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

Production and Characterization of Custard Apple Seed Extract for its Possible Application as Bio-pesticide

A Thesis Submitted to the School of Chemical and Bio Engineering, Addis Ababa Institute of Technology in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemical Engineering (Process Engineering Stream)

By

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This is to certify that the thesis prepared by Assefa Taye, entitled: ***"Production and Characterization of Custard Apple Seed Extract for its Possible Application as Bio-pesticide)"*** and submitted in partial fulfillment of the requirements for the degree of Masters of Science in Chemical Engineering (Process Engineering) complies with the regulation of the University and meets the accepted standards with respect to originality and quality.

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ABSTRACT

The intensive use of synthetic pesticide has undeniably resulted in adverse and sometimes irreparable effects on the environment including human health. Therefore, alternative pest management strategies are desired. Natural pesticides have proven potential for pest management and they are being used throughout the world. To this effect, this thesis investigated the production and characterization of locally available custard apple seed extract for its possible application as natural pesticide using ethanol (97%) as a solvent. The raw seed moisture content, fat content, ash and organic matter content were determined properly. The extraction parameters were extraction time, temperature and ethanol to custard apple seed ratio. A maximum of 12.63 % total extraction yield of pesticide was achieved at the optimal conditions of 4h, 50℃ and 7:1 v/w of extraction time, temperature and ethanol to CAS ratio respectively. The individual and interaction effect of these parameters on the yield of oil was studied and optimum conditions were established by using RSM of Box-Behnken method of Design Expert 7.0.0 software. The characteristics of CAS extract were then evaluated using different analytical methods. Specific gravity, flash point, water solubility, pH value and TVOCs were analyzed with respective methods. The FT-IR analysis indicates presence of C=O group of the unsaturated lactone group. The GC-MS result shows that the presence of uvaricin, annonacin and squamocin. From the statistical analysis method, all the three factors had an impact on the yield directly and extraction time with *temperature, extraction time with ethanol to CAS ratio, temperature with ethanol to CAS ratio had also a significant effect on the yield. Using model optimization of the factors were found the optimum points of extraction time (3.99hr), temperature (49.92℃) and ethanol to CAS ratio (7.04:1 v/w) with 12.63 % yield of product. Generally, the results found in this study showed that CAS extract seems to be a viable alternative option for the production of the natural pesticide.*

Keywords: *Natural pesticides, Custard apple seed, Uvaricin, Annonacin, quamocin and RSM*

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LIST OF ACRONYMS

ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ASE	Accelerated Solvent Extraction
BBD	Box – Behnken Design
CAS	Custard Apple Seed
CIF	Cost, Insurance, and Freight
CL	Confidence Level
DDT	Dichlorodiphenyltrichloroethane
EPA	Environmental Protection Agency
ETB	Ethiopian Birr
FAO	Food and Agriculture Organization of the United Nations
FTIR	Fourier Transform Infra-Red Spectroscope
gm	Gram
GC-MS	Gas Chromatography-Mass Spectrometer
GTP	Growth Transformation Plan
h	Hour
LC_{50}	50% Median Lethal Concentration
LD_{50}	50% Median Lethal Dose
LSD	Least Significant Difference
MIC	Methyl Isocyanide
mL	milliliter
MoFED	Ministry of Finance and Economic Development
NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)

NMA	National Meteorological Agency
NOx	Nitrogen oxides
OECD	Organization for Economic Co-operation and Development
PLE	Pressurized Liquid Extraction
RSM	Response surface methodology
RT	Retention Time
S.D	Standard Deviation
S.G	Specific Gravity
SF	Supercritical Fluid
SFE	Supercritical Fluid Extraction
TCDO	Tetrachlorodecaoxide
TVOCs	Total Volatile Organic Compounds
USA	United States of America
UV	Ultra Violate
WHO	World Health Organization

1. INTRODUCTION

1.1. Background

Future agricultural and rural development is, to a large extent, influenced by the rapidly increasing food demand of 2.5 billion people estimated to swell the world population by 2020. Attaining food sufficiency in a sustainable way is a major challenge for farmers, agro-industries, governments and researcher(Margarita, 2011). Now-days the rapid increase in population and demand of food materials has initiated the large use of pesticides.

For better production and aesthetic value, farmers are using a large amount of insecticides during the entire period of agricultural activity(Punyavthi, 2012). Pesticides are the substances proposed for preventing, destroying, repelling any pest. Use of pesticides has absolutely changed our society and has probably helped save millions from starvation, but it also presents a problem since pesticides may lead to cancer, fatal poisoning, sterility, allergic reactions and other serious infectious diseases. These toxic chemicals are resulting in harmful effects and biological magnification due to which our environment is continuously polluting and fertile lands are getting infertility(Kaur & Garg, 2014). There is no doubt they are providing expected results such as eradication of insects, pests and diseases but they are also killing useful organism present in soil due to which the fertility of the soil is rapidly declining. The conventional farming practices which uses chemical methods to kill both useful and harmful life forms extensively, resulting in the malfunctioning of food chain and food web.

The history of pesticide use is almost as old as human agriculture, and one of the earliest written reports on the use of pesticides can be found in Homer’s Iliad, where he refers to the use of sulfur as a mean for pest control. In 1939 and throughout World War II a major breakthrough in the pesticide field was made; the synthesis of DDT. Dichlorodiphenyltrichloroethane **(**DDT) was discovered by Swiss chemist Paul Müller and was rapidly used worldwide to combat malaria and other pests, and thus became the first of the modern pesticides(Mcafee Alison, 2017).

The start of using chemical pesticides has created a bit of irreparable damages on the global ecology. Developed countries excluded many organochlorine pesticides during the early seventies. Specialists in the National Cancer Institute, USA believe that the rising incidents of Non-Hodgkin's Lymphomas, a form of cancer are due to increased use of organophosphate pesticides and phenoxy herbicides, the swelling effects of these pollutants on the human organism. Toxaphene, Hexachloro cyclohexane, Trichlorophenol, Strobane, Perthane, TCDO, Dieldrin, DOT, 1, 2. dichloro ethane, Heptachlor, Picloram etc. have been confirmed to be lymphatic cancer causing pesticides(Abdul Khader, 1999). Most of the pesticides currently being used have a tendency to survive in plants for a long time and they also go into the food chain.

To maintain the yield of crops, it is necessary to keep the pests away from the crops by using pesticide. Organic agriculture is a fast growing area of agriculture. Insecticides and pesticides with lesser non-target effect and residue problems are what the modem agriculturists' are looking for. Based on WHO study, 80% of the populations living in the developing countries rely exclusively on traditional medicine for their primary health care needs and paste control of which most include the use of plant-based extracts(WHO, 2000). Bio-control is the best technique to cope up with the losses done by the chemicals. In the present investigation, oil is extracted using ethanol as a solvent from custard apple (*annona squmocia*) seeds and used as bio-pesticide to control pests. The oil extracted from custard apple seeds contain acetogenin a group of powerful respiratory inhibiting toxic components, which is responsible to act as a bio-pesticide(Moolchandani et al., 2019).

Previous research reported the main active compound of custard apple seed is annonain and squamocin belonging acetogenin compound. Actogenin compounds can inhibit the action of enzyme NADH in the mitochondria causing the death of larvae, as well as toxic contact and stomach poison to insects(Pandey & Barve, 2011).

The insecticidal activity of the seed is due to the presence of bio-active ingredients. Seeds and peels are by product of custard apple processing still having many bioactive compounds. By products utilization are used to no waste approaches that are environmentally friendly.

1.2. Problem Statement

Pesticides have numerous supportive advantages to the world. These include crop protection, preservation of food and prevention of vector-borne disease. In order to better production and aesthetic value, farmers are using a huge amount of synthetic insecticides during the entire period of growth of crops and vegetables. This activity is creating an adverse effect on the environment, the non-target organisms and the soil fertility because of non-degradability of the synthetic pesticides.

Consumption food with huge amounts of synthetic pesticides over a short period of time would most likely affect the nervous system. Pesticides can store in human body, causing numerous health related problems, such as disrupting the endocrine system, which can influence development, growth, reproduction, and behavior. And also Pesticides can contaminate neighboring ground and surface water. In the same way, dependence on chemical pesticides to manage pest problems has serious environmental failure and caused serious health effects on agricultural employees and rural communities**.**

In order to maintain the crops yield pesticide must be used but not the synthetic pesticide. It should be substituted by bio-pesticide. Pesticides with lesser non-target effect and residue problems are what the modem agriculturist's arc looking for. The pathway for the development of such pesticides can be understood only from naturally occurring compounds having insecticidal effect. This means that there is pressing need to develop safe alternatives to conventional insecticides.

The problem can be overcome by using some natural extract which are available easily, at cheaper rates and more effective than the available synthetic pesticides. The internal core of the ripe custard apple fruit is delicious and of nutritive value. After consumption of edible core, the obtained seeds are discarded as waste as are non-edible. Custard apple seed extract is one of naturally existing pesticide because it contains acetogenin group of powerful respiratory inhibiting toxic component which are responsible to perform as a bio-pesticides. It is eco-friendly, biodegradable and will not cause water or air pollution. Previous researches on pesticide from custard apple seeds were performed by using soxhlet extraction mechanisms, which is difficult to see the effect of temperature and its interaction on a yield and also no more study is conducted in Ethiopian custard apple seeds variety for pesticidal purpose. In this study, the custard apple seed oil was extracted using batch-wise extraction method with ethanol solvent and the extract was further processed to produce bio-pesticide. The product characterized and used as insecticide.

1.3. Objective

1.3.1. General Objective

The general objective of this study was production and characterization of locally available custard apple seed extract for its possible application as natural pesticide.

1.3.2. Specific Objectives

The specific objectives of this study were

- To determine the proximate analysis of the custard apple seeds.
- To extract the custard apple seed oil for pesticide activity using one setup extraction mechanism.
- To develop bio-pesticide from the custard apple seed extract.
- To analyze the physicochemical characteristics of custard apple seed extract pesticide.
- To study and determine the optimum operating extraction parameters (extraction time, temperature, ethanol to custard apple seed ratio).

1.4. Scope of the study

The main focus of this research was directed towards the production and characterization of bio-pesticide from natural plant seed (custard apple) within certain parameters: extraction time, extraction temperature and ethanol to custard apple seed ratio with (2, 3 and 4 hours), (30, 40 and 50℃) and (5:1, 7:1 and 9:1v/w) respectively. The proximate analysis such as moisture content, ash content and crud fat content of the custard apple seed were analyzed with standard methods. The product has been characterized by FTIR analysis, GC-MS analysis, water solubility, flash point, specific gravity, TVOCs and pH values.

In this research the application of the product on different pest on field is not included only focuses on production of the pesticide and characterization.

The individual and interaction effect of parameters on extraction were analyzed using Design Expert 7.0.0 software. The statistical significance of each experimental factor was determined and the corresponding mathematical prediction model was generated.

1.5. Significance of the study

This study is an experimental study of solid-liquid extraction method; bio-pesticide production from custard apple seed.

- This study is to introduce the application and possibility of custard apple seed as pesticide which is natural material.
- Provide a means to exploit and manage local available resources
- To create jobs for those will be engaged in planting/cultivating of the fruit as well as establishing small scale pesticide producing plants.
- Increase the foreign currency by reducing the imported synthetic pesticides.

- It will reduce the risk of disease due to synthetic pesticide usage reduction.
- It used to conserve the aquatic eco-system.

2. LITERATURE REVIEW

2.1. Pesticide

According to FAO (1989) a pesticide is any substance or mixture of substances intended for preventing, mitigating, destroying or controlling any pest including vectors of human or animal diseases, unwanted species of plants or animals causing harm during, or otherwise interfering with, the production, processing, storage, or marketing of food, agricultural commodities, wood and wood products, or animal feedstuffs, or which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies. The term includes chemicals used as growth regulators, fruit thinning agents, defoliants, desiccants, and agents for preventing the premature fall of fruits, and substances applied to crops either before or after harvest to prevent deterioration during storage or transport (Zacharia & James, 1996). Pesticides are the only toxic substances released deliberately into our environment to kill living things.

Dichlorodiphenyltrichloroethane’s (DDT) insecticidal action was discovered by the Swiss chemist Paul Hermann Müller in 1939. DDT was used in the second half of World War II to control malaria and typhus among civilians and troops. The first pesticide synthesized was the dichloro diphenyl trichloroethane in 1874 by the Austrian chemist Othmar Zeidler. In 1998, 20,000 commercial products were registered as «pesticides» by the United State Environmental Agency of Protection(Garcia et al., 2012).

Pesticides destroy, prevent, or repel pests, such as insects, weeds and rodents, but may cause a range of harmful health effects in humans, including cancer, short and long-term injury to the nervous system, lung damage, reproductive dysfunction, and possible dysfunction of the endocrine and immune systems. Thus only the safe and best alternative is to use natural pesticides.

Nowadays, synthetic insecticides are used widely for the control of various insect pests because they can be applied whenever and wherever needed, economical and most important thing is the reliability of control method.

Hence, the production and consumption of pesticides has greatly increased in recent years. The contribution of pesticides to increase agricultural production cannot be denied, but synthetic pesticides have also caused unprecedented ecological damage, also induced serious health hazard among workers during manufacture, formulation and field applications(Ansari & Kumar, 1988).

2.1.1. Pesticide in Ethiopia

Ethiopia economy is based on producing and maintaining crops and farmland. But the country is still not sufficient in food. In order to achieve self-sufficiency in food production modern agricultural technology must be practiced by farmers, together with proper use of pesticides. Ethiopia's current development agenda is guided by a vital strategy called the Growth and Transformation Plan (GTP), which aims to eliminate poverty and reach the level of low middle-income economy by 2025(MoFED, 2016). To realize this growth and transformation plan goal, the government of Ethiopia has prioritized strategic sectors, such as agriculture and industry.

At present, most of pest control products are imported from abroad even though many of the raw materials for formulating pesticides, such as solvents, dust diluents, and packing materials, are locally available(UNIDO, 1989). Adami Tulu pesticide factory is one factory that formulates chemical pesticides within the country which is located at 170 km south of Addis Ababa. This company uses imported active ingredients and solvents to formulate a portion of the pesticides required in Ethiopia. The plant has a capacity to formulate 1500 metric tons of powder and the same quantity liquid pesticide formulations every year(Belay, 2016).

Pesticides are mostly imported for agricultural purposes while some amounts of pesticides are imported for health care and industrial purposes. Large quantities of pesticides are imported annually to Ethiopia. As shown in Table 2-1 below averagely 12,350,915 Kg (more than 12,350 tons) of various pesticides are that are worth more than 1,891,383,697 Ethiopian birr were imported annually.

Table 2-1 Ethiopian pesticide import data

Year	Net Wt. (Kg)	CIF Value (ETB)
2014	12,041,676	1,501,328,913
2015	11,086,290	1,613,531,003
2016	16,126,465	2,229,811,495
2017	15,326,427	2,289,822,927
2018	7,203,719	1,822,424,149
Average	**12,356,915**	**1,891,383,697**

Source: Ethiopian Revenue and Customs Authority

2.1.2. General classifications of pesticides

According to the source of origin pesticides can be classified as natural (Bio-pesticides) and Synthetic (Chemical) pesticides(FEPA, 2004).

a. **Natural (Bio-pesticides):** Bio-pesticides are types of chemicals extracted from natural materials such as plants, animals, bacteria or certain minerals and these chemicals can be used for controlling pests. The most common benefits of natural pesticides are less toxicity, fast biodegradability and target to specific pest, maintain ecological balance(Kumar, 2015). The results of a number of studies have been revealed that natural pesticides are comparatively safe to non-target organisms, natural enemies, pollinators, fish, bird and fish, predators, parasitoids, pollinators, secondary insect pests, wild relatives of crops, and soil biota. The natural (bio-pesticides) are categorized among three major groups such as (i) Microbial pesticides (ii) Plant-Incorporated-Protectants and (iii) Bio-chemical pesticides.
b. **Synthetic (Chemical pesticides):** Synthetic pesticides are toxic chemicals that are made to repel, prevent and kill pests. They also cause significant harm to other, helpful living organisms and generate a serious health and environmental risk.

2.1.3. Further classification of pesticides

Pesticides differ in identity, physical and chemical properties, it's therefore reasonable to have them classified and their properties studied under their respective groups.

I. Classification of pesticides based on the mode of action.

Under this type of classification, pesticides are classified based on the way in which they act to bring about the desired effect. In this way pesticides are classified as contact (nonsystemic) and systemic pesticides. The non-systemic pesticides are those that do not appreciably penetrate plant tissues and consequently not transported within the plant vascular system(Yadav & Devi, 2017).

The non-systemic pesticides will only bring about the desired effect when they come in contact with the targeted pest, hence the name contact pesticides. On the other hand, the systemic pesticides are those which effectively penetrate the plant tissues and move through the plant vascular system in order to bring about the desired effect(Yadav & Devi, 2017).

II. Classification of pesticides based on the targeted pest species

In this type of classification, pesticides are named after the name of the corresponding pest in target such as insecticide, herbicides, rodenticides, fungicides, bactericides, virucides, algaecides, molluscicides and acaricides and miticides.

Insecticides

Insecticide is a pesticide that is used to kill insects, or to disrupt the growth or development of insects. The insecticides available can be classified as follow:

- ✓ **Natural (Bio) insecticide**: These type insecticides are derived from a natural source mostly classified under Plant-based (Plant-incorporated protectants) and mineral oils
- ✓ **Synthetic insecticides:** synthetic insecticides are toxic chemicals that are made to repel, prevent and kill pests synthetically. Generally classified as organic and inorganic insecticide

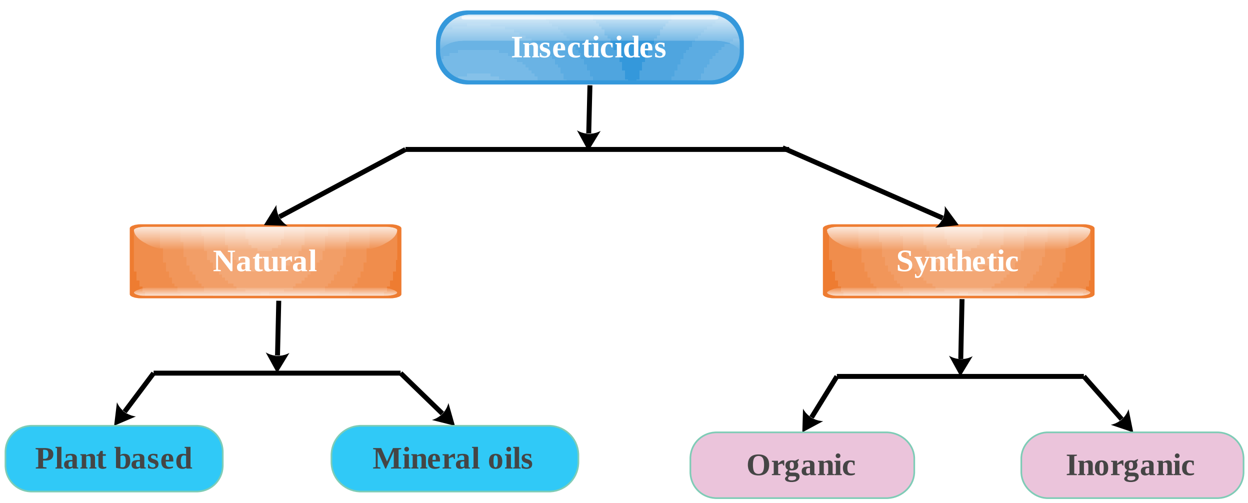


Figure 2-1 Classification of insecticides

Fungicides

Fungicides are chemical substance that abolishes or inhibits the growth of fungi that can cause serious damage in agriculture and used to prevent the growth of molds and mildew(Kumar, 2015).

Herbicides (weed killers)

Herbicides are chemical substances used to control unwanted plants. They are widely used in agriculture, industry, and non-crop areas for weed management(K-State Research, 2015).

Rodenticides

Rodenticides are pesticides that kill rodents. Rodents include rats, mice, squirrels, woodchucks, chipmunks, porcupines, nutria and beavers.

III. Classification of pesticides based on the chemical composition.

Under chemical classification, pesticides are categorized according to the chemical nature of the active ingredients into different families, ranging from organochlorine and organophosphorus compounds to inorganic compounds. The chemical classification of pesticides is by far the most useful classification to researchers in the field of pesticides and environment and to those who search for details. Some of them are discussed below:

Organochlorine

- **Organophosphates:**

Organophosphates pesticides are esters derived from phosphoric acid. In man act on the central nervous system by inhibiting acetyl cholinesterase, an enzyme that modulates the amount and levels of the neurotransmitter acetylcholine, disrupting the nerve impulse by serine phosphorylation of the hydroxyl group in the active site of the enzyme(Garcia et al., 2012).

