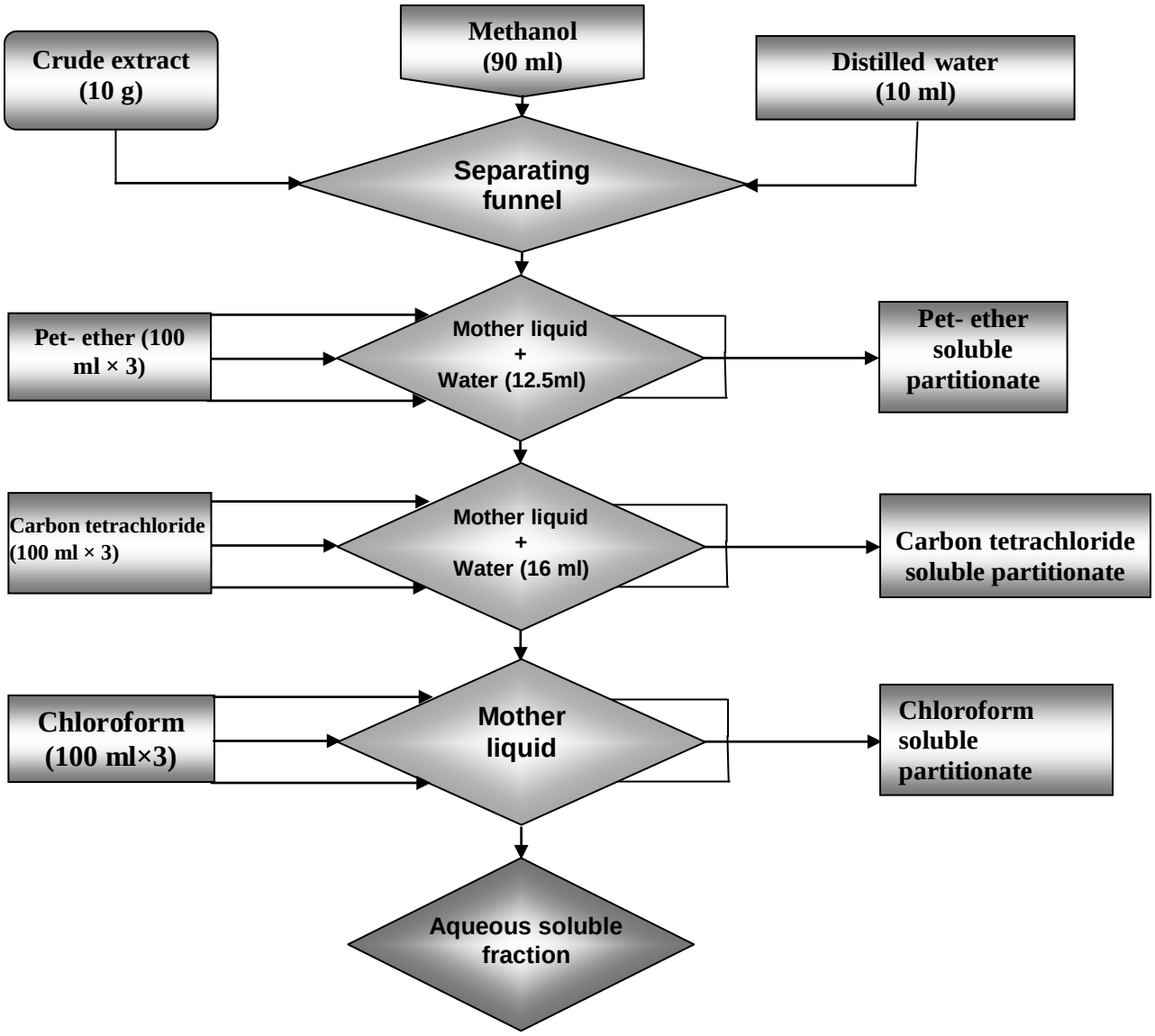


Figure 2.1: Schematic diagram of a modified Kupchan partitioning method

2.4.3 Initial screening of the extractive by thin layer chromatography (TLC)

Thin layer chromatographic technique was used for initial screening of the extractives in different solvent systems. As per TLC reports, petroleum ether soluble fraction (PEFGM) was selected for further chemical investigation.

2.4.4 Investigation of the petroleum ether soluble fraction (PEFGM)

A portion of the PEFGM (850 mg) was subjected to column chromatography (CC) for fractionation.

2.4.5 Investigation of the petroleum ether soluble material

Column Chromatography

A column having 70 cm length and 2.7 cm in diameter was filtered with the silica gel (Kieselgel, mesh 70-230) up to the height of 50 cm under wet packing condition. The column was washed with a few hundred milliliter of solvent to facilitate compact packing. The sample was prepared by adsorbing pet ether soluble materials onto silica gel (as before) and allowed to dry to get a free flowing powder like mass and subsequently applied on top of the adsorbent layer. The column was then eluted with petroleum ether, followed by petroleum ether and ethyl acetate mixtures of increasing polarities and finally with ethyl acetate and methanol in order of increasing polarities. Solvent system used as mobile phase in CC analysis of petroleum ether soluble material and has been listed in Table- 2.2. The elutes were collected in test tubes. A total of 136 fractions were collected each 20 ml.

Table-2.2: Solvent systems used for column chromatography (CC) of petroleum ether (PEFGM) soluble materials

Fraction no.	Solvent systems	Volume collected (ml/test tube)
1-5	Petroleum ether + Ethyl acetate (99 : 1)	20
6-16	Petroleum ether + Ethyl acetate (98 : 2)	20
17-24	Petroleum ether + Ethyl acetate (95 : 5)	20
25-32	Petroleum ether + Ethyl acetate (93 : 7)	20
33-42	Petroleum ether + Ethyl acetate (92.5 : 7.5)	20
43-54	Petroleum ether + Ethyl acetate (90 : 10)	20
55-62	Petroleum ether + Ethyl acetate (85 : 15)	20
63-78	Petroleum ether + Ethyl acetate (80 : 20)	20
79-80	Petroleum ether + Ethyl acetate (70 : 30)	20
81-92	Petroleum ether + Ethyl acetate (60 : 40)	20
93-100	Petroleum ether + Ethyl acetate (50 : 50)	20
101-104	Petroleum ether + Ethyl acetate (30 : 70)	20
105-112	Petroleum ether + Ethyl acetate (20 : 80)	20
113-120	Petroleum ether + Ethyl acetate (100%)	20
121-125	Ethyl acetate + Methanol (99 : 1)	20
126-136	Ethyl acetate + Methanol (95 : 5)	20

2.4.6 Analysis of CC fractions by thin layer chromatography (TLC)

All the CC fractions were screened by TLC using various solvent systems. The plates were monitored under UV light and by spraying with vanillin-sulphuric acid reagent followed by heating at 100-110^{o} C for 5-10 minutes. Depending on the TLC behaviour, fractions 17-22, 42-50, 55-62 and 82-89 were selected for further investigation. Five compounds have been isolated and purified from the different CC fractions by adopting various techniques.

2.4.6.1 Isolation of GM- 022 (166)

The column fractions 17-22 were bulked together as they showed similar TLC feature and subjected to Preparative Thin Layer Chromatography PTLC (Stationary phase:- Silica gel PF_{254}, Mobile phase:- Toluene : Ethyl acetate = 97 : 3, 2 times). 5 plates were developed and two bands formed. From the developed plates a violet colored band (band-1) was visualized after spraying with vanillin-sulfuric acid reagent followed by heating at 110º C for 5 minutes. The band was scrapped on to aluminium foil and eluted initially using a 50:50 mixture of ethyl acetate and chloroform followed by 100 % ethyl acetate. After evaporation of the solvent, white crystals were formed. The compound was checked for purity and termed as GM-022 (2 mg).

2.4.6.2 Isolation of GM- 023 (167)

The column fractions 17-22 were combined together due to their similar TLC feature. This combination of column fractions eluted with 5% EtOAc in petroleum ether was subjected to PTLC using (Toluene : Ethyl acetate =97:3, 2 times). From the developed plates a set of identical bands (band- 2, UV radiation active) were scrapped separately and eluted with conventional process. This compound was given violet color spot after spray with vanillin-sulfuric acid solution by heating at 110°C for 5 minutes. After evaporation of the solvent, white tree branch shaped crystals were formed. The compound, so obtained was designated GM-023 (8.7 mg).

2.4.6.3 Isolation of GM- 029 (168)

The column fractions 42-50 were mixed together due to their identical TLC characteristics.This combination of column fractions eluted with 10% EtOAc in petroleum ether was subjected to PTLC using the solvent system Toluene-Ethyl acetate (95: 5), one violet colored spot was visualized by spraying with vanillin-sulfuric acid followed by heating at 110º C for 5 minutes. The desired band was scrapped from the developed plates and then eluted initially using 50:50 mixture of ethyl acetate and chloroform followed by 100% ethyl acetate. Transparent oily substances was obtained. The compound was checked for purity and termed as GM- 029 (2 mg).

2.4.6.4 Isolation of GM- 032 (169)

The column fractions 55-62 were bulked together as they showed similar TLC feature and subjected to Preparative Thin Layer Chromatography PTLC (Stationary phase:- Silica gel PF_{254}, Mobile phase:- Toluene : Ethyl acetate = 90 : 10, multiple developments, Thickness of plates:- 0.5mm). From the developed plates, a set of identical bands (UV radiation active) was scrapped separately and eluted with conventional process. After evaporation of the solvent, white amorphous particle were formed. It appeared as purple spot on the TLC plate when sprayed with vanillin-sulfuric acid spray reagent by heating at 110º C for 5 minutes. The compound, so obtained was designated GM-032 (8 mg).

2.4.6.5 Isolation of GM- 035 (170)

The silica gel column fractions 82-89 were combined together due to their similar TLC feature and was subjected to PTLC (running with, Toluene-Ethyl acetate = 80 : 20). From the developed plates, a set of identical bands (UV radiation active) was scrapped separately and eluted with conventional process. After evaporation of the solvent, needle shaped crystal were formed. It appeared as violet spot on the TLC plate when sprayed with vanillin-sulfuric acid spray reagent by heating at 110º C for 5 minutes. The compound, so obtained was soluble in Ethyl acetate and Chloroform and was designated as GM-035 (5 mg).

2.5 Chemical investigation of *Glochidion lanceolarium* (Roxb.), Voigt

Plant parts: The fresh stem bark of the plant *Glochidion lanceolarium* (Family-Euphorbiacea)) was selected for phytochemical investigation.

2.5.1 Plant materials

The stem bark of *G. lanceolarium* was collected from Mirpur, Dhaka in the month of April, 2009 and identified by Mr. Sarder Nasir Uddin, Scientific Officer, Bangladesh National Herbarium, Dhaka, where a voucher specimen (DACB-34199) representing this collection has been deposited. Stem bark of this plant was air-dried for several days followed by oven-drying for 24 hours and then ground to a coarse powder.

2.5.2 Preparation of extracts:

The air dried powdered plant material (900 g) was successively cold extracted with methanol (7 days) at room temperature with occasional shaking and stiring. The extractives were filtered through fresh cotton plug and followed by whatman no. 1 filter paper. The filtrate were then concentrated by a Buchii rotavapor at low temperature and pressure and afforded methanol (MEGL) extract (36.8199g). The cold methanol extract (10 g) was subjected to Solvent-Solvent partitioning using the protocol designed by Kupchan and modified by Wagene (Vanwagenen *et al*., 1993). The extract was partioned successively with petroleum ether, carbon tetrachloride and chloroform. Evaporation of solvents afforded petroleum ether (PEFGL, 3.5 g), carbon tetrachloride (CTFGL, 2.6 g), chloroform (CFFGL, 700 mg) and aqueous (AQFGL, 1.9 g).

2.5.3 Initial screening of the extractive by thin layer chromatography (TLC)

Thin layer chromatographic technique was used for initial screening of the extractives in different solvent systems. As per TLC reports, carbon tetrachloride soluble fraction (CTFGL) was selected for further chemical investigation.

2.5.4 Investigation of the carbontetrachloride soluble fraction (CTFGL)

A portion of the carbon tetrachloride soluble fraction (1 g) was subjected to Vacuum Liquid Chromatography (VLC) for fractionation. Then the VLC fractions were analyzed

by TLC. VLC fractions with satisfactory resolution of components were subjected to PTLC or other separation techniques to obtain pure compounds

Vacuum Liquid Chromatography

The column was filled with fine TLC grade silica gel (kieselgel 60H, mesh 70-230) upto a height of three-fourth under reduced pressure. It was washed with petrol to facilitate compact packing. The sample was prepared by adsorbing 1 g of carbon tetrachloride soluble fraction onto silica gel (as before), allowed to dry and subsequently applied on top of the adsorbent layer. The column was then eluted with petroleum ether, followed by petroleum ether and ethyl acetate mixtures of increasing polarities and finally with ethyl acetate and methanol in order of increasing polarities. Solvent systems used as mobile phases in the VLC analysis of CTFGL materials were listed in Table- 2.3. A total of 28 fractions were collected.

Table 2.3: Solvent systems used for VLC analysis of carbon tetrachloride soluble material

Fraction no.	Solvent systems	Volume collected (ml)
1 (A+B)	Petroleum ether- Ethyl acetate (99 : 1)	50 + 50
2 (A+B)	Petroleum ether - Ethyl acetate (98 : 2)	50 + 50
3 (A+B)	Petroleum ether - Ethyl acetate (95 : 5)	50 + 50
4 (A+B)	Petroleum ether - Ethyl acetate (92.5 : 7.5)	50 + 50
5 (A+B)	Petroleum ether - Ethyl acetate (90 : 10)	50 + 50
6 (A+B)	Petroleum ether - Ethyl acetate (88 : 12)	50 + 50
7 (A+B)	Petroleum ether - Ethyl acetate (85 : 15)	50 + 50
8 (A+B)	Petroleum ether - Ethyl acetate (80 : 20)	50 + 50
9 (A+B)	Petroleum ether - Ethyl acetate (75 : 25)	50 + 50
10 (A+B)	Petroleum ether - Ethyl acetate (70 : 30)	50 + 50
11 (A+B)	Petroleum ether - Ethyl acetate (65 : 35)	50 + 50
12 (A+B)	Petroleum ether - Ethyl acetate (50 : 50)	50 + 50
13 (A+B)	Petroleum ether - Ethyl acetate (40 : 60)	50 + 50
14 (A+B)	Petroleum ether - Ethyl acetate (25 : 75)	50 + 50
15 (A+B)	Ethyl acetate (100 %)	50 + 50
16 (A+B)	Ethyl acetate - Methanol (98:2)	50 + 50
17 (A+B)	Ethyl acetate - Methanol (95:5)	50 + 50
28 (A+B)	Ethyl acetate - Methanol (90:10)	50 + 50

2.5.5 Analysis of VLC fractions by TLC:

All the 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^{o}C for 5-10 minutes. Three compounds were isolated.

2.5.5.1 Isolation of GL- 022 (171)

The VLC fractions 5A and 5B were combined together on the basis of TLC analysis. Preparative TLC of the VLC fractions developed with 5% EtOAc in tolune afforded 2.5 mg of GL-022 as white crystal. It appeared as violet spot on the TLC plate when sprayed with vanillin-sulfuric acid spray reagent by heating at 110º C for 5 minutes.

2.5.5.2 Isolation of GL- 023 (172)

Depending on the TLC behavior, fractions 6B and 7A were bulked together. After solvent evaporation the combination was subjected to Preparative Thin Layer Chromatography-PTLC [running with, Toluene-Ethyl acetate = 90:10]. From the developed plates, a set of identical bands (UV radiation active) was scrapped separately and eluted with conventional process. It appeared as purple spot on the TLC plate when sprayed with vanillin-sulfuric acid spray reagent by heating at 110º C for 5 minutes.The compound, so obtained was designated GL-023 (3.5 mg).

2.5.5.3 Isolation of GL- 035 (173)

The VLC fractions 9B and 10(A+B) were mixed together on the basis of the similar TLC feature and subjected to Preparative Thin Layer Chromatography PTLC with 12% EtOAc in toluene yielded 5mg of GL-035. After evaporation of the solvent, oily compound was formed. It appeared purple color spot after spray with vanillin-sulfuric acid solution by heating at 110°C for 5 minutes.

2.6 General experimental procedure

Column chromatography was done on silica gel (60-120 mesh, ASTM) and Vacuum liquid chromatography (VLC) was done over silica gel (Kieselgel 60H, mesh 70-230).

2.6.1 Test for purity of the isolated compounds

The purity of each of the isolated compounds was checked by TLC using different solvent systems. Commercially available plates pre-coated with silica gel (Kieselgel 60 F_{254}, thickness 0.2 mm) on plastic or aluminum sheets were used for this purpose. Thus the developed plates were first observed under UV light at 254 and 366 nm and also sprayed with different spray reagents, particularly with vanillin-sulfuric acid solution followed by heating at 110 ^{o}C for 10 minutes.

2.6.2 Instrumentation

Mass measurements were conducted on a Micromass Q-TOF Ultima Gloval Tandem mass spectrometer. ^{1}H- and ^{13}C- NMR spectra were acquired with a Bruker AMX–400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) spectrometer and the spectra were referenced to the residual non-deuterated solvent signals. *J*-modulated ^{13}C spectra were acquired with a relaxation time (d1) of 4 s.

2.6.3 Properties of isolated compounds

PSB-001 (2-Oxo-14,15-bisnor-3,11*E*-kolavadien-13-one, **162**): Colorless gum, light orange color when sprayed with vanillin-sulfuric; ESIMS: *m/z* 297.2 $[M+Na]^{+}$, $C_{18}H_{26}O_2Na$; ^{1}H NMR (Table- 3.1, Figure 3.3-3.7); ^{13}C NMR (Table-3.2, Figure 3.8-3.10); ESIMS spectrum (Figure 3.13).

PSB-007 (Kolavenic acid, **163**): Colorless gum; ^{1}H NMR (Table-3.3, Figure 3.15-3.16) and ^{13}C NMR (Table-3.4, Figure 3.17-3.18).

PSB-004 (16β-Hydroxycleroda-3,13(14)*Z*-dien-15,16-olide, **164**): Amorphous white mass; ^{1}H NMR (Table- 3.5, Figure 3.20-3.22) ^{13}C NMR (Table-3.6, 3.23-3.24); DEPT spectrum (Figure 3.25).

PSB-008 (16-Oxocleroda-3,13(14)*E*-dien-15-oic acid, **165**): Colorless gum; ^{1}H NMR (Table-3.7, Figure 3.27-3.29).

GM-022 (3-*Epi*-lupeol, **166**): White powder; ^{1}H NMR (Table-3.8, Figure 3.31-3.34).

GM-023 (Lupeol, **167**): White tree branch shaped crystal, violet color when sprayed with vanillin-sulfuric acid; ^{1}H NMR (Table-3.9, Figure 3.36-3.38)

GM-029 (Glochidone, **168**): Oily substance; ^{1}H NMR (Table-3.10, Figure 3.40-3.43).

GM-032 (Glochidonol, **169**): White amorphous particle, purple color on TLC when sprayed with vanillin-sulfuric acid; ^{1}H NMR (Table-3.11, Figure 3.45-3.47); ^{13}C NMR (Table-3.12, Figure 3.48-3.49).

GM-035 (Glochidiol, **170**): Needle shaped crystal, violet spot on TLC when sprayed with vanillin-sulfuric acid followed by heating at 110 ^{o}C for 10 minutes; ^{1}H NMR (Table-3.13, Figure 3.51-3.53).

GL-022 (Epilupeol, **171**): White crystal, ESIMS: *m/z* 427 $[M + H]^{+}$, ($C_{30}H_{50}O$, M = 426); ^{1}H NMR (Table- 3.14, Figure 3.55-3.58); ^{13}C NMR (Table-3.15, Figure 3.59-3.60); ESIMS spectrum (Figure 3.61-3.62).

GL-023 (Glochidonol, **172**): White amorphous particle, purple color on TLC when sprayed with vanillin-sulfuric acid, ESIMS: *m/z* 441 $[M + H]^{+}$, ($C_{30}H_{48}O_2$, M = 440); ^{1}H NMR (Table- 3.16, Figure 3.64-3.67); ESIMS spectrum (Figure 3.68-3.70).

GL-035 (Glochidone, **173**): Oily substance, ESIMS: *m/z* 422.69 $[M + H]^{+}$, ($C_{30}H_{46}O$); ^{1}H NMR (Table- 3.17, Figure 3.72-3.76); ESIMS spectrum (Figure 3.77-3.79).

Chapter-3

Results and Discussion

3.1 Chemical investigation of *Polyalthia simiarum*

Repeated chromatographic separation and purification of the pet ether fraction of the stem bark of *Polyalthia simiarum* afforded four compounds PSB-001, PSB-007, PSB-004 and PSB-008. The structures of the isolated compounds were determined by extensive spectroscopic analysis as well as by comparison with published values.

3.1.1 Characterization of PSB-001 as 2-oxo-14, 15-bisnor-3, 11 *E*-kolavadien-13-one (162)

Compound PSB-001 was obtained from the petroleum ether soluble fraction as a colorless gum. The compound was isolated by preparative TLC method. Spraying the developed plate with vanillin-sulfuric acid followed by heating at 110°C for 5 minutes produced a light orange color.

The ESI mass spectrum of this compound showed the pseudo molecular ion peak for $[M+Na]^+$ at *m/z* 297.2, consistent with the molecular formula, $C_{18}H_{26}O_2$ (Figure: 3.13). The ^{13}C NMR spectral data of the compound (Table-3.2, Figure: 3.8-3.10) displayed 18 carbon resonances, which was in support of the molecular formula. These spectral features indicated that this compound was a bisnor-type diterpenoid (Kijjoa *et al*.,1990). The DEPT spectrum (Figure: 3.11-3.12) indicated that 13 out of the 18 carbons in this compound had attached protons. The ^1H NMR (Table-3.1, Figure: 3.3-3.7) spectrum of this compound exhibited two vicinally coupled doublets (J =16.0 Hz) at δ 6.00 and 6.40, and a three protons singlet at 2.24, characteristic of an acetyl group. This was further supported by a carbonyl group at δ_C 199.4 and two methine carbons at δ_C 156.9 and 130.5, and a methyl carbon at δ_C 27.5 and allowed us to draw the partial structure, -CH=CH-$COCH_3$. These data are in close agreement to those published for a similar side chain in 14,15-bisnor-3,11*E*-kolavadien-13-one (Sashidhara *et al*., 2009; Kijjoa *et al*., 1990). The remaining signals in the NMR spectrum, assignable to the bicyclic diterpenoid moiety included three methyl group resonances (δ_H 0.74, δ_C 16.6; δ_H 0.98, δ_C 12.0; δ_H 1.13, δ_C 18.8), an olefinic methyl at δ_H 1.89 (δ_C 18.0), and an olefinic proton singlet of one proton intensity at δ 5.71. The last signal at δ 5.71 attached to a carbon at

125.9, could be assigned to H-3. An ABX system was also evident from the ^{1}H NMR signals at δ 1.89 (m), 2.13 (br. dd, *J* = 17.6, 3.6 Hz) and 2.32 (br. dd, *J* = 17.6, 14.4 Hz), which could be attributed to the equatorial proton, H-10 and equatorial and axial protons at C-1, respectively. These NMR signals were identical to the protons and carbons of the bicyclic portion of 2-oxokolavens (Bohlmann *et al*., 1981; Hasan *et al*., 1982). The side chain comprising -CH=CH-CO-CH_3 (C_4H_5O) could be linked to the bicyclic part of the diterpenoid skeleton at C-9, as in 2-oxokolava-3,13-dien-15-oic acid (Hasan *et al*., 1982), and 14,15-bisnor-3,11*E*-kolavadien-13-one (Sashidhara *et al*., 2009; Kijjoa *et al*., 1990) to provide 2-oxo-14,15-bisnor-3,11*E*-kolavadien-13-one, which has been reported as a new compound by us (Kabir *et al*., 2010). To the best of our knowledge, only two reports of the occurrence of a C18 bisnor-type clerodane diterpenoid, 14,15-bisnor-3,11*E*-kolavadien-13-one isolated from *P. viridis* (Kijjoa *et al*., 1990) and *P. longifolia* var. *pendulla* (Sashidhara *et al*., 2009) are available in the literature. Thus, 2-oxo-14,15-bisnor-3,11*E*-kolavadien-13-one (**162**) represents the second report of the isolation of any 18-carbon containing bisnor-clerodane diterpenoid from nature.

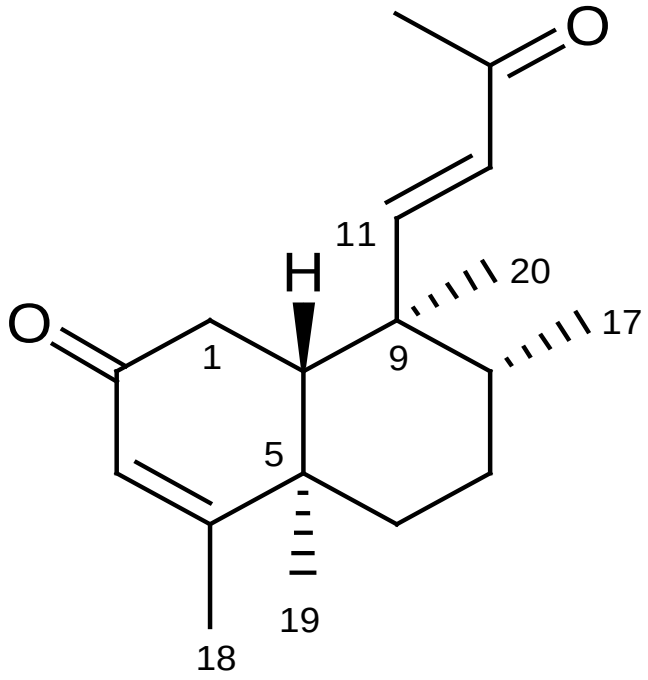
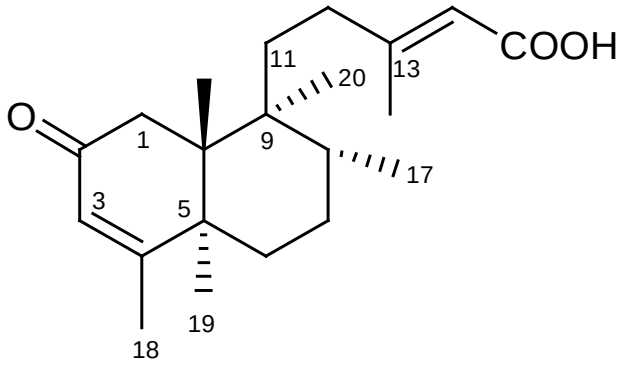
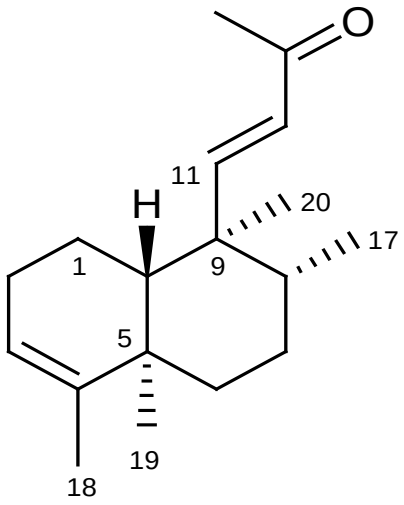


Figure 3.1 Structure of 2-Oxo-14,15-bisnor-3,11*E*-kolavadien-13-one (PSB-001, **162**)

Comparison of the compound 2-oxo-14,15-bisnor-3,11*E*-kolavadien-13-one(**162**) (Figure-3.1) with compounds 2-oxo kolavenic acid (**31**) and 14,15-bisnor-3,11*E*-kolavadien-13-one (**22**).



2-oxo kolavenic acid (**31**)



14,15-bisnor-3,11*E*-kolavadien-13-one (**22**)

Figure 3.2: Structure of 2-oxo kolavenic acid (**31**) and 14,15-bisnor-3,11*E*-kolavadien-13-one (**22**)

Table-3.1: ^{1}H NMR spectral data of PSB-001 (2-oxo-14,15-bisnor-3,11*E*-kolavadien-13-one, 162), 2-oxo kolavenic acid (31) and 14,15-bisnor-3,11,*E*-kolavadien-13-one (22) in $CDCl_3$ (Hasan *et al*., 1994).

Position	PSB-001	2-oxo kolavenic acid	14, 15-bisnor-3, 11, *E*-kolavadien-13-one
	δ_H, multi, *J* in Hz	δ_H, multi, *J* in Hz	δ_H, multi, *J* in Hz
1	2.13, br dd (17.6, 3.6); 2.32, br dd (17.6, 14.4)	2.33, dd (18.0, 4.7); 2.40, dd (18.0, 13.3)	
2			
3	5.71, br s	5.74, br s	5.17, br t (2.0, 1.5)
4			
5			
6		1.90, m; 1.40, dt (12.2, 5.5)	
7		1.30-146, m	
8		1.50, m	
9			
10	1.89, m	1.86, ABX (13.0, 4.7)	
11	6.40, d (16.0)	1.50, m	6.51, d (15.0)
12	6.00, d (16.0)	1.83, br td (3.0, 12.6); 2.05, dt (12.6, 4.6)	6.00, d (15.0)
13			
14	¥	5.68, br s	¥
15	¥		¥
16	2.24, s	2.15, d (6.0)	2.28, s
17	0.74, d (6.0)	0.85, d (6.0)	0.71, d (7.0)
18	1.89, br s	1.89, d (1.0)	1.59, d (1.5)
19	1.13, s	1.12, s	1.01, s
20	0.98, s	0.83, s	0.91, s

¥ bisnor-clerodane diterpenes are 18-carbon compounds, and thus structures **162** and **22** lack C-14 and C-15; the signals for H-10 and H_3-18 in compound **162** were overlapped.

Table-3.2 : ^{13}C NMR spectral data of PSB-001 (2-oxo-14,15-bisnor-3,11*E*-kolavadien-13-one, 162), 2-oxo kolavenic acid (31) and 14,15-bisnor-3,11,*E*-kolavadien-13-one (22) in $CDCl_3$ (Hasan *et al*., 1994).

Position	PSB-001	2-oxo kolavenic acid	14, 15-bisnor-3, 11, *E*-kolavadien-13-one
	δ_c in ppm	δ_c in ppm	δ_c in ppm
1	36.3	35.9	29.5
2	199.4	200.5	20.1
3	125.9	125.3	120.5
4	171.3	171.8, 172.8	143.5
5	39.1	40.2	37.2
6	35.3	34.5, 35.1	36.4
7	26.2	27.1	26.7
8	40.5	36.4	40.6
9	44. 1	39. 0	44.4
10	48.8	46.1	49.8
11	156.9	34.5, 35.1	159.8
12	130.5	35.9	129.6
13	199.4	162. 9	198.5
14	¥	115.8	¥
15	¥	171.8, 172.8	¥
16	27.5	18. 9	27.2
17	16.6	15. 8	16.6
18	18.0	18. 4	17.7
19	18.8	19. 5	19.6
20	12.0	17 .8	12.0

¥ bisnor-clerodane diterpenes are 18-carbon compounds, and thus structures **162** and **22** lack C-14 and C-15.

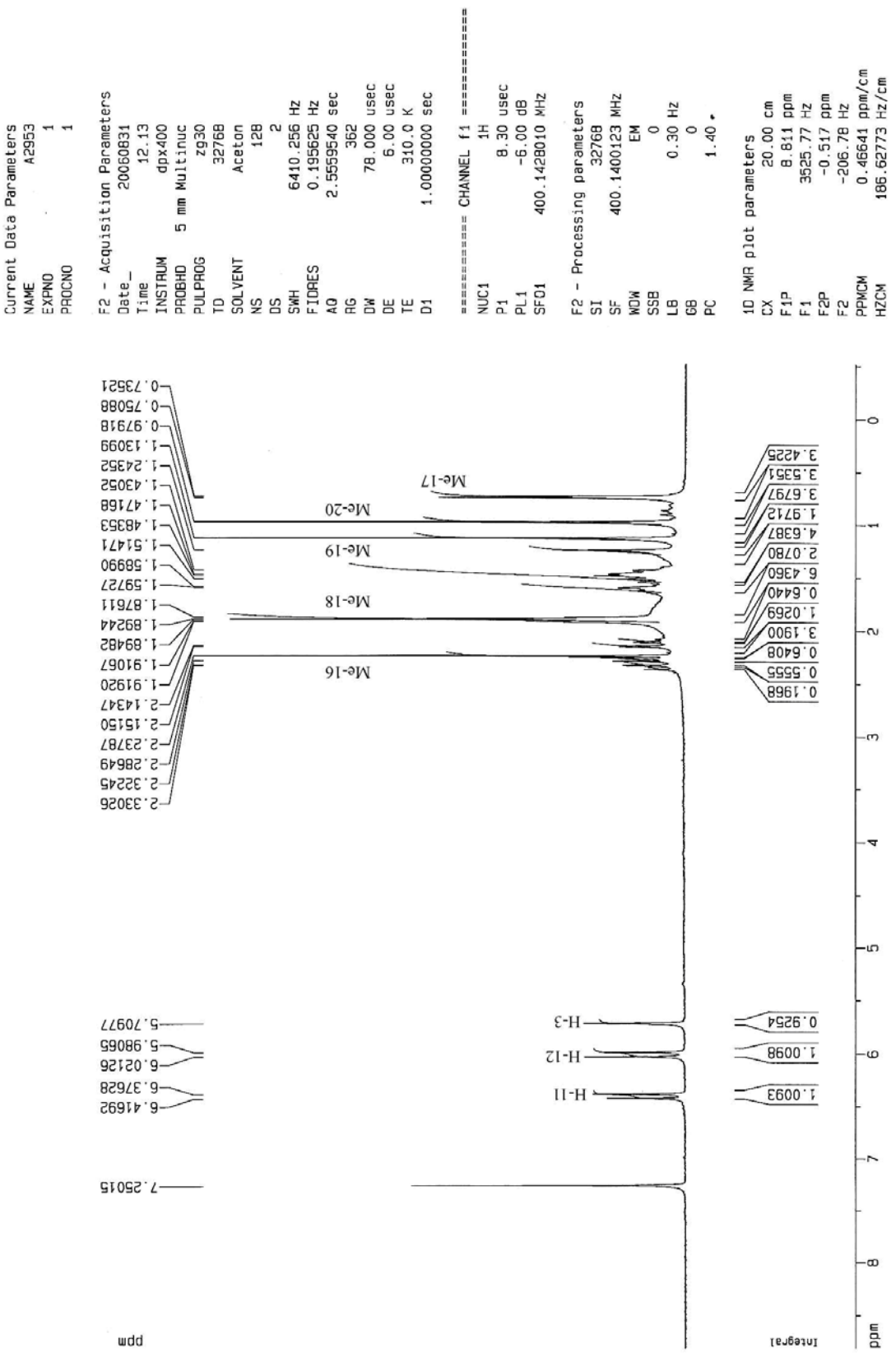


Figure-3.3: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-001 (**162**)

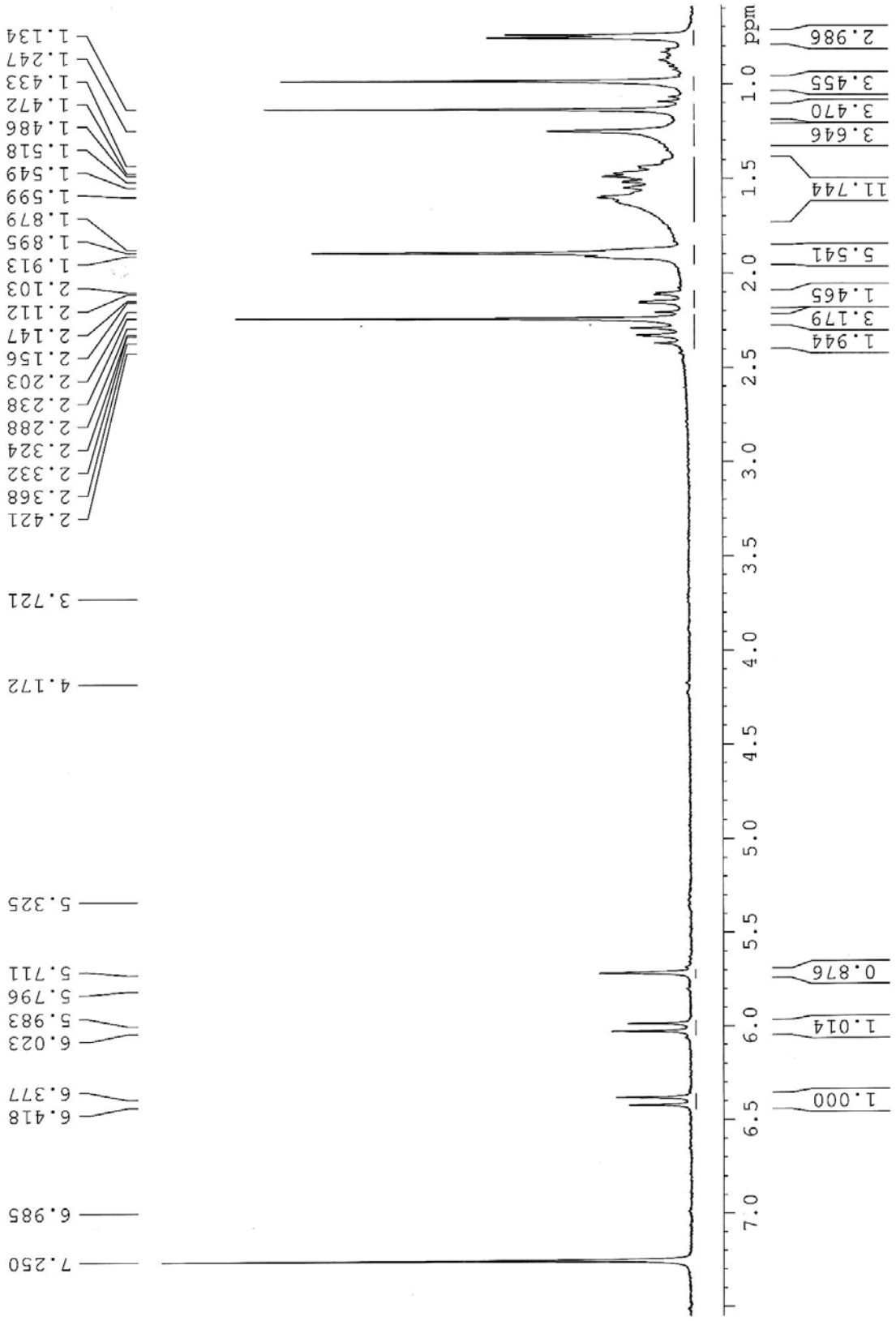


Figure-3.4: Expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-001(**162**)

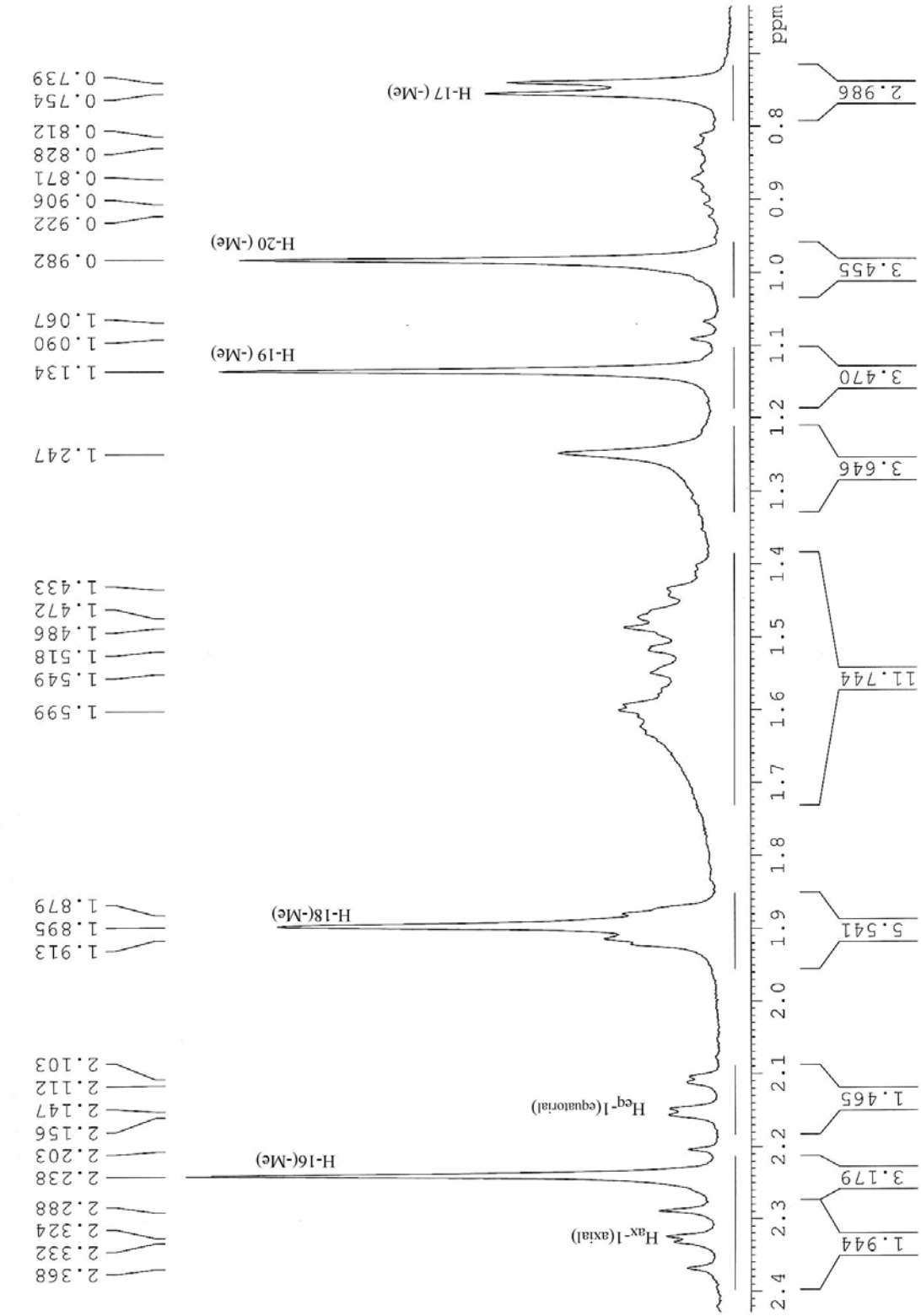


Figure-3.5: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-001 (**162**)

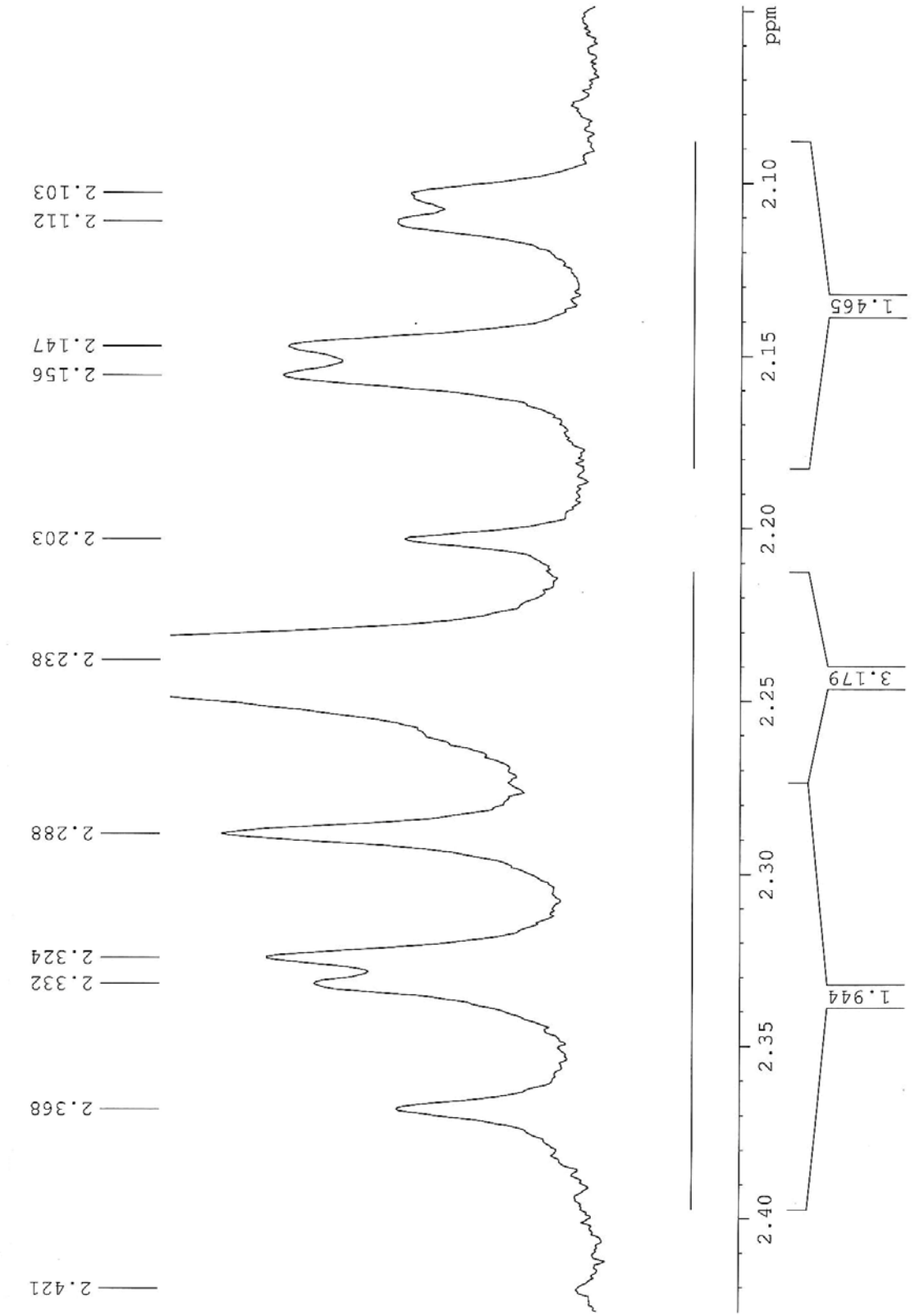


Figure-3.6: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-001 (**162**)

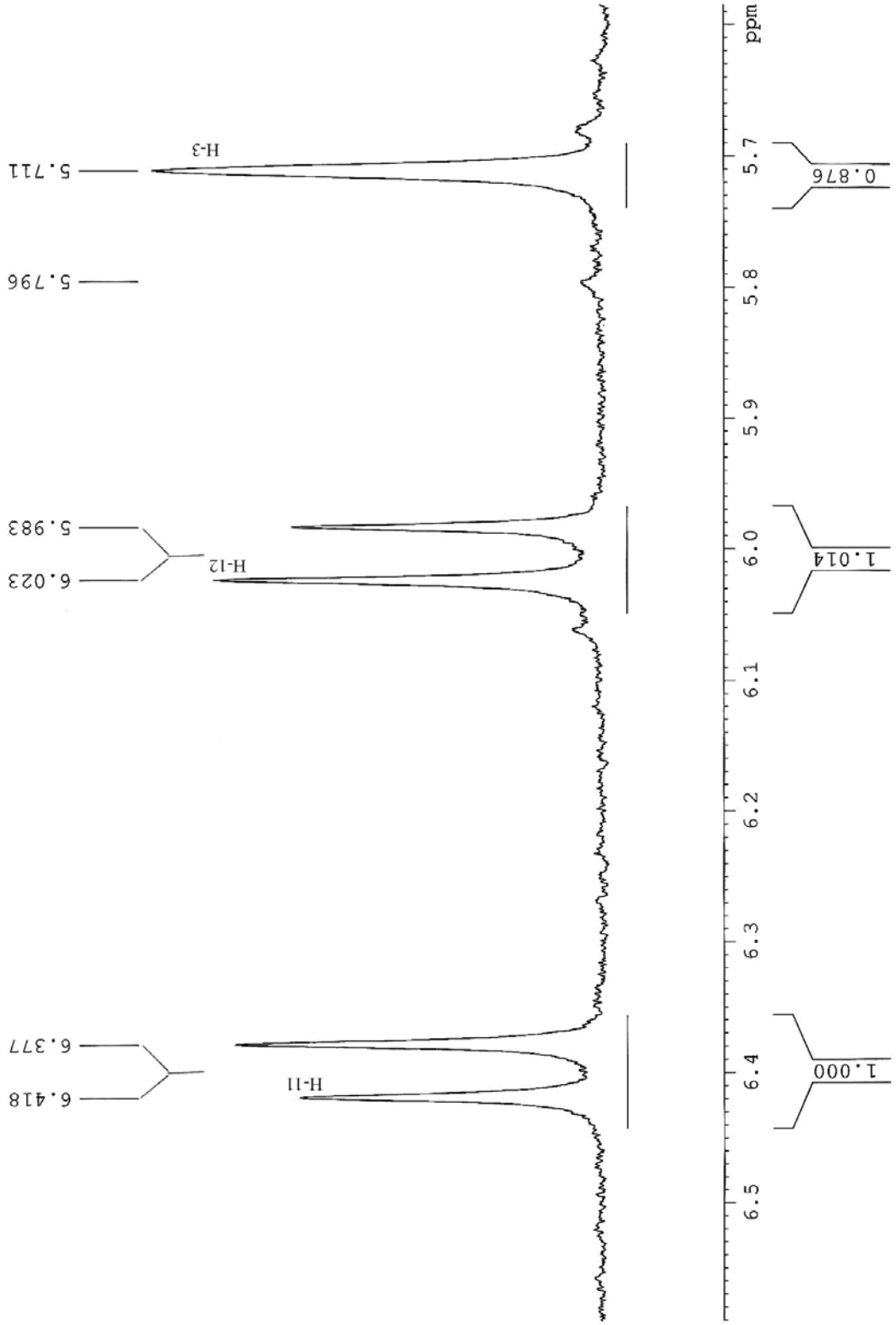


Figure-3.7: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-001 (**162**)

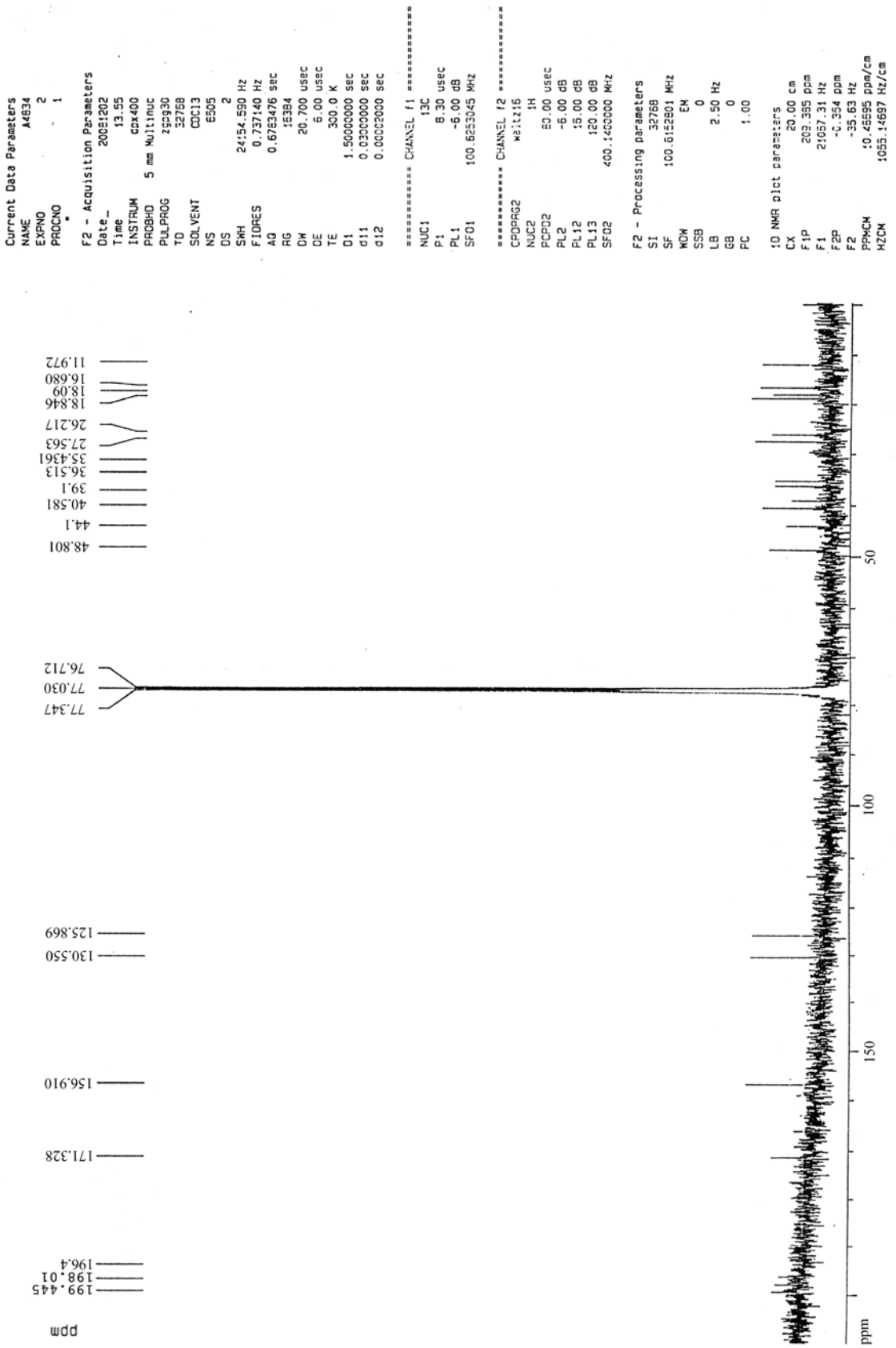


Figure-3.8: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of PSB-001 (**162**)

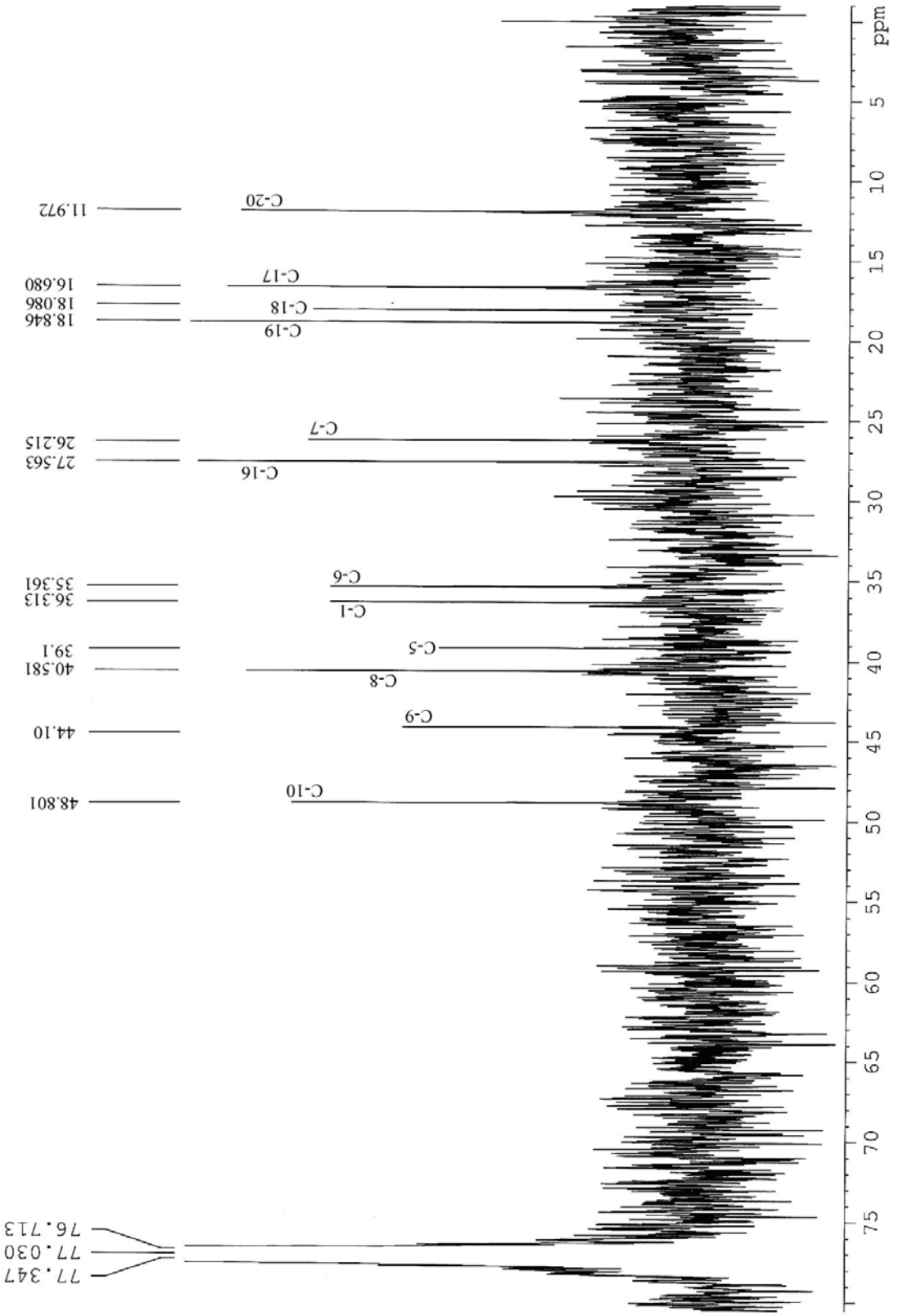


Figure-3.9: Partially expanded ^{13}C NMR (100 MHz, in $CDCl_3$) spectrum of PSB-001 (**162**)

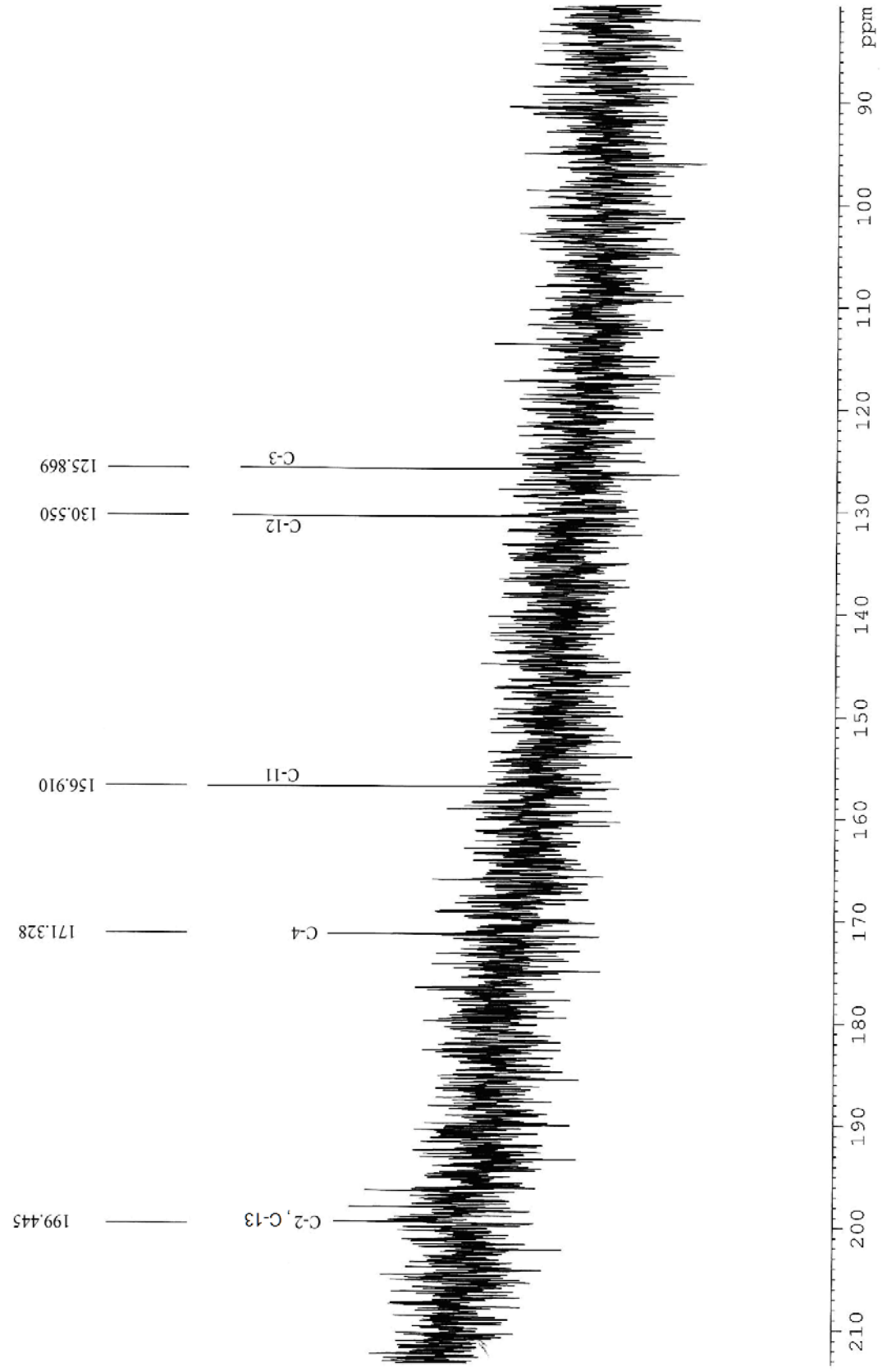


Figure-3.10: Partially expanded ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of PSB-001 (**162**)

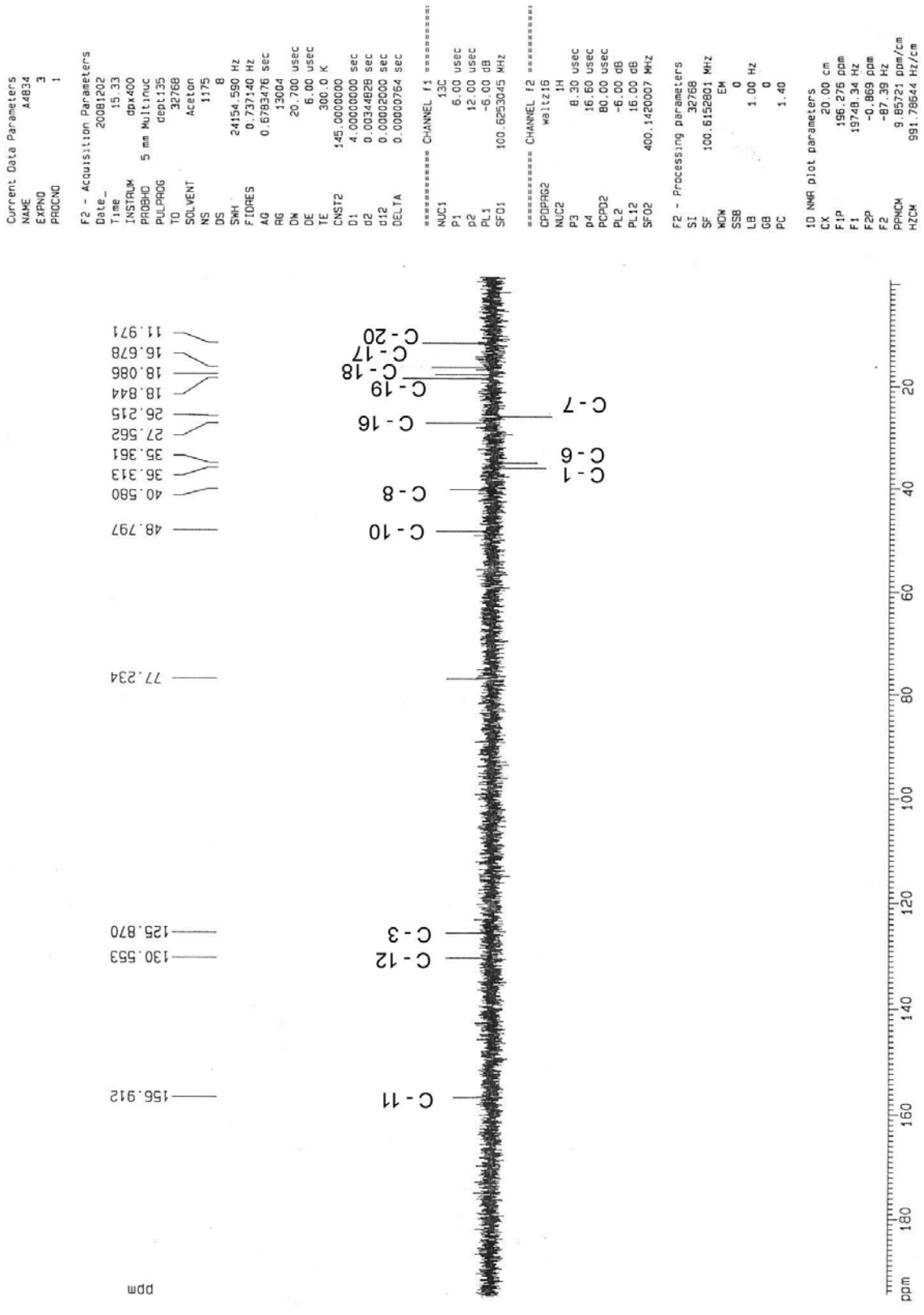


Figure-3.11: DEPT spectrum of PSB-001 (**162**) in $CDCl_3$

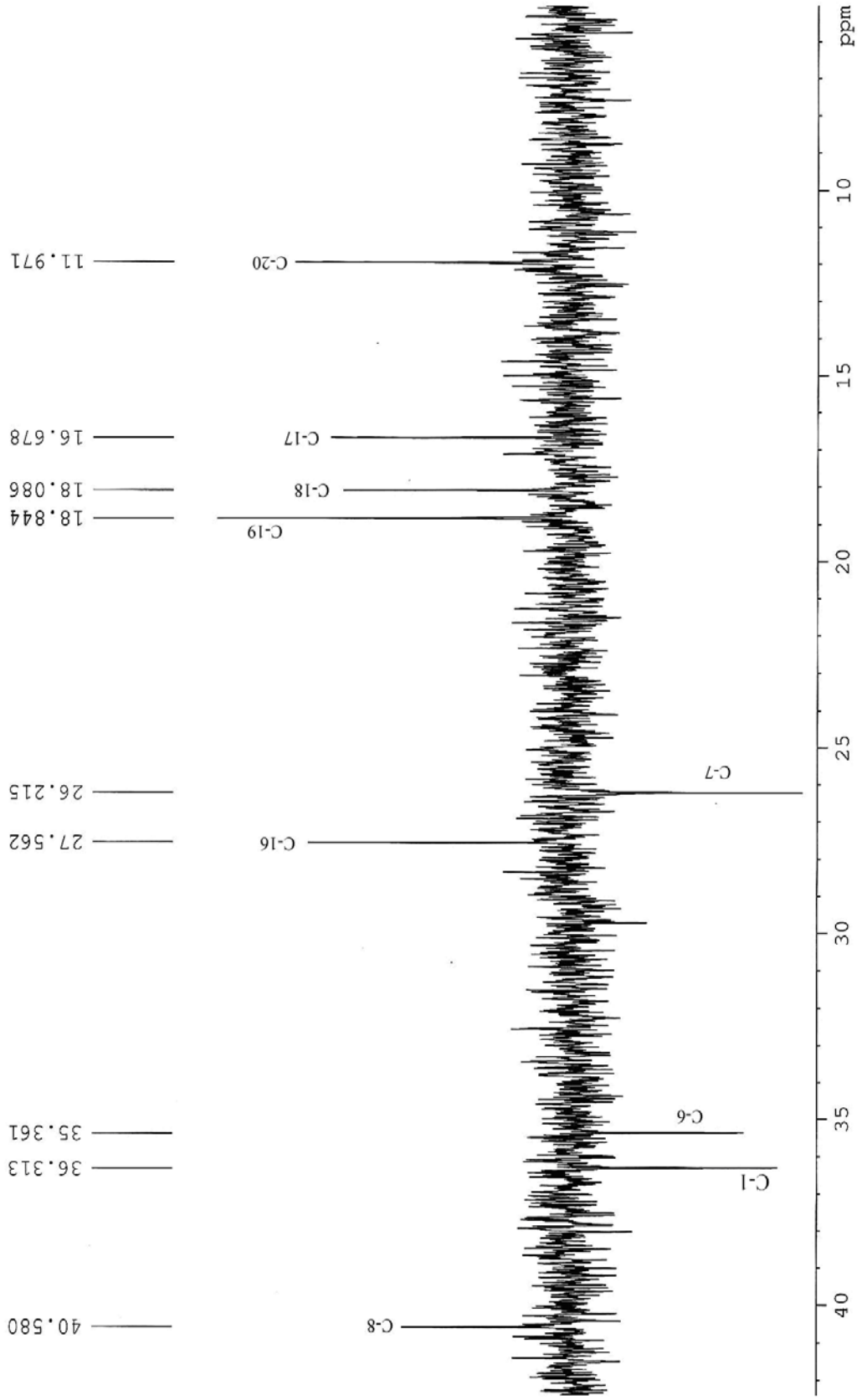


Figure-3.12: Partially expanded DEPT spectrum of PSB-001 (**162**) in $CDCl_3$

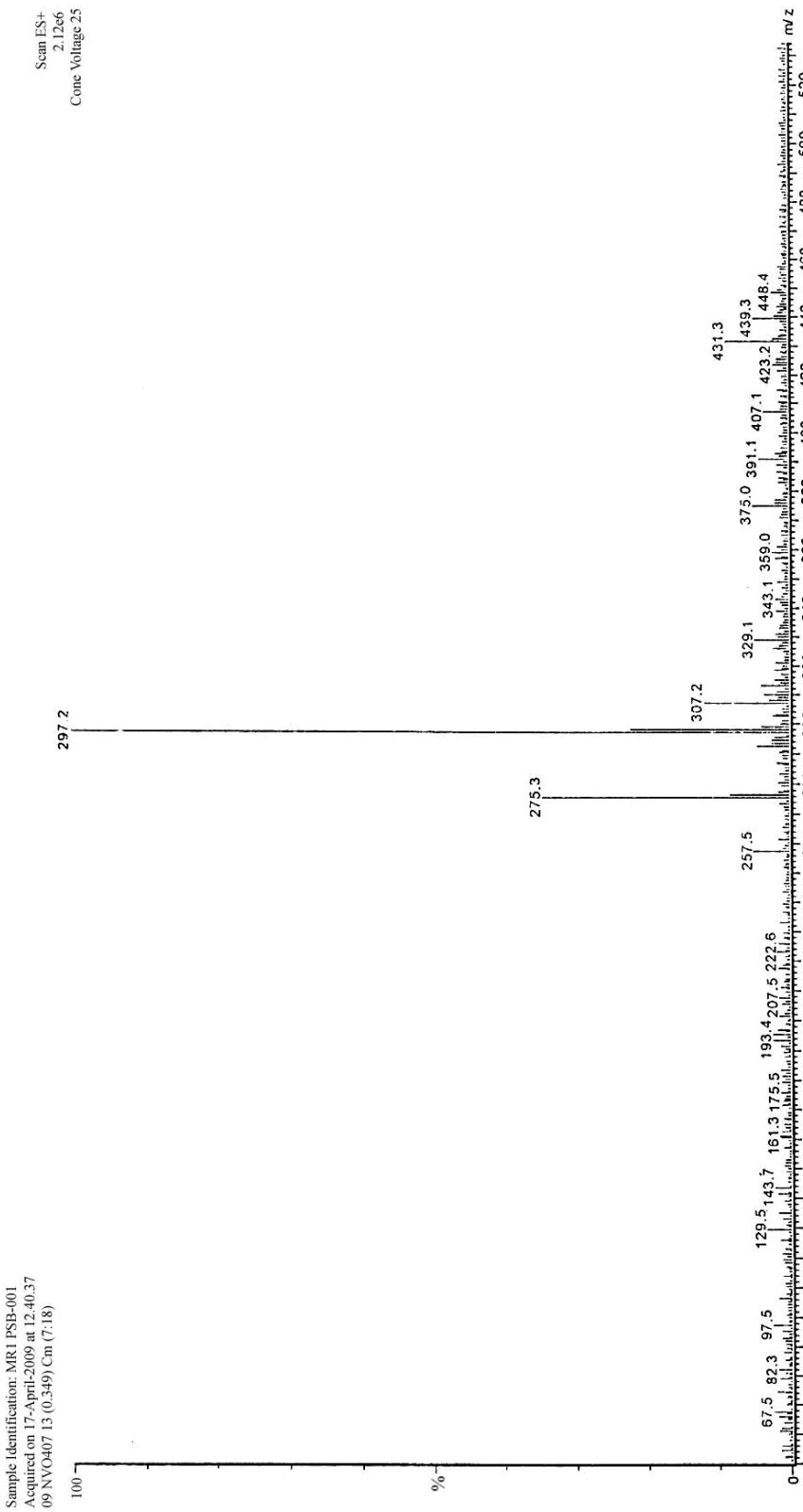


Figure 3.13: ESI MS spectrum of the compound PSB-001 (**162**)

3.1.2 Characterization of PSB-007 as Kolavenic acid (163)

PSB-007 was isolated from column fractions 50-60 of petroleum ether extract by PTLC technique as colorless gum. It was spray active and spraying the developed TLC plate with vanillin-sulfuric acid followed by heating at110°C for 5 minutes produce violet color.

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of PSB-007 (Table-3.3, Figure: 3.15-3.16) revealed the presence of five methyl resonances at δ 0.72 (s, H-20), 0.99 (s, H-19) and 0.81 [(d, *J*=7.08 Hz), H-17] for two tertiary and one secondary methyl one saturated carbons and at δ 1.57 (s, H-18) and 2.16 (s, H-16) for tertiary methyl groups on unsaturated carbons. The latter both exhibited long- range allylic coupling to olefinic protons at δ 5.18 (s, H-3) and 5.67 (s, H-14).

The ^{13}C NMR spectrum (Table-3.4, Figure: 3.17-3.18) showed five downfield resonances at δ 144.4 (C-4, C-13), 164.6 (C-15), 120.4 (C-3) and 114.6 (C-14). The first three due to quarternary carbons and the latter two for tertiary carbons. The signal at δ 164.6 (C-15) must be assigned to the carboxyl group. The spectral features (Table 3.3 and 3.4) are in close agreement to those observed for kolavenic acid (Hasan *et al*., 1994). On this basis, the identity of PSB-007 was confirmed as kolavenic acid. This is the first report of its occurance from *Polyalthia simiarum.*

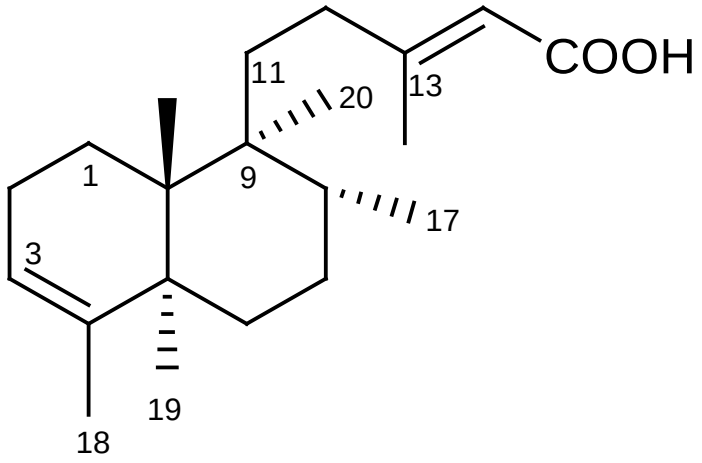


Figure 3.14 Structure of Kolavenic acid (PSB-007, **163**)

Table- 3.3: Comparison between the ^{1}H NMR spectral data of PSB-007 (400 MHz , $CDCl_3$) and kolavenic acid (Hasan *et al*., 1994).

