ADDIS ABABA UNIVERSITY
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Extraction, Optimization, Characterization of Ruta Chalenpensis oil (Tenadam)

A Thesis submitted to the school of Chemical and Bio Engineering in partial fulfillment of the requirements of the Degree of Masters of Science in Chemical and Bio Engineering (Under Process engineering stream)

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This is to certify that the thesis prepared by Keresa Defa, entitled: ***Extraction, Optimization and Characterization of Ruta Chalepensis oil (Tenadam)*** and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Chemical and Bio Engineering (Under Process Engineering stream) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Table of Contents

List of Figures

List of Acronyms and Abbreviations

ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemistry
AOCS	American Oil Chemists Society
RSM	Response Surface Methodology
SFE	Supercritical fluid Extraction
DPPH	2,2-Diphenyl-1-picrylhydrazyl
IE	Inhibition Effect
WGARC	Wando Genet Agricultural Research Center
SNNPE	Southern Nation Nationalities and People of Ethiopia
EIAR	Ethiopian Institute Agricultural Research
DOE	Design of Experiments
TBHQ	Tert-butyl hydroquinone
GAE	Gallic Acid Equivalent
POV	Peroxide Value
SOV	Saponification Value
AV	Acid value
SC-CO_2	Super Critical Carbon dioxide
FFC	Face Centered Cube
FC	Folin ciocalteu Phenol
SFME	Solvent- free micro wav extraction
IC_{50}	Inhibition concentration of 50%
ERCA	Ethiopian Revenues and Custom Authority
2FI	Two Factors Interaction
TPC	Total Phenolic Compound
QE	Quercetin Equivalent
GC-MS	Gas Chromatography-Mass Spectroscopy
BHT	Butylated hydroxytoluene
BHA	Butylated hydroxyanisole

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Lists of Table

Lists of Appendixes

Abstract

In this study extraction, optimization and characterization of Ruta chalepensis oil which has been recognized as good sources of crude oils, essential oils and other important chemicals was investigated. The crude oil was extracted by soxhlet apparatus using hexane as a solvent. The process parameters such as extraction temperature, time, and solid mass to solvent ratio were optimized for maximum yield of crude oil using response surface method. The maximum crude oil yield (16.68 %) was obtained at extraction temperature of $85^{o}C$, extraction time of 6 hrs and solid mass to solvent ratio of 13. Physico - chemical properties of the crude oils studied for the optimum yield show that the oil is a solidifying oil at room temperature having dark green colour with a pleasant smell having melting point of $50^{o}C$, boiling point of 160 ^{o}C, specific gravity of 0.725 and P^{H} 4.49-5.24 and characterized by having acid value 6.73mg KOH/gram, peroxide value 5.023 milliequivalents of active O_2/ kg, iodine value 0.50706 gram I_2 /100g, saponification value 196mg KOH/g. Ruta chalepensis crude oils extracted in incubator shaker using solvents ethanol, methanol, distilled water, diethyl ether and hexane were compared with the Ruta chalepensis crude oil extract using soxhlet extractor of the optimum yield and shows varied value of total phenolic compound and antioxidant activity evaluated using both maximum inhibition effect and IC_{50}. The total phenolic compounds are arranged in decreasing order as follows: aqueous extract> methanol extract> ethanol extract> diethyl ether extract> optimized hexane extract of soxhlet extractor > hexane extract by incubator shaker. Methanolic crude extract has maximum antioxidant activity followed by ethanol crude extract in terms of both maximum inhibition effect and IC_{50}. and Ruta chalepensis crude extract oil extracted using soxhlet extractor has appreciable antioxidant activity than that of aqueous extract, diethyl ether and hexane extract of incubator shaker in terms of IC_{50} .The chemical composition of essential oils extracted from Ruta chalepensis using Clevenger hydro steam distillation and identified using GC-MS have 2-undecanone (methyl nonyl ketone) (31.74), 1-dodecene or α-Dodecene(13.591), Tridecene (13.189), 2-Nonanone (methyl heptyl ketone) (9.573) ,2-tetradecanol (4.856) as the major components and the chemical structure of these compounds possess the functional group of ketones, alkenes which is one type of hydrocarbons and alcohols being dominated by ketones followed by alkenes and alcohols respectively.

Key words: *Response surface methodology, Total Phenolic compound, antioxidant, Ruta chalepensis, essential oil and GC-MS.*

Chapter One

1. Introduction

1.1. Back ground

Ruta chalepensis is originally indigenous to the Mediterranean region and the Canary Islands. It is cultivated in the tropics as a potherb or medicinal plant and has widely become naturalized. In tropical Africa it has been introduced in several countries, including the Cape Verde Islands, Sudan, Ethiopia, Somalia and southern Africa (including South Africa), where it is mostly cultivated in herbal gardens. It has also been naturalized in peninsular Arabia, India, Malaysia, Vietnam and Java. It has furthermore been naturalized in the United States, Mexico, Cuba and Chile. It is cultivated in several countries in tropical Africa where it is used for cooking and medicinal purposes (Jansen, P.C.M., 1981).

Ruta chalepensis (Rutaceae) commonly known as rue is used in the traditional medicine of many countries for the treatment of a variety of diseases (Neuwinger, 2000). The medicinal value of this plant for the treatment of nervous diseases was emphasized by Dioscorides (Stuart, 1979). In Saudi Arabia, a decoction of the aerial parts of the plant is used as an analgesic and antipyretic and for the treatment of rheumatism and mental disorders.

The plant is prescribed in the Indian system of medicine for the treatment of dropsy, neuralgia, rheu-matism and menstrual and other bleeding disorders (Foucaud, 1953). In China, a decoction of the roots of the plant is used as antivenom (McClure and Hwang, 1934). The leaves of this plant infused with vinegar are given to children for the treatment of convulsions and other nervous disorders. An aqueous decoction of the leaves is used for the treatment of fevers in Africa (Kritikar and Basu, 1984). Keeping in view of its widespread use and effectiveness, Stuart (1979) suggested detailed scientific studies on this plant .The medicinal and culinary properties are attributed to the presence of essential oils and other chemicals which are contained in all parts of the plant (Kloos *et al.,* 1978).

Essential oils are volatile and liquid aroma compounds from natural sources, usually plants. They are not oils in a strict sense, but often share with oils a poor solubility in water and often have an odor and used in food flavoring and perfumery. They are natural products obtained from plants which referred to any concentrated, hydrophobic, typically lipophilic liquid of plants that contains highly volatile aroma compounds and carries a distinctive scent, flavor, or essence of the plant. They were formed by varied and complex volatile mixtures of chemical compounds, with predominance of terpene associated to aldehyde, alcohols and ketone. This large and diverse class of oils also is also referred to as volatile oils or ethereal oils. Essential oils are found in diverse parts of plant including leaves, seeds, flowers, roots and barks (Rubiolo et al., 2010).

For the plant, essential oils are thought to be vital for the life of the plant, containing compounds that help to fight parasites and infections. In addition, essential oils may also act as antibacterial , antiviral , antifungal, antioxidant , insecticides. For people, essential oils are used in perfumes, cosmetics, and bath products, for flavoring food and drink, for scenting incense and household cleaning products, and for medicinal purposes. Interest in essential oils has revived in recent decades, with the popularity of aromatherapy, a branch of alternative medicine which claims that the specific aromas carried by essential oils have curative effects.

Essential oils are usually isolated by fragrance extraction techniques such as distillation (including steam distillation), cold pressing, or extraction (maceration). Typically, essential oils are highly complex mixtures of often hundreds of individual aroma compounds. An essential oil is a concentrated hydrophobic liquid containing volatile aroma compounds from plants. Essential oils are also known as volatile oils, ethereal oils, or aetherolea, or simply as the "oil of" the plant from which they were extracted, such as oils of cloves. Oil is “essential" in the sense that it carries a distinctive scent, or essence, of the plant. Essential oils do not form a distinctive category for any medical, pharmacological, or culinary purpose.

By 1550, pharmacists, chemists, and physicians were studying the physical, medicinal and chemical properties of the oils and essential oils. Since then the number and types of individual oils have increased enormously. International markets and industries have evolved to deal solely in essential oils. As a result of 20th century distillation technology, essential oils can be now regarded as industrial raw materials. Their complex mixture of chemical compounds can be separated and the individual compounds can be used as building blocks to introduce particular flavor or aroma into the product. The essential oils can be produced in almost all plant organs such as flowers, buds, stems, leaves, fruits, seeds and roots etc. These are accumulated in secretary cells, cavities, channels, and epidemic cells (Burt, 2004). Almost all odoriferous plants contain essential oils.

The raw material from which essential oils are manufactured may be fresh, partially dehydrated or dried . The extraction of the essential oil depends mainly on the rate of diffusion of the oil through the plant tissues to an exposed surface from where the oil can be removed by a number of processes (Oscan, 2003).

There are different methods, depending upon the stability of the oil, for the extraction of the oil from the plant materials. The essential oils obtained by steam distillation or by cold-pressed are Generally preferred for food and pharmacological applications. Due to the bactericidal and fungicidal properties of essential oils, their pharmaceutical and food uses are becoming increasing important as alternatives to synthetic chemical products to protect the ecological equilibrium (Burt, 2004). The extracted oils can vary in quality, quantity and in the chemical composition depending up on the agro climate, plant organ, age and vegetative cycle stage (Masotti *et al*., 2003)

The complexity of essential oils requires various techniques for studying the chemical profiles of them and some of these techniques are: IR-spectroscopy, UV-spectroscopy, NMR spectroscopy and Gas chromatography-Mass spectroscopy. The increasing importance of oils and essential oils in various domains of human activities including pharmacy, cosmetics, aromatherapy, and food and beverages industry has prompted an extensive need of reliable methods for analyses of oils. The combination of gas chromatography and mass spectrometry (GC-MS) allows rapid and reliable identification of oils components.

Since oils plays great role in perfumes cosmetics, soaps and other products, for flavoring food as spices and drink, and for adding scents to incense and household cleaning products, this thesis is examined extraction, characterization, optimization and evaluating total phenolic compound, detecting the antioxidant effect of oils from *Ruta chalepensis (Tenadem)* which is source of oils including essential oils and other chemicals that is used for the above application.

Anti-oxidant effect is important parameter to check whether the oils have potential to neutralize the free radicals formed in our body due to metabolic activities, in certain fats and oils foods and they play a great role for food preservatives by increasing their shelf life. Natural antioxidant extracted from different plant species have great advantage over the synthetic organic antioxidants because the later are suspected to be the potential cause of cancer (carsogenic substances) that is threatening seriously the global world population

1.2. Problem Statement

Ethiopia is an agrarian country situated in the tropics, the wide geographical distribution enable to have diverse geographical distribution climates which make the country to cultivate different plant species. One of the plants that is cultivated and naturalized in the country is *Ruta chalepensis* (Tenadam). It is widely known for its medicinal and aromatic properties. The aromatic behavior of plants mostly associated with the chemical compositions of the extractive substances. These extractive substances can be classified into fixed oil, resinoids and essential oils. The essential oils which are extracted from spice are used in the manufacture of high quality perfumes and lotions, food flavorings and medicine. *Ruta chalepensis* (Tenadem) is among the well-known plants which consists of different chemicals apart from essential oils that can contribute a lot for industrialization. It is fast growing and easy to cultivate, can survive drought and poor soil and keeps its leaves almost all year round.

Ruta chalepensis (Tenadem) is available in almost part of Ethiopian region including even in the city where people plant it in their garden and use it for flavoring purpose in the coffee and use it for medicinal purpose. Most Ethiopian people plant this herb without having knowledge about its use. Nonetheless largely rural people use leaves of these plants for village medicine, especially to cure headache, to treat swellings, influenza, stomachache, fever, common cold, and teeth ache earache disease. From the literature, it is observed, world science approves the application of this plant for medicinal purpose as internal as well as external treatment. Unlike its medicinal purpose, *Ruta chalepensis* (Tenadam) is also used for production of cosmetics and perfumes, as well as for its antioxidant effect etc.

The Ethiopian community prepare their village medicine traditionally from Ruta leaves but, the concentration of the active constituent's chemical is very week. Extraction of oil from this plants is a very simple process, anyone can prepare it locally at home. Processing these plant in small industrial scale do not require complicated physical equipment and it is cost effective with regard to energy and raw materials usage. Thus, we can extract more concentrated essential oil from *Ruta Chalenpensis* . Despite its vast potential of this plant as a sources of essential oils, dried oils and other chemicals, our country is not utilizing this resource. This may be due to lack of adequate research and knowledge about its potential application in industrial scale.

According to data from Ethiopian Revenues and Custom Authority (ERCA) around 5,5000,000 US dollars import of essential oils and retinoid which are used for application in cosmetics, medicines, perfumes, toilets application from Ireland and France and closely observing, imported essentials and resinoids for 2013 cost about 2,500,000 USD dollars . When we see data annually starting from 2004 up to 2013 there is substantial increase in quantity and value of importing of these essential oils from 23,000,000 USD dollars to 55,000,000 USD dollars. Demand for the various types of oils including essential oils will increase mainly with the expansion and of the food, soft drinks, alcoholic drinks, pharmaceuticals and cosmetics industry and various chemical industries. Due to the favorable climate created for foreign and local investors, a number of food, beverage, pharmaceutical, chemical and cosmetics manufacturing projects are in the pipe line for establishment. Considering the past demand, ERCA puts an annual growth rate of 15%, which is almost equal to the historical trend, to forecast the future demand related to essential oils . In addition , ERCA estimates a consumption level of 18,500,000 kg of cosmetics and this organization shows much emphasis on the production of herbicides, perfumes in the same way . These all above areas highly triggers the production of oils and essential oils .As result, this thesis aims to close the knowledge gab on the oils of Ruta chalepensis and highlight the technology behind there on the production of essential oil.

This thesis specifically addresses the extraction of oils, characterization and optimization of the processing parameters of *Ruta chalepensis* that widely grows with aim of reducing import of essential oils by exploiting the available resource and contribute there by towards the industrialization of the country.

1.3. Objectives

3.1. General Objectives

The main objectives of this thesis work were optimization, characterization and extraction of oil from *Ruta Chalepensis* (Tenadam).

3.2. Specific Objectives

The specific objectives of this work were to:

- Study the effect of extraction time, temperature, and solid to solvent ratio on the yield of oils extracted from *Ruta Chalenpensis* with hexane as solvent by using soxhlet extractor.
- Optimize process parameters (extraction time, temperature, solid mass to solvent ratio) for maximum yield using response surface central composite face centered cubic design.
- Study physico-chemical properties of *Ruta Chalepensis* oil extracts obtained using optimum processing parameters.
- Determine chemical composition of *Ruta Chalepensis* essential oils obtained by steam hydro distillation using gas chromatography- mass spectroscopy detector (GC-MSD).
- Evaluate total phenolic compounds of *Ruta chalepensis* oil and extracts obtained using different solvents.
- Evaluate antioxidant activity of *Ruta chalepensis* extracts obtained using different solvents by DPPH radical scavenging method.

1.4. Significance of study

The application of this project can be applied for both generating knowledge and solving the current problems which is currently prevailing on the utilization of essential oils and oils in our country. It is paramount important in investigating oils and essential oils present in the *Ruta Chalepensis* and brings a great opportunity that brings strategic solution of changing agriculturally produced plants of this plants for industrial application. Organizations like that of cosmetics and perfume industry, pharmaceutical industry ,medicine and other food processing sector will be benefited a lot from this project since the expected result is cross linked with these organizations.

Chapter 2

Literature Review

2.1. Ruta chalepensis

Ruta chalepensis which is locally known as Tenadam is found in the plant kingdom and belongs Rutaceae family. Ruta is old Latin plant name for rue; the same word means also bitterness, unpleasantness, perhaps referring to the strong smell or taste of rue. 'Chalepensis' is originated from or first collected at Aleppo, Syria. There are so many varieties of this plant with different names. Some of these are Ruta bracteosa, Ruta chalepensis L, and Ruta graveolens L (Jansen, P.C.M., 1981). In Ethiopia, this plant do have different names in different places local names like: tenadam, adam, taladdam, gulla (Amarigna); dehn, tenadam (Tigrigna); talatam, talles, dscharta (Oromigna) and Trade names such as rue, herb of grace (English); rue, rue des jardins, grande rue, rue d'Algérie (French); Raute, Weinraute, Gartenraute, Kreuzraute (German). Commercial production of essential oil from Ruta chalepensis and Ruta graveolens is centered in the Mediterranean region. In Ethiopia either dried fruits or fresh or dried twigs with leaves, flowers and fruits are found commonly in the local markets (Jansen , P.C.M., 1981, 1981)

2.2. Geographical Distribution and Plant description

Ruta chalepensis is indigenous to the Mediterranean region and the Canary Islands. It is cultivated in the tropics as a potherb or medicinal plant and has widely become naturalized. In tropical Africa it has been introduced in several countries, including the Cape Verde Islands, Sudan, Ethiopia, Somalia and southern Africa (including South Africa), where it is mostly cultivated in herbal gardens. It has also been naturalized in peninsular Arabia, India, Malaysia, Vietnam and Java. It has furthermore been naturalized in the United States, Mexico, Cuba and Chile. In Africa, the morphologically and chemically related *Ruta graveolens* L. seems only to be present in South Africa. Ruta chalepensis grows well in well-drained sandy or rocky limestone soils and prefers an open sunny position. In Ethiopia it is cultivated between the altitudes 1500-2000 m.

Ruta chalepensis has protandrous flowers. The stamens do not develop all at once but one after the other. At maturity a stamen turns towards the center of the flower, the anther dehisces its pollen and the stamen turns back. In this way the male flowering time is lengthened and the chance of shedding the pollen on a visiting insect increased, as insects usually stand on the ovary to reach the nectar which is present on the disc. Probably because of its strong smell, the flower is often visited by rubbish-visiting flies. At the end of male flowering, the style lengthens and rises above the ovary lobes and the stigma becomes receptive. Cross-pollination is normal, but, as the anthers can approach the stigma because of the wilting process of the stamens, some self-pollination might occur (Jansen, P.C.M., 1981).

2.3. Uses of Ruta Chalepensis

Ruta chalepensis is cultivated in several countries in tropical Africa where it is used for cooking and medicinal purposes. The medicinal and culinary properties are attributed to the presence of essential oils and oils which are contained in all parts of the plant. The tops of the fresh shoots are the most active and should be gathered before the plant flowers. In northern Sudan, a fruit poultice is applied to swellings. In Ethiopia Ruta chalepensis is an important medicinal plant. An aqueous-alcoholic extract of the leaves is drunk as an anti-implantation and uterotonic medicine. A decoction of the pulverized fruits in milk is taken to treat diarrhea. A root decoction in an alcoholic drink, with hot peppers, is taken to treat influenza. Plant sap is taken to treat stomach-ache. A leaf decoction with tea is taken to treat headache, fever and common cold. In southern Africa, the oil obtained from the aerial parts is applied externally as a rub to treat stomach-ache, colic, hysteria, epilepsy and is taken orally as an anthelmintic (El Sayed, K.,200).

Among southern Africa, a decoction of the whole plant in high doses is taken to ease child birth. In South Africa, a leaf decoction of *Ruta chalepensis* or *Ruta graveolens* is taken for the treatment of typhoid and scarlet fever, whereas the leaf juice is given to children suffering from convulsions, fits, jaundice and diarrhea. The crushed leaves are externally applied to treat toothache and earache. A maceration of the leaves is taken to treat cardiac and respiratory diseases, rheumatism, gout and hypertension. Leaves are taken in tea or chewed to treat stomach-ache and headache.

Ruta chalepensis and *Ruta graveolens* which are the most common species of the Ruta have traditionally been used for centuries as a condiment of food and alcoholic beverages in the Mediterranean region, but their use has much declined because of its bitterness. The fragrance of the leaves is strong, characteristically aromatic and sweet. The fruits taste similar, but stronger and somewhat hot.

In Ethiopia fresh leaves of *Ruta chalepensis* are used as a flavorings of a beverage called 'kuti', which is an infusion of coffee leaves; the leaves are also a component of the spicy Capsicum sauce 'berbere'. Washed leaves are added to sour milk to make a local cheese .The plants can be planted to deter dogs and cats, as they hate the smell. The dried and crushed leaves are also an effective insect repellent. Both the herb and its essential oils have been widely used in the past in Europe as an anthelmintic, stomachic, antispasmodic, anti-epileptic, rubefacient, emmenagogue and abortifacient. Excessive use of the herb is dangerous. Its toxic effects are clearly dose-dependent. It is potentially toxic and carcinogenic when consumed orally, and can produce dermatitis when touched. Used internally, the leaves and oil can cause haemorrhages, miscarriage and abortion, and have been used as such since ancient times. It may further cause vomiting, gastroenteritis, swelling of the tongue, coldness of the extremities, and even death. For some people, ingestion. causes increased photosensitivity and can lead to severe sunburn .The oil is used as a flavorings agent and in perfumes and soap scents. Oils, which are rich in methyl nonylketone are used for the preparation of methyl-n-nonyl acetyl aldehyde, widely applied as a synthetic perfume (Jansen ,P.C.M., 1981).
Ruta chalepensis is popularly used for its medicinal and food flavoring properties. Application of the linear, phototoxic furanocoumarins in medicine as found in *Ruta* is well documented, for instance in the treatment of psoriasis. Therefore, *Ruta chalepensis* merits further research on its potential as a local or industrial source (Jansen , P.C.M. *et al.,*1981).

