INVESTIGATION ON ANTIBACTERIAL ACTIVITY OF CERTAIN PLANT PRODUCTS AGAINST FEW PATHOGENIC BACTERIA

A
dissertation thesis submitted to Nirma University in partial fulfilment for the degree
of

MASTER OF SCIENCE
IN
BIOCHEMISTRY

ANUSHA SANGHVI
(08MBC012)

INSTITUTE OF SCIENCE
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NIRMA UNIVERSITY

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Certificate

This is to certify that the thesis entitled "**Investigation on Antibacterial Activity of Certain Plant Products Against Few Pathogenic Bacteria**" submitted to the Institute of Science, in partial fulfilment of the requirement for the award of the Degree of M. Sc. in Biochemistry is a faithful record of bonafide research work carried out by **Ms. Anusha Sanghvi** under my direct guidance and supervision.

No part of this thesis has been submitted for any other degree or diploma. The candidate possesses minimum 80% attendance in the current academic session.

I further certify that any help or information received during this work has been duly acknowledged.

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ANUSHA SANGHVI

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M.Sc. Biochemistry

Under the Guidance

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Anusha Sanghvi

Date :

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Abbreviations

AcN	Acetonitrile
n-BuOH	n-butanol
EtOH	Ethanol
GAE	Galic Acid Equvivalent
HPLC	High Performance Liquid Chromatography
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MH Agar	Mueller-Hinton agar
NA	Nutrient Agar
NCCLS	National Committee For Clinical Laboratory Standards
OD	Optical Density
QE	Quercetin Equvivalent
Rf	Retardation Factor
TLC	Thin-Layer Chromatography
ZOI	Zone of Inhibition

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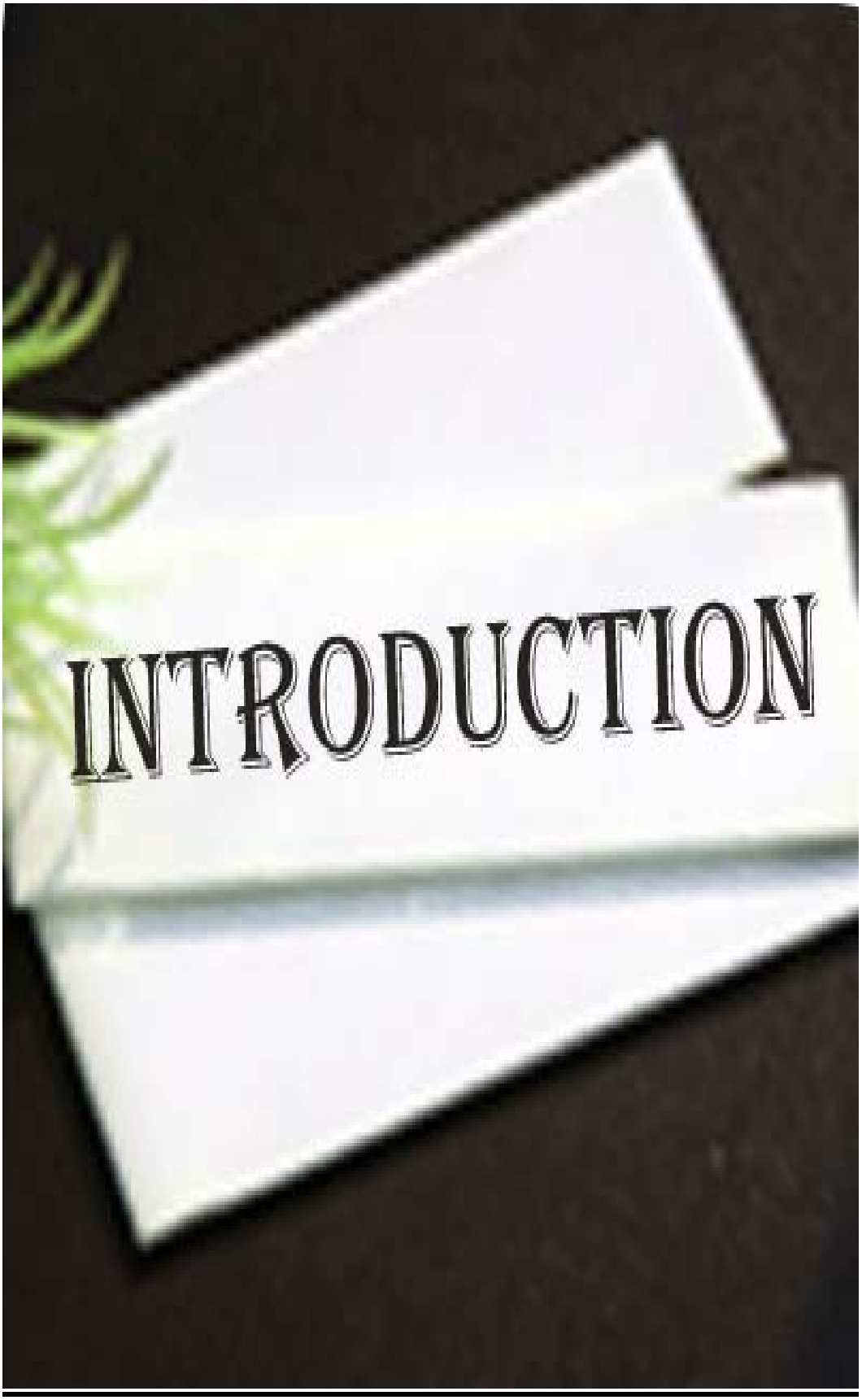
1. ABSTRACT

Extracts of *Syzygium cumini* Linn. Skeels, (Myrtaceae) and *Tamarindus indica,* (Leguminosae) seeds were screened for their potential antibacterial activity. Extracts prepared by microwave assisted extraction (MAE) method were subjected to disc diffusion assay (DDA) against the test organisms followed by broth dilution assay for determination of minimum inhibitory concentration (MIC). Phytochemical analysis of methanol extracts of *S. cumini* and *T. indica* and ethanol extract of *S. cumini* revealed presence of phenols, alkaloids, and flavonoids in them. Quantification of phenols and flavonoids in active extracts was also achieved. Further separation was achieved by TLC and . Fractions separated through TLC were also subjected to broth dilution assay against susceptible organisms.


INTRODUCTION

2. INTRODUCTION

The history of herbal medicine dates back to ancient times when the early man became conscious about his health. Natural products since long have been the primary source of commercial medicines and drug leads. One of the earliest records of the use of natural product is that of Chaulmoogra oil from *Hydnocarpus gaertn*, which was known to be effective in the treatment of leprosy. Such a use was reported in the pharmacopoeia of the Emperor of China between 3000 and 2730 B.C. [Wink, 1998]. Plants have been exploited for their natural products as templates for their new antimicrobial substances [Gibbon, 2008]. Plants produce diverge range of bioactive molecules which makes them a rich source of different types of medicines [Nair, Kalaraiya, and Chanda, 2005]. Quinine from *Cinchona b*ark, morphine and codeine from the latex of the opium poppy, digoxin from *Digitalis l*eaves, atropine (derived from (-)-hyoscyamine) and hyoscine from species of the *Solanaceae c*ontinue to be in clinical use. In the post-war (world war II) years there were relatively few discoveries of new drugs from higher plants with the notable exception of reserpine from the *Rauwolfia s*pecies heralding the age of tranquillisers and also vinblastine from *Catharanthus roseus w*hich was effective in cancer chemotherapy [Kong, *et al.,* 2003]. A new trend in new drug development is the emergence of successful clinical agents emerged from the multidisciplinary research of pharmacology and synthesis, eg., atenolol (betablocker) and captopril (ACE-inhibitor) for the treatment of hypersion, salbutamol (adrenoceptor stimulant) for asthma and the benzodiazepines (hypnotics and anxiolytics) for insomnia and anxiety attacks [Kong, *et al.*, 2003]. Some plant-derived anticancer drugs have received Food and Drug Administration (FDA) approval for commercial production, after a series of long term and painstaking works. They are [Kong, *et al.*, 2003]:

1. Taxol / Paclitaxel : A chemical discovered in the Pacific Yew tree (*Taxus brevifolia*) is now the first drug of choice in several tumour cancers including breast cancer.
2. Vinblastine : A chemical discovered in the Madagascar periwinkle in the 1950s. Vinblastine is the first drug of choice in the treatment of many forms of leukemia and since the 1950's it has increased the survival rate of childhood leukemias by 80 %.
3. Vincristine : Another antileukemic drug discovered in the Madagascar periwinkle. Effective against Hodgkin's disease, resulting in an 80% remission in sufferers of this form of lymph cancer.
4. Topotecan : An analog (synthesized chemical) of a plant alkaloid discovered in the Chinese tree species, *Camptotheca acuminata*, for the treatment of ovarian and small cell lung cancers.

5. Irinotecan : Another chemical analog which has been developed from another plant alkaloid discovered in the same tree, *Camptotheca acuminata*, for the treatment of metastatic colorectal cancer.

Well known examples of plant derived medicines include quinine, morphine, codeine, colchicines, atropine, reserpine and digoxin. Some of the prominent commercial plant-derived medicinal compounds include : colchium, colchicine, bettulinic acid, camptothecin, topotecan (hycamlin), CPTII (irinotecan, Camptosar), 9-aminocaptothecin, delta-9-tetrahydrocanabinol (dronabinol, Marino), beta lapachone, lapachol, podophyllotoxin, etoposide, podophyllinic acid, omblastine (Vellam), viscristine, (leurocristine, Oncovin), vindesine (Velbam), Vincoristine (leurocristine, Oncovin), vindesine (eldisine, Filfesin), vinorelbine (Navelbine), docetaxel (Taxotere), paclitaxel (Taxol), tubocurarine, pilocarpine, and scopolamine [Shrikumar and Ravi, 2007; Dicosmo and Misawa, 1996].

Some of the examples of the antimicrobial compound isolated from plants are [Cowan, 1999]:

- Catechinisolated from *Camellia sinensis* (green tea) was found active against *Shigella, Vibrio, S. mutans.*
- Piperine from *Piper nigrum* was reported to have activity against fungi, *Lactobacillus*, *Micrococcus*, *Escherichia coli*, and *Enterococcus faecalis.*
- Salicylic acids from *Anacardium pulsatilla* (cashew) was reported active against *Propionibacterium acnes* bacteria, fungi.
- Rhein isolated from *Cassia angustifolia* was found active against *S. aureus.*

As per the present scenario, there is an urgent need for the development of drugs due to the increasing occurrence of drug resistance in bacteria. The major cause for failure of treatment of infections worldwide is the emergence of the bacterial strains that exhibit resistance to variety of antibiotics. For example, the treatment of methicillin resistant *Staphylococcus aureus* generally requires vancomycin as a last resort, even enterococcal strains that no longer respond to vancomycin have also been identified [Nowak, *et al.,* 1999]. It is important to investigate plants as alternative sources of antimicrobial compounds for the reason that they may inhibit bacteria by a different mechanism than the presently used antibiotics which may have clinical value in the treatment of resistant microbial strains.

The utilization of whole plant or other crude preparations for therapeutic or experimental reasons can have several advantages and drawbacks [Colegate and Molyneux, 2007] as indicated below:

Drawbacks:

1. Variation in the amount of the active constituent with geographic areas, from one season to another, with different plant parts and morphology, and with climatic and ecological conditions.
2. Co-occurrence of undesirable compounds causing synergistic, antagonistic, or other undesirable, and possibly unpredictable, modulations of the bioactivity.
3. Changes or losses of bioactivity due to variability in collection, storage, and preparation of the raw material.

Advantages:

1. Pure bioactive compound can be administered in reproducible, accurate doses with obvious benefits from an experimental or therapeutic aspect.
2. It can lead to the development of analytical assays for particular compounds or for classes of compounds. This is necessary, for example, in the screening of plants for potential toxicity and for quality control of therapeutic formulations or food for human or animal consumption.
3. It permits the structural determination of bioactive compounds, which may enable the production of synthetic material, incorporation of structural modifications, and rationalization of mechanisms of action. This in turn will lead to reduced dependency on plants, for example, as sources of bioactive compounds and will enable investigations of structure / activity relationships, facilitating the development of new compounds with similar or more desirable bioactivities.

Aim and Objectives of the research

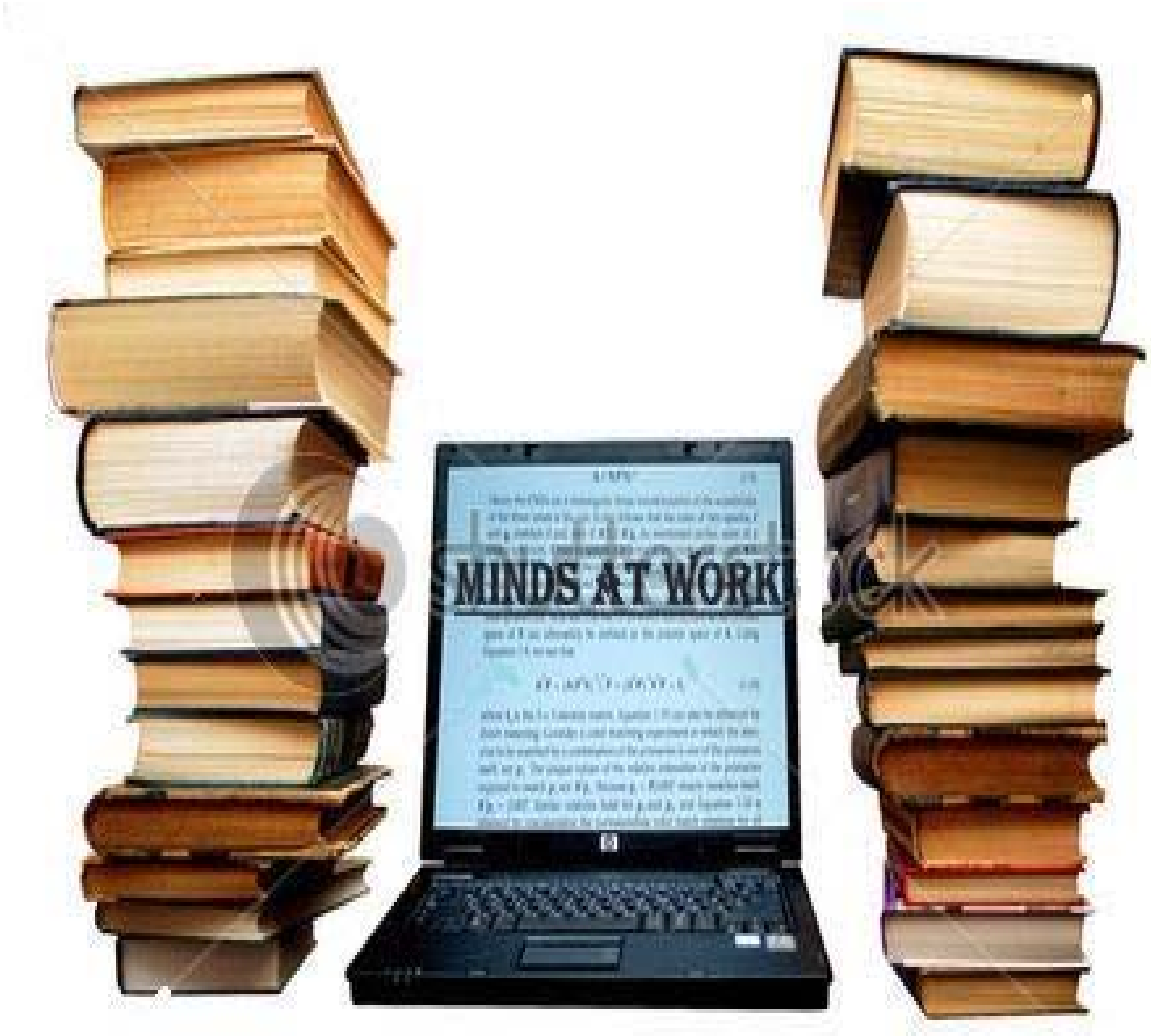
Aim of the present study was to investigate the antibacterial potential of extracts from seeds of two plants *Syzygium cumini* and *Tamarindus indica* against various pathogenic bacteria.
The objectives set were:

- Qualitative screening of crude plant extracts for their antibacterial potential by disc diffusion assay (DDA).
- Determination of minimum inhibitory concentration (MIC) of potent extracts against susceptible organism(s).
- Preliminary characterization of active crude extract(s).
- Fractionation of active extract
- Screening and characterization of the fractions

Results of this study will accord the growing database of knowledge on ethnomedicines and will help to recommend the safe and effective use of traditional herbal remedies. The

screening of these drugs will also augment ever increasing scientific database of medicinal plants, globally.

Review of Literature



3.REVIEW OF LITERATURE

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last two decades, with more intensive studies for natural therapies. The appearance of drug resistant strains as a result of inappropriate and indiscriminate use of antibiotics urges the need for finding better antimicrobial agents. Natural drugs could represent an interesting approach to limit the emergence and spread of these organisms which are difficult to treat. According to World Health Organization [Santos, Oliveira, and Tomassini, 1995] medicinal plants would be the best source to obtain a variety of drugs. In Japan, there are about 600 bacteriologically confirmed cases of shigellosis each year caused by *Shigella sonnei,* which was found to be drug resistant [Hirose, *et al.,* 2005]. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. 25% of all prescriptive medicine owe their origin either directly or indirectly to natural products and in the field of antibiotics and anticancer drugs this figure is near to 60 % [Gibbons, 2008].

Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example, the phenolic compounds which are part of the essential oils [Gislene and Nascimentol, 2000]. Antimicrobial traits of various plants are shown as follows [Evans, 2008]:

- Order conifer: Various essential oils from junipers and pinus species have antibacterial activity.
- Monocotyledons: fresh garlic owes its antibiotic action to alliine, a sulphur-containing amino acid; ginger has antibacterial properties, so too aloe vera gel.
- Dicotyledons: Mouse-earhawkweed has been used clinically for the treatment of Malta fever. Mastic gum is effective in the treatment of gastric ulcers and has been shown to be active in low doses against *Heliobacter pylori,* an organism associated with this condition. Cinnamon extracts have been shown to inhibit the growth and urease activity of the same organism.
- Antiviral agents: It is considered that there are some ten stages in the replication of the HIV virus which could be targeted in search for effective drugs. One such stage of critical importance is the reverse transcription step mediated by enzyme RT and many compounds have now been shown to have HIV_RT inhibitory properties.

