PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION ON *Syzygium aromaticum* (Myrtaceae)

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Dedicated to

My parents

For their unparallel blessing,

Inspiration

&

Guidance to my life

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Declaration by the Research candidate

I, **Sharif Neaz**, hereby declare that the dissertation entitled **“**PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION ON *Syzygium aromaticum* (Myrtaceae)**”**, submitted by me to the Department of Biochemistry & Molecular Biology, University of Dhaka, in partial fulfillment of the requirements for the award of the degree of Masters of Philosophy (M. Phil) is a complete record of original research work carried out by me during the period 2013-2014 under the supervision and guidance of **Prof. Dr. Md. Enamul Haque,** Professor, Department of Biochemistry & Molecular Biology, University of Dhaka and it has not formed the basis for the award of any other Degree/Diploma/Fellowship or other similar title to any candidate of any University.

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Certification

This is to certify that the research work on **"**PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION ON *Syzygium aromaticum* (Myrtaceae)**"** submitted to the Department of Biochemistry & Molecular Biology, University of Dhaka in partial fulfillment of the requirements for the degree of Masters of Philosophy (M Phil) was carried out by Sharif Neaz under or guidance and supervision and that no part of the thesis has been submitted for any other degree. I further certify that all the sources of information and facilities availed of this connection are duly acknowledged.

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Prof. Dr. Md. Enamul Haque

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Abstract

Clove buds were collected, grinded and extracted with chloroform and the solvent was evaporated to get the crude extract. The crude extract was then subjected to vacuum liquid chromatography to get thirty fractions named as VLC fractions 1-30. These VLC fractions were then analyzed by thin layer chromatography to identify promising fraction. Those fractions were then subjected to preparative TLC in order to isolate the compounds. A small portion of crude was washed with petroleum ether, ethyl acetate, methanol and water to get petroleum ether soluble fraction (PESF), ethyl acetate soluble fraction (EASF), methanol soluble fraction (MSF) and water soluble aqueous fraction (AQF). These compounds then identified by ^{1}H NMR, ^{13} C NMR and ATR- FTIR respectively. Compounds isolated from fractions SA-1.1, 1.2, 6, 13.1, 13.2 and 29.30 are characterized as eugenol, octane, eugenol acetate, oleanolic acid, β- Amyrin and gossypetin-o-rhamnopyranoside respectively. SA-1, 3, 6, 7, 8, PESF and crude showed highest antibacterial activity against *Bacillus cereus, Bacillus subtilis, Sarcina lutea, Lactobacillus sp., Micrococcus sp, Streptococcus epidermidis, Vibrio cholerae, Klebsiella sp.*, and SA-1, 3, 6, 7, crude and PESF antifungal activity against *Candida albicans, Aspergillus niger, Saccharomyces cereviseae, Tricophyton sp., Aspergillus flavus, Rhyzopus nigricans*. Total phenolic content of fraction SA-1, 6, PESF, EASF, MSF and AQF was 519.33, 489.00, 427.25, 369.35, 208.73 1nd 114.41 mg GAE/ g of extractives. IC_{50}values of SA-1, 6, PESF, EASF, MSF and AQF was 0.72, 0.17, 4.01, 4.06, 9.10, 10.91 µg/ml. When peripheral and central analgesia were tested, SA-1, 6, PESF and AQF exhibited statistically significant ($p<0.05$)peripheral analgesia and though during first thirty minutes these extracts did not show any central analgesia, but after sixty minutes SA-1, 6, PESF and MSF showed significant ($p<0.05$) analgesia, after ninety minutes PESF, EASF showed significant ($p<0.05$) activity. SA-1, 6, PESF, EASF, MSF and AQF displayed statistically significant ($p<0.05$) thrombolytic effect, SA-1, 6, PESF, EASF, MSF showed statistically significant ($p<0.05$) anesthetic activity at 0.01, 0.02 and 0.03% concentrations on *Channa punctatus*. Crude, SA-1, 3, 4, 6, PESF and AQF showed antidiarrhoeal activity. Crude, SA-1, 3, 4, 6, PESF, EASF, MSF and AQF showed statistically significant ($p<0.05$) antiemetic activity. When crude, SA-1, 3, 6, PESF, EASF, MSF and AQF were tested for antihelminthic activity, paralysis time caused by crude, SA-6, PESF, EASF, MSF, AQF was statistically significant ($p<0.05$), death times caused by crude, PESF, EASF, AQF were also statistically significant ($p<0.05$). All extracts and soluble fractions exhibited statistically significant ($p<0.05$) antipyretic activity only after second, third and fourth hour of brewer yeast administration.

Chapter 1

Introduction

1.1 Rationale of the work

The plant is a biosynthetic laboratory and the remedial phyto-elements produced inside a plant through acascade of biochemical reactions significantly contribute to the traditional and modern medicines. Medicinal plants are plants whose extracts can be used directly or indirectly for the treatment of different ailments. Therefore, the use of traditional medicine and medicinal plants in most developing countries is a basis for the maintenance of good health. Scientists throughout the world are trying to explore the precious assets of medicinal plants to help the suffering humanity. Furthermore, in the world more than 30% of the pharmaceutical preparations are based onplants [1].

However, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants.

The use of medicines from plants in the form of local medicine dates back to 4000-5000 B.C. While the medicinal values of these plants are due to the presence of small doses of active compounds which produces physiological actions in the human and animal body [2]. Some of the important bioactive compounds found in medicinal plants are alkaloids, glycosides, resins, gums, terpenoids, antibiotics, flavonoids, mucilages etc. [3]. Plants can be considered as one of the most important and interesting subjects that should be explored for the discovery and development of newer and safer drug candidates.

There are several familiar approaches for lead searching from the plants and the isolated bioactive compounds are utilized in three basic ways [4]:

1. Unmodified natural plant products where ethnomedical uses suggested clinical efficacy, e.g., Digitalis.

2. Unmodified natural products of which the therapeutic efficacy was only remotely suggested by indigenous plant use, e.g., Vincristine.

3. Modified natural or synthetic substances based on a natural product used in folk medicine, e.g., Aspirin

Secondary plant metabolites that results from plant evolution may be equal or superior to that found insynthetic combinatorial chemical libraries. It was estimated that in 1991 in the United States, for every10, 000 pure compounds (most likely those based on synthesis) that are biologically evaluated (primarily *in vitro*), 20 would be tested in animal models, and 10 of these would be clinically evaluated, and only one would reach U.S. Food and Drug Administration

approval for marketing. Most large pharmaceutical manufacturers and some small biotechnology firms have the ability to screen 1,000 ormore substances per week using high throughput *in vitro* assays. In addition to synthetic compounds from their own programs, some of these companies screen plant, microbial, and marine organisms.

1.2 Necessity of Studying of medicinal plants

There are some very important causes to study medicinal plants; such as-

■ Medicinal plants have played an essential role in the development of human culture, for example religions and different ceremonies. (e.g. Datura has long been associated with the worship of Shiva, the Indian god).

■ Most of the modern medicines are produced indirectly from medicinal plants, for example, aspirin.

■ Plants are directly used as medicines by a majority of cultures around the world, for example, Chinese medicine and Indian medicine.

■ Most food crops have medicinal effects, for example garlic, ginger, turmeric etc.

■ Medicinal plants are resources of new drugs. It is estimated that there are more than 250, 000 flower plant species.

■ studying medicinal plants helps to understand plant toxicity and protect human and animals fromnatural poisons.

Still today, natural products including those from plants are playing an important role in the therapy of diseases. “A study of the 25 best-selling pharmaceutical drugs in 1997 found that 11 of them (42%) were biologicals, natural products or entities derived from natural products, with a total value of US$ 17.5 billion” [5]. So far, about 25% of all drugs prescribed worldwide originate from plants. Moreover, from 252 drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusively from plants and there are a significant number of drugs that were obtained by molecular modification of natural products [6]. Recent approvals of several new plant-derived drugs and semi-synthetic and synthetic drugs based on plant secondary compounds confirm the importance of natural product research. Taxol, for example, an anticancer taxane diterpenoid derived from the relatively scarce Pacific or western yew tree, *Taxus brevifolia* (Taxaceae), has recently been approved for the treatment ofrefractory ovarian cancer and breast cancer. Artemisinin, a Chinese drug isolated from *Artemisia annua* (Asteraceae), and several of its derivatives, newly discovered as effective antimalarial agents, are currently undergoing intensive clinical testing [7]. Considering that only a very small percentage of the 300'000 to 500'000 plant species existing on earth have been minimally evaluated, nature still provides alarge amount of new substances [8].

Of these, only 6% have been screened for biological activity, and reported 15% have been evaluated phytochemically [9].

From very early ages of history, plants and plant products have been the primary source of food, shelter and transport materials, clothing, fragrances, flavors and ingredients of medicinal substances for mankind [10]. In ancient times Hindus, Babylonians, Assyrians, Persians, Romans, Chinese and Greeks as well as the people of old American civilisations like Incas, Mayas and Aztecs used natural resins primarily for embalming and for its incense in cultural ceremonies.

Along with the developments in extraction techniques, the development of chromatographic techniques primarily with planar chromatography (thin layer chromatography (TLC) and other novel analytical methods were introduced to the benefit of scientists. Gaschromatography (GC) in the 1950's had opened a new dimension in the analysis of volatile compounds. In the meantime high performance liquid chromatography (HPLC) was introduced for the fractionation and isolation of more polar and non-volatile compounds.The combination of gas chromatography and mass spectrometry (GC-MS) allows the rapid identification of not only volatile components but also plant extracts, by comparing their mass spectra with available libraries which build up with reference substances recorded under the same experimental parameters. The same principle has been applied in the last decade for liquid chromatography and mass spectrometry (LC-MS) for non-volatile plant constituents. Moreover, the invention of chiral stationary phases for gas chromatography, mostly based on cyclodextrins, has facilitated the identification of the enantiomeric composition of the isolated substances, especially in essential oils. Simultaneously, the advances in spectroscopic methods such as mass spectrometry (MS) and nuclear magnetic resonance (NMR)spectroscopy like ^{1}H NMR, ^{13}C NMR have increased the speed of the identification and structure elucidation of natural products.

The empirical use of plants as medicine can be traced back over five millennia to ancient documents of early civilizations such as in China, Egypt, India, and the Near East, but is certainly as old as mankind. Although indigenous knowledge systems rapidly disappear under the influence of Western culture, the World Health Organization (WHO) estimates that even today 80% of the world's population exclusively relies on traditional medicine. In industrialized countries medicinal plant research had its ups and downs in the last decades. In the following "expedition boom" researchers dispersing all over the world scoured almost impenetrable jungles in search of new medicines in journeys sometimes lasting for years. The industrial revolution and progress made in organic chemistry somehow slowed this development causing a preference of synthetic drugs in western communities. These soon got an excellent reputation, originating from the fact that pure compounds couldeasily be obtained and structural modifications yielding in more active and less toxic and therefore safer drugs could simply be performed. The WHO considers that in Germany for example 90% of the population have used a natural remedy at least once in their life and the global market for herbal medicines currently stands at over US$ 60 billion per annum (2003) and is growing steadily.

However at least 119 chemical substances from 90 plant species are important drugs used all over the world, many of them containing compounds derived from or modelled after naturally occurring lead molecules and 74% of these come from traditional medicinal plants. Of the 252 drugs considered as basic and essential by the WHO, 11% are exclusively of plant origin e.g. digoxin from *Digitalis spp*. (Plantaginaceae), quinine and quinidine from *Cinchona spp*. (Rubiaceae), vincristine and vinblastine from *Catharanthus roseus* (Apocynaceae), or morphine and codeine from *Papaver somniferum* (Papaveraceae). It is further estimated that 60% of anti-cancer and anti-infectious drugs already on the market or under clinical trials are of natural origin. A 22% increase was observed for cancer incidence as well as mortality since 1990, making it to the second leading cause of death in high income countries, surpassed only by cardiovascular diseases [11]. There have been several efforts to discover new anticancer agents of plant origin, the most prominent is the National Cancer Institute (NCI) program initiated by Dr. Jonathan Hartwell assembled a list containing over 3,000 different species of plants traditionally used against cancer describing their uses in considerable detail, remaining today as the only compilation of the ethnomedicinal use of anticancer plants [19]. 10-18 Natural products have served as an important source of drugs since ancient times and about half of the useful drugs today are derived from natural sources. Chemodiversity in nature, e.g. in plants, microorganisms and marine organisms, still offers a valuable source for novel lead discovery, but rapid identification of the bioactive compounds of natural product mixtures remains a critical factor to ensure that this tool of drug discovery can compete with recent developed technologies such as chemical compound libraries and high-throughput screening of combinatorial synthetic efforts. Rapid screening of natural product mixtures requires the availability of a library of reference of natural compounds and methods for simple identification of putative lead structural classes avoiding, to a large extent, the potential for false-positive results. The coupling of chromatographic methods such as high pressure liquid chromatography (HPLC) with diode array detection, mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR) like ^{1}H NMR, ^{13}C NMRwith on-line bioactivity assays, is an important tool for high throughput screening of natural product mixtures. Structure-activity studies of these leads, preferentially combined with computergraphic model building, should result in molecules with optimal activity and bioavailability, fewer side effects and an acceptable therapeutic index and, consequently in good candidates, for the development to new drugs.

Spices and herbs have played a dramatic role in civilization and in the history of nations. The delightful flavour and pungency of spices make them indispensable in the preparation of palatable dishes. In addition, they are reputed to possess several medicinal and pharmacological properties and hence find position in the preparation of a number of medicines.

1.3 Medicinal plant scenario in Bangladesh

"A medicinal plant is a plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor of synthesis of useful drugs." This definition

of medicinal plant has been formulated by WHO (World Health Organization). The total numbers of plants with medicinal properties in the subcontinent are present stands at about 2000. About 450 to 500 of such medicinal plants names so far have been enlisted as growing or available in Bangladesh [12]. Dhaka, Rajshahi, Sylhet and Chittagong division are rich in medicinal plants. The Bangladeshi Herbal medicine market is valued at tk. 3300 million (app. US $ 60 million) at trade price [13]. At the factory level, 5000 tons of medicinal plants are imported annually that cost around 480 million taka [14]. Although modern medicinal science has been developed to a great extent, many rural people of Bangladesh still depend on plant products & herbal remedies for treating their problems.

1.4 Strategies in the search of new natural compounds

To study medicinal plants it is first of all necessary to know which plant to select and what type of biological activity to look for. The selection criteria of plants, which potentially contain new biological agents, based on five principle approaches: the random, the taxonomic, the phytochemical, the ethnomedical and the information-managed approach. In the random approach all available species are collected, irrespective of prior knowledge and experience.

In the taxonomic approach, plants of a specific genus or family are deemed to be of interest, and sought from diverse locations.

The phytochemical (chemo-taxonomic) approach is based on a particular compound type, which is of biological interest. Plants anticipated to produce related compounds are collected. Taxonomic and the phytochemical approach are closely related and can not be clearly divided from each other. In the ethnomedical approach, credence is given to information on the medicinal use of the plant. Based on this information, the plant is collected and evaluated [15]. Much of the traditional medicine clearly reflects real medicinal properties. Indeed, 74% of our plant-derived major medicines were discovered by following up empirical use [16]; Field studies on the use of traditional medicines should be an important additional tool in the selection of plants for further studies. Most of the medicinal plants used are insufficiently described concerning theirphytochemical composition and their biological properties [17].

1.5 Objectives of the study

General objective

-To investigate the phytochemical constituents and biological activities of the buds of S. aromaticum herb.

Specific objectives

-To phytochemically screen the crude extract of the leaves of the herb.

-To isolate pure compounds from chloroform crude extract of the buds of clove herb and characterize them using IR and NMR spectroscopy like ^{1}H NMR, ^{13}C NMR.

-To investigate the biologicial activities of the crude and vacuum liquid chromatography extract, the isolated compounds and the essential oil of the plant buds using some established methods.

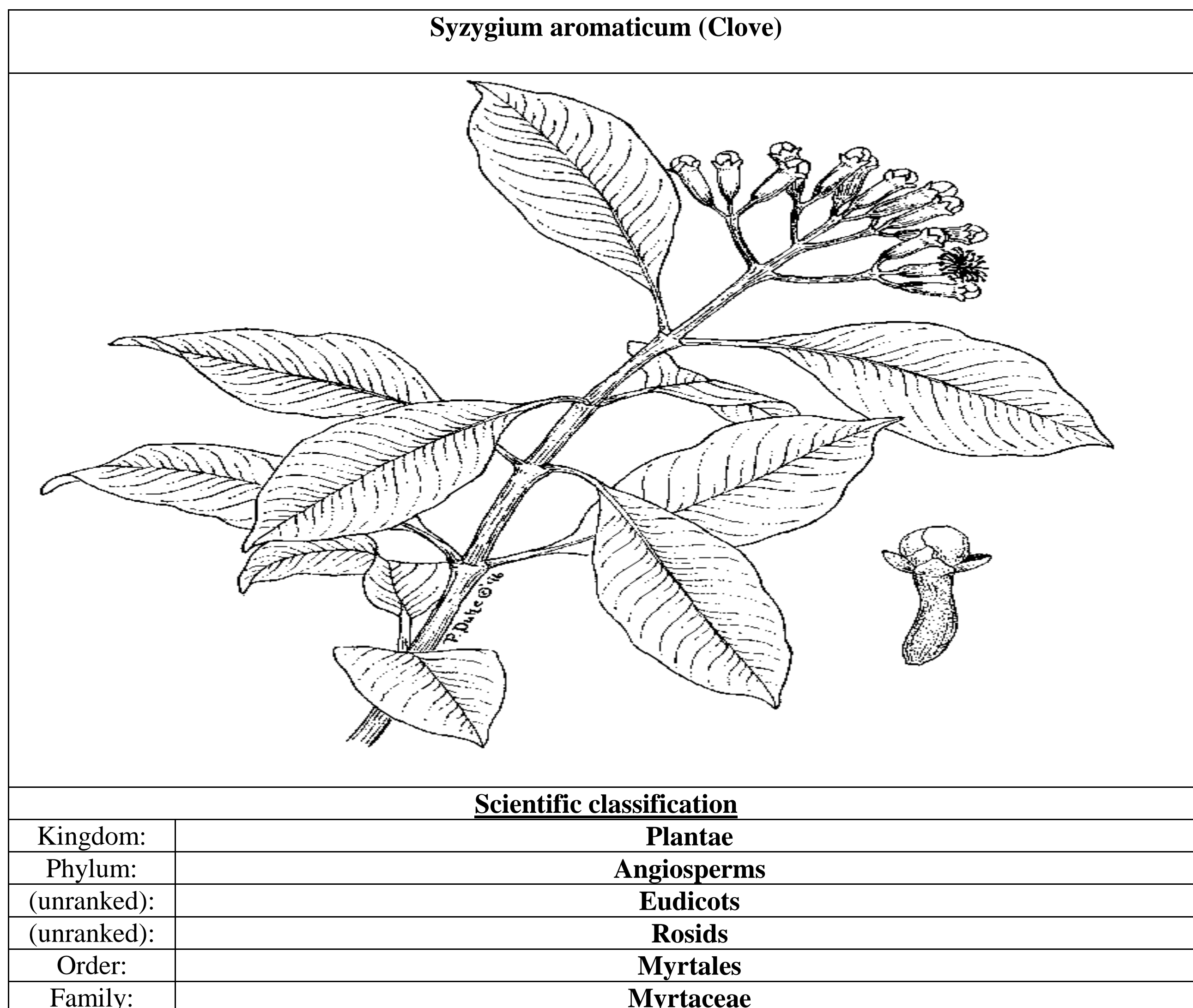
Chapter 2

Literature Review

Cloves (*Syzygium aromaticum*) are the aromatic dried flower buds of a tree in the family Myrtaceae. Cloves are native to the Maluku islands in Indonesia and used as a spice in cuisines all over the world. Cloves are harvested primarily in Indonesia, India, Madagascar, Zanzibar, Pakistan, and Sri Lanka.

The clove tree is an evergreen that grows to a height ranging from 8–12 m, having large leaves and sanguine flowers in numerous groups of terminal clusters. The flower buds are at first of a pale color and gradually become green, after which they develop into a bright red, when they are ready for collecting. Cloves are harvested when 1.5–2 cm long, and consist of a long calyx, terminating in four spreading sepals, and four unopened petals which form a small ball in the center.

Syzygium aromaticum (Clove)



Scientific classification	
Kingdom:	**Plantae**
Phylum:	**Angiosperms**
(unranked):	**Eudicots**
(unranked):	**Rosids**
Order:	**Myrtales**
Family:	**Myrtaceae**

Genus:	***Syzygium***
Species:	*Syzygium aromaticum*
Syzygium aromaticum (L.) Merrill & Perry	
Synonyms	
*Caryophyllus aromaticus*L. *Eugenia aromatica* (L.) Baill. *Eugeniacaryophyllata* Thunb. *Eugenia caryophyllus* (Spreng.) Bullock & S. G. Harrison	

2.1 Taxonomy and nomenclature

The scientific name of clove is *Syzygium aromaticum*. It belongs to the genus *Syzygium*, tribe Syzygieae, and subfamily Myrtoideae of the family Myrtaceae. It is classified in the order of Myrtales, which belong to superorder Rosids, under Eudicots of Dicotyledonae. Clove is an Angiospermic plant and belongs to division of Magnoliophyta in the kingdom Plantae [18].

2.2 Etymology

The name *clove*, as well as Spanish *clavo*, Catalan *clau*, Portuguese *cravinho* and Tagalog *clovas*, ultimately derives from Latin *clavus* nail (because of shape resemblance). The word made its way into English via Old French *clou*. Most Germanic and Germanic-influenced tongues have a different word for clove: German *Nelke*, Norwegian *nellik*, Danish *nellike*, Icelandic *negull*, Swedish *neijlikor*, Yiddish *negelen*, Finnish *neilikka*, Estonian *nelgi* and Sranan *nagri*. These are related to German *Nagel*, nail.

Nail and its cognates in Germanic languages (Old High German *nagal*, English *nail*, Icelandic *nagli*, Swedish *nagel*) basically means either nail of finger or toe or slim pointed piece of metal; the second meaning is younger and does not appear outside the Germanic languages: Old Irish *ingen*, Latin *unguis*means nail, Latvian *nags*means hoof, Greek *onyx* means claw, Sanskrit *anghri* means foot. Cloves are named nail spice in other languages, too; for example, take Russian *gvozdika*which comes from *gvozd'*means nail. As another example, one finds a group of related names in Western to Central Asia: Georgian *mikhak'i*, Azeri *mixək* and Farsi *mikhak* belong to the same kin as Azeri *mıx* and Farsi *mikh*nail. Similar names for nail are found in several Turk languages (Turkish *mıh* and Uighur *mih*), suggesting that the name is ultimately of Altaic origin.

In the same spirit, Hebrew *tsiporen* also has two meanings fingernail and clove, although it is not related to any of the former mentioned languages. In its Old Hebrew form *tsipporen* that word also appears in the Old Testament, but only in the meanings finger nail and tip, point, not in reference to the spice. It is believed that cloves came to the Mediterranean no sooner than the first or second century B.C.

Another unrelated language names cloves as nails: Basque *iltze-kanela* literally means cinnamon nails (*iltzatu* nail); so the spice was named both for its shape and, even if inaccurately, for its fragrance (it can also be called *iltzea* for short). German *Gewürznelke*, Dutch *kruidnagel* or Swedish *kryddnejlikor* are emphatic formations meaning condiment clove. In Old Greek, the clove spice was known as *karyophyllon*, which appears to be a compound of two Greek nouns: *karyon* nut and *phyllon* leaf. Yet such a compound is poorly motivated: Clove is neither nut nor leaf, and does not even look so. Moreover, practically all names of foreign spices in Greek tongue are loans from languages of the trading peoples. The English name derives from Latin *clavus* 'nail' (also the origin of French *clou* and Spanish *clavo*, 'nail') as the buds vaguely resemble small irregular nails in shape.

Cloves are also known under the following names in other languages:

Bengali: Labanga

Chinese: *(pinyin: dīng xiāng)*

Czech: *hřebíček*

Danish: *nelliker*

Dutch: *kruidnagel*

French: *giroflier* (for the tree), *clou de girofle* (for the spice)

German: *Gewürznelkenbaum*

Hindi: *lavang*

Indonesian: *cengkeh* or *cengkih*

Italian: *Chiodi di garofano*

Kannada: *lavanga*

Malayalam: *grampoo*

Marathi: *lavang*

Pashto: *lawang*

Portuguese: *cravo-da-Índia*, *cravo-das-molucas*, and *cravo-de-doce*

Româneşte: *cuişoare*, *cuisoare*

Spanish: *árbol del clavo* or *clavero giroflé* (for the tree), *clavo de olor* (for the spice)

Sinhala: *karabu nati*

Tamil: *kirambu, lavangam*

Telugu: *lavangam*

Türkçe: *karanfil*

Urdu: *laung*, *laong*

Vietnamese: *đinh hương*

2.3 Botany and Uses

Clove (*Syzygium aromaticum* (L.) Merril. & Perry, synonyms are *Eugenia aromaticum* or *E. caryophyllata*) is one of the most ancient and valuable spices of the Orient. It is a member of the family *Myrtaceae*. The clove of commerce is its dried unopened flower buds. The word 'clove' was derived either from the Latin word *clavus*, or the French form *clou*, meaning 'nail'. The buds resemble irregular nails. Tanzania, Indonesia, Madagascar, Cameroon and Sri Lanka are the major clove-exporting countries. In recent years, world production of clove has averaged around 80,000 t a year. Indonesia is the world's largest producer at 50,000–60,000 t per annum. It is used mainly in the preparation of *kretek* cigarettes. Singapore is the entrepot for the clove trade. Saudi Arabia, the USA, France and India are the major importing countries.

Cloves are also an important incense material in Chinese and Japanese culture. Oil of clove is used extensively for flavouring all kinds of food products, such as meats, sausages, baked goods, confectionery, candies, table sauces, pickles, etc. Clove oil is used in aromatherapy and oil of cloves is used widely to treat toothache. It is used in medicine for its antibacterial, antiseptic and antibiotic properties. The oil has many industrial applications and is used extensively in perfumes, soaps and as a clearing agent in histological work. It is an ingredient in many toothpastes and mouthwashes. It is also used for flavouring oral preparations and chewing gums. The chief constituent of the oil, eugenol, is used in the preparation of synthetic vanillin and isoeugenol [19].

The evergreen clove tree originated in the Moluccas, the 'Spice Islands', but has now been introduced into most tropical countries and Madagascar, Zanzibar and Tanzania dominate the world trade. Cloves grow best near the sea, thus the preponderance of island cultivation. Clove trees are fairly small, growing naturally up to 9 m (30 ft), but usually kept at a more accessible height, 5-6 m (16 ½ -20 ft), for cropping. They are conical, looking a little like laurels, but the leaves are longer than laurel, brighter green and although as shiny, they have visible dots containing the aromatic substances. These are released if you bruise or crush the leaves. The trunk, covered with a smooth greyish bark, divides quite low into large branches, at the end of which the crimson flowers grow or would, if they were allowed to reach that stage. The cloves are the unopened, long, yellowish-green flower buds which appear at the end of the rainy season. When these turn pink, just before opening, they are picked by hand or beaten from the tree. The

flowers are then dried in the sun, or gently over heat, for a few days until they are the familiar dark brown colour of the spice.

The clove tree does not produce the spice until aged about five years, and can carry on increasing its yield until it is about 20 years old. The yield of a mature tree is generally around 3 – 4 kg (6 ½ - 8 ¾ lb) fresh buds. When these are dried, the weight reduces to about 1 kg (21/4 lb), and in turn this yields about 15 - 20 per cent essential oil. In the middle of the rainy season, the hot humid atmosphere disperses the fragrance of the clove trees all over the islands where they grow. The lack of epidemics on Penang was attributed to the medicinal scents from the tree (clove is a very strong antiseptic).

2.4 General Composition of *Sysygium aromaticum*

The composition of the clove varies according to the agroclimatic conditions under which it is grown, processed and stored. The dried clove bud contains carbohydrates, fixed oil, steam-volatile oil, resins, tannins, proteins, cellulose, pentosans and mineral elements. Carbohydrates comprise about two-thirds of the weight of the spice [19]. The dried bark and flower buds also contain nutrients like proteins, minerals, vitamins, etc. Nutrient composition of 100 g of clove is indicated in Tables that 61% of clove is carbohydrates, 20% is fat and the rest is contributed by secondary metabolites, vitamins and minerals. Cloves are an excellent source of manganese, a very good source of dietary fibre, vitamin C, vitamin K and ω-3 fatty acids and a good source of calcium and magnesium. Volatile oil can be extracted from the leaf, stem and buds of clove. Volatile oil is present in oval cavities, two or three rows below the epidermis. The major component of the volatile oil is a phenol, namely eugenol. Phenolic activity is greater at the outer glandular regions of the hypanthium than in the inner aerenchymatous spongy tissue.

Table 2.1: Nutritional composition of S. aromaticum (Clove) Source: [19]

Composition	Amount
Water	5.40–6.86 g
Food energy	323 Kcal
Protein	5.98 g
Fat	20.06 g
Carbohydrate	61.22 g
Ash	5.88 g
Ca	0.646 g
P	105 mg
Na	243 mg

K	1102 mg
Fe	8.68 mg
Thiamin	0.115 mg
Riboflavin	0.267 mg
Niacin	1.458 mg
Ascorbic acid	80.81 mg

Table 2.2: Nutrient values and weights for edible portion of clove (*Source*: [19])

Nutrient	**Units**	**Value per 100 g**
Water	g	6.86
Energy	kcal	323
Energy	kj	1350
Protein	g	5.98
Total lipid (fat)	g	20.07
Ash	g	5.88
Carbohydrate, by difference	g	61.21
Fibre, total dietary	g	34.2
Sugars, total	g	2.38
Sucrose	g	0.02
Glucose (dextrose)	g	1.14
Fructose	g	1.07
Galactose	g	0.15
Calcium	mg	646
Iron	mg	8.68
Magnesium	mg	264
Phosphorus	mg	1102
Potassium	mg	1.09
Sodium	mg	5.9 1
Zinc	mg	1.09
Copper	mg	1102
Manganese	mg	1.09

Selenium	mg	5.9 1
Vitamin C, total ascorbic acid	mg	80.8
Fibre, total dietary	g	34
Sugars, total	g	2.38
Sucrose	g	0.02
Calcium	mg	646
Iron	mg	8.68
Magnesium	mg	264
Phosphorus	mg	105
Potassium	mg	1102
Sodium	mg	243
Zinc	mg	1.09
Copper	mg	0.347
Manganese	mg	30.033
Selenium	mcg	5.9
Vitamin C, total ascorbic acid	mg	80.8
Thiamin	mg	0.115
Riboflavin	mg	0.267
Niacin	mg	1.458
Vitamin B6	mg	0.590
Folate, total	mcg	93
Vitamin A	IU	530
Vitamin E (tocopherol)	mg	8.52
Vitamin K (phylloquinone)	mcg	141.8
Fatty acids, total saturated	g	5.438
14:0	g	0.022
16:0	g	3.967
18:0	g	0.847
Fatty acids, total monounsaturated	g	1.471
16:1 undifferentiated	g	0.089
18:1 undifferentiated	g	1.337
20:1	g	0.022
Fatty acids, total	g	7.088
18:2 undifferentiated	g	2.586
18:3 undifferentiated	g	4.257

20:4 undifferentiated	g	0.045
22:5 n-3	g	0.022
Phytosterols	mg	256
β-Carotene	mcg	84
α-Cryptoxanthin	mcg	468

Table 2.3: Physico-chemical properties of S. aromaticum (Clove)

Characteristic	Bud oil	Stem oil	Leaf oil
Colour	Colourless to pale yellow	Yellow to dark brown Straw	coloured or very pale
Specific gravity (25°C)	1.051–1.054	1.050–1.055	1.040–1.054
Optical rotation	-1°35′ to -0°25′	−1°30′ to −0°32′	1°40′ to −0°40′
Refractive index (20°C)	1.531–1.537	1.531–1.539	1.531–1.538
Solubility	Soluble in 1 vol. of Soluble in 70% ethanol	Solubility Soluble in 1–2 vol. 70% ethanol	Soluble in 1.0–1.5 vol. 70% ethanol
Total phenols (%)	91–93	88–93	78–93

Volatiles

Clove yields three types of volatile oil – oil extracted from the leaves, the stem and the buds. These oils differ considerably in yield and quality. The yield and composition of the oil obtained are influenced by its origin, season, variety and quality of raw material, maturity at harvest, pre- and post-distillation treatments and method of distillation. The chief component of the oil is eugenol.

Bud oil

Good-quality clove buds contain 15–20% essential oil. The oil is dominated by eugenol (70–85%), eugenyl acetate (15%) and β-caryophyllene (5–12%), which together makes up 99% of the oil. β-Caryophyllene, which was earlier thought to be an artefact of distillation, was first reported as a constituent of bud oil by Walter [19]. The constituents of the oil also include methylamylketone, methylsalicylate, *α*- and β-humulene, benzaldehyde, β-ylangene and chavicol. The minor constituents like methylamylketone, methylsalicylate, etc., are responsible for the characteristic pleasant odour of cloves. The physico-chemical properties of clove oils are shown in Table. Gopalakrishnan *et al*. [19] characterized six sesquiterpenes, namely: α-cubebene (1.3%), α-copaene (0.4%), β-humulene (9.1%), β- caryophyllene (64.5%), γ-cadinene (2.6%) and δ-cadinene (2.6%), in the hydrocarbon fraction of the freshly distilled Indian clove bud oil. The oil from the Malagasy Republic (Madagascar) was dominated by eugenol (72–73%), eugenyl acetate (6.3–7.8%) and caryophellene (15.7%).

The clove bud and stem oils from Madagascar were also dominated by eugenol, eugenyl acetate and β-caryophyllene. The stem oil contained a higher level of eugenol, whereas the eugenyl acetate content was higher in the bud oil. The oil from clove bud contained 73.5–79.7% eugenol and 4.5– 10.7% eugenyl acetate, while the stem oil contained 76.4–84.8% eugenol and 1.5–8.0%eugenyl acetate. Both contained 7.3–12.4% β-caryophyllene and 1.0–1.4% α-humulene.The essential oils of clove buds ofIndian and Madagascan origins were analysed. The oil from Madagascar was richer in eugenol (82.6%) and eugenyl acetate (6%) compared withthat of India (70 and 2.1%, respectively), whereas the Indian oil contained a higherlevel of *b*-caryophyllene (19.5 against 7.2% in Madagascan oil). The neutral fraction of the bud oil from Madagascar contained β-caryophyllene (75.64%), *a*-humulene (14.12%) and δ-cadinene (2.34%) as the major components[19].Pino *et al*. [19] identified 36 compoundsof the volatile oil of clove buds.The major components of the bud oil were eugenol (69.8%), β- caryophyllene (13%) andeugenyl acetate (16.1%).The chief components of clove oil from various regions are listed in Table 2.2, which indicates quantitative variations of the individual components of the oil from different regions. Zachariah *et al*. [19] reported that clove buds from India contained 12.9–18.5%oil, of which 44–55% was eugenol, whereasthe pedicels contained 3.0–7.7% oil with60.0–72.4% eugenol.Wild uncultivated trees in Molucca yielded 3.0–7.7% bud oil. The oil contained no eugenol and was quite different from the bud oil from cultivated trees [19]. Analysis of clove bud oil extracted with liquid and supercritical carbon dioxide showed significant qualitative and quantitative compositional differences comparedto oil obtained by the conventional hydrodistillation process. The parameters such as pressure, temperature, contact time, etc., affect the extraction of the bud flavour from the spice.Guan *et al*. [19] compared the essential oil obtained by four different extraction techniques; namely, hydro distillation, steam distillation, solvent extraction.

The study showed that temperature had the largest effect on the eugenol content of the extracts and particle size had the maximum effect on oil yield. Among these techniques, the oil obtained by SFE and steam distillation had a desirable, pale yellow colour. Hydrodistilled oil had the lowest content of eugenol and eugenyl acetate. Extraction yield of SFE was twice as high as that obtained by steam and hydrodistillation.The SFE method yielded the highest content of eugenol + eugenol acetate in the oil. Clove oil obtained by steam distillation yielded the highest eugenol content. Hydrodistillation yielded oil with high β-caryophyllene content, whereas the SFE extracted oil had the lowest β-caryophyllene content. GC-MS analysis of the clove oils obtained by different methods showed that the composition of the clove oil was almost similar, but the relative concentration of the identified compounds was apparently different [19].

Leaf oil

Clove leaves yield 3.0–4.8% essential oil. In Zanzibar, oil is distilled from dried fallen leaf or fresh leaf after trimming the upper part of the tree. Crude leaf oil is harsh and woody, with a phenolic, sweet aroma quite different from bud oil. Rectified oil is clear pale yellow in colour with a sweeter, less harsh, drywoody odour close to that of eugenol. The oil contained 94.4%

eugenol followed by β-caryophyllene (2.9%), nerol (0.79%) and β-caryophyllene oxide (0.67%). The leaf oil from Cuba contained 31 volatile compounds. Eugenol (78.1%) and β-caryophyllene (20.5%) were the main constituents in the oil. Cuban leaf oil contained a higher amount of β-caryophyllene compared with that from Little Andaman.The leaf oil from Madagascar contained 22 constituents, the chief constituents being eugenol (82.0%) and β-caryophyllene(13.0%). It contained a higher level of β-caryophyllene compared with bud oil (7.2%). A commercial sample of leaf oil obtained in Germany contained 76.8% eugenol and 17.4% β-caryophyllene as the chief components. The essential oil content during the different stages of leaf growth revealed that the eugenol content in the leaves increased from 38.3 to 95.2% with maturity, while the contents of eugenyl acetate (51.2 to 1.5%) and caryophyllene (6.3 to 0.2%) decreased. Clove bud and leaf oil contain various classes of compounds, e.g. monoterpenes, sesquiterpenes, aldehydes and ketones.

Clove stem oil

Clove stem yields 6% volatile oil. The oil is a pale to light yellow liquid containing 80.2% eugenol and 6.6% β-caryophyllene, besides several minor components. Stem oil is used mainly in flavouring and perfumery and also to adulterate bud oil. Stem oil from Madagascar contains 77.10% eugenol and 11.20% β-caryophyllene as the major compounds.

Fruit oil

Ripe fruits yield 2% of oil, which is comprised of 50–55% eugenol.

Clove bud concrete

Clove bud concreteis another important value-added product from buds, extracted using petroleum ether and benzene. It is olive to pale brown, having a sweet, rich spicy aroma similar to that of dried buds. Clove concrete, on treatment with benzene/ petroleum ether, produces a viscous, olive-green semi-solid (at low temperature), namely bud *absolute*, soluble in alcohols of different proportions. *Absolute* lacks caryophyllene and contains the same constituents as those present in unprocessed bud. Clove oleoresin is an extremely concentrate product, containing all the flavouring ingredients soluble in the solvent used, and is much closer to the original clove odour and flavour. Menon and Narayanan [19] studied the glycosidically bound volatiles in clove leaves and buds using hydrolysing enzymes.When β-glucosidase was used for hydrolysis, eugenol was the major compound liberated from both buds and leaves, with *cis*- and *trans*-isoeugenol, nerolidol and farnesol in minor amounts, whereas hydrolysis using *a*–amyloglucosidase yielded farnesol as the major compound.

Non-volatiles

So far, a few non-volatiles have been isolated from clove, which include tannins, sterols, triterpenes and flavonoids. Wild uncultivated trees of the Moluccas contained the crystalline

compounds eugenone, eugenine, eugenitin and isoeugenitol (Guenther, 1950). Some volatile compounds are listed below.

Tannins

Cloves contain 10–13% tannin, which has the same chemical composition as gallotannic acid. Eugeniin and ellagitannin were isolated from cloves. Tanaka *et al*. [19] isolated eugenol glucoside gallate, a chromone-*C*-glycoside, galloyl and hexahydroxy diphenyl esters of 2, 4, 6-trihydroxy acetophenone- 3-glucopyranoside from clove leaves. Further, two ellagitannins, namely, syzyginin A (1, 2, 3-tri-*O*-galloyl–4, 6-(*S*) - tergalloyl-β-D-glucoside) and syzyginin B, were also isolated from the leaves. *Triterpenes* Cloves contain about 2% of the triterpene, oleanolic acid. Narayanan and Natu [19] isolated maslinic acid from clove buds. From clove, 2*a*-hydroxyoleanolic acid was isolated.

Sterols

Sterols isolated from clove include sitosterol, stigmasterol and campesterol [19].

Flavonoids

Achromone- *C*-glucoside, isobiflorin (5, 7-dihydroxy– 2-methoxychromone-8-*C*-β-D glucopyranoside) and biflorin were isolated from the ethanolic extract of cloves [19]. From the ethanol extract of the seeds, apigenin 6-*C*-[β-D-xylopyranosyl-(1→2″)-β-D-galactopyranoside]-7-*O*-β-D-glucopyranoside and apigenin-6-*C*-[β-D-xylopyranosyl- (1→2″)-β-D galactopyranoside]-7-*O*-β-D-(6-*O*-*p*-coumarylglucopyranoside) were isolated [19] flavonoids, kaempferol and rhamnetin isolated from clove are antioxidants.

2.5Medicinal and Pharmacological Uses of *Syzygium aromaticum*

India's traditional Ayurveda healers have used cloves since ancient times to treat respiratory and digestive ailments. Like many culinary spices, cloves help relax the smooth muscle lining of the digestive tract and eating cloves is said to be aphrodisiac. Aqueous extract of clove flower bud inhibits immediate hypersensitivity in rats by inhibition of histamine release from mast cells *in vivo* and *in vitro* [19]. Cloves are more often used to assist the action of other herbal remedies rather than alone. When not available, all spice is substituted. It is spicy, warming, stimulant, anodyne, anaesthetic (topical), antiemetic, antigriping (added to other herbs), vermifuge, uterine stimulant, stomachic, aromatic, carminative, antiseptic, antiviral, antibacterial, antifungal, antispasmodic, expectorant, aphrodisiac and promotes salivation and digestive juices. The oil is expectorant, anaesthetic, emmenogogue; it affects the kidney, spleen and stomach and has preservative properties. Tea made from clove bud (other herbs/spices can be used or added to cloves, such as all spice, bay, cinnamon and marjoram) has been used to relieve bronchitis, asthma, coughs, a tendency to infection, tuberculosis, altitude sickness, nervous stomach, nausea, diarrhoea, flatulence, indigestion, dyspepsia and gastroenteritis.

In Chinese medicine cloves are used as a kidney tonic (especially for impotence associated with deficient yang), to warm the body, increase circulation and as a digestive aid. They are also used for nausea, vomiting, flatulence, hiccups, stomach chills, fever, caries, toothache, cholera, colic, cracked nipples, diarrhoea, dyspepsia, halitosis (chewing on the whole clove), unusual uterine bleeding, nasal polyps and impotence. The root is used for a weaker effect. The oil is employed for diarrhoea, halitosis, hernia, nausea and toothache. Ethanolic extract (50%) of clove produced a significant and sustained increase in the sexual activity of normal male rats, without any conspicuous gastric ulceration or adverse effects. Thus, the resultant aphrodisiac activity of the extract lends support to claims for its traditional usage in sexual disorders. In traditional Chinese medicine it is used to treat in digestion, diarrhoea, hernia, ringworm and other fungal infections [19].

In Ayurveda, cloves are used to treat respiratory and digestive ailments, flatulence, nausea and vomiting. The medieval German herbalists used cloves as part of an antigout mixture. Clove is believed to have a cooling effect on the stomach. A paste of clove was applied to the forehead for relief from colds. It has powerful local antiseptic and mild anaesthetic actions [19].

Clove bud oil has various biological activities such as antibacterial, antifungal, antioxidant and insecticidal properties. The high level of eugenol present in the essential oil imparts strong biological and antimicrobial activity. Clove oil is an active ingredient in several mouthwash products and a number of over-the-counter toothache pain-relief preparations.

It is also used to disinfect root canals. For toothache, clove tea has been used in combination with chamomile or sage. Eugenol is shown to alleviate neuropathic pain. Eugenol inhibits 5-lipoxygenase activity and leukotriene- C4 in human PMNL cells. Clove oil is used to prepare microscopic slides for viewing. It is used to treat flatulence, colic, indigestion and nausea. Eugenol is used in germicides and perfumes, in the synthesis of vanillin and as a sweetener or intensifier. A recent review by Chaieb *et al*. [19] lists the chemical composition and biological activity of clove essential oil.

Antimicrobial activity

Clove exhibits potent antimicrobial activity against *Bacillus subtilis*, *Escherichia coli* and *Saccharomyces cerevisiae*. Essential oils from clove and eugenol show various degrees of inhibition against *Aspergillus niger, S. cerevisiae, Mycoderma* sp., *Lactobacillus acidophilus* and *B. cereus*, as estimated by the paper disc agar diffusion method.The oil also inhibits the growth of *Fusarium verticilloides*. Clove oil (1% v/w) inhibits *Listeria monocytogenes* in chicken frankfurters.

It has excellent antimicrobial properties and is used in food preservation. Clove extracts show high antifungal activity against *Rhizoctonia solani*. Clove oil and eugenol are reported to possess significant antifungal activity against rye bread spoilage fungi. Clove oil shows antifungal activity against the fungi belonging to *Eurotium*, *Aspergillus* and *Penicillium* species, commonly

causing deterioration of bakery products. Eugenol possesses antifungal activity against *Cladosporium herbarum*, *Penicilliumglabrum*, *P. expansum* and *A. niger*. Clove bud oil causes inhibition of both mycelial growth and aflatoxin production of *A. parasiticus* Clove oil, at concentrations > 100 μg/ml, results in reduction in the aflatoxin production in liquid cultures. Clove oil inhibits the growth and production of fumonisin B1 by *F. proliferatum* [19].

Antioxidant activity

Clove essential oil has the highest antioxidant capability of any essential oil, perhaps some of the highest known for a food or supplement. It has been included in some 'longevity'formulae for this reason. Clove and eugenol possess strong antioxidant activity, which is comparable to the activities of the synthetic antioxidants, BHA and pyrogallol. Essential oil from clove leaf possesses scavenging activity againstthe 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical at concentrations lower than the concentrations of eugenol, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). It also shows a significant inhibitory effect against hydroxyl radicals and acts as an iron chelator. The antioxidant activity of clove bud extract and its major aroma components, eugenol and eugenyl acetate are comparable to that of the natural antioxidant *α*-tocopherol. Eugenol inhibits 5-lipoxygenase activity and leukotriene-C4 in humanPMNL cells [19].

Anti-inflammatory activity

Eugenol, the primary component of clove's volatile oils, functions as an anti-inflammatory substance. In animal studies, the addition of clove extract to diets already high in anti-inflammatory components (likecod liver oil, with its high ω-3 fatty acidcontent) brings a synergistic effect. In some studies, it further reduces inflammatory symptoms by another 15–30%. Clove also contains a variety of flavonoids, including kaempferol and rhamnetin, which also contribute to clove's anti-inflammatory andantioxidant properties. Another constituent of clove oil, *b*-caryophyllene, also contributes to the anti-inflammatory activity.

