Screening of various plant extracts for their anti-microbial activity and estimation of various phytochemicals in respective extracts.

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In

Industrial Biotechnology

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DECLARATION

This is to certify that the major project entitled “screening of various plant extracts for their anti-microbial activity and estimation of various phytochemicals in respective extracts” done by me(2K15/IBT/11) in the partial fulfilment of the requirements for the reward of the degree of Masters in Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the my own work carried out under the guidance of my project supervisor Dr.Jai Gopal Sharma, Associate Professor, Delhi Technological University. The information and data enclosed in this report is original and has been carried out at Quest Diagnostics India Private Limited, Gurgaon, Haryana and has not been submitted elsewhere for honouring of any other degree.

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CERTIFICATE

This is to certify that the major project entitled "screening of various plant extracts for their anti-microbial activity and estimation of various phytochemicals in respective extracts" done by Saloni Mishra (2K15/IBT/11) in partial fulfilment of the requirements for the reward of the degree of Masters of Technology in Industrial Biotechnology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her at Quest Diagnostics India Private Limited, Gurgaon, Haryana. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

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Screening of various plant extracts for their anti-microbial activity and estimation of various phytochemicals in respective extracts

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Abstract

India is one of the 12 mega biodiversity centers having over 45,000 plant species. In the current study, *Cicer arietinum, Mentha viridis and Syzygium aromaticum* were used for preparing aqueous and ethanol extracts for phytochemical analysis of the three plants. Plants are a storehouse of wide variety of secondary metabolites like cardiacglycosides, alkaloids, tannins, terpenoids, coumarins, saponins, phenols and flavonoids are rich in antioxidant activity and these plants were tested this activity. Gram positive bacteria such as *Staphylococcus aureus* is mainly responsible for post-operative wound infections, toxic shock syndrome, etc while Gram negative bacteria such as *Escherichia coli* is present in human intestine and causes lower urinary tract infections, septicaemia, etc. These bacteria were collected from throat swab and their activity was tested on these 3-plant species and *Syzygium aromaticum* showed the best zone of inhibition by *Staphyloccus aureus*. Further the antibiotic activity of these plant species was confirmed through MTT assay and it was found that *Syzygium aromaticum* showed the best antibacterial results. Finally, Thin layer chromatography (TLC) was done in order to find the presence of active compounds and retardation factor was noted down which showed the presence of eugenol, genistein and quercitin. To confirm the presence of these compounds HPLC analysis was done and it was observed that Eugenol was found in *Syzygium aromaticum*, Genistein was found in *Cicer arietinum* and Quercetin was found in *Mentha viridis.*

Keywords: *Cicer arietinum, Mentha viridis, Syzygium aromaticum*, phenols, flavonoids, MTT assay, TLC, HPLC

INTRODUCTION

1.1 Overview

Today the world finds itself in a midst of multiplicity of problems particularly in the area of health care. The situation is alarming because of the emergence of new diseases. Antibacterial resistance among bacterial pathogens in recent time is a critical area of public health concern. The usual causative agents of infectious diseases (especially bacteria) are becoming increasingly resistant to some or most antibiotics. Consequently, evolving new herbal medicine is on the ascendancy. Traditionally herbal medicines have long been using plants to prevent all cure infectious diseases; western medicine is also moving in this direction , plants are storehouses of wide variety of secondary metabolites such as alkaloids, terpenoids, flavonoids and tannins etc. which have demonstrated antimicrobial activity (Gupta *et al.,* 2014).Gram positive bacteria such as *Staphylococcus aureus* is mainly responsible for post-operative wound infections, toxic shock syndrome, endocarditis, osteomyelitis and food poisoning (Benayacheet *et al.,* 2001). Gram negative bacteria such as *Escherichia coli* is present in human intestine and causes lower urinary tract infection, coleocystis or septicemia (Benhassaini *et al.,* 2003; Benjilali *et al.,*1986). Different antibiotics exercise their inhibitory activity on different pathogenic organisms (Chanda and Rakholiya, 2011).

India is one of the 12 mega biodiversity centers having over 45,000 plant species. About 1500 plants with medicinal uses are mentioned in ancient texts and around 800 plants have been used in traditional medicine. The export of herbal medicine from India is negligible despite the fact that the country has a rich traditional knowledge and heritage of herbal medicine (Kamboj, 2000). Antimicrobials that are from a natural source consist of intricate and diverse structures (Potterat and Hamburger, 2006). Characterization of antimicrobials in terms of their phyto-chemical properties has allowed for the categorization of antimicrobials into different classes. The classes are differentiated in terms of the constituents and chemical properties of the antimicrobial compound, e.g. whether the antimicrobial consists of protein, lipids or

carbohydrates, thus the characterization of molecules allows for the identification and characterization of an unknown compound.

Thin layer chromatography (TLC) was frequently used in the 1980's to characterize compounds with respect to their polarity. TLC works on the principles that like forces attract and unlike forces repel each other. It entails the spotting of a sample onto a plate that contains an adsorbent material e.g. silica (polar) and the sample is then moved via the mobile phase (solvent system) (Joseph et al.,1983). This is measured in terms of a constant value known as relative mobility represented by Rf that is calculated from the distance travelled on the plate by the analyte divided by the distance travelled by the solvent front. Visualization of the molecules is not only carried out by assessment of fluorescence but also includes staining and bioautography techniques. Staining of the TLC plates with dyes such as ninhydrin (amino acid stain) and sulphuric acid (carbohydrate detection) (Han and Robyt, 1998) allows for the determination of a compound's structural constituents of namely, lipids, carbohydrates or proteins. Such techniques have been used to characterize various compounds ranging from carbohydrates to peptides to lipids (Mangold, 1961; Poole and Poole, 1989; Friedman, 2004). The bioautography assay is used to detect active compounds in a crude plant extract .Bioautographic techniques incorporate the overlaying of bacterial culture onto the TLC plate. The active analyte prevents the growth of bacteria within its region thus showing the RF region of the antimicrobial component in th**e** crude extract. An inoculated layer of agar is poured over a developed thin layer chromatography (TLC) plate, and lack of bacterial or fungal growth in certain areas identifies the presence and location of antibacterial compounds on the TLC plate.

In this study, the plant species are tested for phytochemical compounds. To test for antibacterial activity the bacteria was collected from throat swab and the plant extracts were screened by disc assay. This antimicrobial activity was confirmed by MTT assay. For finding the active compounds TLC was done and certain Retardation factor (rf) values were obtained.

Further, the confirmation of these active compounds was done by HPLC analysis and it confirmed the presence of Eugenol in *Syzygium aromaticum,* Quercitin was found in *Cicer arietinum* and Genistin was found in *Mentha viridis.*

1.2 Objective

- Three plants were chosen (Chick Pea, Spearmint and Clove bud) for the aqueous and ethanol extract.
- Qualitative phytochemical analysis of chosen plant's extract.
- Isolation and characterization of bacteria
- Antibacterial activity of these plant extract against mouth isolated bacteria
- Characterization of antibacterial activity through TLC-Bioautography technique.
- Estimation of active compounds through HPLC

REVIEW OF LITERATURE

2.1 Medicinal plants – History and context

India has a rich culture of medicinal herbs and spices, which includes about more than 2000 species and has a vast geographical area with high potential abilities for Ayurveda, Unani, Siddha traditional medicines but only very few have been studied chemically and pharmacologically for their potential medicinal value (Sandhu and Heinrich, 2005). Human beings have used plants for the treatment of diverse ailments for thousands of years (Sofowara, 1982; Hill,1989). According to the World Health Organization, most populations still rely on traditional medicines for their psychological and physical health requirements (Rabeand Van Stoden, 2000).

Rural areas of many developing countries still rely on traditional medicine for their primary health care needs and have found a place in day-to-day life. These medicines are relatively safer and cheaper than synthetic or modern medicine (Iwu *et al.,* 1999; Okan *et al.,*2007). People living in rural areas from their personal experience know that these traditional remedies are valuable source of natural products to maintain human health, but they may not understand the science behind these medicines, but knew that some medicinal plants are highly effective only when used at therapeutic doses (Maheshwari *et al.,* 1986; Van Wyk *et al.,* 2000).

Herbal medicines are in great demand in both developed and developing countries as a source of primary health care owing to their attributes having wide biological and medicinal activities, high safety margins and lesser costs. Herbal molecules are safe and would overcome the resistance produced by the pathogens as they exist in a combined form or in a pooled form of more than one molecule in the protoplasm of the plant cell (Lai and Roy, 2004). Even with the advent of modern or allopathic medicine, Balick and Cox (1996) have noted that a number of important modern drugs have been derived from plants used by indigenous people.

Traditional use of medicine is recognized as a way to learn about potential future medicines (Fabricant and Farnsworth, 2001). Plants are used medicinally in different countries

and are a source of many potent and powerful drugs (Srivastava, *et al.,* 1996; Mahesh and Sathish, 2008).

2.2 Herbal medicine

The classical Indian texts include Rig-Veda, Atharvaveda, CharakSamhita and Sushruta Samhita. The herbal medicines / traditional medicaments have therefore been derived from rich traditions of ancient civilizations and scientific heritage (Kamboj, 2000).

Plants are a source of medicinal compounds have continued to play a dominant role Phytochemicals such as vitamins (A, C, E and K), carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, saponins, pigments, enzymes and minerals that have antimicrobial and antioxidant activity (Madhuri and Pandey, 2009).

The specific function of many phytochemicals is still unclear; however, a considerable number of studies have shown that they are involved in the interaction of plants/pests/diseases. Antimicrobial screening of plant extracts and phytochemicals, then, represents a starting point for antimicrobial drug discovery. Phytochemical studies have attracted the attention of plant scientist's due to the development of new and sophisticated techniques. These techniques played a significant role in the search for additional resources of raw material for pharmaceutical industry in the maintenance of human health since ancient times (Saeed and tariq 2008).

Medicinal plants possess immunomodulatory and antioxidant properties, leading to antibacterial activities. They are known to have versatile immunomodulatory activity by stimulating both non-specific and specific immunity.

2.3 Phytochemicals / plant secondary metabolites

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. There are more than thousand known phytochemicals. It is well-known that plant produces these chemicals to protect itself but recent research demonstrates that they can protect humans against diseases. Plants are endowed with various Phytochemical molecules such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites, which are rich in antioxidant

activity. Studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, and antiviral activities. The ingestion of natural antioxidants has beenassociated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing and in recent years, there has been a worldwide trend towards the use of the natural phytochemicals present in berry crops, teas, herbs, oilseeds, beans, fruits and vegetables. (Hendricks *et al.,1998)*

"Phyto" is a Greek word that means plant and phytochemicals are usually related to plant pigments. So, fruits and vegetables that are bright colors — yellow, orange, red, green, blue, and purple - generally contain the most phytochemicals and the most nutrients. Most important of such compounds are alkaloids, tannins, flavonoids, terpenoids, saponins and phenolic compounds. Pharmacists are interested in these compounds because of their therapeutic performance and low toxicity (Inayatullah *et al.,* 2012) Awoyinka *et al.,* (2007). Isolated eight bioactive compounds from the water and ethanol extracts of dried leaf of *Cnidoscolusa conitifolius.* 62 compounds were identified (Chowdhury *et al.,* (2007). Mature and immature leaves and stems of eight plant species belonging to 7 families were screened for alkaloids, saponins, tannins and total phenolics contents (Abdulkabirkhan *et al.* (2009). The leaf, stem and root of Ichnocarpus frutescens were investigated for their photochemical properties. The different solvent extracts of Kirganeliareticulata leaves were screened for their photochemical constituents.

Phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments (Nascimento *et al.,* 2000).

2.4 Antimicrobial activity of Medicinal plants

Medicinal plants as a source for relief from illness can be traced back over five millennia to written documents of the early civilization in China, India and the Near east, but it is doubtless an art as old as mankind. Neanderthals living 60,000 years ago in present day Iraq used plants such as holly back, these plants are still widely used in ethno medicine around the world.

Nair et al., 2005 screened nine plants for potential antibacterial activity. The plant screened ere *Sapinduse marginatus, Hibiscus rosasinesis, Mirabilis Jalapa, Rheo discolor, Nyctanthes arbortristis, Colocasiae sculenta, Gracilaria corticata, Dictyola sp. and Pulicaria wightians.* Antibacterial activity was tested against 6 bacterial strains, *Pseudomonas tetosteroni, Staphylococcus epidermidis, Klebsiella pneumonia, Bacillus subtilius, and Roteus morganii and Micrococcus flavus*. Methods used agar disc diffusion which was used to study the antibacterial activity of all these plant *Pseudomonas testosterones* and *Klebsiella pneumonia* were the more resistant bacterial strains. *Sapindus marinates* showed strong activity against the tested bacterial strains.

Antimicrobial activity of pea extracts has also been explored since chickpea are regarded as a natural source of bioactive molecules. Several by-products of pea have been utilized against human pathogens. The seed, fruit skin and aerial parts of ten registered varieties of *Cicer arietinum* (Chickpea) showed the antibacterial and antifungal activities. Unguilin, a Cyclophilin-like Protein (18 kDa) with anti-Mitogenic, antiviral, and antifungal activities were isolated from black-eyed pea (Ye, X. and T. Ng 2001). The antimicrobial activity of hull extracts from chickpea (Cicer arietinum L.) displayed against Gram negative, as well as Gram-positive bacteria. The present study also showed that legume hulls have good antioxidant activity (Ng, T. 2004; Ye, X. *et al.*, 2000; Kanatt *et al.*, 2011; LadanBarari *et al.*, 2015).

