



ADDIS ABABA UNIVERSITY
ADDIS ABABA INSTITUTE OF TECHNOLOGY
SCHOOL OF CHEMICAL AND BIO ENGINEERING

Extraction and Characterization of Atropine from Jimson Seed (*datura stramonium*) as biopesticide and evaluate its bioactivities against Maize weevil (*Sitophilus Zeamais*)

A Thesis submitted to Addis Ababa Institute of Technology, In Partial Fulfillment of the requirement of degree of Master of Science in Bio-chemical Engineering

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ADDIS ABABA INSTITUTE OF TECHNOLOGY (AAiT)
SCHOOL OF CHEMICAL AND BIO- ENGINEERING

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DECLARATION

I declare that this thesis for the M.Sc. Degree at Addis Ababa University, hereby submitted by me, is my original work and has not previously been submitted for the degree at this or any other university, and that all resources of materials used in this thesis have been duly acknowledged.

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List of acronyms

ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials
Bt.	<i>Bacillus thuringiensis</i>
CO	Carbon monoxide
CV	Coefficient of Variance
DDT	Dichlorodiphenyltrichloroethane
DS	<i>Datura stramonium</i>
EIA	Environmental Impact Assessment
EPA	Environmental Protection Agency
EPID	Ethiopia Pesticide Integration Development
FAO	Food and Agricultural Organization
HPLC	High Performance Liquid Chromatography
ICI	Imperial Chemical Industries
ISO	International Standards Organization
MoA	Ministry of Agriculture
RSPM	Residual Suspended Particulate Matter
RSM	Response Surface Methodology
TEA	Triethylamine
TVOC's	Total Volatile Organic Compound's
TVOCM's	Total Volatile Maters Organic Maters
ULV	Ultra-Low Volume
USD	United State Dollar
UV	Ultra-Violate
WHO	World Health Organization of the united nations

Abstract

The effect of synthetic pesticides on the environment and human health is persistent and very aggressive. A series health problem caused by synthetic pesticides every year in the world to a human being are major treat. The study investigates the extraction of atropine from Jimson seeds for biopesticide application using acid-water extraction method. The parameters used were particle size, extraction temperature, and extraction time. The extraction process stages performed in the experiments are pretreatment, extraction, separation and concentration. The first stage was drying and grinding the raw seed to a particulate size of 550, 750 and 1000 μ m. The raw seed have had 24.241 % (w/w) of moisture content, with ash and organic matter content of 6.607 % and 93.393% (w/w) respectively. Water was prepared in a ratio of 5:1(ml/gm) 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. The characteristics of atropine were then evaluated using different analytical methods. The result found; retention time of sample 5.5-minute, specific gravity of 1.4164 ± 0.07225 , the flash point was $36.06 \pm 0.9932^{\circ}\text{C}$, water solubility was $99.768 \pm 0.0217\%$ (w/v), acidity of atropine was 5.2 ± 0.19 and TVOC's was $0.17426 \pm 0.03\%$ (w/w). Response surface methodology (RSM) of Box – Behnken method was used to justify the factors effect on the yield. The interaction effect of particle size and extraction temperature, particle size and extraction time, and extraction temperature and extraction time had also a significant effect on the yield. Depending on the model optimization design 713 μ m, 45.42 $^{\circ}$ C, and 2.33h of the factors were found the optimum points with 10.811 yield of extract. Finally, the extracted atropine was characterized using UV Spectrophotometer, FTIR, and HPLC. The results obtained from the acid-water extraction method at different parameters confirmed that the yields were maximum and effect of prepared biopesticide is comparable with commercial synthetic pesticide. The result of assessment of the percent of death of maize weevil after 48hrs and 72hrs, the atropine based biopesticide was efficient in small quantity and less time when compared with chemical synthetic. The percentage of death of maize weevil was 41.6% for 48hours and 83.3% for 72hours for atropine based biopesticide.

Keywords: Jimson seed, Atropine, Biopesticide and Maize weevil.

Chapter One

1. Introduction

1.1. Background

FAO defines Pest as any species, strain or biotype of plant, animal, or pathogenic agent, injurious to plants or plant product.¹ Any animal, plant or pathogen, which causes damage annoyances to man, animals, crops or possessions is known to us pest ². Globally losses of food production due to diseases, insects and weeds are estimated to \$220 billion³

Pesticides is a substance or mixture of substances intended to prevent, destroy, repel, or to mitigate any pest including unwelcome species of plants or animals; during production and/or storage, transportation, distribution and elaboration of food ⁴. Pesticides are classified predominantly in to two ways according to the source of origin synthetic (Chemical) or natural (bio-pesticide). In addition pesticides are classified based on application as insecticides, herbicides, fungicides, rodenticide, fumigants, insect replant and so on^{5 6 7}.

Over the next 20 years, crop production will have to increase significantly to meet the needs of a rising human population⁸. Over than 1.6 billion hectares land are used as cropland for production of crop to feed the population⁹. The herbicides, insecticides, rodenticide and fungicides are the widely used chemical pesticides in the world as well as in Ethiopia to produce this amount of crop.

Besides, its simplicity in use pesticides was harmful to humans and have impacts on the biosphere as a whole. When chemical pesticide entered to the environment it can cause serious pollution in both ground and surface water and destroying soil dwelling pests, nematodes, and the pathogens of bacterial and fungal disease ^{10 11}. Effect of pesticide on a human can be in either acute effects, which appear immediately or very soon after exposure, and/or chronic effects, which may manifest themselves many years later and whose origins are often difficult to trace ¹⁰. Pesticides also harm non-target species.

Biopesticides are defined as a mass-produced agent manufactured from a living microorganism or a natural product and used as crop protection tools. According to the active substance biopesticide are may be microorganisms, biochemicals or Plant-Incorporated-Protectants. Biopesticides are

selective, produce little or no toxic residues^{12 13}. Most biopesticides are not as efficacious as conventional chemical pesticides, they are not suited for use as stand-alone treatments. This is due to their shorter persistence in the environment and susceptibility to unfavorable environmental conditions and they are highly selective. However, their selectivity and safety mean that they can contribute meaningfully to incremental improvements in pest control¹⁴. Biopesticide products represent just 2.5% of the total pesticide market¹⁵. Biopesticides sector currently to have a five-year compound annual growth rate of 16% (compared to 3% for synthetic pesticides) that is expected to produce a global market of \$10 billion by 2017¹⁶.

Efficacy of plant extracts may in some cases be dependent on extraction efficiency¹⁷. Maceration involved soaking plant materials (coarse or powdered) in a stoppered container with a solvent and allowed to stand at room temperature for a period of minimum 3 days with frequent agitation. Infusion and decoction use the same principle as maceration; both are soaked in cold or boiled water. Those technique is the easiest and simple method resulted in highest extraction yield. However, organic waste come into an issue as large volume of solvents is used and proper management of the waste is needed. Soxhlet extraction requires a smaller quantity of solvent compared to maceration. However, it has disadvantage such as exposure to hazardous and flammable liquid organic solvents, with potential toxic emissions during extraction. Microwave assisted extraction utilizes microwave energy to facilitate partition of analytes from the sample matrix into the solvent by disrupts hydrogen bonding. Ultrasound-assisted extraction or sonication extraction, accelerated solvent extraction and Supercritical fluid extraction are the most efficient extraction methods and most promising technology in future. They are selected mainly due reduction in extraction time and solvent consumption. But cost for the equipment, energy needed, and limited to small molecule are their major drawbacks¹⁸. Water or inorganic acidic extraction is used, so that the organic acid of alkaloids salt is replaced inorganic acid salt, and increasing its solubility. Polar solvents are more effective in the extraction of bioactive molecules¹⁸. This method was simplest and suitable because it gives lower organic waste.

Jimson, *Datura stramonium*, is belongs to family Solanaceae. The seed was available in most regional parts of Ethiopia. The raise of concept of “weeds compete with crop plants and develop resistance to herbicides” makes jimson best choice for biopesticide production¹⁹. The mode of

action of biopesticide from Atropine were exactly the same to organophosphate pesticide. So, it was the perfect replacement of this chemical pesticide by biopesticide²⁰.

Therefore, the present study was focused on the extraction of Atropine from the Jimson seed for biopesticide case using the water or inorganic acidic extraction and test its bioactivity on maize weevil.

1.2. Statement of the problem

The global pesticide usage has been increased to 3.5 million tonnes and worth around 12 billion USD in the year 2020²¹. According to WHO report of 2008, 25% of the world production of synthetic pesticide applied in developing countries. Because of synthetic pesticide poisoning up to 20,000 people will die in the third world countries each year²². Synthetic pesticides have caused series adverse health and environmental effects and pest develop resistance to pesticides.

In Ethiopia 4,128.10 tons of pesticide is used Annually. This figure puts Ethiopia 57th in the global Rank. 0.2354 Kg/Ha pesticide is applied in field area of farming⁹. In this regard, over 4000 tons of various types of pesticides that are worth more than 20 million USD were imported annually.

Comparing to chemical pesticides bio-pesticides are effective in smaller quantities, decompose quickly and do not cause environmental problems. Jimson based pesticides has fewer prone to pest resistance applicably.

In Ethiopia more than 31.5 million quintals maize produced from 1.994 million hectares of land in 2017. Losses due to post-harvest insect pest of maize specially the maize weevil is estimated to 16.6% of total production within three to six months of storage²³. In Eastern and Southern Africa alone, post-harvest losses are valued at US \$1.6 billion per year, or about 13.5% of total value of grain production²³.

Even if there is some others biopesticide like azadirachtin they are not used for treatment of reduction of soil acidity due to chemical pesticide. But atropine is used for reduction of these soil acidity parallel to biopesticides. So, availability, health precaution and clean economy principle are the reasons practically lean towards to have an optional and preferable pesticide in enriching the soil with organic matter and lowering nitrogen losses by inhibiting nitrification.

1.3. Research question

The following research questions guide this thesis works.

- What are the optimum operating conditions for extraction process?
- What are the physicochemical properties of atropine?
- What are bioactivities of atropine against Maize Weevil (*Sitophilus Granaries*)?

1.4. Objective

1.4.1. General objective

The general objective of this study was to extract the active ingredient atropine from jimson seed (*Datura stramonium*) for biopesticide purpose and assess its bioactivities against Maize Weevil (*Sitophilus Granaries*).

1.4.2. Specific objectives

The specific objectives of this study were:

- To study the optimum operating conditions for extraction process.
- To characterize the physicochemical properties of atropine.
- To evaluate bioactivities of atropine against Maize Weevil (*Sitophilus Granaries*).

1.5. Scope of the study

This thesis study focuses on the extraction of atropine from jimson seed for biopesticide purpose within certain parameters; particle size (550 μ m, 750 μ m and 1000 μ m), extraction temperature (40°C, 45°C and 50°C), extraction time (1.5hr, 2hr and 3hr) respectively. The already extracted active ingredient or atropine should be characterized by UV-Spectro photo meter analysis, HPLC, FTIR, Specific gravity, water solubility, Toxicity test (Acute), pH value, and Total volatile organic compound's test of it so that; to correspond 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 $\alpha = 0.05$.

1.6. Significance of the study

Over 4000 tons of several types of pesticides that are worth more than USD 20 million are imported annually ²⁴. In the future it is noticeable to predict that this amount should be parallel to grow with demand and expense. This shows that the import of huge amount of pesticide was costly. It also has several impacts on the human and environmental. So biopesticide were the best solution to those problems.

The cake produced from the extraction of bio-pesticide is used as bio-fertilizer. In addition, Jimson seed pesticide less prone to pest resistance applicably. Atropine officially used for formulations of agricultural specialties, textile, domestic, veterinary pest management. Jimson seed cake (residue of Jimson 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, generating health hazards due to the presence of synthetic pesticide residues in food and fiber are also major problems. Such problems are not expected from biopesticides. 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.

Those reasons listed above are the major driving forces that suggests the need for bio-pesticide. Mostly, Ethiopia is a peasants' country that with it's about 75% of its total population depends on agriculture and agricultural product income. So, the need for bio-pesticide is unquestionable.

Chapter Two

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 petroleum²⁵. 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 as any insect in the wrong place²⁶. Generally, any organism that spreads disease, causes devastation or creating an annoyance is a pest. Pests like mosquitoes, rodents, and weeds are some examples that are being harmful to human. Not all insects are pests. There are 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 annually²⁷. 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 problem²⁸.

The challenge that has been always facing mankind is a sustaining of a food. A major basis in this challenge is the competition from insect pests. Mostly 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.²⁹

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 production²⁹. 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 is a substance or mixture of substances intended to prevent, destroy, repel, or to mitigate any pest including unwelcome species of plants or animals; during production and/or storage, transportation, distribution and elaboration of food; agricultural products or food for animals; or that may be administered to animals to fight ectoparasites⁴. 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. A pesticide is a toxic chemical substance or a mixture of substances or biological agents that are intentionally released into the environment in order to avert, deter, control and/or kill and destroy populations of insects, weeds, rodents, fungi or other harmful pests³⁰. Pesticides are substance or mixture of substance which differ in their physical, chemical and identical properties from one to other.

Presently, three most popular classifications of pesticides which are widely used is classification based on the mode of entry, pesticide function and the pest organism they kill, the chemical composition of the pesticide. Pesticides are classified predominantly in to two ways according to the source of origin synthetic (Chemical) or natural (bio-pesticide). According to the direct type of pest they can control they can be named as: ³¹ algicides to control algae, antifouling agents to kill or repel organisms that attach to underwater surfaces, antimicrobials to kill microorganisms (such as bacteria and viruses). attractants to attract pests (for example, to lure an insect or rodent to a trap), (However, food is not considered a pesticide when used as an attractant.), molluscicides to kill snails and slugs, nematicides to kill nematodes (microscopic, worm-like organisms that feed on plant roots), ovicides to kill eggs of insects and mites, pheromones to disrupt the mating behavior of insects, repellents to repel pests, including insects (such as mosquitoes) and birds, rodenticides to control mice and other rodents.

Based on toxicity of pesticides, WHO classified them into four classes: extremely dangerous, highly dangerous, moderately dangerous and slightly dangerous^{30 6}.

Table 2: 1- Classification of pesticides Application based

Insecticides	Herbicides	Fungicides	Rodenticide	Fumigants	Insect replant
Pyrethroids	Bipyridyls	Thiocarbamates	Warfaienes	Aluminum &	Diethyltoluami de
Organophosphorus	Chlorophenyl	Dithiocarbamates	Indandione	Zink phosphide	
Carbamates	Glyphosate	Cupric salts		Methyl bromide	
Organochlorines	Acetanilide	Thiabendazoles		Ethylene dibromide	
Manganese compounds	Triazines	Triazoles			
		Dicarboximides			
		Dinitrophenols			
		Organotin compounds			

Source: WHO classification of pesticide press. 2010;(11500775):21-22

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 are⁴:

2.2.1.1. Organophosphate pesticides

Organophosphate pesticides are considered to be one of the broad-spectrum pesticides which control wide range of pests due to their multiple functions. They are characterized with stomach poison, contact poison and fumigant poison leading to nerve poisons. These pesticides are also biodegradable, cause minimum environmental pollution and are slow pest resistance³². An organophosphate pesticide affects the nervous system of the host by disrupting the enzyme that regulates acetylcholine, a neurotransmitter. Most organophosphates are insecticides³³. They were developed during the early 19th century, but their effects on insects, which are alike to their effects on humans, were discovered in 1932. Few are very poisonous (they were used in World War II as

nerve agents). Though, they usually are not persistent in the environment. Example malathion is the commonly known Organophosphate Pesticides.

