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Influence of Extraction Methods on Thermal Properties and Antimicrobial Activity of *Moringa peregrina* Seeds Oil

أثر طرق الإستخلاص على الخصائص الحرارية والفعالية ضد الميكروبات لزيت بذور المورينقا بريقرينا

A Dissertation Submitted in Partial Fulfillment of the Requirements of the Master Degree in Chemistry

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Dedication

To who teach us the deeply meaning of love,

To who teach us how to be strong,

To whom I need him all my life,

To whom I find him when I need help,

To My continuous support in this life,

My brother Mahir Abd Elrhman

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Abstract

The aim of the present study was to investigate the effect of extraction methods on the thermal properties and the antimicrobial activity of *Moringa peregrina* seeds oil. The oil was extracted by cold pressing (CP) and maceration in n-hexane (MH). The yields of the oils were 9.12% % and 21.87% respectively. Except the peroxide value, the chemical properties of the oil extracted by the two methods remained fairly unchanged. Additionally, the results have revealed that the oil which was extracted by cold pressing has higher activity against all tested organisms (*Bacillus subtilis, Staphylococcus aureus Escherichia coli, Pseudomonas aeruginosa, Candida albicans*) in comparison to the one obtained by maceration method. Furthermore, the thermogravimetric analysis has demonstrated significant variations in the onset of degradation between the oil samples extracted with the two methods; 200°C (cold pressing) against 274°C (maceration). The maximum mass loss for the oil extracted by the two methods does not differ significantly whereas the final degradation temperature almost similar. Finally, the differential scanning calorimeter thermograms for the oil extracted by the two methods showed somewhat similar characteristics regarding the melting temperatures and melting enthalpies as well as crystallization temperatures and crystallization enthalpies.

مستخلص البحث

في هذا البحث تمت دراسة أثر طرق الإستخلاص على الخصائص الحرارية والفعالية ضد الميكروبات لزيت بذور المورينقا بريقرينا. تم إستخلاص الزيت بإستخدام طريقة العصر على البارد والغمر في مذيب الهكسان العادي. أوضحت النتائج إن نسبة الزيت هي 9.12% و 21.87% على التوالي. فيماعدا رقم البيروكسيد فان الخصائص الكيميائية لزيت المورينقا المستخلص بالطريقتين لم تتغير بصوره ملحوظة. بالإضافة لذلك فقد بينت النتائج أن زيت المورينقا المستخلص بالعصر على البارد ذو فعاليه ضد الميكروبات (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginos, Candida albicans, Bacillus subtilis,*) مقارنة بالزيت المستخلص عن طريق الغمر بالمذيب. أيضا نتائج التحليل الحراري باستخدام التحليل الوزني الحراري أظهرت تباين معتبر في نقطة بداية التفكك بين عينتي الزيت المستخلصتين بالطريقتين أعلاه وكانت 200°C في حالة العصر و 274°C في حالة الغمر. أعلى قيمة للفقد في الوزن لعينتي الزيت لم تظهر فروقات معتبره بينما كانت درجة حرارة التفكك النهائية متماثلة. أخيرا فان منحنيات مسعر المسح التفاضلي للزيت المستخلص بالطريقتين أظهرت تشابها كبيرا فيما يخص نقطة الإنصهار والمحتوى الحراري المصاحب وكذلك نقطة التجمد والمحتوى الحراري المصاحب لها.

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List of Abbreviations

CP: Cold pressing

MH: Maceration in n-hexane

TG: Thermogravimetric analysis

DTG: Derivative thermogravimetric analysis

DSC: Differential scanning calorimeter analysis

FT-IR: Fourier transform infrared spectroscopy

AV: Acid value

SV: Saponification value

FFA: Free fatty acids

PV: Peroxide value

Chapter One

Introduction and literature review

1.1 Introduction

In recent years, materials that are derived from naturally occuring-renewable resources have found special attention as promising substitutes to synthetic ones that are dependent solely on the dwindling fossil fuels [Ogunniyi, 2006; Sivasamy *et al.,* 2009; Islam *et al.,* 2014]. Vegetable oils besides their exciting and well known uses in foods have recently been given special attention as raw materials, ingredients or precursors for producing and synthesizing numerous materials such as biodiesel, polymers, cosmetics, medicines, and pharmaceutical products [Sivasamy *et al.,* 2009; Islam *et al.,* 2014; Vermaak *et al.,* 2011]. They constitute mainly from triglycerides together with other minor components that include mono- and diacylglycerols, phosphatides, sterols, tocopherols, tocotrienols, pigments, vitamins, phenolic compounds, and wax esters [Nielsen, 2010; Cert *et al.,* 2000; Aluyor *et al.,* 2009]. Yield, quality, and stability of vegetable oils depend besides other factors on the extraction methods. Hydraulic pressing, expeller pressing, direct and indirect ultrasonic extraction, as well as solvent extraction are the most commonly applied methods for extraction of oil from seeds. In addition, combination of the above mentioned methods with enzymatic and ultrasonication treatments to facilitate the extraction/recovery and preserve the quality of the oil have been reported in the literature [Tasan *et al.,* 2011; Shah *et al.,* 2005].

1.1.1 Objective

The main objective of the present study is to examine the influence of the extraction methods (cold pressing and maceration in n-hexane) on the thermal properties and the antimicrobial activity of the seeds oil of *Moringa peregrina*.

1.2 Vegetable oils: definition and sources

Fats and oils (Lipids) are group of substances that, in general, are soluble in most organic solvents such as ether, chloroform, or benzene but are sparingly soluble in water. They have lower densities than water. Generally, fats refer to those lipids that are solid at room temperature and oils generally refer to those lipids that are liquid at room temperature [Nielsen, 2010; Institute of Shortening and Edible Oils, 2006].

Fats and oils occur naturally in a wide range of sources. Hundreds of seeds and fruits bear oil, all animals produce fat, and marine sources also provide oils. The combined largest source of vegetable oils is the seeds of annual plants grown in relatively temperate climates. A second source of vegetable oil is oil-bearing trees. Most of the oil-bearing tree fruits and kernels provide the highest oil yields [O'Brien, 2004].

1.2.1 Major and minor components of vegetable oils

The main components of fats and oils which are called major component are triglycerides (triacylglycerols), accompanied by lower levels of diacylglycerols (diglycerides), monoacylglycerols (monoglycerides) and free fatty acids, and by other minor components [Gunstone, 2013].

The triglycerides are chemical compounds resulting from the combination of one unit of glycerol and three units of fatty acids. They are non-polar, water insoluble substances. When all of the fatty acids in a triglyceride are identical, it is termed a simple triglyceride. The more common forms, however, are the "mixed" triglycerides in which two or three kinds of fatty acids are present in the molecule [Institute of Shortening and Edible Oils, 2006; Johnson and Saikia, 2009; Retief, 2011]

The minor compounds consist about (2-5%) of the vegetable oils. These include mono- and diglycerides, free fatty acids, phosphatides, sterols, phenolic compounds, fat soluble vitamins, tocopherols and tocotrienols, pigments, wax

esters, and fatty alcohols [Institute of Shortening and Edible Oils, 2006; Nielsen, 2010; Cert *et al.*, 2000].

