Phytochemical Screening and Comparative Study of Antibacterial Assays of *Carum roxburghianum* and *Trigonella foenum-graecum* Obtained From Plant Seed

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Dedicated To

My Beloved Family

DECLARATION BY THE RESEARCHER

I hereby somberly declare that the research work embodying the results reported in this thesis entitled **"Phytochemical Screening and Comparative Study of Antibacterial Assays of *Carum roxburghianum* and *Trigonella foenum-graecum* Obtained From Plant Seed"** submitted by the undersigned has been carried out under the supervision of **Ms. Zubaida Marufee Islam**, Lecturer, Biotechnology program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further affirmed that the research work presented here is original and has not been submitted and approved for the award of a degree by this or any other University. It is also to be declared that the research work presented here is based on actual and genuine work carried out by me. Information sources or reference to research works performed by other people or institution have been duly cited and referenced.

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ABSTRACT

To control the infections and microbial food spoilage the application of chemical or synthetic antimicrobial agents is one of the ancient techniques for which an enormous number of microbes are escalating resistance to synthetic antibiotics. Therefore, there has been immense concern in the development of efficient and nontoxic antimicrobial agents from natural sources, such as extracts of plants for skirmishing comprised health condition and food preservation. In this current research work, the crude extracts of *Carum roxburghianum* (radhuni) and *Trigonella foenum-graecum* (fenugreek) were collected using two solvents namely Ethanol and Methanol. Antibacterial effects of ethanol and methanol extracts taken of second, fifth and seventh days and observed on selected eleven bacteria where extracts of different days had different effects on six bacteria for radhuni showing positive result against *Shigella flexinera*, *Salmonella typhi*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus* and *Streptococcus pneumonae* and fenugreek showed positive result only for *Bacillus cereus.* The ethanol extracts of both seeds showed the greatest positive result where Radhuni from the fifth day against *Bacillus subtilis* and Fenugreek from the seventh day against *Bacillus cereus.* Moreover, comparing the antibacterial activities between radhuni and fenugreek seed extract, the zone of inhibition was greater for radhuni than fenugreek against *Bacillus cereus.* The activity index of ethanol extract of day five was the highest against *Shigella flexineri* indicating high sensitivity to the extract. Phytochemical assay such as tests for alkaloids, terpenoids, tannins, saponins, steroids, phenolic compounds, flavonoids and cardiac glycosides was performed on both seed extracts to identify the secondary metabolites present in them. The results achieved from this research can act as a milestone in exploring the new arena of antimicrobial properties of both extracts chiefly for radhuni extracts. This study could be the starting of discovering some novel and less expensive microbial agent against various bacterial species.

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LIST OF ABBREVIATIONS

The following abbreviations have been used throughout the text:

AI	Activity Index
$BaCl_2.H_2O$	Barium chloride dehydrate
BRAC	Bangladesh Rural Advancement Committee
et al	And Others
g	Gram
H_2SO_4	Sulphuric acid
MDR	Multi Drug Resistant Bacteria
Ml	Milliliter
Mm	Millimeter
MRSA	Methicillin resistant *Staphylococcus aureus*
NA	Nutrient Agar
NaCl	Sodium chloride

Chapter One

INTRODUCTION

INTRODUCTION

From ancient time plants have been used as a resource of medicines that form the backbone of human health care. The use of different parts of plants as a source of respite from illness can be traced back over five millennia to written documents of the early civilization in China, India and the near east but it is doubtless an art as old mankind. (War, 1978) Despite incredible advances witnessed in modern medicine through synthesis, correspondingly aromatic plants are persistently getting significance in remedial of diseases and comfort of human. Spices have been used for not only flavor and aroma of the foods but also to endow with antimicrobial properties to treat chronic as well as infectious diseases (Nanasombat *et al*., 2002).

1.1 Description of the plants:

1.1.1 *Carum roxburghianum*:

Carum roxburghianum is an erect, usually much branched herbaceous annual or biennial plant growing 30 – 100 cm tall. It has feathery well developed leaves on long expanded petioles bearing white flowers (flowering: December-February) and occurs in compound umbels. The seed is small, 1 to 1.5mm long and brown in color (Khalsa *et al.,* (2008), p123). It is commercially known as 'Radhuni' in Bengali and close relative to Ajwain and in English it is called similar to 'Wild celery'.

It is a spice which is strongly aromatic and medicinal plant that comes under genus Carum out of 192 species and belonging to the family Apiaceae. It is native to tropical Asian countries such as India, Pakistan, China, and Indonesia, some regions of Africa and also cultivated in Bangladesh. (Minosuke, 1958; Heinrich *et al*., 2003; Solomon *et al.,* 2011).

C. roxburghianum contain 1.8-2% essential oil. Plant oils and extracts have been used for a large range of purposes for several thousands of years. (Paul *et al.,* 2013)) The seeds are eminent for their medicinal properties, typically due to the essential oil. The essential oil,

Radhuni oil, or its emulsion in water, are the main constituents of “grip water” and considered to be aromatic, carminative specially useful in flatulence, collie pain, Anti-diarrheal, Anti-tumor, Anti-oxidant, CNS, Diuretic, Cardiovascular, Hypotensive, vomiting, Spasmolytic and hiccups of infants and children. (Khan *et al.,* 2012). Due to all these benefits, it is one of the important spices used in traditional medicine. But still various other modern medicinal properties are not extensively known and hence mostly used in the preparation of Ayurvedic medicine.

1.1.2 Scientific Classification of *Carum roxburghianum*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Apiales
Family	Apiaceae (Umbelliferae)
Genus	*Trachyspermum* (*Carum)*
Species	*T. roxburghianum (C. roxburghianum)*

Binomial name: *Trachyspermum roxburghianum*

Figure 1.1: The external morphology of Radhuni

Source: Google "*C. roxburghianum*"

Figure 1.2: Radhuni herb

Source: Google "*C. roxburghianum*"

Figure 1.3: Extract of *C. roxburghianum*

1.1.3. *Trigonella foenum-graecum*

Trigonella foenum-graecum is an erect annual herbaceous plant growing 20-50 cm (up to 100 cm) long, survives only one growing season. It has trifoliate well developed petiolated compound leaves bearing yellowish white flower (flowering: April-May) 12-18 mm long (1-2) are in the leaf axils arranging single or little groups of twos. (Simon, J. E *et al,* 1984) The seed is small, 2 to 3mm long and yellowish golden in color with a strong odor. The chemical components of fenugreek seed include saponins, coumarin, fenugreekine, nicotinic acid, phytic acid, scopoletin and trigonelline, iron, vitamins, phosphates and alkaloids (Sheikhlar A, 2013) Commercially it is known as 'Methi' in Bengali and in English it is called 'Fenugreek'.

The plant falls under the family of Fabaceae (Leguminosae) and indigenous to the eastern shores of the Mediterranean Sea, but it is grown in South Asian countries like India, Pakistan and Bangladesh, Morocco, Southern France, Lebanon, Egypt and England. ((Flammang *et al*., 2004) It is one of the most practiced and oldest traditional medicinal plants in Ayurveda. (Basch *et al*., 2003)

Fenugreek herb's seeds acquire toxic oils and other bioactive constituents comprise volatile oils and alkaloids in it have been shown to be toxic to bacteria parasites and fungi. The fenugreek seeds are loaded with dietary fiber that can lower blood sugar levels in diabetes. Moreover, it is extensively used as a galactagogue that is often used to boost up production of milk in lactating women and to cure breast cancer. (Sherif *et al*., 2015) Fenugreek seed is useful for tuberculosis, diabetes, atherosclerosis, constipation, high cholesterol, hyper triglyceridemia and externally it is used as a poultice for abscesses, boils, carbuncles, etc. (Sahay *et al.,* 1994) Due to all these reimbursements, it is one of the ancient condiments used in traditional medicine.

1.1.4 Scientific Classification of *Trigonella foenum-graecum:*

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta - Vascular plants
Superdivision	Spermatophyta - Seed plants
Division	Magnoliophyta - Flowering plants
Class	Magnoliopsida - Dicotyledons
Subclass	Rosidae
Order	Fabales
Family	Fabaceae - Pea family
Genus	*Trigonella* L. - fenugreek
Species	*Trigonella foenum-graecum* L.

Figure 1.4: The external morphology of Fenugreek

Source: Google "*Trigonella foenum-graecum*"

Figure 1.5: Fenugreek herb

Source: Google "*Trigonella foenum-graecum*"

Figure 1.6: Extract of "*Trigonella foenum-graecum*"

1.2 Therapeutic Use of Radhuni and Fenugreek:

Conventionally, various antimicrobial agents and pulp therapeutic agents are being tried in endodontic treatments. But the key drawbacks have been their undesirable adverse effects on the host like immune suppression, hypersensitivity and allergic reactions and continuous evolutions of bacterial resistance to the drugs. On top, the traditional pulp therapeutic agents also have the potential to be carcinogenic, mutagenic, allergic, cytotoxic and other health hazards. (Chandrabhatla *et al*., 2012). Hence, there is a growing concern throughout the world in integrating herbals in health care services by adopting extensive studies to find plant based alternatives for the conventional drugs.

Different antimicrobial activities are shown by different herbal extracts. Plants have a broad range of secondary metabolites such as tannins, alkaloids and flavonoids, saponins which have been bring into being to have antimicrobial properties *in vitro* (Khan *et al*, 2009).

The pharmacological substantiations explains, radhuni's employ in traditional method of medicine to treat diarrhea, abdominal spasm (colic), asthma, bronchitis cough, common cold, dyspepsia, lethargy, loss of consciousness, palpitation, vomiting, pain in bladder and kidneys as well as considered useful as anthelmintic, antigout, antimicrobial, cardiotonic, carminative, condiment, digestive, emmenagogue, stimulant and stomachic (Khan *et al.*, 2012). Essential oil and crystalline substance decreased blood pressure in dogs and rats due to direct action on blood vessels. Fruits left after extraction of essential oil showed marked cardio tonic activity. (Abedin, 2012)

On the other hand, apart from the different usage in bakery products, frozen dairy products, condiments, spices, pickles, and beverages, the other plant; fenugreek is known to have several advantageous health effects. Gastric ulcers can be handled through fenugreek seeds as it sooths the gastro intestine tract The cleansing process of fenugreek makes it a precious plant as it helps purify blood, cleaning lymphatic system, and detoxify the body. Moreover, the seed oil acts as an emollient and makes pores and skin smoother and soft. In ailments like hay fever and sinusitis it can be used. The seeds are considered useful in heart sickness and aphrodisiac and as a galactogogue promoting lactation in breast feeding mother and help in breast cancer (Tiran, 2003). The smallpox patients are also given an infusion of seeds as a cooling agent. As a natural health product, it is competent of treating and curing diseases for which, it has been well thought-out as a potential nutraceutical (Kalra, 2003). Other than these traditional medicinal uses, fenugreek is found to have numerous pharmacological properties such as antidiabetic, antinociceptive, anticarcinogenic, antioxidant, anti-inflammatory, and hypocholesterolemic.

1.3 Antimicrobial Properties of Radhuni and Fenugreek:

For controlling infections and microbial food spoilage, using chemical or synthetic antimicrobial agents is one of the ancient methods. Patrons are more conscious about the safety of foods containing chemical preservative. There is growing evidences about adverse effects of chemical preservatives on human health, so continuous pressure has been developed to trim down the quantity of added preservatives in foods (Bukvicki *et al.*, 2014; Tyagi *et al.*, 2014). Therefore,

there has been immense interest in the development of efficient and nontoxic antimicrobial compounds from natural sources, such as extracts of plants, for food preservation (Shan *et al*., 2007).

The antibacterial activities of the cold aqueous, hot aqueous, ethanol, methanol and acetone extracts of *Carum roxburghianum* (Radhuni) extracts were tested against two pathogenic bacteria namely *Serratia marcescens* and *Bacillus cereus* and one yeast, *Rhodotorula mucilaginosa* by well diffusion method and the finding suggest positive activity against all the species. (Dhiman *et al*, 2015)

In another study, the antimicrobial activity of essential oil of *Carum roxburghianum* was determined by using Agar Diffusion Method and was checked on six bacteria's. These were *Bacillus subtilis, Bacillus lichneformis, Escherichia coli, Micorococcus luteus, Nocardia asterides* and *Salmonella typhimurium* from which *Bacillus subtilis, Nocardia asterides* and *Salmonella typhimurium* showed positive result and rests showed negative activity. (Iqbal *et al,* 2014)

There is an extensive similar another spice which is very close relative of *Carum roxburghianum* and most of the researches have been conducted with that spice named '*Carum Copticum'*. Studies were found that antimicrobial activity of essential oil of *C. copticum* was tested against three pathogenic bacteria and it showed inhibition zone on *S. aureus* (24.00 mm) and *E. coli* (16.75 mm) except *P. aeruginosa.* (Peerakam *et al*, 2014) The effect of aqueous extract of *C. copticum* on several strains of bacteria showed positive antibacterial effect on *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *P. aeruginosa*, *S. typhimurium*, and *Shigella flexneri* (Kaur, *et al,* 2009) Antifungal activity of essential oil of *T. copticum* seeds is also documented against toxigenic *Aspergillus* species. The oil of this plant also is able to inhibit the growth of this parasite. (Alizadeh *et al*, 2010)

The antimicrobial effects of methanolic and aqueous extract of fenugreek seeds was studied by Abdalah against six bacterial species. The extract showed highest activity against *Escherichia coli* while *Proteus vulgaris*, showed lowest response. Moderate response was showed against

Staphylococcus aureus, Pseudomonas Aeuroginos, Streptococcus pyogeenes and *Klebsiella pneumoniae* showed negative response. (Abdalah, 2011)

Another study was conducted with aqueous, ethanol, benzene, chloroform, hexan and petroleum ether extracts of fenugreek and antimicrobial activity was done with agar well diffusion method against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsilla* and showed negative results for all. (Al- abdeen *et al*, 2010)

In a study by Dash et al. antimicrobial activity of crude methanol and acetone extract of fenugreek was tested against four bacteria and the process included disk diffusion method. It was seen that methanol extract of fenugreek seed showed positive results for all four bacteria that include *Pseudomonas* spp.*, Escherichia coli, Shigella dysentiriae* and *Salmonella typhi* and exhibited highest zone of inhibition (10 mm) against *Pseudomonas spp.* Whereas, for acetone extract it gave positive results for three and exhibited highest zone of inhibition (9 mm) against *E. coli*. The negative result came out for *Salmonella typhi.* (Dash *et al*, 2011)

The antimicrobial potential fenugreek extract was tested against *Escherichia coli (E. coli), Proteus vulgaris (P. vulgaris), Staphylococcus aureus (St. aureus),* and *Candida albicans (C. albicans)* using two different solvents: aqueous extractions (cold, hot & boiling) and methanol extractions and were evaluated using two different methods: agar disc diffusion and agar-well diffusion method. The results indicated that only the boiling water extract contains the antimicrobial active ingredients of fenugreek seeds against *St. aureus* only, while both cold water extract and methanol extract did not show any activity against any of those bacteria. (Sherif *et al*, 2015)

Countless of the food-borne pathogens are escalating defiant against antibiotics. With the rise in the emergence of various multidrug resistant microorganisms and the scenario worsening through the indiscriminate use of antibiotics, new and/or alternative antimicrobial compounds must be developed to treat general infections. With the changing patterns of susceptibility and the availability of new antimicrobial agents, continuous updating of knowledge concerning treatment of disease caused by such pathogens is required. (Khan *et al*, 2010)

1.4 Effects of Radhuni and Fenugreek on Selected Bacteria:

With the intension of developing modern novel therapeutics, from last few decades scientists have been focusing on natural extracts to assess the antimicrobial properties. A number of plant systems have been studied by the researchers which exhibited antimicrobial action. Among diverse ranges of herbal extracts, radhuni (very less studied) and fenugreek (more studied) are also two of the candidates that have been tested for its activity against ample range of microorganisms like bacteria, virus, and fungus. Sensitivity of the extracts towards bacteria is reliant not only on the solvent system but also on the type of microorganisms used in the experiments.