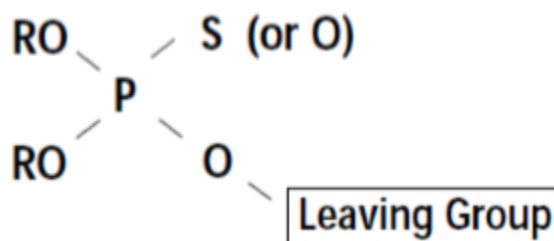


Figure 2: 1- General chemical composition of Organophosphate Pesticides

2.2.1.2. Carbamate pesticides

Carbamates are similar to organophosphates. However, they differ in their origin. Organophosphates are derivatives of phosphoric acid, while carbamates derived from carbamic acid. The working principal of carbamate pesticides is similar to organophosphate pesticides by affecting the transmission of nerve signals resulting in the death of the pest by poisoning. Sometimes, they are also used as stomach and contact poisons as well as fumigant. They can be easily degraded under natural environment with minimum environmental pollution. Some of the widely used insecticides under this group include carbaryl, carbofuran, propoxur⁷.

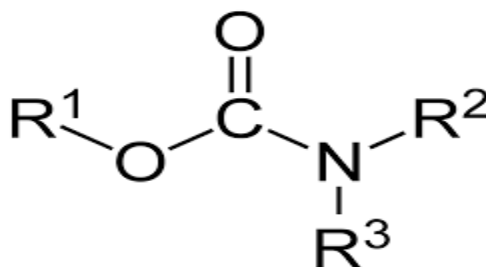


Figure 2: 2- The structural formula of Carbamate Pesticides

2.2.1.3. Organochlorine pesticide

Organochlorine's pesticides (also known as chlorinated hydrocarbons) are organic compounds attached with five or more chlorine atoms. They represent the one of the first group of pesticides ever synthesized and used in agriculture and in public health. Most of them were widely used as

insecticides for the control of a wide range of insects, and they have a long-term residual effect in the environment. Organochlorines may disrupt the nervous system of the insects leading to convulsions and paralysis followed by eventual death.

Organochlorines' commonly used in the past recent 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)³⁴.

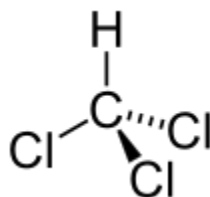


Figure 2: 3- Structural formula of organochlorine pesticide

2.2.1.4. Pyrethroid pesticides

Synthetic pyrethroid pesticides are group of organic pesticide that can be synthesized by duplicating the structure of natural pyrethrin's. Relatively, they are more stable with longer residual effects than natural pyrethrin's and 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.

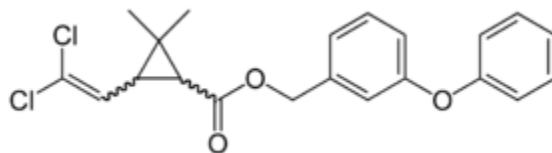


Figure 2: 4- Structural formula of pyrethroid pesticides

2.2.2. Bio - pesticides

According to **FAO** definition, a bio-pesticide is a compound that kills organisms by feature of specific biological properties rather than as a broader chemical poison. The motivation behind replacing conventional or synthetic pesticides with bio-pesticides is that the bio-pesticides are

more selective and biodegradable. Bio-pesticides are derived from natural materials like animals, plants, bacteria, and certain minerals. For example, garlic, mint, Neem, Jimson, 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
- (iii) Bio - chemical pesticides.

2.2.2.1. Microbial pesticides

These consists a microorganism (e.g., a bacterium, fungus, virus or protozoan) as the active ingredient and 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**. 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 larvae³⁵.

2.2.2.2. Plant-incorporated-protectants

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.³⁶ Again, bioactive pesticide ingredients can be extracted from plants and used again to destroy or protect pests.

2.2.2.3. 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 repels³⁷.

2.3. History of pesticide in Ethiopia

Pesticides in agricultural sector were introduced in Ethiopia in the 19640s. Different types of pesticides were imported by both private and public companies for agricultural uses. Since then, the use of pesticide has increased rapidly for crop protection. Recently, Ethiopia has been considered as having the largest accumulations of obsolete pesticides in Africa. It is estimated that there were 1,500 tons of obsolete pesticides³⁸.

Agricultural inputs were introduced to the small holder farmers. In 1972 Regulatory body that specializes in pesticide assessment was established under MoA³¹.

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 2010 E.C was reached about 4200 ton. 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.³⁹

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. Commercial farmers, as the main users of pesticides, account for the use of about 80% of the pesticides imported into Ethiopia. The remaining 20% of the total import is used for small-scale farming, household, health and industrial purposes. Of the total of 4125 metric tons of active ingredients that were used in Ethiopia in 2010, the largest proportion (75%) were herbicides, followed by insecticides (15%) and fungicides (9%).³⁹

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 participated 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 Adami Tulu. The plant has a capacity to formulate 1500 tons of dust and the same quantity liquid formulations every year. Major pesticide formulated include, Malathion, (Ethiolathion 5% Dust and Ethiolathion 50% EC), Endosulfan (Ethiosulfan 25% ULV), Diazinon (Ethiozinone 60% EC), and Fenitrothion (Ethiothrothion 50% EC). The plant imports active ingredients and solvents from foreign countries, mostly from Italy and Israel such as DDT and Pyrethroids (permethrin and deltamethrin) are imported for mosquito net impregnation and are also used for tsetse fly.

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.

2.5.1. Impacts of pesticide on water

Synthetic pesticide residues in water can cause serious pollution in both ground and surface water this may cause for¹⁰: 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 and 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).

2.5.2. Impacts of pesticide on the air

The following table shows some synthetic pesticides related air pollutants and their effects on human and animal health¹⁰.

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

2.5.3. Impacts of pesticide on the soil

Pesticides are presented into the soil and destroying soil dwelling pests, nematodes, and the pathogens of bacterial and fungal disease^{10 11}. 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.

2.5.4. Impacts of pesticide on human health

Use of pesticides creates significant health impacts in all parts of the World. Pesticides effect can be decided broadly into two categories¹⁰: These are, 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 fatal⁴⁰. 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 and diabetes and others.

2.5.5. Impact of pesticide on wild life and non-target species

Pesticides also harm non-target species, and resulted in¹⁰: population decline through the use of pesticides over large areas, reproductive effect such as egg shell thinning, deformity and birth defects, metabolic changes and tumors and cancer. It causes behavioral changes like 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 and toxicity to bees which are pollinators, with adverse effects on the production of certain crops.

Generally, a synthetic pesticide has a short-term benefit on socio-economically and environmentally but with a long-term side effect for our country as well as the world.

2.6. Characteristics of jimson plant

Datura stramonium is mostly known as Angel's trumpet, Locoweed, Jimson weed or *Datura*, it belongs to family Solanaceae.^{41,42}. It grows to a height of 2-4 feet and commonly reaching a diameter of 4-6 feet and they are pubescent (young) and branched plant. The flowers are large and corollas are 6 cm long. The fruit is a large, four ovaled ovate capsule, very thorny and contains numerous blacks to dark brown seeds. The stems are simple, stout and mostly erect. The leaves are large, approximately 20 cm long and oval like with a wavy and coarsely dentate margin. The root is long, thick and tapering and somewhat branched. The species of *Datura* can be found throughout the world. The plant grows in sandy flats, plains, areas up to 2,500 feet above sea level. The origin of *Datura Stramonium* is disputed. *Datura's* habitation of most temperate and Subtropical parts of the world. It is generally grown in April – October⁴³.



(A)

(B)



(C)

(D)

Figure 2: 5- Jimson plant (A) and Jimson seed (B and C) and Jimson plant flower (D)

2.6.1. Chemical constituents of *Datura stramonium*

Plant contains 0.2- 0.6% alkaloids. The main alkaloids are hyoscyamine and hyoscyne (scopolamine). It also contains protein albumin and atropine. Atropine is formed from hyoscyamine by racemization. These alkaloids are usually present in the proportion of about two parts of hyoscyamine to one part of hyoscyne, but in young plants hyoscyne is predominant alkaloid.

The concentration of total alkaloids in the leaves of *Datura stramonium* is 0.2– 0.5%, hyoscyamine being the major compound and scopolamine (hyoscine), apo atropine, tropine, belladonna and hyoscyamine N-oxide minor compounds. Hyoscyamine, atropine and scopolamine are anticholinergics, specifically antimuscarinics.⁴⁴ Atropine is more commonly prepared and used. The seed contains about 17% of a pale-yellow oil.

Table 2: 3- The percentage of various chemical of jimson seed ⁴⁵

Entry	Constituent	(%) of unpurified Jimson seed
1	Nitrogen	3.1
2	Water	7.7
3	Fat	18.1
4	Fiber	17.8
5	Ash	6.6
6	Carbohydrate	31.9
7	Starch	1.1
8	Sugar	2.1
9	Reducing sugar	0.3
10	Glucose	0.13

Source: Friedman, Mendel, Levin, Carol E Composition of Jimson Weed (*Datura stramonium*) Seeds

2.6.2. The main alkaloids found in *Datura stramonium*

More than 70 alkaloids have been identified in the various parts of the plant. Biosynthetically, the main compounds all belong to the tropane alkaloids and are derived from the amino acid ornithine. The toxins in *Datura* are tropane belladonna alkaloids, which consists of strong anticholinergic effects. These alkaloids include: hyoscine (roots); atropine (d, hyoscyamine) hyoscyamine (leaves, roots, seeds), and scopolamine (l-hyoscine), as well as protein and sitosterol. It is reported that hyoscyamine is the predominant alkaloid in DS. Thorn apple leaves contains 0.2%-0.45% of total alkaloids, seeds approximately 0.2%. The tropane alkaloid contains a methylated nitrogen atom (N-CH₃) and include the anticholinergic drugs, atropine, hyoscyamine, and scopolamine as well as the narcotic cocaine.⁴¹

Table 2: 4- List of Compounds Extracted from *Datura stramonium*⁴²

Entry	Compound	sources	Entry	Compound	sources
1	3-acetoxytropane	R, ST	15	3-phenylacetoxy-6,7-epoxytropane	R, ST, L, P
2	3-acetoxy-6-isobutyryloxytropane	R, ST, L, P	16	3-(2'-phenylpropionyloxy) tropane	L
3	3 α -apotropoyloxytropane	R, L, ST	17	3-phenylacetoxy-6-hydroxytropane	R, ST
4	3 β -apotropoyloxytropane	S	18	3 α -phenylacetoxytropane	S
5	Aponorscopolamine	ST, L, P	19	3 β -phenylacetoxytropane	S
6	Apo scopolamine	R, ST, L, P, S	20	Scopoline	S, L, ST, P
7	3-(3-acetoxytropoyloxy) tropane	R, ST, L	21	Scopine	L, P, S
8	3-acetoxy-6-hydroxytropane	R	22	Scopolamine	R, ST, L, P
9	3,6-diacetoxytropane	R	23	3-substituted-6,7-epoxytropanes	R, P, ST, S
10	3,7-dihydroxy-6-tigloyloxytropane	S	24	Tropinone	S
11	3 α ,6 β -ditigloyloxytropane	R, ST	25	3 α -tigloyloxytropane	ST, R
12	3 β ,6 β -ditigloyloxytropane	R	26	3 β -tigloyloxytropane	R
13	3 α ,6 β -ditigloyloxy-7-hydroxytropane	R, ST	27	3 α -tigloyloxy-6-hydroxytropane	R, ST
14	3 β ,6 β -ditigloyloxy-7-hydroxytropane	R	28	3 β -tigloyloxy-6-hydroxytropane	R

Extraction and Characterization of Atropine from Jimson Seed (*Datura stramonium*) as biopesticide and evaluate its bioactivities against Maize weevil (*Sitophilus Zeamais*)

Entry	Compound	sources	Entry	compound	Sources
29	3,7-dihydroxy-6-propionyloxytropane	Plant	42	3-tigloyloxy-6-propionyloxy-7-hydroxytropane	R, ST, L, P
30	6,7-dehydro-3-tigloyloxytropane	Plant	43	3-tigloyloxy-6-propionyloxytropane	R
31	3,7-dihydroxy-6-(2'-methylbutyryloxy) tropane	Plant	44	3-tigloyloxy-6-isobutyryloxytropane	R
32	6,7-dehydroapoaotropine	Plant	45	3-tigloyloxy-6-(2-methylbutyryloxy) tropane	R, ST
33	Hygrine	ST	46	3-tigloyloxy-2-methylbutyryloxytropane	R
34	3-(hydroxy acetoxy) tropane	ST, R	47	3 α -tigloyloxy-6-isovaleryloxy-7-hydroxytropane	R
35	6-hydroxyacetoxytropane	R	48	3 β -tigloyloxy-6-isovaleryloxy-7-hydroxytropane	R
36	3-hydroxy-6-acetoxytropane	R, ST	49	3-tropoyloxy-6-acetoxytropane	R, ST
37	3-hydroxy-6-(2-methylbutyryloxy) tropane	L	50	3,6,7-trisubstitutedtropanes	ST, L
38	3-hydroxy-6-tigloyloxytropane	R, ST, L, p	51	Tropine	R, ST, L, S
39	Hyoscyamine	R, ST, L, S	52	3-tigloyloxy-6,7-dihydroxytropane	R
40	6-hydroxyapoaotropine	R, ST, L	53	3-tigloyloxy-6-methylbutyryloxytropane	R
41	7-hydroxyhyoscyamine	ST, L	54	3-tropoyloxytropane	R, L, S

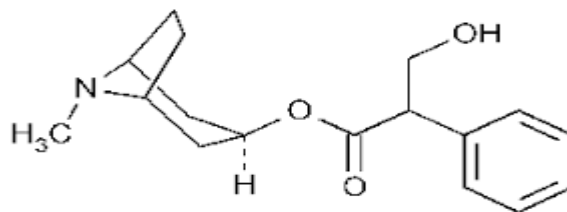
Entry	Compound	sources	Entry	compound	Sources
55	6-hydroxyhyoscyamine	R, ST, L	63	3-tropoyloxy-6,7-epoxynortropane	R
56	Littorina	R, ST, L, S	64	3-tropoyloxy-6,7-epoxytropane	R, L, S
57	Meteloidine	R, P	65	3-tropoyloxy-6-hydroxytropane	R
58	3-monosubstitutedtropanes	R, L	66	3-tropoyloxy-6-tigloyloxytropane	R
59	3-(3'-methoxytropoyloxy) tropane	Plants	67	3-tigloyloxy-6,7-epoxytropane	Plant
60	Methyl scopolamine	Plants	68	3-tigloyloxy-6-isobutyryloxy-7-hydroxytropane	Plant
61	Phenylacetoxytropane	R, ST, L, S	69	3-tropoyloxy-6-isobutyryloxytropane	Plant
62	3 β -tropoyloxy-6 β -isovaleroyloxytropane	Plant			

L = Leaves, R = Roots, S = Seeds, ST = Stems, P = Pericarp

2.6.3. Atropine

Phytochemistry of *Datura stramonium* Nearly more than seventy tropane alkaloids have been detected from *D. stramonium*. The toxins in *Datura* are tropane belladonna alkaloids, which consists of strong anticholinergic effects. These alkaloids include: hyoscyne (roots); atropine (d, lhyoscyamine) hyoscyamine (leaves, roots, seeds), and scopolamine (l-hyoscyne), as well as protein and sitosterol. It is reported that hyoscyamine is the predominant alkaloid in DS.⁴¹

Atropine is a tropane alkaloid extracted from jimsonweed (*Datura stramonium*). Its empirical formula is C₁₇H₂₃NO₃ and its structural formula is:



J7u

Figure 2: 6- Structural formula of Atropine

The IUPAC name of Atropine is benzene acetic acid. Atropine sublimes under high vacuum at 93 to 110 °C and has a melting point of 114 to 116 °C. Atropine has low solubility in water (approximately 1g atropine in 455ml water and 1 g atropine in 90ml water at 80 °C). One gram is soluble in 2 ml alcohol, 2.5ml alcohol at 60°C, 27 ml of glycerol, 25ml ether and 1 ml chloroform. Atropine is optically inactive⁴⁶.

