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SCHOOL OF CHEMICAL AND BIO ENGINEERING

EXTRACTION AND CHARACTERIZATION OF SAPOGENINCHEMICAL FROM AGAVE SISALANA LEAVES AND SEPARATION OF HECOGENIN FOR PHARMACEUTICAL PRODUCT STARTING MATERIALS

BY

Alelign Aliyu Ahmed

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Advisor

Dr.Ing. Zebene Kiflie

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This is to certify that the thesis prepared by Alelign Aliyu, entitled: ***"Extraction and characterization of Sapogenin Chemical from Agavesisalana Leaves and separation of Hecogenin for Pharmaceutical Product starting materials"*** and submitted in partial fulfillments of the requirement for the degree of Master of Science in process engineering complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Approved by the examining Committee:

Advisor	Signature	Date
Dr.Ing. Zebene Kiflie	_____	_____
Internal examiner		
Dr. Sintayehu Nbret	_____	_____
External examiner		
Eng. Gzachewu Shiferaw	_____	_____
School Chairperson		
Dr.Ing. Abubekr Yimam	_____	_____

Declaration

I declare that, this thesis entitled **"extraction and characterization of sapogenin chemical from agavesisalana leaves and separation of hecogenin for pharmaceutical product starting materials"** 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 Information taken from published and unpublished work of others has been acknowledged in the text and a list of references is given.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, Dr. Eng. Zebene Kiflie, instructor in Addis Ababa University, School of Chemical and Bio Engineering.

Name: Alelign Aliyu

Signature: _____

Date of Submission: _____

This thesis has been submitted to my advisor for examination and approval:

Name: Dr. Ing. Zebene Kiflie
Signature: _____
Date: _____

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Abstract

Saponins are of higher molecular, nonvolatile and complex structure compounds; containing sapogenin and sugar. Saponins are extracted and sapogenin is isolated from the agave sisalana leaves, for use as pharmaceutical product starting material. In our country, there is shortage of active pharmaceutical ingredients. Ethiopia currently imports more than 90% active pharmaceutical ingredients, in order to cover its demand. The purpose of this study is to investigate the production possibility of a pharmaceutical starting material from agave leaves.

Saponins were extracted from agave sisalana leaves juice by macerating with ethanol. Sapogenin was then isolated using acid hydrolysis. The yield of sapogenin was maximums at the hydrolysis parameters of 70^{o}C, 4M (Acid concentration) and 3:00hr and 30min and it was minimums at 60^{o}C, 2M and 1:00and 30min. Sapogenin is used for semi synthesis of cortisone and sex hormones drug formulation. Saponin and sapogenin were characterized by both qualitative (saponin foam test and Leiberman –burchard steroid test) and analytical methods (NMR, FTIR, and Spectroscopy photometer). The results of NMR Spectroscopy identified 27carbon atom. UV-Spectroscopy photometers peaks show sapogenin at 430 nm. The functional groups were determined by FTIR spectroscopy. Qualitative Saponin foam and steroid test results showed, that it created foam stable for 30min and its color changed from violate to blue green.

Generally effectively extracts saponin by macerating from sisal leaves juice and isolation of sapogenin by acid hydrolysis of saponin. The yield of sapogenin was characterized by analytical and qualitative methods. The physicochemical characterization result shown as the hydrolysis yield is sapogenin.

Keywords: saponin, sapogenin, *Agave sisalana* leaves, acid hydrolysis

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Chapter one

1. Introduction

Agave sisalana leaves are one of the sources of saponin of hecogenine (K. Hostettmann et al 1995). Sapogenin exist in *Agave sisalana* leaves in the form of secondary metabolites. These are active pharmaceuticals ingredients which are useful for both pharmaceuticals and chemical industries for partial synthesis. The *Agave sisalana* juice mostly contained sugar, organic and inorganic compounds. So, extraction of the organic molecules of steroidal saponin from agave sisalana leaves juice for pharmaceutical products may help to alleviate the shortage of active pharmaceuticals ingredients in Ethiopia.

Saponins are a compound of sugar and sapogenin molecules; they are naturally occurring surface active glycosides with distinctive foaming characteristics .Its, nonvolatile, amorphous and complex compounds. The Sugar portion contains glucose, galactose, xylose, arabinose, fructose and rhamnose, on the other hand the portion of sapogenin includes, hecogenin, tigogenin, neotigogenin, deisogenin, tigogenin etc.

Sapogenins are organic compounds without sugar molecules they are obtained after hydrolysis of saponin either by acid or enzymes. They are very interesting compounds for pharmaceuticals industries, because many pharmaceutical drugs produced from it, when used as active ingredients. Sapogenine is classified into steroidal sapogenine and triterpenoid sapogenine based on the chemical structure. The steroidal saponins contain steroidal structures, as their aglyone. They have C_{27} skeletons in structures; and are organized into six rings(A. Yokosuka et al., 2000).

The steroidal saponin or steroidal sapogenin molecules are used for the semi-synthesis of bio active compounds such as anti-inflammatory, anti-cancer, cardiovascular, anti-microbial and sex hormones, cortisone, vitamins D drugs (A. Yokosuka et al., 2000). Corticosteroid drugs (prednisone, prednisolone, hydrocortisone, dexamethasone and betamethasone) and sex hormone drugs are produced from sapogenin or steroidal saponins which are extracted from agave sisalana leaves and others plants (Y.Al Jasem et al. 2014).

Agave sisalana matured plants contain more hecogenin chemical (steroidal saponine)(D. P.Sharma et al., 2010).

Generally, the bioactive saponin obtained from agave sisalana leaves; when, extracted by using organic solvents, such as ethanol, methanol, hexane etc. Saponins are classified into sugar part (glycone) and non-saccharide (aglycone). Sapogenins or aglycones are produced by hydrolysis of saponin.

1.1. Statement of the problem

Shortage of industries resource have become world's great problems; especially for food factories, chemical factories, pharmaceutical factories and others. Developing countries have wide natural resources, but they do not use them due to absence of processed ingredients from available resources. Mostly their industry use imported raw materials from developed countries.

African countries depend on imported pharmaceutical product, according to studies on local production pharmaceutical and health system in Africa, between the years of 1995-2015GC purchased pharmaceuticals cost of Uganda, Ghana and Ethiopia increased from50-375US$ million,40-250US$ million,50-800US$million respectively; in Kenya purchased cost increased from 298.6US$ million to, 466.4US$ million within 2009- 2013 GC.

Ethiopian pharmaceutical industries covered their requirements by importing 95% of the starting materials and only the rest 5% are obtained from local sources. For example, in 2007, 2008, and 2010 imported from China, Ouman and India and our country spent 6.99US$ million,9.43 US$ million and 11.91US$ million, respectively(M. Ewen et al , 2017).

In addition to this, 85% of the pharmaceutical product (formulated drug) demand is satisfied by imported products; for example, from 2007 to 2009 US$752.27 million is expensed for purchased formulated drug. According to studies on investing in Ethiopia the future pharmaceutical hub of Africa (2015-2030) the demand is expected to grow by 15 % ((Beedemariam, 2011).