To scrutinize antibacterial effect of radhuni and fenugreek extracts eleven different bacteria strains were selected which includes: *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, Klebsiella *sp.*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexinera*, *Staphylococcus aureus*, *Streptococcus pneumonae*, *Enterobacter cloacae* and *Aeromonus hydrophila*

1.4.1 ***Bacillus cereus*** is a gram-positive, rod-shaped, aerobic or facultatively anaerobic, motile, spore-forming, bacterium; widely distributed environmentally. It is mostly connected with food poisoning; but gradually more reported to be a cause of severe and potentially fatal non-gastrointestinal-tract infections due to release of some toxins. (Bottone EJ, 2010)

1.4.2 ***Bacillus subtilis*** is a gram-positive, rod-shaped bacterium; naturally originate in soil, vegetation and within the gastrointestinal tract of human. It grows in the mesophilic temperature range therefore; it has evolved a set of strategies that allow survival under these harsh conditions such as the formation of stress-resistant endospores. (Entrez Genome Project)

1.4.3 ***Escherichia coli*** is a gram negative commensal bacterium of humans and animals. Pathogenic alternates cause intestinal and extra intestinal infections, including gastroenteritis, urinary tract infection; meningitis, peritonitis, and septicemia (D.A *et al*, 2012) Depending on the category of infection therapeutic options vary.

1.4.4 *Klebsiella* spp. is gram negative rods, non-motile, aerobic and facultatively anaerobic organism. They are pervasive all through the environment and also carried by humans. Moreover, they are well-recognized community and nosocomial pathogens causing noteworthy infections. They are a common cause of respiratory and nonrespiratory infections. They are resistant to penicillin and can obtain resistance to third- and fourth-generation cephalosporin. (Bouza E, 2002)

1.4.5 *Pseudomonas aeruginosa* is gram-negative, aerobic, versatile "blue-green pus bacterium" that opportunistically infects people, particularly those who are immune compromised. They seldom cause infection in healthy individual. They generally found in the soil, marshes, and coastal marine habitats. As part of chronic infection *P. aeruginosa* forms biofilms, which shield the encased bacteria from host immune clearance and endow with an impermeable and protective barrier against currently available antimicrobial agents and make it multi drug resistant strain. (Smith D.J *et al.,* 2013)

1.4.6 *Salmonella typhi* is gram-negative enteric bacillus, motile, facultative anaerobe that is usually found in the intestinal tracts of humans and animals causing a serious sickness called 'typhoid fever'. At present, 107 strains of this organism have been isolated; loads of containing varying metabolic characteristics, levels of virulence, and multi-drug resistance genes that obscure treatment in areas that resistance is prevalent. (David, 2003)

1.4.7 *Shigella flexinera* is a gram-negative bacterium that is major cause of diarrheal diseases (shigellosis) and mortality in developing countries in humans. The frequency of strains multiply resistant to ampicillin, trimethoprim-sulfamethoxazole, and streptomycin is causing growing concern till to date. (Ahamed J and Kundu M, 1999)

1.4.8 *Staphylococcus aureus* is a facultative anaerobic, nonmotile, non-spore forming, gram-positive cocci which occur singly, in pairs, and irregular clusters. Humans are generally colonized by this on peripheral skin surfaces and the upper respiratory tract, particularly the

nasal passages. The strains are called methicillin-resistant *Staphylococcus aureus* (MRSA) as they can rapidly build up resistance against antibiotics such as penicillin, methicillin, amoxicillin and oxacillin. (Stapleton and Taylor, 2007)

1.4.9 ***Streptococcus pneumonae*** is a gram-positive, lancet-shaped bacteria which grow in pairs or short chains and well known for causing 'pneumococcal pneumonia'. It is found in nasopharyngeal region. The incidence of pneumococcal disease is highest in infants under 2 years of age and in people over 60 years of age. The pathogen is becoming resistant to antibiotics like penicillin, erythromycin, trimethoprim-sulfamethoxazole, and tetracycline. (Snippe *et al*, 1995)

1.4.10 ***Enterobacter cloacae*** are gram-negative, facultative anaerobic, non-spore-forming, rod-shaped bacteria that live in the mesophilic environment and use their peritrichous flagella for movement. They are important opportunistic and multi resistant bacterial pathogens for humans. Central resistance to ampicillin and narrow-spectrum cephalosporins and exhibits a high frequency of mutation to resistance to expanded-spectrum and broad-spectrum cephalosporins. (Silva R M, *et al*., 1998)

1.4.11 ***Aeromonus hydrophila*** are gram-negative, facultative anaerobic, rod-shaped, nonspore forming bacteria that broadly dispersed in aquatic environments. They attain some virulence factors which are associated with human diseases, such as gastroenteritis, soft-tissue, muscle infections, septicemia, and skin diseases as a result they are fetching the place of enteric pathogens and holding solemn public health concern. (Graevenitz A.V, 2007)

1.5 Selected Phytochemical Properties of Radhuni and Fenugreek:

The medicinal plants are valuable for healing and curing of human diseases because of the existence of phytochemical constituents. These constituents are naturally happening in the

medicinal plants, leaves, vegetables and roots that include defense mechanism and shield from different diseases. Phytochemicals are categorized depending on their function in plant metabolism as both primary and secondary compounds (essential and optional mixes). Chlorophyll, proteins and common sugars are included in primary constituents whereas secondary compounds have alkaloids, terpenoids, steroids, flavonoids, phenolic compounds etc. (Krishnaiah *et al,* 2007)

To examine the existence of alkaloids, terpenoids, tannins, saponins, steroids, phenolic compounds, flavonoids and cardiac glycoside in both radhuni and fenugreek; the phytochemical assay was demonstrated.

1.5.1 Alkaloids: These are a heterogeneous class of secondary metabolites and defined as basic (alkali-like), nitrogen-containing organic constituents. These usually exist as salts of organic acids, glycosides of sugar etc. Extensively used in medicine and provide lead structures for novel synthetic drugs.

1.5.2 Terpenoids: These metabolites are unsaturated hydrocarbons that pursue a diversity of essential functions in growth and development but the majority of terpenoids are being used for more specific chemical interactions and fortification in the abiotic and biotic environment. Human exploit these metabolites conventionally in the food, chemical industries, pharmaceutical. In recent times, in the development of biofuel products these are being employed.

1.5.3 Tannins: These secondary metabolites are polyphenolic substances having high molecular weight; present in various plants artifact. The water-soluble nature enables simple extraction and is handy in diverse applications in the chemical and pharmaceutical industry. Plant parts having tannins comprise wood, leaves, bark, fruit, fruitpods, roots, and plant galls.

1.5.4 Saponins: These are glucosides that found principally from plants as in vegetables, beans, and herbs. These owe foaming aptitude caused by the combination of a hydrophobic (fat-soluble)

sapogenin and a hydrophilic (water-soluble) sugar part. Because of that; these have been used for thousands of years as natural detergents. Moreover, commercially these are introduced in the photography industry as photographic emulsions and an important ingredient in cosmetic and personal care products also being recurrently used in beer and root beer beverages to provide better foaming power.

1.5.5 Steroids: These are the components of membranes in plant system. These form a set of secondary metabolites having low molecular weight and miscellany in their structure and biological functions. These natural products, even though often associated with the venomous consequence on health, posses numerous medicinal applications such as being hormonally vibrant essence that reproduce pregnancy acting as anti fertility, prophylactic mixes, anti inflammatory medications etc.

1.5.6 Phenolic compounds: These are aromatic benzene ring containing chemical compounds which role in plant physiology and interactions with biotic and abiotic environments are difficult to overestimate. But these play a vital function in plant development by providing structural integrity and scaffolding support to plants. These have well proved antioxidant activity which depends on the structure. These also facilitate managing of human pathogenic infections via plants by serving as defenses against herbivores and pathogens.

1.5.7 Flavonoids: These are extensively circulated secondary metabolites and from a various group of phytonutrients (plant chemicals) which are potent antioxidants with anti-inflammatory and immune system benefits. These are considered to serve health benefits through cell signaling pathways thus recently diets rich in flavonoid-containing foods are at times linked with cancer, neurodegenerative and cardiovascular disease prevention.

1.5.8 Cardiac glycoside: These are one of the various glycosides attained primarily from plant resources used vastly being highly explicit and powerful attribute in medicine to boost up the strength of contraction of heart muscle, to regulate heartbeats and for treatment of atrial fibrillation and shudder.

1.6 Objectives and Aim of the Study:

Nevertheless, several synthetic drugs or antibiotics against such microorganisms have manufactured by numerous pharmaceuticals and other medicinal companies but since many eras the escalating number of multi drug resistant bacteria (MDR) is becoming immense concern in fighting comprised health status in medicine world. Laying in such situation; different parts of plants like seeds of radhuni and fenugreek posses' some antimicrobial properties and phytochemical constituents which can definitely light a hope not only in the treatment of infections rooted by bacteria but also in constructing novel antibiotics extorted from these products of nature.

Hence, this study stands by with some precise objectives which include:

- Isolation of ethanol and methanol extracts of radhuni (*Carum roxburghianum*) and fenugreek (*Trigonella foenum-graecum*)
- Observation of the antimicrobial properties of the both seed extracts on eleven selected microorganisms
- Comparison of zone of inhibition of 2^{nd},5^{th} and 7^{th} day seed (radhuni and fenugreek) extracts (ethanol and methanol) in selected microbs
- Comparison of the antimicrobial activity between the both seed extracts
- Determination of selected phytochemical assay

The aim of the study was to examine the antimicrobial activities of radhuni and fenugreek against eleven selected bacteria; on the ethanol and methanol extracts and to ensure the existence of secondary metabolites by executing a number of phytochemical assays.

Chapter Two

MATERIAL AND METHOD

MATERIAL AND METHOD

2.1 Working Laboratory

As the research was a solely laboratory based investigative work so everything in this research was executed in the Microbiology Specialized Research Laboratory, Department of Mathematics and Natural Sciences, BRAC University, Mohakhali, Dhaka. The Laboratory follows Biosafety Label 2 (BSL-2) facility. Therefore, all the microbiological works were accomplished within Biological Safety Cabinet under proper supervision.

2.2 Collection and Reference of Materials

Both of the seeds; radhuni and fenugreek were bought from local market of Dhaka city. All the reagents, chemicals and apparatus being used were from Microbiology Specialized Research Laboratory, Department of Mathematics and Natural Sciences; where few reagents were prepared fresh within the laboratory only. During the research; clinical strains of selected eleven bacteria and several antibiotics were used which were taken from the conserved bacterial stock in the departmental laboratory.

2.3 Processing of the Seeds

Both of the seeds were processed exactly in a same procedure during the whole study. Seeds were sundried for 5-7 days and then to make powder of seeds without any impurities; grinder was used and stored the seeds powder in air tight box separately at the laboratory at room temperature for further works.

2.4 Extraction

Two types of extraction was done with the radhuni and fenugreek seed powder; extracted in ethanol and methanol.

2.4.1 Ethanol

20 gm of radhuni powder was taken; weighing by an electric weighing machine and then put in a beaker with 100 ml of ethanol, the powder was mixed and stirred with a glass rod for about 30 minutes continually to ensure proper mixing. After that, with foil paper the beaker was covered, sealed and kept in a dark chamber for 2 days with proper labeling. Exactly, the same procedure was followed for another two sets of beakers and kept for 5 and 7 days correspondingly.

The stuffs of the beakers were filtered, through Whatman No.1 filter paper right after 2, 5 and 7 days and then concentrated in a water-bath at about 80°C temperature until the contents of the beaker became pourable into a petri-dish that is about 20 ml and then put it back in the water-bath to obtain oily sticky substances which is the product of interest. The dish was taken out of the water-bath and stored in vial (previously washed with ethanol) by means of scooping with spatula. At last, vial was labeled and stored below 10 ℃ in the refrigerator for further use. The whole process was repeated multiple times for the collection of a substantial amount of extracts for the study.

Just like this ethanol extraction process of radhuni seeds, fenugreek seed extract in ethanol was done independently.

2.4.2 Methanol

100 gm of radhuni powder was taken; weighing by an electric weighing machine and then put in a beaker with 500 ml of methanol, the powder was mixed and stirred with a glass rod for about 30 minutes continually to ensure proper mixing. After that, with foil paper the beaker was covered, sealed and kept in a dark chamber for 2 days with proper labeling. Exactly, the same

procedure was followed for another two sets of beakers and kept for 5 and 7 days correspondingly.