1.3 Antimicrobial activity of seeds oil

The antimicrobial activity of vegetable (or seeds) oils have been investigated by a number of authors [Ruttarattanamongkol and Petrasch, 2015; Murthy *et al.*, 2014; Adamu1 *et al.*, 2013; Al Ashaal *et al.*, 2010; Lalas *et al.*, 2012; Zaki *et al.*, 2015; Ogbolu *et al.*, 2007; Tavares *et al.*, 2015; Shah *et al.*, 2013; Singh *et al.*, 2005]. In almost all the reviewed studies, extraction of oils were carried out in a Soxhlet extraction apparatus using either normal hexane or petroleum ether. In conclusion, few articles [Zaki *et al.*, 2015; Shah *et al.*, 2013; Lalas *et al.*, 2012;] [Al Ashaal *et al.*, 2010; Ogbolu *et al.*, 2007; Singh *et al.*, 2005] were reported that the extracted oils have antibacterial and antifungal activities.

The antibacterial activities of sesame oil against *Enterobacter spp., Serratia marscense, Acinetobacter spp., E.coli, Pseudomonas aeruginosa, Klebsiella spp., Salmonella spp, Staphylococcus spp., Streptococcus spp.,* and *Micrococcus spp* was studied by Zaki *et al.* [2015]. The study showed that sesame oil has antibacterial activity on most types of the tested bacteria except *Klebsiella spp., Enterobacter spp., Salmonella spp.,* and *Micrococcus spp.*

In another study by Tavares *et al.* [2015] the antibacterial activity of corn and soy oils against *E. coli* (ATCC10536), *S. aureus* (ATCC25923) and *P. aeroginosa* (ATCC15442) was evaluated. The results did not show any antibacterial activity against the tested strains.

Similar to the previous study, the antibacterial activities of Moringa oleifera seeds oil were investigated against six bacterial strains. Among these were two gram-positive, namely *Staphylococcus aureus* (TISTR 1466) and *Bacillus subtilis* (TISTR008), and four gram-negative, namely *Salmonella typhimurium* (TISTR292), *Enterobactor aerogenes* (TISTR1540), *Pseudomonas aeroginosa* (TISTR 781) and *Escherichia coli* (780). The results showed that the oil was inactive against all bacterial strains tested [Ruttarattanamongkol and Petrasch,

2015;] Similar results were also obtained for *Lavandula bipinnata* seeds oil against several microorganisms [Murthy *et al.*, 2014]

Adamu *et al.* [2013] were studied the antimicrobial activities of the oil of Butyrospermum parkii Seed (Shea Butter). The extraction was done by soxhlet using n-hexane and petroleum ether as solvents. Activity of the crude oil from Butyrospermum parkii seed was tested on microorganism including; *Escherichia coli, Staphylococcus aureus, Pseudomonas aeroginosa* and *Trychophyton rubrum*. The results indicate that the hexane extracted Butyrospermum parkii seeds oil was inactive against *Pseudomonas aeroginosa, Escherichia coli, Trychophyton rubrum* but it shows slight activities against *Stapylococcus auerus*. On the other hand, the petroleum ether extract shows reasonable activity against *Stapylococcus auerus* but it was inactive against other pathogens.

The antimicrobial activities of olive oil against *Salmonella* and *Escherichia coli* were investigated by Shah *et al.* [2013] The findings of this work indicate that the oil was effective against these microorganisms.

In an agreement with Shah *et al.* [2013]; Lalas *et al*. [2012], and Al Ashaal *et al.* [2010] have demonstrated the biological activities of *Moringa peregrina* and Balanites aegyptiaca seeds oils against a number of microorganisms. Lalas *et al.* [2012] were reported the extraction of the seeds oil of *Moringa peregrina* and it is antibacterial activities against two gram positive bacteria: *Staphylococcus aureus* (ATCC 25923), and *S. epidermidis* (ATCC 12228), and four gram negative ones: *Pseudomonas aeroginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047) and *Klebsiella pneumonia* (ATCC 13883), as well as three human pathogenic fungi *Candida albicans* (ATCC 10231), *C. tropicalis* (ATCC 13801) and *C. glabrata* (ATCC 28838). The oil proved effective against all of the tested microorganisms.

Al Ashaal *et al.* [2010] have assessed the antimicrobial activity of the seeds oil of Balanites aegyptiaca fruits (Balanitaceae) against gram-positive bacteria (*Bacillus subtilis* (NRRL4219), *Staphylococcus aureus* (ATCC29213)), gram-

negative bacteria (*Pseudomonas aeuroginosa* (ATCC27953), *Escherichia coli* (ATCC25922)) and Fungus (*Candida albicans* (ATCC90028)). The hexane extracted oil proved effective against all microorganisms under consideration.

The effectiveness of virgin coconut oil as an antifungal agent was determined against fifty two Candida species. Among these were seventeen *C. albicans*, nine *Candida glabrata*, seven each of *Candida tropicalis* and *Candida parapsilosis*, and six each of *Candida stellatoidea* and *Candida krusei*. The authors concluded that coconut oil was active against species of all tested Candida [Ogbolu *et al.*, 2007].

The biological activity of Ocimum sanctum fixed oil was studied by Singh *et al.* [2005]. Extraction of the oil was performed in a Soxhlet extractor using petroleum ether as a solvent. The antibacterial activity of O. sanctum fixed oil was evaluated against *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Micrococcus luteus*, *Bacillus pumilus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The oil showed good antibacterial activity against *Staphylococcus aureus*, *Bacillus pumilus* and *Pseudomonas aeruginosa*. However, the oil was found to be less active against *E. coli, K. pneumoniae, S. typhi* and *S. epidermidis* and inactive against *B. subtilis* and *M. luteus*.

1.4 Thermal properties of vegetable oils

The thermal properties of vegetable oils have been reported by a number of scientific articles [Bagoria *et al.,* 2012; Nehdi *et al.,* 2010; Neto *et al., 2009;* Nyama *et al.,* 2009; Souza *et al.,* 2004; Dweck and Sampaio, 2004; Santos *et al.,* 2004; Besbes *et al.,* 2004; Santos *et al.,*xxxx Santos *et al.,* 2007]. Both thermogravimetric analysis (TGA and DTGA) and differential scanning calorimetry were used to evaluate the thermal properties of different types of vegetable oils. In general, under flowing of air the TGA results revealed three steps of decomposition by almost all oil samples which were attributed to polyunsaturated, mono-unsaturated, and saturated fatty acids. The temperature

range of the decomposition varies observably depends on the type of the oil however the lowest decomposition temperature was found to be around 200 ˚C whereas the highest temperature was around 800˚C. On the other hand, DSC was used to determine the exothermic and the endothermic transitions such as the onset of melting and crystallization, the melting and crystallization enthalpies, polymerization of fatty acids component, and decomposition of unsaturated and saturated fatty acids. It could be concluded that the results have shown significant variations in the values of these transitions between the different vegetable oils.

Soybean oil, sunflower oil and cotton seeds oil with and without antioxidants were studied for their thermal properties (in air atmosphere) using thermogravimetric and derivative thermogravimetric analysis. The TG/DTG curves indicate that the decomposition of these oils take place in three steps begin in the range of 153 ˚C -183 ˚C. These three steps relate to the decomposition of polyunsaturated fatty acids, monounsaturated fatty acids and saturated fatty acids respectively [Bagoria *et al.*, 2012].