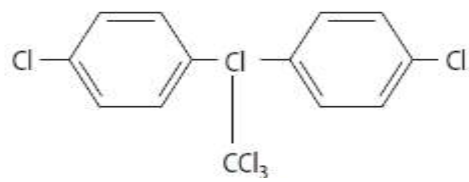
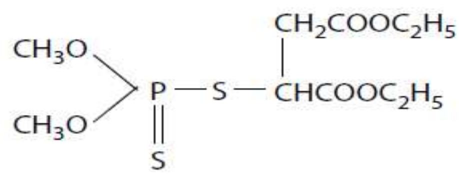
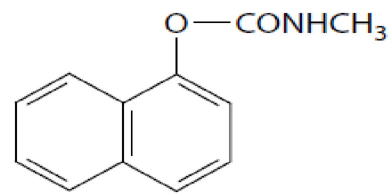
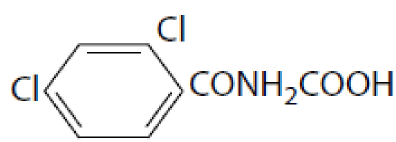
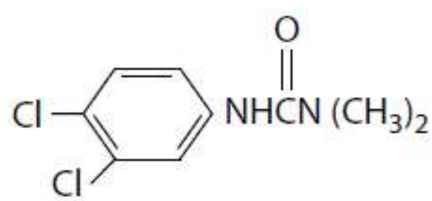
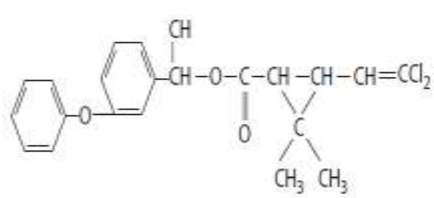
- **Carbamates**

They are esters derived from acids or dimethyl N-methyl carbamic acid that are used as insecticides, herbicides, fungicides and nematicides. Carbamates are less persistent than organochlorines and organophosphates and similarly the latter inhibit acetyl cholinesterase.

- **Pyrethroids**

They originate from natural insecticide derived from pyrethrum extract derived from chrysanthemum flowers, known as pyrethrins.

Table 2-2 Classification of pesticides based on chemical nature

No.	Chemical Type	Example	Structure	Typical action
A	Organochlorine	*p,p′*-DDT		Insecticide
B	Organophosphates	Malathion		Insecticide
C	Carbamates	Carbaryl		Insecticide
D	Dithiocarbamates	Thiram	$(CH3)_2$ N–CS–S–CNSN $(CH_3)_2$	Fungicide
E	Carboxylic acid derivatives	2,4-D		Herbicide
F	Substituted ureas	Diuron		Herbicide
G	Triazines	Simazine		Herbicide
H	Pyrethroids	Cypermethrin		Insecticide

Source: Handbook of pesticides(Leo & Hamir, 2010).

IV. Classification of pesticides based on toxicity criteria

The WHO Recommended Classification of Pesticides by Hazard was approved by the 28th World Health Assembly in 1975 and has since gained wide acceptance. The classification is based primarily on the acute oral and dermal toxicity to the rat since these determinations are standard procedures in toxicology(WHO, 2009).

Table 2-3 WHO classification of pesticide based on toxicity

WHO Class	Toxicity level	LD_{50} for the rat (mg/kg body weight)	
		Oral	Dermal
Ia	Extremely hazardous	< 5	< 50
Ib	Highly hazardous	5–50	50–200
II	Moderately hazardous	50–2000	200–2000
III	Slightly hazardous	Over 2000	Over 2000
U	Unlikely to present acute hazard	5000 or higher	

Source: The WHO Guidelines to Classification of pesticide by hazards 2009

2.1.4. Mode of pesticide poisoning

Pesticides are divided mainly into four groups depending on their mode of poisoning action.

- **Physical Poisons**

Physical poisons pesticides kill living organisms by physical action. For example, endrin penetrates percutaneous through the epidermis of the skin and produces effects that are lethal. Silica and charcoal dusts also interfere in the inhalation of air through the passage that is nasal thereby accumulate in the lungs.

- **Nerve Poisons**

Pesticides such as DDT, methyl isocyanide (MIC), malathion, parathion, diazinon, and systox act as nerve poisons. They initiate extreme nervous excitation, cause the release of excessive neuroactive substances, and disrupt nerve activity.

- **Protoplasmic Poisons**

Pesticides including endrin, ziram, lead arsenate, and sodium arsenite cause precipitation of protein in the body, resulting in liver damage and ultimately death.

- **Respiratory Poisons**

Fumigants such as hydrocyanic gas, methyl bromide, ethylene dichloride, and ethylene dibromideinactivate respiratory enzymes such as oxidases, peroxidases and reductases and ultimately, they cause acute suffocation and block the respiratory tract.

2.1.5. Impact of Pests on agriculture

Agriculture is the science and art of cultivating plants and livestock. It is essential to all aspects of life. Mankind is always in competition with pests for available natural resources and most importantly food and food crops. A pest is any living organism, whether animal, plant or fungus, which is invasive (troublesome) to plants or animals, human or human concerns and livestock.

Insect pests inflict their damage on stored products mainly by direct feeding. Some species feed on the endosperm causing loss of weight and quality, while other species feed on the germ, resulting in poor seed germination and less feasibility(Sallam, 2001). Consequently, due to damage done by insects, grains lose value for marketing, consumption and planting.

2.1.6. Effect of pesticides

In modern agriculture the use of chemicals has significantly increased productivity. But it has also significantly increased the concentration of pesticides in food and in our environment, with related adverse effects on human health(Andersson et al., 2014). Pesticides are important chemicals that are used to mitigate crop damage pests and to improve productivity in agricultural sector. Pesticides are not only an agricultural commodity but find use in non-agricultural regions. But the very nature of the pesticides to kill renders them harmful for the humans and other living things. Their extensive use with insufficient technical advice or research has brought environmental problems such as contaminating our soil, water and food, thus risking our wellbeing(Leo & Hamir, 2010).

Annually there are dozens of million cases of pesticide poisonings worldwide(Andersson et al., 2014). According to estimates by the World Health Organization (WHO) each year between 500,000 and 1 million people are poisoned by pesticides and between 5,000 and 20,000 die(WHO, 1990).

The majority of pesticides are not specifically targeting the pest only and during their application they also affect non-target plants and animals.

2.1.6.1. Potential Impact of Synthetic Pesticide on Human Health

The main means of human exposure to pesticides are through the food chain, air, water, soil, flora and fauna(Aktar et al., 2009). Pesticides are distributed throughout the human body through the bloodstream but can be excreted through urine, skin and exhaled air. Pesticide poisonings remain a serious concern, especially in developing countries, even though these nations account for only 25 per cent of pesticide usage(Human Rights Council, 2017).

There are four common ways pesticides can enter the human body:

I. **Through the skin:** It is called absorption or dermal. The pesticides enter the body through the pores used for releasing sweat from the body. It usually happens when a worker mixes or sprays pesticides or becomes exposed without wearing suitable personal protective clothing.

II. **Through the mouth (ingestion or oral):** This usually happens when eating food and drinks on the field or dust swallowed with saliva or smoking cigarettes. Sometimes it happens by using chemical containers to carry drinking water without cleaning appropriately.

III. **Through the eyes:** Sometimes called ocular. In mixing of pesticides or spraying work activities without appropriate protective clothing, splashes of dust or vapors may enter through the eyes.

IV. **Through respiratory pathways (inhalation):** The pesticides are breathed in as dust, fumes, vapors or gasses especially if not wearing suitable individual protective equipment.

The toxicity of pesticides can vary depending on the type of exposure such as dermal, oral, or respiratory (inhalation). Synthetic pesticides cause: acute and chronic human health effects causing damage to the nerves, damage to the lungs, cancer, sterility, birth defects, allergic reactions and fatal poisoning. Agricultural workers are regularly exposed to toxic pesticides via spray, drift or direct contact with treated crops or soil, from accidental spills or insufficient personal protective equipment. Families of agricultural workers are also exposed to the possibility of being attacked, as workers bring home pesticide residues on their skin, clothing and shoes(Human Rights Council, 2017).

Seasonal and migrant workers are also more susceptible, as they may work temporarily at numerous agricultural sites, reproducing their exposure risk to pesticides. Those living close to industrial agricultural lands and plantations may also be at serious risk of pesticide exposure.

Children are most defenseless to pesticide contamination, as their organs are still developing and, owing to their smaller size, they are exposed to a higher dose per unit of body weight; the levels and activity of key enzymes that detoxify pesticides are much lower in children than in adults. Pregnant women who are exposed to pesticides are at advanced risk of miscarriage, pre-term delivery and birth defects. Studies have regularly found a cocktail of pesticides in umbilical cords and first feces of newborns, proving prenatal exposure.

- **Acute effect:** In general, acute poisoning occurs immediately or shortly after a single episode of exposure or short-term exposure to a pesticide. Acute poisoning generally affects farmers and industrial workers. Acute effects include nerve, skin, and eye irritation and damage, headaches, dizziness, nausea, fatigue, and systemic poisoning - which can sometimes be dramatic, and even occasionally fatal(Kumar, 2015).
- **Chronic effect:** The chronic effects are long term effects and may occur years after even minimal exposure to pesticides in the environment, or result from the pesticide residues which we consume through our food and water(Kumar, 2015). A person may also have chronic toxic effects after being in contact with a small amount of pesticides for days, months or years. Chronic poisoning is more common in the general population.

2.1.6.2. Potential Impacts of Synthetic Pesticide on Environment

As pesticides are designed to be toxic to specific groups of organisms, they can have significant adverse environmental effects on other living creatures as well as diverse media including air, soil, or water(Aktar et al., 2009). Excessive use and misuse of pesticides result in contamination of surrounding soil and water sources, causing loss of biodiversity, destroying beneficial insect populations that act as natural enemies of pests and reducing the nutritional value of food.

- **Impacts on non-target organism:** Chemicals are still the main means of pest and disease control in agriculture, but they may have negative effects on beneficial organisms and on other non-target organisms in the environment. Pesticides can kill non-target organisms in several ways, including direct ingestion of granules, baits, treated seeds, and direct exposure to sprays(Fishel, 2014).

- **Loss of biodiversity:** Biodiversity refers to the diversity of living organisms which exist on earth. Factors affecting biodiversity include the expansion of human habitat, the reduction of wildlife habitats, as well as the negative impact of invasive species, such as weeds and fungi. Pesticides are a major factor affecting biological diversity globally, along with habitat loss and climate change(Isenring, 2010).
- **Impacts on soil micro-flora**: Pesticides contaminate and degrade soil to varying degrees. They enter the soil via spray drift during flora treatment, wash-off from treated leaves of plant, release from granulates or from treated seeds in soil. Persistence of pesticides in soil can vary from few hours to many years in case of organochlorine pesticides and affects soil microorganisms.
- **Impacts on water and air ecosystem**: Many pesticides are not easily degradable, they persist in soil, leach to ground and surface water and contaminate wide-ranging environment. Pesticides can get into water through drift during spraying of pesticide. In some cases pesticides can be applied directly onto water surface for control of mosquitoes and indirectly through surface runoff or drain flow. When pesticides reach water bodies, they are quickly absorbed and accumulated by the bottom sediment, plankton, algae, aquatic invertebrates, aquatic vegetation and fish(Ogunfowokan et al., 2012).

2.2. Natural sources of pesticide

Many natural products are phytotoxic, but few have both the correct physicochemical properties and level of biological activity to be good pesticides. Natural (bio-active pesticide) ingredients can be extracted from plants and used again to destroy or protect pests(Rimando & Duke, 2006). Today due to awareness about the harmful effects of the chemical pesticides, most of the farmers are diverting towards the organic farming.

There are different sources of organic (natural) pesticides throughout the world such as, custard apple seed, neem seed, garlic, mint, papaya, lantana leaf, onion bulb, calotropis leaf, datura leaf etc…(Kumawat et al., 2014)

2.3. Custard apple

Custard apple is a yellowish green fruit of the family of plant species Annona squamosa L (Annonaceae). *Annona* means year's harvest and *squamosa* means scaly referring to the scale like structure of the fruit surface(Nag et al., 2018). Annona squamosa L. is a small, semi-deciduous tree, 3-7 m in height, with a broad, open crown or irregularly spreading branches. Custard apple fruit is considered as one of the delicious and nutritionally valuable fruit and goes by various local names (in Amharic Gishta). The pulp of ripe sugar apple can be processed into juice or other processed products.

Figure 2-2 Custard apple plant and fruit

2.3.1. Custard apple seed

The seeds of a custard apple are dark brown in color and are found inside the flesh of the fruit. In each fruit there are numerous seeds. The seeds contain chemicals known as acetogenins, which are toxic to insects. Bark of custard apple has phytochemical compounds such as alkaloid, tannin, protein, saponin, phenolic compound etc. These compounds have antioxidant activity that scavenging free radical and important for pharmacology(Neha & Dushyant, 2011). Sikdar et al reported that a s the concentration of custard apple seed oil increases from 0.00% to 0.75% the number of mealy bugs decreases to zero within 2 days. Hence, the oil solution of 0.75% is effective to keep away the pests(Sikdar et al., 2016). The custard apple seed kernels contain 14- 49% of whitish or yellowish, non-drying oil with saponification index of 186.40(Chakraverty et al., 2016).

Figure 2-3 Custard apple seed

2.3.2. Custard apple seed cake

The inner core of the ripe fruit is delicious and of nutritive value. After consumption of edible core, the obtained seeds are discarded as waste as are non-edible. Since the Custard apple seeds are available free of cost therefore development of metal adsorbent from them may prove cost effective(Santhi et al., 2010). The Custard apple seeds cake with physical treatment (heating at 500^0C in air free atmosphere) plus chemical treatment (H_2SO4) can be transformed into metal adsorbent(Parate et al., 2016). This shows that the potential of custard apple seed for removal of removal of heavy metals such as Ni (II) ions. Additional the custard apple seed cake can be used as organic fertilizer. This suggests utilization of wastes as value added product.

Figure 2-4 Custard apple seed cake

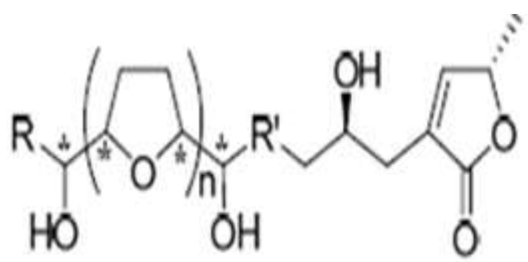
2.3.3. Acetogenin compounds

Acetogenin compounds are a class of polyketide products that are natural in plants of the family Annonaceae. They are characterized by linear 32- or 34-carbon chains and the terminal carboxylic acid is combined with a 2-propanol unit at the C-2 position to form a methyl-substitutedα, β-unsaturated-γ-lactone. containing oxygenated functional groups including hydroxyls, epoxides, ketones, tetrahydrofurans and tetrahydropyrans(Li et al., 2008).

Many acetogenin compounds are characterized by neurotoxicity which is form of toxicity in which a biological, chemical, or physical agent produces an adverse effect on function of the central and peripheral nervous system(Cunha-Oliveira et al., 2008). The bio-active acetogenin (BAA) consists of C-35 or C-37 presumably derived from C-32 or C-34 fatty acids combined with two propanol units.

The active ingredient is the chemical or chemical compound that performs the intended purpose of a substance. In the case of a pesticide, the active ingredient is the material intended to kill the target pest. Acetogenin compounds are the active ingredients present in the custard apple seed extract intended to kill the past.

Previous research reported the main active compound of sugar apple seed is annonain and squamocin belonging acetogenin compound. Acetogenin compounds can inhibit the action of the enzyme NADH in the mitochondria, causing the death of larvae, as well as toxic contact and stomach poison to insects(Djamil et al., 2011).



n=1-3, R, R'=hydrocarbon chain having oxygenated moieties and/or double bond

Figure 2-5 Representative structure of the annonaceous acetogenins

2.3.3.1. Polyketides

Polyketides represent a family of highly structurally diverse compounds of secondary metabolites which either contain alternating carbonyl and methylene groups (-CO-CH_2-), or are derived from precursors which contain such alternating groups. Polyketides that are found in acetogenins are bullatacin, squamocin, molvizarin, uvaricin and annonacin.

- **Bullatacin ($C_{37}H_{66}O_7$):** Sometimes called Trilobacin is bis (tetrahydrofuranoid) fatty acid lactone found in some fruits from Annonaceae family. Have a molecular weight 622.928 g/mol

- **Squamocin:** Classified as squamocin A ($C_{37}H_{66}O_7$) and squamocin B ($C_{35}H_{62}O_7$) based on number of carbon constituents**.**
- **Molvizarin ($C_{35}H_{62}O_7$):** Class of polyketide natural products found in plant of the family Annonaceae.
- **Uvaricin ($C_{39}H_{68}O_7$):** Uvaricin is a bis (tetrahydrofuranoid) fatty acid lactone present in Annonaceae family.
- **Annonacin ($C_{35}H_{62}O_7$):** is one of the bioactive components found in custard apple seed which is with toxic effects, especially in the nervous system.

2.3.4. Custard apple in Ethiopia

Custard apple plant flourishes best in dry and hot weather. Custard apple is most widely cultivated species among the genus *Annona*, which is being grown widely through the tropics and warmer subtropics. It requires tropical and subtropical climate, with summer temperature from 25^0C to 41^0C. It is moderately drought tolerant and requires rainfall about 700 mm and has some resistance to the cold(Chandel, 2017). It also requires light soil and is generally grown on the slope of hills. Ethiopia is located in the tropical and sub-tropical region mostly. The climatic region of Ethiopia is best for cultivation of the custard apple plant. The foremost growing areas of this wild custard apple include Wolega, Gamo Gofa, Jimma, Kefa, as well as Bale zones. It also grows mainly in the Harar region of the eastern parts of the country.

2.4. Extraction Techniques

Extraction is the first step to separate the desired natural products from the raw materials. There are various technologies available to extract the active ingredients of custard apple seed.

2.4.1. One setup extraction/ Extraction with alcohol

This type of extraction uses alcohol (ethanol and methanol) as a solvent and the powdered seed as solute. The alcoholic extract contains the active ingredients. By using moving bed contacting method the powder seed will be agitated for certain hour with an overhead agitator in a mixing settling tank. After decantation of the crude cake, the custard apple extract solution is drained out, filtered and delivered to the next procedure.

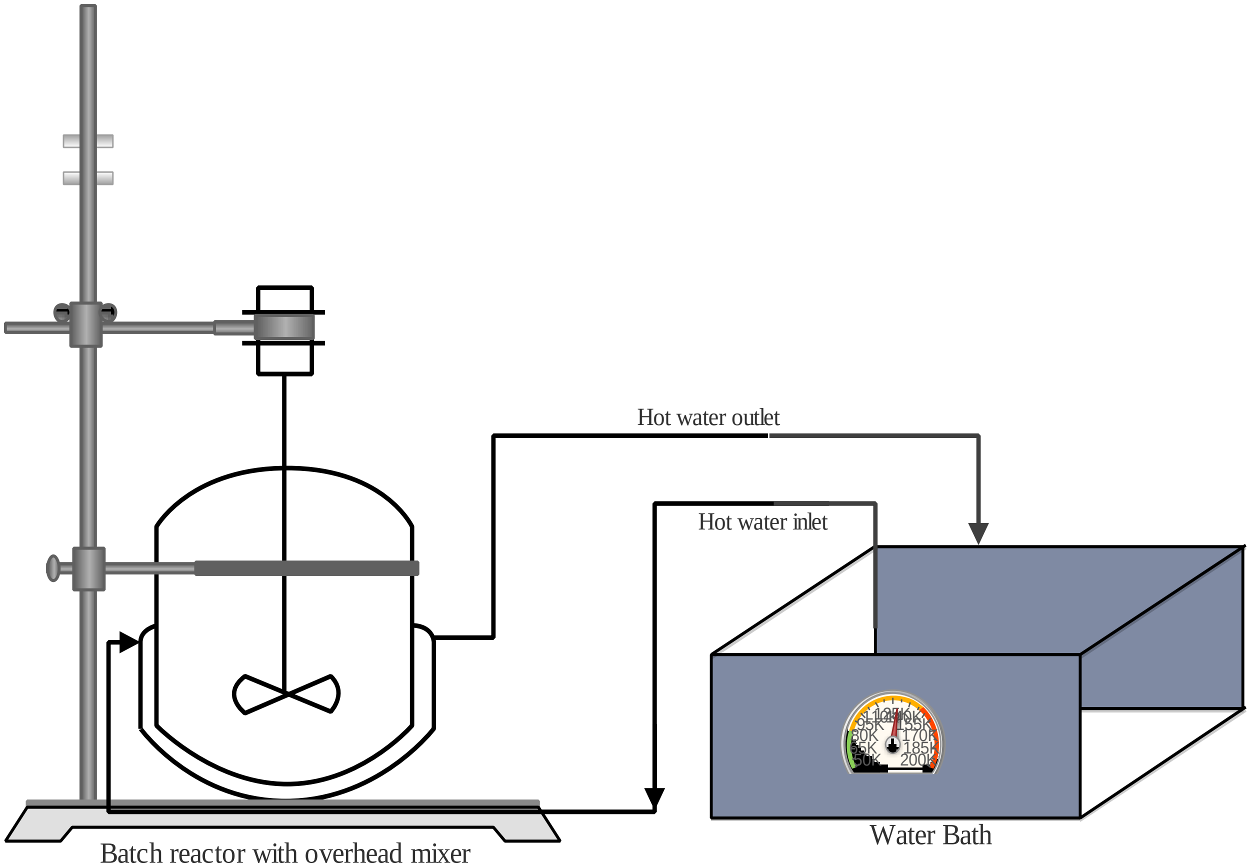


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Figure 2-6 Schematic model of one setup extraction

2.4.2. Supercritical fluid extraction (SFE)

This method uses supercritical fluid (SF) as an extraction instrument for "drawing out" the organic compounds from solid matrices. Commonly used for this purpose is CO_2, as it has relatively low critical temperature (31℃) and low critical pressure (0.73bar)(peter Atkins, Julio de Paula, 2002) . Super critical fluid extraction is not reactive and is reachable in a high degree of purity at low cost.

