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PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF SOME ORCHIDS

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Declaration

I, Gurucharan D.N. hereby declare that this thesis entitled **"PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF SOME ORCHIDS"**, embodies the results of bonafide research work done by me under the guidance of **Dr. Krishnaswamy K.**, Associate Professor, Department of Botany, Sahyadri Science College, Kuvempu University, Shimoga-577203, Karnataka. I further declare that the results of this work have not been previously submitted for any other diploma or degree either in any institution or any other University.

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Dedicated to My

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LIST OF ABBREVIATIONS

Cm	-	Centimeter
Inch	-	Inches
μm	-	Micrometer
^{o}C	-	Degree Centigrade
%	-	Percentage
mm	-	Millimeter
mg	-	Milligram
ml	-	Milliliter
DPPH	-	1, 1-diphenyl-2-picrylhydrazyl.
ABTS	-	2, 2^{1}- azino-bis[3-ethylbenzothiazoline-6-sulphonic acid].
GAE	-	Gallic Acid Equivalent.
SDA	-	Sabourauds Dextrose Agar
NA	-	Nutrient Agar
PDA	-	Potato Dextrose Agar
MBC	-	Minimum Inhibitory Concentration
MIC	-	Minimum Inhibitory Concentration
DMSO	-	Di Methyl Sulphoxide
LCMS	-	Liquid Chromatography Mass Spectroscopy

INTRODUCTION

Orchidaceae is a unique family among plant groups, they constitute a strange, yet interesting clan and in almost all aspects. Orchidaceae stands apart from the rest of the plant families, maintaining intriguing individuality, all its own. The various morphological, physiological and genetic peculiarities, so liberally displayed by this group of plants have stimulated fields of research so vast and varied that Orchidology today has developed into one of the most dynamic branch of Botany (Abraham and Vatsala, 1981).

Orchidaceae is one of the largest families of flowering plants in the world. Many of the estimated 20,000-30,000 species are locally distributed and are generally rare (Atwood, 1986). Orchids are concentrated in three areas, notably tropical America, Indo-Malayan and the Eastern Himalayas (Tremblay, 1997; Benavides *et al*., 2005). About 73% of species are epiphytes, and these make a significant contribution to the epiphytic plant communities in the tropical forests. Orchids are very widely distributed but their largest diversity occurs in the tropics (Dressler, 1981). Orchid species are not to be found in a few isolated islands and Antarctica (Dressler, 1981).

The term orchid goes back to classical Greek times. The scholar Theophrastus, who was one of Plato's students and is often called the father of Botany, described a plant with paired roots that looked like testicles; he gave it the name Orchis from the Greek word for testicle (Arditti, 1966). Later Greek botanist Dioscorides interpreted the description as referring to an orchid. Carolus Linnaeus adopted the name Orchid in species plantarum, published in 1753. The British Botanist Lindley introduced Orchidaceae as the name of family in the year 1836.

Orchidaceae represents a peak in the evolution of monocots and is one of the most successful families of flowering plants as it clear from the wide distribution and the innumerable number of species spread all over the world. It consists of 25000-35000 species belonging to 600-800 genera (Arditti, 1979).

The Orchidaceae, is one of the largest and most diverse families of plants, including somewhere between one tenth and one fourteenth of all flowering plant species. In this actively evolving group, highly specialized adaptation for attracting deceiving

and manipulating insects to achieve cross-pollination have fascinated observers since the time of Darwin (Dressler, 1981).

Taxonomically, orchids are a unique group of plants. They are vastly different vegetatively, yet all species can be tied together by their floral characteristics as members of this enormous family. They are monocotyledonous herbaceous perennial plants. They may be erect grower (monopodial) prostrate (sympodial) with a few climbers. Although the majority of orchids are so-called green plants. In this family, few are saprophytes and leafless plants (Larson, 1980). The exotic and delicate beauty of orchids has attracted the botanists and layman alike. The diversity of forms found in the orchid family is quite amazing. They are represented almost throughout the world in tropical and temperate climates.

The exquisite beauty of flowers, variety of fragrance, brilliance in colours remarkable range of sizes, manifold shapes, and variations in the form, attractive habits and wide distribution in the earth has aroused highest admirations throughout the world (Bose and Bhattacharjee, 1980). Orchids are herbaceous perennials that occur as shrubs, veins and even grasses. They may bear a single flower or many flowers. They are found from the Arctic to the borders of the Antarctic. They grow in tropical rain forests, subtropical plains and alpine meadows, in bogs, moors and desert; in deep valleys and on high mountains. Most of the tropical and sub tropical species are epiphytic, they are independent, non-parasitic plants grow on the tree trunks and limbs of shrubs. It is not uncommon, however to find normally epiphytic species growing on rocks. In the temperate and arctic regions, the genera are belongs to terrestrial species.

Unusual feature of this group are the complex and highly specialized flowers, the minute microscopic seeds, having no endosperm, undifferentiated embryo, the great number of seeds in an orchid capsule. The irregular flowers of orchids developed in connection with insect pollination and the extraordinary beauty of flowers makes orchids the basic of a multi-million dollar floral industry.

The cultivation of orchids is not new. Confucius (551-479BC) mentioned orchids in his writings; he speaks of the fragrance of land in the home, indicating that the Chinese were using orchid flowers to decorate their homes (Withner, 1959). However, the evolution of orchid, culture from the hobbyist to commercial production was very slow. The early Greeks and Romans looked to the orchids more for their medicinal than for their aesthetic qualities. It was not until the 1700s that interest in orchids really began to develop.

Among the ornamental plants orchids are considered as the most elite group and the blossom remain fresh for quite a long period. Thousands of prestigious hybrids have been developed which often boast of gorgeous and colourful bloom with greater vigour. Singapore, Malaysia, Japan, Thailand, Srilanka and other countries earn a good foreign exchange by exporting orchid flowers to the developed countries. Apart from Darjeeling and Kalipong (W.B), Arunachal Pradesh and Shillong are the potential suppliers of rare and hybrid orchids. Besides their great ornamental value, many of the orchids having aromatic compounds for instance Vanillin is made from the terminated poach and seeds of the climbing orchid *Vanilla planifolia*. It is used as flavours in the preparation of sweets, puddy, custards, chocolates etc. Apart from these economic values some of the orchids have medicinal properties (Manibhusan, 1991).

Orchidaceae is a cosmopolitan family and occur even in the frozen areas of Alaska, the snow-covered areas of the Himalayas and sandy deserts of Australia and Africa. New Guinea has more orchids native to the island than any other part of the world. They are abundant in tropical forests, where the majority are epiphytes. In the temperate and arctic regions, the genera are terrestrial. In India the orchids are present in North-East Himalayas, North Eastern Ghats, Western Ghats, Palani, Nilgiri hills, Biligiri hills, Andaman and Nicobar islands and in Rajasthan. Orchids can grow above the sea level from 50-2500mts.

Terrestrial orchids grow on the ground, with their fleshy roots in the soil and require uniform supply of water like ordinary plants. They grow in extra-tropical regions. Epiphytic orchids are the inhabitants of the tropical moist deciduous forest. But few

species are saprophytes, lacking chlorophyll entirely and obtaining their food by absorption from the soil complex organic substances.

According to the diversified habit of growth, vegetative structures of the orchids are modified. The majority of the orchids found in temperate climate are terrestrial in habit. Most of the terrestrial orchids are deciduous and tuberous rooted. The tuber arises from a bud at the base of the stem and is a storage organ. It undergoes a period of rest during the cold and dry season and the leaves die and wither with the advent of the warm. In wet season the bud spouts, produces leaves and flowers.

In tropical rain forest, epiphytes frequently grow on trees or on rocks (lithophytes). They support themselves by means of adventitious roots. Orchids are also divided into monopodial and sympodial types. In the sympodial orchids, the roots are normally produced from rhizome. In general, the roots are cylindrical, often thread like branched and of varying length, some are furnished with numerous root hairs. The roots of epiphytic orchids are whitish, spongy and pulpy coat called velamen covers them. It protects the inner conductive channel of the root, absorbs water from damp air and helps to cling to any surface it comes in contact. The green tip on the root is an important indicator of healthy growth.

Monopodial orchids do not make separate new growth in each season and do not have a rhizome. The single stem increases in height throughout the life period. New leaves appear during growth and the flower appears from the axils of the leaves at the upper portion of the plants. The leaves are arranged into two rows, one opposite the other, or the leaves of one row alternate with those of the other. *Aerides, Phalaenopsis, Rhyncostylis, Sacclobium, Vanda* and allied genera are the examples of monopodial orchids. In some orchid species like *Aerides, Vanda, Luisia,* the leaves are cylindrical, much resembling the stem from which they arise, being terete, dull colour and usually sharp pointed. The leaves are leathery in texture and persistent for several years. In monopodial types, for propagation purpose, one can cut off the top of the plant to have another plant and new roots will arises from the stem, itself. But in the case of sympodial orchids, a portion of the plant including the rhizome is separated.

In sympodial orchids extension of the rhizome develops new growth and each new growth produces its own set of roots. Plants of these groups often have more than one stem. The stems are generally thickened and bulbous and have earned the name pseudobulb, very useful device for storage of water and food, which enable the plants to withstand drought. The pseudobulbs are formed by the swelling and consolidate of the base of each new growth. Sympodial types constitute a large number of orchids. All terrestrial orchids both temperate and tropical, all pseudobulbs species including the epiphytes of the orchids whose stems mature in one season are included in this group. *Epidendrum, Coelogyne* etc. are epiphytic orchids with sympodial growth habit.

The orchid flower exhibit great range of variation in size, shape and colour (Vij, 1986; Manibhusan, 1991). Some of them have an appearance like Ladies slipper (*Cypripedium*). They may assume various shapes of animals and insects, for example, *Ophrys apifera* looks like a bee; *Coeloglossum viride* looks like a minute frog; *Brassia* spike suggests a small collection of colourful spiders; *Peristeria elota* or the dove orchid looks like a small dove and *Bulbophyllum purpureorhachis* simulates a lizard (Mukherjee, 2002).

The flowers are very distinctive and range in size, form, few millimeters to several centimeters in diameter. They exhibit different range of colour and have a wide variety of fragrance. Some of the fragrant orchids are *Malaxis tennuifolia, Aerides odorata, Aerides crispum etc.* The shape of the flower are fantastically varied that many of them inevitably seem to be imitates something else. The orchid flowers are very attractive, colourful and long lasting among the flowering plants for instances, *Grammetophyllum multiforum* holds the floral record by remaining in flower for a full nine months. Among other noteworthy characteristics of orchids are resupination, a twisting of the bud, that occur as the bud becomes a flower and a fungus infection that is necessary for the germination of the orchid seed.

The flowers are simple with seven floral parts, three sepals, three petals and the column or gynostegium. One of the three petals designated as the lip or labellum is the showy part of the flower. The lip has considerable variation in shape. It may be tubular as in *Cattleya,* expanded with warts and protuberances as in *Oncidium* or may

be indistinguishable from mother petals as in *Masdevallia* and even saccate as in the Lady's slipper. It is commonly much more brilliantly coloured than the other five parts and gives to flower its showiness. It serves as a landing place for insects, and is often a definite factor in bringing about pollination. The column is the reproductive part of the orchid flower and it is distinct from other plants. It contains the male (staminate) and female (pistilate) organs of the flower. The pistilate parts consist of a sticky surfaced area called the stigma. In the staminate element the pollen is not separated into dusty minute grains as in normal flowering plants. But they form compact, waxy masses termed, as pollinia that, however contain the pollen grains that are indistinguishable. The number of pollinia may be 2, 4 or 8 on the basis of which the genera are separated.

An insect comes to the orchid flower seeking the nectar, which it contains. In getting the nectar the insects comes in contact with the sticky mass of cells, which becomes firmly cemented to some part of the body, often the eyes or the antennae. When the insects leave the flower it drags the pollinium out. The latter may be in a position so that when the insect enters the next flower, the pollen comes directly in contact with the stigma and pollination is accomplished. In many cases, however, a striking change occurs. The stalk of the pollinium, due to changes in its water content bends through an angle of 90^0 and so, bring the pollen mass into a position which will insure in reaching the surface of the stigma of the next flower visited by the insect. The ovary of the orchid flower is inferior, that is, all the floral organs are borne at the apex of the ovary. This contains an immense number of ovules attached to its wall. After fertilization, the ovules develop into very minute and light seeds.

Orchids are of important primarily for their horticulture and floricultural purposes. Many industries have developed around the world involving this beautiful and unusual plant. It is not only the business, which earns a good profit, but it is also a source of enjoyment for the owner and visitors. Today, floriculture industry is a billion dollar industry in the world and earns a lot of foreign exchange. An orchid plant in flower can easily be kept within a living room in fresh form for many days and is a spot of beauty. Orchids having flowers with persistent perianth in which the segments do not drop as in many other flowers are of highest value for cut flowers.

Some orchid flowers last for one to three months if remain attached to the plant and as cut flower they remain fresh from 1- 4 weeks.

The primary importance of orchid is of ornamental value in their utilization in floriculture. They are used for ornamentation in home, gardens, public establishment festivals etc. No wonder orchid growing has become a flourishing trade in several countries. USA is the largest importer followed by Japan, Germany, France, Italy, Europe and the Netherlands. It is estimated that 50000 plants of Lady slipper orchid and 200 million *Cattleya* flowers are sold annually in American market. The orchid flower industry is run on a co-operative basis in the developing countries like Malaysia, Singapore, Thailand and Srilanka and earn huge sum of revenue. Orchids are sold in Indian market at a low price ranging from Rs. 10-150 each. In Calcutta and Delhi market *Cymbidium* cut flowers fetch Rs 30-120 per spike with 10-15 blooms. The famous vanillin used for flavoring ice creams and delicacies throughout the world comes from the green pods of the terrestrial climbing orchid *Vanilla planifolia* (Jyotsna Devi, 1999).

Many authors have also reported use of orchid as medicine and food in various parts of the world. The genus *Orchis* and *Bletilla* are rich in mucilage. The mucilage rich tubers of *Bletilla* are employed medicinally in China. Most of the species of *Orchis* have tubers, which when properly prepared are capable of yielding sleep. The fleshy succulent tubers are dried and grind and mixed with water. This is a food of great nutritive value and many orchid species are glycosidal plants with alkaloids. They are also reported to induce sterility among the women. In Queenland the native habitants used *Cymbidum madidum* seed as an oral contraceptive and this plant is known as “sterility plant” (Bose & Bhattacharjee, 1980).

India is recognized as a significant producer of orchids in the world. Near about 1,300 species of orchids are found in India which constitutes almost 10% of the world orchid flora with Himalayas as their main home (Medhi & Chakrabarthi, 2009). The Indian subcontinent encompasses different climatic regimes, forest types and habitat conditions providing a favorable environment for accommodating diverse species. Next to Himalaya, the peninsular region of India has a high degree of endemism making it the second richest endemic centre (Jalal and Jayanthi, 2012).

The Western Ghats of India is known to be one of the 34 biodiversity hotspots (also one among eight hottest hotspots of biodiversity) of the world. Western Ghats encompass a wide range of forest types ranging from tropical wet evergreen forests to grasslands and harbor rich flora and fauna evident from the occurrence of several species of flowering plants, butterflies, reptiles, birds, mammals, fishes and amphibians. The forests of Western Ghats are known to be a varietal storehouse of economically important plants. The tropical climate, heavy rainfall from southwest monsoon and favorable soil factors made the area ideal for the rich biodiversity. The Central Western Ghats area of Karnataka covers places viz., Kodagu, Hassan, Chikmagalur, Shivamogga, and Uttara Kannada (Ramachandra *et al*., 2013).

There are many kinds of orchids in India and they are especially plentiful in certain regions of the country. They constitute an integral and significant part of India's wonderful natural heritage. Because of the richness of the orchid flora in the Indian subcontinent, these plants have been studied in the past by well-known botanists. India has a great diversity of climate with many striking contracts of meteorological conditions and characteristics of tropical as well as the temperate zone. In India this family is represented by about 152 genera and 1300 species (Rao, 1979). Out of these nearly 287 species are endemic and they are distributed among 71 genera. This indicates that 22% of orchid species are endemic to India. It is more so with respect to the Western Ghats as more than 46% of the known species of orchids are endemic to this region. In south India (Bhat, 1999) 320 spices occur particularly in the hills of Western Ghats. The Western Ghat regions of Karnataka state harbour about 176 species in 51 genera of orchids (Rao, 2006). Among them 23 species in 13 genera are endemic. In Uttar Kannada district alone 100 species of orchids have been reported which is highest for Karnataka region. Dandeli of the Utter Kannada has high potentialities for a hot spot harboring many species. Similarly, Kudremukh of Chikamagalur district and Kodagu districts are also rich in diversity and density of orchids. Krishnaswamy *et al.* (2004a) reported 203 species and 59 genera of orchids in Karnataka state. Sringeri of Chikmagalur and Agumbe of Shimoga Districts of Karnataka in peninsular India are very rich in Orchid flora (Geetha, 2000).

Shimoga is one of the 27 districts of the Karnataka state and is situated roughly in the mid south–western part of the state. It has an eventful history and has rich cultural traditions. It is bestowed with abundant natural resources (Abhishankar, 1975). The flora of Shimoga district of Karnataka with special reference to the family Orchidaceae is under explored. Reference to literature reveals that the previous work on plants of this region made by Ramaswamy *et al.*, (2001).

Traditional medicine has a long history of serving peoples all over the world. Medicinal plant is an important element in indigenous medicinal systems in India and elsewhere. The ethnobotany and ubiquitous plants provides a rich resource for natural drug research and development. In recent years the use of traditional medicine information in plant research has again received considerable interest. There is a need for basic scientific investigations of medicinal plants using indigenous medical system become imminent.

The use of plant as medicine goes back to early man. Evidence of this early association has found in the graves of Neanderthal man buried 60,000 years back. Pollen analysis indicated that the numerous plants buried with the corpse were all of medicinal value. The earliest known medical document is a 4,000 years old Somerian clay tablet that recorded plant remedies for various illnesses.

William Withering was the first in the medical field to scientifically investigate the folk remedy. He studied foxglove as a treatment for congestive heart failure (dropsy). In 19^{th} centaury scientists begin purifying the active extracts from the medicinal plants. In 1806 Friedrich Sertuner isolated morphine from opium poppy (*Papavar somniferum*). Now a day 75% to 90% of rural population apart from western countries depend upon herbal medicine for their health care. The use of herbal medicine is still continuing in China, India, and other Asian, African and South American countries. In India, herbal medicine dates back several thousand years to the Rig-Veda. This has lead to a system of health care known as Ayurvedic medicine. The *Rauwolfia serpentina,* commonly called as Sarpaghandha, is used for centuries for its sedative effect but it is now used for high blood pressure.

The Orchidaceae, one of the largest family in plant kingdom, comprises more than 30,000 species. The use of orchids in Chinese herbal medicine has a very long history. These orchids are listed in Chinese Meteria Medica (Shen Nung Pen-Tsao Ching).

Beletilla striata: The rhizome of this plant is used against tuberculosis, hemoptysis, gastric and duodenal ulcers and crack on the body. It is also used against Euphoria, blood purification, strengthening and consolidation of lungs as well as pus, boil, abscesses, malignant swellings, and breast cancer. The tuber is used as demulcent, bechic and an expectorant. The tuber is boiled and dried and used for treating flatulence, dyspepsia, dysentery, fever, malignant ulcer, gastrointestinal disorders, hemorrhoids, anthrax, malaria, eye diseases, traumatic injuries, coughs, cheast pain, tuberculosis, vomiting of the blood, silicosis, Tenia, ring worm, tumors and necrosis, gastrorrhagia, enterorrhegia, internal bleeding, inflammation and chopped skin. Powdered root is mixed with oil and has been used as an emollient for burns and skin diseases. Whole plant preparations are tonic and treatment against leucorrhea, hemoptysis and purulent coughs (Kong *et al.,* 2003).

Dendrobium spp. and *Flickngeria* spp.: These are used in stomach ache in Japan. It is used to treat sweating at night, to strengthen kidney and cure impotence. In Korea it is used against impotence and the entire plant was used as an antipyretic, tonic and peptic (Kong *et al.,* 2003).

Dendrobium nobile Lind: It is used as tonic and strengthening medicine. It is used to impart longevity and serve as an aphrodisiac. The stem is used to alleviate thirst, calm, restlessness, accelerate convalescence and reduce dryness of the mouth (Kong *et al.,* 2003).

Gastrodia elata Blume: In China rhizomes, stalks and dried tubers (known as Tiannma) are used to treat headaches, dizziness, blackouts, numbness of the limbs, hemiplegia, epilepsy, limb cramps, spasms, migraine, expulsion of poisonous effluvia, rheumatism, vertigo, neuralgia, facial paralysis, dysphrasia, infantile, convulsions, lumbago, fever and nervous afflictions. It is used to improve circulation and enhance memory. The stalk is considered as aphrodisiacs (Kong *et al.,* 2003).

In North America the native Indians are using orchids as medicine. *Dichdes lindley*, wash is prepared from this orchid and is used for treating eye infections probably conjunctivitis. *Epidendrum sp.* mucilage and pseudobulb is used for treating sores on the lips (Kong *et al.*, 2003).

In India orchids are used in different system of traditional medicine like Ayurveda, Sidda and Unani. The following species found in Karnataka are reported to have medicinal properties. *Acampe praemorsa* is used in rheumatism. *Habenaria* sp. root tubers are used as tonic to cure unconsciousness, vermicide and as blood purifier. *Cymbidium aloifloium* roots and pseudobulb are used as emetic and purgative. *Dendrobium ovatum* stem juice is used to treat stomachache as a result of constipation. *Eulophia nuda* tubers are used to treat tumors and bronchiatis. Halva made from *Flickingeria nodosa* is believed to have the property of astringent, aphrodisiac and expectorant. It is also used in treatment of asthama, bronchiatis, tridosha, throat infection, biliousness and is used as blood purifier. *Luisia zeylanica* stem is used as emollient for boils, abscess and burns. *Nervillia aragona* tubers are used in postnatal treatment. *Satyrium nepalense* tuber is used as tonic in combination with other tubers to cure Malaria and dysentry. *Vanda testacea* roots are used in dyspepsia, bronchitis, inflammation and hiccough in Unani system. In Ayurveda, it is used as tonic to liver and brain and to lessen the inflammation and heal minor fracture (Rao 1998).

The rain forest which is situated near the equator contains a large number of medicinal plants. This tropical rain forest cover only 12 % of land area of earth but it is home of 50% to 90% species of world floral species. Tropical rain forest are the vital source of medicine today and less than 1% of the world's tropical forest plants have been tested for pharmaceutical proprieties. At least 25% of all modern drugs originally came from rain forests. These forests are declining in very fast rate. The species of these forests are disappearing. For instance in 1991 researchers isolated a compound from gum of a twig of Malaysia which blocks the spread of AIDS virus in human being. But when those researchers again visited the same place the tree was disappeared. So there is an immediate need to conserve these plants (Kong *et al.*, 2003).

The present investigation is an attempt to isolate and characterize the active components from the medicinal orchids of Shimoga district since minimal work has been done on the medicinal properties of the orchids in this area. The work has been carried out with the following objectives.

OBJECTIVES

1. To study the occurrence and distribution of orchid species in Shimoga district.
2. To screen the presence of various phytochemicals in two orchids namely *Bulbophyllum neilgherrense* and *Acampe praemorsa*.
3. To evaluate antimicrobial property of solvent extracts on selected pathogens.
4. Activity-guided separation of phytoconstituents and their characterization by Chromatographic and Spectral studies.
5. To determine antimicrobial property of compound characterized.

1.1. INTRODUCTION

Orchids form the most beautiful flowers and comprise a unique group of plants, taxonomically representing the most highly evolved family among monocotyledons with 788 genera and 18,500 species (Mabberley, 1998). Orchidaceae is the second largest family of flowering plants in the World. In India, orchids account for over a thousand species of epiphytic, terrestrial and saprophytic Orchids. The Orchidaceae is a cosmopolitan family distributed throughout the World, barring a few isolated island and frozen continent of Antarctica, and grows in almost all kinds of habitats except the aquatic and marine ecosystem. India is a country with diverse climate and topography. In total, 190 species of Orchids under 54 genera were recorded so far in Eastern Ghats (Gamble & Fischer, 1915), which leads to diversity in natural and biological resources (Rajarajeshwari & Nandakumar, 2015). Orchids constitute an order of royalty in the world of ornamental plants. Economically, they are of immense importance in horticulture. The wild species, on the other hand, have been used as indicators of regions that have a healthy ecosystem (Mishra, 2004).

Orchids rouse our highest admiration not only with their bewildering range of flowers but also their horticultural and many medicinal values. They are rich in alkaloids and other phytochemical contents and are extensively used in indigenous system of medicine for over 3000 years. The parts like leaves, pseudobulbs and flowers of orchids are of great ethnobotanical importance and used for various ailments, for external application and internal administration by the tribal people. The members of Orchidaceae have been reported for their pharmacognostic, antiviral, anticancer, antibacterial and anti-inflammatory activities (Gupta *et al*., 1970; Rastogiand Dhawan, 1990; Kaushik and Kishore, 1997; Kaushik and Kishore, 1995).

1.1.1. Medicinal uses of orchids

Bulbophyllum neilgherrense Wt. constitute the vital herbal medicine of India and China. *B. neilgherrense* is a fleshy native Indian epiphyte reputed to cure stomach ache in traditional Indian medicine. It is a robust epiphytic or lithophytic herb with slender, creeping rhizome. Pseudobulb is conical-ovoid, yellowish green, fleshy with terminal leaf (Rao, 1998).

B. neilgherrense in tribal use has shown to have *Madhura Rasa* and *Lavana Anurasa* (sweet taste and salt as subsidiary taste) with predominance of *Prithvi*, *Ap*and *Agni Mahabhutas* (earth, water and fire proto elements). This evaluation of *Rasa* helps to express sensations produced at tongue level with the drug (Harshitha *et al*., 2013).

B. neilgherrense pseudobulb is used medicinally to treat leucoderma (Harshitha *et al*., 2012), the juice from pseudobulb is used as a restorative of adolescence and also as a tonic (Harshitha *et al*., 2013). The folklore vaidyas of South East Asia use pseudobulb juice as a cooling drink in summer (Maheshwari, 1996).

Tribal claims the use of *B. neilgherrense* in leucoderma, the skin disease for which there is no effective treatment can be brought to practice if proven by different phases of research. Antibacterial effect of this orchid drug is only proven till date (Rosa *et al.*, 2010).

The fine paste of the pseudobulb and leaves of *B.neilgherrense* is administered orally for leucoderma (Rajendran *et al.,* 1997). The *B. albidum* is commonly known as Kalmalpuloravi in Kanikkartribals of Agasthiarmalai Biosphere Reserve, Western Ghats, and Tamil Nadu. Kanikkar tribe uses this plant for strengthening of a weak uterus for conception (Lalitharani. *et al*., 2011).

