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EXTRACTION OF AZADIRACHTIN FROM NEEM SEEDS FOR BIOPESTICIDE PURPOSE

A final thesis Submitted to the Graduate School of Chemical and Bio Engineering, Addis Ababa institute of Technology in Partial Fulfillment of the Requirements for the Degree of Master of Science in Process Engineering

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List of acronyms

Bt	Bacillus thuringiensis
WHO	World Health Organization
USD	United State Dollar
EIA	Environmental Impact Assessment
EPA	Environmental Protection Agency
FAO	Food and Agricultural Organization
V/W	Volume by Weight
TLC	Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
TVOC's	Total Volatile Organic Compound's
pH	Potential Hydrogen
RSM	Response Surface Methodology
DDT	Dichlorodiphenyltrichloroethane
EPID	Ethiopia Pesticide Integration Development
MoA	Ministry of Agriculture
ULV	Ultra-Low Volume
EC	Emulsifiable insecticide
RSPM	Residual Suspended Particulate Matter
SO_2	Sulfur dioxide
NO_2	Nitrogen dioxide

SPM	Suspended Particulate Matter
CO	Carbon monoxide
ISO –	International Standards Organization
Proc.	Proclamation
ICI	Imperial Chemical Industries
UV	Ultra-Violate
IPM	Integrated Pest Management
ASTM	American Society for Testing and Materials
LD_{50}	Lethal Dose of 50% weight loss
LC_{50}	Lethal Concentration of 50% weight loss
VOC'S	Volatile Organic Matter
TVOC's	Total Volatile Maters Organic Maters
R*f*	Retention factor
ANOVA	Analysis of Variance
CV	Coefficient of Varian

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Abstract

The effect of synthetic pesticides on the environment and human health is persistent and very aggressive. About 500,000 people are exposing to a series health problem caused by synthetic pesticides every year in the world. The objective of this study was extraction of azadirachtin from Neem seed for biopesticide application using ethanol as a solvent so that to claim environmental issue and clean economy as an alternative. The four factors analyzed in this study were particle size, extraction temperature, solvent to solid ratio and extraction time. The extraction process was carried out in four main stages namely; pretreatment, extraction, separation and concentration. The first stage was drying and grinding the raw seed to a particulate size of 350, 450 and 550µm. The raw seed have had 20.873% (w/w) of moisture content, with ash and organic matter content of 3.648 ± 0.187% and 96.3515% (w/w) respectively. Ethanol was prepared in a ratio of 5:1, 6:1 and 7:1v/w for extraction. Finally, the ground seed and the solvent were poured to an extraction vessel with overhead stirrer simultaneously and extraction was carried out until the extraction time becomes finished. An optimum yield was found 9.319g from 100g of sample with operational conditions of 350µm, 45^{o}C, 6:1v/w and 4h of particle size, extraction temperature, solvent to solid ratio and extraction time respectively. The characteristics of azadirachtin were then evaluated using different analytical methods. The result found was; Rf value of TLC 0.7079 ± 0.0068, retention time of HPLC 3.03minute, specific gravity of 1.3355 ± 0.0743, the flash point was 35.05 ± 1.0966 ^{o}C, water solubility was 99.708 ± 0.0096% (w/v), acute toxicity of azadirachtin was 2343.0.777 ± 60.2106 mg/kg, acidity of azadiracthin was 4.0533 ± and TVOC's was 0.45095 ± 0.3360% (w/w). RSM of Box – Behnken method was used to justify the factors effect on the yield. From the statistical analysis method, all the factors had an impact on the yield directly. The interaction effect of particle size and solvent to solid ratio, particle size and extraction time, extraction temperature and extraction time and solvent to solid ratio and extraction time had also a significant effect on the yield. Depending on the model optimization design 363.95µm, 44.82^{o}C, 6.62:1(v/w) and 3.68h of the factors were found the optimum points with 9.36399g yield of extract.

***Keywords**: Neem seed, Azadirachtin, TLC, HPLC, TVOC's Acute toxicity and RSM*

1. INTRODUCTION

1.1. Background

Any animal, plant or pathogen, which causes damage annoyances to man, animals, crops or possessions is known to us pest (Chakraborty, 2016). Combating the diseases of plant, destructive animals and weeds to protect crops started along with the starting of agricultural activities by human.

Plant extracts were likely the earliest agricultural bio-pesticides, as history records that nicotine was used to control plum beetles as early as the 17th century (bpia, 2013). Experiments involving biological controls for insect pests in agriculture date back as far as 1835, when Agostine Bassi demonstrated that white-muscadine fungus (Beauveria bassiana) could be used to cause an infectious disease in silkworm. Experiments with mineral oils as plant protectants were also reported in the 19th century. During the rapid institutional expansion of agricultural research during the early 20th century, an ever-growing number of studies and proposal for bio-pesticides were developed.

Very little progress has been made with regard to treatment of diseases until about 1882 when the value of lime and copper sulphate as a fungicide was accidentally observed in France and very quickly resulted in the development of the Bordeaux mixture by Millardet. The sudden discovery of Bordeaux mixture is believed to be the first important landmark in the history of chemical control of plant diseases. Most of the insecticidal organophosphates were developed during the early 19th century, but their effects on insects, which are similar to their effects on humans, were discovered in 1932. While figured back, the discovery of dithiocarbamates in 1934 and introduction of several other pesticides in the late 1960's are considered to be the two most important events in the one hundred years long history of chemical control of plant disease.

Mineral oils as plant protecting agent were also reported in the 19th century. One of the most important discoveries of bio-pesticides was spores of the bacteria Bt, in 1901. Japanese biologist Shigetane Ishiwata isolated Bt from a diseased silkworm. It was rediscovered after ten years, by

Ernst Berliner in Thuringen, Germany in a diseased caterpillar of flour moth. Bt was started to use as natural insecticide in 1920 while commercially became available in 1938, in France as Bt product, Sporeine. After this, till 1999 several bio-pesticides were discovered, developed commercially, appeared in world market, yet toxic synthetic chemical insecticides were continuously leading the market of pesticides in the 20th century as they were cheap.

Until the early 20th Century, cultural and mechanical methods augmented by Pesticides in the Modern World – Pesticides Use and Management a diverse range of organic and inorganic substances derived from plants, animal and minerals dominated pest control. Effective and affordable synthetic pesticides gained ground by the mid-20th Century, due to the maturing chemical industry and environmental concern.

Neem is a member of the Mahogany family Meliaceae and it contains an active ingredient known Azadirachtin, which is a proven natural anti-feedant, growth regulator, anti-ovipository and insect repellent. It is toxic to soft bodied insect larvae. Azadiracthin has proven effectiveness as a pesticide against about 200 insect species and is reported as non-toxic to humans (Csurhes .. , 2016).

In 1963 Indian scientists extensively examined the chemistry of the active principles of Neem. Following the discovery of Neem kernel as a locust feeding deterrent, its chemistry has grown considerably. Several compounds have been isolated and characterized. The main feature is that most of them are chemically similar and biogenetically derivable from a Tetracyclicterpenes. These are also called liminoids bitter principles and occur in other botanical species as well. The unraveling of high complex structural features and biogenetic interrelationship represent classic piece of work on natural product chemistry. From the practical side, these compounds also exhibit a wide variety of biological activity.

In ancient time, it is believed that the seed of Neem tree have a mutagenic property of mosquitoes and house flies (Medical Sanskrit of India). Still, it is in use and have high curative in some urban and rural areas in the developing countries. For example, in the Northern part of Ethiopia (Tigray), particularly in central part, the Neem leave and seed were used to protect the crops from pests by steeping the crops to a 24hr water solution of Neem leaves.

Now, a day due to so many reasons organic or bio-pesticide production largely encouraged by different countries. The reasons that countries encouraging bio-pesticide instead of the synthetic one is; environmental concern, health concern, resource utilization and conservation concern and specific target effect concern of a particular pest.

1.2. Problem Statement

Uses of synthetic pesticides have caused series negative health and environmental effects. In addition, development of resistance by the pests is another drawback associated with the use of synthetic pesticides. Therefore, this work is aimed at developing azadirachtin from Neem seed for bio pesticide purpose as an alternative to synthetic pesticides.

According to WHO report of 2008, 25% of the world production of synthetic pesticide applied in developing countries where 99% of deaths due to pesticides occur. Up to 20,000 people die because of synthetic pesticide poisoning in the third World countries each year (WHO., 2008).

Bio-pesticides are effective in smaller quantities, decompose quickly and do not cause environmental problems than chemical pesticides. Neem based pesticides has less prone to pest resistance applicably.

So, these reasons practically tend to have an optional and preferable pesticide in terms of availability, health precaution and clean economy principle. Furthermore, the cake produced from the extraction of bio-pesticide is used as bio-fertilizer by enriching the soil with organic matter and lowering nitrogen losses by inhibiting nitrification.

1.3. Objective

1.3.1. General Objective

The general objective of this study was to extract the active ingredient named as azadirachtin from Neem seed for biopesticide purpose.

1.3.2. Specific Objectives

The specific objectives of this study were:

- To extract and concentrate the active ingredient called Azadirachtin from Neem seed for bio pesticide purpose.
- To study the optimum operating conditions of seed particulate size (350μm, 450μm and 550μm), extraction temperature (35^{o}C, 40^{o}C and 45^{o}C), extraction time (3hr, 4hr and 5hr) and solvent to solid ratio (5:1v/w, 6:1v/w and 7:1v/w).
- To evaluate and characterize azadirachtin.

1.4. Scope of the study

This thesis study focuses on the extraction of azadirachtin from Neem seed for biopesticide purpose within certain parameters; particle size (350μm, 450 μm and 550 μm), extraction temperature (35^{o}C, 40^{o}C and 45^{o}C), extraction time (3hr, 4hr and 5hr) and solvent to solid ratio (5:1v/w, 6:1v/w and 7:1v/w) respectively. The already extracted active ingredient or azadirachtin should be characterized by TLC analysis, HPLC analysis test, Specific gravity, Flash point, water solubility, Toxicity test (Acute), pH value, and Total volatile organic compound's test of it so that; to harmonize with the environment and check its potential effect.

Box-Behnken method of RSM statistical analysis is used to carry out the experimental design optimized operation condition with a probability of significance 5% or $\alpha = 0.05$.

1.5. Significance of the study

Over 3000 tons of various types of pesticides that are worth more than USD 20 million are imported annually (EPA .. F., 2004). 12 years later it is obvious to predict that this amount should be parallel to grow with demand and expense.

Furthermore, the cake produced from the extraction of bio-pesticide is used as bio-fertilizer. In addition, Neem seed pesticide less prone to pest resistance applicably. Azadirachtin technically used for formulations of agricultural specialties, textile, domestic, veterinary pest management. Neem seed cake (residue of Neem seed extract) when used for soil amendment or added to soil, not only enriches the soil with organic matter but also lowers nitrogen losses by inhibiting nitrification. It also works as a nematicide.

Environmental pollution, creating health hazards due to the presence of synthetic pesticide residues in food and fiber are also major problems. Such problems are not expected from bio-pesticides. This is because, Bio-pesticides are less harmful than chemical pesticides because they do not leave harmful residues, generally target one specific pest or a small number of related pests in contrast to broad spectrum chemical pesticides which affect, apart from the pest, other beneficial insects, birds, mammals or non-target species, effective in smaller quantities,

decompose quickly and do not cause environmental problems and often cheaper than chemical pesticides.

So, those all reasons are the major driving forces that implies the need for bio-pesticide. Principally, Ethiopia is a peasants' country that going to be a middle-income state with it's about 75% of its total population depends on agriculture and agricultural product income. So, the need for bio-pesticide is unquestionable.

2. LITERATURE REVIEW

2.1. Impact of Pests on crop production

Insects are the most diverse species of animals living on earth. Apart from the open ocean, insects can be found in all habitats; swamps, jungles, deserts, even in highly harsh environments such as pools of crude petroleum (Imms, 1964). Less than 0.5 percent of the total numbers of the known insect species are considered as pests, and only a few of these can be a serious menace to people. Insect pests inflict damage to humans, farm animals and crops. Insect pests have been defined by (Williams, 1947) as any insect in the wrong place. Generally, a pest is any organism that spreads disease, causes destruction or creating a nuisance. Some examples of pests that are being harmful to human are mosquitoes, rodents, and weeds. Not all insects are pests. A lot of different kinds of insects eat other pests and are beneficial species for human. Examples of beneficial insects are dragonflies (which feed mainly on mosquitoes) and lady beetles (which eat aphids, scale insects, mites, and other insects).

Herbivorous insects are said to be responsible for destroying one fifth of the world's total crop production annually (Mohamed, 2002). Insect pests are capable of evolving to biotypes that can adapt to new situations, for example, overcome the effect of toxic materials or bypass natural or artificial plant resistant, which further confounds the problem (Roush, 1987).

Sustaining of food has always been a challenge facing mankind. A major cornerstone in this challenge is the competition from insect pests. Particularly in the tropics and sub-tropics, where the climate provides a highly favorable environment for a wide range of insect pests, massive efforts are being required to suppress population densities of the different pests so that to achieve an adequate supply of food.

In developing countries, the problem of competition from insect pests is further complicated with a rapid annual increase in the human population (2.5-3.0 percent) in comparison to a 1.0 percent increase in food production (FAO, 2014). Taking into consideration a sudden problem caused by drought, pre-harvest and post-harvest damage of pests in those places on agricultural products added a serious burden to people's daily life.

2.2. General classification of Pesticides

Pesticides are substance or mixture of substances proposed to prevent, destroy, repel, or to diminish any pest including unwelcome species of animals; during production or storage, transportation and distribution of agricultural products (Sallam, 2006). In addition to this, pesticides might be administered on animals to fight parasites, such as a flea, that lives outside of them as a host. Pesticides are classified predominantly in to two ways. According to the source of origin synthetic (Chemical) or natural (bio-pesticide) and According to the direct type of pest they can control they can be named as: (FEPA .. T., 2004).

Table 2-1 Classification of pesticides Application based

Insecticides	**Herbicides**	**Fungicides**	**Rodenticide**	**Fumigants**	**Insect replant**
Pyrethroids	Bipyridyls	Thiocarbamates	Warfaienes	Aluminum & Zink phosphide	Diethyletoluamide
Organophosphorous	Chlorophenoxy	Dithiocarbamates	Indanodion	Methyle bromide	
Carbamates	Glyphosate	Cupric salts		Ethylene dibromide	
Organochlorines	Acetanilides	Tiabendzoles			
Manganese compounds	Triazines	Triazoles			
		Dicarboximides			
		Dinitrophenols			
		Organotin compounds			

2.2.1. Chemical or Synthetic Pesticides

Chemical pesticides are pesticides formulated synthetically or simple combination of chemicals. They usually classified by their common source or production method. There are four basic types of chemical pesticides that are most commonly used are:

- **Organophosphate Pesticides**

An organophosphate pesticide affects the nervous system of the host by disrupting the enzyme that regulates acetylcholine, a neurotransmitter. Most organophosphates are insecticides (Kazemi, 2012). They were developed during the early 19th century, but their effects on insects, which are similar to their effects on humans, were discovered in 1932. Some are very poisonous (they were used in World War II as nerve agents). However, they usually are not persistent in the environment.

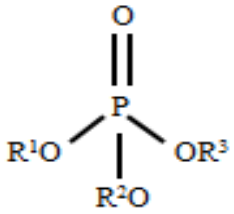


Figure 2-1. Structural formula of Organophosphate Pesticides

- **Carbamate Pesticides**

Carbamates' affect the central nervous system by distracting an enzyme that regulates acetylcholine, a neurotransmitter. The enzyme effects are usually reversible. There are several subgroups within the Carbamate.

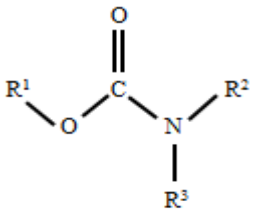


Figure 2-2. Structural formula of Carbamate Pesticides

❖ **Organochlorine pesticide**

Organochlorines' commonly used in the past years as pest and insecticide regulator, but many of them have been removed from the market due to their health and environmental effects and their persistence (e.g. DDT and chlordane) (KutzFw, 1991).

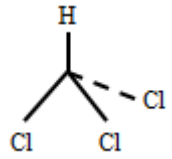


Figure 2-3. Structural formula of Organochlorine Insecticides

❖ **Pyrethroid Pesticides**

Those kinds of pesticides developed as a synthetic version of the naturally occurring pesticide Pyrethrin, which is found in chrysanthemums. They have been modified to increase their stability in the environment. Some synthetic pyrethroids are toxic to the nervous system.

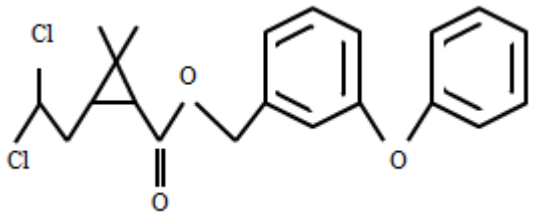


Figure 2-4. Structural formula of Pyrethroid Pesticides

The use of synthetic pesticides has undoubtedly give result in increasing of crop production. However, synthetic pesticides that are used to control plant diseases are doing irreparable harm and damage to our fragile environment. Detrimental health effects, environmental issues, insect resistance or marketing opportunities for organically produced food are well-known arguments against the use of synthetic pesticides. The increasing awareness and concern about the impact of agricultural practices on the environment and in food and fiber production is promoting the concept of sustainable agriculture; thus, raising the thrust for bio-pesticides over synthetic pesticides. The increasing incidence of pesticide resistance is also fueling the search for more

environmentally and toxicologically safe and more selective and efficacious new bio-pesticides. Thus, in the context of all these challenges, the modern pesticides will be needed to meet, there arises the need of environment-friendly pesticides in order to boost agricultural production with the ever escalating world population the use of pesticide is absolute. Generally, there has been an evident shift all over the world from synthetic pesticides to non-synthetic ones; this is largely because of the wide spread awareness of the side effects of these synthetic pesticides not only on plants and soil but also on other living organisms. This is a great opportunity for Bio-pesticide manufacturing. Some basic disadvantages of chemical pesticides are: Persistence in the environment, health hazard to human and non-targeted animals, non-degradability, high environmental contamination /pollution, pest resistance or adaptation and Ecological imbalance as a whole.

2.2.2. Bio - pesticides

According to FAO definition, a bio-pesticide is a compound that kills organisms by virtue of specific biological effects rather than as a broader chemical poison. The rationale behind replacing conventional or synthetic pesticides with bio-pesticides is that the latter are more likely to be selective and biodegradable. Bio-pesticides are derived from natural materials like animals, plants, bacteria, and certain minerals. For example, garlic, mint, Neem, papaya, canola oil, baking soda etc. all have pesticide effect and are considered as bio-pesticide. Almost all the bio- pesticides are categorized among three major groups such as (i) Microbial pesticides (ii) PIPs and (iii) Bio - chemical pesticides.

- **Microbial pesticides**

These consists a microorganism (e.g., a bacterium, fungus, virus or protozoan) as the active ingredient. Microbial pesticides can control many different kinds of pests, although each of the active ingredients is separated relatively for specific target pests. For example, there are fungi that control certain weeds, and other fungi that kill specific insects. The most widely used microbial pesticides are subspecies and strains of Bt. Each strain of this bacterium produces a different mix of proteins, and specifically kills one or a few related species of insect larvae

(Bonaterra, 2014). While some Bt's control moth larvae found on plants, other Bt's are specific for larvae of flies and mosquitoes. The target insect species are determined by whether the particular Bt produces a protein that can bind to a larval gut receptor, thereby causing the insect larvae to starve.

- **Plant-Incorporated-Protectants or PIP's**

Those are pesticide substances that are produced from plant genetic material that has been added or induced to the plant or crop. For example, scientists can take the gene for the Bt pesticide protein, and introduce the gene into the plant's own genetic material. Then the plant, instead of the Bt bacterium, manufactures the substance that destroys the pest. (John, 2017) Again, bio-active pesticide ingredients can be extracted from plants and used again to destroy or protect pests.

- **Biochemical pesticides**

Biochemical pesticides are naturally occurring substances that control pests by non-toxic mechanisms. Conventional pesticides by contrast, are generally synthetic materials that directly kill or inactivate the pest. Biochemical pesticides include substances that alter biological activity of the pest, such as sex pheromones that interfere with mating as well as various scented plant extracts that attract insect pests to traps or repels (Sarwar, 2015).

2.3. History of pesticide in Ethiopia

The startup of using chemical pesticide in Ethiopia date back to 1960's due to the emergence of commercial farms. Because of some large agricultural projects was implemented in different areas of Ethiopia for example, (Chilalo Agricultural Development in 1967, Wolita Agricultural development in 1970 and Minimum Package Projects under EPID in 1971in different areas) from this time (i.e.1967- 1971) Agricultural inputs were introduced to the small holder farmers. In 1972 Regulatory body that specializes in pesticide assessment was established under MoA (FEPA .. T., 2004).

Currently with the development of floriculture and horticulture mechanized farms in the country, the annual importation of pesticides has been expected to be increased correspondingly. The annual importation of synthetic pesticide recorded in 2004[1] was reached about 4200T[2] (FEPA, 2004). The growth in amount of imported pesticides in use and pesticide users is requiring a better choice of subsidized and appropriate pesticide with good pesticide management system.

2.4. Pesticide Import, Production and use in Ethiopia

Ethiopia is one of the agricultural leading economy African countries that use different kinds of pesticides for agricultural, industrial and health care purposes. In most cases pesticides are imported to Ethiopia. However, there is one factory that formulates chemical pesticides within the country (i.e. AdamiTulu pesticide factory).

Pesticides are mainly imported for agricultural purposes while some amounts of pesticides are imported for health care and industrial purposes. Both public and private enterprises are engaged in pesticide importation business. Currently, about 20 organizations are actively involved in importation and sale of pesticides. Large quantities of pesticides are imported annually to Ethiopia. In this regard, over 4000 tons of various types of pesticides that are worth more than 20 million USD were imported annually.

There is one local pesticide formulation plant in AdamiTulu**.** The plant has a capacity to formulate 1500 tons of dust and the same quantity liquid formulations every year (EPA, 1999). Major pesticide formulated include, Malation, (Ethiolathion 5% Dust and Ethiolathion 50% EC), Endosulfan (Ethiosulfan 25% ULV), Diazinon (Ethiozinone 60% EC), and Fenithrothion (Ethiothrothion 50% EC). The plant imports active ingredients and solvents from foreign countries, mostly from Italy and Israel such as DDT and Pyrethoids (permethrin and deltamethrin) are imported for mosquito net impregnation and are also used for tsetse fly.

[1] *Gregorian calendar*

[2] *Tones*

2.5. Major Environmental Impacts of synthetic Pesticides

Besides, its simplicity in use and less harmful to humans and prone pesticides should be reconsidered their impacts on the biosphere as a whole. When pesticide entered to the environment there will be negative impacts on air, water, soil, human beings and animals. It is important to take note of the following impacts that possibly emanate from production, use, handling, and disposal of pesticides in order to come up with a proactive new product development and workable Environmental management system.

- **Impacts on water**

Synthetic pesticide residues in water can cause serious pollution in both ground and surface water this may cause for:

- The death and disturbance of aquatic animals
- Change in the organoleptic properties of water (its odor, taste);
- Negative effect on the process of oxygen formation by phytoplankton, on the vital activities of the inhabitants of the water ecosystems;
- Impacts that transmitted along the food chains, and accumulate in food products;
- Direct toxic action (acute or chronic toxicity) and indirectly (dimensioning of the content of oxygen dissolved in the water, a change in the chemical composition of water, extermination of water insects, etc).

- **Impacts on the air**

The following table shows some synthetic pesticides related air pollutants and their effects on human and animal health.

Table 2-2. Basic air pollutant chemicals and compounds found in synthetic pesticides

Name of pollutant	Impact
RSPM	Respiratory illness, including chronic bronchitis and asthma; heart diseases.
SO_2	Heart diseases; respiratory problems including pulmonary emphysema, cancer, eye burning, headache, etc.
NO_2	Lung irritation, viral infection, airway resistance, chest tightness, etc.
SPM	Pneumoconiosis, restrictive lung diseases, asthma, cancer, etc.
Benzene	It causes immunotoxicity, carcinogenicity, asthma, anemia, unconsciousness etc.
Ozone	Impaired lung function, chest pains, coughing, irritation of eyes, nose etc.
CO	CO poisoning cause cherry lips, unconsciousness, death by asphyxiation etc.
Lead	It causes decreased hemoglobin synthesis, anemia, damage the nervous and renal (kidney) systems etc.