M.longifolia or wild mint is a fast-growing, perennial herb, growing wildly in the northeastern part of Tunisia and cultivated in all regions. The species possesses antimicrobial and antioxidant properties (Emmanuel,Idu *et al.*,2015; Kaur and Kapoor 2002; Dewick *et al.*, 2003). The green mint acquires a very powerful action on the nervous system. The boiled leaves extract has an anti-infectious anti-flatulence effect and anti-inflammatory action (notably of the digestive system), it was counseled in the viral hepatitis and colitis, gastric acidities, aerophagia, to stimulate the digestion; furthermore, it presents some invigorating and stimulating qualities (Saleemand *et al.*, 2000;Kouhila *et al.*, 2001; Kumar and Chattopadhyay 2007; Arumugam *et al.*, 2008).Many researchers have reported the antimicrobial, antifungal, and antioxidant properties of essential oil, In fact, essential oils from mint species have strong antioxidant activity and good antimicrobial activity against *Staphylococcu saureus, Escherichia coli, Candida albicans,* and

Salmonella enteritidis (Mounira Mkaddem *et al.*, 2009)

The aqueous extract of clove causes inhibition of inflammation. The extract can inhibit both acute and chronic inflammation and modulates a cascade of biochemical reactions that propagate and mature the inflammatory response. There is inhibition in the formation of edema and granuloma and the extract is found to decrease elevated levels of several biochemical parameters involved in inflammation and oxidative stress. Eugenol (l-hydroxy-2-methoxy-4allylbenzene), a natural compound available in honey and various plants extracts including clove is exploited for various medicinal applications (Thanekar,Ramachandra and Udgire M, 2013). The clove oil shows antimicrobial activity *against Actino bacillus actinomycetemcomitans, Capnocytophagagingivalis, Fusobacteriumnucleatum, Porphyromonasgingivalis, Prevotell aintermedia, Prevotell amelaninogenica, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, and Escherichia coli.* The antimicrobial action observed for the clove oil suggests its usage as an adjuvant to periodontal therapy. Oral intake of clove suppresses the over growth of *C. albicans*in the alimentary tract including the oral cavity (Alanis ,2005)

Table1 Antimicrobial property of some Indian medicinal plants

SCIENTIFIC NAME	PLANT ORIGIN	SOLVENT	ANTIMICR-OBIAL ACTIVITY	REFERENCES
Withania sominifera	Roots & leaves	Ethyl Acetate, Methanol, Water	*Escherichia coli, Staphylococcus aureus, Salmonella Typhimurium*	Owais *et al,* 2003
	Roots & leaves	Methanol, Hexane, Diethyl ether	*S. typhimurium& E. coli*	Arora *et al,* 2004
Nerium Oleander	Flowers	Hexane	*E. coli, Pseudomonas aeruginosa, S.aureus*	Derwich *et al,* 2010
	Leaves	Chloroformic, Ethnolic, Methanolic	*Bacillus pumillus, Bacillus subtilus, S.aureus, E.coli*	Derwich *et al,* 2010
	Roots Bark	Chloroformic, Ethnolic, Methanolic	*E. coli, B. punillus, B. subtillus, S.aureus, E. coli*	Hussain m & Gorsi m,2004
Lantana	Leaf	Mixture of Dichloromethane and	*P. aeruginosa, E.coli*	Kumar *et al,* 2006

		methanol		
Ficus Sycomoros	Leaves & Stem bark	70% aqueous Ethanol	*S. aureus, Salmonella typhi*	Olusesan *et al,*2010
Eucalyptus camaldulensis	Leaf Leaf	Methanol Aqueous, Acetone, Chloramphenicol	*Klebsiellasp, S. typhi, Yersinia enterocolitica, P. aeruginosa, S. aureus, B. subtilis* *E. coli, K. pneumonia, S.typhi, S. aureus*	Ayepola and adeniyi, 2010 E1 mahmood Muhammad Abubkae,2010
Artemisia herba-alba	Leaf	Methanol	*S.aureus*	Seddik *et al,* 2010
Allium Sativum	Bulbs Bulbs	70% ethanol Water and methanol	*Mycobacterium Tuberculosis* *E. coli, k.pneumoniae, S. typhi, B.cereus, S. Mutans*	Hannam *et al,*2009 Saravanan *et al,* 2010

2.5 *Escherichia coli*

2.5.1 Classification

Escherichia coli is the most commonly encountered member of the family *Enterobacteriaceae* in the normal colonic flora and the most common cause of opportunistic infections (Sherris, 1984). All members of the family *Enterobacteriaceae* are facultative, all ferment glucose and reduce nitrates to nitrites and all are oxidase negative (Sherris, 1984).

Table2 Classification of *E. coli*

Domain	*Bacteria*
Kingdom	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gamma proteobacteria*
Family	*Enterobacteriaceae*
Genus	*Escherichia*
Species	*E. coli*

2.5.2 Morphology and identification

Escherichia coli is gram-negative, non-sporing bacilli with most strains being motile and generally possessing both sex pili and adhesive fimbriae (Mahon and Manuselis, 1995). Because most strains rapidly ferment lactose, colonies grown on MacConkey media are smooth, glossy, and translucent and are rose-pink in colour. Colonies are smooth, circular, 1 – 1.5mm in diameter and yellow opaque if lactose fermenting (blue, if non-lactose fermenting) when grown on cysteine-lactose-electrolyte deficient (CLED) medium (Mackie and McCartney, 1989).

2.5.3 Epidemiology

Strains of Escherichia coli predominate among the aerobic commensal bacteria present in the healthy gut (Mackie and McCartney, 1989).

2.5.4 Infections

Escherichia coli was initially considered a non-harmful member of the colon flora, but is now associated with a wide range of diseases and infections including meningeal, gastrointestinal, urinary tract, wound and bacteremia infections in all age groups. Other Z infections caused by *Escherichia coli* include peritonitis, cholecystitis, septic wounds and bedsores. They may also infect the lower respiratory passages or cause bacteremia and endotoxic shock especially in surgical or debilitated patients (Mackie and McCartney, 1989).

2.5.5 Antimicrobial Susceptibility

Within the community, *Escherichia coli* strains are commonly susceptible to all agents active against the *Enterobacteriaceae*. However, because of the frequent occurrence of R plasmids, strains acquired in hospitals may be resistant to any combination of potentially effective antimicrobics and therapy must therefore be guided by susceptibility testing (Sherris, 1984).

2.6 *Staphylococcus aureus*

2.6.1 Classification

Members of the genus *Staphylococcus* (staphylococci) are Gram-positive cocci that tend to be arranged in grape-like clusters (Ryan and Ray, 2004).

Table3 Classification of *S. aureus*

Domain	*Bacterium*
Kingdom	*Eubacteria*
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Bacillates*
Family	*Staphylococcaceae*
Genus	*Staphylococcus*
Species	*Aureus*

2.6.2 Morphology and identification

Staphylococci are spherical cells about 1 m in diameter arranged in irregular clusters. Single cocci, pairs, tetrads, and chains are also seen in liquid cultures. Young cocci stain strongly gram-positive; on aging, many cells become gram-negative. Staphylococci are non-motile and do not form spores. *Staphylococcus aureus* is a facultative anaerobe that grows at an optimum temperature of 37ºC and an optimum pH of 7.5. *S. aureus* produces white colonies that tend to turn a buff-golden color with time, which is the basis of the species epithet aureus (golden). Most, but not all, strains show a rim of clear β-hemolysis surrounding the colony ((Ryan and Ray, 2004). On nutrient agar, following aerobic incubation for 24 hours at 37ºC, colonies are 1 – 3mm in diameter, have a smooth glistening surface, an entire edge and an opaque pigmented appearance. In most strains, pigmentation is golden with orange, yellow and

cream varieties. On MacConkey agar, colonies are small to medium in size and pink or pink-orange in colour (Mackie and McCartney, 1989).

2.6.3 Epidemiology

Staphylococci are highly successful colonizers of humans and animals. They reside mainly on the skin, particularly in moist areas such as the anterior nares (nose), axilla and groin. Between one-third and three-quarters of individuals carry these organisms at any one time.

Staphylococcal infections occur worldwide and newly emerging hypervirulent or multiresistant strains spread rapidly over wide geographical areas. The bacteria survive in the air, on objects or in dust for days; therefore, they can contaminate environments (such as hospitals) and continue to be transmitted over long periods of time. Some individuals may shed the organism more heavily than others. *Staphylococcal* infections are acquired from either self (endogenous) or external (exogenous) sources (Irving et al., 2006).

2.6.4 Infections

S. aureus causes serious infections of the skin, soft tissues, bone, lung, heart, brain or blood (Irving *et al.,* 2006). Include pneumonia, bacteremia leading to secondary pneumonia and endocarditis, osteomyelitis secondary to bacteremia and septic arthritis, seen in children and in patients with a history of rheumatoid arthritis. Diseases caused by *Staphylococcal* toxins include scalded skin syndrome and toxic shock syndrome (Sherris, 1984).

2.6.5 Antimicrobial Susceptibility

Resistance to penicillin G can be predicted by a positive test for β-lactamase; approximately 90% of *S aureus* produce β-lactamase. Resistance to nafcillin (and oxacillin and methicillin) occurs in about 35% of *S aureus* and approximately 75% of S epidermidis isolate. Alternative antibiotics for resistant organisms (e.g. MRSA) include vancomycin, erythromycin and gentamicin. Some strains become resistant to multiple antibiotics.

2.6.6 Antibiotic resistance

The discovery of antibiotics in the mid-twentieth century revolutionized the management and treatment of infectious disease caused by bacteria. Infections that would normally have been fatal were now curable. Since then, antimicrobial agents (antibiotics and related medicinal drugs acting on bacteria, viruses, fungi and parasites) have saved the lives and eased the suffering of millions of people. Today, antibiotics are crucial not only for the treatment of bacterial infections, but also for prophylactic coverage of high risk patients e.g. those in intensive care, organ transplants, cancer chemotherapy and prenatal care. However, these gains are now seriously jeopardised by the rapid emergence and spread of microbes that are resistant to antimicrobials (www.earto.eu). The mass production of penicillin in 1943 dramatically reduced illness and death from infectious diseases caused by bacteria. However, within four years, bacteria began appearing that could resist the action of penicillin. Pharmaceutical companies fought back by developing other types of antibiotics. After more than 50 years of widespread use of these ―miracle drugs‖, antibiotics are no longer as effective as they once were. Virtually all important bacterial infections in throughout the world are becoming resistant (Johnson, 2006). And even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents (Nascimento *et al.*, 2000). From these microbe's resistant to antibiotics:

2.7 Methicillin-resistant *Staphylococcus aureus (MRSA)*

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections. MRSA infections are very difficult to cure because MRSA strains are resistance against almost all clinically available antibiotics (Adwan and Mhanna, 2008). MRSA infections that are acquired by persons who have not been recently hospitalized or had a medical procedure (such as dialysis, surgery and catheters) are known as Healthcare associated MRSA (HA MRSA) first appeared in the 1960s and has typically been linked to persons with health care associated risk factors such as hospitalization or nursing home care, chronic dialysis, antibiotic treatment, or exposure to invasive devices or procedures. HA MRSA is a highly resistant and important

nosocomial pathogen in both acute care and long-term care settings and causes infections associated with increased morbidity, mortality, and cost when compared to infections due to susceptible strains of *S. aureus* (Cuaresma *et al.,* 2008). Beginning in the 1990s community associated MRSA (CA MRSA) infections emerged in persons having none of the risk factors associated with MRSA in the past. CA MRSA is currently defined as an infection with MRSA in a person who does not have any prior history of a health care exposure such as hospitalization, surgery, permanent intravenous lines or other indwelling device, or hemodialysis.

2.8 Multidrug-resistant *Enterobacteriaceae*

Multidrug-resistant *Enterobacteriaceae,* mostly *Escherichia coli,* produces extended spectrum β lactamases (ESBLs) such as the CTX-M enzymes. These enzymes were named for their greater activity against cefotaxime than other oxyimino-beta-lactam substrates such as ceftazidime, ceftriaxone, or cefepime have emerged within the community setting as an important cause of urinary tract infections (UTIs). Recent reports have also described ESBL-producing *E. coli* as a cause of bloodstream infections associated with these community-onsets of UTI (Darwish and Aburjai, 2010).

Three different plant used in this study.

2.9 *Cicer arietinum*

Figure 1 Plant of chick pea

Table4 Scientific Classification of *Cicer arietinum*

Kingdom	*Plantae*
Subkingdom	*Tracheobionta*
Superdivision	*Spermatophyta*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Subclass	*Rosidae*
Order	*Fabales*
Family	*Fabaceae*
Genus	*Cicer l. – cicer p*
Species	*Cicer arietinum*

A member of the pea and bean family (Leguminosae/Fabaceae), *Cicer arietinum* is one of 43 species in the genus *Cicer*. *Cicer* is Latin for chickpea and is thought to be the origin of the surname Cicero.

Chickpea is the third most important pulse in the world (after beans and peas). Its seeds have been eaten by humans since around 7,000 BC. It is widely cultivated for its nutritious seeds, which are harvested when immature and eaten raw, roasted, or boiled or when mature and dry

processed into flour. Chickpea is a major protein source for poor communities in many parts of the semi-arid tropical areas of Africa and Asia.

Cicer arietinum is not known as a wild plant but is believed to have originated in the central part of the Fertile Crescent (in modern Turkey, Syria and Iran) *Cicer arietinum*

(chickpea) Haryana, India. Chickpea is cultivated in tropical, subtropical and warm temperate zones, including the Mediterranean, the Canary Islands, western and central Asia and northeastern tropical Africa, including Madagascar. It is grown up to 2,500 m above sea level.

Chickpea is valued for its nutritive seeds with high protein content, 25.3-28.9 %. Chickpea seeds are eaten fresh as green vegetables, parched, fried, roasted, and boiled; as snack food, sweet and condiments; seeds are ground and the flour can be used as soup, dhal, and to make bread; prepared with pepper, salt and lemon it is served as a side dish (Saxena, 1990). "Sprouted seeds are eaten as a vegetable or added to salads. Young plants and green pods are eaten like spinach. Animal feed is another use of chickpea in many developing countries In Chile, a cooked chickpea-milk (4:1) mixture was good for feeding infants, effectively controlling diarrhea. Chickpeas yield 21% starch suitable for textile sizing, giving a light finish to silk, wool, and cotton cloth" (Duke, 1981).