The stuffs of the beakers were filtered, through Whatman No.1 filter paper right after 2, 5 and 7 days and then concentrated in a water-bath at about 80°C temperature until the contents of the beaker became pourable into a petri-dish that is about 20 ml and then put it back in the water-bath to obtain oily sticky substances which is the product of interest. The dish was taken out of the water-bath and stored in vial (previously washed with ethanol) by means of scooping with spatula. At last, vial was labeled and stored below 10 ºC in the refrigerator for further use. The whole process was repeated multiple times for the collection of a substantial amount of extracts for the study.

Just like this methanol extraction process of radhuni seeds, fenugreek seed extract in methanol was done discretely.

2.5 Fresh Nutrient Agar (NA) Plates Preparation

For detecting the antimicrobial properties of radhuni and fenugreek seeds, different bacterial culture were required and for that lots of nutrient agar plates were prepared.

Nutrient agar media was freshly prepared by adding 28 gm of nutrient agar powder in 1000 ml of distilled water. In a conical flask, maintaining the proportion at good; the required amount of agar was prepared and placed onto a bunsen burner, until the boiling point was gained; continually stirred with a glass rod. Now, underneath of the conical flask diminutive bubbles formed which rose up and the solution progressively turned crystal clear. The flask was kept to cool down for a while by confiscating from the heat. After that, with aluminum foil; the top of the flask was sealed, labeled and autoclaved for about 1.5 hours to sterilize and remove all impurities. The autoclaved nutrient agar solution was poured vigilantly into labeled petri dishes under the laminar air flow chamber as soon as it was taken out. The petri-dishes were about 20 ml per medium sized plates or 30 ml per large sized plates with proper labeling holding the name of the agar, the initials of the person who made the agar and the date when it was made.

To solidify and store for further use, the plates were kept in the refrigerator. Since then, nutrient agar plates were prepared whenever it was needed keeping everything constant.

The composition of Nutrient agar is as follows:

- 0.5% Peptone
- 0.3% beef extract/yeast extracts
- 1.5% agar
- 0.5% NaCl
- Distilled water
- pH is adjusted to neutral (7.4) at 25 °C.

2.6 0.5% McFarland Standard Solution Preparation

To visually evaluate the turbidity of bacterial suspension McFarland standard solution was used. This solution endows with a reference for standardization of bacterial suspensions that require a standardized inoculum.

To formulate the McFarland standard solution, 0.05 ml of 1.175% barium chloride dehydrate ($BaCl_2.H_2O$) was mixed with 9.95 ml of 1% sulphuric acid (H_2SO_4).

To compose 1 ml of 1.175% barium chloride dehydrate, 0.01175 gm of barium chloride dehydrate is dissolved in 1 ml of distilled water. The laboratory has 97% of sulphuric acid hence it is needed to convert it into 1%, so the formula used to make 1% sulphuric acid (H_2SO_4) from the 97% sulphuric acid is given below:

Concentration 1 x Volume 1 = Concentration 2 x Volume 2

Where,

- Concentration 1 is 97% of sulphuric acid
- Volume 1 is unknown volume of the same acid
- Concentration 2 is 1% sulphuric acid

- Volume 2 is 9.95 ml of the subsequent acid solution.

Now, 0.10258 ml of 97% sulphuric acid is taken and till the volume reaches 9.95 ml, distilled water is added. Then finally to make the standard solution 0.05 ml barium chloride dehydrate is added to the sulfuric acid.

2.7 Saline Solution Preparation

0.9% saline solution was needed in the experiment and to make that 100 ml of distilled water was taken where 0.9 gm of sodium chloride (NaCl) was poured and stirred with glass rod. Multiple test tubes were taken and about10 ml of the saline solution was put in each test tube and autoclaved, with the screw cap opened through 1.5 turns. After removing from autoclave machine, the screw caps were turned fully to shut the mouth of the tube so that the saline does not get contaminated and stored safely for further use upon necessity.

2.8 Bacterial Sub-culturing Process

First of all, selected eleven microorganisms from bacterial stock culture present in the laboratory were taken. Then, inside the laminar hood; subculture was done by streaking method on to the NA plates. Streaking was done by taking a loopful of microbes from the stock culture before which the loop was burned till red hot over a bunsen burner flame, cooled it afterwards and streaked on to the labeled NA plates which were then incubated for 24 hours at 37°C temperature.

2.9 Antimircobial Assay

2.9.1 Inoculation of Test Organisms

It was needed to start with inoculation of test microbes for carrying out the antimicrobial assay. A bacterial suspension was made from freshly prepared 24 hours incubated subculture of the

microbes and the method incorporates in burning the loop till red hot, then a loopful of test bacteria was transferred in test tubes containing 10 ml of 0.9% saline to formulate cell suspension, vortex was done for ensuring homogenous mixing. The concentration of the cells in each test tube was optimized by visually compared with 0.5 McFarland solution by holding both of the tubes against a dark background. It was vital to adjust the turbidity of the suspension with the McFarland solution which was done either by adding more loopful bacteria or adding more saline solution. After that, using an autoclaved cotton bud the cell suspension was instantaneously inoculated on properly labeled NA agar plates to do lawn culture. To make sure there was uniform distribution of organisms in the media; the lawn was done multiple times in each plate by rotating them 90º each time.

2.9.2 Agar or Well Diffusion Method

This is the method used in whole research for accomplishing antimicrobial tests. The traditional method is obtaining the previously lawned nutrient agar plate and aseptically punching holes in the agar using a sterile cork borer but in this experiment the holes were created by the back of an autoclaved micropipette tip. Then marking of the holes were done to pour the samples of seed extracts (radhuni and fenugreek) according to the further works by means of separate autoclaved micropipettes. After that, to compare the result; an appropriate positive control of an antimicrobial disc was placed on each plate and at 37°C temperature for 24 hours the plates were incubated. Next, the positive result for antimicrobial tests depended on the existence of a clear zone around the hole. The entire method was replicated discretely for each eleven organisms for both radhuni and fenugreek seed's ethanol and methanol extracts.

2.9.3 Measuring the Inhibition Zone

The zone of inhibition means the width of clear zone around the well and antibiotic disk. It is the most decisive part of the study which (clear zone) was measured three times in millimeter (mm) with a ruler or scale and was noted down to find the average of the three values which was recorded as well.

2.9.4 Measuring the Activity Index

The activity index of the positive results holding methanolic and ethanolic extracts of radhuni and fenugreek were measured by using the following formula:

Activity Index (AI) = Zone of inhibition of extract/ Zone of inhibition of antibiotics

2.10 Preservation of Selected Bacterial Strains

Preserving the selected bacterial strains within accurate sterile conditions was essential to reuse them for repetitive works for a long period of time and hence selected stocks were prepared from the conserved bacterial stock, sealed with Parafilm to ensure that accurate characteristics of the strains remain unchanged and then stored.

2.11 Phytochemical Tests

In the study, for both radhuni and fenugreek seeds; eight different kinds of phytochemical assays were accomplished individually and exactly with the same process. These were for alkaloids, terpenoids, tannins, saponins, steroids, phenolic compounds, flavonoids and cardiac glycoside.

To do the tests, stock solution was prepared for which; in a beaker around 10 gm of the powdered sample was taken with 100 ml of distilled water. This was boiled for about 10 minutes and was filtered while still hot; filtrate was let to cool down and used to perform further tests in the test tubes.

2.11.1 Tests for Alkaloids: A few drops of picric acid is added slowly to 0.5 ml of filtrate. The presence of alkaloids is confirmed by white or creamy precipitation

2.11.2 Tests for Terpenoids: 2ml of chloroform was added in 5ml of filtrate and then 3 ml of concentrated sulfuric acid was incorporated that forms a layer. The presence of terpenoids in the

filtrate is positively indicated by observing reddish brown precipitates at the interface between the two layers

2.11.3 Test for Tannins: 1 ml of filtrate is diluted with 5ml of distilled water and five to six drops of 10% of ferric chloride is added to it. The presence of tannins in the filtrate is positively indicated by formation of brownish-green or bluish-black precipitate

2.11.4 Tests for Saponins: 2.5ml of filtrate is diluted with 10ml of distilled water and shaken robustly for 2 minutes. The presence of saponins in the filtrate is positively indicated by visibility of frothing in the tube

2.11.5 Tests for Steroids: 2 ml of chloroform and 2 ml of sulphuric acid is added gradually to 2 ml of filtrate. The presence of steroids is confirmed by producing red color in the lower chloroform layer

2.11.6 Tests for Phenolic Compounds: 5% of ferric chloride is added to 5 ml of filtrate. The presence of phenolic compound is positively indicated by turning the extract into dark green color

2.11.7 Tests for Flavonoids: A few drops of 20% sodium hydroxide solution are added in 1 ml of filtrate. The presence of flavonoids in the extract is positively indicated by a change of color to yellow. Then acid was added and extract turning back to its native color reconfirmed the test result.

2.11.8 Tests for Cardiac Glycoside: 2ml of glacial acid including 1 drop of ferric chloride solution; is added to 5ml of filtrate. Then gradually by the side of the test tube; 1 ml of sulphuric acid was added. The presence of cardiac glycoside (deoxysugar characteristics) in the extract is

confirmed by formation of a brown ring at the interface. Moreover, in the glacial acid layer; a violet or greenish ring may be seen below that ring.

Chapter Three

RESULT

RESULTS

3.1 Antibacterial Assay

As mentioned earlier; eleven different bacteria were used for analyzing the antimicrobial properties of ethanolic and methanolic extracts of radhuni and fenugreek. Extracts of radhuni showed noteworthy positive results against six microbes which were *S. flexineri, S. typhi, S. aureus, B. cereus, B. subtilis* and *S. pneumonae* whereas extracts of fenugreek showed positive result only against one bacterium that was *B. cereus*; from the eleven test microbes and negative or nondescript results were given by rest.

During the antimicrobial assay; to evaluate the results accurately, positive control was used in form of antimicrobial discs on petri dishes along with different extracts in different amalgamations and to obtain precise result, three replicates of petri dishes were used for each type of combination.

List of antimicrobial discs to respective microbes are given bellow:

- Kanamycin - *B.subtilis* and *Klebsiella* spp.
- Gentamycin - *E.* coli , *P. auriginosa* and *E. cloacae*
- Amikacin - *A. hydrophila*
- Ampicilin - *S. typhii* and *S. aureus*
- Tetracycline - *B. cereus*
- Chloramphenicol - *S. pneumonae*
- Nitrofurantoin - *S. flexneri*

3.1.1 Ethanol Extraction of Radhuni:

In table 3.1.1 and table 3.1.2, antibacterial effect of ethanol extract of radhuni is shown on 2^{nd}, 5^{th} and 7^{th} day extraction where the zone of inhibition for the four microbes *S. flexineri, S. typhi, S. aureus* and *B. subtilis* – was maximum for the 5^{th} day extracts in all three replicates while other two microbes *B. cereus* and *S. pneumonae* showed maximum zone of inhibition for the 7^{th} day extracts in all three replicates.

Table 3.1.1: Positive antimicrobial effects (average zone of inhibition) with positive controls, produced by ethanol extract of radhuni

Extraction Days	Inhibition Zone (mm)					
	Shigella flexinera		***Salmonella typhi***		***Staphylococcus Aureus***	
	Per trial	**Average**	**Per trial**	**Average**	**Per trial**	**Average**
2nd day	17.30	**17.67**	12.00	**12.40**	14.20	**14.34**
	17.50		12.20		14.30	
	18.20		13.00		14.50	
5th day	18.20	**18.60**	17.90	**18.04**	17.20	**17.34**
	18.40		18.10		17.20	
	19.20		18.10		17.60	
7th day	18.00	**18.44**	15.80	**16.07**	17.00	**17.20**
	18.30		16.20		17.30	
	19.00		16.20		17.30	
Positive Controls	17.70	**17.90**	28.00	**28.17**	22.00	**22.14**
	18.00		28.00		22.20	
	18.00		28.50		22.20	

Table 3.1.2: Positive antimicrobial effects (average zone of inhibition) with positive controls, produced by ethanol extract of radhuni

Extraction Days	Inhibition Zone (mm)					
	Bacillus cereus		***Bacillus subtilis***		***Streptococcus Pneumonae***	
	Per trial	**Average**	**Per trial**	**Average**	**Per trial**	**Average**
2nd day	16.20	**16.57**	14.00	**14.40**	13.20	**13.60**
	16.70		14.30		13.40	
	16.80		14.90		14.20	
5th day	19.30	**19.50**	19.80	**20.20**	15.60	**15.67**
	19.60		20.30		15.60	
	19.60		20.50		15.80	
7th day	19.60	**19.74**	18.00	**18.27**	15.50	**15.84**
	19.80		18.40		16.00	
	19.80		18.40		16.00	
Positive Controls	28.50	**28.77**	24.00	**24.00**	29.00	**29.07**
	28.90		24.00		29.00	
	28.90		24.00		29.20	