One significant discovery in this field to date relates to a series of coumarins - the calanolides. In 1991 calanolidesA and calanolideB were isolated in small yield from the leaves and twigs of the tropical rainforest tree *Calophyllum lanigerum* (Guttiferae) and shown

to possess anti-HIV activity. Sumbul root (*Ferula sumbul,* Umbelliferae), a drug formerly for its stimulant and antispasmodic properties, contains at least 27 coumarins and posssess anti-HIV activity. Also salaspermic acid, a pentasyclic triterpene from the roots of *Triptrygium wilfordii* (Celastraceae) shows inhibition of HIV reverse transcriptase and HIV replication in HG (Harderian Gland) lymphocyte cells [Evans, 2008].

Medicinal plants typically contain mixtures of such chemical compounds that can act individually, additively or in synergy to improve health. A single plant may, for example, contain bitter substances that stimulate digestion, anti inflammatory compounds that reduce swelling and pain, phenolic compounds that act as antioxidants and venotonics, antibacterial and antifungal tannins that act as natural antibiotics, diuretic substances that enhance the elimination of waste product and toxins; alkaloids that enhance mood and give a sense of well-being.

3.1 Secondary Metabolites

Secondary metabolites are the plant products which do not participate directly in growth and development but perform other functions like protection against microbial infection, as attractants for pollinators etc [Buchanan, Gruissen, and Jones, 2004]. It is likely that the plants produce them *de novo* as part of their phytoalexic response on microbial invasion [Gibbons, 2008].

Naturally occurring plant compounds may be divided into three broad categories [Colegate, 2007]. Firstly, there are those compounds which occur in all cells and play a central role in the metabolism and reproduction of those cells. These compounds include the nucleic acids and the common amino acids and sugars. They are known as primary metabolites. Secondly, there are the high-molecular-weight polymeric materials such as cellulose, the lignins and the proteins which form the cellular structures. Finally, there are those compounds that are characteristic of a limited range of species. These are the secondary metabolites. Most primary metabolites exert their biological effect within the cell or organism that is responsible for their production. Secondary metabolites, on the other hand, have often attracted interest because of their biological effect on other organisms [Rishton, 2008]. The biologically active constituents of medicinal and poisonous plants have been studied throughout the development of organic chemistry. Many of these compounds are secondary metabolites. It has been estimated that over 40% of medicines have their origins in these natural products [Hanson, 2003]. A number of screening programmes for bioactive

compounds exist and have led to new drugs, for example taxol, which is used for the treatment of various cancers. [Sharma and Arora, 2006].
Secondary metabolites are categorized as follows:

1) Isoprenoids / Terpenes:

They are large and varied class of hydrocarbons, produced by wide variety of plants. They occur in various forms of mono (iridoid compounds, iridians), di (gibberellins), tri (Steroids) etc. Carotenoids are tetraterpenes. Polymeric isoprene derivatives are a large family of substances of little functional and structural common ground: steroids, carotenoids, gibberellic acids are just some of its members [http://www.biologie.uni-hamburg.de/b-online/e20/20b.htm]. Terpenes show antimicrobial and cytotoxic activity against wide range of organisms including bacteria, fungi, vertebrates and insects.

2) Alkaloids:

They are nitrogen containing heterocyclic compounds common to about 15 to 20% of all vascular plants. They are synthesized by plants from amino acids formed as metabolic byproducts. Their characteristic bitter taste and accompanying toxicity generally help to repel insects and herbivores. Only a few (like caffeine) are derived from purines or pyrimidines, while the large majority is produced from amino acids [http://www.biologie.uni-hamburg.de/b-online/e20/20.htm]

3) Phenolics :

They are aromatic metabolites possessing one or more acidic hydroxyl groups attached to phenol ring. Phenolic compounds have their role in plant growth, development, reproduction and defence. The different classes of phenolic compounds which possess antimicrobial properties include:

- Simple phenols and phenolic acids
- Flavones and flavonoids
- Tannins
- Coumarins
- Xanthones

Flavones and flavonoids - Flavones are phenolic structures containing one carbonyl group. They are hydroxylated phenolic substances that occur as C6–C3 units linked to an aromatic ring conferring a high solubility in methanol and ethanol [Chen, *et. al.,* 2007]. Flavonoids are known to be synthesized by plants in response to microbial infection and are effective antimicrobial substances against a wide array of microorganisms [Dixon, Dey and Lamb,

1983]. Antimicrobial flavonoids have been reported from *Erythrina latissima* [Wanjala, *et al.,* 2002]. Compounds of *Caesalpinia pulcherrima* with antiviral activities were derived from the flavonoid of quercetin [Chiang, *et al.,* 2003]. Moreover, the flavonoids, acacetin-7-*o*-β-D-galactopyranoside from *Chrysanthemum morifolium* was found to be active against HIV [Hu, *et al.,* 1994]. Quercetin is the most abundant of the flavonoids. It is also the building block for other flavonoids [http://www.phytochemicals.info/phytochemicals/quercetin.php]. Quercetin is found to have antimicrobial activity against organisms like *E. coli, S. aureus, P. aeruginosa,* etc [Akroum, *et. al.,* 2009].

Quercetin and other flavonoids appear active against different viruses, including HIV, probably due to the inhibition of reverse transcriptase. Quercetin, at high concentrations, appeared active against different microorganisms including *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus* and *Staphylococcus epidermidis*; *Aspergillus flavus* and *Aspergillus parasiticus*. Additionally, the *in vivo* antimicrobial activity of certain natural substances could be due to their ability to interfere with virulence factors such as lipase [Gatto, *et al.,* 2009].

Tannins - Tannins are generally descriptive of a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solutions, the property known as astringency. Proanthocyanidins (condensed tannins) and hydrolyzable tannins are the two major classes of tannins. Proanthocyanidins are flavonoid polymers, the most common type of tannin found in forage legumes (Reed, 1995]. Hydrolyzable tannins are polymers of gallic and ellagic acid esters. Tannins (tannic acid) are water-soluble polyphenols that are present in many plant foods. [Scalbert, 1991]. Their antimicrobial properties seemed to be associated with the hydrolysis of an ester linkage between gallic acid and polyols hydrolyzed after the ripening of many edible fruits. Eucaglobulin is a new complex of gallotannin and monoterpene of leaves of *Eucalyptus globulus* possessing antibacterial effects [Hou, 2000]. Tannins can be present in high concentration in some plant extracts and they have been shown to inhibit HIV replication and infection of cells [Colegate and Molyneux, 2007]. Another compound which occurs as a free molecule or as a part of a tannin molecule is gallic acid. It seems to have antibacterial, antifungal and antiviral properties [http://www.phytochemicals.info/phytochemicals/gallic-acid.php ; Akiyama H., *et al.*, 2001]. The mode of antimicrobial action of tannins and their ability to inactivate microbial adhesins, enzymes and cell envelop transport proteins also have been studied [Samy and Gopalakrishnakone, 2008].

Major classes of antimicrobial compounds from plants are shown in table 1.

3.2. Microorganisms

Gram- negative bacteria are generally much harder to find “hits” against, presumably as a result of their outer membrane in cell wall which greatly decreases permeability and because they are intrinsically resistant through the expression of membrane bound efflux pumps [Gibbons, 2008].

3.2.1. *Salmonella paratyphi A*

S. paratyphi A is a gram-negative motile aerobic rod belonging to the Enterobacteriaceae family. There is an identification of somatic and flagellar antigens [Peter, *et al.*, 2000 ; Martin, *et al*., 2006]. It causes disease conditions like mild enteric fever, perforation / hemorrhage / ulceration of the intestine, less frequently psychosis, hepatitis, cholecystitis, pneumonitis and pericarditis [http://www.ebi.ac.uk/2can/genomes/bacteria/*Salmonella_paratyphi*.html]. Since 1996, an increasing trend in isolation of *S. paratyphi A* causing enteric fever has been noticed in north India. Between 2001 to 2003 an unusually high rate in isolation of *S. paratyphi A* was reported from Nagpur (46%) and Sevagram (53%). In 2004, between March and August, in Rourkela, Blood culture, phage typing and biotyping for 795 patients suspected to have enteric fever was carried out. A total of 85 Salmonella isolates were obtained from 795 patients of which 47 were *S. paratyphi A.* All 47 isolates of *S. paratyphi A* were susceptible to ciprofloxacin, cephotaxime and ceftriaxone. Chloramphenicol sensitivity was 91.48%. Only three isolates were found multi drug resistant [Bhattacharya and Dash, 2007].

3.2.2 *Staphylococcus epidermidis*

S. epidermidis is gram-positive, facultative anaerobic cocci. Taxonomically, the genus *Staphylococcus* is in the bacterial family Staphylococcaceae. *S. epidermidis* is a critical pathogen of nosocomial blood infections, causing endocarditis and urinary tract infections resulting in significant morbidity and mortality. It also causes infections associated with vascular catheters, cerebrospinal shunts, prosthetic joints, peritoneal catheters, vascular grafts and prosthetic cardiac valve. The organism produces a glycocalyx ‘slime’ that acts as a glue adhering it to plastic and cells, and also exerts resistance to phagocytosis and some antibiotics like cephalosporin, penicillin [Mitra, 2003] methicillin, quinolone and vancomycin [Sarkar, Latif, and Gray, 2006]

S. epidermidis contributes to approximately 65-90% of all staphylococci recovered from human aerobic flora. Healthy individuals can posses up to 24 strains of the species, some of which can survive on a dry surface for long periods [Nilsson, *et al*., 1988].

Table 1 : Major classes of antimicrobial compounds from plants

Class	Subclass	Example(s)	Mode of action
Nitrogen containing	Alkaloids	Berberrine, Piperine	Intercalate into cell wall and / or DNA
	Glucosinolates	Sinigrin	
Terpenoids	Monoterpenes	Capsaicin	Membrane disruption
	Sesquiterpenes		
	Diterpenes		
	Saponin		
Phenolics	Simple phenols	Catechol	Substrate deprivation
	Phenolic acids	Cinnamic acid	
	Flavonoids	Hypericin	Bind to adhesins, complex with cell wall, inactivate enzymes
	Tannins	Ellagitannin	Bind to proteins and enzymes

	Lignans	Nordihydroguaiarctic acid	
	Coumarins	Warfarin	Interact with DNA

[Adapted from Schultz, 2002 ; Cowan, 1999]

Multiple antibiotic-resistant *S. epidermidis* were reported to be involved in nosocomial septicemia [Christensen, *et al.,* 1982]. It is also the most common cause of nosocomial bacteremia in neutropenic patients with cancer [Koll and Brown, 1993] and in bone marrow transplant patients with leukemia [Wade, *et al.,* 1982].

The isolates of *S. epidermidis* from patients suffering from above disease are found to be plasmid mediated methicillin resistant [Lowy, Wexler, and Steigbigel, 1982]. It has been reported that *S. epidermidis* exhibits resistance to ciprofloxacin and vancomycin due to the production of slime over clinical devices [Kotilainen, Nikoskelainen, and Huovinen, 1990]. It is also a common agent of bacterial keratitis. The virulence factor responsible for keratitis is slime production [Nayak, 2007].

A study of neonatal infections conducted in Naples between January 1996 and December 1998 reported in total 184 infections, of which 56 were directly attributed to *S. epidermidis* (30.4%) [Villari, Sarnataro, and Iacuzio, 2000].

3.2.3 *Pseudomonas oleovorans*

P. oleovorans is a gram-negative, methylotrophic bacterium that is a source of rubredoxin (part of the hydroxylation-epoxidation system). It was first isolated in water-oil emulsions used as lubricants and cooling agents for cutting metals. Based on 16S rRNA analysis, *P.oleovorans* has been placed in the *P. aeruginosa* group [Anzai, et al., 2000].

They are straight or slightly curved rod shaped cells that occur singly or in pairs or in short chains. They do not possess pili and normally grow at 28-30°C. [Moore, *et al.,* 2006] When grown on agar, the cells are almost coccoid (0.5×0.8 μm), but the length increases to about 1.5 μm during the exponential phase in broth. Gelatine is not liquefied and starch is hydrolysed by this organism [Garrity, Bell, and Lilburn 2005].

3.2.4 *Vibrio cholerae*

V. cholerae is a gram- negative, small, slightly curved, facultative anaerobic bacterium. They are motile by the means of monotrichous or multitrichous polar flagella [Peter, *et al.,* 2000]. It causes cholera, which is one of the world's most communicable acute intestinal infection

diseases [Madigam, 2006]. *V. cholerae* produces cholera toxin, the model for enterotoxins, whose action on the mucosal epithelium is responsible for the characteristic diarrhoea of the disease cholera. In its extreme manifestation, cholera is one of the most rapidly fatal illnesses known [Todar, 2009].

It has led to an epidemic in Viet Nam between 5th March and 22nd April 2008. There were 2,490 reported cases of severe acute watery diarrhoea out including 377 cases that were positive for *V. Cholerae* [http://www.irinnews.org/report.aspx?ReportId=77970].

In April 1997, a cholera outbreak occurred among 90,000 Rwandan refugees residing in temporary camps in the Democratic Republic of Congo. During the first 22 days of the outbreak, 1521 deaths were recorded, most of which occurred outside of health-care facilities [Todar, 2009].

There has been a sharp increase in number of cholera cases reported to WHO during 2005 with a total of 1,31,943 cases including 2,272 deaths notified from 52 countries (WHO 2006) [Sharma, Patel, and Ramkteke, 2009].

The WHO estimates that during any cholera epidemic, approximately 0.2-1% of the local population will contract the disease. Death rates associated with untreated or poorly treated cholera are often 20% - 50%, can be even over 50% during severe epidemic. However, now-a-days with prompt treatment, death rate may be as low as 1-2% [Choudhury, 2009].

3.2.5 *Bacillus subtilis*

Bacillus subtilis known as the hay bacillus or grass bacillus, is a gram-positive bacterium commonly found in soil. A member of the genus *Bacillus*, *B. subtilis* is rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions.

3.2.6 *Escherichia coli*

E. coli is the head of the large bacterial family, Enterobacteriaceae, the enteric bacteria, which are facultatively anaerobic, gram-negative rods that live in the intestinal tracts of animals in health and disease.

Theodor Escherich first described *E. coli* in 1885, as *Bacterium coli commune,* which he isolated from the faeces of newborns. It was later renamed *Escherichia coli* [Todar, 2009].

Pathogenic strains of *E. coli* are responsible for three types of infections in humans: urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (gastroenteritis).

When ingested, the following strains can cause diarrhoea:

- Enterohaemorrhagic: These strains (eg., type O157:H7ee) produce several cytotoxins, neurotoxins, and enterotoxins, including Shiga toxin, and cause bloody diarrhoea, which, in 2 to 7% of cases, lead to haemolytic-uremic syndrome.
- Enterotoxigenic: These strains can cause watery diarrhoea, particularly in infants and travellers.
- Enteroinvasive: These strains can cause inflammatory diarrhoea.
- Enteropathogenic: These strains can cause watery diarrhoea, particularly in infants.
- Enteroaggregative: Some strains are emerging as potentially important causes of persistent diarrhoea in patients with AIDS and in children in tropical areas [Burke and Cunha, 2009]

Over 700 antigenic types (serotypes) of *E. coli* are recognized based on O, H, and K antigens. In northern India, 302 *E. coli* isolates from human and animal populations were checked for their antibiotic susceptibility and the results obtained showed the prevalence of multidrug resistant strains which accounted 41%. Other isolates were reported to be resistant to ampicillin (43.5%), oxytctracycline (36·4%) and trimethoprim-sulphamethoxazole (9.3%) [Mahipal, *et al.,* 1992].

Similarly, in a recent study in London, *E. coli* isolates from urine samples of different people were tested for their ability to resist various antibiotics, commonly used as empirical oral treatments for urinary tract infections. It was found that out of 11,865 isolates of *E. coli*, only 55% and 40% of isolates showed resistance to ampicillin and trimethoprim respectively. While 94% isolates were susceptible to nitrofurantoin followed by gentamicin (93.7%), and cefpodoxime (92%) [Bean, Krahe, and Wareham, 2008].
According to the U.S. Centres for Disease Control and Prevention (CDC), E. coli is one of the leading causes of food borne illness in the U.S. Yearly, an estimated 76 million Americans fall ill from some form of food-borne illness, 325,000 land in the hospital, and 5,000 die, according to statistics from the U.S. Centre for Disease Control and Prevention (CDC) [http://www.foodpoisoningblog.org/].

3.3 Extraction procedures:

The process of obtaining something from a mixture or compound by chemical or physical or mechanical means is called extraction [wordnetweb.princeton.edu/perl/webwn]. Initial screening of plant for possible antimicrobial activities typically begin by using crude alcohol or aqueous extracts. Since nearly all of the identified components from plants active against

microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction [Cowan, 1999].

One single plant can contain up to several thousand secondary metabolites, making the need for the development of high performance and rapid extraction methods an absolute necessity.

For the past 128 years, Soxhlet extraction has been the most respected among all other conventional techniques as it serves a dual purpose of extraction step for the isolation of phyto-constituents and as a well established model for the comparison of new extraction alternatives. It has a major significant shortcoming of lengthy extraction time which makes it more labour-intensive and limits the number of samples that can be processed which may not be entertained from commercial aspects [Mandal, Mohan and Hemalatha, 2007].
Various extraction procedures are shown alongwith their advantages and limitations in table 2.

3.3.1 Properties of compound to be extracted:

1. Polarity: A general principle is ‘like dissolves like’. Thus non polar solvents will extract out nonpolar substances and polar materials will be extracted out by polar solvents.
2. Effect of varying pH: The pH of the extracting solvent can be adjusted to ensure maximum extraction. It is also important to ensure that the compounds will not break down at pH values employed.