2.7. Atropine based bio-pesticide

The first successful isolation of an alkaloid poison was performed in 1850 by Jean servial Stas, a Belgium chemist, using a solution of acetic acid and ethyl alcohol to extract nicotine from the tissues of the murdered Gustave Fougne. German chemist, Friedrich Otto, the Stasotto method was quickly applied to isolation of numerous alkaloid poisons, including colchicine's, conine, morphine, narcotine and strychnine and this. method is still used today.⁴⁶

Jimson 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 Jimson used for Manufacturing Pesticides, Jimson 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 Jimson based pesticides which differs it from its synthetic counterparts is the fact that it does not leave any residue on the plants.

Jimson 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 no 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 Jimson pesticides manufacturers to cash in on the growing popularity of natural or herbal pesticides.

Jimson seed contains more than 70 different active compounds called tropane alkaloids. The main active ingredient of Jimson seed is Atropine. 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. Like chemical insecticides, it works on the digestive or nervous system, and it is claimed that this does not lead to development of resistance in future generations. Because Atropine 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. Atropine has been considered to be a promising environmentally compatible insect pest control device for plant protection. Moreover, Atropine 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.

2.7.1. Mode of action of atropine-based bio-pesticide

Atropine is commonly classified as an anticholinergic or anti-parasympathetic (parasympatholytic) drug. More precisely, however, it is termed an antimuscarinic agent since it antagonizes the muscarine-like actions of acetylcholine and other choline esters.

Atropine inhibits the muscarinic actions of acetylcholine on structures innervated by postganglionic cholinergic nerves, and on smooth muscles, which respond to endogenous acetylcholine but are not so innervated. As with other antimuscarinic agents, the major action of atropine is a competitive or surmountable antagonism, which can be overcome by increasing the concentration of acetylcholine at receptor sites of the effector organ (e.g., by using anticholinesterase agents, which inhibit the enzymatic destruction of acetylcholine). The receptors irritated by atropine are the peripheral structures that are stimulated or inhibited by muscarine, (i.e., exocrine glands and smooth and cardiac muscle). Responses to postganglionic cholinergic nerve stimulation may also be inhibited by atropine, but this occurs less readily than with responses to injected (exogenous) choline ester²⁰.

Jimson 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 Jimson product, because of the presence of Atropine there is an antimuscarinic agent since it antagonizes the muscarine-like actions of acetylcholine and other choline esters. Because of this impression the insect does not feed on the Jimson treated surface and ability to swallow is also blocked. Secondly it acts as oviposition deterrent i.e., by not allowing the female to deposit eggs comes in very handy when the seeds in storage are coated with Jimson kernel powder and/or Jimson oil. It also acts as insect growth regulator. It is a very interesting property of Atropine and unique in nature, i.e. it works on juvenile hormone⁴⁷.

2.7.1.1. Biological effects of atropine-based pesticide on pest

Jimson products as its pest control action can be displayed at different levels and in different ways to knock out effect of chemical pesticides. Jimson extracts do not show this type of effect on pests but affect them in several other ways.

2.7.1.2. Atropine effect on pest growth regulation

Regulation of the insects' growth is a very interesting property of Jimson products. 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 molting and is governed by an enzyme, ecdysones. When the Jimson components, especially Atropine, enter the body of the larva, the activity of ecdysones is suppressed and the larva fails to molt, remains in the larval stage and ultimately dies. If the concentration of Atropine 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 sterile⁴⁸.

2.7.1.3. Atropine effect of feeding preventive

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 Jimson product, because of the presence of Atropine, there will be an antimuscarinic agent since it antagonizes the muscarine-like actions of acetylcholine and other choline esters. Because of this

impression, the insect does not feed on the Atropine treated surface. Its ability to swallow is also blocked⁴⁹.

2.7.1.4. Oviposition preventive effect of atropine

Another way in which Atropine controls pests is by preventing the females from depositing eggs. This property is known as oviposition prevention, and comes in very handy when the seeds in storage are coated with Atropine or crude Jimson 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.

2.8. Extraction methods of atropine

Jimson seed is a valued raw material which contains not only insecticidal and fungicidal ingredients, but also certain amount of oil. Even the extracted Jimson cake can be used as fertilizer; it might have effects on soil pests⁵⁰. There are several technologies available to extract the active ingredients of Jimson seed. The technique to be used depends mainly on the quality required of the final product, production cost and extract application.

The cost of the extraction method varies accordingly; the higher the quality of atropine required the more expensive of the extraction method. Considering the solubility of the important component atropine it is clear that only polar solvents should be used for extraction. Still, the cold pressed Jimson oil could contain up to 0.6% atropine. 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

The general methods of extraction and isolation of the alkaloids from the plant sources one has to take into consideration the following steps in a sequential manner, namely:⁵¹

- i. Separation of the alkaloid(s) from the main bulk of the non-alkaloidal substances,
- ii. Most of the alkaloid-containing plants, several alkaloids having closely related chemical structures are normally present, such as: the cinchona alkaloids consist of

more than twenty-five alkaloids. There is hardly any known plant source that contains only one alkaloid exclusively,

- iii. Separation of each individual alkaloid from the mixture of alkaloids obtained from a particular plant source (e.g. cinchona bark) using latest separation techniques, for instance, preparative high-performances liquid chromatography, (HPLC) column chromatography, by the help of chromatogram, and high-performance thin-layer chromatography (HPTLC).

Nevertheless, the general methods of isolation of alkaloids largely depend upon several vital factors, for instance:⁵¹

- a. the alkaline nature of most alkaloids,
- b. the ability and ease of formation of alkaloidal salts with acids, and
- c. the relative solubilities of the resulting alkaloidal salts either in polar organic solvents e.g .ethanol, chloroform, isopropanol etc., or in aqueous medium.

Conventional processes essentially associated with the separation as well as isolation of the hundreds of alkaloids from the natural plant sources, the following five steps are most important and vital, namely:⁵¹

- i. **Sample preparation**-The first and foremost step is the sample preparation. The plant material is reduced to a moderately coarse powder by appropriate means using grinders and sieves, to facilitate maximum effective contact of the solvent with the ruptured alkaloid bearing tissues and cells.
- ii. Liberation of free alkaloidal base: the alkaloids invariably occur in the plant sources as the salt of acids, such as: oxalates, tannates etc. Therefore, when the plant substance is exposed to an alkaline medium, the alkaloidal salts are readily converted to the corresponding alkaloid bases.
- iii. Extraction of alkaloidal base with organic solvent
- iv. Purification of crude alkaloidal extract
- v. Fractionation of crude alkaloids

There are several methods available to extract the active ingredients of Jimson seed. The major three types of extraction methods that are discussed below.

2.8.1. Extraction with alcohol

It is one-step extraction. The Jimson seeds are crushed into powder and extracted with ethanol or methanol. The alcohol should have low water content, and its purity is very important. Using the moving-bed contacting method, the grains will be stirred for certain hours by an overhead stirrer in a mixing-settling tank. After decantation of the crude cake, the Jimson solution is drained out, filtered and passed to the next procedure. The dilute Jimson solution will be further evaporated until the concentrated extract has been achieved, and the solvent will be recycled.

The usual pretreatment of the crude with alkali may be avoided completely, because alcohol appears to affect dissolution of not only the alkaloidal salts but also the free bases found in the plant substances. It is, however, believed that alcohol predominantly exerts a hydrolyzing effect upon the alkaloidal tannates and other salts.⁵¹

2.8.2. Extraction of water or acidic

Most alkaloids are present in salt form in the plant. Usually, water or inorganic acidic extraction is used, so that the organic acid of alkaloids salt is replaced inorganic acid salt, and increasing its solubility. Acid extraction method usually use 0.1% to 1% sulfuric acid, hydrochloric acid or acetic acid, tartaric acid solution as a solvent, by boiling method. The advantage of that acidic extraction is changing alkaloids molecules into small molecule organic acid salts of inorganic acids, increasing the solubility in water, and the extraction method is relatively simple⁵².

2.8.3. 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.

So, from different types of existing extraction process, extraction with water or acidic is selected for this thesis depend on following factors: - the simplicity of process, less extraction times, the extraction efficiency, less cost of process because it does not have recovery of the solvent and availability of extraction equipment.

2.9. Benefits and field application of atropine-based pesticides

Atropine 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 jimson seed for pesticidal effect is grinding and soaking the seed for 24 hours minimum and make in use for treatment of wound. Atropine helps the soil to sustain and conditioned since the residue have an ability of nitrogen inhibition. In addition to the pesticidal effect, it is environmentally friendly, since it doesn't leave any residue on the plant and environment. Atropine based pesticide doesn't kill directly the pest; rather it affects the life cycle of the pests like antifeedant 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 Atropine 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.

Biological activity of extracts of *Datura stramonium* are used in traditional systems of medicines. The plant as a whole possesses remarkable biological activities which are summarized below. The leave extract of exhibits antibacterial activity against the microorganism strains of *Salmonella typhi*, *Pseudomonas aeruginosa*, *Proteus vulgaris* in the alcoholic extract of the leaves *Datura stramonium* was found to be most efficient and moderately effective against *Klebsiella pneumonia*, *Staphylococcus-aureus* and *E. coli*. Efficient nematocidal activity of the aqueous extract prepared from the leaf of *Datura stramonium*. The leaf exhibit excellent antifungal activity. The leaf and seed extracts have contact toxicity against *Tribolium castaneum* adults. Exposure of human cancer cells derived to the extract for 48 hours showed that all cancer cell lines were sensitive to cell killing induced by *Datura* aqueous leaf extract exposure with some variability ($p < 0.05$).

Antimicrobial activity crude ethanolic extract of *Datura stramonium*, *Terminalia arjuna* and *Withania somniferous* in cup plate diffusion method for antibacterial and antifungal activity.

Table 2: 5- The traditional uses of *Datura stramonium* in different parts of the world

Entry	Countries	uses of <i>Datura stramonium</i>
1	India	Fruit is burnt and ash is given orally with honey in bronchitis and asthma
2	Pakistan	Green Leaves are used for softening the boils. The juice of flower is useful for earache
3	Bulgaria	It is used as anti-asthmatic, spasmolytic and antiviral remedy
4	Rwanda	It is used for acaricidal activity.
5	Ethiopia	It is used for wound treatment against wound causing bacteria

Source: *Datura stramonium*: An overview of its phytochemistry and pharmacognosy

2.10. Jimson plant 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 Jimson plant, and about 35-45% of the conditions in Ethiopia are appropriate for the jimson plantation. There are various places that Jimson plant has observed in Ethiopia. Particularly, it is found widely in the following regions; Oromia, Gambella, Somali, Southern Nations, Nationalities and Peoples, Sidama and Amara ⁵³.

2.11. Maize weevil (*Sitophilus Zeamais*)

The **maize weevil** (*Sitophilus zeamais*), is a species of beetle in the family Curculionidae. It can be found in numerous tropical areas around the world, and is a major pest of maize. This species attacks both standing crops and stored cereal products including wheat, rice, sorghum, oats, barley, rye, buckwheat peas, and cottonseed. The maize weevil also infests other types of stored, processed cereal products such as pasta, cassava, and various coarse, milled grains. It has even been known to attack fruit while in storage, such as apples.

In Ethiopia, maize (*Zea mays L.*) is one of the major cereal crops grown for its food and feed values. It is one of the most important staple food and cash crops providing calories for the consumers and income for the traders. The production and productivity of maize has increased since the development of high yielding hybrid varieties by the Ethiopian Institute of Agricultural Research (Bako Agricultural Research Center). Thus, maize ranks first in production and productivity since the release of these hybrid varieties⁵⁴. Yet, these hybrid varieties are reported to be highly susceptible to insect pest attacks both in the field and storage⁵⁵.

Traditionally, maize grain is stored by Ethiopian farmers, both in and outdoors for consumption and sell in the later months of the year depending on the quantity produced per household. The stored maize is attacked and damaged by several pests that lead to quality deterioration forcing farmers to sell at reduced prices and below the production cost. Insects are most often considered as the principal cause of maize grain losses⁵⁶. The most important insect pests that cause damage to maize in the field and storage are lepidopterous stalk borers and coleopterous weevils, respectively⁵⁷

The global picture of losses of grain and pulse crops after harvest is estimated to be 10% mostly due to insect pests and this is very serious in developing countries⁵⁸. Maize weevil followed by grain moth, rice weevil and flour beetle were the most important. Significant grain damage (average of 64.50%) associated with losses of up to 80% was caused by the pests within three to six months of storage.



Figure 2: 7- Maize weevil (*Sitophilus zeamais*)

Chapter Three

3. Materials and Methods

3.1. Research materials and chemicals

There are different chemicals and materials used on this study for experimental work. They are listed as follows.