2.4. Properties and Chemical Composition of *Ruta chalepensis*

The compounds isolated from *Ruta chalepensis* and *Ruta graveolens* are essentially the same, although quantitative differences are observed. However, these differences are of the same magnitude as those observed within the same species collected from different sources. Qualitative and quantitative differences are also found for the different parts of the plants. Both species are characterized by the presence of alkaloids (acridone-, quinolone-, furoquinolone-type alkaloids, and quaternary furoquinolines, furano- coumarins) and essential oils.

Chemical composition Ruta chalepensis also contains an essential oil, which can be obtained by Clevenger type steam distillation. It is yellow-green oil tasting and smelling bitter, with bluish-violet fluorescence, present up to 0.6% in plants growing in the wild and up to 0.08% in fresh cultivated plants or 0.1% in dried cultivated plants (Watt & Breyer-Brandwijk, 1962). This oil contains up to 80% methyl-n-heptyl keton and 10% methylnonylketon (in Ruta graveolens the ratio of the substances is exactly the reverse) (Gamier *et al.,* 1961). The yellow colour of the petals is caused by the presence of rutin, which is a rhamno-glucoside of quercetin. Rutin is a substance which has a constrictor action on the capillary bed and decreases the permeability and the fragility of the vessels (Watt & Breyer-Brandwijk, 1962). Fresh leaves contain 390 mg vitamin C per 100 g (Gamier *et al*., 1961). The essential oils from aerial parts of *Ruta chalepensis* plants harvested at different stages of growth in northern India contained 19 components, representing 85.4–93.3% of essential oils. The major components were 2-undecanone (41.3–67.8%), 2-nonanone (5.2–33.6%), 2-nonyl-acetate (2.8–15.3%) and 2-dodecanone (<0.1–11.6%). The essential oils isolated from the *Ruta chalepensis* plant are classified into different functional groups and they are dominated by Non-terpenoid compounds and ketones. This functional groups are found in it are shown Table 2.1.

Table 2.1. Principal chemical classes (%) in the essential oils from wild and cultivated Ruta chalepensis

Principal chemical classes	R. chalepensis (%)	
	Wild plant	Cultivated plant
Hydrocarbon monoterpenes	2.9	6.5
Oxygenated monoterpenes	0.7	0.4
Hydrocarbon Sesquiterpenes	0.5	2.1
Oxygenated Sesquiterpenes	0.1	0.4
Non-terpenoid compounds	90.3	83.2
Ketones	59.4	63.7
Aldehydes	0.8	1.1
Alcohols	5.2	0.7
Esters	23.6	15.4
Others	1.3	2.3

Major compounds isolated from the roots of Ruta chalepensis are the furoquinolin, alkaloids, kokusaginin, skimmianin and graveolin, the acridone alkaloids 1-hydroxy-N-methylacridone and chaloridon, and the furanocoumarin chalepensin(El Sayed, K. *et al.* ,200). From the dried aerial parts, major compounds isolated include the furoquinolin alkaloids kokusaginin, skimmianin, graveolin, γ-fagarin and dictamnin, the acridone alkaloid arborinin and the (furano-)coumarins bergapten (or 5-methoxypsoralen) and chalepensin Bergapten. Chalepensin belong to the family of the linear furanocoumarins, which are known to have phototoxic activity. Dermatitis may arise after plant material containing these compounds comes into direct contact with the skin, if this is immediately followed by exposure to UV-A light, e.g. from the sun (Gunaydin, k .*et al.* ,2005).

Ethanol extracts of air-dried flowering material have been studied in various models. Oral administration at a dose of 500 mg/kg significantly reduced carrageenan-induced oedema in rats. Similar results were reported using the cotton-pellet granuloma test model. Additionally, intraperitoneal administration at 100 mg/kg significantly reduced subcutaneously induced (yeast suspension) fever in mice. However, no analgesic activity was observed in the hot-plate test with mice. Of the isolated compounds, chalepensin at an intraperitoneal dose of 10 mg/kg significantly prolonged hexobarbital-induced sleeping time in mice. A hexane extract of the aerial parts showed strong molluscicidal activity against the schistosomiasis-transmitting snail Bulinus truncatus. Ethanolic extracts furthermore showed in vitro activity against the bacteria Staphylococcus aureus and Pseudomonas vulgaris (disk diffusion assay).

Information on the antifertility effect of *Ruta chalepensis* presents a mixed picture. Whereas oral consumption of the essential oil induces abortion in guinea-pigs and humans, this probably can be attributed to a general toxic effect. The essential oil has no effect on the isolated uterus in (non-) pregnant cats or the isolated oviduct in (non-)pregnant women. An ethanol extract of the plant, however, shows a significant anti-implantation effect, an increased absorption rate and an overall reduced pregnancy ratio in albino rats. Petroleum ether and methanol extracts are reported to have similar results, whereas benzene and chloroform extracts merely have a toxicological effect. The antifertility effect is attributed to the furanocoumarin chalepensin, which has a very narrow therapeutical range. Rutin(7–8% in the dried leaves) and quercetin glycoside containing the disaccharide rutinose as sugar component are responsible for the bitter taste of this plant. It is known for its capillary protection properties.

Extracts of *Ruta chalepensis* have also been shown to possess anti-inflammatory properties and its leaves contain 390 mg vitamin C (Ascorbic acid) per 100 g (Gamier et al., 1961). Ruta chalepensis have Pharmacological properties such as antibacterial, analgesic, anti-inflammator, antidiabetic due to the presence of compound Rutin, quercetin, psoralen, methoxypsoralen. Italso showed significantly higher concentrations of total phenolics (1328.8 mg GAE/100g), highest value of Calcium (122.9 mg/g), potassium contents (52.0mg/g) ,Protein (10.4% dry matter), fat (4.2% dry matter), fibre (20.2 % dry matter) ,ash (8.7% dry matter) and carbohydrate (51.7% dry matter) (Khalil *et al.,*2001).

4.4. Botanical Description of *Ruta chalepensis*

The plant of *Ruta chalepensis* are erect, densely branched sub shrub 0.5–1.5 m high. Leaves spirally arranged, 2–3-pinnatisect, obovate to oblong-obovate in outline, 4–15 cm ×2–9 cm, ultimate segments obovate-lanceolate, about 5–30 mm ×1.5–6 mm, conspicuously glaucous, crenate, translucent glandular punctate, strong smelling, lower leaves more or less petiolate, up to 12.5 cm long, 2(–3)-pinnate; stipules absent. Inflorescence a bracteate cyme, terminal or in the upper leaf axils, often combined into a corymb, bracts cordate-ovate, wider than the subtended branch. Flowers bisexual, 4(–5)-merous, protrandrous, central flowers 5-merous; pedicel 0.5–2 cm long; sepals deltate-ovate, 3–4mm × 2–3 mm, glabrous; petals free, oblong, 4–8 mm long, fringed with cilia not as long as the width of the petal, greenish-yellow outside, yellow inside; anthers twice as many as the petals; ovary superior, almost round, 4–5-lobed, 3–5-celled. Its fruit is a 4-lobed capsule, 5–7mm × 5–8mm, segments acuminate, apically opening, 5–10-seeded having three-edged, kidney-shaped, and dark brown or brownish-black seedling with epigeal germination.

Ruta comprises about 8 species. The botanical identity of Ruta grown in tropical Africa is not always clear. The presence of the related Ruta graveolens in tropical Africa is based on misidentifications, certainly so in Ethiopia. The chemical composition of both species is almost identical. Ruta graveolens is a polyploid. The medicinal and culinary uses in South Africa are similar to those of Ruta chalepensis (Jansen, P.C.M., 1981).

Figure 2.1: Ruta chalepensis (Tenadam) plants.

2.6. Factors Affecting Essential oil Accumulation

Factors that determine the composition and yield of the essential oil obtained are numerous. In some instances it is difficult to segregate these factors from each other, since many are interdependent and influence one another (Terblanche, 2000). These variables may include seasonal and maturity variation, geographical origin, genetic variation, growth stages, part of plant utilized and postharvest drying and storage (Marotti, 1994).

2.6.1. Seasonal and Maturity Variations

These two factors are interlinked with each other, because the specific ontogenic growth stage will differ as the season progresses. There are variations in the chemical profile of essential oils from various plants collected during different seasons. The essential oils yields varied considerably from month-to-month and was also influenced by the micro-environment (sun or shade) in which the plant was growing. Results obtained by Badi (2004) also indicated that timing of harvest is critical to both yield and oil composition.

2.6.2. Geographical Variation

There are many reports in the literature showing the variation in the yield and chemical composition of the essential oil with respect to geographical regions (Uribe- Hernandez, 1992). Chalchat *et al.,*1995 reported variations in the yield and chemical profile of essential oils, collected from different geographical locations, respectively. Such differences could be linked to the varied soil textures and possible adaption response of different populations, resulting in different chemical products being formed, without morphological differences being observed in the plants (Hussain *et al*.,2008). Altitude seems to be another important environmental factor influencing the essential oil content and chemical composition. Climatic factors such as heat and drought were also related to the essential oil profiles obtained (Uribe-Hermandez et *al*., 1992). Moreover, the preference of the plant for these conditions suggest that genetic make-up of the plant, rather than the soil-type in which it is growing, should have a greater influence on the chemical profile of the oil produced (Abdullah, 2009).

2.6.3. Genetic Variation

Genotype is typically defined as “the genetic make-up of an organism, as characterized by its physical appearance or phenotype”, while chemotype is generally defined as “a group of organisms that produce the same chemical profile for a particular class of secondary metabolites”. Variations in chemical profiles were observed from oils produced from specimens from the same population and location, demonstrating the presence of different chemotypes within this species. Genetic makeup of the plant is one of the most important contributors to their essential oil composition.

2.6.4. Other Factors Affecting Yield and Composition of Essential oil

Other factors which affect the growing plants thus leading to variations in oil yield and composition, include part of plant used; post-harvest drying; length of exposure to sunlight; availability of water, height above sea level, plant density, time of sowing and the presence of fungal diseases and insects. The oil composition and yield may also change as a result of the harvesting methods used, the isolation techniques employed, the moisture content of the plants at the time of harvest and the prevailing extraction conditions (Abdullah, 2009). Post-harvest drying of material is an accepted practice in the production of essential oils. Drying methods include exposure to natural air in the shade, sun-drying, as well as drying by blowing warm air over the material. Post-harvest drying is thought to improve oil yield and accelerate distillation, by improving heat transfer, in addition to providing increased loading capacity, due to loss of plant moisture. Further advantages include the reduction of microbial growth and the inhibition of some biochemical reactions in dried material. However, some amount of the oil may be lost during such post-harvest treatment due to volatilization and mechanical damage to oil glands. Essential oil components (including terpenoids) are usually present in the free form, but may also be bound to sugar moieties, usually mono- or disaccharides (Abdullah, 2009).

2.5. Essential oils and their chemistry

2.5.1. Essential oils

Essential oils are natural, volatile, complex plant compounds, oily or lipid-like in nature and frequently characterized by a strong fragrance (Bakkali F.*et al.*, 2008). They have a low solubility in water but are soluble in fats, alcohol, organic solvents and other hydrophobic substances and are generally liquid at room temperature. They are stored in specialized plant cells, usually oil cells or ducts, resin ducts, glands or trichomes (glandular hairs) (Baser *et al.*,2007) and may be extracted from the leaves, flowers, buds, seeds, fruits, roots, wood or bark of plants by a variety of methods, including solvent and supercritical fluid extraction, expression under pressure, fermentation or enfleurage, but either low- or high-pressure steam or hydro-distillation are used predominantly for commercial production(Lahlou, M.,2004). Essential oils make up only a small proportion of the wet weight of plant material, usually approximately 1% or less (Pengelly, A., 2004; Langenheim, 1994). The presence, yield and composition of essential oils may be influenced by many factors, including climate, plant nutrition and stress (Croteau, R., 1986).

In commercial production settings, selection and breeding programmers are often instigated to improve yields and foster desired compositions (Figueiredo et al., 2008). Essential oils are also called ethereal oils, volatile oils, plant oils or aetheroleum. The term ‘essential oil’ groups together a wide range of chemical compounds on the basis of their historic use and method of extraction, usually steam distillation, and belies the variety and complexity of compounds found within them (Obst, J.R, 1998).

Some plant families are particularly well known for their oil-bearing species. These include Apiaceae (also known as Umbelliferae), Asteraceae (also known as Compositae), Cupressaceae, Hypericaceae (sometimes included as a subfamily of the Guttiferae/Clusiaceae), Lamiaceae (previously known as Labiatae), Lauraceae, Fabaceae (also known as Leguminosae), Liliaceae, Myrtaceae, Pinaceae, Piperaceae, Rosaceae, Rutaceae, Santalaceae, Zingiberaceae and Zygophyllaceae (Baser et al., 2008).

Essential oils are often described as secondary plant metabolites. Traditionally, secondary plant metabolites have been all those compounds synthesized by the plant which do not appear to be essential for plant growth and development and/or those compounds without an obvious function (Croteau et al., 2000). They are also not universally synthesized in all plants. In contrast, primary metabolites are produced by all plants, usually have an obvious function and are part of the essential metabolic processes of respiration and photosynthesis (Theis et al., 2003). This artificial and rather simplistic division is also difficult because the natural functions of many secondary plant metabolites are unknown simply because they have never been investigated; this lack of evidence or knowledge is then interpreted as a lack of function (Pichersky *et al.*, 2006). Greater interest in investigation of secondary metabolites in recent years has led to the discovery that they have roles in defense, signaling and as intermediates in secondary metabolism.

2.5.2. Chemistry of Essential Oils

Essential oils are not simple compounds or even simple mixtures of several individual compounds. They may contain up to approximately 100 components, although many contain about 20 to 60 (Pengelly, A., 2004). The compounds found in essential oils are from a variety of chemical classes, predominantly terpenes, but phenylpropanoids and other compounds also occur although at lesser frequency and often, but not always, in smaller proportions (Friedrich, H., 1976). They are all hydrocarbons and their oxygenated derivatives, and they may also contain nitrogen or sulfur. They are generally low-molecular-weight compounds with limited solubility in water (Griffin, S.G. *et al.*, 1999).The classification and nomenclature of essential oil compounds is complicated by the fact that many were isolated and studied before the investigation of systematic chemical nomenclature. Consequently, many are known by nonsystematic or trivial or common names (Obst, J.R., 1998). These are sometimes but not always based on their source, such as eucalyptol, limonene, pinene and thymol, names which hint at historical botanical origins of these compounds. In terms of shedding light on their chemistry, the long history and widespread use of these nonsystematic names further obfuscates the chemical nature and characteristics of essential oils and their components.

2.5.2.1. Terpenes.

Terpenes, also known as isoprene, or terpenoids or isoprenoids when they contain oxygen. They are the largest group of natural compounds, with over 30, 000 known structures (Breitmaier, E., 2006). The name 'terpene' comes from the fact that the first described members of this class were isolated from turpentine, the monoterpene-rich liquid obtained from the resin of various *Pinus* spp. (Breitmaier, E., 2006). Traditionally, terpenes have been regarded as polymers of isoprene (C_5H_8) joined together in a repetitive head-to-tail manner. This is largely a legacy of work by the German chemist Otto Wallach, who was the first to recognize that many terpene compounds could be hypothetically constructed in this fashion (Croteau, R. *et al.*, 2000). This concept, known as the isoprene rule, was the first step in rationalizing the enormous variety of terpenes, since it accounted for the structure of many, but not all, terpenes. However, head-to-head combinations also occur, as do tail-to-tail and head-to-middle combinations (Glaeske, K.*et al.*, 2002). This variation in initial arrangement of the isoprene units coupled with the numerous rearrangements and substitutions that can occur afterwards mean that the isoprene origins of the final compound are often obscured, or at the very least not obvious to the non-chemist. Furthermore, although terpenes may be viewed as polymers of isoprene, the biosynthesis of terpenes does not occur by the successive addition of single isoprene units. Leopold Ruzicka, recipient of the 1939 Nobel Prize in Chemistry, addressed many of the limitations of Wallach's isoprene rule when he proposed the biogenetic isoprene rule (Ruzicka, L., 1953). His concept was revolutionary since it emphasized the single biochemical origin of terpenes rather than the ultimate structure (Little, D.B. *et al.,* 1999) and this approach has proved the most practical. Terpenes are classified by the number of isoprene units from which they were biogenetically derived, even though loss or addition of carbon atoms may have subsequently occurred (Croteau, R.*et al.*, 2000). Therefore, hemi-, mono-, sesqui- and diterpenes contain 1, 2, 3 and 4 isoprene units, respectively. Triterpenes and tetraterpenes contain 6 and 8 isoprene units, respectively. Monoterpenes are the most common terpenes found in essential oils, followed by sesquiterpenes. Many essential oils are composed mainly of monoterpenes and sesquiterpenes and their oxygenated derivatives (Dung, N.T.*et al*, 2008).

2.5.2.1.1Monoterpenes

Monoterpenes are formed when two C_5 isoprene units are joined, yielding a skeleton with the molecular formula $C_{10}H_{16}$ (Pengelly, A., 2004). Despite this initial simplicity, subsequent substitutions, cyclizations and/or isomerizations result in a remarkable number of monoterpenoid structures. Approximately 1500 monoterpenoids have been described (Breitmaier, E., 2006) although not all occur in essential oils. Monoterpenes may be cyclic (that is, ring-forming) or acyclic (also known as linear), regular or irregular, and their derivatives include alcohols, esters, phenols, ketones, lactones, aldehydes and oxides (Keszei, A.*et al.*, 2008). Cyclic monoterpenes include the monocyclic, bicyclic and even tricyclic compounds. The rings are produced in a multistep process called cyclization by enzymes called monoterpene cyclases via the universal intermediate, α-terpinyl cation (McGarvey *et al.*, 1995). Cyclic monoterpenes that contain a benzene ring such as *p*-cymene are known as aromatic monoterpenes and are common components of many essential oils. In this context, the term 'aromatic' refers to the benzene ring, consisting of a ring of delocalized electrons. In many instances the benzene ring makes a significant contribution to the biological activity of the component and to the whole essential oil, especially when a hydroxyl group is attached to the ring, forming a phenol (Shapiro, S.*et al.*, 1998). Use of the term 'aromatic' in this fashion should not be confused with the terms 'aromatic plants' or 'aromatic oils', which are often also used to describe about medicinal plants and essential oils and refer to the aroma or fragrance of plants and oils.

Acyclic monoterpenes found in essential oils may be regular, linear structures in which the head-to-tail arrangement of isoprene units is readily observed, such as the hydrocarbons -myrcene or the *(E)* and *(Z)* isomers of ocimene. Note that the *(E)* and *(Z)* notation for stereoisomers supersedes the *cis–trans* notation for stereoisomer. Other examples of acyclic monoterpenes commonly found in essential oils include geraniol, linalool and citronellol.

Monocyclic monoterpenes include the largest group of naturally occurring monoterpenes (Baser *et al.*, 2007) those that arise from the *p*-menthane skeleton by cyclization of a regular acyclic monoterpenoid. Important monoterpenes in this group include limonene, terpinene and terpinolene, as well as the aromatic hydrocarbon *p*-cymene and its hydroxylated derivatives thymol and carvacrol, both noted for their antimicrobial activity. Other notable compounds in this group are the carbonyls piperitone and pulegone. The biogenesis of bicyclic monoterpenes occurs by the further cyclization of monocyclic monoterpenes. They may be further categorized on the basis of the skeleton from which they are derived, including bornane, carane, camphane, fenchane, pinane and thujane (Croteau, R., 1986). Pinene and -pinene are common important constituents of essential oils, particularly pine oils, and are bicyclic monoterpenes formed by intermolecular rearrangement of the universal intermediate -terpinyl cation, producing the bicyclic structure.

Alternative cyclizations of the terpinyl cation yield the bicyclic skeletons for the bornane-, camphane- and fenchanetype monoterpenes. Thujane monoterpenes come from either the terpinen-4-yl cation or from the sabinyl cation and include thujene, sabinene and thujone. 3-carene, a carane-type bicyclic monoterpene, is a common component of various essential oils including those from *Pistacia lentiscus* L. and *Juniperus* spp. (Castola,V. *et al.*, 2000). Other important members of this group include the cyclic ethers 1, 8-cineole, 1, 4-cineole and ascaridol.