The characteristics and chemical composition of date palm (Phoenix canariensis) seeds and seeds oil were studied by Nehdi *et al.* [2010]. The thermal characteristics of phoenix canariensis seeds oil were measured using a differential scanning calorimeter. The DSC melting curves revealed that phoenix canariensis seeds oil exhibited a melting peak (3.71 °C), a melting enthalpy (62.08 J/g) and an onset temperature (-13.64 °C).

The thermal stability of n-hexane extracted Brazil nut seed kernel oil was assessed by Neto *et al.* [2009] using thermogravimetry, derivative thermogravimetry and differential scanning calorimetry. The oil was found to be stable up to 209 ˚C. The results showed that the process of thermal decomposition of the oil in the air atmosphere occurs in three phases at temperature range from 209 °C to 408 °C, 408 °C to 492 °C and 492 °C to 602 ˚C. These phases attributed to the thermal decomposition of the polyunsaturated

fatty acids, mono-unsaturated fatty acids and saturated fatty acids respectively. The DSC curve revealed exothermic and endothermic transitions. The endothermic transition in the oil corresponds to the thermal decomposition of the saturated and unsaturated fatty acids; exothermic transition is attributed to the process of auto-oxidation.

The thermal properties of petroleum ether extracted oil from five varieties of plant seeds (bittermelon, Kalahari melon, kenaf, pumpkin, and roselle seeds) were evaluated by differential scanning calorimetry. The DSC curves showed that bittermelon seeds oil has the lower onset temperature for crystallization (-12.10 °C) and higher onset temperature for melting process (37.52 °C) comparing with the other seeds oils. The other seed oils onset temperature of crystallization and melting process were as follow: Kalahari melon seed oil, -8.64 °C and -16.48 °C; kenaf seed oil, -9.52 °C and -19.32 °C; pumpkin seed oil, -5.93 °C and -19.90 °C; and roselle seed oil, -9.52 °C and -5.65 °C. This study concluded that generally the vegetable oils which have high content of saturated fatty acids revealed DSC melting and crystallization profiles at higher temperature regions as compared to the oil samples which have high content of unsaturated fatty acids [Nyama *et al.*, 2009].

Thermo-analytic and kinetic parameters of sunflower oils, with and without antioxidants, were evaluated using thermogravimetry/derivative thermogravimetry and differential scanning calorimetry. The results showed that both sunflower oils, with and without antioxidants, had thermal stability up to about 200 °C. The thermogravimetric curves of these oils indicate that thermal decomposition in air atmosphere occurred in three steps at the range of 230 °C to 550 °C with no residue at 800 °C. These three steps were 230 °C to 380 °C, 380 °C to 480 °C and 480 °C to 550 °C which correspond to the decomposition of the polyunsaturated fatty acids, monounsaturated fatty acids and saturated fatty acids respectively. DSC curves for the two oils were similar and contain exothermic and endothermic transitions. The exothermic transitions which occur

at around 340 °C correspond to polymerization of fatty acids component of oil whereas the endothermic ones refer to thermal decomposition of saturated and unsaturated fatty acids. The heat capacities of the sunflower oils were determined from the data obtained by DSC. The authors observed that sunflower oil which contains antioxidant had higher thermal stability compared to other ones. Another good observation is that increasing the frying time produced a decrease in the onset of thermal decomposition temperature in the sunflower oils analyzed [Souza *et al.*, 2004].

The thermal decomposition of canola, sunflower, corn, olive and soybean oils and some of their thermal properties in air atmosphere was evaluated by thermogravimetry, derivative thermogravimetry and differential thermal analysis. The thermal stability of these oils decrease in order of corn oil, sunflower oil, soybean oil, canola oil and olive oil in a range of 306 °C up to 288 °C. The combustion heat of the oils estimated from differential thermal analysis peaks decreases in the following order: olive oil, canola oil, corn oil, soybean oil, and sunflower oil. The thermal decomposition of these oils having four mass loss steps. The study indicates that the lower is the thermal stability of the oil the higher is its heat of combustion except for corn oil which has different thermal composition behavior from the other oils [Dweck and Sampaio, 2004].

Santos *et al.* [2004] were evaluate the thermal stability of corn oil with and without antioxidant, sunflower oil with and without antioxidant, rice oil, soybean oil, rapeseed oil with and without antioxidant and olive oil. The thermal decomposition of all these oils in air atmosphere occurred in three steps (between 200 °C and 600 °C), with no residues after 800 °C. These three steps which occur in the temperature ranges 200 °C to 380 °C, 380 °C to 480 °C and 480 °C to 600 °C due to polyunsaturated fatty acids, monounsaturated fatty acids and saturated fatty acids decomposition respectively. Also the DSC curves of these oils have similar characteristics with endothermic and exothermic

transitions. Exothermic transitions due to polymerization of fatty acids that constitute edible oils. Endothermic transitions are due to thermal decomposition of saturated and unsaturated fatty acids. This study concluded that thermo-analytic properties of edible oil depend on the composition of oil and it is affected by the presence of antioxidant in the oil. From the TG/DTG Curves the stability of these oils comes in order of corn oil with antioxidant, corn oil, sunflower oil with antioxidant, rice oil, soybean oil, rapeseed oil with antioxidant, olive oil, rapeseed oil, sunflower oil respectively, according to the temperature at which the oil start to decompose.

Thermal properties of petroleum ether extracted seed oils of two *date palm* (Phoenix dactylifera L.) cultivars (Deglet Nour and Allig) were analyzed. The DSC curves showed that Deglet Nour seeds oil has a melting peak of -2.66 °C, a melting enthalpy of 67.50 J/g and an onset temperature of -19.01 °C whereas Allig seeds oil has -3.29 °C, 71.87 J/g and -21.72 °C, in the same order [Besbes *et al.*, 2004].

Santos *et al*. were assessed some commercial edible oils for their thermal stability by thermogravimetric and derivative thermogravimetric techniques. The thermogravimetric curves which showed the thermal behavior of the oils indicating that the thermal decomposition profile in air atmosphere of all commercial edible oils under investigation are similar. Also the curves showed that the decomposition start at 200 °C and end at 600 °C consisting of three steps. These steps probably corresponding to polyunsaturated (200 °C to 380 °C), monounsaturated (380 °C to 480 °C) and saturated (480 °C to 600 °C) fatty acids decomposition. The results indicate that these oils can be set in order to their stability as follows: corn with an antioxidant, sunflower with antioxidant, soybean with an antioxidant, rice, soybean+olive, sunflower+olive with an antioxidant, canola and lastly olive according to the initial decomposition temperature. Similar results were obtained by Santos *et al*. [2007] in the study

which included different vegetable oils with and without antioxidants to assess their thermal decomposition.

1.5 *Moringa peregrina* (Forssk.) Fiori

1.5.1 Taxonomical classification [IT IS Report. *Moringaceae*, 2015]

Kingdom: *Plantae*

Class: *Magnoliopsida*

Order: *Brassicales*

Family: *Moringaceae*

Genus: *Moringa*

1.5.2 Description, distribution and uses

Moringa peregrina is small trees up to 5 m high with branches have whip-like and leaves 3-pinnate with numerous stipitate glands on petioles, broadly lanceolate. The seeds of *Moringa peregrina* are without wings, about 13 per fruit. *Moringa peregrina* has a wide geographic range; it is distributed throughout many counties from tropical Africa to the East Indies and also growing from the Dead Sea area sporadically along the Red Sea to northern Somalia and around the Arabian Peninsula to the mouth of the Arabian (Persian) Gulf, Red sea coast Sinai Mountains. In Sudan, it is distributed in several areas including areas of red sea hills, Blue Nile, Kordofan and Darfur [EL Amine, 1990; Osman and Abohassan, 2012; AL-Kahtani and Abou-Arab, 1993].