2.4.3. Soxhlet extraction

In this type of 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. In this technique, fresh solvent comes in contact with the plant material a number of times, until the plant material is completely extracted.

2.4.4. Accelerated solvent extraction (ASE)

Accelerated solvent extraction (ASE) method, commonly known as pressurized liquid extraction (PLE), is comparatively new sample preparation technique, that uses small amounts of water and organic solvents, and is based on the extraction under elevated temperature (up to 200℃) and pressure (up to 200bar) for short period of time, resulting in improved extraction efficiency. This solvent extraction technique was introduced by Dionex Corporation in 1995. It is a fully automated rapid extraction technique for organic compounds from solid and semisolid matrices(Bohlin, 1998). Practically, a used solvent is pumped into an extraction cell containing the sample, which is then taken to an elevated temperature and pressure. Later, the extract is shifted to a collection vial for cleanup and analysis. Solvents of different polarities starting from n -hexane to methanol can be used for this accelerated solvent extraction technique.

3. METHODOLOGY

3.1. Material and Methods

3.1.1. Materials and Equipment's

In this thesis work, the following materials and equipment's was used. These are Custard apple seed, Centrifugal mill (Retsch GmbH, 5657 HAAN), Micro wave oven with UV (SUPRA-SM20BH1), ceramic container (crucible), Measuring cylinder (10ml and 500ml), Batch extractor vessel with overhead mixer and temperature sensor, Petridish, Vacuum pump with Buchner funnel and beaker, Centrifuge, Freeze drier(LFZ/EV), Tray drier (TDC/EV), GC-MS analyzer, FTIR analyzer (PerkinElmer), moisture content analyzer(MB45, OHAUS), Filter paper, Shaker incubator (EXCELLA, E24R), rotary evaporator, water chiller, Ethanol(97%), Muffle furnace (VF2, Vecstar, U.K), Hexane (99.9%), Pycnometer (25ml), potassium bromide, separating funnel.

3.1.2. Experimental setup

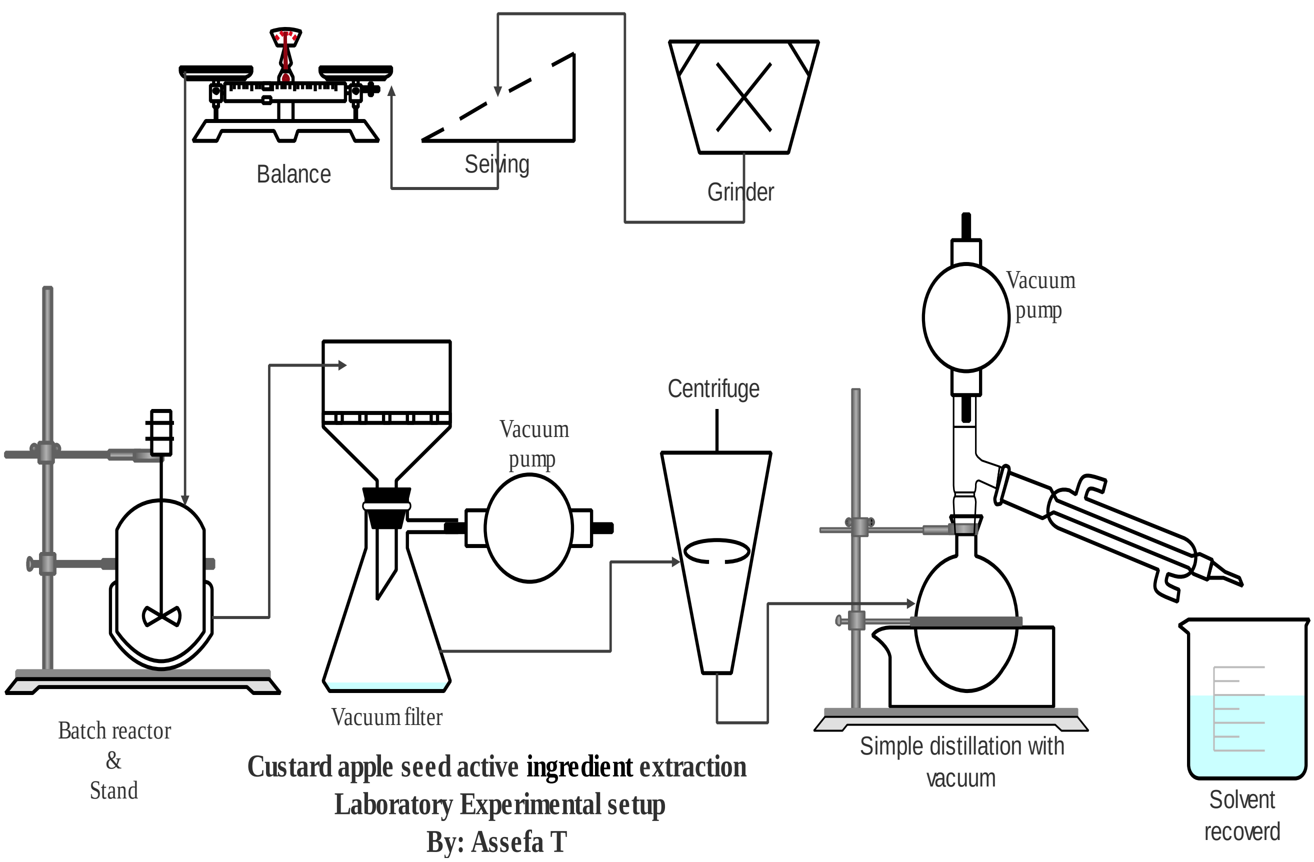


Figure 3-1 Custard apple seed active ingredient extraction laboratory experimental setup

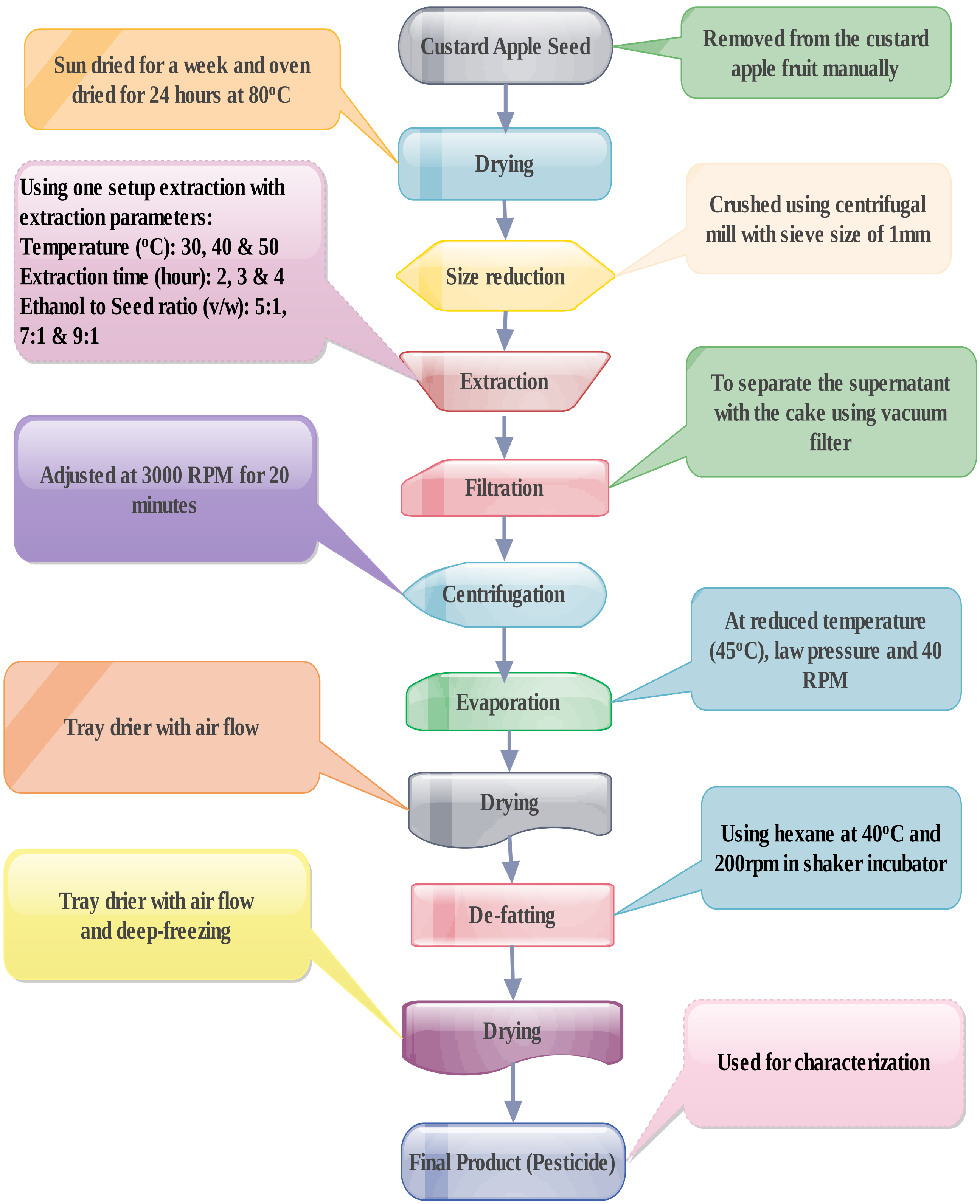


Figure 3-2 Experimental setup for bio-active component extraction and production from CAS

3.1.3. Raw material collection and preparation

The mature custard apple fruits were collected from south west region of Ethiopia, Jimma and Kaffa zones in December 2018. The seeds were removed from the custard apple fruit and collected manually. The collected seed were washed severally with water in order to eradicate impurities and foreign materials. Afterward cleaning the seeds were dried for a week using sun. After the sample was collected and packed in a polyethylene-bag, transportation is carried out by bus to Addis Ababa. The sample was stored at room temperature in Addis Ababa University Institute of Technology, Chemical Engineering laboratory until further processing.

3.1.4. Proximate compositional analysis of custard apple seed

Moisture content, crude fat and ash content were determined according to method of Association of Official Analytical Chemists (AOAC, 1990 & 2000).

- ✓ **Moisture content determination of custard apple seed**

A clean porcelain crucible was dried in an oven at 105 ± 1°C for 20 minutes, cooled in a desiccator and weighed. About 10g of the powder custard apple seed were placed in dried crucible. Then sample containing crucible was placed in an oven to dry at 105± 1°C overnight. This was then followed by cooling in a desiccator and weighing until a constant weight was attained. The moisture determination was done in triplicate and the average value was recorded as percent moisture. The percent moisture content was calculated as:

$$\text{Moisture content}(\%) = \frac{m_2 - (m_3 - m_1)}{m_2} x100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.\ldots\ldots\ldots\ldots\ldots 3.1$$

Where: m_1 = mass of empty dry crucible (gm)

m_2 = initial mass of custard apple seed sample (gm)

m_3 = mass of crucible and sample after drying (gm)

- ✓ **Ash content determination of custard apple seed**

The ash content of the custard apple seed was determined by Association of Official Analytical Chemists (AOAC 1990) standard methods. To determine the ash content, porcelain crucibles (ceramic container) was dried in an oven 105 ± 1°C for 20 minutes, cooled in a desiccator and weighed. The dried custard apple seed sample, which is transferred from moisture content analysis, was placed in a muffle furnace (VF2, Vecstar, U.K.) and burned at temperature of around 550°C for

3 hours. The crucibles was transferred to a desiccator to cool and weighed thereafter. The determination was done in triplicate and the average value was recorded. The percentage of crude ash will be calculated as:

$$\text{Crude ash }(\%) = \frac{W_3 - W_1}{W_2 - W_1} x100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.3.2$$

Where: W_1 = mass of empty dry crucible (gm)

W_2 = dry mass of custard apple seed sample and crucible (gm)

W_3 = mass of crucible and sample after burning (gm)

- ✓ **Crude Fat content determination custard apple seed**

The crud fat content of the custard apple seed was determined by Association of Official Analytical Chemists (AOAC 2000) standard method. The powdered and dried custard apple seed sample was weighed and put in to a thimble made of filter paper. The thimble was then put into soxhlet apparatus. The flask was filled with 250 mL n-hexane and extraction was done by allowing the soxhlet apparatus to reflux for 8 hour. Finally the sample was dried at 100°C in an oven for 1 hour then cooled to room temperature and re-weighed. The difference in the weights gave the fat-soluble material present in the sample. Determinations were done in triplicate and the average value was recorded.

$$\text{Crud Fat }(\%) = \frac{w_3 - w_1}{w_2} x100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots..\ldots\ldots.\ldots.. \ 3.3$$

Where: - w_1 = dry mass of thimble (gm)

w_2 = mass of custard apple seed sample (gm)

w_3 = dry mass of sample and thimble (gm)

3.1.5. Size reduction and sieve analysis

After the custard apple seed dried using sun the seed was placed in an oven at 80^{o}C for 24 hours to remove the moisture, the dried custard seed was milled in Cross Beater Miller (Centrifugal mill) to reduce the particle size and then the milled seed was shaken using vibrating shaker for 10 minutes with amplitude of 20mm with the sieve size of 0.5mm. The powder custard apple seed was kept in an oven dryer to remove the moisture content for 3 hours at 60^{o}C and packed with aluminum foil and polyethylene bag until further processing.

3.2. Optimization studies

During the extraction process three factors (variables) have been selected based on their effects on extraction. These three variables were extraction time, temperature and solvent to seed ratio.

3.2.1. Effects of temperature on extraction yield

Extraction temperature is an important factor that affects the yield of active component and affects the compound stability due to chemical and enzymatic degradation, losses by volatilization or thermal decomposition. Elevated temperature promotes extraction by enhancing both diffusion coefficients and solubility of active ingredients in a solvent. Raised temperatures also increase cell membrane permeability following the breakdown of cellular constituents thereby setting more active ingredients free to be permeable and extractable(Kankara et al., 2014). High temperatures are, however, not always suitable for extracting all sorts of active ingredient compounds because of its breakdown in the activity and equilibrium state of extraction. To extract bio-active component the preferable temperature range is 25-60℃(Moure et al., 2001). Depending on the above facts three temperature levels were selected, i.e. 30 ℃ , 40 ℃ and 50 ℃. T

3.2.2. Effects of extraction time on extraction yield

Extraction time plays significant role in extracting the active ingredient (Kankara et al., 2014). It is obviously longer extraction time could lead to increase in exposure of temperature and this might lead to degradation on the effective biological activity of the component. From this perspective, extraction time is going to be analyzed in three different levels for this study, i.e. 2h, 3h and 4h.

3.2.3. Effects of Solvent to Solid ratio on extraction yield

In one setup extraction method a high solid-to-solvent ratio is expected to be favorable in extraction of active compounds found in custard apple seed. These results were consistent with mass transfer principle where the driving force for mass transfer is considered to be the concentration gradient between the solid and the solvent. A high solid-to-solvent ratio could promote an increasing concentration gradient, resulting in an increase of diffusion rate that allows greater extraction of solids by solvent. Solvent-to-solid ratio of between 5:1 (v/w) and 10:1 (v/w) was found to be favorable for extracting of organic components(Valentin et al., 2016). Based on this fact a solvent-to-solid ratio of 5:1 (v/w), 7:1 (v/w) and 9:1(v/w) were selected for this experiments.

3.3. Experimental Design and Statistical Analysis

Response surface methodology of Box – Behnken method Design-Expert 7.0.0 software was used to determine the optimal conditions of extraction. This Box – Behnken method were used to investigate the effects of three independent factors (variables) with three levels (extraction time, extraction temperature and solvent to powder seed ratio) and to test the second and third order effects of each of this independent variables on the yield. The adequacy of the model was determined by evaluating the lack of fit that was obtained from ANOVA. The statistical significance of the model and model variables were determined with 95% confidence interval (probability level, $\alpha = 0.05$)

3.4. Extraction process

The active ingredients present in the custard apple seed was extracted by using batch-wise (One setup) extraction process. Extraction process were conducted with three extraction time (2, 3 and 4 hours), three ethanol to custard apple meal ratio (5:1, 7:1 and 9:1 v/w), three temperatures (30, 40, 50^{o}C) and constant agitation speed. After the extractions were completed the supernatant and the cake were separated by centrifuge at speeds of 3000 rpm for 20 minutes. For further clarification the supernatant was filtered by using vacuum filter. The mixed solvent was removed from the extract and the supernatant was concentrated with a vacuum rotary evaporator under low pressure, reduced temperature (45°C) and constant rotation (40rpm) to obtain a viscos extract and to recover the ethanol.

3.5. Pesticide production process

The concentrated semi-solid supernatant was dried in a tray dried to remove trace amount ethanol (solvent) remains in custard apple seed extract. During the extraction process ethanol was used as a solvent and ethanol has a capability of dissolving both polar and non-polar substances, because of the presence of both ethyl (C_2H_5) and hydroxyl (OH) groups, which are non-polar and polar respectively. Based on this fact the non-polar fat compounds should be removed from the extract by using a non-polar solvent. The dried product was de-oiled using a non-polar solvent, n-hexane, at 45^{o}C and 200rpm in shaker incubator to remove all the non-polar fat compounds and 20ml of n-hexane was used for each runs. The de-oiled brownish slurry was separated by using separating funnel by means of density difference and the separated slurry was dried again using tray drier with dry air flow rate 60m^3/h for 24hours. The dried pesticide product was further solidified by using freeze drier. Finally the solid pesticide product was tested for its pesticidal properties by standard methods.

3.5.1. Extraction and Production process flow diagram

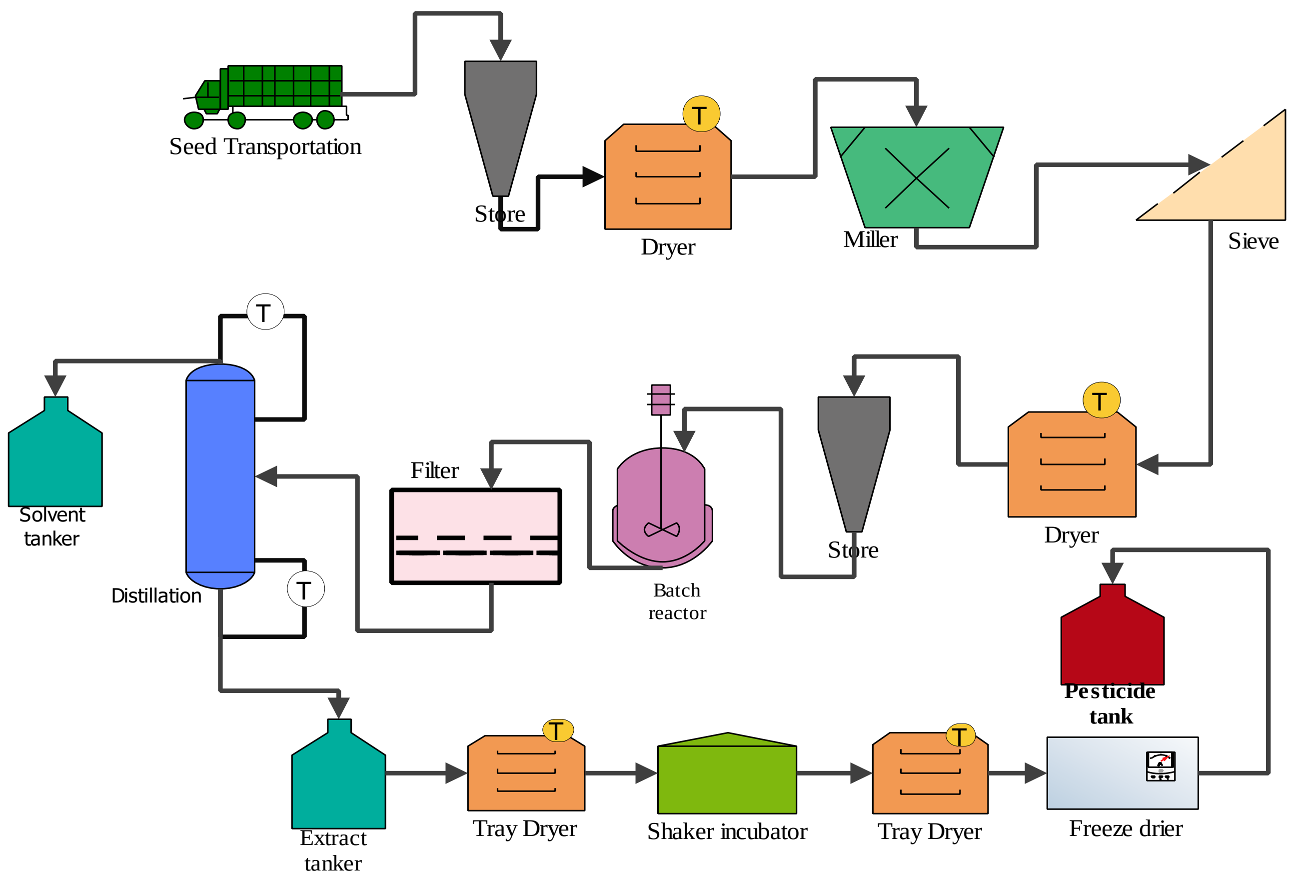


Figure 3-3 Flow diagram of extraction and production

3.6. Physicochemical characterization of product

3.6.1. Determination of Specific Gravity

Specific gravity is the measurement of relative density of the sample with respective to water. The determination was carried out by using Association of Official Analytical Chemists (**AOAC 2000**) standard method. The density of extract was determined by using density bottle method. A clean and dry density bottle (measuring cylinder) of 10ml capacity at 30^0C was weighed in gram. Then the measuring cylinder was filled with water and reweighed at 30^0C. The extract was brought to 30^0C and the water was substituted with this extract after drying the density bottle and weighted again and the specific gravity was determined by dividing the density of the extract by density of water.

$$S.G = \frac{W_2 - W_0}{W_1 - W_0} \quad \ldots\ldots 3.4$$

Where: W_0 = mass of density bottle (10ml measuring cylinder)

W_1 = mass of bottle and water

W_2 = mass of bottle and sample

3.6.2. Determination of Flash Point

The flash point is the lowest temperature at which an applied ignition source was causing the vapors of a sample to ignite. It was used to determine flammability property and reliable classification of the flammability of extract. The flash point analysis is very important characteristics in transportation and safe using of the product to protect accident and hazard in either pre or post application. The flash point was determined by heating a sample of the product in a benzene burner in the presence of oxygen. The flammability of the product was categorized as follow based on the recorded data.