The pseudobulbs of *B. sterile* are used to prepare medicated coconut oil for external application in rheumatism (Shanavaskhan *et al.,* 2012). The whole plant infusion or decoction of *B. odoratissimum* Lindl. is used in fracture healing and treatment of tuberculosis (Rosa *et al*., 2010). Fresh pulp of pseudobulbs of *B.careyanum* (Hook.) is externally applied to burns. Powder of leaves used with honey to induce abortions within 3 months of pregnancy and stimulate recovery from childbirth. Fresh pulp or juice of *B.leopardinum* (Wall.) externally applied to burns. Powder of *B.odoratissimum* (Sm.) is used in treating tuberculosis, chronic inflammation and fractures (Abishkar *et al.*, 2013).

Acampe praemorsa (Roxb.) Blatt. & McCann is a fleshy native Indian epiphyte which is used in rheumatism. It is commonly called as Parajivi, Rasna (Sanskrit). The root powder is used in treating rheumatism and for cooling effect (Abishkar *et al.,* 2013; Subedi *et al*., 2013). In Nepal, tribal people use the *A. premorsa* root powder in

treating rheumatism and for cooling effect (Subedi, A. *et al*., 2013). Half spoon of fresh root paste is mixed with 1gm root paste of *Asparagus racemosus* and the mixture was taken orally on an empty stomach twice a day for 15 days to cure arthritis (Dash *et al*., 2008).

A.praemorsa is used in traditional medicine for the treatment of bone fractures, antityphoid properties (Reddy *et al.,* 2005; Mishra *et al*., 2008) and has anti-inflammatory activity (Bhattacharya *et al*., 2009). It can be applied externally as a poultice in rheumatism and arthritis (Dash *et al.,* 2008; Pullaiah, 2006). It has been reported that Orchids are important in reducing fever, serving as anti-impotence aids, increasing the white blood cell count, treating fatigue and headache and most importantly functioning as anti-cancer agents (Bulpitt, 2005).

The ethnic tribal groups of North Eastern India use the wild orchids as a food. *Cephalanthe raensifolia, Habenaria acuminta, Habenaria susannae, Orchis latifolia, Microstylis wallachii,* and *Pholidata articulata* are some species whose pseudobulbs, roots and rhizomes are taken as food in this region by boiling or preparing curry. They also take it as a nutritious health drink. Stems of *Dendrobium* are given to the cows as feed to increase the milk yield and the pseudobulb of *Cymbidium* is fed to the cattle to improve their health (Medhi and Chakrabarti, 2009).

1.2. MATERIALS AND METHODS

The present work was based on the distribution and taxonomical study of all seven Taluks of Shimoga district, Karnataka state for the period 2008-2010. Care was taken to cover all the different vegetation zones, especially along the Western Ghats. The regular field trips were conducted in different field area, for the identification of commonly available species. The identification of species is based on regional and local floras (Saldanha, & Ramesh, 1984; Ramaswamy *et al*., 2001; Saldanha, 1978).

1.3. RESULTS AND DISCUSSION

1.3.1 Distribution of orchids in Shimoga District

A total of 30 species of orchids belonging to 19 genera were collected in the present investigation, namely *Acampe praemorsa*, *Aerides maculsoum*, *A. crispum*, *A. ringens*, *Bulbophyllum neilgherrense* Wt., *B.fimbriatum*, *B.mysorense*, *Cymbidium*

bicolor, Coelogyne nervosa, Dendrobium macrostachyum, D. ovatum, D. microbulbon, D. herbaceum, D. nutans, Eria mysorensis, Flickingeria nodosa, Habenaria longicorniculata, H. roxburghii, Liparis, Malaxixs rheedii, Nervilia prainiana, N.cruciformae, Oberonia brachyphylla, Polystychia flavasence, Pholidota pallida, Peristylus spiralis, Rhynchostylis retusa, Sarcanthus pauciflorus and Vanda roxburghii.

The present investigation reveals that 30 species of Orchids belonging to 19 genera from the Shimoga district, which speaks well of the richness of orchid diversity in this area. Among these 20 species were categorized as endemic to Western Ghats according to Tewari (1995) & Sarkar (1995). They reported that *A. maculsoum, Dendrobium barbatulum, D. crepidatum, D. microbulbon, Gastrochilus acaulis, Habenaria grandifloriformis, Nervilia aragoana, Platanthera susannae* and *Sirhooker alanceolata* are rare. Similar finding was also noticed in the present investigation except for *A. maculsoum* which was more abundantly found in the study area.

Nature of forest and altitude markedly affect the composition of epiphytic orchids. Several epiphytic orchids thrive well on the common trees found in this zone. Most common orchids growing here are *A. praemorsa, A. maculosum, A. ringens, B. neilgherrense, D. macrostachyum, N. prainiana, R. retusa, V. roxburghii, H. longicorniculata, N. cruciformae, Peristylus spiralis* and *P. pallida* etc.

The present study revealed the distribution pattern and species abundance of orchids in Shimoga region. In the present investigation two orchids were selected namely *Bulbophyllum neilgherrense* Wt. and *Acampe praemorsa* (Roxb.) based on the following criteria.

a. The species abundance in the region.
b. Availability of the plant material.
c. Ethnobotanical significance of the species.

These two orchid species were available near Shimoga and these orchids are not endangered species. *B. neilgherrense* was collected in a village called Kudumallige which is near Thirthahalli, Shimoga district. The *B.neilgherrense* was collected on the *Artocarpus integrifolia* tree. *A.praemorsa* was collected from the *Mangifera indica* tree near Gajanur, Shimoga district.

A. praemorsa

P. flavasence

C. nervosa

D.ovalifolium

B. fimbriatum

A. crispa

A.ringens

D.ovatum

D.macrostychium

S. pauciflorus

P. pallida

C. bicolor

Figure 1: Orchids of Shimoga district

R. retusa

E. mysorensis

B. neilgherrense

O. brachyphylla

Figure 1a: Orchids of Shimoga district

1.3.2 PLANT DESCRIPTION

Bulbophyllum neilgherrense Wight, Icon. Pi. Ind Orient. T. 1650.1851; Hook F., Fl Brit India 5:761. 1860; C.E.C Fischer in Gamble, Flora of Madras 1418. 1928 (repr. Ed 3: 992. 1957); Ahmedullah & Nayar, Endem. Pl Ind Region i: 224. 1987- "KALMEL PULLURUVI". An epiphpytic herbs; pseudobulbs globular conical, 2 to 4cm tall. Leaves are narrowly elliptic to broadly oblong. Flowers are in racemes, many flowered, erect, brownish-yellow or greenish purple, lip entire or toothed auricles; scape stout, shorter or longer than the leaf. Fairly common in the Western Ghats, Konkan- Karnataka region, Travancore and Nilgiris, 800-1000m (Rajendran *et al.,* 1997).

B. neilgherrens is commonly known as Pottlekai in Kannada (Bhat, 2003), Kalmelpullurvi in Malayalam and is abundantly available in Western Ghats (Rajendran *et al*., 1997) and sparsely distributed in Eastern Ghats (Jadhav, 2003). Forests of Karnataka, mainly Udupi (Bhat, 2003), Belgaum (Gamble, 2011), Malabar (Hooker, 1885) are the places from where the species can be traced. The plant is endemic to South India, occurs in plains and in higher elevations up to 900m (Abraham and Vatsala, 1981). The special characteristic of the plant is the presence of pseudobulb for the preservation of water and nutrients. Pseudobulbs are 3.0-3.5 cm long and 2 cm across, smooth, green, four angled. Progressing yellowing of pseudobulbs is observed on ageing. Leaves 10-15cm long, 2-3 cm broad, coriaceous, elliptic to broadly oblong, obtuse at apex, base narrowed tapering into short petiole attached to the pseudobulb (Bhat, 2003; Abraham and Vatsala, 1981). Scape stout, from the base of the pseudobulb, sheathed at the base, jointed and with bract like sheaths at the joints. Flowers in racemes, petals small, pale yellow, lip purple (Cooke, T., 2006).

A. praemorsa is an orchid distributed in India, Burma, and Sri Lanka and is an epiphytic herb. In Karnataka, it is distributed in Belgaum, Kodagu, Chickmagalur, Mysore, Uttara kannada. Flowering and fruiting occurs in April to July. The stem stout, monopodial, covered by persistent leaf bases, roots thick spreading, leaves alternate, distichous, coriaceous, oblong, unequally cleft at the apex or bilobulate, channeled. Flower yellow with transverse purplish streaks, pleasantly scented, fleshy,

fragile, pedicollate, in leaf- opposed corymbose panicle up to 10 cm long, stout pedunculate with sheathing bracts; dorsal sepal oblong, shortly mucronulate, lateral obtuse, both of almost same size, lateral petals spathulate; lip fleshy, creamy yellow, striated on the mid lobe, crenulate along the margin, spurred at base, spur slender and papillose within; column short; pollinia 2, globose, waxy. Fruit almost sessile, longitudinally ribbed, about 6 cm long (Rao, 1998).

Plants epiphytic or lithophytic, monopodial herb.Stems simple or branched, stout, leafy. Leaves distichous, conduplicate, leathery, sometimes somewhat fleshy, apex emarginate or bilobed. Inflorescence axillary, racemose, corymbose, or paniculate; floral bracts persistent, inconspicuous, scale like. Flowers fragile, not resupinate, small, fleshy. Sepals and petals similar, flat to curved. Lip saccate or with a short spur, adnate to column, immovable, rigid, variously lobed, fleshy, papillose, tuberculate, sometimes dentate; spur lacking any internal tongue or median septum. Column short, fleshy, glabrous or papillose; stelidia 2, toothlike; foot absent; anther cap ovoid, apiculate; pollinia 4, united in 2 pairs, globose; caudicle linear, thick; viscidium oval, small; stigma transverse, concave; rostellum short, emarginate. Plants 5–20 long, laxly clothed with many distichous leaves. Leaves oblong, 7–14.5 × 1.4–2.3cm, amplexicaul-sheathing at base, apex obtuse and slightly unequally bilobed. Inflorescence several, leaves opposite or axillary, 1–4 cm, subumbellate, densely many flowered. Flowers 1 cm across, yellow, slightly fragrant, opening widely; sepals and petals yellow with reddish brown transverse stripes, lip white, slightly spotted with purple-red, spur yellow. Dorsal sepal oblong, 0.5 × 0.2 cm, obtuse; lateral sepals falcate oblong, 0.5 × 0.2 cm, obtuse. Petals oblong, as long as dorsal sepal and narrower. Lip inconspicuously 3-lobed, divided into hypochile and epichile; hypochile a narrow margin around entrance of spur; epichile ovate; spur straight, in-side with dense white hairs; column, stout, slightly shortly hairy; capsule erect or ascending, fusiform.

Figure 2: *Bulbophyllum neilgherrense* Wight.

Figure 3: *Acampe praemorsa* (Roxb.) Blatt. & McC.

1.4. CONCLUSION

In the present investigation, two orchid species were selected namely *Bulbophyllum neilgherrense* Wt. and *Acampe praemorsa* (Roxb.). These two orchid species are commonly available near Shimoga and these orchids are not endangered species. For extraction the plant material is needed in handful quantity and these orchids are very common in the collected area and hence found suitable for the extraction work. The literature survey revealed the significant medicinal properties of the orchids selected. During the field visits we have come across several persons who practice the folk medicine and used these orchids in their medicine. It has been shown that the fresh *B.neilgherrense* pseudobulb was used to treat wound healing, head ache and stomach ache by local people (Rosa *et al.*, 2010; Rajendran *et al.*, 1997; Maheshwari, 1996). The *A.praemorsa* (Roxb.) is used in treating rheumatism and for cooling effect (Abishkar *et al.*, 2013) as well as treating bone fractures and typhoid (Reddy *et al.*, 2005; Mishra *et al.*, 2008). A scientific investigation on antimicrobial effect as well as phytochemicals of these two orchids was not studied from this study area. Hence, these two orchids were selected for further investigation.

2.1. INTRODUCTION

Plant-derived substances are of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, pharmaceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Extraction methods involve the separation of medicinally active portions of plant tissues from the inactive/inert components by using selective solvents. During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity (Ncube*et al.,* 2008**;** Tiwari*et al.,*2011).

The purpose of standardized extraction procedures for crude drugs (medicinal plant parts) is to attain the therapeutically desired portions and to eliminate unwanted material by treatment with a selective solvent known as menstrum. The extract thus obtained, after standardization, may be used as medicinal agent as such in the form of tinctures or fluid extracts or further processed to be incorporated in any dosage form such as tablets and capsules. These products contains complex mixture of many medicinal plant metabolites, such as alkaloids, glycosides, terpenoids, flavonoids and lignans (Handa*et al.,* 2008;Tiwari*et al.,*2011).

The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents). For aromatic plants, hydrodistillation techniques (water distillation, steam distillation, water and steam distillation), hydrolytic maceration followed by distillation, expression and enfleurage (cold fat extraction) may be employed. Some of the latest extraction methods for aromatic plants include headspace trapping, solid phase micro-extraction, protoplast extraction, microdistillation, thermomicrodistillation and molecular distillation (Handa*et al.,* 2008;Tiwari*et al.,*2011).

Effect of extracted plant phytochemicals depends on

1. The nature of the plant material
2. Its origin
3. Degree of processing
4. Moisture content
5. Particle size (Ncube *et al.*, 2008)

The variations in different extraction methods that will affect quantity and secondary metabolite composition of an extract depend upon following (Ncube*et al*., 2008).

1. Type of extraction
2. Time of extraction
3. Temperature
4. Nature of solvent
5. Solvent concentration
6. Polarity

Choice of solventsforsuccessful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are quantity ofphytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractants(Eloff, 1998). The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted (Ncube*et al.,* 2008;Das*et al.,* 2010).

The various solvents that are used in the extraction procedures are:

Water: Water is universal solvent, used to extract plant products with antimicrobial activity. Though traditional healers use primarily water but plant extracts from organic solvents have been found to give more consistent antimicrobial activity compared to aqueous extract. Also water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics are only important as antioxidant compound (Das*et al.*,2010).

Alcohol: The higher activity of the ethanolic extracts as compared to the aqueous extract can be attributed to the presence of higher amounts of polyphenols as compared to aqueous extracts. It means that they are more efficient in cell walls and seeds degradation which have unpolar character and cause polyphenols to be released from cells. More useful explanation for the decrease in activity of aqueous extract can be ascribed to the enzyme polyphenol oxidase, which degrade polyphenols in aqueous extracts, whereas in methanol and ethanol they are inactive. Moreover, water is a better medium for the occurrence of the microorganisms as compared to ethanol (Lapornik *et al.*,2005). The higher concentrations of more bioactive flavonoid compounds were detected with ethanol 70% due to its higher polarity than pure ethanol. By adding water to the pure ethanol up to 30% for preparing ethanol 70% the polarity of solvent was increased (Bimakr 2010). Additionally, ethanol was found easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material(Wang, 2010). Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction (Cowan, 1999). Methanol is more polar than ethanol but due to its cytotoxic nature, it is unsuitable for extraction in certain kind of studies as it may lead to incorrect results.

Chloroform: Terpenoid lactones have been obtained by successive extractions of dried barks with hexane, chloroform and methanol with activity concentrating in chloroform fraction. Occasionally tannins and terpenoids will be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents (Cowan, 1999).

Petroleum Ether: Ether is commonly used selectively for the extraction of coumarins and fatty acids (Cowan, 1999).

Extraction procedures

Soxhlet extraction: Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds (Nikhal*et al.,* 2010).

Radhika*et al*.(2013) separated active fraction from *R. retusa* using hexane, chloroform and methanol using solvents soxhlet extraction method and enumerated the antifungal and cytotoxicity activity of the plant.

Shanmugavalli*et al*.(2009) isolated the active ingredient of the *Vanillaplanifolia* extract by using solvents *viz*. ethanol, choloroform, ethylacetate and petroleum ether in a soxhlet apparatus and was resolved by HPLC and the compounds identified were flavonoid and alkaloid in nature.

Sharma*et al*.(2014) extracted bioactive fraction by using ethyl alcohol from *Coelogynecristata* Lindley and showed that its compound coelogin promote osteoprotective activity in ovariectomized estrogen deficient mice.

Jakubska-Busse*et al*.(2014) studied the association between the composition of floral scent emission and its pollination systems and compounds are extracted using three organic solvents differing in hydrophobicity – methanol, methylene chloride and n-hexane.

Kalaiarasan and John (2011) identified six compounds in ethanolic extract by gas chromatography from *Bulbophyllumkaitense*.Keerthiga and Anand (2015) extracted bioactive compound from *Geodorumdensiflorum*using ethanol as solvent.

Ramesh Marasini and Susan Joshi (2012) evaluated antibacterial and antifungal activity of ethanol extract (obtained from soxhlet extraction) of medicinal orchids growing in Nepal. Jagtap*et al*. (2014)separated bioactive principles through the Soxhlet extraction using solvents like water, methanol,chloroform, acetone and IPA. Extracts were evaluated for the phytochemical study, quantitative multi-mineral analysis by Inductively Coupled Plasma (ICP)spectrometry, antioxidant and antimicrobial activities of the tuber extracts of *Habenarialongicorniculata*.

Ruchi*et al*. (2012) extracted active fraction from *Eulophia nuda* Lind through successive extraction with different solvents like aqueous, ethanolic and methanolic with the help of Soxhletapparatus and conducted qualitative phytochemical analysis.

2.2. MATERIALS AND METHODS

2.2.1. Preparation of plant material for extraction

The orchids were collected from forest trees of Gajanur and Kudumallige villages of Shimogadistrict, Karnataka in March-April 2008. The plant was identified at Department of Botany, Sahyadri Science College (Autonomous), Shimoga by referring literature. A fresh specimen weighing 750 g was surface sterilized in 70% ethanol with two drops of surfactant, tween 20 for 1 minute and washed with sterile distilled water for 2-3 times. The air dried specimen was then cut into 5 mm pieces aseptically and shade dried aseptically for 30 days in laboratory. The dried plant material was powdered with help of blender (Jagtap *et al*., 2014).

2.2.2. Extraction method

750 g powder was subjected to soxhlet extraction using Petroleum Ether, Chloroform, Ethyl Alcohol and Water as solvent.The crude extract was obtained by removing the solvent using flash rotary evaporator, similarly aqueous extracts were prepared by hot water extraction. The dried crude extracts were stored in dry air tight glass vials (Priya andKrishnaveni, 2005).

2.2.3. Preliminary phytochemicals screening

Phytochemical tests were carried out for the solvent extracts. Petroleum ether, chloroform, ethanol and aqueous extracts were subjected to routine qualitative chemical analysis to identify thephytochemical constituents. Standard procedures

were followed to identify phytochemicals as described by Sofowara (1993), Harborne (1973), Brindha*et al*., (1982) andLala (1993).Phytochemical examinations were carried out for all the extracts as per the standard methods(NeelapuNeelima*et al*.,2011).

Detection of alkaloids: Extracts were dissolved individually in dilute hydrochloric acid and filtered.

a) **Mayer's Test:**Filtrates were treated with Mayer's reagent (Potassium mercuric iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

b) **Dragendroff's Test:**Filtrates were treated with Dragendroff's reagent (solution of Potassium bismuth iodide). Formation of red precipitate indicates the presence of alkaloids.

c) **Hager's Test:**Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.

d) **Tannic acid test** Alkaloids give buff coloured precipitate with 10% Tannic acidsolution.

Detection of steroids

a) **Salkowski's Test:**Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of concentratedsulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

b) **LibermannBurchard's test:**Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled and concentratedsulphuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols.

Detection of Tannins

a) **Ferric chloride test:**Test solution gives blue greencolour with ferricchloride.

Detection of flavonoids

a) **Alkaline reagent test:**Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

b) **Lead acetate test:**Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

c) **Shinoda test (Magnesium hydrochloride reduction test):** To the test solution, few fragmentsof Magnesium ribbon was added and concentrated hydrochloric acid was added dropwise. Pink scarlet, crimson red oroccasionally green to blue colour appears after fewminutes.

d) **Zinc hydrochloride reduction test:**To the testsolution, a mixture of Zinc dust and concentratedhydrochloric acid was added. Red colour development occurs after fewminutes.

e) **Ferric chloride test:** Test solution when treated with few drops of Ferric chloride solution would result in the formation of blackish red color indicating the presence of flavonoids(Bhandary *et al.,* 2012).

Detection of Triterpenoids

a) **Salkowski test:**Extract in chloroform was treated withfew drops of concentratedsulphuric acid, shaken well and allowed to stand for some time.Red colour appearing at thelower layer indicates the presence of steroids and formationof yellow coloured lower layer indicates the presenceof triterpenoids.

b) **Libermann-Burchard test:**Extract wastreated withfew drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was added from the sides of the test tube. Formation of brown ring at the junction of two layers and theupper layerturnsgreenwhichshowsthepresenceofsteroids and formation of deep red colour indicates thepresence of triterpenoids.

c) Tschugajew test: To 2ml extract in a test tube, 2 ml acetyl chloride and pinch of zinc chloride was added and boiled in water bath. Zinc chloride powder sinks and settled at the bottom of test tube and indicates thepresence oftriterpenoids(Mehatre, 2013).

2.3. RESULTS AND DISCUSSION

It is known that plants are rich in a variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, phenols, steroids, glycosides, saponins and volatile oils. It is necessary to identify the phytochemical components of local medicinal plants usually employed by herbalists in the treatment of diseases (Banso and Adeyemo, 2007).

Solvent extraction is one of the most widely employed methods for preparation of plant extracts. Solvent extraction (solid-liquid extraction) involves the process of leaching (simple physical solution or dissolution process). Leaching is a separation technique that involves removal of soluble solids from a solid mixture by employing a suitable solvent or solvent mixture. Various factors influence the solvent extraction procedure, which includes: the rate of transport of solvent into the material, rate of solubilization of soluble constituents in the solvent, and the rate of transport of solution (extract) out of the insoluble matter. Solvent polarity, vapor pressure, and viscosity are also of importance for effective extraction. In case of plant materials, adequate time is required for diffusion of solvent via plant cell walls for dissolution of soluble constituents and for diffusion of the solution (extract) out to the surface of the cell wall (Houghton and Raman 1998; Singh, 2008; Wijekoon*et al*., 2011).

Soxhlet extraction is a common conventional method used for extracting heat-stable compounds. The Soxhlet extractor consists of a distillation flask, an extractor, and a condenser. The solvent in the distillation flask is heated and the resulting vapor is condensed in the condenser. The condensed solvent from the condenser fills into the thimble-holder containing the sample that needs to be extracted. When the solution in the extractor reaches the overflow level, a siphon aspirates the solution of the thimble-holder and unloads it back into the distillation flask, carrying dissolved solute into the bulk liquid. The solute is left in the distillation flask while the solvent is evaporated,

condensed, and passed back into the sample solid bed. This process is repeated 3 to 5 times or until a complete extraction is achieved (Tandon and Rane, 2008).

It is another common method of extraction which involves successive extraction with solvents of increasing polarity from a non-polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compound could be extracted. Some researchers employ Soxhlet extraction of dried plant material using organic solvent. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds (Das *et al*., 2010).

2.3.1 Yield of extracts in different solvents

270 gram of dried powder of both plants was used for Soxhlet extraction. The yield of extracts is given in the Table 1.

Table 1: Yield of extracts in different solvents

Plant Name	Yield of extracts in Grams			
	Petroleum Ether	**Ethyl Alcohol**	**Water**	**Chloroform**
B.neilgherrense	2.21	5.6	23.0	8.51
A.praemorsa	6.0	7.3	18	10.4

The yield of extracts is different in different solvent based on the composition of phytoconstituents and polarity of the solvent. The basic parameters influencing the quality of an extract are plant part used as starting material,solvent used for extraction and extraction procedure (Ncube *et al.,* 2008). *B.neilgherrense* provided yield of 2.21grams, 5.6 grams, 23.0grams and 8.51 grams from solvent petroleum ether, ethyl alcohol, water and chloroform respectively. *A.praemorsa*provided yield of 6.0 grams, 7.3 grams, 18 grams and 10.4 grams from solvent petroleum ether, ethyl alcohol, water and chloroform respectively. The petroleum ether gave least extract yield in case of both orchidswhereas water gave highest yield in both plants (Figure 4).

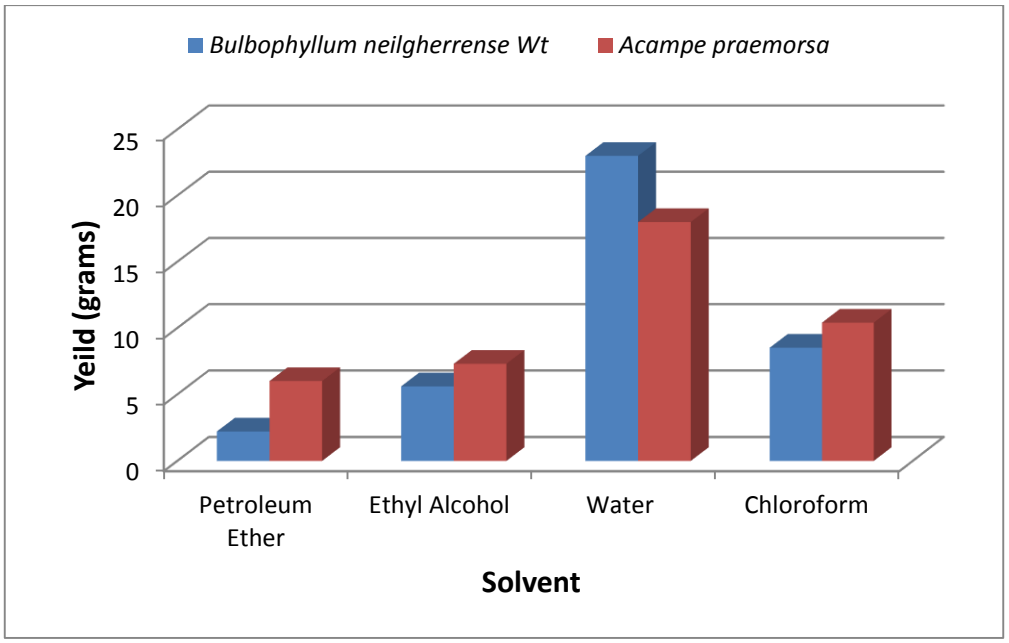


Figure 4: Extract yield in different solvents

2.3.2 Preliminary Phytochemicals Screening

Preliminary phytochemical analysis of four solvent extracts(*viz*.,petroleum ether extract, chloroform extract, ethanol extract and aqueous extract) of *B.neilgherrense* and *A.praemorsa*. are recorded in Table 2 and Table 3.