The Moringa tree has a wide range of use in the areas of agriculture, health, and industry for developing countries. Moringa serves as a medicinal plant, animal fodder, and a food source for humans [Ghodsi *et al.*, 2014].

The main product derived from *Moringa peregrina* is seed oil, called ‘ben oil’. The use of the oil goes back to antiquity and is already referred to in old Egyptian texts and the Bible. The oil is used for cooking, in cosmetics and in medicine. In Yemen the oil is used as a lubricant for small machinery. The seeds are also used as coagulant to purify water, e.g. in Sudan. In southern Sudan and

Yemen *Moringa peregrina* is a bee plant and its leaves are used as fodder. The seeds are used in medicine in the Middle East and Sudan. The oil is used to treat abdominal pain. The tuber of the young plant is eaten in Yemen and Oman. The plant is grown as ornamental in Saudi Arabia and the Middle East. The wood is collected for fuel in the southern Sinai, but it has now become scarce [Munyanziza and Yongabi, 2015].

1.5.3 Previous studies on seeds oil of *M. peregrina*

Tsakins [1998] has studied the physico-chemical characteristics, oxidation, sterol composition, tocopherol composition and the fatty acid composition of *Moringa peregrina* seeds oil from Saudi Arabia. The n-hexane extracted oil content of *Moringa peregrina* seeds was 49.8%. . The results showed that the oil contain high levels of oleic acid (70.52%) followed by 8.9% palmitic, 3.82% stearic and 1.5%gadoleic acid. The contents of different tocopherols (α, γ and δ) in *Moringa peregrina* oil were as follow: 145, 58 and 66mg/kg respectively. Results of some physical and chemical characteristics of extracted oil were: refractive index (40 °C) 1.460, density (24 °C) 0.906, acidity (as oleic) 0.30%, iodine value 69.6, saponification number 185 and peroxide value 0.4 meq/kg. Furthermore, Rancimat test has shown that the oil is stable up to 10.2 h and this value reduced to 8.1 h after degumming. Finally, β-sitosterol was found as the most predominant component of the sterolic fraction of the oil.

The proximate and mineral composition of *Moringa peregrina* seeds and the seeds oil sterols and fatty acids composition were investigated. Protein, fat, moisture and ash contents were found to be 24.1%, 53.5%, 2.4% and 2.6% respectively. The mineral analysis indicated high potassium (630.2 mg/100 g) and phosphorus (620.5 mg/100 g) content. β sitosterol was the most predominant sterol of the oil in percentage of 28.3% followed by stigmasterol 24.54%, campesterol 23.7% and Δ-avenasterol 16.1% . The seeds oil was found to contain high level of unsaturated fatty acids (83.5%) and in particular oleic acid (74.8%). Palmitic (8.9%), stearic (3.1%) and behenic (2.6%) acids were

found to be the predominant saturated fatty acids. The present investigations revealed that *M. peregrine* seeds have a great potential as a food or feed due to its high content of oil, protein, sterols and polyunsaturated fatty acids [AL-Dabbas *et al.*, 2010].

The n-hexane extracted oil from *Moringa peregrina* Seeds were characterized for it is antibacterial activities and resistance to oxidation. The antimicrobial activity was studied against two gram positive bacteria: *Staphylococcus aureus* (ATCC 25923), and *S. epidermidis* (ATCC 12228), and four gram negative ones: *Pseudomonas aeroginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047) and *Klebsiella pneumonia* (ATCC 13883), as well as three human pathogenic fungi *Candida albicans* (ATCC 10231), *C. tropicalis* (ATCC 13801) and *C. glabrata* (ATCC 28838). The result showed that, the oil proved effective against all of the tested microorganisms. The susceptibility to oxidation, as determined by the Rancimat method, proved that *Moringa peregrina* seed oil could resist up to 10.5 hours. Additionally, the oil appeared more resistant to oxidation than extra virgin olive oil (8.9 h) [Lalas *et al.*, 2012;].

Chapter Two

Materials and methods

2.1 Sample collection and pretreatments

The *Moringa peregrina* seeds were collected from their original growing place (Wadi Alkasinger) in the Northern State of Sudan in February 2015.The seeds were cleaned to remove any extraneous materials and dehulled using a hammer. The kernels were crushed and used for cold press extraction and extraction via maceration in n-hexane.

2.2 Chemicals

n-hexane (density = 0.66 g/ml at 20 º C, b.p = 65- 70 º C, minimum assay = 95%), was purchased from SDFCL. Potassium hydroxide pellets (minimum assay = 85%), was purchased from CDH laboratory reagent. Hydrochloric acid (density = 1.18 g/ml at 20 º C, assay = 35-38%), was purchased from Lobe chemie. Phenolphthalein indicator. Sodium hydroxide (assay = 98%), was purchased from Nice Laboratory. Acetic acid glacial (boiling point = 118 at 20 º C, minimum assay = 99.7%), was purchased from Duksan Reagents. Chloroform (density = 1.474-1.480 g/ml at 20 º C, assay = 99.5%), was purchased from Alpha chemika. Potassium iodide (assay = 99%), was purchased from SCP. Sodium thiosulphate (minimum assay = 99%), was purchased from Alpha chemika. Starch.

2.3 Oil extraction

The crushed kernel seeds of *Moringa peregrina* were divided into two portions and the oil was produced by cold press (CP) extraction and via extraction by maceration in n-hexane (MH).

2.3.1 Cold pressing

500g of the ground kernel seeds were exposed to water-vapor (boiled water) and pressed using screw pressing machine. The extracted oil was collected and stored at 4˚C for further analyses.

2.3.2 Maceration in n-Hexane

300 g of the ground kernel seeds was percolated in one liter n-hexane and kept for four days with occasional shaking. The percolates were filtered and concentrated at 60℃ under reduced pressure in a rotary evaporator.

2.4 Determination of physicochemical properties of the oil

2.4.1 Determination of saponification value

2.5 g of the oil was weighed into a 250 ml conical flask. 25 ml of alcoholic potassium hydoxide solution (0.7N) was pipetted into the flask. The flask was connected with air condenser and boiled until fat was completely saponified-as indicated by absence of any oily matter and appearance of clear solution. The flask contents was cooled and titrated with *0.45N* hydrochloric acid using phenolphthalein indicator. Blank determination was conducted along with that on sample. The titration was repeated three times and the saponification value (SV) was calculated using the following equation:

$$SV = 56.1* (B - S) *N/ \text{Wt of sample in gram} \quad (2.1)$$

Where B = volume in mL of *0.45N* HCl required by the blank, *S* = volume in mL of *0.45N* HCl required by the sample and N is normality of HCl solution.

