ASSESSMENT OF THE ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF OIL SEED MEALS AGAINST FOOD-BORNE PATHOGENS, AND THEIR APPLICATION IN POULTRY MEAT PRODUCT

POOJA SHARMA

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in (Food Science and Technology - (Type A 1))
2020

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has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Food Science and Technology - (Type A 1) of Naresuan University

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Title ASSESSMENT OF THE ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF OIL SEED MEALS AGAINST FOOD-BORNE PATHOGENS, AND THEIR APPLICATION IN POULTRY MEAT PRODUCT

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Keywords Oil seed meal; Ultrasound-assisted extraction; Antioxidant; Antimicrobial; Polyphenols; Challenge test

ABSTRACT

This study was undertaken with the aim to identify the efficacy of ultrasound-assisted extraction of *Moringa oleifera* seed meal extract (ME), *Camellia oleifera* seed meal extract (CE) and *Nigella sativa* seed meal extract (NE) as a greener and more natural antioxidant and antibacterial alternative, applicable to the food industry. The first part of the test involved the screening of the seed meals to select the suitable ethanol concentration for phenolic extraction that exhibited optimum antioxidant and antibacterial activities. The sample to solvent ratio of 1:10 w/v was used in this study. From the results, 60% ethanol concentration was selected for the ultrasound-assisted extraction (UAE) of the seed meals, which was performed at different time and power conditions (10min, 20min and 30 min; and 100 W, 200 W and 300 W respectively). The ultrasonic extraction duration of 20 minutes and power of 200 W for ME ($ME_{200/20}$) with 460.86±2.94 mg GAE/g extract, and likewise $CE_{200/30}$ with and $NE_{300/20}$ gave the highest total phenolic content (TPC), and subsequent higher antioxidant activity in the DPPH and FRAP assay, respectively. The The minimum inhibitory concentration (MIC) exhibited by $ME_{200/200}$ and $NE_{300/20}$ against *E. coli* among the gram-negative, was 3.1g/g and 12.5mg/g respectively and MIC against *Bacillus cereus* among the gram-positive bacteria was at 3.125mg/g, for both $ME_{200/200}$ and $NE_{300/20.}$ HPLC results showed improved phenolic

recovery with ultrasound assisted extraction with predominant gallic acid content(158.3±12.5mg/100g extract) in ME, quercetin (28.49±1.64) in CE, and chlorogenic acid (99.00±1.42) in NE.The cellular leakage test confirmed nucleic acid and protein leakage from $ME_{200/200}$, $NE_{300/20}$ and $_{CE300/20}$-treated *E. coli and B. cereus*. Scanning electron microscopy revealed the damage done to the bacterial cell membrane by the antimicrobial extracts. ME showed the highest antibacterial activity amongst the three seed meals in disc diffusion and MIC, and hence was further selected for the inoculation test in chicken sausages with *E. coli* and *B. cereus*. $ME_{200/20}$ at 0%, 1%, 3% and 5% concentrations, dissolved in 1% chitosan solution, were used as antibacterial coating in raw chicken sausages. 3% extract solution showed around 2 log reduction, with *B. cereus*, on the third day of inoculation. Until the 9^{th} day, the bacteria showed suppressed growth of 3.4 and 3.3 log CFU/g for *B. cereus*, and 3.6 and 3.7 Log CFU/g for *E. coli*, at 3% and 5% of the extract concentrations. The results indicate the inactivation activity of ME against both *E. coli* and *B. cereus* in the chicken sausages used as a real food system.

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CHAPTER I
INTRODUCTION

1.1 Background

The extraction method used to extract the bioactive compounds plays a significant role in the percentage recovery as well as retaining the bioactivity of the extract molecules. Solvent extraction with maceration, Soxhlet extraction with less polar solvents like ethanol, methanol, acetone etc. have been widely used to isolate polyphenolic compounds from plant extracts. More recently, lesser time and resources-consuming, and more efficient and automated methods have been introduced in extraction process. One of the methods most used in recent time is the ultrasonic extraction. Adding ultrasonic assistance to the conventional solvent methods of extraction fortifies the rate and extent of mass transfer to and from the interfaces. UAE is sometimes also referred to as a green technique of extraction (Chemat, Vian, & Cravotto, 2012), as it utilizes lesser solvent quantity, lower energy consumption, and lesser time and labour requirements. The principle behind it is the ultrasonic cavitation that can cause cell wall breakage and ultimately, the material transfer. The high-frequency sound damages the plant cell wall, which improves the solvent penetration into the plant cell and the subsequent release of the target compounds.

Microorganisms that can cause diseases via contamination of food during production and delivery (farm-to-fork) process are generally referred to as food-borne pathogen. They can cause infection either directly or by producing exo- and endo-toxins (Rajkovic, 2014). Food-borne disease outbreaks are a global public health problem, and their global burden amount to 1 in 10 people falling ill each year and 420000 deaths (WHO, 2015).

Chicken and its products are widely consumed worldwide; attributable to its higher digestibility, affordable price and better acceptance. Its consumption is increasing by 2.4% annually (Food and Agriculture Organization (FAO), 2012). Poultry sector is the fastest growing industry among all livestock sectors and is estimated to become the largest meat sector by 2021 (www.thepoultrysite.com). With

increasing consumption pattern of chicken meat products, the quality and food safety aspect need to be taken care of simultaneously. Poultry associated food borne illness is recorded worldwide, and the major pathogen are *Salmonella, Campylobacter, Staphylococcus aureus, E. coli* and *Listeria spp*. Epidemiological study suggests that poultry meat is the primary cause of human food poisoning (Mulder, 1999).

Various food preservatives are being used of natural as well as synthetic origin. However, there has been a long debate over the safety of synthetic preservatives, and a simultaneous demand for natural source of food preservation is increasing. Studies have been performed on the invention and identification of plant and animal sources of antimicrobial and antioxidant compounds, and the biopolymers as a new approach to food packaging. The coating agents that act as binder as well as carrier to the antimicrobial substances or the food preservatives are being successfully used in the food industry. These coating agents can be from the plant origin, animal origin, made synthetically in the lab, or the industrial byproducts such as petroleum. However, the use of natural coatings is better preferred because of their non-toxic and biodegradability traits. Chitosan is one of the biopolymers used as coating or biofilm in the food industry, with the food preservatives added to it. Chitosan is the derivative of chitin, found abundantly in the exoskeleton of the crustaceans. Its popularity as a coating agent is increasing because of its biodegradability, non-toxic property as well as digestibility.

Use of natural compounds derived from plants have regained considerable interest in the context of food preservation. Various factors including physical and/ or biological factors play role in food spoilage. The biological factor of food spoilage, which includes microbial contamination is a major cause of food poisoning. Despite various technological enhancement in food value chain process, the risk of food spoilage remains a constant challenge (Gutierrez, Barry-Ryan, & Bourke, 2009). Spoilage due to microbial contamination and growth bears a huge risk to public health.

Agricultural biomass is abundant worldwide, and it can be considered as alternative source of renewable and sustainable materials which can be used as potential materials for different applications. Despite this enormous production of

agricultural biomass, only a small fraction of the total biomass is utilized for different applications. Due to this abundant availability, the agricultural biomass has acquired enormous amount of interest for prospective utilization as food supplement alternatives. Oil seed cakes and meals are the agriculture biomass residue remaining after the mechanical extraction of the oil from oil seeds. The utilization of natural antioxidants of plant seems promising in view of the economic accessibility of byproducts formed in the production of vegetable oils. Byproducts of oil processing contain phenolic compounds of various chemical structures such as tocopherols, carotenoids, flavonoids, lignans, lignins, phenolic acids and tannins (Kreps, Vrbiková, & Schmidt, 2014). These sources of inexpensive natural bioactive compounds could substitute synthetic additives and also play an important role as preservatives in food industry.

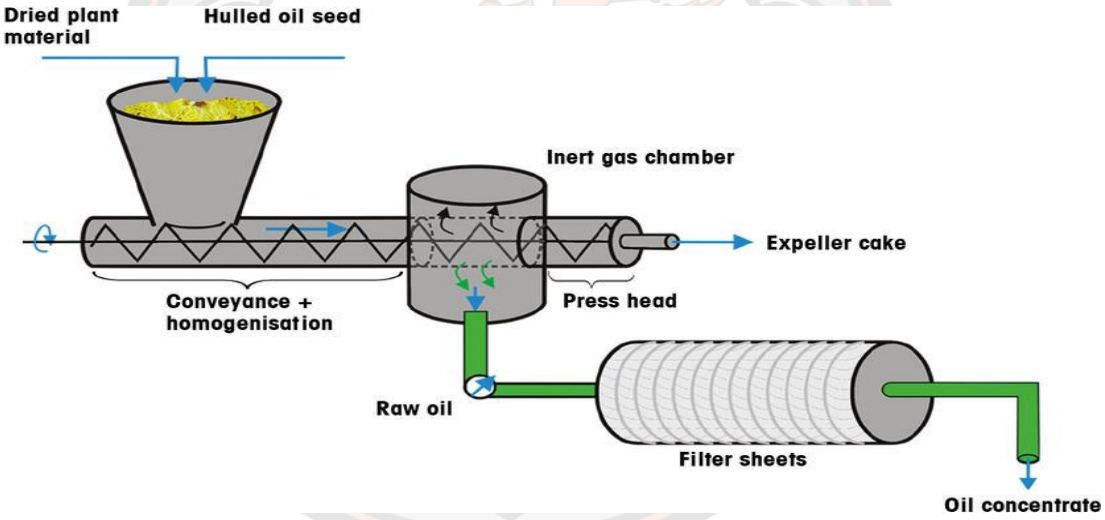


Figure 1. Diagrammatic representation of cold pressed oil extraction and the expulsion of seed cakes/ meals.

The phenolic compounds of different oil seeds have been studied for their antioxidant and antimicrobial properties (Cowan, 1999). The polyphenols of leaves of *M. oleifera,* the oil of *C. oleifera* and seeds of *N. sativa* have been studied for their antioxidative activity. However, lesser is known about the phenolic extracts of the seed cake, and their antimicrobial properties, as much of the study remains to be done on the residual seed meal component.

1.2 Research Objective

Based on the traditional use of the plants, the general aim of the study is to understand the anti-microbial and preservative potential of the oil seed meals of *Moringa oleifera, Camellia oleifera* and *Nigella sativa*, which are the by-products or residues of oil manufacturing industry. The general objective of this study is to valorize the byproduct of the oil industry into premium natural anti-microbial product and food preservative.

Its specific objectives are:

a. To study the effect of ethanol concentration on the antimicrobial and antioxidant properties of the defatted seed meals of *M. oleifera, N. sativa and C. oleifera.*
b. To investigate the effects of ultrasound-assisted ethanolic extraction on the antimicrobial and antioxidant properties of the defatted seed meals.
c. To identify the related mechanism of action of the antimicrobials on selected bacteria.
d. To conduct the microbial challenge test in poultry product to evaluate the anti-microbial property of the extracts.

1.3 Research Significance

The idea of using plant byproducts to identify potential antimicrobials is more sustainable and eco-friendlier. The byproducts of oil industry, which would otherwise have none to nominal value might prove to be the invaluable source of therapeutics to mankind. The study focuses on identifying a valuable component out of the residual materials and make practical implications on the improvement of shelf-life of food.

The study establishes the comparative efficacy of the plant-based phytochemicals against its synthetic antibiotic counterpart. The study also compares the effect of the phenolic extracts of the seed meals on the food-borne pathogen, along with comparing the efficacy of conventional extraction technique with the ultrasound extraction method.

Antimicrobials used to be righteously called the ‘miracle drug’, and the medical science has seen its tremendous growth ever since the discovery of the antimicrobials. It cured many diseases which were previously fatal. However, the microorganisms, that have the history of inhabiting the earth millions of years before the evolution of humans, still prove to be better adapted to the changing environment. They have mutated themselves to become resistant to several antibiotics, and the number is increasing every day. Further aggravating the condition, the discovery of new antibiotics has shrunken since 1980s. This stage of crisis of potent antimicrobials has created havoc worldwide and requires unparalleled attention. Plants have been used for traditional medicine from the primitive days of human existence. Many countries still follow the herbal treatment method for primary ailments, which is believed to cause comparatively lesser possibility of complications (Wichtl, 2004).

Hence, the research will provide the pharmaceutical industry with a potential antimicrobial agent to treat different food-borne illness. Further, the natural antimicrobial product can be used in birds in poultry farm to reduce the bacterial load, and also by the poultry meat processing industries as natural anti-microbial preservatives. Similarly, the demand for natural food preservatives can be addressed by the research.

1.4 Research Scope

The scope of this research is to utilize ultrasound assisted extraction method to obtain the antioxidant and antimicrobial extract and apply it on chicken sausages to further verify the antimicrobial activity of the extracts in real food system. The experimental design has 2 parts. First part of the study was the screening of the seed meal samples for their antimicrobial property against 4 target food-borne pathogenic bacteria. Each of the 3 oil seed meals underwent conventional ethanolic extraction at various concentrations. Each extract was analyzed for the bacterial growth inhibition property using the agar gel disc diffusion and the minimum inhibitory concentration test. Then the best performing ethanol concentration was selected for Part II for the seed meals to undergo ultrasound assisted ethanolic extraction in part two of the experiment. The anti-bacterial and antioxidant properties as well as phenolic contents

were analyzed, and the optimum power and time of ultra-sound treatments were determined. The anti-bacterial properties were analyzed by broth dilution method. The synergistic effect of the two different extracts on food-borne pathogen were assessed using the checkerboard method. The antioxidant properties were analyzed using DPPH assay and FRAP method. The mechanism of action of antibacterial activity on bacterial cell wall damage were determined by the absorbance test at 260 nm for nucleic acids and 280 nm for proteins. The cellular damage caused by the antimicrobials was assessed by scanning electron microscopy. Part two of the research included the microbial challenge test in a chicken sausage model using one extract exhibiting the best performance from part two of the research. The target bacteria used were 2 most susceptible bacteria out of 4 strains, one gram-positive and one gram-negative. The sausages were inoculated with the bateria and coated with the antimicrobial agent from the concentration of 1% to 5%, and stored under refrigeration for 15 days, and the viable cells were counted during this period of storage. A field study on the traditional therapeutic use of the plant parts of the best performing seed meal was performed in Nepal.

1.5 Keywords

Antimicrobial activity, Defatted seed meals, Ultrasonication, Chicken sausages, Microbial challenge test.

1.6 Research Hypothesis

The alternative research hypothesis is that the phenolic extracts of oil seed meals possess anti-microbial property against the food borne pathogen. The alternative hypothesis is tested because the phenolic compounds extracted from several plants, leaves, fruits, fruit peels, plant seeds and seed meals have shown antibacterial properties in the researches. The ultrasound-assisted extraction is one of the most common modern techniques used in food and pharmaceutical industry. This method was applied to the ethanolic extraction of the seed meals. The second part of the study covers the prospective use of the seed-meal extracts as antimicrobial agent in raw chicken sausages as a model food system.

CHAPTER II
REVIEW OF LITERATURE

2.1. Food borne diseases

Foodborne diseases are of both acute and chronic type, commonly transmitted through food and causes considerable morbidity, malaise, post-infection complications as well as mortality in severe cases (World Animal Health Information System (WAHIS), 2007) and are an increasing public health threat worldwide. The typical clinical sign of foodborne disease is gastrointestinal upset. However, neurological, gynaecological, immunological symptoms have also been reported. Furthermore, multiorgan failure and cancer may result from the ingestion of contaminated food (who.int). Poultry meat is often associated with food-borne illness, as they act as an excellent vehicle for disease transmission.

2.2. Food borne pathogen

Bacteria and viruses are the major causes of food-borne illness. Some parasites and chemicals are also associated with the illness. Bacterial contamination of food is one of the major causes of meat spoilage. Some of the important food borne bacteria are *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Campylobacter jejuni* (Mulder, 1999). *Bacillus cereus*, among the gram-positive spore forming bacteria, is also one of the major food-borne pathogens. These are also the major causes of meat spoilage and can contaminate meat straight from the farm or during transportation and storage.

2.2.1. Gram-Negative bacteria

About 69% of the cases of bacterial food-borne disease is caused by gram negative bacteria. It is majorly attributed to the ingestion of viable, gram-negative foodborne pathogenic bacteria. (Greig & Ravel, 2009)or the toxins produced by them. *E. coli and Salmonella* are the two important gram-negative food borne pathogens

frequently causing diseases. They belong to family Enterobacteriaceae. They are rod-shaped, non-spore-forming and facultative anaerobic bacteria.

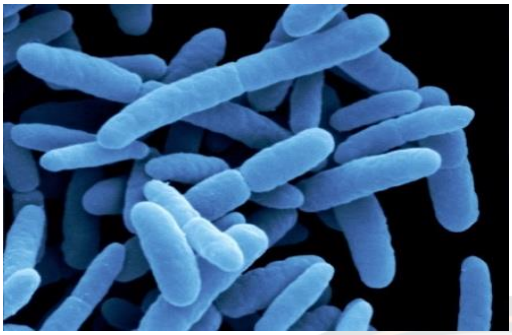


Figure 3. Morphology of *S.* Typhimurium

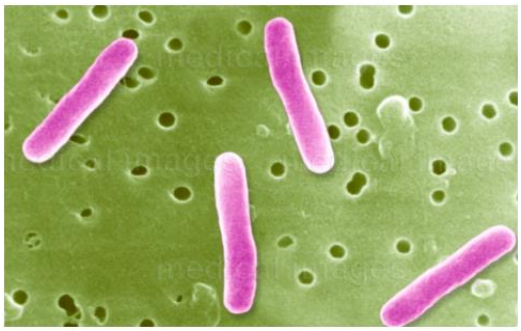


Figure 2. Morphology of *E. coli*

Source: timesdotco.files.wordpress.com

2.2.2. Gram Positive Bacteria

Listeria, Staphylococcus, Bacillus are some of the important gram-positive bacteria causing foodborne illness. *Listeria* can cause potentially hazardous disease called listeriosis, which can range from gastric upset to miscarriage and still-birth (Vázquez-Boland et al., 2001). S*taphylococcus* is an important infectious pathogen capable of causing serious illness, because of its attributes like antibiotic resistance and toxin-mediated virulence. It can survive and grow even in adverse environmental conditions. *Bacillus* is a spore forming type of bacteria and produces toxins. The ailments caused by it can range from gastric symptoms to cutaneous, respiratory and central nervous system involvement (Hoffmaster et al., 2006; Miller et al., 1997; Nishikawa et al., 2009) It remains as a spore in the environment and takes its vegetative form inside the host. Food can get easily contaminated by its spores because of its adhesive nature (Stenfors Arnesen, Fagerlund, & Granum, 2008). All three of these bacteria can survive and grow even in inclement and harsh environmental conditions (Ivanek, Grohn, & Wiedmann, 2006).

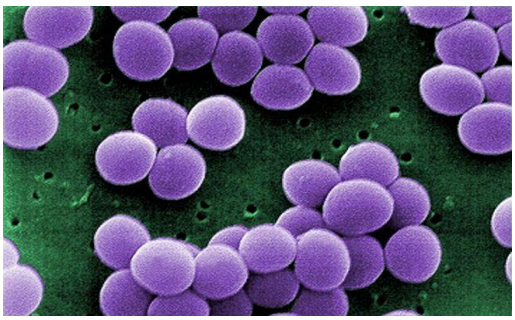


Figure 5. Morphology of *S. aureus*

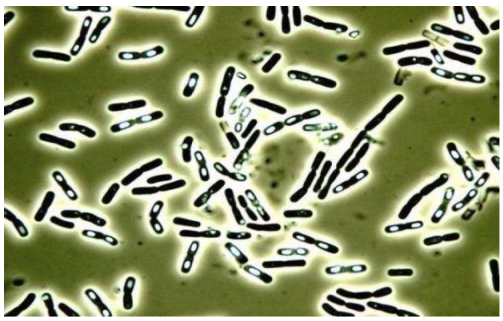


Figure 4. Morphology of B. cereus

Source: alchetron.com; popsci.com

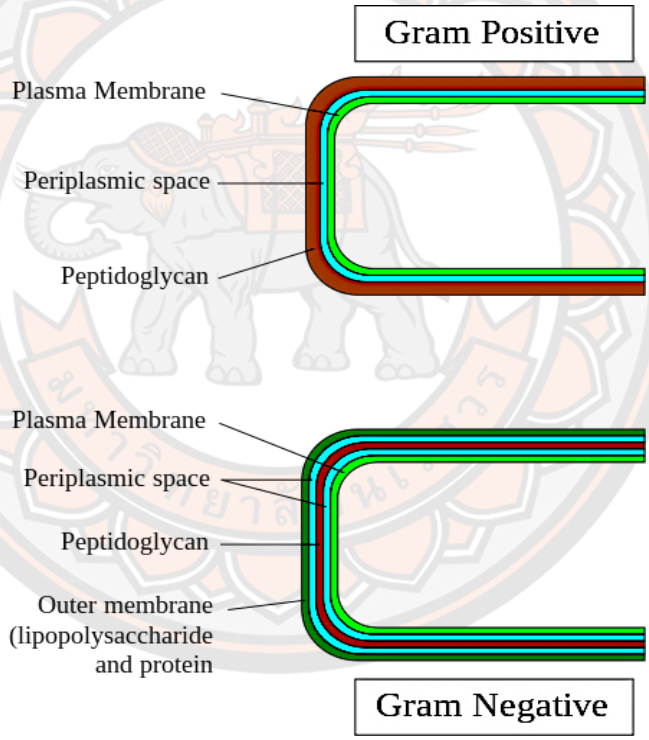


Figure 6. Cell wall structure of gram -positive and gram-negative bacteria.

Source: static.diffen.com

2.3. Poultry meat: microbiological safety aspect

Poultry meat is the second most widely consumed meat (35%) in the world (FAO, 2012) (Figure 7) and is a preferred source of high-quality protein and other nutrients. However, chicken meat is highly perishable with a higher rate of degradation under inadequate storage system, and a comparatively shorter shelf life even under refrigeration (Kozacinski et al., 2012). The microbial contamination is considered the major cause of physical and chemical changes in meat that affect the overall quality of the meat.

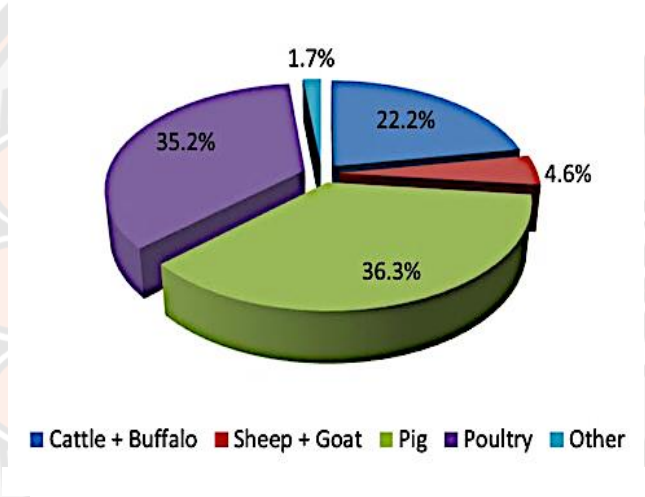


Figure 7. Sources of the world's meat supply

Source: FAO, 2012.

The effects of microbial activity of the spoilage bacteria like *Pseudomonas* are the major causes of quality degradation in poultry meat. The dominant spoilage bacteria under aerobic refrigerated condition is *Pseudomonas spp*. whereas under anaerobic condition it is *Lactobacillus spp*. (Marth, 1998). Psychrophilic counts and anaerobic count of all the samples increased with storage time in the study conducted by El Al, 2005. This increase in microbial activity is further manifested by changes in the odor, flavor, color and pH of the meat (Ingram & Dainty, 1971).

The metabolic activities of the spoilage bacteria are the main causes of spoilage and produce off-odors (Gram et al., 2002). Poultry meat can get contaminated by pathogenic and spoilage bacteria during one or more stages of the

supply value chain process. Storage time is directly related to the microbial growth in poultry meat under continuous refrigeration. In the study conducted by Ghollasi-Mood, Mohsenzadeh, Housaindokht, and Varidi (2017), the Total Viable Counts (TVC) increased with the time of storage, as shown in fig 7. The count increased from initial 5.07 log CFU/g up to 7-8 log CFU/g at the end of shelf life. The rate of growth showed increment with the increase in temperature (Ghollasi-Mood et al., 2017).

2.4. Antimicrobials

Antimicrobials, natural or synthetic, are the compounds that destroy or inhibit the growth of microorganisms, and are categorized as bactericidal or bacteriostatic drug, respectively. The antibiotic was first discovered by Sir Alexander Fleming in 1928, from the fungus Penicillium (Fleming, 1929). Several antibiotics were successfully tested after this, and the world saw a subsequent reduction in human mortality caused by diseases of bacterial origin. Various antimicrobials and their combinations have been extensively used all over the globe. However, the incremental occurrence of antimicrobial resistant bacteria and other microorganisms in recent times, in combination with the shrinking discovery of new antimicrobials after 1980 has created a state of crisis in the medical field (Tanwar, Das, Fatima, & Hameed, 2014).

2.5. Antimicrobial food preservatives

Antimicrobial preservatives are substances which are used in meat to prevent microbial growth and proliferation during the meat value chain process (Melgardt, 2009). They render protection of meat from microbial spoilage under refrigeration. Some frequently used antimicrobial preservatives are Chlorides, Nitrites, Sulfides and Organic acids (Chipley, 2005). However, antimicrobial agents should not be used in food products as a substitute for good manufacturing practice or to mask a perished food product (Ray, 2004).

2.6. Plant based antimicrobial food preservatives

Major concerns of consumers, food industry and food safety authorities are food spoilage and food-borne illness. Various synthetic chemicals are being used by the industry to improve the shelf-life of meat. However, the safety and impact in long-

term use of such chemicals in human health is questionable, as they are time and again associated with the life-threatening conditions in human.

Various physical and chemical preservation methods have been applied to effectively prevent the growth of microorganisms in food. Regardless of the effectiveness, such methods should not compromise the availability of essential nutrients, organoleptic properties, and human health concerns (H. Ye et al., 2013). Furthermore, the changing consumer preference and growing demand for fresh, hygienic, nutrient rich food supply to distant markets has created a necessity to extend the shelf-life of products. Spoilage of poultry meat implies financial setback to the poultry sector and is considered to be the main problem faced by the poultry processing industry (Gram et al., 2002). At the same time, increased awareness about the possible health detrimental impacts of chemicals used in food processing has led to the increased demand of functional foods and sustainable food production and improved food quality. This has subsequently created a high demand for more natural and safer food additives, simultaneously paying concerns to environmental sustainability aspect of food product innovation.

To meet this demand, the search for natural antimicrobial agents that inhibit bacterial growth in food and ensure better quality and shelf-life has attracted considerable attention in recent years (Tajkarimi, Ibrahim, & Cliver, 2010). Fruits, vegetables, seeds and their byproducts are prime sources of natural polyphenols that have received interest in recent years. One of many such studies conducted is presented in Table 1, which shows the data on the coliform count of chicken patties treated with various plant extracts as reported by (Reddy, Vani, Naveen, & Rao, 2017).

Table 1. Coliform count (Log10 CFU/g) values of chicken meat patties as influenced by different treatments during refrigerated storage (4±1oC)

Days of storage	Control	Treatments			Overall mean ± S.E.
		T1	T2	T3	
0	2.31±0.07	2.14±0.01	1.99±0.03	2.19±0.01	$2.16±0.03^{a}$
2	6.24±0.03	1.92±0.02	1.24±0.01	2.82±0.02	$3.05±0.02^{b}$
4	7.98±0.02	2.04±0.01	1.87±0.02	3.28±0.02	$3.79±0.02^{c}$
6	8.81±0.09	3.43±0.02	3.01±0.03	4.01±0.05	$4.81±0.02^{d}$
8	9.01±0.07	4.67±0.09	3.86±0.03	6.08±0.01	$5.91±0.15^{e}$
Overall mean± S.E.	$6.87±0.17^{D}$	$2.84±0.12^{B}$	$2.21±0.12^{A}$	$3.67±0.03^{C}$	

Means bearing at least one common superscript in the same row and in the same column do not differ significantly. (p<0.05)

Treatments: Cheicken meat sausages incorporated with

T1: 2% Rosemary extract

T2: 2% Green tea extract

T3: 0.01% Butylated Hydroxy Anisole

Source: Reddy et al., 2017

A variety of naturally occurring antimicrobial compounds derived from plant sources have been investigated as alternatives to synthetic ones for ensuring food safety and quality, owing to their antimicrobial properties against a broad range of foodborne microorganisms. Several studies have been conducted on the antimicrobial properties of essential oils and extracts of plants which show promising results (Sakkas et al., 2016). The byproducts of oil industry, which are basically discarded and cause a huge burden to environment, require to be studied in deep. Oil derived from the seed extract of various plants have been found to have sufficient antioxidant and antimicrobial properties (Zito et al., 2013). The plant-derived phenolic compounds have been extensively researched for their antimicrobial activities against

a broad spectrum of food spoilage and food poisoning microorganisms (Sanchez Maldonado, Schieber, & Gänzle, 2015).

2.7. Natural antimicrobials in edible coating for meat products

Edible coatings are food grade suspensions which may be delivered by spraying, spreading, or dipping, which upon drying form a clear thin layer over the food surface. Coatings are a particular form of films directly applied to the surface of materials and are regarded as part of the final product (Han & Gennadios, 2005). On the other hand, edible films are obtained from food grade filmogenic suspensions that are usually cast over an inert surface, which after drying can be placed in contact with food surfaces. Films can form pouches, wraps, capsules, bags, or casings through further processing and one of the main differences between films and coatings is their thickness.

The incorporation of natural antimicrobial agents into biobased polymeric matrices called coatings has been investigated in the recent time, as a promising alternative to direct adding of the compounds into the food mix. Different approaches have been used to evaluate the antimicrobial performance of the resulting antimicrobial coatings and films either *in vitro* or *in situ*, when directly applied as coatings on food systems (Aloui & Khwaldia, 2016).

Although some researchers have reported the efficacy of natural antimicrobial agents, when directly added to food systems, in reducing microbial contamination, the rapid diffusion of these agents within the bulk of food, as well as their possible interaction with food components, may decrease their antimicrobial activity during storage and thus limit their practical application in the food industry. Recently the use of edible coatings as polymeric matrices for the entrapment of natural antimicrobial agents has been investigated as a promising alternative to overcome these limitations by lowering the diffusion of active compounds onto food surfaces and hence maintaining their concentrations at a critical level for microbial growth inhibition over long periods of storage (Gyawali & Ibrahim, 2014) (Figure 10). Furthermore, compared to direct application, this approach may impart a highly localized functional effect without affecting its organoleptic properties (Campos et al., 2011). Moreover,

edible coatings may act as a semipermeable barrier providing an additional protection for foods against moisture loss, solute migration, gas exchange, respiration, and oxidative reactions (Quirós-Sauceda, Ayala-Zavala, Olivas, & González-Aguilar, 2014) (Figure 8).