Ethiopia has many medicinal plants that can be used as active pharmaceutical ingredients; by their local name or Amharic name Dama kessi, Endod, Akeraro, Algai (Agave sisalana), Fiyele feji, Yemdir emboy and etc are available for traditional and modern medicine. Agave sisal plant

is used as starting materials for anti-microbial, anti-inflammatory/ anti-allergic, tuberculosis, cardiovascular and steroidal hormonal drugs. Sapogenin material extract from sisal leafs or roots used as active ingredient materials for corticosteroid and steroidal hormones drugs. They are used for treatment of anti-inflammatory, cardiovascular and sexual disorder diseases.

Our country has at least five pharmaceutical industries; for example Addis pharmaceutical factory, Candila pharmaceutical factory,Julphar pharmaceutical factory, Ethiopia pharmaceutical factory, East Africa pharmaceutical factory, and MedTech Ethiopia pharmaceutical factory. From them Addis pharmaceutical factory, Candila and Julphar(Gulf) pharmaceutical factories producing anti-inflammatory, anti-microbial, anti TB, cardiovascular and sex hormone. All of the above pharmaceutical factories cover their required ingredients through importing due to, the absence of active pharmaceutical ingredients product in the local market. In Ethiopia we have many agave sislana plants which can be used for production of sapogenin active pharmaceutical materials, especially in west, north, and south Ethiopia.

1.2. Objective

1.2.1. General Objective

The general objective of this thesis is extraction and characterization of sapogenin chemical from agave sisalana leaves.

1.2.2. Specific objectives

- Extraction of saponin from the sisal leaves juice
- To separate sapogenin from saponin
- To optimize saponin extraction parameters
- To characterize sapogenin.
- To optimize saponin hydrolysis parameters: time, acid concentration, and temperature.

1.3. Significance of the study

- It increases pharmaceutical ingredients: This study increase the number of raw materials which are available to pharmaceuticals precursors. Especially for precursors of anti-inflammatory and cortisone drug.
- It gives information to our country investor or foreign investor about production of pharmaceutical drug from sisal leaves.
- And also partially reduce import cost, when our pharmaceutical factories produce sex hormones (used for balance hormone) and cortisone drug from agave plants.

Chapter two

2. Literature review

2.1. Origin and distribution of *agave sisalana* plants

Several botanical researchers studied on the agave sisalana plants historical origin and distribution. *Agave sisalana* plants are native in Mexico. In 1753, the genus of agave was established by Linnaeus, it contained 136 species , in the agavecea family (S.Sarkar et al., 2018). Agave plants widely grows in Brazil and it distributed into Africans countries Tanzania and Kenya (M.Debnath et al., 2010). Other investigators also studied the distribution of sisal plants into Africa. The sisal plants grows in the other African countries, for example it grows in Uganda, Malawi, Ethiopia, Kenya, Madagascar, South Africa, Angola, Central Africa (D. Kimaro et al., 1994).

Agave Sisal was originally grown in southern Mexico but widely cultivated and naturalized in many other countries. It has been widely introduced in the tropics and subtropics; in India between 1885 and 1892, in Tanzania in 1893, in Brazil at the end of 19th Century, and in Kenya between 1903 and 1908. The first commercial plantings in Brazil were made in the late 1930s and the first sisal fiber exports from there were made in 1948. Until the 1960s, Tanzania was the leading producer of sisal, but Brazil has become the major world producer of sisal, followed by Tanzania, Kenya, Madagascar and Chin (J.Trejo-Torres et al., 2018).

Agave sisalana in Ethiopia and most of African countries cultivate sisal plant for fiber production and cattle feed. In Ethiopia, in the year 1981/1982 about 2,100 tonnes of sisal waste was used as cattle feed (M. Meragiaw et al,.2014).

2.2. Botanical

Botanical properties of *Agave sisalana* plants are very important for plants properties, about leaves, roots and stem height, length, diameters and width. The sisal plant looks like an over grown pineapple plant of height ranging from 1.2-1.5 m with a short bole (J.Trejo-Torres et al., 2018).The crescent shaped leaves with a terminal spine having basal rosette are encircle around the meristem. Sisal plant forms a shallow but tufted, fibrous and spreading root system (J.Santos et al,. 2015). In favorable soil conditions, the roots of plant having diameter between 2–4mm are

spreads horizontally up to 5m from the stem base, but concentrated in the upper 30-40 cm of soil(A.F. Ade-Ajayi, et al 2011). Sisal can grow on poor soils in the regions with evenly distributed annual rainfall of 60–125 cm and temperature ranging from 16-35 ^{0}C. Being a xerophyte plant, it can withstand extreme drought condition (N. Almararaz-Abarca et al,.2013). After 11-12 years of life cycle, it produces a large flowering stalk/ pole of 8-12 m tall and plant dies (M.Mwale et al., 2015). The mean length and weight of bole was found to be 0.57 m and 38.38 kg respectively, whereas mean weight of pole was found to be 21.4 kg. the sisal leaf weight range from 0.275-0.725 kg in addition its length of leaf, width, and thickness, 1.04 m, 0.079 m, and 0.018 m respectively (N. D. Behera et al,2016). Scape 12 to 15 feet high; flowers pale, yellowish-green (M. Hidalgo-Reyes et al., 2015).

2.2.1. *Agave Sisal* plant growth

Agave sisana plants are grows in tropical and sub-tropical region researchers expressed the production of sisal, and it produces by a sexually sterile clone, probably of hybrid origin, due to its general inability to produce seed and by its chromosomes. During matured years, under plantation conditions *Agave sisalana* produces about 220 leaves per plant. Leaves can be harvested after two years of age (KG. Asfaw, 2011).

The roots of the sisal plant feed near the surface, and do not descend to any great depth, in hard clay rarely over 12 inches and rocky soil seldom over18 inches. The point of such a runner reaches the surface beginning with the second year, and forms a bulb. This bulb is known as the sucker or shoots. This sucker takes root and obtains nourishment through the runner from the old plant, and also through its own roots . New suckers continue to appear during the life of the plant. After the third year the lower leaves, 2 to 2 1-2 feet long, assume a horizontal position and show yellowish spots. These are called "mature" leaves. As the plant grows older more leaves mature, which, however," are" longer and wider, attaining at the age of 5 to 7 years a length of from 4 to 6 feet. After three vigorous growths of about 6 years, which may be extended to 7, or even 9 years, after planting, a central stalk or "pole" grows up rapidly. This pole is 4 to 6 inches in diameter at the base, tapers upward, and attains a height of 30 feet. It bears flowers, which fall off and are replaced by 2,000 to 3, 000 bulbs. These bulbs grow to be about 6 inches long .They finally fall to the ground and thus reproduce the plant. When this has been accomplished the

leaves of the plant have become brown and leathery, and the whole mass dies and forms considerable debris. Suckers attached to the plant at this time will throw out a central stalk one inch thick, and from 3 to 6 feet high. Some bear flowers and bulbs; others bear only bulbs, which are usually smaller than those of the mother plant (A.Salum et al 2009).