Anaesthetic effect

Clove oil is used as a safe anaesthetic for aquatic research. Tricaine or MS-222, the only anaesthetic registered in NorthAmerica, is a very effective anaesthetic for several fish species but its application in the field is limited because the US Food and Drug Administration guidelines demand a 21-day withdrawal period after exposure to MS-222 before the fish enters the food chain. In this context, clove oil is found to be an alternative to MS-222 for use as a fish anaesthetic. Exposure of channelcatfish (*Ictalurus punctatus*) to clove oil at a concentration of 100 mg/l induces anaesthesia within 1 min. The fish recovered from a 10 min period of anaesthesia within 4 min after removal from the anaesthetic solution [19].

Clove oil is therefore used as a safe anaesthetic for channel catfish. The anaesthetic effect of clove oil and eugenol for use in aquaculture and aquatic research was also reported. Clove oil

and eugenol were reported as an acceptable anaesthetic for rabbit fish (*Saiganus lineatus*), coral reef fish (*Pomacentrus amboinensis*) and rainbow trout (*Oncorhynchus mykiss*) for use in aquaculture and aquatic research. It was also found to be useful as a crab anaesthetic. *β*-Caryophellene is also reported to be ananaesthetic [19].

Mosquito-repellent activity

Clove oil exhibits repellent activity on *Anopheles albimanus, Aedes aegypti, A. dirus* and *Culex quinquefasciatus.*

Insecticidal activity

Eugenol, isoeugenol and methyl eugenol cause contact toxicity to the storage pathogens, *Sitophilus zeamis* and *Tribolium costaneum*. These compounds have similar toxicity to *S. zeamis* at LD^{50} 30 µg/ mg insect, while for *T. costaneum* the order ofpotency is isoeugenol > eugenol > methyleugenol. The clove leaf and bud oils show potent insecticidal activity against the human headlouse (*Pediculus capitis*).

Antithrombotic activity

Clove oil inhibits human platelet aggregation induced by arachidonic acid (AA), platelet activating factor (PAF) or collagen. Clove oil is a more effective inhibitor for aggregation induced by AA and PAF (IC_{50}: 4 and 6 µM, respectively) than collagen (IC_{50}: 132 µM). It inhibits platelet aggregation and thromboxane synthesis and acts as an antithrombotic agent. Eugenol and acetyl eugenol are more potent than aspirin in inhibiting platelet aggregation induced by arachidonate, adrenaline and collagen. In arachidonate -induced aggregation eugenol is on par with indomethacin [19].

Anticancerous activity

Clove has strong anticancerous properties. The sesquiterpenes, β-cayophyllene, β-cayophyllene epoxide, α-humulene, α-humulene epoxide and eugenol present inclove oil showed potent anticarcinogenic activity by inducing the detoxifying enzyme, glutathione-*S*-transferase, in mouse liver and small intestine [19].

Antiviral activity

Clove is a potent antiviral agent and eugenin isolated from clove buds showed antiviral activity against *Herpes simplex* virus at a concentration of 10 µg /ml [19].

Antipyretic effect

Eugenol, the chief constituent of clove oil, has marked antipyretic activity when given intravenously, intragastrically and centrally to rabbits made febrile by interleukin-1. Eugenol was

more effective in reducing fever than acetaminophen and it reduced fever primarily through a central action similar to that of common antipyretic drugs, such as acetaminophen [19].

<u>Toxicity studies</u>

Cloves can cause local skin irritation, pulmonary oedema, mouth sensitivity and sudden lower airway closure. In addition, smoking clove cigarettes can damage soft tissues and injure the airway linings [19].

2.6 Culinary use

Cloves can be used in cooking either whole or in a ground form, but as they are extremely strong, they are used sparingly. Cloves have historically been used in Indian cuisine (both North Indian and South Indian). In North Indian cuisine, it used in spicy dishes as an ingredient of a mix called *garam masala*, along with other spices, although it is not an everyday ingredient for home cuisine, nor is it used in summer very often. In the Maharashtra region of India it is used sparingly for sweet or spicy dishes, but rarely in everyday cuisine. In Ayurvedic medicine it is considered to have the effect of increasing heat in system, hence the difference of usage by region and season. In south Indian cuisine, it is used extensively in biryani along with "cloves dish" (similar to pilaf, but with the addition of other spices), and it is normally added whole to enhance the presentation and flavor of the rice. Dried cloves are also a key ingredient in Indian masala chai, spiced tea, a special variation of tea popular in some regions, notably Gujarat. In the US, it is often sold under the name of "chai" or "chai tea", as a way of differentiating it from other types of teas sold in the US. In Mexican cuisine, cloves are best known as clavos de olor, and often used together with cumin and cinnamon. In Vietnamese cuisine, cloves are often used to season the broth. In American cooking, it is often used in sweet breads such as pumpkin or zucchini bread along with other sweet spices like nutmeg and cinnamon.Due to the Indonesian influence, the use of cloves is widespread in the Netherlands. Cloves are used in cheeses, often in combination with cumin.

2.7 Traditional medicinal uses

Cloves are used in Indian Ayurvedic medicine, Chinese medicine, and western herbalism and dentistry where the essential oil is used as an anodyne (painkiller) for dental emergencies. Cloves are used as a carminative, to increase hydrochloric acid in the stomach and to improve peristalsis. Cloves are also said to be a natural anthelmintic. The essential oil is used in aromatherapy when stimulation and warming are needed, especially for digestive problems. Topical application over the stomach or abdomen are said to warm the digestive tract. Clove oil, applied to a cavity in a decayed tooth, also relieves toothache. It also helps to decrease infection in the teeth due to its antiseptic properties.In Chinese medicine cloves or *ding xiang* are considered acrid, warm and aromatic, entering the kidney, spleen and stomach meridians, and are notable in their ability to warm the middle, direct stomach downward, to treat hiccough and to

fortify the kidney [19]. Because the herb is so warming it is contraindicated in any persons with fire symptoms and according to classical sources should not be used for anything except cold from yang deficiency. As such it is used in formulas for impotence or clear vaginal discharge from yang deficiency, for morning sickness together with ginseng and patchouli, or for vomiting and diarrhea due to spleen and stomach coldness [19].

Chapter: 3

CHEMICAL INVESTIGATION

3.1 Collection and proper identification of plants

About one kg bud of *Syzygium aromaticum* were collected from local market in August 2013. The plant was identified by a Taxonomist. Bud was collected and then chopped into small pieces followed by air drying for several days.

3.2 Grinding of dry plants

Grinding improves efficiency of extraction by increasing the surface area of plant material. This decreases the amount of solvent needed for extraction as it allows the plant material to pack more densely. Therefore, it is essential to grind samples into finer size for better extraction results. At first these buds were chopped to small pieces with scissor and dried under sun-light for two weeks. After fourteen days of sun light drying, the dried plants were grinded into grinding machine to get fine powder. After grinding the weight of the grinded plants was measured and the weight was about 993 grams. All grinded plants were preserved in an air tight container covered with aluminum foil papers.

Figure 3.1: Grinding machine and electronic balance

3.3 Extraction procedure

Extraction can be done in two ways, cold extraction and hot extraction. In this research the crude is extracted by cold extraction method. About 993.00 g of the powder material was taken in a clean, round bottomed flask (5 liters) and soaked in 2.5 liters of chloroform. The container with its content was sealed with foil and kept for a period of 15 days accompanying occasional

shaking and stirring. The whole mixture was then filtered through filter paper and the mother filtrate was obtained.

3.4 Evaporation

The mother filtrate thus obtained was concentrated at 40 ^{o}C under reduced pressure with a rotatory evaporator. The residual solvent in the extract and compounds were removed under high vacuum. A rotary evaporator is a specially designed instrument for the evaporation of solvent (single-stage or straight distillation) under vacuum. The evaporator consists of a heating bath with a rotating flask, in which the liquid is distributed as a thin film over the hot wall surfaces and can evaporate easily. After evporation crude chloroform extract was obtained.

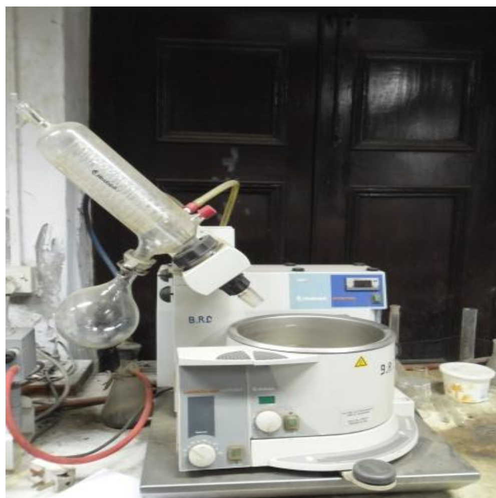


Figure 3.2: Vacuum rotary evaporator

3.5 Chromatographic technique

Chromatographic techniquers are the most useful technique in the isolation and purification of compounds from plant extracts. The advent of relatively new chromatografic media e.g. Sephadex and Polyamide, have improved the range of separations that can be performed.

3.6 Vacuum Liquid Chromatography (VLC) of the crude extract

Vacuum Liquid Chromatography (VLC) is a relatively recent separation technique which involves short column chromatography under reduced pressure, the column being packed with

fine TLC grade silica (Kiesel gel 60G). Details of the method have been published [21]. This technique is used for the initial rapid fractionation of crude extracts.

The column is packed with silica gel (Kiesel gel 60G) undere vacuum. The size of the column and height of the adsorbent layer are dependent upon the amount the amount of extract to be analyzed. The column is initially washed with a non-polar solvent (petroleum ether) to facilitate compact packing. The sample to be separated was adsorbed onto silica gel (Kiesel gel 60, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. The column is then eluted with a number of organic solvents of increasing polarity and the fractions are collected.

Figure 3.3: Crude powder, VLC and flowing column

Table 3.1: VLC fractions collected from column with different solvent

Fraction no.	Solvent system	Volume collected(ml)
1	100% Petroleum Ether (PE)	60.0
2	PE:EA(99:01)	65.0
3	PE:EA(98:03)	125.0
4	PE:EA(95:05)	100.0
5	PE:EA(90:10)	94.0
6	PE:EA(85:15)	92.0
7	PE:EA(80:20)	90.0
8	PE:EA(85:25)	92.0

9	PE:EA(70:30)	90.0
10	PE:EA(65:35)	90.0
11	PE:EA(60:40)	110.0
12	PE:EA(55:45)	90.0
13	PE:EA(50:50)	90.0
14	PE:EA(45:55)	90.0
15	PE:EA(40:60)	90.0
16	PE:EA(35:65)	77.0
17	PE:EA(30:70)	77.0
18	PE:EA(25:75)	77.0
19	PE:EA(20:80)	95.0
20	PE:EA(15:85)	80.0
21	PE:EA(10:90)	90.0
22	PE:EA(05:95)	80.0
23	PE:EA(03:97)	90.0
24	PE:EA(01:99)	95.0
25	100% Ethyl Acetate (EA)	77.0
26	EA:MeOH(99:01)	95.0
27	EA:MeOH(99:02)	105.0
28	EA:MeOH(95:05))	95.0
29	100% Methanol (MeOH)	90.0

Figure 3.4: VLC fractions before drying and after drying

These different fractions were then investigated with different solvent systems on analytical thin layer chromatography over silica gel (Merck kiesel gel 60GF 254) to detect compounds.

3.7 Thin Layer Chromatography(TLC)

Ascending one-dimensional thin layer chromatography technique is used for the initial screening of the extracts and column fractions and checking the purity of isolated compounds. For the latter purpose commercially available precoated silica gel (Kiesel gel 60 GF254) plates are usually used. For initial screening, TLC plates are made on glass plates with silica gel (Kiesel gel 60 GF254). A number of glass plates measuring 20cm x 5cm are throughly washed and dried in an oven. The dried plates are then swabbed with acetone-soaked cotton in order to remove any fatty residue. To make the slurry required amount of silica gel 60 GF-254 and appropriate volume of distilled water (2ml/g of silica gel) are mixed in a conical flask and the flask is gently shaken. The slurry is then evenly distributed over the plates using TLC spreader. After air drying the coated plates are subjected to activate by heating in an oven at 110 ^{o}C for 70 minutes (Stahl,1969;Remington Pharmaceutical sciences,1988.)

Table 3.2: Thickness of TLC plates

Size (cmxcm)	Thickness (mm)
5X20	0.25 & 0.5

Cylindrical glass chamber (TLC tank) with air-tight lid used for the development of chromoplates. The selected solvent system is poured in sufficient quantity into the tank. A smooth sheet of filter paper is introduced into the tank and allowed to soak in the solvent. The tank is then made air-tight and kept for few minutes to saturate the internal atmosphere with the solvent vapour. A small amount of dried extract is dissolved in a suitable solvent to get a solution (approximately 1%) [21]. A small spot of the solution is applied on the activated silica plate with a capillary tube just 1 cm above the lower edge of the plate. The spot is dried with a hot air blower and a straight line is drown 2 cm below the upper edge of the activated plate which marks the upper limit of the solvent flow. The spotted plate is then placed in the tank in such a way as to keep the applied spot above the surface of the solvent system and the can/lid is placed again. The plate is left for development. When the solvent front reaches up to the given mark, the plate is taken out and air-dried. The properly developed plates were viewed under UV light of various wavelengths as well as treated with suitable reagents to detect the presense of compounds.

Preparative thin layer chromatographic technique is routinely used in separation and for purification of the compounds. The principle of preparative TLC is same as that of TLC. Here larger plates (20cm × 20cm) were used.

Table 3.3: Thickness of TLC plate for preperative TLC

Size (cm x cm)	Thickness (mm)
20 X 20	0.75

3.8 Analysis of VLC fractions through TLC

In 1938, Izmailow and Schraiber pioneered the thinlayer chromatography (TLC) method for the analysis of plant material containing alkaloids. The subject matter of their scientific research was an extract of a plant rich in tropane alkaloids. Since then, numerous papers exploring the detection, isolation and quantitative determination of alkaloids by TLC have been published. It has been stated that no other method has delivered so much information on natural products. Preliminary tests are often performed on 5 × 10 cm plates, and these are commercially available coated with silica gel 60 F254. To obtain plates of this size pre-coated with other sorbents it is best to do in small plates.

Glass plates are usually coated with surface-active sorbents, which pick up not only water but also “dirt” from the surrounding atmosphere. This should be removed just as completely as the soluble binder components that can form dirty zones with certain solvent systems (mainly polar).

This purification step is known as “prewashing”. In routine analysis also, e.g. raw materials testing, prewashed plates should always be used, as a dirty front would otherwise appear at the height of the substance zones under investigation.

Methanol is usually used as the prewashing agent. If the cleaning power is insufficient for the solvent system which is to be used later in the chromatographic process, it is a good idea to perform a development using this solvent system, to which neither acids nor bases should normally be added. In the blank chromatography, the plate should be marked, e.g. at the right hand side of the solvent front, to ensure that the prewashing and the subsequent development are performed in the same direction.

3.9 Activation

An example of activation is the drying of silica gel plates for 30 min at 120 °C. This causes the physically adsorbed water from the moisture present in the atmosphere to be expelled from the surface of the silica gel. If a much higher temperature is used, this could cause some release of chemisorbed water and hence an irreversible change in the chromatographic properties of the silica gel.

In adsorption chromatography, high activity leads to high retardation properties of the stationary phase and hence to shorter migration distances of the sample substances than those obtained with less active sorbents. This means that, to achieve reproducible retardation values, a well-defined level of activity of the layer is necessary.

To dry the marked plates, they are removed from the prewashing chamber and lid, in the direction of flow and with the layer at the top, along the slots of the drying rack until they hit the end stops. They are then left for a few minutes in this position in the fume hood before tilting the drying rack until the plates are standing on the end stops in a vertical position. The drying rack and plates are then placed in the drying oven preheated to 120 °C. On completion of the drying process, the hot rack is taken out of the drying oven using insulating gloves or a cloth, tilted back into its original horizontal position and, keeping it in this position, placed in a vacuum desiccator containing self-indicating silica gel. To equalize the pressure, the desiccator tap must be opened briefly. The plates are kept in the desiccator until they are used. To avoid undesired contamination, the use of any type of grease (e.g. ground-glass joint lubricant) is avoided.

An example of activation is the drying of silica gel plates for 30 min at 120 °C. This causes the physically adsorbed water from the moisture present in the atmosphere to be expelled from the surface of the silica gel. If a much higher temperature is used, this could cause some release of chemisorbed water and hence an irreversible change in the chromatographic properties of the silica gel.

3.10 Sample application

Resolution of the chromatographic system is dependent on the size of starting zone (spot) of the solute. If this zone is too large then resolution of components of sample mixture, when it is especially more complex and migration of solvent front is short, cannot be satisfactory. Avoiding pitfalls, concerned with sample application, is crucial for final separation in planar chromatography. Conventional application of sample mixture on the chromatographic plate can be performed with calibrated capillary or microsyringe. More advantageous modes of sample application can be performed with semiautomatic applicator or fully automated device. All these modes can be applied for analytical and preparative separations as well. In the following section some information is presented, which will help to introduce the reader in problems of manual and automatic sample application relevant for analytical and preparative separations.

3.11 Sample Application in Analytical Thin Layer Chromatography

The sample spotting can be performed by hand operation using disposal micropipette or calibrated capillary and microsyringe. However, this operation should be performed with care because the adsorbent layer can be damaged by the tip of capillary or syringe needle when pressed too strong against the layer. The adsorbent layer can be prevented from this damage using special device, in which the capillary is held by the dispenser. Sample application with microsyringe possesses one important advantage is sample volume applied on the chromatographic plate can be conveniently varied depending on requirements of the analysis. Very important variable influencing on the size of the sample spot, when manually applied with capillary or microsyringe, is solvent type of the sample mixture. It is desired that elution strength of this solvent should be as low as possible. When this requirement is fulfilled then it should be

expected the sample dimension to be very small. In the other case circular chromatography is realized during sample application leading to widening of starting zone and diminishing final resolution of sample bands on chromatogram. However, for some compounds it is difficult to find an appropriate solvent which fulfill this requirements.

3.12 Sample Application in Preparative Thin Layer Chromatography

Sample application for preparative separations in planar chromatography usually requires spotting larger volumes of the sample solution on the plate—its solution is usually deposited on almost the whole width of the chromatographic plate in shape of band, streak, or rectangle. Adsorbent layers used for preparative separations are thicker than for analytical separations. This procedure of sample application can be performed manually using capillary or microsyringe. Then the sample solution is spotted side by side on the start line of the chromatographic plate. This mode is tedious and needs a lot of manual operations. Shape of starting band is often not appropriate leading to lower resolution of the zones on final chromatogram. More experience is necessary when sample application is performed by moving tip of pipette or syringe needle over a start line without touching the layer surface.The starting sample zone can be formed in desired shape. When sample mixture is more complex then starting zone should be formed as very narrow band what leads to higher resolution of bands on the chromatogram. Total volume of the sample applied to the chromatographic plate in one run can be equal to 500 mL or even more if several repetitions of this procedure are performed.

In this case volume of sample solution depends on syringe capacity. Especially large quantities of sample can be applied on the chromatographic plate according to the paper. The sample solution is mixed with specified quantity of bulky adsorbent. The solvent is evaporated and remnant (bulky adsorbent with deposited sample on it) is introduced to the start line of the chromatographic plate. This mode was adapted by Nyiredy and Benkö to extraction and separation of components from plant materials. Vertical chamber can be very easily used for band sample application. In the first stage of this procedure the adsorbent layer of the chromatographic plate is fed with sample solution instead of the solvent (the mobile phase). When desired sample volume is introduced then the chromatographic plate is supplied with solvent to precede chromatographic process. This procedure possess two advantages: no sophisticated equipment is necessary to perform sample application for preparative separation and during sample application frontal chromatography is performed, which leads to preliminary separation of the components of the sample mixture.

3.13 Equipments for sample application

1. Eppendorf pipette with 10-µl syringe

2. 5- µl Microcapillary in the holder

3. Application pipette (50 µl)

4. 1-µ l Microcapillary in the holder

5. Unimetrics syringe (50 µl)

3.14 Solvent Systems

Next to the choice of the stationary phase (precoated layer), the choice of solvent system is the factor with the greatest influence on a thin-layer chromatogram. Normally, only one solvent to the mixtures of up to six components are used, and these must have the appearance of single-phasesystems with no sign of cloudiness. The solvent system performs the following main tasks: to dissolve the mixture of substances, to transport the substances to be separated across the sorbent layer, to give Rf values in the medium range, or as near to this as possible, to provide adequate selectivity for the substance mixture to be separated.

They should also fulfill the following requirements:

a. adequate purity,
b. adequate stability,
c. low viscosity,
d. linear partition isotherm,
e. a vapor pressure that is neither very low nor very high,
f. Toxicity that is as low as possible.

In analogy to “precoated layer = stationary phase”, the term “mobile phase” is often used for the solvent system. This is a false analogy in over 90% of cases, especially when solvent mixtures are used. The liquid (i.e. the solvent system) that is placed inthe development chamber can, on migration, release some of its components into thepores of the sorbent where it forms a “liquid stationary phase”. In equilibrium withthis, the mobile phase then forms, this being depleted with respect to certain componentsin comparison to the solvent system. As a result, so-called, and -fronts formalong the migration distance. As many fronts can be formed as there are components of differing polarity in the solvent mixture so that, in coordination with a polar stationary phase, the most polar component of the solvent system becomes concentrated nearto the starting line and the least polar becomes concentrated near to the front.

3.15 Selection of developing chambers for TLC

Capillary saturation means the process of capillary filling of any free volume still remaining in the sorbent layer following pre-loading andthe condition of the layer aftercompletion of the development process.

3.16 Chamber atmosphere

The lid of the TLC chamber must on no account be opened during the chromatographic development process. It is therefore a good idea to leave a viewing slit in the CS filter paper lining to check the height of the solvent system. To produce saturation of the chamber, circular filter papers must *never* be used, as these would not take up solvent over the whole width of the chamber. To line the developing chambers for this purpose, filter papers with good absorption properties for the solvent system should always be used, although if the filter paper is too thick it can become separated from the chamber wall, so that the TLC plate is no longer firmly supported.

3.16 Drying After the Development

After the chromatography, the solvent must be removed from the layer to prevent any subsequent deterioration of the chromatogram obtained due to diffusion effects and so that the solvent will not have any harmful effects on the subsequent detection process or even make it impossible. For this, the plate is placed in a level position in the fume cupboard until the odor of the solvent has disappeared. The plate can sometimes be carefully blown with compressed air, but care must be taken to avoid blowing the solvent across it, as this could lead to an undesired chromatographic effect. With powerful solvent systems containing water or acids, drying with warm air is usually necessary, and the last residues of solvent should therefore be removed using a fan heater. For drying after development: With volatile compounds such as essential oils, it is advisable to avoid the use of high temperatures and simply to allow the TLC plate to dry for a time in a fume cupboard before treating it with a reagent.

3.17 Direct Visual Evaluation

TLC plates are still evaluated with the naked eye, a process which requires minimum expenditure on apparatus and which is still prescribed by the pharmacopoeias. The evaluation can be performed in daylight, using either reflected or transmitted light, or can be assisted by the use of UV equipment providing short- and/or long-wave illumination.

3.18 Detection in Daylight

The simplest method of detecting substances on the TLC plate after chromatography is by the visual detection of spots caused by substances with a color of their own. Here, precoated layers without fluorescence indicators can be used.

3.19 Detection with 254-nm UV Light

Nonselective detections can be performed in short- and long-wave UV light. For this,

UV lamps of the appropriate excitation wavelengths are necessary, and these are supplied in various forms. Darkrooms are very suitable locations for UV lamps combined with documentation equipment, as the evaluation of TLC plates by daylight would not be done here. Certain types of evaluation equipment intended for use in daylight rooms are provided with weak UV lamps. For working with UV lamps: dark glasses must always be worn to prevent eye damage. The fluorescence indicators show their usefulness in short-wave UV light, as all UVactive substances show fluorescence quenching when irradiated by UV light with anexcitation wavelength of 254 nm, appearing as dark spots on a bright background.

3.20 Detection with 365-nm UV Light

If a UV lamp with an excitation wavelength of 365 nm is used for the detection, the background of the layer appears dark, and substances that absorb in the UV region and are thereby excited to emit fluorescence or phosphorescence (luminescence) can be seen as bright spots against this dark background. Examples of self-fluorescence with various colors are given, which show images obtained by the action of 365-nm UV light on greater celandine without additional derivatization.

3.21 Photographs in 254-nm UV Light

When detections are performed in short-wave UV light using pre-coated layers containing a fluorescence indicator, the substances appear as dark zones on a bright yellow-green or pale blue background. We are thus not concerned with the formation of different colors, but only with the accurate representation of small differences in brightness.

3.22 Photographs in 365-nm UV Light

On illumination with long-wave UV light, the fluorescent zones appear bright on adark background. Here, there can be a broad palette of colors. Very great demands with respect to true representation of color are therefore made on the film material. The representation of differences in brightness is here of only minor importance.

3.23 Documentation by Digital Camera

Using digital cameras is also a type of photographic documentation. Nearly all advice for practical use given applies here too.

3.24 Determination of R_f (Retardation Factor) values

The $\mathbf{R_f}$ value indicates the distance travelled by the compound in comparison to the distance travelled by the solvent system used to develop the chromatogram. This value is characteristic for a compound in a specific solvent system and helps in the identification of compounds. The $\mathbf{R_f}$

values of the compounds were calculated on a developed chromatogram using the following formula.

$$R_f = \frac{\text{Distance spot moved (cm)}}{\text{Distance solvent moved (cm)}}$$

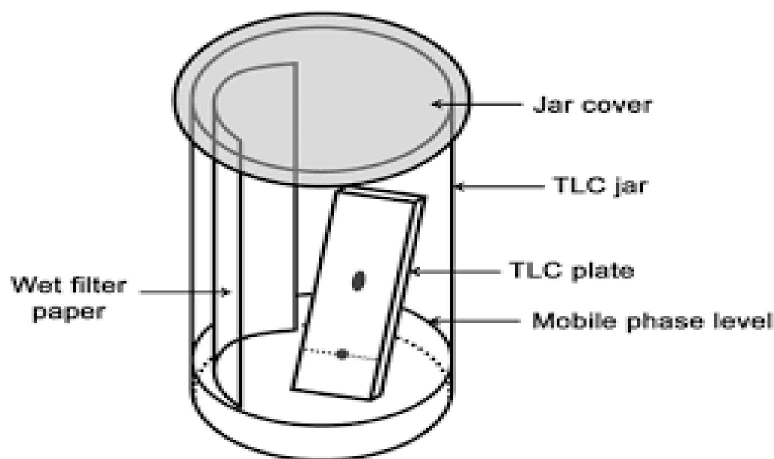


Figure 3.5: Thin Layer Chromatography

3.25 Infrared spectroscopy

Infrared (IR) spectroscopy is the measurement of the vibrational energy changes of a molecule incited by electromagnetic radiation of specific wavelength (1-1000 µm). The covalently bonded atoms of a molecule keep continuously oscillating about an equilibrium position, due to vibrational energy. The movements of these vibrations oscillate with frequencies that comprise the infrared spectral region. When exposed to infrared radiation, energy is absorbed by the molecule, which results in an increase of vibrational motion. The frequency of the absorbed infrared radiation is correlated with the structure of the compound.

A brief overview is warranted. Infrared spectroscopy is the spectroscopy that deals with the infrared region, 14000 cm^{-1} (714 nm) to 10 cm^{-1} (1×10^6 nm), of the electromagnetic spectrum. Moreover, the IR portion of the electromagnetic spectrum is divided into three regions; near infrared, mid-infrared and far-infrared. The near-infrared energy, approximately in the region between 14000-4000 cm^{-1}, can excite overtone or harmonic vibrations. The mid infrared energy, approximately in the region between 4000 cm^{-1} (2500 nm) to 400 cm^{-1} (25000 nm), can be used to study the fundamental vibrations of structures. The far-infrared region, approximately in the region between 400-10 cm^{-1}, can be used to study to rotations of structures. With IR spectroscopy, different functional groups adsorb at different IR bands or regions as thus, this technique can help identify and even quantify organic and inorganic molecules.

Mid-Infrared (IR) spectroscopy is an extremely reliable and well recognized fingerprinting method. Many substances can be characterized, identified and also quantified. One of the strengths of IR spectroscopy is its ability as an analytical technique to obtain spectra from a very wide range of solids, liquids and gases. However, in many cases some form of sample preparation is required in order to obtain a good quality spectrum. Traditionally IR spectrometers have been used to analyze solids, liquids and gases by means of transmitting the infrared radiation directly through the sample. Where the sample is in a liquid or solid form the intensity of the spectral features is determined by the thickness of the sample and typically this sample thickness cannot be more than a few tens of microns. The technique of Attenuated Total Reflectance (ATR) has in recent years revolutionized solid and liquid sample analyses because it combats the most challenging aspects of infrared analyses, namely sample preparation and spectral reproducibility.

3.26 Principles of ATR

An attenuated total reflection accessory operates by measuring the changes that occur in a totally internally reflected infrared beam when the beam comes into contact with a sample (indicated in Figure 4). An infrared beam is directed onto an optically dense crystal with a high refractive index at a certain angle. This internal reflectance creates an evanescent wave that extends beyond the surface of the crystal into the sample held in contact with the crystal. It can be easier to think of this evanescent wave as a bubble of infrared that sits on the surface of the crystal. This evanescent wave protrudes only a few microns (0.5 μ - 5 μ) beyond the crystal surface and into the sample. Consequently, there must be good contact between the sample and the crystal surface. In regions of the infrared spectrum where the sample absorbs energy, the evanescent wave will be attenuated or altered. The attenuated energy from each evanescent wave is passed back to the IR beam, which then exits the opposite end of the crystal and is passed to the detector in the IR spectrometer. The system then generates an infrared spectrum.

For the technique to be successful, the following two requirements must be met:

• The sample must be in direct contact with the ATR crystal, because the evanescent wave or bubble only extends beyond the crystal 0.5 μ - 5 μ.

• The refractive index of the crystal must be significantly greater than that of the sample or else internal reflectance will not occur the light will be transmitted rather than internally reflected in the crystal. Typically, ATR crystals have refractive index values between 2.38 and 4.01 at 2000 cm-1. It is safe to assume that the majority of solids and liquids have much lower refractive indices.

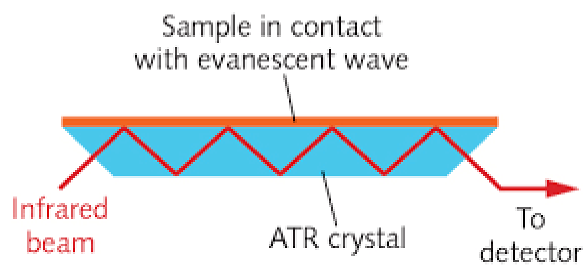


Figure 3.6: ATR cell

3.27 Analyzing solids

Solids are generally best analyzed on the single reflection ATR accessories; diamond being the preferred choice for most applications because of its robustness and durability. After the crystal area has been cleaned and the background collected, the solid material is placed onto the small crystal area. Experience has shown that ideal results from powder samples have been achieved by placing just enough samples to cover the crystal area. The sample height should not be more than a few millimeters.Once the solid has been placed on the crystal area; the pressure arm should be positioned over the crystal/sample area.The pressure arm locks into a precise position above the diamond crystal. Force is applied to the sample, pushing it onto the diamond surface.

3.28 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) spectroscopy is the study of molecular structure through measurement of the interactions of an oscillating electromagnetic field with a collection of nuclei immersed in a strong external magnetic field. The nuclei are the central parts of the atom which are assembled into molecules by bonds formed by electron orbital overlaps. In NMR spectroscopy, the main measurements are only absorption and emission of radio frequency radiation. Quantum mechanics, the field of physics that deals with energy at the atomic level and defines the rules that describe the probability for a photon to be absorbed or emitted under a given set of circumstances.

All nuclei with unpaired protons or neutrons are magnetically active- they have a magnetic field arising from the unpaired nuclear particle. Of greatest interest to an organic chemist is hydrogen (including deuterium) and carbon (the ^{13}C isotope not the ^{12}C isotope which has paired neutrons and protons). B. Placed in an external magnetic field this magnetic field of the nucleus has two stable states, alignment with or against the applied field, which are of slightly different energies (aligned against is higher). The greater the applied field the greater this difference (this is a crucial fact).

Internal (in the molecule) factors which affect (add to or subtract from) the applied magnetic field so as to put the individual nucleus in a different magnetic environment from that felt by another nucleus create differences in the nuclei.

Higher applied magnetic fields will create larger absolute numerical values of the differences between energy states and allow easier distinction between two different nuclei (better resolution).

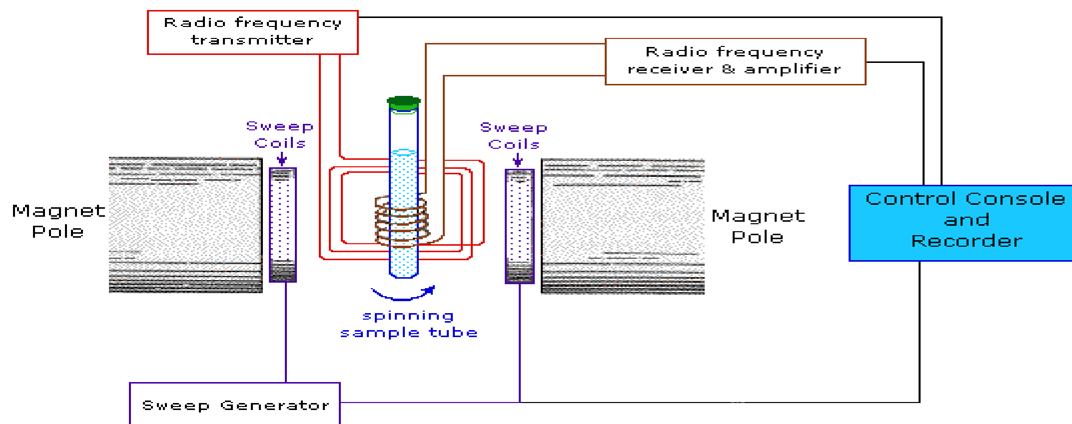


Figure 3.7: Nuclear Magnetic Resonance (NMR)

Electromagnetic radiation of radio frequency wavelengths is of the right energy range to cause the nucleus to move (resonate) between these two energy states. This absorption allows detection of the hydrogen or carbon-13 nucleus. Different nuclei experiencing different magnetic fields and thus different energy differences between states will absorb different radio frequencies or at a particular constant frequency will absorb at different applied magnetic fields and allow us to distinguish between them. This selectivity of energy required to match the energy differences between states is fundamental for all spectroscopies. The energy states are termed quantized. Transitions can occur only when the precise energy corresponding to the energy difference between the states is delivered to the system to excite it to the higher state. So the frequency (or wavelength) of radiation absorbed is specific to that energy transition. When the energy difference between the states changes or is different, the frequency of light absorbed will change.

3.29 Priliminary phytochemicals screening on buds of *S. aromaticum*

The preliminary phytochemical screening for the buds of S. aromaticum was carried out to analyze the presence of compounds namely: Saponins, Anthraquinones, Flavonoids, Phenols, Alkaloids, Tannins, Terpenoids, Steroids, Phlobatannins and Glycosides.

Test for Saponins: To 0.5 g of crude extract 5 mL of distilled water was added and shaken and then heated to boil. Frothing (appearance of creamy miss of small bubbles) showed the presence of saponins [21].

Test for Anthraquinones: To 0.5 g of crude extract 10 mL of benzene was added and shaken and then filtered. 0.5 mL of 10% ammonia solution was added to the filtrate and the mixture was shaken well and the presence of the violet color in the layer phase indicated the presence of the anthraquinones [22].

Test for flavonoids: To 0.5 g portion of crude extract 10 mL of ethyl acetate was added and heated with a steam bath for 3 min. The mixture was filtered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution and a yellow coloration was observed [23].

Test for Phenols: 0.5 g of each of crude extracts extracts were put in a different test tube and treated with a few drops of 2% of FeCl3; bluish green or black coloration indicated the presence of phenols [21].

Test for Alkaloids: 0.5 g of crude extracts was defatted with 5% ethyl ether for 15 min. The defatted sample was extracted for 20 min with 5 mL of aqueous HCl on a boiling water bath. The resulting mixture was centrifuged for 10 min at 3000 rpm. 1 mLof the filtrate was treated with few drops of Mayer's reagent and a second 1 mL with Dragendroff's reagent and turbidity was observed [24].

Test for Tannins: 0.5 g of each crude extract was boiled in 10 mL of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added to give brownish green or a blue black coloration [25].

Test for Terpenoids: 0.5 g of crude powder was separately dissolved in 5 mL of methanol. 2 mL of the extract was treated with 1 mL of 2, 4- dinitrophenyl hydrazine dissolved in 100 mL of 2M HCl. A yellow-orange coloration was observed as an indication of Terpenoids [23].

Test for Steroids: 0.5 g of crude extracts was dissolved in 5 mL of methanol. 1 mL of the extract was treated with 0.5 mL of acetic acid anhydride and cooled in ice. This mixed with 0.5 mL of chloroform and 1 mL of concentrated sulphuric acid was then added carefully by means of a pipette. At the separations level of the two liquids, a reddish-brown ring was formed, as indication of the presence of steroids [21].

Test for Phlobatannins: 2 mL of crude extract was added to 2 mL of 1% HCl and the mixture was boiled. Deposition of a red precipitate was taken as an evidence for the presence of phlobatannins. [26]

Test for Glycosides: 0.5 g of crude extract was dissolved separately in 5 mL of methanol. 10 mL of 50% HCl was added to 2 mL of each extract in test tubes. The mixtures were heated in a boiling water bath for 30 min. 5 mL of Fehling's solution was added and the mixtures were

boiled for 5 min to give a brick red precipitate as an indication for the presence of glycosides [21].

Table 3.4: Priliminary phytochemical screening

Phytochemical tests	**Crude extract of Clove**
Test for Saponins	Present
Test for Anthraquinones	Present
Test for flavonoids	Present
Test for Phenols	Present
Test for Alkaloids	Present
Test for Tannins	present
Test for Terpenoids	Present
Test for Steroids	Present
Test for Phlobatannins	Not present
Test for Glycosides	Present

Chapter 4

StructureElucidation

Result and discussion

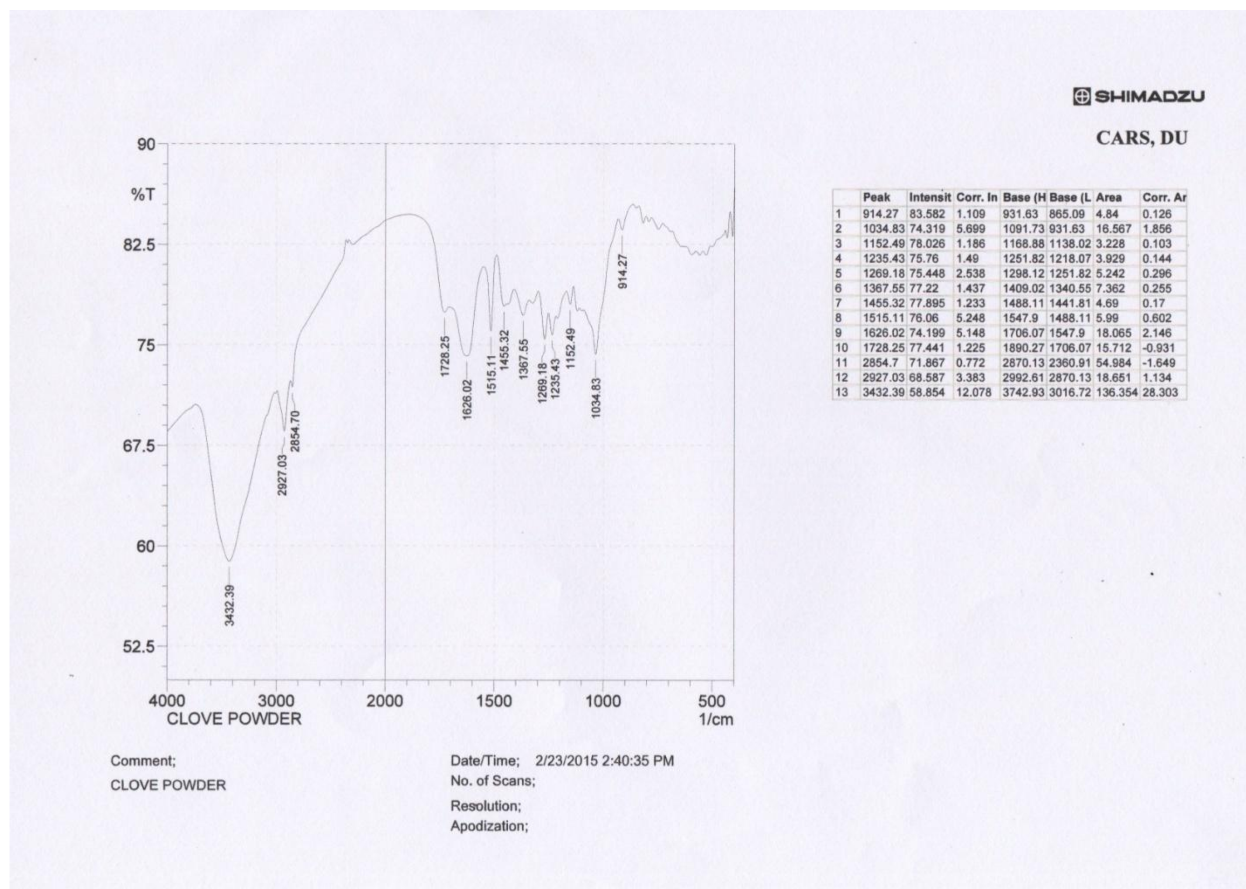
4.1 FTIR-ATR spectrum

In the FTIR-ATR spectrum of the Clove powder, the broad absorption band at 3409 cm^{-1} showed O-H stretching that indicated the presence of hydroxyl group. The strong absorption band at 3019 cm^{-1} showed the presence of the =C-H stretching of alkenes. The weak absorption bands at 2977 cm^{-1} showed the presence of the -C-H stretching (asymmetric stretching) for methyl groups. The absorption band at 2896 cm^{-1} showed the presence of -C-H stretching (symmetrical stretching) of the methylene groups. The weak and broad band at 1630 cm^{-1} showed -C=C- stretching in isolated olefins. The absorption band at 1523 cm^{-1} also showed the presence of -C=C- stretching in the unsubstituted olefins. The strong absorption band at 1218 cm-1 is due to the C-O stretching of the alcoholic region. On the other hand the strong absorption band at 772 cm^{-1} showed the presence of -C-H bending in alkenes. Table 4.1 below shows the principal IR frequencies of clove powder [27].

Table 4.0: FTIR- ATR spectral data of Clove powder

Sl.no	Bond	Functional group	Frequency, cm^{-1}	Intensity	Peak
1	C–H bend	Aromatic C–H out of plane bending	914.27	83.582	s
2	C–H bend	Aromatic C–H out of plane bending	1034.83	74.319	s
3	C–O stretch	alcohols, carboxylic acids, esters, ethers	1152.49	78.026	s
4	C-O stretch	alcohols, carboxylic acids, esters, ethers	1235.43	75.76	s
5	C-O stretch	alcohols, carboxylic acids, esters, ethers	1269.18	75.448	s
6	C–H rock	Alkanes	1367.55	77.22	w, sharp
7	C–H bend	Alkanes	1455.32	77.895	m, sharp
8	C–C stretch (in–ring)	Aromatics	1515.11	76.06	s
9	C=C stretch	olefinic	1626.02	74.199	w, sharp

10	C=O stretch	aldehydes, saturated aliphatic	1728.25	77.441	m
11	C–H stretch	Alkanes	2854.7	71.867	m
12	C–H stretch	Alkanes	2927.03	68.587	s
13	O–H stretch, H–bonded	alcohols, phenols	3432.39	58.854	br,s



	Peak	Intensit	Corr. In	Base (H	Base (L	Area	Corr. Ar
1	914.27	83.582	1.109	931.63	865.09	4.84	0.126
2	1034.83	74.319	5.699	1091.73	931.63	16.567	1.856
3	1152.49	78.026	1.186	1168.88	1138.02	3.228	0.103
4	1235.43	75.76	1.49	1251.82	1218.07	3.929	0.144
5	1269.18	75.448	2.538	1298.12	1251.82	5.242	0.296
6	1367.55	77.22	1.437	1409.02	1340.55	7.362	0.255
7	1455.32	77.895	1.233	1488.11	1441.81	4.69	0.17
8	1515.11	76.06	5.248	1547.9	1488.11	5.99	0.602
9	1626.02	74.199	5.148	1706.07	1547.9	18.065	2.146
10	1728.25	77.441	1.225	1890.27	1706.07	15.712	-0.931
11	2854.7	71.867	0.772	2870.13	2360.91	54.984	-1.649
12	2927.03	68.587	3.383	2992.61	2870.13	18.651	1.134
13	3432.39	58.854	12.078	3742.93	3016.72	136.354	28.303

ATR signal 4.1: ATR- FTIR data of clove powder

Its FT-IR was exactly identical with that of pure eugenol and showed (cm^{-1}): 3514.3 (br,s, OH str.), 3074.7 and 3003.0 (=CH aromatic and olefinic, m-w), 2972.9, 2937.9, 2841.1 (m,C-H str., Aliphatic), 1637.6 (w, sharp, C=C olefinic), 1608, 1513.8, 1459.5 (m-s, sharp, C=C aromatic), 1432.0 (m, sharp, CH_2, aliphatic), 1367.9 (w, sharp, CH_3), 1268.8, 1234.2 (s, C-O str.) [28, 29].

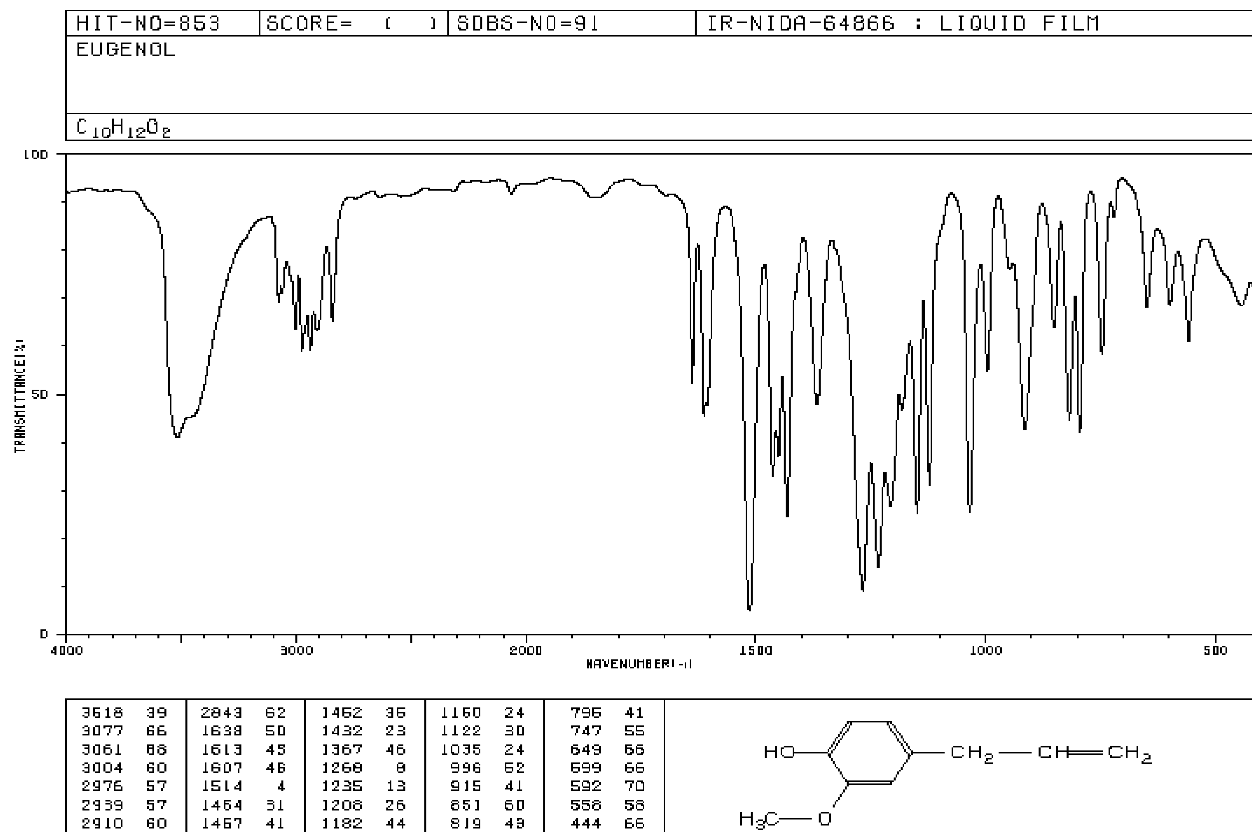


Figure 4.2: IR of liquid film of Euginol.

