EFFECT OF SELECTED BOTANICAL ANTIMICROBIALS FOR ENHANCING POTABILITY OF DRINKING WATER FROM VARIOUS SOURCES

THESIS

SUBMITTED TO THE
MIZORAM UNIVERSITY, AIZAWL

FOR THE DEGREE OF

Doctor of Philosophy

IN

HORTICULTURE, AROMATIC AND MEDICINAL PLANTS

By

Mr. AWADHESH KUMAR

(Ph.D. Reg. No. MZU/Ph.D/293;09-06-2009)

Department of Horticulture, Aromatic and Medicinal Plants,
Mizoram University, Aizawl-796004

2011

Acknowledgements

It's my privilege to express my heartfelt gratitude to my revered teacher Dr. Amritesh Chandra Shukla, *D.Sc*. Associate Professor, Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University, Aizawl, and Professor Anupam Dikshit, *F.N.A.Sc,* Department of Botany, University of Allahabad, who took keen interest at every step and provided able guidance as a supervisor and joint supervisor, respectively, to bring this work up to the mark.

My sincere thanks are due to Dr. B. P. Nautiyal, *Head*- Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University, Aizawl as well as Prof. D.N. Shukla, *Head*- Department of Botany, University of Allahabad for providing me the necessary facilities, and for granting me the permission to work in their Laboratory.

My special thanks to Professor R. P. Tiwari, *Dean* School of Physical Sciences; Professor P. Rinawma, *Dean* School of Earth Sciences & Natural Resources Management as well as Professor H. Lalramnghinglova, *Head* Department of Environmental Sciences, Mizoram University, Aizawl for their invaluable help and psychological support during the course of my research work.

I am also thankful to Prof. B. Gopichand, *Head* Department of Forestry; Dr. N. Senthil Kumar, *Head* Department of Biotechnology; Dr. R.C. Laha, *Head* Department of Botany; Dr. Diwakar Tiwari, *Head* Department of Chemistry; Dr. Shiva Kumar, *Head* Department of Geology; Dr. Lalnilawma, *Head* Department of Extension Education & Rural Development; Dr. B.P. Mishra, *Associate Professor*, Department of Environmental Sciences; Ms. Nuchhungi Khawlhring, *Assistant Professor* and Sri Tridip K. Hazarika, *Assistant Professor*, Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University, Aizawl, for their invaluable help and fruitful discussions during my research work.

Further, I am highly grateful and thankful to the authorities of the Mizoram University, Aizawl for providing support in various ways as and when required during the compilation of the work.

I am grateful to Prof. T. N Lakhanpal, Department of Botany, Shimla University for his valuable suggestions during my research in form of ethanobotanical study.

Technical, Scientific and moral support extended by Dr. Rohit Kumar Mishra, *Research Scientist*, DST, New Delhi and Er. Om Prakash (*M.Tech.*), Project Manager, IIIT, Allahabad during the course of my experimentation and compilation is gratefully acknowledged.

My sincere thanks are due to my colleagues Mr. Amit Kumar Tiwari, Anand Pandey, Mrs. Shalu Saxena, Rajesh Kumar Dwivedi, Lab attendant Sanjay Prajapati and my senior Dr. Ramesh C. Mishra; Dr. K. P. Pandey, Dr. Bharat Kumar Mishra, Dr. Piyush Mishra, Dr. Ahsan Kamran, Dr.

Ravindra Kumar Pandey, for their invaluable help rendered during my research work and in bringing the thesis in final shape.

I am also very thankful to the Director, Genome Diagnostics, Dr. Pradeep Singhal and also pay heartfelt thanks to Dr. Nitin Semwal, Mr. Amit and Mr Sanjeev for their cooperation in the work associated with RT-PCR. I will always be indebted to them for their help.

I am highly thankful to my Grandparents like Sri. L.K. Dikshit, Smt. Savitry Dikshit for their blessings and moral support and Dr. Shalini Dikshit for always encouraging me to work hard and her kind blessings during the course of my research investigation.

It feels fortunate to pay heartfelt thanks to my father Sri Rampati Ram and my mother Smt. Lachi Devi and my siblings Mr. Santosh Kumar, Mrs. Ranjanlata, Amarjeet Kumar, Ambesh Kumar, Brijesh Kumar and Abeeshek Kumar Bharti. I am also thankful for the constant support to my sisters Mrs. Kumkum, Km. Kusumlata and Km. Neelam and Jeejaji Mr. Himanshu for his love and affection.

Thanks are also due to my well-wishers, friends and relatives, who rendered their support during the course of research work.

Finally, with folded hands and bent knees, I bow before the Almighty to thank him for the continuing graces and blessings He showers on me as I tread through scientific path of Service for humanity.

Dt: 23-05-2011

(**Awadhesh Kumar)**
(Ph.D. Reg. No. MZU/ Ph.D./ 293; dt 09.06.2009)
Department of Horticulture,
Aromatic and Medicinal Plants,
School of ES & NRM
Mizoram University, Aizawl

CONTENTS

3.4.2 PCR analysis

3.4.2 a Amplification of water borne bacterial isolates through Real Time PCR

3.4.2 (b) Agarose Gel Electrophoresis

3.4.3 Validation of water borne bacterial isolates by Real Time PCR

3.5 PHYLOGENY OF TESTED BACTERIA

3.5.1 Role of Bioinformatics

3.6 HERBAL ANTIMICROBIALS; COLLECTION AND IDENTIFICATION

3.6.1 Collection, Storage, and Vouchering of Plants

3.6.1 (a) Collection of Plants in the Field

3.6.1 (b) Storage of Plants at Low Temperatures (Drying):

3.6.1 (c) Vouchering of Collected Plants in the Field:

3.6.1 (d) Photographic Records of Plants Collected:

3.6.2 The Extraction Process

Hydro-distillation

3.7 Description of herbal antimicrobials

3.7.1 ***Mentha piperata*** **L.** (White Peppermint)

Historical background:

3.7.2 ***Mentha arvensis*** **L. (Mint, Pudina in Hindi)**

3.7.3 ***Micromeria biflora*** **(Buch. Ham. ex D.Don.) Benth.**

3.7.4 ***Citrus reticulata*** **Blanco. (In Hindi Santra)**

3.8 CHARACTERIZATION OF ESSENTIAL OIL

3.8.1 Gas-Chromatographic analysis

3.8.2 Gas-Chromatographic-Mass Spectrometry

3.8.3 Quantitative analysis by GC

3.8.4 Calculation of Kovats Retention Indices (IR)

3.8.5 Mass Spectroscopy (MS)

3.8.6 Identification of compounds

3.9 PHYSICO-CHEMICAL PROPERTIES OF ESSENTIAL OILS

3.9.1 Specific gravity

3.9.2 Optical rotation

3.9.3 Refractive index

3.9.4 Solubility in 90% alcohol

HIMEDIA
Water Sample
HIMEDIA MS1186-1KT 1kit
PA Coliform Kit

HIMEDIA
HIMEDIA MS1186-1KT 1kit
PA Coliform Kit
Water Sample
Kitgang

LIST OF TABLE

LIST OF FIGURE

LIST OF PLATE

ABBREVIATIONS

DNA	-	Deoxyribonucleic acid
PCR	-	Polymerase chain reaction
RAPD	-	Randomly amplified polymorphic DNA
REA	-	Restriction endonuclease analysis
RFLP	-	Restriction fragment length polymorphism
RNA	-	Ribonucleic acid
rRNA	-	Ribosomal RNA
IPS	-	Intracellular polysacharide
MAb	-	Monoclonal antibody
AP-PCR	-	Arbitrarily primed polymerase chain reaction
0.5xTBE buffer	-	45 mM Tris, 45 mM boric acid, 1 mM EDTA
TE buffer	-	10 mM Tris-HCl, 1 mM EDTA
Tris	-	Tris(hydroxymethyl)aminomethane
UV	-	Ultraviolet
bp	-	Base pair
EDTA	-	Eethylenediaminetetraacetic acid
SDS	-	Sodium dodecyl sulfate
ATCC	-	American Type Culture Collection
MTCC	-	Microbial type culture collection
EA	-	Ethyl acetate
NA	-	Nutrient agar
NB	-	Nutrient broth
D.W	-	Distilled water
CFU	-	Colony forming units
MIC	-	Minimal inhibitory concentration
MBC	-	Minimum inhibitory concentration
IC_{50}	-	Inhibition concentration at 50% concentration
NCCLS	-	National Committee for Clinical Lab. Standards
CLSI	-	Clinical Laboratory Standards Institute

MAP	-	Medicinal and Aromatic plant
µl	-	Micro litre
µg	-	Microgram
g	-	Gram
l	-	Liter
mg	-	Milligram
ml	-	Millilitre
ppm	-	Parts per million
h	-	Hours
lbs	-	Pounds
No.	-	Number
pH	-	$-\log_{10}$ hydrogen ion concentration
sp.	-	Species
spp.	-	More than one species
viz.	-	Namely
wt	-	Weight
yr	-	Year
%	-	Percentage
/	-	Per
<	-	Less than
>	-	Greater than
eg	-	For example
et. al.	-	And other
etc.	-	And the rest
Fig.	-	Figure

Introduction

"Knowledge is the first line of defence towards providing safe drinking water"

LeChevallier, *et al.,* (1999)

Water and plants are the two main sources on earth to continue the life. They are directly linked to each other. The ancient data revealed that the human as well as animal lives properly settled near the banks of rivers, lakes and other water resources, because they got drinking water easily. Wherever the scaricity of any of them occurs, life becomes difficult. But as the population grew rapidly, the fresh drinking water became polluted and several fatal bacterial, viral, protozoans, nematodal and fungal diseases emerged. This is due to rapid pace of urbanization which increased the demand of infrastructure for the better livelihood. This was followed by the fulfillment of basic needs for living as such food, shelter and clothing. Countries with good lot of basic resources such as water developed ahead while still developing countries with poor resources have an increased demand of safe drinking water and other.

Inadequate drinking water supply and quality with poor sanitation are among the world's major cause of preventable morbidity and mortality. According to the World Health Organization (WHO, 2002) estimates, basic hygiene related diseases have a significant impact on human health. Diarrhoeal diseases alone cause 2.2 million of the 3.4 million of the water-related deaths per year. Many of the deaths involve children less than five years of age and the poorest households and communities. The level of sanitation is among most grievous problem is not limited to developing countries, India being the same. (UN - Habitat, 2003).

Water is essential to sustain life, and without it life becomes impossible (WHO, 1997). Water supply is one of the key natural resource bases that are inevitable for sustainability of human and environmental health. There is a strong and direct link between people's health and the development of communities. Gleick, (2002) and the World Health Organization (WHO, 2003) summarized these links as:

poor health reduces life expectancy and educational achievement; it reduces investment and returns from investment (as production, productivity and employment decrease); it reduces parental investment in children (and increases the fertility rate); it increases health inequity and poverty; and it reduces social and political stability.

The 31 percent Indian population have adequate sanitation and only 88 percent population have improved water sources according to 1999 estimates (Human Development Report 2001) but the percentage has fallen down to 28 percent and 84 percent for adequate sanitation and improved water sources in year 2000 (Human Development Report 2004).

Water is an indispensable commodity, which should be easily accessible, adequate in quantity, free of contamination, safe, affordable and available throughout the year in order to sustain life (Al- Khatib, *et al*., 2003). Since water is very important for life, it should be available to all livings; plants, animals, and human. Availability of water implies sufficient quantity and good quality. Adequate supply of quality water is essential to maintain good public and community health since protection of water resources from contamination is the first priority (Daud, *et al*., 2001).

The Fundamental Rights in Article 21 and Article 39 (a) and 39 (b) of the Constitution of India includes the right to clean water. The National Water Policy has assigned highest priority to drinking water supply followed by irrigation, hydropower, and navigation, industrial and other uses. As per the existing norms mentioned in the Ninth Five Year Plan (1997-2002), for rural water supply includes 40 liters of drinking water per capita per day (lpcd) and a public stand post or a hand pump for 250 persons. Further, the sources of water supply should be within 1.6 km. horizontal distance in plains or 100 meters elevation distance in hills. The norm for urban water supply is 125 lpcd excluding sewerage system and 40 lpcd in towns with spot sources. Availability of at least one source for 20 families within a maximum distance of 100 meters has been stipulated (Ramachandraiah, 2001).

According to Peter H. Gleick, (2002) the simplest estimate (from 2000 to 2020) of future deaths from water-related diseases comes from assuming that the

proportion of deaths to total global population experienced today will be maintained in the future. As total population grows, total water-related deaths will grow annually. "To halve, by the year 2015…the proportion of people who are unable to reach or to afford safe drinking water". The 59 and 135 million people, mostly children, will die between now and 2020 from preventable water-related diseases Gleick, (2002). The following figure confirmed the deaths estimate.

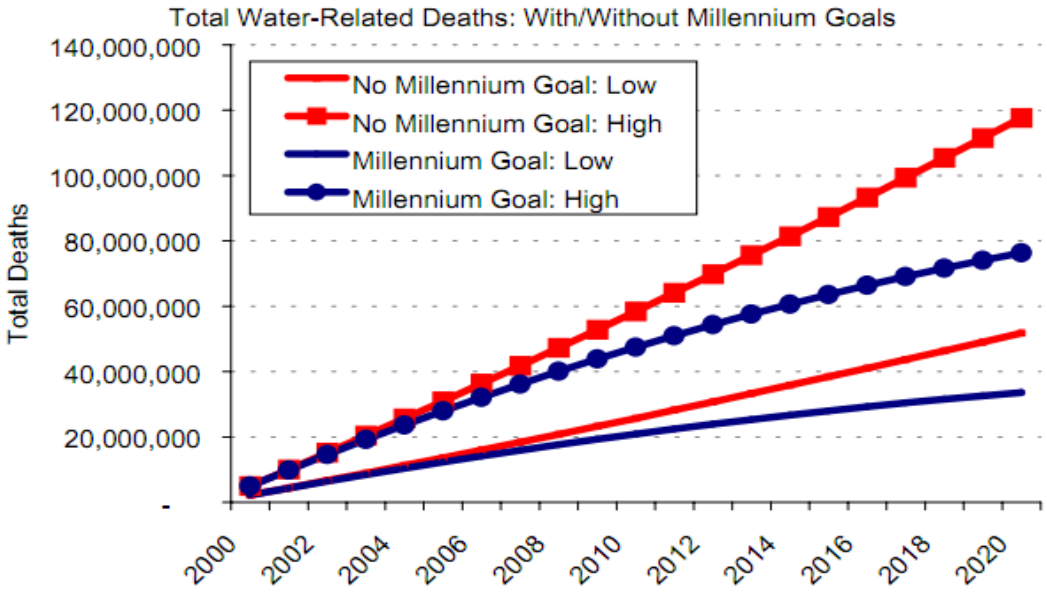


Fig. 1.1 Total water-related deaths between 2000 and 2020. The red lines (top and third from top) show the range of deaths likely to occur without the UN Millennium Goals. The blue lines (second from top and bottom) show the range of deaths even if the Millennium Goals are achieved. This range is 34 to 76 million deaths total by 2020.

Water is one of the most important and sensitive issues in the Middle East, where increasing water deficiency and deterioration of the available water resources are imminent. A major issue is that water resources are very limited and do not meet the existing population's demands, as well as the generations to come (Al-Khatib, *et al*., 2003).

1. Water

Water is naturally occurring chemical entity with empirical formula H_2O. The molecular weight of water is 18 a.m.u. Its molecule is composed of one Oxygen and two Hydrogen atoms linked by covalent bonds and molecules held together by Hydrogen bonds. It is the only chemical which can co-exist in all the three forms of matter at various energy levels. Water being the major head in our atmosphere covers up to 70.9 % of Earth's surface. Its dissolving power can be illustrated by the fact that any substance continues to dissolve in water until it reaches a Saturation point (no more solute can be dissolved in it). Since impurities being present in small quantities, thus remain dissolved in it, making the water look cleaner but unfit for drinking. The hydrological cycle (Figure 1) illustrates the process of contamination and natural purification.

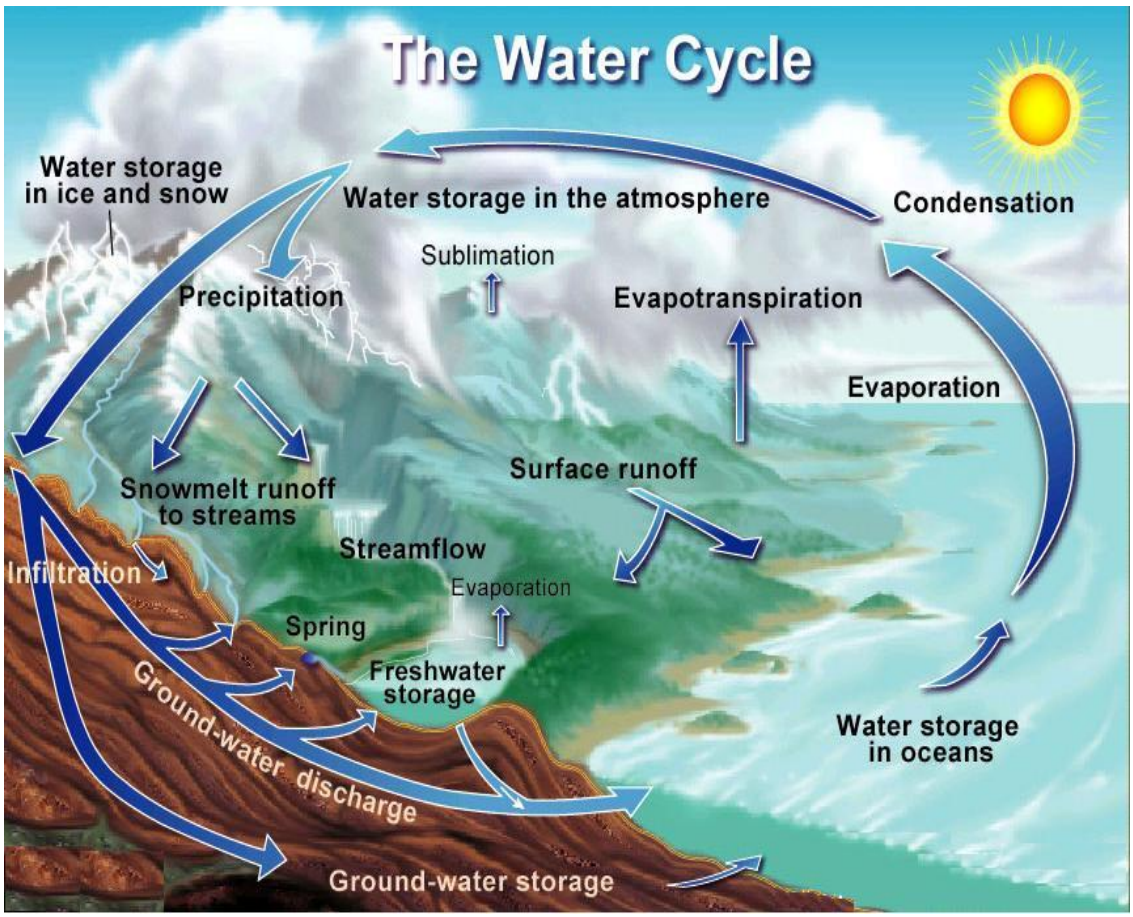


Fig. 1.2 Hydrological cycle: Naturally maintains the water level

1.1 Quality of Drinking Water

A standard for pure drinking water is decided by the regulatory authorities of each country. These prescribe which substances can be in drinking water and up to what maximum amount. The World Health Organization (WHO) (1996) guidelines for determining the purity of water in potable resources are summarized below in the table.

Table 1.1: WHO guideline for patable water

S. No.	Characteristics	Acceptable*	Cause of Rejection**
1.	Turbidity (Units on JTU scale)	2.5	10
2.	Colour (Units on Platinum cobalt scale)	5.0	25
3.	Taste and colour	Unobjectionable	Unobjectionable
4.	pH	7.0 to 8.5	6.5 to 9.2
5.	Total dissolved solids	500	1500
6.	Total hardness (mg/l as $CaCO_3$)	200	600
7.	Chlorides (mg/l as Cl^-)	200	1000
8.	Sulphates (as SO_4^2 mg/l)	200	1000
9.	Fluorides (as F, mg/l)	1.0	1.5
10.	Nitrates (as NO_3, mg/l)	45	100
11.	Calcium (as Ca, mg/l)	75	200
12.	Magnesium (as mg, mg/l)	30	150
13.	Iron (as Fe, mg/l)	0.1	1.0
14.	Manganese (as Mn, mg/l)	0.05	0.5
15.	Bacteria colony (cfu/ml)	Must not be detectable in 100 ml water samples	

*****The figures indicated that under coloun acceptable are the limits upto, which water is generally acceptable to the customers.

******Figures in axcess of those mentioned under acceptable render the water non-acceptable but still may be tolerated in the absence of alternative and better source but upto the limits indicated under column cause for rejection above which the supply will have to be rejected.

(*Source: From Documents of National Drinking Water Mission, Department of Rural Development, Government of India, May 1990; Source: WHO, 1996. Guidelines for drinking-water quality, 2nd ed. Vol. 2 Health criteria and other supporting information, p. 940-949; and WHO, 1998. Addendum to Vol. 2. p. 281-283. Geneva, World Health Organization. Summary tables).*

1.2 Importance of Water in the Diet

Most of us take water for granted, which is one of the major reason for its depletion. For the human body, water is truly a vital resource. When the water in your body is reduced by just 1 percent, you become thirsty. At 5 percent, muscle strength and endurance declines significantly and you become hot and tired.

When the loss reaches 10 percent, delirium and blurred vision occur. A 20 percent reduction results in death. No other substance is as widely involved in the processes and make up of the body. A man's body is about 60 percent water, and a woman's is approximately 50 percent. Did you know that the human brain is about 75 percent water?

Tissue	Percent Water
Blood	83.0
Heart	79.2
Muscle	75.6
Brain	74.8
Skin	72.0
Bone	22.0

Every day, we lose 2-3 quarts of water through urination, sweating and breathing. Since many of the processes within the body rely greatly on water, it is important we replace our fluids regularly to compensate for this loss.

1.3 Identifying Impurities

Water quality depends a lot on its profile; such as physical, chemical and microbial parameters. In order to assess the need for treatment and the appropriate technology, the specific contaminants must be identified and measured.

1.4 General Quantitative Identification

Following are the major quantitative analyses which define water quality.

General Physical and Chemical Parameters

- Turbidity (clarity)
- Taste
- Colour
- Odour
- pH

(i) Turbidity

Turbidity is caused by scattering and absorption of light by suspended material (organic or inorganic) in water, giving it a cloudy appearance. The small size of the particles prevents its rapid settling makes it necessary for its treatment. A candle turbiditimeter is a very basic visual method used to measure highly turbid water. Its results are expressed in Jackson Turbidity Units (JTU). A nephelometer is more useful in low-turbidity water, with results expressed in Nephelometric Turbidity Units (NTU).

(ii) Taste

The taste sense is moderately accurate and able to detect concentrations from a few tenths to several hundred ppm. However, taste often cannot identify particular contaminants. A bad taste may be an indication of harmful contamination in drinking water, but certainly cannot be relied on to detect all harmful contaminants.

(iii) Colour

Colour is contributed primarily by organic material, although some metal ions may also tint water. While not typically a health concern, colour does indicate a certain level of impurities. "True colour" refers to the colour of a sample with its turbidity removed. Turbidity contributes to "apparent" colour.

(iv) Odour

The human nose is the most sensitive odour-detecting device available detecting odour down to parts per billion (ppb). Smell is useful because it provides an early indication of contamination which could be hazardous or at least reduce the aesthetic quality of the water.

(v) pH

The relative acidic or basic level of a solution is measured by pH. The pH is a measure of hydrogen ion concentration in water, specifically the negative logarithm (log). The measurement of pH lies on a scale of 0 to 14 (Figure 2), with a pH of 7.0 being neutral (i.e., neither acidic nor basic), and bears equal numbers of hydroxyl (OH-) and hydrogen (H+) ions; and pH less than 7.0 is acidic; a pH of more than 7.0

is basic. Since pH is expressed in log form, a pH of 6.0 is 10 times more acidic than a pH of 7.0, and a pH of 5.0 is 100 times more acidic than a pH of 7.0. The pH has an effect on many phases of water treatment such as coagulation, chlorination and water softening (WHO, 2002).

1.5 Microbial Quality of Water

WHO states in 1997 that the "infectious diseases caused by pathogenic bacteria, viruses and protozoa or by parasites are the most common and widespread health risk associated with drinking water." Esrey, *et al.,* (1998) surveyed 142 studies on 6 of the major waterborne diseases and estimated that in developing countries (excluding China); there were 875 million cases of diarrhoea and 4.6 million deaths annually in the mid-1980s.

1.6 Water-Borne Pathogens and Diseases

It is caused by intake of contaminated fresh water by pathogenic microorganisms. Several reports have indicated that environmental waters are potential reservoirs and transmitting vehicles for these water-borne pathogens. Contaminated fresh water, used in the preparation of food, can be the source of food borne disease through consumption of the same microorganisms. (Common waterborne diseases: Table 1 and 2).

1.7 Bacterial Contamination

One difficulty of water purity is bacterial contamination and control of bacterial growth. It is a necessary medium for bacterial growth because it carries nutrients. It is an essential component of living cells. Its thermal stability and nutrient availability provides a controlled environment for bacterial growth. Bacteria are unicellular microorganisms of shapes like rods, spheres or spiral etc. Prior to widespread chlorination of drinking water, bacteria like *Escherichia coli*, *Vibrio cholerae*, *Salmonella sps.* and several *Shigella sps.* commonly caused diseases such as cholera, typhoid fever and bacillary dysentery, respectively (Microbe world, 2002).

(i) *Escherichia coli*

E. coli is a facultative Gram negative organism. It is a heterogeneous species comprising many different strains, the vast majority of which are not pathogenic (WHO, 1997). Symptoms include abdominal pain, vomiting, anemia, thrombocytopenia, acute renal injury with bloody urine, seizure and pancreatitis (Atiya, 2003).

(ii) *Vibrio cholerae*

Vibrio is motile, non-spore-forming, slightly curved Gram-negative rods with a single polar flagellum. Symptoms of Asiatic cholera may vary from a mild, watery diarrhoea to an acute sever diarrhoea, with characteristic rice water stools. Illness is caused by the ingestion of viable bacteria, which attack to the small intestine and produce cholera toxin resulting in watery diarrhoea associated with this illness (CDC, 2002).

(iii) *Salmonella typhimurium*

Salmonella is a Gram-negative rod-shaped, non-spore forming organism. It causes salmonellosis, a disease with milder symptoms (CDC, 2002). The organism grows and produces an endotoxin that causes the illness. Acute symptoms include nausea, abdominal cramps, minor diarrhoea, headache, etc. whereas chronic consequences may be identified by arthritic symptoms following 3-4 weeks after onset of acute symptoms.

(iv) *Shigella dysenteriae*

Shigella is also a Gram-negative, non-motile and non-spore forming rod-shaped bacteria. The organism is frequently found in water polluted with human faeces. Shigellosis (bacillary dysentery) is the name of the illness caused by *Shigella dysenteriae* produces shiga toxin (CDC, 2000). The *Shigella sp*. is a highly infectious agent that is transmitted by the contaminated water and faecal-oral route (AWWA, 2000).

(v) Opportunistic Pathogens

Certain bacteria in drinking water deserve particular attention because they are opportunistic pathogens to humans, i.e. they are able to cause infections in susceptible persons. The most important organisms of this type are *Legionella, Aeromonas* and *Pseudomonas aeruginosa* (Curriero, 2001).

1.8 Sources of potable water for drinking purposes

The rural peoples generally depend upon handpumps, ponds, lakes, rivers and other sources, but cities lack such types of sources. Many rural dwellers lack indoor plumbing or nearby outdoor piped water from a safe supply (from wells, boreholes, protected or upland surface water sources, etc.). Often they have to travel considerable distances to reach any water source, regardless of quality, for collection and household use (White, *et. al*., 1972). Whenever these sources are present in cities they became more polluted or unsafe to dring. So the urban people mainly depend upon municipal water supply. Presently, the municipal water supplying pipes get contamination due to corrosion, rusting, effected by feacal contamination and sometimes gets affected by digging the holes in festival times or during construction of buildings. Many urban dwellers are also lack safe water and suffering from various types of bacterial, viral and other microbial illnesses (WHO, 2000; Swerdlow, *et al*., 1992; Ries, *et a*l., 1992; Weber, *et al.*, 1994).

Table 1.2: List of water borne bacterial diseases and their causal organism:

Disease and Transmission	Microbial Agent	Sources of Agent in Water Supply
1. Botulism	*Clostridium botulinum*	Bacteria can enter a wound from contaminated water sources. Can enter the gastrointestinal tract.
2. Campylobacteriosis	Most commonly caused by *Campylobacter jejuni*	Drinking water contaminated with feces
3. Cholera	Spread by the bacterium *Vibrio cholerae*	Drinking water contaminated with the bacterium
4. *E. coli* Infection	Certain strains of *Escherichia coli* (commonly *E. coli*)	Water contaminated with the bacteria
5. *M. marinum* infection	*Mycobacterium marinum*	Naturally occurs in water, most cases from exposure in swimming pools or more frequently aquariums; rare infection since it mostly infects immuno compromised individuals
6. Dysentery	*Shigella dysenteriae*	Water contaminated with the bacterium
7. Legionellosis (two distinct forms: Legionnaires' disease and Pontiac fever)	Caused by bacteria belonging to genus *Legionella* (90% of cases caused by *Legionella pneumophila*)	Contaminated water: the organism thrives in warm aquatic environments.
8. Leptospirosis	Caused by bacterium of genus *Leptospira*	Water contaminated by the animal urine carrying the bacteria
9. Otitis Externa (swimmer's ear)	Caused by a number of bacterial and fungal species.	Swimming in water contaminated by the responsible pathogens
10. Salmonellosis	Caused by many bacteria of genus *Salmonella*	Drinking water contaminated with the bacteria. More common as a food borne illness.

11. Typhoid fever	*Salmonella typhi*	Ingestion of water contaminated with feces
12. Vibrio Illness	*Vibrio vulnificus*, *Vibrio alginolyticus*, and *Vibrio parahaemolyticus*	Can enter wounds from contaminated water. Also got by drinking contaminated water or eating undercooked oysters.

Table 1.3: List of water borne viral diseases and their causal organism

Disease and Transmission	**Microbial Agent**	**Sources of Agent in Water Supply**
1. Adenovirus infection	Adenovirus	Manifests itself in improperly treated water
2. Gastroenteritis	Astrovirus, Calicivirus, Enteric Adenovirus, and Parvovirus	Manifests itself in improperly treated water
3. SARS (Severe Acute Respiratory Syndrome)	Coronavirus	Manifests itself in improperly treated water
4. Hepatitis A	Hepatitis A virus (HAV)	Can manifest itself in water (and food)
5. Poliomyelitis (Polio)	Poliovirus	Enters water through the feces of infected individuals
6. Polyomavirus infection	Two of Polyomavirus: JC virus and BK virus	Very widespread, can manifest itself in water, ~80% of the population has antibodies to Poltomavirus

1.9 Purification of water using physical and chemical disinfectant

S. No.	Boiling water	
1.	**Advantages**	**Disadvantages**
	• Readily available. Well-suited for emergency and temporary disinfection. • Will drive volatile organic chemicals out of water. • Extremely effective disinfectant that will kill even giardia cysts.	• Requires a great deal of heat. • Time to bring water to boil and cool before use. • Can give water stale taste. • Typically limited capacity. • Not an in-line treatment system. • Requires separate storage of treated water.
2.	**Chlorination**	
	• Provides residual disinfectant. • Residual easy to measure. • Chlorine readily available at reasonable cost. • Low electrical requirement. • Can be used for multiple water problems (bacteria, iron, manganese, hydrogen sulfide). • Can treat large volumes of water.	• Requires contact time of 30 minutes for simple chlorination. • Turbidity (cloudy water) can reduce the effectiveness of chlorine. • Gives water a chlorine taste. • May combine with precursors to form THMs. • Does not kill giardia cysts at low levels. **Careful storage and handling of chlorine is required**
3.	**Ultraviolet light**	
	• Does not change taste or odor of water. • Kills bacteria almost immediately. • Compact and easy to use.	• High electrical demand. • No disinfection residual. • Requires pretreatment of cloudy or colored water. • Requires cleaning and new lamp annually.
4.	**Iodine**	•
	• Does not require electricity. • Requires little maintenance. • Provides residual treatment. • Residual easy to measure.	• Health effects of iodine undetermined. • Concentration affected by water temperature. • Gives water a slight straw color at high levels. • Gives water an iodine taste. • Not effective as an algicide

In the lack of safe water, peoples have been described a variety of technologies for treatment of household water which are widely used in different parts of the world. The physical methods, include boiling, heating (fuel and solar), settling,

filtering, exposing to the UV radiation in sunlight, and UV disinfection with lamps. The chemical methods include coagulation-flocculation and precipitation, adsorption, ion exchange and chemical disinfection with germicidal agents (primarily chlorine). According to World Health Organization (WHO, 2002) some water treatment and storage systems use chemicals and other media and materials that can not be easily obtained locally at reasonable cost and require relatively complex and expensive systems and procedures to treat the water.

This is also confirmed by earlier research of Crapper, *et al.,* (1973) and Miller, *et al*., (1984) that the chemicals used for the water purification causes serious health hazards if an error occurs in their administration during the treatment process. Therefore, there is a need to investigate the use of non-chemicals which would be available locally in most developing countries. The economical aspects of some physical and chemical disinfectant are summarized in above table 4.

1.10 Herbal antimicrobials

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies pertinent for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased in India. According to World Health Organization (WHO), medicinal plants would be the best source from which to obtain a variety of drugs (Santos et al. 1995). The 80% of the rural population depends on traditional medicines for their primary health care needs (Akerele, 1993; Nyazena and Kire, 1986).

Therefore, the putative medicinal plants should be investigated to understand their properties, safety and efficacy (Ellof, 1998). Sanskrit writings in India the Sushruta Samhita contains 184 chapters and description of 1120 illnesses, 700 medicinal plants, a detailed study on Anatomy, 64 preparations from mineral sources and 57 preparations based on animal sources. Detailed practical guidance for water purification is given in the famous treatise of Indian Sushruta. Sushruta disclosed that muddy water could be purified with herbs and naturally occurring substances. In India dating from several centuries BC make reference to seeds of the tree *Strychnos potatorum* as a clarifier, Peruvian texts from the 16th and 17th centuries detail the use

by sailors of powdered, roasted grains of *Zea mays* as a means of settling impurities. More recently, Chilean folklore texts from the 19th century refer to water clarification using the sap from the 'tuna' cactus (*Opuntia fiscus indica*) (Sutherland, 1990).

The discovery of new antimicrobial agents marked a new era in the history of medicine. Antibiotics were treated as miracle drug when they first became available over half a centuary ago. However, their popularity rapidly led to overuse. Over the last decade, it has become well known that antibiotics are loosing their effectiveness as bacteria evolve resistance against them and new drugs only rarely reach the market (Cuevas, 2003). It has been well documented that traditional medicinal plants confer cosiderable antimicrobial activity against various microorganisms (Jonathan, *et al*., 2000).

1.11 Screening plants for new antimicrobials-Ethnopharmacology

The concept of ethnopharmacology has evolved from the requirement for studies in light of modern science on the drugs used in the traditional medicine. In its entirety, pharmacology embraces the knowledge of the history, source, chemical and physical properties, compounding, biochemical and physiological effects, mechanism of action, absorption, distribution, biotransformation, excretion and therapeutic and other uses of drugs. A drug is broadly defined as any substance (chemical agent) that affects process of living. Nature can be considered as the ultimate chemist as natural products offer us with an abundant source of novel chemo-types, pharmacophores or lead structures, which could be directly used or derived into ready-made drugs (Lucy and Dasilva, 1999).

The screening of plant extracts and plant products for antimicrobial activity has shown that plants represent a potential source of new anti-infective agents (Salvat, *et al*., 2001). Many commercially proven drugs used in modern medicine were initially used in crude form an unparalleled growth in the plant-derived medicinally useful formulations, drugs and health-care products (Sandhya, 2004). The primary benefits of using plant-derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment. The relatively lower incidence of adverse reactions to plant preparations compared to modern conventional pharmaceuticals, coupled with their reduced cost is

consequently encouraging the consuming public and national health care institutions to consider plant medicines as alternatives to synthetic drugs (Nair, *et al*., 2004).

The traditional use can provide valuable clues for the selection, preparation and indications for use of herbal formulations with specificity in action against the diseases. A systematic approach through experimental and clinical validation of efficacy is required for a plant identified for traditional medicine, as is done in modern medicine; animal toxicity studies is also required to establish the potential adverse effects. There should be adequate data from *in-vivo* and *in-vitro* studies to validate the therapeutic potential claimed (Seth and Sharma, 2004).

Drug discovery from plants has evolved to include numerous interdisciplinary fields and various methods of analysis. The process typically begins with a botanist, ethnobotanist, ethno-pharmacologist, or plant ecologist who collects and identifies the plants of interest. Collection may involve species with known biological activity for which active compound(s) have not been isolated or may involve taxa collected randomly for a large screening programme. Phytochemists (natural product chemists) prepare extracts from the plant materials, subject these extracts to biological screening in pharmacologically relevant assays, and commence the process of isolation and characterization of the active compound(s) through bioassay-directed Dfractionations. Molecular biology has become essential to medicinal plant drug discovery through the determination and implementation of appropriate screening assays directed towards physiologically relevant molecular targets.

As mentioned above, different techniques and interdisciplinary approaches have to be follwed for demonstrating the medicinal properties of a plant, for determining and for identifying the bioactive compounds. Preclinical pharmacological studies and randomized clinical trials form an important part of the biological screening of medicinal plants and verify the experimental data in nature. However, a pharmacological effect observed *in vitro* or in animal models for both safety and efficacy needs to be reconfirmed by clinical studies and the information obtained from the preclinical studies can from the basis for further clinical trials. Biological screening is one way of carrying out preclinical studies, which involves antimicrobial

susceptibility tests to evaluate the effect of plant extracts on disease causing pathogens.

The current research was therefore initiated, if secondary metabolites of plants origin inherit antimicrobial activity against disease causing water borne bacteria such as *Escheirchia coli*, *Vibrio cholerae*, *Salmonella typhimurium* and *Shigella dysenteriae, in-vitro* as well as *in vivo* for possible development of new, effective, ecofriendly and safe natural antimicrobial formulations.

2.1 GENERAL BACTERIOLOGICAL ASPECTS OF DISEASE CAUSING WATER BORNE BACTERIA

The recognition, in the 1800s that bacteria were agents of disease, along with the development of bacteriology as a science made it possible to use bacteria as tools to evaluate water quality and treatment. Essentially, non pathogenic, easily detectable microorganisms were used to 'indicate' that contaminations have taken place and, as such, there was a risk to public health (WHO, 2003). Waterborne diseases: caused by the ingestion of water contaminated by human or animal faeces or urine containing pathogenic bacteria or viruses; include cholera, typhoid, amoebic and bacillary dysentery and other diarrheal diseases.

The pathogenic *E. coli, V. cholerae, S. typhimurium* and *S. dysenteriae* are extremely virulent and can cause severe gastrointestinal illness. *Escherichia coli*, *Vibrio cholerae*, *Salmonella typhimurium* and *Shigella dysenteriae* are mainly isolated from contaminated water and various food products (Bilge, *et al.*, 1996, Call, *et al.*, 2001 and Fratamico, *et al.,* 1995). *E. coli* is one of the most harmful water borne pathogenic bacteria and is alone responsible for many cases of infection and deaths worldwide (Gannon, *et al*., 1997 and Gilbert, *et al*., 2003). The infection is usually self-limiting but the bacterium can cause life-threatening complications, including hemorrhagic colitis and hemolytic uremic syndrome in children and the immunocompromised patients (Gryko, *et al.*, 2006). The genus *Salmonella* are among the major pathogens that cause infections in humans and animals. Most human *Salmonella* infections are thought to be associated with water borne transmission from contaminated water, animal–derived meat and dairy products (Gubala and Proll, 2006). *V. cholerae* is a waterborne pathogen with a major virulence factor identified as cholera enterotoxin (Bilge, *et al*., 1996). Infection is characterized by vomiting and rice-like diarrhea (Ibekwe, *et al*., 2002). Many pathogenic *Shigellae* are noted for high transmission rates and may cause shigellosis with a very low infectious dose. Strains of Shigella produce shiga toxin, similar to the vero-toxin of Shiga-toxin producing *Escherichia coli* (STEC) (Ul-Hassan, *et al.,* 2009). STEC may cause a broad disease

spectrum in humans and are often difficult to distinguish by phenotypic traits from members of *Shigella* (Koneman, *et al*., 1997).

2.1.1 Origin of Gram negative bacteria

A central issue in bacterial phylogeny is to understand how different bacterial phyla are related to each other and their branching order from a common ancestor (Gupta and Griffiths, 2002). As seen, the model predicted with very high degree of accuracy the presence or absence of these indels in various species/genomes (Gupta, 2003). For a number of proteins that are present in virtually all of the sequenced genomes (viz. Hsp70, Hsp60, RpoB, RpoC, GyrB, AlaRS), The ability of the indel model (Fig. 3) to predict with remarkable accuracy (>99%) the presence or absence of these indels in various genomes/species provides compelling evidence that the branching pattern suggested by these indels is reliable (Battista and Rainey, 2001). In case of CTP synthase, the insert was originally indicated to be specific for all proteobacteria (Gupta, 2000). However, the absence of CTP synthase insert in most delta proteobacteria indicates that this insert was introduced in this gene after the divergence of delta proteobacteria and it is a shared characteristic of species from the epsilon, alpha, beta and gamma proteobacterial subdivisions.

The branching order of the bacterial phyla as deduced based on conserved indels, is generally in accordance with phylogenetic trees based on 16S rRNA as well as many other genes and proteins (Brown, *et al*., 2001, Ciccarelli, *et al*. 2006, Olsen, *et al*., 1994, Viale, *et al*., 1994). In most published trees, groups such as Thermotoga, Deinococcus-Thermus, Cyanobacteria and Green nonsulfur bacteria show deep branching, whereas other groups such as Proteobacteria and Chlamydiae and Bacteriodetes-Chlorobi are late branching lineages (Eisen, 1995, Gupta, 1998). A recent phylogenetic study based on concatenated sequences for 31 universally conserved proteins also strongly indicates that the Firmicutes constitute the deepest branching lineage with the bacteria (Ciccarelli, *et al.,* 2006). The deep branching of Firmicutes, Actinobacteria as well Thermotoga, Deinococcus-Thermus is also supported by comparison of gene order arrangements in bacterial genomes (Ludwig and Klenk, 2001). Studies by Lake and coworkers also provide strong evidence that the root of the bacterial tree does not lay within the Gram-negative (or diderm)

bacteria (Lake, *et al.,* 2007). However, this anomaly has now been shown to be due to the very high G+C content of Aquifex rRNA, which is a common characteristic of various hyperthermophilic organisms that results in their clustering with the Archaea and deep branching in the rRNA trees (Meyer and Bansal, 2005).

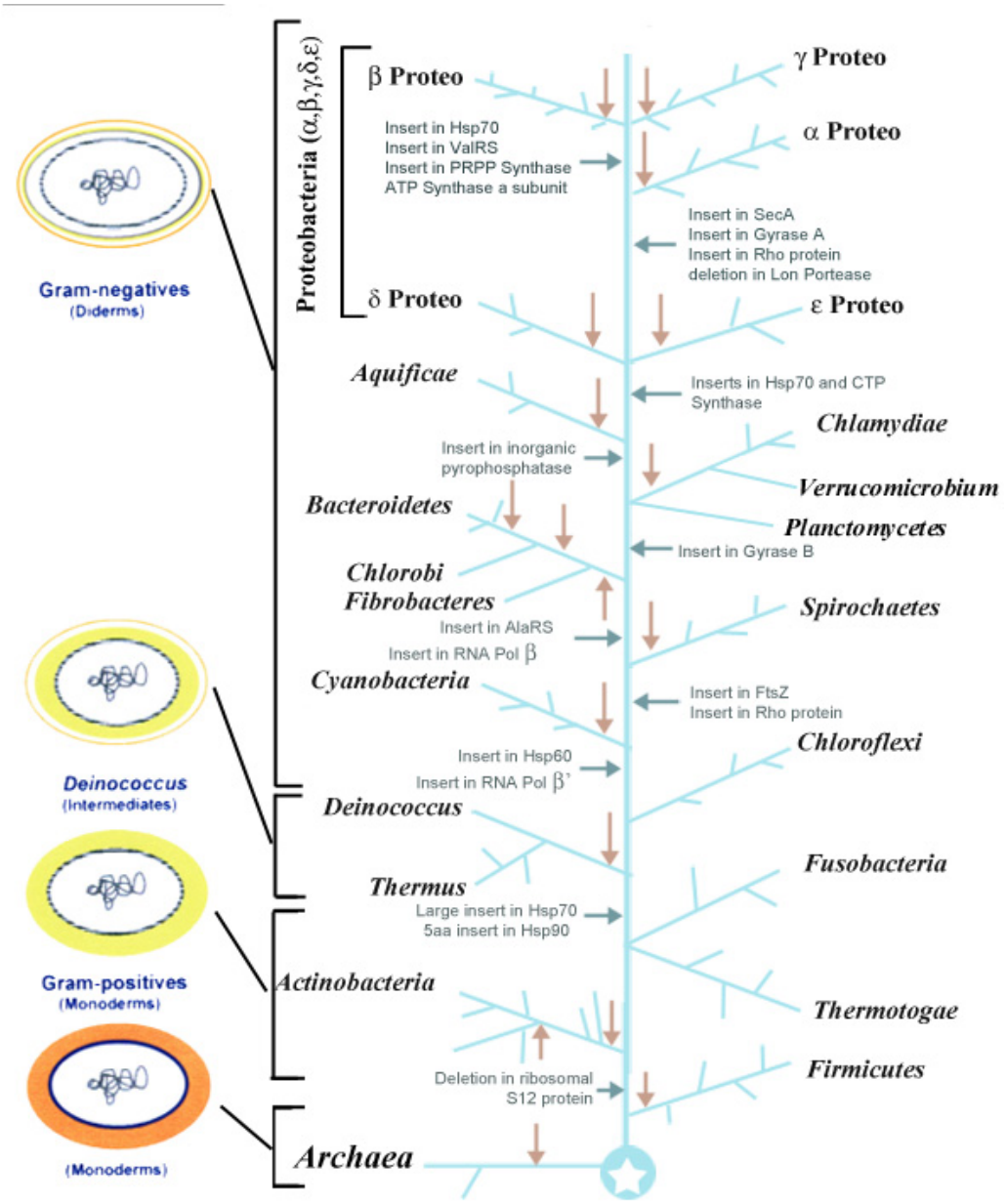


Fig. 2.1 Evolution of Gram negative bacteria: responcible for water borne disease through Reliability and Predictive Power of the Indel Model

2.1.2 Epidemiology

The water borne diseases such as; diarrhoea, dysentery, cholera and typhoid causing bacteria mostly present in contaminated drinking water. A well publicized waterborne out- break of illness caused by *E. coli* O157:H7 occurred in the farming community of Walkerton in Ontario, Canada. The outbreak took place in May 2000 and led to 7 deaths and more than 2300 illnesses (O'Connor, 2002). The epidemiological accounts of diseases causing water borne bacteria are as follows:

(i) *Esxherichia coli*

A limited number of enteropathogenic strains can cause acute diarrhoea. Several classes of enteropathogenic *E. coli* have been identified on the basis of different virulence factors, including enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative E. coli (EAEC) and diffusely adherent E. coli (DAEC). More is known about the first four classes named; the pathogenicity and prevalence of EAEC and DAEC strains are less well established (Nataro and Kaper, 1998). *E. coli* commonly live in the intestines of people and animals worldwide. There are many strains (over 700 serotypes) of *E. coli*. Most of the *E. coli* are normal inhabitants of the small intestine and colon and do not cause disease in the intestines (non-pathogenic). Nevertheless, these non-pathogenic *E. coli* can cause disease if they spread outside of the intestines, for example, into the urinary tract (where they cause bladder or kidney infections), or into the blood stream (sepsis). Other *E. coli* strains (enterovirulent *E. coli* strains or EEC) cause "poisoning" or diarrhoea even though they usually remain within the intestine by producing toxins or intestinal inflammation. The *E. coli* and related bacteria possess the ability to transfer DNA via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population. This process led to the spread of the gene encoding shiga toxin from Shigella to E. coli O157:H7, carried by a bacteriophage (Brüssow, *et al.,* 2004).

New strains of *E. coli* evolve through the natural biological process of mutation and through horizontal gene transfer (Lawrence and Ochman, 1998). Some strains develop traits that can be harmful to a host animal. These virulent strains

typically cause a bout of diarrhoea that is unpleasant in healthy adults and is often lethal to children in the developing world (Nataro and Kaper, 1998). More virulent strains, such as O157:H7 cause serious illness or death in the elderly, the very young or the immunocompromised (Hudault, *et al*., 2001).

(ii) *Vibrio cholerae*

Vibrio spp. are small, curved (comma-shaped), Gram-negative bacteria with a single polar flagellum that causes cholera in humans (Ryan and Ray, 2004). Species are typed according to their O antigens. There are a number of pathogenic species, including *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. *Vibrio cholerae* is the only pathogenic species of significance from freshwater environments (Ogg, *et al*., 1989). While a number of serotypes can cause diarrhoea, only O1 and O139 currently cause the classical cholera symptoms in which a proportion of cases suffer fulminating and severe watery diarrhea (WHO, 2002). The O1 serovar has been further divided into "classical" and "El Tor" biotypes. The classical biotype is considered responsible for the first six cholera pandemics, while the El Tor biotype is responsible for the seventh pandemic that commenced in 1961 (Rhodes, *et al.,* 1985). Strains of *V. cholerae* O1 and O139 that cause cholera produce an enterotoxin (cholera toxin) that alters the ionic fluxes across the intestinal mucosa, resulting in substantial loss of water and electrolytes in liquid stools (Kaper, *et al*., 1995)). Not all strains of serotypes O1 or O139 possess the virulence factors, and they are rarely possessed by non-O1/O139 strains. There are two major biotypes of *V. cholerae* identified by hemagglutination testing, classical and El Tor, and numerous serogroups. The classical biotype is found only in Bangladesh, whereas the El Tor is found throughout the world (Faruque and Nair, 2008).

According to Waldor and Mekalanos (1996) the *V. cholerae* pathogenicity genes code for proteins directly or indirectly involved in the virulence of the bacteria. In *V. cholerae*, most of virulence genes are located in two pathogenicity plasmids, which are organized as prophages:

- CTX (Cholera ToXins) plasmid and

- TCP (Toxin-Coregulated Pilus) plasmid, also named VPI (*V. cholerae* Pathogenicity Island).

The Virulent and epidemic strains of *V. cholerae* require these two genetic elements to cause infections. The CTX plasmid is composed of genes involved in toxin production. This genetic element is a 7- to 9.7-kb segment of DNA flanked by two repetitive sequences (att-like site), resembling a transposon organization. CTX plasmid is divided in two regions: CORE encoding toxins, and RS2 encoding proteins that catalyze the integration and the replication of CTX prophage in the V. cholerae chromosome (Waldor and Mekalanos, 1996, Faruque and Nair, 2002).

(iii) *Salmonella typhimurium*

Salmonella spp. they are motile, Gram-negative bacilli that do not ferment lactose, but most produce hydrogen sulfide or gas from carbohydrate fermentation (Angulo, *et al*. 1997). Originally, they were grouped into more than 2000 species (serotypes) according to their somatic (O) and flagellar (H) antigens (Kauffmann-White classification) (WHO, 2008). It is now considered that this classification is below species level and that there are actually no more than 2–3 species (*Salmonella enterica* or *Salmonella choleraesuis*, *Salmonella bongori* and *Salmonella typhi*), with theserovars being subspecies. All of the enteric pathogens except *S. typhi* are members of the species *S. enterica*. Convention has dictated that subspecies are abbreviated, so that *S. enterica* serovar *Paratyphi* A becomes *S. Paratyphi* A (Escartin, *et al*., 2002). *Salmonella enterica* subspecies *enterica* serotype *typhimurium* is the commonly isolated serotype in industrialized countries (Herikstad, *et al*., 2002). *Escherichia coli* (or, alternatively, thermotolerant coliforms) is a generally reliable index for Salmonella spp. in drinking-water supplies (Pegram, *et al*., 1998).

(iv) *Shigella dysenteriae*

Shigella spp. is a species of the rod-shaped bacterial genus *Shigella* (Ryan and Ray, 2004). *Shigella* can cause shigellosis (bacillary dysentery). Shigellae are Gram-negative, non-spore-forming, facultatively anaerobic, non-motile bacteria (Hale and Keusch, 1996). Shigella spp. can cause serious intestinal diseases, including bacillary dysentery. Over 2 million infections occur each year, resulting in about 600 000 deaths, predominantly in developing countries (Alamanos, *et al*., 2000). Most cases of Shigella infection occur in children under 10 years of age. Members of the

genus have a complex antigenic pattern, and classification is based on their somatic O antigens, many of which are shared with other enteric bacilli, including *E. coli.* There are four species: *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei. S. dysenteriae*, spread by contaminated water and food, causes the most severe dysentery because of its potent and deadly Shiga toxin, but other species may also be dysentery agents (Herold, *et al*., 2004). *Escherichia coli* (or, alternatively, thermotolerant coliforms) is a generally reliable index for Shigella spp. in drinking-water supplies (Pegram, *et al*., 1998).

2.2 Methods for direct detection: Using DNA probes

Bacteria are the very tiny microscopic microorganisms, so their direct identification is very difficult job. Based on their genomic study confirmed the differences in their species and genus, eventhough proper classification can be done. Shigellae are noted for high transmission rates and may cause shigellosis with a very low infectious dose. Some strains of *Shigella* produce shiga toxin, similar to the vero-toxin of Shiga-toxin producing *Escherichia coli* (STEC). STEC may cause a broad disease spectrum in humans and are often difficult to distinguish by phenotypic traits from members of *Shigella* (Koneman, *et al.,* 1997). Although O157:H7 serotype is the dominant STEC in many parts of the world, now it is recognized that STEC strains belong to a very broad range of O: H serotypes (Paton & Paton, 1998). The ability of STEC serogroups other than O157 to cause diarrhea and hemolytic uremic syndrome has also been well documented (Gerber, *et al*., 2002; Werber, *et al*., 2003; Ethelberg, *et al*., 2004). More than 200 serotypes of STEC have been identified and around 160 of these have been recovered from humans with HC and HUS (Brett, *et al*., 2003). STEC are more prevalent in animals and as contaminants of food, therefore humans are probably more exposed to these strains, and some of them have been associated with severe illness (Boerlin, *et al*., 1999; Blanco, *et al*., 2003). Raw milk and beef have been confirmed to be the most likely sources of infection in outbreaks which have occurred during the last decade mainly in Canada, the USA, the UK and Japan (Paton & Paton, 1998; Centers for Disease Control, 2007).

Four specific genes were selected from the three target bacterial pathogens: the rfbE gene of *E. coli*, the ctx gene of *V. cholerae*, the STM4497 gene of *S.*

typhimurium and the ipaH gene of *S. dysenteriae* that transcribed putative cytoplasmic protein. The rfbE gene of *E. coli* encodes the O157 LPS and is therefore unique to the *E. coli* O157 serogroup (Wang, *et al*., 1997). This gene has been identified as a good marker because it is transcribed in all growth phases from early exponential to late stationary phase (Wang, *et al.*, 2002). Only strains of *V. cholerae* that produce cholera toxin have been associated with epidemics and pandemics; therefore, production of cholera toxin has become an important marker for identifying isolates with the potential to cause epidemics.

2.2.1 Typing of diseases causing water borne bacteria

Typing of isolates is applied in epidemiological studies to determine bacterial occurrence and modes of transmission. Typing of isolates is also performed for evaluation of whether certain strains are associated with specific clinical disease conditions and to characterize the heterogeneity of infection, *i.e.*, whether subjects are colonized by one or multiple types of the microorganism. Evaluation criteria for typing methods include typeability (ability to give an outcome for every isolate included), reproducibility (ability to give the same result when repeating the analysis) and discriminatory power (ability to differentiate between unrelated strains) (Arbeit, 1999). Two main types of epidemiological typing systems for microorganisms are available, the phenotypic and the genotypic methods.

2.2.2 Phenotyping

Traditional methods of characterizing bacterial isolates have relied on measurement of characteristics expressed by the microorganisms, such as bacteriocin production and sensitivity to bacteriocins, serotype, biochemical properties, antibiotic resistance and bacteriophage type (Maslow and Mulligan, 1996).

2.2.3 Bacteriocin typing

One of the first epidemiological typing systems for oral streptococci was bacteriocin typing (Kelstrup, *et al.*, 1970). Bacteriocins are proteinaceous substances produced by the bacteria that inhibit the growth of other, mostly closely related, bacteria. The typing is performed by measuring the inhibiting effect on bacterial growth of certain indicator strains, and by measuring the sensitivity of the bacteria to

be typed to bacteriocins from other strains (Jack, *et al*., 1995). Heterogeneity among strains of mutans streptococci within one individual was first shown by bacteriocin typing (Kelstrup, *et al.*, 1970).

2.2.4 Serotyping

Using Ouchterlony immunodiffusion, Bratthall demonstrated five serological groups of mutans streptococci (Bratthall, 1970). A total of eight serotypes were subsequently recognized (Perch, *et al.*, 1974; Beighton, *et al.*, 1981). The classification is based on cell-wall carbohydrate antigen (Table 2.1). Serotyping by immunodiffusion, immunofluorescence or immunoelectrophoresis has been widely applied for typing of mutans streptococci.

2.2.5 Biotyping

Shklair and Keene, (1974) divided mutans streptococci into five biotypes (a-e) on the basis of fermentation characteristics, arginine hydrolysis and bacteriocin sensitivity, and they reported that these biotypes corresponded with the serotypes reported in 1970. Their later scheme (Shklair and Keene, 1976) also includes serotypes *f* and *g*.

Among other phenotypic methods are cellular fatty acid analysis, whole-cell protein analysis and multilocus enzyme electrophoresis (MEE). MEE is based on the relative electrophoretic mobility of metabolite cellular enzymes. MEE has been successfully applied in studies with many organisms, but only one report using MEE in strain identification of mutans streptococci has been published (Gilmour, *et al.,* 1987). Phenotypic typing is, in most cases, relatively inexpensive to perform and typeability of mutans streptococcal isolates is high, however, reproducibility and discriminatory power are usually somewhat poorer (Arbeit, 1999).

2.2.6 Genotyping

For isolate fingerprinting, molecular typing methods have a higher discriminatory ability and reproducibility since these methods do not examine the gene expression but rather the DNA of the microorganisms to be studied (Arbeit, 1999, Olive and Bean, 1999). Among these typing methods are plasmid analysis,

restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP), (including ribotyping), pulsed field gel electrophoresis (PFGE) and arbitrarily primed polymerase

Chain Reaction (AP-PCR).

2.2.7 Plasmid analysis

Plasmids are extrachromosomal circles of DNA that encode many properties, including antimicrobial resistance, many virulence traits and hydrocarbon metabolism (Madigan, *et al.*, 1997a). Plasmid analysis was the first DNA-based technique applied in epidemiological studies on mutans streptococci (Caufield, *et al.*, 1982). Because plasmids are infrequently detected in mutans streptococci, in only 5% of strains (Hamada and Slade, 1980b), plasmid analysis is not applicable to typing of these bacteria.