2.3. *Agave sisalana* plant advantage

The plants of agave sislana have many advantages, it used for medicine,food and fiber (J.D. G. Santos, 2015) ; the sisal juice is the source steroidal sapogenin (JC. Cushman et al2015). the researchers (Debnath et al., 2010). The sisal plant also used for energy source (biofuel) (A.Salum et al 2009) **.**

Agave sisalana is chiefly cultivated for its fiber which is eminently suited for cordage of all kinds and trials have shown that for marine cordage the fiber compares favorably in durability with the Manila hemp obtained from Musa textiles Nees. The leaves are cut for fiber between the third and fourth year. The lowest leaves are cut close to the trunk. Each plant yield 250-300 leaves during its lifetime of 7-8 years. For extraction of the fiber, two methods are employed ; the retting process and the mechanical process. The fiber yield in retting process is about 5.5% and in the mechanical process is about 3-4% based on the weight of green leaves. *Agave Sisal* fiber being strong and more resistant to dampness than other fiber is used for binder twines, ship cordage, webbing and sacking, and as a substitute for Manila hemp which is used for heavier twines, ropes, marine cordage, fish, nets, etc. It is used as a substitute of jute for mats, rugs, sacks for coffee, wagon cover and floor coverage. Preliminary pharmacological investigation shows that the juice of the leaves lowers the blood Pressure in dogs and stimulates their intestinal movements. It possesses ebolic properties and may be used as an abortifacient as it activates the uterine motility. The juice may be administered with Pitocin to intensify the ebolic action. It is a uterine stimulant, emmenagogue, laxative and hypotensive drug. Only 5% of the decortications of the leaves of sisal (*Agave sisalana*) produce a hard fiber that is used for various purposes; the remaining 95% consists of solid waste (mucilage) and waste liquid (juice of the sisal) that are normally discarded by sisal farms. Thus, sisal waste principally contains plant tissue (lignin and cellulose), primary and secondary metabolites, and water, amongst others(N. Monterrosan-Brisson et al,2013)**.**

Agave sisalana plants also used as food and fermented beverages such as Tequila and mezcal, both distilled from agave(JC. Cushman et al2015).

Generally *Agave sisalan* has a wide application, some of its uses are

- Its fiber is used for making cloth and fiber
- Used for soap making
- Has Pharmacological importance such as lowers blood pressure, liver and heart disease skin disease and scalp antimicrobial and for treatment of syphilis (treat by hecogenin)
- It used as bio insecticide
- It's used as fertilizer
- It's used for fungicide, by making dandruff shampoo from its leaf
- When the plant is baked, its rich sources of carbohydrate in form of saccharine hence can be used as food.
- The roots are used for sources of saponnins used to make soap.
- The sap of the plant is used as antiseptic

Agave sisalana to be served as feedstock for biofuel and biogas production (JR.Mielenz et al 2015).

2.3.1. *Agave sisalana* plants medicinal property

Agavesisalana plants ,has pharmacological properties of interest due to the main phytochemicals (Mendes et al., 2016).The active elements are occurred in sisal roots, leaves and stem. The sisal plants are the source of steroidal sapogenins; they are used as a starting material for production of steroidal drugs (N. Monterrosan-Brisson et al,2013).

All these secondary metabolites attribute to the pharmacological properties of the plant. Agave sisalana mostly contain the saponin of hecogenin. Hecogenin occurs in the plant in the form of its glycoside. Sisal waste and the juice expressed from the leaf pulp by decortication of fresh leaves are the commercial source of hecogenin (Debnath et al., 2010).*Agave sisalana* plants have many medicinal advantages, for example it used for; Toothache and wound healing, Lower blood pressure , Liver and heart disease ,Intestinal stimulator and Stop the growth of bacteria in the stomach and intestine (Debnath et al., 2010) and (J.D.G. Santos et al., 2015).

2.4. Chemical composition of sisal leaves juice

Agave sisal leaves contain many solid particle and liquid; it contained organic and inorganic compounds(N. Monterrosan-Brisson et al,2013)). The plants are available for hard fiber production only 2.7-7.3%, the remaining 97.3-92.7% consists of solid waste (mucilage) and waste liquid (juice of the sisal) that are normally discarded by sisal farms. The different phytochemical compounds present in the juice of sisal were characterized by performing chemical reactions that resulted in the development of foam, coloring or precipitation.

Figure 2-1 Composition of sisal leaves

Total solid (%)	16.1±1.5
Volatile solids, VS (% of TS)	83.3±1
Total organic carbon(%)	49.4±0.4
Total inorganic carbon(%)	4.3
Neutral detergent fibers (NDF) (%)	69.0±8
Acid detergent fibers (ADF) (%)	47.4±8.3
Lignin(%)	4.5±2.7
Cellulose(%)	76.7±5.6
Hemicellulose (%)	21.6±7.2

(M.Muthangya et al., 2013)

2.5. Saponin

Saponin is compound of sugar molecules and aglycones; it extracted from plant or small marine animals. Saponins are high-molecular-weight glycosides, consisting of a sugar moiety linked to atriterpen or steroid aglycone. The classical definition of saponin is based on their surface activity; many saponins have detergent properties, give stable foams in water, show hemolytic activity, have a bitter taste and are toxic to fish (k.Hostettmann et al.,1995). Some saponin-containing plants have been employed for hundreds of years as soaps and this fact is reflected in their common names: soapwort (Saponaria officinalis), soaproot (Chlorogalum pomeridianum), and soapbark (Quillaja saponaria), soapberry. Indeed, the name 'saponin' comes from the Latin word sap (soap).

Saponins are natural occurring glycosides whose active portions are soluble in water ,alcohols and both(M. Muthangya, et al., 2013). It classified based on chemical structure into two major groups of steroidal saponin and triterpenoid saponin (Y.Al Jasem et al. 2014).

The investigators, by 1987 studied the structure and source of saponin; the structure of over 360 sapogenins and 750 triterpene glycosides had been elucidated.The most common sources of saponins are the higher plants, but increasing numbers are being found in lower marine animals. So far, they are only been found in the marine phylum Echinodennata and particularly in species of the classes Holothuroidea (sea cucumbers) and Asteroidea(Starfishes) (K. Hostettmann et al , 1995).

Saponins are mostly known by their foaming and hemolytic properties. saponin aglyones are capable of increasing permeability of membrane. The aglycones can cause hemolysis by destroying the membrane of red blood cells, when, releasing hemoglobin(A.Salum et al 2009. In addition to their foaming and hemolysis properties, they are known by its complexity of structure. The complexity of structure is caused by the variability of aglycone structure of the side chain and the attachments of moieties on the aglycone. In case of this complexity saponin are difficult to classified(E.Moghimipour et al ,.2015). It also lowering the cholesterol of humans and animals(E. Moghimipour et al.,2014).

The Secondary metabolites classes of saponins are different from the components of primary metabolism due to the fact they are non-essential for basic metabolic processes in the plant. Plants synthesize diverse secondary metabolites that have been clearly demonstrated to play a role in the adaptation of plants to their environment. The production of these secondary molecules used for the response of external factors including various biotic and abiotic stimuli. There are many different type of saponin present in different plant as secondary metabolites. Agave sisal plant gives one type of saponin that is called hecogenin.

Saponins are the most important bioactive substance it used as starting materials in the pharmaceuticals industries (E. Moghimipour et al.,2014).

2.5.1. Classification of saponin

Saponins are class of glycoside containing sugar moiety and steroidal saponin or triterpene saponin. Saponin is classified into three types, triterpene glycosides, Steroid glycosides and Steroid alkaloid glycoside (K. Hostettmann et al.,1995) and**.** (E. Moghimipour et al.,2014).

We have two type of saponin, they are glycon and aglycone. Glycones are part of sugar content whereas aglycones are part of sapogenin. Chemically saponins are higher molecular weight glycosides in which (1-8 residues) are linked to triterpene or steroidal aglycone moiety. Sapogenin glycosides are divided into 2 types based on the chemical structure of their aglycone (sapogenin). Saponins on hydrolysis yield an aglycone known as sapogenin.