Table 2: Phytoconstituents in*B.neilgherrense*

Sl.No.	Test Name	Petroleum Ether Extract	Alcohol Extract	Aqueous extract	Chloroform Extract
I.	**Test for Alkaloids**				
1.	Mayer's Test	+ve	+ve	+ve	+ve
2.	Dragendroff's Test	-ve	-ve	+ve	+ve
3.	Hager's Test	+ve	+ve	+ve	+ve
4.	Tanic Acid Test	+ve	+ve	+ve	+ve
II.	**Test for Steroids**				
1.	LibermannBurchard's test	+ve	+ve	-ve	+ve
2.	Salkowski's Test	+ve	+ve	-ve	+ve
3.	Test for Cholesterol	+ve	+ve	-ve	+ve
III.	**Test for Tannins**				
1.	Ferric Chloride Test	-ve	+ve	-ve	-ve
IV.	**Test for Flavonoids**				
1.	Ferric Chloride Test	-ve	-ve	-ve	+ve
2.	Shinoda Test	-ve	-ve	-ve	+ve
3.	Alkaline Reagent Test	-ve	-ve	-ve	+ve
4.	Lead Acetate Test	-ve	-ve	-ve	+ve
5.	Zinc Hydrochloride reduction test	-ve	-ve	-ve	+ve
V.	**Test for Triterpenoids**				
1.	Salkowski Test	-ve	+ve	-ve	+ve
2.	Libermann-Burchard test	-ve	-ve	-ve	-ve
3.	Tschugajew test	-ve	-ve	-ve	-ve
4.	Taschugajew's Test	-ve	-ve	-ve	-ve
5.	Hirshuorn Test	-ve	-ve	-ve	-ve

Table 3: Phytoconstituents in*A.praemorsa*

Sl.No.	Test Name	Petroleum Ether Extract	Alcohol Extract	Chloroform Extract
I.	**Test for Alkaloids**			
1.	Mayer's Test	+ve	+ve	+ve
2.	Dragendrof's Test	+ve	+ve	+ve
3.	Hager's Test	+ve	+ve	+ve
4.	Tannic Acid Test	+ve	+ve	+ve
II.	**Test for Steroids**			
1.	LibermannBurchard's test	+ve	+ve	+ve
2.	Salkowski's Test	+ve	+ve	+ve
3.	Test for Cholesterol	+ve	+ve	+ve
III.	**Test for Tannins**			
1.	Ferric Chloride Test	+ve	-ve	+ve
IV.	**Test for Flavonoids**			
1.	Ferric Chloride Test	-ve	+ve	+ve
2.	Shinoda Test	-ve	+ve	+ve
3.	Alkaline Reagent Test	-ve	+ve	+ve
4.	Lead Acetate Test	-ve	+ve	+ve
5.	Zinc Hydrochloride reduction test	-ve	+ve	+ve
V.	**Test for Triterpenoids**			
1.	Salkowski Test	+ve	+ve	+ve
2.	Libermann - Burchard test	+ve	+ve	+ve
3.	Tschugajew test	+ve	+ve	+ve
4.	Taschugajew's Test	+ve	+ve	+ve
5.	Hirshuorn Test	+ve	+ve	+ve

Petroleum ether extractof*B.neilgherrense*contained alkaloid and steroid. Chloroform extract contained alkaloid, steroid, flavonoid and triterpinoid. Alkaloid, steroid, tannin and triterpinoidwere present in ethanol extract andaqueous extract contained only alkaloids. Except chloroform extract, flavonoid was absent in rest of extracts.

Petroleum ether extract of*A.praemorsa*containedalkaloids, steroids, tannins and triterpenoids. Chloroform extract containedalkaloids, steroids, flavonoids, tannins and triterpenoids. Alkaloids, steroids, flavonoids and triterpenoidswere present in ethanol extract.Alkaloids, steroids and triterpenoids were present in all extracts whereas tannin present only in chloroform extract. Flavonoids were detected in chloroform and ethanol extracts.

Orchid phytochemicals are generally categorized as alkaloids, flavonoids, carotenoids, anthocyanins and sterols. Among orchids, *Dendrobium* is the leading genus for phytochemical content and Zhang *et al*. (2003) reviewed 100 compounds from 42 *Dendrobium* species, including 32 alkaloids, 6 coumarins, 15 bibenzyls, 4 fluorenones, 22 phenanthrenes and 7 sesquiterpenoids. Williams (1979) conducted a major survey of leaf flavonoids and surveyed 142 species in 75 genera and found that the most common constituents were flavones C-glycoside and flavonols. To date, more than 2000 orchid species have been screened for their alkaloids and/or flavonoids contents. Altogether, a single familial pattern of flavonoid distribution is not evident in orchids (Jagtap*et al.*,2014).

*Habenarialongicorniculata*revealed the presence of carbohydrates, alkaloids, glycosides,saponins, flavanoids, phenols, vitamin E and vitamin C.The hydrophilic carbohydrates andglycosides were present in waterwhereas andhydrophobic carbohydrates and glycosides weredetected in rest of the organic solvents (Jagtap*et al.*,2014).

Keerthiga and Anand(2014) conducted physicochemical, preliminary phytochemical analysis and antibacterial activity against clinical pathogens of *Geodorum densiflorum*. It showed the presence of alkaloids, tannins, flavonoids, saponins, steroids and terpenoids. Chloroform extract showed the presence of alkaloids,

steroids, flavonoids and triterpenoids. Ethanol and aqueous extracts revealed the presence of alkaloids, steroids, flavonoids, tannins, saponinsand triterpenoids.

Harshita*et al*.(2013) showed the presence of phytoconstituents namely alkaloids, tannins and phenols in water and methanolic extract of the pseudobulb of*B.neilgherrense*. Aqueous extract also showed the presence of saponin, glycosides and reducing sugar. Flavonoids and steroids were also detected in the methanol extract.

The phytochemical analysis of *Vanilla planifolia* has shown the presence chemical constituents namely steroids, terpenoids, glycosides and saponins together with trace amounts of alkaloids and tannins(Shanmugavalli*et al.*,2009).

Preliminary phytochemical analysis of various solvent extracts of leafof *B. kaitense* showed the presence different phytochemicals. The ethanol extract alone contained phenolic compounds, coumarin, quinine and carbohydrate and aqueous extract containedflavonoids, Tannins, coumarin, quinine and carbohydrates (Kalaiyarasan*et al.*,2012).

Methanolic extract of *Cymbidium aloifolium*revealed the presence of tannins, alkaloids, flavonoids, triterpenoids, coumarins, flavones, flavonones and carbohydrates(Radhika*et al*., 2013).

Methanolic extract of *R. retusa*revealed the presence of tannins, alkaloids, steroids, terpenes, flavanoids, triterpenoids, coumarins, flavones, flavanones, anthocyanins, quinones, and carbohydrates (Radhika*et al.,* 2013).

Avasthi*et al.* (2013)demonstratedthe presence of bioactive substances like alkaloids, flavonoids, steroid, tannins, reducing sugars, cardiac glycosides and triterpenoids in the methanolic extract of *Orchislatifolia*.

Bhattacharjee*et al.* (2015) identified alkaloid, terpenoids, flavonoids, phenols, tannins, steroidsand glycosides from the whole plant extracts of *Vanda tessellate*.

In *Dendrobiumcrumenatum* the bioactivecompounds such as saponin, terpenoid, alkaloid, reducingsugar and flavonoid were present in most of the parts of plants

whereas cardiacglycosides were detected in leaves. Alkaloids havenumerous functions and among them are most isanalgesic, anti–inflammatory and antibacterial effects(Sandrasagaran*et al.,* 2014).

Alkaloids are formed as metabolic byproducts and have been reported to be responsible for the antibacterial activity (Doughari, 2006). Steroids and triterpenoids are known for anti-inflammatory, lipolytic and anti-cholesteremicactivities (Chawal*et al.,* 1987). Flavonoids have been referred to as nature's biological response modifiers, because of their inherant ability to modify the body's reaction to allergies and they showed their anti-allergic, anti-inflammatory, antimicrobial and anticancer activities (Aiyelaagbe*et al.,* 2009). Flavonoids are hydroxylated phenolic substance known to synthesized by plants, in response to microbial infection and it should not be surprising that they have been found in-vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cellwalls(Marjorie, 1999). It has also been observed and accepted that the medicinal value of plant lies in the bioactive phytocomponents present in the plants (Veermuthu*et al.,* 2006).

2.4. CONCLUSION

In the present investigation both orchids namely*B.neilgherrense*and *A.praemorsa* containedphytoconstituents such as alkaloids, steroids, flavonoids, tannins and triterpenoids. These groups of compounds are very significant in the field of medicinalproperty of plants. These compounds have shown the anti-inflammatory, lipolytic, anti-cholesteremic, anti-allergic, anti-inflammatory, antimicrobial and anticancer activities.These extracts are further evaluated for antimicrobial property against selected pathogenic microorganisms.

3.1. INTRODUCTION

Orchids are one of the largest and most diverse groups of angiosperms consisting of nearly 25,000 species with more than 850 genera (Stewart & Griffith 1995, Gutierrez, 2010). They are generally cultivated for beautiful flowers and are widely known for their economic importance and very less for their medicinal use. Chinese were the first to present documentary evidence for medicinal uses of orchids. The earliest report for the medicinal use of orchids is from 28th century B.C when Shen-nung described *Bletilla striata* and a *Dendrobium* species in his *Materia Medica*. The literal meaning of the term orchid (*órkhis*) in Greek is testicles and it was Theophrastus who first coined the term as the anatomy of plant resembles testicles (Stewart & Griffith 1995).

The Greek myth of Orchis explains the origin of the plants. Orchis, the son of a nymph and a satyr, came upon a festival of Dionysios (Bacchus) in the forest. He drank too much, and attempted to rape a priestess of Dionysios. For his insult, he was torn apart by the Bacchanalians. His father prayed for him to be restored, but the gods instead changed him into a flower. These flowers were previously called *Orchis*, *Satyrion* (*Satyrion feminina*), or "ballockwort". Orchids have been widely used in traditional Chinese medicine and some them have been subjected for phytochemical and pharmacological studies. India is one of the richest habitats of orchid. India comprise of about 2500 species in 167 genera. In India, some orchids such as *Eulophia campestris*, *Orchis latifolia*, *Vanda roxburgii* have drawn the attention of scientific community because of their medicinal properties (Stuart, 1984; Singh & Duggal, 2009). Medicinal orchids mainly belong to genera *Calanthe*, *Coelogyne*, *Cymbidium*, *Cypipedium*, *Dendrobium*, *Ephemerantha*, *Eria*, *Galeola*, *Gastrodia*, *Gymnadenia*, *Habenaria*, *Ludisia*, *Luisia*, *Nevilia* and *Thunia* (Gutierrez 2010; Szlachetko, 2001). Certain constituent of orchids such as alkaloids, flavonoids etc., suggest medicinal properties. The present review deals with the phytochemistry and medicinal uses of orchids.

A number of members of orchid family are used as potent inhibitor against gram positive and gram negative bacteria and also proved to be a potent antimicrobial agent. Gastrodianin, a protein isolated from orchid *Gastrodia elata* have shown *in-*

vitro activity against plant pathogenic fungi (Gutierrez, 2010). Gastrodianin is homologous to mannose binding proteins of other orchids some of which also displayed *in-vitro* antifungal activity (Wang *et al.*, 2001). The methanolic extract from different parts of orchids has shown antimicrobial activity. The methanolic extract derived from the leaves of *Spiranthus mauritianum* have shown inhibitory effect against gram positive bacteria and also showed anti-inflammatory activity (Matu and van Staden, 2003). The methylene chloride extract from the leaves and stem bark of *Galeola foliate* have shown a broad spectrum antibacterial activity against gram positive and gram negative bacteria, however the extract was found to be inactive against moulds (Khan and Omoloso, 2004). Vanillin, the major flavoring component of vanilla is a membrane active compound which results in dissipation of ion gradients and the inhibition of respiration (Fitzgerald *et al.*, 2004). Vanillin has shown antimicrobial activity against *Escherichia coli, Lactobacillus plantarum* and *Listeria innocua* (Fitzgerald *et al.*, 2004). Antimicrobial activity of vanillin and vanillic acid isolated from *Vanilla planifolia* have been studied against several strains of *Listeria monocytogenes, Listeria innocua, Listeria grayi* and *Listeria seeligeri* and it was found that mixture of vanillin and vanillic acid exhibited additive inhibitory effects particularly at low pH (Delaquis *et al.*,2005). The herb extract from *Bletilla striata* have shown to possess antioxidant and antimicrobial capacity (Luo *et al.*, 2007). The ethanolic extract of seedlings of *Cypripedium macranthos* var. rebunense was found to contain antifungal compounds lusianthrin and chrysin. Lusianthrin maintains the perilous symbiotic association for germination was found to be more potent antifungal compound than chrysin which helps to protect adult plants (Shimura *et al.*, 2007). The methanolic extract from the leaves of *Acanthephippium bicolor* Lindley was found to have antimicrobial activity against *Staphylococcus aureus, Streptococcus faecalis, Bacillus cereus, Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Shigella dysenteriae, Escherichia coli, Microsporum audouinii, Microsporum fulvum, Candida albicans* and *Trichophyton rubrum* (Kala & Senthilkumar, 2010). The gram positive bacteria are found to be more sensitive than gram negative bacteria and fungi (Kala & Senthilkumar, 2010).

Acampe praemorsa is used in traditional medicine for the treatment of bone fractures, antityphoid properties (Reddy *et al.*, 2005; Mishra *et al.*, 2008) and has anti-inflammatory activity (Bhattacharya *et al.*, 2009). It can be applied externally as a poultice in rheumatism and arthritis (Dash *et al.*, 2008; Pullaiah, 2006). It has been reported that Orchids are important in reducing fever, serving as anti-impotence aids, increasing the white blood cell count, treating fatigue and headache and most importantly functioning anti-cancer agents (Bulpitt, 2005). *A. praemorsa* has been reported to contain phenanthropyran derivative, praemorsin (1,7-dihydroxy-3-methoxy-9,10-dihydro phenanthropyran) (Anuradha and Prakasa, 1994) and flavonoids (Maridass *et al.*, 2008). Activity of *A. premorsa* may be due to these constituents. Literature revealed that not much work has been carried out on the antimicrobial activity of *Acampe praemorsa*.

From the primitive period medicinal plants have occupied a distinct place in human life. They have been the backbone of traditional herbal medicines and have been extensively studied because of their pharmacological importance. Orchids are generally known for its beautiful flowers and very less known for its medicinal uses. However, a number of compounds have been isolated from the different parts of the plant which possess medicinal properties. Compounds with antimicrobial, antitumor, anti-inflammatory, antioxidative, antidiabetic, neuroprotective, antiallergic properties have been isolated and tested on animal models but clinical trials with orchid plant parts have not been a regular practice. Emphasis on the clinical trials will provide a new gateway for treatment of diseases with herbal medicines. The orchid components still requires proper study with full experimental trials which will lead to its acceptance as medical recommendations (Singh *et al.*, 2012).

Kalaiyarasan *et al.* (2012) tested *B. kaitense* extracts for the antibacterial activity against Gram positive bacterial like *Streptococcus pneumoniae, Bacillus subtilis* and Gram negative bacteria like *Salmonella typhi ,Salmonella paratyphi, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Enterobacter faecalis, Shigella flexneri, Micrococcus sp.,* and fungal strains namely *Asperigillus fumigatus, Trichophyton rubrum, Microsporum gypseum, Aspergillus flavus, Aspergillus niger and Mucor* sp. The zone of inhibition ranging from 12mm-32mm.

Jagtap *et al.* (2014) conducted *in vitro* antimicrobial activity of *Habenaria longicorniculata* against clinically isolated bacterial and fungal strains *Staphylococcus aureus*, *Pseudomonas aeuroginosa*, *Salmonella typhi*, *Escherichia coli* and *Aspergillus niger* by disc diffusion method. The zone of inhibition ranged from 6-10 mm.

Antimicrobial activity of *Cymbidium aloifolium* solvent extracts was studied by Radhika *et al*. (2013) against ten potential clinical pathogenic bacteria namely *E. coli*, *Proteus vulgaris*, *Xanthomonas sp.*, *Pseudomonas mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus mitis*, *Staphylococcus anginosus* at different concentrations and showed the zone of inhibition ranging from 7mm to 18 mm.

Dendrobium crumenatum methanolic extracts showed 7-10 mm inhibition zone in *in vitro* antimicrobial activity of against the microorganisms namely *E. coli*, *K. pneumoniae*, *Streptococcus pneumoniae*, *S. flexeneri*, *P. aeroginosa*, *Enterobacter aerogenes*, *S. aureus* and *Saccaromyces cerevisiae* (Sandrasagaran *et al*., 2014).

Chowdhury *et al.* (2013) studied antimicrobial activity of *Acampe papilosa*, *Aerides odorata* and *Acampe ochracea* against three antibiotic resistant *E. coli* strains by paper disc method and showed the inhibition zone ranging from 5-8 mm.

Amit Bharal *et al.* (2014) evaluated antimicrobial activity of *Habenaria intermedia*, *Cypripedium cordigerum*, *Malaxis acuminata* and *Satyrium nepalense* solvent extracts against pathogenic microbial strains namely *Pseudomonas aeruginosa* and *Staphylococcus aureus* which showed the inhibition zone ranging from 6-16 mm.

Marasini and Joshi (2012) evaluated the antimicrobial activity of the following orchids; *Eria spicata*, *Bulbophyllum affine*, *Vanda cristata*, *Rynchostylis retusa*, *Dendrobium nobile*, *Dendrobium amoneum*, *Pholidota imbricata*, *Pholidota articulata*, *Coelogyne cristata*. *Coelogyne stricta* and *Coelogyne stricta* against bacteria *Vibrio cholerae*, *Staphylococus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumoniae* observed inhibition zones 8-14mm whereas in antifungal activity against *Candida albicans*, *Rhyzopus stolonifer* and *Mucor* sp. it showed zone of inhibitions 8-10 mm.

Shanmugavalli *et al*. (2009) evaluated antibacterial efficiency of *V. planifolia* against the *E. coli*, *E. coli* mutant K12, *P. vulgaris*, *E. aerogens*, *Bacillus cereus*, *Streptococcus faecalis*, *K. pneumoniae*, *S. typhi*, *S. marcescens* and *P. aeruginosa*. The zone of inhibition ranged between 1.1-8.1mm.

Rashmi *et al.* (2015) evaluated antimicrobial activity of four orchids namely *Luisia zeylanica*, *Pholidota pallida*, *Dendrobium nutantiflorum* and *Coelogyne breviscapa* against four bacteria namely *Bacillus coagulans*, *B. subtilis*, *S. typhi and E. coli*. This showed the inhibition zone ranging from 14 to 22mm.

Bhattacharjee *et al.* (2015) enumerated antimicrobial activity of four solvent extracts of *Vanda tessellata* against bacteria like *S. aureus*, *B.* subtilis, *V. cholerae*, *E. coli*, *K. pneumoniae* and the fungal organisms like *Penicillium sp.*, *Rhyzopus sp.* and *Aspergillus niger*. This extracts showed inhibition zone ranging from 5-15mm.

The study of Shweta *et al*. (2015) showed the varied inhibitory effect of orchids namely *Luisia zeylanica, Dendrobium nutantiflorum*, *Coelogyne breviscapa* and *Pholidota pallida* against *Colletotrichum capsici, Bipolaris sorokiniana, Fusarium oxysporum* and *Curvularia* sp.

3.2. MATERIALS AND METHODS

3.2.1. *In vitro* antibacterial assay

Test bacteria

Gram positive bacteria - *Staphylococcus aureus* NCIM 2079, *Staphylococcus epidermidis* NCIM 2493, *Bacillus subtilis* NCIM 2063, *Bacillus cereus* NCIM 2106, *Streptococcus faecium* NCIM 2098.

Gram negative bacteria - *Escherichia coli* NCIM 2138, *Salmonella typhi* NCIM 2501, *Shigella flexneri* NCIM 5265, *Shigella sonnei*, *Pseudomonas aeruginosa* NCIM 5029, *Proteus mirabilis*- NCIM 2241, *Klebsiella pneumoniae* NCIM 2706, *Klebsiella aerogenes* NCIM 2098, *Enterobacter aerogenes* NCIM 2340, *Vibrio cholerae* NCIM 5316, *Porteus vulgaris* NCIM 2027.

Most of these bacterial cultural were procured from National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pune-411 008, India.

Sample preparation

10% of extracts was prepared by dissolving all four extracts separately in DMSO, which was used for the antibacterial activity. The extracts were tested for the antibacterial activity.

Agar well diffusion assay

The agar well diffusion method was followed to assess the antibacterial property of the extracts **(**Cappuccino, 1996**)**. The 24 hour old Nutrient broth cultures of test bacteria were swab inoculated on the surface of solidified nutrient agar plates. The agar wells of 8mm diameter were made by using sterile cork borer. 200 µl of solvent extracts were dispensed into labeled wells with the help of micropipette. The plates were incubated at 37^{O}C for 24 hours. The zone of inhibition was measured in millimeter scale after incubation (Odunbaku *et al.*, 2008; Akiyama *et al*., 2001; Cowan, 1999). The assay was done in triplicate and result was expressed in Mean±Standard deviation (SD).

Minimum Inhibitory Concentration (MIC)

To assess the minimum inhibitory concentration of all the four extracts, broth dilution test was carried out with different concentrations of extracts *viz*., 1.57, 3.125, 6.25 and 12.5 mg/ml. Series of 5ml nutrient broth tubes were inoculated separately with 1ml of broth cultures of test bacteria namely *V. cholerae* and *B. cereus*. One ml of different concentrations of all the four extracts was transferred separately to each set of test tubes. The tubes were incubated at 37^{o} C for 24 hours. Absorbance was measured at 600nm. Nutrient broth tubes with/without extracts served as controls (Shanmugavalli *et al*., 2009; Farzaneh Lotfi Pour *et al.*, 2008; Cube *et al*, 2008). The MIC of different extracts was tabulated.

Minimum Bactericidal Concentration (MBC)

In this method, subcultures were made from each tube showing no growth into the sterile nutrient agar plates. The plates were incubated at 37oC for 24 hours. The lowest concentration of extract that fails to show any growth on subculture in the plates was taken as the MBC.

3.2.2. *In vitro* antifungal assay

Test fungi

Saccharomyces cerevisiae, Curvularia sp., *Trichophyton cutanium, Aspergillus niger, Aspergillus wentii, Aspergillus pumilus, Fusarium oxysporum, Candida lipolytica, Candida albicans, Candida krusei* and *Cryptococcus neoformens.*

Sample preparation

10% of extracts was prepared by dissolving all four extracts separately in DMSO, which was used for the antifungal activity.

Agar well diffusion assay

Agar well diffusion method was followed but nutrient medium used was Sabourauds Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) (Cappuccino, 1996). The Agar plates were swabbed (Sterile Cotton Swabs) with 72 h old broth culture of the respective fungi. A sterile cork borer was used to punch four wells, each measuring 8 mm in diameter. The wells were added with solvent extracts and the plates allowed for diffusion at room temperature for 2h. The plates were then incubated at 28°C for 48-72 hours. The diameter of the inhibition zones was recorded. The experiment was repeated twice and the average values were calculated. The assay was done in triplicate and result was expressed in Mean±Standard deviation (SD). Antifungal assay was compared with standard antibiotics like Flucanazole and Griseofulvin.

3.3. RESULTS AND DISCUSSION

3.3.1. Antibacterial activity of *B. neilgherrense*

Antibacterial effect of four solvent extracts (*viz*., Petroleum ether extract, Chloroform extract, Ethanol extract and Aqueous extract) of *B. neilgherrense* was tested against 15 bacteria (*viz*., *S. typhi*, *S. flexneri*, *P. vulgaris*, *P. aeruginosa*, *E. aerogenes*, *K.*

aerogenes, *V. cholerae*, *K. pneumoniae*, *S. sonnei*, *S. aureus*, *B. cereus*, *S. epidermidis*, *Streptococcus sp*, *B. subtilis* and *E.coli*) and the results were shown in Table 4 and Figure 5, 6 and 6a.

Table 4: Antibacterial activity of *B. neilgherrense*

Sl. No.	*Test bacteria*	Zone of Inhibition in mm				
		Petroleum ether	**Chloroform**	**Ethanol**	**Water**	**Control**
1.	*Salmonella typhi*,	--	23 ±2.00	21 ±1.52	13 ±0.57	--
2.	*Staphylococcus aureus*	--	22 ±2.02	18 ±0.57	--	--
3.	*Proteus vulgaris*	--	25 ±2.00	--	--	--
4.	*Pseudomonas aeruoginosa*	--	27 ±2.00	27 ±1.52	--	--
5.	*Bacillus cereus*	25 ±2.51	38 ±2.51	18 ±0.57	--	--
6.	*Enterobacter aerogenes*	10 ±0.57	36 ±2.00	12 ±0.57	10 ±0.57	--
7.	*Streptococcus sp*.	21 ±2.02	33 ±2.51	19 ±0.57	13 ±1.52	--
8.	*Staphylococcus epidermidis*	24 ±2.51	47 ±2.51	21 ±1.52	13 ±1.52	
9.	*Shigella flexneri*,	22 ±0.57	28 ±2.00	17 ±1.52	--	--
10.	*Bacillus subtilis*	--	--	31 ±2.51	20 ±0.57	--
11.	*Klebsiella aerogenes*	23 ±2.51	36 ±2.51	13 ±0.57	--	--
12.	*Vibrio cholera*	25 ±2.02	35 ±1.52	16 ±1.52	12 ±0.57	--
13.	*Klebsiella pneumoniae*	--	--	18 ±0.57	--	--
14.	*Shigella sonnei*	19 ±0.57	26 ±1.52	--	--	--
15.	*E. coli*	--	--	17 ±0.57	13 ±0.57	--

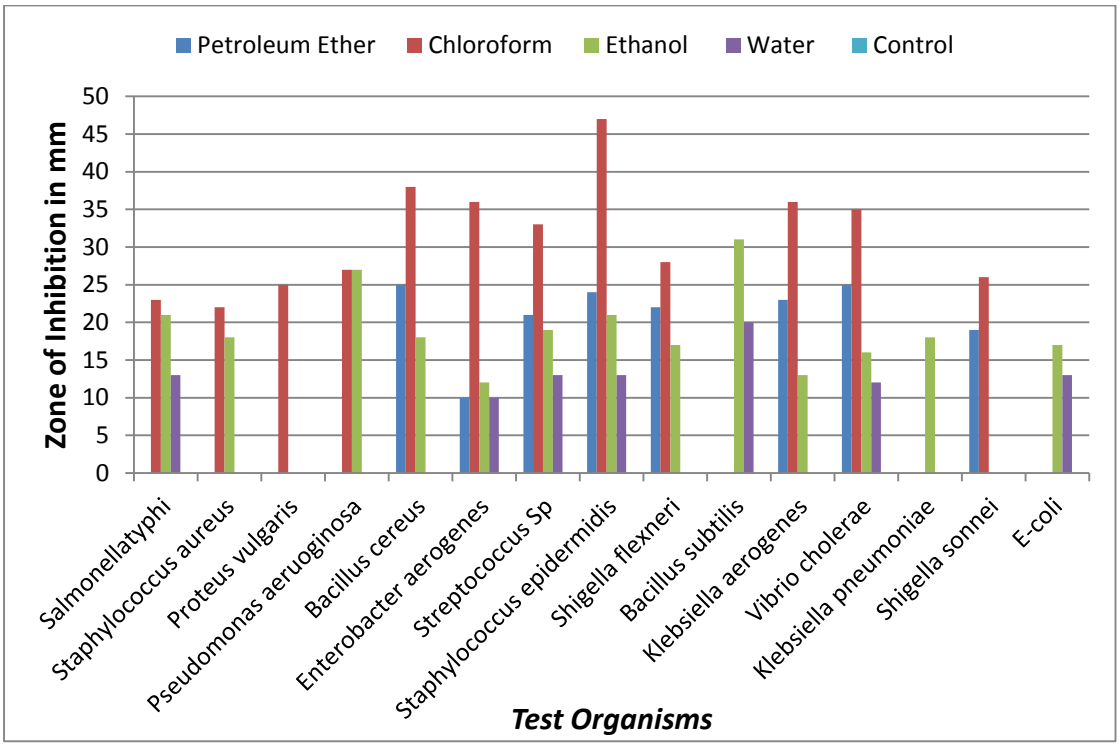


Figure 5: Inhibition of test bacteria by solvent extracts of *B. neilgherrense*

Among extracts chloroform and ethanol extracts inhibited more number of test bacteria. Chloroform extract inhibited *Salmonella typhi*, *Shigella flexneri*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Klebsiella aerogenes*, *Vibrio cholerae*, *Shigella sonnei*, *Staphylococcus aureus*, *Bacillus cereus*, *Staphylococcus epidermidis* and *Streptococcus sp.* Ethanol extract inhibited 13 test bacteria namely *Salmonella typhi*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Klebsiella aerogenes*, *Vibrio cholerae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus cereus*, *Staphylococcus epidermidis*, *Streptococcus* sp., *Bacillus subtilis* and *E.coli*. Petroleum ether extract inhibited 8 test bacteria *viz*., *Bacillus cereus*, *Enterobacter aerogenes*, *Streptococcus* sp., *Vibrio cholerae*, *Shigella flexneri*, *Klebsiella aerogenes*, *Shigella sonnei and Staphylococcus epidermidis*. Aqueous extract inhibited 7 test bacteria (*viz*., *Salmonella typhi*, *Enterobacter aerogenes*, *Vibrio cholerae*, *Staphylococcus epidermidis*, *Streptococcus* sp, *Bacillus subtilis* and *E. coli*.). The zone of inhibition ranged between 10-25 mm, 22-47mm, 12-31mm and 13-20 mm in case of petroleum ether extract, chloroform extract, ethanol extract and aqueous extract respectively.

