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M.SC THESIS ON

EVALUATION OF THE PHYSICOCHEMICAL PROPERTIES, PHYTOCHEMICAL CONSTITUENTS AND ANTIMICROBIAL ACTIVITIES OF FIXED OIL FROM SEEDS OF *Nicandra phaysaloids*

BY

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JUNE, 2019

JIMMA, ETHIOPIA

EVALUATION OF THE PHYSICOCHEMICAL PROPERTIES, PHYTOCHEMICAL CONSTITUENTS AND ANTIMICROBIAL ACTIVITIES OF FIXED OIL FROM SEEDS OF *Nicandra physaloides*

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Abbreviations and Acronyms

DMSO	Dimethyl Sulphoxide
MH	Mueller Hinton Agar
SB	Sabouraud dextrose Bronth
AOAC	Association of Official Analytical Chemist
GC-MS	Gas Chromatography-Mass spectrometry
FAO	Food and Agricultural Organization
WHO	World Health Organization of United Nations
ES	Ethiopian Standard
NIST	National Institute of Standards and Technology

Abstract

Fixed oils of plant seeds used as raw materials in industries to produce commodities for our daily life and contain fatty acids compounds essential for human health. This study was aimed to investigate the yield, phytochemical composition, fatty acid composition, and antimicrobial activity of fixed oil from *Nicandra physaloides* seeds. Phytochemical screening for determination of alkaloids, glycoside, quinones, steroids, terpenoids, etc. was carried out using chemical test methods; antimicrobial activity tests were carried on the bacteria (*S. aureus, E. coli, P. aeruginosa* and *B. subtilis*) and fungi (*Fusarium spp* and *S. cerevisia*) using agar diffusion method. Fatty acid composition of the oil was determined from GC-MS data of the oil methyl ester. Proximate and physicochemical tests were carried following a standard protocol (AOAC). The oil yield was 19.66%. The proximate and physicochemical analysis data for the oil indicated: relative density (0.86 ± 0.01), refractive index at 21 ˚C, (1.48 ± 0.00), moisture content (1.32 ± 0.07), ash contents (1.33 ± 0.04), acid value (1.63 ± 0.00 mg KOH/g), free fatty acid (0.30 ± 0.02 as oleic %), peroxide value (0.49 ± 0.02 meq O_2/kg oil), iodine value (150.77 ± 0.03 g I_2 /100 g oil), saponification value (181.58 ± 4.32 mg KOH/g), ester value (179.95 mg KOH/g) and unsaponifiable matter (0.83). GC-MS analysis of the oil revealed the presences of Palmitic acid (12.88%), Oleic acid (18.40 %), Linoleic acid (63.10%) and α-Linolenic acid (5.65%). The phytochemical screening of the fixed oil showed the presence of alkaloids, glycosides, quinones, steroids, terpenoids and flavonoids. The oil showed an activity against bacteria on *B. subtilis* (10)*, P. aeruginosa* (11)*, E. coli* (12) *and S. aureus* (12)*,* and the fungi *S. Cervisiae* (12) and *Fusarium spp* (7), with zone of inhibition (mm). Therefore, *N. physaloides* seed oil has a potential to use in cosmetic, soap, pharmaceutical industries and as a biofuel oil resource and for medicinal purposes. Further study is needed to study the nutritional compositions of the products of oil extracted from seeds in order to ascertain its usage as foods for animals.

Keywords: *N. physaloides,* Fixed Oil, Physiochemical Properties, Fatty Acid Composition.

1. Introduction

1.1 Background Information

Fixed oils are plant or animal derived non-volatile viscous liquids mostly with yellow color and characteristic odor that can be obtained by different extraction methods (mechanical expression, solvent extraction, supercritical fluid extraction, microwave-assisted extraction, ultrasonic-assisted extraction etc.) [1]. Several plant species are known to produce fixed oils mainly in their seeds and other parts including fruits and nuts. Palm, Cotton, Sunflower, Niger, Canola, Castor, Rape, Jatropha, Soy bean, Peanut and Sesame are few plants containing oil in their seeds [2].

Chemically fixed oils are mainly composed of triglycerides (esters of glycerol and saturated and unsaturated fatty acids such as palmitic, stearic, oleic acids and linoleic and linolenic acids). Other minor constituents such as Complex lipids (phospholipids, sphingolipids, diacylglyceride ethers, glucolipids), lipid vitamins (A, D, and E), Natural hydrocarbons (squalene, short and long chain hydrocarbons, waxes), sterols (phytosterols, cholesterol), alcohols (aliphatic and terpenoic alcohols), phenolic compounds (simple phenols such as gossypol, melanine, quinones, saponins, flavonoids, lignans), photosynthetic pigments (chlorophylls and carotenoids) and sulfur and nitrogen compounds (glycosylated alkaloids, glucosinolates, oxazolidinthiones, isothiocyanates) can also be contained in them [3-6].

Fixed oils possess wide variety of uses such as nutritional, nutraceuticals, industrial, medicinal, production of biodiesel or bio-lubricant and as taxonomic makers in plants [7-12]. Plant oils contain chemicals that play role in food flavor, provide energy and to supplement important nutrients for body and thus are common food staffs recommended. Dietary role of fixed oils are mainly associated with triglycerides which are primarily responsible for taste and texture of foods, energy storage, supplying essential fatty acids, and carrying fat-soluble compounds (such as lipid vitamins, phospholipids, pigments and some important phytochemicals such as lignans). Recently essential fatty acids are also being considered as functional food and nutraceuticals owing to their roles in reducing the risk of serious diseases i.e. prophylactic role, especially cardiovascular diseases, cancer, osteoporosis, diabetes and other health promotion activities related to their complex influence on concentrations of lipoproteins, fluidity of biological

membranes, function of membrane enzymes and receptors, modulation of eicosanoids production, antihypertensive, antiatherogenic, antithrombotic, anti-inflammatory, antiarrhythmic, hypolipidemic effects and their effect on the metabolism of minerals. Moreover eicosapentaenoic acid and docosahexaenoic acid both derived from α-linolenic have been also associated with protection against mental disorders like Alzheimer's disease, aging and dementia, chronic daily headache and with attention-deficit hyperactivity disorder in children [13]. Besides an essential component of human diet some oils can be useful if included in to animal feed [14]. Fatty acids also find importance in various industries such as soaps and detergents, ink, varnish, adhesive, paints, pharmaceuticals or cosmetics as stimulant or laxative, cathartic, lubricant, emollient and solvent in preparation of certain injections. Some fixed oils might have potential to substitute petroleum derived fuels and lubricants and to serve as biofuels and hydraulic fluid [10, 11]. Many plant derived fixed oils and fatty acid components or phytochemicals contained in them were investigated for diverse biological activities as antioxidant, antibacterial, antifungal, insecticidal, antiparasitic activities and most were confirmed to have medicinal potential [14-21]. Studies also show that fatty acids contained in plants to be a good taxonomic biomarkers for making inferences on plant families, such as Sapindaceae, Asteraceae, Boraginaceae, Vochysiaceae, Rubiaceae and Malvaceae [13]. Generally potential application of plant fixed oils are mostly determined from analysis of the oil (it's methyl ester derivative) for percentage oil yield, physical parameters like color & odor, fatty acid or phytochemical composition, proximate analysis such as moisture content, specific gravity, refractive index, viscosity, ash content, PH, crude protein, mineral and vitamin, calorific value and physicochemical parameters such as acid value, saponification value, iodine value, peroxide value etc. among others [22].

Therefore, the objective this study was made to extract fixed oil from the seeds of *N. physaloides* and carry out physicochemical, phytochemical and antimicrobial activity tests of the oil according to standard test protocols. Based on results to be obtained for each parameter determined recommendations were given on possible future commercial value of the oil and use of the oil for human diet, various industrial sectors and/ or as biodiesel.

(a) (b)

Figure 1 (a) *N. physaloides* plant and (b) its seeds (source: shumi's photo gallery)

1.2 Statement of the Problem

With an increase in prices of petroleum products, over depleted natural fossil fuel deposits in the world and increase in diversity of application for plant derived oils or their derivatives there will be an ever expanding market for oilseed crops. This condition will therefore demand continuous effort to discover plant potentially that yield fixed oils. The diversity of plant flora (6.5-7,000 higher plants with 12% endemicity) and communities having close association with plants and their traditional healing power enable Ethiopia to be land of such potential [23].

Infectious diseases caused by pathogenic micro-organisms such as bacteria and fungi are becoming serious threat for human health as drug resistant problems have been emerged almost for all available antimicrobial agents and are getting widespread all over the world. Pools for discovery of new drugs with noble mechanism of action are also getting depleted. The antimicrobial activities reported for some plant derived fixed oils and their constituents will also encourage further screening of oils as a source of new drugs [24].

N. physaloides is one of the seed bearing plant recently introduced as weed to Ethiopia and that has got widespread into most farm lands of the country [25-28]. The plant is known in Ethiopia by different vernacular names such as "*Asangra*" in "Afan Oromo" and in "Amharic" "*Machara*" and "*Madaberiya"* due to the assumption that the plant was introduced from abroad with imported seeds. The plant belongs to the family Solanaceae and is native of Peru but 2currently found widespread in different region of the world and known by names "*Apple of*

Peru" or "*shoo-fly plant*". The plant is perceived to be toxic due to its alkaloidal content but its leave and fruits are reported to be edible after boiling [29]. In folk Tibetan medicine the plant is used for the treatment of diuresis, mydriasis, analgesia, antibacterial and inflammation [30]. Several reports also show potential application of *N. physaloides* as analgesic, vermifuge, antibacterial, antipyretic, diuretic, anti-inflammatory, insecticide and as mydriatic agent and in treatment of hydrophobia, psychosis, epilepsy, rheumatoid arthritis, nasosinusitis, influenza, urinary tract infection, parkinson's disease, sore and furuncle [14-22]. The Powdered leaf of plant is used in traditional treatment of leishmaniasis and skin diseases of horse in "*Amaro*" district in Ethiopia [31]. Several classes of compounds including alkaloids, steroids, triterpenoids, carotenoids, glycosides, saponins and various phenolic compounds have been identified in the different morphological part of the plant. On our preliminary analysis on the seeds of this plant we have confirmed that the plant contains fixed oil.