Tricyclic monoterpenes occur infrequently in essential oils compared to monocyclic and bicyclic monoterpenes. However, pinene oxide and tricyclene are two important examples found in essential oils. Irregular monoterpenes also occur and fall into two categories. The first is the troponoids or substituted cycloheptane monoterpenes. These are thought to be formed by ring expansion of the *p*-menthane skeleton (forming a seven-membered ring structure) and oxygenation of the side chain(s) (Zhao, J., 2007). Many are found in the heartwood of trees in the Cupressaceae family of evergreen shrubs and trees (Bentley, R., 2008). Examples include the thujaplicins (α-, β-and γ-isoforms) and nezukone. β -thujaplicin is also known as hinokitiol .The second category of irregular monoterpenes is formed by joining isoprene units in the less common non-head-to-tail arrangements (Croteau,*et al.*,2000). Compounds in this category include artemisia ketone, chrysanthemol and lavandulol. Many irregular monoterpenes are found in the genus *Artemisia* (Asteraceae) (Bentley, R., 2008).

2.5.2.1.2.Sesquiterpenes

In terms of their frequency in essential oils, sesquiterpenes are the second most common, after the dominant monoterpenes. They are formed from the combination of three isoprene units, giving them the molecular formula $C_{15}H_{24}$. They are a structurally diverse group, all deriving from farnesyl pyrophosphate by various cyclization processes often followed by skeletal rearrangement (Croteau *et al.*, 2000). Of the terpenoids found in essential oils, they are the most structurally diverse, with over 120 different skeletal types (Baser *et al*, 2007). Sesquiterpenes may be linear, branched or cyclic.

Acyclic sesquiterpenes feature in many essential oils and include the isomers nerolidol and farnesol and the α- and β- structural isomers of farnesene. *(E)* Isomers occur more commonly in nature than *(Z)* isomers and *(E)*-nerolidol is also found in many plants (Fugh-Berman *et al.*, 2004). Essential oils containing more than 90% *(E)*-nerolidol have been identified (Limberger *et al.*, 2005). Farnesol is an important component of the commercially important rose flower essential oil (Baser *et al.*, 2007) and of Australian sandalwood oil, *Santalum spicatum* (Piggott *et al.*, 1997).

Irregular acyclic sesquiterpenes have been identified in *Santolina* spp. (Asteraceae) (Ferrari, B. *et al*, 2005). Cyclic sesquiterpenes may be mono-, bi- or tricyclic. Monocyclic sesquiterpenes include abscisic acid, α-bisabolene and its oxygenated derivatives, α- and β-bisabolol, both present at high levels in chamomile (*Matricaria chamomilla*) oils (Ganzera *et al.*, 2006). Bicyclic sesquiterpenes include eudesmol, widdrol, guaiol and the group known as azulenes. Azulenes are responsible for the blue colour of some essential oils, such as chamazulene in chamomile oil (*Ganzera, H.et al*, 2006) and *Artemisia aborescens* oil (Sinico, C. *et al.*, 2005). The bicyclic caryophyllene is present in many essential oils, β-caryophyllene being the most common form, which may also occur as a major component (Henriques, A.T. *et al.,* 1993). Cedrene and santalol are examples of tricyclic sesquiterpenes. Cedrene occurs in many essential oils, including various cedarwood oils derived from *Juniperus* spp., *Cupressus* spp. and *Cedrus* spp. (Adams, R.P. ,1991) , while santalols are important constituents of sandalwood (*Santalum album*) oil (Sangwan, N.S *et al* ., 2001).

2.5.2.1.3. Diterpenes

Most diterpenes in essential oils are formed by the head-to-tail combinations of four isoprene units followed by rearrangement and/or substitutions. They are very common and important components of plant resins (Langenheim, J.H., 2003) but are also found in small quantities in many essential oils. They have the general molecular formula $C_{20}H_{32}$ and so are much heavier than their mono- and sesquiterpenoid counterparts. Their heavier molecular mass relative to the mono- and sesquiterpenes means they require a greater amount of energy to be liberated from plant parts by steam distillation. Their recovery and the concentration obtained from essential oils increases with increasing steam-distillation times (Baser *et al.,* 2007) and can be influenced by the extraction method. For example, supercritical CO2 extraction of essential oils has been shown to increase the concentration of diterpenes recovered from essential oils (Reverchon, E., 1997). As with monoterpenes and sesquiterpenes, they may be acyclic or cyclic.

Acyclic diterpenes include phytol. Phytol forms the hydrophobic side chain of chlorophyll and so is found in the leaves of all green plants (Obst, J.R., 1998). It occurs in many essential oils (Wu *et al.*, 2004). Another important linear diterpene is plaunotol, the main component of the Thai medicinal plant *Croton stellatopilosus* (Euphorbiaceae; formerly known as *C. sublyratus* (Breitmaier, E., 2006). A notable cyclic diterpene in essential oils is the monocyclic camphorene (also known as dimyrcene), a component of camphor oil from the tree *Cinnamonum camphora* (Lauraceae), more commonly known as the camphor laurel. Several isomers of camphorene are found in the essential oils distilled from the leaves and twigs of *P. lentiscus (*Lo Presti *et al., 2008*) and from mastic gum derived from the same plant (Boelens *et al.*, 1991). Bicyclic and tricyclic diterpenes also occur in essential oils. Bicyclic diterpenes found in essential oils fall largely into two structural groups, the labdanes and the clerodanes. Labdane representatives include manool and manoyl oxide while sclareol typifies the clerodane class of bicyclic diterpenes. These components can be found in essential oils such as those from *Salvia* spp. including *Salvia sclarea* or clarysage (Ulubelen, A.*et al.*, 1994). Tricyclic diterpenes that occur in essential oils include phyllocladene and 16- kaurene(Baser *et al.*, 2007) . Phyllocladene constitutes a significant portion of essential oils from *Araucaria* spp. (up to 61%) from the Araucariaceae family and 60% of the essential oil from the ancient

Wollemi pine, *Wollemia nobilis*, from the same family (Brophy *et al.,* 2000) .Tetracyclic and pentacyclic diterpenes also occur in essential oils, although they are minor components (Briggs *et al.*, 1975).

2.5.2.1.4. Norterpenes

Carotenoids (C40) are a class of higher terpenes based on eight isoprene units and are important in plants for several reasons, including their role in photosynthesis (Hirschberg, J., 2001). They do not occur in essential oils. However, they are relevant to oils because when their carbon backbone is cleaved, usually oxidatively, they yield a range of smaller compounds known as apocarotenoids (Kloer *et al.*, 2006) .The most common and widespread group of apocarotenoids occur when carotenoids are cleaved at the 9–10 position, yielding C_{13} products known as norterpenoids or norisoprenoids. These are important minor components of some essential oils, contributing particularly to aroma and flavor (Rodriguez-Bustamante *et al.*, 2007). Examples include β-ionone, the violet-like aroma found naturally in *Boronia megastigma(* Ghisalberti, E.L.,1998), and β-damascone from *Rosa damascena (*Babu *et al.* ,2002)

2.5.2.2. Phenylpropanoids

Phenylpropanoids arise from the aromatic amino acids phenylalanine and to a minor extent tyrosine (Wu *et al.*, 2008).They have a C_6 and C_3 skeleton composed of a six carbon aromatic ring with a three-carbon side chain. The aromatic ring is also known as a benzene ring. When the three-carbon side chain attached to the phenyl ring is shortened by two carbons, benzenoids are formed (Dudareva *et al.*, 2004). The term is often used to include phenylpropanoids (Jirovetz, L.et al., 2003). Only approximately 50 phenylpropanoids have been described. Phenylpropanoids occur in essential oils less frequently and usually less abundantly than terpenoids. However, some of the oils in which phenylpropanoids do occur contain significant proportions of them, such as the eugenol in clove oil, present at 70 to 90% of the oil (Gang *et al.*, 2001), or the methyleugenol-rich chemotype of the root essential oil of *Anemopsis calfornica*, or yerba mansa, containing 59% methyleugenol. Plant families in which phenylpropanoids occur more frequently include Apiaceae (Umbelliferae), Lamiaceae, Myrtaceae, Piperaceae and Rutaceae (Ghisalberti, E.L., 1998). Important phenylpropanoids include the hydroxycinnamic acids, anethole, chavicol, eugenol, and their methylated derivatives, estragol (methyl chavicol) and methyl eugenol, as well as the widely distributed cinnamaldehyde. Myristicin and dillapiole are two other phenylpropanoids that occur commonly in essential oils when phenylpropanoids are present.

2.7. Extraction Technique and Recent technology.

2.7.1. Steam Distillation

Steam distillation, or also known as hydro-distillation, is a special type of distillation for temperature sensitive materials like natural aromatic compounds. Many organic compounds tend to decompose at high sustained temperatures. Separation by normal distillation would then not be an option, so water or steam is introduced into the distillation apparatus. By adding water or steam, the boiling points of the compounds are depressed, allowing them to evaporate at lower temperatures, preferably below the temperatures at which the deterioration of the material becomes appreciable. If the substances to be distilled are very sensitive to heat, steam distillation can also be combined with vacuum distillation. After distillation the vapors are condensed as usual, usually yielding a two-phase system of water and the organic compounds, allowing for simple separation (Wikipedia, 2008). When a mixture of two practically immiscible liquids is heated while being agitated to expose the surfaces of both the liquids to the vapour phase, each constituent independently exerts its own vapour pressure as a function of temperature as if the other constituent were not present. Consequently, the vapour pressure of the whole system increases. Boiling begins when the sum of the partial pressures of the two immiscible liquids just exceeds the atmospheric pressure. In this way, many organic compounds insoluble in water can be purified at a temperature well below the point at which decomposition occurs (Wikipedia, 2008). Inevitably, steam distillation process is the simplest extraction technique. Yet, the process of extracting oil using steam distillation takes several hours with low yield, thus making the process less effective (Mazni, 2007). Steam distillation, hydro distillation and simultaneous distillation extraction methods are known to be the most common methods for the extraction of essential oils. It is known that conventional methods used for the extraction of essential oils from plant materials have some disadvantages mainly concerned with the quality of the final product. Losses of some volatile compounds, low extraction efficiency, degradation of unsaturated or ester compounds through thermal or hydrolytic effects and toxic solvent residue in the extract may be encountered using these extraction methods Moreover, these extraction methods are time-consuming. Therefore, new technologies have been developed for obtaining essential oils (Bayramoglu, 2007).

2.7.2 Ultrasonic Extraction

Ultrasound, the term used to describe sounds ranging from 20 kHz to 1 GHz, is usually generated by a transducer which converts mechanical or electrical energy into high frequency vibrations. Sun, &Wang2008) reported that the enhancement of extraction efficiency of organic compounds using ultrasound is attributed to a phenomenon called cavitation produced in the solvent by the passage of an ultrasonic wave. They found that cavitation bubbles are produced and compressed during the application of ultrasound, allowing higher penetration of the solvent into the raw plant materials and intracellular products released by disrupting the cell walls. Ultrasound has been shown to aid extraction in a number of plant materials by significantly reducing extraction time and increasing maximum extraction yield, respectively

Recent studies have shown that the ultrasound-assisted extraction can enhance the extraction efficiency through acoustic cavitations and some mechanical effects. Acoustic cavitation can disrupt cell walls facilitating solvent to penetrate into the plant material and allowing the intracellular product release. Another mechanical effect caused by ultrasound may also be the agitation of the solvent used for extraction, thus increasing the contact surface area between the solvent and targeted compounds by permitting greater penetration of solvent into the sample matrix. Therefore the main advantages of ultrasound assisted extraction include the reduced extraction time and reduced solvent consumption. In addition, ultrasound-assisted extraction can be carried out at a lower temperature which can avoid thermal damage to the extracts and minimize the loss of bioactive compounds (Zhanget *et al.,* 2007).

2.7.3 Microwave Extraction

Microwaves are high frequency electromagnetic radiation with a typical wavelength of 1mm to 1 m. Many microwaves, both industrial and domestic, operate at a wavelength of around 12.2 cm (or a frequency of 2.45 GHz) to prevent interference with radio transmissions. Microwaves are split into two parts, the electric field and the magnetic field component. These are perpendicular to each other and the direction of propagation (travel) and vary sinusoidally. Microwaves are comparable to light in their characteristics. They are said to have particulate character as well as acting like waves. The 'particles' of microwave energy are known as photons. These photons are absorbed by the molecule in the lower energy state (Eo) and the energy raises and electron to a higher energy level (Ei). Since electrons occupy definite energy levels, changes in these levels are discrete and therefore do not occur continuously. The energy is said to be quantized. Only charged particles are affected by the electric field component of the microwave. If the charged particles or polar molecules are free to move, this causes a current in the material. However, if they are bound strongly within the compound and, consequently, are not mobile within the material, a different effect occurs. The particles re-orientate themselves so that they are in-phase with the electric field. This is known as dielectric polarization.

Microwave heating has been recently used for the isolation and analysis of essential oils (Lucchesi *et al.*, 2007). Solvent-free microwave extraction (SFME) is a new technique which combines microwave heating with dry distillation at atmospheric pressure for the isolation and concentration of the essential oils in fresh plant materials. In SFME method, there is no need to add any solvent or water if fresh plant material is used. If dry plant material is used, the sample is rehydrated by soaking in water for some time and then draining off the excess water.

2.7.4. Solvent Extraction.

Solvent extraction is the process of separating one constituent from a mixture by dissolving it into a solvent in which it is soluble but in which the other constituents of the mixture are not, or at least less soluble. The separation process is performed by the process of percolation, where a solvent is passed through the mixed solids and, thus, the analyte which is soluble in the solvent is extracted into that solvent. If the solvent used is water, then this process can be termed leaching. When the term solvent extraction is used, it is usually taken as meaning extraction involving two or more liquids, i.e. liquid-liquid extraction. In liquid-liquid extraction, the solution containing the desired constituent must be immiscible with the liquid used to extract the desired constituent. When the extraction process has occurred, the phase which contains the extracted analyte is known as the extract phase; while the sample from which the analyte has been removed is named the refined phase. The extraction of an analyte from one phase into a second phase is dependent upon two main factors: solubility and equilibrium. The principle by which solvent extraction is successful is that 'like dissolves like'. That is, to remove a polar solute from a solution a polar solvent should be used. Qualitative predictions can be made on the likely success of an extraction by considering the polarity of the analyte of interest and of the two solvents used. Uncharged solutes are more easily extracted into non polar organic solvents and the less polar the solute the more efficient the extraction process. If the solute is charged, then it is usually best to form an ion-pair with a counter ion and extract the newly formed neutral complex into a non-polar solvent. One of the most important decisions when using any solvent extraction system is the selection of the solvent to be used. The properties which should be considered when choosing the appropriate solvent are: selectivity; distribution coefficients; insolubility; recoverability; density; interfacial tension; chemical reactivity; viscosity; vapour pressure; freezing point; safety and cost (Wikipedia, 2008).

2.7.5 Supercritical Fluid Extraction (SFE)

A supercritical fluid is any substance at a temperature and pressure above its thermodynamic critical point. It can diffuse through solids like a gas, and dissolve materials like a liquid. Additionally, close to the critical point, small changes in pressure or temperature result in large changes in density, allowing many properties to be "tuned". Supercritical fluids are suitable as a substitute for organic solvents in a range of industrial and laboratory processes. Carbon dioxide and water are the most commonly used supercritical fluids, being used for decaffeination and power generation respectively (Wikipedia, 2008). Supercritical fluids extraction (SFE) is an efficient alternative for the extraction of natural substances from foods (Mendes *et al.*, 2003) and (Sun and Temelli, 2006). Supercritical fluids possess excellent extractive properties such as high compressibility, liquid-like density, low viscosity, high diffusivity (Lim *et al.*, 2002). They have been widely used in many industrial applications, i.e. the decaffeination of coffee, the extraction of hops, the synthesis of polymers, the purification and the formation of nano particles (Lim *et al,* 2002; Kopcak and Mohamed, 2005; and Machmudah *et al.*, 2006). For extraction of natural substances, supercritical carbon dioxide (SC-CO2) is generally used. It has a greater ability to diffuse through the ultra-fine complex matrix than conventional organic solvents and can be easily separated from the products by depressurizing process. Furthermore, low critical temperature of carbon dioxide means that the SC-CO2 system could be operated at moderate temperature, preventing the degradation of the substance due to heat induction (Lopez *et al.*, 2004; Vasapollo *et al.*, 2004; Machmudah *et al.*, 2006). As a result, the obtained product is pure and of great quality, and thus safe for use as nutritional additives and for pharmaceutical applications (Mendes et *al.*, 2003).

Table 2.2.Substance useful as supercritical fluids, parameters from (Lopez *et al*, 2004)

Supercritical fluids	Critical temperature (K)	Critical Pressure(bar)	Critical compression factor (Z.)	Acentric Factor (')
Carbon dioxide	304	74	0.274	0.225
Water	647	221	0.235	0.344
Ethane	305	49	0.285	0.099
Ethene	282	50	0.280	0.089
Propane	370	43	0.281	0.153
Xenon	290	58	0.287	0
Ammonia	406	114	0.244	0.250
Nitrous oxide	310	72	0.274	0.165
Fluoroform	299	49	0.259	0.260

Table 2.1.shows the critical parameters of some of the important compounds useful as supercritical fluids. One compound, carbon dioxide, has so far been the most widely used, because of its convenient critical temperature, cheapness, chemical stability, non-flammability, stability in radio-active application and non-toxicity. Large amount CO_2 released accidentally could constitute a working hazard, given its tendency to blanket the ground, but hazard detectors are available. It is an environmentally friendly substitute to other organic solvents. The $C0_2$ that is used is obtained in large quantities as a by-product of fermentation, combustion and ammonia synthesis and would be released into the atmosphere sooner rather than later, if it were not used as a supercritical fluid (Lopez *et al.*, 2004). Its polar character as a solvent is intermediate between a truly non polar solvent such as hexane and weakly polar solvents. Because the molecule is non-polar, it is often classified as a non-polar solvent, but it has some limited affinity with polar solutes because of its large molecular quadrupole. It has a particular affinity for fluorinated compounds and is useful for working with fluorinated metal complexed and fluoropolymers (Lopez *et al.*, 2004)

Carbon Dioxide is not such a good solvent for hydrocarbon polymers and other hydrocarbons of high molar mass. Ethane, ethene and propane become alternatives for these compounds, although they have the disadvantages of being hazardous because of flammability and of being somewhat less environmentally friendly. However, small residues of lower hydrocarbons in foodstuffs and pharmaceuticals are not generally considered a problem. Water has good environmental and other advantages, although its critical parameters are much less convenient and it gives rise to corrosion problems. Supercritical water is being used, at a research level, as a medium for the oxidative destruction of toxic waste. There is a particular interest in both supercritical and near-critical water because of the behavior of its polarity. Ammonia has similar behavior, is often considered and discussed, by not often used. Many halocarbons have the disadvantage of cost or of being environmentally unfriendly. Xenon is expensive, but is useful for small-scale experiments involving spectroscopy because of its transparency in the infrared (Lopez *et al.*, 2004).

2.7.6 Soxhlet Extraction

A Soxhlet extractor is a piece of laboratory apparatus invented in 1879 by Franz von Soxhlet. It was originally designed for the extraction of a lipid from a solid material. However, a Soxhlet extractor is not limited to the extraction of lipids. Typically, a Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance .Normally a solid material containing some of the desired compound is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor.

The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapors cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded (Wikipedia, 2008).

2.7.9 Comparison Between all Extraction Techniques.

In general, a comparison between all extractions techniques discussed previously is tabled to provide clearer picture on extraction technique available for extraction oil. All techniques are compared with hydro distillation, based on numerous researches on each type of extraction. The advantages and disadvantages of each technique are described as below, in Table 2.3.

Table 2.3: Comparison between all Extraction Technique

Techniques	***Advantages***	***Disadvantages***
Steam distillation	-The simplest method -Only requires cheap and simple apparatus setup with only one or two solvents to be used, depending on the parameters of experiments. -Usually performed as control in any extraction experiment.	-Low oil yield (Mazni,2007) -Time consuming -Exposure to heat may cause degradation of thermally labile compounds
Ultrasonic Extraction	-Higher oil yield and lower solvent consumption, compared to conventional method of extraction -Extraction time is significantly shortened compared to hydro distillation -Effective and indeed feasible method for the production of the plant oil does not affect the oil composition too much, based on the extraction of oil from flaxseed (Zhang *et al.*, 2007)	-Expensive equipment
Microwave Extraction	-Shorter extraction times (30 min for SFME method against 4.5 hours for hydro-distillation) (Lucchesi *et al.*, 2004) -Substantial saving of energy, compared to hydro distillation method (Lucchesi *et al.* , 2004)	-Safety concern, in case of any organic solvent leakage, which may cause fire

	-Reduced environmental burden (less CO2 rejected in the atmosphere)	
Solvent extraction	-Simple extraction method -Easy to setup the apparatus	-Longer extraction time - Suitable solvent need to be identified first before proceeding experiment
Supercritical Fluid Extraction (SFE)	-Unique properties of supercritical fluids, making it suitable for aromatic natural compounds -Excellent extractive properties such as high compressibility, liquid-like density, low viscosity, high diffusivity -low critical temperature of carbon dioxide means that the SC-CO2 system could be operated at moderate temperature, preventing the degradation of the substance due to heat induction -high product purity and quality	-Expensive equipment -Complicated process of method development in SFE
Soxhlet Extraction	• Simple extraction method • Easy to setup the apparatus • Only one batch of solvent is recycled, thus saving the solvent consumption and increase the efficiency	Longer extraction time • Suitable solvent need to be identified first before proceeding experiment

2.3. Antioxidant Activity of Plants.

Antioxidants are substances that offer protection against lipid oxidation, react with free radicals, reduce oxidative stress, and stop low density lipoproteins (LDLs) or bad cholesterol from being oxidized. They are substance that has the ability to delay the oxidation of a substrate by inhibiting the initiation or propagation of oxidizing chain reactions caused by free radicals. It plays important roles to prevent fats and oils from becoming rancid and protects human body from detrimental effects of free radicals (Lee S. *et al.,* 2007).