In case of petroleum ether extract, high susceptibility was recorded in case of *Vibrio cholerae and Bacillus cereus* (Zone of inhibition 25 mm) and *Staphylococcus epidermidis* (zone of inhibition 24 mm) while least inhibitory effect was observed against *Enterobacter aerogenes* (Zone of inhibition 10mm). Rest of the test bacteria showed varied degree of inhibition as follows *Klebsiella aerogenes* (Zone of inhibition 23 mm), *Shigella flexneri* (Zone of inhibition 22 mm), *Shigella sonnei* (Zone of inhibition 19 mm), *Streptococcus sp* (zone of inhibition 21 mm) and *Proteus mirabilis* (Zone of inhibition 14 mm). Petroleum ether extract was ineffective against bacteria such as *Salmonella typhi*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus subtilis.*

In case of chloroform extract, high susceptibility was recorded in case of *Staphylococcus epidermidis* (Zone of inhibition 47 mm) while least inhibitory effect was observed against *Staphylococcus aureus* (Zone of inhibition 22 mm). Next to *S. epidermidis*, *Bacillus cereus* was inhibited to high extent (Zone of inhibition 38mm) followed by *Enterobacter aerogenes*, and *Klebsiella aerogenes* (Zone of inhibition 36 mm), *Vibrio cholerae* (Zone of inhibition 35 mm), *Streptococcus* sp (Zone of inhibition 33mm), *Shigella flexneri* (Zone of inhibition 28mm), *Pseudomonas aeruginosa* (Zone of inhibition 27mm), *Shigella sonnei* (Zone of inhibition 26mm), *Proteus vulgaris* (Zone of inhibition 25 mm), *Salmonella typhi* (Zone of inhibition 23mm). Chloroform extract was ineffective against *Klebsiella pneumoniae*, *E. coli* and *Bacillus subtilis.*

In case of ethanol extract, high susceptibility was recorded in case of *Bacillus subtilis* (Zone of inhibition 31 mm) while least inhibitory effect was observed against *Enterobacter aerogenes* (Zone of inhibition 12 mm). Next to *Bacillus subtilis*, high susceptibility was recorded in case of *Pseudomonas aeruginosa* (Zone of inhibition 27 mm) followed by *Salmonella typhi* and *Staphylococcus epidermidis* (Zone of inhibition 21mm), *Streptococcus sp* (Zone of inhibition 19mm), *Bacillus cereus* and *Klebsiella pneumoniae* (Zone of inhibition 18 mm), *E. coli* and *Shigella flexneri* (Zone of inhibition 17mm), *Vibrio cholerae* (Zone of inhibition 16mm), *Klebsiella*

aerogenes (Zone of inhibition 13mm). Ethanol extract was ineffective against *Proteus vulgaris* and *Shigella sonnei*.

In case of aqueous extract, high susceptibility was recorded in case of *Bacillus subtilis* (Zone of inhibition 20 mm) while least inhibitory effect was observed against *Enterobacter aerogenes* (Zone of inhibition 10 mm). Next to B. subtilis, high susceptibility was recorded in case of *Salmonella typhi, Streptococcus sp., Staphylococcus epidermidis* and *E. coli* (Zone of inhibition 12 mm) and *Vibrio cholerae* (Zone of inhibition 12 mm). Aqueous extract was ineffective against *Proteus vulgaris, Shigella sonne*i, *Staphylococcus aureus, Pseudomonas aeruginosa*, *Bacillus cereus, Shigella flexneri, Klebsiella aerogenes, Klebsiella pneumonia* and *Shigella sonnei*.

Enterobacter aerogenes, Streptococcus Sp. *Staphylococcus epidermidis* and *Vibrio cholera* are inhibited by all four extracts. *Bacillus subtilis* and *E.coli* were inhibited by aqueous and ethanol extracts. The control DMSO did not cause inhibition of any bacteria (Table 4).

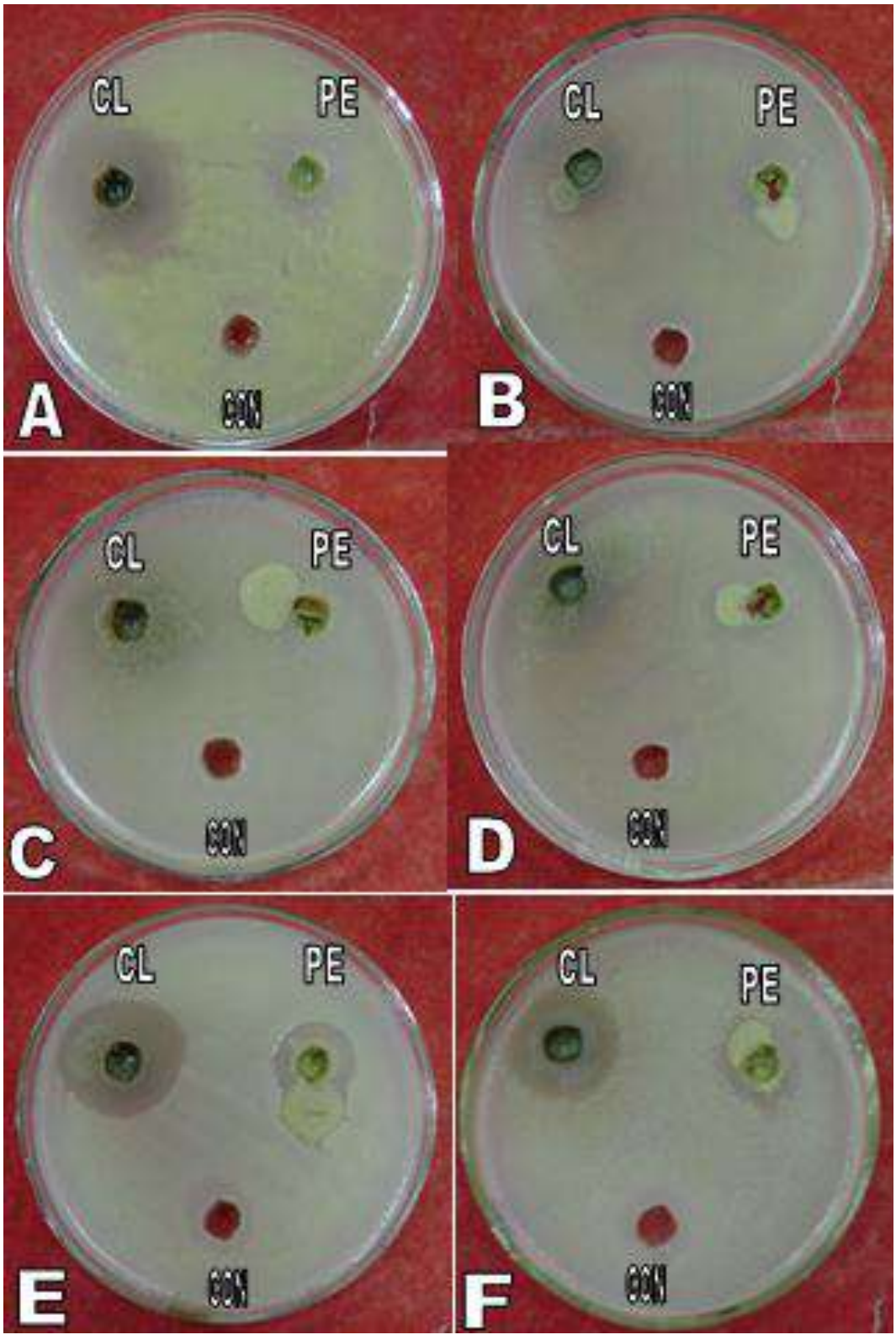


Figure 6: Antibacterial activity of *Bulbophyllumneilgherrense* (A- *K.aerogenes*;B- *V.cholarae* ;C- *K.pneumoniae*;D- *S. epidermidis*;E- *B.cereus*;F- *Streptococcus sp*; PE-Petroleum ether extract;CON-Control)

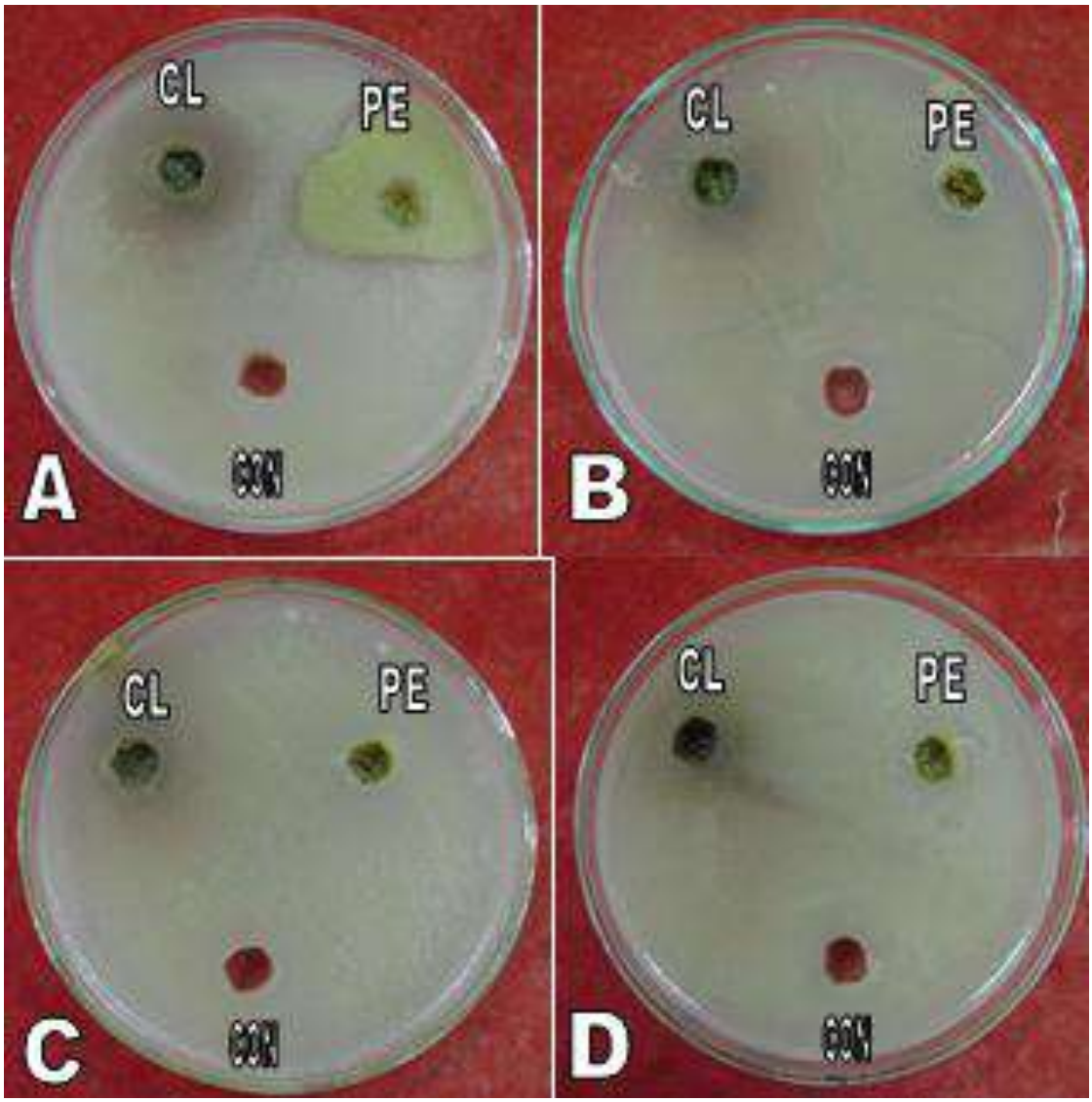


Figure 6a: Antibacterial activity of *Bulbophyllum neilgherrense* (A- *E. aerogenes*; B- *S.flexneri*; C- *S.sonnei* ;CL-chloroform extract; PE-Petroleum ether extract;CON-Control)

3.3.2. Antifungal activity of *B. neilgherrense*

Antifungal effect of four solvent extracts (*viz*., Petroleum ether extract, Chloroform extract, Ethanol extract and Aqueous extract) of *B. neilgherrense* was tested against 10 fungi (*viz*., *Saccharomyces cerevisiae*, *Curvularia* sp., *Trichophyton cutanium*, *Aspergillus niger*, *Aspergillus pumilus*, *Fusarium oxysporum*, *Candida lipolytica*, *Candida albicans*, *Candida krusei and Cryptococcus neoformens*) and the results were depicted in Table 5 and Figure 7 and 8.

Table 5: Antifungal activity of *B. neilgherrense*

Sl. No.	Test Organisms	Zone of Inhibition in mm				
		Petroleum Ether	Chloroform	Ethanol	Water	Control
1.	*Saccharomyces cerevisiae*	--	--	--	--	--
2.	*Curvularia* sp	14 ±2.00	14 ±0.57	15 ±0.57	12 ±1.52	--
3.	*Trichophyton cutanium*	11 ±1.52	14 ±1.52	16 ±0.57	10 ±0.57	--
4.	*Aspergillus niger*	13 ±1.52	14 ±0.57	10 ±0.57	14 ±0.57	--
5.	*Aspergillus pumilus*	11 ±2.00	12 ±0.57	--	14 ±1.52	--
6.	*Fusarium oxysporum*	14 ±1.52	21 ±2.00	16 ±2.00	14 ±0.57	--
7.	*Candida lipolytica*	16 ±2.00	19 ±0.57	14 ±0.57	16 ±0.00	--
8.	*Candida albicans*	24 ±1.52	23 ±2.00	26 ±2.00	25 ±1.52	--
9.	*Candida krusei*	20 ±1.52	19 ±2.00	19 ±0.57	20 ±1.52	--
10.	*Cryptococcus neoformens*	--	--	--	20 ±0.57	--

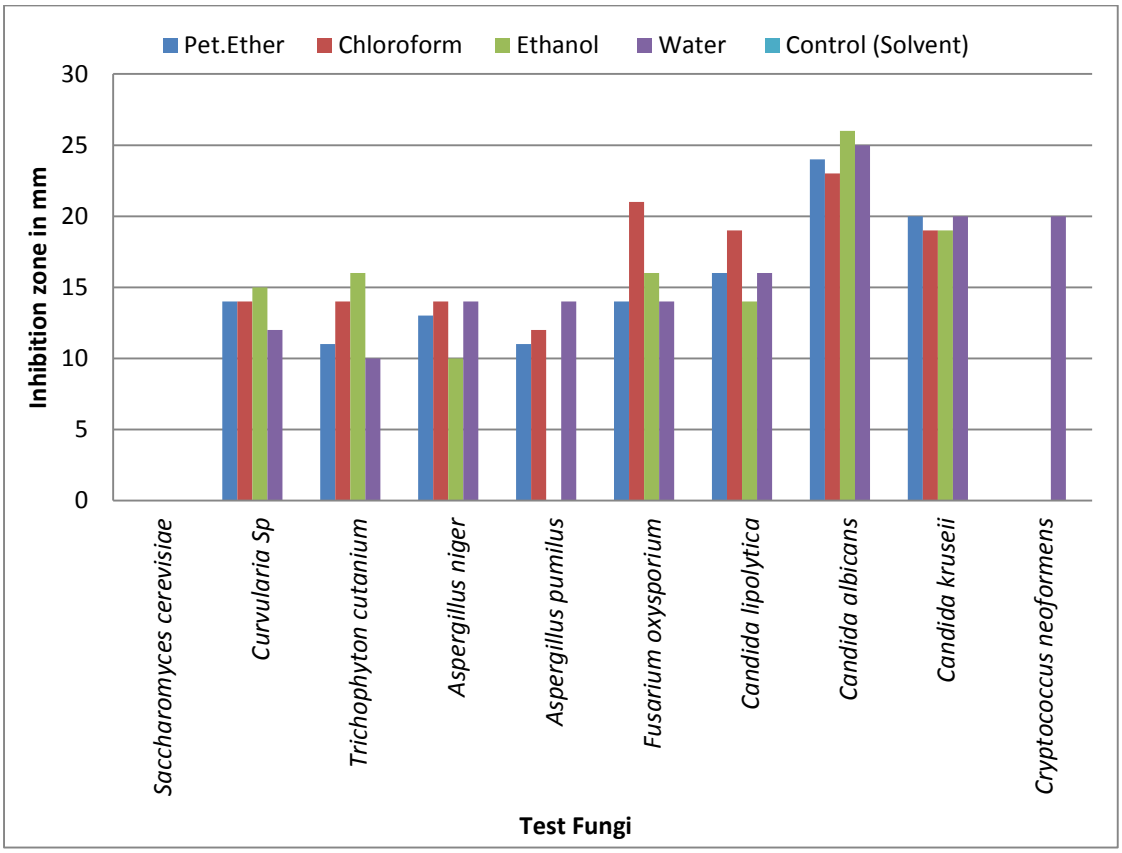


Figure 7: Inhibition of test fungi by extract of *B. neilgherrense*

Among extracts aqueous extract inhibited more number of test fungi (*viz. Curvularia* sp., *Trichophyton cutanium Aspergillus niger*, *Aspergillus pumilus*, *Fusarium oxysporum*, *Candida lipolytica*, *Candida albicans*, *Candida krusei and Cryptococcus neoformens.*) followed by petroleum ether and chloroform (*viz.*, *Curvularia* sp., *Trichophyton cutanium Aspergillus niger*, *Aspergillus pumilus*, *Fusarium oxysporum*, *Candida lipolytica*, *Candida albicans* and *Candida krusei.*) and ethanol extract (*Curvularia* sp., *Trichophyton cutanium Aspergillus niger*, *Fusarium oxysporum*, *Candida lipolytica*, *Candida albicans* and *Candida krusei.*). The Zone of inhibition ranged between 11-24 mm in case of petroleum ether extract, 12-23mm in case of chloroform extract, 10-26mm in case of ethanol extract and 10-25 mm in case of aqueous extract.

In case of petroleum ether extract, high susceptibility was recorded in case of *Candida albicans* (zone of inhibition 24 mm) followed by *Candida krusei* (Zone of inhibition 20 mm), *Candida lipolytica* (zone of inhibition 16 mm), *Fusarium oxysporum* and

Curvularia sp. (zone of inhibition 14mm), *Aspergillus niger* (zone of inhibition 13mm). Least inhibitory effect was observed in case of *Trichophyton cutaneum* and *Aspergillus pumilus* (Zone of inhibition 11mm).

In case of chloroform extract, high susceptibility was recorded in case of *Candida albicans* (Zone of inhibition 23 mm) followed by *Fusarium oxysporum* (zone of inhibition 21 mm), *Candida lipolytica* and *Candida krusei* (zone of inhibition 19 mm) and *Curvularia* sp., *Trichophyton cutanium Aspergillus niger* (zone of inhibition 14 mm). Least inhibitory effect was observed against *Aspergillus pumilus* (zone of inhibition 12 mm). Chloroform extract was ineffective against fungi such as *Saccharomyces cerevisiae* and *Cryptococcus neoformens*.

In case of ethanol extract, high susceptibility was recorded in case of *Candida albicans* (Zone of inhibition 26 mm) while least inhibitory effect was observed against *Aspergillus niger* (zone of inhibition 10 mm). Next to *C. albicans*, high inhibition was observed against *Candida krusei* (zone of inhibition 19 mm) followed by *Trichophyton cutanium* and *Fusarium oxysporum* (zone of inhibition 16 mm), *Curvularia sp.* (zone of inhibition 15 mm) and *Candida lipolytica*(zone of inhibition 14 mm). Ethanol extract was ineffective against fungi such as *Saccharomyces cerevisiae*, *Aspergillus pumilus and Cryptococcus neoformans.*

In case aqueous extract high susceptibility was recorded in case of *Candida albicans* (zone of inhibition 25 mm) while least inhibitory effect was observed against *Trichophyton cutanium* (zone of inhibition 10 mm). Next to *C. albicans*, marked effect was seen against *Cryptococcus neoformans* and *Candida krusei* (zone of inhibition 20 mm) followed by *Candida lipolytica* (zone of inhibition 16 mm), *Aspergillus niger*, *Aspergillus pumilus* and *Fusarium oxysporum* (zone of inhibition 14 mm) and Curvularia sp. (zone of inhibition 12 mm). Only by aqueous extract showed inhibition against *Cryptococcus neoformens* with inhibition zone of inhibition 20mm. Aqueous extract was ineffective against *Saccharomyces cerevisiae*. None of the solvent extracts were shown to inhibit fungi *Saccharomyces cerevisiae*. The control DMSO did not cause inhibition of any bacteria. The standard antifungal antibiotics *viz*., griseofulvin and Flucanazole at 10mg/ml concentration moderately inhibited the test fungi when compared with all the four solvent extracts (Table 6).

Table 6: Antifungal activity of standard antibiotics

Test fungi	Antifungal Antibiotics	
	Griseofulvin	**Flucanazole**
C.kruseii	0	10
C.albicans	0	0
S.cervesiae	12	14
C.lipolytica	0	25
C.neoformans	0	28
A.niger	17	0
A.pumilis	16	0
F. oxysporum	15	12
Curvularia sp.	16	0
T.cutaneum	23	0

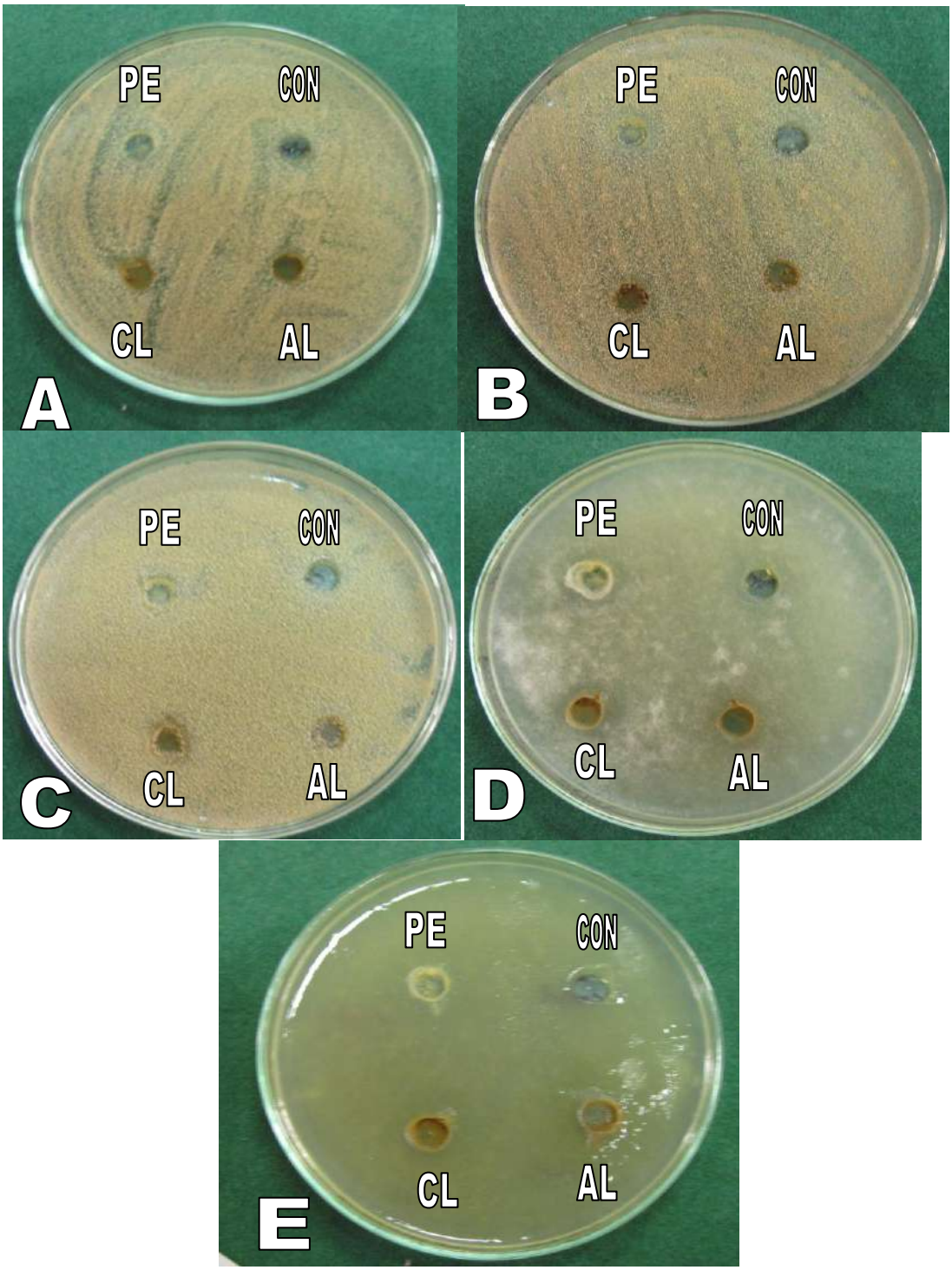


Figure 8: Antifungal activity of *B.neilgherrense* (A- *Aspergillus niger*; B- *A.wentii*; C – *A. pumilus*; D- *Fussarium oxysporium*; E- *Curvularia* Sp ; CL-chloroform extract; PE-Petroleum ether extract;AL- Ethanol extract; CON-Control)

3.3.3. Antibacterial activity of *A. praemorsa*

Antibacterial effect of four solvent extracts (*viz*., Petroleum ether extract, chloroform extract, ethanol extract and aqueous extract) of *A. praemorsa* was tested against 16 bacteria (*viz., Salmonella typhi, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, Bacillus cereus, Enterobacter aerogenes, Streptococcus sp, Staphylococcus epidermidis, Shigella flexneri, Bacillus subtilis, Klebsiella aerogenes, Vibrio cholerae, Klebsiella pneumoniae, Shigella sonnei, Escherichia coli and Proteus mirabilis*) and the results are shown in Table 7 and Figure 9, 10a and 10b.