1.3 Objectives of the Study

General Objective

- To investigate the yield, fatty acid composition, physicochemical properties, phytochemical constituents and antimicrobial activities of fixed oil from *N. physaloides* seeds.

Specific Objectives

- To determine yield of fixed oil of *N. physaloides* obtained by cold maceration petroleum ether extract of its seeds.
- To determine the physicochemical properties of the fixed oil.
- To characterize the fatty acids contained in the fixed oil using GC-MS analysis.
- To screen out the phytochemical constituents of the fixed oil.
- To screen the fixed oil for antimicrobial activities against four bacterial strains (*S. aureus, E. coli, B. subtilis* and *P. aeruginosa*), and two fungal strains (*Fuzarium spp and S. cervisiae*).

1.4 The Significance of the Study

The main significance of the output of this work could be:

- Provide a means to exploit and use valuable local resources.
- Identifying physicochemical properties of the fixed oils of the *N. physaloides* seeds whether it is used or not in a wide variety of consumer goods manufacturing such as detergents, soaps, pharmaceuticals, etc.
- Creating a new awareness for the utilization of available natural resources in regard to fixed oils of the seeds *N. physaloides* in the country for the production of important products locally.
- Proving fatty acid profile of the fixed oil of the seeds of the plant.
- Developing the optional method of control of bacteria & fungus using *N. physaloides* seeds oil.

2. Review of Related Literature

2.1 Botanical Information of the Plant *N. physaloides*

a. The Family Solanaceae

The family Solanaceae is one of the largest and economically most important families of angiosperms, including food, spice and drug plants. In 1979 the estimated number of genera was 83 and 2671 species but the most recent estimate is that the family includes more than 3000 species [32]. These include the edible species such as the potato (*Solanum tuberosum*), eggplant (*Solanum melongena*), tomato (*Solanum lycopersicum*), capsicum (*Capsicum species*) and cape gooseberry (*Physalis peruviana*). This family also includes species that are grown as ornamentals such as those belonging to the genera *Browallia, Brunfelsia, Cestrum, Datura, Nicotiana, Salpiglossis, Solanum, Solandra* and *Nicandra* among others. Well known are the trumpet-like flowers of *Datura s*pecies which are popular as ornamental plants and which can produce flowers of up to 30 cm long in a variety of colors. All of the Solanaceae family plants are toxic in some way. Their toxins range from mild to very high. Many of them, like potatoes and tomatoes are safe for humans, yet not safe for some animals. Jimson Weed (*Datura stramonium*) and mandrake (*Mandragora officinarum*) are highly toxic to humans, yet when used correctly can have substantial medical benefits. Tobacco is another member of the Solanaceae family that is mildly toxic but people usually smoke it. Humans have also learned to use the toxicity of peppers to make spray which is used as discouragement to both humans and animals. Plants in the Solanaceae family are known for possessing a wide range of alkaloids. For humans, these alkaloids can be desirable or toxic [33].

b. The Genus *Nicandra*

Nicandra is a genus of flowering plant in the family Solanaceae, native to western South America, Peru. It was first described by Michel Adanson in 1763. The genus is named for Greek poet Nicander of Colophon, who wrote about plants notably in his poem *Alexipharmaca*, which treats of poisons and their antidotes. As of March 2019, Plants of the World Online accepted three species: *Nicandra john-tyleriana* S.Leiva & Pereyra, *N. physalodes* (L.) Gaertn, and *Nicandra yacheriana* S.Leiva. From 1763 until 2007, when *Nicandra john-tyleriana* was

described, the only species in the genus was *Nicandra physalodes*. A third species, *Nicandra yacheriana*, was described in 2010 [34-38].

c. *N. physalodes* (L.) Gaertn

N. physaloides is a coarse, erect annual plant reaching three to eight feet in height and about half as wide. It has large alternate leaves reaching up to one foot long. The leaves are ovate-cordate in shape. The young plants have dark colored ‘‘dots’’ that decorate the adaxial surfaces of the leaves. They are seen to be small cuticular spikes or trichomes. Their margins are shallowly lobed, bluntly dentate, or undulate. The petiole of each leaf is long and slender, tilting at an upward angle; there are a few hairs near its base, otherwise it is hairless. The flowers are pale blue in color with white throats and are bell-shaped. The flower becomes lantern-like towards the end of its bloom. The fruits appear prickly and are enclosed in papery inflated calyxes. Each fruit has a dry berry in it. The stems are angular and largely hairless. It is native to Peru and it is recognized elsewhere as an exotic species. The plant is known by the common names *Apple of peru* and *shoo-fly* plant. It is kept as an ornamental plant but has a tendency to be weedy and has consequently become a noxious weed in the tropics. The preference is full or partial sun, moist conditions and a loamy fertile well drained soil. Most vegetative growth occurs during the late spring and summer. This species is a summer annual. The size of a plant is variable, depending on soil fertility and availability of moisture. All parts of the plant are mildly poisonous [33].

N. physaloides is one the seed bearing plant recently introduced as weed to Ethiopia and that has got widespread into most farm lands of the country [25-28]. The plant is known in Ethiopia by different vernacular names such as “*Asangra*” in Afan-Oromo and in Amharic as “*Machara”* and “*Madaberiya*” due to the assumption that the plant was introduced from abroad with imported seeds. It is also kept as an ornamental plant. The plant is thought to have insect repellant properties [39].

2.2 Use of *N. physaloides*

a. In Traditional Medicine

A decoction of the seeds is used in the treatment of fevers [46]. The plant is commonly used as an insecticide and pediculicide. It is also used as an insect repellent administered by rubbing exposed skin with the tender stems and foliage. In some communities the plant’s boiled extract is

mixed with milk and set as fly poison [45]. As a traditional folk medicine, it has been used as sedative, expectorant, antipyretic and antidote in China.

b. Nutritional Role

The leave and fruits of *N. physaloides* are reported to be edible after boiling [29]. The seeds of *N. physaloides* can be utilized to extract edible pectin to make jelly. The fruits of *N. physaloides* are rich in protein, carotenoids, vitamin-A and vitamin-C, and antioxidants like lycopene, anthocyanins, chlorophylls and phenols. Oil from its seed was reported to have abundance in linoleic acid [46, 47].

2.3 Previous Work Reported on *N. physaloides*

a. Antimicrobial Activities

N. physaloide has been investigated for various biological activities. The methanolic and aqueous extract of the leaf, fruit, stem and root of *N. physaloides* were active against *Bacillus subtilis*, *Mycobacterium phelei, Proteus mirabilis and Staphylococcus epidermidis.* Both methanolic and aqueous extract of leaves and roots were active against *Candida albicans and Aspergillusflavus* [49].

b. Pesticides Activities

N. physaloide has insecticidal properties [48]. *Nicandrenone* is insecticidal compound which was identified in aqueous extracts as inhibiting the feeding of insect larvae [43]. Its leaf extracts serve as insect antifeedant [45]. The compounds isolated from the petroleum ether extract of *N. physaloides* cause mortality on female two-spotted spider mites but they didn't affect the mite eggs [51].

c. Diuretic Activity

Aqueous and alcoholic extracts of *N. physaloides* leaves have been tested for diuretic activity in rats. They both showed significant increase in excretion of sodium, potassium and chloride ions in the urine in a dose dependent manner. The obtained effect was comparable to that of furosemide. The study supported the presence of effective diuretic constituents in the aqueous and alcoholic extract of *N. physaloides* [49].

d. Various Other Illnesses

Modern researchers have indicated that *N. physaloides* was reported being taken as analgesic, vermifuge, antipyretic, mydriatic and treatments of hydrophobia, psychosis, epilepsy, rheumatoid arthritis, nasosinusitis, influenza, urinary tract infection, sore and furuncle [51]. The plant has also been used as an anthelmintic, antibacterial, anti-inflammatory and as febrifuge and its leaf extracts have an activity to decrease blood sugar, have anti-tumor activity [45].

2.4 Phytochemical Constituents of *N. Physaloides*

a. Alkaloids and Terpenes

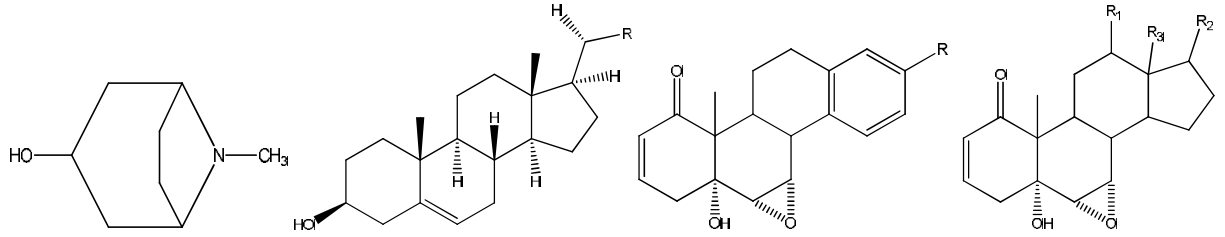
The aqueous and alcoholic extract of the leaves of the plant *N. physaloides* was yield alkaloid tropine (**1**) [40]**.** A terpene loliolide (**11**) was also identified from *N. physaloides* [42].

b. Phystosterol, Aromatic Steroids and Whithanolides

From aerial parts of the plant extract was yielded phyhytosteroids β-sitosterol (**2**) and stigmasterol (**3**) [41]. The aromatic steroids Nic-10 (**4**) and nicandrenone (**5**) were also identified from it [42]. From the aerial parts of the plant *N. physaloides* whithanolide, withanicandrin (**6**) was isolated [69]. From methanolic and aqueous extract of fresh whole plants, Nicaphysalins (ergostane-related compounds, nicaphysalin A, B, C and D) (**7-10**) were isolated [42, 43].