2.4.2 Determination of acid value and free fatty acids

2.35 g of oil were weighed into a 250 ml conical flask and 16.7 ml of ethanol alcohol (previously neutralized by adding 2 mL phenolphthalein solution and enough 0.1N NaOH to produce faint permanent pink) was added to the flask. The conical flask contents were titrated with 0.011 NaOH with vigorous shaking until permanent faint pink color appears and persists ≥ 1 min. The experiment was repeated three times and the acid value and free fatty acid was calculated using the following equations:

$$\text{Acid value (AV)} = (V * N * 40)/\text{Wt of sample} \quad (2.2)$$

$$\%\text{Free Fatty Acid (as oleic)} = AV *0.503 \quad (2.3)$$

Where: V is volume in ml of NaOH required by sample, N is normality of NaOH.

2.4.3 Determination of peroxide value

2.5 g of the sample were weighed into a 250 ml conical flask and 15 ml of glacial acetic acid and chloroform mixture (3:2) were added and stirred. 0.3 ml of saturated potassium iodide solution was added to the flask and shacked for 1 min. 15 ml of distilled water and 0.5 ml of 1 % starch solution were added to the flask contents and titrated against 0.001N sodium thiosulphate solution with vigorous shaking until blue colour just disappeared. Blank determination was conducted. The experiment was repeated three times and the peroxide value was calculated using the following equation:

Peroxide value (meq /kg) = V*N*1000/Wt of sample (2.4)

Where V is mL of sodium thiosulphate required by the sample, N is normality of sodium thiosulphate solution [Association of official analytical chemists, 1980].

2.5 Biological activity of oil

2.5.1 Preparation of bacterial suspensions

One ml aliquots of a 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37º C for 24 hours. The bacterial growth was harvested and washed off with 100 ml sterile normal saline, to produce a suspension containing about 10^8- 10^9 C.F.U/ ml. The suspension was stored in the refrigerator at 4 °C till used.

The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique. Serial dilutions of the stock suspension were made in sterile normal saline solution and 0.02 ml volumes of the appropriate dilution were transferred by micro pipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drops to dry and then incubated at 37 °C for

24 hours. After incubation, the number of developed colonies in each drop was counted. The average number of colonies per drop (0.02 ml) was multiplied by 50 and by the dilution factor to give the viable count of the stock suspension, expressed as the number of colony forming units per ml suspension.

Each time a fresh stock suspension was prepared. All the above experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

2.5.2 Preparation of fungal suspension

The fungal cultures were maintained on Sabouraud dextrose agar, incubated at 25 °C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspension in 100ml of sterile normal saline, and the suspension were stored in the refrigerator until used.

2.5.3 Testing of antibacterial susceptibility

The paper disc diffusion method was used to screen the antibacterial activity of plant extracts and performed by using Mueller Hinton agar (MHA). The experiment was carried out according to the National Committee for Clinical Laboratory Standards Guidelines (NCCLS, 1999). Bacterial suspension was diluted with sterile physiological solution to 10^8cfu/ ml (turbidity = McFarland standard 0.5). One hundred microliters of bacterial suspension were swabbed uniformly on surface of MHA and was allowed to dry for 5 minutes. Sterilized filter paper discs (Whatman No.1, 6 mm in diameter) were placed on the surface of the MHA and soaked with 20 µl of the oil sample. The inoculated plates were incubated at 37 °C for 24 h in the inverted position. The diameters (mm) of the inhibition zones were measured.

2.6 Thermal stability

2.6.1 Thermogravimetric analysis

10 mg of the oil sample were subjected to a temperature range of 30 °C to 700 °C. Analysis was done in the nitrogen atmosphere with flux of 50 ml min^{-1} and a

heating rate of 10 °C min^{-1}. The thermal stability was determined by the analysis of TG/DTG curves that registered mass loss of oil during the heating period.

2.6.2 Differential scanning calorimeter analysis

The DSC curves were obtained in a differential scanning calorimeter, in nitrogen (50 ml min^{-1}) with heating rates of 10 °C min^{-1} and a sample mass of 10 mg in a temperature range of -80 °C to 30 °C.

Samples of oil (8-10 mg) were weighed in aluminum pans; covers were sealed into place and analyzed with DSC instrument. Oil samples were equilibrated at 30 °C for 5 min, cooled to -80 °C at a rate of 5 °C min^{-1}, equilibrated at -80 °C for 3 min and then reheated to 30 °C at a rate of 5 °C min^{-1}. Dry nitrogen was purged in the DSC cell at 50 cm^3 min^{-1}. Thermograms were analyzed and the enthalpy, onset, and offset temperatures of the transitions, and peak temperature were obtained. Triplicate analyses were carried out per sample.

2.7 Fourier transform infrared spectroscopy:

FT-IR spectrum of the oil was obtained using an IR 300 model spectrometer (Thermo Nicolet). A drop of oil was placed between a pair of salt plates. The pair of the plates were inserted into a holder that fits into Infrared spectrophotometer. The scanning was done in the range between 4000 and 500 cm^{-1}. The number of scans was adjusted to 10 scans with resolution of 4 cm^{-1}.

Chapter Three

Results and discussion

The oil content of the *Moringa peregrina* seeds which was extracted by cold pressing (CP) and maceration (MH) methods are shown in Table 4.1.

Table 3.1: Oil content of *Moringa peregrina* seeds

Method of extraction	CP	MH
Oil content	9.12%	21.87%

Obviously, the results revealed that both the cold press and maceration methods are less effective in the extraction of oil seeds as compared to Soxhlet method (using n-hexane) reported by previous studies [Tsaknis, 1998; Salaheldeena *et al.*, 2014]. Tsaknis [1998] has shown that the oil content of *Moringa peregrina* seeds originated from Saudi Arabia was 49.8%. In another study, Salaheldeen *et al.* [2014] have reported that the oil content of *Moringa peregrina* collected from Wadi Alkasinger in the Northern State of Sudan was 38.33%. However, the previous studies on other oil seeds extracted by cold press, maceration, and Soxhlet method with n-hexane have displayed similar trend and in an agreement with above results concerning the efficiency of the extraction method and the oil content [Tsaknis *et al.*, 1998; Lalas *et al.*, 2003].

3.1 Chemical properties of seeds oil of *Moringa peregrina*

The chemical characteristics of the extracted oils are shown in Table 3.2. As could be noticed from the table no significant differences in the reported values are present between the two methods although the macerated samples have shown undetectable level of peroxide value. In the case of the macerated sample, the absence of heat treatment could be the major reason that the oxidation did not take place. On the other hand, cold press of the sample involves pretreatment using water vapour which could be the initiator of the oxidation and probably the major factor in the ease of oxidation.

Table 3.2: Chemical characteristics of *Moringa peregrina* seeds oil

Oil sample	AV (mg KOH/g oil)	SV(mg KOH/g oil)	FFA (%)	PV(meq O_2/Kg oil)
CP	0.481±0.02	206.40 ±3.96	0.242±0.01	0.394±0.004
MH	0.441±0.01	203.24±6.24	0.222±0.01	-

Chemical characteristics of the oil extracted with cold press method was as follows: acid value 0.481, saponification value 206.4, free fatty acid 0.242 and peroxide value 0.394 whereas for oil extracted with maceration method was 0.441, 203.24, 0.222 and not detectable respectively. These results fall within the range of previously reported values [Tsaknis, 1998] and [Ahmed, 2016].

3.2 FT-IR analyses

The FT-IR analyses were performed to examine the variations in the structural characteristics of the oil samples based on different extraction methods.