Protons	**PSB-007**	**Kolavenic acid**
	δ_H, multi, *J* in Hz	**δ_H, multi, *J* in Hz**
H-3	5.18, s	5.17, bs
H-14	5.67, s	5.66, bs
H-16	2.16, s	2.14, d (1.2)
H_3-17	0.81, d (7.08)	0.79, d (6.0)
H_3-18	1.57, s	1.56, d (1.4)
H_3-19	0.99, s	0.97, s
H_3-20	0.72, s	0.80, s

Table-3.4: Comparison between the ^{13}C NMR spectral data of PSB-007 (100 MHz, $CDCl_3$) and kolavenic acid (Hasan *et al*., 1994).

Carbons	PSB-007	Kolavenic acid
	δ_c in ppm	δ_c in ppm
1	17.9	17.9
2	19.9	20.6
3	120.4	120.4
4	144.4	144.5
5	38.2	38.3
6	26.8	27.5
7	36.3	36.8
8	36.8	36.9
9	38.7	38.9
10	46.3	46.7
11	27.4	27.6
12	34.9	35.1
13	144.4	162.9
14	114.6	115.8
15	164.6	172.8
16	18.3	18.9
17	15.9	15.7
18	18.2	18.1
19	17.9	17.8
20	19.4	19.8

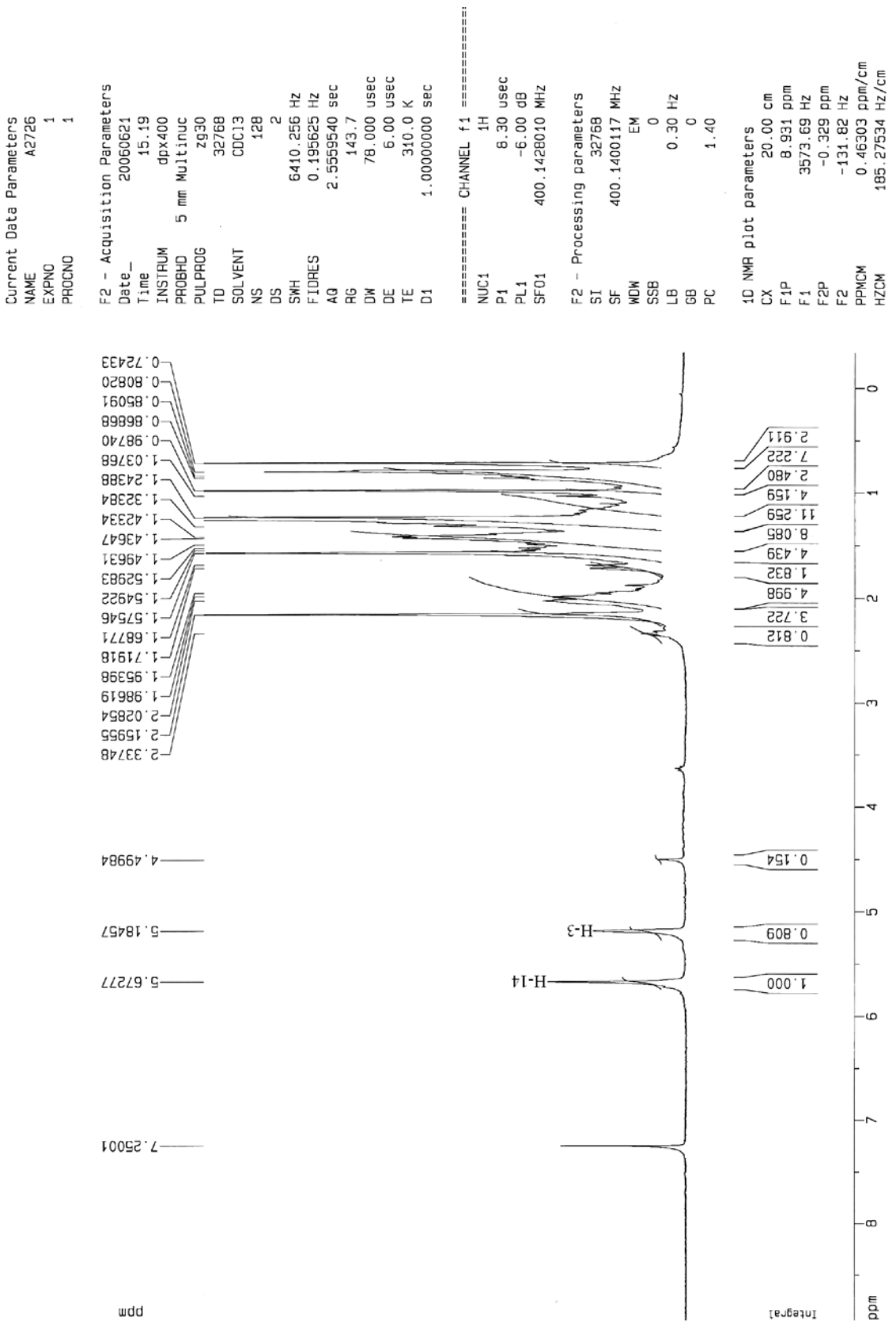


Figure-3.15: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-007 (**163**)

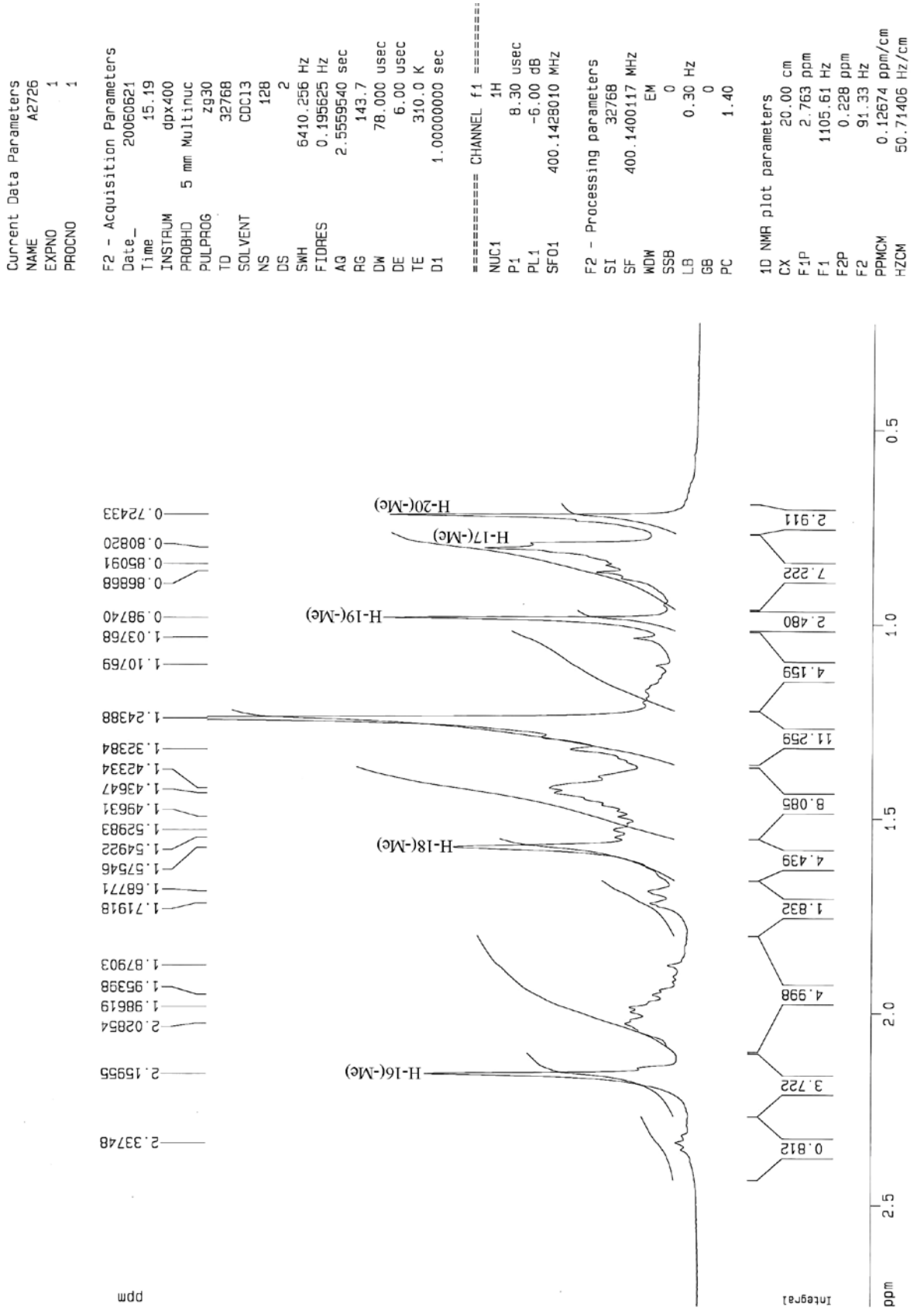


Figure -3.16: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-007 (**163**)

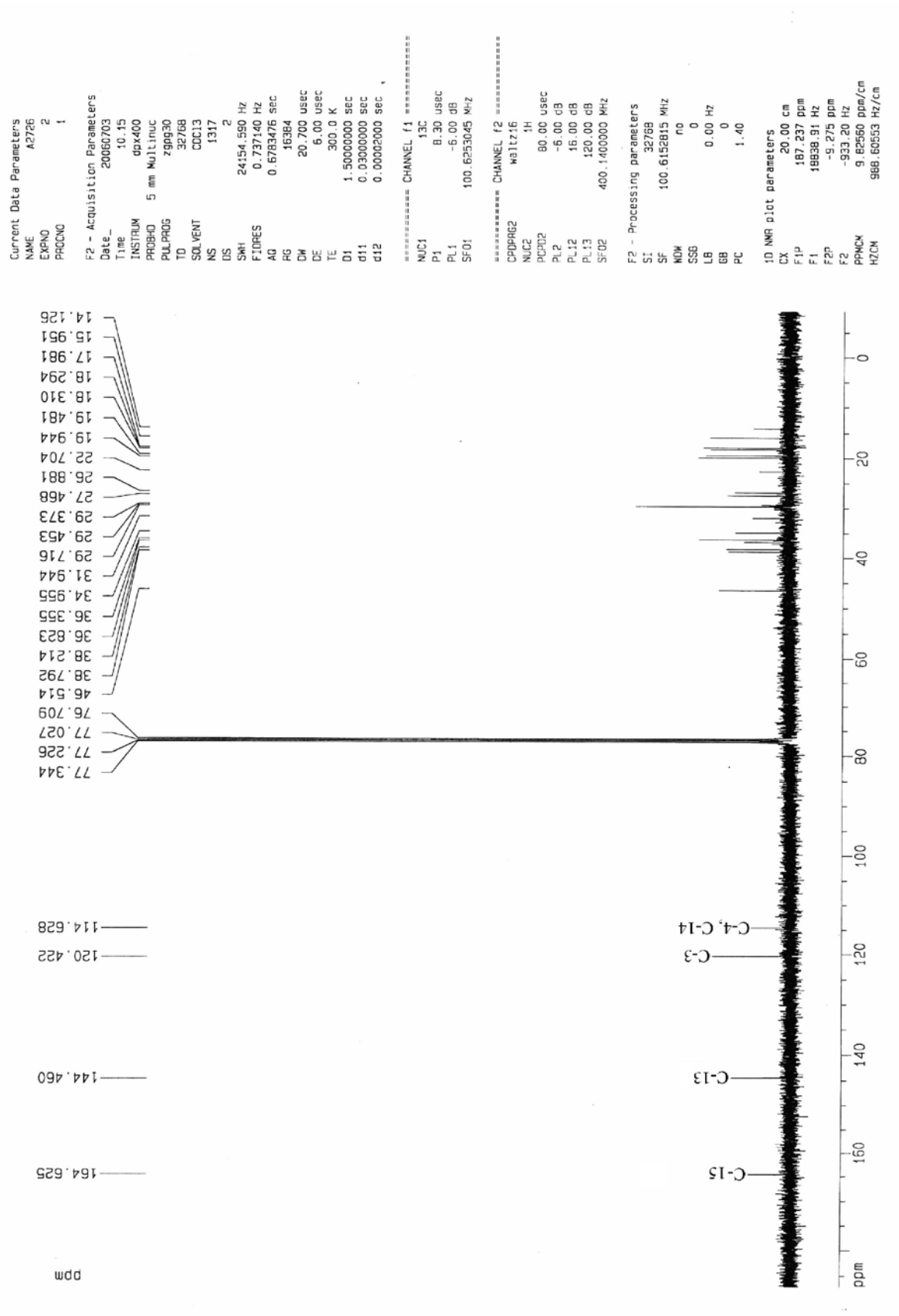


Figure -3.17: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of PSB-007 (**163**)

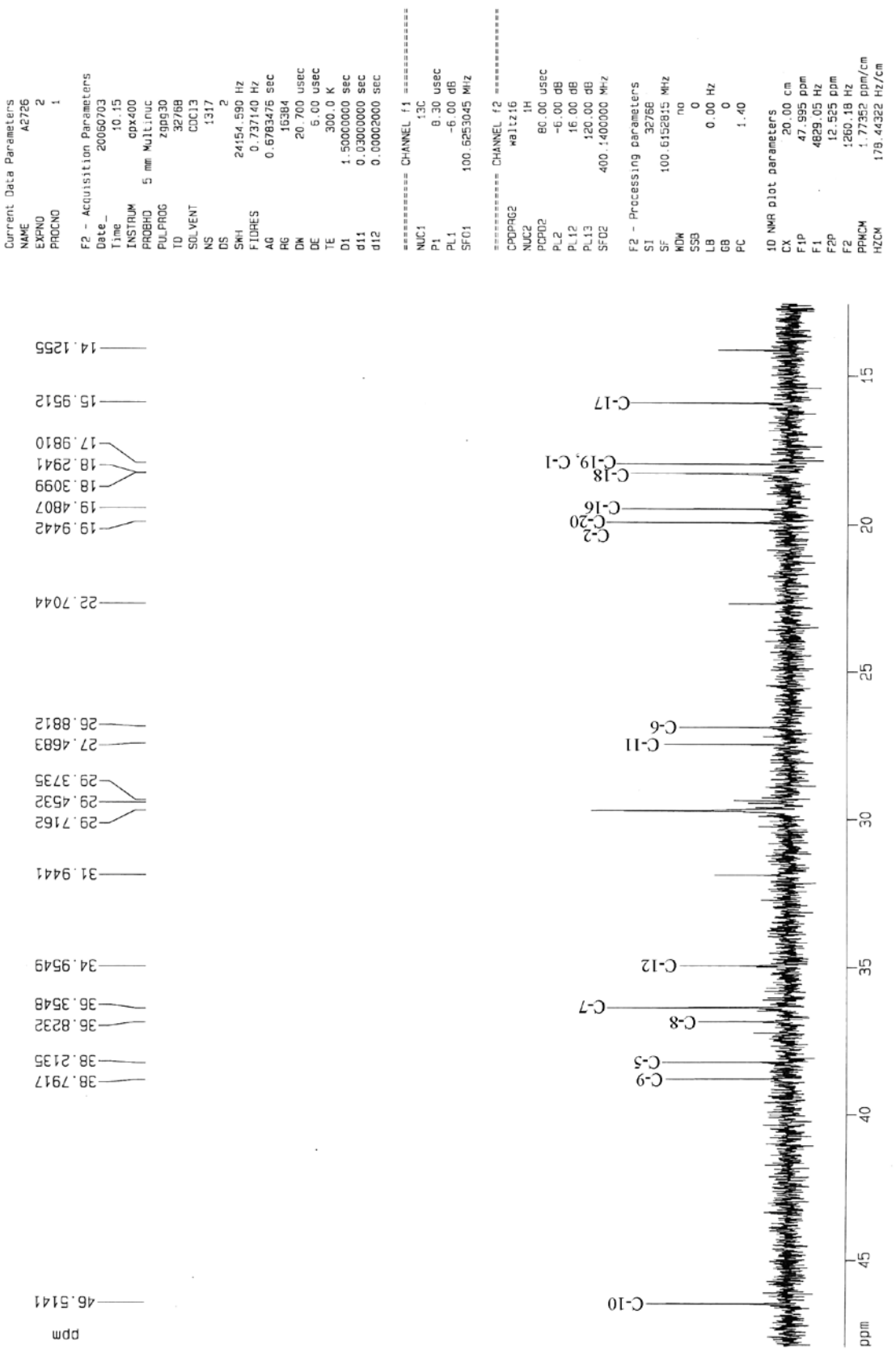


Figure -3.18: Partially expanded ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of PSB-007 (**163**)

3.1.3 Characterization of PSB-004 as 16β-Hydroxy-cleroda-3,13(14)*Z*-dien-15,16-olide (164)

PSB-004 was isolated from column fractions 66-68 of petroleum ether extract of *P. simiarum* by PTLC as amorphous white mass. Spraying the developed TLC plate with vanillin-sulfuric acid followed by heating at 110°C for 5 minutes produced a violet color.

The ^{1}H NMR (400 MHz, $CDCl_3$) of PSB-004 (Table-3.5, Figure: 3.20-3.22) chemical shifts of the C-5 methyl (H-19, δ_H 0.99 s) and the C-9 methyl (H-20, δ_H 0.75 s) indicated the ring junction to have a 5α, 10β-*trans* relative configuration (Seaman *et al*., 1990). This was also supported by consideration of the ^{13}C NMR chemical shift of the C-19 methyl group, which resonated upfield relative to the predicted value for a *cis*-clerodane (Manabe and Nishino, 1986). The ^{1}H and ^{13}C NMR chemical shifts for the C-8 methyl (CH_3-17) and C-6 (^{13}C NMR shift) are indicative of the relative configuration of CH_3-17 in *trans*-clerodanes (Nogueira *et al*., 2001) and corresponding chemical shifts indicate that the CH_3-17 has an α (equatorial) relative configuration.

The ^{13}C NMR (Table-3.6, Figure: 3.23-3.24) of compound PSB-004 displayed 20 carbon resonances. The DEPT (Figure: 3.25) spectrum indicated that 15 out of the 20 carbons in this compound had attached protons. The multiplicities observed from the DEPT spectrum suggested the presence of four methyl groups, six methylenes, three aliphatic methines, one olefinic methines, one methane with a chemical shift typical of a hemiacetal at δ_c 99.1, d (C-16) and five quarternary carbons. The ^{13}C NMR spectrum of PSB-004 comprised a six- carbon side chain [two methylenes at δ_c 27.4 and 34.8 for (C-11 and C-12), a hemiacetal methine (δ_c 99.1 , C-16) , an olefinic methane (δ_c 116.9, C-14), a carbonyl (δ_c 171.6, C-15), and a quarternary olefinic carbon (δ_c 170.6, C-13)], which could be connected to the decalin ring system at C-9. The ^{1}H NMR spectrum contained signals for four downfield methines and four methyl groups as well as a number of signals attributable to methylene proton.

The spectral features (Table 3.5 and 3.6) are in close agreement to those observed for 16β-Hydroxy-cleroda-3,13(14)*Z*-dien-15, 16-olide (Hasan *et al*., 1994). On this basis, the identity of PSB-004 was confirmed as 16β-Hydroxy-cleroda-3,13(14)*Z*-dien-15, 16-olide. This is the first report of this compound from *Polyalthia simiarum.*

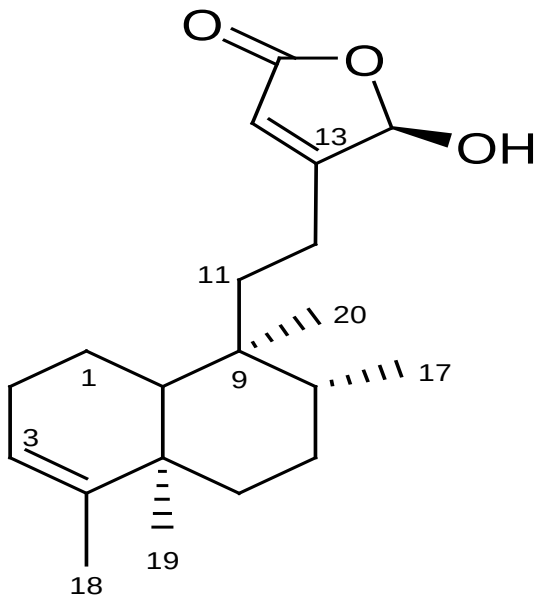


Figure 3.19 Structure of 16β-Hydroxy-cleroda-3,13(14)*Z*-dien-15,16-olide (PSB-004, **164**)

Table-3.5: Comparison between the ^{1}H NMR spectral data of PSB-004 (400 MHz , $CDCl_3$) and 16β-Hydroxy-cleroda- 3,13(14)*Z*-dien-15,16-olide (Hasan *et al*., 1994).

Protons	**PSB-004**	**16β-Hydroxy-cleroda-3,13(14)*Z*-dien-15,16-olide**
	δ_H, multi, *J* in Hz	**δ_H, multi, *J* in Hz**
H-3	5.17, bs	5.16, m
H-14	5.82, s	5.80, bs
H-16	5.99, s	5.65, bs
H_3-17	0.79, dd (4.4, 6.6)	0.79, dd (6.2, 2.5)
H_3-18	1.57, s	1.56, d (1.5)
H_3-19	0.99, s	0.97, s
H_3-20	0.75, s	0.74, s

Table-3.6: Comparison between the ^{13}C NMR spectral data of PSB-004 (100 MH_Z, $CDCl_3$) and 16β-Hydroxy-cleroda- 3,13(14)*Z*-dien-15,16-olide (Hasan *et al*., 1994).

Carbons	PSB-004	16β-Hydroxy-cleroda-3,13(14) *Z*-dien-15,16-olide
	δ_c in ppm	δ_c in ppm
1	18.3	18.2
2	22.7	21.3
3	120.4	120.3
4	144.3	144.2
5	38.2	38.1
6	26.8	26.7
7	36.7	36.6
8	36.3	36.2
9	38.7	38.6
10	46.5	46.4
11	27.4	27.2
12	34.8	34.6
13	170.6	171.1
14	116.9	116.7
15	171.6	172.2
16	99.1	99.4
17	15.9	15.9
18	18.1	18.1
19	17.9	18.0
20	19.9	19.8

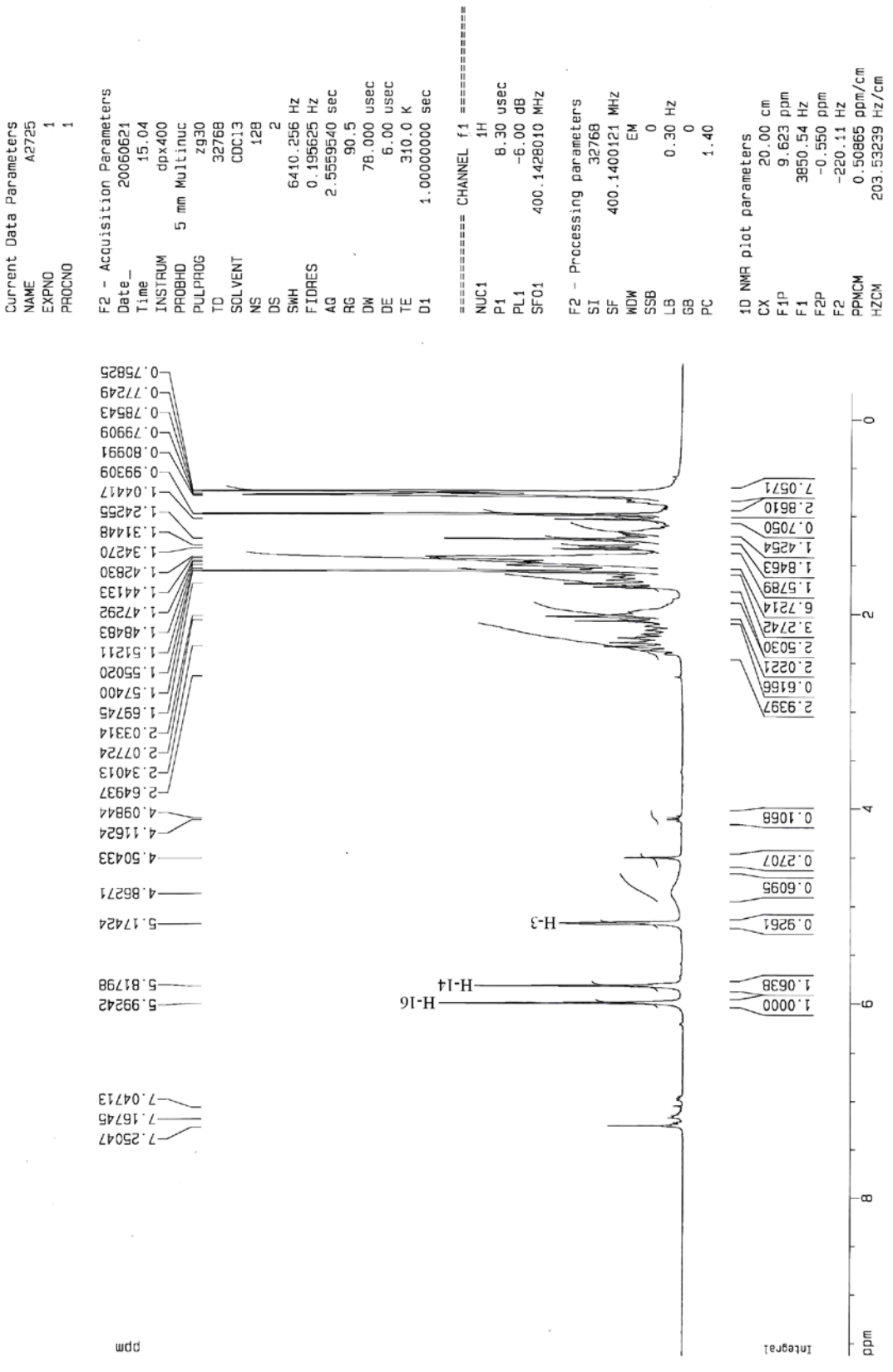


Figure-3.20: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-004 (**164**)

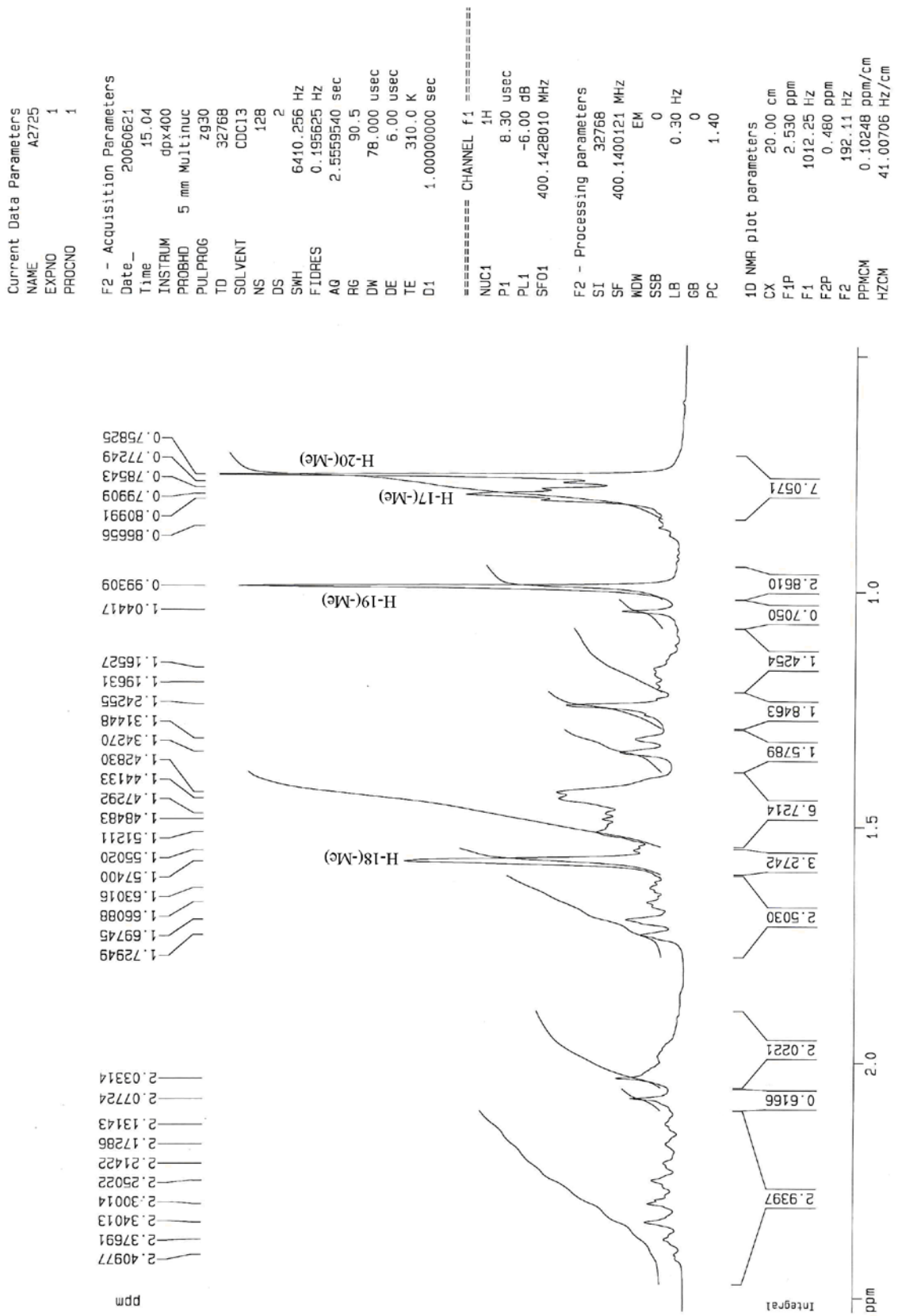


Figure -3.21: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-004 (**164**)

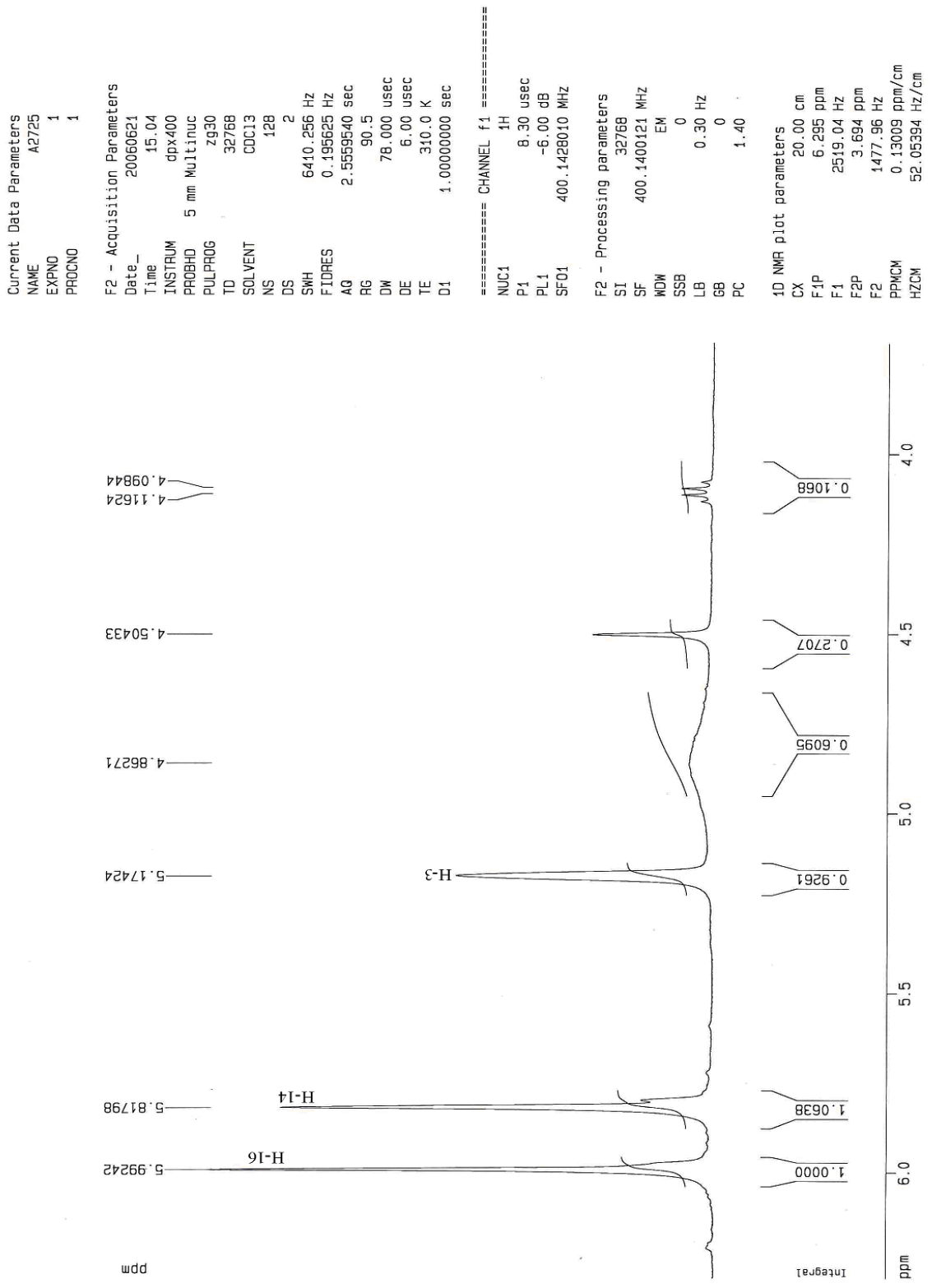


Figure -3.22: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-004 (**164**)

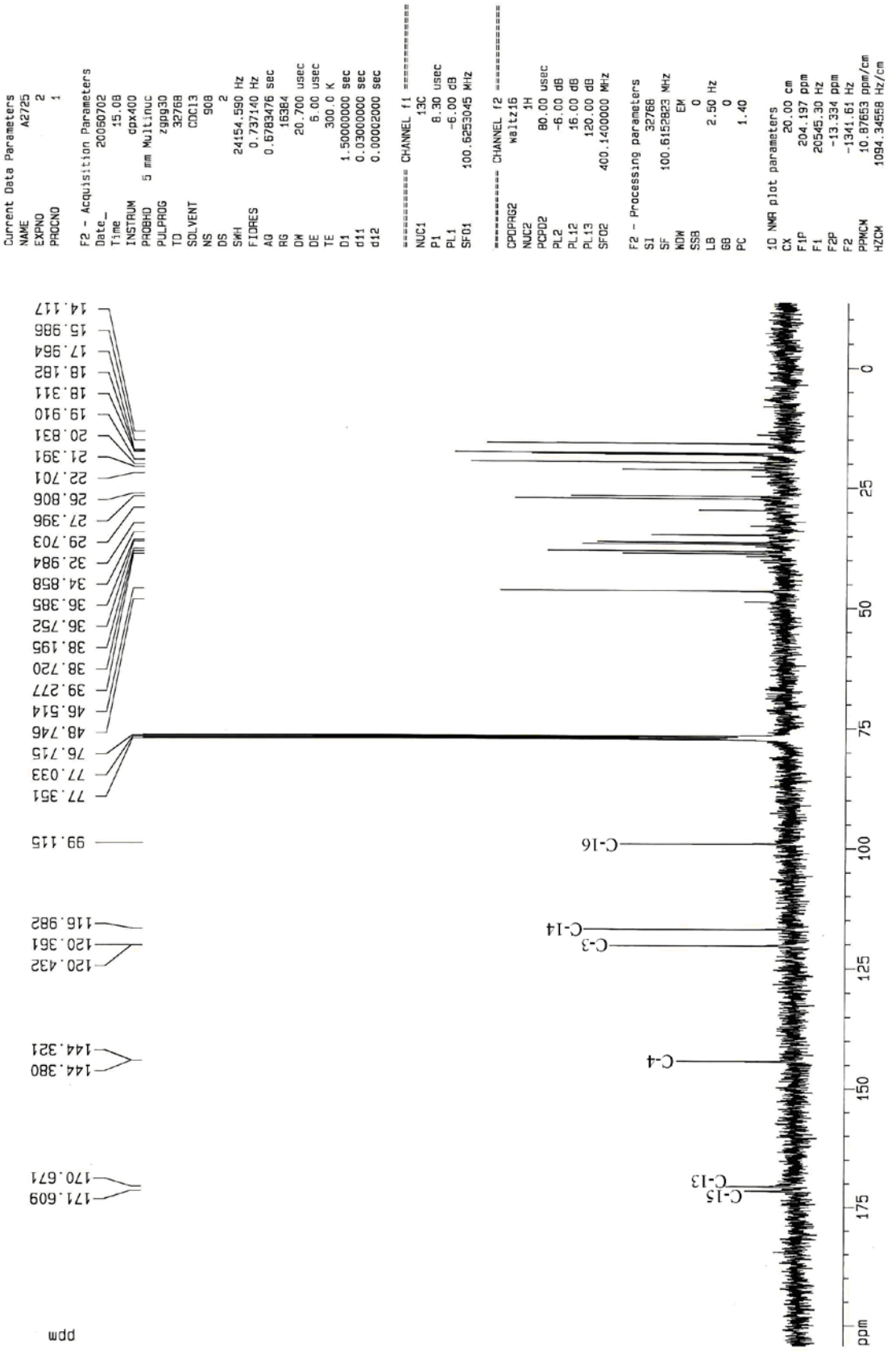


Figure -3.23: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of PSB-004 (**164**)

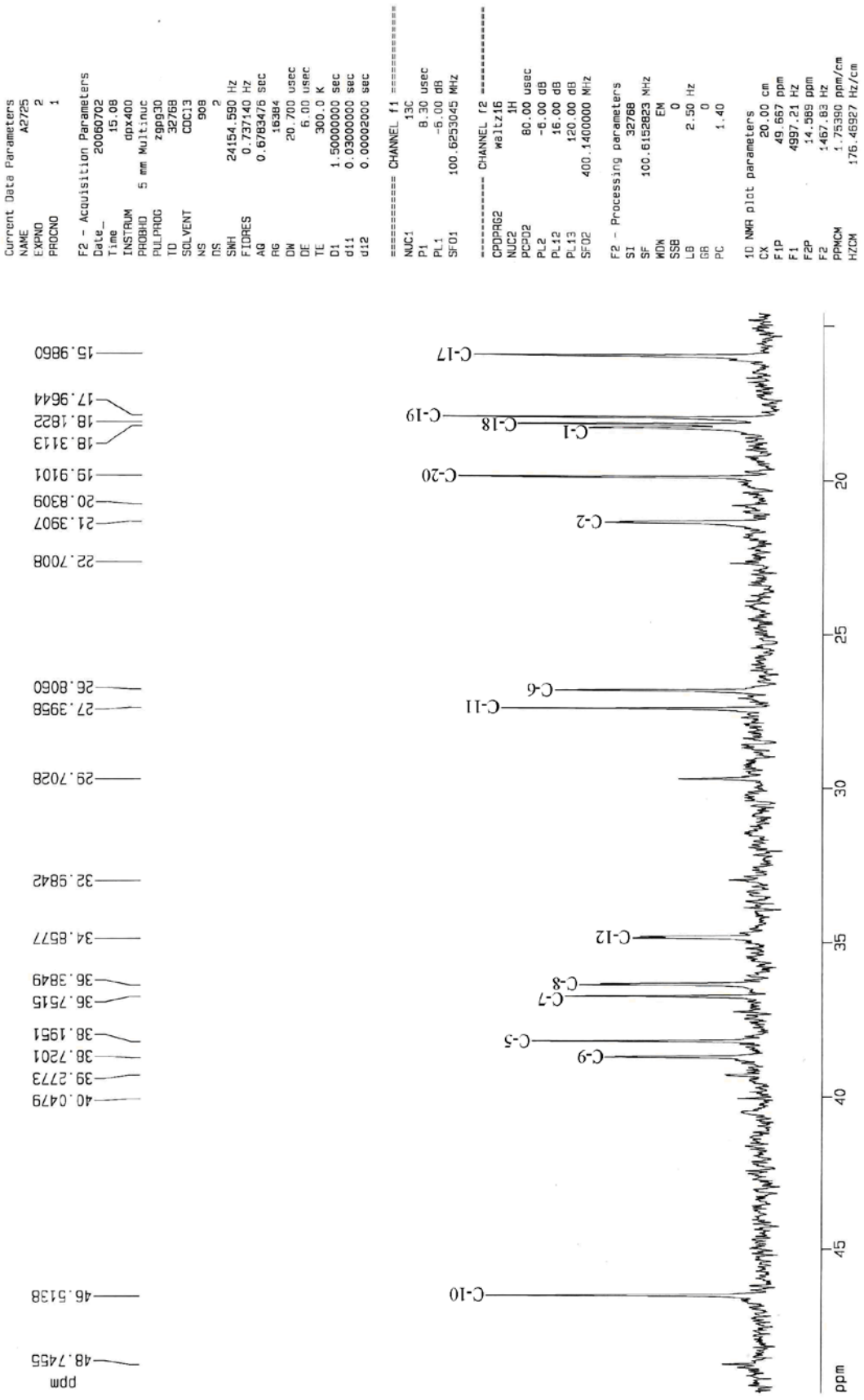


Figure -3.24: Partially expanded ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of PSB-004 (**164**)

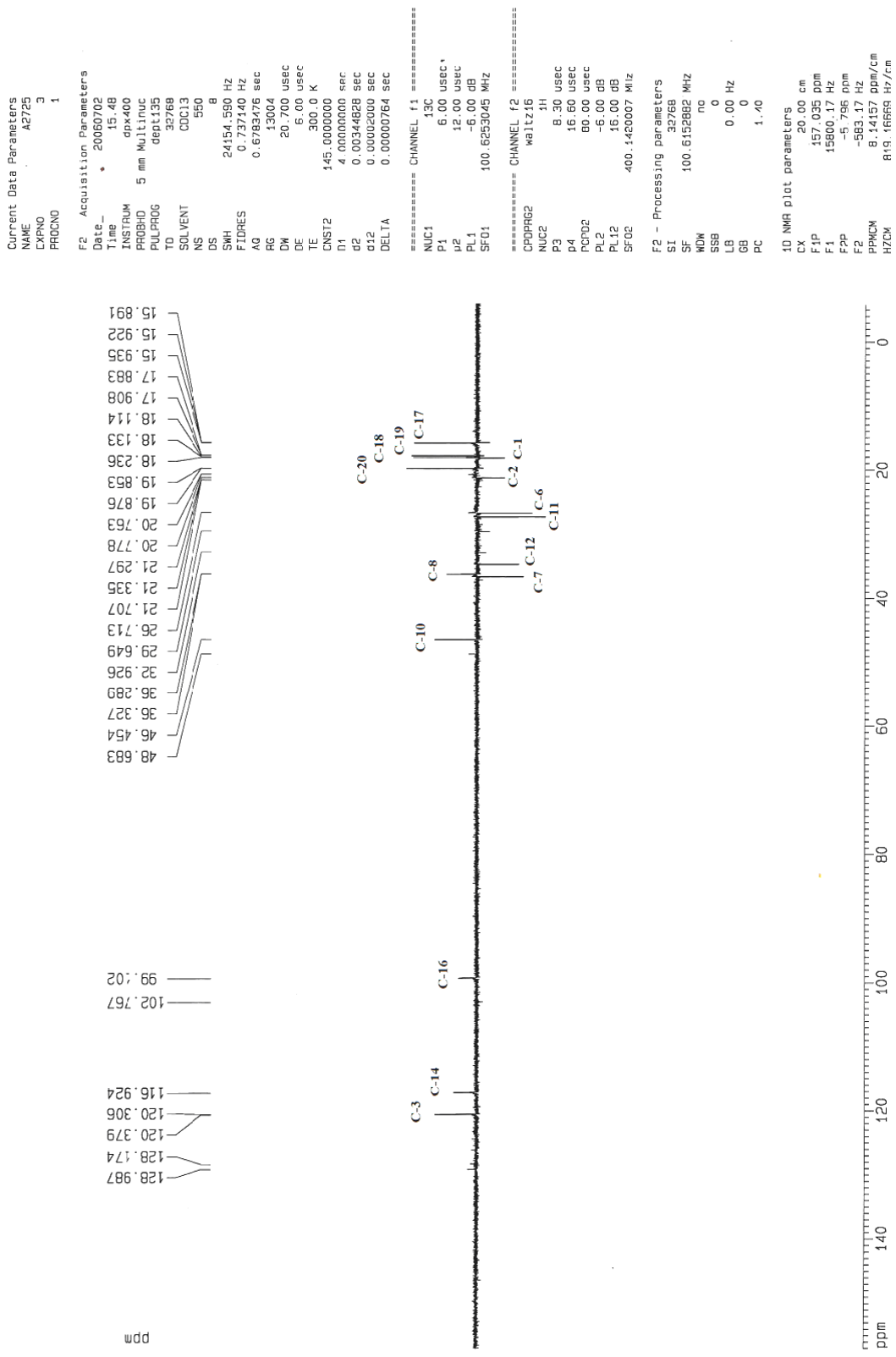


Figure-3.25: DEPT spectrum of PSB-004 (**164**) in $CDCl_3$

3.1.4 Characterization of PSB-008 as 16-oxocleroda-3,13(14)*E*-dien-15-oic acid (165)

PSB-008 was isolated from column fractions 87-102 of petroleum ether extract of *P. simiarum* by PTLC as colorless gum. Spraying the developed TLC plate with vanillin-sulfuric acid followed by heating at 110°C for 5 minutes produce a greenish color.

The ^1H NMR spectrum (400 MHz, $CDCl_3$) of PSB-008 (Table-3.7, Figure: 3.27-3.29) indicated the presence of four signals of methyl groups at δ 1.58, 0.99, 0.68 and 0.84 for H_3-17, H_3-18, H_3-19 and H_3-20 respectively (each s, 3H). Functionalization of ring A corresponded to that PSB-008 as indicated by the broadened singlet of H-3 at δ 5.20, bs allylically coupled to the vinyl methyl of H_3-18 at δ 0.99. The spectrum also revealed an α, β unsaturated carboxylic acid unit and an aldehyde group at δ 9.52 (bs, H-16). The spectrum shows a broad singlet at δ 2.1 (bs, H-2), a multiplate at δ 2.55 (m, , H-12) and a singlet at δ 6.45 (s, H-14). The above assignments were characteristic for a clerodane-type diterpene skeleton. Comparison of the spectral data with published values ((Hasan *et al*., 1994) confirmed the identity of PSB-008 as 16-oxocleroda-3,13(14)*E*-dien-15-oic acid. This is the first report of its occurance from *Polyalthia simiarum*.

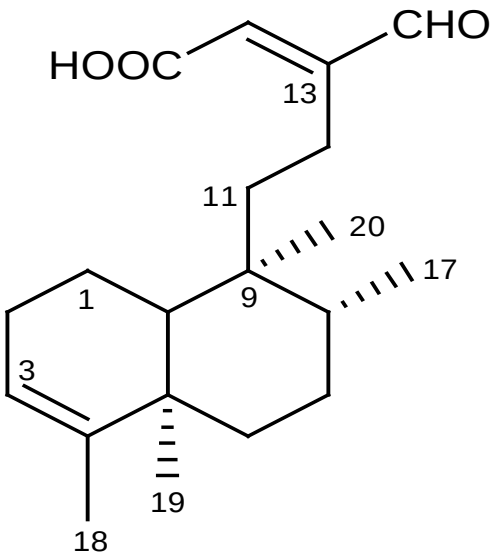


Figure 3.26: Structure of 16-oxocleroda-3,13(14)*E*-dien-15-oic acid (PSB-008, **165**)

Table-3.7: Comparision between the ^{1}H NMR spectral data of PSB-008 (400 MHz, $CDCl_3$) and 16-oxocleroda-3,13(14) *E*-dien-15-oic acid (Hasan *et al*., 1994).

Protons	**PSB-008**	**16-oxocleroda-3,13(14)*E*-dien-15- oic acid**
	δ_H, multi, *J* in Hz	**δ_H, multi, *J* in Hz**
H-2	2.1, bs	2.1, m
H-3	5.20, bs	5.21, bs
H-12	2.55, m	2.52, t (7.0)
H-14	6.45, s	6.54, bs
H-16	9.52, bs	9.54, s
H_3-17	1.58, bs	1.58, d (1.5)
H_3-18	0.99, s	0.98, s
H_3-19	0.68, s	0.67, s
H_3-20	0.84, d (6.4)	0.83, d (6.7)

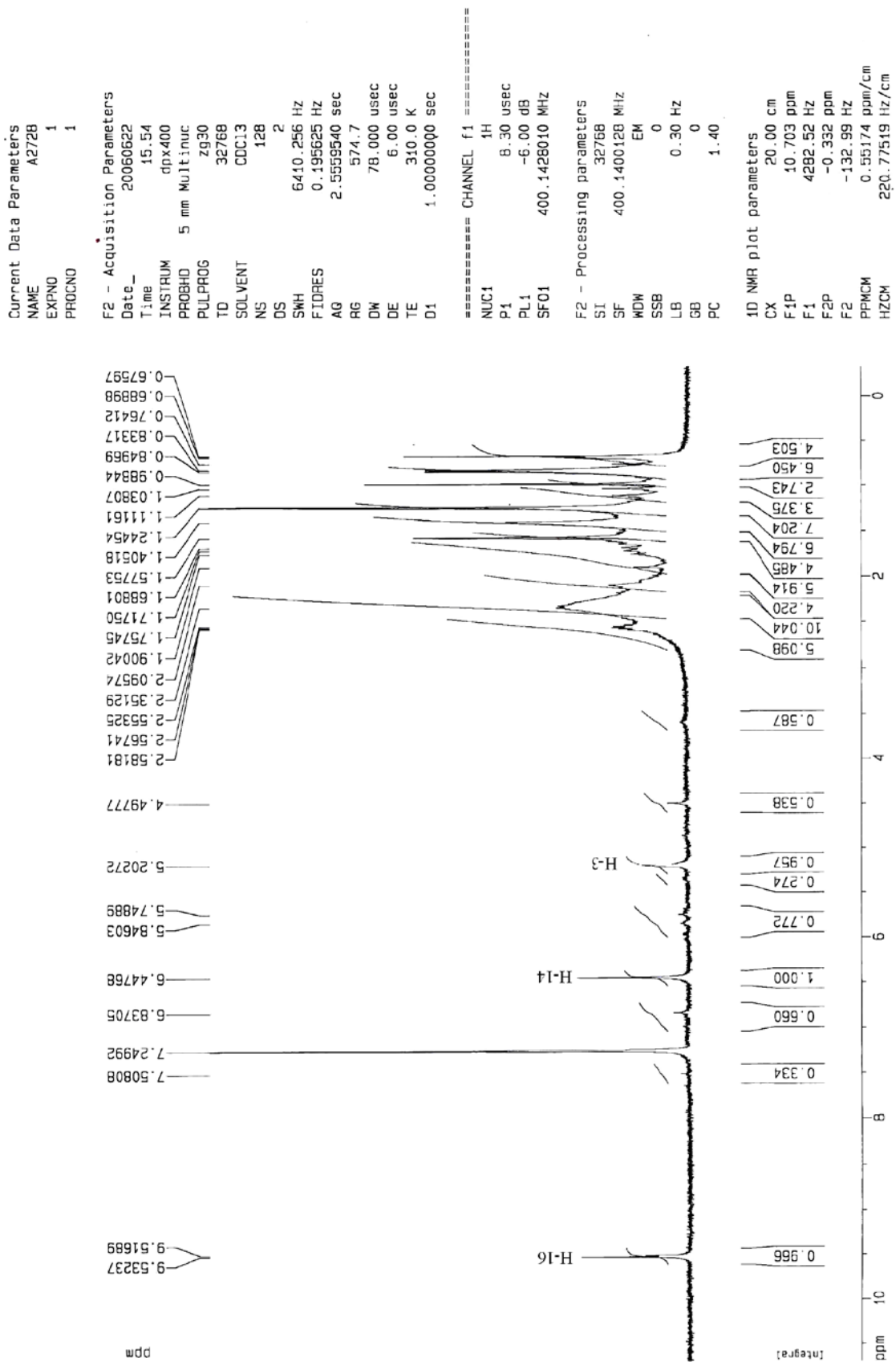


Figure-3.27: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-008 (**165**)

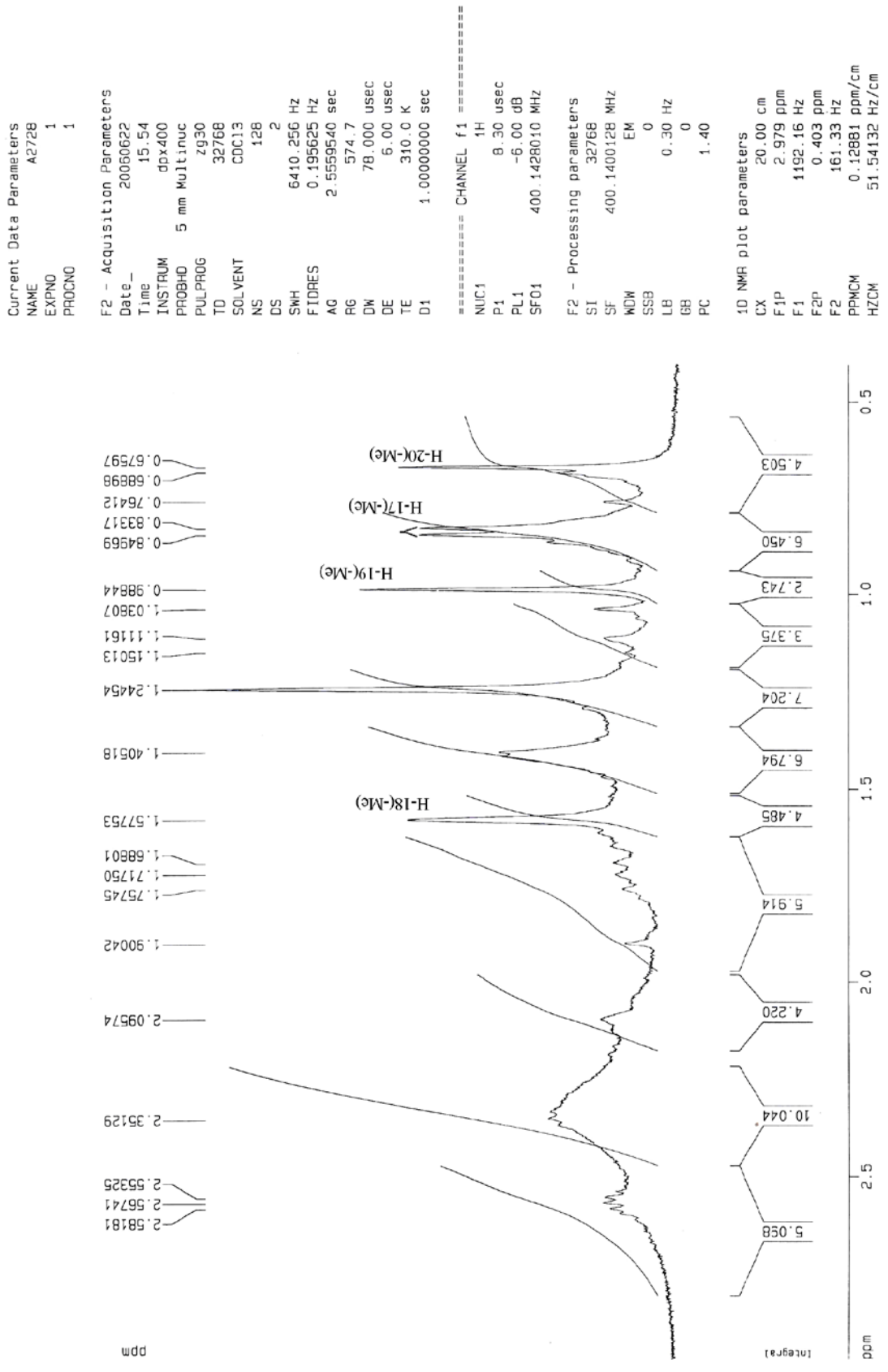


Figure -3.28: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-008 (**165**)

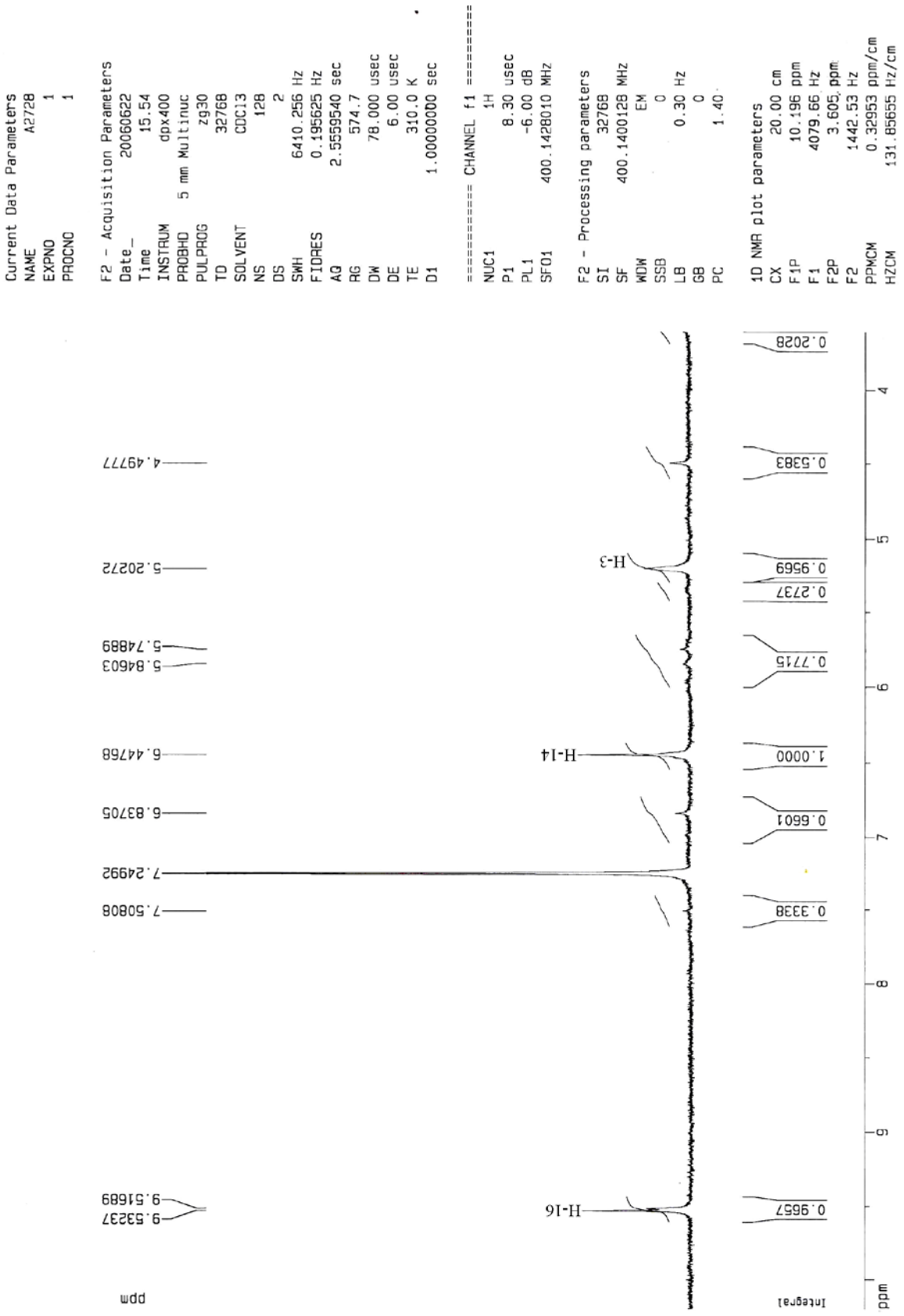


Figure -3.29: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PSB-008 (**165**)

3.2 Chemical investigation of *Glochidion multiloculare*

Repeated chromatographic separation and purification of the pet ether fraction of the stem bark of *Glochidion multiloculare* afforded five compounds GM-022, GM-023, GM-029, GM-032 and GM-035. The structures of the isolated compounds were determined by extensive spectroscopic analysis as well as by comparison with published values.

3.2.1 Characterization of GM-022 as 3-*Epi*-lupeol (166)

GM-022 was isolated as white powder from the petroleum ether extract of the stem bark of *G. multiloculare*. It appeared as a violet spot on TLC, when the developed plate was sprayed with vanillin-sulfuric acid followed by heating at 110˚C for 5 minutes.

The ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound GM-022 (Table-3.8, Figure: 3.31-3.34) showed a trplet (J =2.8 Hz) of one proton intensity at δ 3.39, typical for an oxymethine proton at C-3 of a triterpene type carbon skeleton. The absence of a double doublet and the appearance of a triplet suggested that the hydroxy group was at the α (alpha)-position, thus confirming the β (beta) orientation of C-3 proton (Alam *et al*., 2009). The spectrum displayed a doublet at δ 4.69 (J=2.4) and a multiplet at δ 4.57 assignable to the vinylic protons at C-29. A multiplet of one proton intensity at δ 2.39 could be ascribed to proton at C-19. The spectrum also displayed seven singlets at δ 0.83, 0.94, 0.85, 1.04, 0.96, 0.79 and 1.68 (3H each) for 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. On this basis and by comparing these ^{1}H NMR data with previously published values (Thu *et al.*, 2010; Alam *et al*., 2009), compound GM-022 was identified as 3-*epi*-lupeol. The identity of GM-022 was further substantiated by Co-TLC with an authentic sample.

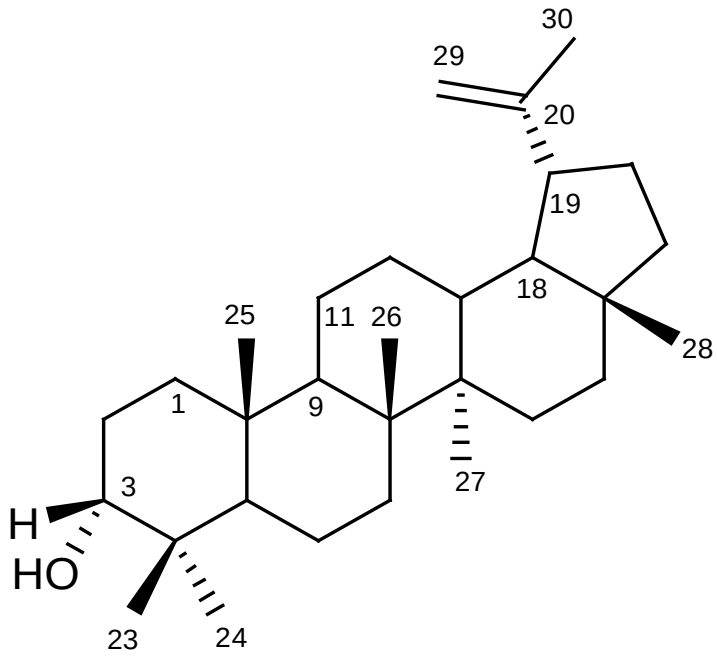


Figure-3.30: Structure of GM-022 (3-*Epi*-lupeol, **166**)

Table 3.8: ^{1}H NMR (500 MHz, $CDCl_3$) spectral data of GM-022 and 3-*epi*-lupeol (Thu *et al.*, 2010; Alam *et al*., 2009).

Protons	**GM-022**	**3-*epi*-lupeol**
	δ_H, multi, *J* in Hz	**δ_H, multi, *J* in Hz**
H-3	3.39, t (2.8)	3.38 br.s
H-19	2.39, m	2.38, ddd (11.0, 11.0, 5.5)
H-29	4.69 H_a, d (2.40); 4.57 H_b, m	4.68 H_a, br. d (1.5); 4.56 H_b, d (1.0)
H_3-23	0.83, s	0.82, s
H_3-24	0.94, s	0.93, s
H_3-25	0.85, s	0.84, s
H_3-26	1.04, s	1.03, s
H_3-27	0.96, s	0.96, s
H_3-28	0.79, s	0.78, s
H_3-30	1.68, s	1.68, s

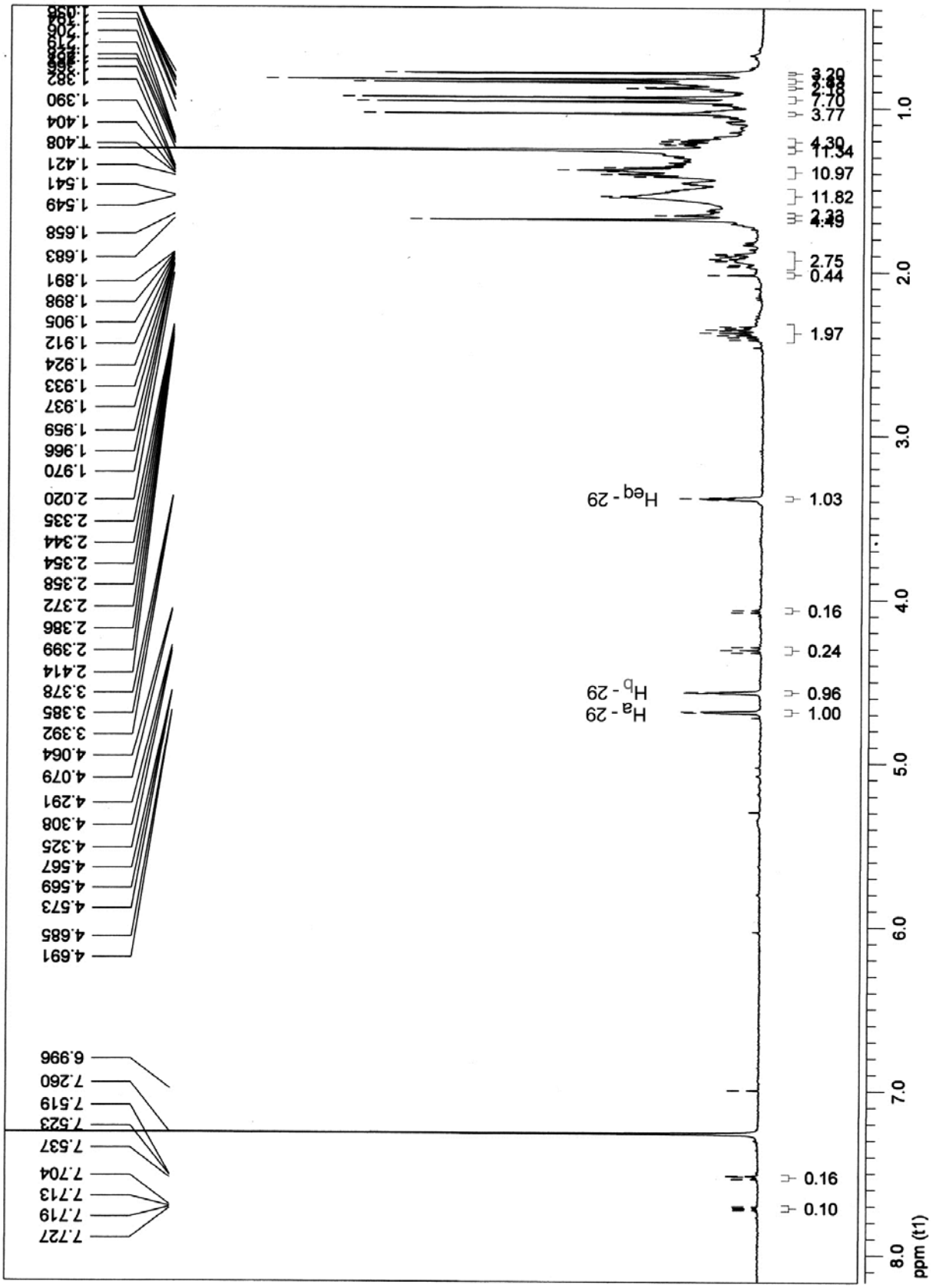


Figure-3.31: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GM-022 (**166**)

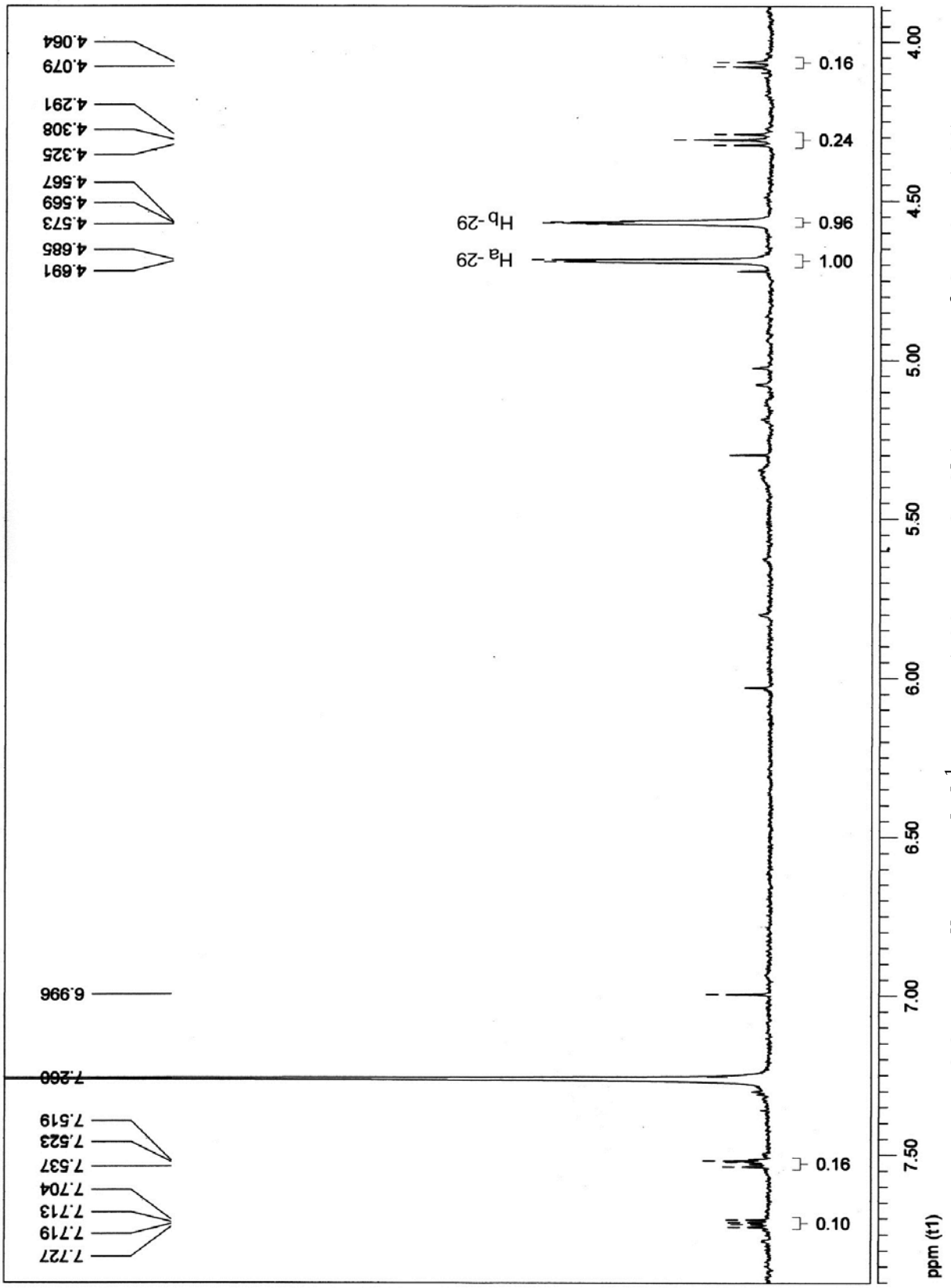


Figure -3.32: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GM-022 (**166**)

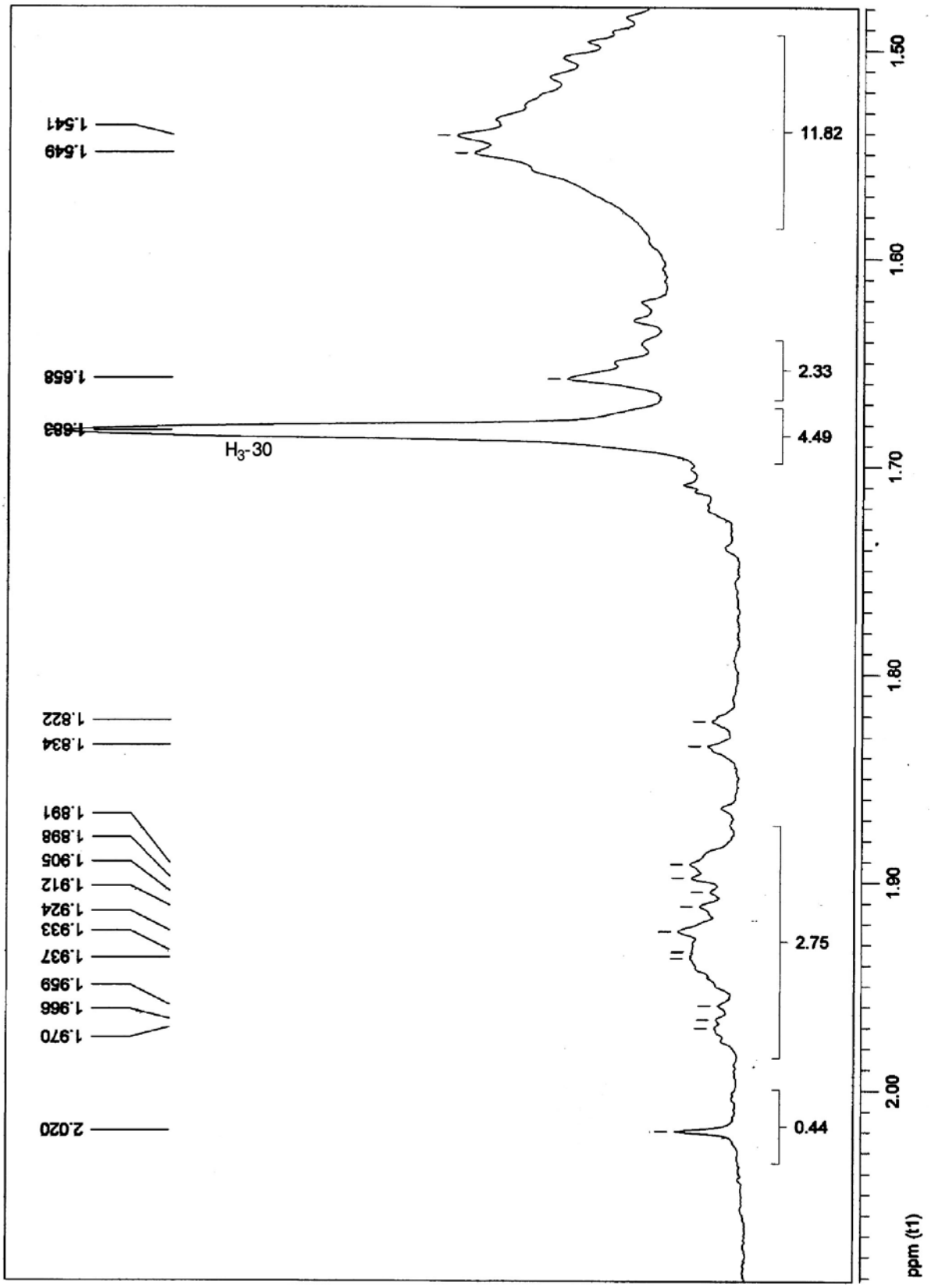


Figure -3.33: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GM-022 (**166**)

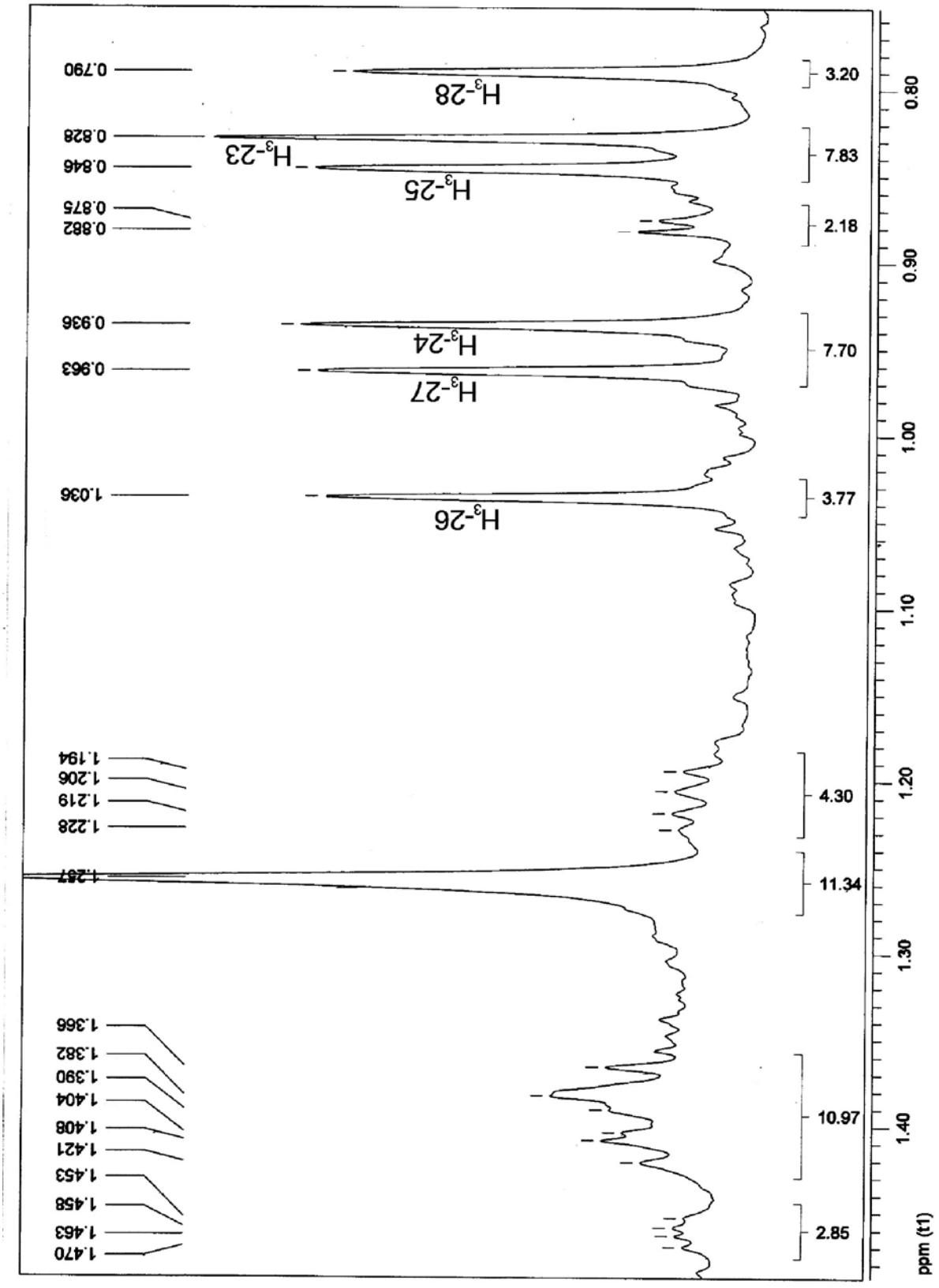


Figure -3.34: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GM-022 (**166**)

3.2.2: Characterization of GM-023 as Lupeol (167)

GM-023 was isolated as white tree branch shaped crystal from the petroleum ether soluble fraction of methanolic extract of the stem bark of *G. multiloculare*. It appeared as a violet spot on TLC, when the developed plate was sprayed with vanillin-sulfuric acid followed by heating at 110˚C for 5 minutes.