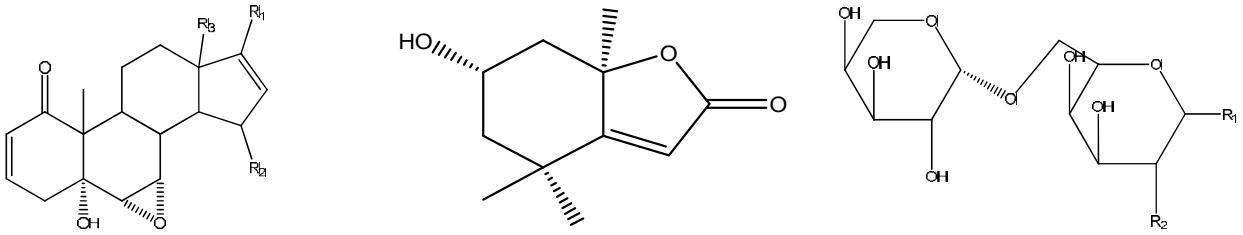
c. Glycosides and Phenolic Amides

Three glycosides (**12**–**14**) were isolated from the fruits of it's by ethanol extraction [44]. From the ethyl acetate extract of the fruits of two phenolic amides were isolated, (7R, 8S)-7-(4-hydroxy-3, 5 dimethoxyphenyl)-8-hydroxymethyl-10-[N-hydrxyphenyl) ethyl] carbamoylethenyl -30 methoxybenzodihydrofuran **(15)** and cis-N-phydroxycinnamoyl-70-methoxyethyltyramine **(16)** [42].



1 **2** R=C_8H_{18} **4** R=R_1=$C_7H_{12}O_3$ **6** R_1=O, R_2= $C_8H_{14}O_2$ & R_3= CH_3

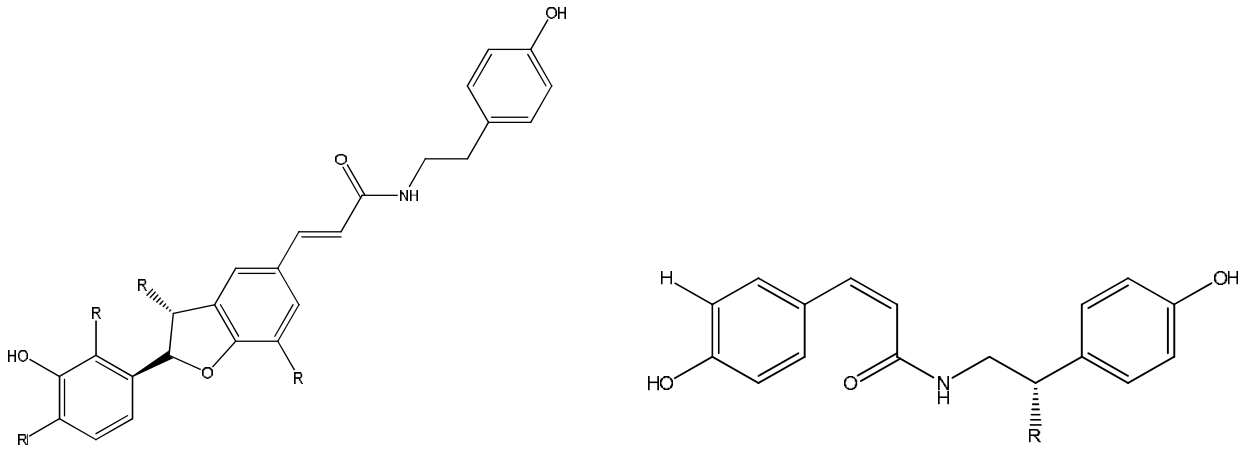
3 R=C_8H_{16} **5** R=R_2=C_2H_4O **7** R_1=OH and R_2=$C_9H_{17}O_3$



8 R_1=$C_9H_{16}O_3$, R_2=H and R_3= H **11** **12** R_1=$C_8H_{10}O$ and R_2=OH

9 R_1=$C_9H_{16}O_3$, R_2=OH and R_3=H **13** R_1= C_8H_{16} and R_2=OH

10 R_1=$C_8H_{13}O_3$, R_2=OH, R_3=CH_3 **14** R_1=$C_6H_{12}O$ and R_2 = $C_7H_{15}O_5$



15 R=OCH_3 **16** **R**=OCH_3

Figure 2 Structures of compounds reported from *N. physaloides*.

2.5 Fixed Oils

2.5.1 Chemistry of Fixed Oils

Fixed oils are oily, non- volatile part of the plant, typically extracted from various parts of plants such as fruits, seeds, or plant seedlings and animals fats. Chemically, they are a combination of triglycerides (esters of glycerol and higher fatty acids) [50]. Fatty acids are divided into saturated acids e.g. lauric; myristic; palmitic; stearic; arachidic and unsaturated acids e.g. oleic; linoleic and linolenic.

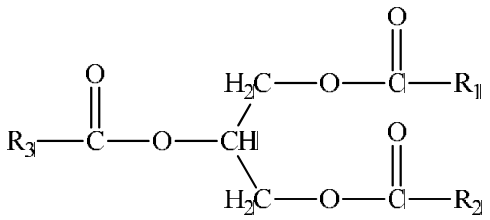


Figure 3 Structure of a typical plant oil triglyceride

Vegetable oils are predominantly triglycerides (95% - 98%) along with complex mixtures of minor compounds (2% - 5%) that constitute wide range of chemical classes; lipid vitamins (A,D,E,& K) , minerals such as potassium, magnesium and calcium, fat, protein, fatty alcohols, waxes, esters, hydrocarbons, volatiles, pigments, phenolic compounds, glyceride compounds, phospholipids and triterpenic acids and phytochemicals [51]. They are called 'fixed' as they have large molecules that don't evaporate like the essential oils. They are also thick, viscous, most of the time yellow colored liquids with characteristic odor, can't be distilled and turn rancid on storage due to free acidity [51].

2.5.2 Physical and Physicochemical Parameters to Characterize Fixed Oils

Various physical and physicochemical parameters are used to characterize fixed oils. The most important ones are density, refractive index, moisture contents, ash contents, acid value, saponification value, iodine value, ester value, peroxide value, unsaponifiable matter etc. [52].

a. Density

The density of a material is a defined as the measured of its mass per unit volume (e.g. in g/ml). The density vegetable oil lower than of water and the differences between vegetables oil are

quite small, particularly amongst the common vegetable oils. Generally, the density of oil decreases with molecular weight, yet increase with unsaturation level [53].

b. Refractive Index

Refractive index is a measure of the deviation of the beam of light as it passes from one medium to another. The refractive index of the seeds oil was determined with a digital refractometer [54]. The oils exhibited different refractive indices. The physicochemical values for oils of the same type were quite similar. This trend suggests that refractive index could be used in the preliminary identification of the oils and fat. The refractive index of fats and oils is sensitive to their composition. In fats, refractive index increases with increasing chain length of fatty acids in the triglycerides or with increasing unsaturation. This makes it an excellent spot test for uniformity of compositions of oils and fats [55].

c. Specific Gravity

Specific gravity or relative density can be defined as the ratio of the density of the substance (liquid) to the density of water. In view of the fact that this is a ratio of densities, it is a plain number not including any units. A Specific gravity (density bottle) bottle is used to calculate relative density. The number indicates that the fatty acids average molecular weight of oil. The specific gravity is proportional to the fatty acids mean chain-length of the oil, as the fatty acid chain-length is proportional to the fatty acid molecular mass. As the temperature increases, the specific gravity of the oil decreases [56].

d. Moisture Content:

The moisture content of seeds is an important factor that affects the yield and quality of the oil extracted. Turbid oil is obtained from seeds with high moisture level; therefore, moisture adjustment of the seed is necessary before pressing. Thus, moisture increases the flow of oil through the pores of the press cake, hence reducing the amount of oil entrained in the cake and increasing the oil yield mostly in mechanical expression [57].

e. Ash Content:

Ash is the inorganic residue remaining after the water and organic matter have been removed by heating in the presence of oxidizing agents, which provides a measure of the total amount of minerals within a food. Analytical techniques for providing information about the total mineral content are based on the fact that the minerals (the analyte) can be distinguished from all the other components (the matrix) within a food in some measurable way. The most

widely used methods are based on the fact that minerals are not destroyed by heating, and that they have a low volatility compared to other food components [57].

f. Acid Value

The acid value is a measure of free fatty acids in the oils derived from the hydrolysis of the triglycerides. This change occurs under unsuitable conditions of treatment and preservation of the oil. These free fatty acids cause rancidity of the oils when the oil is stored for a long time due to the release of free fatty acids. The acid value can be defined as the number of milligrams of Potassium hydroxide (KOH) needed to neutralize the free organic acids in l gram of oils and is determines by titration [58].

g. Peroxide Value

Peroxide value is a measure of the concentration of peroxides and hydro peroxides formed in the initial stages of lipid oxidation. Its values increased during storage. Thus it measures deterioration of oil from oxidation. Mill-equivalents of peroxide per kg of fat/oil are measured by titration with iodide ion are used to quantify peroxide value. Peroxide value is not static and care must be taken in handling and testing samples. It is difficult to provide a specific guideline relating peroxide value to rancidity. High peroxide values are a definite indication of a rancid fat, but moderate values maybe the result of depletion of peroxides after reaching high concentrations [60].

h. Iodine Value

Iodine value is expressed as the number of milligrams absorbed per gram of sample and is determined by titrimetry. Iodine value gives a measure of the average degree of unsaturation of oils and fats: the higher the iodine value, the greater the number of C=C double bonds. The iodine value could also reflect susceptibility of the oil to oxidation as quantity of double bond present in the oil could also account for this property. Oils with iodine value less than 100 g I_2/100 g of oil are non-drying oils; correspondingly, reported that the lower the iodine value the lesser the number of unsaturated bonds; thus the lower the susceptibility of such oil to oxidative rancidity. Oil and fats can be divided as follows based on the iodine value: Drying fats and oils: oils with an iodine value of 130-200, semi drying fats and oils: oils with an iodine value of 100-130, and non-drying fats and oils: oils with an iodine value lower than 100 [57].

i. Saponification Value

Saponification is the alkaline hydrolysis of oils or fats, since one of the products of the alkaline hydrolysis is soap that is potassium or sodium salts of higher fatty acids. The alkaline hydrolysis gives a way of determination of a constant known as Saponification value. Its value can be defined as: "The number of milligrams of potassium hydroxide (KOH) needed to totally saponify one gram of the lipid (oil or fat) that is to neutralize the fatty acid resulting from complete hydrolysis of one gram of lipid (oil or fat)". Its value is an indication of the size or nature of fatty acid chains esterified to glycerol and gives a measure of the average length of the fatty acid chain that makes up a fat. It is inversely proportional to the mean molecular weight of the fatty acid in the glyceride present in the oil [55].

j. Unsaponifiable Matter

The unsaponifiable matter is defined as the substances soluble in oil which after saponification are insoluble in water but soluble in the solvent used for the determination. It includes lipids of natural origin such as sterols, higher aliphatic alcohols, pigments, vitamins and hydrocarbons as well as any foreign organic matter nonvolatile at 100°C e.g (mineral oil) which may be present. Light Petroleum or diethyl ether is used as a solvent but in most cases results will differ according to the solvent selected and generally the use of diethyl ether will give a higher result [61].