Table 7: Antibacterial activity of *A. praemorsa*

Sl. No.	Test bacteria	Zone of Inhibition in mm				
		Petroleum ether	Chloroform	Ethanol	Water	Control (Solvent)
1.	*Salmonellatyphi*	19 ±1.52	20 ±0.57	22 ±2.00	--	--
2.	*Staphylococcus aureus*	13 ±0.57	--	14 ±1.52	--	--
3.	*Proteus vulgaris*	--	--	--	--	--
4.	*Pseudomonas aeruginosa*	--	--	--	--	--
5.	*Bacillus cereus*	27 ±2.00	25 ±2.00	30 ±2.00	--	--
6.	*Enterobacter aerogenes*	15 ±1.52	18 ±0.57	19 ±1.52	--	--
7.	*Streptococcus sp*	14 ±0.57	--	16 ±1.52	--	--
8.	*Staphylococcus epidermidis*	--			--	--
9.	*Shigella flexneri*	18 ±1.52	25 ±2.02	--	--	--
10.	*Bacillus subtilis*	--	16 ±0.57	16 ±1.52	--	--
11.	*Klebsiella aerogenes*	13 ±0.57	20 ±2.00	--	--	--
12.	*Vibrio cholerae*	--	--	--	--	--
13.	*Klebsiella pneumoniae*	--	--	--	--	--
14.	*Shigella sonnei*	17 ±0.57	16 ±1.52	--	--	--
15.	*Escherichia coli*	18 ±0.57	18 ±1.52	26 ±2.00	--	--
16.	*Proteus mirabilis*	14 ±0.57	18 ±1.52	17 ±0.57	--	--

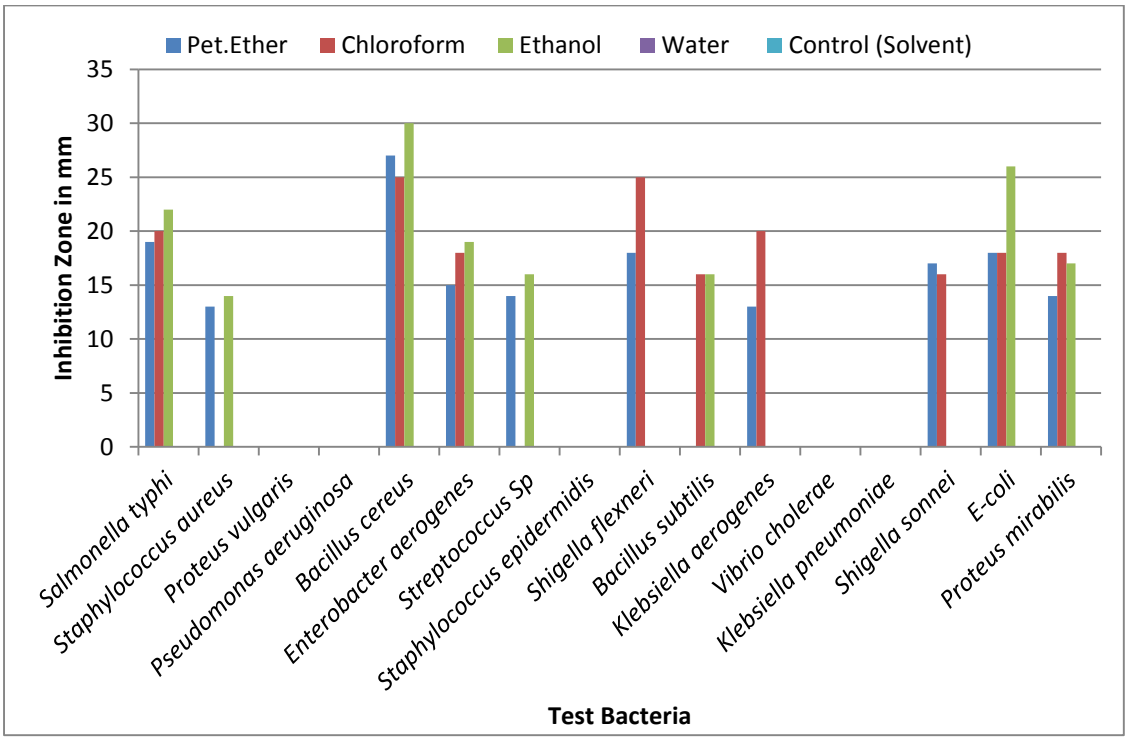


Figure 9: Inhibition of test bacteria by solvent extracts of *Acampe praemorsa*

Among extracts petroleum ether extract inhibited more number of test bacteria (*Salmonella typhi*, *Staphylococcus aureus*, *Bacillus cereus*, *Enterobacter aerogenes*, *Streptococcus sp*, *Shigella flexneri*, *Klebsiella aerogenes*, *Shigella sonnei*, *E. coli and Proteus mirabilis*) followed by chloroform (*Salmonella typhi*, *Bacillus cereus*, *Enterobacter aerogenes*, *Shigella flexneri*, *Bacillus subtilis*, *Klebsiella aerogenes*, *Shigella sonnei*, *E. coli and Proteus mirabilis*) and ethanol extract (*Salmonella typhi*, *Staphylococcus aureus*, *Bacillus cereus*, *Enterobacter aerogenes*, *Streptococcus sp*, *Bacillus subtilis*, *E. coli and Proteus mirabilis*). The Zone of inhibition ranged between 13-27 mm in case of petroleum ether extract, 16-25mm in case of chloroform extract and 14-30 mm in ethanol extract respectively. Aqueous extract did not inhibit any test bacteria.

In case of petroleum ether extract, high susceptibility was recorded in case of *Bacillus cereus* (Zone of inhibition 27 mm) followed by *Salmonella typhi* (Zone of inhibition 19mm), *Shigella flexneri* and *E. coli* (Zone of inhibition 18 mm), *Shigella sonnei* (Zone of inhibition 17 mm), *Enterobacter aerogenes* (Zone of inhibition 15 mm) and *Proteus mirabilis*(Zone of inhibition 14 mm). Least inhibitory effect was observed

against *Staphylococcus aureus* and *Klebsiella aerogenes* (Zone of inhibition 13 mm). Petroleum ether extract was ineffective against *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus epidermidis*, *Bacillus subtilis* and *Klebsiella pneumoniae*.

In case of chloroform extract, high susceptibility was recorded in case of *Bacillus cereus and Shigella flexneri* (Zone of inhibition 25 mm) followed by *Salmonella typhi* and *Klebsiella aerogenes* (Zone of inhibition 20mm), *Enterobacter aerogenes*, *E. coli and Proteus mirabilis* (Zone of inhibition 18mm) and *Shigella sonnei* and *Bacillus subtilis* (Zone of inhibition 16mm). Chloroform extract was ineffective against *Vibrio cholerae*, *Streptococcus* sp., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus vulgaris*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*.

In case of ethanol extract, high susceptibility was recorded in case of *B. cereus* (Zone of inhibition 30 mm) while least inhibitory effect was observed against *Staphylococcus aureus* (Zone of inhibition 14mm). Next to *B. cereus*, marked inhibitory effect was observed against *E. coli* (Zone of inhibition 26 mm), *Salmonella typhi* (Zone of inhibition 22mm), *Enterobacter aerogenes* (Zone of inhibition 19 mm), *Proteus mirabilis* (Zone of inhibition 17 mm), *Streptococcus sp.*, and *Bacillus subtilis*(Zone of inhibition 16 mm). Ethanol extract was ineffective against bacteria such as *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Shigella flexneri*, *Staphylococcus epidermidis*, *Klebsiella aerogenes*, *Klebsiella pneumoniae*, *Shigella sonnei*.

Salmonella typhi, *Bacillus cereus*, *Enterobacter aerogenes*, *E. coli and Proteus mirabilis* were inhibited by petroleum ether, chloroform and ethanol extracts. None of the solvent extracts were shown to inhibit *Proteus vulgaris*, *Staphylococcus epidermidis*, *Vibrio cholerae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The control DMSO did not cause inhibition of any bacteria.

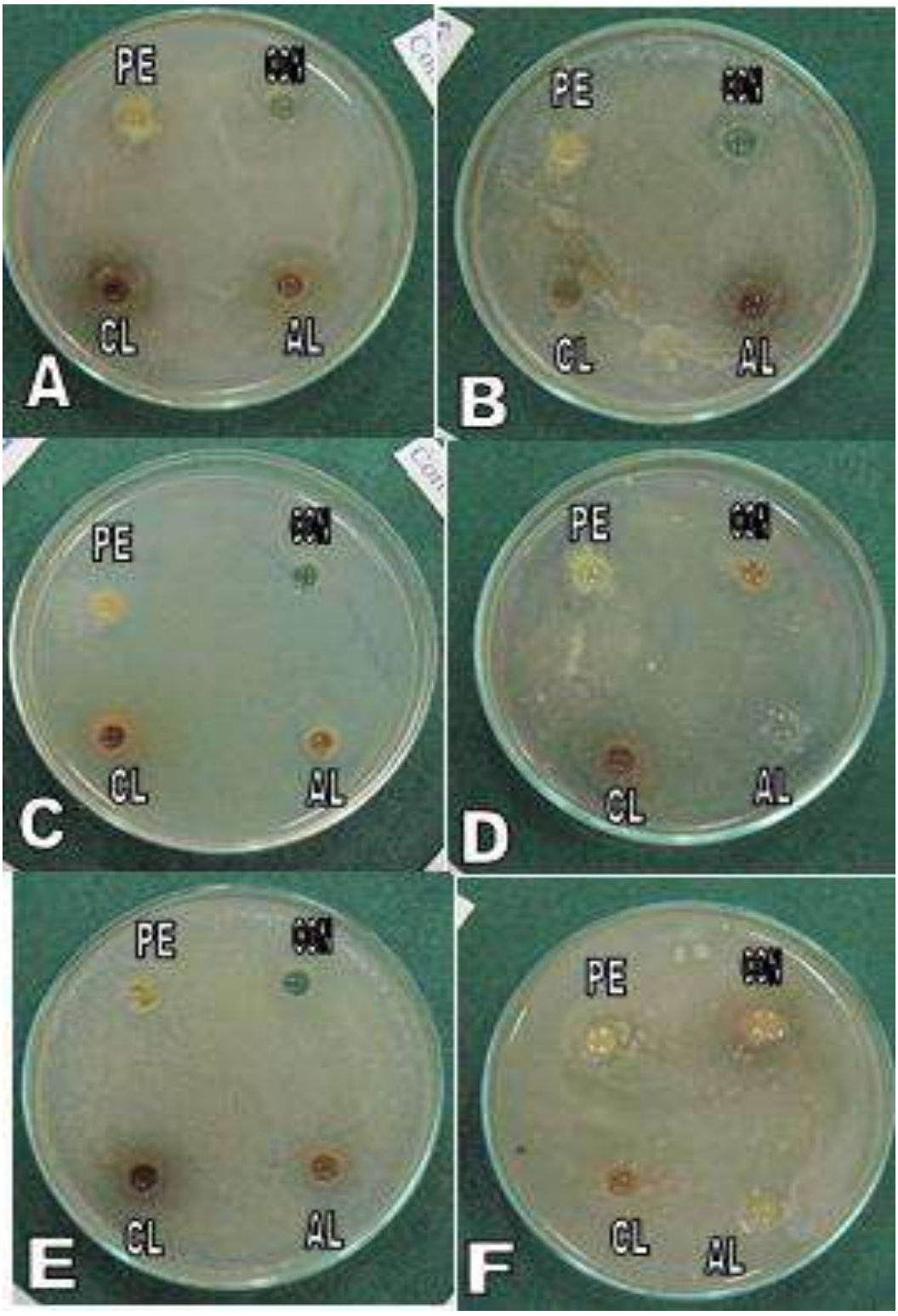


Figure 10: Antibacterial activity of *A. praemorsa* (A- *S. typhi*; B- *S. aureus*,; C-*P. aeroginosa*; D- *B. cereus*; E- *E. aerogenes*; F- *Streptococcus sp*,; PE-Petroleum ether extract; AL- Ethanol extract; CON-Control)

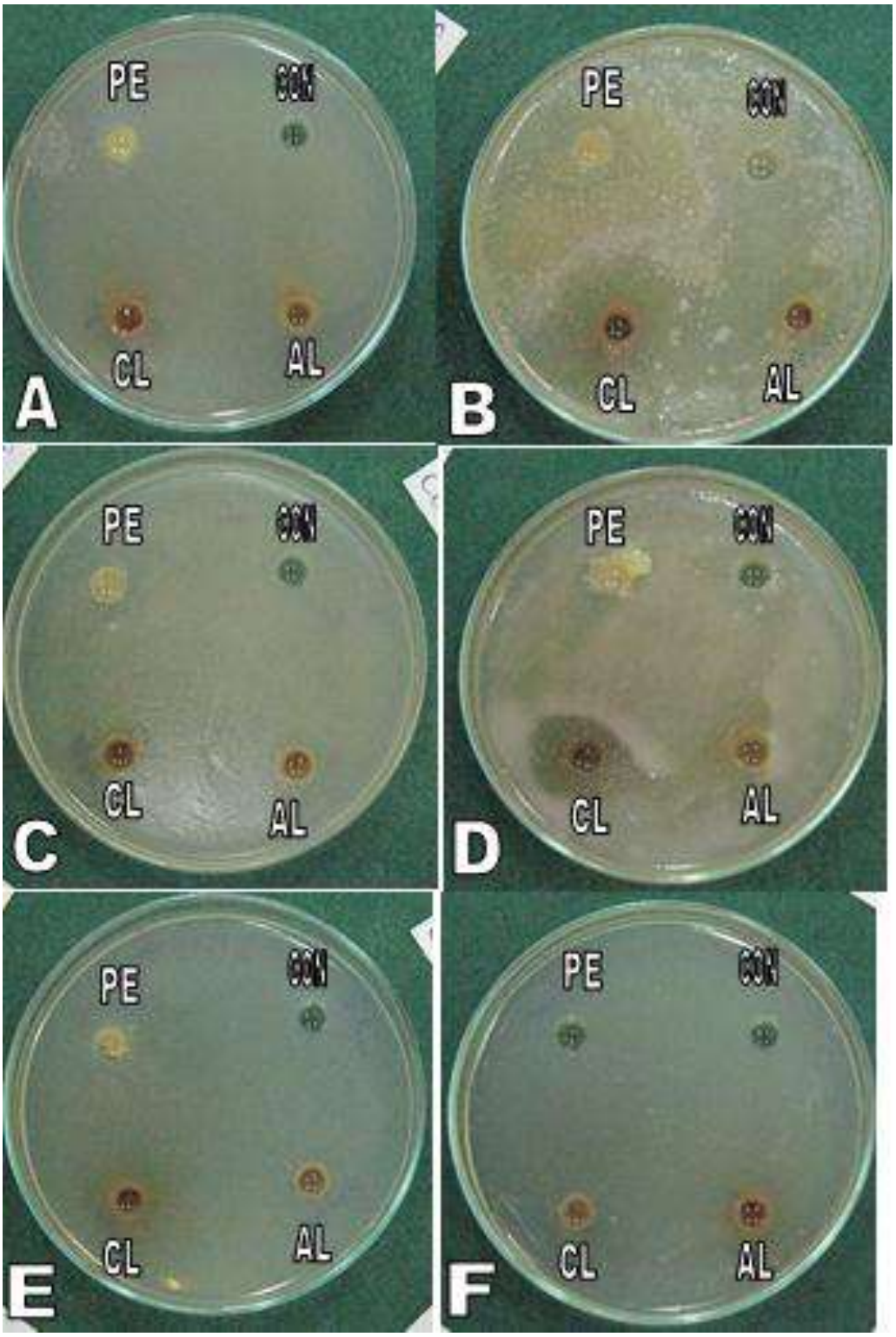


Figure 10a: Antibacterial activity of *A. praemorsa* (A – *S. epidermidis* ; B- *S. flexneri*; C- *B. subtilis*,; D- *K. aeroogenes*,; E- *V. cholera*,; F- *K. pneumoniae*; PE-Petroleum ether extract; AL- Ethanol extract; CON-Control)

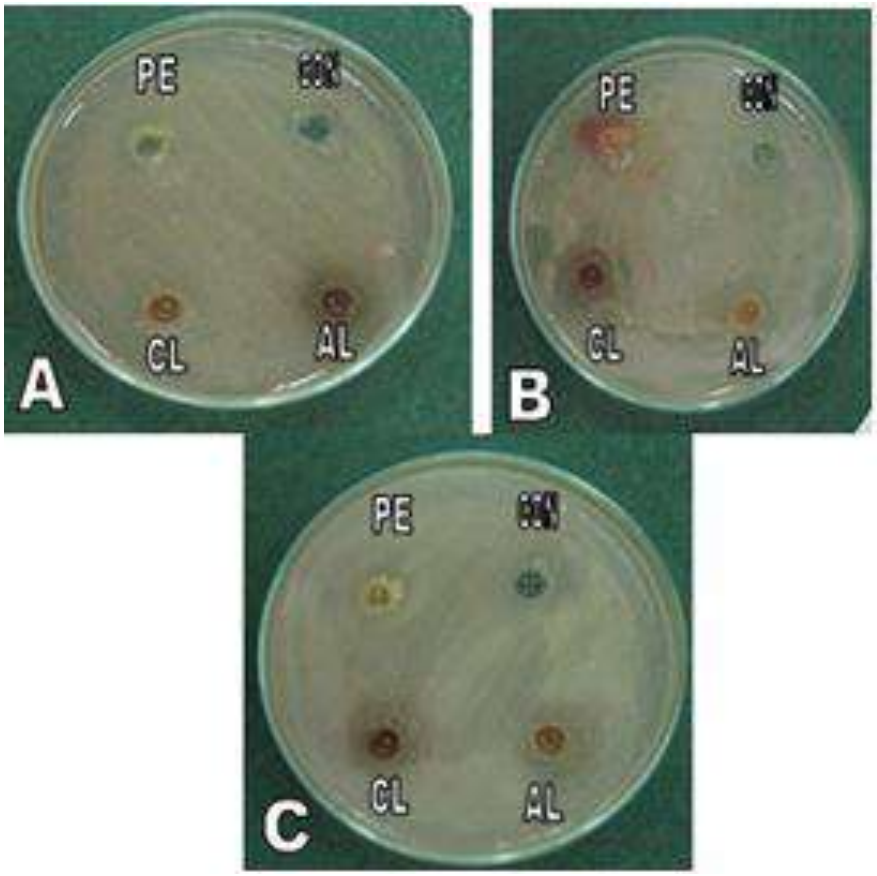


Figure 10b: Antibacterial activity of *A. praemorsa* (A – *S. sonnei*; B- *E-coli* ; C- *P. mirabilis*; PE-Petroleum ether extract; AL- Ethanol extract; CON-Control)

3.3.4. Antifungal activity of *A. praemorsa*

Antifungal effect of four solvent extracts (*viz*., petroleum ether extract, chloroform extract, ethanol extract and aqueous extract) of *Acampe praemorsa* was tested against 11 fungi (*viz*., *Saccharomyces cerevisiae*, *Curvularia* sp, *Trichophyton cutanium*, *Aspergillus niger*, *Aspergillus wentii*, *Aspergillus pumilus*, *Fusarium oxysporum*, *Candida lipolytica*, *Candida albicans*, *Candida krusei* and *Cryptococcus neoformans*) and the results were shown in Table 8 and Figure 11 and 12.

Table 8: Antifungal activity of *A. praemorsa*

Sl. No.	Test fungi	Zone of Inhibition in mm				
		Petroleum Ether	Chloroform	Ethanol	Water	Control (Solvent)
1.	*Saccharomyces cerevisiae*	16 ±1.52	18 ±2.02	19 ±1.52	14 ±1.52	--
2.	*Curvularia* sp	14 ±0.57	--	--	--	--
3.	*Trichophyton cutanium*	18 ±1.52	17 ±0.57	--	--	--
4.	*Aspergillus niger*	--	14 ±0.57	--	--	--
5.	*Aspergillus wentii*	12 ±0.57	15 ±0.57	12 ±0.57	14 ±0.57	--
6.	*Aspergillus pumilus*	13 ±0.57	13 ±0.57	--	10 ±0.57	--
7.	*Fusarium oxysporum*	18 ±1.52	16 ±0.57	--	16 ±1.52	--
8.	*Candida lipolytica*	12 ±0.57	13 ±0.57	16 ±0.57	12 ±0.57	--
9.	*Candida albicans*	18 ±1.52	19 ±1.52	16 ±0.57	15 ±0.57	--
10.	*Candida krusei*	18 ±0.57	16 ±1.52	12 ±0.57	--	--
11.	*Cryptococcus neoformans*	--	--	--	--	--

Among extracts, petroleum ether and chloroform extracts inhibited 9 out of 11 test fungi. Ethanol extract inhibited 5 test fungi and aqueous extract inhibited 6 test fungi. The zone of inhibition ranged between 12-18 mm in case of petroleum ether extract and 13-19 mm in case of chloroform extract, 12-19 mm in case of ethanol extract and 10-16 mm in case of aqueous extract respectively.

Petroleum ether extract inhibited test fungi *viz*., *Saccharomyces cerevisiae*, *Curvularia* sp, *Trichophyton cutanium*, *Aspergillus wentii*, *Aspergillus pumilus*, *Fusarium oxysporum*, *Candida lipolytica*, *Candida albicans* and *Candida krusei*). Chloroform extract inhibited *Saccharomyces cerevisiae*, *Trichophyton cutanium*, *Aspergillus niger*, *Aspergillus wentii*, *Aspergillus pumilus*, *Fusarium oxysporum*, *Candida lipolytica*, *Candida albicans* and *Candida krusei*. Aqueous extract inhibited fungi namely *Saccharomyces cerevisiae*, *Aspergillus wentii*, *Aspergillus pumilus*, *Fusarium oxysporum*, *Candida lipolytica* and *Candida albicans* and ethanol extract inhibited *Saccharomyces cerevisiae*, *Aspergillus wentii*, *Candida lipolytica*, *Candida albicans* and *Candida krusei*,).

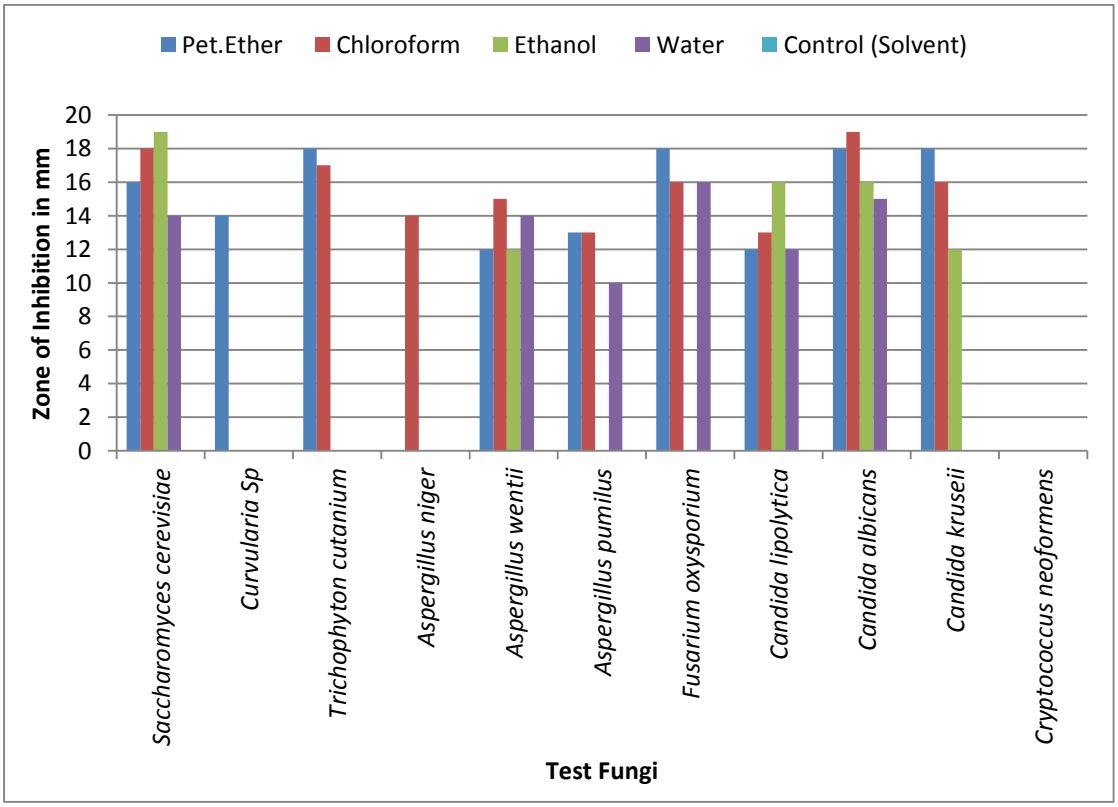


Figure 11: Inhibition of test fungi by solvent extracts of *Acampe praemorsa*

In case of petroleum ether extract, high susceptibility was recorded in case of *Trichophyton cutanium*, *Fusarium oxysporum*, *Candida albicans*, and *Candida krusei*, (Zone of inhibition 18 mm) followed by *Saccharomyces cerevisiae* (zone of inhibition 16 mm), *Curvularia* sp. (Zone of inhibition 14 mm) and *Aspergillus pumilus* (zone of inhibition 13 mm). Least inhibition was observed in case of *Aspergillus wentii* and *Candida lipolytica* (Zone of inhibition 12 mm). *Aspergillus niger* and *Cryptococcus neoformans* were not affected by this extract.

In case of chloroform extract, high susceptibility was recorded in case of *Candida albicans* (Zone of inhibition 19 mm) followed by *Saccharomyces cerevisiae* (Zone of inhibition 18 mm), *Trichophyton cutanium* (Zone of inhibition 17 mm), *Fusarium oxysporum* and *Candida krusei* (Zone of inhibition 16 mm), *Aspergillus wentii* (Zone of inhibition 15 mm), *Aspergillus niger* (Zone of inhibition 14 mm). Least inhibitory effect was observed against *Aspergillus pumilus* and *Candida lipolytica* (Zone of inhibition 13 mm). Chloroform extract was ineffective against *Curvularia* sp. and *Cryptococcus neoformans*.