Table 2 : Various extraction procedures

Extraction procedure	Principle	Advantages	Limitations
Soxhlet extraction [Yrjonen, 2004]	Based on the continous extraction of the solid by repeated boiling-condensation cycle of a solvent in such a way that the extraction fluid is continuously refreshed.	Less expensive, automated and continuous method, with effective extraction yield.	Not suitable for thermally labile compounds as they get hydrolysed, decomposed during the extraction. time consuming in which large amount of solvent and sample is required
Percolation [Wijesekera, 1991]	Continuous process in which saturated solvent is constantly displaced by fresh solvent, and extracts are then collected and pooled.	Usually depletes the plant material completely of the desired components.	Expensive, time consuming procedure with large solvent requirement.
Maceration [Wijesekera, 1991]	Solvent is poured onto the grounded plant material and after certain time interval the extract	Simple method with easy construct.	Low extraction efficiency.

	is strained and washed with fresh solvent to a prescribed weight.		
Microwave assisted extraction [Yrjonen, 2004]	Consist of heating the extract in contact with the sample with microwave energy	Short operation time, reduced solvent consumption, moderate recovery, less sample manipulation.	–
Supercrtical fluid extraction [Christen and Veuthey, 2001]	For extraction of volatile components	Low viscosity, high solute diffusivity, improved mass transfer and reduction of extraction time.	Can lead to degradation of active component(s) as result of lengthy exposure of high temperature
Steam distillation*	Based on heating plant material, either by placing it in water which is brought to the boil or by passing through it.	Useful procedure for extraction of temperature sensitive and aromatic compounds.	–

[*http://en.wikipedia.org.wiki/Steam_distillation]

3. Thermostability: the solubility of the compounds in a solvent increases with increasing temperature and higher temperatures facilitate penetration of the solvent from cellular structures of the organism to be extracted.

3.3.2 Properties of solvents to be used:

For choosing a solvent for extraction, its ability to extract components of a solute has to be considered. There are several basic procedures mentioned in the literature that can be tried and refined, if necessary, although a trial and error approach is often required [Colegate and Molyneux, 2007].

1. Volatility, flammability and boiling point: A low boiling point solvent may be favoured over a higher boiling solvent with the same or similar polarity. The more volatile solvent is important because it have an adequate containment and safe handling procedure s in order to prevent operator and environment. The hazard potential of the solvents must also be considered.
2. Toxicity: Less toxic solvents with similar properties must be used wherever possible.
3. Reactivity: Solvents itself should not react chemically with the compound to be extracted.
4. Cost: When large quantities of solvent are to be used, it may be necessary to use the most economical; solvent that fulfills the extraction and safety criteria required.

Due to the shortcomings of conventional extraction methods, microwave assisted extraction has proved to be more applicable for the extraction of plant material.

3.3.3. Microwave assisted extraction

The extraction principle of MAE is based on microwave energy. Microwave energy is a non-ionizing radiation that causes molecular motion by migration of ions and rotation of dipoles. Many compounds with bioactivity can be extracted with microwave-assisted extraction [Chen, *et.al.,* 2007; Ahuja and Diehl, 2006 ; Sharma, Yelne, and Dennis, 2007]. When the moisture is heated up inside the plant cell due to the microwave effect; it evaporates and generates tremendous pressure on the cell wall due to the swelling of the plant cell. This pressure pushes the cell wall from inside and ruptures it, facilitating leaching out of the active constituents from the ruptured cells to the surrounding solvent thus improving the yield of phytoconstituents [Heemken ; Theobald and Wenclawiak, 1997]. Higher temperature attained by microwave radiation can hydrolyse ether linkages of cellulose, and can convert it into soluble fractions within one to two min. This higher temperature attained by the cell wall, during MAE, enhances the dehydration of cellulose and reduces its mechanical strength which in turn helps solvent to have an easy access easily to compounds inside the cell [Pare and Lapointe, 1990]. There are multiple factors that one must consider which affect the efficiency of MAE [Llompart, *et al.,* 1997 ; Proestos and Komaitis, 2007] such as:

- Solvent nature: Solvent in which target analyte is soluble must be chosen. Polar molecules absorb microwave energy strongly. [Charalampos and Komaitis, 2007]
- Dielectric constant: Solvents with high dielectric constants (e.g. water) can absorb more microwave energy [Charalampos and Komaitis, 2007] effect of microwave energy is strongly dependent on the dielectric susceptibility of both the solvent and solid phase matrix. [Mandal, Mohan and Hemalatha, 2007].
- Dissipation factor: Rate at which water absorbs microwave energy is higher than the rate at which the system can dissipate the heat. This phenomenon accounts for the "superheating" effect which occurs when water is used. So, intense heating may cause degradation of the analyte. This is why it is best to choose a solvent that has high dielectric constant as well as a high dissipation factor to facilitate heat distribution through the matrix. Methanol has a high dielectric constant as well as a high dissipation factor. [Charalampos and Komaitis, 2007]
- Extraction time: Generally by increasing the extraction time, the quality of analytes extracted is increased, although there is a risk that degradation may occur. [Mandal, Mohan, and Hemalatha, 2007].

  The significance of the extraction time can be presumed to be related to the time required for the desorption process to take place [Chen, *et. al.,* 2007].
- Microwave power: A combination of low or moderate power with longer exposure may be a wise approach. Fine powder can enhance the extraction by providing larger surface area, which provides better contact between the plant matrix, and the solvent, also finer particles will allow Improved or much deeper penetration of the microwave. One of the disadvantages associated with the use of finer particles is the difficulty in separation of the matrix from the solvent after microwave irradiation [Mandal, Mohan and Hemalatha, 2007].
- Matrix characteristics: The particle size of extracted material are generally selected in range of 100µm-2mm. Smaller particle size have advantage of large surface area and better penetration power. However at the same time it may interfere with filteration of recovered extract [Wang and Weller, 2006].
- Temperature: Higher temperature does indeed result in improved extraction efficiency since desorption of analyte from active sites in matrix increases. Solvents have high capacity to solubilise analyte at high temperature while surface tension and solvent viscosity, decreasing with temperature because of improved sample immersion in solvent and matrix penetration respectively.

3.3.3.1. Advantages of MAE [Siddig, *et al.,* 2006] **:**

1. Reduced extraction time.

2. Reduced solvent usage.
3. Improved extraction yield.
4. Better accuracy and precision.
5. Provides agitation during extraction, which improves mass transfer phenomenon.

3.3.3.2. Disadvantages of MAE [Mandal, Mohan and Hemalatha, 2007].

1. Amount of sample that can be processed is limited.
2. The usual constituent material of the vessel, PTFE (*polytetraflouro ethylene),* does not allow high solution temperatures.
3. Single-step procedure excludes the addition of reagents or solvents during operation

3.3.4 Storage of prepared extract

Extracts were then stored in autoclaved flat bottom glass vials. Inner side of the vial cap was covered with aluminium foil to prevent the leaching of compounds from the plastic into the extract. Changes in the composition of a mixture may occur the action of living organisms or by chemical or enzymatic processes within the mixture itself. The former case is called deterioration and the latter is known as decomposition. Deterioration occurs most commonly in biological material due to microbiological attack. Decomposition is a problem likely to occur at any stage in the fractionation. The major processes likely to occur are oxidation, hydrolysis, isomerization and polymerization. Increase in exposure to light, oxygen, temperature, in case of hydrolysis, moisture accelerates such processes. In the crude extract some constituents may prevent the decomposition of others ex. Antioxidant compounds such as flavonoids may preserve molecules susceptible to oxidation. Decomposition may also occur due to interaction with the solvents or other materials used in fractionation procedures. The products formed are called artifacts [Houghton and Raman, 1998].

3.4 Antimicrobial susceptibility testing methods

For the effective use of the compound it is necessary to determine its antimicrobial potential by various antimicrobial susceptibility tests (AST). The assay is categorized into broth dilution assay and agar diffusion assay [Coyle, 2005].

Two purposes which antibacterial assays should fulfil are:

(a) To determine whether the compound has antimicrobial activity and

(b) To determine the concentration of the antimicrobial agent needed to inhibit the target microorganism. The activity of antimicrobial compound should be determined and tested under control conditions.

There are a number of conditions on which effectiveness of compound is dependent. This includes the nature of the target microbes, concentration of the test compound, density of the microbes, composition of the media, time in contact with the compound, temperature, pH of media and amount of aeration [Shin, *et al*., 2001]

The agar diffusion method was first utilized by Beijerinck in 1889 for studying the effect of different auxins on bacterial growth. In 1924, ditch plate technique was introduced by Fleming for evaluating antimicrobial qualities of antiseptic solutions, this technique was further modified by Reddish. He had cut wells in agar instead of making ditch and filled the wells with antiseptic solutions. The development of broth dilution technique using turbidity as an end point determination is the Fleming's second contribution to modern AST. The broth dilution method is generally used for determination of minimum inhibitory concentration (MIC). The use of absorbent paper for carrying antimicrobial solutions was introduced by Heatley in 1940. Vincent and Vincent (1944) incorporated penicillin in paper discs for assaying the newly discovered compound. Filter paper discs of 6-6.5 mm were first described by Bondi and co-workers (1947) which are commonly used today. For determining the MIC of antimicrobial agent the use of agar dilution was first described by Schmith and Reymann (in 1940s). 'Breakpoint' technique, a term first used by Ericsson and Sherris (in 1940s) is the modified version of the of the agar dilution technique described by Schmith and Reymann. In 1966, significant progress in standardization of the disc method occurred when Bauer, Kirby and co-workers published their attempt to establish the disc diffusion technique as a practical method of testing with broad application to clinical laboratories. In 1975 this method became the basis of the National Committee for Clinical Laboratory Standards (NCCLS) disc diffusion standards [Wheat, 2001 ; http://www.bsac.org.uk/_db/_documents/Chapter_1.pdf].

***In vitro* Assay for Antimicrobial Activity**

The activity of the antimicrobial compounds can be determined easily by various Antimicrobial Susceptibility Tests (AST). The assay is characterized into broth dilution assay and disc diffusion assay [www.bact.wisc.edu]. Both of these were carried out according to the NCCLS guidelines.

A good antimicrobial assay should fulfil two purposes:

- It should verify whether the compound actually has the desired antimicrobial activity sor not.
- It should indicate the concentration of antimicrobial that will be needed to inhibit the target organism.

Effectiveness of a compound is dependent upon number of conditions like nature of the target microbes, concentration of the microbes, time in contact with the compound, temperature, pH and amount of aeration.

3.4.1. Disc Diffusion Assay (DDA)

Disc diffusion assay (DDA), also known as Kirby-Bauer assay, is a standard procedure used in clinical laboratories to test the susceptibility of bacterial isolates to anti microbial compounds and antibiotics. Antibiotic impregnated discs are put on the surface of the appropriate medium such as Mueller-Hinton that has been inoculated with a pure bacterial suspension. After incubation, zones of inhibition are measured and translated into predetermined category as susceptible, intermediate, or resistant on the basis of the zones of clearance [Jorgensen and Turnidge, 2003]

The zone is influenced by many technical factors such as, rate of diffusion of test substance and bacterial growth, type of culture medium, incubation conditions, inoculums density and age of culture [http://biology.fullerton.edu/biol302/302labf99/quant.html#mcfar **;** Jorgensen and Turnidge, 2003].

The diameter of the zone of inhibition is influenced by the rate of the diffusion of the antimicrobial agent through the agar, which may vary among different drugs depending upon the size of the drug molecule and its hydrophilicity. The zone size, however, is inversely proportional to the MIC [Jorgensen and Turnidge, 2003]

3.4.2. Minimum Inhibitory Concentration (MIC)

A quantitative standard assay often use to gauge the effectiveness of an antimicrobial is the MIC. It is the lowest concentration of the compound that inhibit 80% growth of initial growth of organism.

MIC measurements are influenced by a number of factors including the composition of the medium, the size of the inoculum, the duration of the incubation, and the presence of resistant subpopulations of the organism.

Dilution susceptibility testing methods are used to determine the minimal concentration, usually in micrograms per millilitre, of antimicrobial agent required to inhibit or kill a microorganism [Jorgensen and Turnidge, 2003]. MIC for each dilution is determined in triplicate by microtiter modification of the method. Microtiter trays containing 96 wells are used, each filled with Mueller-Hinton Broth (MHB). Bacterial strains are suspended in saline after overnight growth on agar medium, diluted in MHB and added in equal volume to the wells containing MHB. The final inoculums size is 10^5 -10^6 bacteria/mL. MIC end points are

read as the lowest concentration of antibiotic with no turbidity. It can be performed in microbroth as well as macrobroth dilution methods.

Advantages of microbroth dilution method: Inoculation and reading procedures allow relatively convenient simultaneous testing of several antimicrobial agents against individual isolates [Jorgensen and Turnidge, 2003]. In some cases, agar well diffusion technique is also used as an assay for antimicrobial susceptibility testing.

The most common problem experienced in bioassays is the preparation of the low polarity fraction in an aqueous medium. Ethanol and dimethylsulphoxide (DMSO) are the two most common solvents since they are miscible with water and at concentrations below 3% (v/v) are usually not toxic. It is always desirable to include a DMSO / Ethanol control (negative control) in the experiment. Care should be taken that precipitation does not occur when the solution of the fraction in the solvent is diluted with water to the desired volume.

Observed activity in the total extract may be due to synergism between the components which are separated as a result of the fractionation process.

3.5 Fractionation of bioactive plant extracts

Chromatographic techniques play a very important role in the separation of active constituents from the crude extracts [Ross and Brain, 1997].

The most important chromatographic methods include column chromatography (CC), gas-liquid chromatography (GLC), thin-layer chromatography, and high performance liquid chromatography (HPLC). Many fractions of the compound can be obtained with the help of various chromatographic techniques which can then be tested for biological activity. For further investigation, appropriate tests must be done to identify which fractions possess the antibacterial activity [Williamson, Okpako, and Evans, 1996]

Synergy

Synergy occurs when the effect of two or more components applied together to a biological system is more than the sum of the effects when identical amounts of each constituent. The concentration of the substance in the extract may be considerably less than the expected. If this is the case, then the presence of other active compounds in the extract is likely and synergy may also be occurring. This may be due to the substances acting in different ways, so that, for example, some rate limiting step in the process is eased or overcome. Other mechanisms may exist, such as enhanced penetration to the site of action through effects on gut or membrane permeability. Synergy may explain why the activity of an extract or other

mixture is greater than might be expected from the amount of the compound, which is thought to be major 'active' constituent present.

Synergy can also be related to the considerably less activity of any one of the fraction obtained from a mixture than that of the total mixture [Houghton and Raman, 1998].

With relevance to our work, TLC proved to be the methods of choice.

3.5.1 Thin-layer chromatography

Thin-layer chromatography is a simple, quick and inexpensive procedure that can be used for the analysis of mixtures.
In addition to qualitative detection, TLC also provides semi-quantitative information on the major active constituents of a drug or drug preparation, thus enabling an assessment of drug quality. Also TLC provides a drug fingerprint and therefore is suitable for monitoring the identity and purity of drugs and for detecting adulterations and substitutions [Wagner and Bladt, 2007].

Plates can be visualized, depending on the chemical structure of the compounds under day light, UV-254 nm and 365 nm or by using spray reagents [Wagner and Bladt, 1988]. The effectiveness of the separation depends on the mixture to be separated, the choice of the mobile phase and the adsorption layer [Fritz and Schenk, 1987]. The term retention factor R_f, is commonly used to describe the chromatographic behaviour of sample solutes. The R_f value for each substance is the distance it has moved divided by the distance the solvent front has moved. Usually, the centre of each spot is the point taken for measurement. Comparison of R_f values assist in analyzing complex mixtures qualitatively.

The selection of a solvent for application of the sample can be a critical factor in achieving reproducible chromatography with distortion free zones. In general, the application solvent should be a good solvent for the sample and should be as volatile as possible and more nonpolar [Fried and Sherma, 1994]. Terpenes from *Commiphora molmol* and phenols from *Thymus taxa* are examples of antibacterial components separated through TLC [Horavth, *et al*., 2002 ; Rahman, *et al*., 2008].

3.5.1.1. Advantages of TLC [Touchstone, 1992]:

1. Simplest chromatographic method

2. TLC uses less solvent. Little equilibration is required and only a small amount of solvent is required to develop a chromatogram.

3. Short development time and easy change of mobile phase.

4. Number of samples can be handled per plate of for separation at one time.

3.5.1.2. Problems encountered:

- The observed zones may not be very discrete and tailing is often observed. Tailing may be reduced or completely removed by adding a little water to the mobile phase.
- A large number of natural products are either fluorescent or absorb UV light. In the latter situation they can be observed as dark areas when examined by UV light if they are coated on a fluorescent layer. The compound will absorb some of the UV light before it reaches the fluorescent substance present in the layer and so the fluorescence is reduced, this effect is known as quenching.

The activity of an extract may change over a period of time or after fractionation due to changes in the nature of the chemical compounds present. The more common result of this process is a decrease in activity, but in some cases activity may be enhanced [Houghton and Raman, 1998]. Phases are available which incorporate bonded fluorescent agents and so give a uniform, brightly coloured background when exposed to UV light. These are useful for the examination of TLC separation and are denoted by the code F_{254} or F_{365}, where the numbers represent the wavelength in nanometers at which the strongest fluorescence occurs. Layers focusing at 254 nm are the more frequently used ones [Coopoosamy and Magwa, 2006]

The marked band is carefully scraped off the plate and mixed with an excess volume of a suitable solvent but it should be noted that ethanol, methanol and water can dissolve appreciable amounts of silica gel which appears as a residue when the eluent is concentrated. It is best to use the least polar solvent which will completely elute the substance in question [Stahl, 2005]**.**

Conventional preparative thin layer chromatography has several disadvantages, viz. only about 50 mg of mixture can be separated on each 20×20cm, 1mm-thick plate, removal of each band and subsequent elution is time consuming and silica dust produced is health hazard .in addition, some of the desired compounds will be lost if detection involves spraying an edge of the plate [Houghton and Raman, 1998].

3.5.3 UV-Visible Spectroscopy

The absorption spectra of plant constituents can be measured in very dilute solution against a solvent blank using an automatic recording spectrophotometer. Such spectral measurements are important in the identification of many plant constituents, for monitoring the elutes of chromatographic columns during purification of plant products and for screening crude plant extracts for the presence of particular classes of compounds [Harborne, 2008]. Spectra of compounds within same subclass of compounds are often very similar. This means that

several of the compounds in a plant extract will often have almost identical UV spectra since they are either intermediates or end products of the same biosynthetic family / pathway [Larsen and Hansen, 2007].