Autoxidation of polyunsaturated lipids in foods involves a free radical chain reaction that is initiated by the exposure of lipids to light, heat, ionizing radiation, metal ions, or metalloprotein catalysts. The process of autoxidation involves initiation (production of lipid free radicals), propagation and termination (production of non-radical products) reactions (Shahidi,F., 2004). Oxidative deterioration of lipids may cause the loss of fat soluble vitamins (e.g. vitamin A, D, E and K) and pigments (e.g. chlorophyll and carotenoids) (Gordon, M., 2001), it may also leads to the development of other changes that negatively affect nutritional quality, whole.someness, safety, color, flavor, and texture of foodstuff (Bandoniene, D.*et al.*, 2002). The use of antioxidants in lipid-containing foods is one method used to minimize rancidity, retard the formation of toxic oxidation products, maintain nutritional quality and increase the shelf life of food products (Bandoniene,D. *et al.*,2002).

Synthetic antioxidants such as Ascorbic acid, tert-butylhydroxytoluene, tert-butylhydroxyanisole and tert-butyl hydroquinone (TBHQ) have been used to retard lipid oxidation in foods. However, such synthetic antioxidants are not preferred due to their toxicological effects. For this reason, there is an interest in developing natural antioxidants from plants (Rababah,T.et al, 2004). Among natural antioxidants, phenolic antioxidants that are widely distributed in the plant kingdom (Wettasinghe, M.*et al.*, 1999) ,for example, the phenolic compound p-cymene-2,3diol (2,3-dihydroxy-4-isopropyl-1-methyl- benzene) which was isolated from hexane extract of Ruta chalepensis, showed a relatively strong antioxidant activity and greater than those of α-tocophenol and butylated hydroxyanisole (Turan, M,2003).

Wild plants play an important role in the diet of inhabitants in different parts of the world. These plants tend to be drought-resistant, gathered both in times of abundance and times of need, used in every day cooking and may be an important source of nutrients (Turan, M, 2003). Phenolic compounds are among the best known natural compounds acts as antioxidant and it is found in different plant species extract. One of this plant species is Ruta Chalenpensis and previous work reports that this plant contains the Total phenolic compounds of 1328.8 mg GAE/100g. That is why this project is devoted to determine the Total phenolic compound of Ruta Chalenpensis (Tenadam) using different solvent at the same time and the same extraction method and tried to study the Radical scavenging activity of this extract evaluated using inanition effect of DPPH radical scavenger called 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay. DPPH is commercialized in the radical form due to its stability. This radical shows a strong absorption maximum at 517 nm (purple). In the presence of antioxidants, the colour turns from purple to yellow. Therefore the sole equipment needed for the assay is a UV-Vis spectrophotometer. Initially, DPPH radical was thought to be reduced to the corresponding hydrazine when it reacted with the donating hydrogen substances. However, more recent studies have shown that what occurs is mainly a fast electron transfer from the sample to DPPH radical. The abstraction of hydrogen from the sample by DPPH radical is marginal, because it occurs very slowly and depends on the hydrogen-bond accepting solvent. Methanol and ethanol, solvents generally used for antioxidant ability assays, are strongly hydrogen bond-accepting; therefore the hydrogen-abstracting reaction occurs very slowly (Miguel, M.G, 2010).

2.4. Response Surface Experimental Design.

Experiment is a test or series of tests in which purposeful changes are made to the input variables of a process or system so that we may observe and identify the reasons for changes that may be observed in the output response. It is a series of tests, called runs, in which changes are prepared in the input variables in order to recognize the reasons for changes in the output variables and are used to study the performance of processes and systems (Montgomeery *et al.*, 2010). Design of experiments (DOE) is a powerful technique used to exploring new processes ; gaining the increased knowledge of the existing process and optimizing these process for achieving world class performance .Often engineering experimenters wish to find the conditions under which certain processes obtain the optimum results. That is by careful design of parameters at which the response reaches its optimum. The optimum could be either maximum or minimum of the response (output variables) which is influenced by several independent variables (input variables). One of methodologies for obtaining the optimum results is Response Surface Methodology.

Response surface methodology (RSM) is invented by Box and Wilson and is defined as the collection of mathematical and statistical tools or techniques useful for modeling, analyzing and simultaneously solving problems in which a response of interest is influenced by several variables and the objectives is to optimize this response .Response surface methodology also quantifies the relationship between the controllable input parameters and obtained response surfaces. It is as well know up to date approach for constructing approximation models based on physical experimented observations (Montogomery *et al.*,2010)

The main advantage of RSM is:

- The reduced number of experimental runs needed to provide sufficient information for statistically acceptable results.
- Designing of a series of experiments for adequate and reliable measurement of the response of experiment.
- Developing a mathematical model of the second order response surface with the best fittings.
- Finally finding the optimum set of experimental parameters that produce a maximum or minimum value of the response.

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Chapter Three

Materials and Methods

3.1. Materials

3.1.1. Raw Materials

The plant material used for this project is *Ruta chalepensis* (Local name, Tenadem). The detailed information on this plant species and its botanical description was obtained from Wando Genet Agricultural Research center (WGARC) .The center, WGARC, is one of the fourteen Federal research center under Ethiopian Institute of Agricultural Research and it is found in SNNPE regional state and located at a distance of 267 km from Addis Ababa. WGARC is known by Aromatic, Medicinal, and Bio energy as center of excellence in our country Ethiopia. After botanical description of *Ruta chalepensis* is identified, some of the representative sample is taken from this center and the other is purchased from local people who cultivate this plant and bring it to the local market available at Addis Ababa on morning time. The harvested plant was identified into different parts as stems, leafs, aerial parts including leaf and stems extension found on the upper part of the stems including the flowers parts which are about to flower. The dirty substances harvested along with all the plant part is removed and washed by the tap water to remove all the dust particles tighten on the plant parts. Then all aerial parts of the plant were identified and allowed to be dried under the shaded area for about three weeks to prevent the exposure of these plant parts to the open sun light. The dried aerial parts were reduced in size to the desired particle size by Cross beater Mill. All the procedure used were shown using figure in appendix E.

3.1.2. Chemicals

The chemical used for this projects are: hexane ,ethanol , distilled water, methanol ,diethyl ether, DPPH (2,2-diphenyl-1-picryrazyl ,ascorbic acid, gallic acid , sodium carbonate, folin-Ciocalteau reagent, formic acid, toluene, chloroform, glacial acetic acid, potassium hydroxide, potassium iodide, phenolphthalein indicator, starch indicator, hydrochloric acid and anhydrous sodium sulphate. All these chemicals used in this analysis were analytical grade (more than 98% minimum assay) .

3.2.Methods

3.2.1.The Reason and Range of The Process Parameters Selection

There are many process parameters which may affect the extraction of oil using soxhlet extraction such as Extraction temperature, extraction time, and solid mass to solvent ratio, particle size, solvent type, moisture content of the sample and etc, but it is quite difficult to study the effect of all these parameters. As a result this thesis focused on effect of temperature, extraction time, solid mass to solvent ratio on the yield of the *Ruta chalepensis* oil (Tenadam oil). From the literature survey the effect of particle size on the yield of extractive substances the study was restricted to the particle size of 0.45-0.85 mm for soxhlet extraction. The first experimental parameter selected was Temperature and its level is determined based on the previous works. The starting temperature, 68^{o}C, was determined taking into consideration the normal boiling point of pure analytical grade of organic solvent hexane and the highest level, 85^{o}C, was selected as the maximum possible temperature to keep important components of oils exist in this plant extract from decomposition and further alteration to other undesirable components. The medium temperature, 76.5, is predicted by the statistical reasoning of the optimization process of response surface methodology and always the medium temperature is specified taking the average of the lowest and the highest value of the process parameters and this software find the optimum value based on the data and the type optimization selected.

The second most important parameter selected to see the effect of yield was extraction time and the level selected are 2hrs, 4hrs and 6 hrs. The minimum extraction time was fixed on 2 hrs since much previous works started level of the extraction time from 2 hrs and performing the extraction time below 2hrs doesn't bring the significant value that affect the optimization process. likewise undergoing the extraction time beyond 6 hrs does not bring the value which is much greater than the one obtained at 6 hrs particularly the extraction process which involves the areal parts of the leaf plants and keeping the level of variables between the indicated ranges is well enough to find the optimum extraction time specially for this plants .According to some journal article site, unlike that of the seed plants ,the plant part extract is aerial parts and doing the extraction time above 6 hrs does not bring measureable effect from the one that obtained at 6 hrs (Macid *et al.,*2005). The third process parameters selected was Solid mass to solvent ratio. As the same way solid mass to solvent ratio of 9 is the minimum solvent required to recycle back to the soxhlet extractor of about 500ml and the upper, 13, is fixed based on the previous work (Macid *et al.,* 2005).

3.2.2. The Procedure of Soxhlet Extraction and Yield Calculation.

Different Solid to solvent ratio (SSR) of analytical grade hexane (99% purity) was poured into round bottom flask. 30g of the sample was placed in the materials like thimble which is prepared from the filter papers which have close porosity with the actual thimble and was inserted in the center of the extractor. This material was prepared by folding thick filter paper and any opening which is suspected to allow the entering of the solvent was made fixed not to allow the direct contact of solvent with the sample inside it. After all the soxhlet apparatus set up was finished it was heated by varying the temperature of the heater as 68^0C, 76.5^0C and 85^0C using temperature sensors. When the liquid inside the distilling flask is boiled as a result of heating, the vapor is created on the surface of the solvent and it is enforced to raises up through the distillation arm of the soxhlet apparatus and on its way to the upper part of the distillation arm, the vapor gets the condenser and get condensed. After it condenses, it converted into the liquid and refluxed back on the thimble which contains the sample. Then the solvent is absorbed by the thimble and solvent start extracting the oils from the sample. Finally the extracted oil of the sample to gather with the solvent is returned back to distilling flask through Siphon exit when the level of the solvent is become equal with the Siphon top. This was allowed to continue for two, four, and six hours. The experiment was repeated by placing the same amount of the sample into the thimble again by varying solid to solvent ratio (1:9, 1:11 and 1:13 mg/ml). The weight of oil extracted was determined for each run hours. At the end of the extraction, the resulting mixture (miscella) containing the oil was heated to recover solvent from the oil using rotary evaporator under reduced pressure by vacuum pump, and the yield was calculated using the following formula.

$$\text{yield} = \left(\frac{w_{2-} w_1}{w_0}\right) * 100 \ldots \ldots . . (3.1)$$

where ;

$w_{2=}$ weight of the beaker and oil attached to the Rota evaporator and

$w_{1=}$weight of the beaker

w_0= Sample weight loaded to the thimble (30g).

3.3. Experimental Design

The optimal extraction conditions for yielding *Ruta chalepensis oil* were determined using RSM. The 3-level, 3-factorial Face Centered Cube (FFC) experimental design was used to investigate and validate extraction parameters affecting the extraction yield. A summary of the extraction temperature, extraction time, and Solid mass loaded to solvent ratio of 20 experiments with 6 center points were evaluated. Each experiment was carried out in duplicate. The software Design-Expert (Trial Version 8.0.4, Stat-Ease Inc., and Minneapolis, MN, USA) was employed for experimental design, data analysis, and model building. Statistical significance of the model and model variables was determined at a 5% probability level ($p < 0.05$). Three-dimensional response surface plots were generated by keeping one response variable at its optimal level and plotting that against two factors (independent variables).The axial points were excluded by making it to Face centered Cubic Design since they are out of ranges to which the experimental or process parameters were intended to be studied.

3.4. Characterization *Ruta Chalepensis* (Tenadam) Oils

3.4.1. Physical Properties of *Ruta Chalepensis*

3.4.1.1. Determination of Moisture Content (Method AOAC, 2000*).*

A known weight of the empty beaker and that of the oil sample were measured. The oil in beaker was kept in an oven for 6 hours and maintained at a temperature of 105°C Then, the samples were taken out from oven and placed in a desiccators to cool to room temperature before weighing. These procedures of heating and weighing continued till constant weight reached .Percentage weight of the moisture was calculated as:

$$\text{Moisture content} = \frac{b-c}{b-a} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(3.2}$$

Where:

a = weight of empty beaker
b = weight of beaker + oil before drying
c = weight of beaker + oil after drying.

3.4.1.2. Determination of Melting Point

Since the oil was solid at room temperature, little amount of the oil samples were put into a test tube instead of capillary tube with a thermometer. With this method, the temperatures at which the oil samples turned into liquid (oil) were taken using thermometer (Nayak, B.*S et al.*, 2010).

3.4.1.3. Determination of pH value

2g of the sample was poured into a clean dry 25ml beaker and 13ml of hot distilled water was added to the sample in the beaker and stirred slowly. It was then cooled in a cold-water bath to 25°C. The pH electrode was standardized with buffer solution and the electrode immersed into the sample and the pH value was read and recorded.

3.4.1.3 Determination of Specific Gravity (British Standard of methods, 1988*)*

The density of the oil was determined by using density bottle. A clean and dry bottle of 5ml capacity with stopper was weighed (w_0) and then the bottle was filled with the oil, stopper inserted and reweighed to give (w_1). The oil was substituted with water after washing and drying the bottle and weighed to give (w_2). The expression for specific gravity (Sp.gr) is:

$$\mathrm{Sp.gr} = \frac{w_1 - w_0}{w_2 - w_0} = \frac{\text{mass of a substance}}{\text{mass of an equal volume of water}} \ldots\ldots\ldots\ldots .3.3$$

3.4.1.4. Determination of Boiling Point of the oil

The solid sample oil which is melted properly was poured in to beaker and a thermometer was inserted and placed on a heating mantle, it was observed that the oil in the beaker started circulating leading to boiling of oil and read temperature on thermometer.

3.4.2. Chemical properties *Ruta chalepensis* Oil

Sensory quality evaluation of the product that means the quality of Tenadam oil can be expresses based on the average molecular weight of triglycerol present in the oil and the amount of free fatty acid present in the oil which is determined through determination of Saponification value, acid value, Iodine value and pH-value. Determinations for peroxide, iodine, acidic, saponification, values, and free fatty acid contents were carried out using standard analytical methods.

3.4.2.1. Determination of Saponification Value (AOAC methods, 1997)

2g of the oil samples were weighed into 25ml of 0.5N ethanolic potash in a conical flask. To another flask, $25cm^3$ of the 0.5M ethanolic potash was placed without the oil, this was used as blank. Both flasks were boiled in a water bath with reflux condenser for 30 min with frequent shaking and 2 drops of phenolphthalein indicator were added. Titration was done with 0.5M HCl without delay with vigorous shaking to get the end point. Saponification value (SV) was calculated using the formula:

$$SV = \frac{56.1 * (B - S) * N}{w} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ 3.4$$

where:

56.11 is equivalent mass of the potassium hydroxide, N is The normal solution taken, w is the

B= Average volume in ml of standard hydrochloric acid required for the blank

S=Average volume in ml of standard hydrochloric acid required for the sample.

w=The weight of the sample taken.

3.4.2.3. Determination of Iodine Value (British Standard method, 2000)

0.5g of the sample was dissolved in 10ml of chloroform in a conical flask; 25ml of the Wij solution (excess of iodine monochloride solution in glacial acetic) was added to the chloroform and corked. This was kept in desiccators for 30mins in the dark. A blank was also carried out under the same conditions. When reaction was completed in the flask, which is at the end of time, $15cm^3$ of 10% potassium iodide (KI) solution and 10ml of distilled water was added to each flask and mixed by gentle shaking. The content of both flasks were titrated with 0.1N $Na_2S_2O_3$ to till the colour the sample is changed to pale green and 2 ml of starch solution indicator was added. Titration was continued until the blue-black color was completely discharged. The same procedure was used for blank test and other samples and given as follows:

$$\text{Iodine value} = \frac{12.69 * C * (v_1 - v_2)}{W} \ldots\ldots\ldots\ldots 3.5$$

where,

c = concentration of sodium thiosulphate used;

v_1 = volume of sodium thiosulphate used for blank;

v_2 = volume of sodium thiosulphate used for determination,

w = mass of the sample.

3.4.2.3. Determination of Peroxide Value (AOCS method, 2000)

About 2.5g of each oil sample was added to 30ml of ethanolic acid-chloroform mixture in the ratio of 3:2 respectively and 5ml of saturated potassium iodide (KI) was added. 15ml of sodium thiosulphate ($Na_2S_2O_3$) was also added until a color of the oil had almost disappeared, then 2ml of starch solution (indicator) was added and titration continued to a colorless end point or slightly to milk colour. Peroxide expressed as milliequivalents ratio of peroxide oxygen per Kg sample (meq O_2 /Kg) and expressed as follows:

$$\mathrm{POV} = \frac{(\mathrm{V1}-\mathrm{V0})*\mathrm{C}*1000*\mathrm{T}}{\mathrm{M}} \ldots\ldots\ldots\ldots\ldots (3.6)$$

where:

POV = peroxide value

V1 = consumption of 0.1 mol/l sodium thiosulfate solution in the main test

V0 = consumption of 0.1 mol/l sodium thiosulfate solution in the blank test

C= molar concentration (morality) of the sodium thiosulfate

T = titre of the thiosulfate solution (0.1N)

M = weighed portion of substance in grams

3.4.2.4. Determination of Acid Value (British standard method, 2000)

The oil sample is mixed thoroughly before weighing and about 5g of oil sample was added into a 250 ml conical flask and add 50 ml of freshly neutralized hot ethyl alcohol and about one ml of 1% phenolphthalein indicator solution in ethanol was added to it. The mixture was boiled for about five minutes and it was titrated against standard alkali solution while it was hot with vigorously shaking during the titration. The weight of the oil/fat taken for the estimation and the strength of the alkali used for titration shall be such that the volume of alkali required for the titration does not exceed 10 ml and the weight taken was 5 according to this method . This was calculated using the formula:

$$\text{Acide Value(AV)} = \frac{\mathrm{V} * \mathrm{N}}{\mathrm{W}} * 56 \ldots\ldots\ldots\ldots\ldots\ldots (3.7)$$

where,

V= volume in ml of standard potassium hydroxide or sodium hydroxide used,

N = normality of the potassium hydroxide solution or Sodium hydroxide solution;

56.1= constant called equivalent mass of potassium hydroxide and

W= weight in g of the sample.