Source: EIA guideline on pesticide, FEPA, 2004

- **Impacts on the soil**

Pesticides are introduced into the soil and destroying soil dwelling pests, nematodes, and the pathogens of bacterial and fungal disease (Seymour, 2005). Herbicides are widely introduced into the soil. Pesticides also get into the soil after treatment of the green organs of plants. They

are washed off by atmosphere and precipitate by the mechanism of wind force. Pesticides may get into soil in the form of their residues contained in leaves, roots, etc.

- Depending on the conditions poisonous chemicals may remain in the soil unchanged and retain their toxicity for a more or less prolonged time.
- Persistent use of DDT and its related chemicals can thoroughly undermine the productivity of the soil over time by destroying the microorganisms and nutrients that nourish crops. This decreases agricultural productivity of land and makes it vulnerable to desertification.

- **Impacts on human health**

Use of pesticides creates substantial health impacts in all parts of the World. Pesticides effect can be decided broadly into two categories:

- Acute effects, which appear immediately or very soon after exposure and
- Chronic effects, which may manifest themselves many years later and whose origins are often difficult to trace.

Each year in the world, an estimated 500,000 humans are poisoned by pesticide with 10,000 fatal (WHO, 1996). Pesticide health related impacts may include**:**

- Headache, irritability, dizziness, loss of appetite, nausea, muscle twitching, convulsion, loss of consciousness, and possible death.
- Carcinogenic effects,
- Neurobehavioral effect,
- Reproductive deficits,
- Diabetes and others.

- **Impact on wild life and non-target species**

Pesticides also harm non-target species, and resulted in:

- Population decline through the use of pesticides over large areas
- Reproductive effect such as egg shell thinning, deformity and birth defects
- Metabolic changes
- Tumors and cancer

- Behavioral changes
- Abnormally functioning thyroid glands
- Sub-lethal or lethal poisoning of mammals and other vertebrate animals
- Through extinction of the pest population -losses of food sources for many birds
- Toxicity to bees which are pollinators, with adverse effects on the production of certain crops

Note: generally, a synthetic pesticide has a short term benefit (socio-economically and environmentally) with a long term side effect for our country as well as the world.

2.6. Policies and Legal Frame Work

Understanding the overall importance of issues associated with pesticides, the government of Ethiopia has reacted in so many ways to address the problems. Accordingly, the Federal Democratic Republic government of Ethiopia has developed policies and legal frameworks related to safe production and use of pesticides. Ethiopia has also accepted different international agreements related to pesticides. The following is the highlights of major policies and legal frameworks, which required considerations in safe management of pesticides.

2.6.1. National Policies

- **Environmental Policy of Ethiopia**

The country has approved an environmental policy in 1997. The overall policy goal is to improve and enhance the health and quality of life of Ethiopians through sound management of natural resources and with objective of sustainable development.

- **Proclamation of the Constitution of the Federal Democratic Republic of Ethiopia (Proc. № 1/1995)**

In the constitution of Ethiopia has captured the most important aspects of sustainable development. This has been sufficiently reflected in the provisions govern the right to development (art. 43), where peoples' right to:

- Improved living standards and to sustainable development,

- Consultation and participation regarding matters that may affect their wellbeing,
- sustainable development, and
- Enhanced capacity for development and meet their basic needs provided for; as well as Environmental right (art.44), where people's right to:
- Clean and healthy environment, and
- Proper compensation is recognized.

It is to be noted that any undertaking related to pesticide should aware of these broad rights protected by the constitution.

- **Special decree for Pesticides Registration (Proc.№ 20/1990)**

This decree:

- Covers agricultural, household, public health, and industrial pesticides
- Provides registration and control responsibilities to MoA
- seeks to promote safer pesticide handling and use in the country
- Requires that all pesticides should be registered on the basis of demonstrated product effectiveness and safety for humans, non-target organisms and the environment
- Prohibits importation of highly hazardous, severally restricted or banned pesticides (including most Organochlorines) and
- Obliges that all pesticides must display labels that meet specific MoA label requirements.

The required data that all pesticides must display are:

- Specification
- Common name of the active ingredient, according to ISO standard
- Chemical name
- Empirical and molecular weight
- Chemical and physical properties of the active ingredients
- Formulation products and efficacy data to make the registration complete

The decree also includes requirements for consideration for acceptance, testing procedure and the content of report and information for recommendation to be filled by the researcher.

- **Environmental impact assessment proclamation (Proc. № 299/2002)**

This proclamation requires that major development program, plans and projects of the private and public sectors are subject to EIA before their approval. This proclamation also provides a legal base to harmonize and integrate environmental economic, cultural and social considerations into the planning and decision making process and there by promotes sustainable development.

- **The Environmental Pollution Control (proc. № 300/2002)**

This proclamation aims at eliminating or when not possible, to mitigate pollution as undesirable consequence of social and economic development activities. It further requires among other things for:

- ❖ control of pollution,
- ❖ management of hazardous waste, chemical and radioactive substances,
- ❖ Respect of environmental standards,
- ❖ Punitive and incentive measures etc. are included in the proclamation

- **Public health proclamation (proc. № 200/2000)**

This proclamation:

- ❖ Prohibits discharging of untreated liquid waste generated from septic tanks, seepage pits and industries into water bodies, or water convergences
- ❖ Prohibits the disposal of solid or liquid or any other waste in a manner which contaminates the environment or affect the health of the society, etc.

- **Provisional standards for industrial pollution control in Ethiopia**

The country has provisional effluent and emission standards which specially contains pesticide formulation and production standards.

2.6.2. International Agreements

The policy calls among other things for prevention of pollution. In the sectoral environmental policies that relates to Soil husbandry and sustainable agriculture the emphasis is on:

- The use of biological and cultural methods in an integrated manner to control pest and diseases,
- To safe guard human and environmental health by adequately regulate the agricultural chemicals.

Ethiopia has ratified four international conventions that have importance in pesticides managements. Consideration of these conventions is therefore essential. These conventions include:

- **Rotterdam convention, Prior Informed Consent (proc. № .278/2002)**

The objective of the convention is to promote shared responsibility and cooperative efforts among parties in the international trade of certain banned or severely restricted hazardous chemicals and severely hazardous pesticides formulations in order to protect human health and the environment from potential harms.

- **Basel convention, (proc. № 357/2002) amended (proc. № 356/2002.)**

Objective of the convention include ensuring environmentally safe transfer, disposal of hazardous wastes, and limiting "Toxic trade" in hazardous wastes.

- **Stockholm convention (proc. № 279/2002)**

The objective of this convention is to protect human health and the environment from persistent organic pollutants.

- **Bamako Convention (proc. № 355/2002)**

The convention refers to" the Ban of the Import into Africa and the Control of trans boundary Movement and Management of Hazardous Wastes within Africa" The general obligation in the convention include:

- Hazardous Waste Import Ban,
- Ban on Dumping of Hazardous Wastes at Sea and Internal Waters,
- Parties required further action consistent with the convention in controlling Waste Generation

All these conventions should be taken under consideration for those who are engaged in pesticide production and importation as national instrument implementation for development.

2.7. Characteristics of Neem tree

The Neem tree is drought resistant and thrives normally in areas with sub-arid to sub-humid conditions, with an annual rainfall between 400-1200 mm. Neem is generally evergreen but can shed most of its leaves under dry conditions. Neem is a type of mahogany, a fast growing evergreen tropical to subtropical tree that can reach a height of 50-65 feet or (164m – 197m). Neem can tolerate high summer temperatures (up to 50 °C) but does not tolerate frost or temperatures below 4 °C (leaf fall and death may result). Neem grows best in areas where annual rainfall is 450–1200 mm (with optimum growth where annual rainfall is around 1100 mm), but can tolerate annual rainfall as low as 150 mm if its roots can access ground water within 9–12 m of the ground surface. Once established it is very drought tolerant and can survive 7–8 months dry seasons (Council, 1992). The major parts of the Neem tree and their constituents that can be possibly used as an insecticide are listed below.

Neem seed: Seed of Neem tree have mainly an ability of insect repellent, antifeedant, less toxic (i.e. to the environment) and growth regulatory effects. The water extract of Neem seed is effective as natural insecticides for combating of storage and field pests. The new seed have more repellent effect. Neem oil is an extract from the seeds of the Neem tree. Neem oil works to smother and repel pests. The product suffocates all of the life stages of the insects. Seed kernels of Neem are the source of Azadirachtin and related Limonoids, which has been well known since ancient times as a potent bio-pesticide against a variety of insects.

Neem leaves: Neem leaves are also used in storage of grains as preservative. Twigs of Neem when it is used as green manure after decomposing and widely incorporated in a cultivation

fields. Neem leaf properties. They are used as green leaf manure and also in preparation of compost mealy bugs, whiteflies and scale, among others, as well as mite adults and eggs.

Neem bark: Neem bark has insecticidal properties. Bark in powdered form is also used to control fleas and sucking pests in field cultivation.

Neem root: Neem root has also used to control fleas and sucking pests in field cultivation traditionally.

2.7.1. Composition of Neem seed

Neem plants contain several chemical constituents having insecticidal property. Enormous active ingredients are found in all botanical parts of the Neem tree, but concentrated largely in the seed kernels. A group of limonoids (triterpenoids) including Azadirachtin, Nimbin, Nimbidin, Salanin, Salannol, Quercetin, and Gedunin are reported. Terpenoids from different parts of the Neem plant are also extracted. Out of these, most active and well effective compound is Azadirachtin ($C_{35}H_{44}O_{16}$). The Neem triterpenoids are present in all parts of the plant.

Neem tree protects itself from the multitude of pests with a diverse of pesticidal ingredients. Its main pesticidal effective chemicals are a mixture of 3 or 4 related compounds, and it backs these up with 20 or so others that are minor but nonetheless active in one way or another. Mainly, these compounds belong to a general class of natural products called “triterpenoid” or specifically, “limonoids.” Limonoids So far had verified an ability to block insect growth, affecting a range of species that includes some of the deadliest pests of agriculture and human health. Azadirachtin, Salannin, Meliantriol, and Nimbin are the best known limonoids.

❖ **Azadirachtin ($C_{35}H_{44}O_{16}$)**

One of the first active ingredients isolated from Neem, azadirachtin has proved to be the tree's main agent for battling insects or pests. It appears to cause some 90 percent of the effect on most pests. It does not kill insects at least not immediately. Instead it both repels and disrupts their growth and reproduction.

Research over the past 20 years has shown that it is one of the most effective growth regulators and feeding deterrents ever assayed. It will repel or reduce the feeding of many species of pest insects as well as some nematodes. In fact, it is so potent that a simple trace of its presence prevents some insects from even touching plants. Azadirachtin is structurally similar to insect hormones called "ecdysones" which controls the process of metamorphosis as the insects pass from larva to pupa to adult. It affects the corpus cardiacum, an organ similar to the human pituitary, which controls the secretion of hormones. Azadirachtin blocks the insect's production and release of these vital hormones. Insects then will not molt. This off course breaks their life cycle.

Azadiracthin, found only in Azadirachta species plants it is a complex liminoids from the Neem seeds (Estifania, 2016). Of all the liminoids found in Neem tree, Azadiracthin is the most biologically active pesticidal effect component Neem seeds have 12 to 18 mg/g of azadiracthin, depending on the ecotype of the tree and local conditions. (Kumar, 2008).

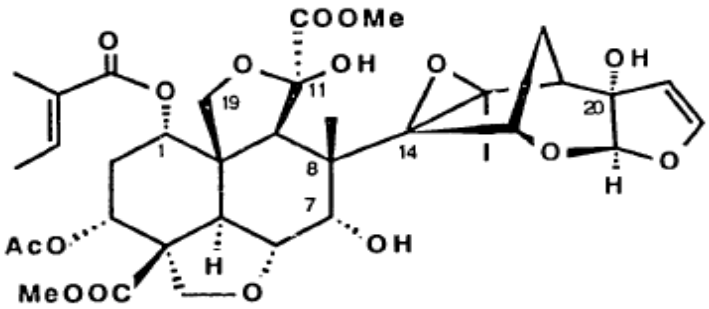


Figure 2-5. Molecular structure of Azadiracthin ($C_{35}H_{44}O_{16}$)

❖ **Meliantriol**

Another feeding inhibitor, meliantriol, is able, in extremely low concentrations, to cause insects to cease eating. The demonstration of its ability to prevent locusts chewing on crops was the first scientific proof for Neem's traditional use for insect control.

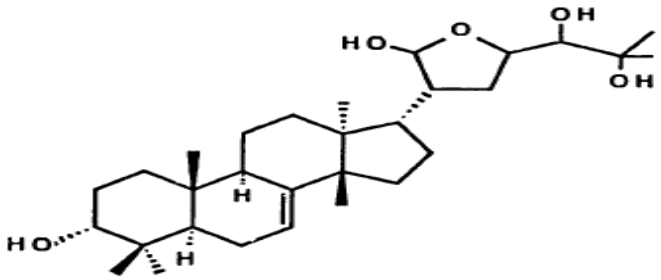


Figure 2-6. Molecular structure of Meliantriol

❖ **Salannin**

The third triterpenoid isolated from Neem is Salanin. Studies indicate that this compound also powerfully inhibits feeding, but does not influence insect molts.

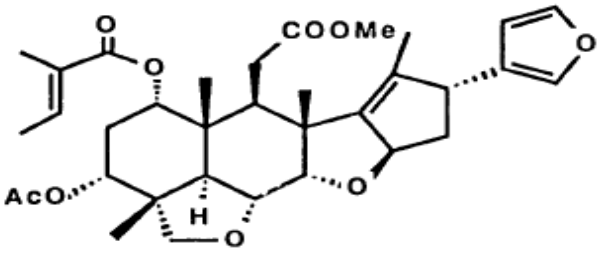


Figure 2-7. Molecular structure of Salanin

❖ **Nimbin and Nimbidin**

Nimbin and Nimbidin have been found to have antiviral activity. They could perhaps open a way to control these and other viral diseases of crops and livestock. Nimbidin is the primary component of the bitter principles obtained when Neem seeds are extracted with alcohol. It occurs in sizable quantities about 2% of the seed.

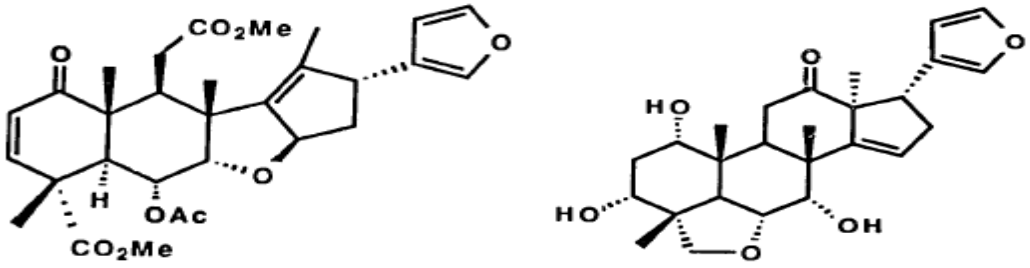


Figure 2-8. Molecular structure of Nimbin and Nimbidin

2.8. Azadirachtin based bio-pesticide

Neem's ability to repel insects was first reported in the scientific literature in 1928 and 1929. Two Indian scientists, Chopra and Husain in 1962, used a 0.001percent aqueous suspension of ground Neem kernels to repel desert locusts. That year, in field tests in New Delhi, S. Pradhan ground tip Neem kernels in water and sprayed the resulting suspension over different crops. He found that, although locusts landed on the plants, they refused to eat anything, sometimes for tip to 3 weeks after the treatment. Furthermore, he noted that Neem kernels were even more potent than the conventional insecticides then available and that Neem's repellency was as important as its toxicity. In neighboring insecticide-treated fields, for instance, the insects also died, but not before consuming the crops.

Neem's IGR effects were independently observed in England and Kenya in 1972. In England, Ruscoe, at that time an employee of the ICI Company, tested azadirachtin on insect pests such as cabbage white butterfly (Pierjs hras. sicae) and cotton strainer bug (Dvsderciis fi'sciats) and noted IGR effects in each case. The azadirachtin was provided by D. Morgan, a Keele University chemist who had been the first to isolate azadirachtin. In Kenya that same year, K. Leuschner, a German graduate student working at the Coffee Research Station in Upper Kiambu observed that a methanolic Neem leaf extract controlled the coffee bug (Antestiopsis orbilalis bechuana) by growth-regulating effects. Most fifth-instar nymphs treated with the extract died during subsequent molts and the few that survived to adulthood had malformed wings and thoraxes.

Neem's fertility reducing effects were first recorded by Steets (another graduate student) and Schmutterer in Germany in 1886. Applying methanolic Neem-kernel extract and azadirachtin to the Mexican bean beetle (Elpilachna varivestis) and the Colorado potato beetle (Leptinotarsa decemlinea/a) they found that females almost stopped laying eggs. Some females had been completely sterilized, and the effect was irreversible.

Neem seed is used as insecticide which protects plants and crops from pests. This is effective against insecticide-resistant pests and yet does not harm the beneficial insects. Parts of Neem used for Manufacturing Pesticides, Neem oil and seed extracts, are known to possess germicidal

and anti-bacterial properties which are useful to protect the plants from different kinds of pests. One of the most important advantages of Neem based pesticides which differs it from its synthetic counterparts is the fact that it does not leave any residue on the plants.

Neem pesticides play a vital role in pest management and hence have been widely used in agriculture. There has been an evident shift all over the world from synthetic pesticides to non-synthetic ones; this is largely because of the wide spread awareness of the side effects of these synthetic pesticides not only on plants and soil but also on other living organisms. This is a great opportunity for Neem pesticides manufacturers to cash in on the growing popularity of natural or herbal pesticides.

Neem seed contains 40 different active compounds called liminoids. The main active ingredient of Neem seed is Azadiracthin. It exhibits antifeedant, insect repellent and insect sterilization properties and has been reported to be non-mutagenic and it appears to have no apparent mammalian toxicity. Insects treated with Azadiracthin during the larval and pupal stages, comprising 60 – 70 percent of their lives, generally die within 3 – 14 days. Unlike chemical insecticides, it works on the insect's hormonal system, not on the digestive or nervous system, and it is claimed that this does not lead to development of resistance in future generations. Because azadiracthin had a multi-modal action, it is unlikely that an insect species would develop resistance based on one mode of action. This is in contrast to most synthetic pesticides which operating on the insect's nervous system and resistance to one chemical leads to resistance to all others with the same reaction pathway. Azadiracthin has been considered to be a promising environmentally compatible insect pest control device for plant protection. Moreover, Azadiracthin has also been found to degrade rapidly due to environmental factors such as UV radiation in sunlight, heat, air moisture, acidity and enzymes present in foliar surfaces.

2.8.1. Mode of Action of azadirachtin based bio-pesticide

Neem bio-pesticide acts as antifeedant i.e. when an insect larva is hungry and it wants to feed on the leaf, and if the leaf is treated with Neem product, because of the presence of azadiracthin there is an antiperistalitic wave in the alimentary canal and this produces something similar to vomiting sensation in the insect. Because of this sensation the insect does not feed on the Neem

treated surface and ability to swallow is also blocked. Secondly it acts as oviposition deterrent i.e. by not allowing the female to deposits eggs comes in very handy when the seeds in storage are coated with Neem kernel powder and/or Neem oil. It also acts as insect growth regulator. It is a very interesting property of azadirachtin and unique in nature, i.e. it works on juvenile hormone (Mathur, 2013).

- **Biological effects of azadirachtin based pesticide on pests**

The action of Neem products as pest control agents can be manifested at different levels and in different ways. This is a very important point to be noted since the farmer would use the knock-out effect of chemical pesticides. Neem extracts do not exhibit this type of effect on pests but affect them in several other ways.

- **Pest growth regulation effect of azadirachtin**

Regulation of the insects' growth is a very interesting property of Neem products which is unique in nature, since the products work on juvenile hormones. The insect larva feeds and as it grows, it sheds its old skin. This particular shedding of old skin is the phenomenon of ecdysis or moulting and is governed by an enzyme, ecdysones. When the Neem components, especially Azadiracthin, enter the body of the larva, the activity of ecdysones is suppressed and the larva fails to moult, remains in the larval stage and ultimately dies. If the concentration of azadiracthin is not high enough, the larva will die only after it has entered the pupal stage. If the concentration is lower still, the adult emerging from the pupa will be 100% malformed, and absolutely sterile. (Denise Porfirio Emerenciano, 2015)

- **Feeding deterrent effect of azadirachtin**

The most important property of azadirachtin is feeding deterrence. When an insect larva sits on a leaf, it will want to feed on it. This particular trigger of feeding is given through the maxillary glands. Peristalsis in the alimentary canal is thus speeded up, and the larva feels hungry and starts feeding on the surface of the leaf. If the leaf is treated with a Neem product, because of the presence of azadiracthin, salanin and melandriol, there will be an anti-peristaltic wave in the alimentary canal which produces something similar to a vomiting sensation in the insect.

Because of this sensation, the insect does not feed on the azadirachtin treated surface. Its ability to swallow is also blocked (Diedhiou, 2015).

- **Oviposition deterrent effect of azadirachtin**

Another way in which azadirachtin controls pests is by preventing the females from depositing eggs. This property is known as oviposition deterrence, and comes in very handy when the seeds in storage are coated with azadirachtin or crude Neem oil. The seeds or grains obtained from the market may already be infested with some insects. Even these grains could be treated with azadirachtin or crude Neem oil. After this treatment, the insects will not feed on them. Further damage to the grains will be halted and the female will be unable to lay its eggs during the egg-laying period of its life cycle. There are also other known modes of action i.e. the formation of chitin or the hard part covering of insects, mating and sexual communication disruption, Larvae and adults of insects are repelled and poisoned and Sterilization of adults (Mahmoud, 2001).

The use of azadirachtin based pesticide does not give immediate results, unlike chemical insecticides. Some patience is required after the application of azadirachtin. Besides its insecticidal and nematicidal properties, Azadirachtin is also a promising agent for control of plant diseases. It has also been demonstrated to possess anti-fungal properties. One of the problems with the use of chemical pesticides has been their impact on "non-target" species. Often they have proven harmful to various other species in the ecosystem that could be beneficial. However, azadirachtin based pesticide is free from such effects. Azadirachtin has been proven to be remarkably compassionate to spiders and also other insects such as bees that pollinate crops and trees, ladybug beetles that consume aphids and wasps which act as parasites on various crop pests. Azadirachtin has to be eaten or drank to be effective. Azadirachtin belongs to an organic molecule class called tetranortriterpenoids. It also does not kill insects, but alters their life process.

2.9. Extraction Methods of azadirachtin

Neem seed is a valuable raw material which contains not only insecticidal and fungicidal ingredients, but also up to 48% oil. Even the extracted Neem cake can be used as fertilizer; it

might have effects on soil pests (BSTIT, 1992). There are various technologies available to extract the active ingredients of Neem seed. The technique to be used depends mainly on the quality required of the final product, productions cost and extract application.

The cost of the extraction method varies accordingly; the higher the level of azadiracthin required the more expensive of the extraction method. Considering the solubility of the leading component azadiracthin, it is clear that only polar solvents should be used for extraction. Still, the cold pressed Neem oil could contain up to 0.6% azadiracthin. Water and alcohol are the best solvents. Often methanol is the preferred alcohol because of the availability and price, but its environmental toxicity of it headed to see another solvent as a choice. The major three types of extraction technologies that are available discussed below.

2.9.1. Extraction with alcohol

It is also called one-step extraction. The Neem seeds are crushed into crude powder and extracted with ethanol or methanol. The alcohol should have low water content, and its quality (purity) is very important.

The alcoholic extracts contain the active ingredients. Using the moving-bed contacting method, the kernels will be stirred for certain hours by an overhead stirrer in a mixing-settling tank. After decantation of the crude cake, the Neem solution is drained out, filtered and passed to the next procedure. The dilute Neem solution will be further evaporated until a specific volume called the concentrated extract has been achieved, and the solvent will be recycled.

Neem kernels contain a large percentage of oil (up to 48%, average 40%), which have its own considerable value for different purposes. Since, the oil can disturb the extraction of the active ingredients, it would be best to remove the oil with solvents such as hexane or by cold pressing with an oil expeller. It is also possible to remove the oil content from the extract by cooling or freezing to separate the oil.

2.9.2. Refined or enriched extraction method

By applying special extraction procedures or further enrichment steps for alcoholic extracts, e.g. by fluid/fluid extraction, unwanted substances (such as residual oil, sugar, etc.) are separated and azadiracthin(s) and other tetranortriterpenoids are converted in an organic phase which is easy to vaporize. After recovery of the solvents a powder extract remains with an azadiracthin content of up to 20% (Gabriela, 2010).