The present investigation was undertaken to screen some of the common seeds against few bacterial pathogens. The aim of this study was to investigate and characterize the crude extract(s) of the seeds of Jamun (*Syzygium cumini* Linn. Skeels) and Imli (*Tamarindus indica*) for their antibacterial activity.

3.6 Experimental plant materials

3.6.1 *Syzygium cumini* Linn. Skeels :

Family: Myrtaceae

Myrtaceae is a plant family widely used in folk medicine in different countries and *Eugenia* and *Syzyium* are among its most important genera [Brito, *et al*., 2007]. Java plum, black plum are synonyms for Syzygium cumini, besides *Eugenia jambolana* Lam. Its vernacular name is Zamune [Khare, 2007].

It is a large or medium sized evergreen tree. Fruits are ovoid or globose, pink, turning blackpurple when fully ripe. Common throughout upper gangetic plain, Bihar, Orissa, especially in moist localities, planted in West Bengal, Deccan and Konkan region, and all forest districts of south India [Bryan, 2000].

The *S. cumini* has medicinal importance as an anti-inflammatory, antiulcergenic and have been reported to show antibacterial activity against gram-positive and gram-negative bacteria [Sharma, Patel and Ramteke, 2009].

Few of the reported applications / activities, and constituents of *S. cumini* are described below:

1. The fruit is astringent, stomachic, carminative, antiscorbutic and diuretic. The juice of a ripe fruit or decoction of the fruit may be administered for the treatment of enlarged spleen, chronic diarrhoea and urine retention [Bryan, 2000].
2. Bark is used in non specific acute diarrhoea and in topical medicine or topical therapy for mild inflammation of the oral-pharyngeal mucosa; externally in mild, superficial inflammation of the skin. The ayurvedic Pharmacopoeia of India recommends the bark in acute diarrhoea and haemorrhagic diseases; the seed in hyperglycaemia and polyuria [Bryan, 2000 ; Khare, 2007]. Chemical composition of bark extracts consists of tannins, resins, terpenes, steroids, saponinic glycosides and flavanols [Sharma, patel and Ramteke, 2009].

3. Leaves are antibacterial and antidysentric [Khare, 2007]. Leaf extracts were found to contain flavonoids [Brito, *et al.,* 2007]. Also heptacosane, nonacosane, octacosane, tricontane, octadecane are reported to be present in leaf extracts.
4. Seeds are used in treatment of diabetes [http://www.ntbg.org/plants/plant_details.php?plantid=10971 ; Gupta, Kaur, and Chander, 2009] diarrhoea, pharyngitis, splenopathy, urinary disorders, ringworm and to strengthen teeth and gums [Khan, *et al.,* 1992]. Seed - claimed applications mentioned in *German Commission E* monograph: in diabetes, also in combination preparations for atonic and spastic constipation, diseases of the pancreas, gastric and pancreatic complaints.

   Constituents of *S. cumini* seeds are fatty oils (30g/kg) including lauric, myristic, palmitic, stearic, oleic, linoleic, malvalic, sterculic and vernolic acid and pytosterols such as β-sitosterol. Further constituents are tannins predominantly corilagin, ellagitannins, ellagic acid, galloyl-galactoside betulinic acid and gallic acid. Additionally, phenolic components like quercetin, ferulic acid, veratrol, flavanone, guajacol and caffeic acid have also been identified [EMA, 1999 ; Karthic, 2008 ; , 1972].

   The presence of gallic acid, hexahydroxydiphenic acid, chebulic acid, and isohexahydroxy diphenic acid as building blocks of tannins in seeds of *S. cumini* has been confirmed from chromatographic studies of the tannin hydrolysates [Bhatia, 1972 ; Sharma, 2008].

   Ethanolic extracts of seeds have been shown to contain gallic acid, ellagic acid, chebulic acid, corilagin and related ellagitannins, 3,6-hexahydroxydiphenoylglucose and its two isomeric forms, galloylglucose and quercetin, by chemical and enzymatic studies on tannase [Bhatia, 1972]. Activities of *S. cumini* extracts may probably be due to the presence of flavonoids in the extract as these substances are known to exert a potent inhibitory effect on variety of enzymes related to cell activation and to the production of inflammatory mediators [Brito, *et. al*., 2007].

3.6.2 *Tamarindus indica*:

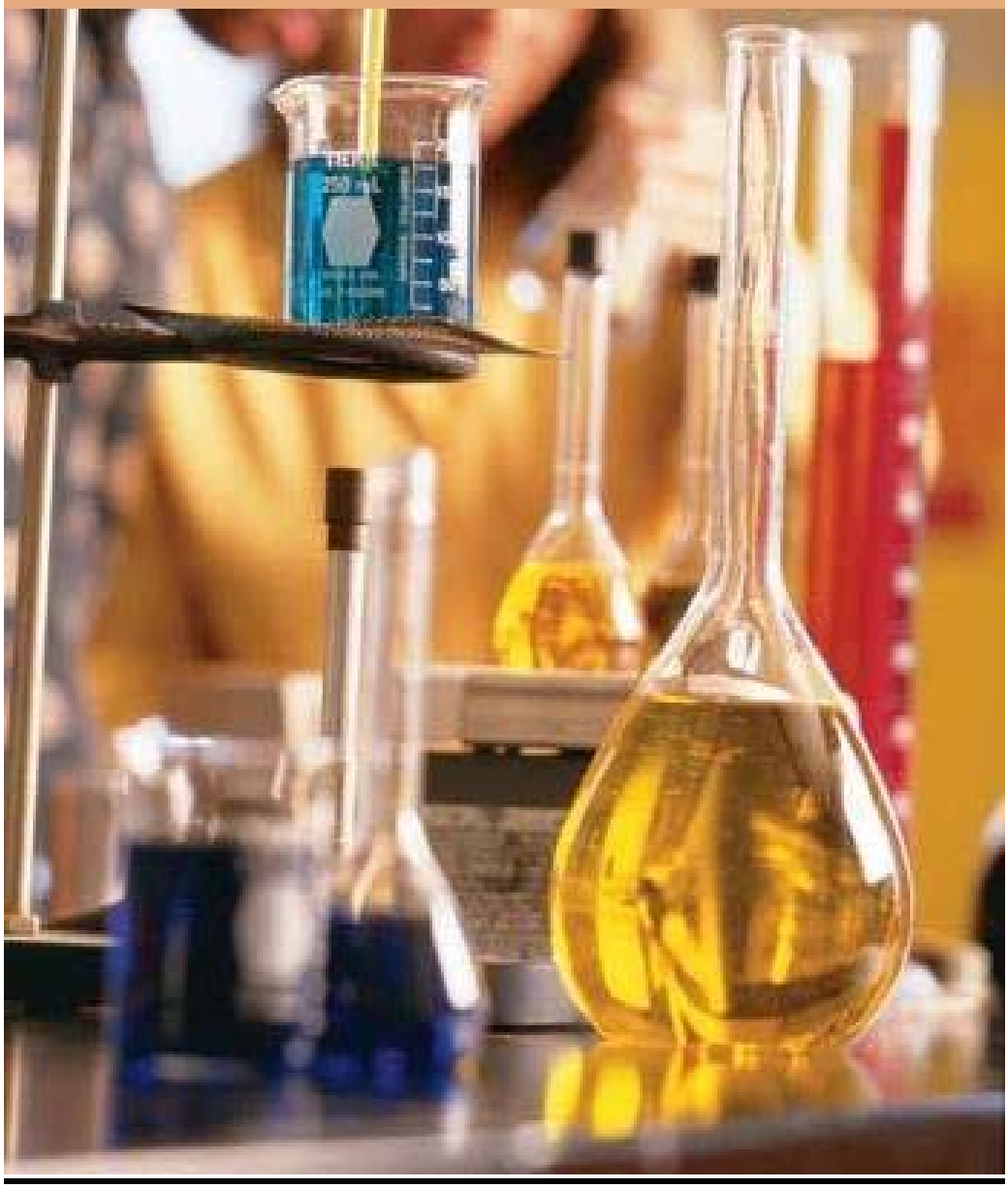
Family: Leguminosae

It is commonly known as Imli. *Tamarindus indica* is a multipurpose tropical fruit tree used primarily for its fruits which are eaten fresh or processed. The species has a wide geographical distribution in the sub-tropics and semiarid tropics and is cultivated in numerous regions.

Reported uses of *T. indica* are as follows:

1. The pulp is applied on inflammations, used in a gargle for sore throat and, mixed with salt, as a liniment for rheumatism. The pulp is said to aid the restoration of sensation in cases of paralysis [Road, Alrahwan, and Rolston, 1998].
2. Seeds are presently gaining importance as an alternative source of protein, rich in some essential amino acids.
3. Tamarind leaves and flowers, dried or boiled, are used as poultices for swollen joints, sprains and boils [Road, Alrahwan and Rolston, 1998].
4. The bark of the tree is regarded as an effective astringent, tonic and febrifuge [Road, Alrahwan and Rolston, 1998].

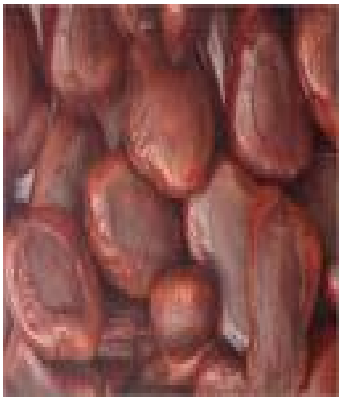
MATERIALS AND METHODS



4. MATERIALS AND METHODS

4.1. Experimental objects

The plant materials selected were seeds of *Syzygium cumini* Linn. Skeels (Myrtaceae) and *Tamarindus indica* (Leguminosae). Seeds were purchased from local market of Ujjain (M.P). They were authenticated for their unambiguous identity by Prof. Y. T. Jasrai, Dept. of Botany, Gujarat University. During collection of seeds care was taken that, it should be fresh, healthy and must be free from any contamination. These seeds were washed with tap water followed by distilled water and air dried. The dried seeds were stored in clean air tight container in dark. The seeds were checked at regular time intervals for any physical or biological damage.



(a)

(b)

Fig 1 : (a) *Tamarindus indica* and (b) *Syzygium cumini seeds.*

4.2. Test microorganisms

Six bacterial strains procured from MTCC (Microbial Type Culture Collection, IMTECH, Chandigarh) were used as test organisms (table 4), in addition to a drug resistant *Salmonella paratyphi A* brought from Gujarat University. All bacterial strains were maintained on nutrient agar slants. All these strains were subcultured every month on nutrient agar media. Paraffin slants were prepared by overlaying agar slants with it. To prepare glycerol stocks, glycerol (10% v/v) was added to the broth inoculated with organisms. Paraffin (Heavy paraffin oil, CDH Pvt. Ltd., New Delhi) stocks and agar slants were preserved in refrigerator at 4°C and glycerol (SD Fine chemicals, New Delhi) stocks were stored at -20°C in deep freezer.

Table 4 : Test Microorganisms

Organism	MTCC number	Remarks
Salmonella paratyphi A	735	Used to make widal antigen
Salmonella paratyphi A (GU)	-	Resistant to nalidixic acid, tetracycline, and co-trimexaxole
Staphylococcus epidermidis	435	Associated with skin lesions
Pseudomonas oleovorans	617	Recommended for assay of antimicrobial agents in aqueous metal working fluids
Escherchia Coli	723	Used as standard strain producing colonization factor antigenI (cfx / I) and heat stable / heat labile entrotoxin
Bacillus subtilis	619	Recommended for assay of penicillin, streptomycin, vancomycin, and is associated with production of weak penicillinase.
Vibrio cholerae	3906	

4.3 Extraction

Clean and dry seeds were crushed with the help of an electric blender (12,000 rpm, 20 s) to a constant particle size. This coarse powder was then extracted with (50%) ethanol (Merck-GR, Mumbai) or methanol (Merck-GR) by the technique of microwave assisted extraction [Kothari, Punjabi and Gupta, 2009].

One gram of seed powder was soaked into 50 mL of respective solvent into a 250 mL flask having glass lid placed loosely on it. This flask was kept in microwave oven (Electrolux, EM30EC90SS) for extraction at 720 W. At a time one flask was kept for extraction and intermittent cooling of 40 s was given to it. The total time for extraction of both the seeds with each solvent is reported in table 5.

Table 5 : Heating and cooling cycles for both solvents during MAE

Solvent	Total Extraction time* (s)	First heating cycle (s)	Cooling time (s)	Reheating time (s)
Methanol	90	10	40	10
Ethanol (50%)	70	40	40	30

*excluding intermittent cooling

After extraction, the contents were centrifuged at a speed of 10,000 rpm (7600g) for 15 minutes. After centrifugation, the extract was subjected to filtration through Whatman paper no.1 (Whatman International Ltd., England). Filtrate was allowed to evaporate in pre-weighed 150 mm diameter petriplates (Borosil). The extract was then reconstituted in the respective solvent for DDA and TLC. For determination of MIC, extracts were reconstituted with DMSO. The extraction efficiency and reconstitution efficiencies of both the seeds are presented in the table 6.

Extraction efficiency for both the seeds in MeOH and EtOH was calculated as:

$$\frac{\text{Weight extracted (mg)} \times 100}{\text{Weight of initial material (mg)}}$$

Reconstitution efficiency was calculated as:

$$\frac{\text{Weight of extract reconstituted (mg)} \times 100}{\text{Total weight of dried extract (mg)}}$$

Reconstitution efficiency is the % of material recovered from 1 g of seed.

Extracts were then stored in autoclaved flat bottom glass vials (15 mL ; Merck) under refrigeration. The vials were protected from light for further use. Inner side of the vial cap was covered with aluminium foil to prevent the leaching of compounds from the plastic into the extract [Houghton and Raman, 1991].

4.4 Antimicrobial Susceptible Testing

4.4.1. Disc diffusion assay

For testing various seed extracts against the test microorganisms the Kirby-Bauer disc assay was performed according to NCCLS guidelines [Jorgensen and Turnidge, 2003]. Sterile petridishes (150 mm) was poured with 60 mL of molten Mueller-Hinton (MH) agar to give a mean depth of 4.00 ± 0.5 mm. Discs of 6 mm diameter made up of Whatman paper no. 1 were exposed to UV radiation for 30 min for sterilization.

The inoculum was prepared in sterile saline (0.85% NaCl solution) from 24 h old bacterial culture and the turbidity of the inoculum was adjusted using sterile saline solution to approximate that of 0.5 McFarland turbidity standard (appendix 7) which is equivalent to 10^6 - 10^8 CFU/mL. Within 15 min of adjusting the inoculums turbidity, 500 µL of the suspension was taken and spread over the plate with the help of sterile glass spreader in order to get a uniform microbial growth. The inoculum was allowed to be absorbed. The discs were dipped in extract of known concentration by keeping dipping time as 10 s and dried for 40 s so as to ensure complete evaporation of the solvent from the disc. Under aseptic condition these discs were put on to the agar surface by applying gentle pressure with the help of a sterile forceps to ensure complete contact of disc with agar. 10 discs were placed at a distance of 12 mm from the periphery of the petridish and the minimum distance between two discs (centre to centre) was kept 24 mm. Disc containing antibiotic spectinomycin (100µg/disc) or streptomycin (10µg/disc) (HiMedia, Mumbai) was used as positive control. A disc soaked into extraction solvent was kept as negative control. Plates were incubated at 35°C in inverted position for 16-24 h. After incubation diameter of the inhibition zone was measured. Studies were performed in triplicates, and mean value was reported.

4.4.2. Minimum inhibitory concentration

The method used for determination of minimum inhibitory concentration was broth dilution method. The method was carried out according to NCCLS guidelines [Jorgensen and Turnidge, 2003].

4.4.2.1. Microbroth dilution method

Microbroth dilution method was performed in 96 well microtitre plates (HiMedia). The extract was reconstituted in DMSO. Bacterial strains taken were tested for percent tolerance of DMSO [appendix 1]. For microbroth dilution, the turbidity of the inoculum which was prepared from 24 h old bacterial culture was visually adjusted using sterile saline solution (0.85% NaCl solution) to approximate that of 0.5 McFarland turbidity standard which is equivalent to 10^6 - 10^8 CFU/mL, various controls and experimentals were prepared and the final volume in each well taken was 200uL. Turbidity controls were kept to nullify the contribution of extract itself towards total turbidity and colour.

Fig 2 : Microwave assisted extraction

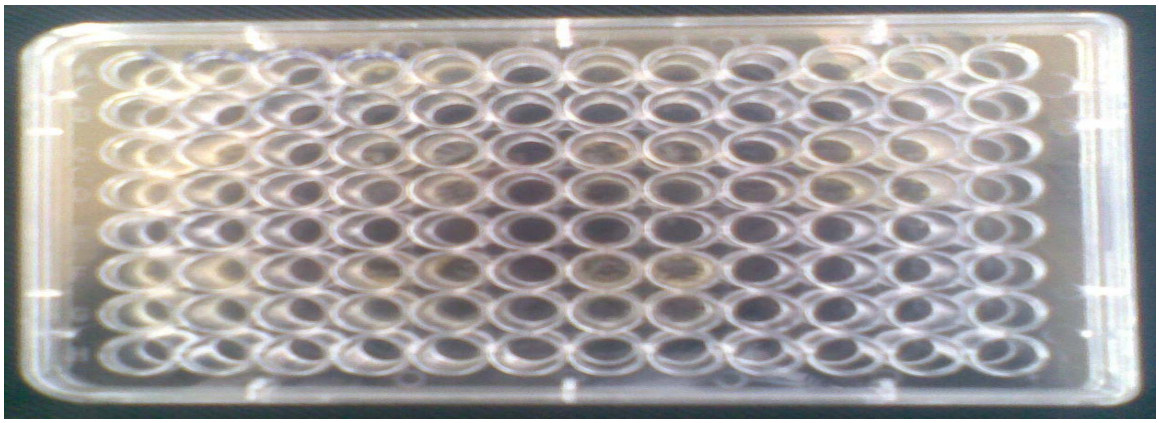


Plate 1 : Microtiter plate

Fig 3 : Microtiter plate reader

- Experimental wells : 148 µL broth + 2 µL extract (various concentrations) + 50 µL inoculum.
- Turbidity Control : 148 µL broth + 2 µL extract + 50 µL saline (0.85%).