This peak confirms the presence of eugenol because eugenol contains an –OH functional group. The IR spectrum compares closely with a reference spectrum for Eugenol, further confirming that the IR spectrum shows the presence of Eugenol.

4.2 Characterization of *Syzygium aromaticum* (SA)-1.1 as eugenol

Eugenol (4-allyl-2-methoxyphenol) is brownish oil. It was isolated from fraction of the chloroform extract of *S. aromaticum*. The IR spectra data clearly indicated the appearance absorption of hydroxyl group at 3418 cm^{-1}, C-O-C linkage of ether around 1030 cm^{-1}, strong absorptions at 1610 and 1510 cm^{-1} were also found from eugenol due to terminal double bond and aromatic moiety.

The structure of eugenol was elucidated by spectroscopic techniques mainly ^{1}H NMR, 13C NMR and IR. The 400 MHz (^{1}HNMR) and 100 MHz (^{13}C NMR) in $CDCl_3$ spectra of eugenol showed the presence of 12 protons in the molecule [30]. The presence of an aromatic protons at δ 6.88 (d, 1H, *J*= 8.5 Hz), 6.70-6.72 (m, 2H) could be assigned to be 1, 2, 4-trisubstituted benzene, together with the singlet of methoxy proton at δ 3.88 (s, 3H) and proton of hydroxyl group at δ 5.59 (br, 1H). The doublet pattern at δ 3.35 (d, 2H-1', J= 6.7 Hz) is attached to aromatic ring. The signal at δ 5.09-5.14 (m, 2H-3') suggested that was methylene proton of terminal double bond, indicating the location of terminal double bond. The ^{13}C NMR displayed of 10 carbon atoms. It indicated three quaternary, four tertiary, two secondary and one methyl carbons. Notably, the chemical shift at 55.7 ppm was assigned to methoxy carbon at para position of benzene ring. The downfield quaternary carbon at 143.84 and 146.39 ppm corresponded to aromatic carbon (C-1, C-2) bearing a hydroxyl and methoxy group while the upfield quaternary carbon (C-4) at 131.85 ppm was allylic group confirmed. It showed the interactions between proton of methoxy group at 3.88 ppm to C-2, H-3 (δ 6.88) to C-1, C-2, C-4, C-5, C-6, H-1' (δ 3.55) to C-2', C-3', C-4, C-5, C-6 as shown in table. Consequently, alkoxy group of 55.7 ppm and allylic group substantiated at C-2 (δ 146.3) and C-4 (δ 131.8). The structure of compound SA-1.1 was finally confirmed by directed comparison of ^{1}H and ^{13}C NMR with the value reported [30].

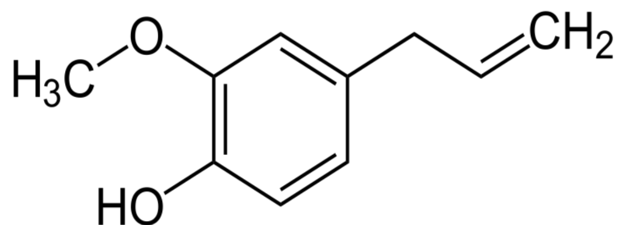
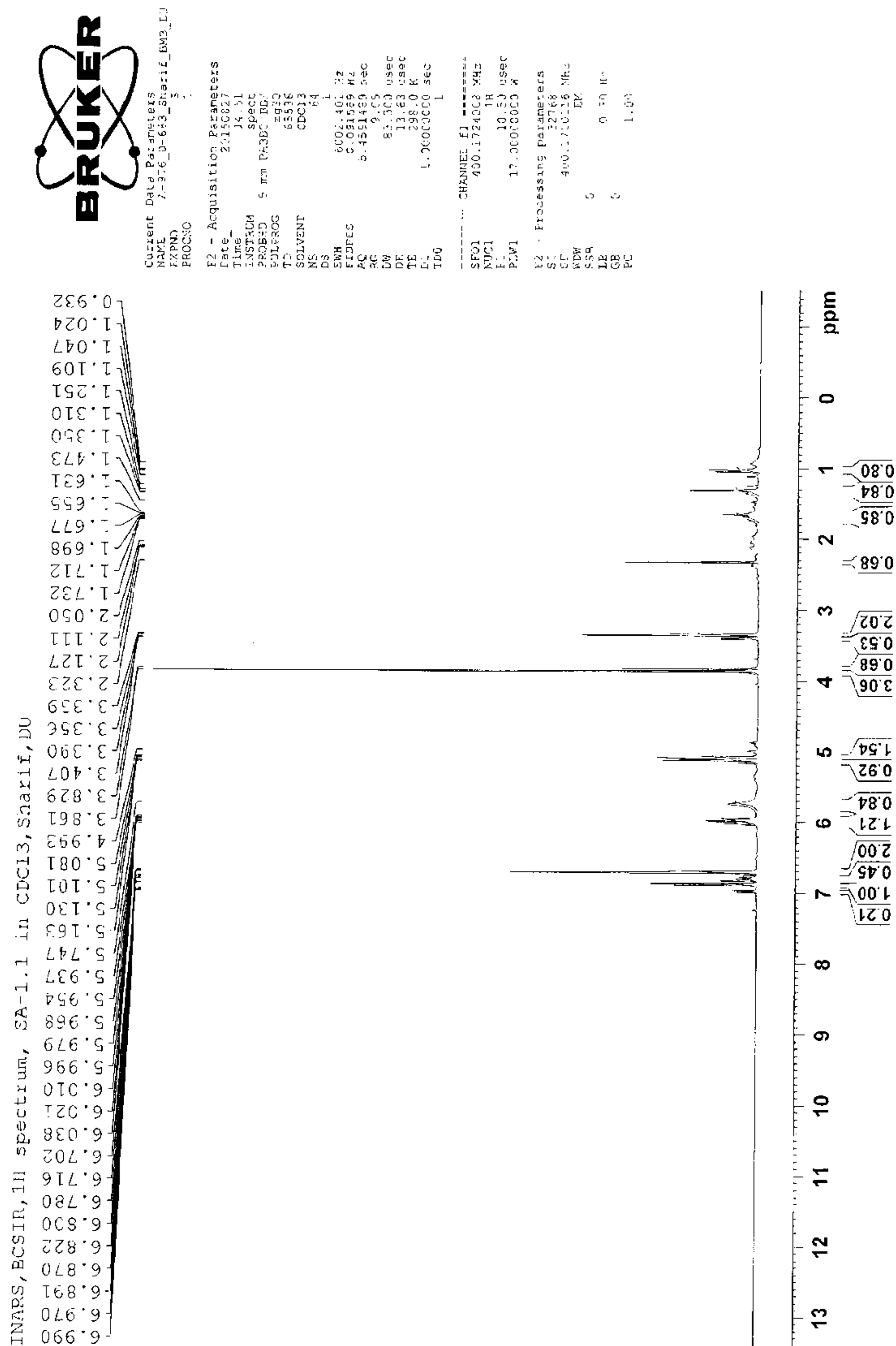


Figure 4.3: Eugenol

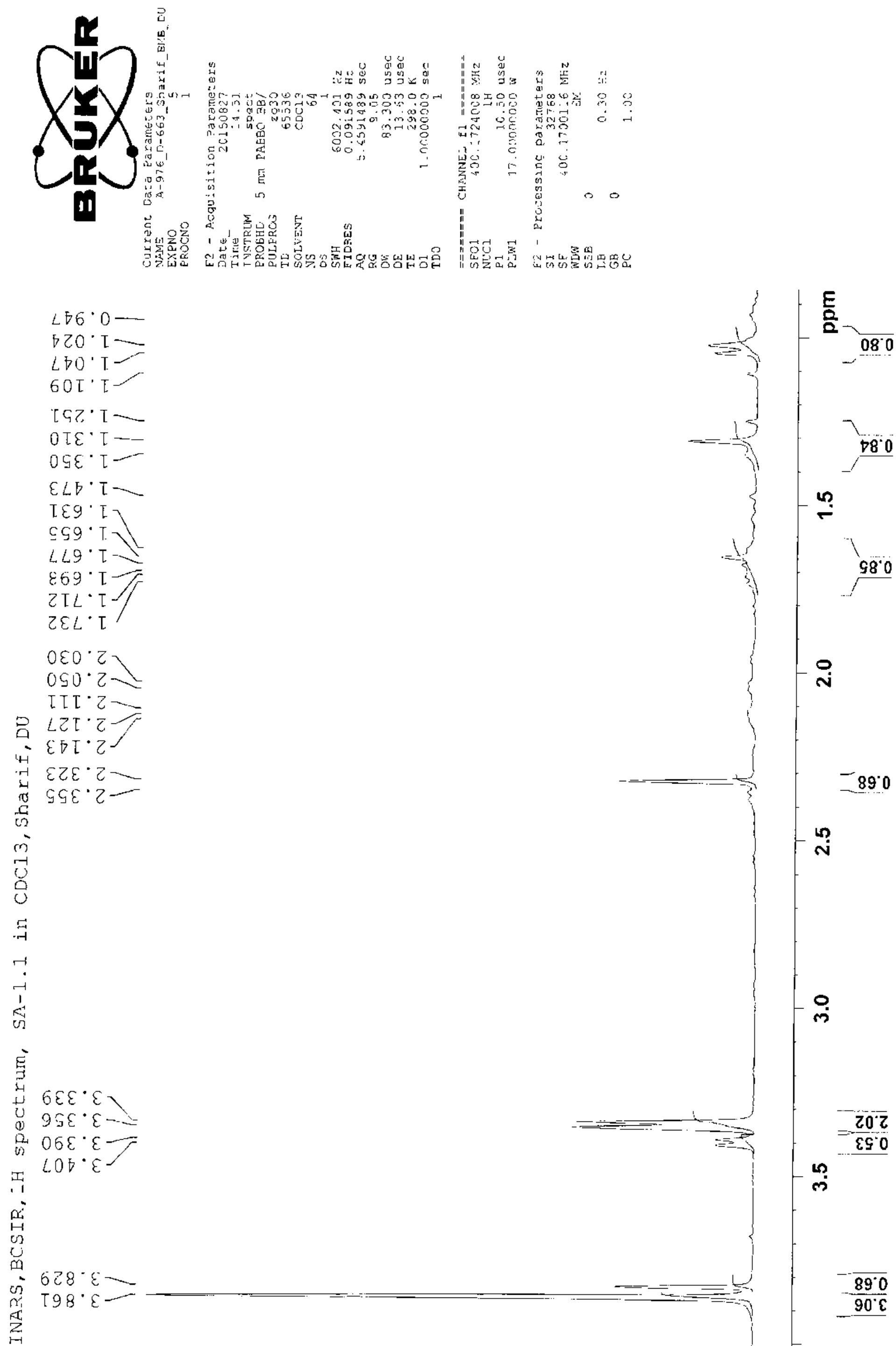
Table 4.1: ^{1}H and ^{13}C NMR data of eugenol ($CDCl_3$) at 400 (^{1}H NMR) and 100 MHz (^{13}C NMR)

Position	**δ_H in ppm in $CDCl_3$**	
	SA-1.1	**Eugenol [30]**
1	-	-
2	-	-
3	6.87-6.89 [d, 1H, J= 8.4 Hz]	6.88 [d, 1H, J = 8.5 Hz]
4	-	-
5, 6	6.70-6.716 [m, 1H]	6.70-6.72 [m, 1H]
1'	3.35 [d, J=6.8 Hz]	3.35 [d, 2H, J = 6.7 Hz]
2'	5.933-6.08 [m, 1H]	5.93-6.04 [m, 1H]
3'	5.08-5.163 [m, 2H]	5.09-5.14 [m, 2H]
O-CH_3	3.861 [s, 3H]	3.88 [s, 3H]
OH	5.74 [br, 1H]	5.59 [br, 1H]

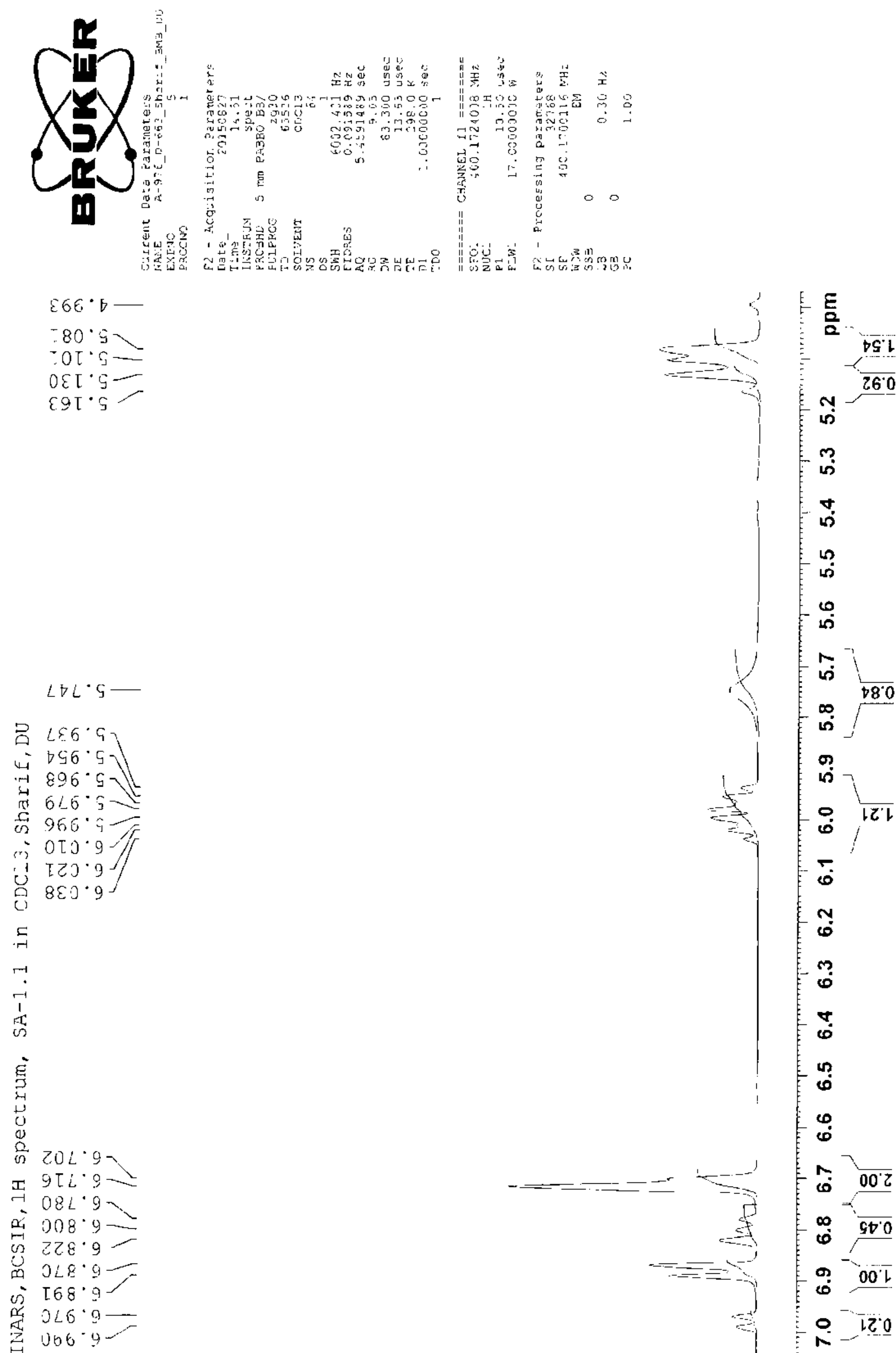
Position	**Chemical shift (δ) in ppm**	
	SA- 1.1	**^{13}C NMR [30]**
1	146.41	146.3
2	143.84	143.8
3	114.26	114.2
4	131.76	131.8
5	111.09	111.0
6	121.06	121.1
1'	39.85	39.8
2'	137.74	137.7
3'	115.35	115.4
O-CH_3	55.69	55.7



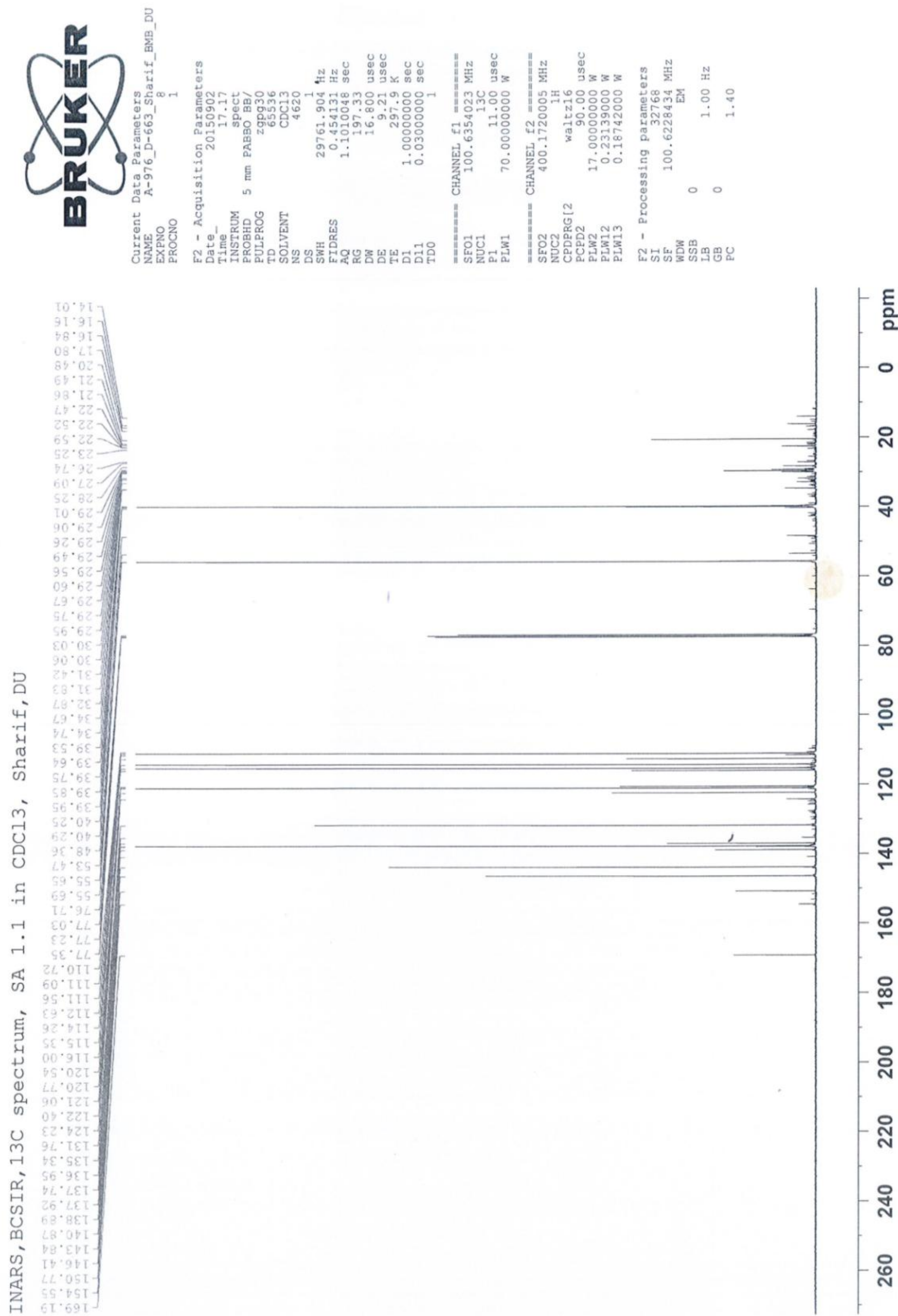
NMR Signal 4.2: ^{1}H NMR signals of SA-1.1



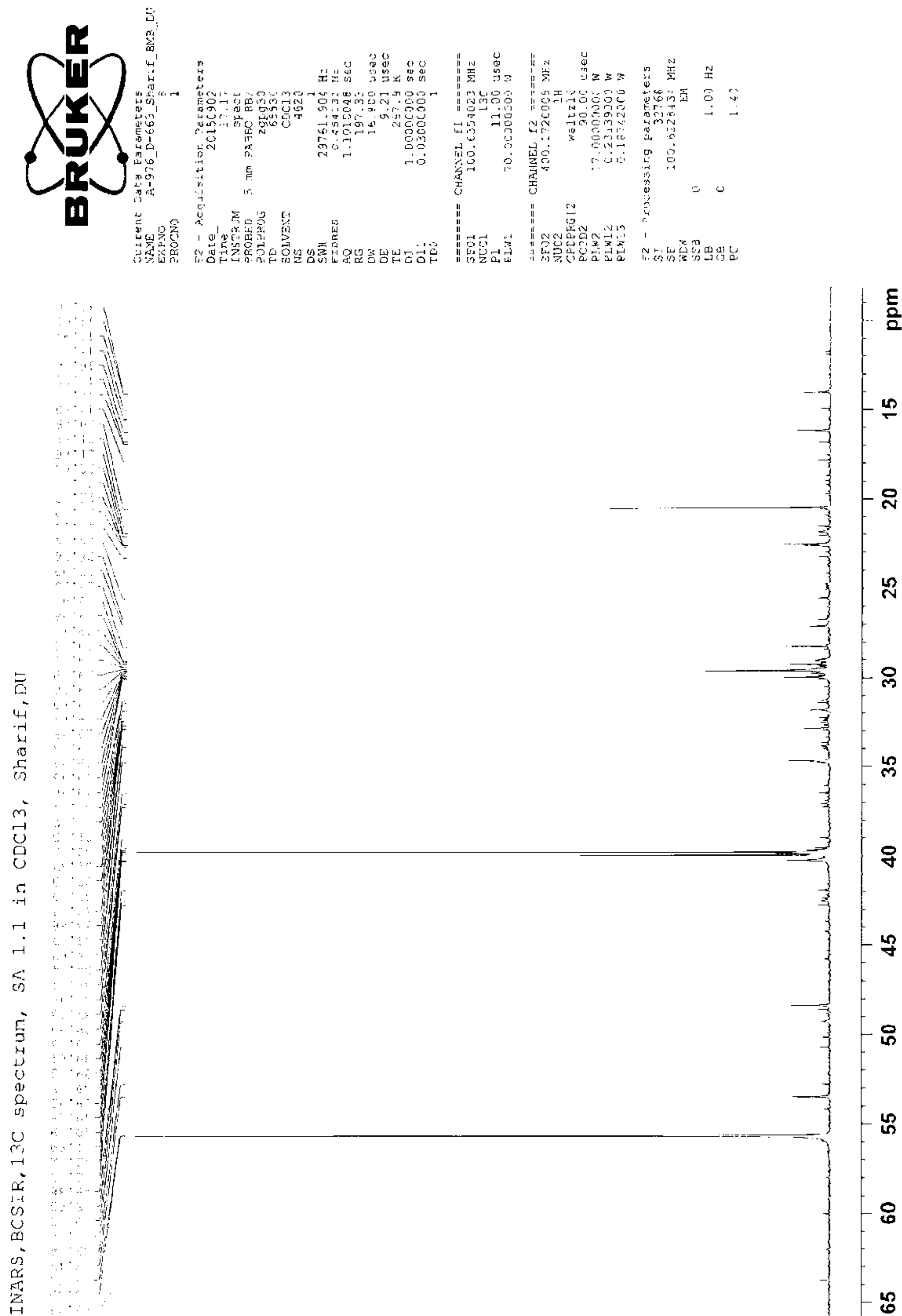
NMR signal 4.3: ^{1}H NMR signals of SA-1.1



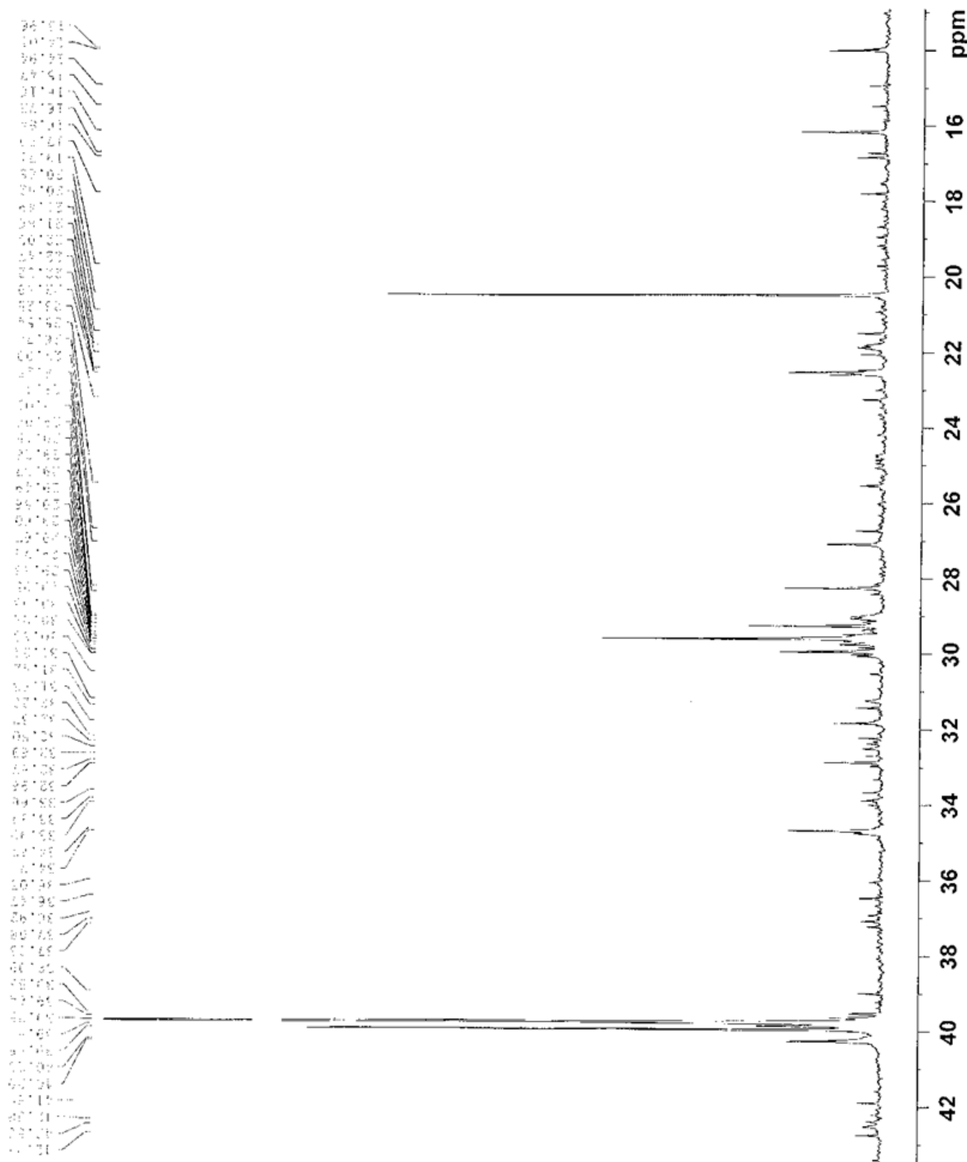
NMR signal 4.4: ^{1}H NMR signals of SA-1.1



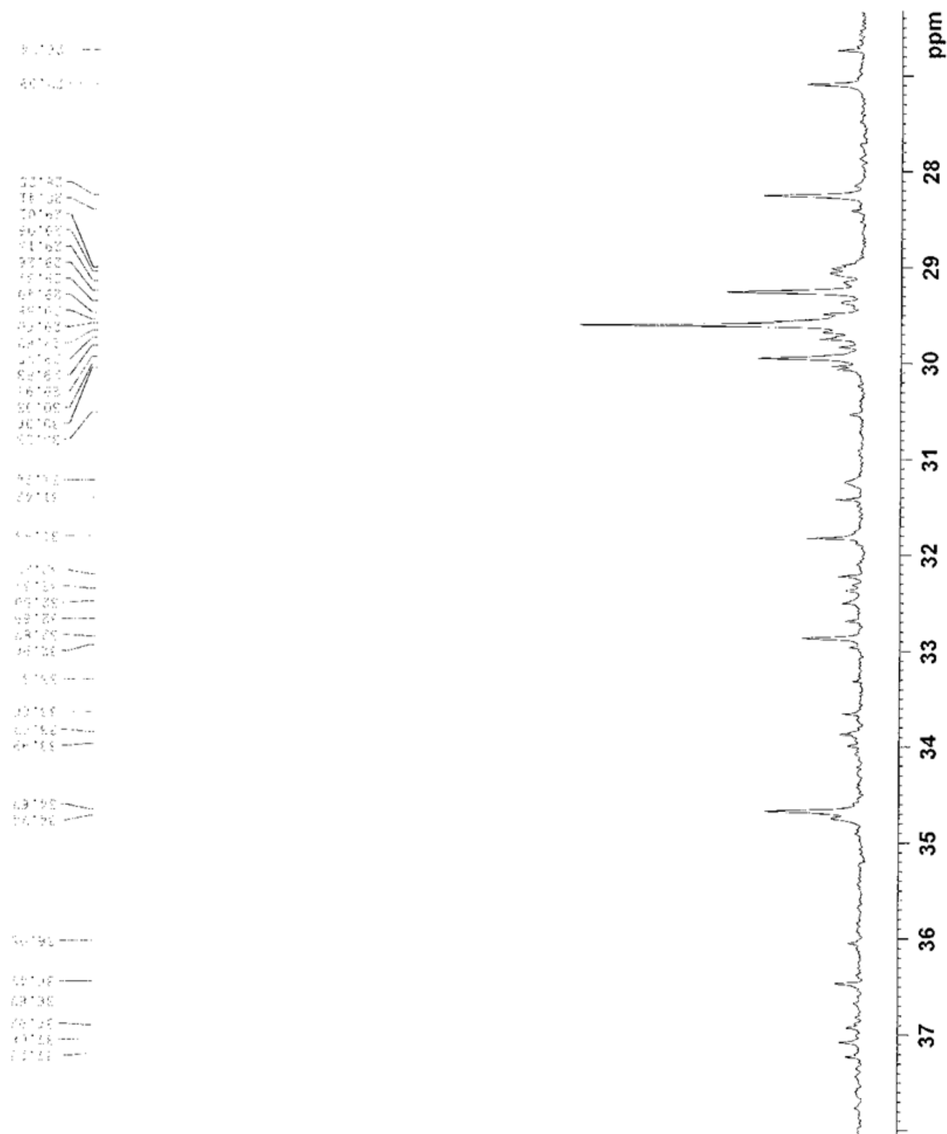
NMR signal 4.5: ^{13}C NMR signals of SA-1.1



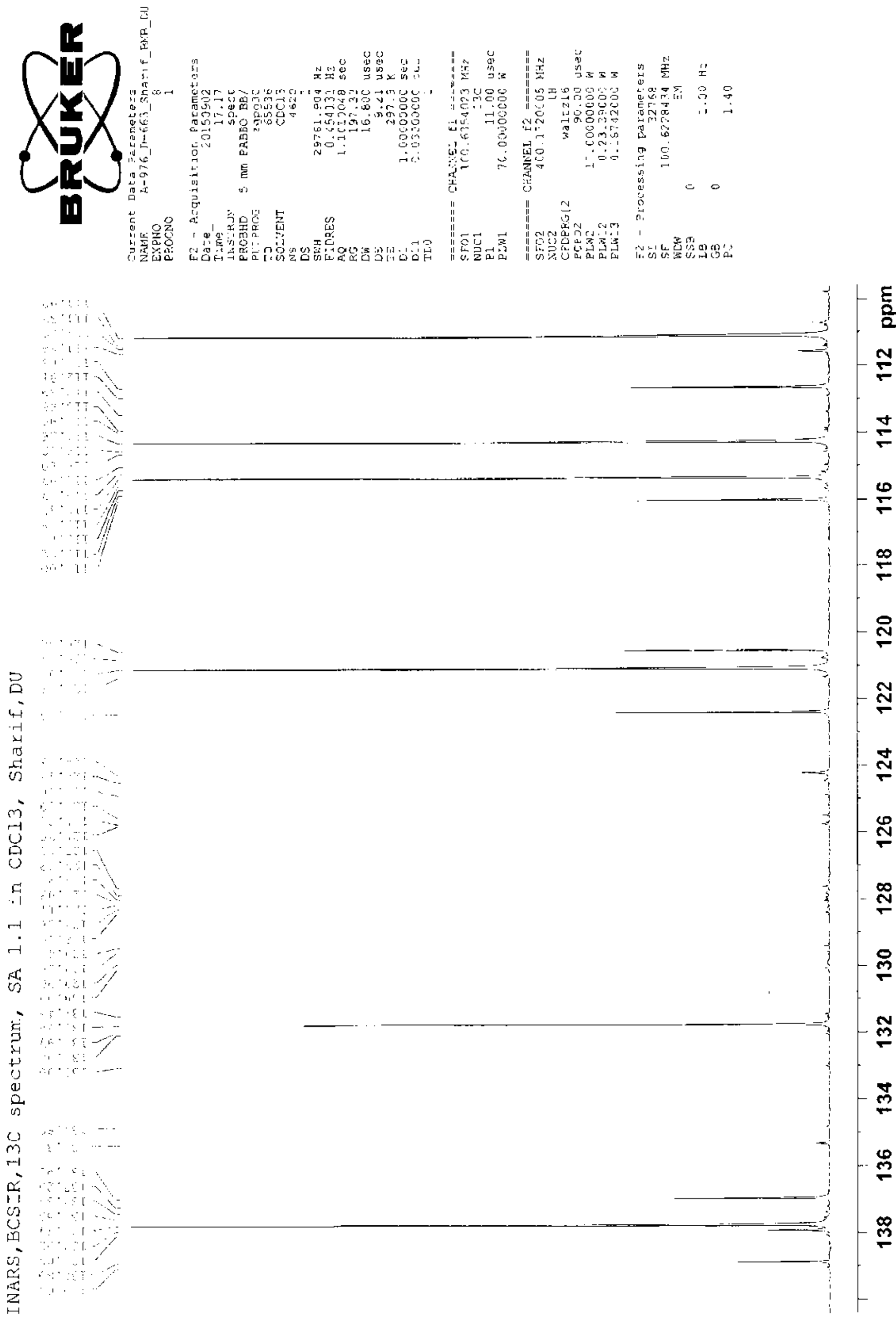
NMR signal 4.6: ^{13}C NMR signals of SA-1.1



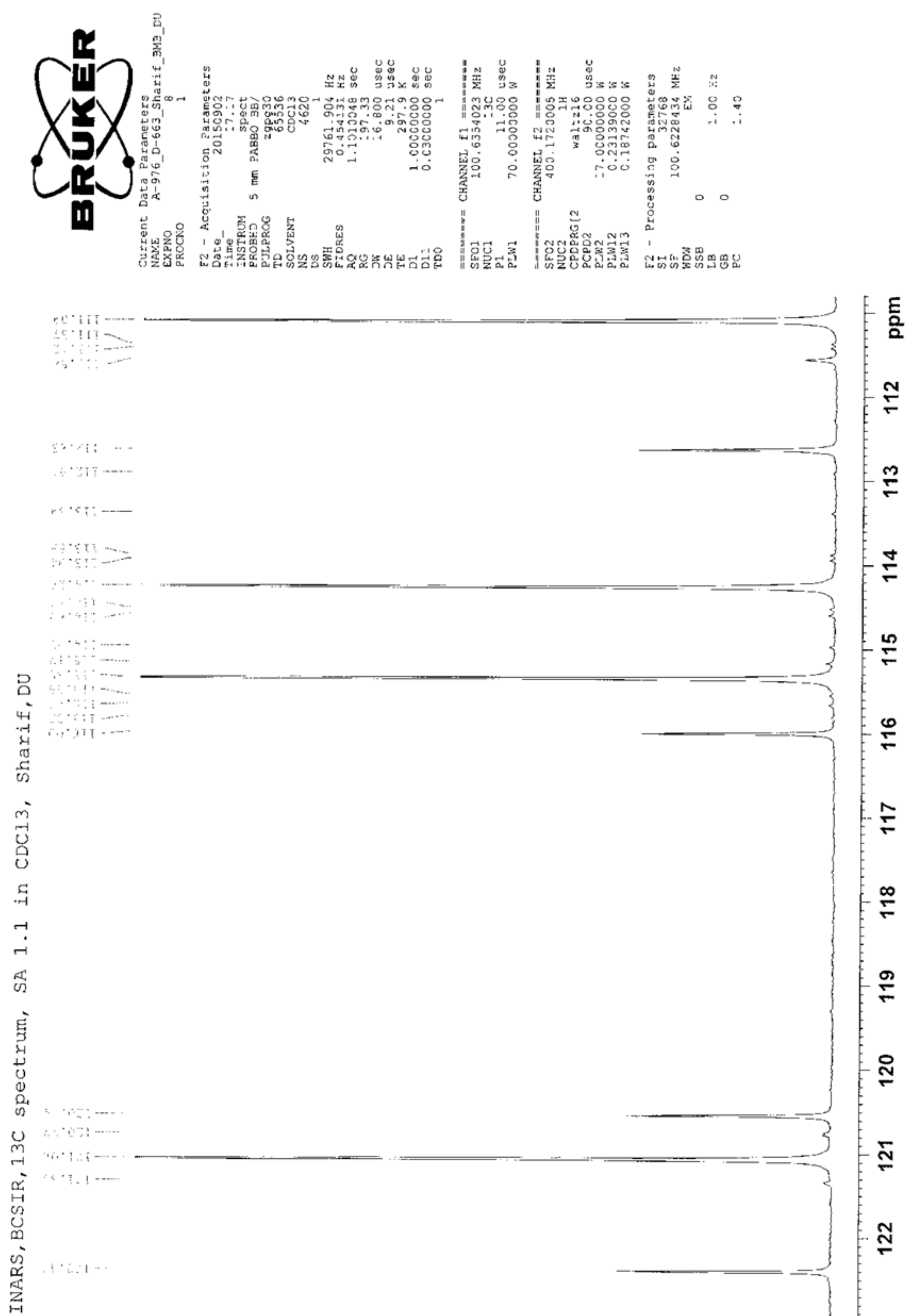
NMR signal 4.7: ^{13}C NMR signals of SA-1.1



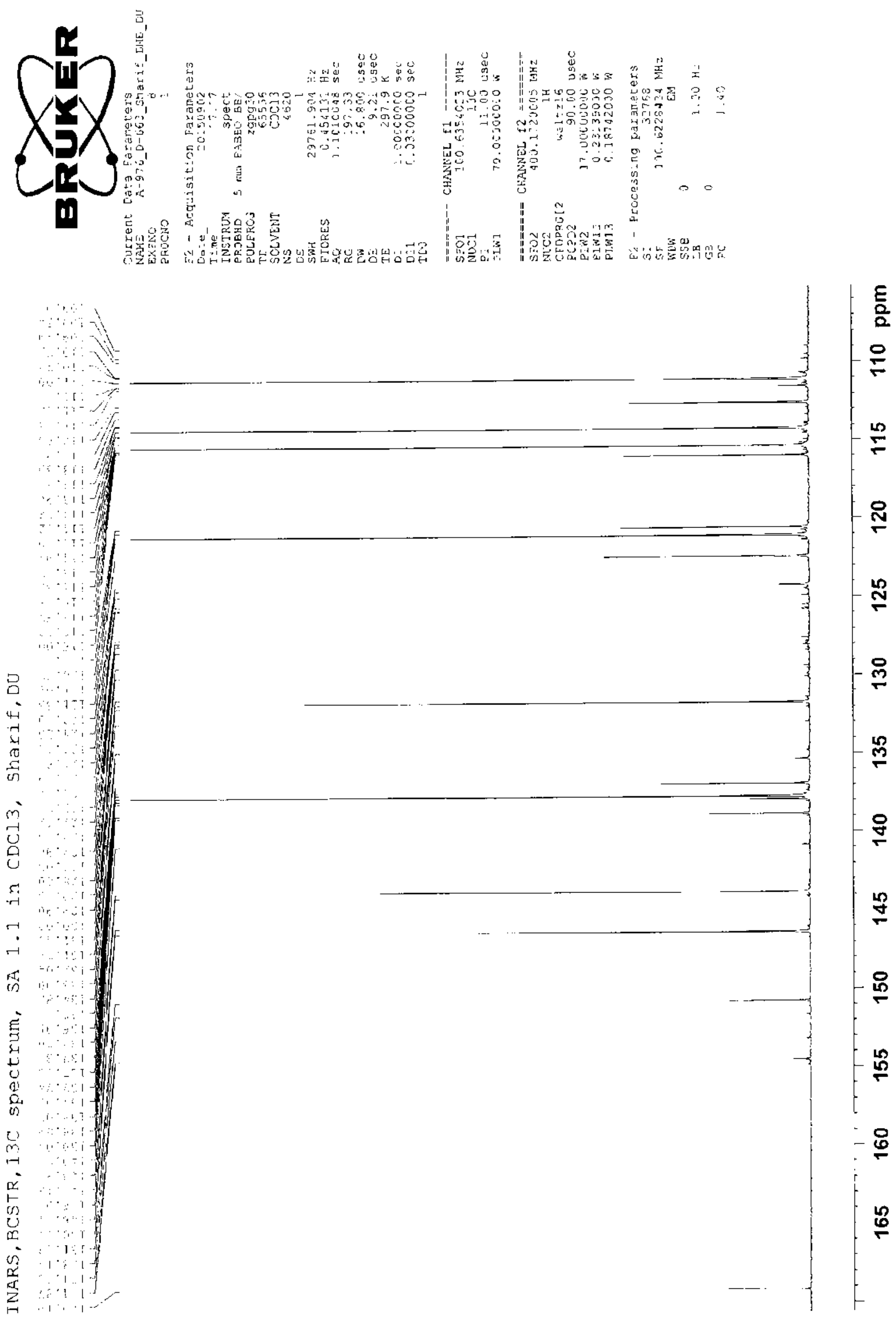
NMR signal 4.8: ^{13}C NMR signals of SA-1.1



NMR signal 4.9: ^{13}C NMR signals of SA-1.1



NMR signal 4.10: ^{13}C NMR signals of SA-1.1



NMR signal 4.11: ^{13}C NMR signals of SA-1.1

4.3 Characterization of SA-1.2 as octane

SA-1.2 was obtained as a colorless amorphous powder from chloroform fraction of S. aromaticum. It was purified from chloroform crude by preparative TLC (Stationary phase-Silica gel F_{254}, Mobile Phase- Toluene: Ethyl-acetate = 90:10). Colorless liquid was obtained which evaporated to yield a colorless amorphous powder. It was found to be soluble in petroleum ether, ethyl acetate and chloroform.

The ^1H NMR spectrum (400 MHz, $CDCl_3$) of SA-1.2 revealed the spectrum displayed a multiplet at δ 0.85 due to the terminal methyl group proton (H_3-8). The spectrum also displayed singlte at δ 1.24, which could be assigned to the methylene protons at C-3 to C-7 and C-2 respectively.

Comparing the ^1H NMR spectral data obtained for SA-1.2 and reported ^1H NMR spectral data of octane; SA-1.2 might be characterized as octane [31].