Chickpea seed has 38-59% carbohydrate, 3% fiber, 4.8-5.5% oil, 3% ash, 0.2% calcium, and 0.3% phosphorus. Digestibility of protein varies from 76-78% and its carbohydrate from 57-60%. (Hulse, 1991, Huisman and van der poel, 1994). Raw whole seeds contain per 100 g: 357 calories, 4.5-15.69% moisture, 14.9-24.6 g protein, 0.8-6.4 % fat, 2.1-11.7 g "Percent fatty acid compositions are: 'Desi': oleic 52.1, linoleic 38.0, myristic 2.74, pactic 5.11, and steatic 2.05; 'Kabuli': oleic 50.3, linoleic 40.0, myristic 2.28, palmitic 5.74, stearic 1.61, and arachidic 0.07%. The leaves contain 4-8% protein" (Duke, 1981).

2.9.1 Traditional Medicinal Uses

Among the food legumes, chickpea is the most hypocholesteremic agent; germinated chickpea was reported to be effective in controlling cholesterol level in rats (Geervani, 19991). "Glandular secretion of the leaves, stems, and pods consists of malic and oxalic acids, giving a sour taste. In India these acids used to be harvested by spreading thin muslin over the crop

during the night. In the morning, the soaked cloth is wrung out, and the acids are collected in bottles. Medicinal applications include use for aphrodisiac, bronchitis, catarrh, cutamenia, cholera, constipation, diarrhea, dyspepsia, flatulence, snakebite, sunstroke, and warts. Acids are supposed to lower the blood cholesterol levels. Seeds are considered antibilious".

2.11 *Mentha viridis*

Figure 2 Plant of spearmint

Table5 Scientific Classification of *Mentha viridis*

Kingdom	*Plantae*
Subkingdom	*Tracheobionta*
Superdivision	*Spermatophyta*
Division	*Sagnoliophyta*
Class	*Magnoliopsida*
Subclass	*Asteridae*
Order	*Lamiales*
Family	*Lamiaceae/labiatae*
Genus	*Mentha l.*
Species	*Mentha viridis*

Mentha (also known as mint, from Greek *Mentha Linear B*(mi-ta) is a genus of plants in the family *Lamiaceae* (mint family) The species are not clearly distinct. *Mentha viridis* Linn (Labiatae) commonly known as spearmint, is a perennial, rhizomatous and herbaceous plant, growing 30 – 100 cm tall, having 5 – 9 cm long and 1.5 – 3 cm broad leaves (Muhammad majid aziz *et al.*, 2014) Due to their tendency to spread unchecked, some mints are considered invasive. The leaves are arranged in opposite pairs, from oblong to lanceolate, often downy, and with a serrated margin. Leaf colors range from dark green and gray-green to purple, blue, and sometimes pale yellow. The flowers are white to purple and produced in false whorls called verticillasters. The corolla is two-lipped with four sub equal lobes, the upper lobe usually the

largest. The fruit is a nutlet, containing one to four seeds. The genus *Mentha L.,* consisting of more than 25-30 species grows widely throughout temperate regions of the world (Gulluce *et al.,*

2007). *Mentha arvensis(M. arvensis), Mentha piperita (M. piperita), Mentha longifolia (M longifolia)* and *Mentha spicata (M. spicata),* commonly known as menthol mint, peppermint, wild mint and spearmint, respectively are frequently cultivated in several countries of East Asia, Europe, America and Australia for the production of essential oils (Pandey *et al.,* 2003; Gulluce *et al.,* 2007).

The essential oils from *Mentha* species have been in use by human being since ancient times (Iscan *et al.,* 2002). The aerial parts of *Mentha* species are commonly used in commercial spice mixtures for many processed foods as well as in herbal teas (Moreno *et al.,* 2002; Kofidis *et al.,* 2006). Moreover, the essential oils from *Mentha* species are generally employed to flavor liqueurs, breads, salads, soups and cheese, as well as an ingredient of cosmetics (Yadegarinia *et al.,* 2006).

Many other hybrids, as well as numerous cultivars, are known. The genus has a sub cosmopolitan distribution across Europe, Africa, Asia, Australia, and North America. Mints are aromatic, almost exclusively perennial, rarely annual, herbs. They have wide-spreading underground and over ground stolons and erect, square, branched stems.

India fulfills 80% of the total mint global demand with a production of 16,000 tons of mint oil (Khanuja, 2005). Essential oils are composed of a number of compounds of different biosynthetic origins ranging from terpenoid hydrocarbons to sulfur compounds (Schenkel, *et al.,*2007)

2.11.1 Traditional medicinal uses

In addition, these have been utilized traditionally for the treatment of many digestive tract diseases due to its carminative, antiemetic spasmodic, analgesic, and anti-inflammatory attributes (Moreno *et al.,* 2002; Gulluce *et al.,* 2007). The genus *mentha* has attracted serious attention

from plant breeders for genetic improvement of quality and yield traits. *Mentha* is usually taken after a meal for its ability to reduce indigestion and colonic spasms by reducing gastrocholic reflux (Prasad M.P 2014) The essential oils of some *Mentha* species including *M. viridis*, *M. piperita*, *M. longifolia* and *M. spicata* are potential candidates for exhibiting antimicrobial, antioxidant and radical-scavenging activities (Dorman *et al.*, 2003; Pandey *et al.*, 2003; Kaur and Kapoor, 2002; Gulluce *et al.*, 2007), which are mainly attributed to the presence of phenolic substances (Hosseinimehr *et al.*, 2007).

2.12 *Syzygium aromaticum* (clove)

Figure 3 Flower of Clove bud and Clovebud

Table6 Scientific classification of *Syzygium aromaticum*

Kingdom	*Plantae*
Subkingdom	*Tracheobionta*
Super division	*Spermatophyte*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Subclass	*Rosidae*
Order	*Myrtales*
Family	*Myrtaceae*

Genus	*Syzygium*
Species	*Syzygium aromaticum*

The botanical name is *Caryophyllus aromaticus* which is derived from the Latin *"clavus"*, which means nail due to its resemblance with the shape. The clove tree is an evergreen that grows up to 8–12 m tall, with large leaves and sanguine flowers grouped in terminal clusters. The flower buds initially have a pale hue, gradually turn green, and then transition to a bright red when ready for harvest. Cloves are harvested at 1.5–2.0 cm long, and consist of a long calyx that terminates in four spreading sepals, and four unopened petals that form a small central ball

They are native to the Maluku Islands in Indonesia; Cloves are commercially harvested primarily in Bangladesh, Indonesia, India, Madagascar, Zanzibar, Pakistan, Sri Lanka, and Tanzania. Cloves are available throughout the year.

The Clove is used as a spice in almost all the world's fare. It has a very major role in spice trade and is highly appreciated for their therapeutic properties. Cloves are an excellent source of manganese. They are also a very good source of dietary fiber, vitamin C, vitamin K, and Ω-3 fatty acids and a good source of magnesium and calcium. Cloves consist of a significant amount of proteins, iron, carbohydrates, calcium, phosphorus, potassium, sodium and hydrochloric acid. They are also rich in vitamins A and C, manganese, and dietary fiber (Kim *et al.,* 1998). The most important constituent of clove is the phenyl propene eugenol due to which it has strong characteristic aroma. Major parts of clove consist of eugenol comprises 70 to 90 % and remaining 15% consist of dry weight (Shobana and Naidu, 2000).

2.12.1 Traditional Medicinal use

Syzygium species (Fam. *Myrtaceae*) have been reported to possess antibacterial and anti-inflammatory activity. It was reported that the buds of *Syzygium aromaticum* (L.) Merr & Perry (clove) were used in folk medicine as diuretic, odontalgic, stomachic, tonicardiac, aromatic condiment properties and condiment with carminative and stimulant activity (AmitPandey & Parul Singh 2011.) Due to their antimicrobial nature, spices are used to improve taste and enhance shelf life. Some of spices are also known to contribute to the self-defense of plants against infectious organisms.Molds, yeast and bacterial growth could be inhibited by the application of clove essential oil (Burt, 2004 Micro-organisms like *Alternaria sp., Aspergillus sp., Canninghamella sp., Lactobacillus sp. Fusarium sp., Clostridium sp; Mucor sp., Salmonella sp. Penicillium sp. Bacillus sp.* could be repressed by using clove essential oil (Soliman and Badeaa, 2002). Extract derived from spices and plants have antimicrobial activity against *Listeria monocytogenes, Salmonella typhimurium, Escherichia coli O157:H7, Shigelladysenteriae, Bacillus subtilis* and *Staphylococcus aureus* at levels between 0.2 and 10µ ml-1 (Burt, 2004). Bud Oil of Clove has natural behavior and the main properties include antioxidant, insecticidal, antifungal and antibacterial properties. By tradition, it has been used in food preservation as flavoring and antimicrobial substance (Velluti *et al.,* 2003)

2.13 Thin layer chromatography

According to the IUPAC, the chromatography is defined as ―a *physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction* .TLC is a chromatographic technique used to separate mixtures . It is performed on a sheet of glass, plastic or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose (blotter paper). This layer of Adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (mobile phase) is drawn up the plate via capillary action. Because different analyte ascends the TLC plate at different rates, separation is achieved (A.I. Vogel, *et al.,* Vogel's Textbook of Practical Organic Chemistry 5 edition 1989). TLC which was popular in the late 1960s and 1970s has been almost completely superseded by LC and GC. However, thanks to recent improvements in instrumentation and especially in detection. TLC is being used more in the recent past, and is the object of an official method (D Barselo, M.C. hennion 1997). The TLC technique has many advantages, single use of the layer simplifies sample preparation procedures; simplicity of development by dipping the plate into a mobile phase in a chamber; high sample through put with low operating cost because multiple samples can be run simultaneously with standards on a single plate using a very low volume of solvent; high resolution through multiple development or two-dimensional (2D)development on a plate with a single adsorbent or dual adsorbents (J Sherma 2005). This technique can be useful for screening important agricultural commodity moving in international trade, and for the domestic food supply. TLC is availability of many sensitive and selective reagents for detection and confirmation without interference of the mobile phase. The ability to repeat detection and quantification at any time with changed parameters because fractions representing the entire sample are stored on the plate (V.Betina 1973).

2.14 Bioautography

Bioautography is a highly efficacious assay for the detection of antimicrobial compounds because it allows localization of activity even in a complex matrix, and therefore facilitates the target-directed isolation of the active constituents (Choma and Grzelak 2011). Bioautography has

enabled rapid progress for quick detection of new antimicrobial compounds from plants and other natural products. This technique allows the localization of antimicrobial activity directly on a chromatographic plate where the organism is applied. The method is fast, cheap, and permits a better bioassay-directed fractionation of bioactive compounds. Bioautography is particularly important to avoid the time-consuming isolation of inactive compounds. TLC- Bioautographic methods combine chromatographic separation and *in situ* activity determination facilitating the localization and target-directed isolation of active constituents in a mixture (Shahverdi *et al.,* 2007).

A number of Bioautographic assays have been developed, which can be divided into three groups (Rios *et al.,* 1988). These include direct bioautography; where the microorganisms grow directly on thin-layer chromatography (TLC) plates, contact bioautography; where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact, and agar overlay or immersion bioautography; where a seeded agar medium is applied onto the TLC plate. The latter technique can be considered as a hybrid of direct and contact bioautography (Islam *et al.,* 2003).

Among the all Bioautographic methods, the most widely applied is direct bioautography (Irena M. choma and EdytaM .grzelak 2010)

2.14.1 Thin –layer chromatography – Direct Bio-autography

The beginnings of coupling microbiological assay with planar chromatography date back to 1946, when (Goodall and Levi 1946) combined paper chromatography method (PC) with contact Bio-Autography detection for the determination of the different *penicillin's.* Fifteen years later, introduced thin-layer chromatography (TLC) in the same field. The methods were described as

simple, reproducible and highly sensitive. The first review of the application of bioautography in paper and thin-layer chromatography was presented by Betina in 1973. The author not only emphasized the advantages of the method, such as rapidity and versatility, but also pointed out the difficulties of quantitative interpretation of the obtained results. The influence of various factors, such as tested microorganisms, medium composition, pH, and solubility of the sample in the culture on the Bioautographic detection was widely discussed in a summary on screening methods for testing antimicrobial activity in natural products (J.L.Rios *et. al.,* 1988). The authors concluded that it is highly difficult to standardize these methods because of their diversity. Henceforth, other studies were done to estimate standardized parameters, which can influence the Bioautographic detection. The broad review on various factors influencing bacterial growth, such as mobile phase and their additives, type of adsorbent, test microorganism, pre-conditioning of TLC plates, living conditions for test bacteria and post-chromatographic detection, as well as on bioautography methods was done by (Botz *et al.,* 2001). Many examples of various applications of TLC-bioautography can be found in the review article by Choma2005. Morlock and Schwack, in the review about hyphenations in planar chromatography, give many examples on bioassays used for (HP) TLC-EDA (G morlack *et. al* 2010). They point to papers on bacterial assays with *Vibrio fischeri, Escherichia coli, Bacillus subtilis and Pseudomonas savastanoi*as well as to papers on Bioautographic fungi assays. The authors state, that the effect-directed analyses (EDAs), such as Bioautographic assays, are especially suitable for selective detection while combining with chromatography (Irena m; Chorma&Edyta; M. Grzelak 2010).

2.15 HPLC analysis

It is an analytical method to separate, identify and quantify each component present in a mixture.

