Investigation of Bitter Leaf (Vernonia amygdalina) Extract as a Potential Substitute for Imported Hops.

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This is to certify that the thesis prepared by Etefa Gurmesa, entitled: *Investigation of Bitter Leaf (Vernonia amygdalina) Extract as a Potential Substitute for Imported Hops* and submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical and Bio Engineering (Under Process Engineering Stream) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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List of Abbreviations

AAS	Atomic Absorption Spectroscopy
ASBC	American Society of Brewing Chemists
ANOVA	Analysis of variance
A.O.A.C	Association of Official Analytical Chemists
DoE	Design of experiments
EBC	European Brewery Convention
EIAR	Ethiopian Institute Agricultural Research
ERCA	Ethiopian Revenues and Custom Authority
GAE	Gallic Acid Equivalent
GFD	General Factorial Design
IOB	Institute of Brewing
IE	Inhibition Effect
IC_{50}	Inhibition concentration of 50%
TPC	Total Phenolic Compound
TPC	Total Flavonoids Compound
2FI	Two Factors Interaction
QE	Quercetin Equivalent

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Abstract

Recently, considerable attention has been focused on the brewing industry that are helping transform Ethiopia's business landscapes with alcoholic beverage industries having a lion's share in terms of investment and market. However, the country`s requirement of hops are met through import. Thus, the need to investigate some Ethiopian plants that could substitute hops in beer brewing. In this paper work bitter leaf (Vernonia amygdalina) was investigated using a reflux condenser extraction unit and appropriate solvent material to obtain the extract. Thereafter, the extract was characterized in order to determine its suitability for use in brewing by comparing it with those of standard commercial hops values. The optimization of process parameters for the bitter leaf extract was investigated using GFD. The three parameters and their ranges namely temperature (80-100^oC), time (1-3hr), and particle size (0.25-1.0mm) were chosen. The individual parameters effect as well as effect of interactions between the process parameters on bitterness level and Iso alpha acid value was analyzed using various graphical representations. The physicochemical analysis of the bitter leaf showed that the sample extract had brewing properties or variables as follows: Iso-alpha acid (mg/l) ranged from 7.99-9.14, alpha acid (mg/l) of 9.51, total resin (%) of 19.72, hard resin (%) of 5.7, soft resin (%) of 14.02, protein content (%) of 27.73% and fat content (%) of 2.74. The Analytical Bitterness Unit (ABU) was found to be adequate and ranged from 16.55-17.1. The AAS results showed that the concentrations of metals of the samples investigated were Calcium (mg/100g) of 25.61±0.048, Magnesium (mg/100g) of 4.92±0.04, Iron (mg/100g) of 9.1±0.07and Zinc (mg/100g) of 2.27±0.052. Based on the comparison, it was observed that the brewing qualities of hops are present in bitter leaf to an appreciable level with variation in the protein and mineral contents. Consequently, the results obtained showed that bitter leaf presents an acceptably high level of their hop properties that is adequate for beer brewing.

Key words: hop substitution, bitter leaf, optimization, bitterness level, Iso-alpha acid, alpha acid, resin.

1. Introduction

1.1. Background

Alcoholic beverages are a part of human dietary culture and have an inseparable relationship with the life of mankind in history. The making and drinking of alcoholic beverages are ways of enhancing the nutritional significance as well as social relationships for human beings. Exactly when mankind started to produce and consume alcoholic beverages is not known but beer is known to have been produced by the Sumerians before 7,000 BC (T. Goldamer., 2008)

Beer is defined as an alcoholic drink made from yeast fermented malt, flavored with hops. Beer production worldwide is a viable industry. Among commercial beverages in 2006, beer ranks fourth in per capita consumption behind carbonated soft drinks, bottled water and coffee followed by milk and fruit drinks in the United States of America. Per capita beer consumption rose rapidly during the second world-war, declined during the 1950s and early 1960s, increased before peaking in the early 1980s and has generally leveled-off thereafter. (Dufour J et al., 2003)

A similar trend is reported of the beer industry in Ethiopia. Ethiopia is attracting more investment as a business and world tourism destination, beverage investment has become the lucrative market for the wine and beer industry and has largely contributed to economic growth recently. This can be attributed to the country's favorable demographics with populous and vibrant youth and growing middle class. This, along with a growing, largely youth population with increased disposable incomes is the constant drive that increased beer consumption in Ethiopia. The brewery companies are helping transform Ethiopia's business landscape with alcoholic beverage industries having a lion's share in terms of investment and market coverage. By the end of the fiscal year, 2015, the country's export revenue from the alcoholic beverage industry will hit 17 million USD (Ethiopian brewery industry, www, 2017)

From medieval times, herbs have been used to flavor and preserve fermented malt liquors but only hop inflorescence is used on a commercial scale today (J.S. Hough et al., 1982). Hop plants are vital to the brewing industry and some of their unique chemicals have the potential to be used in the nutraceutical industry (R.A. Shellie et al., 2009). Hop extracts give beer its bitter taste,

improve foam stability, enhance aroma and flavor and act as antiseptic towards microorganisms (D.R.J. Laws et al., 1981).

1.2. Statement of the Problem

Hop plants are grown throughout the temperate regions of the world and cannot be successfully grown in a tropical country like Ethiopia; hence its importation for brewing in is imperative. Ethiopia is in tropical region and since beer production in Ethiopia has never declined with ready market as consumption rate continues to increase, the importation of hops becomes inevitable. The total beer consumption in Ethiopia is around 5.1 million hectoliter of beer per year. However, the country`s requirement of hops are met through import. The present (2016) demand for hops is estimated at approximately 180,631.5kg per annual. According to data from Ethiopian Revenues and Custom Authority (ERCA) around 7,261,182.67 US dollars import of hops which are used in beer industry from Germany, USA, China, and United Kingdom and this will create a pressure on a foreign currency of our country. When we see data annually from 2006 to 2016 there is substantial increase in quantity from 58,260 kg of net weight to157, 058kg and value of importing of these hops from 1,167,782.68 USD to 7,261,182.67 USD. Thus, the need to investigate some Ethiopian plants that could substitute hops in beer brewing is point of attention to save the much needed foreign currency.

Therefore, the rationale of this research work is to investigate bitter leaf extract as a potential substitute for imported hops in beer production which could be a good source of raw material help to satisfy brewery industry ensuring stability and sustainability. In Ethiopia the plant is used in cleaning the containers used for fermentation purpose. Due to its bitterness, it also can be used as a bittering agent, a hop substitute and for the control of microbial contamination in beer brewing without affecting the quality of malt and also it is used to make honey wine called Teij.

1.3. Objectives

1.3.1. General Objectives

The overall objective of this study was to investigate bitter leaf (Vernonia amygdalina) extract as a potential substitute for imported hops.

1.3.2. Specific Objectives

The specific objectives of this work were to:

1. study proximate, mineral content, phytochemical and anti-oxidant activity of the bitter leaf (Vernonia amygdalina) against the imported commercial hops
2. extract and study the effect of process parameters (extraction time, temperature and particle size) on the Bitterness unit and Iso- alpha acid value of the bitter leaf aqueous extract using a reflux condenser
3. optimize the process parameters (time, temperature and particle size) for the optimum Bitterness unit and Iso- alpha acid value of the extract
4. evaluate the performance of the product against the standard commercial hops

1.4. Significance of the Study

The output of this study will;

- introduce the bitter leaf (Vernonia amygdalina) as a local raw material for use in the beer production.
- have a foreign exchange saving effect to the country by substituting the current imports, can create job opportunity for many persons, create forward and backward linkage with the manufacturing sector and also generates income for the government in terms of tax revenue and payroll tax.
- helps to satisfy brewery industry ensuring stability and sustainability of input raw material.

2. Literature Review

2.1. Hops

Hop is one of the ingredients used for bittering, flavoring and aroma imparting agent for beer brewing. It is also used as bacteriostatic activity to inhibit the growth of most microorganisms (Simpson and Smith, 1992). The bittering characteristics of hop are widely used to balance the sweetness of the malt in one hand and its essential oils help to impart special flavor/aroma to beer in another hand. The hop resins (bitter) are the most valuable and most characteristic components of hops. They give beer its bitter taste, improve foam stability and preservative properties to the beer (Laws, 1983). Based on degree of solubility, hop resins are further subdivided into as hard and soft resins. Soft resins contribute a lot as flavoring agent and also help to preserve beer for relatively long period of time in comparison with that of hard resins. Soft resins are hexane soluble while the totals resin is soluble in cold methanol. The most important property of the resin is, of course, its bittering value (Kunze, 1996).

Hops, the female flowers of the hop plant (Humulus lupulus) are grown in the temperate region of the world, solely to meet the demands of the brewing industry (Hough et al., 1982; Grant, 1977). In most tropical countries, the hops are imported. With the expansion of the brewing industry in Africa, huge amounts of money are therefore being spent by developing countries for the importation of hops. In Ethiopia, gesho (Rhamnus prinoides) is particularly used to provide a special aroma and flavor (Shale and Gashe, 1991; Berhanu Abegaz et al., 1999). The chemical substances such as emodin, physcion, rhamnazin, prinoidin, and many other emodin-derived compounds were reported from Rhamnus prinoides (Abegaz and Kebede, 1995). Among different chemical substances found in Rhamnus prinoides, naphthalenic glucoside, geshoidin (Van Staden and Drewes, 1994) is the basic bittering agent for beverages. Although gesho may have antibacterial effect against some groups of bacteria, its main purpose in the process is believed to impart the typical bitter taste to tella (Ashenafi, 2006).

Apart from bittering and flavoring properties of hops, they are widely used for their oils (Tinseth, 1994) to impart characteristic aroma for beer production (Goldammer, 2000). In brief, essential

oil with the range of 0.5 to 5% and the volatile resins are together responsible for the aroma in hops (Versele and Keukeleire, 1991).

2.2. Hop Chemistry

Because of the presence of the oil- and resin-rich lupulin glands, the overall composition of fresh, dried hop cones shows them to be unlike that of other plant material, though the leafy nature of the hop petals ensures the presence of such ubiquitous substances as proteins and carbohydrates. In an excellent and thoroughly comprehensive review of hop chemistry in 1967, Stevens (Stevens, R., 1967) quotes a typical analysis as being:

Table 2.1: Components of whole hop

Component	Amount (%)
Resins	15%
Proteins	15%
Monosaccharides	2%
Tannins (polyphenols)	4%
Pectins	2%
Steam volatile oils	0.5%
Ash	8%
Moisture	10%
Cellulose	43%

Source: Comprehensive review of hop chemistry in 1967, Stevens (Stevens, R., 1967)

No doubt this analysis was for a hop of quite modest resin content by today’s standards, because some of the modern hop varieties contain nearly twice the amount quoted above (Verzele, M., 1986). Of the aforementioned components, the lupulin glands contain virtually all of the hop resins (in the form of a-acids, b-acids, desoxy-a-acids, and uncharacterized “soft resins, the essential oils and most of the hop cone fats and waxes (comprising perhaps 5% of the total and included previously under cellulose, etc). Seeded hops will contain a relatively large proportion of fats and nonvolatile “fixed” oils.

Other constituents of hops include varying amounts of tannins, carbohydrates, protein, lipids and waxes. However, the most important constituents are the hop resins from which are derived the principal bitter substances in beer and other non-alcoholic beverage and the oil which is responsible for hop character.

The brewing value of the hop is found in its resins and essential oils. (Peacock, 2009) put it that the brewing value of the hop is found in hop resins and essential oils that are contained in the lupulun glands of the female hop cone. These contain bitter resins and ethereal oils which supply bittering and aroma components of beer. Hop resins are the most valuable and most characteristics components of hops. They give beer its bitter taste, improve foam stability and act as antiseptics towards microorganisms (Hudson, 1970).

In the traditional brewing process, hops are boiled with wort in a copper vessel for 1-2 hours, during which the resins go into solution and are isomerized to produce the bitter principles of beer.

Hop resins are sub-divided into hard and soft based on their solubility. Alpha and Beta acids are two compounds present in the soft resins and are responsible for bitterness. Alpha acids are the precursors of beer bitterness since they are converted into Iso alpha acids in the brew kettle. They are therefore responsible for about 90% of the bitterness in beer (Westwood, 1994)

2.3. Hop Resins

The so-called “hard” and “soft” resins are primary subclasses of the “total” resin content of hops. These classes of resins are defined by their propensity to dissolve in various solvents, the hard resins (mostly xanthohumol and oxidation products of a- and b-acids) being more polar than the soft resins (mainly a-acids, b-acids, and desoxy-a-acids):

Total Resins – soluble in diethyl ether and methanol and consisting of the:

- Hard resins — insoluble in hexane
- Soft resins — soluble in hexane

2.3.1. Soft Resins

The resinous fraction of fresh hops contains mostly the a-acids and b-acids, each of which consists of analogous series of closely related homologs. Together with the desoxy-a-acids, they constitute the major portion of the192 Handbook of Brewing chemistry, (Verzele, M., 1986). Tannins (polyphenols) so-called "soft resin" fraction of hops. The close similarities between these compounds arise as a result of the proposed common pathway to their formation (Fung, S.Y et al., 1994).

In their native state, the hop resin acids exist within the lupulin glands as fully protonated, non ionized species. In this form, they are virtually insoluble in water, but they dissolve readily into methanol or less polar solvents such as dichloromethane (methylene chloride), hexane, or diethyl ether. At room temperature, a preparation of a-acids will be a pasty, yellow solid, while b-acids, also yellow unless highly purified, will be substantially harder and may become semi crystalline. When slowly heated, both become progressively more mobile, though the temperature has to be raised to more than about 60°C before the b-acids will flow easily.

2.3.2. Hard Resins

In a fresh hop, the amount of the "hard resin" is quite small and is mostly made up of the yellow/ orange-colored prenylflavonoid, xanthohumol. However, during storage, the hard resin fraction increases as the alpha- acids and beta-acids from which they are mostly derived decline. With the exception of xanthohumol, the precise chemical identity of the hard resins is not well understood (and may include dimers and trimers), but generally they are more polar and therefore more water-soluble compounds and will tend to pass more readily into the beer than their precursors.

2.4. Hop Oils

Dependent on variety, hops contain from about 0.4 to 2.5 ml/100 g of steam volatile, essential oils. As a general rule, high-alpha, bittering hops contain more oil on a dry weight basis than do lower-alpha aroma hops, but this is really no more than a reflection of the greater amount of lupulin present in the former. Of much more significance is the spectrum of components, which

is primarily genetically determined and varies substantially between varieties (Kovacˇevicˇ, M. and Kacˇ, M., 2002). Gas chromatographic analysis of hydro distilled oils from freshly harvested and dried hops reveals a vast number of compounds, but the chromatographic patterns are distinctive for each variety and generally enable reliable identifications to be made (Peacock, V.E. and McCarty, P., 1992; Walsh, A., 1998)

Hops are typically added to Wort in 1-3 stages during the boil: Bittering, Flavor and Aroma. These stages have to do with what role they are playing in your beer, and are not associated with a specific type of hop. In other words, the same hop variety might be used for bittering, flavor and aroma. Not all beers will have 3 additions; some may have only one, some may have up to 5 or 6 additions. All beers do have at least one hop addition for bitterness, to balance the sweetness of the malt.

There are no specific types of hops for specific addition times. Meaning Cascade can be used as bittering, flavoring, or aroma hops. It is not uncommon that your bittering and aroma hops are the same. When you have a kit that comes with 2 oz. of Cascade hops and wonder which one if for bitter, and which one is for aroma, they are the same. 1 oz. gets added at the beginning of the boil, while the other ounce gets added right at the end of the boil.

You want to add hops at different times to give your beer more complexity. If you just added bittering hops, the beer would be fine, but would be missing something. By adding the aroma hops, you are adding another dimension to your beer. If you used only aroma hops, your beer would be lacking bitterness. Not enough alpha acids from the hops would be isomerized in your boil.

Most recipes will tell you when to add your hops either in time from the start of the boil, or time that is left in the boil. For example, you might have a beer that is supposed to boil for a total of 60 minutes. The directions might tell you to add the bittering hops 30 minutes into or 30 minutes left of the boil.

Bittering: Bittering hops are added once the wort has been collected in the kettle (or after you've added the malt extract) and a rolling boil has been achieved. They are usually boiled for 60 minutes, although some recipes call for as little as 30 minutes. All beers have some bittering

hops. The main reason for this is that without the bitterness from the hops, your beer would taste syrupy-sweet. Another benefit is that hops are a natural preservative and will help your beer to keep for a longer time or for extended aging periods.

Flavoring: Flavoring hops are generally added with between 15 and 30 minutes remaining in the boil. In this time frame, very little of the bitterness will be extracted from the hops, but that crisp hoppy flavor will be imparted. Again, these may be the same as your bittering or aroma hops, it is the time that they are boiled that makes the difference.

Aroma: Hop oils that are responsible for aroma are extremely volatile and will be driven off in the steam of your boil almost immediately. Therefore, aroma hops must not be boiled for long. They are typically added during the last 5 minutes of the boil, or at flame out (when the kettle is removed from the heat). Adding hops at flame out will produce the maximum amount of aroma.

2.5. Tropical Hop Substitutes for Beer brewing

Some pioneer work showed that leaves of the vegetable, Gongronema latifolium (utazi) have great potential as substitute for hops in tropical beer brewing. It was found out that this plant possessed some antiseptic properties against beer spoilage microorganisms. The chemical properties of beer brewed using this plant did not differ much from that brewed with hops though their organoleptic differences were pronounced. The authors however did not characterize the vegetables as they only used it for brewing and sensory analysis (Ajebesone and Aina., 2004) carried out proximate analysis of four tropical plants used for food in Africa namely Azadirachta indica (neem), Garcinia kola (bitter kola), Gongronema latifolium (heckel) and Vernonia amygdalina (bitter leaf). Those authors concluded that these tropical vegetables can serve as substitutes for hops in tropical beer brewing.

The use of bitter leaf as substitute for hops in the Nigerian brewing industry has been chronicled by Adama (Adama et al., 2011) Azadirachta indica is used in some parts of Nigeria for treatment of malaria while Garcinia kola is used in some areas for the treatment of stomach ache and gastritis. Vernonia amygdalina and Gongronema latifolium are widely consumed as vegetables. One thing common to all the four plants is that they are bitter, like hops, but thrive in tropical regions, unlike hops (Ajebesone P.E. and Aina J.O., 2004).

2.5.1. Bitter leaf (V. amygdalina)

V. amygdalina occurs naturally along rivers and lakes, in forests margins, woodland and grassland up to 2800 m altitude, in regions where mean annual rainfall is 750-2000mm (Ofori et al., 2013). V. amygdalina can be commonly found along drainage lines and in natural forests or at home and commercial plantations (Alem and Woldemariam, 2009). It requires full sunlight and prefers humid environment. It grows on all soil types but prefers humus-rich soils (Ofori et al., 2013).

Figure 2.1: Bitter leaf (Vernonia amygdalina)

The variety of secondary metabolites extracted from V. amygdalina, explains well the diversity of the biological activities of this plant extract. Leaf extract of V. amygdalina was found to contain reducing sugar, polyphenolics, terpenoids, saponins, alkaloids, cardiac glycosides, steroids or triterpenes, anthraquinone and coumarins without cyanogenic glycoside (Ayoola et al., 2008).

However, only tannins, glycosides and saponins without flavanoids could be obtained from its root and stem bark extracts (Nduagu et al., 2008). All of these phytochemicals contributed to anticorrosion (Odiongenyi et al., 2009) and antifungal effect (Nduagu et al., 2008) of V. amygdalina while its bitter taste was reported to be due to the presence of anti nutritional factors such as alkaloids, saponins, tannins and glycosides (Arhoghro et al., 2009). Phenolic compounds identified in V. amygdalina can be grouped into flavonoids, tannins (Salawu and Akindahunsi, 2007).