2.5.3 Potential Uses of Fixed Oil of Plant Origin

Crude or refined plant derived oils and their chemical constituents possess wide variety of applications. Some of their most important uses are summarize in the following sections.

a. Food and Feed Application of Plant Oils

The largest proportion of plant oils is consumed as food and feed, and the oils used in these markets contain various proportions of the five common, nutritionally important fatty acids; palmitic, stearic, oleic, linoleic and α-linolenic acids. The properties of oils depend greatly on their fatty acid composition, and certain compositions are desirable for specific end uses. For example, cooking oils generally need to contain a higher proportion of mono-unsaturated fatty acids (such as oleic acid), which are more stable under high temperature, while margarine and spreads are often rich in saturated fatty acids (e.g. palmitic and stearic acids) [57].

b. Plant Oils as Functional Foods/Nutraceuticals

Plant oils are the source of essential fatty acids. Linoleic acid and α-linolenic acid are the two parent essential fatty acid. These acids must be supplied in the diet because they are required by the human body and cannot be endogenously synthesized. Recently, essential fatty acids (EFAs) have been considered as functional food and nutraceuticals. A lot of research studies have documented their significant roles in many biochemical pathways resulting in cardioprotective effect because of their considerable antiatherogenic, antithrombotic, anti-inflammatory, antiarrhytmic, hypolipidemic effect, because of the potential of reducing the risk of serious diseases, especially cardiovascular diseases, cancer, osteoporosis, diabetes and other health promotion activities following from their complex influence on concentrations of lipoproteins, fluidity of biological membranes, function of membraned enzymes and receptors, modulation of eicosanoids production, blood pressure regulation, and finally, on the metabolism of minerals [62].

c. Plant Oils as Alternative to Diesel Fuels and Lubricants

The use of plant oils in diesel engines is nearly as old as the diesel engine itself. The fuel and energy crises of the late 1970's and early 1980's as well as the accompanying concerns about the depletion of the world's nonrenewable resources provided the incentives to seek alternatives to conventional, petroleum-based fuels. In this context, plant oils as fuel for diesel engines were remembered. They now occupy a prominent position in the development of alternative fuels [63]. There has been a constant demand for environmentally friendly or ''green'' lubricants. A significant lubricant market of some nine million metric tons per year of industrial and automotive lubricants exists. Currently, plant oils are providing fraction of the lubricant market. They are already in use as lubricants due to their superior lubricity, good anticorrosion, better viscosity-temperature characteristics and low evaporation loss in industrial applications such as rolling, cutting, drawing, quenching operations, and greases either alone or in combination with mineral oils [63].

d. Plant oils as Biologically Active Agents

The antimicrobial properties of medium-chain fatty acids derived from plants are considered valuable therapeutic alternatives to treat various diseases caused by microorganisms. The antibacterial activities of long-chain unsaturated fatty acids have been well known for many years. Fatty acids function as the key ingredients of antimicrobial food additives which inhibit

the growth of unwanted microorganisms. Linoleic and oleic acids are antibacterial components in the herbs (*Helichrysum pedunculatum and Schotia brachypetala*) used for dressing wounds during male circumcision rituals in South Africa. Fixed oils from *Thymus maroccanus* and *T. broussonetii* are able to disrupt the biomass and inhibit the metabolic activity of preformed bio films of distinct *Candida spp*. and nosocomial infections acquired from hospital settings [59]. The plant oils are also being sold for insect and mite control. Among these are canola oil, refined edible vegetable oil obtained from the seeds of two species of rape plants (*Brassica napus* L. and *B.campestris* L.) [59].

e. Plant Oils Industrial Raw Material

Plant-derived oils can also be important industrial feedstocks in the manufactory of soap, paints, varnishes and pharmaceuticals (suppositories, tablet coating, and emulsifying agents) [51].

f. Uses of Plant Oils in Cosmetics

Plant oils used in cosmetics contain a range of fatty acids which contribute several beneficial properties in cosmetic and personal care products. Linoleic acid is the most frequently used fatty acid in cosmetic products. Linoleic acid deficiency will cause various signs. The skin dries out and becomes scaly, nails crack, and hair loss as well as transepidermal water loss increases. Linoleic acid moisturizes the skin, aids in the healing process of dermatoses and sunburns and is used for the treatment of acne vulgaris. Plants containing linoleic acid may be beneficial in acne lesion reduction as the anti-inflammatory effects have been shown to inhibit Propionibacterium acnes. Many plants containing high levels of linoleic and linolenic acids are used in the treatment of acne. Oleic acid is reported to be an effective percutaneous absorption enhancer. It markedly enhanced the penetration of tenoxicam, a non-steroidal anti-inflammatory drug, by as much as 15% and is reported to increase diffusivity and partitioning as well as the fluidity and flux by interaction with subcutaneous lipids. Of the unsaturated fatty acids, Skin permeation enhancement effects were also recorded for palmitic acid (most potent), linoleic, lauric, myristic and stearic acids and oleic acid, all of which are present in various seed oils [64].

2.5.4 Methods of Extraction of Fixed Oil

Fixed oils are normally extracted from various plant parts but the seeds are generally good sources of these products [65]. There are three main techniques that have been identified for

extraction of oil: mechanical extraction, chemical or solvent extraction, and enzymatic extraction. Besides, accelerated solvent extraction (ASE), supercritical fluid extraction (SFE) as well as microwave-assisted extraction (MAE) method is frequently used. It has been observed that mechanical pressing and solvent extraction are the most commonly used methods for commercial oil extraction.

a. Mechanical Oil Extraction

Prior to the discovery of any other oil extraction method oil was extracted mechanically or cold pressed. It is the simple process of heating the plant material to low temperatures and then physically pressing the oil out. Today mechanical expression is used mainly for citrus peels and is unpopular due to the low extraction yield. It is the most conventional technique. A manual ram press or an engine driven screw press can be used. It has been found that engine driven screw press can extract 68–80% of the available oil while the ram presses only achieved 60–65% [57].

b. Solvent Extraction

The solvent extraction is chemical oil extraction method which is the most popular method of extraction of oil because of its high percentage of oil recovery from seeds [57]. When performed at low temperature, solvent extraction has another advantage over screw-pressing because it gives better quality oil. It is basically a mass transfer operation involving diffusion of a suitable solvent into oil-bearing cells of the raw material, resulting in an oil-solvent solution (miscella). The oil is then distilled and the solvent evaporated leaving the oil behind. This process leaves minimum oil residual in the cake compared to mechanical [57].

Common solvent extraction uses a pure organic or mixed organics to extract the valuable extracts from the plant material. Typical solvents include ethyl acetate, diethyl ether, methanol, ethanol, petroleum ether, isopropyl alcohol, carbon tetrachloride and hexane. The chemical extraction using n-hexane method results in the highest oil yield which makes it the most commonly used solvent. There are, however, some disadvantages associated with the solvent extraction technique. Solvent residues often contaminate the product; therefore, with solvent extraction effective separation of the extracted oil from the solvent is necessary to remove any solvent which may contaminate the oils [57].

Mechanical and solvent extraction methods are time and solvent consuming, in addition to being thermally unsafe. These shortcomings can be overcome by the use of alternative modern methods such as microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), supercritical fluid extraction (SFE) etc. [66].

c. Microwave-Assisted Extraction (MAE)

MAE is one of the modern techniques for isolating vegetable oils from oilseeds. The method is superior to many other thermal methods used for the purpose of extracting high quality vegetable oils. Oilseed is treated in the microwave oven, which uses radio waves to convey energy and convert it to heat at a frequency range of about 300 MHz to 300GHz. The use of microwave radiation in oilseeds results in the rupture of cell membranes, making it possible to obtain higher extraction yield and an increase in mass transfer coefficients [1].

d. Ultrasonic-Assisted Extraction (UAE)

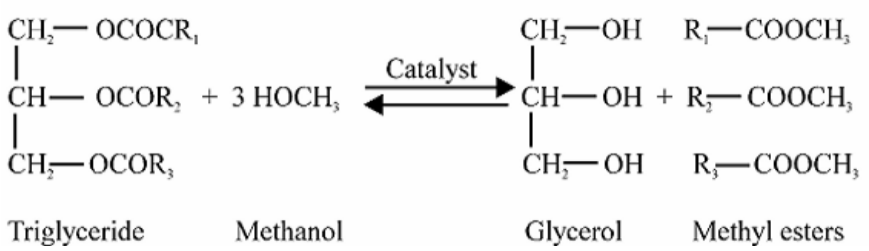
UAE is a new modern technique which makes use of ultrasonic sound waves to increase vibration and heat, resulting in the destruction of rigid plant cell walls, thereby enhancing contact between solvent and the plant material. When coupled with solvent extraction, the UAE method represents a modern way of increasing extracted oil yield by making plant cell walls thinner, and thus enhancing the interaction of the solvent. This technique has been applied by a number of researchers. They reported that conventional solvent extraction lasted 12 hours, whereas the UAE method lasted only 30 minutes. This makes the UAE more suitable in terms of reduced time lag and yield. It has the potential to be used in oil extraction processes to improve efficiency and reduce the process time, which may have a significant impact on edible oil industry [1].

e. Others

Other innovative techniques for the extraction of oilseeds include supercritical fluid extraction (SFE), microwave -assisted hydrodistillation (MAHD), pressurized liquid extraction, soxtec and a host of others. These methods, together with MAE and UAE, have been successfully used to effectively reduce the major shortcomings of the conventional methods of oil extraction [1].

2.5.5 Conversion Fixed Oils into their Methyl Esters

Transesterification is a chemical reaction between triglycerides (oil /fats) and methanol, which form immiscible phases when they are in a reaction vessel, the reaction takes place at contact surface between oil and methanol (Scheme 1). It consists of a number of consecutive, reversible reactions. Triglycerides are converted step wise to diglycerides, monoglycerides and finally glycerol liberating a mole of ester in each step [67]. The reactions are reversible, the equilibrium lies towards the production of fatty acid ester and glycerol.