2.2.8 Restriction endonuclease analysis (REA)

In restriction endonuclease analysis (REA), bacterial chromosomal DNA is cut with a restriction endonuclease and separated by gel electrophoresis. The restriction endonucleases are enzymes that cut the DNA chain at specific recognition sequences. The restriction enzymes are nowadays synthetically fabricated, but were originally isolated from bacteria, with their original function being defence against other bacteria. After separation by gel electrophoresis, gels are stained with ethidium bromide and detected under UV light, whereby the banding patterns obtained for different strains are compared. Often the process results in fingerprints with many bands, thus the interpretation of the REA profiles can be complicated.

2.2.9 Restriction fragment length polymorphism (RFLP), including ribotyping

After cleaving the chromosomal DNA of the microorganisms to be studied, the separation products can be labelled with either DNA or RNA probes in the Southern blot technique (Southern, 1975). The use of a probe derived from the *Escherichia coli* ribosomal operon was introduced by Grimont and Grimont (1986). They had discovered that variations of the genes encoding ribosomal ribonucleic acid (rRNA), and variations in sites flanking those loci, could serve as a means of typing

strains since ribosomal sequences are highly conserved. In ribotyping an isolate, after the gel electrophoresis of the cleaved DNA, the fragments are hybridized with the rRNA probe. When detecting the hybrids, every fragment containing a ribosomal gene will be highlighted. The banding patterns obtained in ribotyping include only a small number of bands, thus rendering comparison of fingerprints among isolates easier than comparing REA patterns. The term ribotyping was introduced in 1988 (Stull, *et al.*, 1988).

2.2.10 Pulsed field gel electrophoresis (PFGE)

In pulsed field gel electrophoresis, a variation of agarose gel electrophoresis, the orientation of the electric field across the gel is changed periodically ("pulsed"), thus larger bacterial DNA fragments can be analysed than by REA (Arbeit, 1999). PFGE is considered the "gold standard" of molecular typing methods, with excellent discriminatory power and reproducibility (Arbeit, 1999; Olive and Bean, 1999).

2.3 Herbal antimicrobials against water borne bacterial pathogens

Since ancient times, the need for pure water resulted in the development of water purification methods. Ancient civilizations that developed early water purification methods include those located in Africa, Asia, particularly India, and the Middle East, and Europe (Nadkarni, 1976). Between 4000 B.C. and 1000 A.D., different natural minerals were used to purify water. To disinfect water, many ancient cultures would use copper, iron or hot sand in conjunction with boiling it. Herbs were often used in well filtration, such as amla, which is high in vitamin C, and khus. Plants were sometimes used to purify water, such as water lily roots and the seeds of the nirmali (*Strychnos potatorum*) (Kirtikar and Basu, 1972). In ancient Egypt, aluminum sulfate, iron sulfate or a mix of the two was used to extract suspended solids. In Greece, a fabric bag, called the Hippocrates Sleeve, was used to strain water before boiling it. In ancient India, sand and gravel were used to filter water before boiling it. This method was from the Sanskrit manuscript called the Susruta Samhita.Certain metals disrupt bacteria cycles, including copper. In ancient India, brass, an alloy of copper and zinc and sometimes with other metals, was used to store water (Nadkarni, 1976). It is estimated that there are 250,000 to 500,000 species of plants on Earth (Boris, 1996). A relatively small percentage of (1 to 10%) of these, are

used as foods by both humans and other animals species. It is possible that even more are used for medicinal puposes (Moerman, 1996).

Bourke, (1895) Reported that the, earliest reference found for the use of prickly pear cactus in water purification dates to the late 19th century and likely was based on observations of peoples living in the Rio Grande Valley. “Cut into strips and thrown into a bucket of turbid water, the nopal will cause the sedimentary matters in suspension to be precipitated to the bottom. This expedient was resorted to with success during our expedition to explore the Black Hills of Dakota in 1875.

Remington, *et al.,* (1918), Gupta and Chaudhuri, (1992) reported that *Strychnos potatorum* L. f. (syns *Strychnos heterodoxa* Gilg, *Strychnos stuhlmannii* Gilg) Cleaning Nut Tree, Indian Gum Nut. The name of the plant refers to its traditional use as a water purifier: one of the dried nuts S. potatorum is rubbed hard for a short time around the inside of the earthenware water pot, on settling, the water is left pure and tasteless.

Tripathi, *et al.,* (1976) Studied that Nirmali seeds and nuts have long been used in crushed form to clarify muddy water in India. An extract from the seeds was found to be an anionic polyelectrolyte which was effective as a coagulant and coagulant aid in the clarification of natural turbid water. The two main groups on the polymer were carboxyl and hydroxyl. It was an efficient flocculant for turbidity that was of an inorganic, hydrophobic nature. The extract performed poorly as a flocculant and coagulant for natural biocolloids, such as bacteria.

Nadkarni, *et al.,* (1976) in ayurvedic medicine, basil leaves ground with water are applied to bad boils and an infusion of the leaves is given against fever, diarrhoea and other gastric diseases in children and adults.

Jahn, (1981) Reported that the, Surjana seed has a cationic polymer of water-soluble basic polypeptides with a molecular weight of 6000 to 7000 Daltons. Ndabigengesere *et al.*, (1995) reported that the active ingre- dients in an aqueous *Moringa oleifera* (Surjana seed) extract are dimeric proteins with a molecular weight of 13,000 Daltons. Therefore Surjana seed can be considered to contain low molecular weight cationic polymer.

Wood, (1988) Shown that the, gingerols have analgesic, sedative, antipyretic, antibacterial and gastrointestinal tract motility effects. Ginger has the capacity to eliminate harmful bacteria, such as *Escherichia coli,* responsible for most of the diarrhoea, especially in children.

Coles and Coles, (1989) Explorered that in Northern Europe used *sphagnum* moss to keep food from spoiling and to keep drinking water fresh. In fact, *sphagnum* moss is what allowed the Vikings to travel to Iceland, Greenland and North America by presser- ving their food and water during long ocean voyages.

Sutherland, *et al.,* (1990), David, (2004) they showed that natural material of plant origin to clarify turbid surface waters is not a new idea. Sanskrit writings in India dating from several centuries BC make reference to seeds of the tree *Strychnos potatorum* as a clarifier, Peruvian texts from the 16th and 17th centuries detail the use by sailors of powdered, roasted grains of *Zea mays* as a means of settling impurities. More recently, Chilean folklore texts from the 19th century refer to water clarification using the sap from the *'tuna' cactus* (*Opuntia fiscus indica*) however, of all the plant materials that have been investigated over the years, the seeds from *M. oleifera* have been shown to be one of the most effective as a primary coagulant for water treatment.

Biddlestone, *et al.,* (1991) reported that a number of investigations are in progress worldwide into the ability of aquatic plants to purify wastewaters from domestic, industrial and agricultural sources. At the University of Birmingham, Phragmites are being studied for the treatment of agricultural effluents which are significantly more polluting than sewage. Two reed beds have been constructed on farms and some initial results are given in the paper.

Tobiason, *et al.,* (1993) reported that head loss development in a filter is just as important as filtrate tur- bidity to evaluate the filter performance. Based on volume of filtered water produced it was observed that the head loss development in the case of Surjana seed and maize as coagulant aids were comparable with suspension using alum alone, whereas, it was more in the case of Nirmali seeds. The difference observed in head loss development by different coagulant aids may be due to the difference in the sizes of flocs formed. The active ingredient in Surjana seed is a low

molecular weight polymer and in maize it is starch (nonionic polymer). It seems that the flocs present in the influent for filtration treated by maize and Surjana seeds were larger, as for the same mass deposition in the filter bed, larger flocs/particles cause less head loss in comparison to smaller ones.

Bhole, (1995) reported that ten edible materials as mentioned below were selected as natural coagulants. The Soyabean, Cowpea, Kidney bean, Rice, Tamarind, Maize, *Manila tamarind*, Horse gram, *Tinospora* and Elephant apple (or Wood apple) taken in study. Turbidities of 25, 100, 200, 400, 800 and 1600 NTU were prepared out of bentonite clay. The relative performance of these materials was compared with that of alum. Natural coagulants were also used as coagulant aids in conjunction with alum. The effect of variation of pH in the range of 5.5-9 and the sequence of addition of alum and coagulant aid were also studied.

Al -Samawi and Shokralla, (1996) reported that the coagulants play an important role in the treatment or water and waste water, and the treatment and disposal of sludge. A natural indigenous coagulant is suggested as a substitute for alum or as an aid for alum. Okra extract derived from Okra seeds old tips, sap, plant stalk, and roots is suggested in this study. Laboratory jar tests are carried out on clay suspension to assess the coagulation power of Okra extract in comparison with that of alum. A widely reported Indian grown natural coagulant (Nirmali seeds) was utilized in this study and the results obtained with this natural coagulation are compared to that of our Okra extract. The results of this study have shown that Okra extract is a powerful polyelectrolyte coagulant whether it is used as a primary or as a coagulant aid in relation to alum. It performed much better than alum at higher turbidity levels. A significant reduction in alum dosages was achieved when using Okra extract as a coagulation aid.

Dalsgaard, *et al.,* (1996) investigated the vibriocidal properties of juice from lime (*Citrus aurantifolia*) fruits added to well water, tap water, and food samples.The results showed that during epidemics of cholera in areas without safe sources of drinking water, juice from citrus fruits added to water and food in palatable concentrations may be appropriate measures in reducing the transmission of cholera.

Pattnaik, *et al.,* (1997) Reported that the principal active constituents of *Mentha piperita* are the essential oils, which comprise about 1% of the herb. The oils are dominated by monoterpenes, mainly menthol, menthone, and their derivatives (e.g., isomenthone, neomenthol, acetylmenthol, pulegone). These essential oils dilate peripheral blood vessels and inhibit bacteria. Its oils especially menthol have a broad spectrum antibacterial activity since Gram +ve and Gram -ve bacteria were found susceptible to the oils.

Valsaraj, *et al.,* (1997) reported that the leaf extracts of *Mentha piperita* exhibited antibacterial activity only in ethyl acetate, petroleum ether, choloroform, menthanol and aqueous extracts against the bacteria tested in agar well diffusion method at 50 µl -100 µl concentration.

Fleming, (1998) Reported that *Mentha piperita* shows significant activity as because the leaf contains many potent compounds such as menthol, menthone, menthyl acetate, menthofuran, and limnone.

Deans and Baratta, (1998) showed that the compounds from *Mentha piperita* possess potent antimicrobial activity and suggesting that the *Mentha piperita* leaf extracts should contains the effective active constituents responsible for eliminating the bacterial pathogens.

Environmental Research Laboratory, (1999) reported that roots of *Acorus calamus* Linn. (Buch), roots of *Arnebia nobills* Rachinger (ratangot), leaves of *Azadirachta indica* A. Juss. (neem), *Anaphalis unefolia* Hook F., *Eclipta alba* (bhangra), *Hypericum ysorense heyne* and *Moringa oleifera* Lam. (shevga) were scrutinized against *Shigella dysentriae, E.Coli, Salmonella typhi, Vibrio cholerae* and *Pseudomonas pyocyaneus* for their antimicrobial activity.

Foster, (2000) investigated that Ginger eases both diarrhoea and constipation; hence it should have impact on the growth of bacterias which mainly causes diarrhoea and nausea.

Iscan, *et al.,* (2002) *Mentha piperita* were found more potent against various pathogenic bacterial strains.

Mimica-Dukic, *et al.,* (2003) Reported that few essential oils obtained from different Mentha species i.e. *Mentha longifolia*. L., *Mentha aquatica* and *Mentha piperita*. L. exhibited broad-spectrum antibacterial activity against disease pathogens.

Mimica, *et al.,* (2003) and Forster, (1996) Peppermint oil or peppermint tea is often used to treat gas and indigestion; it may also increase the flow of bile from the gall bladder.

Mandloi, *et al.,* (2004) reported that surjana (*Moringa oleifera*) seed, maize (*Zea mays*) and chitosan were used in direct filtration of Bilaoli lake water and evaluated for their efficiency in removing turbidity and microorganisms from water. The experiments with these natural coagulants gave filtered water turbidity less than or almost equal to 1NTU and thereby met the turbidity criteria for drinking water as per WHO guidelines. The head loss development across the filter bed with chitosan was more than that of alum, while with maize it was comparable to that of alum. With *M. oleifera* seeds the head loss was much less in comparison to alum. The average most probable number (MPN) reductions obtained with *M. oleifera* seeds, maize and chitosan were 97.35%, 95.4% and 87.1% respectively, whereas, with alum it was only 7.7%.

Saeed and Tariq, (2005) Reported that the juices of leaves and stem of *Mentha piperita* (peppermint), skin and seeds of *Pisum sativum* (garden pea), skin and pulp of *Momordica charantia* (bitter melon) were screened for antibacterial activities against 56 isolates belonging to 11 different species of Gram-negative bacilli: *Escherichia coli* (19), *Klebsiella pneumoniae* (11), *Pseudomonas aeruginosa* (9), *Salmonella typhi* (3), *Salmonella paratyphi* A (1), *Salmonella paratyphi* B (1), *Proteus mirabilis* (5), *Proteus vulgaris* (1), *Enterobacter aerogenes* (4), *Shigella dysenteriae* (1), and *Yersinia enterocolitica* (1). The screening was performed by well diffusion method. Leaves of *M. piperita* exhibited highest antibacterial activity (average zone of inhibition 17.24 mm ± 0.87 SD) while stem of *M. piperita* exhibited least antibacterial activity (average zone of inhibition 15.82 mm ± 3.56 SD). The skin and seeds of *P. sativum*, skin and pulp of *M. charantia* exhibited good antibacterial activity with average zone of inhibition of 16.30 mm ± 2.02 SD, 16.39 mm ± 3.16 SD, 16.16 mm ± 2.17 SD and 15.88 mm ± 2.24 SD respectively.

Scavroni, *et al.,* (2005) Peppermint is very beneficial and important plant. It is widely used in food, cosmetics and medicines.

Adnan, (2006) reported that the GC/MS analysis of essential oil of *Micromeria* biflora ssp. *arabica* K. showed 39 peaks. The antimicrobial activity of oil tested against the *Bacillus subtillis* ATCC 6633, *Escherichia coli* 25922, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 2913, *Candida albicans* ATCC 90028 and *Mycobacterium smegmatis* ATCC 35797.

Aboaba, *et al.,* (2006) found that the sixteen antibacterial preparation of the ethnol and aqueous extracts of four edible plants like as *Entanda Africana* (bark), *Terminalia avicennoides* (bark), *Mitragyna stipulosa* (bark), *Lannae acida* (stem and bark), were screened for their inhibitory effects on ten strians of *E.coli*.

Sarac and Ugur, (2007) have reported that the Lamiaceae is one of the most diverse and widespread plant families in terms of ethnomedicine and its medicinal value is manly based on the volatile oils concentration. It is well documented that some plants belonging to this family possess antimicrobial properties however the reported aspects are not constant.

Bupesh, *et al.,* (2007) evaluated that the antibacterial activity in the leaf extracts of *Mentha piperita* L. against pathogenic bacteria like *Bacillus subtilis*, *Pseudomonas aureus, Pseudomonas aerogenosa, Serratia marcesens* and *Streptococcus aureus*. The aqueous as well as organic extracts of the leaves were found to posses' strong antibacterial activity against a range of pathogenic bacteria as revealed by in vitro agar well diffusion method. The ethyl acetate leaf extract of *Mentha piperita* showed pronounced inhibition than chloroform, petroleum ether and water, leaf extracts being more on *Bacillus subtilis*, *Pseudomonas aerogenosa* than *Streptococcus aureus*, *Pseudomonas aureus* and *Serratia marcesens*.

Soković, *et al.,* (2007) The chemical composition and antibacterial activity of essential oils from 10 aromatic plants *Matricaria chamommilla*, *Mentha piperita*, *M. spicata*, *Lavandula angustifolia*, *Ocimum basilicum*, *Thymus vulgaris*, *Origanum vulgare*, *Salvia officinalis*, *Citrus limon* and *C. aurantium* have been determined. Antibacterial activity of these oils and their components; i.e. linalyl acetate, linalool,

limonene, α-pinene, β-pinene, 1, 8-cineole, camphor, carvacrol, thymol and menthol were assayed against a variety of human pathogenic bacteria.

Garrett, (2007) showed that *Sphagnum* is reusable, making it even more cost effective; a single filter could last for years. For many places in the world, where people can't afford the water filters they need, *Sphagnum* could save lives.

Viuda-Martos, *et al.,* (2007) reported that the effectiveness of the essential oils (EOs) of lemon (*Citrus lemon* L.), mandarin (*C. reticulata* L.), grapefruit (*C. paradisi* L.) and orange (*C. sinensis* L.) to inhibit the growth of some bacteria commonly used in the food industry, (*Lactobacillus curvatus,* L. sakei, *Staphylococcus carnosus* and *S. xylosus*) or related to food spoilage (Enterobacter gergoviae and *E. amnigenus*). The agar disc diffusion method was used to determine the antibacterial activities of the oils. All EO studied had some inhibitory effect on the six bacteria tested. Lemon EO showed the highest inhibition effect upon *S. carnosus*, E. *gergoviae* and *E. amnigenus* while grapefruit EO showed the highest inhibition effect upon *S. xylosus, L. curvatus* and *L. sakei*. Orange and mandarin EOs had the lowest inhibition effect upon the six bacteria tested.

David, *et al.,* (2008) worked on potential in water purification and conditioning is realized, we can expect to see the cultivation, harvesting and marketing of *Sphagnum* moss to become an industry of its own.

Gülay Kirbaşlar, *et al.,* (2009). Worked on the samples of the Citrus fruits viz., lemon (*Citrus limon* (L.) Burm. f.), grapefruit (*Citrus paradisi* Macfayden), bergamot (*Citrus bergamia* Risso et Poit.), bitter orange (*Citrus aurantium* L.), sweet orange (*Citrus sinensis* (L.) Osbeck), mandarin (*Citrus reticulata* Blanco). The antimicrobial activities of Turkish Citrus peel oils were evaluated using the disk diffusion method toward 9 bacteria and the results compared with those for penicillin-g, ampicillin, cefotaxime, vancomycine, oflaxacin and tetracycline. The *Citrus* peel oils showed strong antimicrobial activity against the test organisms. Lemon and bergamot peel oils have a little higher activity than the other Citrus peel oils.

Tao, *et al.,* (2009) studied the chemical composition of the essential oil obtained from the peel of Ponkan (*Citrus reticulata* Blanco) was analyzed by GC/MS. The dominant

components of Ponkan oil were monoterpene hydrocarbons (limonene 75.75%, 3-carene 10.12%, β-myrcene 5.12%, α-pinene 1.79%, β-pinene 1.20%, terpinolene 0.80%, sabinene 0.68, and other minor constituents, which represented 95.96% of the total oil. The antimicrobial activity of the essential oil was measured by the disc diffusion method. Among the tested microbes, the oil was very active against *Bacillus subtilis*, with an average inhibition zone of 36.38 mm. Although *Aspergillus flavus* was more resistant to the oil, as compared with *Escherichia coli* and *Staphylococcus aureus,* it could also be easily inhibited by the oil.

Sadgir, *et al.,* (2010) reported that the *Ocimum sanctum* herb, which is experimented, is naturally available throughout India. At the end of dry season, villagers have to collect the required plant materials for water purification for rainy season.

Audrey, *et.al.,* (2010) Studied newly derived water purification methods have improved the water quality in developing countries; few have been accepted and maintained for long-term use. In an effort to implement a material that will meet community needs, two fractions of mucilage gum were extracted from the *Opuntia ficus-indica* cactus and tested as flocculation agents against sediment and bacterial contamination. As diatomic ions are known to affect both mucilage and promote cell aggregation, CaCl2 was studied in conjunction and compared with mucilage as a bacteria removal method. To evaluate performance, ion-rich waters that mimic natural water bodies were prepared. Column tests containing suspensions of the sediment kaolin exhibited particle flocculation and settling rates up to 13.2 cm/min with mucilage versus control settling rates of 0.5 cm/min. *Bacillus cereus* tests displayed flocculation and improved settling times with mucilage concentrations lower than 5 ppm and removal rates between 97 and 98% were observed for high bacteria concentration tests (>108 cells/ml). This natural material not only displays water purification abilities, but it is also affordable, renewable and readily available.

Lina, *et al.,* (2010) studied at qualitative composition and antibacterial activity of six essential oils obtained from plants cultivated in the Colombian Andes (*Mentha spicata*, *Mentha piperita*, *Ocimum basilicum*, *Salvia officinalis*, *Rosmarinus officinalis* and *Thymus vulgaris*) and a commercial essential oil of *Origanum vulgare* subsp. *hirtum* were investigated. The antibacterial activity of the essential oils against

Escherichia coli, *Salmonella enteritidis*, *Salmonella typhimurium*, *Lactobacillus acidophilus* and *Bifidobacterium breve* was measured as the minimum bactericidal concentration (MBC) using the agar dilution method. The chemical analysis revealed the presence of 16-28 compounds in each oils corresponding mainly to phenols, oxygenated and hydrocarbon monoterpenes. The present study shows that the antimicrobial potential of essential oils depends not only on the chemical composition of the oil but also on the targeted microorganism. This has important practical implications for essential oils intended to be used as feed additives with antibacterial properties for animal nutrition or pharmaceutical products with natural compounds.

Mubarakali, *et al.,* (2011) Reported that bioreduction of silver nitrate (AgNO(3)) and chloroauric acid (HAuCl(4)) for the synthesis of silver and gold nanoparticles respectively with the plant extract, *Mentha piperita* (Lamiaceae). The plant extract is mixed with $AgNO_3$ and $HAuCl_2$, incubated and studied synthesis of nanoparticles using UV-Vis spectroscopy. The nanoparticles were characterized by FTIR, SEM equipped with EDS. The silver nanoparticles synthesized were generally found to be spherical in shape with 90nm, whereas the synthesized gold nanoparticles were found to be 150nm. The results showed that the leaf extract of menthol is very good bioreductant for the synthesis of silver and gold nanoparticles and synthesized nanoparticles active against clinically isolated human pathogens, *Staphylococcus aureus* and *Escherichia coli*.

Table 2.1: List of plants and their traditional uses

S. No.	Species	Local name (plant parts used)	Traditional uses
1	*Albizia lebbeck*	Chhatim (Bark)	Diarrheoa, piles (Kala, *et al.,* 2004)
2	*Terminalia chebula*	Haritaki (Fruit)	Diarrheoa, indigestion, diabetes (Kala, 2005)
3	*Syzygium cumini*	Jam (Seed)	Diarrheoa, dysentery, piles, indigestion, diabetes (Maikhuri, *et al.,* 2000)
4	*Solanum nigrum*	Kakamachi (Fruit)	Diarrheoa, inflammation (Mukherjee, *et al.,* 1998)
5	*Picorrhiza kurroa*	Kutki (Rhizomes)	Cholera, diarrheoa, dyspepsia (Muthu, *et al*., 2006)
6	*Butea monosperma*	Palash (Flower)	Diarrhea, piles, inflammation, skin disease (Nautiyal, *et al*.,2000)

7	*Saraca. indica*	Ashok (Flower)	Gastrointestinal disorder (Maikhuri, *et al.,* 2000)
8	*Aegle marmelos*	Bel (Leaves)	Cholera, diarrheoa, gastritis, vomiting, diabetes (Maikhuri, *et al.,* 2000)
9	*Withania somnifera*	Aswagandha (root)	Diarrhea, dyspepsia, gastrointestinal disorder (Nautiyal, *et al*., 2000)

2.4 DESCRIPTION OF SECONDARY PLANT METABOLITES

Secondary metabolites are unique to plants or a group of plants, it was initially thought they were of use to a plant but morphine produced by poppies is of no use to the plant but is of use in medicine to help heal people who are suffering. Some of these, such as the fat and oils are important food reserves that are deposited in specialized tissues and cells only at certain times in the life cycle.

Essential oils are important agro-based industrial products. They find wide application in various types of industries, manufacturing food products, beverages, pharmaceuticals, cosmetics and paints. The essential oils are also known as ethereal oils and defined, as the oils obtained by steam distillation of plants (Bernthsen, 1941). Some what more detailed is the definition of Parry (1922) who states that for all practical purposes, the essential oils may be defined as odouriferous bodies of an oily nature, obtained almost exclusively from plant sources, generally liquid at ordinary temperature and volatile without decomposition. They are actually a group of heterogeneous volatile fragrant compounds found especially in plants. The term essential oils are misleading (Anderson, 1953) as it suggests that these compounds are very important to the plants in which they occur. Actually the name is derived from the fact that they are the substances found in essences or volatile compounds, which give many plants their characteristic odour.

2.5 Mode of action of Plant secondary metabolites

The antimicrobial properties of essential oils and their components have been reviewed in the past (Koedam, 1977a, b; Shelef, 1983; Nychas, 1995), the mechanism of action has not been studied in great detail (Lambert, *et al.,* 2001). Considering the large number of different groups of chemical compounds present

in EOs, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell (Skandamis, *et al.,* 2001; Carson, *et al.,* 2002). The locations or mechanisms in the bacterial cell thought to be sites of action for EO components are indicated in Fig. 2.2. Not all of these mechanisms are separate targets; some are affected as a consequence of another mechanism being targeted.

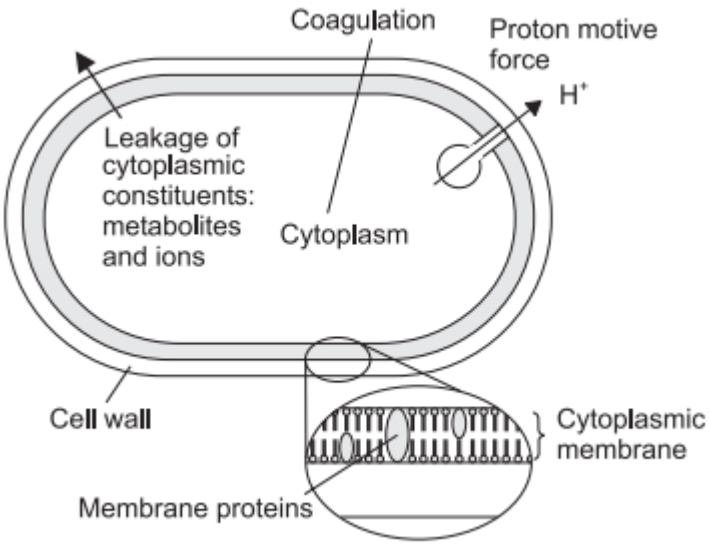


Fig. 2.2: Antimicrobial activity of oil at bacterial cell

An important characteristic of EOs and their components is their hydrophobicity, which enables themto partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering those more permeable (Knobloch, *et al.,* 1986; Sikkema, *et al.,* 1994). Leakage of ions and other cell contents can then occur (Oosterhaven, *et al.,* 1995; Gustafson, *et al.,* 1998; Helander, *et al.,* 1998; Cox, *et al.,* 2000; Lambert, *et al.,* 2001; Skandamis, *et al.,* 2001; Carson, *et al.,* 2002; Ultee, *et al.,* 2002). Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, exten- sive loss of cell contents or the exit of critical molecules and ions will lead to death (Denyer and Hugo, 1991a). There is some evidence from studies with tea tree oil and E. coli that cell death may occur before lysis (Gustafson, *et al.,* 1998).

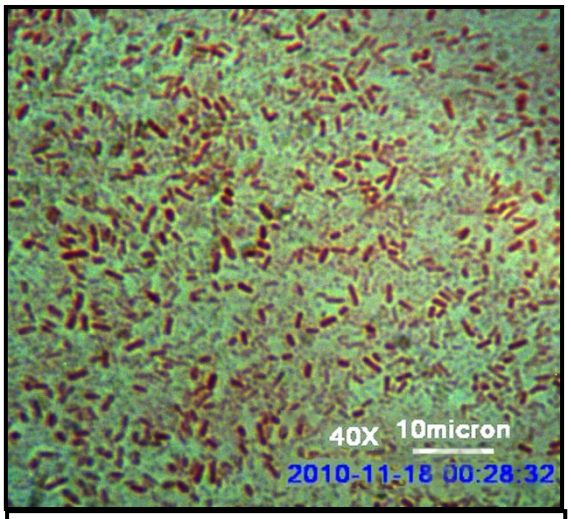
2.6 Systemic position of disease causing water borne bacterial pathogens

The drinking water is acceptably one of the major vehicles of transmission several infectious diseases affecting humans mainly bacterial infections which are widely widespread in developing countries (WHO, 2009). According to Bergey's Manual of Systematic Bacteriology, Second Edition, 2001 (Edited by George M. Garrity, 2001 springer-verlag, New York) the general characteristics of tested pathogens are summarized as:

2.6.1 *Escherichia coli*

Classification

Domain - Bacteria
Phylum - Proteobacteria
Class - Gammaproteobacteria
Order - Enterobacteriales
Family - Enterobacteriaceae
Genus - ***Escherichia***
Species - ***coli***



Escherichia coli

Escherichia coli commonly abbreviated E. coli; named after Theodor Escherich (1885) is a Gram-negative rod-shaped bacterium that is commonly present in large numbers in the normal intestinal flora of humans and animals, where it generally causes no harm. However, in other parts of the body *E. coli* can cause serious disease, such as urinary tract infections, bacteraemia and meningitis. A limited number of enteropathogenic strains can cause acute diarrhoea. Several classes of enteropathogenic *E. coli* have been identified on the basis of different virulence factors. *E. coli* is facultative anaerobic and non-sporulating. Cells are typically rod-shaped and are about 2 micrometres (μm) long and 0.5 μm in diameter, with a cell volume of 0.6 - 0.7 $(\mu m)^3$.

Human health effects

Virulent strains, such as pathogenic *E. coli* cause diarrhoea that ranges from mild and non-bloody to highly bloody, which is indistinguishable from haemorrhagic

colitis. Between 2% and 7% of cases can develop the potentially fatal haemolytic uraemic syndrome (HUS), which is characterized by acute renal failure and haemolytic anaemia. According to WHO (2008), children under 5 years of age are at most risk of developing haemolytic uraemic syndrome. The infectivity of this *E.coli* strains is considerably higher than that of the other strains. As few as 100 *E.coli* trains can cause infection. The region behind cause of diseases is that, it produces heat-labile or heat-stable *E. coli* enterotoxin or both toxins simultaneously, and is an important cause of diarrhoea in developing countries, especially in young children. Symptoms of *E.coli* infection include mild watery diarrhoea, abdominal cramps, nausea and headache. Infection with *E.coli* has been associated with severe, chronic, non-bloody diarrhoea, vomiting and fever in infants. Enterohaemorrhagic infections are rare in developed countries, but occur commonly in developing countries, with infants presenting with malnutrition, weight loss and growth retardation. Enterohaemorrhagic causes watery and occasionally bloody diarrhoea where the mechanism similar to that of Shigella. These virulent strains typically cause a bout of diarrhoea that is unpleasant in healthy adults and is often lethal to children in the developing world

Source and occurrence

E. coli normally colonizes an infant's gastrointestinal tract within 40 hours of birth, arriving with water or food or with the individuals handling the child. *E. coli* are enteric organisms, it can be spread through contact with cattle and sheep and, to a lesser extent, goats, pigs and chickens but the major source was contaminated drinking water. The latter have also been associated with raw vegetables, such as bean sprouts. *E. coli* are not always confined to the intestine, and their ability to survive for brief periods outside the body makes them an ideal indicator organism to test environmental water samples for fecal contamination.

Routes of exposure

Infection is associated with person-to-person transmission, consumption of contaminated water, contact with animals and food. Person-to-person transmissions are particularly prevalent in communities where there is close contact between individuals, such as nursing homes and day care centres.

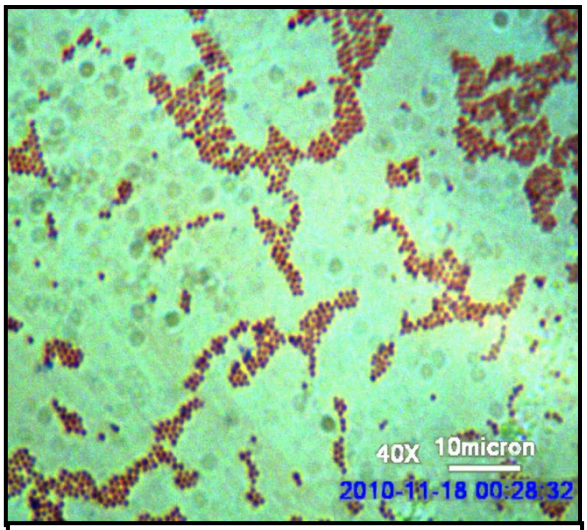
Significance in drinking-water

Waterborne transmission of pathogenic *E. coli* has been well documented for recreational waters and contaminated drinking-water. A well publicized waterborne out-break of illness caused by *E. coli* O157:H7 occurred in the farming community of Walkerton in Ontario, Canada. The outbreak took place in May 2000 and led to 7 deaths and more than 2300 illnesses. The drinking-water supply was contaminated by rainwater runoff containing cattle excreta. Within a WSP, control measures that can be applied to manage potential risk from enteropathogenic *E. coli* include protection of raw water supplies from animal and human waste, adequate treatment and protection of water during distribution.

2.6.2 *Vibrio cholerae*

Classification

Domain - Bacteria
Phylum - Proteobacteria
Class - Gammaproteobacteria
Order - Vibrionales
Family - Vibrionaceae
Genus - ***Vibrio***
Species - ***cholerae***



Vibrio cholerae

The cholera causing *Vibrio cholerae* is small, curved (comma-shaped), Gram-negative bacteria with a single polar flagellum. There are a number of pathogenic species, including *V. cholerae, V. parahaemolyticus* and *V. vulnificus*. *Vibrio cholerae* is the only pathogenic species of significance from freshwater environments. While a number of serotypes can cause diarrhoea, only O1 and O139 currently cause the classical cholera symptoms in which a proportion of cases suffer fulminating and severe watery diarrhoea. Strains of *V. cholerae* O1 and O139 that cause cholera produce an enterotoxin (cholera toxin) that alters the ionic fluxes across the intestinal mucosa, resulting in substantial loss of water and electrolytes in liquid stools (WHO,

2008). Other factors associated with infection are an adhesion factor and an attachment pilus.

Human health effects

Cholera outbreaks continue to occur in many areas of the developing world. Symptoms are caused by heat-labile cholera enterotoxin. The initial symptoms of cholera are an increase in peristalses followed by loose, watery and mucus-flecked "rice-water" stools that may cause a patient to lose as much as 10–15 litres of liquid per day. Decreasing gastric acidity by administration of sodium bicarbonate reduces the infective dose of *V. cholerae.* The case fatality rates vary according to facilities and awareness. About 60% of untreated patients may die as a result of severe dehydration and loss of electrolytes, but well-known diarrhoeal disease control programmes can be reduce fatalities to less than 1% (WHO, 2008).

Source and occurrence

The toxigenic *V. cholerae* is widely distributed in water environments. Humans are a traditional source of toxigenic *V. cholerae;* in the presence of disease, the organism can be detected in fresh water as well as sewage. The disease causing *V. cholerae* has also been found in association with live copepods as well as other aquatic organisms, including molluscs, crustaceans, plants, algae and cyanobacteria. Some times *V.cholerae* has been isolated from birds and herbivores in areas far away from marine and coastal waters. The occurrence of *V.cholerae* decreases as water temperatures fall below 20 °C.

Routes of exposure

The Cholera is mainly transmitted by the faecal–oral route, and the infection is primarily contracted by the ingestion of faecally contaminated water and food. The most important source of transmission of *Vibrio cholerae* is contaminated drinking water followed by food (contaminated during or after preparation e.g. milk, cooked rice, eggs, chicken, potatoes etc) and fruits and vegetables (WHO, 2000). The high numbers required to cause infection make person-to-person contact an unlikely route of transmission. A clinical case or an asymptomatic carrier and the environment in

which the sources of drinking water supply plays the most important role to transmission of cholera (WHO, 1970).

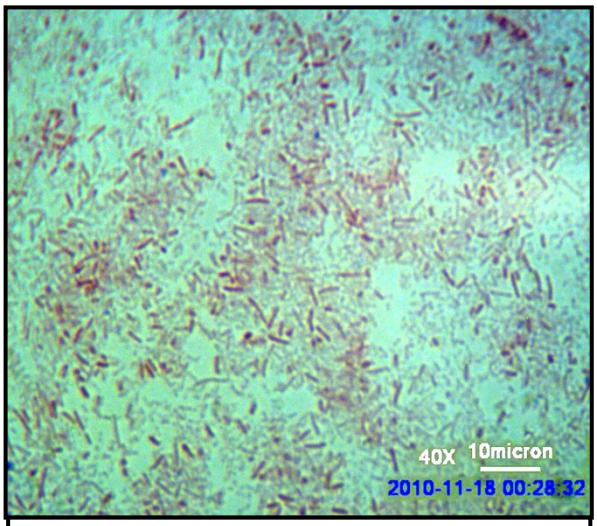
Significance in drinking-water

The contamination of water due to poor sanitation is largely responsible for transmission, but this does not fully explain the seasonality of recurrence, and factors other than poor sanitation must play a role. The presence of the pathogenic *V. cholerae* in drinking-water supplies is of major public health importance and can have serious health and economic implications in the affected communities. It is highly sensitive to disinfection processes.

2.6.3 *Salmonella typhimurium*

Classification

Domain - Bacteria
Phylum - Proteobacteria
Class - Gammaproteobacteria
Order - Enterobacteriales
Family - Enterobacteriacae
Genus - ***Salmonella***
Species - ***typhimurium***



Salmonella typhimurium

The *Salmonella typhimurium* belong to the family Enterobacteriaceae. They are motile, Gram-negative bacilli that do not ferment lactose, but most produce hydrogen sulfide or gas from carbohydrate fermentation. It is now considered that the classification of species level showed that there are actually no more than 2–3 species (*Salmonella enterica* or *Salmonella choleraesuis*, *Salmonella bongori* and *Salmonella typhi*), with the serovars being subspecies.

Human health effects

The infections of *Salmonella typhimurium* is typically cause four clinical manifestations: gastroenteritis (ranging from mild to fulminant diarrhoea, nausea and vomiting), bacteraemia or septicaemia (high spiking fever with positive blood cultures), typhoid fever / enteric fever (sustained fever with or without diarrhoea). Based on enteric illness, *Salmonella* spp. can be divided into two reasonably distinct

groups: the typhoidal species/serovars (*Salmonella typhi* and *S. paratyphi*) and the remaining non-typhoidal species/serovars. The symptoms of non- typhoidal gastroenteritis appear from 6 to 72h after ingestion of contaminated water. The diarrhoea lasts 3–5 days and is accompanied by fever and abdominal pain (WHO, 2008). Usually this disease is self-limiting. The incubation period for typhoid fever can be 1–14 days, but it is usually 3–5 days. It is a more severe illness and can be fatal.

Source and occurrence

The *Salmonella typhimurium* is widely distributed in the environment, but some species or serovars show host specificity. The *S. typhimurium* infect humans and also a wide range of animals, including poultry, cows, pigs, sheep, birds and even reptiles. The pathogens typically gain entry into water systems (supply water) through faecal contamination from sewage discharges, livestock and wild animals. Contamination has been detected in a wide variety of stored water, foods and milk.

Routes of exposure

The infectious *Salmonella typhimurium* is spread by the faecal–oral route. The infectious serovars are first and foremost connected with person-to-person contact (but it is uncommon). The main way of spreading of typhoid fever is consumption of a variety of contaminated water, foods and exposure to animals.

Significance in drinking-water

The waterborne typhoid fever outbreaks have distressing public health implications. The transmission, of *S. typhimurium*, has been associated with the consumption of contaminated water supplies and surface water supplies. The main illness associated with a communal rainwater supply, bird faeces were implicated as a source of contamination. The *Salmonella typhimurium* is relatively sensitive to disinfection.

2.6.4 *Shigella dysenteriae*

Classification

Domain - Bacteria

Phylum - Proteobacteria

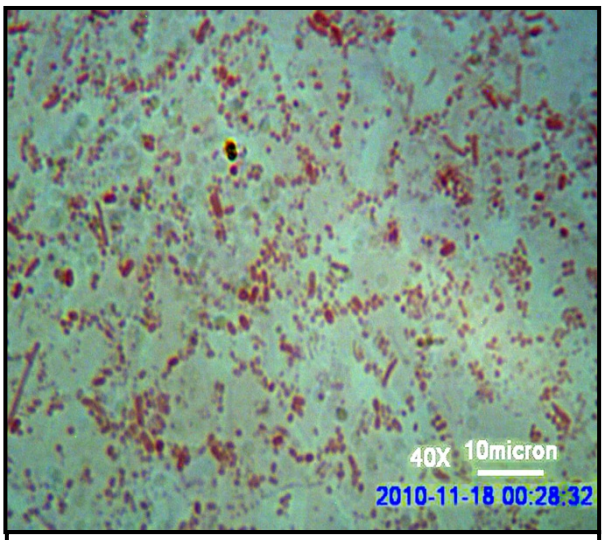
Class - Gammaproteobacteria

Order - Enterobacteriales

Family - Enterobacteriacae

Genus - ***Shigella***

Species - ***dysenteriae***



Shigella dysenteriae

The *Shigella dysenteriae* is Gram-negative, non-spore-forming, non-motile, rod-like members of the family Enterobacteriaceae; generally grow in the presence or absence of oxygen. There are four species: *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* which are very fatal for human health.

Human health effects

The *Shigella dysenteriae* causes serious intestinal diseases, including bacillary dysentery. Over 2 million infections occur each year, resulting in about 600 000 deaths, predominantly in developing countries (WHO, 2008). In most cases of *Shigella* infection occurs in children under 10 years of age. The incubation period for shigellosis is usually 24-72h. However, the ingestion of as few as 10-100 organisms may lead to infection, which is substantially less than the infective dose of most other enteric bacteria. Normally, abdominal cramps, fever and watery diarrhoea occur early in the disease. The infectious *S. dysenteriae*, clinical manifestations may proceed to an ulceration process, with bloody diarrhoea and high concentrations of neutrofils in the stool. The production of Shiga toxin by the pathogen plays an important role in this outcome.

Source and occurrence

The humans and other higher primates are the best fit natural hosts for *Shigella*. The bacteria remain localized in the intestinal epithelial cells of their hosts. The epidemics of shigellosis occur in crowded communities and where hygiene is poor and sources of water supllies contaminated with feacaly contaminated (WHO,

2008). The shigellosis is associated with day care centres, prisons and psychiatric institutions, Military field groups and travellers to areas with poor sanitation are also prone to infection.

Routes of exposure

The infection of *Shigella dysenteriae* (enteric pathogens) mostly transmitted by the faecal-oral route through person-to-person contact, contaminated water and food. The flies have also been identified as a good transmission vector from contaminated faecal waste.

Significance in drinking-water

There are a large number of waterborne outbreaks of shigellosis have been recorded. It is not particularly stable in water environments; their presence in drinking-water indicates recent human faecal pollution. The control of infection of *Shigella dysenteriae* in drinking-water supplies is of special public health importance in view of the severity of the disease caused. It is relatively sensitive to disinfection.

Materials and Methods

3.1 SAMPLE COLLECTION, ISOLATION AND PURIFICATION OF DISEASE CAUSING WATER BORNE BACTERIA

3.1.1 Experimental site and duration of study

The initial study was carried out in the Department of Horiticulture Aromatic and Medicinal Plants, Mizoram University, Aizawl, and molecular work carried out in the Biological Product Laboratory, Department of Botany, University of Allahabad, during the period extending from March 2009 to March 2011.

3.1.2 Cleaning and sterilization of glassware

Glassware which used in this study like, bottles, petriplates, pipettes, conical as well as round bottom flasks, culture tubes and beakers, etc were cleaned with suitable detergents or solution (Appendix 2). After that air dried the glassware wrapped with paper and then put it into oven at 180ºC for 3 hours to sterilize.

3.1.3 Area of sample collection

(i). Plain area (Allahabad)

The water samples were collected from different municipal water supply sources of Allahabad city (North central part of India) (Plate 1) these are:

- AU Campus
- Katra, Allahabad
- Daraganj, Allahabad.
- Kidganj, Allahabad.
- Chowk, Allahabad
- Tagore Town
- Civil Lines, Allahabad (A random site)

(ii). Hilly area (Mizoram)

From different municipal water supply sources the water samples collected from Aizawl (Capital of Mizoram State, North East region of India) and adjoining areas.

- MZU Campus- Tanhril, Zarkawt etc (Mizoram)
- Zarkawt
- Kulikown
- Ramhlun
- Chaltlang

3.1.4 Samples collection

The Water samples were collected randomly from different places around city. 200 ml water samples from each place of municipal water supply sources were collected in sterilized ziplock bag (plastic bag) or glass jar.

Sampling Procedures

- The water sample was taken from a distribution-system tap without attachments, select sites of tap that is supplying water from a service pipe directly connected with the main, and is not, for example, served from a cistern or storage tank.
- The tap was opened fully and water was allowed to flow for 2 or 3 min or for a time sufficient to permit clearing the service line. Reduced water flow was used for filling bottle without splashing. Tap cleanliness was also considered for collection of water sample.
- Removed the ziplock or stopper just before sampling and avoided touching the inside of the bag or cap.
- During sampling by hand, gloves were used and bottle was holded at its base. Plunged it (opening downward) below the water surface near to tap and then filled the required amount of water.
- The ziplock bag was handled carefully so that its opening did not touch to any other material during sampling because the surface film may contain greater numbers of fecal coliform bacteria which may contaminate the water sample.
- Sampling of thoses water samples was avoided, when municipal water supply was about to abondonned, because water remaining in the pipes already contained bacterial contaminants due to rusting or other sources.
- Some space in the sample container (an inch or so) was left while collecting samples, to allow mixing of the sample before-pipetting.

- Sampling from leaking taps that allow water to flow over the outside of the tap was avoided.
- After collection the ziplock opening or stopper was tightly closed.

The water samples were handled aseptically in sterile ziplock bag, labeled and kept in an ice-box during transportation to the Department of Horticulture Aromatic and Medicinal Plants, Mizoram University, Aizawl and Biological Product Laboratory at the Department of Botany in University of Allahabad.

Water temperature was measured at each water sampling site prior to sample collection. pH and turbidity were measured after samples arrived at the laboratory. The pH meter was rinsed with distilled water between each sampling. Samples were returned to the laboratory eight hours after collection and processed immediately.

3.1.5 Detection of Total Coliform

Detection of Coliform bacteria was performed using following methods:

Detection of water borne coliform bacteria completed with PA Coliform Kit (HIMEDIA).

Testing Procedure (the Quick and Easy way)

Kit contains-

1. One sterile disposable bottle-100 ml capacity,
2. Dehydrated medium-3X concentration,
3. One ziplock bag

How to use the Kit:

- Collected 200 ml water samples in ziplock bag carried out in laboratory as soon as possible.
- Then transfer the 100 ml water into the sterile disposable bottle under properly sterilized chamber.
- Now, add entire quantity of dehydrated medium (PA Broth) slowly to water by swirling to dissolve the powder completely.
- After dissolution, incubate the bottle for 24-48 hours at 30-35ºC.

- Observe the colour change of the medium from reddish purple to yellow.
- Colour change indicating the presence of Coliform bacteria.

Isolation of Bacteria:

- Firstly took the bottle which showed the presence of coilform bacteria.
- Prepared the petridishes containing the Nutrient agar.
- Dipped the sterilized cotton swabs into the bottle containing the Coliform bacteria.
- Now streaked directly on Nutrient Agar plate medium (Appendix 1).
- Incubate the plates at the incubation temperature of 35-37^{o}C for 24 houres.
- Different types of bacterial colony have seen onto the NA plate indicating the various types of Coliform bacteria present into the collected municipal water sample.

3.1.6 Detection of Total Coliform using Membrane filtration equipment:

Before use the Membrane filtration equipment, assemble filtration units and check for leaks. Discard units if inside surfaces are scratched. Wash and rinse filtration assemblies thoroughly after use, wrap in nontoxic paper or foil, and sterilize.

Membrane filtration equipment used as follows:

- Properly set the filtration unit after sterilization.
- Took collected tap water samples.
- Put100 ml of tap water into the upper flask of unit.
- Start the suction pump, which suck the air from inside of lower flask, during this process a pressure would be arised to filetr the water.
- Membrane filter having pore size 0.45 µm and 47mm diameter should be used for filtration.
- During filtration the bacterial contaminants stick on the upper side of the membrane filter.
- Now, the membranes were then transferred aseptically to Nutrient agar plates.
- Incubate these plates at 35-37^{o}C for 24 houres.
- Count the number of colony forming units and report per 250 ml or per 100 ml.

3.1.7 Purification of water borne bacterial isolates

The technique commonly used for isolation of discrete colonies initially required the number of organisms into the inoculum to be reduced. The resulting decrease in the population size ensures that in the following inoculation; individual cell would be sufficiently far apart on the surface of the agar medium to effect a separation of different species present. The techniques commonly employed to obtain pure culture are:

Streak plate method

It was rapid qualitative isolation method, essentially a dilution technique that involves spreading a loopful of culture over the surface of a nutrient agar plate (Fig. 3.1)

- First of all the loop was resterilized in a flame and touched to the end of streak A to collect a small amount of organisms.
- Again, the loop was dragged to attenuate the organisms and resterilized.
- Same procedure was successively repeated for streaks C and D.
- By the end of streak D, individual colonies should be apparent.
- After complition of streak the petriplates properly closed and kept into incubation chamber for 24 hrs at 37°C.

Then after the plates, were evaluated for cultural purity. Morphologically divergent colonies in the final streak were one sign that the colony isolated from the original spread plate was formed by more than one type of organism.

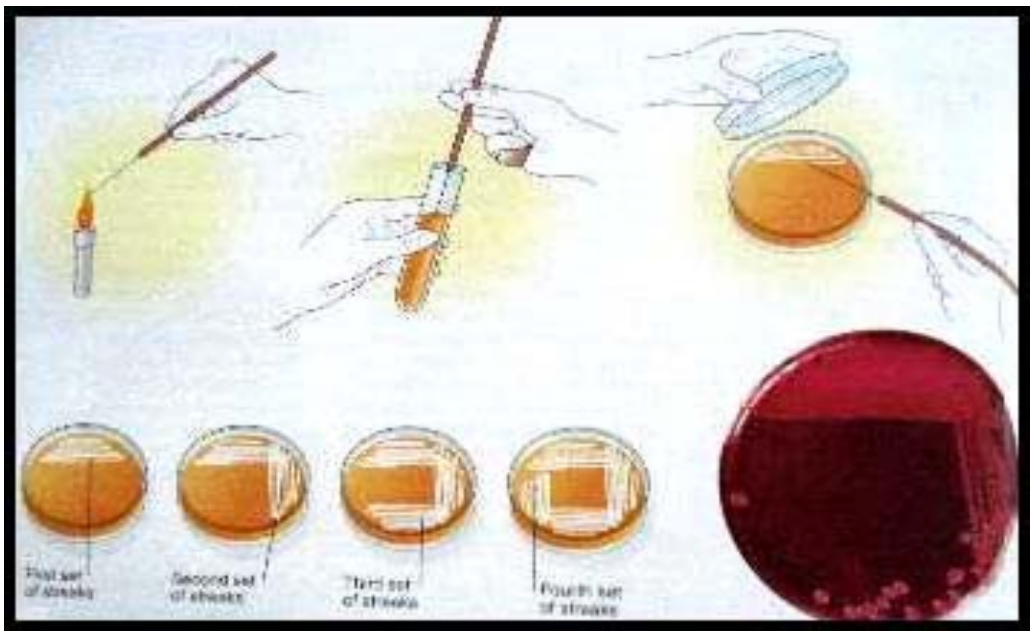


Fig. 3.1 Purification of water borne bacteria by streak plate method

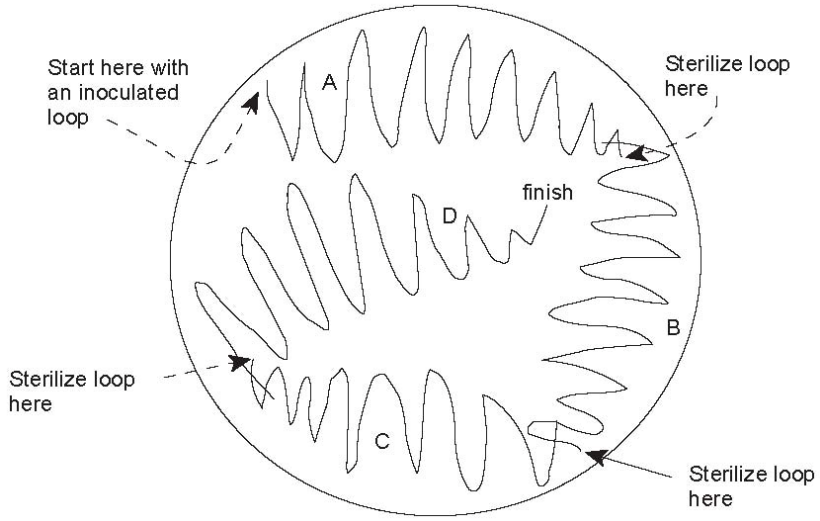


Fig 3.2: Isolating single colonies of a bacterium by making a streak plate.

Quadrant streak:

- Placed a loopful culture on the agar surface over area 1 near edge of the plate. Applied the loop lightly.
- Flamed the loop, cooled it and made 5 or 6 streaks from point 1 to 2

- Flamed the loop again, cooled it, and made 6 or 7 streaks from area 2 to area 3.
- Again flamed the loop and cooled then made as many streaks as possible from area 3 into area 4, using up the reminder of the plate surface.
- Now properly flamed the loop before putting it aside.
- Foe successes, it is important that the loop be sterilized between streaks (Let the loop cool so as not to kill the organisms on it.) Also, take care so as to catch the end of the previous streak, crossing it only once. Otherwise, too many organisms may adhrere to loop.

3.1.8 Revival of standard lyophilized culture of water borne bacteria from MTCC (Microbial type culture collection) Chandigarh, India, and ATCC (American type culture collection) Bangalore, India

1) ***Escherichia coli*** **MTCC 723**
2) ***Vibrio cholerae*** **MTCC 3906**
3) ***Salmonella typhimurium*** **MTCC 98**
4) ***Shigella dysenteriae*** **ATCC 23513**

- Care should be taken in opening the ampoule as the contents are in a vacuum.
- Marked on the ampoule near the middle of the cotton wool with a sharp file.
- Disinfected the surface around the mark with alcohol.
- Wraped thick cotton around the ampoule and broken at the marked area.
- Gently removed the pointed top of the ampoule. Snap opening will draw the cotton plug to one end; hasty opening will release fine particles of dried organisms in to the air of the laboratory.
- Carefully removed the cotton plug and about 0.3 to 0.4 ml of specified medium to make a suspension of the culture. Avoid frothing or creating aerosols.
- Streaked a few drops of the suspension on to recommended medium (solidified with agar) in a petriplate.
- Incubated at recommended temperature and conditions for the proper culture.
- If proper condition followed, showed the good results, growth of the culture should be visible within a few days.

- All the remains of the original ampoule should be treated as infected and autoclaved before discarding.

3.2 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF WATER BORNE BACTERIAL ISOLATES

On the basis different culture medium such as Nutirent agar, MacConkey agar and tripticase soya agar etc, the 19 isolates of *Escherichia coli* were isolated with one standard *Escherichia coli* MTCC 723, 9 isolates of *Vibrio cholerae* with one standard *V. cholerae* MTCC 3906, 16 isolates of *Salmonella typhimurium* standard is *S. typhimurium* MTCC 98 and 12 isolates of *Shigella dysenteriae* with one standard ATCC 23513 were obtained from the many different municipal water supply sources. The newly isolated water borne bacterial pathogens were identified through their cultural, morphological and biochemical characteristics (Mackie and McCartney, 1956) (Plate 3).

3.2.1 Culture characteristics

Water borne bacterial isolates were streaked on Nutrient agar as well as MacConkey agar, and Citrate agar for colony morphology (Plate 3 & 4).

3.2.2 Morphological characteristics

For the morphological study water borne bacterial isolates were subjected to Gram's stain and observed under 100X objective in oil immersion microscope (Plate-3).

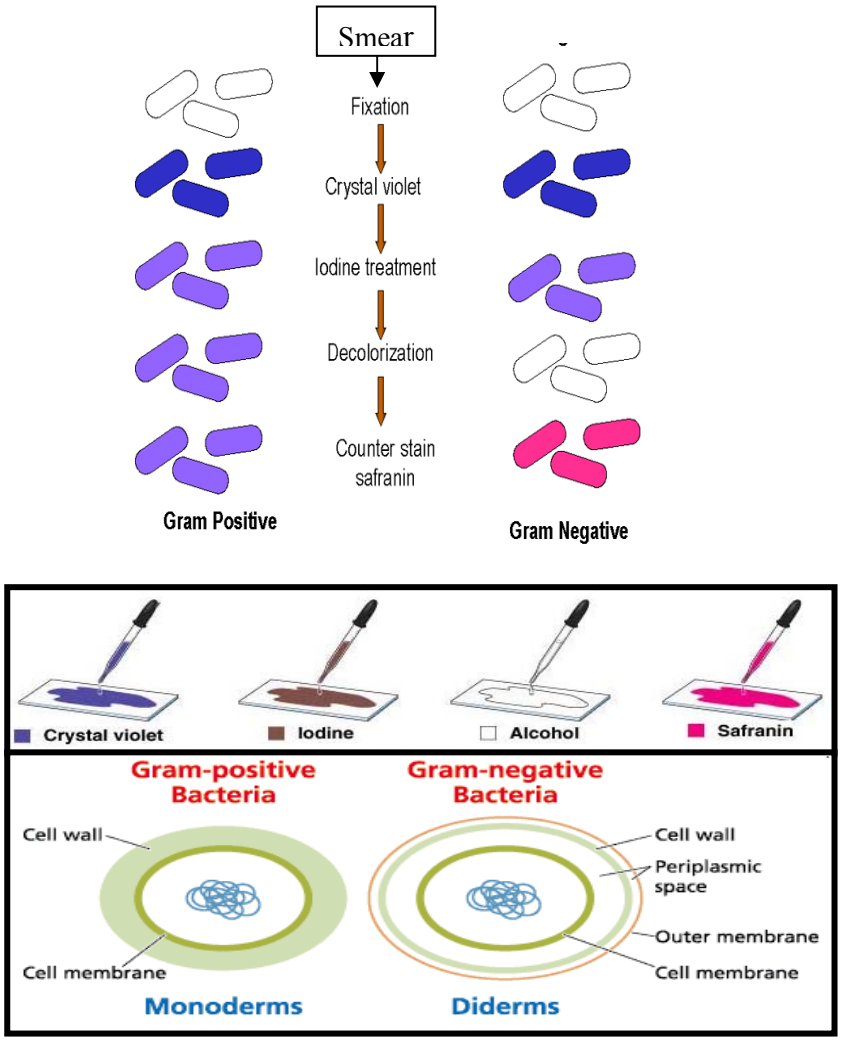


Fig. 3.3: General step in the Gram's stain procedure and the resultant staining of Gram-positive and Gram-negative bacteria.

3.3 BIOCHEMICAL CHARACTERISTICS

The biochemical technique was performed for the proper identification of the isolated bacterial pathogens as prescribed in Bergeys Manual of Systematic Bacteriology. The standards strains were taken for their reference. The types of biochemical tests were employed was elaborated below:

3.3.1 MacConkey agar test

MacConkey agar is used for the selective isolation and identification of Enterobacteriaceae from feces, urine, waste water and foods. It is also a selective and

diifferntial medium for the isolation of enteric gram-negative bacteria. The inocula of isolated as well as standard strains were streaked directoly on the medium or inoculated first into an enrichment agar such as MacConkey agar and incubate at 30-35°C for 18-24 hours, as indicated by the (Aneja, 2003).

