Chapter 2
Chemical profiling & bioactivity studies
Medicinal and aromatic plants (MAPs) have been used for centuries as remedies for human diseases because they contain components of therapeutic value. It has been estimated by WHO that 80% of the population, the majority of this in the developing countries, still rely on plant-based medicine for primary health care needs (WHO 1993). For this purpose, various strategies have been developed, e.g. biological screening, isolation, as well as clinical trials for a variety of plants. Following the advent of modern medicine, herbal medicine suffered a setback, but during the last two or three decades the advances in phytochemistry and in the identification of the plant compounds, providing effective against certain chronic disease. This awakening has led to a sudden demand for herbal medicine. Worldwide as well as in the developing countries, the most humans died due to infectious bacterial diseases (Nathan 2004). The bacterial organisms including both Gram positive and Gram negative ones are the main cause of severe infections in humans, because they have the ability to survive in harsh conditions due to their multiple environmental habitats (Ahameethunisa & Hopper 2010). Nowadays, multiple drug resistance is developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases (Gupta et al. 2008). In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression, and allergic reactions (Werner et al. 1999). On the other hand, environmental safety is a major concern in many countries, and the application of synthetic agrochemicals runs the risk of causing unacceptable environmental damage, such as health hazards to humans, toxicity to useful non-targeted animals and environmental pollution. The use of natural products would be a helpful way to reduce this risk, and also the situation requires searching for new antimicrobial substances. The screening of the active compounds from plants has lead to the discovery of new medicinal drugs which show efficient protection and treatment roles against various diseases, including cancer (Kumar et al. 2004; Sheeja & Kuttam 2007). Therefore, there is a need to develop alternative antimicrobial drugs from medicinal plants for the treatment of infectious diseases.

The term ‘Natural Products’ in the broadest sense should represent all the chemical compounds, occurring in nature (Krishnaswamy 1999). However, by convention and practice, the term is now used to refer only to the organic compounds occurring in nature. The boundaries are further defined by restricting the term to the secondary metabolites, leaving out the primary metabolites and whose biochemical functions are more or less well known and therefore covered under biochemistry.

The use of natural products with therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products were the main sources of drugs (De Pasquale 1984). Plants are a rich source of secondary metabolites that have medicinal and aromatic
properties. According to some estimates, at least ~100,000 such secondary metabolites are now known to occur in 50,000 plant species and ~4,000 new secondary metabolites are being discovered every year from a variety of plant species (Verpoorte et al. 1999; Gomez-Galera et al. 2007). For thousands of years these natural plant products have been utilized for human healthcare in the form of drugs, antioxidants, flavors, fragrances, dyes, insecticides and pheromones. During the last century, however, the use of synthetic drugs led to a decline in the use of plant-derived compounds, so that at one time it was believed by many that the synthetic drugs would perhaps completely replace the use of traditional plant-derived medicines. However, in recent years, a resurgence of the use of herbal drugs has one again been witnessed, firstly because the synthetic drugs have been found to be hazardous in many cases, and secondly because there is growing awareness that the plant derived-medicines have none of the side effects that are so common in the case of synthetic drugs. Recently, the world market for herbal medicines including herbal products and raw materials is actually growing at an annual rate of 5-15% (Kumar et al. 2008). This is an indication of a possible growing demand for plant-derived drugs in coming years.

**Essential Oils:**

The essential oils also known as volatile or ethereal oils, are defined as, the oils obtained by the steam distillation of plants. From the view point of practical applications, these materials may be defined as odiferous bodies of an oily nature, obtained almost exclusively from vegetable organs: flowers, leaves, barks, woods, roots, rhizomes, fruits, and seeds (Burt 2004; Celiktas et al. 2006; Skocibusic et al. 2006). An essential is generally identified with the name of the source plant. Essential oils are generally liquid, aromatic and possess pleasant odour and essence. The term “essential oil” is often used in cosmetics and perfume industries as synonymous with perfume oil, base or, “compound”.

Chemically, the essential oils are a complex and highly variable mixture of constituents that belong to two groups: terpenoids and aromatic compounds. The name terpene is derived from the English word “Turpentine” (Guenther 1985). The terpenes are the unsaturated hydrocarbons which have a distinct architectural and chemical relation to the simple isoprene molecule (CH2==C (CH3)—CH==CH2).

**Sources and Isolation of Essential Oils:**

Essential oils are isolated from different aromatic plants generally distributed in Mediterranean and tropical countries across the world where they are esteemed as an imperative component of the native medicine systems. These essential oils can be produced in almost all plant organs such as flowers, buds, stems, leaves, fruits, seeds and roots etc. These are accumulated in secretary cells, cavities, channels, and epidermic cells (Burt 2004; Chalchat & Ozcan 2008; Hussain et al. 2008; Anwar et al. 2009a). Almost all odoriferous plants contain essential oils. The raw material
from which essential oils are manufactured may be fresh, partially dehydrated or dried (Ozcan 2003; Asekun et al. 2007; Hussain et al. 2008).

The extraction of the essential oil depends mainly on the rate of diffusion of the oil through the plant tissues to an exposed surface from where the oil can be removed by a number of processes. There are different methods, depending upon the stability of the oil, for the extraction of the oil from the plant materials. Steam distillation and hydrodistillation are still in use today as the most important processes for obtaining essential oils from the plants (Baker et al. 2000; Kulisic et al. 2004; Masango 2004; Sokovic & Van Griensven 2006). Other methods employed for isolation of essential oils include the uses of liquid carbon dioxide or microwaves, low or high pressure distillation employing boiling water or hot steam (Bousbia et al. 2009; Donelian et al. 2009). The essential oils obtained by steam distillation or by expression are generally preferred for food and pharmacological applications.

The complexity of the essential oils is a real challenge for determining their reliable and accurate compositional data. The rapid advances in spectroscopic and chromatographic techniques have totally changed the picture of chemical study of essential oils. Many techniques have been used for studying the chemical profiles of essential oil e.g. IR-spectroscopy, UV-spectroscopy, NMR spectroscopy and gas chromatography (Bakkali et al. 2008; Hussain et al. 2008). The increasing importance of essential oils in various domains of human activities including pharmacy, perfumery, cosmetics, aromatherapy, and food and beverages industry has prompted an extensive need of reliable methods for analyses of essential oils. These requirements have been satisfactorily fulfilled by GC and GC-MS techniques (Salzer 1977; Wilkins & Madsen 1991; Daferera et al. 2000; Juliano et al. 2000; Jerkovic et al. 2001; Delaquis et al. 2002; Burt 2004). Gas chromatography has been proved to be an efficient method for the characterization of essential oils (Bakkali et al. 2008; Anwar et al. 2009b). The combination of gas chromatography and mass spectrometry (GC-MS) allows rapid and reliable identification of essential oils components (Yadegarinia et al. 2006; Gulluce et al. 2007; Anwar et al. 2009a).

The yield and the quality of the essential oil are considerably affected by processing methods used for their handling and storage (Barroso 2006; Van Vuuren et al. 2007). The essential oils are enclosed in oil glands present in the cellular structure of the plant materials. Although essential oils may be produced from an endemic population, there can be several reasons why the composition and thus, the essential oil quality from aromatic plants might differ greatly. Genetic, physiological and environmental factors as well as processing conditions may play an important role while defining the chemistry and chemical composition of essential oils (Lawrence 1992; Juliani et al. 2002; Mactavish & Harris 2002; Masotti et al. 2003; Angioni et al. 2006). Moreover, the influence of environmental factors, maturity factors on chemotypic differentiation
and physico-chemical variations of the essential oils of many plants have also been reported in the literature (Hussain et al. 2008; Anwar et al. 2009a).

**Recent trends for Uses of Essential Oils:**

Essential oils obtained from various plant species are recently gaining much scientific and public interest because of their multifarious uses and diverse biological activities (Anon 2002; Miura et al. 2002; Bowles 2003; Allahverdiyev et al. 2004; Burt 2004; Jie et al. 2007; Souza et al. 2007). A large number of herbal plants have been screened for their potential essential oil and exploited for commercial applications (Burt 2004; DeSousa et al. 2004; Busatta et al. 2008; Maksimovic et al. 2008; Mohammadreza 2008). The essential oils find wide and varied application in many industries such as cosmetics and perfumes, beverages and ice creams, confectionary and backed food products, etc. for the scenting and flavouring of consumer’s finished products (Burt 2004; Guenther 1985). Currently, about 300 essential oils, out of approximately 3,000 are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries (Sivropoulou et al. 1996; Sivropoulou et al. 1997; Burt 2004; Delamare et al. 2007). Some of the essential oils or their bioactive components such as limonene, geranyl acetate or carvone are also used as an important ingredient in tooth pastes, hygienic products. These also act as food preservers and additives, as well as employed for the treatment of different ailments in the folk medicine systems.

Now a day, some herbal-based essential oils are also used in aromatherapy as they are believed to exhibit certain medicinal benefits for curing organ dysfunction or systemic disorder (Perry et al. 1999; Silva et al. 2000; Hajhashemi et al. 2003). In view of the multiple uses of essential oils, it is imperative to build up better understanding regarding their mechanism of action and biological activities for novel applications in our environment, health and agriculture systems (Gustafson et al. 1998; Carson & Riley 2003). Recent scientific reports have also focused on the antioxidant principles and biological activities of essential oils (Skocibusic et al., 2006; Yuenyongsawad & Tewtrakul 2005; Tepe et al. 2007; Hussain et al. 2008; Anwar et al. 2009b). The essential oils have shown potential as anti-bacterial agents, disinfectants, anti-fungal agents, insecticides and as herbicides (Skocibusic et al. 2006; Bozin et al. 2006; Maksimovic et al. 2007; Van Vuuren et al. 2007). The most thoroughly examined are antimicrobial properties of essential oils, which in many ways are better than those exhibited by synthetic antibiotics due to their wider spectrum of activity. Bactericidal properties of essential oils may also be used for disinfection of air. The utilization of essential oils for the fungus-free storage of food has also been focused (Burt 2004; Devlieghere et al. 2004; Holley & Patel 2005). Essential oils of some spices and herbs such as sage, oregano, thyme, and Satureja etc. have shown their antioxidant potential (Ruberto & Baratta 2000; Rota et al. 2004; Rota et al. 2008) and thus can be used as natural antioxidants for the protection of fats/oils and related
products (Burt 2004; Sacchetti et al. 2005; Bozin et al. 2006). Recently, the uses of natural antioxidants are becoming very popular in food and preventive medicine due to the claims that they are safer and have disease–preventing and health promoting attributes. Research is now in progress to exploring the applications of some essential oils for therapeutic uses and management of infectious diseases as an alternative to standard drugs remedies (Bozin et al. 2006; Celiktas et al. 2007; Kelen & Tepe 2008; Politeo et al. 2007; Sokovic & Van Griensven 2006).