Table 3-1 Classification of pesticide based on flammability

Flammability	Flash point (℃)
Extremely flammable	Below 0
Highly flammable	Below 21
Flammable	Below 55

3.6.3. Determination of Water Solubility

Solubility is a measure of the amount of Pesticide (chemical) that can dissolve in water at specific temperature. The solubility in water is specified in units of mass per volume of solution (mg/L or parts per million) at controlled pH and room temperature. Testing water solubility property is very vital for the product. Since, the final product is going to be used by dissolving in water and other organic solvents and spray on field, it should not left a residue over the crop. The water solubility test was conducted based on OECD method (Shake-Flask method) (OECD, 1995). One gram sample was dissolved in a 20ml of water at 25℃ for 20minutes with randomly selected 5 samples. The dissolved sample was filtered using filter paper and the undissolved sample remains in the filter paper. The filter paper dried in an oven and weighed. Finally the solubility of the sample was calculated. Based on the data the solubility level classified as follow:

Table 3-2 Pesticide classification based on solubility

Level	Solubility (mg/L or ppm)
Low water solubility	< 10
Moderate water solubility	10-1,000
High water solubility	> 1,000

Source: Fate and Transport of Organic Chemicals in the Environment: A practical guide 1995

3.6.4. Determination of pH Value

pH value is a logarithmic scale used to specify the acidity or basicity of an aqueous solution.
0.5g of the sample was taken and placed in a clean dry 25 ml beaker and 13 ml of hot distilled water was added to the sample in the beaker and stirred slowly. Then stirred sample was cooled in a cold water bath to 25^0C. Five samples were randomly selected for pH determination. The pH electrode was standardized with a buffer solutions first and then the electrode immersed in to the sample and the pH was read and recorded.

3.6.5. Determination Total Volatile Organic Compounds (TVOCs)

Volatile organic compounds (VOCs) refer to any compound of carbon excluding carbon monoxide, carbon dioxide, carbonic acid, metallic carbides or carbonates, and ammonium carbonate which participates in atmospheric photochemical reactions and that have a high vapor pressure at ordinary room temperature(Voorspuij, 1999). Volatile organic compounds (VOCs) are environmentally important to be determined because they participate in atmospheric photochemical reactions, when released into the atmosphere can react with sunlight and nitrogen oxides (NOx) to form tropospheric (lowest layer) ozone (smog), and they also may have important health implications(Dudley & Griffit, 2014).

Ethiopia is one of the largest Africa countries and known as a wide variety of landscapes. In the 2007 National Meteorological Agency (NMA) report, temperature data are considered, and for the 1951–2006 intervals, increasing rates of 0.13℃ per decade and 0.37℃ per decade for mean annual minimum and mean annual maximum temperature, respectively, are presented(Fazzini et al., 2015). This indicates that the temperature of Ethiopia is between 24 and 32 ℃. The average temperature of Ethiopia becomes 28℃. Therefore, the TVOC's present in custard apple seed extract were analyzed in a universally accepted ambient temperature (25 ℃).

The total volatile component present in the extract was determined by using microwave oven with a UV light output sources up to 164w and 25 ℃ temperature was used to measure the TVOC in 24 hours and then the mass difference was analyzed as follow.

$$\mathrm{TVOC}(\%) = \frac{\mathrm{M_0} - \mathrm{M_1}}{\mathrm{M_0}} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.\ldots\ldots\ldots\ldots\ldots..3.5$$

Where:-

M_o = initial mass of sample (gm)

M_1 = final mass of sample (gm)

3.6.6. Fourier Transform Infra-Red Spectroscope (FTIR) analysis

The functional groups present in the extract was detected by using FTIR analyzer was used. The wave number region for the analysis was between 4000–400cm^{-1} (in the mid infrared range). The potassium bromide (KBr) IR grade cell was washed with ethanol, cleaned and dried. 100mg of custard apple seed extract samples was weighed and spread on the potassium bromide cell and then placed at the light chamber of the spectrophotometer. The absorption spectrum was obtained after three minutes scan time. This analysis was carried out in Addis Ababa University, Collage of Natural Science, Chemistry department laboratory, by using Spectrum 65 FT-IR (Perkin Elmer).

3.6.7. Gas chromatography-mass spectrometry (GC-MS) analysis

Gas chromatography mass spectrometry (GC/MS) is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified. The GC-MS was employed with helium as the carrier gas at a constant flow of 1 mL/min. The oven temperature started at 75 °C and remained at this temperature for 3 min increasing to 120 °C at 25 °C/min ramp rate and then increased to 300 °C at 5 °C/min ramp, holding at 300 °C for 11 min. Injection port was adjusted at 250 °C and splits injection mode was used. Total GC-MS running time was 35 minutes. The relative percentage amount of each component was calculated by comparing its average peak area to the entire areas. This analysis was carried with gas chromatography (Agilent, 7890B) coupled with a mass spectrometry (Agilent, 5977A MSD).

4. RESULT AND DISCUSSION

4.1. Proximate analysis of custard apple seed

4.1.1. Moisture content determination

The moisture content is the amount of water in the seed and is frequently expressed as a percentage of the weight of the original sample. It can be expressed on either a wet weight basis or on a dry weight basis. In this case the moisture content was expressed on a dry weight basis. The importance of studying the moisture content is that a small change in seed moisture content has huge impact on the storage life of the seeds. And also the moisture content affects the extraction yield of the bio-active component by blocking the diffusivity of the solvent towards the surface of the seed(Edwards, 1943). The other advantage of knowing the moisture content is to check the requirement of pre-drying for grinding of the seed. In triplicate analysis of the moisture content the following data's were obtained.

Table 4-1 Moisture content analysis of custard apple seed

Run	Empty crucible mass (gm)	Sample mass (gm)	Dry sample & Crucible mass	Dry sample Mass (gm)	Moisture content (%)	Mean ± S.D
1	21.8	10	31.18	9.38	6.2	
2	21.8	10	31.21	9.41	5.9	6.067 ± 0.125
3	21.4	10	30.79	9.39	6.1	

$$\text{Moisture content}(\%) = \frac{m_2 - (m_3 - m_1)}{m_2} x100$$

The moisture content was calculated using the above formula for each runs and the mean ± S.D (6.067 ± 0.125) moisture content was recorded.

Additionally the moisture content the custard apple seed was analyzed using MB45 (OHAUS) moisture analyzer. The following result was recorded.

$$\text{Moisture content}(\%) = \frac{6.05 + 6.18}{2} = 6.115\%$$

The amount of moisture content observed by using the above two methods are comparatively the same. This result shows that the amount of moisture removed the custard apple seed was about 6.067

± 0.125 % from initial weight. As shown in the result above the amount of moisture content is low. This indicates that the seed requires small energy and time to remove the moisture.

4.1.2. Ash content determination

Ash is the inorganic residue remaining after the water and organic matter have been removed by heating in the presence of oxidizing agents. This provides a measure of the total amount of minerals within the seed, such as Ca, Na, K and Cl.

Table 4-2 Ash content analysis of custard apple seed

Run	Empty crucible mass (gm)	Dry sample & Crucible mass (gm)	Dry sample mass (gm)	Burned sample & crucible Mass (gm)	Burned sample Mass (gm)	Ash Content (%)	Mean ± S.D
1	21.8	31.18	9.38	22.15	0.35	3.73	
2	21.8	31.21	9.41	22.13	0.33	3.51	3.513 ± 0.176
3	21.4	30.79	9.39	21.71	0.31	3.30	

$$\text{Crude ash } (\%) = \frac{W_3 - W_1}{W_2 - W_1} x100$$

The amount of ash content in a sample indicates the total amount of nonorganic matters or compounds present in a sample (mostly metallic oxides). Applicably, these compounds are harmful by leaving non-volatile and non-decomposable matters on the environment. These cause different health problems on different floral and faunas. For this reason, the amount of ash content should be less as much as possible. From the laboratory result of a triplicated custard apple seed sample as shown above Mean ash content ± S.D (3.513 ± 0.176**)** was recorded.

Organic matter is matter that contains a large amount of carbon-based compounds and it is formed by living organisms. Organic matters are biodegradable and environmental sustainable. So, the need for high amount of organic matter is very vital environmentally and applicably.

Based on the above observed ash content the organic matter constituent of the seed was calculated and record as follow

$$\text{Mean} \pm \text{S.D organic matter}(\%) = 96.487 \pm 0.176\%$$

The custard apple seed has about 96.487 ± 0.176 percent organic compounds that are easily decomposable and degradable. At the same time they are harmless to the environment and its living things.

4.1.3. Crude fat content determination

To determine the crude fat content of the custard apple seed soxhlet extraction apparatus was used with n-hexane solvent. The solvent is heated and volatilized, then is condensed above the sample continually. Fat content is measured by weight loss of sample or weight of fat removed.

Table 4-3 Crud fat content analysis of custard apple seed

Run	Mass of sample (gm)	Dry mass of thimble (gm)	Dry mass of sample and thimble after extraction (gm)	Mass of oil extracted (gm)	Fat content (%)	Mean ± S.D
1	15	1.7	13.25	3.45	23.15	
2	15	1.4	13.1	3.3	22.08	22.3± 0.624
3	15	1.45	13.2	3.25	21.67	

The fat content of custard apple seed kernel obtained in this study was 22.3± 0.624%. The result is within the range as it is reported by Chakraverty (14- 49%) (Chakraverty et al., 2016). These results indicated very clearly that the seeds from custard apple plant form a potential source of oils and fats. Therefore, from an economic point of view, the production of oil from custard apple seed could be of interest for different purposes.

Table 4-4 Comparison of obtained proximate analysis with reported values

No	Particulars	Obtained values	Reported values	
			Ref.1 (Lokhande et al., 2013)	Ref.2 (Mariod et al., 2010)
1	Moisture content	6.067 ± 0.125	6.40±0.3	6.7 ± 0.2
2	Ash content	3.513 ± 0.176	1.77±0.2	2.2 ± 0.1
3	Fat content	22.3± 0.624	28.03±0.5	26.8 ± 0.4

Note: Values are means ± standard deviation (S.D) of triplicate determinations

The observed result is comparatively close with the reported data with slight differences. This difference may be due to the variety of the plant and area of cultivation.

4.2. Solvent Recovery

Ethanol (97%) was used to extract the active ingredient of the custard apple seed. The extracting solvent and the active ingredients were separated by using Rotary vacuum evaporator. A total of 5950mL (5.95L) ethanol was used for 17 experiments. From total ethanol used 5332mL (5.332L) ethanol was recovered. The percent recovery was calculated as follow:

$$\%\text{Ethanol recovery} = \frac{5332}{5950} \text{x}100 = 89.61\%$$

The extraction procedure should provide improved recoveries (at least 80% efficient), give higher sample throughput (sufficiently selective), consume less organic solvent, and require minimum cleanup before the determination(Leo & Hamir, 2010). Therefore 89.61% ethanol recovery is reasonable. In an economic point of view this recovering or recycling of solvent reduces liquid waste disposal cost, fresh solvent purchase cost and storage costs. In addition to that it reduces environmental impact.

4.3. Pesticide production process

4.3.1. De-Fating Process

The de-fating process was carried out by using non-polar solvent (n-hexane) and shaker incubator with constant temperature (45 ℃) and 200rpm. During this process 28.7% fat was removed from the total extract. This result shows that the custard apple seed has potential to produce fates for different purposes. The de-fatted product was dried using tray drier and then freeze drier was used for further drying. After drying the product mass was measured and recorded. The final product was taken to characterization.

4.4. Yield of custard apple seed extract for pesticidal activity

The yield of the bio-active ingredients from custard apple seed using one setup extraction method with further improvement process steps and well-defined operating parameters was found reasonable different in magnitude. These operating parameters are extraction time, temperature and ethanol to CAS ratio. The reason why this difference in magnitude could be possibly happened was due to individual and interactive effect of the factors.

From 50gm of powdered custard apple seed a maximum yield was found 6.314gm (12.63%) with operational parameters of extraction time, temperature and ethanol to CAS ratio of 4h, 50 ℃ and 7:1v/w respectively. In contrast, the minimum yield 3.381gm (6.76%) was found from the same amount of powdered custard apple seed with operational parameters of extraction time, temperature and ethanol to CAS ratio of 3h, 30 ℃ and 9:1v/w respectively.

4.5. Physicochemical characterization of product

4.5.1. Specific gravity determination

Specific Gravity (SG) is used to describe the weight or density of a sample as compared to the density of the same volume of water at a specified temperature. The density of extract was determined using density bottle method.

Table 4-5 Specific gravity analysis of custard apple seed extract

Run	Mass of 10ml measuring cylinder (gm)	Mass of cylinder + water (gm)	Mass of cylinder + extract (gm)	Specific gravity	Mean S.G ± S.D
1	36.79	45.54	37.84	0.831	
2	36.79	46.63	39.32	0.843	
3	36.79	46.62	38.28	0.821	0.837 ± 0.0151
4	36.79	46.47	40.12	0.863	
5	36.79	46.68	38.51	0.825	

The experimental result of specific gravity 0.837± 0.0151 shows that the presence of an organic matters in the sample, due to the fact that most of organic matters have a specific gravity less than one.

4.5.2. Flash point determination

Flash point is the lowest temperature causes the vapors of a specimen of the sample to ignite under specified condition of atmospheric pressure. The necessary significance of this test has been very important in controlling accident and hazard before and after use of pesticide on field. Pesticides should have a well-constructed store that is properly maintained with a safe system of work for those who access it and transport. Consequently, the producer, supplier and end users should be noticed about the flammability and possible accident that could be occurred during storage and transportation.

From this point of view, the result of five randomly selected samples was tested for flash point using benzene burner and digital thermometer and the result found 121.56 ± 1.381℃.

Table 4-6 Flash Point analysis of custard apple seed extract

Run	Flash Point (℃)	Mean Flash Point ± S.D
1	122.9	
2	119.8	
3	123.4	121.56 ± 1.381
4	120.5	
5	121.2	

4.5.3. Water solubility determination

Solubility is the amount of substance that dissolves in a unit volume of liquid substance to form a saturated solution under specified conditions of temperature and pressure. Using the above method, the result found from the test was stated below.

Table 4-7 Water solubility analysis of custard apple seed extract

Run	Water solubility (mg/L or ppm)	Mean W.S ± S.D
1	324.30	
2	312.50	
3	332.10	323.06 ± 7.08
4	328.50	
5	317.90	

This method helps to determine the disintegration and relative dissolution of custard apple seed extract in water; since the product becomes expected to be dissolve in water and consistently disperse. The time, temperatures and pressure were set to be constant i.e. 20minutes, 25℃ and atmospheric pressure respectively.

Based on the obtained result i.e. 323.06 ± 7.08 mg/L the solubility of the product was categorized under moderate water solubility as stated in Table: 3-2 because the result was found in between 10 and 1,000mg/L. In general, a high water-soluble insecticide will kill insect pests more rapidly(Cloyd, 2018). But being high water soluble has a high risk of contaminating ground and surface water. Regarding to environmental issue being moderately water soluble is advantageous. The result moderate water solubility show the possibility of dissolving in a water and spray on field, but is better to use organic solvent to dissolve the pesticide to apply on a field well.

4.5.4. pH value determination

The tendency of liberating hydrogen ion or hydroxide ion when custard apple seed extract dissolves in water was measured directly by a calibrated pH meter. This tendency could be strongly affect the environment as well as the end user. Based on the nature of the soil this acidity or alkalinity might cause soil infertility. Five randomly selected samples were used to confirm the pH value of custard apple seed extract. The result found was recorded below:

Table 4-8 pH value analysis of custard apple seed extract

Run	pH value	Mean pH value ± S.D
1	4.87	
2	4.79	
3	4.60	4.770 ± 0.1083
4	4.70	
5	4.89	

Since, the final product is going to dissolve in water to spray on afield the pH of dissolving water should not be alkaline (pH higher than 7). Because the pesticide undergo a chemical reaction in the presence of alkaline water and also the pesticide is hydrolyzed and rendered ineffective. A pH value of 4 to 7 is recommended for mixing most pesticides and spray solution(Wayne, 2018). From the observed result the pH value was found to be 4.770 ± 0.1083 which is in the recommended range. This also minimizes the buffering solution cost which is used to adjust the pH of spray solution to a prescribed level and keeps it there.

4.5.5. Total volatile organic compounds (TVOCs) Analysis

Volatile organic compounds are organic chemicals that have a high vapor pressure at normal room temperature. They are a major concern of the Environmental Protection Agency (EPA) and air quality boards across the world and have been found to be a major contributing factor to the formation of ozone, a common air pollutant that has been proven to be a public health hazard(Ultralast, 1999).

The TVOCs analysis was performed using five randomly selected samples and the mean ± S.D result was recorded.

Table 4-9 TVOCS analysis of custard apple seed extract

Run	Initial mass of sample (gm)	Final mass of sample (gm)	TVOCs (%)	Mean TVOCs ±SD
1	1.00	0.9941	0.59	
2	1.00	0.9933	0.67	
3	1.00	0.9957	0.43	0.464 ± 0.147
4	1.00	0.9973	0.27	
5	1.00	0.9964	0.36	

From the result above, the amount of TVOC's per 24 hours with a 164w and 25℃ has been found 0.464 ± 0.147%. The total volatile compounds were expected to be less as much as possible and the observed result is reasonable. This shows that the contribution for Ozone (O_3) formation, which is photochemical reactions between volatile organic compounds (VOCs) and nitrogen oxides (NO_x), is less.

4.5.6. Fourier Transform Infra-Red Spectroscope (FT-IR) analysis

In this analysis, Fourier transform infrared radiation (FT- IR) was used with wave numbers from 400cm^{-1} to 4000cm^{-1}.

Concentrated semi-solid custard apple seed extract sample were mixed with potassium bromide for pellet formation. Then the pellet were placed in FT-IR (65 FT-IR, PerkinElmer) for determination its functional groups.

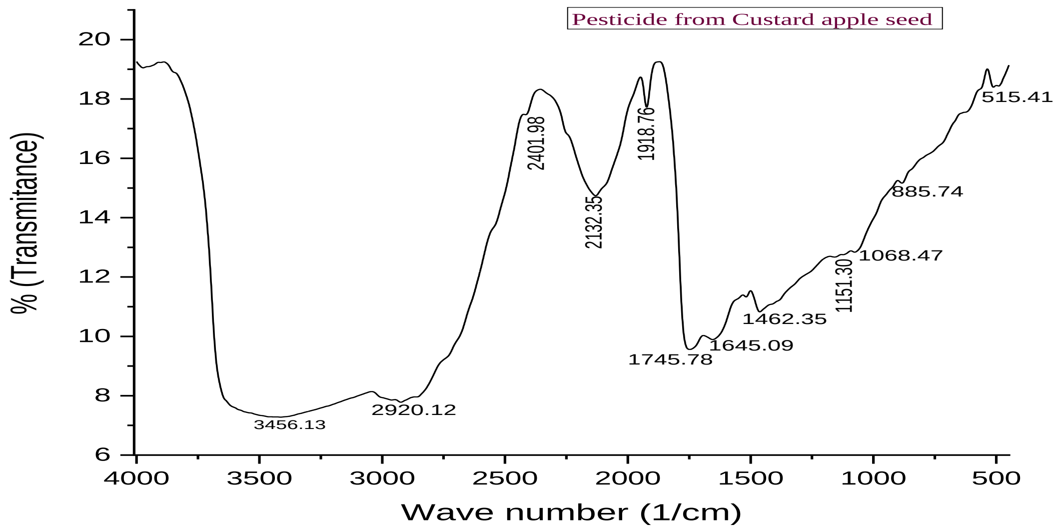


Figure 4-1 FT-IR spectrum of custard apple seed extract

The above FTIR analysis figure the peak values indicate the appropriate functional groups that are presents in the custard apple seed extract. The vertical axis is percent Transmittance (%T) and the horizontal axis is the wave number in cm^{-1}. The peak values and the functional groups are presented below

Table 4-10 Functional group interpretation by FT-IR

Wave number (cm^{-1})	Functional group Present
515.41	C-Br stretch shows the presence of alkyl halides
885.74	C-H bend
1068.47	C-N show the presence of amines
1151.30	O-C and (2 bands) and C-N shows the presence of carboxylic acid and amines respectively
1462.35	C-H bend
1645.09	C=C and C=O stretch shows the presence of alkenes and amides respectively
1745.78	C=O show the presence of ester and lactones groups
1918.76	C=C alkenes symmetric stretch
2132.35	C=C alkyne

2401.98	C=O show the presence of carbonyl compounds
2920.12	C-H shows the presence of alkanes and carboxylic acid overlapped
3456.13	N-H and O-H group presence shows high concentrations of amides amines, phenol and alcohols

As shown in the above table, the FT-IR analysis of the seed extract of custard apple (Annona squamosal*)* obtained many peaks which represented the presence of various functional groups which includes amides, amines, phenols, alcohols, alkanes, alkenes, carboxylic acids, esters, lactose, alkyl halides etc.