3.3.2 Citrate utilization test

The medium contains the dye bromo-thymol blue which is blue in the alkaline and yellow in the acid. At neutral pH the dye is half yellow and half blue making a green color. The green color or the uninoculated medium is an optical illusion; there is no green. Citrate is supplied to the medium as sodium citrate, as the organism uses citrate, sodium ions remain in the medium thereby making the medium basic and ultimately a blue color develops due to utilization of citrate.

The inoculum of each bacterial isolates, were streaked over the surface of simmon citrate agar (Appendix 1.9) slants. The slants were incubated at 37°C for 24-48 hours. Appearance of blue colouration along the lines of growth was assigned as positive test and absence of coluration was assigned as negative test (Plate 4).

3.3.3 Urease degradation test

This test was performed by growing the test bacterial pathogens on urea agar medium containing the pH indicator phenol red (pH 6.8). The inoculum was inoculated in urease broth (Appendix 1.5) and then incubated at 37ºC for 24 to 48 hours. Appearance of pink colour on urease broth assigned positive test. No change in colour was assigned as negative test (Table 16).

3.3.4 Amylase production test (Starch hydrolysis)

The properly inoculated bacterial culture plates were incubated for 24-72 hours at 37ºC. In this test presence or absence of starch in the medium was determined by using iodine solution as an indicator. The typical positive starch hydrolysis reaction (i.e. Clear zone surrounding the bacterial colonies) by the production of exoenzyme amylase, which was diffused in the medium, surrounded the growth. A negative reaction showed coloration of the medium (Appendix 1.8) (Plate 4).

3.3.5 Haemolysis in blood agar

Blood agar (Appendix 1.9) is a differential medium used for the cultivation of tested bacterial pathogens. Then, observed the blood agar plates surrounding the colonies due to no lysis of RBC's indicates gamma haemolysis; appearance of greenish yellow surrounding the colonies due to the reduction of haemoglobin to methaemoglobin indicates alpha-haemolysis; and formation of a clear zone around the colonies due to complete destruction and use of haemoglobin by the bacteria indicates beta-haemolysis or frequently the pathogens (Plate 4).

3.3.6 KB003: Hi25^{TM} Enterobacteriaceae identification kit (Himedia)

The isolated bacterial cultures were identified with their biochemical profile and validated through standard strains such as *Escherichia coli* MTCC 723, *Vibrio cholerae* MTCC 3906, *Salmonella typhimurium* MTCC 98 and *Shigella dysenteriae* ATCC 23513 by using redymade biochemical identification test kit (Plate 5). The KB003: Hi25^{TM} Enterobacteriaceae identification kit includes:

1) ONPG (o–nitrophenyl-β–D-galactopyranoside)
2) Lysine utilization
3) Ornithine utilization
4) Urease
5) Phenylanine deamination
6) Nitrate reduction
7) H_2S production
8) Citrate utilization
9) Voges proskauer's
10) Methyl red
11) Indole
12) Malonate utilization
13) Esculin hydrolysis
14) Arabinose
15) Xylose
16) Adonitol
17) Rhaminose
18) Cellobiose
19) Melibiose
20) Sacchrose
21) Raffinose
22) Trehalose
23) Glucose
24) Lactose
25) Oxidas

(i) Voges proskauer's

Some organisms have the ability to produce a neutral end product acetyl methyl carbinol (acetoin) from glucose utilization. This can be detected by addition of 1-2 drops of Barritt Reagent A (R029) and 1-2 drops of Barritt Reagent B (R030). A positive test is indicated by pinkish red colour within 2-5 minutes. No change in colour indicates negative test (Appendix 2.3).

(ii) Esculin hydrolysis

The esculin test is a measure of the ability of an organism to hydrolyze the glycoside esculin to esculetin and glucose. Esculetin (6, 7- dihydroxycoumarin) will react with iron salts to form a black precipitate. The production of a black color in the presence of iron salts, for example ferric citrate, is a positive reaction. A tan or light gray color is a negative result.

(iii) Indole test

In this test inoculums of each bacteria inoculated into trptone broth, indole production during the reaction is detected by adding the Kovac's reagents (dimethylaminobenzaldehyde) which produces a cherry-red reagent layer. Here the tryptophan, an essential amino acid is oxidized by some bacteria by the enzyme tryptophanase resulting in the formation of indole, pyruvic acid and ammonia

(iv) ONPG test

Two enzymes, Permease and β-galactosidase are required for Lactose fermentation. True non-lactose fermenters are devoid of both enzymes; however some organisms may lack permease but possess the enzyme β- galactosidase. ONPG (o–nitrophenyl -β –D- galactopyranoside) is structurally similar to lactose. In the presence of β-galactosidase, ONPG is cleaved into galactose and o-nitrophenol, a yellow compound. Since members of Enterobacteriaceae are routinely grouped according to their abilities to ferment lactose, the ONPG test is especially useful in rapidly identifying cryptic lactose fermentation. Development of a yellow colour, when ONPG Discs (DD008) is placed on 8 - 24 hours growth and incubated further for a minimum of 1 hour at 35°C, indicates positive reaction.

3.3.7 KB009 HiCarbohydrate^{TM} Kit (Himedia)

The isolated bacterial culture were identified with help their reference standard strains of *Escherichia coli* MTCC 723, *Vibrio cholerae* MTCC 3906, *Salmonella typhimurium* MTCC 98 and *Shigella dysenteriae* ATCC 23513 using redymade test kit (Plate 6). KB009 HiCarbohydrate^{TM} Kit includes:-

1) Lactose
2) Xylose
3) Maltose
4) Fructose
5) Dextose
6) Galactose
7) Raffinose
8) Trehalose
9) Melibiose
10) Sucrose
11) L-Arabinose
12) Mannose
13) Insulin
14) Sodiumgluconate
15) Glycerol
16) Salicin
17) Dulcitol
18) Inositol
19) Sorbitol
20) Mannitol
21) Arabitol
22) Erythritol
23) α–Methyl-D-glucoside
24) Rhaminose
25) Cellobiose
26) Melezitose
27) α–Methyl-D-mannoside
28) xylitol
29) ONPG
30) Esculin hydrolysis
31) D-Arabinose
32) Citrate utilization
33) Malonate utilization
34) Sorbose

3.3.8 Carbohydrate utilization test

Each HiCarbohydrate^{TM} Kit is a standrized colorimetric identification system utilizing thirty five carbohydrate utilization tests. Specific carbohydrates were added to basal media, which contains phenol red as indicator. On fermentation of carbohydrate, acid was liberated which lowers down the pH of medium and this change of colour was indicated by pH indicator dye. Positive test was indicated by colour change to yellow due to acid reaction. No change in colour or red/pink colour indicates negative reaction.

3.4 MOLECULAR CHARACTERIZATION OF WATER BORNE BACTERIAL ISOLATES

The isolated bacterial strains from tap water (municipal supply sourses) were carried out under molecular characterization as well as boichemical characterizations were made with each strandard strains of bacterial pathogens.

3.4.1 Genomic DNA extraction Kit *AccuPrep*®

AccuPrep® Genomic DNA Extraction Kit rapidly and conveniently extracts an average of 6µg of total DNA from 10^4-10^8 bacterial cultured cells. *AccuPrep*® Genomic DNA Extraction Kit employs glass fibers, fixed in column that specifically binds DNA in the presence of a chaotropic salt. Proteins and other contaminants are eliminated through a series of short wash and spin steps. Finally, genomic DNA is eluted by a low salt solution. The process do not require phenol/chloroform extraction, alcohols precipitation, or other burden some steps.

Advantages:

- The DNA was extracted promptly and more conveniently.
- For improving the efficiency and reproducibility of PCR the contaminants, such as proteins and nucleases was completely removed, because, it may interfere with PCR reactions.
- Avoid precipitation and used of organic solvent to minimized the damage of DNA.

- Now, isolated DNA was ready to use in various applications or stored at 4^{o}C for further use.

Extraction of bacterial genomic DNA by using Kit *AccuPrep*® (Appendix 2.5)

- Added 20µl of Proteinase K to a clean 1.5 ml tubes.
- Applied 200µl of 10^{4}-10^{8} cultured cells to the tube containing proteinase K.
- (If the sample volume was less than 200µl, make the total volume 200µl by adding Phosphate Buffer Saline).
- Added 200µl of Binding buffer (GC) to the sample and mixed immediately by vortex mixer. (You must completely resuspend the sample to achived maximum lysis efficiency).
- Incubated at 60ºC for 10 min.
- Added 100µl of Isopropanol and mix well by pippetting.
- (After this step, briefly spin down to get the drops clinging under the lid. Don't vortex as that might reduced DNA yield).
- Carefully transferred the lysate in to the upper reservoir of the Binding column tube (fit in a 2 ml tube) with out wetting the rim.
- Closed the tube and centrifuged at 8,000 rpm for 1 min. must close each binding column tube to avoid aerosol formation during centrifugation, centrifuged again at a higher speed (>100, 000 rpm) untill the binding column tube was empty.
- Open the tube and transferred the Binding column tube to a new 2ml tube for filtration.
- Added 500 µl of Washing buffer 1 (W1) without wetting the rim, closed the tube, and centrifuged at 8, 000 rpm for 1 min.
- Open the tube and poured the solution from the 2ml tube in to a disposal bottle.
- Carefully added 500 µl of Washing buffer 2 (W2) without wetting the rim, close the tube, and centrifuged at 8, 000 rpm for 1 min.

- Centrifuged once more at 12,000 rpm for 1 min to completely remove ethanol, and checked that there was no droplet clinging to the bottom of Binding column tube.
- (Residual W2 in the Binding column tube may cause problems in later application)
- Transferred the Binding column tube to a new 1.5 ml tube for elution (supplied), add 200µl of Elution buffer (EL, or nuclease-free water) onto Binding column tube, and waited for at least 1 min. at RT (15-25 ºC) until EL completely absorbed in to the glass fiber of Binding column tube.
- (To increase DNA yield, you should wait for 5 min after adding Elution buffer (EL). The volume of EL added can be adjusted from 50µl to 100µl. A smaller volume will result in a concentrated solution, but total yield may be reduced.)
- Centrifuged at 8, 000 rpm for 1 min to elute.
- (About 180µl-200µl of eluent was obtained when using 200µl of Elution buffer (or nuclease-free water). For an improved yield, eluted the sample twice and use concentration process.)

The eluted genomic DNA was stable and was used directely, or stored at 4ºC for further analysis. The to be storage for DNA long-term, then eluted with Elution buffer (EL) and stored at -20ºC, because DNA stored in water was subject to acid hydrolysis.

About 6µl of DNA in 200µl of eluent (30 mg/µl) with an A260/A280 ratio of 1.6-1.9 were typically obtained from 200µl of bacterial culture (-5x 10^6).

3.4.2 PCR analysis

The PCR analysis was employed to know the species-specific identification of biochemically characterized 10 isolates of water borne bacterial pathogens. The primers and probes were desined by the Genome diagonistic Pvt. Ltd. New Delhi. The primer sequences were used in research, as follows:

Sequences primer pairs for *Escherichia coli* MTCC 723

E. coli rfbE gene 239 bp F: GTGCTTTTGATATTTTTCCGAGTAC

R: TTTATATCACGAAAACGTGAAATTG

Sequences primer pairs for *V.cholerae* MTCC 3906

V. cholerae ctx gene 432 bp F: ATTTGTTAGG CACGATGATG

R: ATCGATGATCTTGGAGCATTC

Sequences primer pairs for *Salmonella typhimurium* MTCC 98

S. typhimurium STM4497 gene 360 bp F: TTAACGAGGATTCAATGTCG

R: TTATTTGACCGCGTCTGTCA

Sequences primer pairs for *Shigella dysenteriae* ATCC 23513

S. dysenteriae ipaH gene 884 bp F: CCTTGACCGCCTTTCCGATA

R: AATCAGTTTTCCCGATGCAG

3.4.2 (a) Amplification of water borne bacterial isolates through Real Time PCR

Each PCR reaction consisted of 10 mM Tris-HCl buffer (pH 8.3), 1.5mM $MgCl_2$, 50mM KCl, 200µM each of dATP, dTTP, dGTP, and dCTP or use 2.5X Universal Hot Start PCR Premix Cat. No. 110145 (Professional Biotech India Ltd., New Delhi) description was given below. 1µM primers 1U *Taq* DNA polymerase and 2µl of supernatant from the DNA extraction.

The conditions for was PCR as follows:

- Denaturation at 95°C for 30 seconds,
- Annealing at 59ºC for 30 seconds, and
- Extension at 72ºC for one minute.

- The amplification process was repeated for 25 cycles by using a machine the Roter gene 6600, Corbett Research, Australia.

2.5X Universal Hot Start PCR-PREMIX for PCR/Real Time PCR

(Mg final Concentration 2.5mM): Cat No.110145

The 2.5 X Hot Start PCR Pre-mix (1 ml × 10, for 500 reactions of 50µl) was an optimized; ready to use PCR mixture of Hot Start *Taq* DNA Polymerase, PCR buffer, $MgCl_2$ and dNTP's. The 2.5 X Hot Start PCR pre-mix contains all components for PCR amplification, Real Time PCR applications, except DNA template and primers. It was tested for absence of endo nucleases and exo nucleases and also tested for amplification of single gene copy.

Molecular grade water

It was supplied in 1.5 ml × 10 quantities, which were RNAs and DNAs free. It was advised to aliquot the molecular grade water.

Storage Conditions

The recommended stored temperature was -20 °C.

Table 3.1: Dilution procedures of various gradients for amplification of RT-PCR.

Components	50 µl reaction Volume		25 µl reaction volume	
	Volume	**Final Conc.**	**Volume**	**Final Conc.**
2.5 X Hot Start PCR Premix	20 µl	1 X	10 µl	1 X
Forward primer	Variable	0.1-1 µM	Variable	0.1-1 µM
Reverse Primer	Variable	0.1-1 µM	Variable	0.1-1 µM
Template DNA	Variable	10pg-1 µg	Variable	10pg-1 µg
Sterile Deionized water	Up to 50 µl	----	Up to25 µl	----

-- It was advisable to use 20 µl of extracted template DNA in case low yield was expected for 50 µl reaction volumes. In case of High yields the volumes were adjusted as per the yield of DNA.

3.4.2 (b) Agarose Gel Electrophoresis

The authenticity of DNA was judged through gel analysis in following steps.

- The 100 ml Agarose gel (1.0%) was casted in 0.5X TBE (Tris Borate EDTA) buffer containing 0.5 µl/ml of Ethidum Bromide. (Appendix 2.6)
- In each well, 2 µl of DNA per sample was loaded.
- For each gel, a control marker consisting of a 100 bp ladder was used for size verification.
- Electrophoresis was conducted at about 50 V for 1 hr.
- After electrophoresis, DNA was visualised under light using transilluminator (Gel Documentation system, Model- Bio-vision Vilber Lourmate, France).
- The visible DNA images were captured with attached camera to the system.
- The presence of single compact band at the corresponding position of 100 bp DNA fragment ladder indicates high molecular weight of the isolated DNA.
- The images were saved for the further molecular analysis.

3.4.3 Validation of water borne bacterial isolates by Real Time PCR

The validation of identified different water borne bacterial isolates using TaqMan RT-PCR assay for complete and the virtual quantification.

3.5 PHYLOGENY OF TESTED BACTERIA

3.5.1 Role of Bioinformatics

The *in silico* study was conducted for evolutionary relationship of above 4 tested water borne bacterial pathogens on the basis of their toxin production as well as some common protein which are responsible for the vital activities such as enzyme production, toxin production etc. This work was carried out with help of Bioinformatical Tool.

In the present, *in-silico* prediction study, first of all Protein sequences was collected from National Center for Biotechnology Information (NCBI) database on the basis of toxin production and common protien. The protein sequences of each bacterial pathogen considered as the base, number of protein sequences collected from NCBI database. Now, collected protein sequences inserted into FASTA format were cladogram as well as phylogram of each pathogens analysed through computer based

programme commonly known as ClustalW (EBI) for prediction of phylogenetic relationship.

The protein sequences of E.coli was as follows:

>gi|47155005|emb|CAE85204.1| YeeV protein [*Escherichia coli*]
MNTLPDTHVREASRCPSPVTIWQTLLTRLLDQHYGLTLNDTPFADERVIEQHIEAGISLCDAVN
FLVEKYALVRTDQPGFSACTRSQLINSIDILRARRATGLMTRDNYRMVNNITQGKHPEAQQ

On the basis of **YeeV protein** of *Escherichia coli* several strains of *Vibrio cholerae*, *Salmonella typhimurium*, and *Shigella dysenteriae* were found and with help of above process construct the Phylogram and Cladogram to know the Phylogeny (evolutionary tree) of pathogens.

The protein sequences of tested bacterial pathogen are as follows:

>gi|15601778|ref|NP_233409.1| glucosamine-6-phosphate deaminase [*Vibrio cholerae* O1 biovar eltor str. N16961]
MRLIPLKAAAQVGKWAAAHIVKRINEFQPTAERPFVLGLPTGGTPLATYKALIEMHKAGEVSF
KHVVTFNMDEYVGLAADHPESYRSFMYNNFFNHIDIQEENINLLNGNTDDHEAECKRYEDKI
KSYGKINLFMGGVGNDGHIAFNEPASSLSSRTRIKTLTEDTRIANSRFFDGDINQVPKYALTIGV
GTLLDAQEIMILVTGHNKALALQAAVEGSVNHLWTVSALQLHPKAVIVCDEPSTQELKVKTV
KYFTELEAKNIVGF

>gi|153212201|ref|ZP_01947996.1| glucosamine-6-phosphate isomerase [*Vibrio cholerae* 1587]
MRLIPLKAAAQVGKWAAAHIVKRINEFQPTAERPFVLGLPTGGTPLATYKALIEMHKAGEVSF
KHVVTFNMDEYVGLAADHPESYRSFMYNNFFNHIDIQEENINLLNGNTDDHEAECKRYEDKI
KSYGKINLFMGGVGNDGHIAFNEPASSLSSRTRIKTLTEDTRIANSRFFDGDINQVPKYALTIGV
GTLLDAQEIMILVTGHNKALALQAAVEGSVNHLWTVSALQLHPKAVIVCDEPSTQELKVKTV
KYFAELEAKNIVGF

>**gi|254285300|ref|ZP_04960265.1| putative transcriptional regulator ToxR [*Vibrio cholerae* AM-19226]**
MYKIDQKILSSDSPFLTDSISREQIKLGTHEHLVLLALCEQAGKLIDKETLIKKGWPGKFVTDSS
LTQAVRNIRAYLNDDGKSQKHIKTIAKKGYLIESKFVTLIQDFDKNQISNLQQETKKESFGKKY
ILFLSILVQLLFIIYSTYKIYPITTVAIDKEIYPILSYQQDYIYIYSDDFQYSEQLGVELIHSLSAEGI
SPERLYIMLNKETVSYSFIEKNGKSKNRIIFTAGTKDYKKIREHIINEIKI

>gi|229515146|ref|ZP_04404606.1| hypothetical protein VCB_002803 [*Vibrio cholerae* TMA 21]
MISKMYKIDQKILSSDSPFLTDSISREQIKLGTHEHLVLLALCEQAGKLIDKETLIKKGWPGKFV
TDSSLTQAVRNIRAYLNDDGKSQKHIKTIAKKGYLIESKFVTLIQDFDKNQISNLQQETKKESF
GKKYILFLSIFVQILFIIYSINKIYPITTVAIDKEIYPILSYQQDYIYIYSDDFQYSEQLGVELIHSLS
AEGISPERLYIMLNKETVSYSFIEKNGKSKNRIIFTAGTKDYKKIREHIINEIKI

>gi|254224921|ref|ZP_04918536.1| putative transcriptional regulator ToxR [*Vibrio cholerae* V51]
MYKIDQKILSSDSPFLTDSLSREQIKLGTHEHLVLLALCEQAGKLIDKETLIKKGWPGKFVTDSS
LTQAVRNIRAYLNDDGKSQKHIKTIAKKGYLIESKFVTLIQDFDKNQISNLQQETKKESFGKKE
SFGKKYILFLSILVQLLFIIYSIYKIYPITTVAIDKEIYPILSYQQDYIYIYSDDFQYSEQLGVELIHS
LSAERISPERLYIMLNKETVSYSFIEKNGKSKNRIIFTAGTKDYKKIREHIINEIKI

>gi|153830704|ref|ZP_01983371.1| iron(III) ABC transporter, periplasmic iron-compound-binding protein [*Vibrio cholerae* 623-39]
MCLMSLWNVRLRNICRSKAVFGWFAALCLFALPVQAQIVLTDSQGTHTFAEVPQRVVVLNW
DLLEQVLELGIQPVGAPELSSYAQWVVQPALPSSVQDIGTRTEPNLEKIAALKPDVILAAGPQQ
DLLATLGRIAPVVYLPNFSEQDNAAQVAISHFKTLATLFGQEAVAQQKLEAMYARFAKLKAS
LQHAFGDTLPAVVTLRFANPTSVFLYTENSTPQYVLEHLGLSSALPQPPKEWGIVQKRLSELQH
VEQGYVLYFLPFAEEKKVQKSVLWRAMPFVQAGRVNSVRSVWSYGGAMSLRYSAEAITESL
LAVAPQS

>gi|153800826|ref|ZP_01955412.1| iron(III) ABC transporter, periplasmic iron-compound-binding protein [*Vibrio cholerae* MZO-3]
MCLMSLWNVRLRNICQPKAVFGWLAALCLFALSVQAQIVLTDSQGTHTFAEVPQRVVVLNW
DLLEQVLELGIQPVGAPELSSYVQWVVQPEVPSSVQDIGTRTEPNLEKIAALKPDVILAAGPQQ
DLLATLGRIAPVVYLPNFAEQDNAAQVAISHFKTLATLFGKEAVAQQKLEAMYARFAELKAS
LQHAFGDTLPAVVTLRFANPTSVFLYTENSTPQYVLEHLGLSSALPQPPKEWGIVQKRLSELQH
VEQGYVLYFLPFAEEKKVQKSVLWRAMPFVQAGRVNSVRSVWSYGGAMSLRYSAEAITESL
LAVAPQS

>gi|229529289|ref|ZP_04418679.1| peptide transport system permease protein SapB [*Vibrio cholerae* 12129(1)]
MFVYTVRKFNLFIITLLILTMVGHSLARFDPHSPWTLIGFWQGWSSYLVQLMEFNFGLNKNGV
PIIHELLVVFPATIELCTIAFILSLLVGIPIGTLAGMRQGKWLDTIISFISMSGYSAPIFWLALMMI
MAFSLHFPVFPVAGRYDLLYQIDHVTGFALIDAFLSQSPYRSQALQSVIEHLTLPCLVLALAPTT
QVIGQMRASVAEVMNQNYIRAAKIKGLSNYQIVTQHVLRNAIPPMIPKFGVQLSSMLTLAIITE
SIFNWPGIGRWLLDALANRDFMSIQAGVIVVGTLVLTANILSDLIGAAANPLVRKEWYVKR

>gi|121730203|ref|ZP_01682592.1| transcription termination factor Rho [*Vibrio cholerae* V52]
RGDPTGSITRLARAYNTVVPASGKVLTGGVDANALHRPKRFFGAARNVEEGGSLTIIATALVD
TGSKMDEVIYEEFKGTGNMELHLNRKIAEKRVFPAIDFNRSGTRREELLTKTDELQKMWILRK
IVHPMGETDAMEFLIDKLAMTKTNDEFFDAMRRQ

>gi|229523772|ref|ZP_04413177.1| peptide transport system permease protein SapB [*Vibrio cholerae* bv. *albensis* VL426]
MFVYTVRKFNLFIITLLILTMVGYSLARFDPHSPWTLIGFWQGWSSYLVQLMEFNFGLNKNGV
PIIHELLVVFPATIELCTIAFILSLLVGIPIGTLAGMRQGKWLDTIISFISMSGYSAPIFWLALMMI
MAFSLHFPVFPVAGRYDLLYQIDHVTGFALIDAFLSQSPYRSQALQSVIEHLTLPCLVLALAPTT
QVIGQMRASVAEVMNQNYIRAAKIRGFSNYQIVTQHVLRNAIPPMIPKFGVQLSSMLTLAIITE
SIFNWPGIGRWLLDALANRDFMSIQAGVIVVGTLVLTANILSDLIGAAANPLVRKEWYVKR

>gi|194449129|ref|YP_002048313.1| hypothetical protein SeHA_C4676 [*Salmonella enterica* subsp. *enterica* serovar Heidelberg str. SL476]
MKTLPDTHVREASRCPSPVTIWQILLSRLLDQHYGLTLNDTPFADERVIEQHIEAGISLCDAVNF
LVEKYALVRTDQPGFNACTHSQLINSIDILRARRATGLMTRDNYRTVNDITQGKHPEAKQ

>gi|161615569|ref|YP_001589534.1| hypothetical protein SPAB_03343 [*Salmonella enterica* subsp. *enterica* serovar Paratyphi B str. SPB7]
MQILPSLPPGATSSHPTPVGIWQTLLSHLLQQHYGLMLNDTPFANDGVIEQHINAGISLCDALN
GIVEKYDLVRTDRPGFSIAVQSPFITRIDILRARKACGLMKRRGYRAVTDITTGRYSGVAR

>gi|213161112|ref|ZP_03346822.1| CP4-6 prophage; toxin of the YkfI-YafW toxin-antitoxin system [*Salmonella enterica* subsp. *enterica* serovar Typhi str. E00-7866]

MQTQSLSPTREASSRPSPVEIWQRLLGRLLDRHYGLTLNDTPFCDEAVIQEHINAGITLADAINF
LVDKYELVRIDRRGVSRQEQSPYLRAVDILRARQATGLLRQGHNLSTR

>gi|200387753|ref|ZP_03214365.1| conserved hypothetical protein [*Salmonella enterica* subsp. *enterica* serovar Virchow str. SL491]
MQTQYNHPHRATPSQPSPVEIWQKLLTHLLAKHYGLELSDTPFSVEKVIQEHIDAGITLANAV
NFIVEKYELVRIDRKGFSWQEQSPYLRAVDILRARQATGLLRRQRYLAAH

>gi|194471406|ref|ZP_03077390.1| toxin of the YeeV-YeeU toxin-antitoxin system [*Salmonella enterica* subsp. *enterica* serovar Kentucky str. CVM29188]
MLLTRLLDQHYGLTLNDTPFSDKTVIQEYINAGVSLSDAVNFLVEKYGLVRIDRKGFSWQEQS
PYLSVVDILQARRSTGLLKTDVK

>gi|213620682|ref|ZP_03373465.1| hypothetical protein SentesTyp_25602 [*Salmonella enterica* subsp. *enterica* serovar Typhi str. E98-2068]
MLVQATGHPQRLHRFGLTVDTRTDEVHEALVAELGAAFLSAALGLPGAMLSRLDVAPWVTY
LQGDPWRLFRAAEAARKAMMWLKERRPSMTTVEMWQKMASLILETHYGFSLDDTTLGCRS
VVERHIECGITPLMAINALARIYQWERYDQPQRSLFINEAGPDSEILTLSEIRPELLTCYRVPVPS
GIPDRKAEAVQVESLPLLLAPAVSEGKSAANDDGPDDPDGNDNVVALPWAARRGKENPHIHR
FVSIFNGIAPHESRWQVFSDFVHMAACSLYNAVHRDPDFEADYMRRVSHYSAEDANNMARL
LSEVVMGLEFSPTDFLGRIYMISGLGNFHNAQYFTPYSVSYAMARMTLSDRIPELSSGERDFIT
VSDPASGAGSMVVALAEAMLEAGFNPQKQMVAYCVDIDPVASMMCYIQLSLMGIPAIVATG
NSLTVEIKREMATPMFVLGRWHHRWQADRTRKAA

>gi|16763059|ref|NP_458676.1| hypothetical protein STY4592 [*Salmonella enterica* subsp. *enterica* serovar Typhi str. CT18]
MNTSLHPDDISRFISGRLISSLAAGQVPWRGTIPGLPEHALTGVPFTGINVLLLWQAMQQRSLR
SGRWLTGDDLRQLGGQVRSGEKPVTLVRYRPSLSLFKVINPEQCDGLPDTLQPGWPLPPRPQP
SLNVIRDLLQNSGVPVIHRDNVLPVYRALHDRIELPPVASYVGEETYWQDILNLLVQATGHPQ
RLHRFGLTVDTRTDEVHEALVAELGAAFLSAALGLPGAMLSRLDVAPWVTYLQGDPWRLFR
AAEAARKAMMWLKERRPSMTTVEMWQKMASLILETHYGFSLDDTTLGCRSVVERHIECGITP
LMAINALARIYQWERYDQPQRSLFINEAGPDSEILTLSEIRPELLTCYRVPVPSGIPDRKAEAVQ
VESLPLLLAPAVSEGKSAANDDGPDDPDGNDNVVALPWAARRGKENPHIHRFVSIFNGIAPHE
SRWQVFSDFVHMAACSLYNAVHRDPDFEADYMRRVSHYSAEDANNMARLLSEVVMGLEFS
PTDFLGRIYMISGLGNFHNAQYFTPYSVSYAMARMTLSDRIPELSSGERDFITVSDPASGAGSM
VVALAEAMLEAGFNPQKQMVAYCVDIDPVASMMCYIQLSLMGIPAIVATGNSLTVEIKREMA
TPMFVLGRWHHRWQADRTRKAA

>gi|213162340|ref|ZP_03348050.1| hypothetical protein Salmoneentericaenterica_21035 [*Salmonella enterica* subsp. *enterica* serovar Typhi str. E00-7866]
DTRTDEVHEALVAELGAAFLSAALGLPGAMLSRLDVAPWVTYLQGDPWRLFRAAEAARKA
MMWLKERRPSMTTVEMWQKMASLILETHYGFSLDDTTLGCRSVVERHIECGITPLMAINALA
RIYQWERYDQPQRSLFINEAGPDSEILTLSEIRPELLTCYRVPVPSGIPDRKAEAVQVESLPLLLA
PAVSEGKSAANDDG

>gi|213865304|ref|ZP_03387423.1| hypothetical protein SentesT_36419 [*Salmonella enterica* subsp. *enterica* serovar Typhi str. M223]
MASLILETHYGFSLDDTTLGCRSVVERHIECGITPLMAINALARIYQWERYDQPQRSLFINEAGP
DSEILTLSEIRPELLTCYRVPVPSGIPDRKAEAVQVESLPLLLAPAVSEGKSAANDDGPDDPDGN
DNVVALPWAARRGKENPHIHRFVSIFNGIAPHESRWQVFSDFVHMAACSLYNAVHRDPDFEA
DYMRRVSHYSAEDANNMARLLSEVVMGLEFSPTDFLGRIYMISGLGNFHNAQYFTPYSVSYA

MARMTLSDRIPELSSGERDFITVSDPASGAGSMVVALAEAMLEAGFNPQKQMVAYCVDIDPV
ASMMCYIQLSLMGIPAIVATGNSLTVEIKREMATPMFVLGRWHHRWQADRTRKAA

>gi|213419622|ref|ZP_03352688.1| hypothetical protein Salmonentericaenterica_18236 [*Salmonella enterica* subsp. *enterica* serovar Typhi str. E01-6750]
MNTSLHPDDISRFISGRLISSLAAGQVPWRGTIPGLPEHALTGVPFTGINVLLLWQAMQQRSLR
SGRWLTGDDLRQLGGQVRSGEKPVTLVRYRPSLSLFKVINPEQCDGLPDTLQPGWPLPPRPQP
SLNVIRDLLQNSGVPVIHRDNVLPVYRALHDRIELPPVASYVGEETYWQDILNLLVQATGHPQ
RLHRFGLTVDTRTDEVHEALVAELGAAFLSAALGLPGAMLSRLDVAPWVTYLQGDPWRLFR
AAEAARKAMMWLKERRPSMTTVEMWQKMASLILETHYGFSLDDTTLGCRSVVERHIECGITP

>gi|82776316|ref|YP_402665.1| hypothetical protein SDY_1009 [*Shigella dysenteriae* Sd197]
MNTLPDTHVREASGCPSPVTIWQTLLTRLLDQHYGLTLNDTPFADERVIEQHIEAGISLCDAVN
FLVEKYALVRTDQPGFSACTRSPLINSIDILRARRVTGLMTRDNYRTVNNITLGKHPEAK

>gi|194432386|ref|ZP_03064673.1| conserved hypothetical protein [*Shigella dysenteriae* 1012]
MKTLSDTHVREVSRCPSPVTIWQTLLIRLLDQHYGLTLNDTPFVDERVIEQHIEAGISLCDAVN
FLVEKYALVRTDQPGFSTCPRSQLINSIDILRARRATGLMTRDNYRTVNNITLGKYPEAK

>gi|309786787|ref|ZP_07681407.1| conserved hypothetical protein [*Shigella dysenteriae* 1617]
MIEQHIEAGISLCDAVNFLVEKYALVRTDQPGFSACTRSPLINSIDILRARRVTGLMTRDNYRT
VNNITLGKHPEAK

>gi|309787455|ref|ZP_07682067.1| hypothetical protein SD1617_3951 [*Shigella dysenteriae* 1617]
MDERVIEQHIEAGISLCDAVNFLVEKYALVRTDQPGFSAGA

>gi|309785480|ref|ZP_07680111.1| intracellular protease 1 [*Shigella dysenteriae* 1617]
MSKKIAVLITDEFEDSEFTSPADEFRKAGHEVITIEKQSGKTVKGKKGEASVTIDKSIDEVTPAE
FDALLLPGGHSPDYLRGDNRFVTFTRDFVNSGKPVFAICHGPQLLISADVIRGRKLTAVKPIIID
VKNAGAEFYDQEVVVDKDQLVTSRTPDDLPAFNREALRLLGA

>gi|194433787|ref|ZP_03066062.1| intracellular peptidase, PfpI family [*Shigella dysenteriae* 1012]
MSKKIAVLITDEFEDSEFTSPADEFRKAGHEVITIEKQAGKTVKGKKGEASVTIDKSIDEVTPAE
FDALLLPGGHSPDYLRGDNRFVTFTRDFVNSGKPVFAICHGPQLLISADVIRGRKLTAVKPIIID
VKNAGAEFYDQEVVVDKDQLVTSRTPDDLPAFNREALRLLGA

>gi|82778467|ref|YP_404816.1| hypothetical protein SDY_3332 [*Shigella dysenteriae* Sd197]
MRNSPHPMQQRGGSMSKKIAVLITDEFEDSEFTSPADEFRKAGHEVITIEKQAGKTVKGKKGE
ASVTIDKSIDEVTPAEFDALLLPGGHSPDYLRGDNRFVTFTRDFVNSGKPVFAICHGPQLLISAD
VIRGRKLTAVKPIIIDVKNAGAEFYDQEVVVDKDQLVTSRTPDDLPAFNREALRLLGA

>gi|320175520|gb|EFW50616.1| General stress protein 18 [*Shigella dysenteriae* CDC 74-1112]

MQQRGGSMSKKIAVLISDEFEDSEFTSPADEFRKAGHEVITIEKQAGKTVKGKKGEASVTIDKS
IDEVTPAEFDALLLPGGHSPDYLRGDNRFVTFTRDFVNSGKPVFAICHGPQLLISADVIRGRKLT
AVKPIIIDVKNAGAEFYDQEVVVDKDQLVTSRTPDDLPAFNREALRLLGA

>gi|309787092|ref|ZP_07681704.1| phosphoheptose isomerase [*Shigella dysenteriae* 1617]
MYQDLIRNELNEAAETLANFLKDDANIHAIQRAAVLLADSFKAGGKVLSCGNGGSHCDAMHF
AEELTGRYRENRPGYPAIAISDVSHISCVGNDFGFNDIFSRYVEAVGREGDVLLGISTSGNSAN
VIKAIAAARERGMKVITLTGKDGGKMADTADIEIRVPHFGYADRIQEIHIKVIHILIQLIEKEMV
K

>gi|194432766|ref|ZP_03065051.1| phosphoheptose isomerase [*Shigella dysenteriae* 1012]
MYQDLIRNELNEAAETLANFLKDDANIHAIQRAAVLLADSFKAGGKVLSCGNGGSHCDAMHF
AEELTGRYRENRPGYPAIAISDVSHISCVGNDFGFNDIFSRYVEAVGREGDVLLGISTSGNSAN
VIKAIAAARERGMKVITLTGKDGGKMAGTADIEIRVPHFGYADRIQEIHIKVIHILIQLIEKEMV
K

>gi|82775613|ref|YP_401960.1| phosphoheptose isomerase [*Shigella dysenteriae* Sd197]
MYQDLIRNELNEAAETLANFLKDDANIHAIQRAAVLLADSFKAGGKVLSCGNGGSHCDAMHF
AEELTGRYRENRPGYPAIAISDVSHISCVGNDFGFNDIFSRYVEAVGCEGDVLLGISTSGNSAN
VIKAIAAARERGMKVITLTGKDGGKMADTADIEIRVPHFGYADRIQEIHIKVIHILIQLIEKEMV
K

>gi|4063860|gb|AAC98501.1| qac delta E [*Salmonella typhimurium* DT104]
MKGWLFLVIAIVGEVIATSALKSSEGFTKLAPSAVVIIGYGIAFYFLSLVLKSIPVGVAYAVWSG
LGVVIITAIAWLLHGQKLDAWGFVGMGLIIAAFLLARSPSWKSLRRPTPW

>gi|82776677|ref|YP_403026.1| Shiga toxin subunit B precursor [*Shigella dysenteriae* Sd197]
MKKTLLIAASLSFFSASALATPDCVTGKVEYTKYNDDDTFTVKVGDKELFTNRWNLQSLLLS
AQITGMTVTIKTNACHNGGGFSEVIFR

>gi|166788581|dbj|BAG06739.1| cholera toxin B subunit [*Vibrio phage* CTX]
MIKLKFGVFFTVLLSSAYAHGTPQNITDLCAEYHNTQIHTLNDKIFSYTESLAGKREMAIITFKN
GATFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTEAKVEKLCVWNNKTPHAIAAISMAN

The same processes were applied to other three bacteria such as *Vibrio cholerae, Salmonella typhimurium* and *Shigella dysenteriae* to found their evolutionary history under the class Gammaproteobacteria.

The Protein sequence of *Vibrio cholerae* was on the basis of cholera toxin:

>gi|166788581|dbj|BAG06739.1| cholera toxin B subunit [*Vibrio phage* CTX]

MIKLKFGVFFTVLLSSAYAHGTPQNITDLCAEYHNTQIHTLNDKIFSYTESLAGKREMAIITFKN

GATFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTEAKVEKLCVWNNKTPHAIAAISMAN

The Protein sequence of *Salmonella typhimurium* was taken on the basis of gene resistant:

>gi|4063860|gb|AAC98501.1| qac delta E [*Salmonella typhimurium* DT104]

MKGWLFLVIAIVGEVIATSALKSSEGFTKLAPSAVVIIGYGIAFYFLSLVLKSIPVGVAYAVWSG
LGVVIITAIAWLLHGQKLDAWGFVGMGLIIAAFLLARSPSWKSLRRPTPW

The Protein sequence of *Shigella dysenteriae* was taken on the basis of shiga toxin:

>gi|82776677|ref|YP_403026.1| Shiga toxin subunit B precursor [*Shigella dysenteriae* Sd197]

MKKTLLIAASLSFFSASALATPDCVTGKVEYTKYNDDDTFTVKVGDKELFTNRWNLQSLLLS
AQITGMTVTIKTNACHNGGGFSEVIFR

To get the evolutionary relation among all Firmicutes, the possible sequences were taken with BLAST application available at NCBI. BLAST records with zero E-value were submitted to CLUSTALW at EBI to get the phylogenetic information.

3.6 HERBAL ANTIMICROBIALS; COLLECTION AND IDENTIFICATION

Botanicals antimicrobials were collected from variMus parts of India *viz.* Uttar Pradesh, Kinnaur and Shimla (Himachal Pradesh), Almora and Nainital (Uttrakhand), Amarkantak (Madhya Pradesh), Nagpur (Maharastra) Aizawl and kolasib (Mizoram) and Gangtok, Chhangu Lake (Sikkim) based on their ethnomedicinal importance and literature survey from various libraries, on talk with traditional healers. The plant identification were made with the floras (Hooker, 1872-1892; Duthie, 1960; Maheshwari, 1963; Santapau, 1967; Srivastava, 1976 and the authentic herbarium specimens lodged in the herbarium of Botanical survey of India (BSI), Allahabad, and Sikkim. The collected botanicals were screened out against water borne bacterial pathogens for their antimicrobial investigation (Plate 7 and 8).

Table 3.2: List of collected herbal antimicrobials used in present investigation

S. No.	Name of antimicrobials herbs	Common name	Family	Oil Code	Extraction of essential oil from
1.	*Aegle marmelos* Linn. Correa	Bael	Rutaceae	Am-1	Leaves
2.	*Cinnamomum tamala* (Buch.-Ham.) Nees & Eberm.	Tejpatta	Lauraceae	Ct-2	Leaves
3.	*Citrus reticulate* L.	Santra	Rutaceae	Cr-8	Fruit peels
4.	*Cymbopogon flexuosus* (Steud.) Watson	Lemongrass	Poaceae	Cf-4	Leaves
5.	*Foeniculum vulgare* Miller	Souf	Apiaceae	Fv-6	Seed
6.	*Micromeria biflora* (Buch.-Ham. ex D.Don.) Benth	Satureja	Lamiaceae	Mm-7	Leaves
7.	*Cedrus deodara* (Roxb.) G.Don	Deodar	Pinaceae	Cd-3	Leaves
8.	*Mentha piperata* L.	Peppermint	Lamiaceae	Mp-9	Leaves
9.	*Mentha spicata* L.	Spearmint	Lamiaceae	Ms-10	Leaves
10.	*Mentha arvensis* L.	Corn Mint	Lamiaceae	Ma-11	Leaves
11.	*Moringa oleifera* Lam.	Drumstick	Moringaceae	Mo-5	Leaves and Seed
12.	*Occimum americanum* L.	Wild basil	Lamiaceae	Og-12	Leaves
13.	*Pogostemon cablin* Benth.	Patchouli	Lamiaceae	Pc-13	Leaves
14.	*Psidium guajava* L.	Guava	Myrtaceae	Pg-14	Leaves
15.	*Pogostemon benghalensis* L.		Lamiaceae	Pb-15	Leaves

3.6.1 Collection, Storage, and Vouchering of Plants

For isolation of secondary metabolites, the first steps are to identify the plants of interest and to preserve the compounds within them. During early finding, it is not normally known which of the collected plants actually have useful compounds. Therefore, it is essential to be able to keep complete records of all collected specimens.

3.6.1 (a) Collection of Plants in the Field

When the plants were collected in the field for antimicrobial investigation, then the following important things are as follows:

- Wear field clothes, and cover yourself head to toe.

- A notepad (Field diary, waterproof paper is excellent) and pencil to recorded information about the collecting site location, soil conditions, ecological habitat, date of collection, plant identity.
- A good-quality portable digital camera/handycam for recording geographical positions and elevations to kept a photographic record of each plant.
- A pocket-size field guide (with photos, drawings, and good, usable identification keys) to the local flora and a hand lens also with us, which became helpful to identify each plant.

3.6.1 (b) Storage of Plants at Low Temperatures (Drying)

Some times, collected plants used directly for extraction of secondary metabolites but generally dried first. The preservation was necessary to prevent the degradation of the collected plant material or any enzymatic changes that vary or degrade naturally occurring metabolites. This technique was especially important for molecular biological studies. So, kept the plants material at low temperature, basically the shed dry. This process carried as soon as possible after collection. The aim of drying of collected plants material is to reduce the water content, because most plants contain nearly 70 to 80% moisture when harvested. Thus dehydrated plant can be stored for longer time.

3.6.1 (c) Vouchering of Collected Plants in the Field

Vouchering process (detailed records of the collected plant material) was helpful, when some time if a particular collected pants material have contained compounds of interest. This became true when searching for new medicinal compounds. So, accurate and lasting records are absolutely essential.

3.6.1 (d) Photographic Records of Plants Collected

The photographic records of plants that were collected became essential either for plant identification or for recollection of a particular plant. So the photographs were taken during field collection.

3.6.2 The Extraction Process

In the resent study the actual extraction is performed using techniques that depend on the knowledge of several chemical as well as physical properties of the desired compounds. These are coefficients in water or organic solvents, relative polarity of the molecule, stability of the molecule, as well as the temperature employed during the extraction process.

Hydro-distillation

In hydro-distillation, Clevenger apparatus was commonly used for the extraction of secondary metabolites.

Clevenger apparatus is a glass apparatus invented by Clevenger (1928) was commonly used for extraction of the volatile plant secondary metabolites/ essential oil (Plate 7 and 8). The technique behind this is hydro-distillation and the steps involved can be summarized as follows:

1. Collected the fresh plant material (leaves, stems, seeds and roots etc.) and washed it properly with tap water.
2. Chopped & weigh the plant material
3. Loaded the plant material into the flask and filled the water in a manner so that it could not be more than 50% of the total volume of the flask.
4. Adjust the temperature; it should be not more than 30-40^{o}C. If the temperature becames more than this the chances of degrading the compound will be higher.
5. Now, run the apparatus continuously, for 4-6 hrs for proper extraction of the volatile constituents.
6. Volatile fraction in the form of essential oil was dehydrated over anhydrous Sodium sulphate and stored in air tight sterilized voils.

3.7 Description of herbal antimicrobials

Based on antimicrobial screening, 4 botanicals antimicrobials were found to be effective against water borne bacteria *Escherichia coli* MTCC 723, *Vibrio cholerae* MTCC 3906, *Salminella typhimurium* MTCC 98, and *Shigella dysenteriae* ATCC 23513. Primarily, the antimicrobial tests were performed by using disc diffusion method (Plate 10). If the plants essential oils, found to be effective then their MIC

(minimum inhibitory concentration), IC_{50} and MBC (minimum bactericidal concentration) determined using the Spectrophotometer commonly known as Elisa reader the model name is Spectramax plus^{384} (Molecular Devices, USA).

Out of above 15 screened antimicrobial plants the *Mentha piperita* L. and *Mentha arvensis* L., *Micromeria biflora* and *Citrus reticulata* has been chosen for the present investigation. Description of plants was given below:

***Mentha piperita* L.** (White Peppermint)

Classification/Taxonomical position

Kingdom	:	Plantae
Class	:	Dicotyledons
Sub class	:	Gamopetalae
Series	:	Bicarpellate
Order	:	Lamiales
Family	:	Lamiaceae
Genus	:	*Mentha*
Species	:	*piperita*
Author	**:**	L.
Range	:	Asia and Britain

***Mentha piperata* L.**

Historical background:

Mentha piperita L. is a native of Europe, highly aromatic. The ancient Egyptians, Greeks and Romans knew it as flavouring for food and as medicine. It was first cultivated in England commercially around 1750 while its aerial parts have been widely used for their medicinal effects. Peppermint is widely known to relieve digestive ailments, being a popular remedy for at least two centuries.

At the herbarium of the English botanist John Ray (1628-1705) it can be found one of the oldest specimens of peppermint. In 1721, *Mentha piperita* became the official item of Materia Medica in the London Pharmacopoeia as *Mentha piperitis sapore* (Fluckiger 1879, Herbalgram, American Botanical Council, 1996).

Ellingwood, in 1902, in the book "Materia medica and Therapeutics – Chicago Medical Press: recommends peppermint water (distillate) for "flatulent colic, gastrodynia, nausea, vomiting, intestine spasmodic pain, hiccough, palpitation from indigestion, griping, cholera morbus, cholera infantum, irritability of the stomach, diarrhoea with abdominal pain, nervous headache, painful gonorrhoea".

In the Indian Materia Medica, leaves of *Mentha piperita* L., in infusion, are used in cases of vomiting, gastric colic, cholera, diarrhoea, flatulence, weak digestion, hiccup and palpitation of the heart (Nadkarni's – reprint of third edition – 1982).

Physical characteristics:

Mentha piperita L. (peppermint) is a medicinally important plant that belongs to the family Lamiaceae (Kirethekar and Basu, 1985). It is hardy to zone 3 and was not frost tender. Peppermint is a non-native herbaceous plant, it is a perennial, which can reach 100 cm in height (40 inches) has four-sided stem. The flowering time is from August to October. The flowers are hermaphrodite (have both male and female organs) and pollinated by Insects. The leaves are stalked opposite and toothed. The flower are irregular in shape, they are pinkish or purplish (Clark and Menory, 1980).

Cultivation

The plant fully succeeds in moist soils and situations so long as the soil is not too dry (Chittendon, 1956). The plant prefers light (sandy), medium (loamy) and heavy (clay) soils and grows in heavy clay soil. It can be grow in semi-shade (light woodland) or no shade generally requires moist soil. The plant prefers acid, neutral and basic (alkaline) soils. A sunny position is best for the production of essential oils, but the plant also succeeds in partial shade. It is often grown in the herb garden and also commercially for its essential oil. The whole plant is a pleasant aroma of peppermint. Most mints have fairly aggressive spreading roots and, unless you have the space to let them roam, they need to be restrained by some means such as planting them in containers that are buried in the soil. They can hybridize freely with other members of this genus. The flowers are very attractive to bees and butterflies. It is a good companion for growing near cabbages and tomatoes, helping to keep them free of

insect pests (Riotte, 1978). It produces a better quality essential oil if the plant is grown in dry ground. Members of this genus are rarely if ever troubled by browsing deer (Thomas, 1990).

Edible Uses: An essential oil from the leaves and flowers is used as flavouring in sweets, chewing gum, ice cream etc. (Facciola, 1990). An herb tea is made from the fresh or dried leaves (Facciola, 1990).

Medicinal uses

White peppermint is a very important and commonly used remedy, being employed by allopathic doctors as well as herbalists (Launert, 1981). It is also widely used as a domestic remedy. A tea made from the leaves is used traditionally in the treatment of fevers, headaches, digestive disorders (especially flatulence) and various minor ailments (Bown, 1995). The herb is abortifacient, anodyne, antiseptic, antispasmodic, carminative, cholagogue, diaphoretic, refrigerant, antistomachic, tonic and vasodilator (Bown, 1995). An infusion is used in the treatment of irritable bowel syndrome, digestive problems, spastic colon *etc.* (Chevallier, 1996). Externally a lotion was applied to the skin to relieve pain and reduce sensitivity (Chevallier, 1996). The leaves and stems can be used fresh or dried; they are harvested for drying in August as the flowers start to open (Grieve, 1984). The essential oil in the leaves is antiseptic and strongly antimicrobial, though it is toxic in large doses. When diluted it is used as an inhalant and chest rub for respiratory infections (Chevallier, 1996). The essential oil is used in aromatherapy. Its keyword was 'Cooling' (Westwood, 1993).

Other uses

The essential oil obtained from the whole plant used in perfumery (Tanaka, 1976). It is also an constituent of oral hygiene preparations, toiletries etc (Bown, 1995). Peppermint leaves are used as a component of pot-pourri (Bown, 1995). They were formerly used as a strewing herb. The plant repels insects, rats etc. (Holtom and Hylton, 1979). Rats and mice intensely dislike the smell of mint. The plant is therefore used in homes as a strewing herb and had also been spread in granaries to keep the rodents off the grain (Phillips and Foy, 1990).

3.7.1 *Mentha arvensis* L. (Mint, Pudina in Hindi)

Classification/Taxonomical position

Kingdom	:	Plantae
Class	:	Dicotyledons
Sub class	:	Gamopetalae
Series	:	Bicarpellate
Order	:	Lamiales
Family	:	Lamiaceae
Genus	:	*Mentha*
Species	:	*arvensis*
Author	**:**	L.
Range	:	Asia, U.S.A

***Mentha arvensis* L.**

Habitat

Plant easly rows on sandy and clay loams with periodic water-soaking indicator of damp soil conditions with moist to wet ground. It is due to from fibrous roots, often with adventitious roots from lower nodes, hollow. The plant well distributed to Western Europe, the Himalayas, India and China. European part of the former USSR, the Caucasus, Central Asia, Western Siberia, Eastern Siberia (Shiskin, 1954).

Morphology

Aromatic perennial from creeping rhizomes with fleshy surface and underground shoots. Stem dull green, somewhat purple-splotched, prostrate to erect, square, often ramified, 15-60 cm high. Cotyledons broad, rounded triangular. Leaves horizontally opposed, ovate or elliptical, petiolate, slightly serrate to notched along margin. Flowering time is June to September; Flowers are lilac or rose-lilac (Nikitin, 1983).

Edible Uses:

The leaves of wild mint are edible, raw or cooked. Having a quite strong minty flavor with a slight bitterness, they are used as a flavoring in salads or cooked foods.

A herb tea can be made from the fresh or dried leaves. The North American tribes used the leaves to make tea or beverages, to spice pemmican and soups, and to add flavor to certain meats in cooking. An essential oil from the plant is used as a flavoring in sweets and beverages (Nikitin, 1983).

Medicinal Uses:

Mint is often used as a domestic herbal remedy, being valued especially for its antiseptic properties and its beneficial effect on the digestion. The whole plant is anaesthetic, antispasmodic, antiseptic, aromatic, and has agents that counteract inflammation, that relieve and remove gas from the digestive system, promote secretion of milk, relieve fever and thirst, give strength and tone to the stomach, and is a stimulant.

North American Indians made a cold infusion of the plant as a lotion for fever and influensa. A compound infusion was taken and poultice was applied to the chest for pneumonia. A decoction of plant parts was taken for stomach pain, colds, swellings, headaches, diarrhea, and fevers. Dried leaves were chewed and swallowed for chest pains and heart ailments. Fresh leaves were put in the nostrils for colds. An infusion of leaves and stems was taken for vomiting, colds, pains, swellings, fevers, headaches, to prevent influensa, for stomach troubles and indigestion. Leaves were used for carious teeth and in the sweatbath for rheumatism.

Other Uses:

The plant is used as an insect repellent. Rats and mice intensely dislike the smell of mint. The plant was therefore used in homes as a strewing herb and has also been spread in granaries to keep the rodents off the grain. The leaves also repel various insects. Native people used leaves and stems as perfume to deodorize houses. Leaves were powdered and sprinkled on meat and berries as a bug repellant. Plants were boiled with traps to destroy the human scent.

Economic significance

Weed of grain and tilled crops; occurs in kitchen gardens, fallow lands, along roads and irrigation ditches. Control measures include multiple-field crop rotation, removing stubble, pre-winter plowing, chemical weeding (Agaev, 1988).

3.7.2 *Micromeria biflora* (Buch. Ham. ex D.Don.) Benth.

Classification/Taxonomical position

Micromeria biflora

Kingdom	**:**	Plantae
Class	:	Dicotyledons
Sub class	:	Gamopetalae
Series	:	Bicarpellatae
Order	:	Lamiales
Family	:	Lamiaceae
Genus	:	*Micromeria*
Species	:	*biflora*
Binomial Name	:	*Micromeria biflora*
Author	**:**	(Buch.-Ham. ex D.Don.) Benth.
Range	:	E. Asia from Himalayas to Bhutan and Myanmar

Physical characteristics

Micromeria biflora is hardy to zone 0, flowers from June to August, and the seeds ripened from August to September. The flowers are hermaphrodite (have both male and female organs) and are insect-pollinated. The plant prefers light (sandy) and medium (loamy) soils, requires well-drained soil and can grow in nutritionally poor soil, acid, neutral and basic (alkaline) soils. It does not grow in the shade. It required dry soil.

Cultivation

Prefered an open position in a well-drained soil succeeds in poor soils (Hulxey, 1992) and required a sunny position (Brickell, 1990).

Edible uses

The flowers and leaves are used as a tea. A powder of the dried flowers and leaves is used as flavouring in soups and curries.

Medicinal uses

A paste of the root was pressed between the jaws to treat toothache (Manandhar, 2002). The plant was rubbed and the aroma inhaled to treat nose bleeds (Manandhar, 2002). A paste of the plant was used as a poultice to treat wounds (Manandhar, 2002). The juice of the plant is taken internally and also inhaled in the treatment of sinusitis.

3.7.3 *Citrus reticulata* Blanco.

(In Hindi Santra)

Classification/Taxonomical position

Kingdom	**:**	Plantae
Class	:	Dicotyledon
Sub Class	:	Polypetalae
Series	:	Disciflorae
Order	:	Geranials
Family	:	Rutaceae
Genus	:	*Citrus*
Species	:	*reticulata*.
Range	:	E. Asia from

Citrus reticulata

The importance of *Citrus* fruits in preventing scurvy was scientifically proved in1756 by john Lind. Later in the century, the use limes or lemons became such an established custom on British ships that sailors came to be called “limeys” by the people from other countries and some times are so called even today. All C*itrus* fruits contain vitamin C. *Citrus* contains the most important species, sweet, bitter and mandarin oranges, lemon, lime, grapefruit, citron and the shaddock or pummelo. All these are excellent source of vitamin C and fruit acids, and are appreciated all over the world for their fruity sweet taste and refreshing qualities. The cultivated species of

Citrus are believed to be the native of Southeast Asia (Eastern India, Indochina, Southern China and Philippines).

In India *Citrus* fruits are commercially grown in Maharashtra, Andhra Pradesh, Karnataka and Assam, the first two accounting for nearly 50 per cent of the total Indian Production.

Citrus species are long-lived, much branched, evergreen, small aromatic trees or shrub. They are often spiny with the spines arising in the axils of the leaves, which are rather rough and leathery and dotted with glands. The leaves are apparently simple but in reality they are unifoliate compound, having been derived from trifoliate compound leaves by the reduction of two lateral leaflets. The flowers are white or purplish pink, often scented and are either solitary axillary or clustered in small terminal cymes.

Citrus is essentially a subtropical fruit and its cultivation is undertaken from sea level to nearly 760 m. On the whole, C*itrus* orchards do well in dry climates with annual rainfall of between 75 to 125 cm. They can not tolerate high winds; windbreak, therefore, should be provided wherever necessary. They can not withstand frost, tangerine being the most resistant. *Citrus* species are unsuited to the very humid tropics as high atmospheric humidity increases the incidence of pests and diseases. In general, they are grown on fertile, light loamy soils.

3.8 CHARACTERIZATION OF ESSENTIAL OIL

3.8.1 Gas-Chromatographic analysis

The Gas-Chromatographic (GC) analysis of the oil was performed on a Perkin-Elmer GC 8500, using a fused silica capillary column (25 m X 0.55 mm, film thickness 0.245 μm), coated with dimethyl siloxane (BP-1). The oven temperature was programmed from 60^0 C to 220^0 C at 5^0 C/min, then held isothermally at 220^0 C; detector temperature, 300^0 C; carrier gas-nitrogen at a inlet pressure of psi; split, 1: 80 (Plate 9).

3.8.2 Gas-Chromatographic-Mass Spectrometry

GC-MS data were obtained on a Shimadzu QP-2000 Mass Spectrometer instrument at 70 eV and 250^0 C. GC column: Ulbon HR- 1 (equivalent to OV –1), fused silica capillary column (0.25 mm X 50m, film thickness 0.25 μm). The initial temperature was 100^0C for 7mm, and then heated at 5^0C/ min to 250^0 C. Carrier gas, Helium was used at flow rate of 2 ml /min.

3.8.3 Quantitative analysis by GC

The Quantitive GC analysis showed the number of component in sample, such as their retention time and approximate boiling points. The time at which the particular components pass through the column was called the compounds Retention Time (RT). Retention Time was measured from the point of injection of the sample to the top of the compound peak and was usually reported in minutes.

3.8.4 Calculation of Kovats Retention Indices (IR)

The oil was separately spiked with a standard mixture of homologous n-alkane series (C9-C28) and then analysed by GC under the above-mentioned conditions. Retention indices were directly obtained by applying Kovats procedure (Kovats, 1965; Jennings and Shibamoto, 1980).