2.5.1.1. Botanical Description of Vernonia amygdalina

According to (Erasto et al., 2006), the botanical overview of Vernonia amygdalina belongs to the family Asteraceae. The leaves are dark green coloured with a characteristic odour and a bitter taste (Bosch et al, 2005). Flower heads thistle like, small, creamy white, 10 mm long, grouped in dense heads, axillary and terminal, forming large flat clusters, 15 cm in diameter, sweetly scented (Ofori et al., 2013).

Vernonia is a genus of about 1000 species of forbs and shrubs in the family Asteraceae. Some species are known as Ironweed. The genus is named after English botanist William Vernon. Vernonia amygdalina, commonly called bitter leaf, is a perennial shrub of 2-5 m in height that grows throughout tropical Africa. It belongs to the family Asteraceae, has a rough bark with dense black straits, and elliptic leaves that are about 6 mm in length. The leaves are green and have characteristic odor and bitter taste (Ofori et al., 2013).

2.5.1.2. Major Uses and Functions

The leaves are used for human consumption and washed before eating to get rid of the bitter taste. They are used as vegetable and stimulate the digestive system, as well as they reduce fever (Argheore, et al., 1998). Furthermore, are they used as local medicine against leech, which are transmitting bilharzias. Free living chimpanzees eat the leaves, if they have attacked by parasites. Vernonia amygdalina is also used, instead of hops to make beer in Nigeria (Anonymous, 2000).

According to the research carried out by (Okoh et al., 1995), the high cost of importing conventional brewing ingredients into some tropical countries where they cannot be produced has led to the adoption of locally available products in their place. The possibility of using locally available substitutes instead of hops has also been investigated. One possible hop substitute is Vernonia amygdalina, known as "bitter leaf", which is widely grown in Nigeria as a food vegetable, and resembles the hop not only in its bitter flavour but also in its antimicrobial properties.

In Ethiopia the plant is used in cleaning the containers used for fermentation purpose. Due to its bitterness, it also can be used as a bittering agent, a hop substitute and for the control of

microbial contamination in beer brewing without affecting the quality of malt. In Ethiopia, it is used to make honey wine called Teij (Eleyinmi et al., 2004; Kasolo and Temu, 2008). The leaves are used for human consumption and washed before eating to get rid of the bitter taste. They are used as vegetable and stimulate the digestive system (Ofori et al., 2013).

2.5.1.3. Health benefits of Bitter Leaf (Vernonia amygdalina)

The plant is used for treatment of jaundice, diarrhea, Hepatitis B and C, cancer; diabetes and tuberculosis with the development of bitter leaf based dietary supplements have shown great promise in chemical studies (Muanya, 2013). Polyherbal preparations with bitter leaf as the active ingredients strengthen the immune system through many cytokines and chemokines regulations (Muanya, 2013). The herb not only lowers the body sugar level sufficiently, it also plays a role in the repair of pancreas. If 10 handfuls of fresh leaves are squeezed in 10 liters of water and consumed two glasses thrice a day for a month, diabetes is cured.

Adding bitter leaf to your healthy diet may reduce the risk of chronic diseases like breast cancer and type 2, diabetes. Bitter leaf is a traditional ingredient in Africa cuisine. Bitter leaf can reduce bad and total cholesterol. It is an abundant source of oxidation increasing (antioxidants). The antioxidant properties of bitter leaf make a healthy disease fighting addition to human health. Staying physically active, eating a low fat diet and maintaining a healthy weight can reduce risk of breast cancer in women. Additionally, eating bitter leaf may combat breast cancer cell growth (Edeoja et al., 2005; Ejoh et al., 2005).

Bitter leaf is an abundant source of the poly unsaturated fatty acids, linoleic and linolenic acid and these poly unsaturated fatty acids have been found to be protective against cardiovascular disease (Tapsell, 2006). Bitter leaf is a real wonder of nature (Adodo, 2009). It is very useful in the care of the liver and kidney. It is a multi-healer. It should be taken always for good health (Swee et al., 2010). It is a body cleanser, it cures pile. It is a wonder plant that is known to cure almost all human health problems. It soothes inflamed joints and eradicates pains common with arthritis or rheumatism patients (Okoli et al.,2007). Bitter leaf is also employed in zoo pharmacology for example, in the wild, chimpanzees have been observed to ingest the leaves when suffering from parasitic infections. Ethno botanically, the leaves of bitter leaf could be consumed either as vegetable (macerated leaves in soups) or aqueous extracts as tonics for

various illnesses (Sabiu and Wudii, 2011). Many herbalists and naturopathic doctors recommend aqueous extracts of bitter leaf for their patients for emesis, loss of appetite induced ambrosia, dysentery and other gastrointestinal tract problems (Schippers, 2000).

2.6. Hop Analysis

2.6.1. General

Over the course of the last century, a variety of methods have been developed for the analysis of hops and hop products, primarily, of course, with the objective of obtaining a useful measure of the bittering power of hops.

Many of these methods have been subjected to collaborative testing and subsequent approval by brewing organizations, and are published as recommended methods. These methods, many of which have been adopted as international methods, should generally be preferred for use by the brewer who is not thoroughly familiar with the subject, though it is true to say that improved methods are often to be found in the literature, and also that there are specific areas that are poorly or not at all covered. The most comprehensive and widely used analytical compilations are:

1. ASBC(American Society of Brewing Chemists) Methods of Analysis
2. Analytical-EBC(European Brewery Convention)
3. Methods of Analysis of the Institute of Brewing, (now the Institute of Brewing & Distilling)

2.6.2. Hop Resin Analysis

Methods for the analysis of hop resins in hops and hop products fall mostly into one of the following categories:

1. Classification into "hard," "soft," and other resin fractions via solvent dissolution
2. Polarographic methods (for a-acids)
3. Lead conductometric value (LCV or CLV) methods (for a-acids)
4. Spectrophotometric methods (for a-acids, b-acids, and hop storage index)
5. HPLC methods (for a-acids, b-acids, and iso-a-acids)
6. Capillary electrophoresis (for a-acids and b-acids)
7. Near infrared (NIR) methods (for moisture, a-acids, b-acids, and hop oil)

2.6.2.1. Spectrophotometric Methods

The spectral characteristics of dissolved a- and b-acids are dependent upon their degree of dissociation and are substantially different from one another, especially in alkaline solution. This fact is used as the basis for an analytical method in which the absorbance of a dilute, alkaline solution in methanol of organic, solvent-extracted hops is first measured at three different wavelengths in the UV region (Alderton, G., et al 1954). The values obtained are then entered into regression equations that determine the original content in the hop sample of not only the a-acids, but also that of the b-acids. The method is especially popular in the U.S., where it forms the basis for most commercial transactions. In the official ASBC method (Hops 6, known colloquially as "ASBC Spectro"), the primary extraction is done with toluene and the residual hop material separated simply by allowing the extraction mixture to stand for about 30 min. The toluene solution is next diluted into alkaline methanol prior to reading in a 1-cm path length cell in a variable wavelength spectrophotometer. Readings are then taken at 275, 325, and 355 nm and inserted into regression equations:

$$\%\ \text{alpha acids} = D^*[-19.07A_{275} + 73.79A_{325} - 51.56A_{355}] \quad (1)$$

3. Materials and Methods

The study consisted of five separate but related stages: (i) carrying out proximate, mineral content, phytochemical and anti-oxidant activity of the bitter leaf (Vernonia amygdalina) against the imported commercial hops (ii) extract and study the effect of process parameters (extraction time, temperature and particle size) on the bitterness level and Iso- alpha acid value of the bitter leaf aqueous extract using a reflux condenser, (iii) optimization of the bitter leaf extract process parameters (time, temperature and particle size) (iv) extraction of resinous component of bitter leaf and (v) evaluation of the bitter leaf as a commercial hops.

3.1. Experiment Location

Sample preparation (drying, size reduction and particle size distribution of the raw material) were conducted in the laboratory of School of Chemical and Bio Engineering Addis Ababa University, Institute of Technology. While, proximate composition, mineral content, phytochemical and anti oxidant activity of the sample has been carried out at the Center of Food Science and Nutrition, Addis Ababa University. The experiment on extraction process has been carried out in the Laboratory of Ethiopian Environment and Forest Research Institute, Forest Product Utilization Research Center.

3.2. Experimental Materials

The materials used were bitter leaf locally known as 'grawa' which were collected from Billa town, Western Wollega Zone while other chemicals and solvents will be purchased from the local market in Addis Ababa. The chemical used for this thesis work are: methanol, n-hexane, Iso-octane ,ethanol , distilled water, diethyl ether, DPPH (2,2-diphenyl-1-picryrazyl ,ascorbic acid, gallic acid, sodium carbonate, Folin-Ciocalteaus reagent, glacial acetic acid, hydrogen peroxide, boric acid, sulfuric acid and hydrochloric acid. All these chemicals used in this analysis were analytical grade.

3.3. Experimental procedure

3.3.1. Design of experiments (DoE)

The first task before conducting the experiments was selection of potential parameters to be varied. The three main factors selected in this study were extraction temperature, extraction time and particle size. The levels of the selected factors are determined from the literature research and are presented in Table 3.1.

Table 3.1: Factors and Levels of variables used for General Factorial Design

Factors	Levels		
Temperature (^{o}c)	80	90	100
Extraction Time (hr)	1	2	3
Particle size (mm)	0.25 - 0.5	0.5 - 0.75	0.75 – 1

The experiment performed as a completely randomized design with three main factors at three levels and two response variables. The two response variables were the bitterness level and Iso alpha acid value. The experimental design used was a General Factorial Design (GFD). General factorial design required 27 runs for modeling a response. Details of the experimental

Table 3.2: General Factorial Design and combination of process variables

Run order	Temperature [^{o}C]	Time [hr]	Particle size [mm]
1	80	1	0.25 - 0.5
2	90	1	0.25 -0.5
3	100	1	0.25 -0.5
4	80	2	0.25 -0.5
5	90	2	0.25 -0.5
6	100	2	0.25 -0.5
7	80	3	0.25 -0.5
8	90	3	0.25 -0.5
9	100	3	0.25 -0.5
10	80	1	0.5-0.75
11	90	1	0.5-0.75
12	100	1	0.5-0.75
13	80	2	0.5-0.75
14	90	2	0.5-0.75
15	100	2	0.5-0.75
16	80	3	0.5-0.75

17	90	3	0.5-0.75
18	100	3	0.5-0.75
19	80	1	0.75-1
20	90	1	0.75-1
21	100	1	0.75-1
22	80	2	0.75-1
23	90	2	0.75-1
24	100	2	0.75-1
25	80	3	0.75-1
26	90	3	0.75-1
27	100	3	0.75-1

runs with the set of input parameters that were conducted are given in Table 3.2. Design expert software was used to design the experiment and randomize the runs. Randomization ensures that the conditions in one run neither depend on the conditions of the previous runs nor predict the conditions in the subsequent runs. Randomization is essential for drawing conclusions from the experiment, in correct, unambiguous and defensible manner.

3.3.2. Sample Preparation

Freshly bitter leaves were collected from western wollega zone area, Oromia regional state of Ethiopia. The collected leaves were de-stalked and thoroughly washed with distilled water and screened to remove dirt, soil particles and foreign bodies. They were then dry at ambient temperature ($25^{0}C$) for five days to eliminate the moisture content to 10 -12%. (Adama, K.K., 2011)

The dried leaves were then crushed and sorted using a sieve analysis into particle size of 1-0.75mm, 0.75 - 0.5mm and 0.5 - 0.25mm. The size reduction was done in order to increase the surface area for contact with the solvent because the particle of a soluble material is surrounded by a matrix of insoluble matter and thus, the size reduction will allow the solvent to penetrate and diffuse into the particle to allow the extract to diffuse out accordingly. The resulting powders were then put into HDPE (high density polyethylene) bags, heat sealed and then stored in airtight metal containers, desiccator (Ajebesone P.E. and Aina J.O,.2004).

Generally, two kilograms (2.0 kg) of the plant material thus prepared and stored in a desiccator for the rest of the experiment and 20g of the resulting powders were then used to obtain the extracts by steeping procedure according to their assigned to treatment groups at random.

3.3.3. Preparation of Bitter leaf extract (Reflux Condenser)

In the process, 20 grams of the granulated sample of particle size 1- 0.75mm, 0.75 - 0.5mm and 0.5 - 0.25mm was measured into a round bottom flask which contained 200ml of distilled water. The mixture will rigorously agitated by swirling the flask (Adama et al., 2011).

A reflux condenser was mounted and fitted onto the conical flask. Then the condenser was connected to a tap water source. The vent of the flask will make air-tight to prevent the escape of the evaporating steam. The set-up was held tight with a retort-stand and the mixture placed on a water bath and the thermostat adjusted to maintain a constant heating rate at temperatures of 80 ^{o}C, 90°C and 100°C. The mixture was allowed to boil for the extraction time of 1hr, 2hr and 3hrs. The vapor from the boiling solvent was made to condense and return to the mixture by means of a reflux condenser which will mount on the flask through which water was constantly flowing. After the extraction had been completed, the heater will be switched off, the solution allowed to cooled and afterward filtered using a filter paper placed on a funnel in a beaker.

This procedure was repeated several times using fresh samples of same mass to obtain sufficient quantity of the extract for analysis. The extract will be used to determine Analytical Bitterness Level and Iso-alpha acid values.

3.4. Parameters analysis

3.4.1 Determination of Alpha acid and Resin

The resin values for the bitter leaf (Vernonia amygdalina) were determined by the method described by (Hough et al., 1983 and Lob, 1977.) while Alpha Acid was calculated using (AOAC, 2000) and ASBC 1976 methods.

3.4.1.1. Alpha Acid Determination

Twenty (20) grams of the granulated bitter leaf sample was added to 100ml of cold methanol in a conical flask and the mixture was thoroughly agitated. The resulting solution was then centrifuged at 2,500 radiant per minute for 20 minutes and the extract decanted. The extract was then acidified with 10ml of 0.002N HCl and its absorbance read at 355nm, 325nm and 275nm respectively using a spectrophotometer. The spectrophotometer was switched on and calibrated using a blank (pure methanol). The sample was then inserted into the curette and the absorbance read at 275nm and recorded. The curette was then rinsed. The procedure was repeated and the absorbance read at 325nm and 355nm respectively. The Alpha Acid calculated using AOAC (2000) and ASBC 1976 methods

$$\text{Alpha Acid (mg/L)} = 73.79(A_{325}) - 51.56\,(A_{355}) - 19.07\,(A_{275}) \qquad (1)$$

3.4.1.2. Resin Determination

Total Resin: In this process, 20 g of the sample was dissolved in 100 ml of cold methanol in a conical bottom flask and the mixture was vigorously agitated by swirling the flask. Thereafter, the solution was filtered. The filtrate containing the resin was then dried to a constant weight over water bath at 50^0C. The total resin was calculated as a percentage of the original sample weight.

Soft Resin: With regard to resin determination, 20 grams of the sample was dissolved in 20 ml of n-hexane thoroughly stirred and filtered using filter paper. Filtrate was dried to a constant weight at 50^0C. The soft resin was calculated as the percentage of the original weight of sample dissolved in the n-hexane.

Hard Resin: Hard resin was determined by subtracting soft resin from total resin.

3.4.2. Determination of Analytical Bitterness Level and Iso alpha acid value

3.4.2.1. Determination of Analytical Bitterness Level

Five (5) milliliters of the extract obtained from direct extraction (a reflux condenser) of the bitter leaf extract was acidified with 0.5ml of 6N HCl. This was subsequently extracted with 10ml of Iso-octane in a shaker. The mixture was then agitated for 20 minutes and thereafter centrifuged for 10 minutes. The absorbance of the Iso-octane extract was then determine at 275nm using a spectrophotometer. The analytical bitterness level values for the extract were determined by the method described by (Hough et al., 1983)

$$\text{Bitterness level in ABU} = A_{275} * 50 \qquad (2)$$

3.4.2.2. Determination of Iso-Alpha Acid

Fifteen (15) milliliters of sample water extract obtained from direct extraction (a reflux condenser) of the bitter leaf extract was acidified with 0.5ml of 6N HCl and mixed with 15ml of pure Iso-octane in a shaker. 10ml of the Iso-octane extract was then washed with 10ml of a mixture of methanol and 4N HCl (68:32, v/v). Subsequently, 5ml of the washed Iso-octane layer was diluted with 5ml of alkaline methanol and its absorbance read at 255nm. The Iso-alpha acid (mg/L) was then computed according to (AOAC 2000) method of analysis.

$$\text{Iso-Alpha Acid (mg/L)} = A_{255}(96.15) + 0.4 \qquad (3)$$

3.4.3. Proximate composition

3.4.3.1. Determination of Moisture Content

A crucible was washed and dried in an oven at 105°C for 1h and placed in a desiccator to cool. The weight of the crucible (W_1) was measured. 5gm of the prepared Samples were weighted in this dry crucible (W_2), dried in a oven at 105°C for 5 hour and reweighted again (W_3). All weights are measured in grams. The moisture content was determined by the following relationship:

$$\text{Moisture content (MC)} = \frac{W2 - W3}{W2 - W1} \times 100 \qquad (4)$$

3.4.3.2. Determination of Total Ash

The apparatus used for total ash analysis were crucible and muffle furnace. A crucible was washed and dried in an oven at 105°C for 2h and placed in a desiccator to cool. The weight of the crucible (W_1) was measured. Samples were weighted in this dry crucible (W_2), dried at 550°C for 5 hour in a muffle furnace until a white or grayish ash is formed. The crucible was placed in a desiccator, cooled and weighed until constant weight reached (W_3). The ash content was calculated on dry mass basis by the following relationship:

$$\%\ \text{Ash} = \frac{W2 - W}{W1 - W} \times 100 \qquad (5)$$

3.4.3.3. Determination of Fiber Content

Crude fiber was determined using fiber tec after digesting a known weight of sample in refluxing 1.25 % sulfuric acid and 1.25 % sodium hydroxide (AOAC, 2000). About one gram of sample was weighed into a pre-dried crucible which contained one gram of celite sand for the purpose of simplifying filtration (W_1). Then the crucible with its content was placed in the fibertic and the sample was digested with 1.25 % of sulfuric acid for 30 min and followed by digestion with 1.25% of sodium hydroxide for 30 min. Then after, the crucibles were taken-off and dried in oven at 130°C for 2hrs. Then cooled in desiccator and weighed as (W_2). Finally the crucibles were placed in muffle-furnace and the sample was ashed at 525°C for 3hrs. Cooled to room temperature in desiccator and weighed as (W_3). The crude fiber content was calculated by using the following formula.

$$\text{Crude Fiber (g/100g)} = \frac{W2 - W3}{W1} * 100 \qquad (6)$$

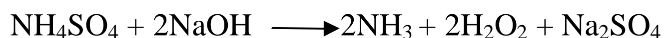
3.4.3.4. Determination of Crude Protein

Protein (N×6.25) is determined by Kjeldahl method. In this method, proteins and other organic food components in a sample are digested with sulfuric acid in the presence of catalysts. Then, the total organic nitrogen is converted to ammonium sulfate. The digest is neutralized with alkali

and distilled into a boric acid solution. The borate anions formed are titrated with standardized HCl, and are converted to nitrogen in the sample. The result of the analysis represents the crude protein content of the food (A.O.A.C. 2000).