**Aims and Objectives of the Present Study:**

Although a considerable amount of work has been done and data generated on the chemical composition of essential oils of Lamiaceae family (Burt 2004; Delamare et al. 2007; Politeo et al. 2007; Rota et al. 2008) however, to the best of our knowledge there are no earlier reports yet available regarding the detailed chemical characterization and evaluation of biological activities of essential oils from plants of *Ocimum* species, native to West Bengal, India. The present work was therefore undertaken with the main objective to isolate and characterize the essential oils of selected *Ocimum* species growing in West Bengal for their detailed chemical constituents and biological activities. The following were the main objectives of the present study:-

1. Investigation of essential oils yields and chemical characteristics with respect to West Bengal condition.
2. Appraisal of the biological (antimicrobial) activities of the essential oils and methanol extracts.
In taxonomically genus *Ocimum* is difficult, due to the interference of man with selection, cultivation and hybridization. Pushpangadan and Bradu (1995) recognize more than 150 species in the genus. However, most of their taxa are essentially based on leaf morphology and colour, which frequently depend on environmental conditions. More recently, Paton et al. (1999) proposed that only 65 species of *Ocimum* should be retained, and that other attributions should be considered synonyms or false attribution.

The still existing uncertainly in the classification within the genus depends on the fact that the species identification relied on morphological characters whose expression is known to be affected by developmental and environmental factors. To assist in classification a system of standardized descriptors based on volatile oil has proposed by Lawrence (1993) and Grayer et al. (1996) that classified the different basil chemotypes on the basis of the prevalent aromatic compound or the components major than 20%, respectively.

Several aroma compounds can be found in chemotypes of *Ocimum* species such as citral, linalool, methylchavicol, and methylcinnamate and are traded in the international essential oil market. These chemotypes are commonly known by names based on geographical origins. Although essential oils in different basil cultivars are variable, prevalent components are monotherpenes and phenylpropanoids (Tateo 1989; Marotii et al. 1996). Many *Ocimum* species contain primary monotherpene derivatives such as limonene, camphor, 1,8-cineole, linalool and geraniol (Charles & Simon 1992; Martins et al. 1999). Other, including primarily contain phenoyl derivatives, such as eugenol, methyleugenol, chavicol, estragole, methyl-cinnamate, often combined with various amounts of linalool (Werker et al. 1993; Miele et al. 2001). Because chemotype classification based on just one major volatile oils is problematic, frequently one plant contains two or more compounds in nearly equal amounts. It is more convenient to consider the overall oil profile of major constituents, by identifying them above a fixed threshold level.

About 116 different genotypes of *Ocimum* have been screened for their essential oil and these in turn have also been analyzed for the chemical constituents (Grayer et al. 1996; Vieira et al. 2003); Marotti et al. (1996) studied essential oil of Italian cultivars having different morphological features, and determined three different chemotypes: ‘linalool’, ‘linaloolemethyl chavicol’ and ‘linalooleeugenol’. Similarly, Lachowicz et al. (1997) defined essential oil composition of basil varieties from Australia containing methyl chavicol, linalool, methyl cinnamate, a mixture of linalool/methyl cinnamate, and linalool/methyl chavicol. They finding, the variation in the oil composition could be attributed to differences in soil conditions and altitude. In the oils, obtained from aerial parts of *O. basilicum* grown in Colombia and Bulgaria, linalool and methyl cinnamate are reported as major components of volatile oils respectively (Vina et al. 2003; Jirovetz et al. 2001). Linalool and methyl eugenol are the main components of
the essential oils of *O. basilicum* cultivated in Mali (Chalchat et al. 1999) Guinea (Keita et al. 2000). Authors are finding, these differences may be probably due to different environmental and genetic factors, different chemotypes and the nutritional status of the plant as well as other factors that can influence the oil composition.

Telci et al. (2006) studied the identifying the essential oil composition of 18 Turkish basils (*Ocimum basilicum* L.) using GC and GC-MS. In their study, seven different chemotypes were identified. They finding, Methyl-chavicol and high citral contents as a new and major chemotypes in the Turkish basils. Sajjidi et al. (2006), also found that methyl-chavicol is a dominant constituent in basils in Iran.

Recently, Hakkim et al. (2008) investigated chemical composition of eight selected *Ocimum* species. In their experiment, leaves of these plants were extracted using methanol and quantitative analysis of phenolic constituents was determined using HPLC. They finding, *Ocimum* species has led to 11 phenolic compounds (rosmarinic acid, lithospermic acid, vanillic acid, *p*-coumaric acid, hydroxybenzoic acid, syringic acid, caffeic acid, ferulic acid, cinnamic acid, hydroxyl phenyl-lactic acid, and sinapic acid). It may be pointed that reinvestigation is necessary in certain cases as the identify of the specimens in doubtful. Volatiles in the essential oils of *Ocimum* species are mainly derived either from the phenylpropanoid or mevalonic acid metabolism. Biogenetic pathways and the formation of volatiles, such as terpenes from the isoprenoid metabolism and phenylpropanoids from the cinnamic acid metabolism, have been studied and discussed intensively during the last 30-40 years (Schreier 1984). In the last decade, new valuable tools, based on DNA analysis, have been made available for taxonomic studied (Powell et al. 1996; Karp et al. 1998; Cordeiro et al. 2003; Barcaccia et al. 2003). The use of PCR-based tools allows detection of DNA polymorphism at random or specific loci in the genome. Their use has been instrumental in solving controversial taxon attributions by comparing genotypes independently from phenotypes. By identifying polymorphic sequence in the genomic DNA, these tools allow phylogenetic (Heun et al. 1997) and taxonomic (Winfield et al. 2003) studies, as well as cultivar and clone identification (Stallen et al. 2000; Labra et al. 2001; Labra et al. 2003; Vos et al. 1995).

**Antibacterial Activity of Ocimum basilicum L.**

*Studies Involving Diffusion Methods:*

Lahariya & Rao (1979) studied the antimicrobial effectiveness of the essential oil of *O. basilicum* tested in vitro against 10 different microorganisms. They found that this essential oil was more active than the reference, streptomycin, in inhibiting the growth of *Bacillus pumilus*, but it had no activity against *Bacillus mycoides*, *Pseudomonas mangiferae indica*, *Staphylococcus albus*, and *Vibrio cholerae*. The oil was found to be most effective against *Bacillus anthracis* and less effective against *Bacillus subtilis* and *Salmonella paratyphi*. In addition, it had certain activity
against all of the tested fungi, including *Microsporum gypseum*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Penicillium lilacinum* but was less active than the reference, griseofulvin.

Prasad et al. (1986) studied the antimicrobial activity of essential oils of *O. basilicum* (French), *O. basilicum* (Indian), and *O. basilicum* (Niazbo), which are rich in linalool, methyl chavicol, and methyl cinnamate, respectively, against 11 Gram-positive and seven Gram-negative bacteria. They found that these oils were more effective against Gram-positive than against Gram-negative bacteria. For example, all of the Gram-positive bacteria *Bacillus scharolyticus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Bacillus thurengiensis*, *Micrococcus glutamicus*, and *Sarcina lutea* were inhibited by each of these Basil essential oils. However, the Gram-negative strain *Salmonella welteVreden* only was suppressed by all of the oils. Prasad et al. (1986) also found that methyl cinnamate type basil essential oil inhibited all of the 13 tested fungi and only *Histoplasma capsulatum* grew in the presence of linalool type basil oil. *Candida albicans*, *H. capsulatum*, and *Sporotrichum schenckii* were found to be resistant to methyl chavicol type basil oil. Sinha & Gulati (1990) found that each of these basil essential oils was also effective against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Salmonella paratyphi*, *Shigella boydii*, and *Proteus Vulgaris*. All basil essential oils showed also an antifungal effect on *C. albicans* and *S. schenckii*, with methyl chavicol type basil essential oil being highly effective. This effect has been predominantly associated with the main constituents, linalool and methyl chavicol.

Deans & Ritchie (1987) screened 50 plant essential oils (including basil) for their antibacterial properties against 25 genera of bacteria by using the agar diffusion technique. They found that most of the bacteria, including *Aeromonas hydrophila*, *B. subtilis*, *BreVibacterium linens*, *Brocothrix thermosphacta*, *Erwinia carotoVora*, *E. coli*, *Lueconostoc cremoris*, *S. aureus*, *Streptococcus faecalis*, and *Yersinia enterocolitica*, show a reasonably broad sensitivity to undiluted basil essential oil. Gangrade et al. (1989) examined the antibacterial properties of the linalool and the methyl cinnamate types of the essential oils of *O. basilicum*, in the pure state and at four dilutions (1:10, 1:100, 1:1000, and 1:10000) prepared with DMSO against four major bacterial species. They found that both essential oils had an inhibitory activity against *S. aureus* and *E. coli* at all dilutions. The dilution of either oil with DMSO beyond 1:1000 resulted in no inhibition against *Streptococcus pyogens*. Basil essential oil also showed an inhibition against *Bacillus cereus*, *Lactobacillus acidophilus*, *A. niger*, and *S. cereVisiae*, as determined by the paper disk agar diffusion method, both at ambient temperature and at 37 °C (Meena et al. 1994). These results were expanded and supported by Aboul Ela et al. (1996) and Elgayyar et al. (2001), who showed that basil essential oil has antibacterial and antifungal activity against *S. aureus*, *E. coli*, and *A. niger*. 
Baratta et al. (1998) reported that the methyl chavicol type of basil essential oil showed a significant activity against the growth of *S. aureus* food poisoning organisms. They also reported that *B. subtilis*, *B. thermosphacta*, *E. carotoVora*, *B. linens*, and *P. aeruginosa* were resistant to undiluted basil essential oil. These findings contradict those obtained by Dean and Ritchie (1987) and Lachowicz et al. (1998), presumably because of the different chemotypes of basil essential oil used in the two studies. In two publications, Lis-Balchin & Deans (1997) and Lis-Balchin et al. (1998) described the relationship between the bioactivity and the chemical composition of commercial essential oils, including that of the methyl chavicol type basil essential oil. The authors reported that a strong bioactivity was observed when the major component was eugenol and a less pronounced one when the main constituents were geraniol, citronellol, and linalool. Methyl chavicol has not shown a strong antimicrobial activity. The findings of Lis-Balchin et al. (1998) contradict those of Baratta et al. (1998), Reuveni et al. (1984), and Sinha & Gulati (1990).

Essential oils extracted by hydrodistillation from five different varieties of *O. basilicum* L. plants (Anise, Bush, Cinnamon, Dark Opal, and a commercial sample of dried basil) in Australia were examined by the agar well diffusion method for their antimicrobial activity against a wide range of food-borne Gram-positive and Gram-negative bacteria, yeasts, and molds. All five essential oils of basil showed antimicrobial activity against 20 out of 24 tested microorganisms including *A. hydrophila*, *B. cereus*, *B. subtilis*, *B. thermosphacta*, *E. coli*, *Lactobacillus plantarum*, *Listeria monocytogenes*, *Mucor piriformis*, *Penicillium candidum*, *Penicillium expansum*, *S. cereVisiae*, *Salmonella typhimurium*, *S. aureus*, *Candida colliculosa*, *Candida formata*, *Candida humicola*, and *Zygosaccharomyces bailli*. In addition, the spectrum of antimicrobial activity did not vary greatly between oils from the different varieties of basil, except for *Enterococcus faecalis* that was found to be resistant to Cinnamon basil oil but sensitive to the other four basil oils. *Pseudomonas* species were found to be resistant to all of the tested oils (Lachowicz et al. 1998).