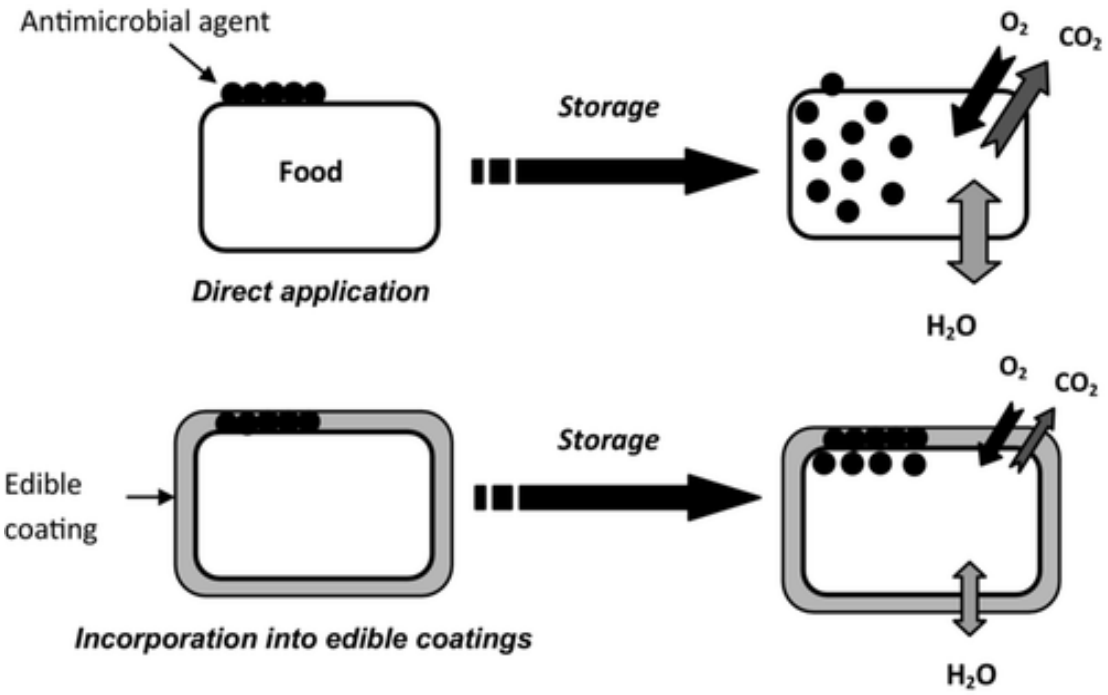


Figure 8. Functional properties of an edible coating.

Source: Quirós-Sauceda et al., 2014.

Edible coatings are thin layers prepared from naturally occurring polymers and applied on food surfaces by different mechanical procedures, such as spraying, brushing, and dipping (Dhall, 2013), or by electrostatic deposition (Poverenov et al., 2014). Overall, the functional properties of edible coatings depend on different factors including coating characteristics (composition, chemical structure, viscosity of the coating solutions, coating thickness, degree of crosslinking), coating processing conditions (temperature, pH, type of solvent), and type and concentration of additives (emulsifiers, plasticizers, or cross-linking agents). Recently, the performance of edible coatings was improved by the incorporation of different bioactive compounds, mainly

antimicrobial agents that may not only increase antimicrobial properties but also reduce biochemical deteriorations caused by processing, such as texture breakdown, enzymatic browning, and off-flavors development (Valdés, Burgos, Jiménez, & Garrigós, 2015). In this context, several studies have investigated the application of edible coatings as natural antimicrobial delivery systems to extend shelf life of highly perishable foodstuff, mainly fresh and minimally processed fruits and meat products.

Edible coatings incorporated with natural antimicrobial agents have been investigated in the meat industry for their ability to increase microbial safety and extend shelf life of meat products by reducing oxygen and moisture transmission, limiting microbial contamination and discoloration, and preserving texture, color, and flavor (Gennadios et al., 1997). Various biopolymers, including polysaccharides and proteins, have been investigated as effective antimicrobial delivery systems to curb undesirable effects in seafood products. Ariaii, Tavakolipour, Rezaei, Elhami Rad, and Bahram (2015) studied the effect of methylcellulose-based coatings incorporating 1.5% of *Pimpinella affinis* essential oil on the quality and shelf life of fresh silver carp (*Hypophthalmicthys molitrix*) fillets during storage at 4 °C for 20 d. They highlighted the ability of these coatings to reduce microbial growth and to extend shelf life of silver fillets up to 12 days without affecting their color, odor, texture, or overall acceptability. Likewise, Heydari, Bavandi, and Javadian (2015) reported the effectiveness of NaAlg coatings incorporating 1% horsemint essential oil in controlling microbial deterioration and limiting lipid oxidation of bighead carp (*Aristichthys nobilis*) fillets. Recently, Hosseini et al. (2016) found that fish gelatin-based coatings enriched with 1.2% oregano essential oil significantly reduced volatile base formations in rainbow trout (*Oncorhynchus mykiss*) stored at 4 °C for 16 days, and resulted in a 1.05 CFU/g reduction of psychrotrophic bacteria counts.

Several studies have demonstrated the high potential of edible coatings carrying different natural antimicrobial agents to preserve poultry, meat, and seafood products. (Fernández-Pan, Carrión-Granda, & Maté, 2014) reported the ability of whey protein isolate-based coatings enriched with oregano oil at a concentration of 20 g/kg to extend the refrigerated shelf life of chicken breast from 6 to 13 days, while at the same time, maintaining total mesophilic aerobic, LAB, and *Pseudomonas* spp

counts below the microbiological critical limits established for distribution and consumption. In agreement with these findings, (Aşik & Candoğan, 2014) reported the effectiveness of chitosan coatings incorporating garlic oil in reducing aerobic bacteria counts and extending the refrigerated shelf life of shrimp meat by 2 days.

Thus, antimicrobial packaging can be a promising tool for protecting meat products from pathogens contamination by preventing microbial growth and improve the quality and safety of meat products (Sánchez-Ortega et al., 2014). The gradual release of an antimicrobial substance from a packaging film to the food surface for extended period may render it more advantageous than incorporating the antimicrobial directly into foods (M. Ye, Neetoo, & Chen, 2008).

2.7.1. Application of Antimicrobial Edible Coatings

An edible coating material can be applied directly over food in liquid form by using different techniques, such as immersion, spraying, etc. (Kang, Kim, You, Lacroix, & Han, 2013). The edibility of films and coatings is only achieved when all components including biopolymers, plasticizers and other additives are food-grade ingredients, while all of the involved processes and equipment should be also acceptable for food processing (Falguera, Quintero, Jiménez, Muñoz, & Ibarz, 2011). The main coating techniques are described below (Valdés, Ramos, Beltrán, Jiménez, & Garrigós, 2017).

Spraying

Spray technique in packaging has drawn interest of the food industries because of the high quality of the final product and a potential cost reduction pertaining to the simplicity of its operation. The spray method provides uniformity of the coating, thickness control and minimizes possibility of multilayer build-up (Ustunol, 2009). The most important benefits that come with the spray technique is that the spraying systems do not contaminate the coating solution, allow temperature control and can offer automation for continuous production. The temperature directly affects the performance of the antimicrobial agents due to their high volatility and degradation. In addition, the thickness control is very important to establish the amount of

antimicrobial agent necessary to be released (Ramos, Fernandes, Silva, Pintado, & Malcata, 2012).

This technique allows less viscous coating solutions to be sprayed at high pressures (Ramos et al., 2012). The drop-size distribution of a sprayed coating solution can be up to 20 μm, whereas electrospraying can produce uniform particles lower than 100 nm from polymer and biopolymer solutions.

Spraying converts bulk liquids into droplets, and its advantages can be summarized as mentioned below:

- Increase in the liquid surface area, which is an important issue in processes where rapid vaporization is required. In fact, in antimicrobial applications, it is important to obtain homogeneous coatings where the additive is available to release quickly to the surrounding environment.
- The formation of an even surface, since the droplets dispersion generates coatings with homogeneous spatial patterns and controlled thicknesses. This is essential to evaluate the kinetics release of the antimicrobial additive.
- Cost reduction, since spraying techniques are usually fast and efficient processes in terms of solvent and material consumption.

Dipping

The dipping method has been successfully used to form coatings on fruits, vegetables and meat products (Lu, Ding, Ye, & Liu, 2010). Film thickness in the final product is an important aspect of the coating solution, which depends on the properties of the solution, such as density, viscosity and surface tension (Tavassoli-Kafrani, Shekarchizadeh, & Masoudpour-Behabadi, 2016), as dipping techniques are able to form thick coating layers (Dhanapal et al., 2012). In this method, a membranous film is formed over the product surface by direct dipping of the product into the aqueous coating formulation and further air-drying. This process can be divided into three steps (Schneller, Waser, Kosec, & Payne, 2013):

- Immersion and dwelling: The substrate is immersed into the precursor solution at a constant speed followed by dwelling to ensure that interaction of the substrate with the coating solution is enough for complete wetting.
- Deposition: A thin layer of the precursor solution is formed on the food surface by deposition. The liquid excess drains from the surface and is removed.
- Evaporation: The solvent excess evaporates from the fluid, forming the thin film.

It is important to take into consideration the density and viscosity of the solution while using this method of dipping. The coating solution must be dilute, because a significant residual coating material might remain in the surface. The optimal amounts of coating solution cannot be easily controlled by dipping, and a further processing step to dry off surplus solution is needed, requiring extra time and hindering industrial applications of this technique (Andrade, Skurtys, & Osorio, 2012).

Different examples of the use of the dipping technique for edible coating processing have been reported. Sodium acetate solution (10 g/L), was used for dipping the oysters, resulting in a coating with sodium alginate (40 g/L), with further use of modified atmosphere packaging (MAP) conditions (Costa, Conte, & Del Nobile, 2014) and a significant shelf-life extension was achieved.

Spreading

This method, also known as brushing, utilizes controlled spreading of a suspension on the surface. This method is considered a valid alternative for the preparation of films with dimensions larger than those prepared by casting procedures. The thickness of the coating suspension can be controlled by a blade attached to the lower part of the spreading device, and the film drying is held on the support itself, by circulation of hot air. This method can be applied to the production of polysaccharides and protein-based films. Two parameters can be used to characterize the spreading of liquid droplets: the wetting degree and the spreading rate (Khan & Nasef, 2009). In this sense, contact angle measurements are commonly used

to evaluate the degree of spreading/wettability of a surface by a particular liquid. Spreading is affected by several factors, such as the substrate properties (surface roughness and geometry), system conditions, such as temperature and relative humidity, and liquid properties (viscosity, surface tension and density) (G. Kumar & Prabhu, 2007).

Thus, we can summarize that coating methods are very diverse, and their selection depends on the purpose of coating, the desired product, coating thickness, solution rheology and the drying technique.

2.7.2. Chitosan as a coating material

Chitosan is one of the most widely researched polymers for its biopharmaceutical and biomedical applications including food science and technology. Chitosan is a deacetylated derivative of chitin. It is a cationic polysaccharide consisting of (1,4)-linked-2- amino-deoxy-b-D-glucan (Elsabee & Abdou, 2013). Chitin is the second-most abundant polysaccharides available after cellulose. It consists of three kinds of reactive functional groups, an amino group at C-2, and both the primary and secondary hydroxyl groups at the, C-3, and C-6 positions, respectively. Chitin is predominantly available in marine crustaceans as a major constituent of the exoskeleton, and is also preset in certain fungi as a component of the cell walls. Crabs, shrimps, lobsters and oysters are consumed every year as sources of protein rich sea food. The external shells and other non-edible parts of these crustaceans constitute about half of the body mass, and are usually discarded as waste (Hamed, Özogul, & Regenstein, 2016), which has gathered immense interest in recent times as a source of chitin for its multipurpose use in food industry, medics as well as cosmetics, in the form of chitosan.

Chitosan is approved for its use as an additive in food by the United States Food and Drug Administration (USFDA), and is a Generally Recognized as Safe (GRAS) food additive (Bellich, D'Agostino, Semeraro, Gamini, & Cesàro, 2016). It has been strongly indicated as a suitable functional material and the most appropriate biopolymer for production of edible films and coatings because of its excellent

biocompatibility, non-toxicity, biodegradability and film-forming abilities (Karimnezhad, Razavilar, Anvar, & Eskandari, 2017). Recently, chitosan-based edible films enriched with natural antimicrobial and antioxidant components like plant extracts and essential oils are being practiced in food industries. Composite films of chitosan with other biopolymers such as polysaccharides or proteins have been used for fabrication. solution-casting, layer-by-layer, extrusion and other techniques, and studied for their functional and antimicrobial properties and applicability as food packaging materials. Chitosan composites with numerous natural antioxidants, antimicrobial components, and nanomaterials have also attracted significant research focus in recent years (Kumar et al., 2020). Chitosan have been reported by Siripatrawan and Noipha (2012), as a suitable coating incorporated with green tea extracts to enhance the shelf life of pork sausages.

Chitosan matrices act as carriers of active compounds such as essential oil, fruit extracts, and other phytochemicals. The use of chitosan as a biopolymer for food packaging applications prolong shelf-life of various foods and food products. Chitosan being biocompatible, biodegradable and environment friendly, its use can reduce the use of synthetic plastics and additives and help promote healthy food and healthy environment (S. Kumar, Mukherjee, & Dutta, 2020).

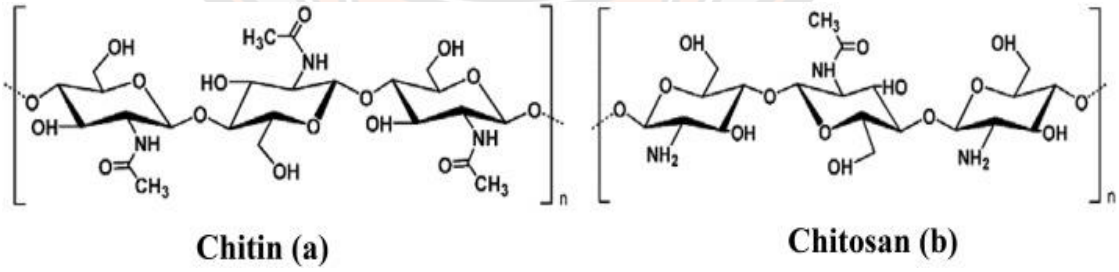


Figure 9: Chemical structure of chitin (a) and chitosan (b).

2.8. Plant Phenolic compounds

Phenolics are the abundantly occurring secondary metabolites of plants. They are the aromatic compounds with one or more hydroxyl bonds (-OH) attached

(Harborne et. al., 1989). A single unit of this hydroxylated aromatic ring is called a phenol. They can be simple phenols or polyphenols, depending on the number of aromatic rings or phenols they possess. There are a variety of phenolic compounds found in nature, and can be broadly classified as: phenolic acids, flavonoids, isoflavonoids, hydroxycinnamic acid, stilbenes, lignans, tannins etc.

The classification tree of polyphenols is shown in Figure 10. In plants they play the role of providing resistance against pathogens and predators and helping seed germination (Ross & Kasum, 2002). The most abundantly occurring plant phenols in nature are the flavonoids (Whiting, 2001).

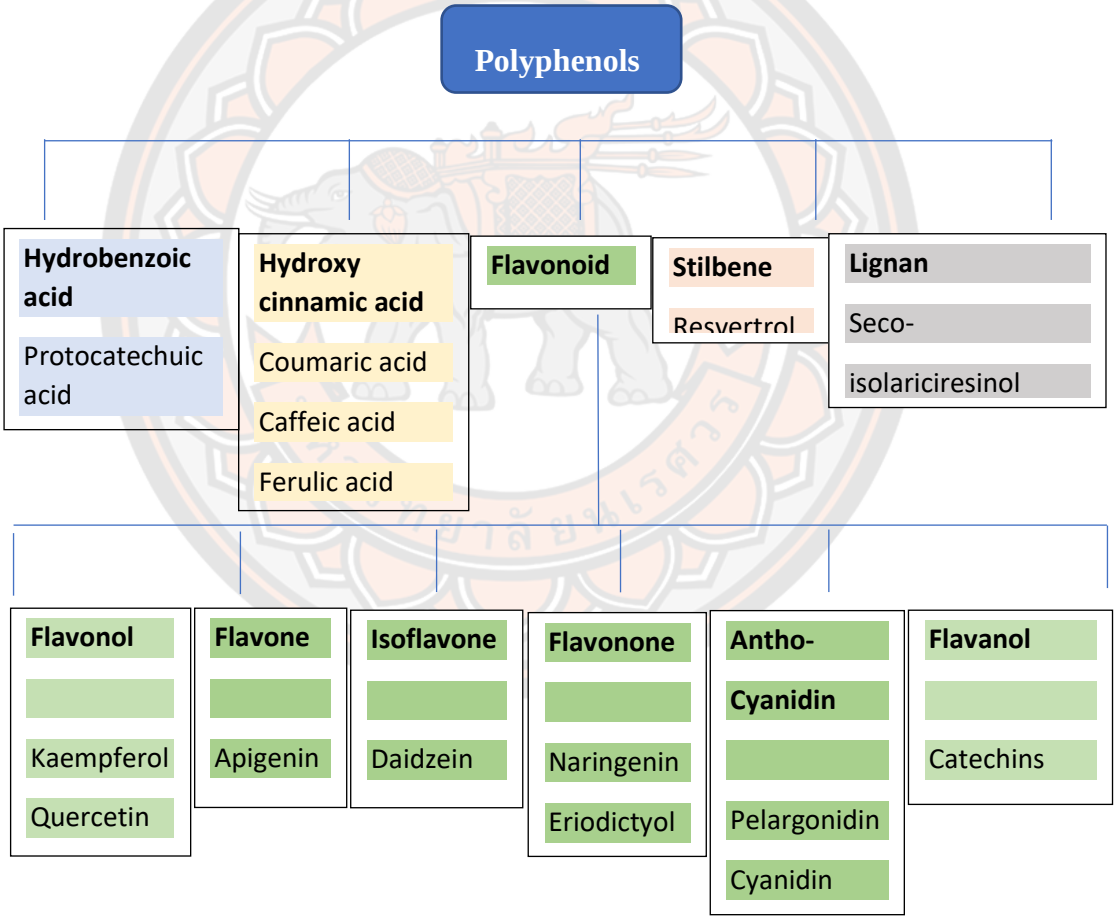


Figure 10. Classification of polyphenols

Polyphenols are available in almost all fruits, vegetables and plant-based beverages. Incorporating polyphenol rich food to diets have been regarded beneficial to human health. Although polyphenols were previously associated with toxicity, the more recent epidemiological studies suggest that dietary polyphenols could help maintain gut-health, reduce the risk of cardiovascular disease, prevent cancer, obesity, and type 2 diabetes, subside the rate of brain aging and Alzheimer's disease (Anuradha, 2013; Perez-Vizcaino, Duarte, Jimenez, Santos-Buelga, & Osuna, 2009). Furthermore, plant polyphenols have been found to exert several effects of pharmaceutical importance such as food preservative, antioxidant, anti-inflammatory, antiallergic, antimutagenic activities (Chirumbolo, 2014; Cueva et al., 2010). Plant polyphenols have also exhibited antimicrobial effects against a broad spectrum of pathogenic and spoilage bacteria. Various studies suggest that the plant polyphenols possess the potential to be used as natural preservatives for food (Davidson, Sofos, & Branen, 2005).

2.9. Antimicrobial susceptibility testing methods

Watts et al. (2008) suggest the following tests for antimicrobial susceptibility.

- Disc diffusion
- Broth dilution
- Agar dilution

2.9.1. Disc diffusion method

Disc diffusion refers to the diffusion of an antimicrobial agent of a specified concentration from discs, tablets or strips, into the solid culture medium that has been inoculated with the isolate in a pure culture. Disc diffusion is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disc.

The diffusion of the antimicrobial agent into the culture media results in a gradient of the antimicrobial around the disc, which is known as the zone of inhibition. The diameter of this zone of inhibition is related to minimum inhibitory concentration (MIC) for that particular bacterium/ antimicrobial combination; the

zone of inhibition correlates inversely with the MIC of the test bacterium. Generally, the larger the zone of inhibition, the lower the concentration of antimicrobial required to inhibit the growth of the organisms. However, this depends on the concentration of antibiotic in the disk and its diffusibility.

2.9.2. Broth dilution

The broth microdilution as well as broth macrodilution is a useful technique for determining minimum Inhibitory Concentration (MIC) of large numbers of test samples. MIC in microbiology is the lowest concentration of antimicrobials that completely inhibits the visible growth of microorganisms after overnight incubation. MIC is carried out in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and in antimicrobial research to monitor the activity of new antimicrobial agent. The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in tubes or microdilution wells as detected by the unaided eye (Wayne, 2012). For the determination of MIC endpoint, viewing devices can facilitate reading microdilution tests and recording results with high ability to discern growth in the wells. Moreover, several colorimetric methods based on the use of dye reagents have been developed.

Some studies have used indicators like tetrazolium salts or resazurin dye (Umesh et al., 2005) or spectrophotometry to determine presence of growth (Devienne & Raddi, 2002). For spectrophotometric method the absorbance is usually at 620 nm measured against the blank (Salie, Eagles, & Leng, 1996). Concentration with sharp decline in absorbance value (Devienne & Raddi, 2002) or the lowest concentration which gives a zero-absorbance reading (Salie et al., 1996) is the MIC of the plant extract or the test phytochemical.

2.9.3. Agar dilution

The agar dilution method involves the incorporation of varying desired concentrations of the antimicrobial agent into an agar medium (molten agar medium), habitually using serial two-fold dilutions, followed by the inoculation of a defined microbial inoculum onto the agar plate surface. The MIC endpoint is recorded as the

lowest concentration of antimicrobial agent that completely inhibits growth under suitable incubation conditions.

2.10. Mechanism of antibacterial action

The anti-microbial mode of action can be broadly categorized as:

- Bacteriostatic action
- Bactericidal action

The mechanism by which the anti-microbials exert these properties are enlisted as follows:

2.10.1. Bacteriostatic action

- Interference with protein synthesis by binding to 30s ribosomal sub-unit and blocking the attachment of transfer-RNA (tRNA). eg. Tetracyclines, Aminoglycosides, Streptomycin, Amikacin etc)
- Interference with protein synthesis by binding to 50s ribosomal sub-unit. Eg. Macrolides (chloramphenicol).

2.10.2. Bactericidal action

- Interference with cell wall synthesis:
  They block the peptidoglycan synthesis in growing bacteria. E.g. β-lactams.
- Interference with cytoplasmic membrane:
  They bind to the cytoplasmic membrane and disrupt and destabilize it. This cause the cytoplasm to leak out of the cell resulting in cell death. E.g. Polymyxin.

Increase in membrane permeability and subsequent loss of membrane integrity of the bacteria is a major factor in the mechanism of antimicrobial action. Various phenolic compounds are generally presumed to exert their antibacterial property by damaging the cytoplasmic membrane of bacterial cells. This phenomenon is attributed to the presence of hydroxyl groups of the phenols (Gyawali & Ibrahim, 2014). The

hydrophobic phenolic groups attach to the lipid bilayer, thus disrupting the lipid–protein interaction and increasing membrane permeability. This further causes alterations in membrane structure and hence cause the leakage of intracellular constituents (Char, Guerrero, & Alzamora, 2010). The cell integrity of the bacteria thus collapses causing cell death. The mechanisms accountable for antibacterial property of phenols include: disruption of bacterial cell membranes, inhibition of nucleic acid synthesis, interaction with cellular enzymes, inhibition of energy metabolism (Cushnie & Lamb, 2011). The mechanism has also been linked to metal (iron, Fe) ion chelation (Wong & Kitts, 2006).

A diagrammatic representation of the antimicrobial action of natural preservatives as created by Lee and Paik (2016), is presented in Figure 11.

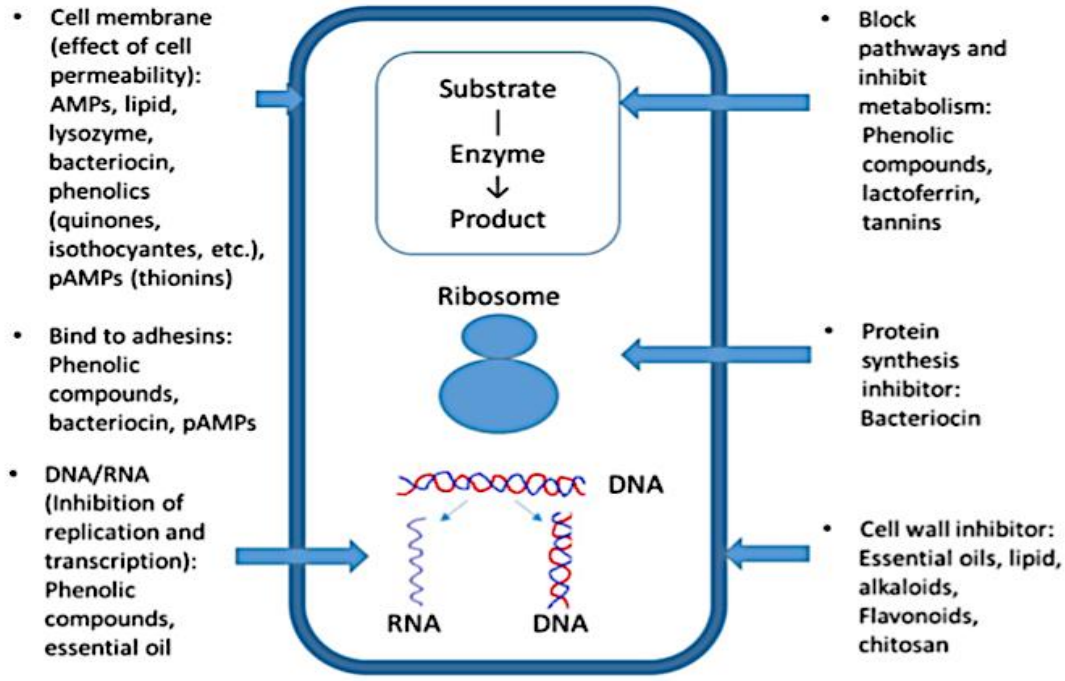


Figure 11. Mechanism of antimicrobial activity of various natural preservatives

Source: Lee and Paik, 2016

2.11. The antioxidant activity of phenolic compounds

Reactive oxygen species (ROS) are generated as a result of partial reduction of oxygen which leads to the formation of radical oxygen species such as $O_2^{\bullet-}$ (superoxide anion), $HO^{\bullet}$ (hydroxyl radical), $RO^{\bullet}$ (alkoxyl) and $ROO^{\bullet}$ (peroxyl) radicals that are generated during lipid peroxidation (Lewoyehu & Amare, 2019) . These radicals initiate chain reaction causing cellular damage or death (H. Sun, Mu, Song, Ma, & Mu, 2018). Antioxidants scavenge these free radicals and inhibit the oxidation process. Phenolic compounds exert the antioxidant activity by direct scavenging of ROS, inhibition of enzymes involved in oxidative stress, regeneration of other antioxidant (α-tocopherol), chelation of metal ions that are responsible for ROS production and, finally, stimulation of endogenous antioxidant defense systems. The two main approaches by which phenolic compounds exert antioxidant activity, the H-atom transfer and electron transfer (Figure 12).

In the H-atom transfer (HAT)-based method, the phenol antioxidant donates an H-atom to an unstable free radical ($R^{\bullet}$) to form stable compounds (Craft et al., 2012). The ability of an antioxidant to scavenge free radicals by hydrogen donation HAT-based methods are more relevant to the radical chain-breaking antioxidant capacity.

In the electron transfer (ET), the phenoxyl radical is produced by single-electron transfer from the phenol antioxidant.These reducing effects are characterized by two important physicochemical parameters, the bond dissociation energy (BDE) of the O–H bond and the ionization potential (IP) of the phenolic compounds that quantify the HAT and ET, respectively. The lower the BDE and the IP, the stronger is the reducing activity of a phenolic compound. It is very difficult to distinguish between HAT and SET reactions. In most situations, these two reactions take place simultaneously, and the mechanism of the reaction is determined by the antioxidant's structure and solubility, the partition coefficient and solvent polarity. Examples of HAT-based assays include ABTS and ORAC, and examples of SET-based assays include DPPH and FRAP Source: Shalabay and Shanab, 2013.

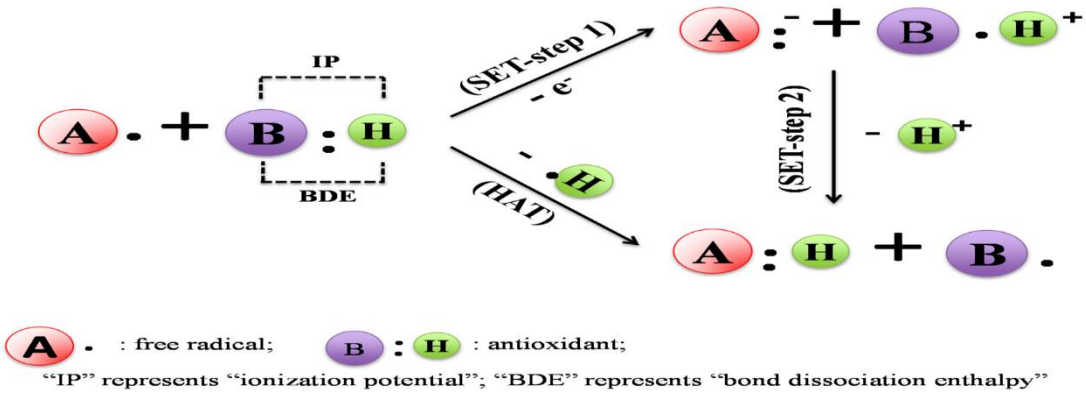


Figure 12. Mechanisms of antioxidant reacting with free radical: electron transfer (ET) and hydrogen atom abstraction (HAT).

Source: Liang and Kitts, 2014

2.11.1. Methods to evaluate the antioxidant activity of phenolic compounds

2.11.1.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a strong, purple, stable free radical that, after electron transfer or hydrogen quenching from phenolic antioxidants, turns into a colorless hydrazine (DPPH-H) (Cerretani & Bendini, 2010). This is the most frequently used assay for the evaluation of the free radical-scavenging capacity of plant extracts. The reaction mechanism involves the H- transfer from a phenolic compound to the DPPH radical (Figure 13). Interaction of the DPPH radical (purple-coloured) with a phenolic compound, which is able to neutralize its free radical character, leads to the formation of yellow colorless hydrazine and the resulting effect can be quantified spectrophotometrically at 515 nm (Bujor, 2016).

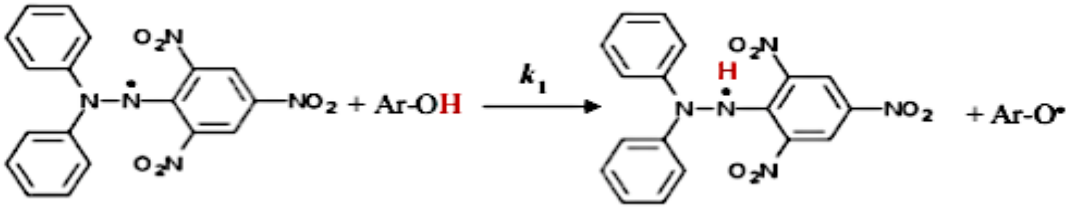


Figure 13. The H-transfer reaction from a phenolic compound (Ar-OH) to DPPH.

In the DPPH test, the antioxidant activity of phenolic compounds is generally quantified by their EC50 values (concentration necessary to reduce 50% of DPPH) or their stoichiometry (number of DPPH molecules reduced by one molecule of antioxidant) (Goupy, Bautista-Ortin, Fulcrand, & Dangles, 2009; Vučić et al., 2013) and also as micromoles Trolox, Ascorbic acid equivalents (Abdulkadir, Nashriyah, Hasan, & Jahan, 2016).

2.11.1.2. Folin–Ciocalteu redox method

The Folin-Ciocalteu (FC) method is based on a single electron transfer mechanism and is used to quantify the contents in total phenolic compounds in plant extracts using gallic acid as a standard. Since its mechanism is an oxidation/ reduction reaction, the FC method can be also be considered as a method for quantification of the antioxidant capacity. The FC method involves the reduction of the molybdenum component in the phosphotungstic-phosphomolybdic complexing reagent according to the following reaction (Craft et al., 2012).

$$Mo^{6+}\ (\text{yellow}) + ArOH \rightarrow Mo^{5+} + [ArOH]^{\bullet+}$$

The Total Phenolic Compounds are estimated after reaction of the sample with diluted Folin-Ciocalteu reagent (a mixture of sodium molybdate, sodium tungstate and other reagents) and sodium carbonate. The reaction with phenolic compounds produces a blue color which typically absorbs at 765 nm.

2.11.1.3. Other methods

Other popular methods for evaluation of the antioxidant activity of phenolic compounds are oxygen radical absorbance capacity (ORAC), ferric ion reducing antioxidant power (FRAP), and Trolox equivalent antioxidant capacity (TEAC) assays (Craft et al., 2012).