Scheme 1 The mechanism for transesterification of triglycerides

3. Materials and Methods

3.1 Materials and Methods

The seeds of the *N. physaloides* were collected and prepared them for fixed oil extraction by solvent petroleum ether and it was subjected to the analysis of different physicochemical parameters, phytochemical screening and antimicrobial activities according to standard procedures in the following paragraphs.

3.2 Chemicals and Apparatus

Instruments and apparatus such as electronic balance (WT50001NF), GC-MS (QP/5050A), muffle furnace (CARBOLITE, CWF 11/5/301), drying oven (Memmet, UNB 100), rotary evaporator (heidolph Laborata 4000), electrical grinder, desiccator, bunsen burner, electrical heater (stuart CB162), refractometer (RFM 330's), separatory funnel, reflux condenser, conical flask, were used during the analysis of the sample.

Analytical grade reagents and chemicals used during the analysis of the sample, were; H_2SO_4, HCl, HNO_3, KOH, NaOH, NH_4OH, $FeCl_3$, Mayer's reagent, Hanus reagent, Ethanol, CCl_4, $Na_2S_2O_3.5H_2O$, KI, glacial acetic acids, DMSO chloroform, petroleum ether, acetone, methanol.

3.3 Plant Material Collection and Preparation

The seeds of *N. physaloides* were collected from Oromia region Illu-Ababor zone, Darimu District, Western Ethiopia from November up to March, 2018. The plant seeds were identified by a botanist in the Department of Biology of Jimma University for the study. The sample fruits of the seeds were allowed for shade-air dry for days until the seeds cases well dried. The seeds were separated from its seed pods and foreign materials like dust particles before milling by electrical grinder. The fine powder form the seeds collected in plastic bags and stored in the laboratory for extraction.

3.4 Extraction of Fixed Oil

About1kg of powdered sample seeds were taken and added to round bottomed flask and then macerated it with the solvent petroleum ether for 24 hr. After 24 hr., the mixture was filtered with cotton followed by filter paper. The solvent of the solution was evaporated using rotary evaporator and oil was collected in amber colored vials, and then sealed with Teflon caps and

stored in laboratory until analysis [71]. Solvent was freed from the oil obtained after extraction was placed on table for days and the mass of oil was recorded and expressed as percent oil content calculated as below [40].

$$\% \text{ Oil content} = \frac{\text{Weight of oil}}{\text{Weight of sample}} \times 1oo$$

3.5 Physical & Physicochemical Analysis of the Oil

Physicochemical properties of the oil such as percentage oil yield, relative density, refractive index, ash content, acid value, free fatty acid, iodine value, peroxide value, unsaponification value, saponification value and Ester value were determined respectively using standard procedure of the AOAC [69].

a. Moisture Content

A crucible was washed and dried in the oven, after cooling in the desiccator and it was weighed (W_1). 2.0 g of the sample was carefully weighed in the crucible and the weight was taken as (W_2). The crucible containing the sample was then placed in an oven at temperature of 105°C for 1 hour. It was cooled and weighed. The crucible was then introduced into the oven again and process of cooling and weighing continued at intervals until a constant weight was obtained (W_3). Percentage moisture content was calculated as follow [40].

$$\% \text{ Moisture content} = \frac{w2-w3}{w2-w1}$$

b. Ash Content

A crucible was washed and dried in an oven then it was cooled in a desiccator and weighed. 2.0 g of the sample was weighed in the crucible containing the sample and was heated gently on a Bunsen burner until the smoke was ceased. It was then transferred to a muffle furnace and heated at a temperature of 550 °C – 570 °C for 2 hours to burn all organic matter. The crucible was taken out of the muffle furnace after a white was observed and placed in the desiccators to cool and was weighed [40].

$$\% \text{ Ash Contents} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \text{x } 100$$

c. Relative Density

A specific density bottle was washed, dried and weighed (W_1). It was filled with distilled water and weighed (W_2). The water was poured off and the bottle was dried to its previous constant weight and then filled with the oil sample and weighed (W_3) [40].

$$\text{Relative density} = \frac{W3 - W1}{W2 - W1}$$

d. Refractive Index

Refractive index was determined on the instrument RFM 330's refractometer by using refractive index of distilled water, which is 1.3330 at 21°C as a standard. The prism was cleaned with clean tissue paper wetted by distilled water to remove old sample then dried with fresh clean tissue paper. Few drops of oil was placed on the prism, closed the prisms and allowed to stand for 1-2 min, adjusted the instrument and lighted to obtain the most distinct reading and determined the refractive index. Refractive index of oil increases with the increase in unsaturation and also chain length of fatty acid [74].

e. Iodine Value

Five grams of oil sample was weighed in 250 mL conical flask and then 25 mL of carbon tetrachloride was added to oil sample and content was mixed well. 25 mL of Hanus reagent was added to the solution, swirled for proper mixing, and kept in the flask in dark for half an hour. After standing, 15 mL of potassium iodide solution was added and then 100 mL of distilled water was added into the mixture and 1 mL starch indicator solution was added to the sample solution. The sample solution was titrated with 0.01 N of sodium thiosulphate solution; then, at the end, blue color was formed and then disappeared after thorough shaking. The blank determination was carried in the same manner as test sample but without oil. The iodine value was estimated using the following formula [75].

$$\text{Iodine value} = ((A\text{-}B) \times (Na_2S_2O_3 .5H_2O) \times 12.69/Q$$

Where, A = volume of 0.01 N in $Na_2S_2O_3.5H_2O$ solution was used for the black titration.

B = Volume of 0.0 l N of $Na_2S_2O_3$ $5H_2O$ solution was used for the Sample titration.

Q= Weight in gram of the oil sample

12.69 = Conversion factor.

N = Normality

f. Saponification Value

A 0.1 N KOH solution was prepared with 95 % ethanol and distilled water. 5 g of oil sample was weighed in a conical flask, the flask was connected to an air condenser and boiled until the oil was completely saponified, cooled and titrated with 0.5 M HCl using phenolphthalein as indicator [76].

$$\text{Saponification value} = \frac{A-B}{Q} \times 28.05$$

Where, A = Volume of 0.5M of Hydrochloric acid was used in the blank titration.

B = Volume of 0.5M of Hydrochloric acid was used in the sample titration.

Q = Weight in grams of the oil sample.

28.05 = Conversion Factor

g. Peroxide Value

2.0 g of the oil sample was transferred into 250 cm^3 flask and 1g of powdered potassium iodide (KI) and a solvent mixture (2:1 of glacial acetic and trichloromethane) was then added. The solution was then placed on a water bath for a few minutes for complete dissolution. 20 cm^3 of 50% potassium iodide was introduced and the sample was titrated with 0.1M $Na_2S_2O_3$. The indicator was a regular starch solution. Blank experiment was done similarly [40].

$$\text{Peroxide value (meq } O_2\text{/kg oil)} = (S\text{-}B) \times W \times N$$

Where, S = Volume of sodium thiosulphate consumed by the sample oil

B = Volume of sodium thiosulphate used for blank

W = Weight of oil sample

N = the normality of sodium thiosulphate

h. Acid Value

100 ml of ethanol was heated with 10g of oil sample in a 250 ml beaker until the mixture began to boil. The heating was stopped and the solution was titrated with 0.1 N KOH solution, using two drops of phenolphthalein as indicator with consistent shaking for which a permanent pink color was obtained at the end point. The Acid value was calculated using the expression [77].

$$\text{Acid value} = V \times N \times 56.1 / W$$

Where, V= Volume of standard KOH solution in ml

N=Normality of standard KOH solution.

W=Weight of oil sample in grams.

i. Unsaponifiable Matter

The unsaponifiable matter was determined following the standard method as below. In brief, 30 mL of ethanol and 5 mL of 50% aqueous KOH were added to 5 mL of oil. The mixture was refluxed for one hour in boiling water bath and then transferred to a separating funnel. Extraction of unsaponifiable matter was done using petroleum ether (500 mL), washed with distilled water (1000 mL) and finally evaporated to dryness at 105 ^{o} C using oven. And then the residue was weighted and dissolved it the in 50 ml of warm neutral ethanol, containing a few drop of phenolphthalein indicator solution and titrated it with 0.1 N NaOH solutions. The unsaponifiable matter was calculated as a percent of oil as followed [54].

Calculations

Weight in g of the acids in the extracts (as oleic acid) = B = 0.282 x N x V

Where, V = Volume in ml of standard NaOH solution.

N = Normality of standard NaOH solution.

% of Unsaponifiable matter, by mass = 100 (A – B)/W

Where, A = Weight in g of the residue.

B = Weight in g of the fatty acids in the extracts.

W= Weight in g of the material taken for the test

3.6 Fatty Acids Constituents the Oil

a. Transesterification of the Oil

About 100 g of *N. physaloides* seed oil was weighed and transferred it in to 100 mL conical flasks. About 1 g of KOH (1% by weight of the oil) was weighed and dissolved it in 36.36 g anhydrous methanol. The KOH in methanol was dissolved completely and transferred it slowly to the sample in a conical flask with stirring and continually stirred for 120 minutes to complete the reaction. After completion of the reaction, the reaction mixture was transferred to a separating funnel and kept it to stand overnight for phase separation. The ester phase was decanted from the mixture and transferred it to another separating funnel for further washing to remove any traces of methanol excess and solution residual catalyst with water which was sprayed into the top of the separating funnel at a low velocity. During the washing, some of the ester formed an emulsion with the water; a time of 24-48 hours was required for the water phase containing alcohol, catalyst, and emulsified ester to settle and the ester phase to become clear [67].

b. GC-MS Analysis of the Seeds Oil

GC-MS analysis of the seeds oil was performed on a Shimadzu model (GC-MS-QP/5050A) instrument. Analytes were separated on a 30 m × 0.32 mm nonpolar capillary column with a phase thickness of 1.0 μm and was interfaced with a quadrupole mass spectrometer. The injector and interface temperature was kept at 275 °C and 300 °C respectively and the temperature was programmed to increase from 70 °C to 270 °C at a rate of 3 °C /min. Helium was used as the carrier gas with a linear velocity of 74.6 cm/s and the total flow rate was 39.0 ml/min. The MS operating parameters was: ionization voltage 70 eV, scan rate 500 amu/sec [73].