Nascimento et al. (2000) found *P. aeruginosa* to be susceptible to basil essential oil (containing linalool, methyl chavicol, and eugenol). Rai et al. (1999) examined the antifungal activity of the essential oils of 10 plant species (including *O. basilicum*), grown in Chhindwara, India, against five *Fusarium* species. They found that the essential oils of basil (methyl cinnamate-rich type) were active against all *Fusarium* species and especially active against *Fusarium acuminatum*, *Fusarium solani*, *Fusarium pallidoroseum*, and *Fusarium chlamydosporum*.

The antimicrobial activity of the individual principle constituents of basil essential oil (linalool, methyl chavicol, eugenol, and methyl cinnamate) was also studied. Knobloch et al. (1989) evaluated the antimicrobial activity of essential oil components against Gram-negative bacteria (e.g., *Enterobacter aerogenas* and *P. Vulgaris*), Gram-positive bacteria (e.g., *S. aureus* and *B. subtilis*), and fungi (e.g., *Aspergillus flaVus*, *A. niger*, *A. ochraceus*, and *P. expansum*). They found that linalool, with its high water solubility, had a significant antimicrobial activity as
compared to cinnamaldehyde, citral, geraniol, eugenol, and menthol whereas methyl chavicol, with its lower water solubility, had a low antimicrobial activity. The solubility in water of essential oil constituents is directly related to their ability to penetrate the cell walls of a bacterium or fungus. Thus, the antimicrobial activity of essential oils is due to their solubility in the phospholipid bilayer of cell membranes (1989). It was also reported that the antibacterial activities of monoterpenic alcohols (including linalool, nerol, citronellol, and geraniol) are more effective than their antifungal activity. Meena & Sethi (1994) found that eugenol has an inhibitory effect against A. niger, L. acidophilus, and S. cerevisiae. Kim et al. (1995) studied the antibacterial activity of some essential oil components (including linalool and eugenol) against five food-borne pathogens (E. coli, E. coli O157: H7, S. typhimurium, L. monocytogenes, and Vibrio Vulnificus). They found that eugenol showed a dose-related increase in the zone of inhibition against the five strains, whereas linalool exhibited a similar effect against all tested strains except for L. monocytogenes. Linalool inhibited the growth of L. monocytogenes, but the difference in the zone size between the test concentrations (5, 10, 15, and 20% v/v) was not significant.

Pattnaik et al. (1997) studied the antibacterial properties of the aromatic constituents of essential oils. The results of the disk diffusion assays showed that linalool was the most effective compound and retarded 17 out of 18 bacterial strains (only VR-6, a Pseudomonas, is resistant), followed by cineole, geraniol, menthol, and citral. They also found that the MIC values of the essential oils were usually lower than those of their constituents. One possible reason for this result could be the synergistic action of the constituents in the oils. Mazzanti et al. (1998) found that linalool was the active compound that completely inhibited the growth of all yeasts (seven strains of C. albicans, Candida krusei, and Candida tropicalis), S. aureus and E. coli. Authentic pure linalool showed a similar antibacterial spectrum to that of basil essential oils. However, pure methyl chavicol exhibited a much narrower antibacterial spectrum, with an activity against only eight out of the 24 strains of organism tested (Lachowicz et al.1998). This result is in contradiction with the findings of Wan et al. (1998), although the same parameters and the same experimental technique were used. A possible explanation might be batch to batch variations (Lis-Balchin et al.1997) or a difference in sources of compounds.

Scora & Scora (1998) investigated the fungicidal effect of volatile compounds, with the main basil essential oil components, against three Penicillium species. It is known that phenolic compounds such as carvacrol, thymol, and eugenol possess a major fungicidal effect. Etherified compounds such as anethole, methyl chavicol, and safrole exhibit less fungicidal action while monoterpenic hydrocarbons such as limonene and α-myrcene have almost no effect. As noted by Knobloch et al. (1989), the variation in the fungicidal action of essential oil components seems to rely on their water solubility and lipophilic properties (i.e., their ability to penetrate the chitin-
based cell walls of fungal hyphae). Specific functional groups and the interference of membrane-associated enzyme proteins may also affect the results (Knobloch et al. 1989; Kurita et al. 1981). Recently, Dorman & Deans (2000) reported on the antibacterial activity of 21 plant volatile oil components (including eugenol and linalool) against 25 bacterial strains by the agar well diffusion technique. Eugenol exhibited the widest spectrum of activity against 24 out of 25 bacteria, except for *L. cremoris*, followed by linalool (against 23 strains, except *L. cremoris* and *P. aeruginosa*). These results contradict those obtained by Lachowicz et al. (1998) and Wan et al. (1998) who used the same technique and found that linalool inhibited *L. cremoris*. The components with phenolic structures, including eugenol, were highly active against the test microorganisms. Members of this class are known as either bacteriocidal or bacteriostatic agents, depending on the concentration (1993). These components are strongly active despite their relatively low solubility in water (Kim et al. 1995; Knobloch et al. 1989; Suresh et al. 1992). Alcohols are known to possess bacteriocidal rather than bacteriostatic activity against vegetative cells. The tertiary alcohol, linalool, is active against the test microorganisms, potentially acting as either a protein denaturing agent (Pelczar et al. 1993) or as a solvent dehydrating agent. Knobloch et al. (1989) demonstrated the relationship between water solubility of terpenoids and their antimicrobial activity on whole cells. The solubility of essential oils and their terpenoid compounds in water should therefore be taken into consideration when studying the action of these compounds on the membrane-catalyzed functions within the cell wall that acts as a physical barrier.

**Studies Involving Dilution Methods:**

**Agar Dilution**

Dube et al. (1989) studied the antifungal activity of the essential oil of *O. basilicum* by an agar dilution method. They showed that the essential oils of basil at a concentration of 1.5 mL/L completely suppress the mycelial growth of 22 species of fungi, including the mycotoxin-producing strains of *A. flavus* and *Aspergillus paralyticus*. In addition, the lethal dose of the oil was found to be four times less than that of Agrozim, Bavistin, and Emison and six times less than Sulfex and Celphos. The oil of *O. basilicum* is evidently a potent mycotoxic agent endowed with the ability to kill aflatoxin-producing strains. Therefore, this oil is more effective and preferable, being natural, over synthetic fungicides.

Hammer et al. (1999) studied the antimicrobial activity of a large number of essential oils and other plant extracts (including basil essential oils) against a diverse range of organisms using either the agar dilution or the broth microdilution method. The MICs of basil essential oils obtained by the agar dilution method ranged from 0.5 to >2.0% v/v. The essential oils of basil inhibited all tested organisms at concentrations below 2.0% v/v except for *E. faecalis*, *P. aeruginosa*, and *Serratia marcescens*. Kurita et al. (1981) examined the antifungal activity of 47
kinds of essential oils and several related compounds against seven fungi. The results suggest that secondary alcohols (e.g., 2-octanol, L-menthol, borneol) and tertiary alcohols (e.g., linalool) possess a markedly lower antifungal activity as compared to primary alcohols such as cinnamyl alcohol, geraniol, and citronellol. The antifungal activity of eugenol (4-allyl-guaiacol), a phenolic compound, was found to be 8-10 times higher than that of guaiacol (o-methoxyphenol) and 3-4 times higher than that of creosol (4-methylguaiacol). From the molecular structure, it is clear that the addition of alkyl or alkenyl group(s) to the benzene ring of either phenol or guaiacol enhances the antimicrobial activity. The activity of these phenolic compounds appeared to depend on the size of the added alkyl or alkenyl group, where the larger the size of the alkyl or alkenyl group, the stronger the antimicrobial activity (Knobloch et al. 1989; Kurita et al. 1981; Pelczar et al. 1993). Because alkyl or alkenyl groups are hydrophobic, these results indicate that a hydrophobicity above a minimum extent is required for phenolic compounds to show a potent antimicrobial effect. Reuveni et al. (1984) studied the percentage of inhibition of principle constituents of basil on *R. nigricans* and *F. oxysporum*. They found that both linalool and methyl chavicol had the highest percentage of inhibition (100%) against *R. nigricans* while a value of 38.1% only was found for eugenol. The reverse was found for *F. oxysporum* where the percentage inhibition of eugenol was highest (100%), while for linalool and methyl chavicol the values were only 26.4 and 30.3%, respectively. In addition, the antimicrobial activities of basil essential oils of different chemotypes were predominantly related to their main components (Reuveni et al. 1984; Farag et al. 1989). This is in agreement with the results of Pattnaik et al. (1997) working with citral, a major antifungal component in lemongrass. The study by Karapinar and Aktug (1987) on the inhibition of food-borne pathogens by four spice components showed that eugenol is the most effective inhibitor against *S. typhimurium*, *S. aureus*, and *Vibrio parahaemolyticus*. Moleyar & Narasimham (1992) studied the antibacterial activity of 15 essential oil components against the food-borne pathogens: *Staphylococcus* sp., *Micrococcus* sp., *Bacillus* sp., and *Enterobacter* sp., using an agar plate technique. Cinnamic aldehyde was found to be the most active compound, followed by citral, geraniol, eugenol, and menthol. Linalool was found to exhibit only a slight antibacterial activity.

**Broth Dilution:**

Different researchers used the broth dilution method reporting the following results: Hitokoto et al. (1980) claimed that basil leaves showed a complete inhibition of ochratoxin A, in the production of *A. ochraceus*, and a partial inhibition of the growth and toxin production of *A. flavus* and *Aspergillus Versicolor* and the growth of *A. ochraceus*. Basilico & Basilio (1990) investigated the inhibitory effects of some spice essential oils, including the essential oil of basil (*O. basilicum*), on *A. ochraceus* growth and ochratoxin A production. They reported that at a level of 1000 ppm, only basil affected the fungal growth and the production of ochratoxin A up
to 7 days but permitted mold growth afterward. This is in agreement with the work of Hitokoto et al. (1980). Lis-Balchin et al. (1998) studied the antifungal activity of the methyl chavicol type of basil essential oil against three fungi and found that the oil exhibited 94, 76, and 71% of inhibition on *A. niger*, *A. ochraceus*, and *Fusarium culmorum*, respectively. These results are in agreement with those of Baratta et al. (1998) who worked with the agar well diffusion method and found that methyl chavicol type basil essential oil shows 93.1% inhibition on *A. niger*. Amvam Zollo et al. (1998) concluded from the MIC results of the oil, as determined by the broth microdilution method after 7 days of incubation, that *O. basilicum* essential oil has an important antifungal activity. The oil was fungicidal against *C. albicans* and *A. flavus* at 5000 ppm, but it was not fungistatic on *Cryptococcus neoformans* up to 1250 ppm. Recently, Ozcan & Erkmen (2001) studied the antifungal activity of basil essential oil collected in Turkey. They found the oil to be ineffective on *S. cerevisiae*, *A. niger*, and *Rhizopus oryzae*, contrary to the findings of Dube et al. (1989), Meena & Sethi (1994), and Prasad et al. (1986). This contradiction might be due to the different chemotype of sweet basil or due to different test methods. Smith-Palmer et al. (1998) examined the antimicrobial properties of 21 plant essential oils and two essences (including basil essential oil) against five predominant food-borne pathogens: *Campylobacter jejuni*, *Salmonella enteritidis*, *L. monocytogenes*, *S. aureus*, and *E. coli*. The results of bacteriocidal and bacteriostatic concentrations showed that the two Gram-positive bacteria, *S. aureus* and *L. monocytogenes*, are more sensitive to inhibition by plant essential oils than the three Gram-negative bacteria. This is in agreement with the results of Prasad et al. (1986). Thus, in general, lower bacteriostatic and bacteriocidal concentrations are required for basil essential oil against *S. aureus* and *L. monocytogenes*. It is not completely clear why Gram-negative bacteria should be less susceptible, but it may be associated with the outer membrane of Gram-negative bacteria that endows the bacterial surface with strong hydrophilicity and acts as a strong permeability barrier (Nikaido et al. 1985). Fyfe et al. (1998) studied the inhibition of *L. monocytogenes* and *S. enteritidis* by combinations of plant essential oils with either benzoic acid or methylparaben (ester of *p*-hydroxybenzoic acid). This work highlighted the fact that the essential oil of basil at 0.2% v/v in the broth is a potent inhibitor of both strains where cells are undetectable (<10 colony forming units (cfu)/mL) at 4, 8, 24, and 48 h. Even after 1 h only of exposure, there were only 3.4 and 1.4 log cfu/mL of the cultures *L. monocytogenes* and *S. enteritidis*, respectively. Fyfe et al. (1998) suggested that the properties of basil essential oil should be determined in both a broth and a food system. Lachowicz et al. (1998) reported that a synergistic antibacterial effect was found when a combination of 5% w/v of sodium chloride (NaCl) and 0.1% v/v Anise basil essential oil in MRS broth (pH 6.2) was used. This system completely suppressed the growth of *Lactobacillus curvatus* up to 99 h (which was the time for growth detection) as compared to the Anise basil essential oil (51.4 h) or 5% NaCl (28.3 h).
alone. The work of Mejlholm & Dalgaard (1982) showed that 0.1% v/v basil essential oil resulted in over 85% reduction in the growth rate (RGR) of *Photobacterium phosphoreum* in a liquid medium at 2 and 15 °C.