The method relies on the pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component present in the sample interacts differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as the flow out the column. HPLC-UV method has been successfully used for determination of eugenol in *Syzygium*

aromaticum (Clove) by using NDBD-F as a labelling reagent. (Higashi et al., 2012). Several studies have reported pharmacological mode of action of eugenol from medicinal plants such as *Pimpinella anisum* (leaf), *Anethum sowa Roxb* (leaf), *Salvadora persica* (leaf), *Alpinia galanga wild* (rhizome), *Vetiveria zizanioides* (root) and *Ocimum sanctum* (leaf) in experimental animal systems as hepatoprotective agent, vaso-relaxing action (Nishijima et al., 1999), as an attractant

to fruit fly(Saedi et al.,2012) and etc. The effect of quercetin (Mentha viridis) on erythrocyte Na+/K+ ATPase and sodium hydrogen exchanger (NHE) activity in normal and type 2 diabetic subjects a significant ($p<0.02$) increase in the activities of Na+/K+ ATPase and a decrease ($p< 0.01$) in NHE were observed in type 2 diabetic subjects compared to normal. In vitro treatment with quercetin caused inhibition of both Na+/K + ATPase and NHE. The inhibitory effect of quercetin was concentration dependent. The effect of quercetin on Na+/K+ ATPase and NHE may be explained due to a direct effect of this compound on plasma membrane leading to a change in membrane fluidity. (Int J Cancer 120(3):451–458). The objective of this study was therefore to evaluate the presence of the isoflavones daidzein, glycitein, genistein and their conjugated forms in grains and leaves of several leguminous plants utilized largely in Brazilian cuisine. Grains used in Peruvian cuisine were also analyzed. After extracting phenolic compounds with methanol (80%), isoflavones as detected by reversed-phase high-performance liquid chromatography/diode-array detector were only found in chickpeas and soybean. Chickpea (Cicer arietinum) extracts showed only the isoflavone genistein at 31 µg/g defatted flour. (Isoflavones. Chapman & Hall, London 1993; 117–238).

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Sample Collection

The plant materials used in this study consisted *of Cicer arietinum, Mentha viridis and Syzygium aromaticum*. The leaves of Cicer arietinum and Mentha viridis were used and for Syzygium aromaticum (Bud) part was used. These plants were collected from Sector 17, Local market area and were tested at Quest Diagnostics Laboratory, Gurgaon,given in (Table 3.1)

Table7 Plant materials used in this study

Plant/Part used	Place	Time of collection
Cicer arietinum/ leaves, Stem and Root	West U.P. Deoband Market	February/March
Mentha viridis/ leaves, Stem and Root	Noida U.P. Market	February/March
Syzygium aromaticum/		

Clove bud	Noida U.P.	February/March
	Market	

3.1.2 Chemicals

All chemicals and solvents used were of analytical grade and obtained from Merck, SD-fine and Qualigens. Standards and reagents were procured from SRL (Mumbai, India).

3.1.3. Equipments

- 37°C incubator
- Centrifuge
- Laminar Air Flow
- Microscope
- Weighing Balance
- Autoclave
- Water Bath
- Refrigerator
- Hot Air Oven

3.1.4. Accessories

- Petri plates
- Glass rod
- Conical flasks
- Measuring cylinder
- Test tube
- Micropipette or tips (100–1,000ml)
- Glass Slide
- Capillary tube
- TLC Sprayer

3.2 Methods

3.2.1 Qualitative phytochemicals analysis of three plants

3.2.1.1 Preparation of plant extracts

Five gram of each plant samples (leaves, stem, root and bud) were weight separately Crushed each plant samples with the help of mortar and pestle with 20 ml of aqueous and ethanol solution. The samples were filtered with the help of muslin cloth and the undissolved particle is discarded and filtrate was kept at 4^{o} C for the experimental procedure.

3.2.1.2 Test for phlobatannins

Deposition of a red precipitate when a plants extract of each plant sample was boiled with 1 %

aqueous hydrochloric acid was taken as evidence for the phlobatannins.

3.2.1.3 Test for cardiac glycosides

1 ml of each extract was treated with 0.5 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 0.25ml of concentrated sulphuric acid. A brown ring at the interface formed. A violet ring may appear below the ring while in the acetic acid layer, a greenish ring may be formed.

3.2.1.4 Test for Saponins

The test solution was shaken with water. And noted for the stable froth formation indicates the presence of saponins.

3.2.1.5 Test for Phenols

To 1 ml of the extract, 2ml of distilled water was added followed by few drops of 10% aqueous ferric chloride. Appearance of blue or green colour indicates the presence of phenols.

3.2.1.6 Test for Flavonoids

1 ml of the extract, a few drops of dilute sodium hydroxide was added. An intense yellow

color was produced in the plant extract, which become colorless on addition of a few drops of dilute acid indicates the presence of flavonoids.

3.2.1.7 Test for proteins (Biuret test)

Equal volume of 5% solution of sodium hydroxide and 1% copper sulphate were added. Appearance of pink or purple colour indicates the presence of proteins and Free amino acids.

3.2.1.8 Test for carbohydrates (Fehling's test)

1 ml of Fehling's A (copper sulphate in distilled water) and Fehling's B (potassium tartrate and sodium hydroxide in distilled water) solution were mixed and boiled for one minute. Equal volume of extract was added and heated on boiling water bath for 5-10 min. Appearance of a yellow coloured first and then brick red coloured precipitates revealed the presence of reducing sugars.

3.3 Isolation and identification of *S. aureus* and *E. coli* from mouth Samples

3.3.1 Preparation of Nutrient media

- Nutrient Agar Medium (NAM):

Sodium chloride	-	5 g
Peptone	-	5 g
Beef extract	-	3 g
Agar	-	15 g
Distilled water	-	1000 ml
pH	-	6.8.

- Nutrient Broth:

Sodium chloride	-	5 g
Peptone,	-	5 g
Beef extract	-	3 g
Distilled water	-	1000 ml
pH	-	6.8.

3.3.2 Procedure

- Saliva samples were obtained from mouth.
- The samples were spread on plates containing nutrient agar media.
- The plates were incubated at 37^{O} C for 24-48 hrs.
- Two different colonies identified according to morphology shape, size and colour.
- These colonies are sub cultured into a fresh NA Medium
- The plates were incubated at 37^{O} C for 24-48 hrs.

3.3.3 Gram staining

The most important differential stain used in bacteriology is the Gram stain, named after Dr. Christian Gram. It divides bacterial cells into two major groups, gram-positive and gram-negative, which makes it an essential tool for classification and differentiation of microorganisms. The Gram stain reaction is based on the difference in the chemical composition of bacterial cell walls. Gram-positive cells have a thick peptidoglycan layer whereas the peptidoglycan layer in gram negative cells is much thinner and surrounded by outer lipid-containing layers. Peptidoglycan is mainly a polysaccharide composed of two chemical subunits found only in the bacterial cell wall. These subunits are N- acetyl glucosamine and N- acetyl muramic acid. As adjacent layers of peptidoglycan are formed, they are cross linked by short chains of peptides by means of a Tran's peptidase enzyme, resulting in the shape and rigidity of the cell wall. Early experiments have shown that if the gram-positive cell is denuded of its cell wall by the action of lysozyme or penicillin, the gram-positive cell will stain gram negative.

3.3.4 Procedure

- One clean glass slide was taken.
- A smear was prepared by placing a drop of water on the slide and then transferring Microorganism to the drop of water with a sterile cooled loop, It was mixed and spread by means a circular motion of the inoculating loop.
- Smear was air dried and heat fixed.
- Smear was gently flooded with crystal violet for 1 min.
- Gently washed with tap water.
- Smear was gently flooded with Gram's iodine and left for 1 min.
- Gently washed with tap water.
- Decolorized with 95% ethyl alcohol reagent. It was added drop by drop until no further violet colour comes out.
- Gently washed with tap water.
- Counterstained with safranin for 45 seconds.
- Gently washed with tap water.
- It was dried with bibulous paper and examined under Microscope.

3.4 Biochemical test

3.4.1 Catalase Test

3.4.1.1 Principle

Used to test for the presence of enzyme catalase, hydrogen peroxide (H_2O_2) is formed as an end product of the aerobic breakdown of sugars. When H_2O_2 accumulates, it becomes toxic to the

organism. Catalase decomposes H_2O_2 and enables the organism to survive. Only obligate anaerobes lack this enzyme.

Catalase

$$2\ H_2O_2 \longrightarrow 2H_2O + O_2$$

3.4.1.2 Procedure

- Streak nutrient agar slant with the organism.
- Incubate at optimum temperature for 24-48 hours.
- Place a few drops of 3% H_2O_2 on the slant culture.

3.5.1.3 Interpretation

- Positive- Bubbling (O_2 gas is liberated from the H_2O_2).
- Negative- No bubbling.

3.4.2 Starch Hydrolysis

3.4.2.1 Principle

Used to determine the ability of an organism to hydrolyze (break down) starch.

The enzyme amylase breaks starch down into components more easily metabolized by the organism.

$$\text{Starch} \xrightarrow{\text{Amylase}} \text{Maltose+Glucose+Dextrin}$$

3.4.2.2 Starch agar plate

Beef extract	-	3 g
Soluble starch	-	10 g
pH	-	7.5
Agar	-	12 g
Distilled water	-	1000 ml

3.4.2.3 Procedure

- Make a single streak of the organism on a starch agar plate
- Incubate at optimal temperature for 24-48 hours
- Drop a small amount of KI (Gram's Iodine) onto the plate and rotate the plate gently. (Iodine is an indicator of starch; in the presence of starch the iodine will turn blue/black)

3.4.2.4 Interpretation

- Positive-a zone of clearing appears adjacent to the streak line.
- Negative-No clearing; only a blue/black area surrounding the streak line.

3.4.3. Citrate Utilization (Simmons Citrate Agar)

3.4.3.1. Principle

Used to determine if an organism is capable of using citrate as the sole source of carbon with production of the enzyme citratase

Citratase

Citrate ⟶ Oxaloacetate and Acetate

Oxaloacetate ⟶ Pyruvate + CO_2

Pyruvate ⟶ Acetate + Formate

The media contains sodium citrate as the carbon source, and ammonium salts the nitrogen source, with bromothymol blue as the pH indicator. An organism that uses citrate breaks down the ammonium salts to ammonia, which creates an alkaline pH.

3.4.3.2 Simmons's Citrate agar

Sodium chloride	-	5 g
Sodium citrate	-	2 g
Ammonium dihydrogen phosphate	-	1 g
Dipotassium phosphate	-	0.2 g
Magnesium sulphate	-	0.2 g
Bromothymol blue	-	0.08 g
pH	-	6.9

Agar	-	15 g
Distilled water	-	1000 ml

3.4.3.3 Procedure

- Stab and streak Simmons citrate agar slant with the organism.
- Incubate at the optimum temperature for 24-48 hours.

3.4.3.4 Interpretation

- Positive- Alkaline pH causes media to change from green to Prussian blue.
- Negative- No color change.

3.4.4 Carbohydrate utilization (lactose and sucrose)

3.4.4.1 Principle

Used to determine the ability of an organism to ferment a specific carbohydrate, Phenol Red is used as an indicator in the media. At a neutral pH, the media is red at a pH of less than 7, the media is yellow. Fermentation of the carbohydrate produces acid, causing the media to change from red to yellow. The inverted tube in the broth, called a Durham tube, captures some of the gas the organism produces, allowing production to be seen (if it ferments, gas will be produced).

3.4.4.2 Procedure

- Inoculate each media with the organism
- Incubate at the optimum temperature for 24-48 hours

3.4.4.3 Interpretation

- Positive- Media turns yellow (fermentation has occurred) and gas produced
- Negative- Media remains red (no fermentation). Continue incubation of negative tubes for up to 2 weeks to detect slow fermenters.

3.4.5 Casein Hydrolysis (Skim milk agar)

3.4.5.1 Principle

Used to determine the ability of an organism to produce the enzyme caseinase, which hydrolyzes (breaks down) casein (a white protein in milk) to more soluble products?

3.4.5.2 Skim milk plate

Skim milk powder	-	50 g
Peptone	-	5 g
pH	-	7.0
Agar	-	15 g
Distilled water	-	1000 ml

3.4.5.3 Procedure

- Make a single streak of the organism on a skim milk agar plate.
- Incubate at the optimum temperature for 24-48 hours.

3.4.5.4 Interpretation

- Positive- A zone of clearing occurs along the streak line.
- Negative- No zone of clearing (note- compare results with the Litmus milk test).

3.4.6 Urease Test (Urea Broth)

3.4.6.1 Principle

Used to determine the ability of an organism to split urea to form ammonia (an alkaline end product) by the action of the enzyme urease, Media also contains the pH indicator phenol red, which turns an intense pink at alkaline pH.

$$\text{Urea} \xrightarrow{\text{Urease}} 2\ \text{Ammonia} + CO_2$$

3.4.6.2 Urea plate

Peptone	-	1g
Dextrose	-	1 g
NaCl	-	5 g
Potassium phosphate	-	2 g
Phenol red	-	0.012 g
pH	-	6.7
Agar	-	15 g
Distilled water	-	1000 ml

Autoclave

Then add Urea - 20 g

3.4.6.3 Procedure

- Inoculate urea broth with the organism.
- Incubate at optimum temperature for 24-48 hours.

3.4.6.4 Interpretation

- Positive- Intense pink/red color.

- Negative- No color change.

(Note- Continue incubation of negative tubes for a total of 7 days to check for slow urease producers)

3.5 Antibacterial activity of plant extracts

3.5.1 Plant extraction procedure

According to the Gupta *et al.,* 2013 with some modifications)

- Fresh leaves were taken.
- Leaves were kept air dried under shade
- Kept for 1hr. at 40°C in hot air oven for complete dry.
- With the help of mortar and pistil the leaves were cursed properly in powdered form.
- The powdered leaves were weighed with the help of weighing machine
- Added the ethanol and aqueous solution in 1:4 (w/v) ratio
- Vortex it for 2- 4 minutes for proper mixing
- leave the sample for proper extraction for overnight
- Collect the supernatant after centrifuge 10000 rpm for 10 minutes
- This process was repeated twice; addition of 1:4 (w/v) ratio of ethanol and aqueous solution.
- Taken the supernatant in petriplate for proper evaporation
- Samples were dried and weighed with the help of weighing machine

- Made the final concentration of each plant sample and stored at 4°C until use.

.

3.5.2 Preparation of plant extracts standard concentrations

Scarabs the plant crude extracted material from petriplate and weighed the crude extract and dissolved in respective solvent and make the concentration 100 mg/ ml.

3.5.3 Disk Diffusion Assay

Suspensions of testing microorganisms were spread on Nutrient Agar (NA) medium. The filter paper discs (5mm in diameter) were dipped in plant extracts (concentration 100 mg/ml) and placed in nutrient agar plate. The plates were subsequently incubated at 37°C for 24 Hrs. After incubation the growth inhibition zone were quantified by measuring the diameter of the zone of inhibition in mm (Kumar *et al.,* 2009).