After that microtitre plate was incubated aseptically at 35°C for 16 to 24 h. Then the readings were taken in microplate reader (BIO RAD 680) at 655 nm, 5 s shaking. Each extract was tested for broad to narrow range until the exact MIC value is determined. Results obtained are shown in table 9.

Because addition of the inoculum results in dilution so, all final drug concentrations must be prepared accordingly. All wells were inoculated within 30 min of inoculum's preparation [Jorgensen and Turnidge, 2003]. The readings of negative control were considered as 100% for calculation of percent inhibition, in order to nullify inhibitory effect of DMSO / methanol itself.

4.4.2.2. MIC of standard antibiotics

MIC test were also performed using HiComb™ MIC strips of standard antibiotics: Ofloxacin and Streptomycin. There were 2 strips A and B for each antibiotic. Strip A carried a concentration gradient from 64 µg/ mL to 0.01 µg/ mL and strip B, 8 µg/ mL to 0.001 µg/ mL. Each strip is made of an inert material, with 8 extensions that carry the discs of 4 mm, resembling the 'tooth' of a comb. Towards the stem of the strip, MIC reading scale in µg/mL is given. A defined concentration of antibiotic was loaded on each of the disc as to form a gradient when placed on agar plates. When applied to the agar surface, the antibiotic instantaneously diffused into the surrounding. The gradient remains stable after diffusion, and

the zone of inhibition created took the form of an ellipse. This method generated the MIC values of a given antibiotic in µg/mL, which inhibited the growth of a particular microorganism under the specific set of experimental conditions.

4.5. Characterization of crude extracts

4.5.1. Qualitative tests

Phytochemical screening of the extracts with significant antimicrobial activity was achieved by employing various qualitative tests as described below:

4.5.1.1. Test for alkaloids

Dragendorff reagent was used to test the presence of alkaloids. The presence of alkaloids was indicated by the appearance of yellow precipitate when few drops of reagent was added to the solution [Eloff, 2004].

4.5.1.2. Test for flavonoids

An aqueous solution of the extract is treated with 10% ammonium hydroxide solution. Yellow florescence indicates the presence of flavonoids [Wagner, Bladt and Zgainski, 1983].

4.5.1.3. Test for Phenols

Ferric chloride was used to test the presence of phenols. The presence of phenols was indicated by appearance of green colouration when the reagent was added to extract [Raman, 2006].

4.5.2. Quantitative estimation

4.5.2.1. Total phenolic estimation

Folin-ciocalteu method was applied to determine total phenolic content of the sample. The method was described by Singleton and Rossi. Briefly it is 0.2ml of 10% v/v FC reagent was added to 0.1ml of sample extract, reconstituted in 95% methanol. Then it was vortex for 5 m followed by addition of 0.8ml of 700 mM of sodium carbonate. This reaction mixture was then incubated for 2 h at room temperature. Absorbance was then measured at 765 nm.

Calibration curve was prepared by gallic acid at concentration 0.4 to 1.6 mM in 95% methanol. Total phenolic content is expressed in Gallic Acid Equvivalents (GAE) in mg/g of dry extract

4.5.2.2. Total flavonoid estimation

Aluminium chloride colorimetric method was used for flavonoid determination [Chang, *et. al.,* 2002]. For estimation, 0.5 ml of extract reconstituted in methanol were separately mixed with 1.5 ml methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. Reaction mixture was allowed to stand at room temperature for 30 m: the absorbance of reaction mixture was measured at 415 nm.

The calibration curve was prepared by preparing quercetin solution at concentration 12.5 to 110 µg /ml in methanol as shown. Total flavonoid content is expressed in Quercetin Equvivalents (QE) in mg/g of dry extract.

Fig 4 : Standard curve of Folin-Ciocalteu assay for total phenolics

Fig 5 : Standard curve for flavonoid estimation

4.5.3. UV- visible spectroscopy

For this purpose, double beam UV-visible spectrophotometer was used to generate spectra of various potent crude extracts. While obtaining the spectra, pure solvent was used as a blank in the reference cell. The cut-off value of different solvents (appendix 3) was taken into consideration for selecting the wavelength range for scanning. Extract was diluted to 10 times with respective solvent before subjecting it to spectrophotometer, as the extract was highly turbid and coloured. The compounds present in the potent extracts were predicted on the basis of the absorption maxima obtained in the spectra [Harborne, 1998 ; Seigler, 1998 ; Raman, 2006 ; Bhat, *et al*., 2005 ; Scott, 1964 ; Cseke, *et al*., 2006].

4.6. Chromatography

4.6.1. Thin-layer chromatography (TLC)

TLC of plant extracts was done on 20 x 20 cm TLC silica gel 60 F_{254} plates (Merck) of 0.25 mm thickness to achieve the separation. Extracts were equilibrated for 15 min at room temperature before loading. The spots were loaded 2.0 cm above the lower edge of the plate and 1.5 cm was left from each side. Twelve spots were loaded on each plate having distance of 1.5 cm in between. Three µL of the extract having concentration of 72 mg/ mL was loaded per spot, so the amount of extract loaded per spot was 220 µg and per plate it was 2640 µg. In

few preparative plates used, extract was loaded in band form (57spots), per plate 9177 µg per plate. Later band pattern was followed on analytical plate with same concentration as loaded on previous analytical plate as indicated in experimental setup below. The solvent system used for separation was n-butanol : water (1:1) [Harborne, 1998]. The glass chamber of 25 x 25 x 14 cm (Merck) was saturated for 1.5 h and the plates were placed in that saturated chamber for 2 h for separation. The volume of solvent put into the chamber was 100 mL and solvent level was kept 2.5 cm. The separation experiment was performed at room temperature. While the plates kept into the chamber for separation silicon grease was applied on the corners of the lid in order to avoid evaporation of solvent. After 1 h the plates were taken out of the chamber and solvent front was marked by sharp pencil, usually solvent ran up to 8.5 cm. Plates were allowed to dry at room temperature. The plates were then examined under ultraviolet (UV) light. Examination of the plates was done at UV- 254 and 365 nm and daylight. Fluorescent spots were marked with a scalpel washed with methanol. The retardation factor (R_f) i.e. the distance travelled by the solute divided by the distance travelled by the solvent from the origin was calculated and tabulated.

Sample was applied with care avoiding diffuse bands, as that would have decreased the resolution of the components. Non-destructive detection method was used, for the bands separated by preparative layer chromatography [Houghton and Raman, 1998]. Quercetin (SD Fine chemicals, New Delhi) was run as a marker along with samples.

4.6.1.1. Experimental setup for TLC [adapted from Stahl, 2007]

1. Adsorbent layer : Silica gel 60 F_{254} plates (Merck)
2. Layer thickness: 0.25 mm (analytical), 0.5mm (preparative plate)
3. Layer size: 20×20 cm
4. Chamber type: Glass chamber, 25 x 25 x 14 cm (Merck)
5. Separation technique: Ascending
6. Chamber saturation state: Minimum 2 h
7. Length of run: 8.5 cm
8. Solvent composition and total volume: n-butanol : water (1:1) ; 150mL
9. Purity data for solvent components: 99.8%
10. Solvent and solution concentration used for applications: methanol: 3µL of 70.37mg/ml.
11. Margin between start point and plate edge: 2.0 cm above the lower edge of the plate and 1.5 cm was left from each side.
12. Amount applied : 7260 µg per plate
13. Band length : 17 cm

4.6.1.2. Recovery of separated components

After separation, it was seen that 7 fractions were obtained from methanol extract of *S. cumini*. The separated constituents were recovered by scraping off the adsorbent at the appropriate places on the developed plate, and the powder was reconstituted in methanol. The powder was subjected to centrifugation (Eppendorf 5417R) at 14,000 rpm for 15 min. This step was carried out twice to ensure maximum removal of the adsorbent. Glass vials (15mL, Merck) containing separated components were stored in refrigerator.

4.6.1.3. Assay for separated components

After separation all the separated components were tested for antibacterial activity applying the same method of broth dilution as that for crude extract. The results of experiment are reported in table 14 and 15.

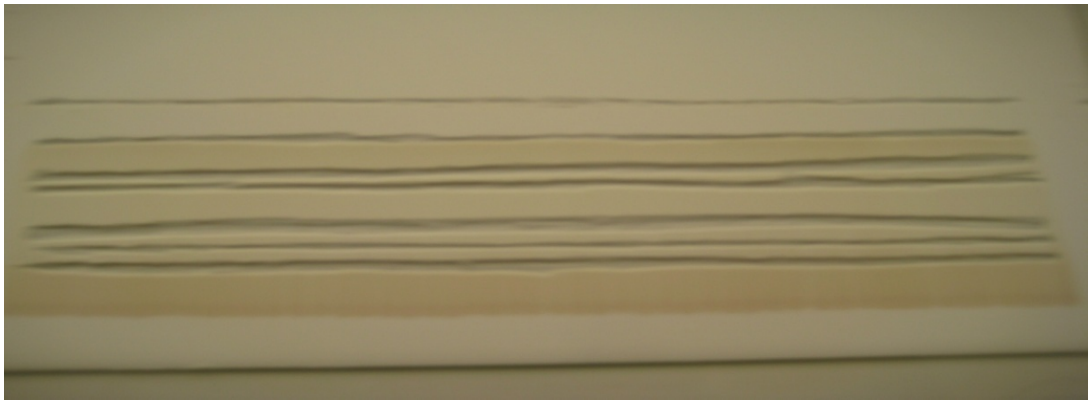


Plate 2 : Scrapped TLC plate ; sample applied as bands

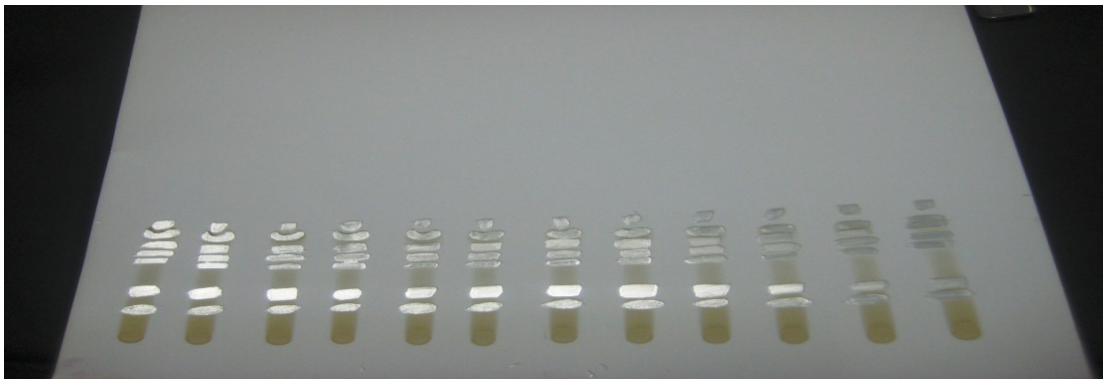
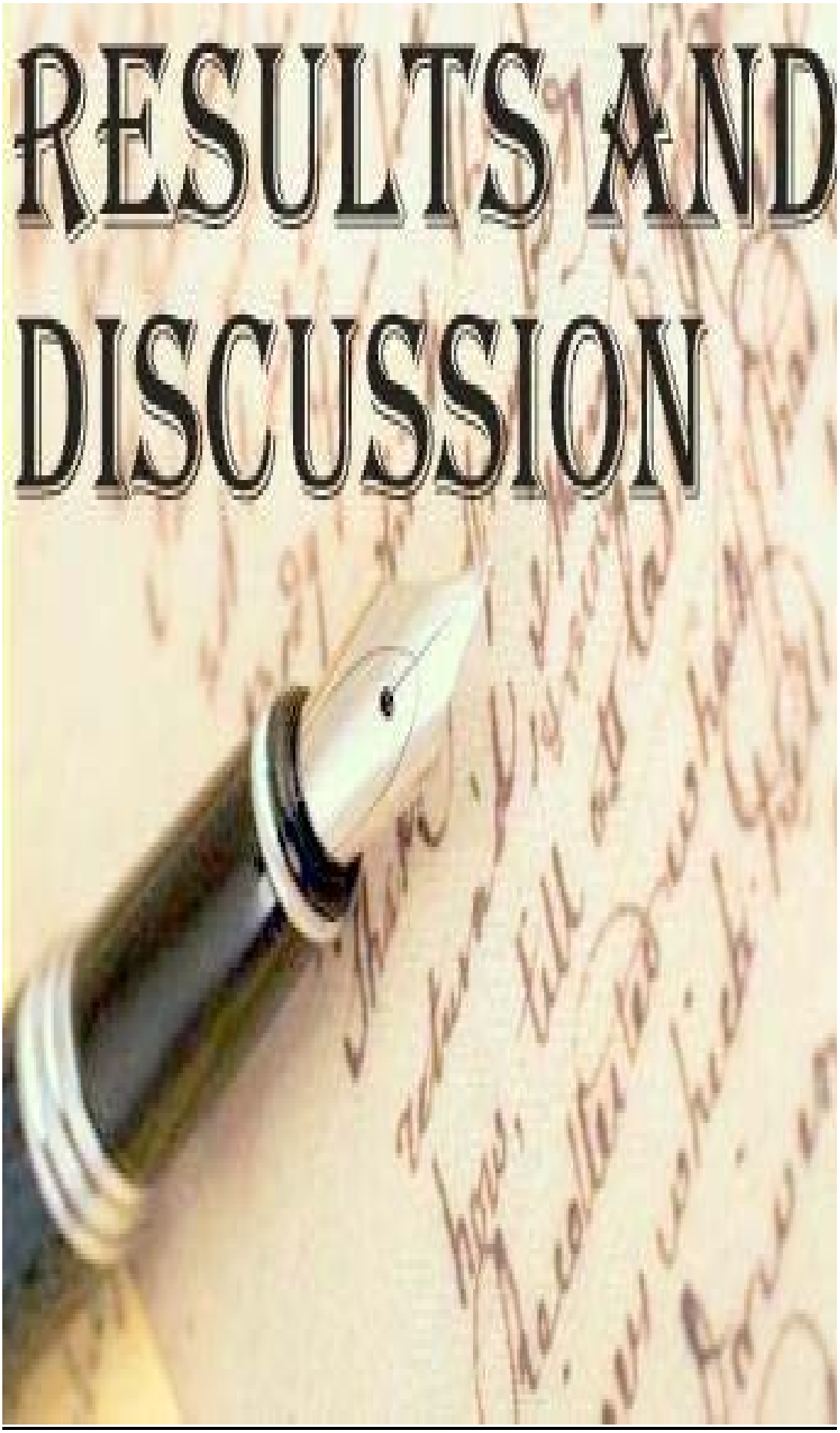


Plate 3 : Scrapped TLC plate ; sample applied as individual spots



5. Results and Discussion

5.1 Extraction

Extraction and reconstitution efficiency for both the seeds in ethanol and methanol are reported in table 6.

Table 6 : Extraction and Reconstitution efficiency in various solvents

	S. cumini **(Methanol)**	***S. cumini*** **(Ethanol - 50%)**	***T. indica*** **(Methanol)**
Extraction Efficiency (%)	11	21.5	13
Reconstitution Efficiency in same solvent (%)	84.57	81	83.30
Final Concentration (mg/mL)	70.37	250	37.03
Reconstitution Efficiency (DMSO) (%)	90	-	-
Final Concentration (mg/mL)	50	-	-

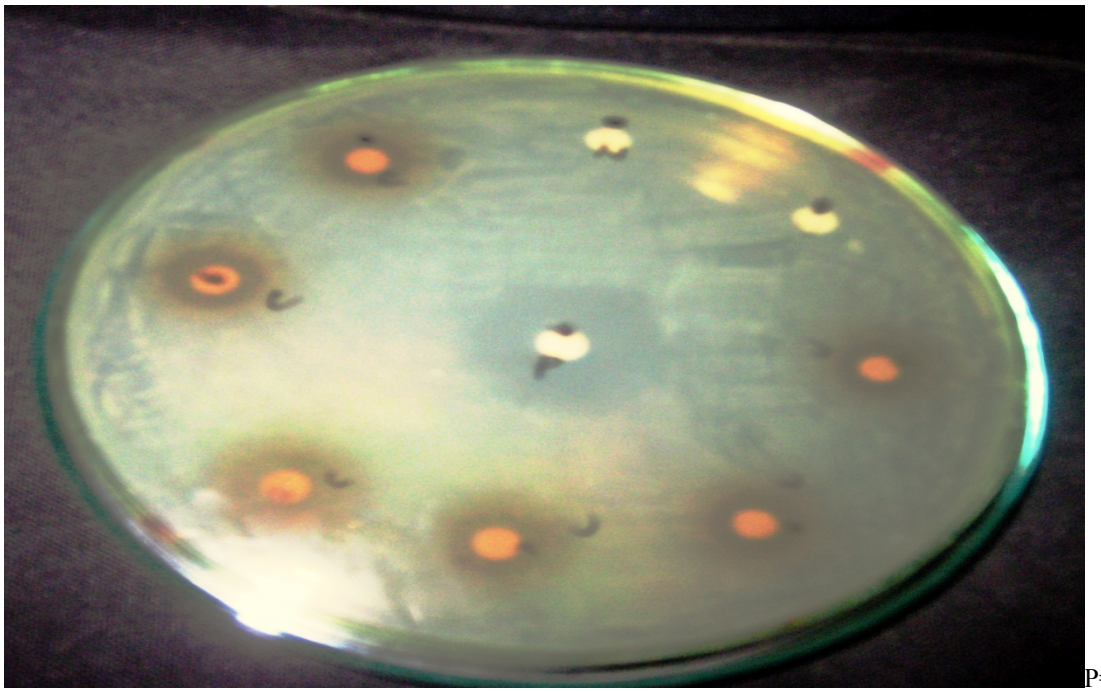
Highest (21.5%) and lowest (11%) extraction efficiency was achieved when *S. cumini* seeds were extracted in ethanol and methanol, respectively. However, methanol proved slightly better over ethanol with respect to reconstitution efficiency. Efficient reconstitution is necessary in order to take full advantage of high extraction efficiency.