ORAC measures antioxidant inhibition of peroxyl-radical-induced oxidations and reflects classical radical chain-breaking antioxidant activity by H-atom transfer

mechanism (Ou et al.2001). In the basic protocol of ORAC assay, the peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product which is quantitated by fluorescence (Karadag, Ozcelik, & Saner, 2009). Antioxidant capacity is measured by a decreased rate and amount of product formed over time. The advantage of this method is that it can be adapted to detect both hydrophilic and hydrophobic antioxidants by altering the radical source and solvent (Prior, 2015). FRAP assay is based on the ability of phenolics to reduce the yellow colored ferric tripyridyltriazine complex (Fe(III)-TPTZ) to blue colored ferrous complex (Fe(II)-TPTZ) by the action of electron-donating antioxidants (Benzie & Strain, 1996). The resulting blue color measured spectrophotometrically at5 93 nm is taken as linearly related to the total reducing capacity of electron-donating antioxidants. One FRAP unit is defined as the reduction of 1 mol of Fe(III) to Fe(II) (Huang, Ou, & Prior, 2005).

2.12. *Moringa oleifera*

M. oleifera comes under Moringaceae family and is popularly known as the drumstick tree. Its long slender pods are used as vegetables (drumstick) (Figure 14). It is considered as one of the most useful trees in the world because most of the parts of this plant can be used as food, in medicine and for industrial raw material (Anwar, Latif, Ashraf, & Gilani, 2007).

Figure 14. *M. oleifera* tree with pods (drumstick)

2.12.1. Local names

The local names include: Shigru, Shobhanjana (Sanskrit), Saijan (Nepali), Shajna(Hindi), Makhokom(Thai), Drum stick tree, Horseradish tree, Ben oil tree, Benzoil tree (English) (Ramachandran, Peter, & Gopalakrishnan, 1980).

2.12.2. Botanical nomenclature

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliphyta

Class: Magnoliopsida

Subclass: Dilleniidae

Order: Brassicales

Family: Moringaceae

Genus: *Moringa*

Species: *Moringa oleifera*

Source: Kumar et al., 2010; Muhammad et al., 2016

2.12.3. Distribution

Moringa is a single species of the family Moringaceae, and *M. oleifera* is the most widely cultivated species of its family. It is an indigenous plant of the sub-Himalayan region of Asia and is widely distributed in different Asian and African countries (Ramachandran et al., 1980). It grows in the tropical and sub-tropical climate of around 25-35^{o}C. The height of the full-grown tree can range from 5 to 10 m.

2.12.4. Nutritional and Therapeutic properties

M. oleifera is a highly nutritious plant. Leaves, pods, bark, seeds are edible and are used in many countries as food, oil, medicine etc. (Prabhu et al., 2011). The leaves are the most widely used part of the tree for its rich nutritional composition. It is rich in minerals like Potassium, calcium, Phosphorus, Iron. It is also a good source of Vitamin A, D and several essential amino acids. The vitamin A composition in leaves is about 23,000 IU, which is far more than that found in green leafy as well as colorful vegetables like spinach, broccoli, carrots etc. (Amaya et al.,1992). High amount of essential amino acids like methionine and cysteine (43.6g/kg protein) resemble to the. milk of human and cow, as well as chicken eggs (WHO, 1985).

The tree also bears pharmacological potential and is used worldwide in traditional medicine (TH, Joshi, Desai, Desai, & Tirgar, 2012). *M. oleifera* has also been extensively tested for its antioxidant activity. Radio-protective properties were demonstrated by the methanolic extracts of dried leaves of *M. oleifera* in an in-vivo study conducted by (Rao, Devi, & Kamath, 2001). Detoxification and anti-carcinogenic properties have been reported from the oral administration of aqueous acholic extract of the green pods, by improving the enzyme levels like b5, P450 cytochrome catalases, reductase, glutathione-peroxidase etc, that play role in detoxifying the xenobiotic substances or chemicals (Bharali, Tabassum, & Azad, 2003).

The high-quality protein content and the relative lack of anti-nutritional factors and low toxicity (LD_{50} value of 512.8mg/kg of the aqueous extract) (Berger, Habs, Jahn, & Schmahl, 1984) are the desirable attributes of *Moringa oleifera* as animal feed.

2.12.5. *M. oleifera* seed

Moringa seed kernels contain 22-40% of crude fat (Ghazali & Mohammed, 2011). It is commercially known as Ben oil and is edible. Ben oil is also used in

Figure 15. *M. oleifera* seeds

manufacturing cosmetics and perfume (Ghazali & Mohammed, 2011). The edible oil resembles olive oil in taste and aroma. The seeds are known to have antimicrobial activity and are utilized for waste-water treatment. The seed powder is used in water treatment because of its strong coagulating property that causes sedimentation of suspended undesired particles (Anwar et al., 2007).

2.12.5.1. M. oleifera seed components

Seed of *M. oleifera* is a significant source of fats, proteins, vitamins, minerals, and other nutrients with low toxicity (Saini, Shetty, Prakash, & Giridhar, 2014).
In the study conducted by Compaoré, Nikièma, Bassolé, Savadogo, and Mouecoucou (2011) (Table 1), lipid content was shown to be about 43.56±0.03% which was comparable to that of peanuts, rapeseed, sunflower and mustard (Grosso, Zygadlo, Lamarque, Maestri, & Guzmán, 1997; SAEED & CHERYAN, 1988; Sheehan, Atherton, Broadbent, & Pritchard, 1991; Zhou, He, Yu, & Mukherjee, 1990). The same research showed a high protein content of 35.37±0.07%. However, with geographical ecozones and cultivation practices, the chemical composition of the seeds can have considerable variation.

Table 2. Proximate analysis of the seeds of Moringa oleifera seed

Analysis (g/100g DM)	***Moringa oleifera* seeds**
Moisture	2.14±0.01
Ash	4.98±0.04
Protein	35.37±0.07
Lipid	43.56±0.03
Total carbohydrate	9.17±0.25
Crude Fibre	4.70±0.2

Source: Compaoré et al., 2011

2.12.5.2. Moringa oleifera seed meal

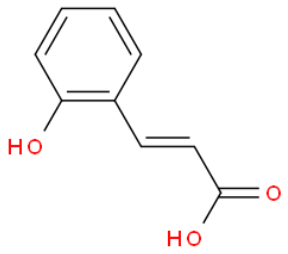
Moringa seed meal, as shown in Figure 16, is the byproduct of the oil seed extraction process. It is rich in protein minerals and polyphenols. Antioxidant activity of the seed. meal have been extensively studied (G. Singh, Negi, & Radha, 2013; Ogbunugafor et al., 2011).The seed extract of *Moringa oleifera* has also been successfully tested for its broad-spectrum antibacterial activity(Auwal et al., 2013).

Figure 16. *M. oleifera* seed meal

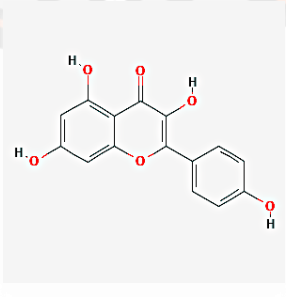
2.12.5.3. Phenolic compounds

The methanol extract of the seeds of *Moringa oleifera* showed antibacterial activity against *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Cladosporium cladosporioides and Penicillium sclerotigenum* (Oluduro, Aderiye, Connolly, Akintayo, & Famurewa, 2010). Three compounds, 4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate, methyl N-4-(α-L-rhamnopyranosyloxy)benzyl carbamate, and 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl thiocarboxamide were identified from the extract of *Moringa oleifera* seed, which exhibited potent antimicrobial activity against some commonly occurring pathogens at the concentration of 5 mg/L. Benzyl isothiocyanate, isolated from the methanol extract of *Moringa oleifera* seed, showed effective antimicrobial activity against the following bacteria: *Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa* (Rim Jeon, Ha Lee, Ha Shin, Sang Kwon, & Sung Hwang, 2014). The minimum inhibitory concentration (MIC) test of the free and bound phenolic extract showed 1.91mg/ml and 1.4mg/ml value respectively against *E. coli*; 1.17mg/ml an 0.6 mg/ml against *Staphylococcus aureus* and 1.4 mg/ml and 0.69 mg/ml against Bacillus cereus respectively (G. Singh et al., 2013). A short polypeptide called 4 (ά – L -rhamnosyloxy) benzyl-isothiocyanate was attributed for the antimicrobial property of M. oleifera seeds (Guevara et al., 1999).

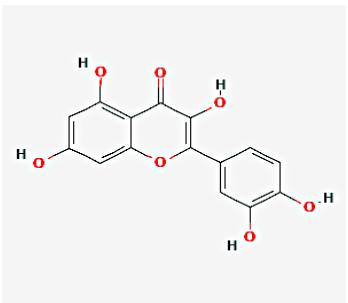
Some of the frequently isolated phenolic compounds are shown in Figure 17.



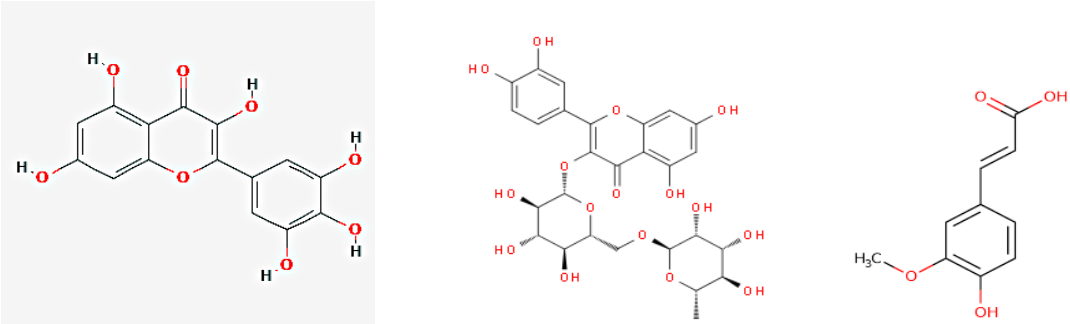
o-coumaric acid



Kaempferol



Quercetin



Myricetin Rutin Ferulic acid

Figure 17. Major polyphenolic compounds isolated from the alcohol-extract of *M. oleifera* seeds

2.13. *Nigella sativa*

N. sativa is an annual herbaceous plant, native to Southern Europe, and Southwest Asia (Khare, 2004). The plant is grown for its seed which is commonly known as black seed or black cumin. These seeds are used as condiments, toppings in bakeries and naan. They are also used in traditional medicine round the globe for the treatment and prevention of several ailments. (Goreja, 2003). Such biological activity of the seeds has been attributed to thymoquinone, an active ingredient present in the essential oil of *Nigella sativa* (Venkatachallam, Pattekhan, Divakar, & Kadimi, 2010).

Figure 18. *N. sativa* in bloom

2.13.1. Local names

English fennel flower, black caraway, black cumin, nutmeg flower, Roman coriander (English), kalonji (Hindi), Mungrelo (Nepali), Ketzah (Hebrew).

2.13.2. Botanical nomenclature

Kingdom	Plantae
Subkingdom	Viridiplantae
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Super-order	Ranunculanae
Order	Ranunculanes
Family	Ranunculaceae
Genus	*Nigella* L.
Species	*Nigella sativa* L.

Source: http://www.itis.gov

2.13.3. Distribution

N. sativa is a native plant of southern Europe, North Africa, south and southwest Asia. *N. sativa* is one of the most ancient known domesticated plants and is believed to have been mentioned in the old testament. It has been extensively documented for its traditional use as an important medicinal plant and spice since ancient times (Padhye, Banerjee, Ahmad, Mohammad, & Sarkar, 2008). Its seeds were reportedly found in Egyptian Pharaoh Tutankhamon's tomb (Zohary & Hopf, 2000) to assist in his afterlife.

Figure 19. *N. sativa* seeds

Black cumin is grown worldwide – especially in the Middle East, Mediterranean regions, Southern Europe, Syria, Saudi Arabia, Turkey, India and Pakistan and is used as condiments and traditional medicine (Chevallier, 1996).

12.13.4. Chemical composition and therapeutic properties of seeds

The chemical composition of the black cumin seed has been studied by various researchers. In a study conducted by (Hajhashemi, Ghannadi, & Jafarabadi, 2004), twenty different compounds were identified in the oil. The major components identified were para-cymene (37.3%) and thymoquinone (13.7%). Thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ) and thymol (THY) were the major phenolic compounds obtained by super-critical carbon di-oxide, among the forty-seven volatile compounds identified in the oil of black cumin seed (Venkatachallam et al., 2010). The seed coat of black cumin predominantly possesses volatile oil components and nigellidine and nigellicine alkaloids. Compounds such as dopamine and other important metabolites are present in the inner tissue layer of seed (Botnick et al., 2012).

Black cumin seeds have been used as condiment and for medicine since ancient times (Chevallier, 1996). The plant is known to have been used since the Babylonian era to treat external wounds and to relieve gastric upsets (Levey, 1966). Hippocrates and Galen described its use to treat various health conditions, including rhinitis. Similarly, Pedanius Dioscorides reported its use as food and medicine to treat head and tooth aches, eye infection, dermatitis and leprosy. It is also described to be used as anthelmintic, menstruation regulator, diuretic and milk flow enhancer (Al-Shaibani, Phulan, Arijo, Qureshi, & Kumbher, 2008; Benhelima, Kaid-Omar, Hemida, Benmahdi, & Addou, 2016; Padhye et al., 2008). This seed is

also popular in Islam and has been referred to as a panacea in the holy book of *Sahih al-Bukhari* (Butt & Sultan, 2010). *N. sativa* seeds are used as traditional medicine to treat various maladies related to respiratory problems, hepatic and digestive disorders including diarrhea (Butt & Sultan, 2010). It has been reported to have anti-cancer, anti-inflammatory, anti-hypertensive and immune-modulator properties (Ramadan, 2007). Black cumin seeds are also commonly used as a condiment and are used in confectionaries and top-dressing.

12.13.4.1. Phenolic Compounds

Very few researches have been published on isolation and identification of phenolic compounds of *N. sativa*. Among those, (C. C. Toma, Olah, Vlase, Mogosan, & Mocan, 2015) identified quercetin, quercitrin and kaempferol as the major polyphenols and two phenolic acids: p-coumarinic and ferulic acid in the seeds of N. sativa. Sinapinic acid and kaempferol were identified as the major phenolic components of the seed extracts (Topcagic, Cavar Zeljkovic, Karalija, Galijasevic, & Sofic, 2017). The major components identified were para-cymene (37.3%) and thymoquinone (13.7%). Thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ) and thymol (THY) were the major phenolic compounds obtained by super-critical carbon di-oxide, among the fourty-seven volatile compounds identified in the oil of black cumin seed (Venkatachallam et al., 2010). Thymoquinone obtained from nigella shows MIC at 0.5 mg/ml for *E. coli* and *S. typhimurium.* The MIC is 0.003 mg/ml for *S. aureus* (Chaieb, Kouidhi, Jrah, Mahdouani, & Bakhrouf, 2011).

Figure 20. *N. sativa* and its extract

The crude methanolic extracts and water fraction of black cumin seedcake contains hydroxybenzoic, syringic and p-coumaric acids. p-Coumaric acid was the

most abundantly available phenolic acid in both the methanolic extracts and the water fraction, contributing about 66.8% and 72.1% to the total phenolic content, respectively (Mariod, Ibrahim, Ismail, & Ismail, 2009).

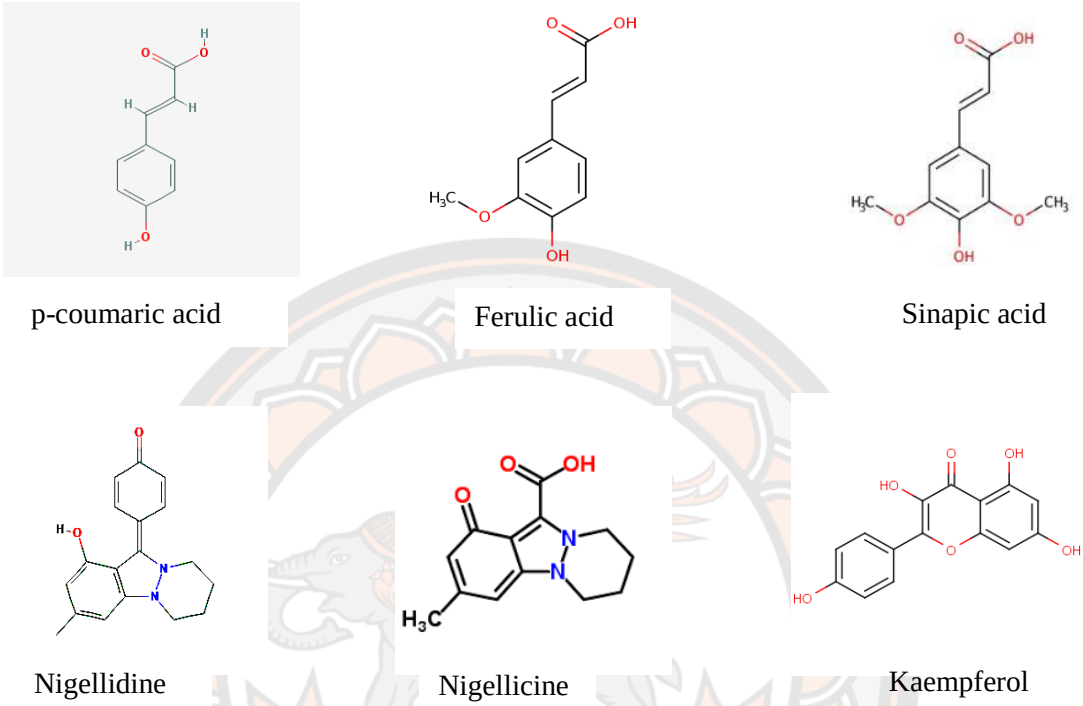


Figure 21. Major phenolic compounds isolated from *Nigella sativa*

2.14. *Camelia oleifera*

Figure 22. *C. oleifera* on bloom

C. oleifera is a tree grown for its seeds. Tea oil is extracted from the seeds. The tree is commonly called the tea-oil tree and is the most important edible oil tree grown in China (Zhang, Wang, Wu, Xu, & Chen, 2011). 55-71% of tea-oil can be extracted from the tea oil seed depending on the method of extraction (Wu, Johnson, & Jung, 2009; Zhang et al., 2011). Tea oil has a better essential fatty acid profile than in palm, rapeseed and peanut oil (J. Ma, Ye, Rui, Chen, & Zhang, 2011). Tea oil has been referred to as eastern olive oil (Long and Wang, 2008) because of the similarity of the unsaturated fatty acids profile (>90%) in both of them. The total unsaturated fatty acid was derived to range between 75-91% as compared to 87% in olive oil (J. Ma et al., 2011).

The tea oil is used as cooking oil in China. Its use is extended to cosmetics as well as therapy. The deoiled tea seed meal contains a substantial amount of saponin compounds, and it has been used extensively in aquaculture to selectively remove predaceous fish and insects in shrimp ponds (Tang, 1961). The use of tea seed meal as molluscicide in rice field showed promising results (Kijprayoon et. al., 2014).

2.14.1. Botanical nomenclature

Kingdom: Plantae
Phylum: Tracheophyta
Class: Magnoliopsida
Order: Theales
Family: Theaceae
Genus: *Camellia*
Species: *Camellia oleifera* Abel.

2.14.2. Distribution

C. oleifera is the native of China, where it is widely used as cooking oil. It is distributed in southern China, and the cultivation is known to have started 2000 years back (Lin et al., 2018).

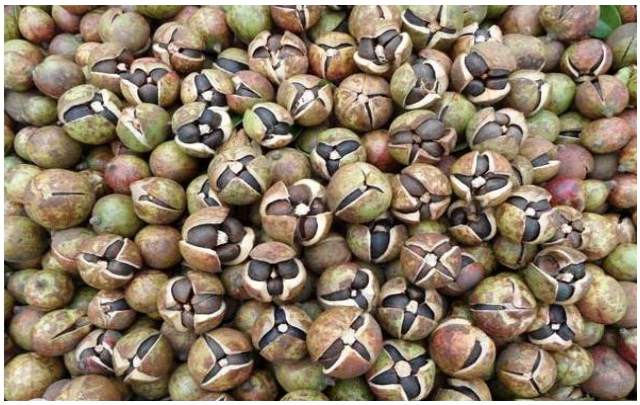


Figure 23. *C. oleifera* seeds

It is also cultivated in some parts of Laos, Myanmar and Vietnam (iucnredlist.org). It is a hardy wooden tree and can even grow on unfertilized barren land. The first harvest of fruits (seeds) can be made after eight years of initial planting, and the production continues until 80 subsequent years. *C. oleifera* seed is mainly used to produce edible oils, such as camellia oil, tea seed oil, or oil-tea camellia seed oil in China.

2.14.3. Chemical composition and therapeutic properties

The major product of *C. oleifera* seeds is oil, also called Camellia oil or tea oil. Tea oil accounts for about 50% of dry weight of kernel. The unsaturated fatty acids are predominant in the oil accounting for approximately 90% of the total fatty acid content. The major unsaturated fatty acids in tea oil are oleic acid (18:1) (74-87%) which is a monounsaturated fatty acid (MUFA), and linoleic acid (18:2) (7-14%), which is a polyunsaturated fatty acid (PUFA). (Yang et al., 2016). These unsaturated fatty acids are considered beneficial for human health. Besides oleic acid and linoleic acid, tea oil contains several other fatty acids, including stearic, palmitic and linolenic acids (J. Ma et al., 2011). The flavonoid (1-3%), saponin (10-14%), and polysaccharide (15-20%) were detected in *C. oleifera* seeds (Chen, 2007) .

Tea oil is used in traditional medicine in China as an emollient and anti-wrinkle cream for skin. It is also known to have been used to prevent as well as heal gastric upsets and ulcers (Cheng et al., 2014), to treat burns (Lee & Yen, 2006). It is reported that tea oil reduces serum triglycerides and can increase high-density

lipoproteins (HDL) (good cholesterol) in humans (J. Y. Gao, 1993). This reduces the risk of heart disease. According to the research conducted at Clemson University (Chen, 2007), camellia oil and camellia oil meal exhibited antiproliferative activities against cancer cells of human (uterus cancer, breast cancer, and colon cancer). Kaempferol, kaempferol glyosidic flavonoids, saponins, and five-ring triterpenes are ascribed to the antiproliferative and antioxidative properties of *C. oleifera* seed meal. A dehydro-triterpenic hydrocarbon called squalene, found in both olive oil and camellia oil (Lee & Yen, 2006) is also known to have antitumor and anticancer effect.

2.14.4. Phenolic compounds

Polyphenols found in *C. oleifera* and tea (*Camellia sinensis*) are similar in composition. Catechin, epigallocatechin-gallate, epigallocatechin, epicatechin-gallate, gallocatechin, and epigallocatechin have reported in the seeds of *C. oleifera* (Fang, Du, Luo, & Jin, 2015). The MIC of the phenolic extracts of *C. oleifera* was 0.125 mg/ml, 0.25 mg/ml and 0.063mg/ml for *E. coli*, *S. aureus* and *B. cereus* respectively (Hu, Nie, Huang, Li, & Xie, 2012). Kaempferols and gallic acid was isolated from the defatted seed meal of the *C. oleifera*. The high content of kaempferol and its derivatives in the seed cake of *C. oleifera* can be regarded as a good source of health promoting flavonoid compounds (D.-F. Gao et al., 2011) . However, little is known about the anti-inflammatory effects of flavonoids from the C. oleifera seeds and requires further investigation.

Flavonoids are members of a class of natural compounds widely distributed in the plant kingdom, and possess many bioactivities including antioxidation, antibacterial, antiviral, and protective effects from many diseases such as cancer, cardiovascular, and inflammation. Four compounds in total flavonoid fraction were identified in the 80% ethanolic extract of *C. oleifera* seeds by semi-permeative HPLC. The compunds as shown in Figure 24, were kaemferol-3-O-[2-O-β-D-glucopyranosyl-6-O-L-rhamnopyra-nosyl]-β-D-glucopyranoside (A), kaempferol-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-O-α-L-rhamnopyranoside (B), kaemferol-3-O- [2-O-β-D-xylopyranosyl - 6-O-α -L-rhamnopyranosyl] - β-D-

glucopyran-oside (C), and kaemferol-3-O-α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside (D) (Liu et al., 2014).

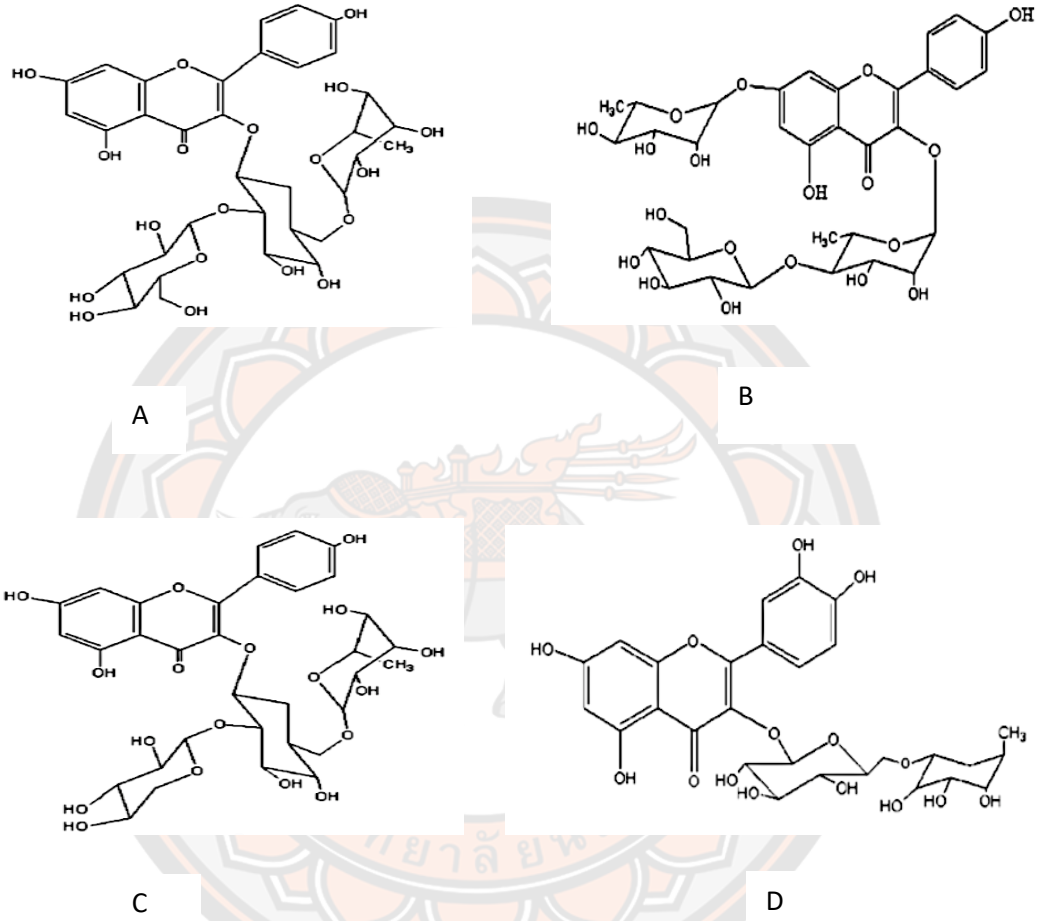


Figure 24. The chemical structures of four Kaempferol compounds isolated by 80% ethanolic extraction of C. oleifera seeds by Liu et al. (2014).

The antimicrobial activity of the three selected seed meals in different literature is given below in Table 3.

Table 3. The Minimum Inhibitory concentrations (MICs) of the different bioactive compounds isolated from the seed extracts, expressed as mg/ml

Serial No.	Sample	*E. coli*	*Salmonella sp*	*S. aureus*	*B. cereus*	References
I	***M. oleifera***					
a	Free phenolic extract	1.91	NA	1.17	1.4	Singh et al.,2013
b	Bound Phenolic extract	1.4	NA	0.6	0.69	
II	***N. sativa***					
a	Thymoquinone	0.5	0.5	0.008	0.008	Chaieb et al., 2011
b	Thymoquinone	0.8	08	.003	NA	Halawani, 2009
c	Thymohydroquinone	1.6	0.8	0.4	NA	Halawani, 2009
d	Seed extract (EtOH)			0.15		Yassen, 2017
III	***C. oleifera***					
a	Saponin mix	0.2	0.2	0.15	NA	Khan et al., 2018
b	Phenolic component	0.125	NA	0.250	0.063	Hu et al., 2008

2.15. Extraction Methods

Selecting the extraction method to ensure the highest efficacy depends on the nature of the ingredients and the way it is processed, the thermal stability and polarity of the compounds (Shirsath, Sonawane, & Gogate, 2012). Polarity of a compound is one of the major factors determining its solubility. Organic compounds tend to have higher affinity towards organic solvents, because of their corresponding polarity. Commonly used organic solvents are ether, acetone, alcohol, hexane etc. Likewise, more polar compounds are more soluble in polar solvents like water. The properties of different solvents at 25^{o}C is shown in Table 4.

Table 4. Properties of different solvents at 25ºC

Type of solvent	Polarity index	Surface tension (mN/cm)	Vapor pressure (mm/Hg)	Viscosity (cP)
Ethanol	5.2	23.7	59.02	1.2
Methanol	5.1	22.6	127.05	0.6
Acetone	5.1	23.7	229.52	0.32
Water	9	72.8	23.8	0.89

Source: www.eastman.com

2.15.1. Solvent extraction

Solvent extraction is a popular method of extraction of polyphenols. Liquid-liquid and solid-liquid extraction are the most commonly used for polyphenolic and simple phenolic compounds in natural plants. Solvent extraction is one of the techniques to separate compounds based on their relative solubilities. It is a widely used method in laboratories as well as in industries The most common methods are: liquid- liquid extraction (LLE), applied to liquid samples; liquid-solid extraction (LSE), where a solvent is used to extract a compound form solid sample; supercritical fluid extraction (SFE), which uses a supercritical fluid as solvents (Moldoveanu & David, 2015). Several extraction methods using solvents with different polarity have been suggested for the extraction of phenolic compounds (Goli, Barzegar, & Sahari, 2005). Table 5 shows some important solvents used for phenolics extraction in sunflower seed meal.

The solvents used for extraction are water (hot, cold), alcohol (methanol, ethanol), acetone, diethyl ether, and ethyl acetate. However, depending upon the polarity of the phenolic acids, pure organic solvents or their diluted counterparts can be used (Stalikas, 2007). Ethanol is the most common bio-solvent, obtained by the fermentation of sugar-rich materials such as sugar beet and cereals. Although inflammable and potentially explosive, ethanol is used on a large scale because it is

easily available in high purity, it has a low price and it is completely biodegradable (Chemat et al., 2012).

The conventional methods of solvent extraction have the drawbacks of being time consuming, labour intensive, higher energy consuming, diluting the extracts, or even damaging the bioactive compounds due to the length of extraction and higher temperature. To overcome such challenges, the newer and more effective methods of extraction are combined solvent- enzymatic extraction, acid hydrolysis, alkali hydrolysis, microwave assisted extraction, ultrasound-assisted extraction, CO_2 extraction, supercritical fluid extraction etc (De Monte et al., 2014).

Table 5. Comparison of different solvents of extraction phenolics in sunflower seed meal.

Extraction method Conventional Extraction	TPC mg/100g	FRSA (%)
80% Methanol	1684.64±0.66	89.52±0.39
80% Ethanol	1003.66±0.59	86.24±0.81
80% Acetone	1802.76±0.47	90.63±0.61

TPC= Total Phenolic Content
FRSA= Free Radical Scavenging Activity
Source: Taha et al., 2011

2.15.2. Ultrasound-assisted extraction (UAE)

The ultra-sound assisted extraction method has gained the most attention in recent days as one of the most effective ways of extraction attributable to its comparative simplicity, cost effectiveness, higher recovery rate, reduced extraction time and solvent concentration (Chen et al., 2010).