3.8.5 Mass Spectroscopy (MS)

It is an analytical technique of phytochemical analysis for the identification of plant constituents/ essential oils having wide spectral range to analyze small molecule upto protein characterization (Siuzdak, 2003).

The Mass spectroscopic analysis was run by E1 (Electron Impact Ionwasation) at 70ev by MSD (Mass Selective Detector). It is generally used to quantify the known compounds as well as identify the unknown compounds. MS is a technique of separating the ions in accordance with their masses. After the process of identification it can simply resolve the chemical structure of the compound/molecule with their properties. The detection of the compounds performed by the MS required very

minute, nearly 10^{-12} g, 10^{-15} moles, quantity of the compounds having mass 1000 Daltons, so, any compounds can be identified from a chemically complex mixtures present in very low concentrations and it can be separated individually on the basis of their masses.

3.8.6 Identification of compounds

The compound were identified by comparing the retention indices of the peaks on the BP-1 column with literature values, computer matching against the library spectra built up using pure substances and components of known essential oils, and finally confirmed by comparison of mass spectra with published data. The relative amounts of individual components were based on peak areas obtained without FID response factor correction.

3.9 PHYSICO-CHEMICAL PROPERTIES OF ESSENTIAL OILS

Various physico-chemical properties of the selected oils were determined using the techniques as recommended by Langenau, (1948). These provided important criteria for standardizing the oil, determining its quality and purity (Table 4.14).

3.9.1 Specific gravity

Specific gravity was the ratio of the weight of the oil to the weight of an equal volume of water. The Pycnometer was cleaned with chromic acid and properly sterilized in hot air oven were taken, filled with double distilled water and weighed. The pycnometer was then emptied, rinsed several times with alcohol and finally with ether. The ether fumes were removed by putting the Pycnometer in hot air oven. The weight of the emptied Pycnometer was recorded. It was taken filled with oil and weighed. The weights of water and oil were determined. The specific gravity of the oil was calculated by the following formula:

$$SG = \frac{\text{Weight of the oil}}{\text{Weight of an equal volume of water}}$$

According to "*United States Pharmacopoeia*" and the "*National Formulary*" the specific gravity was represented at 25^0C. To convert specific gravity determined at room temperature to 25^0C, a correction factor of 0.008/^{0}C was used. If the room temperature was higher than 25^0C, the correction factor was added. On the other hand, if the room temperature was lower than 25^0C, the correction factor was subtracted from the original value. The specific gravity (SG) of each essential oil at 25^0C is presented in (Table 4.14).

3.9.2 Optical rotation

When the solution of an essential oil was placed in a beam of polarised light, it possesses the property of rotating its planes. That property was known as specific rotation. Rotation due to pure oil was known as optical rotation. Specific rotation was temperature dependent.

10 ml of absolute alcohol was pipetted to a flask containing weighted amount of essential oil was swirled properly. The percentage of the solution was calculated. Now a Polarimeter tube (10 cm) containing the known concentration of the oil solution was placed in the trough of the Polarimeter (Lippich type) between polariser and analyzer. The analyzer was slowly turned until both the halves of the field viewed through the telescope, showed equal intensities of illumination. At the proper setting, a small rotation to right or left caused pronounced inequalities in the intensities of illumination of the two halves of the field. Direction of rotation was determined. If the analyzer was turned counter clockwise from zero position to obtain the final reading, the rotation was laevorotatory (-); if clock wise dextrorotatory (+). The eyepiece of the telescope was adjusted to give a clear sharpline between the two halves of the field. Rotation was determined by means of protractor by reading the degree directly and the minutes with the aid of either of the two fixed verniers. The movable magnifying glasses help in obtaining accuracy.

Specific rotation was calculated by following formula:

$$[\alpha]_{t^0}^{D} = \frac{\alpha \times 100}{l \times c}$$

Where,

$[\alpha]_{t^0}^{D}$ = specific rotation at temperature (28±2^{0}C) using sodium light.

α = observed rotation

l = length of the column (Polarimeter tube) in decimeter (10 cm = 1 decimeter)

c = concentration of the solution in percentage.

Specific rotation of solution of each essential oil is represented in the Table 4.18

3.9.3 Refractive index

When a beam of light enters a denser medium, it bends towards the normal.

According to law of refraction:

$$\frac{Sin\, i}{Sin\, e} = \frac{N}{n}$$

Where,

i = angle of incidence

e = angle of refraction

n = index of refraction of the less dense medium

N = index of refraction of the denser medium

Refractometer used for determining the refractive index

Refractometer (Abb'e type) was placed in such a position that day light can readily be obtained for illumination. The prisms of refractometer were cleaned with alcohol and then with ether. Then charged the instrument, the double prism was opened by means of the screw head (clamp) and one drop of the oil was placed in between the prisms. The prisms were then closed firmly by clamp. The alidade was moved backward or forward until the field of vision was divided into a light and dark zone. The dividing line (border line) would not be a sharp line but a band of colour-the solar spectrum. The colours are eliminated by rotating the screw head of the compensator until a sharp colourless line was obtained. The border line was adjusted so that it falls on the point of intersection of the cross hairs. The refractive index was read directly on the scale of the sector. The whole process as adopted for determining the refractive index of each oil and results obtained are presented in Table 4.18.

3.9.4 Solubility in 90% alcohol

This property played a significant role in determining the quality of oil. 1 ml of the oil was introduced into 10 ml glass toppered cylinder (calibrated to 0.1 ml). 90% alcohol was then pipetted into it drop by drop with concurrent shaking of the cylinder after each addition. The volume of alcohol used to obtain a clear solution was recorded.

3.9.5 Solubility in different organic solvents

Besides the earlier described physico-chemical properties, the solubility of the oils in different organic solvents was also determined. 1 ml of each the oil was introduced separately to a glass stoppered tube (10 ml) so as to prepare a set of 12 tubes for the each oil. In this way the solubility of each the oil in 1:1 ratio with respect to the following 12 different organic solvents was observed and recorded in (Table 4.15).

3.10 *IN-VITRO* STUDY OF HERBAL PLANT ESSENTIAL OILS AGAINST TEST PATHOGENS

The method were performed to determine the antimicrobial activity of essential oils was performed by the Disc diffusion method and Broth microdilution method against water borne bacterial pathogens in order to evaluate their virtues required for an ideal antimicrobial agent.

1) **Disc diffusion method:**
2) **Broth microdilution method:**

3.10.1 Disc diffusion method

The disc diffusion technique is also referred as “Kirby-Bauer Technique”, it is the most common technique used to determine the effectiveness of plants secondary metabolites for killing or inhibiting the growth of infectious pathogens. The In this technique, the paper discs impregnated with different antibacterial plant secondary metabolites were carefully placed on the surface of media (Nutrient agar or Mueller-

Hinton agar) which has recently inoculated with the bacteria. During incubation at suitable temperature the bacteria rapidly grow on the surface of media, commonly called as "lawn". As the bacteria grow the secondary metabolites diffuses out from the paper disk and the growth of the bacterial cells in the lawn may be affected. Around the disc the zone of growth inhibition clearly appeared. The diameter of zone around the disc confirmed that the plant secondary metabolites are susceptible to tested bacterium, have a good antibacterial agent (Plate 10).

Following materials required for disc diffusion:

- Mueller-Hinton agar plate or Nutrient agar plate.
- bacterial culture in broth, 18-20 hours old
- fresh culture should be preferred
- plants secondary metabolites (essential oils)
- forceps
- 95% ethanol in a beaker
- paper disks (sterilized) 4 or 6 mm in diameter
- sterile cotton swabs
- ruler, millimeter
- gloves

Procedure:

- Properly cleaned the surface of the inoculation chamber with 95% alcohol then sterilized with UV light for given time before proceed the experiment.
- Now, at the bottom of Mueller-Hinton agar plates label the name of the bacterium, date and write evenly spaced capital letters A-D or E about 20 mm from the plate edge. The gloves should be bear in both hands.
- From fresh cultured plate, the bacterium inoculums inoculated into Mueller-Hinton broth in order to obtain a concentration of 1×10^7 CFU/ml (0.5 McFarland used for the turbidity match).

- Then with non-dominant hand hold the tube of sterilized swabs, curl the little finger around the cotton plug and remove it with the dominant hand.
- From swabs tube took one swab; care not to touch the end of other. Replaced the plug and put it into tube stand. Remember do not put the plug down.
- Same as above, pick up the Mueller-Hinton broth tube remove the plug and quickly pass the opening of the tube through the flame three times.
- Cotton swab inserted into broth culture and pressed the swab inside the tube to taken excess medium. Replace the plug and put the tube into stand.
- Inoculated it into entire surface of Mueller-Hinton agar plate in back-and-forth motion.
- Again dipped the swab in the broth culture and inoculated on agar surface in second direction. Repeat it in third time in third direction this results in a "lawn" of bacteria on the agar surface.
- Discard the contaminated swab in the biohazard container. Allow the plate lid on dry agar surface for 5 minutes.
- Picked-up the sterile paper disc from its container with sterilized forceps.
- Then dipped the edge of the paper disc into the stock solution of plant secondary metabolites (different concentrations of plant secondary metabolites prepared in the DMSO such as (50 mg/ml) and allowed to saturating the disc through capillary action.
- Now gently placed the saturated disc over its letter code by applying some light pressure, because it prevent the disc from falling off when the plate is inverted during incubation.
- Repeated the same process for remain plates with different concentrations of plant secondary metabolites, but flamed the forceps for each new solution.
- After completion of the experiment placed all the inoculated plate upside-down in the incubator and adjust the temperature at ±35°C for 18-24 hours.
- Measured the diameter (in mm) of any zone of growth inhibition around a disc after incubation. The diameter of inhibition zones was measured in millimeters using a Hi-media Antibiotic Zone Reader.

- Finally, discarded the plate into biohazard container for proper sterilization.

3.10.2 Broth micro dilution method

For evaluating the minimum inhibitory concentration, IC_{50} values and minimum bactericidal concentration, the Broth microdilution methods were used. In which the 96 well microtitre culture paltes were required. Currently it is the most widely used protocol and it is standardized by the National Committee for Clinical Laboratory Standards (NCCLS) now known as Clinical Laboratory Standards Institute (CLSI). This method was done due to the most clinically relevant and reproducible standard for testing bacterial cell susceptibility to antimicrobial agents. Microtitre technique is a very sensitive, modern, rapid, automated, economical and quantitative. *in-vitro* antimicrobial testing method (Plate 11). The method was as follows:

Procedure:

- Properly cleaned and sterilized the surface of laminar air flow.
- Labeled the organisms name and date on the 96-well Microtitre plates.
- 100 µl of Muller Hinton broth filled into each well of the 96-well Microtitre culture plates.
- Again 100 µl medium added into 2nd column to maintain total volume 200 µl, this entire column called as "media control".
- Then in 3rd column, again added 90 µl medium while in the 4th column add 80µl of medium.
- Now, 10 µl of drug (from stock solution made by desired concentration of plant secondary metabolite dissolved into per ml of DMSO) carefully add into the wells in 3rd column (called "Drug control). So final volume become 200 µl.
- 20 µl of drug also added to the wells of 4th column to get net volume 200 µl.
- The drug mixed thoroughly using a multichannel micropipette into 4th column.
- Now from 4th column 100 µl are transfered to 5th column and from fifth to 6th, 7th, 8th upto 11th column respectively. This process known as serial dilution of drug

concentration to get the 2500, 1250, 625, 312.5, 156.25, 78.12, 39.06, 19.53 concentration sequence.

- ❖ Then 100 µl of bacterial inoculums having CFU 1 × 10^7 cells/ ml are added to column number 1st to 12th except 2nd and 3rd column.
- ❖ So, in 12th column the final volume is 200 µl containing only 100 µl media + 100 µl of bacterial inoculum having CFU 1 × 10^7 cells/ ml, it is called as "positive control".
- ❖ In 1st column when the 100 µl of bacterial inoculum having CFU 1 × 10^7 cells/ ml going to be adedd, mix 2-3 drops of formaldehyde then it should be add into 1st column, it is marked as "negative control".
- ❖ Plates were incubated in a moist chamber at 37^{0}C for 24 hrs.
- ❖ Absorbance was recortded at 492 nm using Microtitre plate reader.

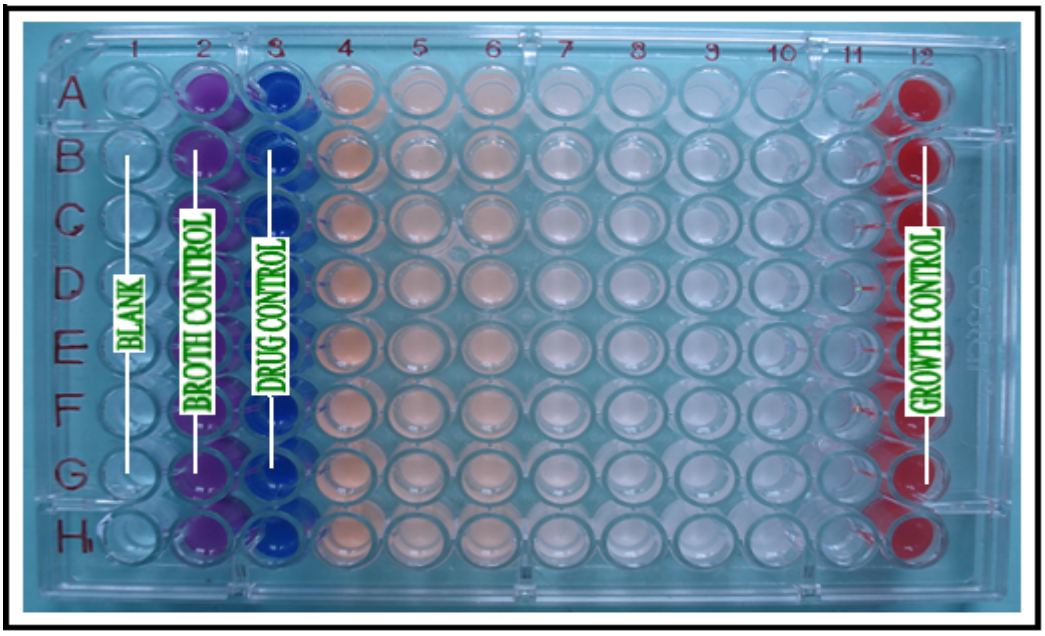


Fig. 3.4: CLSI protocol of drug testing in 96 well

3.10.2 (a) Testing protocol

(i) Negative control

The test organism was added to the media at a CFU of 1 x 10^7 cells/ ml and 100µl of 40% Formaldehyde was added to kill the cells. 100µl of the culture suspension was added to each well of 1^{st} column, served as the negative control.

(ii) Media control

200µl of media was added to each well of 2^{nd} column. No organism and no drug were added. It was to checkout the contamination in the media.

(iii) Drug control

The highest testing concentration of the drug was used along with media for drug control. Some drugs absorbed at the test wavelength and some drugs were turbid. The optical density values of the control were subtracted from the optical density (O.D.) values obtained by culturing the test organism with different concentrations of the drug under consideration so that the exact percent inhibition by the drug can be obtained.

(iv) Growth control

100µl of culture suspension at a CFU of 1 x 10^7 cells/ ml and 100µl of media was added to each well of 12^{th} column, served as a positive control.

3.10.2 (b) Preparation of antimicrobial drug stock solution

All the antimicrobial agents used in this study were water insoluble, so for preparing their stocks, DMSO i.e. Dimethyl Sulphoxide was used.

(i) Preparation of natural antimicrobial stock solution

10-mg or higher concentration of essential oil was weighed and dissolved in 1ml DMSO. The stock solution was aliquoted and stored at -20^0C.

(ii) Preparation of synthetic antimicrobial stock solution

1mg of synthetic antimicrobial was weighed and dissolved in 1ml DMSO. The stock solution was aliquoted and stored at -20^{0}C.

Table 3.3: Different concentration of drug stock solution

Drug stock solution	**CLSI Broth microdilution method (Drug concentration in µg/ml from 4th well to 11th well)**							
	4th	**5th**	**6th**	**7th**	**8th**	**9th**	**10th**	**11th**
1mg/ml	50	25	12.5	6.25	3.125	1.563	0.781	0.391
10mg/ml	500	250	125	62.5	31.25	15.625	7.8125	3.90625
20mg/ml	1000	500	250	125	62.5	31.25	15.625	7.8125
30mg/ml	1500	750	375	187.5	93.75	46.875	23.4375	11.718
40mg/ml	2000	1000	500	250	125	62.5	31.25	15.625
50mg/ml	2500	1250	625	312.5	156.25	78.125	39.062	19.5312

3.10.2 (c) Preparation of drug testing medium

21gm of Muller Hinton broth was weighed and dissolved in 1000ml SDW (Single Distilled Water).

- The pH of the medium was maintained at 7.0 using HCl or NaOH.
- media properly autoclaved at, 15 lbs (= 121^{0}C) for 15 min.

3.10.2 (d) Preparation of normal saline Compositions was

NaCl : 8.5 gm

Distilled water : 1000 ml

Preparation: Contents properly mix and then autoclaved at 15 lbs (= 121^{0}C) for 15 min.

3.10.2 (e) McFarland standard

Recent collaborative efforts have found that an inoculum in the range of $0.5x10^3$ to $2.5x10^3$ per ml produced the most interlaboratory agreement and this value had been adopted in the proposed NCCLS (National Committee for Clinical Laboratory Standards). According to NCCLS standard prepared an inoculum of above

range, the best method followed was the matching of turbidity of the inoculum suspension at 492 nm with 0.5 McFarland standards. The absorbance of 0.5 McFarland was equal to the absorbance of the inoculum suspension containing $1x10^7$ cells per ml for bacterial isolates.

(i) Preparation of McFarland standard stocks

- 1% H_2SO_4: 2.04ml dissolved in 197.96 ml TDW (Triple Distilled Water)
- 1% $BaCl_2$: 0.1 gm dissolved in 10ml TDW.

Optical density was recorded at 530nm. McFarland was diluted 10 times and corresponding O.D was recorded. The bacterial culture suspension was prepared and matched with 0.4, 0.5, 0.6, 0.7, McFarland and CFU were determined.

Note: McFarland solution should be discarded every 2 months and should be stored at 4^0C.

Table 3.4: Standard McFarland preparations

McFarland	1% H_2SO_4	1% $BaCl_2$
1	9.9	0.1
2	9.8	0.2
3	9.7	0.3
4	9.6	0.4
5	9.5	0.5
6	9.4	0.6
7	9.3	0.7
8	9.2	0.8
9	9.1	0.9
10	9.0	1.0

(ii) Procedure for CFU count

1. Nutrient agar plates were prepared.
2. Normal saline was poured into a culture tube in which bacteria was grown and transferred into a centrifuge tube.
3. The content of the tube was vortexed thoroughly.
4. Serial 10 fold dilutions of the cell suspension were prepared.

5. From each dilution 200µl of inoculum was taken and spread on Nutrient agar plates. Two plates were used for each dilution. Incubate the plates at 35^0C for 24 hrs in an inverted position.

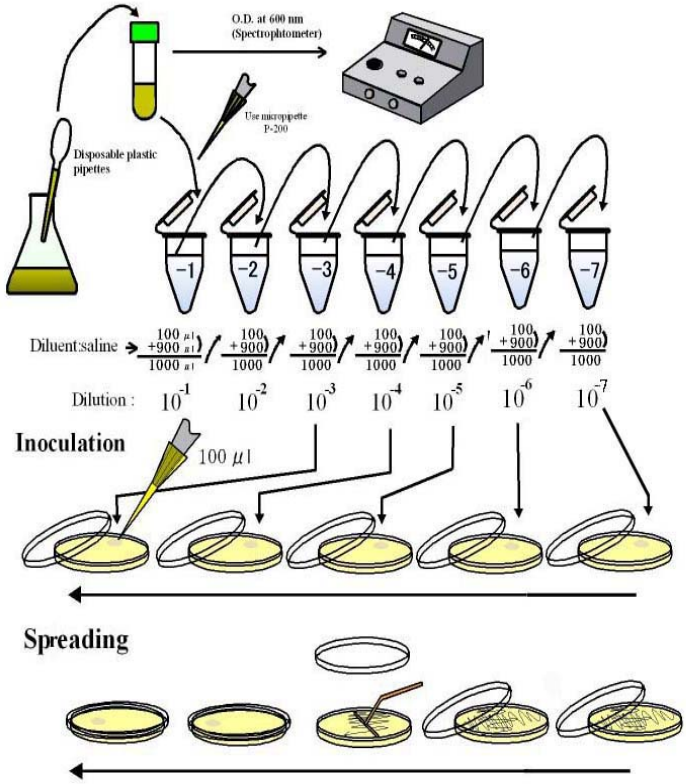


Fig. 3.5: Serial dilution of inoculum for Colony forming unit (CFU) estimation

3.10.3 Determination of Minimum Inhibitory Concentration (MIC) and IC_{50} by Broth micro dilution method recommended by (CLSI) new name of NCCLS

The antimicrobial activity of compounds was determined by Broth micro dilution method as described by Clinical Laboratory Standards Institute (CLSI) new name of NCCLS in Mueller Hinton broth. All the dental caries causing bacterial isolates with MTCC standard isolates were maintained on Blood agar at 37^oC. The 96-well tissue culture plates were used for twofold serial dilution. The proper growth control, drug control and the blank was adjusted onto the plate. Compounds were dissolved in 5-10% DMSO at a concentration of 1mg/ml or higher in case of synthetic and 10mg/ml or higher in case of natural antimicrobials, 20 µl of drug was added to

96-well tissue culture plate having 180µl Mueller Hinton broth. So the maximum concentration of the test compound was 50µg/ml, 500µg/ml and higher respectively. From here the solution was serially diluted resulting into the half of the concentration of test compounds and then bacterial inoculum was added and kept for incubation at 37^{o}C in a moist, dark chamber, and MIC and IC_{50} values was recorded spectrophotometrically at 492 nm after 24 hrs (Plate 11).

National Committee for Clinical Laboratory Standards (NCCLS) (2003). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. *Approved standard M7–A6. NCCLS, Wayne, Pa.*

3.10.4 Nature of antimicrobial activity, bacteristatic/bactericidal

To determine Minimum bactericidal concentration (MBC) and Minimum static concentration (MSC), Aseptically 100 µl aliquot of inoculum was taken from those wells which not showed any turbidity in incubated 96 well plate treated with essential oil. After poured on to Nutrient agar plates incubated for 24 hours at 37°C. MBC was defined as the lowest concentration of the essential oil at which 99.99% or more of the initial inoculum was killed. If there was no growth, it means the concentration was cidal.

3.10.5 Synergestic effect of bioactive essential oil

The antimicrobial activity in form of synergestic effect of the bioactive essential oils of *Micromeria biflora* and *Citrus reticulata* were determined. The combinations of same ratio (20 mg/ml) of both oils were prepared. The the efficacy of synergism were evaluated through Broth microdilution method (NCCLS, 2003), and MIC, $IC_{50,}$ MBC values were recorded spectrophotometrically at 492 nm after 24 hrs.

Results

4.1 SAMPLE COLLECTION, ISOLATION AND PURIFICATION OF WATER BORNE BACTERIAL ISOLATES

4.1.1 Consumption of different water sources for drinking purposes in Allahabad city and Aizawl city

To know the consumption of types of water such as, municipal supply water, stored cintax water and other sources (handpumps, wells, oozing of water from hills, ponds, rivers etc.), the survey has been done in Allahabad city (Palin area) and Aizawl city (Hilly area). So, based on survey the result drawn as the majority of peoples were consumed municipal water directly in Allahabad city. While the peoples mainly dependent on the municipal water supply in Aizawl city.

Table 4.1: Consumption rate of different water sources in both cities

S. No.	Types of water	Allahabad city (%)	Aizawl city (%)
1.	Municipal water	91%	28%
2.	Stored cintax water	4%	62%
3.	Other sources	5%	10%

4.1.2 The bacteriolgical analysis of municipal water samples from different places of Allahabad city

A total number of 140 water samples (randomly selected and 20 samples from each places) were collected from the 7 places around the Allahabad city, were tested for the presence of coliforms bacterial pathogens through rapid water testing kit. During the investigation, the test results showed presence of Coliforms in 56 (40%) water samples table 4.3: and fig 4.1.

Table 4.2: Municipal water sample analysis by rapid water test kits in different places around Allahabad city

S. No.	Name of places	Total no. of tested	Positive for coliforms	Percentage (%)
1.	Civil lines	20	14	70%
2.	Daraganj	20	11	55%
3.	Kitganj	20	9	45%
4.	Chowk	20	8	40%
5.	Katra	20	6	30%
6.	Teliarganj	20	6	30%
7.	AU campus	20	4	20%
Total		**140**	**58**	**41.42%**

The bacteriological analysis of drinking municipal water samples showed that the supply water of Civil lines was highly contaminated (13 out of 20 (65%).

4.1.3 The bacteriolgical analysis of municipal water samples from different places of Aizawl city

The same things and process were applied in the 5 places around Aizawl city. Here, the total number of 75 water samples (randomly selected and 15 samples from each places) of municipal sources were collected and were tested for presence of coliforms bacteria. The test results showed that 29 (38.66%) water samples have contaminated with Coliform bacteria.

Table 4.3: Municipal water sample analysis by rapid water test kits in different places around Aizawl city

S. No.	Name of places	Total no. of tested	Positive for coliforms	Percentage (%)
1.	Zarkwt	15	9	60%
2.	Kulikown	15	7	46.66%
3.	MZU campus, Tanhril	15	6	40%
4.	Ramhlun	15	5	33.33%
5.	Chaltlang	15	3	13.33%
Total		**75**	**29**	**38.66%**

In Aizawl city, the bacteriological analysis of drinking municipal water samples showed that supply of Zarkawt was highly contaminated (9 out of 15(60%).

Table 4.4: Description of the Collection of Samples from various sites of Allahabad

Name of the Site	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
Civil Lines	3	61.96±2.01[a]	3.4828	53.3084	70.6116	58.16	65.00
Daraganj	3	51.37±0.92[b]	1.5986	47.4054	55.3479	50.00	53.13
Tagoretown	3	44.66±0.88[c]	1.5275	40.8721	48.4612	43.00	46.00
Kitganj	3	40.41±1.12[d]	1.9433	35.5860	45.2406	38.71	42.53
Katra	3	28.33±0.99[e]	1.7168	24.0653	32.5947	26.57	30.00
Chowk	3	30.15±1.21[e]	2.1042	24.9262	35.3804	28.13	32.33
A.U.Campus	3	20.12±1.15[f]	1.9927	15.1698	25.0702	18.19	22.17
Total	21	39.57±3.05	13.8680	33.2617	45.8869	18.19	65.00

VAR00002
Duncan

Collection sites	N	Subset for alpha = .05	e	d	c	b	a
		F					
AU Campus	3	20.1200					
Chowk	3		28.3300				
Katra	3		30.1533				
Kitganj	3			40.4133			
Tagoretown	3				44.6667		
Daraganj	3					51.3767	
Civil Lines	3						61.9600
Sig.		1.000	.315	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Table 4.5: Description of the Collection of Samples from various sites of Aizawl

Name of the Site	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
Zarkawt	3	59.67±1.45[a]	2.5166	53.4151	65.9183	57.00	62.00
Kulikown	3	46.51±1.04[b]	1.7945	42.0555	50.9711	44.65	48.23
MZU Campus	3	40.00±1.73[c]	3.0000	32.5476	47.4524	37.00	43.00
Ramhlun	3	34.75±0.81[d]	1.4002	31.2684	38.2249	33.36	36.16
Chatlang	3	14.56±0.91[e]	1.5752	10.6470	18.4730	13.00	16.15
Total	15	39.10±3.94	15.4590	30.5364	47.6583	13.00	62.00

VAR00002
Duncan

Collection sites	N	Subset for alpha = .05	d	c	b	a
		e				
Chatlung	3	14.5600				
Ramhlun	3		34.7467			
MZU Campus	3			40.0000		
Kulikown	3				46.5133	
Zarkawt	3					59.6667
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

4.1.4 Prevalence of bacterial isolates on the basis of Gram's reaction

The CFU count of the water samples clearly revealed that the percentage of *E. coli* was very high. The data was also confirmed through the Gram's reaction of the bacterial colonies isolated from the specific medium. Based on Gram reaction the highest concentration of *E.coli* were, obtained in collected water samples. The confirmation bacterial isolates through Gram's reaction were shown in (Table. 4.6).

Table 4.6: Incidence of bacterial isolates on the basis of morphology

1.		Bacterial Isolates from water of Allahabad City					
Total sample collected	**No. of Positive shwoed**	**No. number of colonies isolated**	**Bacterial isolates suspected on the basis of morphology (Gram's reaction)**				
			Escherichia spp.	*Vibrio* spp.	*Salmonella* spp.	*Shigella* spp.	Others
140	**56**	**299**	**78**	**40**	**58**	**64**	**59**
2.		**Bacterial Isolates from water of Aizawl City**					
			Escherichia spp.	*Vibrio* spp.	*Salmonella* spp.	*Shigella* spp.	Others
75	**29**	**167**	**53**	**27**	**39**	**48**	**30**
Total = 215	**85**	**466**	**131**	**67**	**97**	**112**	**89**

4.2 IDENTIFICATION OF WATER BORNE BACTERIAL ISOLATES

In present study, 131 isolates of *Escherichia* sp. and 67 isolates of *Vibrio* sp., 97 of isolates of *Salmonella* sp. and 112 isolates of Shigella sp. were identified as main dominant disease causing water borne bacteria. The 6 isolates were randomaly selected from each isolated species of bacteria for their biochemical charachterization. The confirmation was done with their standards strains such as *E. coli* MTCC 723, *V. cholerae* MTCC 3906, *S. typhimurium* MTCC 98 and *S. dysenteriae* ATCC 23513 respectively.

4.2.1 Cultural characteristics of selected bacterial isolates

The cultural characteristics of *E. coli* MTCC 723, *V. cholerae* MTCC 3906, *S. typhimurium* MTCC 98 and *S. dysenteriae* ATCC 23513 were shown in (plate 3) in MacConkey agar, Nutrient agar etc The cultural characteristics of the each randomly selected bacterial isolates (6 isolates) was namely represented as; EC1, EC15, EC30, EC45, EC60, EC75; VC1, VC8, VC16, VC24, VC32, VC40; ST1, ST11, ST22, ST33, ST44, ST55 AND SD1, SD12, SD24, SD36, SD48, SD60 respectively.

4.2.2 Morohological characteristics of selected bacterial isolates

All the selected isolates bacterial pathogens showed similar morphology as like their standards.

4.2.3 Biochemical characteristics of selected bacterial isolates

The Biochemical characterization of the isolated water borne bacterial species has been done with help of routine biochemical testing as well as biochemical testing Kits. Some specific agar media were used such as MacConkey agar, Starch Hydrolysis, ONPG, Lysine utilization, Ureage nitrate reduction, Citrate utilization and Voges Proskauer's. to confirmed the water borne bacterial isolates. Based on observations table has been drawn and following symbols have been assigned for results (Table 4.7).

+ = Positive (more than 90%); - = Negative (more than 90%); V = 11 - 89%

Table 4.7: Morphological & biochemical characteristics of water borne bacterial isolates and match with their standardstrians

S. No.	Characteristics	Water borne bacterial isolates						MTCC 723	Water borne bacterial isolates						MTCC 3906
		EC1	EC15	EC30	EC45	EC60	EC75		VC1	VC8	VC16	VC24	VC23	VC40	
1.	Grams reaction	_	_	_	_	_	_	_	_	_	_	_	_	_	_
2.	Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
3.	Growth at 37°C	good	good	good	good	good	good	good	good	good	good	Good	good	good	good
4.	MacKonkey agar	+	-	+	-	+	+	+	-	+	+	+	+	-	+
5.	Starch Hydrolysis	-	+	-	+	-	-	-	-	+	-	+	+	-	+
8.	Haemolysis on Blood Agar	+	-	+	-	+	+	+	-	+	-	+	+	+	+
9.	ONPG	+	+	+	_	+	+	+	-	+	-	+	+	-	+
10.	Lysine utilization	+	-	+	+	+	+	+	V	+	-	+	+	-	+
11.	Ornithine utilization	V	_	V	-	V	V	V	-	+	V	+	+	-	+
12.	Ureage	-	-	-	-	-	-	-	+	-	+	-	-	+	-
13.	Phenylaline Deamination	-	+	-	+	-	-	-	+	-	+	-	-	V	-
14.	Nitrate reduction	+	-	+	-	+	+	+	-	+	+	+	+	+	+
15.	H_2S Production	-	+	-	+	-	-	-	+	-	+	-	-	+	-
16.	Citrate utilization	-	-	-	+	-	-	-	-	+	+	+	+	-	+
17.	Voges Proskauer's	-	-	-	+	-	-	-	V	+	-	+	+	-	+
18.	Methyl red	+	+	+	+	+	+	+	-	V	-	V	V	+	V
19.	Indole	+	-	+	-	+	+	+	+	+	-	+	+	-	+
20.	Malonate utilization	-	-	-	-	-	-	-	-	V	V	V	V	-	V
21.	Esculin hydrolysis	V	-	V	-	V	V	V	-	-	-	-	-	-	-
22.	Arabinose	+	+	+	-	+	+	+	+	-	+	-	-	+	-
23.	Xylose	+	-	+	-	+	+	+	-	-	+	-	-	-	-
24.	Adonitol	-	+	-	+	-	-	-	+	-	+	-	-	+	-
25.	Rhamnose	V	+	V	+	V	V	V	+	-	+	-	-	+	-
26.	Cellobiose	-	-	-	+	-	-	-	V	-	+	-	-	V	-
27.	Melibiose	V	V	V	V	V	V	V	-	V	-	V	V	-	V
28.	Saccharose	V	-	V	-	V	V	V	V	+	V	+	+	V	+
29.	Raffinose	V	+	V	+	V	V	V	-	V	-	V	V	-	V
30.	Trehalose	+	V	+	-	+	+	+	-	+	-	+	+	+	+
31.	Glucose	+	-	+	-	+	+	+	-	+	-	+	+	-	+
32.	Lactose	+	-	+	+	+	+	+	-	+	+	+	+	-	+
Identification		*E. coli*		*E. coli*		*E. coli*	*E. coli*	*E. coli*		Vc		Vc	Vc		Vc
S. No.	Characteristics	Water borne bacterial isolates						MTCC	Water borne bacterial isolates						ATCC

		ST1	ST11	ST22	ST33	ST44	ST55	98	SD1	SD12	SD24	SD36	SD48	SD60	32513
1.	Grams reaction	_	_	_	_	_	_	_	_	_	_	_	_	_	_
2.	Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
3.	Growth at 37°C	good	good	good	good	good	good	good	good	good	good	Good	good	good	good
4.	MacKonkey agar	+	+	+	-	+	-	+	+	+	-	+	-	+	+
5.	Starch Hydrolysis	+	-	+	-	V	-	+	+	+	-	V	-	+	+
8.	Haemolysis on Blood Agar	+	-	+	-	+	-	+	+	+	-	+	-	+	+
9.	ONPG	-	+	-	-	+	+	-	-	-	+	-	+	-	-
10.	Lysine utilization	+	-	+	-	-	-	+	-	-	+	-	-	-	-
11.	Ornithine utilization	+	V	+	-	-	V	+	-	-	-	-	+	-	-
12.	Ureage	-	+	-	+	-	+	-	-	-	-	-	V	-	-
13.	Phenylaline Deamination	-	-	-	V	+	-	-	-	-	V	-	-	-	-
14.	Nitrate reduction	+	-	+	+	V	-	+	+	+	-	+	+	+	+
15.	H_2S Production	+	V	+	_	+	V	+	-	-	+	-	+	-	-
16.	Citrate utilization	+	-	+	-	+	-	+	-	-	+	-	V	-	-
17.	Voges Proskauer's	-	-	-	+	-	-	-	-	-	V	-	-	-	-
18.	Methyl red	+	-	+	-	-	-	+	+	+	-	+	-	+	+
19.	Indole	-	V	-	V	+	V	-	V	V	V	V	-	V	V
20.	Malonate utilization	-	+	-	-	-	+	-	-	-	+	-	+	-	-
21.	Esculin hydrolysis	-	-	-	+	-	-	-	-	-	-	-	+	-	-
22.	Arabinose	+	-	+	-	-	-	+	V	V	-	V	-	V	V
23.	Xylose	+	-	+	-	+	-	+	-	-	+	-	+	-	-
24.	Adonitol	-	V	-	+	-	V	-	-	-	+	-	-	-	-
25.	Rhamnose	+	-	+	-	+	-	+	-	-	+	-	+	-	-
26.	Cellobiose	-	-	-	V	+	-	-	-	-	+	-	+	-	-
27.	Melibiose	+	-	+	-	V	-	+	V	V	-	V	+	V	V
28.	Saccharose	-	+	-	+	-	+	-	-	-	+	-	-	-	-
29.	Raffinose	-	+	-	+	V	+	-	V	V	+	V	-	V	V
30.	Trehalose	-	V	-	-	-	V	-	V	V	-	V	+	V	V
31.	Glucose	+	_	+	_	+	_	+	+	+	-	+	+	+	+
32.	Lactose	-	+	-	V	-	+	-	-	-	+	-	_	-	-
Identification		ST		ST		ST	-	ST	SD	SD		SD		SD	SD

4.3 IDENTIFICATION OF WATER BORNE BACTERIAL ISOLATES ON THE BASIS OF GENE SPECIFIC PRIMER

The DNA were extracted from the new isolated bacteria which showd the positive biochemical characteristics and with relative standard strains. Based on their specific gene such as from *E. coli* rfbE gene 239 bp, from *V. cholerae* ctx gene 432 bp, *S. typhimurium* STM4497 gene 360 bp and from *S. dysenteriae* ipaH gene 884 bp. The isolated new bacterial isolates showed the maximum similarity with the DNA amplification of standard strains through Real Time-PCR.

14 Hilly Street Mortlake NSW 2137 Australia
T +61 2 9736 1320
F +61 2 9736 1364
W www.corbettlifescience.com

Quantitation Report

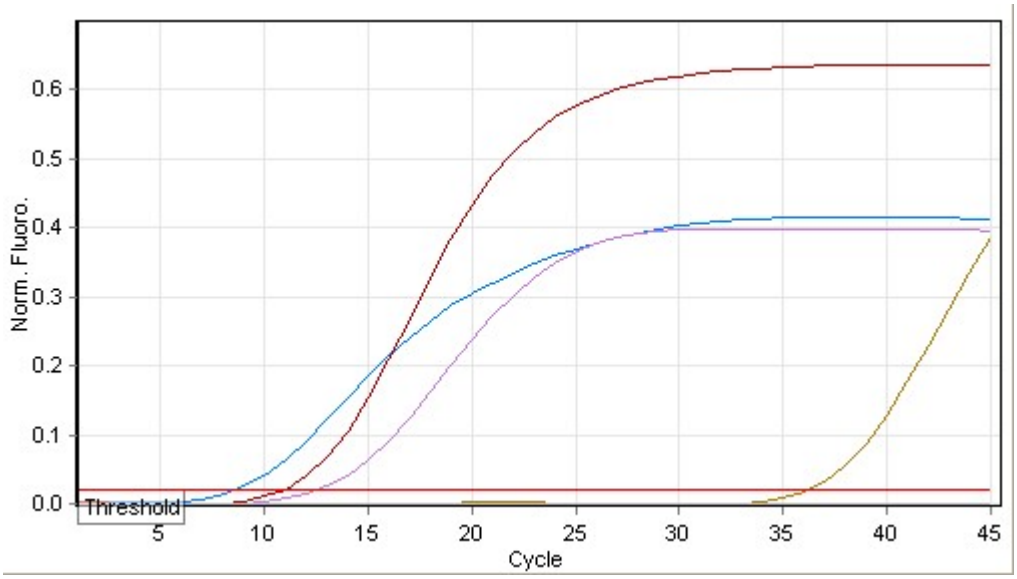
Experiment Information

Run Name	Awadhesh Samples using UNIVERSAL ENTRIC PROBE & JOE 301010 (4367)
Run Start	10/30/2010 1:59:58 PM
Run Finish	10/30/2010 3:32:09 PM
Operator	BPLAU
Notes	real time pcr
Run On Software Version	Rotor-Gene 1.7.87
Run Signature	The Run Signature is valid.
Gain Green	8.
Gain Yellow	10.

Quantitation Information

Threshold	0.020
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
No Template Control Threshold	10%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light

Quantitation data for Cycling A.Green



No.	Colour	Name	Type	Ct
1		*E. coli* MTCC with,PKS ECOLI F1, R1, PKS PAN ENT P1 PROBE	Unknown	NEG (NTC)
2		*E. coli* ATCC WITH PKS ECOLI F1, R1, PKS PAN ENT P1 PROBE	Unknown	NEG (NTC)
3		NC	NTC	NEG (NTC)
4		*S. typhimurium* with PKS TYPHM F1, R1 & PKS PAN ENT PI PROBE	Unknown	NEG (NTC)
5		NC	NTC	NEG (NTC)
8		*V. cholerae* with, PKS VICH F1, R1 & PKS PAN ENT P1 PROBE	Unknown	NEG (NTC)
9		NC	NTC	NEG (NTC)
10		*S. dysenteriae* with WITH PKS SHIG F1, R1 & PKS PAN ENT P1 PROBE	Unknown	NEG (NTC)
11		NC	NTC	NEG (NTC)
16		*S. Dysenteriae* with WITH PKS SHIG F1, R1 & SHIG P1 FAM PROBE	Unknown	NEG (NTC)
17		NC	NTC	NEG (NTC)
20		*S. typhimurium* with PKS SAL F1, R1 & PKS SAL PROBE	Unknown	10.83
21		NC	NTC	36.12
26		*V.cholerae* with PKS VICH PAN F1, R1 & PKS V.CH-PAN P1 PROBE	Unknown	NEG (NTC)
27		NC	NTC	NEG (NTC)

No.	Colour	Name	Type	Ct
28		*V.cholerae* with PKS VICH F1, R1 & PKS V.CHPAN P1 PROBE	Unknown	NEG (NTC)
29		NC	NTC	NEG (NTC)

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

4.4 ROLE OF BIOINFORMATICS IN PHYLOGENITIC RELATIONSHIP OF BACTERIAL ISOLATES

The Database record for protein, toxin as well as specific gene on NCBI (National Center for Biotechnology Information) got the various strains of *E.coli, V. cholerae, S. typhimurium* and *S.dysenteriae.* With the help of computer based bioinformatical tool; the ClustalW, the interpretation of their phylogenetic evaluation were made and their blast results was shown as follows:

In *E.coli*, YeeV protein taken as the base with their reference number was >gi|47155005|emb|CAE85204.1| YeeV protein [*Escherichia coli*] and found 11 strains of S. *dysenteriae*, 10 strains of *S. typhimuriun* and 10 strains of *V. cholerae*. (Schneider *et, al.,* 2004).

Same as in case of *V. cholerae*, the cholera toxin taken as the base with assertion number was >gi|166788581|dbj|BAG06739.1| cholera toxin B subunit [Vibrio phage CTX] and found 10 strains of *S. typhimurium*, 2 strains of *E.coli* and 10 strains of *S. dysenteriae* (Ehara, *et al*., 2008).

The bla_{CARB-2} resistant gene made as base in case of *S. Typhimurium* with their assertion no. >gi|4063860|gb|AAC98501.1| qac delta E [*Salmonella typhimurium* DT104] and found 11 strains of *E.coli*, 11 strains of *V.cholerae* and 11strains of *S. dysenteriae* (Briggs, C. E. and Fratamico, P. M., 1999)

Similarly shiga toxin as the base and with assertion number >gi|82776677|ref|YP_403026.1| Shiga toxin subunit B precursor [*Shigella dysenteriae*

Sd197] in case of *S. dysenteriae* and found 7 strains of *E.coli*, 10 strains of *V. cholerae* and 1 strains of *S. typhimurium* (Yang, *et al*., 2005).

On the basis of above strains the blast were made and the phylogenetic evaluation drawn as:

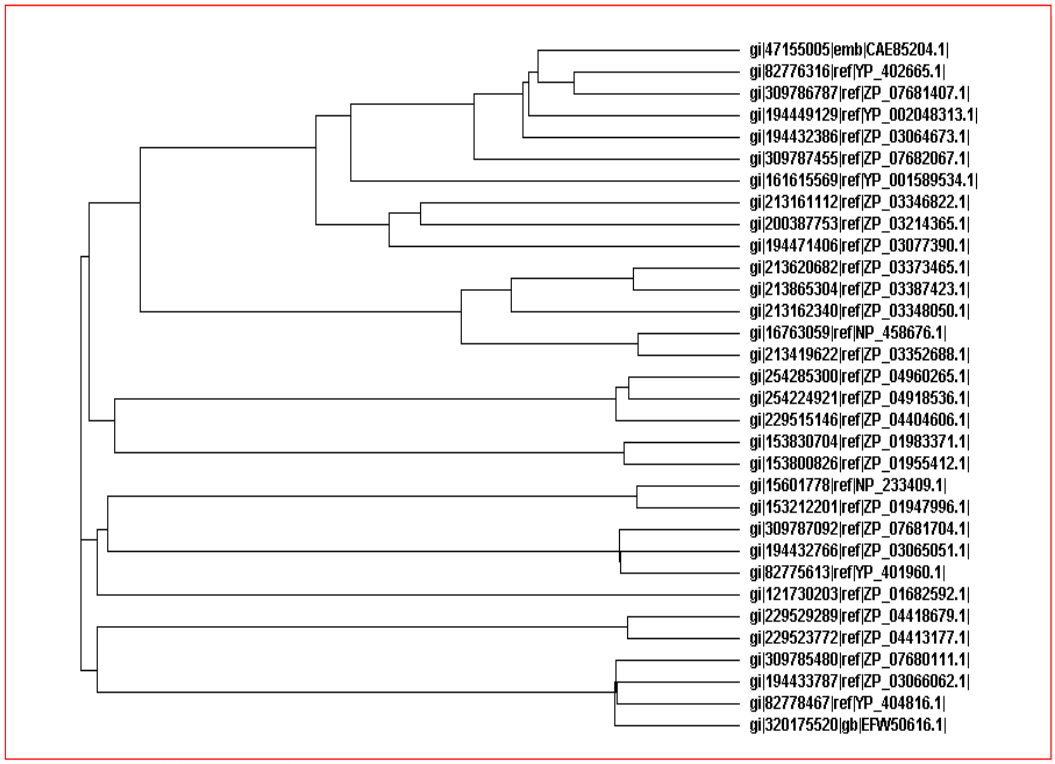


Fig. 4.2: Cladogram showed the phylogeny of *E. coli* with *V. cholerae, S.typhimurium* and *S. dysenteriae* on the basis of Yeev protein.

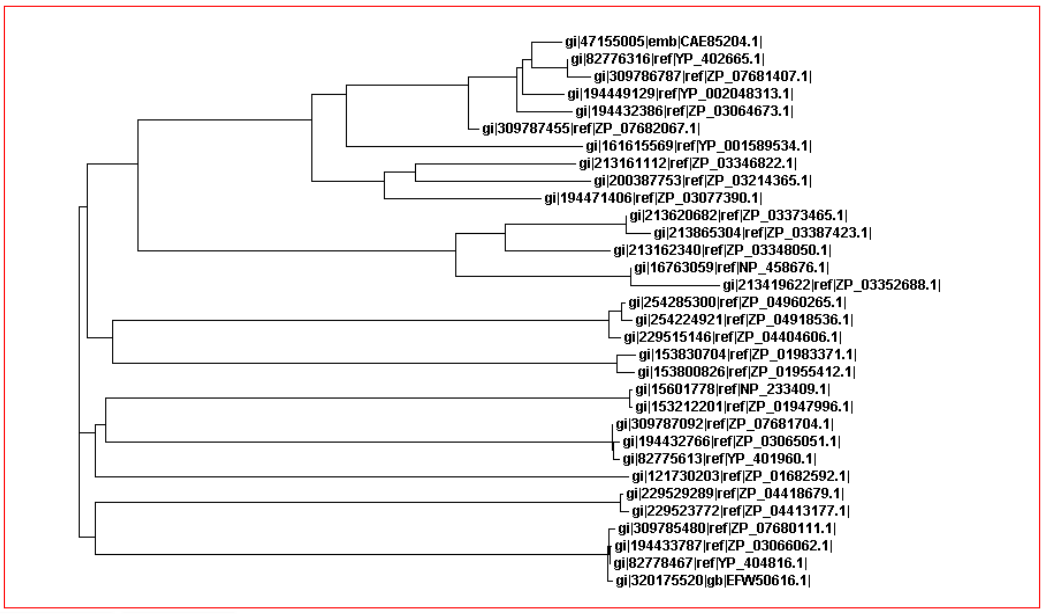


Fig. 4.3: Phylogram of *E. coli* with *V. cholerae*, *S. typhimurium* and *S. dysenteriae*

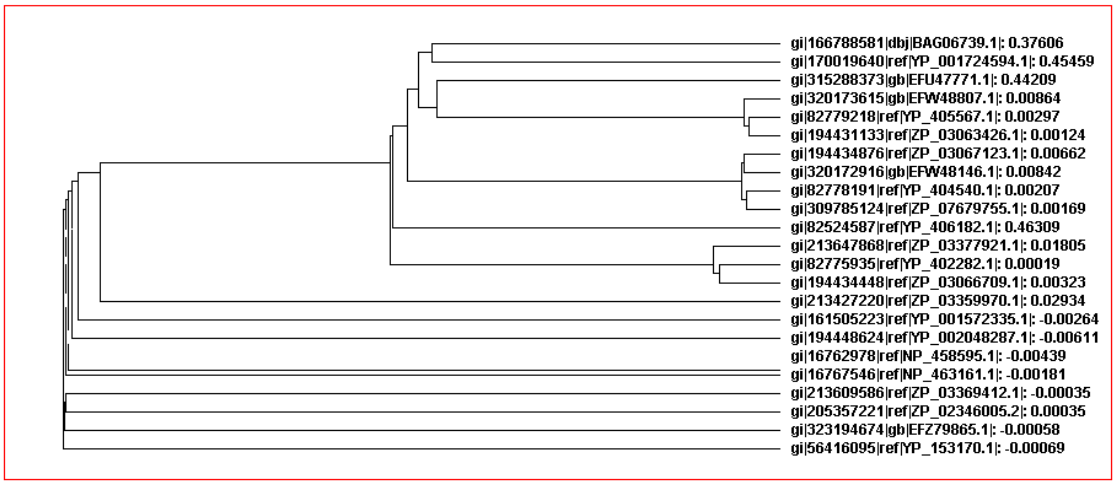


Fig. 4.4: Cladogram showed the phylogeny of *V. cholerae* with *E. coli, S. typhimurium* and *S. dysenteriae* on the basis of cholera toxin.

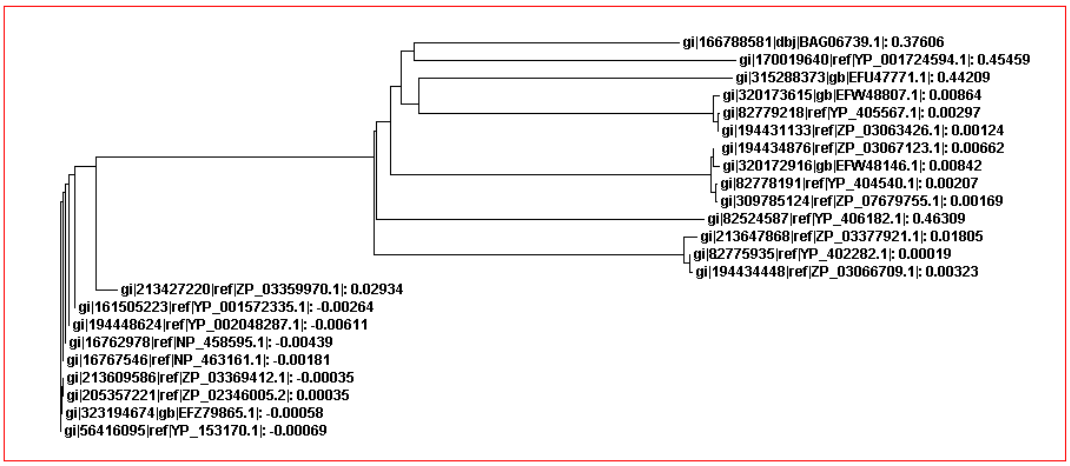


Fig. 4.5: Phylogram of *V. cholerae* with *E. coli*, *S. typhimurium* and *S. dysenteriae*

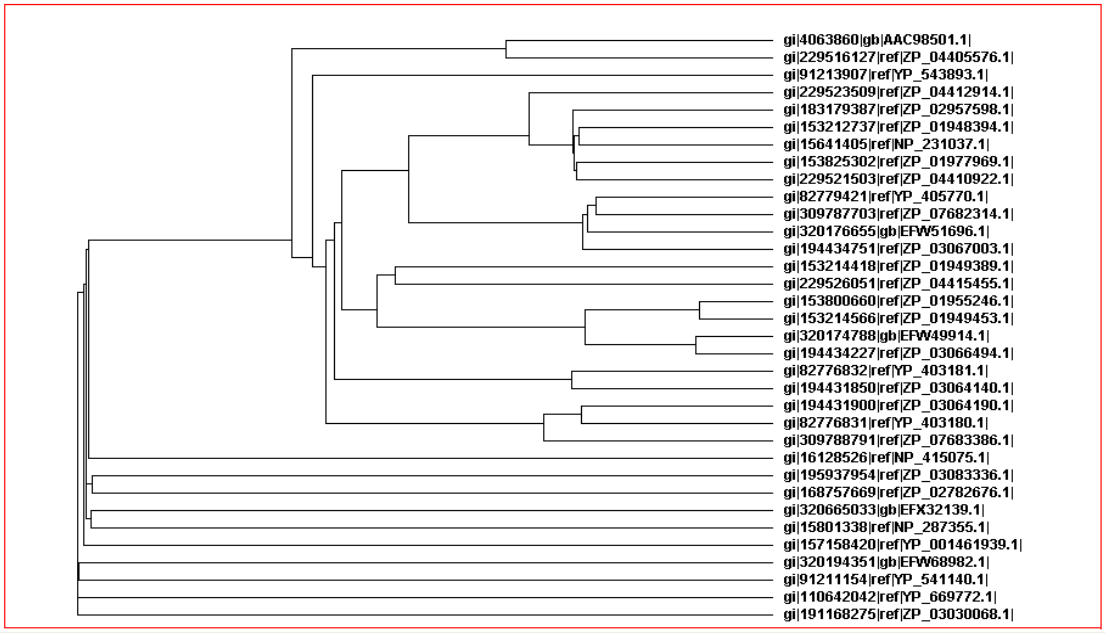


Fig. 4.6: Cladogram showed the phylogeny of *S. typhimurium* with *E. coli, V. cholerae* and *S. dysenteriae* on the basis of resistance gene

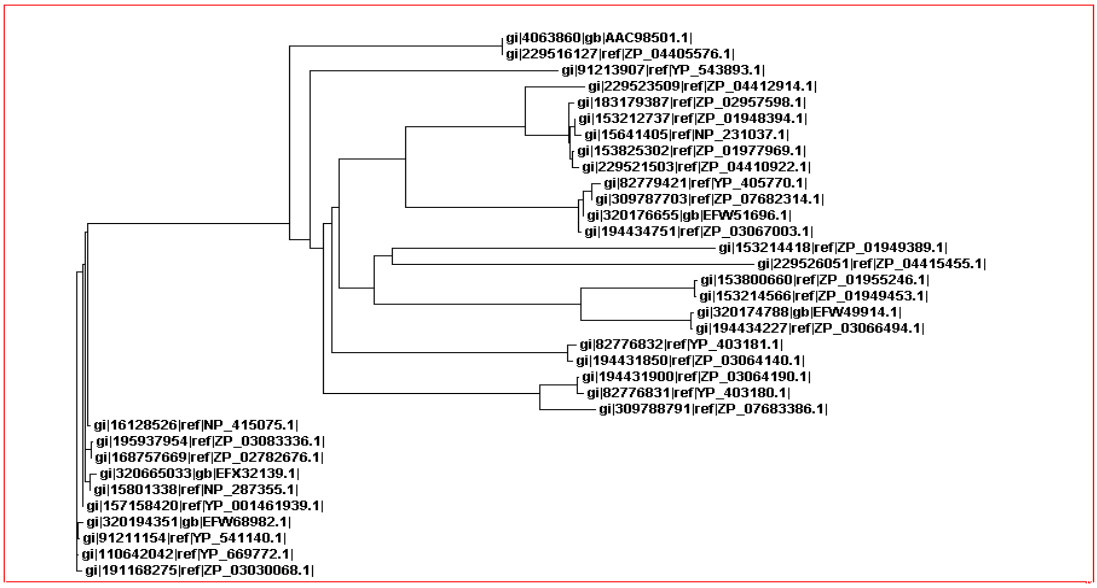


Fig. 4.7: Phylogram of *S. typhimurium* with *E. coli*, *V. cholerae* and *S. dysenteriae*

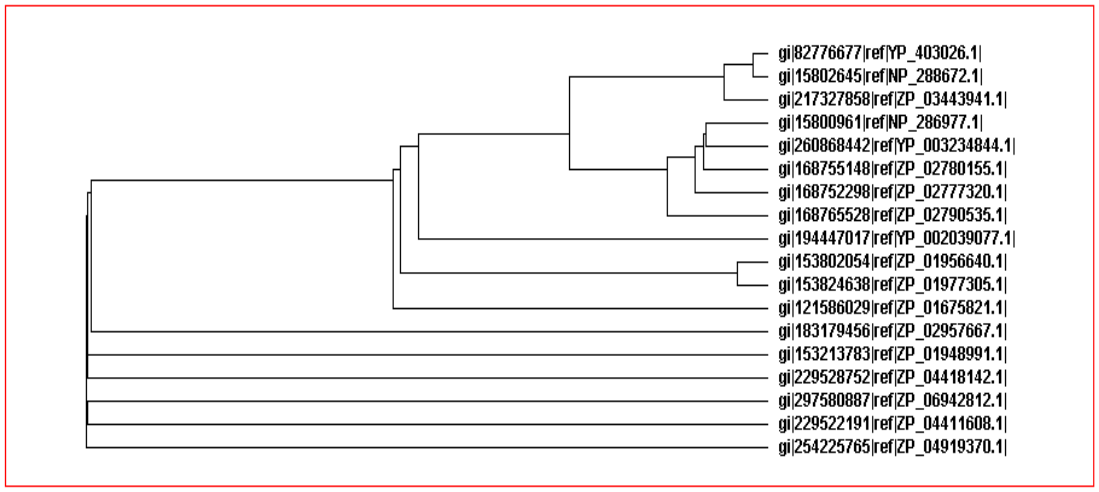


Fig. 4.8: Cladogram showed the phylogeny of *S. dysenteriae* with *E. coli, V. cholerae* and *S. dysenteriae* on the basis of Shiga toxin

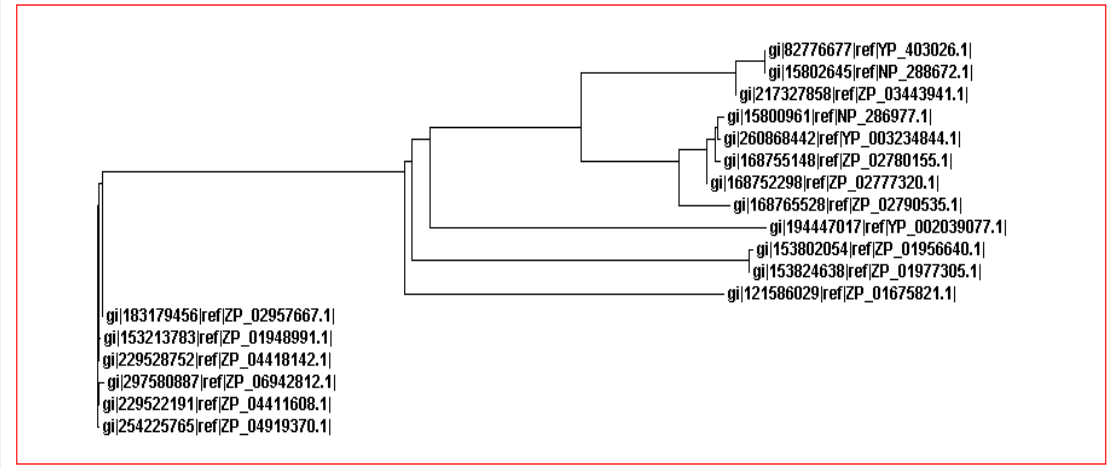


Fig. 4.9: Phylogram of *S. dysenteriae* with *E. coli*, *V. cholerae* and *S. typhimurium*

4.5 SCREENING ANTIMICROBIAL ACTIVITY OF SECONDARY METABOLITES (ESSENTIAL OILS) FROM COLLECTED PLANTS AGAINST TEST PATHOGENS

The screening of antimicrobial activity of essential oils were carried out by disk diffusion using Kirby-Bauer Method.