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound GM-023 (Table-3.9, Figure: 3.36-3.38) showed a double doublet (J =11.2, 4.8 Hz) of one proton intensity at δ 3.18, typical for an oxymethine proton at C-3 of a triterpene. The splitting pattern of this proton confirmed the β (beta) orientation of the C-3 oxygenated substituent. A one proton doublet of triplets at δ 2.38, (J =11.2, 5.6) is indicative of H-19. The spectrum displayed two broad singlets at δ 4.67 and 4.58 (1H each) assignable to the vinylic protons at C-29. It also showed seven singlets at δ 0.96, 0.75, 0.82, 1.02, 0.93, and 0.78, including a downfield signal at 1.67 (3H each) assignable to methyl group 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. By comparing the ^{1}H NMR data of compound GM-023 with previously published data (Thu *et al*., 2010; Aratanechemuge *et al*., 2004), it was identified as lupeol. The identity of GM-023 was further substantiated by co-TLC with an authentic sample of lupeol, previously isolated in our laboratory.

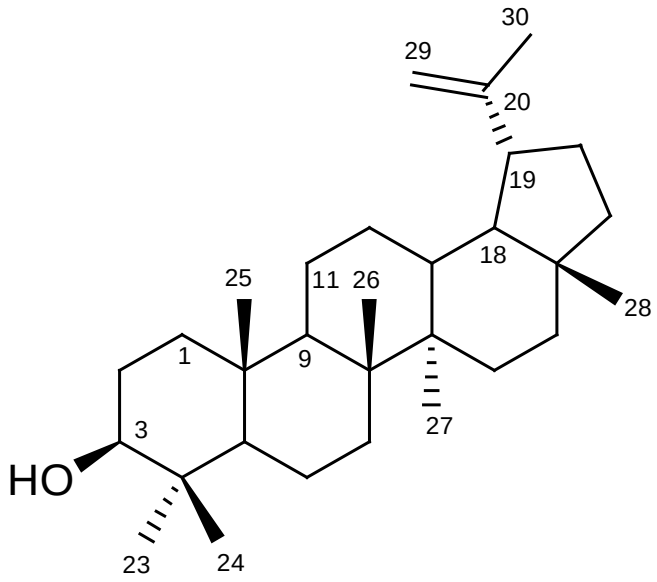


Figure 3.35: Structure of GM-023 (Lupeol, **167**)

Table 3.9: ^{1}H NMR (400 MHz, $CDCl_3$) spectral data of GM-023 and Lupeol (Thu *et al*., 2010; Aratanechemuge *et al.*, 2004).

Protons	**GM-023**	**Lupeol**
	δ_H, multi, *J* in Hz	**δ_H, multi, *J* in Hz**
H-3	3.18, dd (11.2, 4.8)	3.19, dd (11.5, 5.0)
H-19	2.38, dt (11.2, 5.6)	2.38, ddd (11.0, 11.0, 5.5)
H-29	4.67 H_a, br. s; 4.58 H_b, br. s	4.69 H_a, d (2.0); 4.56 H_b, d (1.0)
H_3-23	0.96, s	0.97, s
H_3-24	0.75, s	0.76, s
H_3-25	0.82, s	0.83, s
H_3-26	1.02, s	1.03, s
H_3-27	0.93, s	0.95, s
H_3-28	0.78, s	0.79, s
H_3-30	1.67, s	1.68, s

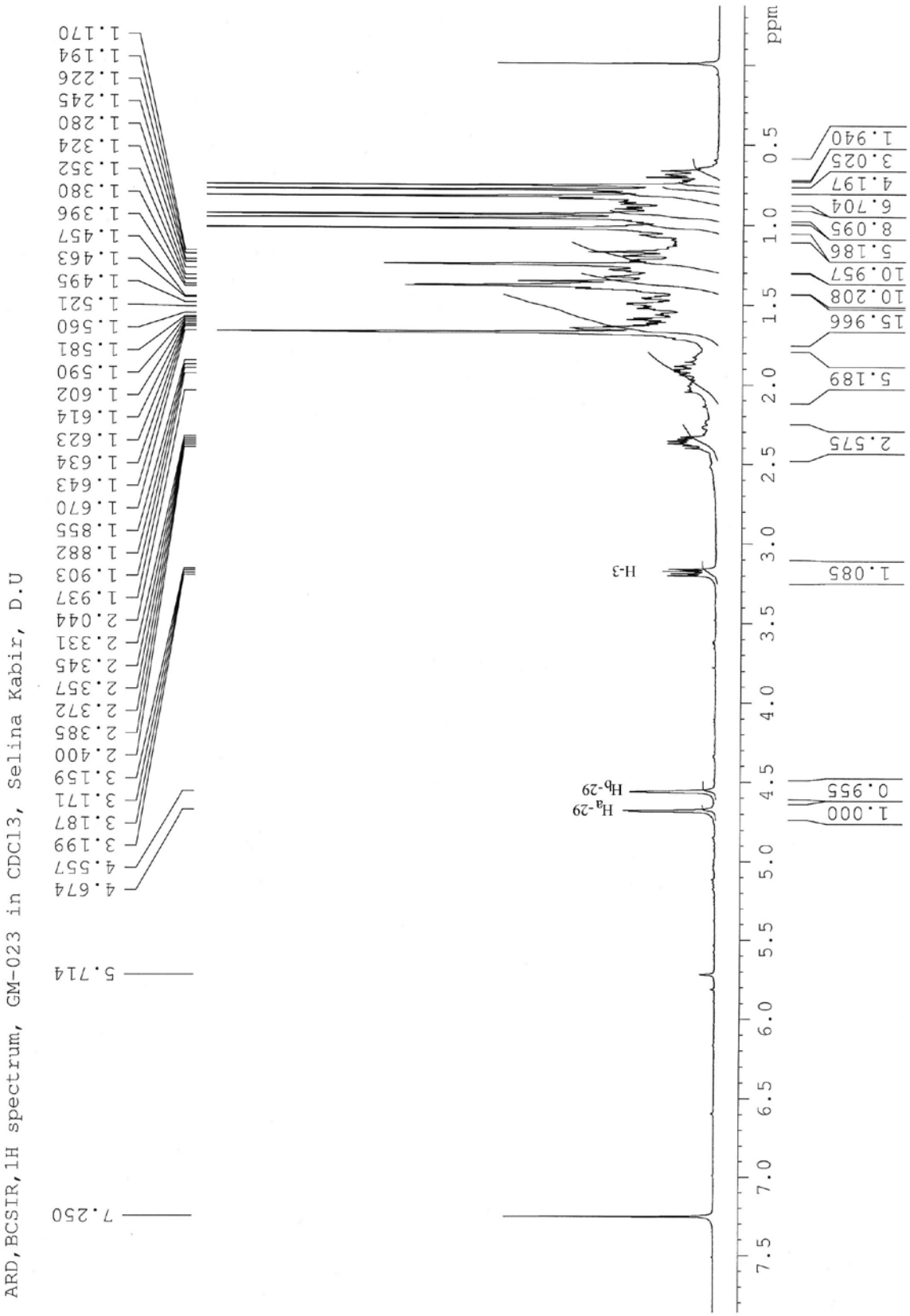


Figure-3.36: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of GM-023 (**167**)

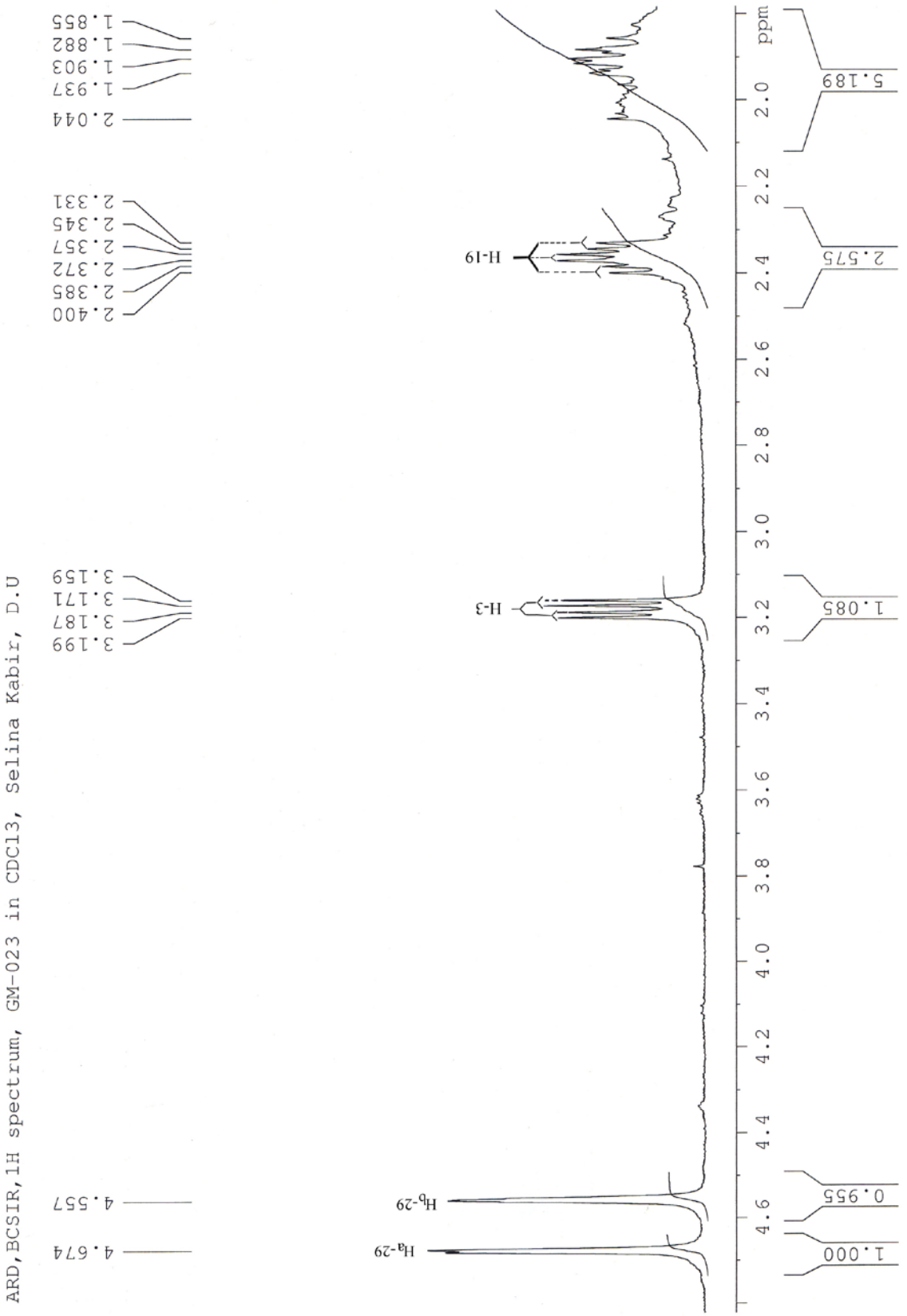


Figure -3.37: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of GM-023 (**167**)

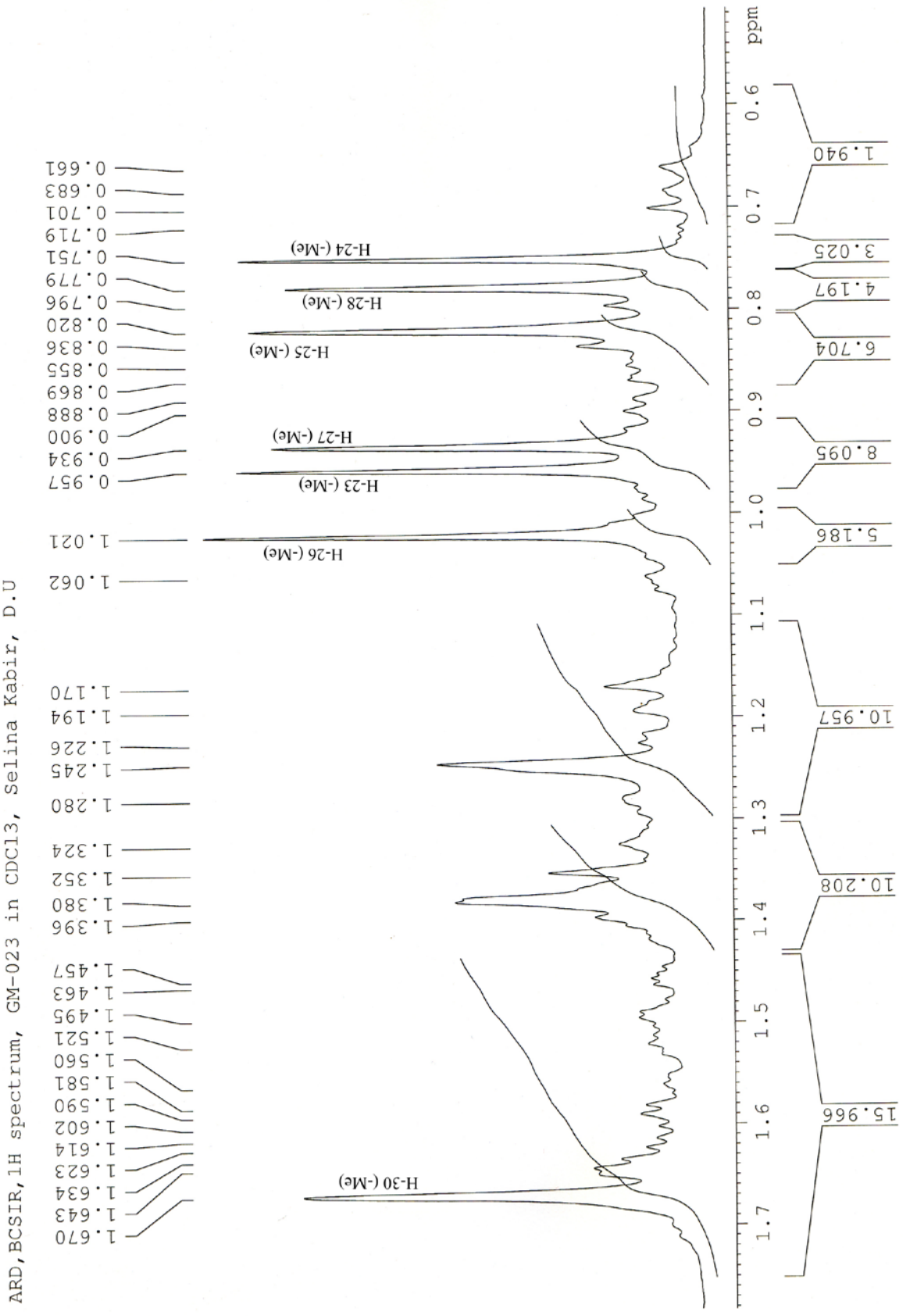


Figure -3.38: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of GM-023 (**167**)

3.2.3: Characterization of GM-029 as Glochidone (168)

GM-029 was isolated as transparent oily substance from petroleum ether soluble fraction of the stem bark of *G. multiloculare*. It appeared as a violet spot on TLC, when the developed plate was sprayed with vanillin-sulfuric acid followed by heating at 110˚C for 5 minutes.

The ^{1}H NMR ($CDCl_3$, 500 MHz) spectrum of GM-029 (Table-3.10, Figure: 3.40-3.43) exhibited a doublet at δ 7.08, integrating one proton, is indicative of H-1. A doublet at δ 5.77, d (10.0) is assignable to H-2. A doublet of triplets at δ 2.39 (10.8, 7.2), integrating one proton is indicative of H-19. The spectrum revealed two doublets at δ 4.68, d (2.4) and 4.56, d (1.2) assignable to protons at C-29. The spectrum also displayed seven singlets at δ 1.04, 1.06, 1.10, 1.09, 0.93, 0.78 and 1.67 (3H each) for 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, characteristic for glochidone (Hui *et al.*, 1976; Neto *et al.*, 1995). On this basis, the identity of GM-029 was confirmed as glochidone (**168**)

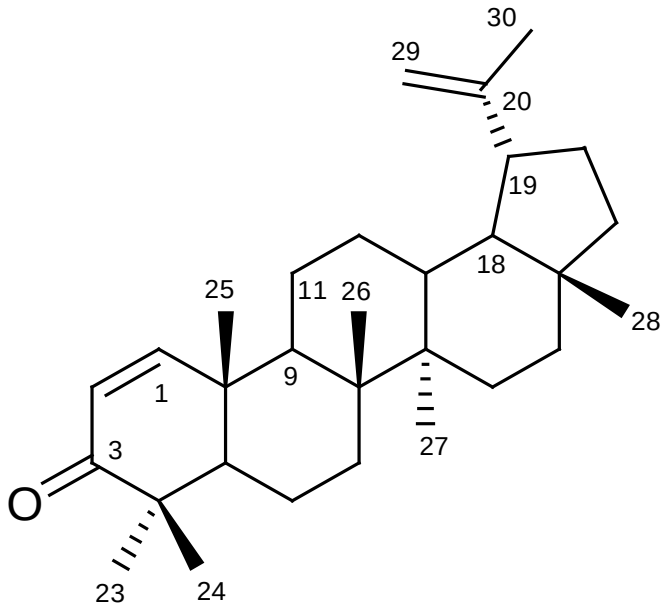


Figure 3.39: Structure of GM-029 (Glochidone, **168**)

Table 3.10: ^{1}H NMR (500 MHz, $CDCl_3$) spectral data of GM-029 and Glochidone (Hui *et al*., 1976; Neto *et al*., 1995).

Protons	**GM-029**	**Glochidone**
	δ_H, multi, *J* in Hz	**δ_H, multi, *J* in Hz**
H-1	7.08, d (10.4)	7.12, d (10.4)
H-2	5.77, d (10.0)	5.89, d (10.4)
H-19	2.39, dt (10.8, 7.2)	
H-29	4.68 H_a, d (2.4); 4.56 H_b, d (1.2)	
H_3-23	1.04, s	1.07, s
H_3-24	1.06, s	1.07, s
H_3-25	1.10, s	1.11, s
H_3-26	1.09, s	1.11, s
H_3-27	0.93, s	0.96, s
H_3-28	0.78, s	0.80, s
H_3-30	1.67, s	1.68, s

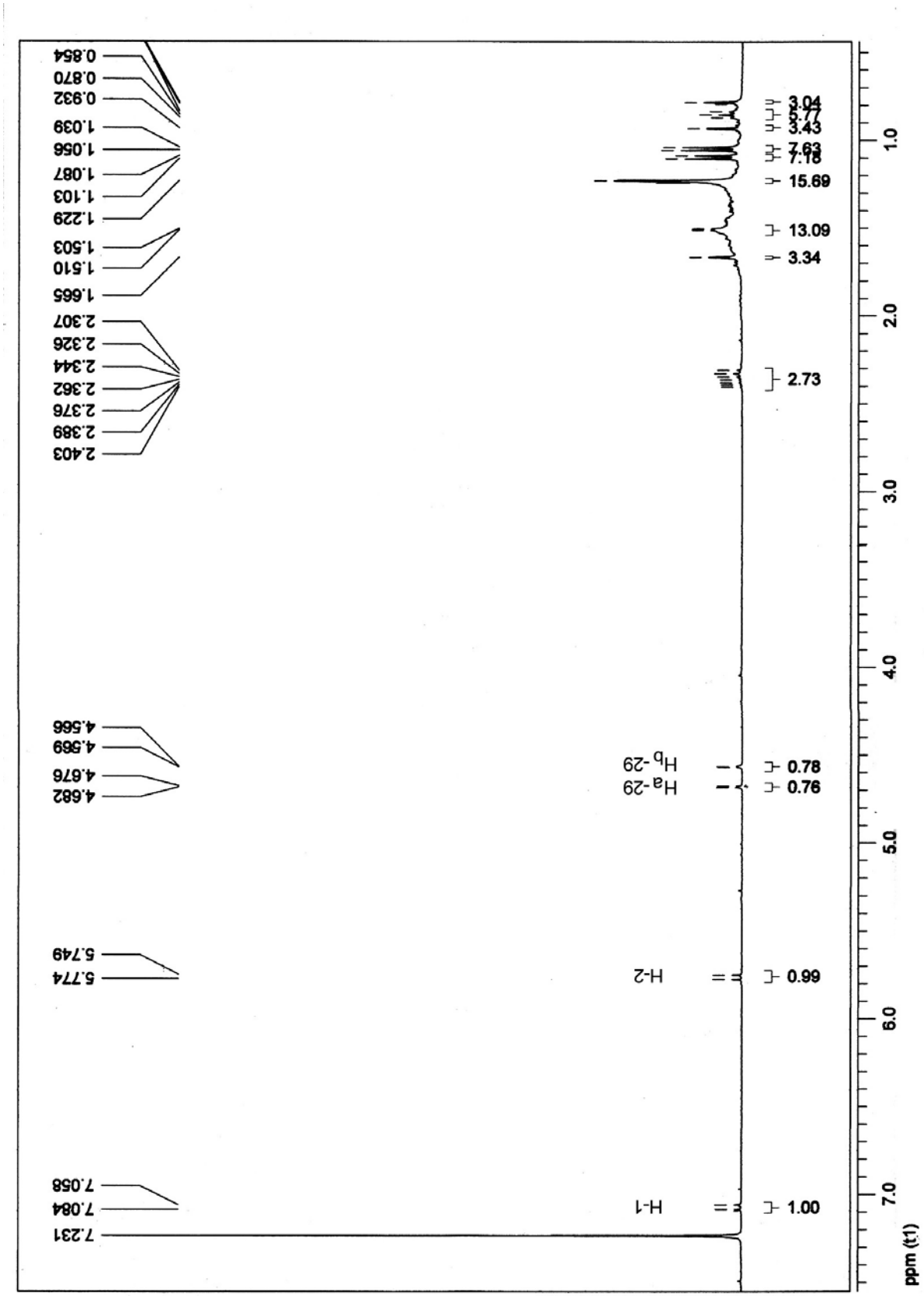


Figure-3.40: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GM-029 (**168**)

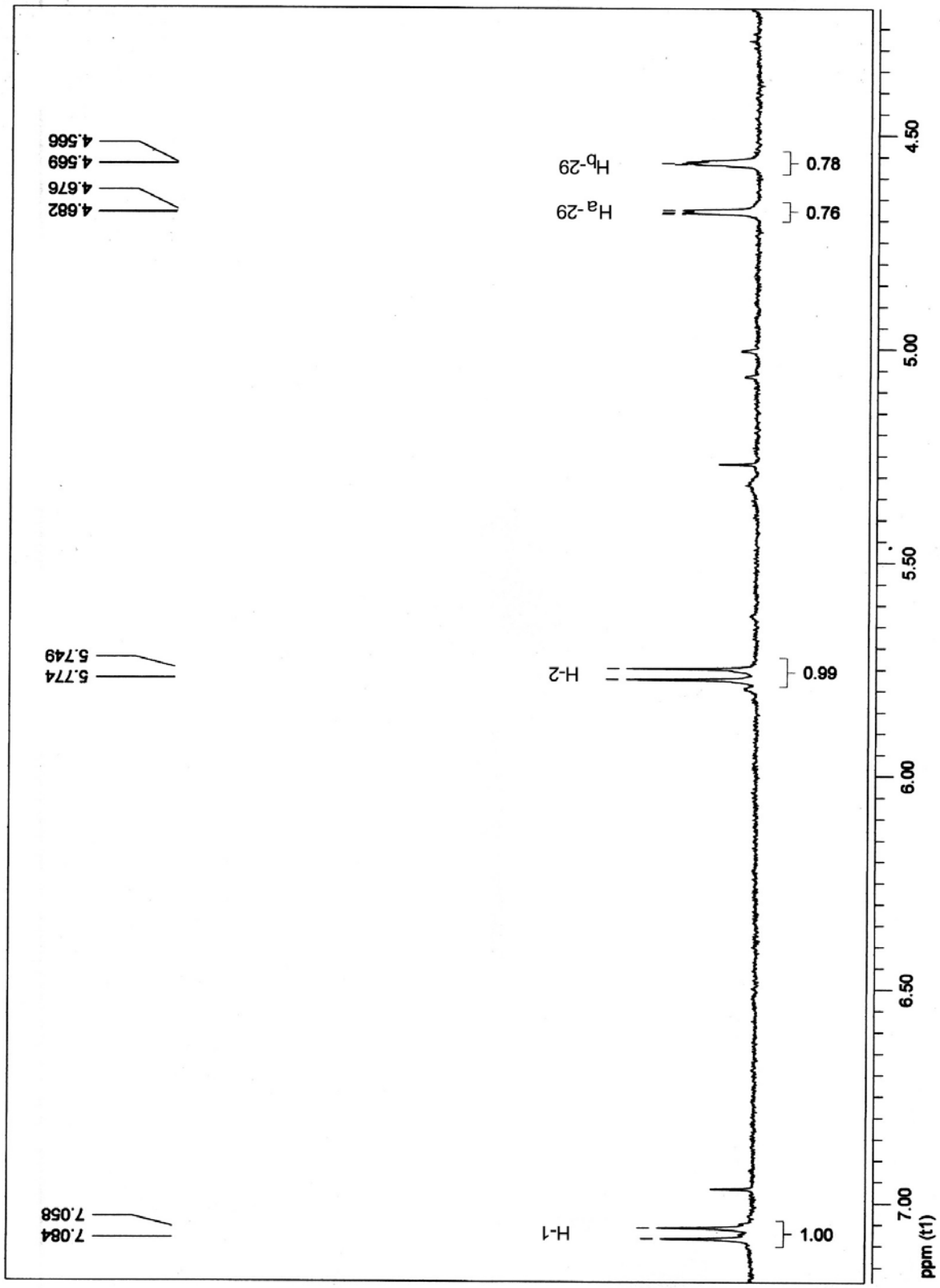


Figure -3.41: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GM-029 (**168**)

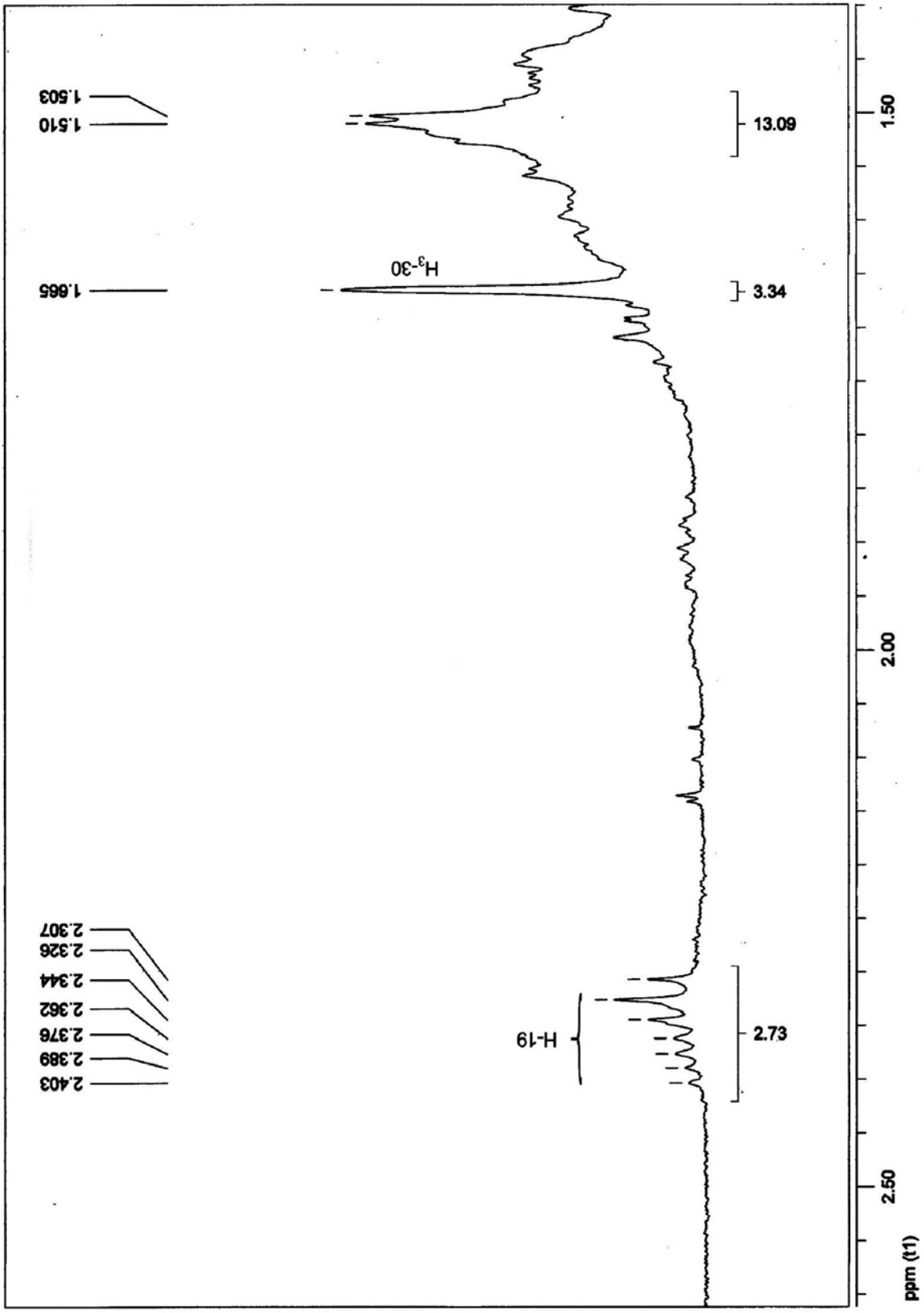


Figure -3.42: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GM-029 (**168**)

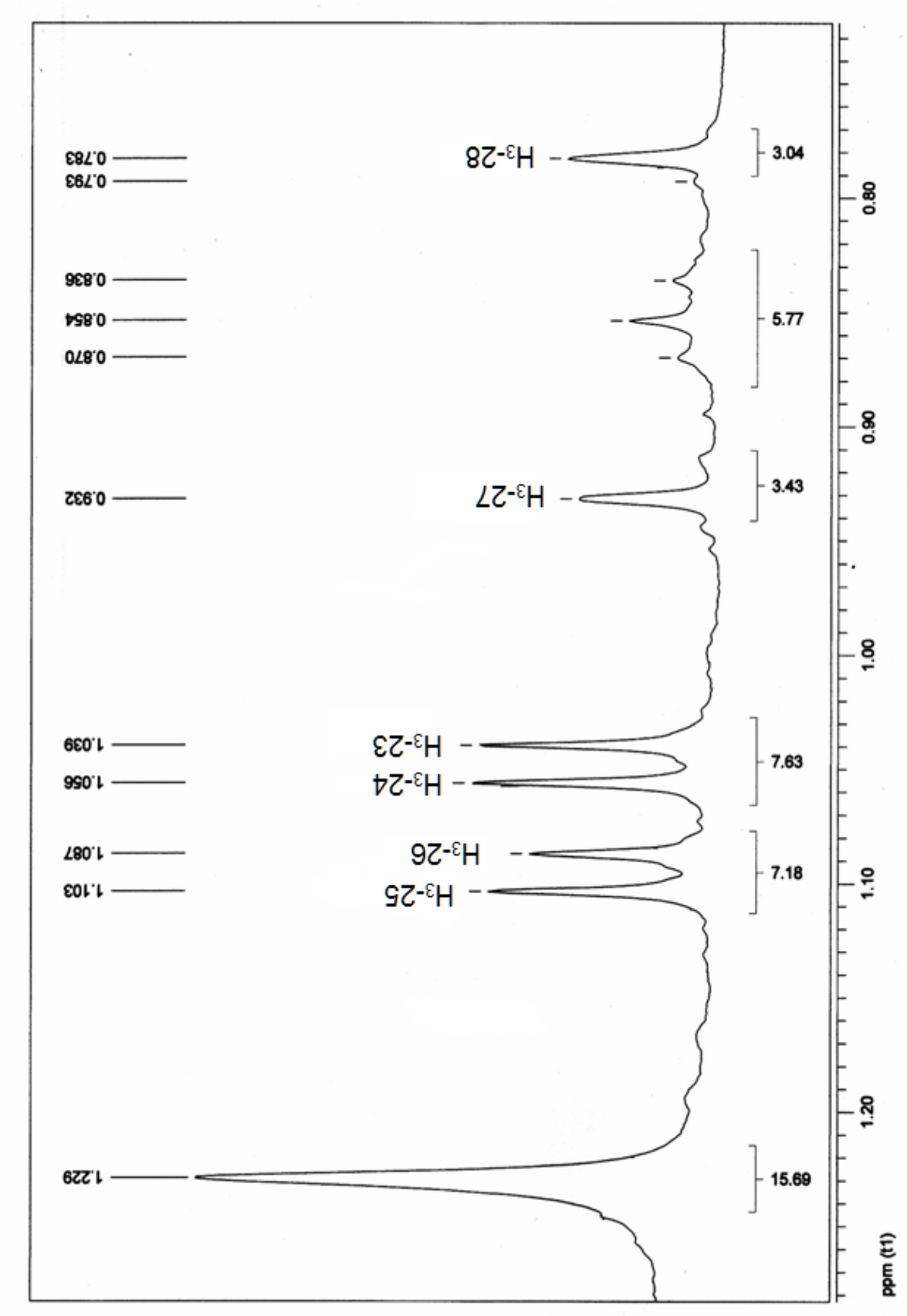


Figure -3.43: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GM-029 (**168**)

3.2.4: Characterization of GM-032 as Glochidonol (169)

GM-032 was isolated as white amorphous particle from the petroleum ether soluble fraction of a methanolic extract of the stem bark of *G. multiloculare*. It appeared as a purple spot on TLC, when the developed plate was sprayed with vanillin-sulfuric acid followed by heating at 110˚C for 5 minutes.

The ^{1}H NMR (Table-3.11, Figure: 3.45-3.47) and ^{13}C NMR (Table-3.12, Figure: 3.48-3.49) spectrum ($CDCl_3$, 400 MHz) exhibited typical signals for 50 protons and 30 carbons including seven tertiary methyls, one oximethine and one terminal disubstituted double bond. The ^{13}C NMR showed the presence of a carbonyl group at δ_c 215.7 (C-3).

The methyl group resonances at δ 1.05, 1.03, 0.83, 1.05, 0.97, 0.79 and 1.69 were attributed to H_3-23, H_3-24, H_3-25, H_3-26, H_3-27, H_3-28 and H_3-30 respectively. The spectrum displayed two singlet at δ 4.67 and 4.56 (1H each) assignable to protons at C-29. A doublet of triplets at δ 2.38 (J_t=11.2, J_d=6.0) integrating one proton, is indicative of H-19. A double doublets at δ 3.89, integrating one proton, is indicative of Hα-1. Two doublets of doublets at δ 2.98 (14.1, 8.0) and 2.21 (14.4, 3.6) are assignable to H_{ax}-2 and H_{eq}-2 respectively. The spectral features (Table-3.11 and 3.12) are in close agreement to those observed for glochidonol (Hui *et al*., 1976; Thu *et al.*, 2010). On this basis, the identity of GM-032 was confirmed as glochidonol. The identity of GM-032 was further substantiated by co-TLC with an authentic sample in our laboratory.

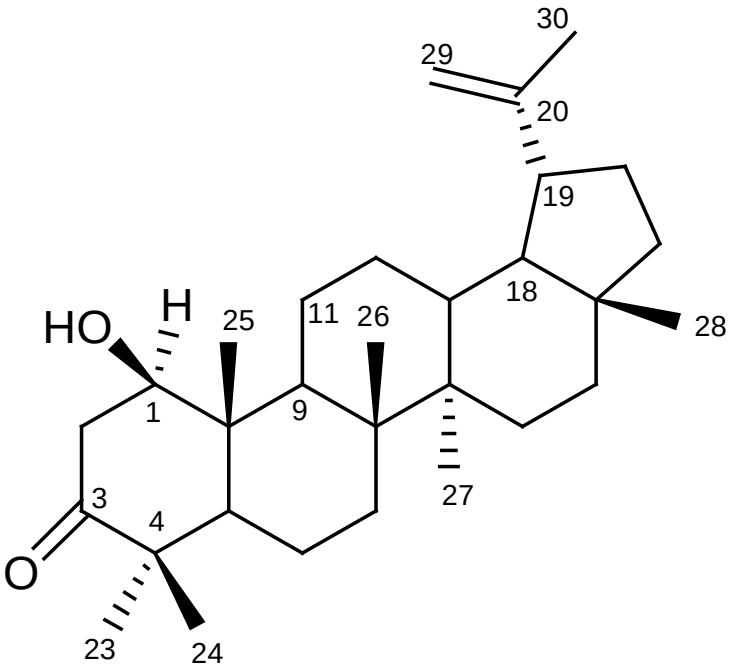


Figure 3.44: Structure of GM-032 (Glochidonol, **169**)

Table 3.11: ^{1}H NMR spectral data of GM-032 and Glochidonol (Hui *et al.*, 1976; Thu *et al.*, 2010).

Protons	**GM-032**	**Glochidonol**
	δ_H, multi, *J* in Hz	**δ_H, multi, *J* in Hz**
H-1	3.89 H_α, dd (8.0, 3.6)	3.89, m
H-2	2.98 H_{ax}, dd (14.4, 8.0); 2.21 Heq, dd (14.4, 3.6)	3.00 H_a, dd (14.5, 8.0); 2.23 H_b, dd (14.5, 4.0)
H-5		1.36
H-7		1.31 H_a, 1.45 H_b
H-9		1.51
H-13		1.71
H-16		1.41 H_a, 1.51 H_b
H-18		1.41
H-19	2.38, dt (11.2, 6.0)	2.37, ddd (11.0, 11.0, 5.5)
H-21		1.26 H_a, 1.92 H_b
H-22		1.21 H_a, 1.42 H_b
H-29	4.67 H_a, br. s; 4.56 H_b, br. s	4.56 H_a, br. s; 4.68 H_b, d (1.5)
H_3-23	1.05, s	1.06, s
H_3-24	1.03, s	1.06, s
H_3-25	0.83, s	0.84, s
H_3-26	1.05, s	1.05, s
H_3-27	0.97, s	0.98, s
H_3-28	0.79, s	0.80, s
H_3-30	1.69, s	1.68, s

Table 3.12: ^{13}C NMR spectral data of GM-032 and Glochidonol (Thu *et al.*, 2010).

Carbons	GM-032 δ_c in ppm	Glochidonol δ_c in ppm
C-1	79.6	79.6
C-2	45.1	45.1
C-3	215.7	215.5
C-4	47.1	47.1
C-5	51.4	51.5
C-6	19.6	19.6
C-7	32.9	33.0
C-8	42.9	43.0
C-9	50.7	50.8
C-10	42.9	43.0
C-11	23.1	23.1
C-12	25.2	25.3
C-13	38.0	38.0
C-14	41.1	41.2
C-15	27.5	27.5
C-16	35.5	35.6
C17	42.9	43.0
C-18	48.3	48.3
C-19	47.9	47.9
C-20	150.7	150.7
C-21	29.8	29.9
C-22	40.0	40.0
C-23	27.9	27.9
C-24	19.8	19.9
C-25	11.8	11.7
C-26	15.9	16.0
C-27	14.4	14.5
C-28	18.0	18.0
C-29	109.5	109.4
C-30	19.3	19.3

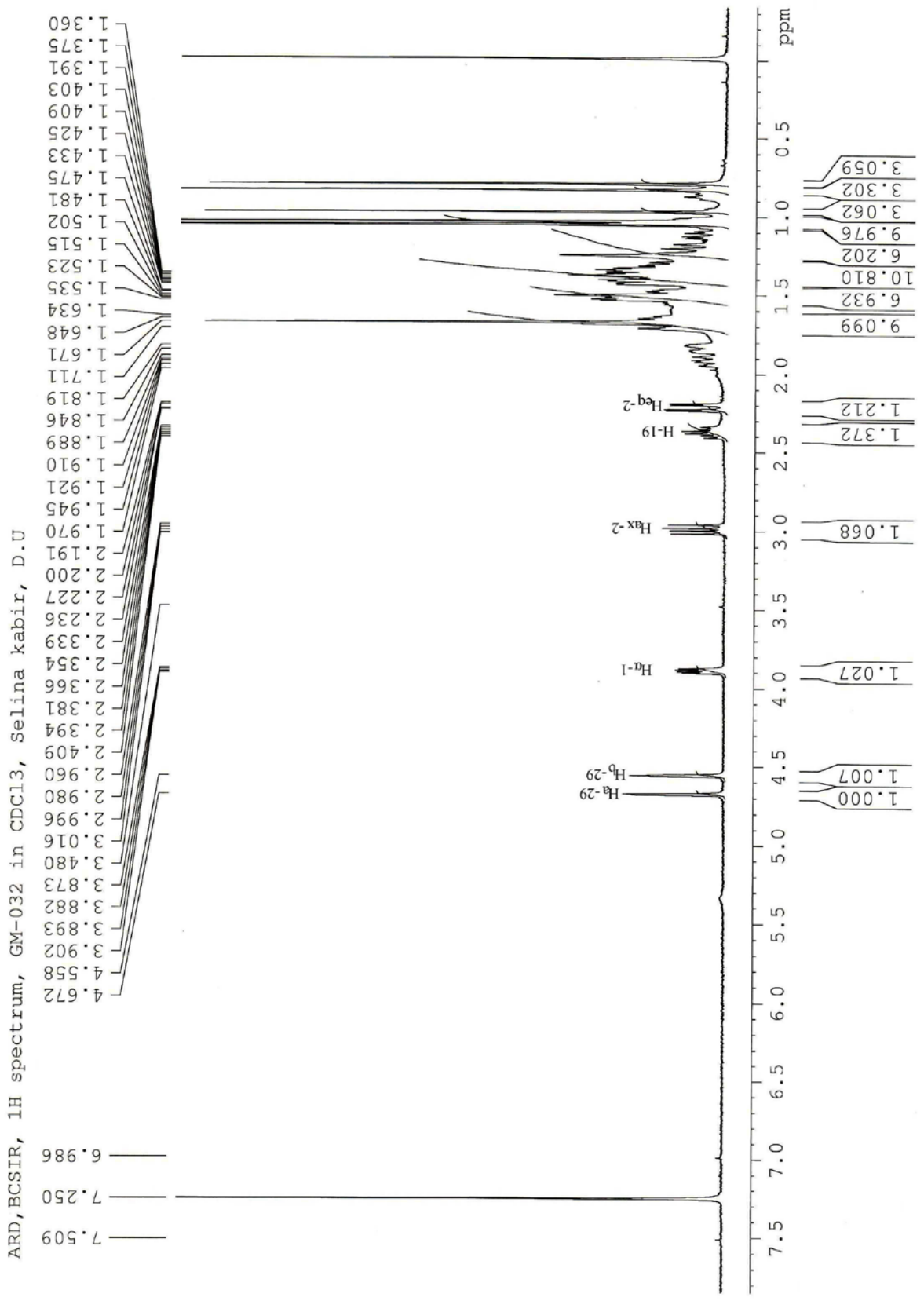


Figure-3.45: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of GM-032 (**169**)

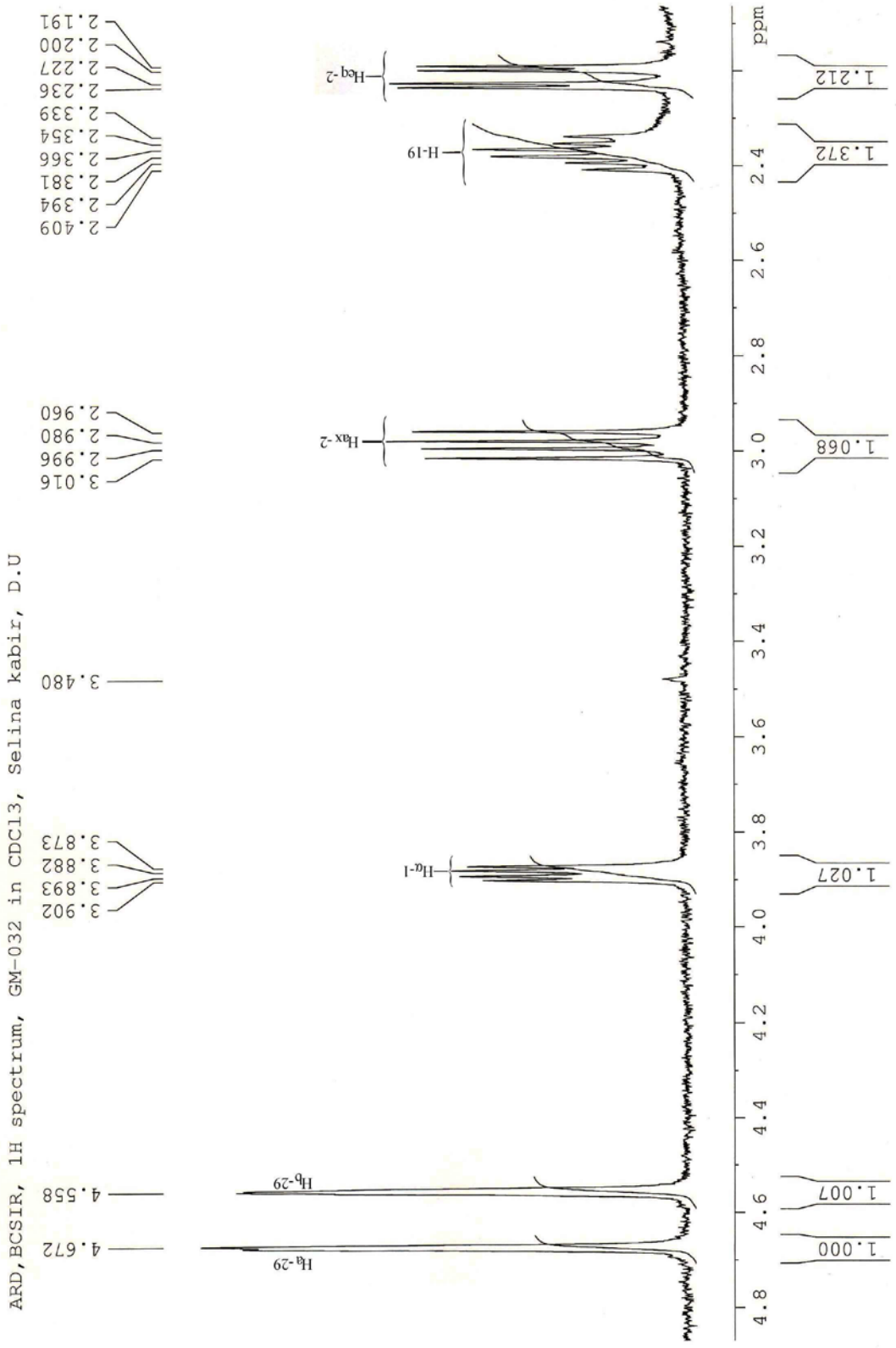


Figure -3.46: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of GM-032 (**169**)

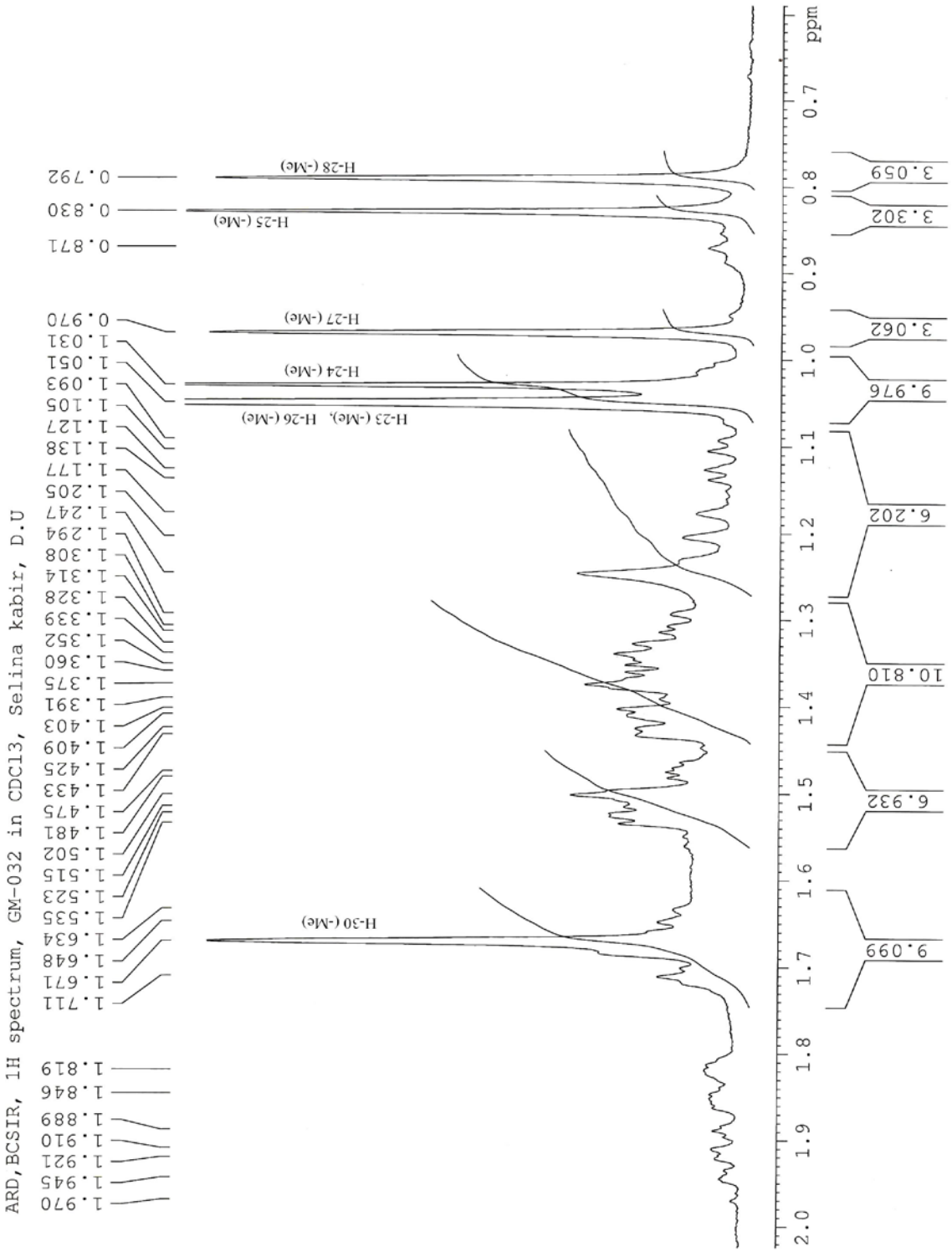


Figure -3.47: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of GM-032 (**169**)

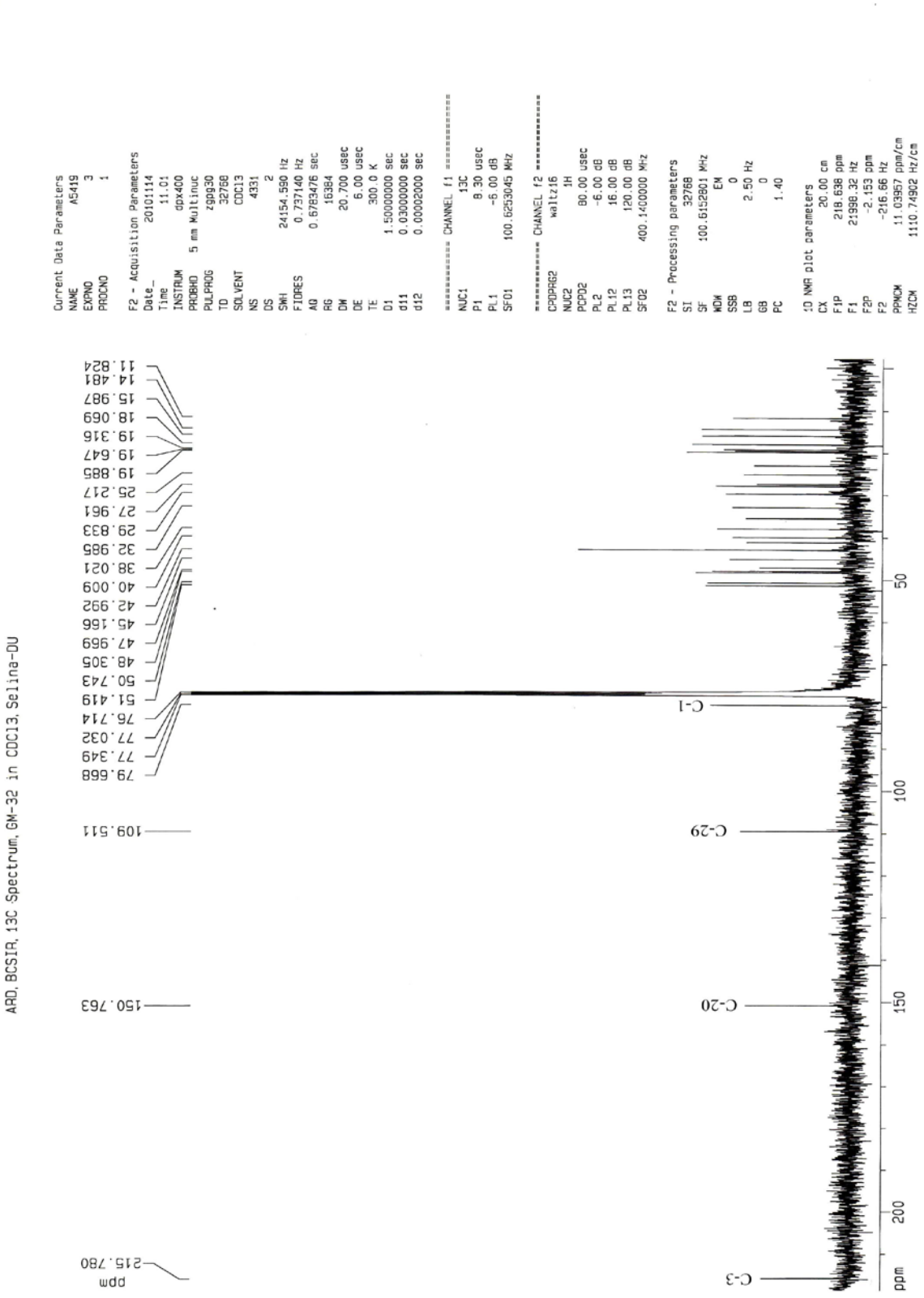


Figure -3.48: ^{13}C NMR (400 MHz, $CDCl_3$) spectrum of GM-032 (**169**)

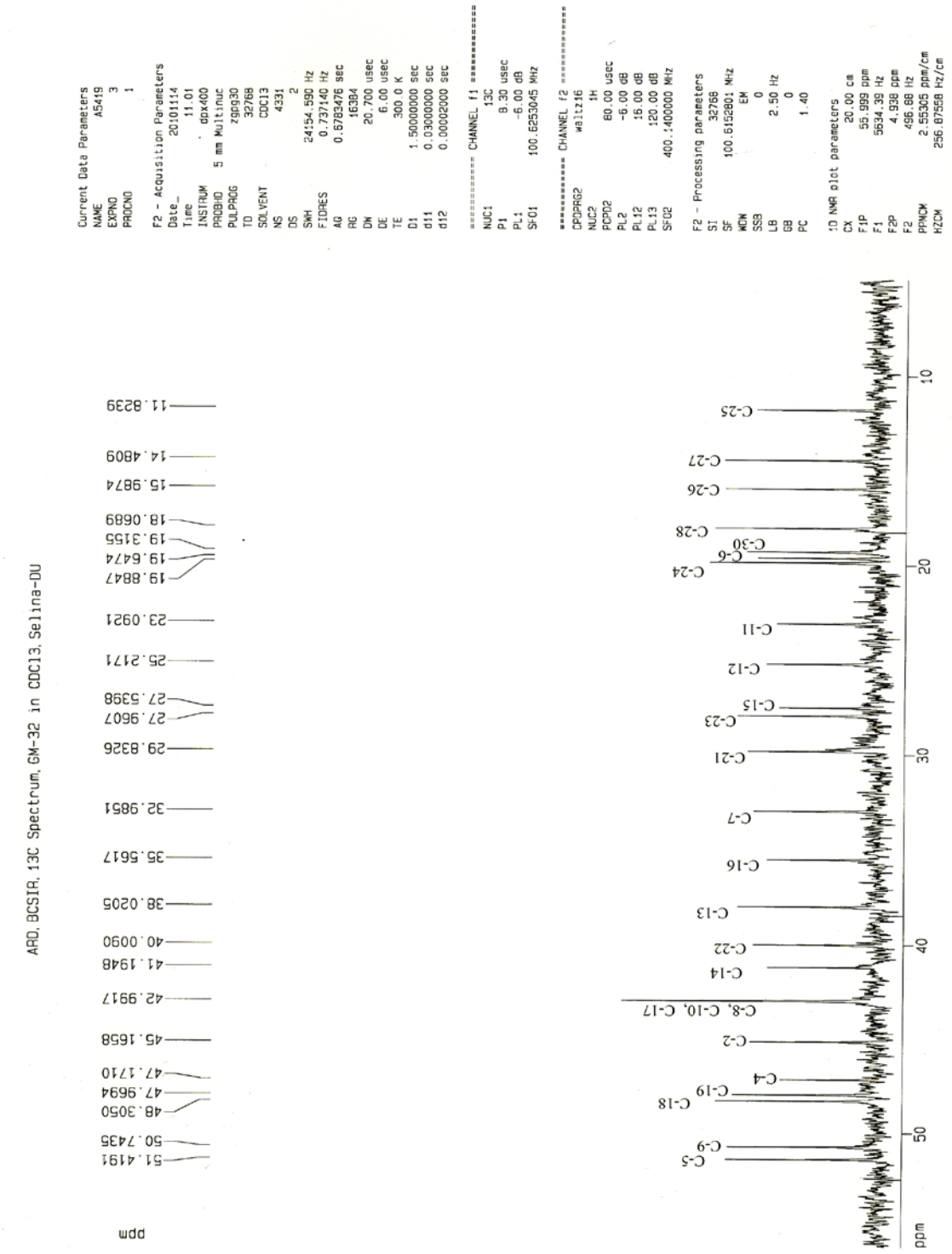


Figure -3.49: Partially expanded ^{13}C NMR (400 MHz, $CDCl_3$) spectrum of GM-032 (**169**)

3.2.5: Characterization of GM-035 as Glochidiol (170)

GM-035 was isolated as needle shaped crystals from the petroleum ether soluble fraction of methanolic extract of the stem bark of *G. multiloculare*. It appeared as a violet spot on TLC (Silica gel PF_{254}) when the developed plate was sprayed with vanillin-sulfuric acid followed by heating at 110°C for 5-10 minutes. It was found to be soluble in ethyl acetate and chloroform.

The ^{1}H NMR spectrum (400 MHZ, $CDCl_3$) of compound GM-035 (Table-3.13, Figure: 3.51-3.53) showed seven methyl group resonances at δ 0.94, 0.74, 0.90, 1.04, 0.94, 0.78 and 1.66 were attributed to 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 spectrum displayed two singlets at δ 4.67 and 4.55 (1H each) assignable to protons at C-29. A triple doublets at δ 2.36, integrating one proton, is indicative of proton at C-19. A multiplet δ 3.42, is assignable to proton at C-1. The spectrum showed a double doublets at δ 3.23, integrating one proton, is indicative of H-3. The spectral features (Table-3.13) are in close agreement to those observed for glochidiol (Hui *et al.*, 1976). On this basis, the identity of GM-035 was confirmed as glochidiol (**170**).

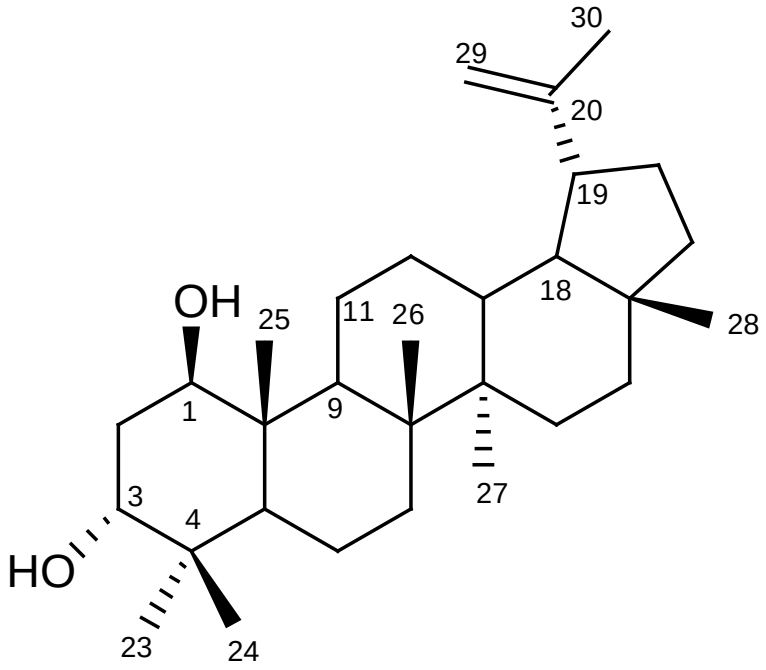


Figure 3.50: Structure of GM-035 (Glochidiol, **170**)

Table 3.13: ^{1}H NMR spectral data of GM-035 and Glochidiol (Hui *et al*., 1976)

Protons	**GM-035**	**Glochidiol**
	δ_H, multi, *J* in Hz	**δ_H, multi, *J* in Hz**
H-1	3.42 Hax, m	
H-3	3.23, dd (12.0, 4.4)	
H-19	2.36, ddd (10.8, 10.8, 5.6)	
H-29	4.67 H_a, bs; 4.55 H_b, bs	
H_3-23	0.94, s	0.90, s
H_3-24	0.74, s	0.82, s
H_3-25	0.90, s	0.90, s
H_3-26	1.04, s	1.04, s
H_3-27	0.94, s	0.97, s
H_3-28	0.78, s	0.78, s
H_3-30	1.66, s	

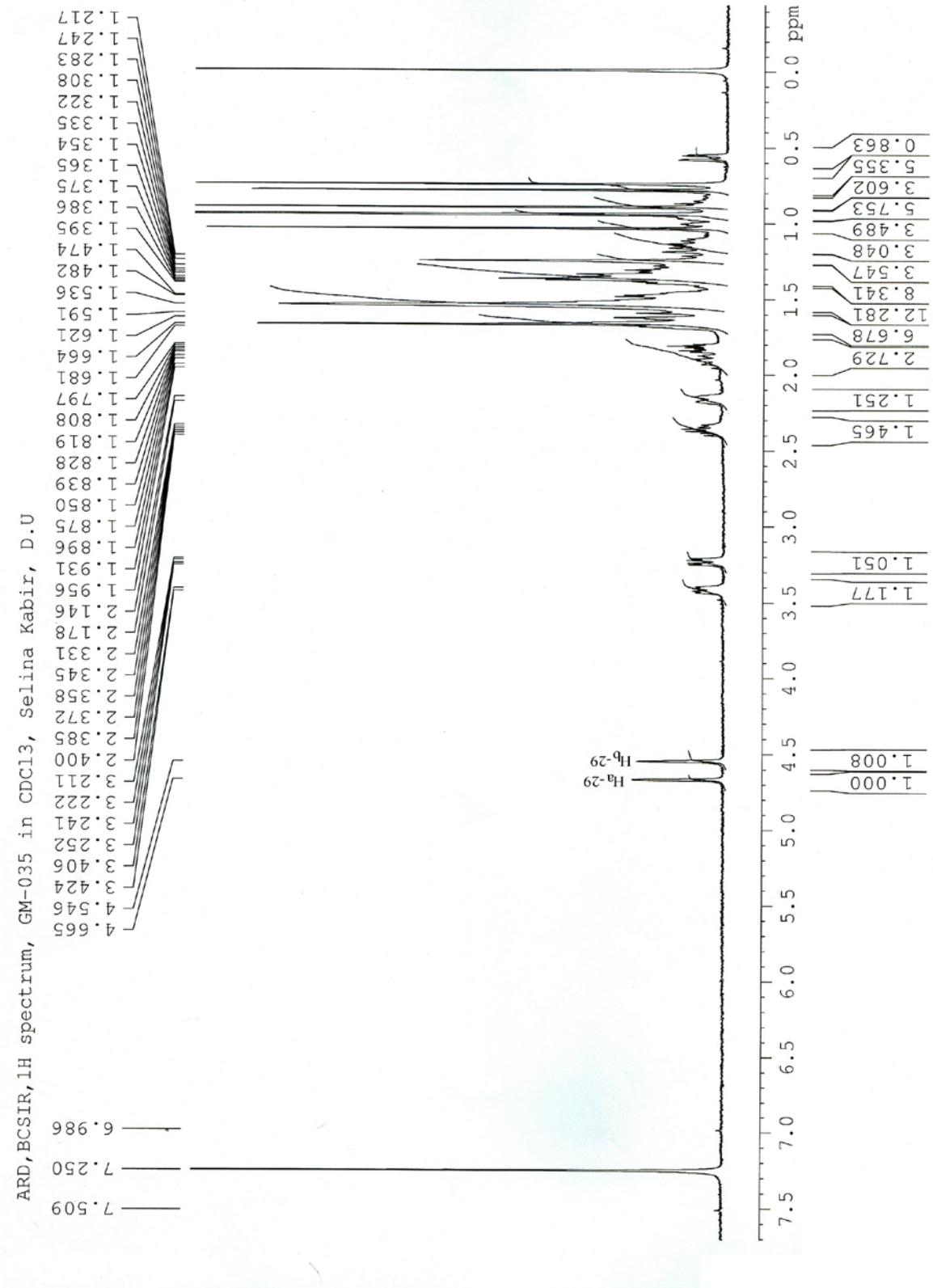


Figure-3.51: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of GM-035 (**170**)

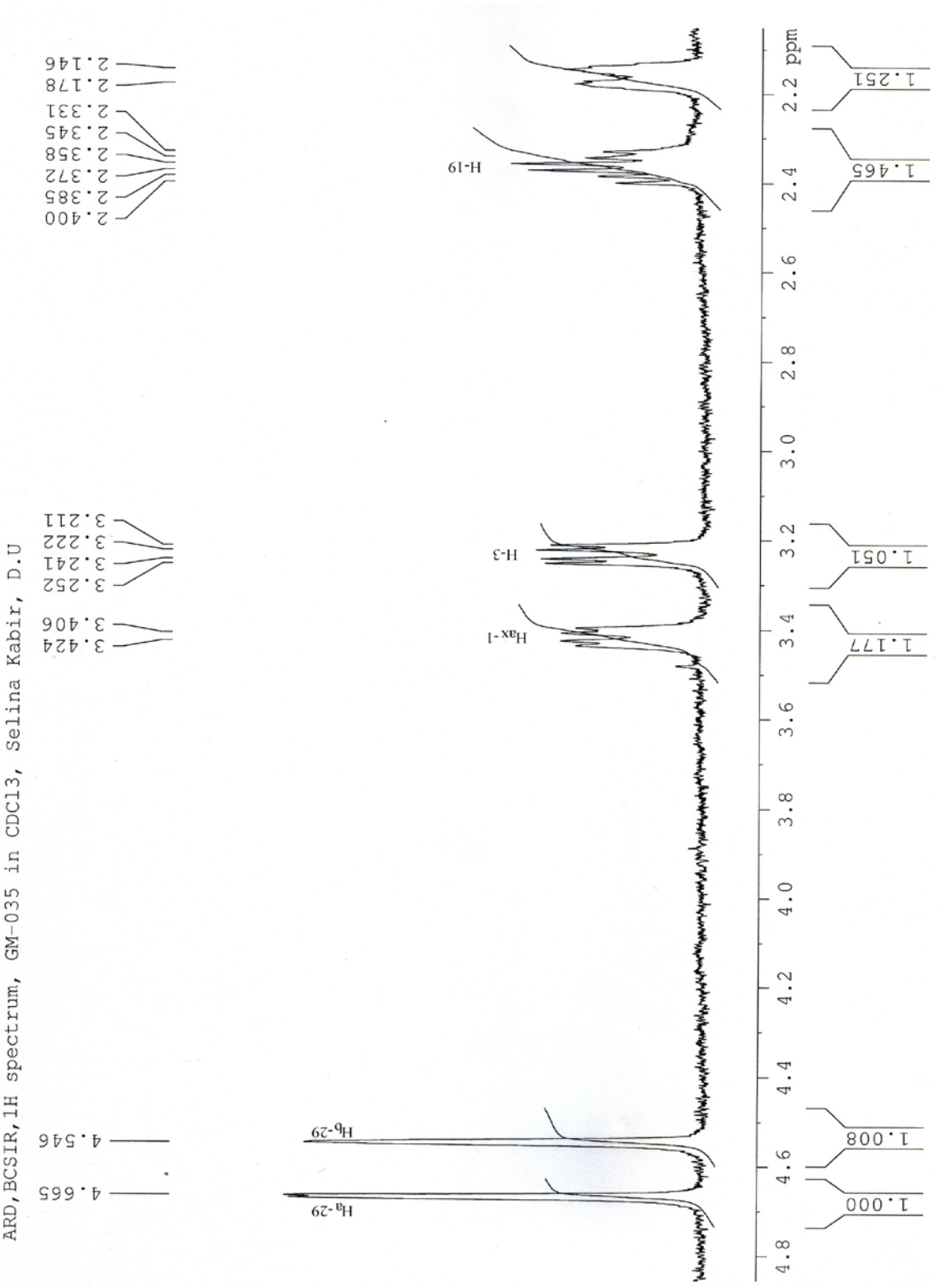


Figure -3.52: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of GM-035 (**170**)

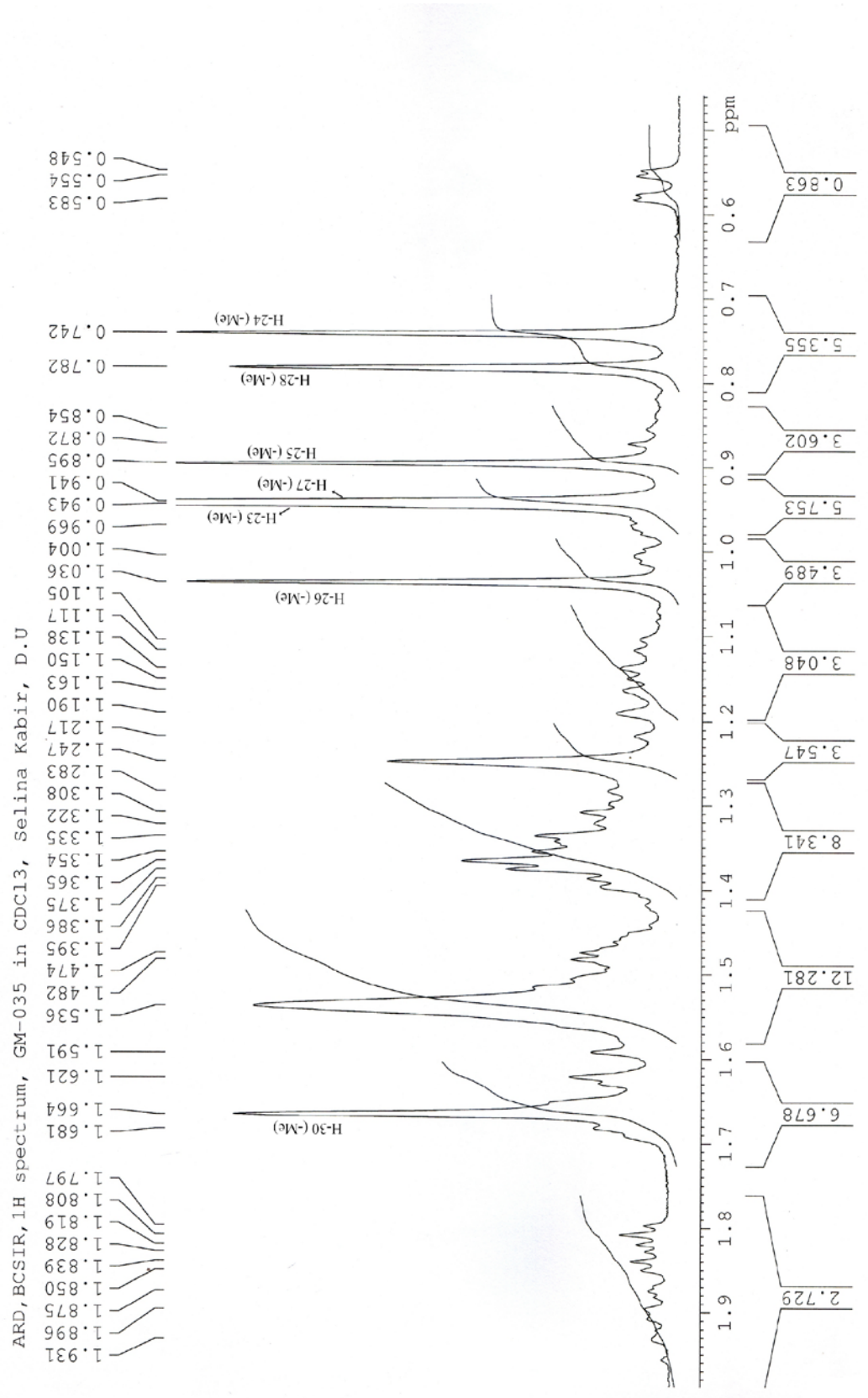


Figure -3.53: Partially expanded ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of GM-035 (**170**)

3.3 Chemical investigation of *Glochidion lanceolarium*

Three compounds GL-022, GL-023 and GL-035 have been isolated from the carbon tetrachloride soluble material of the stem bark of *Glochidion lanceolarium* by using different chromatographic techniques.