This sort of extraction technology is used by most plants processing commercial Neem products on a large scale. Some company has developed a procedure starting from simple water extraction, but continuing with a fluid/fluid extraction with a special combined solvent and formulation liquid, which concentrates the extract of the non-water phase and formulates them for application in the field.

2.9.3. Soxhlet extraction

The solid is placed in a porous thimble in the chamber and the extracting solvent in the boiling flask below. The solvent is heated at reflux temperature, and the distillate as it drops from the condenser, collects in the chamber. By coming in contact with the solid in the thimble the liquid effects the extraction. After the chamber fills to the level of the upper reach of the siphon arm, the solution empties from the chamber into boiling flask by siphoning action. This process may be continued automatically and without attendance for as long as is necessary for effective removal of the desired component, which will then be contained in the solvent in the boiling flask.

2.10. Benefits and field application of azadirachtin based Pesticides

Azadirachtin based pesticide has many benefits since it is natural, it has a great advantage in application field as well as environmental effect. The old way of using Neem seed for pesticidal effect is grinding and soaking the seed for 24 hours minimum and make in use. This helps the soil to nourish and conditioned since the residue have an ability of nitrogen inhibition. In addition to the pesticidal effect, it is environmental friendly, since it doesn’t leave any residue on the plant and environment.

Another important benefit of Azadirachtin based pesticide is, it doesn't kill directly the pest; rather it affects the life cycle of the pests like anti feedant properties, pest repellent properties and menstrual cycle disruption of the pest. These helps to protect the plants from being eaten and pest reproduction controller.

Pests generally do not develop a resistance to Azadirachtin based pesticides because of its biological effect rather than its chemical effect on the pest. Biological based pesticides are generally water soluble and usually less toxic than conventional chemical pesticides. With a specific pest target effect.

Generally, bio-pesticides are effective in very small quantities and decompose quickly, thereby resulting lower exposures and avoiding pollution problems. Bio-pesticides leave less / no residues in water, soil and organic materials on which they dissolve. When used as a component of IPM programs, bio-pesticides can greatly decrease the use of conventional chemical pesticides, while crop yields remain high. Azadirachtin based bio-pesticide; chemically a tetranorterpenoid component of Neem acts on the mitotic cells and blocks the microtubule polymerization. (Tripathi, 2015) Certain activities of genes and proteins are also altered by azadirachtin. As a botanical insecticide azadirachtin is effective against many biological processes. It may cause a reduction in feeding habit, suspend the molting process, larvae and pupae death and also cause sterility in the emerging adults, this all depend on the given dose. Apart from azadirachtin, many other components of Neem also have insecticidal properties. For this reason, the already extracted and dried Azadirachtin based bio – pesticide is going to dissolve in a required amount and dispersed directly to the crop fields.

2.11. Neem Tree availability in Ethiopia

Ethiopia is located in the tropical and sub-tropical region mostly. For this reason, the weather condition is suitable for the growth of Neem tree, and about 35-45% of the conditions in Ethiopia are appropriate for tree plantation. There are about 25 places that Neem tree has observed in Ethiopia. Particularly, it is found widely in the following regions; Gambella, Somali, and Amara (Centre, 2002).

3. MATERIALS AND METHODS

3.1. Materials and Chemicals

The major chemicals and materials used to accomplish this study were listed below. The table below states all the major materials and chemicals required for this study.

Table 3-1. Chemicals and Materials needed

№	Chemical	№	Materials
1	Matured Neem seed	1	Oven (DAS 42000, 224.2007)
2	Ethanol, 97% purity	2	Attrition mill (Retsch GmbH, 5657 HAAN)
3	Acetonirile, 99.9%	3	Batch extractor vessel with overhead mixer and Temperature sensor (armfield CEX Chemical Reactor Service, Batch reactor).
4	Methanol, HPLC grade		
5	Potassium Hydroxide	4	Measuring cylinder (500ml)
6	Hexane, 99.9%	5	Chiller (PN:91A0A12E,107900199)
7	Ethylacetate, 99.9%	6	Petridish (90mm diameter)
8	Isopropylealchol	7	Rotary vacuum evaporator (RVO 400, INGOS)
9	Iodine	8	Vacuum pump with funnel and beaker (KIF, Lab)
10	TLC, cellulosic	9	Centrifuge (Andreas HettichD72 centrifuge, Germany)
		10	Tray drier (BLOSEC, N 11/2006)
		11	HPLC with C18- column
		12	Pycnometer (25 ml)

	13	Filter paper, 0.22µm of diameter 90mm
	14	Shaker incubator (EXCELLA, E24R)
	15	TLC paper with aluminum stand
	16	Micro wave oven with UV (SUPRA-SM20BH1)
	17	Bomb calorimeter (cussons, P6310/417)

3.2. Methods

3.2.1. Experimental setup

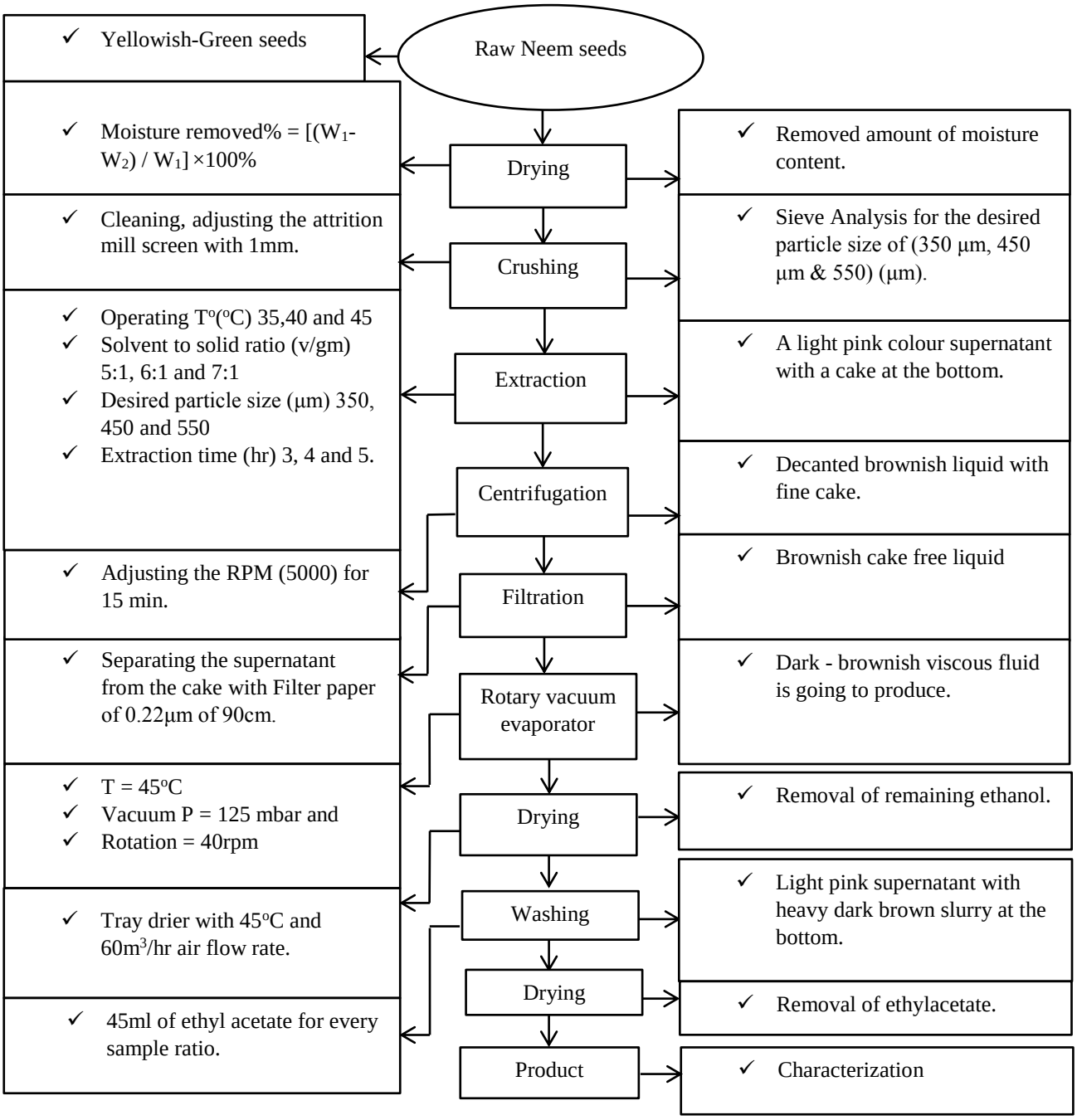


Figure 3-1. Experimental setup of azadirachtin extraction from Neem seed

3.2.2. Raw material acquisition and preparation

Twenty kg of matured seed in a light-yellow color stage were collected from Tigray regional state Mekelle city manually in January, 2017. After the sample was collected and packed in a polyethylene bag, transportation is carried out by bus to Addis Ababa. A local transport is used to deliver the raw sample to AAiT Chemical and Bio Engineering Department laboratory. The sample should be stored at room temperature open air before use.

- **Moisture content of Neem seed**

After the sample is decorticated its stem, moisture content analysis is carried out as ASTM D 2974-87 Standard Test Methods for Moisture, Ash, and Organic Matter of Peat and Other Organic Soils procedure by 45^{o}C in an oven (DAS 42000, 224.2007) for four days, so that the moisture amount is going to be determined every 24 hours.

Figure 3-2. Moisture content removal of Neem seed

$$\text{Moisture content}\,(\%) = \frac{W_1 - W_2}{W_1} \times 100\% \quad \text{..... Equation 3.1}$$

Where:

W_1 - initial weight of sample, (g)

W_2 – final weight of sample, (g)

The dried sample was then milled by an attrition miller **(**Retsch GmbH, 5657 HAAN**)** and made ready for further Ash content analysis before extraction.

- **Ash content of Neem seed**

Ash content of an organic sample is determined by igniting the oven dried sample from the moisture content determination in a muffle furnace (LHT 04/17, SN 262014) at 750^{o}C (Method-D). The substance remaining after ignition is the ash. It is expressed as a percentage of the mass of the oven-dried sample. This sample characterization is carried out according to ASTM D 2974-87 Standard Test Methods for Moisture, Ash, and Organic Matter of Peat and Other Organic Soils.

$$\text{Ash content}\,(\%) = \frac{W_3}{W_2} \times 100\% \quad \text{..... Equation 3.2}$$

Where:

W_2 = Ash (g)

W_3 = Oven dried specimen (g)

$$\text{Organic matter}\,(\%) = 100 - \text{Ash content},\% \quad \text{.....Equation 3.3}$$

Figure 3-3. Muffle furnace and desiccator with crucible

Finally, the dried sample that was milled by an attrition miller and analyzed moisture and ash content, was sealed by polyethylene bag and ready for further extraction process.

3.3. Experimental factors

- **Effects of particle size on extraction**

A highly exposed surface area of fine particles size is expected to give the highest concentration of a given yield. However, the small amount yield in the crude liquid extract is due to the rapid solvent evaporation which affects diffusivity of the required active ingredient as more solvent is needed to penetrate deep inside the inner structure and to extract the bio-active constituent (Saiful, 2014). From this, this experimental tendency undertakes particle size as one extraction factor so that, the effect could be defined.

- **Effects of Solvent to Solid ratio on extraction**

A high solid-to-solvent ratio is expected to be favorable in extraction of active compounds. These results were consistent with mass transfer principle where the driving force for mass transfer is considered to be the concentration gradient between the solid and the solvent. A high solid-to-solvent ratio could promote an increasing concentration gradient, resulting in an increase of diffusion rate that allows greater extraction of solids by solvent.

The chance of bio-active components coming into contact with extracting solvent expanded with increase amount of extracting solvent and will not continue to increase once equilibrium is reached (Wong, 2013). Overall, the main effect of the solid-to-solvent ratio is to modify the solubility and equilibrium constant and thus increase the extraction yields to a maximum at the optimum solid-to-solvent ratio. For this reason, solvent to solid ratio is taken as one factor and going to be analyzed here.

- **Effects of temperature on extraction**

Elevated temperature promotes extraction by enhancing both diffusion coefficients and solubility of active ingredients. Raised temperatures also increase cell membrane permeability following the breakdown of cellular constituents thereby setting more active ingredients free to

be permeable and extractable (Sulaiman S. K., 2014). High temperatures are, however, not always suitable for extracting all sorts of active ingredient compounds because of its breakdown in the activity and equilibrium state of extraction.

- **Effects of extraction time on extraction**

Extraction time plays significant role in extracting the active ingredient (Sulaiman S. K., 2014). It is obviously longer extraction time could lead to increase in exposure of temperature and this might lead to degradation on the effective biological activity of the component. From this perspective, extraction time is going to be analyzed in three different levels for this study.

3.3.1. Extraction Method

The attrition milled seed was sorted by its particulate size of 350µm, 450µm and 550µm in a polyethylene plastic bag. A proportionate solvent (Ethanol) to solid (seed) ratio (5:1v/w, 6:1v/w and 7:1 v/w) will be prepared as desired. The water chiller as a hot circulate was adjusted (35^{o}C, 40^{o}C and 45^{o}C) to the required temperature. Then, simultaneously the seed and the solvent were added to an overhead batch extraction vessel (armfield CEX) for further extraction. After adding the solvent and the seed in to the extraction vessel, mixing parameters were set to a constant revolution of 100rpm and extraction time as required (i.e. 3hr, 4hr and 5hr).

After extraction, Azadirachtin and some polar limonoids was separated as supernatant from the cake by centrifugation for 15 minutes at 5000rpm. For further clarification, the supernatant was vacuum infiltrated. The infiltrated supernatant was kept in 500ml plastic flask and made ready for rotary vacuum evaporation process. Rotary vacuum evaporator made the supernatant to concentrate with an operation condition of 40rpm, 45^{o}C and 125mbar of rotation, temperature and vacuum pressure respectively to yield a dark brownish semi solid material.

The concentrated dark brownish extract was dried in a tray drier for 48hours continuously with an air flow rate of 60m^{3}/hr. The concentrated extracted were then de-oiled using hexane at 45^{o}C with 200rpm in a shaker incubator. The defatted brownish slurry was separated by a means of density difference in a funnel. The brownish heavy slurry was dried in a tray drier for 48hours continuously with an air flow rate of 60m^{3}/hr again.

The dried sample was washed with ethyl acetate of 45ml in a shaker incubator with 45^{o}C and 200rpm to dissolve the trace amount of other terpenoids. At the end, a light pink supernatant was separated (i.e. ethyl acetate and dissolved unwanted terpenoids) were removed from the solid (Azadirachtin) by decantation principle. Finally, a deep-freezing principle was carried out to solidify the extract in a deep freeze refrigerator so that handling becomes easy in that state.

(a) (b) (c)

Figure 3-4. Raw and dried seed (a) Extracted seed (b) and Azadirachtin (c)

3.4. Flow diagram of Azadirachtin extraction

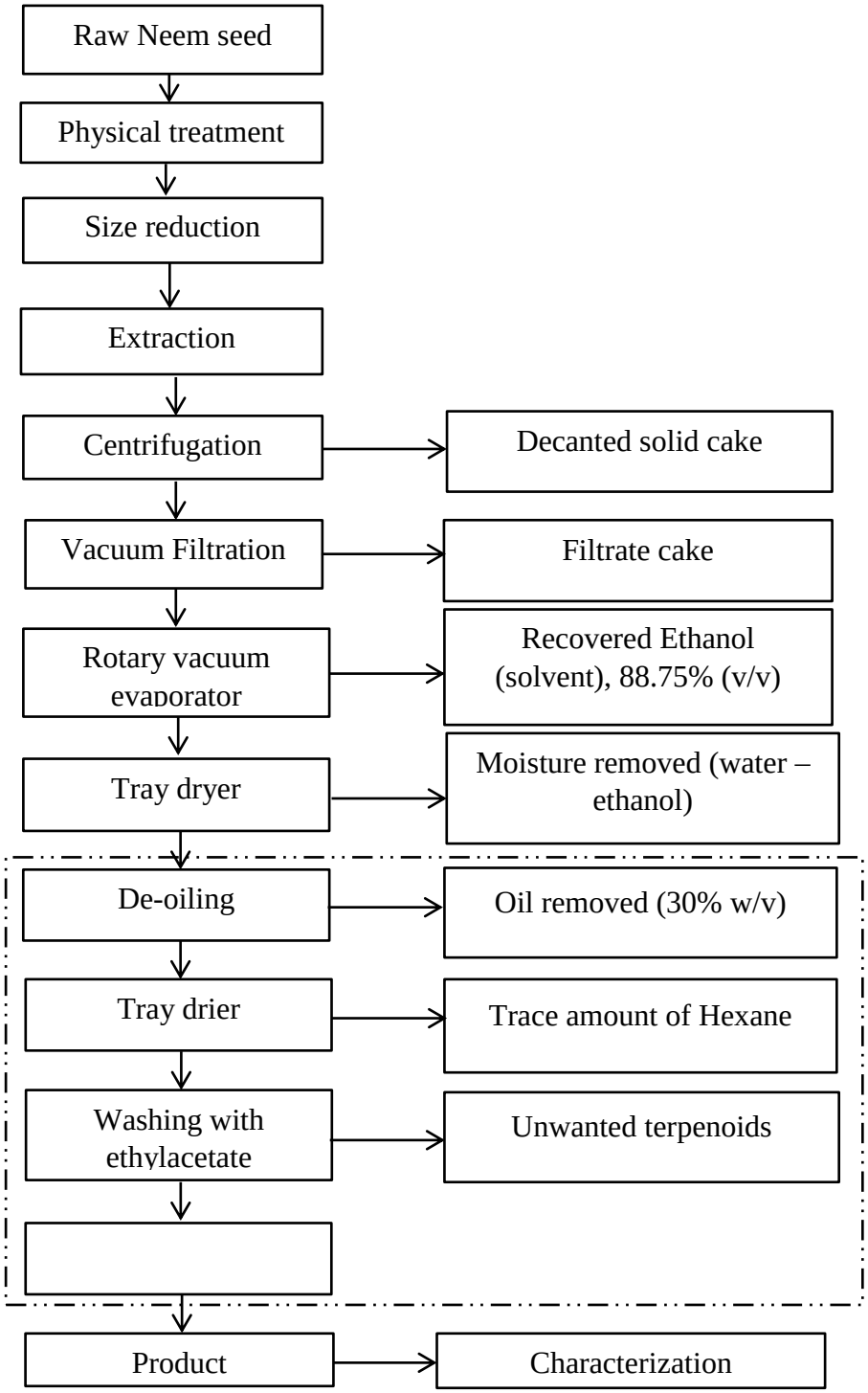


Figure 3-5. Flow Diagram of Azadirachtin Extraction

3.5. Physico – chemical characterization of extract

3.5.1. Thin Layer Chromatography (TLC) Analysis

This characterization analysis simply indicates the qualitative characteristics of the compound i.e. azadirachtin with respect to other compounds or elements extracted together and present simultaneously; and can possibly dissolve in the same mobile phase. The test was undertaken by a small amount of extract and dissolves it in ethanol. Then, spot the sample on a TLC plate and place it in a beaker consisting Iso-propyl alcohol: hexane (2.5:17.5) ratio as mobile phase. Run till $3/4^{th}$ of the plate distance, and then place it in Iodine chamber. It gives two pale yellow colored spots (Supriya Dubhashi, Mar, 2013). The upper spot indicates azadirachtin and the below spot indicates nimbin. This was because of the density difference of nimbin and azadirachtin. The R*f* (Retention factor) of azadirachtin was calculated then by dividing the distance traveled by azadirachtin to the mobile phase (i.e. isopropyl alcohol and hexane) distance travelled. The value of R*f* simply indicates as the presence of Azadirachtin in the extract. So, R*f* value is calculated as:

$$Rf = \frac{\text{Distance travelled by component}}{\text{Distance travelled by solvent}} \quad \text{.....Equation 3.4}$$

3.5.2. HPLC analysis test

The HPLC analysis is basically a quantitative analysis test that is used to specify the concentration of a given compound or element. It was performed with a C18 column (100 × 3 mm 3 Omnispher C18) equipped instrument, maintained at 30°C and a UV-visible detector (λ = 215 nm). The mobile phase consisted of acetonitrile/water at a flow rate of 0.8 mL/min. The injection volume was 20 µL. The mobile phase flow rate gradient programming was: 20% acetonitrile from 0 to 5 min increased from 20 to 65% acetonirile from 5 to15 min and maintained at 65% for 5 min more (Dubhashi., 2013). The R*t* of every compound is different from each other in the same detectable UV wave length. From this context using HPLC the R*t* value of azadirachtin will be analyzed.

3.5.3. Specific gravity

The specific gravity is determined by specific gravity bottle known as Pycnometer method using IS: 1460-2000 method depending on the Archimedes principle of displacement. This was conducted at room temperature and pressure. Specific gravity is the measurement of relative density of the sample with respective to water. This indicates that, the physical property of the sample immersion or flotation when it gains an opportunity to contact with water.

$$\text{Density of sample} = \frac{W_1}{V_1 - W_2/\rho} \quad \text{.....Equation 3.5}$$

From this the specific gravity of the sample can be calculated simply by dividing its density to the density of water.

Where: -

W_1 - Mass of sample (g)

V_1 - Volume of Pycnometer (cm^3)

W_2 - Mass of Hexane (g) and ρ – Density of Hexane, i.e. 0.6554 (g/cm^3)

3.5.4. Flash point

A key property of determining the flammability of a product is the flash point. The flash point is the lowest temperature at which an applied ignition source will cause the vapors of a sample to ignite. Therefore, it is a measure of the tendency of a sample to form a flammable mixture with air. This characteristic analysis is very important in transportation and safe using of the extract to protect accident and hazard in either pre or post application. As a side note, the value of the flash point is used for the classification of flammable and combustible materials needed for safety. The flash point was determined by heating a sample of the extract in a bomb calorimeter in the presence of oxygen. Generally, there are three main categories of product flammability these are; extremely flammable (Flash point below 0^oC), highly flammable (Flash point below 21^oC) and flammable (Flash point below 55^oC).

3.5.5. Water solubility

Solubility is defined as the amount of substance that passes into solution to achieve a saturated solution at constant temperature and pressure. Solubility is expressed in terms of maximum volume or mass of the solute that dissolve in a given volume or mass of a solvent. This property is very crucial for this test. Since, the final extract is going to be used by dissolving in water and spray on field, it should not left a residue over the crop. The more soluble mater in the solvent means the more dispersing material on the environment (Fleming, 4th, eddition).

$$\text{Solubility } (\%) = \frac{W_1 - W_2}{V_w} \times 100\% \quad \text{.....Equation 3.6}$$

Where: -

W_1 – Mass of sample before dissolution (g)

W_2 – Mass of sample after dissolution and (g)

V_w – Volume of solvent (i.e. water) (mL)

3.5.6. Acute Toxicity test

According to the WHO Classification 1996, pesticides have been classified into four categories: That are extremely hazardous, highly hazardous, moderately hazardous and slightly hazardous, depending on their acute oral and dermal LD_{50}. Values for solids in both oral and dermal tests is always lower in all four categories of acute toxicity levels. In the same way, solid pesticides have lower acute toxicity than liquid pesticides.

This test was carried out in Ethiopian Public Health institute (pastor) laboratory in a host animal i.e. rabbit (Oryctologus Cuniculus). The hosts were registered with a coded name of H –1, H – 2 and H – 3 with 4.5724kg, 4.9038kg and 4.8471kg initial mass respectively: and was feed 0.643gm of azadirachtin per day every host animal consecutively for 14 days.