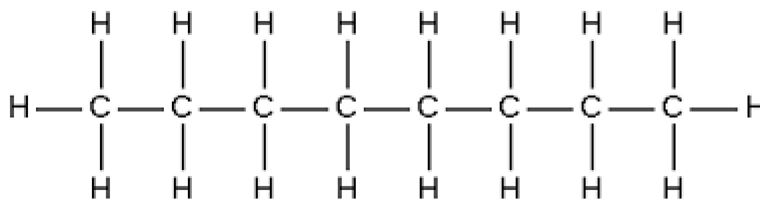
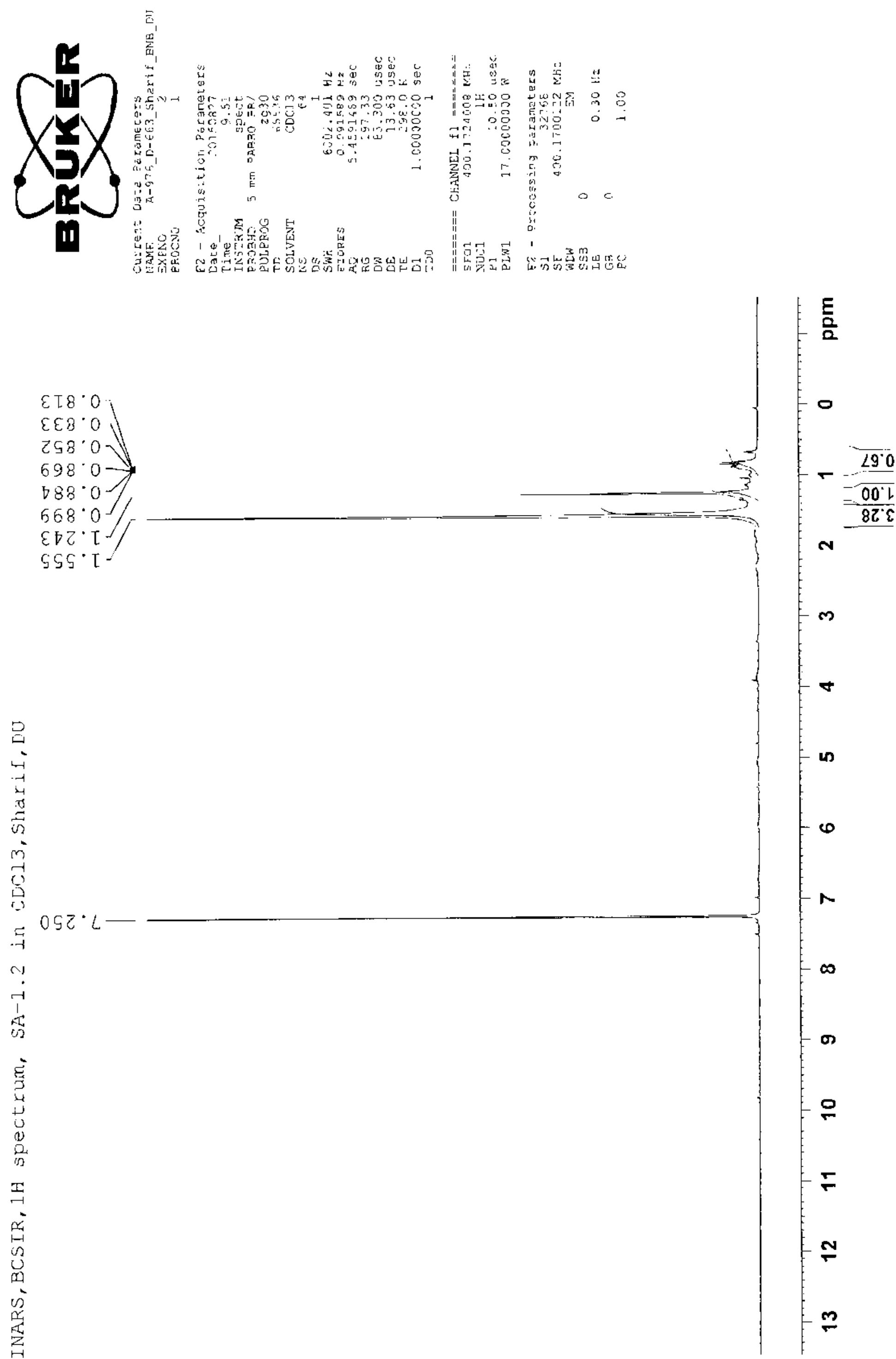


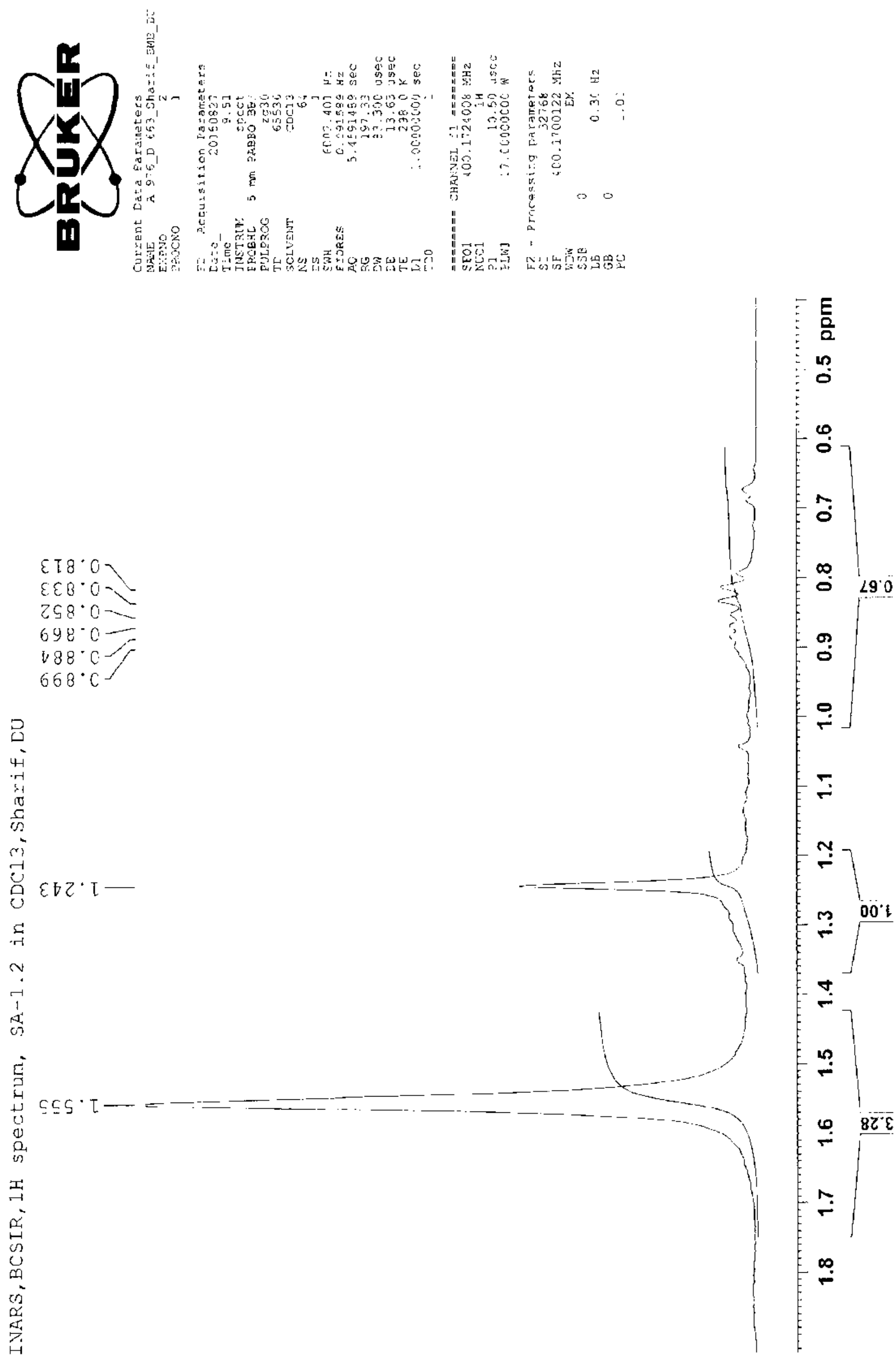
Figure 4.3: Octane

Table 4.2: Comparison between ^1H NMR spectral data of SA-1.2 (400 MHz) and Octane (31) in $CDCl_3$

Positions	**δ_H in ppm in $CDCl_3$**	
	SA-1.2	**Octane (31)**
H_3-8	0.85	0.815- 0.883 (3H, m)
H_2-3 to H_2-7	1.243	1.26 (10H, s)



NMR signal 4.12: ^{1}H NMR signals of SA-1.2



NMR signal 4.13: ^{1}H NMR signals of SA-1.2

4.4 Characterization of SA-6 as Euginol Acetate

Euginol acetate is also known as Acetyleugenol or eugenyl acetate. Its' molecular Weight is 206.241. Melting point: 26° C, boiling point: 281-286° C (lit.), density :1.08 g/mL at 25 ° C. **Other names:** Eugenol acetate; Acetyl eugenol; Phenol, 2-methoxy-4-(2-propenyl), acetate; Aceteugenol; Phenol, 4-allyl-2-methoxy-, acetate; 1,3,4-Eugenol acetate; Aceto eugenol; 1-Acetoxy-2-methoxy-4-allylbenzene; 4-Allyl-2-methoxyphenol acetate; 4-Allyl-2-methoxyphenyl acetate; NSC 1242; Phenol, 2-methoxy-4-(2-propen-1-yl)-, 1-acetate.

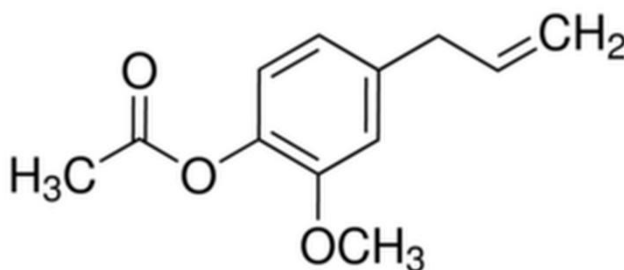
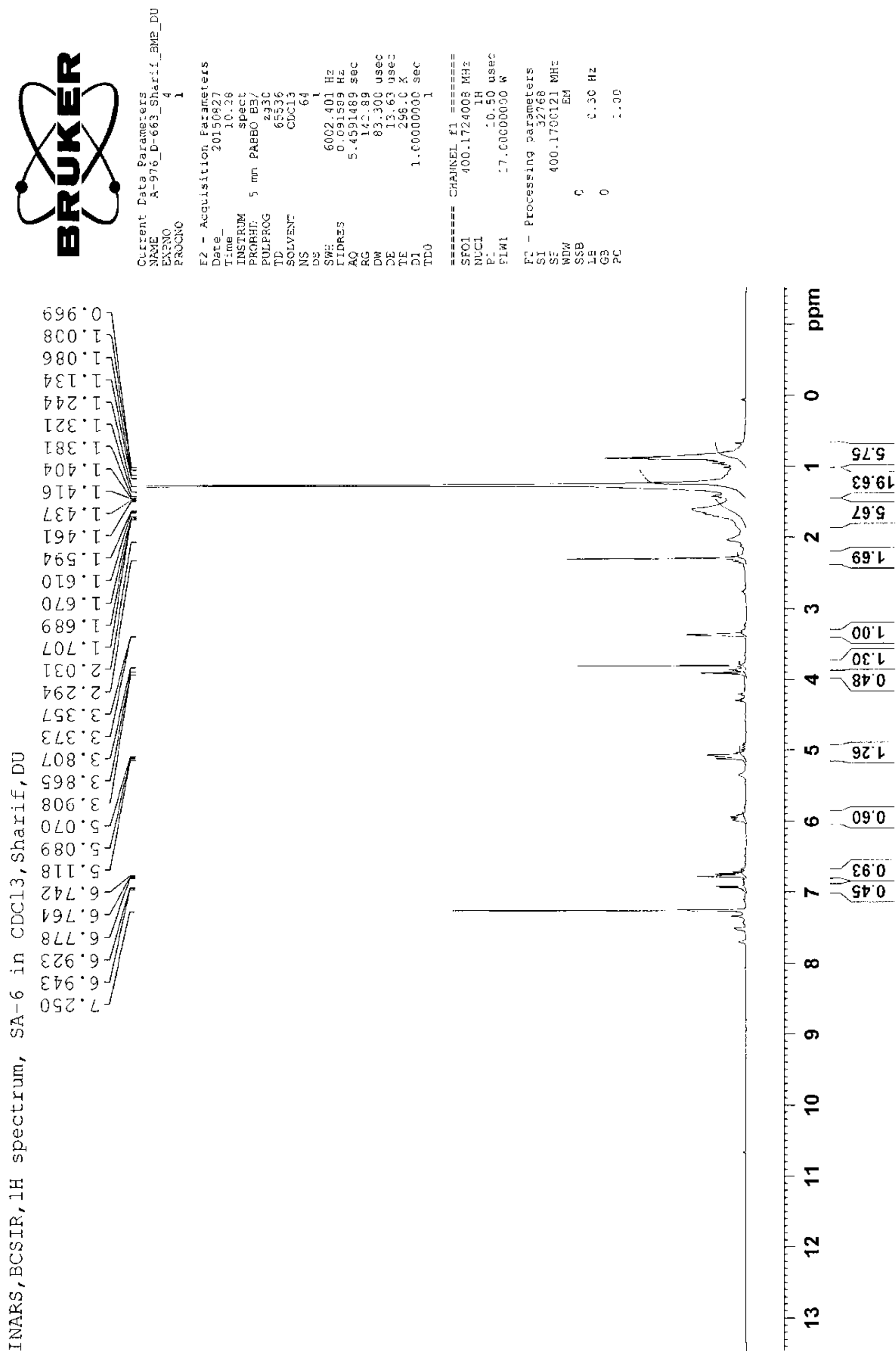


Figure 4.4: Euginol Acetate

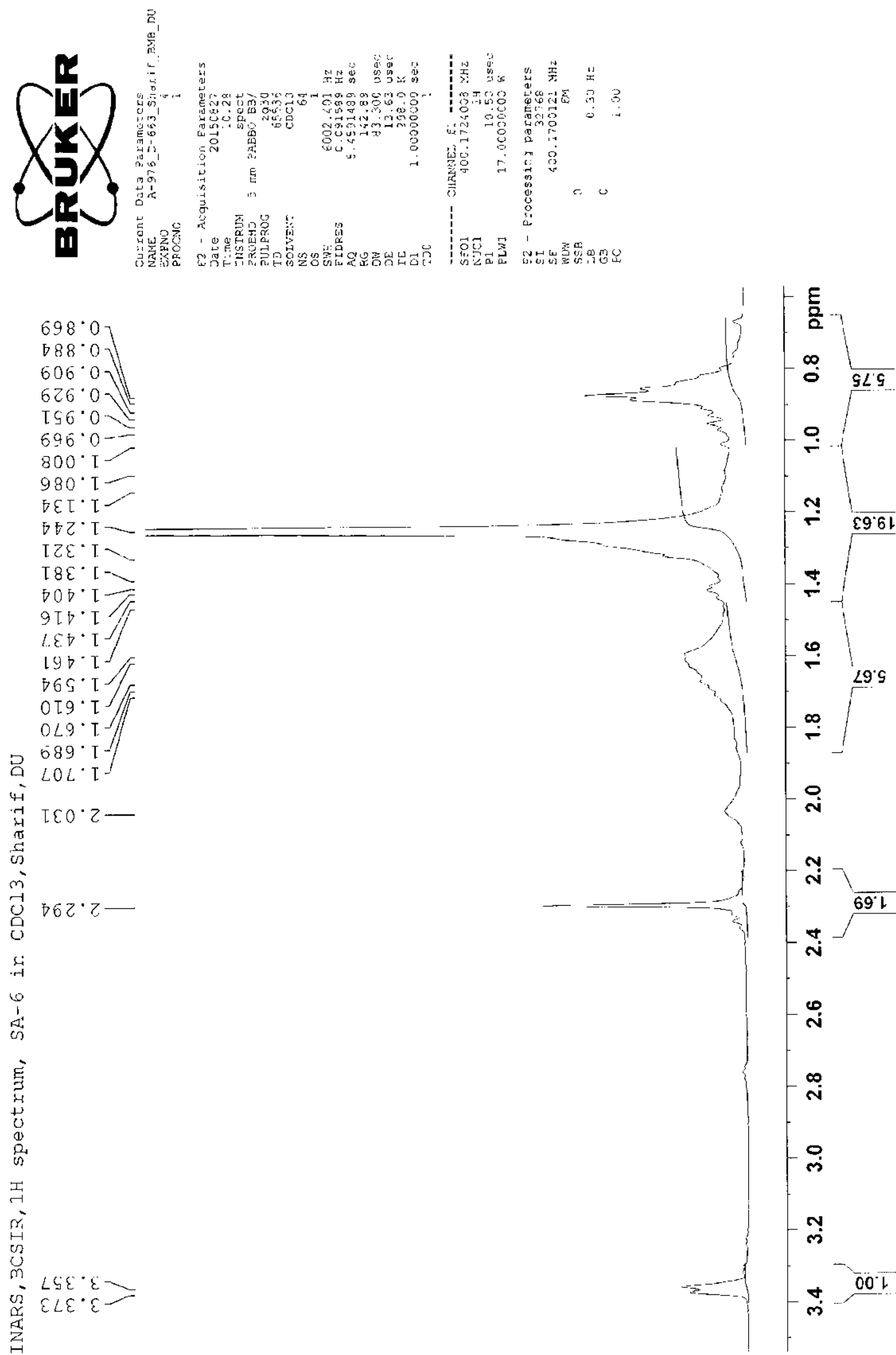
Comparing the ^{1}H NMR spectral data obtained for SA-6 and reported ^{1}H NMR spectral data of Euginol acetate; SA-6 might be characterized as Euginol acetate [32].

Table 4.3: Comparison between ^{1}H NMR spectral data of SA-6 (400 MHz) and Euginol acetate [32] in $CDCl_3$

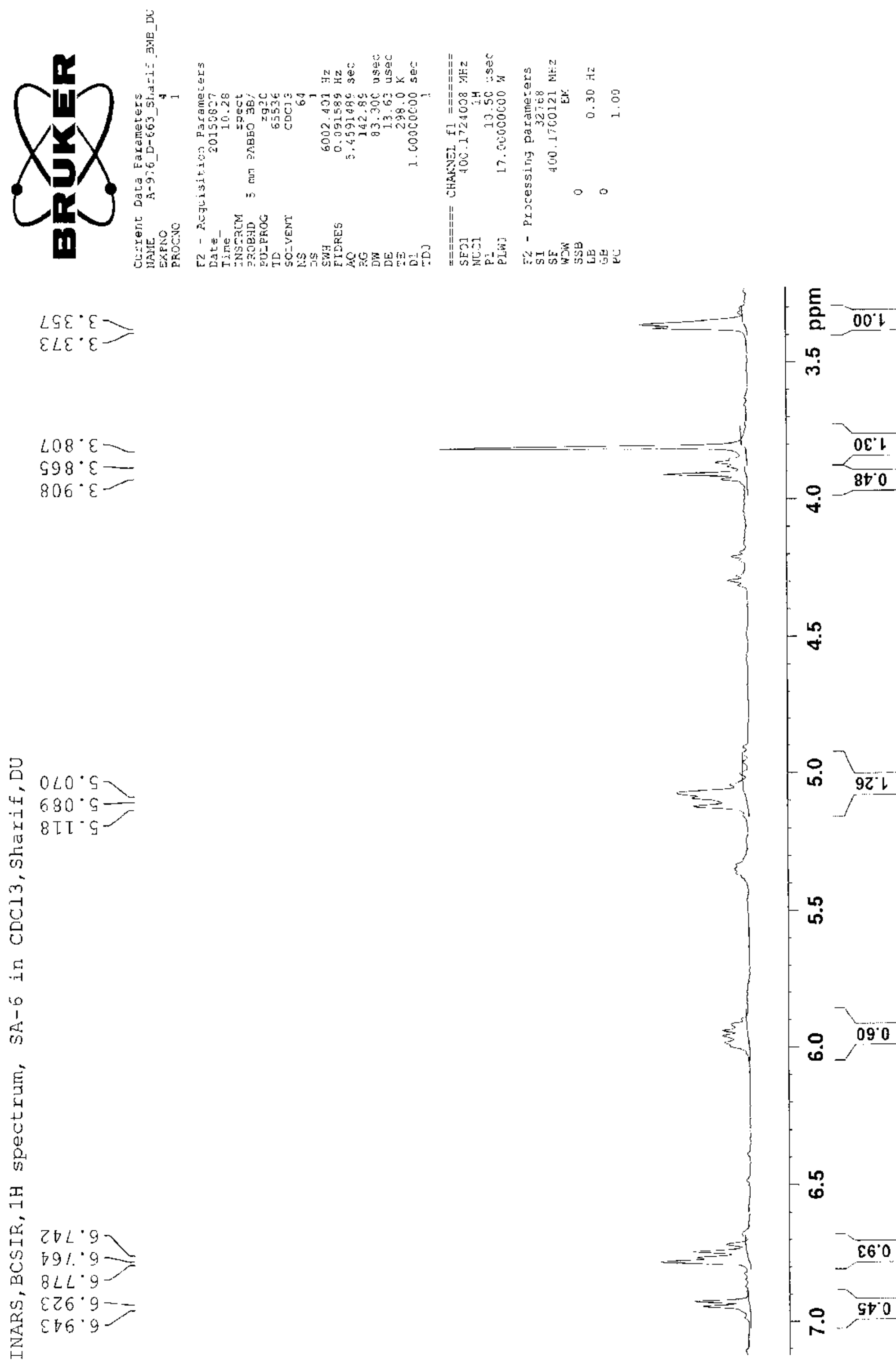
Positions	**δ_H in ppm in $CDCl_3$**	
	SA-6	**Euginol Acetate [32]**
CH_3COO	2.29 (3H, s)	2.31 (3H, s)
H-1	3.373 (2H, d, J= 6.4 Hz)	3.38 (2H, d, J=6.7 Hz)
OCH_3	3.80 (3H, s)	3.82 (3H, s)
H-3’	5.11 (2H, m)	5.10 (2H, m)
H-2’	5.97 (1H, m)	5.97 (1H, m)
H-3 and H-5	6.77 (2H, m)	6.77 (2H, m)
H-6	6.943 (1H, d, J=8.0 Hz)	6.95 (1H, d, J=7.9 Hz)



NMR signal 4.14: ^{1}H NMR signals of SA-6



NMR signal 4.15: ^{1}H NMR signals of SA-6



NMR signal 4.16: ^{1}H NMR signals of SA-6

4.4 Characterization of SA-13.1 as Oleanolic Acid

It is a white amorphous powder, also named (3β)-3-Hydroxy olean-12-en-28-oic acid. Its' molecular formula is $C_{30}H_{48}O_3$, molecular weight is 456.71 and melting point is 310°C. It is a pentacyclic triterpenoid present in many species of the plant kingdom. It exists in almost 190 species of medicinal herbs and plants in the form of the free acid or aglycones for triterpenoid saponins. It is a compound that appears to have multiple and diverse pharmacological activities in animals and humans. It has three functional groups, viz., a secondary hydroxyl at C-3, an olefinic bond at C-12 and a carboxylic acid at C-17. Synthesis of a number of derivatives of these functional groups has been carried out.

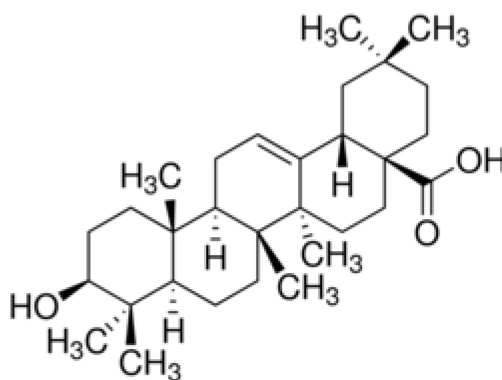
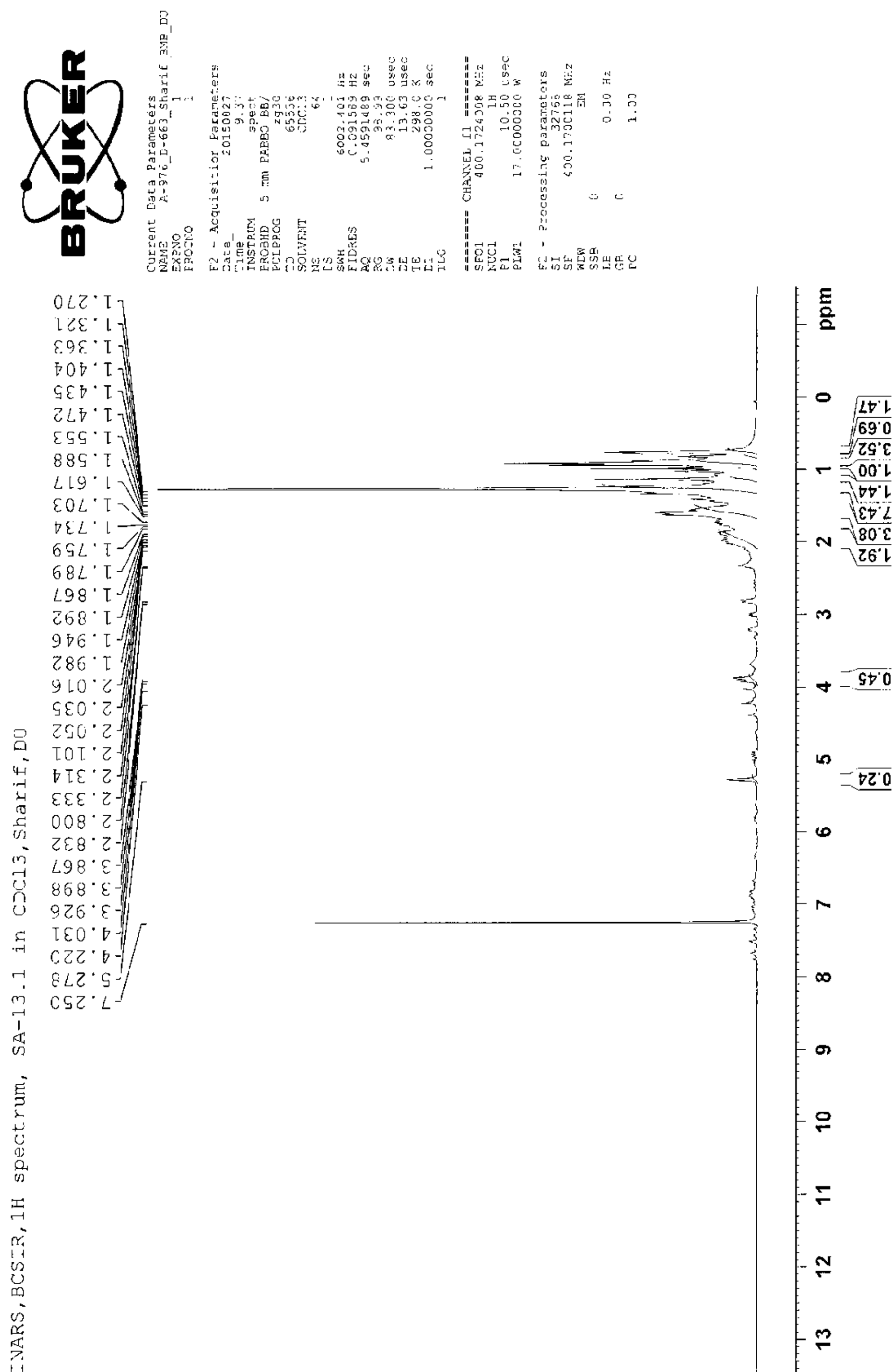


Figure 4.5: Oleanolic Acid

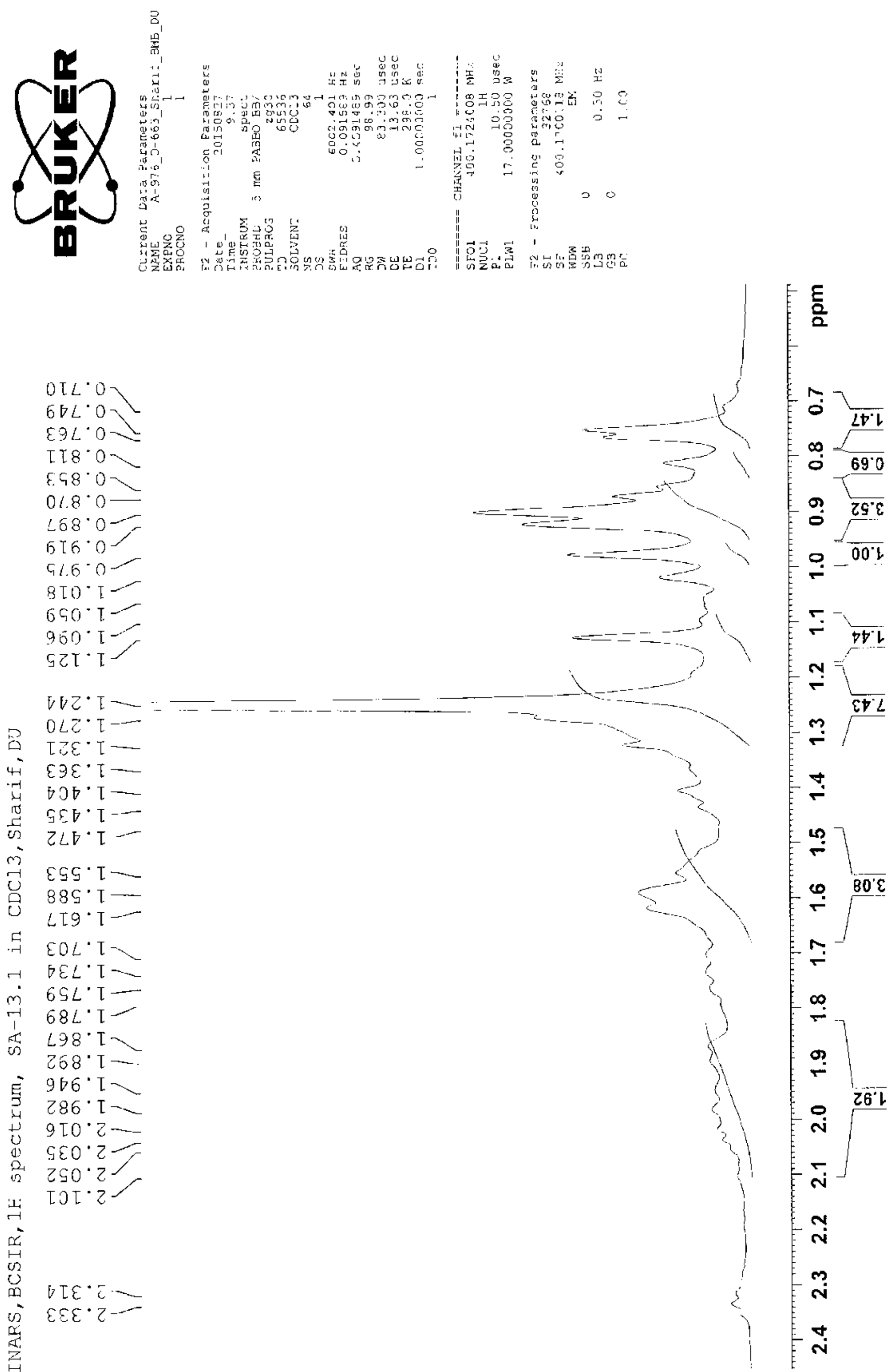
Based oncomparisons with published data (33, 34), the purified compound was identified as (3β)-3-hydroxyolean-12-en-28-oic acid (oleanolic acid, OA). The structure was confirmed by the signal at 2.04 attributable to CH3 of the acetoxy group in the lH-NMR spectrum. The deshielding observed for H-3 from 3.21 to 4.49 ppm confirms its assignment due to the anisotropic field of the acetate carbonyl.

Table 4.4: Comparison between ^{1}H NMR spectral data of SA-13.1 (400 MHz) and oleanolic acid, [33, 34] in $CDCl_3$.

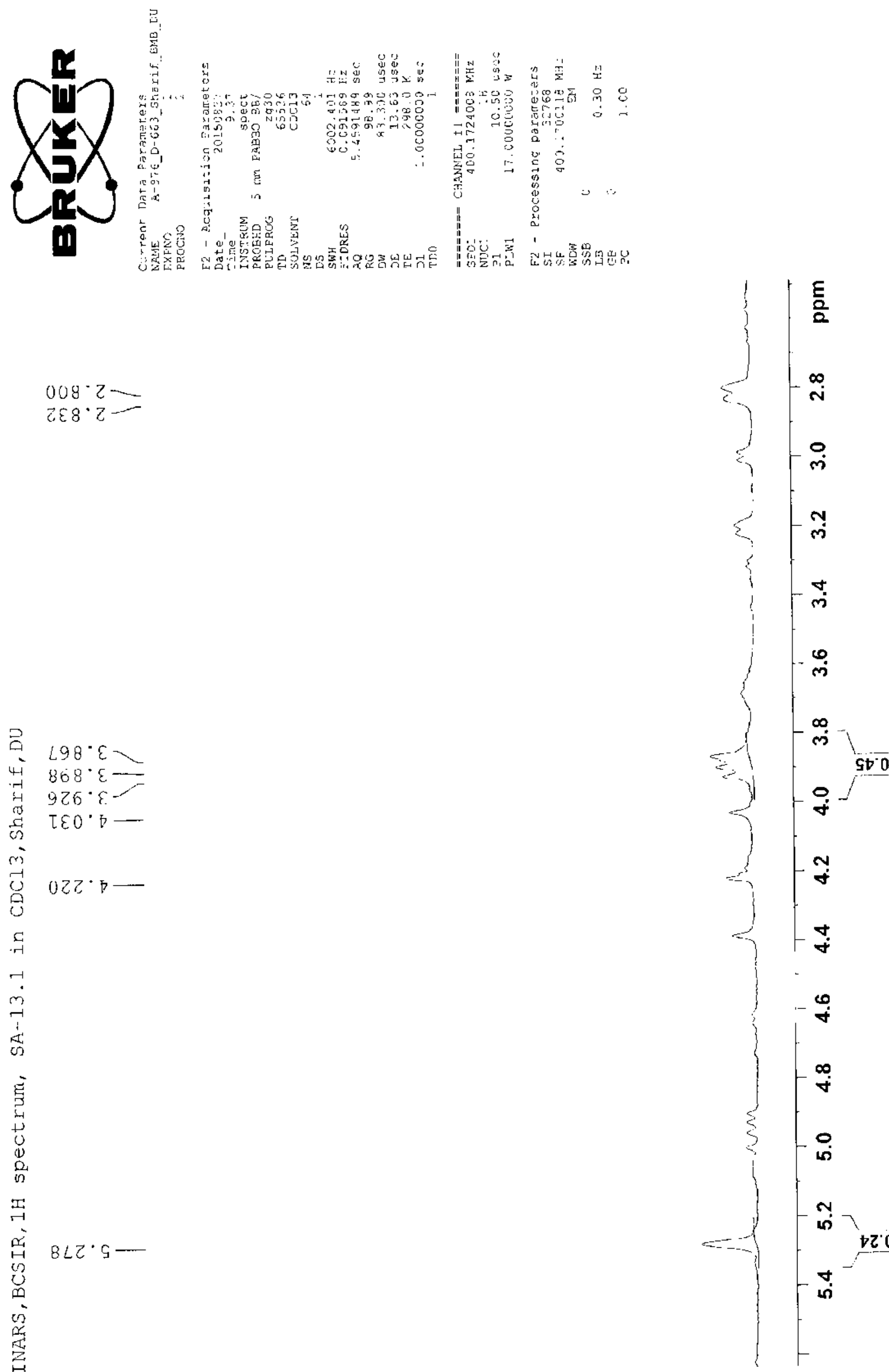
Positions	**δ_H in ppm in $CDCl_3$**	
	SA-13.1	**Oleanolic acid [33, 34]**
H-12	5.27 (1H, m)	5.27 (1H, m, H-12)
	3.20 (1H, dd, J= 14.0, 6.0 Hz)	3.25 (1H, dd, *J* = 15.0, 5.9 Hz)
	2.80 (1H, dd, J=12.8, 5.4)	2.81 (1H, dd, *J* = 12.3, 5.5 Hz)
	1.24 (3H, s)	1.13 (3H, s)
	1.059 (3H, s)	1.06 (3H, s)
	0.975 (3H, s)	0.98 (3H, s)
	0.919 (3H, s)	0.92 (3H, s)
	0.897 (3H, s)	0.90 (3H, s)
	0.763 (3H, s)	0.77 (3H, s)
	0.749 (3H, s)	0.74 (3H, s)



NMR signal 4.17: ^{1}H NMR signals of SA-13.1



NMR signal 4.18: ^{1}H NMR signals of SA-13.1



NMR signal 4.19: ^{1}H NMR signals of SA-13.1

4.5 Characterization of SA-13.2 as β-amyrin

The chemical structure of β-amyrin is (3β-hydroxy-olean-12-en-3-ol).β-amyrinis a white crystalline powder**,** molecular formula is $C_{30}H_{50}O$**,** molecular weight is 426.73**,** and melting point is 197°C. Also known β-Amyrenol, amyrin, 3-β-hydroxyolean-12-ene, (3-β) - olean-12-en-3-ol. Its melting point is 197-187.5 °C, molar mass is 426.73 g·mol^{-1} and chemical formula is $C_{30}H_{50}O$.

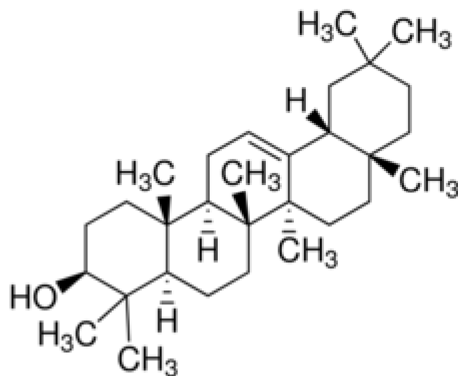
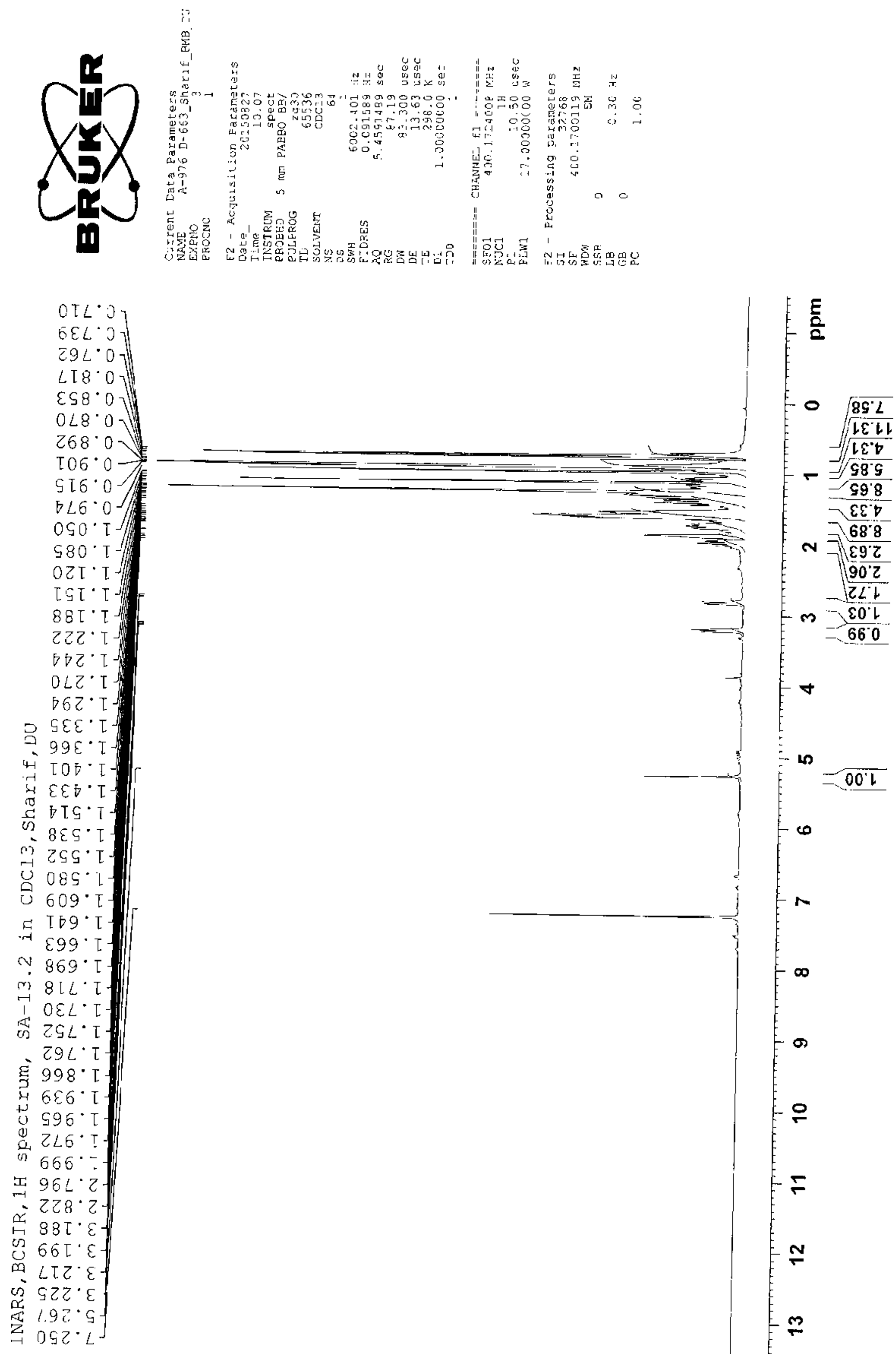


Figure 4.6: Structure of β amyrin

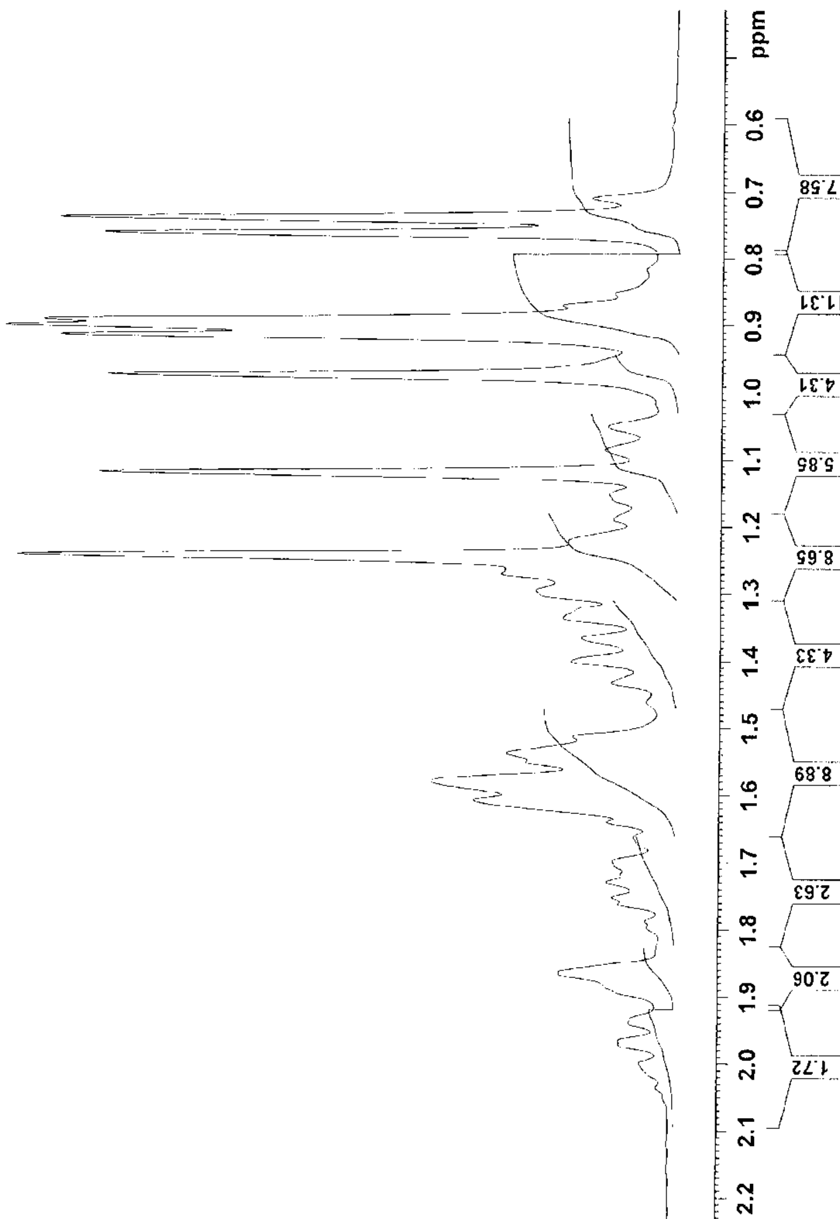
Table 4.5: ^{1}H NMR Spectral Data of β-amyrin

Position	**δ_H in ppm in $CDCl_3$**	
	SA- 13.2	**β- Amyrin [35]**
1	--	--
2	--	--
3	3.18 (dd, J= 4.4; 10.8 Hz)	3.15(dd,J=4.4;10.8 Hz)
4	--	--
5	0.71 (d, J=10.0)	0.68(d,J=11.0)
6	--	--
7	--	--
8	--	--
9	--	--
10	--	--
11	--	--
12	5.12 (t, J=3.2)	5.12(t,J=3.2)
13	--	--

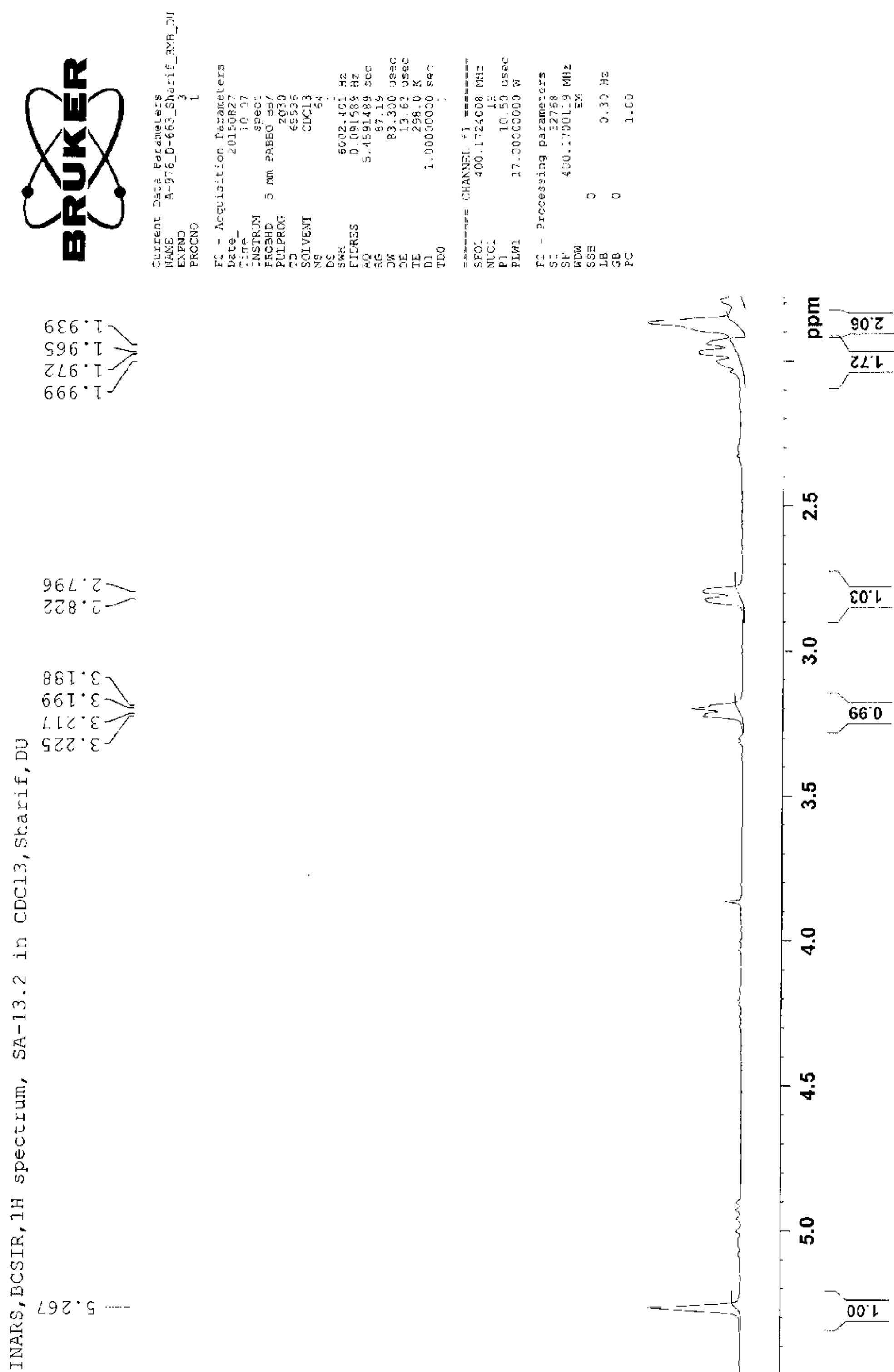
14	--	--
15	1.91 (td,J=4.0;14.0Hδ)	1.89 (td, J=4.0;14.0 Hδ)
16	1.70 (td, J=4.0;13.0Hβ)	1.70 (td, J=4.3;13.5 Hβ)
17	--	--
18	--	--
19	1.93(dd, J=4.0;13.0Hβ)	1.93(dd,J=4.0;13.7Hβ)
20	--	--
21	--	--
22	1.797 m	1.80m
23	0.762 s	0.77s
24	0.901 s	0.90s
25	0.739 s	0.73s
26	0.915 s	0.93s
27	1.188 s	1.19s
28	1.12 s	1.07s
29	0.892 s	0.87s
30	0.762 s	0.80s



NMR signal 4.20: ^{1}H NMR signals of SA-13.2



NMR signal 4.21: ^{1}H NMR signals of SA-13.2



NMR signal 4.22: ^{1}H NMR signals of SA-13.2

4.6 Characterization of SA-29.30 as Gossypetin 7-O-Rhamnopyranoside (Rhodiolgin) [36]

Its' Chemical formula is $C_{21}H_{20}O_{10}$. It is also known as Rhodiolgin;Gossypetin 7-rhamnoside;Gossypetin-7-O-α-rhaMnopyranoside;Gossypetin-7-O-L-rhamnopyranoside;7-[(6-Deoxy-alpha-L-mannopyranosyl)oxy]-2-(3,4-dihydroxyphenyl)-3,5,8-trihydroxy-4H-1-benzopyran-4-one.