In case of ethanol extract, *Saccharomyces cerevisiae* was inhibited to high extent (zone of inhibition 19 mm) followed by *Candida lipolytica* and *Candida albicans* (zone of inhibition 16 mm). *Candida krusei* and *Aspergillus wentii* were inhibited to least exent (zone of inhibition 12 mm). *Curvularia* sp., *Trichophyton cutanium*, *Aspergillus niger*, *Aspergillus pumilus*, *Fusarium oxysporum* and *Cryptococcus neoformans* were not inhibited.

In case of aqueous extract, *Fusarium oxysporum* displayed marked susceptibility (zone of inhibition 16 mm) followed by *Candida albicans* (zone of inhibition 15 mm), *Saccharomyces cerevisiae* and *Aspergillus wentii* (zone of inhibition 14 mm), *Candida lipolytica* (zone of inhibition 12 mm). *Aspergillus pumilus* exhibited least susceptibility to aqueous extract (zone of inhibition 10 mm). *Curvularia* sp., *Trichophyton cutanium*, *Aspergillus niger*, *Candida krusei* and *Cryptococcus neoformans* are not inhibited**.**

Saccharomyces cerevisiae, *Aspergillus wentii*, *Candida lipolytica* and *Candida albicans* were inhibited by all solvent extracts. None of the solvent extracts were

shown to inhibit *Cryptococcus neoformans*. The control DMSO did not cause inhibition of any fungi.

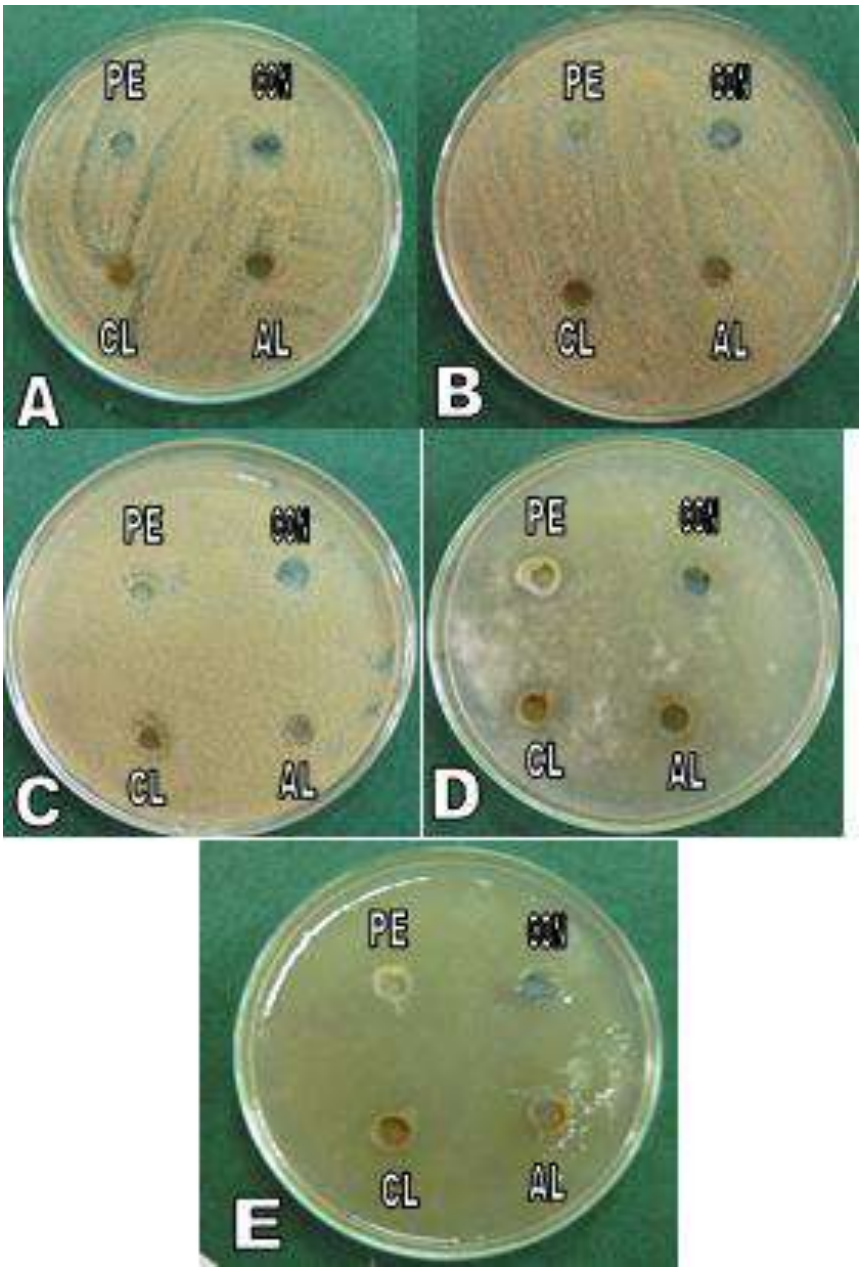


Figure 12: Antifungal activity of *A. praemorsa*(A – *A. niger* ; B- *A. wentii*; C- *A. pumilus*; D- *Fussarium oxysporium*; E- *Curvularia* sp; PE-Petroleum ether extract; AL- Ethanol extract; CON-Control)

MIC and MBC of chloroform extract of *B. neilgherrense*

Minimum inhibiting concentrations (Table 9) and minimum bacterial concentration (Table 10) of the solvent extracts was performed on selected organism which slowed best results in preliminary screening assay.

Table 9: MIC of chloroform extract of *B. neilgherrense*

Solvent extracts	MIC (mg/ml)	
	V .cholerae	***B. cereus***
Alcohol	1.564	1.564
Chloroform	3.125	3.125
Petroleum ether	6.25	3.125
Aqueous	6.125	12.5

Table 10: MBC of chloroform extract of *B. neilgherrense*

Test bacteria	Solvent Extracts			
	Petroleum ether extract (6.25 mg/ml**)**	**Aqueous extract (12.5**mg/ml**)**	**Chloroform extract (3.125** mg/ml**)**	**Alcohol extract (3.125** mg/ml**)**
B.cereus	++	+	+	+++
	Petroleum ether extract (12.5 mg/ml**)**	**Aqueous extract (12.5** mg/ml**)**	**Chloroform extract(6.25**mg/ml**)**	**Alcohol extract (3.125** mg/ml**)**
V.cholerae	++	+	+	+++

Nature has provided rich resources of herbal products named from plants as phytomedicines. Orchids have been used traditionally for treating different ailments like Tuberculosis, Jaundice, Syphilis, Arthritis, Leucoderma, stomach diseases and chest pain. The present investigation focused on antimicrobial efficacy of the orchids available in malnad region of shimoga. The inhibitory potential was observed in petroleum ether extract of *B. neilgherrense* and petroleum ether and ethanol extract *A. praemorsa*.

In case of *B. neilgherrense*, gram positive bacteria were prominently than gram negative bacteria. Spore forming species such as *B. cereus* was suppressed by the chloroform extract with zone of inhibition 38mm, followed by *Streptococcus sp* with zone of inhibition 33mm and *Staphylococcus aureus* with inhibition zone 22mm. Gram negative bacteria like *Vibrio cholerae*, *E. aerogenes* and *K. aerogenes* being most affected. Antibacterial activity of *A. praemorsa* again bacterial test pathogens was moderate with zone of inhibition from 13 -30mm. Here also *B.cereus* was inhibited to the maximum extent when compared with other bacterial pathogens. Chloroform solvent was found to be best in extracting the antibacterial constituents from both orchid varieties which was supported by the inhibition of more number of bacterial pathogens.

Antifungal activity of extracts of petroleum ether, chloroform, ethanol and water was moderate when compared with antibacterial activity of these extracts. Interestingly, all the solvent extracts were capable of suppressing the test yeasts and molds except *S. cerevisiae* and *C.neoformans*. Marked inhibition was observed in case of *C. albicans* with zone of inhibition 26mm which was better than the standard antibiotic fluconazole. Significant finding in antifungal activity of extracts of *B. neilgherrense* was inhibition of both yeast and molds in which some were plant pathogens, human skin pathogens and spoilage fungi. In future these extracts may be useful in exploiting novel antifungal agents from these orchids.

In case of *A. praemorsa*, antifungal activity was lesser in all the four solvent extracts tested on all the tested fungal pathogen. Highest zone of inhibition 19mm was observed in dermatophytic yeasts *C. albicans T. cutanium*, while *C. neoformens* was unaffected by all the extracts.

B. neilgherrense was best in showing antimicrobial activity when compared to *A. praemorsa*. Broad spectrum of activity was exhibited by *B. neilgherrense* extracts by inhibiting both bacterial and fungal pathogens. Moderate effect was recorded in solvent extracts of *A.praemorsa*.

Plants are known to possess pain reveling and healing constituents like alkaloids, flavonoids tannins and phenol compounds. These metabolites are commonly found in several of orchids (Radhika *et al.*, 2013). Possible cause of antimicrobial potential was assessed by subjecting the extracts to phytochemical assays which revealed the possible phytoconstituents responsible for antimicrobial activity. Most of the extracts of both orchid varieties showed the presence of alkaloids, steroids, flavonoids, tannins and triterpenoids. The observed inhibitory efficacies of solvent extracts of *B. neilgherrense* and *A. praemorsa* could be ascribed to the presence of the phytochemicals which have been detected in the extracts.

In a study, Umamageswary *et al.* (2014) showed that leaf, root and stem extracts of *D. onemenatum* possess moderate antimicrobial activity against selected pathogens. The study of Priya & Krishnaveni (2005) revealed antimicrobial activity of the psuodobulb of *B. neilgherrense*. Overall, *A. praemorsa* was less effective in exhibiting antibacterial activity when compared to *B. neilgherrense*.

Since strong antibacterial activity was shown by the petroleum ether and chloroform extracts, which were further subjected for separation of components by TLC, HPLC and LCMS studies. Similar studies were conducted on *Orchis latifolia* by Anupama sharma *et al.* (2013). Antimicrobial activity of *B. kaitense* (an endermic species in South India) revealed similar findings as in the present work that chloroform and ethanol extracts were best in extracting the antibacterial constituents and can be used as herbal and scientific medicine in treating microbial infections in humans (Kalaiyarasan *et al.*, 2012).

3.4. CONCLUSION

Evaluating plants from the traditional Indian system of medicine provides as with classes as how to these plants can be used in the treatment of disease (Behera, 2013). Plants produce a diverse range of bioactive molecules, making them rich source of different types of medicines. The medicinal value of plants lies in chemical substances. Most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds. Phytomedicines derived from plants have shown great promise in the treatment of various diseases (Vinayaka *et al*., 2009). Antimicrobial activity of tannins (Funatogawa *et al*., 2004), flavonoids (Baharaminejad *et al*., 2007), saponins (Baharaminejad *et al*., 2007), terpenoids (Tincusi *et al*., 2002), alkaloids (Faizi *et al*., 2003) have been documented. In the present study, most of these phytoconstituents have been detected in the solvent extracts which may account for the antimicrobial activity. The findings of present research also augment that ethnomedicinal orchids could be used as an alternative sources of therapeutic agents in future. *B. niligherense* and *A. praemorsa* are the less studied orchids found in South India.

4.1. INTRODUCTION

Living cells generate free radicals and other reactive oxygen species (ROS) as a result of physiological and biochemical processes. To some extent, free radicals are needed for several important physiological functions. However, in excess, free radicals can cause oxidative damage to biomolecules such as lipids, proteins and DNA, leading to many chronic diseases, such as cancer, diabetes, aging, and cardiovascular and neurodegenerative diseases in humans. To counteract this adverse effect caused by ROS, cells have evolved a variety of defense systems based on both water-soluble and lipid-soluble antioxidant species, and on antioxidant enzymes. Plants are endowed with free radical scavenging species, such as vitamins, terpenoids, phenolic acids,tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites which exert marked antioxidant activity. Studies have shown that several of these compounds exhibit anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, antiviral and other activities. The ingestion of plants containing such natural antioxidants is beneficial and has been associated with reduced risks of developing diseases/disorders such as cancer, cardiovascular disease, diabetes, and other diseases. In recent years, much focus has been devoted to use the natural phytochemicals present in berry crops, teas, herbs, oilseeds, beans, fruits and vegetables (Tilak *et al*., 2004; Kaviarasan *et al*., 2007; Aiyegoro and Okoh, 2010; Ebrahimzadeh *et al*., 2010; Kekuda *et al*., 2012; Pavithra *et al*., 2013).

Luo *et al*. (2011) studied the antioxidant activity of *Dendrobium denneanum* and found that the water-extracted polysaccharide DDP purified with AB-8 and ADS-7 column chromatography exhibited strong scavenging ability on DPPH radical. The antioxidant effect of DDP was similar to that of vitamin C.

Mukherjee *et al.* (2012) investigated antiglycation and antioxidant activity of *Dendrobium aqueum* Lindl. The result showed a dose dependent DPPH free-radical scavenging and a significant antiglycation potential.

Krishnasree and Andallu (2013) found out that the fresh *Vanilla fragrans* bean extracts showed better antioxidant activity than vanilla essence. Vanilla essence had lesser radical scavenging efficiency indicating lesser biochemicals and phytochemicals than in vanilla pods and according to them, this is due to the loss of phytochemicals during processing.

Banerjee *et al.* (2015) screened different solvent extracts of *Dendrobium ochreatum* which showed the presence of flavonoids, alkaloids, triterpenoids, steroids for antioxidant activity. The presence of these phytoconstituents in single or in combination might be responsible for the observed antioxidant potential.

Uddin *et al.* (2015) showed antioxidant and cholinesterase inhibitory activities of polyphenols from chloroform extract of *V. roxburghii*. Chloroform extract of *V. roxburghii,* possibly due to its polyphenolic compounds; possess a combination of antioxidant properties and cholinesterase inhibitory activities which support its traditional utilization in Bangladesh in the treatment of Alzheimer's disease.

Moretti *et al*. (2013) evaluated the chemical composition, the antiradical properties, genotoxic/antigenotoxic effects of the extracts of *Dendrobium speciosum* leaves and stem. The stem extract showed a small amount of polyphenols and flavonoids that might accounted for the little antioxidant activity of the extract.

Methanol extract of *S. pauciflorus* was shown to exhibit antimicrobial, antioxidant, anthelmintic and insecticidal activity. The observed biological activities might be attributed to the secondary metabolites contained in the extract (Kiran *et al.*, 2013).

The methanolic and ethyl acetate extracts of *Eria alba* whole plant showed significant antioxidant capacity which was found to be 85.65% and 90.03% respectively. When compared to BHT as standard, while the concentration of crude extract and petroleum ether extract was 50.452% and 25.125 % (Singh *et al.*, 2016).

Kshirsagar *et al.* (2010) isolated two phenanthrenes from *Eulophia ochreata* namely 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7- diol and 5,7-Dimethoxyphenanthrene -2, 6-dio. Both compounds were obtained by free radical scavenging activity-guided fractionation of the extract.

In a study, Rashmi *et al.* (2015) have shown scavenging of DPPH free radicals by extracts of *P. pallida*, *L. zeylanica*, *D. nutantiflorum* and *Coelogyne breviscapa*.

Extract of pseudobulb of *P. pallida* has shown highest DPPH scavenging activity in the hot methanolic extract and highest ABTS scavenging activity in the cold methanolic extract (Nagananda *et al.,* 2014).

Yang *et al.*(2007) reported the occurance of 2-glucosyloxycinnamic acids from *Dendrobium* and the three isolated major compounds *cis*-melilotoside, *trans*-melilotoside and dihydromelilotoside showed potent radical scavenging activity.

Singh *et al*. (2015) showed strong antioxidant activity of the methanol extract of *Dendrobium denudans* which may be due to presence of high phenols and flavonoids content.

Kuppusamy *et al*. (2015) demonstrated significant antimicrobial and antioxidant activity. Aqueous extracts of *C.nudiflora* showed significant antioxidant and antimicrobial activity.

4.2. MATERIALS AND METHODS

4.2.1. Antiradical activity of solvent extracts

4.2.1.1. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity

In this assay, 2ml of different concentrations of solvent extracts and reference standard ascorbic acid (2.5-80µg/ml of methanol) was mixed with 2ml of DPPH solution (0.002% in methanol).The tubes were incubated for 30 minutes at room temperature in dark. The absorbance was read at 517 nm in a UV-Visible spectrophotometer. The absorbance of the DPPH control (2ml of DPPH+2ml of methanol) was noted. The scavenging activity of each concentration of solvent extracts was calculated using the formula:

Scavenging activity (%) = [(A–B) /A] x100, where A is absorbance of DPPH and B is absorbance of DPPH and extract/standard combination (Pavithra *et al*., 2013). IC_{50} value was calculated for each extract. IC_{50} value represents the concentration of extract/standard required to scavenge 50% of free radicals. The IC_{50}values were calculated by Origin 6.0 software.

4.2.1.2. ABTS(2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid]) radical scavenging activity

The ABTS radical was generated by mixing 7mM ABTS stock solution with 2.45mM potassium persulfate. The mixture was kept in the dark for 12–16 hours at room temperature. The resulting solution was diluted with distilled water to an absorbance of 0.70 at 730nm.1ml of each concentration of solvent extracts and ascorbic acid (2.5-80µg/ml) was added to 3ml of ABTS solution. The tubes were incubated for 30 minutes at room temperature. The absorbance of each tube was read at 730nm in a UV-Visible spectrophotometer. The radical scavenging activity was calculated using the formula:

Scavenging activity (%) = (A – B / A) x 100, where A is the absorbance of the ABTS solution without extract/standard and B is the absorbance of ABTS solution in the presence of extract/standard (Rakesh *et al*., 2013). IC_{50} value was calculated for each extract. IC_{50} value represents the concentration of extract/standard required to scavenge 50% of free radicals. The IC_{50}values were calculated by Origin 6.0 software (Pellegrini *et al.,* 1999).

4.2.2. Ferric reducing activity of solvent extracts

Various concentrations of solvent extracts and ascorbic acid (2.5-80 µg/ml) in 1 ml of methanol were mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (1%) in separate and labelled tubes. The tubes were placed in water bath for 20 minutes at 50^{o}C, cooled rapidly and mixed with 2.5 mL of trichloroacetic acid (10%) and 0.5 ml of ferric chloride (0.1%). The amount of iron (II)-ferricyanide complex formed was determined by measuring the formation of Perl's Prussian blue at 700 nm after 10 minutes. The increase in absorbance indicates increased reducing power (Pavithra *et al*., 2013).

4.2.3. Total phenolic content of solvent extracts

Folin-Ciocalteau reagent (FCR) method was used. A dilute concentration of extract (0.5 ml) was mixed with 0.5 ml of FC reagent (1:1) and 2 ml of sodium carbonate (7%). The tubes were allowed to stand for 30 minutes and the optical density was measured at 765nm in a UV-Vis spectrophotometer. A standard curve was plotted

using different concentrations of Gallic acid (standard, 0-1000 µg/ml).The total phenolic content of solvent extracts was expressed as µg Gallic acid equivalents (GAE) from the graph (Pavithra *et al*., 2013).

4.3. RESULTS AND DISCUSSION

4.3.1. Antiradical activity of solvent extracts

4.3.1.1. DPPH free radical scavenging activity of solvent extracts

Dose dependent scavenging of DPPH free radicals by solvent extracts and ascorbic acid was observed. Scavenging potential was in the order Ethanol extractextract (IC_{50} value 7.42µg/ml) >Chloroform extract (IC_{50} value 15.63µg/ml) > Petroleum ether extract (IC_{50} value 33.52µg/ml). Efficacy of ascorbic acid to scavenge radicals was marked (IC_{50} value 0.69µg/ml) when compared to solvent extracts (Table 11and Figure 13).

Dose dependent scavenging of DPPH free radicals. Scavenging potential of *B. neilgherrense* ethanol extract showed the higher degree of activity of 38.46% in concentration of 2.5 µg/ml, 50.41% in concentration of 5 µg/ml, 58.14% in concentration of 10 µg/ml, 62.31% in concentration of 20 µg/ml, 69.41% in concentration of 40 µg/ml and 78.66% in concentration of 80 µg/ml. The activity was higher when compare to other solvent extracts but lesser than that of Ascorbic acid standard.

Chloroform extract of *B. neilgherrense* showed activity of 30.98% in concentration of 2.5 µg/ml, 36.14% in concentration of 5 µg/ml, 41.48% in concentration of 10 µg/ml, 56.25% in concentration of 20 µg/ml, 63.14% in concentration of 40 µg/ml and 74.56% in concentration of 80 µg/ml. The activity was higher when compare to petroleum ether extract but lesser than that of Ascorbic acid standard and ethanol extract.

Petroleum ether extract of *B. neilgherrense* Wt. showed least activity of 22.89% in concentration of 2.5 µg/ml, 28.36% in concentration of 5 µg/ml, 36.14% in concentration of 10 µg/ml, 43.14% in concentration of 20 µg/ml, 52.66% in concentration of 40 µg/ml and 63.14% in concentration of 80 µg/ml. The activity was less when compare to other solvent extract and Ascorbic acid standard.

Ascorbic acid standard Showed higher activity than all the solvent extracts, that is 65.36% in concentration of 2.5 µg/ml, 68.11% in concentration of 5 µg/ml, 74.56% in concentration of 10 µg/ml, 82.14% in concentration of 20 µg/ml, 91.24% in concentration of 40 µg/ml and 94.16% in concentration of 80 µg/ml.

Table 11: DPPH free radical scavenging activity of solvent extracts

Concentration (µg/ml)	Radical scavenging activity (%)			
	Petroleum ether extract	Chloroform extract	Ethanol extract	Ascorbic acid
2.5	22.89	30.98	38.46	65.36
5.0	28.36	36.14	50.41	68.11
10.0	36.14	41.48	58.14	74.56
20.0	43.14	56.25	62.31	82.14
40.0	52.66	63.14	69.41	91.24
80.0	63.14	74.56	78.66	94.16

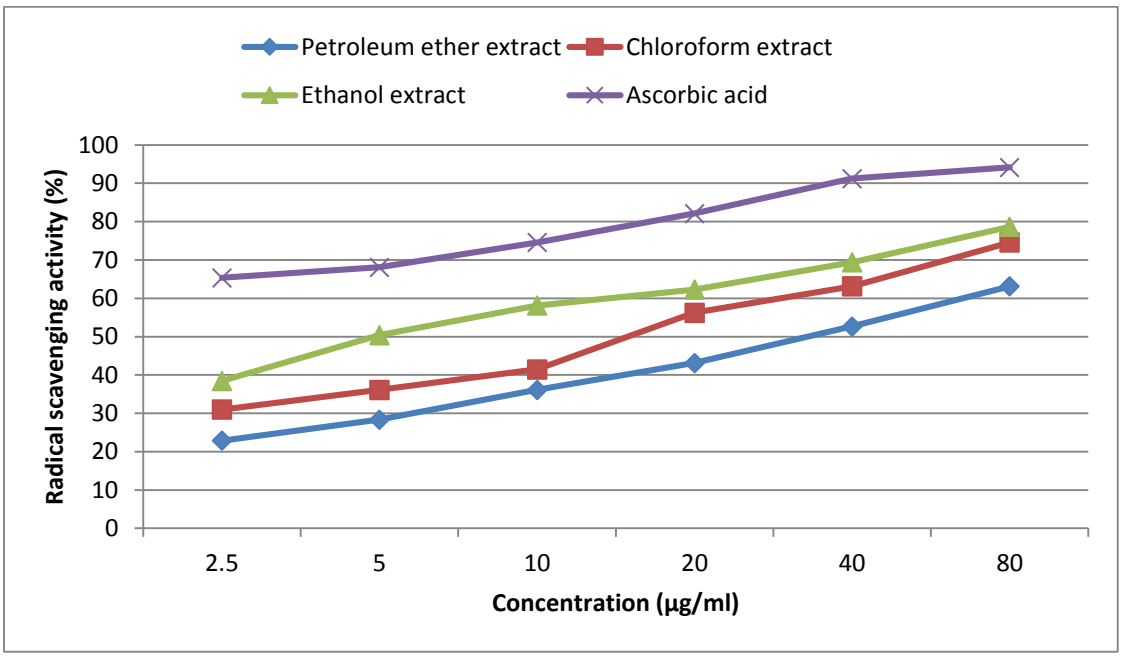


Figure 13: Scavenging of DPPH radicals by solvent extracts

4.3.1.2. ABTS radical scavenging activity of solvent extracts

The result of scavenging effect by solvent extracts on ABTS radicals is shown in Table 12 and Figure 14.The extracts scavenged radicals in concentration dependent manner. Here also, the scavenging potential of solvent extracts was in the order Ethanol extract (IC_{50} value 9.36µg/ml) >Chloroform extract (IC_{50} value 25.94µg/ml) > Petroleum ether extract (IC_{50} value 31.62µg/ml). Ascorbic acid scavenged radicals to higher extent than solvent extracts (IC_{50} value 0.83µg/ml).

Dose dependent scavenging of ABTS radicals Scavenging potential of *B. neilgherrense* Wt. Ethanol extract showed the higher degree of activity of 38.69% in concentration of 2.5 µg/ml, 46.58% in concentration of 5 µg/ml, 52.66% in concentration of 10 µg/ml, 62.14% in concentration of 20 µg/ml, 68.66% in concentration of 40 µg/ml and 82.69% in concentration of 80 µg/ml. The activity was higher when compare to other solvent extracts but lesser than that of Ascorbic acid standard.

Chloroform extract of *B. neilgherrense* Wt. showed activity of 33.18% in concentration of 2.5 µg/ml, 40.14% in concentration of 5 µg/ml, 46.26% in concentration of 10 µg/ml, 50.16% in concentration of 20 µg/ml, 54.68% in concentration of 40 µg/ml and 66.49% in concentration of 80 µg/ml. The activity was higher when compare to petroleum ether extract but lesser than that of Ascorbic acid standard and ethanol extract.

Petroleum ether extract of *B. neilgherrense* Wt. showed least activity of 33.14% in concentration of 2.5 µg/ml, 38.69% in concentration of 5 µg/ml, 44.16% in concentration of 10 µg/ml, 48.21% in concentration of 20 µg/ml, 51.44% in concentration of 40 µg/ml and 61.33% in concentration of 80 µg/ml. The activity was less when compare to other solvent extract and Ascorbic acid standard.

Ascorbic acid standard, Showed higher activity than all the solvent extracts that is 63.55% in concentration of 2.5 µg/ml, 70.66% in concentration of 5 µg/ml, 80.14% in concentration of 10 µg/ml, 88.24% in concentration of 20 µg/ml, 94.62% in concentration of 40 µg/ml and 96.66% in concentration of 80 µg/ml.