5.2 Antibacterial Assay

5.2.1. Disc Diffusion Assay

Results of antibacterial activity tested by DDA presented in table 7 show that among all test organisms, *S. epidermidis* was found to be the most susceptible against all three extracts. Both the extracts of *S. cumini* can be called as having broad spectrum of activity as they are active against gram-positive as well as gram-negative bacteria [Talero, 2008]. The methanol extract of *S. cumini* exerted same magnitude of activity against both *V. cholerae* and *P.*

oleovorans. Since this extract was active against five out of seven organisms, it was chosen for further studies.



P= positive control (ofloxacin); N= negative control; C = extract

Plate 4. Methanol extract of *S. cumini* seeds against *S. epidermidis.*

Table 7 : Disc diffusion assay of *S. cumini* and *T.indica* seed extracts

Organism	ZOI (mm) *(Mean ± SD)*			Positive control (Spectinomycin)[#]
	(S.cumini)		*(T. indica)*	
	(MeOH) (70.37mg/mL)	(EtOH-50%) (250mg/mL)	(MeOH) (37.03mg/mL)	
S. epidermidis	**14±0**	**15±2.4**	10±0.57	18±0
V. cholerae	12±1.8	12±1.0	0	20±2.2*
P. oleovorans	12±0.70	14±1.0	-	20±0
S. paratyphi A	FI	-	-	18±0
E. coli	13±1.0	13±1.8	-	28±1.8
S. paratyphi (resistant)	0	-	-	22
Bacillus subtilis	0	-	-	21
Proportion index	71.42	100	50	

- Assays were performed in triplicates; ZOI = Zone of inhibition, FI = Faint Inhibition, - indicate not performed. [#]spectinomycin (100 µg/ disc), *streptomycin (10 µg/disc) – HiMedia, Mumbai.
- Figures in bold indicate the highest recorded value.

Proportion index was calculated by the formula given below and results are reported in table 7 [Borgio, Thorat, and Lonkar, 2008]

$$\text{Proportion index (\%)} = \frac{\text{No. of positive results}}{\text{Total number of tests}} \times 100$$

Table 8 : Activity index of different extracts against various organisms

Organism	*S.cumini*		*T.indica*
	MeOH	EtOH	Meoh
S. epidermidis	0.77	0.83	0.55
V. cholerae	0.60	-	0
P. oleovarans	0.60	-	-
S. paratyphi A	0	-	-
E. coli	0.46	0.46	-
S. paratyphi A (GU)	0	-	-
B. subtilis	0	-	-

5.2.2. Broth Dilution Assay

Results of broth dilution assay are shown in table 9.

5.2.3 Total activity

Total activity of an extract was calculated by the following formula [Eloff, 2004] :

$$\text{Total activity (mL/g)} = \frac{\%\ \text{extraction efficiency}}{\text{MIC (mg/mL)}} \times 10$$

Total activity is a measure of the amount of material extracted from a plant in relation to the MIC of the extract, fraction or isolated compound. Total activity is expressed in mL/g which is an indication of the degree to which active extracts of one gram can be diluted and still inhibit the growth of the test organisms. From table 16 it can be found that total activity of methanol extract of *S. cumini* was higher for *S. epidermidis* than methanol extract of the same seed for *P. oleovorans* and *V. cholerae.*

Table 10 : MIC, IC_{50}, and total activity of methanol extract of *S. cumini*

Organism	IC_{50} (µg/mL)	MIC (µg/mL)			Total activity (mL/g)	Average total activity (mL/g)
		Extract	ofloxacin	streptomycin		
S. epidermidis	>387	494	0.15	0.1	222.67	226.60
P. oleovorans	400	656	0.15	-	167.68	
V. cholerae	>225	380	0.004	-	289.47	
E. coli	~ 724	-	-	-	-	

Highest susceptibility was registered by *V. cholera*e. Based on MIC values *V. cholerae* is 1.3 and 1.72 times more susceptible than *S. epidermidis* and *P. oleovorans*, respectively.

Plate 5 : Disc diffusion assay of MIC strip of standard antibiotic

Table 11 : Comparative efficacy of crude methanol extract of *S. cumini* seeds against different test organisms.

Organism	Concentration (µg/mL) (A)	% Inhibition (B)	Ratio (A/B)
V. cholerae	395	89.73	4.40
E. coli	724	54.63	13.25
S. epidermidis	600	87	6.89
P. oleovorans	682	99	6.88

Table 11 records the ratio of concentration of crude extract to magnitude of inhibition at that concentration. Lower the value of this ratio, better is the efficacy. Thus, *V. cholerae* proves to be the most susceptible, and *E. coli* the least. Susceptibility of remaining two organisms to this particular extract is almost identical.

5.3. Characterization

5.3.1. Phytochemical Investigation

The potent extracts as identified from the antimicrobial assays were further subjected to various qualitative and quantitative tests for detecting the presence of various classes of secondary metabolites.

5.3.1.1. Qualitative Screening

Phytochemical tests of methanol and ethanol extracts of *S. cumini* and *T. indica* seeds revealed the presence of phenols, alkaloids, and flavonoids. Presence of phenols in ethanol extract of *S. cumini* seeds was also reported by Sagrawat, *et al*. Antimicrobial activity of phenolics has been reported by Charalampos and Komaitis (2007). Compounds which are soluble in methanol and ethanol include lectins, alkaloids, quassinoids, flavones, polyphenols, tannins, and saponins [Ahmed, I., Aqil, F. and Owis, M., 2006].

5.3.1.2. Quantitative Estimation

Table 12. Quantification of flavonoids and phenols

	Extract	*S. cumini*	*T. indica*
Total Flavonoid (µg/mLQE/mg of dry extract)	Methanol	27.38	**29.48**
	Ethanol	2.20	-
Total Phenols (mgGAE/mg of dry extract)	Methanol	76	**494**
	Ethanol	360	-

Antibacterial activity of these extracts could be due to phenols, flavonoids and alkaloids as reported from qualitative analysis and these metabolites are also suggested to be antibacterial in nature [Cowan, M. M., 1999].

The mechanism of antibacterial activity of alkaloids is due to their ability to intercalate with DNA. Phenol inhibits bacterial growth by substrate deprivation and membrane disruption. The antibacterial activity of flavonoids may probably be due to their ability to complex with extracellular and soluble proteins and due to formation of complex with cell wall.

Methanol extract of *S. cumini* contains more flavonoids and lesser total phenols than ethanolic extract of same seed. Thus the antibacterial activity of the former may be attributed largely to flavanoid class of compounds. Flavonoids are effective antimicrobial substances against a wide array of microorganisms [Dixon, Dey and Lamb, 1983]. Antimicrobial flavonoids have been reported from *Erythrina latissima* [Wanjala, *et al.,* 2002].

5.3.2. Chromatographic Separation by TLC

The colour and R_f values were recorded under UV light at 365 as well as 254 nm. Results of TLC are reported in table 13. A fraction corresponding to R_f value 0.71/0.72 was found to be commonly present in both the extracts subjected to TLC, which may be due to polarity of both the solvents used for extraction being somewhat similar.

Table 13 : Results of chromatographic separation of extracts of *S.cumini* seeds by TLC

Extract	Fr. No.	Appearance of band		R_f	hR_f
Methanol extract		**365nm**	**254nm**		
	1	Translucent	Not visible	0.12	12
	2	Translucent	Not visible	0.27	27
	3	Blue	Not visible	0.38	38
	4	Pink	Not visible	0.62	62
	5	Dark pink	Not visible	0.71	71
	6	Not visible	Brown	0.78	78
	7	Faint pink	Brown	0.87	87
Ethanol extract	1	Light blue	Not visible	0.65	65
	2	Pink	Not visible	0.72	72
	3	Blue	Not visible	0.91	91
Quercetin		Faint pink	Brown	0.88	88

In daylight only fraction 7 of methanol extract was visible as a brown coloured component.

Plate 6 : TLC plate under daylight ; sample applied as individual spots

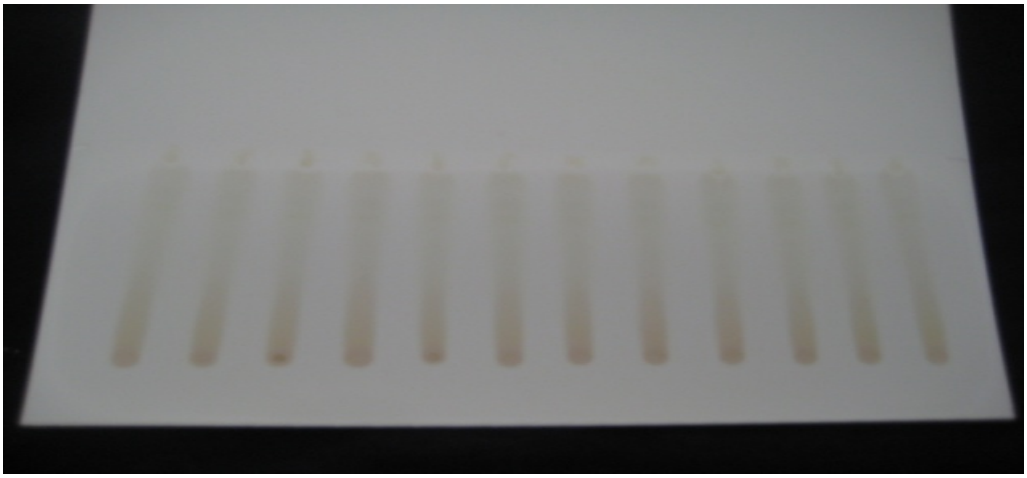


Plate 7 : TLC plate under daylight ; sample applied as individual spots

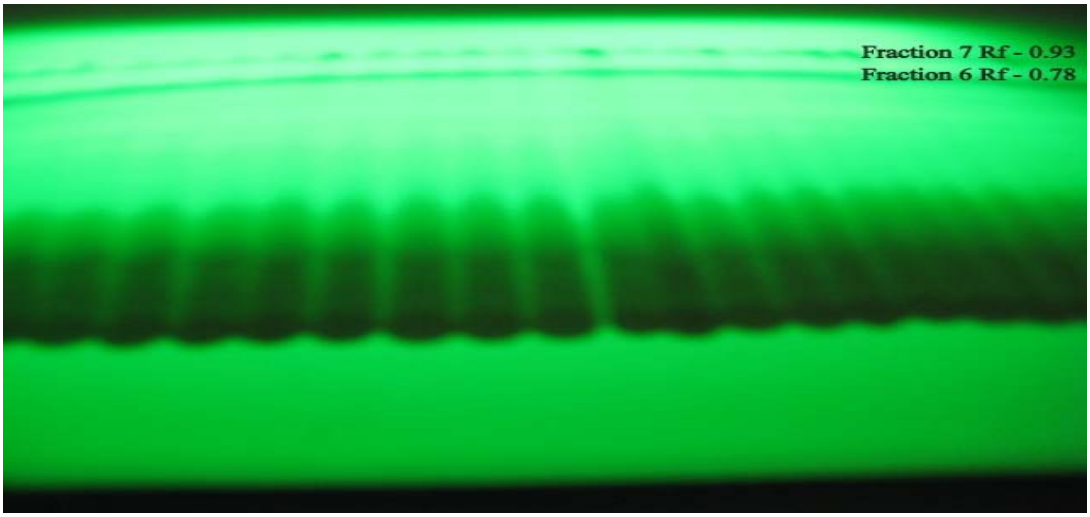


Plate 8 : TLC plate under 254 nm ; sample applied in band form

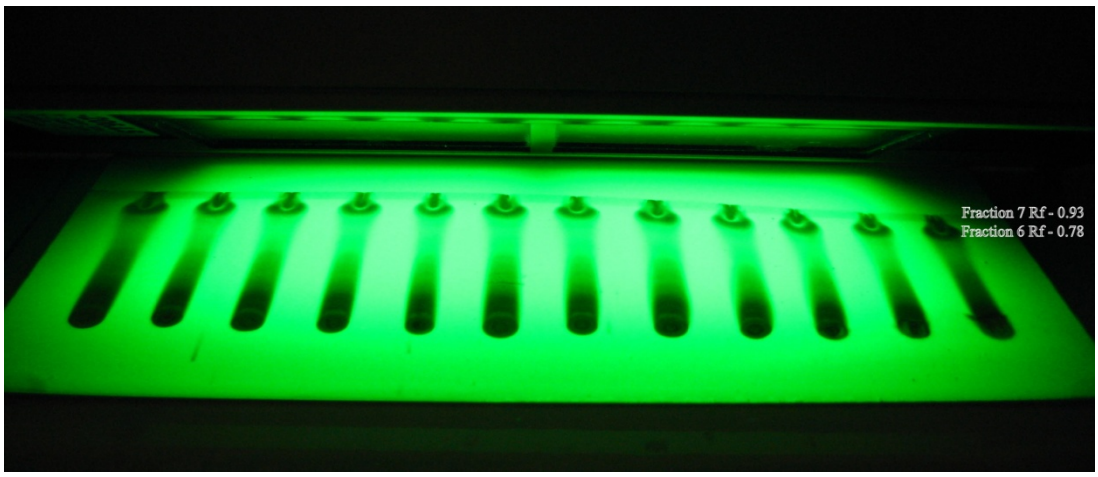


Plate 9 : TLC plate under 254 nm ; sample applied as individual spots

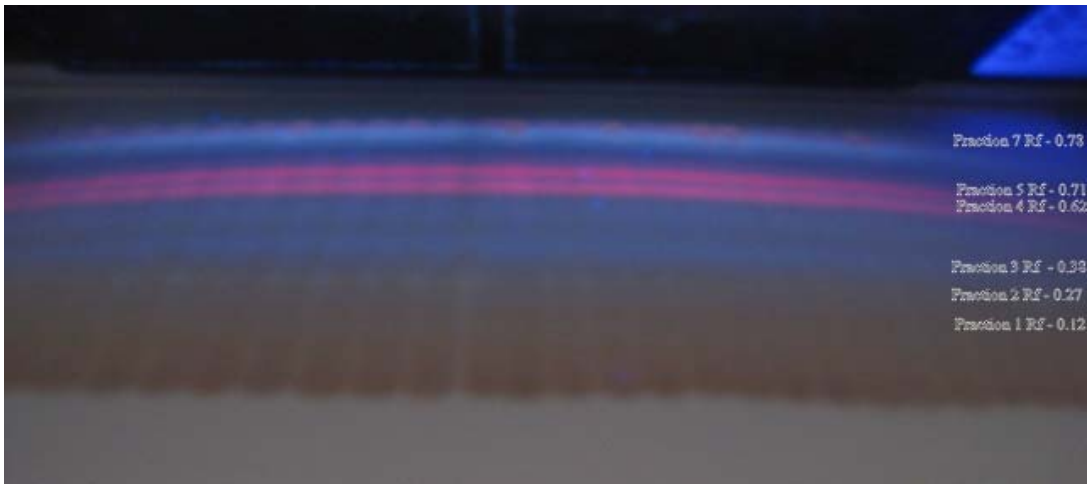


Plate 10 : TLC plate under 356 nm ; sample applied in band form

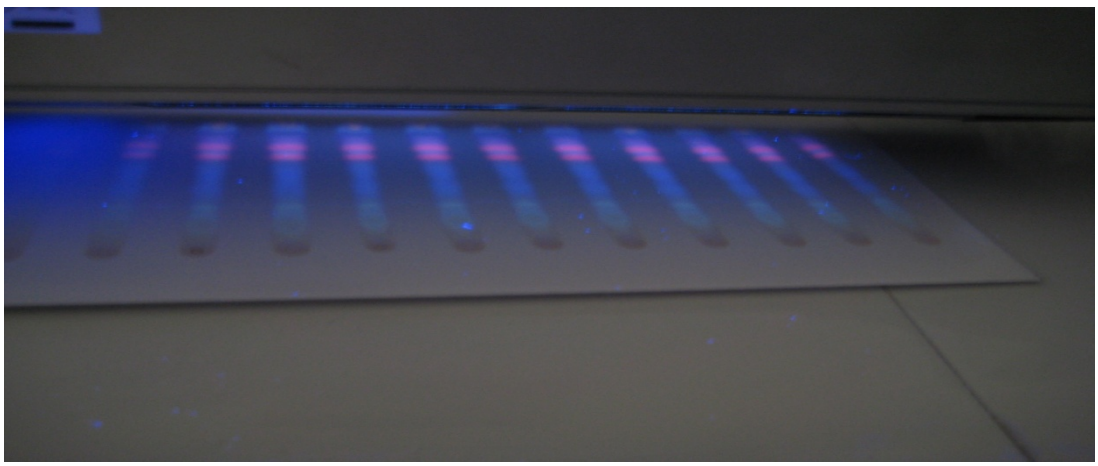


Plate 11 : TLC plate under 356 nm ; sample applied as individual spots

Plate 12 : TLC plates of ethanolic extract of *S. cumini at* 365 nm

Plate 13 : TLC plates of Ethanolic extract of *S. cumini* at 254 nm

Fractions separated on TLC plate were subjected to bioassay by broth dilution method, whose results are presented in table 14 – 16.

5.3.3. Bioassay of separated fractions

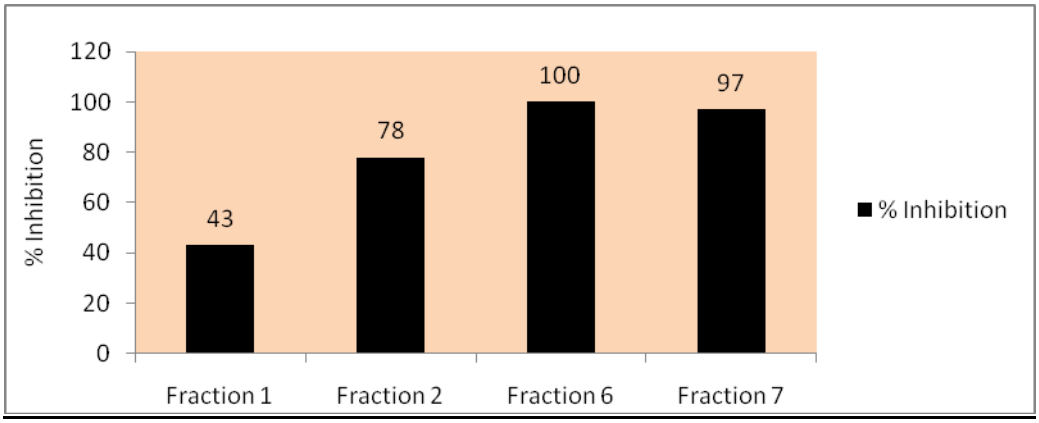


Fig 8 : Inhibition potential of different fractions at same concentration (186µg/mL) against *P. oleovorans.*

As is evident from the graph, the inhibition potential of fraction 6 and 7 is almost equal whereas that of fraction 1 is less than half of fraction 6.