3.5. Procedures of Total Phenolic Compounds Determination in *Ruta chalepensis* oil Crude Extract.

The method of determination of the total phenolic compound involves the identification materials and chemicals, instrumentation, reagents and solution preparation, preparation of the Gallic acid calibration curve standards and saturated carbonate solution. The materials and chemicals required are: extracted sample, Folin ciocalteu Phenol (FC) stored in the dark and discarded if the reagents become visibly green, saturated sodium carbonate (NaCO3), distilled water, 100 volumetric flasks, vortex mixer, Gallic acid calibration standards, micro pipettes and whatman No1 filter paper. Instrumentation required are UV-Vis high performance Spectrophotometer which have a capacity of measuring absorbance of the solution at about 765nm and Cuvettes (1-cm, 2 ml plastic or glass). For reagents and solution preparation deionized or distilled water was used in all Recipes and protocol steps. The Procedures of total phenolic compounds determination is based on the method developed by Singleton and Rossi with some modification (Singleton and Rossi, 1965) and involves three parts. These are:

i) Preparation of different standard concentration of Gallic acid:

0.05g of Gallic acid was dissolved in 1 ml methanol and diluted to 10 ml with water (5g/liter final (stock)).From this stock solution,0.01,0.1,0.15,0.2 ,0.25 ,0.3,0.4 and 0.5ml was dissolved and diluted with water of 10ml to create standards of 5, 50, 75, 100, 125 ,150,200 and 250 of mg/liter solution (ppm) by serial dilution in test tubes which was washed properly by concentric nitric acid and distilled water. These solutions were stored at 4^oC in refrigerator.

ii) **Preparation of saturated sodium carbonates solution and Folin-ciocalteu phenol reagents (FC).**

20g of anhydrous sodium carbonate was dissolved in 80 ml water and brought to boil (saturated). After cooling few crystals of sodium carbonate was added and it was sat for 24 hours at room temperature. The solution was filtered through whatman No.1 filter paper and water was added till it reaches 100ml label and finally it was stored indefinitely at room temperature. Folin ciocalteu phenol (FC) reagent was prepared by taking about 1 ml of its stock solution and diluted with 10 ml of Methanol .Then the colour of the solution was noted whether it was visibly green or yellow since the solution should be yellow in colour for full its potency .

iii) **Procedures for formulation of Calibration curve for Gallic acid and determination of Total phenolic compounds of Tenadam oil extract of different solvents.**

Different concentration of Gallic acid was taken from 0.05g/ml by serial dilution to 10ml with distilled water to reach at Concentration of 0.01, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, and 0.5 mg/ml of Methanol solution. Then, 0.03 mg of each samples extracted using incubator shaker at 60 ^{o}C using solvent ratio of 1:13 of different solvent for about 6 hrs is taken and dissolved in 1 ml of methanol solution. Different concentration of the sample extracted with different solvent as stated above were taken from the stock solution of 0.05 mg/ml and more diluted solution were prepared to search for the concentration of the extracted sample to which the exact value of the maximum possible concentration is included in the interval of calibration curve standard generated by measuring the absorbance of the concentration using High performance UV-Viss Near Infrared spectrophotometer. In each of the concentration taken 1ml of the concentration of Folin-ciocalteu phenol was added to the sample, standard and blank and the test tubes were incubated for 8 minutes after they were mixed by Vortex mixer .Then 1ml of Saturated sodium carbonate was added to each of the test tubes incubated for 8 minutes and mixture in the test tubes were adjusted to 10ml with distilled water and again incubated for about 90 minutes at room temperature. Finally the Total Phenolic compound for each extracted sample with different solvent was measured as Equivalent of Gallic acid and their amount was compared with the optimum Ruta *Chalenpensis oil* (Tenadam).

3.6. Antioxidant Determination of *Ruta Chalenpensis* oils.

The antioxidant activity of the crude extract of *Ruta Chalenpensis* oil using hexane and other solvents like ethanol, methanol, diethyl ether and distilled water is evaluated or estimated using the method of Kirby and Schmidt (2004) with slight modification.

The method of Kirby and Schmidt uses DPPH (2, 2-diphenyl-1-hydraine) as radical scavenging ability. For measuring free radical scavenging ability using this method, the methods are grouped in two groups, according to the chemical reactions involved: hydrogen atom transfer reaction-based methods and single electron transfer reaction-based methods. This radical shows a strong absorption maximum at 517 nm (purple). In the presence of antioxidants, the colour turns from purple to yellow.

First, 0.004% DPPH was prepared by measuring 0.01g of DPPH in 250ml methanol in volumetric flask and about 3 mg/ml of standards of Ascorbic acid was prepared by dissolving 3mg of ascorbic acid in 1ml methanol and to have more solution for successive uses, the standard solution was further dissolved by measuring 150 mg of ascorbic acid in 50ml keeping the ratio stated in the Kirby and Schmidt method.

Secondly, about 4ml of 0.004% solution of DPPH radical solution in methanol was mixed with 1 ml of various concentrations (0.2-2mg/ml) of a extracts in solvent extracted and with a vortex mixer. 10 mg of extract/ml of methanol solution is prepared as stock solution and using micro pipette 20μl, 30μl,60μl, 90μl,120μ,150μl, 180μl, 200μ, were taken from stock solution as doublet and added to the test tube which was washed with distilled water and kept in oven at 105^{o}C to remove water droplet from the surface of test tube which may interfere with absorbance of the extracted samples. Then contents of the test tubes are mixed with the vortex mixer for about 20s to allow the further mixing of DPPH with extracts and to facilitate the reaction between the two by homogenizing the samples.

Thirdly, all the test tube was incubated for about 30 minutes in the dark at room temperature. Incubating the samples for 30 minutes in dark was required to allow the reaction between the extract and DPPH and to prevent the absorbance of light by DPPH that may interfere with the actual absorbance of the sample and DPPH solution. Fourthly, scavenging capacity was read spectrophotometrically by monitoring the decrease in absorbance at 517nm using high performance UV-Vis spectrophotometer and inhibition of free radical DPPH in percent (I %) was calculated in the following way:

$$\mathrm{IE} = \left(\frac{\mathrm{Acontrol} - \mathrm{A\ sample}}{\mathrm{A\ sample}}\right) \mathrm{x}100 \ldots\ldots\ldots\ldots\ldots 3.7$$

Where:

A $_{\text{control}}$ is the absorbance of the control reaction (containing all reagents except the test compound)

A $_{\text{sample}}$ absorbance of the test compound.

Finally the estimation of the scavenging will be carried out in doublet and the results were reported as a mean average of the two parallel measurements plus or minus their standard deviation along with their IC_{50} value.

3.7. Essential Oil Isolation Procedure

Aerial parts of *Ruta chalepensis* plants used in this research were collected at flowering stage in a morning time and air-dried at room temperature. About 100 g of the sample was subjected to hydro-distillation for 5 hours in a Clevenger-type apparatus. The yield was calculated as the percentage of the ratio of weight of the essential oils collected over the water to the weight of the sample fed to the Clevenger steam distillation. The essential oils, collected over water, were dried over anhydrous sodium sulfate and stored at 5 °C until analysis.

3.8. Gas Chromatography–Mass Spectroscopy Analysis of *Ruta chalepensis* essential oils

Gas chromatography-mass spectrometry (GC-MS) analysis were performed using an Agilent Technologies 7820A Gas chromatograph system equipped with a HP-5 capillary column (30 m x 0.25; coating thickness, 0.25 μm) and a Agilent technologies 5977E Mass spectroscopy ion trap detector .Analytical conditions were as follows: injector and transfer line temperature, 220 and 240 ^{O}C, respectively; oven temperature, programmed from 60 to 240 ^{o}C at 3 ^{o}C/min; carrier gas, helium at 1mL/min; injection, 5μL (10 % hexane solution); and split ratio, 1:30. Identification of the constituents was based on comparison of the retention time along with their percentage peak areas and quantitative data was obtained from electronic integration of area percentages without the use of correction factors. All chemical compositions were identified searching through mass\hunter\library\NIST11.L and mass\hunter\library\W9N11.L and comparing with other previous work,

Chapter Four

Results and Discussion

The present research work was planned to study the effect of the process parameters on the yield, optimizing the yield of *Ruta Chalenpensis* oil, characterizing physiochemical properties, evaluating total phenolics content and antioxidant extract using soxhlet extractor and incubator shaker, characterizing essential oils components of the oil extracted using Clevenger steam distillation. The oil was extracted using soxhlet extractor with optimized parameters and the essential oil component was obtained using the oil obtained from Clevenger steam distillation. The essential oil component was characterized from the oil yield obtained from the steam distillation since all the extract extracted using soxhlet extraction is difficult to say all are essential oils.

4.1. Result Obtained From Soxhlet Extraction of Ruta Chalenpensis Oils

The extraction parameters such as temperature, extraction time, and solid mass to solvent ratio are some of the factors which are very important to produce both quality and optimum amount of oil. The parameters selected for entire work are temperature, extraction time, solid mass to solvent ratio. Using these parameters the extraction yield was used to evaluate the performance of soxhlet extraction for the extraction of *Ruta chalepensis* oils. Extract yield was the ratio of the extracted weight to the dry sample weight. Selecting these three parameters, there were about 20 numbers of experimental run as per by the central composite design of optimization process of FCC (face centered cube with 6 center points). This optimization process is the best method for optimization process than the other when the variables of study are more than three and there is constraints in raw materials used and expensive market price of the chemical reagent used to do all the experimental runs (Montogomery,2001). These 20 numbers of experimental run with its level were performed as duplicate .The yield obtained for maximizing of the yield were taken as the mean of the these two replicates plus standard deviations. The individual replicates of raw data were written in appendix A and one can refer them. The yield from these two replicates by different combination as per the Design Expert output by AOAC (2000) official method 972.28 are tabulated in table below. The figure obtained on the yield from this experimental result is highly related with the previous research on the Ruta chalepensis in which the yield vary from 9.27-18.06 (Fakhfakh et al. , 2012

Table 4.1.Yield of *Ruta chalepensis* (Tenadam) oil extract using soxhlet extractor

Run	Extraction Temperature	Extraction time	Solid mass to solvent ratio	Yield (%)
1	76.50	4.00	9.000	11.500±1.697
2	68.00	2.00	13.00	10.580 ±1.513
3	76.50	2.00	11.00	12.203±0.150
4	85.00	4.00	11.00	14.493±1.369
5	76.50	4.00	11.00	13.000±0.590
6	68.00	4.00	11.00	10.973±1.420
7	76.50	4.00	11.00	12.540±1.020
8	85.00	6.00	13.00	16.768±0.164
9	85.00	6.00	9.00	15.150±0.430
10	76.50	6.00	11.00	13.500±1.456
11	68.00	6.00	9.00	9.820±1.980
12	76.50	4.00	11.00	13.220±0.954
13	85.00	2.00	9.00	12.976 ±0.397
14	68.00	2.00	9.00	8.980±0.764
15	76.50	4.00	11.00	12.911±1.090
16	68.00	6.00	13.00	12.976±1.190
17	76.50	4.00	13.00	13.672±1.379
18	76.50	4.00	11.00	12.476±1.32
19	85.00	2.00	13.00	13.220±1.881
20	76.50	4.00	11.00	12.910±0.890

4.1.1.Data Analysis for Optimization of *Ruta Chalepensis* oil Extracts Using Soxhlet Extractor.

4.1.1.1 Selecting Model

Selection of the best model to describe the analysis of yield is the starting point before analyzing, modeling, checking the statistical significance of terms by analysis of Variance for the response (ANOVA) and before optimization of the process parameters. There are about five known model equation that can model the responses of the variables in Design expert software. These are: mean, Linear, FI, Quadratic and Cubic model equation. The model selection process is based on the greater value of F-value, significance of the model, whether the lack of fitness is significant (higher value of lack of fitness) or not (lower value of lack of fitness) and close proximity or reasonable agreement of Predicted R-square is with that of adjusted R-square (Montgomery, 2010). The following table shows sum of squares, mean square, Adjusted R-square, Predicted R-square, F-value ,p-value (prob>F),significance model, and lack of fitness of different models when the response (yield) is evaluated within their respective ANOVA analysis. This information is taken from appendix C which shows ANOVA of the models discussed before.

Table 4.2. Model Summary Statistics.

Model	Sum of squares	Mean square	F-value	AdjR-Squared	PredR-Squared	significance model,	Significance Of lack of fitness
Mean	0	-	-	0.0000	-0.1080	Significant	Significant
Linear	55.17	18.39	1157.15	0.9946	0.9921	Significant	Not significant
2FI	55.22	9.20	587.74	0.9946	0.9845	Significant	Not significant
Quadratic	55.27	6.14	397	0.9947	0.9860	Significant	Not significant
Cubic	55.33	4.26	297.08	0.9951	0.2576	Significant	Not significant

As it is shown in the above table the linear model have greater value of F-value, close proximity of predicted R-square with that of Adjusted R-square, significance model and non-significance lack of fitness. Therefore, it is the linear model that was suggested based the design program of the above information for the response and to test for its adequacy and to describe its variation within the independent variables. Then, using the linear model and when the significance of F- value was judged at 95 % confidence interval, the F-value of 1157.15 implies the model is significant. Values of " Prob>F less 0.05 than indicate model terms are significant. In this case A, B and C are significant model terms. Values greater than 0.100 indicates that the model terms are not significant. From Appendix C of Post ANOVA analysis, the "Pred R-Squared" of 0.9921 is in reasonable agreement with the" Adj R-Squared" of 0.9946. "Adeq precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In the linear model of this analysis the signal to noise ratio is 105.281 indicates an adequate signal and all the data analysis result show that the data are statistically significant. when all these are fulfilled this model can navigate the design space (Montogomery,2001).

4.1.1.2. Correlation Matrix for the Optimization of *Ruta Chalenpensis* oil Extract

Correlation matrix is one of the most common and useful statistics. The correlation coefficient measures the strength of the linear relationship between the variables. The correlation coefficient is always between-1 and +1.The closer the correlation is +/-1,the closer to the perfect the linear relationship

Table 4.3: correletion matrix of Factors [Pearson's] for process optimization of *Ruta Chalepensis* oil using Design Expert- RSM**.**

	A	B	C
A	1.00	0.000	0.000
B	0.000	1.00	0.000
C	0.000	0.000	1.000

Where A=Temperature, B=Extraction time, c=Solid mass to solvent ratio

According to correlation matrix of factors for optimization all diagonal values are always 1 and for factors-to-factors correlation, off-diagonal value is closer to zero are better.

4.1.1.3. Model Equation for Optimization of Ruta Chalenpensis oil Extract

The final equations in terms of factors are can be expressed in two different ways .These are the model equation in terms of the coded variables and in terms of the actual factors.

The final equations in terms of the coded variables are:

$$\text{Yield}=+12.71+1.84*A+1.11*B+0.95*C \text{......} \quad 4.1$$

The final equation in terms of Actual factors:

$$\text{Yield}=-11.28700+0.21666*\text{Temperature}+0.55400*\text{Extractiontime}+0.47365*\text{ solid mass to solvent ratio}\text{..}4.2$$

4.1.1.4. Diagnostic Test for The Response.

Diagnosing of the statistical properties of the model after post ANOVA analysis is the necessary part before going for examining the model graphs and optimization. There are different ways of diagnostic details test provided by Design-Expert. Some of them are Normal probability plot of studentized residuals, Predicted versus actual, studentized residuals versus factor, Box-Cox plot, leverage, etc. The most important diagnostic are the normal probability plot of the residuals and predicted versus actual. When we take Normal probability plot of studentized residuals for the diagnosing for model adequacy checking, the data points should be approximately linear and a non-linear pattern (look for an S-shaped curve) indicates non-normality in the error term. The same is true for Predicted versus actual plot curve. The data points should be linear and all the entire response data should nicely line up on the linear curve generated when predicted versus actual data of the response is plotted. Furthermore, the relationship between actual values and predicted values (Fig.4.2) showed that the actual values are distributed relatively near to the straight line, indicating good fitness of the model. For riduals versus run plot graph all the data should be connected with one another and should form visible pattern. The figure below shows how precisely The yield of the Tenadam oil of crude extract is modeled using linear model since all the points are lined up nicely as discussed before and the test for yield from normality is insignificant when we refer to Figure 4.1. Indeed, from figure 4.2, the actual data were almost aligned with the predicted values and this indicates the validity of the model equations. Again in Fig 4.3, the plot of studentized residuals versus run order was tested and the residuals were scattered randomly with visible pattern around ±3.00. This was an indication of better fitness of the model with the experimental data.

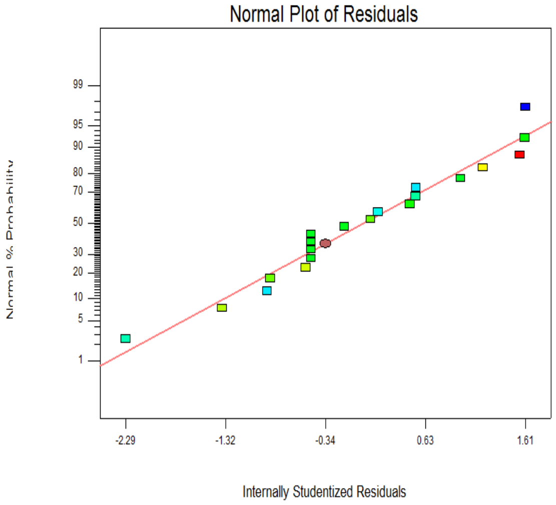

Figure.4.1:Normal plot of Residuals

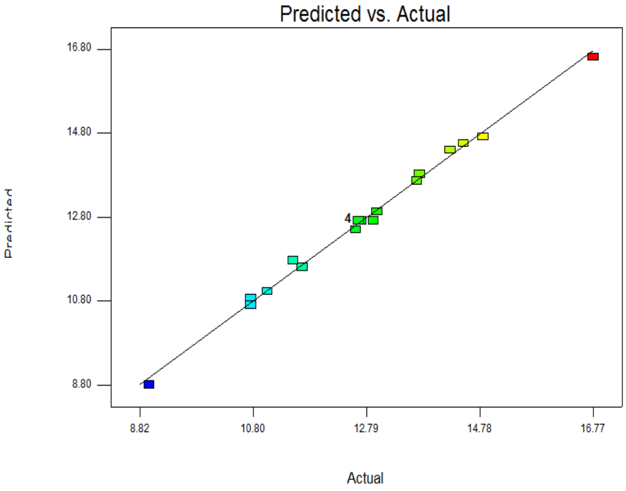

Figure 4.2: Predicted versus actual plot

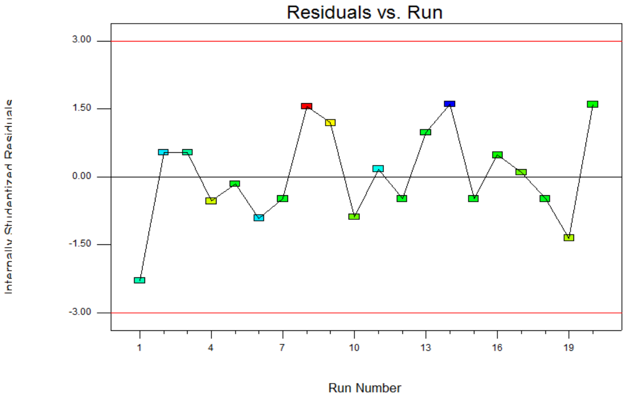

Figure 4.3.Risuduals versus run

4.1.2. Effect of Processing Variables on the Yield (Response Surface Plot)

The effect of different parameters on the yield are given in Table 4.1. To avoid visualization of variables in response to processing variables, series of three dimensional response surface were drawn using design Expert Soft Ware (stat-ease,200). Since the present study involves three variables, it was necessary to fix the value of one variable in order to see the effect of two variables on the response. That means, using surface response plots of the linear model, the relationships between the extraction parameters and the response (extraction yield) could be better understood by holding one variable constant and studying the relationship between the other two variables. The non-variant was set at optimum and put in equation 4.1 and 4.2 above. The relationships developed between dependent and independent variables were used to plot response surface and representative plots as shown in Figure 4.4.

As it can be visualized from the effect of the processing variables on yield (response surface plots) in Figure 4.4 that the yield of *Ruta Chalenpensis* oil exhibits linear increase trend by various temperature and extraction time combination., but when we closely observe the linear relationship of the effect of temperature on the yield, an increase temperature result in increase of the yield than increasing extraction time. This fact can be understood from more steepness of the linear relationship of temperature with that of yield than that of extraction time. Likewise, when the effects of experimental parameters of temperature- SSR on the yield are studied, they show the linear increase in on the yield of Ruta *Chalenpensis* oil and the steepness is large for Temperature. In all combination increasing Temperature followed by more steepness of the linear relationship along with increasing the other two variables. This findings show that maintaining temperature at constant value highly affect the amount of oil produced and this variable should take special consideration when the extraction of the oil from the plant parts is needed. Indeed, we can say the extraction temperature is the most significant factor than the other two variables . In addition since the extraction time and SSR affects the yield of the oil extracted this variables also should be considered .As one can undrestand from all combination of variables increasing one value also increase the other values and all of them are positively correlated and there is no any variables that are negatively correlated.

The increasing temperature could improve the extraction yield by enhancing the solubility of the extraction compounds. Based on the surface plots generated graphs, the yield was found to enhance with increasing temperature. This is because of rising the temperature, both the diffusion coefficient and the solubility of the oil into the solvent is enhanced, thus heat treatment improves the extraction oil. The higher extraction temperatures, the easier to break the molecule inside the sample; as a result, the yield also gets high.