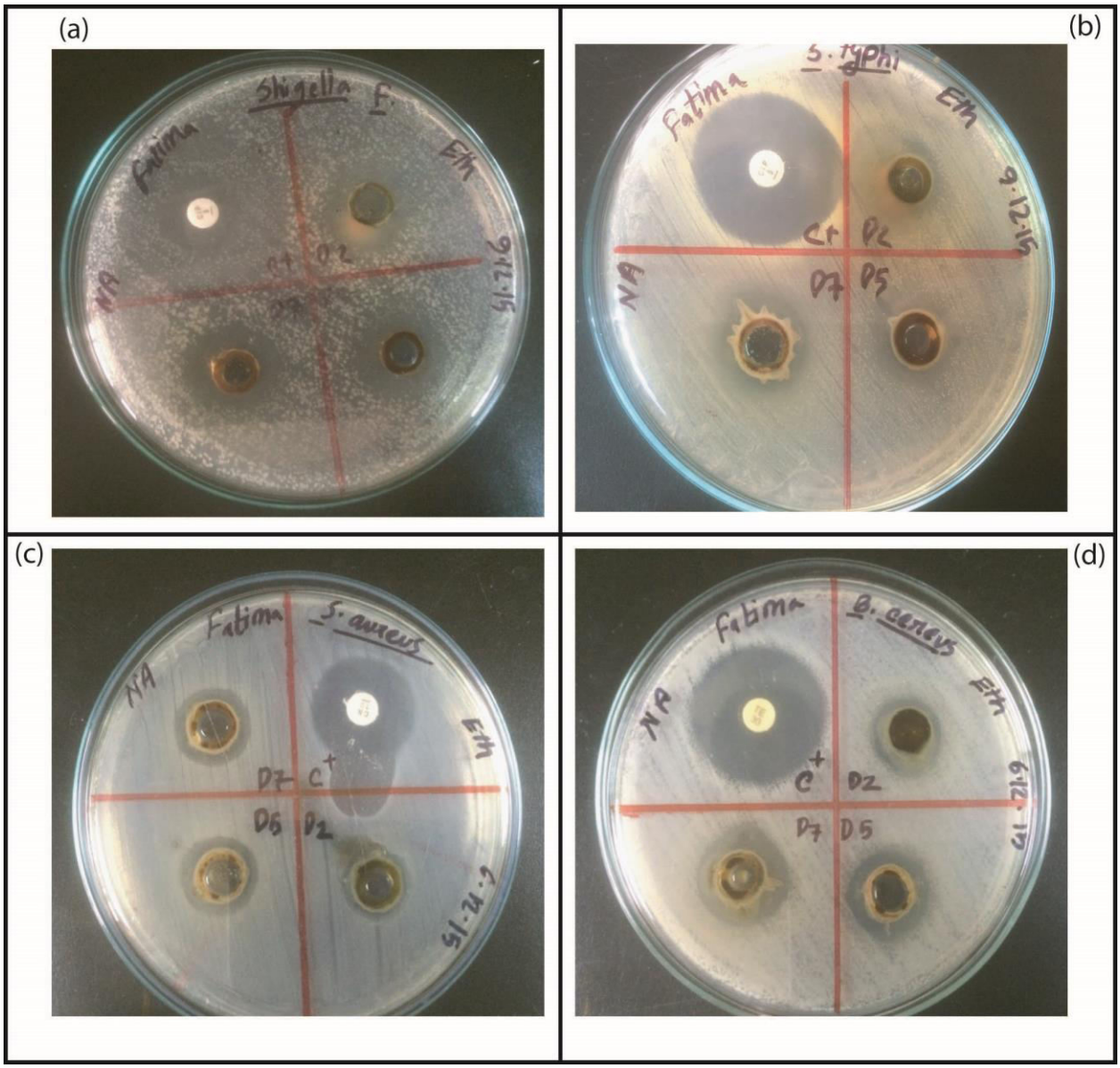

Figure 3.1.1: The antibacterial effect of Radhuni seed; 2nd, 5th and 7th day ethanol extracts Of (a) *S. flexineri,* (b) *S. typhi,* (c) *S. aureus,* and (d) *B. cereus*

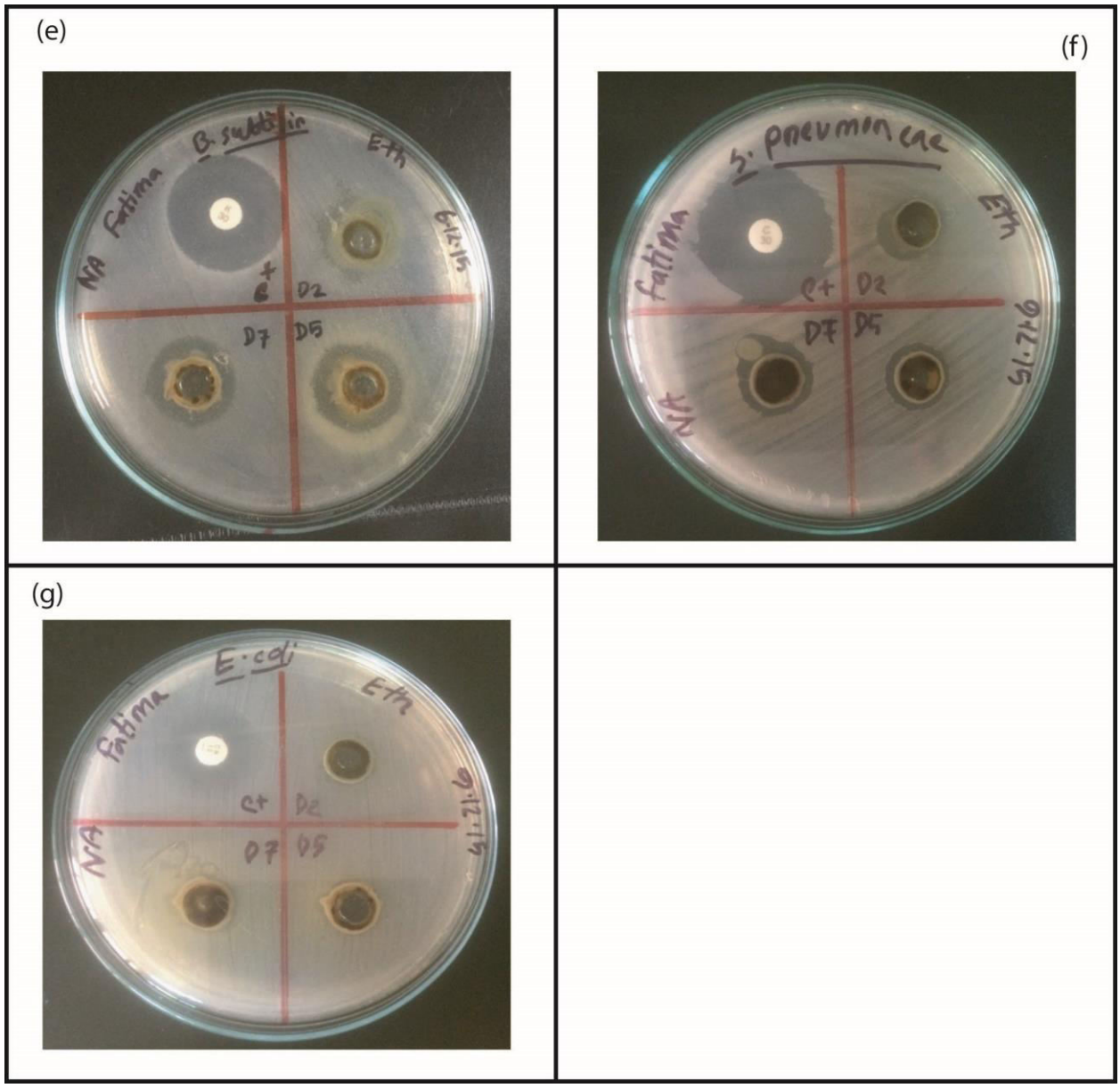

Figure 3.1.2: The antibacterial effect of Radhuni seed; 2nd, 5th and 7th day ethanol extracts of (e) *B. subtilis,* (f) *S. pneumonae* and (g) *E.coli* (Negative result)

3.1.2 Methanol Extraction of Radhuni:

In table 3.2.1 and table 3.2.2, antibacterial effect of methanol extract of radhuni is shown on 2^{nd}, 5^{th} and 7^{th} day extraction where the zone of inhibition for the five microbes *S. flexineri, S. typhi, S. aureus*, *B. subtilis* and *S. pneumonae* – was maximum for the 5^{th} day extracts in all three replicates while only one microbe *B. cereus* showed maximum zone of inhibition for the 7^{th} day extracts in all three replicates.

Table 3.2.1: Positive antimicrobial effects (average zone of inhibition) with positive controls, produced by methanol extract of radhuni

Extraction Days	Inhibition Zone (mm)					
	Shigella flexinera		*Salmonella typhi*		*Staphylococcus Aureus*	
	Per trial	**Average**	**Per trial**	**Average**	**Per trial**	**Average**
2nd day	16.00	**16.94**	0.00	**0.00**	13.30	**14.23**
	17.20		0.00		14.20	
	17.60		0.00		15.20	
5th day	18.00	**18.77**	17.40	**17.80**	16.60	**17.06**
	19.30		18.00		17.00	
	19.00		18.00		17.60	
7th day	17.00	**17.40**	15.40	**15.86**	15.50	**16.23**
	17.20		16.00		16.00	
	18.00		16.20		17.20	
Positive Controls	18.00	**18.20**	28.00	**28.07**	22.00	**22.00**
	18.00		28.00		22.00	
	18.60		28.20		22.00	

Table 3.2.2: Positive antimicrobial effects (average zone of inhibition) with positive controls, produced by methanol extract of radhuni

Extraction Days	Inhibition Zone (mm)					
	Bacillus cereus		***Bacillus subtilis***		***Streptococcus Pneumonae***	
	Per trial	**Average**	**Per trial**	**Average**	**Per trial**	**Average**
2nd day	11.00	**11.70**	10.00	**10.20**	0.00	**0.00**
	11.20		10.30		0.00	
	12.90		10.30		0.00	
5th day	18.50	**18.84**	19.40	**19.87**	17.60	**17.80**
	19.00		20.00		17.60	
	19.00		20.20		18.20	
7th day	18.70	**19.03**	17.00	**17.54**	14.50	**14.84**
	18.90		17.40		15.00	
	19.50		18.20		15.00	
Positive Controls	28.00	**28.08**	24.00	**24.07**	28.00	**28.70**
	28.00		24.00		29.00	
	28.20		24.20		29.00	

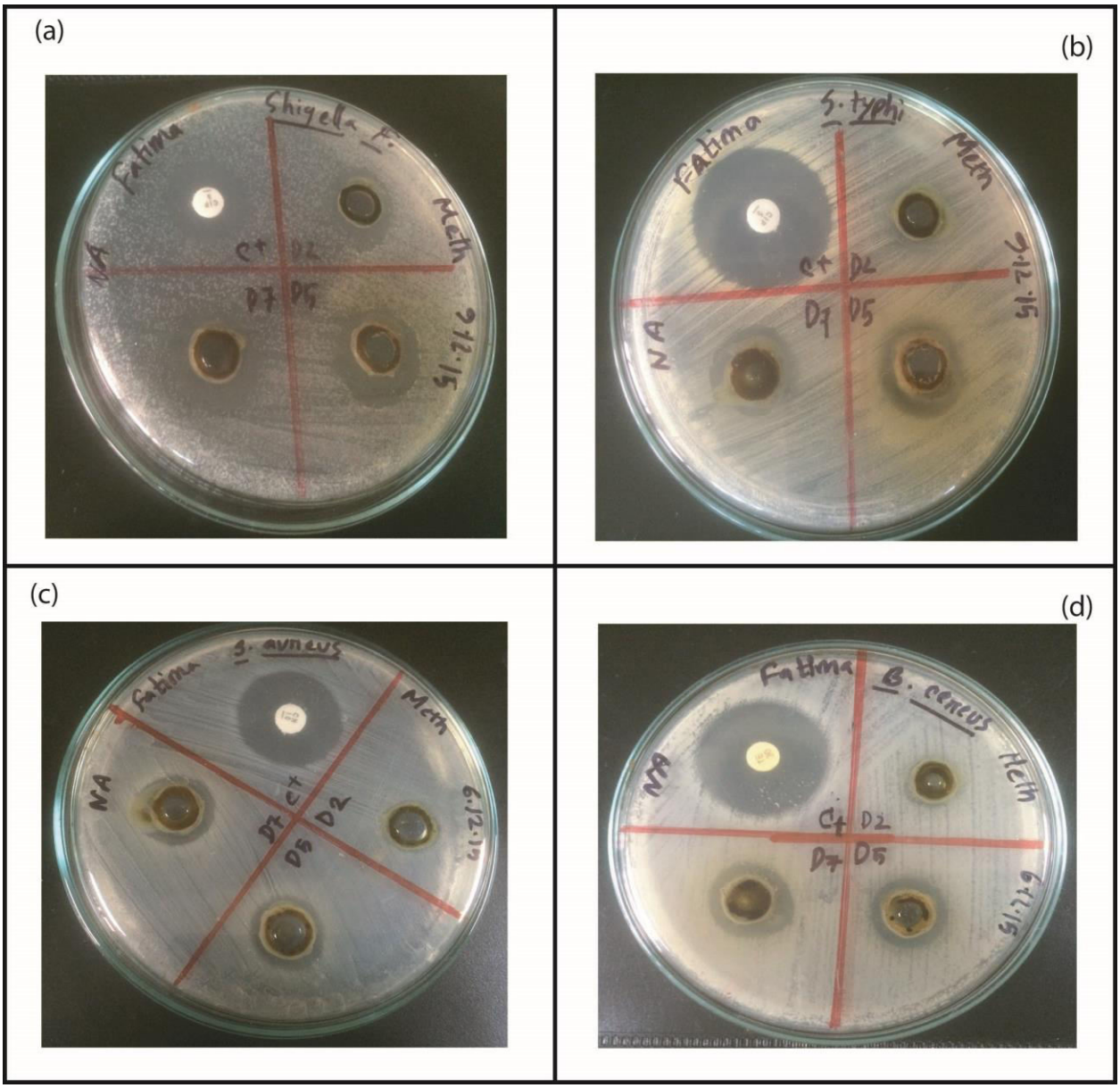

Figure 3.2.1: The antibacterial effect of Radhuni seed; 2nd, 5th and 7th day methanol extracts Of (a) *S. flexineri,* (b) *S. typhi,* (c) *S. aureus,* and (d) *B. cereus*