Absorption peak of the α,β-unsaturated γ-lactone group contained in acetogenin molecules are in wave number range of 1740 - 1760 cm^{-1}(Mulia et al., 2014). In this study the absorption peak is seen at wave numbers around 1745.78cm^{-1} that is assigned to the vibration of C=O group of the unsaturated lactone group. This result indicates that the IR absorption peaks are due annonaceous acetogenins compounds.

4.5.7. Gas Chromatography- Mass Spectrum (GC-MS) analysis

Gas chromatography-mass spectrometry (GC-MS) is the most universal analytical technique for the identification and quantitation of organic substances within a test sample(Sparkman et al., 2011). The product composition was analyzed by using a gas chromatography (Agilent, 7890B) coupled with a mass spectrometry (Agilent, 5977A MSD).

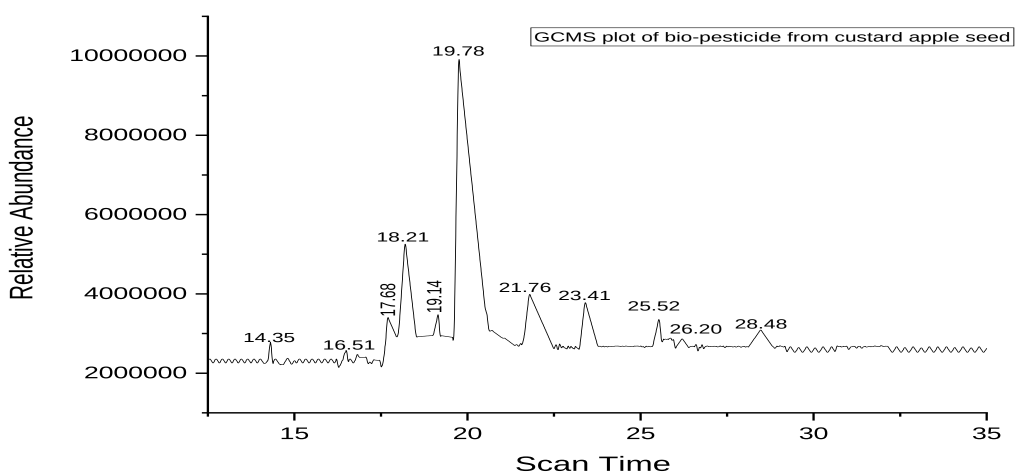


Figure 4-2 GC-MS plot of bio-pesticide from custard apple seed

Table 4-11 GC-MS analysis of pesticide from custard apple seed

S/N	Name	Formula	RT	Area	Area %
1	3-Buten-2-one-4-(2,5,6,6-tetramethyl-2cyclohexen-1-yl)	$C_{14}H_{22}O$	14.35	3359889	4.87
2	Butyl octyl phthalate	$C_{20}H_{30}O_4$	16.51	2055949	2.98
3	Ethyl hexadecanoate	$C_{18}H_{36}O_2$	17.68	4229183	6.13
4	Hexadecanal dsiallyl acetal	$C_{22}H_{42}O_2$	18.21	12301195	17.83
5	Uvaricin	$C_{39}H_{68}O_7$	19.14	2752763	3.99
6	Ethyl oleate	$C_{20}H_{38}O_2$	19.78	19531511	28.31
7	Palmitaldehyde	$C_{26}H_{54}O_2$	21.76	6781870	9.83
8	Oxalic acid, butyl 2-isopropylphenyl ester	$C_{15}H_{20}O_4$	23.41	6160946	8.93
9	Annonacin	$C_{35}H_{64}O_7$	25.52	5119174	7.42
10	Squamocin	$C_{37}H_{66}O_7$	26.20	2545789	3.69
11	4, 4 Dimethylcholesterol	$C_{29}H_{50}O$	28.48	3946317	5.72

The GC-MS analysis shows that eleven phytochemicals were identified. From this result uvaricin, annonacin and squamocin with retention time (RT) 19.14, 25.52 and 26.20 minutes respectively were bioactive annonaceous acetogenin compounds.

4.4. Experimental design analysis results

Response surface methodology (RSM) of Box – Behnken Design (BBD) of Design-Expert 7.0.0 software was used to determine the optimal conditions of extraction process. RSM is a mathematical modeling tool used to predict the output relationship with respect to the multi input parameters. The model predicts the value of the unknown output for any desirable input.

Box - Behnken Design (BBD) uses the method of least-squares regression to fit the data to a certain model. The adequacy of the model was determined by evaluating the lack of fit; that was obtained from ANOVA.

The statistical significance of the model and model variables were determined at 95% confidence interval ($\alpha = 0.05$).

The result found after the data was put to Design-Expert software in a standard order, there has been found a single model that can be possibly fit or satisfy the factors significance. A quadratic model was suggested, even though it has lower R-Squared (R^2) and adjusted R-Squared (Adj-R^2) values than a cubic model. This is because the cubic model is aliased, which means that the effects of each variable that cause different signals become indistinguishable.

In RSM, If p-value of lack of fit, $p>0.05$ (non-significant) it implies that the proposed model fit the experimental data and the independent variables or parameters has considerable effects on the response. Based on this fact, the lack of fit (p-value = 0.6722) of quadratic models has been found best fitted model among all with 67.22% of lack of fit.

The individual factors coded name (A, B and C), factors low and high coded value (-1 and 1), type of factor (numeric), mean and standard deviation of the study parameters were show in the table below. The model standard deviation becomes 0.758 found a least square error of the model. The model uses polynomial mathematical correlation to evaluate the data and to fit the result observed.

Table 4-12 Design summery table

Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded	Mean	Std. Dev.
A	Extraction time	Hour	Numeric	2.00	4.00	-1.000	1.000	3.000	0.686
B	Temperature	℃	Numeric	30.00	50.00	-1.000	1.000	40.000	6.860
C	Ethanol to CAS ratio	v/w	Numeric	5.00	9.00	-1.000	1.000	7.000	1.372

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
Y1	Yield	Gram	17	polynomial	3.381	6.314	4.379	0.758	1.867	None	Quadratic

Table 4-13 Analysis of variance (ANOVA) table

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	9.72	9	1.08	178.53	< 0.0001	significant
A-Extraction Time	2.39	1	2.39	395.59	< 0.0001	
B-Temperature	1.12	1	1.12	184.44	< 0.0001	
C-Ethanol to CAS ratio	0.21	1	0.21	34.06	0.0006	

AB	0.37	1	0.37	61.39	0.0001	
AC	0.51	1	0.51	83.90	< 0.0001	
BC	1.82	1	1.82	300.08	< 0.0110	
A^2	1.68	1	1.68	277.48	< 0.0001	
B^2	1.43	1	1.43	236.49	< 0.0001	
C^2	0.096	1	0.096	15.87	0.0053	
Residual	0.042	7	6.051E-003			
Lack of Fit	0.012	3	4.147E-003	0.55	0.6722	not significant
Pure Error	0.030	4	7.479E-003			
Cor Total	9.76	16				

From the ANOVA result of the design expert the Model F-value of 178.53 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise (personal error or disturbance). The predicted response fits well with those of experimentally obtained responses. All the factors, i.e. extraction time, temperature, ethanol to custard apple seed ratio had a significant effect individually on the yield this indicates that, the variation in magnitude on the individual factors directly affects the yield.

Additionally the interaction or combination effect of extraction time with temperature(AB), extraction time with ethanol to custard apple seed ratio(AC), temperature with ethanol to custard apple seed ratio (BC), the square of extraction time (A^2), the square of temperature (B^2) and the square of ethanol to CAS ratio (C^2)were affect the yield simultaneously.

Lack-of-Fit is the variation due to the model inadequacy. The "Lack of Fit F-value" of 0.55 implies the Lack of Fit is not significant relative to the pure error. There is a 67.22% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good because we want the model to fit. This non significance lack of fit shows the proposed model fit the experimental data and the independent variables or parameters have a desirable effect on the response. This conclusion was deducted basically by comparing the p – value of the individual and combined factors with the percent of probability of the model (i.e. $\alpha = 0.05$). The lesser the p – value than α value has implied the larger significance effect of the factor on the response.

4.4.1. Development of Model Equation

A model equation is a representative equation in which it represents the whole model with a single mathematical relation that helps to maximize yield and optimize operating conditions. The software package Design-Expert 7.0.0 was used to determine the statistical significance of each experimental factor and to generate the corresponding mathematical prediction models.

Final model equation in terms of coded factors

$$\text{Yield} = 3.88 + 0.55\text{x}(A) + 0.37\text{x}(B) + 0.16\text{x}(C) + 0.30\text{x}(A\text{x}B) + 0.36\text{ x }(A\text{ x }C) + 0.67\text{ x }(B\text{ x }C) + 0.63\text{x }(A^2) + 0.58\text{ x }(B^2) - 0.15\text{ x }(C^2)$$

Final model equation in terms of actual factors

$$\begin{aligned}\text{Yield} = {} & 30.17299 - 5.70773\text{ x (Extraction Time)} - 0.75627\text{ x (Temperature)} \\ & - 1.27304\text{ x (Ethanol to CAS ratio)} \\ & + 0.030475\text{ x (Extraction Time x Temperature)} \\ & + 0.17812\text{ x(Extraction Time x Ethanol to CAS ratio)} \\ & + 0.033687\text{ x (Temperature x Ethanol to CAS ratio)} \\ & + 0.63148\text{ x (Extraction Time\^{}2)} + 5.82975E - 003\text{ x (Temperature\^{}2)} \\ & - 0.037756\text{ (Ethanol to CAS ratio \^{}2)}\end{aligned}$$

4.4.2. Checking of data and adequacy of model

Table 4-14 Model adequacy table

Std. Dev.	0.078	R-Squared	0.9957
Mean	4.38	Adj R-Squared	0.9901
C.V. %	1.78	Pred R-Squared	0.9748
PRESS	0.25	Adeq Precision	48.516

The above value shows that adequate signal for the model can be used to direct the design space. The "Pred R-Squared" of 0.9748 is in reasonable agreement with the "Adj R-Squared" of 0.9901; i.e.

the difference is less than 0.2. The value of correlation coefficient (R^2) of the model was 0.9957 which expresses the quality of fit. A high R^2 indicates that the variation could be accounted for by the data satisfactorily fitting the model. This shows that only 1.43% of the total variation in the outcome parameter assessed is unexplained by the observed model, and expresses well enough quadratic fits to navigate the design space. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this case ratio of 48.516 indicates an adequate signal.

Coefficient of variation (CV) is a measure expressing the standard deviation as a percentage of the mean, smaller values of CV give better reproducibility. The coefficient of variation (CV) of less than 10 (i.e 1.78) indicated that the model was reproducible. The PRESS (Predicted Residual Sum of Squares) measure of how a particular model fits each point in the design. In this case the PRESS value was 0.25.

The R^2 should be at least 0.80 for a good fit of a model even R^2 values depends on the nature of the analysis. The R^2 value obtained in the present study for these response variables was higher than 0.80, indicating that the regression models explained the extraction process well.

All the above stated statistical parameters show the reliability of the models.

4.4.2.1. Normality of the data

Normality of the data was done by means of normal probability plot. The normal probability plot of the residuals for yield is shown in Figure: 4-3, which reveals that the residuals are falling on the straight line. This means that the errors are distributed normally. Therefore the normality assumption was satisfied as the residual plot approximated along a straight line.

Figure: 4-4 indicates the relationship between the actual and predicted values of the yield. This figure shows that the developed model is adequate. Because, the residuals in the prediction of each response are small, with the residuals have tendency to be close to the diagonal line.

Figure: 4-5 presents a plot of residuals versus the predicted response. The general impression is that the residuals distribute randomly on the display, telling that he variance of the original observation is constant for all values of Y. Figure: 4-3 and Figure: 4-5 are satisfactory, based on this two plots we conclude that the empirical model is adequate to describe the active ingredient yield by response surface.

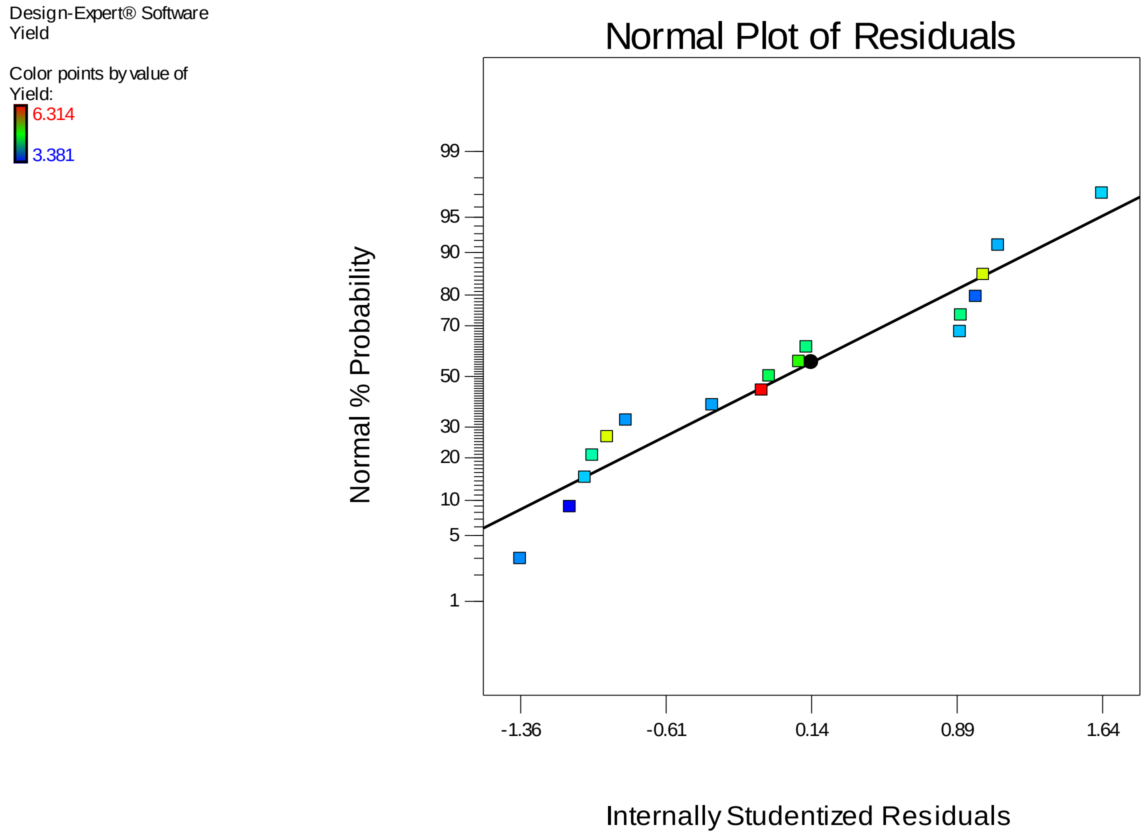


Figure 4-3 Normal probability of internally studentized residuals (Normality of the data) plot

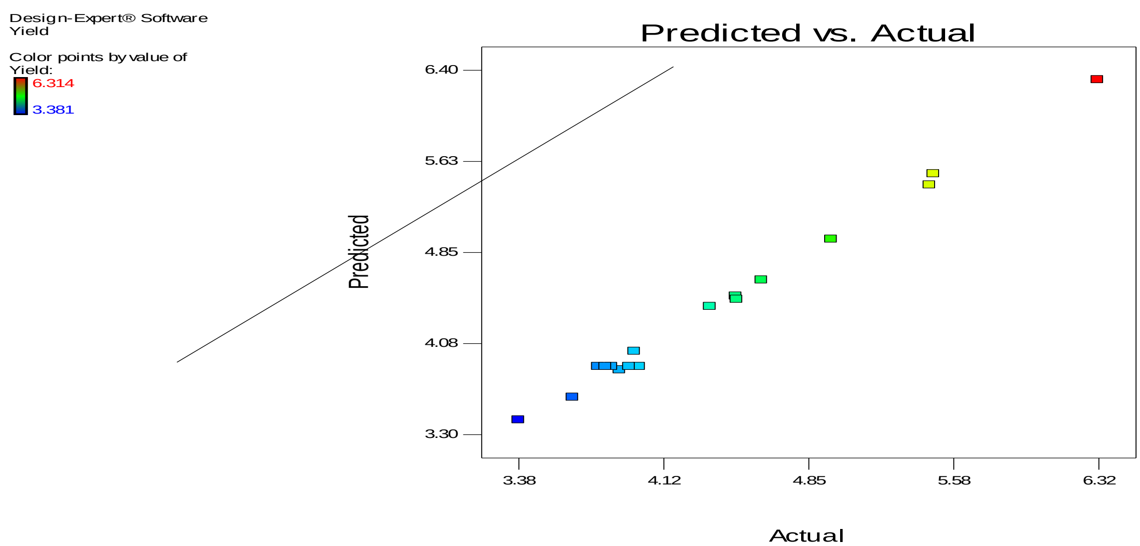


Figure 4-4 Experimental yield vs. the predicted yield under optimum extraction conditions

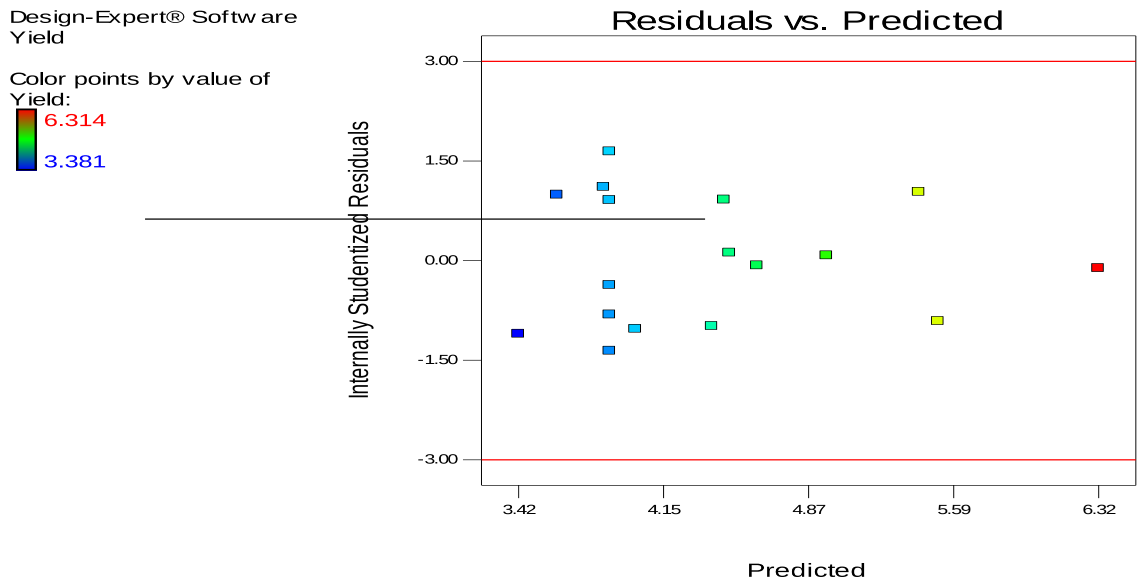


Figure 4-5 Plot of internally studentized residuals vs. predicted response

4.4.2.2. Independency of the data

The independency of the data was tested by plotting a graph between the residuals and the run order. The residual plot for yield is shown in Figure: 4-6 which reveals that there was no predictable pattern observed because all the run residues lay on or between the levels of -3 to 3.