- ✓ Chemicals used are: -
 - Sulfuric acid
 - Ammonium carbonate
 - Methanol
 - Acetic anhydride
 - Acetyl chloride
 - Dichloromethane
 - Triethylamine (TEA)
 - Aqueous sodium bicarbonate solution
- ✓ Materials needed
 - Oven
 - Measuring cylinder (250ml)
 - Pipette
 - Batch extractor vessel with overhead mixer and Temperature sensor
 - Tray drier
 - Water Bath
 - Pycnometer (25 ml)
 - Filter paper, 0.22 μ m of diameter 90mm
 - Bomb calorimeter
 - UV spectroscopy
 - FTIR
 - HPLC chromatography

3.2. Process Methods

3.2.1. Experimental setup

The experimental setup for the extraction of atropine from jimson seed were discussed in below figure starting from the pretreatment of raw material to final concentration of the product as follows

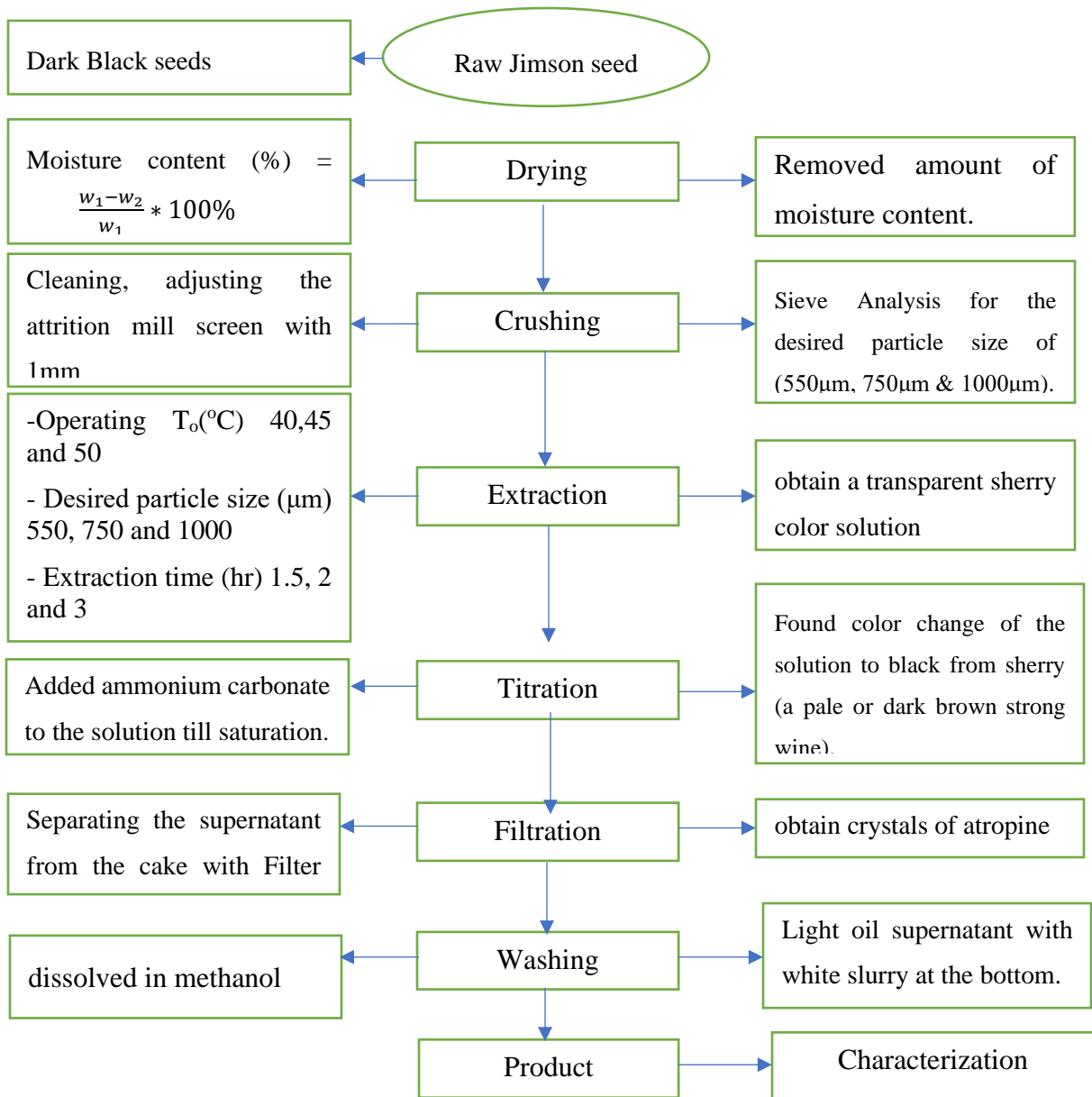


Figure 3: 1- Experimental setup of atropine extraction from Jimson seed

3.2.2. Raw material acquisition and preparation

A matured seed in a dark color stage were collected from Oromia regional state Nekemte city manually in Nov, 2019. After the sample was collected manually using sterilized scissor and glove and then packed in a polyethylene bag and, transported to AAiT Chemical and Bio Engineering Department laboratory. The sample was stored at room temperature open air before use.

3.2.3. Moisture content of jimson seeds

Moisture content analysis was carried out following ASTM D 2974-87 Standard Test Methods by heating at 55°C in an oven (DAS 42000, 224.2007) for days, so that the moisture amount was determined every 3hours.



Figure 3: 2- Moisture content removal of Jimson seed

$$\text{Moisture content (\%)} = \frac{w_1 - w_2}{w_1} * 100\% \dots\dots\dots 3.1$$

w_1 -initial weight of sample, (g)

w_2 - final weight of sample, (g)

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

3.2.4. Ash content of jimson seed

The sample characterization was carried out according to ASTM D 2974-87 Standard Test Methods. Ash content of an organic sample was determined by burning the oven dried sample in

a muffle furnace (LHT 04/17, SN 262014) at 750°C (MethodD). The substance remaining after burning was the ash. It is expressed as a percentage of the mass of the oven-dried sample.



Figure 3: 3- Furnace and desiccator

$$\text{Ash content (\%)} = \frac{w_2}{w_1} * 100 \dots\dots\dots 3.2$$

Where:

$W_1 = \text{Ash (g)}$

$W_2 = \text{Oven dried specimen (g)}$

$$\text{Organic Matter} = 100 - \text{Ash content (\%)} \dots\dots\dots 3.3$$

Finally, after measuring the moisture and ash content, the samples were dried and milled by an attrition miller was sealed by polyethylene bag and ready for further extraction process.

3.3. Experimental factors

3.3.1. Particle size

The highest concentration of a given yield was expected as surface area of fine particles size are highly exposed to solvent. 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

constituent⁵⁹. Particle size of 550 μ m, 750 μ m and 1000 μ m was selected as one extraction factor for this experimental work because of the jimson seed has higher amount oil and difficult to reduce its size beyond this.

3.3.2. Extraction time

Extraction time plays significant role in extracting the active ingredient⁶⁰. The longer extraction time could lead to increase in exposure of temperature which lead to degradation on the effective biological activity of the component. Extraction time of 1.5hr, 2.5hr and 3hr was selected as the second factor that affect the yield because of oily content of jimson seed was extracted between of 1hr-3hr⁴⁶.

3.3.3. Extraction temperature

An extraction was promoted as the extraction temperature increases. Both the solubility of active ingredients and diffusion coefficients are enhanced as temperature increases. It also increases cell membrane permeability following the breakdown of cellular constituents thereby setting more active ingredients free to be permeable and extractable⁶⁰. However, high temperatures are not always suitable for extracting all sorts of active ingredient compounds. Rather it destructs the required active component. The temperature 40°C, 45°C and 50°C was selected for the experimental process⁴⁶.

3.3.4. Extraction process and steps

The Jimson Seed after attrition milled, it was sorted by its particulate size in a polyethylene plastic bag. A proportionate solvent (water) to solid (seed) ratio 5:1ml/gm will be prepared as desired. The water bath as a hot circulate was adjusted to the 40°C, 45°C and 50°C temperature. Then, simultaneously the seed and the solvent were added to a beaker for further extraction. After adding the solvent and the seed in to the extraction vessel, mixing parameters were set to a constant revolution of 400rpm and extraction time of 1.5hr, 2.5hr and 3hr as required. Sulfuric acid was added to change organic acid of alkaloids salt is to inorganic acid salt.

After extraction, the solution was separated as supernatant from the cake by centrifugation for 15 minutes at 5000rpm. Atropine and some polar limonoids were goes for titration by ammonium

carbonate to regulate the pH of the solution. For further clarification, the supernatant was vacuum infiltrated⁴⁶. The separated sample was washed with methanol of 45ml. At the end Light oil supernatant with white slurry at the bottom were obtained. The supernatant was removed by pipette. The white slurry at the bottom was goes for acylation process. And finally, the product is characterized.

3.4. Acylation of atropine

The O-acyl derivative of atropine was found by consumption of starting material (Light oil supernatant with white slurry at the bottom) immediately after addition of Triethylamine followed by acetic anhydride. Both triethylamine (TEA) and diethylamide (DEA) can be used as base for the acetylating of Atropine⁴⁶.

3.4.1. Procedure for acetylation of atropine

Acetylation of atropine was carried out by using acetic anhydride and Acetyl chloride. A 0.5 gm of Atropine was dissolved in 20 ml of Dichloromethane (DCM), to this solution TEA is added as base, at cold temp added 1 equivalent of acetic anhydride. The resulting reaction mixture was stirred at room temp for two hours. Stopped the reaction, added aqueous sodium bicarbonate solution and extracted with DCM, repeated the extraction for 2 times separated the organic and aqueous layers⁴⁶.

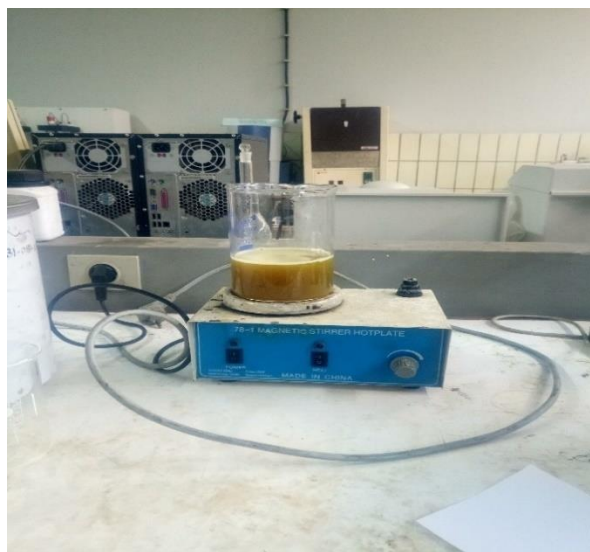


Figure 3: 4- Acetylation of atropine

3.6. Physio – chemical characterization of Atropine

3.6.1. High-performance liquid chromatography (HPLC) of sample

High-performance liquid chromatography is sensitive and selective for the determination of atropine in biological specimens. The method consists of two steps: the first one is the extraction with dichloromethane of atropine from biological specimens and the second one was HPLC analysis of atropine. The t_R for atropine could be varied by changing either the acetonitrile-water ratio in the mobile phase or the pH of the mobile phase. This method can be applied to the monitoring of atropine in biological samples. A 400 μ l volume of standard or sample was placed into a 10 x 100 tapered glass centrifuge tube. To the tube were added 200 μ l of 0.4 M sodium hydroxide and 1.5 ml of dichloromethane. The mixture was vortex mixed for 1 min, then centrifuged at 1400 g for 5 min. The bottom layer was transferred to another 10 x 100 mm tube. This tube was immersed in a water bath at 50°C, and the dichloromethane was evaporated with an air stream. The residue was dissolved in 400 μ l of the mobile phase, and 200 μ l or less were injected into the chromatograph⁶¹.

3.6.2. UV-vis spectrum analysis of atropine

A variety of techniques can be used to determine and estimate the presences of Atropine in plant extract. UV-VIS spectroscopic is simple, cost-effective and rapid tests for detecting Atropine. UV-visible spectroscopy uses light in the visible ranges or its adjacent ranges. The extract was scanned at wave length ranging from 300 to 800 nm using UV 3200 Spectrophotometer and the characteristic peaks were detected. The absorbance at those wavelengths were recorded.

3.6.3. Fourier transform infrared (FTIR) analysis of atropine

Fourier transform infrared (FTIR) is the preferred method of infrared spectroscopy. When IR radiation is passed through a sample, some radiation is absorbed by the sample and some passes through (is transmitted). The resulting signal at the detector is a spectrum representing a molecular ‘fingerprint’ of the sample. The usefulness of infrared spectroscopy arises because different chemical structures (molecules) produce different spectral fingerprints⁶².

Infrared is a powerful identification tool for functional groups because of the similar absorption frequencies for those groups in different molecules. The actual frequency is affected by the

environment, so the reference chart shows wide bands rather than specific frequencies. The identification of functional groups is a cornerstone of IR spectroscopy and organic chemistry⁶².

3.6.4. Specific gravity

The measurement of relative density of the sample with respect to water is called Specific gravity. It is determined by specific gravity bottle known as Pycnometer method using ⁶³IS: 1460-000 method depending on the Archimedes principle of displacement which was conducted at room temperature and pressure. This indicates that, the physical property of the sample immersion or flotation when it gains an opportunity to contact with water.

$$\text{Density of the sample} = \frac{W_1}{V - \frac{W_2}{\rho}} \dots \dots \dots 3.4$$

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 (cm³)

W_2 - Mass of Hexane (g) and ρ - Density of Hexane, i.e. 0.6554 (g/cm³)

3.6.5. Flash point

The flash point is a key property of determining the flammability of a product and it is the lowest temperature at which an applied ignition source will cause the vapors of a sample to ignite. Flash point is a measure of the tendency of a sample to form a flammable mixture with air. 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°C), highly flammable (Flash point below 21°C) and flammable (Flash point below 55°C)⁶⁴.

3.6.6. Water solubility

The amount of substance that passes into solution to achieve a saturated solution at constant temperature and pressure and which is expressed in terms of maximum volume or mass of the solute that dissolve in a given volume or mass of a solvent is called Solubility. 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 leave a residue over the crop. The more soluble matter in the solvent means the more dispersing material on the environment⁶⁵.

$$\text{Solubility (\%)} = \frac{w_1 - w_2}{v_w} * 100 \dots\dots\dots 3.5$$

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)

3.6.7. pH value

A numeric scale used in specifying the acidity or basicity of an aqueous solution is a pH. Since, the extract is applied simply 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. 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.

3.6.8. Total volatile organic compounds analysis

Volatile organic compounds (VOCs) are any chemical or compound based on carbon chains or rings with a vapor pressure greater than 0.01kpa at 298.15 K (25°C), that participate in photochemical reaction⁶⁶. Because of they produce smoke when they react with nitrogen oxides in photochemical reactions Volatile organic compounds (VOC's) are environmentally important to be determined. Also, may have important health implications. International Environmental Protection Agencies or EPA regulation agencies are designed to control smoke formation by limiting VOC and nitrogen oxide emissions. The TVOC's present in atropine were analyzed in 25°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 Solar Energy). Considering this, a microwave oven with a UV light output sources up to 700w and 25 to 225°C temperature gradient regulator was used to measure the TVOC in 24 hours and then analyzed the mass difference.

$$\text{TVOC (\%)} = \frac{\text{Initial mass of the sample} - \text{Final mass of the sample (gm)}}{\text{Initial mass of the sample}} * 100\% \dots\dots\dots 3.6$$

3.7. Biopesticide formulation

The biological activity of a pesticide, biological in nature, is determined by its active ingredient (Atropine). Pesticide products very rarely consist of pure technical material. The atropine-based pesticide is usually formulated with other materials and this is the product as sold, but it may be further diluted in use. Formulations improve the properties of a pesticide for handling, storage, application and may substantially influence effectiveness and safety.

Atropine in their “raw” or unformulated state are not usually suitable for pest control. This active ingredient may not mix well with water, may be unstable, and may be difficult to handle and transport. For these reasons, inert substances, such as clays and solvents are added, to improve application effectiveness, safety, handling, and storage. Inert ingredients do not possess pesticidal activity and are added to serve as a carrier for the atropine.