The use of ultrasound-assisted extraction (UAE) for extraction of polyphenols from the plant matrix could be applied for the development of more efficient and less time, labor and energy consuming procedures, that can be operated at room temperature and atmospheric pressure without requiring sophisticated laboratory

procedures. Hence, UAE can contribute to achieving the principles of green extraction (Chemat et al., 2012), while being used for the extraction of the natural bioactive compounds from the plant by-products. The principle abiding the ultrasonic solvent extraction is the ultrasonic cavitation that creates shear forces that can cause cell wall breakage thus improving the material transfer. Figure 25 is the pictorial depiction of the principle of ultrasound activity. Additionally, high-frequency sound disrupts the plant cell wall thereby enhancing solvent penetration into the plant material and facilitating the release of the bioactive materials. However, the antioxidants and lipids present in fat-rich products was reported to have degraded when extracted ultrasonically, ultimately deteriorating the flavour and composition of these foods (Pingret, Fabiano-Tixier, & Chemat, 2013). Recently, the UAE processes have frequently been applied to recover polyphenolic compounds from various plant seeds by liquid solvent extraction (Wen, Yang, Cui, You, & Zhao, 2012). UAE is applicable for the extraction of thermolabile and unstable compounds and is commonly employed as the extraction method of choice for many types of natural products (Chemat et al., 2017). Jovanović et al. reported a higher yield of polyphenols from *Thymus serpyllum* L. when UAE was used at an optimized condition (50% ethanol as solvent; 1:30 solid-to-solvent ratio; 0.3 mm particle size and 15 min extraction time) than maceration and heat-assisted extraction methods (Jovanović et al., 2017).

Solvent extraction from dried material involves:

i) Soaking the plant material in solvent to facilitate swelling and hydration processes ii) Ultrasound increases the swelling index and hydration of the vegetal cells. This increases the porosity of the cell wall. This further improves diffusion of compounds and enhances mass transfer (M. Toma, Vinatoru, Paniwnyk, & Mason, 2001). Ultrasonication may also help the breakage of cell walls thus facilitating the washing-out process (Vinatoru, 2001). Use of ultrasound results in ‘cavitation’ or the formation, growth, and collapse of gas–vapor filled bubbles in a liquid medium. The implosion of the cavitation bubbles is supposed to destruct the cell wall of the solid matrix and thus assist in better extraction (Veillet, Tomao, & Chemat, 2010).

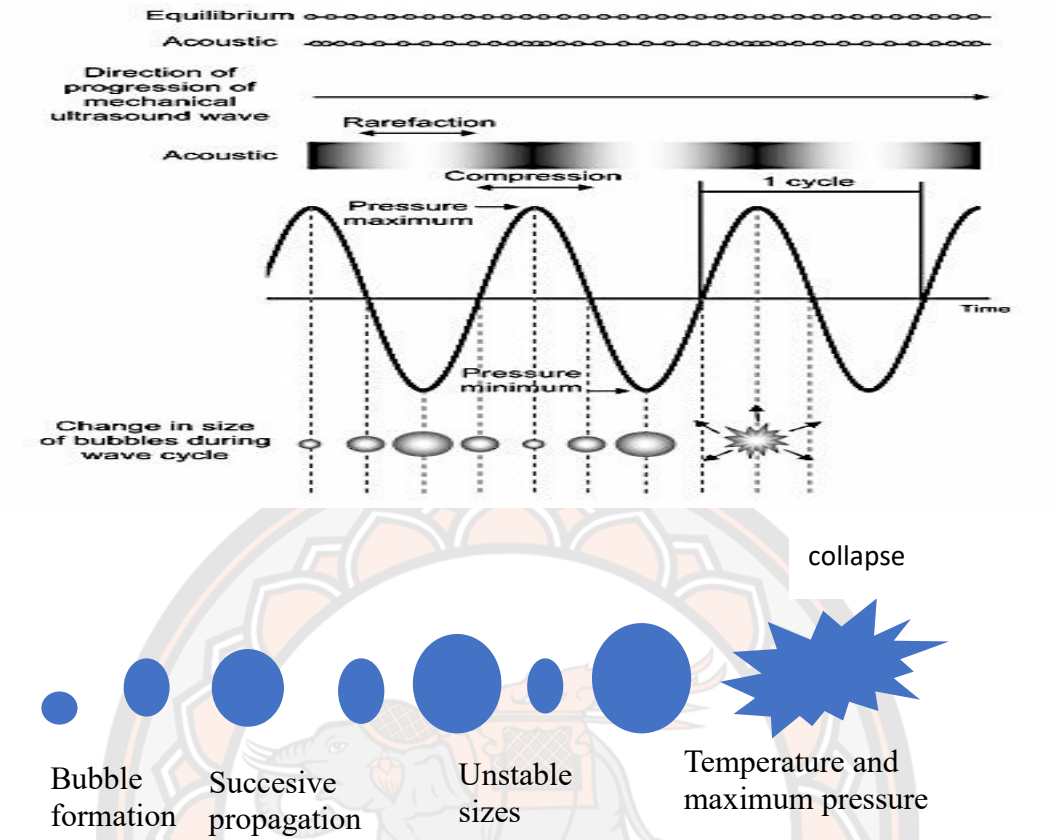


Figure 25. The propagation of ultrasonic wave in the particles causing them to implode

However, to the best of our knowledge, the impact of the UAE conditions such as ultrasound power, sonication time on the antioxidant and antimicrobial capacities, in vitro and in real food matrix of the ultrasound assisted extracts of *M. oleifera*, *C. oleifera* as well as *N. sativa*, have not been reported yet.

This study aims to (a) provide UAE to extract the bioactive compounds from oil industry waste (oil seed meals); (b) identify the targeted compounds (phenolics) in obtained extracts by using HPLC; (c) compare the results between conventional and ultrasound assisted extracts; (d) define optimum parameters for the UAE to obtain maximal antimicrobial and antioxidant activity of the bioactive compounds in extracts; and (e) use the best performing extract as an antibacterial preservative in real food matrix.

CHAPTER III
RESEARCH METHODS

3.1. Research Requirements

Oil-seed meal samples: (the seed meals were kindly provided by Lopburi Cold-Pressed Vegetable Oil Store Lopburi and Patpat, Chiang Rai, Thailand)

- *Moringa oleifera* seed meal (MoSM)
- *Camelia oleifera* seed meal (CSM)
- *Nigella sativa* seed meal (NSM)

Poultry product:

- Raw chicken sausages, without added preservatives, were purchased from Little Munchy Co. Ltd., Thailand.

Chemicals:

1) Agar (Difco, USA)
2) Acetate buffer (Sigma-Aldrich, USA)
3) Amoxicillin 500 mg (Sigma-Aldrich, USA)
4) Ascorbic acid (Labscan, Ireland)
5) Dimethyl sulfoxide (DMSO)
6) Distilled water
7) DPPH (2, 2-Diphenyl-1-Picrylhydrazyl)
8) Egg yolk emulsion (Himedia, India)
9) Eosine methylene blue agar (Himedia, India)
10) Ethanol 95-99% (Lab scan, Ireland)
11) Ferric Chloride (Sigma-Aldrich)
12) Ferrous Sulphate Heptahydrate (Sigma-Aldrich, USA)
13) Folin Ciocalteu's phenol reagent (Sigma-Aldrich, USA)
14) Gallic acid (Sigma-Aldrich, USA)
15) Glacial Acetic acid (Sigma-Aldrich, USA)
16) Hydrochloric acid (Labscan, Ireland)
17) Mueller Hinton agar (Becton, Dickinson & Co., France)

18) Mannitol yolk polymyxin B agar base (Himedia, India)
19) Phosphate buffer (pH 7) (Himedia, India)
20) Phosphate Buffer saline (pH 7.2) (Himedia, India)
21) Polymixin B (Himedia, India)
22) Sodium carbonate (Na_2CO_3 75%) (Lobachemie, India)
23) Tetramethoxy-propane (1,1,33- TMP)
24) TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine (Sigma-Aldrich, USA)
25) Tryptic Soy Broth (Merck, Germany)

Instruments:

1) Aluminum foil 18", Dimond
2) Beaker, 50, 100, 200, 250ml
3) Bottle (amber) 10 ml
4) Bottle (clear) 100 ml
5) Centrifuge (Eppendorf)
6) Erlenmeyer flask
7) Filter paper (Whatmann no. 1)
8) Freeze Dryer (Labconco No: 79340-01 USA)
9) Glass funnel
10) Incubator (Esco, Singapore)
11) Laminar flow cabinet
12) Micropipettes
13) Microplate reader (Drawell, Thailand).
14) Petri plates,
15) Pipettes 1, 5, 10 ml
16) Refrigerator
17) Rotary Vacuum Evaporator (Buchi R-114, Japan)
18) Round bottom flask 250 ml
19) Shaker (N-biotex NB-101MH 25 India)
20) Spectrophotometer
21) Test tubes
22) Ultrasonic Homogenizer, Model 300VT (Biologics Incorporation)
23) Vacuum packaging machine (Fresh sealer, Thailand)

24) Volumetric flask

25) Vortex (VELP Scientifica, Italy)

Personal Protective Equipment

1) Disposable hair cap
2) Face masks
3) Gloves
4) Goggles
5) Lab coat

Bacteria: the strains were bought from Thailand Institute of Scientific and Technological research (TISTR).

Bacillus cereus, NBRC 13494

Escherichia coli, TISTR 527

Salmonella Typhimurium, DMST 1724

Staphylococcus aureus, TISTR 2329

3.2. Research framework

The research is planned to be carried out in two different parts. A flow diagram is presented below (Figure 26).

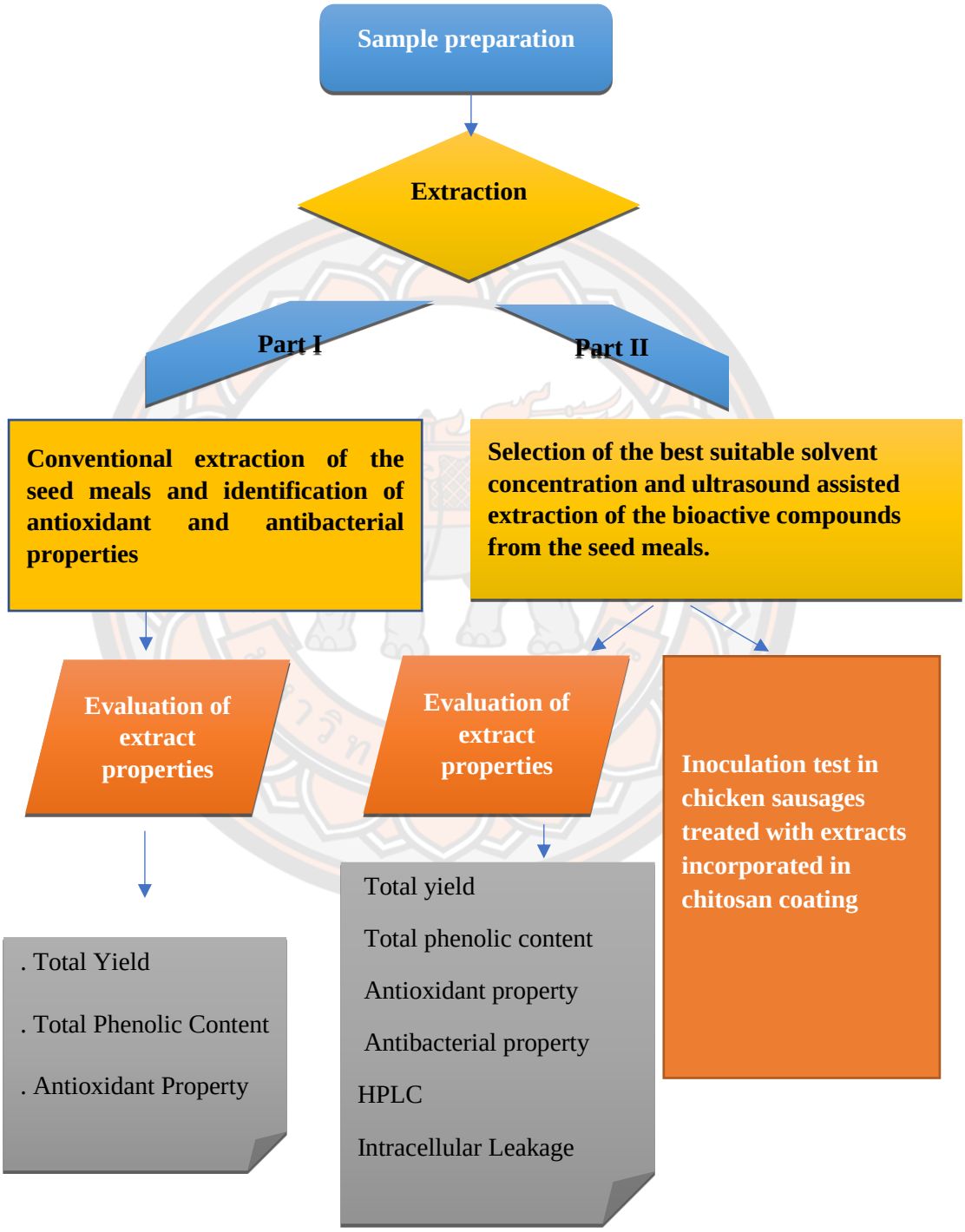


Figure 26. Flow diagram depicting the research framework.

Part I: Screening of the samples for the optimum ethanol concentration for extraction with conventional method

This part deals with the preparation of the received seed meals for the study, the method used for extraction and the *in-vitro* application of the extracts for the antioxidant and antimicrobial property analysis. The suitable solvent concentration for bioactive compound extraction was determined in this part of the study. The detailed methods and protocols are as described below:

3.3. Sample preparation

The oil seed meals were screened for visible impurities. The meals were then crushed in mortar and pestle. The crushed samples were ground in a mixer grinder. The ground powder was sieved through 40 mm mesh. The seed meal powder was vacuum sealed and kept under refrigeration at -20^{o}C until further use.

3.4. Solvent extraction

To extract the phenolic contents of the two seed meals, ethanol at different concentrations 0%, 60%, 80% and 100% ethanol were used. The study aims to use the phenolic extract on raw chicken sausages for its antimicrobial property assessment. Thus, keeping several past reports into consideration, and the fact that ethanol is a safer choice of solvent and is also enlisted as GRAS by USFDA, and is also more economicalto use. The sample to solvent ratio was maintained at 1:10 w/v and extracted for 24 hours in a shaker at 150 rpm. The extraction was performed in triplicate. The solution was filtered first through a 40mm sieve and then through Whatman filter paper no. 1. The obtained liquid extract was evaporated by rotary evaporator to separate the extract from the solvent. Then the extract was freeze dried and stored at -20^{o}C until further use.

3.5. Percent yield

The yield of the extract after freeze drying was recorded as the percentage of the sample used. The ethanolic extracts obtained from MoSM, CSM and NSM were respectively named ME, CE and NE. The following formula gives the percent yield.

% Yield = (dry weight of extract) / (dry weight of raw material) x 100

3.6. Antimicrobial property assessment

3.6.1. Bacterial culture preparation

The pure colony of *E. coli*, *S.* Typhimurium, *S. aureus* and *B. cereus* were grown in freshly prepared Tryptic Soy Broth media for 24 hours under incubation at 37^{o}C. The suspensions were diluted to obtain the optic density of 0.1 at 630 nm using the microplate reader. Then this dilution was spread on the sterile tryptic soy agar plate and incubated at 37^{o}C for 24 hours. The total plate count was performed to get the colony forming unit. The dilution was adjusted to 10^{6} cfu/ml.

3.6.2. Concentration of the extract

The original stock concentration of 100 mg of extracts diluted in 1ml DMSO was prepared for the study.

3.6.3. Agar gel disc diffusion

100 µl of the bacterial suspension was spread to the TS agar plates. Sterile paper discs of about 6 mm diameter were inserted to the microbe inoculated plates. 6 µl of the extract was dropped onto the discs. Amoxicillin was used as positive control and DMSO as negative control for the study (Figure 27). The plates were incubated at 37^{o}C for 24 hours. All treatments were performed in triplicate. The diameter of inhibition zone created by the discs was recorded in mm ±SD.

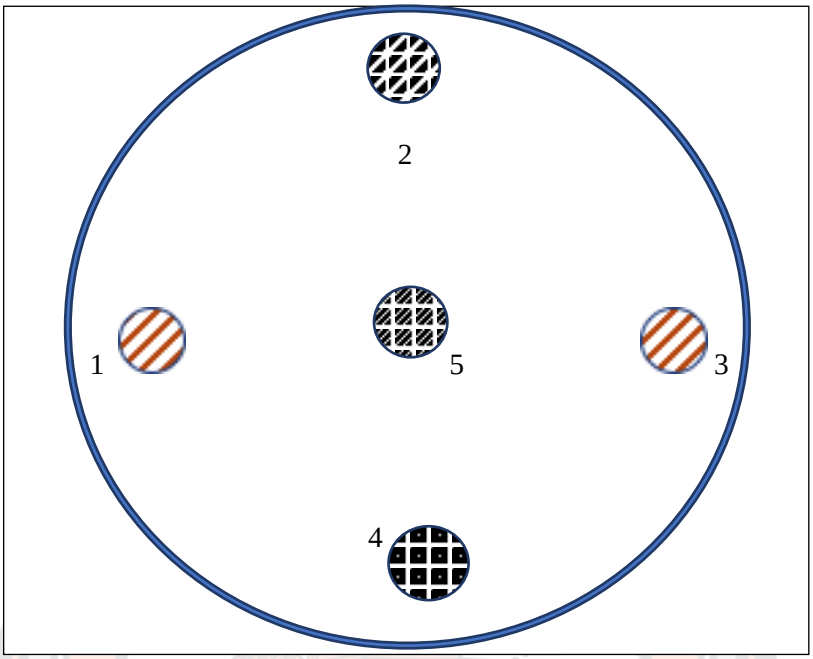


Figure 27. Diagrammatic representation of the agar gel disc diffusion test.

Discs 1,2,3 = antimicrobial extracts

Disc 4 = Negative control (DMSO)

Disc 5 = Positive control (Amoxicilin)

Part II: Selection of the best suitable solvent concentration and ultrasound assisted extraction of the bioactive compounds from the seed meals.

The second part of the study deals with the ultrasonic extraction of the selected seed meals with the suitable ethanolic concentration as a solvent, which was predermined in the Part I. The bioactive properties were compared with the conventional extracts. This further includes the identification of the phenolic compounds, antimicrobial property analysis, and inoculation test of the extracts in the food matrix. The detailed methodology of the tests is given below.

3.7. Ultrasound assisted extraction

The seed meals were freshly prepared again for extraction., as stated in 3.3. The extraction of the samples was done using the Ultrasonic Homogenizer 300VT,

Biologics. Inc. with solvent (ethanol), and the variables: time of extraction and the power of the machine. The optimum solvent concentration was determined from the screening test. All the treatments were performed in triplicate. The ultrasonic treatment groups along with their codes is presented in Table 6.

Table 6. The variables of Ultrasound assisted extraction of the seed-meals and treatment code.

Power	Time	Treatment Code		
		ME	**CE**	**NE**
100 W	10 min	$ME_{100/10}$	$CE_{100/10}$	$NE_{100/10}$
	20 min	$ME_{100/20}$	$CE_{100/20}$	$NE_{100/20}$
	30 min	$ME_{100/30}$	$CE_{100/30}$	$NE_{100/30}$
200 W	10 min	$ME_{200/10}$	$CE_{200/10}$	$NE_{200/10}$
	20 min	$ME_{200/20}$	$CE_{200/20}$	$NE_{200/20}$
	30 min	$ME_{200/30}$	$CE_{200/30}$	$NE_{200/30}$
300 W	10 min	$ME_{300/10}$	$CE_{300/10}$	$NE_{300/10}$
	20 min	$ME_{300/20}$	$CE_{300/20}$	$NE_{300/20}$
	30 min	$ME_{300/30}$	$CE_{300/30}$	$NE_{300/30}$

3.8. Antimicrobial property assessment

3.8.1. Minimum Inhibitory Concentration

Minimum inhibitory concentrations are regarded as the gold standard for antibacterial susceptibility test. MICs are performed in diagnostic centers when several anti-biotics show resistances against the pathogen. MIC is the minimum concentration of antimicrobial agent that can inhibit the visual growth of microorganisms (Andrews, 2001). The procedure of Y. Sun, Dong, Sun, Ma, and Shang (2015) will be followed with modification. The microbial culture in MH broth was diluted to 10^6 cfu/ml and then inoculated (50 µl) to serially diluted concentrations of the extract (50 µl). The stock extract solution was initially serially diluted with MHB to 25 mg/ml and diluted two-fold serially, in the 96-well microplates (Figure 28). The samples were incubated overnight at 37^{o}C. The value

of the dilution of the extract in which no visible bacterial growth can be seen, spectrophotometrically, was the interpretation of minimum inhibitory concentration of extract. The seed meal samples whose extracts exhibited better inhibition to the bacteria were selected for the application of the extracts in poultry meat to observe and analyze the antibacterial activity in real food system.

25 12.5 6.25 3.12 1.5 0.75 0.37 (mg/ml)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 28. The 96 micro-well plate with serial dilution of the anti-bacterial agent.

3.8.2. Fractional Inhibitory Concentration (FIC)

The interaction between the two extracts of meals with each of the four bacteria were analyzed using the checkerboard test (Q. Ma, Davidson, & Zhong, 2013; Mackay, Milne, & Gould, 2000).The data obtained from the checkerboard test were analyzed in terms of the fractional inhibitory concentration index (FIC).

The antimicrobials were serially diluted and mixed together to obtain a fixed amount of the antimicrobial A and increasing amounts of antimicrobial B in each row, and vice versa in each column. Eight two-fold serial dilutions (from 2MIC to MIC/64) of each antimicrobial solution were prepared. A 25-µl aliquot of extract A dilutions were added to wells maintained in row, and 25 µl of extract B dilutions were added in column, to obtain various concentration combinations of the two antimicrobials. 50 µl

of fresh bacterial suspension (10^6 CFU/ml) were added to each well and incubated at 37^0C for 24 hours. The Fractional Inhibitory Concentration Index (FICi) were calculated according to the method provided by Mackay et al. (2000). The formula is:

$$\text{FIC index} = \frac{\text{MIC}_{A+B}}{\text{MIC}_A} + \frac{\text{MIC}_{B+A}}{\text{MIC}_B}$$

Where, MIC_A is the MIC of antimicrobial A, MIC_B is the MIC of antimicrobial B and MIC_{A+B} and MIC_{B+A} is the MIC of antimicrobial A in combination with antimicrobial B and vice-versa. The interpretation of the FIC index is given in Table 7.

Table 7. Interpretation of the value obtained for FIC index

FIC Index	Explanation
≤ 0.5	Synergism
>0.5-1	Additive effect
>1-2	No effect
>2	Antagonism

3.8.3. Effect of the extract on bacterial cell membrane leakage

The mechanism by which the plant extracts show the antimicrobial property in bacterial cell were analyzed. The initial site of damage is the cell membrane of the bacteria. This leads to the collapse of the cell integrity and subsequent exudation of vital intracellular constituents (Juven et al., 1994; Ultee et al., 1999). The cytoplasmic leakage analysis of the representative gram positive (*B. cereus*) and gram negative (*E. coli*) bacteria including nucleic acids and proteins under the presence of the extracts was measured by the following method (Singh et al., 2016). The bacterial suspension was inoculated into Mueller Hinton Broth (MHB) medium added with a different concentration of the ME extracted by the optimized condition at 1/2MIC, 1MIC, 2MIC. Bacterial suspension in MHB without ME was used as the control, then cultured at 37ºC. After 24 h of incubation, the bacterial suspension was obtained and

centrifuged at 6000 rpm for 10 min at 4ºC, and the supernatant was filtrated by 0.22 µm syringe filter (Verticlean™, Vertical Chromatography). The filtrate was measured at the absorption of 260 nm and 280 nm by ultraviolet spectrophotometer for the detection of nucleic acids and proteins, respectively. The measurement of absorbance was made by the spectrophotometer at 260nm for nucleic acids and 280 nm for proteins. The extrusion of DNA and the cell protein into the extracellular matrix was analyzed using the spectrophotometer at specified wavelengths.

3.8.4. Scanning electron microscopy

The morphological changes of the extracts-treated bacteria were observed by SEM (Chen et al., 2018). The prepared *B. cereus* suspension was treated with the extracts at the final concentration of previously determined MIC and the mixtures were incubated at 35°C for 24 h. The mixture was centrifuged at 6000 rpm at preset temperature of 4ºC for 10 min and the precipitated cells were washed with 0.1 M phosphate buffered saline (PBS) at pH 7.4, and then fixed with 2.5% glutaraldehyde overnight at 4ºC. The cells were dehydrated with an ethanol series of 30%, 50%, 70%, 80% and 90% for 15 min at each graduation, and then twice for 15 min with 100% ethanol. The cells were then critical point dried in liquid CO_2 and coated with gold by cathodic spraying (Leica EM CPD300, Leica Microsystems, India). The final sample was morphologically examined under a scanning electron microscope (JSM-IT-500HR, JEOL ltd., Japan) and compared with the untreated bacterial cells.

3.8.5. The microbial inoculation tests

Challenge test is performed by the meat industry and researchers to evaluate product's shelf life (McMeekin et al., 1997). The NACMCF (2010) guidelines were followed for the test. The inoculation test was carried out with the extract that showed the best response in previous tests, against the most susceptible bacteria. Chicken sausages of Little Munchy Co. Ltd., which has no added preservatives was procured for the test. All the steps of the inoculation test were performed under aseptic conditions.

3.8.5.1. Preparation of the dipping solution

Chitosan solution was prepared according to the protocol described by Khalifa et al. (2016) with some modifications. 1 g chitosan was dispersed to 100 ml of 1% acetic acid (v/v) at 40^{o}C and stirred in a magnetic stirrer for 12 hours. 2% glycerol was added to the solution as a plasticizer and stirred for 12 more hours. The seed meal extract was added to this chitosan solution at 1%, 3% and 5% concentration (w/v). Chitosan solution without the extract was used as the negative control and butylated hydroxy anisole (BHA) was used as the positive control.

3.8.5.2. Bacterial inoculation

The chicken sausages (25 g) were inoculated with the inoculum concentration at 10^4 cfu /g of two selected susceptible bacteria, of Gram positive and Gram-negative type each, based on previous antibacterial tests. Inoculated sausages were allowed a 15-minute drying period in a biosafety cabinet to enable bacterial attachment.

3.8.5.3. Antibacterial coating

The inoculated sausages were coated with the antibacterial chitosan solution at 1%, 3% and 5% concentration, and the positive and the negative control. Then the antibacterial coating over the sausages were allowed to dry for 30 minutes under laminar flow cabinet. The sausages were then kept in sterile zip-lock bags and kept under refrigeration at 4^{o}C for 15 days. The test for the total viable count were done using selective media on 0, 3, 6, 9, 12 and 15 days were recorded.

3.8.5.4. The antibacterial activity test on the inoculated sausages

To prepare for the bacterial survival count on each aforementioned test days, the treated sausages were homogenized in a stomacher (25g sausages + 225 ml phosphate buffer saline). The homogenates were serially diluted until the sixth dilution and spread on selective media, Eosin Methylene Blue (EMB) agar for enumerating *E. coli*, and Mannitol Egg yolk Polymyxin (MYP) agar for *B. cereus*. The inoculated plates were incubated at 35^{0}C for 24 hours for the total plate count

(FAO, 2007). All the dilutions (100 microliter) were poured and spread onto the selective media and incubated for 24 hours at 37ºC.

3.9. Total phenolic content

The amount of TPC in each extract was determined according to the Folin–Ciocalteu method (Chandler and Dodds,1993). 1 mL of the prepared samples in methanol was added to test tubes with 2.5 mL of Folin–Ciocalteu's reagent. 2mL of sodium carbonate (7% w/v) was added after 5 minutes. The tubes were shaken and kept in dark at room temperature for 30 min. Then, the tubes were warmed in water bath at 45ºC for 15 minutes. Absorption at 765 nm against a blank was measured using an UV/VIS Spectrophotometer. The standard curve was used, and the total phenolic compounds content was calculated and expressed as gallic acid equivalent in mg/g of extracts.

3.10. Antioxidant property assessment

The free radical scavenging activity of the extracts was assessed using a stable free radical DPPH and the Ferric Reducing Antioxidant Power (FRAP) Assay.

3.10.1. Free Radical Scavenging Assay (DPPH)

The assay was performed according to methodology described by Abdulkadir et. al., 2015 with some modification.

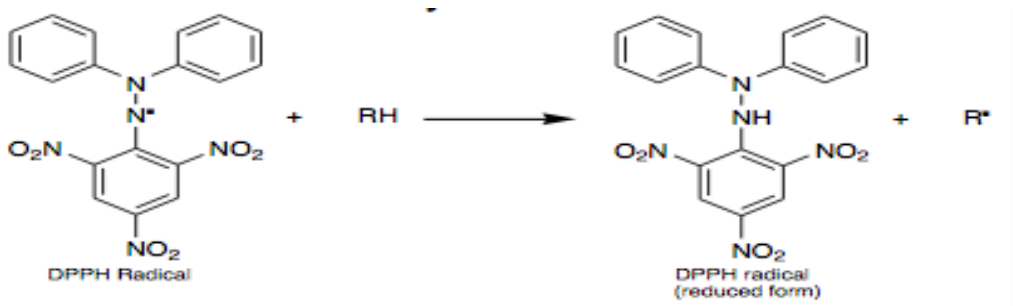


Figure 29. DPPH reaction with the RH antioxidant.

One ml of the extracts dissolved in methanol was mixed in 2 ml of DPPH in methanol (0.1Mm) in a test tube. The mix was shaken before placing the tubes in the dark for 30 minutes, and measurement was taken at the absorbance at 517 nm using spectrophotometer. In the presence of the antioxidant, DPPH takes up the hydrogen ion from the antioxidant and gets reduced. This reaction triggers the change in color (from deep violet to light yellow). The control solution was solvent (2 mL) mixed with DPPH radical solution (1 mL). Ascorbic acid was used as the standard, and the values were expressed as mg AAE/ g dry weight.

3.10.2. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was based on the method of (Benzie & Strain, 1996) with modification. The FRAP reagent was prepared at 1:1:10 ratio of three solutions, viz:

- 20ml of 20mM $FeCl_3$ (0.108g FeCl3 dissolved in 20 ml distilled water),
- 20ml of 10mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) solution (0.31 g TPTZ + 100 ml of 40mM HCl) and
- 200ml of 300mM acetate buffer at pH 3.6.

24 ml distilled water was added to the mixture and incubated in water bath at 37^0C for 10 minutes before use. 50 µl of the extract was mixed with 3 ml FRAP reagent and incubated at 37^0C for 4 minutes. The absorbance was measured against blank at 593 nm. Distilled water was used in place of the extract, with 3 ml FRAP reagent, to create the blank solution. The standard solution was created with ascorbic acid by mixing 3 ml FRAP reagent and 50 ul Ascorbic acid, in place of the sample, in various concentrations of 0.1 to 1 mM (Teh, Bekhit, & Birch, 2014).

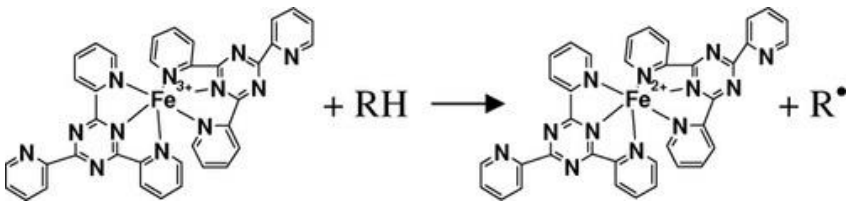


Figure 30. Reduction of Fe3+-(TPTZ)2 complex to a Fe2+-(TPTZ)2 complex by antioxidant RH.