Table 3-2. Chemical Toxicity classification of WHO

		Oral		Dermal	
	Class	Solids	Liquids	Solids	Liquids
1.	Extremely Hazardous	5 or less	20 or less	10 or less	40 or less
2.	Highly	5 - 50	20 - 200	10 – 100	40 - 100
3.	Moderately	50 - 500	200 - 2000	100 - 1000	100 - 4000
4.	Slightly	500 - 2000	2000 - 3000	Over 1000	Over 4000

Source: Guide line to classification of pesticide2000-2002 (revised): LD_{50} (mg / kg body weight)

The Globally Harmonized System of WHO defines acute toxicity as, those adverse effects occurring following oral or dermal administration of a single dose of a substance, or multiple doses given minimum within in 24 hours, or an inhalation exposure of minimum of 4 hours. Contextually this is derived by applying various concentrations of pesticide to a test subject's eyes, skin, and mouth as air we breathe. Data from these trials are converted into LD_{50} or LC_{50} values. These values are the doses or concentrations at which 50 percent of the animals tested die. Lower values are more toxic than higher values. LD_{50} and LC_{50} values are expressed:

$$LD_{50} = \frac{\text{Chemical weight (mg)}}{\text{Animal weight (kg)}} \quad \text{Equation 3.7}$$

$$LC_{50} = \frac{\text{Chemical particles (mg)}}{\text{air or water particles (L)}} \quad \text{Equation 3.8}$$

In this study, acute toxicity of extract was assessed with respect to the initial weight of a rabbit (Oryctologus Cuniculus) before dose and after dose by direct feeding; and calculating the mass difference of the host after 14 days. Then the amount of extract used divided to the mass difference of the host that degrades gives the LD_{50}. The raw data was then interpreted in terms

of LD_{50} and compared with the specified criteria under WHO's classification for toxic chemicals.

3.5.7. pH value

pH is a numeric scale used in specifying the acidity or basicity of an aqueous solution. Since, the extract is applied merely by spraying in an aqueous solution form on the crops; the possibility of remaining on the crops as residue, and penetrating the outer layer of earth surface so that to pollute the ground water has high implication. Due to this reason, the pH value of the extract was measured by dissolving 500mg of extract in 10ml of deionized water then directly immersing the rod to the solution and read the quantity directly at room temperature and 1atm.

3.5.8. Total volatile organic compounds Analysis

Volatile organic compounds or VOC's are environmentally important to be determined because they produce smog when they react with nitrogen oxides in photochemical reactions, and they also may have important health implications. International Environmental Protection Agencies or EPA regulation agencies are designed to control smog formation by limiting VOC and nitrogen oxide emissions. Generally, VOCs' are any chemical or compound based on carbon chains or rings with a vapour pressure greater than 0.01kpa at 298.15 K (25^{o}C), that participate in photochemical reaction (Australian Government, 2015/2016).

Recorded data's' about temperature in Ethiopia (1951 – 2006 E.C.) with an increment of 0.13^{o}C per decade indicates that between 24 and 32^{o}C i.e. the average temperature of Ethiopia becomes 28^{o}C (Massimiliano, 2015). So, the TVOC's present in azadirachtin were analyzed in 25^{o}C (since it is a universally accepted ambient temperature) and the average UV photo light energy of sun in 24 hours per meter square is 164watt (Basics of SolarEnergy). Considering this, a microwave oven with a UV light output sources up to 700w and 25 to 225 ^{o}C temperature gradient regulator was used to measure the TVOC in 24 hours and then analyzed the mass difference.

$$\text{TVOC}(\%) = \frac{\text{Initial mass of sample - Final mass of sample (gm)}}{\text{Initial mass of sample (gm)}} \times 100 \quad \text{……..…..…Equation 3.9}$$

4. RESULTS AND DISCUSSIONS

4.1. Results and discussion on pre-extraction of Neem seed

4.1.1. Moisture content of Neem seeds

The data collected from the analysis is listed below. From this result, the amount of moisture content removed can be calculated as:

$$\text{Moisture content}\,(\%) = \frac{60.0000 \;-\; 47.4762}{60.0000}$$

$$= 20.873\,\%$$

This result indicates that the amount of moisture removed from the seed was about 21% of its initial weight. Basically, the amount of moisture present in the seed blocks the diffusivity of the solvent towards the seed surface and prohibits extraction kinetics. "Neem Seed with higher moisture content yields less bioactive extract as compared to those with lower moisture content" (Orhevba, 2013). Generally, as it was shown from the data above the last 24 hours moisture content removed was about 0.7%. Since, the amount of moisture content was decreased consequently and left in small quantity of not aggressively affects the process i.e. less than 1%. In addition, drying of the sample beyond this might cause degradation of bioactive component and burning.

4.1.2. Ash contents of Neem seeds

The amount of ash content in a sample designates the total amount of nonorganic maters or compounds present in a sample (mostly metallic oxides). Applicably, these compounds are harmful by leaving nonvolatile and non-decomposable metallic compounds on the environment. These causes different health problems on different floral and faunas. For this reason, the amount of ash content should be less as much as possible. From the laboratory result of a replicated seed sample, the ash content can be calculated:

From this the mean standard Ash content of Neem seed is:

$$(\text{Ash content},\%) = 3.648 \pm 0.187\%$$

An organic matter is anything that contains carbon compounds that were formed by living organisms. Organic matters are biodegradable and environmental sustainable. So, the need for high amount of organic matter is very important environmentally and applicably. From this, the amount of organic matter in Neem seed was calculated as:

The mean standard organic matter constituent of a Neem seed was:

$$\text{Organic matter} = 96.3515 \pm 0.1865$$

The seed has about 96.3515 ± 0.1865 of organic compounds that are easily decomposable and degradable. Simultaneously, harmless to the environment and its living things.

4.2. Physico – chemical characteristics of Extract

4.2.1. Thin Layer Chromatography (TLC) Analysis

TLC measurement is a qualitative test used to determine the presence of a certain compound or element with respect to another elements or compounds found simultaneously by dissolving in a mobile phase. The standard R*f* value of Azadirachtin is 0.70 (Mukunda, 2016). The result shown below was found from TLC analysis of extract in with a cellulose coated aluminum stand stationary phase and hexane and isopropyl defined ratio mobile phase.

The result of seven randomly selected samples was simply indicating the presence of azadirachtin with an Rf value of 0.7079 ± 0.0068. Eventhough, the result has a nearer proximate value to the literature, there has been an error occurred here that was happened due to measurement and calibration of instrument. Here, there is also a trace amount of nimbin present mixing with azadirachtin. This can be simply shown in the TLC plate by spotting the dark yellow color below azadirachtin (i.e. light yellow) spot.

Table 4-1. TLC analysis results of azadirachtin

Sample number	Concentration (gm/ml)	Distance covered (cm)	$Rf = \frac{\text{Distance covered by component}}{\text{Distance covered by solvent}}$	Mean ± SD of R*f* value
1	0.05	SD[3] = 12.1 CD[4] = 8.4	0.6942	0.7079 ± 0.0068
2	0.045	SD = 11.72 CD = 7.82	0.7143	
3	0.04	SD = 11.2 CD = 8.0	0.7107	
4	0.035	SD = 11.1 CD = 7.7	0.7045	
5	0.03	SD = 10.4 CD = 7.4	0.7115	
6	0.025	SD = 9.7 CD = 6.9	0.7113	
7	0.02	SD = 9.68 CD = 6.82	0.7045	

4.2.2. HPLC analysis test

The graph above implied that, the peak of the first move when 0.05mg/mL of 20µL azadirachtin was injected and made the pumps of mobile phases (acetonitrile: water) to flow 20% and 80% respectively for the first 5 minutes at constant temperature of 30ºC. Detection was happened at

[3] Solvent distance covered

[4] Component distance covered

3.03minute with 212.28nm wave length. Again, when the flow rate of the mobile phase pumps increased to 65% and 35% another peak was happening in 8.6minute with a wave length of 213.6nm. These two peaks resemble that the wave length of azadirachtin at 30^{o}C. The retention time of azadirachtin at the above parameters was found 2.756 minute (Kaushik, 2003). Retention time found from this result was 3.03 minute for the first peak and 3.6 minute. The second peak retention time was slightly longer than the first one. This is because of the smaller polar diffusivity of azadirachtin happened due to the decrease in water flow rate and it was expected. Generally, eventhough some impurity was observed since the retention time was longer a bit, but it can be concluded that azadirachtin was detected sufficiently under the HPLC UV detector.

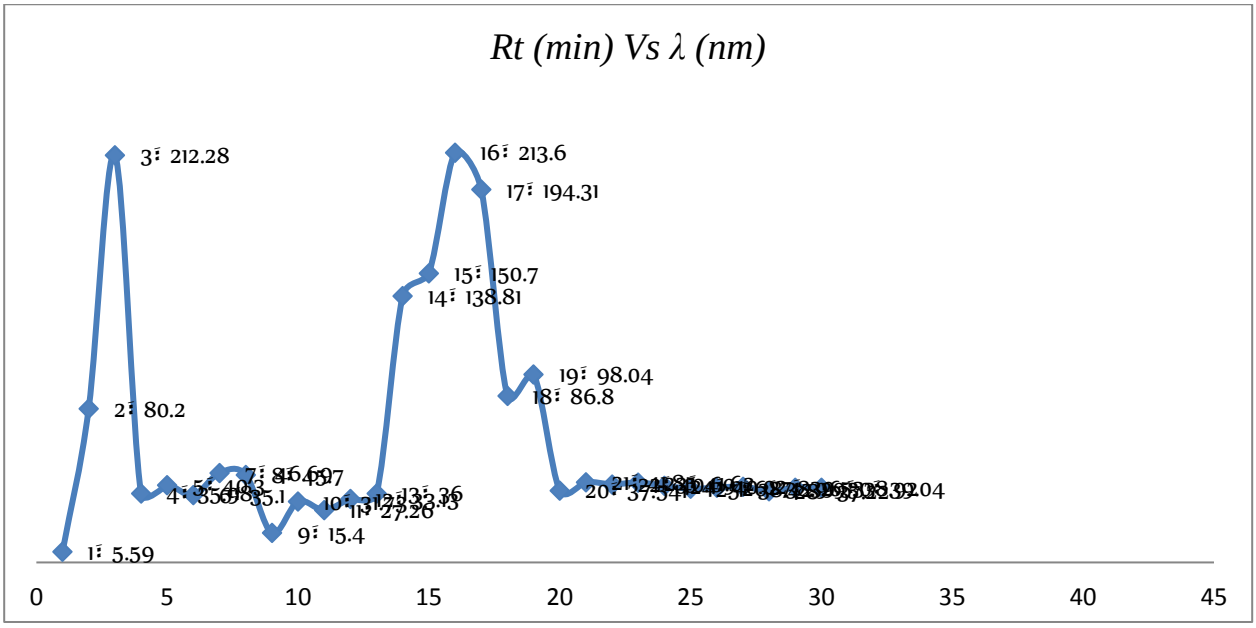


Figure 4-1. HPLC Analysis graph

4.2.3. Specific gravity

Fundamentally, this test was done because of the media in which the extract has been going to dissolve becomes water. Comparatively the specific gravity of the sample should not be smaller than water, so that solubility facilitates deply. The result found from the analysis of randomly selected six samples has indicated that, the density of the extract was found slightly higher than

the density of water (i.e. 1.3355 ± 0.0743). This simply helps the extract both, to submerse rather than float when it mixed with water and enhances the dissociation due to diffusion and surface attraction with water during solubilization; and has great factor not to be easily disperse by wind after use on field.

Table 4-2. Specific gravity analysis result of azadirachtin

Sample Number	Density (gm/cm^3)	Sp.gr	Mean Sp.gr ± SD
1	1.2572	1.2572	1.3355 ± 0.0743
2	1.4613	1.4613	
3	1.2908	1.2908	
4	1.4120	1.4120	
5	1.3041	1.3041	
6	1.2875	1.2875	

4.2.4. Flash point

Flash point is the lowest temperature causes the vapors of a specimen of the sample to ignite under specified conditions of test (i.e. at pressure of 101.3 kPa or 760 mm Hg). The fundamental significance of this test has been very important in controlling accident and hazard before use (i.e. in storage time) and after use on field (i.e. ignition temperature might cause crop firing). So, the end users should be noticed about the flammability and possible accident that could be occurring.

From this point of view, the result of six randomly selected samples was tested tire flash point using bomb calorimeter and found the result 35.05 ± 1.0966^oC. The result found to be under the classification of flammable components (i.e. the ignition temperature is below 55^oC).

Table 4-3. Flash point analysis result of azadirachtin

Sample number	Flash point (°C)	Mean flash point ± SD (°C)
1	35.2	35.05 ± 1.0966°C
2	37.1	
3	35.5	
4	34	
5	34.7	
6	33.8	

4.2.5. Water solubility

This method helps to determine the disintegration and relative dissolution of azadirachtin in water; Since azadirachtin becomes expected to be dissolve in water and uniformly disperse. The time and temperature at which azadirachtin disintegrates and dissolves was set to be constant i.e. 20minutes, 25°C and atmospheric pressure. Using the above method, the result found from the test were stated below. The result implied that 99.708 ± 0.0069% of the initial mass dissolved smoothly. Meanwhile, the rest remains undissolved with the given parameter.

Table 4-4. Solubility analysis result of azadirachtin

Sample Number	$\text{Solubility} = \frac{\text{mass of specimen remain in filter paper}}{\text{initial mass of specimen}} \times 100\%$	Mean solubility ± SD (%)
1	99.71	99.708 ± 0.0069
2	99.71	
3	99.70	
4	99.72	
5	99.70	
6	99.71	

4.2.6. Acute toxicity test

The total dose of the hosts collectively was 27gm per 14 days. The individual host consumption per day was 643mg. The result obtained from the analysis is listed below. The practical connotation of this test was, to notify the toxicity (specifically Acute in oral) according to the WHO classification of pesticide so that to compare with the synthetic one. From this perspective, a randomly chosen six samples was in use to calculate the lethal dose of the hosts that can be possibly make them to loss 50% of their weight. The result was recorded 2276.7806 ± 64.1001. This means that, the amount of azadirachtin that could be possibly make the host to loss 50% of their weight becomes 2276.7806 ±64.1001mg of azadirachtin/kg of host. According to the WHO classification of pesticide toxicity the result falls under slightly toxic category of liquid oral dose, since the field application of azadirachtin becomes expected in liquid phase.

Table 4-5. Acute Toxicity analysis result of azadirachtin

Host Animal code	Host initial mass (kg)	Mass loss of host (kg)	$LD_{50} = \frac{\text{Chemical weight (mg)}}{\text{Animal weight (kg)}}$	Mean LD_{50} ± SD (mg/kg)
H - 1	4.5724	0.7220	2357.4195	2276.7806 ± 64.1001
H - 2	4.9038	0.9431	2272.3256	
H - 3	4.8471	0.7573	2200.5966	

4.2.7. pH value

The tendency of liberating hydrogen ion or hydroxide ion when azadirachtin dissolves in water was measured directly by a pH meter. This tendency could be vigorously affect the environment as well as the end user if it goes to much high or low. The liberation of both ions might cause soil infertility depending up on the natural behaviour of the soil. In addition to this, the pH value of azadirachtin has great implication on the target pest depending on the physiological and

enzymatic pattern. Six randomly selected samples were used to affirm the pH value of azadirachtin. The result found is listed below:

Table 4-6. pH analysis result of azadirachtin

Sample Number	pH value	Mean pH value ± SD
1	4.14	4.0533 ± 0.06263
2	4.04	
3	3.97	
4	4.10	
5	4.09	
6	3.98	

The result implies that the pH value of azadirachtin has been found in the range of 4.0533 ± 0.06263. If categorizing would be necessary azadirachtin becomes acidic depending on the magnitude.

4.2.8. Total volatile organic compounds Analysis

VOCs are a health hazard resulting in eye, nose, and throat irritation, headaches, loss of coordination, nausea, damage to liver, kidney, and central nervous system. Some organics can cause cancer in animals; some are suspected or known to cause cancer in humans. Key signs or symptoms associated with exposure to VOC's include conjunctival irritation, nose and throat discomfort, headache, allergic skin reaction, dyspnea, declines in serum cholinesterase levels, nausea, emesis, epistaxis, fatigue, and dizziness. The ability of organic chemicals to cause health effects varies greatly from those that are highly toxic, to those with no known health effect. As with other pollutants, the extent and nature of the health effect will depend on many factors including level of exposure and length of time exposed. Eye and respiratory tract irritation, headaches, dizziness, visual disorders, and memory impairment are among the immediate symptoms that some people have experienced soon after exposure to some organics. There has been no standard set for VOCs in non-industrial settings. There are so many VOC's. Some of

the compounds have been recognized as a specific health risk with specific environmental problems. Here the amount of TVOC is calculated in percent of mass difference from a randomly selected six specimens. The result found from the analysis is listed below.

Table 4-7. TVOC's analysis result of azadirachtin

Sample Number	Initial mass (gm)	Final mass (gm)	$TVOC(\%) = \frac{\Delta m}{m_i}$	Mean TVOC ± SD (%)
1	1.1018	1.1006	1.0893	0.45095 ± 0.3360
2	1.3073	1.3047	0.1989	
3	0.9853	0.9784	0.7003	
4	0.9525	0.9509	0.1680	
5	2.3529	2.3457	0.3060	
6	1.6445	1.6405	0.2432	

From the result above, the amount of TVOC's per 24 hours with a 165 w and 25^{o}C has been found 0.45095 ± 0.3360%.

Note: **-** The result is expressed relative to the initial mass of specimen before analysis.

4.3. Result of azadirachtin extraction

The yield of azadirachtin from Neem seed using batch reactor with further enrichment process steps and defined operation parameters (i.e. particle size, Extraction temperature, solvent to solid ratio and extraction time) was found quite different in magnitude. The reason why this could be possibly happened was back to the factors individual and interactive effect.

It can be simply observed from the yield result, TLC and HPLC test analysis result that; concentration of azadirachtin was increased simultaneously with an increment of yield quantity. This means, the more yield of an extract found have had been at the same time more concentration than the lower yield extract.

From this aspect, from 100gm of ground Neem seed an adequate yield was found 9.319gm as optimal with operational conditions of 350µm, 45ºC, 6:1v/w and 4hr of particle size, extraction temperature, solvent to solid ratio and extraction time respectively. In contrary 6.168gm of yield was found the least from the same amount of ground Neem seed with operational parameters of 550µm, 40ºC, 6:1v/w and 3hr particle size, extraction temperature, solvent to solid ratio and extraction hour.

4.4. Experimental design analysis results

RSM of Box – Behnken method Design-Expert 7.0.0 software was used to determine the optimal conditions of Azadirachtin extraction. A Box – Behnken method is used to investigate the effects of four independent factors with three levels (particle size, extraction temperature, extraction time and solvent to solid ratio). Box - Behnken method uses the method of least-squares regression to fit the data to a certain model. The adequacy of the model is going to be determined by evaluating the lack of fit, obtained from ANOVA that will be generated by it. Statistical significance of the model and model variables will be determined at 5% probability level ($\alpha = 0.05$).

The result found after the data was put to Design-Expert in order of sequence, there has been found a single model that can be possibly fit or satisfy the factors significance. This possible choice of model was a Quadratic model that fits the given data. Lack of fit is a measure of all the factors effect on the response. The higher the lack of fit implies the highest fitting possibility of the model or the higher impact of factors on the response.

Depending on this fact, the lack of fit of Quadratic models has been found best fitted model among all with 0.9329 or 93.29% of lack of fit.So, quadratic model with 93.29% of fiting becomes the best choice of analysis for this case.

Table 4-8. Sequential Model Sum of Squares

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F	
Mean vs Total	1926.04	1	1926.04			
Linear vs Mean	13.70	4	3.42	18.71	<0.0001	
2FI vs Linear	1.83	6	0.31	2.15	0.0972	
Quadratic vs 2FI	*1.63*	*4*	*0.41*	*6.14*	*0.0045*	*Suggested*
Cubic vs Quadratic	0.34	8	0.042	0.43	0.8689	Aliased
Residual	0.59	6	0.099			
Total	1944.13	29	67.04			

The table below shows the individual factors coded name (A, B, C and D), factor low and high coded value (-1 and 1), type of factor (numeric), mean and standard deviation of the study parameters. What can be observed from the data above was, the model best choice becomes quadratic with a certain standard deviation error of each factor. The model standard deviation becomes 0.79 found a least square error of the model. The model uses polynomial mathematical correlation to analyze the data and to fit the result found.

Table 4-9. Design Summary table

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
Y1	Yield	(gm)	29	Polynomial	6.17	9.32	8.15	0.79	1.51	None	Quadratic

Factor	***Name***	***unit***	***Type of factor***	***Low Coded***	***High Coded***	***Mean***	***Std. Dev.***
A	Particle-Size	µm	Numeric	-1.000	1.000	450.000	64.327
B	Temperature	^{o}C	Numeric	-1.000	1.000	40.000	3.216
C	Solvent - Solid ratio	(v/w)	Numeric	-1.000	1.000	6.000	0.643
D	Extraction time	Hour	Numeric	-1.000	1.000	4.000	0.643

From the ANOVA result of the design expert all the factors, i.e. particle size, temperature, solvent to solid ratio and extraction time had a significant effect individually on the yield this indicates that, the variation in magnitude on the individual factors directly affects the yield parallel or not. In addition to this, the interaction or combination effect of particle size with solvent to sold ratio, particle size with extraction time, temperature with extraction time and solvent to solid ratio with extraction time was significantly affect the response at the same time. This conclusion was deducted basically by comparing the p – value of the individual and combined factors with the percent of probability of the model (i.e. $\alpha = 0.05$ or 5%). The lesser the p – value than α value has implied the greater significance effect of the factor on the response. In the reverse, the higher the p –value causes a lower possible effect of the factor on the response and lower model fitting possibility.

Generally, the basic connotation of ANOVA was to approve and disprove the impact of individual and combined factors on the yield. This could be justified from the significance value of factors and lack of fit of the model.

Table 4-10. Analysis of variance table ANOVA

Source	***Sum of Squares***	***df***	***Mean Square***	***F Value***	***p-value Prob > F***	
Model	*17.159144*	*14*	*1.225653144*	*18.47079668*	*< 0.0001*	*Significant*
A-Particle-Size	9.5440436	1	9.544043603	143.8303241	< 0.0001	
B-Temperature	0.78229027	1	0.782290268	11.78924441	0.0040	
C-Solvent–Solid ratio	1.80497633	1	1.804976333	27.20129348	0.0001	
D-Extraction time	1.56421302	1	1.564213021	23.57295033	0.0003	
AB	0.10390952	1	0.103909523	1.565933782	0.2313	
AC	0.43665664	1	0.43665664	6.580488173	0.0224	
AD	0.4109451	1	0.410945103	6.193011028	0.0260	
BC	0.13675204	1	0.13675204	2.060875982	0.1731	
BD	0.34117281	1	0.34117281	5.141530978	0.0397	
CD	0.40500496	1	0.40500496	6.103492092	0.0270	
A^2	1.1025214	1	1.102521405	16.6151809	0.0011	
B^2	0.03727967	1	0.03727967	0.561810825	0.4659	
C^2	0.00035744	1	0.000357444	0.005386733	0.9425	
D^2	0.45762737	1	0.457627366	6.896520495	0.0199	
Residual	0.92898776	14	0.066356268			
Lack of Fit	*0.41511125*	*10*	*0.041511125*	*0.323121407*	*0.9329*	*Not significant*
Pure Error	0.51387651	4	0.128469127			
Cor Total	18.0881318	28				

4.4.1. Development of Model Equation

A model equation is a representative equation in which it represents the whole model with a single mathimatical relation that helps to maximize response (in this case yield) and the operating conditions. The model equation of azadirachtin extraction from Neem seed that was developed by the software has been shown below in the table. Practically, from the equation it can be easily observed that, increment in individual factors also made the yield to increase parallely. In other hand, eventhough the combined factors effect of particle size with solvent to solid ratio, particle size with extraction time, extraction temperature with extraction time and solvent to solid ratio with extraction time affects the yield vigrously; extraction temperature with solvent to solid ratio, extraction temperature with extraction tme and solvent to solid ratio with extraction time made the yield to fall down counterpartly. Finally, doubling the operating temperature can only cause yield icrement.

Table 4-11. Model equation in Coded and Actual factor form

Final model equation in terms of coded factors:	**Final model equation in terms of actual factors:**
Yield = +8.40 -0.89 × [A] +0.26 × [B] +0.39 × [C] +0.36 × [D] -0.16 × [A × B] +0.33 × [A × C] +0.32 × [A × D] -0.18 × [B × C] -0.29 × [B × D] -0.32 × [C × D] -0.41 × $[A]^2$ +0.076 × $[B]^2$ +7.423E - 003 × $[C]^2$ -0.27 ×$[D]^2$	Yield = -17.85392 +8.43573E-003 × [Particle-Size] +0.40905 × [Temperature] +1.56395 × [Solvent - Solid ratio] +5.28919 × [Extraction time] -3.22350E-004 × [Particle-Size × Temperature] +3.30400E – 003 ×[Particle-Size × Solvent - Solid ratio] +3.20525E-003 × [Particle-Size × Extraction time] -0.036980 × [Temperature × Solvent - Solid ratio] -0.058410 × [Temperature × Extraction time] -0.31820 × [Solvent - Solid ratio × Extraction time] -4.12277E - 005 × $[Particle\text{-}Size]^2$ +3.03243E – 003 × $[Temperature]^2$ +7.42333E – 003 × $[Solvent - Solid\ ratio]^2$ -0.26561 × $[Extraction\ time]^2$

4.4.2. Model Adequacy Checking

From ANOVA results for responses which are presented in the above figures confirms the adequacy of the quadratic model (the Model Prob > F is less than 0.05). Adeq-precision measures the signal to noise ratio and a ratio greater than 4 is desirable. As a result, Adeq-Precision for the extraction of azadirachtin from Neem seed with four independent factors was found 17.398.