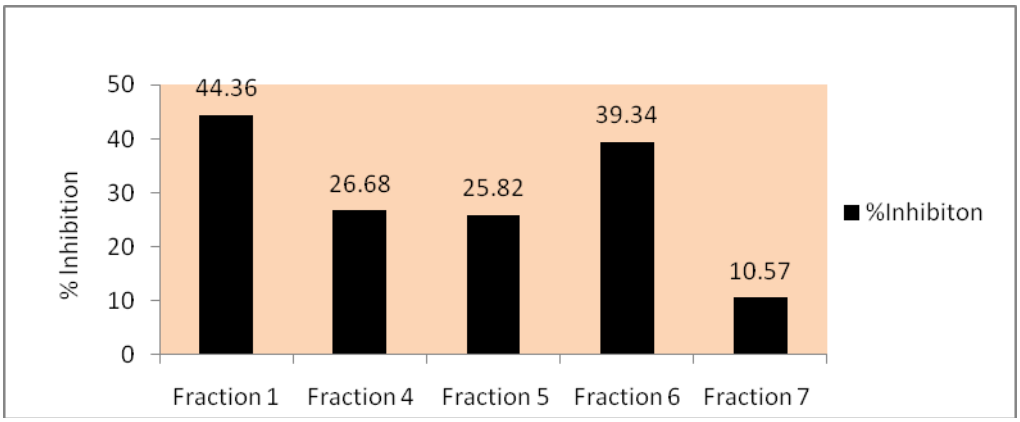


Fig 9: Inhibition potential of different fractions at same concentration (140 µg/mL) against *S. epidermidis.*

As evident from fig 9, the inhibition potential of fraction 1 is greater than the other fractions against *S. epidermidis* at concentration of 140 µg/ mL.

Fraction 6 is showing appreciable activity in relation to other fractions, against both the organisms tested. Fraction 7 is showing good activity against *P. oleovorans* but poor against *S. epidermidis.* Fraction 1 is more effective against *S. epidermidis* than *P. oleovorans,* as it requires higher concentration against the latter to achieve same degree of inhibition against the former.

Table 16: Summary of broth dilution assay of TLC fractions

Fraction 1 and fraction 7 respectively recorded average total activity 2.54 and 2.38 times higher than that of crude.

From the table 17, it can be said that fraction 6 is the most potent against *S. epidermidis* as well as *P. oleovorans*, as it required lesser concentration to achieve a particular degree of inhibition of the test organism. Fractions have been arranged in decreasing order of potency. Fraction 6 is 3.69 and 1.91 times more potent than crude extract against *P.oleovorans* and *S. epidermidis,* respectively. Though the crude extract is almost equally effective against both the test organisms, fraction 6 is 1.93 times more effective against *P. oleovorans* than it is against *S. epidermidis.* Both the organisms exhibited varying degree of susceptibility when tested against the same fraction, except fraction 3.

Table 17 records relative contribution of different fractions towards activity of the crude preparation. Relative contribution of each fraction was calculated by considering the (A/B) value of crude preparation (table 17) as 100%.

Table 18 : Relative Contribution of different fractions towards activity of the crude preparation.

Fraction no	% contribution of the fraction	
	P. oleovorans	***S. epidermidis***
1	68.03	44.56
2	72.39	-12.62
3	46.52	46.59
4	65.27	33.68
5	59.02	38.18
6	72.97	47.76
7	72.1	27

Fraction 6 seems to be contributing maximum towards antibacterial property of the crude preparation against *S. epidermidis*. Whereas fraction 2, 6 and 7 seem to be making equal contribution towards activity of the crude preparation against *P. oleovorans.* Fraction 2 seems to be contributing negligibly towards crude preparation's activity against *S. epidermidis* (gram- positive), which is in striking contrast to its contribution in crude preparation's activity against *P. oleovorans* (gram-negative). Fraction 3 seems to be equally contributing towards extract's activity against both the test organisms. It may be one of the components responsible for extract's broad spectrum activity. From table 18, it may be predicted that each fraction when tested individually exerts a magnitude of activity different than when it is tested in presence of other fractions (which is the case when test is made on crude preparation). Observed activity in the total extract may be due to synergism between

components which are separated as a result of the fractionation process. It is necessary to test combinations of the fractions to see if this is the case. Synergy occurs when the effect of two or more components applied together to a biological system is more than the sum of the effects when identical amounts of each constituents are used separately [Houghton and Raman, 1998].

Total activity

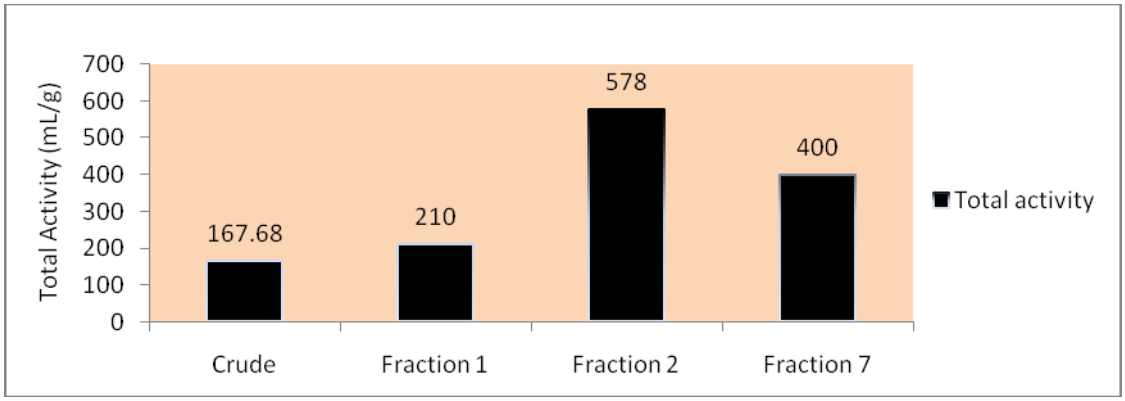


Fig 10 : Comparative total activity against *P. oleovorans*

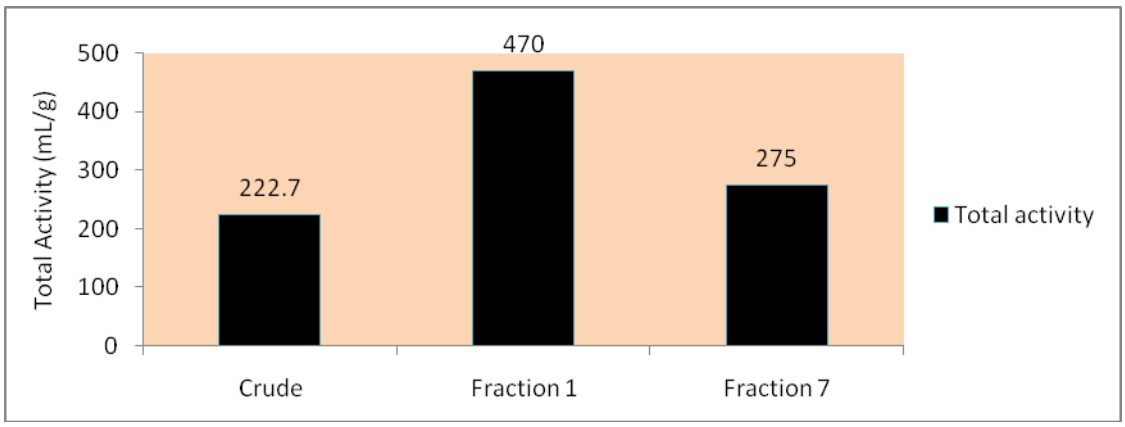


Fig 11: Comparative total activity against *S. epidermidis*

Total activity is an indication of the degree to which active extract of 1 g can be diluted and still inhibit the growth if the test organism. In case of *P. oleovorans*, it can be inferred from fig 10, that the total activity of fraction 2 is more as compared to total activity of other fractions as well as of crude. The increasing order of potency with respect to total activity is

as follows: crude < fraction 1 < fraction 7 < fraction 2. Fraction 2 is more than thrice active than the crude.

Similarly, from the graph it can be found that the total activity of fraction 1 against *S. epidermidis* is higher than fraction 7. Hence, fraction 1 when diluted, can be more potent in inhibiting the growth of test organism than the diluted fraction 7. The increasing order of the potency with respect to total activity is as follows: crude < fraction 7 < fraction 1. Fraction 1 is more than twice active than the crude.

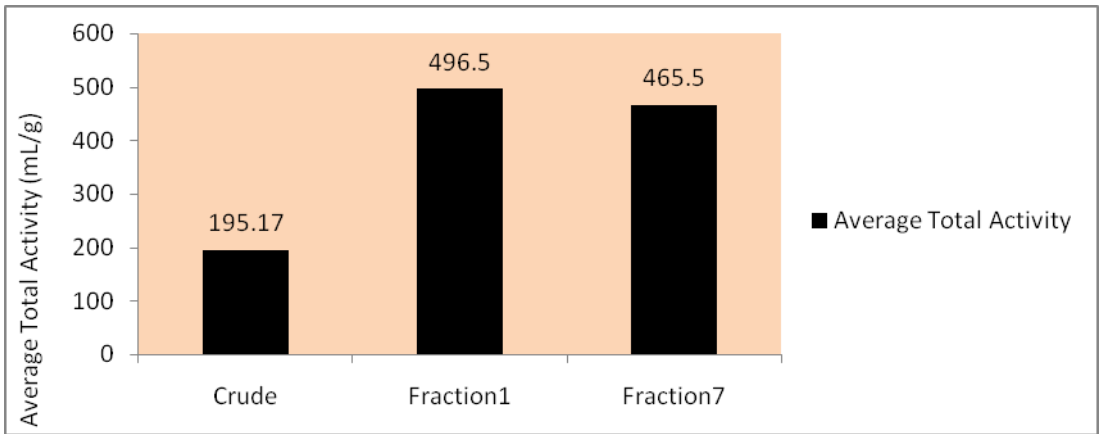


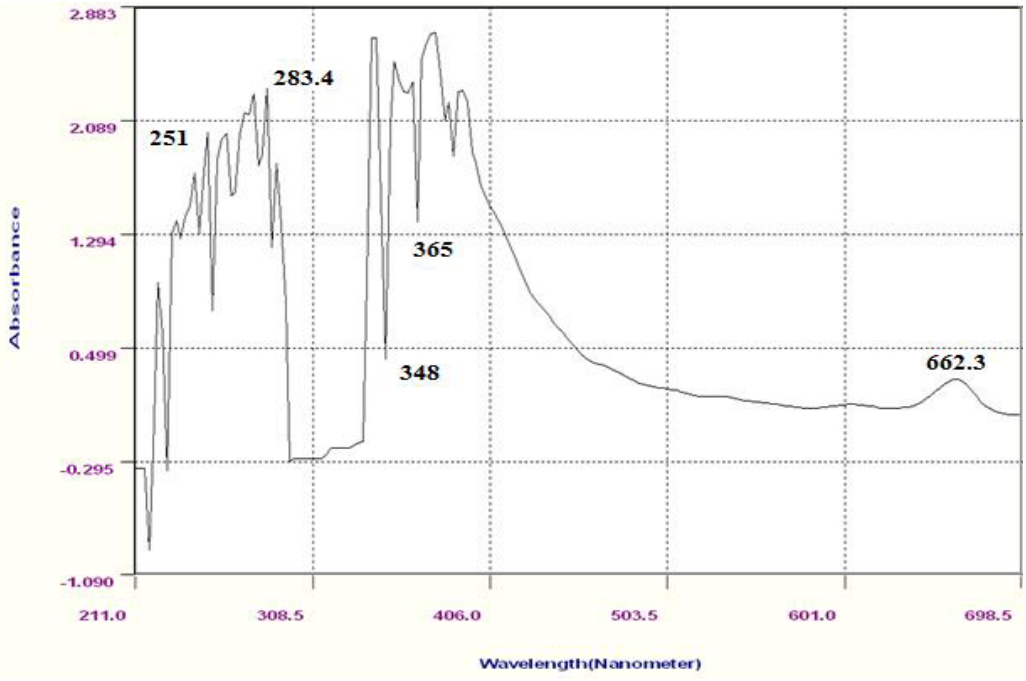
Fig 12 : Comparison of average total activity against *P. oleovorans* and *S. epidermidis.*

As per the calculation of average total activity of *S. cumini* methanol extract against both the organisms it was seen that fraction 1 and 7 respectively registered 2.54 and 2.38 times higher activity than the crude.