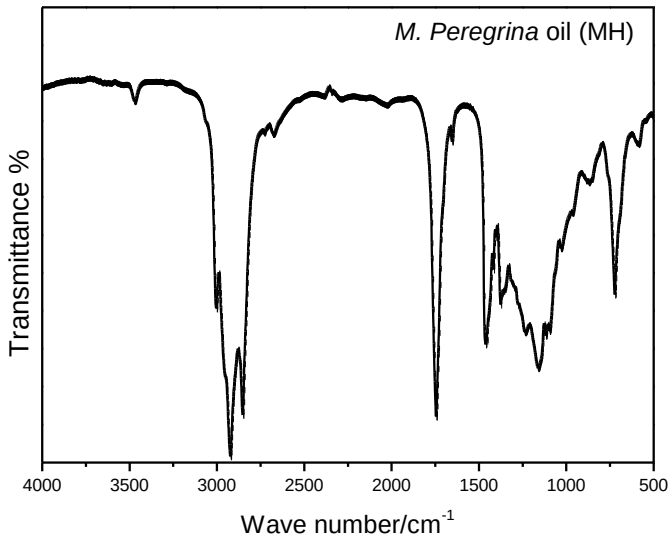

Figure3.1: IR spectrum of *Moringa peregrina* seeds oil which extracted by maceration method

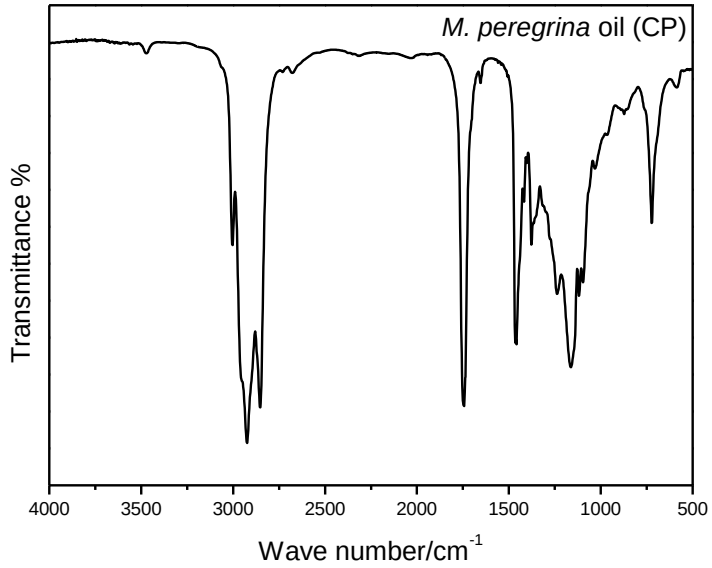

Figure3.2: IR spectrum of *Moringa peregrina* seeds oil which extracted by cold press method

The characteristics peaks of the oil samples and their corresponding wave number are shown in table (3.3).

Table 3.3: The characteristic peaks of the oil samples and their corresponding wave number.

Wave number (cm^{-1})	Functional group
3003 cm^{-1}	-CH stretching vibration of double bond
2924 cm^{-1}, 2853 cm^{-1}	-CH (sp^3 hybridized) stretching vibrations of hydrocarbons
1744 cm^{-1}(for cp) and 1745 cm^{-1} (for MH)	Stretching vibration of carbonyl group C=O
1456 cm^{-1}(for MH), 1458 cm^{-1} (for CP) and 1377 cm^{-1}	-CH bending vibration of aliphatic groups
1163 cm^{-1}	C-O stretching vibration of ester bond

In conclusion, the FT-IR analyses showed that there are no significant differences in the absorption peaks between the oils extracted by cold press and maceration methods.

3.3 Biological activity of oil

The antimicrobial activity of *Moringa peregrina* oil seeds extracted by cold press (CP) and maceration (MH) methods was assessed against two gram positive (+ve) bacterial (Bacillus subtilis, Staphylococcus aureus), two gram negative (–ve) bacteria (Escherichia coli, Pseudomonas aeruginosa) and one fungal microorganism (Candida albicans).The oil sample which was extracted by cold press method showed higher activity against tested organisms compared to oil sample obtained by maceration method. The magnitude of cold press extracted oil inhibition zones against the tested organisms was as follows: Bacillus subtilis (18 mm), Staphylococcus aureus (12 mm), Escherichia coli (10 mm), Pseudomonas aeruginosa (12 mm) and Candida albicans (18 mm), whereas for oil which extracted by maceration method as follows: Bacillus subtilis (10 mm), Staphylococcus aureus (-), Escherichia coli (10 mm), Pseudomonas aeruginosa (11 mm) and Candida albicans (12 mm).

Escherichia coli proved the least resistant (10mm) while Candida albicans was the most resistant for both oil samples. Lalas *et al*. [2012] also reported *Staphylococcus aureus, S. epidermidis, Pseudomonas aeruginosa, Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Candida albicans, C. tropicalis* and *C. glabrata* be sensitive to *Moringa peregrina* seeds oil.

Table 3.4: The antimicrobial activity of *Moringa peregrina* oil seeds

microorganism		inhibition zones for CP /mm	inhibition zones for H /mm
Gram positive bacteria	*Bacillus subtilis* NCTC 8236	18	10
	Staphylococcus aureus ATCC 25923	12	-
Gram negative bacteria	*Escherichia coli* ATCC 25922	10	10
	Pseudomonas aeruginosa ATCC 27853	12	11
Fungal microorganism	*Candida albicans* ATCC 7596	18	12

3.4 Thermal stability of *Moringa peregrina* oil

The thermal stabilities of the extracted oils were investigated under flow of nitrogen and the mass loss was plotted against temperature. Figures 3.3 and 3.4 show the thermograms (TG & DTG) of *Moringa peregrina* oil which extracted by cold press method. Figures 3 and 4 show that the highest temperature below which the cold press extracted oil is stable and no significant degradation takes place is 200 °C. Furthermore, the TG&DTG curves reveal that 99.892% of the mass loss of the oil occurs in a single step that starts above 200 °C (T_{onset} temperature) and up to 470 °C (T_{offset} temperature). In addition, Figure 4 displayed that the temperature at which the maximum mass loss occurred for cold press extracted oil was 409.53 °C. On the other hand, Figures 5 and 6 demonstrate that 99.22% of the mass loss of the oil extracted by maceration

occurs in a single step between 273.95 °C (T_{onset} temperature) and 478.10 °C (T_{offset} temperature). The temperature at which the maximum mass loss occurred was 418.73 °C (Figure 3.6). It could be noticed from above that the onset of degradation of the oil extracted with the two methods (cold press and maceration, respectively) varies significantly, 200 °C against 273.95 °C. This might be due to the extraction conditions that involve the exposure of the sample to water vapour in case of cold press prior to pressing. This process probably raised the level of moisture in the oil and hence accelerates the oxidation process or rancidity.