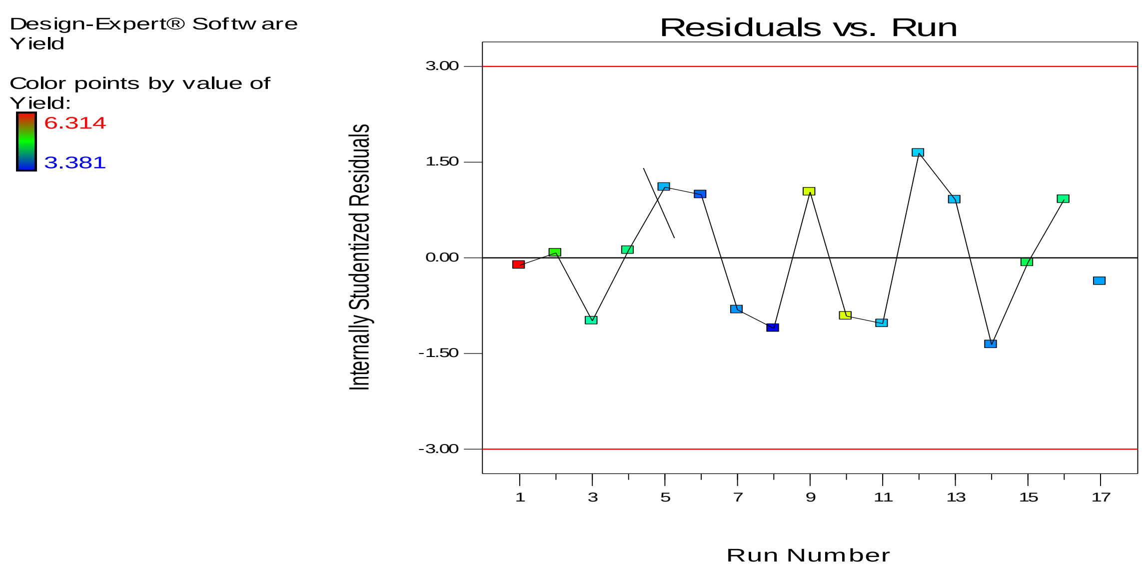


Figure 4-6 Residual vs. Run plot

4.4.3. Validation of the model

To compare the predicted result with the practical value, the rechecking experiment was performed using the extraction condition. The value obtained from laboratory experiments, demonstrated the validity of the response surface methodology model, since there was no significant ($p > 0.05$) differences. The tough correlation between the experimental and the predicted results confirmed that the response model was adequate to reflect the expected extraction condition.

Table 4-15 Predicted and experimental values of the response at the extraction condition

	Actual parameters (Variables)			Extraction yield (g/50g)	
Standard Order	Extraction time (hour)	Temperature (℃)	Ethanol to CAS ratio (v/w)	Actual Value	Predicted Value
1	2.00	30.00	7:1	4.482	4.48
2	4.00	30.00	7:1	4.965	4.96
3	2.00	50.00	7:1	4.612	4.62
4	4.00	50.00	7:1	6.314	6.32
5	2.00	40.00	5:1	3.968	4.01
6	4.00	40.00	5:1	4.351	4.39
7	2.00	40.00	9:1	3.655	3.62
8	4.00	40.00	9:1	5.463	5.42
9	3.00	30.00	5:1	4.486	4.45
10	3.00	50.00	5:1	3.893	3.85
11	3.00	30.00	9:1	3.381	3.42
12	3.00	50.00	9:1	5.483	5.52
13	3.00	40.00	7:1	3.853	3.88
14	3.00	40.00	7:1	3.993	3.88
15	3.00	40.00	7:1	3.784	3.88
16	3.00	40.00	7:1	3.942	3.88
17	3.00	40.00	7:1	3.822	3.88

4.4.4. Individual Effect of Factors on Yield

The factors used in this analysis were extraction time, temperature and ethanol to custard apple seed (CAS) ratio. These factors had a great impact on the yield and examined individually to determine their effects.

4.4.4.1. Effect of extraction time on yield

Extraction time is the time required to extract the active ingredient present in the custard apple seed. Extraction time plays significant role on the yield. In a general way, extraction rate will be increased along with extraction time increase. As shown in the Figure 4-7 the yield increases proportionally with the increase of extraction time from 2 hour to 4 hours. These are by making to dissolve unnecessary components like solutes in the solvent and enhancing impurity; and affect the biological activity of the active bioactive ingredient by exposing the solute to a higher temperature. The yield was slightly declined when extraction time increase from 2 to 2:45 hour and increases when increased from 2:45 to 4 hour. The decrease in a yield may be happens due to a short period of contact time between the solute and the solvent. The maximum yield was found when the extraction process was going to 4 hours and it was 5.05728gm with least significant difference (LSD) of 0.156692

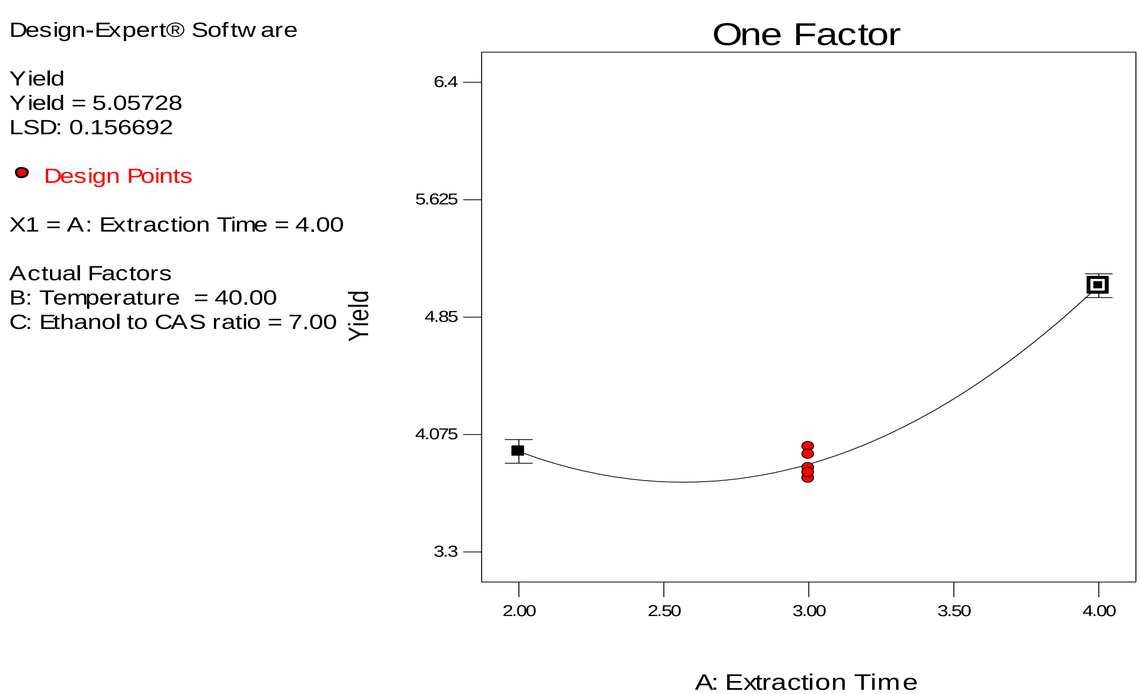


Figure 4-7 A plot of effect of extraction time on yield

4.4.4.2. Effect of temperature on yield

Temperature has a great influence on the extraction yield of the active component. But higher extraction temperature could produce degradation of bioactive constituents. To overcome such problems the choice of extraction temperature plays a significant role depending on the final application of the product. Therefore, it is better to use a temperature below 60℃ to extract bio active components in order to resolve degradation of the bioactive components, because the product is temperature sensitive. In this thesis work extraction temperature was chosen 30, 40 and 50℃ because the custard apple seed extract is bioactive and used as bio-pesticide.

Figure: 4-8 shows the effect of temperature on the yield of bio-pesticide production. During the process the other parameters are kept constant. It is observed that the yield slightly decreases when temperature is increased from 30℃ to 37.5℃ and increases when increased from 37.5℃ to 50℃. The reason is that as the extraction temperature increased, the rate of solubility of phytochemical compounds was increasing and gave high rate of extraction. For the range 30℃ to 37.5℃ the decrease in a yield may be due to the rate of diffusion of phytochemical compounds towards the solvent. From the present investigation, optimum temperature was found to be 50℃ with 4.83534gm bio-pesticide yield.

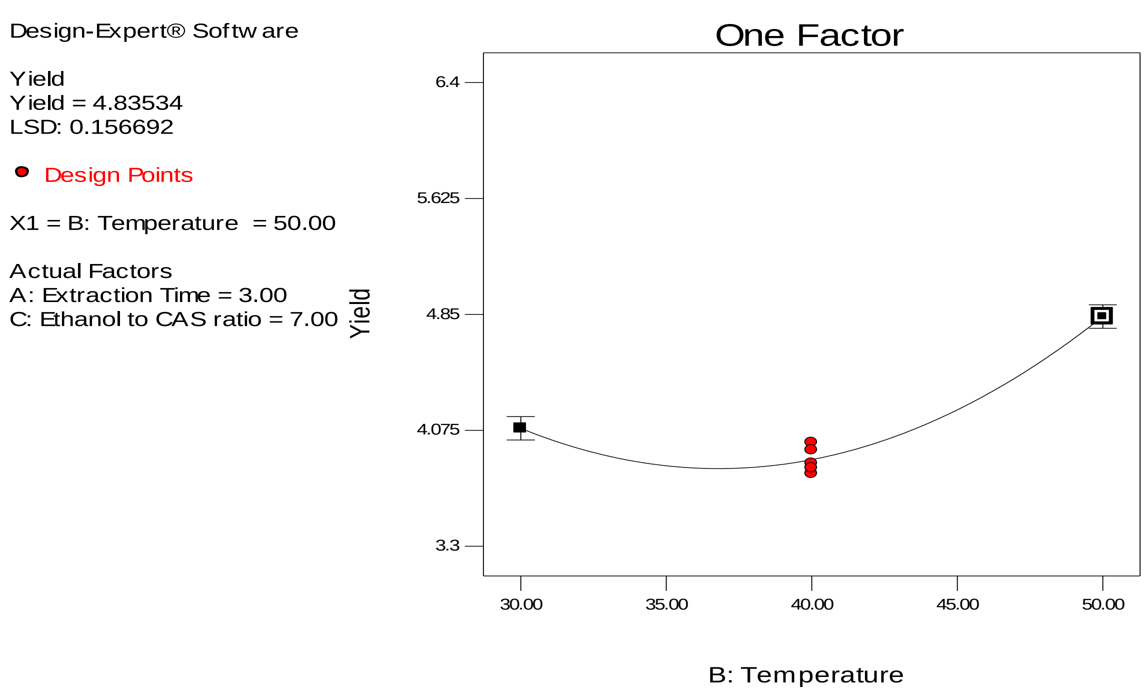


Figure 4-8 A plot of effect of temperature on yield

4.4.4.3. Effect of ethanol to custard apple seed (CAS) ratio on yield

Generally, different ratio of solvent to solid significantly affect extract yield(Sheng et al., 2011).
As shown in the Figure: 4-9 the extract yield is increasing with increase of ethanol to custard apple seed ratio from 5:1 to 9:1 v/w. If ratio of ethanol to custard apple seed is too small, the active ingredient in the seed cannot be completely extracted up. When ratio of ethanol to custard apple seed is increased, the extraction rate of active ingredient also increases. However, this will cause high process cost when ratio of ethanol to custard apple seed is too large. Therefore, suitable ratio of ethanol to custard apple seed should be selected for extraction of active ingredient. Based on these facts, the ratio of ethanol to custard apple seed of 7:1 was adopted in the thesis work.

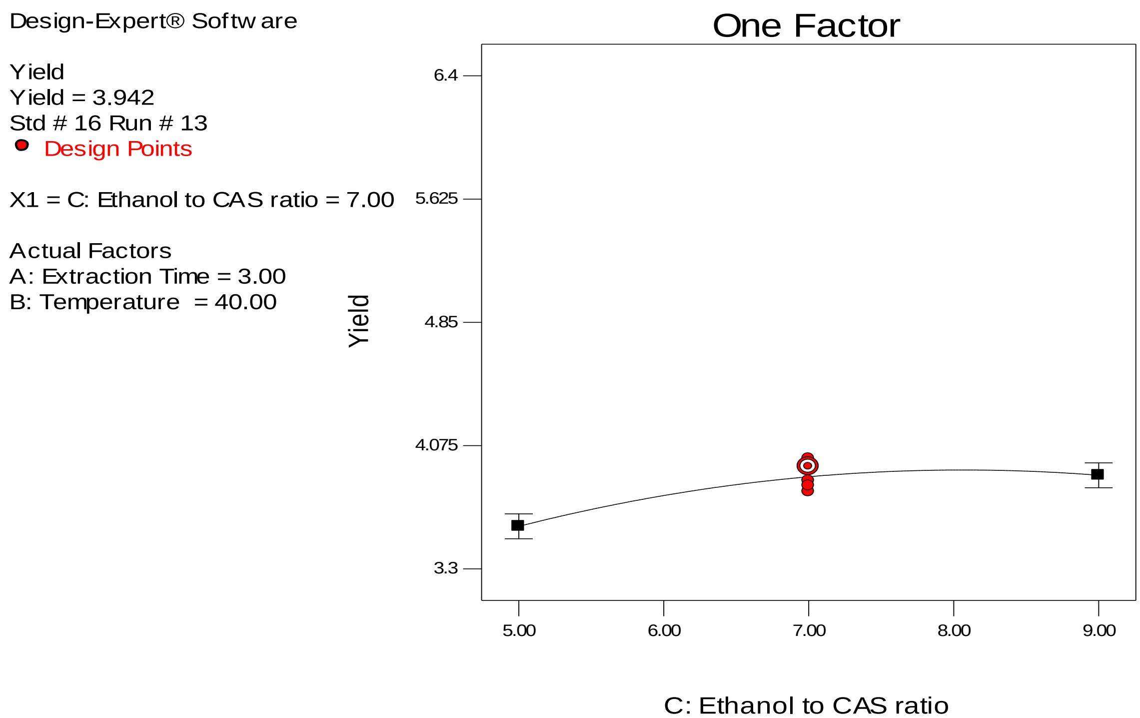


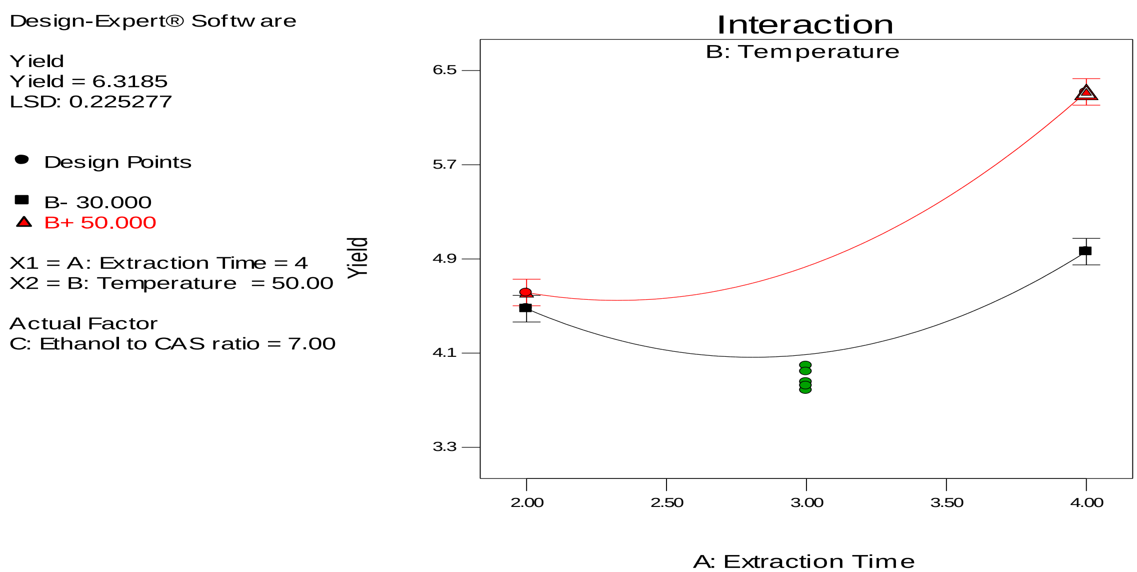
Figure 4-9 A plot of effect of ethanol to custard apple seed on yield

4.4.5. Interaction Effect of Factors on Yield

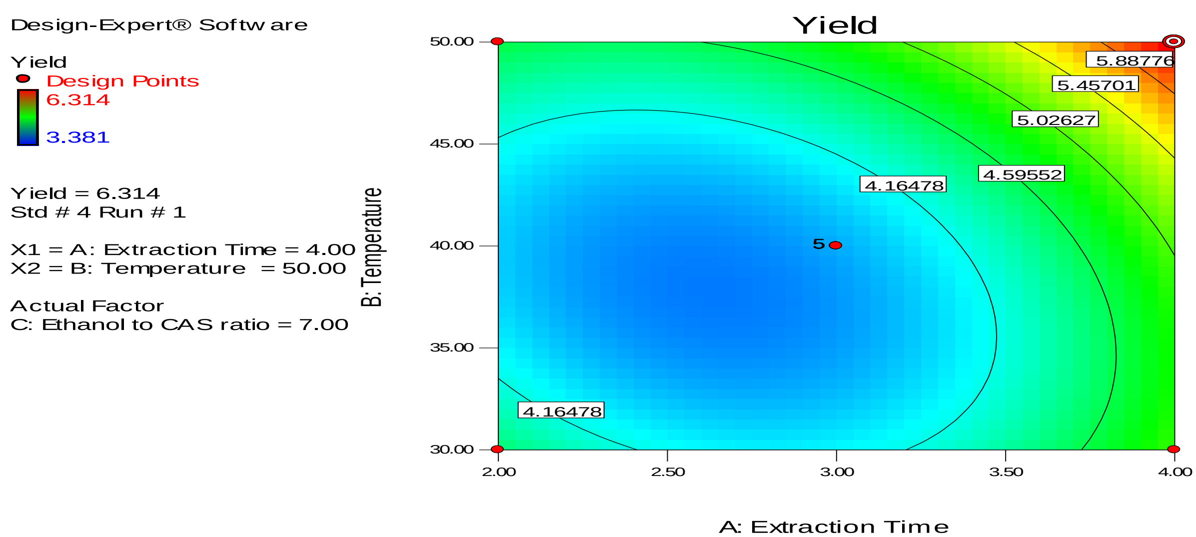
Interaction effects represent the combined effects of factors on the dependent response. When an interaction effect was present, the impact of one factor depends on the level of the other factor. The ability to estimate and test interaction effects is one of the advantage of ANOVA. Response surfaces were plotted using Design-Expert version 7.0.0 software to study the effects of parameters and their interactions on yield.

4.4.5.1. Effect of extraction time with temperature (AB) on yield

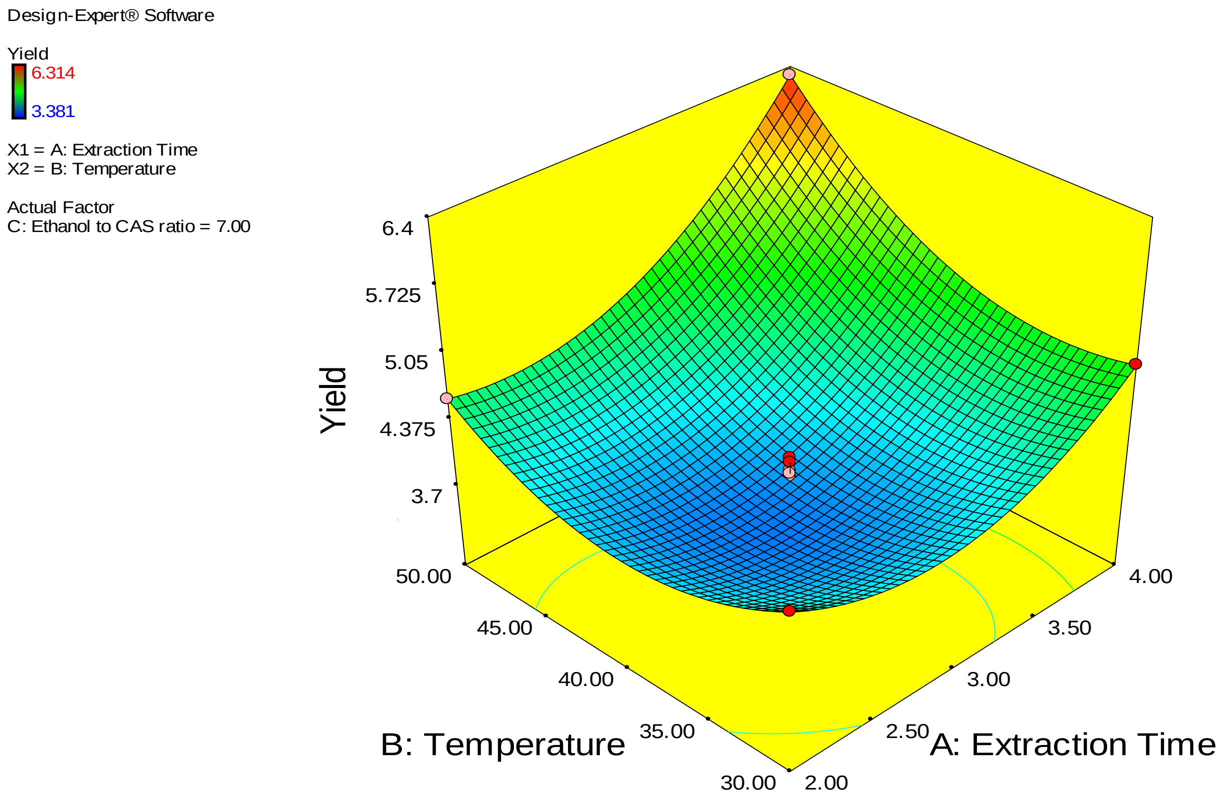
Figure: 4-10 shows the interaction effect of extraction time with temperate on yield of extract. At low levels of extraction time and temperature, there is virtually no difference on yield. As extraction time increases the gap between low level of temperature and high level of temperature plots gets bigger and bigger, because the yield increases more when the temperature increases from 30℃ to 50℃ with the extraction time from 2 to 4hour and the maximum yield found was 6.3185gm. So, the interactive factor of extraction time and temperature was significantly affecting the yield.