The difference between the two classes lies in the fact that the steroid saponins have three methyl groups removed (i.e. they are molecules with 27 C-atoms), whereas in the triterpenoid saponins all 30C-atoms retained (M. Thakur et al 2011). On the other hand hostettman and A.marson, saponin classified into three according to the type of genin presents. The aglycone or non-saccharide portion of the saponin molecule is called the genin or sapogenin (K. Hostettmann et al,1995).

The saponins can be divided into three major classes

(1) triterpene sapogenin

(2) Steroid sapogenin

(3) Steroid alkaloid sapogenin

2.5.1.1. Aglycone (sapogenin)

Based on the chemical structure of sapogenin, it classified into two main groups that are steroidal sapogenin and tripinoidal sapogenin (M. Thakur et al 2011).

A non-sugar portion of saponin that is, typically obtained by hydrolysis, has either a complex terpenoid or a steroidal structure, and in the latter case forms a practicable starting point in the synthesis of steroid hormones.

The aglycones are normally hydroxylase at C-3 and certain methyl groups are frequently oxidized to hydroxyl methyl, aldehyde or carboxyl functionalities. When an acid moiety is esterified to the aglycone , the term ester saponin is often used for the respective glycosiste (K. Hostettmann et al,1995). Example of aglycone, are tigogenin, hecogenin, neogitogenin, and

tokorogenin. According to the nature of aglycone, saponins are also classified into two groups, which are neutral saponins (steroids) and acid saponin (triterpenoids).

a. Steroidal sapogenins

Steroidal sapogenins are aglycone non sugar portion of the saponin compound used for the semi synthesis of bioactive compounds. Steroidal saponins are mainly compounds containing 27 carbon atoms forming the core structures: spirostan (16β,22:22α,26- diepoxy-cholestan) and furostan (16β,22-epoxycholestan) (D.Kregi.et al., 2017). Agave sisalana is a very important resource of hard fiber and steroidal compounds (HA. Deshapande et al 2014).

The sapogenins of steroid saponins are C_{27} steroids carrying a spiroskeleton side-chain, and are derived from the same tetracyclic ring system found in sterols, bile acids, and cardiac aglyoons (M.Marrelli et al 2016).

Nourish the cardiac muscle, slow down platelet aggregation, increase coronary flow, improve peripheral circulation as well as show decline in cholesterol level and triglyceride in blood are some biological activates of steroidal saponin. Steroid hormones are produced from steroidal sapogenins; for example sex hormone, progestogen and cortical hormone. Steroidal saponins which extracted from any different medicinal plants have many pharmacological advantages because, several drugs are produced from steroidal saponin used for treatment of some diseases such as , anti-cardiovascular, anti-inflammatory, sexual disorder treatments antifungal, cytotoxic and antitumor, anti-arthritic(D. Tewari et al., 2018)and (MS.Mohammed et al.,2014).The reason that saponins are considered steroidal in case of that they have some positive impact on levels of testosterone; the male hormone that increases muscle mass, fat burning and libido. The way this may work is by increasing the amount of good cholesterol in the blood. There is a link here, seeing as sex hormones are actually made in the body from cholesterol.It is possible to increase the amount of steroid hormones, when by raising good cholesterol (adam sinicki in nutrition).

b. Tripenoidal saponin

This type of saponins is the most widely distributed in the plant kingdom. The term triterpene is meaning three monoterpenes (10 carbon atoms) of 30 carbon atoms distributed as six isoprene molecules (M. Marrelli et al 2016)**.** They are almost invariably pentacyclic compounds, e.g.

terpenoid sapogenin, with few functional groups and thus difficult to attack chemically for structural deduction (PK. Gupta et al 2010).

The sapogenins of triterpenoid saponins consist of a 30 carbon pentacyclio skeleton which in principle is divisible into isopentane units (isoprene rule). The triterpenoid sapogenins are conveniently sub-divided into three groups based on their relationships with bet-amyrin , alpha-amyrin and lupeol. The triterpene saponins can be mono, bi- or even tridesmosidic. One sugar chain is often attached at C-3 and a second is frequently found to be esterified to the carboxyl group at C-17 of the aglycone (except certain dammarane glycosides and lanostane glycosides which have asecond or even athird glycosidically bound sugar chain).

c. Steroid alkaloids

Steroid alkaloids are the largest group of secondary chemical constituents made largely of ammonia compounds comprising basically of nitrogen bases synthesized from amino acid building blocks with various radicals replacing one or more of the hydrogen atoms in the peptide ring, most containing oxygen (KC. Youn et al., 1996). There are two classes of steroid alkaloid sapogenin anidans and the spirosolans (K. Hostettmann et al 1995).

2.5.1.2. Glycone (sugar part)

The monosaccharide moieties are attached at C-3 or C-26 carbons atom. They are includes abroad spectrum of simple sugar like: D-glucose, D-galactose, D-fructose, 3-methyl- D-glucose, D-xylose, L-arabinose, L-rhamnose etc**.**

2.5.2. Source of saponin

Saponins occur in, many plants especially in, vegetables, fruits, seeds and sea cucumbers.

Saponin producing plants are found in various geo- graphical regions and climatic zones around the world. These include annual and biennial herbs, grasses, perennial evergreen, shrubs, trees, and wild and cultivated species (A.Faizal et al, 2013).

Saponins are widely distributed in the plant kingdom. In 1927, kofler had listed 472 saponin-containing plants (L.Kofler et al 1927). In 1970, Gubanov systematically investigates on 1730 plants from his investigation 76% of the plants contained saponin. Saponins occur in plants which are used as human food: soybeans, chick peas, peanuts, mung beans, broad beans, kidney

beans, lentils, garden peas, spinach, oats, aubergines, asparagus, fenugreek, garlic, sugar beet, potatoes, green peppers, tomatoes, onions, tea, cassava, yams and in leguminous forage species (such as alfalfa) (N. Monterrosan-Brisson et al,2013) and (M. Saxena et al., 2013). Kitagawa in1984 has studied on the occurrences and isolation of soyasaponin from a number of forage and cover crops, including Trifolium spp Medicago spp., Astragalus spp. and Vicia saliva. Finally, saponins are also present in numerous herbal remedies. Saponin content depends on factors such as the cultivar, the age, the physiological state and the geographical location of the plant. There can be considerable variation in composition and quantity of saponins in vegetable material from different places, as documented for Lonicera japontca (Caprifoliaceae) (Y.Vaghasiva et al 2011) The steroidal saponins are mainly found in monocotyledons (such as in the family's Agavaceae, Dioscoreaceae and Liliaceae), triterpenoid saponins mostly are present in dicotyledonsile (Fabaceae, Araliaceae and Caryophyllaceae).

Plant material often contains triterpene saponins in considerable amounts. Thus, primula root contains about 5-10 % saponin, licorice root between 2% and 12% glycyrrhizin, quillaia bark up to 10% of a saponin mixture and the seeds of the horse chestnut up to 13% aescine. In other words, the concentration of saponins in plants is high when compared with other secondary metabolites.

2.5.3. Saponin chemical, physical and biological properties

The physical and chemical properties saponins are foaming properties, bitter taste, surface active properties, amorphous property, hemolytic property, anti-coagulant, anti-carcinogenic and antioxidant (J.S.Negi et al., 2013).