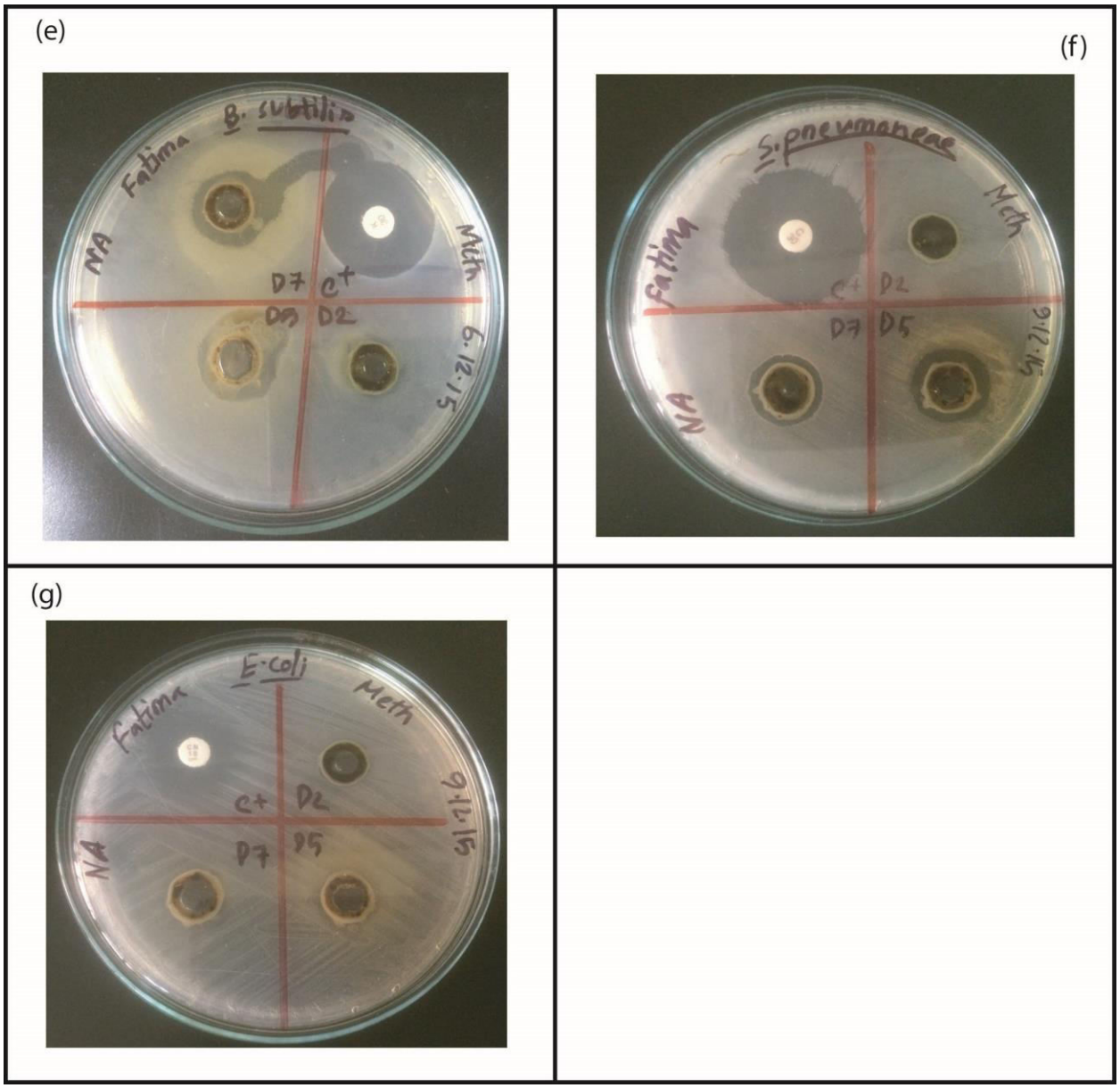

Figure 3.2.2: The antibacterial effect of Radhuni seed; 2nd, 5th and 7th day methanol extracts of (e) *B. subtilis,* (f) *S. pneumonae* and (g) *E.coli* (Negative result)

3.1.3 Ethanol and Methanol Extraction of Fenugreek

In table 3.3 antibacterial effect of ethanol and methanol extract of fenugreek is shown on 2^{nd}, 5^{th} and 7^{th} day extraction where the zone of inhibition was found only one bacterium within all eleven tested microbes, which was ***Bacillus cereus***

Table 3.3: Positive antimicrobial effects (average zone of inhibition) with positive controls, produced by ethanol and methanol extract of fenugreek

Extraction Days	Inhibition Zone (mm) of *Bacillus cereus*			
	Ethanol		**Methanol**	
	Per trial	**Average**	**Per trial**	**Average**
2nd day	16.50	**17.07**	17.00	**18.00**
	17.20		18.30	
	17.50		18.70	
5th day	17.90	**18.54**	11.40	**11.87**
	18.50		12.00	
	19.20		12.20	
7th day	20.20	**20.47**	18.90	**19.10**
	20.50		19.00	
	20.70		19.40	
Positive Controls	29.00	**29.08**	25.00	**25.07**
	29.00		25.00	
	29.20		25.20	

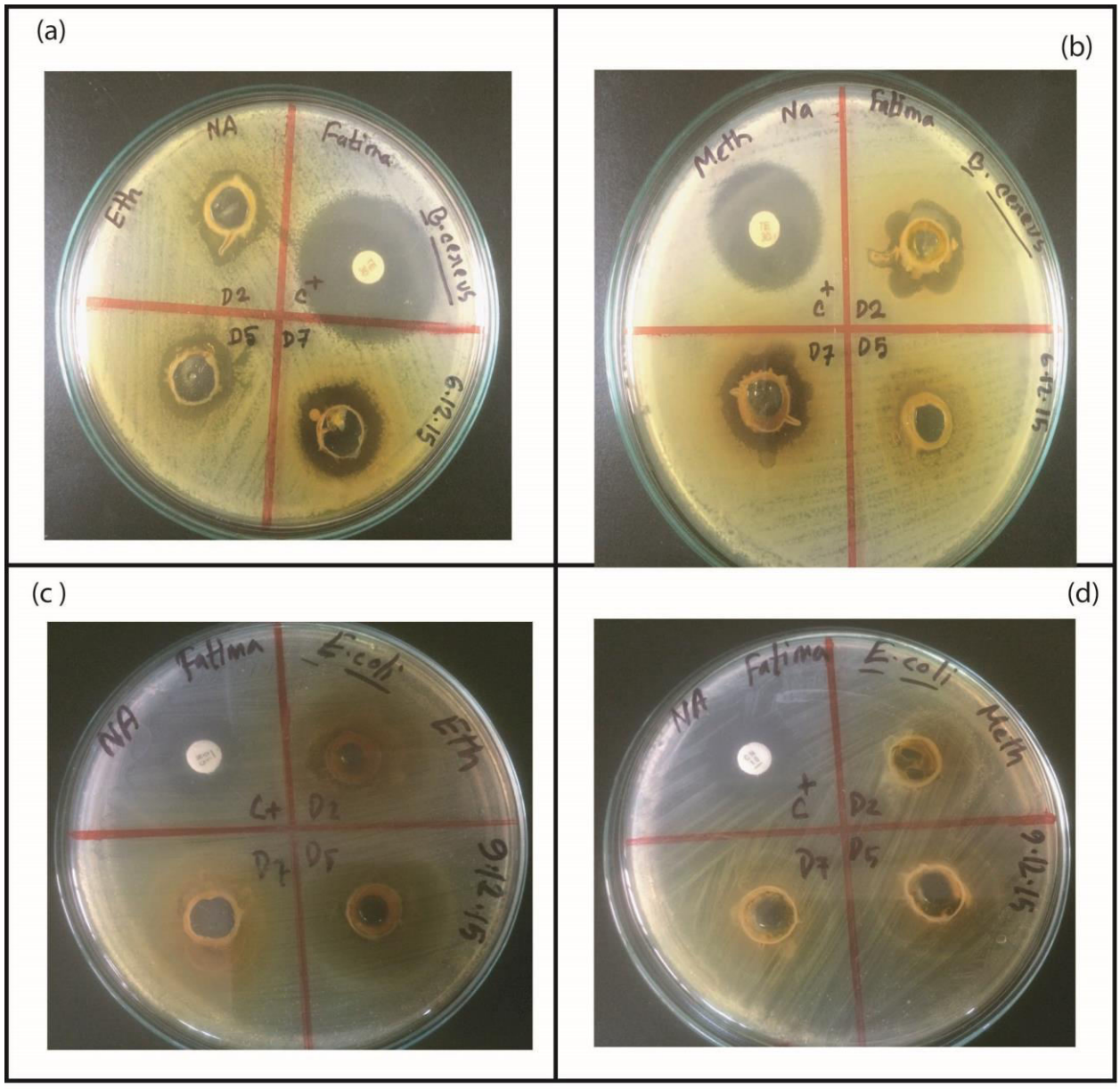

Figure 3.3: The antibacterial effect of fenugreek seed; 2nd, 5th and 7th day ethanol and methanol extracts of (a/b) *B. cereus* and (c/d) *E.coli* (Negative result)

3.1.4 Comparison of Different Extraction:

3.1.4.1 By Means of Different Days:

Radhuni Seed Extraction: Antibacterial effect of **2nd** day of extracts of ethanol and methanol is shown in table **3.1.1**, **3.1.2**, **3.2.1** and **3.2.2** where it is observed that in ethanol extract the average zone of inhibition for *S. typhi* (table 3.1.1) is lowest positive result where *S. flexineri* (table 3.1.1) found having highest positive result. For methanol extract, the average zone of inhibition is unremarkable for *S. typhi* and *S. pneumonae* while *S. flexineri* (table 3.2.1) is maximum among all six microbes which showed positive results.

Antibacterial effect of **5th** day extracts of ethanol and methanol is shown again in table **3.1.1**, **3.1.2**, **3.2.1** and **3.2.2** where it is observed that in ethanol extract the average zone of inhibition is lowest for *S. pneumonae* (table 3.1.2) and *B. subtilis* (table 3.1.2) is maximum. For methanol extract, the average zone of inhibition is lowest for *S. aureus* (table 3.2.1) and highest for *B. subtilis* (table 3.2.2) amongst all six microbes which showed positive results.

Antibacterial effect of **7th** day extracts of ethanol and methanol is shown in table **3.1.1**, **3.1.2**, **3.2.1** and **3.2.2** where it is observed that in ethanol extract the average zone of inhibition is lowest for *S. pneumonae* (table 3.1.2) and highest for *B. cereus* (table 3.1.2) and in methanol extract the average zone of inhibition is lowest for *S. pneumonae* (table 3.2.2) and highest against *B. cereus* (table 3.2.2) from all six microbes which showed positive results.

In a nutshell, it is observed that the average zone of inhibition for ethanol extraction of all the six microbes – 5th day extract of *B. subtilis* (table 3.1.2) was maximum and 2nd day extract of *S. typhi* (table 3.1.1) was lowest. And the average zone of inhibition for *B. subtilis* (table 3.2.2) was also maximum for methanol extract from the 5th day where *S. typhi* and *S. pneumonae* showed no mark able result from 2nd day extract.

Fenugreek Seed Extraction: From all eleven tested microbes, fenugreek showed positive zone of inhibition against *B. cereus* only where zone of inhibition was maximum for both ethanol and methanol extract from the 7th day and lowest from the 2nd day for ethanol and 5th day for methanol (table 3.3).

3.1.4.2 By Means of Different Solvents (Ethanol and Methanol):

In radhuni seed extracts, considering all the tables **3.1.1**, **3.1.2**, **3.2.1** and **3.2.2** it is observed that the average zone of inhibition for all the six microbes – *S. flexineri, S. typhi, S. aureus, B. cereus, B. subtilis* and *S. pneumonae*– was maximum for ethanol from which *B. subtilis* showed highest average zone of inhibition that is **20.20** mm for 5^{th} day extract of *B. subtilis* and unremarkable result or lowest average zone of inhibition was for methanol that is **0.00** mm from the 2^{th} day extract of *S. typhi* and *S. pneumonae*

3.1.5 Comparison between Positive Antibacterial Activities of Radhuni and Fenugreek

In table **3.4** antibacterial effect of ethanol and methanol extracts of radhuni and fenugreek is shown where the zone of inhibition was found commonly by both the seed extracts was in only one bacterium ***Bacillus cereus***; within all eleven tested microbes. So by comparing both seed's ethanol and methanol extracts; radhuni showed maximum zone of inhibition in all three replicates.

Table 3.4: Positive antimicrobial effects (average zone of inhibition) with positive controls, produced by ethanol and methanol extract of radhuni and fenugreek

Extracts	Inhibition Zone (mm) of *Bacillus cereus*			
	Radhuni		Fenugreek	
	Per trial	Average	Per trial	Average
Ethanol	19.00	**19.14**	18.90	**18.97**
	19.20		19.00	
	19.20		19.00	
Positive Controls	26.90	**26.97**	26.90	**26.97**
	27.00		27.00	
	27.00		27.00	
Methanol	17.80	**18.20**	17.70	**18.00**
	18.30		18.00	
	18.50		18.30	
Positive Controls	25.00	**25.14**	25.00	**25.14**
	25.20		25.20	
	25.20		25.20	

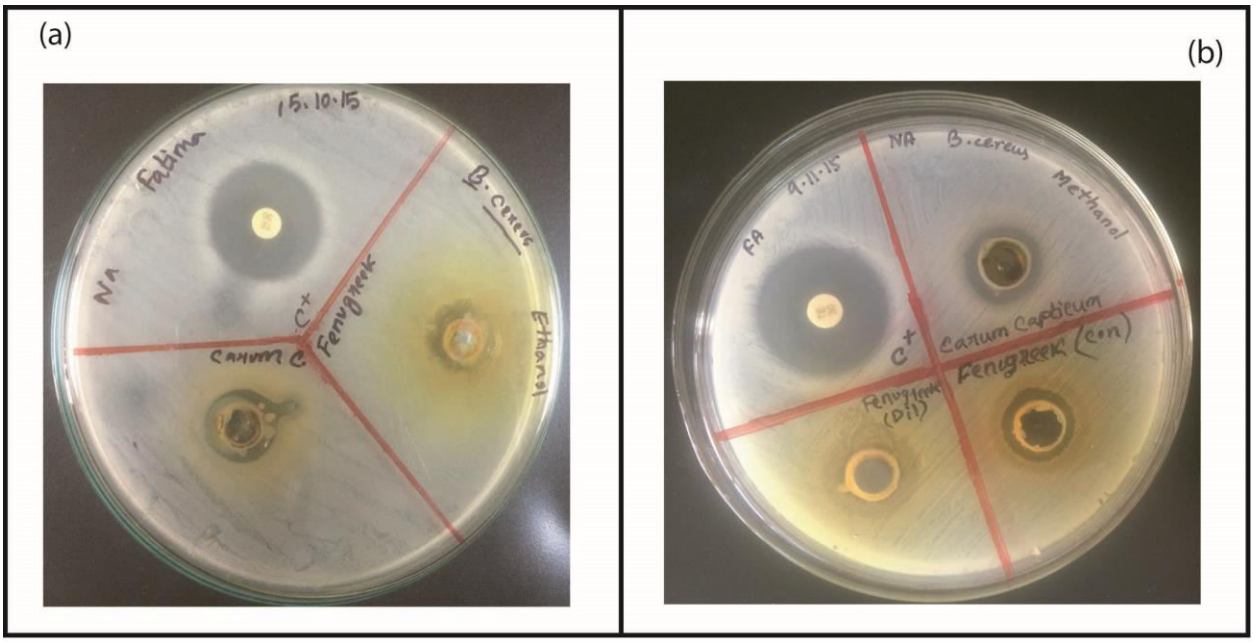

Figure 3.4: Comparison between the antibacterial effect of (a) radhuni and (b) fenugreek seed on ethanol and methanol extracts of *B. cereus*