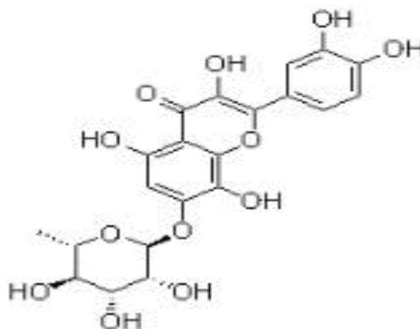
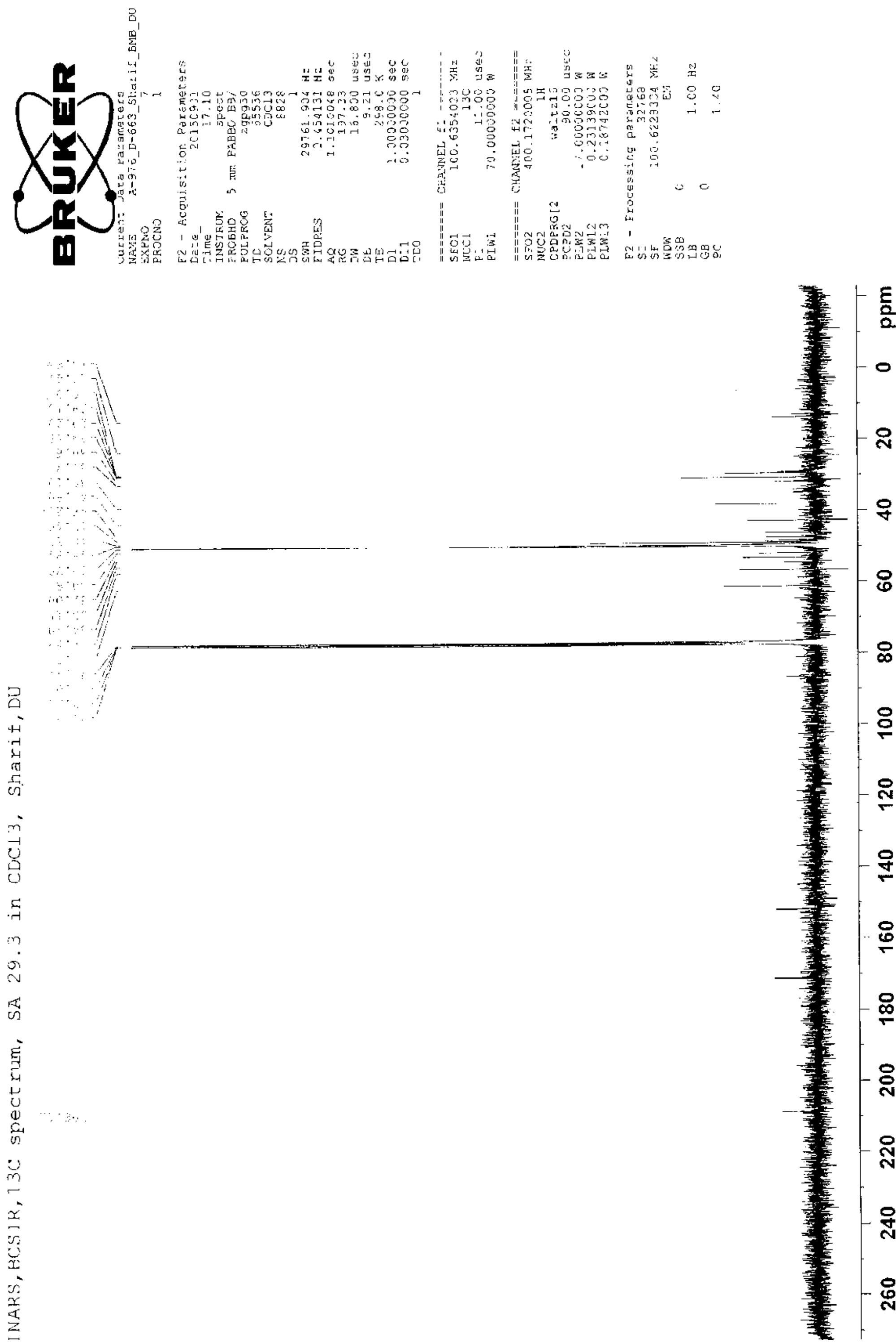


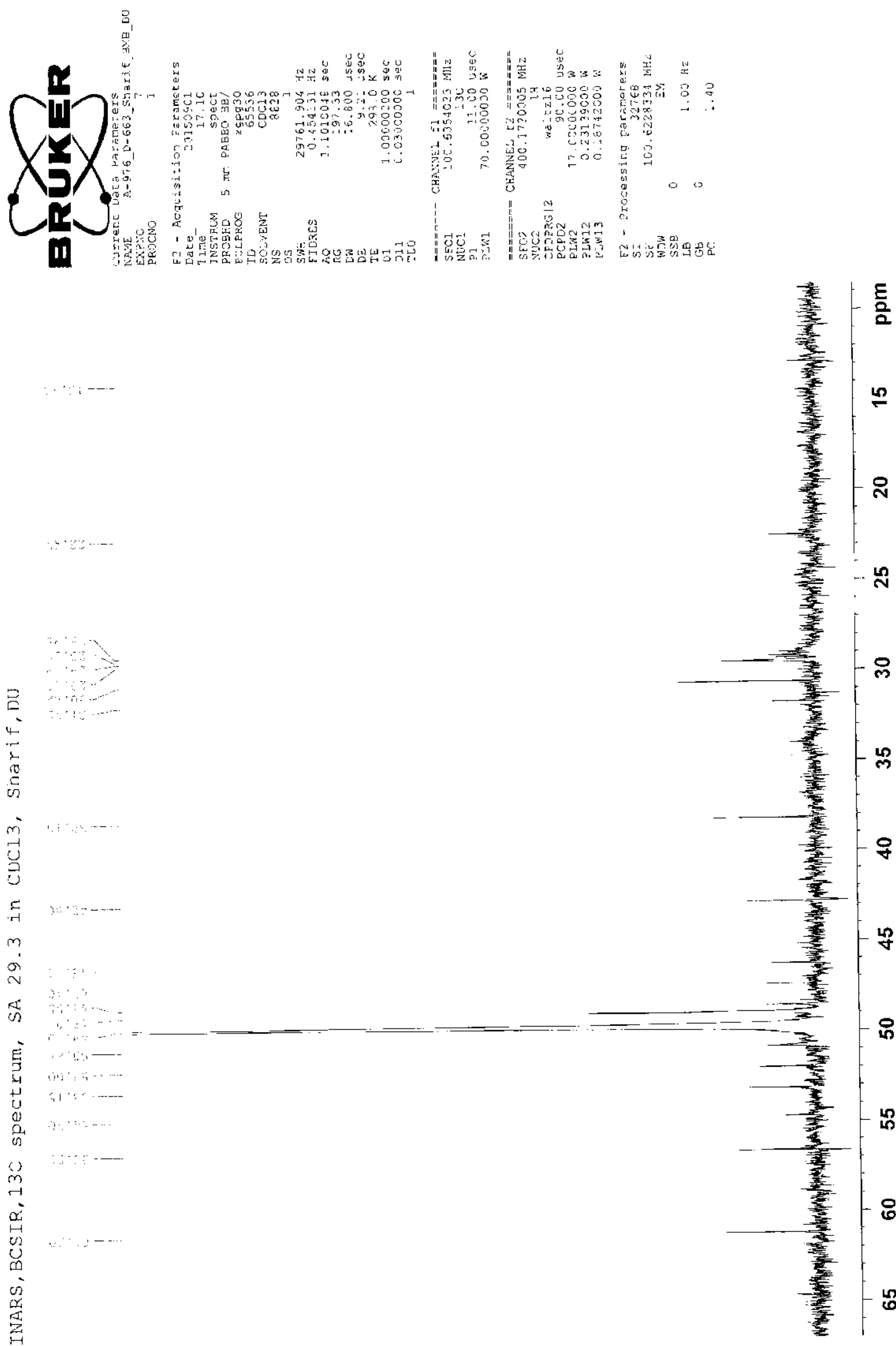
Figure 4.7: Rhodiolgin

Table 4.6: Comparison between ^{1}H NMR spectral data of SA-13.1 (400 MHz) and oleanolic acid, [33, 34] in $CDCl_3$

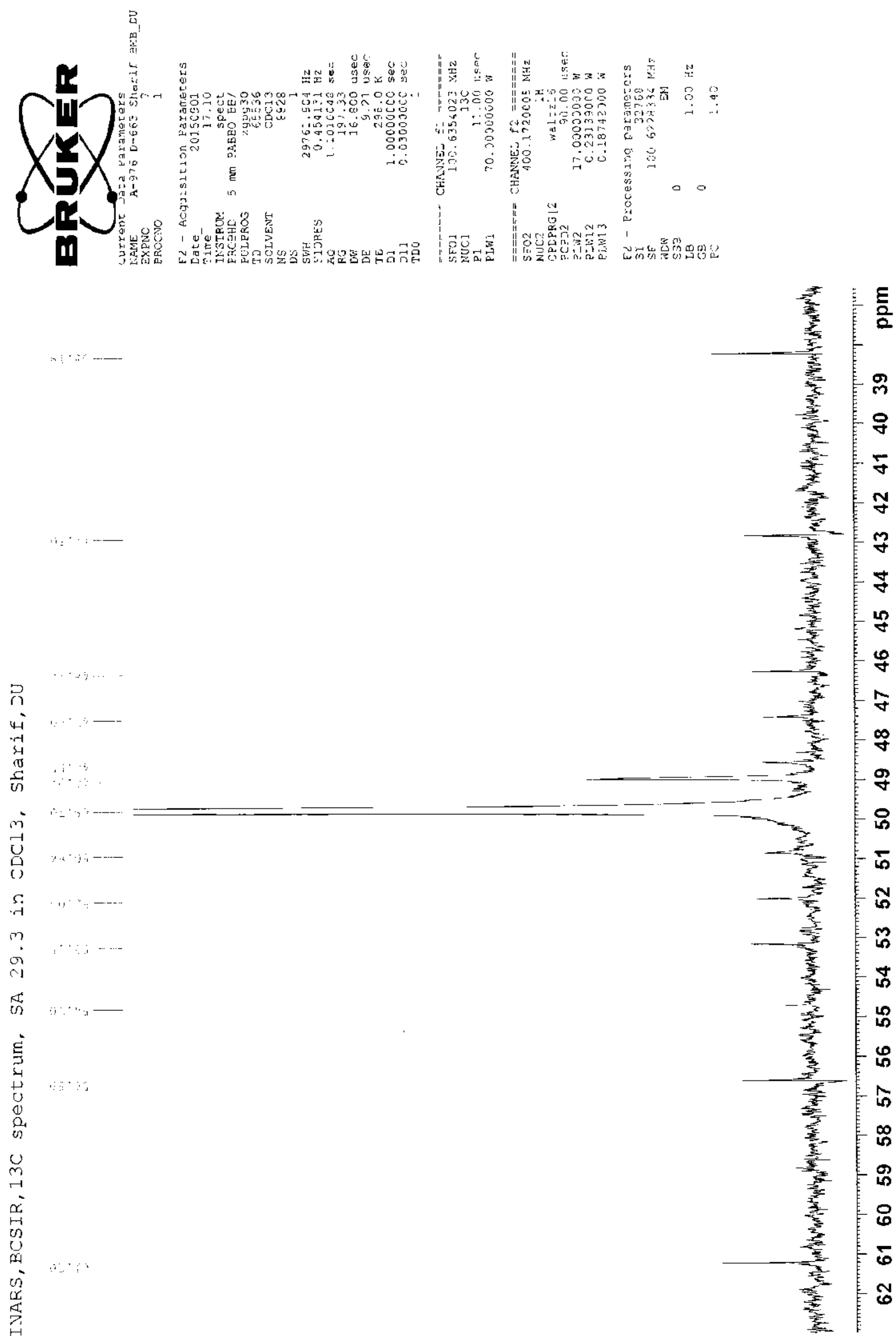
Position	Chemical shift (δ) in ppm	
	SA- 29.30	**^{13}C NMR [36]**
C=O	208	--
COOR Carboxylic ester & Lactone	170.90	170.961
Ar–C–O– Heteroaromatic compound	153.00	153.160
– CH_2–	61.20	60.139
	53.15	53.913
	52.00	52.055
	50.84	50.198
	49.70	49.746
= CH–	48.94	48.823
	48.55	47.880
	47.40	47.458
	46.24	46.988
	42.80	43.760
	38.19	38.767
– CH–	22.47	20.506
– CH_2–	13.83	13.97



NMR signal 4.23: ^{13}C NMR signals of SA-29.30



NMR signal 4.24: ^{13}C NMR signals of SA-29.30



NMR signal 4.25: ^{13}C NMR signals of SA-29.30

Chapter 5

BIOLOGICAL INVESTIGATION

5.1 Antimicrobial activity

5.2 Antioxidant activity

5.3 Brine Shrimp Lethality Bioassay

5.4 Analgesic activity

5.5 Thrombolytic activity

5.6 Anesthetic activity

5.7 Antidiarrhoeal activity

5.8 Antiemetic activity

5.9 Anti-helminthic activity

5.10 Antipyretic activity

Chapter 5.1

Antimicrobial Activity

5.1.1 Introduction of antimicrobial screening

The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. Recently there has been increased dialogue related to natural antimicrobials as topical actives & preservatives in the personal care industry. Synthetic compounds long accepted as effective in controlling microbial growth have come under scientific & regulatory scrutiny. These efforts are mainly driven by safety & environmental concerns, & the increased incidence of antibiotic resistant microbial strains. Natural alternatives derived from botanicals are therefore being explored by researchers around the world. Multi-functionality is an additional advantage of natural extracts. Several of them offer anti-inflammatory, immunological and wound healing support as well. According to World Health Organization medicinal plants would be the best source to obtain a variety of drugs developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiently [37]. Resistance of pathogens to antimicrobial compounds has lethal effects as the development of drug resistance outpaces the development of new drugs. Infectious diseases, a leading cause of untimely death worldwide, have become a global concern. The clinical effectiveness of many existing antibiotics is being threatened by rapid emergence of multidrug resistant pathogens. Many infectious diseases have been identified to be treated with herbal products throughout the history of mankind. Natural products provide enormous opportunities for the development of new drugs, especially antimicrobials, which can have therapeutic potential to treat infectious diseases [38]. There is a continuous need to discover new antimicrobial compounds with suitable chemical structures and novel mode of actions against pathogens. Antimicrobial compounds of plant origin have an enormous therapeutic potential to treat many infectious diseases [39].

Bacteria and fungi are responsible for many infectious diseases. The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. Essential oils have been known for centuries for their biological activities and have been widely evaluated against various biological targets [40] such as bactericidal, virucidal, fungicidal, antiparasitic, insecticidal, anticancer agents, cholesterol lowering agents, cosmetics and other pharmaceutical applications. These antimicrobial activities of essential oils have been resulted after screening of a wide range of plant species. Essential oils can be a valuable source to explore their antibacterial properties against multidrug resistant human pathogens. The essential oils have already proved to exert strong synergistic effects when used in combination with less effective antibiotics. Mostly plant derived essential oils consist of chemical components such as terpenoids including monoterpenes, sesquiterpenes and their oxygenated derivatives. These compounds have the ability to easily diffuse across cell membrane to induce biological reactions. Chemical analysis has shown that the composition of essential oils

from different plant species varies significantly. Even within the same species, plants belonging to different geographical sources, the composition of essential oils can differ reasonably [41]. Clove buds essential oil induced antimicrobial activity due to high level of eugenol and eucalyptol components [42]. Eugenol (4-allyl-2-methoxy phenol), in addition to clove also present in cinnamon oil is active against fungi, viruses and many pathogenic bacteria such as *E. coli, Listeria monocytogenes, Campylobacter jejuni, S enterica, S aureus, Lactobacillus sakei,* and *Helicobacter pylori* [42].

Plants are the natural reservoir of many antimicrobial agents. In recent times, traditional medicine as an alternative form of health care and to overcome microbial resistance has led the researchers to investigate the antimicrobial activity of medicinal plants.

The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth. This ability may be estimated by any of the following three methods:

1. Disc diffusion method
2. Serial dilution method
3. Bioautographic method

But there is no standardized method for expressing the results of antimicrobial screening. Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz. the extraction methods, inoculums volume, culture medium composition, P^H and incubation temperature can influence the results. Among the above mentioned techniques the disc diffusion method [43] is a widely accepted in vitro investigation for preliminary screening of test agents which may possess antimicrobial activity.

Determination of antibacterial activity

It is essentially a quantitative test indicating the sensitivity or resistance of the microorganism to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method.

5.1.2 Experimental design

Kirby-Bauer antibiotic testing (KB testing or disk diffusion antibiotic sensitivity testing) is a test, which uses antibiotic-impregnated wafers to test whether particular bacteriaare susceptible to specific antibiotics. Known quantities of bacteria are grown on agar plates in the presence of thin wafers containing relevant antibiotics. Density of each microbial suspension was adjusted equal to that of 10^8 CFU/ ml (standardized by 0.5 McFarland standards) and used as the inoculum for performing agar well diffusion assay. The suspension was then diluted 1:100 in Mueller-Hinton

broth to get 10^6 CFU/ml. If the bacteria are susceptible to a particular antibiotic, an area of clearing surrounds the wafer where bacteria are not capable of growing (called a zone of inhibition).

The bacteria in question are swabbed uniformly across a culture plate. A filter-paper disk, impregnated with the compound to be tested, is then placed on the surface of the agar. The compound diffuses from the filter paper into the agar. The concentration of the compound will be highest next to the disk, and will decrease as distance from the disk increases. If the compound is effective against bacteria at a certain concentration, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. This is the zone of inhibition. This along with the rate of antibiotic diffusion is used to estimate the bacteria's sensitivity to that particular antibiotic. In general, larger zones correlate with smaller minimum inhibitory concentration (MIC) of antibiotic for those bacteria. Inhibition produced by the test is compared with that produced by known concentration of a reference compound. This information can be used to choose appropriate antibiotics to combat a particular infection.

5.1.3 Apparatus and reagents

Filter paper discs, Nutrient agar medium, Petri dishes, Sterile cotton, Micropipette, Incubating loop, Sterile forceps, Screw cap tube, Autoclave, Laminar air flow hood, Spirit burner, Refrigerator, Incubator, Chloroform and methanol, Nose mask and hand gloves.

5.1.4 Preparation of Mcfarland standard:

0.05 mL (50 µl) of 0.048 M $BaCl_2$ (1.17% W/V $BaCl_2$ in H_2O) was added to 9.95 mL of 0.18 M H_2SO_4 (1% V/V) in a test tube with constant stirring. The tube was then sealed tightly to prevent loss of evaporation. The standard may be stored for six months.

5.1.5 Test samples

Table 5.1.1: List of test samples

Plant part	sample code	Test sample
Extracts and soluble fractions of *S. aromaticum*	Crude, SA-1,2,3,4,5,6,7,8,9, 10,11,12,13,14,15,16,17,18,19,20, 21,22,23,24,25,26,27, 28,29,30	
	PESF	Petroleum Ether Soluble Fraction
	EASF	Ethyl Acetate Soluble Fraction
	MSF	Methanolic Fraction
	AQF	Aqueous Fraction

5.1.6 Test organisms

The bacterial and fungal strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Sciences (INFS), University of Dhaka. Both gram positive and gram negative organisms were taken for the test and they are listed in the table 4.2.

Table5.1.2: List of test organisms

Gram positive Bacteria	Gram negative Bacteria	Fungi
Bacillus cereus	*Vibrio mimicus*	*Candida albicans*
Staphylococcus aureus	*Vibrio cholera*	*Aspergillus niger*
Bacillus subtilis	*Salmonela typhi*	*Saccharromyces cervisieae*
Bacillus megaterium	*Shigella boydii*	*Trichophyton sp.*
Sarcina lutea	*Acenetobacter sp.*	*Aspergillus flavus*
Lactobacillus sp.	*Shigella dysentereae*	
S. mutans	*Pseudomonus sp.*	
S. salivarius	*Klebsiella sp.*	
S. epidermidis	*Escherchia coli*	
Micrococcus sp.	*Vibrio parahaemolyticus*	
L. monocyogenes	*S.paratyphi*	
	P. aruginosa	

5.1.7 Composition of culture media

The following media was used normally to demonstrate the antimicrobial activity and to make subculture of the test organism.

Table 5.1.3: Composition of Nutrient agar media

Ingredients	Amount
Peptone	1.0g
Sodium Chloride(NaCl)	0.5g
Yeast extract	0.5g
Agar	1.5g
Distilled water	100.0mL
p^H	7.4 at 25°C

Table 5.1.4: Composition of Nutrient broth media

Ingredients	Amount
Beef extract	**0**.3g
Peptone	0.5g
Distilled water	100.0mL
p^H	7.4 at 25°C

5.1.8 Preparation of the 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 water bath to make a clean solution. The P^H (at 25°C) was adjusted at 7.2-7.6 using NaOH or HCl. 10.0mL and 5.0mL of the medium was then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15 lbs. pressure at 121°C for 20 minutes. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

5.1.9 Sterilization procedure

In order to avoid any type of contamination and cross contamination by the test organism 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. Petri dishes and other glass wares were sterilized by autoclaving at a temperature of 121°C and a pressure of 15 lbs/sq. inch for 20minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized by UV light.

5.1.10 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°C for their optimum growth. These fresh cultures were used for the sensitivity test.

5.1.11 Preparation of the test plate

The test organisms were transferred from the subculture to the test tubes containing about 10.0 mL of melted and 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 bacterial and fungal suspension was immediately transferred to the sterilized petridish. The petridish were rotated several times clockwise and anticlockwise to assure homogenous distribution of the organisms in the media.

5.1.12 Preparation of the discs

Measured amount of each test sample (specified in table) was dissolved in specific volume of solvent (chloroform or methanol) to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBl, Cockville, USA) filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test sample and dried.

Standard ciprofloxacin (10.0 µg/disc) discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by test sample. Blank discs were used as

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

5.1.13 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 plate's pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at $40^{o}C$ for about 24 hour's upside-down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at $37^{o}C$ for 24 hours. The crude extract, VLC fraction of *S. aromaticum*, ethyl acetate, petroleum ether and methanol and aqueous soluble fractions were used in this investigation.

5.1.14 Result and discussion of in vitro antibacterial screening of different test samples of *S. aromaticum*

The VLC extract of plant *S. aromaticum* and different partitionates i.e. petroleum ether soluble fractions (PESF), ethyl acetate soluble fraction (EASF), methanol partitionates of the crude extract of plant stem of *S. aromaticum* were subjected to antimicrobial screening. The zone of inhibition expressed as mean ± Standard deviation (SD).

Table 5.1.5:- Antibacterial activity of test samples against gram positive bacteria

Test Microorganism (Gram Positive Bacteria)	**Mean diameter of zone of inhibition (mm) ± Standard Deviation (SD)**									
	Ciprofloxacine	**SA-1**	**SA-3**	**SA-6**	**SA-7**	**SA-10**	**SA-11**	**SA-12**	**Crude Extract**	**PESF**
Bacillus cereus	18±0.1	22±0.12	24±0.11	14±	12±	--	--	--	20±0.22	15±0.21
S. aureus	19±0.13	14±0.19	18±0.21	16±0.13	12±0.21	--	--	--	16±0.11	20±0.16
B. subtilis	18±1.0	20±0.1	18±0.2	18±0.1	8±0.1	--	--	--	12±0.1	16±0.3
B. megaterium	15± 0.11	12±0.21	12±0.23	12±0.18	10±0.11	--	--	--	14±0.16	12±0.21
Sarcina lutea	20± 0.11	24±0.12	18±0.43	18±0.15	10±0.22	8±0.22	--	--	16±0.21	20±0.11
Lactobacillus sp.	14± 0.31	18±0.33	17±0.10	16±0.11	18±0.26	--	--	--	13±0.31	13±0.43

Streptococcus mutans	15± 0.53	13±0.26	14±0.22	13±0.41	13±0.42	--	--	--	10±0.44	13±0.56
Streptococcus salivarius	10± 0.86	9±0.16	10±0.19	9±0.55	9±0.25	--	--	--	11±0.7	13±0.47
Micrococus sp.	11± 0.55	17±0.55	16±0.5	16±0.36	16±0.7	--	--	--	18±0.88	17±0.78
S. epidermidis	11± 0.59	15±0.87	14±0.41	13±0.22	13±0.6	--	--	--	18±0.34	20±0.18
Listeria monocytogenes	14±0.66	20±0.1	19±0.44	18±0.66	18±0.77	--	--	--	18±0.41	20±0.66

Table 5.1.6:- Antibacterial activity of test samples against gram negative bacteria

Test Microorganism (Gram Negative Bacteria)	**Diameter of zone of inhibition (mm)**									
	Ciprofloxacine	**SA-1**	**SA-3**	**SA-6**	**SA-7**	**SA-10**	**SA-11**	**SA-12**	**Crude Extract**	**PESF**
Vibrio minicus	**19±0.22**	**18±0.31**	**16±0.09**	**20±0.17**	**--**	**--**	**--**	**--**	**14±0.33**	**15±0.11**
V. cholarae	**21±0.10**	**22±0.44**	**21±0.11**	**22±0.23**	**20±0.45**	**--**	**--**	**--**	**18±0.43**	**23±0.11**
Salmonella typhi	**20±0.13**	**16±0.91**	**16±0.11**	**12±0.4**	**--**	**--**	**--**	**--**	**12±0.77**	**16±0.27**
S. paratyphi	**20±0.41**	**18±0.22**	**18±0.19**	**18±0.33**	**12±0.54**	**8±0.19**	**--**	**8±0.11**	**20±0.14**	**16±0.19**
Shigella boydii	**22±0.10**	**16±0.21**	**16±0.14**	**14±0.33**	**12±0.19**	**--**	**--**	**--**	**14±0.42**	**15±0.23**

S. dysentareae	**18±0.22**	**18±0.44**	**20±0.35**	**16±0.11**	**12±0.44**	**--**	**--**	**8±0.49**	**14±0.55**	**22±0.56**
Pseudomonas sp.	**17±0.11**	**20±0.47**	**18±0.77**	**24±0.66**	**12±0.37**	**8±0.66**	**--**	**--**	**20±0.44**	**15±0.13**
Klebsiella sp.	**15±0.33**	**15±0.55**	**13±0.55**	**14±0.54**	**13±0.22**	**--**	**--**	**--**	**12±0.88**	**16±0.45**
E.coli	**18±0.46**	**14±0.88**	**18±0.57**	**12±0.98**	**8±0.11**	**--**	**--**	**--**	**16±0.92**	**16±0.74**
P. aruginosa	**12±0.45**	**10±0.28**	**11±0.20**	**11±0.90**	**12±0.66**	**--**	**--**	**--**	**14±0.91**	**11±0.55**
Acenatobacter sp.	**--**	**7±0.11**	**7±0.86**	**6±0.81**	**6±0.61**	**--**	**--**	**--**	**18±0.91**	**20±0.4**
V. parahaemolyticus	**--**	**16±0.31**	**18±0.65**	**14±0.37**	**--**	**--**	**--**	**--**	**12±0.1**	**16±0.9**

All bacteria were resistant to SA-10, SA-11 & SA-12. *Bacillus cereus, B. subtilis, Sarcina lutea, Lactobacillus sp. Micrococus sp. S. epidermidis, V. cholarae, S. dysentarae, Klebsiella sp.* and *Listeria monocytogenes* were sensitive against SA-1.*Bacillus cereus, Micrococus sp. S. epidermidis, S. salivarius, V. cholarae, S. dysentarae, Pseudomonas sp. E. coli* and *Listeria monocytogenes* were sensitive against SA-3. *Bacillus subtilis, Micrococus sp. S. epidermidis, Lactobacillus sp. S. salivarius, V. cholarae, S. dysentarae, Pseudomonas sp. E. coli* and *Listeria monocytogenes* were sensitive against SA-3. *Micrococus sp. S. epidermidis, Lactobacillus sp., V. cholarae, V. minicus, Pseudomonas sp.*and *Listeria monocytogenes* were sensitive against SA-7. Bacillus *cereus, Micrococus sp. S. epidermidis, S. paratyphi, P. aruginosa, S. salivarius* and *Listeria monocytogenes* were sensitive against Crude extract. *S. aureus, S. lutea, Micrococus sp. S. epidermidis, Pseudomonas sp. P. aruginosa* and *Listeria monocytogenes*were sensitive

against PESF. EASF, MSF and AQF were also tested for antibacterial activity but since those samples showed no activity are not included in the table.

Determination of antifungal activity

5.1.15 Introduction

The presence and growth of fungi in food may cause spoilage and result in a reduction in quality and quantity. Some *Aspergillus* species are responsible for many cases of food and feed contamination [44]. *Aspergillus Xavus* and *Aspergillus parasiticus* are able to produce aflatoxins in food and feedstuffs [45]. Aflatoxins are known to be potent hepatocarcinogens in animals and humans [45]. Therefore, the presence of toxigenic fungi and mycotoxins in foods and grains stored for long periods of time presents a potential hazard to human and animal health. Considerable interest has developed on the preservation of foods by the use of essential oils to effectively retard growth and mycotoxin production.

During the last few years, due to the increasing development of drug resistance to antifungal agents in human dermatophytic fungi, demand for searching novel antifungal agents is being increased. The plant essential oils as natural substances could represent a potential source of new antifungal agent. The antifungal activity of *S. aromaticum* extracts were performed by using the disc diffusion method. The testing procedure was the same as that for antibacterial activity test. The only difference was that the period of incubation were 48 hours at room temperature.

5.1.16 Materials and Methods:

Candida albicans
Aspergillus niger
S. cerevisieae
Trichophyton sp.
Aspergillus flavus
Rhyzopus nigricans
Candida albicans

Table 5.1.7: Ingredient of PDA media

Ingredients	Amount
Potato	20g
Distilled water	100 ml
Glucose	2g
pH	5.6
Agar	2g

5.1.17 Preparation of media

100g sliced potatoes were boiled for about 30 minutes with 200ml of distilled water. The extract

was then cooled, decanted and filtered. 10 g dextrose was dissolved in 100ml of distilled water and soluble filter starch was dissolved to it. The P^H of this medium was adjusted to 5.6 then 4g agar was added and was adjusted the volume to 200ml by adding distilled water and sterilized by using an autoclave. Preparation of test plates, preparation of discs, and preparation of test samples placement of disc diffusion and incubation processes were same as the antibacterial screening.

Inocula were prepared according to Shehata et al., method [46]. For dermatophytes, inocula were prepared by counting conidia harvested from ~7-day-old potato dextrose agar cultures in a haemocytometer, followed by adjustment to the appropriate final density. Results were read after 48 h incubations for Candida and Aspergillus species and 4 day incubations for dermatophytes. Microbial tests were carried out every 48 h on PDA medium. The plates were incubated at 25 °C for 3–5 days. Grisofulvin was used as potive control and discs soaked with chloroform and then dried were used as negative control.

5.1.18 Results and Discussion

Antifungal action

Table 5.1.8: Antifungal activity of clove crude and fractions

Test Fungus	**Diameter of zone of inhibition (mm)**									
	Grisofulvin	**SA-1**	**SA-3**	**SA-6**	**SA-7**	**SA-10**	**SA-11**	**SA-12**	**Crude Extract**	**PESF**
Candida albicans	12±0.33	22±0.27	16±0.44	24±0.11	18±0.19	12±0.8	10±0.8	8±0.71	16±0.77	20±0.2
Aspergillus niger	12±0.10	18±0.44	16±0.19	16±0.12	8±0.1	--	--	--	18±0.11	16±0.1
S. cerevisiae	8±0.10	16±0.68	18±0.10	14±0.54	10±0.1	--	--	--	12±0.2	15±0.13
Trichophyton sp.	7±0.10	20±0.81	19±0.30	5±0.7	--	--	--	8±0.41	21±0.24	19±0.18
Aspergillus flavus	6±0.10	10±0.66	10±0.17	9±0.2	--	--	--	--	10±0.3	12±0.19
Rhyzopus nigricans	8.9±0.10	7±0.11	7±0.33	6±0.22	4±0.18	--	--	8±0.33	8±0.22	7±0.18

5.1.19 Antifungal activity of the crude extract and fractions

The crude extracts and 30 VLC fractions were investigated against fungi. This crude extract was used in concentration 3µg /disc and the activity observed is list above in table. The chloroform

extract and its fraction 1, 3, 6, crude and PESF have shown promising zone of inhibition against all the fungi, fraction 7 has shown promising zone of inhibition against only to *Trichophyton sp. Aspergillus flavus*. Fraction 10, 11 have shown promising zone of inhibition against *Candida albicans* fungi, fraction 12 have shown promising zone of inhibition against only to *Trichophyton sp, Aspergillus flavus* fungi. The Grisofulvin showed antifungal activity.

5.1.20 Conclusion

The chloroform extract was tested for antibacterial and antifungal activity against a number of Gram positive and Gram negative bacteria as well as some fungi. Standard disc of ciprofloxacin (10μg/disc) was used for comparison purpose. From the above result, it can be reported that the crude extracts of *S. aromaticum* has antimicrobial activity and it can be concluded that further investigation on this plant may contribute to the field of medicine. Compounds also showed better antibacterial and antifungal activity against different type of bacteria and fungi.

The large spectrum of activity of clove oil and eugenol in acting on *Candida, Aspergillus* and dermatophytes, the fungicidal effects observed is due to the impairment in the biosynthesis of ergosterol all agree with the mechanism of action proposed: lesion of the cytoplasmic membrane. Cox [47] proposed that different modes of action are involved in the antimicrobial activity of essential oils. The activity may, in part, be due to their hydrophobicity, responsible for their partition into the lipid bilayer of the cell membrane, leading to an alteration of permeability and a consequent leakage of cell contents. In bacteria, permeabilization of the membranes is associated with ion loss and reduction of membrane potential, collapse of the proton pump and depletion of the ATP pool. Recent investigations on the antimicrobial action of some EOs showed disruption of the bacterial and fungal membrane [47]. All these reports suggest that this antimicrobial mechanism is due to membrane damage and our results further confirm this point of view. All of these data confirm that clove essential oil compromises the structural and functional integrity of cytoplasmic membranes.

In conclusion, the findings of the present study indicate that the clove oil from S. aromaticum has interesting potential as a therapeutic option against fungi that are pathogenic to humans. Clove oil is a broad-spectrum agent which inhibited not only dermatophytes, Aspergillus and Candida species (such as *C. albicans, C. tropicalis and C. parapsilosis*), but also fluconazole-resistant *C. albicans* isolates, *C. krusei*, which is intrinsically resistant to fluconazole, and *C. glabrata*, whose resistance is easily inducible.

Clove oil might be useful in the clinical management of candidosis, particularly mucocutaneous presentations such as vulvovaginal candidosis, considering its fungicidal activity and the inhibition of germ tube formation.The water extract of *Syzygium aromaticum* (clove) was reported to have antibacterial activity against *Vibrio cholera, Staphylococcus aureus* and *Shigella dysenteriae* [47]. But this study was done to determine the antimicrobial activity of chloroform and ethanol extract of clove against some multi-drug resistant (MDR) pathogenic

organism. Among 11 isolates of bacteria 100 % bacteria were resistant to chloroform extract of clove. All the isolates were sensitive to ethanol extract except *Shigella sonnei, Klebsiella sp.* and *Salmonella typhi*. The ethanol extract of clove was most effective against *Staphylococcus aureus, Shigella dysenteriae, Shigella boydii* and *Bacillus cereus. Vibrio cholera* showed sensitivity on this extract. Water extract of clove sensitivity in *Vibrio cholera* classical and *Vibrio cholerae* El Tor was reported by Hoque et al., (2005).

Chapter: 5.2

Antioxidant activity

5.2.1 Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated by many redox processes [48]. However, the excessive amounts of ROS and RNS can attack important biological molecules such as carbohydrates, proteins, lipids, DNA and RNA, which lead to cell death and tissue damage. The flower buds of *Syzygium aromaticum* (L.) Merr. & Perry. (Family Myrtaceae), commonly known as clove, is a well known food flavor and a popular remedy for dental disorders, respiratory disorders, headache and soar throat in traditional medicines of Australia, and Asian countries [49]. Clove buds comprise around 20% of volatile oil rich in eugenol. Clove oil has been listed as a 'Generally regarded as safe' substance by the United States Food and Drug Administration when administered at levels not exceeding 1500 ppm in all food categories [50]. Clove bud essential oil still remain a research priority due to their wide range of pharmacological and biological activities such as antioxidant [51], antibacterial, antifungicidal, antiviral, and anesthetic effects. However, these informations are still limited. In this study, the chemical composition and antioxidant activities of essential oil from the clove buds toward various oxidative stresses in vitro are investigated in order to provide a reference for the comprehensive development and utilization of clove buds.

The aim of this study is to investigate the DPPH radical scavenging, activities of clove fractions. In addition, the objective of the present study is to also clarify the antioxidant and radical scavenging mechanisms of clove oil. Furthermore, an important goal of this research is to investigate the in vitro antioxidative effects of clove oil as compare with commercial and standard antioxidants such as BHA, BHT, α-tocopherol and trolox commonly used by the food and pharmaceutical industry.

Many extensive studies on antioxidant agents and oxidative stress indicate their close relationship with aging [48]. External factors (cigarette smoking, excessive exercise and the environment) increasingly affect the cellular balance. The misbalance of excessive production of pro-oxidant agents or free radicals causes cell damage. Thus, the increase of reactive oxygen radical species (ROS, NO•, HO•, ROO•, O2•−) produced by mitochondria, the main organelle involved in the respiratory process, generates many structural changes to the primary metabolites that cause many human diseases. Diabetes [52], cardiovascular disease, carcinogenesis and Alzheimer's dementia [53] are related to ROS generation. On the other hand, free radical oxidation of the lipid components in food due to the chain reaction of lipid peroxidation is a major strategic problem for food manufacturers [54]. Thus, there is an urgent need for the development of novel antioxidant agents, of both natural and synthetic origins.

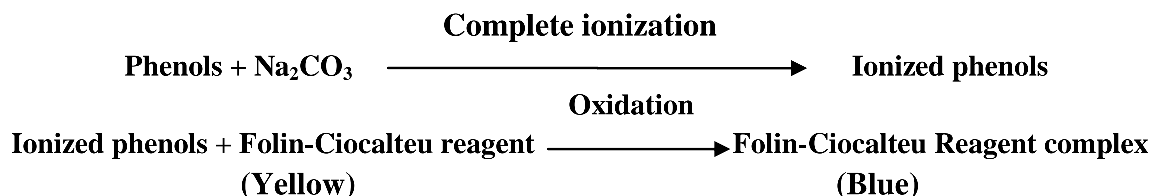
Natural products are emerging as the leading alternative to prevent some diseases produced by free radicals, either in humans, food and cosmetics. The best-known antioxidant agents are vitamin E (α-tocopherol), vitamin C (ascorbic acid) and several phenolic compounds found in food products. Plant or fruit extracts and essential oils (EO) also contain many different antioxidant molecules [54].

5.2.2 Assays for total phenolics

Plants synthesize compounds with biological activity, namely antioxidant, as secondary products, which are mainly phenolic compounds serving in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to avoid oxidative damage. Phenolics are secondary plant metabolites ranging from simple structures with one aromatic ring to complex polymers such as tannins and lignins [55]. The interests in phenolic compounds, particularly flavanoids and tannins have considerably increased in recent years because of their broad spectrum of chemical and diverse biological properties [55]. In addition to their antioxidant properties, these compounds have been reported to be potential candidates in lowering cardiovascular diseases [56] and anticarcinogenic activities [57], antiallergenic, anti-arthrogenic, anti-inflammatory, antimicrobial and antithrombotic effects [57]. Plant phenolics, in particular phenolic acids, tannins and flavonoids are known to be potent antioxidants and occur in vegetables, fruits, nuts, seeds, roots and barks [58]. In the case of phenolic compounds, the ability of the phenolics to act as antioxidants depends on the redox potential of their phenolic hydroxyl groups that allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they have a metal chelation potential.

5.2.3 Principle:

In the alkaline condition phenols ionize completely. When Folic-Ciocalteu reagent is used in this ionized phenolic solution the reagent will readily oxidize the phenols. Usual color of Folin-Ciocalteu reagent is yellow and after the oxidation process the solution becomes blue. The intensity of the color change is measured in a spectrophotometer at 760 nm. The absorbance value will reflect the total phenolic content of the compound.



5.2.4 Experimental method:

Total phenolic content of *S.aromaticum* extractives was measured employing the method as

described by Skerget *et al*., [59] involving Folin-Ciocalteu reagent as oxiding agent and gallic acid as standard (Majhenic *et al*., 2007) [60].

5.2.5 Sample preparation:

2 mg of the extractives was taken and dissolved in the distilled water toget a sample concentration of 2 mg/ml in every case. The sample along with their concentration for the total phenolic content measurement is given in the table:

Table 5.2.1: Test samples for total phenolic content determination

Plant part	Sample code	Test Sample	Calculated amount (mg/ml)
Buds of S.aromaticum	PESF	Petrolium Ether Soluble fraction	2.0
	MSF	Methanol soluble fraction	2.0
	EASF	Ethyl Acetate Soluble Fraction	2.0
	AQF	Aqueous fraction	2.0

5.2.6 Total phenolic compound analysis:

To 0.5 ml extract solution (conc. 2 mg/ml), 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of Na_2CO_3 (7.5 % w/v) solution were added. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 760 nm by UV-spectrophotometer and using the standard curve prepared from gallic acid solution with different concentration, the total phenols content of the sample was measured. The phenolic contents of the sample were expressed as mg of GAE (gallic acid equivalent) / g of the extract.

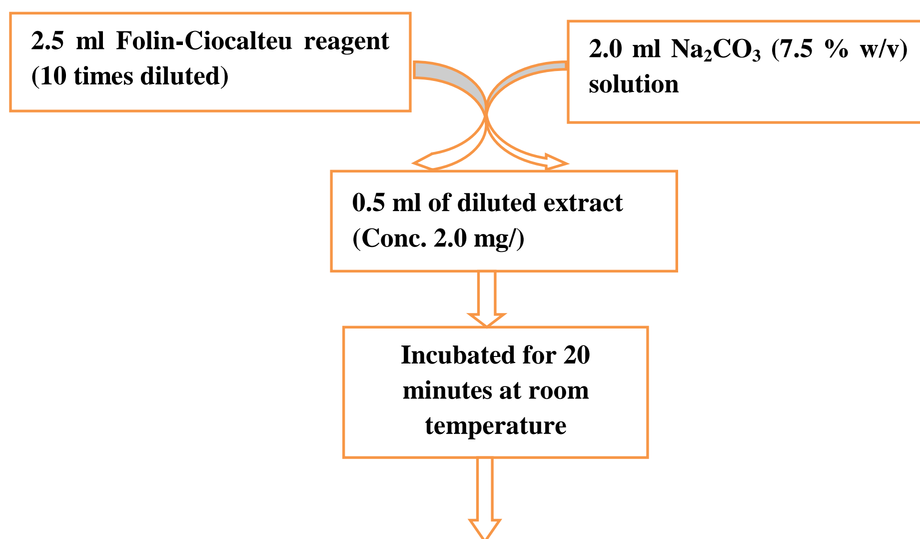


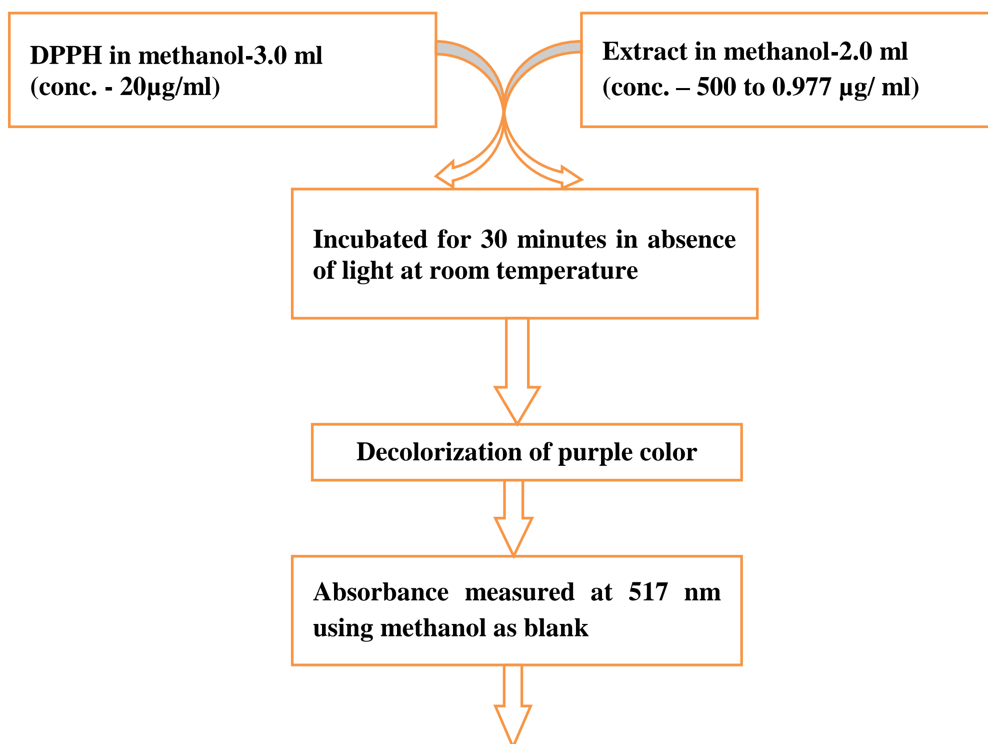
Figure 5.2.1: Schematic representation of the total phenolic content determination

5.2.7Antioxidant activity: DPPH assay

The free radical scavenging activities (antioxidant capacity) of the plant extracts on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were estimated by the method of Brand-Williams *et al*., [61].2.0 ml of a methanol solution of the extract at different concentration were mixed with 3.0 ml of a DPPH methanol solution (20µg/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 (BHT) and ascorbic acid (ASA) by a UV spectrophotometer.

5.2.8 Materials & methods:

DPPH was used to evaluate the free radical scavenging activity (antioxidant potential) of various compounds and medicinal plants.