Liquid formulations are formulated carried out by mixed with water. Out of liquid formulation Ready-to-Use Low Concentration Solutions are suitable for atropine. Low-concentrate RTU formulations are ready to use and require no further dilution before application. They consist of a small amount of Atropine (often 1% or less per unit volume) dissolved in water. They usually do not stain fabrics nor have unpleasant odors. They are especially useful for structural and institutional pests and for household use.

Chapter Four

4. Results and Discussions

In this chapter the result of experimental work starting from pre-extraction to the final product characterization with their discussion was presented.

4.1. Pre-extraction of Jimson seed

4.1.1. Moisture content of Jimson seed

The result of the amount of moisture content removed after 3hour was 24.241 %. Jimson Seed with higher moisture content yields less bioactive extract as compared to those with lower moisture content⁶⁷. Jimson Seed with 24% moisture content yields less extract. This was because of the amount of moisture present in the seed blocks the diffusivity of the solvent towards the seed surface and prohibits extraction. The moisture content removed in the next 3hours was about 0.9%. The amount of moisture content was decreased consequently and left in small quantity that not 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 content of Jimson seed

From the laboratory result of a replicated seed sample, the ash content of Jimson seed was 6.607%. The amount of ash content in a sample entitles the total amount of nonorganic matters or compounds present in a sample (mostly metallic oxides). Applicably, these compounds are causes different health problems on different plant and animals if they are high in amount. For this reason, the amount of ash content should be less as much as possible. For plant extraction ash content less than 8% was recommended⁶⁸. So, amount of ash content for Jimson seed was acceptable.

4.1.3. Organic matter

Anything that contains carbon compounds that were formed by living organisms is an organic matter. They are biodegradable and environmentally sustainable. So, the need for high amount of organic matter is very important environmentally and applicably. The amount of organic matter in Jimson seed was 93.393%. The seed has about 93.393% organic compounds that are easily decomposable and degradable¹⁰.

4.2. Acylation of atropine

After Acetylation hydrogen atom is substituted for an acetyl group ($\text{CH}_3\text{C}=\text{O}$ group) in atropine. The products formed in acetylation reactions typically have an acetoxy functional group. When the hydrogen atom belonging to an alcohol group replaced with an acetyl group in an acetylation reaction, an ester is formed as the product. For such reactions (where the reactant compound contains free hydroxyl groups), the most commonly used acetylating agent is acetic anhydride.

Its effect was observed on the FTIR result that the absorption at 3369.25 nm is due to the C-O stretch that are present in the extract and the absorption at 1656.63nm and 1387.69 nm was due to the stretching of hydroxyl groups that are present in the extract. Small peaks are observed due to acylation process.

4.3. Physio – chemical characterization of Atropine

4.3.1. High-performance liquid chromatography of atropine

Calibration graphs from spiked samples were found to be linear over the concentration range 0-6.5#g/ml for atropine ($r = 1.698$). The limit of detection at a signal-to-noise ratio of 5:1 was 8.5 ng/ml for atropine, when a 200- μl sample was injected into the column. The highest height at 297nm and retention time 5.5minute shows the atropine were dominant on the solution.

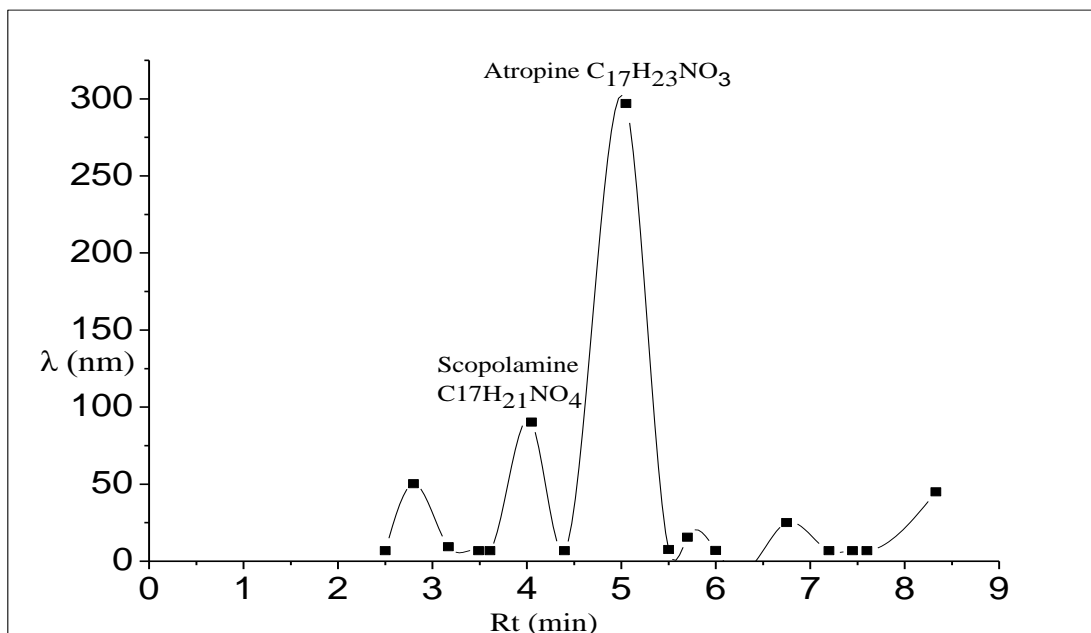


Figure 4: 1- Chromatograms of atropine solution

4.3.2. UV-Visible spectrum analysis of atropine

The qualitative UV-VIS profile was taken at the wavelength of 300 nm to 800 nm due to the sharpness of the peaks and proper baseline. The obtained data of UV-VIS spectroscopic analysis was given as follows in table. The concentration of atropine in the extract were calculated using linear line equation $y=mx+b$.

Table 4: 1- Data obtained from UV-VIS spectroscopic analysis

Concentration (µg/ml)	Absorption
0	0
2	0.225
4	0.308
6	0.656
8	1.301
10	1.562

The given data were linearized and the concentration of the atropine were calculated from the graph using $y = 0.1053x - 0.1559$. where x is for concentration.

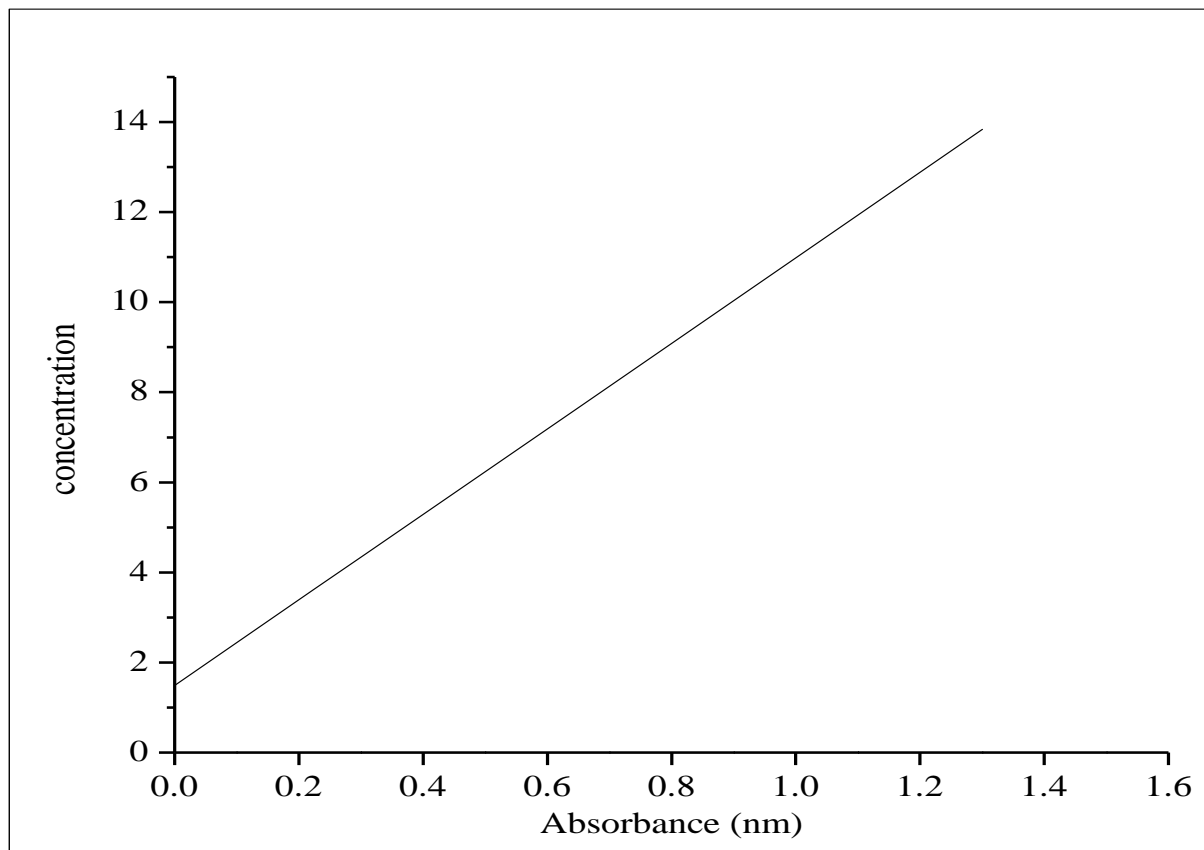


Figure 4: 2- UV-VIS spectroscopic analysis graph

4.3.3. Fourier transform infrared (FTIR) analysis of atropine

FTIR spectrum of the atropine was measured on Spectrum 65FT-IR (PerkinElmer) in the range $4000-400\text{ cm}^{-1}$. The region $2750-1750\text{nm}$ is attributes to an over tone structure vibration. Because it remains unaffected by different treatments, its integrated value is used as a reference band to normalize all other intensities. The absorption at 3369.25 nm is due to the C-O stretch that are present in the extract. The band at 2966.34 nm is due to the symmetric stretching of saturated carbon. The band at 2882.36 nm is due to C=C stretching associated with the aromatic skeletal mode of the extracts. The absorption at 1656.63nm and 1387.69 nm was due to the stretching of

hydroxyl groups that are present in the extract. Small peaks are observed due to acylation process. A prominent band at 1102.75 and 1035.77 nm can be allotted to C-O stretching. The band at 900.80 nm is assigned to the bending mode of absorbed water, since plant extracts are known to have a strong affinity for water. A band at 649.86 nm represent the aromatic H out of plane bending. The small peaks may be attributed to the compounds present in small.

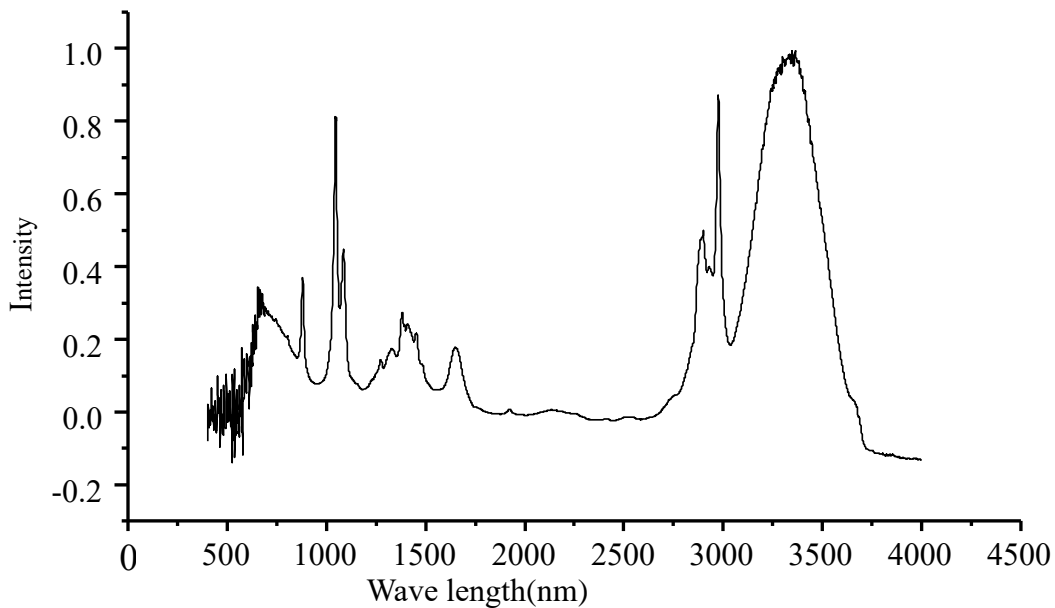


Figure 4: 3- FTIR spectra of Atropine

4.3.4. Specific gravity

The test was done in the water as a media in which the extract has been going to dissolve. The specific gravity of the sample should be greater than that of media. So that solubility facilitates intensely.

The average density of the extract was found 1.4164 ± 0.07225 which was higher than the density of water. This indicates the extract submerge rather than float when it mixed with water and increases 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.

4.3.5. Flash point

The result of samples tested for flash point using bomb calorimeter are found averagely $36.06 \pm 0.9932^{\circ}\text{C}$. The result found to be under th0065 classification of flammable components (i.e. the ignition temperature is below 55°C)⁶⁴. This helps 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.

4.3.6. Water solubility

Water solubility method helps to determine the disintegration and relative dissolution of atropine in water. Atropine expected to be dissolved in water and uniformly disperse. The temperature and pressure at which atropine disintegrates and dissolves was set to 25°C and atmospheric pressure⁶⁹. The solubility calculated from the sample were found $99.768 \pm 0.0217\%$.

The result implied that $99.768 \pm 0.0217\%$ of the initial mass of sample is dissolved easily⁶⁹.

4.3.7. pH value

The tendency of liberating hydrogen ion or hydroxide ion when atropine dissolved in water was measured directly by a pH meter. This tendency could be vigorously affecting the environment and 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 behavior of the soil. The pH value of atropine has great meaning on the targeted pest depending on the physiological and enzymatic pattern.

The result implies that the pH value of atropine has been found in the range of 5.2 ± 0.18708 . If categorizing would be necessary atropine becomes acidic depending on the magnitude.

4.3.8. 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. The ability of organic chemicals to cause health effects varies greatly from highly toxic, to those with no known health effect depending on many factors like level of exposure and length of time exposed. There

has been no standard set for VOCs in non-industrial settings. 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 specimens. The result found from the analysis is listed below.

The amount of TVOC's the sample has been found $0.17426 \pm 0.0311\%$. Eye and respiratory tract irritation, headaches, dizziness, visual disorders, and memory impairment that end users have experienced soon after exposure to product were very few.

4.4. Atropine extraction

The yield of atropine from Jimson seed using batch reactor with further improvement process steps and defined operation parameters such as particle size, extraction temperature, and extraction time was found quite different in extent. This is because of individual factors and interactive effect.

The HPLC and FTIR test analysis show that there is an increment of yield quantity and concentration of atropine simultaneously. This shows that the more concentration yield extract is found due to the more yield of an extract.

The optimum yield was found to be 10.811gm at operational conditions 713.4 μ m, 45.4°C, and 2.3hr of particle size, extraction temperature, and extraction time respectively. Moreover, and the minimum yield was found to be 8.5272 gm at operational parameters of 1000 μ m, 40°C, and 2.5hr.