3.3.1 Characterization of GL-022 as Epilupeol (171) an analogue of GM-022

GL-022 was isolated as white crystal from the carbon tetrachloride soluble fraction of the stem bark of *G. lanceolarium*. It appeared as a violet spot on TLC, when the developed plate was sprayed with vanillin-sulfuric acid followed by heating at 110˚C for 5 minutes.

The high-resolation ESI mass spectrum of GL-022 (Figure:3.61-3.62) showed the pseudo-molecular ion peak, $[M + H]^+$ at *m/z*, 427 which was consistent with a molecular formula ($C_{30}H_{50}O$, M = 426) for this compound.

The ^1H NMR (Table-3.14, Figure: 3.55-3.58) and ^{13}C NMR (Table-3.15, Figure: 3.59-3.60) spectrum revealed typical signals for 50 protons and 30 carbons including seven tertiary methyls, one oximethine and one terminal disubstituted double bond (Thu *et al.*, 2010).

The ^1H NMR ($CDCl_3$, 500 MHz) spectrum of GM-022 (Figure: 3.31-3.34) represents similar features of that reported for 3-*epi* Lupeol (Thu *et al.*, 2010; Alam *et al*., 2009).

The ^1H NMR spectrum (500 MHz, $CDCl_3$) of compound GL-022 (Table-3.14, Figure: 3.55-3.58) showed a trplet (J =2.8) of one proton intensity at δ 3.39 typical for an oxymethine proton at C-3 of a triterpene type carbon skeleton. The absence of a double doublet and the appearance of a triplet suggested that the hydroxy group was at the α (alpha)-position, thus confirming the β (beta) orientation of C-3 proton (Alam *et al*., 2009). The spectrum displayed a doublet at δ 4.69 (J=2.4) and a double doublet at δ 4.57 (2.4, 1.6) assignable to the vinylic protons at C-29. Triple doublet at δ 2.39 (11.2, 11.2, 6.0) could be ascribed to proton at C-19. The spectrum also displayed seven singlets at δ 0.83, 0.94, 0.85, 1.04, 0.96, 0.79 and 1.68 (3H each) for 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. These spectral features of ^1H NMR are in close agreement to those observed

for GM-022 (Table-3.8). On this basis and by comparing these ^{1}H NMR (Table-3.14) and ^{13}C NMR (Table-3.15) data with literature values (Thu *et al.*, 2010; Alam *et al.*, 2009), compound GL-022 was identified as epilupeol. The identity of GL-022 was further substantiated by co-TLC with an authentic sample.

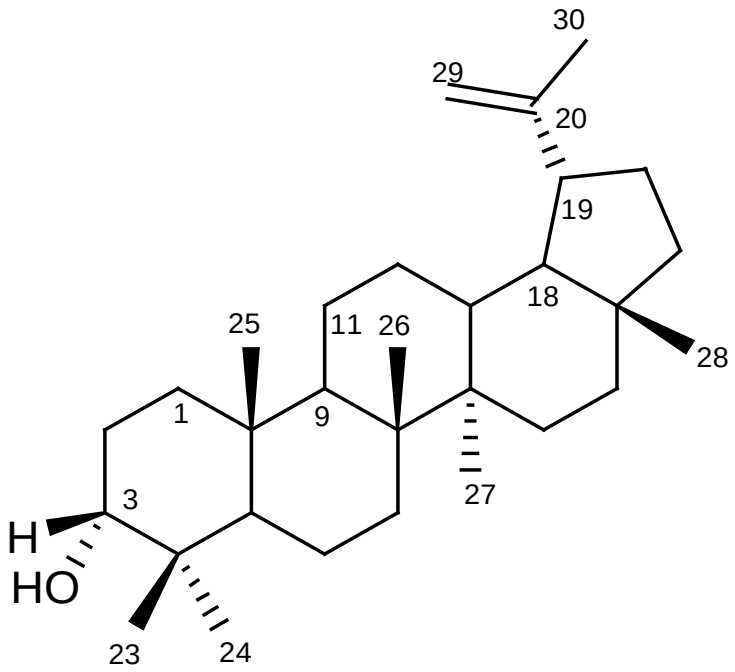


Figure-3.54: Structure of GL-022 (Epilupeol, **171**)

Table 3.14: ^{1}H NMR (500 MHz, $CDCl_3$) spectral data of GL-022 and Epilupeol (Thu *et al.*, 2010; Alam *et al*., 2009).

Protons	**GL-022**	**Epilupeol**
	δ_H, multi, *J* in Hz	**δ_H, multi, *J* in Hz**
H-3	3.39, t (2.8)	3.38 br.s
H-19	2.39, ddd (11.2, 11.2, 6.0)	2.38, ddd (11.0, 11.0, 5.5)
H-29	4.69 H_a, d (2.4); 4.57 H_b, dd (2.4, 1.6)	4.68 H_a, br. d (1.5); 4.56 H_b, d (1.0)
H_3-23	0.83, s	0.82, s
H_3-24	0.94, s	0.93, s
H_3-25	0.85, s	0.84, s
H_3-26	1.04, s	1.03, s
H_3-27	0.96, s	0.96, s
H_3-28	0.79, s	0.78, s
H_3-30	1.68, s	1.68, s

Table 3.15: ^{13}C NMR ($CDCl_3$, 100 MHz) spectral data of GL-022 and Epilupeol (Thu *et al.*, 2010).

Carbons	GL-022 δ_c in ppm	Epilupeol δ_c in ppm
C-1	33.2	33.2
C-2	25.4	25.4
C-3	76.2	76.2
C-4	38.0	37.5
C-5	49.7	49.0
C-6	18.3	18.3
C-7	34.1	34.1
C-8	40.0	41.0
C-9	50.2	50.2
C-10	38.0	37.3
C-11	19.2	20.8
C-12	25.1	25.1
C-13	38.0	38.0
C-14	43.0	43.0
C-15	27.4	27.4
C-16	35.6	35.6
C17	42.9	42.9
C-18	49.0	48.3
C-19	49.0	48.0
C-20	150.9	150.9
C-21	28.2	29.8
C-22	40.0	40.0
C-23	27.4	28.2
C-24	22.1	22.1
C-25	15.9	16.0
C-26	15.9	15.9
C-27	14.6	14.6
C-28	18.0	18.0
C-29	109.2	109.3
C-30	19.3	19.3

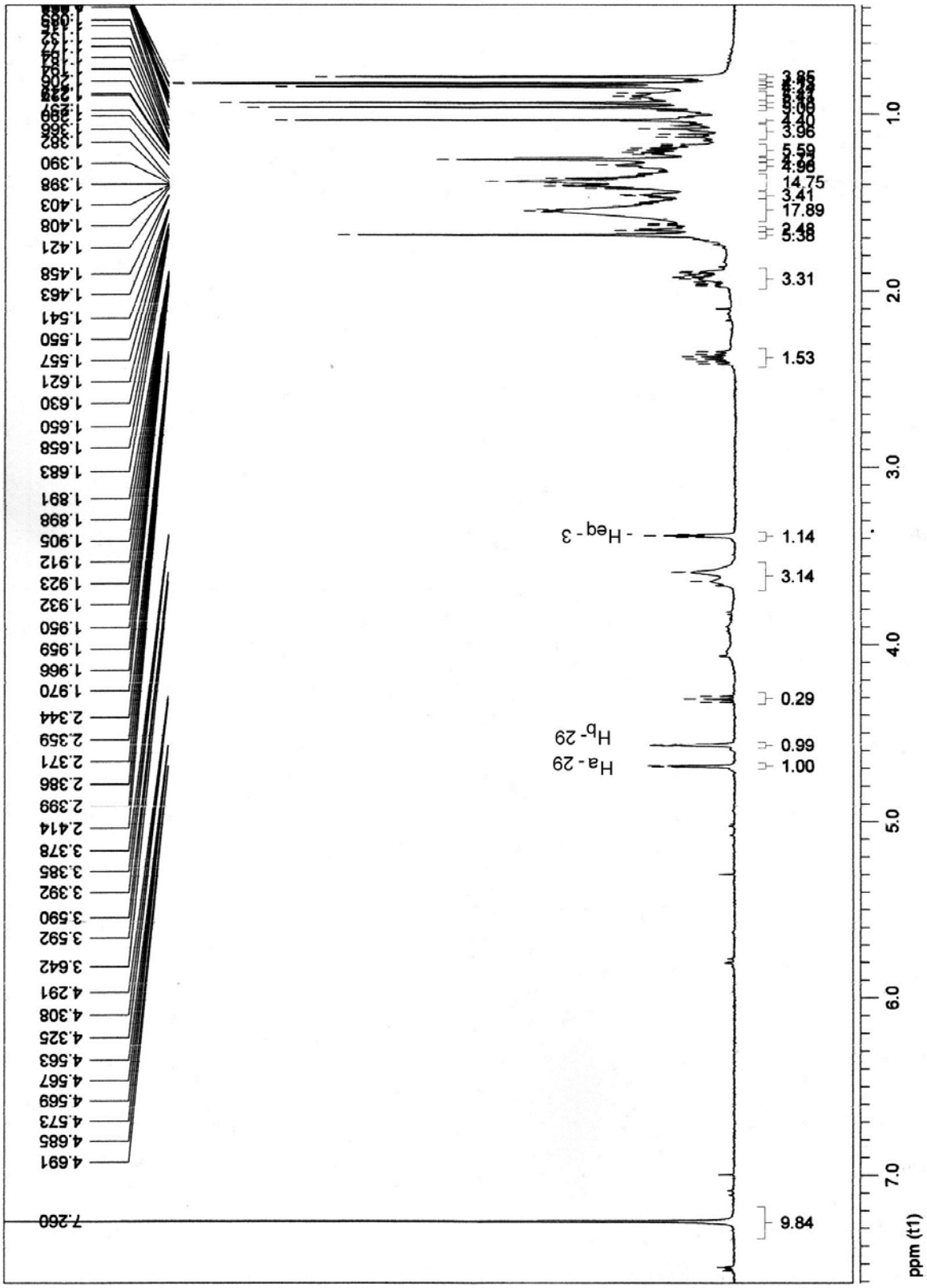


Figure-3.55: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-022 (**171**)

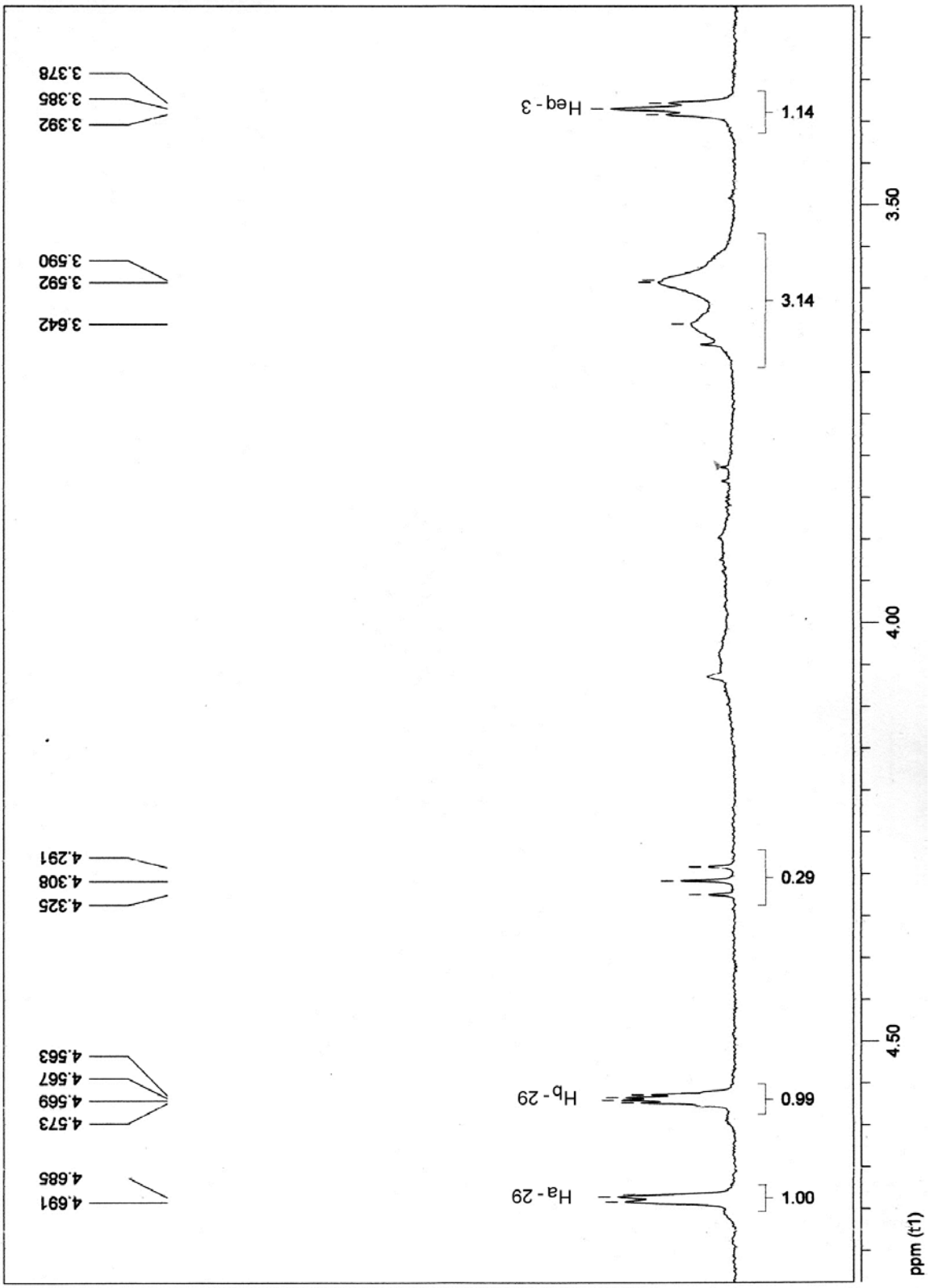


Figure -3.56: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-022 (**171**)

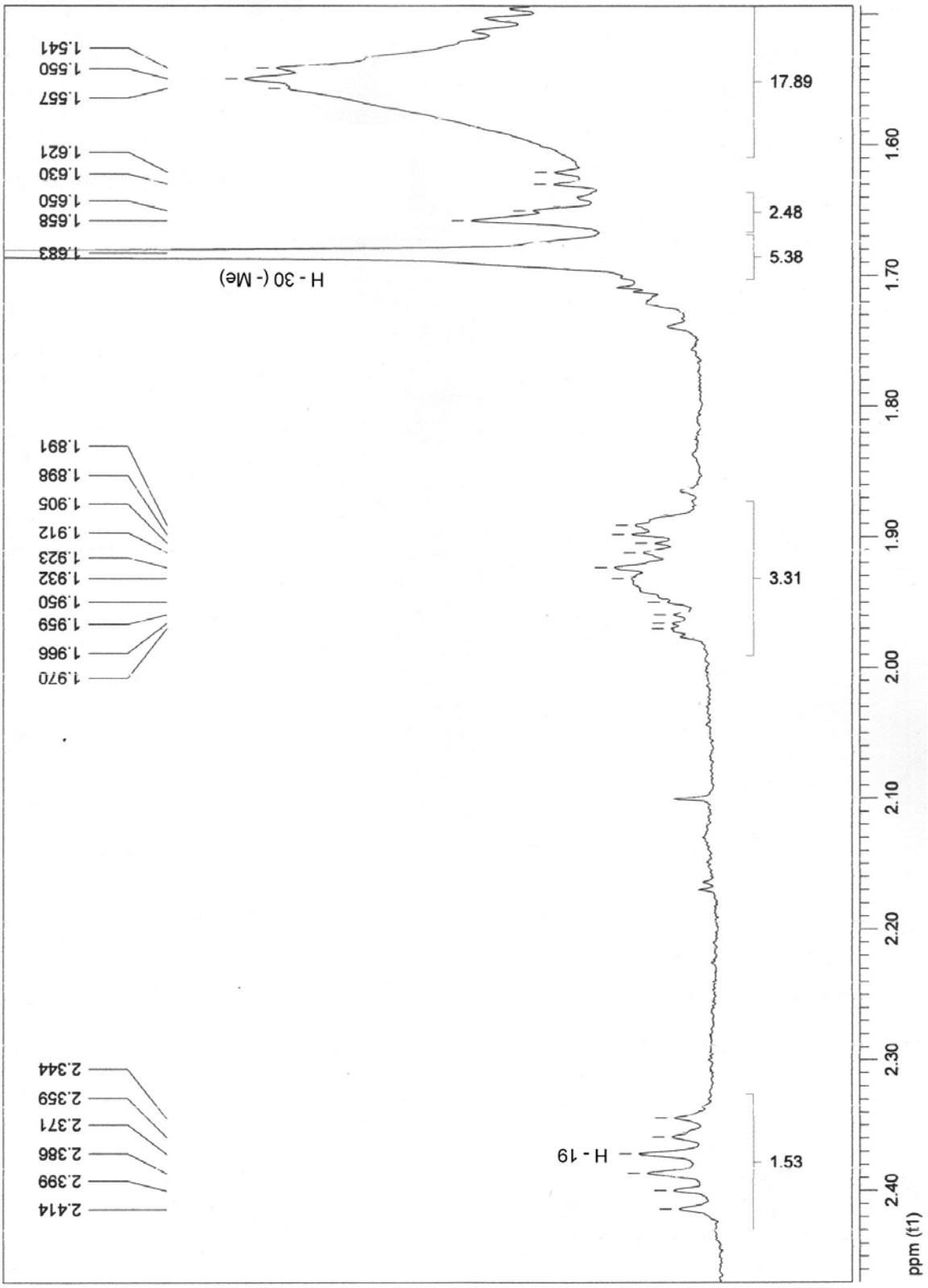


Figure -3.57: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-022 (**171**)

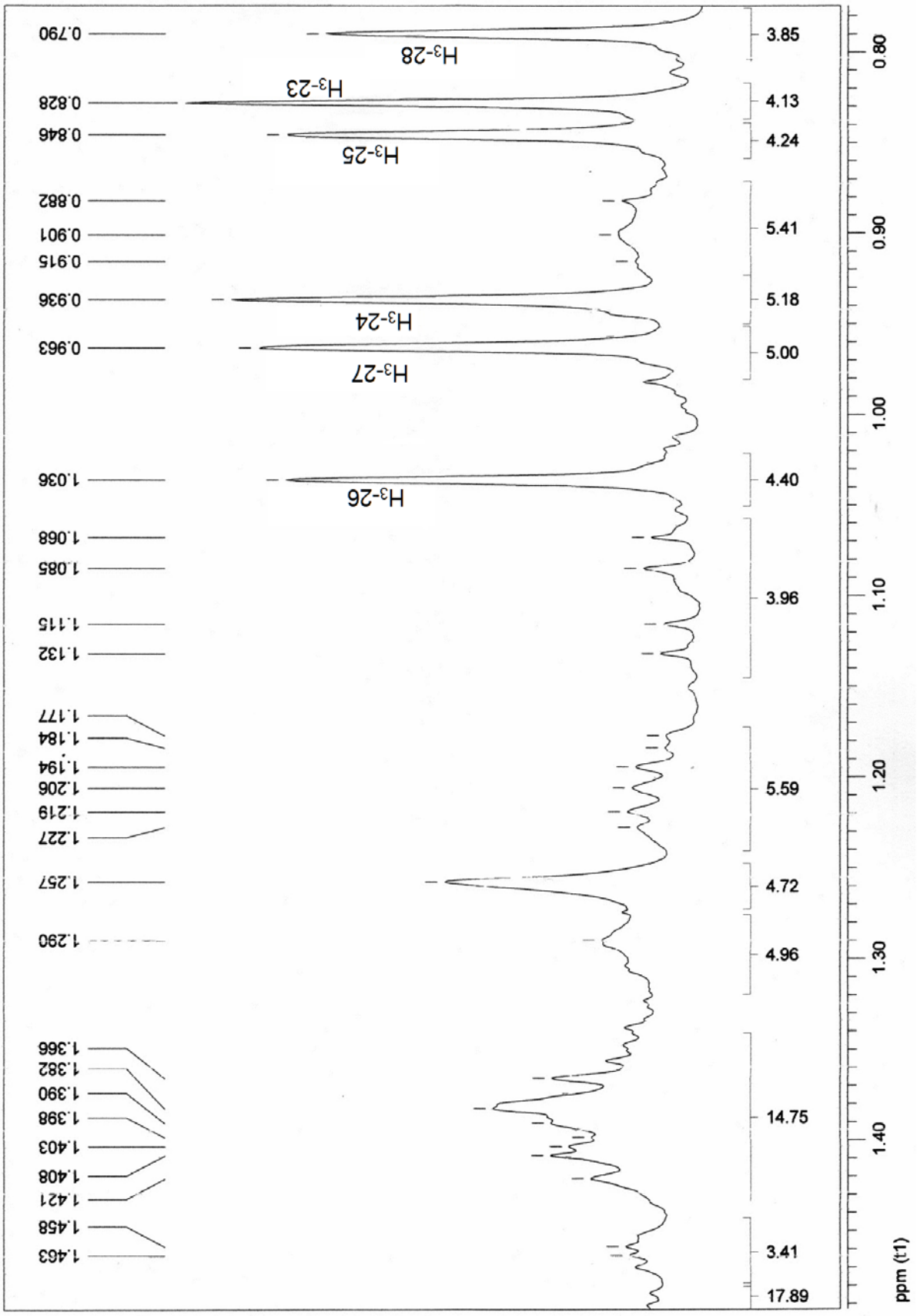


Figure -3.58: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-022 (**171**)

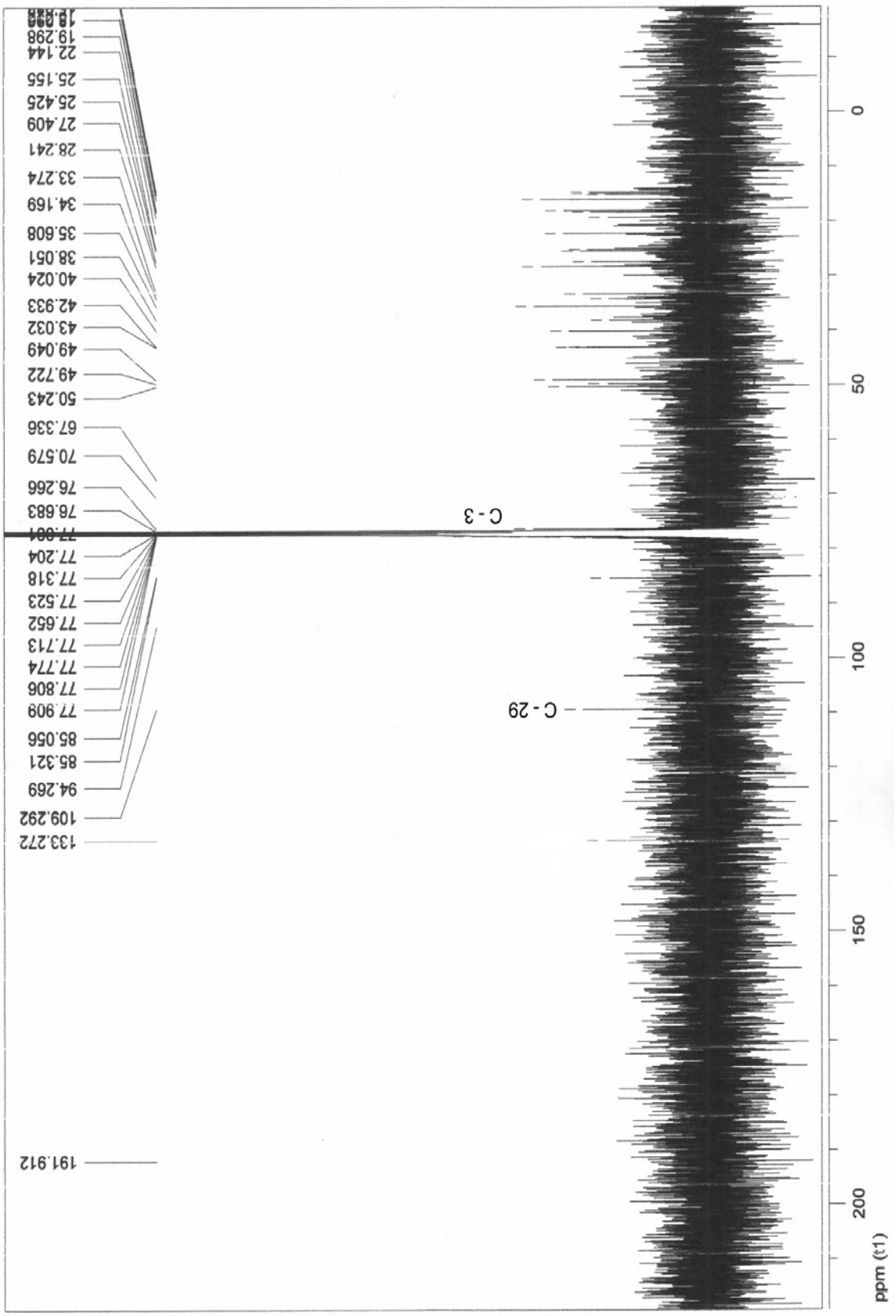


Figure -3.59: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of GL-022 (**171**)

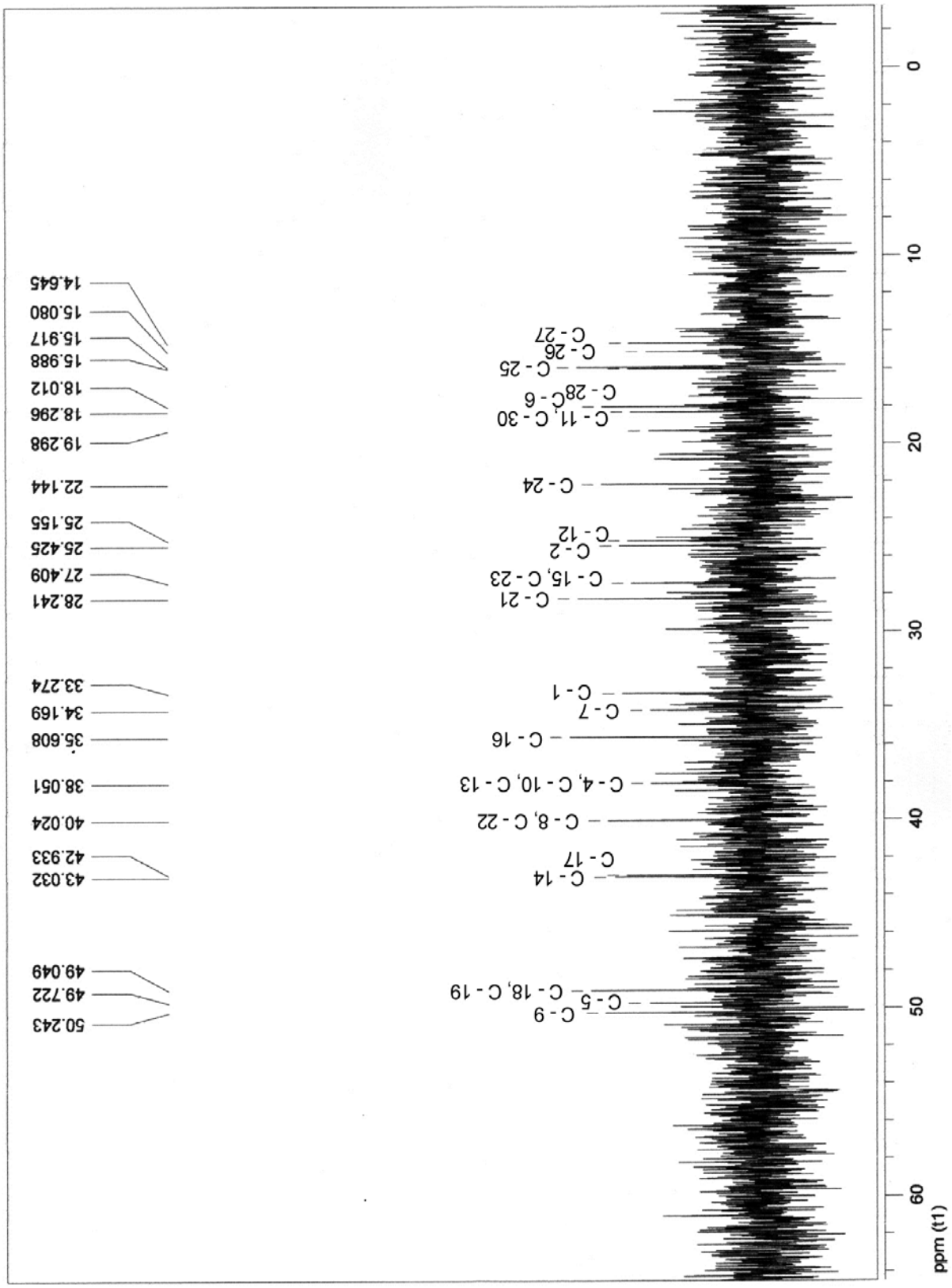


Figure -3.60: Partially expanded ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of GL-022 (**171**)

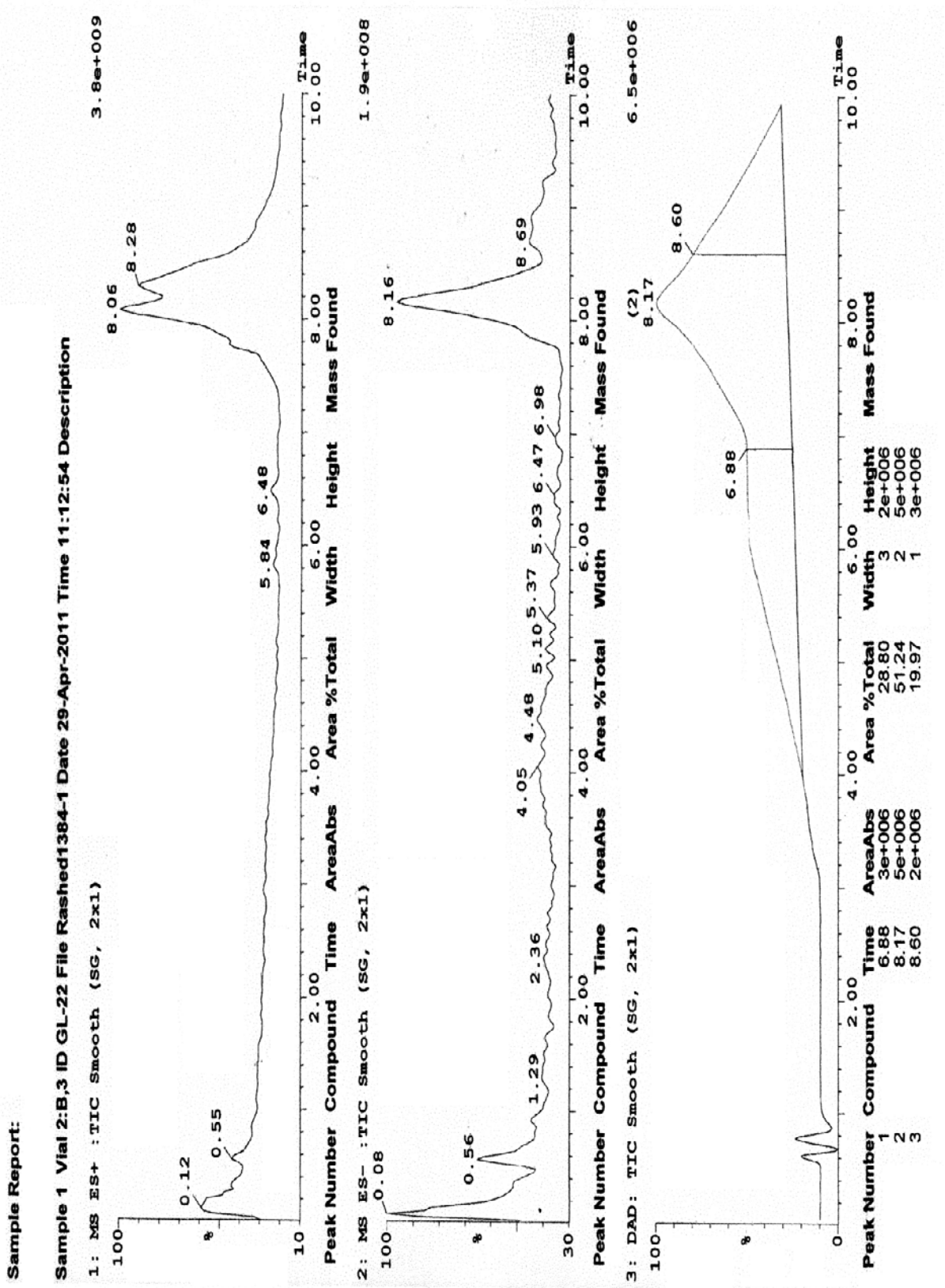


Figure 3.61: Electrospray mass spectrum of compound GL-022 (**171**)

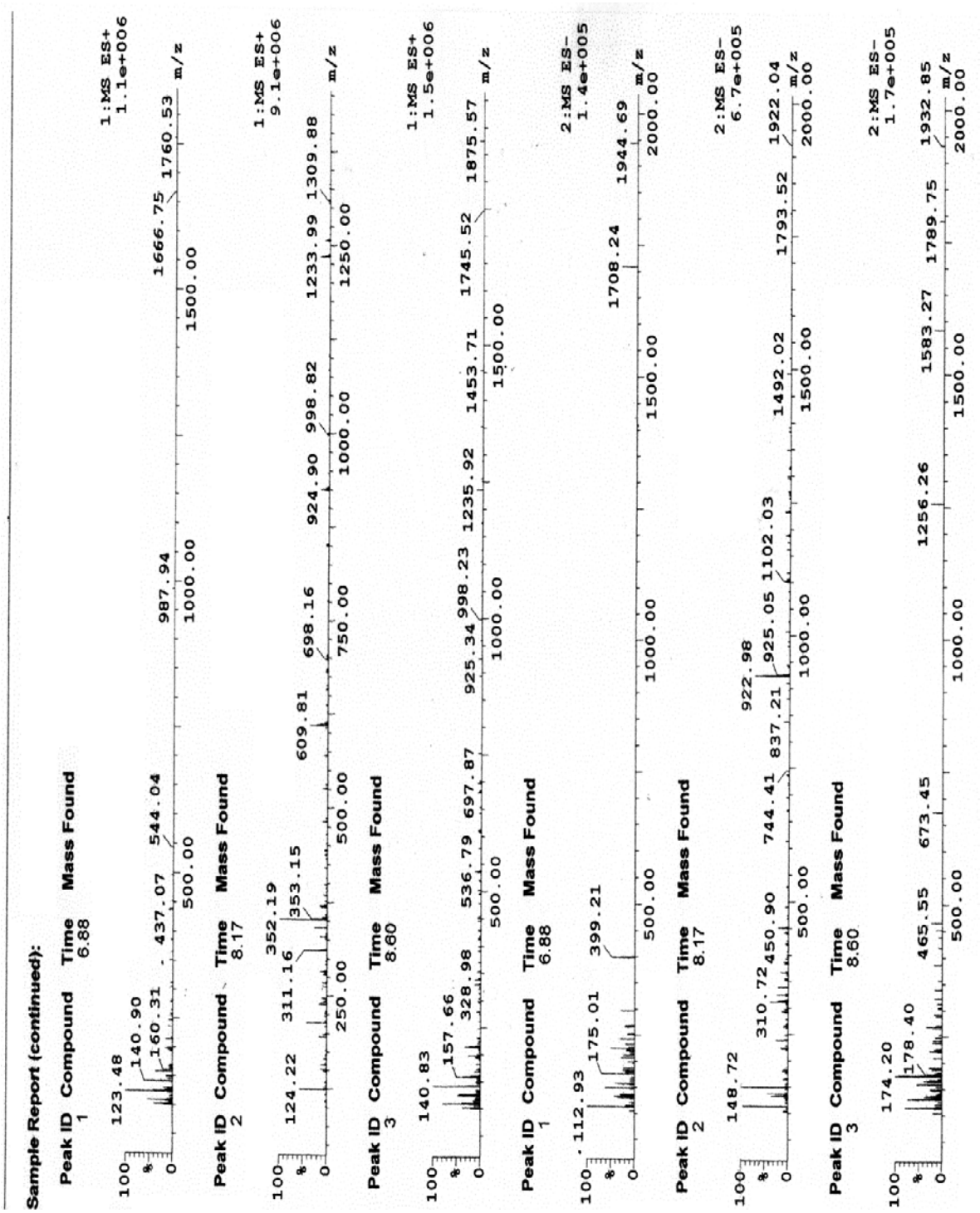


Figure 3.62: Expanded Electrospray Mass spectrum of compound GL-022 (**171**)

3.3.2: Characterization of GL-023 as Glochidonol (172) an analogue of GM-032

GL-023 was isolated as white crystal from the carbon tetrachloride soluble fraction of the stem bark of *G. lanceolarium* by VLC method. It appeared as a purple spot on TLC, when the developed plate was sprayed with vanillin-sulfuric acid followed by heating at 110˚C for 5 minutes.

The high-resolation ESI mass spectrum of GL-023 (Figure: 3.68-3.70) showed the pseudo-molecular ion peak, $[M + H]^+$ at *m/z*, 441 which was consistent with a molecular formula ($C_{30}H_{48}O_2$, M = 440) for this compound (Thu *et al.*, 2010).

The ^1H NMR ($CDCl_3$, 500 MHz) spectrum of GL-023 (Table-3.16, Figure: 3.64-3.67) displayed methyl group resonances at δ 1.06, 1.04, 0.84, 1.06, 0.98, 0.80 and 1.68 were attributed to H_3-23, H_3-24, H_3-25, H_3-26, H_3-27, H_3-28 and H_3-30 respectively. The spectrum showed a doublet at δ 4.69 (2.0) and a singlet at 4.57 assignable to protons at C-29. A doublet of triplets at δ 2.38, (11.2, 6.0) integrating one proton intensity is indicative of H-19. A double doublets at δ 3.89 (8.0, 3.6) integrating one proton, is indicative of Hα-1. Two doublets of doublets at δ 2.99 (14.4, 8.0) and δ 2.21 (14.4, 3.6) are assignable to H_{ax}-2 and H_{eq}-2 respectively. These spectral features of ^1H NMR are in close agreement to those observed for GM-032 (Table-3.11). On this basis and by comparing these ^1H NMR (Table-3.16, Figure: 3.64-3.67) data with literature values (Hui *et al*., 1976; Thu *et al.*, 2010), compound GL-023 was identified as glochidonol. The identity of GL-023 was further substantiated by co-TLC with an authentic sample.

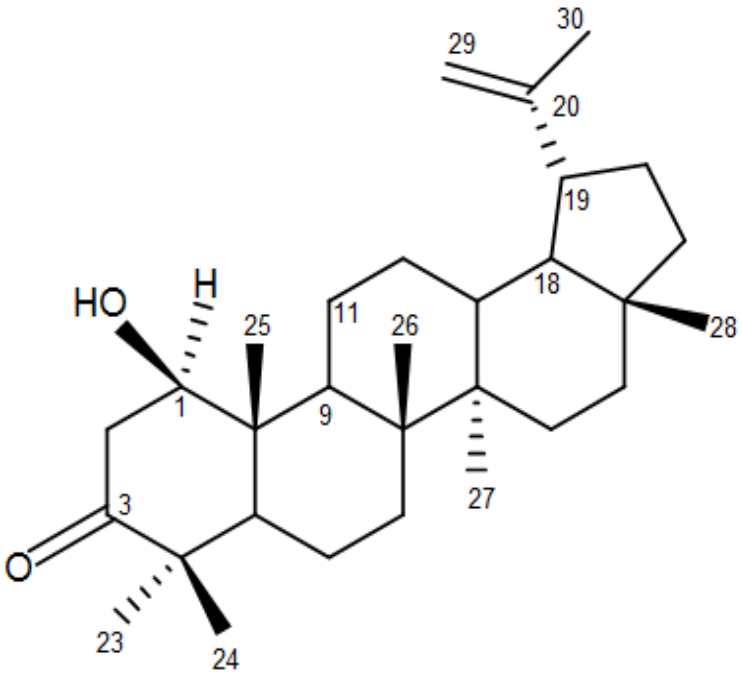


Figure 3.63: Structure of GL-023 (Glochidonol, **172**)

Table 3.16: ^{1}H NMR (500 MHz, $CDCl_3$) spectral data of GL-023 and Glochidonol (Hui *et al.*, 1976; Thu *et al.*, 2010).

Protons	**GL-023**	**Glochidonol**
	δ_H, multi, *J* in Hz	**δ_H, multi, *J* in Hz**
H-1	3.89 $H_{\alpha,}$ dd (8.0, 3.6)	3.89, m
H-2	2.99 H_{ax}, dd (14.4, 8.0); 2.21 Heq, dd (14.4, 3.6)	3.00 H_a, dd (14.5, 8.0); 2.23 H_b, dd (14.5, 4.0)
H-5		1.36
v-7		1.31 H_a, 1.45 H_b
H-9		1.51
H-13		1.71
H-16		1.41 H_a, 1.51 H_b
H-18		1.41
H-19	2.38, dt (11.2, 5.6)	2.37, ddd (11.0, 11.0, 5.5)
H-21		1.26 H_a, 1.92 H_b
H-22		1.21 H_a, 1.42 H_b
H-29	4.69 H_a, d (2.0); 4.57 H_b, br. s	4.56 H_a, br. s; 4.68 H_b, d (1.5)
H_3-23	1.06, s	1.06, s
H_3-24	1.04, s	1.06, s
H_3-25	0.84, s	0.84, s
H_3-26	1.06, s	1.05, s
H_3-27	0.98, s	0.98, s
H_3-28	0.80, s	0.80, s
H_3-30	1.68, s	1.68, s

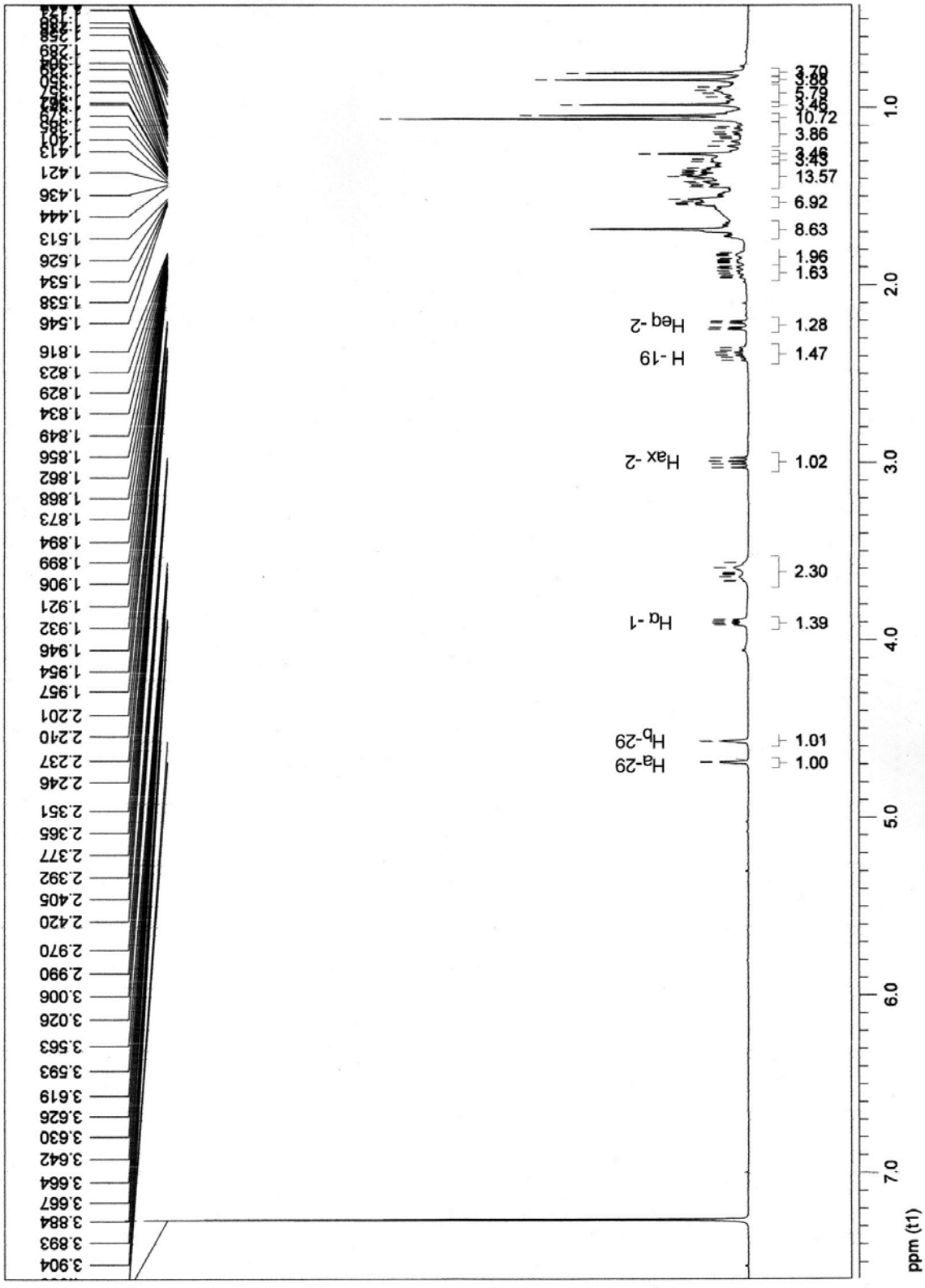


Figure-3.64: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-023 (**172**)

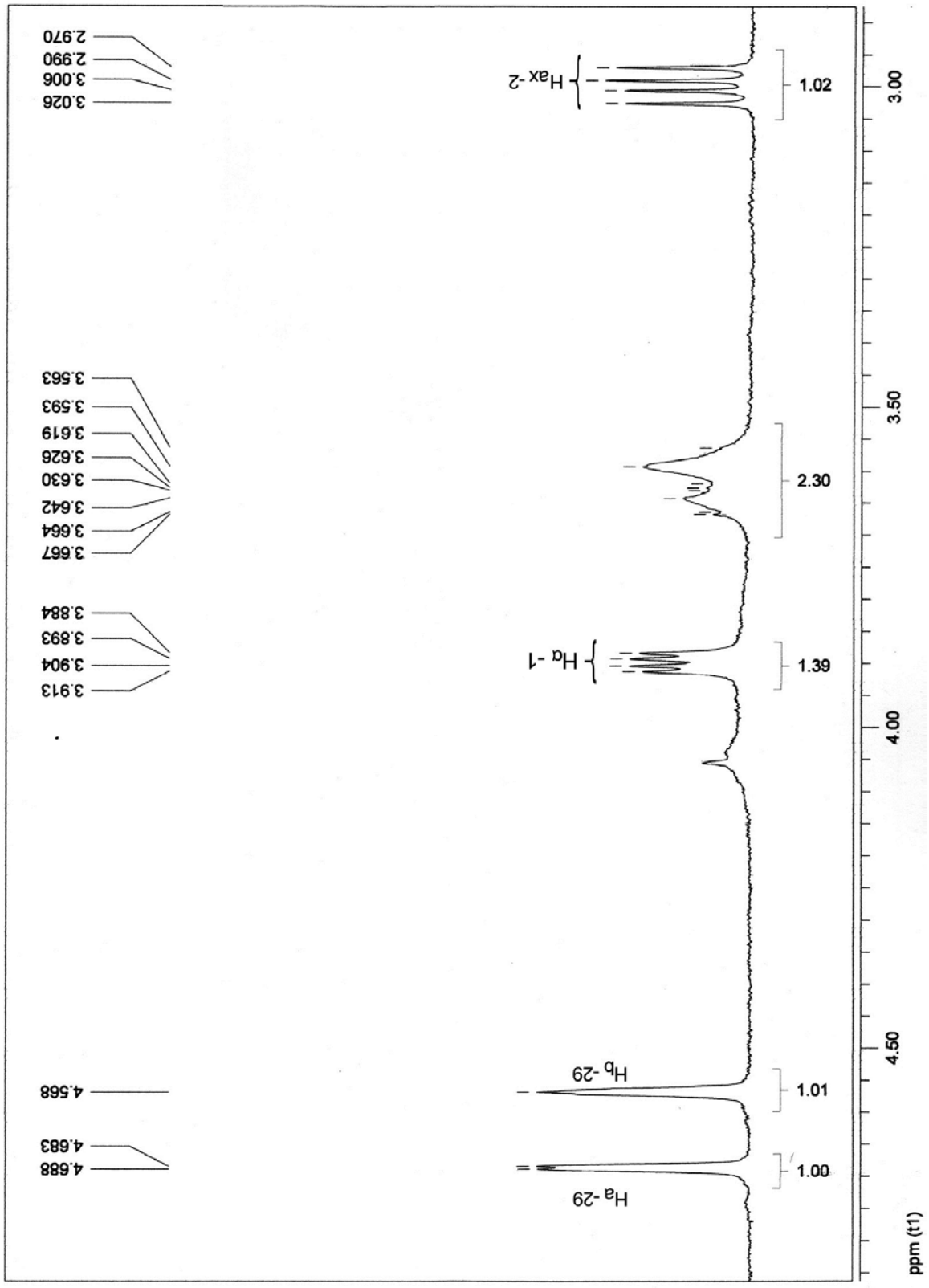


Figure -3.65: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-023 (**172**)

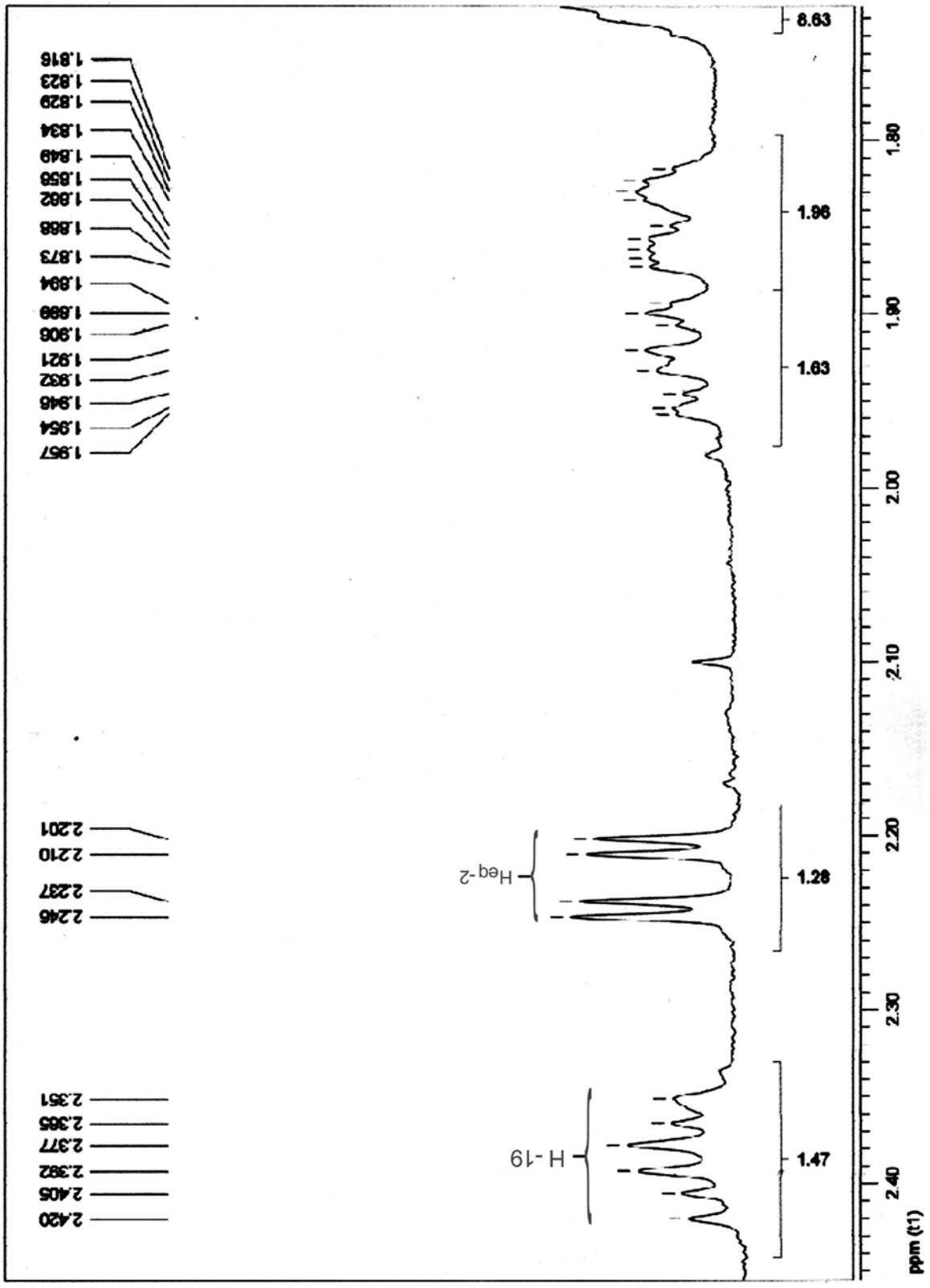


Figure -3.66: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-023 (**172**)

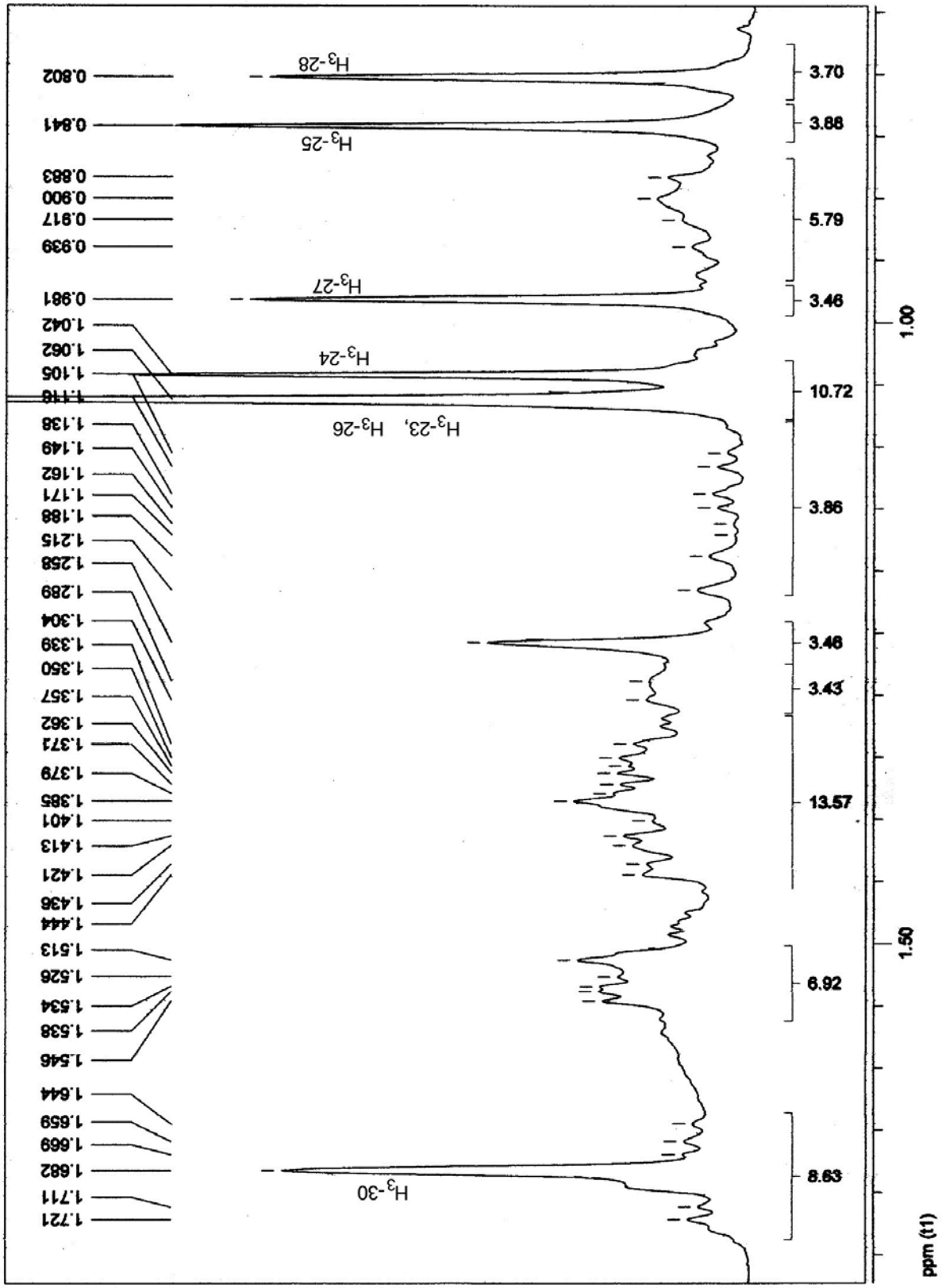


Figure -3.67: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-023 (**172**)

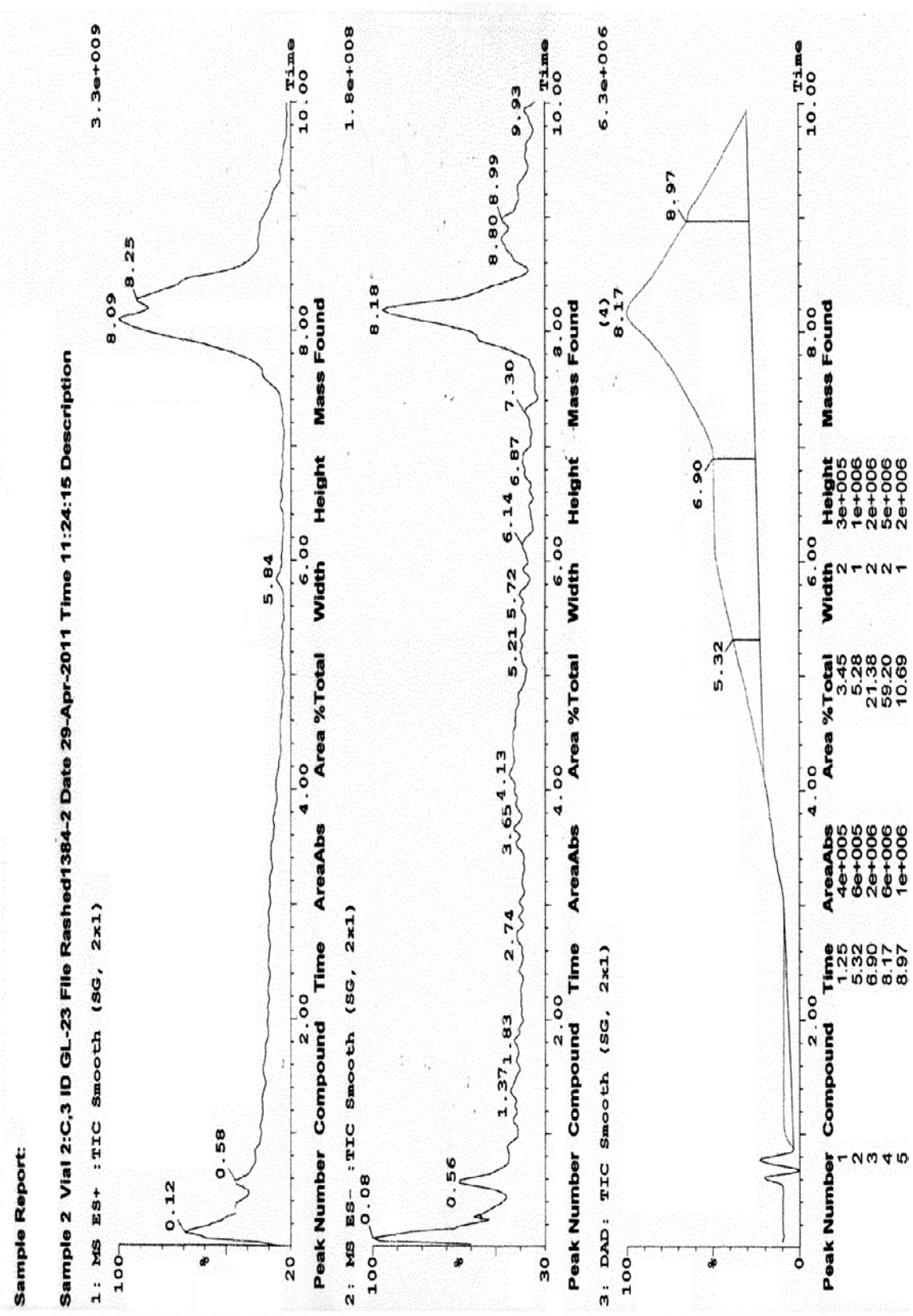


Figure 3.68: Electrospray mass spectrum of compound GL-023 (**172**

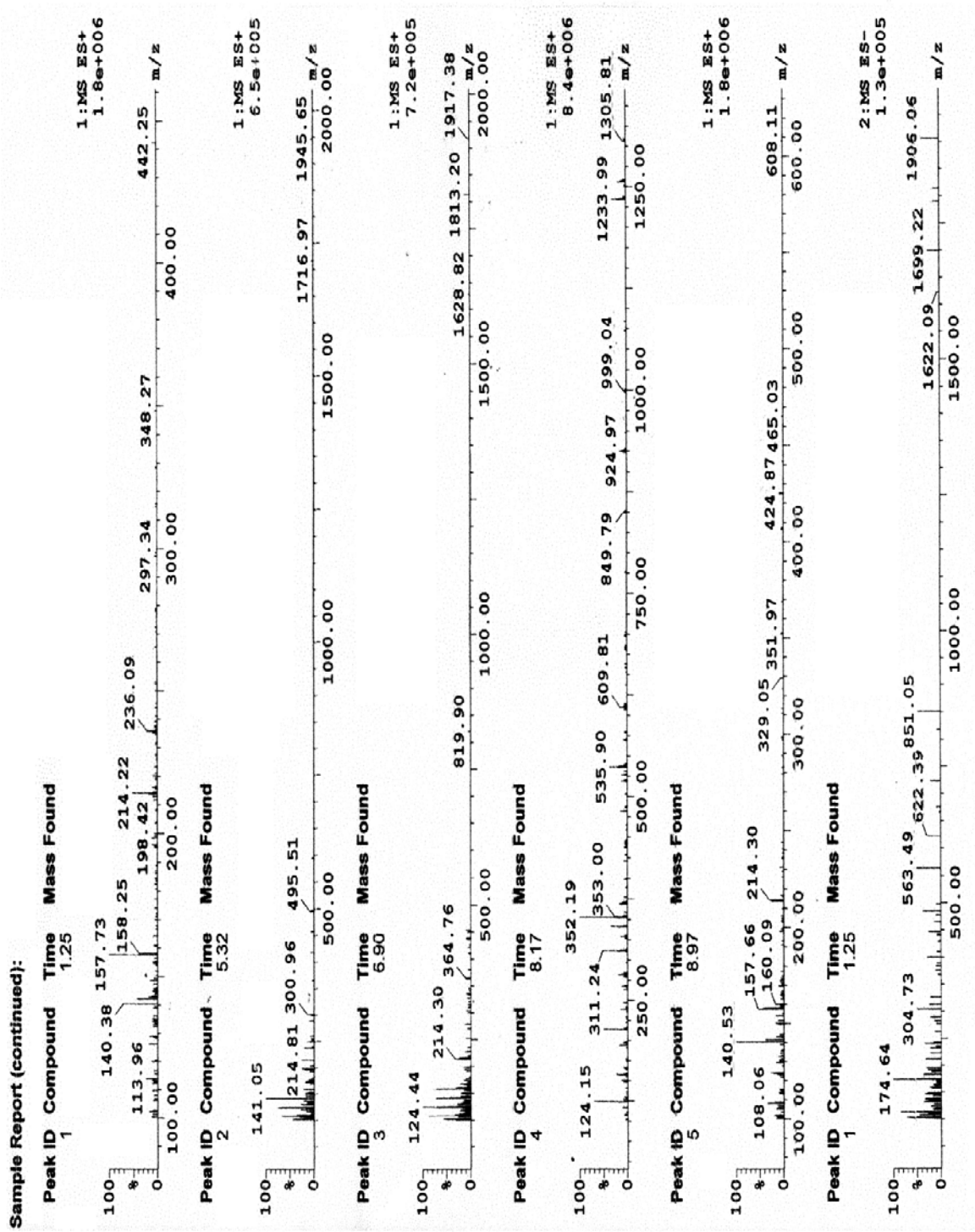


Figure 3.69: Expanded Electrospray Mass spectrum of compound GL-023 (**172**

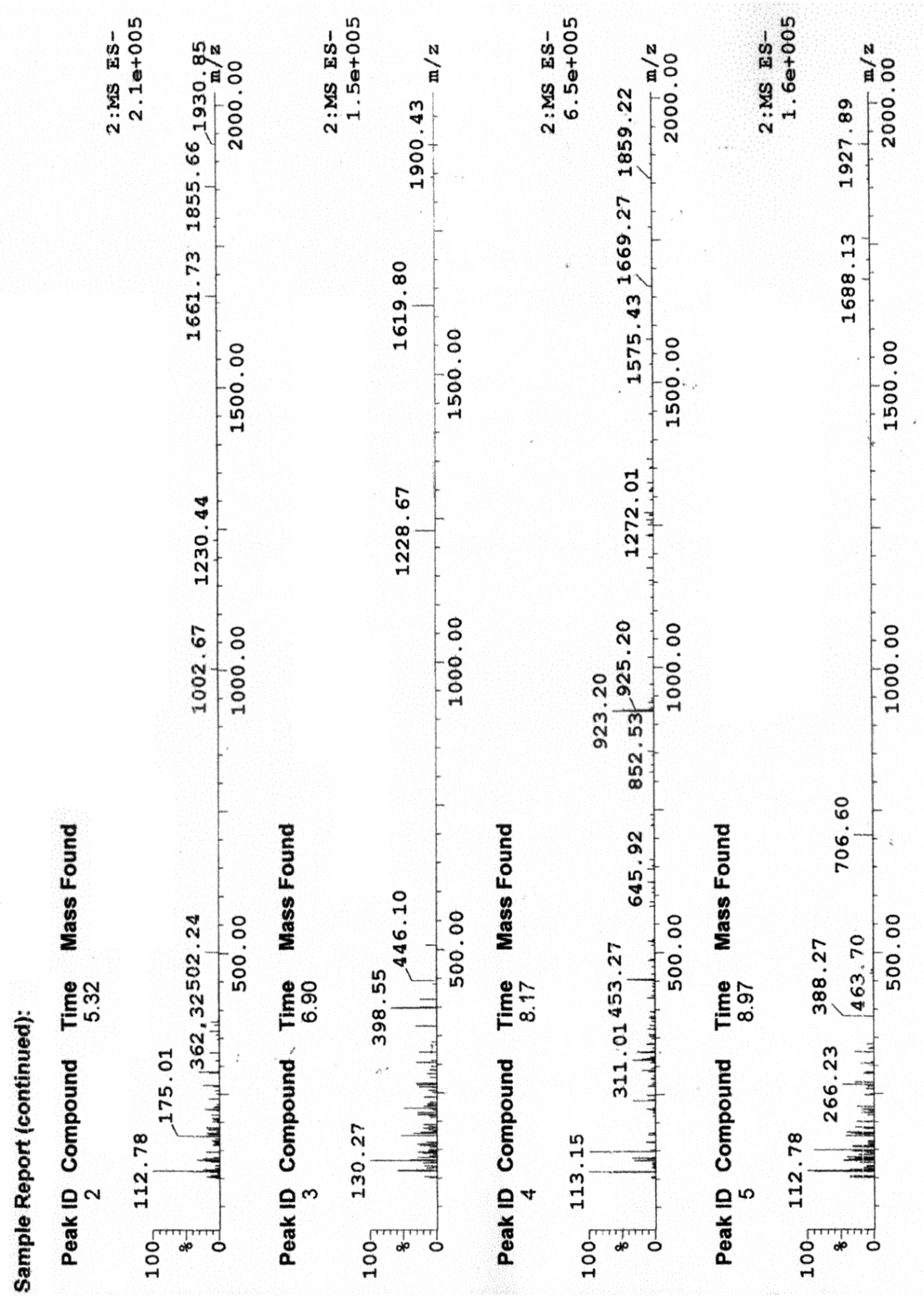


Figure 3.70: Expanded Electrospray Mass spectrum of compound GL-023 (**172**)

3.3.3 Characterization of GL-035 as Glochidone (173) an analogue of GM-029

GL-035 was isolated as oily substance from carbon tetrachloride soluble fraction of the stem bark of *G. lanceolarium* by Vacuum Liquid Chromatographic method. It appeared as a purple spot on TLC, when the developed plate was sprayed with vanillin-sulfuric acid followed by heating at 110˚C for 5 minutes.

The high-resolation ESI mass spectrum of GL-035 (Figure: 3.77-3.79) showed the pseudo-molecular ion peak at *m/z* 422.69 which was consistent with a molecular formula, $C_{30}H_{46}O$ for this compound.

The ^{1}H NMR (500 MHz, $CDCl_3$,) spectrum of GM-029 (Figures: 3.40-3.43) represents similar features of that reported for glochidon (Hui *et al.*, 1976; Neto *et al.*, 1995).

The ^{1}H NMR ($CDCl_3$, 500 MHz) spectrum of GL-035 (Table-3.17, Figure: 3.72-3.76) showed a doublet at δ 7.10, integrating for one proton, is indicative of H-1. A doublet at δ 5.79, d (10.0) was assigned to H-2. A doublet of triplets at δ 2.40, dt (11.0, 6.0), integrating one proton was indicative of H-19. The spectrum exhibited two doublets at δ 4.71, d (2.0) and 4.59, m was assigned to protons at C-29. The spectrum also displayed seven singlets at δ 1.07, 1.08, 1.13, 1.12, 0.96, 0.81 and 1.69 (3H each) for 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. These spectral features are in close agreement to those observed for glochidone (Hui *et al.*, 1976; Neto *et al.*, 1995).

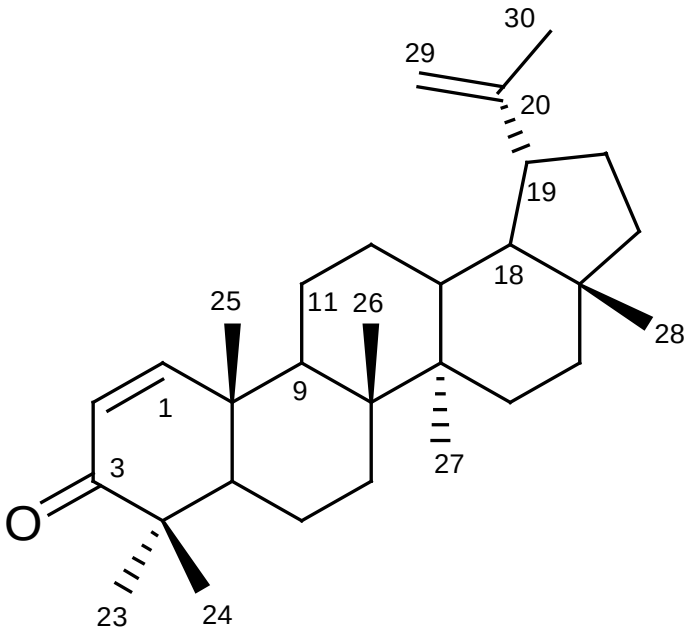


Figure 3.71: Structure of GL-035 (Glochidone, **173**)

Table-3.17: ^{1}H NMR spectral data of GL-035 and Glochidone (Hui *et al*., 1976; Neto *et al*., 1995).