Calculation of IC_{50} value from the graph plotted

Figure 5.2.2: Schematic representation of the method of assaying free radical scavenging activity

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

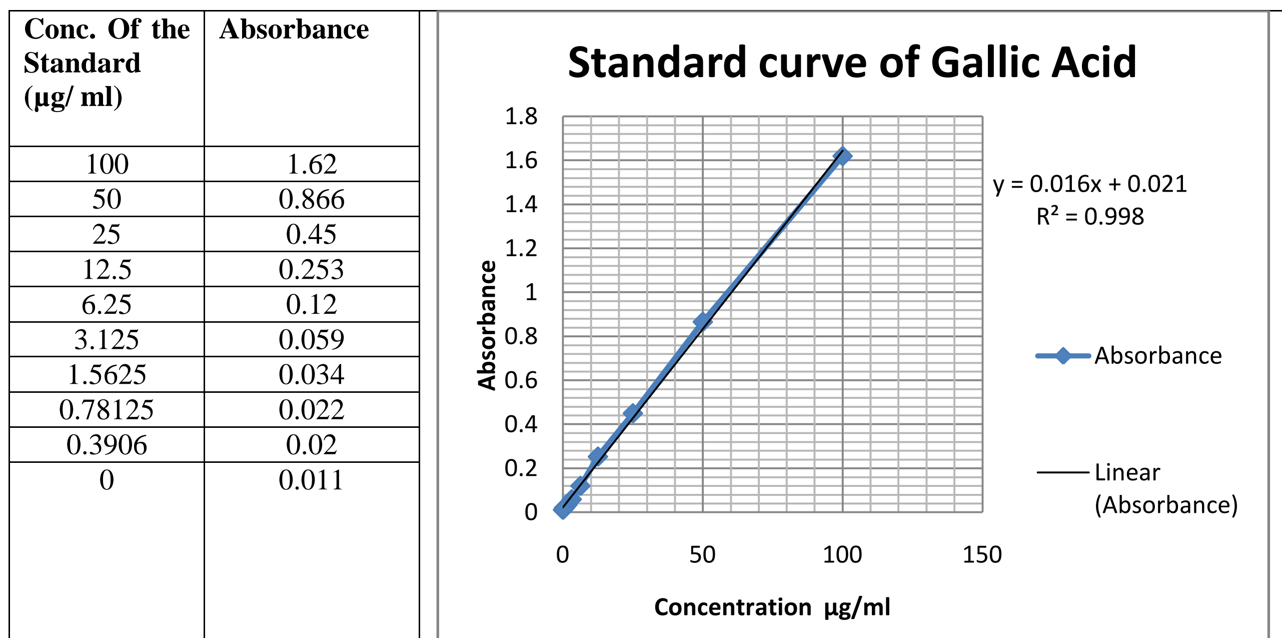
$$(I\ \%) = (1 - A_{sample}/A_{blank}) \times 100$$

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 inhibition percentage extract concentration.

5.2.9Result and Discussion: Total phenolic content (TPC)

The extracts of *S. aromaticum* partitionates i.e. VLC fraction 1, fraction 6, petroleum ether soluble fraction (PESF), ethyl acetate soluble fraction (EASF) and aqueous (AQF) fractions were subjected to total phenolic content determination.The amount of total phenolic content differs in different extractives and ranged from 114.41 mg of GAE / g of extractives to 519.33 mg of extractives of *S. aromaticum*bud. Among all extractives, the highest phenolic content was found in SA-1 (519.33 mg of GAE / g of extractives) followed by SA-6 (489.00 mg of GAE / g of extractives).

Table 5.2.2: Standard curve preparation by using Gallic acid

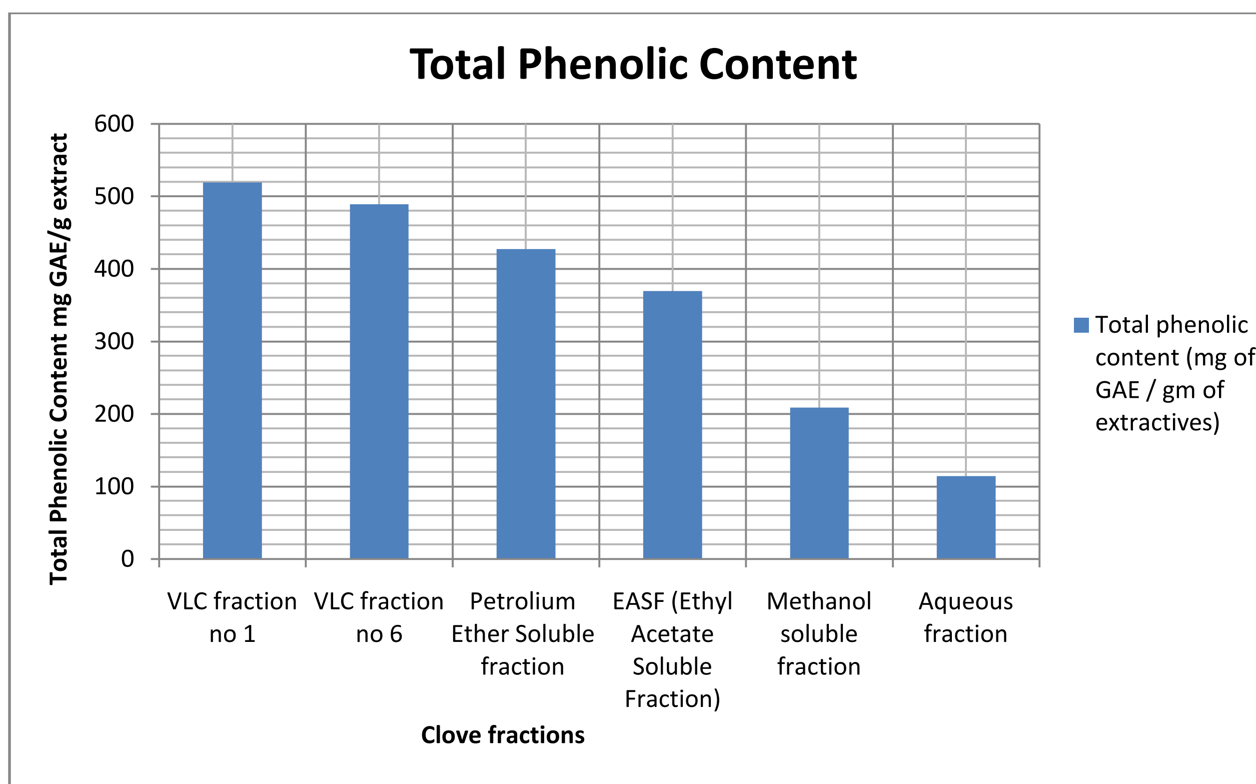


Conc. Of the Standard (µg/ ml)	Absorbance
100	1.62
50	0.866
25	0.45
12.5	0.253
6.25	0.12
3.125	0.059
1.5625	0.034
0.78125	0.022
0.3906	0.02
0	0.011

Table 5.2.3: test samples for total phenolic content determination

Plant part	Sample code	Test sample	Total phenolic content (mg of GAE / g of extractives)
Buds of S.aromaticum	SA-1	VLC fraction no 1	519.33
	SA-6	VLC fraction no 6	489.00
	PESF	Petrolium Ether Soluble fraction	427.25
	EASF	EASF (Ethyl Acetate Soluble Fraction)	369.35
	MSF	Methanol soluble fraction	208.73
	AQF	Aqueous fraction	114.41

Figure 5.2.3: Total phenolic content of clove fractions



5.2.10 Free radical scavenging activity (DPPH):

In the investigation, SA-1 and SA-2 showed the highest free radical scavenging activity with IC_{50} value 4.16 and 3.47μg/ml for leaves of *S.aromaticum*. At the same time PESF, EASF, MSF

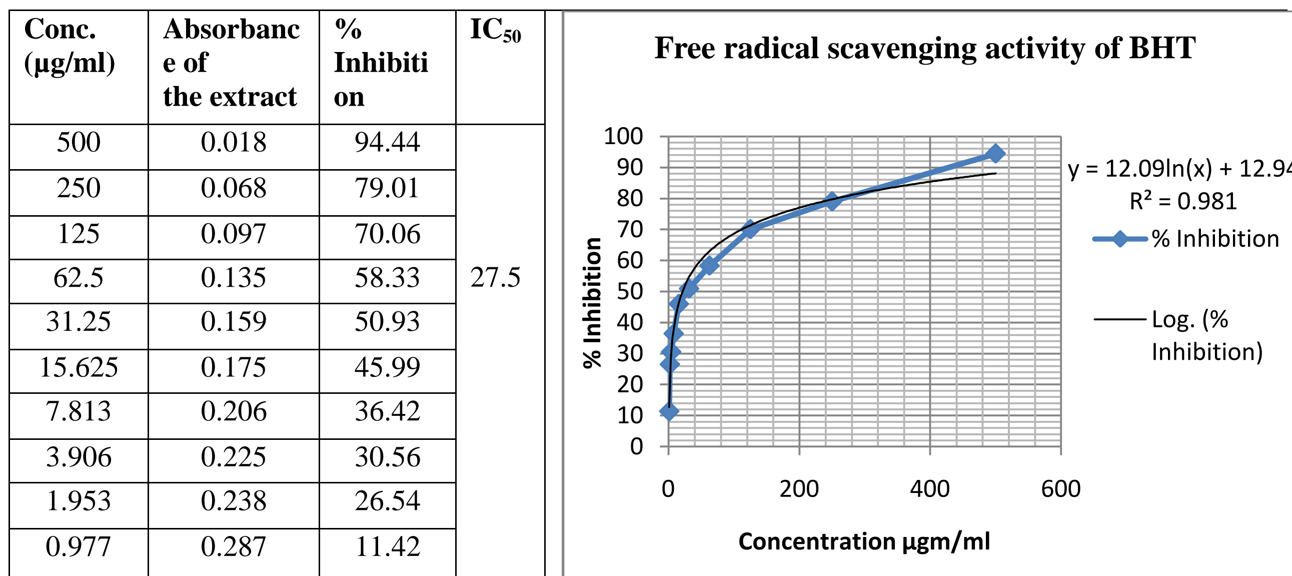
and AQF also exhibited antioxidant potential having IC_{50} value 6.09, 10.92, 9.6 and 5.79 µg/ml respectively.

Table 5.2.4: IC_{50} values of the standard and partitionates of Buds of *S.aromaticum*

Plant part	**Sample code**	**Test sample**	**IC_{50} (µg/ml)**
Buds of S.aromaticum	SA-1	VLC fraction no 1	4.16
	SA-6	VLC fraction no 6	3.47
	PESF	Petrolium Ether Soluble fraction	6.09
	EASF	EASF (Ethyl Acetate Soluble Fraction)	10.92
	MSF	Methanol soluble fraction	9.6
	AQF	Aqueous fraction	5.79

Table 5.2.5: IC_{50} value of tert-butyl-1-hydroxytolune (BHT)

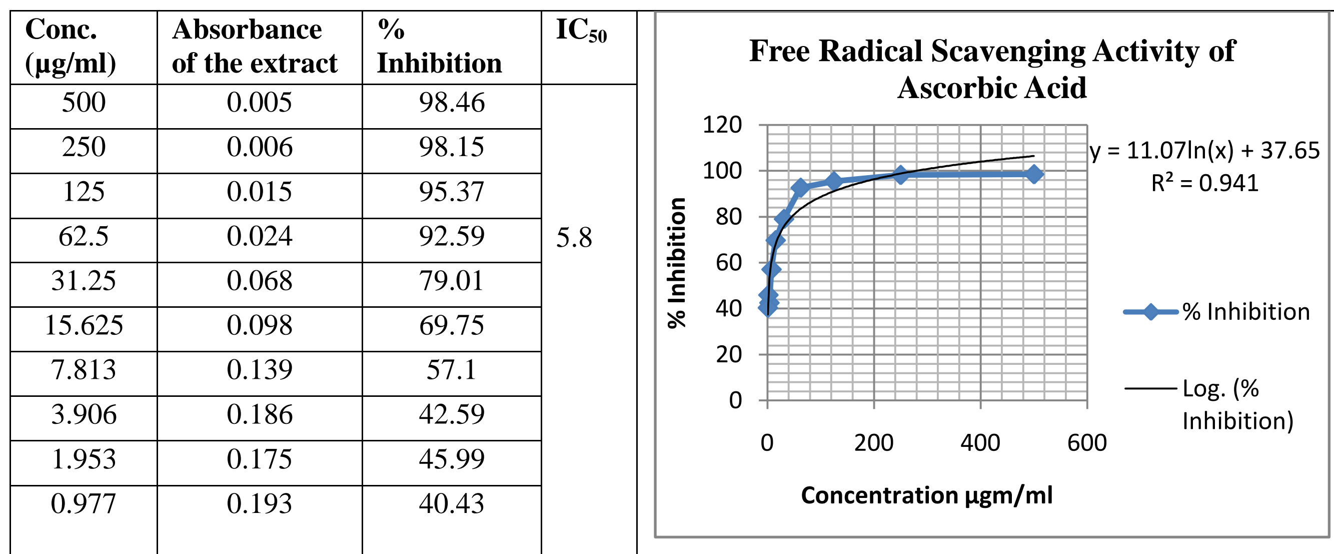
Conc. (µg/ml)	**Absorbance of the extract**	**% Inhibition**	**IC_{50}**
500	0.018	94.44	27.5
250	0.068	79.01	
125	0.097	70.06	
62.5	0.135	58.33	
31.25	0.159	50.93	
15.625	0.175	45.99	
7.813	0.206	36.42	
3.906	0.225	30.56	
1.953	0.238	26.54	
0.977	0.287	11.42	



Absorbance of the blank: 0.325

Table 5.2.6: IC_{50} value of Ascorbic acid (ASA)

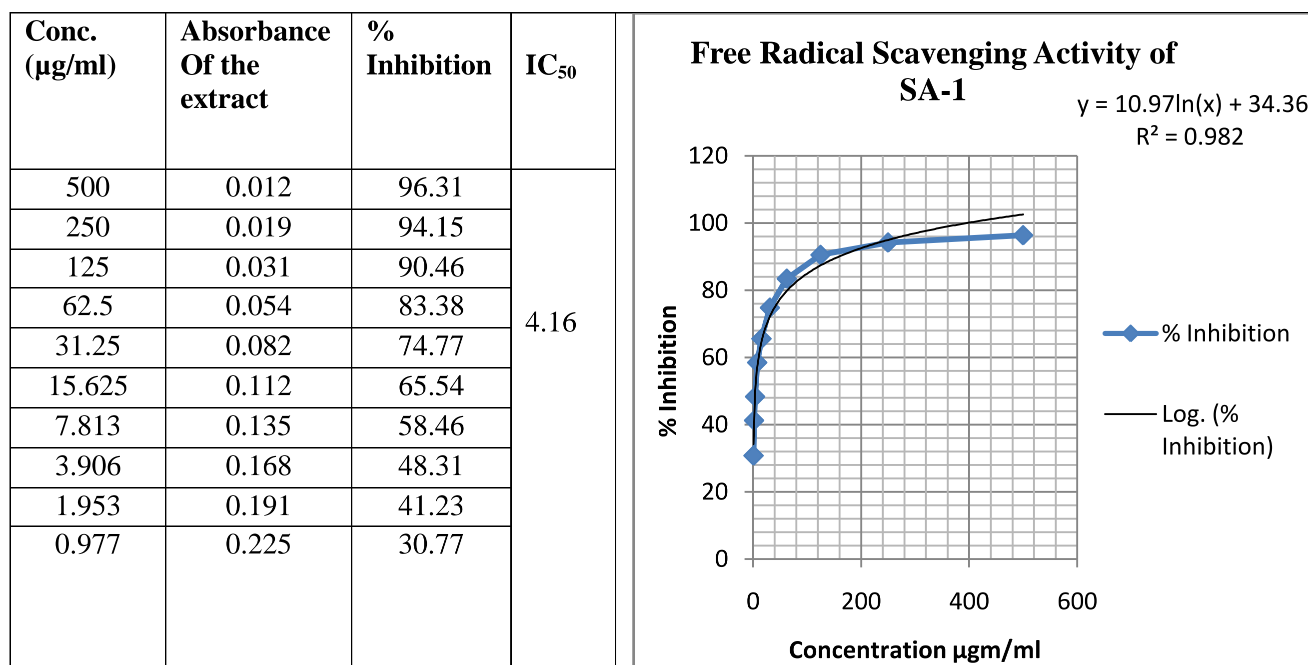
Conc. (µg/ml)	Absorbance of the extract	% Inhibition	IC_{50}
500	0.005	98.46	
250	0.006	98.15	
125	0.015	95.37	
62.5	0.024	92.59	5.8
31.25	0.068	79.01	
15.625	0.098	69.75	
7.813	0.139	57.1	
3.906	0.186	42.59	
1.953	0.175	45.99	
0.977	0.193	40.43	



Absorbance of the blank: 0.325

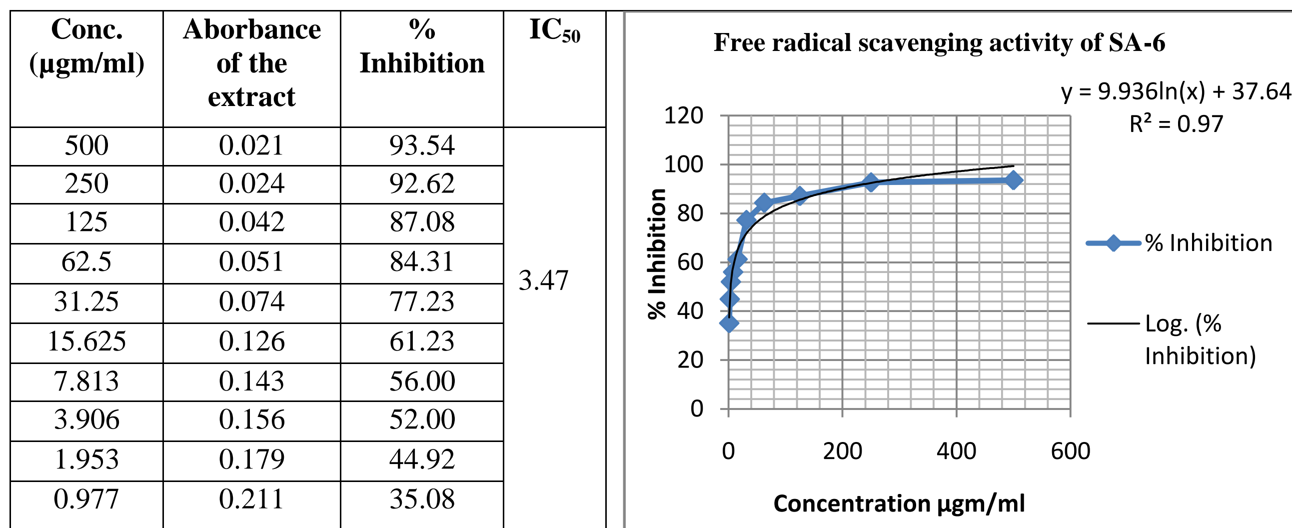
Table 5.2.7: IC_{50} value of SA-1 extract of bud of *S. aromaticum*

Conc. (µg/ml)	Absorbance Of the extract	% Inhibition	IC_{50}
500	0.012	96.31	
250	0.019	94.15	
125	0.031	90.46	
62.5	0.054	83.38	
31.25	0.082	74.77	4.16
15.625	0.112	65.54	
7.813	0.135	58.46	
3.906	0.168	48.31	
1.953	0.191	41.23	
0.977	0.225	30.77	



Absorbance of the blank: 0.325

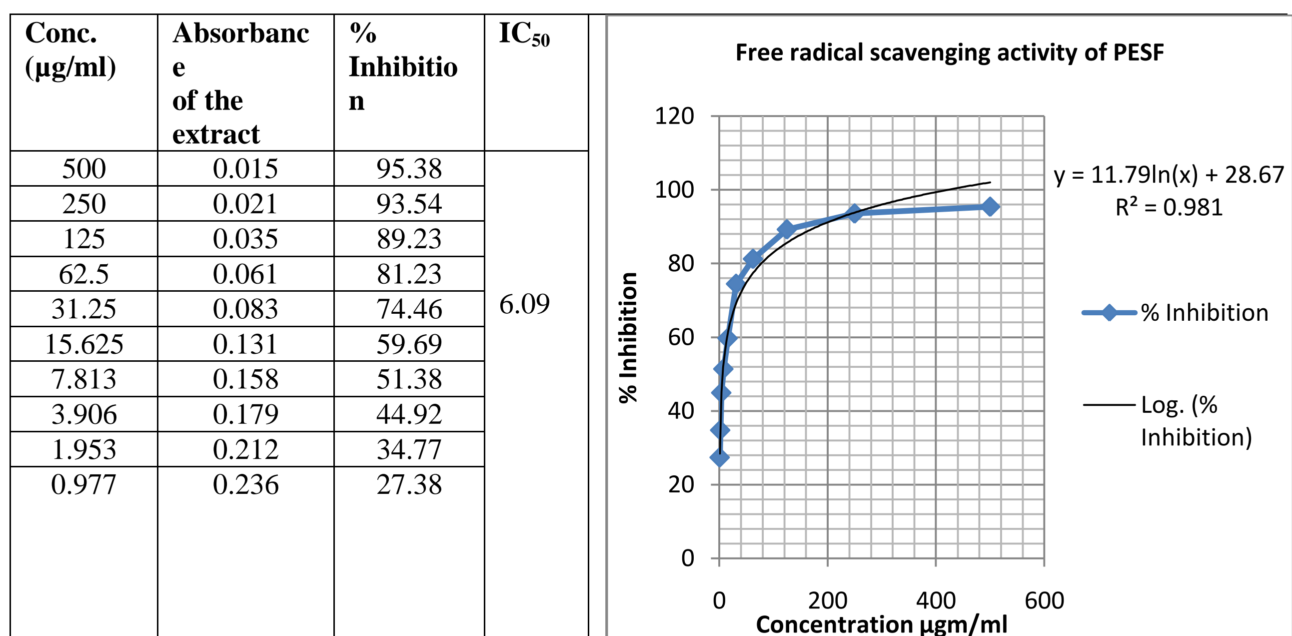
Table 5.2.8: IC_{50} value of SA-6 of bud of *S.aromaticum*



Conc. (µgm/ml)	Aborbance of the extract	% Inhibition	IC_{50}
500	0.021	93.54	3.47
250	0.024	92.62	
125	0.042	87.08	
62.5	0.051	84.31	
31.25	0.074	77.23	
15.625	0.126	61.23	
7.813	0.143	56.00	
3.906	0.156	52.00	
1.953	0.179	44.92	
0.977	0.211	35.08	

Absorbance of the blank: 0.325

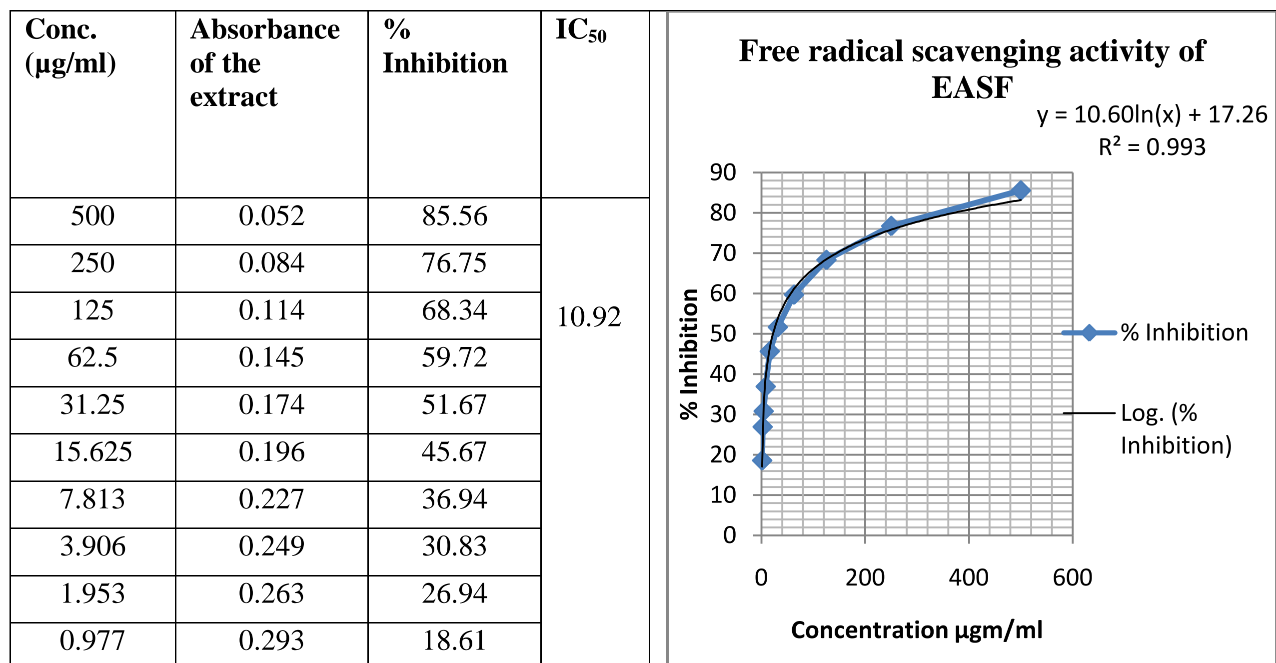
Table 5.2.9: IC_{50} value of Petrolium Ether Soluble Fraction (PESF) of bud of *S.aromaticum*



Conc. (µg/ml)	Absorbance of the extract	% Inhibition	IC_{50}
500	0.015	95.38	6.09
250	0.021	93.54	
125	0.035	89.23	
62.5	0.061	81.23	
31.25	0.083	74.46	
15.625	0.131	59.69	
7.813	0.158	51.38	
3.906	0.179	44.92	
1.953	0.212	34.77	
0.977	0.236	27.38	

Absorbance of the blank: 0.325

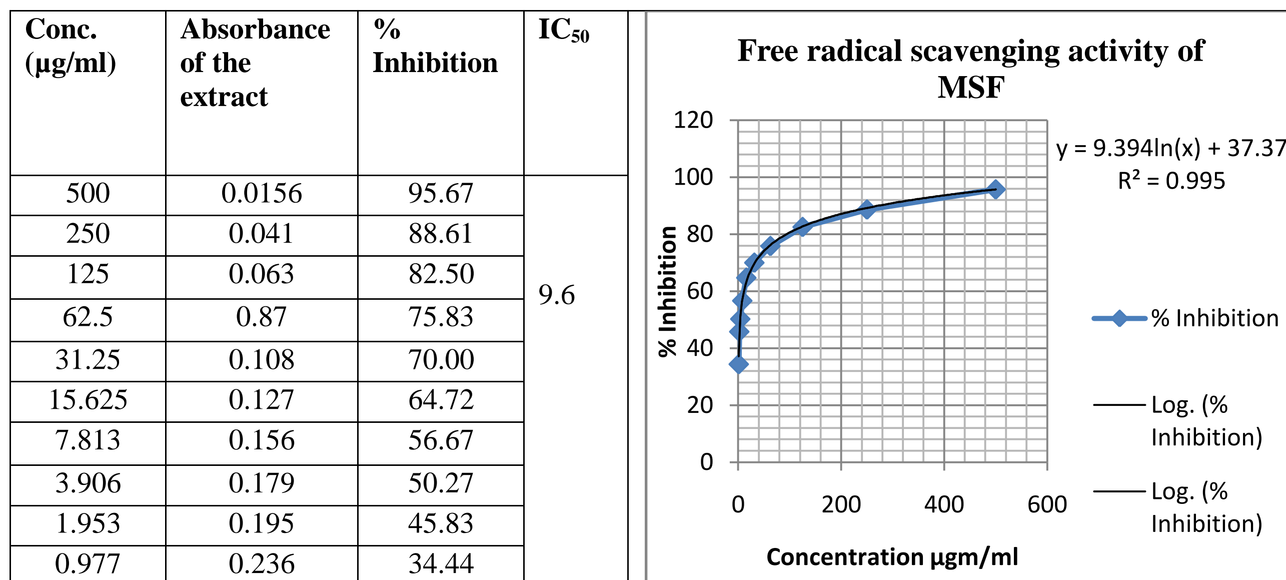
Table 5.2.10: IC_{50} value of Ethyl Acetate Fraction (EASF) of bud of *S.aromaticum*



Conc. (µg/ml)	**Absorbance of the extract**	**% Inhibition**	**IC_{50}**
500	0.052	85.56	10.92
250	0.084	76.75	
125	0.114	68.34	
62.5	0.145	59.72	
31.25	0.174	51.67	
15.625	0.196	45.67	
7.813	0.227	36.94	
3.906	0.249	30.83	
1.953	0.263	26.94	
0.977	0.293	18.61	

Absorbance of the blank: 0.325

Table 5.2.11: IC_{50} value of Methanol Soluble Fraction (MSF) of bud of *S.aromaticum*

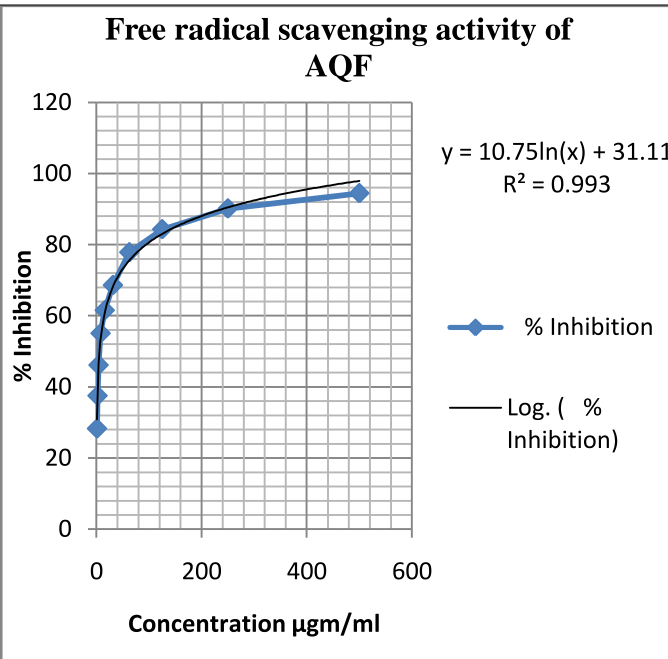


Conc. (µg/ml)	**Absorbance of the extract**	**% Inhibition**	**IC_{50}**
500	0.0156	95.67	9.6
250	0.041	88.61	
125	0.063	82.50	
62.5	0.87	75.83	
31.25	0.108	70.00	
15.625	0.127	64.72	
7.813	0.156	56.67	
3.906	0.179	50.27	
1.953	0.195	45.83	
0.977	0.236	34.44	

Absorbance of the blank: 0.325

Table 5.2.12: IC_{50} value of the aqueous Fraction (AQF) of leaves of *S.aromaticum*

Conc. (µg/ml)	**Absorbance of the extract**	**% Inhibition**	**IC_{50}**
500	0.018	94.46	5.79
250	0.032	90.15	
125	0.051	84.31	
62.5	0.072	77.85	
31.25	0.102	68.62	
15.625	0.125	61.54	
7.813	0.146	55.08	
3.906	0.175	46.15	
1.953	0.203	37.54	
0.977	0.233	28.31	



Absorbance of the blank: 0.325

5.2.11 Conclusion: SA-1 shows IC_{50} at 4.16, SA-6 shows IC $_{50}$ at 3.47 µg/ml. PESF shows 6.09, EASF shows 10.92, MSF shows 9.6 and AQF shows 5.79 µg/ ml as IC_{50}. Among these fractions SA-6 shows lowest IC_{50}value.

Chapter: 5.3

Brine shrimp lethality bioassay

5.3.1 Introduction:

Bioactive compounds are always toxic to living body at same higher doses and justices the statement pharmacology is toxicology at higher doses and toxicology is simply pharmacology at lower doses. Brine shrimp lethality bioassay is a rapid and comprehensive bioassay for the bioactive compounds of the natural and synthetic origin. By this method natural product extract, fraction as well as the pure compounds can be tested for their bioactivity. In this method, in vivo lethality is simple zoological organism [Brine shrimp] is used as a favorable for screening and fractionation in the discovery of new bioactive natural product.

This bioassay indicates preliminary cytotoxicity as well as a wide range of pharmacological activities such as antibacterial, antifungal, pesticidal and antitumor etc. of the compounds. Generally the LD_{50} values for cytotoxicities are one tenth of LC_{50} values in the brine shrimp lethality test.

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

5.3.2 Principle:

Brine shrimp eggs are hatched in simulated sea water to get nauplii. By the addition of calculated amount of dimethylsulphoxide (DMSO), desired concentration of the test sample is prepared. 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 premarked vials through micropipette. The vials are then left for 24 hours. Survivors are counted after 24 hours.

5.3.3 Experimental procedure:

The experiment was done by following the method illustrated by Meyer *et al.,*[62]

5.3.4 Preparation of sea water

76 g of the iodine free salt was weighted, dissolved in 2 liter of distilled water and filtered off to get clear solution.

5.3.5 Hatching of Brine shrimps

Artemia salina leach [Brine shrimp eggs] collected from pet shop was used as the test organisms.

Sea water was taken in a small tank and shrimp eggs were added to one side of the tank and then this side was covered. Two days were allowed to hatch shrimp and tob maturen das nauplii. Constant oxygen supply was carried out through the hatching time. The hatched shrimp were attracted to the lamp through the perforated dam and they were taken for the experiment. With the help of Pasteur pipette, 10 living shrimps were taken to each of the test tubes containing 5 ml of sea water.

Figure 5.3.1: Hatching of *A. salina*

5.3.6 Preparation of test solution with samples of experimental plant

Measured amount of sample was dissolved in specific volume of DMSO to obtain the desired concentration of the prepared solution as 400, 200, 100, 50, 25, 12.50, 6.25, 3.13, 1.56, 0.00(µg/ml) were prepared from this solution by serial dilution with DMSO.

5.3.7 Preparation of control group

Control group were used in cytotoxicity study to validate the test method and ensure that the result obtained are only due to the activity of the test.

5.3.8 Counting of nauplii

After 24 hours, the test tubes were observed and the number of survived nauplii in each tube was counted and results were noted. The percentage of lethality of brine shrimp nauplii was calculated for each concentration of sample.

5.3.9 Materials

The following materials are needed for cytotoxicity test.

Table 5.3.1: Materials needed for cytotoxicity test

Materials	Collection, amount and functions
Artemia salina leach	Brine shrimp eggs from aquarium
Sea salt	NaCl
Small tank	With perforated dividing dam to hatch Brine shrimp , cover
Pipettes	5ml
Micropipette	200µl
Test tubes	10 per sample + control
Table lamps	To attract shrimp

Table 5.3.2: Test sample of *S.aromaticum*

Plant part	Sample code	Test Sample	Calculated Amount (mg)
Buds of S.aromaticum	SA-1	VLC fraction no 1	4.0
	SA-6	VLC fraction no 6	4.0
	PESF	Petrolium Ether Soluble fraction	4.0
	MSF	Methanol soluble fraction	4.0
	EASF	EASF (Ethyl Acetate Soluble Fraction)	4.0
	AQF	Aqueous fraction	4.0

5.3.10 Results and Discussion of Brine shrimp lethality Bioassay

Bioactive compounds are almost always toxic at higher dose. Thus, *in vivo* lethality in a simple zoological organism can be used as a convenient informant for screening and fractionation in the discovery of new bioactive natural products. Crude extract of chloroform, methanol, ethyl acetate and petroleum ether soluble fractions were screened by brine shrimp lethality bioassay for probable cytotoxic activity.It appears from the result that all the test samples were lethal to brine shrimp nauplii. In the present bioactivity study, all the crude extracts, pre-ether, carbon tetrachloride, dichloromethane, ethyl acetate and aqueous soluble fractions of chloroform extract showed positive results indicating that the test samples were biologically active. Each of the test samples showed different mortality rate at different concentrations. Plotting of log of concentration versus percent of mortality for all test samples showed an approximate linear correlation. From the graph, the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) was determined for the samples.The lethal concentration LC_{50} of the test samples after 24 hours was obtained by a plot of percentage of the Shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best fit-line was obtained from the curve data by means of regression analysis.

Vincristine sulfate (VS) was used as positive control and the lethal concentrations ($LC_{50)}$ were found 0.45µg/mL for vincristine sulfate. Comparing with the positive control gave significant mortality and the LC_{50} values of the different extractives were compared to this positive control. The positive control group show non linear mortality rates at lower concentrations and linear rates at higher concentrations. There was no mortality in the negative control groups indicating the test as a valid one and the results obtained are only due to the activity of the test

agents.Comparing with the positive control Vincristine sulfate signifies that cytotoxicity exhibited by the crude extract of chloroform, ethyl acetate, methanol, petroleum ether and water soluble fractions are promising and they might have antitumor or pesticidal compounds. Due to time constrain, further analysis was not performed. However, its further study with specific case may establish its potentiality.

Table 5.3.3: Effect of Vincristine sulphate (positive control) on shrimp nauplii

Conc. (µg/ml)	**Log_{10} Conc.**	**% Mortality**	**LC_{50}**
0		20	0.45
0.0390	-1.4089	20	
0.078125	-1.1-72	30	
0.15625	-0.8061	30	
0.3125	-0.5051	40	
0.625	-0.2014	50	
1.25	0.09691	70	
2.5	0.39794	80	
5	0.6989	80	
10	1.00	90	
20	1.3010	100	

Figure 5.3.2: Graphical representation of the effect of Vincristin Sulphate on shrimp nauplii

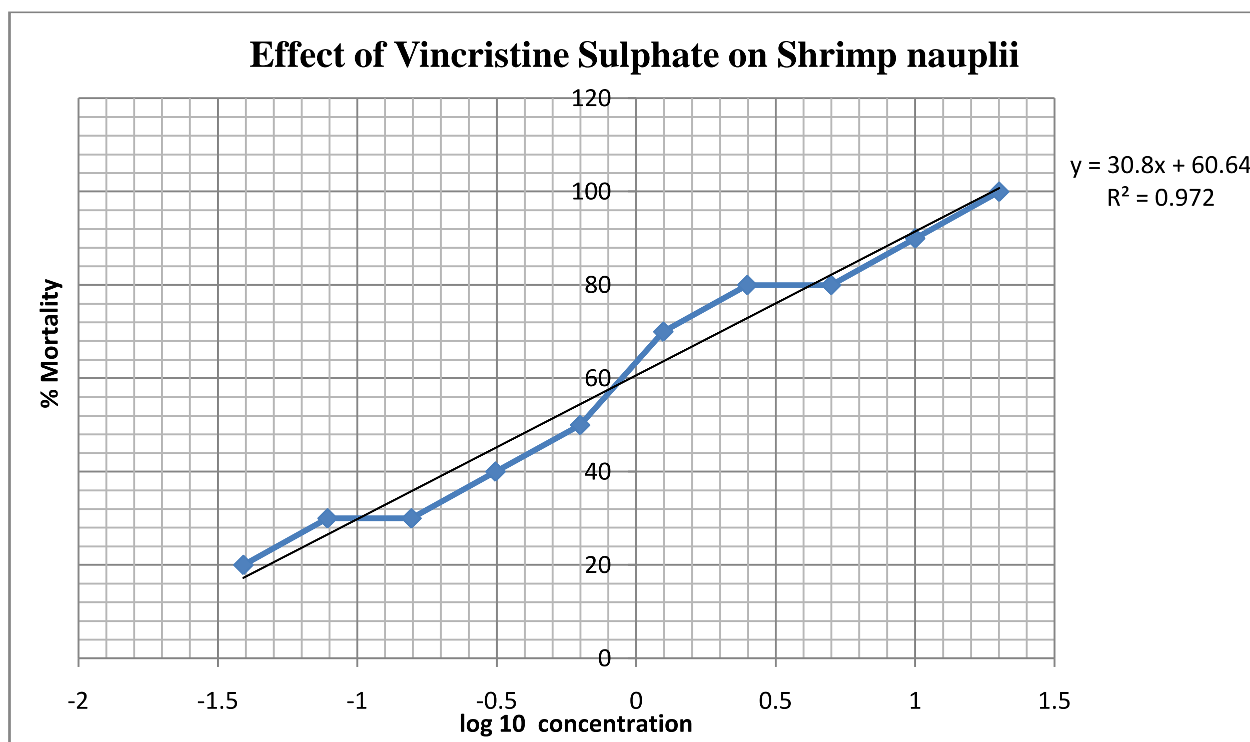


Table 5.3.4: Effect of SA-1 of buds of *S.aromaticum* on shrimp nauplii

Conc. (µg/ml)	Log_{10} Conc.	% Mortality			Mean± SD	LC_{50}
		1st	2nd	3rd		
0		0	0	0	0	0.72
1.56	0.194	60	50	50	53.33± 5.77	
3.125	0.495	60	60	70	63.33± 5.77	
6.25	0.796	60	70	70	66.67±5.77	
12.50	1.097	80	70	70	73.33±5.77	
25	1.398	100	80	90	90± 10	
50	1.699	100	100	100	100	
100	2.000	90	100	100	96.67± 5.77	
200	2.301	100	100	100	100	
400	2.602	100	100	100	100	

Figure 5.3.3: Graphical representation of the effect of SA-1 of buds of *S. aromaticum* on shrimp nauplii

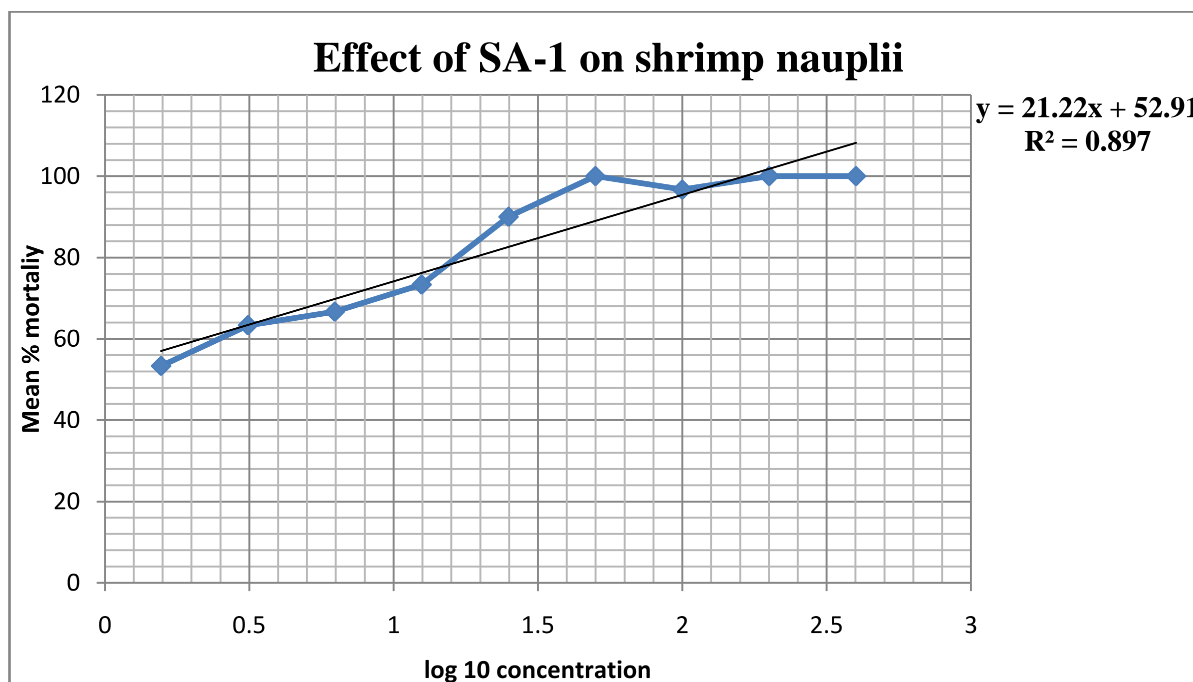


Table 5.3.5: Effect of SA-6 of buds of *S.aromaticum* on shrimp nauplii

Conc. (µg/ml)	Log_{10} Conc.	% Mortality			Mean ± SD	LC_{50}
		1st	2nd	3rd		
0		0	0	0	0	0.17
1.56	0.194	70	70	60	66.67± 5.77	
3.125	0.495	70	60	70	66.67± 5.77	
6.25	0.796	80	70	70	73.33± 5.77	
12.50	1.097	70	90	80	80± 10	
25	1.398	80	80	80	80	
50	1.699	100	100	100	100	
100	2.000	90	100	100	96.67± 5.77	
200	2.301	100	100	100	100	
400	2.602	100	90	100	96.67± 5.77	

Figure 5.3.4: Graphical representation of the effect of SA-6 of buds of *S. aromaticum* on shrimp nauplii

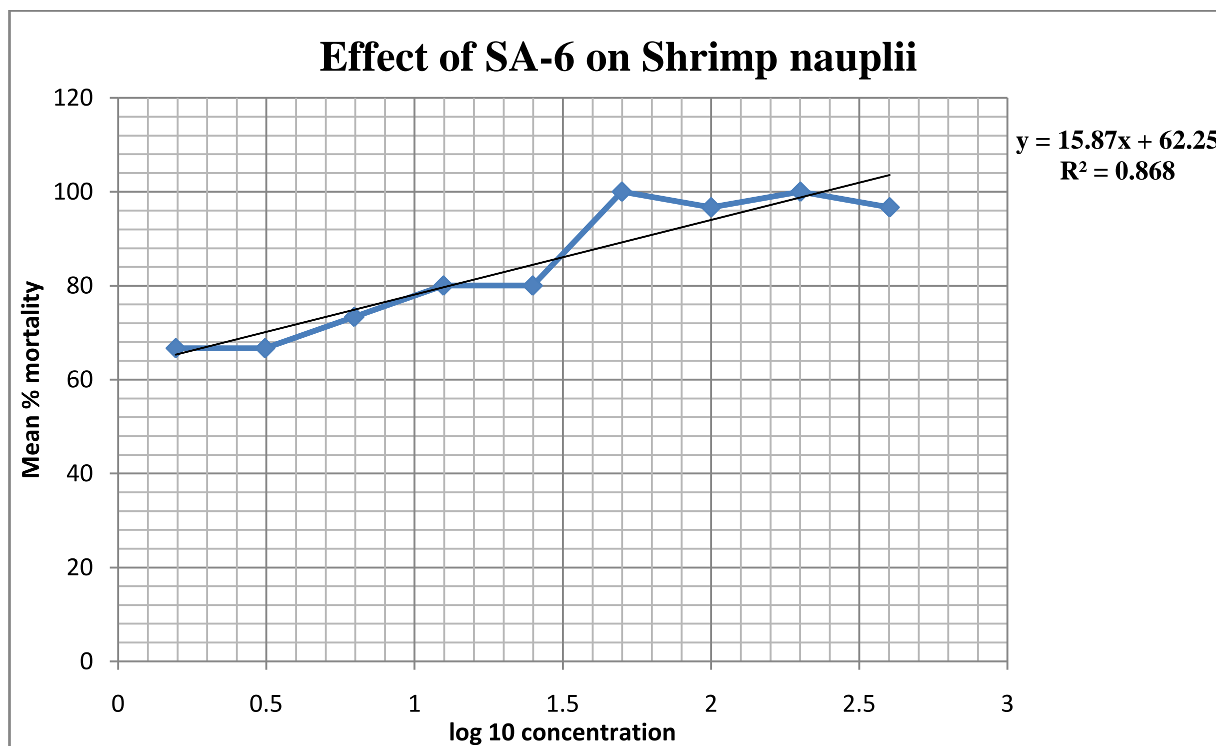


Table 5.3.6: Effect of PESF (Petrolium Ether Soluble Fraction of buds of *S.aromaticum* on shrimp nauplii