Koga et al. (1999) studied the bacteriocidal activity of basil and sage essential oils against a range of bacteria, including *V. parahaemolyticus*, by viable count determination. Using this method, they were able to compare the bacteriocidal activity in both the exponential and the stationary growth phases. Their findings show that Gram-positive bacteria exhibit higher resistance to basil essential oil than Gram-negative bacteria. *S. aureus*, *M. luteus*, and *B. subtilis* show very high resistance to the essential oil of basil. The viability of these three strains treated with 1% v/v this essential oil was above 90%. *L. monocytogenes* and *B. cereus* were more sensitive to basil essential oil than other Gram-positive bacteria. Nearly all Gram-negative bacteria exhibited high sensitivity to basil essential oil. In particular, *Vibrio* species and *Aerobacter hydrophila* had very high sensitivities to this oil. The viability of these strains treated with 0.01% v/v basil essential oil ranged from 0.014 to 3.64% (Koga et al. 1999). There is a partial disparity between the results of Koga et al. (1999) and those of Elgayyar et al. (2001), Prasad et al. (1986), and Smith-Palmer et al. (1998).

According to Mahmoud (1994), the antifungal action and antiaflatoxigenic properties of certain essential oil constituents (including linalool and eugenol) could be determined on a toxigenic strain of *A. flavus*. Similarly, myrcene, ocimene, α-3-carene, and linalool appeared to cause slight enhancement of both growth and aflatoxin production. Initially, no growth or aflatoxin production in the presence of eugenol for up to 8 days was observed. This can be attributed to the presence of the aromatic moiety and the phenolic-hydroxy group of eugenol; the latter is known to be reactive and forms hydrogen bonds with active sites on target enzymes (Farag et al. 1989). After 8 days, there was a poor vegetative growth, accompanied by remarkably high concentrations of aflatoxin. This observation is in accordance with the observation made by Basilico & Basilico (1999), despite the different species tested.

Kim et al. (1995) used a liquid culture assay and found that eugenol possesses a potent inhibitory/bacteriocidal activity against the five bacterial strains: *E. coli*, *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, and *V. Vulnificus*, followed by linalool. Wan et al. (58) determined later the effect of BSL and BMC on the growth of *A. hydrophila* and *P. fluorescens*. The effect of BMC (0.1 and 1% v/v) on resting cells (105 cfu/mL) of *A. hydrophila* and *P. fluorescens* in saline (0.9% w/v NaCl) was also determined after treatment at 20 °C for 10 min. The addition of either 0.1 or 1% v/v BMC caused a decrease in the viable count of *A. hydrophila* to levels below the detection limit (<1 cfu/mL). BMC at 0.1% v/v showed no effect while at the level of 1% it was bacteriocidal also to *P. fluorescens* resting cells.
Studies Involving Microatmosphere Method:

Caccioni et al. (1997) studied the antifungal activity of natural volatile compounds (including methyl chavicol) by monitoring their vapor pressures. Methyl chavicol appeared to be active against *P. expansum* and *Botrytis cinerea*, when added to the liquid substrate or when introduced directly into the headspace. In the latter case, it was active at much lower doses. Methyl chavicol was less effective in the vapor phase than hexanal at the same temperature and concentration. At the same dose in the headspace, hexanal induced fungistasis at a level approximately 10-fold higher than that of methyl chavicol. The vapor pressure, at a given temperature, of a specific volatile molecule in a biological system can be used as an indirect measure of its “actual hydrophobicity”. It is inversely related to its capacity to form links with the sheath of water molecules surrounding its polar groups (Caccioni et al. 1997; Guerzoni et al. 1997). The reason for it is that the tendency of a molecule to pass into the vapor phase is associated with the level of its interaction with water and various solutes (Caccioni et al. 1997; Gardini et al. 1997). At a constant concentration and temperature, the higher the vapor pressure, the lower the steric hindrance, due to the linked water molecules, and the higher is the hydrophobicity (Caccioni et al. 1997). However, the ability of a potentially active molecule to interact with the hydrophobic cell membranes can be regarded as a result of its intrinsic hydrophobicity, which increases with the hydrocarbon chain length and/or with the presence of double bonds (Caccioni et al. 1997; Knobloch et al. 1989) and with its “actual hydrophobicity” (Guerzoni et al. 1997). Thus, because of its higher volatility, hexanal has proven to be biologically more active than methyl chavicol, even though methyl chavicol is more hydrophobic (Caccioni et al. 1997).

Adiguzel et al. (2005) studied the antimicrobial effectiveness of the Ethanol, methanol and hexane extracts essential oil of *O. basilicum* tested *in vitro* against 146 microbial organisms belonging to 55 bacteria, and four fungi, and a yeast species were using a disk-diffusion and minimal inhibition concentration (MIC) method. They found that all three extract of essential oil were different in terms of their antibacterial activities. The hexane extract showed a stronger and broader spectrum of antibacterial activity, then methanol and ethanol extracts, which inhibited 10, 9 and 6% of the 146 bacterial strains tested, respectively. The minimal inhibition zones (MIC) of the hexane, methanol, and ethanol extracts ranged from 125 to 250 µl/ml, 62.50 to 500 µl/ml, and 125 to 250 µl/ml, respectively.

Gupta et al. (2009) investigated the antimicrobial activity of *O. basilicum* were extracted with different organic solvents (ethanol, methanol, ethyl acetate and hexane) by using agar well diffusion method against *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Pseudomonas aeruginosa, Escherischia coli*, and *Salmonella typh*. They reported that methanol extract prominent effect on the tested microorganisms. However, *Salmonella typhi* was found to be most resistant against the extracts. The MIC and MBC of methanol extract against *Staphylococcus*
*Staphylococcus aureus*, *Bacillus subtilis* and *Bacillus cereus* were found to be 0.625 and 1.250 mg/mL, respectively whereas it was 1.250 and 2.500 mg/mL against *Escherichia coli*. The results provide evidence that *Ocimum basilicum* might be prominent sources of preservatives in food products.

Tomar et al. (2009) studied the antimicrobial activity of different extracts from the dried leaves and flowering tope against both gram positive and gram negative bacterial strains using standard disk diffusion method. Using this method, they were comparing aqueous, organic and Hydrolacholic extracts against microorganisms. Their finding showed that Hydroalcoholic extract significant inhibitory effects and broad spectrum antibacterial activity against antibiotic resistant gram negative bacteria including *E. coli* and *Pseudomonas aeruginosa*.

Recently, Rattanachaikunsopon et al. (2010) reported on the antibacterial activity against reference and clinical strains of *Salmonella Enteritidis* using Swab paper disc method in nine different essential oils. In their experiment, all the oils inhibited the bacterial strains with various degree of inhibition and also most effective essential oil in inhibiting of *S. Enteritidis*, the chemical composition of basil oil was investigated. Based on GC and GC-MS analysis, 40 components were identified, and the major oil constituents were linalool (65.35%). Among all of the tested bacteria S. Enteritidis SE3 was most sensitive strain to all the essential oils.

Anand et al. (2011) investigated the essential oil composition and antibacterial activity of volatile compounds, against Gram +ve bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*) and Gram -ve bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) as well as yeast *Candida albicans*. Their finding showed the best MIC against *C. albicans*.

**Antibacterial activity of Ocimum gratissimum L.**

The antibacterial effect of *O. gratissimum* oil is frequently reported (El-Said et al. 1969; Grover & Rao 1977; Ramanoeilina et al. 1987; Janssen et al. 1989; Thomas 1989; Jedlickova et al. 1992; Ilori et al. 1996; Ndounga & Ouamba 1997). El-Said et al. (1969) found that *O. gratissimum* oil was active against *E.coli*, *Klebsiella aerogenes*, *Proteus spp.*, *Salmonella spp.*, *Shigella sonneil*, *Bacillus subtilis*, *Sarcina lutea* and *Staphylococcus aureus*. The oil showed no activity against *Pseudomonas aeruginosa*, an organism which is known to be resistant to many antibacterial agents. Grover & Rao (1977) found that the oil of *O. gratissimum* was active against a number of bacteria. The oil was not active against *Klebsiella pneumonias*. Ramanoeilina et al. (1987) report that the oil of *O. gratissimum* was tested on eight referred stains of bacteria commonly used for antibiotic measurements and also on twelve other enterophogenic bacteria strains and showed a large spectrum action. Janssen et al. (1989) determined the maximum inhibitory dilution (MID) of *O. gratissimum* oil against *Escherichia coli* and *Staphylococcus aureus*, and the result were 1:1600 and 1:3200, respectively.
According to earlier reports (EI-Said et al. 1969; Grover & Rao 1977) *O. gratissimum* oil showed no antibacterial activity against *Pseudomonas aeruginosa* and *Klebsiella pneumonia*, but Ndounga & Ouamba (1997) showed that the oil had a low inhibitory effect on *P. aeruginosa* (+) and a quite good inhibitory effect on *K. pneumonia* (+++). These opposite results are probably due to different techniques used for the testing and different oil compositions. EI-Said et al. (1969) used drops of oil on the surface of seeded agar plates, which were incubated and zones of inhibition measured. Grover & Rao (1977) used the disc diffusion method with discs clipped in the essential oil and measured inhibition zones. Ndounga & Ouamba (1977) also used the disc diffusion method. Janssen et al. (1989) state that a combination of techniques should be used to obtain optimum information of the antibacterial activity. The biogram technique should be applied to investigate which constituent of an oil might be responsible for an observed activity, and the dilution technique to determine the maximum inhibitory dilution-value, which is a more fundamental parameter of antimicrobial activity than the inhibition diameter obtained by the agar overlay technique. The minimum inhibitory concentration of *O. gratissimum* oil was 312.5 µg/ml for *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* sp., *Serratia marcescens*, *Klebsiella pneumonia* and *Proteus vulgaris* and 625 µg/ml for *Streptococcus faecalis* and *Pseudomonas aeruginosa* (Ndounga & Ouamba 1997).