Protons	**GL-035**	**Glochidone**
	δ_H, multi, *J* in Hz	**δ_H, multi, *J* in Hz**
H-1	7.10, d (10.0)	7.12, d (10.4)
H-2	5.79, d (10.0)	5.89, d (10.4)
H-19	2.40, dt (11.0, 6.0)	
H-29	4.71 H_a, d (2.0); 4.59 H_b, m	
H_3-23	1.07, s	1.07, s
H_3-24	1.08, s	1.07, s
H_3-25	1.13, s	1.11, s
H_3-26	1.12, s	1.11, s
H_3-27	0.96, s	0.96, s
H_3-28	0.81, s	0.80, s
H_3-30	1.69, s	1.68, s

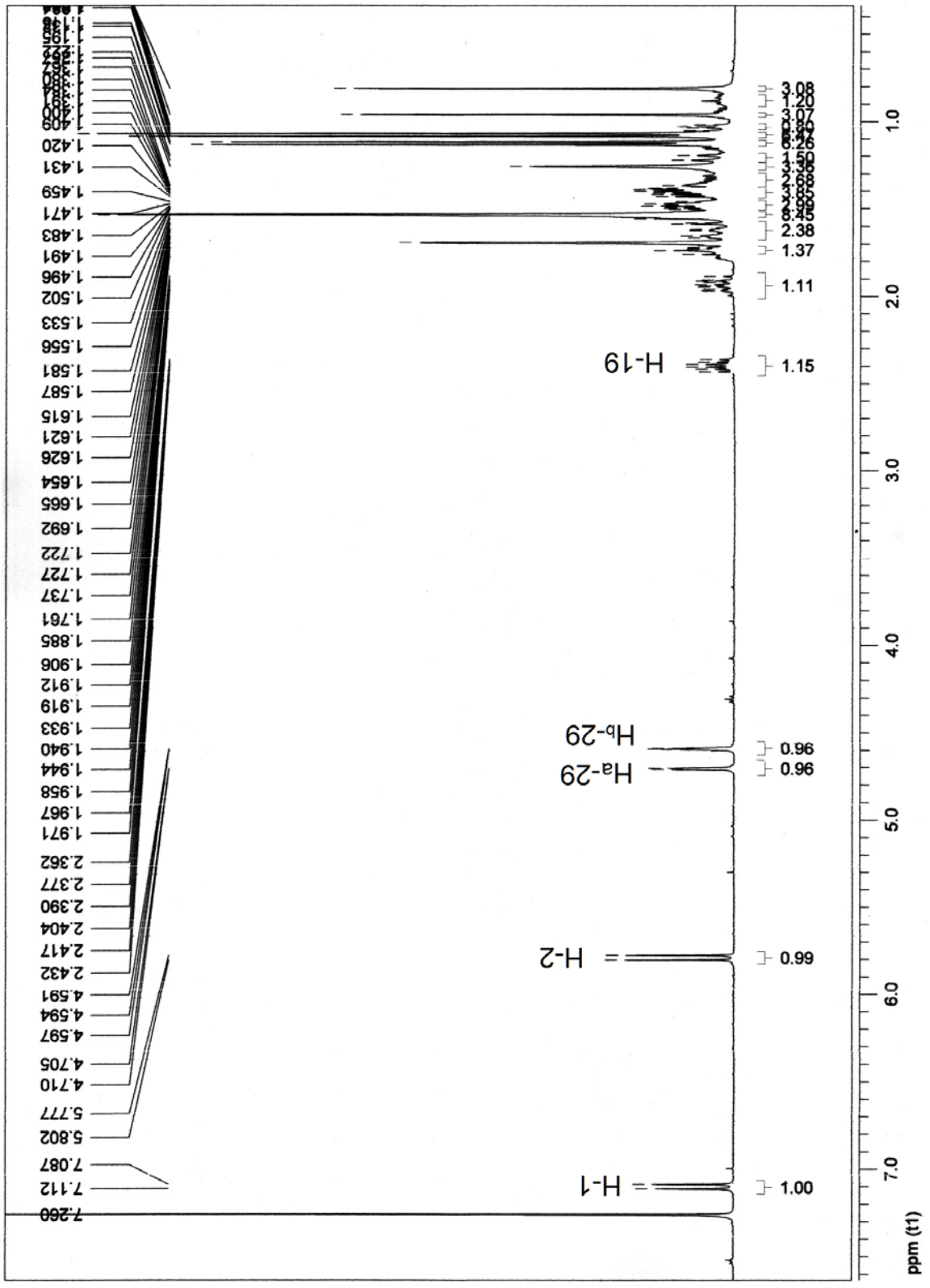


Figure-3.72: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-035 (**173**)

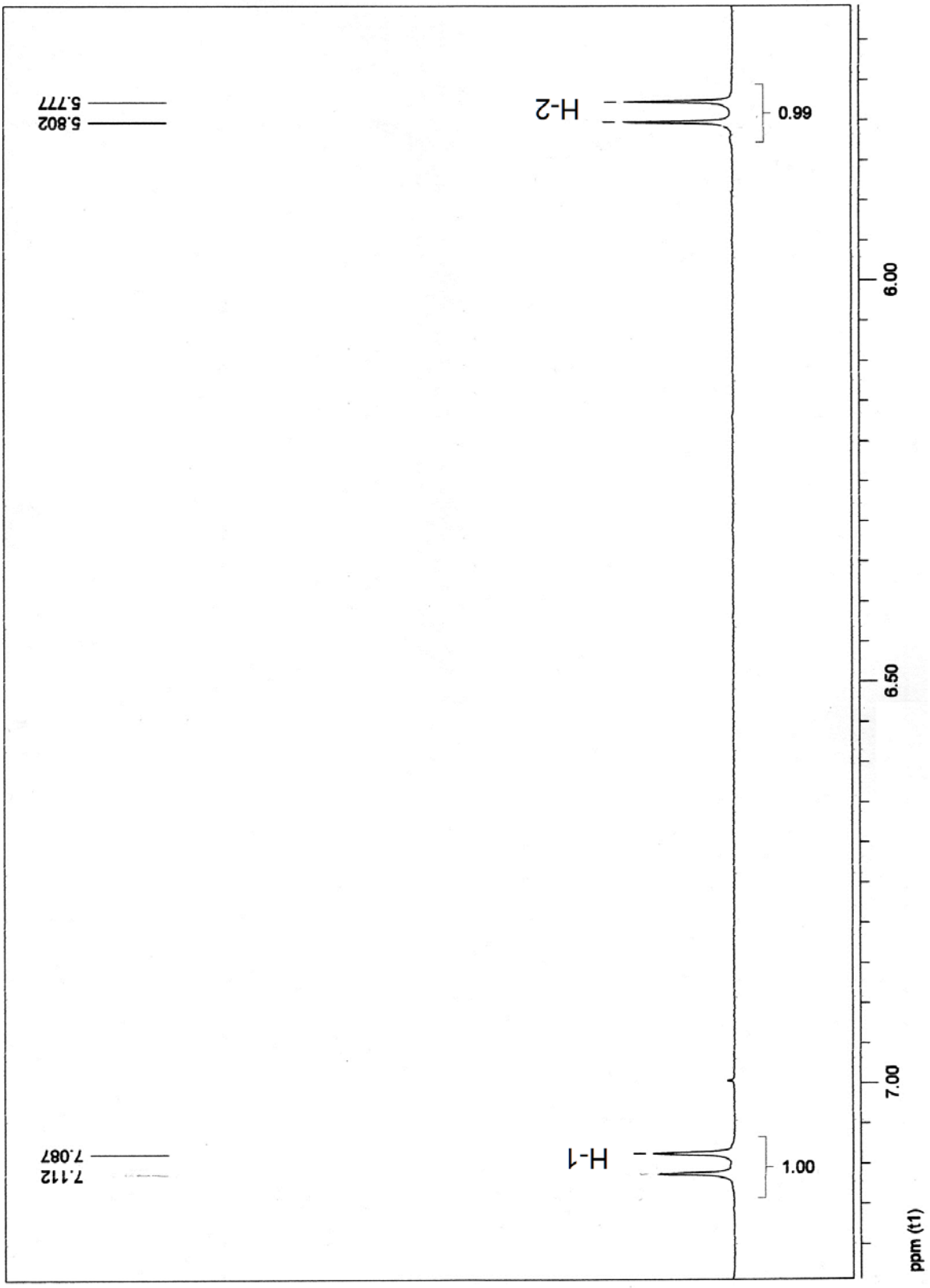


Figure -3.73: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-035 (**173**)

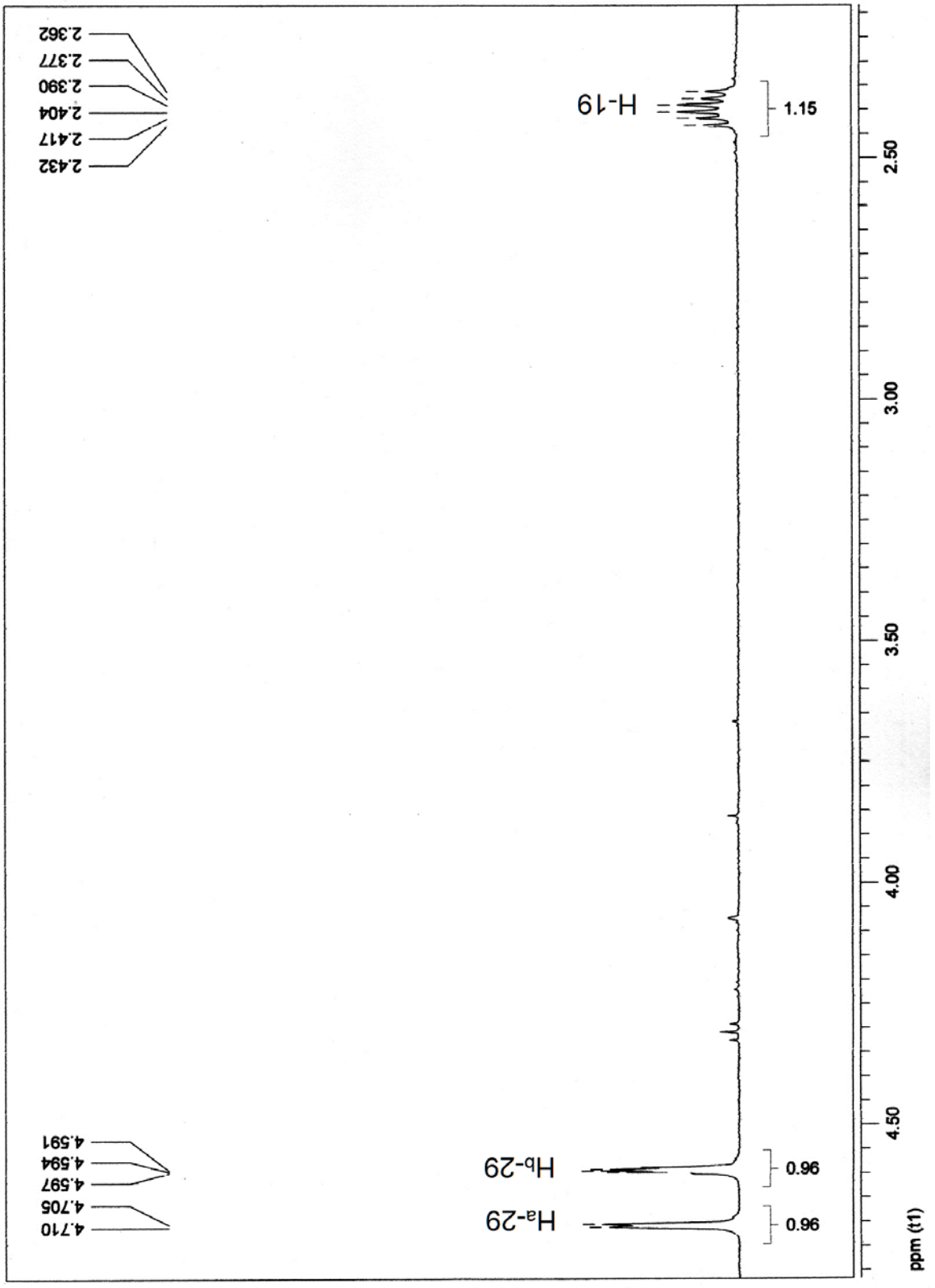


Figure -3.74: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-035 (**173**)

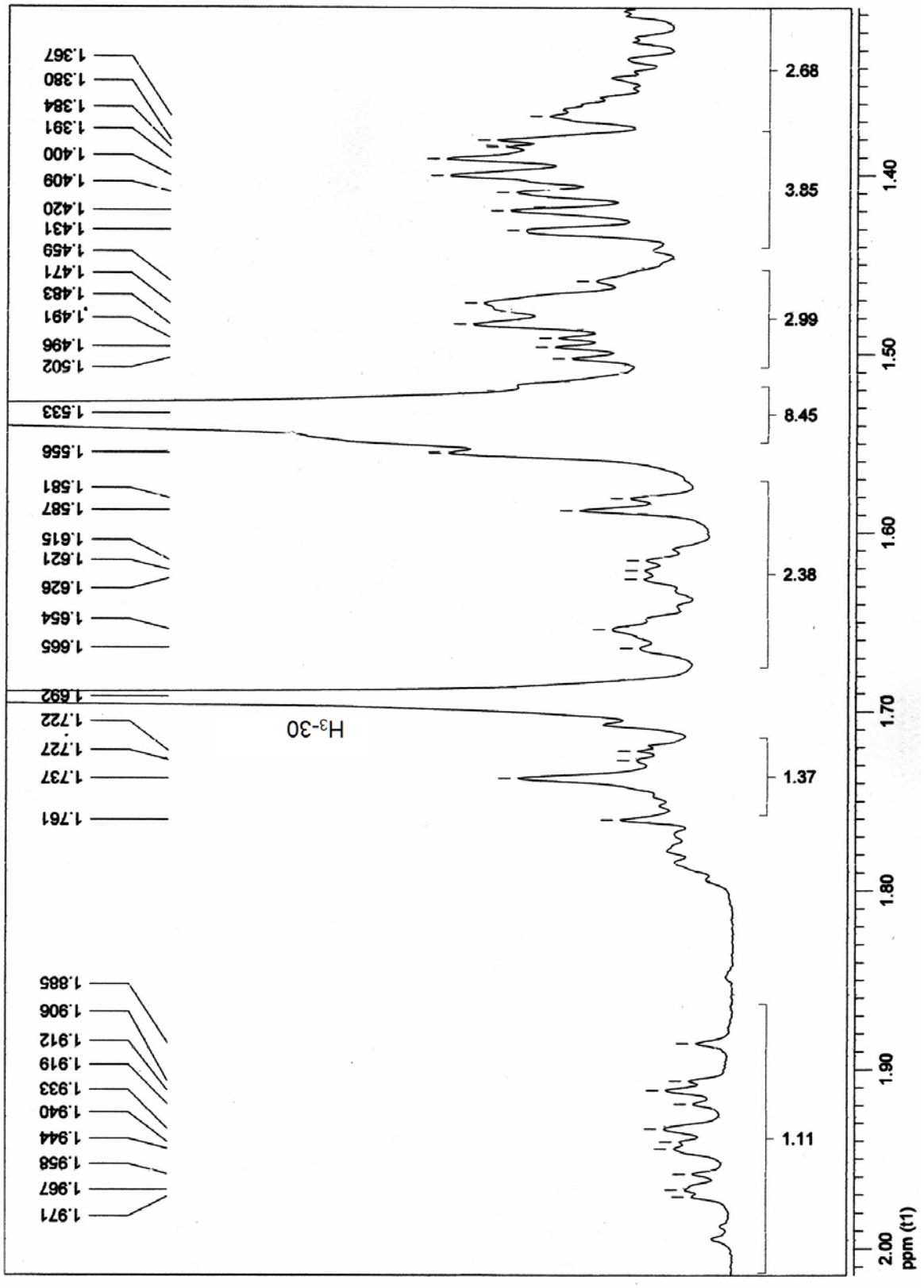


Figure -3.75: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-035 (**173**)

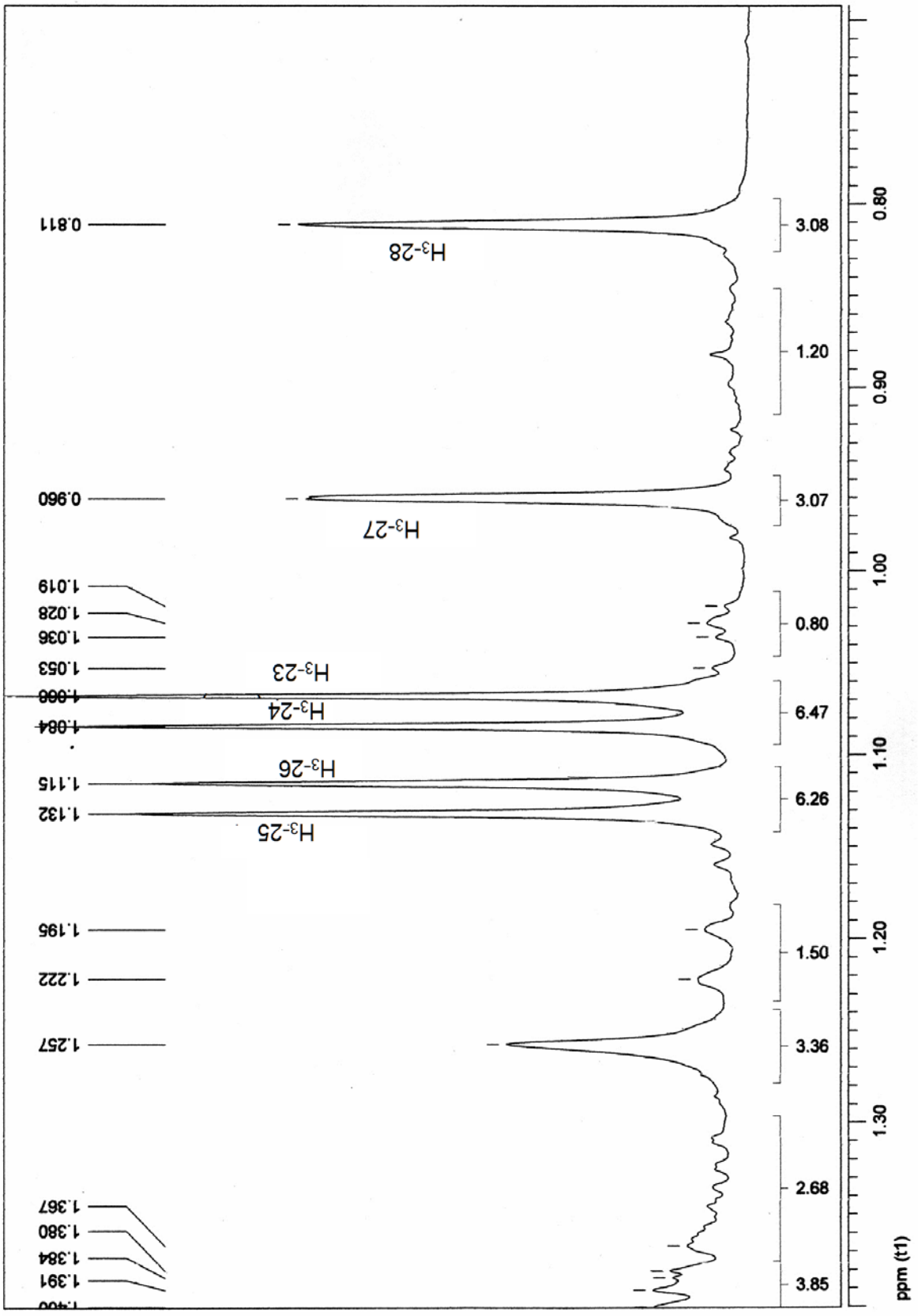


Figure -3.76: Partially expanded ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of GL-035 (**173**)

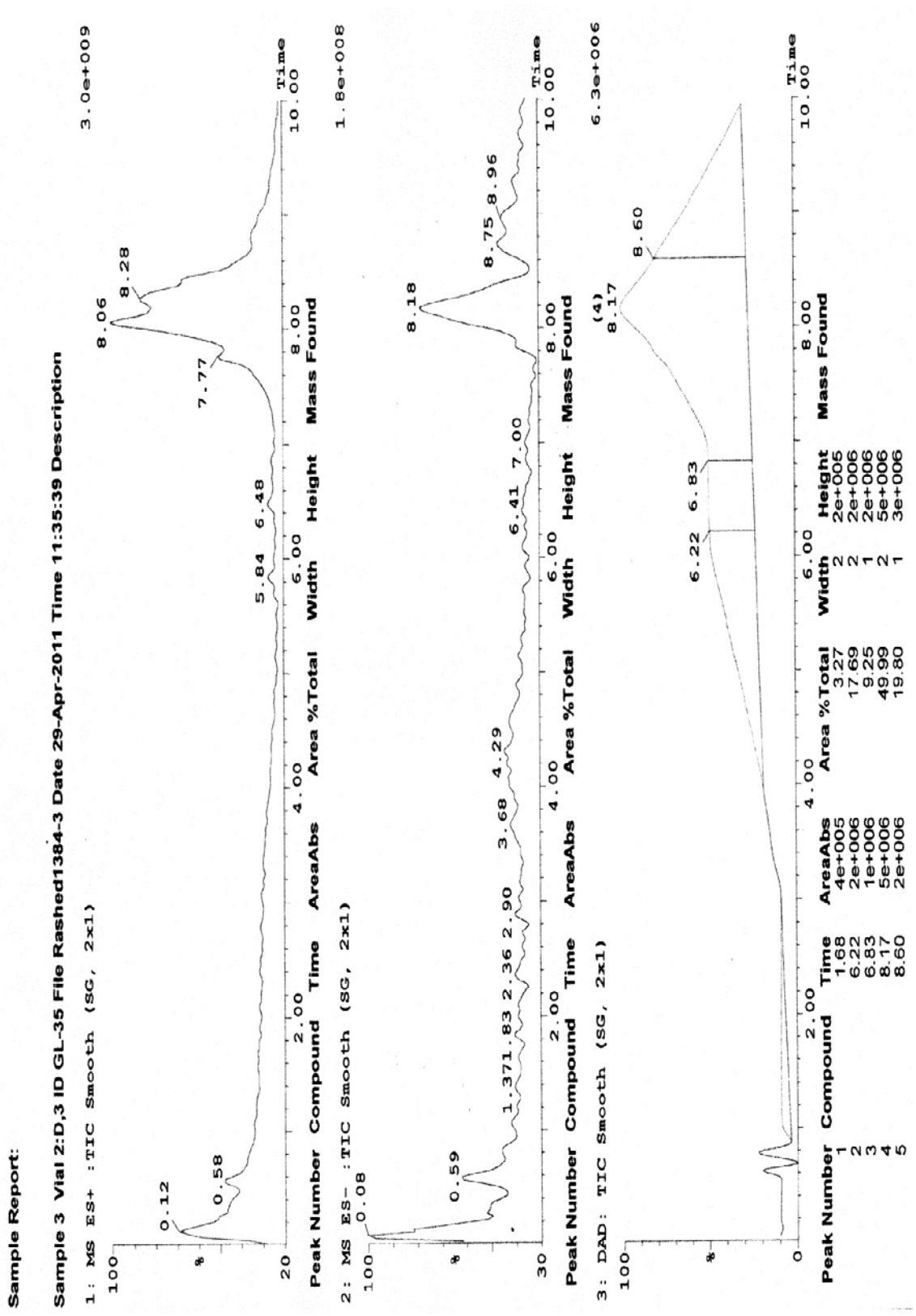


Figure 3.77: Electrospray mass spectrum of compound GL-035 (**173**)

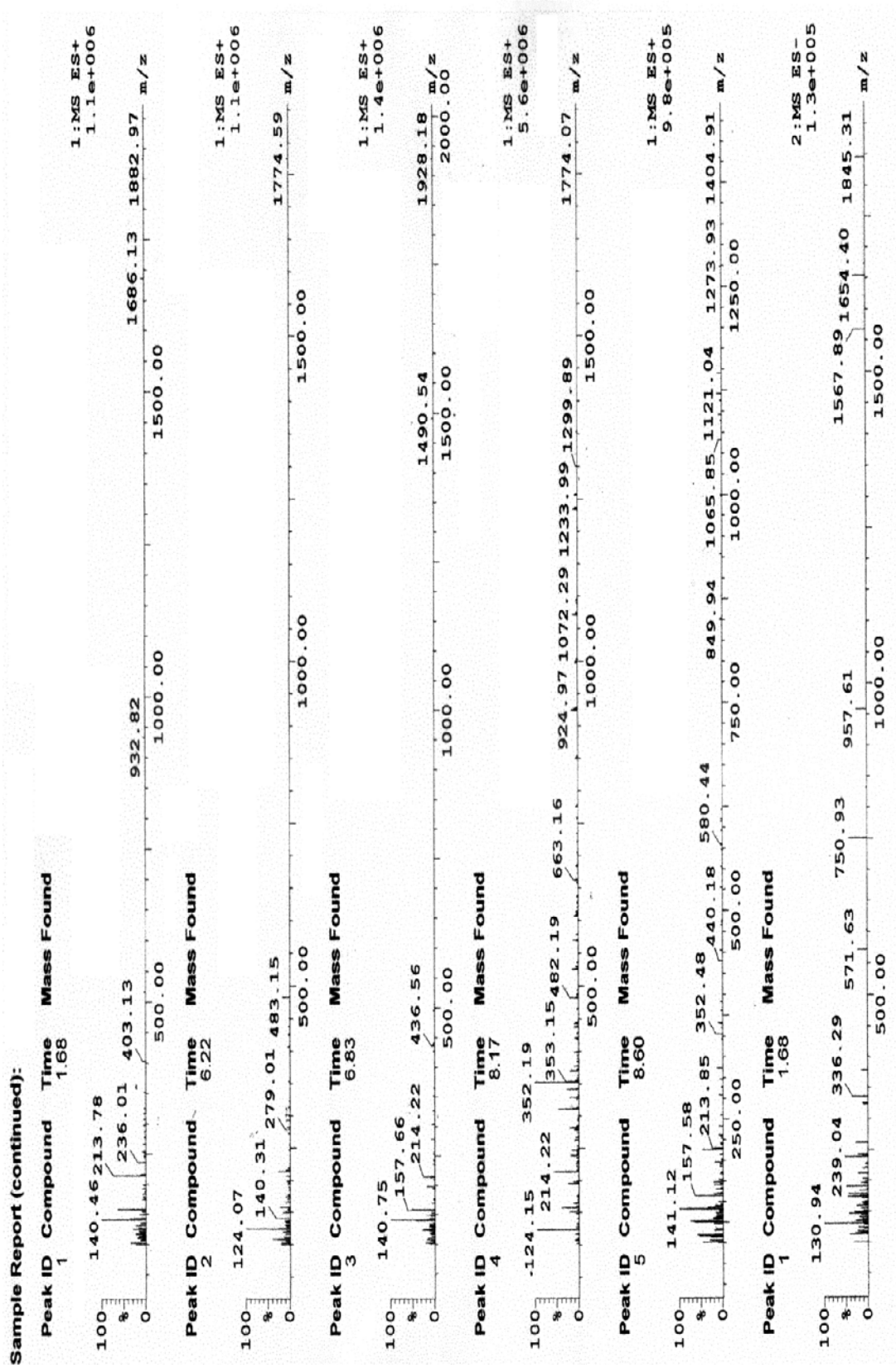


Figure 3.78: Expanded Electrospray Mass spectrum of compound GL-035 (**173**)

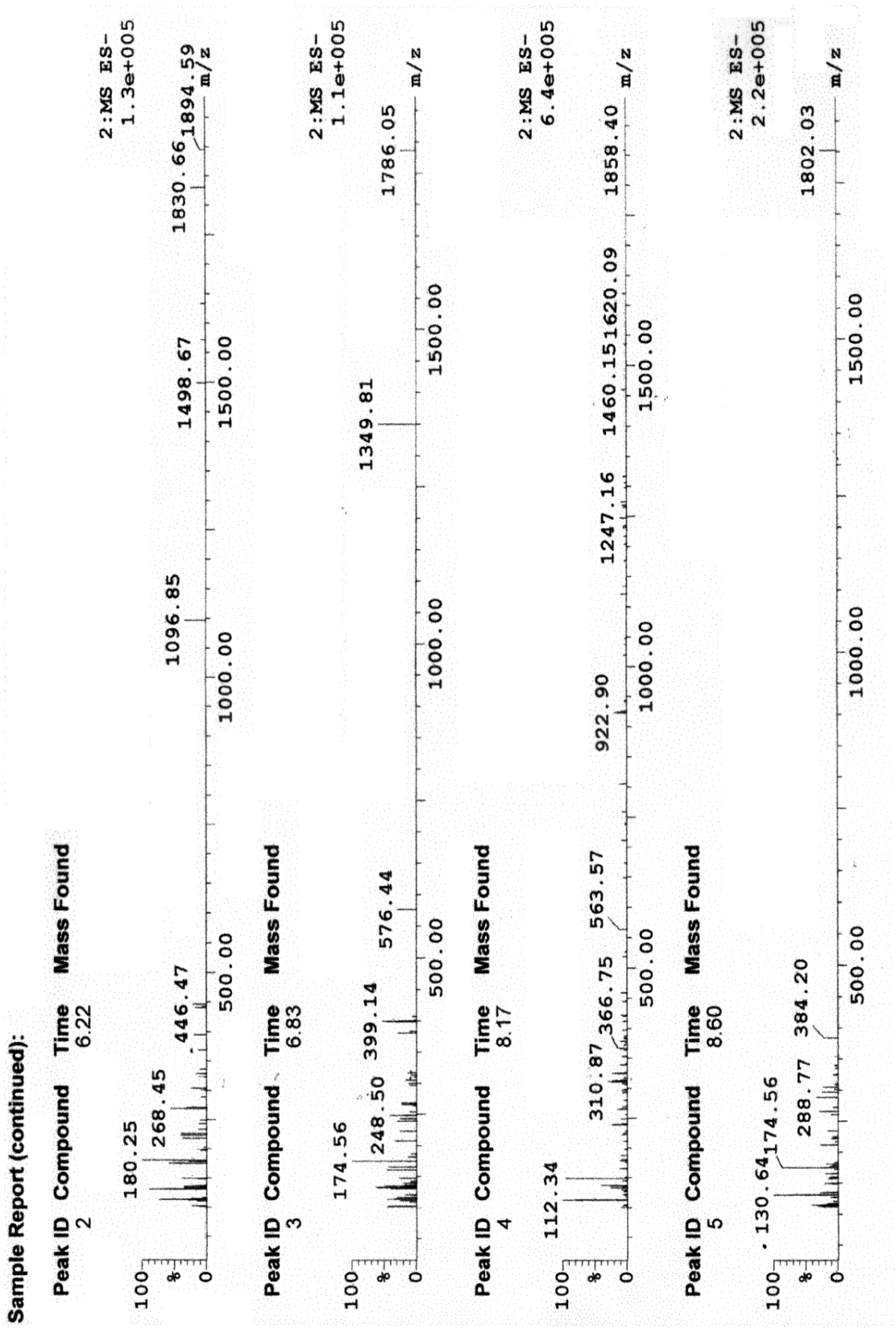


Figure 3.79: Expanded Electrospray Mass spectrum of compound GL-035 (**173**)

CHAPTER 4
Anti-cancer Activity

4.1 Introduction

Cancer is an unregulated, disorganized proliferation of cell growth. A tumor is malignant or cancerous, if it has the properties of invasiveness and metastasis. Invasiveness refers to the tendency of a tumor to enter surrounding tissue, breaking through the basal laminas that define the boundaries of the tissue, their by often entering the body's circulatory system. Metastasis refers to the tendency of a tumor to migrate to other areas of his body and establish areas of proliferation away from the side of initial appearance. Cancer is now the second leading cause of death in the world. Exposure of a cell to a carcinogen such as: certain viruses, certain chemicals or radiation leads to DNA alteration that inactivates the tumor suppressive gene or activates "**Proto-oncogene"**. Tumor suppressive genes are growth regulatory genes, which upon mutation can no longer control cell growth.

Proto-oncogenes are involved in cellular proliferation that by mutation or altered context of expression become oncogenes. The products of transforming genes cause in appropriate cell growth. Cancer may affect people at all ages, even fetuses but risk for the more common varieties tends to increase with age cancer causes about 13% of all death. Accordingly to the American cancer society 7.6% million people are being died from cancer in the world during 2007 (Parkin *et al*., 2002; Hanahan and Wdinberg , 2000; Cancer Research UK, 2007; WHO., 2007; American Cancer Society, 2007; Bedard, 2007).

4.2 Types of cancer

There are over 200 different types of cancer. Cancer can develop in any organ of body. There are over 60 different organs in the body where cancers can grow (Hanahan and Wdinberg, 2000).

Table-4.1 Types of cancers.

Types of Cancer	Description	Cause of cancer	Treatment	References
Blood cancer or leukemia	The bone marrow produces large number of abnormal white cell. It occurs due to mutation in blood forming cells & other generative tissues.	Ionizing radiation, arsenic, alkylating cytotoxic drugs, chloramphenicol & phenylbutazone are responsible for leukemia.RNA viruses can also act in causation of leukemia.	Chemotherapy, some drugs like chlorambucil, fludarabine, vincristine, doxorubicin, methotrexate and purine antagonists are used in leukemia. Steroid treatment with prednisolone (deltacortril) is also used. Radio therapy is also used.	Thonston *et al*., 2004; Kipps *et al*., 2006; Puci-H. *et al*., 2006; Liesveld and lichtman, 2006; Green *et al.*, 2004
Bone cancer	It is uncommon mesenchymal cancer that arises from bone & cartiladge or from other soft tissues of the bodies.	It is not yet clear to scientist but there are some risk factors that may put a person at increased risk .a small number of bone cancer due to heredity such as children with hereditary retinoblastoma (an uncommon cancer of the eye) are at a higher risk of developing osteosarcoma.	Chemotherapy, therapeutic agents like cisplatin, doxorubicin, ifosfamide & methotrexate are used.	Going *et al*., 2003
Brain tumor	It is an abnormal tissue in the brain. Unlike other tumors it is spread by local extension & rarely metastasizes out side the brain.		Surgery, chemotherapeutic regimen (combination approach like procarbazine, CCNU & vencristine) & radiation are used.	Moulignier and Antoine *et al*., 1994
Breast cancer	It is the most common malignancy in the women of the western world	The two most important risk factors have been identified Particularly first degree relatives were affected & the age at first successful pregnancy. The second factor is genetic, ionization radiation, obesity, estrogens, alcohol consumption early menarche or late menopause.	Surgeries, radiotherapy, chemotherapy, hormonal therapy, herceptin, are used. Adjuvant chemotherapy (tamoxifen, cyclophosphamide, methotrexate & 5-fluorouracil) Is also used.	Holmberg and Anderson *et al*., 2004; Hansen, 2004; Mahady *et al*.,2002; Yah[a] and Sellick[b], 2006; Moulies *et al*., 2008; [ab]Vogel *et al*., 2006; Chelbowski *et al*.,2006
Cervical cancer	It is disease in which the cells of the cervix become abnormal & start to grow uncontrollably forming tumors.	Infection with the common human papillomavirus (HPV) is a cause of approximately 90% of all cervical cancer.	Chemotherapy, Surgery, or radiation treatments are used. Common drugs like cisplatin, ifosfamide & fluorouracil are also used. Biological therapy with interferon is the other popular therapeutic application.	[ab]Lowy and Schiller, 2006; [abcd]Canavan and Doshi, 2006

Colon & colorectal cancer	Pancreatic, gastric & colorectal cancers are major cause's morbidity & mortality world wide.		Chemotherapy, Surgery, radiation therapy & immunotherapy are four standard colon cancer treatments. Several novel agents such as capecitabine, oxaliplatin & irinotecan either alone or in combination have proved to be effective chemotherapeutic agents to be used.	Sica, 2006; Mololits *et al*., 2005; Baron *et al*., 2006; Sinha *et al*., 2005
GIT cancer	It includes cancer of the esophagus, gallbladder, liver, pancreases & stomach cancer.		Chemotherapy, Surgery, Adjuvant chemotherapy. The chemotherapeutic agents are BCL – XL VFGF, gemcitabine capecitabine, oxaliplatin & irinotecan.	Eduardo diaz-Rubio, 2005; Zhu *et al*., 2005
Liver Cancer	Cancerous (malignant) tumors of the liver can be primary cancer or secondary met static cancer. Primary cancer starts in the liver it self while secondary cancer starts in another part of the body & spread to the liver.	Cirrhosis of the liver is the most frequent cause of this type of cancer. Infection with either the hepatitis B or C virus can lead to liver cancer. Aflatoxin (a poison) is an important cause of hepatoma.	Surgery is done by lobectomy liver transplantation & tumor ablation**.** Radiotherapy, cryosurgery & chemoembolisation are the other treatment for liver cancer.	Fruix and Sherman, 2005; Larsson and Wolk, 2007
Lung Cancer	Cancer that arises in pulmonary system.	Cigarette smoking, asbestos have a higher risk of lung cancer.	Chemotherapy, Surgery, radiation therapy, drugs used to treat lung cancer mitomycin, eyefosfamide & cisplatin.	Gorlova, 2007; [ab]Catelinois *et al*., 2007
Ovarian cancer	Cancer that generates in tissue of the ovary.	Uses of oral contraceptives estrogen replacement & menopausal hormone are the high risk factors foe this cancer.	Chemotherapy, Surgery, radiation therapy, immunotherapy & vaccine therapy.	Brinton *et al*., 2005
Skin Cancer	It occurs in the soft epithelial tissue of the body.	UV light from the sun is the main cause of skin cancer. The regular use of sun lamps & sun beds increased the risk of developing skin cancer. other rare possible causes are over exposure to certain chemicals at work, including coal tar, soot, pitch, asphalt, waxes, hair dyes, cutting oils & arsenic	Surgery, cry therapy & radiotherapy are also used as effective treatment in skin cancer. Vindesine, doxorubicin, cyclophosphamide are the common agent often used to treat this cancer.	Grant *et al*., 2005

4.3 Currently used chemotherapeutic drugs & their uses

Table-4.2 Some chemotherapeutic drugs &their uses

Generic name	Brand name	uses
Paclitaxel	Taxol	Ovarian cancer
Tamoxifen	Nolvadex	Breast cancer
Prednisone	Metricorten	Acute leukemia
Streptozocin	Zanosar	Islet cell carcinoma
Asparaginase	Elspar	Lymphocytic leukemia
Decarbazine	DTIC-Dome	Hodgkin's Disease
Ifosfamide	I-fex	Germ cell Testicular
Levamisole	Ergamisol	Colon cancer
Plicamycin	Mithracin	Testicular cancer

4.4 Antineoplastic activity of different crude extracts

4.4.1 Introduction

An essential part of drug development is the testing of potential new compounds against animal tumors both in *vitro* and in *vivo*. In *vitro* tests determine whether the compound has any effect against neoplasm or not and in *vivo* tests determine dose response curves on animals bearing transplanted tumor. This gives an indication of the effects of the new drugs not only on the tumor but also on the host, thereby indicating its toxicity and therapeutic index.

4.4.2 Instruments and Apparatus

Hemometer

The hemoglobin concentration was measured with Hellige Sahli's Hemometer No. 304-B Hellige, USA.

Microscope

Samples on the haemocytometer were visualized with binocular microscope with magnification of 10x, 40 x and 100x.

Haemocytometer

Tumor cells, Red blood cells, WBC were counted with the help of Haemocytometer. The subsequent cell concentration per ml was determined using the following procedure:

$$\text{Cells per ml} = \frac{\text{The average count per square} \times \text{Dilution factor}}{\text{Depth of fluid under cover slip} \times \text{Area counted}}$$

$$= \frac{\text{The average count per square} \times \text{Dilution factor}}{(0.1\text{mm}) \times (1\text{mm})^2}$$

$$= \frac{\text{The average count per square} \times \text{Dilution factor}}{(0.1) \times (1\text{mm})^3}$$

$$= \frac{\text{The average count per square} \times \text{Dilution factor}}{(0.1) \times (1\mu\text{L})}$$

$$= \frac{\text{The average count per square} \times \text{Dilution factor}}{(0.1) \times 10^{-3}\ \text{mL}}$$

$$= \text{The average count per square} \times \text{Dilution factor} \times 10^4.$$

4.5 Experimental Animal

Swiss Albino male mice of 6-8 weeks of age, weighing 25-30 grams were collected from International Center for Diarrheal Diseases Research, Bangladesh (ICDDRB).

Mice are used as common experimental animals in biological studies primarily because they are mammals and thus share a high degree of homology with human. There are much additional benefits of using mice in laboratory research. Mice are small, inexpensive, easily maintained and can reproduce quickly.

Figure-4.1 Swiss Albino mice

4.5.1 Animal care

(i) Cage: Mice were kept in iron cages with wood dust bedding which was changed once a week.

(ii) Temperature, light and humidity: A constant room temperature of 28-30^{0}C and a controlled day length, 14 hours light and 10 hours dark were maintained in the laboratory.

(iii) Food: Pellet diet was collected from ICDDRB, Dhaka. The nutrient composition of the diet is given in the table- 4.3.

Table-4.3 The nutrient composition of the diet (Per hundred grams of diet)

Nutrient	Grams
Starch	66
Casein	20
Fat	8
Standard vitamins	2
Salt	4
Total = 100	

4.6 Experimental Tumor Model

Transplantable tumor (Ehrlich's Ascites Carcinoma) used in this thesis were obtained from Indian Institute of Chemical Biology (IICB), Calcutta 700032, West Bengle India and were maintained in Swiss Albino mice by intraperitoneal (i.p) transplantation at the laboratory of the Department of Applied Chemistry & Chemical Engineering, University of Rajshahi, Rajshahi, Bangladesh.

4.6.1 Ehrlich's Ascites Carcinoma (EAC) cell line

In 1970, Ehrlich located these tumors in the mammary gland of a white mouse and thus the tumor was named after him. The present form Ehrlich ascites carcinoma cell has been developed by Loewenthal and John from one of the several lines of carcinoma. The later arises from spontaneous epithelial tumors probably of mammary gland origin (Satosker and Bhandarker, 1993).

External surface of EAC cell is covered with a thin cell membrane as revealed by the electron microscopic examination of ultra thin section. The membrane matrix of Ehrlich

ascites tumor cells are specially strong (Donald *et al*., 1960). Extensive studies on the morphology of normal and cancer cells have shown that both the surface and intracellular membranes have 'unit membrane' structure, a biomolecular lipid leaflet lined on both sides of protein and or polysaccharide material (Pachiro *et al*., 1925).

EAC cell being a cancer cell also possess the same type of structure. Tumor can be grown subcutaneously as solid form, but the present ascitic form is produced by infecting tumor cell suspension into the mouse peritoneal cavity. The ascitic tumor develops as a milky white fluid containing rounded tumor cells. One million of tumor cells multiply to yield about 25-100 million tumor cells/ml. Host survives for 14-30 days (Satosker and Bhandarker, 1993; Donald *et al*., 1960; Pachiro *et al*., 1925).

4.7 Anti-cancer activity test of *Polyalthia simiarum*

4.7.1 Methods

4.7.2 Chemicals

Sodium chloride, propylene glycol, trypan blue, methyl violet, sodium sulphate, methylene blue was collected from Merck Limited, Mumbai, India. All other chemicals and reagents used were of highest analytical grade.

4.7.3 Preparation of extracts

The air dried and powdered plant material (700 g) was extracted in a Soxhlet apparatus with ethyl acetate (60-80^{0}C). The extract was filtered through a fresh cotton plug followed by Whatman no.1 filter paper. The filtrates were then concentrated with a Buchii rotavapor at low temperature and pressure to afford ethyl acetate extract (EA, 2.5g).

4.7.4 Animal

Swiss albino mice (25-30g) and Wistar rats (175-250 g) of both sexes were used for assessing the biological activity. The animals were maintained under standard laboratory conditions and

had free access to food and water *ad libitum.* The animals were allowed to acclimatize to the environment for 7 days prior to experimental session. The animals were divided into different groups, each consisting of five animals which were fasted overnight prior to the experiments. Experiments on animals were performed in accordance with guidelines of the Institutional Animal Ethics Committee, Department of Applied Chemistry & Chemical Engineering, University of Rajshahi, Rajshahi, Bangladesh.

4.7.5 Acute toxicity

The acute oral toxicity of plant in male Swiss albino mice was studied as per established protocol (Lorke 1983).

4.7.6 *In vivo* antitumor activity

4.7.6.1 Transplantation of tumor

The EAC cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation of 2×10^6 cells per mouse after every 10 days. Ascitic fluid was drawn out from EAC tumor bearing mouse at the log phase (days 7–8 of cell implantation) of the tumor cells. Each animal received 0.1 ml of tumor cell suspension containing 2×10^6 tumor cells intraperitoneally.

4.7.6.2 Treatment schedule

60 Swiss albino mice were divided into five groups (n = 12) and given food and water *ad libitum*. All the animals in each groups except Group-I received EAC cells (2×10^6 cells/mouse i.p.). This was taken as day '0'. Group-I served as normal saline control (5 ml/kg i.p.) and Group-II served as EAC control. After 24-h of EAC transplantation, Group-III and Group-IV received EA extract of *P. simiarum* stem bark at 50 mg/kg and 25 mg/kg i.p. for nine consecutive days, respectively. Group-V received reference drug Bleomycin (0.3 mg/kg i.p) for nine consecutive days (Rana and Khanam, 2002). In this experiment Bleomycin used as positive control. After 24 hours of last dose and 18 h of fasting, 6 animals from each group were sacrificed by cervical dislocation to measure antitumor and hematological parameters and the rest were kept with food and water *ad libitum* to check percentage increase in life span of the tumor bearing host. The antitumor

activity of the extract of *P. simiarum* was measured in EAC animals with respect to the following parameters.

4.7.6.3 Determination of tumor volume and weight

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and weighed immediately.

Figure-4.2 Tumor weight measurement

4.7.6.4 Tumor cell count

The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer's counting chamber and the numbers of cells in the 64 small squares were counted.

4.7.6.5 Viable/nonviable tumor cell count

The viability and non viability of the cells were checked by trypan blue assay. The cells were stained with trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those that took the dye were nonviable. These viable and nonviable cells were counted using the following equation:

Cell count = (Number of cells × dilution factor)/ (Area × thickness of liquid film)

4.7.6.6 Determination of median survival time and percentage increase in life span

The mortality was monitored by recording percentage increase in life span (% ILS) and median survival time (MST) (Sur and Ganguly, 1994).

4.7.6.7 Body weight

Body weights of the experimental mice were recorded both in the treated and control group at the beginning of the experiment (day 0) and sequentially on every 5^{th} day during the treatment period.

4.7.6.8 Hematological parameters

Collected blood was used for the estimation of hemoglobin (Hb) content, red blood cell (RBC) and white blood cell count (Armour *et al*., 1965).

4.7.6.9 Statistical analysis

All data are expressed as mean ± S.E.M. (n = 6 mice per groups). Statistical significance (p) calculated by Student's t test. $P<0.001$ and <0.05 were considered to be statistically significant.

4.8 Results

4.8.1 Phytochemical screening

The phytoconstituents present in the EA extract of *P.simiarum* were identified by various chemical tests which showed the presence of alkaloids, terpenoids, phenolic and flavonoid compounds and steroids (table- 4.4).

4.8.2 Acute toxicity studies

The acute toxicity study was conducted to establish the therapeutic index, i.e., the ratio between the pharmacologically effective dose and the lethal dose on the same strain and species. The extracts of *P. simiarum* were safe up to a dose of 1000 mg/kg (p.o.) body weight. Behavior of the animals was closely observed for the first 3h then at an interval of every 4h during the next 48h. The extract did not cause mortality in mice and rats during 48h of observation but little behavioral changes, locomotors ataxia, diarrhea and

weight loss were observed. Food and water intake had no significant difference among the group studied.

4.8.3 Tumor growth and survival parameters

Antitumor activity of extract against EAC tumor bearing mice was assessed by the parameters such as tumor volume, tumor weight, cell count (viable and non viable), mean survival time and % increase of life span. The results are shown in table 4.5. The tumor volume, tumor weight and viable cell count were found to be significantly ($p<0.001$) increased and non-viable cell count was significantly ($p<0.001$) low in EAC control animals when compared with normal control animals. Administration of EA extract at dose of 50mg/kg and 25mg/kg significantly ($p<0.05$) decreased the tumor volume, tumor weight and viable cell count. Furthermore, the median survival time was increased to 27 ± 0.40 and 25± 0.57 (% ILS = 22.12 and 13.63) on administration of crude extract at 50 mg/kg and 25mg/kg, respectively. All these results clearly indicate that the EA extract had a capacity to inhibit the growth of solid tumor induced by EAC cell line in experimental animals.

4.8.4 Hematological parameters

Hematological parameters (Table-4.6) of tumor bearing mice on 14 day were found to be significantly altered compared to the normal group. The total WBC count was found to be increased with a reduction of Hb content of RBC. The total number of RBC showed a modest change. At the same time the EA extract at 50 mg/kg and 25mg/kg b.wt. restored all the altered hematological parameters to almost close to normal.

Table-4.4 Result of chemical group tests of the EA extract of *Polyalthia simiarum*.

Extract	Triterpene	Diterpene	Flavonoid	Phenol	Sterol	Alkaloid
Polyalthia simiarum(EA)	+	+++	+++	++	+++	++

EA: Ethyl acetate extract; (+): Present; (-): Absent; (+ + +): Reaction intensity is high; (+ +): Reaction intensity is medium; (+): Reaction intensity is normal.

Table-4.5 Effect of the EA extract of *Polyalthia simiarum* on tumor volume, tumor weight, mean survival time (MST), percentage increase life span (% ILS), viable and non-viable tumor cell count in EAC bearing mice.

Parameter	EAC control	EA extract (50mg/Kg b.wt.)	E A extract (25mg/Kg b.wt.)	Bleomycin (Positive control)
Tumor volume (ml)	3.2 ± 0.21	0.79 ± 0.34*	1.70±0.028**	0.52 ± 0.21*
Tumor weight (g)	3.70 ± 0.24	1.22 ± 0.21*	2.31± 0.026**	0.51 ± 0.11*
MST (days)	22 ± 0.12	27 ± 0.40	25 ± 0.57	44.6 ± 0.12
% ILS	00.0	22.12	13.63	96.81
Viable cell (x 10^7 cell/ml)	8.1 ± 0.22	0.23 ± 0.05*	0.56 ±0.024**	0.5 ± 0.05*
Non-viablecell(x10^7 cell/ml)	0.5 ± 0.24	0.76 ± 0.54*	1.13 ±0.049**	3.3 ± 0.05*
Total cell (x 10^7 cell/ml)	8.6 ± 0.15	0.99 ± 0.21*	1.69 ±0.026**	3.8 ± 0.05*
Viable %	94.18	23.23	33.13	13.15
Non-viable %	5.82	76.76	66.86	86.85

Each point represent the mean ± SEM. (n = 6 mice per group), *p<0.05 statistically significant when compared with EAC control group.

Table-4.6 Effect of the EA extract of *Polyalthia simiarum* on hematological parameter in EAC bearing mice.

Treatment	RBC (cell x 10^3/mm^3)	WBC (cell x 10^3/mm^3)	Hemoglobin (g %)
Normal control	5.39 ± 0.12	3.92 ± 0.32	13.90 ± 3.1
EAC control	3.91 ± 0.80*	5.84 ± 0.52*	4.95 ± 1.80*
EA extract (50mg/kg)	4.83 ± 0.66**	4.89 ± 0.32**	7.93 ± 1.62**
EA extract (25mg/kg)	3.02±0.03**	3.03±0.28**	4.25±0.144**
Bleomycin (0.3 mg/kg) (Positive control)	5.18 ± 0.12**	3.15 ± 0.83**	12.89 ± 2.93**

Each point represent the mean ± SEM. (n = 6 mice per group), *p<0.001 statistically significant when compared with control group, **p<0.005 statistically significant when compared with EAC control group.

4.8.5 Discussion

In EAC tumor bearing mice, a regular rapid increase in ascetic tumor volume was observed. Ascetic fluid is the direct nutritional source for tumor cells and a rapid increase in ascetic fluid with tumor growth would be a way to meet the nutritional requirement of tumor cells (Prasad and Giri, 1994). Treatment with EA extract of *P. simiarum* reduced the intraperitonial tumor burden, thereby reducing the tumor volume, tumor weight, viable tumor cell count and increased the life span of the tumor bearing mice. The steadfast criteria for judging the potency of any anticancer drug are prolongation of life span of animals (Clarkson and Burchenal, 1965). It can therefore be inferred that the EA extract increased the life span of EAC bearing mice may be due to decrease the nutritional fluid volume and delay the cell division (Sur *et al*., 1997).

Reduction in viable cell count and increased non viable cell count towards normal in tumor host suggest antitumor effect against EAC cell in mice. These demonstrated that the EA extract have direct relationship with tumor cells as these tumor cells are absorbed the anticancer drug by direct absorption in peritoneal cavity and this anticancer agent

lysis the cells by direct cytotoxic mechanism (Kennedy *et al*., 2001). Anemia and myelosuppression have been frequently observed in ascites carcinoma (Hogland, 1982). Anemia is encountered in ascites carcinoma mainly due to iron deficiency, either by haemolytic or myelopathic conditions which finally lead to reduced RBC number (Gupta *et al*., 2007). Treatment with EA extract brought back the hemoglobin content, RBC and WBC count more or less to normal levels, thus supporting its haematopoietic protective activity without inducing myelotoxicity, the most common side effects of cancer chemotherapy.

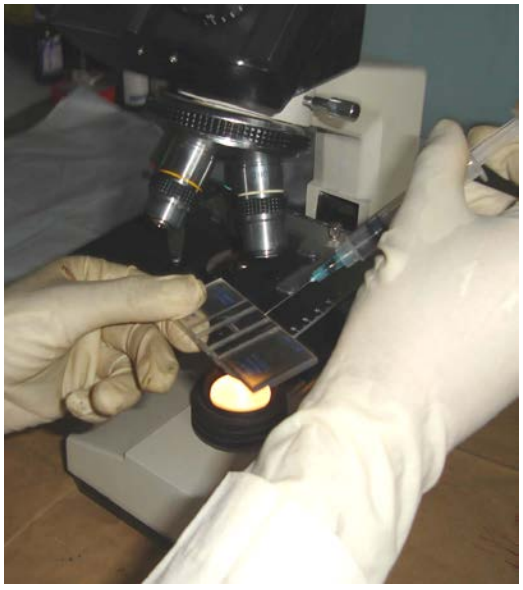


Figure-4.3 (a**)** EAC cell counting by hemocytometer

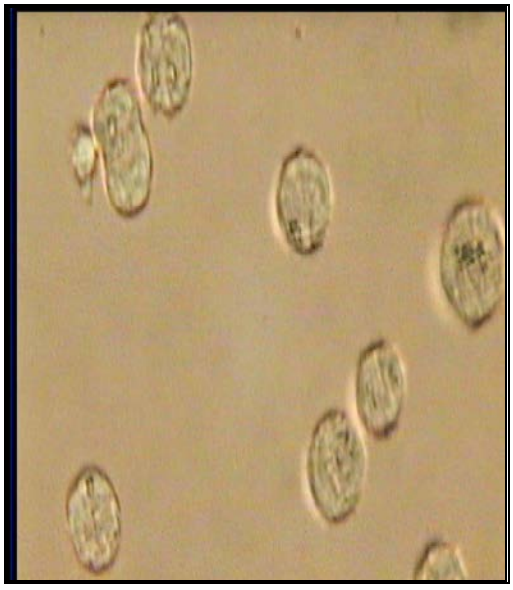


Figure-4.3 (b) EAC cell of control mice

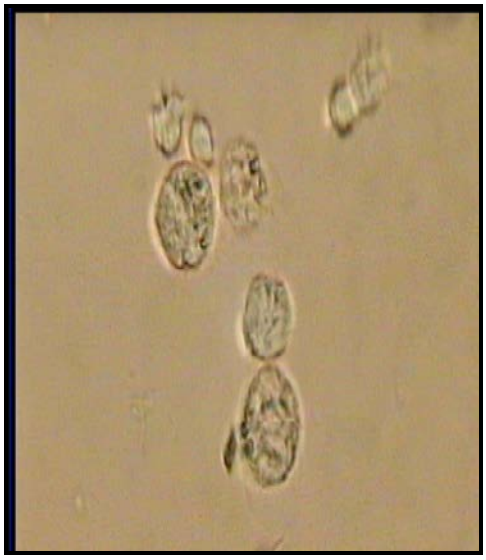
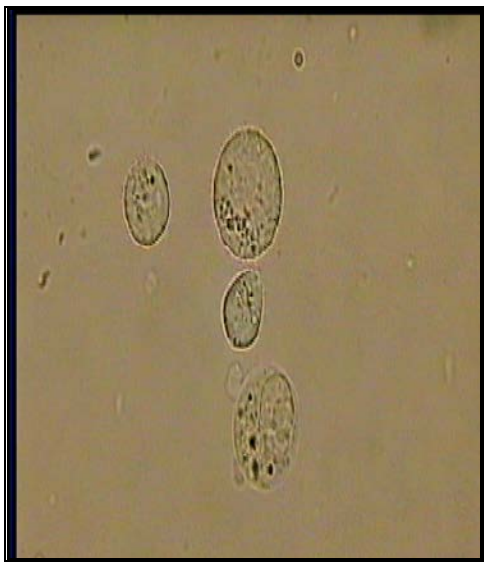


Figure-4.3 (c) EAC cell of mice treated with EA-PSB 50 mg/kg(b.wt.)



Figur-4.3 (d) EAC cell of mice treated with EA-PSB 25mg/kg(b.wt.)

Preliminary phytochemical studies indicated the presence of alkaloid, steroids, phenolic and flavonoid compounds in EA extract of *P. simiarum.* A number of scientific reports indicated that certain terpenoids, steroids and phenolic compounds and flavonoids have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis (Blois, 2002). Phytosterols are able to be incorporated into the cell membrane, alter membrane fluidity and the activity of membrane bound enzymes. They alter signal transduction in pathways leading to tumor growth and stimulate apoptosis in tumor cell lines. They also have shown to enhance *in vitro* human peripheral blood lymphocyte and T-cell proliferation in vitro, which suggests a possible stimulation of the immune system function (Jones *et al.,* 2009). The anticancer activities of EA extract of *P. simiarum* are probably due to the presence of alkaloid, phenolic compounds, flavonoids as well as terpenoids.

4.9 Conclusion

In present study, it was observed that the EA extract of *P. simiarum* exhibited anti-cancer activity significantly reduced tumor growth, viability of tumor cells, normalized the hematological profiles and increased life span as compared with those of EAC control mice. Further chemical and biological studies are underway made to isolate the bioactive compounds.

4.10 Anti-cancer activity test of *Glochidion multiloculare*

4.10.1 Methods

4.10.2 Preparation of extracts

The air dried powdered plant material (1000 g) was successively cold extracted with methanol (7 days) at room temperature with occasional shaking and stiring. The extractives were filtered through fresh cotton plug and followed by whatman no. 1 filter paper. The filtrate were then concentrated by a Buchii rotavapor at low temperature and pressure and afforded methanol extract (41.7398 g). The cold methanol extract (10 g) was subjected to Solvent-Solvent partitioning using the protocol designed by Kupchan and modified by Wagene (Vanwagenen, 1993). The extract was portioned successively with petroleum ether, carbontetrachloride and chloroform.

4.10.3 *In vivo* antitumor activity

4.10.3.1 Treatment schedule

84 Swiss albino mice were divided into seven groups (n = 12) and given food and water *ad libitum*. All the animals in each groups except Group-I received EAC cells (2×10^6 cells/mouse i.p.). This was taken as day '0'. Group-I served as normal saline control (5 ml/kg i.p.) and Group-II served as EAC control. After 24-h of EAC transplantation, Group-III, IV, V, and VI received Methanol, Pet ether, Carbontetrachloride, Chloride extracts of *G. multiloculare* stem bark at 20mg/kg i.p for nine consecutive days, respectively. Group-VII received reference drug Bleomycin (0.3 mg/kg i.p,) for nine

consecutive days (Rana and Khanam, 2002). In this experiment Bleomycin used as positive control. After 24 hours of last dose and 18 h of fasting, 6 animals from each group were sacrificed by cervical dislocation to measure antitumor and hematological parameters and the rest were kept with food and water *ad libitum* to check percentage increase in life span of the tumor bearing host. The antitumor activity of the extractives of *G. multiloculare* were measured in EAC animals with respect to the following parameters.

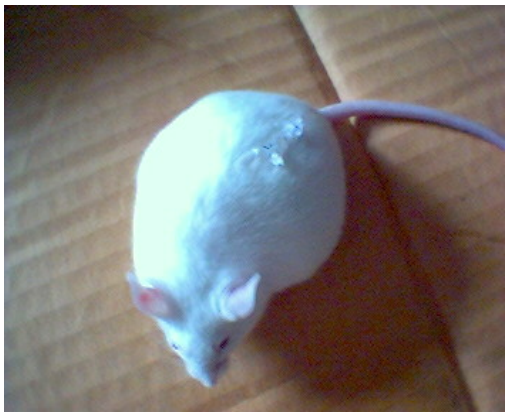


Fig-4.4 Only EAC cell bearing mice

Fig-4.5 Mice treated with Petroleum ether fraction of GM at the dose of 20 mg/kg. (6 days).

Fig-4.6 Mice treated with Carbontetrachloride fraction of GM at the dose of 20 mg/kg. (6 days).

Fig-4.7 Mice treated with Chloroform fraction of GM at the at the dose of 20 mg/kg. (6 days).

4.11 Results

4.11.1 Phytochemical screening

The phytoconstituents present in the different extractives of *G. multiloculare* were identified by various chemical tests which showed the presence of terpenoids, phenolic and steroid compounds and (table 4.7).

4.11.2 Acute toxicity studies

The acute toxicity study was conducted to establish the therapeutic index, i.e., the ratio between the pharmacologically effective dose and the lethal dose on the same strain and species. The different extractives of *G. multiloculare* were safe up to a dose of 500 mg/kg (p.o.) body weight. Behavior of the animals were closely observed for the first 3h then at an interval of every 4h during the next 48h. The extractives did not cause mortality in mice and rats during 48h of observation but little behavioral changes, locomotors ataxia, diarrhea and weight loss were observed. Food and water intake had no significant difference among the group studied.

4.11.3 Tumor growth and survival parameters

Antitumor activities of GM Methanol, GM Pet ether, GM Carbontetrachloride and GM Chloroform extracts against EAC tumor bearing mice was assessed by the parameters such as tumor volume, tumor weight, cell count (viable and non viable), mean survival time and % increase of life span. The results are shown in table -4.8. The tumor volume, tumor weight and viable cell count were found to be significantly ($p<0.001$) increased and non-viable cell count was significantly ($p<0.001$) low in EAC control animals when compared with normal control animals. Administration of the extractives at dose of 20mg/kg significantly ($p<0.05$) decreased the tumor volume, tumor weight and viable cell count. Furthermore, the median survival time was increased to 24±0.12, 26± 0.40, 21±0.12 and 27±0.42 (% ILS = 22.2, 27.2, 19.81 and 29.01) on administration of GM Methanol, GM Pet ether, GM Carbontetrachloride and GM Chloroform extracts at 20

mg/kg b.wt, respectively. All these results clearly indicate that the extractives had a capacity to inhibit the growth of solid tumor induced by EAC cell line in experimental animals.

4.11.4 Hematological parameters

Hematological parameters (Table-4.9) of tumor bearing mice on 14 day were found to be significantly altered compared to the normal group. The total WBC count was found to be increased with a reduction of Hb content of RBC. The total number of RBC showed a modest change. At the same time the extractives at 20 mg/kg b.wt. restored all the altered hematological parameters to almost close to normal.

Table-4.7 Result of chemical group tests of different extracts of *Glochidion multiloculare*

Extracts	Triterpene	Diterpene	Phenol	Sterol
Glochidion multiloculare	+	+++	++	+++

(+): Present; (-): Absent; (+ + +): Reaction intensity is high; (+ +): Reaction intensity is medium; (+): Reaction intensity is normal.

Table-4.8 Effect of the GM Methanol, GM Pet Ether, GM Carbontetrachloride, GM Chloroform extracts of *Glochidion multiloculare* on tumor volume, tumor weight, mean survival time (MST), percentage increase life span (% ILS), viable and non-viable tumor cell count in EAC bearing mice.

Parameter	**GM Methanol**	**GM Pet Ether**	**GM CCl_4**	**GM $CHCl_3$**	**EAC control**	**Bleomycin (Positive control)**
Tumor volume (ml)	0.89± 0.21	0.76±0 .34*	1.12 ± 0.21*	0.72±0.34*	3.5± 0.21	0.4± 0.21*
Tumor weight (g)	1.82 ± 0.24	1.25± 0.21*	2.01 ± 0.11*	1.22±0.21*	3.89±0.24	0.43±0.11*
MST (days)	24 ± 0.12	26±0.40	21 ± 0.12	27±0.42	20 ± 0.12	48.6± 0.12
% ILS	22.2	27.2	19.81	29.01%	00.0	94.81
Viable cell (x 10^7 cell/ml)	0.96 ± 0.22*	0.66±0.05*	1.52 ± 0.05*	0.50±0.05*	8.1 ± 0.22	0.5 ± 0.05*
Non-viable cell (x 10^7 cell/ml)	2.21 ± 0.24*	2.13± 0.54*	2.07 ± 0.05*	2.01±0.54*	0.5 ± 0.24	3.3 ± 0.05*
Total cell (x 10^7 cell/ml)	3.18 ± 0.15	2.79 ± 0.21	3.59 ± 0.05	2.51±0.21	8.6 ± 0.15	3.8 ± 0.05*
Viable %	30.51	23.66	42.34	23.31%	94.18	13.15
Non-viable %	69.49	76.34	57.66	76.84	5.82	86.85

Each point represent the mean ± SEM. (n = 6 mice per group), $^*p<0.05$ statistically significant when compared with EAC control group.

Table-4.9 Effect of the extractives of *Glochidion multiloculare* on hematological parameter in EAC bearing mice.

Treatment	RBC (cell x $10^3/mm^3$)	WBC (cell x $10^3/mm^3$)	Hemoglobin (g %)
Normal control	5.69 ± 0.12	3.92 ± 0.32	13.90 ± 3.1
EAC control	3.91 ± 0.80*	5.94 ± 0.52*	4.95 ± 1.80*
Bleomycin (0.3 mg/kg) (Positive control)	5.18 ± 0.12**	3.35 ± 0.83**	12.89 ± 2.93**
GM Methanol (20 mg/kg)	4.37 ± 0.16**	4.59 ± 0.12**	7.93 ± 1.62**
GM Pet Ether(20 mg/kg)	4.83 ± 0.61**	4.49 ± 0.39**	8.73 ± 1.01**
GM CCl_4(20 mg/kg)	4.01 ± 0.29**	4.97 ± 0.32**	7.23 ± 1.79**
GM $CHCl_3$(20 mg/kg)	4.81± 0.61**	4.44± 0.39**	8.50± 1.00**

Each point represent the mean ± SEM. (n = 6 mice per group), *p<0.05 statistically significant when compared with EAC control group.

4.11.5 Discussion

In EAC tumor bearing mice, a regular rapid increase in ascetic tumor volume was observed. Ascetic fluid is the direct nutritional source for tumor cells and a rapid increase in ascetic fluid with tumor growth would be a way to meet the nutritional requirement of tumor cells (Prasad and Giri, 1994). Treatment with different extracts of *G. multiloculare* reduced the intraperitonial tumor burden, thereby reducing the tumor volume, tumor weight, viable tumor cell count and increased the life span of the tumor bearing mice. The steadfast criteria for judging the potency of any anticancer drug are prolongation of life span of animals (Clarkson and Burchenal, 1965). It can therefore be inferred that the

extractives increased the life span of EAC bearing mice may be due to decrease the nutritional fluid volume and delay the cell division (Sur *et al*., 1997).

Reduction in viable cell count and increased non viable cell count towards normal in tumor host suggest antitumor effect against EAC cell in mice. These demonstrated that the extractives have direct relationship with tumor cells as these tumor cells are absorbed the anticancer drug by direct absorption in peritoneal cavity and this anticancer agent lysis the cells by direct cytotoxic mechanism (Kennedy *et al*., 2001). Anemia and myelosuppression have been frequently observed in ascites carcinoma (Hogland, 1982). Anemia is encountered in ascites carcinoma mainly due to iron deficiency, either by haemolytic or myelopathic conditions which finally lead to reduced RBC number (Gupta *et al*., 2007). Treatment with the extractives brought back the hemoglobin content, RBC and WBC count more or less to normal levels, thus supporting its haematopoietic protective activity without inducing myelotoxicity, the most common side effects of cancer chemotherapy.

Preliminary phytochemical studies indicated the presence of steroids, phenolic and terpenoid compounds in the extractives of *G. multiloculare.* A number of scientific reports indicated that certain terpenoids, steroids and phenolic compounds have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis (Blois, 2002). Phytosterols are able to be incorporated into the cell membrane, alter membrane fluidity and the activity of membrane bound enzymes. They alter signal transduction in pathways leading to tumor growth and stimulate apoptosis in tumor cell lines. They also have shown to enhance *in vitro* human peripheral blood lymphocyte and T-cell proliferation in vitro, which suggests a possible stimulation of the immune system function (Jones *et al.,* 2009). The anticancer activity of the extractives of *G. multiloculare* are probably due to the presence of phenolic and terpenoid compounds present in the extractives.

4.12 Conclusion

In present study, it was observed that the extractives (GM Methanol, GM Pet ether, GM Carbontetrachloride and GM Chloroform) of *G. multiloculare* exhibited anti-cancer activity as well as significantly reduced tumor growth, viability of tumor cells, normalized the hematological profiles and increased life span as compared with those of EAC control mice.Among these extractives GM Pet ether and GM Chloroform extracts have shown almost same highest anti-cancer activity whereas GM Methanol and GM Carbon tetrachloride exhibited moderate activity. Further chemical and biological studies are underway made to isolate the bioactive compounds.

4.13 Anti-cancer activity test of *Glochidion lanceolarium*

4.13.1 Methods

4.13.2 Preparation of extracts

The air dried powdered plant material (900 g) was successively cold extracted with methanol (7 days) at room temperature with occasional shaking and stiring. The extractives were filtered through fresh cotton plug and followed by whatman no. 1 filter paper. The filtrate were then concentrated by a Buchii rotavapor at low temperature and pressure and afforded methanol extract (36.8199 g). The cold methanol extract (10 g) was subjected to Solvent-Solvent partitioning using the protocol designed by Kupchan and modified by Wagene (Vanwagenen, 1993). The extract was portioned successively with petroleum ether, carbontetrachloride and chloroform.

4.13.3 *In vivo* antitumor activity

4.13.3.1 Treatment schedule

72 Swiss albino mice were divided into six groups (n = 12) and given food and water *ad libitum*. All the animals in each groups except Group-I received EAC cells (2×10^6 cells/mouse i.p.). This was taken as day '0'. Group-I served as normal saline control (5 ml/kg i.p.) and Group-II served as EAC control. After 24-h of EAC transplantation,

Group-III, IV, and Group-V received GL Methanol, GL Pet ether, GL Carbontetrachloride extracts of *G. lanceolarium* stem bark at 20 mg/kg b.wt. i.p. for nine consecutive days, respectively. Group-VI received reference drug Bleomycin (0.3 mg/kg i.p) for nine consecutive days (Rana and Khanam, 2002). Here Bleomycin used as positive control. After 24 hours of last dose and 18 h of fasting, 6 animals from each group were sacrificed by cervical dislocation to measure antitumor and hematological parameters and the rest were kept with food and water *ad libitum* to check percentage increase in life span of the tumor bearing host. The antitumor activity of the extractives of *G. lanceolarium* was measured in EAC animals with respect to the following parameters.

4.14 Results

4.14.1 Phytochemical screening

The phytoconstituents present in the different extractives of *G. lanceolarium* were identified by various chemical tests which showed the presence of terpenoids, phenolic and steroid compounds and (table-4.10).

4.14.2 Acute toxicity studies

The acute toxicity study was conducted to establish the therapeutic index, i.e., the ratio between the pharmacologically effective dose and the lethal dose on the same strain and species. The different extractives of *G. lanceolarium* were safe up to a dose of 500 mg/kg (p.o.) body weight. Behavior of the animals was closely observed for the first 3h then at an interval of every 4h during the next 48h. The extractives did not cause mortality in mice and rats during 48h of observation but little behavioral changes, locomotors ataxia, diarrhea and weight loss were observed. Food and water intake had no significant difference among the group studied.

4.14.3 Tumor growth and survival parameters

Antitumor activities of GL Methanol, GL Pet ether and GL Carbontetrachloride extracts against EAC tumor bearing mice was assessed by the parameters such as tumor volume,

tumor weight, cell count (viable and non viable), mean survival time and % increase of life span. The results are shown in table-4.11. The tumor volume, tumor weight and viable cell count were found to be significantly (p<0.001) increased and non-viable cell count was significantly (p<0.001) low in EAC control animals when compared with normal control animals. Administration of the extractives at dose of 20 mg/kg significantly (p<0.05) decreased the tumor volume, tumor weight and viable cell count. Furthermore, the median survival time was increased to 25±0.32, 26± 0.10 and 23±0.19 (% ILS = 24.6, 30.3, and 20.5) on administration of GL Methanol, GL Pet ether and GL Carbontetrachloride extracts at 20 mg/kg b.wt, respectively. All these results clearly indicate that the extractives had a capacity to inhibit the growth of solid tumor induced by EAC cell line in experimental animals.

4.14.4 Hematological parameters

Hematological parameters (Table-4.12) of tumor bearing mice on 14 day were found to be significantly altered compared to the normal group. The total WBC count was found to be increased with a reduction of Hb content of RBC. The total number of RBC showed a modest change. At the same time the extractives at 20 mg/kg b.wt. restored all the altered hematological parameters to almost close to normal.

Table-4.10 Result of chemical group tests of different extracts of *Glochidion lanceolarium*

Extracts	Triterpene	Diterpene	Phenol	Sterol
Glochidion lanceolarium	+	+++	++	+++

(+): Present; (-): Absent; (+ + +): Reaction intensity is high; (+ +): Reaction intensity is medium; (+): Reaction intensity is normal.

Table-4.11 Effect of the GL Methanol, GL Pet Ether, GL Carbontetrachloride extracts of *Glochidion lanceolarium* on tumor volume, tumor weight, mean survival time (MST), percentage increase life span (% ILS), viable and non-viable tumor cell count in EAC bearing mice.

Parameter	**GL Methanol**	**GL Pet Ether**	**GL CCl_4**	**EAC control**	**Bleomycin (Positive control)**
Tumor volume (ml)	0.79 ± 0.19	0.70 ± 0.14*	0.85 ± 0.41*	3.5 ± 0.21	0.49 ± 0.21*
Tumor weight (g)	1.32 ± 0.29	1.05 ± 0.11*	1.81 ± 0.31*	3.89 ± 0.24	0.43 ± 0.11*
MST (days)	25 ± 0.32	26 ± 0.10	23 ± 0.19	20 ± 0.12	48.6 ± 0.12
% ILS	24.6	30.3	20.5	00.0	94.81
Viable cell (x 10^7 cell/ml)	0.91 ± 0.32*	0.64 ± 0.15*	1.02± 0.25*	8.1 ± 0.22	0.5 ± 0.05*
Non-viable cell (x 10^7 cell/ml)	2.17 ± 0.23*	2.25 ± 0.59*	2.17 ± 0.15*	0.5 ± 0.24	3.3 ± 0.05*
Total cell (x 10^7 cell/ml)	3.08 ± 0.35	2.89 ± 0.21	3.19 ± 0.25	8.6 ± 0.15	3.8 ± 0.05*
Viable %	29.55	22.15	31.98	94.18	13.15
Non-viable %	70.45	77.85	68.02	5.82	86.85

Each point represent the mean ± SEM. (n = 6 mice per group), *$p<0.05$ statistically significant when compared with EAC control group.

Table-4.12 Effect of the extractives of *Glochidion lanceolarium* on hematological parameter in EAC bearing mice.

Treatment	RBC (cell x 10^3/mm^3)	WBC (cell x 10^3/mm^3)	Hemoglobin (g %)
Normal control	5.69 ± 0.12	3.92 ± 0.32	13.90 ± 3.1
EAC control	3.91 ± 0.80*	5.94 ± 0.52*	4.95 ± 1.80*
Bleomycin (0.3 mg/kg) (Positive control)	5.18 ± 0.12**	3.35 ± 0.83**	12.89 ± 2.93**
GL Methanol(20 mg/kg)	4.51 ± 0.31**	4.38 ± 0.49*	8.01 ± 2.19**
GL Pet Ether(20 mg/kg)	4.91 ± 0.71**	4.01 ± 0.11**	8.99 ± 1.90**
GL CCl_4 (20 mg/kg)	4.12 ± 0.15*	4.91 ± 0.91**	7.93 ± 2.01**

Each point represent the mean ± SEM. (n = 6 mice per group), *p<0.05 statistically significant when compared with EAC control group.