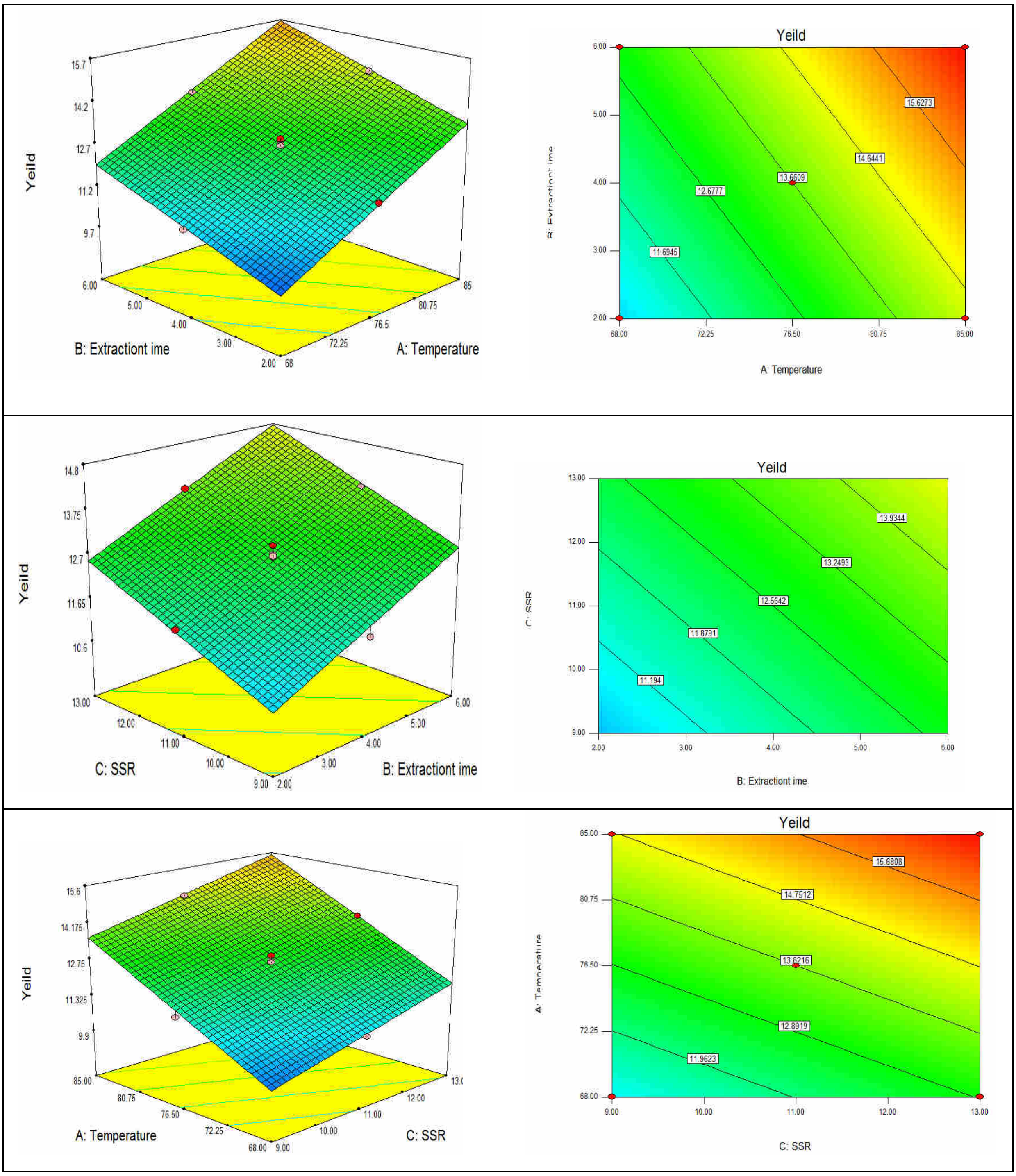

Figure 4.4.effect of processing variables on Yield (Response surface plot

4.1.3. Optimum Condition for The Yield of Oil and The Model Validation

Optimization was carried out after observing all the effects of the extraction variables. The proficiency of ‘point optimization’ was used for all variables. Table 4: shows the optimum working conditions (ultimate goals, high and low limits) of the response and factors (Extraction temperature, Solid mass to Solvent ratio (SSR) and Extraction time) employed during the optimization analysis. In view of the optimization, the targeted criterion was maximized for the Yield of Ruta Chalenpensis while the factors values were set in the range studied.

A set of solutions were generated by the Design-Expert 7.1.1 version software to determine the optimum conditions of the process which would select the values of high composite desirability and maximum response. The software searched for a combination of factors that simultaneously satisfied the requirements placed on the ultimate goals of response and each of the factors (Temperature, Solid mass to Solvent ratio, Extraction time) which give the highest composite desirability (0.998) . The predicted and experimental values of the yield under the optimum conditions are also presented. The very small deviation, between the predicted and experimental values of yield in percentage of *Ruta chalepensis* oil indicates that the model (eq 4.1) is suitable and sufficient to predict the oil extraction process using the soxhlet extraction in the range of variables studied.

Table 4.4 working conditions of response and factors for optimization.

Variables	Ultimate goal	Experimental range	
		Lower limit	Upper limit
Extraction Temperature	In the range	68	85
Extraction time	In the range	2	6
Solid mass to Solvent ratio (g/mL)	In the range	9	13
The Percentage yield of oil	Maximize	8.98	16.768

When we check the validation of the model for the optimization results, an experiment was performed under predicted conditions by the developed model. The model predicted 16.605 yield of the oil at Extraction temperature of 85 0C, Extraction time of 6 hrs, and Solid mass to solvent ratio of 13. The experimental value obtained at these conditions is 16.768 and is closely in agreement with the result obtained from the model and hence validated the findings of the optimization.

Table 4.5. Optimum conditions and model validation

Variables	Optimum results
Extraction Temperature (OC)	85
Extraction Time(hours)	6 hours
Solid mass to solvent ratio (mg/ml)	13
Percentage yield of the oil (%)	16.768
Desirability	0.998
Experimental percentage of the oil yield	16.605
Deviation in percent	0.163

4.2. Result on Physico-Chemical Properties of *Ruta Chalenpensis* oil.

All the physico-chemical properties of the *Ruta Chalenpensis* oil extracted using Soxhlet extraction was evaluated for the optimum yield of oil produced based on the selected optimum parameters.

4.2.1. Physical Properties.

Determining the physical properties of the oil helps to maintain the quality of the oils. The physical properties of the oils which are studied for these specific projects are moisture content, PH of the oils boiling point and melting point and Specific gravity .These are summarized in the following table.

Table 4.6.the physical properties of *Ruta chalepensis* oil extracted using hexane as Organic solvent.

Physical properties	Their value
Melting point	$50\,^{o}$ C
Boiling point	$160\,^{o}$ C
PH of the oil	4.49-5.4
Specific Gravity	0.725
The color of the oil	Dark green
Moisture content	0.28%
Odor	Pleasant smell

4.2.2. Chemical Properties of Ruta chalepensis (Tenadam) Oil

The chemical properties of the oils which is done by analyzing the Acid value, Peroxide value, Iodine value, Free acid value, and saponification value helps to know the sensory quality evaluation of the product since some of these properties give the indication for the product that is susceptible to rancidity or deterioration of the oil components and the other (iodine value) determine the amount of un saturation degree in the oil component that is the primary condition for the rancidity of the oils product .Some of the results of chemical properties of *Ruta chalepensis* oil evaluated were discussed as follows.

4.2.2.1. Saponification Value

The saponification value is the number of mg of potassium hydroxide required to saponify 1 gram of oil/fat**.** An analytical importance of saponification value is used as an index of mean molecular weight of the fatty acids of glycerides comprising a fat. Lower saponification value means larger the molecular weight of fatty acids in the glycerides and vice-versa. Saponification value of oil serve as important parameters in determining the suitability of oil in soap making. Oils obtained gave a clear solution in water; this type of oil is grouped among those yielding soaps of soft consistency. In this specific study the saponification value of *Ruta chalepensins* oils is 196mg of KOH/g. According American Oil Chemists Society (AOCS), this value is in agreement with the standard interval to which the saponification value of crude and refined oil is founded. AOCS determines this interval to be 190 - 200 mg/g KOH of the crude or refined oils. From this value we can deduce that large value of saponification value which is almost approach to 200mg/g of KOH shows that the crude extract of the *Ruta chalepensins* oils extracted using hexane probably can be used as source of oils which can be used for soap production and good flavoring may also contribute for perfume and cosmetics production process as an additive ingredients. In addition, other previous work suggests that larger saponification value of oils and fats is good indicative in the use of the oil in production of liquid soap, shampoos and lather shaving creams (Bailey, E.A., 1954).

4.2.2.2. Acidic Value

The acid value is defined as the number of milligrams of potassium hydroxide required to neutralize the free fatty acids present in one gram of fat. It is a relative measure of rancidity as free fatty acids which are normally formed during decomposition of oil glycerides. The value is also expressed as percent of free fatty acids calculated as oleic acid. The analytical importance of a value is a measure of the amount of fatty acids which have been liberated by hydrolysis from the glycerides due to the action of moisture, temperature and/or lypolytic enzyme lipase. The low acid value suggests that the oil may be of advantage for paint making and the two values finally suggest that the oils are edible. For this specific *Ruta chalepensins* oil extract, the acid value evaluated using equation 3.4 and the result is 6.73mg of KOH/g of the oil sample. This value is small when we compare its value with that of the one reported according to AOCS or ISO 3961:1998. According to this data the maximum acid value of oils and fats sample should be less than 10mg/g to resist rancidity when the oils sample is exposed to the condition which favors oxidation. According to previous work the lower the acid value of oil, the fewer free fatty acids it contains which make it less exposed to the phenomenon of rancidification (Roger et al., 2010). Therefore, *Ruta chalepensis* oil is not susceptible to rancidification.

4.2.2.3. Peroxide Value

The peroxide value helps in determining which oil will be easily susceptible to oxidative rancidity. It is determined by measuring the iodine liberated from potassium iodide by peroxide, using sodium thiosulfate solution as the titrant. The sample oil is treated in solution with a mixture of acetic acid and a suitable organic solvent and then with a solution of potassium iodide. The liberated iodine is titrated with a standard solution of sodium thiosulfate. Peroxides and similar products which oxidize potassium iodide under the conditions of the test will contribute to the peroxide value. Peroxide values are expressed either in milliequivalents of peroxide/kg or millimoles of peroxide or oxygen. The peroxide index, measured in milliequivalents of active oxygen per kilogram, determines the initial oxidation of oil in a quality analysis. Fats and oils becomes oxidized when it comes into contact with the air. This is due to the fact that the unsaturated fatty acids (monounsaturated and polyunsaturated) take oxygen and give rise to the formation of peroxides, one of the main products of oxidation. On reacting with another unsaturated fatty acid, these peroxides transform into hydroperoxides which, in turn, are oxidized and give rise to the aldehydes and ketones that are responsible in this case for the rancidity of the oils and fats.

The peroxide index indicates the quality of life attributed to oils from the moment it was produced to when it was packaged. Among the refined oils this parameter is not indicative of anything, given that during the refinement process any product resulting from oxidation is eliminated and, therefore, so is any trait indicative of its age. Oxidation is an inevitable, natural process. However, it appears later on among the oils that present a high percentage of oleic acid and high poly phenol content (natural antioxidants). Using equation 3.6, the peroxide value of *Ruta chalepensis* oil is 5 milliequivalents of active oxygen/kg oil. This value is reported as milliequivalents of active oxygen taking oxygen as reference since it is mostly substance that bring oxidation on oil by further oxidation to peroxides which is the active form of radicals. According to AOCS, Cd 8b-90, The maximum peroxide value of crude oil that can be tolerated should be less than 25 meq/kg of the sample oil to resist oxidation that makes the oils rancid. The determined value of POV of *Ruta chalepensis* oil less than the maximum possible value reported by AOCS and this oil is not susceptible oxidation that facilitates rancidity.

4.2.2.4. Iodine Value

The iodine value of an oil/fat is the number of grams of iodine absorbed by 100g of the oil/fat, when determined by using Wij solution. In chemistry The iodine value (or "iodine adsorption value" or "iodine number" or "iodine index") is the mass of iodine in grams that is consumed by 100 grams of chemical substances. Iodine numbers are often used to determine the amount of unsaturation in fatty acids in the form of multiple bonds which is most of the time double bonds and can react with iodine compounds. The iodine value is a measure of the unsaturation of oil. The higher the iodine value the more multiple bonds are present, which consequently reflects the reactivity of the oil and the oils are unsaturated. The smaller value shows that the oil is dominated by saturated bonds (Thomas Alfred ,2002). The value of Iodine value calculated using equation 3.4 was 0.5076 grams of I_2/100gram the oil sample. AOCS Cd 1-25 reports that the maximum iodine value for the crude oil and finished to resist oxidation should be less than 0.75 gram of I_2/100gram and the figure shows that the crude extract of Ruta chalepensis oil has less amount of iodine value or multiple bonds that is susceptible for oxidation.

4.3. Results on Total Phenolic Compounds (TPC) Determination.

Gallic acid was taken as standard since it is the most known synthetic organic compound possessing maximum amount of total phenolic compound and any other phenolic compound extracted from Aromatic and medicinal plant is evaluated using ascorbic acid as standard and Folin-Ciocalteu reagent. The Folin-Ciocalteu reagent is sensitive to reducing compounds including poly- phenols, there by producing a blue color upon reaction. This blue colour is measured spectrophotometrically. Thus total phenolic content can be determined (Savitree M.*et al,* 2004) .In order to use it as standard, appropriate concentration of this acid is taken and its absorbance is measured by spectrophotometer at 725nm and the curve generated from the plot of absorbance versus concentration should give linear relationship with minimum regression coefficient of 0.933.Then the appropriate calibration curve equation is generated in the form of y=bx+a where y is absorbance in nanometer (nm), b is the slope and x is the variable represents Gallic acid in millimole (mM). The equation of the curve is used to find the total phenolic compound by measuring the absorbance of plant extract by taking the maximum possible absorbance measured within the interval of absorbance measured by the ascorbic acid. Using the procedure 3.5, the absorbance measured to have calibration curve of Gallic acid as standard at different concentration and its corresponding curve are given below.

Table 4.7. Absorbance of Standard Compound (Gallic Acid) at different concentration.

Concentration (mg/ml)	Absorbance λmax=725 nm		
	The first trial	the second trial	average
0.01	0.097	0.073	0.085
0.05	0.1167	0.185	0.1585
0.1	0.23	0.2304	0.2302
0.15	0.3089	0.2713	0.2901
0.2	0.345	0.3645	0.35475
0.25	0.4052	0.4033	0.40425
0.3	0.5023	0.5089	0.5056
0.4	0.6305	0.6248	0.62765
0.5	0.756	0.7049	0.73045

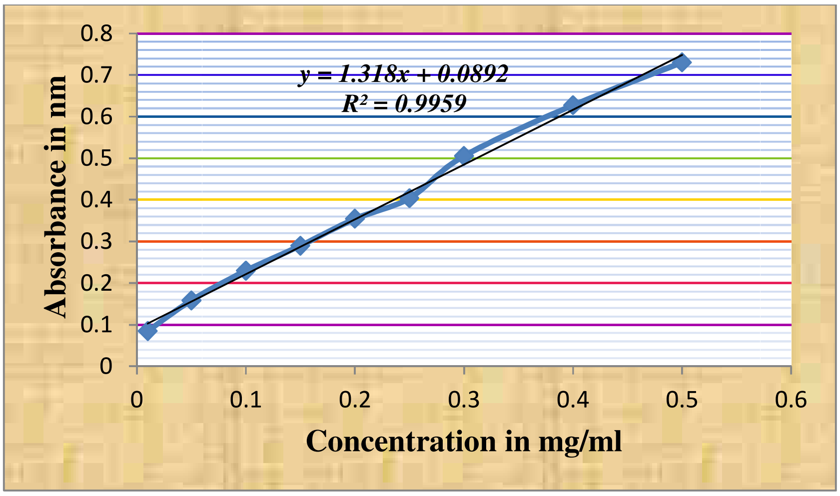

Figure 4.6.Calibration curve of Gallic acid as standard

From figure 4.6 we deduced that absorbance versus concentration of the calibration curve is straight line having equation of the curve $y = 1.318x + 0.0892$ with Regression coefficient of $R^2 = 0.9959$. This large number of R^2 shows the two parameters are linearly related and the calibration curve is successfully obtained. This implies also we can use it for determining the total phenolic compound of *Ruta Chalepensis* extract extracted using different solvent and its value is determined by putting the maximum absorbance of each extract with the limit of the standard (0.085-0.73045) in place of y and the value of x is solved which corresponds the total phenolic compound in terms of milligram equivalent of Gallic acid or millimole of equivalent Gallic acid. Based on the above reason, Gallic acid was used as calibration standard, and results were calculated as Gallic acid equivalent (GAE) (mg/g dry weight basis) and its value is given in the table below.

Table 4.8.The maximum absorbance of different Ruta chalepensis crude extract and their corresponding total phenolic compounds.

Crude Ruta Chalenpensis oil extract in	*Maximum absorbance measured (nm)*	*Total phenol compound in milligram equivalent of Gallic acid /gram of dry weight.*
Ethanol	0.5094	10.81
Methanol	0.6254	13.74
Aqueous (distilled water)	0.70894	15.674
Diethyl ether	0.4650	9.686
Hexane	0.2086	3.20
Optimized yield using soxhlet extractor	0.2452	4.127

From Table 4.7, we can see that the total phenolic compound of *Ruta Chalepensis* crude extract is highly affected by the type of solvent used to extract them and it is maximum for polar solvents and minimum for non-polar solvent. Aqueous crude extract oil of *Ruta Chalepensis* have greater number of total phenolic compound and that of hexane crude extract oil extracted using incubator shaker have less number of TPC Than that of the one extracted and optimized from Soxhlet extractor having the same extraction time and the same solvent to feed ratio. From these findings we can understand that TPC found in this plant is polar in nature and they are highly extracted by the Polar solvents. The TPC of aqueous and methanol crude extract of *Ruta chalepensis* for this specific study is closer with that of the one evaluated by previous work. This previous work reported that the TPC of methanol extract to be 13.288mg GAE/g of dry weight, 21.18 for aqueous extract (*Alali, F. et al., 2007*) and 11.58 for ethanol extract (Fakhfakh *et al.*, 2012). From these two previous works and this specific study we understand that aqueous extract have maximum TCP than ethanol and methanol extracts. For other solvents there is no previous work done on them which is similar with the method used for TPC determination in this specific study and their discussion is related with only the data we get in this specific study. Diethyl ether which is known as the solvent having intermediate polarity between polar and non-polar shows maximum TPC than hexane. Based on the data of Table 4.7, we can arrange the Ruta chalepensis extract as the following decreasing order: Aqueous extract>Methanol extract>Ethanol extract> Diethyl extract>optimized hexane extract using soxhlet extract> hexane extract by incubator shaker.

4.4. Antioxidant Activity of Ruta chalepensis Crude Oil Extract.

Antioxidant activity of *Ruta chalepensis* oil crude extract was evaluated using the procedure 3.6 taking Ascorbic acid as standard. Ascorbic acid is one of the known synthetic organic compound which shows the greatest antioxidant activity by scavenging the stable radical of DPPH and added to different food products especially oils to suppress oxidation process takes place due to the presence of multiple bonds or unsaturation. Antioxidant activity of all the *Ruta chalepensis* crude extract were evaluated taking the all amount of ascorbic acid used for determining its antioxidant activity and the table below shows the results of antioxidant potential of Ascorbic acid.

Table 4.9: The prepared Solution of standard (Ascorbic acid) at different concentration for Spectroscopy

Run order	Volume of standard solution taken from methanol solution in μl	Concentration of the standard solutions taken in mg/ml	Volume of the methanol taken in μl	DPPH solution (0.004%) μl	Absorbance in nm	Inhibition effect (IE) in %	mean of the IE from the two duplicate and ± SD
Blank	0	0	100	4000	0.8633	0.00	0.00
1	20	0.06	980	4000	0.6368	26.23	27.93±2.70
1	20	0.06	980	4000	0.6075	29.63	
3	30	0.09	970	4000	0.4287	50.34	50.57±0.01
3	30	0.09	970	4000	0.4248	50.79	
4	60	0.18	940	4000	0.2925	66.11	67.39±1.81
4	60	0.18	940	4000	0.2705	68.67	
5	90	0.27	910	4000	0.1258	85.42	87.93±0.02
5	90	0.27	910	4000	0.1042	87.93	
6	120	0.36	880	4000	0.0481	94.42	94.5±0.035
6	120	0.36	880	4000	0.0486	94.37	
7	150	0.45	850	4000	0.0345	96.01	96.65±0.10
7	150	0.45	850	4000	0.0342	96.04	
8	180	0.54	820	4000	0.0353	95.91	95.88±0.04
8	180	0.54	820	4000	0.0358	95.85	
9	200	0.6	800	4000	0.0398	95.38	95.32±0.08
9	200	0.6	800	4000	0.0409	95.26	

Table 4.9 shows that ascorbic acid is the strong antioxidant having maximum inhibition effect of 96.025 and the Inhibition effect increase in the first phase until the maximum value of IE is achieved and become decrease with further addition of ascorbic acid. The volume of crude extract of *Ruta chalepensis* which is the same as the volume of the standard (20,30,60,90,120,150,180,200 μl) were taken and its antioxidant activity was evaluated taking all other required reagents which are the same as that of ascorbic acid. Inhibition effect of all *Ruta chalepensis* crude extract was expressed as the average IE of the two duplicate and their corresponding values are presented in Table 4.10 and Figure 4.7 and it was drafted from the detailed data generated using the procedure 3.2 and all their data including all experimental result were written in appendix D.