3.10.3. High performance liquid chromatography (HPLC)

Phenolic compounds of ME, CE and NE obtained from the optimum UAE condition were identified using HPLC according to Nour, Trandafir, and Cosmulescu (2013). All chemicals and solvents used were HPLC spectral grade, and obtained from Sigma (St. louis, USA), Merck–Shcuchrdt (Munich, Germany). All phenolic compounds were qualitatively and quantitatively measured using Shimadzu model HPLC system (pump: LC-10AD vp, system controller: SCL-10A vp, column oven: CTO-10AD vp, UV/vis Detector: SPD-10A vp, Low pressure gradient unit: FCV-10ALvp, Degasser: DGU-14A) equipped with a ODS Hypersil (250X4 mm, 5µm, Thermo, USA). The mobile phase consisted of 1% aqueous acetic acid solution (A) and methanol (B). Samples were eluted with the following gradient of mobile phase B: started with 10% for 27 min, from 10 to 40% for 28 min and hold to 40% for 5 min, from 40 to 44% for 2 min hold to 44% for 8 min and from 44 to 10% for 2 min hold to 10% for 3 min. The UV-detector wavelength was 254 nm (sinapic acid, protocatechuic acid, vanillic acid, syringic acid and quercetin hydrate) and 300 nm (caffeic acid, gallic acid, chlorogenic acid, ferulic acid and p-coumaric acid). The flow rate and injection volume were 1 ml/min and 20 µl, respectively. Column oven temperature was 40°C. The phenolic compound standards were prepared in 1% aqueous acetic acid solution (0.5, 1, 5 and 10 mg/l). Phenolic compounds of the extract were identified by comparing their relative retention times with those of the standard mixture chromatogram. The concentration of an individual compound was calculated using the regression equation of their concentration and peak area, and then converted to mg phenolic/100 g extract.

3.10.4. Interview about the ethnomedical uses

Interview about the ethnomedical uses of the best performing seed meal in the rural community of Nepal was conducted, and the report is presented in APPENDIX F.

3.11. Statistical Analysis

The experiment was conducted in triplicate for each treatment, and the data were recorded as the average value ± standard deviation. SPSS software was used for statistical analysis. Duncan's one-way multiple comparisons was used to determine significant differences among treatments at $P < 0.05$.

CHAPTER IV
RESULTS AND DISCUSSION

Part I. Conventional extraction of the seed meals and identification of antioxidant and antibacterial properties

The results of the Part I of the study includes the analysis of the chemical composition of the seed meals. The results of the conventional maceration technique for the extraction and the *in-vitro* application of the extracts for the antioxidant and antimicrobial property analysis is presented below. The suitable solvent concentration for bioactive compound extraction was determined for the second part of this study. The results and analysis are discussed below.

4.1. Chemical composition of the seed meals

Proximate analysis of *M. oleifera* seed meal (MoSM), *C. oleifera* seed meal (CSM) and *N. sativa* seed meal (NSM) are given in Table 8. Hassan et al. (2016) reported that decorticated MoSM contained 92% dry matter, 44.14% crude protein, 18.9% ether extract (fat), 3.9% crude fiber and 4.6% ash. The moisture content of *C. oleifera* seed meal (CSM) was 5.71%, fiber 23.63%, protein 8.64%, fat 11.72%, carbohydrates 71.29% and ash 2.64%. Proximate analysis of *N. sativa* seed meal (NSM) showed 5.78% moisture, 5.6% fiber, 31.3% protein, 15.36% fat, 40.78% carbohydrate, and 6.77% ash. Thilakarathne, Madushanka, and Navaratne (2018) reported the NSM moisture content of 4.56%, crude protein 18.44% fat 15.69%, fiber 7.69%, total carbohydrate 28.16% and ash 5.31%, where the protein content seems lower than that reported in this study (18.44%). Atta (2003) concluded that *N. sativa* seeds possess protein in high quantity.

Table 8. Proximate analysis of MoSM, CSM, and NSM seed meals on dry weight basis

Components (%)	MoSM	CSM	NSM
Moisture	6.79±0.01	5.71±0.02	5.78±0.06
Fiber	28.95±0.07	23.63±0.07	5.60±0.14
Protein	30.14±0.00	8.64±0.01	31.30±0.01
Fat	12.06±0.01	11.72±0.02	15.36±0.01
Other Carbs	17.89±0.03	71.29±0.04	40.78±0.04
Ash	3.99±0.01	2.64±0.01	6.77±0.02

Data are presented as mean±SD (n=3)

4.2. Total yield

The yield of the seed meal extracts is shown in Table 9. The highest total yield of all the three extracts was recorded with distilled water as the solvent. The highest yield was recorded for distilled water (DW) extraction of NSM at 17.94%. DW extract of CSM, followed at 14.10%, succeeded by the DW extract of MoSM at 12.17%. The better yield with water as a solvent is possibly because of higher solubility of proteins in water compared to ethanol (Dubie, Stancik, Morra, & Nindo, 2013).

C. oleifera water extraction in Yu and He (2018) is reported to be 8.04%, which is lesser than the value obtained in this study (14.10%). Likewise, the ethanolic extraction at 63% gave 7.28% yield, which is in agreement with this study where 60% ethanolic extraction produced 6.12% yield.

Table 9. Effect of different ethanol concentration as a solvent on the extraction yield of three different seed meals

Ethanol concentration	Yield %		
	ME	**CE**	**NE**
0%	$12.17\pm0.56^{a, C}$	$14.10\pm0.47^{a, B}$	$17.94\pm0.75^{ab, A}$
60%	$6.12\pm0.26^{b, C}$	$11.19\pm0.28^{b, B}$	$14.45\pm0.19^{c, A}$
80%	$5.54\pm0.39^{b, C}$	$10.62\pm0.33^{b, B}$	$19.40\pm0.74^{a, A}$
100%	$6.58\pm0.35^{b, B}$	$6.85\pm0.19^{c, B}$	$16.72\pm0.54^{b, A}$

Data are presented as mean ± SD (n=3, $p<0.05$). Different superscripts in lowercase represent significantly different values among different concentrations of each seed meal and different superscripts in uppercase represent significantly different values among different seed meals.

4.3. Antioxidant activity of the seed meal extracts

4.3.1. Total phenolic content (TPC)

Total phenolic contents of different ethanolic extract concentration of ME, CE and NE is given in Table 10. The resuts showed that the water extracted ME yielded the highest TPC at 131.59 mg gallic acid equivalent (GAE) per gram of freeze-dried ME weight, followed by 80% ethanolic extract. The least TPC was obtained by 100% ethanol, and the amount was significantly lower than all the treatment conditions at 12.48 mg GAE/ g dry extract.

CE extracted with 60% and 80% ethanol yielded the highest TPC at 186.02 mg GAE/g and 185.35 mg GAE/g dry extract respectively. The lowest TPC was obtained from CE extracted with 100% ethanol.

NE also showed the highest TPC yield with 60% ethanol (132.63 mg GAE/g extract. The results show that for different seed extracts, the solvents for optimum TPC yield can vary, and this variation is possibly because of the different phenolic acids present in them that act different with different polar solvents. However, it is

clear from the data obtained that for all the seed meal extracts, the aqueous solution of ethanol yields better quantity of TPC than the pure ethanol. The low solubility of the polyphenols in absolute organic solvents is due to the stronger hydrogen bonds between polyphenols and protein. The addition of water to organic solvents causes the weakening of the hydrogen bonds in aqueous solutions thus increasing the solubility (Siprad et al., 1981). (Venkatesan, Choi, & Kim, 2019) in their study with *Pinus densiflora* bark extract reported that the highest TPC was obtained by 20% ethanol in water, followed by 20% acetonitrile and 40% aqueous ethanol. The least TPC was obtained by 100% ethanol, which is in agreement with the present study. Also, among all the treatment groups, CE extracted with 60% ethanol gave the highest TPC (186.02 g GAE/g extract). In a study conducted by (Gaafar, Ibrahim, Asker, Moustafa, & Salama, 2016), the cold-water extraction of MoSM yielded total phenolic content (TPC) of 15.76 mg gallic acid equivalent per gram dry weight of the extract, which is lower than that obtained in the present study. This variation in TPC content could probably be because of the difference between extraction protocol, storage condition, seasonal and spatial variation of the seed trees grown etc.

Table 10. Total phenolic content (TPC) of the different seed meal extracts that were extracted conventionally with different concentrations of ethanol

Ethanol concentration	**TPC** (mg GAE/g)		
	ME	**CE**	**NE**
0%	$131.59\pm0.15^{a, B}$	$134.80\pm1.17^{b, A}$	$102.85\pm0.94^{b, C}$
60%	$101.82\pm0.39^{c, C}$	186.02 ± 0.09^{aA}	$132.63\pm0.71^{a, B}$
80%	$115.12\pm0.15^{b, B}$	$185.35\pm0.82^{a, A}$	$57.95\pm1.89^{c, C}$
100%	$12.48\pm0.89^{d, C}$	$119.47\pm1.55^{c, A}$	$51.32\pm0.09^{d, B}$

Data are presented as mean ± SD, (n=3, $p<0.05$).

Different superscripts in lowercase represent significantly different values among different concentrations of each seed meal and different superscripts in uppercase represent significantly different values among different seed meals

Phenolic compounds are well known as secondary metabolites abundantly occurring in plants, and act as a defense mechanism against environmental stresses including harmful solar radiation and pathogens (Yang et al., 2018). These compounds render the harmful effects of free radicals ineffective by their structural properties such as hydrogen donation, reduction, metal chelation, and singlet oxygen quenching (Choe & Min, 2009; Mathew, Abraham, & Zakaria, 2015). They are also reported to exhaust the free radical chain reaction by forming a relatively stable phenoxy-radical intermediate compound (Leopoldini, Marino, Russo, & Toscano, 2004). Hence, the polyphenols are generally attributed for the antioxidant property of plant extracts. There are several key factors that play a pivotal role in the successful extraction of polyphenolic compounds with potential antioxidant activity from plant sources. The selection of solvent is the most significant among all (Ngo, Scarlett, Bowyer, Ngo, & Vuong, 2017). The aqueous solution of organic solvents is numerously reported as the most suitable solvents for the extraction of phenolic compounds from plant materials. Extraction from different plant material depends on the target compound of interest, which further depends on solvent type of the combination of solvents for optimum extraction. While acetone at 50% with water could extract the highest TPC from *Camellia sinensis*, *Lippia javanica*, and *Ilex paraguariensis*, 50% of ethanol with water is required to get the highest TPC from *Cuphea carthagenensis* (Bhebhe, Füller, Chipurura, & Muchuweti, 2016).

4.3.2. (2, 2-Diphenyl-1-Picrylhydrazyl) DPPH scavenging activity

The DPPH assay was performed for the seed meal extracts isolated with different concentration of ethanolic solvents and the data obtained are presented in Table 11. The highest radical scavenging activity was obtained for ME with 80%, CE with 60% and 80%, and NE with 60% aqueous ethanol. Absolute ethanol showed the least antioxidant activity with ME (3.40 mg AAE/g dry extract and CE (6.39 mg AAE/g dry extract), and second least antioxidant activity with NE. The overall

highest radical scavenging activity was recorded of CE extracted with 60% and 80% ethanol (7.52 and 7.49 mg/ AAE g dry extract respectively), among all the seed meal extracts and solvent concentrations.

The DPPH assay measures the hydrogen-donating activity of the antioxidant substances. It is a spectrophotometric method based on quenching of stable colored radicals (violet) by the antioxidant substances and forming colorless molecules (Huang et al., 2005). Truong et al. (2019) reported that ethanol resulted in lower phenolic content (3.60 mg GAE/g DW) than distilled water (5.95 mg GAE/g DW). However, ethanol extracted higher alkaloids (1.34 mg AE/g DW) as well as terpenoids (0.97%, w/w) when compared to the distilled water extract (0.16 mg AE/g DW and 0.43%, w/w, respectively). This result is similar to the result of *Moringa oleifera* seed meal extract of this study (Table 10), where higher phenolic content was obtained with water extraction than ethanol at all the concentrations, however the ethanolic extracts portrayed a better antioxidant power than the aqueous extract.

Table 11. Antioxidant activity of the different seed meal extracts that were extracted conventionally with different concentrations of ethanol.

Ethanol concentration	Antioxidant activity (DPPH; mg AAE/g extract)		
	ME	CE	NE
0%	$4.53\pm0.01^{c, C}$	$6.61\pm0.00^{b, B}$	$6.85\pm0.01^{b, A}$
60%	$4.84\pm0.01^{b, C}$	$7.52\pm0.01^{a, A}$	$7.34\pm0.00^{a, B}$
80%	$5.66\pm0.01^{a, B}$	$7.49\pm0.00^{a, A}$	$5.40\pm0.00^{d, C}$
100%	$3.40\pm0.00^{d, C}$	$6.39\pm0.00^{c, A}$	$6.25\pm0.01^{c, B}$

Data are presented as mean ± SD (n=3, $p<0.05$). Different superscripts in lowercase represent significantly different values among different concentrations of each seed meal and different superscripts in uppercase represent significantly different values among different seed meals.

4.3.3. Ferric reducing antioxidant property (FRAP) of the conventionally extracted seed meals

The ferric reducing ability of the three seed meal extracts was performed for further understanding of the antioxidant activity, which utilizes electron transfer method. The ability to reduce the ferric ion confirms the antioxidant property of the substances under study (Pandey & Rizvi, 2012). The results showed that 60% aqueous ethanol had the highest ferric reducing property for all ME, CE and NE (5.82, 6.27 and 6.18 mg AAE/g dry extract respectively) (Table 12). The least significant antioxidant activity was shown by absolute ethanol extracts for all three seed meals. The results are in coordination with the DPPH assay results of this study.

Table 12. Antioxidant activity of the different seed meal extracts that were extracted conventionally with different concentrations of ethanol.

Ethanol concentration	Antioxidant activity (FRAP; mg AAE /g extract)		
	ME	CE	NE
0%	$5.04\pm0.02^{c, C}$	$5.59\pm001^{c, A}$	$5.19\pm0.03^{c, B}$
60%	$5.82\pm0.01^{a, C}$	$6.27\pm0.02^{a, A}$	$6.18\pm0.01^{a, B}$
80%	$5.34\pm0.11^{b, C}$	$5.97\pm0.08^{b, A}$	$5.83\pm0.03^{b, B}$
100%	$4.80\pm0.00^{d, B}$	$5.40\pm0.02^{d, A}$	$4.88\pm0.06^{d, B}$

Data are presented as mean ± SD (n=3, $p<0.05$). Different superscripts in lowercase represent significantly different values among different concentrations of each seed meal and different superscripts in uppercase represent significantly different values among different seed meals.

4.3.4. Correlation of TPC with antioxidant activity of seed meal extracts

TPC of CE showed strong positive correlation with DPPH and FRAP assay (R^2= 0.99 and R^2=0.9 respectively). High correlation between TPC and antioxidant activity have been well-established previously by several studies, including (Clarke, Ting, Wiart, & Fry, 2013), (Augusto et al., 2014) in recent times. However, TPC of

NE showed moderate positive correlation (0.72) in DPPH assay and low positive correlation in FRAP assay (R^2=0.49), whereas TPC of ME showed lowest positive correlation with DPPH and FRAP assay (R^2=0.36, R^2=0.38) respectively. This result suggests that TPC may not be the critical factor determining the DPPH activity of ME, and FRAP activity of both ME and NE. This result suggests the presence of water-soluble phenolic compounds that do not necessarily impart antioxidant activity, as compared to those extracted with aqueous ethanol, which is also in agreement with the findings of (Dubie et al., 2013), who further suggests that water might extract undesirable compounds, thus lowering the yield of specific antioxidants. Folin–Ciocalteu method is useful to make a crude estimate of TPC of plant extracts, and may not be specific to polyphenols. The reagent may react to various interfering compounds, thus showing an elevated apparent phenolic concentration (Prior, Wu, & Schaich, 2005). The total phenolic content does not necessarily incorporate all the antioxidants that may be present in an extract (Sricharoen, Techawongstein, & Chanthai, 2015; Tawaha, Alali, Gharaibeh, Mohammad, & El-Elimat, 2007). Hence, this may explain the differing correlation between total phenolic content and antioxidant activity of different seed meals in this study.

4.4. Antimicrobial effects of the seed meal extract

4.4.1. Clear zone

The agar disc diffusion method was used to evaluate the antibacterial activity of the ethanolic seed meal extracts. The results are shown in Table 13. ME and CE extracted with 60% and 80% ethanol, and NE extracted with 80% and 100% ethanol showed an overall better inhibition against the bacterial strains, and all three samples showed similar antibacterial activity against the Gram-negative bacteria. In addition, NE at 60, 80 and 100% ethanolic extraction showed the best response against *S. aureus*, the Gram-positive bacteria. The result is supported by the finding of Mohammed (2012) who reported that *N. sativa* alcoholic extracts worked well with gram positive bacteria. Likewise, ME showed a good response against *B. cereus* at 60% ethanolic extraction. The results are in accord with the findings of Singh, Negi, and Radha (2013), who reported the antibacterial property of *M. oleifera* defatted seeds against *Bacillus cereus* at the concentration of 1.4 mg/ml. Gaafar et al. (2016)

suggested that the antimicrobial property exhibited by ME is possibly because of the phytochemicals like polyphenols.

Table 13. Inhibition zone (mm) of seed meals extracted with ethanol at different concentrations against pathogenic bacteria

Extract sample	Ethanol %	Inhibition zone (mm)			
		EC	ST	SA	BA
ME	0%	$6.25\pm0.35^{c, C}$	$6.25\pm0.35^{bc, C}$	$10\pm0.00^{c, B}$	$14\pm1.41^{ab, A}$
	60%	$6.75\pm0.35^{bc, C}$	$7.25\pm0.35^{a, B}$	$14.5\pm0.70^{a, A}$	$14.75\pm1.06^{a, A}$
	80%	$7.5\pm0.71^{ab, C}$	$6.75\pm0.35^{ab, D}$	$13\pm1.41^{ab, A}$	$12\pm0.00^{c, B}$
	100%	$6.25\pm0.35^{c, C}$	$6.50+0.00^{bc, C}$	$11.5\pm0.71^{bc, B}$	$13\pm1.41^{bc, A}$
CE	0%	$6.25\pm0.35^{c, B}$	$6.00\pm0.00^{c, C}$	$6.75\pm0.35^{d, A}$	$6.5\pm0.00^{e, AB}$
	60%	$7.5\pm0.00^{ab, A}$	$6.5\pm0.00^{bc, B}$	$8\pm0.71^{d, A}$	$6.5\pm0.00^{e, B}$
	80%	$7\pm0.00^{abc, AB}$	$6.75\pm0.35^{ab, B}$	$7.5\pm1.42^{d, A}$	$6.75\pm0.35^{e, B}$
	100%	$6.75\pm0.35^{bc, B}$	$6.5+0.00^{bc, B}$	$7\pm0.70^{d, A}$	$7\pm0.00^{de, A}$
NE	0%	$7.25\pm0.35^{abc, A}$	$6.5\pm0.00^{bc, B}$	$6.75\pm0.35^{d, B}$	$6.75+0.35^{e, B}$
	60%	$8.00\pm0.70^{a, A}$	$6.5\pm0.00^{bc, B}$	$8\pm0.71^{d, A}$	$8.25\pm0.35^{d, A}$
	80%	$7.25\pm1.06^{abc, B}$	$7.25\pm0.35^{a, B}$	$7.25\pm1.06^{d, B}$	$8.5\pm0.71^{cd, A}$
	100%	$6.75\pm0.35^{bc, B}$	$6.35\pm0.21^{bc, B}$	$7\pm0.00^{d, AB}$	$7.75\pm0.35^{de, A}$

EC= *E. coli*, ST= *S.* Typhimurium, BC= *B. cereus*, SA= *S. aureus*

Data are presented as mean ± SD (n=3, $p<0.05$). Different superscripts in lowercase represent significantly different values among different concentrations of seed meals and different superscripts in uppercase represent significantly different values among different bacteria.

Aqueous ethanolic solution from 60-80% showed better inhibition zones among all seed meals, compared to the aqueous extract. This suggests that the bioactive phenolic compounds, including flavonoids (Panche, Diwan, & Chandra, 2016), phenolic acids and polyphenols (Alberto, Rinsdahl Canavosio, De Nadra, & Cristina, 2006) have antimicrobial properties.

Gram positive bacteria were relatively more susceptible to ME. The susceptibility differences between these two bacterial groups may be due to the difference in cell wall structure, with the outer membrane of the Gram-negative cell wall, acting as a barrier to many compounds, including antibiotics (Russel 1995). Nevertheless, 60% ethanolic CE and NE had a similar susceptibility to *E. coli*, which suggests that some bioactive compounds can alter the outer membrane structure of gram-negative bacteria (La Storia et al., 2011; Lambert, Skandamis, Coote, & Nychas, 2001). On the contrary, Bouarab Chibane, Degraeve, Ferhout, Bouajila, and Oulahal (2019) reported no clear correlation between Gram-staining and bacterial susceptibility to polyphenols.

4.5. Summary Part I

Based on the data obtained in Part I of this study, we can summarize the results as follows:

- Concentration of ethanol plays a major role in isolating the bio-active compounds from the tested oil seed meals.
- The ethanolic extracts of the tested oil seed meals bear antioxidant as well as antimicrobial activity against food borne pathogen.
- Aqueous ethanolic solution from 60-80% have higher antioxidant and antibacterial activity of the seed meals.

Part II. Selection of the best suitable solvent concentration and ultrasound assisted extraction of the bioactive compounds from the seed meals

The data obtained from the conventional ethanolic extraction for antioxidant and antibacterial activity (Part I) suggested that the optimum ethanolic concentration was the 60% aqueous solution of ethanol. Thus, for the ultrasonic extraction, 60% ethanol was selected for the extraction of the seed meals for further tests. The ultrasound extraction of the seed meals was performed at 3 power-conditions: 100W, 200W, 300W, and 3 time-conditions: 10, 20 and 30 minutes. The data obtained for the bioactive properties were compared with that of the conventional extracts. The list of phenolic compounds is presented in comparison with the corresponding conventional extracts. The results including the antioxidant and antimicrobial property analysis,

and inoculation test of the extracts in the food matrix and the analysis and discussions of the respective tests are given below.

4.6. Total yield of UAE extracts

The total yield of the UAE extracts is given in Table 14. Comparing the yield of the conventional seed meals extracted with 60% ethanol, almost all the treatment conditions (except $NE_{100/10}$) of the ultrasound extraction showed significantly higher yields, and this result is in agreement with (Chemat et al., 2017). According to Qi and Zhang (2014), when ultrasound was used for extraction of CE with water, the yield was found to be 21.32%, as compared to the yield from conventional extraction of 8.04% (Yu and He, 2018). Likewise, the yield of ultrasonic CE extracted with 70% ethanol was shown to be 18.54%. The highest yield of CE recorded in our study was 17.41% by $CE_{200/20}$. This supports our finding that the ultrasonic extraction of seed meals gives a higher yield because of the cavitation of bubbles and implosion which causes the cell wall breakage and hence the better penetration of the solvent leading to the better extraction (Veillet et al., 2010).

Among the three different power treatments for ultrasound extraction, at 100 W, the yield of all the three seed meal extracts invariably increased when the time of extraction was increased from 10 minutes to 20 minutes. This increase in yield was significantly greater in case of NE. A similar trend of increase in yield with the prolongation of extraction time from 10 minutes to 20 minutes of extraction duration was seen in the remaining 200 W and 300 W ultrasound treatment as well. However, further increasing the time of extraction from 20 minutes to 30 minutes did not significantly increase the yield in all the three seed meal extracts. Hence, it can be derived from the results that 20 minutes of extraction was the optimum duration for UAE for all three seed meal extracts.

Extracting the seed meals for 20 minutes at three different power conditions, viz 100 W, 200 W and 300 W showed a statistically insignificant changes in the total yield. Further, at 300 W, increasing the duration of extraction from 20 minutes to 30 minutes decreased the total yield. This shows that the duration of UAE is an important

factor determining the total yield of extraction. The highest yield among all the ultrasound treatments was given by $NE_{300/10}$ (21.34%).

Table 14. Yield of seed meal extracts under ultrasound extraction and comparison with the conventional extract yield

Power (W)	Time (min)	Yield (g/100 g seed meal)		
		ME	CE	NE
100	10	$10.41\pm1.93^{a, A}$	$14.63\pm0.38^{a, B}$	$10.48\pm3.53^{b, B}$
	20	$11.73\pm2.10^{a, B}$	$14.71\pm0.33^{a, B}$	$17.35\pm1.74^{a, A}$
	30	$9.94\pm0.12^{a, C}$	$14.84\pm1.03^{a, B}$	$16.46\pm0.00^{a, B}$
200	10	$10.14\pm1.01^{b, A}$	$16.49\pm0.87^{a, A}$	$18.88\pm0.89^{a, A}$
	20	$11.99\pm0.21^{a, A}$	$17.41\pm0.19^{a, A}$	$18.48\pm2.46^{a, A}$
	30	$13.20\pm0.50^{a, A}$	$17.36\pm0.04^{a, A}$	$18.41\pm0.69^{a, A}$
300	10	$9.75\pm0.74^{b, A}$	$15.07\pm0.15^{b, AB}$	$21.34\pm1.33^{a, A}$
	20	$12.82\pm0.10^{a, A}$	$17.10\pm0.01^{a, A}$	$20.13\pm0.65^{ab, A}$
	30	$11.94\pm0.00^{a, B}$	$15.20\pm0.25^{b, B}$	$17.12\pm0.66^{b, AB}$
Conv		6.12 ± 0.26	11.19 ± 0.28	14.45 ± 0.19

Conv= Conventional seed meal extracts, 60% ethanol.

Data are presented as mean ± SD, (n=3, $p<0.05$)

Lowercase letters represent significantly different values among extraction times, and uppercase letters represent significantly different values among ultrasonic power, within each extract.

4.7. Antioxidant activity of the ultrasound assisted seed meal extracts

Total phenolic content of the ultrasound extracted seed meal extracts was measured by Folin Ciocalteu method. The antioxidant activity was assessed by DPPH assay and FRAP methods.

4.7.1. Determination of the total phenolic content

The TPC yield by UAE of all the seed meals was significantly higher than TPC obtained from conventional extraction (Table 15). UAE could be efficiently used for the extraction of polyphenolic compounds from plant materials (Anaya-Esparza, Ramos-Aguirre, Zamora-Gasga, Yahia, & Montalvo-González, 2018). The mechanical effects generated by UAE cause the morphological alteration of the plant cell walls, thus creating a more porous surface that facilitates the extraction. This enhances the diffusion of intracellular components into the solution. The ultrasonic waves also accelerate the molecular motion of the solvent as well as the extract and effectively combine the components in the solution in substantially reduced extraction time (Hernández et al., 2019).

Among the power treatments, at all 100 W, 200 W and 300 W, increasing the duration of ultrasonic extraction from 10 minutes to 20 minutes invariably increased the total phenolic content of all the three seed meal extracts, ME, CE and NE. Further increase of extraction duration from 20 minutes to 30 minutes further increased the TPC at 100 W for ME and NE, but the TPC of CE decreased. However, at 200 W and 300 W, the increase in the times of extraction from 20 minutes to 30 minutes remained significantly constant for ME, and significantly reduced for CE and NE. The highest TPC among all the treatment groups was obtained for CE extracted for 30 minutes at 200 W power (522.56 mg GAE/g). This finding is in accordance with (Savic Gajic et al., 2019) who reported that increasing the UAE time from 20 minutes to 30 minutes increased TPC from 2.81 g/100g dry plant material (dpm) to 3.00 g/100 g dpm, when extracted with 60% alcohol at 40^{o}C, and also concluded that extraction time was the most important factor of phenolic extraction. Effect of extraction power on phenolic yield was studied by (Maran, Manikandan, Nivetha, & Dinesh, 2017), who concluded that the extraction of polyphenols depends on extraction duration, and that the yield was optimum in the first 20 minutes of UAE.

Most of the polyphenols are released early from the broken plant cells during the period of extraction, because ultrasound enhances the release of intracellular bioactive compounds into the exterior solvent. However, longer ultrasonic extraction time might induce the degradation polyphenols due to increased cavitation of the solvents leading to asymmetric collapse of micro-bubbles that can damage the

compounds dissolved in the solution (Maran et al., 2017; Tiwari, O'Donnell, & Cullen, 2009). The results of our study are in agreement with (Rostagno, Palma, & Barroso, 2007), which concluded that 20 min of ultrasonication was sufficient to extract phenolics from soy beverages.

Table 15. Effect of UAE seed meal extracts on the total phenolic contents (TPC)

Power (W)	Time (min)	TPC (mg GAE/g)		
		ME	CE	NE
100	10	380.28±3.88$^{b, B}$	476.04±2.34$^{c, C}$	391.32±0.94$^{c, C}$
	20	443.87±2.93$^{a, B}$	502.14±2.24$^{a, C}$	473.15±1.56$^{b, C}$
	30	448.02±2.24$^{a, A}$	482.25±3.45$^{b, C}$	494.48±2.84$^{a, A}$
200	10	382.35±0.94$^{c, B}$	492.41±2.18$^{c, B}$	402.50±1.29$^{c, B}$
	20	460.86±2.94$^{a, A}$	510.23±1.00$^{b, B}$	483.50±2.70$^{a, B}$
	30	449.05±2.51$^{b, A}$	522.24±1.01$^{a, A}$	449.32±1.84$^{b, B}$
300	10	415.70±4.97$^{b, A}$	516.44±2.72$^{a, A}$	430.89±1.56$^{b, A}$
	20	421.50±0.95$^{a, C}$	516.44±1.24$^{a, A}$	490.96±2.54$^{a, A}$
	30	446.36±2.58$^{a, A}$	507.12±1.88$^{b, B}$	437.31±2.80$^{b, C}$
Conv		101.82±0.39	186.03±0.09	132.63±0.71

Conv= Conventional seed meal extracts, 60% ethanol

Data are presented as mean ± SD (n=3, p<0.05)

Lowercase letters represent significantly different values among extraction times, and uppercase letters represent significantly different values among ultrasonic power, within each extract.

4.7.2. DPPH radical scavenging assay

The radical scavenging activity as tested by DPPH assay showed a distinct effect of duration of extraction as well as the role of appropriate power of the ultrasound extraction to obtain the optimum activity. The data is shown in Table 16. All the UAE treatments showed significantly higher DPPH radical scavenging activity than of conventional extraction, and this result is in agreement with Dadi, Emire, Hagos, and Eun (2019). The highest radical scavenging activity was observed

with CE extracted for 30 minutes at 200 W ultrasonic power (13.21mg AAE/g freeze-dried extract).

At all the ultrasound power treatments i.e. 100 W, 200 W and 300 W, increasing the time of extraction from 10 to 20 minutes invariably increased the radical scavenging activity of all the three extracts, imitating the results of TPC. Prolongation of extraction time from 20 minutes to 30 minutes at 100W power further increased the antioxidant activity of ME and NE significantly, but remained constant for CE. Likewise, at 200 W the increase in duration of ultrasound extraction from 20 minutes to 30 minutes decreased the antioxidant activity of ME, but increased in case of CE and NE. Again, at 300 W, when the time was increased from 20 minutes to 30 minutes, the antioxidant activity remained constant for ME, increased for CE, and decreased for NE.

From the above results, it can be implied that the optimum extraction time for ME and NE was 20 minutes and that for CE was 30 minutes. So again, keeping the extraction time constant at 20 minutes, increasing UAE power from 100 W to 200 W significantly increased the antioxidant activity of ME, whereas remained constant for NE. Further increasing the power to 300 W significantly reduced the antioxidant activity of ME while it increased for NE.

Tracking the effect of power in the extraction of CE the optimum duration of 30 minutes, increasing the power from 100 W to 200 W significantly increased the antioxidant activity. Further increasing the power to 300 W, however, significantly decreased the antioxidant activity. Thus, it can be concluded that 200W power for 20 minutes; 300 W power for 20 minutes; and 200 W for 30 minutes, were the optimum extraction duration for ME, NE and CE respectively.