Nakamura et al. (1999) studied the antimicrobial activity of essential oils of *O. gratissimum*, against both gram positive *Staphylococcus aureus* (ATCC 25923) and gram negative (*Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella* sp., *Salmonella enteritidis*, and *Shigella flexineri*) bacterial using diffusion techniques on solid method. In their experiment, they are finding that gram-negative bacteria belonging to the genera *Proteus*, *Klebsiella*, *Salmonella*, *Escherichia*, and *Shigella* were inhibited by EO with MICs ranging from 3 to 12 mg/ml. The minimum bactericidal concentration of EO was within a twofold dilution of the MIC for this organism. The MICs of the reference drugs used in this study were similar to or higher (Ramonoelina et al. 1987; Janssen et al. 1989). These differences may be explained by susceptibility testing conditions, physicochemical characteristics of the oil, and even strain-to-strain differences. *In vivo* data may be helpful in determining the potential usefulness of the essential oil from *O. gratissimum*.

Ngassoum et al. (2003) investigated the antimicrobial activity of *O. gratissimum* L. oils. In their experiment, all the bacteria were susceptible on a different scale to the undiluted oils. The inhibition zone of the undiluted oil of *O. gratissimum* L. is more extensive than that of the other oil. The most susceptible strains are *B. cereus* and *E. faecalis*. The least sensible strains are *B. subtilis*, *C. glutamicum* and *E. coli*, while the other ones show a medium susceptibility. The susceptibilities of the strains changed with the dilution of the essential oils with Tween 80. Using a dilution of 1/30 of essential oils all the strains have practically no susceptibility any
more, expect \textit{B. subtilis}. The pure, undiluted essential oils of fresh leaves of \textit{O. gratissimum} showed the most extensive inhibition zones and are, therefore, very effective antimicrobial systems.

Adebolu et al. (2005) investigated the antibacterial activity of different extracts from the leaves of \textit{Ocimum gratissimum} against \textit{Staphylococcus aureus}, \textit{Escherichia coli}, \textit{Salmonella typhi} and \textit{Salmonella typhimurium}, pathogenic bacteria that cause diarrhea. In their experiment, they were comparing cold water extract (CWE), hot water extract (HWE) and steam distillation extract (SDE). Their finding showed only steam distillation extract has inhibitory effects on the selected bacteria and the minimum inhibitory concentration (MIC) ranged from 0.1\% for \textit{S. aureus} to 0.01\% for \textit{E. coli} and \textit{S. typhimurium}, and 0.001\% for \textit{S. typhi}.

Junaid et al. (2006) studied the antimicrobial efficacy of cold and hot water, hexane and methanolic extracts of fresh and dried leaf of \textit{Ocimum gratissimum} against \textit{Salmonella typhimurium}, \textit{E. coli}, \textit{Yersinia enterocolitica}, \textit{Bacillus cereus}, and \textit{Aeromonas hydrophila}, using the Agar gel diffusion method. The results of agar gel diffusion method showed that cold water extracts of the fresh leaf was most potent, inhibiting all isolates with diameter zones of inhibition ranging from 5 mm to 18 mm, followed by hexane extract of the fresh leaf with zone range of 6mm to 14 mm, but \textit{E. coli} showed no resistance to the hexane extract, methanol extract of the fresh leaf showed no inhibitory effect on all isolates. The extracts inhibited the growth of the bacterial isolates in a concentration dependent manner with MICs ranging between (12.5 - 150) mg/ml, while MBCs gave a range of (3.13 - 100) mg/ml. In their experiment, they finding \textit{in vitro} evidence that might justify \textit{O. gratissimum} as a good candidate medicinal plant for further investigations, and that the active principles of the plant may be more polar in nature.

Matasyoh et al. (2007) studied the antimicrobial activity of hydro-distilled volatile oils from the leaves of \textit{O. gratissimum} L. from 13 populations of different silvicultural zones. They are finding that the best antimicrobial activity in gram positive bacteria (\textit{Staphylococcus aureus}) and gram negative bacteria (\textit{Pseudomonas aeruginosa} and \textit{Proteus mirabilis}) from the eastern Kenya oil (Meru district). Meru oil was overall the best and its effectiveness was consistent on nearly all the microbes tested.

Mbata et al. (2007) studied the antimicrobial activity of \textit{O. gratissimum} against \textit{psychrophils} and heat resistant organisms. In their experiment, they were finding that the \textit{O. gratissimum} oils have properties that can inhibit the growth of \textit{psychrophils} and heat resistant organisms and suggested that the plant and its derivatives can be used for the primary purpose of flavouring foods and for antimicrobial activities.

\textbf{Antibacterial activity of \textit{Ocimum kilimandscharicum} Guerke:}

The essential oil of \textit{O. kilimandscharicum} was active against a number of bacteria, all of them gram positive. No effect was found against the tested gram negative bacteria (Prasad et al. 1986).
Deo et al. (2011) studied the antimicrobial and antifungal activity of crude methanolic and aqueous extracts of *Ocimum kilimandscharicum* against gram positive, gram negative bacteria and to find the zone of inhibition and to set a HPLC profile or fingerprint of these extracts. In their experiment, results showed that the crude methanolic extract of *Ocimum kilimandscharicum* strong antimicrobial activity against *S. aureus, E. coli* and *C. albicans* at higher concentration, same as that shown by the standard for *C. albicans*. It showed moderate activity against *B. subtilis*. The crude aqueous extracts of *Ocimum kilimandscharicum* showed moderate activity against the gram positive and gram negative organisms and strong activity against *C. albicans* at higher concentration, same as that shown by the standard for *C. albicans*.

**Antibacterial activity of Ocimum sanctum:**

The oil of *O. sanctum* is surprisingly little investigated with respect to the antibacterial activity considering its extensive use and the research going on in other areas. The essential oil of *O. sanctum* was active against a number of bacteria, but it showed no activity against *Klebsiella pneumoniae*. Prasad et al. (1986) found that *O.sanctum* oil was not active against *Salmonella saintpaul, Salmonella* spp. and *Pseudomonas* sp. Compared to the oil of *O.gratissimum* it seemed to have a higher antibacterial activity. Even 0.2% dilutions of the oil were active against most of the tested bacteria, except for *Pseudomonas aeruginosa* (Grover & Rao 1977).

Baskaran (2008) studied the antibacterial activity of acetone, benzene and chloroform extracts of *O. sanctum* oil in different bacterial strains (*E.coli, Bacillus subtilis, Staphylococcus aureus* and *Klebsiella pneumonia*) by using agar disc diffusion method. The results of agar disc diffusion method showed good antibacterial activity, but the acetone extract didn’t show any specific activity. In their experiment, they finding the presence of the phytochemicals signify the potential of *Ocimum sanctum* as a source of therapeutic agents and may provide leads in the ongoing search for antimicrobial agent from plants.

Joshi et al. (2009) investigated the antibacterial activity of ethanol extract of *O. sanctum* against bacterial strains, namely *Bacillus subtilis, Bacillus cereus, Bacillus thuringiensis, Staphylococcus aureus, Pseudomonas* spp, *Proteus* spp, *Salmonella* Typhi, *Escherichia coli, Shigella dysenteriae, Klebsiella pneumoniae*. using agar well diffusion method. In their experiment, results showed ethanol leaf extracts were more active against Gram-positive bacteria than against Gram-negative bacteria. Most susceptible bacteria were *B. subtilis*, followed by *S. aureus*, while the most resistant bacteria were *E.coli*, followed by *Shigella dysenteriae, Klebsiella pneumoniae* and *Salmonella typhi*.

Parag et al. (2010) studied the antimicrobial activity of *O. scantum* against *Escherichia Coli, Salmonella typhi, Pseudomonas pyocyaneus, Vibrio Cholerae, Shigella dysenteriae* and *Proteus Vulgaris* within specified contact time. They finding *O. sanctum* is effective against Escherichia Coli with increase in specified contact time.
Mishra & Mishra (2011) investigated the antibacterial activity of aqueous extract, chloroform extract, alcohol extract from leaves of *O. Sanctum* against the selected bacteria (*E. coli, P. aeruginosa, S. typhimurium* and *S. aureus*). In their experiment, *E. coli, P. aeruginosa, S. typhimurium* and *S. aureus* were not found resistant against the *Ocimum* extract since reduction in optical density were observed from 0.20 to 0.85. Chloroform extract were found most effective against *P. aeruginosa* were 0.85 reduction in O.D were observed. They finding the Extract from *O. Sanctum* were equally effective against the gram negative and gram positive bacteria.
Collection of plant material:

The five species of *Ocimum* namely *Ocimum basilicum* L., *Ocimum kilimandscharicum* Guerke, *Ocimum gratissimum* L., *Ocimum canum* Sims and *Ocimum tenuiflorum* L. (green type) were collected from medicinal and aromatic plant garden, Department of Botany, University of Kalyani, Kalyani, West Bengal, India, in the month of August of 2011, which is located at 22°57′ N latitude, 88°22′ E longitude with an average altitude of 9.75 m above mean sea level. The taxonomic identification of plant material was confirmed by Dr. G. G. Maity, Professor of Taxonomist, Taxonomy and Plant systematic Unit, Department of Botany, University of Kalyani. The voucher specimens (143, 148, 149, 150 and 163 KUH respectively) was deposited and preserved in the Department of Botany, University of Kalyani, Kalyani, and West Bengal, India, for reference.

Preparation of extracts

Equipments:

1. Soxhlet extractors.
2. Rotary evaporator (Buchi Rotavapor R-200, Buchi Labortechnik AG, Flawil, Switzerland).

Chemicals and reagents:

1. Methanol.
2. Anhydrous sodium sulfate (NO$_2$SO$_4$).
3. Authentic standard compounds.

Grinding of the plant materials:

All the plant samples, i.e. leaves, were dried under shade and ground in a grinder with a 2 mm diameter mesh for the laboratory. Exposure to sunlight was avoided to prevent the loss of active components.

Extraction of essential oil by methanolic solvent:

The dried and powdered leaves (100 gm) were extracted by hot extraction process using a soxhlet extraction device with solvent methanol (1:5 w/v) for 72 hours at a temperature not exceeding the boiling point of the solvent (Lin et al. 1999). The extracts were filtered using Whatman filter paper (No. 1) and then concentrated in vacuo at 40°C using a rotary evaporator (Buchi Rotavapor R-200). The resulting crude extract were then lyophilized and kept in the dark in a refrigerator at + 4 °C until tested.
Extraction of essential oil by water distillation:
The air-dried and ground aerial parts of the plants collected were submitted to water distillation for 2.5 h using a Clevenger-type apparatus (Clevenger et al. 1928). Distillates of essential oils were dried over anhydrous sodium sulfate, filtered and stored at +4°C until analyzed.

Analysis and chemical characteristics of Essential Oil

Infrared absorption spectra (IR spectrum):
IR spectrums (fingerprints) were recorded using Fourier Transform Infrared spectrophotometer on a Perkin Elmer-120-000A over the range 4000-450 cm\(^{-1}\) to analyses the compounds that has been isolated in this study from the methanolic extracts.