Identification of its constituents was accomplished by comparison of the mass spectra of the data obtained with the database on Mass Hunter in the Library of NIST14.L.

3.7 Phytochemical Screening

The qualitative phytochemical screenings of petroleum ether extracts of seeds of *N. physaloides* were carried out by using standard procedures as follows.

a. Test for Alkaloids

Two drops of Mayer's reagent was added to a few ml the oil of seeds of *N. physaloides* of along the side of test tube. Appearance of white creamy precipitate indicates the presence of alkaloids [78].

b. Test for Saponins

The sample of 50 mg oil of *N. physaloides* was diluted with distilled water and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 minutes. A 2 cm layer of foam indicates the presence of saponins [67].

c. Test for Flavonoids

An aqueous solution of the oil was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavonoids [78].

d. Test for Phenols

The sample of 0.25 g of was dissolved in 10 ml distilled water and filtered. 1% aqueous Iron chloride solution was added to the filtrate. The appearance of intense green, purple, blue or black color indicated the presence of tannins in the test samples [79].

e. Test for Glycosides

The sample of 5 ml oil of the seeds of *N. physaloides* was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution and 1ml of concentrated H_2SO_4. A brown ring at the interface indicates the presence of cardiac glycosides [80].

f. Test for Terpenoids

Two ml of chloroform and 3 ml of concentrated H_2SO_4 was added to 5 ml oil of the seeds of *N. physaloides* in the test tube. Formation of yellow colour ring at the interface of the two liquids that turns reddish brown color after two minutes, showed the presence of terpenoids [80].

g. Test for Quinones

One ml oil of seeds of *N. physaloides* was treated separately with alcoholic potassium hydroxide solution in the test tube. Quinines give coloration ranging from red to blue [80].

h. Test for Steroids

The sample of 0.5 g oil of seeds of *N. physaloides* was mixed with 2 ml of acetic anhydride followed by 2 ml of H_2SO_4 in the test tube. The color changed from violet to blue or green in some samples indicated the presence of steroids [79].

3.8 Antibacterial and Antifungal Activity Tests

For the experiments, six microorganisms including Gram-positive bacteria (*S. aureus* (ATTC25923) and *B. subtilis* (ATTC6633), Gram-negative bacteria *E. coli* (ATTC25922) and *P. aeruginosa* (ATTC27853) and fungal Strains (*Fuzarium spp and S. cervisiae*) were used. These micro-organisms were provided from the Department of microbiology (Jimma University).

The microorganism working stocks was enriched on a tube containing of Mueller-Hinton broth for bacteria and Sabouraud dextrose broth for fungi, then incubated at 37 °C for 18-24 h for bacteria and 24-48h for fungi. The cultures were used for the antimicrobial activity of various *N. physaloides* seeds solvent extract used in this study. The overnight cultures of the microorganisms were streaked across the entire media surface disk plates. Each of the disk plates partitioned in six regions, for petroleum ether, chloroform, acetone and methanol *N. physaloides* seeds extract sample, one for negative control and the center the positive control. Sterile filter discs of diameter 6 mm of Whatman Paper No.1 were impregnated with 0.2 mg/ml by each crude extract sample, which was prepared by 0.2 mg of each solvent extracts dissolved in 1 ml DMSO and controls. Gentamicin was used as a positive control for bacterial and Micandzole the fungal micro-organisms. The negative control was DMSO. The dishes were incubated at 37 °C for 18-24 h for bacterial and 24-48 h for fungi strains. The diameters of the zones of inhibition around each of the discs were taken as measure of the antimicrobial activity [81].

4. Results and Discussions

The fixed oil of *N. physaloides* seeds was extracted by solvent petroleum ether and it was subjected to the analysis of different physicochemical parameters, phytochemical screening and antimicrobial activities. These are discussed in the following sections.

4.1 Percentage Yield of the Oil

Oil extracted from seeds *N. physaloides* using petroleum ether as a solvent was collected separately and the percentage was calculated as 19.66%. This indicates a good oil yield to be used as a raw material in industries. The yield is higher than the value reported in soybeans oil with value 18% and cotton seed oil with value 14%. The value is lower than the oil yield reported in palm oil (63%), coconut oil (70 %) and groundnut oil (45%) respectively. This may be because of the high moisture content, because for a good oil yield, the moisture content must be very low. This may be because of the high moisture content, because for a good oil yield, the moisture content must be very low [56].

4.2 Physical Characteristics of the Oil

The oil form *N. physaloides* seed is yellowish-green in color and has liquid consistency at room temperature which may be due to the presence of unsaturated fatty acids.

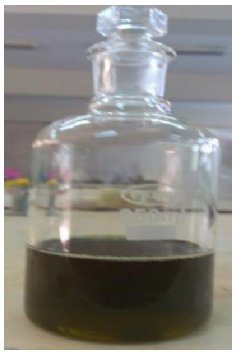


Figure 4 Physical appearance of fixed oil of seeds of *N. physaloide*

Others physical characteristics of the oil are summarized on Table 1. The moisture content in the *N. physaloides* seeds oil was 1.32, but the acceptable limit is < 0.2 for edible oil. The slight high moisture content the oil may affect the stability and result to a shorter shelf life of the oil [56]. Ash content is a measure of the total amount of minerals within oil sample. The amount of total inorganic residues in *N. physaloides* seeds oil is 1.33 which is not in the range of the acceptable

limits (1.5-2.5%) for edible oil [56]. This may indicate that the oil contains less essential minerals elements. Relative density of the *N. physaloides* seeds oil obtained is 0.86; this indicates that oil is nearly less than the level advised by FAO/WHO (0.919-0.925) [82]. Refractive index is an indication of the level of unsaturation and chain length of oil. The refractive index at 21^0C of *N. physaloides* seeds oil is found to be 1.48 which was slightly above the level recommended by FAO/WHO (1.466-1.470). This signifies that the oil probably contain highly unsaturated or long chain fatty acids in their triglycerides [82].

Table 1 Physical Properties of *N. physaloides* Seeds oil

Properties	Value
Physical state (at room T^0)	Liquid
Color	Yellowish-green
Refractive index (21^0C)	1.48 ± 0.00
Relative density	0.86 ± 0.01
Moisture content (%)	1.32 ± 0.07
Ash content (%)	1.33 ± 0.04
Each data is mean ± STD of triplicate measurements	

4.3 Physicochemical Properties of the Oil

The physicochemical characteristics of the oil are summarized on Table 2.

Table 2 Chemical Properties of *N. physaloides* Seeds oil

Properties	Value
Acid value(mg KOH/g)	1.63 ± 0.00
Free fatty acid (as oleic %)	0.30 ± 0.02
Peroxide value (meq O_2/kg oil)	0.49 ± 0.02
Iodine number (g I_2 /100 g)	150.77 ± 0.03
Saponification value (mg KOH/g)	181.58 ± 4.32
Unsaponifiable matter (%)	0.83 ± 0.28
Ester value, (S.V - A.V) oil mg KOH/g	179.95 ± 4.32
Each data is mean of three replicates + standard deviation	

The physicochemical properties of the oil were carried out according to standard protocols. These properties of the oils showed that its utilities as raw materials in industries and for human consumption. Therefore, these properties of *N. physaloides* Seeds oil are discussed in the following paragraphs.

Acid value of the oil shows the presence of free fatty acids in the oils. Free fatty acids cause rancidity of the oils. Oils become rancid when stored for a long time due to the release of free fatty acids. The quality of such oils can be determined by the acid value of oils. The acid value of N. physaloides seeds oil was 1.63 mg KOH/g oil. The permissible level of it for all edible oils should be below 0.6 mg KOH/g of oil, according to FAO/WHO recommendation, which shows a slight high value than the permissible range for edible oil but it signifies a maximum purity and suitability of the it for soap production but edibility of the oil is very low and not recommended for consumption and further treatment is necessary to lower its acid content [58].

The peroxide value is a quality scale for determining the freshness of lipids (edible oils or fats). The lower the peroxide value, the fresher is the lipid (edible oils or fats). Its value should be below 10 meq/kg oil according to FAO/WHO standards for edible vegetable oils [83]. Therefore, the low peroxide value 0.49 of obtained from *N. physaloides* seeds oil (Table 2) indicates that the oil can be kept for a very long period of time.

Iodine value is the mass of iodine in grams that is consumed by 100g of unsaturation contained in fatty acids [55]. The results show in Table 2 below, *N. physaloides* seeds oil has the highest iodine value of 150.77g/100g, indicating that the fatty acids presence are unsaturated and which makes it a drying oil that well suited for industries making oil paints and inks.

Saponification is the alkaline hydrolysis of oils or fats, since one of the products of the alkaline hydrolysis is soap that is potassium or sodium salts of higher fatty acids. The alkaline hydrolysis gives a way of determination of a constant known as Saponification value. Its value is an indication of the size or nature of fatty acid chains esterified to glycerol and gives a measure of the average length of the fatty acid chain that makes up a fat. It is inversely proportional to the mean molecular weight of the fatty acid in the glyceride present in the oil [55]. The results in Table 2 below showed that the saponification value for *N. physaloides* seeds oil 181.58 mg KOH/g. The ester value of an oil sample was carried out, which is the

saponification value minus the acid value of oil and its value is 179.95. The highest saponification value indicates a high content of triacylglycerol or triglycerides, consistent with the high ester value, and also indicate that the oil has the potential to be used for cosmetic industry [84].

The unsaponifiable matter is defined as the substances soluble in an oil which after saponification are insoluble in water but soluble in the solvent used for the determination [58]. The results in Table 2 above *N. physaloides* seeds oil showed a low unsaponifiable matter of 0.83 %, which is in an acceptable range according to set by FOA/WHO as standards for edible oils.

4.4 Fatty Acid Compositions of *N. physaloides* Seeds Oil

The chromatogram for analysis of oil extracted from *N. physaloides* seeds revealed that the oil contains 4 different compounds as presented in figure 6 below. Of 11.435, 13.305, 13.367 and 13.568 with component at 13.37 retention times accounting for highest concentration (63.08%) and compound with retention time 13.568 with the lowest concentration (5.65 %).