2.5.3.1. Saponin foaming and hemolysis properties

Saponins are secondary metabolites; it occurs in plant or marine animals and haves hemolysis activities of red blood cells. The first characterization of saponin is strong foaming ability in aqueous solution. Amphiphilic nature of sapogenins in case of the occurrence of lipophilic moieties and hydrophilic; Thus amphiphilic nature causes the saponins has surface active compounds with foaming, to interact with cell membrane and lytic action on erythrocytes membrane(A.N. Mohammad et al 2014)and (J.H.Lorent et al 2014) . several investigators

reported, saponins were characterized and approved conformations by the foam creation and red blood cells hemolysis(E. Rami et al ., 2013).

2.5.3.2. Saponin medicinal properties

Saponin used for the limited the growth of the microbial; they are affected the growth of fungi , bacteria and other microbial .Many researchers' studies on the: Anti-microbial activities of saponin, anti-cancer, Anti-inflammatory and Anti-cardiovascular activity(Y.Al Jasem et al. 2014) .

2.5.3.2.1. Anti-microbial activities

Several investigators studied on the plants including *agave sisalana* their phytochemicals for anti-microbial. The antimicrobial property of the agave sisal plant leaf extracts against some strains of microorganisms was evaluated. Thus, the phytochemical with adequate antimicrobial efficacy proved that the plant may be adapted for treatment of some infections caused by microorganisms (GA. Shallangwa et al.,2011). The saponin extracted from agave sisalana leaves or roots are available for anti-microbial , anti-cancer and for other treatments (J. Santos et al., 2009). Steroidal saponin extract from croton polyandrop leaves important and interested due to, their relationship with various anabolic hormones including sex hormones, they are shown anti-bacterial activities (Fernandes et al ,2013).

2.5.3.2.2. Antifungal activity

The triterpenoid saponins exhibit antifungal activities and prevent important crop loss in modern agriculture and horticulture. They exert strong antifungal activity against a broad range of fungi types and stages. Saponins prevent the synthesis of ecdysteroid, the protease inhibitors, or are cytotoxic to certain fungi (S.Garai, 2016). Nine steroidal saponine isolated from asparagus plants for antifungal (Shimoyama and Bokaldu et al, 1996).The saponin isolated from Capsicum frutescens, exhibited antifungal activity against Candida sppand Aspergillus fumigatus, with MICs ranging from 4.0 to 16 mg mL-L. investigated on the antifungal activity of C-27 steroidal saponins against Candida albicans, c.glabrata,c. krusei, Cryptococcus neoformans, and Aspergillus fumigatus (yang et al, 2006).. These saponins showed significant activity against C. neoformansand A. fumigates that was comparable to the positive control amphotericin. Studied the antimicrobial activity of saponins extract of Sorghum Bicolor against three pathogens are

Escherichia coli, Staphylococcus aureus and C. albicans. The saponins inhibited the growth of the S. aureus, but not had inhibitory effect on E. coli and C.albicans. They demonstrated that ineffectiveness of the saponins from S. bicolor on gram-negative bacteria and fungus may be as a result of the protective effect of the microbial membranes. The saponins may not be able to penetrate the cell membranes of the microorganisms.

2.5.3.2.3. Anticancer activity

Natural compounds isolated from medicinal plants, are promising plants for anti-cancer activities. Secondary metabolites like saponin available for anti-cancer.Saponinstype of plant-derived secondary metabolites, are glycosides containing aglycones of triterpene sapogenins or steroidal sapogenins (XiaoHuang Xu et al.,2016). Other investigators also, supported the secondary metabolites, saponine used for anti-cancer; for example Asharaf stated : over 3000 plant species have been used for the cancer treatments. Natural substances, secondary metabolites , like sapogenin or steroidal saponine are available for anti-cancer (A.Abdul Kadir et al., 2013)

Steroid saponins isolated from the rhizome of polyphylla plants, used for anticancer activities(Yan etal.,2009).The saponins showed anticancer activity against lung a ofdenocarcinoma cell line, both in vitro and in vivo. They demonstrated that the saponins could be regarded as promising drugs for cancer therapy. Investigated antitumor activity of polysaccharides and saponin extracted from sea cucumber(Su et al.,2011).These results indicated that the in vitro anti-tumor effect of saponins is more potent than polysaccharides. Several reports have been demonstrated that plants saponins can reduce the risk of colorectal cancer. Studied the apoptotic effect of crude saponins isolated from the roots of Platycodon grandiflorum in HT-29 human colon cancer cells (Kim et al, 2008). Their results showed saponins could inhibit HT-29 cell proliferation and induce apoptosis. The apoptosis was induced by DNA fragmentation and poly ADP-ribose polymerase (PARP) cleavage. Prepared and evaluated saponin loaded chitosan nanoparticles as a cancer therapeutic agent for an enhanced and sustained release (Rejinold et al, 2011). They extracted saponin from Sapindus emarginatus and evaluated the cytotoxicity of the nanoparticles at different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1mg/ml) on mouse fibroblast cell line (L929), mouse embryonic fibroblast cell line (NIH-

3T3), oral cancer cell line (KB) and prostate cancer cell line (PC3).The nanosaponin showed specific toxicity on prostate and oral cancer cells, while did not show any toxicity on normal L929 and NIH-3T3 cells. Many studies demonstrated that the induction of cell death in cancer cells by anticancer saponins appeared in a dose and time-dependent manner.

2.5.3.2.4. Anti-cardiovascular activity

Saponins exert various pharmacological effects, including cardiovascular protective activity (Xiao-Huang Xu et al,2016)**.** It has been reported that ingestion of saponin containing food decrease cholesterol levels in the bloodstream and as a results decrease the risk of cardiovascular diseases. It was also, reported that ginseng saponins decrease blood cholesterol levels in rabbits by increasing cholesterol excretion through bile acid formation. Consumption of saponin from Solanum anguivifruit lead to reduction in the risk of hyperlipidemic symptoms and heart diseases. It has been previously reported that the total saponins extracted from G. glabra and Q. saponaria were capable of forming complex with cholesterol (S. Snehal et al 2015).

2.5.3.2.5. Anti-inflammatory activity

Inflammation is action of response or a complex pathophysiological process mediated by a variety of signaling molecules produced by leukocytes, macrophages and mast cells as well as by the activation of complement factors, which bring about edema formation as a result of extravasation of fluid and proteins and accumulation of leukocytes at the inflammatory site. Secondary metabolites are available for the treatments of anti- inflammatory agents. Saponins isolated from about 50 plants showed anti-inflammatory activity against several experimental models of inflammation in mice and rats (E. Moghimipour et al., 2015).

The aqueous leaf extract of Agave sisalana showed some anti-inflammatory and analgesic properties. The 100 or 200 mg/kg body weight doses reduced pain and inhibited inflammation remarkably. Other researchers study the activity of saponin on anti-inflammatory which is extract from plants and seeds. Patel et al. in 2012 studied anti-inflammatory activity of saponin isolated from the spesia populnea (L.) leaves. According to their results, the saponin showed potent anti-inflammatory activity on acute and chronic inflammation models. They demonstrated that mechanisms for anti-inflammatory activity might be associated with the inhibition of prostaglandin and histamine. Investigated on the anti-inflammatory activity of a saponin-

containing fraction derived from methanolic extract of Gleditsia caspica fruits (MB. Nayeli et al,2013). The saponin could significantly inhibit the progression of the inflammation in the treated animals. That the inhibitory effect of saponin could be due to inhibition affect the enzyme cyclooxygenase and subsequent inhibition.