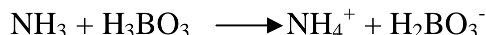
Sample preparation for digestion: 0.5 g of sample was weighed in a Tecator tube and placed in the tecator rack. Then 6 ml of sulfuric acid mixture was added by using pipette and mixed with the sample immediately. Then 3.5 ml of 30% hydrogen peroxide was added step by step till the reaction stops. As soon as the reaction has ceased, the tube was hand-shaken for a few minutes and is put back into the rack. Then, 3 g of catalyst (K_2SO_4 with a mixture of $CuSO_4$ 100 to 0.5 ratios) was added and the mixture was allowed to stand for 5-15 min before digestion.

Digestion: The tube in the rack was lowered into the digester in the fume hood at 370°C. The digestion was continued until clear solution appeared (3-4 hrs) in the fume hood. After digestion was completed, the content in the flask was diluted by water and concentrated sodium hydroxide (40%) was added to neutralize the acid and to make the solution slightly alkaline.



$$NH_4SO_4 + 2NaOH \longrightarrow 2NH_3 + 2H_2O_2 + Na_2SO_4$$

The ammonia was then distilled into a receiving flask that consisted solution of excess boric acid (4%). The solution was titrated with standard acid (0.1HCL) until the color changes to a reddish color. The total nitrogen content was latter calculated using the following formula



$$NH_3 + H_3BO_3 \longrightarrow NH_4^+ + H_2BO_3^-$$

The total protein was calculated using the following formula:-

$$\text{Total Nitrogen (\%)} = \frac{0.1*(Vt-Vb)*14*100}{W*1000} \qquad (7)$$

$$\text{Crude Protein (\%)} = \text{Total Nitrogen (\%)} \times 6.25$$

Where: Vt &V_b = volume of HCl consumed during titration and to neutralize the blank

0.1= Normality of the HCl, 14 = Eq. wt of Nitrogen

W = weight of sample on dry basis

6.25 = Conversion factor from total nitrogen to crude protein

3.4.3.5. Determination of Crude Fat

Fat was determined by exhaustively extracting the sample in petroleum ether (boiling point, 40 to 60°C) in Soxhlet extractor (Method No.4.5.01) (AOAC 2000). A 2.00g (M) of the sample was

weighed into an extraction thimble and covered with absorbent cotton. Then 50 ml solvent (petroleum ether) was added to a pre-weighed cup (M_1). Both the thimble and the cup were attached to the extraction unit .The extraction process continue for 4 hrs and then this flask with its content was removed from the Soxhlet and placed into drying oven at 92°C for 30min, and placed into desiccators for 30 min. Finally the mass of each flask together with its fat contents was measured as (M_2). Then the total content of the fat was calculated using the following formula.

$$\% \text{ Crude Fat} = \frac{M2-M1}{M} * 100 \tag{8}$$

3.4.3.6. Determination of Carbohydrate

The total and available carbohydrate content was determined by difference. Addition of the percentages of crude protein, crude fat and ash was subtracted from 100.

Carbohydrate (g/100g) = [100-(Protein + Fat +Ash in grams)] (9)

3.4.4. Mineral Analysis

Minerals analyzed in this study were Calcium, Magnesium, Iron, and Zinc. The mineral content of the samples were determined by the method described by (Dickman and Bray, 1940). For estimation of minerals the ash was dissolved by 7ml of 6NHCl at low temperature on hot-plate for about 2hrs. 15ml of 3 NHCl was added and the crucibles were heated on the hot plate until the solution just boiled. Then, 10ml of 3HCl were added and heated on a hot plate until the solution boils. The digest was cooled and filtered through a filter paper (42mm, Whatmann) in to a 50ml volumetric flask. Then ml 3MHCl was added to dishes and heated to dissolve the residue in the dishes and then transferred to the volumetric flask. Then the filter paper was washed thoroughly and the washing was collected in the flask made to the mark. Then, after the mineral concentration was determined by AAS for calcium determination 5 ml of 10% lanthanum chloride solution was added to the flask. Then diluted to 50ml mark with distilled water. The blank was prepared by taking the same amount of reagents through the steps all of the above without the sample. The instrument was set and optimized based on the instruction given in the

manual. The calibration solutions and the reagent blank solutions were measured first. Then the samples were run following the calibration values. The calibration curve was prepared for the required metal by plotting the absorption values against the metal concentration in ppm. The mineral content of each sample was calculated using given formula (carvalho, 2009)

$$\text{Mineral (mg/100g)} = \frac{(\text{Conc.Sample} - \text{Conc.blank}) * \text{Vol.solution}}{10 * \text{weight sample}} \quad (10)$$

3.4.5. Phytochemical screening and anti oxidant activity of bitter leaf

3.4.5.1. Tannin

The amount of tannin was determined by the vanillin assay of (Burns 1971) as modified by (Maxson and Rooney 1972). The method was based on 1% HCl in methanol extraction of total tannin and determination using vanillin reagent. The extraction was done by weighing one gram of sample in a screw cap test tube and adding 10ml 1% HCl in methanol to the tube containing sample. After this the tube was put on mechanical shaker for 24 hrs at room temperature and centrifuged at 3000× g for 20 min. Then, one ml of the supernatant was taken and mixed with five ml of vanillin-HCl reagent in another test tube. The mixture was put at rest for 20 min in order for the reactions to complete. Then, the absorbance of the sample and blank (1 ml of supernatant with 1% HCl in methanol) were read at 500 nm using a UV-Vis spectrophotometer.

Standard solutions were prepared: About 40 mg D-Catechin was weighed and dissolved in 100 ml of 1% HCl in methanol (stock solution). A serious of (0, 200, 400, 600, 800 and 1000µl) of stock solution was taken in a test tube. The volume of each tube was adjusted to one ml with 1% HCl in methanol and five milliliter of vanillin-HCl reagent was added in each tube. The reaction was completed in 20 min and the absorbance was later read at 500 nm. The calibration curve (absorbance Vs concentration) is plotted using excel and the slope and intercept were calculated after adjusting for the blank zero (Embaby, 2010).

$$\text{Tannin (mg/g)} = \frac{[(\text{Ab} - \text{As}) - \text{Intercept}] * 10}{\text{Slope} * \text{Wt.sample} * d} \quad (11)$$

Where: As, A_b = absorbance of sample & blank, d = Density of solution (0.791 g/ml)

3.4.5.2. Phytic acid

The phytate content in the sample was determined according to the method described by (Adeniyi et al,.2009). One gram of dried sample was extracted with 10ml 0.2NHCl for 1hr at ambient temperature and centrifuged (3000 rpm) for 30 min. The clear supernatant was used for the phytate estimation. Two milliliter of wade reagent (0.03% solution of $FeCl_3.6H2O$ containing 0.3% sulfosalicylic acid in distilled water) was added to three of the sample solution and the mixture was centrifuged. The absorbance at 500 nm was measured using spectrophotometer. The phytate concentration was calculated from the difference between the absorbance of the blank (3ml of 0.2N HCl and 2ml of wade reagent) and that of the assayed sample. The level of phytate in the sample was calculated using phytic acid standard curve and the results were expressed as phytic acids in mg/100g dry weight.

To prepare the phytic acid standard curve, serial dilution of 4-45mg/ml phytic acid in 0.2NHCl were prepared. Three milliliter of the standards was pipette into 15ml centrifuge tubes with 3ml of 0.2NHCl as blank. To each tube one ml of the wade reagent was added and the solution was mixed on a vortex mixer for 5 second. The mixture was centrifuged for 10min and the supernatant read at 500nm by using water as a blank.

$$\text{Phytic acid in } \mu g/g = \frac{[(As - Ab) - \text{Intercept}] * 10}{\text{Slope} * W * 3} \quad (12)$$

Where: As= Sample absorbance,

Ab= Blank absorbance, W= Weight of sample

3.4.5.3. Alkaloid

The alkaloid content was determined gravimetrically by the method of Haborne, (1973) as cited in (Adeniy, et al., 2009). Five gram of each sample was weighed using analytical balance. Then the sample was dispersed into 50ml of 10% acetic acid solution in ethanol. The mixture was well shaken and then allowed to stand for about 4hrs before it was filtered. The filtrate was then evaporated to one quarter of its original volume on a hot plate. Concentrated ammonium hydroxide was added drop wise in order to precipitate the alkaloids. A pre-weighed filter paper

was used to filter the precipitate and the precipitate was washed with 1% ammonium hydroxide solution followed by drying in an oven maintaining at $60^{O}C$ for 30min. Then it was transferred in to desiccators to cool and then reweighed until a constant weight was obtained. The weight of the alkaloid was determined by weight difference of the filter paper and expressed as a percentage of the sample weight analyzed using the following formula. The experiment was repeated three times for each sample type and the reading recorded as the average of the triplicates.

$$\text{Alkaloid (\%)} = \frac{W2-W1}{\text{Weight of sample}} * 100 \qquad (13)$$

Where W1 = weight of empty filter paper

W2 = weight of filter paper + alkaloid precipitate

3.4.5.4. Antioxidant activity of Vernonia amygdalina

The antioxidant activity of the crude extract of bitter leaf (Vernonia amygdalina) using methanol is evaluated or estimated using the method of (Kirby and Schmidt 2004) with slight modification.

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

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

Secondly, about 4ml of 0.004% solution of DPPH radical solution in methanol was mixed with 1 ml of various concentrations (0.2-2mg/ml) of extracts in solvent extracted and with a vortex mixer. 10 mg of extract/ml of methanol solution is prepared as stock solution and using

micro pipette 10μl , 15μl, 20μl, 40μl, 60μl, 80μl,120μ,160μl, 180μl,200μl, were taken from stock solution as doublet and added to the test tube which was washed with distilled water and kept in oven at 105°C to remove water droplet from the surface of test tube which may interfere with absorbance of the extracted samples. Then contents of the test tubes are mixed with the vortex mixer for about 20s to allow the further mixing of DPPH with extracts and to facilitate the reaction between the two by homogenizing the samples.

Thirdly, all the test tube was incubated for about 30 minutes in the dark at room temperature. Incubating the samples for 30 minutes in dark was required to allow the reaction between the extract and DPPH and to prevent the absorbance of light by DPPH that may interfere with the actual absorbance of the sample and DPPH solution.

Fourthly, scavenging capacity was read using spectrophotometer by monitoring the decrease in absorbance at 517nm using high performance UV-Vis spectrophotometer and inhibition of free radical DPPH in percent (I %) was calculated in the following way:

$$\text{IE} = \left(\frac{\text{Acontrol} - \text{A sample}}{\text{A sample}}\right) \text{x100} \tag{14}$$

Where:

A_{control} is the absorbance of the control reaction (containing all reagents except the test compound)

A_{sample} absorbance of the test compound

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

3.4.5.5. Total phenolic Compound of Bitter leaf (V.amygdalina)

The total content of polyphenols of samples was estimated using the method described by (Martin et al. 1999). Stock solution of Gallic acid (20, 40, 60, 80, 100, 120, 140, 160 μg/ml) were prepared in methanol for preparation of standard solutions. Five gram of sample was mixed with 50ml of methanol in the 50ml of conical flask and placed on mechanical shaker for 4hrs in order to get methanol extract. The extracted methanol was filtered and the residues of samples were extracted again using the same set up by adding 25ml of methanol.

Then the filtrate was evaporated on rotary vapor shaker to get the residue of polyphenols content and afterward the residues were dissolved using 50mg/1ml calculated extraction volume. After sample preparation 160μl that was make up by 840μl water of approximately diluted sample and a standard solution of gallic acid were added to 15 ml of test tube containing 7ml of distilled water and the control or the blank reagent were prepared using distilled water. 1ml of Folin-Ciocalteaus phenol reagent was added to the mixture and then well shaken for 2sec.

After 5min, 1ml of a 7% sodium carbonate solution was added to the mixture. After putting the solution in the dark place for 90min as the reaction was completed, and then the absorbance was measured at 725nm. The total phenolic content of the samples were calculated using the following equation.

$$TPC = \frac{(\text{Sample Abs} - \text{Intercept}) \text{ x Sample extraction volume}}{\text{Slope x mass test sample x 10,000 x wt in dry matter}} * 100 \quad (15)$$

3.5. Statistical analysis

The optimal extraction conditions for analytical bitterness unit and Iso- alpha acid were determined using General Factorial Design. The 3-Factor, 3-Level and 2-replicate experimental design were used to investigate and validate extraction parameters affecting the responses. A summary of the extraction time, temperature, and particle size of 27(twenty seven) experiments with 2(two) replications were evaluated. The software Design-Expert (Trial Version 6.0.8) was employed for experimental design, data analysis, and model building. Statistical significance of the model and model variables was determined at a 5% probability level ($p < 0.05$). Significance of the result was set from analysis of variance (ANOVA).

4. Results and Discussion

4.1. Experimental results

The experimental values of bitterness level and Iso alpha acid obtained under different conditions were computed according to method described by (Hough et al., 1983; A.O.A.C.2000) and presented in Table 4.1. A total of 27 experimental run with its levels were performed in duplicate. The responses obtained for maximizing of the bitterness level and Iso alpha acid value were taken as the mean of these two replicates plus standard deviations. The individual replicates of raw data were written in appendix 1 and one can refer them. The figure obtained on the responses from this experimental result is highly related with the previous research on the bitter leaf extract in which the bitterness level and Iso alpha acid values are 14 and 8.52 respectively (Ajebesone P.E. and Aina J.O., 2004; Adama et al., 2011). These results were input into the Design Expert software version 6.0.8.for further analysis.

Table 4.1: Bitterness level and Iso-alpha acid values of bitter leaf extract

Std	Factor 1 A:Temprature [°C]	Factor 2 B:Extraction Time [hr]	Factor 3 C:Particle Size [mm]	Response-1 Bitternes Unit [ABU]	Response-2 Iso-alpha acid Value [mg/l]
1	80.00	1.00	0.5-0.25	16.55	8.09
2	90.00	1.00	0.5-0.25	16.65	8.28
3	100.00	1.00	0.5-0.25	16.75	8.57
4	80.00	2.00	0.5-0.25	16.65	8.28
5	90.00	2.00	0.5-0.25	16.75	8.47
6	100.00	2.00	0.5-0.25	16.85	8.76
7	80.00	3.00	0.5-0.25	16.8	8.47
8	90.00	3.00	0.5-0.25	16.85	8.66
9	100.00	3.00	0.5-0.25	17.00	9.05
10	80.00	1.00	0.75-0.5	16.60	8.18
11	90.00	1.00	0.75-0.5	16.75	8.38
12	100.00	1.00	0.75-0.5	16.85	8.66
13	80.00	2.00	0.75-0.5	16.75	8.38
14	90.00	2.00	0.75-0.5	16.9	8.47
15	100.00	2.00	0.75-0.5	17.05	8.95
16	80.00	3.00	0.75-0.5	16.95	8.57
17	90.00	3.00	0.75-0.5	17.05	8.76
18	100.00	3.00	0.75-0.5	17.1	9.14
19	80.00	1.00	1.0-0.75	16.5	7.99
20	90.00	1.00	1.0-0.75	16.65	8.18
21	100.00	1.00	1.0-0.75	16.7	8.47
22	80.00	2.00	1.0-0.75	16.65	8.18
23	90.00	2.00	1.0-0.75	16.7	8.38
24	100.00	2.00	1.0-0.75	16.9	8.86
25	80.00	3.00	1.0-0.75	16.75	8.28
26	90.00	3.00	1.0-0.75	16.8	8.57
27	100.00	3.00	1.0-0.75	16.9	9.05

4.2. Development of empirical models

In the present study, empirical models for the output responses (bitterness level and Iso alpha acid) in terms of the process parameters in actual and coded factors were developed by using the GFD. The sequential model fitting for bitterness level and Iso alpha acid of the samples prepared are given at Appendix 3. Two tests were carried out to determine the adequate model. These included Sequential model Sum of Squares and Model Summary Statistics. From Table 4.1 and Table 4.2, it was found that quadratic model was the most suitable model for the present study, because quadratic model had highR^2, adjustedR^2, predictedR^2 and low PRESS for both responses. The Sequential model Sum of Squares also showed that quadratic model was the highest order polynomial where the additional terms were significant as the PRESS value of cubic model could not be defined in the Model Summary Statistics for both responses. Besides, it can be observed that quadratic model had close proximity or reasonable agreement of Predicted R-square is with that of adjusted R-square.

The regression coefficients of the developed model are determined from the regression analysis. From the two tables it is observed that the quadratic model is the best fit model for both responses in terms of its significance and for this experimental design, the second order (quadratic) model is suggested, as the p-value of this model is also smaller than that of other models. The two developed equations regardless of significant and insignificant model terms for bitterness level and Iso alpha acid respectively are given at Appendix 4. The developed models are further used for optimization of the bitter leaf extract process.

4.3. Adequacy check for the developed models

Usually, it is essential to confirm first whether the fitted model provides an adequate approximation of the actual values or not. The adequacy of the model was checked by analysis of variance (ANOVA) and some diagnostic plots. Analysis of variance (ANOVA) is employed to test the significance of the developed models. Table 4.2and Table 4.3 shows the summary of the analysis of variance (ANOVA) of the two responses (bitterness level and Iso alpha acid) respectively. The detail ANOVA for the two responses is given at Appendix 4. The F-value is measure of variation of the data about the mean. Generally, the calculated F value should be

several times greater than the tabulated value, if the model is a good prediction of their experimental results and the estimated factors effects are real (Montgomery 2000). Also the high F-value and a very low probability indicate that the present models are in a good prediction of the experimental results. The p-value serves as a tool for checking the significance of each of the coefficients. The pattern of interaction between the variables is indicated by these coefficients. The variables with low probability levels contribute to the model, whereas the others can be neglected and eliminated from the model.