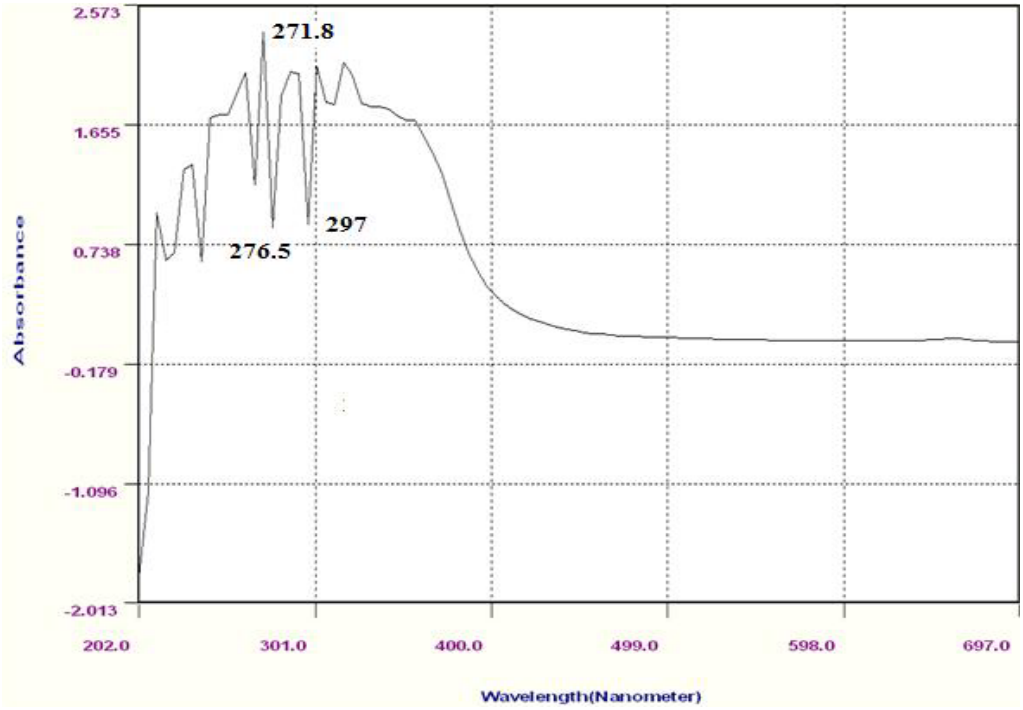
5.3.2. UV-visible spectra

Spectrum1 : Ethanol extract of *S. cumini* seeds

Spectrum2 : Methanol extract of *S. cumini* seed

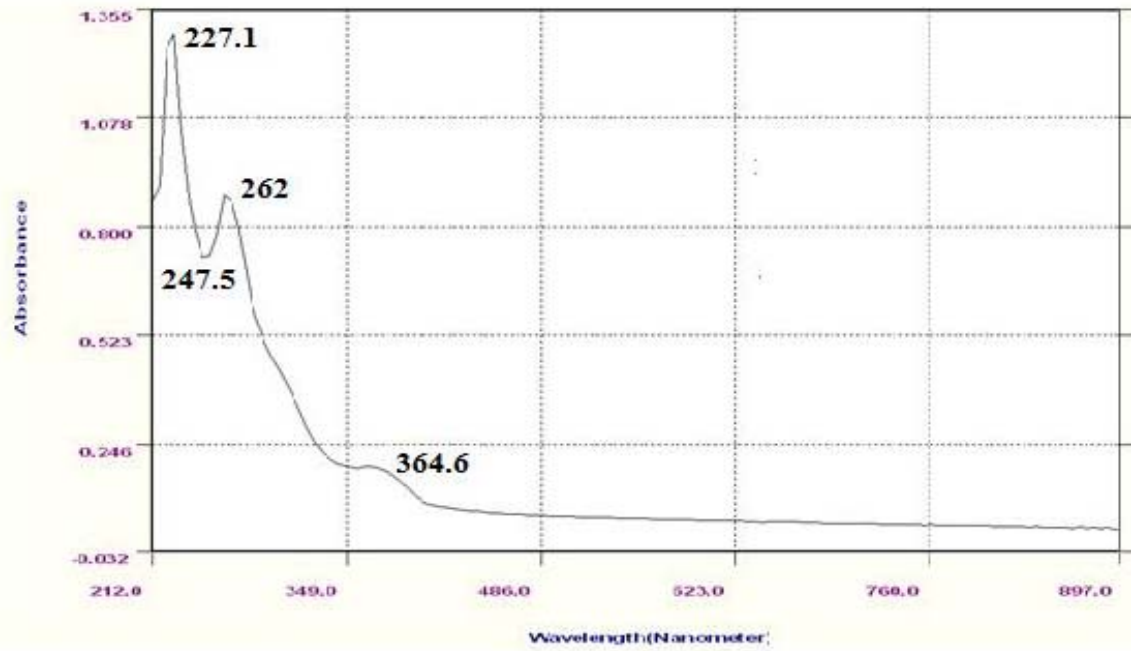


Spectrum3 : Fraction 1 of methanol extract of *S. cumini*

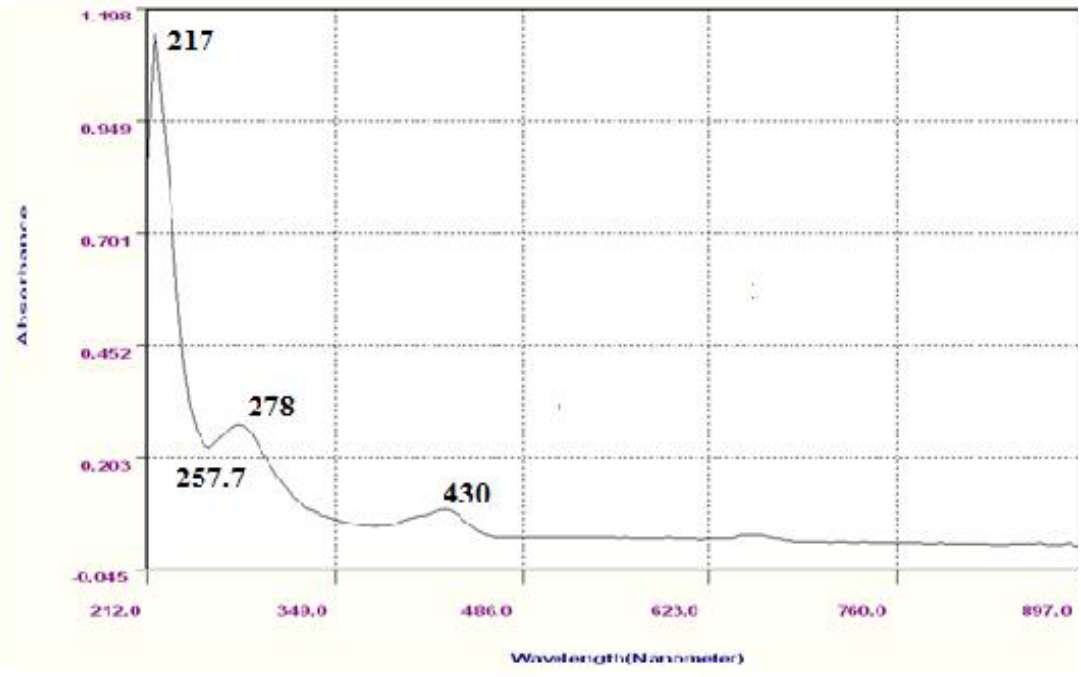


Spectrum4 : Fraction 2 of methanol extract of *S. cumini*

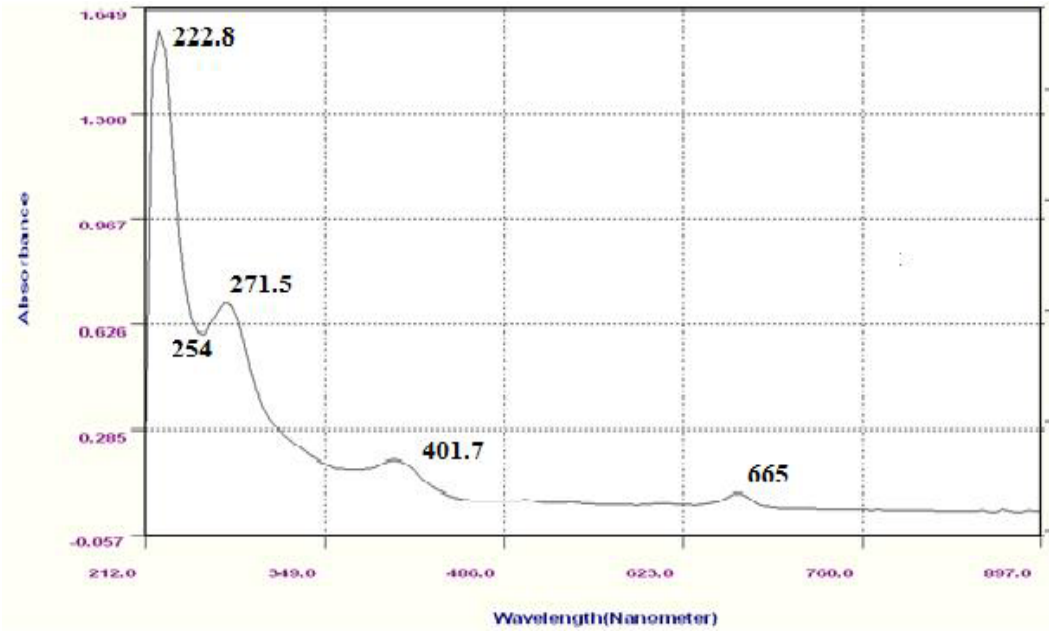
Spectrum5 : Fraction 3 of methanol extract of *S. cumini*



Spectrum6 : Fraction 4 of methanol extract of *S. cumini*

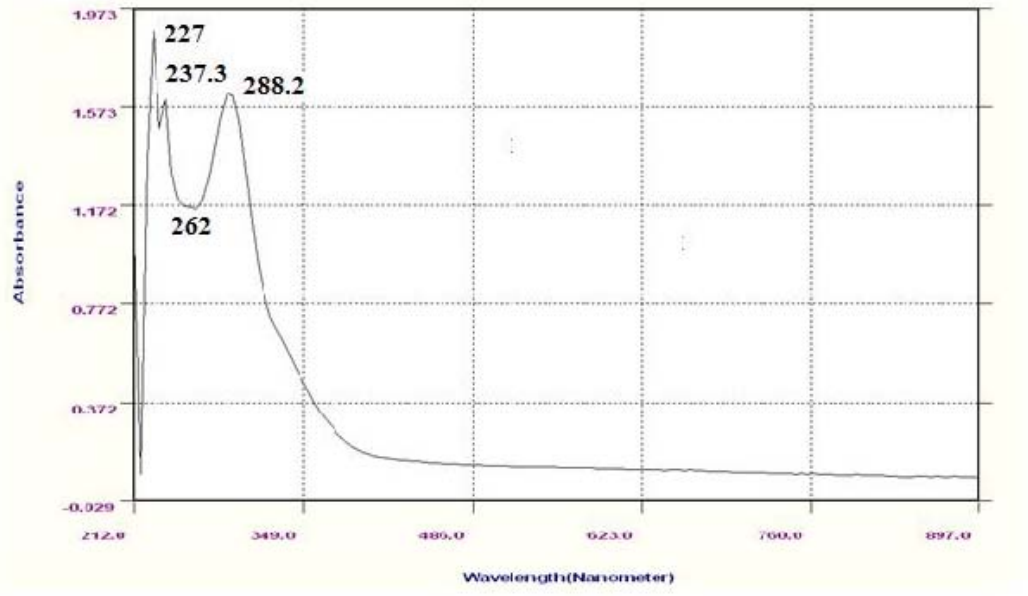


Spectrum7 : Fraction 5 of methanol extract of *S. cumini*



Spectrum8 : Fraction 6 of methanol extract of *S. cumini*

Spectra 9 : Fraction 7 of methanol extract of *S. cumini*



As shown in above spectra, the λ_{max} values of methanol and ethanol extract of *S. cumini* seeds and separated fractions of methanol extract of *S. cumini* falls in the absorption range of flavonols and phenols (appendix 6). This is in accordance to the results of phytochemical tests. UV-visible spectrum of fraction 1 and fraction 2 shows absorption peak at 271-272 which matches with that of gallic acid. Similarly, UV-visible spectrum of fraction 3-7 shows absorption in the range of 220-280 which falls in the absorption range of flavonols (appendix 6).

6. FINAL COMMENTS

- All the three extracts tested were found to be active against one or more test organism(s). Both ethanol and methanol extracts of *S. cumini* seeds were found to have a broad spectrum of activity. *V. cholerae* proved to be the most susceptible organism against methanol extract of *S. cumini.*

- When different fractions of methanol extract of *S. cumini* separated on TLC plate were subjected to bioactivity testing, all of them were found to have varying degree of antibacterial activity. Fraction 6 was found to be appreciably active against *S. epidermidis* and *P. oleovorans.* Testing two or more of these fractions in different combinations may yield further useful information such as that on synergy.

- UV-visible characterization and phytochemical screening revealed the presence of phenols, alkaloids, and flavonoids in all the three extracts tested. HPLC analysis (alongwith TLC and UV- visible spectra) indicated presence of gallic acid and quercetin in methanol extract of *S. cumini,* which have been reported in literature to possess antibacterial activity.

- It will be useful to carry out further investigations on ethanol extract of *S. cumini,* focusing on its fractionation. It is also needed to make an effort for identification of all the remaining fractions of methanol extract of *S. cumini.*

7. APPENDICES

Appendix 1 : Effect of DMSO on bacterial growth

Organisms	**DMSO**					
	% of growth as compared to positive growth control					
	1%	2%	3%	4%	5%	6%
S. paratyphi A	100	100	93	50	33	12
S. epidermidis	98	97	85	37	21	15
V. cholerae	100	100	100	49	46	43
P. oleovorans	100	100	100	42	35	33

[Wadhwani, *et. al.,* 2009]

Appendix 2 : Effect of Methanol on bacterial growth

Organisms	Methanol					
	% of growth as compared to control					
	1%	2%	3%	4%	5%	6%
S. paratyphi A	88	86	82	17	15	10
S. epidermidis	100	100	97	43	32	26
V. cholerae	98	92	90	63	61	59
P. oleovorans	100	100	86	83	79	33

[Wadhwani, *et. al.*, 2009]

Appendix 3 : Cut-off values of different solvents

Solvent	Methanol	Water	Ethanol	Acetone	Chloroform	Acetonitrile	Ethyl acetate
Wavelength (nm)	210	195	210	230	245	190	280

[Harborne, 1998 ; Siegler, 1998 ; Raman, 2006 ; Bhatt, *et. al.*, 2005, Scott, 1964 ; Sceke, *et. al*., 2006]

Appendix 4 : Properties of organic solvents

Solvents	**Solubility in water (g/100mL)**	**M.P (ºC)**	**B.P (ºC)**	**M.W (D)**	**D (g/mL)**
Acetone	Miscible	-94.3	56.2	58.08	0.786
Water	100		100	18	1.000
Chloroform	0.795	-63.7	61.7	119.38	1.498
Ethanol	Miscible	-114.1	78.5	46.07	0.789
Methanol	Miscible	-98	64.4	32.04	0.791

***Appendix 5 : Dissipation factor and dielectric constants for some solvents commonly used in MAE**

Solvent	Dielectric constant (ε)	Dielectric loss (tan δ) x 10^{-4}
Water	78.3	1570
Methanol	32.6	6400
Ethanol	24.3	2500
Acetone	20.7	-
Hexane	1.89	-

*Determined at 20°C; [Mandal V; Mohan, Y. and Hemalatha, S., 2007].

Appendix 6: Absorption maxima of different secondary metabolites

Class of secondary metabolites	Name of compound	λ_{max} (nm)
Phenols	Catechol	279 (Et)
	Hydroquinone	295 (Et)
	Orcinol	276, 282 (Et)
	Resorcinol	276, 283 (Et)
Alkaloids		250-303
Flavaonids		280-400
Phenolic acids	Gallic acid	272 (Et)

	Salicylic acid	235, 305 (Et)
Flavonols	Aurones	370-340
	Anthocynidine	270-280, 465-550
	Chalcones add yellow flavanols.	220-270, 340-390

[Harborne, 1998 ; Seigler, 1998 ; Raman, 2006 ; Bhatt, *et. al*., 2005 ; Scott, 1964 ; Cseke, *et. al*., 2006]

Appendix 7 : McFarland Standards

0.5 McFarland Standard : Add 0.5 mL of 0.048 M BaCl2 (SD Fine Chemicals, Mumbai) (1.17% w/v BaCl2.2H2O) to 99.5 mL of 0.18 M H2SO4 (1% w/v) with constant stirring. Record the O.D of the solution; it should be in the range of 0.08 to 0.1 at 625 nm. It should be stored in dark, away from sunlight.

McFarland Scale	**No. Bacteria (x 10^6 / mL)**
1	**300**
2	**600**
3	**900**
4	**1200**
5	**1500**
6	**1800**
7	**2100**
8	**2400**
9	**2700**
10	**3000**

[http://biology.fullerton.edu/biol302/302labf99/quant.html#mcfar]

Appendix 8 : Reagent and media preparation:

- 700 mM Sodium carbonate : 74.193g of Sodium carbonate is added to 1000 mL distilled water.
- 10% v/v FC reagant : 1mL 2 N FC reagent is diluted upto 10mL. This reagent is carcinogenic hence precautions were taken while handling.
- 10% Ammonium hydroxide (SD Fine Chemicals, Mumbai) : 10 mL ammonium hydroxide in 90 mL distilled water.

- ❖ **Nutrient agar :** 40 g nutrient agar (HiMedia, Mumbai) in 1000 mL distilled water.
- ❖ **Mueller Hinton agar :** 38 g of MH agar (HiMedia, Mumbai) in 1000 mL distilled water.
- ❖ **Mueller Hinton broth** : 21 g of MH broth (HiMedia, Mumbai) in 1000 mL distilled water.

All the media were sterilized by autoclaving at 121°C, 15 psi for 15 min.

All the media contained vegetable peptones.

Appendix 9 : Definitions

Extraction efficiency : The amount of the target compound transferred into the solvent) [Chen L. *et.al.,* 2007]

Minimum inhibitory concentration (MIC): The lowest concentration of test agent that inhibits the visible growth of test organism during overnight incubation [Jorgenson and Ferraro, 1998 ; Greenwood, Slack, and Peutherer, 2002]

IC $_{50}$: The concentration of a compound needed to reduce population growth of organisms by 50% in vitro [Barile, 2004].

Total activity: Total activity is a measure of the amount of material extracted from plant in relation to the MIC of the extract, fraction or isolated compound [Eloff, 2004].

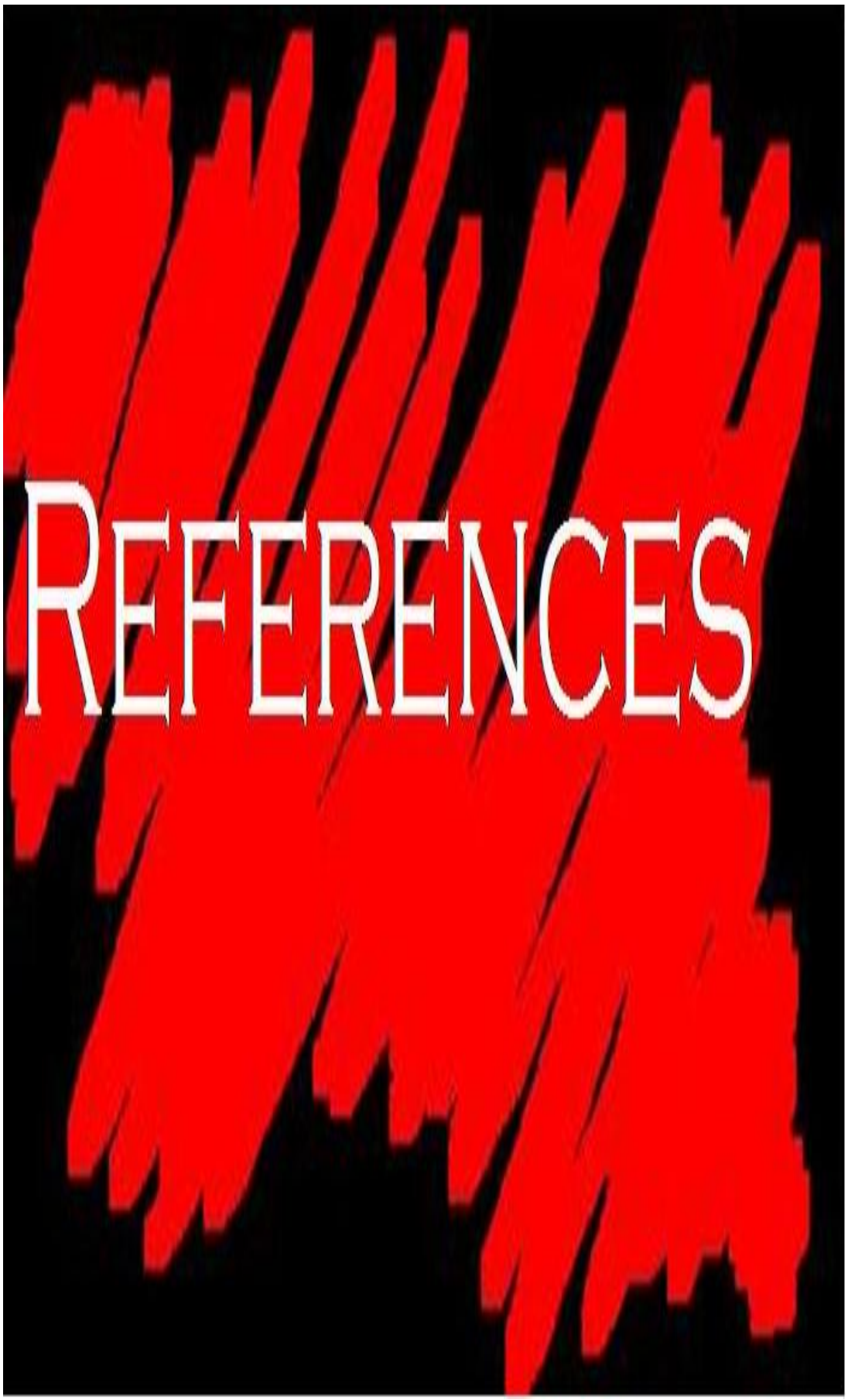
Synergistic activity: Synergistic activity is the enhanced performance of the mixture of two or more components compared to the simple additive performance of the components at the same concentration [Houghton and Raman, 1998].

Retardation factor (R_f) : The retardation factor (R_f) i.e. the distance travelled by the solute divided by the distance travelled by the solvent from the origin where sample was loaded [Stahl, 2005].

hR_f : R_f value multiplied by 100 is referred to as hR_f. R_f value is quoted in the form of hR_f as a whole number is used to describe qualititative description of thin-layer chromatograms [Deinstrop, 2007].

Cut-off value : The cut-off value is the UV absorbance value of a particular solvent [Schmidt, 2005].

Broad Spectrum : A word used to denote drugs that affect many different types of bacteria, both gram- positive and gram- negative [Talaro, 2008].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