Table 12: ABTS radical scavenging activity of solvent extracts

Concentration (µg/ml)	Radical scavenging activity (%)			
	Petroleum ether extract	Chloroform extract	Ethanol extract	Ascorbic acid
2.5	33.14	33.18	38.69	63.55
5.0	38.69	40.14	46.58	70.66
10.0	44.16	46.26	52.66	80.14
20.0	48.21	50.16	62.14	88.24
40.0	51.44	54.68	68.66	94.62
80.0	61.33	66.49	82.69	96.66

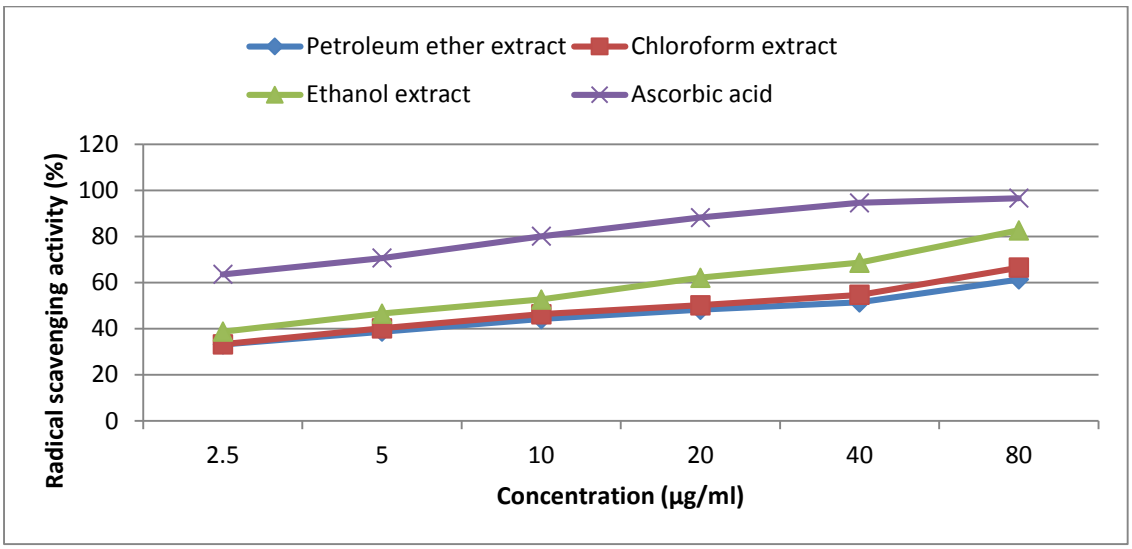


Figure 14: Scavenging of ABTS radicals by solvent extracts

4.3.2. Ferric reducing activity of solvent extracts

The result of ferric reducing efficacy of extracts and ascorbic acid is shown in Table 13 and Figure 15.The absorbance was found to increase on increasing the concentration of extract/standard. Ferric reducing potential was in the order Ethanol extract>Chloroform extract> Petroleum ether extract. Ascorbic acid exhibited high reducing activity than that of solvent extracts.

Dose dependent Ferric reducing potential of *B. neilgherrense* Wt. Ethanol extract showed the higher degree of activity of optical density 0.24 in concentration of 2.5 µg/ml, 0.31 in concentration of 5 µg/ml, 50.36 in concentration of 10 µg/ml, 0.40 in concentration of 20 µg/ml, 0.44 in concentration of 40 µg/ml and 0.51 in concentration of 80 µg/ml. The activity was higher when compare to other solvent extracts but lesser than that of Ascorbic acid standard.

Chloroform extract of *B. neilgherrense* Wt. showed activity of 0.18 in concentration of 2.5 µg/ml, 0.22 in concentration of 5 µg/ml, 0.24in concentration of 10 µg/ml, 0.31 in concentration of 20 µg/ml, 0.38 in concentration of 40 µg/ml and 0.48 in concentration of 80 µg/ml. The activity was higher when compare to petroleum ether extract but lesser than that of Ascorbic acid standard and ethanol extract.

Petroleum ether extract of *B. neilgherrense* Wt. showed least activity of 0.12 in concentration of 2.5 µg/ml, 0.18 in concentration of 5 µg/ml, 0.21 in concentration of 10 µg/ml, 0.26 in concentration of 20 µg/ml, 0.34 in concentration of 40 µg/ml and 0.39 in concentration of 80 µg/ml. The activity was less when compare to other solvent extract and Ascorbic acid standard.

Ascorbic acid standard, Showed higher activity than all the solvent extracts that is 0.38 in concentration of 2.5 µg/ml, 0.44 in concentration of 5 µg/ml, 0.51 in concentration of 10 µg/ml, 0.56 in concentration of 20 µg/ml, 0.63 in concentration of 40 µg/ml and 0.68 in concentration of 80 µg/ml.

Table 13: Ferric reducing activity of solvent extracts

Concentration (µg/ml)	Optical density at 700nm			
	Petroleum ether extract	Chloroform extract	Ethanol extract	Ascorbic acid
2.5	0.12	0.18	0.24	0.38
5.0	0.18	0.22	0.31	0.44
10.0	0.21	0.24	0.36	0.51
20.0	0.26	0.31	0.40	0.56
40.0	0.34	0.38	0.44	0.63
80.0	0.39	0.48	0.51	0.68

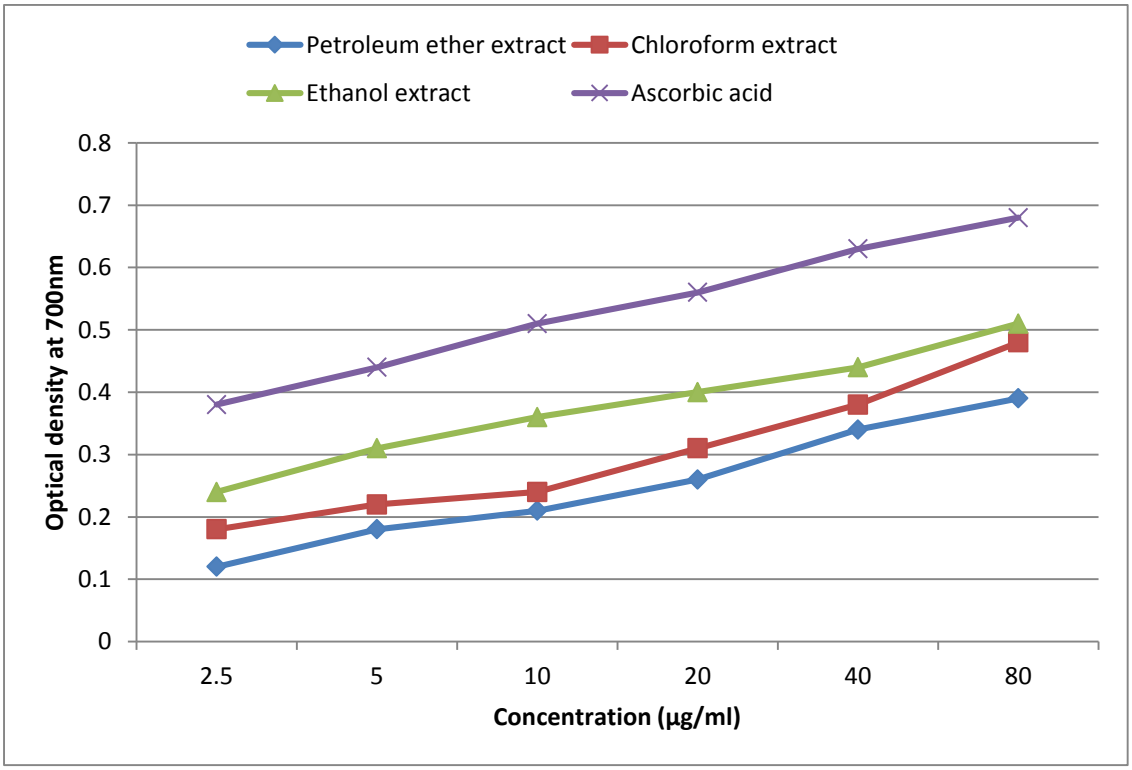


Figure 15: Ferric reducing activity of solvent extracts

4.3.3. Total phenolic content of solvent extracts

The content of total phenolics in solvent extracts was estimated by FCR method. The phenolic content was higher in Ethanol extract followed by chloroform and petroleum ether extracts **(Table)**. The total phenolic compounds in the Petroleum ether extracts was 28.65 mg GAE/g, Chloroform extract was 44.13 mg GAE/g and ethanol extract contains maximum of 59.36 µg Gallic acid equivalents per gram.

Table 14: Total phenolic content of solvent extracts

Extract	Total phenolic content (mg GAE/g)
Petroleum ether	28.65
Chloroform	44.13
Methanol	59.36

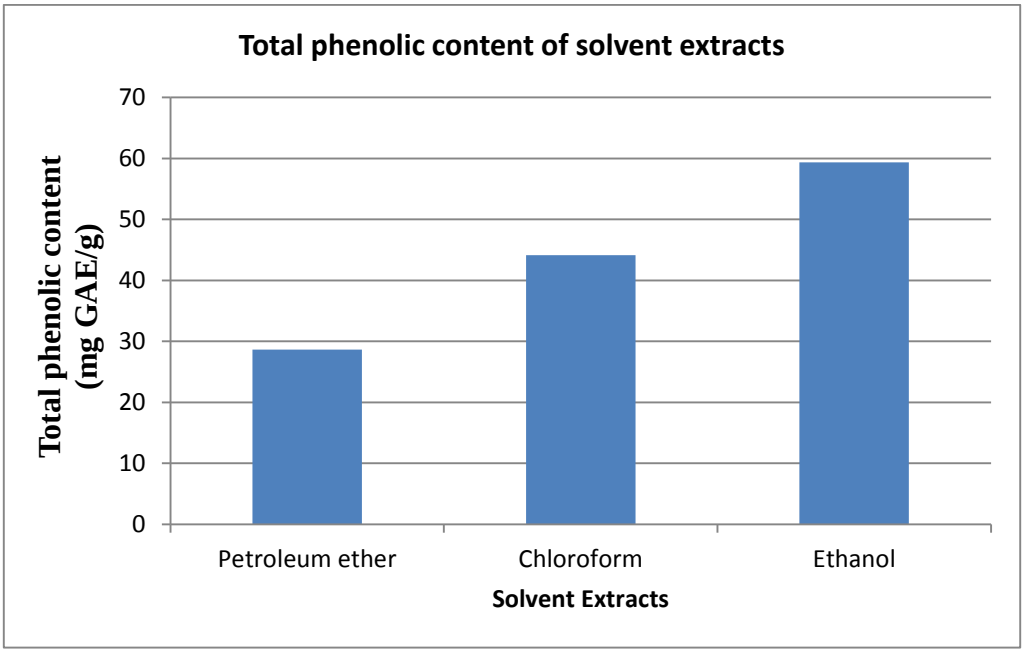


Figure 16: Content of total phenolics in solvent extracts

DPPH is a stable, organic, nitrogen centred free radical and shows maximum absorption at 517nm in alcoholic solutions. On accepting an electron or hydrogen atom, it becomes a stable diamagnetic molecule. This assay is simple, rapid and one of the most widely used *in vitro*antiradical assays for determining the radical

scavenging effect of various types of samples including plant extracts. In the presence of an antioxidant (extract) capable of donating an hydrogen atom, the free radical nature of DPPH is lost and the color (purple) changes to yellow (diphenylpicrylhydrazine). This assay has been used to evaluate radical scavenging potential of several plants including orchids (Dasgupta and De, 2004; Elmastas *et al*., 2006; Coruh *et al*., 2007; Kaviarasan *et al*., 2007; Choi *et al*., 2007; Gulcin *et al*., 2011; Kekuda *et al*., 2012). In this study, the decrease in DPPH absorption in the presence of varying concentrations of solvent extracts and ascorbic acid has been monitored at 517nm. It can be noticed that the extracts at high concentrations showed significant decrease in the absorption of DPPH radicals. Highest scavenging of radical was observed in case of Ethanol extract followed by chloroform and petroleum ether extracts. Ethanol extract was shown to scavenge radicals more effectively as evidenced by lower IC_{50} values. However, the scavenging potential of solvent extracts was lower when compared to ascorbic acid. Although the scavenging abilities of flower extracts were lesser than that of ascorbic acid, it was evident that the extracts showed hydrogen donating ability and therefore the extracts could serve as free radical scavengers, acting possibly as primary antioxidants (Chung *et al*., 2006).

Like DPPH assay, the scavenging of ABTS radicals is another commonly used radical scavenging assay. However, this method needs to generate the free radical. The ABTS radical is generated by reacting the ABTS salt with a strong oxidizing agent which may be potassium permanganate or potassium persulfate. An antioxidant species can reduce the blue-green ABTS radical solution to colorless neutral form by suppression of its characteristic long wavelength absorption spectrum (Shalaby and Shanab, 2013). This assay is widely used to determine radical scavenging activity of several kinds of samples including plant extracts (Wan *et al*., 2011; Rakesh *et al*., 2013; Shalaby and Shanab, 2013). In the present study, the radical scavenging potential of solvent extracts was evaluated by ABTS radical scavenging method. The Ethanol extract scavenged ABTS radicals more efficiently in a dose dependent manner. The scavenging of ABTS radicals by ascorbic acid was more marked than solvent extracts. Although solvent extracts displayed lesser radical scavenging potential than that of ascorbic acid, it is evident that the leaf extract has the electron donating property and hence the extract could serve as free radical scavengers.

The reducing properties of antioxidant species are generally associated with the presence of compounds calledreductones. Ferric reducing assay has been employed by several researchers in order to evaluate antioxidant activity of compounds (Yuan *et al*., 2005; Kim *et al*., 2006; Barros *et al*., 2008; Gulcin *et al*., 2011; Kekuda *et al*., 2012; Pavithra *et al*., 2013). In this assay, the presence of reductants (antioxidants) in the samples would result in the reduction of Fe^{+3} to Fe^{+2} by donating an electron (Chung *et al*., 2006). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Hsu *et al*., 2006).In the present study, it was observed that the reducing powers of all solvent extracts increased with the increase of their concentrations. Ethanol extract displayed greater reducing power than other extracts. It is evident that the solvent extracts possess reductive potential and could serve as electron donors, terminating the radical chain reactions (Chung *et al*., 2006).

Polyphenolic compounds of plant kingdom are one of the most effective antioxidative constituents and hence it is important to estimate phenolic contents of extracts in order to assess their contribution to antioxidant activity (Choi *et al*., 2007). In the present study, total phenolic content of solvent extracts was estimated by FCR method and expressed as mg GAE/g of dry extract. FCR method is one of the oldest and most commonly used colorimetric assays for estimating total phenolic content of a variety of substancesincluding plant extracts. Phenolics reacts with FCR only under basic conditions (adjusted by sodium carbonate solution to pH 10) to form blue complex which has maximum absorption near 750nm. Despite the undefined chemical nature of FCR, the total phenols assay by FCR is convenient, simple, and reproducible. As a result, a large data has been accumulated, and it has become a routine assay in studying the phenolic antioxidants (Tilak *et al*., 2004; Dasgupta and De, 2004; Huang *et al*., 2005; Chung *et al*., 2006; Coruh *et al*., 2007; Kekuda *et al*., 2012; Pavithra *et al*., 2013). The total phenolic content, as estimated in this study was higher in Ethanol extract followed by chloroform and petroleum ether extracts. In this study, a positive correlation was observed between antioxidant activity and total phenolic content of extracts. Ethanol extract containing high phenolics displayed marked antioxidant activity. Similar correlations were observed in earlier studies by researchers such as Tilak *et al*. (2004), Coruh *et al*. (2007), Kekuda *et al*. (2012), Pavithra *et al*. (2013)

where a direct correlation between phenolic content and antioxidant activity was observed.

4.4. CONCLUSION

In the present study, extracts were shown to display dose dependent scavenging of free radicals and ferric reducing activity. The observed bioactivities could be ascribed to the presence of polyphenolic compounds in the solvent extracts. A positive correlation was observed between the phenolic content and the radical scavenging and ferric reducing activity. Methanol extract containing high phenolic content was shown to display marked activity while petroleum ether extract revealed least activity and was found to contain less phenolic content. This positive correlation was supported by the result of several researches.

5.1. INTRODUCTION

Secondary metabolites are chemical compounds derived from living organisms namely plants, animals and microorganisms. The study of natural products involves isolation of compounds in a pure form and investigation of their structure, process of formation, use of the compound, and the role played in the organism. Secondary metabolites are known to function primarily in defense against predators and pathogens and in provide reproductive advantage as intraspecific and interspecific attractants. These metabolitescan also act to create competitive advantage as poisons of rival species. Many plant terpenoids are toxins and feeding deterrents to herbivores or are attractants, and many possess pharmacological activities. Phenolic compounds play important roles in plants. Tannins, lignans, flavonoids, and some simple phenolic compounds serve as defenses against herbivores and pathogens. Some phenolic compounds have allelopathic activity and may adversely influence the growth of neighboring plants. Throughout evolution, plants have developed defenses against herbivory and microbial attack and produced other natural products to foster competitiveness (Tesso, 2005; Cowan, 1999). The study of these natural products has a number of rewards. An intensive study on natural products has led to the discovery of a huge number of drugs useful for the treatment of various ailments and contributed to the development of separation science and technology, spectroscopic methods for structure elucidation and synthetic methodologies that now make up the basics of analytical organic chemistry (Tesso, 2005).

A number of phytochemicals have been isolated and characterized from several orchids. Most of these phytochemicals have shown to display various bioactivities. Takagi *et al*. (1983) isolated five antibacterial compounds from *Bletilla stricta*. Majumdar and Sen (1987) isolated Moscatilin, a bibnezyl derivative from the Orchid *Dendrobium moscutum*. Majumdar and Chatterjee (1989) isolated crepaditin, bibenzyl derivative from the orchid *Dendorbium cripidatum*. Li *et al*. (1991) isolated shibunidine, shihuminine and dendrophenol, shihunidine and shihunine inhibits Na+, K+ and ATPase in the rat kidney. Talapatra *et al*. (1992) isolated Defigenin, a diogenin derivative from *Dendrobium fimbriatum*. Majumdar & Pal (1992) isolated rotundatin a new 9, 10-dihydrophenantherene derivative from Dendrobium rotunatum.

Majumdar & Pal (1993) isolated two bibenzyle dierivates cumulating and fristin, from *Dendrobium cumulatum* and *Bulbophyllum triste* respectively. Yumki *et al*. (1993) isolated three dihydorphenanthropyrans from *Bletilla striata*. Saito *et al*. (1994) isolated an acylated anthocynin from the red flower of *Dendrobium*. Chen *et al*. (1994) found out that moscatilin, moscatin and moscatilin diacetate isolated from *Dendrobium loddigesii* can inhibit the aggregation of rabbit platelets induced by arachidonic acid and collagen. Saito *et al*. (1995) isolated acylated cyanidinglycosides in the purple-red flowers of *Bletilla striata*. Majmder & Ghosal, (1993, 1996) isolated four stilebenoids from the orchids *Arundina bambusifolia* and *Agrostophyllum khasiyanume*. Similarly Yamki & Honda (1996) isolated seven stilbenoids from *Dendrobium catile*.

Miyajawa *et al*. (1997) isolated gigantol from *Dendrobium nobile* and that shows the antimutagenic property. Tatsuzawa *et al*. (1998) found out that flavon-C-glycoside are most common in tropical and subtropical species of the Epidendroid and Vandoid sub family and flavonol glycosides are found in Neottioids orchid. Miyazawa *et al*. (1999) isolated moscatilin from *Dendrobium nobile* and the compound showed antimutagenic property. In the year 2000, Honda & Yamki isolated three phenanthrene from *Dendrobium plicatile*. Fan *et al*. (2001) studied chemical constituents from *Dendrobiumdensiflorum*. Majumder *et al*. (2001) isolated phenanthrene derivatives from *Coelogyne cristata*. William *et al*. (2002) found out that a new flavonol is present along with some known flavonls and acylated anthocynins in the *Dendrobium pompadour*. Chi-Kuan Ho & Chieh-Chih Chen (2003) isolated Moscatilin from the orchid *Dendrobium loddigsii* and tested for anti-cancerous property. Zhi-Ming *et al*. (2005) isolated two triterpines from the orchid *Pholiodata yunnaensis*. Guan *et al*. (2005) studied chemical components and pharmalogical activities of geobiotic type medicinal plants in orchidaceae family. Liu *et al*. (2005) isolated two phenanthraquinones from *Cypripedium tibeticum*. Xue *et al*. (2006) isolated mono-, bi- triphenanthrenens from the tubers of *Cremastra appendiculata*.

In the present study, the chloroform extract of pseudobulb of *B. neilgherrense* exhibited marked antibacterial, antifungal and antioxidant activities. Hence, the chloroform extract was subjected for isolation and identification of bioactive principles present in the extract.

5.2. MATERIALS AND METHODS

5.2.1. Isolation of active compound from *B. neilgherrense*

In present investigation, Thin layer chromatography (TLC) and conventional Column chromatography techniques were adopted to isolate pure components from the chloroform extract of *B.neilgherrense*.

5.2.1.1.Thin Layer Chromatography (TLC)

Different solvent systems were tried for developing TLC for the identification of constituents present in the chloroform extract. One showing maximum and clear separation was selected as mobile phase for the study. The different solvent systems used were as follows,

- Petroleum ether: Ethyl acetate
- Petroleum ether: Chloroform
- Ethyl acetate: Chloroform
- Chloroform: Ethanol

The sample was dissolved in chloroform and spotted on a pre-coated TLC plate. The chromatogram was developed using the above solvent system and the spot were visualized under iodine vapor (detecting agent). After a number of trials, it was observed that, the mixture of Petroleum ether: Chloroform in the ratio of 1:1 by volume showed clear separation of components.

5.2.1.2. Column Chromatography

The chloroform extract of *B. neilgherrense* was subjected to Column chromatography for the isolation of pure compounds using Petroleum ether: Chloroform solvent system in different ratios. The isolation of pure component was done by Column chromatography using Silica gel 100-200 mesh size.For the chromatographic separation, a column of length 55cm and diameter of 6 cm was cleaned and dried.

Stationary phase was prepared by dissolving 300g of (100-200mesh). In 500 ml of freshly distilled petroleum ether and loaded in the column. The column was tapped gently to ensure uniform package. A small quantity of solvent was allowed to remain at the top of the column to avoid drying or cracking of the column. The column was left for overnight before charging extract for complete saturation and removal of air bubbles to make the bed static.

Chloroform extract (8g) was dissolved in minimal amount of chloroform and the solution is blended with activated Silica gel (10g). The mixture was dried well and loaded over the stationary phase in the column. The column was eluted with petroleum ether and chloroformmixture of various proportions as mobile phase. During elution the polarity of mobile phase was increased gradually by varying the proportions of petroleum ether and chloroform. The elution is monitored by TLC and the eluted fractions were collected in 100 ml portions. The fractions with similar spots were pooled together. Four fractions were obtained from different ratios of solvent system (Table 15). Disc diffusion method was employed to determine antimicrobial activity of purified fractions against *Aspergillus niger*, *Cryptococcus neoformans* and *Bacillus subtilis*. Only one fraction (fraction 6 named as DNG-01 now onwards) showed antimicrobial effect. Hence, the fraction DNG-01 was subjected to characterization (structural elucidation) by various analytical techniques.

5.2.2. Characterization of DNG-01

5.2.2.1.Elemental analysis

The elemental analysis of the isolated fraction DNG-01 was carried to know the percentage composition of the element present in the compound.

5.2.2.2. Infrared spectrum

The infrared spectrum of the compound was recorded in the range of 4000-400cm^{-1} on Shimadzu, Japan.

5.2.2.3. Proton Magnetic Resonance spectrum (^{1}H NMR)

The magnetic resonance spectrum of the compound DNG-01 was recorded on AV 400 FT NMR Spectrophotometer (400MHz) using TMS as an internal standard. The fraction DNG-01 was prepared by dissolving the compound in DMSO.

5.2.2.4.^{13}C Nuclear Magnetic Resonance spectrum (^{13}C NMR)

The fraction DNG-01 was subjected to ^{13}C-NMR in Bruker BioSpin model. The fraction was dissolved in DMSO.

5.2.2.5.Liquid Chromatography-Mass Spectrum (LC-MS)

The purity of the fraction DNG-01 was tested using The LCMS 2010 Shimadzu, Japan. The following conditions were maintained in running the sample on LCMS 2010, Shimadzu, Japan.

HPLC Conditions

1. Column- C18
2. Mobile phase- Methanol:water (90:10)
3. Rate of flow- 0.2 ml/min
4. Sample dissolved in methanol
5. Injected volume- 5µl
6. UV-Vis wavelength- 254 nm

Mass spectroscopy Conditions

Probe:

APCI: Atmospheric pressure chemical ionization mainly used for non polar compounds to analyze.

ESI: Electron spray ionization mainly used for polar compounds to analyze.

+ ionization: which gives protonated M+1 values

- ionization: which gives protonated M-1 values

+ or – ionization and the type of probes can be identified in data file name

In presence of halogens the values will show M and M+2 in positive: M and M-2in negative.

5.3. RESULTS AND DISCUSSION

5.3.1. Thin Layer Chromatography

Chromatography is the method of choice in handling the problem of isolation of an interested compound from a complex mixture. TLC involves the use of a particulate sorbant spread on an inert sheet of glass, plastic, or metal as a stationary phase. The mobile phase travel up carrying the sample that was initially spotted on the sorbant just above the solvent level. Depending on the nature of the stationary phase, the separation can be either partition or adsorption chromatography. The advantage of TLC is that the samples do not have to undergo the extensive cleanup steps, and the ability to detect a wide range of compounds, using reactive spray reagents (Tesso, 2005). In the present study, we optimized the solvent system for resolution compounds present in chloroform extract of *B. neilgherrense*. Petroleum ether and chloroform mixture appear promising in the separation of components.

5.3.2. Column chromatography

It consists of a column of particulate matterfor e.g., silica or alumina that has a solvent passed through it at atmospheric, medium or low pressure. The separation can be liquid/solid (adsorption) or liquid/liquid (partition). The columns are usually made up of glass or plastic with sinter frits to hold the packing. Most systems rely on gravity to push the solvent through, but medium pressure pumps are oftenemployed in flash method. The sample is dissolved in solvent and applied to the top of the column or alternatively adsorbed on a coarse silica gel. The solvent elutes the sample through the column, allowing the components to separate. The solvent is usually changed stepwise, and fractions are collected according to the separation required, with the eluting products usually monitored by TLC. The advantage is that no expensive equipment is required, and the technique can be scaled up tohandle sample sizes approaching gram amounts (Tesso, 2005). In the present study, four fractions were obtained during column chromatographic separation of chloroform extract of *B. neilgherrense* (Table 15).

Table 15: Column Chromatographic details of extract of *B. neilgherrense*

Fractions	Solvent System Ratio Petroleum ether:Chloroform	Colour of compound	Yield in mg
1.	100:0	No Residue	---
2.	90:10	No Residue	---
3.	80:20	No Residue	---
4.	70:30	Yellowish green	150mg
5.	60:40	No Residue	---
6.	50:50	Reddish Brown	700mg
7.	40:60	No Residue	---
8.	30:70	Light Brown	15mg
9.	20:80	No Residue	---
10.	10:90	Brown Solid	100 mg
11.	0:100	No Residue	---

5.3.3. Antimicrobial activity of purified fractions

Out of 4 fractions, the fraction 6 (reddish brown in color and 700mg yield) only displayed antimicrobial activity. The result of antimicrobial activity of fractions against bacteria and fungi is shown in Table 16.