Gas chromatography analysis:
The essential oils were analyzed using a Hewlett-Packard gas chromatograph model 6890, equipped with flame ionization detector (FID) and HP-5MS capillary column (30 m x 0.25 mm, film thickness 0.25 \(\mu\)m). Injector and detector temperatures were set at 250 and 280°C, respectively. Column oven temperature was programmed from 60°C to 280°C at the rate of 10°C min\(^{-1}\); initial and final temperatures were held for 5 minutes. Helium was used as the carrier gas at 15 P.S.I. inlet pressure. A sample of 1.0 \(\mu\)L was injected, using slit mode (split ratio, 1:10). Quantification was completed by built-in data-handling software supplied by the manufacturer of the gas chromatograph. The results (composition) were reported as a relative percentage of the total peak area.

Gas chromatography/mass spectrometry (GC-MS) analysis:
GC–MS analysis of the essential oils was performed using an Agilent-Technologies (Santa Clara, USA) 6890N Network gas chromatographic (GC) system, equipped with an Agilent-Technologies 5975 inert XL Mass selective detector and Agilent-Technologies 7683B series auto injector. Compounds were separated on HP-5 MS capillary column (30 m x 0.25 mm, film thickness 0.25 \(\mu\)m). A sample of 1.0 \(\mu\)L was injected using the split mode (split ratio 1:10). For GC/MS detection, an electron ionization system, with ionization energy of 70 eV, was used. Column oven temperature program was the same as previously used in GC analysis. Helium was used as the carrier gas at 15 P.S.I. inlet pressure. Mass scanning range was 50 –550 \(m/z\) while injector and MS transfer line temperatures were set at 250 and 280°C, respectively.

Compounds identification:
The identification of the components was based on comparison of their GC retention indices (RI) and mass spectra with those of the authentic standard compounds run previously under same condition.
Quantitative estimation of the essential oils:

The percentage of essential oils was measured following the method of Dey (2009). After detecting the existence of the same in the test sample by comparing the retention time of the GC peaks of the test samples and that of the standard authentic ones.

Before GC analysis a stock concentration was prepared both for the authentic samples and test samples. For example, the stock concentration of authentic sample ‘X’ was prepared as M ppm and the concentration of the test samples was prepared as N ppm. Both the stock concentration was prepared by dissolving them into HPLC, n-hexane. If the injected volume for the test sample and authentic sample being 1μl, then the percentage (%) of ‘X’ available in the test sample could be calculated by the following formula:

\[
\text{The concentration (\%) of ‘X’ in the test sample} = \frac{M \times A_2 \times 100}{A_1 \times N}
\]

Where, M= Standard stock concentration (ppm)

A1= Area of the standard authentic sample (obtained from chromatogram)

A2= Area of the test sample (obtained from the chromatogram)

N= Stock concentration of the test sample (ppm)

Studies on Antibacterial activity:

The methanol extracts and essential oils were individually tested against a panel of microorganisms including Gram positive *Bacillus subtilis* (MTCC 441), *Micrococcus luteus* (MTCC 2522), Gram negative *Pseudomonas aeruginosa* (MTCC 741), *Shigella dysenteriae* (Clinical isolate), *Escherichia coli* (MTCC 443), *Vibrio cholera* (MTCC 3904) and *Shigella flexneri* (MTCC 1457). All the bacterial strains except *Shigella dysenteriae* were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. The reference strains of bacteria were maintained on nutrient agar (Hi Media, India) slants at 4°C with a subculture period of 30 days.

Materials:

Nutrient Agar (Hi-Media, India), Dimethyl sulphoxide (DMSO), sterile Petri dishes, analytical balance, Incubator, Becton Dickinson 2ml sterilized syringes, autoclave.

Preparation of McFarland standard:

The turbidity standard was prepared by mixing 0.5 ml of 1.75% (w/v) BaCl\(_2\),2H\(_2\)O with 99.5 mL of 1% H\(_2\)SO\(_4\),BaSO\(_4\) (v/v). The standard was taken in screw cap test tube to compare the turbidity. The bacterial cultures of selected strains were grown overnight and were subsequently mixed with physiological saline. Turbidity was corrected by adding sterile saline until McFarland 0.5 BaSO\(_4\) turbidity standard 10\(^8\) Colony Forming Unit (CFU) per ml was achieved. These inocula were used for seeding of the nutrient agar.
**Disc diffusion method:**

The antibacterial activity of the essential oils was determined by disc diffusion method (Murray et al., 1995). Briefly, 100 μL of suspension containing $10^8$ colony-forming units (CFU)/mL of bacteria cells were spread on petri plates containing nutrient agar medium (50 mL media/plate). The paper discs (6 mm in diameter) were separately impregnated with 10 μL of essential oils or 30 mg/ml extracts (300 μg/disc) placed on the inoculated agar. Gentamicin (10 μg/disc) was used as a positive reference and DMSO were used as a negative control. Plates were kept at 4 °C for 1h. The plates were incubated at 37 °C for 24 hours. The antibacterial activity was evaluated by measuring the zone of inhibition, the diameters of these zones being measured in millimeters against the test organisms.

**Micro-well dilution assay:**

The minimal inhibitory concentration (MIC) values were followed with the bacteria strains sensitive to the essential oils and/or extracts in disc diffusion assay. The inocula of the bacterial strains were prepared from 12 h broth cultures and the suspensions were adjusted to 0.5 McFarland standard turbidity. The essential oils and extracts of *Ocimum* spp. dissolved in 10% dimethylsulfoxide (DMSO), were first diluted to the highest concentration (500 μg/ml) to be tested, and then twofold serial dilutions were made in order to obtain a concentration range from 7.8 to 500 μg/ml in 10 ml sterile test tubes containing nutrient broth. MIC values of the extracts against bacterial strains were determined based on a micro well dilution method as previously described (Sokmen et al. 2004). The 96-well plates were prepared by dispensing into each well 95 μl of nutrient broth and 5 μl of the inoculum. A volume of 100 μl from the stock solutions of *Ocimum* spp. essential oil and extracts initially prepared at the concentration of 500 μg/ml was added into the first wells. Then, 100 μl from their serial dilutions was transferred into six consecutive wells. The last well containing 195 μl of nutrient broth without compound and 5 μl of the inoculum on each strip was used as negative control. The final volume in each well was 200 μl. The plate was covered with a sterile plate sealer and then incubated at the appropriate temperature 37°C for 24 h. The bacterial growth was determined by absorbance measured at 600 nm using the ELx 800 universal microplate reader (Biotek Instrument Inc., USA) and confirmed by plating 5 μl samples from clear wells on nutrient agar medium. The MIC was defined as the lowest concentration of the compound to inhibit the growth of microorganisms.
**Results**

**Chemical characteristics of Methanol extracts using IR spectrum:**

In the IR spectrum of *O. basilicum* L., a broad band at 3368.58 cm\(^{-1}\) is observed which indicates the presence of a hydroxyl group (-OH group) (Fig. 2.1.). Since the band is broad it is clear that –OH involving the hydrogen bonding. There is a presence of almost sharp peak at 1606.97 cm\(^{-1}\). This may arise due to presence of salt or any derivative of acid group. The peak at 1606.97 cm\(^{-1}\) also corresponding to –C=C- \text{str.}\ which is the part of aromatic compound. The peaks at the range from 1409.78 cm\(^{-1}\) to 1116.01 cm\(^{-1}\) appear either due to C-O \text{str.}\ of acid entity or O-H \text{def.}\ A sharp peak at 1057.85 cm\(^{-1}\) appears which may indicate the presence of O-H \text{def.}\ (coupled) or any C-O \text{str.}\ of carbohydrate type entity.

In *O. kilimandscharicum* Guerke, peak at 3369.66 cm\(^{-1}\) corresponds to presence of O-H group which involves in H- bonding (Fig. 2.2.). The peak at 1603.23 cm\(^{-1}\) arises due to presence of C=C \text{str.}\ where –C=C- group is a part of aromatic compound. Bands at 1410.35 cm\(^{-1}\) and 1269.37 cm\(^{-1}\) appear due to presence of C-O \text{str.}\ of acid group. They may also appear due to different mode of O-H \text{str.}\ The peak at 1069.77 cm\(^{-1}\) appears due to O-H \text{def.}\ A sharp peak at 773.28 cm\(^{-1}\) is observed. It may appear due to presence of C-H \text{str.}\ of aromatic compounds.

In *O. gratissimum* L., the broad band at 3391.72 cm\(^{-1}\) corresponds to –O-H group (hydroxyl) (Fig. 2.3.). The broad band indicates that –O-H group is not free, i.e. it involving in H- bonding. The peak 1606.78 cm\(^{-1}\) indicates the presence of –C=C- \text{str.}\ of aromatic entity or it may also appears from derivative of acid group due to C-O- \text{str.}\ Sharp peak at 1053.02 cm\(^{-1}\) suggests the presence of various type of group, such as C-O-\text{str.}\ of acetal or hemiacetal group or O-H group, which in turn further confirms the presence of O-H- group. Hence the bands at 1516.02 cm\(^{-1}\) to 1264.87 cm\(^{-1}\) appear due to presence of C-O- \text{str.}\ of any kind of acid entity and O-H \text{def.}\ In *O. canum* Sims., Peak at 3392.02 cm\(^{-1}\) confirms the presence of O-H group (Fig. 2.4.). The peak is quite broad which suggests that, O-H group is involved in H- bonding. Peak at 1606.86 cm\(^{-1}\) appears due to C=C \text{str.}\ of aromatic entity. The peaks at 1410.96 cm\(^{-1}\) and 1265.91 cm\(^{-1}\) also indicate the C-O \text{str.}\ of any kind of acid fragment and O-H \text{def.}\ The sharp peak at 1053.18 cm\(^{-1}\) appears due to C-O \text{str.}\ of any kind of carbohydrate type molecule. Sharp peak at 773.42 cm\(^{-1}\) confirms the C-H \text{str.}\ of aromatic compounds.

In *O. tenuiflorum* L., broad peak at 3391.64 cm\(^{-1}\) appears due to O-H \text{str.}\ and broadness is ascribed to H- bonded O-H group (Fig. 2.5.). Peak at 1623.02 cm\(^{-1}\) appears due to C=C \text{str.}\ of aromatic species. Peaks at 1410.38 cm\(^{-1}\) and 1267.05 cm\(^{-1}\) indicate the C-O \text{str.}\ of any kind of derivative generated from acid. A sharp peak is also observed at 1059.03 cm\(^{-1}\). This may arise
due to –O-H def. The peaks at 818.53 cm\(^{-1}\), 775.07 cm\(^{-1}\) and 616.97 cm\(^{-1}\) are originated from C-H str. of aromatic compounds. Thus is has been observed from the above spectral features of all compounds it can be concluded that, all compounds contain aromatic entity. At the same time all compounds consist of –O-H groups, which involves in H- bonding. Further it has been observed that there is a weak peak at 1688 cm\(^{-1}\) in spectrum of \textit{O. gratissimum} L., which suggests the presence of amide group. At the same time various derivative may presence in all compounds which can be confirmed from peaks generated in the ranging 1200 cm\(^{-1}\) to 1400 cm\(^{-1}\).