This shows that the different factors affecting ultrasound-assisted extraction may vary with the compounds to be extracted (Chemat et al., 2017). This can be attributed to the difference in the structural composition of the plant cells that can possibly affect the efficiency of UAE. Therefore, it is important to find optimum time and power of UAE that can improve the extraction efficiency as well as the compounds' bioactivity (Dadi et al., 2019). The optimum ultrasonic conditions vary

with the samples used; however, a distinct pattern of time and power conditions required for optimum activity can be observed in several studies (Bamba et al., 2018; Dadi et al., 2019) as well as this study. Duration of extraction plays an important role in the extraction process. For example, lesser time (10 minutes) of extraction is insufficient for optimum bioactivity of the extracts. However, increasing the time to 20 minutes increased the bioactivity in all the extracts in this study. In accordance to this study with ME and NE, 20 minutes of UAE at 40ºC with 70% ethanol was found to be optimal condition for *Moringa stenopetala* leaves extraction by (Dadi et al., 2019). However, further prolonging the time of extraction may degrade the phenolic compounds, leading to the decrease of the extraction yield and hence the bioactivity (Carrera, Ruiz-Rodríguez, Palma, & Barroso, 2012). The extraction duration of 30 minutes was optimum for extracting bioactive polyphenols with ethanol from *Phyllanthus urinaria*, although the UAE power used was high at 500 W, which was out of the scope of our study. UAE power above 300 W was not considered in this study because the bioactivity of the extracts extracted at this power condition was comparatively lesser than those extracted at 200 W power. Overall, the results obtained in this study for power condition (200 W) and extraction duration (20 minutes to 30 minutes) is in agreement with several studies as the optimum condition for the extraction of polyphenols from different plant samples (Dadi et al., 2019; Maran et al., 2017).

Table 16. Effect of UAE seed meal extracts on the antioxidant activity measured by DPPH assay

Power (W)	Time (min)	DPPH (mg AAE/g)		
		ME	CE	NE
100	10	8.87±0.05^{cC}	7.62±0.02$^{b, B}$	9.14±0.02$^{c, B}$
	20	10.18±0.02^{bC}	9.86±0.02$^{a, C}$	10.54±0.04$^{b, B}$
	30	11.07±0.05^{aA}	9.87±0.01$^{a, C}$	10.99±0.01$^{c, A}$
200	10	8.95±0.02^{cB}	10.60±0.01$^{c, A}$	9.31±0.13$^{c, B}$
	20	11.18±0.02^{aA}	11.50±0.06$^{b, B}$	10.56±0.02$^{b, B}$
	30	11.09±0.01^{bA}	13.21±0.04$^{a, A}$	10.86±0.03$^{a, A}$
300	10	10.07±0.01^{bA}	10.67±0.02$^{c, A}$	10.31±0.0$^{c, A}$
	20	10.98±0.01^{aB}	12.40±0.01$^{b, A}$	11.35±0.01$^{a, A}$
	30	11.02±0.02^{aA}	12.81±0.01$^{a, B}$	10.80±0.03$^{b, A}$
Conv		4.84±0.02	7.52±0.01	7.34±0.00

Conv= Conventional seed meal extracts, 60% ethanol.

Data are presented as mean ± SD, (n=3, p<0.05). Lowercase letters represent significantly different values among extraction times, and
uppercase letters represent significantly different values among ultrasonic power, within each seed meal extract sample.

4.7.3. Ferric reducing antioxidant property (FRAP)

The ferric reducing activity of the seed meal extracts is shown in Table 17. Almost all the UAE treatments showed higher ferric reducing activity than the conventional extracts. The highest FRAP was exhibited by CE extracted at 200W ultrasound power for 20 minutes ($CE_{200/20}$) Similar to the pattern shown by TPC and DPPH, the FRAP assay also showed increase in antioxidant activity with the increase in time of extraction from 10 minutes to 20 minutes at 100 W and 200 W of all the seed meal extracts, ME, CE and NE. NE showed increase in antioxidant activity even at 300W, but ME and CE showed no significant increase with the increase in time of extraction from 10 minutes to 20 minutes. Further increase in time from 20 minutes to 30 minutes did not create significant difference in antioxidant activity of ME and CE at 100 W and 200 W ultrasonic power, but significantly decreased when the power

was increased to 300 W. The antioxidant activity of NE increased at 100 W as well as at 200 W when time of extraction was further increased from 20 minutes to 30 minutes. However, at 300 W ultrasound extraction, this increase in extraction duration from 20 minutes to 30 minutes significantly reduced the antioxidant activity. This shows the different activity of polyphenols of different samples in relation to power and extraction duration of ultrasound.

Table 17. Effect of UAE seed meal extracts on the antioxidant activity measured by Ferric reducing power (FRAP) as compared to the conventional extracts.

Power (W)	Time (min)	FRAP (mg AAE/g)		
		ME	CE	NE
100	10	$7.15\pm0.20^{b, A}$	$7.55\pm0.90^{b, B}$	$5.54\pm1.92^{c, A}$
	20	$9.11\pm0.07^{a, A}$	$8.97\pm0.54^{a, A}$	$6.32\pm0.12^{b, C}$
	30	$9.06\pm0.84^{a, A}$	$9.20\pm0.10^{a, A}$	$9.06\pm0.84^{a, A}$
200	10	$8.15\pm0.20^{b, B}$	$8.88\pm0.09^{a, A}$	$5.62\pm0.04^{b, A}$
	20	$9.20\pm0.03^{a, A}$	$9.18\pm0.05^{a, A}$	$7.74\pm0.43^{a, B}$
	30	$8.91\pm0.48^{a, A}$	$9.63\pm0.17^{a, A}$	$8.60\pm0.48^{a, B}$
300	10	$8.87\pm0.20^{a, C}$	$9.25\pm0.03^{a, A}$	$5.56\pm0.04^{c, A}$
	20	$9.15\pm0.07^{a, A}$	$9.04\pm0.62^{a, A}$	$9.47\pm0.40^{a, A}$
	30	$7.55\pm0.51^{b, B}$	$7.92\pm0.83^{b, B}$	$7.55\pm0.51^{b, C}$
Conv		5.82±0.21	6.27±0.02	6.18±0.01

Conv= Conventional seed meal extracts, 60% ethanol

Data are presented as mean ± SD (n=3, $p<0.05$). Lowercase letters represent significantly different values among extraction times, and

uppercase letters represent significantly different values among ultrasonic power, within each seed meal extract sample.

Based on the data obtained from the antioxidant activity of the ultrasound extracted seed meal extracts, it can be concluded that the efficacy of UAE is superior over the conventional maceration technique of extraction. The total extraction yields as well as the total phenolic content increased with the use of UAE, with the time of extraction being drastically reduced. The study indicates that shorter time (10 minutes) was insufficient for optimal extraction and increasing the time to 20 minutes increased the yield of phenolic content. Also, the power of 200 W and 300 W showed higher yield of the bioactive compounds. Further increase in time and power of extraction to 30 minutes and 300 W respectively, did not significantly improve the yield and antioxidant performance of any of the three seed meal extracts. This indicates that the higher power and longer extraction duration caused the ultrasonic degradation of the bioactive compounds of the extracts (Maran et al., 2017).

4.8. Antibacterial activity of the ultrasound extracted seed meals

4.8.1. Zone of inhibition

The antibacterial activity of the seed meal extracts was measured through agar gel disc diffusion test. The extracts of three different seed meal extracts extracted at different ultrasound power and duration of extraction were examined for the zone of inhibition against 4 different bacteria, *E. coli*, *S.* Typhimurium, *B. cereus* and *S. aureus*. Sterilized 6 mm discs were placed on the surface of tryptic soy agar discs and were imbibed with 100 mg/ml of different extract solutions prepared in DMSO. As a general principle, the zone of inhibition more than 6mm was considered to have antibacterial property. Broad spectrum antibiotic Amoxycillin was used as positive control. The test result of ME is shown in Table 18.

The zone of inhibition of all the treatment conditions were significantly higher than that of conventional for all the tested bacteria. ME interestingly showed significantly bigger inhibition zones than those made by amoxycillin against gram positive *B. cereus* and *S. aureus.*

ME showed potent antibacterial activity against all the 4 bacteria. Among the ultrasound extracted extracts, the largest inhibition zone was exhibited by the $ME_{200/20}$, which was 9.87mm, 9.75mm, 19.75mm and 19.25mm against *E. coli*, *S.* Typhimurium, *B. cereus* and *S. aureus* respectively.

Table 18. Effect of ME on the zone of inhibition exhibited against the tested bacteria

Power (W)	Time (min)	Inhibition zone (mm)			
		EC	ST	BC	SA
100	10	8.00±0.57$^{ef, C}$	7.5±0.57$^{d, C}$	11.37±0.75$^{d, B}$	16.37±1.10$^{bc, A}$
	20	8.75±0.5$^{cde, C}$	8.75±0.5$^{c, C}$	19.5±1.00$^{a, A}$	16.75±1.55$^{bc, B}$
	30	8.25±0.5$^{f, D}$	9±0.81$^{c, C}$	16.25±2.06$^{b, A}$	15.25±0.95$^{c, A}$
200	10	8.75±0.5$^{cde, B}$	8.75±0.29$^{c, B}$	18.25±0.5$^{ab, A}$	18.25±0.5$^{ab, A}$
	20	9.87±0.47$^{b, B}$	9.75±0.5$^{b, B}$	19.75±0.28$^{a, A}$	19.25±0.95$^{a, A}$
	30	9.5±0.57$^{bcd, B}$	9±0.00$^{c, B}$	17.00±1.15$^{b, A}$	16.12±1.03$^{bc, A}$
300	10	8.75±0.5$^{cde, B}$	8.75±0.29$^{c, B}$	17.00±2.3$^{b, A}$	16.87±0.62$^{b, A}$
	20	9.62±0.48$^{bc, B}$	8.87±0.48$^{c, B}$	18.25±1.5$^{ab, A}$	16.62±1.10$^{bc, A}$
	30	8.62±0.48$^{de, B}$	8.75±0.29$^{c, B}$	16.50±0.57$^{b, A}$	15.25±0.5$^{c, A}$
ME_{Conv}		6.75±0.35$^{f, B}$	7.25±0.35$^{d, B}$	14.75±1.06$^{c, A}$	14.50±0.70$^{d, A}$
Amoxicilin		13±1.15$^{a, A}$	13.25±1.5$^{a, A}$	10.50±0.58$^{e, B}$	10.25±0.5$^{e, B}$

Data are presented as mean ± SD, (n=3, p<0.05)

Uppercase letters represent significant difference among different bacteria in each ultrasound treatment group.

Lowercase letters represent significant difference among different treatments within each bacterial treatment.

C. oleifera seed meal extract (CE) exhibited moderate antibacterial activity against the tested bacteria, as shown in Table 19. Against *E. coli*, the ultrasound extracted $CE_{300/10}$ and $CE_{300/20}$ showed significantly bigger inhibition zone (8.75mm and 8.50 mm respectively) as compared to that of the conventional extract (7.50 mm). With *S.* Typhimurium, no significant difference in the antibacterial activity was observed between the conventional extracts and UAE extracts. $CE_{200/30}$ and $CE_{300/10}$ and $CE_{300/20}$ showed significantly bigger inhibition zones (7.25 mm) than the conventional CE (6.50 mm) against *B. cereus*. Likewise, with *S. aureus*, $CE_{200/30}$ and at $CE_{300/10}$ showed significantly higher inhibition (9 mm) as compared to the

conventional (8 mm). however, the inhibition exhibited by CE was lesser than that shown by amoxycillin.

Table 19. Effect of CE on the zone of inhibition exhibited against the tested bacteria.

Power (W)	Time (min)	Inhibition zone (mm)			
		EC	ST	BC	SA
100	10	$6.75\pm0.35^{de, A}$	$6.75\pm0.35^{c, A}$	$6.00\pm0.00^{d, A}$	$6.00\pm0.00^{d, A}$
	20	$7.00\pm0.00^{de, A}$	$7.0\pm0.35^{bc, A}$	$6.25\pm0.35^{cd, A}$	$6.50\pm0.00^{d, A}$
	30	$6.25\pm0.35^{e, A}$	$6.55\pm0.07^{c, A}$	$6.50\pm0.00^{c, A}$	$6.25\pm0.35^{d, A}$
200	10	$8.25\pm0.35^{bc, A}$	$7.0\pm0.0^{bc, B}$	$7.0\pm0.0^{bc, B}$	$7.5\pm0.7^{cd, AB}$
	20	$7.50\pm0.7^{cd, AB}$	$7.0\pm0.0^{bc, B}$	$6.75\pm0.35^{c, B}$	$8.00\pm0.0^{c, A}$
	30	$6.55\pm0.07^{e, B}$	$6.5\pm0.00^{c, B}$	$7.25\pm0.35^{ab, B}$	$9.00\pm0.00^{a, A}$
300	10	$8.75\pm0.35^{b, A}$	$6.25\pm0.35^{c, B}$	$7.25\pm1.06^{ab, B}$	$9.00\pm0.00^{a, A}$
	20	$8.50\pm0.71^{b, A}$	$6.75\pm0.35^{c, B}$	$7.25\pm0.35^{ab, B}$	$7.5\pm0.7^{cd, AB}$
	30	$6.50\pm0.00^{e, A}$	$6.25\pm035^{c, A}$	$6.50\pm0.00^{c, A}$	$6.75\pm0.35^{d, A}$
CE_{Conv}		$7.50\pm0.0^{cd, AB}$	$6.50\pm0.00^{c, B}$	$6.50\pm0.00^{c, B}$	$8.00\pm0.71^{c, A}$
Amoxicillin		$11.0\pm0.71^{a, A}$	$8.50\pm0.35^{a, C}$	$8.33\pm0.71^{a, C}$	$10\pm0.71^{a, B}$

Data are presented as mean ± SD, (n=3, $p<0.05$)

Uppercase letters represent significant difference among different bacteria in each ultrasound treatment group.

Lowercase letters represent significant difference among different treatments within each bacterial treatment.

NE also showed impressive bacterial inhibition in the disc diffusion test against the tested bacteria (Table 20). All the treatments groups showed similar antibacterial response against *E. coli*, and the UAE were also not different in antibacterial activity with the conventional extract, and the $NE_{100/20}$ showed similar antibacterial response (8.12 mm) as that shown by amoxycillin (9.15 mm). Similar results were obtained for *S.* Typhimurium where all the UAE NE showed similar antibacterial activity against the aforementioned bacteria. However, the antibacterial inhibition was significantly better than that exhibited by the conventional NE (6.50 mm) but were significantly lesser when compared to the antibacterial activity of amoxycillin (12 mm). $NE_{300/20}$ exhibited the highest inhibition against *B. cereus* with 9 mm of inhibition zone, which however was significantly similar to the inhibition shown by negative control, the conventional NE (8.25 mm) as well as the positive control (amoxycillin). Most of the ultrasound extracted NEs exhibited similar antibacterial activity against *S. aureus*, and the results were not significantly different from that of the conventionally extracted NE. $NE_{100/20}$ and $NE_{100/30}$ exhibited similar inhibition (8.75 and 9.25 mm respectively) with that of amoxycillin (9.50 mm), which showed the highest inhibition activity against *S. aureus*.

Table 20. Effect of NE on the zone of inhibition exhibited against the tested bacteria.

Power (W)	Time (min)	Inhibition zone (mm)			
		EC	ST	BC	SA
100	10	$7.87\pm0.17^{b, AB}$	$7.50\pm0.70^{b, B}$	$7.25\pm0.35^{c, B}$	$8.37\pm0.17^{bcde, A}$
	20	$8.12\pm0.17^{ab, A}$	$7.25\pm0.35^{b, B}$	$7.25\pm0.00^{c, B}$	$8.75\pm0.0^{abc, A}$
	30	$8.00\pm0.0^{b, A}$	$7.25\pm0.35^{b, A}$	$8.5\pm1.41^{abc, A}$	$9.25\pm0.35^{ab, A}$
200	10	$7.50\pm0.35^{b, A}$	$8.00\pm1.41^{b, A}$	$8.87\pm0.88^{ab, A}$	$7.25\pm0.35^{cde, A}$
	20	$7.87\pm0.17^{b, A}$	$8.25\pm0.35^{b, A}$	$7.37\pm0.18^{bc, A}$	$7.25\pm0.70^{cde, A}$
	30	$7.87\pm0.53^{b, A}$	$7.25\pm0.35^{b, A}$	$8.37\pm0.88^{abc, A}$	$8.00\pm0.0^{bcde, A}$
300	10	$7.12\pm0.88^{b, A}$	$7.75\pm0.0^{b, A}$	$8.62\pm0.17^{abc, A}$	$7.12\pm0.17^{de, A}$
	20	$7.50\pm0.70^{b, B}$	$7.75\pm00^{b, B}$	$9.00\pm0.35^{a, A}$	$6.50\pm0.35^{e, B}$
	30	$7.62\pm0.17^{b, A}$	$7.50\pm0.7^{b, A}$	$8.62\pm0.17^{abc, A}$	$8.37\pm0.88^{bcde, A}$
NE_{Conv}		$8.00+0.70^{b, A}$	$6.50\pm0.0^{c, B}$	$8.25\pm0.35^{abc, A}$	$8.00+0.7^{bcde, A}$
Amoxicillin		$9.15\pm0.91^{a, B}$	$12.0\pm2.82^{a, A}$	$8.95\pm1.49^{ab, B}$	$9.50\pm2.12^{a, B}$

Data are presented as mean ± SD, (n=3, $p<0.05$).

Uppercase letters represent significant difference among different bacteria in each ultrasound treatment group.

Lowercase letters represent significant difference among different treatments within each bacterial treatment.

4.8.2. Broth dilution test for minimum inhibitory concentration (MIC)

MIC test for the UAE seed meal extracts was performed against the four selected bacteria, *E. coli*, *S.* Typhimurium, *B. cereus* and *S. aureus*. The initial extract concentration was maintained at 25g/ml and was serially diluted two-fold using MHB. Respective bacteria at 10^6 CFU/ml were inoculated to the extract solution and incubated for 24 hours at 37ºC. The results of ME, CE and NE are shown in tables 21, 22 and 23, respectively.

ME showed considerable antibacterial activity in broth dilution test. The lowest concentration that showed the bacterial inhibition was ME extracted at 200W

ultrasound power for the duration of 20 minutes ($ME_{200/20}$). The lowest concentration showing bacterial inhibition was 3.125 mg/ml against *E. coli*, which was considerably lower than the inhibition concentration of the conventional ME of 25 mg/ml. Against *S.* Typhimurium, the MIC value was 12.5 mg/ml with $ME_{200/20}$. Again, with *B. cereus* as well as *S. aureus*, the treatment group exhibiting bacterial inhibition and the minimum concentration was $ME_{200/20}$, and the MIC for *B. cereus* was 3.125 mg/ml and for *S. aureus* was 6.25 mg/ml. The minimum antibacterial concentration of all the seed meal extracts were lower than their conventional counterparts. Hence, it can be concluded that ME extracted at 200W for the duration of 20 minutes is the optimum UAE condition for ME that exhibited the highest antibacterial activity. The results of the disc diffusion test of ME also support this result.

Table 21. Minimum inhibitory concentration (MIC) of ME against the tested bacteria.

Power (W)	Time (min)	MIC (mg/ml)			
		EC	**ST**	**BC**	**SA**
100	10	25	25	25	12.5
	20	25	25	6.3	12.5
	30	25	25	12.5	>25
200	10	12.5	25	12.5	12.5
	20	3.1	12.5	3.1	6.3
	30	12.5	25	25	25
300	10	6.3	12.5	25	25
	20	12.5	12.5	6.3	12.5
	30	25	12.5	6.3	25
ME_{Conv}		25	25	12.5	25

CEs showed moderate antibacterial activity in the broth diffusion test in the tested concentration of the extracts (Table 22). The minimum concentration exhibiting bacterial inhibition against *E. coli* was 12.5 mg/ml, which was exhibited by CE extracted at 300W ultrasound power for all 10, 20 and 30 minutes of extraction. Against *S.* Typhimurium, CE exhibited bacterial inhibition at 25 mg/ml extract concentration and above. The lowest concentration that showed inhibition against B. cereus 6.25 mg/ml and was exhibited by CE extracted at 300W ultrasound power, extracted for the duration of 20 minutes ($CE_{300/20}$). For *S. aureus*, the MIC was 25 mg/ ml and above. Hence, it can be concluded from the data that the UAE treatment condition exhibiting the highest antibacterial activity in broth diffusion is the $CE_{300/20}$ and showed better inhibition against *E. coli* and *B. cereus* among the four tested bacteria.

Table 22. Minimum inhibitory concentration (MIC) of CE against the tested bacteria

Power (W)	Time (min)	MIC (mg/ml)			
		EC	**ST**	**BC**	**SA**
100	10	25	>25	25	25
	20	25	>25	25	25
	30	>25	>25	25	25
200	10	25	25	25	25
	20	25	25	25	25
	30	25	25	12.5	25
300	10	12.5	>25	12.5	>25
	20	12.5	25	6.3	25
	30	12.5	>25	12.5	>25
CE_{Conv}		>25	>25	25	25

NEs also showed moderate antibacterial activity in the broth diffusion test for minimum inhibitory concentration of the extract against the tested bacteria, as shown in Table 23. The minimum concentration exhibiting bacterial inhibition against *E. coli* was 12.5 mg/ml, which was exhibited by $NE_{300/20}$, $NE_{100/30}$, and $NE_{200/10}$. Against *S.* Typhimurium, NE exhibited bacterial inhibition at 25 mg/ml extract concentration and above. The lowest concentration that showed inhibition against B. cereus was 6.3 mg/ml and was exhibited by$NE_{300/20}$. For *S. aureus*, the MIC was 25 mg/ml and above. Hence, it can be concluded from the data that the UAE treatment condition exhibiting the best antibacterial activity in broth diffusion is the $NE_{300/20}$ and showed better inhibition against both *E. coli* and *B. cereus*.

Table 23. Minimum inhibitory concentration (MIC) of NE against the tested bacteria

Power (W)	Time (min)	MIC (mg/ml)			
		EC	ST	BC	SA
100	10	25	25	12.5	25
	20	25	25	12.5	12.5
	30	12.5	25	25	12.5
200	10	12.5	25	6.3	12.5
	20	25	25	12.5	12.5
	30	25	25	12.5	12.5
300	10	25	25	12.5	25
	20	12.5	12.5	3.1	12.5
	30	25	12.5	6.3	12.5
NE_{Conv}		>25	>25	25	25

From the data obtained from the antibacterial activity of the extracts, it is evident that certain UAE conditions improved the antibacterial activity of the extracts as compared to the conventional extracts. Similar results have been reported the extracts showed better antibacterial activity against *E. coli* among the gram-negative bacteria, and *B. cereus* among the gram-positive. The minimum inhibitory concentration for bacterial inhibition was at 3.1 mg/ ml of $ME_{200/20}$ against both *E. coli* and *B. cereus*. The least MIC among the CEs was of $CE_{300/20}$ which was 12.5 mg/ ml against *E. coli* and 6.3mg/ml against *B. cereus*. $NE_{300/20}$ showed MIC of 12.5 mg/ml against *E. coli* and 3.1mg/ ml against *B. cereus*. Interestingly, the zone of inhibition exhibited by ME against *B. cereus* was significantly higher (19.75±0.3 mm) than antibiotic amoxycillin (10.5±0.6 mm). Similar pattern of lower antibiotic resistance by Gram-positive bacteria against moringa seed extract was reported by (Singh et al., 2013; Viera, Mourão, Ângelo, Costa, & Vieira, 2010; Yolmeh, Habibi-Najafi, Shakouri, & Hosseini, 2015). The lesser susceptibility of the gram-negative bacteria can be attributed to the outer phospholipid and lipopolysaccharide bilayer (Glauert & Thornley, 1969) that renders them lesser penetrable than the gram-positive ones which lack this outer membrane.

The effect of power and time as the factors of UAE is evident in the antibacterial activity of the extracts. 20 minutes of extraction time was optimum for all the three seed meal extracts. Likewise, 200W for ME and 300W for CE and NE were found to be optimum conditions for UAE. Therefore, $ME_{200/20}$, $NE_{300/20}$ and $CE_{300/20}$ were selected for the remaining studies related to the extracts' antibaterial activity. Likweise, as per the research design, two most susceptible bacteria, gram positive and gram negative each, i.e., *B. cereus and E. coli* were selected as the representative bacteria for the upcoming tests.

4.8.3. HPLC analysis of the extracts

For the identification of phenolic compounds of UAE optimized seed meal extracts using HPLC, $ME_{200/20}$, $CE_{300/20}$, $NE_{300/20}$ were selected based on their superior antimicrobial bioactivity compared to other UAE treatment groups. The results were compared with the conventional 60% ethanolic extract of the respective seed meals, as presented in Table 25. The phenolic compounds (gallic acid, vanillic acid,

quercetin, sinapic acid, chlorogenic acid, p-coumaric acid, protocatechuic acid, protocatechualdehyde, syringic acid, ferulic acid and caffeic acid) were identified from ME and the findings were similar to that of (Singh et al., 2013). $ME_{200/20}$ gave higher quantities, than the conventional ME, of all the phenolic compounds on test. Gallic acid was the predominant polyphenol found in the seed meal extracts, followed by vanillic acid, quercetin and sinapic acid. The quantity of gallic acid and vanillic acid obtained from $ME_{200/20}$ was 2.27 times (158.3 mg/100 g) and 2.08 times (89.74 mg/100 g) higher, respectively, than the value obtained from the conventional ME (69.73 mg/100 g and 158.3 mg/100 g respectively). In CEs, quercetin was the most predominant polyphenol, followed by chlorogenic acid. Ultrasonic intervention increased quercetin from 22.04 mg/100 g in CE_{Conv} to 28.49 mg/100 g extract in $CE_{300/20}$. Chlorogenic acid was the predominant phenolic acid amongst the NEs, whose quantity was 63.73 mg/100g extract in NE_{Conv} and 99 mg/100g extract in $NE_{300/20}$ and was the highest amount of chlorogenic acid among all the three seed meal extracts. Sinapic acid was the second most prevalent phenolic acid in the NEs. NE_{Conv} had 51.12mg/100 g extract, and $NE_{300/20}$ had 67.80mg/100 g extract. All of the polyphenols were isolated in higher amount in $NE_{300/20}$ than NE_{Conv}, which proves the improved phenolics extraction with the intervention of UAE.

This finding supports the results of TPC, DPPH and FRAP assay of this study (Tables 15, 16, 17) where the ultrasound extracts performed better in the antioxidant activity than the conventional extracts, and thus attributes this improved antioxidant activity to the increased release of phenolic compounds when ultrasound is used to the seed meal extraction process. Additionally, caffeic acid was detected only in $ME_{200/20,}$ and was not detected in ME_{conv}.Also, protocatechuic, vanillic and *p*-coumaric acids showed antibacterial activity against *E. coli*, *Pasteurella multocida* and *Neisseria gonorrhoeae* (Alves et al., 2013). Several studies on antibacterial activities of similar phenolic compounds as isolated in this study are enlisted in Table 24.

Table 24. Polyphenols associated with antibacterial activity

Polyphenols	Target bacteria	References
Ferulic acid, Gallic acid	*E. coli, S. aureus*	Borges et al., (2013)
Catechin, Gallic acid	*B. cereus, S. aureus, E. coli*	Diaz- Gômez et al., (2013)
Coumarin	*B. subtilis*	M. De Souza et al., (2005)
Caffeic acid	*S. aureus*	Kepa et al., (2018)
Chlorogenic acid	*E. coli, S. enteritidis*	Mujtaba et al., ((2017)
Caffeic acid, p-coumaric acid	*S. aureus*	Stojkovic et al., 2013
Sinapic acid	*E. coli, B. subtilis, S. aureus*	Engels et al., (2012)

Table 25. The major phenolic compounds identified with HPLC from conventional seed meal extracts and compared with the respective UAE extracts of optimized conditions

Phenolic constituents	Content (mg/100 g extract) ±SD					
	ME_{Conv}	**CE_{Conv}**	**NE_{Conv}**	**$ME_{200/20}$**	**$CE_{300/20}$**	**$NE_{300/20}$**
Gallic acid	69.73±5.75	6.53±0.76	8.31±0.45	158.3±12.58	9.01±0.34	11.23±0.21
Vanillic acid	43.06±0.90	ND	ND	89.74±1.62	ND	ND
Quercetin	25.48±2.60	22.04±0.54	29.67±0.90	42.66±0.82	28.49±1.64	34.87±0.14
Sinapic acid	24.83±5.59	ND	51.12±1.01	26.70±1.55	ND	67.80±2.28
Chlorogenic acid	14.39±1.55	15.10±0.09	63.73±1.49	17.83±1.01	22.26±0.06	99.00±1.42
p-Coumaric acid	7.00±0.89	4.92±0.18	7.15±0.08	11.21±1.38	8.23±0.23	7.57±0.11
Protocatechuic acid	5.83±0.64	ND	ND	11.72±0.12	ND	ND
Protocatechualdehyde	4.96±0.09	-	-	6.18±0.38	-	-
Ferulic acid	2.43±0.27	9.79±0.23	9.79±0.23	4.75±0.48	8.78±0.72	2.26±0.14
Caffeic acid	ND	7.52±0.13	4.53±0.39	4.64±0.32	15.72±0.22	4.98±0.07
Syringic acid	-	ND	ND	-	ND	ND

ME_{Conv} = Conventionally extracted ME with 60% ethanol

CE_{Conv} = Conventionally extracted ME with 60% ethanol

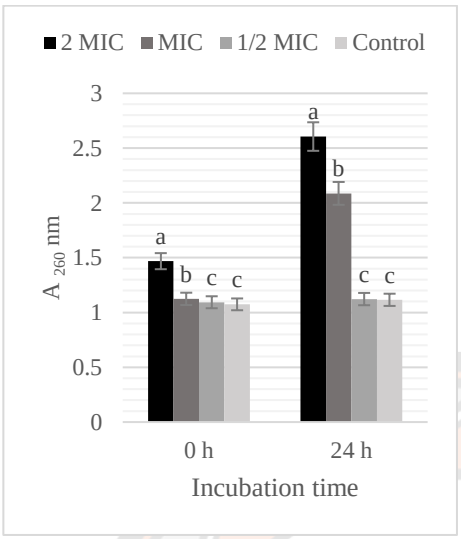
NE_{Conv} = Conventionally extracted ME with 60% ethanol

ND= Not detected

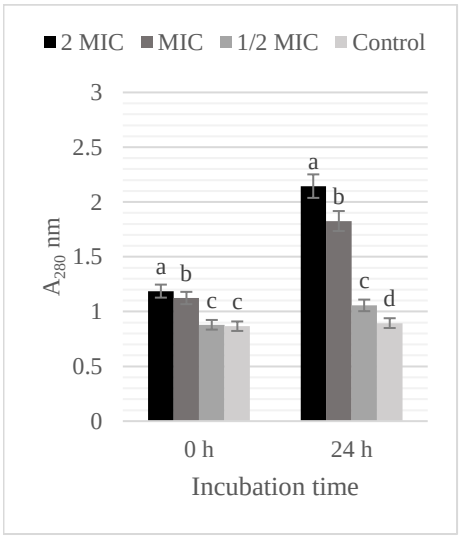
4.8.4. Bacterial cell leakage test

The cytoplasmic leakage study was conducted using UV-spectrophotometry of *E. coli* and *B. cereus* at 260 and 280 nm wavelengths for nucleic acid content and protein content respectively. The measurements obtained are shown in Figure 31. The values given are the measurements after 24 hours of incubation with $ME_{200/20}$, $NE_{300/20}$ and $CE_{300/20}$ at three different concentrations, subtracted from the control for each treatment. The results indicate the alteration in the cell membrane permeability and consequent cellular leakage at all the extract concentrations, which can be attributed to the antibacterial effect of the extract samples. Similar results have been reported in Al-Habib, Al-Saleh, Safer, and Afzal (2010) in which grape seed oil extract when used against *S. aureus* caused the cell to swell, and the intracellular constituents were reduced. Several other studies have also reported that the membrane-active mechanism of phytochemicals can cause severe damage and disruption of the cell membrane. Therefore, membrane integrity was chosen as a parameter to explore the antibacterial mechanism of the seed meal extracts. Cell membrane integrity plays an important role in maintaining homeostasis and protecting against external influence (Sánchez, García, & Heredia, 2010). Thus, any external influence that damages the cell membrane structure could adversely affect cellular viability and cause eventual cell death (Cox, Mann, & Markham, 2001). The results indicate the alteration in the cell membrane permeability and consequent cellular leakage at all the extract concentrations, which indicates that the cell membrane could be the primary target of the seed meal extracts in their antibacterial action. Also, increase in extract concentration increased the cytoplasmic constituents' leakage which indicated that at higher concentration, the antibacterial activity of the extracts might be due to bactericidal damage to the membrane.