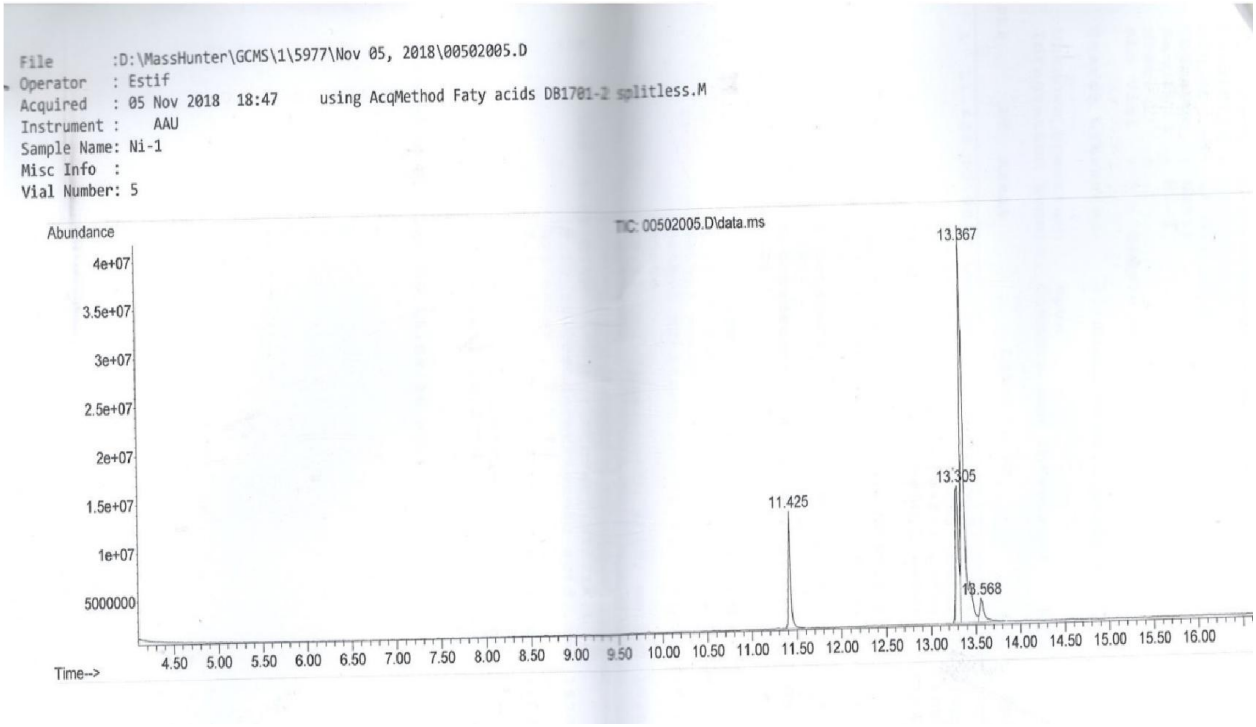


Figure 5 The GC-MS chromatogram of the *N. physaloides* Seeds oil

Table 3 Probable compounds form *N. physaloides* seeds oil

No.	RT	% Area	Probable Compounds Names	Quality
1	11.425	12.88	Hexadecanoic acid, methyl ester	98
			Hexadecanoic acid, methyl ester	98
			Hexadecanoic acid, methyl ester	97
2	13.305	18.40	9-Octadecenoic acid (Z)-, methyl ester	99
			8-Octadecenoic acid, methyl ester (E)-	99
			6-Octadecenoic acid, methyl ester (Z)-	99
3	13.367	63.08	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	99
			Methyl 10-trans, 12-cis- Octadecadienoate	99
			10,13-Octadecadienoic acid, methyl ester	99
4	13.568	5.65	9,12,15 Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	98
			9,12,15 Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	90
			9,12,15 Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	90

Identification of the four fatty acids was done by comparing the mass spectra of each component with the database on Mass Hunter in the Library of NIST14.L. And then the compositions of the oil were identified with quality of comparison about 98-99% match.

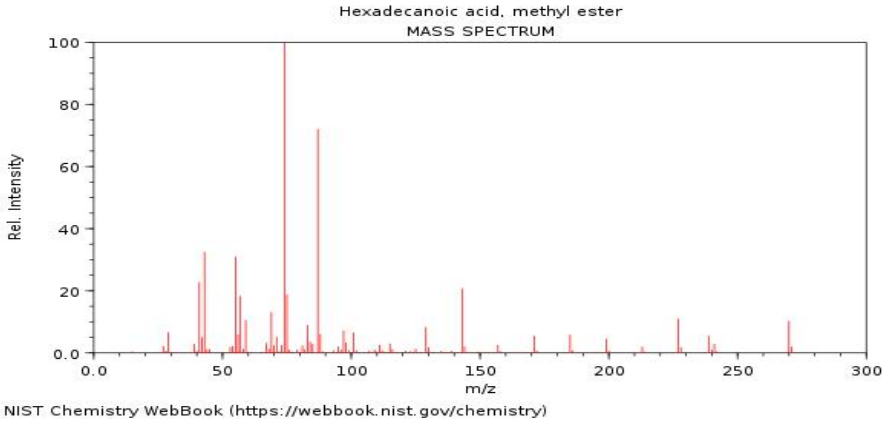


Figure 6 Mass spectrum of Hexadecanoic acid, methyl ester

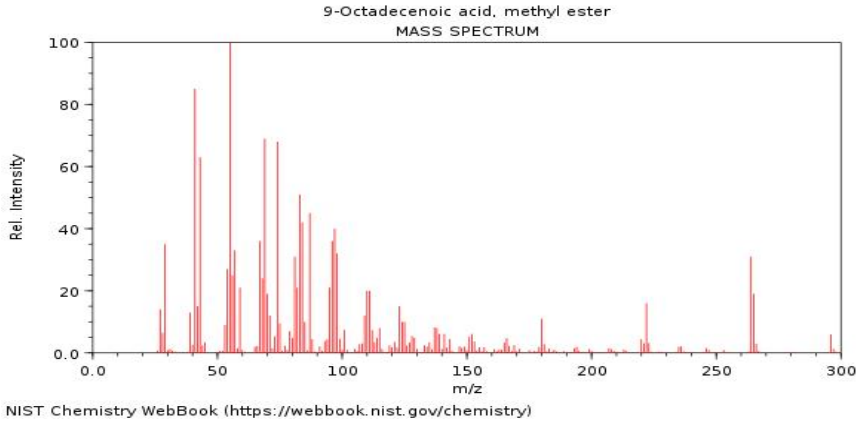


Figure 7 Mass spectrum 9-Octadecanoic acid, methyl ester

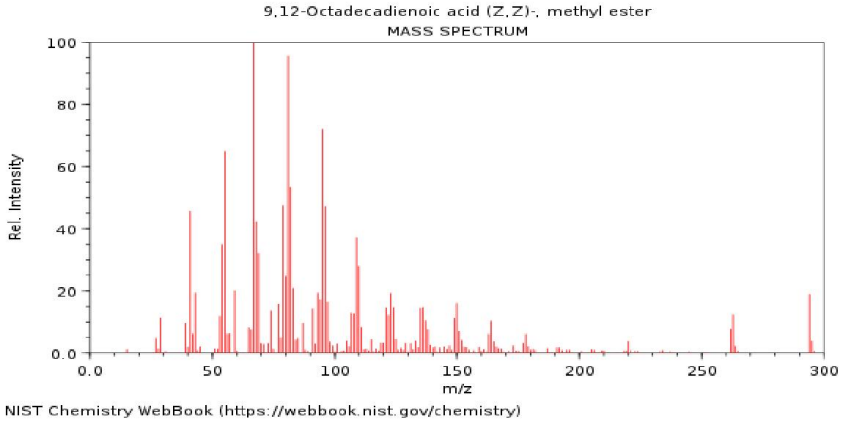


Figure 8 Mass spectrum 9, 12-Octadecadienoic acid (Z, Z)-, methyl ester

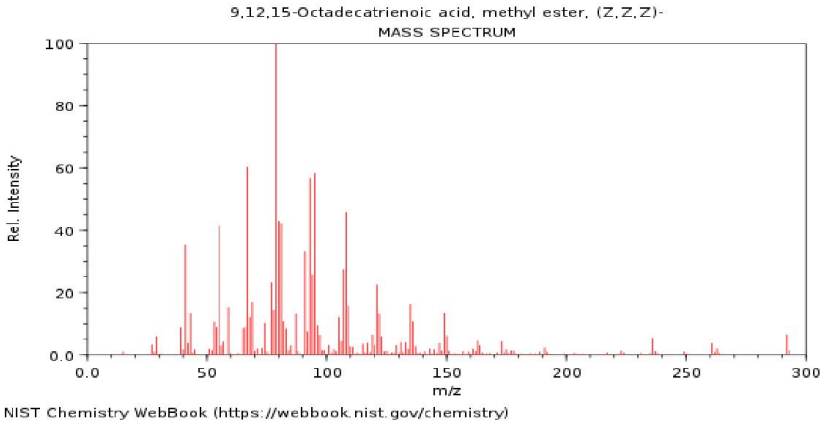


Figure 9 Mass spectrum 9, 12, 15-Octadecatrienoic acid (Z, Z, Z)-, methyl ester

Table 4 Fatty acid compositions *N.physaloides* Seeds oil

Common name	Systematic name	RT	Area %	General Formula	Numerical symbol
Palmitic acid, methyl ester	Hexadecanoic acid, methyl ester	11.425	12.88	$C_{17}H_{34}O_2$	C16:0
Oleic acid, methyl ester	9-Octadecenoic acid (Z)-, methyl ester	13.305	18.40	$C_{19}H_{36}O_2$	C18:1
Linoleic acid, methyl ester	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	13.367	63.08	$C_{19}H_{34}O_2$	C18:2
α-Linolenic acid, methyl ester	9,12,15 Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	13.568	5.65	$C_{19}H_{32}O_2$	C18:3

Based on the list of candidate compounds obtained from NIST Library (Table 3) on comparison the four fatty acids constituents in the oil were identified to be Linoleic acid (63.08%), Oleic acid (18.40 %), palmitic acid (12.88%) and α-Linolenic acid (5.65%). Unsaturated fatty acids represented 87.13% of the total studied fatty acids. However, linoleic acid and oleic acid represent 93.50% of the unsaturated fatty acids.