2.6. Saponins in plant defense

For many secondary metabolites, synthesis is stimulated upon challenge by biotic or abiotic stresses. Although this is a strategy to combat pathogens and build protection in a cost-effective way, some saponins are produced independent from external signals and contribute to the innate immunity. These saponins are referred to as phytoanticipins as they are present in unchallenged plants. The downside of accumulating saponins as a first defense is not only that it consumes substantial amounts of energy, but also that it allows pathogens to develop tolerance. This is avoided when saponin precursors accumulate and saponin content increases as the result of chemical modifications of precursor molecules, which are stimulated upon pathogen infection (Morrissey and Osbourn et al,1999). For instance, the saponin content may rise due to partial or complete hydrolysis of stored precursors as part of plant defense mechanism or by pathogen controlled degradation (Szakiel et al, 2011).

2.6.1.Chemical structure of saponin and sapogenin

The chemical structure of sapogenin defines the classification of saponins as triterpenoid saponins (30 carbon atoms) and steroidal saponins (27 carbon atoms with a 6-ring spirostane or a 5-ring furostane skeleton). According to the carbon skeleton of the aglycon, saponins are sometimes further classified into 12 main classes, namely the: dammaranes, tirucallanes, lupanes, hopanes, oleananes, 23-nor oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes, and steroid (Vincken et al, 2007).

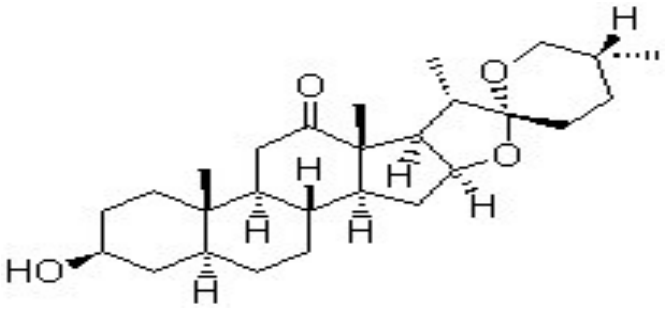

Figure 2-2: Hecogenin (3beta –hydroxy-5-alpha –spirotan-12-one; 5-alpha-spiostan-3 beta –ol-12-one)

Saponins are compounds of sapogenin (aglycone) and sugar (glycon). Saponins with the carbohydrate or oligosaccharide groups attached at the C-3 position are monodesmosidic, while saponins with carbohydrates attached at both the C-3 and C-26 or C-28 positions are bidesmosidic;fish (K. Hostettmann et al., 1995).The variety of a glycones, carbohydrates, and different attachment positions result in numerous types of saponins. The carbohydrate chains of saponins usually include: D-glucose, D-galactose, L-rhamnose, L-arabinose, D-xylose, D-apiose, D-fucose, and D-glucuronic acid(Wilkins, Miles, Kock, Erasmus, & Basson, 1996).

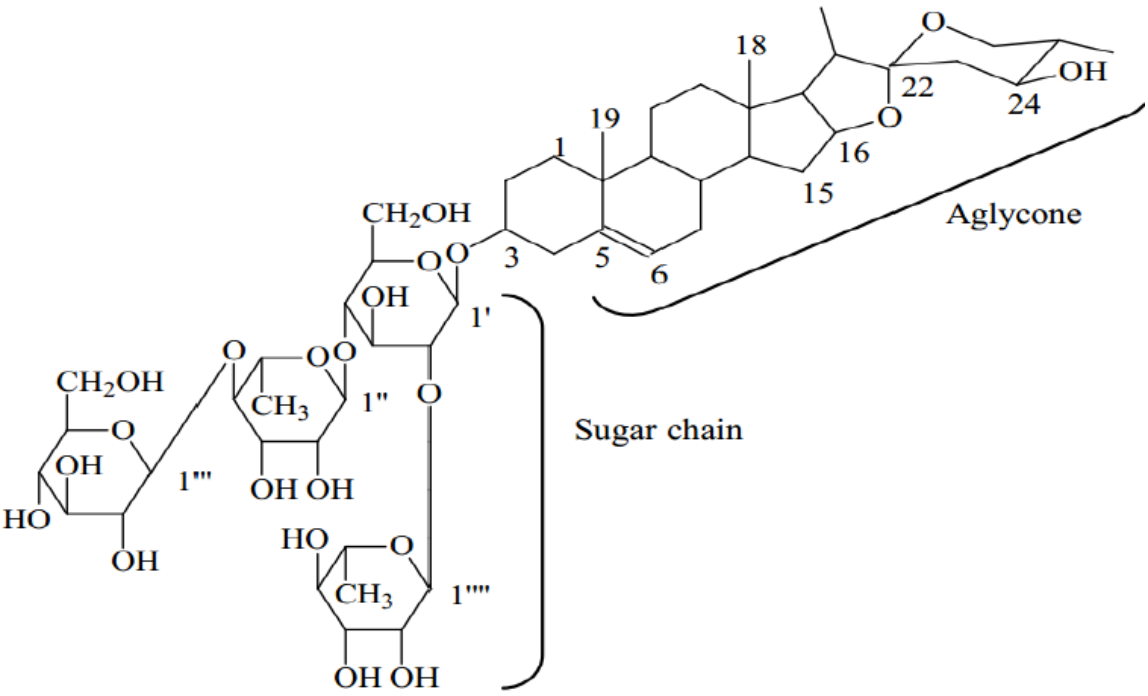

Figure 2-3: Saponin chemical structure

The steroidal saponins usually show furostanol or spirostanol form. Additionally, both steroidal and triterpene saponins may contain other functional groups: –OH, –COOH, $–CH_3$ that gives them additional diversity (J.S.Negi et al., 2013)

The chemical structure of saponins may be transformed during storage or hydrolysis of saponin resulting in isolation of sapogenin and sugar portion. The linkages between the sugar chain and the aglycones as well as between the sugar residues can undergo hydrolysis during acid or base treatment, hydro thrombolysis or enzymatic/microbial Transformations, resulting in the formation of aglycones, prosapogenins (partially hydrolyzedsaponins), and sugar residues. Therefore, the selection of extraction and isolation of sapogenin methods are appropriate to storage of plant material and it is a key part of each efficient technology.

2.7. Steroidal saponin and bio active substance

Steroidal sapogenins oraglycones are non-sugar portion of the saponin compound, used for the semi synthesis of bioactive compounds (P.Savjani et al2015). For example, compounds used for bioactive compounds manufacture include the following smilagenin, sarsasapogenin, diosgenin, yamogenin, tigogenin, neotigogenin (J.Santos et al 2014). Among these steroidal sapogenin,diosgenin , sarsasapogenin and hecogenin are particularly important . The usefulness of hecogenin as asynthetic starting material is due to the presence of an oxygen atom in the C-12 position that can be moved to the C-11 position. this makes it possible to introduce the 9-11double bound required for the synthesis of corticosteroids (Pereira et al.,2005). Steroidal sapogenin are available for the derivatives corticosteroids such as prednisone , dexamethasone , triamcinolone, sexual hormones and steroid diuretics (J.Santos, et al 2014).