Table 4.2: ANOVA results of the quadratic regression model for bitterness level

Source	Sum of squares	DF	Mean square	F-value	P-value	
Model	0.61	7	0.087	80.13	<0.0001	significant
A	0.21	1	0.21	197.48	<0.0001	
B	0.26	1	0.26	242.07	<0.0001	
C	2.450E-003	1	2.450E-003	2.27	0.1486	
C^2	0.12	1	0.12	114.95	<0.0001	
AB	3.675E-003	1	3.675E-003	0.0808	3.40	
AC	1.333E-004	1	1.333E-004	0.7293	0.12	
BC	6.750E-004	1	6.750E-004	0.62	0.4391	
Residual	0.021	19	1.081E-003			
Core Total	0.63	26				

R^2=0.9684; Pred.R^2=0.9308; Adj.R^2=0.9552; and Adeq. Precision=34.104

Values of P >F less than 0.0500 indicates model terms are significant. In the present study A, B, C^2 for the response bitterness level and A, B, C, A^2, C^2, AB, AC for the response Iso alpha acid are significant model terms. High F-values and non significant lack of fit relative to the pure error indicated that models were a good fit. The coefficients of determination (coefficient of correlation), R^2 was high for all responses,

Table 4.3: ANOVA results of the quadratic regression model for Iso alpha acid

Source	Sum of squares	DF	Mean square	F-value	P-value	
Model	2.44	8	0.3	191.24	< 0.0001	significant
A	1.44	1	1.44	905.59	< 0.0001	
B	0.78	1	0.78	490.35	< 0.0001	
C	0.025	1	0.025	15.80	0.000009	
A^2	0.049	1	0.049	31.06	< 0.0001	
B^2	1.712E-004	1	1.712E-004	0.11	0.7468	
C^2	0.11	1	0.11	67.16	< 0.0001	
AB	0.019	1	0.019	12.09	0.0027	
AC	0.012	1	0.012	7.74	0.0123	
Residual	0.029	18	1.593E-003			
Core Total	2.47	26				

R^2= 0.9884; Pred.R^2=0.9729; Adj.R^2=0.9832; and Adeq. Precision=51.46

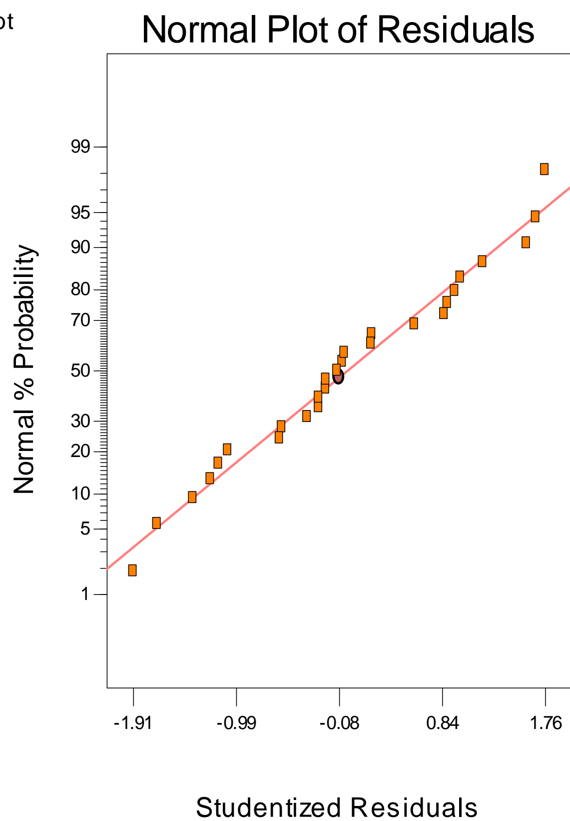
a value > 0.75 indicates the aptness of the model. For a good statistical model, the R^2 value should be close to one (Montgomery 2000). The value of R^2 (correlation coefficient) for both responses is very high and close to one which indicates a good agreement between experimental and predicted values. The predicted R^2 is in a reasonable agreement with the adjustedR^2 for both responses. Also, a ratio of adequate precision greater than 4 is desirable. In the present study the ratio for all responses were greater than 4, which is (34.104 for bitterness level and 51.460 for Iso alpha acid) indicates an adequate signal

Additionally, the developed response model for bitterness level and Iso alpha acid value has been checked by using residual analysis. Residuals are usually considered as components of variations, imprecisely fitted to the model and subsequently it is predicted that they behave according to a normal distribution feature. For the evaluation of normality of the residuals, a graphical visualization of the normal probability plot is considered as the proper method.

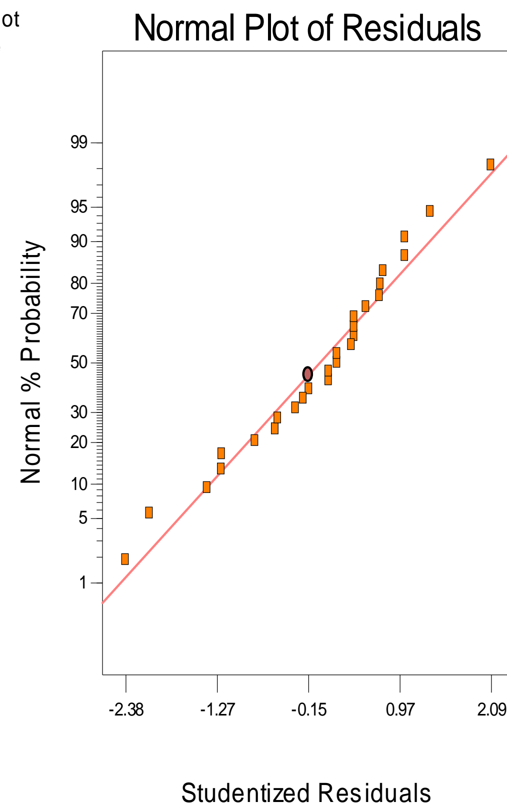
The normal plots for bitterness level and Iso alpha acid value are shown in Figure 4.1 (a) & (b). It can be observed that, the data are spread approximately in a straight line, which shows a good correlation between experimental and predicted values for the responses. The plots of observed versus predicted values shows minimal variation between the observed and fitted values for all

responses (Figure 4.1(c) and (d)). From the above ANOVA and analysis of residual plots for the two responses, the model does not reveal inadequacy and this model were used to navigate the design space to find the optimized conditions.

a)

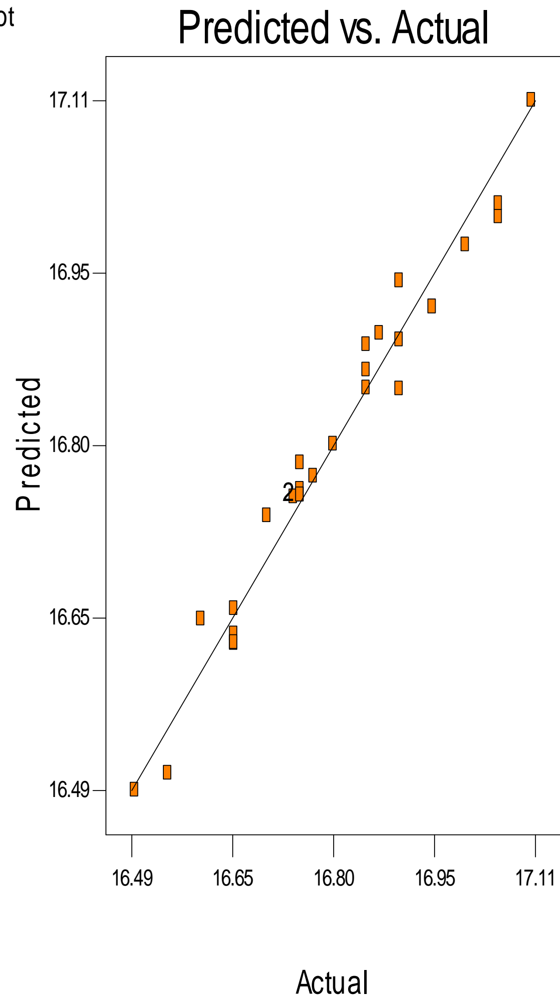


b)



DESIGN-EXPERT Plot
Bitternes Unit

c)



DESIGN-EXPERT Plot
Iso-alpha acid Value

d)

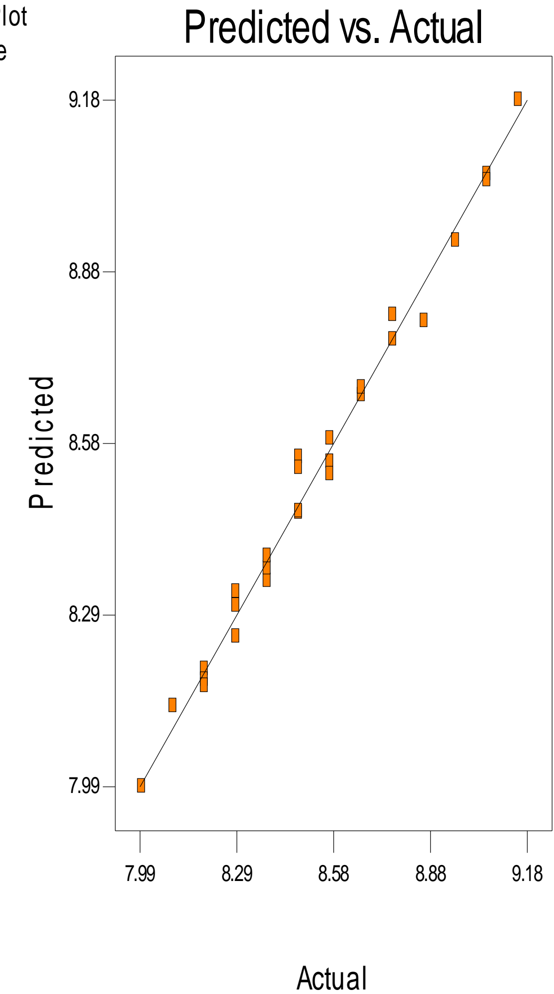


Figure 4.1: Normal probability and actual versus predicted plots for the two responses; (a) Normal probability plot for bitterness level, (b)Normal probability plot for Iso-alpha acid,(c) Actual versus predicted for bitterness level, and (d) Actual versus predicted for Iso-alpha acid.

4.4. Interpretation of the developed models

In the subsequent headings, whenever direct effect, interaction effect or a comparison between any two input parameters is being discussed and the third parameter would be on its center point. General Factorial Design was used to estimate the effect of the three process variables on the value of bitterness level and Iso alpha acid. Perturbation, contour and 3D surface plots were drawn by using GFD to investigate the effect of all the factors on the responses. The inferences so obtained are discussed below.

4.4.1. Effect of process parameters on bitterness level

4.4.1.1. Direct effects

It is evident from Figure 4.2 that, the extraction temperature and time (A and B) has a positive effect on bitterness level. Thus, increasing in extraction temperature and contact time resulted in an increase of bitterness level of the extract. This behavior is due to the fact that, the increasing temperature and contact time could improve the extraction yield by enhancing the solubility of the

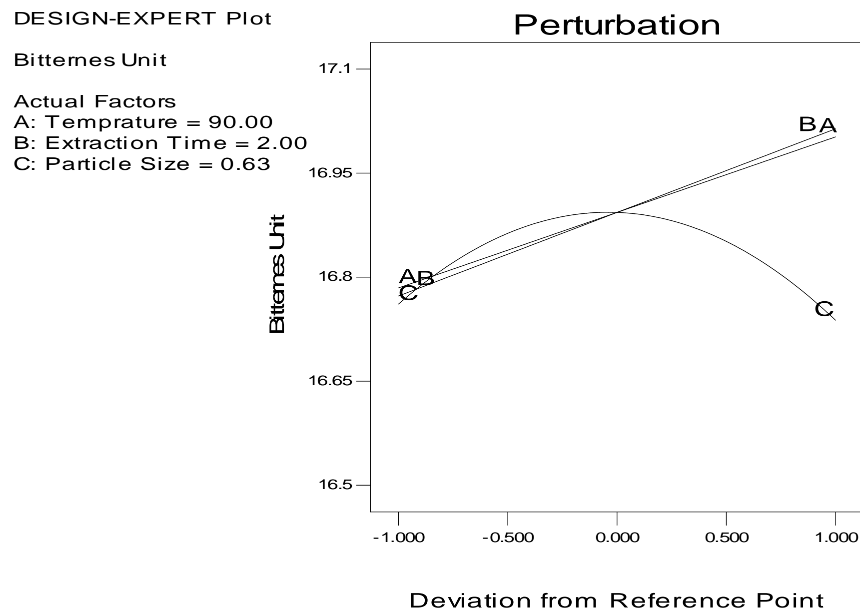


Figure 4.2: Perturbation plot showing the effect of all factors on bitterness level

extraction compound. The higher the extraction temperatures, the easier to break the molecule of the leaf; as a result, the bitterness level also gets high. While, in the case of particle size (C) the result demonstrate that increasing the particle size until it reaches its center value would result increasing in the bitterness level of the extract, the bitterness level of extract then starts to drop as the variable tend to increase above the center limit. Such behavior could be attributed to the following reasons. As particle size get higher and higher, it decrease the surface area for contact with the solvent because the particle of a soluble material is surrounded by a matrix of insoluble matter and thus, the size will not allow the solvent to penetrate and diffuse into the particle to allow the extract to diffuse out accordingly.

Generally, as the results indicate neither too high nor too low particle size is recommended for the extraction process.

4.4.1.2. Interaction effects

The other benefit of perturbation plot is for selecting axes and constants in contour and 3D plots. From the above perturbation plot it can be noticed that the curve for A and B (temperature and time) are almost constant and in this section only the interaction effect of c (particle size) is plotted, while the plot of the other interaction effects for both responses is given at Appendix 5. The interaction effects of these variables on the bitterness level of bitter leaf extracts were studied by plotting 3D surface and counter curves against the two independent variables, while keeping constant the temperature and time at its center value. The three dimensional surface and counter plots for bitterness level as a function of temperature and particle size are shown in Figure 4.3 and Figure 4.4 respectively using the design expert statistical software, 6.0.8 trial version. The response plots in Figures are part of a parabolic cylinder, exhibiting a minimum and maximum ridge, respectively in the investigated domain.

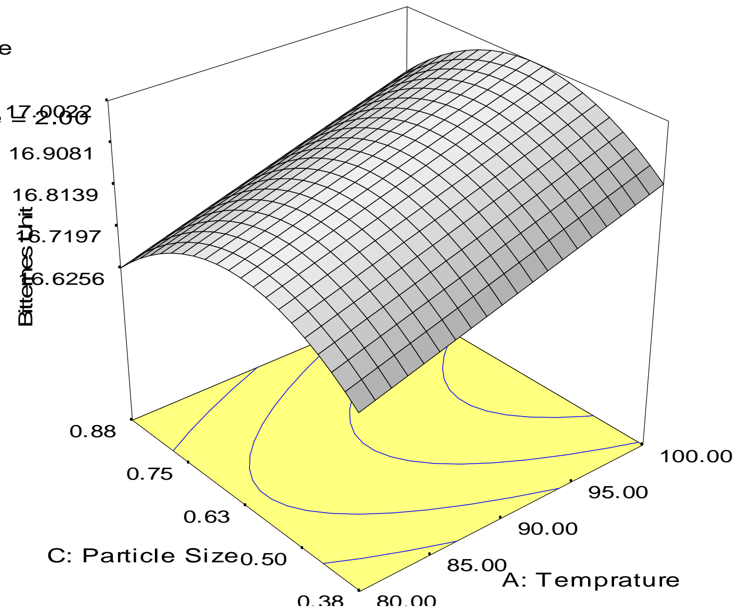


Figure 4.3: Response surface plot for the effect of temperature and particle size on bitterness level

It can be observed that bitterness level of the extract increased with increasing extraction temperature. From the figure it can also be noticed that at lower value of particle size the value of bitterness level was also low and bitterness level was increased as the particle size was increased, maximum bitterness level was observed at particle size of 0.63mm and bitterness level was decreased as the particle size was further increased.

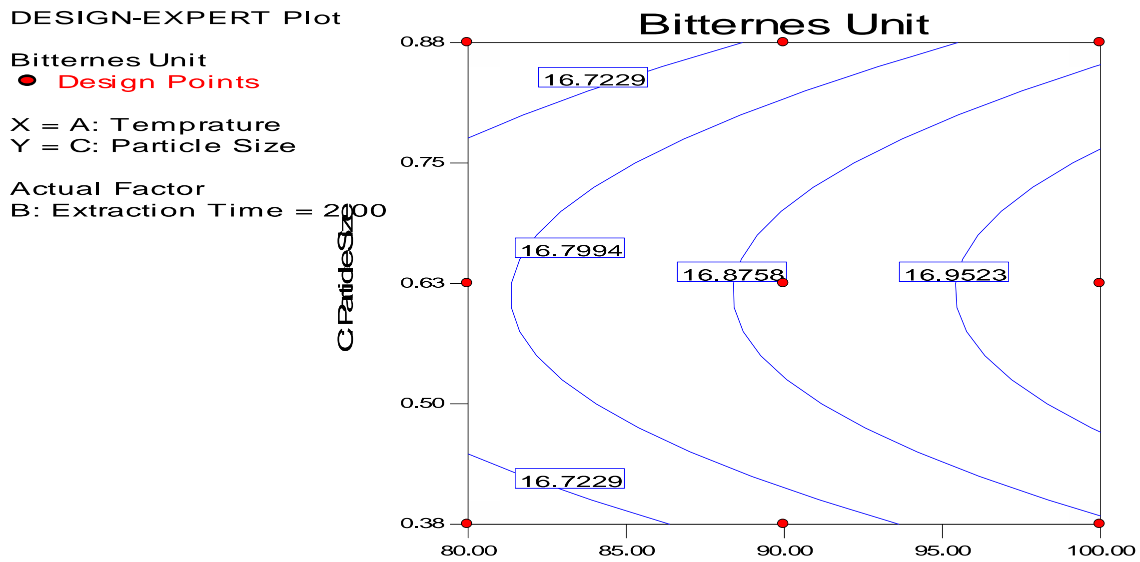


Figure 4.4: Contour plot showing the effect of particle size and temperature on bitterness unit

4.4.2. Effect of process parameters on Iso alpha acid value

4.4.2.1 Direct effects

The perturbation diagram for the Iso alpha acid value with respect to the three input process factors is shown in Figure 4.5 where the influence of a process variable around a specific point in the design range is illustrated by this perturbation plot. In this method the re-

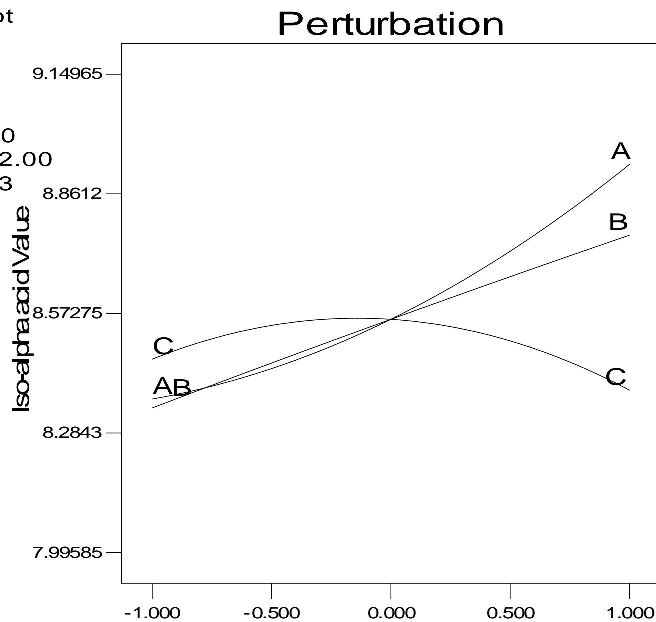


Figure 4.5: Perturbation plot showing the effect of all factors on Iso alpha acid value

sponse (the value of Iso alpha acid) is plotted with respect to only one variable of the overall process, one at a time over its range considering the additional process variables as remaining constant at their center point. It can be observed from Figure 4.5, that initially at a lower value of the particle size the value of Iso alpha acid is low and as the particle size was increased also the quantity of Iso alpha acid is increased and decreased in Iso alpha acid is observed as the particle size was further increased.

In the case of extraction time and temperature, Iso alpha acid value was increased sharply as extraction time is increased. This phenomenon is attributed to the fact that, at high temperature and contact time could improve the isomerization of alpha acid into Iso alpha acid by enhancing the solubility of the extraction compound. The higher the extraction temperatures and contact time, the easier to break the molecule of the leaf; as a result, isomerization was high.