4.14.5 Discussion

In EAC tumor bearing mice, a regular rapid increase in ascetic tumor volume was observed. Ascetic fluid is the direct nutritional source for tumor cells and a rapid increase in ascetic fluid with tumor growth would be a way to meet the nutritional requirement of tumor cells (Prasad and Giri, 1994). Treatment with different extracts of *G. lanceolarium* reduced the intraperitonial tumor burden, thereby reducing the tumor volume, tumor weight, viable tumor cell count and increased the life span of the tumor bearing mice. The steadfast criteria for judging the potency of any anticancer drug are prolongation of life span of animals (Clarkson and Burchenal, 1965). It can therefore be inferred that the extractives increased the life span of EAC bearing mice may be due to decrease the nutritional fluid volume and delay the cell division (Sur *et al*., 1997).

Reduction in viable cell count and increased non viable cell count towards normal in tumor host suggest antitumor effect against EAC cell in mice. These demonstrated that

the extractives have direct relationship with tumor cells as these tumor cells are absorbed the anticancer drug by direct absorption in peritoneal cavity and this anticancer agent lysis the cells by direct cytotoxic mechanism (Kennedy *et al*., 2001). Anemia and myelosuppression have been frequently observed in ascites carcinoma (Hogland, 1982). Anemia is encountered in ascites carcinoma mainly due to iron deficiency, either by haemolytic or myelopathic conditions which finally lead to reduced RBC number (Gupta *et al*., 2007). Treatment with the extractives brought back the hemoglobin content, RBC and WBC count more or less to normal levels, thus supporting its haematopoietic protective activity without inducing myelotoxicity, the most common side effects of cancer chemotherapy.

Preliminary phytochemical studies indicated the presence of steroids, phenolic and terpenoid compounds in the extractives of *G .lanceolarium.* A number of scientific reports indicated that certain terpenoids, steroids and phenolic compounds have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis (Blois, 2002). Phytosterols are able to be incorporated into the cell membrane, alter membrane fluidity and the activity of membrane bound enzymes. They alter signal transduction in pathways leading to tumor growth and stimulate apoptosis in tumor cell lines. They also have shown to enhance *in vitro* human peripheral blood lymphocyte and T-cell proliferation in vitro, which suggests a possible stimulation of the immune system function (Jones *et al*., 2009). The anticancer activity of the extractives of *G. lanceolarium* are probably due to the presence of phenolic and terpenoid compounds present in the extractives.

4.15 Conclusion

In present study, it was observed that the extractives (GL Methanol, GL Pet ether and GL Carbontetrachloride) of *G. lanceolarium* exhibited anti-cancer activity as well as significantly reduced tumor growth, viability of tumor cells, normalized the hematological profiles and increased life span as compared with those of EAC control mice. Among these extractives GL Pet ether extracts has shown highest anti-cancer activity whereas GL Methanol, GL Carbontetrachloride exhibited moderate activity. Further chemical and biological studies are underway made to isolate the bioactive compounds.

CHAPTER 5

Antioxidant Activity

5.1 Introduction

The largest parts of the diseases are mainly linked to oxidative stress due to free radicals (Gutteridgde, 1995). Antioxidants can interact with the oxidation process by reacting with free radicals, chelation, catalyzing metals, and also by acting as oxygen scavengers (Buyukokuroglu *et al.*, 2001).

Literature reviews have shown that there was much effort to invent medicine to overcoming the death. But until recently the actual cause of aging was not known. There is considerable recent evidence that free radical induce oxidative damage to biomolecules. This damage causes aging, diabetes, cancer, malaria, neurodegenerative diseases and other pathological events in living organisms (Halliwell *et al.* 1992). Antioxidants which scavenge free radicals are known to posses an important role in preventing these free radical induced-diseases. There is an increasing interest in the antioxidant effects of compounds derived from plants, which could be relevant in relations to their nutritional incidence and their role in health and diseases (Steinmetz *et al.*, 1996; Aruoma, 1998; Bandoniene *et al.*, 2000; Pieroni *et al.*, 2002; Couladis *et al.*, 2003). A number of reports on the isolation and testing of plant derived antioxidants have been described during the past decade. Natural antioxidants constitute a broad range of substances including phenolic or nitrogen containing compounds and carotenoids (Shahidi *et al.*, 1992; Velioglu *et al.*, 1998; Pietta *et al.*, 1998). The medicinal properties of plants have been investigated throughout the world, due to their potent antioxidant activities, minimum or no side effects and economic viability (Auudy *et al.*, 2003).

Lipid peroxidation is one of the main reasons for deterioration of food products during processing and storage. Synthetic antioxidant such as *tert*-butyl-1-hydroxitoluene (TBHT), *tert*-butylhydroquinone (TBHQ), butylated hydroxianisole (BHA) and propyl gallate (PG) are widely used as food additives to increase shelf life, especially lipid and lipid containing products by retarding the process of lipid peroxidation. However, TBHT and BHA are known to have not only toxic and carcinogenic effects on humans (Ito *et al.*, 1986; Wichi, 1988), but also abnormal effects on enzyme systems (Inatani *et al.* 1983).

Thus, the interest in natural antioxidant, especially of plant origin, has greatly increased in recent years (Jayaprakasha *et al.*, 2000). Plant polyphenols have been studied largely because of the possibility that they might underlie the protective effects afforded by fruit and vegetable intake against cancer and others chronic diseases (Elena *et al.*, 2006).

5.2 Antioxidants: The free radical scavengers

Oxygen is the highest necessary substance for human life. But it is a Jeckyl and Hyde (both pleasant and unpleasant) element. We need it for critical body functions, such as respiration and immune response, but the element's dark side is a reactive chemical nature that can damage body cells. The perpetrators of this "oxidative damage" are various oxygen-containing molecules, most of which are different types of *free radicals*-unstable, highly energized molecules that contain an unpaired electron.

Free radicals cause depletion of immune system ,antioxidants change in gene expression and induce abnormal proteins and contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, diabetes, cancer and AIDS (Hela and Abdullah, 2010). In normal metabolic condition, oxidant and antioxidant levels are maintained in balance within humans for sustaining optimal physiological conditions (Temple, 2000). However, overproduction of free radical and reactive oxygen species (ROS) would assault on important biological molecules such as DNA, protein or lipid leading to many degenerative diseases (Suja *et al*., 2004). According to the estimates of the WHO, more than 80% of people in developing countries depend on traditional medicine for their primary health needs. A recent survey shows that more than 60% of cancer patients use vitamins or herbs as therapy (Madhuri *et al*., 2008; Sivalokanathan *et al*., 2005). The extensive use of herbal preparations and medicinal plants has provided some of the most important sources of lead compounds for the pharmaceutical industries. Furthermore, over a 100 new products are in the process of clinical development, particularly as anti-cancer agents and anit-infectives (Hafidh *et al*., 2009). It has been shown that antioxidant rich diets can reduce oxidative damage to DNA, thus preventing a critical step in the onset of carcinogenesis and the impact of antioxidants on mutagenesis and carcinogenesis has been well established (Zhang *et al*., 2008; Meyskens and Szabo, 2005). Various free radicals are also responsible for the induction of short term algesia

(Chung, 2004). Moreover, free radicals play an important role in the pathogenesis of inflammation (Winrow *et al*., 1993). For several years, many researchers have been investigated powerful and nontoxic antioxidants from natural sources, especially edible or medicinal plants to prevent the reactive species related disorders in human as well as replace the synthetic compounds which are in use may have carcinogenic activity and harmful to the lungs and liver (Rechner *et al*., 2002).

5.2.1 Antioxidant Protection

As a defense against oxidative damage, the body normally maintains a variety of mechanisms to prevent such damage while allowing the use of oxygen for normal functions. Such "antioxidant protection" derives from sources both inside the body (endogenous) and outside the body (exogenous). Endogenous antioxidants include molecules and enzymes that neutralize free radicals and other reactive oxygen species, as well as metal-binding proteins that sequester iron and copper atoms (which can promote certain oxidative reactions, if free). The body also makes several key antioxidant enzymes that help "recycle," or regenerate, other antioxidants (such as vitamin C and vitamin E) that have been altered by their protective activity.

Exogenous antioxidants obtained from the diet also play an important role in the body's antioxidant defense. These include vitamin C, vitamin E, carotenoids such as beta-carotene and lycopene, and other *phytonutrients*, or substances found in fruits, vegetables, and other plant foods that provide health benefits. Vitamin C (ascorbic acid), which is water-soluble, and vitamin E (tocopherol), which is fat-soluble, are especially effective antioxidants because they quench a variety of reactive oxygen species and are quickly regenerated back to their active form after they neutralize free radicals.

Morever, recent years have witnessed a renewed interest in plants as pharmaceuticals. This interest has been focused particularly on the adoption of extracts of plants, for self-medication by the general people. Within this context, considerable interest has arisen in the possibility that the impact of several major diseases may be either ameliorated or prevented by improving the dietary intake of natural nutrients with antioxidant properties, such as vitamin E, vitamin C, β-carotene and plant phenolics like tannins and flavonoids. The use of plant extracts in traditional medicine by old Indian and Chinese people have

been going on from ancient time. Herbalism and folk medicine, both ancient and modern, have been the source of much useful therapy (Rashid *et al*., 1997).

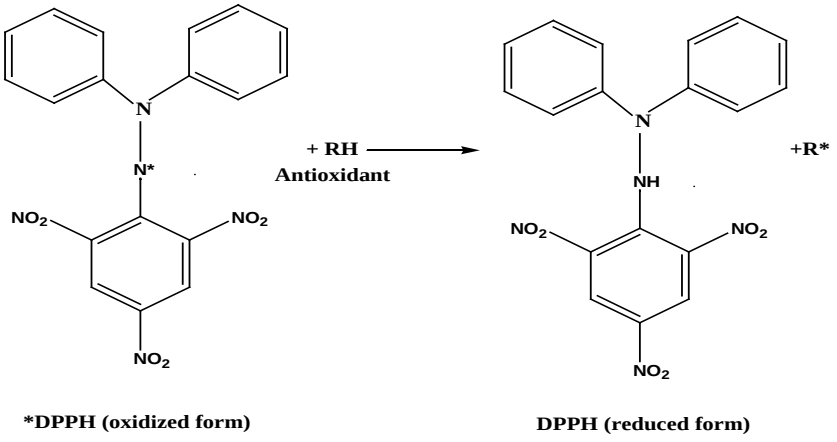
The purpose of this study was to evaluate extractives as well as isolated compounds as new potential sources of natural antioxidants and phenolic compounds.

5.2.2 Antioxidant activity: DPPH assay

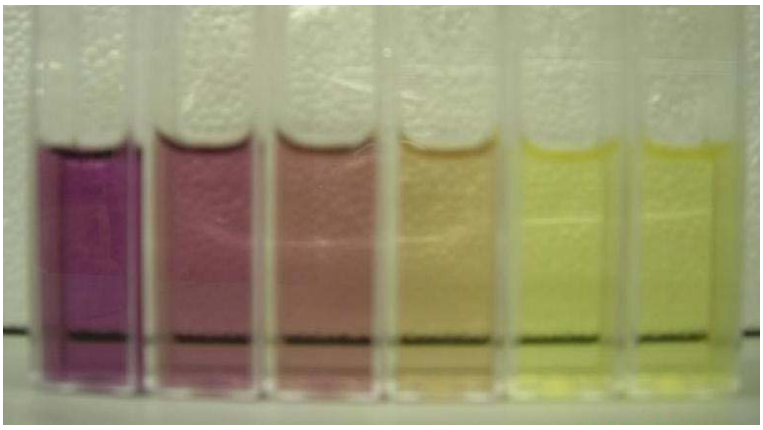
5.2.3 Principle

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

Here 2.0 ml of a methanol solution of the extract at different concentration were mixed with 3.0 ml of a DPPH methanol solution (20 μg/ml). The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extract as compared to that of *tert*-butyl-1-hydroxytoluene (TBHT) by a UV spectrophotometer. The reaction mechanism is shown below:



- DPPH = 2,2-diphenyl-1-picrylhydrazyl



Color variation of DPPH solution after samples treatment

5.2.4 Materials and Methods

DPPH was used to evaluate the free radical scavenging activity (antioxidant potential) of various compounds and medicinal plants (Choi *et al.*, 2000; Desmarchelier *et al.*, 1997).

5.2.4.1 Materials and preparation of materials

2,2-diphenyl-1-picryldrazyl (DPPH)	Beaker (100 & 200 ml)
tert-butyl-1-hydroxytoluene (TBHT)	Test tube
Distilled water	Pipette (5 ml)
Methanol	Micropipette (50-200 μl)
UV-spectrophotometer	Amber reagent bottle
Beaker (100 & 200 ml)	Weighing balance
Test tube	Exts. of related plant

Table 5.1: Test samples of experimental plants

Plant	Test samples (fraction/compound)	Code	Amount (mg)
P. simiarum (stem bark/ Compounds)	Petroleum ether soluble fraction	PEFPSB	2.00
	Ethyl acetate soluble fraction	EAFPSB	2.00
	16β-Hydroxy-cleroda-3, 13 (14) *Z*-dien 15, 16-olide (**164**)	PSB-004	2.00
	Kolavenic acid (**163**)	PSB-007	2.00
G. multiloculare (stem bark)	Petroleum ether soluble fraction	PEFGM	2.00
	Carbon tetrachloride soluble fraction	CTFGM	2.00
	Chloroform soluble fraction	CFFGM	2.00
	Methanol soluble fraction	MEGM	2.00
	Aqueous soluble fraction	AQFGM	2.00
G. lanceolarium (stem bark)	Petroleum ether soluble fraction	PEFGML	2.00
	Carbon tetrachloride soluble fraction	CTFGL	2.00
	Chloroform soluble fraction	CFFGL	2.00
	Methanol soluble fraction	MEGL	2.00
	Aqueous soluble fraction	AQFGL	2.00

5.2.4.2 Control preparation for antioxidant activity measurement

Tert-butyl-1-hydroxytoluene (TBHT) was used as positive control. Calculated amount of TBHT was dissolved in methanol to get a mother solution having concentration of 1000 µg/ml. Serial dilution was made using the mother solution to get different concentrations ranging from 500.0 to 0.977 µg/ml.

5.2.4.3 DPPH solution preparation

20 mg DPPH powder was weighed and dissolved in methanol to get a DPPH solution having a concentration 20 µg/ml. The solution was prepared in the amber colored reagent bottle and kept in the light proof box.

5.2.4.4 Test sample preparation

Calculated amount of different extractives were measured and dissolved in methanol to get a mother solution (1000 µg/ml). Serial dilution of the mother solution provided different concentrations from 500.0 to 0.977 µg/ml which were kept in the dark flasks.

5.2.4.5 Methods

- 2.0 ml of a methanol solution of the extract at different concentration (500 to 0.977 µg/ml) were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml).
- After 30 min of reaction period at room temperature in dark place, the absorbance was measured at 517 nm against methanol as blank by using a suitable spectrophotometer.
- Inhibition of free radical DPPH in percent (I%) was calculated as follows: $(I\%) = (1 - A_{sample}/A_{blank}) \times 100$

  Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test material).
- Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted by inhibition percentage against extract/compound concentration (Figure 4.1).

The experiments were carried out in triplicate and the result was expressed as mean ± SD in every cases.

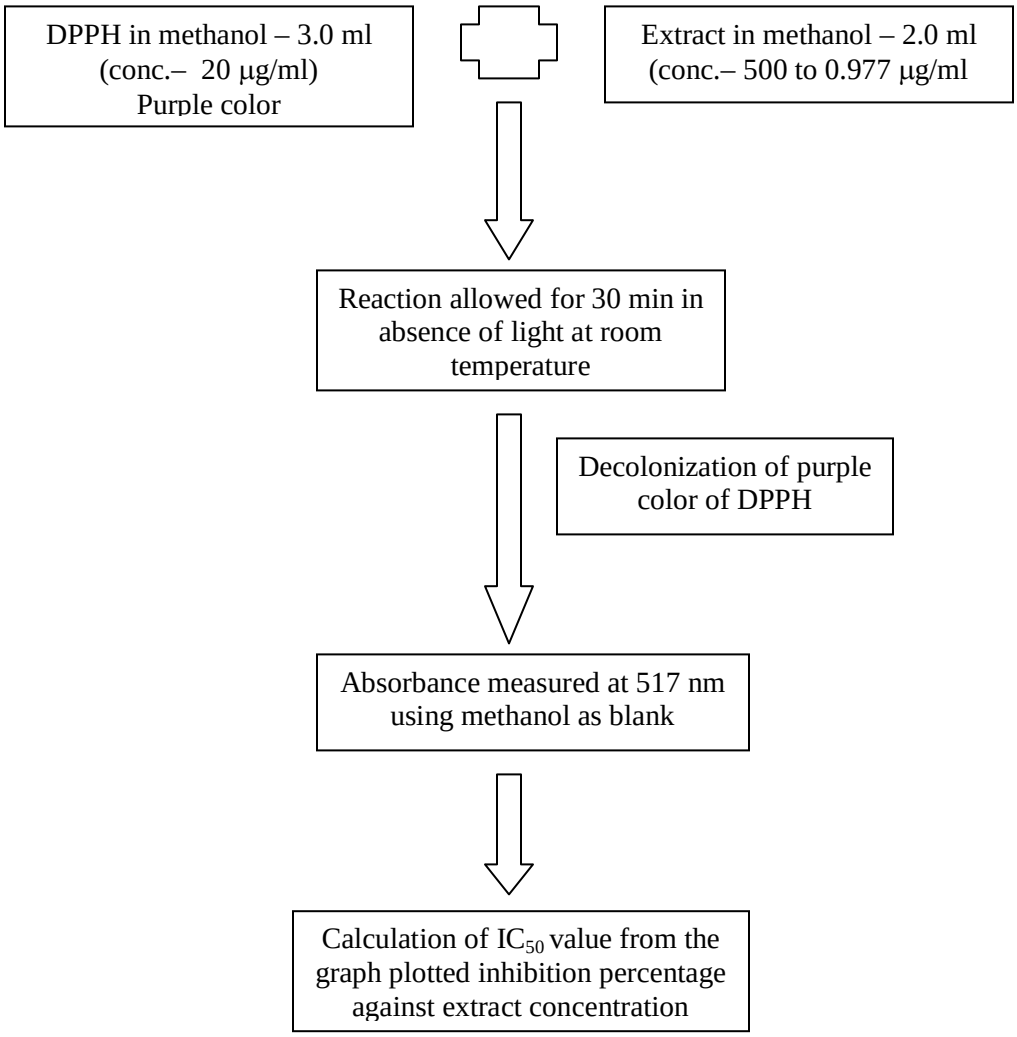


Figure 5.1: Schematic representation of the method of assaying free radical scavenging activity

5.3 Results and Discussion

5.3.1 *Polyalthia simiarum*

Different fractionates and pure compounds of stem bark of *P. simiarum* were subjected to free radical scavenging activity assay by the method of Brand –Williams *et al.*, 1995. Here, *tert*-butyl-1-hydroxytoluene (TBHT) was used as reference standard.

In this investigation, the Petroleum ether soluble fraction (PEFPSB) showed the highest free radical scavenging activity with IC_{50} value **21.5 g/ml**. At the same time the Ethyl acetate soluble fraction (EAFPSB) and pure compound PSB-004 also exhibit moderate antioxidant potential having IC_{50} values 24.5 and 23.5 µg/ml respectively. Another pure compound PSB-007 exhibit low antioxidant activity having IC_{50} value 62 µg/ml. The IC_{50} value for the TBHT was found to be 9 µg/ml (Table 5.2).

Table 5.2: List of IC_{50} values of standard and the test samples of *P. simiarum*

Test samples	**IC_{50} (µg/ml)[#]**
TBHT (Standard)	9 ± 0.02
PEFPSB	21.5 ±0.707
EAFPSB	24.5 ±0.30
PSB-004 (**164**)	23.5 ±0.15
PSB-007 (**163**)	62 ±1.53

[#] The values of IC_{50} are expressed as mean±SD (n=3)

Table 5.3: List of absorbance against concentrations and IC_{50} value of tert-butyl- 1-hydroxytoluene (TBHT)

TBHT (Standard)				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.395	500	0.038	97.215	9
	250	0.027	93.165	
	125	0.048	87.848	
	62.5	0.085	78.481	
	31.25	0.121	69.367	
	15.625	0.179	54.683	
	7.8125	0.208	47.342	
	3.90625	0.252	36.203	
	1.953125	0.265	32.911	
	0.976563	0.311	21.666	

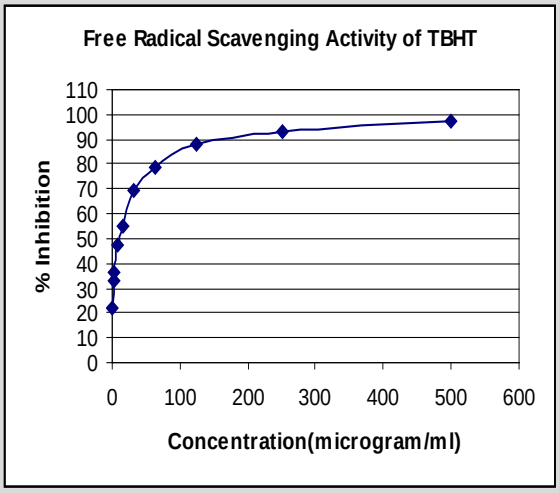


Figure 5.2: Chart for IC_{50} value of TBHT

Table 5.4: IC_{50} value of PEFPSB (Petroleum ether fraction of *P. simiarum* bark)

PEFPSB				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.395	500	0.081	79.303	21.5
	250	0.112	71.582.	
	125	0.145	63.291	
	62.5	0.163	56.974	
	31.25	0.186	53.215	
	15.625	0.216	45.316	
	7.8125	0.235	40.189	
	3.90625	0.264	33.164	
	1.953125	0.299	24.303	
	0.976563	0.312	21.012	

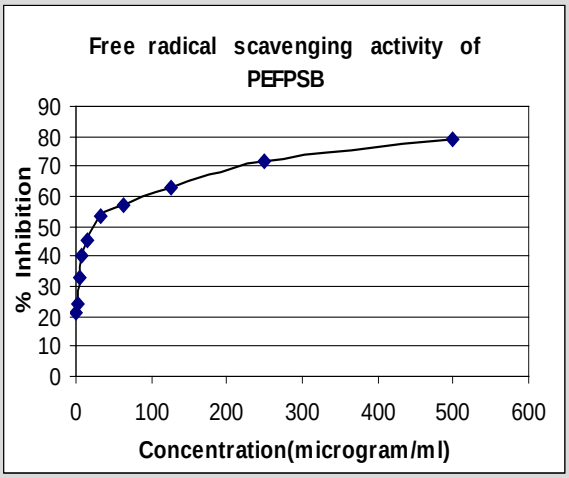


Figure 5.3: Chart for IC_{50} value of PEFPSB of *P. simiarum*

Table 5.5: IC_{50} value of EAFPSB (Ethyl acetate fraction of *P. simiarum* bark)

EAFPSB				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.395	500	0.036	90.886	24.5
	250	0.030	92.405	
	125	0.078	80.253	
	62.5	0.158	60	
	31.25	0.185	53.165	
	15.625	0.225	43.038	
	7.8125	0..249	36.962	
	3.90625	0.267	32.405	
	1.953125	0.272	31.139	
	0.976563	0.301	23.797	

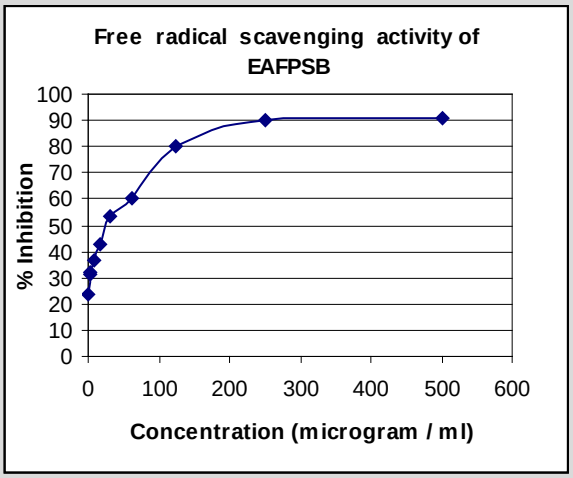


Figure 5.4: Chart for IC_{50} value of EAFPSB of *P. simiarum*

Table 5.6: IC_{50} value of pure compound PSB-004 (16β-Hydroxy-cleroda-3, 13 (14)*Z*-dien 15, 16-olide, 164) of *P. simiarum* bark

PSB-004 (164)				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.395	500	0.074	81.341	23.5
	250	0.081	79.303.	
	125	0.120	69.62	
	62.5	0.158	60	
	31.25	0.185	53.215	
	15.625	0.219	44.455	
	7.8125	0.249	36.936	
	3.90625	0.303	23.911	
	1.953125	0.321	19.645	
	0.976563	0.355	10.126	

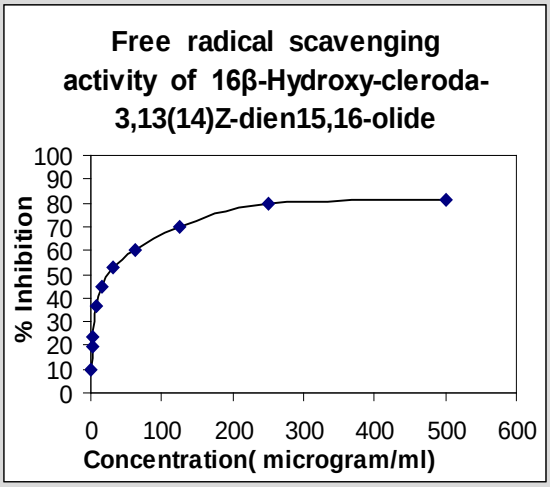


Figure 5.5: Chart for IC_{50} value of compound of *P. simiarum*

Table 5.7: IC_{50} value of pure compound PSB-007 (Kolavenic acid, 163) of *P. simiarum* bark

PSB-007 (163)					
Absorbance of the control reaction	Conc. (μg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (μg/ml)	
0.395	500	0.030	92.405	62	
	250	0.025	93.671		
	125	0.087	77.975		
	62.5	0.201	49.114		
	31.25	0.246	37.722		
	15.625	0.281	28.861		
	7.8125	0.288	27.089		
	3.90625	0.287	27.342		
	1.953125	0.293	25.823		
	0.976563	0.295	25.316		

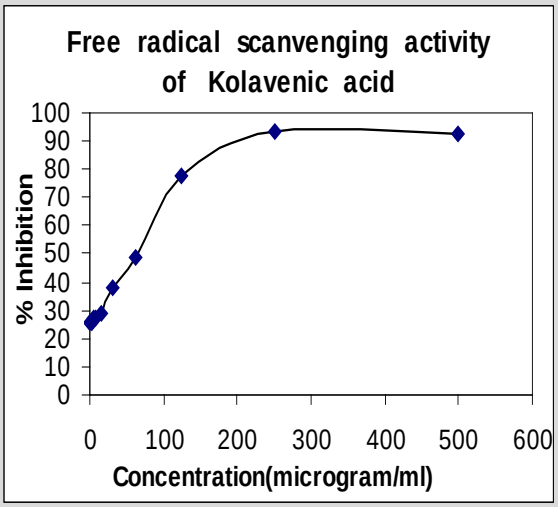


Figure 5.6: Chart for IC_{50} value of compound of *P. simiarum*

5.3.2 *Glochidion multiloculare*

Free radical scavenging activities of different fractionates of *G. multiloculare* have been examined. The obtained results have been listed in Table 5.8. The IC_{50} value for the standard (TBHT) was found to be 15.97 μg/ml. Methanol soluble extract and Petroleum ether soluble fractions exhibit significant antioxidant capacity having IC_{50} value of **16.40 μg/ml** and **19.85 μg/ml**, At the same time Carbontetrachloride, Chloroform and Aqueous soluble fractionates exhibit moderate antioxidant activities having IC_{50} values of 27.41, 32.30 and 38.17 μg/ml respectively (Table 5.8).

Table 5.8: List of IC_{50} values and equation of regression lines of standard and test samples of *G. multiloculare*

Test samples	IC_{50} (µg/ml)[#]	Equation of Regression line	R^2
TBHT	15.97 ± 0.02	y = 15.164Ln(x) + 7.9873	0.9641
PEFGM	19.85 ± 1.65	y = 11.568Ln(x) + 15.436	0.9809
CTFGM	27.41 ± 0.29	y = 10.15Ln(x) + 16.39	0.9796
CFFGM	32.30 ± 1.22	y = 10.1Ln(x) + 14.903	0.9895
MEGM	16.40 ± 1.35	y = 12.279Ln(x) + 15.661	0.9851
AQFGM	38.17 ± 1.53	y = 11.08Ln(x) + 9.6451	0.9709

[#]The values of IC_{50} are expressed as mean±SD (n=3)

Table 5.9: IC_{50} value of TBHT (*tert*-butyl-1-hydroxytoluene)

TBHT (Standard)				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.445	500	0.028	93.708	15.97
	250	0.032	92.809	
	125	0.062	85.843	
	62.5	0.095	78.652	
	31.25	0.150	66.292	
	15.625	0.227	48.989	
	7.8125	0.310	30.337	
	3.90625	0.348	21.798	
	1.953125	0.361	18.876	
	0.976563	0.391	12.135	

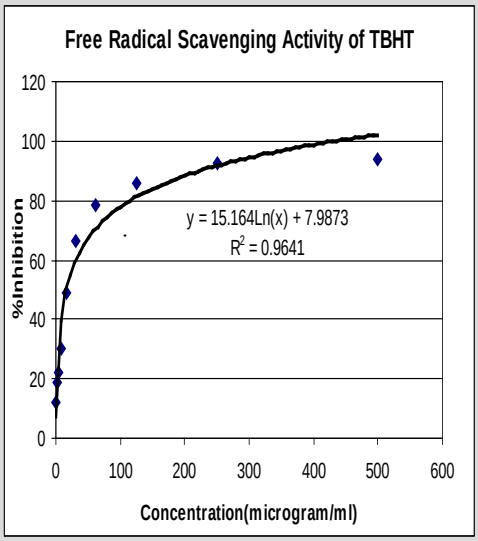


Figure 5.7: Chart for IC_{50} value of TBHT

Table 5.10: IC_{50} value of PEFGM (Petroleum ether fraction of *G. multiloculare*)

PEFGM				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.445	500	0.074	83.371	19.85
	250	0.092	79.326	
	125	0.125	71.910	
	62.5	0.149	66.517	
	31.25	0.172	61.348	
	15.625	0.223	47.865	
	7.8125	0.288	35.281	
	3.90625	0.321	27.865	
	1.953125	0.352	20.899	
	0.976563	0.364	18.202	

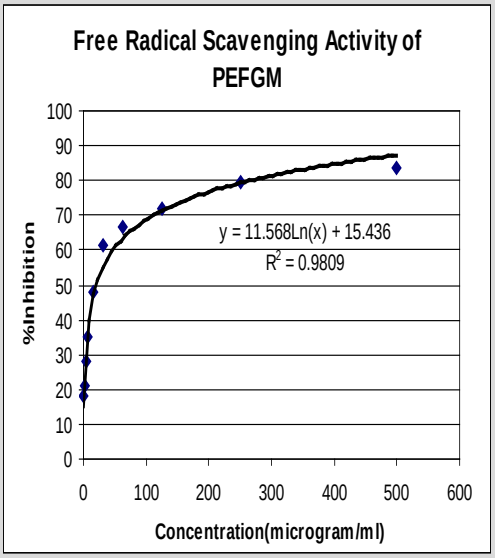


Figure 5.8 : Chart for IC_{50} value of PEFGM of *G. multiloculare*

Table 5.11: IC_{50} value of CTFGM (Carbon tetrachloride fraction of *G. multiloculare*)

CTFGM				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.445	500	0.108	75.731	27.41
	250	0.136	69.438	
	125	0.142	68.090	
	62.5	0.155	65.169	
	31.25	0.217	51.236	
	15.625	0.252	43.371	
	7.8125	0.277	37.753	
	3.90625	0.305	31.461	
	1.953125	0.348	21.798	
	0.976563	0.382	14.157	

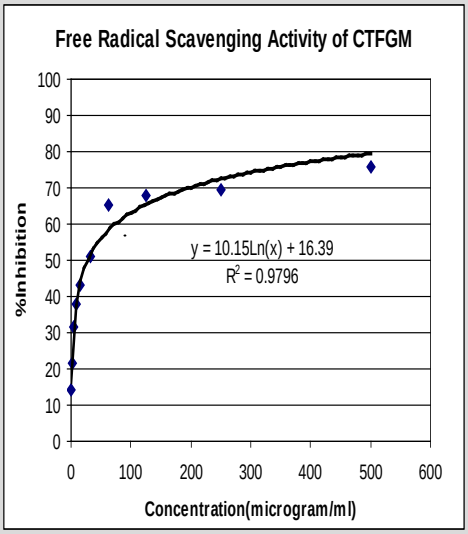


Figure 5.9: Chart for IC_{50} value of CTFGM of *G. multiloculare*

Table 5.12: IC_{50} value of CFFGM (Chloroform fraction of *G. multiloculare*)

CFFGM				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.445	500	0.107	75.955	32.30
	250	0.136	69.438	
	125	0.152	65.843	
	62.5	0.172	61.348	
	31.25	0.231	48.09	
	15.625	0.268	39.775	
	7.8125	0.285	35.955	
	3.90625	0.315	29.213	
	1.953125	0.348	21.798	
	0.976563	0.381	14.382	

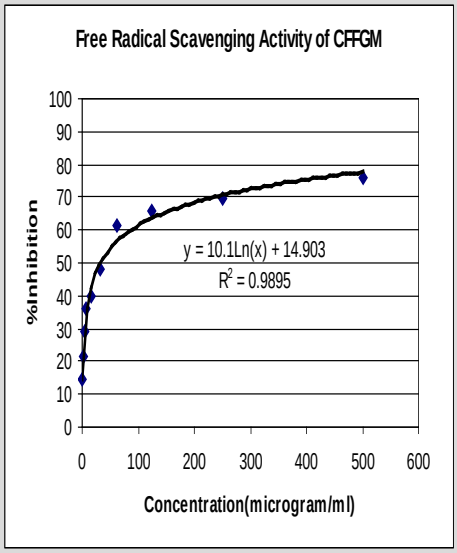


Figure 5.10: Chart for IC_{50} value of CFFGM of *G. multiloculare*

Table 5.13: IC_{50} value of MEGM (Methanol extract of *G. multiloculare*)

MEGM				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.445	500	0.052	88.315	16.40
	250	0.075	83.146	
	125	0.112	74.831	
	62.5	0.127	71.461	
	31.25	0.163	63.371	
	15.625	0.239	46.292	
	7.8125	0.278	37.528	
	3.90625	0.298	33.034	
	1.953125	0.335	24.719	
	0.976563	0.382	14.157	

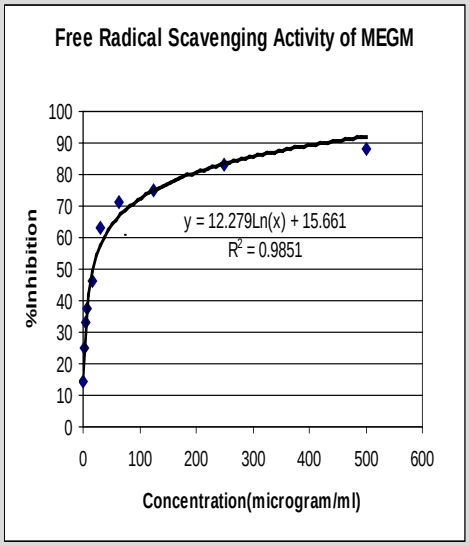


Figure 5.11: Chart for IC_{50} value of MEGM of *G. multiloculare*

Table 5.14: IC_{50} value of AQFGM (Aquous fraction of *G. multiloculare*)

AQFGM				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.445	500	0.097	78.202	38.17
	250	0.123	72.360	
	125	0.141	68.315	
	62.5	0.188	57.753	
	31.25	0.252	43.371	
	15.625	0.301	32.360	
	7.8125	0.315	29.213	
	3.90625	0.330	25.843	
	1.953125	0.362	18.652	
	0.976563	0.385	13.483	

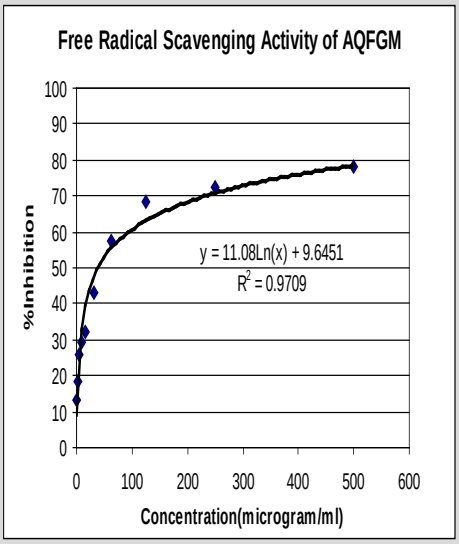


Figure 5.12: Chart for IC_{50} value of AQFGM of *G. multiloculare*

5.3.3 *Glochidion lanceolarium*

Five fractions from *G. lanceolarium* were subjected to assay for free radical scavenging activity. In this study, the Methanol soluble extract, Petroleum ether soluble and Carbontetrachloride soluble fractions showed the highest free radical scavenging activities with IC_{50} values of **18.32**, **20.29** and **22.24 µg/ml** respectively. At the same time Chloroform soluble fraction exhibit moderate antioxidant potential having IC_{50} value 34.19 µg/ml. The Aquous soluble fraction exhibit low antioxidant activity having IC_{50} value 67.15 µg/ml. IC_{50} value for TBHT was found to be 15.24 µg/ml (Table 5.15).

Table 5.15: List of IC_{50} values and equation of regression lines of standard and the test samples of *G. lanceolarium*

Test samples	IC_{50} (µg/ml)[#]	Equation of Regression line	R^2
TBHT	15.24 ± 0.63	y = 14.471Ln(x) + 10.578	0.9456
PEFGL	20.29 ± 0.79	y = 11.133Ln(x) + 16.536	0.9817
CTFGL	22.24 ± 2.02	y = 12.466Ln(x) + 11.333	0.9835
CFFGL	34.19 ± 1.14	y = 9.5255Ln(x) + 16.355	0.9724
MEGL	18.32 ± 0.76	y = 13.14Ln(x) + 11.764	0.965
AQFGL	67.15 ± 1.65	y = 9.1608Ln(x) + 11.46	0.982

[#] The values of IC_{50} are expressed as mean±SD (n=3)

Table 5.16: IC_{50} value of TBHT (*tert*-butyl-1-hydroxytoluene)

TBHT (Standard)				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.475	500	0.036	92.421	15.24
	250	0.037	92.211	
	125	0.067	85.895	
	62.5	0.095	79.579	
	31.25	0.162	65.895	
	15.625	0.250	47.368	
	7.8125	0.335	29.473	
	3.90625	0.371	21.895	
	1.953125	0.376	20.842	
	0.976563	0.388	18.316	

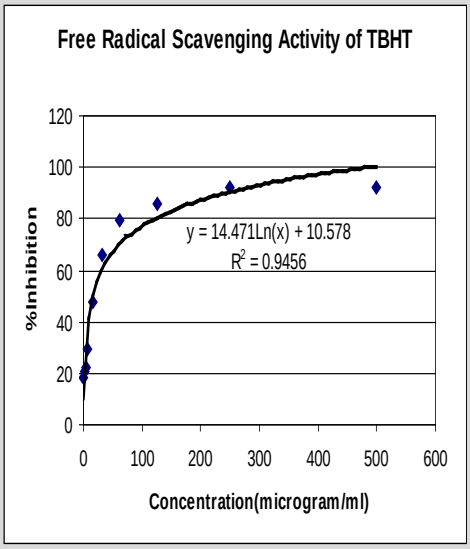


Figure 5.13: Chart for IC_{50} value of TBHT

Table 5.17: IC_{50} value of PEFGL (Petroleum ether fraction of *G. lanceolarium*)

PEFGL				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.475	500	0.045	90.526	20.29
	250	0.097	79.579	
	125	0.142	70.105	
	62.5	0.195	58.947	
	31.25	0.232	51.158	
	15.625	0.255	46.316	
	7.8125	0.304	36	
	3.90625	0.330	30.526	
	1.953125	0.350	26.316	
	0.976563	0.377	20.632	

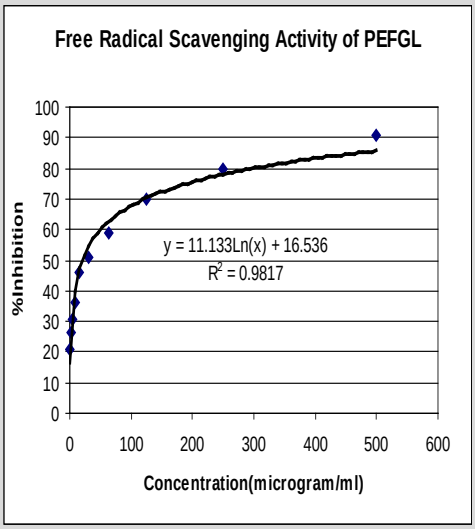


Figure 5.14: Chart for IC_{50} value of PEFGL of *G. lanceolarium*

Table 5.18: IC_{50} value of CTFGL (Carbon tetrachloride fraction of *G. lanceolarium)*

CTFGL				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.475	500	0.065	86.316	22.24
	250	0.105	77.895	
	125	0.125	73.684	
	62.5	0.161	66.105	
	31.25	0.190	60	
	15.625	0.258	45.684	
	7.8125	0.326	31.368	
	3.90625	0.356	25.053	
	1.953125	0.377	20.632	
	0.976563	0.415	12.632	

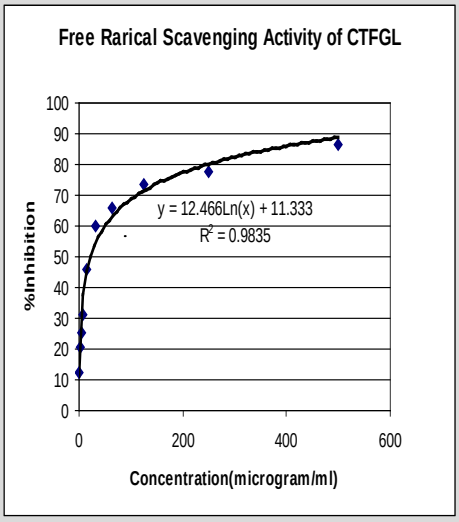


Figure 5.15: Chart for IC_{50} value of CTFGL of *G. lanceolarium*

Table 5.19: IC_{50} value of CFFGL (Chloroform fraction of *G. lanceolarium*)

CFFGL				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.475	500	0.121	74.526	34.19
	250	0.135	71.579	
	125	0.165	65.263	
	62.5	0.220	53.684	
	31.25	0.265	44.211	
	15.625	0.285	40	
	7.8125	0.301	36.632	
	3.90625	0.305	35.789	
	1.953125	0.364	23.368	
	0.976563	0.411	13.474	

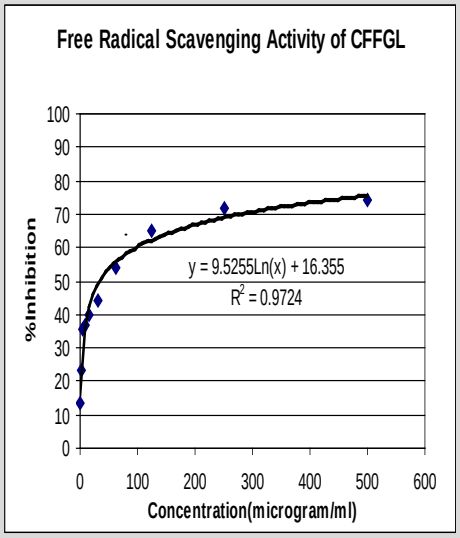


Figure 5.16: Chart for IC_{50} value of CFFGL of *G. lanceolarium*

Table 5.20: IC_{50} value of MEGL (Methanol extract of *G. lanceolarium*)

MEGL				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.475	500	0.058	87.789	18.32
	250	0.063	86.737	
	125	0.076	84	
	62.5	0.149	68.632	
	31.25	0.206	56.632	
	15.625	0.261	45.053	
	7.8125	0.325	31.579	
	3.90625	0.355	25.263	
	1.953125	0.376	20.842	
	0.976563	0.388	18.316	

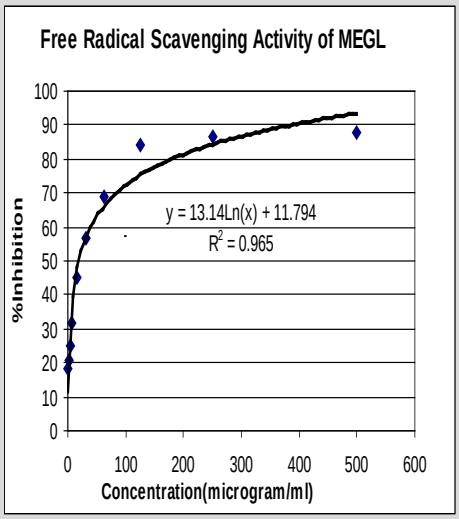


Figure 5.17: Chart for IC_{50} value of MEGL of *G. lanceolarium*

Table 5.21: IC_{50} value of AQFGL (Aqous fraction of *G. lanceolarium*)

AQFGL				
Absorbance of the control reaction	Conc. (µg/ml)	Absorbance of the test sample	% Inhibition	IC_{50} (µg/ml)
0.475	500	0.166	65.053	67.15
	250	0.178	62.526	
	125	0.188	60.421	
	62.5	0.230	51.579	
	31.25	0.269	43.368	
	15.625	0.321	32.421	
	7.8125	0.338	28.842	
	3.90625	0.363	23.579	
	1.953125	0.390	17.865	
	0.976563	0.415	12.632	

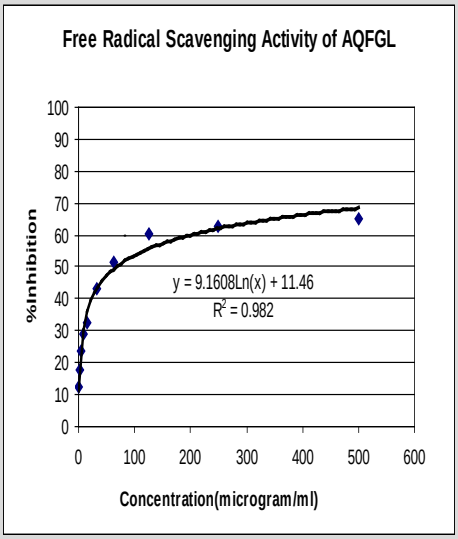


Figure 5.18: Chart for IC_{50} value of AQFGL of *G. lanceolarium*

5.4 Conclusion

All the fractions and pure compounds of the investigated plants were subjected to free radical scavenging activity by the method of Brand-Williams et al., 1995. Here, *tert*-butyl-1-hydroxytoluene (TBHT) and ascorbic acid was used as reference standard. In this study, the **MEGM, MEGL, PEFGM, PEFGL** and **PEFPSB** showed highest free radical scavenging activity with IC_{50} values **16.40, 18.32, 19.85, 20.29** and **21.5** µg/ml respectively. The CTFGL, compound PSB-004 and EAFPSB exhibit promising antioxidant capacity having IC_{50} values of 22.24, 23.5 and 24.5 µg/ml respectively**.** In this investigation, the CTFGM, CFFGM, CFFGL and AQFGM showed moderate free radical scavenging activity with IC_{50} values of 27.41, 32.30, 34.19 and 38.17 µg/ml respectively. At the same time, compound PSB-007 and fraction AQFGL exhibit low antioxidant activity having IC_{50} values 62, and **67.15** µg/ml respectively. In this investigation, some of the fractions from *P. simiarum, G. multiloculare* and *G. lanceolarium* demonstrate highest antioxidant activity whereas one of the compounds isolated from *P. simiarum* revealed significant free radical scavenging activity. On the other hand the another compound from the same plant showed poor antioxidant activity.

CHAPTER 6

Brine Shrimp Lethality Bioassay

6.1 Introduction

Brine shrimp lethality bioassay is a rapid general bioassay for the bioactive compound of the natural and synthetic origin (Meyer *et al*., 1982; Persoone, 1980). By this method, natural product extracts, fractions as well as the pure compounds can be tested for their bioactivity. Bioactive compounds are almost always toxic in high doses. In this method, *in vitro* lethality in a simple zoological organism (Brine shrimp nauplii) is used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. The bioassay is indicative of biotoxicity, anticancer, antiviral, pesticidal and a wide range of pharmacological activities of the compounds (McLaughlin, 1998).

Brine shrimp lethality bioassay technique stands superior to other cytotoxicity testing procedures because it is rapid, inexpensive and requires no special equipment. It utilizes a large number of organisms for statistical validation and a relatively small amount of sample. Furthermore, unlike other methods, it does not require animal serum.

6.2 Principle

Brine shrimp eggs are hatched in simulated sea water to get nauplii. Desired solution of the test sample is prepared by the addition of calculated amount of dimethylsulfoxide (DMSO). The nauplii are counted by visual inspection and are taken in vials containing 5 ml of simulated sea water. Then samples of different concentrations are added to the marked test tubes through micropipette. The tubes are left for 24 hours and then the survivors are counted.

6.3 Materials

6.3.1 Materials for test

i. *Artemia salina* leach (brine shrimp eggs)

ii. salt (NaCl)

iii. Small tank with perforated dividing dam to hatch the shrimp

iv. Lamp to attract shrimps

v. Pipettes

vi. Micropipette

vii. Glass vials

viii. Magnifying glass

ix. Test samples of experimental plants (Table 5.1)

x. Vincristine sulfate etc.

6.3.2 Test samples

Table -6.1 List of test samples of experimental plants

Plant	Test samples	Code	Amount (mg)
Polyalthia simiarum	Petroleum ether soluble fraction of stem bark	PEFPSB	4.00
	Ethyl acetate soluble fraction of stem bark	EAFPSB	4.00
Glochidion multiloculare	Methanol soluble bark extract	MeEGM	4.00
	Petroleum ether soluble fraction of MeEGM	PEFGM	4.00
	Carbon tetrachloride soluble fraction of MeEGM	CTFGM	4.00
	Chloroform soluble fraction of MeEGM	CFFGM	4.00
	Aqueous soluble fraction of MeEGM	AQFGM	4.00
Glochidion lanceolarium	Methanol soluble bark extract	MeEGL	4.00
	Petroleum ether soluble fraction of MeEGL	PEFGL	4.00
	Carbon tetrachloride soluble fraction of MeEGL	CTFGL	4.00
	Chloroform soluble fraction of MeEGL	CFFGL	4.00
	Aqueous soluble fraction of MeEGL	AQFGL	4.00
Compounds	16β-Hydroxy-cleroda-3,13(14)*Z*-dien15,16-olide	PSB-004	2.00
	Kolavenic acid	PSB-007	2.00

6.4 Methods

6.4.1 Preparation of seawater

38 g of sea salt (marketed NaCl) was weighed, dissolved in one liter of distilled water and filtered off to get clear solution.

6.4.2 Hatching of brine shrimps

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank (as a hatcher). The shrimp eggs were incubated in the hatcher at 28-30^{o}C with strong aeration for constant oxygen supply was provided through out the hatching time. About 24 hours were allowed to hatch the shrimp and to be matured as nauplii. The hatched shrimps were attracted to the lamp through the perforated dam and they were taken for experiment.

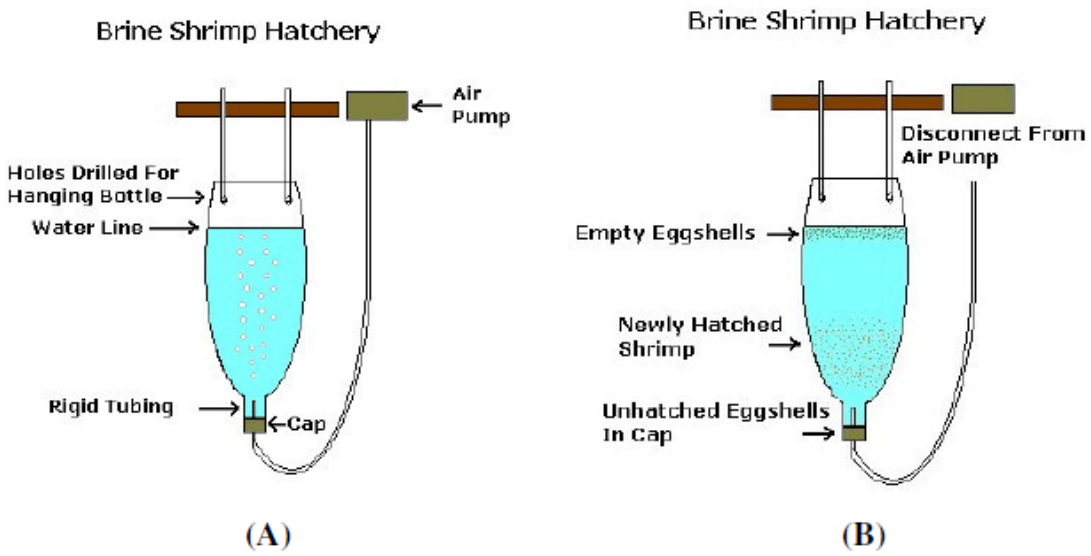


Figure- 6.1: (A) and (B) Hatching of Brine Shrimps

6.4.3 Application of brine shrimp nauplii

With the help of a Pasteur pipette 10 living shrimps were added to each of the vials containing 5 ml of seawater.

6.4.4 Preparation of test solutions of experimental extractives

Measured amount of each sample was dissolved in 100 µl of DMSO in a vial to get stock solution. Then 50 µl of solution was added to test tube each containing 5 ml of seawater and 10 shrimp nauplii. Thus, the final concentration of samples in the No.1 test tube was 400 µg/ml. Then a series of solutions of 0varying concentrations (every time half than previous) were prepared from stock solution by serial dilution method. In each case, fresh

50 µl DMSO was added to vial (total volume 100 µl; then shaking it) and from it, 50 µl of sample was taken to test tube. Thus, the concentrations of the obtained solution in the test tubes were as:

Sl no.	For 4 mg sample	Sl no.	For 2 mg sample	Sl no.	For 0.2 mg VS
1.	400 µg/ml	1.	200	1.	20 µg/ml
2.	200 ,,	2.	100	2.	10 ,,
3.	100 ,,	3.	50	3.	5 ,,
4.	50 ,,	4.	25	4.	2.5 ,,
5.	25 ,,	5.	12.5	5.	1.25 ,,
6.	12.5 ,,	6.	6.25	6.	0.625 ,,
7.	6.25 ,,	7.	3.125	7.	0.3125 ,,
8.	3.125 ,,	8.	1.5625	8.	0.15625 ,,
9.	1.5625 ,,	9.	0.786125	9.	0.078125 ,,
10.	0.78125 ,,	10.	0.3930625	10.	0.0390625 ,,

6.4.5 Preparation of control group

Control groups are used in cytotoxicity study to validate the test method and ensure that the results obtained are only due to the activity of test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used.

i. Positive control
ii. Negative control

6.4.5.1 Preparation of the positive control group

The use of positive control in cytotoxicity study is a widely accepted technique and the result of the test agent is compared with the result obtained for the positive control (vincristine sulfate). Measured amount of vincristine sulfate (0.2 mg) was dissolved in DMSO to get an initial concentration 20 µg/ml from which serial dilutions were made using DMSO to get solution of gradual lower concentration. The positive control solution were added to the premarked vials containing 10 living shrimp *nauplii* in 5 ml simulated seawater to get positive control group.

6.4.5.2 Preparation of the negative control group

DMSO (100 µl) was added to each of three premarked vials containing 5 ml of simulated seawater and 10 shrimps to use as control group. If the shrimps in these vials show a rapid mortality rate, then the test is considered to be invalid as the *nauplii* died due to some reasons other than the cytotoxicity of the test compounds.

6.4.5.3 Counting of nauplii

After 24 hours, the vials were observed using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of mortality of the brine shrimp *nauplii* was calculated for each concentration.

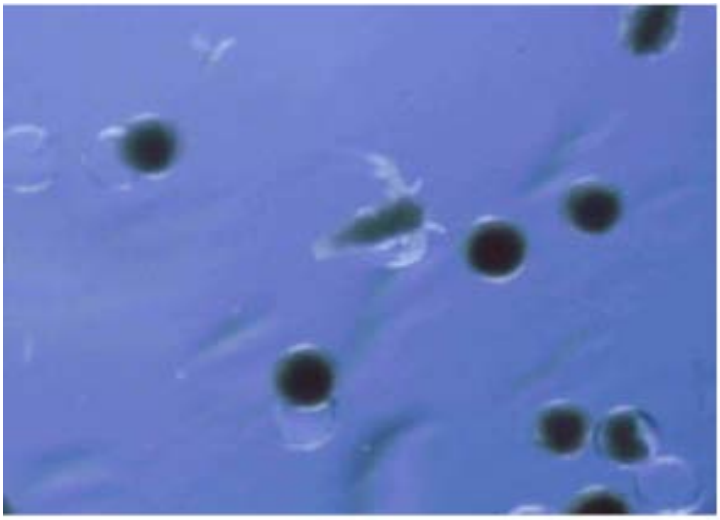


Figure- 6.2: Brine Shrimp Nauplii

6.5 Analysis of data

The concentration-mortality data were analyzed statistically by using excel worksheet analysis/probit analysis and linear regression modeling of binary response variables with the help of a laptop computer. The effectiveness or the concentration mortality relationship of plant product is usually expressed as median lethal concentration (LC_{50}) value. This represents the concentration of the chemical agent that produces death in half of the test subjects after a certain exposure period.

In this bioactivity study all the crude extracts and different fractions showed positive results indicating that the test samples are biologically active. Each of the test samples showed different mortality rates at different concentrations. Plotting of logarithm of concentration versus percent mortality for all test samples showed an approximate linear correlation. From the graphs, the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) was determined for each sample. Ther0e was no mortality in the control groups.

6.6 Results and discussion

Bioactive compounds are almost always toxic at higher dose. Pharmacology is simply toxicology at higher dose, or toxicology is simply pharmacology at lower dose. Thus, *in*

vitro lethality in a simple zoological organism can be used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. The results of cytotoxical lethality assay of *P. simiarum, G. multiloculare* and *G. lanceolarium* were discussed successively in the following sections.

6.6.1 *Polyalthia simiarum*

Fllowing the procedure of Meyer *et al.* 1982, the lethality of the Petroleum ether soluble fraction (PEFPSB) and Ethyl acetate soluble fraction (EAFPSB) of *Polyalthia simiarum* stem bark were subjected to brine shrimp lethality bioassay. LC_{50} values were found to be 1.91 and 3.65 µg/ml respectively. The result are summarized in Table 6.2 and 6.3 (Figure 6.4 and 6.5). The Petroleum ether soluble fraction (PEFPSB) exhibited highest cytotoxicity with LC_{50} value of 1.91 µg/ml. The positive control drug, vincristine sulfate showed LC_{50} at 0.32 µg/ml (Table: 6.3 and 6.4, Fig: 6.3). From the experiment, it is clearly evident that both the fractions were highly cytotoxic. Comparison with the activity of positive control vincristine, the cytotoxicity exhibited by the fractions might have antitumor or pesticidal compounds. However, this can not be confirmed without further studies and specific tests.

Table- 6.2 Effect of Petroleum ether and Ethylacetate soluble fractions of *P. simiarum* on brine shrimp nauplii

Polyalthia simiarum					
Conc. (C) (µg/ml)	**LOG C**	**% Mortality**		**LC_{50} / (µg/ml)**	
		PEFPSB	**EAFPSB**	**PEFPSB**	**EAFPSB**
400	2.602	90	100	1.91	3.65
200	2.3010	90	100		
100	2.0000	80	100		
50	1.6989	80	80		
25	1.3979	80	80		
12.5	1.0969	70	70		
6.25	0.7958	60	60		
3.125	0.4948	60	50		
1.5625	0.1938	40	40		
0.78125	-0.1075	40	20		

Table- 6.3 LC_{50} data of test samples of *P. simiarum*

Test samples	Regression line	R^2	LC_{50} (µg/ml)
VS (Positive control)	y = 33.623x + 66.812	0.9548	0.32
PEFPSB	y = 19.531x + 44.635	0.9227	1.91
EAFPSB	y = 29.397x + 33.327	0.9499	3.65
PSB-004	y = 32.417x + 38.317	0.9475	2.29
PSB-007	y = 34.632x + 17.221	0.9537	8.84

Table- 6.4 Effect of Vincristine sulfate on brine shrimp nauplii

Conc (C) (µg/ml)	Log C	% Mortality (µg/ml)	LC_{50} (µg/ml)
20	1.301	100	0.32
10	1	100	
5	0.698	100	
2.5	0.397	90	
1.25	0.096	70	
0.625	-0.204	60	
0.3125	-0.505	40	
0.15625	-0.806	40	
0.07812	-1.1072	30	
0.0390	-1.4089	20	

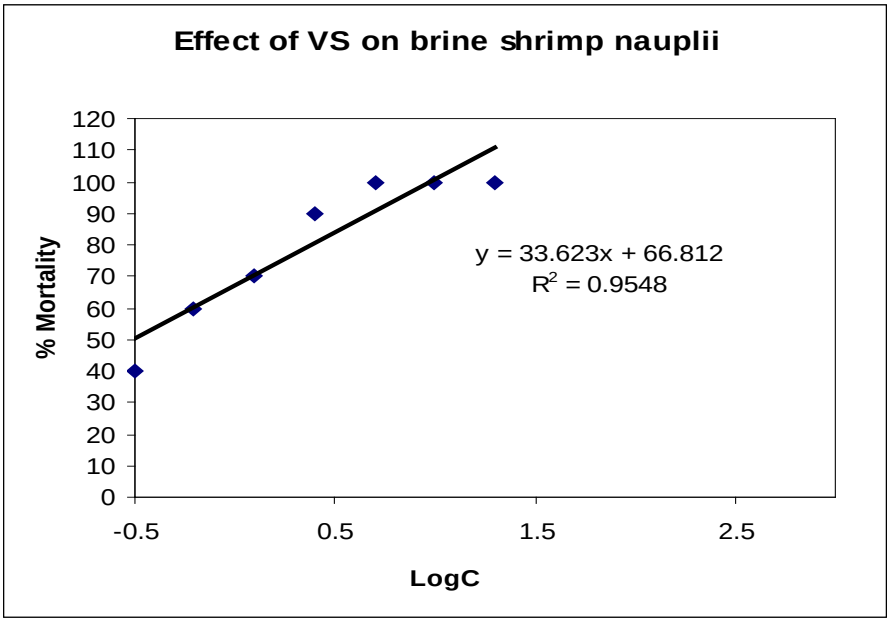


Fig 6.3: Effect of Vincristine sulfate on brine shrimp nauplii

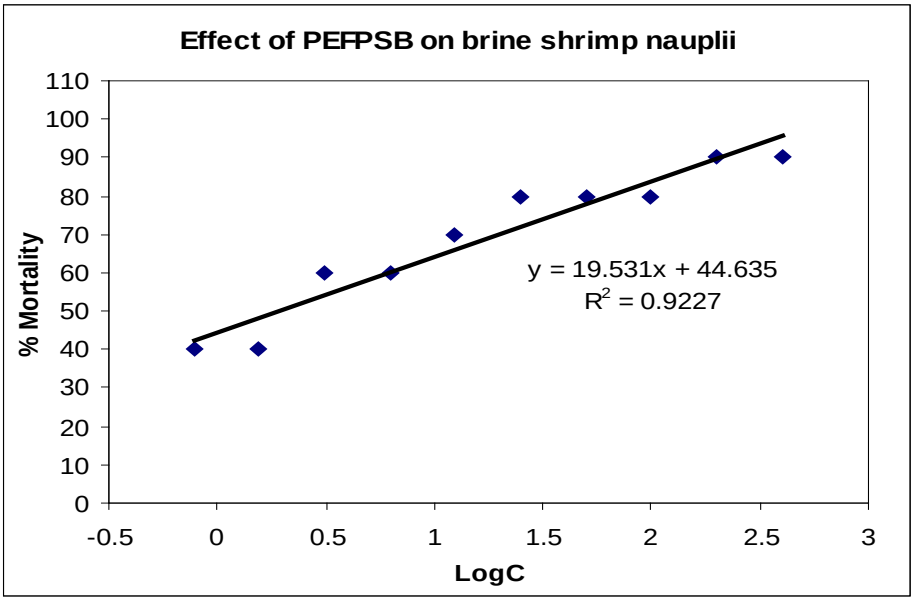


Fig 6.4: Effect of Petroleum ether soluble fraction on brine shrimp nauplii

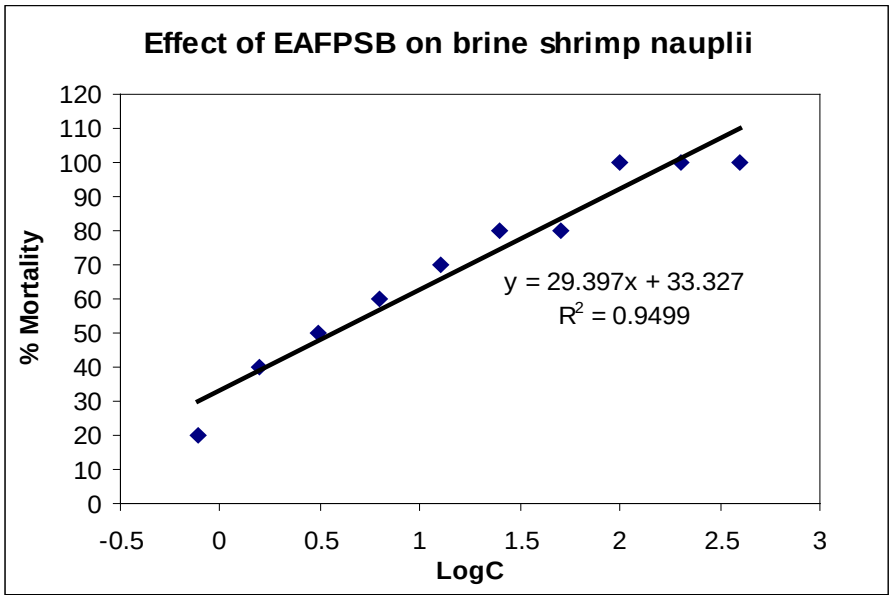


Fig 6.5: Effect of Etthyl acetate soluble fraction on brine shrimp nauplii

6.6.2 *Glochidion multiloculare*

The lethality of the Petroleum ether (PEFGM), Carbon tetrachloride (CTFGM), Chloroform (CFFGM) and water soluble (AQFGM) fractions of the methanolic extract as well as crude methanol soluble bark extract (MeEGM) of *G. multiloculare* to brine shrimp was investigated. The degree of lethality was directly proportional to the

concentration of the extract ranging from the lowest concentration (0.78125 µg/ml) to the highest concentration (400 µg/ ml). The LC_{50} obtained from the best-fit line slope were determined and summarized in Table 6.5 and 6.6 (Figure: 6.6, 6.7, 6.8, 6.9 and 6.10).

The LC_{50} values of PEFGM, CTFGM, CFFGM, MeEGM and AQFGM were found to be 3.11, 4.96, 7.56, 9.23 and 16.32 µg/ml, respectively (Table: 6.5 and 6.6). The positive control drug, vincristine sulfate showed LC_{50} at 0.32 µg/ml (Table:6.6) **PEFGM** exhibit significant mortality whereas CTFGM and CFFGM showed moderate activity.

Table- 6.5 Effect of methanol extract, petroleum ether, carbon tetrachloride, chloroform and aqueous soluble fractions of *G. multiloculare* on brine shrimp nauplii

Glochidion multiloculare											
Conc. (C) (µg/ml)	Log C	% Mortality					LC_{50} (µg/ml)				
		PEF GM	**CTF GM**	**CFF GM**	**MeE GM**	**AQF GM**	**PEF GM**	**CTF GM**	**CFF GM**	**MeE GM**	**AQF GM**
400	2.602	100	100	100	100	90	3.11	4.96	7.56	9.23	16.32
200	2.3010	100	100	100	100	80					
100	2.0000	100	100	100	80	70					
50	1.6989	100	90	100	80	70					
25	1.3979	90	90	60	70	50					
12.5	1.0969	90	70	50	70	50					
6.25	0.7958	60	60	40	50	40					
3.125	0.4948	50	40	30	20	30					
1.5625	0.1938	30	30	30	20	20					
0.78125	-0.1075	20	10	20	10	10					

Table- 6.6 LC_{50} data of test samples of *G. multiloculare*

Test samples	Regression line	R^2	LC_{50} (µg/ml)
VS (Positive control)	y = 33.623x + 66.812	0.9548	0.32
PEFGM	y = 31.813x + 34.313	0.8558	3.11
CTFGM	y = 34.431x + 26.048	0.9144	4.96
CFFGM	y = 35.236x + 19.043	0.9089	7.56
MeEGM	y = 35.437x + 15.792	0.9387	9.23
AQFGM	y = 28.793x + 15.081	0.9852	16.32

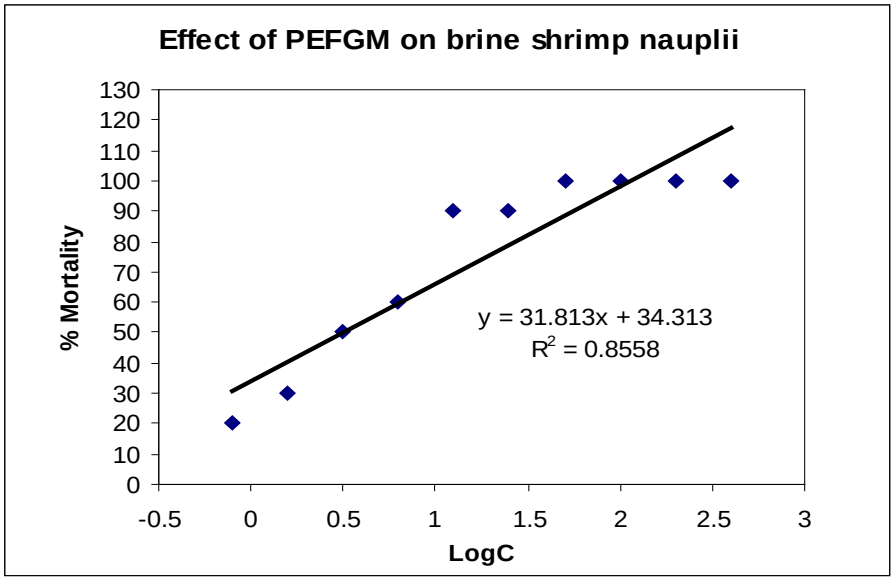


Fig 6.6: Effect of Petroleum ether soluble fraction of *G. multiloculare* on brine shrimp nauplii

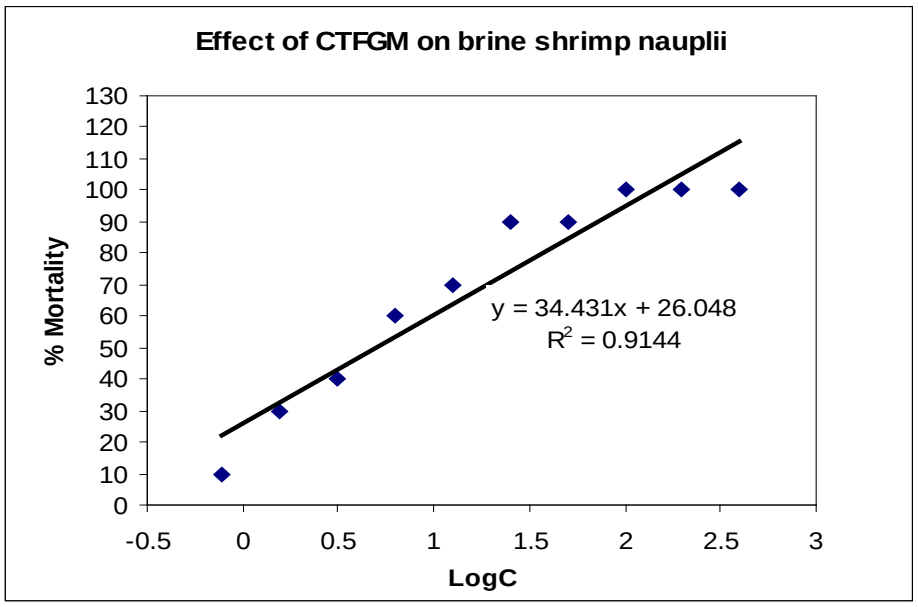


Fig 6.7: Effect of Carbontetrachloride soluble fraction of *G. multiloculare* on brine shrimp nauplii

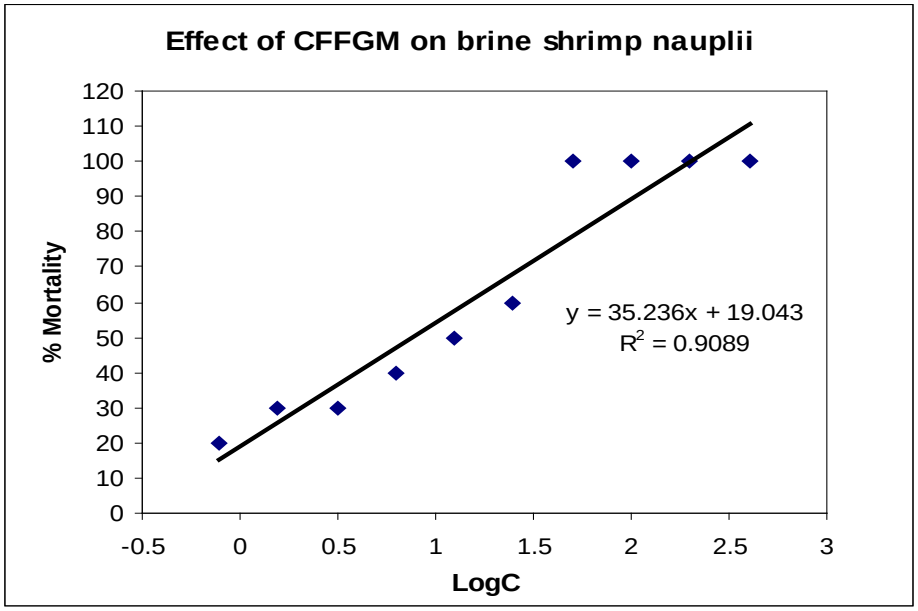


Fig 6.8: Effect of Chloroform soluble fraction of *G. multiloculare* on brine shrimp nauplii

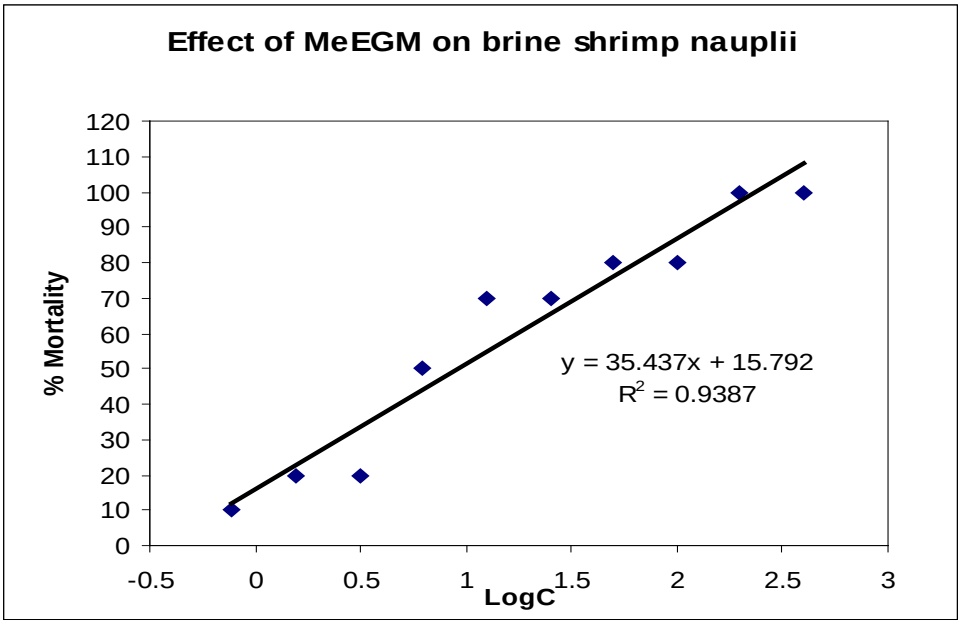


Fig 6.9: Effect of Methanol soluble extract of *G. multiloculare* on brine shrimp nauplii

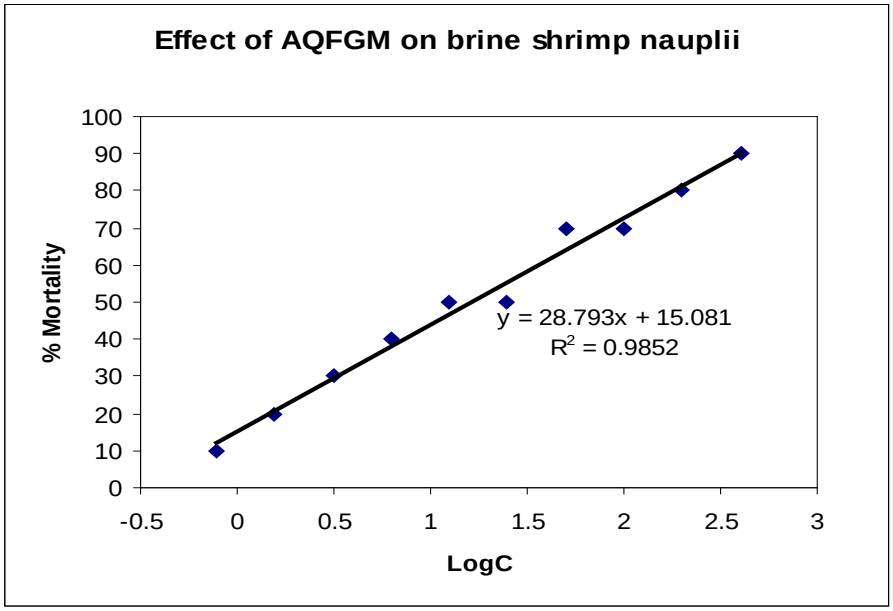


Fig 6.10: Effect of Aqeuous soluble fraction of *G. multiloculare* on brine shrimp nauplii

6.6.3 *Glochidion lanceolarium*

Following the procedure of Meyer, the lethality of the Petroleum ether (PEFGL), Carbon tetrachloride (CTFGL), Chloroform (CFFGL) and water soluble (AQFGL) fractions of the methanolic extract as well as crude methanol soluble bark extract (MeEGL) of *G. lanceolarium* to brine shrimp was investigated. The degree of lethality was directly proportional to the concentration of the extract ranging from the lowest concentration (0.78125 µg/ml) to the highest concentration (400 µg/ ml). The LC_{50} obtained from the best-fit line slope were determined and summarized in Table 6.7 and 6.8 (Figure: 6.11, 6.12, 6.13, 6.14 and 6.15).