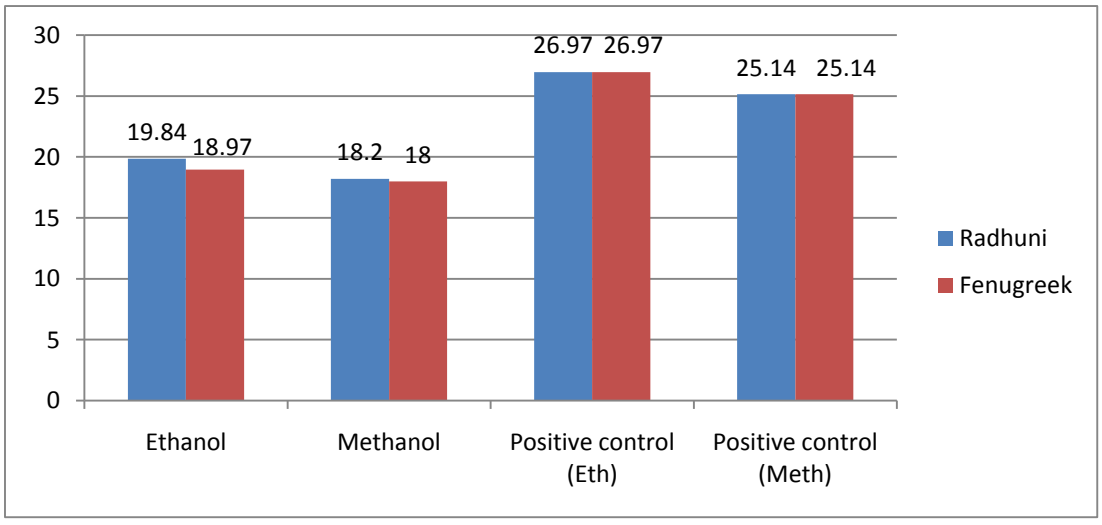

Figure 3.5: Average zones of inhibition produced by radhuni and fenugreek in ethanol and methanol extraction, along with the positive controls for *B. cereus*

3.1.6 Comparison of Activity Index:

The following formula is used for both radhuni and fenugreek seeds' ethanol and methanol extracts to calculate the activity index values.

Activity Index (AI) = zone of inhibition of extracts/ zone of inhibition of the antibiotics

For radhuni seed extracts, among six microbes the activity index (AI) values for *B. cereus* and *S. pneumonae* was maximum in ethanol extract from the 7th day where rest four were maximum in ethanol extract from the 5th day. From all the AI values, the 5th day ethanol extract of *S. flexineri* has got the highest AI value. This is shown in figure 3.6

For fenugreek seed extracts, the activity index (AI) value for *B. cereus* was maximum in methanol extract from the 7th day. This is shown in figure 3.7

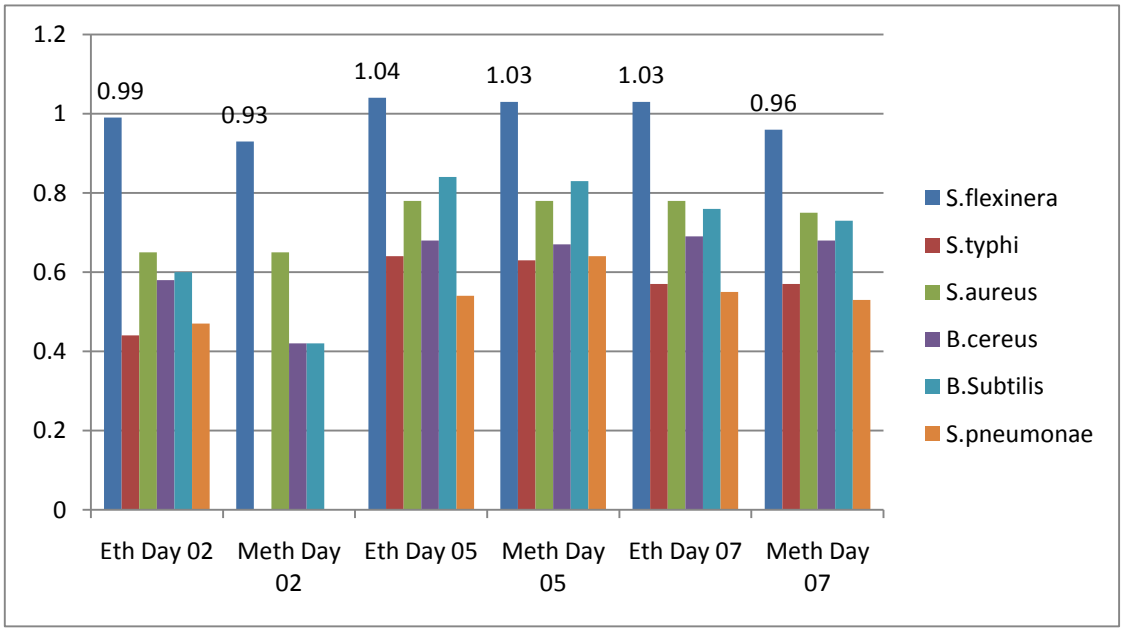

Figure 3.6: Activity Index of the ethanol and methanol extracts of radhuni seeds that showed positive antibacterial effects

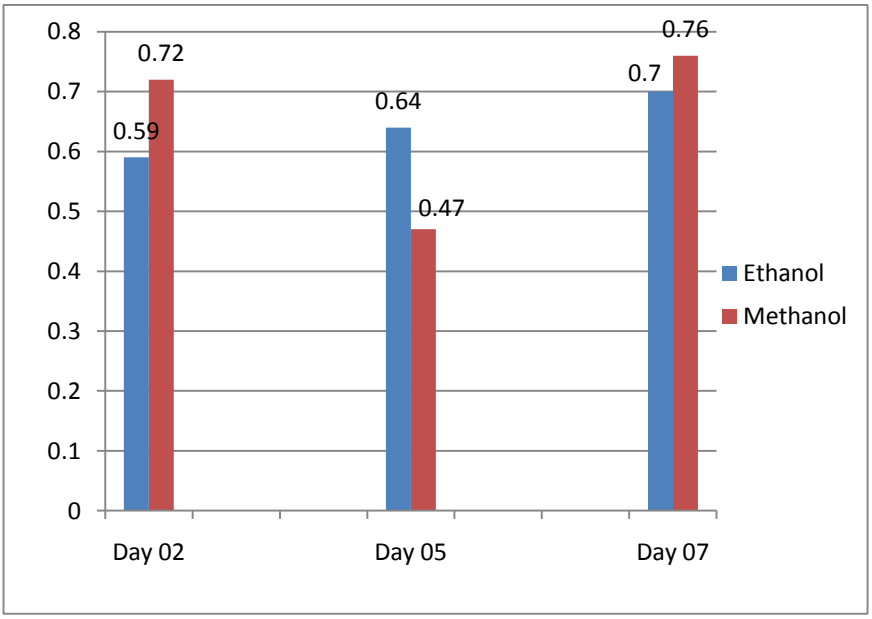

Figure 3.7: Activity Index of *B. cereus* in ethanol and methanol extracts of fenugreek seeds which showed only positive antibacterial effects

3.2 Results of Phytochemical Assay

For testing various phytochemicals found in both seed extracts, screening was done using various chemicals and reagents. The crude extracts were tested for the presence or absence of secondary metabolites such as alkaloids, terpenoids, tannins, saponins, steroids, phenolic compounds, flavonoids and cardiac glycoside. The results obtained from the phytochemical assay are shown below.

Alkaloids: Tube 1 having crude extract; tube 2, 3 and 4 includes the extract along with picric acid. The turbid effect on the top layer is not present in the mixture which indicates negative results. Tube 5 contains only picric acid.

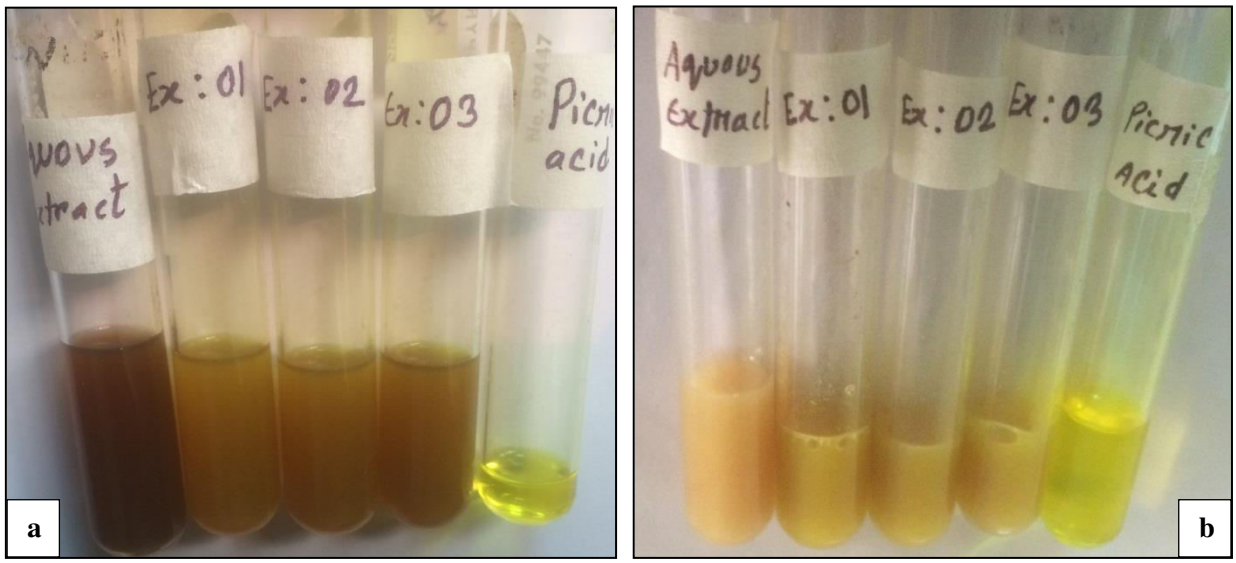

Figure 3.8: Alcaloids test with (a) Radhuni seed extracts and (b) Fenugreek seed extracts

Terpenoids: Tube 1 having crude extract; tube 2, 3 and 4 includes the extract along with chloroform and sulphuric acid presenting the reddish brown color. Tube 5 contains only chloroform and sulphuric acid.

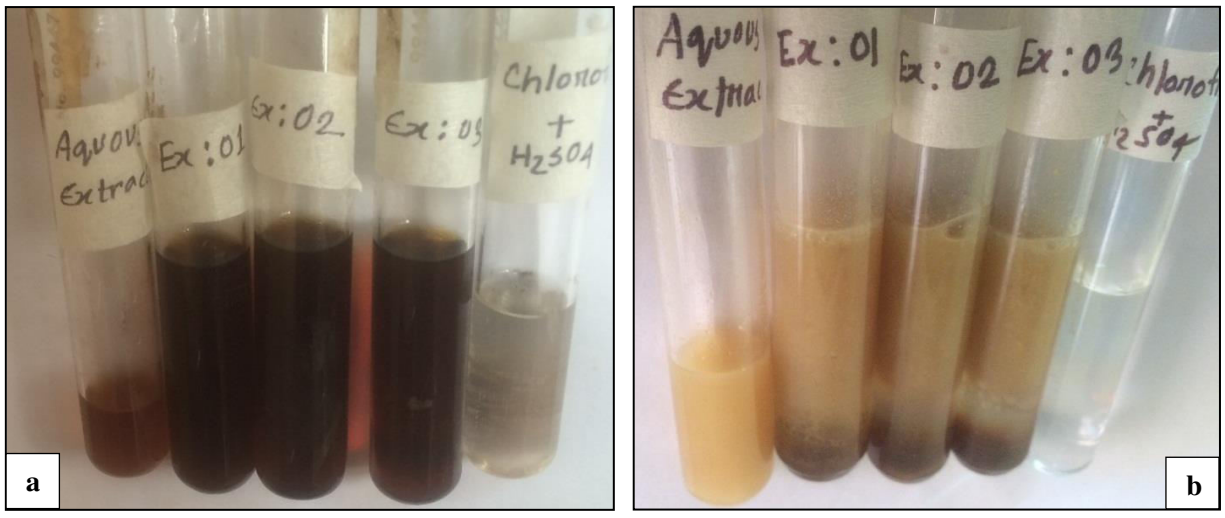

Figure 3.9: Terpenoids test with (a) Radhuni seed extracts and (b) Fenugreek seed extracts

Tannins: Tube 1 having crude extract; tube 2, 3 and 4 includes the extract with few drops of 10% ferric chloride which presenting the bluish black color. Tube 5 contains only 10% ferric chloride.