In the present investigation, 15 essential oils, extracted from different parts of the plants, are listed in (Table 3.2). The stock solution (50mg/ml) of each oil samples were used against water borne bacterial pathogens such as *Escherichia coli* MTCC 723, *Vibrio cholerae* MTCC 3906, *Salmonella typhimurium* MTCC 98 and *Shigella dysenteriae* ATCC 23513.

4.5.1 Antimicrobial activity of plant essential oils against tested bacterial pathogens

Out of 15 plant essential oils, 4 essential oils namely *Mentha piperita, Mentha arvensis, Micromeria biflora* and *citrus reticulata* were found to be more effective against all tested pathogens. The maximum inhibition zone of *Mentha piperita* essential oil (19 mm) of inhibition was observed (Plate 10) in the case of *Mentha arvensis* essential oil (20 mm), *Micromeria biflora* (24 mm) and Citrus reticulata (25 mm) inhibition zone was observed.

Table 4.8: Antimicrobial screening from collected plant extracted essential oils against water borne bacterial pathogens

S. No.	Name of antimicrobials herbs	Common name	Family	Oil Code	Extraction of essential oil from
1.	*Aegle marmelos* Linn. Correa	Bael	Rutaceae	Am-1	Leaves
2.	*Cinnamomum tamala* (Buch.-Ham.) Nees & Eberm.	Tejpatta	Lauraceae	Ct-2	Leaves
3.	*Cedrus deodara* (Roxb.) G.Don	Deodar	Pinaceae	Cd-3	Leaves
4.	*Cymbopogon flexuosus* (Steud.) Watson	Lemongrass	Poaceae	Cf-4	Leaves
5.	*Moringa oleifera* Lam.	Drumstick	Moringaceae	Mo-5	Leaves and Seed
6.	*Foeniculum vulgare* Miller	Souf	Apiaceae	Fv-6	Seed
7.	*Micromeria biflora* (Buch. Ham. ex D.Don.) Benth	Satureja	Lamiaceae	Mm-7	Leaves
8.	*Citrus reticulate* L.	Santra	Rutaceae	Cr-8	Fruit peels
9.	*Mentha piperata* L.	Peppermint	Lamiaceae	Mp-9	Leaves
10.	*Mentha spicata* L.	Spearmint	Lamiaceae	Ms-10	Leaves
11.	*Mentha arvensis* L.	Corn Mint	Lamiaceae	Ma-11	Leaves
12.	*Occimum americanum* L.	Wild basil	Lamiaceae	Og-12	Leaves
13.	*Pogostemon cablin* Benth.	Patchouli	Lamiaceae	Pc-13	Leaves
14.	*Psidium guajava* L.	Guava	Myrtaceae	Pg-14	Leaves
15.	*Pogostemon benghalensis*		Lamiaceae	Pb-15	Leaves

4.6 CHARACTERIZATION OF THE BIOACTIVE ESSENTIAL OILS

In the present investigation, 4 plants essential oil such as *Mentha piperita, Mentha arvensis*, *Micromeria biflora* and *Citrus reticulata* were taken for the screening against water borne bacterial pathogens. The essential oils were extracted and their GC-MS analysis showed the various constituents.

4.6.1 Identification of compounds from *Mentha piperata* L. essential oil

The GC-MS analysis of *Mentha piperata* L. essential oil resulted total of 14 components (Table 4.9). Out of 14 components 2 compounds i.e. Menthol 37.20% and Menthone 22.52% were the major compounds.

Table 4.9: Chemical analysis of *Mentha piperata* essential oil

S. No.	Components	%
1	α- pinene	0.68
2	β- pinene	1.12
3	d- limonene	3.31
4	Sabinene	0.38
5	Isomenthol	0.44
6	**Menthol**	**37.20**
7	Neomenthol	3.56
8	Isomenthone	4.70
9	Isopulegone	0.09
10	**Menthone**	**22.52**
11	Pulegone	3.70
12	Isomethyl acetate	0.06
13	methyl acetate	4.18
14	1,8- cineole	4.68

4.6.2 Identification of compounds from *Mentha arvensis* L. essential oil

The GC-MS analysis of the *Mentha arvensis* essential oil showed that 8 compounds, but out of 8 compounds, the percentage of L-Menthone (29.41) and menthol (21.33%) was found greater than others compounds (Table 4.10).

Table 4.10: Chemical analysis of *Mentha arvensis* essential oil

S. No.	Compound	Peak Area (%)
1	Eucalyptol	6.91
2	α- Phellandrene	3.2
3	**L-Menthone**	**29.41**
4	Isomethone	3.82
5	Linalool	2.2
6	neo-Menthol	4.7
7	**Menthol**	**21.33**
8	Iso-menthone	6.98

4.6.3 GC/MS analysis of *Micromeria biflora* essential oil

Gas-Chromatographic (GC) analysis of the oil was performed, out of 22 peaks, 3 peakes No. 7, 12 and 16 are major ones and shown Table 4.11 and Plate 9.

Table 4.11: Chemical analysis of *Micromeria biflora* essential oil

Micromeria biflora		
Peak	**Time (min)**	**Area (%)**
1	14.045	2.51
2	14.594	4.51
3	14.810	0.16
4	16.209	0.17
5	16.980	0.20
6	18.782	0.44
7	**19.133**	**10.41**
8	19.529	4.05
9	19.811	1.35
10	20.050	0.04
11	21.106	0.16
12	**21.249**	**44.36**
13	21.548	5.85
14	21.699	1.62
15	22.357	0.32
16	**22.515**	**13.33**
17	22.952	0.54
18	24.585	0.28
19	25.361	0.04
20	26.078	9.19
21	26.531	0.14
22	29.358	0.34

4.6.4 Identification of compounds from *Citrus reticulata* L. essential oil

The monoterpene fraction was predominant, accounting for more than 89.6%, where Limonene was the most abundant component (80.3%), followed by γ-terpinene (4.7%), myrcene (2.1%) and α-pinene (1.2) shown in (Table 4.12).

Table 4.12: Volatile components of *Citrus reticulata* Blanco (Ponkan) peel oil

Peak no.	Components	Amount (w/w) %
1	*α*-Pinene	1.2
2	Camphene	*
3	*β*-Pinene	0.5
4	Sabinene	0.5
5	**Myrcene**	**2.1**
6	*α*-Phellandrene	**
7	*α*-Terpinene	**
8	**Limonene**	**80.3**
9	*β*-Phellandrene	nq
10	(*Z*)-*β*-Ocimene	**
11	***γ*-Terpinene**	**4.7**
12	*p*-cymene	0.1
13	Terpinolene	0.2
14	Octanal	1.0
15	Nonanal	**
16	(*E*)-Sabinene hydrate	*
17	*δ*-Elemene	**
18	Citronellal	0.1
19	Decanal	0.3
20	Linalool	0.6
21	Octanol	**
22	*β*-Elemene	0.1
23	*α*-Terpineol	
24	Perilaldehyde	0.1
25	2,4-Decadienal	
26	Germacrene B	0.1

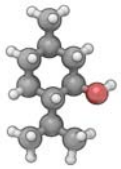
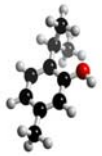
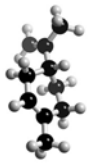
* - Peak area detected less than 0.005%

4.6.5 Description of major compounds form tested plant essential oils

The identification of isolated compounds were made by comparing the retention indices of the peaks on the BP-1 column with literature values, computer matching of stored library spectra which was previously built up same pure substances and components of known essential oils. The final confirmation carried out by comparison of mass spectra with published data. Based on above application, it was concluded that essential oil of *Mentha piperata* have Menthol, as their major compound. Similarly in case of *Mentha arvensis*, the second major compound was

menthol, however in case of *Micromeria biflora*, Thymol was the major compound and Limonene was rhe mojor compound in *Citrus reticulata* Table 4.13.

Table 4.13: Major constituent from bioactive plant essential oils

S. No.	Bioactive essential oils	Code	Name of Major Compound	Mol. formula	Molar mass	Composition	Crystal structure
1.	*Mentha piperata*	Mp-9	Menthol	$C_{10}H_{20}O$	156.27 g/mol	37.20%	
2.	*Mentha arvensis*	Mv-11	Menthol	$C_{10}H_{20}O$	156.27 g/mol	21.33%	
3.	*Micromeria biflora*	Mm-7	Thymol	$C_{10}H_{14}O$	150.22 g/mol	54%	
4.	*Citrus reticulata*	Cr-8	Limonene	$C_{10}H_{16}$	136.24 g/mol	80.3	

4.7 PHYSICO-CHEMICAL PROPERTIES OF THE ESSENTIAL OILS

The essential oils obtained from the 2 species of *Mentha* plant were studied under various parameters of physio-chemical properties as; Plant height, Oil yield, Colour, Specific gravity, Optical rotation, Refractive index and Solubility in 90% alcohol. The result was given in the Table 4.14.

Table 4.14: Physio-chemical properties of the 2 selected essential oils

Parameter studies	*Mentha piperata*	*Mentha arvensis*	*Micromeria biflora*	*Citrus reticulata*
Plant height	30-90 Cm	10-60 Cm	30 Cm	2 to 6 M
Oil yield	0.1-1%	0.1-2%	0.03-0.07%	0.6-0.9%
Appearence	Colourless or pale yellow	Slightly thick pale yellow liquid	Yellowish brown	transparent white in colour
Specific gravity at 25^0C	0.900 to 0.912	0.894 - 0.901	0.8913 to 0.91260	0.8450
Optical rotation	-16 to -30	-15.50 to -45	-3 to -25	$+73^0.5'$
Refractive index at 20^0C	1.460 to 1.467	1.459 - 1.475	1.468 to 1.488	1.476 to 1.481
Solubility in 90 % alcohol	Soluble	Soluble	Soluble	Soluble

4.7.1 Solubility in different organic solvents

The solubility of the oils in different organic solvents was also determined. The essential oils of both plants were soluble in different organic solvents however; in case of water it was found to be insoluble. In this way the solubility of each the oil in 1:1 ratio with respect to the following 12 different organic solvents was observed and recorded in Table 4.15.

Table 4.15: Solubility of selected essential oils and their active constituent, in different organic solvents (1: 1) ratio.

S. No.	Organic Solvents	*Mentha piperata*	*Mentha arvensis*	*Micromeria biflora*	*Citrus reticulata*
1.	Hexane	+	+	+	+
2.	Petroleum ether	+	+	+	+
3.	Benzene	+	+	+	+
4.	Chloroform	+	+	+	+
5.	Carbon tetrachloride	+	+	+	+
6.	Solvent ether	+	+	+	+
7.	N-Butanol	+	+	+	+
8.	Propanol	+	+	+	+
9.	Methanol	+	+	+	+
10.	Ethanol	+	+	+	+
11.	Acetone	+	+	+	+
12.	DMSO (dimethyl sulphoxide)	+	+	+	+

+ indicate solubility
- indicate insolubility

4.8 *IN-VITRO* STUDY OF THE SELECTED ESSENTIAL OILS AGAINST THE TESTED BACTERIAL PATHOGENS

4.8.1 Antimicrobial activities of bioactive essential oils and its active constituents against water borne bacterial isolates

According to NCCLS (2003) antimicrobial activities of selected bioactive essential oils were tested and the results were given in the form Minimum inhibitory concentration (MIC), IC_{50} and minimum bactericidal concentration (MBC). The efficacy of the oil of *Mentha piperiata* against the tested bacterial pathogens was recorded and shown in table-4.16 and Fig-4.10

Table 4.16: Antimicrobial activity of *Mentha piperita* against *Escherichia coli* MTCC723

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	A4	0.131	0.130	0.002	1.2	81.262
		B4	0.129				81.577
BL2	1.250	A5	0.318	0.313	0.006	2.0	54.472
		B5	0.309				55.719
BL3	0.625	A6	0.437	0.437	0.001	0.1	37.425
		B6	0.438				37.310
BL4	0.313	A7	0.522	0.522	0.001	0.2	25.190
		B7	0.521				25.377
BL5	0.156	A8	0.711	0.692	0.027	3.9	-1.814
		B8	0.672				3.687
BL6	0.078	A9	0.710	0.739	0.041	5.6	-1.642
		B9	0.768				-9.980
BL7	0.039	A10	0.790	0.812	0.031	3.8	-13.160
		B10	0.834				-19.463
BL8	0.020	A11	0.652	0.644	0.012	1.8	6.552
		B11	0.636				8.887

Based on the observations recorded in table-4.16 as well as fig-4.10 the minimum inhibitory concentration of the essential oil of *Mentha piperita* against *E. coli* was found to be 2.58 mg/ml but it was bactericidal at 5.11 mg/ ml. However, the IC_{50} value was recorded as 0.928 mg/ml.

Table 4.17: Antimicrobial activity of *Mentha piperita* against *Salmonella typhimurium* MTCC 98

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	E4	0.203	0.201	0.002	1.2	70.947
		F4	0.200				71.420
BL2	1.250	E5	0.229	0.243	0.020	8.1	67.151
		F5	0.257				63.140
BL3	0.625	E6	0.535	0.558	0.033	6.0	23.414
		F6	0.582				16.681
BL4	0.313	E7	0.804	0.809	0.008	0.9	-15.194
		F7	0.815				-16.727
BL5	0.156	E8	0.884	0.858	0.036	4.2	-26.655
		F8	0.833				-19.292
BL6	0.078	E9	1.129	1.097	0.045	4.1	-61.682
		F9	1.065				-52.556
BL7	0.039	E10	0.939	0.997	0.082	8.2	-34.520
		F10	1.055				-51.152
BL8	0.020	E11	1.096	1.063	0.047	4.4	-57.026
		F11	1.030				-47.542

Based on the observations recorded in table-4.17 as well as fig-4.10 the minimum inhibitory concentration of the essential oil of *Mentha piperita* against *S. typhimurium* was found to be 3.84 mg/ml but it was bactericidal at 4.60 mg/ ml. However, the IC_{50} value was recorded as 1.034 mg/ml.

Table 4.18: Antimicrobial activity of *Mentha piperita* against *Vibrio cholerae* 3906

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	C4	0.264	0.203	0.086	42.7	62.208
		D4	0.142				79.729
BL2	1.250	C5	0.146	0.170	0.034	19.8	79.113
		D5	0.193				72.322
BL3	0.625	C6	0.101	0.133	0.046	34.5	85.574
		D6	0.166				76.248
BL4	0.313	C7	0.094	0.094	0.001	0.9	86.491
		D7	0.093				86.663
BL5	0.156	C8	0.653	0.390	0.372	95.5	6.423
		D8	0.127				81.849
BL6	0.078	C9	0.682	0.416	0.376	90.3	2.312
		D9	0.151				78.425
BL7	0.039	C10	0.680	0.434	0.348	80.3	2.584
		D10	0.188				73.139
BL8	0.020	C11	0.237	0.215	0.031	14.2	66.062
		D11	0.194				72.265

Based on the observations recorded in table-4.18 as well as fig-4.11 the minimum inhibitory concentration of the essential oil of *Mentha piperita* against *V. cholerae* was found to be 2.89 mg/ml but it was bactericidal at 5.10 mg/ ml. However, the IC_{50} value was recorded as 0.951 mg/ml.

Table 4.19: Antimicrobial activity of *Mentha piperita* against *Shigella dysenteriae* MTCC23513

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	G4	0.217	0.163	0.077	47.1	68.856
		H4	0.109				84.413
BL2	1.250	G5	0.073	0.107	0.048	44.6	89.499
		H5	0.141				79.829
BL3	0.625	G6	0.308	0.329	0.030	9.2	55.891
		H6	0.351				49.745
BL4	0.313	G7	0.428	0.456	0.041	8.9	38.757
		H7	0.485				30.505
BL5	0.156	G8	0.580	0.608	0.040	6.5	16.853
		H8	0.636				8.830
BL6	0.078	G9	0.817	0.722	0.134	18.5	-16.985
		H9	0.628				10.076
BL7	0.039	G10	0.664	0.702	0.054	7.7	4.848
		H10	0.740				-6.040
BL8	0.020	G11	0.662	0.736	0.105	14.2	5.191
		H11	0.810				-16.040

Based on the observations recorded in table-4.19 as well as fig-4.11 the minimum inhibitory concentration of the essential oil of *Mentha piperita* against *S. dysenteriae* was found to be 1.92 mg/ml but it was bactericidal at 2.80 mg/ ml. However, the IC_{50} value was recorded as 0.483 mg/ml.

Table 4.20: Antimicrobial activity of *Mentha arvensis* against *Escherichia coli* MTCC 723

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	G4	0.189	0.195	0.008	4.0	82.278
		H4	0.200				81.239
BL2	1.250	G5	0.703	0.770	0.094	12.3	34.137
		H5	0.837				21.622
BL3	0.625	G6	0.954	0.989	0.050	5.0	10.671
		H6	1.024				4.057
BL4	0.313	G7	1.126	1.159	0.047	4.1	-5.460
		H7	1.193				-11.746
BL5	0.156	G8	1.111	1.189	0.109	9.2	-4.111
		H8	1.266				-18.594
BL6	0.078	G9	1.244	1.264	0.028	2.2	-16.570
		H9	1.284				-20.299
BL7	0.039	G10	1.165	1.220	0.078	6.4	-9.161
		H10	1.275				-19.437
BL8	0.020	G11	1.162	1.169	0.009	0.7	-8.898
		H11	1.175				-10.032

Based on the observations recorded in table-4.20 as well as fig-4.12 the minimum inhibitory concentration of the essential oil of *Mentha arvensis* against *E. coli* was found to be 2.46 mg/ml but it was bactericidal at 5.81 mg/ ml. However, the IC_{50} value was recorded as 1.742 mg/ml.

Table 4.21: Antimicrobial activity of *Mentha arvensis* against *Vibrio cholerae* MTCC 3906

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	G4	0.128	0.147	0.027	18.4	85.215
		H4	0.166				80.779
BL2	1.250	G5	0.206	0.175	0.043	24.7	76.122
		H5	0.145				83.223
BL3	0.625	G6	0.173	0.189	0.023	12.2	80.003
		H6	0.205				76.227
BL4	0.313	G7	0.360	0.364	0.006	1.6	58.284
		H7	0.369				57.311
BL5	0.156	G8	0.765	0.792	0.039	4.9	11.430
		H8	0.820				5.036
BL6	0.078	G9	0.874	0.906	0.046	5.0	-1.219
		H9	0.938				-8.679
BL7	0.039	G10	0.744	0.872	0.181	20.8	13.862
		H10	1.000				-15.814
BL8	0.020	G11	0.719	0.864	0.206	23.8	16.735
		H11	1.010				-16.996

Based on the observations recorded in table-4.21 as well as fig-4.13 the minimum inhibitory concentration of the essential oil of *Mentha arvensis* against *V. cholerae* was found to be 0.792 mg/ml but it was bactericidal at 2.86 mg/ ml. However, the IC_{50} value was recorded as 0.283 mg/ml.

Table 4.22: Antimicrobial activity of *Mentha arvensis* against *Salmonella typhimurium* MTCC 98

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	G4	0.294	0.262	0.046	17.4	71.233
		H4	0.230				77.533
BL2	1.250	G5	0.432	0.456	0.034	7.5	57.723
		H5	0.480				53.018
BL3	0.625	G6	0.502	0.542	0.056	10.4	50.876
		H6	0.582				43.098
BL4	0.313	G7	0.639	0.715	0.107	15.0	37.464
		H7	0.791				22.633
BL5	0.156	G8	0.797	0.855	0.081	9.5	22.027
		H8	0.912				10.757
BL6	0.078	G9	0.897	0.845	0.074	8.8	12.244
		H9	0.792				22.486
BL7	0.039	G10	0.868	0.888	0.029	3.2	15.110
		H10	0.908				11.139
BL8	0.020	G11	0.894	0.921	0.038	4.1	12.498
		H11	0.948				7.284

Based on the observations recorded in table-4.22 as well as fig-4.14 the minimum inhibitory concentration of the essential oil of *Mentha arvensis* against *S. typhimurium* was found to be 3.72 mg/ml but it was bactericidal at 6.10 mg/ ml. However, the IC_{50} value was recorded as 0.850 mg/ml.

Table 4.23: Antimicrobial activity of *Mentha arvensis* against *Shigella dysenteriae* ATCC 23513

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	G4	0.161	0.187	0.036	19.4	85.950
		H4	0.213				81.487
BL2	1.250	G5	0.217	0.207	0.014	6.7	81.130
		H5	0.197				82.844
BL3	0.625	G6	0.413	0.406	0.010	2.4	64.076
		H6	0.399				65.286
BL4	0.313	G7	0.483	0.574	0.128	22.4	57.942
		H7	0.665				42.133
BL5	0.156	G8	0.718	0.746	0.038	5.1	37.487
		H8	0.773				32.771
BL6	0.078	G9	0.968	0.988	0.029	2.9	15.804
		H9	1.008				12.263
BL7	0.039	G10	0.855	0.959	0.147	15.3	25.619
		H10	1.063				7.547
BL8	0.020	G11	0.866	0.960	0.132	13.8	24.618
		H11	1.053				8.365

Based on the observations recorded in table-4.23 as well as fig-4.15 the minimum inhibitory concentration of the essential oil of *Mentha arvensis* against *S. dysenteriae* was found to be 1.32 mg/ml but it was bactericidal at 3.01 mg/ ml. However, the IC_{50} value was recorded as 0.320 mg/ml.

Table 4.24: Antimicrobial activity of *Micromeria biflora* against *Escherichia coli* MTCC 723

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	E4	0.402	0.379	0.032	8.3	50.381
		F4	0.357				55.902
BL2	1.250	E5	0.063	0.069	0.009	13.6	92.251
		F5	0.076				90.609
BL3	0.625	E6	0.046	0.055	0.013	22.8	94.289
		F6	0.064				92.091
BL4	0.313	E7	0.050	0.036	0.020	56.0	93.857
		F7	0.022				97.340
BL5	0.156	E8	0.273	0.296	0.033	11.1	66.265
		F8	0.320				60.497
BL6	0.078	E9	0.511	0.462	0.070	15.1	36.906
		F9	0.413				49.047
BL7	0.039	E10	0.652	0.632	0.028	4.4	19.516
		F10	0.612				24.382
BL8	0.020	E11	0.902	0.752	0.213	28.3	-11.460
		F11	0.601				25.729

Based on the observations recorded in table-4.24 as well as fig-4.16 the minimum inhibitory concentration of the essential oil of *M.biflora* against *E.coli* was found to be 0.308 mg/ml but it was bactericidal at 2.80 mg/ ml. However, the IC_{50} value was recorded as 0.091 mg/ml.

Table 4.25: Antimicrobial activity of *Micromeria biflora* against *Salmonella typhimurium* MTCC 98

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	E4	0.488	0.354	0.189	53.3	52.287
		F4	0.220				78.425
BL2	1.250	E5	0.090	0.061	0.041	67.0	91.215
		F5	0.032				96.861
BL3	0.625	E6	0.022	0.005	0.023	431.5	97.869
		F6	-0.011				101.079
BL4	0.313	E7	-0.027	-0.032	0.006	20.1	102.664
		F7	-0.036				103.545
BL5	0.156	E8	0.384	0.357	0.038	10.7	62.386
		F8	0.330				67.690
BL6	0.078	E9	0.692	0.648	0.061	9.4	32.315
		F9	0.605				40.789
BL7	0.039	E10	0.790	0.749	0.058	7.7	22.705
		F10	0.708				30.730
BL8	0.020	E11	1.009	0.935	0.104	11.1	1.304
		F11	0.862				15.660

Based on the observations recorded in table-4.25 as well as fig-4.17 the minimum inhibitory concentration of the essential oil of *M.biflora* against *S. typhimurium* was found to be 0.202 mg/ml but it was bactericidal at 2.96 mg/ ml. However, the IC_{50} value was recorded as 0.104 mg/ml.

Table 4.26: Antimicrobial activity of *Micromeria biflora* against *Shigella dysenteriae* ATCC 23513

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	E4	0.366	0.212	0.217	102.4	69.978
		F4	0.059				95.193
BL2	1.250	E5	0.068	0.105	0.052	49.5	94.388
		F5	0.142				88.345
BL3	0.625	E6	0.020	0.036	0.022	62.4	98.354
		F6	0.052				95.751
BL4	0.313	E7	0.036	0.017	0.027	158.7	97.081
		F7	-0.002				100.168
BL5	0.156	E8	0.481	0.342	0.197	57.4	60.478
		F8	0.203				83.312
BL6	0.078	E9	0.885	0.691	0.274	39.6	27.348
		F9	0.498				59.132
BL7	0.039	E10	0.989	0.868	0.172	19.8	18.760
		F10	0.746				38.720
BL8	0.020	E11	1.048	0.936	0.158	16.8	13.990
		F11	0.825				32.299

Based on the observations recorded in table-4.26 as well as fig-4.18 the minimum inhibitory concentration of the essential oil of *M. biflora* against *S. dysenteriae* was found to be 0.178 mg/ml but it was bactericidal at 2.60 mg/ ml. However, the IC_{50} value was recorded as 0.094 mg/ml.

Table 4.27: Antimicrobial activity of *Citrus reticulata* against *Vibrio Cholerae* MTCC 3906

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	E4	0.131	0.249	0.167	67.0	85.468
		F4	0.367				59.280
BL2	1.250	E5	0.470	0.297	0.243	81.9	47.894
		F5	0.125				86.100
BL3	0.625	E6	0.786	0.810	0.033	4.1	12.729
		F6	0.833				7.536
BL4	0.313	E7	0.887	0.886	0.001	0.1	1.566
		F7	0.886				1.699
BL5	0.156	E8	0.993	0.980	0.019	1.9	-10.230
		F8	0.967				-7.289
BL6	0.078	E9	1.014	1.025	0.016	1.6	-12.505
		F9	1.036				-15.012
BL7	0.039	E10	1.001	1.013	0.018	1.7	-11.073
		F10	1.026				-13.836
BL8	0.020	E11	0.937	0.977	0.056	5.7	-3.993
		F11	1.016				-12.738

Based on the observations recorded in table-4.27 as well as fig-4.19 the minimum inhibitory concentration of the essential oil of *C. reticulata* against *V. cholerae* was found to be 8.06 mg/ml but it was bactericidal at 12.1mg/ ml. However, the IC_{50} value was recorded as 4.19 mg/ml.

Table 4.28: Antimicrobial activity of *Citrus reticulata* against *Salmonella typhimurium* MTCC 98

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	G4	0.462	0.475	0.018	3.8	56.697
		H4	0.488				54.289
BL2	1.250	G5	0.628	0.660	0.045	6.8	41.143
		H5	0.692				35.156
BL3	0.625	G6	0.737	0.768	0.044	5.8	30.977
		H6	0.799				25.111
BL4	0.313	G7	0.829	0.853	0.033	3.9	22.291
		H7	0.876				17.878
BL5	0.156	G8	0.851	0.846	0.007	0.9	20.258
		H8	0.841				21.213
BL6	0.078	G9	0.804	0.800	0.007	0.9	24.624
		H9	0.795				25.533
BL7	0.039	G10	0.904	0.920	0.023	2.5	15.310
		H10	0.936				12.312
BL8	0.020	G11	0.975	0.985	0.014	1.4	8.630
		H11	0.995				6.756

Based on the observations recorded in table-4.28 as well as fig-4.20 the minimum inhibitory concentration of the essential oil of *C. reticulata* against *S. typhimurium* was found to be 5.18 mg/ml but it was bactericidal at 8.49 mg/ ml. However, the IC_{50} value was recorded as 2.09 mg/ml.

Table 4.29: Antimicrobial activity of *Citrus reticulata* against *Shigella dysenteriae* ATCC 23513

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	G4	0.447	0.460	0.019	4.2	65.311
		H4	0.474				63.198
BL2	1.250	G5	0.872	0.945	0.103	10.9	32.262
		H5	1.018				20.937
BL3	0.625	G6	1.043	1.097	0.076	6.9	18.972
		H6	1.151				10.599
BL4	0.313	G7	1.076	1.123	0.067	5.9	16.401
		H7	1.170				9.092
BL5	0.156	G8	1.036	1.150	0.162	14.1	19.555
		H8	1.265				1.752
BL6	0.078	G9	1.173	1.168	0.008	0.7	8.875
		H9	1.162				9.753
BL7	0.039	G10	1.200	1.221	0.029	2.4	6.793
		H10	1.241				3.609
BL8	0.020	G11	1.266	1.263	0.005	0.4	1.651
		H11	1.259				2.226

Based on the observations recorded in table-4.29 as well as fig-4.21 the minimum inhibitory concentration of the essential oil of *C. reticulata* against *S. dysenteriae* was found to be 2.99 mg/ml but it was bactericidal at 4.89 mg/ ml. However, the IC_{50} value was recorded as 2.05 mg/ml.

Synergistic effect of combined oil

Activity of combined oil of *M. biflora* and *C. reticulata* also determined by the broth micro dialutuion method showed the great antimicrobial activity aginast the *E. coli* and *V.cholerae*. Their Minimum inhibitory concentration and (MIC) and IC_{50} were shown in table 4.30 and fig. 4.22.

Table 4.30: Synergistic (antimicrobial) activity of combined oil against *E.coli* MTCC 723

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	C4	0.338	0.401	0.089	22.1	35.074
		D4	0.464				11.048
BL2	1.250	C5	0.110	0.132	0.031	23.7	78.980
		D5	0.154				70.517
BL3	0.625	C6	0.075	0.090	0.022	24.1	85.601
		D6	0.106				79.671
BL4	0.313	C7	0.048	0.057	0.013	23.0	90.820
		D7	0.066				87.251
BL5	0.156	C8	0.184	0.114	0.099	86.7	64.607
		D8	0.044				91.511
BL6	0.078	C9	0.385	0.366	0.026	7.0	26.208
		D9	0.348				33.174
BL7	0.039	C10	0.435	0.426	0.012	2.9	16.613
		D10	0.417				19.914
BL8	0.020	C11	0.408	0.435	0.039	9.0	21.776
		D11	0.463				11.183

Based on the observations recorded in table-4.30 as well as fig-4.22 the minimum inhibitory concentration of the combined oil against *E. coli was* found to be 0.15 mg/ml but it was bactericidal at 3.09 mg/ ml. However, the IC_{50} value was recorded as 0.081 mg/ml.

Table 4.31: Synergistic (antimicrobial) activity of combined oil against *Vibrio cholerae* MTCC 3906

Sample	Concentration	Wells	Values	Mean Values	Std.Dev.	CV%	%inhibition
BL1	2.500	G4	0.255	0.344	0.126	36.5	50.982
		H4	0.433				16.844
BL2	1.250	G5	0.189	0.167	0.031	18.5	63.647
		H5	0.146				72.072
BL3	0.625	G6	0.093	0.086	0.010	12.1	82.166
		H6	0.078				84.967
BL4	0.313	G7	0.071	0.057	0.020	35.1	86.426
		H7	0.043				91.818
BL5	0.156	G8	0.052	0.049	0.004	8.5	90.033
		H8	0.046				91.166
BL6	0.078	G9	0.334	0.337	0.004	1.2	35.976
		H9	0.339				34.863
BL7	0.039	G10	0.387	0.402	0.021	5.2	25.729
		H10	0.416				20.106
BL8	0.020	G11	0.395	0.415	0.029	6.9	24.270
		H11	0.435				16.479

Based on the observations recorded in table-4.31 as well as fig-4.22 the minimum inhibitory concentration of the combined oil against *V. cholerae* was found to be 0.149 mg/ml but it was bactericidal at 2.97 mg/ ml. However, the IC_{50} value was recorded as 0.080 mg/ml.

Discussion

The potable water supplies vary from large urban system serving populations to small community systems providing water to very small populations. In most countries, they include community sources as well as piped means of water supply. This potable water generally obtained from surface sources as well as ground water such as- rivers, streams and lakes. Such natural water supplies are likely to be polluted with domestic and industrial water. Water is used for bathing, washing clothes, washing utensils and flushing toilets. But most of water taken into the houses may be returned as waste water through drainage system. The potential of pathogenic organisms, water can be in danger to health and life. The malfunctions most frequently transmitted through water are those which cause infections of the intestinal tract, caused by typhoid and paratyphoid bacteria, dysentery (Bacillary) diarrhoea and cholera bacteria.

The present study entitled "*Effect of selected botanical antimicrobials for enhancing potability of drinking water from various sources*" was carried out at Department of Horticulture Aromatic and Medicinal Plants, Mizoram University, Aizawl and Biological product laboratory, Department of Botany, University of Allahabad during year 2009–2011.

Consumption of different water sources for drinking purposes in Allahabad city and Aizawl city

Based on survey the result has been drawn that majority of peoples consumed municipal water directly (91%) in Allahabad city. Similarily the peoples in Aizawl city mainly dependent on the municipal water supply. But, the majority of peoples consumed stored syntex water (62%) because the supply of municipal water was limited to 2 times in a week only.

The bacteriolgical analysis of municipal water samples from different places of Allahabad and Aizawl city

In the present study the area were selected on the basis of density of populations of two city (Allahabad and Aizawl). Out of 140 water samples which was

collected from the 7 places around the city. The bacteriological analysis of drinking municipal water samples showed that 13 out of 20 (65%) of Civil lines, 10 out of 20 (50%) of Daraganj and 9 out of 20 (45%) of Tagore town, 6 out of 20 (30%) of Katra**,** 6 out of 20 (30%) of Chowk, 4 out of 20 (20%) of AU campus water samples were contaminated with coliforms bacteria.

The same case were applied for the Aizawl city, where total number of 75 water samples around city such as Zarkwt (60%) Kulikown (46.66%) MZU campus, Tanhril (40%) Ramhlun (33.33%) and Chaltlang (13.33%) showed the contamination respectivily.

Analysis of Variance (ANOVA) among %age of Contamination among the Water Samples form various sites

The sites for collection of the Samples were selected according to their environmental condition, geographic position, climate, etc. Thus, Allahabad (U. P.) which is located in plain areas with tropical climate with a lot of variation in temperature throughout the year and Aizawl (Mizoram) with location in hilly area and moderately cold climate throughout the year was a better choice for investigation for the researcher as it was comfortable for collection of the samples, had better suited environmental conditions as well as socio- economical parameters were also having variability.

The first site of collection i.e. Allahabad was divided in seven groups of collection sites namely, 1) Civil Lines; 2) Daraganj; 3) Tagoretown; 4) Kitganj; 5) Katra; 6) Chowk and 7) AU Campus. The sites chosen covered the major part of the city. In all the sites, samples were collected in triplicate. A total of 21 sets of samples were thus collected form 7 sites. Further samples were selected randomly and were subjected for evaluation of contamination level in the potable water. This was done through PA Coliform Kit (Himedia). For the estimation of CFU count, the membrane filter technique was applied.

Results obtained were expressed in %age contamination of water samples. The results obtained were subjected to ANOVA Test for the measurement of Variance in the Sample within the group and between the group. It showed significant results in

all the sites except Katra and Chowk sites which were non-significant with same ANOVA coefficient **e.** The contamination level was maximum in the Civil Lines area and minimum in the AU Campus area. The variance in the level of contamination of potable water between the groups may be analysed as such there was a lot of variation in the hygeinicity of the sample area as well as the socio- economic background of the peoples of that area.

Another site of collection was Aizawl, Mizoram with 5 different groups of collection sites namely, 1) Zarkawt; 2) Kulikown; 3) MZU Campus; 4) Ramhlun and 5) Chatlung. Similart criteria were set for selection of the sites for the Water collection. Thus, water was collected form a total of 15 collection sites of the city.

The results of Aizawl also showed variance between the groups when obtained data was subjected to ANOVA analysis. The %age contamination in Water samples were variable significantly. Also non of the group was found insignificant in the ANOVA coefficients obtained. Such a significant result was observed due to variance within the group and between the groups.

Prevalence of bacterial isolates on the basis of Gram's reaction

The basic and general method *viz.,* Gram reaction were applied for the identification of bacterial isolates. It is the scientifically approved and an easy method for bacterial identification. Based on Gram reaction the highest concentration of *E.coli* (78 + 53 =131) were obtained in collected water samples from Allahabad city and Aizawl city followed by *S. dysenteriae* (64 = 48 = 112) and *S. typhimurium* (58 = 39 = 97). It was found that where the *E. coli* bacteria were found in drinking water the other two bacteria were also present in bulk.

Identification of water borne bacterial isolates

The specific medium was uesd for the growth of particular organism. Such as the *S. typhimurium,* and *Shigella dysentriae* growing in MacConkey Agar showed the smooth, colourless colonies, 2-4 mm and 2-3 mm diameter (WHO, 2010). Based on this the 6 isolates were randomaly selected from each isolated species of bacteria for their biochemical characterization. The random selection made the data easy to for discussion. The Gram reaction and biochemical

identification confirmed that all tested pathogens were found abundantly in drinking water which causes the water borne bacterial diseases. The percentage of *E.coli* was found to be high in each water samples. So the cases of diarrhoeal diseases were more common in both places. Similar observations were made by the Rajendran, *et al.,* (2006).

Identification of water borne bacterial isolates on the basis of gene specific primer

Based on their specific gene such as from *E. coli* rfbE gene 239 bp, from *V. cholerae* ctx gene 432 bp, *S. typhimurium* STM4497 gene 360 bp and from *S. dysenteriae* ipaH gene 884 bp. The isolated new bacterial isolates showed the maximum similarity with the DNA amplification of standard strains through Real Time-PCR.

Role of bioinformatics in phylogenitic relationship of bacterial isolates

The computer based bioinformatical tool was used for detection of phylogenetic evolution of target organisms which showed their accurate origin. In the present study, specific genes were taken to evaluate the phylogeny of tested pathogens. Based on the YeeV protein in *E.coli*, CTX cholera toxin in *V. cholerae*, bla_{CARB-2} resistant gene in *S. typhimurium* and shiga toxin (ipaH gene) in *S. dysenteriae* showed close relation in their phylogeny. When the *E. coli* cladogram and phylogram tree were constructed with the *V. cholerae, S. typhimurium* and *S. dysenteriae,* it was found that the *E.coli* was very close to *S. dysenteriae* in its origin, and *S. dysenteriae* with *S. typhimurium, E.coli* and *V.cholerae* the same case have been found. So, it confirmed that *S. dysenteriae* have same origin as that of *E. coli* as predicted by blast analysis in CLUSTALW software, when YeeV gene of *E. coli* and ipaH gene of *S. dysentriae* were blasted separately with other bacteria in blast.

CHARACTERIZATION OF THE BIOACTIVE ESSENTIAL OILS

In the present investigation, 4 plant essential oil namely *Mentha piperita, Mentha arvensis*, *Micromeria biflora* and *Citrus reticulata* were taken for the screening their antibacterial against water borne bacterial pathogens.

The GC-MS analysis of *Mentha piperata* L. essential oil showed a total of 14 components (Table 4.16). Out of 14 components; Menthol 37.20% and Menthone 22.52% were the major compounds. Kjonaas and Croteau (1983) and Dwivedi *et al*., (2004)reported that the volatile oil of mature *Mentha piperata* (peppermint) leaves contained the oxygenated p-menthane monoterpenes l-menthol (47%) and l-menthone (24%) as well as very low levels of the monoterpene olefins limonene (1%) and terpinolene (0.1%), as major components. Previous investigations on *M. piperita* oil composition are consistent with our results in which menthone and menthol was found to be the major compounds (Lawrence, 1997; Gerherman, *et al.,* 2000; Aflatuni, *et al.,* 2000; Stojanova, *et al.,* 2000; Maffei, 1999; Rohloff, 1999; Chalchat, *et al*., 1997; Spencer, *et al*., 1997).

On the basis of GC-MS analysis of *Mentha arvensis* L. essential oil found 8 compounds, but out of 8 compounds, the percentage of L-Menthone (29.41) and menthol (21.33%) was greater than others compounds.

Adnan (2006) was obtained *Micromeria biflora* ssp. Arabica K. Walth, essential oil by hydrodistillation method and analyzed by GC-MS, 30 components were identified representing 98.2% of the total oil. The major constituents were trans-caryophyllene (43.7%), caryophyllene oxide (18.0%), spathulenol (8.5%), α-humulene (4.6%), α-myrcene (3.1%), and germacrene-D (3.1%). However in the present study, composition and relative percentages of essential oil of *Micromeria biflora* was determined. 4 major constituents were identified with high content of Thymol 54%, Isothymol 9.9%, Gurjurene 3.3% and β-caryophyllene 6.6% with RetentionTime (RT) 8.06, 8.83, 10.20 and 12.86 respectively. The present study GC-MS analysis of *Micromeria biflora* essential oil, chemical constituents were quite different due to different agroclimatic changes.

Citrus reticulata L. essential oil have the monoterpene fraction was predominant, accounting for more than 89.6%, where Limonene was the most abundant component (80.3%), followed by *γ*-terpinene (4.7%), myrcene (2.1%) and *α*-pinene (1.2).

IN-VITRO STUDY OF THE SELECTED ESSENTIAL OILS AGAINST THE TESTED BACTERIAL PATHOGENS

Antimicrobial activities of bioactive essential oils and its active constituents against water borne bacterial isolates

Antimicrobial activities of selected bioactive essential oils were tested and the results were given in the form Minimum inhibitory concentration (MIC), IC_{50} and minimum bactericidal concentration (MBC). *In vivo* studies may be required to confirm the validity of some of the results obtained. The need for a standard, reproducible method for assessing oils has been stressed by several authors (Carson, *et al*., 1995; Mann and Markham, 1998). In view of this, many methods have been developed specifically for determining the antimicrobial activity of essential oils (Remmal, *et al*., 1993; Carson, *et al*., 1995; Smith and Navilliat, 1997; Mann and Markham, 1998). The benefits of basing new methods on preexisting, conventional assays such as the NCCLS methods are that these assays tend to be more readily accepted by regulatory bodies (Carson, *et al*., 1995; Smith and Navilliat, 1997).

The minimum inhibitory concentration of the essential oil of *Mentha piperita* against *E. coli* was found to be 2.58 mg/ml but it was bactericidal at 5.11 mg/ ml. However, the IC_{50} value was recorded as 0.928 mg/ml. Where as in case of *V. cholerae* the minimum inhibitory concentration of the essential oil of *Mentha piperita* was found to be 2.89 mg/ml but it was bactericidal at 5.10 mg/ ml. and IC_{50} value was 0.951 mg/ml. Fleming (1998) reported that, due to many potent compounds such as menthol, menthone, limonene, etc., in *M. piperita* showed significant anti-microbial activity. These compounds have higher medicinal value especially in the treatment of dyspepsia, epigastric bloating, impaired digestion. Deans and Baratta (1998) also investigated that the compounds from *M. piperita* possess anti-microbial activity and suggesting that the *M. piperita* leaf extracts should contains the effective active constituents responsible for eliminating the bacterial pathogens**.** The effect of components of essential oil on cell membrane integrity of gram positive and gram negative bacteria has been previously reported (Cox, *et al*., 2000; Oussalah, *et al*., 2006. Mahboubi and Haghi (2008) screened the antimicrobial activity of essential oil from flowering aerial parts of Iranian *M. pulegium* L. against different

microorganisms. They reported significant activity against gram positive bacteria with MIC values in the range of 0.25-4 µl/ml whereas the least susceptible were gram negative bacteria, especially *E.coli*. The essential oil of *Mentha piperita* was earliar found to display good to excellent antimicrobial activities (MIC values 1-8 µl/ml) against *E.coli. S. aureus* and *C. albicans* (Yadegarina, *et al*., 2006). Mimica- Duke, *et al*., (2003) reported low MIC (4 µl/ml) of *M. piperita* oil for *E.coli* and 8 um/ml for *C. albicans*. Hammer, *et al*., (1999) reported cidal activity of *M. piperita* oil at 0.25% (v/v) equivalent to 25 µl/ml for *E. coli* and *C. albicans* and 12 µl/ml for *S. aureus.* The similar results were observed by many researchers earliar (Takahashi, *et al*., 2004l; Bakkali, *et al*., 2008).

Similarly the minimum inhibitory concentration of the essential oil of *Mentha piperita* against *S. typhimurium* was found to be 3.84 mg/ml but it was bactericidal at 4.60 mg/ ml. and their IC_{50} value was recorded as 1.034 mg/ml. In another study the highest MIC value (15.6 µg/ml) was observed against *P.aeruginosa, S. pyogenes, B.subtilis, E.coli* K-12, *S. mutans* and *Salmonella typhimurium* while *S. faecalis, S. aureus* and *L. acidophilus* ranked next (MIC 31.2 µg /ml (Mathur, *et al*., 2011).

The minimum inhibitory concentration of the essential oil of *Mentha piperita* against *S. dysenteriae* was found to be 1.92 mg/ml but it was bactericidal at 2.80 mg/ ml. and the IC_{50} value was 0.483 mg/ml. Results associated with the antimicrobial effects of essential oil of *M. piperita* L. (Peppermint oil) were similar to the previous finding in the literature (Sow, *et al*., 1995; Pattnaik, *et al*., 1996, 1998; Mimica-Dukic, *et al*., 2003).

The minimum inhibitory concentration of the essential oil of *Mentha arvensis* against *E. coli* was found to be 2.46 mg/ml and it was bactericidal at 5.81 mg/ ml. with the IC_{50} value 1.742 mg/ml. Several studies (Pattnaik et al., 1997; Sartoratto et al., 2004) have shown that different *Mentha* species also inhibited the growth of bacterial pathogens. Earlier studies suggested that the antibacterial activity of *M. arvensis* L. essential oil was probably due to their major component (Alves, *et al.,0* 2009). The MIC of the essential oil of *Mentha arvensis* against *V. cholerae* was found to be 0.792 mg/ml having bactericidal at 2.86 mg/ ml concentration and the IC_{50} value 0.283 mg/ml.

While in case of *S. typhimurium* the MIC of the essential oil of *Mentha arvensis* was found to be 3.72 mg/ml and their bactericidal at 6.10 mg/ ml. IC_{50} value was 0.850 mg/ml. The oil of *Mentha arvensis* showed the minimum inhibitory concentration against *S. dysenteriae* was 1.32 mg/ml and their bactericidal at 3.01 mg/ ml. with IC_{50} value was 0.320 mg/ml. The efficacy of *Mentha arvensis* oil were varried and supported the work with Zaika, (1988) according him the antimicrobial activity of many essential oils has been previously reviewed and classified as strong, medium or week. Essential oils are potential sources of novel antimicrobial compounds especially against bacterial pathogens (Prabuseenivasan, *et al.*, 2006).

The minimum inhibitory concentration of the essential oil of *M.biflora* against *E.coli* was found to be 0.308 mg/ml but the bactericidal concentration was 2.80 mg/ ml. with IC_{50} value was recorded as 0.091 mg/ml. The MIC of the essential oil of *M.biflora* against *S. typhimurium* was found to be 0.202 mg/ml, bactericidal at 2.96 mg/ ml. and IC_{50} value was 0.104 mg/ml. The essential oil of *M. biflora* showed the highest minimum inhibitory concentration against *S. dysenteriae* (MIC 0.178 mg/ml) but the bactericidal at 2.60 mg/ml. However, their IC_{50} value was recorded as 0.094 mg/ml. Duru et al., (2004) worked on the antimicrobial activity of five different extractions and pulegone, which is the main component of *Micromeria cilicica*, has been evaluated *in vitro* against nine bacterial species and one yeast) which are known to cause infections in humans. The oil of *M. biflora* was not effective against the *V.cholerae.* The efficacy of oil according to their minimum inhibitory concentration the oil was found to be very active against *S. dysenteriae*< *E.coli*< *S. typhimurium* respectively.

In case of *Citrus reticulata* the minimum inhibitory concentration of the essential oil of against *V. cholerae* was found to be 8.06 mg/ml having 12.1 mg/ ml was bactericidal concentration and the IC_{50} value was 4.19 mg/ml. While the minimum inhibitory concentration of the essential oil of *C. reticulata* against *S. typhimurium* was found to be 5.18 mg/ml but it was bactericidal at 8.49 mg/ ml. with the IC_{50} value was recorded as 2.09 mg/ml. But in case of *S. dysenteriae* the minimum inhibitory concentration of the essential oil of *C. reticulata* was found to be 2.99 mg/ml but it was bactericidal at 4.89 mg/ ml. with the IC_{50} value was recorded as 2.05 mg/ml. Here the oil was found to be not effective against the *E.coli.* Tao et al., (2009)

reported that antimicrobial activity of C. reticulate against E.coli and S. aureus etc by disc diffusion method and found that oil of C. reticulta used for control of these microorganism.

Synergistic effect of combined oil

Research on synergism is very limited and few studies have been reported (Nascimento, *et al*., 2000, Aburjai, *et al*., 2001, Aqil, *et al*., 2005). Activity of combined oil of *M. biflora* and *C. reticulata* also determined by the broth micro dialutuion method showed the great antimicrobial activity aginast the *E. coli* and *V.cholerae*. Essential oils may be considered to be used in combination with standard topical and antibiotic therapies (Settineri and Krassner, 2003). Thus, in our research, the minimum inhibitory concentration of the combined oil against *E. coli was* found to be 0.15 mg/ml and it was bactericidal at 3.09 mg/ ml. However, the IC_{50} value was recorded as 0.081 mg/ml. while in case of the minimum inhibitory concentration of the combined oil against *V. cholerae* was found to be 0.149 mg/ml but it was bactericidal at 2.97 mg/ ml and the IC_{50} value was recorded as 0.080 mg/ml. Considering the MIC values, of *M. biflora* oil the percentage of inhibition of essential oil was better for *E*. coli (ATCC) than *E*. coli (MTCC). These differences found in between the same strains of *E.coli* i.e., *E.coli* (ATCC) and *E.coli* (MTCC) was only due to the differences in their physiochemical and environmental conditions. The differences in their cell wall chemistry are also responsible for the alteration in MIC values. The oil has greater activity against *S.dysenteriae* (ATCC) and has moderate activity against *E.coli* (ATCC). According to Adnan, (2006) the oil of *M. biflora* spp. *arabica* was tested against a range of microorganisms but it have no activity at 1000µl/mL, but in present study the essential oil has no activity only in case of *V.cholerae* (MTCC). Similarly the essential oil of *C.reticulata* has showed the activity against above tested water borne bacterial pathogens. The oil has more activeness against *E.coli* (ATCC) and showed low activity against *V.cholerae* (MTCC), but here the oil didn't show the any activity against *E.coli* (MTCC). Finally, it was observed that the essential oils of *M. biflora* and *C.reticulata* did not showed an inhibitory activity at all the concentrations, as can be seen, the inhibitory effect increased with increasing concentrations. Comparatively the efficacy of essential oils was determined on the basis of percentage of inhibition which recorded in form of minimum

inhibitory concentration through the Spectra max-plus^{384} (ELISA reader). Then the oil of *M. biflora* was found to be more active than the oil of *C. reticulata*.

Two sets of experiment have been conducted in present study, in the first set both oil active against different pathogens separately and their MIC values was high. However in case of mixture oil, the inhibition activity increases due to synergistic effects and comparatively their MIC values lower when the oil used separately. In spite of this mixture oil active against *E.coli* (MTCC) and *V.cholerae* (MTCC). This variation in antibacterial activities of essential oil may be related to the concentration and nature of contents, to the respective composition, the functional groups, the structural configuration of the components and their possible synergistic interaction (Chang, *et al*., 2001). In other synergism study, Betoni, *et al*., (2006) found that plant extracts with weak action on *S. aureus* growth, such as lemongrass, is an important data since it showed a synergism profile similar to that of the clove extract, considered the most efficient *S. aureus* growth inhibitor in this study. The researchers should investigate the synergistic capacity of plant extracts or other natural products, independent of the antimicrobial activity they have. Further, the results of the present study seem to be promising and may enhance the natural product uses, showing the potential of these plants in the treatment of infectious diseases caused by water borne bacterial pathogens.

Thus, the tested botanicals after successful toxicological, organoleptic and pharmacological investigations can be developed as the formulation for enhancing the potability of drinking water.

Summary

Water-water everywhere, but potable water usually nowhere or very precious. Potable water is very much essential for keeping the body healthy so that we can have healthy mind which will lead to excellence.

Available or existing methods of having potable water such as RO (Reverse Osmosis), Aqua guard, filter etc. are beyond the reach of common man which is forming the larger masses. Chlorine tablets used for chlorination by inactivating or eliminating the water borne pathogens is the chemical means for making the water potable. Larger doses of chlorine are reported to have toxic effects. With the objective of exploiting botanical diversity particularly of North-eastern region against water borne microbes by secondary metabolites extracted in the form of essential oils of 15 aromatic plants using Clevenger's Apparatus have been tested against *Escherichia coli* MTCC 723, *Vibrio cholerae* MTCC 3906, *Salmonella typhimurium* MTCC 98 and *Shigella dysentriae* ATCC 2513 using primarily Disc diffusion method which is basically a qualitative technique.

4 essential oils extracted from *Mentha piperita, Mentha arvensis, Micromeria biflora* and *Citrus reticulata* were found to be more effective against all tested pathogen exhibiting 19, 20, 24 and 25 mm zone of Inhibition respectively.

Quantitative antimicrobial testing of the aforementioned 4 plants have been made following NCCLS method renamed as CLSI method which is globally accepted using ELISA Reader of Molecular Devices USA model SpectramaxPlus^{384} and the observations were summarized in the following table:

Plant	***Water-borne Pathogens***							
	E. coli		***V. cholerae***		***S. dysentriae***		***S. typhimurium***	
	IC_{50}	**MIC**	**IC_{50}**	**MIC**	**IC_{50}**	**MIC**	**IC_{50}**	**MIC**
M. piperita	0.928	2.584	0.951	2.893	1.034	3.840	0.483	1.920
M. arvensis	1.742	2.461	0.283	0.729	0.850	3.722	0.320	1.322
M. biflora	0.091	0.308	i.a	i.a	0.104	0.202	0.940	0.178
C. reticulata	I.a	I.a	4.190	8.060	2.090	5.18	2.051	2.990
Combined oil	0.081	0.150	0.080	0.149	NC	NC	NC	NC

i.a- in active, NC- Not considerd

The dysentery causing organism was effectively controlled by *M. piperita* and *M. biflora* oils at the MIC of 1.520 mg/ml and 0.178 mg/ml respectively while cholera causing organism has been inactivated by *M. arvensis* at the MIC of 0.729 mg/ml. It was found quite interesting that *V. cholerae* and *E. coli* could not be controlled effectively by *M. biflora* and *C. reticulata* alone, but when they were mixed together in the ratio of 1:1, then synergistically exhibited MIC of 0.150 and 0.149 mg/ml against *E. coli* and *V. cholerae* respectively.

CHARACTERIZATION OF THE BIOACTIVE ESSENTIAL OILS

The effective oils were characterized by GC-MS Spectroscopy. The present investigation involved 4 plants essential oil many *Mentha piperita, Mentha arvensis*, *Micromeria biflora* and *Citrus reticulata* were taken for the screening against water borne bacterial pathogens. The essential oils were extracted and their GC-MS analysis showed the various constituents.

The GC-MS analysis of *Mentha piperita* L. essential oil resulted total of 14 components. Out of 14 components 2 compounds i.e. Menthol 37.20% and Menthone 22.52% was the major compounds.

The monoterpene fraction was predominant, accounting for more than 89.6%, where Limonene was the most abundant component (80.3%), followed by γ-terpinene (4.7%), myrcene (2.1%) and α-pinene (1.2).

The identification of isolated compounds were made by comparing the retention indices of the peaks on the BP-1 column with literature values, computer matching of stored library spectra which was previously built up same pure substances and components of known essential oils. The final confirmation carried out by comparison of mass spectra with published data. Based on above application, it was concluded that essential oil of *Mentha piperita* have Menthol, as their major compound. Similarly in case of *Mentha arvensis*, the second major compound was menthol, however in case of *Micromeria biflora*, Thymol was the major compound and Limonene was the major compound in *C. reticulata.*

Molecular explanation of Phylogeny vis a vis Antimicrobial activity

To find out why these secondary metabolites (Essential oil) of the aromatic plants exhibited variable MICs against water borne bacterial pathogens, bioinformatical tool has been taken into consideration to minimize the cost of experimentation as well as to reduce the time also.

The Database record for protein, toxin as well as specific gene on NCBI (National Center for Biotechnology Information) got the various strains of *E.coli, V. cholerae, S. typhimurium* and *S.dysenteriae.* With the help of computer based bioinformatical tool; the ClustalW, the interpretation of their phylogenetic evaluation were made and their blast results was shown as follows:

In *E.coli*, YeeV protein taken as the base with their reference number was >gi|47155005|emb|CAE85204.1| YeeV protein [*Escherichia coli*] and found 11 strains of S. *dysenteriae*, 10 strains of *S. typhimuriun* and 10 strains of *V. cholerae*.

Same as in case of *V. cholerae*, the cholera toxin taken as the base with assertion number was >gi|166788581|dbj|BAG06739.1| cholera toxin B subunit [Vibrio phage CTX] and found 10 strains of *S. typhimurium*, 2 strains of *E.coli* and 10 strains of *S. dysenteriae*.

The bla_{CARB-2} resistant gene made as base in case of *S. typhimurium* with their assertion no. >gi|4063860|gb|AAC98501.1| qac delta E [*Salmonella typhimurium* DT104] and found 11 strains of *E.coli*, 11 strains of *V. cholerae* and 11strains of *S. dysenteriae*. Similarly shiga toxin as the base and with assertion number >gi|82776677|ref|YP_403026.1| Shiga toxin subunit B precursor [*Shigella dysenteriae* Sd197] in case of *S. dysenteriae* and found 7 strains of *E.coli*, 10 strains of *V. cholerae* and 1 strains of *S. typhimurium*.

Molecular explanation at gene/protein level is that similar resemblance in gene/protein sequencing showed almost similar MIC of the secondary metabolites of the effective aromatic plants.

Thus, the tested botanicals after successful toxicological, organoleptic and pharmacological investigations can be developed as the formulation for enhancing the potability of drinking water.

Bibliography

Aboaba, O.O., Smith, S.I. and Olude, F.O. (2006) Antibacterial Effect of Edible plant Extract on *Escherichia coli* 0157:H7. *Pakistan Journal of Nutrition 5(4):* 325-327.

Aburjal T, Darwish RM, Al-Khalil S, Mahgzah A, and Al-Abbdi A (2001) Screening of antibiotic resistant inhibitors from local plant materials against two different strains of Pseudomonas aeruginosa. *J Ethnopharmacol* 76: 39-44.

Adnan J. Al-Rahaily (2006) Composition of Essential oil of *Micromeria biflora* ssp *arabica* K. Walth *Pak. J. of Biological Sci.* 9(14): 2726-2728.