The LC_{50} values of PEFGL, CTFGL, CFFGL, MeEGL and AQFGL were found to be 3.09, 6.70, 9.18, 14.24 and 17.68 µg/ml, respectively (Table: 6.7 and 6.8). The positive control drug, vincristine sulfate showed LC_{50} at 0.32 µg/ml (Table:6.8) **PEFGL** exhibit significant mortality whereas CTFGL and CFFGL showed moderate activity.

Table- 6.7: Effect of methanol extract, petroleum ether, carbon tetrachloride, chloroform and aqueous soluble fractions of *G. lanceolarium* on brine shrimp nauplii.

Glochidion lanceolarium											
Conc.(C) (µg/ml)	Log C	% Mortality					LC_{50} (µg/ml)				
		PEF GL	**CTF GL**	**CFF GL**	**MeE GL**	**AQF GL**	**PEF GL**	**CTF GL**	**CFF GL**	**MeE GL**	**AQF GL**
400	2.602	100	100	100	100	90	3.09	6.70	9.18	14.24	17.68
200	2.3010	100	100	100	90	80					
100	2.0000	100	100	90	70	60					
50	1.6989	90	80	80	70	60					
25	1.3979	90	60	60	50	60					
12.5	1.0969	80	60	50	50	40					
6.25	0.7958	60	50	40	40	40					
3.125	0.4948	50	40	30	30	30					
1.5625	0.1938	40	30	30	20	30					
0.78125	-0.1075	20	20	20	10	10					

Table- 6.8: LC_{50} data of test samples of *G. lanceolarium*

Test samples	Regression line	R^2	LC_{50} (µg/ml)
VS (Positive control)	y = 33.623x + 66.812	0.9548	0.32
PEFGL	y = 30.001x + 35.574	0.9079	3.09
CTFGL	y = 32.216x + 23.811	0.9649	6.70
CFFGL	y = 31.612x + 19.564	0.9713	9.18
MeEGL	y = 32.014x + 13.062	0.9809	14.24
AQFGL	y = 26.175x + 17.346	0.9484	17.68

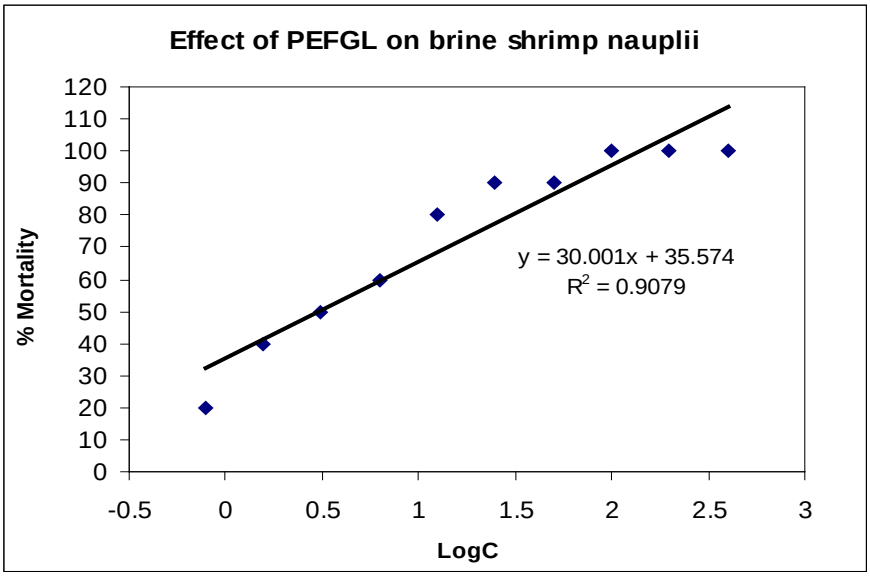


Fig 6.11: Effect of Petroleum ether soluble fraction of *G. lanceolarium* on brine shrimp nauplii

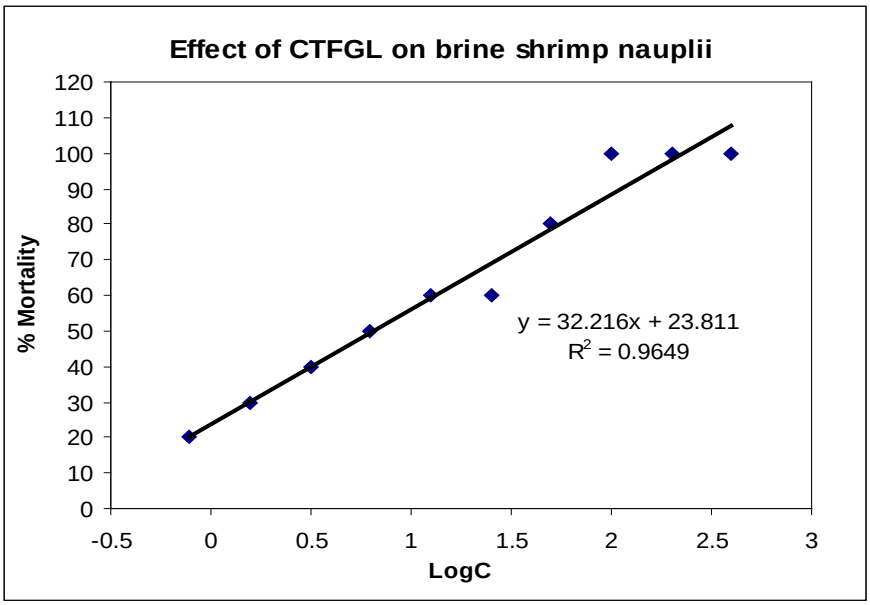


Fig 6.12: Effect of Carbontetrachloride soluble fraction of *G. lanceolarium* on brine shrimp nauplii

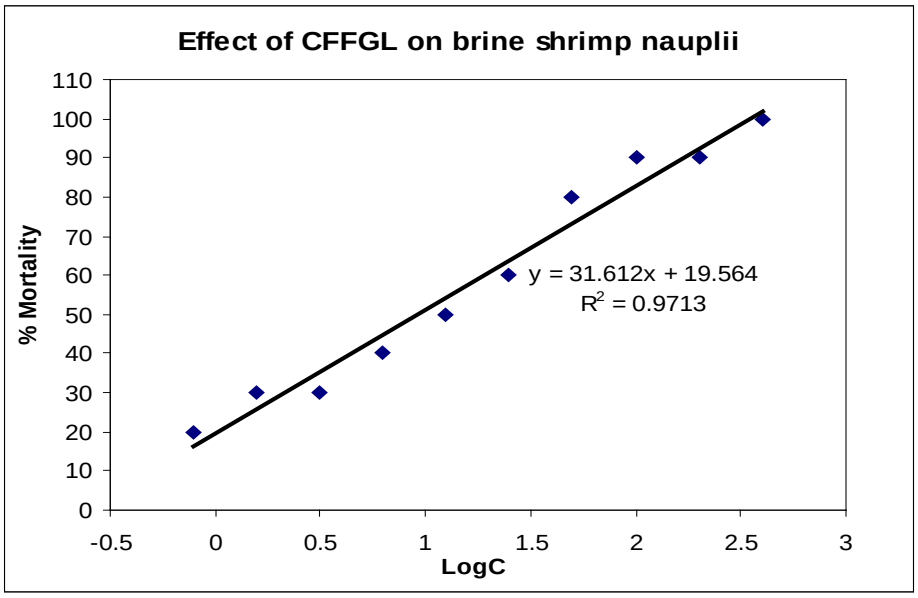


Fig 6.13: Effect of Chloroform soluble fraction of *G. lanceolarium* on brine shrimp nauplii

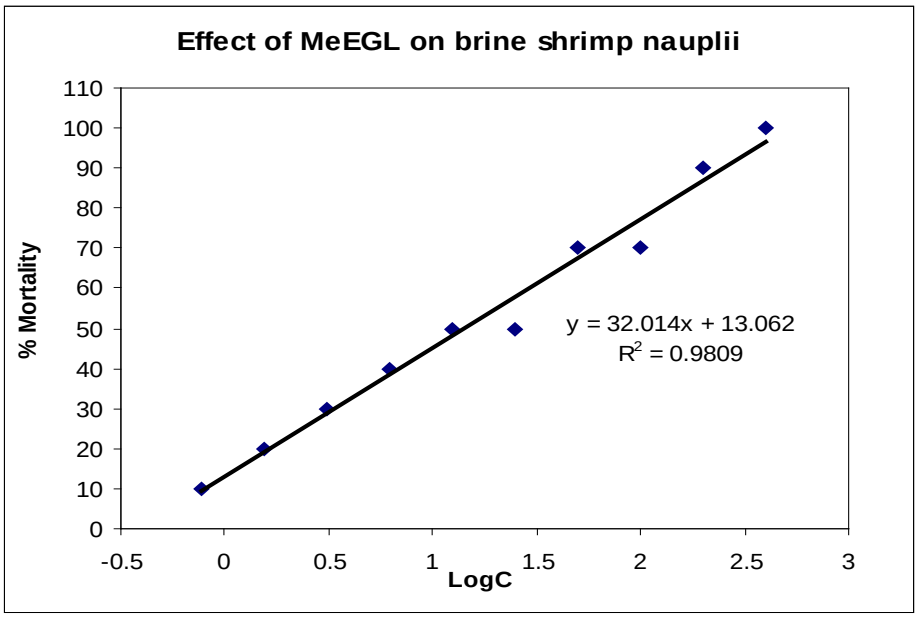


Fig 6.14: Effect of Methanol soluble extract of *G. lanceolarium* on brine shrimp nauplii

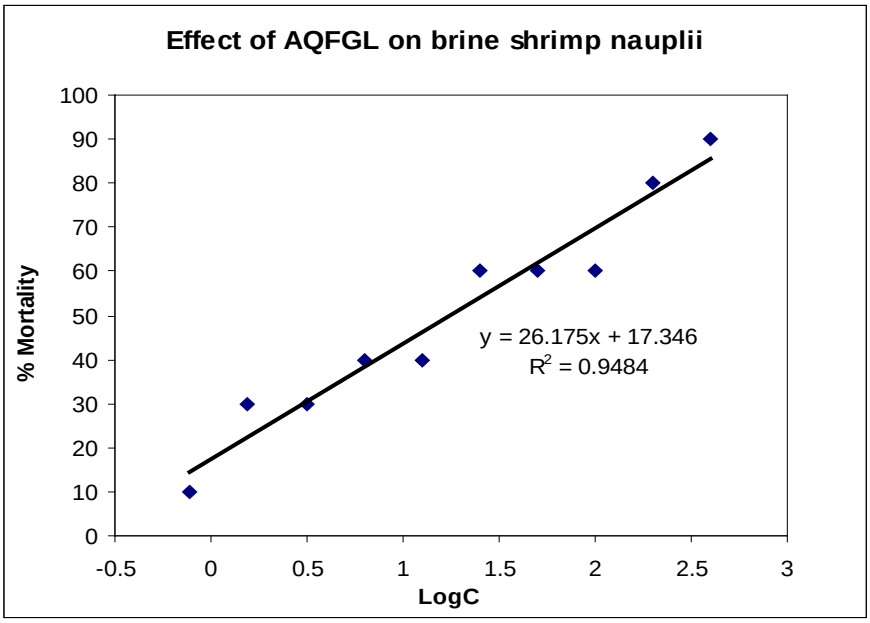


Fig 6.15: Effect of Aqeous soluble fraction of *G. lanceolarium* on brine shrimp nauplii

6.6.4 Isolated Compounds

Two pure compounds of the stem bark of *Polyalthia simiarum* were screened by brine shrimp lethality bioassay for probable cytotoxic activity. In this bioassay, the pure compounds showed different mortality rates at different concentrations (Table: 6.9 and Fig: 6.16), indicating that the compounds are biologically active. Compound **PSB-004 (**16β-Hydroxy-cleroda-3,13(14)*Z*-dien 15,16-olide, **164**) exhibited promising cytotoxic activity with LC_{50} value of 2.29 μg/ml, whereas the compound **PSB-007** (Kolavenic acid, **163**) showed moderate activity with LC_{50} value of 8.84 μg/ml.

Table- 6.9: Effect of pure compounds of *P. simiarum* (PSB-004 and PSB-007) on brine shrimp nauplii

Polyalthia simiarum					
Conc. (C) (μg/ml)	**LOG C**	**% Mortality**		**LC_{50} / (μg/ml)**	
		PSB-004	**PSB-007**	**PSB-004**	**PSB-007**
200	2.3010	100	100	2.29±0.18	8.84±0.50
100	2.0000	100	100		
50	1.6989	100	70		
25	1.3979	90	60		
12.5	1.0969	80	50		
6.25	0.7958	70	40		
3.125	0.4948	60	30		
1.5625	0.1938	40	20		
0.786125	-0.1075	30	20		
0.3930625	-0.408	20	10		

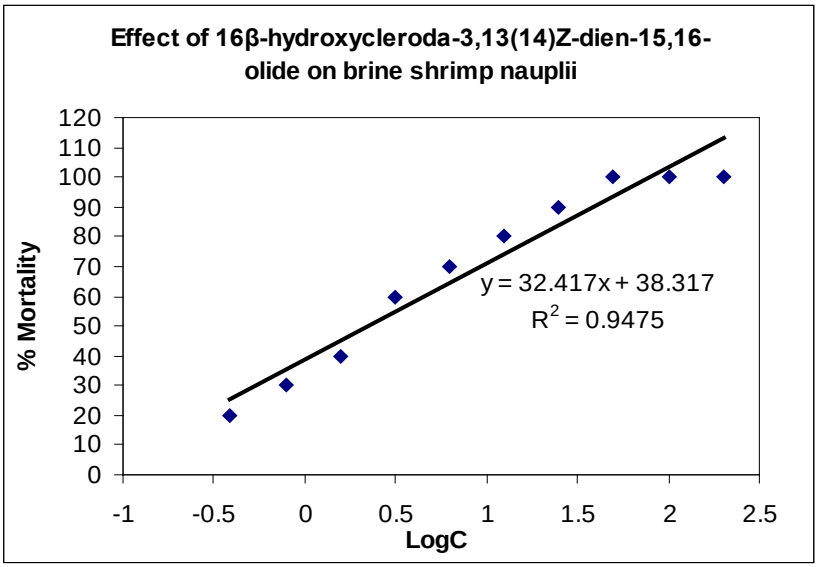


Fig 6.16: Effect of Pure compound (PSB-004) of *G. lanceolarium* on brine shrimp nauplii

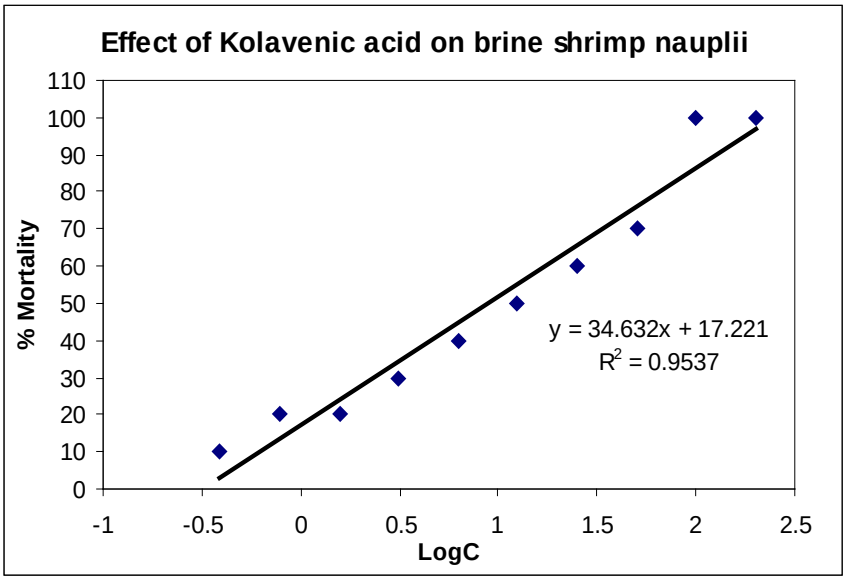


Fig 6.17: Effect of Pure compound (PSB-007) of *G. lanceolarium* on brine shrimp nauplii

6.7 Conclusion

The crude extracts, fractions of the plants; *Polyalthia simiarum, Glochidion multiloculare* and *Glochidion lanceolarium* and two pure compounds were screened by brine shrimp lethality bioassay for probable cytotoxic activity. In this bioassay, the crude extracts, fractions 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.

CHAPTER 7

Antimicrobial Screening

7.1 Introduction

Microbial infection is one of main causes of human diseases in the world and it is accounting for approximately one-half of all deaths in tropical countries. Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is that mortality rates due to infectious disease are actually increasing in developed countries, such as the United States. Deaths from infectious diseases, was ranked 5th in 1981, and has become the 3rd leading cause of death in 1992, with an increase of 58%. It is estimated that infectious disease is the underlying cause of death in 8% of the deaths occurring in the United States. This is really alarming as it was once believed that we would estimate infectious disease by the end of the millennium. The increases are attributed to increase in respiratory tract infections and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosicomial and community acquired infections. Again, the most dramatic increases are occurring in the 25-44 year old age group (Pinner *et al.*, 1996). Microbes are responsible for many infectious diseases. The increasing clinical importance of drug resistant microbial pathogens has lent additional urgency to antimicrobial research.

These negative health trends call for a renewed interest in infectious diseases in the medical and public health communities and renewed strategies on treatment and prevention. It is this last solution that would encompass the development of new antimicrobial agents (Fauci, 2001).

The antimicrobial screening, which is the first stage of antimicrobial drug research, is performed to ascertain the susceptibility of various microbes (bacteria and fungi) to any agent. This measures the ability of each test sample to inhibit the *in vitro* microbial growth and can be estimated by any of the following three methods.

i) Disc diffusion method

ii) Serial dilution method

iii) Bioautographic method

Actually there is no standardized method for expressing the results of antimicrobial screening (Ayafor *et al.*, 1982). Some investigators use the diameter of zone of inhibition and/or the minimum weight of extractive to inhibit (MIC) the growth of microorganisms. However, a great number of factors viz., the extraction methods (Nadir *et al*., 1986), inoculums volume, culture medium composition (Bauer *et al*., 1966), pH (Leaven *et al*., 1979) and incubation temperature (Barry, 1980) can influence the results.Among the mentioned techniques, the disc diffusion technique (Bauer *et al*., 1966) is a widely accepted *in vitro* investigation for preliminary screening of test agents, which may possess any antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between baceriostatic and bactericidal activity can be made by this method (Roland, 1982).

7.2 Principle of disc diffusion method

In diffusion method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Solutions of known concentration (μg/ml) of the test samples are made by dissolving measured amount of the samples in definite volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) are then impregnated with known amounts of the test substances using micropipette. Discs containing the test material are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic (kanamycin) discs and blank discs (impregnated with solvents) are used as positive and negative control. These plates are then kept at low temperature (4^oC) for 24 hours to allow maximum diffusion. During this time dried discs absorb water from the surrounding media and then the test materials are dissolved and diffused out of the media. The diffusion occurs according to the physical law that controls the diffusion of molecules through agar gel (Barry, 1980). As a result there is a gradual change of test materials concentration in the media surrounding the discs. The plates are then inverted and incubated at 37^oC for 24 hours to allow maximum growth of the organisms. If the test materials possess antimicrobial property, it inhibited microbial growth in the media surrounding the discs and thereby yielded a clear, distinct area as 'Zone of Inhibition'. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimeter (Bauer *et al*., 1966).

7.3 Experimental

In the present study all the crude extracts as well as partitionates were tested for antimicrobial activity by disc diffusion method.

7.3.1 Apparatus and Reagents

Filter paper discs	Autoclave
Nutrient agar medium	Laminar air flow hood (BASSAIRE, Type 03VB, AOQC)
Petridishes	Spirit burner
Sterile cotton	Refrigerator
Micropipette	Incubator
Inoculating loop	Ethanol
Sterile forceps	Chloroform
Screw cap test tubes	Nose mask and hand gloves

7.3.2 Test Organisms

The bacterial strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. Both gram positive and gram-negative organisms were taken for the test and they are listed in Table 6.1.

Table 7.1: Test microorganisms used in screening

Gram positive bacteria	Gram negative bacteria	Fungi
Bacillus cereus	*Escherichia coli*	*Candida albicans*
B. megaterium	*Pseudomonas aeruginosa*	*Aspergillus niger*
B. subtilis	*Salmonella typhi*	*Sacharomyces cerevaceae*
Sarcina lutea	*S. paratyphi*	
Staphylococcus aureus	*Shigella boydii*	
	Sh. dysenteriae	
	Vibrio mimicus	
	V. parahemolyticus	

7.3.3 Test samples

Table 7.2: List of plant samples for microbial studies

Plant	Test samples	Code	Amount (mg)
Polyalthia simiarum	Petroleum ether soluble fraction of stem bark	PEFPSB	8.00
	Ethyl acetate soluble fraction of stem bark	EAFPSB	8.00
Glochidion multiloculare	Methanol soluble bark extract	MeEGM	8.00
	Petroleum ether soluble fraction of MeEGM	PEFGM	8.00
	Carbon tetrachloride soluble fraction of MeEGM	CTFGM	8.00
	Chloroform soluble fraction of MeEGM	CFFGM	8.00
	Aqueous soluble fraction of MeEGM	AQFGM	8.00
Glochidion lanceolarium	Petroleum ether soluble fraction of MeEGL	PEFGL	8.00
	Carbon tetrachloride soluble fraction of MeEGL	CTFGL	8.00
	Chloroform soluble fraction of MeEGL	CFFGL	8.00
Compounds **(**Bark of *P. simiarum)*	16β-Hydroxy-cleroda-3,13(14) *Z*-dien15,16-olide (**164**)	PSB-004	2.00
	Kolavenic acid (**163**)	PSB-007	2.00

7.3.4 Culture medium

Nutrient agar medium (DIFCO) was used for making plates on which the sensitivity of the organisms to the tests materials were carried out. This medium was also used to prepare fresh cultures.

Composition of Nutrient Agar Medium

Ingredients	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
pH (at 25°C)	7.2-7.6

7.3.5 Preparation of medium

To prepare required volume of this medium, calculated amount of each of the constituents was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The pH (at 25^{o}C) was adjusted to 7.2-7.6 using NaOH or HCl. Then 10 ml and 5 ml of the medium were then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized.

Sterilization procedures

In order to avoid any type of contamination by the test organisms the antimicrobial screening was done in laminar hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petridishes, capped test (medium containing) and other glassware were sterilized by autoclaving at a temperature of 121^{o}C and a pressure of 15 lbs./sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized by UV light.

Preparation of subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37^{0}C for their optimum growth. These fresh cultures were used for the sensitivity test.

Preparation of the test plates

The test organisms were transferred from the subculture to the test tubes containing about 10 ml of sterilized agar medium with the help of a sterilized transfer loop in an aseptic

area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The microbial suspension was immediately transferred to the sterilized Petri dishes. The Petri dishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

7.3.6 Preparation of discs

Three types of discs were used for antimicrobial screening.

Standard discs: These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. In this investigation, kanamycin (30 μg/disc) standard disc was used as the reference (Positive control).

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

Sample discs: Measured amount of each test samples (specified in Table 6.2) was dissolved in specific volume of solvent (methanol or chloroform) to obtain the desired concentration in an aseptic condition. Sterilized metricel (BBL, Cocksvillie, USA) filter paper was taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

7.3.7 Diffusion and Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test microbes. The plates were then kept in a refrigerator at 4^{o}C for about 24 hours to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37^{o}C for 24 hours.

7.3.8 Determination of the Zone of Inhibition

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the antimicrobial activity of the test materials was determined by

measuring the diameter of the zone of inhibition in millimeter with transparent scale (Figure 6.1-6.2).

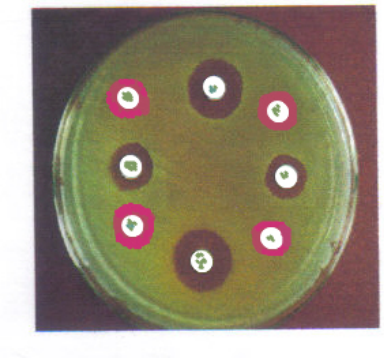


Fig.- 7.1 Clear zone of inhibition

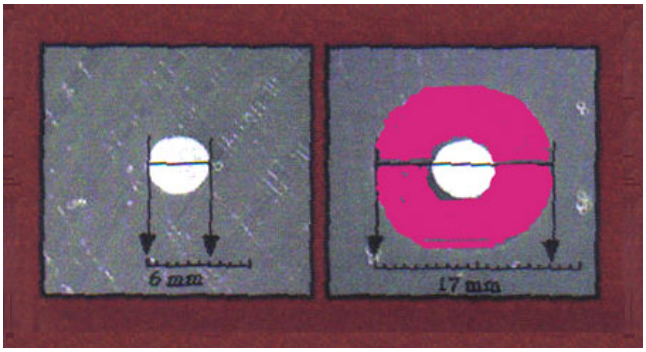


Fig.- 7.2 Determination of clear zone of inhibition

7.4 Results and Discussion

Crude extracts, and various partitionates of crude extracts (400 µg/disc) were tested for antimicrobial activity against a number of both gram positive and gram negative bacteria. Standard disc of kanamycin (30 µg/disc) was used for comparison purpose.

7.4.1 *Polyalthia simiarum*

The crude petroleum ether (PEFPSB) and ethyl acetate (EAFPSB) fractions showed promising antibacterial activity with the average zone of inhibition of **20-28** mm and **21-28** mm, respectively, at 400µg/disc. The pet-ether fraction showed the highest activity against the growth of *B. megaterium* having the zone of inhibition of **28** mm. Besides the growth of *B. cereus* (25 mm), *B. subtilis* (25 mm), *S. sutea* (25 mm), *S. boydii* (24 mm), *S. aureus* (24 mm), *P. aeruginasa* (23 mm), *S. paratyphi* (23 mm), *S. dysenteriae* (23 mm) exhibited prominent activity. In the case of fungi, the average zone of inhibition was found to be **25-28** mm. At the same time, the ethyl acetate extracts also inhibited the growth of *B. megaterium* (27 mm), *S. lutea* (27mm), *B. subtilies* (25 mm), *p. aeruginosa* (25mm), *S. paratyphi* (25 mm), *S.* b*oydii* (25 mm), *S. dysenteriae* (25 mm), *B.* cereus (23 mm), *S. aureus* (23 mm) and *V. mimicus* (23 mm) significantly. The same fraction also exhibited high inhibitory activity against the growth of fungal strains.

Table-7.3: Antimicrobial activity of *P. simiarum* fractions (400 µg/disc) and kanamycin (30 µg/disc).

Test microorganisms	Diameter of zone of inhibition (mm)		
	PEFPSB	EAFPSB	KAN (Standard)
Gram positive bacteria			
Bacillus cereus	25	23	35
B. megaterium	28	27	38
B. subtilis	25	25	36
Staphylococcus aureus	24	23	35
Sarcina lutea	25	27	36
Gram negative bacteria			
Escherichia coli	--	--	37
Pseudomonas aeruginosa	23	25	27
Salmonella paratyphi	23	25	36
S. typhi	20	21	35
Shigella dysenteriae	24	25	36
Vibrio mimicus	21	23	37
V. parahemolyticus	21	21	35
Fungi			
Candida albicans	27	28	35
Aspergillus niger	25	25	35
Sacharomyces cerevaceae	25	28	38

KAN: standard kanamycin disc (30 µg/disc); a diameter of zone of inhibition less than 8 mm was considered inactive.

7.4.2 *Glochidion multiloculare*

The methanolic extract of the stem bark (MeEGM) of *G. multiloculare* as well as its petroleum ether (PEFGM), carbon tetrachloride (CTFGM), chloroform (CFFGM) and aqueous (AQFGM) soluble fractions were subjected to microbiological screening. In this test, the CFFGM of the methanolic extract appeared to be moderate in terms of both zone of inhibition and spectrum of activity.

In this study, the zones of inhibition produced by the CFFGM, PEFGM and CTFGM were ranged from 9-12 mm, 8-10 mm and 7-9 mm respectively (Table-7.4). The CFFGM showed moderate inhibitory activity against *B. subtilis* having the zone size **12 mm** and against *Escherichia coli and Escherichia coli* were **11 mm**.

This fraction also showed mild inhibitory activity against *Bacillus cereus, B. megaterium, Sarcina lutea, Pseudomonas aeruginosa, S. typhi, Shigella dysenteriae,* Sh. boydii*, V. parahemolyticus,* Sacharomyces cerevaceae (10 mm each). At the same time, the petether soluble fraction demonstrated mild inhibitory activity against *V. parahemolyticus, Candida albicans, Aspergillus niger ,* Sacharomyces cerevaceae (10 mm each). The CTFGM and AQFGM of *G. multiloculare* exhibit the least activity against any microbe. The MeEGM showed no activity.

Table 7.4: Antimicrobial activity of test samples of *G. multiloculare*

Test microorganisms	Diameter of zone of inhibition (mm)					
	PEFGM	CTFGM	CFFGM	MeEGM	AQFGM	KAN (Standard)
Gram positive bacteria						
Bacillus cereus	09	08	10	--	--	33
B. megaterium	09	08	10	--	--	33
B. subtilis	09	09	12	--	07	33
Sarcina lutea	09	08	10	--	--	33
Gram negative bacteria						
Escherichia coli	08	09	11	--	--	33
Pseudomonas aeruginosa	08	08	10	--	07	33
Escherichia coli	08	09	11	--	07	33
S. typhi	08	08	10	--	--	33
Shigella dysenteriae	08	08	10	--	07	33
Sh. boydii	09	07	10	--	--	33
Vibrio mimicus	09	07	09	--	07	32
V. parahemolyticus	10	08	10	--	--	33
Fungi						
Candida albicans	10	08	09	--	--	32
Aspergillus niger	10	09	09	--	07	33
Sacharomyces cerevaceae	10	09	10	--	08	33

KAN: standard kanamycin disc (30 µg/disc); a diameter of zone of inhibition less than 8 mm was considered inactive.

7.4.3 *Glochidion lanceolarium*

The petroleum ether fraction(PEFGL) of *G. lanceolarium* was screened against 12 test bacteria and 3 fungi. This fraction showed no activity against all the test bacteria and the fungi cited in Table-7.5. The chloroform fraction (CFFGL) was screened against 12 test bacteria and 3 fungi. The zone of inhibition was **07-08** mm.This fraction showed poor activity against the test bacteria *Bcillus cereus, B. megaterium, B. subtilis, Staphylococcus aureus, Sarcina lutea, Escherichia coli, Pseudomonas aeruginosa,*

Salmonella paratyphi, S. typhi, Shigella boydii, Vibrio mimicus, V. parahemolyticus and fungi *Candida albicans, Aspergillus niger, Sacharomyces cerevaceae.*

The carbontetrachloride fraction (CTFGL) was screened against 12 test bacteria and 3 fungi. The zone of inhibition was **07-10**mm. The fraction exhibited moderate activity against the test bacteria *S. typhi* and *V. parahemolyticus* and also the fungi *Candida albicans, Aspergillus niger.* On the other hand, the bacteria *Bcillus cereus, B. megaterium, B. subtilis, Staphylococcus aureus, Sarcina lutea, Escherichia coli, Pseudomonas aeruginosa, Salmonella paratyphi, Shigella boydii, Vibrio mimicus,* and the fungus *Sacharomyces cerevacae* were found to be poor active to this extract.

Table 7.5: Antimicrobial activity of test samples of *G. lanceolarium*

Test microorganisms	**Diameter of zone of inhibition (mm)**			
	PEFGL	**CFFGL**	**CTFGL**	**KAN (Standard)**
Gram positive bacteria				
Bacillus cereus	--	7	7	34
B. megaterium	--	8	9	34
B. subtilis	--	7	7	34
Staphylococcus aureus	--	7	7	34
Sarcina lutea	--	7	8	33
Gram negative bacteria				
Escherichia coli	--	7	9	33
Pseudomonas aeruginosa	--	7	7	33
Salmonella paratyphi	--	7	8	33
S. typhi	--	7	10	34
Shigella dysenteriae	--	7	7	34
Vibrio mimicus	--	7	8	34
V. parahemolyticus	--	7	10	34
Fungi				
Candida albicans	--	7	10	34
Aspergillus niger	--	7	9	34
Sacharomyces cerevaceae	--	**7**	7	34

KAN: standard kanamycin disc (30 µg/disc); a diameter of zone of inhibition less than 8 mm was considered inactive.

7.4.4 Pure compounds of *Polyalthia simiarum*

In case of pure compounds, PSB-004 (**164**) demonstrated moderate antimicrobial activities against 12 test bacteria and 3 fungi, having the zone of inhibition **10-14** mm. Compound PSB-007 (**163)** showed poor activity against test bacteria and fungi (Table-7.6).

Table-7.6: Antimicrobial activity of Pure compounds (PSB-004 and PSB-007) of *P. simiarum* (200 µg/disc) and kanamycin (30 µg/disc).

Test microorganisms	Diameter of zone of inhibition (mm)		
	PSB-004	PSB-007	KAN (Standard)
Gram positive bacteria			
Bacillus cereus	10	--	35
B. megaterium	10	--	35
B. subtilis	13	--	36
Staphylococcus aureus	13	--	36
Sarcina lutea	14	7	36
Gram negative bacteria			
Escherichia coli	13	7	36
Pseudomonas aeruginosa	14	--	36
Salmonella paratyphi	14	7	36
S. typhi	14	7	35
Shigella dysenteriae	13	7	36
Vibrio mimicus	14	7	35
V. parahemolyticus	14	7	35
Fungi			
Candida albicans	14	7	35
Aspergillus niger	14	7	35
Sacharomyces cerevaceae	14	7	35

KAN: standard kanamycin disc (30 µg/disc); a diameter of zone of inhibition less than 8 mm was considered inactive.

7.5 Conclusion

The present study was commenced in a view to evaluate the biological activities of the extractives from three selected plants *viz. Polyalthia simiarum, Glochidion multiloculare and Glochidion lanceolarium* as well as to isolate compounds having medicinal properties. The present study was conducted to justify the medicinal properties of these plants and to isolate bioactive compounds. For doing so a number of fractions and isolated two compounds have been tested for their biological responses through antibacterial study and screening. Some extractives and pure compounds showed mild to significant responses. But further higher research is necessary to establish the chemical structures and pharmacological properties of the compounds.

CHAPTER 8

Analgesic Activity

8.1 Introduction

Pain has been defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Hossain *et al*., 2011). The direct and indirect action of chemical mediators, such as arachidonic acid metabolites (prostaglandins and leukotrienes), peptides, serotonin, acetylcholine, cytokines, nitric oxide, among others, which can be produced or released following tissue injury or by exogenous irritants (formalin, acetic acid), are responsible for the multiplicity of events that occur during pain transmission, in both the peripheral and central nervous systems (Alam *et al*., 2012).

8.2 Analgesic Activity of *Polyalthia simiarum*

8.2.1 Materials and Methods

8.2.2 Chemicals

Diclofenac-Na, Nalbuphine and Indomethacin were collected from Square Pharmaceuticals Ltd., Bangladesh. All chemicals and reagents were of highest analytical grade.

8.2.3 Preparation of extract

The air dried and powdered plant material (750 g) was extracted in a Soxhlet apparatus with ethyl acetate and pet ether (60-80°C). The extract was filtered through a fresh cotton plug followed by Whatman no.1 filter paper. The filtrate was then concentrated with a Buchii rotavapor at low temperature and pressure to afford ethyl acetate extract (EA, 3.5 g) and pet ether (PE, 2.25 g).

8.2.4 Animal

Swiss albino mice (25-30 g) and Wister rats (175-250 g) of both sexes were used for assessing the biological activity. The animals were maintained under standard laboratory conditions and had free access to food and water *ad libitum.* The animals were allowed to acclimatize to the environment for 7 days prior to experimental session. The animals

were divided into different groups, each consisting of six animals which were fasted overnight prior to the experiments. Experiments with the animals were performed in accordance with guidelines of the Institutional Animal Ethics Committee, Department of Pharmacy, BRAC University, Bangladesh.

8.2.5 Acute toxicity

The acute oral toxicity of the plant extract in Swiss albino mice was studied as per established protocol (Riaz *et al*., 2010).

8.3 Analgesic activity

8.3.1 Tail flick test

The animals were divided into six groups with six mice in each group.

Group- I= animals received vehicle (1% Tween 80 in water, 10 mL kg^{-1} body weight).
Group –II= animals were treated with 50 mg/kg body weight (p.o.) of the PE extract of *P. simiarum*
Group- III= animals were treated with 50 mg/kg body weight (p.o.) of the EA extract of *P. simiarum*

Group –IV= animals were treated with 100 mg/kg body weight (p.o.) of the PE extract of *P. simiarum*

Group –V= animals were treated with 100 mg/kg body weight (p.o.) of the EA extract of *P. simiarum*

Group –VI= animals received nalbuphine at 5 mg kg^{-1} body weight.

From 1-2 cm of the tail of mice was immersed in warm water kept at constant temperature of 60°C. The reaction time was the time taken by the mice to deflect their tails. The first reading was discarded and the reaction time was recorded as a mean of the next three readings. A latency period of 20 second was defined as complete analgesia and the measurement was then stopped to avoid injury to mice. The latent period of the tail-flick response was determined before and 0, 30, 60 and 90 min after the oral administration of drugs (Toma *et al*., 2003).

8.3.2 Acetic acid-induced writhing test

The analgesic activity of the samples was also studied using acetic acid-induced writhing model in mice. Test samples and vehicle were administered orally 30 min before intraperitoneal administration of 0.7% acetic acid but Diclofenac-Na was administered intraperitonially 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for specific contraction of body referred to as 'writhing' for the next 10 min (Ghule *et al*., 2011).

The animals were divided into six groups with six mice in each group.

Group-I= animals received vehicle (1% Tween 80 in water).

Group- II= animals were treated with 50 mg/kg body weight (p.o.) of the PS-PE

Group- III= animals were treated with 50 mg/kg body weight (p.o.) of the PS-EA.

Group- IV= animals were treated with 100 mg/kg body weight (p.o.) of the PS-PE

Group- V= animals were treated with 100 mg/kg body weight (p.o.) of the PS-EA.

Group VI= animals received Diclofenac Na 10 mg/kg body weight (p.o.).

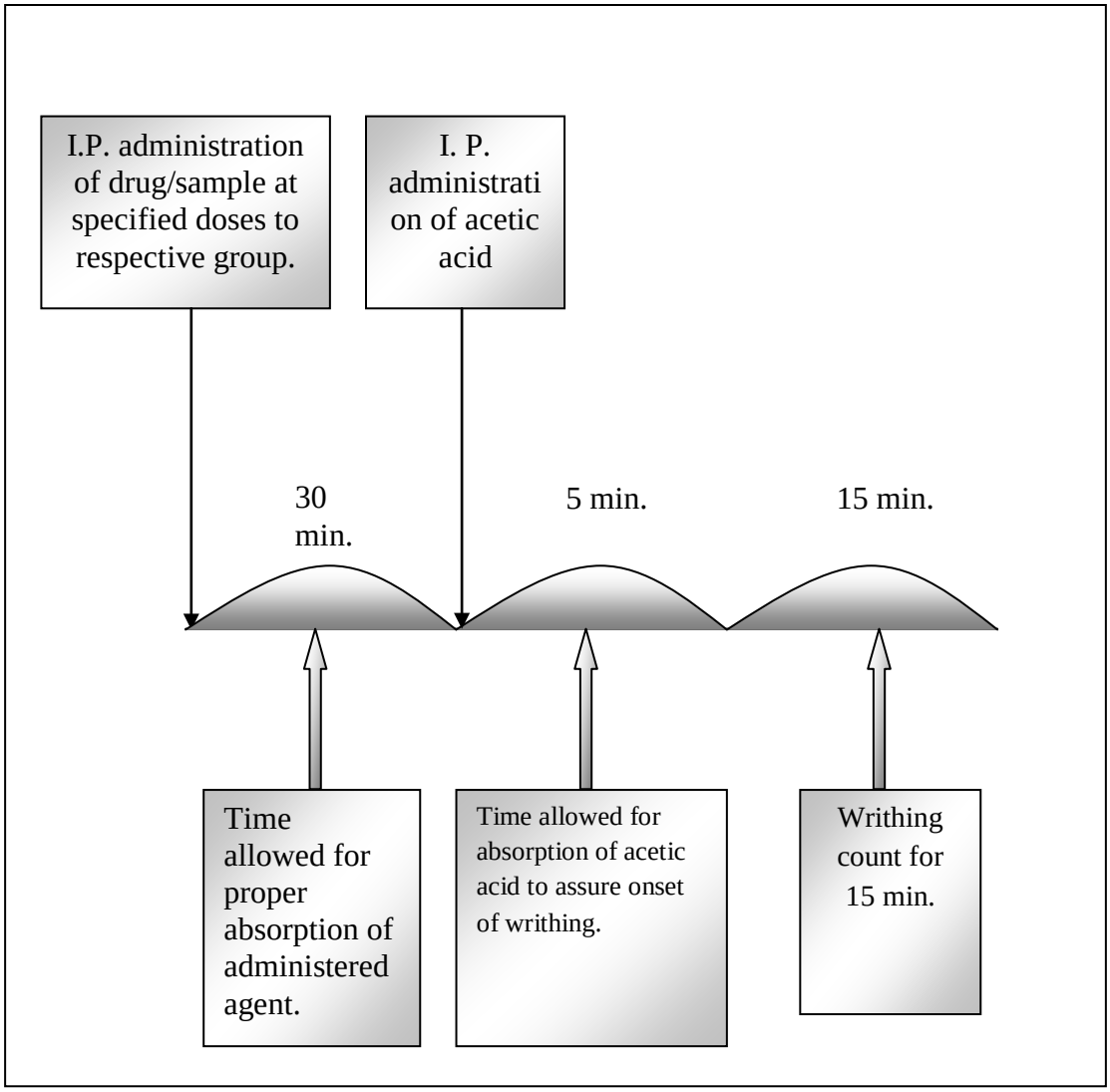
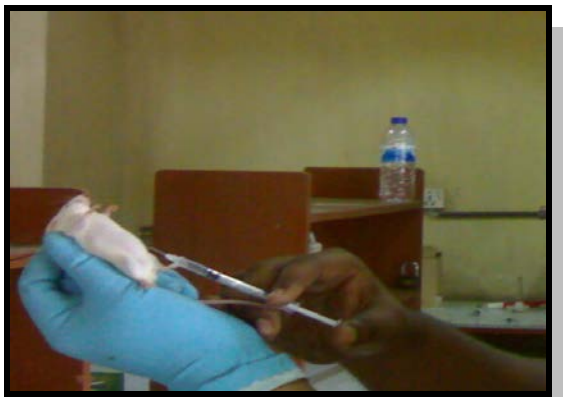


Figure 8.1: Schematic representation of acetic acid induced writhing of mice for investigation of analgesic activity

(A)



(B)

(C)

(D)

Figure- 8.2 (A): Housing of test animal, (B) Intraperitoneal administration of acetic acid, (C) Oral administration of test sample and (D) Writing effect of mice

8.4: Statistical analysis

Analgesic data are expressed as mean ± S.E.M. (n = 6 mice per groups). Statistical significance (p) calculated ANOVA followed by Dunnett's-T test $^{*}P<0.01$ and $^{**}P<0.001$ were considered to be statistically significant.

8.5: Results

8.5.1 Preliminary phytochemical investigation

The phytoconstituents present in the EA and PE extract of *P.simiarum* were identified by various chemical tests which showed the presence of alkaloids, terpenoids, phenolic and flavonoid compounds and steroids (Table-8.1).

8.5.2 Acute toxicity study

The acute toxicity study was conducted to establish the therapeutic index, i.e., the ratio between the pharmacologically effective dose and the lethal dose on the same strain and species. The extracts of *P. simiarum* were safe up to a dose of 1000 mg/kg (p.o.) body weight.

8.5.3: Analgesic activity

8.5.3.1: Tail flick method

The tail withdrawal reflex time following administration of the EA and PE were found to increase with increasing dose of the sample. The result was statistically significant (*p<0.01-0.001) and was comparable to the reference drug nalbuphine (Figure-8.3). As noted, nalbuphine, the reference narcotic analgesic drug (5 mg/kg, p.o.) exhibited significant and paramount analgesic effects (supra spinal), the tail immersion (spinal) test; whereas, EA and PE (for both extract 50 and 100 mg/kg, p.o.) also produced a statistically significant but lesser in degree antinociceptive response to that of nalbuphine in this test suggesting that the plant extracts may act as a narcotic analgesic.

8.5.3.2: Writhing test method

The Table- 8.2 shows the effects of the extract of on acetic acid-induced writhing in mice. oral administration of both doses of EA and PE significantly (**p<0.001) inhibited writhing response induced by acetic acid in a dose dependent manner.

Table-8.1: Result of chemical group tests of the EA and PE extract of *Polyalthia simiarum*.

Extract	Triterpene	Diterpene	Flavonoid	Phenol	Sterol	Alkaloid
EA extract	+	+++	+++	++	+++	++
PE extract	++	++	+	+	+++	+

EA: Ethyl acetate; PE: Petroleum ether; (+): Present; (-): Absent; (+ + +): Reaction intensity is high; (+ +): Reaction intensity is medium; (+): Reaction intensity is normal.

Table-8.2: Effect of extracts of *P. simiarum* on tail withdrawal reflex of mice induced by tail flick method.

Treatment	Tail flict time			
Group	0 MIN	30 MIN	60 MIN	90 MIN
I	2.34±0.22	2.44±0.30	2.39±0.33	2.46±0.44
II- Petether (50mg/kg)	2.35±0.29	2.42±0.20	2.55±0.26	3.10±0.31
III-Ethyl acetate (50 mg/kg)	2.36±0.33	2.45±0.37	2.45±0.37	3.25±0.30
IV- Petether (100 mg/kg)	2.34±0.27	2.34±0.27	3.55±0.22	4.36±0.38
V- Ethyl acetate (100 mg/kg)	2.33±0.27	3.19±0.22	3.73±0.33	4.48±0.28
VI- Control (Nalbuphine 5 mg/kg)	2.36±0.27	3.56±0.33	4.13±0.20	5.07±0.19

Values are mean ± SEM, (n = 6); Statistical significance (p) calculated ANOVA followed by Dunnett's-T test $^{*}P<0.01$ and $^{**}P<0.001$ were considered to be statistically significant.

Table-8.3 Effect of extracts of *Polyalthia simiarum* on acetic acid-induced writhing in mice.

Group	Dose (mg/kg body Wt.)	Writhing no.	% inhibition of writhing no.
I		30.0 ± 2.57	
II	50	25.0±2.15	16.66
III	50	19.0±1.5	36.66
IV	100	18.0 ± 1.15 **	40.0
V	100	13.0 ± 0.57 **	58.06
VI	10	9.0 ±1.57 **	70.96

Values are mean ± SEM, (n = 6); ** p<0.001, Dunnett's-T test as compared to vehicle control.

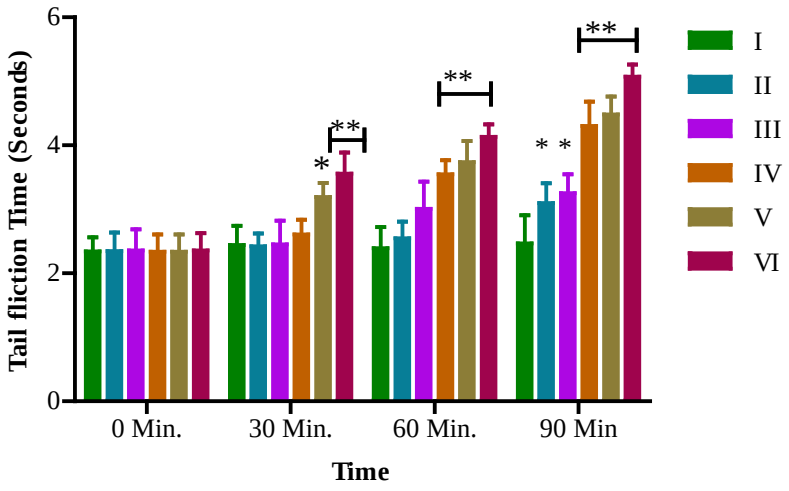


Figure- 8.3: Effects of the PS on tail withdrawal reflex of mice induced by tail immersion method. Values are mean ±SEM, (n = 6); *p<0.01 and **p<0.001, Dunnett's-T test

8.5.4 Discussion

The tail flick method is commonly used for assessing central antinociceptive response. The method is further distinguished by their tendency to respond to the pain stimuli conducting through neuronal pathways as tail immersion mediates a spinal reflex to nociceptive stimuli (Chapman *et al*.,1985). Narcotic analgesics inhibit both peripheral and central mechanism of pain, while non steroidal anti-inflammatory drugs inhibit only peripheral pain (Elisabetsky *et al*., 1995; Pal *et al*., 1999). As noted, nalbuphine, the reference narcotic analgesic drug (5 mg/kg, p.o.) exhibited significant and paramount analgesic effects in both the hot plate (supra spinal) as well as the tail flick (spinal) test; whereas, EA (50 and 100 mg/kg, p.o.) and PE (50 and 100 mg/kg, p.o.) also produced a statistically significant but lesser in degree antinociceptive response to that of nalbuphine in both test suggesting that the plant extract may act as a narcotic analgesic. However, the mechanism (s) behind the central analgesic response of EA and PE in both tested methods is not completely understood and may need further investigation.

On the otherhand, acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics and represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free

arachidonic acid from the tissue phospholipids (Ahmed *et al*., 2006). The response is thought to be mediated by peritoneal mast cells (Ribeiro *et al*., 2000), acid sensing ion channels (Voilley, 2004) and the prostaglandin pathways (Hossain *et al*., 2006). The organic acid has also been postulated to act indirectly by inducing the release of endogenous mediators, which stimulates the nociceptive neurons that are sensitive to NSAIDs and narcotics (Adzu *et al*., 2003). It is well known that non-steroidal anti-inflammatory and analgesic drugs mitigate the inflammatory pain by inhibiting the formation of pain mediators at the peripheral target sites where prostaglandins and bradykinin are proposed to play a significant role in the pain process (Hirose *et al*., 1984).

In addition, it was suggested that non narcotic analgesics produce their action by interfering with the local reaction to peritoneal irritation thereby reducing the intensity of afferent nervous stimulation in the acetic acid induced writhing test, a model of visceral pain (Vogel, 1997). Therefore, it is likely that EA and PE might have exerted its peripheral antinociceptive action by interfering with the local reaction caused by the irritant or by inhibiting the synthesis, release and/or antagonizing the action of pain mediators at the target sites and this response in agreement with the previous studies of *D. indica*, leaves (Bose *et al*., 2010). The above findings clearly demonstrated that both central and peripheral mechanisms are involved in the antinociceptive action of EA and PE.

8.6 Conclusion

Our results indicate that the EA and PE extracts of *P. simiarum* showed analgesic activities in both Tail flick and Acetic acid induced methods at 50- and 100 mg/kg body weight. Among all the extracts, the EA extract showed a dose dependent and comparable analgesic activity in both the tested methods. Therefore, the EA and PE extracts of *P. simiarum* were capable to exhibit moderate analgesic activities. However, further studies are necessary to examine underlying mechanisms of analgesic effects and to isolate the active compounds responsible for these pharmacological activities.

8.7 Analgesic activity of *Glochidion multiloculare*

8.7.1 Materials and Methods

8.7.2 Preparation of extract

The air dried powdered plant material (1000 g) was successively cold extracted with methanol (7 days) at room temperature with occasional shaking and stirring. The extractives were filtered through fresh cotton plug and followed by whatman no. 1 filter paper. The filtrate were then concentrated by a Buchii rotavapor at low temperature and pressure and afforded methanol (MEGM) extract (41.7398g). The cold methanol extract (10 g) was subjected to Solvent-Solvent partitioning using the protocol designed by Kupchan and modified by Wagene (Vanwagenen, 1993). The extract was portioned successively with petroleum ether (PEFGM), carbon tetrachloride (CTFGM) and chloroform (CHFGM).

8.7.3 Acute toxicity

The acute oral toxicity of the plant extract in Swiss albino mice was studied as per established protocol (Riaz *et al*., 2010).

8.8 Analgesic activity

8.8.1 Tail flick test

The animals were divided into six groups with six mice in each group.

Group- I= animals received vehicle (1% Tween 80 in water, 10 mL kg^{-1} body weight).

Group- II= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the MEGM of *G. multiloculare*.

Group –III= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the PEFGM of *G. multiloculare*.

Group- IV= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the CTFGM of *G. multiloculare*.

Group –V= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the CHFGM of *G. multiloculare*.

Group – VI = animals received Diclofenac-Na at 10 mg kg^{-1} body weight.

From 1-2 cm of the tail of mice was immersed in warm water kept at constant temperature of 60°C. The reaction time was the time taken by the mice to deflect their tails. The first reading was discarded and the reaction time was recorded as a mean of the next three readings. A latency period of 20 second was defined as complete analgesia and the measurement was then stopped to avoid injury to mice. The latent period of the tail-flick response was determined before and 0, 30, 60 and 90 min after the oral administration of drugs (Toma *et al*., 2003).

8.8.2 Acetic acid-induced writhing test

The analgesic activity of the samples was also studied using acetic acid-induced writhing model in mice. Test samples and vehicle were administered orally 30 min before intraperitoneal administration of 0.7% acetic acid but Diclofenac-Na was administered intraperitonially 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for specific contraction of body referred to as 'writhing' for the next 10 min (Ghule *et al*., 2011).

The animals were divided into six groups with six mice in each group.

Group- I= animals received vehicle (1% Tween 80 in water, 10 mL kg^{-1} body weight).

Group- II= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the MEGM of *G. multiloculare*.

Group –III= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the PEFGM of *G. multiloculare*.

Group- IV= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the CTFGM of *G. multiloculare*.

Group –V= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the CHFGM of *G. multiloculare*.

Group – VI = animals received Diclofenac-Na at 10 mg kg^{-1} body weight.

8.9 Statistical analysis

Analgesic data are expressed as mean ± S.E.M. (n = 6 mice per groups). Statistical significance (p) calculated ANOVA followed by Dunnett's-T test $P<0.01$ and $P<0.001$ were considered to be statistically significant.

8.10 Results

8.10.1 Acute toxicity studies

The acute toxicity study was conducted to establish the therapeutic index, i.e., the ratio between the pharmacologically effective dose and the lethal dose on the same strain and species. The extract/fractions of *G. lanceolarium* was safe up to a dose of 500 mg/kg (p.o.) body weight which agrees with the previous study (Rahman *et al*., 2011).

8.10.2 Tail flick test

The tail withdrawal reflex time following administration of the MEGM, PEFGM, CTFGM and CHFGM were found to statistically significant (*p<0.01 and **p<0.001) and was comparable to the reference drug Diclofenac-Na (Table-8.4).

8.10.3 Writhing test method

Table-8.5 shows the effects of the extract/fractions on acetic acid-induced writhing in mice. The oral administration of extract/fractions significantly (*p<0.01 and **p<0.001) inhibited (% inhibition of writhing of MEGM, PEFGM, CTFGM and CHFGM were 70, 66.36, 83.25 and 84.09, respectively) writhing response induced by acetic acid.

Table-8.4: Effects of the *G. multiloculare* on tail withdrawal reflex of mice induced by tail flick method.

Treatment Group	Dose (mg/kg body wt.)	Tail flick time			
		0 Min	30 Min	60 Min	90 Min
Vehicle control (1% Tween-80)	-	1.30±0.11	1.35±0.12	1.43±0.05	1.40±0.11
Standard (Diclofenac-Na)	10	1.40±0.02	2.26±0.25**	3.13±0.10**	3.06±20**
MEGM	100	1.20±0.10	1.38±0.10**	2.31±0.15**	1.48±0.10*
PEFGM	100	1.6±0.20	1.80±0.26**	2.30±0.11**	1.53±0.08*
CTFGM	100	1.6±0.12	2.05±0.04**	2.34±0.03**	1.17±0.07*
CHFGM	100	1.45±0.011	1.53±0.21**	1.91±0.16**	1.23±0.10*

Values are mean±SEM, (n = 6);*p<0.01 and **p<0.001, Dunnett's-T test as compared to control.

Effects of the *G. multiloculare* on acetic acid-induced writhing in mice.

Table-8.5: Effects of the *G. multiloculare* on acetic acid-induced writhing in mice.

Treatment Group	Dose (mg/kg body wt.)	Writhing no.	% Inhibition of Writhing no.
Vehicle control (1% Tween-80)		110.0±10.23	-
Standard (Diclifenac-Na)	10	15.0±3.25**	86.36
MEGM	100	33.0 ± 8.57**	70
PEFGM	100	37.0± 5.57**	66.36
CTFGM	100	18.42 ±5.0**	83.25
CHFGM	100	17.5±4.50**	84.09

Values are mean±SEM, (n = 6);*p<0.01 and **p<0.001, Dunnett's-T test as compared to control.

8.10.4 Discussion

The brain and spinal cord play a major role in central pain mechanisms. The dorsal horn of the spinal cord is endowed with several neurotransmitters and receptors including substance P, somatostatin, neuropeptide Y, inhibitory amino acid, nitric oxide, endogenous opioids, and the monoamines which are the major targets for pain and inflammation (McCurdy and Scully, 2005). The tail immersion test was considered to be selective to examine compounds acting through opioid receptor; all the extract/fractions increased pain threshold which means basal latency, which indicates that it may act *via* centrally mediated analgesic mechanism. Narcotic analgesics inhibit both peripheral and central mechanism of pain, while nonsteroidal anti-inflammatory drugs inhibit only peripheral pain (Elisabetsky *et al*., 1995). The extract /fraction inhibit pain with both mechanisms, suggesting that the plant extract may act as a narcotic analgesic. On the otherhand, acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics and represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from the tissue phospholipid (Ahmed *et al*., 2006). The response is thought to be mediated by peritoneal mast cells (Ribeiro *et al*., 2000), acid sensing ion channels

(Voilley, 2004) and the prostaglandin pathways (Hossain *et al*., 2006). The organic acid has also been postulated to act indirectly by inducing the release of endogenous mediators, which stimulates the nociceptive neurons that are sensitive to NSAIDs and narcotics (Adzu *et al*., 2003).

Taken all together, this is an indication that the extract/fractions of *G. multiloculare* can be used to manage central as well as peripheral pain. However, Kerstein et al. (Kerstein et al., 2009) suggest that, the inhibitory effect to pain response is due to inhibit the increase of the intracellular Ca2+ ion through the TRPA1 (Transient Receptor Potential Ankyrin 1), a member of the transient receptor potential family of cation channel that trigger the analgesic action. So it is likely that the extract/fractions may contain substances that affect the metabolism of Ca2+ ions. Tannins are also found to have a contribution in antinociceptive activity (Ramprasath *et al*., 2006). So it can be assume that cyclooxygenase (COX) inhibitory activity along with antioxidant activity may reduce the production of free arachidonic acid from phospholipid or may inhibit the enzyme system responsible for the synthesis of prostaglandins and ultimately relieve pain sensation.

8.11: Conclusion

On the basis of our results, we conclude that both extract and fractions (MEGM, PEFGM, CTFGM and CHFGM) at the dose of 100 mg/kg b.w., produced significant increase in pain threshold in tail flick method whereas significantly reduced the writhing caused by acetic acid induced method. Therefore, the MEGM, PEFGM, CTFGM and CHFGM of *G. multiloculare* was capable to exhibit moderate analgesic activities. However, further studies are necessary to examine underlying mechanisms of these effects and to isolate the active compounds responsible for these pharmacological activities.

8.12 Analgesic Activity of *Glochidion lanceolarium*

8.12.1 Materials and Methods

8.12.2 Preparation of extract

The air dried powdered plant material (900 g) was successively cold extracted with methanol (7 days) at room temperature with occasional shaking and stiring. The extractives were filtered through fresh cotton plug and followed by whatman no. 1 filter paper. The filtrate were then concentrated by a Buchii rotavapor at low temperature and pressure and afforded methanol (MEGL) extract (36.8199 g). The cold methanol extract (10 g) was subjected to Solvent-Solvent partitioning using the protocol designed by Kupchan and modified by Wagene (Vanwagenen, 1993). The extract was partioned successively with petroleum ether (PEFGL) and carbontetrachloride (CTFGL).

8.12.3 Acute toxicity

The acute oral toxicity of the plant extract in Swiss albino mice was studied as per established protocol (Riaz *et al*., 2010).

8.13 Analgesic activity

8.13.1 Tail flick test

The animals were divided into five groups with six mice in each group.

Group- I= animals received vehicle (1% Tween 80 in water, 10 mL kg^{-1} body weight).

Group- II= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the MEGL of *G. lanceolarium*.

Group –III= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the PEFGL of *G. lanceolarium*.

Group- IV= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the CTFGL of *G. lanceolarium*.

Group –V= animals received Diclofenac-Na at 10 mg kg^{-1} body weight.

From 1-2 cm of the tail of mice was immersed in warm water kept at constant temperature of 60°C. The reaction time was the time taken by the mice to deflect their tails. The first reading was discarded and the reaction time was recorded as a mean of the

next three readings. A latency period of 20 second was defined as complete analgesia and the measurement was then stopped to avoid injury to mice. The latent period of the tail-flick response was determined before and 0, 30, 60 and 90 min after the oral administration of drugs (Toma *et al*., 2003).

8.13.2 Acetic acid-induced writhing test

The analgesic activity of the samples was also studied using acetic acid-induced writhing model in mice. Test samples and vehicle were administered orally 30 min before intraperitoneal administration of 0.7% acetic acid but Diclofenac-Na was administered intraperitonially 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for specific contraction of body referred to as 'writhing' for the next 10 min (Ghule *et al*., 2011).

The animals were divided into five groups with six mice in each group.

Group- I= animals received vehicle (1% Tween 80 in water, 10 mL kg^{-1} body weight).

Group- II= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the MEGL of *G. lanceolarium*.

Group –III= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the PEFGL of *G. lanceolarium*.

Group- IV= animals were treated with 100 mg kg^{-1} body weight (p.o.) of the CTFGL of *G. lanceolarium*.

Group –V= animals received Diclofenac-Na at 10 mg kg^{-1} body weight.

8.14 Statistical analysis

Analgesic data are expressed as mean ± S.E.M. (n = 6 mice per groups). Statistical significance (p) calculated ANOVA followed by Dunnett's-T test $P<0.01$ and $P<0.001$ were considered to be statistically significant.

8.15 Results

8.15.1 Acute toxicity studies

The acute toxicity study was conducted to establish the therapeutic index, i.e., the ratio between the pharmacologically effective dose and the lethal dose on the same strain and species. The extract/fractions of *G. lanceolarium* was safe up to a dose of 500 mg/kg (p.o.) body weight which agrees with the previous study (Rahman *et al*., 2011).

8.15.2 Tail flick test

The tail withdrawal reflex time following administration of the MEGL, PEFGL and CTFGL were found to statistically significant (*p<0.01 and **p<0.001) and was comparable to the reference drug Diclofenac-Na (Table-8.6).

8.15.3 Writhing test method

Table-8.7 shows the effects of the extract/fractions on acetic acid-induced writhing in mice. The oral administration of extract/fractions significantly (*p<0.01 and **p<0.001) inhibited (% inhibition of writhing of MEGL, PEFGL and CTFGL were 54.54, 39.09 and 68.18, respectively) writhing response induced by acetic acid .

Table-8.6: Effects of the *G.lanceolarium* on tail withdrawal reflex of mice induced by tail flick method.

Treatment Group	Dose (mg/kg body wt.)	Tail flick time			
		0 Min	30 Min	60 Min	90 Min
Vehicle control (1% tween- 80)	-	1.30±0.11	1.35±0.12	1.43±0.05	1.40±0.11
Standard (Diclofenac- Na)	10	1.40±0.02	2.26±0.25**	3.13±0.10**	3.06±20**
MEGL	100	1.35±0.25	1.53±0.10**	2.30±0.15**	1.11±0.03*
PEFGL	100	1.30±0.25	2.15±0.42**	2.43±0.5**	1.57±0.25*
CTFGL	100	1.30±0.1	1.43±0.01**	2.33±0.5**	1.07±0.16*

Values are mean±SEM, (n = 6);*p<0.01 and **p<0.001,Dunnett's-T test as compared to control.

Table-8.7: Effects of the *G. lanceolarium* on acetic acid-induced writhing in mice.

Treatment Group	Dose (mg/kg) body wt.	Writhing no.	% Inhibition of Writhing no.
Vehicle control (1% tween 80)		110.0±10.23	-
Standard (Diclofenac-Na)	10	15.0±3.25**	86.36
MEGL	100	50.0 ± 7.15**	54.54
PEFGL	100	67.0± 8.48**	39.09
CTFGL	100	35.0 ±6.57**	68.18

Values are mean±SEM, (n = 6);*p<0.01 and **p<0.001,Dunnett's-T test as compared to control.

8.15.4 Discussion

The tail flick method is generally used for evaluating central antinociceptive response. The method is further distinguished by their tendency to respond to the pain stimuli conducting through neuronal pathways as tail immersion mediates a spinal reflex to nociceptive stimuli (Chapman *et al*., 1985). On the otherhand, acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics and represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from the tissue phospholipids (Ahmed *et al*., 2006). The response is thought to be mediated by peritoneal mast cells (Ribeiro *et al*., 2000), acid sensing ion channels (Voilley, 2004) and the prostaglandin pathways (Hossain *et al*., 2006). The organic acid has also been postulated to act indirectly by inducing the release of endogenous mediators, which stimulates the nociceptive neurons that are sensitive to NSAIDs and narcotics (Adzu *et al*., 2003).

Taken all together, this is an indication that the extract/fractions of G. lanceolarium can be used to manage central as well as peripheral pain. However, Kerstein et al. (Kerstein et al., 2009) suggest that, the inhibitory effect to pain response is due to inhibit the increase of the intracellular Ca2+ ion through the TRPA1 (Transient Receptor Potential Ankyrin 1), a member of the transient receptor potential family of cation channel that trigger the analgesic action. So it is likely that the extract/fractions may contain substances that affect the metabolism of Ca^{2+} ions. Tannins are also found to have a contribution in

antinociceptive activity (Ramprasath *et al*., 2006). So it can be assume that cyclooxygenase (COX) inhibitory activity along with antioxidant activity may reduce the production of free arachidonic acid from phospholipid or may inhibit the enzyme system responsible for the synthesis of prostaglandins and ultimately relieve pain sensation.

8.16 Conclusion

Based on the results of the present study, we conclude that the both extract and fractions (MEGL, PEFGL and CTFGL) at the dose of 100 mg/kg b.w., produced significant increase in pain threshold in tail flick method whereas significantly reduced the writhing caused by acetic acid induced method. Among all the extract/fractions the CTFGL showed comperable best analgesic activity in both the tested methods. However, further studies are necessary to examine underlying mechanisms of these effects and to isolate the active compounds responsible for these pharmacological activities.

CHAPTER 9

Anti-Inflamatory Activity

9.1 Introduction

Inflammation results in the liberation of endogenous mediators like histamine, serotonin, bradykinin, prostaglandins etc. Prostaglandins are ubiquitous substances that indicate and modulate cell and tissue responses involved in inflammation (Ravikiran *et al*., 2012). Most of the anti-inflammatory drugs now available are potential inhibitors of cyclooxygenase (COX) pathway of arachidonic acid metabolism. Hence for treating inflammatory diseases analgesic and anti-inflammatory agents are required (Anilkumar, 2010). Non steroidal anti-inflammatory drugs (NSAIDs) are the most clinically important medicine (Conforti *et al*., 2009) but severe adverse effects (Chowdhury *et al*., 2009) and tolerance and dependence induced by opiates, use of these drugs have not been successful in all the cases. Therefore, new anti-inflammatory drugs are needed as alternatives to NSAIDs and opiates. Medicinal plants are believed to be an important source of new chemical substances.

In this experiment, carragennan-induced rat hind paw edema was used as the animal model of acute inflammation. Administration of Carrageenan in the sub-plantar region of rat's hind paw leads to the formation of edema in situ due to localized inflammation. About one hour prior to the administration of carrrageenan solution, experimental animals received test materials and standard anti-inflammatory drug at appropriate doses. The volume of rat's paw was measured each hour up to four hours by plethysmometer (Ugo Basile, Italy). The average percentage of increase in paw volume with time was calculated and compared against the control group. Percent inhibition was calculated using the formula-

$$\% \text{ inhibition} = \frac{V_c - V_t}{V_c} \times 100$$

Where, V_c and V_t represent the average edema volume of control and treated animals respectively.

9.2 Animal used for anti-inflammatory method

Male Wister rats weighing 175-250g used in this experiment were collected from InternationalCenter for Diarrheal Diseases and Research, Bangladesh (ICDDR, B). All the animals were acclimatized four month prior to the experiments. The animals were housed under standard laboratory conditions (relative humidity 55-65%, room temperature 25.0 ± 2^0C, and 12 hrs light dark cycle).

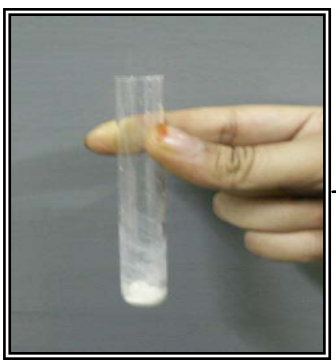
The animals were fed with standard diet from InternationalCenter for Diarrheal Diseases and Research, Bangladesh (ICDDR, B) and had free access to filtered water. . The overall nutrient composition of the diet was 36.2% carbohydrate, 20.9% protein, 4.4% fat and 38.5% fibre with metabolisable energy content of 1.18 MJ/100 g (282 Kcal/100 g).

Figure-9.1: Long Evans Rat

9.2.1 Appliances

- **a.** Digital Balance (Model BL31, BOECKEL Co. Germany)
- **b.** Refrigerator (Model DF8520, Ilshin Lab. Co. Korea)
- **c.** Glass Beakers
- **d.** Petri dishes & glass wrought
- **e.** Safety rat handling gloves
- **f.** Mortar & pestle.
- **g.** Hypodermic Syringes
- **h.** Holder & test tube
- **i.** Hot water bath.
- **j.** Plethysmometer (Model 7141, UGO- BASILE, Italy)

9.2.2 Preparation of Carrageenan 1% suspension



9.2 (A): Carrageenan powder

9.2 (B): Addition of distilled water

9.2 (C): Dissolving powder in water bath

9.2 (D): Preserving in tube

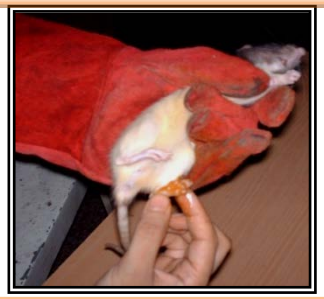
9.2 (E): Dissolved carrageenan in hot water

Carageenan powder was suspended in 5 ml saline to make 1% suspension.The test tube containing carageenan powder and water was kept in water bath for proper homogenization. Then the carrageenan 1% suspension was preserved. The tube was kept in hot water (50±2˚c) containing beaker; otherwise the suspension would be transformed into a jelly like compound.

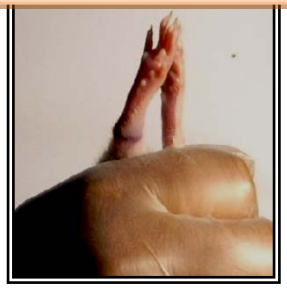
9.2.3 Study Design

Rat handling (front view)

Rat handling (back view)

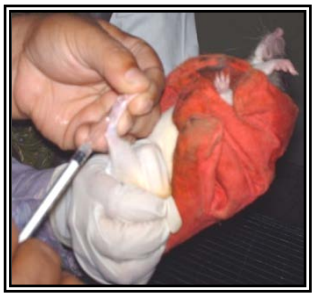


Feeding Samples

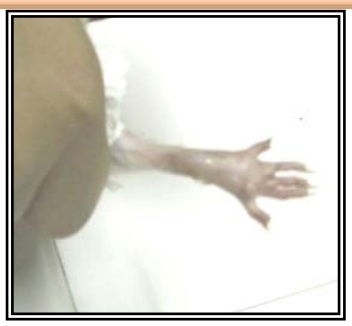


Marking Left paw

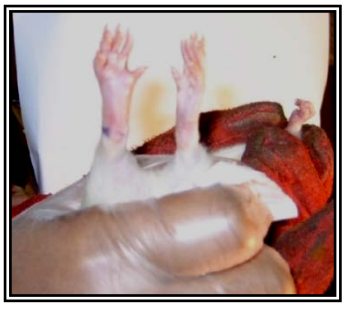
Syringe was filled with 0.1ml carageenan suspension



Injected into rat's left paw



Inflamed paw after injection



Difference between inflamed paw and normal paw

Figure-9.3: Several steps followed during measuring the paw volume

9.3 Anti-inflamatory Activity of *Polyalthia simiarum*

9.3.1 Materials and Methods

9.3.2 Chemicals

Diclofenac-Na, Nalbuphine and Indomethacin were collected from Square Pharmaceuticals Ltd., Bangladesh. All chemicals and reagents were of highest analytical grade.