Table 4: 2- RSM of Box – Behnken method model extraction factors and yield of atropine

		Factor 1	Factor 2	Factor 3	Response 1
Std	Run	A: Particle size Micrometer	B: Temperature (Degree Celsius)	C: Time (Hour)	Yield (Gm)
1	15	550	40	2.25	10.2928
2	5	1000	40	2.25	8.5272
3	4	550	50	2.25	10.6905
4	2	1000	50	2.25	9.3161
5	9	550	45	1.5	10.4993
6	6	1000	45	1.5	8.9012
7	3	550	45	3	10.4494
8	12	1000	45	3	8.9072
9	17	775	40	1.5	9.1358
10	10	775	50	1.5	10.6021
11	8	775	40	3	9.9878
12	7	775	50	3	9.7075
13	11	775	45	2.25	10.6232
14	13	775	45	2.25	10.623
15	16	775	45	2.25	10.6244
16	14	775	45	2.25	10.6237
17	1	775	45	2.25	10.6236

4.5. Experimental design analysis

To determine the optimal conditions of Atropine extraction RSM of Box – Behnken method Design-Expert 11.0.0 software was used. It is used to investigate the effects of three independent factors with three levels (particle size, extraction temperature and extraction time). Least squares regression method is used to fit the data to a certain model. The adequacy of the model is determined by evaluating the lack of fit that will be generated by ANOVA. Statistical significance

of the model and model variables will be determined at 5% probability level ($\alpha = 0.05$). The result found from experiment was inserted to Design-Expert software. A single model that can be possibly fit or satisfy the factors significance has been found. 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 0.8214 or 82.14% of lack of fit. So, quadratic model with 82.14% of fitting becomes the best choice of analysis for this case.

Table 4: 3- Sequential Model Sum of Squares

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	1702.70	1	1702.70			
Linear vs Mean	5.63	3	1.88	6.87	0.0052	
2FI vs Linear	0.8017	3	0.2672	0.9704	0.4445	
Quadratic vs 2FI	2.75	3	0.9180	4.476	< 0.0001	Suggested
Cubic vs Quadratic	2.675E-07	3	8.917E-08	0.3054	0.8214	Aliased
Residual	1.168E-06	4	2.920E-07			
Total	1711.89	17	100.70			

From the ANOVA result shows that all the factors, i.e., particle size, temperature, and extraction time had a significant effect on the yield. This indicates that, the variation in magnitude on the individual factors directly affects the yield parallel or not. The interaction effect of particle size with extraction temperature, particle size with extraction time and temperature with extraction time was significantly affect the response at the same time. The comparison of the p-value of the individual and combined factors held with the percent of probability of the model (i.e. $\alpha = 0.05$ or 5%). If the p-value is less than α value there is the greater significance effect of the factor on the

response and vice versa. Generally, the basic implication of ANOVA was to approve and disprove the impact of individual and combined factors on the yield which could be justified from the significance value of factors and lack of fit of the model.

Table 4: 4- Independent variables and levels used in the Box-Behnken Design

Factor	Name	Units	Type	Minimum	Maximum	Mean	Std. Dev.
A	Particle size	Micrometer	Numeric	550.00	1000.00	775.00	159.10
B	Temperature	Degree Celsius	Numeric	40.00	50.00	45.00	3.54
C	Time	Hour	Numeric	1.50	3.00	2.25	0.5303

4.5.1. Statistical analysis for the yield of Atropine

This design of the experiment helps us to optimize the process parameters for more than two levels and minimize an experimental error that is increased experimental accuracy. Initially Box-Behnken experimental designs were used to obtain the model equation. As usual, the experiments were performed in random order to avoid systematic error. Significance of the result was set from analysis of variance (ANOVA). The symbol terms in ANOVA were listed below: -

A = Particle size,

B = Extraction temperature,

C = Extraction time

AB = the interaction between particle size and extraction temperature

AC = the interaction between particle size and extraction time

BC = the interaction between extraction temperature and extraction time

Table 4.5 showed that the effect of particle size, extraction temperature and extraction time and also the interaction effect between particle size and extraction temperature, between particle size and extraction time and extraction temperature and extraction time.

The P values were used as a tool to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables. The larger the magnitude of F-test value and the smaller the magnitude of P-values, the higher the significance of corresponding coefficient. The model terms are significant if the Values of P less than 0.05.

In this case the three factors which are the particle size, extraction temperature and extraction time are significant model terms and have a high effect on the yield of the product. Values greater than 0.0500 indicate the model terms are not significant. Therefore, the interaction effects between all three factors are significant.

Table 4: 5- ANOVA of fitted model for the yield of atropine

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	9.19	9	1.02	4.980	< 0.0001	Significant
A-Particle size	4.93	1	4.93	2.404	< 0.0001	
B-Temperature	0.7037	1	0.7037	3.431	< 0.0001	
C-Time	0.0009	1	0.0009	4560.76	< 0.0001	
AB	0.0383	1	0.0383	1.866	< 0.0001	
AC	0.0008	1	0.0008	3809.42	< 0.0001	
BC	0.7627	1	0.7627	3.719	< 0.0001	
A ²	1.24	1	1.24	6.053	< 0.0001	
B ²	0.5888	1	0.5888	2.871	< 0.0001	
C ²	0.6448	1	0.6448	3.144E	< 0.0001	
Residual	1.435E-06	7	2.051E-07			
Lack of Fit	2.675E-07	3	8.917E-08	0.3054	0.8214	not significant
Pure Error	1.168E-06	4	2.920E-07			
Cor Total	9.19	16				

4.5.2. Development of model equation

A representative equation in which it represents the whole model with a single mathematical relation is a model equation. It helps to maximize response and the operating conditions. The model equation of atropine extraction from Jimson seed that was developed by the software has been shown below in the table.

Table 4: 6- Model equation in Coded and Actual factor form

Final Equation in Terms of Coded Factors	Final Equation in Terms of Actual Factors
Yield =	Yield =
+10.62	-38.17657
-0.7850 A	+0.009037 Particle size
+0.2966 B	+1.60016 Temperature
-0.0108 C	+8.29182 Time
+0.0978 AB	+0.000087 Particle size * Temperature
+0.0140 AC	+0.000083 Particle size * Time
-0.4367 BC	-0.116440 Temperature * Time
-0.5430 A ²	-0.000011 Particle size ²
-0.3740 B ²	-0.014958 Temperature ²
-0.3913 C ²	-0.695693 Time ²

Practically, from the equation it can be easily observed that, increment in individual factors also made the yield to increase parallelly. In other hand, even though the combined factors affect the yield strongly. Doubling the operating temperature can only cause yield increment.

4.5.3. Model adequacy checking

The results for responses which are presented in the above Figures confirms the adequacy of the quadratic model that means 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 atropine from Jimson seed with three independent factors was found 6228.358.

Table 4: 7- Model adequacy table

Std. Dev.	0.0005	R²	1.0000
Mean	10.01	Adjusted R²	1.0000
C.V. %	0.0045	Predicted R²	1.0000
		Adeq Precision	6228.3581

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 1.0000, and expresses well enough quadratic fits to navigate the design space. A model is good fit if R^2 should be at least 0.80. In this study $R^2 = 1.000 > 0.80$. This indicates is good fitness with the experimental result.

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 atropine that the data was normally distributed. The model can be considered reproducible if CV of the model is greater than 10%.

According to Table 4.7, CV was found 0.0045% so the model was considered not reproducible. This means RSM of Box-Behnken analysis is used to identify values of the independent variables that produce optimum extraction yield as response.

To find the maximum response each independent variable was individually increased or decreased in an attempt. Then, the combination of these optimum variables was selected as the condition for obtaining the overall maximum value.

Design-Expert® Software

Yield

Color points by value of Yield:

8.5272  10.6905

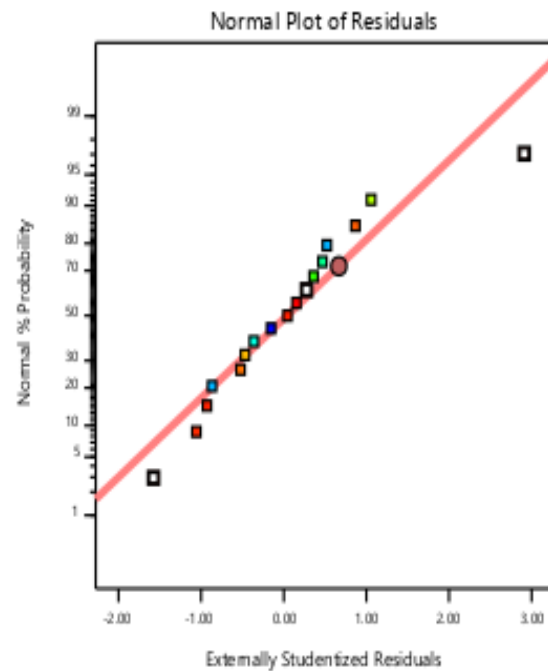


Figure 4: 4- Residual Normality) plot of the model

4.6. Effect of factors on yield

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

4.6.1. Effect of particle size on yield

The graph of particle size versus yield was resembled very declining linear plot. It was simply observed that the yield of atropine decreases with a corresponding increment in particle size. This is due to the principle of diffusion in which smaller particle size has a higher surface area to volume ratio and this increases the extraction kinetics practically.

The extraction of atropine has higher yield in a particulate size of 550 μ m than the rest. Considering particle size, the optimum yield found was 10.6905g/ 100g of atropine per seed. Antagonistic to this result the yield was found declining from 8.5272g in 1000 μ m to 8.9072 μ m of particle size.

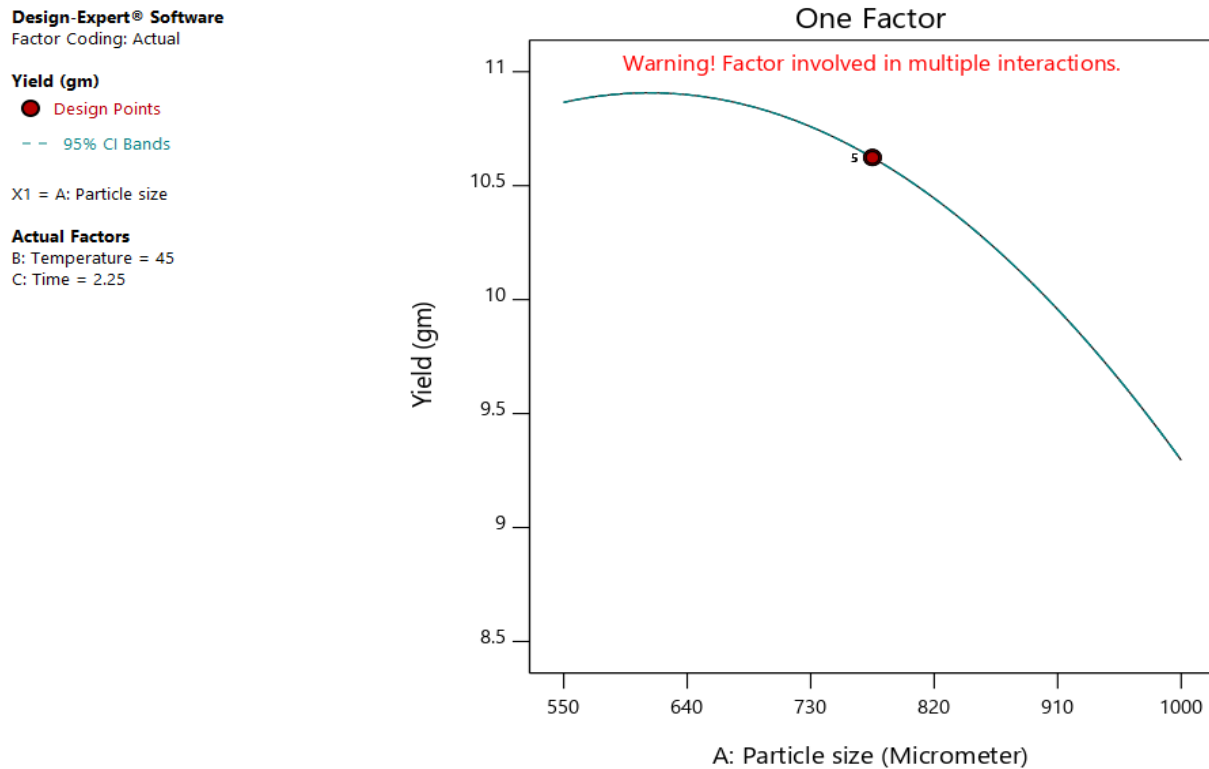


Figure 4: 5- Effect of particle size on yield

4.6.2. Effect of temperature on yield

A bioactive component was degraded at higher extraction temperature. Such problems were solved by careful choice of extraction temperature which gives the final required 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 40, 45 and 50°C. The yield of atropine increases with increasing temperature in small amount. From the figure below the maximum yield was found at the maximum operational temperature of 50°C that is 10.6905g. Respective with an increase in temperature gradient, atropine yield was increased simultaneously from 8.5272g to 10.6905g for 40°C and 50°C.

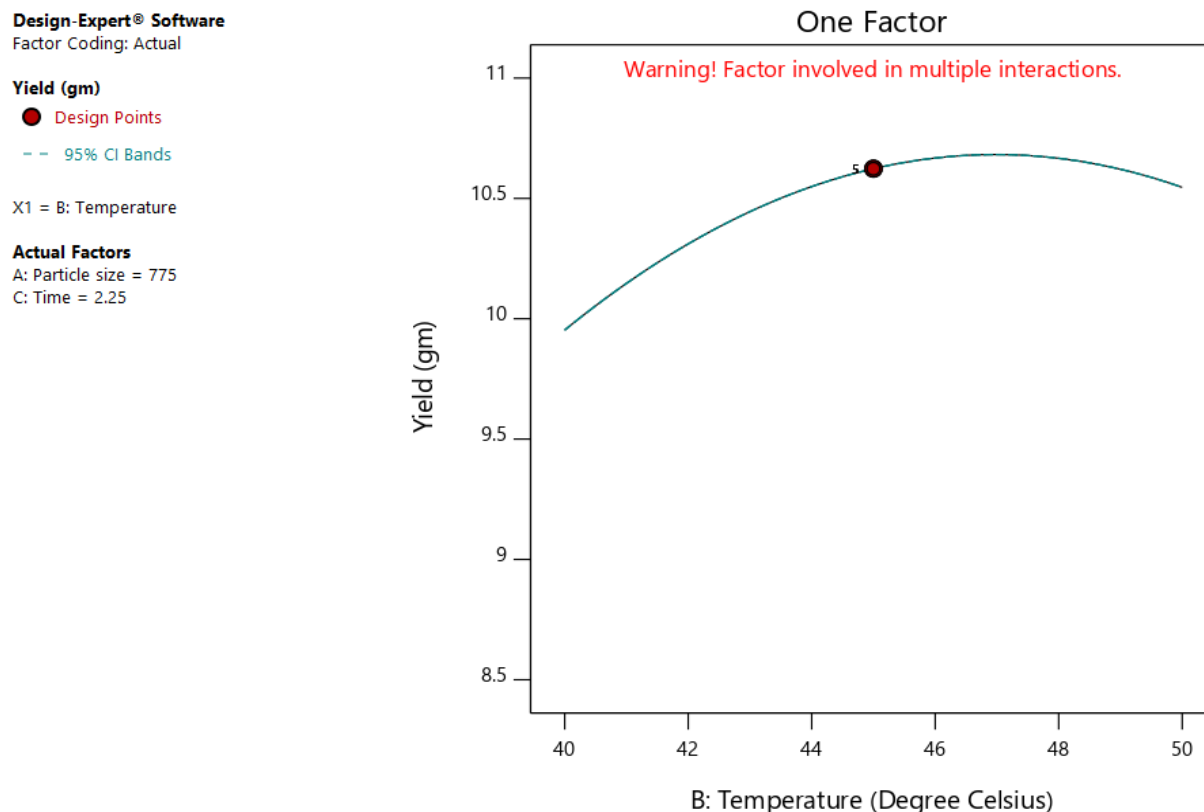


Figure 4: 6- Effect of temperature on yield

4.6.3. Effect of extraction time on yield

Extraction is increasing simultaneously with an increment of extraction time mostly. But further increment of time may dissolve unnecessary solutes in the solvent which enhancing impurity; and affect the biological activity of the active ingredient. The graph below indicates that the raise of yield was found with comparative increment of extraction time. In this study the extraction time varies from 90minutes to 180 minutes. Time which is below 90 minutes of extraction time yields small mass of atropine. The maximum yield was found when the extraction process was going for 3 hours and it was 10.4494. The yield was declined proportionally to 9.9878g and 8.9072g. This was takes place due to a short period of contact hour between the solid and the solvent.