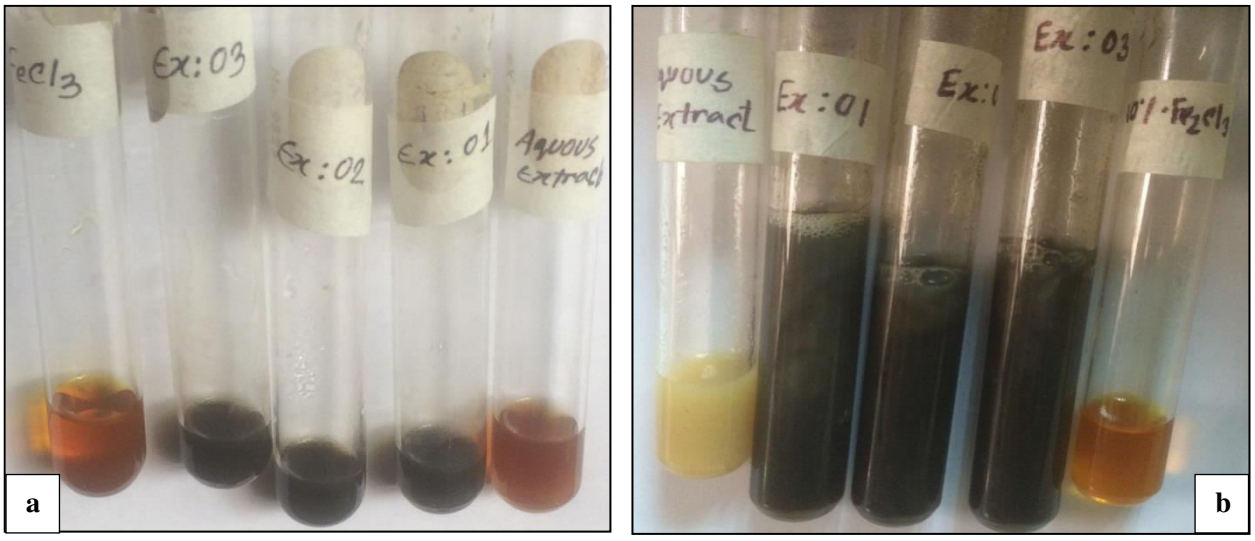

Figure 3.10: Tannins test with (a) Radhuni seed extracts and (b) Fenugreek seed extracts

Saponins: Tube 1 having crude extract; tube 2, 3 and 4 includes the extract with 10 ml of distilled water creating a frothing upon shaking the tubes. Tube 5 contains only distilled water.

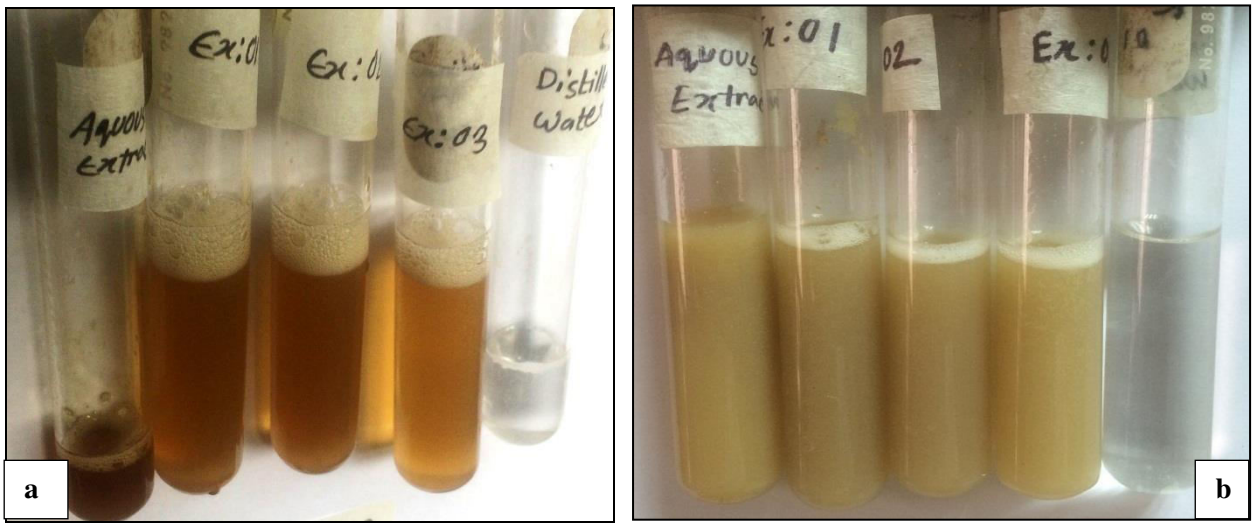

Figure 3.11: Saponins test with (a) Radhuni seed extracts and (b) Fenugreek seed extracts

Steroids: Tube 1 having crude extract; tube 2, 3 and 4 includes the extract with 2ml chloroform and 2ml sulphuric acid presenting the reddish brown color.Tube 5 contains only chloroform and sulphuric acid.

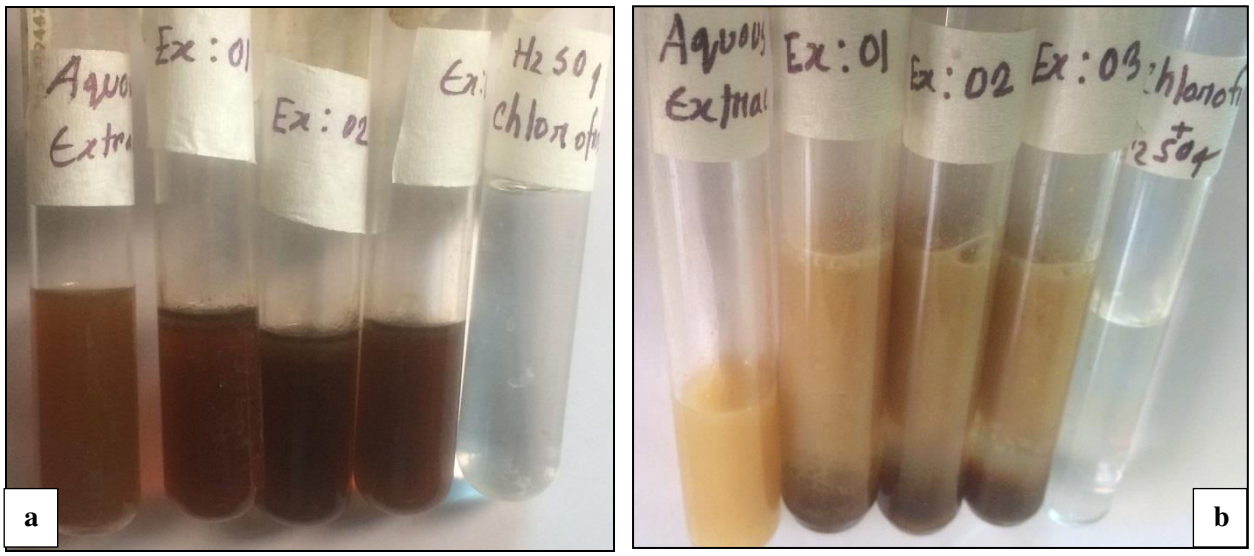

Figure 3.12: Steroids test with (a) Radhuni seed extracts and (b) Fenugreek seed extracts

Phenolic Compounds: Tube 1 having crude extract; tube 2, 3 and 4 includes the extract with few drops of 5% ferric chloride which presenting the bluish black color. Tube 5 contains only 5% ferric chloride.

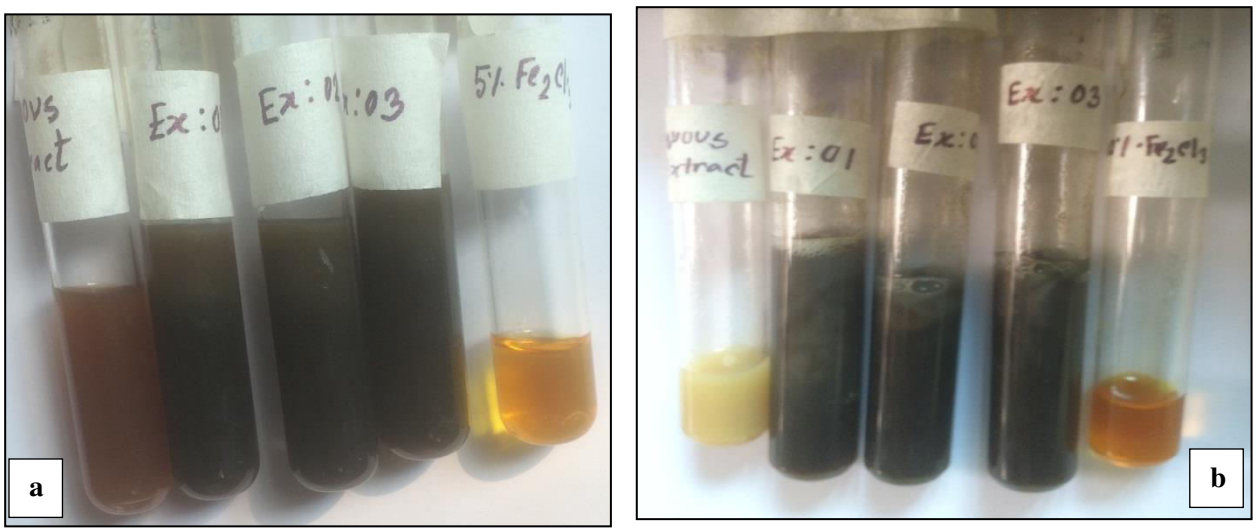

Figure 3.13: Phenolic compound test with (a) Radhuni seed extracts and (b) Fenugreek seed extracts

Flavonoids: Tube 1 having crude extract; tube 2, 3 and 4 includes the extract with 20% sodium hydroxide solution presenting the reddish yellow color. Tube 5 contains only 20% sodium hydroxide solution.

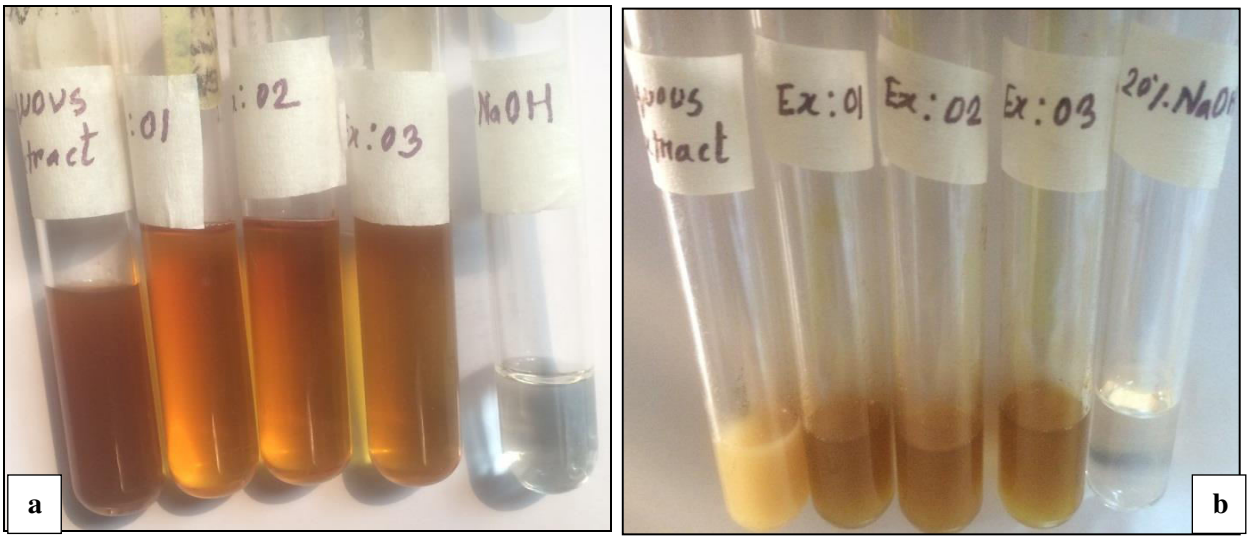

Figure 3.14: Flavonoids test with (a) Radhuni seed extracts and (b) Fenugreek seed extracts

Cardiac Glycoside: Tube 1 having crude extract; tube 2, 3 and 4 includes the extract with 2 ml glacial acid, 1 ml of concentrated sulphuric acid and a drop of ferric chloride presenting a brown ring on the interface. Tube 5 contains 2 ml glacial acid, 1 ml of concentrated sulphuric acid and a drop of ferric chloride.

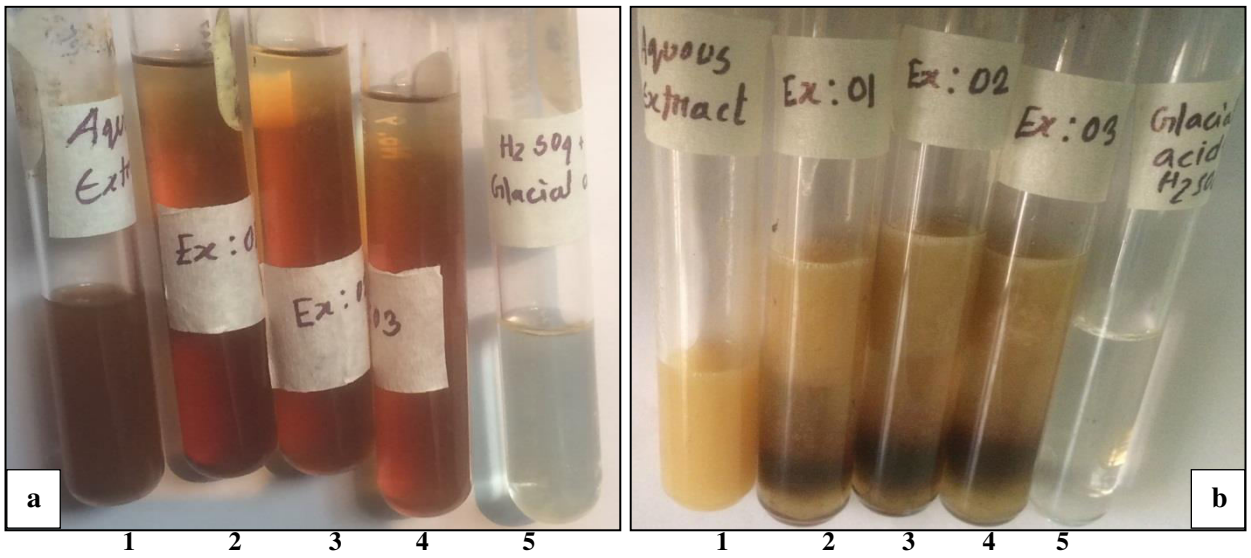

Figure 3.15: Cardiac glycosidetest with (a) Radhuni seed extracts and (b) Fenugreek seed extracts

Chapter Four

DISCUSSION

DISCUSSION

Today's medical world is countering the two most enormous challenges which are antibiotic toxicity and multi drug resistant pathogens that impinging on directly not only the pharmaceutical and medicine industries but also the food and beverage industries. The unrestrained employ of chemical preservatives in food industry over the decades has led to emergence of microbial resistance to classic antimicrobial agents which has become a severe health concern (Kiessling *et al.*, 2002). The rising inclinations of multidrug resistance within copious groups of microorganisms against diverse classes of antibiotics led loads of researchers to build up competent drugs from plant sources to defy multidrug resistant strains. For the development and synthesis of novel antimicrobial agents; different parts of plants have created a great contribution to human health and well-being. In addition, various phytochemicals are affluent sources of antioxidants and endow with defense mechanisms to plants against predation by infectious organisms and insects (Deans & Ritchie, 1987). A number of studies have reported a high correlation between antimicrobial efficacy and the level of different phytochemicals present in certain herb and spice.