Adnan J. Al-Rehaily. (2006) Composition of essential oil of *Micromeria Biflora* ssp. *arabica* K. walth. *Pak. J. of Bio. Sciec*; 9(14): 2726-2728.

Agaev ,M.G. (1988) Main field-weed plants of the Leningrad Region. The catalogue of VIR world collection, Issue 468. Leningrad: VIR. 68-70. (In Russian)

Al - Samawi, A. A. and Shokralla, E. M. (1996) Investigation into an indigenous natural coagulant . J. Envir. Sci. Health,31(8),1881-1897.

Alamanos Y et al. (2000) A community waterborne outbreak of gastro-enteritis attributed to Shigella sonnei. *Epidemiology and Infection,* 125:499–503.

Alves TM, Salvia AF, Brandao M, Grandi TS, Smania EF and Junior AS,. (2009) Biological screening of Brazilian medicinal plants. Memorias do Instituto Oswaldo Cruz,; 93: 67-73.

Angulo FJ et al. (1997) A community waterborne outbreak of salmonellosis and the effectiveness of a boil water order. American Journal of Public Health, 87:580–584.

Anon. (1994) The Microbiology of Water, Part 1-Drmkmg Water. Report on Public Health and Medical subjects. No. 71 Methods for the examination of waters and associated materials. London. Stationery Office.

Anon. (1996) Guidelines for Drinking Water Quality World Health Organisation International Programme on Chemical Safety Geneva WHO.

Anon. (1998) Cryptosportdtum in Water Supplies. Third Report of the Group of Experts to: Department of the Environment, Transport and the Regions & Department of Health.

Anon. (1999) Anglian Water pleads guilty to charges of supplying water unfit for human consumption Press Notice 110, 8 February Department of the Environment, Transport and the Regions.

Anonymous. (1996) Weeds on sugar beet. Berlin: Hoehst Schering AgrEvo GmbH. 242 pp. (In German, English, Russian, Ukrainian)

Arbeit, R.D. (1999). Laboratory procedures for the epidemiologic analysis of microorganisms. In:Manual of clinical microbiology. ASCE 19: 520–539

Atherton F, Newman CPS, and Casemore DP. (1995) An outbreak of waterborne cryptospondiosis associated with a public water supply in the UK. *Epidemiol Infect* 115. 123-31.

Audrey L. B., Stroot, J. M., Lim, D. V. Stroot, P. G. and Alcantar, N. A. (2010). Removal of Sediment and Bacteria from Water Using Green Chemistry, *Environ. Sci. Technol*, 44 (9), 3514–3519.

Battista, J. R. and Rainey, F. A. (2001). Family I. Deinococcaceae Brooks and Murray 1981, 356, VP emend. Rainey, Nobre, Schumann, Stackebrandt and da Costa 1997, 513. In "Bergey's Manual of Systematic Bacteriology vol.1, The Archaea and the Deeply Branching and Phototrophic Bacteria" (D. R. Boone and R. W. Castenholz, Eds.), Springer, New York.

Betoni, J. E. C., Mantovani, R. P., Barbosa, L.N., Stasi, L. C. Di and Junior A. F. (2006) Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. 101(4): 387-390,

Bhole, A. G. (1995) Relative evaluation of a few natural coagulants ,Aqua- Journal of Water Supply: Research and Technology ,44(6), 284-290.

Biddlestone A. J., Gray K. R. and Thurairajan, K.(1991) A botanical approach to the treatment of wastewaters. Journal of biotechnology , 17(3),209-220.

Bilge SS, Vary J, James C, Dowell SF and Tarr PI. (1996)Role of the Escherichia coli O157:H7 side chain in adherence and analysis of an rfb locus. Infect Immun;64: 4795–801.

Boris, R.P. (1996) Natural products research: perspectives from a major pharmaceutical company. *J. Ethnopharmocol*. 51:29-38.

Bourke, John G. (1895) "The Folk-Foods of the Rio Grande Valley and of Northern Mexico." *The Journal of American Folklore* 8(28): 41-71.

Briggs, C. E. and Fratamico, P. M. (1999) Molecular characterization of an antibiotic resistance gene cluster of Salmonella typhimurium DT104, Antimicrob. Agents Chemother. 43 (4), 846-849 (1999)

Brown, J. R., Douady, C. J., Italia, M. J., Marshall, W. E., and Stanhope, M. J. (2001). Universal trees based on large combined protein sequence data sets. Nat.Genet.28, 281-285.

Brugha A, Vipond IB, and Evans MR . (1999) A community outbreak of food borne small round-structured virus gastroenteritis caused by a contaminated water supply. *Epidemiol Infect*; 122. 145-54.

Brüssow H, Canchaya C, Hardt WD (September 2004). "Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion". Microbiol. Mol. Biol. Rev. 68 (3): 560–602.

Bupesh, G., Amutha, C., Nandgopal, S., Ganeshkumar, A., sureshkumar, P. and Murali, S.K. (2007) *Acta agriculturae Slovenica*, 89(1), 73 - 79.

Call DR, Brockman FJ, and Chandler DP. (2001) Detecting and genotyping Escherichia coli O157:H7 using multiplexed PCR and nucleic acid micro arrays. *Int J Food Microbiol*;67:71–80.

Carson, C.F., Mee, B.J. and Riley, T.V (2002) Mechanism of action of Melaleuca alternifolia (tea tree) oil on Staphylococcus aureus determined by time-kill, lysis, leakage and salt tolerance assays and electron microscopy. Antimicrobial Agents and Chemotherapy 46 (6), 1914–1920.

Caufield, P.W. and Walker, T.M. (1982). Genetic diversity within *Streptococcus mutans* evident from chromosomal DNA restriction fragment polymorphism. *J Clin Microbiol.;* 27: 274-278.

Cavalier-Smith, T. (2002). The neomuran origin of archaebacteria, the negibacterial root of the universal tree and bacterial megaclassification. Int.J.Syst.Evol.Microbiol.52, 7-76.

Chang S T, Chen PF, Chang SC. (2001) Antibacterial activity of leaf essential oils and their constituents from *Cinnamomum osmophloeum*. *Journal of Ethnopharmacology* 77: 123–127.

Ciccarelli, F. D., Doerks, T., von Mering, C., Creevey, C. J., Snel, B., and Bork, P. (2006). Toward automatic reconstruction of a highly resolved tree of life. Science311, 1283-1287.

Constantine, C.L., Hales, D., and Dawson DJ.(1994) Outbreak of giardiasis caused by a contaminated private supply in the Worcester area. In: Berts WB, Casemore D, Fncker C, Smith H, Watkins J (Eds) Protozoan Parasites and Water London: Royal Society of Chemistry,; 50-2.

Cosentino, S., Tuberoso, C.I.G., Pisano, B., Satta, M., Mascia, V., Arzedi, E., and Palmas, F., 1999. In vitro antimicrobial activity and chemical composition of Sardinian Thymus essential oils. *Letters in Applied Microbiology* 29, 130– 135.

Council Directive 80/778/EEC of 15 July 1980 relating to the quality of water intended for human consumption. Official] EC 1996; L229- 11-9.

Council Directive of 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Official J EC 1998; L330: 32-54.

Cox, S.D., Mann, C.M., Markham, J.L., Bell, H.C., Gustafson, J.E., Warmington, J.R. and Wyllie, S.G. (2000) The mode of antimicrobial action of essential oil of Melaleuca alternifola (tea tree oil). *Journal of Applied Microbiology* 88, 170–175.

Dalsgaard A., Reichert, P., Mortensen, H. F., Sandstrom, A., Kofoed, E., Larsen, J. L. and Molbak, K. (1996) "Application of lime (Citrus aurantifolia) juice to

drinking water and food as a cholera-preventive measure" Journal of food, 60(11) 1329-1333.

Daubin, V., Gouy, M., and Perriere, G. (2002) A phylogenomic approach to bacterial phylogeny: evidence of a core of genes sharing a common history. Genome Res.12, 1080-1090.

David Odee (2004) *Moringa oleifera* Canadian Biotechnical Division Kenya Forestry Research Institute and Moringa Medical research institute.

David R. Knighton, M.D. and Fiegel, B.S (2008) "Sphagnum Moss, Bacterial Biofilm and Water Quality" Water Conditioning and Purification Magazine.

Davidson, P.M., (1997) Chemical preservatives and natural antimi- crobial compounds. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), *Food Microbiolog*y: Fundamentals and Frontiers. ASM, Washington, pp. 520–556.

Deans, S.G., Baratta, M.T. (1998) Antimicrobial & Antioxidant properties of some essential oils. Flau.Fragrance, 235-244.

Denyer, S.P., and Hugo, W.B. (1991b). Mechanisms of antibacterial action A summary. In: Denyer, S.P., Hugo, W.B. (Eds.), Mechanisms of Action of Chemical Biocides. Blackwell, Oxford, pp. 331– 334.

Denyer, S.P., Hugo, and W.B. (1991a). Biocide-induced damage to the bacterial cytoplasmic membrane. In: Denyer, S.P., Hugo, W.B. (Eds.), Mechanisms of Action of Chemical Biocides. *The Society for Applied Bacteriology*, Technical Series No 27. Oxford Blackwell Scientific Publication, Oxford, pp. 171– 188.

Dorman, H.J.D., Deans and S.G., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology* 88, 308– 316

Dorman, H.J.D., Deansand S.G., (2000) Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology* 88, 308– 316

Duru, M. E.,, Öztürk, M., gur A. U. and C, Özgür. (2004) The constituents of essential oil and in vitro antimicrobial activity of Micromeria cilicica from Turkey . *Journal of Ethnopharmacology* 94 43–48

Ehara, M., Nguyen, M. B., Nguyen, T. D., Ngo, C. T., Le,H. T., Nguyen, T. H. and Iwami, M. (2008) Epidemic strains of Vibrio cholerae O1, biotype El Tor with CTX prophage integrated into both chromosomes, Published Only in Database.

Eisen, J. A. (1995) The RecA protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of RecAs and 16S rRNAs from the same species. J.Mol.Evol.41, 1105-1123.

Environmental Research Laboratory, Economically & Environmentally Safe Approach for Purification of Drinking Water in the Rural Households, Environmental Research Laboratory, Lucknow, India (1999)Foster S. Ginger - Zingiber officinale.htm.2000: 1-3.

Escartin EF et al. (2002) Potential Salmonella transmission from ornamental fountains. Journal of Environmental Health, 65:9–12.

Farag, R.S., Daw, Z.Y., Hewedi, F.M., El-Baroty, and G.S.A. (1989) Antimicrobial activity of some Egyptian spice essential oils. *Journal of Food Protection* 52 (9), 665– 667.

Faruque, Shah M.; and Nair, Balakrish, eds (2008) *Vibrio cholerae:* Genomics and Molecular Biology. *Caister Academic Press*. 978-1-904455-33-2.

Faruque, SM; and Nair,GB (2002) "Molecular ecology of toxigenic Vibrio cholerae". *Microbiology and immunology* 46 (2): 59–66. PMID 11939579.

Fleming T, 1(998) PDR for Herbal medicines: Medical Economic Company, JNC.

Forster S. 1996: Peppermint: *Mentha piperita,* American Botanical Council – Botanical

Fratamico PM, Sackitey SK, Wiedmann M, and Deng MY. (1995) Detection of Escherichia coli O157:H7 by multiplex PCR. *J Clin Microbiol*;33:2188–91.

Furtado C, Adak GK, Stuart JM, Wall PG, Evans HS, Casemore DP. Outbreaks of waterborne infectious intestinal disease in England and Wales, 1992-5. *Epidemiol Infect*; 121: 109-19.

Gannon VPJ, D'souka S, Graham T, King RK, Rahn K, Read S. (1997) Use of flagellar H7 gene as a target in multiplex PCR assays and improved specificity

in identification of enterohemorrhagic Escherichia coli strains. *J Clin Microbiol*;35:656–62.

Garrett, A. (2007) A Sphagnum Solution for Safe Water. Taiwan International Science Fair Canada

Gilbert C, Winters D, O'Leary A, Slavik M. (2003)Development of a triplex PCR assay for the specific detection of Campylobacter jejuni, Salmonella spp. *Mol Cell Probes;*17:135–38.

Gogarten, J. P., Doolittle, W. F., and Lawrence, J. G. (2002) Prokaryotic evolution in light of gene transfer. Mol.Biol.Evol.19, 2226-2238.

Gray, M.J. (1997) Assessment of water supply and associated matters in relation to the incidence of cryptospondiosis in West Herts and North London in February and March.

Griffiths, E. and Gupta, R. S. (2004) Signature sequences in diverse proteins provide evidence for the late divergence of the order Aquificales. International Microbiol.7, 41-52.

Griffiths, E. and Gupta, R. S. (2007) Phylogeney and signature sequences provide evidence that Verrucomicrobia ar the closest free-living relatives of the parasitic chlamydiae. Microbiology (in press).

Grimont, F. and Grimont, P.A.D. (1986). Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann Inst Pasteur/Microbiol.*; 137B: 165-175.

Gryko R, Sobieszczanska BM, Stopa PJ, and Bartoszcze MA. (2006) Comparison of multiplex PCR, and an immunochromatographic method sensitivity for the detection of Escherichia coli O157:H7 in minced beef. *Pol J Microbiol*;51;121–9.

Gubala AJ, and Proll DF. (2002) Molecular-beacon multiplex real-time PCR assay for detection of Vibrio cholerae. Appl Environ Microbiol 72:6424-28.

Gülay Kirbaşlar, F., Aydin Tavman, Başaran Dülger and Gülen Türker (2009).Antimicrobial activity of Turkish *Citrus* Peel Oils. *Pak. J. Bot*., 41(6): 3207-3212

Gupta, A. and Chaudhuri, M. (1992) Domestic water purification for developing countries. *Aqua* 45(5): 290-298.

Gupta, R. S. (1998) Protein Phylogenies and Signature Sequences: A Reappraisal of Evolutionary Relationships Among Archaebacteria, Eubacteria, and Eukaryotes. Microbiol.*Mol.Biol.Rev*.62, 1435-1491.

Gupta, R. S. (1998) What are archaebacteria: Life's third domain or monoderm prokaryotes related to Gram-positive bacteria? A new proposal for the classification of prokaryotic organisms. *Mol.Microbiol*.29, 695-708.

Gupta, R. S. (2000) The phylogeny of Proteobacteria: relationships to other eubacterial phyla and eukaryotes. *FEMS Microbiol.Rev*.24, 367-402.

Gupta, R. S. (2001) The branching order and phylogenetic placement of species from completed bacterial genomes, based on conserved indels found in various proteins. *Inter.Microbiol*.4, 187-202.

Gupta, R. S. (2002) Phylogeny of Bacteria: Are we now close to understanding it? ASM News68, 284-291.

Gupta, R. S. (2003) Evolutionary Relationships Among Photosynthetic Bacteria. *Photosynth.Res*.76, 173-183.

Gupta, R. S. (2004) The Phylogeny and Signature Sequences characteristics of Fibrobacters, Chlorobi and Bacteroidetes. *Crit.Rev.Microbiol*.30, 123-143.

Gupta, R. S. (2005) Molecular Sequences and the Early History of Life. In "Microbial Phylogeny and Evolution: Concepts and Controversies" (J. Sapp, Ed.), Oxford University Press, New York.

Gupta, R. S. and Griffiths, E. (2002) Critical Issues in Bacterial Phylogenies. Theor.Popul.Biol.61, 423-434.

Gupta, R. S., Bustard, K., Falah, M., and Singh, D. (1997) Sequencing of heat shock protein 70 (DnaK) homologs from Deinococcus proteolyticus and Thermomicrobium roseum and their integration in a protein-based phylogeny of prokaryotes. *J.Bacteriol*.179, 345-357.

Gustafson, J.E., Liew, Y.C., Chew, S., Markham, J.L., Bell, H.C., Wyllie, S.G., Warmington, J.R., 1998. Effects of tea tree oil on Escherichia coli. Letters in Applied Microbiology 26, 194–198.

Hale TL, and Keusch GT (1996) Shigella. In: Baron's Medical Microbiology (Baron S et al., eds.) (4th ed.). Univ of Texas Medical Branch. ISBN 0-9631172-1-1.

Hamada, S. and Slade, H.D. (1980b). Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev.;* 44: 331-384.

Helander, I.M., Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm,T., Pol, I., Smid, E.J., Gorris, L.G.M., and Von Wright, A. (1998) Characterization of the action of selected ssential oil components on Gram-negative bacteria. *Journal of Agricultural and Food Chemistry* 46, 3590–3595.

Herikstad H, Motarjemi Y, and Tauxe RV. (2002) Salmonella surveillance: a global survey of public health serotyping. *Appl Environ Microbiol* 129:1–8.

Herold S, Karch H and Schmidt H (2004) "Shiga toxin-encoding bacteriophages--genomes in motion". Int. J. Med. Microbiol. 294 (2-3): 115–21. doi:10.1016/j.ijmm.2004.06.023. PMID 15493821.

Hooker, J.D. (1872-1892). The flora of British India. William counter and sons Ltd., London.

Hudault S, Guignot J, Servin AL (July 2001). "Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection". Gut 49 (1): 47–55.

Hunter, P.R. and Quigley, C. (1998) Investigation of an outbreak of cryptospondiosis associated with treated surface water finds limits to the value of case control studies. Communicable Dts Public Health; 1: 234-8.

Ibekwe AM, Watt PM, Grieve CM, Sharma VK, Lyons SR. (2002) Multiplex fluorogenic real-time PCR for detection and quantification of Escherichia coli O157:H7 in dairy wastewater wetlands. Appl Environ Microbiol;68:4853–62.

Iscan, G., N. Kirimer, M. Kurkcuoglu, K.H. Baser and F. Demirci (2002) Antimicrobial screening of *Mentha piperita* essential oils. J. Agr. Food Chem., 50:3943-3946.

Jack, R.W., Tagg, J.R. and Ray, B. (1995). Bacteriocins of gram-positive bacteria. *Microbiol Rev.;* 59: 171-200.

Jackson SG, Goodbrand RB, Johnson RP et al. Eschenchia colt O157 H7 diarrhoea associated with well water and infected cattle on an Ontario farm. Epidemiol Infect 1998; 120: 17-20

Jahn, S.A. (1981) Traditional water purification in tropical developing countries existing methods and potential applications. German Agency for Technical Cooperation (GTZ), Federal Republic of Germany.

Jones IG, and Roworth M.(1996)An outbreak of Escherichia coll O157 and campylobacteriosis associated with contamination of a drinking water supply. Public Health; 110: 277-82.

Juliano, C., Mattana, A. and Usai, M., 2000. Composition and in vitro antimicrobial activity of the essential oil of Thymus herbabarona Loisel growing wild in Sardinia. *Journal of Essential Oil Research* 12, 516–522.

Kaneko M Chlonnaaon of pathogenic E. coll O157. (1998) Water Set Technol; 38: 141^1

Kaper JB, Morris JGand Levine MM (1995) Cholera. Clinical Microbiology Reviews, 8:48–86.

Kelstrup, J., Richmond, S., West, C. and Gibbons, R.J. (1970). Fingerprinting human oral streptococci by bacteriocin production and sensitivity. *Arch Oral Biol.;* 15: 1109-1116.

Kirtikar K. R. and Basu B. D. (1972) Indian Medicinal plants Interna-tional Book Distributors, Allahabad, India, 2nd Ed., Vol. I to IV.

Knobloch, K., Pauli, A., Iberl, B., Weigand, H. and Weis, N (1989) Antibacterial and antifungal properties of essential oil components. *Journal of Essential Oil Research* 119– 128.

Knobloch, K., Weigand, H., Weis, N., Schwarm, H.-M., and Vigen- schow, H. (1986) Action of terpenoids on energy metabolism. *In: Brunke, E.J.* (Ed.), Progress in Essential Oil Research: 16th International Symposium on Essential Oils. De Gruyter, Berlin, pp. 429– 445.

Koch, A. L. (2003) Were Gram-positive rods the first bacteria? Trends Microbiol.11, 166-170.

Koedam, A., 1977a. Antimikrobielle Wirksamkeit a ¨therischer O ¨ le: Eine Literaturarbeit 1960 –1976—Fortsetzung und Schluss. Riechstoffe, Aromen, Kosmetica 27 (2), 36– 41.

Koedam, A., 1977b. Antimikrobielle Wirksamkeit a ¨therischer O ¨ le: Eine Literaturarbeit 1960– 1976—Teil I. Riechstoffe, Aromen, Kosmetica 27 (1), 8– 11.

Koplan JP et al. (1978) Contaminated roof-collected rainwater as a possible cause of an outbreak of salmonellosis. *Journal of Hygiene*, 81:303–309

Kunisawa, T. (2005) Dichotomy of major bacterial phyla inferred from gene arrangement comparisons. *J.Theor.Biol*.

Lake, J. A., Herbold, C. W., Rivera, M. C., Servin, J. A., and Skophammer, R. G. (2007) Rooting the tree of life using nonubiquitous genes. Mol.Biol.Evol.24, 130-136.

Lambert,R.J.W.,Skandamis,P.N.,Coote,P. and Nychas,G.-J.E., (2001) A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology* 91, 453–462.

Lawrence, J. G., Ochman, H. (1998) "Molecular archaeology of the Escherichia coli genome". PNAS 95 (16): 9413–9417. doi:10.1073/pnas.95.16.9413. JSTOR 45488. PMC..

Lina P Roldán, M.V., Gonzalo J Díaz, M.V. and Jennifer M Duringer, P.C. (2010) Composition and antibacterial activity of essential oils obtained from plants of the Lamiaceae family against pathogenic and beneficial bacteria, Rev Colomb Cienc Pecu, 23:451-461.

Ludwig, W. and Klenk, H.-P. (2001) Overview: A phylogenetic backbone and taxonomic framework for prokaryotic systamatics. In "Bergey's Manual of Systematic Bacteriology" (D. R. Boone and R. W. Castenholz, Eds.), Springer-Verlag, Berlin.

Madigan, M.T., Martinko, J.M. and Parker, J. (1997a). Plasmids. In: Brock biology of microorganisms.eds. *Prentice Hall: Englewood Cliffs, N.J;* pp. 328-333.

Maslow, J. (1996). Mulligan ME. Epidemiologic typing systems. *Infect Control Hosp Epid.;* 17: 595-604.

Meyer, T. E. and Bansal, A. K. (2005) Stabilization against hyperthermal denaturation through increased CG content can explain the discrepancy between whole genome and 16S rRNA analyses. Biochemistry44, 11458-11465.

Mimica Dukic, N., Bozin, B., Sokovic, M., Mihailovic, B. Matavulj, M. (2003)Antimicrobial and antioxidant activities of three *Mentha* species essential oils. Planta Medica. 69: 413-419.

Mimica-Dukic, Bozin, N., B., Sokovic, M., Mihajlovic B. and Matavuli, M. (2003) Antimicrobial and antioxidant activities of three *Mentha* species essential oils. Planta Med., 69: 413-419.

Moerman, D.E. (1996) An analysis of food plants and drug plants of native North America. *J. Ethnophamocol*. 52:1-22

Mubarakali, D., Thajuddin, N., Jeganathan, K. and Gunasekaran, M. (2011) Plant extract mediated synthesis of silver and gold nanoparticles and its antibacterial activity against clinically isolated pathogens. *Colloids and surfaces B Biointerfaces*, 85(2), 360-365.

Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C., Yolken, R.H., eds. *7th ed. ASM Press: Washington, D. C.*; 116-137.

Nadkarni, K. M. (1976) The Indian Materia Medica, Popular Prakashan, Mumbai, India, Vol. I and II, 1st Ed.

Nataro JP, and Kaper JB (1998) Diarrheagenic *Escherichia coli*. *Clinical Microbiology*

Nataro, J.P. and Kaper, J. B. (1998) "Diarrheagenic *Escherichia coli*". *Clin. Microbiol. Rev. 11 (1),* 142–201.

Nikitin V.V. (1983). Weed plants of the USSR flora. Leningrad: Nauka. 314 p. (In Russian)

Nychas, G.J.E., (1995) Natural antimicrobials from plants. In: Gould, G.W. (Ed.), New Methods of Food Preservation. Blackie Academic and Professional, London, pp. 58– 89.

O'Connor DR (2002) Report of the Walkerton Inquiry: The events of May 2000 and

Ogg JE, Ryder RA, Smith HL (1989) Isolation of Vibrio cholerae from aquatic birds in Colorado and Utah. *Applied and Environmental Microbiology*, 55:95–99.

Olive, D.M. and Bean, P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol.;* 37: 1661-1669.

Olsen, G. J. and Woese, C. R. (1997) Archaeal genomics: An overview. Cell89, 991-994.

Olsen, G. J., Woese, C. R., and Overbeek, R. (1994) The winds of (evolutionary) change: breathing new life into microbiology. *J.Bacteriol.176*, 1-6.

Oosterhaven, K., Poolman, B and Smid, E.J (1995) S-carvone as a natural potato sprout inhibiting, fungistatic and bacteristatic compound. Industrial Crops and Products 4, 23–31

Pattnaik S, Subramanyam VR, Bapaji M and Kole CR. (1997) Antibacterial and antifungal activity of aromatic constituents of essential oils. *Microbios*,; 89: 39-46.

Pattnaik, S., Subramanyam, V.R., Bapaji, M. and Kole, C.R. (1997). Antibacterial and antifungal activity of aeromatic constituents of essential oils. Microbios., 89: 39-46.

Pegram GC, Rollins N and Espay Q (1998) Estimating the cost of diarrhoea and epidemic

Prabuseenivasan S, Jayakumar M, and Ignacimuthu S. (2006) In vitro antibacterial activity of some plant essential oils. BMC Complementary and Alternative Medicine,; 6: 39.

Raymond, J., Zhaxybayeva, O., Gogarten, J. P., Gerdes, S. Y., and Blankenship, R. E. (2002) Whole-genome analysis of photosynthetic prokaryotes. Science298, 1616-1620.

Remington JP, Wood HC, Sadtler SP, LaWall CH, Kraemer H, and Anderson JF (1918) The Dispensatory of the United States of America. 20th edn. Philadelphia: JB Lippincott. *Reviews*, 11:142–201.

Rhodes JB, Schweitzer D, Ogg JE (1985) Isolation of non-O1 Vibrio cholerae associated with enteric disease of herbivores in western Colorado. Journal of Clinical Microbiology, 22:572–575.

Ryan KJ, andRay CG (2004) Sherris Medical Microbiology (4th ed.). McGraw Hill. ISBN 0838585299.

Ryan KJ; and Ray CG, (2004) Sherris Medical Microbiology (4th ed.). McGraw Hill.

Sadgir et. al.(2010) Antibacterial Activity of Ocimum sanctum Linn. and its application in Water Purification, Research *Journal of Chemistry and Environment*.14(3): 46-50.

Saeed, S. and Tariq, P. (2005) Antibacterial activities of *Mentha piperita, Pisum sativum* and *Momordica charantia*, Pak. J. Bot., 37(4): 997-1001.

Sandhya, W. (2004). Global health care challemges: Indian experiences and new prescriptions. *Electronic Journal of Biotechnology*, 7(3), 2004.

Sarac, N., Ugur, A. (2007) Antimicrobial activities and usage in folkloric medicine of some Lamiaceae species growing in Mugla, Turkey. EurAsia J BioSci, 1, 4, 28/34.

Sartoratto A, Machado ALM, Delarmelina C, Figueira GM, Duarte MCT and Rehder VLG. (2004) Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. *Braz. J. Microbiol*,; 34 (4): 1517-1520.

Scavroni, J., Boaro, C.S.F., Marques, M.O.M. and Ferreira. L.C. (2005) Yield and composition of the essential oil of *Mentha piperita* L. (Lamiaceae) grown with biosolid. Brazilian Journal of Plant Physiology, 17(4): 1677-1679.

Schneider, G., Dobrindt, U., Bruggemann, H., Nagy, G., Janke, B., Blum-Oehler, G., Buchrieser, C., Gottschalk, G., Emody, L. and Hacker, J. (2004) The pathogenicity island-associated K15 capsule determinant exhibits a novel genetic structure and correlates with virulence in uropathogenic Escherichia coli strain 536, *Infect. Immun. 72 (10),* 5993-6001.

Shatalkin, A. I. (2004) Highest level of division in classification of organisms. 3. Monodermata and Didermata. *Zh.Obshch.Biol*.65, 195-210.

Shelef, L.A., (1983) Antimicrobial effects of spices. Journal of Food Safety 6, 29– 44.

Shishkin, B.K., ed. (1954) Flora of the USSR. Moscow-Leningrad: AN SSSR. V. 21: 604-605. (In Russian) Family - Lamiaceae

Sikkema, J., De Bont, J.A.M., and Poolman, B1994. Interactions of cyclic hydrocarbons with biological membranes. *Journal of Biological Chemistry* 269 (11), 8022–8028.

Sikkema, J., De Bont, J.A.M., Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbiological Reviews* 59 (2), 201– 222.

Skandamis, P.N., and Nychas, G.-J.E. (2001) Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. *Journal of Applied Microbiology* 91, 1011 –1022.

Skophammer, R. G., Herbold, C. W., Rivera, M. C., Servin, J. A., and Lake, J. A. (2006) Evidence that the root of the tree of life is not within the Archaea. *Mol.Biol.Evol.*23, 1648-1651.

Soković, M., Marin, P. D., Brkić, D. and Leo J. L. D. Griensven (2007)Chemical Composition and Antibacterial Activity of Essential Oils of Ten Aromatic Plants against Human Pathogenic Bacteria, Food ©2007 Global Science Books.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol.;* 98: 503-517.

Stull, T.L., Li Puma, J.J. and Edlind, T.D. (1988). A broad-spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. *J Infect Dis.;* 157: 280-286.

Sutherland, J.P., Folkard, G.K. and Grant, W.D.(1990) Natural coagulants for appropriate water treatment: a novel approach, *Waterlines 8*(4):30-32

Tao, N. G., Liu, Y. J., Tang, Y. F., Zhang, J. H., Zhang, M. L. and Zeng, H. Y. (2009) Essential oil composition and antimicrobial activity of *Citrus reticulate.* Chemistry of Natural Compounds. 45 (3).

The Private Water Supplies Regulations 1991 Statutory Instrument No 2790 London: HMSO, 1991

The Water Industry Act 1991. London- HMSO, 1991

The Water Supply (Water Quality) Regulations 1989. Statutory Instrument No. 1147. London HMSO, 1989.

Thoroski, J., Blank, G., and Biliaderis, C. 1989. Eugenol induced inhibition of extracellular enzyme production by Bacillus cereus. *Journal of Food Protection* 52 (6), 399– 403.

Tillett HE, de Louvois J, and Wall PG. (1998) Surveillance of outbreaks of waterborne infectious disease categorising levels of evidence. *Epidemiol Infect* 120: 37-42.

Tobiason JE, Jahnson GS, Westerhoff PK, Vigneswaran B. (1993) Particles size

Tripathi, P.N., Chaudhuri, M., and Bokil, S. (1976) Nirmali Seed--A Naturally Occurring Coagulant *Indian Journal of Environmental Health*,18(4), 272-281.

Ultee, A.(2000). Bactericidal action of carvacrol towards the food pathogen Bacillus cereus. A Case Study of a Novel Approach to Mild Food Preservation. Wageningen University, Wageningen, The Netherlands. 97 pp.

Valsaraj, R., Pushpangadan, P., Smith, U.W., Adsersen, A. and Nyman, U. (1997) Antimicrobial screening of selected medicinal plants from India. J. of Ethnopharmacology, 58: 75-83.

Viale, A. M., Arakaki, A. K., Soncini, F. C., and Ferreyra, R. G. (1994) Evolutionary relationships among eubacterial groups as inferred from GroEL (chaperonin) sequence comparisons. *Int.J.Syst.Bacteriol*.44, 527-533.

Viuda-Martos, M. Ruiz-Navajas, Y., Fernández-López, J. and Perez-Álvarez, J. (2008) Antibacterial activity of Lemon (Citrus Lemon L Mandarin (*Citrus reticulata* L.), Grapefruit (*Citrus paradisi* L.) And Orange (*Citrus sinensis* L. Essential Oils. *Journal of Food Safety* 28: 567–576.

Waldor, M. K.; Mekalanos, J. J. (1996) "Lysogenic Conversion by a Filamentous Phage Encoding Cholera Toxin". Science 272 (5270): 1910–4. doi:10.1126/science.272.5270.1910. PMID 8658163.

Wang G, Clark CG and Rodgers FG. (2002)Detection of Escherichia coli of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and the components of the type 2 Shiga toxin family by multiplex PCR. *J Clin Microbiol*;40: 3613–9.

Wang RF, Cao WW, and Cernaglia CE. (1997) A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. *J Appl Microbiol*;83:727–36.

WHO (2002) Vibrio cholerae.In: Guidelines for drinking-water quality, 2nd ed. Addendum: Microbiological agents in drinking water. Geneva,World Health Organization, pp. 119–142.

Wood, C.D. (1988) Comparison of efficacy of ginger with various antimicrobial sickness drugs. *Clinical Research Practices and Drug Regulatory Affairs* 6(2): 129 – 136

Yang, F., Yang, J., Zhang, X., Chen, L., *et al.* (2005) Genome dynamics and diversity of Shigella species, the etiologic agents of bacillary dysentery*, Nucleic Acids Res. 33(19),* 6445-6458

Zaika LL. (1988) Spices and herbs: their antibacterial activity and its determination. *J Food Saf,*; 23: 97-118.

APPENDIX-1
GROWTH MEDIUM

1.1 Nutrient Agar

- Beef Extract = 3.0 g
- Peptone = 5.0 g
- Sodium chloride = 5.0 g
- Agar = 20 g
- Distilled water = 1000 ml
- pH = 7.2

Reference: - Mackie, T.J. and Mac Cartney, J.E. Handbook of practical bacteriology, 9th ed. 1956.

1.2 Nutrient Broth

- Beef Extract = 3.0 g
- Peptone = 5.0 g
- Sodium chloride = 5.0 g
- Distilled water = 1000 ml
- pH = 7.2

1.3 Muellar Hinton Agar No.2 (Hi Media M-1084)

- Casein acid hydrolysate = 17.50 g
- Beef heart infusion = 2.0 g
- Starch, soluble = 1.50 g
- Agar = 17.0 g
- Distilled water = 1000 ml
- pH = 7.2

1.4 Muellar Hinton Broth (Hi Media M-391)

- Beef infusion from = 300.0 g
- Hydrolysate = 17.50 g
- Starch = 1.50 g
- Distilled water = 1000 ml
- pH = 7.4 ± 0.2

1.5 Urea Broth (Hi Media M-111A) (Filter Sterilizable)

- Monopotassium phosphate = 9.10 g
- Dipotassium phosphate = 9.50 g
- Yeast extract = 0.10 g
- Phenol red = 0.01 g
- Urea = 20.0 g
- pH = 6.8 ± 0.2

Sterilization without autoclaving using Millipore

1.6 Nutrient Gelatin (Hi Media M-060S)

- Peptic digest of animal tissue = 5.0 g
- Meat extract = 3.0 g
- Gelatin = 120.0 g
- Sodium chloride = 30.0 g
- pH = 7.0 ± 0.2

1.7 Starch Agar (Hi Media M-107S)

- Peptic digest of animal tissue = 5.0 g
- Meat extract = 3.0 g
- Starch, soluble = 2.0 g
- Agar = 15.0 g
- pH = 7.2 ± 0.1

1.8 Blood Agar

- Nutrient agar = 100 g
- Sheep (blood) = 5 to 7.5 ml

Sterilized defibrinated sheep blood was added 5 to 7.5 ml per 100 ml of cooled medium. Mix thoroughly and pour.

Reference: - Mackie, T.J and Mc cartney, J.E., Handbook of Practical Bacteriology, 9th ed., 1956,

1.9 Simmons Citrate Agar (Hi Media M-099S)

- Magnesium sulphate = 0.2 g
- Ammonium dihydrogen phosphate = 1.0 g
- Dipotassium phosphate = 1.0 g
- Sodium citrate = 2.0 g
- Sodium chloride = 5.0g
- Bromo thymol blue = 0.08 g
- Agar = 15 g
- Distilled water = 1000 ml
- pH = 6.8 ± 0.1

All ingredients were dissolved and autoclaved at 121°C for 15 min.

Reference: - Mod. From Simmons, J.S. 1926.*J.Infect.Dis*.39.209.

1.10 MacConkey's Agar

- Peptone = 20.0 g
- Sodium chloride = 5.0g
- Bile salt = 1.5 g
- Lactose = 10.0 g
- Neutral Red Solution (1% aqueous) = 10.0 ml
- Crystal violet = 0.001 g
- Agar = 13.5 g
- Distilled water = 1000 ml
- pH = 7.1 ± 0.1

All ingredients were dissolved and autoclaved at 121°C for 15 min.

Reference: - Experimental microbiology by K.R.Aneja.

APPENDTIX-2
REAGENTS AND INDICATORS

2.1 Cleaning Solution for Glass wares

- Sodium dichromate = 25.0 g
- Sulphuric acid (Conc.) = 50.0 ml
- Distilled water = 1000.0 ml

Dissolved the dichromate crystals in 50 ml of water. Allow it cool to room temperature and add slowly to the preparation.

2.2 Gram's Iodine (for detection of starch)

- Iodine = 1.0 g
- Potassium Iodide = 2.9 g
- Distilled water = 300 ml

Using mortar and pestle made homogeneous solution of iodine and iodide. Content was transferred into reagent bottle and volume made up to 300 ml with distilled water. Solution was stored in a glass-stoppered brown bottle.

Reference: - Experimental microbiology by K.R.Aneja.

2.3 α-napthol solution (for VP test)

VP Reagent I

- α-napthol = 5.0
- Ethyl alcohol (abs.) = 95 ml

α-napthol was dissolved in ethyl alcohol with constant stirring.

VP Reagent II

- 40% KOH

2.4 Bromothymol blue (for Simmons's citrate agar)

- Bromothymol blue =0.4 g
- Ethyl alcohol (95%) =500 ml
- Distilled water =500 ml

Bromothymol blue and water was added in alcohol and filtered through whatman filter paper no.1.

2.5 Genomic DNA Extraction Kit *AccuPrep*®:

- Washing Buffer1 (W1) = 40 ml
- Washing Buffer1 (W1) = 20 ml
- Tissue Lysis Buffer (TL) = 25 ml
- Binding Buffer (GC) = 25 ml
- Elution Buffer = 30 ml

For Agarose gel electrophoresis

2.6 50X stock of TAE:

- 242 g Tris base
- 57.1 ml glacial acetic acid
- 100 ml 0.5 M EDTA pH 8.0
- water to make 1000 ml

2.7 Ethidium bromide

- Stock 5 mg/ ml in water

2.8 6X loading dye

- 0.25 % bromophenol blue µl
- 0.25% Xylene cynol
- 30% glycerol in H_2O

2.9 KB003: Hi25™ *Enterobacteriaceae* Identification Kit

- ONPG
- Lysine Utilization
- Ornithine Utilization

- Urease
- Phenylalanine Deamination
- Nitrate Reduction
- H2S Production
- Citrate Utilization
- Voges proskauer’s
- Methyl Red
- Indole
- Malonate Utilization

2.10 Hi media carbohydrate fermentation test kit KB009A

- Lactose
- Xylose
- Maltose
- Fructose
- Dextose
- Galactose
- Roffinose
- Trehalose
- Melibiose
- Sucrose
- L-Arabinose
- Mannose

APPENDIX-3
BIODATA

Name	:	**Awadhesh Kumar**
Date of Birth	:	1 March 1985
Gender	:	Male
Marital status	:	Single
Nationality	:	Indian
Father's Name	:	Sri. R. P. Ram
Mother's Name	:	Smt. Lachi Devi
Linguistic Abilities	:	Hindi & English
Permanent Address	:	Ambedkar Nagar Bhiti, P.O, Mau Nath Bhanjan, Distt: Mau (U.P.) Pin: 229001

PRESENT STATUS

- **Research Scholar, Department of Horticulture Aromatic and Medicinal Plants, Mizoram University, Aizawl-769004**

ACADEMIC QUALIFICATION

- M.Sc. Botany from University of Allahabad, Allahabad with 64.27% in 2008.
- B.Sc. Bio. Group from University Allahabad, Allahabad with 56.96 % in 2006.
- Intermediate from CBSE, Jawahar Navodaya Vidyalaya Mau (Dist),with59.2% in 2003
- 10^{th} from CBSE, Jawahar Navodaya Vidyalaya Mau (Dist), with 67.2 % in 2001

AWARDS

- 2011: Certificate of Appreciation Award (Plant Science Section) at 98^{th} Indian Science Congress (Plant Sciences) at SRM, University, Chennai.

TRAINING/EXPERIENCES

- National Level workshop cum Training Programme on Recent Advances in Medicinal & Aromatic Plants. April 11-25, 2011; Department of Horticulture Aromatic and Medicinal Plants, Mizoram University, Aizawl-769004.

RESEARCH PAPER PUBLISH/ COMMUNICATED IN NAT./ INT. JOURNALS

1. **Amritesh C. Shukla**, **A. Kumar**, Rohit K. Mishra**,** Om Prakash, A. Dikshit and N. Shukla (2011).'Turmeric' An age-Old Panacea for many Ills can be a potential source of Antidermatophytic Agent. *Int. Journal of Experimental Sciences* USA 2011, 2(6).
2. **Awadhesh Kumar**, Rohit K. Mishra**,** Shalu Srivastava, Amit K. Tiwari, Rita Gupta Anand Pandey, **Amritesh Chandra Shukla** and Anupam Dikshit (2011). Antibacterial activity of *Trachyspermum ammi* L. essential oil with special reference to phylogenetic analysis of water borne bacterial pathogens. *Advances in Environmental Biology* USA 2011.
3. **Awadhesh Kumar**, Rohit K. Mishra, **Amritesh C. Shukla** and Anupam Dikshit FNASc. (2011). *In-vitro* antimicrobial activity of medicinal plants against water borne bacteria with their phylogenetic evaluation *NAT ACAD SCI LETT* (Accepted)
4. Amit Kumar Tiwari, Rohit Kumar Mishra**, Awadhesh Kumar**, Anand Pandey, A.K. Bajaj and Anupam Dikshit (2011). A comparative novel method of Antifungal susceptibility for *Malassezia furfur* and Modification of Culture medium by adding lipid supplement *Journal of Phytology*, 3(3): 44-55.
5. Rohit Kumar Mishra, **Awadhesh Kumar**, **Amritesh Chandra Shukla**, Pravin Tiwari and Anupam Dikshit (2010). Quantitative and Rapid Antibacterial assay of *Micromeria biflora* Benth. leaf essential oil against dental caries causing bacteria using phylogenetic approach. *Int. Journal of Ecobiotechnology* 2(4): 22-26.
6. Shalu Srivastava, Rohit K. Mishra, **Awadhesh Kumar**, Amit K. Tiwari and Anupam Dikshit (2010).Water borne bacterial contaminants and their

management using phylogenetic approach *NAT ACAD SCI LETT, VOL.33, No. 11&12*.

RESEARCH PAPER PRESENTED IN CONFERENCES NATIONAL / INT.

1. Rohit Kumar Mishra**,** Amit Kumar Tiwari, **Awadhesh Kumar**, Anand Pandey and Anupam Dikshit (2011). Biotechnological approach: Isolation and Characterization of potent cariogenic bacteria from saliva of local people at Allahabad. National Symposium on Recent Researches in Physical and Biological Sciences, K.N.Institute of Physical and Social Sciences, Sultanpur (U.P.) The Indian Academy of Sciences, India (NASI), Allahabad
2. Srivastava, S., **Kumar A.,** Mishra, R.K. *et. al.*, (2011). Water borne bacterial contaminants and their management by essential oil of *Mentha arvensis* L. using Phylogenetic approach. 98th Indian Science Congress (Plant Sciences) at SRM, University, Chennai, pp-42-43.
3. **Kumar, A.,** Mishra, R.K. *et. al.*, (2011). Antibacterial activity of *Trachyspermum ammi* L. and its major active constituents against water borne pathogens. 98th Indian Science Congress (Plant Sciences) at SRM, University, Chennai, pp-37-38 (Best Poster Award).
4. Tiwari, A.K., **Kumar, A.,** Mishra, R.K., *et. al.,* (2011). Design Herbal *Aloe vera* cosmetic for skin care and cure of Pityriasis versicolar. 98th Indian Science Congress (Plant Sciences) at SRM, University, Chennai, pp-37-38.
5. **Kumar, A.,** Srivastava, S., Mishra, R.K., Shukla, A.C. and Dikshit, A. (2010). Studies on anti-bacterial activity of major active constituents from essential oils with special reference to water borne pathogens. National Conference on BIOPROSPECTING access for sustainable development Organized by Applied Mechanics Department (Biotechnology) Motilal Nehru National Institute of Technology (MNIT), Allahabad-211004
6. Srivastava, S., **Kumar, A.,** Mishra, R.K**.,** Shukla , A.C. and Dikshit , A. (2009). Botanicals: An eco-friendly method for water purification. National Seminar on Biotechnology & Microbiology in Human welfare: The Indian Scenario at Modi

Institute of Technology and Science (Deemed University), Sikar, Jaipur, Rajasthan 26-27 Sep. 2009.

7. Balachander[1], S., Srivastava[2], S., **Kumar[2], A.,** Mishra[2], R.K**., Shukla[3], A.C.,** Jayaprada[1], R. and Dikshit[2]A. (2009). Isolation and characterization of chicken feather degradating bacteria. National Seminar on Biotechnology & Microbiology in Human welfare: The Indian Scenario at Modi Institute of Technology and Science (Deemed University), Sikar, Jaipur, Rajasthan 26-27 Sep. 2009.
8. **Kumar, A.,** Mishra, R.K., Mishra, P., Tiwari, A.K. and Dikshit, A. (2009). Studies on botanicals to make water potable in rural area. National Conference on Natural Resources Management at Dept. of Horticulture & Medicinal and Aromatic Plants, Mizoram University, Aizwal, 24-25 Mar. 2009.
9. Tiwari, A.K., Mishra, R.K., **Kumar, A.** and Dikshit, A. (2009)**.** Exploration of Herbals Cosmetics Against Human Head Lice International Conference on "Current Trends in Biotechnology and its implications in Agriculture & Technology" at SVBPUA&T February 19-21.2009. Modipuram, Meerut.
10. Mishra, R.K., Tiwari, A.K., **Kumar, A.,** Mishra, B.K., Mishra, P., Kamran, A., Pandey, R.K. and Dikshit, A. (2009). Antimicrobial screening of plant essential oils against dental caries causing bacteria National Conference at Department of Botany, DDU Gorakhpur University, 30-31Jan.2009, Gorakhpur.

SYMPOSIUM/WORKSHOP/CONFERENCES ATTENDED: NATIONAL/ INT.

1. **14-15 Feb. 2011:** National Symposium on Recent Researches in Physical and Biological Sciences, K.N.Institute of Physical and Social Sciences, Sultanpur (U.P.) The Indian Academy of Sciences, India (NASI), Allahabad
2. **8-9 Feb. 2011:** "Recent Advances in Mycological Research" 37th Annual Meeting of the Mycological Society of India**.**
3. **3-7 Jan. 2011:** 98th Indian Science Congress Meeting and Int. Conference at SRM, University, Chennai.
4. **10-12 Nov. 2010:** XXXIIInd All India Botanical Conference & International Symposium at Shivaji University, Kolhapur Universty, Kolhapur, 416004.
5. **20-21 Feb. 2010:** National Conference on BIOPROSPECTING access for sustainable development Organized by Applied Mechanics Department (Biotechnology) Motilal Nehru National Institute of Technology, Allahabad-211004

6. **6-7 Feb. 2010:** National National Symposium on Health and Sanitation: Rural Perspective Organized by The Institute of Applied Sciences Allahabad
7. **28-30 Dec. 2009:** XXXII All India Botanical Conference & International Symposium KUVEMPU University, Shankaraghatta Shimoga Karnataka- 577451.
8. **26-27 Sep. 2009:** National Seminar on Biotechnology & Microbiology in Human welfare: The Indian Scenario at Modi Institute of Technology and Science (Deemed University), Sikar, Jaipur, Rajasthan
9. **24-25 March 2009:** National Conference on Natural Resources Management at Dept. of Horticulture & Medicinal and Aromatic Plants, Mizoram University, Aizwal.
10. **17-19 Dec. 2008:** 31st IBS Conference & International Conference, Department of Botany, University of Allahabad, Allahabad-211002.

AREA OF INTEREST

- Medicinal and Aromatic Plants
- Isolation of DNA & RNA from Bacteria, Unicellular Fungus and Viruses
- Working on RT-PCR, Gel Doc. and Elisa reader (Spectra Max plus 384) etc.
- Herbal antimicrobials
- Clinical Microbiology
- Bioinformatics in case of *in-silico* study
- Pharmaceutical Biotechnology.
- Biofertilizer and Biopesticides.

Other Interesting Activities:

- Rural development through Sri Sathya Sai Village Integrated Programmed.
- Photography and Working on Computer.
- Playing Cricket and Reading Books.

Date: 23-05-2011 **(Awadhesh Kumar)**

Place: Aizawl

Journal of Experimental Sciences 2011, 2(7): 04-10
ISSN: 2218-1768
www.scholarjournals.org

journal of experimental sciences

www.jexpsciences.com

JES-Life Sciences

'Turmeric' An Age-Old Panacea for many ills can be a Potential Source of Antidermatophytic Agent

Amritesh C. Shukla[1*], Awadhesh Kumar[2], Archana Verma[3], R. K. Mishra[4], A. Dikshit[5] , Om Prakash[6] and Neetu Shukla[7]

[1]*Associate Professor, Department of Horticulture, Aromatic & Medicinal Plants, Mizoram University, Aizawl-796 004, India*
[2]*Research Scholar, Department of Horticulture, Aromatic & Medicinal Plants, Mizoram University, Aizawl-796 004, India*
[3]*DST- Women Scientist, Department of Horticulture, Aromatic & Medicinal Plants, Mizoram University, Aizawl-796 004, India*
[4]*DST- Research Scientist, Biological Product Laboratory, Department of Botany, University of Allahabad-211 002, India*
[5]*Professor & Director, Biological Product Laboratory, Department of Botany, University of Allahabad-211 002, India*
[6]Project Manager, Indian Institute of Information Technology (IIIT), Allahabad- 211002, India
[7]*Resident Doctor, Ekta Institute of Child Health, Raipur-492 001, India*

Article Info

Article History

Received : 13-03-2011
Revised : 12-05-2011
Accepted : 17-05-2011

*Corresponding Author

Tel : +91-389-2330273
Fax : +91-389-2330214

Email:
amritesh.shukla@rediffmail.com

Abstract

Antimicrobial evaluation of the essential oil(s) of some spp. of *Curcuma* viz., *Curcuma angustifolia, C. aromatica* and *C. zedoaria* – "an age old panacea for many ills", were screened against three common dermatophytic fungi causing ringworm infection in human beings. The essential oil of *Curcuma aromatica* Salisb.(Family- *Zingiberaceae*) was found strongest toxicant against the test fungi. The minimum inhibitory concentration (MIC) of the oil was 1.8µl/ml against *Epidermophyton floccosum* and *Trichophyton rubrum,* and 1.6µl/ml against *Microsporum gypseum*; however, it was fungicidal at 2.0 µl/ml against *E. floccosum* and *T. rubrum*, and 1.8 µl/ml against *M. gypseum,* respectively. The efficacy contains heavy doses of inoculums (25 discs of 5 mm each.). The (MKT) of the oil was 30 sec against *E. floccosum* & *Microsporum gypseum* and 20 sec against *T. rubrum*, while, its MFCs required 6.30 hrs against *E. floccosum* & *Microsporum gypseum* and 5.30 hrs against *T. rubrum.* The oils efficacy was thermo stable up to 100 ^{0}C and for 36 months of storage, the maximum unit taken into consideration. Moreover, the oil of *C. aromatica* did not exhibit any adverse effect on mammalian skin up to 5% conc. Relationship of the dermatophytes to the toxicity of the oil vis-a vis phylogeny using molecular data of the pathogens have also been discussed. Further, the clinical trial of the oil in the form of ointment (at 1% v/v conc) to topical testing on patients, attending out patient department (OPD) of MLN Medical College, Allahabad is still in progress.

Key Words: Antimicrobial activity, Dermatophtes, Medicinal plants, Minimum inhibitory concentration, Herbal drug

Introduction

The World Health Organization (WHO) estimated that 80% of the population of developing countries rely on traditional medicine mostly plant drugs, for their primary health care needs [1]. Medicinal plants being natural, non-narcotic, having no side effects, safe, cost effective, preventive and curative therapies which could be useful in achieving the goal of "Health for all" in a coat effective manner. Demand for medicinal plants is increasing in both developing and developed countries but 90% malarial la harvested from wild sources without applying scientific management hence many species are under threat to become extinct [1, 2].

In fact the traditional herbal remedies led the Scientists to the development of numerous modern drugs [3-5]. At this point the discovery of reserpine from *Rauvolfia serpentine* can be cited as an example of how a plant utilized by the indigenous people eventually becomes the source of one of the most important pharmaceuticals of the world [1].

Keeping these views in mind, in the present investigations, a scientific attempt has been made to explore the possibilities of *Curcuma* spp, as a protecting measurement against the ringworm infection on human beings.

Materials & Methods

In vitro investigation

(a). Extraction and Isolation of Essential oil:

The essential oil(s) were extracted separately from the fresh leaves of *Curcuma angustifolia, C. aromatica* and *C. zedoaria* (Family- Zingiberaceae) by hydro distillation using Clevenger's apparatus [6]. A clear light yellow colored oily layer was obtained on the top of the aqueous distillate, later which was separated and dried over anhydrous sodium sulphate. The oils thus obtained were subjected to various antimicrobial investigations.

(b). In- vitro antimicrobial investigations of the essential oil(s):

The minimum effective concentration (MEC) of the oil against some common human pathogenic fungi *Epidermophyton floccosum* Hartz, *Microsporum gypseum*

(Bodin) Guiart et Grigorakis and *Trichophyton rubrum* Castellani, was determined by using the technique of Shahi et. al. [10], with a slight modification. Two sets were maintained; one for the treatment set and another for the control. The treatment set at different concentration of the oil was prepared by mixing the required quantity of the oil samples in acetone (2% of the total quantity of the medium) and then added in pre-sterilized sabourad dextrose agar medium (SDA). In control set, sterilized water (in place of the oil) and acetone were used in the medium in appropriate amount. The fungi-static/ fungicidal (MSC/ MCC) action of the oil was tested by aseptically re-inoculating the fungi in culture tubes containing sabourad dextrose broth (Table 1-3). The data recorded was the mean of triplicates, repeated twice. The percentage of fungal growth inhibition (FGI) was calculated as per formula:

$$FGI\ (\%) = \frac{Dc - Dt}{Dc} \times 100$$

Where, Dc indicates colony diameter in control set, &
Dt indicates colony diameter in treatment sets.

Table-1: Minimum effective concentration of four different spp of Curcuma against some common human pathogenic fungi

Curcuma spp	Human Pathogenic Fungi		
	Epidermophyton floccosum	*Microsporum gypsum*	*Trichophyton rubrum*
Curcuma angustifolia	2.6 µl/ml	2.2 µl/ml	2.4 µl/ml
C. aromatica	1.8 µl/ml	1.6 µl/ml	1.8 µl/ml
C. zedoaria	2.2 µl/ml	1.8 µl/ml	2.0 µl/ml

Table- 2: Minimum effective concentration of the oil of *Curcuma aromatica* against test fungi

Concentration (µl/ml)	Human Pathogenic Fungi		
	Epidermophyton floccosum	*Microsporum gypsum*	*Trichophyton rubrum*
2.0	100^{C}	100^{C}	100^{C}
1.8	100^{s}	100^{C}	100^{s}
1.6	92	100^{s}	96
1.4	88	60	80
1.2	60	--	76
1.0	--	--	60

^{c} indicates cidal and ^{s} indicates static

Table- 3: Detailed in-vitro investigations of *Curcuma aromatica* against the test fungi

Properties studied	Observations		
	Epidermophyton floccosum	*Microsporum gypsum*	*Trichophyton rubrum*
Minimum Inhibitory Concentration			
MEC *	1.8 µl/ml	1.6 µl/ml	1.8 µl/ml
MFC	2.0 µl/ml	1.8 µl/ml	2.0 µl/ml
Minimum Killing Time			
Pure oil	30 sec	30 sec	20 sec
MFC	6.30 hrs	6.30 hrs	5.30 hrs
Inoculum Density (25 disc, 5mm diam)	No Growth	No Growth	No Growth
Thermostability (up to 100 ^{0}C)	No Growth	No Growth	No Growth
Effect of Storage (36 months)	No Growth	No Growth	No Growth

*MEC *indicates Minimum Effective Conc.;* MFC *indicates Minimum Fungicidal Concentration*

(c). Effect of Inoculums Density:

The effect of inoculums density on the minimum cidal concentration (MCCs) of the oil against the test fungi was determined using the method of [20]. Mycelial discs of 5mm diam of 7-day oil cultures were inoculated in culture tubes containing oil at their respective MCCs. In controls, sterilized water were used in place of the oil and run simultaneously. The numbers of mycelial discs in the treatment as well as control sets were increased progressively up to 25 discs, in multiply of five. Observations were recorded up to seventh day of incubation. Absence of mycelial growth in treatment sets up to 7th day exhibited the oil potential against heavy doses of inoculums (Table- 3).

(d). Effect of some Physical Factors:

Effect of some physical factors viz., temperature (40, 60 and 80^{0} C respectively) and autoclaving (up to 15 lb/ sq inch pressure for 30 min) on efficacy of the oil, at minimum cidal concentration, was also determined. It was determined following the method of [7, 8]. Samples of oil in small vials, each contains 1ml, were exposed at 40, 60 and 80^{0} C in hot water bath, respectively. Further, the oil's efficacy was tested against the test fungi at their respective MCCs (Table- 3).

(e). Minimum Killing Time:

The MKT of the pure oil and their respective MCCs of *C. aromatica* against the test fungi was determined by using the method of [9], (Table-4).