Table 4.10: Inhibition effect of all crude extract of Ruta chalepensis oil crude extract evaluated using different solvent on DPPH assay.

Conc. of the sample taken from 10 mg/ml	Inhibition effect of different extract in different solvents					
	Ethanol Extract	Methanol Extract	Aqueous extract	Diethyl ether extract	Hexane Extract	Optimized yield of soxhlet extract
0.2	14.27±0.02	20.88±0.01	14.18±1.73	14.07±2.46	16.63±2.83	6.05±0.02
0.3	24.94±0.28	38.65±1.04	21.03±0.45	32.17±1.03	21.86±1.13	12.8±0.25
0.6	51.37.±1.61	57.56±0.49	29.81±0.03	44.25±0.04	39.23±0.01	36.71±0.67
0.9	62.41±0.99	84.34±0.57	45.15±0.27	47.17±1.00	57.36±1.07	49.95±0.28
1.2	80.04±1.82	88.78±0.07	56.51±0.02	54.91±0.46	63.08±4.23	71.86±0.35
1.5	86.79±1.12	79.79±0.72	62.92±0.77	61.27±0.13	65.82±0.84	73.98±0.98
1.8	87.98±0.10	79.13±0.31	80.8±0.03	68.95±0.23	60.04±0.19	72.36±2.35
2	86.31±0.25	78.55±0.65	77.19±0.09	60.69±0.84	55.66±0.93	69.83±0.43

The graph below shows the inhibition effect of different crude extract of *Ruta chalepensis* and its oils form soxhlet extractor.

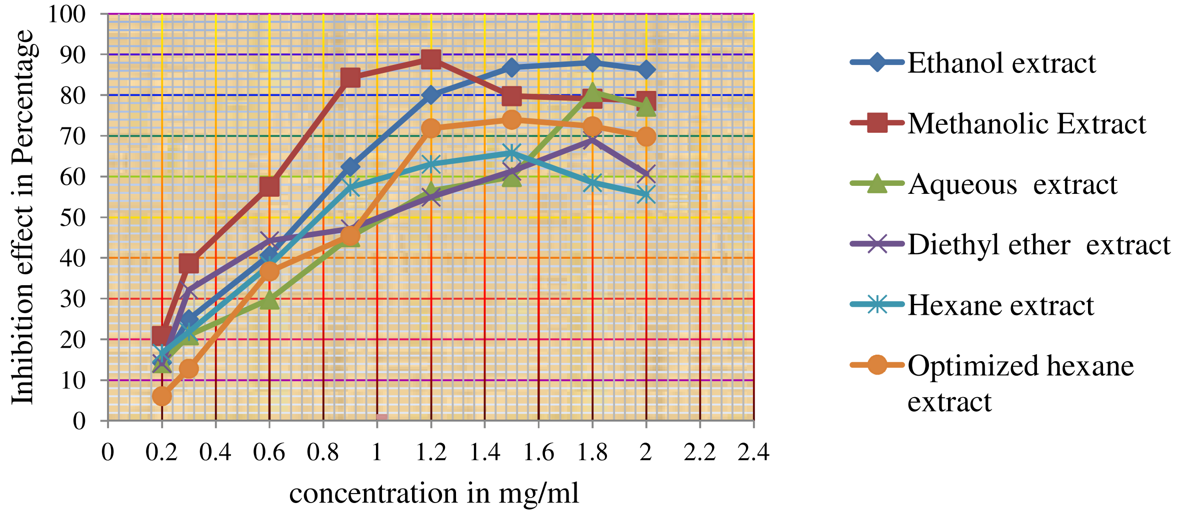

Figure 4.7 Inhibition effect of different extract of Ruta chalepensis crude extract oil.

From Figure 4.7 and Table 4.9-10 we can see that different extract of *Ruta chalepensis* using different solvents show different DPPH scavenging activity. The nature of the graph of Inhibition effect of antioxidant activity versus volume of the sample or the concentration of the sample get increase in the first phase until it reaches the maximum inhibition effect and starts slight decrease with further addition of the sample extract. Natures of these graph looks like that of the standard antioxidant of Ascorbic acid. The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability. When a solution of DPPH is mixed with that of a substance, it can generate a hydrogen atom. This results in the reduced form of DPPH (non-radical) with the change of the violet color and the color changed to yellow when it arrives at maximum inhibition effect and further addition of the extract sample can't get no more unstable radical to stabilize and brings increase in absorbance that decrease the Inhibition effect . DPPH scavenging activity is usually presented by IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. Therefore, extract concentrations providing 50% inhibition (IC_{50}) were calculated using the data plotted in Figure 4.7. Lower IC_{50} value reflects better DPPH radical-scavenging activity and has strong antioxidant potential. The maximum inhibition effect, the volume of the sample and its concentration at which maximum IE occurs and IC_{50} of different crude of extract given below.

Table 4.11.Maximum Inhibition effect and IC_{50} of different crude extract oils of *Ruta chalepensis*

Type of crude extract	Maximum IE in percentage	Volume of maximum IE μl	Concentration of maximum IE occurs mg/ml	IC_{50} in micro liter	IC_{50} concentration In mg /ml
Ethanol extract	87.00	180	1.8	72	0.72
Methanol extract	88.78	120	1.2	52	0.52
Aqueous extract	80.8	180	1.8	100	1
Diethyl ether extract	68.95	180	1.8	102	0.102
Hexane extract	65.82	150	1.5	82	0.82
Hexane oil extract by soxhlet extractor	73.98	150	1.5	96	0.96

From Table 4.10, we can understand that polar solvent extract (methanol and ethanol) have strong antioxidant when we compare them with their maximum inhibition effect and this two solvents shows minimum IC_{50} than the other solvents. There is a significant linear relation between antioxidant activity and total phenolic content for methanol extracts. These results suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the investigated plant species. These results were consistent with the findings of various research groups, who reported positive correlations between total phenolic content and antioxidant activity. A plant with high-antioxidant activity, for which phenolic content versus antioxidant activity falls above the regression line is a type of plant that should be investigated for novel antioxidants (Middleton *et al*,1994) . It is well known that even if phenolic substances primarily contribute for antioxidant of Aromatic and Medicinal plants, flavonoids, phenolic acids, and tannins also contribute directly to the antioxidant capacity of plants. In fact, phenolic compounds exhibit considerable free radical-scavenging activities (through their reactivity as hydrogen- or electron-donating agents) and metal ion-chelating properties, preventing metal-induced free radical formation (Rice-Evans *et al*, 1996). Water is strong polar solvent than the other solvents and it has greater total phenolic compound than the others, but shows maximum inhibition effect at maximum volume when we compare with that of hexane extract and have almost equal IC_{50} with that of diethyl ether. Previous work reports that Both ethanol (347.33 milligram of equivalent Quercetin /gram of dry weight or mg QE/g extract) and methanol extracts (323.12 mg QE/g extract of *Ruta chalepensis* have maximum amount of total flovonoid contents than aqueous extract (87.12 mg QE/g extract) where Total Flavonoids is determined taking Quercetin as standard compound and this may be the reason why aqueous extract of *Ruta Chalepensis* fail to have strong antioxidant since this compound also share their contribution for antioxidant activity of aromatic and medicinal plants (Fakhfakh *et al.*,2012). For other solvents (Diethyl ether and Hexane) there are no previous work and all the scientific justification of their antioxidant activity of the *Ruta chalepensis* plant were based on experimental data of this study . From table 4.10 we can see that hexane crude extract of Ruta Chalepensis oil extracted both in incubator shaker and Soxhlet extractor have appreciable antioxidant activity than that of Diethyl ether and aqueous extract when we compare them with their IC_{50} value. Methanol extract have relatively strongest antioxidant activity than the others solvents being followed by ethanol extract when we compare their antioxidant potential either in their maximum IE and their IC_{50}.

4.9. Chemical Composition of Ruta Chalepensis Essential oils.

The yield of essential oils of *Ruta chalepensis* collected is found to be 1.18±0.07 and it is below the yield evaluated by the previous works in different places. The yield of the *Ruta chalepensis* collected from Tunisia and investigated with the same isolation procedure of essential oils was reported as 2.32±0.2 (Fakhfakh et al.,2012). In this study the volatile compounds of essential oils of *Ruta chalepensis* extracted and investigated using GC-MS was identified searching through mass\hunter\library\NIST11.L and mass\hunter\library\W9N11.L were shown in table 4.12. The components were identified based on their elution or retention time on the HP-5MS column. Quantitative evaluation was based on percentages areas of their peak keeping minimum quality of the each component above 90 %. The chemical distributions of *Rut chalepensis* volatile compounds identified can be separated on the basis of their chemical structures into ketones, esters, acids, alcohols, aldehydes, hydrocarbons and diterpenes.

A survey of the literature shows that essential oils of different aerial parts of *Ruta chalepensis* plants grown at different locations have shown wide range of variations even in their major constituents. In fact, a composition dominated by 2-undecanone was found in the essential oil of *Ruta chalepensis* from Turkey (66%), Iran (52%) and (41 to 68%). However, essential oil of the plant from Italy showed that 2-undecanone and 2-nonanone were major compounds. Moreover, 2-undecanone (26 to 44%) and 2-nonanol (28 to 40%) were found to be the main compounds in essential oils of growing wild *R. chalepensis* from the center of Tunisia (Baser *et al.*, 1996; Rustaiyan *et al.*, 2002; Bagchi *et al.*, 2003; Ntalli et al., 2011; Saidani-Tounsi *et al.*, 2011). Previous works clearly indicate the occurrence of a new chemotype of *Ruta chalepensis* growing of Tunisia containing octyl acetate (28.5 to 33.16%), 2-undecanone (22.6 to 23.8%) and 2-nonanone (14.1 to 16.97%) as the major constituents of the essential oil. It is also to note that other functionalized compounds such as isomaturnin was detected for the first time in the *Ruta chalepensis* essential oil of Tunisia.

According Table 4.12 of this study, 2-undecanone (methyl nonyl ketone) (31.74), 1-dodecene (α-Dodecene; Dodec-1-ene; Dodecene-1; Adacene 12; Dodecylene) (13.591), Tridecene (13.189), 2-Nonanone (methyl heptyl ketone) (9.573) , 2-tetradecanol (4.856) are the major components Ruta chalepensis and the chemical structure of these compounds possess the fuctional group of ketones, alkenes which is one type of hydrocarbons and alcohols. Essential components identified in this study was dominated by ketones followed by alkenes and alcohols which are similar with previous work discussed earlier ,but their percentage were differ from the previous work and this may be the result of geological variation dependent of essential oils and different in amount of essential oils taken for GC-MS analysis which is not clearly discussed in previous works.

Ficusin (6-Hydroxy-5-Benzofuranacrylic acid d-lactone) (4.186) which belongs to one of a group of furanocoumarins occurring naturally in Rutaceae plant species is the other components exist in maximum amount next to 2-tetradecanol (4.856). These compound have various medicinal application and none of the previous work discussed above shows the existence of this compound in essential oils of *Ruta chalepensis* plant

Table 4.12. The chemical composition of Ruta chalepensis essential oil .

Compounds	Retention time (minutes)	(%) of total	Minimum quality
(E)-2-Hexenal	4.070	0.06	98
3-(Z)-Hexenol	4.117	0.018	91
α-Pinene (2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene)	4.322	0.004	97
Camphene	6.469	0.02	97
Benzaldehyde	6.807	0.014	96
Isopropyl tiglate (2-methyl-, 1-methylethyl ester)	7.246	0.019	92
β-Pinene (4,7,7-trimethylbicyclo[3.1.1]hept-3-ene)	7.337	0.03	95
Octanal	8.205	0.024	90
Anisole (1-methoxy-2-methylbenzene)	8.457	0.039	97
p-Cymene (4-Isopropyltoluene, 2,3diol (2,3-dihydroxy-4-isopropyl-1-methyl- benzene)	8.987	0.011	93
D-limonene	9.136	0.056	98
1,8-Epoxy-p-menthane (Eucalyptol)	9.239	0.087	99
beta-ocimene (3,7-dimethylocta-1,3,6-triene)	9.896	0.015	92
γ-Terpine (methyl-4-propan-2-ylcyclohexa-1,4-diene)	10.296	0.013	95
1-octanol	10.772	1.0701	91
2-Nonanone (methyl heptyl ketone)	11.728	9.573	97
Nonal (Nonanaldehyde)	12.161	0.378	91
1,7,7-Trimethyl-bicyclo(2,2,1)heptan-2-one (TMPH)	13.803	0.093	98
2-methoxy -3-isopropyl-(5 or6)-methyl pyridine	14.515	0.019	93
1-Nonanol	15.017	0.187	90
3-Cyclohexen-1-ol (L-4-terpineneol) or 4-methyl-1-(1-methylethyl)-	15.238	0.029	91

2-Decanone (Methyl octyl ketone)	15.974	2.066	97
Estragole (1-methoxy-4-(2-propenyl)-benzene)	16.179	0.040	99
2-tridecanol(tridecan-2-ol)	16.323	0.125	90
Decanal (Decyl aldehyde, caprinaldehyde)	16.52	0.074	91
1-dodecene (α-Dodecene; Dodec-1-ene; Dodecene-1; Adacene 12; Dodecylene)	18.04	13.591	90
2-undecanone (methyl nonyl ketone)	18.796	31.74	90
Cyclodecane	19.512	0.056	93
pregeijerene (1,5-dimethylcyclodeca-1,5,7-triene)	20.082	1.856	97
2-tetradecanol	20.824	4.856	90
4-ethenyl-1 2-dimethoxybenethiol	21.794	0.606	96
Ethyl ,ester	22.724	0.033	97
2-dodecanone	24.716	1.754	96
Tridecene	26.436	13.189	90
trans-β-Ionone (4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-3-buten-2-one)	28.406	0.056	98
2-Tridecanone	28.859	1.669	97
1-undecene	29.12	0.325	94
α-Farnesene	29.352	0.029	90
Cis -α-Bisabolene	30.682	0.058	99
Cyclo hexa methanol	30.894	0.051	91
4-(3,4-methylenedioxyphenyl)-2-butanone	32.668	0.38	98
1-Tridecene	34.081	0.42	93
Linolenic acid	35.406	0.052	93
1,3-Benzodioxole-5-Propanoic acid, ethyl esther	36.373	0.049	90
Tetradecanal	37.124	0.056	91
Phenanthrene	38.951	0.042	96
Ficusin (Psoralen)	40.688	4.186	97
Hexadecanoic acid (Methyl ester)	44.405	0.023	96

And others compounds which have minimum quality less than 90% and those which are not reported as essential oils

Chapter Five

Conclusion and Recommendation

5.1. Conclusion

This work is aimed at extraction, optimization and characterization of *Ruta chalepensis* oil. The extraction was done by soxhlet extractor using hexane as a solvent taking temperature, extraction time and solid mass to solvent ratio as the main factors and the response variables was yield. The results reveal that minimum yield of the *Ruta Chalepensis* oil is 8.98 percent by mass obtained at extraction temperature of 68 ^oC, extraction time of 2hrs and Solid mass to solvent ratio of 9 and the maximum yield is 16.78 percent by mass which found at extraction temperature of 85^oC, extraction time of 6 hours and solid mass to solvent ratio of 13. The yield of the *Ruta chalepensis* oil is varied in the range of 8.98-16.78.

The linear model selected for optimizing the experimental variables shows that the optimum yield is found to be 16.78% with the desirability criteria of 0.998 which corresponds to the maximum yield obtained. The optimum value of the process parameters optimized were: temperature 85 ^oC, Extraction time 6hrs and solid mass to solvent ratio of 13 keeping the criteria of the optimization in the range of the level selected for experimental variables.

The physical properties of the oil for the optimum yield from soxhlet extractor show that the oil is the drying solidifying oil at room temperature having dark green colour of a pleasant smell, with properties- melting point of 50^oC, boiling point of 120 ^oC, Specific gravity of 0.725 and P^H 4.49-5.24. *Ruta chalepensis* oil is also characterized by having acidic value (6.73mg KOH/gram of the sample), peroxide value (5.023milliequaivalentof active O_2/ kg of oil), iodine value (0.50706 gram I_2 /100gram of the oil$_)$, and Saponification value (196mg KOH/gram of the oil). The value of chemical properties of the *Ruta Chalepensis* such as Acidic value, peroxide value, iodine value shows that the oil is not susceptible to oxidation that brings oil deterioration.

Ruta chalepensis crude oil extracted in incubator shaker using solvents ethanol, methanol, distilled water, diethyl ether and hexane were compared with the *Ruta chalepensis* crude oil extract using soxhlet extractor of the optimum yield showed variation in amount of total phenolic compound and Antioxidant activity evaluated using both maximum inhibition effect and IC_{50}. The Total Phenolic compound of these solvents are arranged in decreasing order as follows : aqueous extract> ethanol extract> ethanol extract> diethyl extract> optimized hexane extract of soxhlet extractor > hexane extract by incubator shaker .Methanol crude extract has maximum antioxidant activity followed by Ethanol crude extract in terms of both maximum inhibition effect and IC_{50}. The crude extract oil extracted using soxhlet extractor has appreciable antioxidant activity than that of aqueous extract, diethyl ether and hexane extract of incubator shaker in terms of IC_{50}.

The chemical composition of essential oils extracted from *Ruta chalepensis* using Clevenger hydro steam distillation and identified using GC-MS have 2-undecanone (methyl nonyl ketone) (31.74), 1-dodecene (α-Dodecene; Dodec-1-ene; Dodecene-1; Adacene 12; Dodecylene) (13.591), Tridecene (13.189), 2-Nonanone (methyl heptyl ketone) (9.573) , 2-tetradecanol (4.856) as the major components and the chemical structure of these compounds possess the fuctional group of ketones, alkenes which is one type of hydrocarbons and alcohols being dominated by ketones followed by alkenes and alcohols respectively.

5.2. Recommendation

- In Ethiopia, there is a huge potential of botanic resources from which essential oils having commercial values can be extracted. For these, the government should give due emphasis to exploit and devise ways to properly utilize the bio resources.
- National research institute should give attention to research and development in the area essential oil production and utilization.
- Government and private sectors should take a motive to create awareness for farmers about industrial utilization and economic values of essential oils to promote the plantation of plants used for the production of essential oils of which Ruta chalepsis is one among these.

Recommended Further Studies

The following recommendations are made based on a holistic review of the subject area for production of essential oil from Ruta chalepensis:

- Studies on evaluation of *Ruta chalepensis* extract for its preservative effect on various food products.
- Studies on evaluation of *Ruta chalepensis* essential oil extracted by using steam distillation for the production of perfume, cosmetics formulation, drug formulation and other relevant products.
- Studies on optimization of processing variables to maximize the antioxidant potential of Ruta chalepensis crude extracts.
- Studies on Techno – Economic evaluation of Ruta chalepensis essential oil production

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Appendix- A: *The experimental result from Soxhlet extraction using Hexane as organic solvent*

Run	Temperature	Extraction time	Solid to solvent ratio	Yield on trial -1	Yield on trial -2	Yield =μ±sd
1	76.50	4.00	9.00	12.700	10.300	11.500±1.697
2	68.00	2.00	13.00	9.510	11.650	10.580±1.513
3	76.50	2.00	11.00	12.053	12.353	12.203±0.150
4	85.00	4.00	11.00	14.733	11.723	14.493±1.369
5	76.50	4.00	11.00	13.300	12.700	13.000±0.590
6	68.00	4.00	11.00	10.223	12.233	10.973±1.420
7	76.50	4.00	11.00	13.560	11.520	12.540±1.020
8	85.00	6.00	13.00	16.888	16.648	16.768±0.164
9	85.00	6.00	9.00	15.460	14.840	15.150±0.430
10	76.50	6.00	11.00	12.470	14.530	13.500±1.456
11	68.00	6.00	9.00	11.220	8.420	9.820±1.980
12	76.50	4.00	11.00	12.800	13.640	13.220±0.954
13	85.00	2.00	9.00	13.256	12.695	12.976 ±0.397
14	68.00	2.00	9.00	9.520	8.440	8.980±0.764
15	76.50	4.00	11.00	11.561	14.261	12.911±1.090
16	68.00	6.00	13.00	11.786	14.166	12.976±1.190
17	76.50	4.00	13.00	14.902	12.442	13.672±1.379
18	76.50	4.00	11.00	11.156	13.796	12.476±1.32
19	85.00	2.00	13.00	14.500	11.840	13.220±1.881
20	76.50	4.00	11.00	12.020	13.800	12.910±0.890

Where μ is the mean of the two measurements sd is standard deviation of the two measurements.