3.6 Thin layer chromatography

3.6.1 Developing Silica gel, TLC plate

TLC plate is prepared by using approximate 1:2 (w/v) silica powder (polar) and distilled water and is continuously stirred using glass rod to form slurry, once slurry is viscous and thin were poured on TLC glass plates, and then allowed to solidify at room temperature. Further it is allowed to dry in hot oven incubator at 110° C for 1 hrs. This developing of plates is essential step as it removes excess of water used to make silica plate. If these droplets are not removed they may interfere with the compound separation and regularized pours will not obtain, which may hamper separation of compounds.

3.6.2 Spotting of sample

- Lightly put a spot with a pencil above 1 cm from the end of plate.
- While putting the spot be care full not to scratch the surface of the plate too deeply.
- Rinse capillary tube with acetone between use
- Lightly press against a piece of paper towel and allow capillary action to with draw all solvent
- Made each sample spot as small as possible (less than about 3mm diameter).
- Simply tab and immediately release the sample.

3.6.3 Development Chamber

- The prepared plate is developed in a closed, pre-saturated chamber using an ascending mobile solvent (isopropyl alcohol: chloroform).
- The solvent is then evaporated before development by placing the plate in the mobile phase at a level below the applied sample.
- When the solvent front nears the top edge of the stationary phase after 15 minutes up to several hours, the plate is removed from the solvent reservoir.
- The sample ascends the plate by capillary action of the mobile phase and the various Components of the sample are retarded in proportion to their interaction.
- Separations in TLC involve distributing a mixture of two or more substances between a stationary phase and a mobile phase.
- Components of the samples will separate on the stationary phase according to how much they adsorb on the stationary phase versus how much they dissolve in the mobile phase.

3.6.4 Visualization

The constituents of a sample are identified by simultaneously running standards with the unknown. After development is complete and the plate dried, the solute bands must be located by using:

- If the spots can be seen, outline them with a pencil.
- Calculated the R_f value.

$$R_f = \frac{\text{Distance from start to centre of substance spot}}{\text{Distance from spot to solvent front}}$$

3.7 Bio-autography

Bioautography technique of Nostro *et al.* (2000) was used with some modifications for the detection of antimicrobial components present in the leaf extracts, after separating these components on TLC plates as described above. At the end of TLC run, 1% inoculums of *S.aureus* and *E. coli* containing Nutrient agar medium were poured on TLC plates. After solidification of the Nutrient agar medium on the TLC plates, they were incubated for 24 h at 37 °C. Subsequently, the bioautogram was sprayed with a 1% aqueous solution of 3(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) based on the reduction of MTT by mitochondrial dehydrogenase of viable cells resulting in a blue formazan product after incubation. It was then incubated at 30°C for few minutes. Zones in which bacterial growth was

inhibited failed to take the bluish stain and indicated the presence of active compounds in that area of TLC plate.

3.8 HPLC

It is an analytical method to separate, identify and quantify each component present in a mixture.

It is a form of liquid chromatography to separate the compounds dissolved in solution.The diluent used in High-Performance Liquid Chromatography is isopropyl alcohol:chloroform in the 9:1 ratio.

3.8.1 *Syzygium aromaticum*

Diluent- Methanol:Acetonitrile (70:30)

Mobile phase sample- Methanol:Acetonitrile (70:30)

Detector used- UV-VISIBLE Detector

Sample preparation-

1. 1g of sample was taken in 10mL volumetric flask.

2. 5ml of Diluent was added and was shaken for approximately 1 min and made upto volume with methanol up to 10 ml.

3. Clear extract was transferred into a clean auto-sampler vial and inject 20ul onto HPLC.

3.8.2 *Cicer arietinum*

Diluent- Methanol:Water (70:30)

Mobile phase sample- Methanol:Water (70:30)

Detector used- UV-VISIBLE Detector

Sample preparation-

1. 1g of sample was taken in 10mL volumetric flask .

2. 5ml of Diluent was added and was shaken for approximately 1 min and made upto volume with methanol up to 10 ml .

3. Clear extract was transferred into a clean auto-sampler vial and inject 20ul onto HPLC.

3.8.3 *Mentha viridis*

Diluent- Methanol:Acetonitrile (50:50)

Mobile phase sample- Methanol:Acetonitrile (50:50)

Detector used- UV-VISIBLE Detector

Sample preparation-

1. 1g of sample was taken in 10ml volumetric flask.

2. 5ml of Diluent was added and was shaken for approximately 1 min and made upto volume with methanol up to 10 ml.

3. Clear extract was transferred into a clean auto-sampler vial and inject 20ul onto HPLC.

RESULTS AND DISCUSSIONS

4. Qualitative phytochemicals analysis of three plants

Table8 Qualitative Phytochemical analysis of chick pea aqueous and ethanol extract

S. No	Phytochemical tests	aqueous extracts		Ethanol extract	
		Leaf	Stem	Leaf	Stem
1.	Test for Carbohydrate (Fehling's Test)	+	_	+	+
2.	Test for Proteins (Biuret Test)	+	+	+	+
3.	Test for Flavanoids	+	+	+	+
4..	Test for Saponins	+	+	+	+
5.	Test for Cardiac glycosides	+	+	+	+
6.	Test for Phylobatannins	+	+	+	-
7.	Test for Phenols	+	+	+	+

Present = +

Absent s= -

Table9 Qualitative Phytochemical analysis of spearmint aqueous and ethanol extracts

S. No	Phytochemical tests	Aqueous Extract		Ethanol extracts	
		Leaf	Stem	Leaf	Stem
1.	Test for Proteins (Biuret Test)	+	+	+	+
2.	Test for Carbohydrates (Fehling' Test)	_	_	_	_
3.	Test for Flavonoids	+	+	+	+
4.	Test for Saponins	+	+	+	+
5.	Test for Cardiac glycosides	+	+	+	+
6.	Test for Phylobatannins	_	_	_	_
7.	Test for Phenols	_	_	_	_

Present = +

Absent = -

Table10 Phytochemical analysis of Clove bud of aqueous and ethanol extracts

S. No	Phytochemical tests	Clove bud	
		Aqueous extracts	**ethanol extracts**
1.	Test for Carbohydrates (Fehling's Test)	+	+
2.	Test for Proteins (Biuret test)	_	_
3.	Test for Flavonoids	+	+
4.	Test for Saponins	_	_
5.	Test for Cardiac glycosides	+	+
6.	Test for Phylobatannins	_	_
7.	Test for Phenols	+	+

Present = +

Absent = -

In extraction of Chick pea, Spearmint, Clove bud with aqueous and ethanol extract, the study indicated that presence or absence of certain important compounds in an aqueous and ethanol extract is determined by colour reactions of the compounds with specific chemicals formation of precipitate and clear colour at interface.

In chick pea strong amount of tannin, saponins, flavonoids, coumarins, phenol, cardiac glycoside were present. Fix oil and fat were present in moderate amount, sugar and phlobatannins were absent they give negative result with chick pea.

In mint terpenoid, tannin, steroid, fixed oil, cardiac glycosides, coumarins were present in strong amount and flavonoids and saponins and protein were present in moderate amount sugar, phlobatannins and phenol give negative result with Spearmint.

In Clove bud cardiac glycoside, flavonoids, fixed oil, phenol, terpenoids were present in strong amount and carbohydrate was present in moderate amount, coumarins, saponins, steroid, phlobatannins and fat were absent.

Table11 Biochemical test for identification of *S. aureus* and *E. coli*

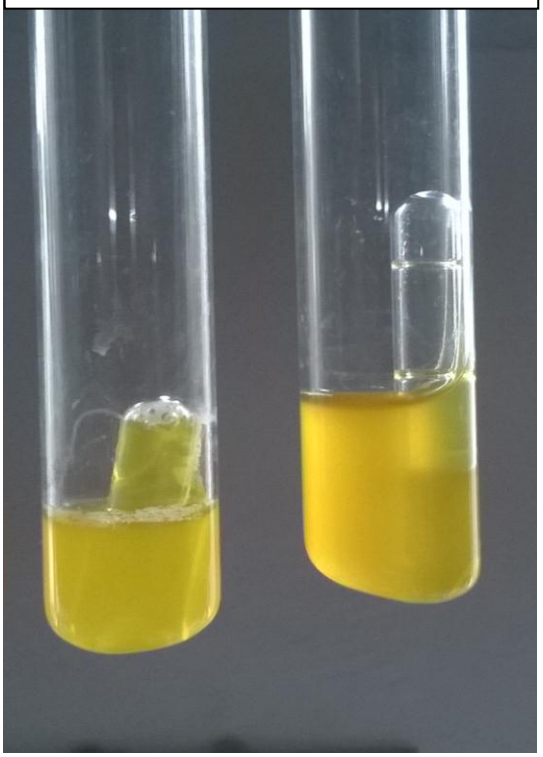
Biochemical test	**Bacteria 1**	**Bacteria 2**
Gram staining	Positive	Negative
Starch hydrolysis	Negative	Negative
Catalase test	Positive	Positive
Urease test	Negative	Negative
Carbohydrate utilization **Sucrose** **Lactose**	 Positive Positive	 Positive Positive
Casein test	Positive	Negative

Citrate test	Positive	Negative

A diverse range of biochemical reagents are known for the identification of certain metabolisms and to differentiate between bacteria. Classical biochemical tests are often used to identify microorganisms; the results are seen by color change. In most cases, detection is based on the reaction of an enzyme with a certain substrate. At the end, a color change gives a result that leads to identify the unknown organism by doing gram staining it is confirmed that the bacteria 1 is gram positive and bacteria 2 is gram negative and by doing biochemical testing concluded that the bacteria 1 is *S.aureus* and bacteria 2 is *E.coli.*

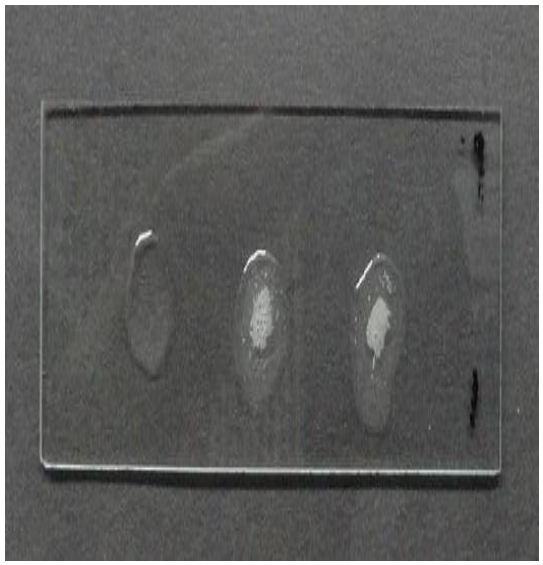
Figure 4 Carbohydrates, Saponins, Catalase, Protein, Urease, Citrate, Starch Hydrolysis, Gram staining Test on *E. coli* and *S.aureus*