Conc. (µg/ml)	Log_{10} Conc.	% Mortality			Mean ± SD	LC_{50}
		1st	2nd	3rd		
0		0	0	0	0	4.01
1.56	0.194	30	40	40	66.67± 5.77	
3.125	0.495	40	40	50	66.67± 5.77	
6.25	0.796	50	50	50	73.33	
12.50	1.097	70	60	60	80± 5.77	
25	1.398	80	80	80	80	
50	1.699	90	100	100	100± 5.77	
100	2.000	90	100	90	96.67± 5.77	
200	2.301	100	100	100	100	
400	2.602	100	90	100	96.67± 5.77	

Figure 5.3.5: Graphical representation of the effect of PESF of buds of *S. aromaticum* on shrimp nauplii

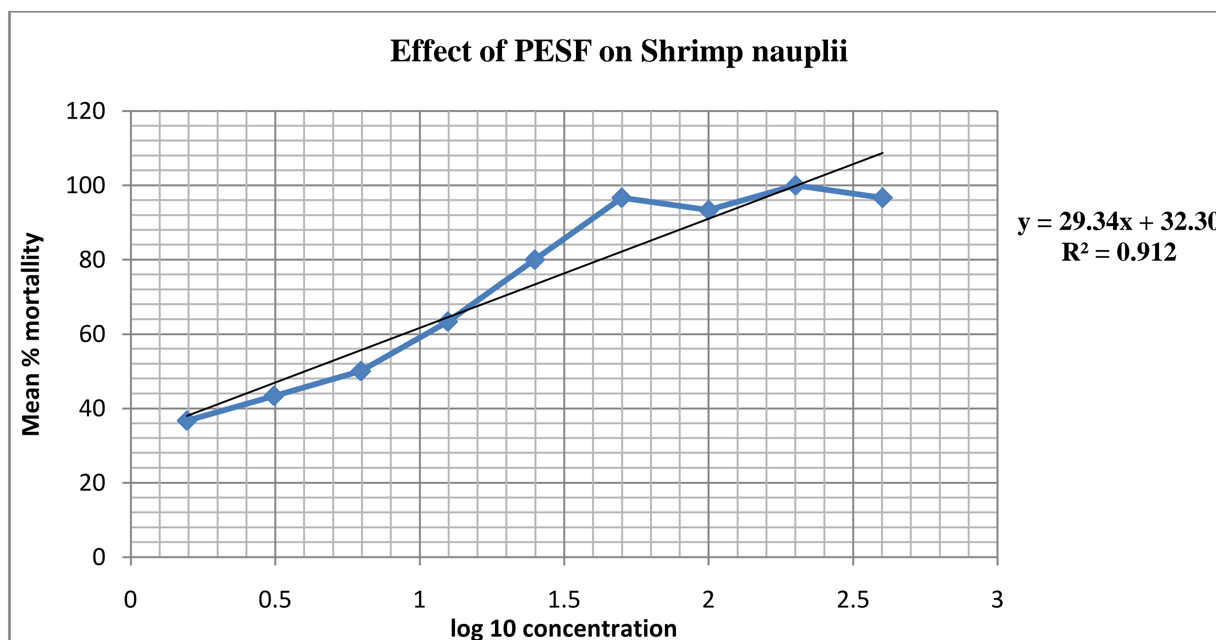


Table 5.3.7: Effect of EASF of buds of *S. aromaticum* on shrimp nauplii

Conc. (µg/ml)	Log_{10} Conc.	% Mortality			Mean ± SD	LC_{50}
		1st	2nd	3rd		
0		0	0	0	0	4.6
1.56	0.194	30	30	40	33.33± 5.77	
3.125	0.495	40	40	50	43.33± 5.77	
6.25	0.796	50	50	50	50	
12.50	1.097	60	60	60	60	
25	1.398	80	80	80	80	
50	1.699	90	90	100	93.33± 5.77	
100	2.000	90	90	90	90	
200	2.301	100	100	100	100	
400	2.602	100	90	100	96.66± 5.77	

Figure 5.3.6: Graphical representation of the effect of EASF of buds of *S. aromaticum* on shrimp nauplii

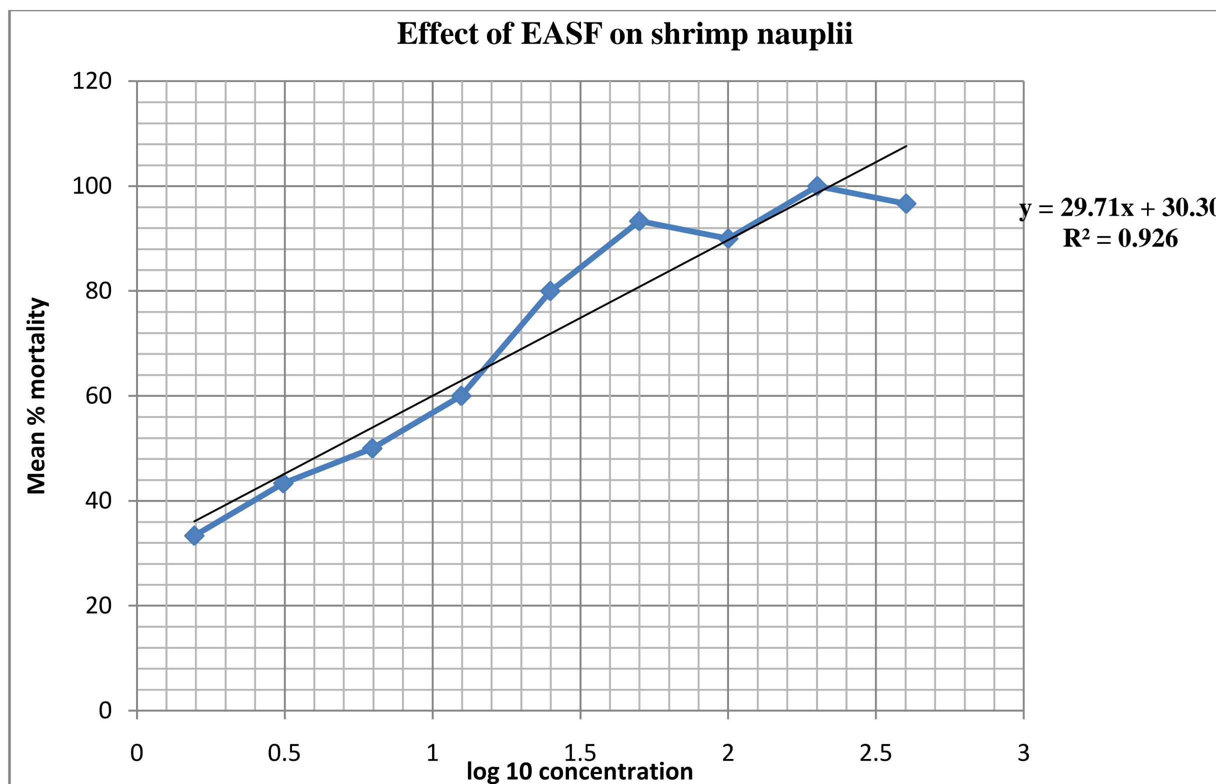


Table 5.3.8: Effect of MSF of buds of *S. aromaticum* on shrimp nauplii

Conc. (µg/ml)	Log_{10} Conc.	% Mortality			Mean ± SD	LC_{50}
		1st	2nd	3rd		
0		0	0	0	0	9.10
1.56	0.194	20	30	20	23.33± 5.77	
3.125	0.495	30	30	40	33.33± 5.77	
6.25	0.796	40	30	40	36.67± 5.77	
12.50	1.097	50	50	50	50	
25	1.398	70	80	70	73.33± 5.77	
50	1.699	90	80	80	83.33± 5.77	
100	2.000	90	90	100	93.33± 5.77	
200	2.301	100	100	100	100	
400	2.602	100	90	100	96.67	

Figure 5.3.7: Graphical representation of the effect of MSF of buds of *S. aromaticum* on shrimp nauplii

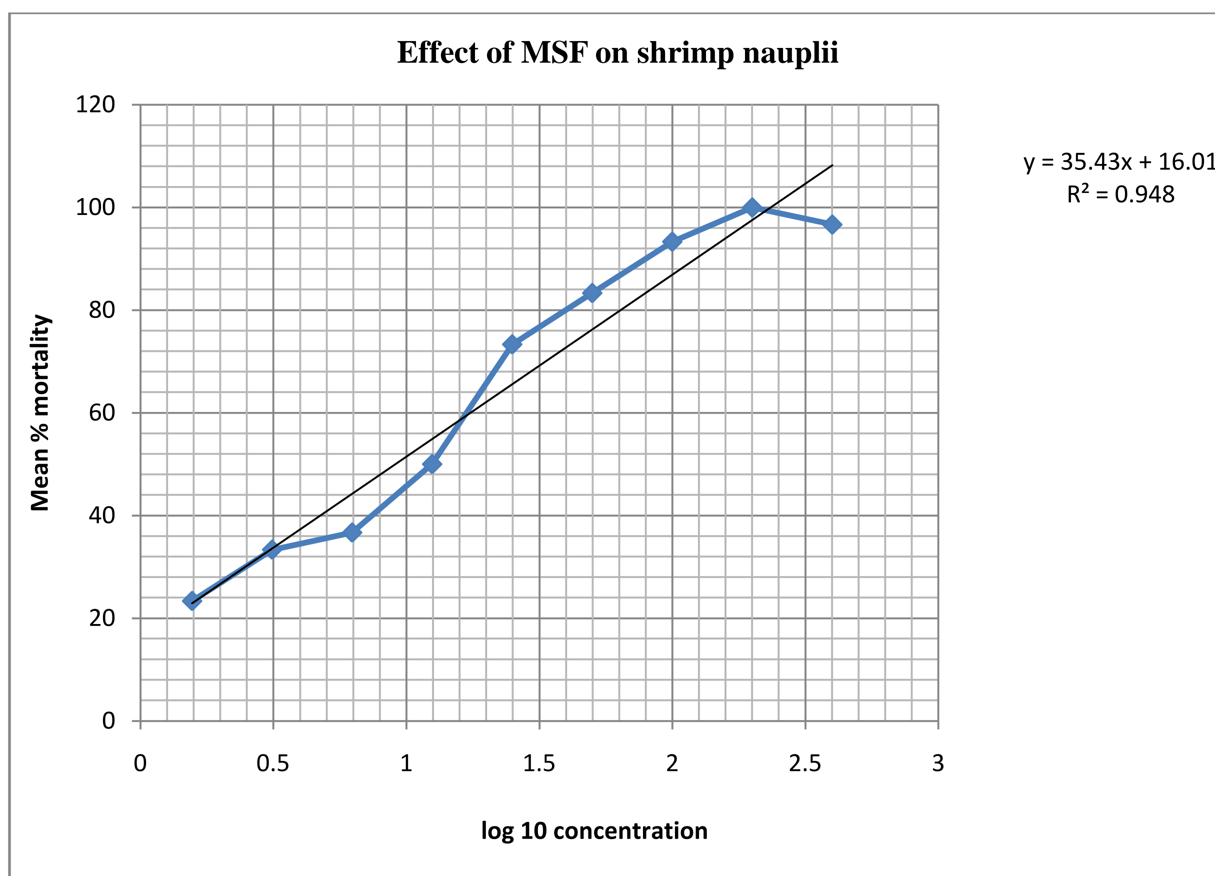
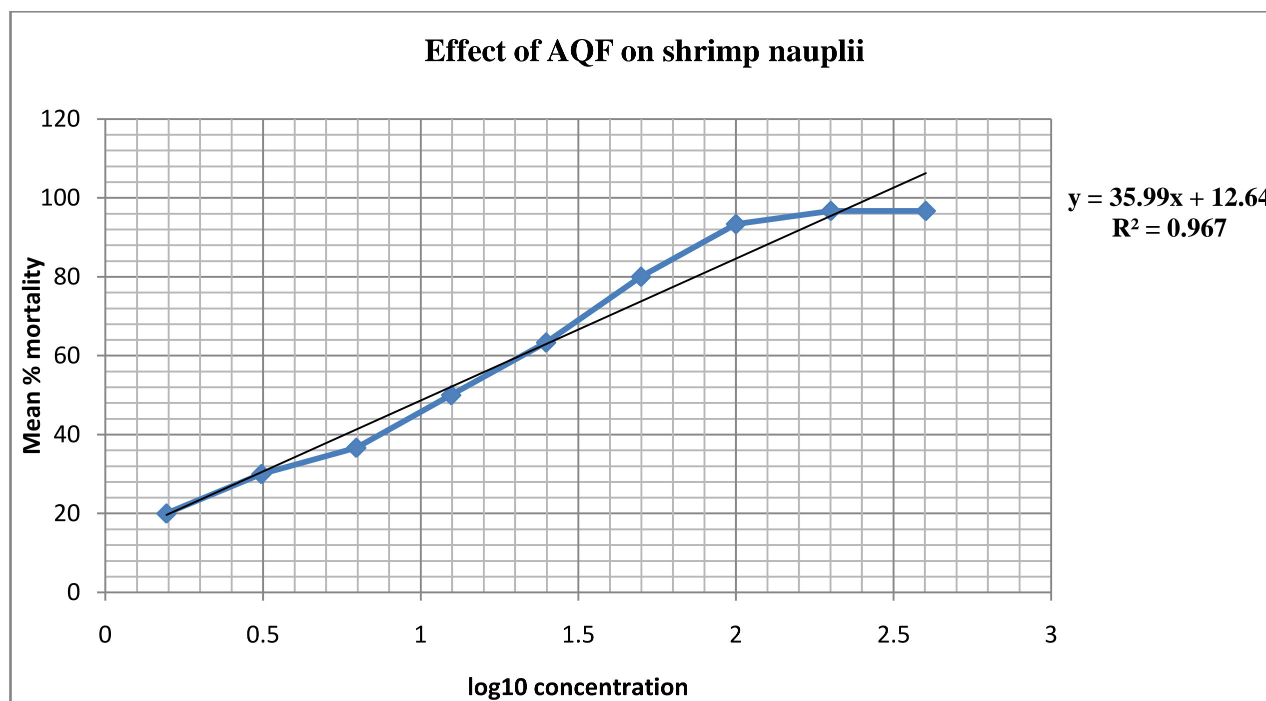


Table 5.3.9: Effect of AQF of buds of *S. aromaticum* on shrimp nauplii

Conc. (µg/ml)	Log_{10} Conc.	% Mortality			Mean ± SD	LC_{50}
		1st	2nd	3rd		
0		0	0	0	0	10.91
1.56	0.194	20	20	20	20	
3.125	0.495	30	30	30	30	
6.25	0.796	40	30	40	36.67± 5.77	
12.50	1.097	50	50	50	50	
25	1.398	60	60	70	63.33± 5.77	
50	1.699	80	80	80	80	
100	2.000	90	90	100	93.33± 5.77	
200	2.301	90	100	100	96.67± 5.77	
400	2.602	100	90	100	96.67± 5.77	

Figure 5.3.8: Graphical representation of the effect of AQF of buds of *S. aromaticum* on shrimp nauplii



5.3.11 Conclusion:

In the present bioactivity study Clove extract different partitionate i.e. petroleum ether, ethyl acetate, methanol and aqueous soluble fractions showed that samples are biologically active.

Vincristine sulfate (VS) was used as positive control and the LC_{50} was found 0.45μg/ml for vs. Compared with the negative control vs (positive control) gave significant mortality and LC_{50}values of the different extractives were compared to this positive control.The LC_{50} values of SA-1, SA-6, PESF, EASF, MSF and AQP were found to be 0.72 μg/ ml, 0.17 μg/ ml, 4.01 μg/ml, μg/ml and μg/ml respectively revealed significant lethality whereas AQE revealed moderate activity.Considering the potential bioactivity, the plant materials can further be studied extensively to find out their unexplored efficacy and to rationalize their uses as traditional medicines.

Chapter: 5.4

Evaluation of analgesic activity

5.4.1 Introduction

Pain is an unpleasant sensation and a very common phenomenon. There is no doubt that pain acts as a warning signal against disturbances either in the body or in the external environment of an individual. The principal objective of the treatment of pain is to remove or abolish the cause of pain. But it is not always possible to do so; hence, analgesics are used for the symptomatic treatment of pain. Opioids are the most potent and commonly used group of analgesic drugs e.g. Morphine and Pethidine. But their analgesic action is associated with a degree of adverse drug reactions, most of which are dose dependent.

Increased pain in response to noxious stimulation following peripheral tissue injury depends on an increase in the sensitivity of primary afferent nociceptors at the site of injury (peripheral sensitization) and on an increase in the excitability of neurons in the CNS (central sensitization).

Most of the synthetic drugs used at present for analgesic and anti-nociceptive effect have many side & toxic effects. Plants still represent a large untapped stimulation source of structurally novel compounds that might serve as lead for the development of novel drugs. Many medicines of plant origin with analgesic and an anti-nociceptive activity had been used since long time without any adverse effect.

5.4.2 Principle

Analgesic and anti-inflammatory drugs are one of the most products that used in many of disease for relief of pain and inflammation. Most analgesic and anti-inflammatory drugs available in the market, still present a wide range of many problems such as efficacy and undesired effects including GIT disorders and other unwanted effects [63], that limit their clinical usefulness and remain to be solved and leaving an open door for new and better compounds [63]. This situation highlights the need for advent of safe, novel and effective analgesic and anti-inflammatory compounds. Nonsteroidal anti-inflammatory drugs (NSAIDs) reduce pain and edema by suppressing the formation of prostaglandins, by inhibiting the activity of the enzyme Cyclooxygenase (COX-1 and COX-2). However, prostaglandins are key mediators of several components of GI mucosal defense, so suppression of synthesis of prostaglandins (PGs) by NSAIDs greatly reduces the resistance of the mucosa toinjury as well as interfering with repair processes. Selective COX-2 inhibitors were thought to be the solution to this conundrum as it is required that NSAIDs suppress prostaglandin synthesis at sites of inflammation, and not in the GI tract. However, it is now clear that both COX-1 and COX-2 isoforms contribute to mucosal defense. Selective COX-2 inhibitors elicit less GI damage and bleeding than conventional NSAIDs, although the magnitude of this reduction continues to be contested in the literature. As

widely reported in the lay-press, the selective COX-2 inhibitors also cause significant adverse effects in the renal and cardiovascular systems, possibly more serious than those caused by conventional NSAIDs. The market for NSAIDs is expanding rapidly because of an aging population in developed countries and the associated increase in the prevalence of diseases like arthritis. Use of Aspirin is also increasing because of its utility in reducing the incidence of a number of common disorders including stroke, myocardial infarction, Alzheimer's disease and cancer. However, their use is limited by their significant side effects upon the stomach and the kidney. Their side effects as well as their therapeutic actions are related to their ability to inhibit cyclooxygenase enzymes involved in the first step of the arachidonic acid cascade. In addition, the damaging effect of some NSAIDs upon the stomach and intestine is in part due to their acidic nature, as with indomethacin, ibuprofen, diclofenac, naproxene, aspirin, etc. Although basic NSAIDs such as glafenine and floctafenine are expected to be devoid of the primary insult effect, their damaging effect upon the stomach and kidney is still prominent as they inhibit prostaglandin biosynthesis as strongly as indomethacin. In the recent years, several novel approaches for reducing the GI toxicity of NSAIDs with promising results have been reported. These mainly involve structural modification of existing NSAIDs such that inhibition of COX is maintained, but other attributes are added that diminish GI (and other) toxicity, and in some cases boost efficacyand/or potency. The mortality rate for NSAID induced GI bleeding is 5-10% in the world population. In this dissertation, two types of analgesic activity has been evaluated-

- Peripheral Analgesic Activity
- Central Analgesic Activity

5.4.3 Peripheral Analgesic Activity

Peripheral analgesic activity can be evaluated by acetic acid induced writhing. In this experiment the formalin test was carried out according to standard methods. The animals received different experimental doses of compound which is supposed to lessen the number of writhing within in a given time frame with respect to the control group. The writhing responses inhibition of positive control was taken as standard and compared with test samples and control. The peripheral analgesic activity is estimated by Kaneria et al., method [64].

5.4.4 Experimental animal

Swiss-albino mice of either sex, aged 4-5 weeks were used for the experiment.

5.4.5 Experiment design

Swiss-albino mice weighing 22-28 gm were acclimated after arrival for 1-2 weeks in a temperature controlled room with a 12-h light/dark cycle and allowed free access to standard laboratory food and water. On day of experiment non fasted animals were weighed; six groups are formed having five experimental animals in each.

5.4.6 Preparation of test materials

Some VLC fractions of *Syzygium aromaticum* which were presumed to contain specific compound those analyzed by TLC and different soluble fractions (PESF, MSF, EASF and AF) were used in a dose of 300mg/kg body weight. The drug used for peripheral analgesic activity was acetyl salicylic acid sodium in a dose of 50 mg/kg-body weight. A control group was maintained in which mice were given 1% Tween-80 in normal saline.

5.4.7 Procedure

At zero hour test samples, controls and acetyl salicylic acid sodium were administered orally by means of long needle with a ball-shaped end. Test compounds were dosed orally one hr. before one hour of the acetic acid injection. Acetic acid was injected by intraperitoneal route. Numbers of writhes after 5 min interval of acetic acid injection for 15 min. are recorded.

5.4.8 Mechanism of pain induction in Acetic acid induced writhing method

Intraperitonial administration of acetic acid causes localized inflammation in mice. Following inflammation, there is biogenesis of prostaglandins (cyclooxygenase pathway) & leukotrienes (lipooxigenase pathway). The released prostaglandin, mainly prostacyclin (PGI2) & prostaglandin E have been reported responsible for pain sensation.

5.4.9 Counting of writhing

Each mouse of all groups was observed individually for counting the number of writhing response they made in 5 minutes just after the subcutaneous administration of acetic acid solution. Percent inhibition is calculated by following equation-

$$\% \text{ Inhibition} = \frac{\text{(Control writhing response} - \text{test writhing response)}}{\text{Control writhing response}} \times 100$$

Figure 5.4.1: Albino mouse

5.4.10 Result

Table 5.4.1: Writhing response to clove fractions

Group& dose	Writhing response (sec.)	Average (sec) ± SD	% Inhibition	P value & Significance
Control	25	31 ± 7.96	---	---
	28			
	28			
	29			
	45			
Std. Acetyl Salicylic Acid	13	15.6 ± 1.67	49.68	0.002 Significant
	17			
	16			
	17			
	15			
SA-1	18	16.6 ± 1.34	46.45	0.004 Significant
	15			
	16			
	16			
	18			
SA-6	19	18.6 ± 1.81	40.00	0.009 Significant
	19			
	21			
	16			
	18			
PESF	20	22.0 ± 3.08	29.03	0.04 Significant
	22			
	22			
	27			
	19			
EASF	26	26.6 ± 3.13	14.19	0.28 Non-significant
	26			
	29			
	22			
	30			
MSF	32	26.8 ± 3.34	13.54	0.30 Non-significant
	28			
	24			
	24			
	26			
AQF	18	19.8 ± 2.38	36.12	0.01 Significant
	19			
	19			
	19			
	24			

Statistical evaluation of the data confirmed that all the soluble fractions of clove extracts of *S. aromaticum* showed extremely significant peripheral analgesic activity with percent inhibition of writhing responces of 46.45 %. All the extracts of cloves and VLC fractions except EASF and MSF show significant peripheral analgesic activity.

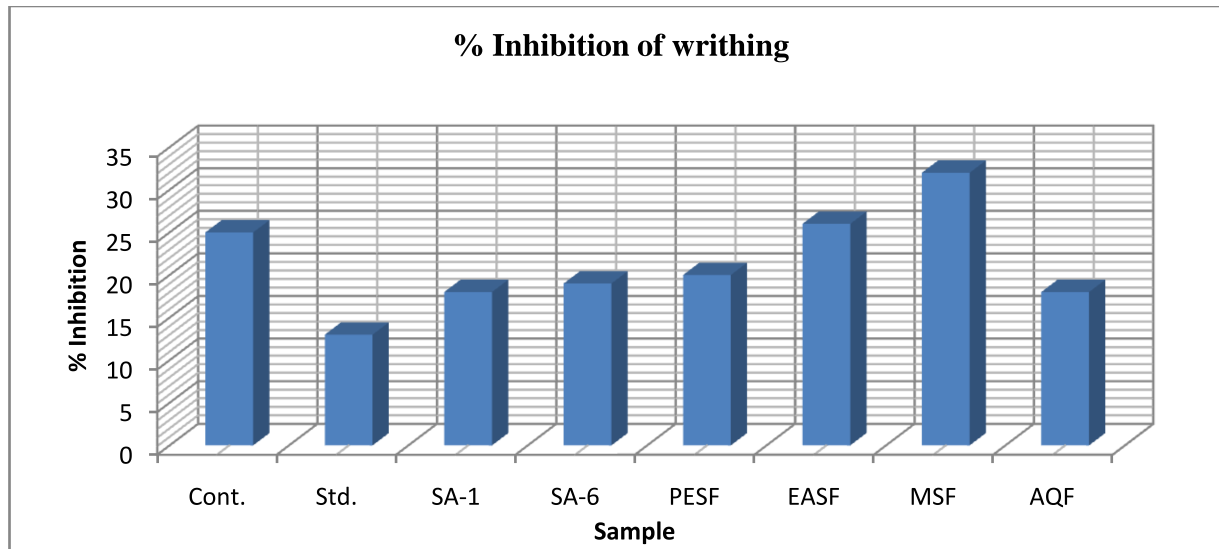


Figure 5.4.2: % inhibition of writhing

5.4.11 Statistical analysis

Data were analyzed by standard t-test using. At 95% confidence interval $p<0.05$ was considered statistically significant.

5.4.12 Discussion

The purpose of this study was to objectively assess the effectiveness of *S. aromaticum* (flower bud) in treating two different types of nociception using the rat formalin test. Most reported studies used licking and biting as the measure of assessing nociception. Results of our studies with *S. aromaticum* (flower buds) are consistent with other studies using opioid type analgesic and NSAIDs. From this study, it was found that in nociception state the (flower buds) of *Caryophyllus aromaticus* shows significant decrease in nociception in both the phases. Moreover, it also shows reduction of nociception in both phases by the standard drug. Receiving all the literature available in support the of *S. aromaticum* (flower buds) as analgesic, it was found that *S.aromaticum* (flower buds)has beneficial effect in reducing nociception. Physiological properties of *S. aromaticum* (flower buds) thus holds the unmyelinated fiber projections to the spinal cord thus hope of new generation of drugs.
However, there is need for further studies on experimental animals and human beings that may more definitive and sure data regarding its usefulness, exact mode of action for its better economic and therapeutic utilization. Hence *S. aromaticum* (flower buds) may have a promising

role in the management of pain especially in countries where conventional treatment is not easily accessible to the general population.

5.4.13 Conclusion

SA-1, SA-6, PESF and AQF shows statistically significant peripheral analgesic activity but EASF, MSF shows non-significant activity.

5.4.14Central Analgesic Activity

Central analgesic activity can be evaluated by Tail Immersion Test. Tail Withdrawal Reflexes Induced by Tail Immersion Test. But tail immersion test is also another parameter foranalgesic activity. The procedure is based on the observation that morphine-like drugs are selectively capable of prolonging the reaction time of the typical tail-withdrawal reflex in rats induced by immersing the end of the tail in warm water. The ameliorative effect of tail withdrawal response was observed in a dose dependent manner. The maximum analgesic effect was shown at higher dose (200mg/kg, p.o.) which was comparable to that of morphine (10mg/kg, p.o). This activity was performed by the use of Olaleye SB et al. method [65].

5.4.15 Experimental animal

Swiss-albino mice of either sex, aged 4-5 weeks were used for the experiment.

5.4.16 Experiment design [65]

Swiss-albino mice weighing 22-28 g were acclimated after arrival for 1-2 weeks in a temperature controlled room with a 12-h light/dark cycle and allowed free access to standard laboratory food and water. On day of experiment non fasted animals were weighed; Healthy albino mice weighing about 30-40 g were taken. They were then placed into individual restraining cages leaving the tail11 hanging out freely. The animals are then allowed to adapt in the cages for 30 minutes before testing.

5.4.17 Preparation of test materials

Some VLC fractions of *Syzygium aromaticum* which were presumed to contain specific compound those analyzed by TLC and different soluble fractions (PESF, MSF, EASF and AQF) were used in a dose of 300 mg/kg body weight. The drug used for central analgesic activity was Morphin in a dose of 10 mg/kg-body weight. A control group was maintained in which mice were given 1% Tween-80 in normal saline.

5.4.18 Procedure

The lower 3.5 cm portion of the tail was immersed in a cup of freshly filled water of exactly

55±5°C. Prior to analgesic experiment, the animals were screened for the sensitivity test by immersing the tail of the mice gently in hot water maintained at 55±5^{0}C. The animals withdrawing the tail from hot water within 5 seconds were selected for the study. The selected mice were then divided into 8 groups having 5in each group and numbered. The animals marked group I received orally 1ml/100 g of body weight of 0.5 %w/v solution of sodium lauryl sulphate and served as control. The animals marked group II received orally 25mg/kg body weight of diclofenac sodium in 0.5%w/v suspension of sodium lauryl sulphate and served as standard. The animals marked test group III to VI received 420 mg/kg body weight of extracts of *Syzygium aromaticum* were screened for analgesic activity. After the drug was administered the reaction time was recorded at an interval of 0, 30, 60, 90 minutes (Table 5.4.2). The latency to withdraw the tail was recorded in sec with a stopwatch and a cut-off maximum latency of 7 second was established in order to prevent tissue damage.

5.4.19 Countings of tail immersion

Each mouse of all groups was observed individually for immersing time, and then the average value was calculated. The percent time elongation of tail immersing was calculated with respect to the control. The higher is the elongation percentage of the extracted fraction, the greater its central analgesic activity. The central analgesic activities of the test sample were compared with respect to Morphin. Percent time elongation is calculated by following equation-

$$\textbf{\% Time elongation} = \frac{\textbf{(Average time of tail flicking of test sample – Average time of tail immersing of the control group)}}{\textbf{Average time of tail flicking of the control group}} \textbf{ X 100}$$

5.4. 20 Statistical analysis

Data were analyzed by unpaired t-test using SPSS. At 95% confidence interval $p<0.05$ was considered statistically significant.

5.4.21 Result

Table 5.4.2: Central analgesic activity of clove fractions

Clove extracts / Time (min)	Control	Std. Morphin	SA-1	SA-6	PESF	EASF	MSF	AQF
30	4.26	9.60	6.11	6.00	6.10	5.00	4.36	4.87
	4.20	8.79	6.23	6.29	5.66	5.29	4.30	4.90
	4.21	8.90	6.91	6.16	6.29	5.39	4.00	5.10
	3.90	8.30	7.02	6.30	6.89	5.20	4.56	5.10
	3.69	8.70	7.90	6.50	6.91	5.20	3.66	4.90

Mean time of immersion ± SD	4.052 ± 0.24	8.858 ± 0.47	6.834 ± 0.71	6.25 ± 0.18	6.37 ±	5.216 ±	4.176 ±	4.974 ±
% Elongation	---	118.6	68.65	54.24	57.2	28.72	3.06	22.75
P value	---	0.001 Significant	0.0007 Significant	0.116 Non-significant	0.648 Non-ignificant	0.001 Significant	0.0002 Significant	0.0013 Significant
60	3.01	7.00	5.96	5.11	5.00	4.19	3.39	3.16
	4.60	7.98	5.80	5.00	5.09	4.11	3.48	3.14
	4.10	7.88	5.91	5.06	5.06	3.21	3.91	3.21
	3.90	7.61	5.46	4.94	4.98	4.60	3.00	3.19
	3.89	7.37	5.12	4.86	4.79	3.00	3.06	3.18
Mean time of immersion ± SD	3.9 ± 0.57	7.568 ± 0.39	5.65 ± 0.35	4.994 ± 0.09	4.984 ± 0.11	4.022 ± 0.68	3.368 ± 0.36	3.176 ± 0.02
% Elongation	---	94.05	44.87	28.05	27.79	3.12	-13.64	-18.56
P value	---	0.006 Significant	0.027 Significant	0.0040 Significant	0.887 Non-significant	0.0032 Significant	0.047 Significant	0.276 Non-Significant
90	2.50	6.40	3.40	3.92	3.19	2.09	3.00	2.0
	2.19	6.00	3.90	3.89	3.16	2.00	2.99	2.61
	2.08	5.92	9.29	3.91	2.11	2.01	1.98	2.22
	2.00	5.96	3.26	3.90	3.10	2.69	1.84	2.31
	2.92	5.29	3.11	3.81	2.98	2.41	3.06	1.56
Mean time of immersion ± SD	2.338± 0.37	5.914 ± 0.39	4.592 ± 2.64	3.886 ± 0.04	2.908 ± 0.45	2.24 ± 0.30	2.574 ± 0.60	2.14 ± 0.39
% Elongation	---	152.95	96.4	66.21	24.37	-4.19	10.09	-8.4
P value	---	0.0043 Significant	0.300 Non-significant	0.56 Non-significant	0.001Non-significant	0.025 Significant	0.30 Non-Significant	0.21 Non-significact

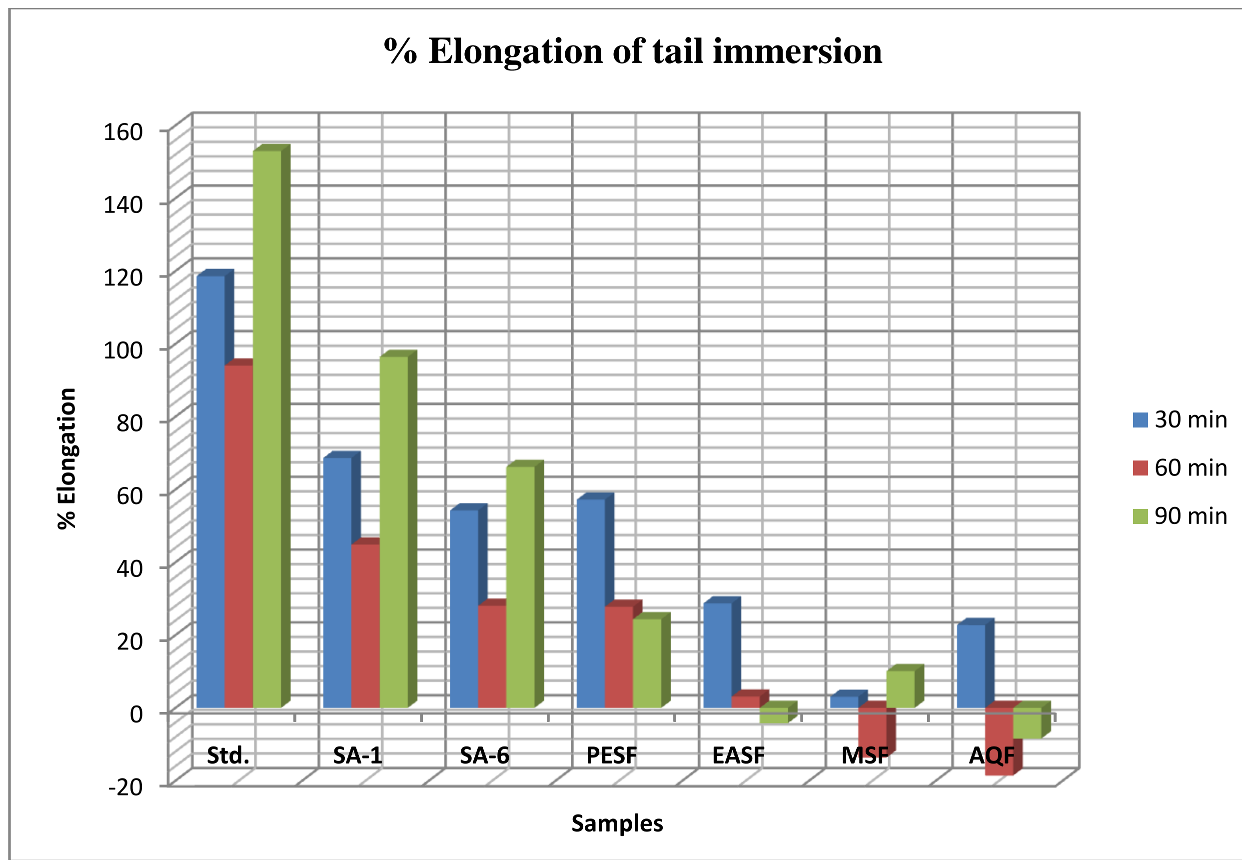


Figure 5.4.3: % elongation of tail immersion

5.4.22 Discussion

In tail-flick model, the methanol extract from *S. aromaticum* exhibited significant analgesic activity by increasing the reaction time of the rats compared to control (saline treated rats) at all time points, except at 60 min. Sodium salicylate and morphine sulfate were used as reference drugs, which are considered mild and moderate to severe analgesics, respectively. In comparison with control, morphine produced the most significant antinociception effect during all observation times, followed by the extract, while no significant analgesic effect was observed for sodium salicylate. The tail-flick method is based on the observation that morphine-like compounds are selectively able to prolong the reaction time of typical tail-withdrawal effect in rats. This method is also useful in differentiating central opioid-like analgesics from peripheral analgesics. Analgesic drugs which are centrally acting elevate pain threshold of animals towards heat and pressure. Therefore, the analgesic effect of the extract on this pain-state model indicates that it might be centrally acting. Both treatments produced comparable reaction times, suggesting that the *S. aromaticum* could be a better natural alternative for mild pain relief.

5.4.23 Conclusion

After 30 minutes SA-1, EASF, MSF, AQF shows statistically significant central analgesic

activity but SA-6 and PESF shows non-significant activity. After 60 minutes SA-1, SA-6, EASF, MSF and AQF show significant central analgesia. Only PESF shows non-significance after 90 minutes.

Chapter: 5.5

Thrombolytic Activity

5.5.1 Introduction

Atherothrombotic diseases such as myocardial orcerebral infarction are serious consequences of the thrombus formed in blood vessels [66, 67]. Thrombolytic agents such as tissue plasminogen activator, urokinase, streptokinase (SK), etc. are used to dissolve the already formed clots in the blood vessels. However, these drugs have certain limitations which cause serious and sometimes fatal consequences including hemorrhage, severe anaphylactic reaction, lacked specificity, etc. Moreover, as a result of immunogenicity multiple treatments with SK in a given patient are restricted [68]. Agents from plant source are expected to be less antigenic and cheaper. Considerable efforts have been directed towards the discovery and development of natural products from various plant and animal sources which have antiplatelet [69, 70], anticoagulant [71], antithrombotic and thrombolytic activities [72]. Epidemiologic studies have provided evidence that foods with experimentally proved antithrombotic effect could reduce risk of thrombosis. Herbs showing thrombolytic activity have been studied and some significant observations have been reported. These plants and herbs are widely used in traditional medicine or as spices. The organic solvent soluble extracts of these plants or spices are reported to have different pharmacological, biological and microbiological activities; the thrombolytic activity was performed by Md. R. Al-Mamun et al., method [73].

5.5.2 Experimental procedure

Streptokinase: The commercially available lyophilized streptokinase (SK) of 1,500,000 I.U per vial was used as positive control. The whole lyophilized powder of the vial was to be dissolved in 100 ml water to get 1,500,000 I.U. of SK solution, which is the recommended dose for myocardial patients. We took one tenth of the powder in 10 ml of water for each time to make 1,500,000 I.U. of SK from where 100 µl was used for *in vitro* thrombolysis.

5.5.3 Preparation of clots

Six milliliters of blood were withdrawn from healthy human volunteers (n=9) irrespective of gender following the guidelines set up by the local ethical committee. The blood was distributed into 12 previously weighed microcentrifuge tubes (0.5 ml to each centrifuge tube) to form clots, and they were then centrifuged at 2000 rpm for 5 min to let the serum separate above for easy removal from the centrifuge tube. The centrifuge tubes were then incubated in simulated body temperature i.e. at 37°C for 45 min in heat controlled incubator.

5.5.4 Clot lysis

The thrombolytic activity in terms of in vitro clot lysis was carried as reported earlier [10].

Briefly, 6 ml of blood was withdrawn from healthy human volunteers. The blood was distributed into 12 pre-weighed centrifuge tubes (0.5 ml to each centrifuge tube) and they were then centrifuged at 2,000 rpm for 5 min. The centrifuge-tubes were then incubated in simulated body temperature i.e. at 37°C for 45 min in heat controlled incubator. After 45 min, blood clot was formed at the bottom of each centrifuge tube. The serum was removed from each centrifuge tube above the clot carefully and completely without disrupting the clot. After removing the serum, the tubes that contained clot (but no serum) were weighed again. One hundred µl of each aqueous extractive was added in each microcentrifuge tubes, where streptokinase and distilled water were applied as positive and negative control respectively. All the 12 centrifuge-tubes were then again incubated at 37°C for 90 min to see their clot dissolving capacity. After 90 min the centrifuge-tubes were taken out of the incubator and the dissolved clot along with the applied agents (extract solution or streptokinase preparation or distilled water) were removed carefully and completely from the centrifuge tubes. Then the centrifuge tubes that contained undissolved clot were weighed. The weights of the clot before and after the clot lysed by the applied extract solutions, streptokinase and distilled water were calculated from the differences in the weights. The weight loss due to thrombolytic activity of each applied agent was calculated in percentage. The ability of the extracts to dissolve clot in percentage of weight loss were compared with that of standard and blank. The experiment was repeated 3 times with the blood samples of 3 volunteers. The equivalent doses of streptokinase for the highest activity showed by the plants and spices were also measured.

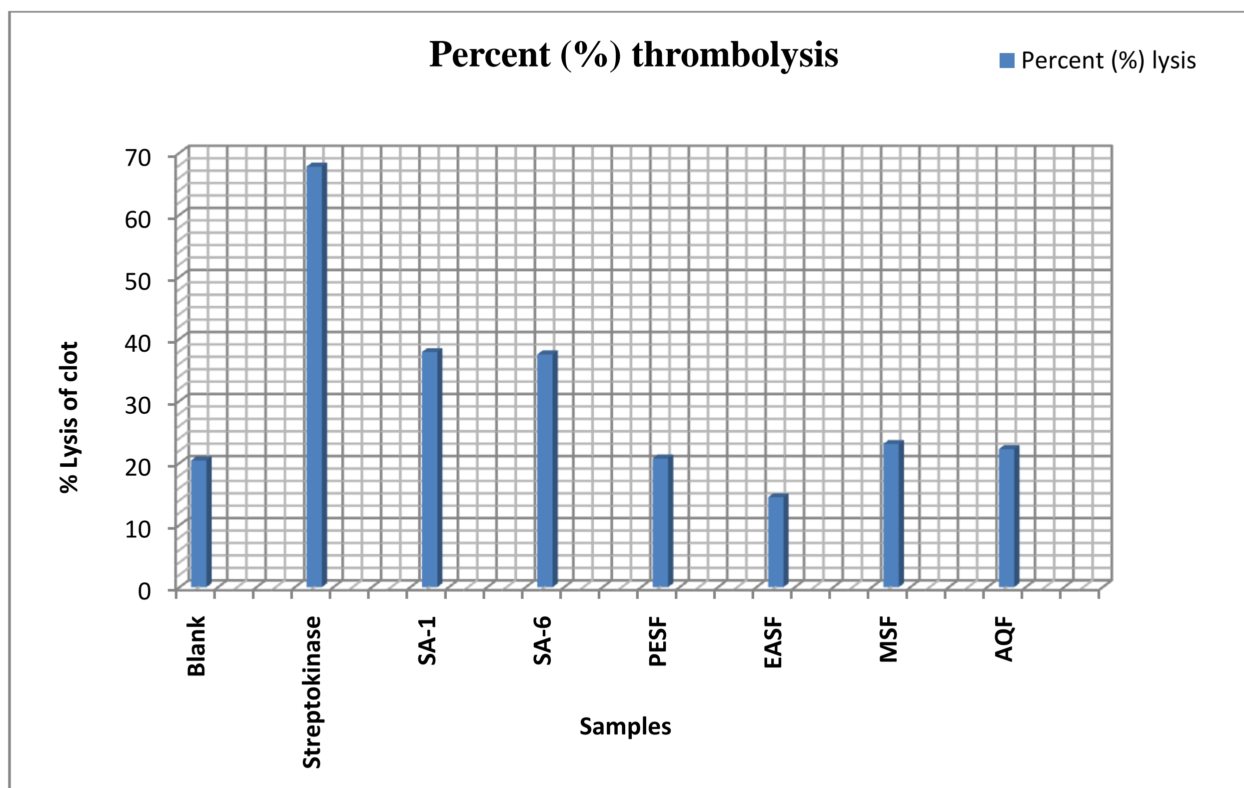
5.5.5 Result

Table 5.5.1: Clot lysis by clove fractions

Samples & fractions	Weight of empty eppendorf tube W1 gm	Weight of clot+eppendorf tube before clot disruption W2 gm	Weight of clot+eppendorf tube after clot disruption W3 gm	Weight of clot before lysis W4=W2-W1 gm	Mean Weight of clot before lysis ± SD W4’	Weight of lysis clot W5= W2-W3 gm	Mean Weight of lysis clot ± SD W5’	P value & Significance	Percent (%) lysis (W5’/W4’)X 100
Blank	0.787	1.394	1.369	0.607	0.607 ± 0.0005	0.024	0.025 ± 0.001	----	4.11
	0.788	1.396	1.370	0.608		0.026			
	0.787	1.394	1.370	0.607		0.024			
Streptok-inase	0.788	1.426	0.996	0.638	0.639 ± 0.001	0.430	0.433 ± 0.003	0.0002 Significant	67.76
	0.788	1.428	0.995	0.640		0.433			
	0.789	1.428	0.992	0.639		0.436			
SA-1	0.789	1.371	1.151	0.582	0.586 ±	0.220	0.222	0.0005	37.88

	0.789	1.371	1.147	0.582	0.006	0.224	± 0.002	Significant	
	0.778	1.372	1.148	0.594		0.224			
SA-6	0.779	1.370	1.150	0.591	0.592 ± 0.001	0.220	0.222 ± 0.002	0.0005 Significant	37.50
	0.778	1.370	1.146	0.592		0.224			
	0.779	1.372	1.148	0.593		0.224			
PESF	0.779	1.367	1.242	0.588	0.588 ± 0.002	0.125	0.122 ± 0.004	0.007 Significant	20.74
	0.778	1.369	1.252	0.591		0.117			
	0.779	1.366	1.242	0.587		0.124			
EASF	0.774	1.314	1.243	0.540	0.539 ± 0.001	0.071	0.078 ± 0.006	0.0001 Significant	14.48
	0.774	1.314	1.233	0.540		0.081			
	0.778	1.318	1.233	0.538		0.083			
MSF	0.798	1.332	1.208	0.534	0.546 ± 0.013	0.124	0.126 ± 0.002	0.001 Significant	23.07
	0.788	1.332	1.206	0.544		0.126			
	0.780	1.341	1.213	0.561		0.128			
AQF	0.789	1.346	1.225	0.557	0.553 ± 0.003	0.121	0.123 ± 0.003	0.004 Significant	22.24
	0.789	1.343	1.215	0.554		0.128			
	0.788	1.338	1.216	0.550		0.122			

Figure 5.5.1: % thrombolysis of clove fractions



5.5.6 Statistical analysis

The significance between % clot lysis by streptokinase and herbal extract by means of difference

in weight was tested were analyzed by unpaired t-test using SPSS. At 95% confidence interval $p<0.05$ was considered statistically significant.