4.4.2.2 Interaction effects

Similarly, the curve for B (extraction time) is almost constant as for the response Iso alpha acid. The 3D surface and counter graphs of Iso alpha acid as a function of temperature and particle size are shown in Figure 4.6 and Figure 4.7 respectively, shows that the graphs are curvilinear profile as the empirical model developed is quadratic. It can be seen that increase

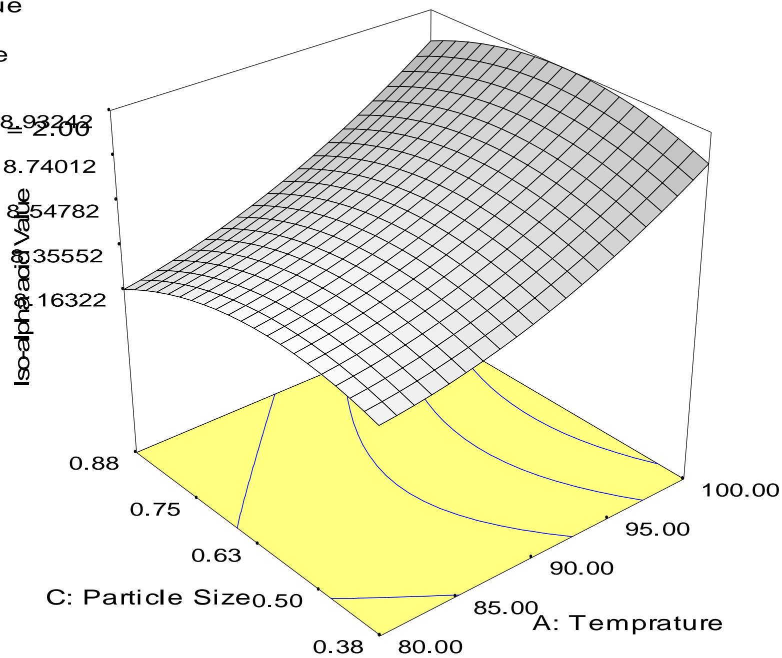


Figure 4.6: Response surface plot for the effect of moisture content and worm stocking density on Iso alpha acid

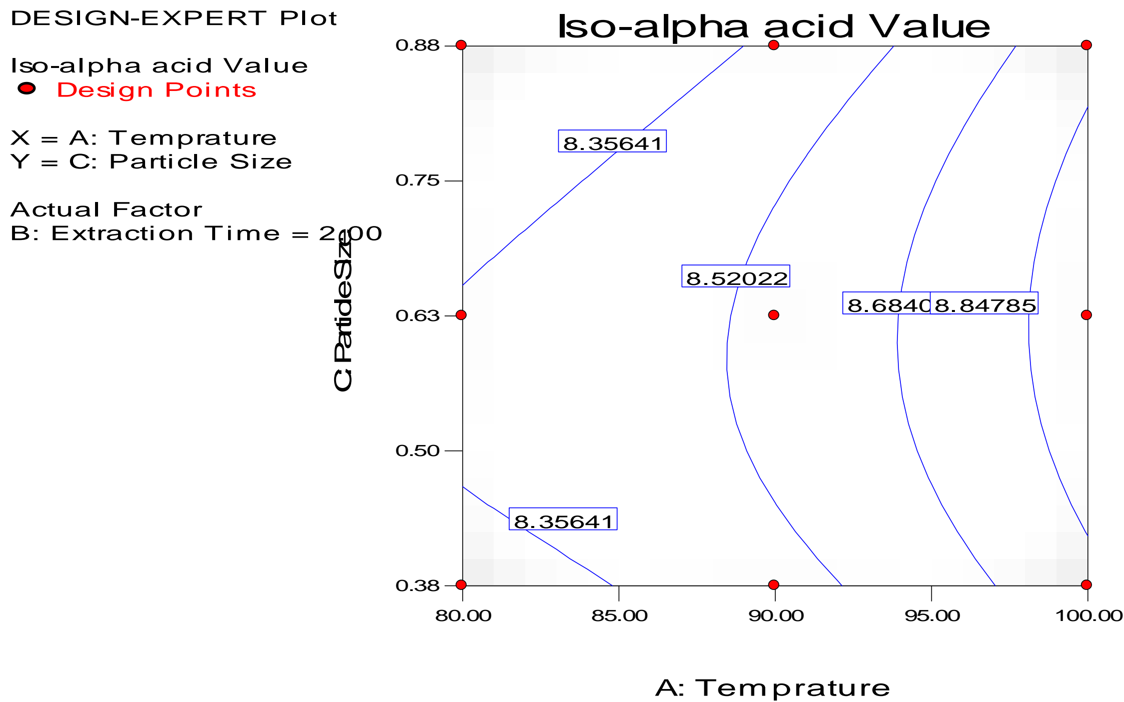


Figure 4.7: Contour plot showing the effect of moisture content and worm stocking density on Iso alpha acid value

Particle size until 0.63mm leads to a sharp increase in Iso alpha acid, then Iso alpha acid was decreased sharply as the particle size was further increased. And it also shows that as the temperature was increased, Iso alpha acid also increased sharply.

4.5. Optimization

One of the primary objectives of the present study was to find the optimum process parameters for maximizing the value of Bitterness level and Iso alpha acid value of bitter leaf extract. The process variables such as temperature, time and particle size have been optimized using General Factorial experimental design and their output values are executed using design-expert software 6.0.8. In optimizing the bitter leaf extraction process, the temperature, time and particle size are a set of process parameters held to be "in range" while the Bitterness level and Iso alpha acid value are set of responses that need to be "maximized". Table 4.4 shows the summary of factors/responses and goals and the corresponding set of specific objectives that will optimize the process condition. Table 4.4

exhibits the desired combinations of process parameters that would provide the highest responses. Numerical optimization

Table 4.4: constraints applied for optimization.

Factors/responses	Ultimate goal	Experimental range	
		Lower limit	Upper limit
Extraction Temperature(°C)	In the range	80	100
Extraction time(hr)	In the range	1	3
Particle size(mm)	In the range	0.25	1
Bitterness level (ABU)	Maximize	16.5	17.1
Iso alpha acid value(mg/l)	Maximize	7.99585	9.14965

was used to optimize any combination of one or more goals. The goals may be apply either factors or responses. The model capable of predicting the maximum Bitterness level and Iso alpha acid value showed that the optimum values of the process variables were extraction temperature of 100°C, extraction time of 3hr and Particle size of 0.6mm. Under these conditions, the predicted bitterness level and Iso alpha acid values were 17.1ABU and 9.15mg/l respectively. Desirability function was used to identify the optimum levels of factors and to get maximum desirable responses. The optimized batch was selected with maximum combined desirability value i.e. 1.00 using Design Expert version 6.0.8. The desirability histogram of the optimized responses for the extraction condition is shown in Figure 4.8.

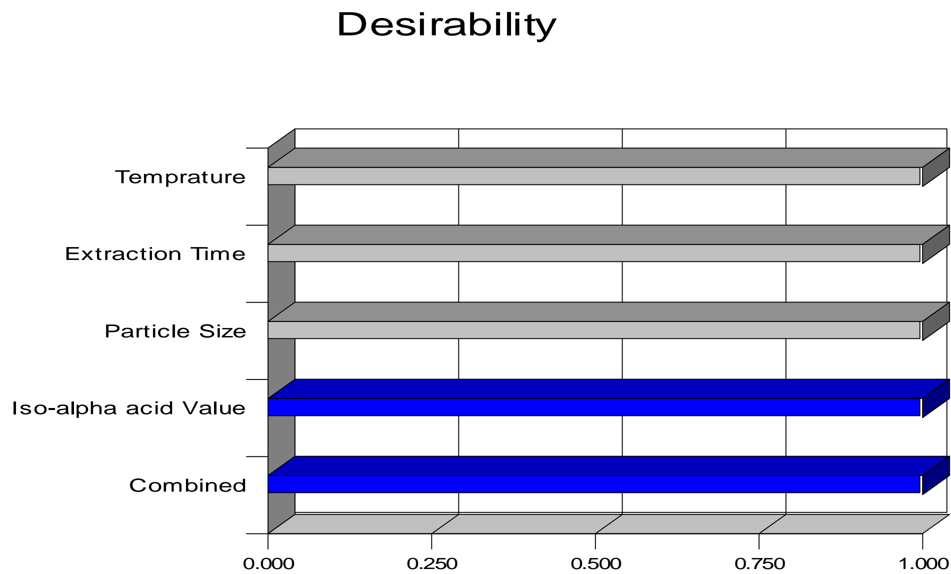


Figure 4.8: Desirability plot of optimization solution for the responses

The histogram shows how well each variables/responses satisfied the criteria and values near one are good. The ramp plot of the optimized responses for the extraction condition is shown in Figure 4.9. The ramp display combines the individual graphs for easier interpretation.

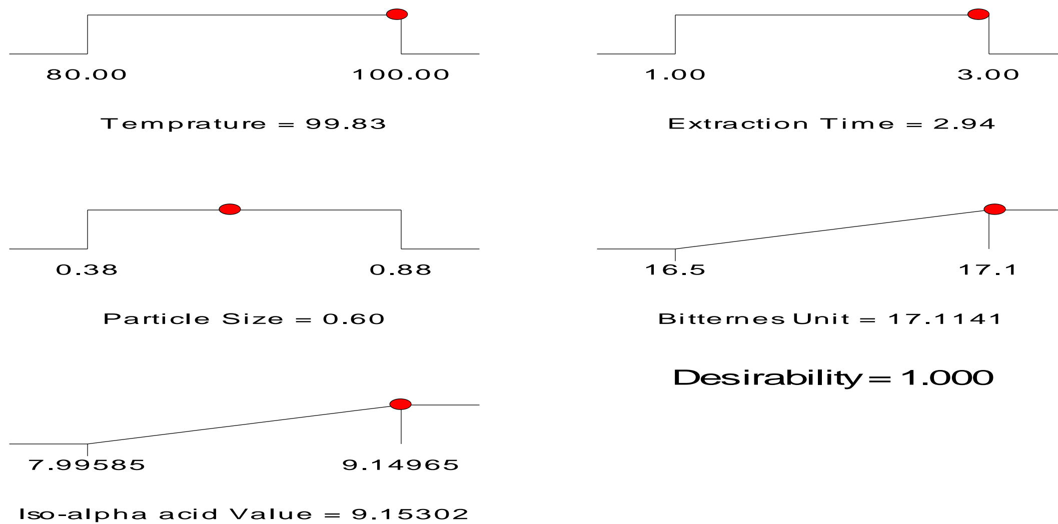


Figure 4.9: Ramp plot of optimization solution for the responses

The dot on each ramp reflects the factor setting or response prediction for that process.

4.6. Validation of the developed models

In order to validate the developed models, confirmation experiment was carried out with bitter leaf extract condition chosen from the optimization results, at 100°C for extraction temperature, 3hr for extraction time and 0.5-0.75mm for particle size. Table 4.5 summarizes the actual experimental values, the predicted values and the percentages of error. The validation results demonstrated that the models developed are quite accurate as the percentages of error in prediction were in a good agreement.

Table 4.5: Validation test results

Temperature [°C]	Time [hr]	Particle size [mm]		Bitterness level	Iso alpha acid
100	3	0.5-0.75	Actual	17.10	9.15
100	3	0.5-0.75	Predicted	17.10	9.18
			Error %	0	0.32

4.7. Proximate analysis of bitter leaf (Vernonia amygdalina)

Based on the chemical analysis using standard methods (A.O.A.C.2000) the calculated proximate compositions of the sample material were tabulated below (Table 4.6). Moisture content, ash, and crude fat values of the leaf were comparable to those of commercial hops.

Table 4.6: Proximate analysis of Vernonia amygdalina compared with that of Hop

Components	Composition (%)	
	Bitter leaf	Hop leaf (control)
Moisture	9.7 ± 0.01	10.00
Ash	7.0 ± 0.09	8.00
Crude Protein	27.73 ±0.07	15.00
Crude Fat	2.74 ±0.02	3.00
Fiber	14.05 ±0.04	3.86
Total Carbohydrate	62.52±0.14	

Values are means of duplicate determinations ± standard deviation.

However, the analyzed bitter leaf extract had the higher protein (27.73%) and fiber content (14.05%) which was about 1.8 and 3.64 times the value for commercial hops respectively (Table 4.6). From literature, high protein content was not desirable as it could lead the problem of haze in the brewing of alcoholic drinks. (Okafor and Anichie, 1983) reported that beer brewed with bitter leaf had a poor foam head because it contain high amount of fat (7%) but in this case (Table 4.6) the amount of crude fat was comparable with that of hop leaf which was 2.74 and this implies that bitter leaf has a good foam head and this partially contradicts what was reported by the authors.

4.8. Alpha acid and Resin Components of bitter leaf (Vernonia amygdalina)

According to (Kunze, 1996), the amount of hop resin was ranged from 14 - 21% with the mean value of 18.5%. In this case, the total resin of the bitter leaf is 19.72% and was found to be within the range. Resins are the most valuable and most characteristic components for beer production. They give beer its bitter taste, improve foam stability and act as antiseptics against microorganisms (Kunze, 1996).

Table 4.7: Alpha acid and Resin components of V. amygdalina compared with that of Hop

Sample	% Compositions			
	Alpha acid	soft resin	hard resin	Total resin
Bitter leaf (V. amygdalina)	9.51±0.03	14.02±0.02	5.7±0.01	19.72±0.03
Hop leaf (Control)	11.5	16	2	18.5

Values are means of duplicate determinations ± standard deviation

Total resins are further divided into soft and hard resins. Soft resin is normally hexane soluble and easily separated from hard resin (which is insoluble in hexane) (Kunze, 1996). In this study, soft resin (14.02%) of bitter leaf powder was significantly ($P \leq 0.05$) less than hop leaf (16%) used as control (Table 4.7). On the other hand, the hard resin (5.7%) was significantly ($P \leq 0.05$) greater than that of hop leaf (2%). The hard resins are generally accepted as the oxidation products of the soft resins (Grant, 1977). These values suggest that the bitter leaf will oxidize or deteriorate faster than commercial hops. In this investigation, in general the result obtained for Alpha acid, soft resin, hard resin and total resin bitter leaf powder was comparable to those of standard hops values used for beer production.

4.9. Phytochemical Components of Vernonia amygdalina

Table 4.8 shows that the analyzed bitter leaf extract had the higher tannin content which was about 2.7 times the value for hop leaf used as a control. Tannins (commonly referred to as tannic acids) are polyphenols present in many plant foods that form colloidal solution in water (Buttler and Bailey, 1973). These solutions have astringent (mouth puckering) taste. Tannins are involved in the formation of haze in beer and also contribute to its taste and colour.

Table 4.8: Phytochemical Components of Bitter leaf (V. amygdalina) with that of Hop

Phytochemical	Content (mg/100gm)	
	Bitter leaf (V. amygdalina)	hop leaf (control)
Tannins	9.67±0.01	3.6
Phytate	2.893±0.04	4
Alkaloid	2.25 ±0.04	4

Values are means of duplicate determinations ± standard deviation

The values for phytochemical obtained during this study were less than the value obtained by the previous works, but this difference was not significant it was consistent with the finding of (Udochukwu, et al., 2015). The author reported that ethanolic extract of Vernonia amygdalina contains tannins (9.62mg/100g), alkaloid (2.16 mg/100g) and Phytate (3.95 mg/100g).

In this study, tannin (14.02%) of bitter leaf extract was significantly ($P \leq 0.05$) greater than hop leaf (3.6%) used as control (Table 4.8). On the other hand, the phytate (2.89%) and alkaloid (2.25%) was significantly ($P \leq 0.05$) less than that of hop leaf (4%). In this investigation, in general the result obtained for Phytate and alkaloid of bitter leaf extract was comparable to those of standard hops values used for beer production.

4.10. Total Phenolic Compounds (TPC) Determination

Gallic acid was taken as standard since it is the most known synthetic organic compound possessing maximum amount of total phenolic compound and any other phenolic compound extracted from aromatic and medicinal plant is evaluated using Gallic acid as standard and Folin-Ciocalteaus reagent. The Folin-Ciocalteaus reagent is sensitive to reducing compounds including poly- phenols, there by producing a blue color upon reaction. This blue colour is measured spectrophotometrically. Thus total phenolic content can be determined (Savitree M.et al, 2004).

In order to use it as standard, appropriate concentration of this acid is taken and its absorbance is measured by spectrophotometer at 765nm and the curve generated from the plot of absorbance versus concentration should give linear relationship with minimum regression coefficient of 0.933.Then the appropriate calibration curve equation is generated in the form of y=bx+a where y is absorbance in nanometer (nm), b is the slope and x is the variable represents Gallic acid in millimole (mM). The equation of the curve is used to find the total phenolic compound by measuring the absorbance of plant extract by taking the maximum possible absorbance measured within the interval of absorbance measured by the ascorbic acid. Using the method described by (Martin et al. 1999), the absorbance measured to have calibration curve of Gallic acid as standard at different concentration and its corresponding curve are given below.

Table 4.9: Absorbance of Standard Compound (Gallic Acid) at different concentration

Concentration (mg/ml)	Absorbance λmax=765 nm		
	The first trial	The second trial	Average + std.dev
Blank	0.003	0.003	0.003 ± 0.00
0.1	0.079	0.081	0.080 ± 0.0014
0.2	0.206	0.221	0.214 ± 0.011
0.4	0.410	0.441	0.425 ± 0.022
0.6	0.621	0.627	0.624 ± 0.004
0.8	0.834	0.836	0.835 ± 0.030

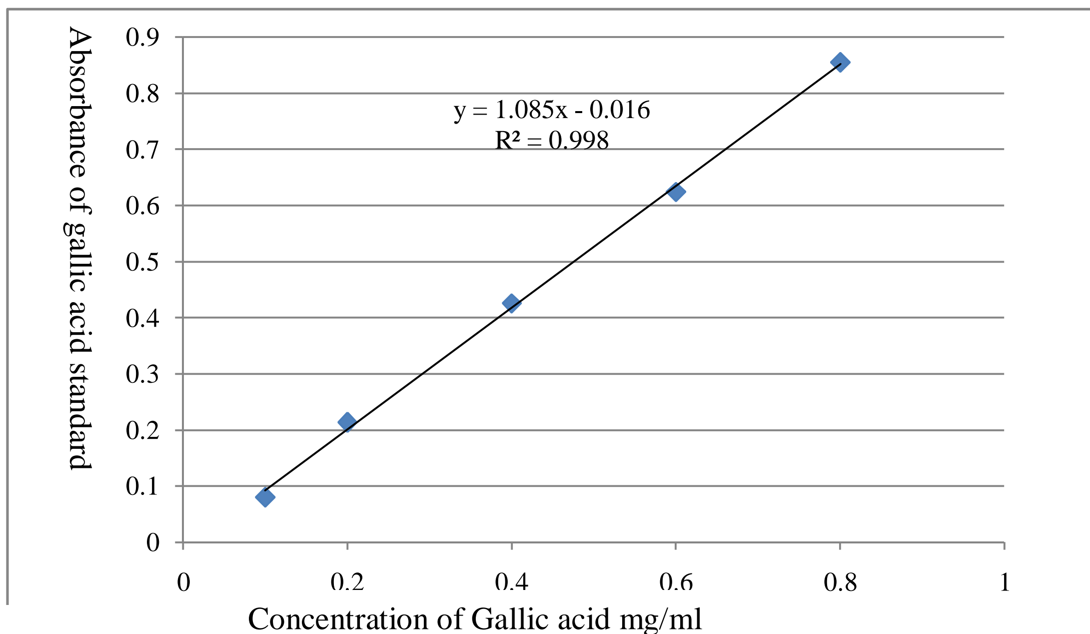


Figure 4.10: Calibration curve of Gallic acid as standard

We deduced that absorbance versus concentration of the calibration curve is straight line having equation of the curve y = 1.085x -0.016 with Regression coefficient of R^2 = 0.998(Figure 4.10). This large number of R^2 shows the two parameters are linearly related and the calibration curve is successfully obtained. This implies also we can use it for determining the total phenolic compound of bitter leaf (V.amygdalina) extracted using methanol solvent and its value is determined by putting the maximum absorbance of each extract with the limit of the standard (0.08-0.855) in place of y and the value of x is solved which corresponds the total phenolic compound in terms of milligram equivalent of Gallic acid or millimole of equivalent Gallic acid. Based on the above reason, Gallic acid was used as calibration standard, and results were calculated as Gallic acid equivalent (GAE) (mg/g dry weight basis) and its value is given in the table below

The maximum absorbance reading in gallic standard solution is 0.836(Table 4.9) but in table 4.10 the maximum absorbance of the extracted solution of bitter leaf (Vernonia amygdalina) is 1.191 at 250μL. If the sample has an absorbance reading above 500mg/l (w/v Gallic acid stock solution for standard preparation) standard it must be diluted and the absorbance re measured. This implies that methanol extract of bitter leaf have greater number of total phenolic compound.