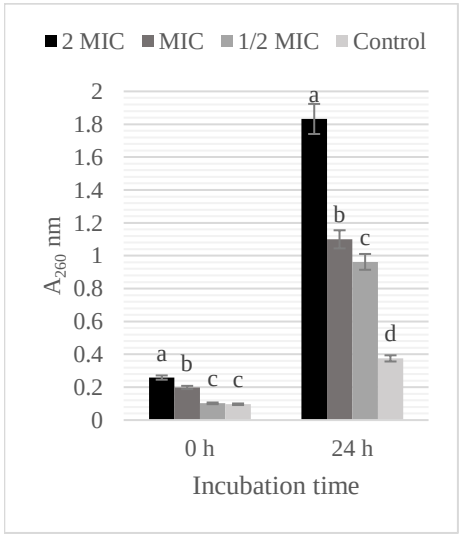
A1



A2



A3



A4

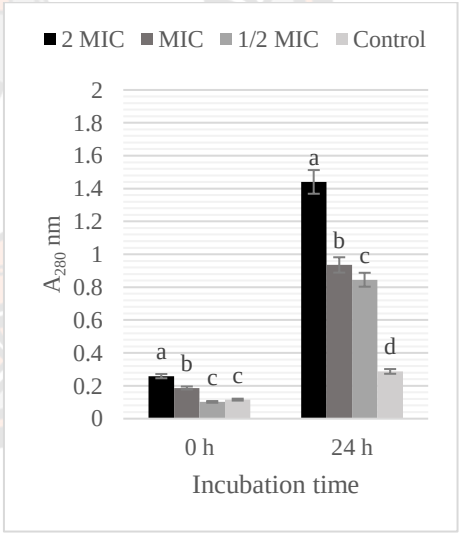


Figure 31. The effect of ME (2MIC, MIC and ½ MIC) at different concentrations on the release of 260 nm-absorbing particles (nucleic acids) and 280 nm-absorbing particles (protein) of *E. coli* and *B. cereus*

A1, A2= ME + *E. coli*, A3, A4=ME + *B. cereus*

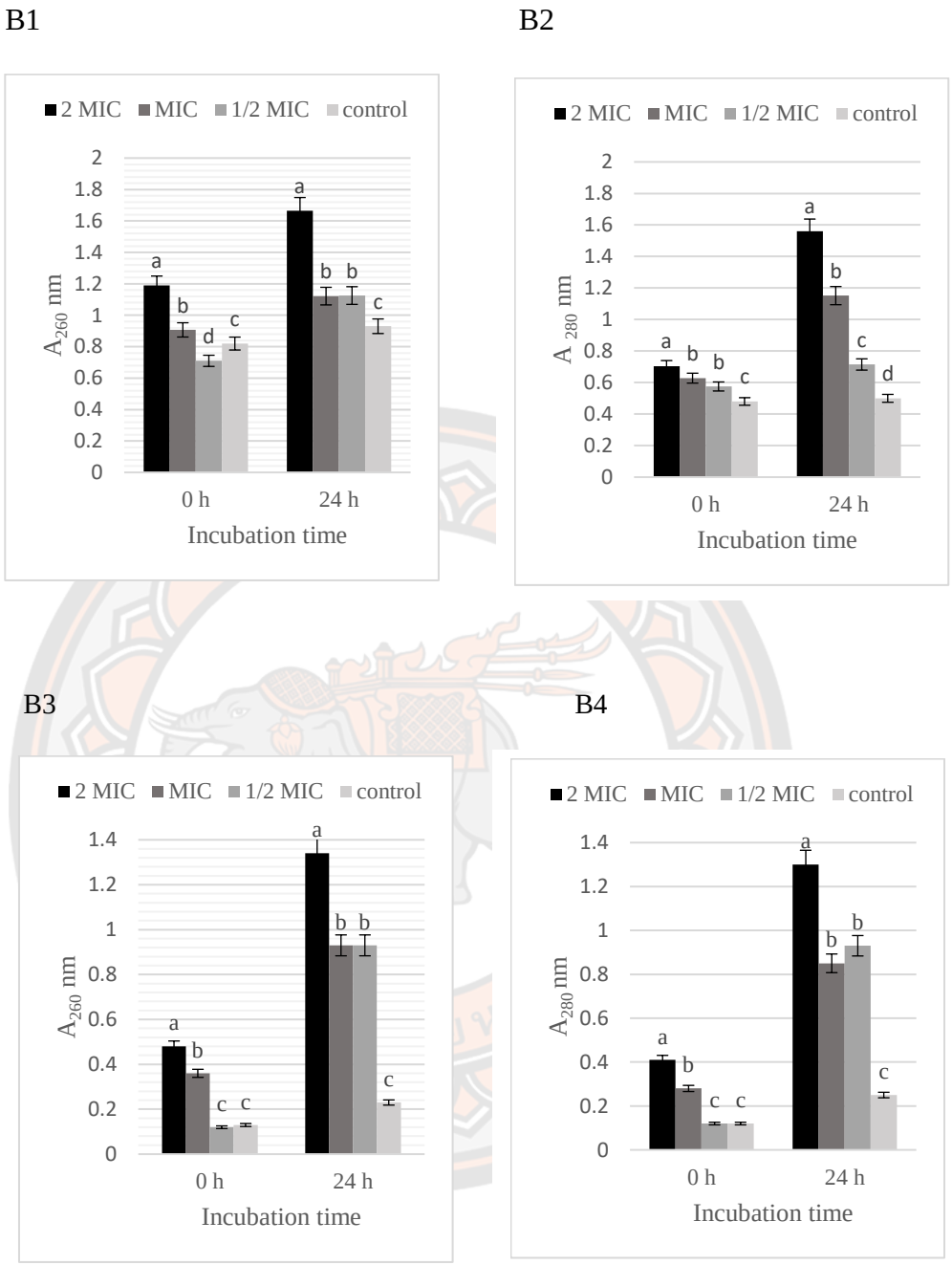


Figure 32. The effect of NE (2MIC, MIC and ½ MIC) at different concentrations on the release of 260 nm-absorbing particles (nucleic acids) and 280 nm-absorbing particles (protein) of E. coli and B. cereus

B1, B2= NE + E. coli, B3, B4= NE + B. cereus,

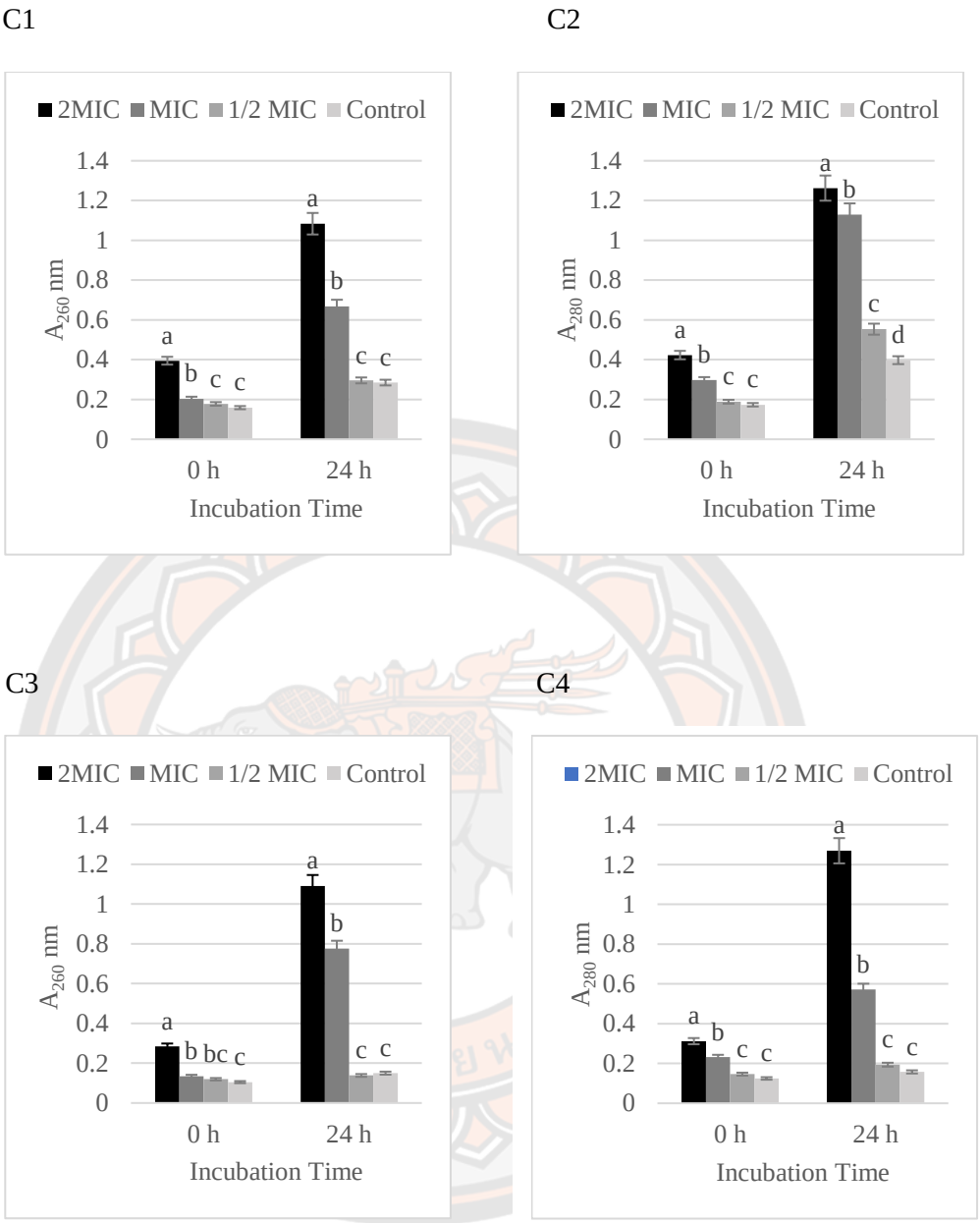


Figure 33. The effect of CE (2MIC, MIC and ½ MIC) at different concentrations on the release of 260 nm-absorbing particles (nucleic acids) and 280 nm-absorbing particles (protein) of E. coli and B. cereus

C1, C2= CE + E. coli, C3, C4= CE + B. cereus.

Based on the data of antibacterial activity, early from Part I of the study until the intracellular leakage test, it is obvious that the extracts are more effective against *B. cereus*. Therefore, *B. cereus* was selected as a representative bacteria for the following SEM test. Also, $ME_{200/20}$ and $NE_{300/20}$ showed superior antibacterial effect among the three seed meal extracts, and thus were selected for the study of the combined antibacterial activity against *E. coli* and *B. cereus*, performed by FICi method.

4.8.5. Morphological alteration of bacterial cells

The micrographs from SEM tests reveal a considerable morphological alteration in the cell membranes of *B. cereus* NBRC 13494 as shown in Figure 28. The outer linings of the bacterial cells treated with ME look disoriented and disrupted as compared to the control which shows the smooth and fuller bacterial cell body with uniform rod-shaped growth. Similar pattern of bacterial cell wall damage was seen in CE-treated *B. cereus*, where the cells looked rough, broken, pitted and perforated. In Figure 32, it can be clearly seen that the ME, CE and NE had individually caused irreversible damage to the bacterial cell wall, inflicting pores onto the surface. This possibly created a favorable environment for the bioactive compounds of the extracts to enter into the bacterial cell and damage further, and finally cause the cell to collapse. The most commonly reported mechanism of action of phenolics against bacteria is based on their accumulation at the surface of bacteria (Negi, 2012). Polyphenols are known to cause perforations in outer cell wall (Gyawali & Ibrahim, 2014) and hence cause the leakage of intracellular constituents (Char et al., 2010). This property of polyphenols is also supported by the results obtained in our study from the spectrophotometry and the SEM tests. Thus, it can be concluded that the MIC value of the polyphenols of ME, CE and NE damaged the cellular integrity of *B. cereus* and *E. coli* and caused the cytoplasmic components to leak out. This study shows that exposure to the seed meal extracts was able to reduce viable cells of both *B. cereus* and *E. coli*. Previous reports have confirmed the inhibitory effects of various plant-based phytochemicals against foodborne pathogenic bacteria (Bouarab Chibane et al., 2019). In the study by (Alves et al., 2013), 2,4- dihydroxybenzoic, protocatechuic, vanillic and *p*-coumaric acids showed antibacterial activity against *Escherichia coli*, *Pasteurella multocida* and *Neisseria gonorrhoeae*. Similarly, gallic acid, catechin, chlorogenic acid, ferulic acid, quercetin etc showed antibacterial property against *B. cereus*, *E. coli*, *S. aureus*, *S. epidermis* and *M. albican* (Fu, Lu, & Zhou, 2016) . These phenolic compounds were also isolated by HPLC in

this study as shown in Table 24. This suggests that the antibacterial activity of the tested extracts could be beause of the effect of the phenolic compounds present in the crude extracts.

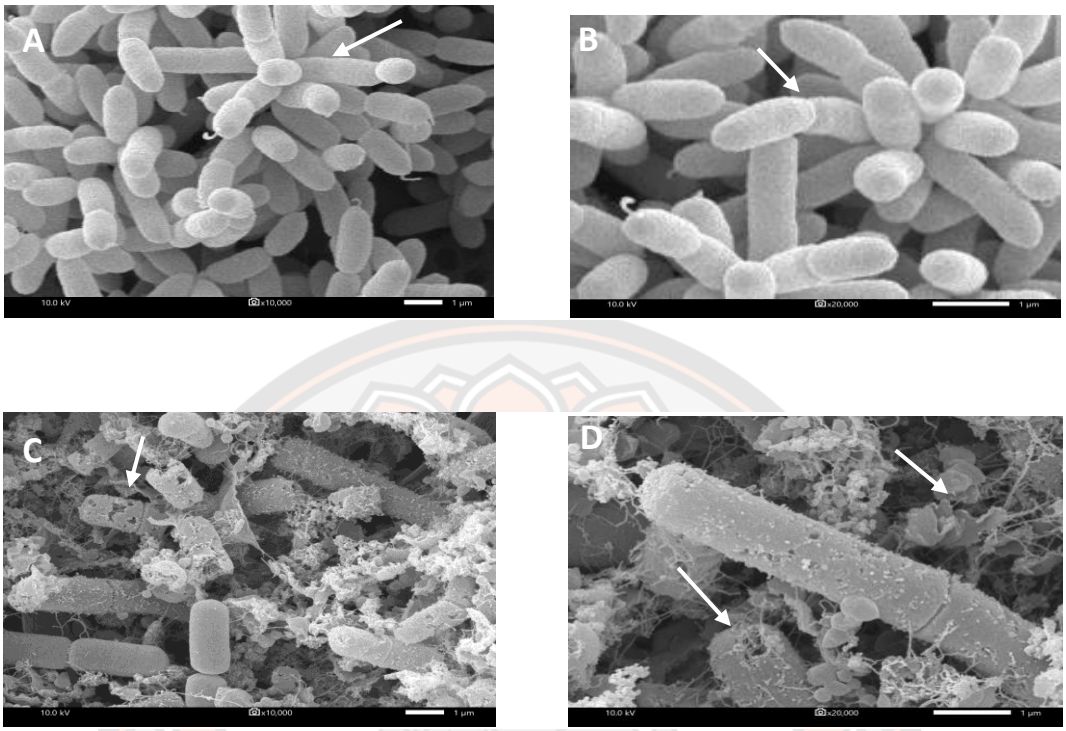


Figure 34. SEM of *B. cereus* without extract at (10000x) (A) and (20000x) (B); *B. cereus* treated with $ME_{200/20}$ at MIC (10000x) (C) and (20000x) (D)

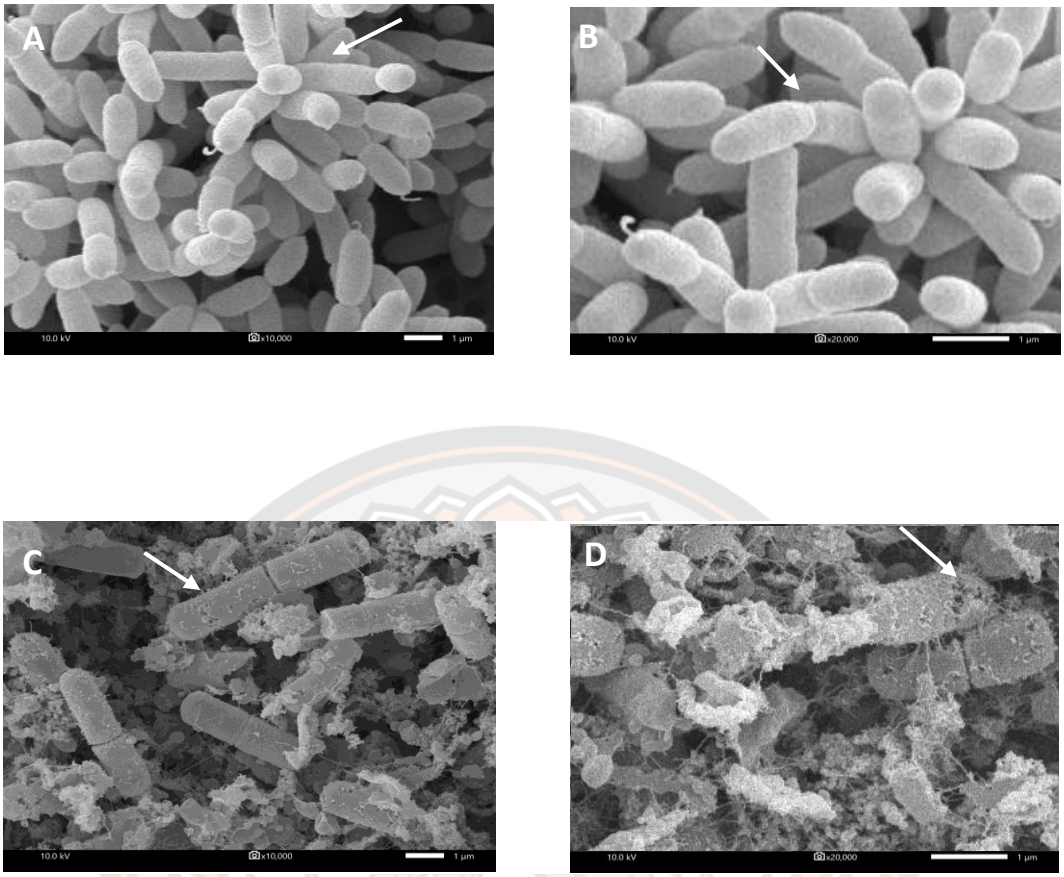


Figure 35. SEM of *B. cereus* without extract at (10000x) (A) and (20000x) (B); *B. cereus* treated with $NE_{300/20}$ at MIC (10000x) (E) and (20000x) (F)

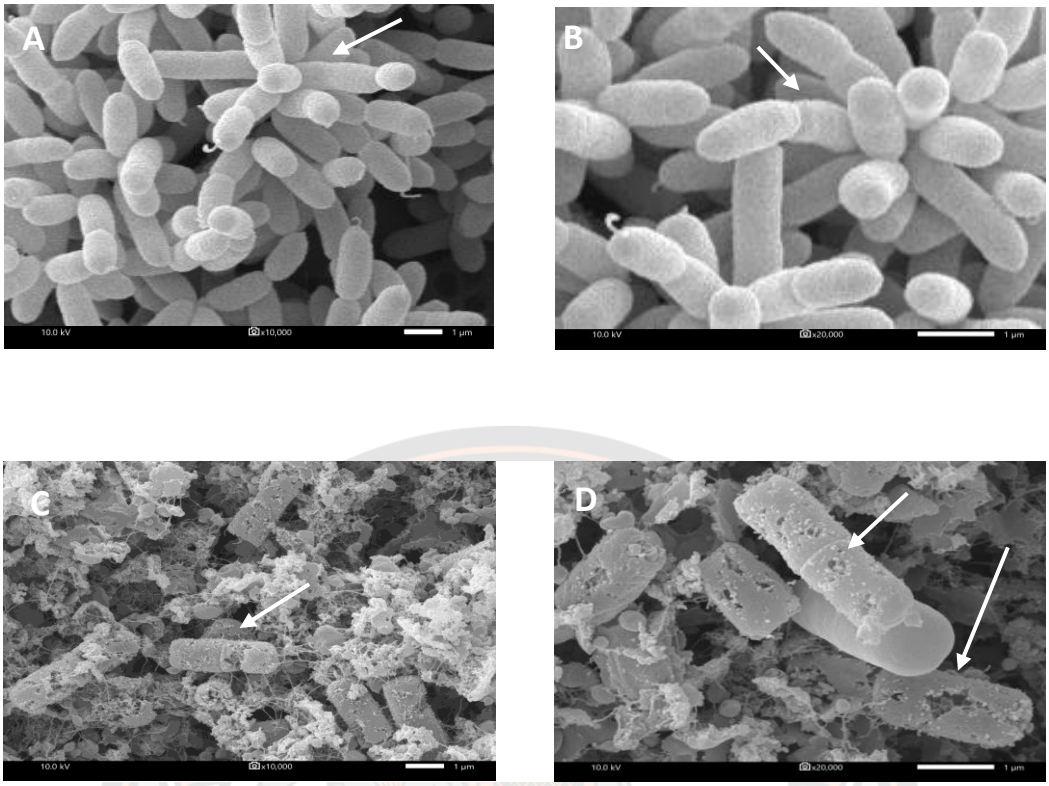


Figure 36. SEM of *B. cereus* without extract at (10000x) (A) and (20000x) (B); *B. cereus* treated with $CE_{300/20}$ at MIC (10000x) (C) and (20000x) (D).

4.8.6. Fractional Inhibitory Concentration (FIC)

The combined effect of the selected $ME_{200/20}$ and $NE_{300/20}$ were tested against each of the two *E. coli* and *B. cereus*. The combined MIC of ME (MIC_{A+B}) and NE (MIC_{B+A}) against *E. coli* was 3.12 mg/ ml and 1.5 mg/ml respectively (Table 25). Similarly, against *B. cereus*, MIC_{A+B} was 1.5 mg/ml, and MIC_{B+A} was 3.12 mg/ml. Using the formula to identify FIC, the FIC index of 1.12 was obtained for *E. coli* and 1.47 for *B. cereus*. The results showed no additive effect from the combination of extracts.

Table 26. The combined effect of ME and NE on the bacterial inhibition against E. coli and B. cereus

Bacteria	MIC_A	MIC_B	MIC_{A+B}	MIC_{B+A}	FIC Index	Interpretation
E. coli	12.5	3.12	1.5	3.12	1.12	No additive effect
B. cereus	3.12	3.12	3.12	1.5	1.47	No additive effect

As the selected extracts didn't have the additive effect, and $ME_{200/20}$ had better antibacterial activity between the two extracts, and *B. cereus* the most susceptible bacteria, $ME_{200/20}$ was selected for the bacterial inoculation test against *B. cereus*, which was the final test of the study.

All the MEs had a pleasant pale golden color that would probably not interfere much with the original color of the sausages. Regarding the acceptance of ME to be incorporated in food from the toxicological point of view, (Ajibade, Arowolo, & Olayemi, 2013) have reported no adverse effect of the methanol extract of *M. oleifera* seeds at the concentration lower than 3000 g/kg, in a toxicological study conducted in rats, and concluded that the extract was safe to be used for nutritional and medicinal purposes.

4.8.7. Bacterial inoculation test in raw chicken sausages coated with ME

The inoculation test was carried out with *B. cereus* and *E. coli* separately at different concentrations (1%, 3%, 5%) of $ME_{200/20}$, incorporated in the 1% chitosan coating, and the controls, positive (Butylated hydroxy toluene) and negative (chitosan coating without the extract). The sausages were inoculated with the bacterial concentration of log 4 per gram and the data were recorded starting from 0 day and continued on day-3, day-6, day-9, day-12 and day-15 day of refrigerated storage at 4°C. The results are shown in Figure 33 and 34. The bacterial load decreased in the ME treated sausages until the third day of refrigerated storage, in contrast to the control where the bacterial count increased straight from the day of inoculation. Even the zero-day record of the plate count in selective media showed more than 1 Log reduction in the inoculated bacterial concentration at all the ME concentrations, including the results of the positive control. The trend of bacterial inhibition was

similar with both the bacteria, however, the colony count for *B. cereus* was comparatively lesser. The 3rd day recording saw the lowest CFU at 2.9 Log CFU/g of *B. cereus*, and 3 Log CFU/g of *E. coli* in the inoculated sausages treated with ME concentrations of 3% and 5%. From 6th day onwards the bacterial count started increasing and reached Log CFU 7.3 with 1% ME, as compared to 8.0 Log CFU/g of the negative control, in the 15th day count, and the values were significantly different from each other. The ME concentration at 3% and 5% showed significantly better inhibition compared to the remaining treatments and were not significantly different to each other until day 15 for *E. coli*, and until day 12 for *B. cereus*. Yemiş and Candoğan (2017) also reported around 1.01-1.34 Log CFU/g reduction of *E. coli* 0157:H7 on day-1 of inoculation with oregano and thyme essential oil incorporated in soy-based edible coating, which is similar to the results of our study. Similar results were reported by Hong et al. (2009) where the *E. coli* 0157:H7 count decreased by l log CFU/g in the inoculated pork loin, incorporated with grapefruit seed extract in a gelatin-based coating. In agreement with this study, Bazargani-Gilani, Aliakbarlu, and Tajik (2015) reported the efficacy of chitosan-based coatings enriched with 2% *Zataria multiflora* essential oil in controlling microbial growth and extending shelf life of chicken breast previously dipped in pomegranate juice by 15 days during refrigerated storage. Similarly, chitosan-based coatings supplemented with antimicrobial agents have shown great potential for preserving seafood products and extending their shelf life. (Jasour, Ehsani, Mehryar, & Naghibi, 2015) reported the ability of the lactoperoxidase system incorporated into chitosan coatings to increase shelf life of trout fillets and maintaining their sensory attributes at high acceptability until the 16th days of storage at 4 °C. Aşik and Candoğan (2014) also reported the effectiveness of chitosan coatings incorporated with garlic oil in reducing aerobic bacterial counts and extending the refrigerated shelf life of shrimp meat by 2 days.

Thus, the challenge test in this study indicates the bactericidal effect of ME in real food system, which opens up the arena for ME to be considered as an effective novel natural antibacterial food preservative that can inhibit the growth of pathogenic bacteria responsible for causing food-borne diseases.

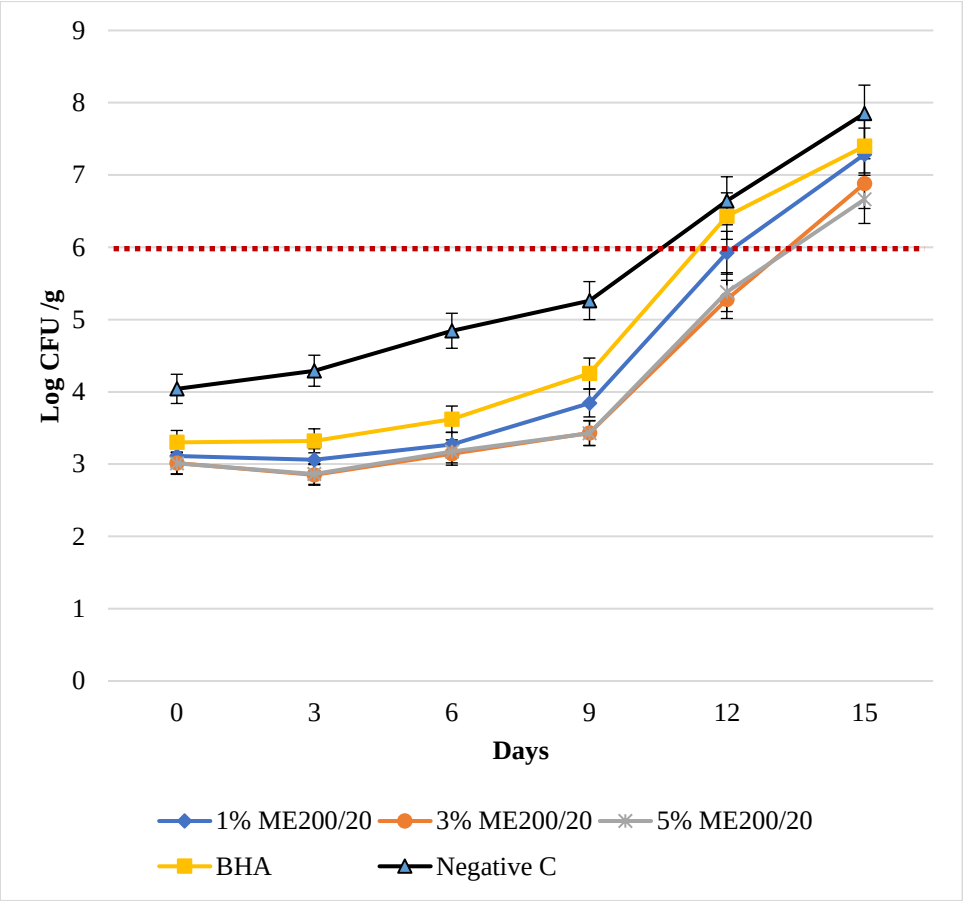


Figure 37. The viable cells of *B. cereus* treated with $ME_{200/20}$ at 1-5% concentrations in the chicken sausages stored under refrigeration at 4°C for 15 days.

Note: The red dotted line at 6 Log CFU/g refers to the level of *B. cereus* considered hazardous to health by USFDA (2001).

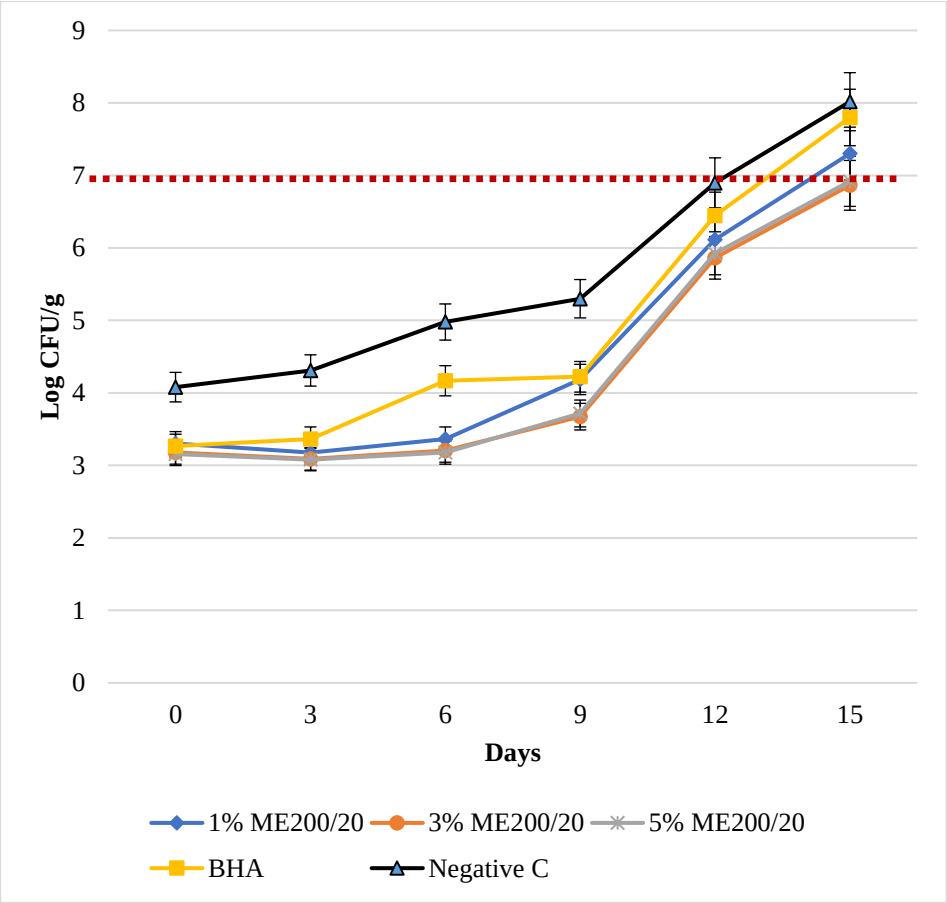


Figure 38. The viable cells of *E. coli* treated with ME$_{200/20}$ at 1-5% concentrations in the chicken sausages stored under refrigeration at 4^{o}C for 15 days.