Table- 4: Minimum killing time of the oil of *Curcuma aromatica* against test fungi

	Mycelial Growth Inhibition (%)					
Minimum	*Epidermophyton floccosum*		*Microsporum gypseum*		*Trichophyton rubrum*	
Killing Time (MKT)	P.O.	MFC	P.O.	MFC	P.O.	M.F.C.
7.0 hrs	100	100	100	100	100	100
6.30	100	100	100	100	100	100
6.0	100	60	100	80	100	100
5.30	100	---	100	---	100	100
5.0	100		100		100	80
2.30	100		100		100	---
2.0	100		100		100	
1.30	100		100		100	
1.0	100		100		100	
30 min	100		100		100	
15.0	100		100		100	
5 .0	100		100		100	
60 sec	100		100		100	
30	100		100		100	
20	90		80		100	
10	60	---	70	---	88	---

*P.O. *indicates Pure Oil;* MFC *indicates Minimum Fungicidal Concentration*

Table-5: Fungi toxic spectrum of the oil of *Curcuma aromatica* against some common pathogenic fungi

Fungi Tested	Lethal Conc (2.0 µl/ml)	Hyper Lethal Conc (4.0 µl/ml
Human Pathogens		
Microsporum auddouinii	100[s]	100[c]
M. canis	100[s]	100[c]
M. nanum	100[c]	100[c]
Trichophyton mentagrophytes	100[c]	100[c]
T. tonsurans	100[c]	100[c]
T. violaceum	100[c]	100[c]
Plant Pathogens		
Aspergillus parasiticus	100[s]	100[c]
Cladosporium cladosporioides	100[c]	100[c]
Curvularia lunata	100[c]	100[c]
Colletotrichum capsici	100[c]	100[c]
C. falcatum	100[c]	100[c]
Fusarium oxysporum	100[c]	100[c]
F. udum	100[c]	100[c]
Helminthosporium maydis	100[c]	100[c]
H. oryzae	100[c]	100[c]
Penicillium implicatum	100[c]	100[c]
P. minio-luteum	100[c]	100[c]

[s] indicates static; [c] indicates cidal in nature

Table- 6: Comparative MECs of the oil of *Curcuma aromatica* with some Synthetic Antifungals

Oil & Trade Name of Antifungal Drugs	Active Ingredients	Minimum Effective Concentration (µl/ml)		
		Epidermophyton floccosum	*Microsporum gypseum*	*Trichophyton rubrum*
Curcuma aromatica	Essential oil	1.8	1.6	1.8
Dactrine	Miconazole nitrate	6.0	6.0	6.0
Nizaral	Ketoconazole	6.0	0.5	5.0
Tenaderm	Tolnaftate	2.0	1.5	0.8

Table- 7: Comparative Efficacy of the oil of *Curcuma aromatica* with some Synthetic Antifungal Drugs

Antimycotic Drugs	Drugs %	Cost (Rs.)		Adverse Effects	Expiry Duration (months)	Environmental impact
		ointment/g	lotion/ ml			
C. aromatica	1%v/v	0.90	0.70	No adverse effects	24-36	Renewable, biodegradable, non-residual toxicity.
Dactrine	2%w/w	2.80	---	Occasionally produced gastrointestinal side effects viz., nausea, vomiting, diarrhea	35	Non-renewable, non-biodegradable and residual toxicity
Nizaral	2%w/w	3.75	3.17	Adverse reaction observed were mainly burning, irritation. Drug may block testosterone synthesis	24	-----do-----
Tenaderm	1%w/v	1.06	1.30	Adverse effects were fever, nausea, vomiting, diarrhoea & skin rash, rarely produced irritation	24	-----do-----
Batrafine	1%w/v	1.50	1.60	----do----	24	----do----

(f). Fungi-toxic Spectrum:

The fungi-toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 2.0 µl/ml and 4.0 µl/ml respectively) was determined against some common human pathogenic fungi viz., *Microsporum auddouinii* Gruby*, M. canis* Bodin*, M. nanum* Fuentes*, Trichophyton mentagrophytes* (Robin) Blanchard*, T. tonsurans* Malmstem, and *T. violaceum* Bodin. This was done by using the method of [9], (Table-5).

Besides, the oil's efficacy was also tested against some plant pathogenic fungi viz., *Aspergillus parasiticus* Speare, *Cladosporium cladosporioides* (Fresenius) de Vries, *Curvularia lunata* (Wakker) Boedijin, *Colletotrichum capsici* (Syd.) Butler & Bisby, *C. falcatum* Went, *Fusarium oxysporum* Schlecht, *F. udum* de vries, *Helminthosporium maydis* Nisikado & Miyakel, *H. oryzae* Breda de Haan, *Penicillium implicatum* Biourge and *P. minio-luteum* Dierckx; by using the technique of [7] (Table-5).

(g). Comparison with some Synthetic Fungicides:

The comparative efficacy of oil of *C. aromatica* with some synthetic antifungal drugs was carried out by comparing MECs. This was done by using the method of [10], (Table-6 & 7).

All the experiments were repeated twice and each contained three replicates; the data presented in the tables are the mean values.

(h). Statistical analysis:

Analysis of variance (ANOVA) was used to determine the significance ($P \leq 0.05$) of the data obtained in all experiments. All results were determined to be within the 95% confidence level for reproducibility. The ANOVA was computed using the SPSS version 16.0 software package.

(i). Phylogenetic study of dermatophytes

To find out the reason why the *Curcuma aromatica* is more effective against certain pathogenic fungi, phylogenetic relationship of the dermatophytes were studied including the genera *Trichophyton*, *Microsporum*, and *Epidermophyton* and identified the species using the base pair sequences of ITS1 [11], Fig. 1. The ITS1 sequences of the standard strains used in this study and members of the *Trichophyton* spp complex (*T. rubrum*, accession no. AB011453; *Microsporum gypsem*, accession no. AB017177 and *Epidermatophyton floccosum*, accession no. AB017181), were aligned using the Clustal W computer program [12,13] and GENETYX-MAC 10.1 software (Software Development Co., Ltd., Tokyo, Japan). Phylogenetic trees were then constructed by the DNA maximum-likelihood (ML) method in the PHYLIP program (Phylogeny Inference Package), version 3.5c [14], and the neighbor-joining (NJ) method in the NJPLOT program [15]. Bootstrap [14] analysis with the Clustal W program was performed by taking 1,000 random samples from the multiple alignments.

Results

On comparing the minimum effective concentration (MEC) of oils of *Curcuma angustifolia, C. aromatica* and *C. zedoaria* against the test fungi, the MEC of the oil of *C. aromatica* was found most effective (Table- 1).

The MEC of *Curcuma aromatica* oil was 1.6 µl/ml against *M. gypseum,* 1.8 µl/ml against *T. rubrum* and *E. floccosum;* however, it was fungicidal at 1.8 µl/ml against *M. gypseum,* and 2.0 µl/ml against *E. floccosum* and *T. rubrum*, and respectively (Table- 2).

The oil's efficacy contains heavy doses of inoculums (i.e. up to 25 discs, each of 5mm), thermo stable up to 80^0 C and

also persisted after autoclaving at 15 lb/ sq inch pressure for 30 min. (Table- 3).

The pure oil kills the test fungi within 30 second; however, its MCC ranges 5.30 to 6.30 hrs to kill all the fungi (Table- 4).

Fungi toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 2.0 µl/ml and 4.0 µl/ml), against some common pathogenic fungi reveals that the oil contains a broad fungicidal spectrum (Table- 5).

Furthermore, on comparing MECs of the oil with some synthetic antifungal drugs, MECs of the oil was more active than Dactrine, Nizaral and Tenaderm (Table- 6 & 7).

The phylogenetic relationship of dermatophytic genera *Trichophyton*, *Microsporum*, and *Epidermophyton* were basis of their ITS1 sequences. The NJ tree was constructed with data for standard strains of dermatophytes (11) demonstrated by using internal transcribed spacer 1 (ITS1) region ribosomal DNA sequences. *Trichophyton* spp. and *Microsporum* spp. form a cluster in the phylogenetic tree with *Epidermophyton*. All strains were successfully identified by comparison of their base sequences with those in the ITS1 DNA sequence database [11] NJ tree of dermatophytes is shown in Fig. 2.

The relationship of the toxicity of the essential oil vis-à-vis phylogeny was analysed using molecular data. The effectiveness of the oil was equal in dermatophytes that are close in phylogenetic tree (Fig. 2).

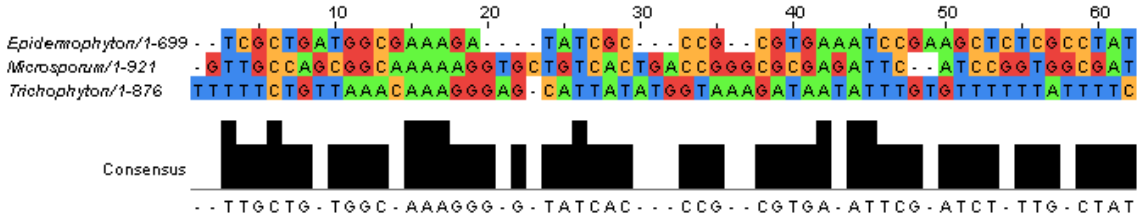


Fig. 1: Alignment of ITS1 sequences of standard strains of dermatophytes

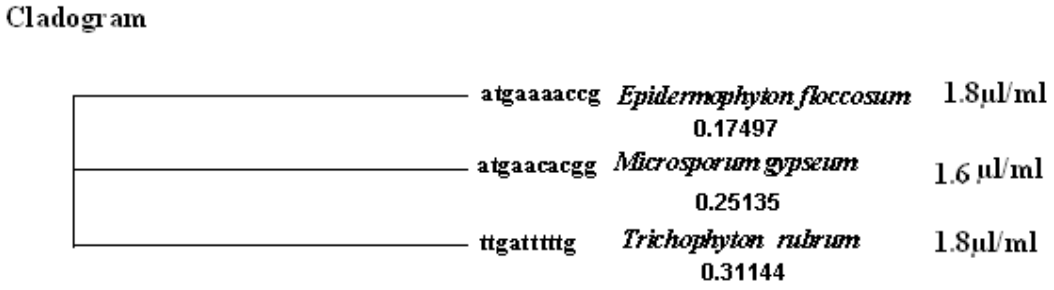


Fig. 2: Result of Cladogram (Neigbour Joining Tree plot) standard strains of dermatophytes on the basis of their ITS1 sequences.

Discussions

Essential oils obtained from the leaves of *Cymbopogon martini* var. motia [16], *Hyptis leucodendron* [17]; *Alpinia galangal* [18] was found to contain fungistatic activity. However, some essential oils, *Cymbopogon flexuosus* [19]; *Eucalyptus* oil [20]; *Citrus sinensis* [21] and *Homalomena aromatica* [22] prove to have fungistatic action at lower concentration and fungicidal action at higher concentration. Similarly, in the present investigation the oil of *Curcuma aromatica* showed fungistatic activity at the lower concentration 1.8 µl/ml against *E. floccosum* and *T. rubrum*, and 1.6 µl/ml against *M. gypseum;* and fungicidal at the higher concentration 2.0 µl/ml against *E. floccosum* & *T. rubrum*, and 1.8 µl/ml against *M. gypseum,* respectively. The fungicidal efficacy of the oil persisted heavy inoculums density with quick killing activity as well as having an edge over some synthetic antifungals viz., Dactrine, Nizaral, Tenaderm.

A fungicide must not be affected by extreme temperatures. Only a few workers have studied the effect of temperature on antifungal activity of the essential oils. [23] reported the oil of *Pepromia pellucida* was active up to 80 ^{0}C; Shahi et al., [24] reported *C. flexuosus* activity up to 100 ^{0}C, and Shukla et al., [22] reported the oil's efficacy of *H. aromatica* up to 80 ^{0}C. Similarly, in the present investigation the oil of *C. aromatica* was not only thermostable up to 80^{0}C but also autoclavable up to 15 lb/ sq inch pressure for 30 min.

A substance may behave as a strong fungicidal against certain fungi yet may be ineffective against the other pathogens. Therefore, a clear picture about the toxicity of a fungicide comes only after it is tested against the large number of fungi. The literature showed that essential oils have been found to exhibit narrow or wide range of activity [22, 25-27], but in the present study the oil of *C. aromatica* exhibited broad antifungal spectrum.

The effectiveness of the oil was equal to those dermatophytes which are close in phylogenetic tree. To understand the relationship of the DNA sequences of the tested fungal strains and their variable response to the different concentrations of active fractions (extracted in the form of essential oil from the leaf of *Curcuma aromatica*) have been critically analyzed. Further, evaluation of the phylogenetic analysis and identification system, both of which are based on

ITS1 rDNA sequences, are continuing in our laboratory with other species and strains.

A toxicant should be tested under both *in vitro* and *in vivo* conditions in order to prove its potential as promising antifungals for the control of disease. Since, detailed *in vitro* studies on the essential oil of *C. aromatica* indicate their potentiality to be as ideal antifungal agent against the dermatophytic fungi; hence, the same was further subjected for detailed *in vivo* investigations as well as clinical trials in the form of ointment (at 1% V/V conc.), which is still in progress.

Conclusions

The preliminary *in vitro* investigations revels that the oil of *Curcuma aromatica*, due to its strong fungicidal efficacy, inhibiting heavy doses of inocula, quick killing activity, broad fungicidal spectrum, long shelf life, and having an edge over some synthetic antifungal, can be used successfully in the form of broad spectrum herbal anti-dermatophytic agent(s). The commercial viability of the same can be determined after detailed *in vivo* as well as successful multi central clinical trials, which is in progress.

Acknowledgements

The authors are thankful to the Head, Department of Horticulture, Aromatic & Medicinal Plants, Mizoram University, Aizawl as well as Head, Department of Botany, University of Allahabad for providing various research facilities; Prof. A.K. Bajaj, Dermatology Department, MLN Medical College, Allahabad and Prof. Uma Banerjee, Division of Microbiology, All India Institute of Medical Sciences, New Delhi for providing their microbial expertise. Further, Department of Science and Technology, New Delhi (Ref No. SR/ WOS-A/ LS-04/ 2009; dt. 29.7.2009) is also thankfully acknowledged for financial assistance.

References

[1] WHO. 2002. WHO traditional medicine strategy 2002-2005.

[2] United Nations Educational Scientific and Cultural Organization UNESCO. 1996. Culture and health orientation texts World decade for cultural Development, 1988-1997, document, pp. 129.

[3] Igbinosa, O. O., E. O. Igbinosa and O. A. Aiyegoro. 2009. Antimicrobial activity and phytochemical screening of stem bark extracts from *Jatropha curcas* (Linn). Afr. J. Pharm. Pharmacol. 3(2):058-062.

[4] Nweze, E. L., J. I. Okafor and O. Njokn. 2004. Antimicrobial activities of methanolic extracts of *Trema guinensis* (Schumm and Thorn) and *Morinda Lucida* Benth used in Nigeria. Biol. Res. 2:39-46.

[5] Ekpo, M. A and P. C. Etim. 2009. Antimicrobial activity of ethanolic and aqueous extracts of *Sida acuta* on microorganisms from skin infections. J.Med. Plants Res. 3(9): 621-624

[6] Clevenger, J.F. 1928. Apparatus for the determination of volatile oil. J. Am. Pharm. Assoc. 17: 346.

[7] Shahi, S. K., M. Patra, A. C. Shukla and A. Dikshit. 2001. Botanical drug for therapy against fungal infections in human beings. Nat Acad of Sci Letters. 24(5-12):73-78.

[8] Shukla, A. C., S. K. Shahi, A. Dikshit and V.C. Saksena 2001. Plant product as a fumigant for the management of stored product pests. Proc. Int. Conf. on Controlled Atmosphere and Fumigation in Stored Products, USA (*Eds.*Donahaye, EJ., Navarro, S. and Leesch, JG) pp 125-132.

[9] Shahi, S. K., A. C. Shukla, A. Dikshit and D. K. Uperti. 2001. Broad-spectrum antifungal properties of the lichen, *Heterodermia leucomela*. The Lichenologist, U.K. 33(2):177-179.

[10] Shahi, S. K., A. C. Shukla, A.K. Bajaj, G. Medgely and A. Diksit. 1999. Broad spectrum antimycotic drug for the control of fungal infection in human beings. Current Science. 76(6): 836- 839.

[11] Makimura, K., Y. Tamura, T. Mochizuki, A. Hasegawa, Y. Tajiri, R. Hanazawa, K. Uchida, H. Saito and H. Yamaguchi. 1999. Phylogenetic Classification and Species Identification of Dermatophyte Strains Based on DNA Sequences of Nuclear Ribosomal Internal Transcribed Spacer 1 Regions. J Clin Microbiol. 37(4):920-924

[12] Makimura, K., T. Mochizuki, A. Hasagawa, K. Uchida and H. Yamaguchi. 1998. Phylogenetic classification of *Trichophyton mentagrophytes* complex strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. J Clin Microbiol. 36: 2629-2633.

[13] Higgins, D. 1996. Clustal W, Version 1.6. Cambridge, United Kingdom: The European Bioinformatics Institute.

[14] Felsenstein, J. 1985. Confidence limits on phylogenies:an approach using the bootstrap. Evolution. 39:783-791.

[15] Gouy, M. 1995. NJPLOT. Lyon, France: University of Lyon 1.

[16] Dikshit, A. 1980. Fungitoxic evaluation of some plants and the active product (*Cedrus* oil). *Ph D. thesis*, University of Gorakhpur, Gorakhpur (India).

[17] Dubey, N.K., N. Kishore, and S.K. Singh, 1983. Antifungal properties of the volatile fraction of *Melaleuca leucadendron.* Trop. Agric. (Trinidad) 60 (3): 227-228.

[18] Tripathi, N.N., N.K. Dubey, R.D. Dikshit and S.N. Dixit, 1983. Fungitoxicity properties of *Alpinia galangal.* Trop. Plant Sci.Res. I(1) : 49-52.

[19] Pandey, M. C., J. R. Sharma and A. Dikshit. 1996. Antifungal evaluation of the essential oil of *Cymbopogon pendulus* (Nees ex Steud.) Wats. cv. Praman. Flavor and Fragrance Journal. 11:257-260.

[20] Shahi, S. K., A. C. Shukla, A. K. Bajaj, U. Banerjee, D. Rimek, G. Medgely, and A. Diksit. 2000. Broad spectrum herbal therapy against superficial fungal infections. Skin Pharmacol Appl Skin Physiol. 13:60-64.

[21] Patra, M., S.K. Shahi and A. Dikshit, 2003. Utilization of pericarp of Citrus sinensis oil for the development of natural antifungal against nail infection. Current Science. 84(12): 1512-1515.

[22] Shukla, A.C, N. Shukla, A. Dikshit and H. Lalramnghinglova, 2009. Ethno Medicinal Approach to Drug Development: Present Status and Future Prospects. Planta Med, USA 75: 963.

[23] Singh, A. K., A. Dikshit and S.N. Dixit. 1983. Antifungal studies of *Peperomia pellucida*. Beitr. Biol. Pflanzen. 58:357-368.

[24] Shahi, S.K., M. Patra, A.C.Shukla and A. Dikshit, 2003. Use of essential oil as biopesticide against post harvest spoilage in fruits, *Mallus pumilo.* Bio-Control 48: 223-232 (Netherlands).

[25] Singh, A.K., A. Dikshit, M.L. Sharma and S.N. Dixit, 1980.

Fungitoxic activity of some essential oils. Eco. Bot. 34 (2): 186-190

[26] Pandey, D.K., N.N. Tripathi, R.D. Tripathi and S.N. Dixit, 1982. Fungitoxic and phytotoxic properties of the essential oil of *Caesulia* axillaries Roxb. (Composite). Angew Botanik. 56 : 259-267.

[27] Dubey, N.K., N. Kishore and S.K. Singh, 1983. Antifungal properties of the volatile fraction of *Melaleuca leucadendron.* Trop. Agric. (Trinidad) 60 (3): 227-228.

Advances in Environmental Biology, 5(6): 1271-1278, 2011
ISSN 1995-0756

ORIGINAL ARTICLE

Role of Phylogenetic Analysis for Anti-bacterial Activity of Essential Oil of *Trachyspermum Ammi* L. Against Water Borne Pathogens

[1]Awadhesh Kumar, [1]Rohit K. Mishra, [1]Shalu Srivastava, [1]Amit K. Tiwari, [1]Anand Pandey, [2]Amritesh Chandra Shukla and [1]Anupam Dikshit

[1]Biological Product Laboratory, Department of Botany, University of Allahabad Allahabad-211002, India.
[2]Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University, Tanhril, Aizawl-796 009, India.

Awadhesh Kumar, Rohit K. Mishra, Shalu Srivastava, Amit K. Tiwari, Anand Pandey, Amritesh Chandra Shukla and Anupam Dikshit: Role of Phylogenetic Analysis for Anti-bacterial Activity of Essential Oil of *Trachyspermum Ammi* L. Against Water Borne Pathogens

ABSTRACT

In developing countries the unsafe drinking water is the main cause of several human fatal diseases of bacterial origin. The phylogenetic relationships of five water borne bacterial pathogens were determined with help of ClustalW computer program using their ITS1 sequences of the aligned standard strains. In the present study the essential oil of *Trachyspermum ammi* L. extracted by hydrodistilation process and their bio-active constituents were analyzed by the Gas Chromatography and Mass Spectroscopy. *In vitro* antibacterial activity of essential oil was tested against *Escherichia coli* (ATCC-25922); *Escherichia coli* (MTCC-723); *Vibrio Cholerae* (MTCC-3906); *Salmonella typhimurium* (MTCC- 98) and *Shigella dysenteriae* (ATCC-23513) using Broth Microdilution method recommended by CLSI (NCCLS). The essential oil of *T. ammi* exhibited the minimum inhibitory concentration (MIC) against *E. coli* (ATCC) 0.087 mg/ml, *V. cholerae* 0.107 mg/ml, *S. typhimurium* 0.109 mg/ml, *E. coli* (MTCC) 0.128 mg/ml, *S. dysenteriae* 0.162 mg/ml respectively. The *in vitro* susceptibility testing indicated that the essential oil of T. ammi L. was most potent antibacterial tool for water borne bacterial diseases originated by contaminated drinking water.

Key words: Bacterial origin, ClustalW, MIC, NCCLS, Susceptibility,

Introduction

Water is one of the key natural resource that is inevitable for sustainability of human and environmental health. There is a strong and direct link between people's health and the development of communities. Gleick [1] and the World Health Organization [2] summarized these links as: poor health reduces life expectancy and educational achievement; it reduces investment and returns from investment (as production, productivity and employment decrease); it reduces parental investment in children (and increases the fertility rate); it increases health inequity and poverty; and it reduces social and political stability. Inadequate water services together with sanitation to the rural poor are among the most serious challenges facing the developing world. Every year, approximately 3.4 million people die due to water-borne diseases, with the greatest health burden falling on children [3].

India is amongst those developing countries, where still vast population resides in the rural areas. They are basically dependent on the supply of untreated water, and mostly on river, ponds and dug wells. This water is unsafe for drinking purposes having a lot of water borne disease causing bacteria such as *Escherichia coli, Vibrio cholerae, Salmonella typhi, Salmonella typhimurium Shigella dysenteriae,*

Corresponding Author
Anupam Dikshit, Biological Product Laboratory, Department of Botany, University of Allahabad Allahabad-211002, India.
E-mail: anupambplau@rediffmail.com

Staphylococcus aureus and *Klebsiella pneumoniae* etc. These enteropathogenic bacteria in water are responsible for a variety of diseases like cholera, typhoid, dysenteries, bacillary dysentery, etc. in human and livestock [4]. Indian subcontinent is a known hot spot for diarrhoeal epidemics as evident by the number of epidemics reported [5]. Ten major water-borne diseases are responsible for over 28 billion disease episodes annually in developing countries [6]. In 1994, an estimated 14 million people had no access to clean water. Although initiatives were taken and improvement measures implemented, 7 million of the 14 million people in rural areas lack safe and clean water [7].

Indian systems of medicine have been developing since the first human civilizations in the Indian subcontinent, Charaka, the ancient Hindu physician and the author of the Great Ayurvedic treatise, "Charaka Samhita", devoted an entire chapter to Atisara which means diarrhoea, which he attributed to diminution of Agni or power of digestion [8]. Around 70% of population in India relies on these systems for primary health care. The proportion of use of plants in the different Indian systems of medicine is: Ayurveda 2000, Siddha 1300, Unani 1000, Homeopathy 800, Tibetan 500, Modern 200 and folk 4500 [9]. Plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies pertinent for natural therapies. According to the World Health Organization (WHO) medicinal plants would be the best source from which to obtain a variety of drugs [10]. According to [11] and [12], 80% of the rural population depends on traditional medicines for their primary healthcare needs. Therefore, putative medicinal plants should be investigated to understand their properties, safety, and efficacy [13]. Plant essential oils are a potentially useful source of antimicrobial compounds. Botanicals, such as essential oils and plant extracts, have been studied for their antimicrobial activities [14]. Other than antibacterial and antiviral effects, most EOs investigated possesses insecticidal, antifungal, acaricidal, cytotoxic and antioxidant properties [15]. Due to the potential for antimicrobial and antioxidant properties [16,17] of *Trachyspermum ammi* L. (Sprague), it had been found to be of strong inhibitory use against a wide range of water-borne disease causing enteropathogenic bacteria. The medicinal plant *T. ammi* is one of the most famous medicinal plants in the treatment of a large number of human ailments being mentioned in Ayurveda, Sushrita Samhita and Charaka Samhita [18].

The aim of present study was to compare antimicrobial activity of essential oils against common water-borne diseases causing bacteria, including *E. coli, V. cholerae, S. typhimurium,* and *S. dysenteriae*. Evaluating minimum inhibitory concentration and GC/MS analysis of the main components of the oil was done for their contribution as alternative products for microbial control in drinking water causing diseases.

Materials and methods

Test organisms:

Two bacterial strains of *E. coli* (ATCC 25922), *E. coli* (MTCC 723) and *V. cholerae* (MTCC 3906) *S. typhimurium* (MTCC 98) and *S. dysenteriae* (ATCC 23513) isolates obtained from Microbial type culture collection (MTCC), Chandigarh and American Type Culture Collection (ATCC), Bangalore, India were stored at 4°C. These microorganisms were selected for assay study, as these are common enteropathogens causing water-borne diseases. The pure cultures were maintained by routine sub-culturing at one-week interval in nutrient agar slants.

Essential oil:

The essential oil of medicinal plant *T. ammi* L. (Sprague) family lamiaceae was used in this study. The oil was extracted through Clevenger's apparatus [19] and dried over anhydrous sodium sulphate. Basically the plant materials (250g) were cut into small pieces and placed it into flasks (5L) with normal water (2L). Through steam distillation continuous extraction head was attached to the flask. After (3h) the oil was isolated. The oil thus obtained was subjected for isolation and identification of their active constituents.

GC-MS analysis:

Gas Chromatographic analysis of the oil of *T. ammi* was performed on a Perkin- Elmer GC 8500, using a fused capillary column (25m x 0.55 mm i.d., film thickness 0.25µm), coated with dimethtyl siloxane (BP-1). The oven temperature was adjusted at 60°C to 220°C at 5°C/min. then held isothermal at 220°C for 15 min. injector temperature, 250°C, detector temperature, 300°C, carrier gas, nitrogen at a linear velocity of 10 psi: split, 1:80. GC-MS data were obtained on a Shimazdu QP-2000 mass spectrometer at 70Ev and 250°C. GC column: Ulbon HR-1 equivalent to OV-1, fused silica capillary column 0.25 mm x 50m, film thickness 0.25 um. The 100°C for 7 min.was initial temperature and heated at 5°C/min to 250°C. Carrier gas helium at a flow rate of 2ml/min.The percentage composition of *T. ammi* L. (Sprague) leaves oil clearly indicated Thymol as its major constituent as shown in the Fig. 1.

Preparation and Standardization of Inoculum:

The cultures of strain considered for study were maintained on NA (nutrient agar) slants at 4ºC. After pure growth a loopful bacterial culture of each test organism were transferred in 5ml of Muller Hinton Broth (MHB) and incubated at 35- 37ºC for 20-24 hours. The turbidity of the full growing bacterial suspension was compared and matched with the turbidity of 0.5 McFarland units. McFarland standards are prepared by adding sulfuric acid, (0.18 M 99.5 mL) to an aqueous solution of barium chloride, (0.048 M 0.5 mL) which results in the formation of a suspended barium sulfate precipitate. The McFarland 0.5 standard corresponds approximately to a homogeneous suspension of 1.5 x 10^7 cells per mL.

Minimum Inhibitory Concentration Test (MIC):

The minimum inhibitory concentration (MIC) values were evaluated using the Broth Microdilution method according to standard methods [20]. Sterile 96-well microtitre plates with lids (SPL) were used for the assay of bacterial strains cultured overnight at 37^0C in Muller Hinton Broth (MHB). Stock solutions of the EO were prepared in 40mg/ml in dimethyl sulfoxide (DMSO). Dilution series, using MHB, were prepared from 10 to 0.01 mg/ml. From stock solution, 100µl were transferred into 96-well microtitre plates, followed by adding 90µl and 80µl of MHB in each wells of rows number 3 to 4. Then, added 10µl and 20µl essential oil in each well of rows number 3 and 4 respectively. Serial dilutions were carried out from rows 4 to 11 and excess broth (100µl) was discarded from row 11. Inoculums of 100µl were added to each well, making the final volume in each well 200µl. The inocula of each bacterial strain were prepared using a 16 h Broth culture and suspensions were adjusted to 0.5 McFarland standards turbidity. Row 12 wells, consisting of MHB 5.0% (v/v), DMSO and microorganisms was the growth control; and row 1 wells, containing MHB, inoculum and formaldehyde served as the negative control. After incubation at 37^0C for 22–24 h, the microorganism growth inhibition was evaluated by measuring O.D. at 630nm, using an ELISA reader (Spectramax Plus^{384}, Molecular Devices Corporation, USA). The MICs was defined as lowest concentration of essential oil, showing no visible bacterial growth after incubation time [21,22]. All tests were performed in triplicate.

Minimum Bactericidal Concentration (MBC):

Aseptically 100 µl aliquot of inoculum was taken from those wells which not showed any turbidity in incubated 96 well plate treated with essential oil. After poured on to Nutrient agar plates incubated for 24 hours at 37°C. MBC was defined as the lowest concentration of the essential oil at which 99.99% or more of the initial inoculum was killed. If there was no growth, it means the concentration was cidal.

Phylogenetic Relationship Between Pathogenic Bacterial Strains:

Using the Clustal W computer program [23] and GENETYX-MAC 10.1 software (Software Development Co., Ltd., Tokyo, Japan, the phylogenetic analysis of both the mentioned strains of *E. coli*, *V. cholerae*, *S. typhimurium* and *S. dysenteriae* was done. Phylogenetic trees were then constructed by the DNA maximum-likelihood (ML) method in the PHYLIP program (Phylogeny Inference Package), version 3.5c [24] and the neighbor-joining (NJ) [25] method in the NJPLOT program [26]. Bootstrap analysis with the Clustal W program was performed. With all these data into consideration, the relation between the dependence of effectiveness of the essential oil on these pathogens in terms of phylogeny was studied.

Nucleotide Sequence Accession Numbers:

For the phylogenetic analysis, data in the form of nucleotide sequences was obtained from GenBank nucleotide sequence database [27]. The ITS1 sequences of the standard strains used in the study with accession no. X80724; *E. coli* [28], accession no. J01859; *E. coli* [29] accession no. X76337; *V. cholerae* [30], accession no. X80681; *S. typhimurium* [28] and *S. dysenteriae* [31] accession no. GU271883 were aligned.

Results and discussion

The essential oil of *T. ammi* L., having the major active constituent's thymol 80.70%, α- pinene 7.90% and p-cymene 11.40% showed broad spectrum antimicrobial activitiy.

It was used against gram-negative enteropathogenic bacteria, *E. coli (ATCC25922*), *E. coli (MTCC723), V. cholerae (MTCC3906), S. typhimurium* (MTCC 98) and *S. dysenteriae* (ATCC 29026). The test was performed by the Broth microdilution method [20]. The antibacterial potency was quantitatively assessed in accordance to the MICs as well as IC_{50} values obtained. The data of the study clearly indicated that the essential oil showed great minimum inhibitory concentrations (MICs) against *E. coli* (ATCC) MIC: 0.087 mg/ml and $IC_{50:}$ 0.085 mg/ml, *V. cholerae* MIC: 0.107 mg/ml and IC_{50}: 0.074 mg/ml, *S. typhimurium* MIC: 0.109 mg/ml, IC50: 0.007mg/ml, *E. coli* (MTCC) MIC: 0.128 mg/ml, IC_{50}: 0.092 and in case of S. *dysenteriae* MIC: 0.162 mg/ml, IC_{50}: 0.155 mg/ ml were recorded.

However, the activeness of the essential oil over the studied bacteria was observed according to their phylogenetic position.

The phylogenetic study of ITS1 sequences (fig. 2) of 16S rRNA of the above mentioned pathogenic bacteria clearly revealed that both the strain of *E.*

coli i.e. *E. coli* (ATCC) and *E. coli* (MTCC) have a similar origin and *V.cholerae* also parallel to same the phylogenetic line. *S. typhimurium* is near to *V. cholerae* but, *S. dysenteriae* is away from the *E. coli* as well as *V. cholerae* in their phylogenetic tree. Although all above pathogens are gram negative but differences in their phylogeny as seen in fig.3. So Activeness of essential oil also varied. The variation in MICs for *E.coli* (ATCC) and *E.coli* (MTCC) were observed due to differences in their geographical origin. The similar activity of the same concentration of the essential oil against *S. typhimurium and V. cholerae* validates the phylogenetic analysis. Apart from these, *S. dysenteriae* remarkably had shown a lot of difference in its phylogeny. So a large variation occurred in its MICs value as well as IC_{50} value at the tested concentration of essential oil.

On the basis of the MICs, it was inferred that the antibacterial activity of the essential oil was more potent against *E. coli* (ATCC) but, for *S. dysenteriae* found less effective. Considering the MICs values, the percentage of inhibition of essential oil was better for *E. coli* (ATCC) than *E. coli* (MTCC).

The data confirmed that, at the low concentration the bacterial growth is enhanced but, concentrations from 1.25 to 0.313mg/ml is quite more effective (fig. 4).

The bacterial growth inhibition is variable at concentration 0.078mg/ml. The growth of *E.coli* (ATCC), *E.coli* (MTCC) and *V.cholerae* are increaesd at concentration of 0.039mg/ml. The average percentage growth inhibition indicated that the, a higher concentration of 0.320 exhibited bactericidal behaviour (Table 2).

The large number of different groups of chemical compounds present in EO, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell [32]. An important characteristic of EOs and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable leading to leakage of ions and other cell contents [33].

The previous work suggest that essential oils possessing the strongest antibacterial properties against water borne pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol (2-methoxy-4-(2-propenyl) phenol) and thymol [34,35,36,37]. Thus, the inference was drawn that the strong antibacterial activity of essesntial oil of *T. ammi* L. is due to the thymol, it was found to be its major active constituent. Mechanism of action of thymol would therefore be similar to other phenolics; which is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force (PMF), electron flow, active transport and coagulation of cell contents[38,39]. Oil toxicity not affected membranes only but also affects cell wall leading to disrupt and thicken, in polar regions and breakdown in some parts which resulted in leakage of cytoplasmic materials [40]. According to another study, the thymol decreased the intracellular ATP pool of *E.coli* and also increased extracellular ATP, indicating disruptive action on the cytoplasmic membrane [41]. It was often reported that Gram-negative bacteria were more resistant to the essential oils present in plants [42] because LPS avoids the accumulation of the oils on the cell membrane, which is in agreement with the work of [43]. The results obtained in our study showed that *E. coli (ATCC)* was found to be more sensitive than *S. dysenteriae*. *E. coli* have a multi-layered structure [44] with many intermembranous preoteins meant for their permeability along with lipopolysaccride. Therefore, on basis of present phylogenetic study, the result can be drawn that the lipopolysaccharides (LPS) present in pathogenic bacterial cell wall were found to be very sensitive in *E. coli* (ATCC) 0.087 mg/ml, than *V. cholerae* 0.107 mg/ml, *S. typhimurium* 0.109 mg/ml, *E. coli* (MTCC) 0.128mg/ml and *S. dysenteriae* 0.162 mg/ml. These differences found between the activities of the EO against the tested pathogens were not only due to differences in their phylogeny, difference in antibacterial activities of essential oil may be related to the concentration and nature of contents, to the respective composition, the functional groups, the structural configuration of the components and their possible synergistic interaction [45].

Conclusion:

The water contamination is a very serious and fatal health problem for the public, but may be better controlled by the use of natural preservatives. The study showed that the essential oil of *Trachyspermum ammi* L. possessed good inhibitory activity against the tested enteropathogenic bacteria. The major active constituent of the oil was found to be thymol (phenolic compound), which was responsible for its strong antibacterial activity. The comparably cheaper, safer and reasonably good antibacterial activity; thus, supports its traditional use for water borne bacterial diseases in the rural areas. The presence of most general phytochemicals in *T. ammi* might be responsible for their therapeutic effects. It further reflects a hope for the development of many more novel chemotherapeutic agents or templates from such plants which in future may serve for the production of synthetically improved therapeutic agents.

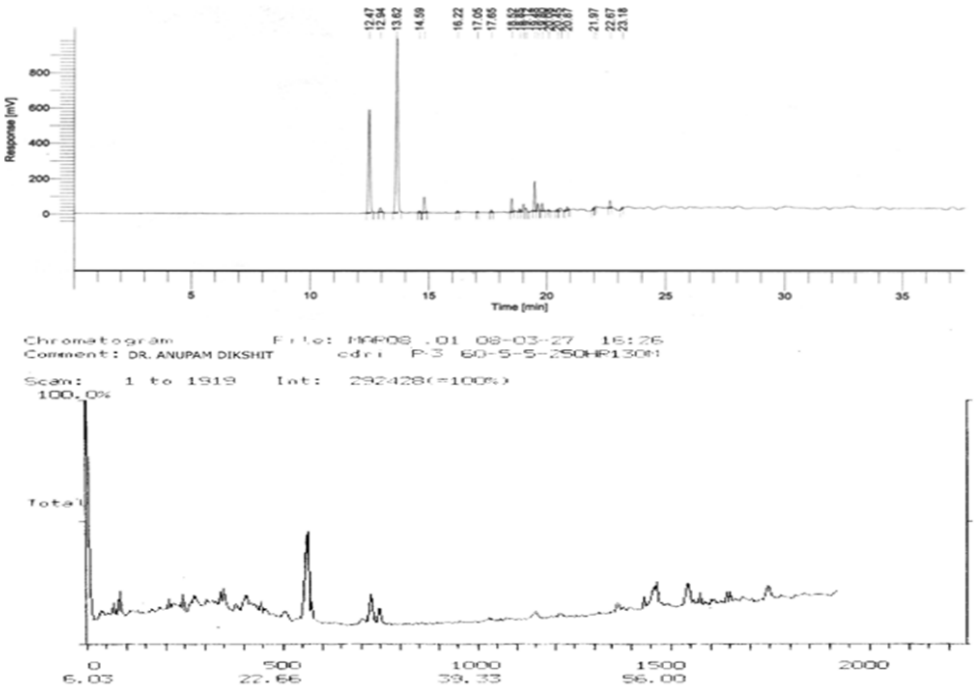


Fig. 1: GC/MS chromatograms of *Trachyspermum ammi* L.. leaf essential oil

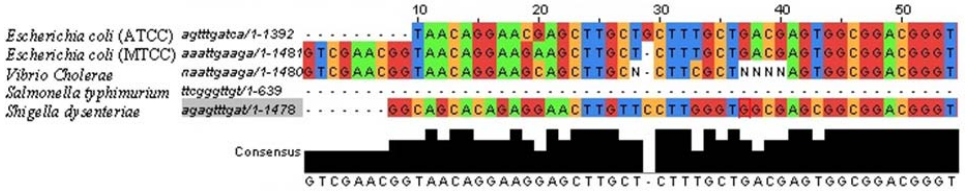


Fig. 2: Alignment of ITS1, 16S rRNA sequences of water borne bacterial pathogens.

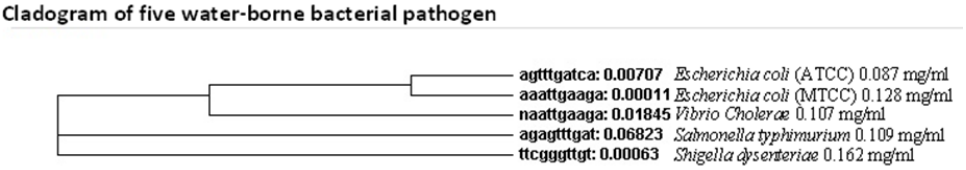


Fig. 3: Results of cladogram (Neigbour Joining Tree plot) of water borne bacterial pathogens on the basis of their ITS1 sequences. The N.J. tree was constructed with data for standard strains of water borne bacterial pathogens.

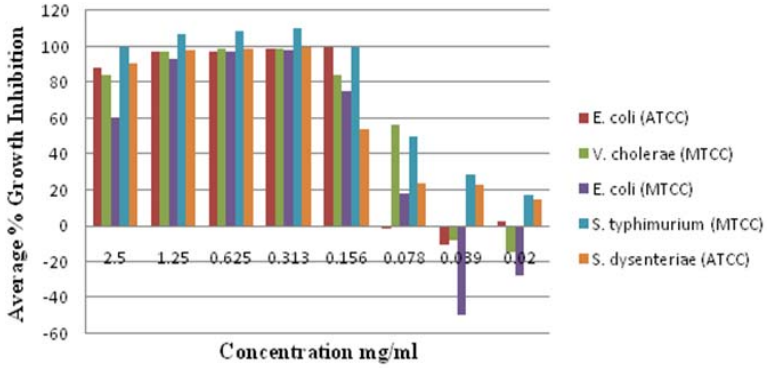


Fig. 4: Percentage growth inhibition of five water borne bacterial pathogens using various concentrations of *Trachyspermum ammi* L. leaf essential oil.

Table 1: Major chemical compound of *Trachyspermum ammi* L.. leaf essential oil

S. No.	Name of Active constituents	% Active constituents
1	Thymol	80.70
2	α-pinene	7.90
3	ρ-cymene	11.40

Table 2: Minimum Inhibitory Concentration (MIC) and IC_{50} of *Trachyspermum ammi* L. leaf essential oil against water borne bacterial pathogens.

S. No.	Water borne bacterial pathogens	IC_{50}	MIC	MBC
1	*E. coli* (ATCC25922)	0.085	0.087	0.175
2	*E. coli* (MTCC723)	0.092	0.128	0.260
3	*V. cholerae* (MTCC3906)	0.074	0.107	0.212
4	*S. typhimurium*(MTCC 98)	0.007	0.109	0.220
5	*S. dysenteriae* (ATCC 29026)	0.155	0.162	0.320

Acknowledgment

This innovative research was supported by the Department of Science and Technology (DST) Ministry of Science and Technology, Government of India, and we are thankful to Head, Department of Botany, University of Allahabad for providing laboratory facility. The authors are also thankful to the authorities of Mizoram University, Aizawl for providing various types of facilities during the course of research.

References

1. Gleick, P.H., 2002. Estimated deaths from water related diseases 2000-2020, Research Report, 15th August 2002. California: Pacific Institute for Studies in Development, Environment, and Security.
2. WHO, 2003. The Global Water Supply and sanitation Assessment 2000, Retrieved 28 December 2004.
3. UN-Habitat, 2003. Water and Sanitation in the World's Cities. Local Action for Global Goals, London: Earthscan.
4. Ashbolt, N.J., 2004. Microbial contamination of drinking water and disease outcome in developing regions, Toxicology., 198: 229-238.
5. Kelkar, S.D., P.G. Ray and N. Shinde, 2004. An epidemic of rotavirus diarrhoea in Jawhar–Taluk, Thane district, Maharashtra, India, December 2000–January 2001. Epi-demiology and Infection., 132: 337-341.
6. Walsh, J.A., 1990. Estimating the burden of illness in the tropics, In K. S. Warren & A. A. F. Mahoud, (Eds.), Tropical and geographical medicines (2nd Ed.) New York: Mc-Graw Hill.
7. Duse, A.G., M.P. da silver and I. Zietsman, 2003. Coping with hygiene in South Africa, a water scarce country, Int. J. Environ. Health res., 13: 95- 105.
8. Sharma, R.K., V.B. Dash, 1997. Agnivesa's Caraka Samhita based on Cakrapani Datta's Ayurveda Dipika, vol. IV. Chowkhamba Sanskrit Series, Varanasi., 204-242.
9. Pandey, M.M., S. Rastogi and A.K. Rawat, 2008. Indian Herbal Drug for General Healthcare: An Overview. The Int J of Alter Med., 6: 1.
10. Santos, P.R.V., A.C.X. Oliveira and T.C.B. Tomassini, 1995. Controle microbi´ogico de produtos fitoter´apicos. Rev Farm Bioqu´ım., 31: 35-8.
11. Akerele, O., 1993. Nature's medical botany: Do not throw it away, World Health Forum, 14: 390-95.
12. Nyazena, Z.N., F.C. Kire, 1986. The interaction between traditional medicine remedies used for gastrointestinal diseases as conventional drugs. East Afr Med J., 63: 258-62.
13. Ellof, J.N., 1998. Which extractant should be used for the screening and isolation of antimicrobial components from plants. J Ethnopharmacol., 60: 1-6.
14. Tepe, B., E. Donmez, M. Unlu, F. Candan, D. Daferera and G. Vardar-Unlu, 2004. Antimicrobial and antioxidative activities of the essential oils and methanol extracts of *Salvia cryptantha* (montbret et aucher ex benth.) and *Salvia multicaulis* (vahl). Food Chem., 84: 519-525.
15. Sacchetti, G., S. Maietti, M. Muzzoli, M. Scaglianti, S. Manfredini, M. Radice, 2005. Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. Food Chem., 91: 621-632.
16. Bandonıene, D., P.R. Venskutonıs, D. Gruzdıene and M. Murkovıc, 2002. Antioxidative activity of sage (*Salvia officinalis* L.), savory (*Satureja hortensis* L.) and borage (*Borago officinalis* L.) extracts in rapeseed oil. European J of Lipid Sci and Tech., 104: 286-292.
17. Exarchou, V., N. Nenadis, M. Tsımıdou, I.P. Gerothanassis, A. Troganis and D. Boskou, 2002. Antioxidant activities and phenolic composition of extracts from Greek oregano, Greek sage, and summer savory. J of Agri and Food Chem., 50: 5294-5299.

18. Pathak, A.K., N. Nainwal, M. Goyal, R. Singh, V. Mishra, S. Nayak, P. Bansal and V. Gupta, 2010. Pharmacological activity of *Trachyspermum ammi*: A Review. J of Pharm Res., 3(4): 895-899.
19. Clevenger, J.F., 1928. Apparatus for the determination of volatile oil. J Am Pharm. Assoc, 17: 346-346.
20. NCCLS, 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically (5th Ed.), Approved standard, M7-A5. Pennsylvania: Wayne.
21. Hammer, K.A., C.F. Carson and T.V. Riley, 1999. Antimicrobial activity of essential oils and other plant extracts. J of Appl Microbiol., 86: 985-990.
22. Delaquis, P.J., K. Stanich, B. Girard and G. Mazza, 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. Int.J. Food Microbiol., 74: 101-109.
23. Higgins, D., 1996. Clustal W, Version 1.6. Cambridge, United Kingdom. The European Bioinformatics Institute.
24. Felsenstein, J., 1995. PHYLIP (Phylogeny Inference Package), version 3.5c. Seattle, University of Washington.
25. Saito, N and M. Nei, 1995. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol., 4: 406-425.
26. Maidak, B.L., N. Larsen. M.J. McCaughey, R. Overbeek, G.J. Olsen, K. Fogel, J. Blandy and C.R. Woese, 1994. The Ribosomal Database Project, Nucleic Acids Res., 22: 3485-3487.
27. Cilia, V., B. Lafay and R. Christen, 1996. Sequence heterogeneities among 16S ribosomal RNA sequences, and their effect on phylogenetic analyses at the species level. Mol. Biol. Evol., 13(3): 451-461.
28. Aznar, R., W. Ludwig, R.I. Amann and K.H. Schleifer, 1994. Sequence determination of rRNA genes of pathogenic *Vibrio* species and whole-cell identification of *Vibrio vulnificus* with rRNA-targeted oligonucleotide probes. Int. J. Syst. Bacteriol., 44(2): 330-337.
29. Ehresmann, C., P. Stiegler, P. Fellner, J.P. Ebel, 1972. The determination of the primary structure of the 16S ribosomal RNA of *Escherichia coli*. 2. Nucleotide sequences of products from partial enzymatic hydrolysis. Biochimie., 54(7): 901-967.
30. Drancourt, M., C. Bollet, A. Carta and P. Rousselier, 2001. Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov, *Raoultella terrigena* comb. nov. and *Raoultella planticola* comb, Nov. Int. J. Syst. Evol. Microbiol., 51(3): 925-932.
31. Zerzghi, H., J.P. Brooks, C.P. Gerba and I.L. Pepper, 2010. Influence of long-term land application of Class B biosolids on soil bacterial diversity. J. Appl. Microbiol, 109(2): 698-706.
32. Carson, C.F., B.J. Mee, T.V. Riley, 2002. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on Staphylococcus aureus determined by time-kill, lysis, leakage and salt tolerance assays and electron microscopy. Antimicro Agents and Chemo., 46(6): 1914-1920.
33. Sikkema, J., J.A.M. De Bont and B., Poolman, 1994. Interactions of cyclic hydrocarbons with biological membranes, J of Bio Chem., 269(11): 8022-8028.
34. Cosentino, S., C.I.G. Tuberoso, B. Pisano, M. Satta, V. Mascia, E. Arzedi and F. Palmas, 1999. In vitro antimicrobial activity and chemical composition of Sardinian Thymus essential oils. Lett in Appl Microbiol., 29: 130-135.
35. Dorman, H.J.D. and S.G. Deans, 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J. of Appl. Microbiol., 88: 308-316.
36. Juliano, C., A. Mattana and M. Usai, 2000. Composition and in vitro antimicrobial activity of the essential oil of Thymus herba-barona Loisel growing wild in Sardinia, J of Essent Oil Res., 12: 516-522.
37. Lambert, R.J.W., P.N. Skandamis, P. Coote and G.-J.E. Nychas, 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J of Appl Microbiol., 91: 453-462.
38. Sikkema, J., J.A.M. De Bont and B. Poolman, 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiolog Rev., 59(2): 201-222.
39. Davidson, P.M., 1997. Chemical preservatives and natural antimicrobial compounds, In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), Food Microbiology: Fundamentals and Frontiers, ASM, Washington., 520-556.
40. El-Meleigy, M.A., M.E. Ahmed., R.A. Arafa., N.A. Ebrahim and E.E. El-Kholany, 2010. Cytotoxicity of four essential oils on some human and bacterial cells. J of appl sci in environm sanit., 5(2): 143-159.
41. Helander, I.M., H.L. Alakomi, K.L. Kala, T.M. Sandholm, I. Pol, E. J. Smid, L.G.M. Gorris and A.V. Wright, 1998. Characterization of the Action of Selected Essential Oil Components on Gram-Negative Bacteria. J. Agric. Food Chem., 46: 3590-3595.
42. Smith-Palmer, A., J. Stewart and L. Fyfe, 1998. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens, Lett in Food Microbiol., 26: 118-122.
43. BeziT, N., M. SkobibuniT, V. DunkiT and A. RadoniT, 2003. Composition and antimicrobial activity of *Achillea clavennae* L. essential oil. Phytoth Res., 17: 1037-1040.

44. Essawi, T. and M. Srour, 2000. Screening of some Palestinian medicinal plants for antibacterial activity, J of Ethnopharma., 70: 343-349.

45. Chang, S.T., P.F. Chen and S.C. Chang, 2001. Antibacterial activity of leaf essential oils and their constituents from *Cinnamomum osmophloeum*. J of Ethnopharma., 77: 123-127.

Journal of Ecobiotechnology 2/4: 22-26, 2010
ISSN 2077-0464

Journal of Ecobiotechnology
http://journal-ecobiotechnology.com/

Quantitative and Rapid Antibacterial Assay of *Micromeria biflora* Benth. Leaf Essential Oil Against Dental Caries Causing Bacteria Using Phylogenetic Approach

Rohit Kumar Mishra[1], Awadhesh Kumar[1], Amritesh Chandra Shukla[2], Pravin Tiwari[3] and Anupam Dikshit[1]*

[1]Biological Product Laboratory, Dept. of Botany, University of Allahabad, Allahabad-211002
[2]Dept. of Horticulture, Aromatic & Medicinal Plants, Mizoram University, Aizawl-796009, India
[3]Dental Surgeon, Saumya Dental Clinic, Taigore Town, Allahabad, India

**Corresponding author, Email: anupambplau@rediffmail.com, Tel: +919335108519*

Keywords

Antibacterial activity
Dental caries
Essential oil
Micromeria biflora Benth
Phylogenetic Analysis
Streptococcus mutans

Abstract

The phylogenetic relationship of four dental caries causing bacterial pathogens has been studied using ITS1 sequences of the standard strains were aligned by using the ClustalW computer program. The essential oil obtained from the leaves of *Micromeria biflora* Benth., obtained by hydrodistillaton. The chemical compositions of the essential oil from *Micromeria biflora* Benth was analyzed by gas chromatography-mass spectrometry (GC-MS). The GC/MS analysis showed eight major active constituents in the leaf essential oil of *Micromeria biflora* Benth. The antibacterial activity of the oil was evaluated against four dental caries causing bacteria such as *Streptococcus mutans* (MTCC 890); *Lactobacillus acidophilus* (MTCC 447); *Streptococcus mitis* (MTCC 2695) and *Streptococcus salivarius* (MTCC 1938) using broth microdilution method recommended by Clinical Laboratory Standards Institute (CLSI) formely (NCCLS). It's showed excellent activity against *Streptococcus mutans* with their Minimum inhibition concentration (MIC) 0.15 mg/ml and (IC_{50}) 0.10 mg/ml and less effective against *Lactobacillus acidophilus*. The essential oil of *Micromeria biflora* Benth from leaf has played a significant role against dental caries causing bacteria. Relationships of the dental caries causing pathogens to the toxicity of the oil vis-à-vis phylogeny using molecular data of pathogens have also been discussed.

1. Introduction

Dental caries is a multifactorial infectious disease, usually associated with increased numbers of *Streptococcus mutans* at the site of the disease. In addition, other microflora like *Lactobacillus acidophilus, Streptococcus salivarius, Streptococcus mitis* and *Streptococcus sanguis* are also involved in the process of causing dental caries. Estimation of the salivary levels of this organism may be useful for assessing caries risk in patients and for monitoring their response to preventive measures (1). *Streptococcus mutans* is an important component of the biofilms on teeth (dental plaque) associated with many forms of dental caries. *Steptococcus mutans* adheres firmly to the smooth tooth surfaces and produces sticky water insoluble dextran from dietary sucrose, forming plaque, which facilitates the accumulation of microorganisms. *Streptococcus mutans* and other organisms in the plaque produce organic acids such as lactic acid that gradually destroy the enamel and form a cavity (2).

The *Micromeria biflora* Benth, known as Indian wild Thyme, belongs to family Lamiaceae found in tropical, temperate Himalayas and Western Ghats. It is hardy to zone 0, flowers from June to August, and the seeds ripened from August to September. The flowers are hermaphrodite (have both male and female organs) and are insect-pollinated. A paste of the root was pressed between the jaws to treat toothache (3). The plant was rubbed and the aroma inhaled to treat nose bleeds (3). A paste of the plant was used as a poultice to treat wounds (3). The juice of the plant is taken internally and also inhaled in the treatment of sinusitis. The objective of this study was to investigate toxicity of the *Micromeria biflora* Benth. leaf essential oil vis-à-vis phylogeny using molecular data of pathogens. In particular, 16S rDNA sequences have been widely used to construct bacterial phylogenetic relationships (4, 5).

2. Materials and Method

Collection of plant materials and extraction of essential oil

The essential oil was extracted from the fresh leaves of *Micromeria biflora* Benth. collected from the Himanchal Pradesh, India by hydro-distillatation using Clevenger's apparatus (6). A clear dark

reddish yellow coloured oily layer was separated and dried with anhydrous sodium sulphate.

Physio-chemical properties

The essential oils obtained from *Micromeria biflora* Benth was studied on various parameters of physio-chemical properties such as Plant height, Oil yield, Colour, Specific gravity, Optical rotation, Refractive index and Solubility in 90% alcohol. The results are given in the Table 2.

GC-MS analysis

Gas Chromatography analysis of the oil was performed on a Perkin- Elmer GC 8500, using a fused capillary column (25m x 0.55 mm i.d., film thickness 0.25um), coated with dimethtyl siloxane (BP-1). The oven temperature was programmed at 60°C to 220°C at 5°C/min. then held isothermal at 220°C for 15 min. injector temperature, 250°C, detector temperature, 300°C, carrier gas, nitrogen at a linear velocity of 10 psi: split, 1:80

GC-MS data were obtained on a Shimazdu QP-2000 mass spectrometer at 70 ev and 250°C. GC column: Ulbon HR-1 equivalent to OV-1, fused silica capillary column 0.25 mm x 50m, film thickness 0.25 um. The initial temperature was 100°C for 7 min. and heated at 5°C/min to 250°C. Carrier gas helium at a flow rate of 2ml/min.The percentage composition of *Micromeria biflora* Benth leaves oil is given in the Table1.

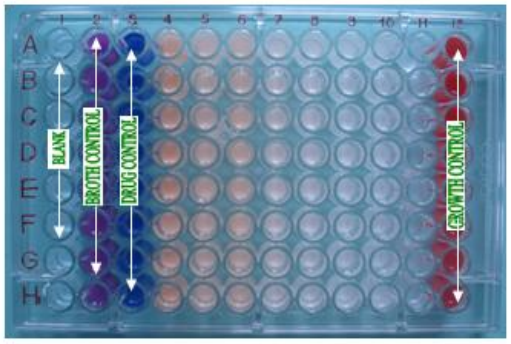
Dental caries causing bacterial pathogens

Four dental caries causing bacterial pathogens were selected for this study; *Streptococcus mutans* (MTCC 890); *Lactobacillus acidophilus* (MTCC 447); *Streptococcus mitis* (MTCC 2695) and *Streptococcus salivarius* (MTCC 1938). Cultures were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. The cultures of bacteria were maintained on Nutrient agar slants at 4ºC throughout the study used as stock cultures.

Determination of minimum inhibitory concentration (MIC) and IC_{50} by broth micro dilution method

The antimicrobial activity of compounds was determined by broth micro dilution method recommended by Clinical Laboratory Standards Institute (CLSI) formerly NCCLS (7) using Mueller Hinton Broth. All the standard water borne bacterial cultures were maintained on Nutrient agar at 37°C. The 96-well tissue culture plates were used for twofold serial dilution. The proper growth control, drug control and the blank was adjusted onto the plate. Essential oil of *Micromeria biflora* Benth was dissolved in 5-10% DMSO at a concentration of 50mg/ml stock solution in case of natural antimicrobials, 20 µl of drug was added into 4th well of 96-well tissue culture plate horizontally having 180µl Mueller Hinton Broth (Fig. 1). So the maximum concentration of the test essential oil was 2.5mg/ml. From here the solution was serially diluted upto 4th well to 11th well resulting into the half of the concentration of test essential oil. The bacterial inoculum was prepared at 0.5 McFarland standards; the absorbance was equal to the inoculum suspension containing $1x10^7$ cells per ml for bacterial isolates. Then standard bacterial inoculums was added and kept for incubation at 37°C in a moist chamber. The Minimum Inhibitory Concentration (MIC) and Inhibitory Concentration at 50% (IC_{50}) was recorded spectrophotometrically at 492 nm using SpectraMaxplus 384 after 24 hrs incubation.

Figure 1. Format of drug testing by broth micro dilution method



Determination of minimum bactericidal concentration (MBC)

100 µl aliquot of inoculum was taken aseptically from incubated 96 well plate those well did not show turbidity and poured on to Nutrient agar plates then incubated for 24 hours at 37°C. MBC was defined as the lowest concentration of the essential oil at which 99.99% or more of the initial inoculum was killed. If there was no growth, it means the concentration was cidal. The number of surviving organisms was determined by viability counts. All tests were performed in triplicate.

Phylogenetic study

To find out the reason; why the essential oil is more effective against dental caries causing bacteria, phylogenetic relationship have been studied including *Streptococcus mutans*; *Lactobacillus acidophilus*; *Streptococcus mitis* and *Streptococcus salivarius* using the Clustal W computer program (8) and GENETYX-MAC 10.1 software (Software Development Co., Ltd., Tokyo, Japan). Phylogenetic trees were then constructed by the DNA maximum-likelihood (ML) method in the PHYLIP program (Phylogeny Inference Package), version 3.5c (9) and the neighbor-joining (NJ) (10), method in the NJPLOT program (11), Bootstrap analysis with the Clustal W program was performed.

Nucleotide sequence accession numbers

Data for the phylogenetic analysis were obtained from sequences contained in the GenBank nucleotide sequence database (12). The ITS1 sequences of the standard strains used in this study *Streptococcus mutans* (accession no. AF204255); *Lactobacillus acidophilus* (accession no. HM162411); *Streptococcus mitis* (accession no. NC013853) and *Streptococcus salivarius* (accession no. S41233) were aligned (4,5,13,14).