Radhuni and fenugreek are such spices that come from the plant that falls under the family Apiaceae and Fabaceae respectively; which have loads of restorative properties other than simply being used as household cooking or flavoring agents. *Carum roxburghianum* (radhuni) is used in conventional system of medicine to treat diarrhea, abdominal spasm (colic), asthma, bronchitis cough, common cold, dyspepsia, lethargy, loss of consciousness, palpitation, vomiting, pain in bladder and kidneys as well as considered useful as anthelmintic, antigout, antimicrobial, cardiotonic, carminative, condiment, digestive, diuretic, emmenagogue, stimulant and stomachic (Khan, M. *et al.,*2012). The other seed *Trigonella foenum-graecum* (fenugreek) also has a long history of traditional medical uses in Ayurvedic and Chinese medicine and has been used for numerous indications, including labor induction, aiding digestion, hypoglycemic and anti-hyperlipidemic properties, lowers blood pressure, relieves congestion, reduces inflammation and fights infection, contains natural expectorant properties and an excellent source of selenium.

(Sheikhlar, A. 2013). For all these vast therapeutic properties, both of these seeds can be the products of interest in the research area to work with.

A study from Pakistan raveled the positive antimicrobial effect of essential oil of radhuni seed on three bacteria *Bacillus subtilis, Nocardia asterides* and *Salmonella typhimurium* out of tested six microbes which were *Bacillus subtilis, Bacillus lichneformis, Escherichia coli, Micorococcus luteus, Nocardia asterides* and *Salmonella typhimurium.* The extract was carried out through Hydrodistillation process and antimicrobial assay was determined by using Agar Diffusion Method (Iqbal *et al,* 2014). In this research, using agar diffusion method, the zone of inhibition was also found for both ethanol and methanol extracts of day 2, 5 and 7 where highest was for *Bacillus subtilis* from the ethanol extract of 5th day and lowest was for *Salmonella typhimurium* from the ethanol extract of 2nd day where *Escherichia coli* did not show any zone of inhibition which represents the exact coherence with the research done by Iqbal *et al.* But the extraction process was different here, where extraction was done via conventional crude method using solvents like ethanol and methanol. Another research finding indicated positive results against two bacteria namely *Serratia marcescens*, *Bacillus cereus* and one yeast, *Rhodotorula mucilaginosa* where the radhuni seed extraction was done in the cold aqueous, hot aqueous, ethanol, methanol and acetone where hot and cold aqueous extracts of plants possessed less antimicrobial activities in comparison to organic extracts (Dhiman *et al,* 2015). In this study both ethanol and methanol extracts showed positive result against *Bacillus cereus* as well which is similar to the findings of Dhiman *et al*.

In an another study by Bhuiyan showed that extract of fenugreek seed done by hydrodistillation method and antimicrobial assay was done by disc diffusion method on Mueller-Hinton medium against ten potential pathogenic bacteria which were *Bacillus subtili*s, *B. cereus, Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *S. sonnei*, *Salmonella typhi*, *S. paratyphi, Staphylococcus aureus* and *Vibrio cholerae* and six phytopathogenic fungi which were *Alternaria alternata*, *Botryodiplodia theobromae*, *Colletotrichum corchori*, *Curvularia lunata*, *Fusarium equiseti* and *Macrophomina phaseolina* and extract showed positive result against all these microbes (Bhuiyan *et al.,* 2009). In this study, eleven bacteria were tested from that

against six pathogenic bacteria both the ethanol and methanol crude extract of radhuni seed gave positive results for those five bacteria *Bacillus subtili*s, *B. cereus, Pseudomonas aeruginosa*, *Salmonella typhi*, and *Staphylococcus aureus* which are common with this research and showing positive response but *Escherichia coli* was resistant here which is contradictory to the study done by Bhuiyan *et al.* The variation in extraction process, media and bacterial strain could be some of the several reasons for this. In addition, antifungal effects of radhuni seed extracts were not in the purview of this study, but may be considered by other researchers.

A research indicated potential antimicrobial activity of radhuni seed extracts by hydro-distillation, against against *S. aureus* and *E.coli* except *P.aeruginosa* (Chansakaow *et al,* 2014) where in this study; zone of inhibition is found in *P.aeruginosa* along with *S. aureus* except *E.coli* and the extracts were in methanol and ethanol. Although, very less research has been done so far with the radhuni seeds, there are numerous researches that have been conducted with another seed which is very closely related to radhuni named 'ajwan'. The result of phytochemical screening of ajwan can be compared with phytochemical assay of radhuni seed extract in this study. Different phytochemicals have been found to acquire different functions for which it was necessary to do phytochemical assay of radhuni seed extract in this study. In a research '*Carum Copticum'*(ajwan) seed extract was done with the help of Soxhlet apparatus in different solvents included petroleum ether, diethyl ether, chloroform, ethyl acetate, acetone, ethanol and methanol. Phytochemical for various constituents including was analyzed. Positive results came for alkaloids, amino acids, sterols, terpenes, glycosides and proteins while negative results was for phenols, flavonoids, resins and sugars. (Khan *et al.* 2010). In this research, the crude aqueous extract of radhuni seeds was obtained through conventional method. Phytochemical screening was done for alkaloids, terpenoids, tannins, saponins, steroids, phenolic compounds, flavonoids and cardiac glycoside and all tested positive. The result varied because the seeds are not exactly the same and the extract is different as well but as closely similar seeds so comparative results could be taken in consideration.

A handful number of researches have been done with fenugreek seeds. A study was done with aqueous and methnol seed extract of fenugreek where extract was concentrated in a rotary

evaporator at 50 °C. In the Muller Hinton agar, agar diffusion method was used to mesurmant the antibacterial activity on bacterial isolates of *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeuroginosa, Escherichia coli, Proteus vulgaris* and *Klebsiella pneumoniae.* The extract showed highest activity against *Escherichia coli* while moderate response was showed against *Staphylococcus aureus, Pseudomonas Aeuroginos, Streptococcus pyogeenes* and *Proteus vulgaris* showed lowest response. *Klebsiella pneumoniae* showed negative response. (Abdalah, 2011). Another study was conducted with aqueous, ethanol, benzene, chloroform, hexan and petroleum ether extracts of fenugreek and antimicrobial activity was done with agar well diffusion method against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsilla* and showed negative results for all. (Nasrulla *et al,* 2010). In this research the ethanolic and the methanolic extract of fenugreek showed negative results for *Pseudomonas aeuroginosa, Escherichia coli. Staphylococcus aureus* and *Klebsiella pneumoniae* which were taken from eleven tested bacteria. Only *B. cereus* gave zone of inhibition for fenugreek extracts in nutrient agar media. Here, the extract was concentrated in water bath at 80 °C.

The antibacterial activity of *T. foenum-graecum* leaves extracts by agar well diffusion method was found maximum on *Serratia marcescens* with a zone of inhibition of 12.33±0.57 mm by aqueous extract followed by inhibition of *Bacillus cereus* (ZOI = 11.50±0.50 mm) by the methanol extract. Results of the antifungal activity showed that methanol extract showed a maximum zone of inhibition against *Trichoderma viridae* while *Pseudomonas aeuroginosa, Escherichia coli. Aspergillus niger, Aspergillusflavus, Aspergillus oryzae* and *Penicillium chrysogenum* showed negative result. (Dharjiya D. *et al*, 2016). In this study, only *B. cereus* gave highest zone of inhibition of 20.47 mm for the 7^{th} day methanol extract while lowest zone of inhibition of 11.87 mm was for the 5^{th} day ethanol extract within the eleven test microbes where *Pseudomonas aeuroginosa* and *Escherichia coli* also showed negative results. In a study the antibacterial assay of fenugreek seeds ethanol extract was done against *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella* spp. where the result was negative for all (Faraj, 2014). The result is exactly similar to this study as in this study all of these does not give any zone of inhibition.

In a study, fenugreek seed's phytochemical assay was carried out for alkaloids, flavonoids, tannins, saponins, starch, essential oil and steroids and the result was positive for all (Swati, *et al,* 2014). In another research with fenugreed seed's phytochemical screening was done for alkaloids, tannins, saponins, sterols, phenolic compounds, flavonoids and cardiac glycoside where except tannins all gave positive result. (Dharjiya D. *et al*, 2016). But in this study, phytochemical screening was done for alkaloids, terpenoids, tannins, saponins, sterols, phenolic compounds, flavonoids and cardiac glycosides where it is found that except alkaloids all other phytochemicals are present in fenugreek seed extract.

It is actually very difficult to compare directly all of the information of different studies with this research due to a number of factors, such as variability in composition or content of active agents between plants due to origin from various geographical regions, harvesting seasons, growth and drying conditions, or using plant material of diverse maturity. Other factors which manipulate the outcome of antimicrobial testing engross differences in experimental design including inoculums size, growth phase, strain susceptibility, culture medium used, etc. Moreover, powdered seed extracts might have considerable loss of antibacterial activity in comparison to extracts prepared by using crushed seeds, which could be attributed to inactivation of the active antibacterial substances by the heat generated during grinding of the seeds (using an electric blender) (Arora and Kaur, 2009).

In this research comparison between radhuni seed extracts and fenugreek seed extracts had been analyzed. The fact that fenugreek showed antimicrobial activity only against *B. cereus*, the comparing was done only on this pathogen where radhuni extract has the larger zone of inhibition which indicates that radhuni seed has more potential activity than fenugreek seed.

Another important fact of the research is the activity index (AI) values of radhuni and fenugreek seeds from different extraction methods. By quantitatively compared to the respective standard antibiotics, AI values are used to discover the prospective of antimicrobial activity of an extract. A research conducted by Dharjiya D. *et al*, showed AI value of *B. cereus* and *P. aeruginosa* was

0.761 and 0.633 respectively for fenugreek leave extract in methanol. And in this study only *B. cereus* gave zone of inhibition hence AI value of this is highest for the 7th day methanol extract which is 0.76 and lowest for the 5th day methanol extract which is 0.47.

For Radhuni seed extract 5th day methanol extract of *S. flexineri* is higher than that 5th day methanol extract of *B. subtilis* which has highest zone of inhibition. The reason behind this is; the zone of inhibition of kanamycin against *B. subtilis* was larger than that of nitrofurantoin against *S. flexneri*. Moreover, elevated AI values mean that the extracts have a potential activity against the bacteria in comparison with the standard antibiotics.

The purpose of this study was to collect crude extracts in ethanol and methanol and scrutinize the presence of antimicrobial properties along with phytoconstituents in radhuni and fenugreek seeds. Antibacterial tests were done with various days of extraction like day 2, 5 and 7 and different solvent like ethanol and methanol; which is a very unique technique adopted in this research and which showed significance difference in result which could be due to the activity of these seeds extracts on their solubility in different solvents, Moreover, comparison between two seed extract's antibacterial property also revealed the potentiality of seeds. Antibacterial tests show that the plant extracts may be used effectively as an antibiotic agent against microorganisms such as *S. flexineri, S. typhi, S. aureus, B. cereus, B. subtilis* and *S. pneumonae* where the phytochemical analysis of the extract revealed the presence of alkaloids, terpenoids, tannins, saponins, steroids, phenolic compounds, flavonoids and cardiac glycoside in radhuni and fenugreek seed extracts where fenugureek did not contain any alkaloids only. Determination of the antibacterial potencies and identification of phytoconstituents to evaluate and formulate chemotherapeutic agents could be the future frontier.

From the study, it is expected that the findings of this study may inspire, motivate and help other researchers to work further and deeper with these household condiments like radhuni and fenugreek. Moreover, these could work as less expensive antimicrobial agents to do clinical trials

by alternating the commercial antibiotics with extracts of these two seeds or in amalgamation with different spices' extracts incorporating analogous antibacterial properties which will definitely be an advantage for people of developing countries like Bangladesh.

In conclusion it can be stated that, according to this research, *Carum roxburghianum* undeniably posses excellent potency of antimicrobial activity against diverse pathogenic microorganisms where *Trigonella foenum-graecum* showed very little influence in antimicrobial activity. The traditional use of radhuni seeds for infectious diseases is promising against various bacteria and disease causing pathogens and for developing new antimicrobial root canal irrigating solutions whereas fenugreek seed is considered as less potential than radhuni.

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APPENDIX – I

Reagents

1. **10% Ferric chloride**

   1 g of Ferric chloride in 10 ml distilled water

2. **5% Ferric chloride**

   1 g of Ferric chloride in 20 ml distilled water

3. **20% sodium hydroxide solution**

   2 g of sodium hydroxide in 10 ml distilled water

4. **Glacial acid**

   Acetic acid

APPENDIX – II

Instruments

The important equipment used through the study are listed below:

Instrument	Manufacturer
Autoclave	SAARC
Freeze (-20ºC)	Siemens
Incubator	SAARC
Micropipette (10-100µl)	Eppendorf, Germany
Micropipette (20-200 µl)	Eppendorf, Germany
Oven, Model :MH6548SR	LG, China
Refrigerator (4ºC) Model: 0636	Samsung
Safety Cabinet Class II Microbiological	SAARC
Vortex Mixture	VWR International
Water Bath	Korea
Weighing Balance	ADAM EQUIPMENTTM, United Kingdom