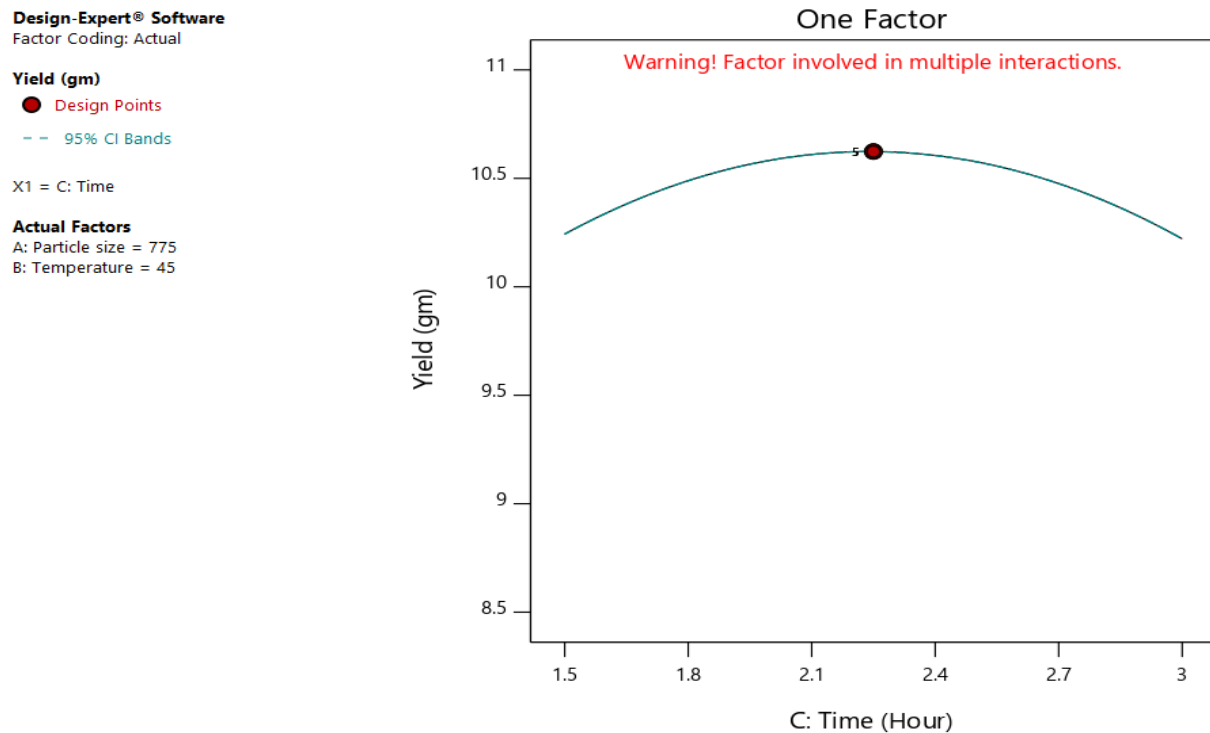


Figure 4: 7- Effect of extraction time on yield

4.7. Interaction effect of factors on yield

A combination effect of two or more individual factors that can be possibly affects the response in the same or different way is an interaction effect. 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%.

4.7.1. Effect of particle size and temperature on yield

The Figure below indicates that, the interaction between particle size and extraction temperature was significantly affect the response. From the ANOVA Table, the p-value of particle size and extraction temperature was <0.0001. This means the p-value of the interaction is less than the p-value of the model design. Generally, there was combined effect of particle size and extraction temperature observed on the yield. So, the yield was dependent of particle size and extraction time influence at the same time. The proportionate increment of particle size and extraction temperature was significantly affecting the yield. When extraction temperature goes from 40 to 50°C and particle size goes from 1000 to 550µm yield is increased.

Extraction and Characterization of Atropine from Jimson Seed (*datura stramonium*) as biopesticide and evaluate its bioactivities against Maize weevil (*Sitophilus Zeamais*)

Design-Expert® Software
Factor Coding: Actual

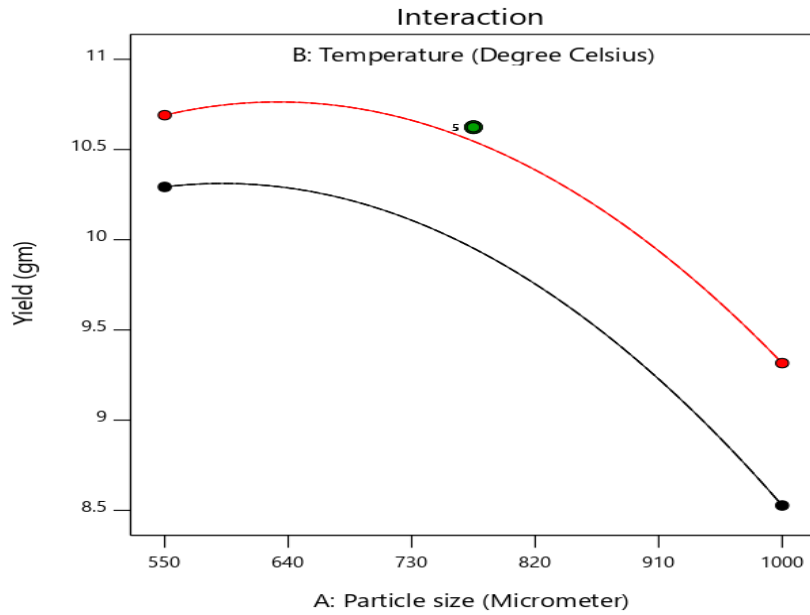
Yield (gm)

● Design Points
-- 95% CI Bands

X1 = A: Particle size
X2 = B: Temperature

Actual Factor
C: Time = 2.25

B- 40
B+ 50



(a)

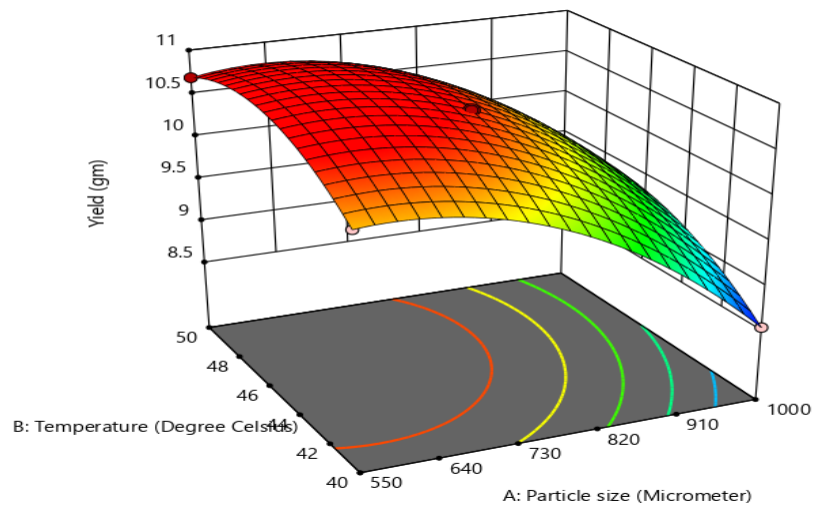
Design-Expert® Software
Factor Coding: Actual

Yield (gm)

● Design points above predicted value
○ Design points below predicted value
8.5272 10.6905

X1 = A: Particle size
X2 = B: Temperature

Actual Factor
C: Time = 2.25



(b)

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

4.7.2. Effect of particle size and extraction time on yield

The graph below shows the interaction of the particle size and extraction time. The extraction gives higher yield when particle size gradually decreased from 1000 μ m to 550 μ m and extraction time increased from 1.5hour to 3hour. The result found at these both operational conditions were 10.6745g. The major observable issue here is the interaction of these factors was concurrently; i.e., when extraction time increases and particle size decreases the yield was interacted at the same point of interaction. This shows there is significant interaction between the factors at that point.

Design-Expert® Software
Factor Coding: Actual

Yield (gm)

● Design Points

-- 95% CI Bands

X1 = A: Particle size

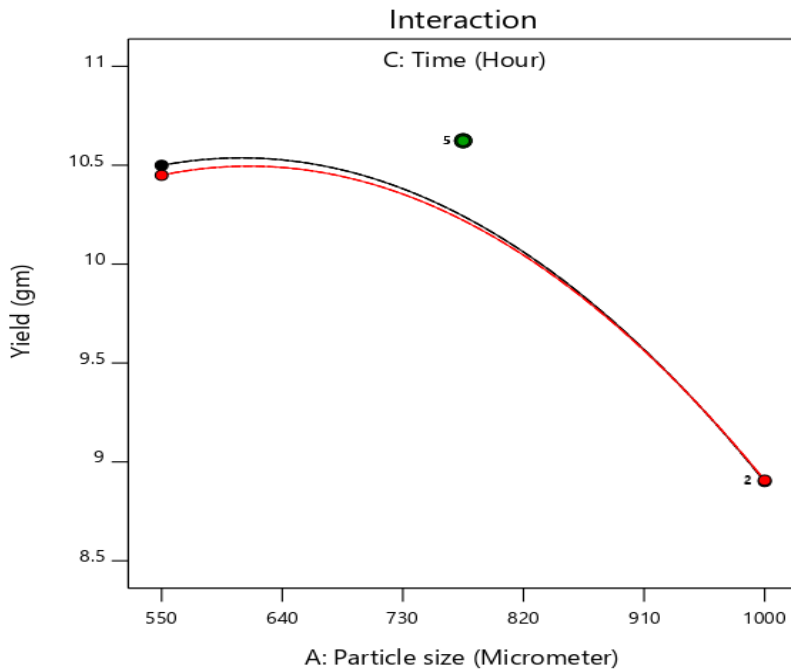
X2 = C: Time

Actual Factor

B: Temperature = 45

C- 1.5

C+ 3



(a)

Design-Expert® Software
Factor Coding: Actual

Yield (gm)

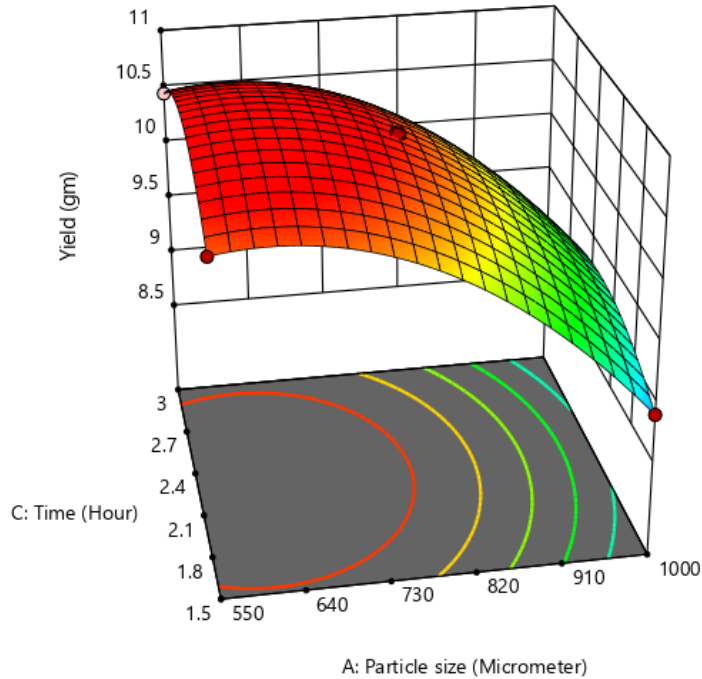
● Design points above predicted value

○ Design points below predicted value

8.5272  10.6905

X1 = A: Particle size
X2 = C: Time

Actual Factor
B: Temperature = 45



(b)

Figure 4: 9- Interaction (a) and 3D (b) plots of particle size and extraction time

4.7.3. 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 1.5 to 3 hour with a maximum yield of 10.6745g. So, the interactive factor of temperature and extraction time was significantly affecting the yield.