**Essential oil composition using GC-MS and GC-FID:**

The percentage of chemical composition of the essential oils of five species of \textit{Ocimum} namely \textit{Ocimum basilicum} L., \textit{Ocimum kilimandscharicum} Guerke, \textit{Ocimum gratissimum} L. \textit{Ocimum canum} Sims. and \textit{Ocimum tenuiflorum} L. (green type) has been summarized in Table 2.1. in the order of the retention times of the constituents. The yields of the essential oils obtained from dry leaves are 1.77%, 1.92%, 1.63%, 1.81% and 1.73% (v/w) respectively. The main components in the \textit{Ocimum basilicum} L. essential oils were identified to be geraniol (34.89%), citral (23.51%), linalool (2.21%) and eugenol (1.33%) (Fig. 2.6.). The major constituents identified in the essential oils of \textit{Ocimum kilimandscharicum} Guerke were camphor (21.65%), eugenol (9.65%), cineole (2.07%) and citral (1.23%) (Fig. 2.7.). Eugenol (47.45 %) was the main constituent in the essential oil of \textit{Ocimum gratissimum} L. followed by citronellal (3.56%), cineole (1.97%), geraniol (1.52%) and vanillin (1.52%) (Fig. 2.8.). In \textit{Ocimum canum} Sims., essential oils were identified to be Camphor (3.47%), Cineole (1.44%), vanillin (1.03%) (Fig. 2.9.). Whereas, in \textit{Ocimum tenuiflorum} L. (green type) eugenol (8.81%), citronellal (1.44%) and vanillin (1.16%) respectively (Fig. 2.10.).

**Evaluation of antibacterial activities of essential oils and methanolic plant extracts:**

The methanol extracts and essential oils of the five species of \textit{Ocimum} were individually tested against a panel of microorganisms selected. Bacterial strains were cultured overnight at 37 °C in nutrient agar (NA) medium. Following antibacterial assays were employed for the determination of antibacterial potential of methanol extracts and essential oils.
Table 2.1.: Percentage composition of the essential oils of five species of *Ocimum* cultivated in West Bengal

<table>
<thead>
<tr>
<th>Compound</th>
<th>&quot;Retention indices (RI) in min.</th>
<th>( % ) of essential oil ( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{O. basilicum L.} )</td>
<td>( \text{O. kilimandscharicum Guerke} )</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>4.76</td>
<td>0.23</td>
</tr>
<tr>
<td>Camphor</td>
<td>4.87</td>
<td>0.64</td>
</tr>
<tr>
<td>Citral</td>
<td>5.26</td>
<td>23.51</td>
</tr>
<tr>
<td>Geraniol</td>
<td>5.76</td>
<td>34.89</td>
</tr>
<tr>
<td>Cineole</td>
<td>5.83</td>
<td>0.05</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>5.92</td>
<td>0.19</td>
</tr>
<tr>
<td>Citronellal</td>
<td>5.96</td>
<td>0.59</td>
</tr>
<tr>
<td>Eugenol</td>
<td>6.57</td>
<td>1.33</td>
</tr>
<tr>
<td>Vanillin</td>
<td>6.72</td>
<td>0.27</td>
</tr>
<tr>
<td>Linalool</td>
<td>7.41</td>
<td>2.21</td>
</tr>
</tbody>
</table>

\(^a\) Identification of oil components was based on their relative retention indices (retention times) with those of authentic standards

\(^b\) Quantitative estimation was done by analysis of FID area percent data
Fig. 2.1. FT-IR spectrum of methanol *Ocimum basilicum* L. leaf extract
Fig. 2.2: FT-IR spectrum of methanol *Ocimum kilimandscharicum* Guerke leaf extract.

Fig. 2.3: FT-IR spectrum of methanol *Ocimum gratissimum* L. leaf extract.
Fig. 2.4: FT-IR spectrum of methanol *Ocimum cornum* Sims. leaf extract.

Fig. 2.5: FT-IR spectrum of methanol *Ocimum tenuiflorum* L. leaf extract.
Fig. 2.6: Chromatograms GC analysis of essential oils from *Ocimum basilicum* L.

Fig. 2.7: Chromatograms GC analysis of essential oils from *Ocimum kilimandscharicum* Guerke.
Fig. 2.8: Chromatograms GC analysis of essential oils from *Ocimum gratissimum* L.

Fig. 2.9: Chromatograms GC analysis of essential oils from *Ocimum canum* Simô.
Fig. 2.10. Chromatograms GC analysis of essential oils from *Ocimum tenuiflorum* L.
Disc diffusion test:
The antibacterial activity of the five species of *Ocimum* essential oils and methanol extracts by disc diffusion method has been summarized in the Table 2.2. (Fig. 2.11.). The inhibitory action was observed in terms of diameter of inhibition zone formed around each disc caused by the disc diffusion of antimicrobial substances from the paper discs into the surrounding medium. The results revealed that the plant extracts showed significant antibacterial activity with varying magnitudes. The essential oils exhibited the maximum antibacterial activity as compared to the methanol extract tested. However these effects were less pronounced compared to antibiotic control except *P. aeruginosa* and *V. cholera*. The essential oils of *O. basilicum*, *O. kilimandscharicum*, and *O. gratissimum* had a great potential of antibacterial activities against all 7 bacteria tested, whereas those of *O. canum* and *O. tenuiflorum* had substantial activities against 5 and 6 bacteria tested. Among the Gram positive bacteria, *B. subtilis* and Gram negative bacteria, *E. coli* was the most sensitive to all the methanol extracts. The highest antibacterial activity (24 mm) was shown by the essential oil extract of *O. kilimandscharicum* Guerke against *B. subtilis* and the lowest activity (7 mm) was observed in the methanol extract of *O. kilimandscharicum* Guerke and *O. gratissimum* L. against *B. subtilis* and *V. cholera*. The microbial strains *M. luteus*, *P. aeruginosa*, *S. dysenteriae* and *S. flexneri* were resistant to the methanol extracts of all *Ocimum* species.

The highest antibacterial activity of *O. basilicum* L. was seen in the essential oil extract (23 mm) of *P. aeruginosa* and the lowest activity was found in the methanol extract (8 mm) against *B. subtilis*. In *O. kilimandscharicum* Guerke, the highest antibacterial activity was observed in the essential oil extract (24 mm) of *B. subtilis* and the lowest activity was noticed in the methanol extract (7 mm) against *V. cholera*. The highest antibacterial activity of *O. gratissimum* L. was seen in the essential oil extract (22 mm) of *P. aeruginosa* and the lowest activity was detected in the methanol extract (7 mm) against *B. subtilis*. In *O. canum* Sims., the highest antibacterial activity was examined in the essential oil extract (18 mm) of *S. dysenteriae* and *V. cholera*. The lowest activity was studied in the essential oil extract (14 mm) against *M. luteus*. The highest antibacterial activity of *O. tenuiflorum* L. was found in the essential oil extract (22 mm) of *B. subtilis* and the lowest activity was observed in the oil extract (9 mm) against *E. coli*.
**Minimum inhibitory concentration (MIC):**

The minimum inhibitory concentrations of essential oils of the five *Ocimum* species against different pathogenic bacteria are depicted in Table 2.3. MIC test was done selectively against seven organisms that were found susceptible in disc diffusion assay. The results in Table 2.3. interpreted as the lowest concentrations that inhibit the visible microbial growth. The maximal inhibition zones and MIC values for bacterial strains, which were sensitive to the essential oils of the five species of *Ocimum*, were in the range of 9–24 mm and 15.62–500 μg/ml (Table 2.2. & 2.3.). The results from the disc diffusion method followed by MIC indicated that *O. kilimandscharicum* Guerke and *O. basilicum* L. showed maximum antibacterial activity with larger inhibition zones (16-24 and 16-23 mm) with smaller MIC values (15.62-125.00 μg/ml) against selected strains of bacteria, respectively (Table 2.3.). In *O. gratissimum* L., also exhibited appreciable antibacterial activity. The inhibition zone and MIC values of *O. gratissimum* essential oil were 15-22 mm and 31.50-125 μg/ml against selected strains of bacteria, respectively. *Ocimum canum* Sims. and *Ocimum tenuiflorum* L. essential oil was comparatively less active with smaller inhibition zone and larger MIC values. The inhibition zones of *O. canum* Sims. and *O. tenuiflorum* L. essential oil were 15-18 and 9-22 mm, respectively. Whereas, MIC values of these oils were 62.50-250 and 62.50-500 μg/ml, against strains of bacteria, respectively. MIC values also showed that essential oils were able to inhibit both type (i.e. gram positive and gram negative) of bacteria at lower concentrations. The lowest MIC for *O. basilicum* L. essential oil was recorded against *B. subtilis* (15.62 μg/ml), *E. coli* (15.62 μg/ml) and *S. flexneri* (15.62 μg/ml), whereas in *O. kilimandscharicum* Guerke lowest MIC against *B. subtilis* (15.62 μg/ml) and in *O. gratissimum* L. lowest MIC against *E. coli* (31.50 μg/ml) as detailed in Table 2. Overall, the tested *O. basilicum* L., *O. kilimandscharicum* Guerke and *O. gratissimum* L. essential oils showed high antibacterial activity against all pathogenic bacteria including Gram-negatives ones than the other two essential oils i.e. *O. canum* Sims and *O. tenuiflorum* L.
Table 2.2.: Antibacterial activity of the essential oil and methanol extract of *Ocimum* ssp. against the bacterial strains tested based on disc diffusion method

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Inhibition zone in diameter (mm) around the discs impregnated with 10 µl of essential oils and extracts (300 µg/disc)</th>
<th>Gentamicin (10µg/disc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Essential oil (10 µl/disc)</td>
<td>MeOH extracts</td>
</tr>
<tr>
<td></td>
<td>E1            E2            E3            E4            E5</td>
<td>M1          M2          M3          M4          M5</td>
</tr>
<tr>
<td><em>B. subtilis</em> (MTCC 441)</td>
<td>22            24            18            15            22</td>
<td>8            8            7            -            -</td>
</tr>
<tr>
<td><em>M. luteus</em> (MTCC 1541)</td>
<td>20            16            16            14            18</td>
<td>-            -            -            -            -</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (MTCC 741)</td>
<td>23            21            22            15            14</td>
<td>-            -            -            -            -</td>
</tr>
<tr>
<td><em>S. dysenteriae</em> (Clinical isolate)</td>
<td>16            16            15            18            -</td>
<td>-            -            -            -            -</td>
</tr>
<tr>
<td><em>E. coli</em> (MTCC 443)</td>
<td>16            17            21            -            9</td>
<td>10           8            8            -            -</td>
</tr>
<tr>
<td><em>V. cholera</em> (MTCC 3904)</td>
<td>22            16            21            18            18</td>
<td>10           7            -            -            -</td>
</tr>
<tr>
<td><em>S. flexneri</em> (MTCC 1457)</td>
<td>18            18            15            -            21</td>
<td>-            -            -            -            -</td>
</tr>
</tbody>
</table>

- E1 to E5: Essential oils. E1-*Ocimum basilicum* L.; E2-*Ocimum kilimandscharicum* Guerke; E3-*Ocimum gratissimum* L.; E4-*Ocimum canum* Sims. and E5-*Ocimum tenuiflorum* L. (green type).
- M1 to M5: Methanol extracts, M1-*Ocimum basilicum* L.; M2-*Ocimum kilimandscharicum* Guerke; M3-*Ocimum gratissimum* L.; M4-*Ocimum canum* Sims. and M5-*Ocimum tenuiflorum* L. (green type).
- Inhibition zone in diameter (mm) around the discs impregnated with 10 µl of essential oil.
- Inhibition zone in diameter (mm) around the discs impregnated with extracts (300 µg/disc).