As the table 4.10 below indicates the sample is analyzed in triplicate and diluted from 250µL to 100µL and 40µL because sample absorbance is greater than Gallic acid standard. At 40µL the sample absorbance reading is 0.346 which is in between the limit of the standard 0.08-0.855(Table 13)

Table 4.10: Absorbance of the methanol extract of bitter leaf

	Sample absorbance at 765nm			
Sample volume	1st trial	2nd trial	3rd trial	Average + std.dev
250µL	1.141	1.186	1.191	1.186± 0.0275
100µL	1.099	0.911	1.029	1.029±0.095
40µL	0.328	0.346	0.353	0.346±0.0128

Values are means of duplicate + standard deviation

The TPC of methanol extract of bitter leaf for this specific study is closer with that of the one evaluated by previous work and were 136.9mg GAE/g of dry weight.

4.11. Antioxidant activity of Vernonia amygdalina

Antioxidant activity of Vernonia amygdalina extract was evaluated using he method of (Kirby and Schmidt 2004) with slight modification taking Ascorbic acid as standard. Ascorbic acid is one of the known synthetic organic compounds which shows the greatest antioxidant activity by scavenging the stable radical of DPPH and added to different food products especially oils to suppress oxidation process takes place due to the presence of multiple bonds or un-saturation. Antioxidant activity of all the Vernonia amygdalina crude extract were evaluated taking the all amount of ascorbic acid used for determining its antioxidant activity and the table below shows the results of antioxidant potential of Ascorbic acid.

Table 4.11: The prepared Solution of standard (Ascorbic acid) at different concentration for Spectroscopy

Run order	Volume of standard solution taken from methanol solution in μl	Volume of the methanol taken in μl	DPPH solution (0.004%) μl	Absorbance in nm	Inhibition effect (IE) in %	Mean of the IE from the two duplicate and ± SD
Blank	0	1000	4000	1.423	0.00	0.00
2	5	995	4000	1.322	7.09	7.24±0.198
2	5	995	4000	1.318	7.37	
3	10	990	4000	1.276	10.33	11.1±1.09
3	10	990	4000	1.254	11.87	
4	20	980	4000	1.188	16.51	17.27±1.07
4	20	980	4000	1.166	18.03	
5	40	960	4000	1.123	21.08	21.39±0.447
5	40	960	4000	1.114	21.71	
6	60	940	4000	0.962	32.39	32.43±0.04
6	60	940	4000	0.961	32.41	
7	80	920	4000	0.711	50.10	50.07±0.04
7	80	920	4000	0.716	50.03	
8	120	880	4000	0.195	86.29	89.14±4.02
8	120	840	4000	0.114	91.96	
9	160	840	4000	0.041	97.11	97.13±0.01
9	160	880	4000	0.040	97.13	
10	200	800	4000	0.042	97.04	97.08±0.05
10	200	800	4000	0.040	97.12	

Table 4.11 shows that ascorbic acid is the strong antioxidant having maximum inhibition effect of 97.11 and the Inhibition effect decrease in the first phase until the maximum value of IE is achieved and become increase with further addition of ascorbic acid. The volume of extract of bitter leaf (Vernonia amygdalina) which is the same as the volume of the standard (5, 10, 20, 40, 80, 100, 120, 160, 200μl) were taken and its antioxidant activity was evaluated taking all other required reagents which are the same as that of ascorbic acid. Inhibition effect of Vernonia amygdalina methanol extract was expressed as the average IE of the two duplicate and their corresponding values are presented in Table 4.12 and Figure 4.11.

Table 4.12: Inhibition effect of methanol extract of V. amygdalina evaluated using DPPH assay.

Volume of methanol extract of the sample in μl	Absorbance		Inhibition effect		Inhibition effect Mean + st.dev
	Trial-1	Trial-2			
Blank	0.868	0.868	0.00	0.00	0.00
20	0.801	0.807	7.72	7.03	7.3±0.01
30	0.756	0.721	12.90	16.9	14.9±2.6
60	0.636	0.607	26.23	29.63	27.93±2.70
90	0.428	0.424	50.34	50.79	50.57±0.01
120	0.292	0.270	66.11	68.67	67.39±1.81
150	0.125	0.104	85.42	87.93	87.93±0.02
180	0.048	0.048	94.42	94.37	94.5±0.035
200	0.034	0.034	96.01	96.04	96.65±0.10
230	0.035	0.035	95.91	95.85	95.88±0.04

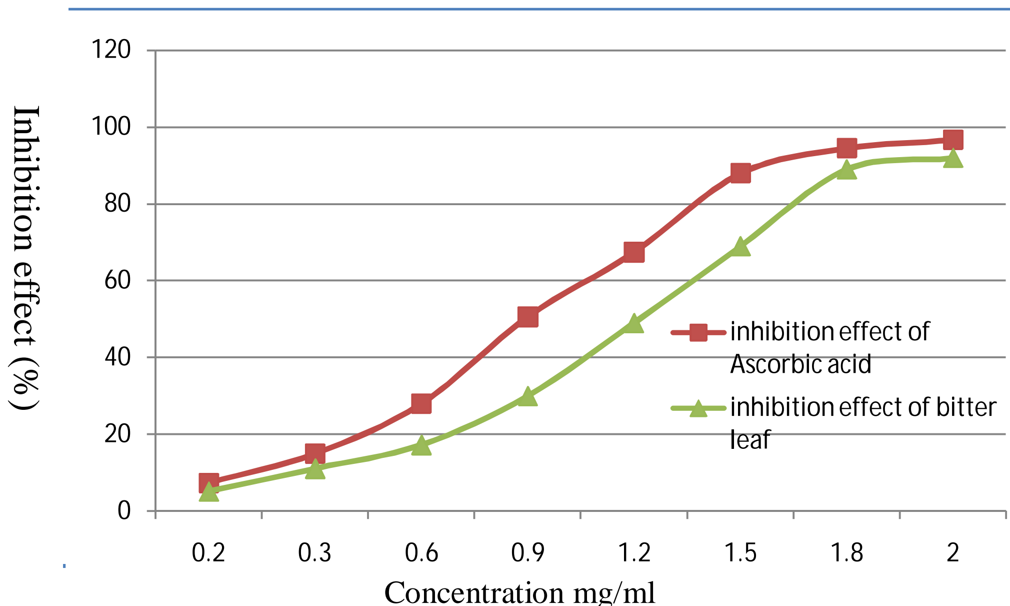


Figure 4.11: inhibition effect of ascorbicacid and bitter leaf

From Figure 4.11 and Table 4.12 we can see that the extract of bitter leaf (Vernonia amygdalina) using methanol solvents show different DPPH scavenging activity. Extract of bitter leaf (Vernonia amygdalina) is the strong antioxidant having maximum inhibition effect of 96.65. The nature of the graph of Inhibition effect of antioxidant activity versus concentration of the sample get increase in the first phase until it reaches the maximum inhibition effect and starts slight decrease with further addition of the sample extract. Natures of these graph looks like that of the standard antioxidant of Ascorbic acid. The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability. When a solution of DPPH is mixed with that of a substance, it can generate a hydrogen atom. This results in the reduced form of DPPH (non-radical) with the change of the violet color and the color changed to yellow when it arrives at maximum inhibition effect and further addition of the extract sample can't get no more unstable radical to stabilize and brings increase in absorbance that decrease the Inhibition effect. DPPH scavenging activity is usually presented by IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. Therefore, extract concentrations providing 50% inhibition (IC_{50}) were calculated using the data plotted in Figure 4.11. Lower IC_{50} value reflects better DPPH radical-scavenging activity and has strong antioxidant potential.

4.12. Determination of mineral content

The AAS results showed that(Table 4.13) the concentrations of metals of the samples investigated were Calcium (mg/100g) of 25.61 ± 0.048, Magnesium (mg/100g) of 4.92 ± 0.04, Iron (mg/100g) of 9.1 ± 0.07and Zinc (mg/100g) of 2.27 ± 0.052.

Table 4.13: mineral content of the bitter leaf sample and hop

Sample	Mineral concentration (mg/100g)			
	Ca	Mg	Fe	Zn
V. amygdalina	25.61±0.048	4.92±0.04	9.1±0.07	2.27±0.052
Hop leaf (control)	17.8	21.13	0.815	1.98

Values are means of duplicate determination ± standard deviation

In this study, Calcium (25.61mg/100g), Zinc (9.1 mg/100g) and Iron (2.27 mg/100g) of bitter leaf extract was significantly ($P \leq 0.05$) greater than hop leaf (17.8 mg/100g), (0.815 mg/100g) and (1.98 mg/100g) used as control respectively (Table 4.13).

From literature, high calcium content was not desirable because calcium reacts with phosphates forming precipitates leading to the release of hydrogen ions and in turn lowering of the pH of the mash. This lowering of the pH is critical because it provides an environment for alpha-amylase, beta amylase and proteolytic enzymes (Bamforth, 2006). Zinc plays an important role in fermentation and has a positive action on protein synthesis and yeast growth. It also impacts flocculation and stabilizes foam, i.e. promotes lacing and iron in large amounts can give a metallic taste to beer. Iron salts have a negative action at concentrations above 3.2mg/L during wort production, preventing complete saccharification, resulting in turbid worts, and hampering yeast activity (Moll, 1979).

On the other hand, the Magnesium (4.9225.61mg/100g) was significantly ($P \leq 0.05$) less than that of hop leaf (21.1325.61mg/100g) (Table 4.13). Magnesium ions react similarly to calcium ions, but since magnesium salts are much more soluble, the effect on wort pH is of little consequence. Magnesium carbonate reportedly gives more astringent bitterness than calcium carbonate (Stewart and Russel, 1985). Calcium and magnesium chlorides give body, palate fullness, and soft sweet flavour to beer.

5. Conclusion and Recommendation

5.1. Conclusion

The results of the present study indicated that bitter leaf (Vernonia amygdalina) locally known as grawa could be used as a hop substitutes in beer brewing. Based on the comparison, it was observed that the brewing qualities of hops are present in bitter leaf to an appreciable level with variation in the protein and metal content. Thus, the quality of the bitter leaf extract from the analysis carried out can be said to be good since there were close similarities in properties of the standard commercial hops and the bitter leaf properties.

Optimization of bitter leaf extract process parameters has been carried out in order to attain maximum amount of bitterness level and Iso alpha acid value. The process parameters (extraction temperature, extraction time and particle size) were selected and optimized to produce high quality bitter leaf extract. Design Expert software was used to develop design of experiment and analyze the results. General Factorial Design (GFD) method was used to optimize the process condition. In summary, the following conclusions can be drawn:

- For this particular study the quadratic model was the best fit model for all responses as the p-value of this model was smaller than the other models and had the highest p-value for Lack of Fit Tests.
- Among the three variables studied, ANOVA showed that, extraction time is found to be the most important parameter influencing the bitterness level and Iso alpha acid value followed by extraction temperature, while particle size has the least effect relative to the two factors.
- The bitterness level and Iso alpha acid values of the bitter leaf extract increased with increase of extraction temperature and time but initially at a lower value of the particle size the value of bitterness level and Iso alpha acid was low and as the particle size was increased also the value of bitterness level and Iso alpha acid was increased and decreased in bitterness level and Iso alpha acid is observed as the particle size was further increased.
- Maximum values for bitterness level and Iso alpha acid of the extract is17.1ABUand 9.1mg/l respectively were achieved under the optimum conditions; temperature of 100°C, time of 3hr and particle size of 0.5mm-0.75mm with high value of combined desirability, i.e. 1.0.

Generally, it can be safely concluded that bitter leaf presents a potential substitute for hops in beer brewing.

5.2. Recommendation

The present study has enabled to confirm that bitter leaf can be used as a potential to substitute for imported hops, which could contribute to save the much needed foreign exchange. However, during the present study, there had been some constraints or limitations. So I strongly advice the following recommended tasks should be considered to strengthen the paper. These are;

- The mineral and protein content of bitter leaf was considered high in comparism to the commercial hops, this could further be investigated or blended with hops in order to create a basis for use of local raw materials to reduce the quantity of hops imported annually and thus save the much needed foreign exchange while encouraging local enterprises and initiatives.
- Studies on evaluation of bitter leaf (Vernonia amygdalina) essential oil extracted by using steam distillation for the flavor and aroma of beer and
- Studies on Techno – Economic evaluation of bitter leaf (Vernonia amygdalina) extract.

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Appendix 1: The experimental result from direct extraction (a reflux condenser)

Run	Temp. [^oC]	Extr.Time [hr]	Particle Size [mm]	Response 1 Bitterness Unit					Response 2 Iso alpha acid value				
				Abs@275nm		Bitterness Unit		IBU	Abs@255nm		Iso alpha acid		
				Trial-1	Trial-2	Trial-1	Trial-2	μ±sd	Trial-1	Trial-2	Trial-1	Trial-2	μ±sd
1	80	1	0.25-0.5	0.324	0.336	16.2	16.8	16.5±0.42	0.071	0.189	7.23	8.96	8.092±1.22
2	90	1	0.25-0.5	0.329	0.337	16.45	16.85	16.65±0.28	0.079	0.085	7.99	8.57	8.284±0.41
3	100	1	0.25-0.5	0.332	0.339	16.6	16.9	16.75±0.21	0.081	0.089	8.18	8.95	8.573±0.54
4	80	2	0.25-0.5	0.328	0.338	16.4	16.9	16.65±0.35	0.084	0.080	8.47	8.09	8.284±0.27
5	90	2	0.25-0.5	0.331	0.339	16.55	16.95	16.75±0.28	0.083	0.085	8.38	8.57	8.476±0.14
6	100	2	0.25-0.5	0.337	0.337	16.85	16.85	16.85±0	0.082	0.092	8.28	9.25	8.765±0.67
7	80	3	0.25-0.5	0.330	0.342	16.5	17.1	16.8±0.42	0.080	0.088	8.09	8.86	8.47±0.544
8	90	3	0.25-0.5	0.341	0.333	16.05	17.65	16.85±0.28	0.087	0.085	8.77	8.57	8.66±0.14
9	100	3	0.25-0.5	0.345	0.335	16.25	16.75	17.0±0.35	0.088	0.092	8.86	9.24	9.05±0.27
10	80	1	0.5-0.75	0.330	0.334	16.5	16.7	16.6±0.14	0.084	0.078	8.47	7.89	8.18±0.41
11	90	1	0.5-0.75	0.336	0.335	16.8	16.75	16.77±0.03	0.079	0.087	7.99	8.76	8.38±0.54
12	100	1	0.5-0.75	0.337	0.338	16.85	16.9	16.875±0.03	0.082	0.090	8.28	9.05	8.66±0.54
13	80	2	0.5-0.75	0.339	0.331	16.95	16.55	16.75±0.28	0.082	0.084	8.28	8.47	8.38±0.14
14	90	2	0.5-0.75	0.323	0.353	16.15	17.65	16.90±1.06	0.083	0.084	8.38	8.48	8.47±0.07
15	100	2	0.5-0.75	0.333	0.333	16.65	17.45	17.05±0.56	0.089	0.087	8.76	8.96	8.95±0.14
16	80	3	0.5-0.75	0.334	0.346	16.7	17.3	16.95±0.42	0.084	0.086	8.47	8.67	8.57±0.14
17	90	3	0.5-0.75	0.338	0.344	16.9	17.2	17.05±0.21	0.087	0.087	8.76	8.76	8.76±0
18	100	3	0.5-0.75	0.346	0.338	17.3	16.9	17.1±0.28	0.092	0.090	9.24	9.05	9.15±0.16
19	80	1	1-0.75	0.327	0.333	16.35	16.65	17.5±0.21	0.076	0.082	7.70	8.28	7.99±0.06
20	90	1	1-0.75	0.334	0.332	16.7	16.6	16.65±0.07	0.081	0.082	8.18	8.28	8.18±0.06
21	100	1	1-0.75	0.334	0.334	16.7	16.7	16.70±0	0.084	0.083	8.38	8.48	8.47±1.22
22	80	2	1-0.75	0.327	0.339	16.35	16.95	16.65±0.42	0.090	0.072	9.05	7.32	8.18±1.08
23	90	2	1-0.75	0.330	0.338	16.5	16.9	16.70±0.28	0.091	0.075	9.14	7.61	8.38±1.22
24	100	2	1-0.75	0.331	0.345	16.55	17.25	16.90±0.49	0.079	0.097	7.99	9.72	8.88±1.22
25	80	3	1-0.75	0.332	0.338	16.6	16.9	16.75±0.21	0.089	0.075	8.95	7.61	8.28±0.95
26	90	3	1-0.75	0.330	0.344	16.5	17.2	16.85±0.49	0.079	0.091	7.99	9.15	8.57±0.81
27	100	3	1-0.75	0.331	0.345	16.55	17.25	16.90±0.49	0.086	0.094	8.66	9.44	9.05±0.54

Appendix 3: Sequential Model and Model Summary Statistics

Response-1 : Bitternes Unit
***** WARNING: The Cubic Model is Aliased! *****

Sequential Model Sum of Squares

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Mean	7618.13	1	7618.13			
Linear	0.48	3	0.16	24.53	< 0.0001	
2FI	4.483E-003	3	1.494E-003	0.21	0.8907	
Quadratic	0.12	3	0.041	34.47	< 0.0001	Suggested
Cubic	0.011	7	1.561E-003	1.64	0.2303	Aliased
Residual	9.512E-003	10	9.512E-004			
Total	7618.75	27	282.18			

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

Model Summary Statistics

Source	Std. Dev.	Adjusted R-Squared	Predicted R-Squared	R-Squared	PRESS	
Linear	0.081	0.7619	0.7308	0.6850	0.20	
2FI	0.085	0.7690	0.6997	0.6041	0.25	
Quadratic	0.035	0.9674	0.9501	0.9165	0.052	Suggested
Cubic	0.031	0.9848	0.9605	0.8840	0.073	Aliased

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

Response-2: Iso-alpha acid Value
***** WARNING: The Cubic Model is Aliased! *****

Sequential Model Sum of Squares

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Mean	1962.91	1	1962.91			
Linear	2.25	3	0.75	79.49	< 0.0001	
2FI	0.032	3	0.011	1.14	0.3584	
Quadratic	0.16	3	0.052	30.96	< 0.0001	Suggested
Cubic	0.013	7	1.908E-003	1.25	0.3637	Aliased
Residual	0.015	10	1.532E-003			
Total1	965.38	27	72.79			

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

Model Summary Statistics

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	0.097	0.9120	0.9006	0.8806	0.29	
2FI	0.096	0.9248	0.9023	0.8775	0.30	
Quadratic	0.041	0.9884	0.9822	0.9689	0.077	Suggested
Cubic	0.039	0.9938	0.9838	0.9589	0.10	Aliased

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

Appendix 4: ANOVA for the two responses (Bitterness Unit and Iso alpha acid)

Response-1: Bitterness Unit

ANOVA for Response Surface Reduced Quadratic Model

Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.61	7	0.087	80.13	< 0.0001	significant
A	0.21	1	0.21	197.48	< 0.0001	
B	0.26	1	0.26	242.07	< 0.0001	
C	2.450E-003	1	2.450E-003	2.27	0.1486	
C^2	0.12	1	0.12	114.95	< 0.0001	
AB	3.675E-003	1	3.675E-003	3.40	0.0808	
AC	1.333E-004	1	1.333E-004	0.12	0.7293	
BC	6.750E-004	1	6.750E-004	0.62	0.4391	
Residual	0.021	19	1.081E-003			
Cor Total	0.63	26				

The Model F-value of 80.13 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C^2 are significant model terms.

Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Std. Dev.	0.033	R-Squared	0.9672
Mean	16.80	Adj R-Squared	0.9552
C.V.	0.20	Pred R-Squared	0.9308
PRESS	0.043	Adeq Precision	34.104

The "Pred R-Squared" of 0.9308 is in reasonable agreement with the "Adj R-Squared" of 0.9552. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 34.104 indicates an adequate signal. This model can be used to navigate the design space.

Factor	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	16.89	1	0.011	16.87	16.92	
A-Temprature	0.11	1	7.748E-003	0.093	0.13	1.00
B-Extraction Time	0.12	1	7.748E-003	0.10	0.14	1.00
C-Particle Size	-0.012	1	7.748E-003	-0.028	4.551E-003	1.00
C^2	-0.14	1	0.013	-0.17	-0.12	1.00
AB	-0.018	1	9.490E-003	-0.037	2.363E-003	1.00
AC	3.333E-003	1	9.490E-003	-0.017	0.023	1.00
BC	-7.500E-003	1	9.490E-003	-0.027	0.012	1.00

Final Equation in Terms of Coded Factors:

Bitternes Unit =
+16.89
+0.11 * A
+0.12 * B
-0.012 * C
-0.14 * C^2
-0.018 * A * B
+3.333E-003 * A * C
-7.500E-003 * B * C

Final Equation in Terms of Actual Factors:

Bitternes Unit =
+14.52458
+0.013556 * Temprature
+0.29681 * Extraction Time
+2.77111 * Particle Size
-2.30222 * Particle $Size^2$
-1.75000E-003 * Temprature * Extraction Time
+1.33333E-003 * Temprature * Particle Size
-0.030000 * Extraction Time * Particle Size

Diagnostics Case Statistics of bitterness level

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	16.55	16.51	0.040	0.472	1.675	0.314	1.766	23
2	16.65	16.63	0.017	0.250	0.595	0.015	0.585	20
3	16.75	16.76	-6.111E-003	0.472	-0.256	0.007	-0.249	9
4	16.65	16.66	-5.556E-003	0.250	-0.195	0.002	-0.190	17
5	16.75	16.76	-0.011	0.111	-0.358	0.002	-0.350	26
6	16.85	16.87	-0.017	0.250	-0.585	0.014	-0.575	22
7	16.80	16.80	-1.111E-003	0.472	-0.047	0.000	-0.045	10
8	16.85	16.89	-0.039	0.250	-1.376	0.079	-1.411	2
9	17.00	16.98	0.023	0.472	0.954	0.102	0.951	13
10	16.60	16.65	-0.046	0.306	-1.693	0.158	-1.789	15
11	16.77	16.77	-2.778E-003	0.167	-0.093	0.000	-0.090	11
12	16.87	16.90	-0.029	0.306	-1.065	0.062	-1.069	4
13	16.75	16.78	-0.034	0.167	-1.148	0.033	-1.158	24
14	16.90	16.89	6.667E-003	0.111	0.215	0.001	0.210	16
15	17.05	17.00	0.048	0.167	1.592	0.063	1.665	1
16	16.95	16.92	0.028	0.306	1.004	0.055	1.004	6
17	17.05	17.01	0.036	0.167	1.203	0.036	1.219	14
18	17.10	17.11	-5.278E-003	0.306	-0.193	0.002	-0.188	25
19	16.50	16.49	5.000E-003	0.472	0.209	0.005	0.204	5
20	16.65	16.62	0.025	0.250	0.888	0.033	0.883	12
21	16.74	16.75	-0.014	0.472	-0.605	0.041	-0.594	3
22	16.65	16.63	0.024	0.250	0.859	0.031	0.852	19
23	16.70	16.74	-0.038	0.111	-1.219	0.023	-1.236	18
24	16.90	16.85	0.050	0.250	1.756	0.129	1.868	7
25	16.75	16.76	-6.111E-003	0.472	-0.256	0.007	-0.249	27
26	16.85	16.85	-8.333E-004	0.250	-0.029	0.000	-0.028	21
27	16.90	16.95	-0.046	0.472	-1.907	0.407	-2.065	8

Response-2: Iso-alpha acid Value

ANOVA for Response Surface Reduced Quadratic Model

Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	2.44	8	0.30	191.24	< 0.0001	significant
A	1.44	1	1.44	905.59	< 0.0001	
B	0.78	1	0.78	490.35	< 0.0001	
C	0.025	1	0.025	15.80	0.0009	
A^2	0.049	1	0.049	31.06	< 0.0001	
B^2	1.712E-004	1	1.712E-004	0.11	0.7468	
C^2	0.11	1	0.11	67.16	< 0.0001	
AB	0.019	1	0.019	12.09	0.0027	
AC	0.012	1	0.012	7.74	0.0123	
Residual	0.029	18	1.593E-003			
Cor Total	2.47	26				

The Model F-value of 191.24 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, A^2, C^2, AB, AC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Std. Dev.	0.040	R-Squared	0.9884
Mean	8.53	Adj R-Squared	0.9832
C.V.	0.47	Pred R-Squared	0.9729
PRESS	0.067	Adeq Precision	51.460

The "Pred R-Squared" of 0.9729 is in reasonable agreement with the "Adj R-Squared" of 0.9832. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 51.460 indicates an adequate signal. This model can be used to navigate the design space.

Factor	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	8.56	1	0.020	8.52	8.60	
A-Temprature	0.28	1	9.408E-003	0.26	0.30	1.00
B-Extraction Time	0.21	1	9.408E-003	0.19	0.23	1.00
C-Particle Size	-0.037	1	9.408E-003	-0.057	-0.018	1.00
A^2	0.091	1	0.016	0.057	0.13	1.00
B^2	-5.342E-003	1	0.016	-0.040	0.029	1.00
C^2	-0.13	1	0.016	-0.17	-0.099	1.00
AB	0.040	1	0.012	0.016	0.064	1.00
AC	0.032	1	0.012	7.843E-003	0.056	1.00

Final Equation in Terms of Coded Factors:

Iso-alpha acid Value	=
+8.56	
+0.28	* A
+0.21	* B
-0.037	* C
+0.091	* A^2
-5.342E-003	* B^2
-0.13	* C^2
+0.040	* A * B
+0.032	* A * C

Final Equation in Terms of Actual Factors:

Iso-alpha acid Value	=
+13.62908	
-0.15117	* Temprature
-0.13087	* Extraction Time
+1.36747	* Particle Size
+9.08083E-004	* Temprature^2
-5.34167E-003	* Extraction Time^2
-2.13667	* Particle Size^2
+4.00625E-003	* Temprature * Extraction Time
+0.012820	* Temprature * Particle Size

Diagnostics Case Statistics of Iso alpha acid

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	8.09	8.13	-0.037	0.426	-1.207	0.120	-1.224	23
2	8.28	8.25	0.036	0.259	1.037	0.042	1.039	20
3	8.57	8.55	0.022	0.426	0.736	0.045	0.726	9
4	8.28	8.30	-0.018	0.343	-0.550	0.018	-0.539	17
5	8.48	8.46	0.014	0.259	0.415	0.007	0.405	26
6	8.77	8.80	-0.039	0.343	-1.210	0.085	-1.227	22
7	8.48	8.47	0.012	0.426	0.383	0.012	0.373	10
8	8.67	8.67	3.561E-003	0.259	0.104	0.000	0.101	2
9	9.05	9.05	6.232E-003	0.426	0.206	0.004	0.201	13
10	8.19	8.19	-4.451E-003	0.343	-0.138	0.001	-0.134	15
11	8.38	8.34	0.036	0.259	1.037	0.042	1.039	11
12	8.67	8.68	-9.793E-003	0.343	-0.303	0.005	-0.295	4
13	8.38	8.37	0.014	0.259	0.415	0.007	0.405	24
14	8.48	8.56	-0.082	0.259	-2.384	0.221	-2.801	16
15	8.96	8.93	0.025	0.259	0.726	0.020	0.716	1
16	8.57	8.53	0.044	0.343	1.348	0.105	1.382	6
17	8.77	8.76	3.561E-003	0.259	0.104	0.000	0.101	14
18	9.15	9.18	-0.026	0.343	-0.798	0.037	-0.789	25
19	8.00	7.99	6.232E-003	0.426	0.206	0.004	0.201	5
20	8.19	8.17	0.014	0.259	0.415	0.007	0.405	12
21	8.48	8.54	-0.063	0.426	-2.090	0.360	-2.334	3
22	8.19	8.16	0.025	0.343	0.770	0.034	0.761	19
23	8.38	8.39	-7.122E-003	0.259	-0.207	0.002	-0.202	18
24	8.86	8.79	0.068	0.343	2.091	0.253	2.335	7
25	8.28	8.33	-0.042	0.426	-1.384	0.158	-1.422	27
26	8.57	8.59	-0.018	0.259	-0.518	0.010	-0.508	21
27	9.05	9.04	0.017	0.426	0.559	0.026	0.548	8

Appendix 5: 3D and contour plots for the three responses (bitterness unit and Iso alpha acid)

1. Contour and 3D plots for the response bitterness unit

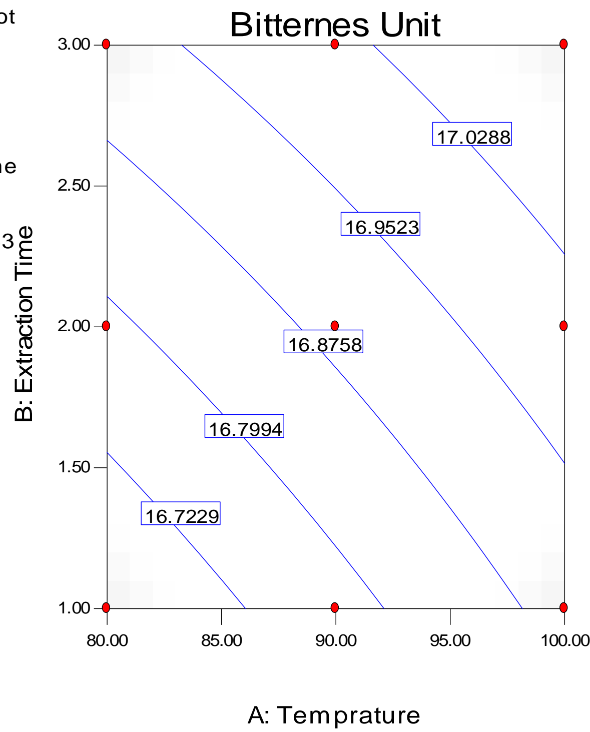
DESIGN-EXPERT Plot

Bitternes Unit
● Design Points

X = A: Temprature
Y = B: Extraction Time

Actual Factor
C: Particle Size = 0.63

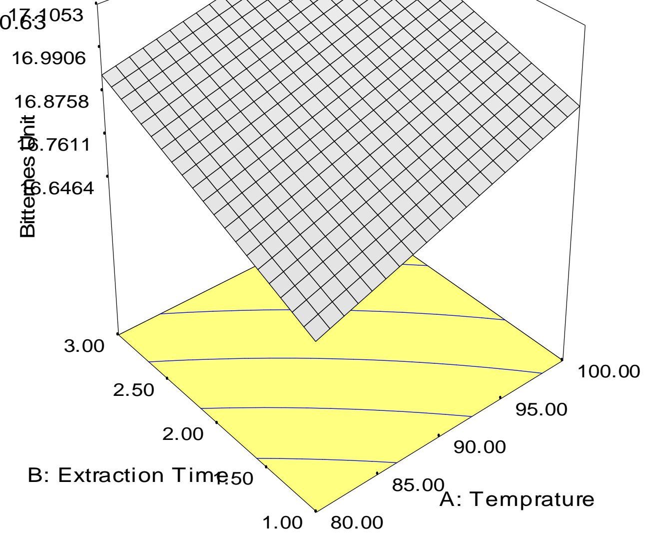
a)



DESIGN-EXPERT Plot

Bitternes Unit
X = A: Temprature
Y = B: Extraction Time

Actual Factor
C: Particle Size = 0.63



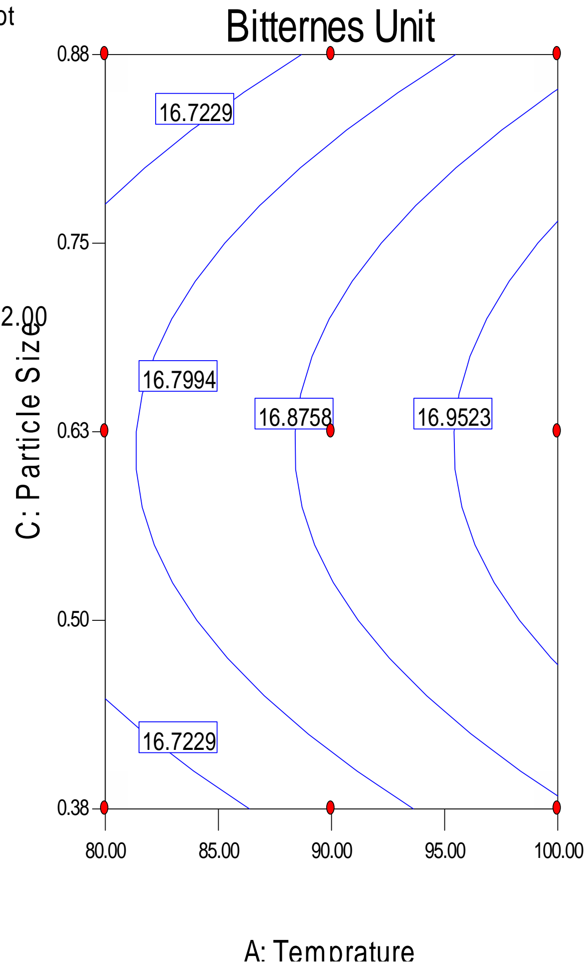
DESIGN-EXPERT Plot

Bitternes Unit
● Design Points

X = A: Temprature
Y = C: Particle Size

Actual Factor
B: Extraction Time = 2.00

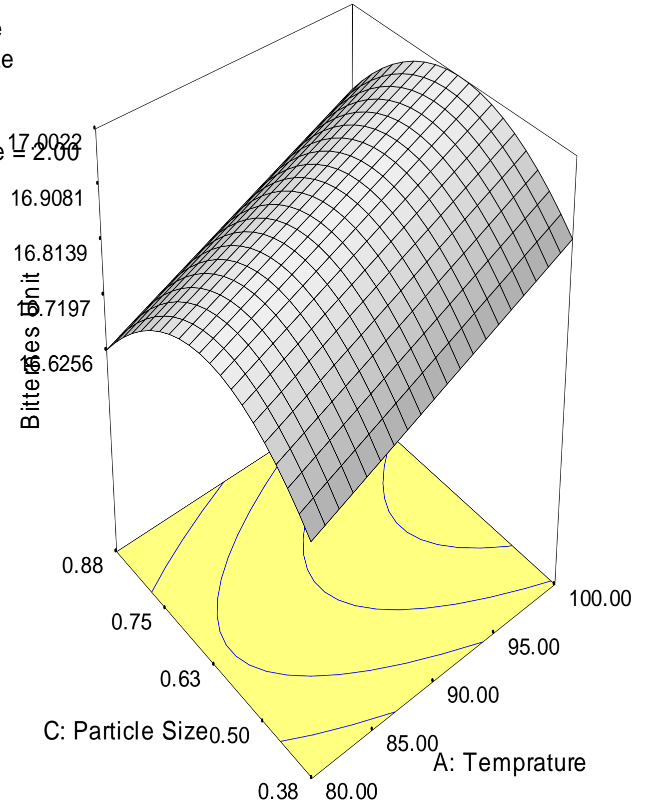
b)



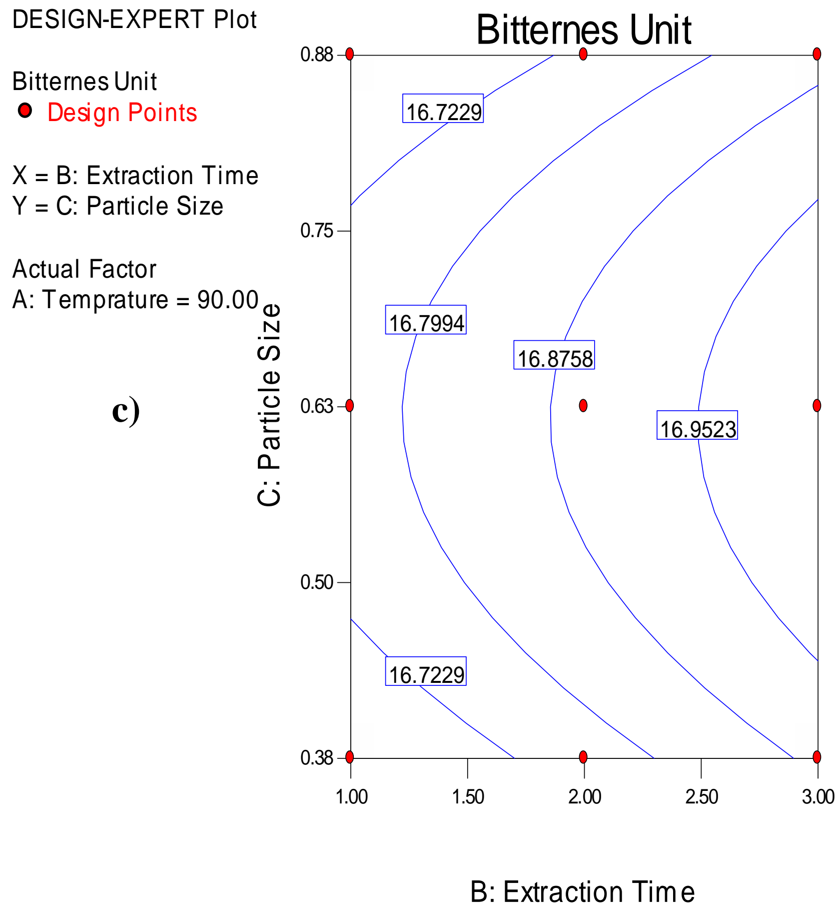
DESIGN-EXPERT Plot

Bitternes Unit
X = A: Temprature
Y = C: Particle Size

Actual Factor
B: Extraction Time = 2.00



c)