Carbohydrate

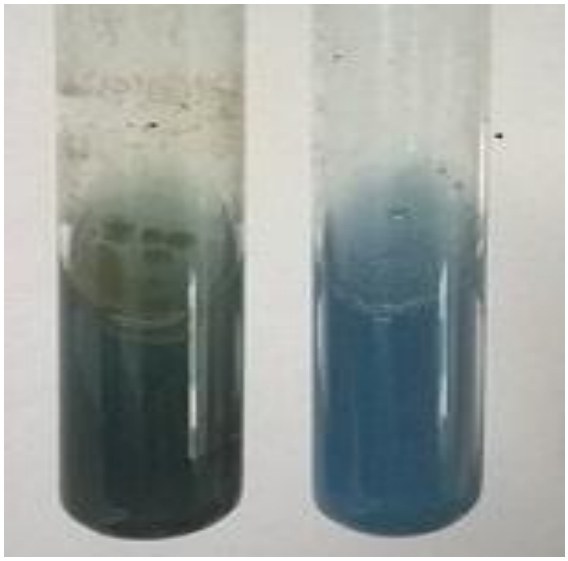


Casein

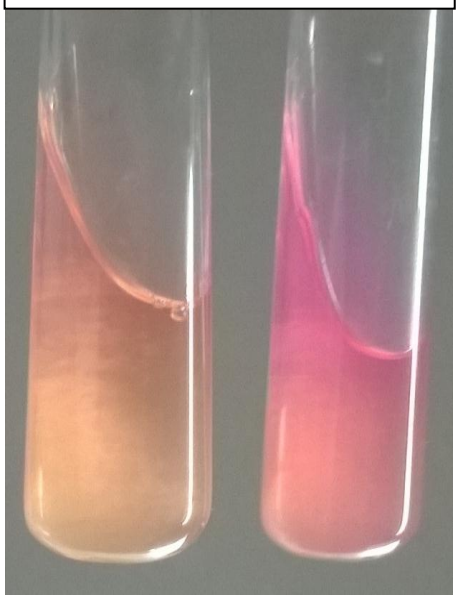
Catalase



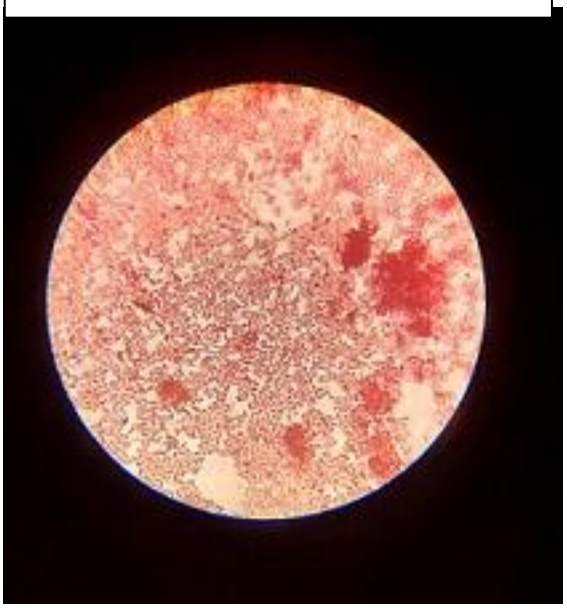
Citrate



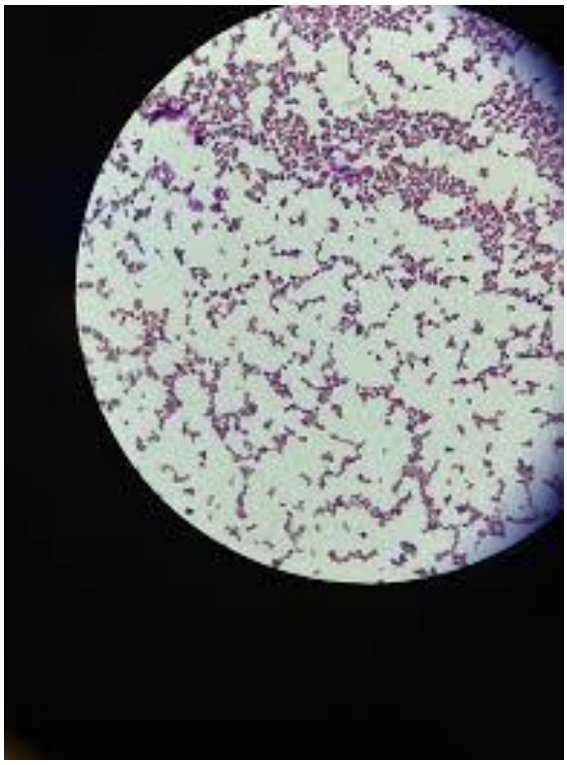
Urease



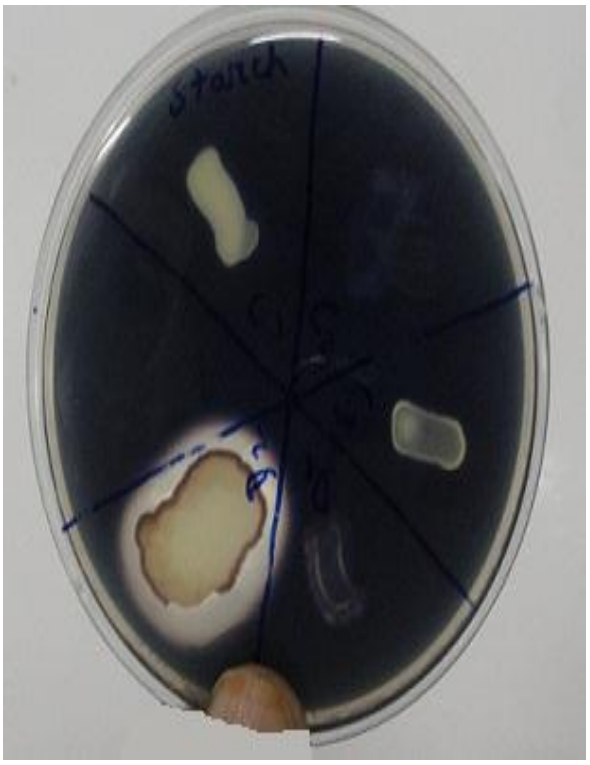
Escherichia coli –Gram staining



Staphylococcus aureus- Gram Staining



Starch hydrolysis



4.5 Antibacterial activity of plant extracts

Table12 Antibacterial activity of different plant aqueous extracts by disk diffusion method (Mean ±SD)

S. No.	Microorganisms	Antibacterial Activity (Zone of inhibition in mm)				
		Chickpea	Spearmint	Clove	CAM	D/W
1	*E. coli*	10.67±0.29	11.5±0.5	11±0.00	18.5±0.5	00
2	*S. aureus*	10.5±0.5	10±0.00	14.5±0.5	14.5±0.5	00

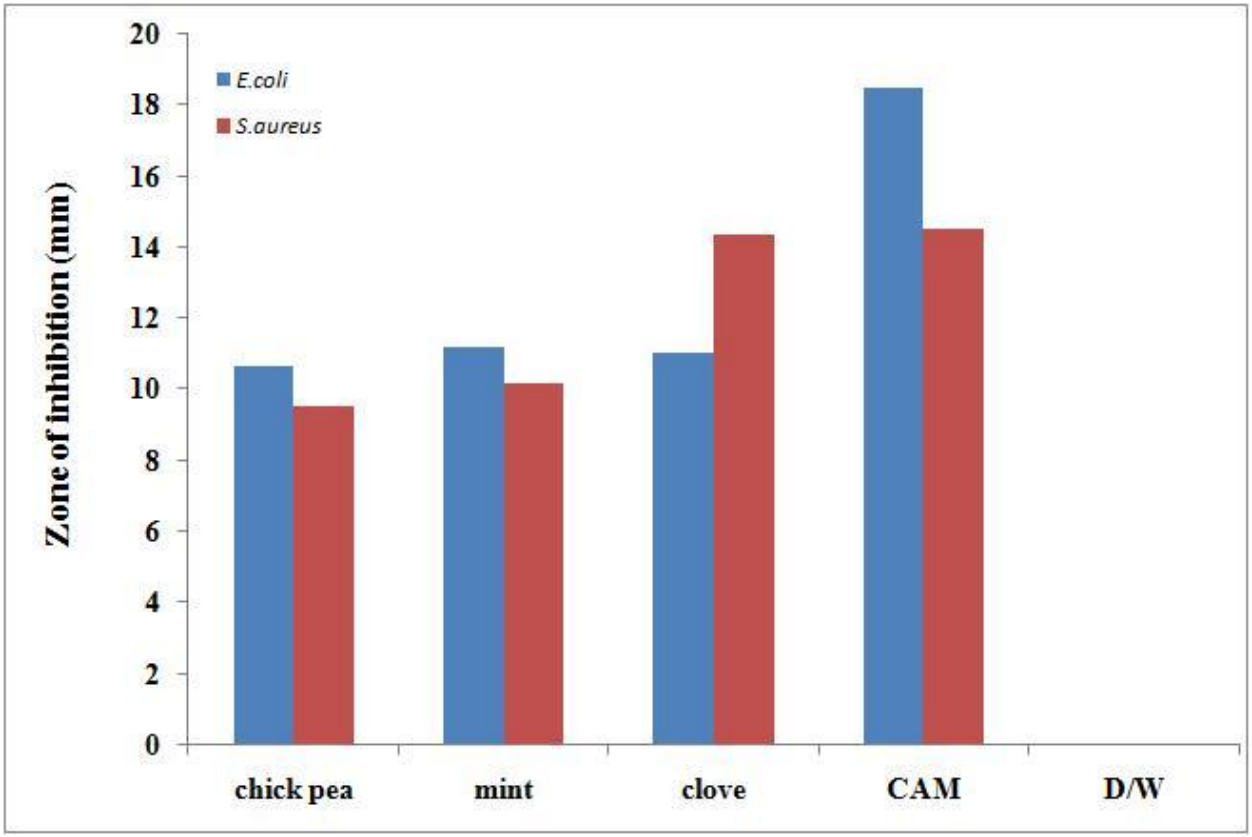


Figure 5 Graph of zone of inhibition for antibacterial activity of different plant aqueous extracts by disk diffusion method

CAM - Chloramphenicol

D/W - Distilled Water

Table13 Antibacterial activity of different plant ethanol extracts by disk diffusion methods (Mean ±SD)

S. No.	Microorganisms	Antibacterial Activity (Zone of inhibition in mm)				
		Chickpea	Spearmint	Clove	CAM	D/W
1	*E. coli*	8.5±0.5	14.5±0.5	10.5±0.5	36.5±0.5	00
2	*S. aureus*	7 ±0.00	11.5±0.5	17.00±0.00	14.5±0.5	00

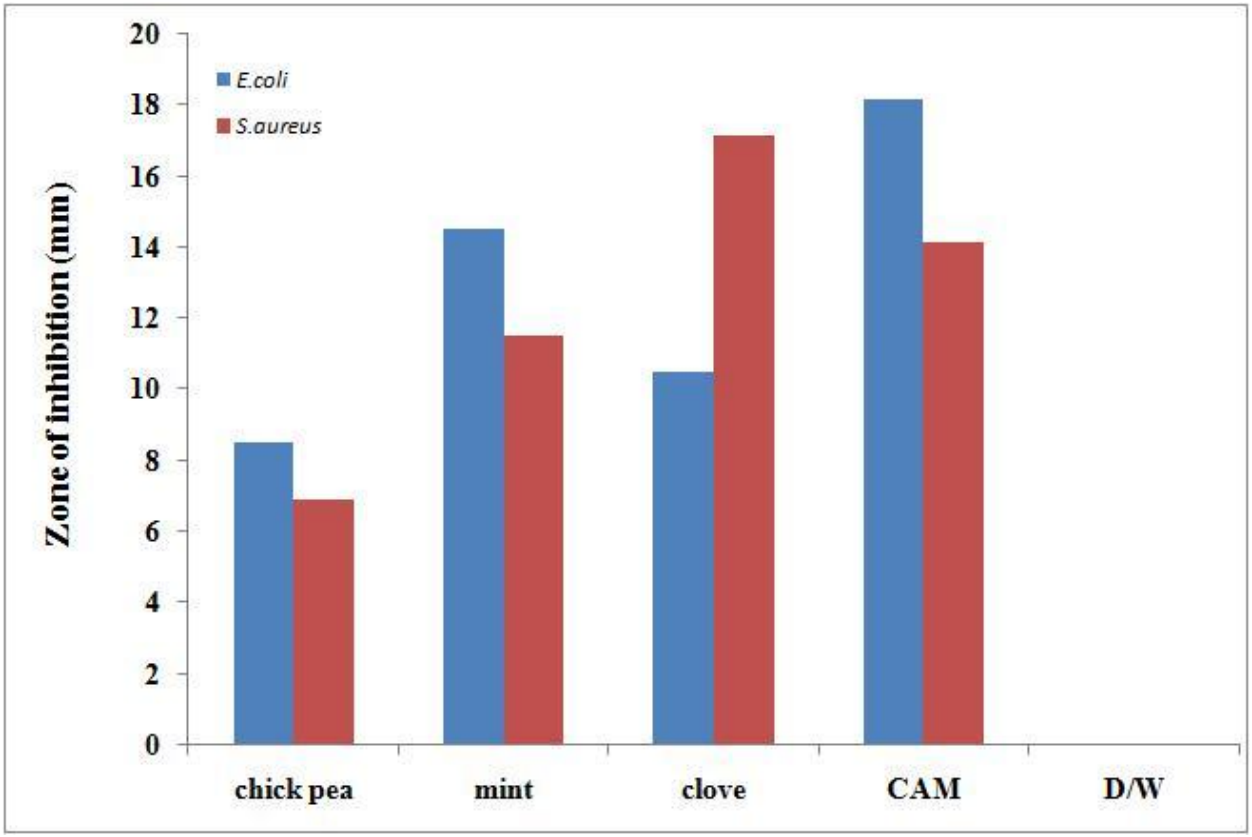


Figure 6 Graph of zone of inhibition for antibacterial activity of different plant ethanol extracts by disk diffusion method

CAM - Chloramphenicol

D/W - Distilled Wate

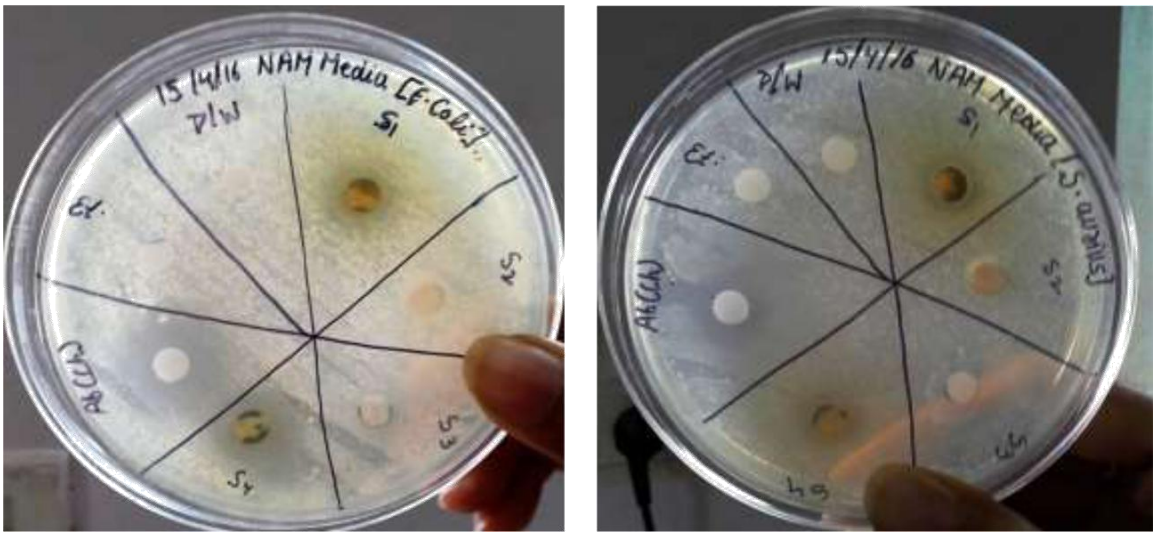


Figure 7 Antibacterial activity of Chick pea and Spearmint against *E.coli* and *S.aureus*