Table 4-12. Model adequacy table

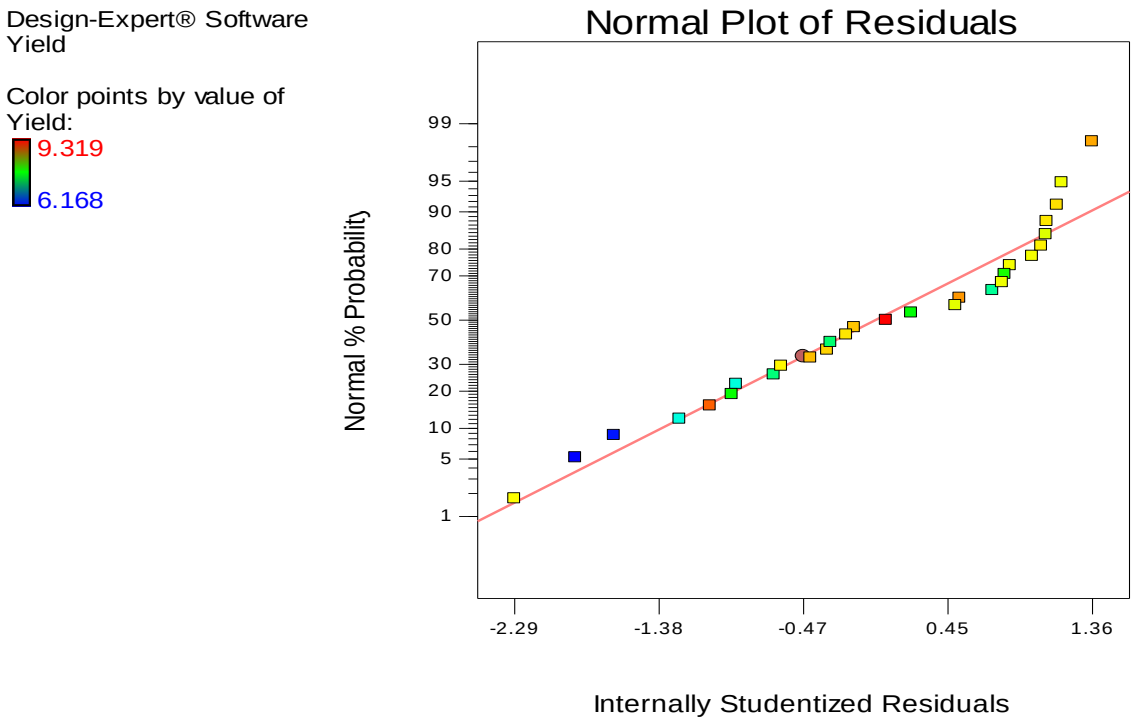
Std. Dev.	0.26	R-Squared	0.9486
Mean	8.15	Adj R-Squared	0.8973
C.V. %	3.16	Pred R-Squared	0.8234
PRESS	3.19	Adeq Precision	17.398

This value indicates that adequate signal for the model can be used to steer the design space. The value of correlation coefficient (R^2) of the model was 0.9486. This indicates that only 0.9486% of the total variation could not be explained by the observed model, and expresses well enough quadratic fits to navigate the design space. The R^2 should be at least 0.80 for a good fit of a model. The R^2 value obtained in the present study for these response variables was higher than 0.80, indicating that the regression models explained the extraction well. A normal probability plot of residuals indicates whether the residuals follow a normal distribution, in which case the points will follow a straight line. Some scattering is expected even with normal data. It can therefore be concluded from the normal probability plot figure 4.1 (a) of the studentized residuals for the extraction of azadirachtin that the data was normally distributed.

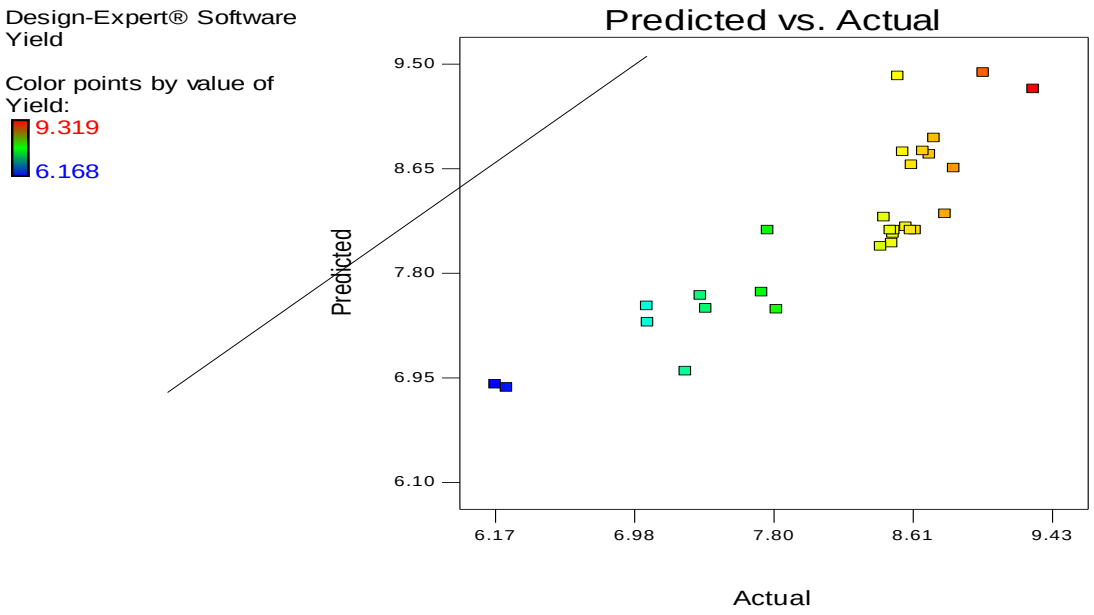
The predicted versus actual value or accuracy plot of the response presented in Fig. 4.1. (b) indicates a good agreement between real data and the data obtained from the model. The CV Fig.4.2. (d) is the ratio of the standard error of estimate to the mean value of the observed

response defined the reproducibility of the model. If CV of the model is greater than 10%, then the model can be considered reproducible. According to Table 4.13, the model was considered not reproducible since, CV was found 3.16%. This means RSM of Box-Behnken analysis is used to identify values of the independent variables that produce optimum extraction yield as response.

Each independent variable was individually increased or decreased in an attempt to find the maximum response. Then, the combination of these optimum variables was selected as the condition for obtaining the overall maximum value. The optimization of experimental condition was identified by considering a higher constraint factor values for this design space only.

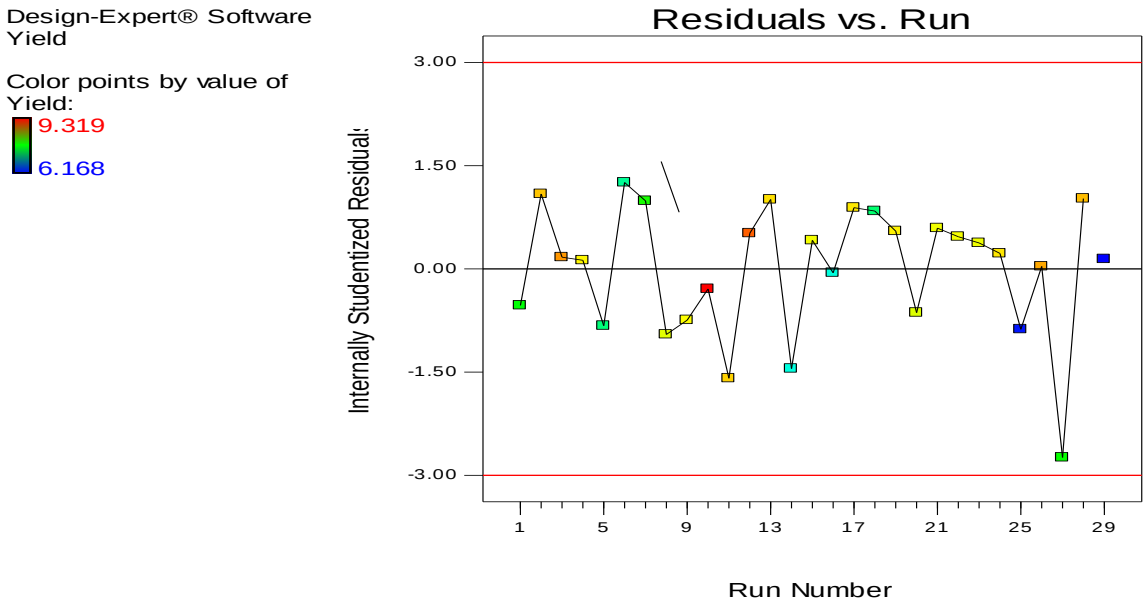


(a)

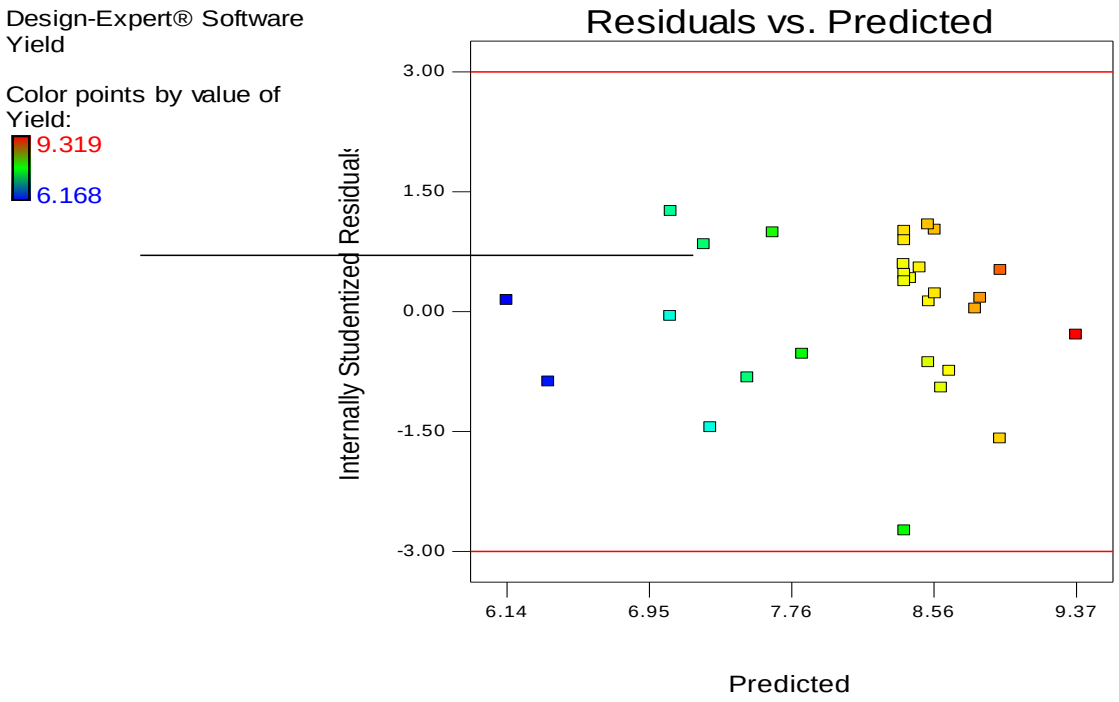


(b)

Figure 4-2. Residual Normality (a) and accuracy (b) plot of the model



(c)



(d)

Figure 4-3. Residual autocorrelation (c) and Variance (d) plot model

4.5. Effect of individual factors on yield

The factors used in this analysis were particle size, extraction time, extraction temperature and solvent to solid ratio. These factors had a great impact on the response (yield) and analyzed individually to ascertain their effects.

4.5.1. Effect of particle size on yield

The graph of particle size verses yield was resembled very declining linear plot. It was simply observed that the yield of azadirachtin downfalls or decreases with a corresponding increment in particle size. This is simply happened due to the principle of diffusion. Since optimally reduced size has a higher surface area to volume ratio and this increases the extraction kinetics practically. The extraction of azadirachtin has higher yield in a particulate size of 350µm than the rest. Considering particle size the optimum yield found was 9.04137g/ 100g of azadirachtin

per seed. Antagonistic to this result the yield was found declining from 7.7641g in 450 µm to 7.25774µm of particle size.

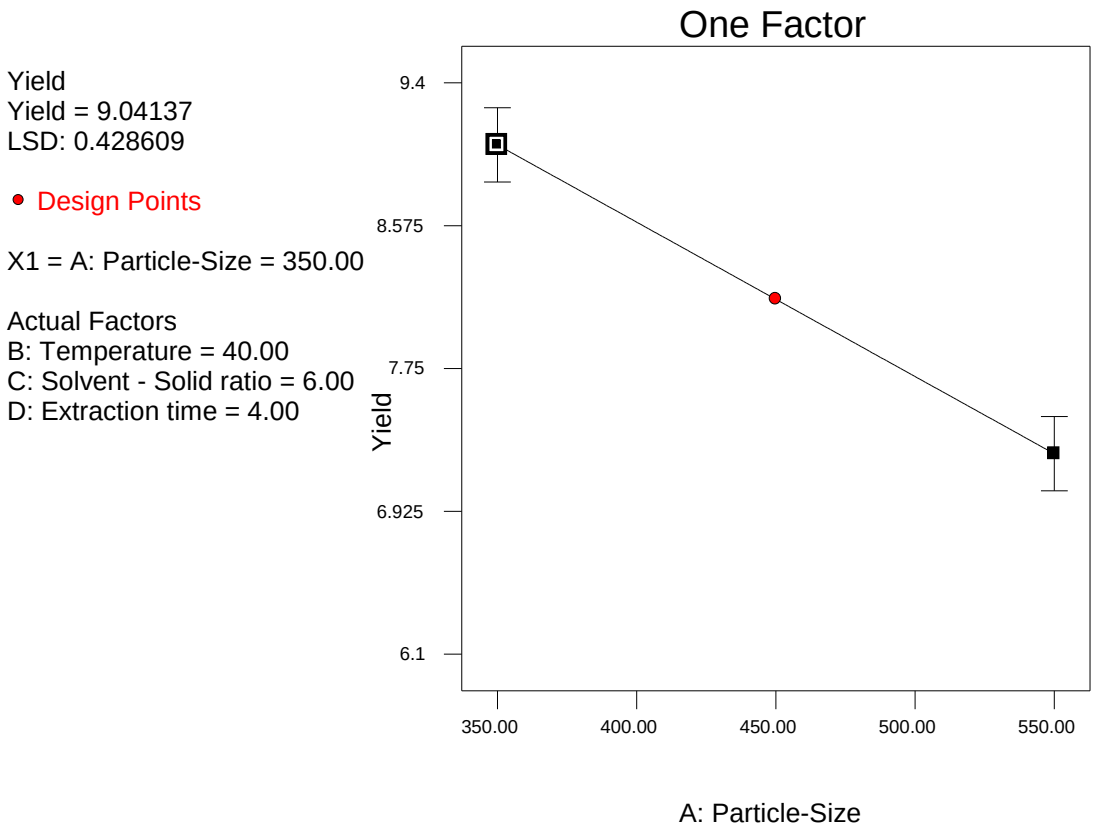


Figure 4-4. Effect of particle size on yield

4.5.2. Effect of Temperature on yield

It is obviously known that; higher extraction temperature could create degradation of bioactive components. To uphold such problems the choice of extraction temperature plays a major role depending on the final application of the yield. But, an optimum temperature helps extraction to be high by softening the outer part of the solid and breakdown the strong molecular force of compounds. In this case extraction temperature was chosen 35, 40 and 45 ^{o}C because the biological activity of azadirachtin becomes very important when it is necessary to use as biopesticide.

From the figure below the maximum yield was found at the maximum operational temperature of 45^{o}C that is 8.40488g. Respective with an increase in temperature gradient, azadirachtin yield was increased simultaneously from 7.89423g to 8.4824g for 35^{o}C and 40^{o}C.

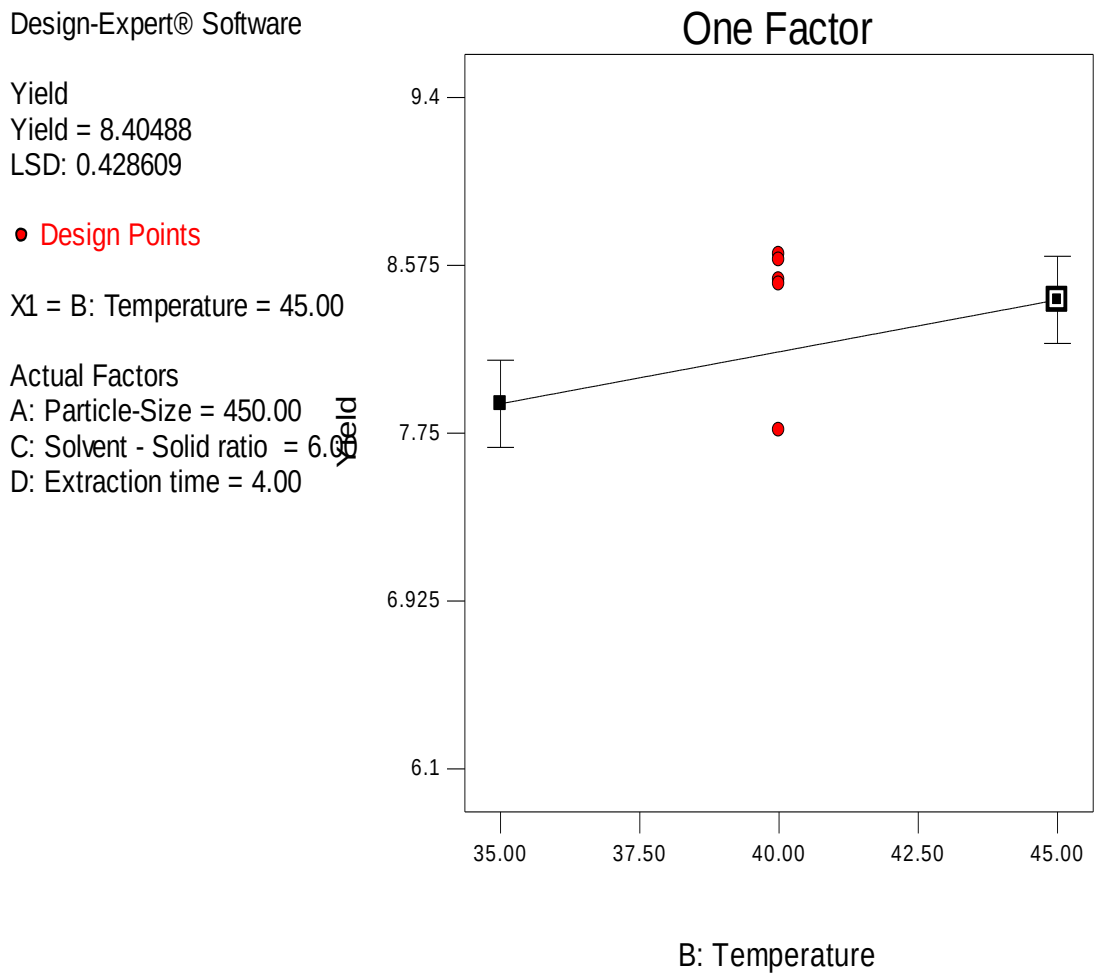


Figure 4-5. Effect of temperature on yield

4.5.3. Effect of solvent to solid ratio on yield

The effect of solvent to solid ratio is directly related with the rate of dissociation between the solute and the solvent. This depends on the solute saturation or amount of solution that can be possibly dissolves in it under certain parameters. The impact of limited solvent is obviously

inadequate extraction process and yield. While, excess solvent usage steered to burst the outer surface of the seed wall so that extraction becomes disrupted; and extraction of other dissociable components is a major side effect when solvent becomes high quantitatively. This causes impurity of extract. From the graphical representation below, the amount of yield was increased with an increment of solvent to solid ratio 8.53739g. The small amount of azadirachtin was found 7.76172g and 8.628g in 5:1 and 6:1 v/w ratio. This was happened due to the rapid solvent evaporation which affected diffusivity of the active ingredient (azadirachtin) as more solvent is needed to penetrate.

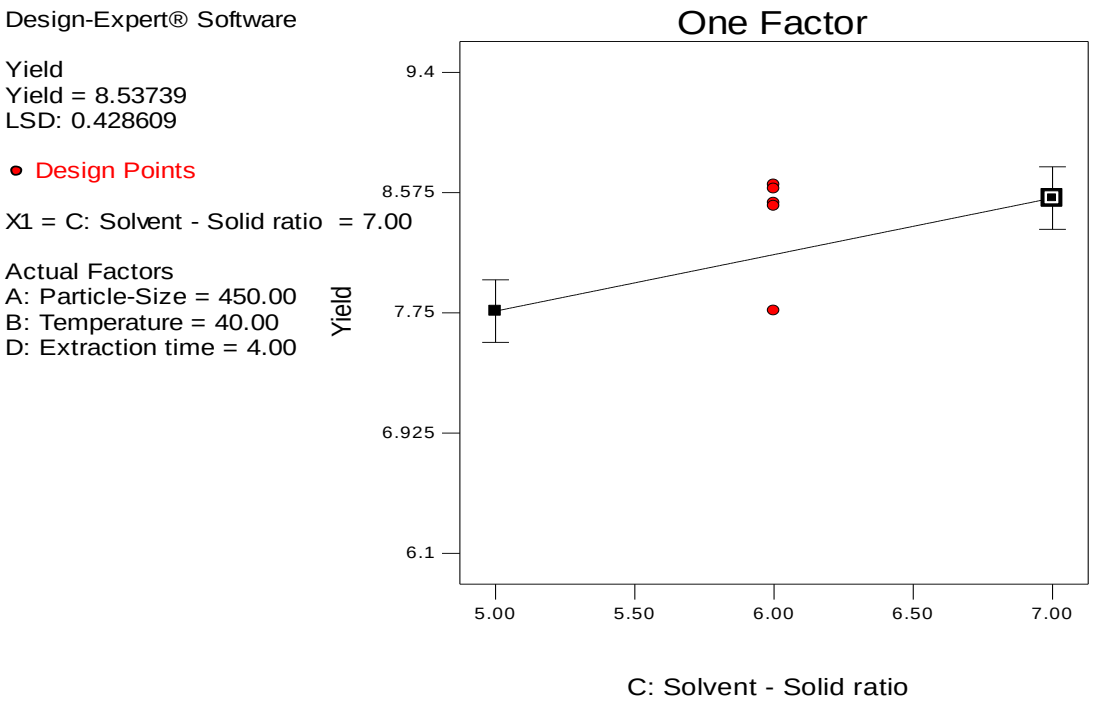


Figure 4-6. Effect of solvent to solid ratio on yield

4.5.4. Effect of extraction time on yield

Extraction time has also a great effect on the yield by dually. These are by making to dissolve unnecessary like solutes in the solvent and enhancing impurity; and affect the biological activity

of the active ingredient by exposing the solute to a higher temperature. Most of the time extraction is increasing simultaneously with an increment of extraction time. From this context, the plot below was shown; the raise of yield was happened due to a comparative increment of extraction time. The maximum yield was found when the extraction process was going for 5 hours and it was 8.5106g. The yield was declined proportionally to 8.5031g and 7.78851g. This was take place due to a short period of contact hour between the solid and the solvent.