(a)



(b)

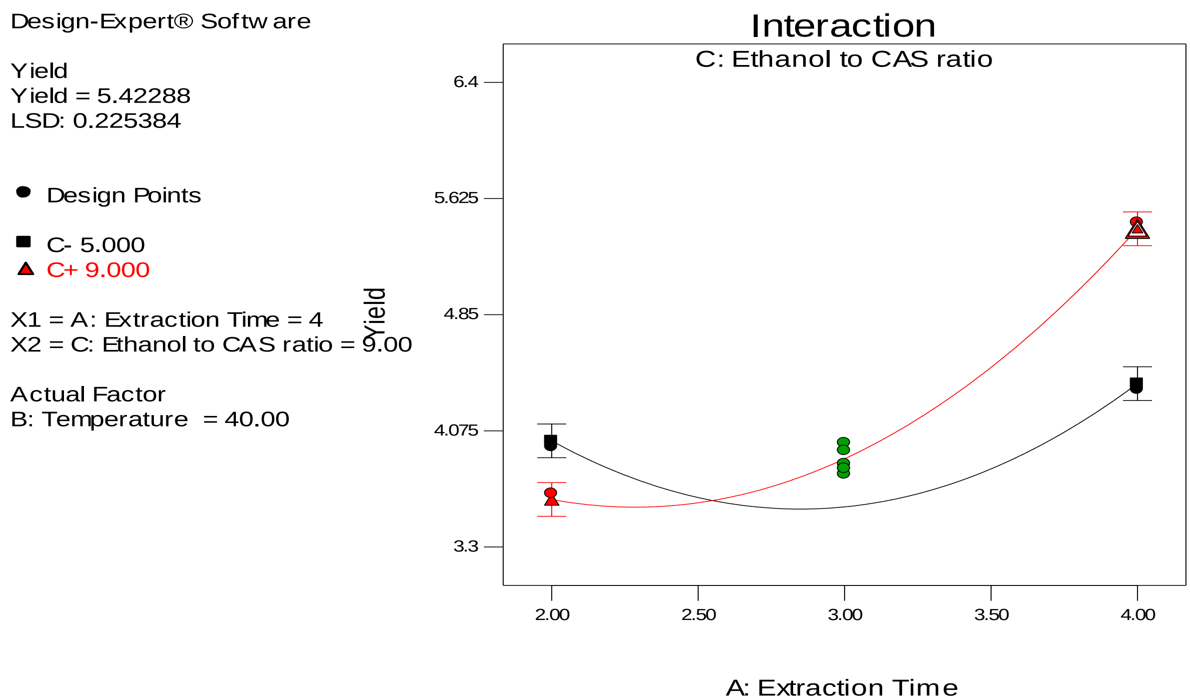


(c)

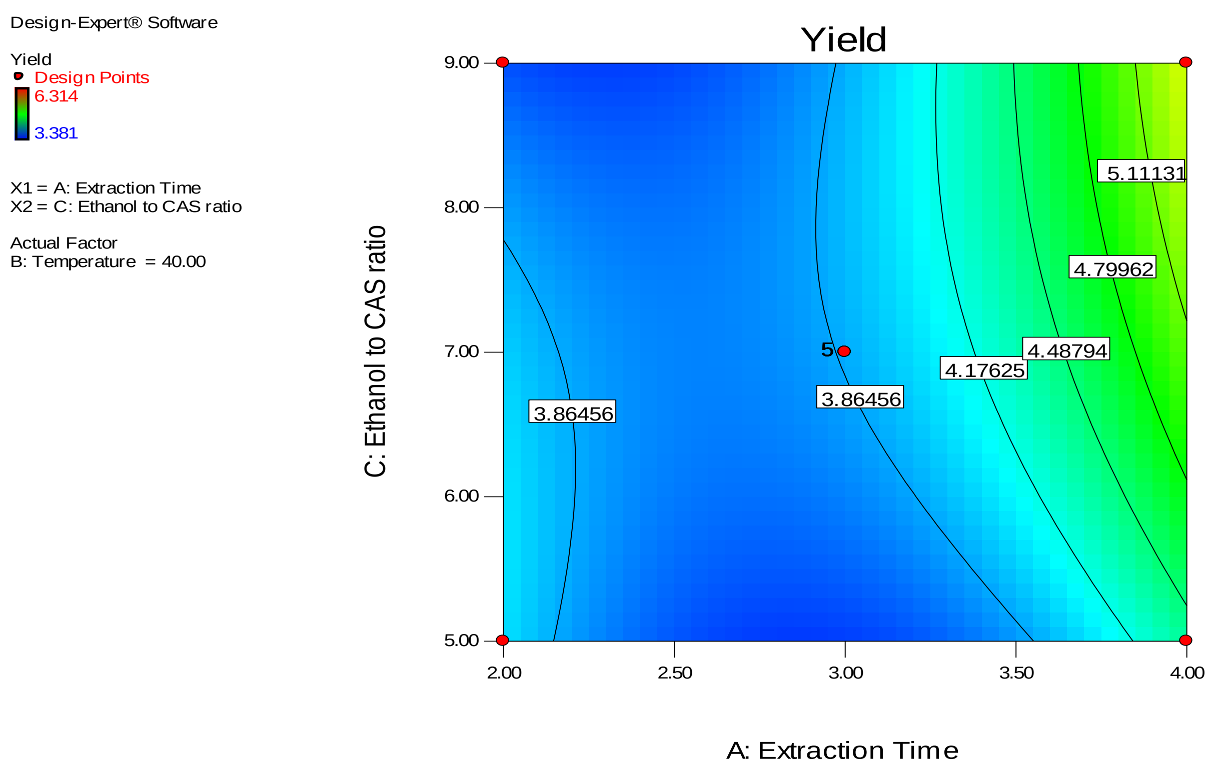
Figure 4-10 Interaction (a) Contour (b) and 3D (c) plots of extraction time and temperature

4.4.5.2. Effect of extraction time with ethanol to CAS ratio (AC) on yield

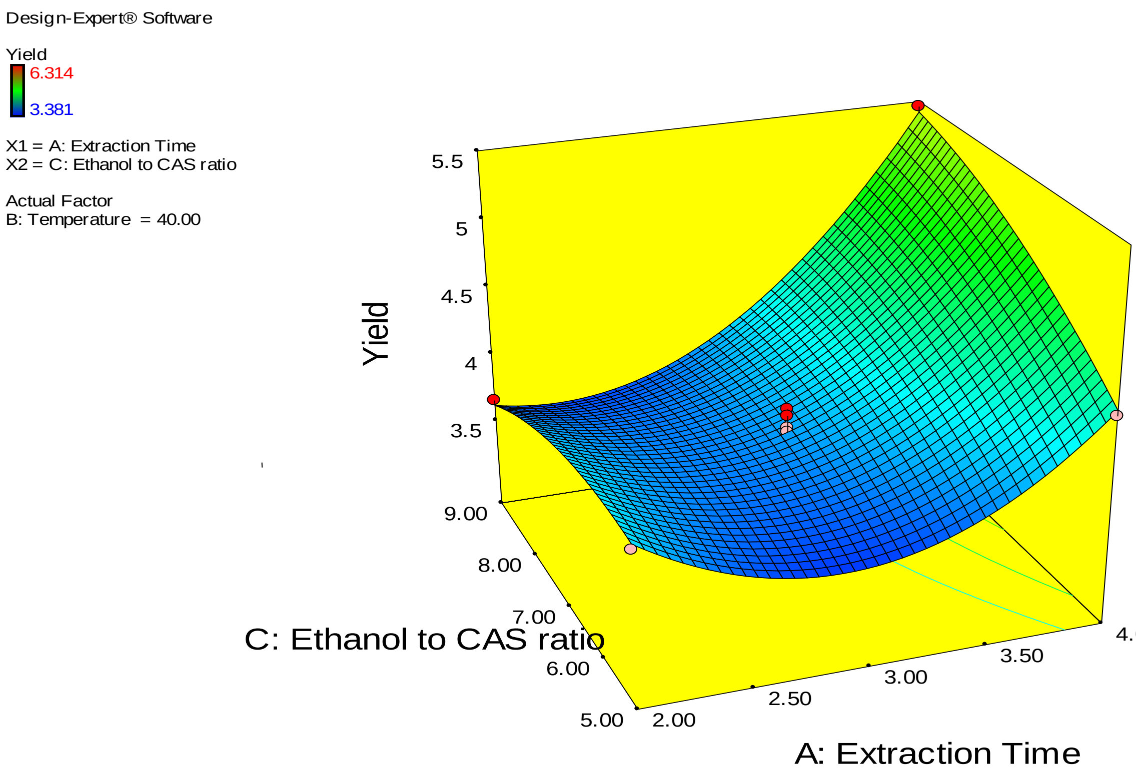
Figure: 4-11 below indicates the interaction effect between extraction time and ethanol to custard apple seed ratio. On the interaction plot shown below, you can see that there is no a significant difference between the two ethanol tow custard apple seed ratio levels, when the extraction time is set at the low level. However, there is a significant difference between the low and high level of ethanol to CAS ratio when the time is set at the high level (right side of the plot). A maximum yield (5.42288gm) was found at 9:1ethanol to CAS ratio and extraction time 4 hour. This shows that there is an interaction effect between extraction time and ethanol to custard apple seed ratio.


Design-Expert® Softw are
Yield
Yield = 5.42288
LSD: 0.225384
Design Points
C- 5.000
C+ 9.000
X1 = A: Extraction Time = 4
X2 = C: Ethanol to CAS ratio = 9.00
Actual Factor
B: Temperature = 40.00
Interaction
C: Ethanol to CAS ratio
Yield
6.4
5.625
4.85
4.075
3.3
2.00
2.50
3.00
3.50
4.00
A: Extraction Time

(a)


Design-Expert® Software
Yield
Design Points
6.314
3.381
X1 = A: Extraction Time
X2 = C: Ethanol to CAS ratio
Actual Factor
B: Temperature = 40.00
Yield
C: Ethanol to CAS ratio
9.00
8.00
7.00
6.00
5.00
5.11131
4.79962
4.48794
4.17625
3.86456
3.86456
5
2.00
2.50
3.00
3.50
4.00
A: Extraction Time

(b)

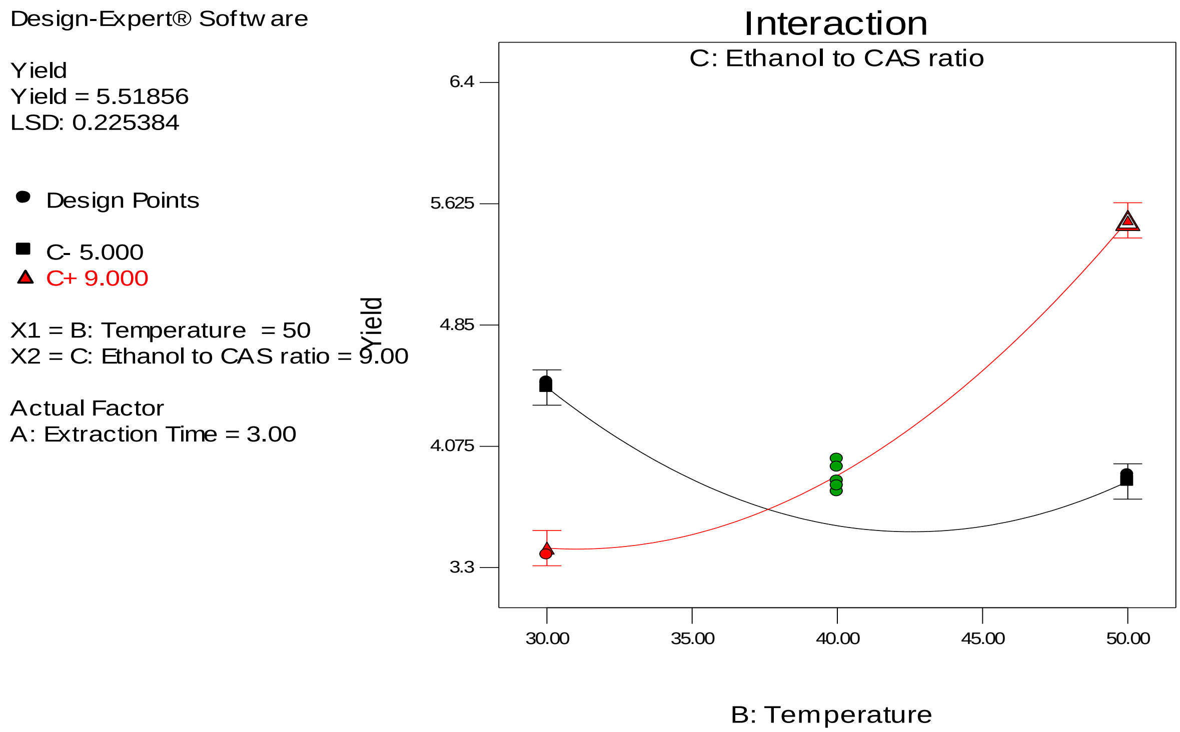


(c)

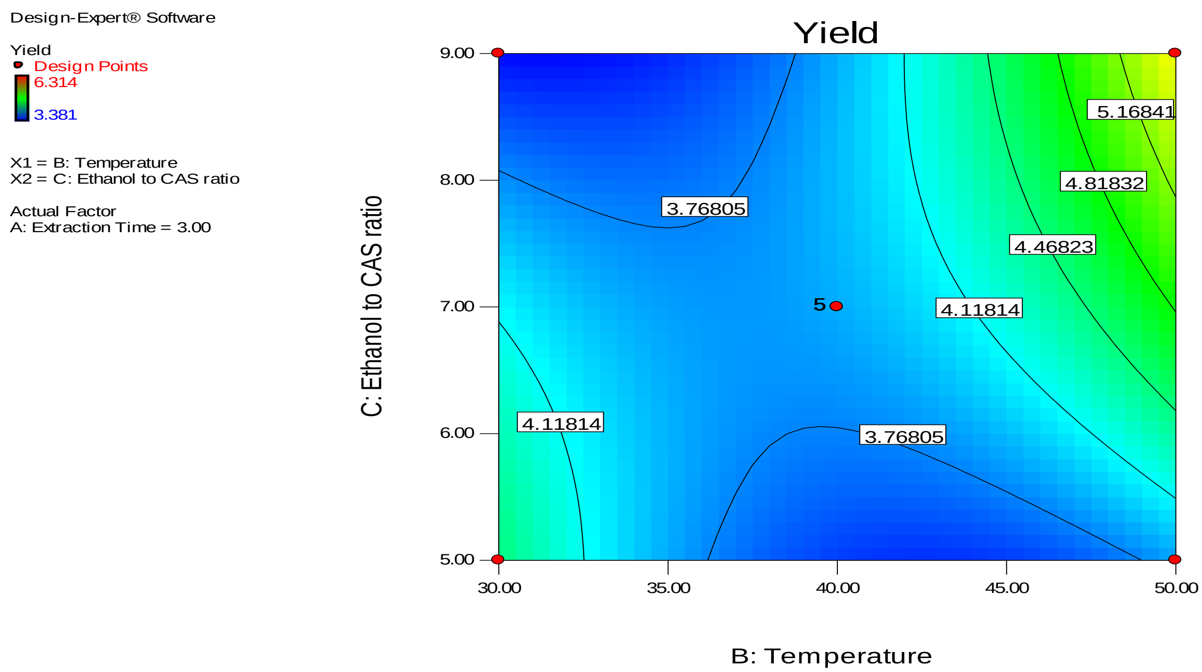
Figure 4-11 Interaction (a) Contour (b) and 3D (c) plots of extraction time and ethanol to CAS ratio

4.4.5.3. Effect of temperature with ethanol to CAS ratio (BC) on yield

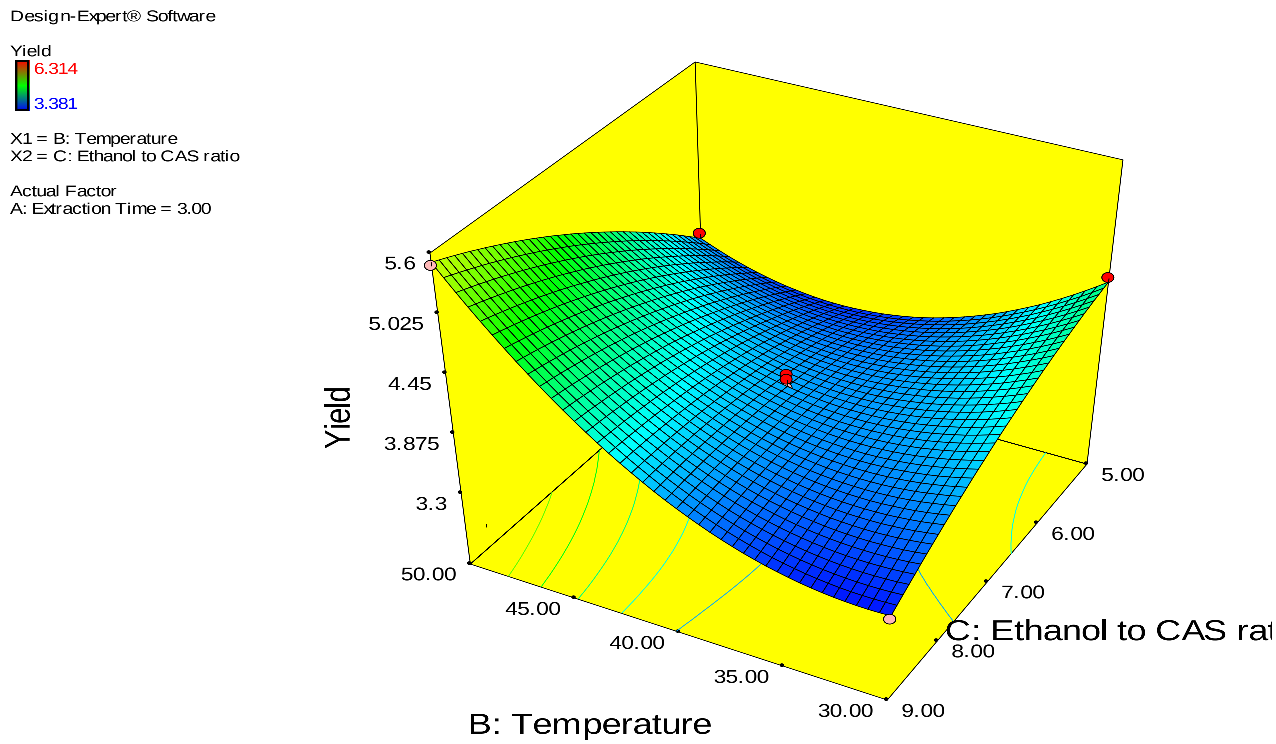
The figure 4-12 below shows the interaction effects of temperature and ethanol to custard apple seed ratio on yield of extract. This type of interaction is called crossover (Dis-ordinal) interaction. Crossover interactions often indicate that a factor has one kind of effect in one condition and the opposite kind of effect in another condition. For the temperature below 38℃ the low level of ethanol to custard apple seed ratio favor over the higher level by giving a maximum yield of 4.4505gm. However, for the temperature above 38℃ high level of ethanol to custard apple seed ratio favor over the low level of ethanol to custard apple seed ratio with maximum yield of 5.5185gm.


Design-Expert® Softw are
Yield
Yield = 5.51856
LSD: 0.225384
Design Points
C- 5.000
C+ 9.000
X1 = B: Temperature = 50
X2 = C: Ethanol to CAS ratio = 9.00
Actual Factor
A: Extraction Time = 3.00
Interaction
C: Ethanol to CAS ratio
Yield
6.4
5.625
4.85
4.075
3.3
30.00
35.00
40.00
45.00
50.00
B: Temperature

(a)


Design-Expert® Software
Yield
Design Points
6.314
3.381
X1 = B: Temperature
X2 = C: Ethanol to CAS ratio
Actual Factor
A: Extraction Time = 3.00
Yield
C: Ethanol to CAS ratio
9.00
8.00
7.00
6.00
5.00
5.16841
4.81832
4.46823
4.11814
3.76805
5
30.00
35.00
40.00
45.00
50.00
B: Temperature

(b)



(c)

Figure 4-12 Interaction (a) Contour (b) and 3D (c) plots of ethanol to CAS ratio and temperature

4.4.6. Determination of optimum conditions of extraction parameters

For three decades, numerous mathematical programming methods have been developed to solve optimization problems. However, until now, there has not been a single totally efficient and robust method to cover all optimization problems that arise in the different engineering fields(Nana Sarfo, 2010).

Most engineering application design problems involve the choice of design variable values that well describe the behavior of a system. In this research, the desirability function approach was applied to determine the optimum combination of extraction time, temperature and ethanol to custard apple seed ratio for the production of bio-pesticide with respect to its yield. The assumptions were to develop a product which would have maximum yield.

Response Surface Method was used to find the optimum response. The optimization of “pesticide production from natural plant seed (Custard apple)” using Box Behnken Design method has found 27 possible Solutions. In general, the response surface can be visualized graphically. The responses predicted by the Design expert-7.0.0 software for these optimum process conditions resulted pesticide yield 6.3141gm.