Where,

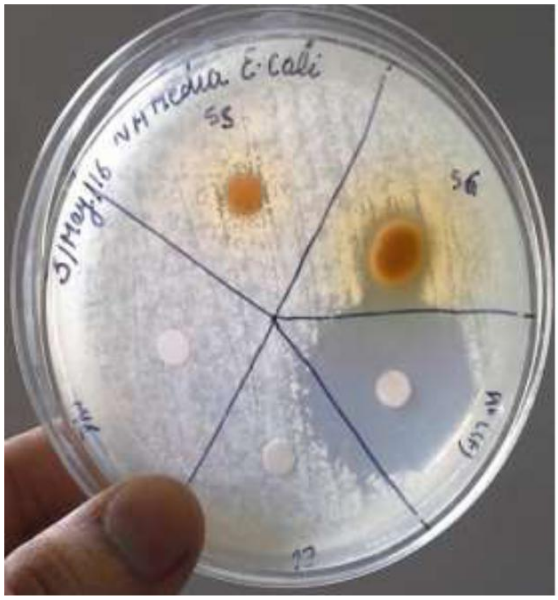
S1= Chickpea *Staphylococcus aureus*

S2= Chickpea *E.coli*

S3= Spearmint *Staphylococcus aureus*

S4= Spearmint *E.coli*

Control= CAM (Chloramphenicol), Ethanol, Distilled water



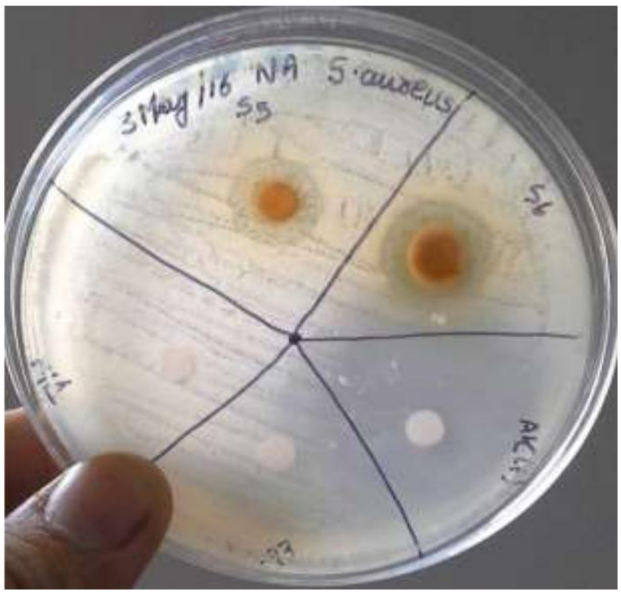


Figure 8 Antibacterial activity of Clove bud against *E.coli* and *S.aureus*

Where,

S5= Clove bud *Staphylococcus aureus*

S6= Clove bud *E. coli*

Control= CAM(Chloramphenicol), Ethanol, Distilled water

Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. The World Health Organization estimates that plant extract or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population. In the present work, the extracts obtained from Chick pea, spearmint and clove show strong activity against most of the tested bacterial strains. The results were compared with standard antibiotic drugs. In this screening work, extracts of Chick pea, spearmint and clove were found to be not inactive against any organism, such as Gram-positive and Gram-negative.

The highest antibacterial activity with aqueous extract in *E.coli* was seen in spearmint (14.5mm) and the lowest activity was seen in chickpea (10.67mm), but in aqueous extract with

S.aureus the highest activity with was clove (14.33mm) and the lowest activity was with chick pea (9.5mm), but with ethanol extract in *E.coli* the highest activity was shown by spearmint(14.5mm) and lowest activity was with chick pea (8.5mm) and with *S. aureus* with ethanol extract the highest activity with was clove (17.16mm) and lowest with was chick pea (6.9mm)

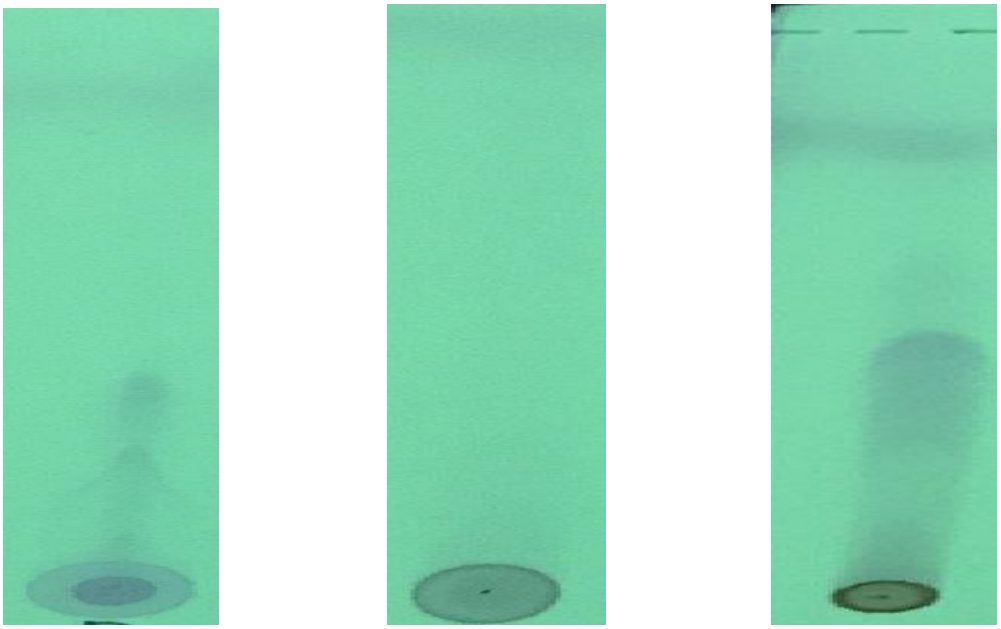
And the result was compared with standard antibiotic drug i.e. CAM (Chloramphenicol), distilled water and ethanol disk gave no zone of inhibition.

Antibacterial activity test was performed in triplicate way and then there mean and standard deviation was calculated.

4.5. Thin layer chromatography

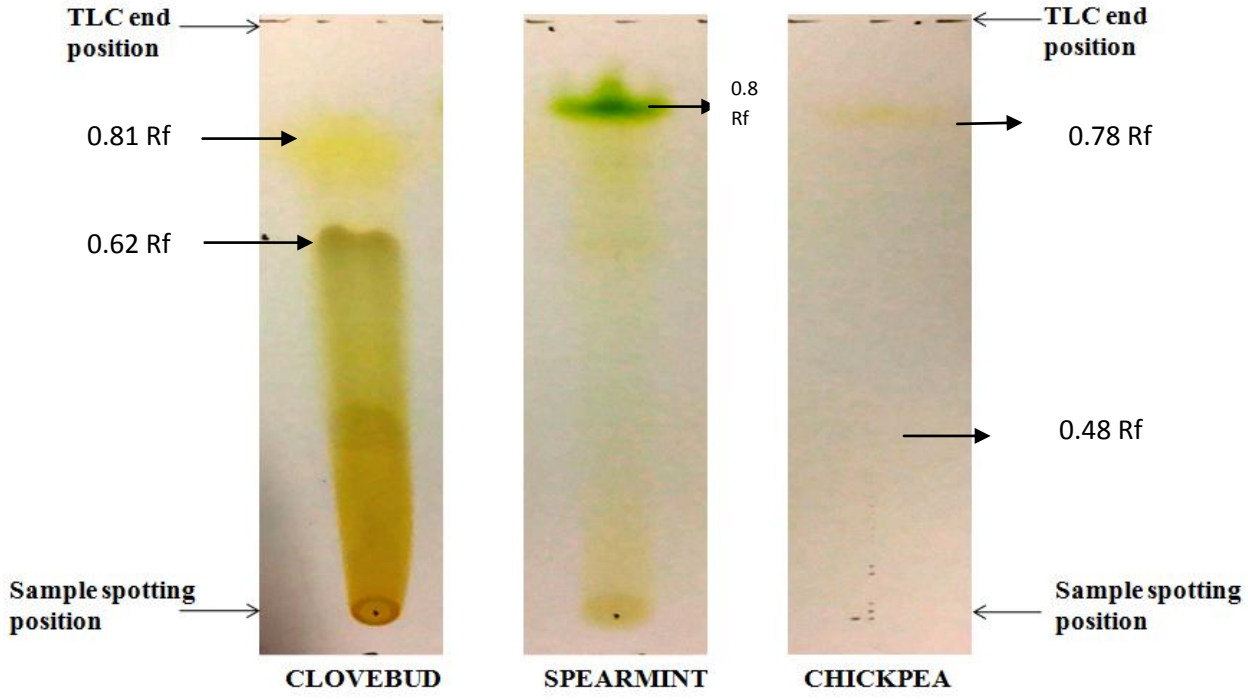
Figure 9 Plant extracts in water

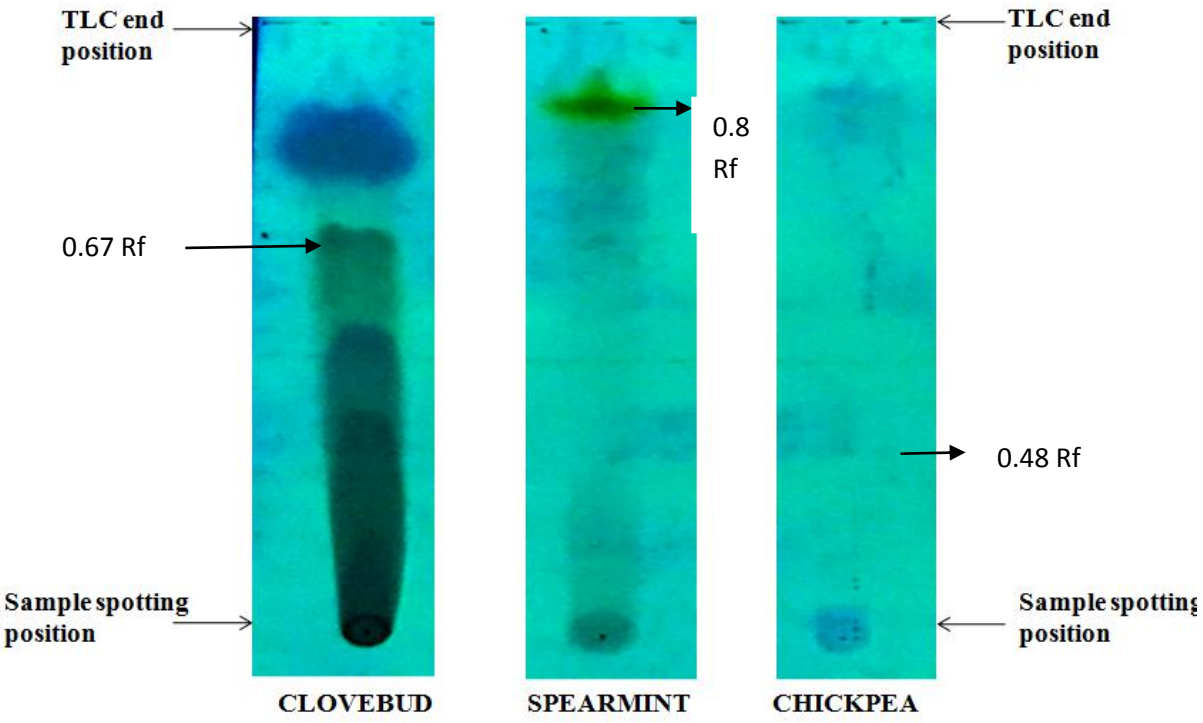
CLOVEBUD **SPEARMINT** **CHICKPEA**

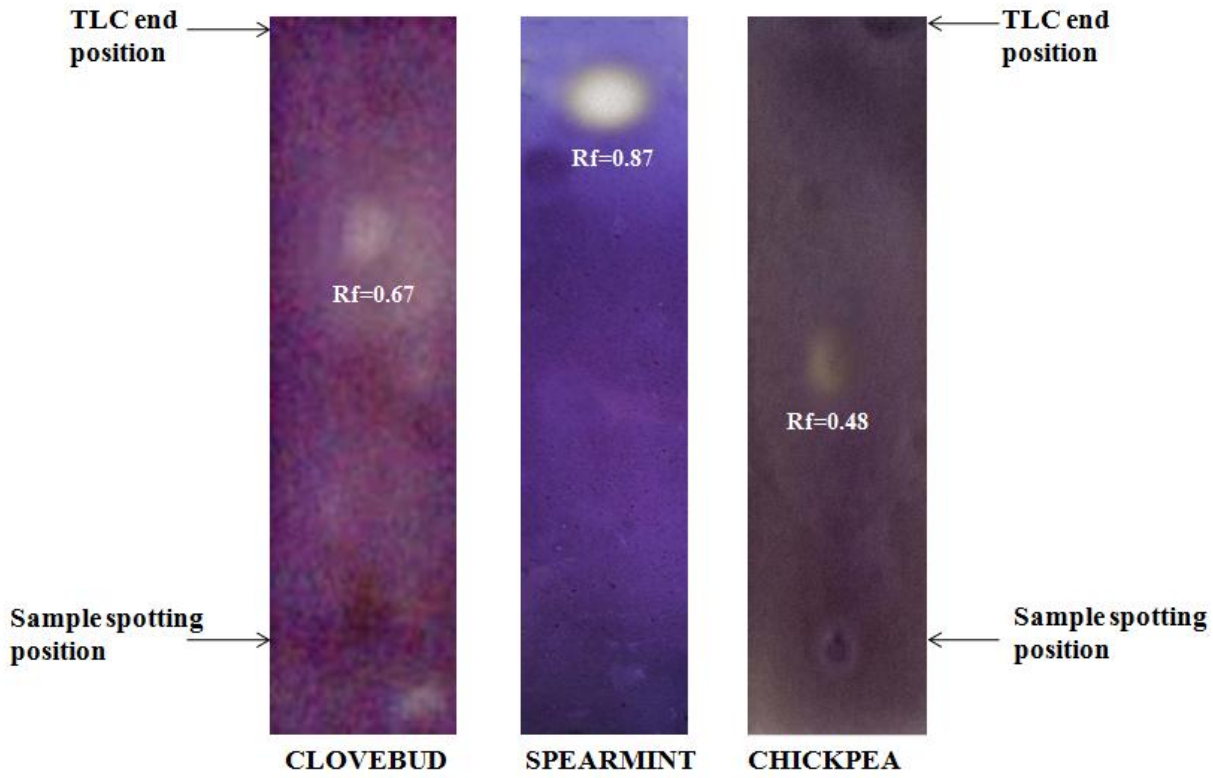


CLOVEBUD **SPEARMINT** **CHICKPEA**

Figure 10 Plant extracts in ethanol




TLC end position
0.67 Rf
Sample spotting position
CLOVEBUD
0.8 Rf
SPEARMINT
TLC end position
0.48 Rf
Sample spotting position
CHICKPEA



For TLC, the sample which was taken (aqueous extract and ethanol extract) of chick pea, mint and clove and the solvent which was used in TLC is isopropyl alcohol: chloroform9:1 (v/v) but the more and clear separation is with ethanol extract because all the contents release is extracted properly the ethanol.