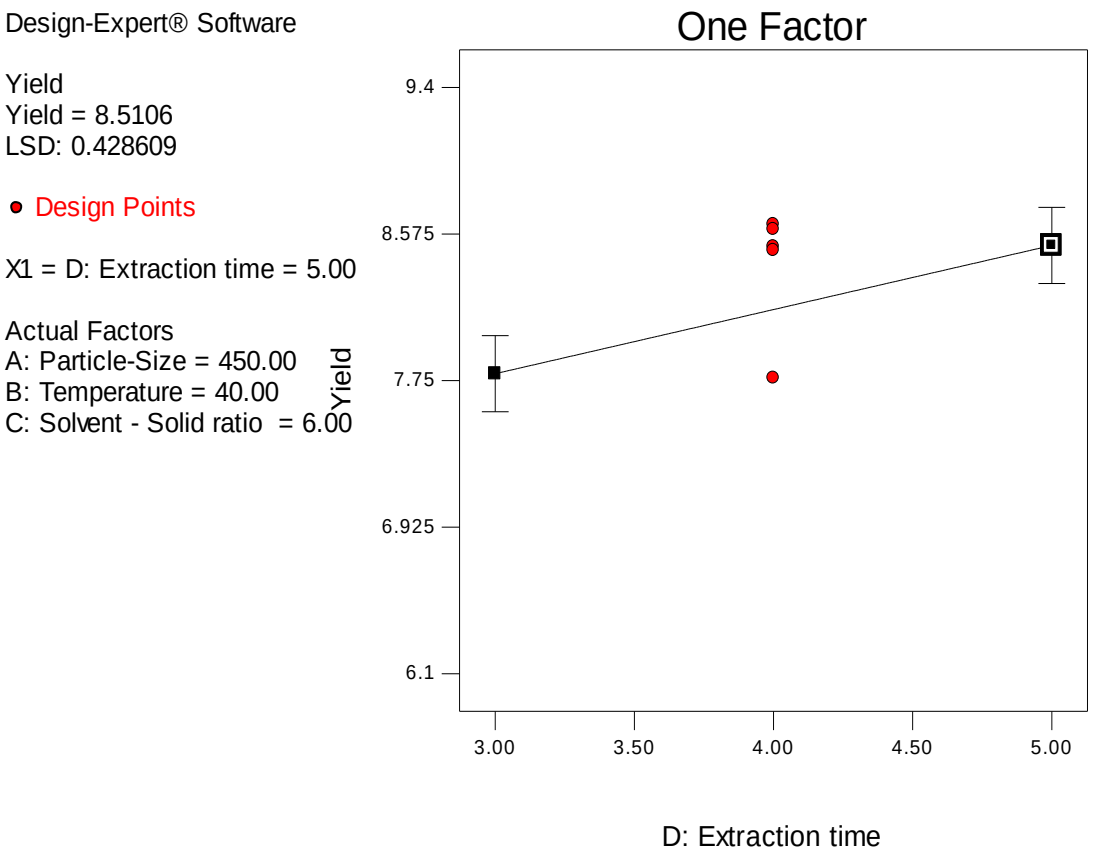


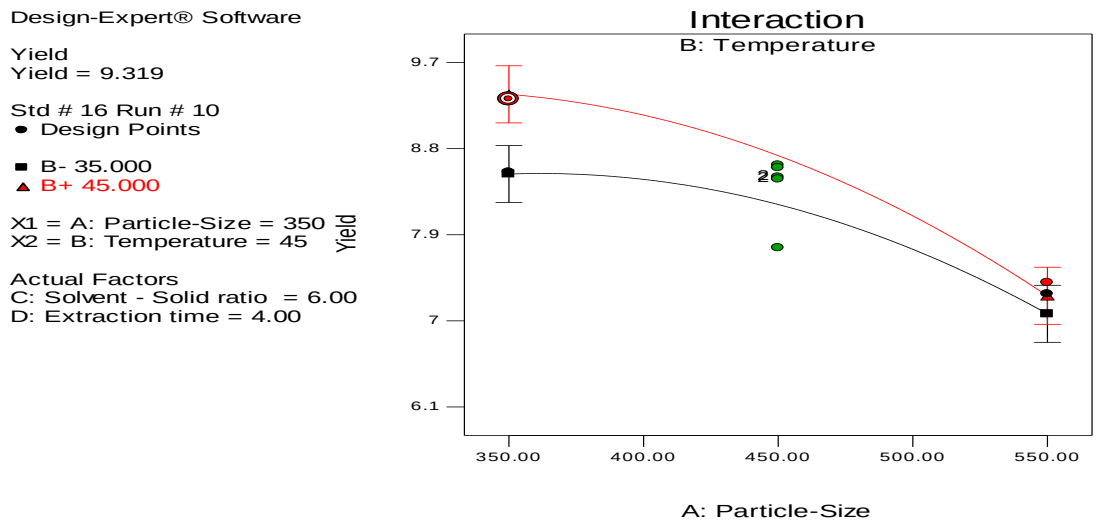
Figure 4-7. Effect of extraction time on yield

4.6. Interaction Effect of Factors on Yield

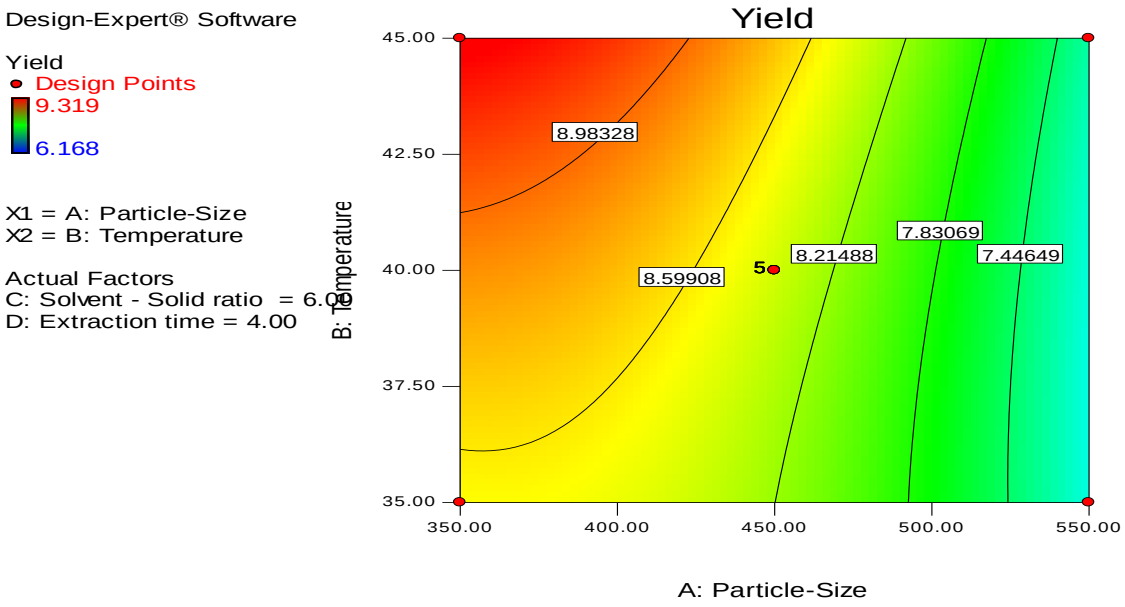
An interaction effect is a combination effect of two or more individual factors that can be possibly affects the response in the same or different way. Interaction effects should always under consideration if and only if the P-value of the combined factors found to be less than 0.05 or 5% (i.e. the probability of the model).

4.6.1. Effect of particle size and temperature on Yield

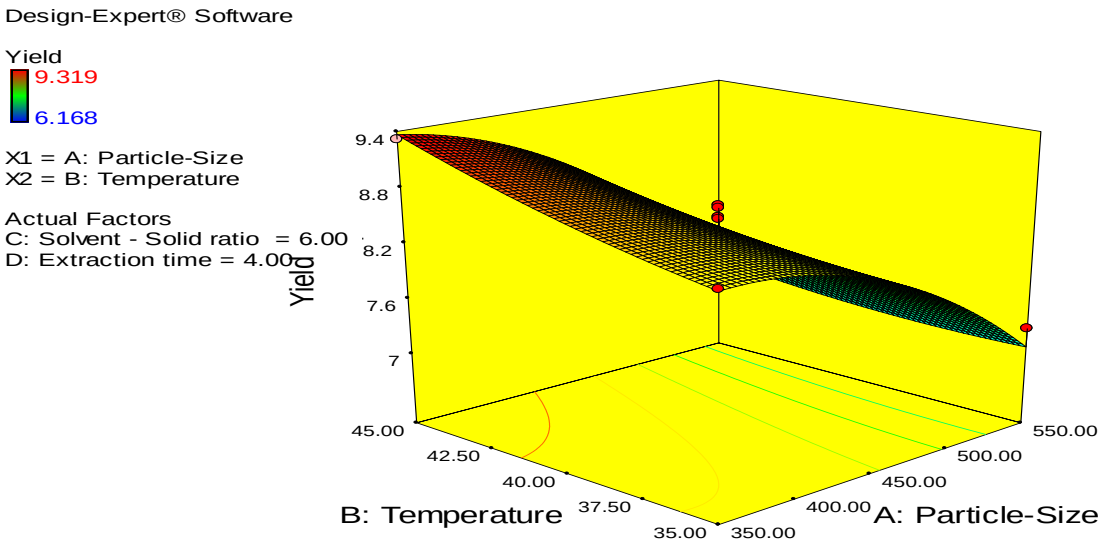
The figure below indicates that, the interaction between particle size and extraction temperature was not significant or couldn't affect the response. From the ANOVA table, the p-value of particle size and extraction temperature was 0.2313. This means the p-value of the interaction is greater than the p-value of the model design. Generally, there was no combined effect of particle size and extraction temperature observed on the yield. So, the yield was independent of particle size and extraction time influence at the same time. The proportionate increment of particle size and extraction temperature was significantly affects the yield individually. When extraction temperature goes from 35 to 45^{o}C and particle size goes down from 550 to 350µm the yield implied a parallel increment of 8.5551g to 9.3119g at 350µm; the yield was simply observed it was not interacted to a single value.



(a)



(b)

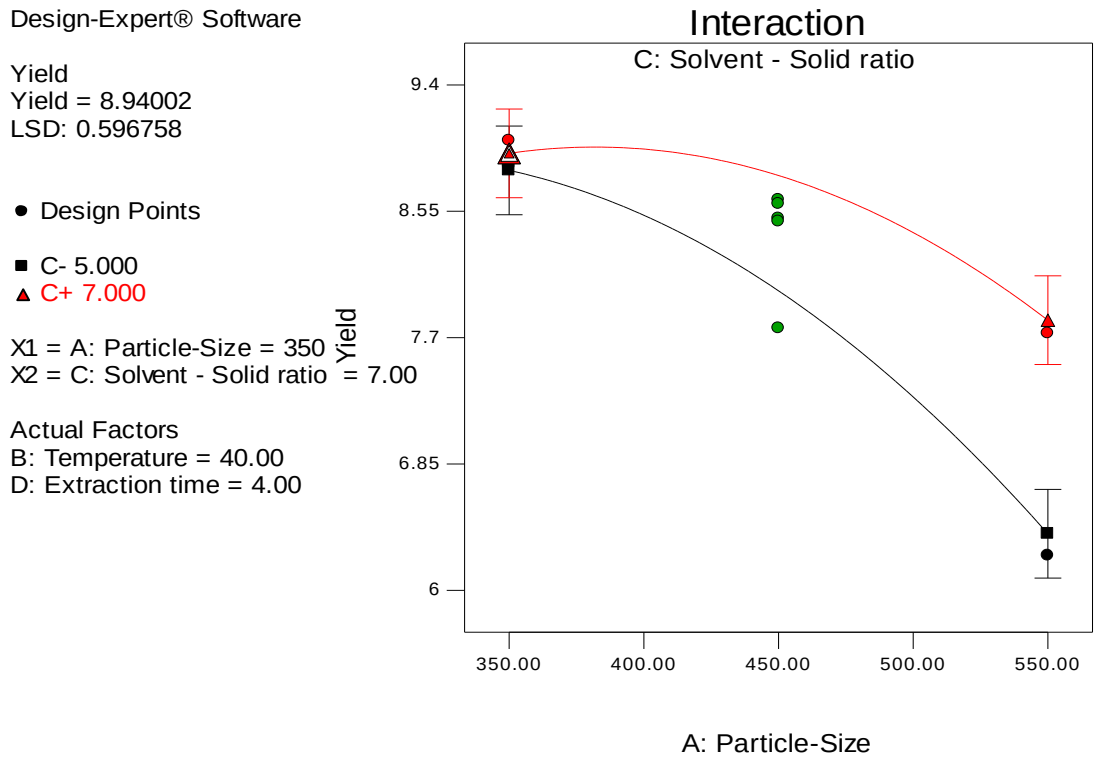


(c)

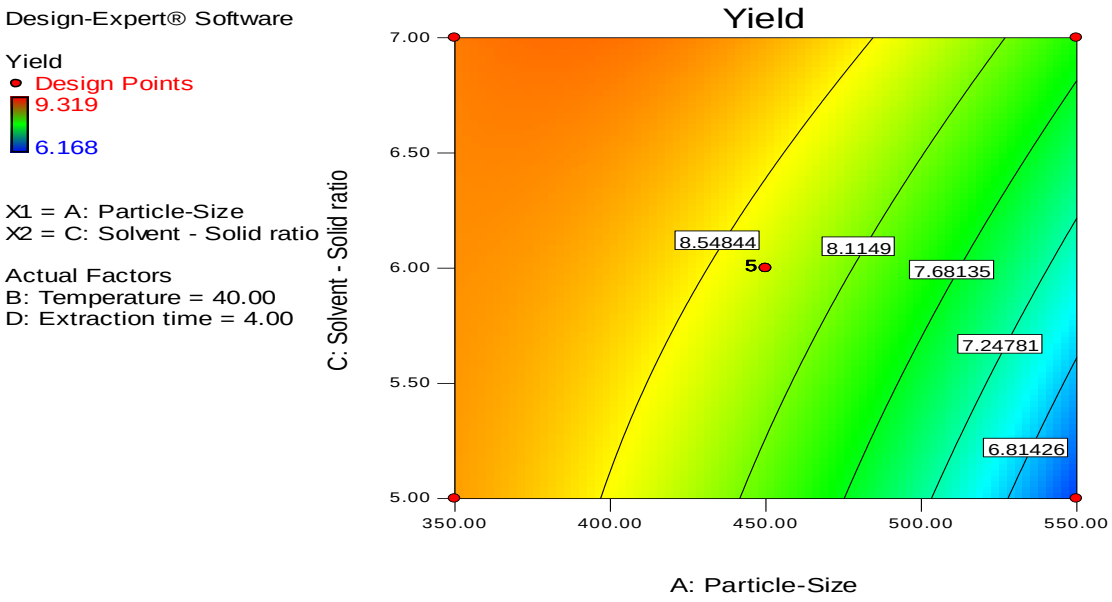
Figure 4-8. Interaction (a) Contour (b) and 3D (c) plots of particle size and extraction temperature

4.6.2. Effect of particle size and solvent to solid ratio on Yield

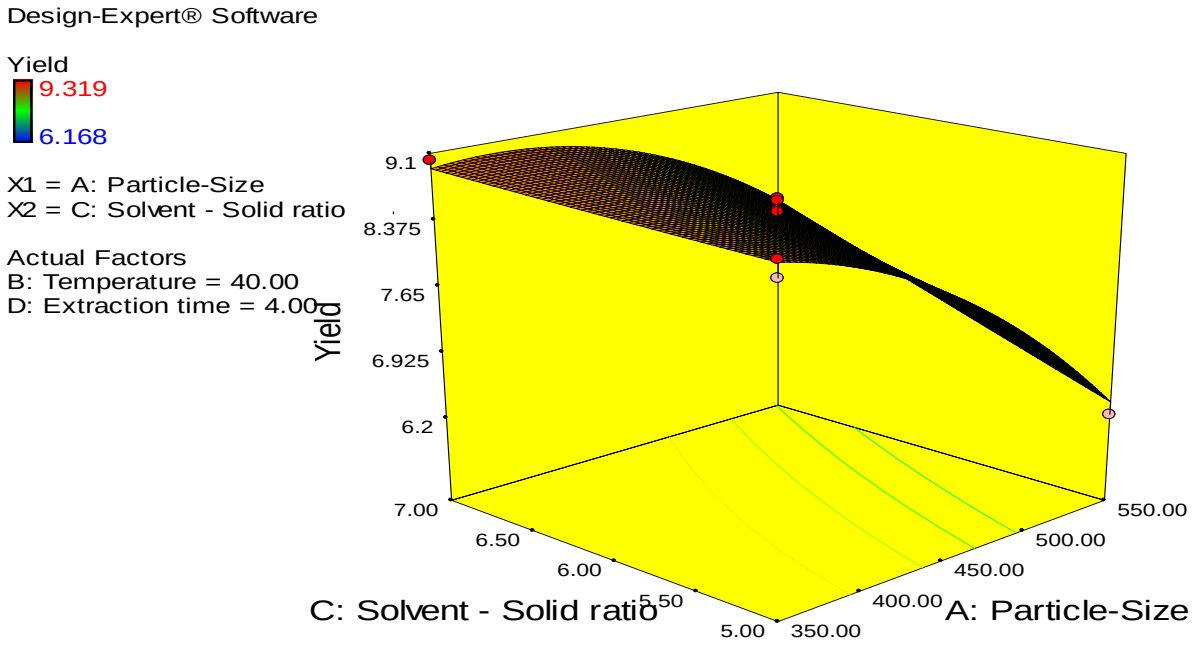
Particle size and solvent to solid ratio affects the yield interactively. This means, a proportionate increment of these factors causeed an increment in the yield simultaneously. The p-value of the interaction was found 0.0224 that is less than 0.05 indicates the interaction effect has effective cooperatively. The figure below entailed the increment in solvent to solid ratio and decrease in particle size to 350µm from550µm the yield was increased simultaneously to 8.94002g a single interactive value. This figured out, both the factors affect the yield simultaneously and finally interacted to a single value of yield.



(a)



(b)

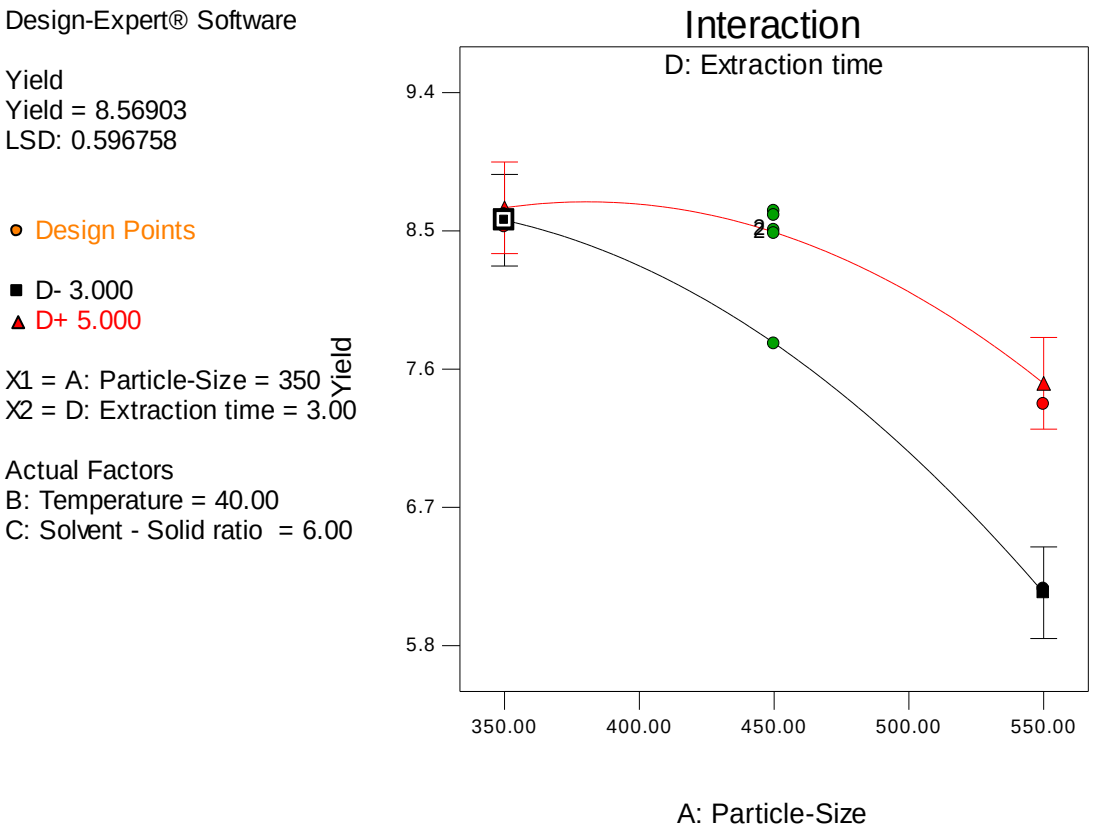


(c)

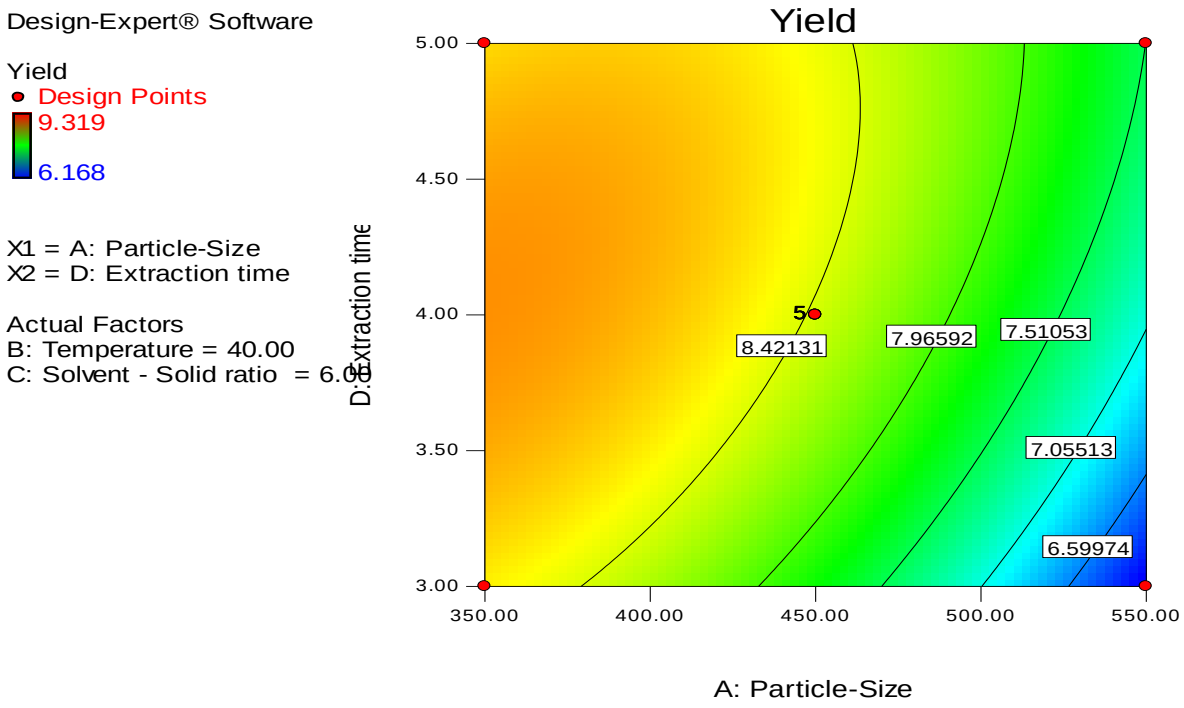
Figure 4-9. Interaction (a) Contour (b) and 3D (c) plots of Particle size and solvent to solid ratio

4.6.3. Effect of particle size and extraction time on Yield

Fundamentally, the plot indicated below shows the interaction of the two factors (particle size and extraction time) was happened when particle size gradually decreased to 350µm from 550µm and extraction time increased to 5hour from 3hour. The result found at these both operational conditions were 8.56903g. The major observable issue here is the interaction of these factors was concurrently; i.e. when extraction increases and particle size decreases the yield was interacted at the same point of interaction.