Appendix B: Fit summary model

Response 1 Yeild Transform: None
**** WARNING: The Cubic Model is Aliased! ****

Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	3232.6	1	3232.69			
Linear vs Mean	55.17	3	18.39	1157.15	< 0.0001	Suggested
2FI vs Linear	0.051	3	0.017	1.08	0.3920	
Quadratic vs 2FI	0.049	3	0.016	1.06	0.4093	
Cubic vs Quadratic	0.069	4	0.017	1.20	0.4016	Aliased
Residual	0.086	6	0.014			
Total	3288.11	20	164.41			

"Sequential Model Sum of Squares [Type I]": Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Linear	0.20	11	0.018	1.75	0.2800	Suggested
2FI	0.15	8	0.019	1.80	0.2688	
Quadratic	0.10	5	0.020	1.94	0.2421	
Cubic	0.033	1	0.033	3.18	0.1345	Aliased
Pure Error	0.053		50.011			

"Lack of Fit Tests": Want the selected model to have insignificant lack-of-fit.

Model Summary Statistics

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	0.13	0.9954	0.9946	0.9921	0.44	Suggested
2FI	0.13	0.9963	0.9946	0.9845	0.86	
Quadratic	0.12	0.9972	0.9947	0.9860	0.77	
Cubic	0.12	0.9984	0.995	0.2576	41.14	Aliased

"Model Summary Statistics": Focus on the model maximizing the "Adjusted R-Squared" and the "Predicted R-Squared".

Appendix-C:Analysis of Variance (ANOVA) for Response Surface Linear Model

Source	*Sum of Squares*	*df*	*Mean Square*	*F Value*	*p-value Prob > F*	
Model	55.17	3	18.39	1157.15	< 0.0001	Significant
A-Temperature	33.91	1	33.91	2134.20	<0.0001	
B-Extraction time	12.28	1	12.28	772.55	< 0.0001	
C-SSR	8.97	1	8.97	564.70	< 0.0001	
Residual	0.25	16	0.016			
Lack of Fit	0.20	11	0.018	1.75	0.2800	Not significant
Pure Error	0.053	5	0.011			
Core Total	55.42	19				

The Model F-value of 1157.15 implies the model is significant. There is only a 0.01% chance that a "Model F- Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C is significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The "Lack of Fit F-value" of 1.75 implies the Lack of Fit is not significant relative to the pure error. There is a 28.00% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit

Std.Dev.	0.13	R- Squared	0.9954
		AdjR-Squared	0.9946
Mean	12.71		
C.V.%	0.99	Pred R-Squared	0.9921
PRESS	0.44	Adeq Precision	138.247

The "Pred R-Squared" of 0.9921 is in reasonable agreement with the "Adj R-Squared" of 0.9946."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 138.247 indicates an adequate signal. This model can be used to navigate the design space.

Factor	*Coefficient Estimate*	*df*	*Standard error*	*95% CI Low*	*95% CI High*	*VIF*
Intercept	12.71	1	0.028	12.65	12.77	
A-Temperature	1.84	1	0.040	1.76	1.93	1.00
B-Extraction time	1.11	1	0.040	1.02	1.19	1.00
C-SSR	0.95	1	0.040	0.86	1.03	1.00

Final Equation in Terms of Coded Factors:

Yeild =+12.71+1.84 * A+1.1B* +0.95 * C

Final Equation in Terms of Actual Factors:

Yeild =-11.28700+0.21666 * Temperatur+0.55400 * Extractiont ime+0.47365*SSR

Appendix-D: *Detailed experimental procedures and raw data on antioxidant determination*

Concentration of the sample taken in mg/ml	Absorbance (A) measured and Inhibition effect (IE) of Ruta chalepensis oil extracted using different solvent measured at 515nm											
	Ethanol Extract		Methanol extract		Aqueous extract		Diethyl ether extract		Hexane extract		Optimized hexane extract	
	A	IE (%)	A	IE (%)	A	IE (%)	A	IE (%)	A	IE (%)	A	IE (%)
Blank	0.8546	0.00	0.6780	0.00	0.8287	0.00	0.7047	0.00	0.8940	0.00	0.7996	0.0
0.2	0.7118	14.28	0.5414	20.14	0.7011	15.40	0.6177	12.34	0.7635	14.6	0.7510	6.07
0.2	0.7121	14.25	0.5314	20.14	0.7213	12.96	0.5933	15.80	0.7271	18.67	0.7513	6.04
AVG.	0.7120	14.27	0.5364	20.88	0.7112	14.18	0.6055	14.07	0.7453	16.63	0.7512	6.05
0.3	0.6416	24.92	0.4210	37.91	0.6570	20.72	0.4831	31.44	0.6914	22.66	0.6986	12.63
0.3	0.6413	24.96	0.4110	39.38	0.6517	21.36	0.4729	32.89	0.7058	21.05	0.6958	12.98
AVG	0.6415	24.94	0.4161	38.65	0.6544	21.03	0.478	32.17	0.6986	21.86	0.6972	12.8
0.6	0.4058	40.81	0.2850	57.9	0.5815	29.83	0.3931	44.21	0.5412	39.46	0.5099	36.23
0.6	0.4008	52.51	0.2901	57.21	0.5818	29.79	0.3927	44.27	0.5631	39.46	0.5023	37.18
AVG.	0.4033	40.66	0.3466	57.56	0.5817	29.81	0.3929	44.25	0.5522	38.23	0.5061	36.71
0.9	0.3273	61.70	0.1035	84.73	0.4560	44.97	0.3773	46.45	0.3744	58.12	0.4742	40.69
0.9	0.3153	63.11	0.1089	83.93	0.4529	45.35	0.3673	47.87	0.3879	56.61	0.3979	50.24
AVG.	0.3213	62.41	0.1062	84.34	0.4545	45.15	0.3723	47.17	0.3812	57.36	0.4361	45.46
1.2	0.1816	78.75	0.0764	88.73	0.3603	56.52	0.3154	55.24	0.3031	66.09	0.2244	71.90
1.2	0.1596	81.32	0.0757	88.83	0.3605	56.49	0.3200	54.59	0.3570	60.07	0.2256	71.85
AVG.	0.1706	80.04	0.0761	88.78	0.3604	56.51	0.3177	54.91	0.3305	63.03	0.225	71.86
1.5	0.1197	85.99	0.1335	80.31	0.3616	56.37	0.2735	61.18	0.3108	65.23	0.2026	74.66
1.5	0.1061	87.58	0.1405	79.28	0.3028	63.46	0.2723	61.36	0.3002	66.42	0.2136	73.28
AVG.	0.1132	86.79	0.137	79.79	0.3322	59.91	0.2729	61.27	0.3055	65.82	0.2081	73.98
1.8	0.1021	88.05	0.143	78.90	0.1589	80.82	0.2199	68.79	0.3561	60.17	0.2360	70.4
1.8	0.1033	87.91	0.1400	79.35	0.1592	80.78	0.2177	69.11	0.3853	59.90	0.2101	73.73
AVG.	0.1027	87.98	0.1415	79.13	0.1591	80.80	0.2188	68.95	0.3707	58.5	0.221	72.36
2	0.1132	86.75	0.1486	78.08	0.1886	77.25	0.2728	61.28	0.3906	56.31	0.2436	69.53
2	0.1102	87.11	0.1422	79.01	0.1896	77.12	0.2812	60.09	0.4023	55.00	0.2387	70.15
AVG.	0.117	86.31	0.1454	78.55	0.1891	77.19	0.277	60.69	0.3965	55.65	0.2412	69.83

AVG: average

Appendix E:Some of Photos from laboratory works

- *Steps used in raw material preparation*

1.plant material collection and identification of aerial parts

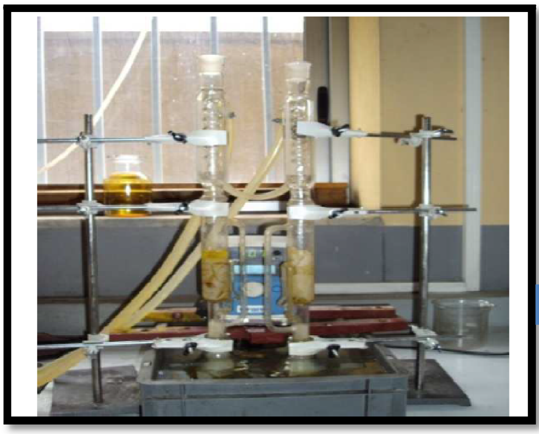
2. Exposing the aerial parts of plant to the shadow area

3.The dried aerial parts to be ready for grinding using Cross beater Mill

4.Grinding of the dried aerial parts using Cross beater Mill.

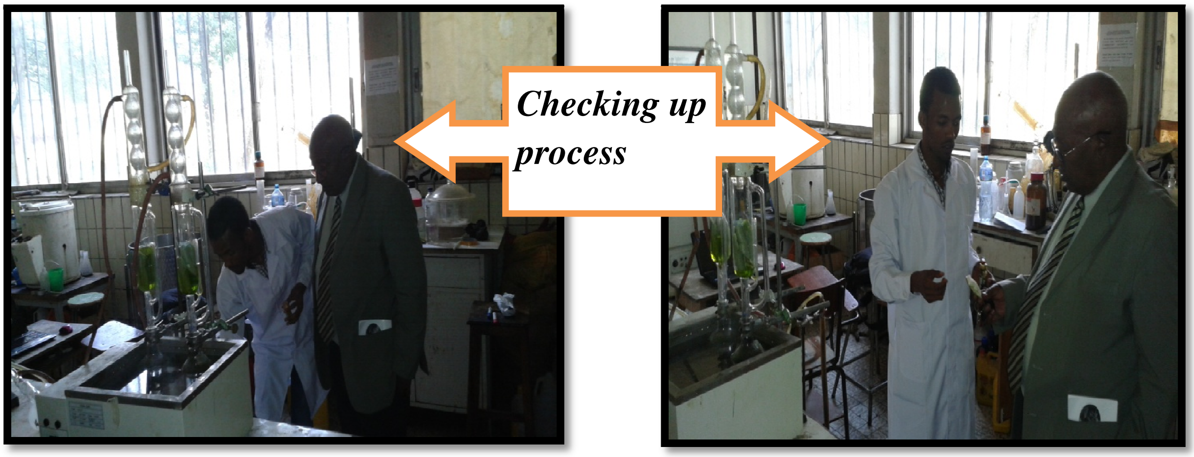
5. Separating grounded plant parts into the desired particle size.

- *Some of photos of soxhlet extraction*



setting up soxhlet extractor together with the condenser.

Concentrating the oil with Rotary evaporator

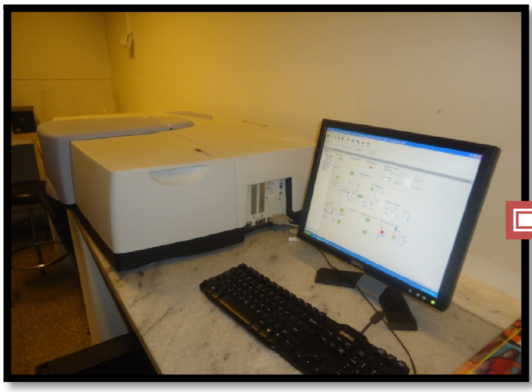


Checking Experimental set up and oil extracted by Ing.Geachew Shifaraw (Principal Advisor)

Some of photos of Antioxidant determination of Ruta chalepensis crude extract

comparing of blank sample with that of the other sample

Incubating the tubes at room temperature for about 30 minutes.



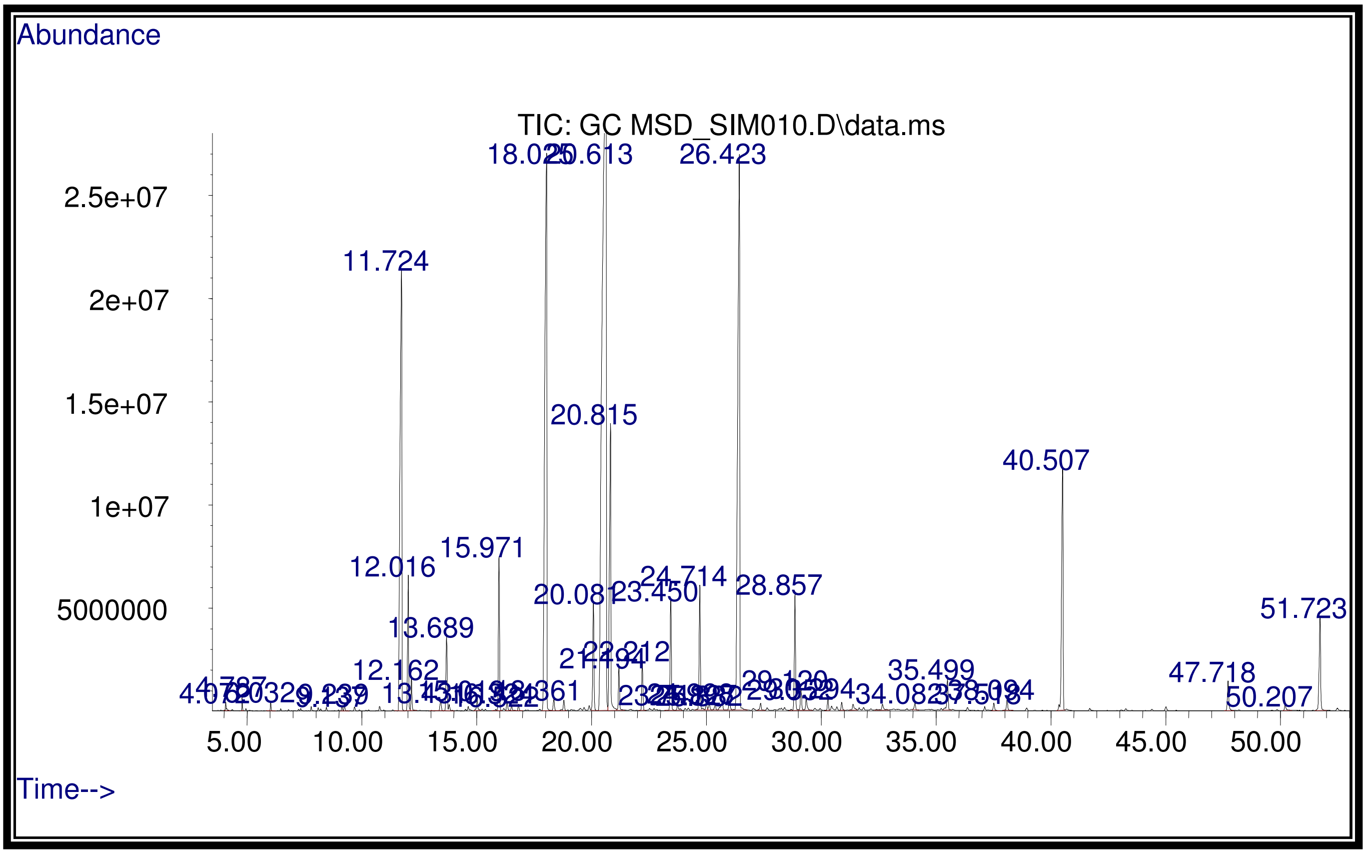
Calibaratinhigh performance UV-Vis NIR spectrophotometer

inserting each of the samples to UV-Vis NIR spectrophotometer

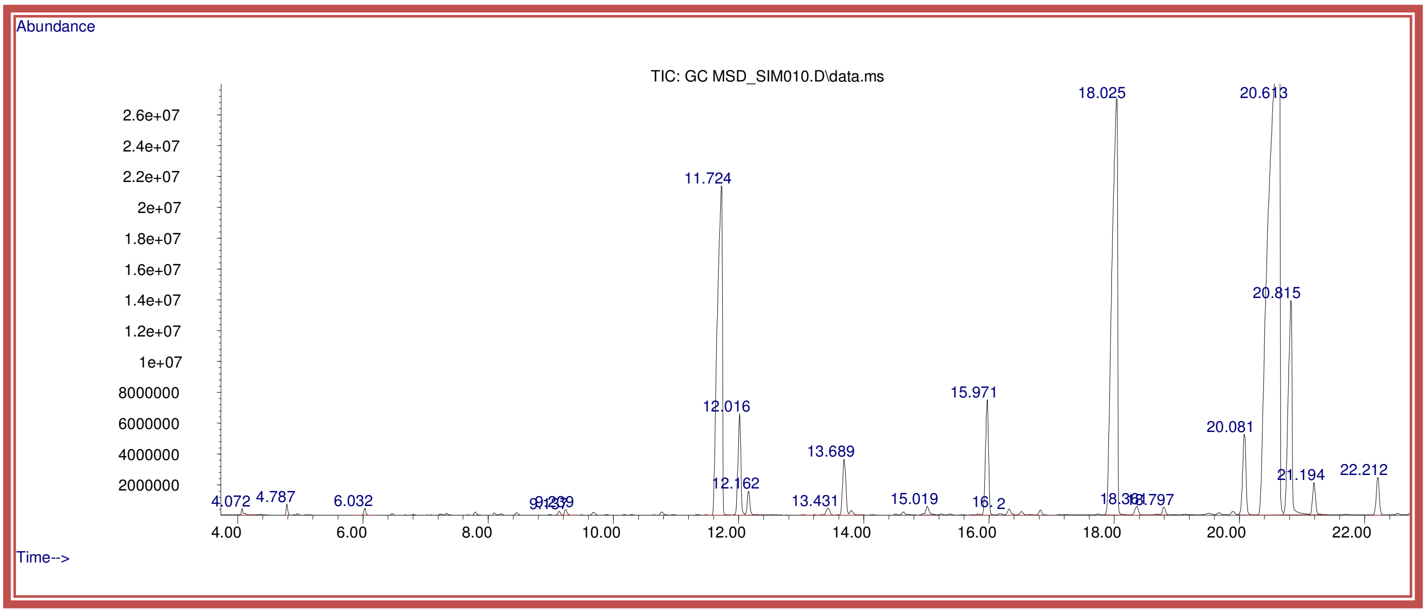
Measuring the absorbance of each solution of test tubes

Appendix F: Chromatogram of Gas Chromatography-Mass spectroscopy Detector

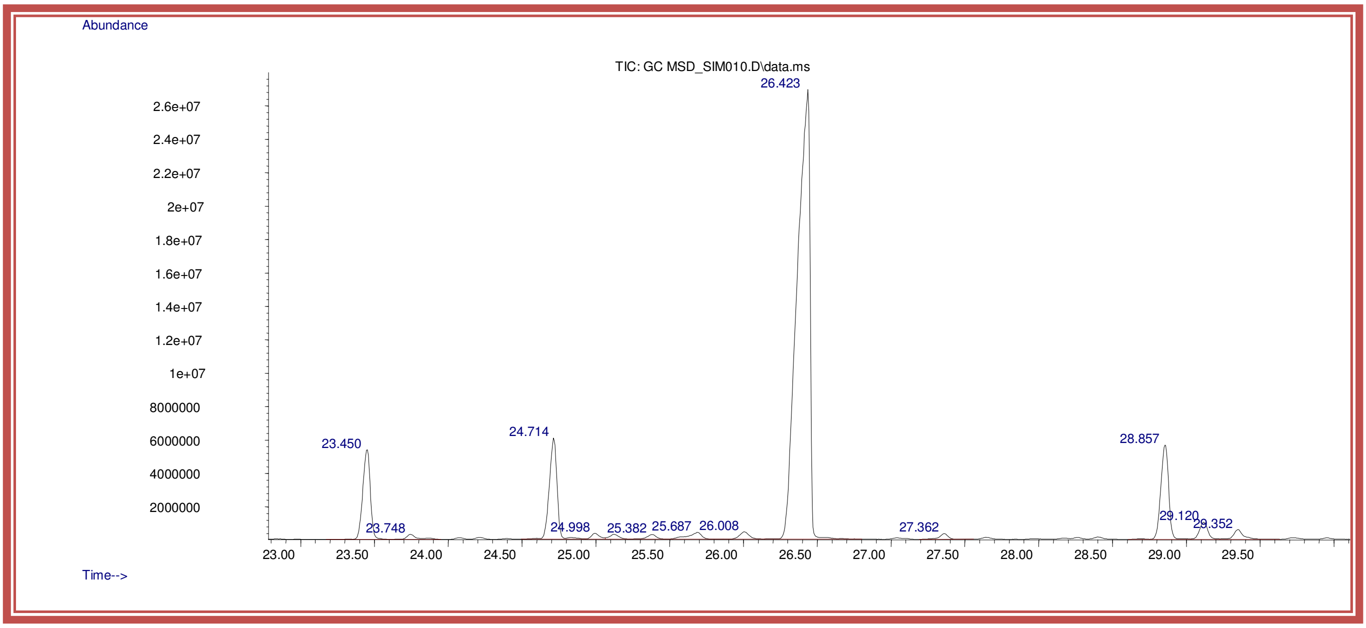
a) *The whole chromatogram of the sample analyzed*



b) The chromatogram up to 22 minutes for further visibility



c) The chromatogram picture from 22 minutes up to 29.5minutes for further visibility



d) The chromatogram picture from 30 minutes up to 52minutes for further visibility