Note: The red dotted line at 6 Log CFU/g refers to the level of enteroheamorrhagic *E. coli* considered hazardous to health by USFDA (2001).

In order to be suitable for use in the food industry, the seed meal extracts should exhibit high biological activity. Thus, an appropriate method for the preparation of crude seed meal extracts is of key importance. In our study we compared the antioxidant and antimicrobial activity of three different seed meal extracts obtained using two different methods: the conventional extraction and, ultrasound-assisted extraction (UAE). We observed that extracts obtained by ultrasound-assisted extraction (UAE) had higher antimicrobial activity than the

conventional extracts. This could be due to the higher overall extraction yield and higher contents of phenols in these extracts. 20 minutes of UAE increased the extraction of phenols and its bioactivity in all the three seed meal extracts. The content of phenols and flavonoids determines the antimicrobial and antioxidative activity of plant extracts. In an extensive research on 22 polyphenolic compounds and 26 plant extracts by Taguri, Tanaka, and Kouno (2006), the positive effect of the pure polyphenols and the plant extracts on antibacterial activity against most of the 26 tested bacteria, including *S. aureus, B. cereus*, and bacteria of Enterobacteriaceae family. Gallic acid, catechin, protocatechuic acid and caffeic acid were some of the polyphenols that showed antibacterial activity in the above-mentioned study, which were also identified in the seed meal extracts in this study. Thus, this study was successful in developing a prospective food antimicrobial and antioxidant extract by the valorization of the byproducts of oil industry.

CHAPTER V
CONCLUSION

Considering the numerous advantages of UAE, such as reduced power consumption, reduced extraction time, higher antimicrobial and antioxidant activity, this method should be considered as an effective alternative method for the extraction of phenolic compounds from Moringa seed wastes. To the best of our knowledge, this is the first study which used UAE to utilize *M. oleifera, N. sativa and C. oleifera* oil seed byproducts for the extraction of bioactive compounds that exhibited antimicrobial activity in the food matrix. The data of antioxidant as well as antibacterial property of ultrasound extracted seed meals clearly indicates the superior efficacy of UAE over the conventional maceration technique of extraction. The total extraction yields as well as the total phenolic content increased with the UAE intervention. Also, the time of extraction is drastically reduced with the use of UAE. The study shows that shorter time (10 minutes) was insufficient for optimal extraction and increasing the time to 20 minutes gave the highest yield of phenolic content. Also, the power of 200 W showed optimal yield of the bioactive compounds. Further increase in time and power of extraction to 30 minutes and 300 W respectively, did not significantly improve the yield and antioxidant performance, probably because the further increase caused the ultrasonic degradation of the bioactive compounds of the seed meal extracts (Maran & Priya, 2014).

This study shows a sustainable approach in extracting substantial antimicrobial and antioxidant bioactive compounds from the seed meals using ultrasound assisted extraction over the conventional method, with reduced time and energy consumption. Ethanol concentration of 60% v/v showed the highest bioactivity of the extracts for all the three seed meal extracts. The minimum inhibitory concentration was marked at 3.125mg/g of the $ME_{200/20}$ for both *B. cereus* NBRC 13494 and *E. coli* TISTR 527. The cell membrane damage caused by the extracts was assessed by spectrophotometry and SEM. The inoculation test suggests that 3% $ME_{200/20}$ extract in 1% chitosan solution coated over the chicken sausages, inoculated with 4 log CFU/g of the aforementioned bacteria, can reduce the bacterial

concentration by at least 1 log and also suppress the growth for 6 to 9 days under refrigeration.

Thus, it can be concluded that the method of extraction plays a significant role in the bioactive functions of the seed meal extracts. Duration of extraction and the ultrasound power and their combined effect of ultrasound assisted extraction are the important factors affecting the antioxidant as well as the antibacterial activity of the seed meal extracts. UAE condition of 20 minutes and 300 W power of both NE and CE showed optimal antioxidant and antibacterial activity amongst their UAE treatment groups. Likewise, 20 minutes of extraction time and 200 W power showed better results in both antioxidant and anti-microbial property assessment and can be concluded as the best suitable condition for the extraction of these seed meals. ME showed a good antibacterial activity in real food system, and hence proved its potential to be used as a prospective food preservative. Further study of the antioxidant as well as antibacterial property of the isolated polyphenols from the seed meals is recommended.

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APPENDICES

APPENDIX A

2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay; Protocol

Sample stock: take 1 g sample and dissolve in 25 ml MeOH

Filter through Whatman no. 4 paper

Prepare DPPH solution: 0.00788 g DPPH + 100 ml MeOH

Take 1 ml sample solution

Mix with 2 ml DPPH solution

Keep in dark for 30 minutes

Measure with spectrophotometer @517 nm

Standard curve (Ascorbic acid)

0.1 g ascorbic acid (AA) + 100 ml MeOH

dilute to 10-40 ppm AA solution

Control: DPPH solution 2 ml+ solvent (MeOH) 1 ml

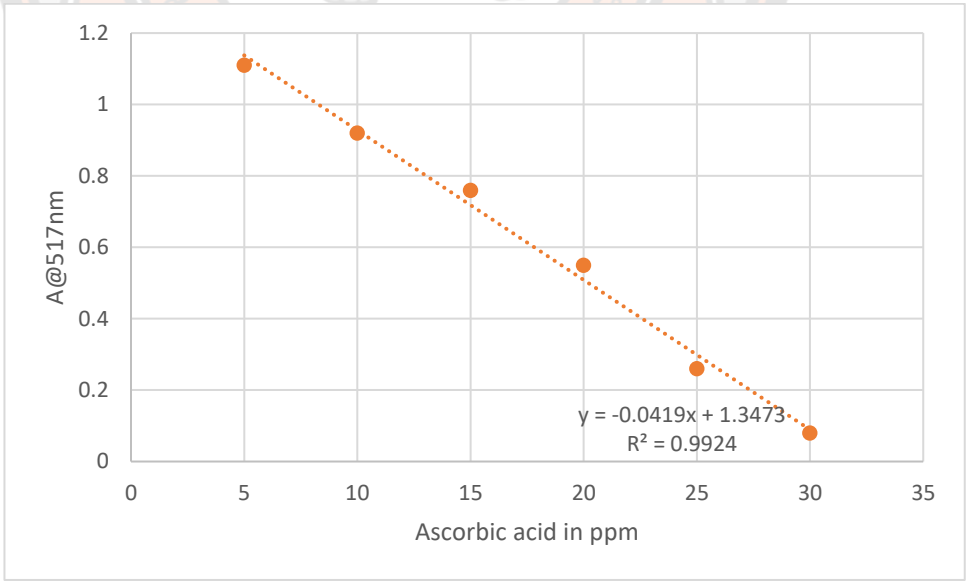


Figure 1. The ascorbic acid standard curve for the test of radical scavenging property of the extracts by DPPH assay.

APPENDIX B

Folin- Ciocalteu method for the test of Total phenolic content; Protocol

Sample preparation: 1 g extract in 25 ml methanol

Folin Ciocalteu reagent (FCR), diluted: 10 ml FCR in 90 ml DW

Na2CO3, diluted: 7.5 g in 100 ml DW

Take 2 ml of sample solution

Add 5 ml of FC reagent

Add 4 ml of Na2CO3

Wait for 10 minutes

Incubate in hot water bath at 45oC for 15 minutes

Measure with spectrophotometer at 765 nm

Control: solvent (methanol) 2 ml + FC reagent 5 ml + Na2 CO3 4 ml

Incubate at 45oC for 15 minutes

Standard curve (with Gallic acid)
Prepare 1000 ppm gallic acid (GA) solution (Dissolve 0.1 g gallic acid in 100 ml methanol)
Make the dilution to obtain 10 to 70 ppm of GA.

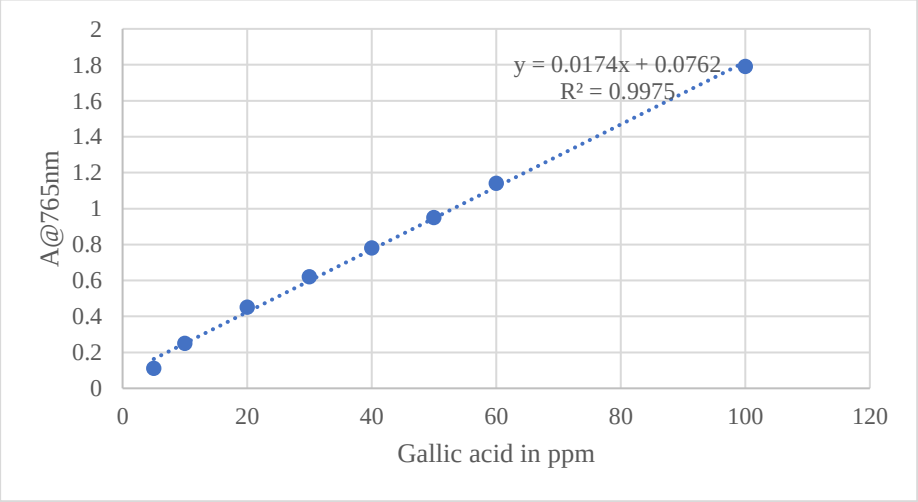


Figure 2. The gallic acid standard curve for the test of total phenolic content of the extracts

APPENDIX C

Ferric reducing antioxidant property (FRAP); Protocol

Prepare solutions:

1. Acetate buffer - 300 mM, pH 3.6. (store at 4^{o}C)
   - 3.1 g sodium acetate $3H_2O$ + 500 ml Distilled water
   - 16 ml glacial acetic acid
   - Add distilled to make the volume of 1 litre
2. Diluted HCl- 40 mM
   - 3.33 ml conc. HCl
   - Add distilled water to make volume of 1 litre
3. TPTZ – 10 mM
   - 0.031 g TPTZ in 10 ml of solution 2 (40 mM HCl) @ 50^{o}C water bath
4. Ferric chloride - 20 mM
   - 0.135g $FeCl_3.6H_2O$ in 25 ml DW.

Standard curve (Ascorbic acid)

- Prepare 100 ppm acetic acid (AA) solution
- Dilute to 10-50 ppm of AA solution

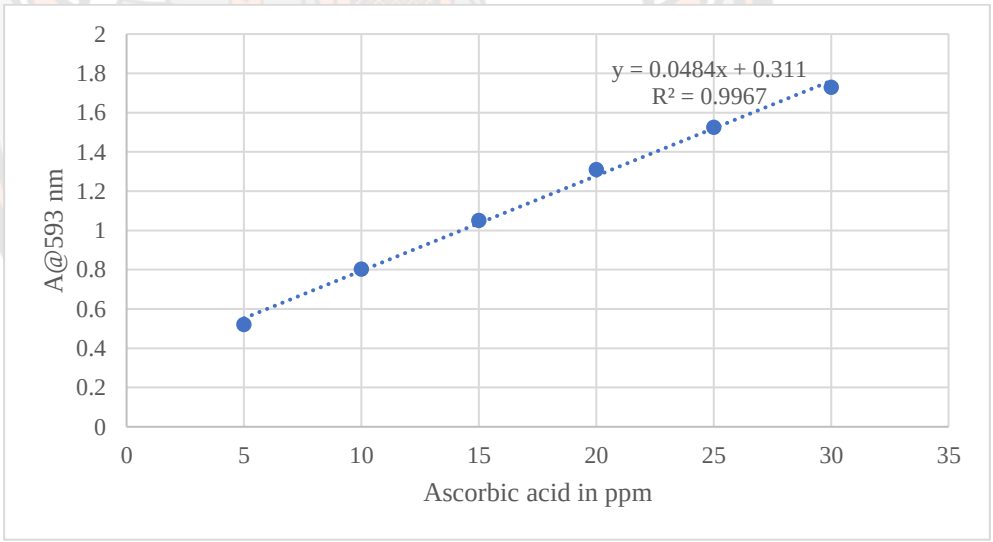
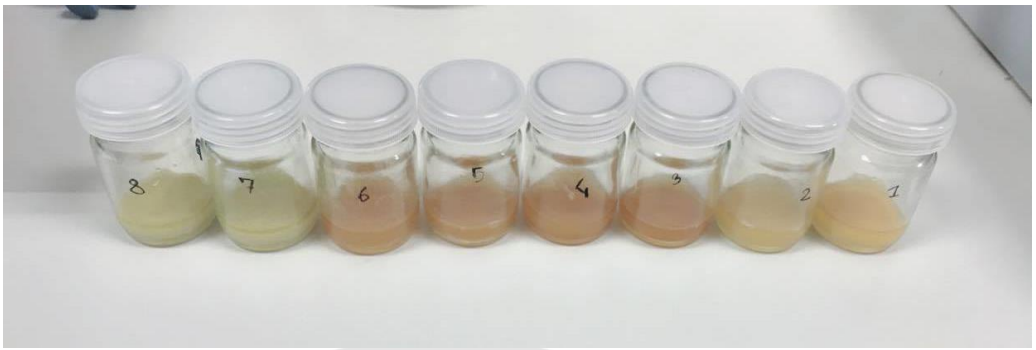


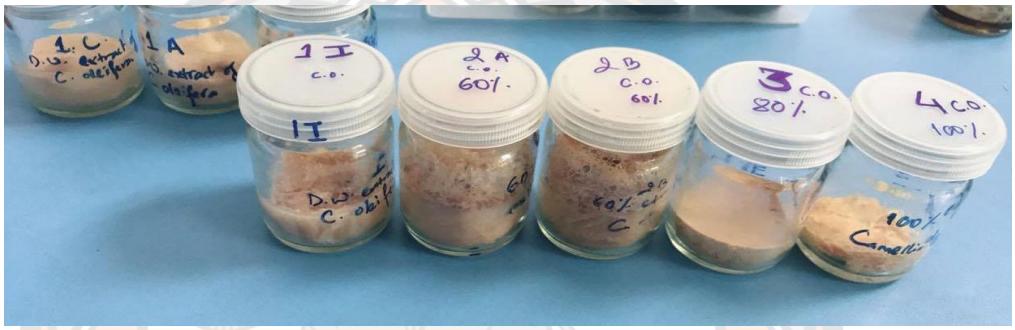
Figure 3. The ascorbic acid standard curve for the test of ferric reducing antioxidant assay.

APPENDIX D

a



b



c

Fig. 4. (a) ME extracted with 0-100% ethanol, after rotary vacuum evaporation, ready to be freeze-dried.

(b) Freeze-dried CE extracted with 0-100% ethanol.

(c) Freeze-dried NE extracted with 0-100% ethanol.

APPENDIX E
Some pictures of the zone of inhibition test of the extracts

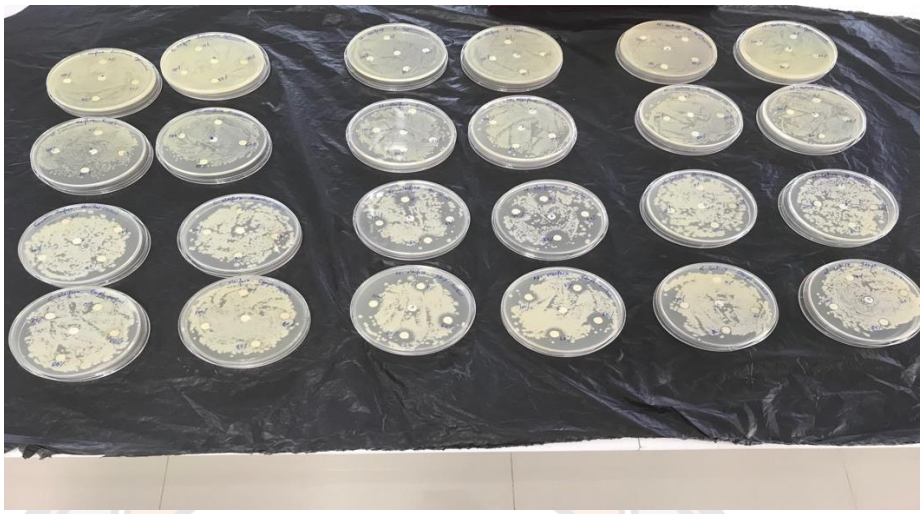


Figure 5. Clear zones exhibited by conventional seed meal extracts of C. *oleifera*, *M. oleifera* and *N. sativa* against *S. Typhimurium*, *E. coli*, *B. cereus* and *S. aureus*.

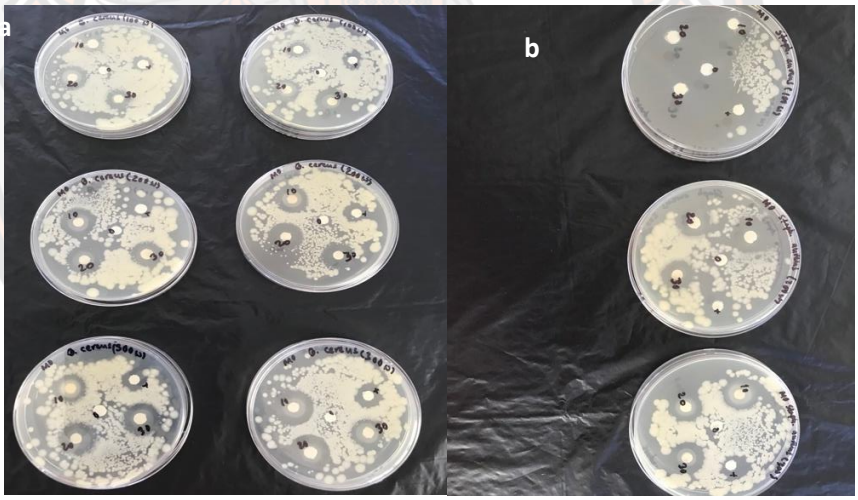


Figure 5. Clear zones of UAE ME against B. cereus (a) and S. aureus (b).

Figure 6. Clear zones exhibited by UAE NE against *E. coli*, *S. Typhimurium*, *B. cereus*, *L. innocua*

APPENDIX F

Interview with the rural community about the ethnomedicinal knowledge and contemporary uses of *Moringa oleifera* in Gorkha district of Nepal

Background

Moringa oleifera belongs to the monogenetic family Moringaceae (order Brassicales). This family has 13 species (viz; *M. arborea*, *M. rivae, M. borziana*, *M. pygmaea, M. longituba, M. stenopetala*, *M. ruspoliana, M. ovalifolia, M. drouhardii, M. hildebrandi, M. peregrine, M. concanensis* (Paliwal and Sharma, 2011). Among these, *M. oleifera* is the most used and studied.

M. oleifera (MO) is indigenous to South Asia, mainly in the sub-Himalayan regions. It is has grown to become naturalized in Nepal, India, Pakistan, Afghanistan, Bangladesh, Sri Lanka, South and Central America, West Indies, and some parts of south east Asia (Kumssa et al 2017, Durgesh et al. 2013). It is also commonly known as drum-stick tree and horse-radish tree. It is fast-growing multipurpose tree that can grow in diverse ecological conditions (Figure 1). This was mentioned in *Sushruta Samhita* that was written around 1st century AD, however it is believed to have been cultivated for 5000 years in the sub-Himalayan region (Foidl et al., 2001, Sujatha and Patel, 2017). Moringa is used for industrial, nutritive and therapeutic medicinal plant. Its seeds are used in wastewater treatment due to their coagulant properties. Its nutraceutical uses corresponds to the entire plant being rich in protein, vitamins, mineral, and carbohydrate content. Moringa leaves are rich in minerals such as iron, potassium, and calcium as well as vitamins, essential amino acids, and a number of glycosides. The seeds have high content (42%) of edible oil that also has medicinal uses.

M. oleifera is known under different local names (Table 1). Names are mostly derived from its fruits (pods) and leaves and their nutritive value and beneficial traits to human health. The aim of this study was to bring out the ethnic knowledge and medicinal and contemporary use of *M. oleifera* plant parts by the people of Gorkha district in Nepal.

Figure 1. *Moringa oleifera* trees grown in Fujel of Gorkha district.

Table 1. Local names of M. oleifera in Nepal.

Local name	**Meaning**
Sital chini	Cool sugar
Saijan	Life-giver
Sajeevan	Life-giver
Shovanjana	Auspicious tree

Relevance of the study

All parts of *Moringa oleifera* are therapeutically valuable with multiple uses in treating several diseases and ailments. Some of the most frequent usage are for body ache, pyrexia, respiratory problems (Price, 1985). It is also known to be hepatoprotective, cardioprotective, anti-diabetic, anti-inflammatory, antimicrobial properties and is also useful in certain skin conditions (Ganatra et. al., 2012), and is used since prehistoric times in Ayurveda. This study was performed with the aim to

obtain ethnobotanical knowhow on the local knowledge and uses of *M. oleifera*. Table 1 is the compilation of the literature review on the researches conducted on different plant parts of *M. oleifera.*

Table 1. Therapeutic properties of *M. oleifera* reported by various authors.

Therapeutic Properties	Parts of the plant used	References
Anti-inflammatory	Roots, seeds	Sulaiman et al., (2008)
Antioxidant	seed	Luqman et al., (2010)
Immune modulation	roots	Ndiae et al., (2001)
Diabetes mellitus	leaves	Kumari, (2010) Jaiswal et al., (2009)
Antibacterial	seeds	Singh et al., (2013)
Diarrhoea	roots	Gopalakrishnan et al., (2016)
Arthritis	roots	Gopalakrishnan et al., (2016)
Rheumatism	seeds	(Sujatha, 2017)
Snake bite	seeds	Raut et al., (2018)
Virility	seeds	Zade et. al., (2013)
Anti-caner	leaves	Jung, (2014)

Materials and Methods

Gorkha district of Nepal was selected for the study. Semi-structured interview was conducted with the local dwellers, Moringa-growing farmers and the related industries personnel and a discussion was held with selected people based on the knowledge about the use of the plant in Fujel, Gordi and Gorbung villages of Gorkha district of Nepal. The use value for different use categories of *Moringa oleifera* and its parts were estimated. The study was backed up by relevant literature review about the ethnomedicinal as well as contemporary uses and researches done on medicinal and nutraceutical aspects of *M. oleifera*.

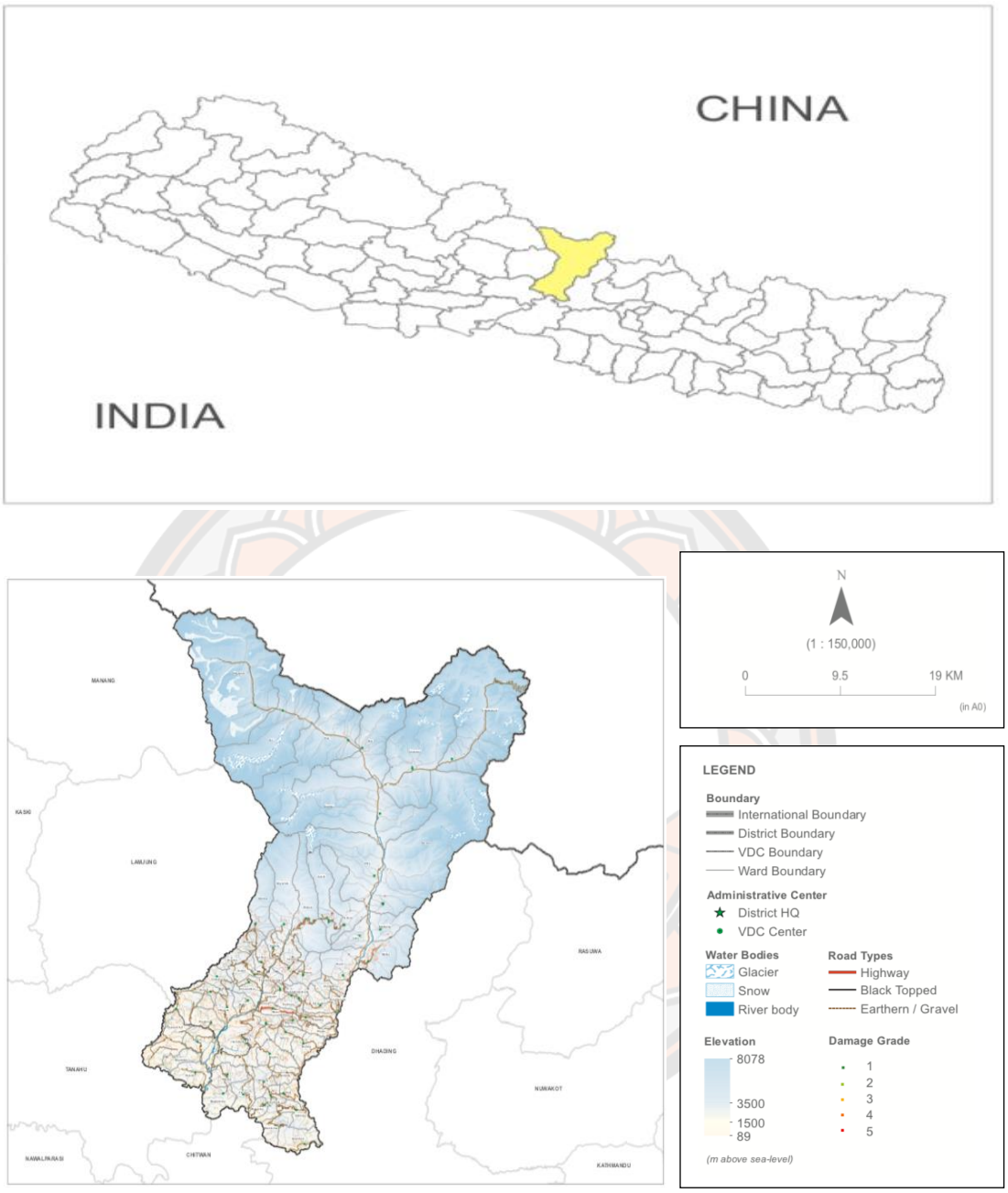


Figure 2. Map of Nepal: Administrative division with Gorkha district marked in yellow (a); Map of Gorkha district (b)

The process involved visiting several villages, communities and discussing with many individuals about their knowhow and practical use of Moringa in their day-to-day life. This study was thus undertaken to illustrate the ethno-medicinal and other possible uses of moringa in Nepal. Purposive sampling was used in the selection of participants so as to create an in- depth focus on the issues important to the study (Raj

et al., 2018). From this area where commercial cultivation of Moringa is practiced, 20 respondents including traditional medicinal practitioners and industry personnel were selected randomly for personal interview through open-ended questionnaire. The interview covered aspects like plant parts used as ethnomedicines, method of administration and dosage and therapy, and the contemporary industrial uses of Moringa.

Results

The leaves and the seeds were the plant parts most utilized for food, medicine, fodder and biofertilizers. Leaves are the most extensively used by the industry to prepare herbal supplements in tablets or powder form. Moringa leaf tea is also getting popularity in Nepalese market because of the underlying health benefits. The seed-meals when used as coagulants or water sanitizers was known to sanitize 90-99% impurities (Price, 1985). The geographical distribution pattern shows that the *Moringa oleifera* is well distributed in the mid-hills and sub-tropical areas of Nepal, and more and more people are taking interest in the cultivation of this 'miracle tree'. Seeds are used in ayurvedic medicine for arthritis, anemia and sexual disability. Pods which are known as drumsticks are used as vegetables in their succulent stage. Commercial cultivation of moringa has started in the last decade, and the industry is in its growing stage in the country. Leaves are harvested 5 times a year, in the succulent and green stages. The oil of moringa is not produced inside the country, and is sparsely imported from India for ayurvedic purposes. Furthermore, few locals reported that the dried seeds were collected by a group of middlemen who then export the seeds to India. Other uses included the use of moringa leaves as livestock fodder.

Gorkha Organic Herbal farm located in Gorbung, Gorkha was visited. The farm is spread in 38 acres. 85 herbal species of plants including Moringa is cultivated in the farm. Moringa is cultivated in 4 acres. It sells 200 kg per year of dried leaves to India, and 1500 kg fresh leaves per year to Gorkha Ayurved Company, also located in Gorkha. Gorkha Ayurved Co. produces herbal tea of Moringa and sells around 800 kg Moringa tea per year. The tea is known to have antarthritic, anticancer and

antidiabetic properties, and is high on demand in the domestic as well as international market.

Table 2. Ethnomedicinal knowledge on *M. oleifera* in order of frequency of use

Used in case of ailments	Parts of the plant used	Type of preparation	Method of administration
Inflammatory condition of skin	Leaves	Paste	Topical
Headache	Leaves	Paste, Tablets, water extracts	Topical, oral and nasal
Dermatological conditions	Seed	Oil	Topical
Diabetes	Leaves		Oral
Indigestion, gut infection	seeds	cooked	Oral
Muscle wasting	Flowers	Used with honey	Oral
Conjunctivitis	Leaves	Water extract	Eye drop
Anorexia	Flowers	cooked	oral
Scurvy	Seed	Seed flour	Oral
Hypertension	Leaves, roots	flour	Oral
Snake bite	bark	paste	Topical

Traditionally, the plant is used as antispasmodic, stimulant, expectorant and diuretic. Fresh root has the taste of horse-radish, and hence the name. Its gum is bland and mucilaginous. The seed are acrid and serves as stimulant. The bark is emmenogogue, abortifacient, antifungal and antibacterial. Its flowers are claimed to be cholagogue, stimulant, tonic and diuretic and useful to increase the flow of bile. The plant is also used as cardiac circulatory tonic and antiseptic. The pods are believed to be antipyretic, anthelmintic, antidiabetic. The root juice is employed as cardiac tonic, antiepileptic, used for nervous debility, asthma, enlarged liver, spleen. Almost every part of plant is of value for food. Seed is said to be eaten like a peanut in Malaya. The foliage is consumed as greens, in salads, in vegetable curries, as

pickles and for seasoning. Seeds yield 38-40% of non-drying oil, known as Ben oil, used in arts and for lubricating watches and other delicate machinery. The oil is clear, sweet and odorless, does not turn rancid; consequently, it is edible and useful in the manufacture of perfumes and hairdressings. *M. oleifera* wood yield blue dye. Leaves and young branches are also relished by livestock (Tripathi et al., 2016).

Conclusion

M. oleifera is the indigenous tree species of the sub-Himalayan region, and is traditionally well-known for its medicinal values. However, commercial cultivation has recently taken pace in various sub-tropical region all around the country, with the increased awareness about its health and nutritional benefits. The ethno-medicinal use of this plant gives an interesting edge to the study and opens up the scope of research in the field of alternative medicine.

ANNEX

I. Questionnaire interview for ethnomedicinal use of *Moringa oleifera* and its seeds.

I …….. (name of informant) hereby give my full consent to participate in this study and declare that to the best of my knowledge the information that I have provided are true, accurate and complete.
Date…… Signature/ Thumb impression……

Informant's details
Name……. Gender……
Age……..
Address……
……
About *Moringa oleifera*
Local name of the plant……..
Plant part used……..
Cultivated for
Medicinal uses …….

……

……

……
Other uses of seed and seed meal
……

……
……

II. Questionnaire Interview with the *Moringa oleifera* oil industry

I …….. (name of informant) hereby give my full consent to participate in this study and declare that to the best of my knowledge the information that I have provided are true, accurate and complete.
Date…… Signature/ Thumb impression……

1. Name of the industry……
2. Name of respondent……..
3. Years of operation…….
4. No. of staffs
   …….Men ……Women
5. Capacity (per day)……..
6. Type of extraction……..
7. Types of waste
   Solid. Liquid. Chemical
8. Quantity of waste (S/L/C)…… ……..
   ……
9. Waste management
   ☐ Compost. ☐ Incinerate. ☐ Landfill. ☐ Sell ☐ Others……
10. % or amount of seed meal sold……. Price per kg…….
11. Purpose of purchase of seed meal……..
12. Knowhow about the medicinal value of seed meal
    ☐ Yes ☐ No
    If yes, then what medicinal purpose do you know of?
    .....
    .....
13. Willingness to promote the use of byproduct
    ☐ Yes ☐ No.