(a)



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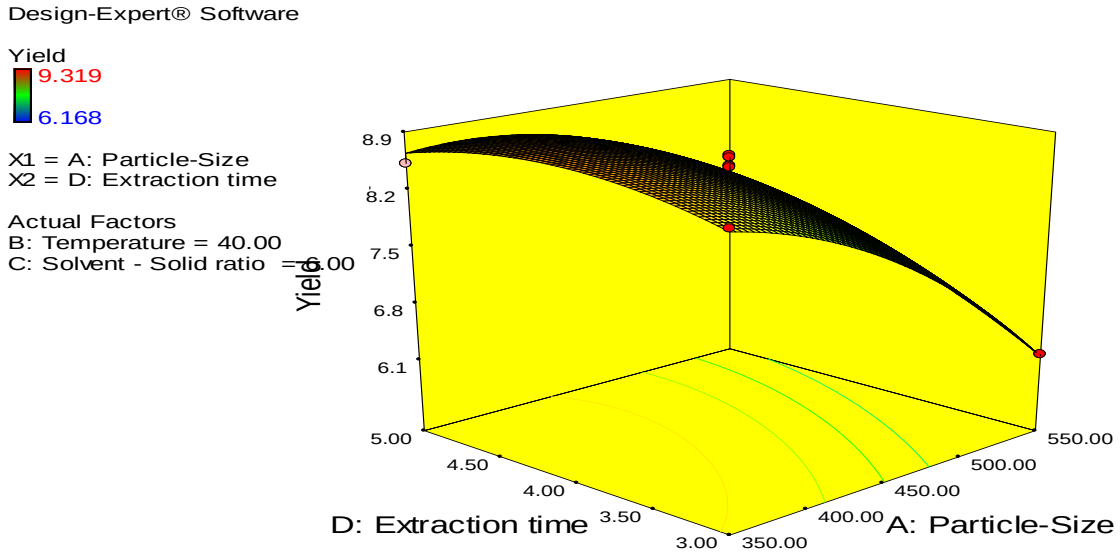
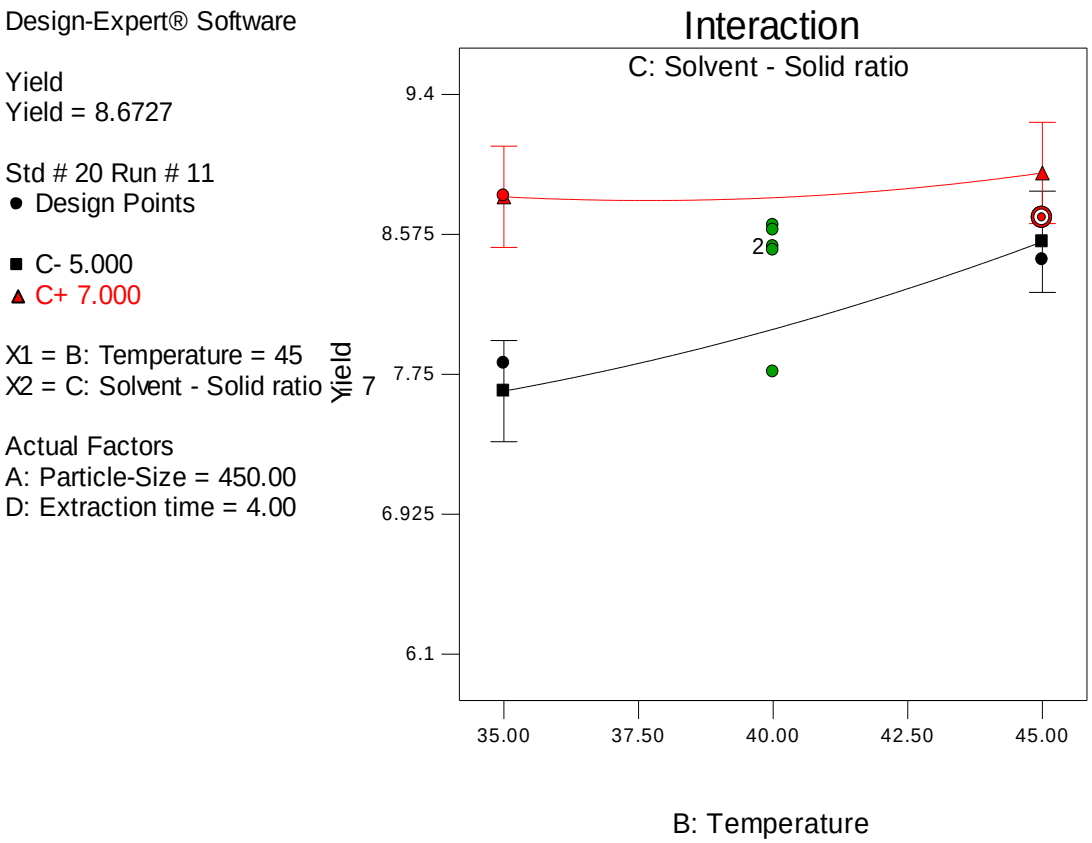


(c)

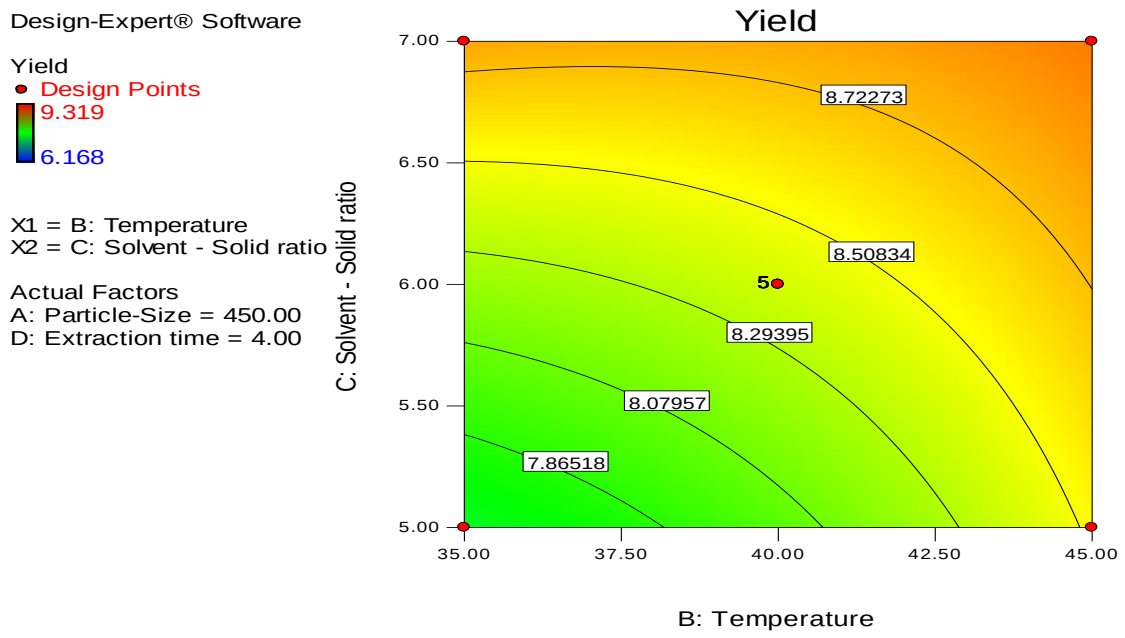
Figure 4-10. Interaction (a) Contour (b) and 3D (c) plots of particle size and extraction time

4.6.4. Effect of temperature and solvent to solid ratio on Yield

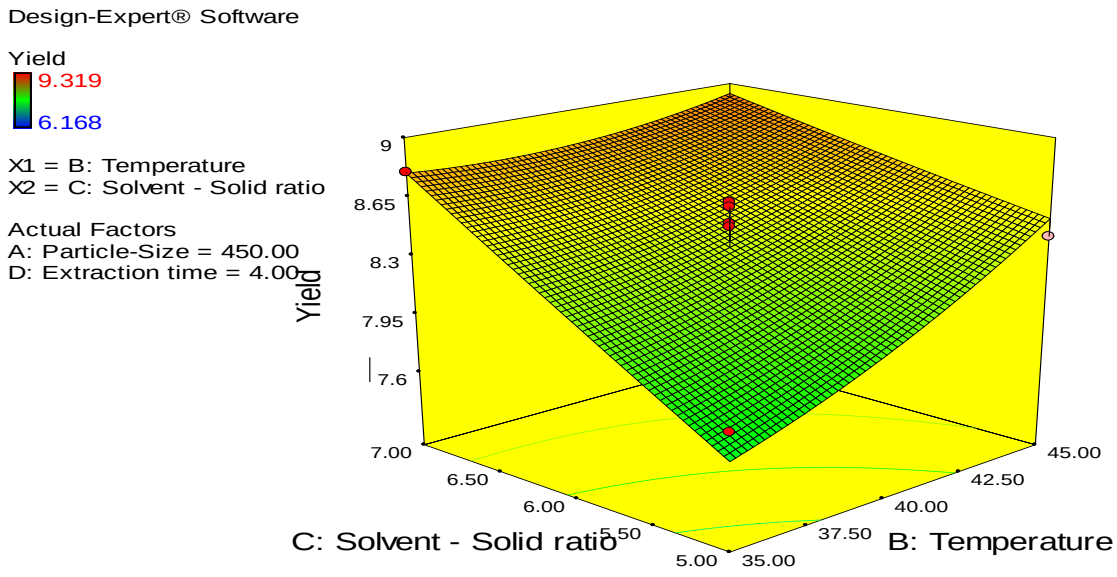
The alpha value of the interaction effect between temperature and solvent to solid ratio was found 0.1731. This figure indicates, it is greater than the value of probability (i.e. 0.05) of the model. What can simply conclude from this figure was the interaction is not validated and nor has interactive effect. In other word, the yield found at 45ºC of extraction temperature was 8.93711g using 7:1v/w of solvent to solid ratio and 8.53125g using 5:1v/w of solvent to solid ratio. Factors should have single yield value so that they could interact each other and affect the response (yield).



(a)



(b)

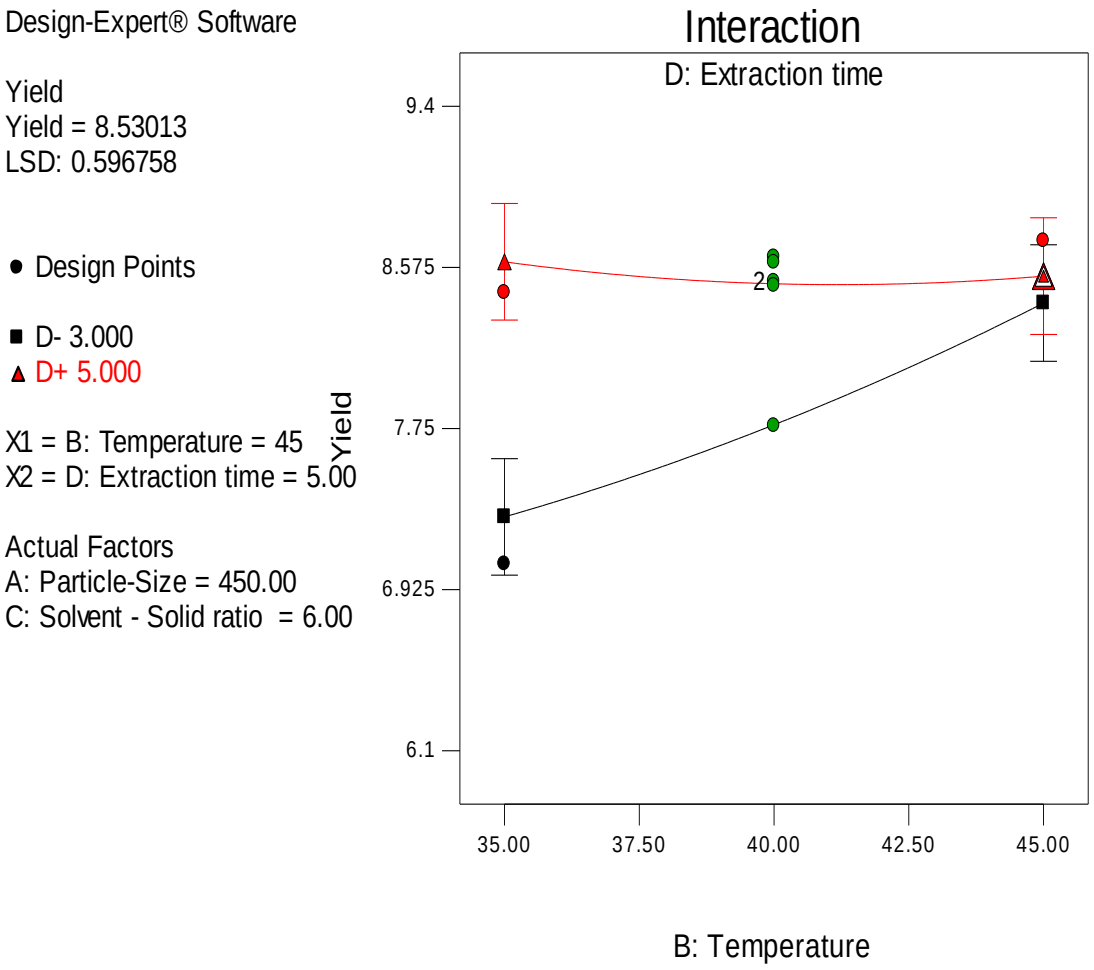


(c)

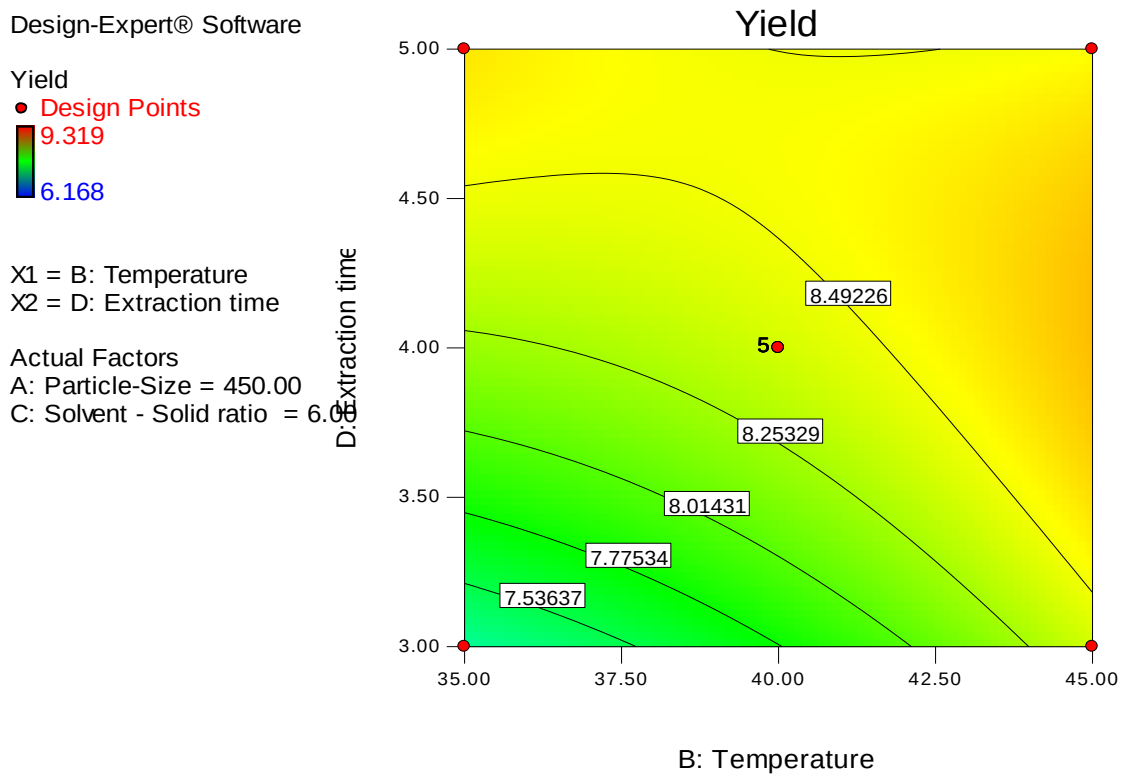
Figure 4-11. Interaction (a) Contour (b) and 3D (c) plots of temperature and solvent to solid ratio

4.6.5. Effect of temperature and extraction time on Yield

From the interaction graph (a) of below, the point of interaction is indicated well at the center of the two lines. This indicates the yield was significantly increased in the time of extraction starting from 3 to 5 hour with a maximum yield of 8.53013g. So, the interactive factor of temperature and extraction time was significantly affecting the yield.



(a)



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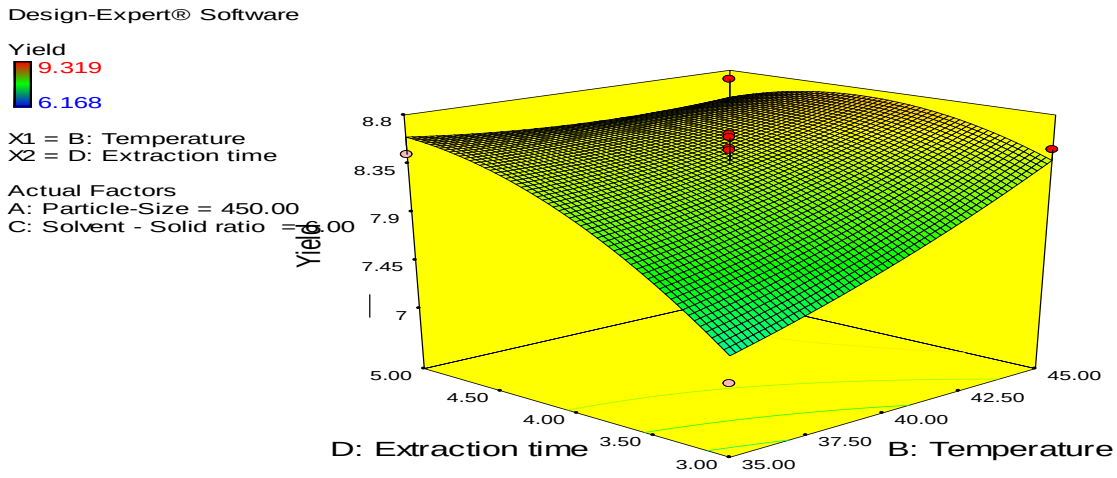
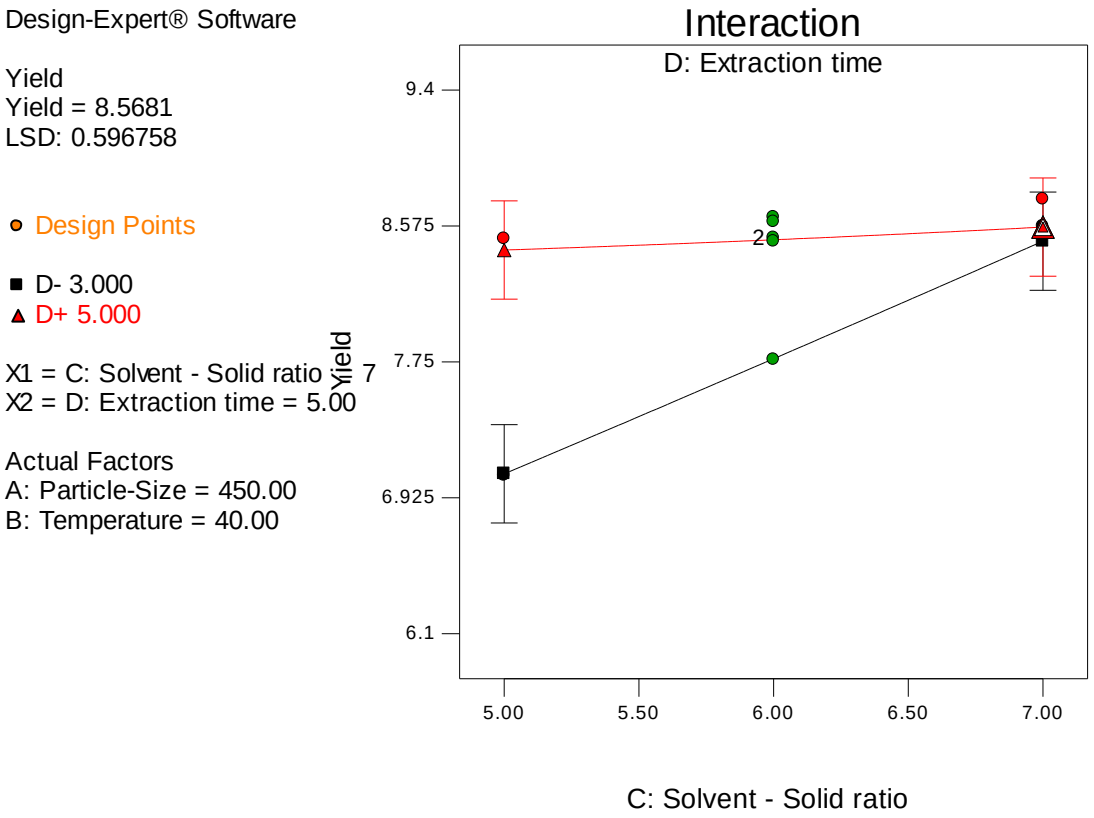


(c)

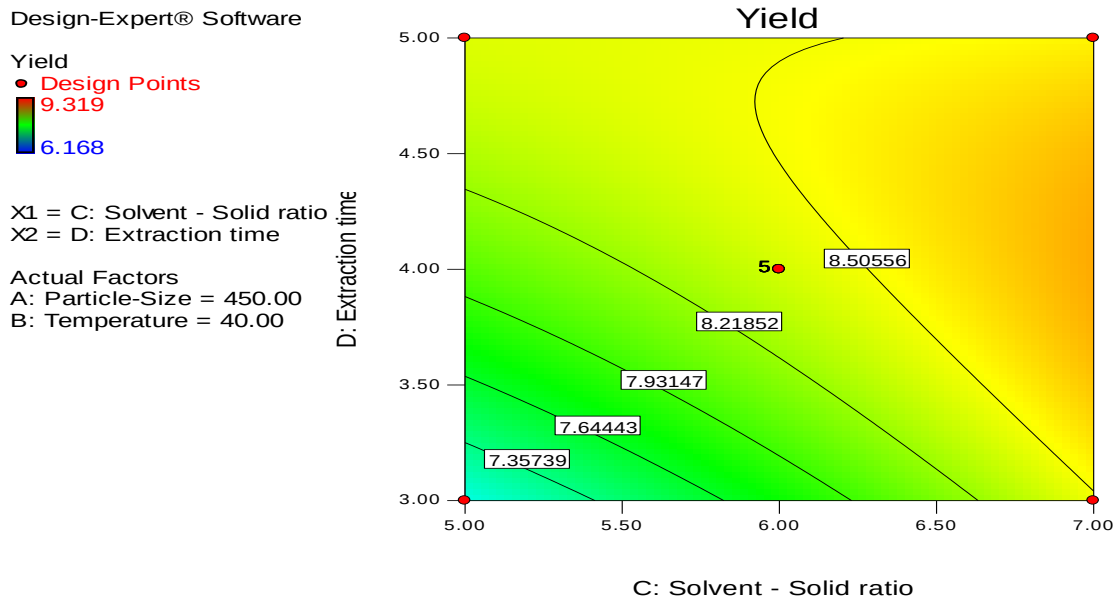
Figure 4-12. Interaction (a) Contour (b) and 3D (c) plots of temperature and extraction time

4.6.6. Effect of solvent to solid ratio and extraction time on Yield

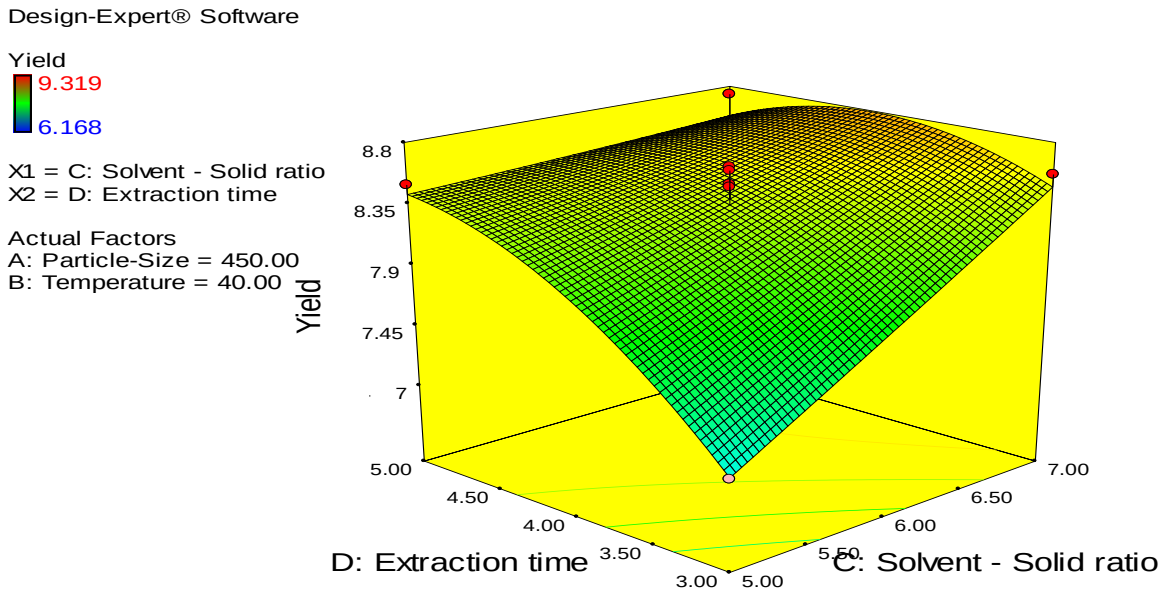
The graph below indicates that there is an interaction between solvent to solid ratio and extraction time. Highest yield was observed at higher solvent to solid ratio and simultaneously at the lower and higher extraction time. What can be simply observed is the amount of solvent makes the difference on yield but there was a visible interaction of both factors. The amount of yield found in these factors operational condition was 8.5681g.