Table 16: Antimicrobial activity of fractions obtained

Fractions/control	Zone of inhibition in mm		
	B. subtilis	***A. niger***	***C. neoformans***
Fraction 4	0	0	0
Fraction 6 (DNG-01)	28	30	30
Fraction 8	0	0	0
Fraction 10	0	0	0
DMSO	0	0	0

5.3.4. Elemental analysis of DNG-01

The result of elemental analysis of the fraction DNG-01 is shown in Table 17.

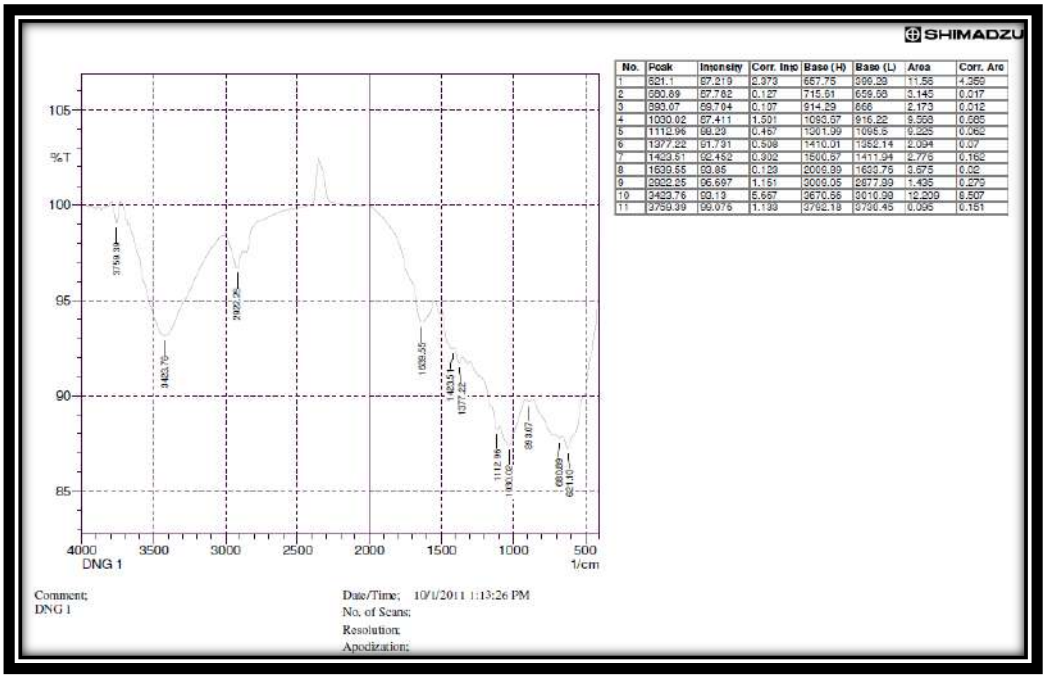
Table 17:Elemental analysis of DNG-01

Fraction	Elemental analysis	
	C	H
DNG-01	74.01%	11.9%

The elemental analysis of the compound indicated the following elemental composition. Carbon= 74.01% and Hydrogen= 11.91%.

5.3.5. IR spectrum

Infrared spectroscopy is a type of spectroscopy that deals about the vibration of atoms in a molecule. Transition of atoms between the vibrational levels result in the vibrational spectra which gives an insight in to the discrete motion of the atoms in the molecular system. Functional groups have characteristic vibrational frequencies make the spectra as one of the most reliable methods for understanding the structure of molecules (Tesso, 2005). In the present study, the infrared spectrum exhibits a broad stretching frequency at 3424cm^{-1} for hydroxyl group (Figure 17).



No.	Peak	Intensity	Corr. Int.	Base (H)	Base (L)	Area	Corr. Area
1	621.1	87.219	2.373	667.75	599.39	11.56	4.359
2	680.89	87.782	0.127	715.91	659.68	3.145	0.217
3	699.07	89.704	0.107	914.39	666	2.173	0.212
4	1030.02	87.411	1.501	1093.57	916.22	9.558	0.685
5	1112.96	98.23	0.467	1201.99	1095.5	0.925	0.062
6	1377.22	91.791	0.508	1410.01	1352.14	2.094	0.07
7	1423.51	92.452	0.302	1500.67	1411.94	2.776	0.162
8	1639.55	93.85	0.128	2000.99	1639.75	3.675	0.02
9	2922.25	86.697	1.151	3000.05	2877.99	1.435	0.279
10	3423.76	93.13	5.667	3670.56	3010.99	12.209	6.507
11	3759.39	99.075	1.133	3792.18	3700.45	0.095	0.151

Figure 17: Infrared spectrum

5.3.6. Proton Magnetic Resonance spectrum (^{1}H NMR)

^{1}H NMR is a plot of signals that arise from absorption during an NMR experiment by the different protons in a compound under study as a function of frequency. The area under the plots provides information about the number of protons in the molecule, the position of the signals reveals information regarding the chemical and electronic environment of the protons, and the splitting pattern provides information about the number of neighboring (vicinal or geminal) protons (Derome, 1987; Abraham et al., 1988; Tesse, 2005). The fraction DNG-01 in its ^{1}H-NMR spectrum exhibits a two overlapped triplets peak at δ 0.71 indicating the presence of two methyl groups adjacent to methylene groups. The strong signal at δ 1.00 is due to long chain methylene groups. The signals at δ 2.00 and 2.30 are due to methoxy protons adjacent to CH-OH groups. The couple of signals in between δ 3.00 to 4.00 are due to a methoxy group and a secondary methylene carbon atom (Figure 18 and Figure 19).

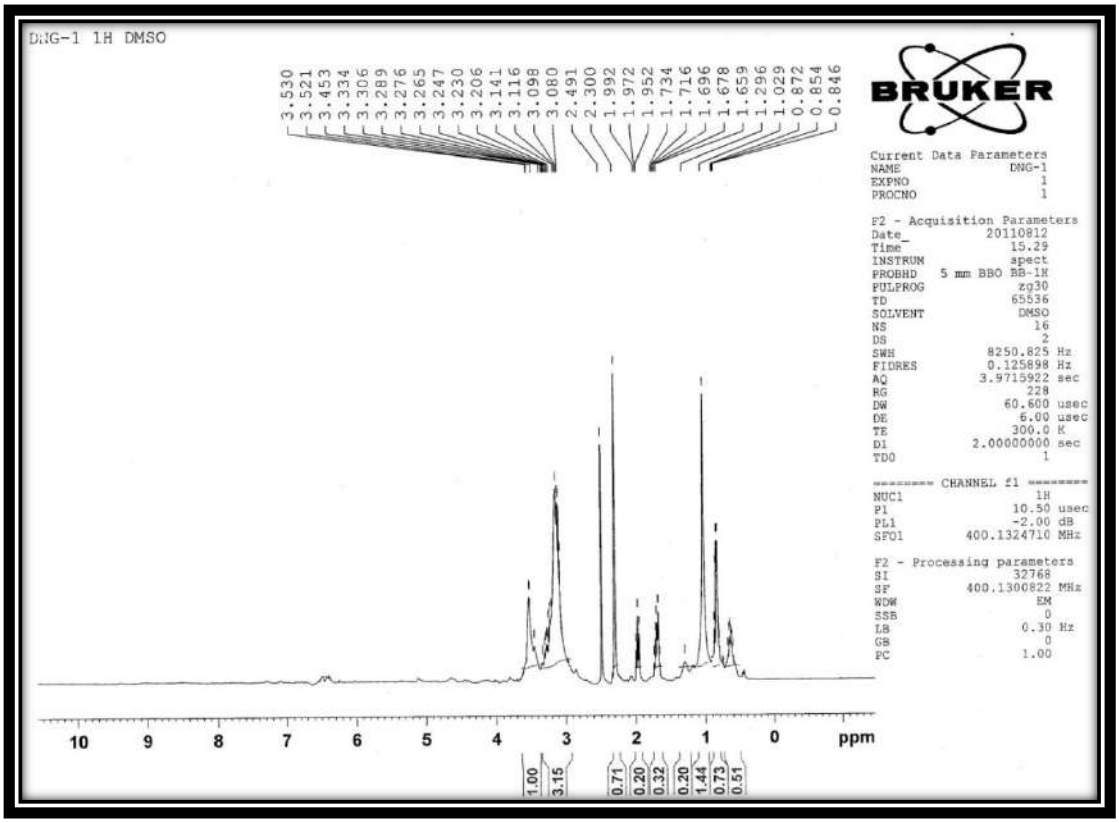


Figure 18: Proton Magnetic Resonance spectrum (^{1}H NMR)

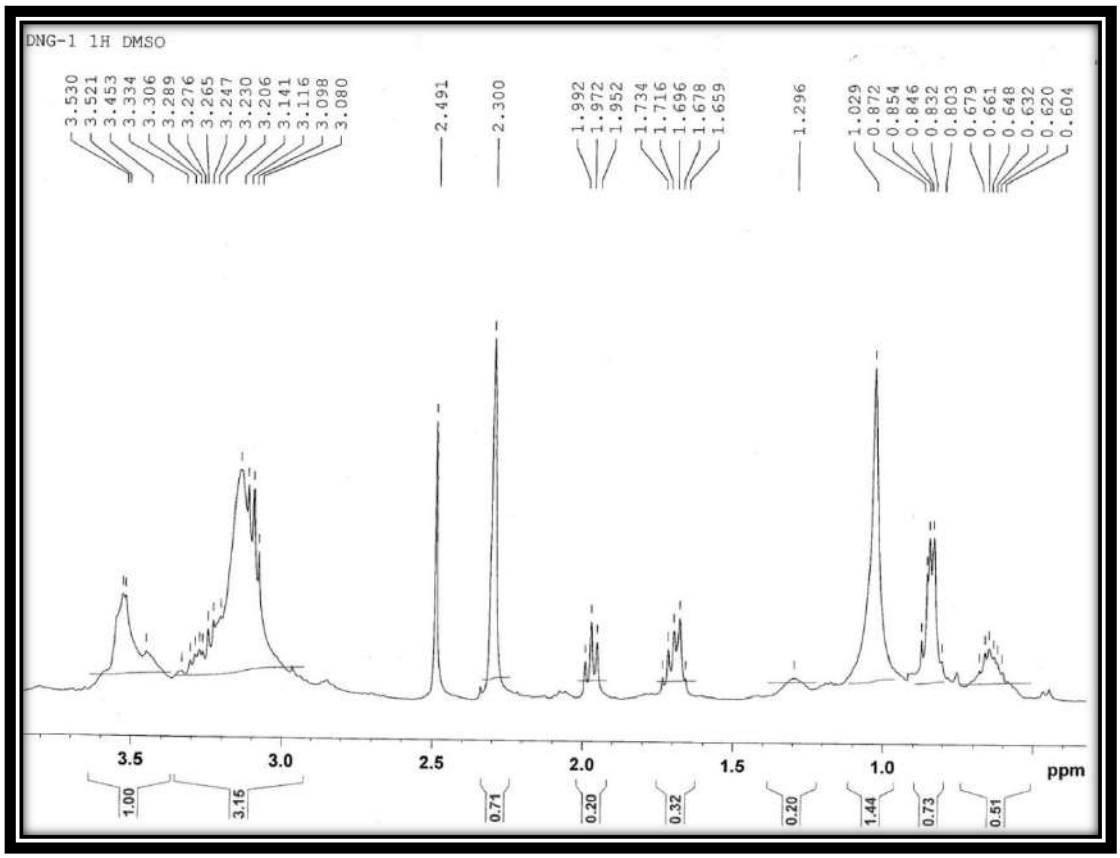


Figure 19: Expanded Proton Magnetic Resonance spectrum (^{1}H NMR)

5.3.7. ^{13}C Nuclear Magnetic Resonance spectrum (^{13}C NMR)

Similar to ^{1}H NMR, C NMR is a plot of signals that arises from the different carbons as a function of chemical shift. The signals in ^{13}C-NMR normally appear as singlets due to the decoupling of the attached protons. Different techniques of recording of the 1D carbon NMR has been developed so that it is possible to differentiate between the various types of carbons such as the primary, secondary, tertiary and quaternary from the 1D ^{13}CNMR plot ((Derome, 1987; Abraham et al., 1988; Tesse, 2005). In the ^{13}C NMR (Figure 20 and 21), complementing the ^{1}H-NMR spectrum the ^{13}C NMR spectrum exhibits signals at δ 74.67 suggesting the presence of a secondary carbon atom under a hydroxyl group. The signal at δ 55.99 is due to the methoxy carbon atom. The signal at δ 48.00 is due to the carbon atom of the chain attached to the methylene group. The signals at δ 13.86 and 14.02 are due to terminal methyl group carbon atoms. Rest of the signals at δ 18.50, 28.60, 28.96, 30.07, 31.23 and 38.98 are due to the carbon atoms of the long chain methylene groups.

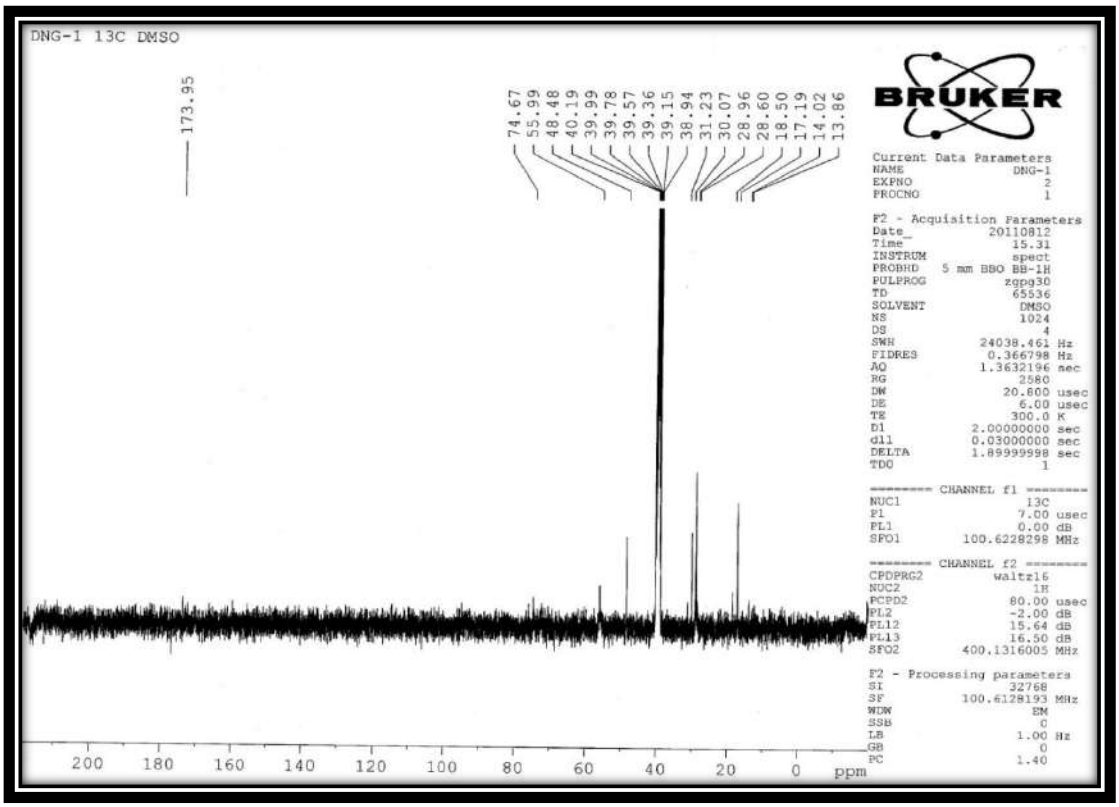


Figure 20: ^{13}C Nuclear Magnetic Resonance spectrum (^{13}C NMR)

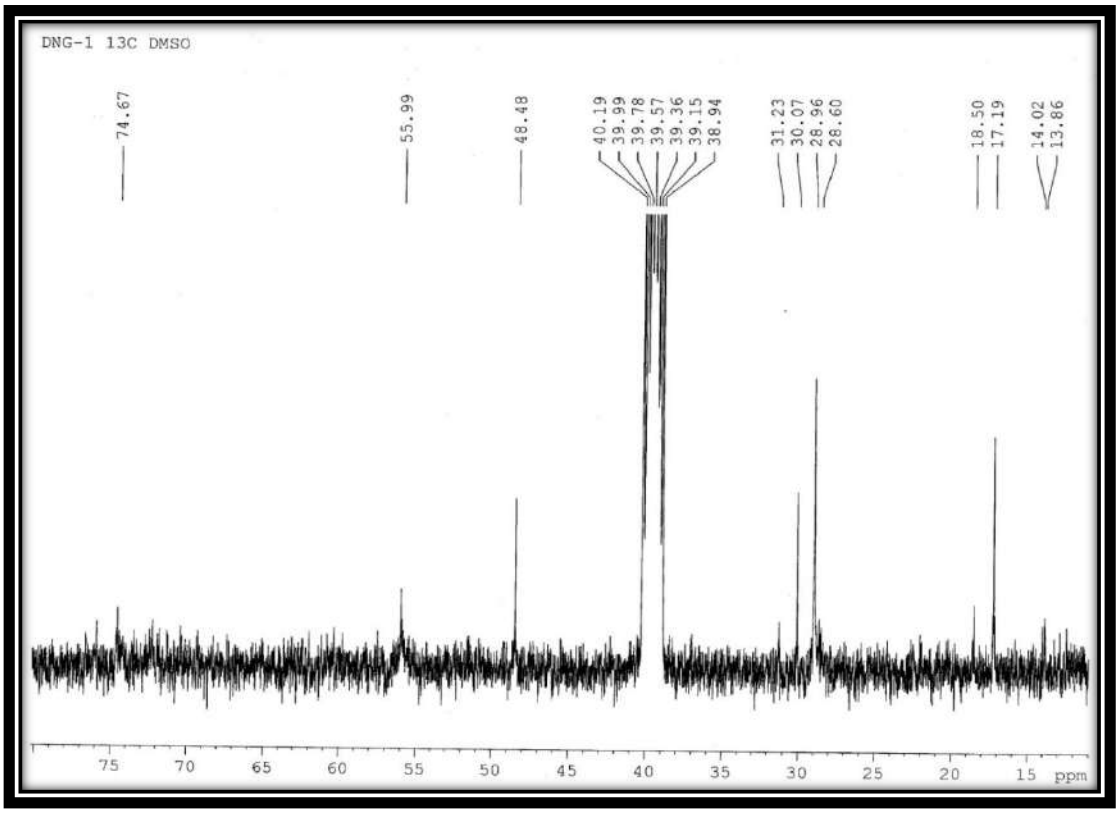


Figure 21: Expanded ^{13}C Nuclear Magnetic Resonance spectrum (^{13}C NMR)

5.3.8. Liquid chromatography-mass spectrometry

LC-MS is an important analytical technique which couples high resolution chromatographic separation with sensitive and specific mass spectrometric detection. This includes high performance liquid chromatography (HPLC)-MS, capillary electrophoresis (CE)-MS and more recently capillary electrochromatography (CEC)-MS. The technique is fast developing, in particular mass spectrometry area, with highly improved sensitivity and resolution (Lim and Lord, 2002). In the present study, liquid chromatography showed a single peak indicating the purity of the fraction. The ESI-MS positive mode mass spectrum shows a strong peak at m/z 258 for the $[M+H]^+$ peak suggesting a molecular weight of 257 to the compound (Figure 22).

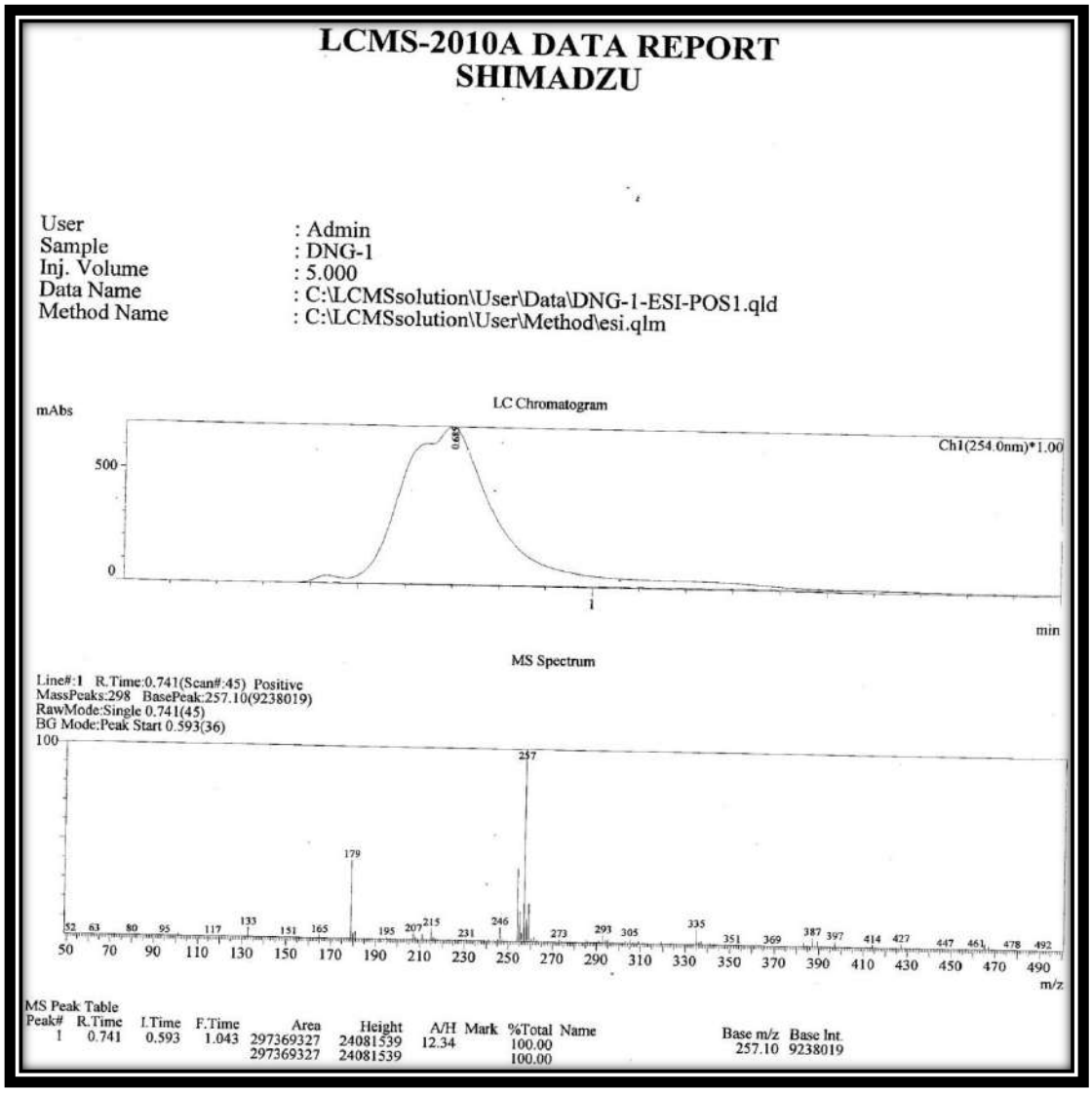


Figure 22: Liquid Chromatography Mass Spectrum (LCMS)

5.4. Conclusion (Elucidation of structure)

On the basis of spectral data, the formula of DNG-01 can be suggested as CH_3-$(CH_2)_6$-CH (OH)-CH (OCH_3)-$(CH_2)_5$-CH_3. The molecular formula is $C_{16}H_{34}O_2$ and the molecular weight is 258. The IUPAC name is 7-methoxytetradecan-8-ol. The suggested structure of DNG-01 is shown in Figure 23.

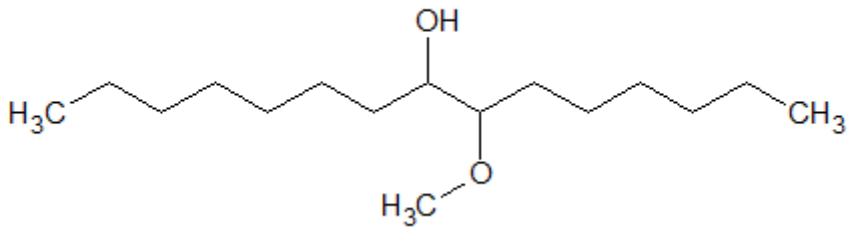


Figure 23: Structure of DNG-01

SUMMARY

- In the present work we conducted a random survey of commonly available orchids and their distribution in Shimoga district of Karnataka for the period of 2008 to 2010. During the survey we have found around 30 species of Orchids belonging to 20 genera.

- Based on the criteria such as medicinal property of orchids, distribution, availability of the plant in sufficient quantity, we have selected two orchids namely *Acampe praemorsa* and *Bulbophyllum neilgherrense*.

- Antimicrobial assay of four solvent extract (Petroleum ether, Chloroform, Ethanol and water) of both orchid species was tested by Agar well diffusion assay against 15 species of human pathogenic bacteria and 10 species of pathogenic fungi. In the antimicrobial assay the chloroform extract of *B. neilgherrense* stand out with high degree of inhibition against most of the test bacteria and fungi.

- The radical scavenging and ferric reducing activity of solvent extracts of *B. neilgherrense* revealed dose dependent activity. Ethanol extract displayed marked scavenging activity followed by chloroform and petroleum ether extracts. A positive correlation was observed between phenolic content of extracts and the scavenging and reducing activity.

- The chloroform extract displaying marked antimicrobial activity was further subjected to recovery of bioactive principles. Thin layer chromatography (TLC) and column chromatography was performed to isolate the active antimicrobial fraction. During the column chromatography we got four fractions and in that fraction-2 was having high degree of purity and was found to display antimicrobial activity.

- The antimicrobially activity fraction-6 (DNG-01) was further subjected to the elemental analysis and spectral analysis *viz*., Infrared Spectroscopy, ^{13}C NMR Spectroscopy, ^{1}H NMR Spectroscopy and Liquid Chromatography and Mass Spectroscopy(LCMS). Based on the elemental analysis and the spectral studies we suggested the name of the fraction DNG-01 as 7-methoxytetradecan-8-ol with molecular formula $C_{16}H_{34}O_2$ and molecular weight 258.

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APPENDIX

MEDIA COMPOSITION

Composition of Nutrient Agar

Ingredients	Quantity (gram)
Beef Extract	3.0
Peptone	5.0
Agar	15.0
Distilled water	1000ml
Final pH	7.0 ± 0.2

Composition of Potato Dextrose Agar

Ingredients	Quantity (gram)
Potato infusion	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled water	1000ml
Final pH	5.6 ± 0.2

Composition of Sabourauds Dextrose Agar

Ingredients	Quantity (gram)
Dextrose (Glucose)	40 gm
Peptone	10 gm
Agar	15 gm
Distilled Water	1000 ml
Final pH	5.6 ± 0.2