The operation conditions of the chosen optimized process method were; 3.99 hour, 49.92℃ and 7.04:1v/w of extraction time, temperature and ethanol to custard apple seed ratio respectively.

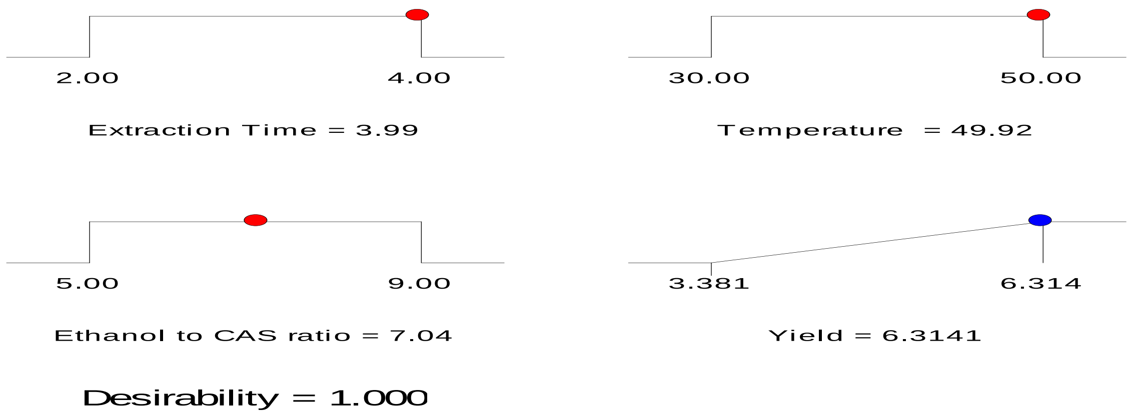


Figure 4-13 Ramp representation of optimal conditions for production

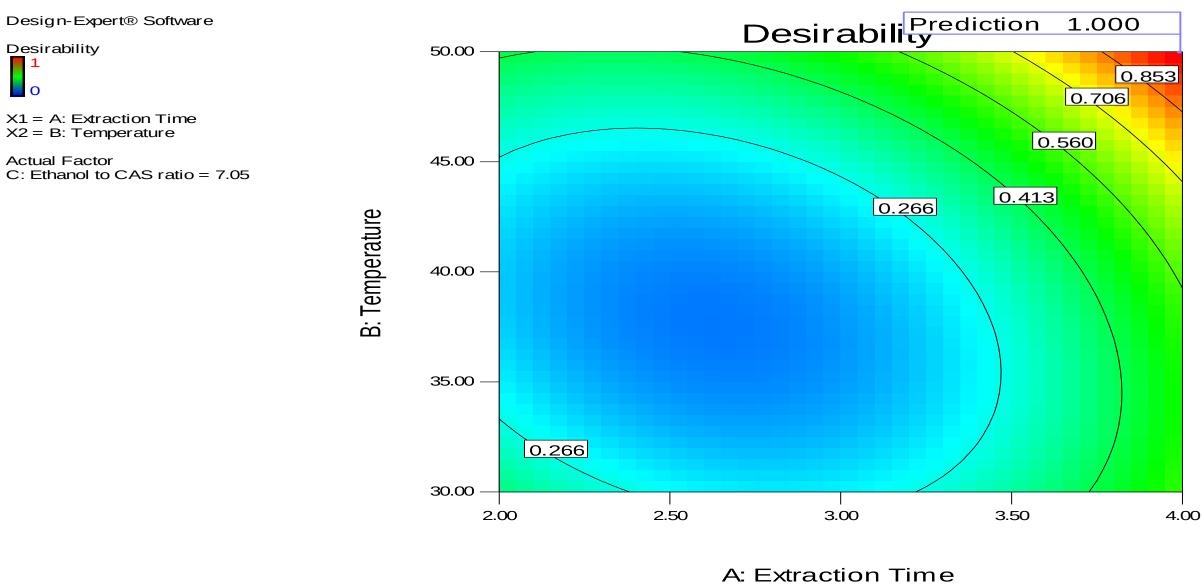


Figure 4-14 Optimized contour plot of Model Desirability

4.4.7. Box-Cox power transformation

The Box-Cox power transformation is a useful data transformation technique used to stabilize variance, make the data more normal distribution. By Using 95% confidence interval it is possible to determine whether a transformation is appropriate or not. For the Box-Cox transformation, a λ value of 1 is equivalent to using the original data. Therefore, if the confidence interval for the optimal lambda (λ) includes 1, then transformation is not necessary. If the confidence interval for λ does not include 1, a transformation is appropriate(Douglas, 2001).

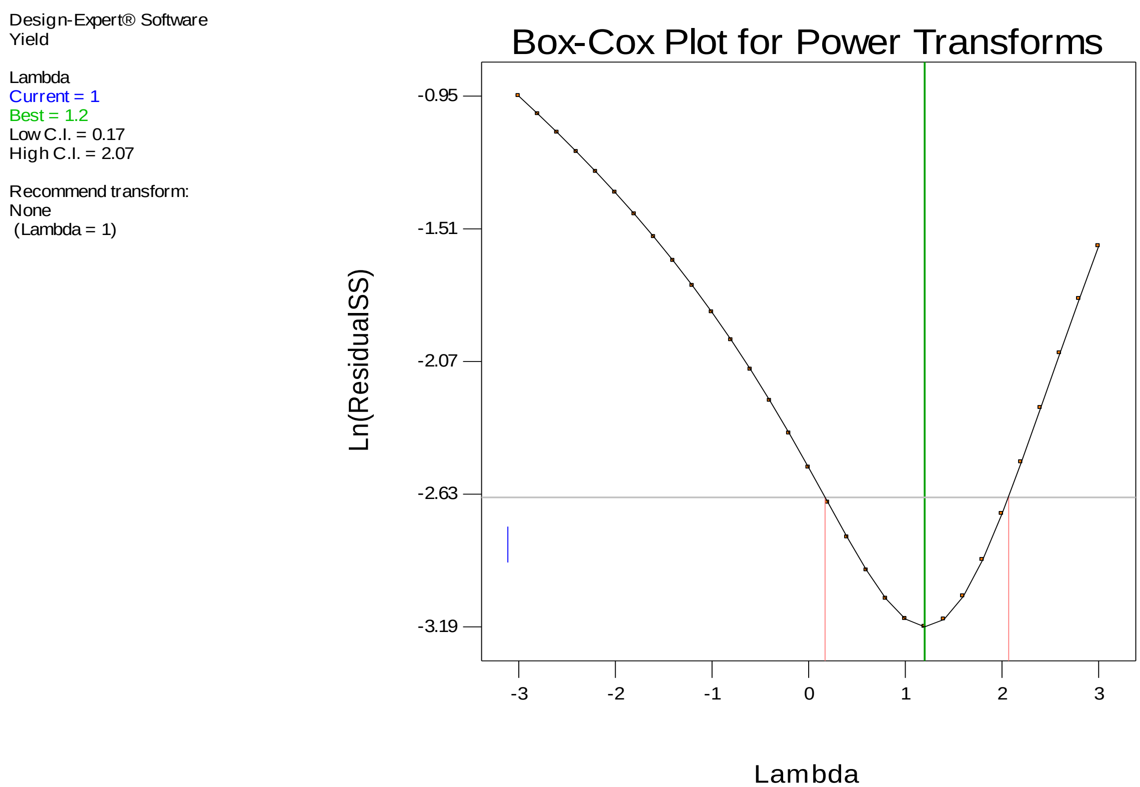


Figure 4-15 Model power transform plot

From the Box-Cox plot above the lower confidence level (CL) is 0.17and the higher confidence level (CL) is 2.07. The confidence interval for the optimal λ includes 1. Therefore the transformation is not necessary.

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Over the past 70 years, crop protection has relied heavily on synthetic chemical pesticides, but their availability is now deteriorating as a result of new legislation and the evolution of resistance in pest populations. A custard apple see (Annona squamosa L) has a promising bio-pesticide effect in crop production and protection from many pests because it contains main active compound called annonain, uvaricin and squamosin which, belongs to acetogenin compound. These acetogenin compounds are toxic to insects. Since, it is natural product this makes more preferable choice than synthetic pesticides. The present study shows the possible use of Annona squamosa seed extracts as a source of bio-insecticidal.

In this study the extraction process were carried out with three independent variables i.e. extraction time, temperature and ethanol to custard apple seed ratio of (2, 3 and 4hr), (30, 40 and 50℃) and (5:1, 7:1 and 9:1 v/w) respectively and their individual and interaction effects on yield were analyzed. Response surface methodology (RSM) of Box- Behnken Design method was used to check the model significance and yield optimization. All the individual factors (extraction time, temperature and ethanol to CAS ratio) had a significant effect individually on the yield of production. Also the interaction effects (extraction time with temperature, extraction time with ethanol to CAS ratio and temperature with ethanol to CAS ratio) were affecting the yield simultaneously.

From the proximate analysis of the custard apple seed the moisture content, fat content, ash content and organic matter content of 6.067 ± 0.125%, 22.3± 0.624%, 3.513 ± 0.176 and 96.487 ± 0.176% (w/w) respectively were observed. Characterization of the product indicates that, specific gravity 0.837 ± 0.0151, flash point121.56 ± 1.381 ℃, water solubility 323.06 ± 7.08 (mg/L), pH value 4.770 ± 0.1083 and TVOCs 0.464 ± 0.147%. FT-IR analysis showed that the presence of lactone group, which are in acetogenin compounds, at wave number of 1745.78 cm^{-1}. In GC-MS analysis result annonain, uvaricin and squamosin were identified at retention time (RT) of 26.20, 19.14, 25.25 minutes respectively. These components are belongs to acetogenin compounds, which are toxic to insects.

Maximum yield from 50gm of sample 6.314gm product was obtained at extraction time 4hour, temperature 50℃ and ethanol to custard apple seed ratio 7:1(v/w). Using RSM of Box-Behnken design an optimized yield of 6.3141gm was obtained at extraction time (3.99hr), temperature (49.92℃) and ethanol to custard apple seed ratio (7.04:1 v/w). Based on the above discussed facts this study proves and conclude that the chosen method of production and optimization was efficient, and reliable to use custard apple seed extract as bio-pesticide and promising alternative to chemical pesticide.

5.2. Recommendations

Recommendations that can be given are:

- Acute and chronic toxicity should be studied with the integration of hematologists and toxicologists by using a host animal preferably using female rat.
- On field test of custard apple seed extract as bio-pesticide should be mandatorily carried out so that the effective concentration for the target pest must be known.
- Need further research on active ingredients in custard apple's plant, so that the ingredients can be used well for the benefit of the society.
- The results of this research is expected to be applied on different types of pests to overcome loses due to pests, particularly on various types of insects.
- The half-life, which is the measure of the persistence of a Pesticide, should be studied in order to understand the time required for that the pesticide to degrade to one half of its previous concentration.

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Appendixes – A

Table A-1 RSM of Box-Behnken Design model extraction factors and yield of product

Std Order	Run Order	Block	Factor 1 A:Extraction Time (Hour)	Factor 2 B:Temperature (℃)	Factor 3 C:Ethanol to CAS ratio (v/w)	Response 1 Yield (gm)
1	4	Block 1	2.00	30.00	7:1	4.482
2	2	Block 1	4.00	30.00	7:1	4.965
3	15	Block 1	2.00	50.00	7:1	4.612
4	**1**	**Block 1**	**4.00**	**50.00**	**7:1**	**6.314**
5	11	Block 1	2.00	40.00	5:1	3.968
6	3	Block 1	4.00	40.00	5:1	4.351
7	6	Block 1	2.00	40.00	9:1	3.655
8	9	Block 1	4.00	40.00	9:1	5.463
9	16	Block 1	3.00	30.00	5:1	4.486
10	5	Block 1	3.00	50.00	5:1	3.893
11	**8**	**Block 1**	**3.00**	**30.00**	**9**:1	**3.381**
12	10	Block 1	3.00	50.00	9:1	5.483
13	17	Block 1	3.00	40.00	7:1	3.853
14	12	Block 1	3.00	40.00	7:1	3.993
15	14	Block 1	3.00	40.00	7:1	3.784
16	13	Block 1	3.00	40.00	7:1	3.942
17	7	Block 1	3.00	40.00	7:1	3.822

Table A-2 Extraction yield, % Oil removed and Pesticide yield

Std Order	Extraction Time (Hour)	Temperature (°C)	Ethanol to CAS ratio (v/w)	Yield of extract (gm)	Oil removed (gm)	Oil % removed	Yield (Pesticide) (gm)	% Yield (pesticide)
1	2.00	30.00	7:1	6.276	1.794	28.59	4.482	8.96
2	4.00	30.00	7:1	6.974	2.009	28.81	4.965	9.93
3	2.00	50.00	7:1	6.458	1.846	28.59	4.612	9.22
4	4.00	50.00	7:1	8.846	2.532	28.62	6.314	12.63
5	2.00	40.00	5:1	5.575	1.607	28.83	3.968	7.94
6	4.00	40.00	5:1	6.112	1.761	28.81	4.351	8.70
7	2.00	40.00	9:1	5.136	1.481	28.84	3.655	7.31
8	4.00	40.00	9:1	7.652	2.189	28.61	5.463	10.93
9	3.00	30.00	5:1	6.283	1.797	28.60	4.486	8.98
10	3.00	50.00	5:1	5.470	1.577	28.83	3.893	7.79
11	3.00	30.00	9:1	4.752	1.371	28.85	3.381	6.77
12	3.00	50.00	9:1	7.680	2.197	28.61	5.483	10.97
13	3.00	40.00	7:1	5.414	1.561	28.83	3.853	7.71
14	3.00	40.00	7:1	5.620	1.627	28.95	3.993	7.99
15	3.00	40.00	7:1	5.327	1.543	28.97	3.784	7.57
16	3.00	40.00	7:1	5.539	1.597	28.83	3.942	7.88
17	3.00	40.00	7:1	5.350	1.528	28.56	3.822	7.64

Table A-3 Constraints of optimization

Name	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
Extraction time	is in range	2	4	1	1	3
Temperature	is in range	30	50	1	1	3
Ethanol to CAS ratio	is in range	5:1	9:1	1	1	3
Yield	maximize	3.381	6.314	1	1	3

Table A-4 Desirable maximum operation conditions that can be possibly yield 100%

Number	Extraction Time	Temperature	Ethanol to CAS ratio	Yield	Desirability	
1	4.00	49.08	7.36:1	6.34382	1.000	
2	3.98	47.42	8.87:1	6.6497	1.000	
3	3.99	48.02	8.27:1	6.55539	1.000	
4	3.99	49.97	7.07:1	6.34123	1.000	
5	3.91	49.79	7.54:1	6.3834	1.000	
6	3.99	49.64	7.33:1	6.42276	1.000	
7	**3.99**	**49.92**	**7.04:1**	**6.3141**	**1.000**	**Selected**
8	3.97	49.20	7.78:1	6.51811	1.000	
9	4.00	49.63	7.14:1	6.32498	1.000	
10	3.79	49.99	8.99:1	6.86331	1.000	
11	3.92	48.59	8.85:1	6.77443	1.000	
12	3.99	46.06	8.99:1	6.43899	1.000	
13	4.00	49.16	7.38:1	6.36928	1.000	
14	3.90	49.09	7.81:1	6.37763	1.000	
15	3.78	49.94	8.88:1	6.78479	1.000	
16	3.96	49.70	7.62:1	6.52732	1.000	
17	3.88	48.52	8.83:1	6.66004	1.000	
18	3.89	49.51	8.16:1	6.60245	1.000	
19	3.97	49.73	7.51:1	6.49808	1.000	
20	2.00	30.00	5.00:1	5.19594	0.619	
21	2.00	30.20	5.00:1	5.16036	0.607	
22	4.00	37.85	9.00:1	5.15927	0.606	
23	4.00	30.00	5.97:1	5.00279	0.553	
24	4.00	30.00	5.95:1	5.00279	0.553	
25	2.10	50.00	9.00:1	4.94256	0.532	
26	2.00	50.00	9.00:1	4.94192	0.532	
27	2.00	30.00	6.27:1	4.77648	0.476	

Table A-5 Experimental and Predicted Values

Standard Order	Actual parameters (Variables)			Production yield (gm/50gm)		
	Extraction time (hour)	Temperature (℃)	Ethanol to CAS ratio (v/w)	Actual Value	Predicted Value	Residue
1	2.00	30.00	7:1	4.482	4.48	0.0045
2	4.00	30.00	7:1	4.965	4.96	0.003
3	2.00	50.00	7:1	4.612	4.62	-0.003
4	4.00	50.00	7:1	6.314	6.32	-0.0045
5	2.00	40.00	5:1	3.968	4.01	-0.040
6	4.00	40.00	5:1	4.351	4.39	-0.038
7	2.00	40.00	9:1	3.655	3.62	0.038
8	4.00	40.00	9:1	5.463	5.42	0.040
9	3.00	30.00	5:1	4.486	4.45	0.035
10	3.00	50.00	5:1	3.893	3.85	0.043
11	3.00	30.00	9:1	3.381	3.42	-0.043
12	3.00	50.00	9:1	5.483	5.52	-0.036
13	3.00	40.00	7:1	3.853	3.88	-0.026
14	3.00	40.00	7:1	3.993	3.88	0.11
15	3.00	40.00	7:1	3.784	3.88	-0.095
16	3.00	40.00	7:1	3.942	3.88	0.063
17	3.00	40.00	7:1	3.822	3.88	-0.057

Appendixes - B

Figure B-1 Custard apple fruit and seed

Figure B-2 Sun and oven drying of custard apple seed

Figure B-3 Size reduction of custard apple seed

Figure B-4 Extraction of the active ingredient

Figure B-5 Filtration and centrifugation of the extract

Figure B-6 Filtrate and cake

Figure B-7 Solvent recovery

Figure B-8 Concentrated semi solid product

Figure B-9 Flash point determination

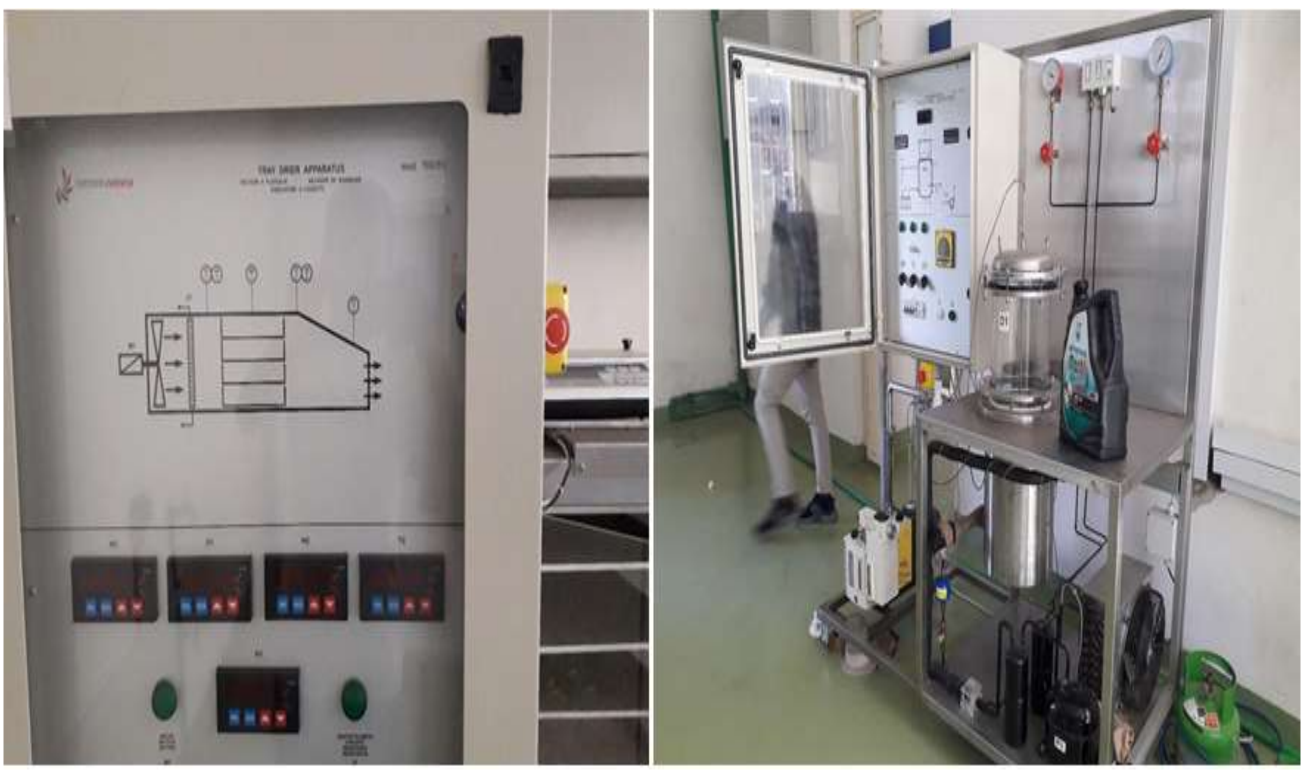

Figure B-10 Tray and Freeze drying apparatus

Figure B-11 Shaker incubator and Moisture content analyzer