Sterols, steroidal sapogenins, steroidal alkaloids and alkaloidal amines were derived from Agave sisalana, and these substances derived from plant sources provide the starting material for steroid production (Z. Anwar et al., 2017). In China, Hecogenin and tigogenin from Agave species are used as industrial precursors for the partial synthesis of steroidal drugs. Hecogenin, a saponin of A.sisalana was transformed to cortisone , which defines the process of manufacturing of the cortisone from hecogenin. This has been found in the leaves of several other Agave species and is a possible source of the production of cortisone, but cultivars selected particularly for sisal

fiber show reduced hecogenin. It has also been shown that hecogenin is most abundant (0.235%) in the leaves of the old plant (D. Tewari et al., 2014).

Contraceptive drug such as Anordin and dinordin, prepared with steroids derived from the sisal plants Agave sisilana and Agave Americana have been used for their antifertility effects. These agents, whose anti- fertility properties have been confirmed by scientists in Sweden and the United States, constitute a new family of contraceptives with the great advantage of having to be taken only once or twice a month instead of the 20 times per month necessary with the ordinary pill (Y. Al Jasem et al. 2014).

Steroidal saponins could noticeably nourish the cardiac muscle, slow down platelet aggregation, increase coronary flow, improve peripheral circulation as well as show decline in cholesterol level and triglyceride in blood (Z. Anwar et al., 2017).They often possess properties such as froth forming, hemolytic activity, toxicity to fish, and complex formation with cholesterol. Some of the steroidal saponins isolated recently have been shown to be antidiabetic, antitumor, antitussive, and platelet aggregation inhibitor (Y.Mimaki et al. 2003). Bioactive compounds are essential and nonessential compounds (e.g., vitamins or polyphenols) that occur in nature, are part of the food chain, and can be shown to have an effect on human health (Biesalski et al., 2009).

Generally Steroidal saponins are of great pharmaceutical importance because of their relationship to compounds such as the sex hormones, cortisone, diuretic steroids, vitamin D and the cardiac glycosides (Y.Al Jasem et al. 2014).

2.7.1.Hecogenin

Hecogenin is aglycone or sapogenin which is separated from saponin by hydrolysis. Hydrolysis breakdown the bond between sugar (glycone) and sapogenin (aglycone) (G.Petre et al,2017)**.** In 1989, researchers isolated a mixture of saponins from the sisal juice cultivated. These saponin showed the identical sapogenin (hecogenin) and different sequences on the sugar position in the glycoside chain of each one. The dried fermented residues of leaf-juices and with the fresh leaves from *Agave sisalana* have found new saponins with other sapogenins.

It is used as a base chemical in the manufacture of steroid drugs and hormones. Hecogenin, $C_{27}H_{12}O_4$, a steroidal sapogenin is present in the leaves of many Agave plants along with tigogenin. The ratio of hecogenin to tigogenin varies considerably with the season and the age of the plant. In bulbils and leaves of young plants, tigogenin is the predominant sapogenin, while in leaves of mature plants, hecogenin is predominant. The tigogenin yield from mature leaves is always low, regardless of origin, and is usually 0.1% of the dry weight. Hecogenin is usually high in plants of Africa and generally low in plants of other places (MS. Murthy et al 1981).

In the1940s, steroidal sapogenins achieved great economic importance because of their transformation into pharmaceutically valuable derivatives such as corticosteroids (prednisone, dexamethasone, betamethasone, triamcinolone, and others), sexual hormones, and steroid diuretics.

A previous study has shown sapogenin extraction from plants that are commonly used as food for human and animal. Some investigators have worked on Saponin extraction from agave sisalana. For example researchers (Chigodi, Samoei, & Muthangya, 2013, Pereira, 2005, Debnath Pandey, Sharma, Thakur, & Lal, 2010, Jener David Gonçalves Santos, Vieira, Braz-Filho, & Branco, 2015, (Addisu & Assefa, 2016 &Murthy, Vaiish, & Rajagopalan, 1981) they were investigated on the erxtraction and characterezation of saponin from diferrent plants. However, the aforementioned studies have not studied the effect of saponin extraction parameters and the extraction efficiency. In addition, in our country, *Agave sisalana* has only been used for fiber production and there are no studies on extraction of saponin or sapogenin from the *Agave sisalana* plant leaves for pharmaceutical product starting materials.

2.8. Extraction

Extraction is a process in which one or more components are separated selectively from a liquid or solid mixture, the feed (Phase), by means of a liquid immiscible solvent (Phase 2). The transfer of the components from the feed to the solvent is controlled by the solubility behavior of each component in the corresponding phase.

2.8.1. Method of extraction

- Liquid- Liquid extraction: extraction of solute from liquid phase by using liquid organic solvent. Thus, the saponin extract from sisal leaves juice using ethanol.

- Solid liquid extraction: the extraction of solute from solid materials by using liquid organic solvent. In this method, saponin is extracted from the dried and small sized sisal leaves by using an organic solvent.

2.8.2. Extraction of juice from Sisal extraction

Mechanical pressing: sisal juice is extracted by pressing the fresh Agave sisalana leaves

There are two types simple manual press machine: that are Embtapa sisal juice and unespsisal juice machine.

Embrapa sisal juice press: Steps of Extraction by embrapa sisal juice press

First the reduced sisal enters into a cylindrical shell after preparation the machine for operates and Cover the above part of the cylinder by the plate then closed other open part. Connect to electricity and exerted pressure by using manual preparation method. The juice is collected through a tunnel and pipe at the bottom of the cylinder.

Unesp sisal juice press: First the reduced sisal enters into a cylindrical shell after preparation the machine for operates. Then Cover the above part of the cylinder by the plate and closed other open part. Connect to electricity and exerted pressure by using manual preparation method. The juice is collected through a tunnel and pipe at the bottom of the cylinder.

Steps of extraction by unesp sisal juice press: the extraction process carried out by the same

Principles of operation both pressing machine produce or gives the same amount of Juice.

The advantage of embrapa sisal juice press is Easier for operation, Extracts more juice due to the use of hydraulic press and Easier for transport. Its disadvantage is mechanically its more robust, heavy and operates more slowly.

Advantage of unesp sisal juice press is Lighter, Simple for manufacture and faster to operate.

Its disadvantage is extracts less juice than the embrapa

The selection of the best pressing machine is based on the speed of the operation and machine cost.

2.8.3. Extraction of saponin from *agave sisalana* leaves juice

Extraction techniques employed in saponin extraction includes conventional and green technology.

2.8.3.1. Conventional extraction method

Based on the solubility of solute, it will be extract from plant materials into solvent. So it often utilizes a large quantity of solvent to extract the desired solute and sometimes aide with elevated temperature by heating, and mechanical stirring or shaking. Convectional extraction method has at least three major methods that are maceration, reflux and sucking and subsequent extraction **Maceration:** The extraction of saponins by maceration is the famous method using the ordinary solvent-like alcohols and n-butanol. It is a solid– liquid interface extraction where saponin's compounds inside the plant material can easily extract by immersion or soaking the plant materials in a suitable specific solvent for a period of time with or without stirring or shaking (M.Mohamed et al., 2019).

Reflux and sucking: In sucking extraction, organic components in solid samples are extracted from the matrix by continuously washing the solid with a volatile solvent in a specialized piece of glass war. This is the most common method for extraction of organic compounds from solid samples. Heating solution to boiling point and then returning the condensed vapor to the original flask.

Subsequent extraction: The method is performed on plant materials using two extraction methods (either sucking with maceration or maceration with reflux) subsequently. Using this method may be lead to highly purify the extract before subjecting to analytical analysis for isolation and identification of saponin.