Table 2.3.: The MIC values of essential oils of the five species of *Ocimum* against the bacterial strains tested in microdilution assay (µg/ml)

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Essential oil</th>
<th>Gentamicin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>O. basilicum</em></td>
<td><em>O. kilimandscharicum</em></td>
</tr>
<tr>
<td><em>B. subtilis</em> (MTCC 441)</td>
<td>15.62</td>
<td>15.62</td>
</tr>
<tr>
<td><em>M. luteus</em> (MTCC 1541)</td>
<td>31.25</td>
<td>62.50</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (MTCC 741)</td>
<td>62.50</td>
<td>62.50</td>
</tr>
<tr>
<td><em>S. dysenteriae</em> (Clinical isolate)</td>
<td>125</td>
<td>250</td>
</tr>
<tr>
<td><em>E. coli</em> (MTCC 443)</td>
<td>15.62</td>
<td>31.50</td>
</tr>
<tr>
<td><em>V. cholera</em> (MTCC 3904)</td>
<td>31.25</td>
<td>125</td>
</tr>
<tr>
<td><em>S. flexneri</em> (MTCC 1457)</td>
<td>15.62</td>
<td>62.50</td>
</tr>
</tbody>
</table>

nt: not tested
IR Spectroscopy:

IR Spectroscopy is an analysis tool able to detect characteristic vibrational modes of individual chemical groups and bonds. It has been used to examine and provide important data on a wide variety of biological molecules. Due to its holistic nature, FT-IR has been recognized as a valuable tool for metabolic fingerprinting/footprinting, owing to its ability to analyse carbohydrates, amino acids, fatty acids, lipids, protein and polysaccharides simultaneously (Ellis et al. 2002; Kaderbhai et al. 2003; Harrigan et al. 2004). The extracted and purified GAGs produced spectra indicating the presence of methyl groups, carboxyl groups and sulphate. While not as specific and sensitive as some techniques, such as GC-ToF-MS (gas chromatography-time of flight-mass spectrometry) (O’Hagan et al. 2005), the rapidity and reproducibility of FT-IR cannot be overstressed. One potential disadvantage of FT-IR in the mid-IR is that the absorption of water is very intense, but this problem can be overcome in one of several ways such as dehydration of samples, subtraction of the water signal, or by application of attenuated total reflectance (ATR) (Ellis et al. 2002; Winder & Goodacre 2004). Since the GAGs obtained from the respective tissues of squid and cuttlefish were hygroscopic in nature the IR spectra was taken by applying ATR-FT-IR. In addition, another measurement is made with biochemical information spread across the whole of the IR spectrum, validated and robust chemometrics must be used in order to turn data into information (Ruiz-Calero et al. 2002).

Essential Oil composition:

The essential oils contents (100 g of dry plant materials) of different species of *Ocimum* are listed in Table 2.1. The minimum and maximum essential oil yields were acquired from leaves of *O. gratissimum* L. (1.63 g 100g⁻¹) and *O. kilimandscharicum* Guerke (1.92 g 100g⁻¹), respectively. The main constituents of *O. basilicum* were geraniol (34.89%), citral (23.51%), linalool (2.21%), and eugenol (1.33%); *O. kilimandscharicum* camphor (21.65%), eugenol (9.65%), cineole (2.07%), and citral (1.23%); *O. gratissimum* eugenol (47.45%), citronellal (3.56%), cineole (1.97%), geraniol (1.52%), and vanillin (1.52%); *O. canum* Sims. camphor (3.47%), cineole (1.44%), vanillin (1.03%), and *O. tenuiflorum* (green type) eugenol (8.81), citronellal (1.44%), and vanillin (1.16%), respectively.

There are some reports on the chemical composition of the oils isolated from *Ocimum species* grown in diverse climate of different countries (Silva et al. 2004; Trevisan et al. 2006; Zheljazkov et al. 2008; Chalchat & Ozcan 2008). The essential oil composition of *O. basilicum*, *O. gratissimum* and *O. kilimandscharicum* obtained from plants grown in northern India, was found to be rich in *O. basilicum* methyl chavicol and linalool (21.9%); *O. gratissimum* eugenol (72.2%), 1.8-cineole (7.6%), germacrene D (2.7%) and β-caryophyllene (1.7%); and *O.
kilimandscharicum camphor (64.9%), limonene (8.7%), in accordance with previous report (Padalia & Verma 2011). Similar results were found with O. gratissimum and O. kilimandscharicum in sub tropical India (Verma et al. 2011). In the oils obtained from the aerial part of O. basilicum grown in Colombia and Bulgaria, linalool and methyl cinnamate were reported as the major components of volatile oils, respectively (Jirovetz & Buchbauer 2001; Vina & Murillo 2003). It is interesting that the oils extracted from Ocimum basilicum L. collected from Bangladesh contain linalool and geraniol as their main constituents (Mondello et al. 2002), and the authors concluded that the oil composition could be dependent on the climatic conditions. The chemical composition of essential oils of Ocimun species shows a large interspecies variability and, within the same species, it seems to depend on the genetic characteristics of the plant and on the conditions under which it has grown. In the present study, our findings on the major components of O. kilimandscharicum and O. gratissimum oils were in agreement with the previous report (Verma et al. 2011). According to the literature, the major compounds concerned of O. basilicum, O. canum, and O. tenuiflorum (green type) are different. The observed differences may be due to the different environmental and genetic factors, different chemotypes, and the nutritional status of the plants as well as other factors that can influence the oil compositions.

**Antibacterial activity of essential oils and methanol extracts (Disc diffusion test):**

The *in vitro* antibacterial activities of the five species of Ocimum essential oils and methanol extracts against the microorganism employed and their potentials were qualitatively and quantitatively assessed by the presence or absence of inhibition zones and the zones diameters, MIC values being showed in Tables 2.2. & 2.3. According to the result given in Table 3, the essential oils of O. basilicum, O. kilimandscharicum, and O. gratissimum had a great potential of antibacterial activities (inhibition diameters ranged from 15–24 mm) against all 7 bacteria tested, whereas those of O. canum and O. tenuiflorum had substantial activities (inhibition diameters ranged from 9 mm to 22 mm) against 5 and 6 bacteria tested.

On the other hand, the fractions of the methanol extracts of O. basilicum, O. kilimandscharicum, and O. gratissimum were also found to be effective against B. subtilis, E. coli, and V. cholera out of 7 bacterial species examined (inhibition diameters ranged from 7–10 mm), respectively, probably due to the presence of similar compounds in these methanol fractions, whereas those of Ocimum canum Sims and Ocimum tenuiflorum L. plants showed no antibacterial activities (Table 2.3.). This disagreement can be explained by that the better extraction of antimicrobial compounds from various medicinal plants may require different solvents. When compared to the methanol extracts, the essential oils exhibited a stronger and broader activity as compared to the methanol extract tested. Based on these results of chemical composition of the essential oils, it is possible to conclude that the antibacterial nature of the essential oils studied is apparently related
to their high phenolic contents, particularly oxygenated terpenoids and phenolic terpenes, and this finding is in agreement with previous reports (Burt 2004; Gallucci et al. 2009; Bassole & Juliani 2012). This claim is further supported by our findings indicating high contents of terpenoids such as citral, geraniol, eugenol, and camphor in the oils (Table 17). The findings in this study support the observations of some other researchers about Ocimum species containing some substances with antibacterial properties (Prasad et al. 1986; Nakamura et al. 1999; Adebolu & Oladimeji 2005; Adiguzel et al. 2005; Moghaddam et al. 2011; Verma et al. 2011). However, it is difficult to compare the data with the literature because several variables influence the results, such as the environmental and climatic conditions of the plant and the choice of the extraction method and antimicrobial test. Moreover, standard criteria for the evaluation of the plant activity are missing and therefore the results obtained by different authors are widely different (Recio et al. 1989; Vanden Burghe et al. 1991). This is the first study to provide data about the extracts and essential oils of the five species of Ocimum plants possessing potential antibacterial activities as evaluated against seven microorganisms. The results indicate that the essential oils of Ocimum species can be used as a natural source that may lead to their use as safe alternatives to synthetic antimicrobial drugs. In addition, the data in the present study support the use of Ocimum species as additives in foods, and as traditional remedies for the treatment of infectious diseases.

**Minimum inhibitory concentration (MIC):**

All the essential oils tested were subjected to MIC studies against all the microorganisms. The results in Table 2.3. interpreted as the lowest concentrations that inhibit the visible microbial growth. The maximal inhibition zones and MIC values for bacterial strains, which were sensitive to the essential oils of the five species of Ocimum, were in the range of 9–24 mm and 15.62–500 μg/ml (Table 2.2. & 2.3.). Based on these results, it is possible to conclude that the essential oils have a stronger and broader spectrum of antimicrobial activity as compared to the methanol extracts tested. This observation confirmed the evidence given in a previous study reporting that the essential oils from medicinal plants contain more antimicrobial substances than other extracts such as water, methanol, ethanol, and hexane extracts (Ahmad et al. 1998; Eloff 1998). Our results also indicated in the present study that the O. basilicum, O. kilimandscharicum, and O. gratissimum essential oils were more active against all pathogenic bacteria including Gram-negatives ones than the other two essential oils, probably owing to the high levels of phenolic compounds in the former ones (Table 2.2.).

According to a number of studies, the Gram positive bacteria are more sensitive to essential oil than Gram-negative bacteria. A possible explanation may reside in the possession of an outer membrane, surrounding the cell wall of Gram-negative bacteria, thus it is logical to expect that these bacteria will be less susceptible to the antibacterial activity of essential oil. This outer
membrane may restrict the diffusion of hydrophobic compounds through its lipopolysaccharide covering, presenting a barrier to the penetration of numerous antibiotic molecule, and is also associated with the enzymes in the periplasmic space, which are capable of breaking down the molecules introduced from the outside (Nikaido 1994; Gao et al. 1999). Gram-positive bacteria do not have such an outer membrane and cell wall structure. The antibacterial substances can easily destroy their bacterial cell wall and cytoplasmic membrane and cause leakage of the cytoplasm and its coagulation (Kalemba & Kunicka 2003). However, the current findings show a remarkable activity against all gram-negative bacteria. The antibacterial activities of *O. basilicum*, *O. kilimandscharicum*, and *O. gratissimum* leaves extracts essential oils may be due to high contents of tannins and phenolic constituents. The most active constituents (essential oils) rich in phenolic compounds are widely reported to possess high levels of antimicrobial activity (Prasad et al. 1986; Nakamura et al. 1999; Dorman & Deans 2000), which has been confirmed and extended in the present study, although antimicrobial activities of phenolic compounds may involve multiple modes of action. The mode of action of antimicrobial agents also depends on the type of microorganisms and is mainly related to their cell wall structure and the outer membrane arrangement. Medicinal plants contain complex phenolics and the mechanism of action of each phenolic compound against various bacteria is also very complicated (Burt 2004). Therefore, it is necessary to investigate further and understand the relationship between the antibacterial activity and chemical structure of each phenolic compound in the extracts tested.