(a)



(b)



(c)

Figure 4-13. Interaction (a) Contour (b) and 3D (c) plots of the solvent to solid ratio and extraction time

4.7. Selection of optimum extraction conditions

Response surface methodology of Box-Behnken method was used for developing, improving, and optimizing processes. It also has important applications in designing, developing, and formulating of new products, as well as to improve existing product designs. The optimization of "azadirachtin extraction from Neem seed for biopesticide application" using this method has found 30 possible Solutions. Selection of an optimum condition is not only considering the extract yield, the software follows the actual extraction path so that material, energy and time become utilized properly. Based on this concept the best solution choosen by the method of numerica optimization was the one with higher desirability (i.e. 100%) efficiency. The operation conditions of the choosen optimized process methode was; 350.08µm, 44.94ºC, 6.11:1v/w and 3.89hr of particle size, extraction temperature, solvent to solid ratio and extraction hour. The optimized solution is summarized as follows:

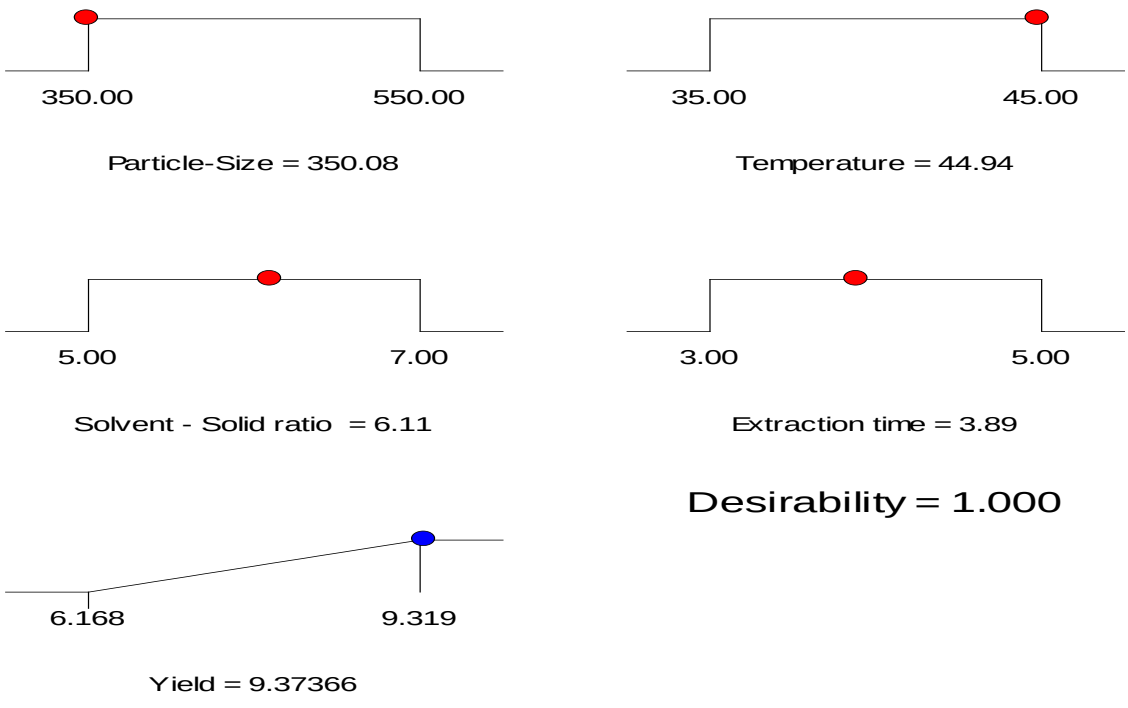


Figure 4-14. Ramp representation of optimal conditions for azadirachtin

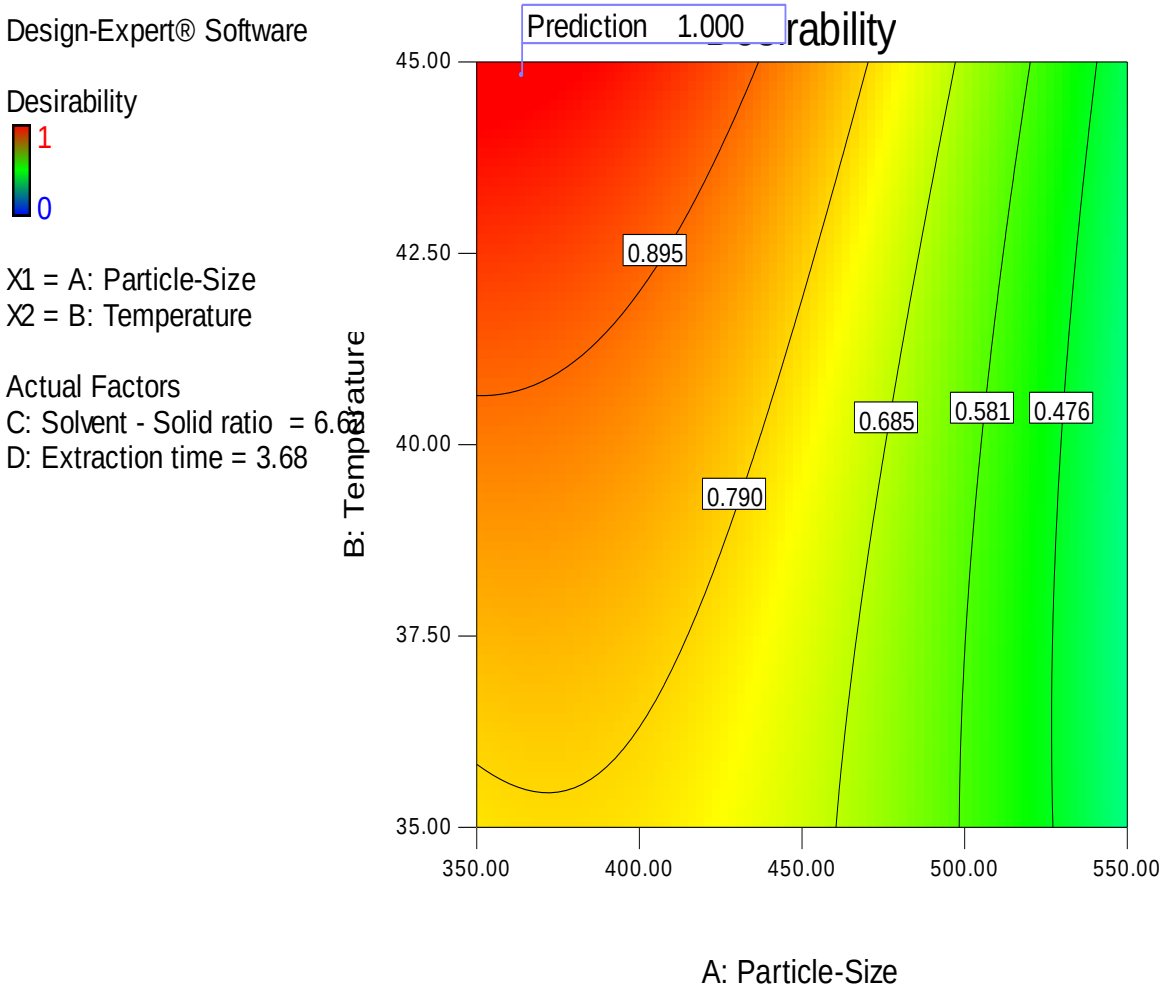


Figure 4-15. Optimized contour plot of Model Desirability

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Neem seed has a promising biopesticide effect in crop production and protection from many pests beside its highly environmental friendly behaviour. Its most abundance, easy applicability and less health hazard effect makes more preferable choice than synthetic pesticides.

Extraction of azadirachtin from Neem seed was carried out in four main stages. These are pretreatment, extraction, separation and concentration. In this study four variables i.e. particle size (350, 450 and 550μm), extraction temperature (35, 40 and 45^{o}C), solvent to solid ratio (5:1, 6:1 and 7:1 v/w) and extraction time (3, 4 and 5 hours) were used as a factor and studied their effect on the yield. Significance checking and yield optimization was implemented using RSM of Box-Behnken method. The result from ANOVA implied, all the individual factors and a combination of particle size - solvent to solid ratio, particle size – extraction time, extraction temperature - extraction time and solvent to solid ratio – extraction time had had a significant effect on the extraction yield of azadirachtin.

Characterization of the extract indicates that, moisture content of the seed was 20.873% (w/w), ash content 3.648 ± 0.187% (w/w), organic matter 96.3515 ± 0.1865% (w/w), TLC *Rf* value 0.7079 ± 0.0068, HPLC retention time of 3.03minute, specific gravity 1.3355 ± 0.0743, flash point 35.05 ± 1.0966^{o}C, water solubility 99.708 ± 0.0069% (w/v), Acute toxicity 2343.0777 ± 60.2106 mg/kg, pH value 4.0533 ± 0.06263 and TVOC of 0.45095 ± 0.3360% (w/w).

An optimal yield of azadirachtin was obtained at 350μm, 45^{o}C, 6:1v/w and 4hr of particle size, extraction temperature, solvent to solid ratio and extraction time respectively it is 9.319g. This means at lower particle size, medium solvent to solid ratio and extraction time and maximum extraction temperature. Depending, on the result of Box-Behnken method an optimized yield of 9.37366g concentration was found at 350.08μm, 45.00^{o}C, 6.11v/w and 3.89 hour from 100gm of ground Neem seed. Based on these facts it can be concluded that, it is evident that the chosen method of extraction and optimization was efficient, and reliable to use azadirachtin for pesticide purpose.

5.2. Recommendations

The major drawbacks to be recommended and requires further study in this area are:

On field test of azadirachtin as biopesticide should be mandatorily carried out so that the effective concentration must be known.

Concentration of azadirachtin must be analyzed using known concentration of azadirachtin (pure) by using calibration curve method using HPLC. The lack of pure azadirachtin in the market limits this study from determining the concentration.

The optimization of the extraction process using Box-Behnken method of experimental design was analyzed by considering a higher constraint factor values. Since, the coefficient of variance was less than 10%, optimization was representing for this design space only. If reproducibility of the model is needed a full factorial experiment with replicate should be undertake to consider as an operational design rather than a space design.

Appendixes - A

Table. A-1. RSM of Box – Behnken method model extraction factors and yield of azadirachtin

Std. Nº	*Run Nº*	*Block*	*Particle size (µm)*	*Extraction temperature (^oC)*	*Solvent – Solid ratio (v/w)*	*Extraction time (hr)*	*Yield (gm)*
1	21	Block 1	450.00	45.00	6.00:1	3.00	8.4905
2	20	Block 1	450.00	45.00	5.00:1	4.00	8.4253
3	9	Block 1	350.00	40.00	6.00:1	5.00	8.5263
4	3	Block 1	350.00	40.00	5.00:1	4.00	8.8534
5	28	Block 1	450.00	40.00	7.00:1	5.00	8.7379
6	12	Block 1	350.00	40.00	7.00:1	4.00	9.026
7	15	Block 1	450.00	40.00	5.00:1	5.00	8.4979
8	5	Block 1	550.00	40.00	6.00:1	5.00	7.37
9	6	Block 1	550.00	35.00	6.00:1	4.00	7.2819
10	1	Block 1	550.00	40.00	7.00:1	4.00	7.7288
11	22	Block 1	450.00	40.00	6.00:1	4.00	8.5031
12	26	Block 1	450.00	35.00	7.00:1	4.00	8.8021
13	13	Block 1	450.00	40.00	6.00:1	4.00	8.628
14	16	Block 1	450.00	40.00	5.00:1	3.00	7.0608
15	7	Block 1	450.00	35.00	5.00:1	4.00	7.8151
16	***10***	***Block 1***	***350.00***	***45.00***	***6.00:1***	***4.00***	***9.319***
17	2	Block 1	450.00	45.00	6.00:1	5.00	8.711
18	19	Block 1	450.00	40.00	7.00:1	3.00	8.5736
19	23	Block 1	450.00	40.00	6.00:1	4.00	8.4824
20	11	Block 1	450.00	45.00	7.00:1	4.00	8.6727
21	4	Block 1	350.00	35.00	6.00:1	4.00	8.5551
22	18	Block 1	550.00	45.00	6.00:1	4.00	7.4011

23	14	Block 1	450.00	35.00	6.00:1	3.00	7.0564
24	25	Block 1	550.00	40.00	5.00:1	4.00	6.2346
25	17	Block 1	450.00	40.00	6.00:1	4.00	8.6005
26	24	Block 1	350.00	40.00	6.00:1	3.00	8.6064
27	8	Block 1	450.00	35.00	6.00:1	5.00	8.4451
28	29	Block 1	550.00	40.00	6.00:1	3.00	6.168
29	27	Block 1	450.00	40.00	6.00:1	4.00	7.7641

Table. A-2. Desirable maximum operation conditions that can be possibly yield 100%

Particle-Size	**Temperature**	**Solvent - Solid ratio**	**Extraction time**	**Yield**	**Desirability**	
350.08	***44.94***	***6.11***	***3.89***	***9.37366***	***1.000***	***Selected***
351.39	44.90	5.05	3.38	9.33319	1.000	
353.53	44.50	5.61	4.00	9.34353	1.000	
352.78	43.81	6.96	3.49	9.34051	1.000	
359.15	44.99	6.26	3.80	9.36698	1.000	
364.07	44.88	5.20	3.79	9.35672	1.000	
370.19	44.44	6.67	3.27	9.34778	1.000	
356.71	44.80	5.04	4.82	9.32061	1.000	
356.16	45.00	5.10	4.54	9.40671	1.000	
359.50	43.99	6.79	3.41	9.34311	1.000	
355.43	44.49	5.65	3.93	9.335	1.000	
361.27	44.44	6.33	3.38	9.32946	1.000	
374.46	44.83	6.90	3.79	9.32199	1.000	
357.25	44.28	6.92	3.53	9.37136	1.000	
357.48	44.66	5.17	4.63	9.32081	1.000	

354.40	44.80	6.90	3.72	9.36938	1.000	
355.38	44.69	6.30	3.82	9.33857	1.000	
360.41	44.82	5.92	3.81	9.35113	1.000	
354.59	44.66	5.22	4.23	9.39417	1.000	
372.30	44.91	5.04	4.32	9.35558	1.000	
355.32	44.38	6.72	3.23	9.39915	1.000	
360.50	44.96	5.75	4.12	9.33856	1.000	
368.35	45.00	7.00	4.10	9.20067	0.962	
383.57	42.13	6.99	3.04	9.10589	0.932	
350.00	44.13	5.00	3.03	9.00998	0.902	
416.26	35.00	7.00	4.42	8.93273	0.877	
420.66	35.00	7.00	4.48	8.93257	0.877	
412.19	35.03	7.00	4.34	8.92992	0.877	
414.67	35.00	7.00	4.64	8.91948	0.873	
462.03	35.00	7.00	4.91	8.86172	0.855	

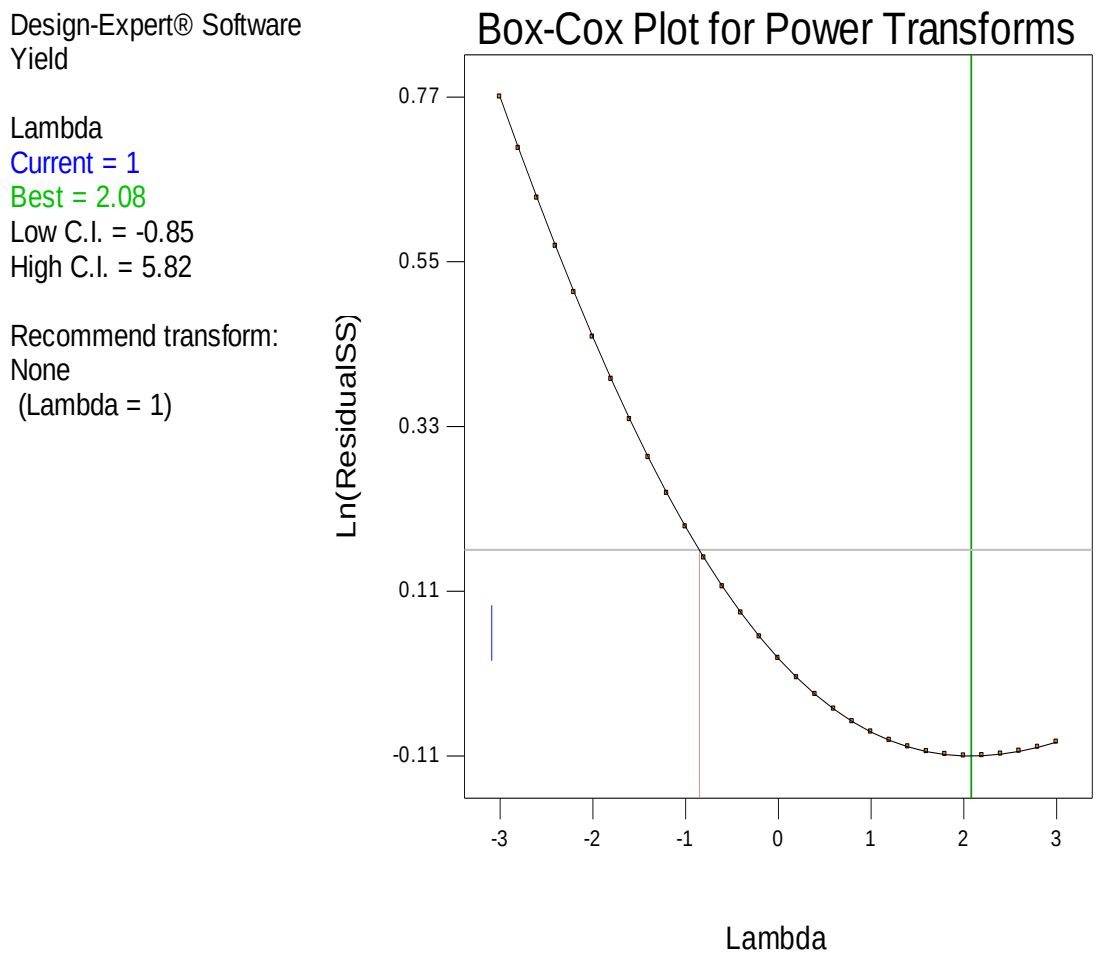


Fig. A – 3. Model power transform plot

N.B. If the ratio of maximum to minimum yield becomes greater than 10 power transforms is required. For ratio, less than 3 power transforms have little effect. The ratio of this model is 1.51086.

Table. A - 4. HPLC Analysis result

Peak	***Start***	***Rt (min)***	***End***	***Height (nm)***	***Area***	***Area %***
1	0	0.321	0.469	5.59	1052.3	6.16
2	1.818	1.845	2.046	80.2	14275	8.3
3	2.976	3.03	3.164	212.28	6875.1	4
4	4.819	4.927	4.967	35.98	2285.65	1.33
5	5.869	5.909	5.963	40.3	2876.6	12.5
6	5.963	5.99	6.017	35.1	2543.77	4.53
7	6.017	6.057	6.219	46.69	2128.6	12.37
8	6.299	6.326	6.421	45.7	2715.37	1.58
9	7.295	7.349	7.425	15.4	1589.6	9.24
10	7.645	7.672	7.699	31.73	2093.18	1.22
11	7.791	7.82	7.879	27.26	4913.74	2.86
12	7.908	7.941	7.968	33.13	3641.34	2.12
13	8.049	8.076	8.103	36	2901.06	1.69
14	8.291	8.358	8.385	138.81	1973.67	1.15
15	8.475	8.52	8.56	150.7	4265.89	2.48
16	8.56	8.627	8.708	213.6	2526.7	14.69
17	8.816	8.843	8.893	194.31	6699.41	3.9
18	8.908	8.991	9.018	86.8	6328.3	3.68
19	9.018	9.045	9.152	98.04	1589.7	9.24
20	9.865	9.906	9.973	37.541	3405	19.8
21	9.973	10	10.161	41.85	5742.26	3.34
22	10.161	10.215	10.266	40.69	2541.62	1.48
23	10.461	11.511	10.565	41.62	4495.27	2.61
24	10.565	12.619	10.644	40.02	3618.9	2.1
25	10.65	12.727	10.819	38.42	4208.59	2.45

26	10.848	13.928	11.036	38.96	26002.3	15.12
27	11.24	14.278	11.406	39.53	12855.9	7.47
28	11.996	15.032	12.072	37.22	1962.14	1.14
29	12.481	16.516	12.55	38.92	1948.15	1.13
30	12.46	17.485	13.542	39.04	2059	1.2

Appendixes – B

Fig. B -1. Drying of Neem seed

Fig. B -2. Size reduction and Sieving

Fig. B – 3. Extraction of Azadirachtin

Fig. B – 4. Separation of supernatant from cake

Fig. B - 5. Solvent recovery and drying of crude azadirachtin

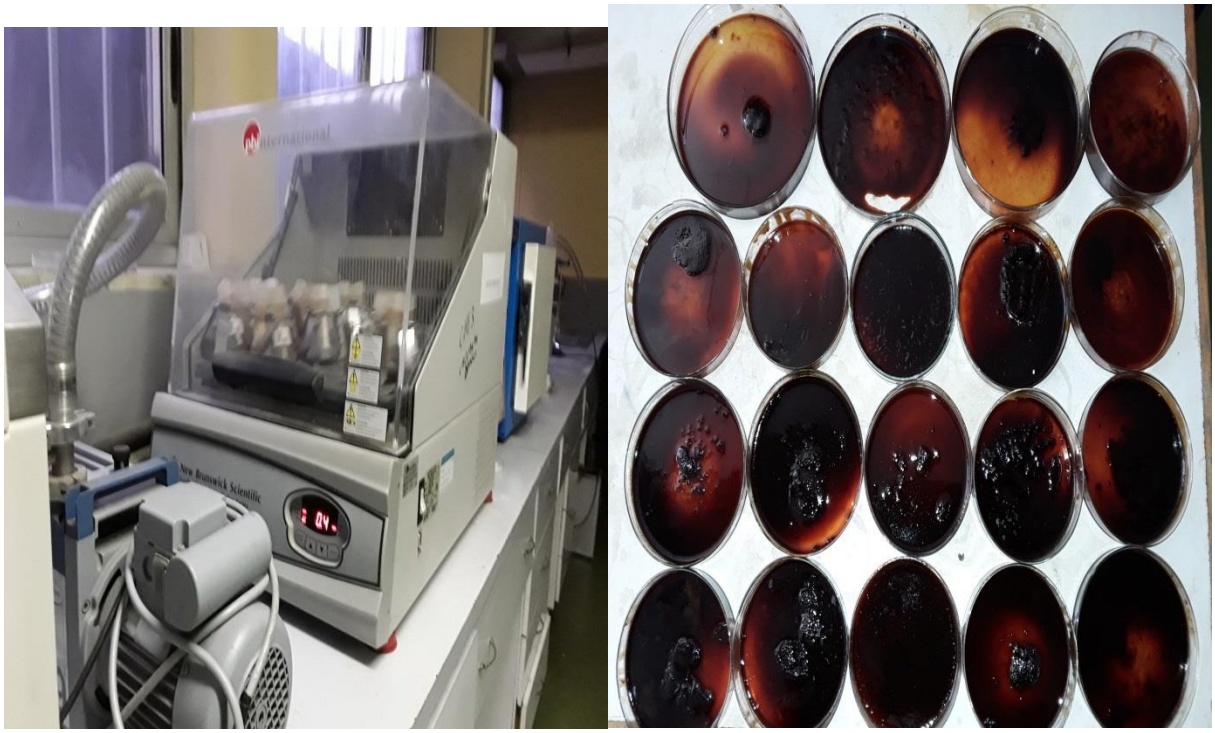


Fig. B -6. Washing with ethylacetate and drying of sample

Fig. B – 7. Deep freeze-drying refrigerator and dried Azadirachtin

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