3. Results and Discussion

Plant essential oils and extracts have been used for many thousands of years (15) especially in food preservation, pharmaceuticals, alternative medicine and natural therapies (16).

In the present study, composition and relative percentages of essential oil of *Micromeria biflora* was determined. 4 major constituents were identified with high content of Thymol 54%, Iso thymol 9.9%, Gurjurene 3.3% and β-caryophyllene 6.6% with RetentionTime (RT) 8.06, 8.83, 10.20 and 12.86 respectively. However, earlier studies suggested that the *Micromeria biflora* sp. Arabica K. Walth, essential oil was analyzed by GC-MS (17), 30 components were identified representing 98.2% of the total oil. The major constituents were trans-caryophyllene (43.7%), caryophyllene oxide (18.0%), spathulenol (8.5%), α-humulene (4.6%), α-myrcene (3.1%), and germacrene-D (3.1%) The present investigation of GC-MS analysis of *Micromeria biflora* essential oil, chemical constituents were quite different due to different agroclimatic changes. The major components and their retention times are summarized in Table 1.

Table 1. Mass Spectoscopy analysis of *Micromeria biflora* Benth leaf essential oil

Peak No.	R. Time (Scan)	I. Scan-F. Scan	Area	Height	Major Compounds	%Total
1	8.06 (62)	59-79	603564	113551	Thymol	54.0
2	8.83 (85)	82-92	110841	40442	Iso-thymol	9.9
3	10.20 (126)	124-130	36581	8317	Gurjurene	3.3
4	12.86 (206)	204-210	74151	21178	β-caryophyllene	6.6

Physio-chemical properties of *Micromeria biflora* Benth leaf essential oil showed various parameters of such as Plant height, Oil yield, Colour, Specific gravity, Optical rotation, Refractive index and Solubility in 90% alcohol. The results are given in the Table 2.

Table 2. Physio-chemical properties of *Micromeria biflora* Benth leaf essential oil

S. No.	Parameter studies	*Micromeria biflora*
1.	Plant height	30 Cm
2.	Oil yield	0.03-0.07%
3.	Appearence	dark reddish yellow
4.	Specific gravity at 25°C	0.8913 to 0.91260
5.	Optical rotation	-3 to -25
6.	Refractive index at 20°C	1.468 to 1.488
7.	Solubility in 90% alcohal	Soluble

Filoche *et al.* (18) reported that essentail oil of *Cinnamon* showed antimicrobial potency (1.25–2.5 mg/ml) against *Streptococcus mutans* and *Lactobacillus plantarum*. However, in the present antibacterial activity of *Micromeria biflora* Benth leaf essential oil were assayed *in vitro* by a broth micro-dilution method against four dental caries causing bacteria such as *Streptococcus mutans* (MTCC 890); *Lactobacillus acidophilus* (MTCC 447); *Streptococcus mitis* (MTCC 2695) and *Streptococcus salivarius* (MTCC 1938) .

According to the results, *Micromeria biflora* Benth leaf essential oil was found to be active against all dental caries causing bacteria. The strongest antibacterial activity was seen against *Streptococcus mutans* with a Minimum inhibitory concentration (MIC) value 0.15 mg/ml and IC_{50} value 0.10 mg/ml. While Minimum inhibitory concentration (MIC) value of *Lactobacillus acidophilus*; *Streptococcus mitis* and *Streptococcus salivarius was* 0.35 mg/ml, 0.20 mg/ml and 0.19 mg/ml respectively. These results are shown in Table 3. Nascimento *et al.,* (19) also

reported that the *Hyptis pectinata* essential oil exhibited considerable inhibitory effect against either all the clinical isolates obtained from patients' saliva or the ATCC strains tested, with minimum inhibitory and bactericidal concentrations of 200µg/mL.

Table 3. Anticaries activity of *Micromeria biflora* Benth leaf essential oil

S. No.	Caries causing bacteria	Antibacterial activity in mg/ml		
		MIC	IC_{50}	MBC
1.	*Streptococcus mutans*	0.15	0.10	0.19
2.	*Lactobacillus acidophilus*	0.35	0.18	0.38
3.	*Streptococcus mitis*	0. 20	0. 16	0.38
4.	*Streptococcus salivarius*	0. 19	0. 15	0.38

The phylogenetic relationships of dental caries causing bacteria were demonstrated by using internal transcribed spacer 1 (ITS1) obtained from GenBank. Alignment of the 16S rRNA nucleotide sequence, adjusted to 1,435 bases, was performed by the computer program MegAlign (DNASTAR Inc.). On the Phylogenetic analysis a clear picture can be drawn as shown in Fig. 2 and 3 *Streptococcus mutans and Streptococcus salvarius* belong to same genetical stock so; they were close to each other. That is the reason the Minimum Inhibitory Concentration (MIC) of test essential oil against *S. mutans* (0.15mg/ml) and *S. salvarius* (0.19mg/ml) reflect almost similar toxicity.

Figure 2. Alignment of ITS1, 16S rDNA sequences of dental caries causing bacterial pathogens

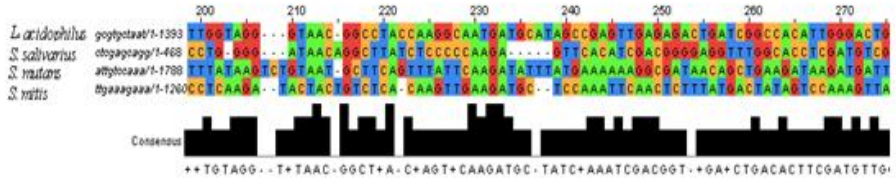


Figure 3. Results of cladogram (Neigbour Joining Tree plot) of dental caries causing bacteria using standard ITS1 sequences

However, *Lactobacillus acidophilus* ***and*** *Streptococcus mitis* found to be closer to each other with their closed MIC *0.35 mg/ml, 0.20*mg/ml. Results are showed in Fig. 2 and 3. An important characteristic of essential oils and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (20,21). Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death (22).

4. Conclusions

In the present study clearly demonstrates that the leaf essential oil of *M. biflora* Benth was exhibited potent bactericidal action and as a therapeutic remedy against dental caries causing bacteria. The effectiveness of the oil was equal to those caries causing bacteria which are close in phylogenetic tree. As such, in future the oil can be used as a potential source of effective and cheap herbal formulation after undergoing successful multicentral topical testing.

Acknowledgements

The authors are very grateful to Department of Science and Technology for financial support and Head, Department of Botany, University of Allahabad for giving me this opportunity to perform experimental work successfully.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the manuscript.

References

1. Hardie, J.M. 1992. Oral microbiology: current concepts in the microbiology of dental caries and periodontal disease. Br. Dent. J. 172:271-278.
2. Islam, B., Khan, S.N., Khan, A.U. 2007. Dental caries: from infection to prevention. Med. Sci. Monit; 13:196-203.
3. Manandhar, N.P. 2002. *Plants and People of Nepal* Timber Press. Oregon, ISBN 0-88192-527-6
4. Denapaite, D., Brueckner, R., Nuhn, M., Reichmann, P., Henrich, B., Maurer, P., Schaehle, Y., Selbmann, P., Zimmermann,W., Wambutt, R., Hakenbeck, R. 2010. The Genome of *Streptococcus mitis* B6- Evolution by Gene Transfer. National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA
5. Pebay, M., Colmin, C., Guedon, G., De Gasperi, C., Decaris, B., Simonet, J.M. 1992. Detection of intraspecific DNA polymorphism in *Streptococcus salivarius* subsp. thermophilus by a homologous rDNA probe. Res. Microbiol. 143:(1),37-46.
6. Clevenger, J.F. 1928. Apparatus for the determination of volatile oil. J Am Pharm. Assoc. 17:346-346.
7. National committee for clinical laboratory standards 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically (5th ed.). Approved standard, M7-A5. Pennsylvania:Wayne.
8. Higgins, D. Clustal, W. 1996. Version 1.6. Cambridge, United Kingdom: The European Bioinformatics Institute.
9. Felsenstein, J. 1995. PHYLIP (Phylogeny Inference Package), version 3.5c. Seattle: University of Washington.
10. Saito, N. Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 4: 406-425.
11. Gouy, M. 1995. NJPLOT. Lyon, France: University of Lyon 1.
12. Maidak, B.L., Larsen, N., McCaughey, M.J., Overbeek, R., Olsen, G.J., Fogel, K., Blandy, J., Woese, C.R. 1994. The Ribosomal Database Project. Nucleic Acids Res. 22: 3485–3487.
13. Goodman, S.D. Gao, Q. 1999. *S. mutans* GS5 gtfB upstream and gtfC downstream sequences Basic Science, University of Southern California, School of Dentistry, 925 West 34th Street, Room 4108, Los Angeles, CA 90089
14. Wang,W. Zhou, Z. 2010. Autochthonous gut *Lactobacillus* in zebrafish (Danio rerio). Feed Research Institute, Chinese Academy of Agricultural Sciences, Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, No.12 Zhongguancun South Street, Beijing, Beijing 100081, China
15. Jones, F.A. 1996. Herbs-useful plants. Their role in history and today. European Journal of Gastroenterology Hepatol. 8:1227-1231.
16. Lis-Balchin, M. Deans, S.G. 1997. Bioactivity of selected plant essential oils against *Listeria monocytogenes*. Journal of Applied Bacteriology 82:759-762.
17. Adnan, J. 2006. Composition of the Essential oil of *Micromeria biflora* ssp. arabica K. Walth *Pakistan Journal of Biological Sciences* Vol. 9, No. 14.
18. Filoche, S.K., Soma, K., Sissons, C.H. 2005. Antimicrobial effects of essential oils in combination with chlorhexidine digluconate. Oral Microbiology and Immunology, 20 (4):221-225.
19. Nascimento, P.F.C., Alviano, W.S., Arrigoni-Blank, M.F., Azevedo, V.G., Alviano, D.S., Bolognese, A.M., Trindade, R.C. 2007. *Hyptis pectinata* essential oil: chemical composition and anti-*Streptococcus mutans* activity. Oral Diseases (Online Early Arti.). doi:10.1111/j.1601-0825.2007.01405.x
20. Knobloch, K., Weigand, H., Weis, N., Schwarm, H.M., Vigenschow, H. 1986. Action of terpenoids on energy metabolism. In Progress in Essential Oil Research: 16th International Symposium on Essential Oils Edited by: Brunke EJ. De Gruyter, Berlin; 16 429-445.
21. Sikkema, J., Bont, J.A.M., Poolman, B. 1994. Interactions of cyclic hydrocarbons with biological membranes. J Biol Chem. 269:8022-8028.
22. Denyer, S.P. Hugo, W.B. 1991. Biocide-induced damage to the bacterial cytoplasmic membrane. In *Mechanisms of Action of Chemical Biocides* Edited by: Denyer SP, Hugo WB. The Society for Applied Bacteriology, Technical Series No 27. Oxford Blackwell Scientific Publication, Oxford; 27:171-188.

Please Cite This Article As: Rohit Kumar Mishra, Awadhesh Kumar, Amritesh Chandra Shukla, Pravin Tiwari and Anupam Dikshit. 2010. Quantitative and Rapid Antibacterial Assay of *Micromeria biflora* Benth. Leaf Essential Oil Against Dental Caries Causing Bacteria Using Phylogenetic Approach. J. Ecobiotechnol. 2(4):22-26.

ISSN 0974 – 5211

Journal of Natural Products
Volume 4 (2011)
www.JournalofNaturalProducts.com

Research Paper

Broad spectrum antimycotic plant as a potential source of therapeutic agent

Amritesh Chandra Shukla[1*], Kumar Pankaj Pandey[2], Rohit Kumar Mishra[2], Anupam Dikshit[2], Neetu Shukla[3]

[1] Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University, Tanhril, Aizawl-796 009, India

[2] Botany Department, Biological Product Lab, University of Allahabad- 211002

[3] Ekta Institute of Child Health, Raipur- 492 001, India

* Corresponding Author

(Received 20 March 2010; Revised 16 April - 08 October 2010; Accepted 09 October 2010)

ABSTRACT

Antimicrobial evaluation of the essential oil(s) of some spp. of *Curcuma* viz., *Curcuma angustifolia*, *C. aromatica*, *C. domestica* and *C. zedoaria* –were screened against three common dermatophytic fungi causing ringworm infection in human beings. The essential oil of *Curcuma domestica* Valet. (Family- *Zingiberaceae*) was found strongest toxicant against the test fungi. The minimum inhibitory concentration (MIC) of the oil was 1.6µl/ml against *Epidermophyton floccosum* and 1.4µl/ml against *Microsporum gypseum* and *Trichophyton rubrum*; however, it was fungicidal at 1.6 µl/ml against *M. gypseum* and *T. rubrum*, and 2.0 µl/ml against *E. floccosum*, respectively. The efficacy contains heavy doses of inoculums (25 discs of 5 mm each). The (MKT) of the oil was 30 sec against *E. floccosum* & *Microsporum gypseum* and 20 sec against *T. rubrum*, while, its MFCs required 6.30 hrs against *E. floccosum* & *Microsporum gypseum* and 5.30 hr against *T. rubrum.* The oils efficacy was thermo stable up to 80 ^{0}C and for 36 months of storage, the maximum unit taken into consideration. Moreover, the oil of *C. domestica* did not exhibit any adverse effect on mammalian skin up to 5% conc. The clinical trial of the oil in the form of ointment (at 1% V/V conc.) to topical testing on patients, attending outpatient department (OPD) of MLN Medical College, Allahabad is still in progress.

Keywords: Antimicrobial activity; Dermatophtes; Medicinal plants; MIC; Herbal drug.

INTRODUCTION

Fungal infections in human beings are a major problem in tropical and subtropical countries due to prevailing humidity and temperature regimes. The superficial fungal infection or dermatomycoses is the disease caused by a group of fungi known as dermatophytes. It involves superficial infections of keratinized tissue in human beings. Clinical surveys carried out in India have showed that ringworm is one of the most common dermatomycoses caused by the species of *Epidermophyton floccosum*, *Microsporum* and *Trichophyton*. Although there are number of synthetic

antifungal are available in market but majority of them are fungi static in nature (Roxburg and Borrie, 1973).

In recent years there has been a gradual revival of interest in the use of medicinal plants because herbal medicines have been reported to be safe and without any adverse side effects. Recent researches reveled that some products of plants origin have been investigated to be an effective source of chemotherapeutic agents without undesirable side effects and with strong fungicidal activity. Consequently, in the present investigations, attempts have been made to explore the possibilities of *Curcuma* spp, as a protecting measurement against ringworm infections in human beings.

MATERIALS AND METHODS

In vitro investigation

Extraction and Isolation of Essential oil*:* The essential oils were extracted separately from the fresh leaves of *Curcuma angustifolia*, *C. aromatica*, *C. domestica* and *C. zedoaria* (Family- *Zingiberaceae*) by hydro distillation using Clevenger's apparatus (Clevenger, 1928). A clear light yellow colored oily layer was obtained on the top of the aqueous distillate, later which was separated and dried over anhydrous sodium sulphate. The oils thus obtained were subjected to various antimicrobial investigations.

In-vitro antimicrobial investigations of the essential oil*:* The minimum effective concentration (MEC) of the oil against some common human pathogenic fungi *Epidermophyton floccosum* Hartz, *Microsporum gypseum* (Bodin) Guiart et Grigorakis and *Trichophyton rubrum* Castellani, was determined by using the technique of Shahi et al., (2001), with a slight modification. Two sets were maintained; one for the treatment set and another for the control. The treatment set at different concentration of the oil was prepared by mixing the required quantity of the oil samples in acetone (2% of the total quantity of the medium) and then added in pre-sterilized sabourad dextrose agar medium (SDA). In control set, sterilized water (in place of the oil) and acetone were used in the medium in appropriate amount. The fungi-static/ fungicidal (MSC/ MCC) action of the oil was tested by aseptically re-inoculating the fungi in culture tubes containing sabourad dextrose broth (Table 1-3). The data recorded was the mean of triplicates, repeated twice. The percentage of fungal growth inhibition (FGI) was calculated as per formula:

$$\text{FGI (\%)} = \frac{Dc - Dt}{Dc} \times 100$$

- Dc indicates colony diameter in control set, &
- Dt indicates colony diameter in treatment sets.

Effect of Inoculums Density*:* The effect of inoculums density on the minimum cidal concentration (MCCs) of the oil against the test fungi was determined using the method of Shukla et al., (2001). Mycelial discs of 5mm diam of 7-day oil cultures were inoculated in culture tubes containing oil at their respective MCCs. In controls, sterilized water were used in place of the oil and run simultaneously. The numbers of mycelial discs in the treatment as well as control sets were increased progressively up to 25 discs, in multiply of five. Observations were recorded up to seventh day of incubation. Absence of mycelial growth in treatment sets up to 7^{th} day exhibited the oil potential against heavy doses of inoculums (Table- 3).

Effect of some Physical Factors*:* Effect of some physical factors viz., temperature (40, 60 and 80 ^{0}C respectively) and autoclaving (up to 15 lb/ sq inch pressure for 30

min) on efficacy of the oil, at minimum cidal concentration, was also determined. It was determined following the method of Shukla et al., (2001) and Shahi et al., (2001). Samples of oil in small vials, each contains 1ml, were exposed at 40, 60 and 80^0 C in hot water bath, respectively. Further, the oil's efficacy was tested against the test fungi at their respective MCCs (Table- 3).

Minimum Killing Time*:* The MKT of the pure oil and their respective MCCs of *C. domestica* against the test fungi was determined by using the method of Shahi, et al. (1999) (Table-4).

Fungi-toxic Spectrum*:* The fungi-toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 2.0 µl/ml and 4.0 µl/ml respectively) was determined against some common human pathogenic fungi viz., *Microsporum auddouinii* Gruby, *M. canis* Bodin, *M. nanum* Fuentes, *Trichophyton mentagrophytes* (Robin) Blanchard, *T. tonsurans* Malmstem, and *T. violaceum* Bodin. This was done by using the method of Shahi et al., (2001) (Table-5).

Besides, the oil's efficacy was also tested against some plant pathogenic fungi viz., *Aspergillus parasiticus* Speare, *Cladosporium cladosporioides* (Fresenius) de Vries, *Curvularia lunata* (Wakker) Boedijin, *Colletotrichum capsici* (Syd.) Butler & Bisby, *C. falcatum* Went, *Fusarium oxysporum* Schlecht, *F. udum* de vries, *Helminthosporium maydis* Nisikado & Miyakel, *H. oryzae* Breda de Haan, *Penicillium implicatum* Biourge and *P. minio-luteum* Dierckx; by using the technique of Shukla et al., (2001) (Table-5).

Comparison with some Synthetic Fungicides*:* The comparative efficacy of oil of *C. domestica* with some synthetic antifungal drugs was carried out by comparing MECs. This was done by using the method of Shahi, et al., (1999) (Table-6 & 7).

All the experiments were repeated twice and each contained three replicates; the data presented in the tables are the mean values.

Statistical analysis: Analysis of variance (ANOVA) was used to determine the significance ($P \leq 0.05$) of the data obtained in all experiments. All results were determined to be within the 95% confidence level for reproducibility. The ANOVA was computed using the SPSS version 16.0 software package.

RESULTS

On comparing the minimum effective concentration (MEC) of oils of *Curcuma angustifolia, C. aromatica, C. domestica* and *C. zedoaria* against the test fungi, the MEC of the oil of *C. domestica* was found most effective (Table- 1).

The MEC of *Curcuma domestica* oil was 1.4 µl/ml against *M. gypseum* and *T. rubrum*, and 1.6 µl/ml against *E. floccosum;* however, it was fungicidal at 1.6 µl/ml against *M. gypseum* and *T. rubrum*, and 2.0 µl/ml against *E. floccosum*, respectively (Table- 2).

The oil's efficacy contains heavy doses of inoculums (i.e. up to 25 discs, each of 5mm), thermo stable up to 80^0 C and also persisted after autoclaving at 15 lb/ sq inch pressure for 30 min (Table-3).

The pure oil kills the test fungi within 30 second; however, its MCC ranges 5.30 to 6.30 hrs to kill all the fungi (Table- 4).

Fungi toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 2.0 µl/ml and 4.0 µl/ml), against some common pathogenic fungi reveals that the oil contains a broad fungicidal spectrum (Table- 5).

Furthermore, on comparing MECs of the oil with some synthetic antifungals, MECs of the oil was more active than Dactrine, Nizaral and Tenaderm (Table- 6 & 7).

DISCUSSIONS

Essential oils obtained from the leaves of *Cymbopogon martini* var. motia (Dikshit, et al., 1980), *Hyptis leucodendron* (Dubey, et al., 1983); *Alpinia galangal* (Tripathi, et al., 1983) was found to contain fungistatic activity. However, some essential oils, *Cymbopogon flexuosus* (Pandey, et al., 1996); *Eucalyptus* oil (Shahi, et al., 2000); *C. flexuosus* (Shahi, et al., 2003); and *Homalomena aromatica* (Shukla, et al., 2009) prove to have fungistatic action at lower concentration and fungicidal action at higher concentration. Similarly, in the present investigation the oil of *Curcuma domestica* showed fungistatic activity at the lower concentration 1.4 µl/ml against *M. gypseum* and *T. rubrum*, and 1.6 µl/ml against *E. floccosum;* and fungicidal at the higher concentration 1.6 µl/ml against *M. gypseum* & *T. rubrum*, and 2.0 µl/ml against *E. floccosum*, respectively. The fungicidal efficacy of the oil persisted heavy inoculums density with quick killing activity as well as having an edge over some synthetic antifungals viz., Dactrine, Nizaral, Tenaderm.

A fungicide must not be affected by extreme temperatures. A few workers have studied the effect of temperature on antifungal activity of the essential oils. Singh et al., (1984) reported the oil of *Pepromia pellucida* was active up to 80 ^{0}C; Shahi et al., (2003) reported *C. flexuosus* activity up to 100 ^{0}C, and Shukla et al., (2009) reported the oil's efficacy of *H. aromatica* up to 80 ^{0}C. Similarly, in the present investigation the oil of *C. domestica* was not only thermostable up to 80 ^{0}C but also autoclavable up to 15 lb/ sq inch pressure for 30 min.

A substance may behave as a strong fungicidal against certain fungi yet may be ineffective against the other pathogens. Therefore, a clear picture about the toxicity of a fungicide comes only after it is tested against the large number of fungi. The literature showed that essential oils have been found to exhibit narrow or wide range of activity (Singh, et al., 1980; Pandey, et al., 1982; Dubey, et al., 1983), but in the present study the oil of *C. domestica* exhibited broad antifungal spectrum.

A toxicant should be tested under both *in vitro* and *in vivo* conditions in order to prove its potential as promising antifungals for the control of disease. Since, detailed *in vitro* studies on the essential oil of *C. domestica* indicate their potentiality to be as ideal antifungal agent against the dermatophytic fungi; hence, the same was further subjected for detailed *in vivo* investigations as well as clinical trials in the form of ointment (at 1% V/V conc.), which is still in progress.

CONCLUSIONS

The preliminary *in vitro* investigations revels that the oil of *Curcuma domestica*, due to its strong fungicidal efficacy, inhibiting heavy doses of inocula, quick killing activity, broad fungicidal spectrum, long shelf life, and having an edge over some synthetic antifungal, can be used successfully in the form of broad spectrum herbal anti-dermatophytic agents. The commercial viability of the same can be determined after detailed *in vivo* as well as successful multi central clinical trials, which is in progress.

Acknowledgements: The authors are thankful to the Head, Department of Botany, University of Allahabad for providing the research facilities; to Prof. A.K. Bajaj, Former-Head Dept of Dermatology, MLN Medical College, Allahabad and to Dr. Uma Banerjee, Division of Microbiology, All India Institute of Medical Sciences, New Delhi for providing their microbial expertise, as well as to the Department of Science & Technology, New Delhi (Ref. No. SR/ FTP/ LS- 227/ 2000) for financial assistance. The authors are also thankful to the authorities of the Mizoram University, Aizawl, for providing various kinds of support during the course of research.

REFERENCES

Clevenger, J.F., (1928): Apparatus for the determination of volatile oil. *J. Am. Pharm. Assoc*., 17:346.

Dikshit, A., Shahi, S.K., Pandey, K.P., Patra, M., Shukla, A.C., (2004): Aromatic plants: a source of natural chemotherapeutants. *National Academy of Science Letters, India*, 27 (5&6): 145-164.

Dikshit, A., Shahi, S.K., Pandey, K.P., Patra, M., Shukla, A.C., (2004): Aromatic plants: a source of natural chemotherapeutants. *National Academy of Science Letters, India*, 27 (5&6): 145-164**.**

Dikshit, A., Singh, A.K. , Dixit, S.N., (1980): Antifungal properties of *Palmarosa* oil. *Ann. Applied Biol.*, 97(supp): 34-35.

Dikshit, A., Srivastava, O.P., Hussain, A., (1985): Effect of some essential oils on *Trichophyton mentagrophytes in vitro* and experimental ringworm. *J. Antibact Antifung Agents*, 13:57-61.

Dubey, N.K., Kishore, N., Singh, S.N., (1983): Antifungal properties of the volatile fraction of *Melaleuca leucodendron. Tropical Agriculture* (Trinidad), 60: 227-228.

Ganesan, S., Venkateshan, G., Banumathy, N., (2006): Medicinal plants used by ethnic group Thottianaickans of Semmalai hills (reserved forest), Tiruchirappali district, Tamil Nadu. *Indian J. Traditional Knowledge*, 5:253–258.

Garber, R.H., Houston, B.R., (1959): An inhibitor of *Verticillium alboatrum* in cotton seed. *Phytopathology*, 49: 449-450.

Grover, G.S., Rao, J.T., (1978): In vitro antimicrobial studies of the essential oil of *Eugenia jambolana*. *Indian Drugs*, 15: 143-144.

Grover, R.K., Moore, J.D., (1962): Toxicometric studies of fungicides against brown rot organisms *Sclerotinia fructicola* and *S. laxa*. *Phytopathology*, 52: 876- 880.

Jadhav, D., (2006): Ethnomedicinal plants used by *Bhil* tribe of Bibdod, Madhya Pradesh. *Indian J. Traditional Knowledge*, 5:268–270.

Jain, S.K., (1991): Dictionary of Indian Folk Medicine and Ethanobotany (*Eds*). Deep Publications, New Delhi, India.

Kirtikar, K.R., Basu, B.D., (1935): Indian Medicinal Plants, Vol 1-4. International Book Distributers, Dehradun.

Lalramnghinglova, H., (2003): Ethno- Medicinal Plants of Mizoram (*eds*), Publisher Bishen Singh Mahendra Pal Singh, Dehradun.

Lalramnglinglova, J.H., (1996): Ethnobotany of Mizoram-a preliminary survey. *J. Econ. Taxon. Bot. Add. Ser*., 12:439-450.

Langenau, I.E.E., (1948): The examination and analysis of essential oils, synthetics and isolates. Guenther (*Eds*). The Essential Oil. Robert E. Krieger Publishing Co. Huntington, New York.

Nehrash, A.K., (1961): The antimicrobial properties of cultivated radish. The antimicrobial activity of extracts and essential oils from cultivated & wild radish. *J. Mircrobial.*, 23:32-37.

Pandey, D.K., Tripathi, N.N., Tripathi, R.D., Dixit, S.N., (1982): Fungitoxic and phytotoxic properties of the essential oil of *Caesulia axillaris*, Roxb. (Compositae). *Angew. Botanik.*, 56: 259-267.

Pandey, D.K., Tripathi, S.N., Tripathi, R.D., Dixit, S.N. (1982): Fungitoxic and phytotoxic properties of the essential oil of *Caesulia axillaries* Roxb. *Angew*. *Botanic*., 56: 256-257.

Pandey, M.C., Sharma, J.R., Dikshit, A., (1996): Antifungal evaluation of the essential oil of *Cymbopogon pendulus* (Nees ex Steud.) Wats var. Praman. *Flavour and Fragrance Journal*, 11:257-260.

Roxburgh, A.C., Borrie, P., (1973): Roxburgh's common skin disease (12th eds). The English Language Book Society and H.K. Lewis and Co. Ltd. London.

Shahi, S.K., Patra, M., Shukla, A.C., Dikshit, A., (2003): Use of essential oil as biopesticide against post harvest spoilage in fruits, *Mallus pumilo*. *Bio-Control*, 48:223-232.

Shahi, S.K., Patra, M., Shukla, A.C., Dikshit, A., (2001): Botanical drug for therapy against fungal infections in human beings. *Nat. Acad. Sci. Letters*, 24(5-12):73-78.

Shahi, S.K., Shukla, A.C., Bajaj, A.K, Banerjee, U, Rimek, D., Medgely, G., Diksit, A., (2000): Broad spectrum herbal therapy against superficial fungal infections. *Skin Pharmacol. Appl. Skin. Physiol*., 13: 60-64.

Shahi, S.K., Shukla, A.C., Bajaj, A.K, Medgely, G., Diksit, A., (1999): Broad spectrum antimycotic drug for the control of fungal infection in human beings. *Current Science*, 76(6): 836- 839.

Shahi, S.K., Shukla, A. C., Dikshit, S., Diksit, A., (1997): Modified spore germination inhibition technique for evaluation of candidate fungitoxicant (*Eucalyptus* spp.) H.W Dehne et al., (Ed). Proc.of the 4th Int. Symposium on Diagnosis and Identification of Plant Pathogens, Kluwer Academic Publishers, Netherlands, pp. 257-263.

Shahi, S.K., Shukla, A.C., Dikshit, A., Uperti, D.K., (2001): Broad-spectrum antifungal properties of the lichen, *Heterodermia leucomela*. *The Lichenologist*, 33(2): 177-179.

Sharma, P.K., Chauhan, N.S., Lal, B., (2005): Studies on plant associated indigenous knowledge among the *Malanis* of Kullu district, Himachal Pradesh. *Indian J. Traditional Knowledge*, 4:409-411.

Shukla, A.C, Shukla, N, Dikshit, A., Lalramnghinglova, H., (2009): Ethno Medicinal Approach to Drug Development: Present Status and Future Prospects. *Planta Med.*, 75: 963.

Shukla, A.C., Shahi, S.K., Dikshit, A., (2000): Epicarp of *Citrus sinensis*: a potential source of natural pesticide. *Indian Phytopathology*, 53(3): 318-322.

Shukla, A.C., Shahi, S.K., Dikshit, A., Saksena, V.C., (2001): Plant product as a fumigant for the management of stored product pests. Proc. Int. Conf. on Controlled Atmosphere and Fumigation in Stored Products, USA (*Eds.* Donahaye E.J., Navarro S., Leesch, J.G.) pp. 125-132.

Singh, A.K., Dikshit, A., Dixit, S.N., (1980): Fungitoxic activity of some essential oils. *Economic Botany*, 34: 186-190.

Singh, A.K., Dikshit, A., Dixit, S.N., (1984): Antifungal studies of *Peperomia pellucida*. *Beitr. Biol. Pflanzen*., 58: 357-368.

Singh, R., Shukla, A.C., Prasad, L., (2007): Antifungal Screening of some higher plants against storage fungi. *Progressive Agriculture*, 7 (1-2): 128- 131.

Tripathi, N.N., Dubey, N.K., Dihshit, A., Tripathi, R.D., Dixit, S.N., (1983): Fungitoxic properties of *Alpinia galangal. Trop. Plant Sci. Res.*, 1: 49-52.

Table-1: Minimum effective concentration of four different species of *Curcuma* against some common human pathogenic fungi.

***Curcuma* spp**	**Human Pathogenic Fungi**		
	Epidermophyton floccosum	*Microsporum gypsum*	*Trichophyton rubrum*
C. angustifolia	2.6 µl/ml	2.2 µl/ml	2.4 µl/ml
C. aromatica	1.8 µl/ml	1.6 µl/ml	1.8 µl/ml
C. domestica	1.6 µl/ml	1.4 µl/ml	1.4 µl/ml
C. zedoaria	2.2 µl/ml	1.8 µl/ml	2.0 µl/ml

Table- 2: Minimum effective concentration of the oil of *Curcuma domestica* against test fungi.

Concentration (µl/ml)	**Human Pathogenic Fungi**		
	Epidermophyton floccosum	*Microsporum gypsum*	*Trichophyton rubrum*
2.0	100^{C}	100^{C}	100^{C}
1.8	100^{s}	100^{C}	100^{C}
1.6	100^{s}	100^{s}	100^{C}
1.4	88	60	100^{s}
1.2	60	--	80
1.0	--	--	60

- ^{c} indicates cidal and ^{s} indicates static.

Table- 3: Detailed *in-vitro* investigations of *Curcuma domestica* against the test fungi.

Properties studied	**Observations**		
	Epidermophyton floccosum	*Microsporum gypsum*	*Trichophyton rubrum*
Minimum Inhibitory Concentration			
MEC (µl/ml)	1.6 µl/ml	1.4 µl/ml	1.4 µl/ml
MFC (µl/ml)	2.0 µl/ml	1.6 µl/ml	1.6 µl/ml
Minimum Killing Time			
Pure oil	30 sec	30 sec	20 sec
MFC	6.30 hrs	6.30 hrs	5.30 hrs
Inoculum Density (25 disc, 5mm diam)	No Growth	No Growth	No Growth
Thermostability (up to 100 ^{0}C)	No Growth	No Growth	No Growth
Effect of Storage (36 months)	No Growth	No Growth	No Growth

- ***MEC** indicates Minimum Effective Conc.; **MFC** indicates Minimum Fungicidal Concentration.

Table- 4: Minimum killing time of the oil of *Curcuma domestica* against test fungi.

Mycelial Growth Inhibition (%)						
Minimum Killing Time (MKT)	*Epidermophyton floccosum*		*Microsporum gypseum*		*Trichophyton rubrum*	
	P.O.	MFC	P.O.	MFC	P.O.	M.F.C.
7.0	100	100	100	100	100	100
6.30	100	100	100	100	100	100
6.0	100	60	100	80	100	100
5.30	100	---	100	---	100	100
5.0	100		100		100	80
2.30	100		100		100	---
2.0	100		100		100	
1.30	100		100		100	
1.00	100		100		100	
30 min	100		100		100	
15 min	100		100		100	
5 min	100		100		100	
60 sec	100		100		100	
30 sec	100		100		100	
20 sec	90		80		100	
10 sec	60	---	70	---	88	---

- *P.O. indicates Pure Oil; MFC indicates Minimum Fungicidal Concentration.

Table-5: Fungi toxic spectrum of the oil of *Curcuma domestica* against some common pathogenic fungi.

Fungi Tested	**Lethal Concentration** (2.0 µl/ml)	**Hyper Lethal Concentration** (4.0 µl/ml)
Human Pathogens		
Microsporum auddouinii	100[s]	100[c]
M. canis	100[s]	100[c]
M. nanum	100[c]	100[c]
Trichophyton mentagrophytes	100[c]	100[c]
T. tonsurans	100[c]	100[c]
T. violaceum	100[c]	100[c]
Plant Pathogens		
Aspergillus parasiticus	100[s]	100[c]
Cladosporium cladosporioides	100[c]	100[c]
Curvularia lunata	100[c]	100[c]
Colletotrichum capsici	100[c]	100[c]
C. falcatum	100[c]	100[c]
Fusarium oxysporum	100[c]	100[c]
F. udum	100[c]	100[c]
Helminthosporium maydis	100[c]	100[c]
H. oryzae	100[c]	100[c]
Penicillium implicatum	100[c]	100[c]
P. minio-luteum	100[c]	100[c]

- [s] indicates static; [c] indicates cidal in nature.

Table- 6: Comparative MECs of the oil of *Curcuma domestica* with some synthetic anti-fungal.

Oil & Trade Name of Antifungal Drugs	Active Ingredients	Minimum Effective Concentration (µl/ml)		
		Epidermophyton floccosum	*Microsporum gypseum*	*Trichophyton rubrum*
Curcuma domestica	Essential oil	1.6	1.4	1.4
Dactrine	Miconazole nitrate	6.0	6.0	6.0
Nizaral	Ketoconazole	6.0	0.5	5.0
Tenaderm	Tolnaftate	2.0	1.5	0.8

Table- 7: Comparative Efficacy of the oil of *Curcuma domestica* with some synthetic antifungal drugs.

Antimycotic Drugs	Drugs %	Cost (Rs.)		Adverse Effects	Expiry Duration (months)	Environmental impact
		ointment/gm	lotion/ml			
C. domestica	1%v/v	0.90	0.70	No adverse effects	24-36	Renewable, biodegradable, non-residual toxicity.
Dactrine	2%w/w	2.80	-	Occasionally produced gastrointestinal side effects viz., nausea, vomiting, diarrhea	36	Non-renewable, non-biodegradable and residual toxicity
Nizaral	2%w/w	3.75	3.17	Adverse reaction observed were mainly burning, irritation. Drug may block testosterone synthesis	24	-----do-----
Tenaderm	1%w/v	1.06	1.30	Adverse effects were fever, nausea, vomiting, diarrhoea & skin rash, rarely produced irritation	24	-----do----
Batrafine	1%w/v	1.50	1.60	----do----	24	-----do----

Journal of Phytology 2011, 3(3): 44-52
Microbiology
ISSN: 2075-6240
Available Online: www.journal-phytology.com

REGULAR ARTICLE

A COMPARATIVE NOVEL METHOD OF ANTIFUNGAL SUSCEPTIBILITY FOR *MALASSEZIA FURFUR* AND MODIFICATION OF CULTURE MEDIUM BY ADDING LIPID SUPPLEMENT

Amit Kumar Tiwari[1*], Rohit Kumar Mishra[1], Awadhesh Kumar[1], Shalu Srivastava[1], Anupam Dikshit[1], Anand Pandey[1], and K Bajaj[2]

[1]*Biological Product Lab, Department of Botany, University of Allahabad, 211002*
[2]*Department of Dermatology and Venereal Diseases, M.L.N. Medical College, Allahabad, India*

SUMMARY

Introduction of new antifungal compounds has increased the demand for method of *in vitro* testing. The present study has proposed a new method of studying antifungal activity of different compounds by using 96 well plates. The infection by different species of *Malassezia* is quite common in tropical country like India. *Malassezia* being a eukaryote, the treatments against it may also adversely affect the patient. Hence, as an alternative, cheap, affordable, ecofriendly, botanicals may be used. In this background, a comparative study on the efficacy of synthetic ingredients (on the basis of their performance in the market) and botanicals was carried out *in-vitro* against *Malassezia furfur* (MTCC 1765).Evaluation of Minimum Inhibitory Concentrations (MICs) of two standard antifungal drugs (Ketoconazole and Fluconazole) available in the market against *Malassezia furfur* and their comparison with botanicals was done using broth microdilution method recommended by Clinical Laboratory Standard Institute (CLSI) with slight modifications. The present work is also an attempt to standardized culture medium for growing *Malassezia* species by overlay of sterile cotton seed oil with principal medium. Cotton seed oil can be used as supplement with principal medium for best growth of *Malassezia*.

Keywords: Botanicals, *Malassezia furfur,* Minimum Inhibitory Concentrations, Synthetics

Amit Kumar Tiwari et al. A Comparative Novel Method of Antifungal Susceptibility for *Malassezia furfur* and Modification of Culture Medium by Adding Lipid Supplement. J. Phytol 3/3 (2011) 44-52.
*Corresponding Author, Email: amittara7@gmail.com

1. Introduction

The genus *Malassezia* includes 10 anthropophilic and obligatory lipophilic species (*M. globosa*, *M. restricta*, *M. slooffiae*, *M. obtusa*, *M. furfur*, *M. sympodialis*, *M. japonica*, *M. yamatoensis*, *M. dermatis*, *M. nana*) and 3 zoophilic species *M. pachydermatis*, *M. caprae*, *M. equine*(1). *Malassezia* species have been associated with a number of diseases of human skin, such as pityriasis versicolor, seborrheic dermatitis, dandruff, folliculitis, atopic dermatitis, and psoriasis etc. (2).

Pityriasis versicolor is mild chronic superficial infection of the stratum corneum characterized by patchy and scaly discoloration of the skin.

Seborrhea, dandruff and seborrheic dermatitis are three closely related terms. Seborrhea literally means a state of oiliness of skin. The skin is shiny and oily looking, particularly on the face. Dandruff (also called *Pityriasis capitis*) means scaliness of the scalp skin without signs of inflammation. Dandruff is so common that it can be considered physiological. It represents desquamation of the skin surface, due to separation of layers of stratum corneum, which is a continuous process, in the form of scales. The status of dandruff being amphibious a disease/disorder, and relatively less medical intervention is sought after for the treatment but dandruff is the most commercially exploited skin and scalp disorder/disease by personal care industries(3).

Malassezia formerly called *Pityrosporum* is a yeast causing infection of skin and scalp (4).

The infection of the scalp clinically represents as Dandruff (5). Dandruff is occurring in at least 40-50% and Seborrheic dermatitis (SD) 1-3% of the general population (6, 7).The vast majority of recent data support a direct causal link between *Malassezia* and D/SD. The factors that support are, firstly antifungal treatment are found most effective in treating disease and secondly, an improvement SD/D is accompanied by a reduction in *Malassezia* levels on the scalp (1,8-13). Warm and humid atmosphere, overcrowding and poor personal hygiene are ideally suited for the growth of *Malassezia* (14*).* Magnitude of problem is presumed to be high in Allahabad, India due to warm and moist weather.

Although a variety of antifungal agents are available in the market for the treatment of dandruff, seborrheic dermatitis, pityriasis versicolor, folliculitis, atopic dermatitis, but complete control is far from reach. Most of the available drugs are expensive and have side effects. The treatments that are available have certain limitations, either due to poor efficacies or due to compliance issue.

India being a developing country is losing much of its foreign exchange in buying formulations and readymade drugs from other developed countries. Patients uncomfortable or unsatisfied with synthetic pills and prescriptions may consider turning to herbal remedies extracted from plants, roots, seeds and fruits. Herbal remedies have been used throughout human history. Recent medical evidences support and clearly define the benefits of herbal medicine. Many modern prescriptions contain herbal extracts or a synthesized equivalent. India's rich natural resources and knowledge of traditional medicine have important role in modern health care system (15,16). The Indian System of Medicine has identified 1,500 medicinal plants, of which 500 are commonly used. The main objective is to use medicinal plants against common prevalent diseases.

2. Materials and Methods

Collection and maintenance of the culture

Pure culture of *Malassezia furfur* (MTCC-1765) was obtained from Institute of Microbial technology, Chandigarh, India. The Sabouraud-Dextrose Agar (SDA) medium and Dutta and Dikshit Modified culture medium were selected and prepared for sub culturing of *Malassezia* (17*).*

These strains were routinely cultured on Sabouraud Dextrose Agar (SDA) slants and modified culture medium at 35^0 C and the pH was adjusted to 5.8 prior to autoclaving at 15 lbs, 121^{o}C for 15 min. All the cultures were maintained at 34 ± 2^{o}C in incubator for four days. *In vitro* investigation of *Malassezia furfur* by culturing (medium + oil as lipid source) was carried out in Biological Product Lab, Department of Botany, and University of Allahabad for standardization of medium. Coconut oil, Cotton seed oil, Til oil and Olive oil used as carbon source. Morphological and physiological structure on the basis of literature as well as microscopic examination of colony was also done.

Collection of plant and extraction of essential oil

Plants were selected on the basis of their ethno-medicinal importance and literature surveyed from various libraries in Allahabad, NBRI, Lucknow and internet sites. The selected plants were identified with the help of flora (Hooker, 1872-1892; Bailey 1958; Srivastava, 1976 and Singh, 1989) and authentic herbarium of Botanical Survey of India, Allahabad and the *Duthie* Herbarium, Department of Botany, University of Allahabad.

Micromeria biflora Benth. collected from Kinnor, Himanchal Pradesh during botanical excursion tour was selected for the antifungal testing, identified by Botanical survey of India (BSI) Allahabad, and extraction of oil was done using Clevenger type apparatus(18). *Syzygium aromaticum* oil was extracted and on the basis of GC-MS analysis found that major component was Eugenol and it was taken for antifungal susceptibility testing. Eugenol was purchased from Central drug house (P) LTD, New Delhi (Batch No 01077, Product No.028374).

Antifungal susceptibility testing

For determination of Minimum Inhibition Concentrations (MICs), media and their components were prepared as per

recommendation of NCCLS; 2002(19) {currently known as CLSI} with slight modification

- Inoculum = $0.5x10^3$-$2.5 x10^3$ CFU/ml
- Medium = RPMI 1640
- pH = 7
- MOPS = 0.165M
- Temp = 35°C
- Duration of incubation = 72H

In 96 well plates four drugs were taken, each have 2 replicate Fluconazole, Ketoconazole, Eugenol and *Micromeria biflora* Benth essential oil respectively. All dilutions were prepared in DMSO (Dimethyl sulfoxide), the final concentration of DMSO per inoculum was as described by the CLSI. The antimalassezia activity of drugs was determined by broth microdilution method recommended by Clinical Laboratory Standards Institute (CLSI) using BPL Modified medium Broth. Culture was maintained on BPL modified medium and Modified Lemming Notman agar medium (MLNA). The 96-well microtiter plates were used for twofold serial dilution. 20 µl of stock sol. of sample drugs was added into 4th well of microtiter plate horizontally having 180µl RPMI 1640 medium. So the maximum concentration of the sample drug was 2.5mg/ml. From here the solution was serially diluted up to 4th well to 11th well resulting into the half of the concentration of the test essential oil.

The yeast inoculum was prepared at 0.5 McFarland standards; the absorbance was equal to the inoculum suspension containing $1x10^6$. Then standard yeast inoculum was added and kept for incubation at 32°C in a moist chamber. The Minimum Inhibitory Concentration (MIC) and Inhibitory Concentration at 50% (IC_{50}) was recorded spectrophotometrically at 530 nm using SpectraMaxplus^{384} after 72 hrs incubation.

For synthetic drug, stock solution was made 1mg/ml and for natural drug, stock solution was made 40mg/ml. In the case of natural 40mg oil/active constituents have been taken and dissolved in 960 µl DMSO and for synthetic 1mg dissolved in 999 µl DMSO.

Fig.1. 96 well plate format for drug testing by serial dilution method.AB drug -1,CD drug-2 EF drug-3,GH drug 4

The MIC end-points for each antifungal agent were defined as the first concentration where spectrophotometrically 80% or more reduction was measured.

3. Results

The recorded results are presented in the form of Tables 1 and 2, Fig. 1, 2, 3 and 4. *Malassezia furfur* (MTCC 1765) developed as white to cream colour and smooth pasty colony on modified medium (as shown in fig.-3). Excellent growth was reported in modified culture medium. Growth of *Malassezia* on the basis of different carbon sources (cotton seed oil, coconut oil, til oil, olive oil) was also observed and was found best growth on cotton seed oil supplemented with principal medium. Based on morphological characters the purified colonies were identified. *Malassezia* is able to exist both in yeast and mycelial forms. The fungus is dimorphic, occurring as a saprophytic yeast form and a parasitic mycelial form. Yeast is the prime form isolated *in vitro* from the culture media. Yeast like conidia are predominant structures.

Malassezia colonies grow rapidly and mature in five days at 30-37˚C. It undergoes asexual reproduction by enteroblastic budding from a characteristic broad base. The daughter cell separate by fission, leaving a bud scar or collarette through which daughter cell emerge. These cells are globose to ellipsoidal in shape. Sexual spores do not exist.

MICs of Fluconazole, Ketoconazole, Eugenol and *Micromeria biflora* essential oil against *Malassezia furfur* were found 10.538 mg/ml, 6.438 mg /ml, 6.956 mg/ml, and 8.928 mg/ml respectively.

Table-1 MICs of Fluconazole, Ketoconazole, Eugenol and *Micromeria* essential oil against *Malassezia furfur* MTCC-1765

Minimum Inhibitory Concentration				
STRAIN	Fluconazole	Ketoconazole	Eugenol	*Micromeria*
MTCC-1765	10.538 mg/ml	6.438 mg /ml	6.956 mg/ml	8.928 mg/ml

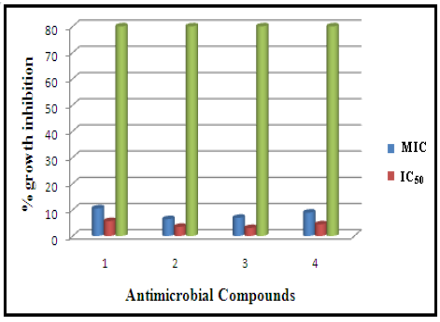


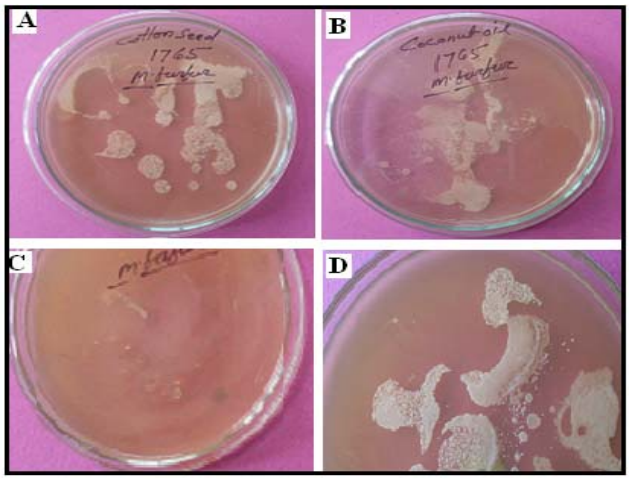
Fig.-2 Graphical representation of antimicrobial activity of candidate compounds against *Malassezia furfur* MTCC-1765

Table -2 Table show IC-50 of four drugs

IC 50 Determinations of four Drugs				
STRAIN	Fluconazole	Ketoconazole	Eugenol	*Micromeria*
MTCC-1765	5.689 mg/ml	3.515 mg /ml	3.044 mg/ml	4.466 mg/ml

Fig. 3: Growth of *Malassezia furfur* (MTCC, 1765) on Dutta and Dikshit Modified Culture Medium Dorsal and ventral view of colony

Fig. 4: Growth of *Malassezia furfur* on Dutta and Dikshit modified culture medium with different Lipid supplement (A) Cotton seed oil (B) Coconut oil (C) Til oil (D) Cotton seed oil .Excellent growth reported on principal medium supplemented with cotton seed oil



4. Discussion

The method used is economical, accurate, rapid, cheaper and comparative for four drugs, one organism and vise-versa in single plate (Fig.-1). The modified susceptibility method can be also used for unicellular yeast and other *Malassezia* species.

One study reported from India, showed MIC of Ketoconazole 2.5 µg/ml, Fluconazole 2.5 µg/ml, Clove oil 1000 µg/ml, Coleus oil 25 mg/ml, and Basil oil 10 mg/ml was effective against *Malassezia furfur* by disc diffusion method (20). Another study done by Miranda *et al.*,in which MIC ranges were <0.03-4 microg/ml for Ketoconazole and <0.125 to >64 microgram/ml for Fluconazole against *Malassezia* (21*)*.In our current study, Eugenol and *Micromeria biflora* oil recorded a very good activity among the herbal ingredients. MIC of Eugenol is very close to Ketoconazole which is a popular synthetic antifungal.

The three major groups of drugs in clinical use are polyenes, azoles & pyrimidines. With the exception of 5-FC, the azoles and polyene antifungal drugs in common usage are directed in same way against Ergosterol, the major sterol in fungal plasma membrane. Ergosterol in fungal membrane contributes to a variety of cellular functions. It is important for the fluidity & integrity of the membrane and for the proper function of membrane bound enzymes including chitin synthetase, which is important for proper cell growth and division. For azole drugs mode of action several lines of evidences suggest that the primary target of azoles is heme protein which co-catalyses cytochrome P450 14α-demethymation of lanosterol. Inhibition of 14α-demethylase leads to depletion of Ergosterol and accumulation of sterol precursors, including 14α-methylated sterols resulting in the formation of a plasma membrane with altered structure and function. However, a significant proportion of patients have experienced azole treatment failure due to the development of drug resistance in *Malassezia* species.

Ketoconazole is an imidazole derivative, broad spectrum antimycotic agent that is active against *Pityrosporum ovale* and is effective against many fungi both *in-vivo* and *in-vitro* (22, 23). It is also effective in many dermatomycoses, including pityriasis versicolor (24).

However, in very severe cases of dandruff, Ketoconazole based shampoos are preferred despite their relatively higher costs.

Herbal ingredients like tea tree oil, rosemary oil, coleus oil, clove oil, pepper extract, neem extract, and basil extract also recorded anti-pityrosporum activity, but their MIC are much higher than the synthetic ingredients.

Synthetic drugs have several side effects and resistance to antifungal drugs .The synthetic antifungals are very popular and drug of choice due to their easy availability, but the reports of increasing number of side effects on the patients, cannot be ignored in anyway. It is estimated that there are over 7800 medicinal drug manufacturing unit in India, which are estimated to consume about 2000 tons of herbs annually (25). According to a recent estimate of World Health Organization (WHO), 60-80% of the world population especially in developing countries relies on traditional medicine or plant based drugs for their primary health care needs (26, 27). The main objective is to use medicinal plants against popular prevalent diseases. In tropical countries like India, fungal infections are of common occurrence. Identifying yeast as the causative agent creates the potential for being able to control this condition with antifungal agents. Dandruff and seborrheic dermatitis patients may require regular, long-term use of therapeutic agents. It is important that the treatments be formulated so as to be aesthetically and cosmetically acceptable to the patient. As such, the study was done mainly to discover the potential active ingredients from the selected plants active against the test pathogen. Eugenol has highest potential as antimalassezia activity and *Micromeria biflora* essential oil also possess significant activity against *Malassezia* causing dandruff, seborrheic dermatitis, pityriasis versicolor, folliculitis, and atopic dermatitis.

One of the important achievement of the present work is standardization of culture medium for growing *Malassezia* species by adding Cotton seed oil as lipid source in medium composition. Hence, it is suggested that modified medium can be further modified by supplementing cotton seed oil. Use of Cotton seed oil for culturing *Malassezia* is a new report and Cotton seed oil can be used as lipid source for excellent growth of *Malassezia*. This work is also important due to the first report of antimalassezia activity of Eugenol and *Micromeria biflora* essential oil. These ingredients can be exploited for its antimalssezia activity individually or in combination.

Eugenol and *Micromeria biflora* essential oil can be used as antifungal drugs against *Malassezia* spp. causing Dandruff, Seborrrheic Dermatitis, and Pityriasis versicolor in Humans. After undergoing detailed bimolecular characterization, formulation of dosages, clinical studies, safety and efficacy is in progress.

Acknowledgements

Thanks to Head, Deptt of Botany, University of Allahabad for providing the necessary facilities and to Department of Science and Technology, New Delhi, India for financial assistance.

References

1. Cabañes FJ, Theelen B, Castellá G, Boekhout T. Two new lipid-dependent Malassezia species from domestic animals. FEMS Yeast Res 2007; 7:1064-1076.
2. Gupta AK, Kohli Y. Prevalence of *Malassezia* species on various body sites in clinically healthy subjects representing different age groups. Med Mycol 2004; 42: 35-42.
3. Ranganathan S, Mukhopadhyay T. Dandruff: The most commercially exploited skin disease. Indian J Dermatol 2010; 55:130-4.
4. Shuster S. The aetiology of dandruff and the mode of action of therapeutic agents. Br J Dermatol 1984; 111:235–242.
5. Noble WC, Midgley G. Scalp carriage of Pityrosporum species: the effect of physiological maturity, sex and race. Sabouraudia 1978; 16:229-232.
6. Warner RR, Schwartz JR, Boissy Y, Dawson TL. Dandruff has an altered stratum corneum ultrastructure that is

improved with zinc pyrithione shampoo. J Am Acad Dermatol 2001; 45:897–903.
7. Ashbee HR, Evans EG. Immunology of diseases associated with *Malassezia* species. Clin Micro Rev 2002; 15:21–57.
8. Ahn KJ, Ashbee HR. Determination of minimum inhibitory concentrations of several azole antifungals for *Malassezia furfur*. Ann Dermatol 1996. 8:187-194.
9. Bulmer AC, Bulmer GS. The antifungal action of Dandruff shampoos. Mycopathologia1999;147(2):63-5.
10. Hammer KA, Carson CF, Riley TV. *In vitro* activity of *Melaleuca alternifolia* (tea tree) oil against dermatophytes and other filamentous fungi. J. Antimicrob. Chemotherapy 2002; 50, 195–199.
11. Gupta AK, Boekhout T, Theelen B, Summerbell RC, Batra R. Identification and typing of *Malassezia* species by amplified fragment length polymorphism and sequence analyses of the internal transcribed spacer and large-subunit regions of ribosomal DNA. J Clin Microbiol 2004; 42: 4253-4260.
12. Vijayakumar R, Muthukumar C, Kumar T, Saravanamuthu R. Characterization of *Malassezia Furfur* and its control by using plant extracts. Indian journal of Dermatology 2006; 51(2) 145-148.
13. Piérard GE, Arrese JE, Piérard-Franchimont C, De Doncker P. Prolonged effects of antidandruff shampoos- time to recurrence of *Malassezia ovalis* colonization of skin. Intl. J Cosm Sci 1997; 19:111-7.
14. Rippon JW. Medical mycology: In *The Pathogenic Fungi and the Pathogenic Actinomycetes*, 1982. 2nd edn. Philadelphia, PA: W. B. Saunders
15. Cardin CW.Isolated Dandruff".Book chapter in Baran and Maibach. Cosmetic Dermatology 2000; p193-200.
16. Hickman J, Wang X, King B, Cardin CW. Dandruff part I: Scalp disease prevalance in Caucasians, African Americans and Chinese and the effect of shampoo frequency on scalp health.Poster#124 presented at the American Academy of Dermatology Meeting 2002;New Orleans.
17. Dutta S, Dikshit A. Modified Culture Medium and process for the preparation thereof. Patent No-217897, 2008; Govt of India, New Delhi.
18. Clevenger JF. Apparatus for the determination of volatile oil. J Am Pharm. Assoc1928; 17: 346-346
19. National Committee for Clinical Laboratory Standards. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi: Approved Standard M38-A. 2002; NCCLS, Wayne, PA, USA.
20. Prabhamanju M, Shankar SG, Babu K, Ranjith MS. Herbal vs. chemical substances as antidandruff ingredients: which are more effective in the management of Dandruff? - An overview Egyptian Dermatology Online Journal 2009; 5 (2):8.
21. Miranda KC, DeAraujo CR, Costa CR , Passos XS, DeFatima , Lisboa FO, DeRosario ,Rodriguez SM. Antifungal activities of azole agents against the Malassezia species. Internat. J. Antimicr. Agents 2007;29 (3), pp. 281-284.
22. San Philippo A, English JC. An overview of Medicated shampoos used in Dandruff treatment, P&T 2006;31(7): 396-400.
23. Heel RC, Brogden RN, Carmin L, Morley PA, Speight TM, Avery GS. Ketoconazole: a review of its therapeutic efficacy in superficial and systemic fungal infections. Drugs 1982; 23: 1- 36.
24. Faergemann, J, Djirv L.Tinea versicolor: treatment and prophylaxis with ketoconazole. 1982 Cutis, 30: 542- 550.982; 23: 1- 36.
25. Rama Krishnappa K. Biodiversity and Ecosystem Approach in Agriculture, Forestry and Fisheries. Impact of Cultivation and Gathering of Medicinal plants on biodiversity: Case studies from India. 2003 United Nations Publications New York, USA. p. 210-215.
26. Farnsworth NR, Akerle O, Bringle AS , Soejarto DD, Guo G. Medicinal plants in therapy. Bulletin of the World Health organization. 1985; 63: 965-981.
27. World Health Organization, 2002. WHO. Traditional Medicine Strategy 2002-2005. Document available www.traditional policy.html.