In chick pea leaf with ethanol extract two bands were observed and the Rf value is (0.78 and 0.81) and in aqueous extract there is no band visualize.

In mint leaf with ethanol extract a single band was observed and the Rf value is (0.85) and in aqueous extract no band was observed.

In clove bud with ethanol extract two bands were observed and R_f value is (0.62 and 0.81) and in aqueous extract no clear spot was found.

Bioautography was done in order to confirm the presence of bacteria whose presence was indicated by disc diffusion assay. Bioautography, the spray which was used MTT assay and when this was sprayed the color changes to yellow or navy blue.

Only ethanol extracts showed the positive results for bioautography.

In bioautography there is a clear zone of inhibition on TLC-bioautography plate in chick pea and spearmint against the *E. coli* bacteria. And, with cove bud only *S.aureus* showed positive result for Bio-autography.

4.6. Results of HPLC analysis

Eugenol standard chromatogram

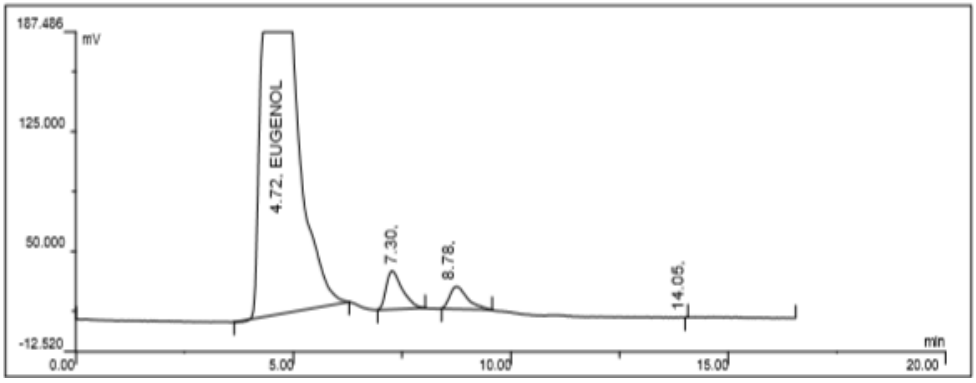


Figure 11 Chromatogram of 1mg/ml eugenol standard

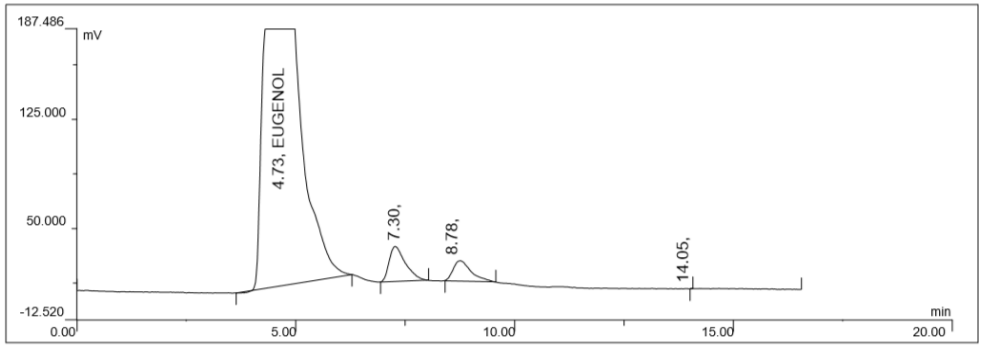


Figure 12 Clovebud TLC extract

Genistein standard chromatogram

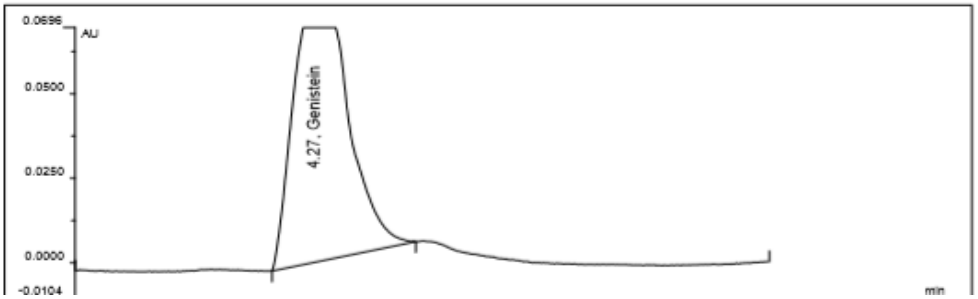


Figure 13 Chromatogram of 1 mg/ml Genistein standard

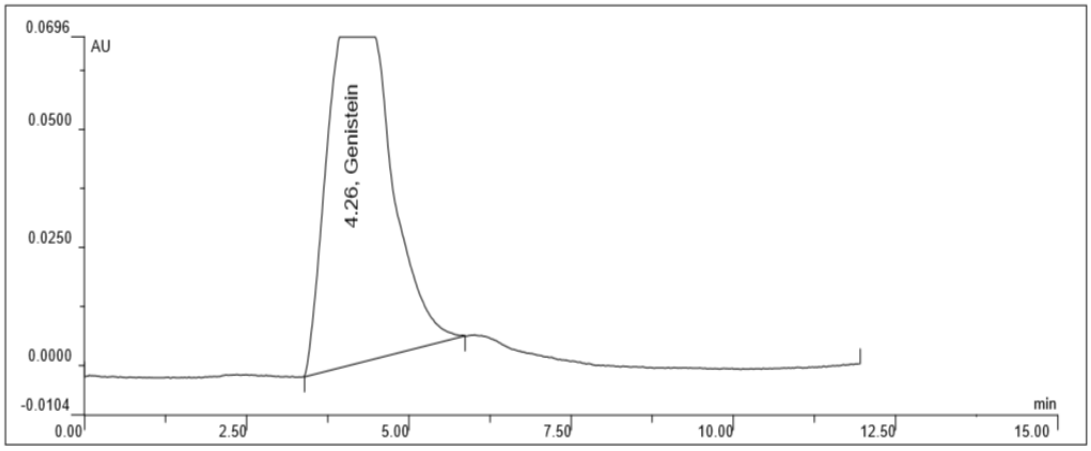


Figure 14 Genistein TLC extract

Quercitin standard chromatogram

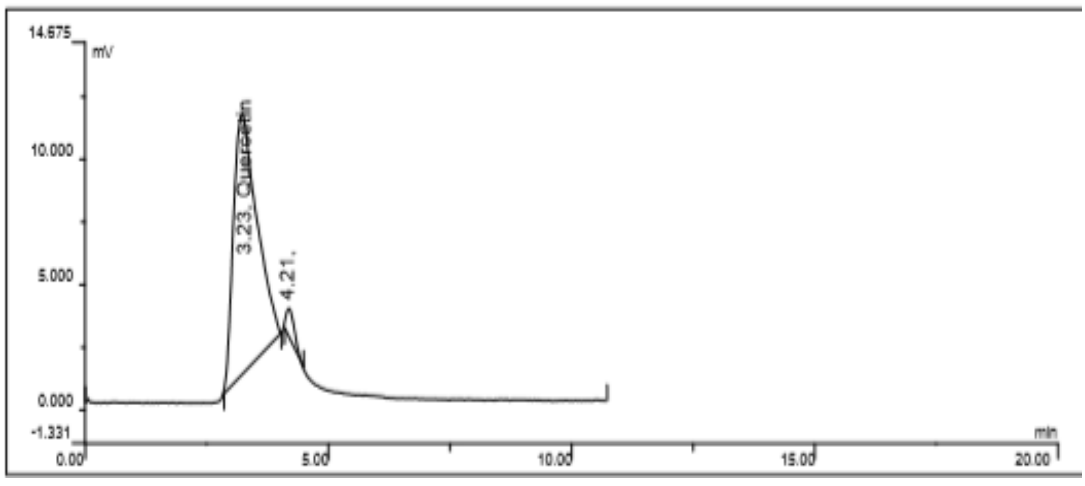


Figure 15 Chromatogram of 1 mg/ml Quercitin standard

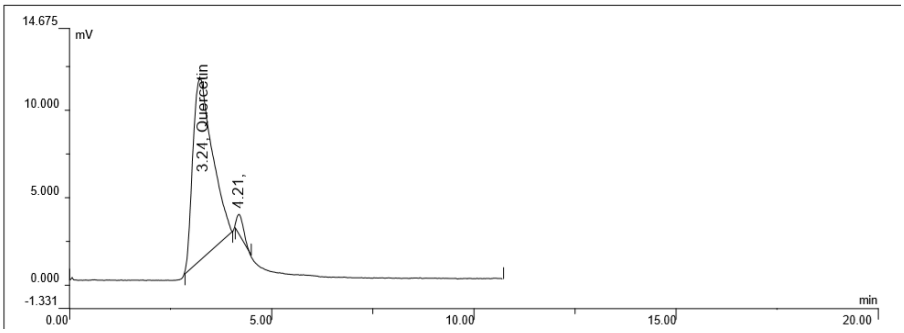


Figure 16 Quercitin TLC extract

On analyzing the extracts for phytochemicals, it was found that eugenol was found in *Syzygium aromaticum* and had a run time of (4.73) and area of (94.09%).

Genistein was found in *Cicer arietinum* and had a run time of (4.26) and area of (100%) and Quercitin was found in *Mentha viridis* and had a runtime of (3.24) and area of (95.73%).

Concentration of sample present in each compound-

Concentration of sample= Concentration of the standard /peak area of the standard *Peak area of the sample

Amount of Eugenol present in the sample= 4.71mg/ml

Amount Genistein present in the sample= 4.01mg/ml

Amount of Quercetin present in the sample= 3.21mg/ml

CONCLUSIONS AND FUTURE PROSPECTS

Plants are a source of large number of drugs which is having antibiotic properties in the traditional system and are also used extensively by the tribal people worldwide. *Cicer arietinum, Mentha viridis* and clove bud were chosen for the experiment as they have been known to have various medicinal values. *Mentha viridis* and *Syzygium aromaticum* oil is one of the most popular widely used essential oils in food products, cosmetics, pharmaceuticals etc. Aqueous and ethanol extracts were prepared for qualitative phytochemical evaluation of *Cicer arietinum, Mentha viridis* (leaf and Stem) and clove bud were carried out.

Phytochemical analysis indicated the presence of various secondary metabolites viz. flavonoids, tannins, proteins, fixed oils, Coumarins, etc. These compounds are present in all three plants but in *Cicer arietinum* (leaf) shows high presence of phenol in *Mentha* phenol and phlobatannins were absent and in clove bud proteins steroid, saponins, and phlobatannins were absent. Different Phytochemicals have different biological activity viz. antimicrobials, anticancerous and antidiabetic.

Biochemical tests are the tests used for the identification of bacterial species based on the differences in the biochemical activities of different bacteria such as physiology, carbohydrate metabolism, protein metabolism, fat metabolism, production of certain enzymes, ability to utilize a particular compound etc. help them to be identified by the biochemical tests. By doing the biochemical testing identified bacteria 1(*S. aureus*) and bacteria 2 (*E. coli*). It is widely used by biologists and chemists to test for the presence of gram-positive bacteria and gram-negative bacteria.

The extracts of medicinal plant were prepared in ethanol and aqueous extract. The antibacterial activities of extract were evaluated using disk diffusion method; the inhibitory zones were recorded in millimetres. Antibacterial activity of chick pea is 10.5mm in this the aqueous extract gave the clearer zone of inhibition with ethanol there is a very slight inhibition. In mint with ethanol extract got 14.5 mm inhibition and with aqueous extract it was 11.5 mm. Antimicrobial activity of clove is very high and zone of inhibition which was formed is of 17mm. Extract of clove were reported to show significant inhibition of bacterial growth. It was found to be more effective in *E. coli* and *S.aureus.*

Thin layer chromatography studies on extracts revealed the presence of a number of compounds with different retention factor (R_f). The values are calculated and it should be always less than one.

In TLC bioautography were also done to identify the active fractions responsible for the antimicrobial activities. The leaf extracts of chick pea mint and clove followed by bioautography using *E. coli and S.aureus* demonstrated that under polar running solvent system (isopropyl alcohol: chloroform) the bactericidal activity resolves into several areas on the TLC plate. Where killing of the *E. coli and S. aureus* is clearly visible in the form of colorless spots; these spots are areas on TLC plates that failed to take MTT stain due to absence of live bacteria in there. When the Bioautography was performed and zones of Inhibition was seen. Results showed the presence of number of bactericidal components. It suggests being a source of antibacterial compounds for human health care.

Further when TLC was performed, there were several spots obtained which indicated the presence of active compounds in the extract. Only ethanol extracts showed positive results for the active compounds. So, they were confirmed by HPLC analysis to show that Eugenol was found in *Syzygium aromaticum*, Genistein was found in *Cicer arietinum* and Quercetin was found in *Mentha viridis.*

Clove has been shown to inhibit the growth of *Aspergillus flavus* and *Penicillum citrinum* and production of mycotoxins in rice. Further studies can be carried at large scale for controlling mycotoxins in food and feed during various stages of processing an also used as preservatives during storage.

The concentration of flavanoids and phenols is less in Chickpea and they have important applications for instance they are stimulants, appetizers and have anthelmintic properties which elevate thirst and burn sensation. So, the concentration of these compounds can be increased by adding various phytohormones or by making genetic transformations.

Spearmint is used for its aromatic oil known as oil of spearmint. It is the most abundant compound in spearmint oil known as R-(-) carvone, which gives spearmint its distinctive smell. Other compounds present in spearmint oil are limonene, dihydrocarvone and 1,8-cineol. Used s flavoring agent in toothpaste and confectionary and also in shampoos and soaps. Also used as fumigant, an effective insecticide against adult moths.

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