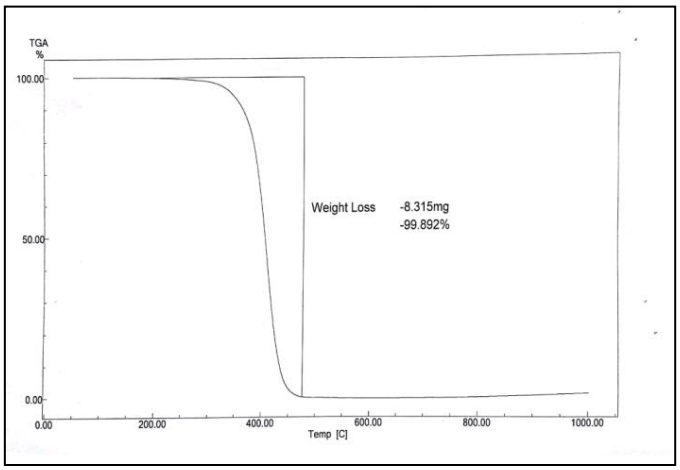

Figure 3.3: TG curve of *Moringa peregrina* seeds oil which extracted by cold press method

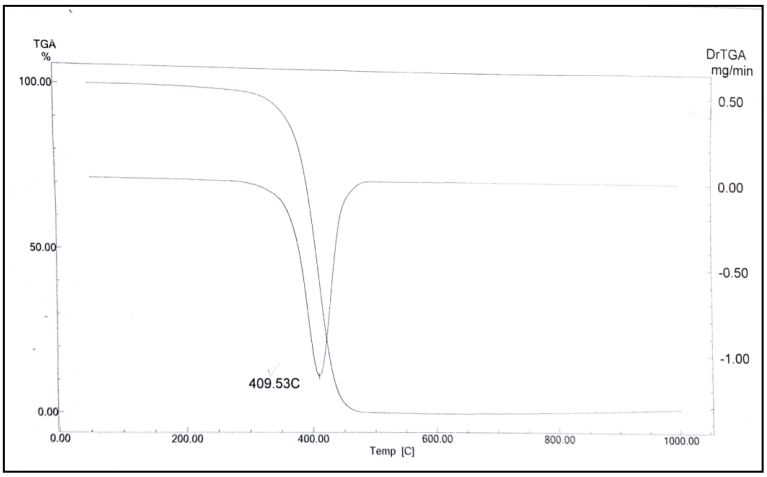

Figure 3.4: DTG curve of oil extracted with cold press method

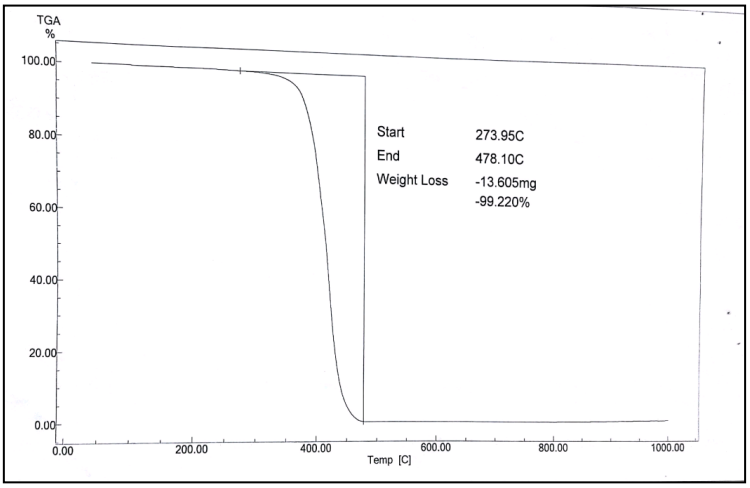

Figure 3.5: Thermogram of *Moringa peregrina* seeds oil which extracted by maceration method

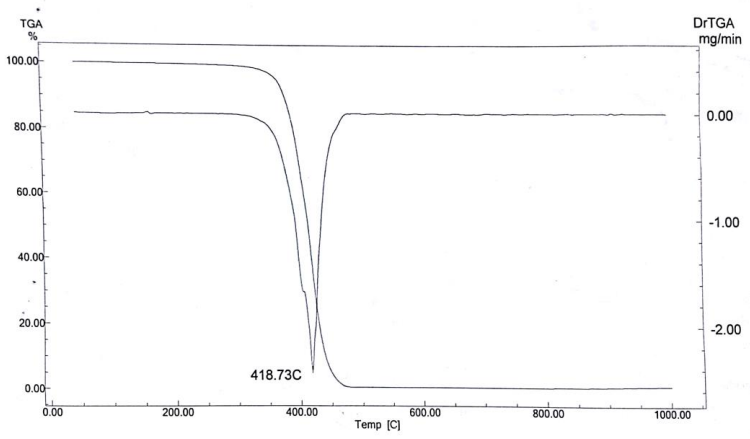

Figure 3.6: DTG curve of oil extracted with maceration method

According to the previous studies [Bagoria *et al.*, 2012; Jayadas and Nair, 2006; Neto *et al., 2009*], the decomposition of vegetable oils under nitrogen atmosphere (Thermal stability) takes place in one step whereas in air atmosphere (oxidative stability) there are three steps. The single step decomposition of the vegetable oils in nitrogen atmosphere involves the breaking down of the oxygenated hydrocarbon present in it into volatile lower molecular hydrocarbons, carbon dioxide and carbon monoxide. One the other hand, the three distinct steps in the degradation of vegetable oils under oxygen atmosphere

attributed to the thermal decomposition of the polyunsaturated fatty acids, mono-unsaturated fatty acids and saturated fatty acids respectively.

The DSC thermogram for *Moringa peregrina* seeds oil which extracted by cold press method is shown in figure 3.5. As can be seen the heating curve of the cold pressed oil sample has shown two distinguished endothermic peaks at -6.92˚C (-61.71J/g) and 10.31˚C (-6.22J/g). Additionally, the crystallization curve has displayed three exothermic peaks at -49.07˚C (2.47J/g), -8.29˚C (13.57J/g) and 8.42˚C (13.57J/g). The heating profile of maceration extracted oil, on the other hand, has also exhibited two peaks having the following characteristics (Figure 3.6): at -7.26˚C (-58.20J/g) and 10.52˚C (-4.91J/g). Furthermore, the crystallization thermogram of the oil extracted by maceration have revealed two exothermic peaks at -56.61˚C (11.28J/g) and -43.82˚C (7.43J/g). These findings reveal that the lowest onsets of crystallization of cold pressed and macerated samples were -49.07˚C and -56.61˚C whereas the highest onsets of melting were 10.31˚C and 10.52˚C respectively.