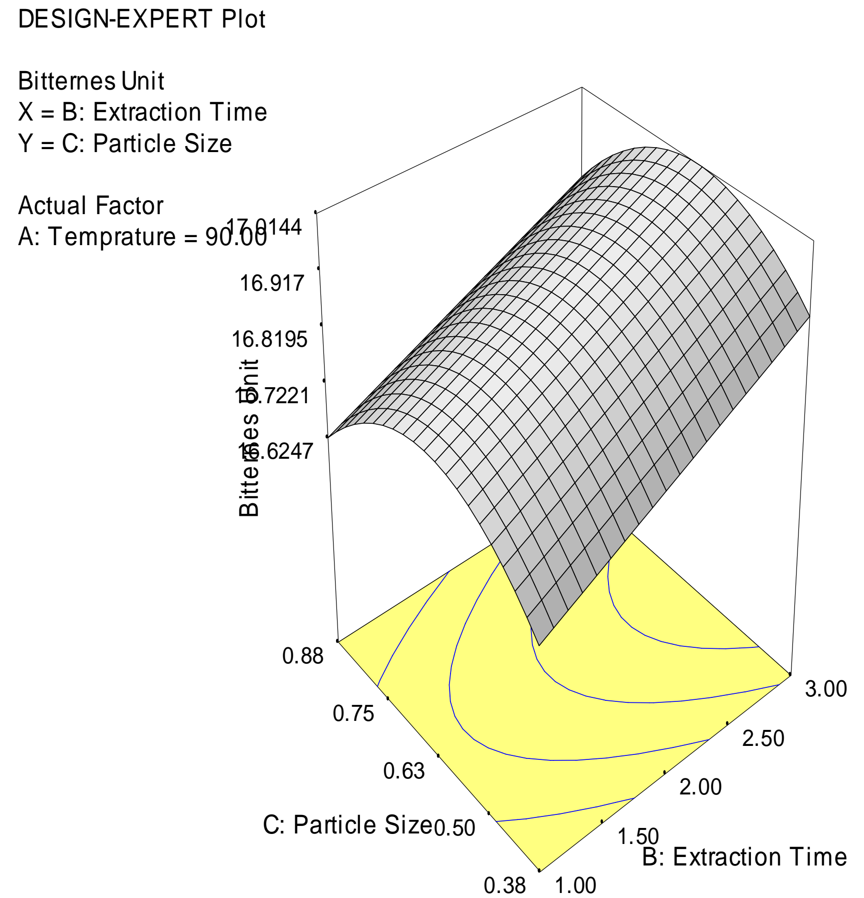


Figure A5-1: Contour and response surface plots of bitterness unit of the sample extracted as a function of (a) temperature and extraction time, particle size fixed at 0.63 mm (b) temperature and particle size, extraction time fixed at 2hr and (c) extraction time and particle size, temperature fixed at 90°C

2. Contour and 3D plots for the response Iso alpha acid

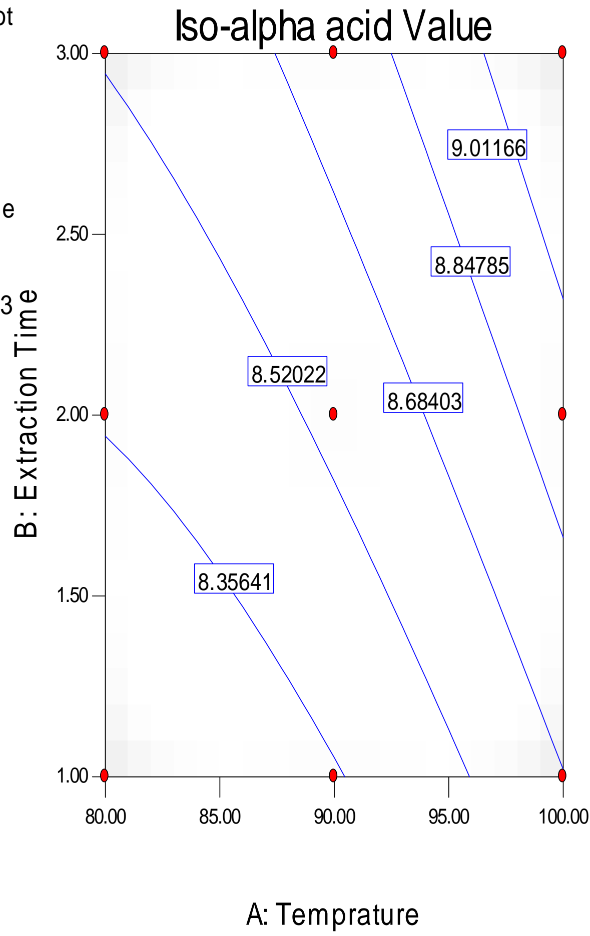
DESIGN-EXPERT Plot

Iso-alpha acid Value
● Design Points

X = A: Temprature
Y = B: Extraction Time

Actual Factor
C: Particle Size = 0.63

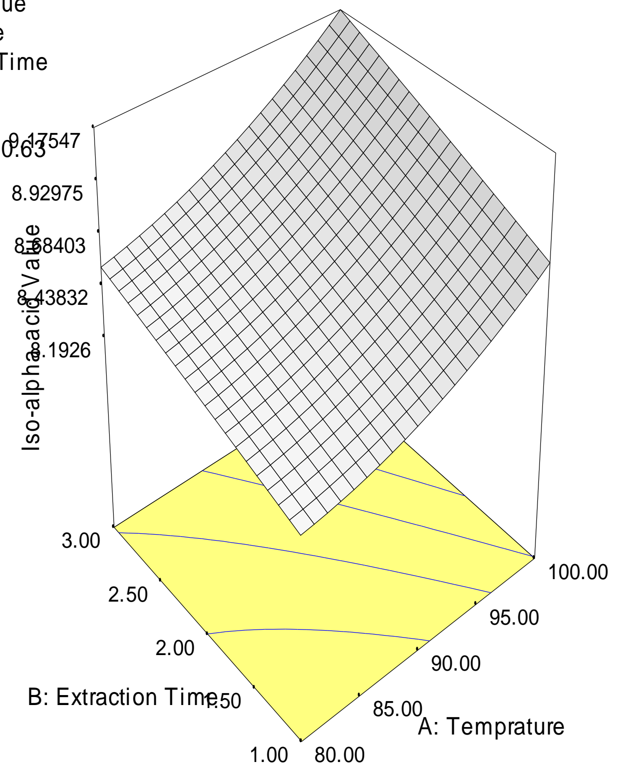
a)



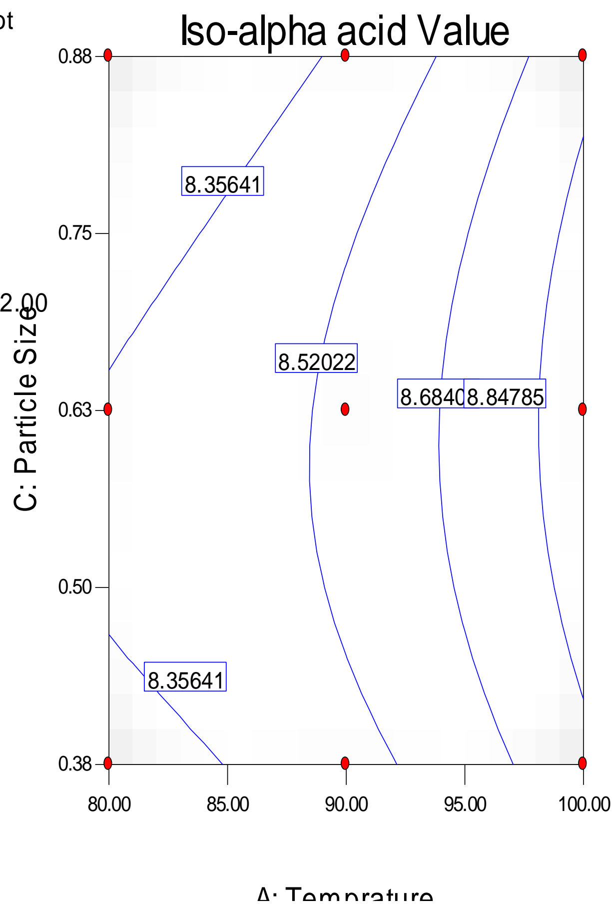
DESIGN-EXPERT Plot

Iso-alpha acid Value
X = A: Temprature
Y = B: Extraction Time

Actual Factor
C: Particle Size = 0.63



b)



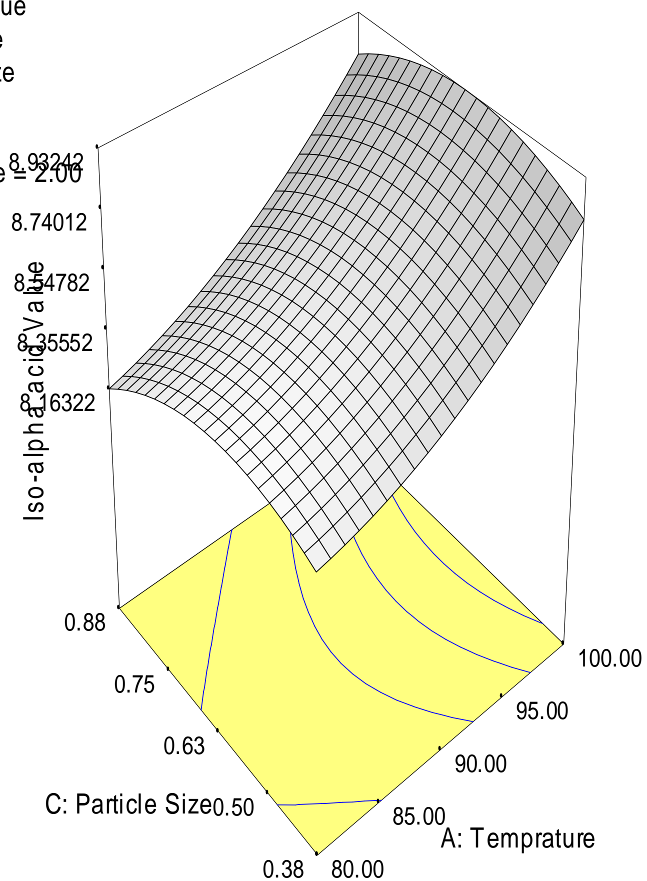


Figure A5-2: Contour and response surface plots of Iso alpha acid of the samples studied as a function of (a) temperature and extraction time, particle size fixed at 0.63 mm (b) temperature and particle size, extraction time fixed at 2hr.

Appendix-6: standards and calibration curve

Table A6-1: Absorbance of the standard and sample total phenolic compound

<table>
<tr><td rowspan="2">Concentration (mg/ml)</td><td colspan="3">Absorbance λmax=765 nm</td></tr>
<tr><td>1st trial</td><td>2nd trial</td><td>Average ± std.dev</td></tr>
<tr><td>Blank</td><td>0.003</td><td>0.003</td><td>0.003 ± 0.00</td></tr>
<tr><td>20</td><td>0.079</td><td>0.081</td><td>0.080 ± 0.0014</td></tr>
<tr><td>40</td><td>0.206</td><td>0.221</td><td>0.214 ± 0.011</td></tr>
<tr><td>80</td><td>0.410</td><td>0.441</td><td>0.425 ± 0.022</td></tr>
<tr><td>120</td><td>0.621</td><td>0.627</td><td>0.624 ± 0.004</td></tr>
<tr><td>160</td><td>0.834</td><td>0.836</td><td>0.855 ± 0.030</td></tr>
</table>

<table>
<tr><td colspan="5">Absorbance of the Sample at 765nm</td></tr>
<tr><td>Volume</td><td>1st trial</td><td>2nd trial</td><td>3rd trial</td><td>Mean ± st.dev</td></tr>
<tr><td>250µL</td><td>1.141</td><td>1.186</td><td>1.191</td><td>1.186±0.0275</td></tr>
<tr><td>100µL</td><td>1.099</td><td>0.911</td><td>1.029</td><td>1.029±0.095</td></tr>
<tr><td>100µL</td><td>0.328</td><td>0.346</td><td>0.353</td><td>0.346±0.0129</td></tr>
</table>

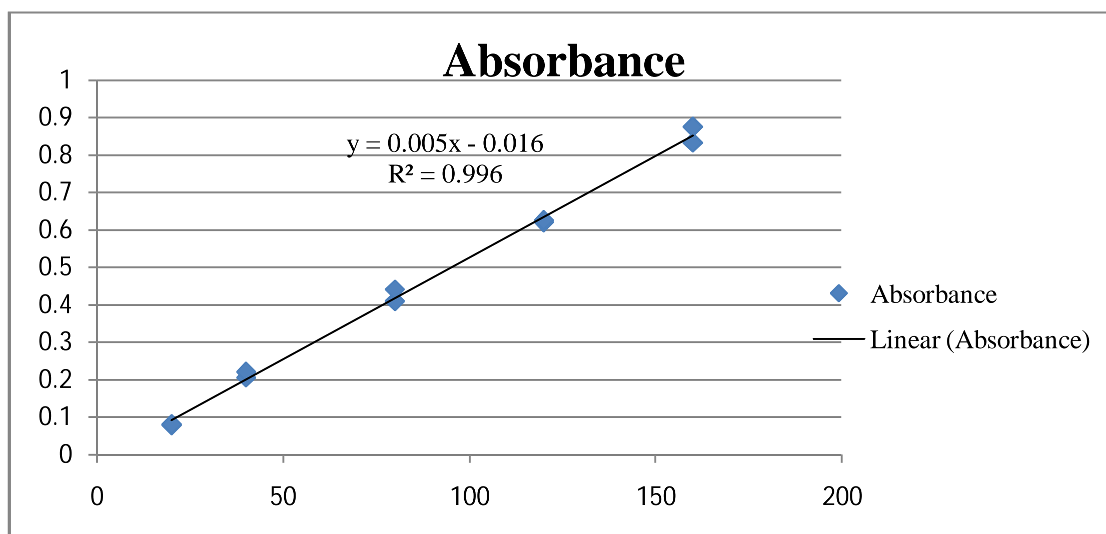


Figure A6-1: Calibration curve of total phenol

Table A6-2: standard and sample absorbance of phytic acid

Phytic Concentration (ppm)	Absorbance λmax = 500 nm		
	1st trial	2nd trial	Average + std.dev
Blank	0.395	0.395	0.395 ± 0.00
5	0.312	0.316	0.312 ± 0.0014
9	0.269	0.273	0.214 ± 0.011
18	0.209	0.215	0.212 ± 0.022
27	0.147	0.155	0.151 ± 0.004
36	0.090	0.096	0.093 ± 0.030
Absorbance of the sample at 500nm			
Bitter leaf	0.127	0.128	0.1275

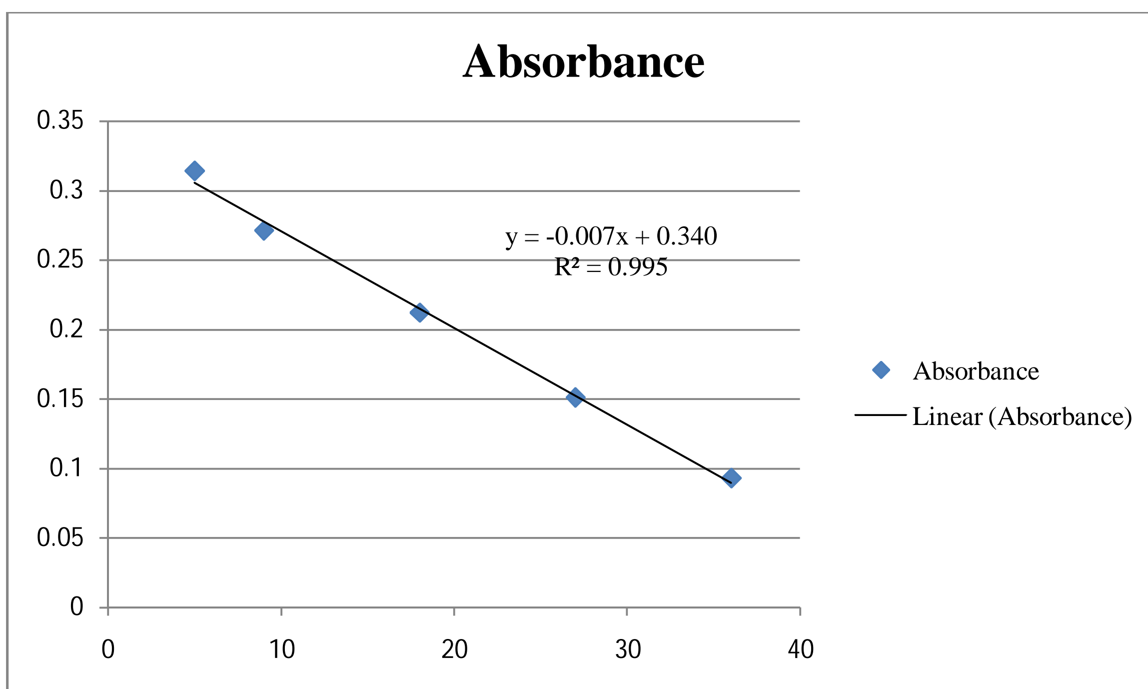


Figure A6-2: Calibration curve of phytic acid

Table A6-2: standard and sample absorbance of tannin

Decatchein Concentration	Absorbance λmax=500 nm		
	1st trial	2nd trial	Average + std.dev
Blank	0.0	0.0	0.395 ± 0.00
0.2	0.033	0.028	0.0305 ± 0.003
0.4	0.071	0.079	0.075 ± 0.005
0.6	0.102	0.107	0.1045 ± 0.003
0.8	0.127	0.125	0.126 ± 0.001
1	0.154	0.143	0.1485 ± 0.007
Absorbance of the sample at 500nm			
Sample	1st trial	2nd trial	Mean ± st.dev
Bitter leaf	0.114	0.130	0.122 ± 0.01

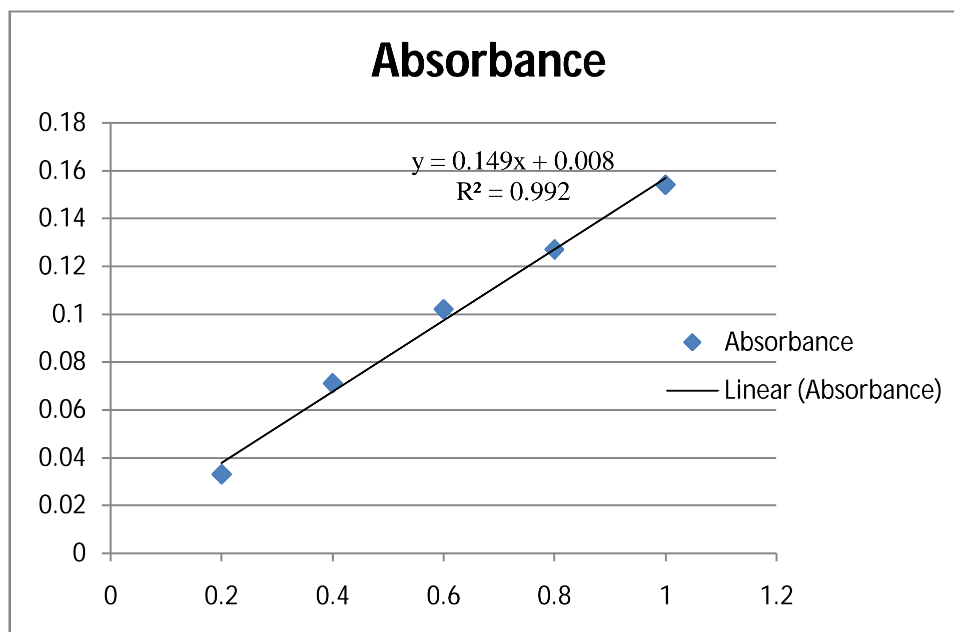


Figure A6-2: Calibration curve of tannin

Appendix-7: pictures during the research work

100
ml
B
TESTED
3bar

Declaration

I, the undersigned, declare that this thesis entitled "Investigation of Bitter Leaf (Vernonia amygdalina) Extract as a Potential Substitute for Imported Hops" has not been submitted in any form for another degree, diploma or an award at any university or other institution of the tertiary education. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature and discussions. Information taken from published and unpublished work of others has been acknowledged in the text and a list of references is given. The work was under the guidance of Teshome Worku (Asst. Prof.) instructor in Addis Ababa University, School of Chemical and Bio Engineering.

Etefa Gurmesa Negeri
Name

Signature

Addis Ababa University, Addis Ababa Institute of Technology
Place of Submission

July, 2017
Date of Submission

This thesis has been submitted to the University with my approval as the University advisor.

Mr. Teshome Worku (Asst. professor)
Advisor

Signature

July, 2017
Date