5.5.7 Results

Addition of 100 µl SK as a positive control (1,500,000 I.U.) to the clots along with 90 min of incubation at 37°C showed 67.76% clot lysis. Clots when treated with 100 µl sterile distilled water (negative control) showed only negligible clot lysis (4.11%). Among the bud *S. aromaticum* gave thrombolytic activity of SA-1, SA-6, PESF, EASF, MSF & AQF are 37.88, 37.50, 20.74, 14.48, 23.07 and 22.24 respectively (Table 1). Clearly *S.aromaticum* showed potent *in vitro* clot lysis activity and are seemed to be potentsources for further investigation to find the responsiblelead compounds for the thrombolytic activity.

5.5.8 Discussion

This study evaluated the thrombolytic potential of some plants and herbs available in Bangladesh. Herbal preparations are used since ancient times for the treatment of diseases. Phytopharmacological investigation has lead to discovery plant derived drugs, which are effective in remedial of certain diseases, and renewed the interest in herbal medicines. About 30% of the pharmaceuticals are prepared from plants worldwide [68, 72]. A number of studies have been conducted by various researchers to find out the herbs and natural food sources and their supplements having antithrombotic (anticoagulant and antiplatelet) effect and there is evidence that consuming such food leads to prevention of coronary events and stroke. Although there are several thrombolytic drugs including those obtained two plants are seemed to potent sources for further investigation to find the responsible lead compounds for thrombolytic activity. Based on these results we suggest that to take *S. aromaticum*as preventive treatment by prospective atherothrombotic patients in crude form is safe, sincethey are already in use as spices, and no toxic effect isreported and thus can contribute in improvement of thepatients suffering from atherothrombotic diseases. By recombinant DNA technology, but side effects related to some of these drugs that lead to further complications have been reported.

5.5.9 Conclusion

Although the beneficial effects of thrombolytic therapy are now well established and the biochemical mechanisms of thrombolytic therapy have been elucidated, but the search for alternative and complimentary therapy is still continuing due to some reasons including availability and diversity of natural resources, easy access and affordability. Based on these results we suggest that to take *S.aromaticum* as preventive treatment by prospectiveatherothrombotic patients in crude form is safe, sincethey are already in use as spices, and no toxic effect isreported and thus can contribute in improvement of thepatients suffering from atherothrombotic diseases.

Chapter 5.6

Anesthetic Activity

5.6.1 Introduction

The need for some means of immobilizing in aquatic animals without harm to the subject has long been recognized. Documents show that basic and applied researches have focused on immobilization of fish as the most applicable aquatics subjected to aquaculture. Anesthesia as a method of immobilizing is now a common practice in fish. The use of anesthetics could possibly improve transport survival; however, to date anesthetic agents have not been briefly evaluated for use in shrimp such as what have been defined for fish. Anaesthesia abolishes pain in fish and induces a calming effect followed by loss of equilibrium, mobility and consciousness [74]. Anaesthetics in fish farms are used to minimize motility during handling and transport. This may reduce susceptibility to pathogens and infection [75]. According to many authors, an ideal anaesthetics fulfils such requirements as are a quick induction of anaesthesia together with its gradual recovery, anaesthetics must not be toxic to fish, must not develop any problems neither in attendance workers, must be quickly metabolized and excreted from organism, must not produce any rezidues and must be cheap [73]. Anaesthetics are also used in fish during artificial spawning, weighing, tagging, grading and surgical procedures [76]. Anaesthesia in fish may be produced by different agents, mainly tricaine methanesulphonate (MS-222), quinaldine sulphate, benzocaine and phenoxyethanol, which are hazardous, expensive and not very effective [77]. Clove oil is considered to be a potential fish anaesthetic [78]. This oily substance is distilled from buds, leaves and stems of clove tree (*Eugenia aromatica)*. The main chemical ingredient of clove oil is eugenol (70-98%) which is reported to possess high antibacterial and antifungal activity [77]. It is non-carcinogenic and non-mutagenic [78]. Eugenol has been successfully used as an anaesthetic in rabbitfish [79]; gold fish, crucian carp and Indian major carps. The anesthetic activity is measured by Matin et al., method [80].

5.6.2 Experimental prosedure

Live fishes (*Channa punctatus*) were collected from the local fish market of and transported to the laboratory using polyethylene bag with clean water aerated by agitation with fingers during transportation. The fish were then kept in plastic buckets in the laboratory and acclimatized in laboratory conditions for a week. Prior to measurement, an individual fish was caught with a fine mesh scoop net and its length recorded in centimetres using a steel measuring scale. Water temperature of the plastic containers was recorded daily with a thermometer and was found within desirable range (29 to 30°C).

Table 5.6.1: Stages of anesthesia

Sl. no	Stage	Characteristic behavior
0	1	Reactive to external stimuli; muscle tone normal
1	2	Sedation ,Onset of erratic opercular movement
2	3	Partial loss of equilibrium and erratic swimming
3	4	Total loss of equilibrium
4	5	Medullary collapse: Respiratory movement or opercular activity cease and fish death
5	Recovery	Ability to remain upright, regain control of equilibrium and normal swimming behavior

5.6.3 Experimental design: [80]

The fish was anaesthesized with three different concentrations of fractions. To prepare anaesthetic stock solution, 10 ml of corresponding clove fractions were taken in a volumetric flask and 90 ml of ethyl alcohol was added to prepare a stock solution of 10% clove oil. To make 0.01%, 0.02% and 0.03% clove oil, 1 ml, 2 ml and 3 ml of stock clove oil solution was made up to one litre of distilled water.

Table 5.6.2: Fractions used for anesthesia

Fish	Clove (fractions)	Clove solutions (%)	Observed parameter
Channa punctatus	Crude extract	0.01, 0.02, 0.03	1. Induction period 2. Recovery period
	VLC fractions SA-1 & SA-6	0.01, 0.02, 0.03	
	Soluble fractions (PESF, EASF, MSF & AQF)	0.01, 0.02, 0.03	

5.6.4 Induction and recovery period

Time taken from putting the fish into the induction tray until it became immobile was considered induction period. The recovery period extended from transferring the fish into the recovery tank until reappearance of mobility. When the fish were put into induction tray containing clove oil, they became excited and hypermotile followed by bubbling. The gill and fin movement progressively decreased, the fish lost equilibrium and started swimming laterally. Finally the fish became immobile with full loss of equilibrium and consciousness. After transfer to recovery tray reappearance of gill movement was noticed first. This was followed by fin and tail movement.

The fish started moving laterally. Gradually full equilibrium was regained and normal behaviour was restored. Similar behavioural changes during induction and recovery from anaesthesia have been reported elsewhere [81].

5.6.5 Result

Table 5.6.3: Mean induction & recovery period of clove extracts

Clove extracts	**Mean induction period ± SD**			**Mean recovery period ± SD**		
	0.01%	**0.02%**	**0.03%**	**0.01%**	**0.02%**	**0.03%**
Crude extract	14.9± 1.2	6.9 ± 1.0	3.9 ± 1.9	10.8 ± 1.1	11.5 ± 1.2	25.9 ± 2.5
SA-1	11.2 ± 1.1	4.3 ± 1.9	2.6 ± 1.9	9.2 ± 1.3	10.2 ± 0.9	18.6 ± 2.1
SA-6	11.8 ± 1.0	5.2 ± 1.4	2.6 ± 1.3	9.2 ± 2.3	10.3 ± 1.3	18.9 ± 1.3
PESF	12.3 ± 1.4	5.2 ± 1.0	2.8 ± 1.0	10± 2.1	10.8 ± 1.3	20.6 ± 1.3
EASF	13.1 ± 1.4	5.9 ± 1.1	3.9 ± 1.0	10.1 ± 1.1	11.2 ± 1.0	24.2 ± 1.0
MSF	11.8 ± 1.0	4.0 ± 1.1	2.8 ± 1.4	9± 2.4	10.4 ± 1.6	19.6 ± 1.9
P value & significancs	< 0.01 Significant	< 0.01 Significant	<0.01 Significant	< 0.01 Significant	> 0.05 Nonsignificant	<0.01 Significant

5.6.6 Statistical analysis: Statistical analysis of data was performed by one-way analysis of variance (ANOVA) to test the significance of variation between the treatment means by using SPSS programme. Induction and recovery period with different concentrations of clove oil of *Channa punctatus* is presented in Table 5.6.3. The differences between induction times in *Channa punctatus* concentrations were significant (P<0.01).The differences between recovery period between concentration 0.01 % to 0.03 % and 0.02 % to 0.03 % in *Channa punctatus* were significant (P<0.01).

5.6.7 Discussion

The field trials in this study indicate clove oil is an effective anaesthetic for handling adult *Channa punctatus*. Rapid increase of intensive aquaculture as a result of high demand for fish as a food source and employment of the technological advances applied, has made a new demand on the necessary chemicals. Nowadays, there are strict control in chemicals used in aquaculture, particularly with regard to their safety and efficacy [82]. Anesthetics are among important and broadly used veterinary medicines. Practices that require fish handling are a common source of stress in aquaculture operations and research activities [83]. To solve this problem, fish handlers have employed the use of anesthetics, added to water, to immobilize fish, reduce stress levels, and prevent mortality. The use of anesthetics facilitates work with fish at the research level and is required for invasive studies such as surgical preparations for physiological investigations, where the fish must be held immobile for extended periods of time. Anesthetics reduce stress responsiveness by causing a depression in nervous function [84]. However the type of anesthetic, the dosage and the time it can affect the physiology of the fish are the important points [85]. There are various types of anesthetics; the one most commonly used are tricaine methanesulfonate (MS-222, TMS) and it's the only anesthetic drug currently approved by the FDA for use on food fish. Clove oil is a dark-brown liquid, a distillate of flowers, stalks and

leaves of the clove tree *Eugenia aromatica* [85] having a mild anesthetic effect in human since antiquity [85] and fish. Eugenol, the active ingredient of clove oil, inhibits the synthesis of prostaglandin H (PHS), which accounts for the analgesic effect of clove oil [86, 87].
Keene et al. [87] showed clove oil is much less expensive than other chemicals including MS222. Most chemical anesthetics leave residues in fish tissues that require a certain withdrawal time before the animal could be released into the environment. He also described the induction of clove oil anaesthesis is faster compared with MS 222 anaesthetics at the same concentration; nevertheless the recovery period is 6 - 10 times longer. Therefore, alternatives are needed in cases when fish must be released immediately, for example, during spawning, biopsy, surgery or after implanting elastomer tags.

5.6.8 Conclusion

Clove oil does appear to have promise as an effective and safe anaesthetic for use on food fishes. However, until further studies are conducted regarding physiological effects, it should be used with caution and at the lowest concentrations necessary to induce anaesthesia.

Chapter 5.7

Antidiarrhoeal Activity

5.7.1 Introduction

Diarrheal diseases are a major problem in Third World countries and are responsible for the death of millions of people each year [88]. Diarrheoa is an alteration in normal bowel movement and is characterized by an increase in the water content, volume, or frequency of stools [89]. Plants have long been a very important source of new drugs. Many plant species have been screened for substances with therapeutic activity. Medicinal plants are a promising source of antidiarrheal drugs [90]. For this reason, international organizations including the World Health Organization (WHO) have encouraged studies pertaining to the treatment and prevention of diarrheal diseases using traditional medical practices [91]. The antidiarrhoeal activity was exhibited by Meite et al method [92].

5.7.2 Experimental animal

Albino Wistar rats (weighing 150 - 200 g) of both sexes, were housed in standard metal cages. They were provided with food and water *ad libitum*, and allowed a one-week acclimatization period prior to the study. Food was withdrawn 18 h before antidiarrheal experiments but water was allowed.

5.7.3 Experimental design

Diarrhoea was induced in mice by castor oil. Fifty five (55) rats were fasted for 18 h and divided into eleven groups of five animals each. The plant extract (250 mg/kg body weight) were administered orally to groups 3, 4, 5, 6, 7, 8, 9, 10 and 11 respectively. The first group received normal saline (5 ml/kg body weight) and served as control, while the second group received the standard drug, loperamide (5 mg/kg body weight). One hour later, all the animals received 2 ml/rat of castor oil orally. The animals were kept in separate cages with a transparent plastic container beneath the cage to collect faeces. The severity of diarrheoa was assessed each hour for the period of 6 h. The total number of faeces (both diarrheal and non-diarrheal) expelled were compared with the control group. The total score of diarrheal faeces for the control group was considered as 100%. The results were expressed as a percentage of inhibition of diarrheoa.

% Inhibition of defecation= [(A-B)/A] X100

Where A indicated mean number of defecation caused by castor oil;

B indicated mean number of defecation caused by drug or extract.

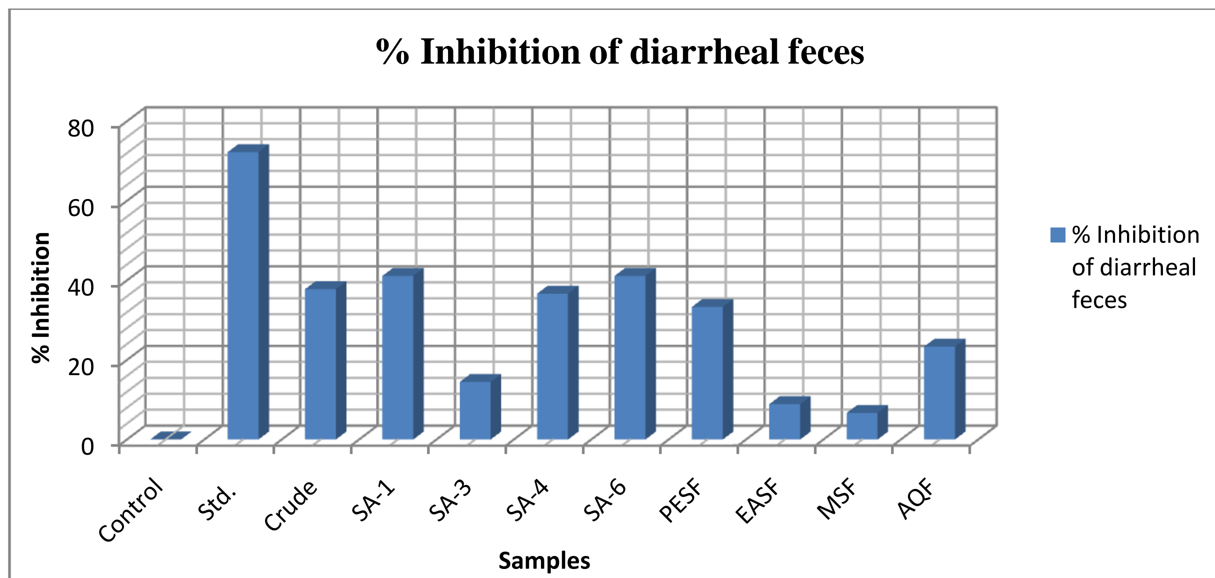
Table 5.7.1: Number of diarrhoeal feces reduction by clove fractions

	Number of diarrhoeal feces										
	Control	**Std.**	**Crude**	**SA-1**	**SA-3**	**SA-4**	**SA-6**	**PESF**	**EASF**	**MSF**	**AQF**
	19	5	11	10	15	12	12	13	18	16	14
	18	6	12	11	15	12	10	12	16	17	14
	18	4	11	11	17	12	11	12	18	17	13
	16	5	11	9	17	11	9	10	14	17	14
	19	5	11	12	13	10	11	13	16	17	14
Mean ± SD	18 ± 1.22	5 ± 0.70	11.2 ± 0.44	10.6± 1.14	15.4 ±1.67	11.4 ± 0.89	10.6 ± 1.14	12 ±1.22	16.4 ± 1.67	16.8 ± 0.44	13.8 ± 0.44

Table 5.7.2: % inhibition of clove fractions

	Control	**Std.**	**Crude**	**SA-1**	**SA-3**	**SA-4**	**SA-6**	**PESF**	**EASF**	**MSF**	**AQF**
% Inhibition of diarrheal feces	---	72.22	37.77	41.11	14.45	36.66	41.11	33.33	8.88	6.67	23.34

Figure 5.7.1: % inhibition of diarrhoeal feces



5.7.4 Statistical analysis

Data were analyzed by Data were analyzed by unpaired t-test using. At 95% confidence interval

$p<0.05$ was considered statistically significant.

5.7.5 Result

Control	**Std. (Loparamide)**	**Crude**	**SA-1**
---	$p<0.05$ Significant	$p<0.05$ Significant	$p<0.05$ Significant
SA-3	**SA-4**	**SA-6**	**PESF**
$p<0.05$ Significant	$p<0.05$ Significant	$p<0.05$ Significant	$p<0.05$ Significant
EASF	**MSF**	**AQF**	
$p<0.14$ Insignificant	$p<0.12$ Insignificant	$p<0.05$ Significant	

5.7.6 Discussion

Antidiarrheal properties of medicinal plants were found to be due to tannins, flavonoids, alkaloids, saponins, reducing sugar, sterols and/or terpenes [90]. The antidiarrheal activity of flavonoids has been ascribed to their ability to inhibit intestinal motility and hydro-electrolytic secretions [91] which are altered in this intestinal condition. *In vitro* and *in vivo* experiments have shown that flavonoids are able to inhibit the intestinal secretory response induced by prostaglandins E2 [93]. In addition, flavonoids present antioxidant properties [95] which are presumed to be responsible for the inhibitory effects exerted upon several enzymes including those involved in the arachidonic acid metabolism [95].

5.7.7 Conclusion

Clove fraction SA-1, 3, 4, 6, PESF, EASF & AQF showed significant antidiarrhoeal activity compared to Loparamide.

Chapter 5.8

Antiemetic activity

5.8.1 Introduction

Nausea and vomiting may be manifestation of a wide variety of conditions, including pregnancy, obstruction, peptic ulcer, drug toxicity, myocardial infarction, renal failure, and hepatitis. Vomiting can follow the administration of many drugs particularly cancer chemotherapeutic agents. Symptoms may occur upon emergence from general anaesthesia and often accompany infectious and non infectious gastrointestinal disorders. In cancer chemotherapy, drug induced nausea and vomiting may occur so regularly that anticipatory vomiting occurs when patients return for treatment before the chemotherapeutic agent is given. If not controlled, the discomfort associated with drug- induced emesis does not only affect the quality of life but can lead to rejection of potentially curative anti-neoplastic treatment. In addition uncontrolled vomiting can produce dehydration, profound metabolic imbalances, and nutrient depletion. Chemotherapeutic agents or their metabolites can directly activate the medullary chemoreceptor trigger zone or vomiting center or act peripherally by causing cell damage in the gastro-intestinal tract and releasing serotonin from enterochromaffin cells of the small intestinal mucosa. The released serotonin activates 5-HT3 receptors on vagal and splanchnic afferent fibres, which then carry sensory signals to the medulla, leading to the emetic response. Current anti-emetic agents are costly and possess serious side effects which include sedation, extral-pyramidal symptoms, severe headache, hyperglycemia in patients with diabetes mellitus, hence the need to search for safe, effective and affordable alternative anti-emetic agent. The antiemetic activity is studied by Yang et al method [96].

5.8.2 Experimental animal & design

The one day old chicks were divided into eleven groups of five chicks each and each chick was kept in a large beaker at 25° C for 20 min. Group 1 served as the control and was treated with 10 ml normal saline/kg. Groups 2 received Chlorpromazine was used as a standard drug (150 mg/kg. b. w). Group 3, 4, 5, 6, 7, 8, 9, 10 & 11 received SA-1, 3, 4, 6, crude, PESF, EASF, MSF & AQF administered at a dose of 150 mg / kg abdominally and volume of 10 ml / kg to the test animal on the basis of their body weights. After 10 minutes copper sulphate was administered orally at 50 mg / kg body weight, then the number of retching (an emetic action without emitting gastric material) was observed during next ten minutes.

The percent inhibition was calculated by the following formula [97]:

Inhibition (%) = (A-B/ A) X 100

Where,

A = Frequency of retching in control group & B = Frequency of retching in test group

Table 5.8.1: Antiemetic activity of clove fraction

	Control	**Std.**	**SA-1**	**SA-3**	**SA-4**	**SA-6**	**Crude**	**PESF**	**EASF**	**MSF**	**AQF**
Dose (mg/kg body weight)	**---**	**100**	**150**	**150**	**150**	**150**	**150**	**150**	**150**	**150**	**150**
	63	13	10	13	19	11	18	10	45	48	41
	61	15	9	15	19	11	17	9	40	47	40
	59	14	11	14	20	10	18	12	45	49	38
	61	14	11	14	21	11	18	13	43	48	38
	64	13	11	14	19	10	18	13	43	48	36
Mean ± SD	61.6± 1.94	13.8 ± 0.83	10.4 ± 0.89	14 ± 0.70	19.6 ± 0.89	10.6 ± 0.54	17.8 ± 0.44	11.4 ± 1.81	43.2 ± 2.04	48 ± 0.70	38.6 ± 1.94
% inhibition of emesis	---	77.59	83.11	77.27	68.18	82.79	71.10	81.49	29.87	22.07	37.33

Figure 5.8.1: Mean number of retches of clove fractions

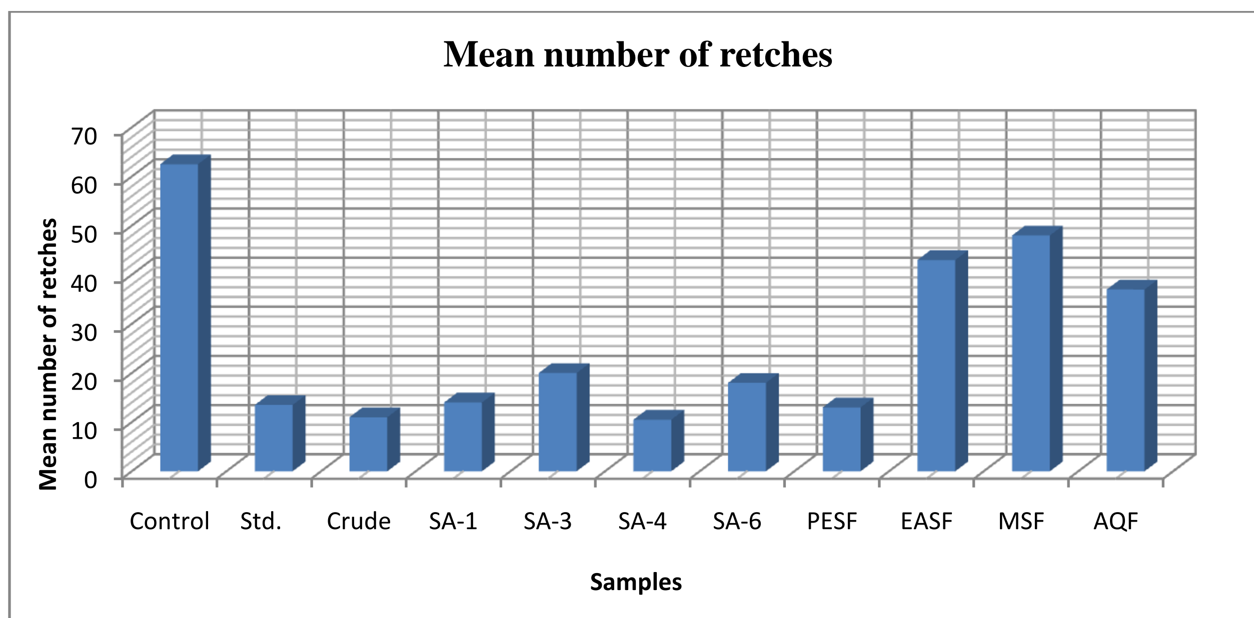
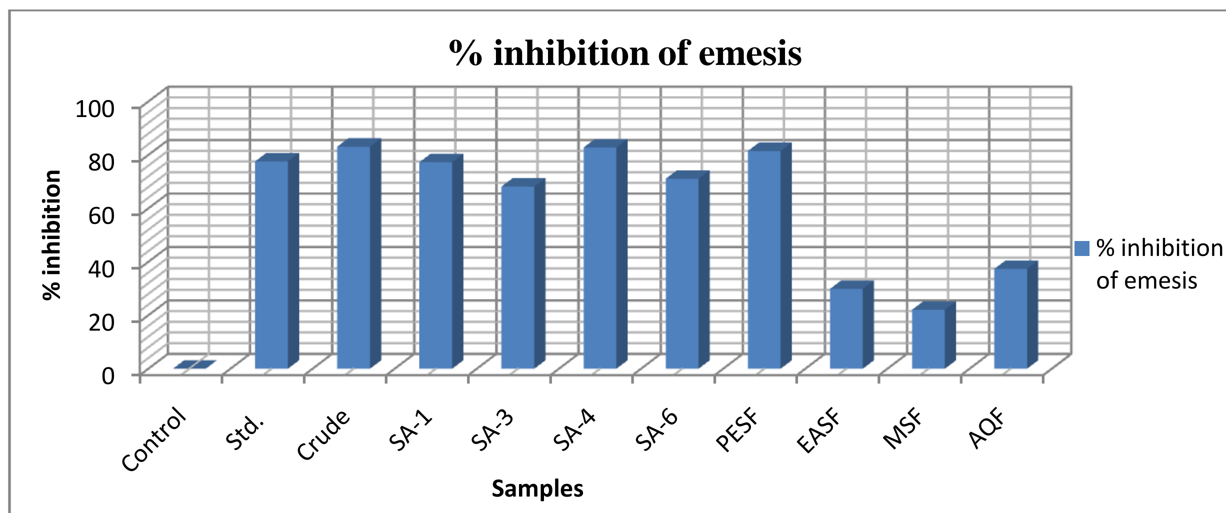


Figure 5.8.2: % inhibition of emesis



5.8.3 Statistical analysis

Data were analyzed by one-way ANOVA followed by Dunnett's t-test using. At 95% confidence interval $p<0.05$ was considered statistically significant.

5.8.4 Result

Control	**Std. (Chlorpromazine)**	**Crude**	**SA-1**
---	p<0.05 Significant	p<0.05 Significant	p<0.05 Significant
SA-3	**SA-4**	**SA-6**	**PESF**
p<0.05 Significant	p<0.05 Significant	p<0.05 Significant	p<0.05 Significant
EASF	**MSF**	**AQF**	
p<0.01 Significant	p<0.01 Significant	p<0.05 Significant	

5.8.5 Discussion

Although the results are significant but the mode of action is not known. However, as the oral copper sulphate induces emesis by peripheral action, and the extracts were able to effectively prevent its effect, it could be implied that these extracts have a peripheral anti-emetic action. From chemical point of view, *S. aromaticum* contain polyphenols and showed highest activity as compared to Chlorpromazine. Therefore, it may be said that polyphenol contents may play some role in anti-emetic effect. Further studies are required to determine the exact mode of action and the active compounds responsible for this effect.

5.8.6 Conclusion

On the basis of these results it can be concluded that all the extracts have anti-emetic potential

and are comparable with that of chlorpromazine (the reference drug).

Chapter 5.9

Anti-helminthic activity

5.9.1 Introduction

Helminth infections are among the most widespread infections in humans, distressing a huge population of the world. Although the majority of infections due to helminths are generally restricted to tropical regions and cause enormous hazard to health and contribute to the prevalence of undernourishment, anaemia, eosinophilia and pneumonia [98]. Parasitic diseases cause ruthless morbidity affecting principally population in endemic areas [99]. The gastro-intestinal helminthes becomes resistant to currently available anthelmintic drugs therefore there is a foremost problem in treatment of helminthic diseases [100]. Hence there is an increasing demand towards natural anthelmintics.

The anthelmintic drugs show their effects on the human body and their regular activities by causing helminthiasis which is a very severe parasitic disease. Mostly population and the stock parasites produce the resistance against the helminths parasites which causes morbidity and mortality [101]. Antihelminthics or anthelmintics are drugs that expel parasitic worms (helminthes) from the body by killing them. It also called Vermifuges (stunning) or Vermicides (Killing) [102]. The antihelminthic activity is estimated by Ajaiyeoba et al method [103].

5.9.2 Experimental animal

Adult earthworms (*Pheretima posthuma)* were used to evaluate anthelmintic activity in *vitro*. Earthworms were collected near the swampy water.

5.9.3 Experimental design

The assay was performed in *vitro* using adult earthworm (*Pheretima posthuma)* owing to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings for preliminary evaluation anthelmintic activity [104]. Test samples of the extract was prepared at the concentrations, 10 mg/ ml and five worms i.e. *Pheretima posthuma* of approximately equal size (same type) were placed in each nine cm Petri dish containing 25 ml of above test solution of extracts. Piperazine citrate (10 mg/ml) was used as reference standard and distilled water as control [105]. This procedure was adopted for all three different types of worms. All the test solution and standard drug solution were prepared freshly before starting the experiments. Observations were made for the time taken for paralysis was noted when no movement of any sort could be observed except when the worms were shaken vigorously. Time for death of worms were recorded after ascertaining that worms neither moved when shaken vigorously nor when dipped in warm water (50° C). All the results were shown in Table.5.9.1 and expressed as a mean ± SEM of five worms in each group.

Table 5.9.1: Paralysis and death time of clove fractions

Paralysis time									
	Std.	**Crude**	**SA-1**	**SA-3**	**SA-6**	**PESF**	**EASF**	**MSF**	**AQF**
	8	36	4.5	10	4	49	22	4.5	48
	7.5	35	9	12	9	48	19	7	48
	9.5	35	7.5	9	5.5	42	19	4	40
	8	33	6	11	5	38	21	4	50
	9	35	7	11	5	39	21	5.5	50
Mean paralysis time± SD	8.4 ± 0.82	34.8 ± 1.09	6.8 ± 1.68	10.6 ± 1.14	5.7 ± 1.92	43.2 ± 5.06	20.4 ± 1.34	5 ± 1.27	47.2 ± 4.14
P value & significance	---	< 0.01 Significant	0.33 Nonsignificant	0.07 Nonsignificant	< 0.01 Significant	< 0.01 Significant	< 0.01 Significant	< 0.01 Significant	< 0.01 Significant
Death time									
	Std.	**Crude**	**SA-1**	**SA-3**	**SA-6**	**PESF**	**EASF**	**MSF**	**AQF**
	15	58	11.5	17	10	56	41	11.5	49
	12	59	11	16.5	12	52	41.5	12	50
	17	58	10.5	13	17	48	39	17	42
	13	55	11	19	12	48	36.5	11	51
	15	67	13	21	12	46	42	12.5	51.5

Mean death time ± SD	14.4 ±1.94	59.4 ± 4.50	11.4 ± 0.96	17.3 ± 2.99	12.6 ± 2.60	50 ± 4.0	40 ± 2.26	12.8 ± 2.41	48.7 ± 3.86
P value & significance	---	<0.05 Sig	0.40 Non significant	0.44 Non significant	0.25 non significant	<0.05 Significant	<0.05 significant	0.88 Non significant	<0.05 Significant

Figure 5.9.1: Mean paralysis time of clove fractions

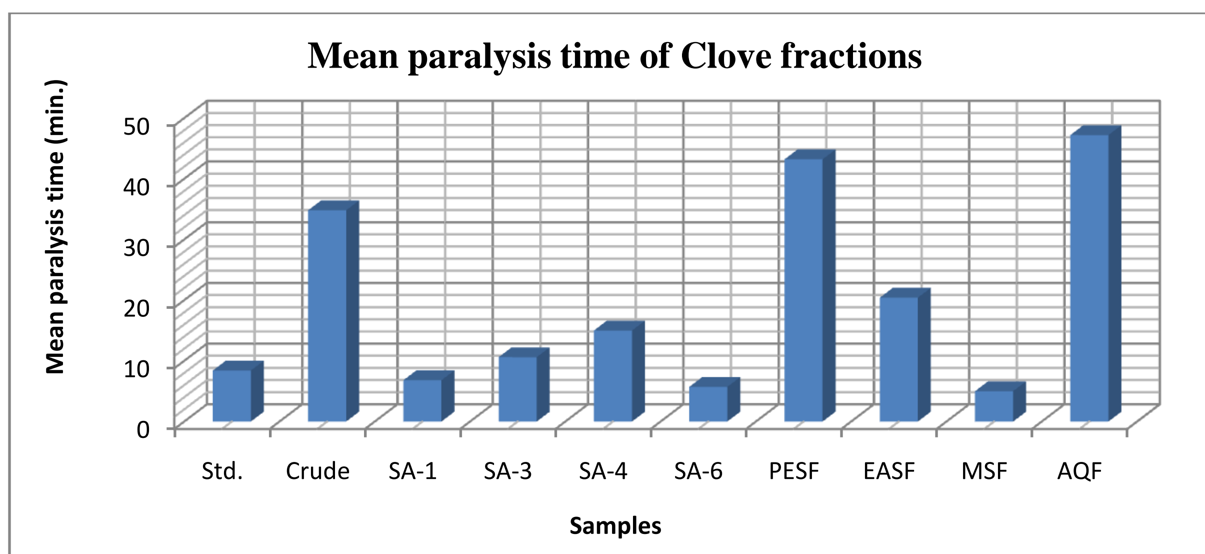
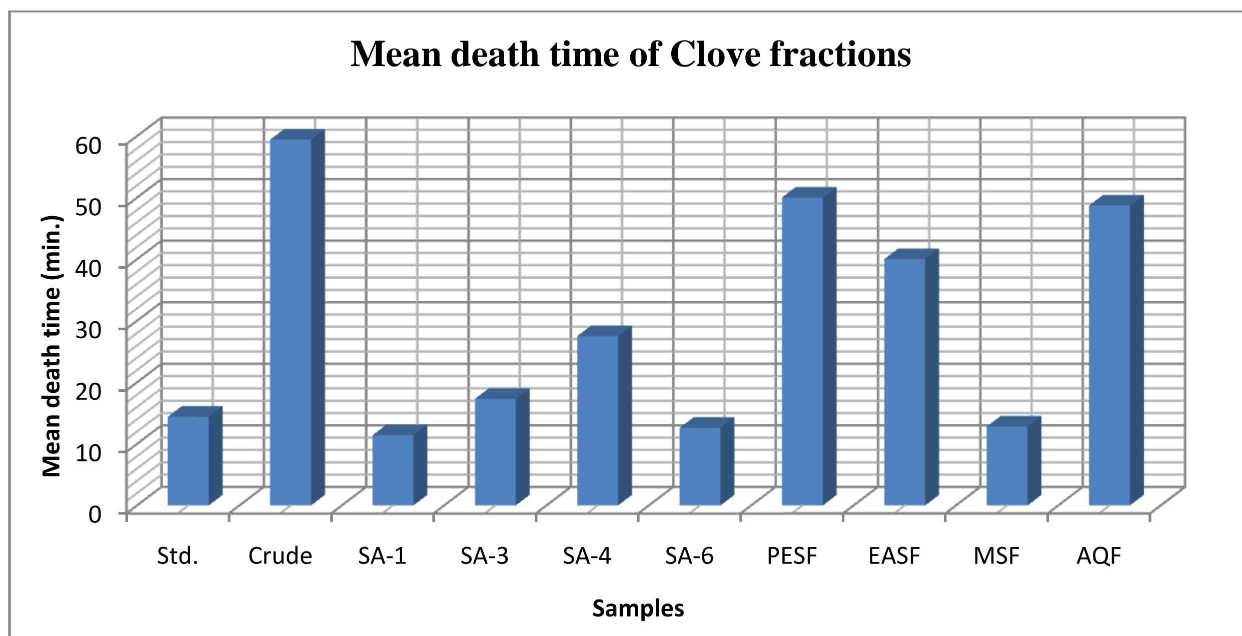


Figure 5.9.2: Mean death time of clove fractions



5.9.4 Discussion

The observed *in vitro* antihelminthic activity of different soluble fractions of test plant indicates the potential of the plants to exhibit high morbidity and mortality on the test organisms, particularly at the 20 mg/ml concentrations used. This activity compares significantly with the standard chemical agent piperazine (of common use among the local populace) at same concentration. The significance of this assay is the evidence (from available results) that the plant extracts used have shown great promises in ethno- medicinal uses as both antimicrobial and antihelminthic compounds and should in addition to their established uses especially as spices, be further standardized and incorporated into capsules, tablets or other formulations for easy acceptability to the local populace.

5.9.5 Conclusion

From the observations made, higher concentration of extract produced paralytic effect much earlier and the time to death was shorter for all worms. Aqueous extract showed anthelmintic activity in dose-dependent manner giving shortest time of paralysis (P) and death (D) with 50 mg/ ml concentration, for all three types of worms. Extract exhibited potent activity.

Chapter 5.10

Antipyretic activity

5.10.1 Introduction

Hyperpyrexia or fever is usually caused as a secondary impact of infection, tissue damage, inflammation, graft rejection and malignant tumors or other diseased states. Typically, the infected or damaged tissue initiates increased formation of pro-inflammatory mediators, including cytokines such as interleukin 1β, α, β and TNF-α, which generally increase the synthesis of prostaglandin E2 (PGE2) near pre-optic hypothalamus area and thus triggering the hypothalamus to elevate the body temperature [106]. Membrane stabilization is a possible mechanism of action for the anti-inflammatory activity. There are many anti-inflammatory drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs) to treat the consequences of inflammation. The effect of these drugs including herbal preparation on the stabilization of erythrocyte membrane exposed to hypotonic and heat has been studied extensively. But these studies showed that, these drugs are not free from adverse effects, as they are responsible for intestinal side effects and mucosal erosions that can progress into ulcers [107]. For these reasons, many researchers have focused on medicinal plants for finding natural anti-inflammatory drugs.

Anipyretic activity is performed by Paul et al method [108].

5.10.2 Experimental design

Test animals

Healthy albino mice (100-120g) were used to assess the antipyretic. The animals were acclimatized in the lab, where they were maintain on standard animal pellet and water ad libitum. The rats for antipyretic study were selected based on the measured basal anal temperature (using a thermistor probe) not exceeding 37°C and achieving anal temperature elevation of at least 0.1°C in response to intra peritoneal (i.p) administration of 15% w/v *Saccharomyces cereviseae* at dose of 10ml/kg body weight. The animals for the antipyretic studies were divided into five groups of the animals each and fasted overnight.

5.10.3 Preparation and Administration of Drug

All the standards and tested samples were prepared using sterile normal saline solution which also served as control in the different experiments. Oral (p. o) administration was carried out using a canula-syringe assemblage, and hypodermic syringe-needle assembly used for intra-peritoneal (i. p) administration.

5.10.4 Antipyretic study Baker's Yeast-Induced pyrexia

Adopting standard procedure as described by Paul *et al*., [108] previous study, albino mice

earlier selected and induced pyrexia were randomly divided into five groups of animals each. The animals were allowed to starve overnight in their respective cages. Twenty hours after the administration of 15% *Saccharomyces cereviseae* (Baker's yeast suspension) the anal temperature of each animal was measured to determine the initial temperature by insertion of digital thermometer probe to a depth of 1.5-2.0 cm into the rectum of the rats. Only animals which developed satisfactory pyrexia (1°C or more increase in rectal temperature) after twenty four hours were used. Fever induced animals were divided into five groups of five animals in each group. Group I served as pyrexia control receive normal saline. Group II to receive standard drugs paracetamol at an oral dose (P.O) while group III to IX received 100 and 200mg/kg oral dose of each soluble fractionsof clove respectively. Rectal temperatures of all micewere recorded using digital thermometer four hours after the administration of the doses.

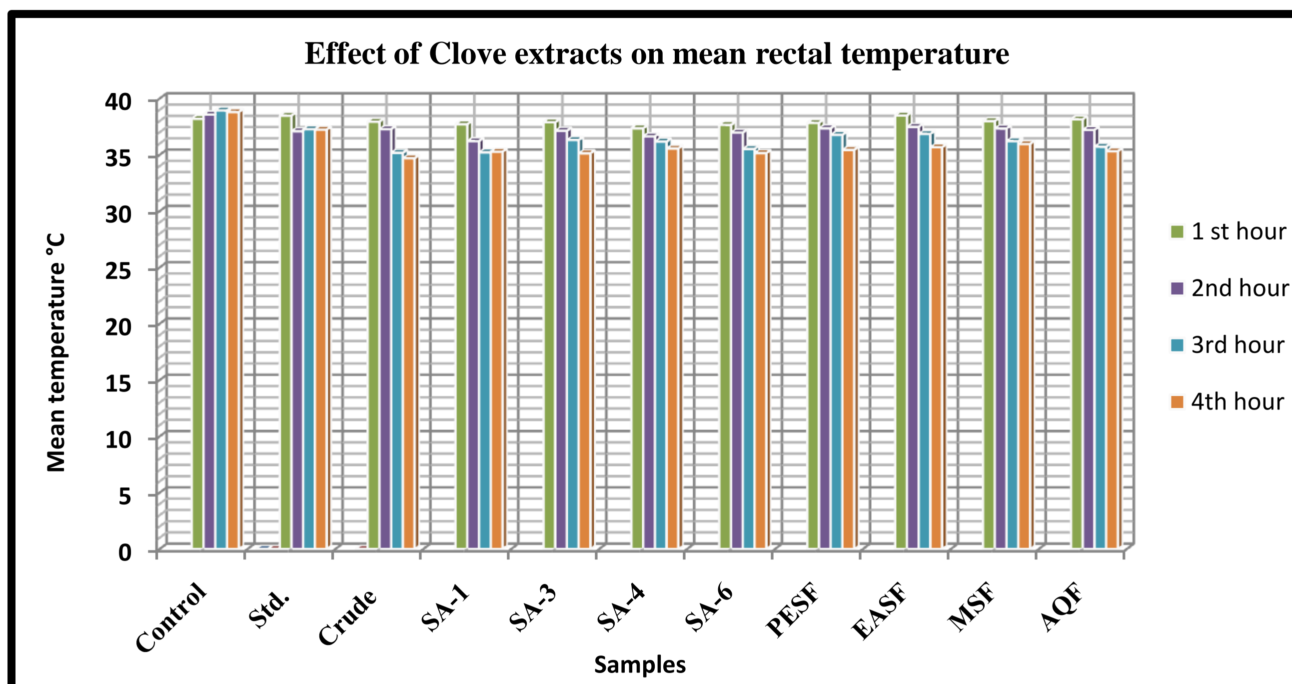
5.10.5 Result:

Table 5.10.1: Antipyretic activity of clove fraction

Treatment	**Control**	**Std. Paracetamol 500 ml/kg**	**Crude 50ml/kg**	**SA-1**	**SA-6**	**PESF**	**EASF**	**MSF**	**AQF**
1st hour	37	38.8	37	37	38.6	37.5	38.6	37.6	37.8
	38	38	38.8	38.2	37.5	37.8	38	37.8	38.5
	39	37.9	38.9	38.1	37	37.9	38.9	38.7	38.5
	38	38.8	37	37.2	38	38	38.5	37.9	38
	39	38.9	38	38.1	37.2	38	38.5	38	38
Mean± SD	38.2± 0.83	38.48± 0.48	37.94± 0.92	37.72± 0.57	37.66± 0.64	37.84± 0.20	38.5± 0.32	38± 0.41	38.16± 0.32
P value	----	0.53 Non-significant	0.65 Non-significant	0.32 Non-significant	0.28 Non-significant	0.37 Non-significant	0.47 Non-significant	0.64 Non-significant	0.92 Non-significant
2nd hour	38.9	37.5	37.4	36.1	37	37.5	37	37	37.8
	38	37.8	37	36.5	36.9	37	37.2	37.7	37.8

	38	36.8	37.3	36.5	36.9	37.5	37.5	37.5	37
	38.5	36.9	37.4	36	37.2	37.3	37.9	37	36
	39.5	36.5	37.3	36	36.9	37.5	37.8	37.5	37.5
Mean± SD	38.58 ± 0.63	37.1± 0.53	37.28± 0.16	36.22± 0.25	36.98± 0.13	37.36± 0.21	37.48± 0.38	37.34± 0.32	37.22± 0.75
P value	----	0.004 Significant	0.002 Significant	0.03 Significant	0.0005 Significant	0.003 Significant	0.01 Significant	0.004 Significant	0.01 Significant
3rd hour	38.7	37	35	35	35.5	36.5	36	36.6	35.9
	39	37.5	35.4	35.5	35.5	36.8	37.5	36.5	36
	39.5	37.4	35.6	35.4	35.7	36.8	37.8	36	36.5
	39	37	34.9	35	35	36.9	36.5	36	35.2
	38.5	37.5	35	35.2	35.9	36.9	36.5	36	35
Mean± SD	38.94 ± 0.37	37.28± 0.25	35.18± 0.30	35.22± 0.22	35.52± 0.33	36.78± 0.16	36.86± 0.75	36.22± 0.30	35.72± 0.61
P value	----	0.026 Significant	0.001 Significant	0.002 Significant	0.003 Significant	0.0005 Significant	0.0005 Significant	0.003 Significant	0.02 Significant
4th hour	38.5	37.3	34	35.2	34.9	35.5	35.4	36.1	35
	39	37.1	34.5	35.1	35	35.5	35.9	36	35.9
	39.5	37.2	34	35.6	35.6	35	35.8	35.9	34.6
	38	37.1	34	35.5	35.6	35.4	35.6	35.9	35.1
	39	37.5	37	35	34.7	35.8	35.7	35.9	36
Mean± SD	38.8± 0.57	37.24± 0.16	34.7± 1.30	35.28± 0.25	35.16± 0.41	35.44± 0.28	35.68± 0.19	35.96± 0.08	35.32± 0.60
P value	----	0.0003 Significant	0.0001 Significant	0.003 Significant	0.007 Significant	0.006 Significant	0.006 Significant	0.01 Significant	0.009 Significant

Figure 5.10.1: Effect of clove fractions on mean rectal temperature



5.10.6 Statistical analysis

The statistical analysis of antipyretic study using student t-test analysis indicated $P \leq 0.001$ as level of significance in the Baker's yeast induced anal temperature. The t-test analysis showed temperature after 1st hour of clove all of the extracts showed no significant activity at all but after the 2nd, 3rd and 4th hour of administration all of the compounds and solvent soluble extracts (i.e. SA-1, SA-6, PESF, EASF, MSF and AQF) of *S. aromaticum* were more effective than the standard drug and control with significant value of ($P \leq 0.05$).

5.10.7 Discussion

In this study, different solvent soluble fraction of this crude chloroform extract and compounds of *S. aromaticum* activity against pyrexia, the baker's yeast (*Saccharomyces cereviseae*) was used to act as pyrogen inform of an exogenous stimulus. This evaluated body temperature and intensified the process of lipid per oxidation, which indicated that increase of oxidative stress causes pyrexia. The supplementation of standard drugs, control and ethanolic extract of *Eugenia aromatica* decrease the lipid per oxidation processes [45]. According to a review article of clove as champion spice, *Eugenia aromatica* contains flavonoid that has antioxidant activity. Thus, its antioxidant activity may be one of the possible mechanisms to reduce the elevated body temperature [46]. The chloroform extract may reduce prostagladin PGE2 by its action on cycloxygenase (COX-2), after been inhibited by the arachidonic acid pathway. Such inhibitory usually decrease elevated body temperature. The antipyretic test indicated that the ethanolic extract of *Syzygium aromaticum*was able to significantly ($P\leq 0.001$) decrease the yeast induced

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and temperature elevation in the animals. This showed that extracts of *S. aromaticum* were more effective in reducing fever than standard paracetamol, or acetic salicylic acid and placebo which also showed its possession and inhibitory activity against arachidonic acid pathway.

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