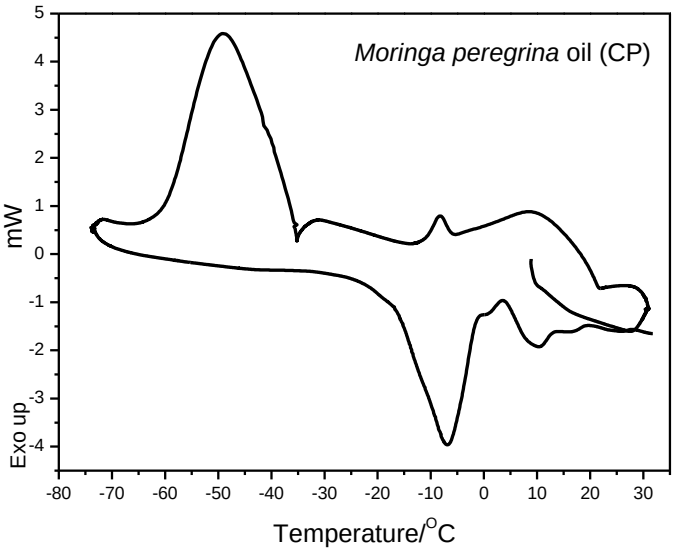

Figure 3.7: DSC curve of *Moringa peregrina* oil extracted by cold press method

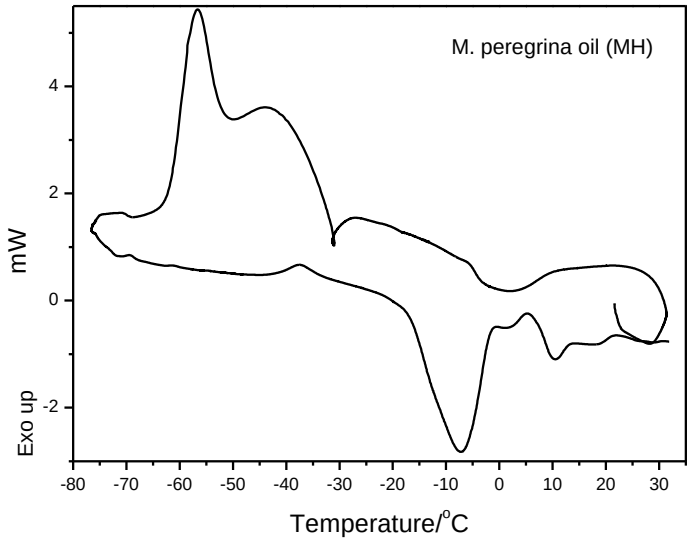

Figure 3.8: DSC curve of *Moringa peregrina* oil extracted by maceration method

Conclusion

In this study, the results have shown that the method of extraction has significant influences on the properties and stability of the extracted *Moringa peregrina* seeds oil. Regarding the antimicrobial activity of the oil, cold pressing proves to be better than maceration in n-hexane for all tested micro-organisms. Additionally, the extraction method has observable effects on the onset of degradation of the oil and does not affect the maximum as well as the final degradation temperatures. DSC results did not reveal any significant changes in the melting and crystallization behavior of the oil.

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Appendix

Figure A.1: *Moringa peregrina* seeds

Figure A.2: Kernels of *Moringa peregrina* seeds

Figure A.3: *Moringa peregrina* oil extracted by maceration

Figure A.4: *Moringa peregrina* oil extracted by cold press

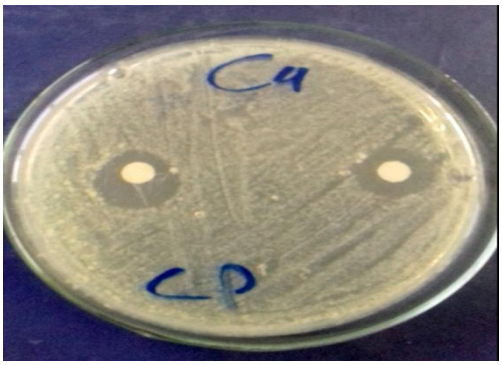

Figure A.5: Inhibition zone of *Moringa peregrina* seeds oil which extracted by cold press against Candida albicans

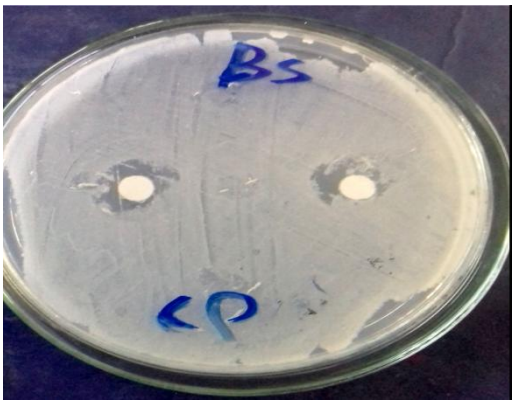

Figure A.6: Inhibition zone of *Moringa peregrina* seeds oil which extracted by cold press against Bacillus subtilis

Figure A.7: Inhibition zone of *Moringa peregrina* seeds oil which extracted by cold press against Staphylococcus aureus

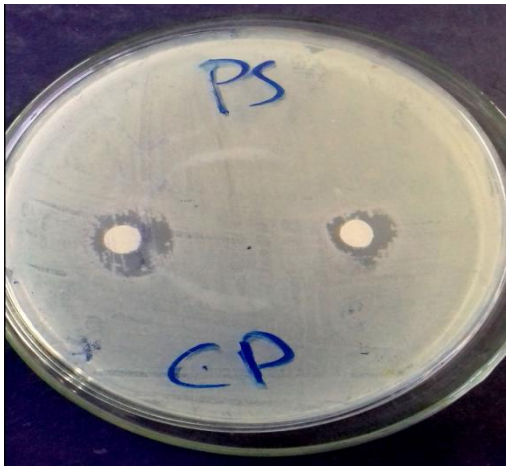

Figure A.8: Inhibition zone of *Moringa peregrina* seeds oil which extracted by cold press against Pseudomonas aeruginosa

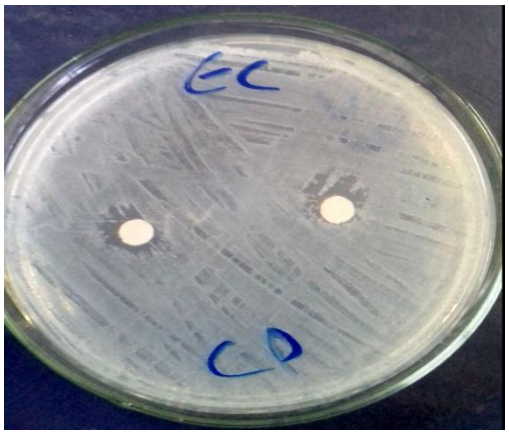

Figure A.9: Inhibition zone of *Moringa peregrina* seeds oil which extracted by cold press against Escherichia coli

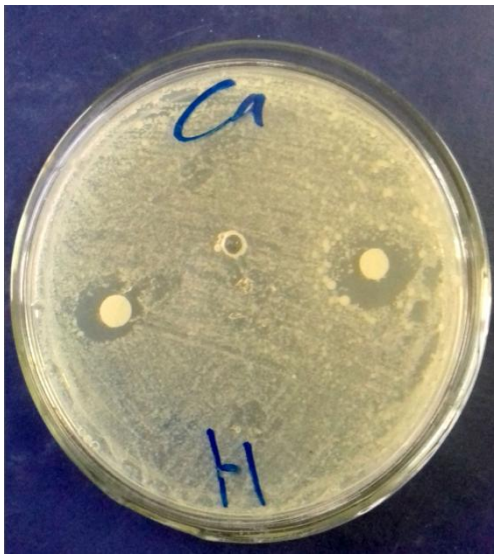

Figure A.10: Inhibition zone of *Moringa peregrina* seeds oil which extracted by maceration in n-hexane against Candida albicans

Figure A.11: Inhibition zone of *Moringa peregrina* seeds oil which extracted by maceration in n-hexane against Staphylococcus aureus

Figure A.12: Inhibition zone of *Moringa peregrina* seeds oil which extracted by maceration in n-hexane against Bacillus subtilis

Figure A.13: Inhibition zone of *Moringa peregrina* seeds oil which extracted by maceration in n-hexane against Pseudomonas aeruginosa

Figure A.14: Inhibition zone of *Moringa peregrina* seeds oil which extracted by maceration in n-hexane against Escherichia coli