Extraction and Characterization of Atropine from Jimson Seed (*datura stramonium*) as biopesticide and evaluate its bioactivities against Maize weevil (*Sitophilus Zeamais*)

Design-Expert® Software
Factor Coding: Actual

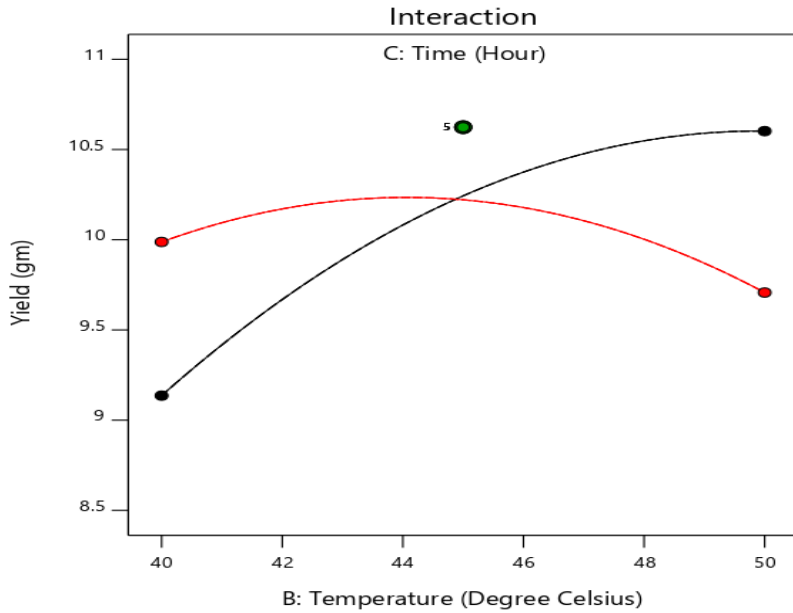
Yield (gm)

● Design Points
-- 95% CI Bands

X1 = B: Temperature
X2 = C: Time

Actual Factor
A: Particle size = 775

C- 1.5
C+ 3



(a)

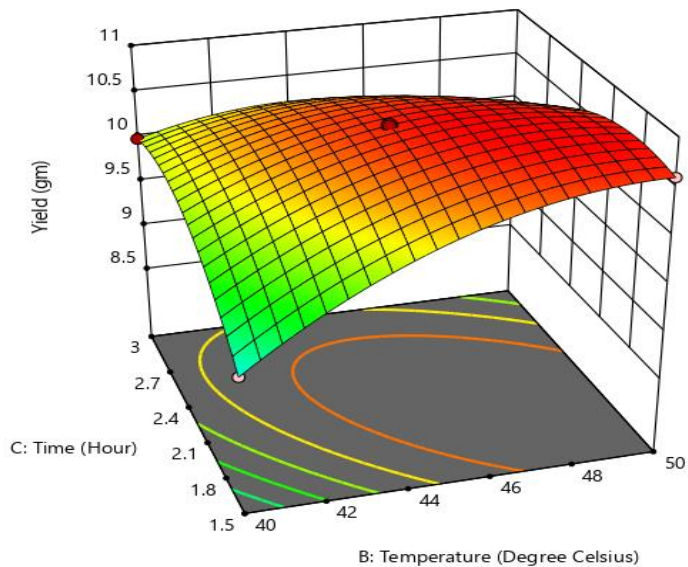
Design-Expert® Software
Factor Coding: Actual

Yield (gm)

● Design points above predicted value
○ Design points below predicted value
8.5272 10.6905

X1 = B: Temperature
X2 = C: Time

Actual Factor
A: Particle size = 775

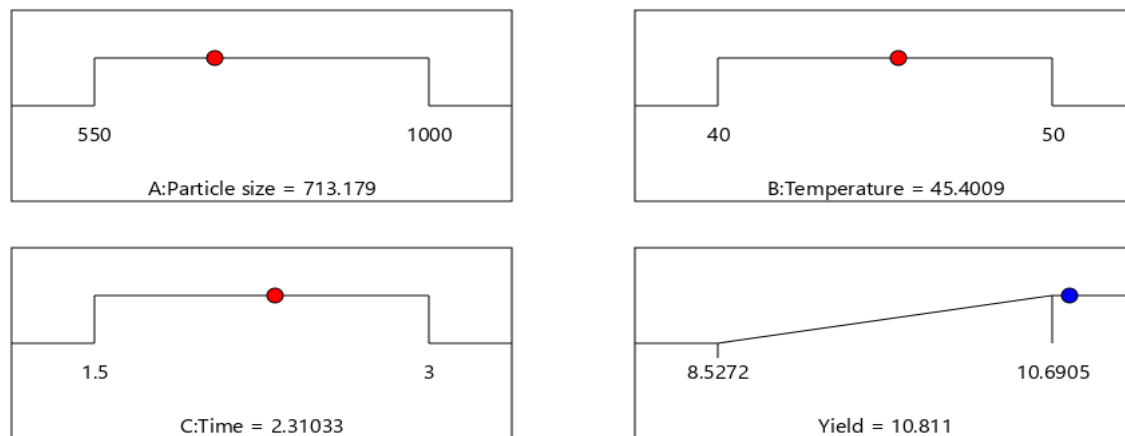


(b)

Figure 4: 10- Interaction (a) 3D (b) plots of temperature and extraction time

4.8. 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. 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 chosen by the method of numerical optimization was the one with higher desirability (i.e. 100%) efficiency. The operation conditions of the chosen optimized process method were; 713.179 μ m, 45.4 $^{\circ}$ C and 2.31hr of particle size, extraction temperature and extraction hour and the experiment were done twice on those parameters to check the consistence of the yield produced. The optimized solution is summarized as follows:



Desirability = 1.000
Solution 1 out of 100

Figure 4: 11- Ramp representation of optimal conditions for atropine

4.9. Biopesticide formulations

Liquid atropine based biopesticide formulations are carried out by mixed with water. Out of liquid formulation Ready-to-Use Low Concentration Solutions are suitable for atropine. Low-concentrate RTU formulations are consist of a small amount of Atropine (often 1% or less per unit volume) dissolved in water.

4.10. Evaluation of Atropine based bio-pesticide in Maize Weevil (*Sitophilus Granarius*)

Since most soil insecticides used against larval rootworms are either organophosphates (Chlorpyrifos, Terbufos, and methyl parathion) or carbamates (carbofuran) or pyrethroid insecticide (tefluthrin). But all this are poisoning and harmful to human, nontarget animal and increase soil acidity. Since atropine act in the same system to those pesticide its was most promising replacement for it.

The efficiency of the biopesticide depend on its concentration, population of maize weevils and time (duration). So, these factors are the most criteria for selection of dilution ratio. Atropine based biopesticide is dissolved on water and applied to the Maize Weevil (*Sitophilus Granarius*) and mortality was assessed after 12 hours but the effect of the bio-pesticide was observed after 48 hours. So here the effect is presented for 48 and 72 hours.

Table 4: 8- effect of atropine based biopesticide on maize weevil

Dilution rate (gm of atropine/ml of water)	Initial number of maize weevils	Number of maize weevils after 48 hours	Percent death of maize weevil after 48 hours	Number of maize weevils after 72 hours	Percent of death of maize weevil after 72 hours
1:100	13	8	38.5	4	69.5
1.5:100	10	6	40	3	70
2:100	15	9	40	4	73.33
2.5:100	10	6	40	2	80
3:100	12	7	41.6	2	83.3

As seen from the table below as the amount of gram of atropine increase in 100ml of water increase the percent of death of maize weevil also increase. So further increase amount atropine in the dilution ratio increases the concentration of biopesticide.

Comparing the effects of the bio-pesticide to those synthetic pesticide on maize weevil after 48 hours and 72 hours the result was presented on below table. In these comparisons five chemical pesticide on market used as pesticide against maize weevil is compared with atropine based biopesticide on depend on percentage of death of after their application.

Table 4: 9 comparison of effect of biopesticide with synthetic pesticide on maize weevil

Pesticide	Percent of death after 48hours	Percent death of after 72hours
Chlorpyrifos	27.8	35.2
Terbufos	35.7	54.3
methyl parathion	40.4	63.5
Carbofuran	16.18	21.5
Tefluthrin	52.5	69.6
Atropine	41.6	83.3

From the result above the atropine based biopesticide was efficient in small quantity and less time when compared with chemical synthetic.

Chapter Five

5. Conclusions and Recommendation

5.1. Conclusions

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

Extraction of atropine from Jimson seed was carried out in four main stages. These are pretreatment, extraction, separation and concentration. In this study three variables i.e., particle size (550, 750 and 1000 μ m), extraction temperature (40, 45 and 50°C), and extraction time (1.5, 2 and 3 hours) were used as a factor and studied their effect on the yield. Significance ANOVA analysis and optimization was performed using RSM of Box-Behnken method due to the shorter time for experiment. The result from ANOVA implied, all the individual factors and a combination of particle size – extraction temperature, particle size – extraction time, and extraction temperature - extraction time had a significant effect on the yield of atropine.

Characterization of the extract indicates that, moisture content of the seed was 24.241 % (w/w), ash content $3.979 \pm 0.187\%$ (w/w), organic matter 93.393% (w/w), HPLC retention time of 5.5-minute, specific gravity 1.4164 ± 0.07225 , flash point $36.06 \pm 0.9932^\circ\text{C}$, water solubility 99.768 ± 0.0217 (w/v), pH value 5.2 ± 0.18708 and TVOC of $0.17426 \pm 0.0311\%$ (w/w).

Depending, on the result of Box-Behnken method an optimized yield of 10.811g concentration was found at 713.179 μ m, 45.4°C and 2.31hr from 100gm of ground Jimson 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 atropine for pesticide purpose.

From the result of assessment of the percent of death of maize weevil after 48hrs and 72hrs, the atropine based biopesticide was efficient in small quantity and less time when compared with chemical synthetic. The percentage of death of maize weevil was 41.6% for 48hours and 83.3% for 72hours for atropine based biopesticide.

5.2. Recommendation

If some further research work is carried out to explore the potential of atropine for biopesticide, recommended studies are listed below:

- Concentration of atropine must be analyzed using known concentration of atropine (pure) by using calibration curve method using HPLC. The lack of pure atropine in the market limits this study from determining the concentration.
- Reserches should be done on the different extraction process and pesticide formulation
- The effect of acetylation should be studied in detail
- The optimization of the extraction process using Box-Behnken method of experimental design was analyzed by considering a higher constraint factor value. 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.
- It is recommended to perform preliminary design, economic feasibility study and establish economic scale for production of biopesticide from Jimson seeds.

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Appendixes – A

Table. A - 1. HPLC Analysis result

Peak	Peak Start	Rt (min)	End	Height λ (nm)	Area
1	1	0	2.5	0.598	6.78
2	2	2.6	2.8	2.95	50.3
3	3	3.05	3.17	3.2	9.29
4	4	3.4	3.49	3.53	6.79
5	5	3.6	3.61	3.9	6.8
6	6	4.1	4.3	4.49	90.3
7	7	4.5	4.6	4.9	6.795
8	8	4.95	5.05	5.45	297
9	9	5.47	5.5	5.59	7.5
10	10	5.62	5.7	5.82	15.6
11	11	5.93	6	6.13	7.01
12	12	6.15	6.3	6.45	6.78
13	13	6.5	6.75	6.98	6.81
14	14	7.01	7.2	7.35	6.795
15	15	7.41	7.45	7.53	6.73
16	16	7.55	7.6	8.01	6.75
17	17	8.1	8.33	8.5	6.75



Fig A-1 Acid-Water extraction



FigA-2 Filtration of clear supernatant

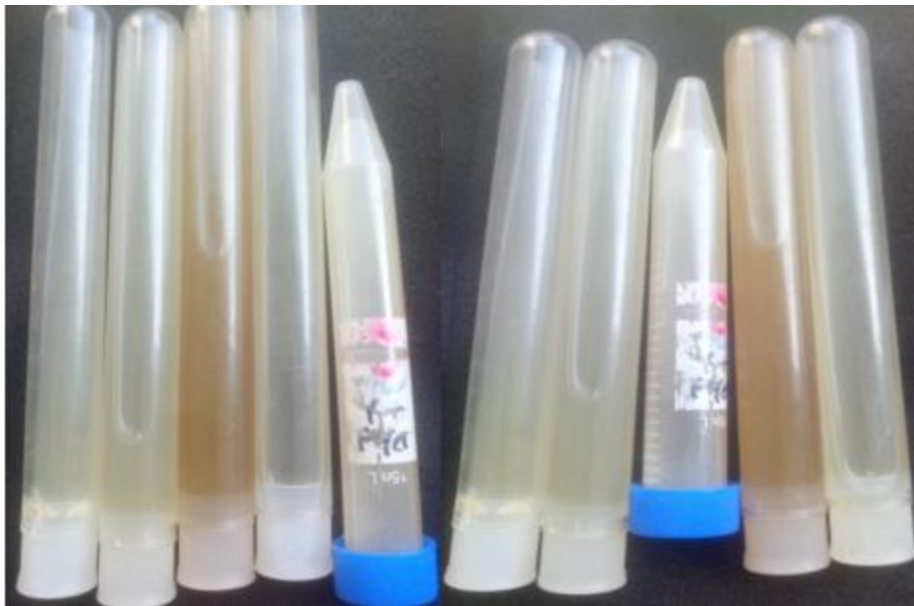


Fig A-3 the solution with white slurry at the bottom

Extraction and Characterization of Atropine from Jimson Seed (*datura stramonium*) as biopesticide and evaluate its bioactivities against Maize weevil (*Sitophilus Zeamais*)

```

PE IR          SUBTECH    SPECTRUM    ASCII      PEDS      4.00
-1
V2, ATR correction ( Leta, AAIT).asc
20/11/11
13:49:43.00
20/11/11
13:50:03.00
Administrator
Sample 011 By Administrator Date Wednesday, November 11 2020
400.000000
#HDR
-1
-1
#GR
cm-1
log(1/R)
1.0
0.0
4000.000000
-1.000000
3601
8
1.156372
-0.190664
#DATA
4000.000000 0.010432
3999.000000 0.010564
3998.000000 0.010321
3997.000000 0.009961
3996.000000 0.009845
3995.000000 0.009986
3994.000000 0.010210
3993.000000 0.010502
3992.000000 0.010847
3991.000000 0.010964

```

Figure A-3 the result of FTIR

Design-Expert® Software
Factor Coding: Actual

All Responses



X1 = A: Particle size
X2 = B: Temperature

Actual Factor
C: Time = 1.94438

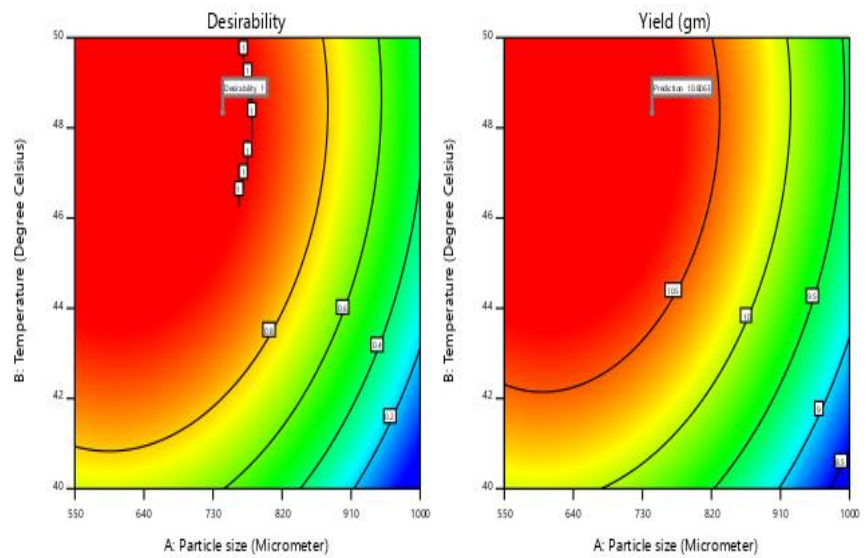


Figure A-4. Optimized contour plot of Model Desirability

Design-Expert® Software

Yield

Color points by value of Yield:

8.5272  10.6905

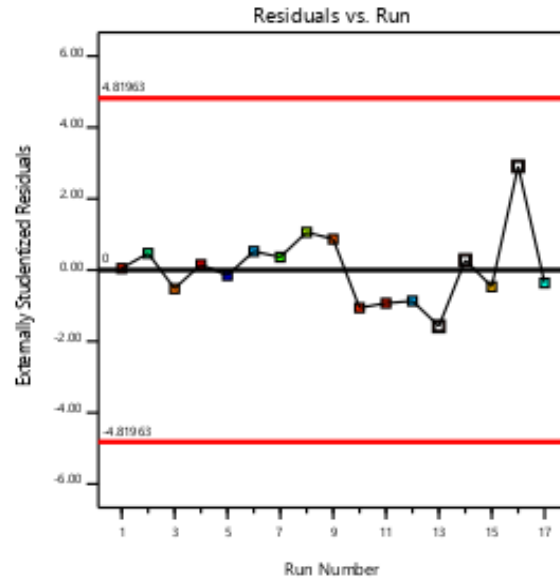


Fig A-4 Residual autocorrelation plot model