Vegetable oils containing the most common essential fatty acids have more unsaturated fatty acids such as Linoleic acid and α-Linolenic acid are than saturated fatty acids are more desirable as food and are found to lowering blood serum cholesterol [54]. In addition, since the oil contained high percentage of linoleic as seen in table 3 which is one of the most important essential polyunsaturated fatty acid in human food, therefore, its presence in the body prevents distinct heart vascular diseases in the body [89]. Characterization of high level of linoleic acid in *N. physaloides* seed oil also signifies its potential use in cosmetic products as skin moisturizer, to aids healing process of dermatoses and sunburns, treatment of acne vulgaris and as vehicle for topical delivery of pharmaceutical agents.

4.5 Phytochemicals Screening of *N. physaloides* Seeds Oil

The study phytochemical screening of the oil was planned to perform in seeking of fulfilled the antimicrobial activity of the oil if not active against the microorganisms. And the study revealed

that presence of alkaloids, steroids, flavonoids, quinones, glycosides and terpenoids whereas saponins and tannins are absent in oil.

The present study regarding the qualitative analysis of the petroleum ether extracts of *N. physaloides* seeds showed that the richness of the seed with phytochemicals (Table 5) and it is in agreement with the previous findings of the various researchers, those that had done phytochemicals on the other morphological parts of the plant *N. physaloides* [27-29].

Table 5 Phytochemicals screening of the *N. physaloides* seed oil

No	Phytochemical Constituents	Indication	No	Phytochemical Constituents	Indication
1	Alkaloids	+	5	Glycosides	+
2	Saponins	-	6	Quinones	+
3	Steroids	+	7	Terpenoids	+
4	Flavonoids	+	8	Phenolics	+

Key: Present (+) and absent (-)

4.6 Antimicrobial Activity of *N. physaloides* Seeds Oil

The oil of seeds of the plant *N. physaloides* was screened for antimicrobial activity against six standard test organisms; two Gram-positive bacteria- *S. aureus*, *B. subtilis*, two Gram-negative bacteria- *E. coli, P. aeruginosa* and two fungal strains- *Fusarium spp and S. cervisiae* [83].

Table 6 Antimicrobial activity tests of *N. physaloides* Seeds oil (0.2 mg/ml) with control drugs

Solution Test	Zones of inhibition (mm)					
	S. aureus	*B.subtilis*	*E.coli*	*P. aeruginosa.*	*Fusarium spp*	*S. cervisiae*
Oil	12	10	12	11	7	12
G	21	20	20	21	-	-
D	Na	Na	Na	Na	Na	Na
M	-	-	-	-	20	20

Key: G- Gentamycin, D- DMSO, M-Micandazone, Na, Not active.

The oil has showed low activity on all bacteria and fungi. The diameters of zone of growth inhibition was 10-12 (for bacteria), 7-12 (for fungi) as shown on table 6.The activity shown on *S. Cervisiae* was slightly higher than *Fusarium spp* but activities on both Gram-positive and Gram-negative bacteria were comparable.

The observed low antibacterial and antifungal activity seeds oil of *N. physaloides* may be attributed to its high content of unsaturated fatty acids (linoleic, linolenic and oleic acids). Studies also indicate effectiveness of plants containing linoleic and linolenic acids for reduction of acne lesion associated with their inhibitory effect on Propionibacterium a causative agent for acni. Similar studies carried on antibacterial activity of linoleic and oleic acids derived from *H. pedunculatum,* actuivity was only revealed against the Gram-positive bacterial species but not on Gram-negative species. [64]. But in our case, the oil showed activity against both types of bacteria and also tested fungi.

In addition our phytochemical screening data showed the presence of active compounds which may also account for the observed antimicrobial activity. Alkaloids and flavonoids are anti-oxidants and free radical scavengers, which prevent oxidative cell damage, and also anti-microbial activity [85]. Quinones are highly reactive colored aromatic rings, which have a similar activity to flavonoids as they are able to inactivate proteins through binding irreversibly to its nucleophilic amino acids. And also terpenoids have an antimicrobial effect upon bacteria such as *Listeria monocytogenes*, fungi, viruses, and protozoa [86]. Glycosides are known to lower the blood pressure according to many reports [87]. Plant steroids are used in the cosmetic, soap and pharmaceutical industries to produces creams, soaps and CDX ointments. They served as treatment against inflammatory diseases [88].

5. Conclusion and Recommendations

5.1 Conclusion

This study was aimed on the extraction of *N. physaloides* seed oil using petroleum ether as solvent. In addition, the physicochemical properties, phytochemical screenings and antimicrobial activities were carried out on the oil following standard test protocols. The fatty acid composition of the oil was also determined from GC-MS data of the oil methyl ester.

The oil yield obtained was 19.66%, which is good amount to be used as a raw material in industries. Physical and Physicochemical characteristics of the extracted oil were; relative density (0.86), refractive index at 21 ˚C, (1.48), moisture content (1.32), ash contents (1.33), acid value (1.63 mg KOH/g), free fatty acid (0.30 as oleic %), peroxide value (0.49 meq O_2/kg oil), iodine value (150.77 g I_2 /100 g oil) saponification value (181.58 mg KOH/g), ester value (179.95 mg KOH/g) and unsaponifiable matter (0.83), respectively. The oil contained Linoleic acid (63.08%), Oleic acid (18.40 %), palmitic acid (12.88%) and α-Linolenic acid (5.65%). The phytochemical screening of the oil revealed the presence of active compounds such as alkaloids, glycosides, quinones, steroids, terpenoids, and flavonoids and in addition, the oil was shown weak activity (7-12 mm) against bacteria (*S. aureus, B. subtilis, E. coli and P. aeruginosa*) and fungi (*Fuzarium spp and S. cervisiae*).

The high saponification value and high ester values indicate that the oil has the potential to be used for cosmetic and soap industries. The high iodine value and high amount of unsaturated fatty acids (87.13%) indicating that the oil will have high drying characteristics making it suitable raw material in making oil paints and inks as well as suitability for human consumption(but not regularly), in cosmetic and pharmaceutical production. But low oil yield (19.66%) and low content of Oleic acid (18.40 %) limits its candidacy for biofuel.

Generally, from the results obtained it can be concluded the seed of notorious weed in Ethiopia (*N. physaloids)* to have potential as a raw material for fixed oil which can be used as a raw material food, industries , cosmetic and medicinal purposes.

5.2 Recommendations

Thought the study of physicochemical properties, phytochemical constituents and its antimicrobial activities of the fixed oil revealed its utility as raw material in food, industries, cosmetic and medicinal purposes. Further studies are recommended.

1. Nutritional constituents of the seeds oil and its solvent extraction byproduct in order to ascertain its usage as foods for animals.
2. The bioactive compounds of the seeds oil and the seeds shall be needed to identify in spectroscopic methods.
3. Identification of toxic compounds in seeds oil shall be needed to ascertain it whether it is suitable for human consumption or not.

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Appendix

Appendix 1 Mass spectra of unknown fatty acids components of *N.physaloides* seeds oil

Library Search Report

Data Path : D:\MassHunter\GCMS\1\5977\Nov 05, 2018\
Data File : 00502005.D
Acq On : 05 Nov 2018 18:47
Operator : Estif
Sample : Ni-1
Misc :
ALS Vial : 5 Sample Multiplier: 1

Search Libraries: D:\MassHunter\Library\NIST14.L Minimum Quality: 80

Unknown Spectrum: Apex
Integration Events: ChemStation Integrator - autoint1.e

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1	11.425	12.88	D:\MassHunter\Library\NIST14.L			
			Hexadecanoic acid, methyl ester	130813	000112-39-0	98
			Hexadecanoic acid, methyl ester	130820	000112-39-0	98
			Hexadecanoic acid, methyl ester	130818	000112-39-0	97
2	13.305	18.40	D:\MassHunter\Library\NIST14.L			
			9-Octadecenoic acid (Z)-, methyl e ster	155750	000112-62-9	99
			8-Octadecenoic acid, methyl ester, (E)-	155753	026528-50-7	99
			6-Octadecenoic acid, methyl ester, (Z)-	155752	002777-58-4	99
3	13.367	63.08	D:\MassHunter\Library\NIST14.L			
			9,12-Octadecadienoic acid (Z,Z)-, methyl ester	153890	000112-63-0	99
			Methyl 10-trans,12-cis-octadecadie noate	153874	1000336-44-2	99
			10,13-Octadecadienoic acid, methyl ester	153881	056554-62-2	99
4	13.568	5.65	D:\MassHunter\Library\NIST14.L			
			9,12,15-Octadecatrienoic acid, met hyl ester, (Z,Z,Z)-	152042	000301-00-8	98
			9,12,15-Octadecatrienoic acid, met hyl ester, (Z,Z,Z)-	152041	000301-00-8	90
			9,12,15-Octadecatrienoic acid, met hyl ester, (Z,Z,Z)-	152040	000301-00-8	90

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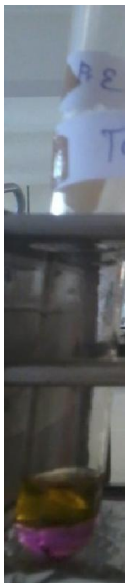
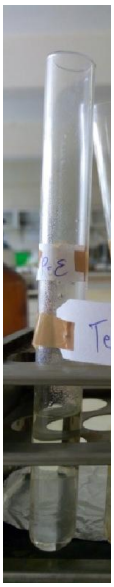
Appendix 2 Phytochemical screening of *N.physaloides* seeds oil


Alkaloides


Steroides


Quinones


Glycosides


Terpenoides


Flavonoides

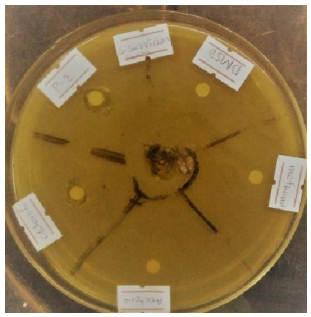
Appendix 3 Antimicrobial activities of *N.physaoides* seeds oil


E. coli


B. subtilis


P. aeruginosa


S. aureus


S. Cervisiae


Fusarium spp