9.3.3 Preparation of extract

The air dried and powdered plant material (750 g) was extracted in a Soxhlet apparatus with ethyl acetate and pet ether (60-80°C). The extract was filtered through a fresh cotton plug followed by Whatman no.1 filter paper. The filtrate was then concentrated with a Buchii rotavapor at low temperature and pressure to afford ethyl acetate extract (EA, 3.5 g) and pet ether (PE, 2.25 g).

9.4 Carrageenan induced paw edema test in rats

Male Wister rats (175-250 g) were divided into six groups of six animals each. The test groups received 50 and 100 mg/kg, *p.o.* of each extract. The reference group received indomethacin (10 mg/kg, *p.o.*) while the control group received 3 mL/kg of distilled water. After 1 h, 0.1 mL, 1% w/v carrageenan suspension in normal saline was injected into the sub plantar tissue of the left hind paw (Winter *et al*., 1962). The paw volume was measured at 1, 2, 3 and 4 h after carrageenan injection using a micrometer screw gauge. The percentage inhibition of the inflammation was calculated from the formula: % inhibition = $(1 - D_t/D_0) \times 100$. Whereas D_0 was the average inflammation (hind paw edema) of the control group of rats at a given time, D_t was the average inflammation of the drug treated (i.e. extract/fractions or reference indomethacin) rats at the same time (Gupta *et al*., 2005).

9.5 Statistical analysis

Anti- inflammatory data are expressed as mean ± S.E.M. (n = 6 mice per groups). Statistical significance (p) calculated ANOVA followed by Dunnett's-T test $^{*}P<0.01$ and $^{**}P<0.001$ were considered to be statistically significant.

9.6 Results

9.6.1 Preliminary phytochemical investigation

The phytoconstituents present in the EA and PE extract of *P. simiarum* were identified by various chemical tests which showed the presence of alkaloids, terpenoids, phenolic and flavonoid compounds and steroids (Table -9.1).

To the carrageenan induced paw edema in mice, the EA and PE extract showed dose dependent inhibition on paw edema compared to the control group (Figure-9.4).

Table-9.1: Result of chemical group tests of the EA and PE extract of *Polyalthia simiarum*.

Extract	Triterpene	Diterpene	Flavonoid	Phenol	Sterol	Alkaloid
EA extract	+	+++	+++	++	+++	++
PE extract	++	++	+	+	+++	+

EA: Ethyl acetate; PE: Petroleum ether; (+): Present; (-): Absent; (+ + +): Reaction intensity is high; (+ +): Reaction intensity is medium; (+): Reaction intensity is normal.

Table-9.2: Effects of the extracts of *Polyalthia simiarum* on carrageenan induced paw edema in mice.

Group	Dose	1h	2h	3h	4h
Group V Standard	10	19.29±1.14	20.97±0.60	33.33±0.98	46.79±0.88
Group II	100	2.85±0.36	11.88±0.53	24.83±0.53	32.69±1.41
Group I	50	1.97±0.04	7.56±0.61	15.12±1.16	23.15±1.14
Group IV	100	17.14±1.11	18.88±1.40	31.13±0.59	39.10±2.02
Group III	50	10.54±1.08	12.32±1.11	20.45±1.59	27.65±1.26

Anti- inflammatory data are expressed as mean ± S.E.M. (n = 6 mice per groups).

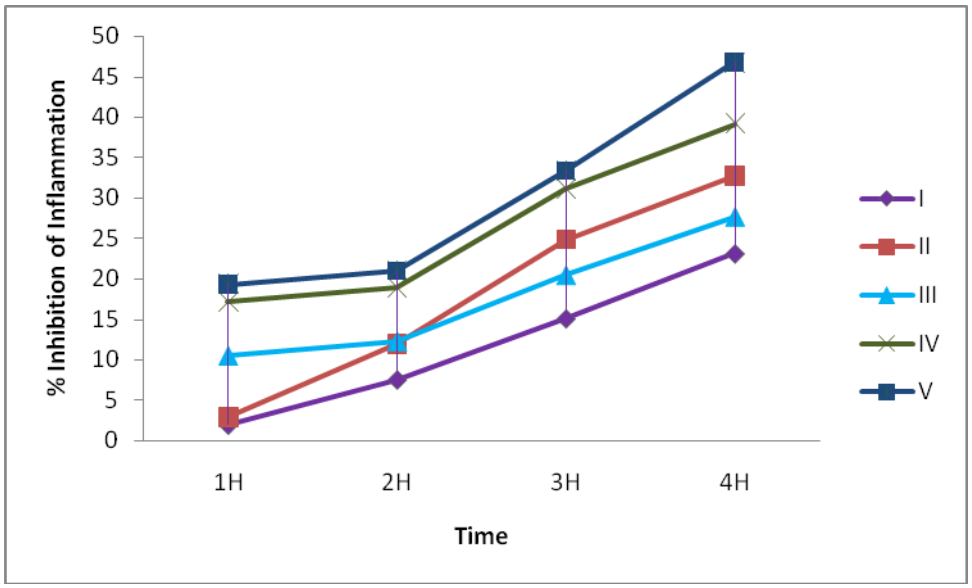


Figure-9.4: Inhibition of inflammation by different extracts (Group-I and Group-II animal treated with 50 and 100 mg/kg body weight of PS-PE extract (p.o), Group-III and Group-IV animal treated with 50 and 100 mg/kg body weight of PS-EA extract (p.o) and Group-V received indomethacin 10 mg/kg body weight (p.o.).

9.6.2 Discussion

Carrageenan induced oedema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1 – 2 h) of the carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (Kaushik *et al*., 2012). Since the extracts significantly inhibited paw edema induced by carrageenan in the second phase and this finding suggests a possible inhibition of cyclooxygenase synthesis by the extract and this effect is similar to that produced by non-steroidal anti-inflammatory drugs such as indomethacin, whose mechanism of action is inhibition of the cyclooxygenase enzyme.

Phytochemical analysis showed that the extract contained alkaloids, phenolic compounds, sterols, diterpenes, triterpenes and flavonoids. Flavonoids and phenolic compounds have all been associated with various degrees of anti-inflammatory (Wang *et*

al., 2008). Therefore, the anti-inflammatory effects observed in this study are perhaps due to the activity of one or more of the identified classes of compounds. Because an essential database on the chemical profile of *P. simiarum* extract is established in which flavonoids and triterpenoids are the major constituents, this information should be considered for future purification of analgesic and anti-inflammatory active compounds from this natural source. Our results indicate that *P. simiarum* extracts have both effective analgesic and anti-inflammatory activity.

9.7 Conclusion

Based on the results of the present study, we conclude that the EA and PE extracts showed anti-inflammatory activities at 50- and 100 mg/kg body weight. the EA extract reduced the paw edema considerably (27.5% and 39.10% inhibition after 4h), in dose dependent manner when compared to carrageenan induced control rat. Therefore, the EA and PE extracts of *P. simiarum* were capable to exhibit moderate antiinflammatory activity. However, further studies are necessary to examine underlying mechanisms of anti inflammatory effects and to isolate the active compounds responsible for this pharmacological activity.

9.8 Anti-inflammatory activity of *Glochidion multiloculare*

9.8.1 Materials and Methods

9.8.2 Preparation of extract

The air dried powdered plant material (1000 g) was successively cold extracted with methanol (7 days) at room temperature with occasional shaking and stirring. The extractives were filtered through fresh cotton plug and followed by whatman no. 1 filter paper. The filtrate were then concentrated by a Buchii rotavapor at low temperature and pressure and afforded methanol (MEGM) extract (41.7398 g). The cold methanol extract (10 g) was subjected to Solvent-Solvent partitioning using the protocol designed by Kupchan and modified by Wagene (Vanwagenen, 1993). The extract was portioned successively with petroleum ether (PEFGM), carbon tetrachloride (CTFGM) and chloroform (CHFGM).

9.9 Carrageenan induced paw edema test in mice

Male Wister rats (175-250 g) or mice were divided into six groups of six animals each. The test groups received 100 mg/kg, *p.o.* of each extract/fractions. The reference group received indomethacin (10 mg/kg, *p.o.*) while the control group received 3 mL/kg of 1% tween 80 in water. After 1 h, 0.1 mL, 1% w/v carrageenan suspension in normal saline was injected into the sub plantar tissue of the left hind paw (Winter *et al*., 1962). The paw volume was measured at 1, 2, 3 and 4 h after carrageenan injection using a micrometer screw gauge. The percentage inhibition of the inflammation was calculated from the formula: % inhibition = $(1 - D_t/D_0) \times 100$. Whereas D_0 was the average inflammation (hind paw edema) of the control group of mice at a given time, D_t was the average inflammation of the drug treated (i.e. extract/fractions or reference indomethacin) mice at the same time (Gupta *et al*., 2005).

9.10 Statistical analysis

Anti inflammatory data are expressed as mean ± S.E.M. (n = 6 mice per groups). Statistical significance (p) calculated ANOVA followed by Dunnett's-T test $P<0.01$ and $P<0.001$ were considered to be statistically significant.

9.11 Results

9.11.1 Anti- inflammatory activity

To the carrageenan induced paw edema in mice, the extract/fraction showed inhibition on paw edema compared to the control group (Table -9.3).

Table -9.3: Effects of the *G. multiloculare* on carrageenan induced paw edema in mice.

Treatment Group	Dose (mg/kg body wt.)	Paw edema volume (cm)			
		1H	2H	3H	4H
Vehicle control (1% Tween-80)		1.23±0.11	1.4±0.12**	1.53±0.05**	1.46±0.11.0**
Standard (Indomethacin)	10	1.23±0.023	1.16±0.25**	1.13±0.10**	1.06±0.20**
MEGM	100	1.3±0.07	1.2±0.05**	1.0±0.07**	0.9±0.045**
PEFGM	100	1.25±0.04	1.15±0.07**	1.0±0.04**	0.95±0.5**
CTFGM	100	1.25±0.32	1.2±0.07**	1.0±0.25**	0.9±0.07**
CHFGM	100	1.3±0.45	1.15±0.05**	0.95±0.02**	0.85±0.05**

Values are mean±SEM, (n = 6);*p<0.01 and **p<0.001, Dunnett's-T test as compared to control.

9.11.2 Discission

Carrageenan induced oedema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1 – 2 h) of the carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (Kaushik *et al*., 2012). Since the extract/fractions significantly inhibited paw edema induced by carrageenan in the second phase and this finding suggests a possible inhibition of cyclooxygenase synthesis by the extract/fractions and this effect is similar to that produced by non-steroidal anti-inflammatory drugs such as indomethacin, whose mechanism of action is inhibition of the cyclooxygenase enzyme.

9.12 Conclusion

On the basis of our results, we conclude that the MEGM, PEFGM, CTFGM and CHFGM showed anti-inflammatory activities at the dose 100 mg/kg body weight. Therefore, the MEGM, PEFGM, CTFGM and CHFGM of *G. multiloculare* was capable to exhibit moderate anti-inflammatory activities. However, further studies are necessary to examine underlying mechanism of this effect and to isolate the active compounds responsible for this pharmacological activity.

9.13 Anti-inflammatory activity of *Glochidion lanceolarium*

9.13.1 Materials and Methods

9.13.2 Preparation of extract

The air dried powdered plant material (900 g) was successively cold extracted with methanol (7 days) at room temperature with occasional shaking and stiring. The extractives were filtered through fresh cotton plug and followed by whatman no. 1 filter paper. The filtrate were then concentrated by a Buchii rotavapor at low temperature and pressure and afforded methanol (MEGL) extract (36.8199 g). The cold methanol extract (10 g) was subjected to Solvent-Solvent partitioning using the protocol designed by Kupchan and modified by Wagene (Vanwagenen, 1993). The extract was partioned successively with petroleum ether (PEFGL) and carbontetrachloride (CTFGL).

9.14 Anti-inflammatory activity

9.14.1 Carrageenan induced paw edema test in mice

Male Wister rats (175-250 g) or mice were divided into five groups of six animals each. The test groups received 100 mg/kg, *p.o.* of each extract/fractions. The reference group received indomethacin (10 mg/kg, *p.o.*) while the control group received 3 mL/kg of 1% tween 80 in water. After 1 h, 0.1 mL, 1% w/v carrageenan suspension in normal saline was injected into the sub plantar tissue of the left hind paw (Winter *et al*., 1962). The paw volume was measured at 1, 2, 3 and 4 h after carrageenan injection using a micrometer screw gauge. The percentage inhibition of the inflammation was calculated from the

formula: % inhibition = $(1 - D_t/D_0) \times 100$. Whereas D_0 was the average inflammation (hind paw edema) of the control group of mice at a given time, D_t was the average inflammation of the drug treated (i.e. extract/fractions or reference indomethacin) mice at the same time (Gupta *et al*., 2005).

9.15 Statistical analysis

Statistical significance (p) calculated ANOVA followed by Dunnett's-T test $P<0.01$ and $P<0.001$ were considered to be statistically significant.

9.16 Results

9.16.1 Anti- inflammatory activity

To the carrageenan induced paw edema in mice, the extract/fraction showed inhibition on paw edema compared to the control group (Table- 9.4).

Table-9.4: Effects of the *G. lanceolarium* on carrageenan induced paw edema in mice.

Treatment Group	Dose (mg/kg body wt.)	Paw edema volume (cm)			
		1H	2H	3H	4H
Vehicle control (1% tween 80)		1.23±0.11	1.4±0.12**	1.53±0.05**	1.46±0.11.0**
Standard (Indomethacin)	10	1.23±0.023	1.16±0.25**	1.13±0.10**	1.06±0.20**
MEGL	100	1.45±0.07	1.25±0.06**	1.05±0.07**	0.95±0.05**
PEFGL	100	1.25±0.5	1.23±0.5**	1.1±0.12.0**	1.0±0.10.0**
CTFGL	100	1.35±0.02	1.1±0.07**	1.0±0.02**	0.85±0.07**

Values are mean±SEM, (n = 6);*p<0.01 and **p<0.001,Dunnett's-T test as compared to control.

9.16.2 Discussion

Carrageenan induced oedema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1 – 2 h) of the carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (Kaushik *et al*., 2012). Since the extract/fractions significantly inhibited paw edema induced by carrageenan in the second phase and this finding suggests a possible inhibition of cyclooxygenase synthesis by the extract/fractions and this effect is similar to that produced by non-steroidal anti-inflammatory drugs such as indomethacin, whose mechanism of action is inhibition of the cyclooxygenase enzyme.

9.17 Conclusion

Based on the results of the present study, we conclude that the MEGL, PEFGL and CTFGL showed anti-inflammatory activities at the dose 100 mg/kg body weight. Among all the extract/fractions the CTFGL reduced the paw edema (0.85± 0.07, after 4h), when compared to carrageenan induced control mice. Therefore, the MEGL, PEFGL and CTFGL of *G. lanceolarium* was capable to exhibit moderate antiinflammatory activity.However, further studies are necessary to examine underlying mechanism of this effects and to isolate the active compounds responsible for this pharmacological activity.

REFERENCES

Adesogan, I. K and Durodola, J. I. (1976) *Phytochemistry*, **15**, 1311.

Adzu, B., Amos, S., Kapu, SD., Gamaniel, KS. (2003) Anti-inflammatory and anti-nociceptive effects of *Sphaeranthus senegalensis. J Ethnopharmacol*, **84**, 169-174.

Ahmed, F., Hossain, MH., Rahman, AA., Shahid, IZ. (2006) Antinociceptive and sedative effects of the bark of *Cerbera odollam* Gaertn. *J Ori Pharm Exp Med.*, **6**, 344- 348.

Ahmed, Z.U. *et al.* (2008) *Glochidion*. In: Ahmed, Z.U., Hassan, M.A., Begum, Z.N.T., Khondker, M., Kabir, S.M.H., Ahmad, M., Ahmed, A.T.A., Rahman, A.K.A. and Haque, E.U. (eds.). *Encyclopedia of flora and fauna of Bangladesh,* Vol.**7**. *Angiosperms: Dicotyledons (Balsaminaceae - Euphorbiaceae)*. Asiatic Society of Bangladesh, Dhaka. pp. 435.

Alam, F., Rahman, MS., Alam, MS., Hossain, MK. and Rashid, MA. (2009, June) Phytochemical and Biological investigations of Phoenix paladusa Roxb., *Dhaka Univ. J. Pharm. Sci*. **8** (1), 7-10,

Alam, MB., Rahman, MS., Hasan, M., Khan, MM., Nahar, K., Sultana, S. (2012) Antinoeieeptive and antioxidant activities of the *Dillenia indica* Bark. *Int J Pharmaco*, DOI: 10.3923/ijp.2012.

Alkofahi, A., Smith, D. L., McLaughlin, J. L. (1988) *Experientia*, **44**, 83.

American Cancer Society. (2007) Report sees 7.6 million global 2007 Cancer death, Reuters.

Anilkumar, M. (2010) Ethnomedicinal plants as Anti-inflammatory and Analgesic agents. *Ethnomedicine: A Source of Complementary Therapeutics.* Research Signpost, Kerala, India, 267-293.

Armour, DFE., Blood, FR., Belden, DA. (1965) The Manual for Laboratory Works in Mammalian Physiology. University of Chicago Press, United State.

Aruoma, O. I. (1998) Free radicals, oxidative stress and antioxidants in human health and disease. *J. Am oil Chem Soc.* **75**, 199-212.

Aratanechemuge, Hibasami, H., Sanpin, K., Katsuzaki, H., Imai, K., Komiya, T. (2004) Induction of apoptosis by lupeol isolated from mokumen(*Gossampinus malabarica L. Merr)* in human promyelotic leukemia HL-60 cells. *Journal of Oncology reports,* **11**, 289-292.

Arvigo, R. and Balick, M. (1993) *Rainforest Remedies,* Lotus press, Twin Lakes.

Asolkar, LV., Kakkar, K., Charkre, OJ. (1992) Second supplement to Glossary of Indian Medicinal Plants with Active Principles, Publication and Information Directorate (CSIR), New Delhi, 329-36.

Auudy, B., Ferreira, B., Blasina, F., Lafon, L., Arredondo, F., Dajas, F. and Tripathi, P. C. (2003) *J. Oil. Res.* **9**, 247.

Ayafor, J. F., Sondengam, B. L. and Nagadju, B. T. (1982) *Planta Med*. **44**, 193.

Baker, D., Mocek, U. and Garr, C. (2000) New leads for for pharmaceutical and Agrochemical Industries. *Biodiversity*.

Bandoniene, D., Pukals, k. A., Venkutonis, P. K. and Gruzdiene, D. (2000) Preliminary screening of antioxidant activity of some plants extracts in repressed oil. *Food Res Int.* **33**, 786-791.

Baron, J., Sandler, R., Bresalier, R., Quan, H., Riddel, R., Lanas, A., Bolognese, J., Oxenius, B., Horgan, K., Loftus, S., Mortar, D. (2006) A randomized trial off rofecosib for the chemoprevention of colorectal adenomas. *Gastroenterology*; **131** (6), 1674-82.

Barry, A. L. (1980) *Procedures for testing antimicrobial agents in agar mediaIn: Antibiotics in Laboratory medicines* (V. Lorian. Ed), Williams and Wilkins Co., Baltimore, USA, pp. 1-123.

Bauer, A. W., Kirby, W. M. M., Sherris, J. C. and Turck, M. **(1966)** Antibiotic susceptibility testing by a standardized single disc method, *Am. J. Clin. Pathol.* **45**, 493-496.

Bedard, PL., Krzyzanowska, MK., Pintilie, M., Tannock, IF. (2007) Statistical Power of negative randomized controlled trails presented at American Society for Clinical Oncology Annual Meetings. *J. Clin Oncol*, **25** (23): 3482-7.

Bhatt, P. K., Hanuman, J. B. and Sabata, B. K. (1988) *Phytochemistry*, **27**(4), 1212-16.

Blois, MS. (2002) Antioxidant determination by the use of a stable free radical. *Nature* **26**, 1199-1200.

Boakye-Yiadom, K., Tiagbe, N. I. Y. and Ayim, J. S.K. (1977) *Lioydia*, **4**: 543.

Bohlmann, F., Zdero, C., King, RM., Robinson, H. (1981) Thirteen kolavane derivatives from *Symphyopappus* species, *Phytochemistry*, **20**, 1657-1663.

Bose, U., Gunasekaran, K., Bala, V., Rahman, AA. (2010) Evaluation of Phytochemical and Pharmacological properties of *Dillenia indica* Linn. Leaves. *J Pharmacol Toxicol*, **5**, 222-228.

Brand-Williams, W., Cuvelier, M. E. and Berset, C. (1995) Use of free radical method to evaluate antioxidant activity. *Lebanese Wiss Technol.* **28**, 25-30.

Brinton, L.A.I., Moghissi, K.S., Skokie, B., Westhoff, C.L., Lamb, E.J. (2005) Ovulation induction and Cancer risk. *Fertil. Steril*, **83** (2), 262-74.

Brockmann, H., Bohnsack, G., Franck, B., Grone, H., Muxfeldt H. and Suling, C. (1956) Zur Constitution der Actinomycine, *Angew. Chem.*, **68**, 70.

Burkhill, I. H. (1966) *A Dictionary of the Economic Plants of the Malay Peninsula*, Ministry of Agriculture, Kuala Lumpur, Malaysia, P-1115.

Buyukokuroglu, M. E., Gulcin, I., Oktay, M. and Kufrevioglu, O. I. (2001) *Pharmacol. Res.* **44**, 491.

[abcd] Canavan, TP., Doshi, NR. (2006) Cervical Cancer. *Am Fam physician* **61** (5), 1369-76.

Cancer Research UK. (2007) UK cancer incidence statistics by age

Catalogue of Life: (2007) Annual Checklist, Species 2000 & ITIS Catalogue of Life Hierarchy, Edition 1 (2007) (accessed through GBIF data portal, http://data.gbif.org/species/browse/taxon/14737287, 2010-07-25).

[ab]Catelinois, O., Rogel, A., Laurier, D., *et al*. (2006) Lung Cancer attributable to Indoor Radon exposure in France: Impact of the Risk models and uncertainty analysis. Environmental Health perspectives, National Institute of Environmental Health Science, **114** (9), 1361-1366.

Cave, A., Guinaudeau, H., Ramahatra, A., Razafindrazaka, J. (1978) Alkaloids of Annonaceae. XVIII: alkaloids from trunk bark of *Polyalthia suaveolens* Engl. and Diels, *Planta Med.*, **33**, 243-250.

Chang, H. M. (1986) *Pharmacology and Applications of Chinese Metirica Medica*, **1** & **2**, World Scientific Publishing, Singapore.

Chang, F. R., Chen, C. Y., Hsieh, T. J., Cho, C. P. and Wu, Y. C. (2000) Chemical constituents from Annonaglabra III. J. *Chin. Soc.*, **47**, 913-920.

Chang, F. R., Hwang, T. L., Yang, Y. L., Li, C. E., Wu, C. C., Hamad H., Hsieh, W.B., Wu, Y. C. (2006) Anti-Inflammatory and Cytotoxic Diterpanes from Formosan *Polyalthia longifolia* var. pendula, *Planta Medica*, **72**, 1344-1347.

Chakrabarty, M. and Patra, A. (1990) 6, 7-dimethoxyonychine and other alkaloids of *Polyalthia longifolia. Indian J. Chem.* Sect. B, **29B**, 394-395.

Chakrabarty, M., Nath, A. C. (1992) A new clerodane-type butenolide diterpene from the bark of *Polyalthia longifolia, J. Nat. Prod.*, **55**, 256-258.

Chapman, CR., Casey, KI., Dubner, R., Foley, KM., Gracely, RH., Reading, AE. (1985) Pain measurement: an overview. *Pain,* **22**, 1-31.

Chelbowski, RT., Black burn, GL., Theomson, CA., *et al*. (2006) Dietary fat reduction and breast cancer out come: interim efficacy results from the woman's intervention nutrition study. *J. Natt, cancer Ins*, **98** (24), 1767-76.

Chen, C. Y., Chang, F. R., Shih, Y. C., Hsieh, T. J., Chia, Y. C., Tseng, H. Y., Chen, H. C., Chen, S. J., Hsu, M. C., Wu, Y. C. (2000) Cytotoxic constituents of *Polyalthia longifolia* var. pendula, *J. Nat. Prod.,* **63**, 1475-1478.

Choi, H. Y., Jhun, B. O., Lim, I. M., Chung, S. H., Kyung, E. J. and Park, D. K. (2000) Application of flow injection-chemilumineacence to the study of radical scavenging activity in plants. *Phytother. Res.* **14**, 250-253.

Chopra, R. N., Nayer, S. L. and Chopra, I .C. (1953) *Glossary of Indian Medicinal Plants*, C. S. I. R. New Delhi, India.

Chowdhury, MA., Abdellatif, KRA., Don, Y., Das, D., Suresh, MR., Knaus, EE.(2009) Synthesis of celecoxib analogues possessing a N-Difluoromethyl-1,2-dihydropyrid-2-one s-Lipoxygenase pharmacophore: Biological evaluation as dual inhibitors of cyclooxygenases and 5-Lipoxygenase with anti-inflammatory activity. *J Med Chem*, **52**, 1525-1529.

Chung, J.M. (2004) The role of reactive oxygen species (ROS) in persistent pain. *Mol. Intev*., **4**, 248-250.

Clarkson, B.D. and Burchenal, J.H. (1965) Preliminary screening of antineoplastic drugs. *Prog. Clin. Cancer*, **1**, 625-629.

Cronquist, A. (1981) *An Integrated System of Classification of Flowering Plants*. Columbia University press, New York. pp. Xiii –XViii.

Cobb, J.P. and Walkler, D.G. (1958) Actinomycin D on tissue cultures of normal and neoplastic cells, *J. Nat. Cancer Inst*., **21** (2), 263.

Conforti, F., Sosa, S., Marrelli, M., Menichini, F., Statti, GA., Uzunov, D., *et al*. (2009) The protective ability of Mediterranean dietary plants against the oxidative damage: the role of radical oxygen species in inflammation and the polyphenol, flavonoid and sterol contents. *Food Chem*, **112**, 587-597.

Connolly, J. D. and Overton, K. H. (1972) Chemistry of Terpenes and Terpenoids; Newman, A.A., ed.; p207, Academic Press, London.

Connolly, J. D., Haque, E., Kadir, A. A. (1996) Two 7,7'-bisdehydro aporphine alkaloids from *Polyalthia bullata. Phytochemistry*, **43**, 295-297.

Cordell, G. A. (1981) *Introduction to Alkaloids-A biogenetic Approach*, 406, John Wiley and Sons, New York.

Couladis, M., Tzakou, O., Verykokidou, E. and Harvala, C. (2003) Screening of some Greek aromatic plants for antioxidant activity. *Phytother Res.* **17**, 194-195.

Cox, P. A. (1994) *The ethnobotanical approach to drug discovery*: *Strengths and limitations.* In: Ethnobotany and the Search for New Drugs, (Ciba Foundation Symposium 185), p.25. Wiley, Chichester.

Dahiru, D. and Obidoa, O. (2007) Evaluation of the antioxidant effects of *Zizyphus mauritiana* Lam. Leaf extracts against chronic ethanolinduced hepatotoxicity in rat liver. *Afr. J. Trad. Comp. Alter. Med*., **5**: 39-45.

Dan, S., Dan, S. S., Mukhopadhyay, P., Mukherjee, M. (1985) Chemical investigation of some Annonaceae species. *Int. J. Crude Drug Res.*, **23**, 73-76.

Dashora, N., Sodde, V., Bhagat, J., Prabhu, K.S. and Lobo, R. (2010) Antitumor activity of Dendrophthoe falcate against Ehrlich Ascites Carcinoma in swiss albino mice. Pharm. Crops., **2**: 1-7.

Desmarchelier, C., Repetto, M., Coussio, J., Liesuy, S. and Ciccia, G. (1997) Antioxidant and prooxidant activities in aqueous extracts of Argentine plants. *Int. J. Pharmacog*. **35**, 116-120

Dev, S. (1997) *Curr. Sci*. **73**, 909.

Donald, F.H., Wallach, J.S and Bricker, L. (1960) *Can Res*, **20**, 397-403.

Eduardo diaz-Rubio. (2005) *Journal of Clinical Oncology*, **23 (**10), 2425-2426.

EI-Razek, M. A., Chang, F. R., Yang, Y.L., Li, C. E., Hung, H. C., Kuo, Y.H., Wu, Y. C. (2003) A natural pyrone derivative from the woods of *Polyalthia longifolia* var. pendula, *Chin. Pharma*. *J*., **55**, 279-285.

Elena, R., Cristina, A. L., Ramon, E. and Rosa, M. L. (2006) *Clinical Chemistry*, **52**, 749.

Elisabetsky E, Amador TA, Albuquerque RR., Nunes DS, Cavalho ADCT. (1995) Analgesic activity of *Psychotria colorata* (Willd. ex R. and S.) Muell. Arg. alkaloids. *J Ethnopharmacol.,* **48** (2), 77-83.

Enamul, M. H., Muttaquin, A. And Rahman, M. (1998) Sesquiterpene and Aromatic ester from the stem bark of *Miliusa velutina*. *Dhaka Univ. J. Biol. Sci.* **7** (1), 93-97.

Encyclipedia of Life: (2010) *Glochidion multiloculare* (Rottler ex Willd.) Voigt, http://www.eol.org/pages/1143204, 2010-07-25).

Eschenmoser, A., Ruzicka, L., Jeger, O., Arigoni, D. (1955) *Helv. Chim. Acta* **38:** 1890-1904.

Evans, W.C. (1996) Trease and Evans Pharmacognosy. 14th Ed., W.B. Saunders Company, London, 224-228, 293-309, 542-575.

Evans, WC. (2007) *Pharmacognosy*, 5th Ed., Elsiever Publication, Delhi, 394.

Fang, X. P., Anderson. J. E., McLaughlin, J. L. (1990) *J. Chem. Soc.* Parkin Trans. **1**, 1655.

Fang, X. P., Alkofahi, A., McLaughlin, J. L. (1991). *Heterocycles*, **32**, 1.

Fansworth, N. R., Akele, O., Bingel, A. S., Soejarto, D. D. and Guo, Z. (1985) *Bull. WHO*, **63**, 965.

Fauci, A. S. (2001) Infectious Diseases Considerations for 21st Century, *Clin. Infec. Diseases*, **32**(5), 675-685.

Ferdous, AJ., Islam, MO., Hasan, CM., Islam, SN. (1992) I*n vitro* antimicrobial activity of lanuginosine and oxostephanine, *Fitoterapia*, **63**, 549–550.

Fruix, J., Sherman, M. (2005) Practice guidelines Committee. American association for the study of liver Disease. Management of hepatocellular carcinoma. *Hepatology*, **42** (5),1208-36.

GBIF Open Geospatial Consortium services: (2010) Density of data for Glochidion, registered within the GBIF network index, http://data.gbif.org/species/14737287, 2010-07-25).

Ghani, A. (1998) Medicinal plants of Bangladesh: chemical constituents and uses. Asiatic Society of Bangladesh, Dhaka, Bangladesh, 260-262.

Ghule, RS., Venkatnarayanan, R., Thakare, SP., Jain, H., Ghule, PR. (2011) Analgesic activity of *Cuscuta campestris* Yuncker a parasitic plant grown on *Nerium indicum* Mill. *J of Adv Phar Educ & Res.,* **1**, 45-51.

Glochidion. (2010, July 10) In *Wikipedia, the free encyclopedia*. Retrieved July 26, 2010, from http://en.wikipedia.org/wiki/Glochidion.

Grant, WB., Garland, CF., Holick, MF. (2005) Comparisons of estimated economic burdens due to insufficient solar ultraviolet irradiance and vitamin D and excess solar UV irradiance for the United States. *Photochem photobiol*, **81** (6),1276-86.

Green, JP., *et al*. (2004) Acute Myeloid Leukemia in adults. In lee, GR. *et al*., (eds.), Wintrobes Clinical Hematology; (11th Eds.), **2**, 2097-2142.

Guinaudeau, H., Ramahatra, A., Leboeue, M., Cave, A. (1978) Alkaloids of Annonaceae. XVIII: alkaloids from *Polyalthia oligoperma* Diels and *Polyalthia emarginata* Diels, *Plant. Med. Phytother.*, **12**, 166-172.

Gupta, M., Mazunder, UK., Sambath, KR., Gomath, P., Rajeshwar, Y., Kakoti, BB., *et al*. (2005) Anti-inflammatory, analgesic and antipyretic effects of methanol extract from Bauhina racemosa stem bark in animal models. *J Ethnopharmacol*, **98**, 267-273.

Gupta, M., Mazumder, UK., Halder, PK., Chandi, CK., Manikandan, L., Senthil, GP. (2007) Anticancer activity of *Indigofera aspalathoides* and *Wedelia calendulaceae* in swiss albino mice. *Iran J Pharm Res*., **6**, 141-145.

Gutteridgde, J. M. C. (1995) *Free Radic. Res. Comm.* **19**, 141.

Going, S. *et al*. (2003) *Osteoporosis Int*; **14**, 634-43.

Gonzalez, M. C., Sentandreu, M. A., Rao, K. S., Zafra-Polo, M. C., Cortes, D. (1996) Prenylated benzopyran derivatives from two *Polyalthia* species. *Phytochemistry*, **43**, 1361-1364.

Gonzales, G.F. and Valerio, L.G. (2006) Medicinal plants from Peru: a review of plants as potential agents against cancer. *Anticancer Agent Med. Chem*., **6** (5): 429–444.

Gorlova, OY., Weng, SF., Zhang, Y., *et al*. (2007) Aggregation of Cancer among relation of never Smoking lung Cancer Patients. *International Journal of Cancer*; **121** (7), 111-118.

Goyal, M. M., Gupta, A. (1985) Pentacyclic triterpenes and phytosterols from *Polyalthia longifolia,* Benth, and Hook. F., *Indian Drugs*, **22**, 658.

Goyal, M. M., Gupta, A. (1986) Isolation and characterization of triterpenoidal fraction from the leaves of *Polyalthia suberosa Thw, Acta Cienc. Indica, Chem.*, **12**, 152-154.

Hafidh, R. R., Abas, F., Abdulamir, A. S., Jahanshiri, F., Bakar, F. A. and Sekawi, Z. (2009) A review: Cancer research of natural products in Asia. *Int. J. Cancer Res*., **5**: 69-82.

Haider, S. S. (1988) M-Pharm. Thesis Dept of Pharmacy, Univ. of , Dhaka.

Halliwell, B., Gutteridge, J. M. C. and Cross, C. E. (1992) Free radicals, antioxidants and human disease: Where are we now? *J. Lab. Clin. Med.* **119,** 598-620.

Hammoniere, M., Fournet, A., Leboeut, M., Borquet, A., Cave, A. (1976) Polycarpol, a new triterpene from *Polyalthia oliveri* Engl. and *meiocarpidium lepideotum* Engl. and Diels, Annonaceae, *C. R. Hebd. Seances Acad. Sci., Ser. C.,* **282**, 1045-1047.

Hammonniere, M., Leboeuf, M., Cave, A. (1977) Alkaloids from Annonaceae. Part 15. Aporphine alkaloids and terpenoid compounds from *Polyalthia oliveri, Phytochemistry*, **16**, 1029-1034.

Hanahan, D., Wdinberg, RA. (2000) The hallmarks of cancer. *Cell*, **100** (1), 57-70.

Hansen, LB. (2004) Non-hormonal therapies. *The journal of family practice*, **52,** 4.

Hara, N., Asaki, H., Fujimoto, Y., Gupta, Y. K., Singh, A. K., Sahai, M. (1995) Clerodane and ent-halimane diterpenes from *Polyalthia longifolia, Phytochemistry*, **38**, 189-194.

Hasan, CM., Healey, TM., Waterman, PG. (1982) Kolavane and kaurane diterpenes from the stem bark of *Xylopia aethiopica*, *Phytochemistry*, **21**, 1365-1368.

Hasan, C. M., Islam, S. N. and Zaman, N. (1988a) Studies on the Antimicrobial activity of an extract of *Annona reticulate*. *Bangladesh J. Microbiol.* **5** (1), 11.

Hasan, C. M., Islam, S. N. and Ahsan, M. (1988b) Antimicrobial activity of stem bark of *Polyalthia longifolia*. *Dhaka Univ. Studies*, Part E **4** (1), 63.

Hasan, C. M., Hossain, M. A., Rashid, M. A., Connolly, J. D. (1994) Chemical and biological studies on the annonaceous species of Bangladesh 7. Constituents from *Polyalthia longifolia* var pendula, *Fitoterapia*, **65**, 283-284.

Hasan, C. M., Connolly, J. D., Haque, M. E. and Hossain, S. (1994) *Phytochemistry*, **36** (5), 1337-1338.

Hasan, C. M., Connolly, J.D., Hoque, M.E. Haider, S. S. (1994a) Constituents of the stem bark of *Artabotrys odoritissimus*. *Fitoterapia*, LXV (No. 1), 92-93.

Hasan, C. M., Haider, S. S. and Rashid, M.A. (1994b) *Fitoterapia*, LXV (No. **2**), 175-176.

Hasan, C. M., Connolly, J .D., Mia, M. Y., Rashid, M. A. (1994c) *Phytochemistry*, **37** (6), 1763-1764.

Hasan, C. M., Islam, M. O., Rashid, M. A. (1995) Clerodane diterpene from *Polyalthia longifolia* var. pendula, *Pharmazie*, **50**, 227-228.

Hao, X. J., Yang, X. S., Zhang, Z., Shang, L. J. (1995) Clerodane diterpenes from *Polyalthia cheliensis*, *Phytochemistry*, **39**, 447-448.

Harrigan, G. G., Gunatilaka, A .A. L., David Kingston, G. I., Chan, G. W. and Johnsom, R. K. (1994) Isolation of bioactive and other oxoaporphine alkaloids from *Miliusa Banacea*. *J. Nat. Prod*. **57**, 68-73.

Hela, A. E, Abdullah, A. (2010) Antioxidant and antimicrobial activities of methanol extracts of some Verbena species: In vitro evaluation of antioxidant and antimicrobial activity in relation to polyphenolic content. *J. Appl. Sci. Res.*, **6** (6), 683-9.

Henry, D.W., Adriamycin (NCS-123127) and its analogs (1974) *Cancer Chemother. Rept*., Pt **2** (4), 5.

Hirose, K., Jyoyama, H., Kojima,Y. (1984) Pharmacological properties of 2-[44-(2-thiazolyloxy)-phenyl]-propionic acid (480156-5), a new non-steroidal anti-inflammatory agent. *Arzneim ittelf Forsch/Drug Res,*, **34**, 280-286.

Hoffmann, P., Kathriarachchi, H. S. and Wurdack, K. J. (2006) A Phylogenetic Classification of Phyllanthaceae. Kew Bulletin **61** (1): 37-53.

Hoffmann, P. (2007) "Phyllanthaceae" In: Heywood, V. H., Brummitt, R. K., Seberg, O. and Culham, A. *Flowering Plant Families of the World.* Firefly Books: Ontario, Canada, 250-252.

Hogland, HC. (1982) Hematological complications of cancer chemotherapy. *Semin Oncol* **9**, 95-102.

Holmberg, L., Anderson, H. (2004) Hormonal replacement therapy after breast cancer. *the Lancet*; **363,** 410-411.

Hossain, M. S. (1991) M-Pharm. Thesis, Dept. of Pharmacy, Univ. of Dhaka.

Hossain, MM., Ali, MS., Saha, A., Alimuzzaman, M. (2006) Antinociceptive activity of whole plant extracts of *Paederia foetida*. *Dhaka Univ J Pharm Sci.*, **5**, 67-69.

Hossain, MS., Alam, MB., Chowdhury, NS., Asadujjaman, M., Zahan, R., Islam, MM., *et al*. (2011) Antioxidant, analgesic and anti-inflammatory activities of the herb *Eclipta prostrata*. *J Pharmacol Toxicol*, **6** (5), 468-480.

Hufford, C. D., Sharma, A. S. and Oguntimein, B. O. (1980) *J. of Pharm. Sci*. **69**, 1180.

Hufford, C. D. and Oguntimein, B. O. (1980) *Phytochemisrty,* **19**, 2036.

Hufford, C. D., Oguntimein, B. O. and Baker, J. K. (1981) *J. Am. Chem. Soc*. **46**, 3073.

Hui, W. and Li, M. (1976) Lupene triterpenoids from *Glochidion eriocarpum*. *Phytochemistry*, **15**: 561-562.

Hui, Y. H., Rupp, J. K., Lui, Y. M., Anderson, J. E., Smith, D. L., Chang, C.J., McLaughlin, J. L. (1989) *J. of Nat. Prod.* **52**, 463.

Hutchinson, J. (1973) "Euphorbiaceae" In: *The Families of Flowering Plants, Third Edition,* Oxford University Press: London, 329-330.

IUBMB: (2005) International Union of Biochemistry and Molecular Biology http://www.iubmb.org/

Inatani, R., N., Nakatani and Fuwa, H. (1983) Antioxidative effects of the constituents of rosemary (*Rosmarinus officinalis*) and their derivatives. *Agricultural and Biological Chemistry*, **47,** 521-528.

Isaacs, J. (2002) *Aboriginal Food and Herbal Medicine*, New Holland Press, Sydney.

Ito, N., Hiroze, M., Fukushina, G., Tauda, H. and Tatematsu, M. (1986) Studies on antioxidants; their carcinogenic and modifying effects on chemical carcinogenesis. *Food Chemistry Toxicology*, **24,** 1071-1081.

Jayaprakasha, G. K. and Jagomohan, R, I. (2000) Hale and antioxidant activity, **56**, 1018.

Jeena, KJ., Joy, KL., Kuttan, R. (1999) Effect of *Emblica officinalis*, *Phyllanthus amarus* and *Picrorrhiza kurroa* on N-nitrosodiethylamine induced hepatocarcinogenesis. Cancer letters, **136** (1): 11-16.

Johns, S.R. *et al*. (1967) *Australian Journal of Chemistry*, **3**, 135.

Jones, J., H., Peter, Abumweis and Suhad, S. (2009) Phytosterols as functional food ingredients: linkages to cardiovascular disease and cancer. *Curr. Opin. Clin. Nutr. Metab. Care*., **12** (2), 147-151.

Jossang, A., Leboeuf, M., Cave, A. (1982) A new type of isoquinoline alkaloids, the bisaporphines. *Terahedron Lett.*, **23**, 5147-5150

Jossang, A., Leboeuf, M., Cabalion, P., Cave, A. (1983) Alkaloids of the Annonaceae. XLV : Alkaloids of *Polyalthia nitidissima. Planta Med.,* **49**, 20-24.

Jossang, A., Leboeuf, M., Cave, A., Sevenet, T., Padmawinata, K. (1984) Alkaloids of the Annonaceae. L. Alkaloids from *Polyalthia cauliflora, J. Nat. Prod.*, **47**, 504-513.

Kabir, S., Rahman, M. S., Chowdhury, A. M. S., Hasan, C. M. and Rashid, M. A. (2010) An unusual bisnor-clerodane diterpenoid from *Polyalthia simiarum*. Nat Prod Commun, **5** (10), 1543-1546.

Kakisawa, H., Chen, Y. P., Hsu, H. Y. (1972) Lignans in flower buds of *Magnolia fargesii. Phytochemisy*, **11**, 2289-2293.

Kapoor, N., Bedi, K. L., Tikoo, M. K., Kapoor, S. K. and Sarin, Y. K. (1990) *Journal of Oil Technological Association of India,* **22** (3), 65-66.

Kanokmedhakul, S., Kanokmedhakul, K., Yodbuddee, D., Phonkerd, N. (2003) New antimalarial bis-dehydroaporphine alkaloids from *Polyalthia debilis*, *Journal of Natural Products*, **66**, 616-619.

Kanokmedhakul, S., Kanokmedhakul, K., Kantikeaw, L, Phonkerd, N. (2006) 2-Substituted Furans from the Roots of *Polyalthia evecta. J. Nat. Prod.,* **69**, 68-72.

Kanokmedhakul, S., Kanokmedhakul, K., Lekphrom, R. (2007) Bioactive constituents of the roots of *Polyalthia cerasoides*, *Journal of Natural Products*, **70**, 1536-1538.

Kar, A. and Jain, S. R. (1971) *Flavour Ind.* **2**, 111.

Kathiriya, A., Das, K., Kumar, E.P. and Mathai, K.B. (2010) Evaluation of antitumor and antioxidant activity of *Oxalis corniculata* Linn. against Ehrlich Ascites Carcinoma on mice. Iran. J. Cancer Prev., **4**: 157-165.

Kathriarachchi, H. S., Hoffmann, P., Samuel, R., Wurdack, K. J. and Chase, M. W. (2005) Molecular phylogenetics of Phyllanthaceae inferred from five genes (plastid atpB, matK, 3'ndhF, rbcL, and nuclear PHYC). *Molecular Phylogenetics and Evolution,* **36** (1): 112–134.

Kathriarachchi, H. S., Samuel, R., Hoffmann, P., Mlinarec, J., Wurdack, K. J., Ralimanana, H., Stuessy, T. F. and Chase, M. W. (2006) Phylogenetics of tribe Phyllantheae (Phyllanthaceae) based on nrITS and plastid matK DNA sequence data. American Journal of Botany, **93** (4): 637-655.

Kaushik, D., Kumar, A., Kaushik, P., Rana, AC. (2012) Analgesic and antiinflammatory activity of *Pinus roxburghii* Sarg., doi: 10.1155/2012/245431.

Keay, R. W. J., Onochie, C. F. A. and Standfield, D. P. (1964) *The Nigerian Trees,* V-**1**. 42 Federal Dept. of Forest Research, Nigeria.

Kennedy, DO., Kokima, A., Hasuma, T., Yano, Y., Otani, S., Matsui-Yuasa, I. (2001) Growth inhibitory effect of green tea extract and (-)-epigallocatechin in Ehrlich ascites tumor cells involves a cellular thiol-dependent activation of mitogenic activated protein kinase. *Chem Biol Interact* **134**, 113-133.

Kerstein, P.C., Camino, del D., Moran, M.M., &. Stucky, C.L. (2009). Pharmacological blockade of TRPA1 inhibits mechanical firing in nociceptors. Molecular Pain, 5, 19-25.

Khanam, M., Rahman, MM. (2002) Annonaceae, in: Khan MS, Rahman MM. (Eds), *Flora of Bangladesh,* Vol. **52**, Bangladesh National Herbarium, Ministry of Environment and Forest, Government of the People's Republic of Bangladesh. 52.

Kiem, PV., Thu, VK., Yen, PH., Nhiem, NX., Tung, NH., Cuong, NX., Minh, CV., Huong, HT., Hyun, JH., Kang, HK., Kim, YH. (2009, Jan.) New triterpenoid saponins from *Glochidion eriocarpum* and their cytotoxic activity. *Chem Pharm Bull* (Tokyo), **57** (1): 102-5.

Kijjoa, A., Pinto, M. M. M., Pinho, P. M. M., Tantisewie, B., Herz, W. (1990) Clerodane derivatives from *Polyalthia viridis, Phytochemistry*, **29**, 653-655.

Kijjoa, A., Pinto, M. M. M., Pinho, P. M. M., Herz, W. (1993) Clerodanes from *Polyalthia viridis, Phytochemistry*, **34**, 457-460.

Kipps, TJ. (2006) Chronic lymphocytic leukemia and related diseases. In lichtman, MA. *et al*., (eds.) Williams Hematology, (7^{th} Ed.) 1343-1383.

Kirtikar, KR., Basu, BD. (1980) *Indian Medicinal Plants*, Vol. **1**, Singh B, Singh MP (Eds), Dehra Dun, India, 72-75.

Kirtikar and Basu. (1980) *Indian Medicinal Plants*, Vol-**1**. Published by Singh, B. and Singh, M. P. (2^{nd} Ed.), India, 60-73.

Kumar, R. S., Jayakar, B. and Rajkapoor, B. (2007) Antitumar activities of *Indigofera trita* on Ehrlich ascires carcinoma induced mice. Int. J. Cancer Res., **3**: 180-185.

Kuper, H., Boffetta, P. and Adami, H.O. (2002) Tobacco use and cancer causation: association by tumour type. J. Int. Med., **252** (3): 206-224.

Koduru, S., Grierson, D. S., Venter, M. V. and Afolavan, A. J. (2006) *In vitro* antitumor activity *Solanum aculeastrum* berries on three carcinoma cells. Int. J. Cancer Res., **2**: 397-402.

Larsson, Sc., Wolk, A. (2007) Coffee consumption and Risk of liver Cancer: a metal analysis. *Gastroenterology*, **132** (5), 1740-5.

Lavault, M., Guinaudeau, H., Bruneton, J., Sevenet T., Hadi, H. A. (1990) (-)-Thaipetaline, a tetrahydroprotoberberine from *Malaysian* Annonaceae. *Phytochemistry*, **29**, 3845-3847.

Lasswell, W. L. and Hufford, C. D. (1977a) *J. Org. Chem.* **42**, 1295.

Leaven (1979) Thesis, University of Dhaka.

Li, HY., Sun, NJ., Kashiwada, Y., Sun, L., Snider, JV., Cosentino, LM., Lee, KH. (1993) Anti- AIDS agents, 9. Suberosol, a new C31 lanostane-type triterpene and anti-HIV principle from *Polyalthia suberosa*, *Journal of Natural Products*, **56**, 1130-1133.

Liesveld, JL., Lichtman, MA. (2006) Acute myclogenous leukemia. In lichtman, MA. *et al*., (eds. 1) William Hematology; (7^{th} Eds.), 1183-1236.

Lorke, DA. (1983) A new approach to practical acute toxicity testing. *Arch Toxicol*, **54**, 275-287.

[ab]Lowy, DR., Schille,r JT. (2006) Prophylactic human papilloma virus vaccines. *J. Clin. Invest*; **116** (5), 1167-7.

Ma, X., Lee, L-S., Chai, H.-B., Zaw, K., Farnsworth, N. R., Soejarto, D.D., Cordell, G. A., Pezzuto, J. M., Kinghorn, A. D. (1994) Cytotoxic clerodane diterpenes from *Polyalthia barnesii*, *Phytochemistry*, **37**, 1659-1662.

Madhuri, S., Pandey, Govind. (2008) Some dietary agricultural plants with anticancer properties. *Plant Arch*, 8, 13–16.

Mahady, GB., Fabricant, D., Chadwick, LR., Diets, B. (2002) An alternative therapy for menopause. *Nutrition in Clinical Care*, **5**, 283-9.

McCurdy, C. R. and Scully, S. S. (2005) Analgesic substances derivedfrom natural products (natureceuticals), *Life Sciences*, **78 (**5), 476–484.

McLaughlin, J. L and Rogers, L. L. (1998) The use of biological assays to evaluate botanicals. *Drug Infor. J.,* **32**, 513-524.

Manabe S, Nishino C. (1986) *Tetrahedron*, **42,** 3461-3470.

Menon, M. K. and Kar, A. (1970) *Indian J. Exp. Biol.* **8**, 151.

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobsen, J. B., Nicholsand, D. E. and Mclaughlin, J. L. (1982) Brine shrimp; a convenient general bioassay for active plant constituents. *Planta Med.* **45**, 31-34.

Meyskens, F.L. and Szabo, E. (2005) Diet and cancer: the disconnect between epidemiology and randomized clinical trials. Cancer Epidemiol. Biomarkers, **14**, 1366-1369.

Mololits, S., Ullenhag, G., and Mellstedt, H. (2005) Therapeutic Vaccination in patients with gastrointestinal malnancies. A review of immunological and clinical results, *Ann. One*, **16** (6) 847-872.

Montgomery, J.A. and Struck, R.I. (1973) In: Progress in Drug Research. *Jacker E. (ed.), Birkhauser-Verlag, Basel*, **17**, p.320.

Moulies, S., Dennise, Sgroi. (2008) Re-evaluating early breast neoplasia. *Breast Cancer research*, **10**, 302.

Moulignier, Antoine, *et al*. (1994) Cerebral Glial Tumors and Human Immunodeficiency Virus-1 infection. *Cancer*, **74** (2) 686-87.

Nadir (1986) Thesis, University of Dhaka.

Neto, JO., Agostinho, SM., Das, MF., Vieira, PC., Vilela, EF. (1995) *Phytochemistry*, **38** (2), 397-401.

Newman, A. A. (1972) Chemistry of Terpenes; Academic Press, London.

Newman, D.J., Cragg G.M. and Snader, K.M. (2003) Natural products as sources of new drugs over the period 1981-2002. J. Nat. Prod., **66**: 1022-1037.

Nelson, D.A., Tan, T.T., Rabson, A.B., Anderson, D., Degenhardt, K. and White, E. (2004) Hypoxia and defective apoptosis drive genomic instability and tumorigenesis. Gene. Dev., **18** (17): 2095-2107.

Nogueira RT, Shepherd GJ, Laverde A, Marsaioli AJ, Imamura PM. (2001) Phytochemistry, **58**, 1153-1157. [Pubmed]

Ogata, T., Higuchi, H., Mochida, S., Matsumoto, H., Kato, A., Endo, T., Kaji, A., Kaji, H. (1992) HIV-1 reverse transcriptase inhibitor from *Phyllanthus niruri*. AIDS research and human retroviruses, **8** (11): 1937-1944.

Okarie, D. A. (1980) A new class of indolosesquiterpene alkaloids from *Polyalthia suaveolen. Tetrahedron*, **36**, 2005-2008.

Okorie, D. A. (1981) Polyavolinamide, an indolosesquiterpene alkaloids from *Polyalthia suaveolens. Phytochemistry*, **20**, 2575-2578.

Otsuka, H., Hirata, E., Takushi, A., Shinzato, T., Takeda, Y., Bando, M. and Kido M. (2000, Apr.) Glochidionolactones A-F: butenolide glucosides from leaves of *Glochidion zeylanicum* (Gaertn) A. Juss. *Chem. Pharm. Bull* (Tokoyo)., **48** (4): 547-51.

Otsuka, H., Hirata, I., Shinzato, T. and Takeda, Y. (2001, Jul.) Glochiflavanosides A-D: flavanol glucosides from the leaves of *Glochidion zeylanicum* (Gaertn) A. Juss. *Chem. Pharm. Bull* (Tokoyo)., **49** (7): 921-3.

Otsuka, H., Takeda, Y., Hirata, E., Shinzato, T. and Bando, M. (2004, May) Glochidiolide, isoglochidiolide, acuminaminoside, and glochidacuminosides A-D from the leaves of *Glochidion acuminatum* Muell. *Chem. Pharm. Bull* (Tokoyo). **52** (5): 591-6.

Ott, M., Thyagarajan, SP, Gupta, S. (1997) *Phyllanthus amarus* suppresses hepatitis B virus by interrupting interactions between HBV enhancer I and cellular transcription factors. European journal of clinical investigation. **27** (11): 908-915.

Pachiro, G., Zumi, Y., Higushi-Zawa's and Gann. (1925) *Cancer Res*, **47**, 525-8.

Padmaa, MP., Khosa, RL. (2009) Phytoconstituents from the genus *Polyalthia* - a review, *Journal of Pharmaceutical Research* **2**, 594-605.

Parkin, D., Bray, F., Ferlay, J., Pisami, P. (2002) Global Cancer Statistics, CA *Cancer J Clin*, **55** (2), 74-108.

Pal, S., Sen, T., Chaudhuri, AK. (1999) Neuropsychopharmacological profile of the methanolic fraction of *Bryophyllum pinnatum* leaf extract. *J Pharm Pharmacol*, **51**, 313-318.

Patoomratana, T., Manat, P., Viehai, R., Wanpen, T., Samaisukh, S., Chalobon, Y., Thawatchai, M. (2001) 2P-Substituted furans from *Polyalthia suberosa. Planta Medica*, **67**, 572-575.

Persoone, G. (1980) Proceeding of the International Symposium on brine shrimp, Artemia saline vols. 1-3, Universa Press, Witterin, Belgium.

Phadnis, A. P., Patwardhan, S. A., Dhaneshwar, N. N., Tavale, S. S., Row, T. N. Guru. (1988) Clerodane diterpenoids from *Polyalthia longifolia. Phytochemistry*, **27**, 2899-2901.

Pieroni, A., Janiak, V., Durr, C. M., Ludeke, S., Trachsel, E. and Heinrich, M. (2002) Invitro antioxidant activity of non-cultivated vegetables of ethnic Albanians in Southern Italy. *Phytother Res*. **16,** 467-473.

Pietta, A., Sionetti, P. and Mauri, P. (1998) Antioxidant activity of selected medicinal plants. *J. Agric. Food Chem.* **46**, 4487-4490.

Pinner, R., Teutch, S., Simonson, L., Klug, L., Glaber, J., Clarke, M. and Berkelman, R. (1996) Trends in infectious diseases mortality in the United States. *J. Am. Med. Assoc*. **275**, 189-193.

Prasad, SB., Giri, A. (1994) Antitumor effect of cisplatin against murine ascites Dalton's lymphoma. *Indian J Exp Biol* **32**, 155-162.

Pruesapan, K., Telford, I. R. H., Bruhl, J. J., Draisma, S. G. A. and Welzen, P. C. V. (2008) Delimitation of Sauropus (Phyllanthaceae) Based on Plastid matK and Nuclear Ribosomal ITS DNA Sequence Data. *Annals of Botany*, **102** (6): 1007-1018.

Puapairoj P, Naengchomnong W, Kijjoa A, Pinto MM, Pedro M, Nascimento MS, Silva AM, Herz W. (2005, Mar.) Cytotoxic activity of lupane-type triterpenes from *Glochidion sphaerogynum* and *Glochidion eriocarpum* two of which induce apoptosis. *Planta Med*., **71** (3): 208-13.

Puci-H. (2006) Acute lympho blastic leukemia. In lichtman, MA. *et al*., (eds.), Williams Hematology; (7^{th} Eds.), 1321-1383.

Rahman, M.O. (2008) *Glochidion multiloculare*. In: Ahmed, Z.U., Hassan, M.A., Begum, Z.N.T., Khondker, M., Kabir, S.M.H., Ahmad, M., Ahmed, A.T.A., Rahman, A.K.A. and Haque, E.U. (eds.). *Encyclopedia of flora and fauna of Bangladesh*, Vol.**7**. *Angiosperms: Dicotyledons (Balsaminaceae - Euphorbiaceae)*. Asiatic Society of Bangladesh, Dhaka. pp. 440-441.

Rahman, M.O. (2008) *Glochidion lanceolarium*. In: Ahmed, Z.U., Hassan, M.A., Begum, Z.N.T., Khondker, M., Kabir, S.M.H., Ahmad, M., Ahmed, A.T.A., Rahman, A.K.A. and Haque, E.U. (eds.). *Encyclopedia of flora and fauna of Bangladesh*, Vol.**7**. *Angiosperms: Dicotyledons (Balsaminaceae - Euphorbiaceae)*. Asiatic Society of Bangladesh, Dhaka. pp. 439-440.

Rahman, MH., Alam, MB., Chowdhury, NS., Jha, MK., Hasan, M., Khan, MM., Rahman, MS., Haque, ME. (2011) Antioxidant, analgesic and toxic potentiality of *Stephania japonica* (Thunb.) Miers. *Leaf. Int J Pharmacol*, **7**, 257-62.

Rajeshkumar, NV., Joy, KL., Kuttan, G., Ramsewak, RS., Nair, MG., Kuttan, R. (2002) Antitumour and anticarcinogenic activity of *Phyllanthus amarus* extract. Journal of ethnopharmacology. **81** (1): 17-22.

Ramprasath**,** V**.**R., Shanthi, P., Sachdanandam, P**.** (2006). Immunomodulatory and anti-inflammatory effects of *Semecarpus anacardium* LINN. Nut milk extract in experimental inflammatory conditions. *Biological and Pharmaceutical Bulletin,* **29**, 693-700.

Rana, AYKMM., Khanam, JA. (2002) *Aristolochia indica* whole plant extract as an antineoplastic agent. *J Med Sci* **2**, 202-205.

Rashid, MA., Hossain, MA., Hasan, CM., Reza, MS. (1996) Antimicrobial diterpenes from *Polyalthia longifolia* var. *pendulla* (Annonancae), *Phytotherapy Research*, **10**, 79- 81.

Rashid, M. A., Hasan, C. M., Choudhury, S. A. R., Begum, B. and Rahman, S. (1997) Ethnopharmacological investigation of medicinal plants of Bangladesh. *Bangladesh J. Physiol. Pharmacol.* **12,** 25-29.

Ravikiran, G., Elumalai, A., Eswaraiah, MC., Naresh, V. (2012) An annual review of anti inflammatory medicinal plants. *Inter J of Phytotherapy*, **2** (1), 23-27.

Rechner, AR., Kuhnle, G., Bremner, P., Hubbard, GP., Moore, KP., Rice-Evans CA. (2002) The metabolic fate of dietary polyphenols in humans. *Free Radic Biol Med*, **33**, 220-235.

Riaz, A., Khan, RA., Ahmed, S., Afroz, S. (2010) Assessment of acute toxicity and reproductive capability of a herbal combination. *Pak J Pharm Sci*, **23** (3), 291-294.

Ribeiro, RA., Vale, ML., Thomazzi, SM., Paschoalato, AB., Poole, S., Ferreira, SH., *et al*. (2000) Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. *Eur J Pharmacol*, **387**, 111-118.

Richomme, P., Godet, M. C., Foussard, F., Toupet, L., Sevenet, T., Bruneton, J. (1991) A novel leishmanicidal labdane from *Polyalthia macropoda*, *Planta Med.*, **57**, 552-554.

Roland, R. (1982) *Antibiotics, An Introduction*, F. Hoffmann La. Roche, and Co. Basle, Switzerland, P. 70-71.

Royal Botanic Gardens, Kew: (2010) Molecular Phylogenetics of Euphorbiaceae *sensu lato*, http://www.kew.org/science/directory/projects/MolecPhylogEuphSL.html, (2010-07-25).

Sadiq, Y., Alexander, A.B. and Abdulkarim, A. (2009) Effect of *Zizyphus mauritiania* (L.) seed extracts on spatial recognition memory of rats as measured by the Y-maze test. J. Nat. Prod. **2**: 31-39.

Saleem, R., Ahmed, M., Ahmed, S. L, Azeem, M., Khan, R. A., Rasool, N., Saleem, H., Noor, F., Faizi, S. (2005) Hypotensive Activity and Toxicology of Constituents from Root Bark of *Polyalthia longifolia* var. pendula, *Phytotherapy res.*, **19**, 881-884.

Sam, T. W., Chews, S. Y., Sabirin, M., Gan, E. K., Dzulkifli, R. and Mohammad. A. L. (1987) *Tetrahedron Letters*, **28**, 2541.

Samuel, R., Kathriarachchi, H. S., Hoffmann, P., Barfuss, M. H. J., Wurdack, K. J., Davis, C. C. and Chase, M. W. (2005) Molecular phylogenetics of Phyllanthaceae: evidence from plastid matK and nuclear PHYC sequences. *American Journal of Botany* **92** (1): 132-141.

Sashidhara, KV., Singh, SP., Sarkar, J., Sinha, S. (2009) Cytotoxic clerodane diterpenoids from the leaves of *Polyalthia longifolia*, *Natural Product Research: formerly Natural Product Letters*, 1-8.

Sashidhara, KV., Singh, SP., Shukla, PK. (2009) Antimicrobial evaluation of clerodane diterpenes from *Polyalthia longifolia* var.*pendulla*, *Natural Product Communications*, **4**, 327-330.

Satosker, R. S. and Bhandarker, S. D. (1993) Pharmacology and pharmacotherapeutics, (13^{th} Ed.), 720-729.

Schultes, R. E. and Raffauf, R. F. (1990) *The Healing Forest: Medicinal and Toxic Plants of the Northwest Amazonia.* Dioscorides Press, Portland, 581.634

Seaman, R., Bohlmann, R., Zdero, C., Mabry, TJ. (1990) Diterpenes of Flowering Plants: Compositae (Asteraceae) Springer-Verlag; New York, 638.

Shaheen, F., Ali, K. R., Soobia, A., Ahmad, S., Saima, T., Aqeel, A. (2003) New antimicrobial alkaloids from the roots of *Polyalthia longifolia* var. pendula. *Planta Medica*, **69**, 350-355.

Shahidi, F., Janitha, P. K. and Wanasundara, P. D. (1992) Phenolic antioxidants. *Crit Rev Food Sci nutr*. **32**, 67-103.

Shahnaz, S. (1986). Phytochemical studies of two Annonaceous species. M-Pharm, Thesis. Dept. of Pharm. Univ. of Dhaka.

Sica, D.A. (2006) Angiogenesis Inhibitors and Hypertension An emerging Issue, *J. Clin. Oncol*, **24** (9), 1329-1331.

Sinha, R., Peters, U., Cross, AJ., *et at*. (2005) Meat, meat cooking methods and presentation and risk fro colorectal adenoma. *Cancer Res*, **65** (17), 8034-41.

Sivalokanathan, S., Ilayaraja, M., Balasubramanium, MP. (2005) Efficacy of *Terminalia arjuna* (Roxb.) on N-nitrosodiethylamine induced hepatocellular carcinoma in rats. *Indian J Exp Biol*, **43**, 264–67.

Somdej, K., Kwanjai, K., Daungrudee, Y., Nutchanat, P. (2003) New Antimalarial Bis-dehydroaporphine Alkaloids from *Polyalthia debilis. J. Nat. Prod.*, **66**, 616-619.

Steinmetz, K. A. and Potter, J. D. (1996) Vegetables, fruit, and cancer prevention, A review. *J Am Diet Assoc*. **96,** 1027-1039.

Strobel, G., Daisy, B., Castillo, U. and Harper, J. (2004) *J. Nat. prod.* **67,** 257.

Suja, KP., Jayalekshmy, A., Arumughan, C. (2004) Free radical scavenging behavior of antioxidant compounds of sesame (*Sesamum indicum* L.) in DPPH center dot system. *J Agric Food Chem*, 52, 912-915.

Sur, P., Ganguly, DK. (1994) Tea plant roots extract (TRE) as an antineoplastic agent. *Planta Med* **60**, 106-109.

Sur, P., Bag, SP., Khanam, JA. (1997) Choroacetohydroxamic acid as antitumor agent against Ehrlich ascites carcinoma in mice. *Neoplasma* ,**44**, 197-201.

Talapatra, S.K., Bhattacharya, S., Maiti, B.C., Talapatra, B. (1973) *Chem. Ind. (London)*, **21**: 1033-1034.

Takhtajan, A. (1969) *Flowering Plants-origin and Dispersal*, Oliver and Boyd. Edinburge.

Talapatra, S. K., Basu, D., Deb, T., Goswami, S., Talapatra, B. (1985) *Ind. J. Chem.* **24B**, 29.

Tamayo-Castillo, G., Jakupovic, J., Bohlmann, F., King, R. M. (1989) *ent*-clerodane derivatives and other constituents from representatives of the *subgenus ageratina*. *Phytochemistry***, 28**, 139-141.

Tanaka, R., Kinouchi, Y., Wada, S., Tokuda, H. (2004, Dec.) Potential anti-tumor promoting activity of lupane-type triterpenoids from the stem bark of *Glochidion zeylanicum* and *Phyllanthus flexuosus*. Planta Med., **70** (12):1234-6.

Temple, NJ. (2000) Antioxidants and disease: more questions than answers. *Nutr Res*, **20**, 449-459.

Thu, V. K., Kiem, P. V., Minh, C. V., Yen, P. H., Cuong, N. X. and Huong, H. T. (2010) A new flavan glucoside from *Glochidion eriocarpum*, *Journal of Chemistry*, **48** (1), 125-131.

Thonston, JB. (2004) Chronic Lymphocytic Leukemia. In Green, JP. *et al*. (eds.) Wintrobes, Clinical Hematology; (11th Ed.), **2**, 2429-2463.

Toma, W., Graciosa, JS., Hiruma-Lima, CA., Andrade, FDP., Vilegas, W., Souza-Brita, ARM. (2003) Evaluations of the analgesic and antiedematogenic activities of *Quassia amara* bark extract. *J Ethnopharmacol*, **85**, 19-23.

Trease, G.E. and Evans, W.C. (1989) A Textbook of Pharmacognosy. 13th Edn., Bailliere Tindall Ltd., London, ISBN: 0702013617.

Trease and Evans (1993) Pharmacognosy. 13th Edn., Bailliere Tindall, London, 177.

Tuchinda, P., Pohmakotr, M., Munyoo, B., Reutrakul, V., Santisuk, T. (2000) An azaanthracene alkaloid from *Polyalthia suberosa.* *Phytochemistry*, **53**, 1079-1082.

Tuchinda, P., Munyoo, B., Pohmakotr, M., Thinapong, P., Sophasan, S., Santisuk, T., Reutrakul, V. (2006) Cytotoxic Styryl-Lactones from the Leaves and Twigs of *Polyalthia crassa.* *J. Nat. Prod.* , **69**, 1728-1733.

Tyler, V. E. (1986) *Econ. Bot.* **40**, p 279.

Vanwagenen, B. C., Larsen, R., Cardellina, J. H., Randazzo, D., Lidert, Z. C., and Swithenbank, C. (1993) *J. Org. Chem.*, **58**, 335.

Velioglu, Y. S., Mazza, G., Gao, Y. L. and Oomah, B. D. (1998) Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *J. Agric. Food Chem.* **46**, 4413-4417.

Venkateswaran, PS., Millman, I., Blumberg, BS. (1987) Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: in vitro and in

vivo studies. Proceedings of the National Academy of Sciences of the United States of America, **84** (1): 274.

Vogel, HG., Vogel, WH. (1997) *Drug Discovery and Evaluation–Pharmacological Assays*. Springer-Verlag, Berlin Heidelberg, 1231.

[ab]Vogel, V., Costantiono, J., Wickerham, D., Croninw, Cecehini, R., Atkins, J., Bevers, T., Fehrenbacherl, Pajon, E., Wade, J., Robindoux, A., Margolese, R., James, J., Lippmans, Runowiez, C., Gann, P., Reis, S., Mccaskill-steven, SW., Ford, L., Jordan, V., Wolmark, N. (2006) Effects of tamoxifen Vs raloxifene on the risk of developing invasive breast cancer and other disease out comes; the NSABP study of tamoxifen and raloxifene (STAR) p-2 trial. *JAMA*, **295** (23), 2727-41.

Voilley, N. (2004) Acid-sensing ion channels (ASICs): New targets for the analgesic effects of Non-Steroid Anti-Inflammatory Drugs (NSAIDs). *Curr Drug Targets Inflamm Allergy,* **3**, 71-79.

Wang, JR. Zhou H, Jiang ZH, Wong YF, Liu L. (2008) In vivo anti-inflammatory and analgesic activities of a purified saponin fraction derived from the root of Ilex pubescens. *Bio Pharmaceu Bull*, **31**, 643-650.

Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P. and McPhail, A. T. (1971) Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia. J. A m. Chem. Soc*. **93,** 2325-2327.

Warthen, D., Gooden, E. L. and Jacobson, M. (1969) *J. Pharm. Sci*., **58**, 637.

Webster, G. L. (1994) Classification of the Euphorbiaceae. *Annals of the Missouri Botanical Garden*, **81** (1): 3-32.

WHO. (2007) Cancer World Health Organization.

Wichi, H. P. (1988). *Food and Chemical Toxicology*, **26**, 717.

Winrow, V.R., Winyard, P.G., Morris, C.J. *et al*. (1993) Free radicals in inflammation: second messengers and mediators of tissue destruction. *Br. Med. Bull*., **49**, 506-522.

Winter, CA., Risley, EA., Nuss, GW. (1962) Carrageenan induced oedema in hind paw of the rats as an assay for anti-inflammatory drugs. *Pro Soc Exp Biol Med*, **111**, 544-547.

Wu, Y. C. (1989) Azafluorene and aporphine alkaloids from *Polyalthia longifolia Heterocycles*, **29**, 463-475.

Wu, Y. C., Duh, C. Y., Wang, S. K., Chen, K. S., Yang, T. H. (1990) Two new natural azafluorene alkaloids and a cytotoxic aporphine alkaloid from *Polyalthia longifolia. J. Nat. Prod.***, 53**, 1327-1331.

World of Science: (1980) The world of plants, published by Bay Books, London.

Yah[a], H., Sellick[b], K. (2006) Quality of life Chinese patients newly diagnosed with breast Cancer: a longitudinal study, **41** (3), 309-319.

Yang, J., Yang, XD., Yang, S., Zhao, JF. and Li, L. (2007) *Chinese Journal of Chinese Materia Medica*, **32**, 593-6.

Zhang, Y., Seeram, N.P., Lee, R., Feng, L. and Heber, D. (2008) Isolation and identification of strawberry phenolics with antioxidant and human cancer cell antiproliferative properties. *J. Agric. Food Chem*., **56,** 670-675.

Zhao, G., Jung, J. H., Smith, D. L., Wood, K. V., McLaughlin, J. L. (1991) Cytotoxic clerodane diterpenes from *Polyalthia longifolia*, *Planta Medica*, **57**, 380-383.

Zhu, W. M., Li, S. L., Shen, Y. M., Ning, X.J., Zhao, B.T., Hao, X. J. (2002) The sesquiterpenoid from *Polyalthia cheliensis* Hu., *Heterocycles*, **57**, 529-534.

Zhu, H., Guo, W., Zhang, L., Davis, J.J., Teraishi, F., Wu, W., Cao, X., Daniel, J. (2005) Bc1-XL small interfering RNA suppresses the proliferation of 5-fluorouracil-resistant human conon cancer cells. *Molecular Cancer Therapy*, **4** (3), 451-456.

List of publications

Kabir, S., Hossain, M. A., Chowdhury, A. M. S. and Rashid, M. A. (2009) Antimicrobial and Cytotoxic Activities of the Crude Extracts of *Glochidion lanceolarium. J. Sci. Foundation*, **7** (1), 127-130.

Kabir, S., Rahman, M. S., Chowdhury, A. M. S., Hasan, C. M. and Rashid, M. A. (2010) An unusual bisnor-clerodane diterpenoid from *Polyalthia simiarum*. Nat Prod Commun, **5** (10), 1543-1546.

Kabir, S., Chowdhury, A. M. S. and Rashid, M. A. (2011) Antimicrobial and cytotoxic activities of the crude extracts of *Polyalthia simiarum. Dhaka Univ J Sci.,* **59**, 199-200.

Kabir, S., Chowdhury, A. M. S. and Rashid, M. A. and Hasan, C. M. (2012) Antimicrobial and Cytotoxic Activities of the Extracts of *Glochidion multiloculare*. *J Sci. Foundation,* **10** (1), 1-11 [www.banglajol.info/index.php/JSF].

Kabir, S., Zahan, R., Mosaddik, A., Chowdhury, A. M. S., Hasan, C. M. and Rashid, M. A. (2013) Antitumor and antioxidant activities of *Polyalthia simiarum* (Buch.-Ham. Ex Hook. F. & Thomson) Hook. F. & Thomson. *Turk J Pharm Sci*, **10** (3), 405-414.