2.8.3.2. Green extraction methods (Modern extraction methods)

Involve less hazardous chemical synthesis, safer chemicals, energy efficiency, and pollution prevention. Example of green extraction methods: micro-wave assisted, ultrasound assisted, accelerated solvent extraction.

Microwave-assisted extraction: Microwaves are non-ionizing electromagnetic waves with a frequency range from 0.3 to 300 GHz. Microwaves are able to penetrate into biomaterials and generate heat by interacting with polar molecules such as water inside the materials. The water content of a plant material is responsible for the absorption of microwave energy which leads to internal superheating and cell structure disruption, and consequently, facilitates the diffusion of bioactive compound from the plant matrix.

Ultrasound-assisted extraction: The phenomenon of ultrasound creates cavitation bubbles in the solvent to denature the plant cell wall when the bubbles collapse at rare fraction resulted in a greater extraction yield of bioactive compounds. Ultrasonic extraction uses ultrasonic vibrations to extract samples with polar solvents in an ultrasonic bath.

Accelerated solvent extraction: It is an automated rapid extraction technique that uses minimal solvent at elevated temperature and pressure. These processes are usually completed in 15–25 min using only 15–45 ml consumption of solvent. Using increased temperature enhances the solubility and mass transfer of solute to solvent, and elevated pressure keeps the solvent below its boiling point, enabling fast, safe, and efficient extraction of material from the plant.

Supercritical fluid extraction and Subcritical water extraction are also included in modern extraction methods.

For laboratory analysis conventional method is best because, it is less cost and easy.

2.8.4.3. Choice organic solvent

Many organic solvents are available for extraction of saponin; for example, buthanol, methanol, ethanol, hexane, acetone, benzene, ethyl acetate, etc. solvents with low solubility in the aqueous phase, high volatility, high purity, Compatibility with choice of chromatographic analysis, Availability, Selectivity, Density difference, Toxicity, Ease of recovery and Cost are usuly preferred. Among the solvents, ethanol is less costly, less toxic, easy to recover, more selective and has good immiscibility with feed.

2.8.5. Isolation methods of sapogenin from glycon

2.8.5.1. Hydrolysis of saponin

Hydrolysis means splitting of a chemical compound into two or more new compounds by reacting with water **.**all natural glycosides are hydrolyses into sugar and other organic compounds by boiling with mineral acid or microorganism (enzyme).separation of sapogenin from saponin is carried out by hydrolysis method.

a. Acid hydrolysis

In organic chemistry, acid hydrolysis is a process in which a aprotic acid is used to catalyze the cleavage of a chemical bond via a nucleophile substitution reaction, with the addition of the

elements of water. The chemical bound of saponine breakdown by using hydrochloric acid or sulfuric acid and boil at specified temperature with specific time .For example saponine hydrolysis by 3M hydrochloric acid at 70^{o}C for 3:00 hr. effectively separated sapogenin and sugar.

b. Enzyme hydrolysis

Enzyme hydrolysis means breakdown of saponine into sapogenin and sugar by using microorganisms (enzymes) due to addition of water. Emulsion, myrosinase, amylase and lipase are type of enzymes which are used for enzyme hydrolysis.

Section of best hydrolysis methods: Acid hydrolysis is selective, because of; it is easy, time saving, effective and easily avoids it by addition of base, neutralization reaction.

c. **Thermolysis:** Thermal degradation of saponin into components of sapogenin and sugar. It is the breakdown of saponin compound by the action of heat.

Chapter Three

3. Materials and method

3.1.1. Saponin preparation

10 kg of sisal leaves were purchased from south Wello Dessie; the leaves were washed using distilled water and then cut into pieces in order to facilitate the subsequent pressing leaves by rotary roller miller. After prepare raw material, 8kg of sisal leaves pressed by roller miller and produced 6L juice.

Saponin extract from sisal juice was carried out with in two 1000 ml conical flask each contained 250ml juice and 450 ml ethanol (97%). The ethanol and sisal juice mixture were waited for 20hr at room temperature. After 20hr, small solid waste materials and precipitate (polysaccharides) were filtered by filter paper. Next, the residual solid materials removal carried out by processes centrifugation. For the centrifugation the liquid of the sample added into 15ml centrifuged tube and it centrifuged at 6000rpm for 10min; the solid waste materials formed cake on the wall of tube and it removed from the tube wall. Then the pure liquid sample measured and it entered into round bottom flask for evaporation of solvent. It concentrated by vacuum rotary evaporator at 50^0C, after ethanol removed obtained 34ml of viscose saponin.

3.1.2. Sapogenin (hecogenin) preparation

Separate sapogenin from sugar carried out by heated saponin with acid solution. First Prepared 3M hydrochloric acid and sodium hydroxide solution; they used for hydrolysis and neutralization respectively. Measured 72 g of hydrochloric acid and it diluted by 500ml distilled water with in 500ml volumetric flask. It used for break down the bound of saponin. After hydrochloric acid solution prepared, next measured 160g of sodium hydro oxide and then it diluted by 1000ml of distilled water with in1000ml, for removed acidity from hydrolyzed sample. To hydrolyze by added 15 ml of hydrochloric acid into 34ml of viscose saponin with in round bottom flask, and

then it refluxed for 2hr and 30min at 70^{o}C. After boiled transferred from rounded bottom flask into cylindrical beaker then,it cooled at room temperature.

Remove acid from hydrolyzed sample added solution of sodium hydroxide solution until the p^{H} reaches neutral; its P^{H} measured by P^{H} meter. During that neutralization process, salt and water produced and salt removed by washed frequently with distilled water. Finally the sapogenin formed cake at the bottom part of cylindrical beaker and its weight was130.9mg of sapogenin (0.77% of 17g of saponin).

3.1.3. Hecogenin Separation

First measured 25 ml of ethyl acetate and it added into 200 mg of powder sapogenin with in 100ml cylindrical beaker; then it waited for 6hr. Finally it entered into 100ml round bottom flask and ethyl acetate removed by evaporation at 45^{0}C.

3.2. Determination of the amounts of saponin from different *Agave sisalana* species, by volumetric analysis

After collects, the different agave sisal leaves species from S. Wello, washed with distilled water. Next, extracted its juice, by mechanical pressed and collected within three cylindrical beakers, then added equal amount of ethanol and waited for 20hr.

The other procedure of saponin extraction was similar to the above saponin extraction method

3.3. Determination of Saponin moisture, ash, and extracted value of Saponin

3.3.1. Moisture content

Moisture content is the amount of liquid moisture mass found in the total mass of the saponin. Moisture content of saponins was determined by using weight difference method (initial and final weight difference method). The known weight of wet saponin was dried in oven dryer for a time of 5:00hr, at temperature of 105ºC. The dried sample was weighed. The difference between the mass of wet and dry sample is the moisture content of saponin.

Moisture content (%) $= \left(\frac{loss\ in\ weight}{weight\ of\ sample}\right) \times 100$ ……………………………………….. (3.1)

3.3.2. Ash content of saponin

Ash content represents the inorganic residue (minerals) remaining after ignition and complete oxidation of organic matter. The total ash content equals the weight of the ash divided by the weight of the original sample multiplied by 100%.

Weighted 5g of saponin and taken with cleaned porcelain dishes, it weighed accurately. Hot air oven method was applied to remove the moisture. Then the sample would burn on a gas burner. This was done to prevent the loss of sample in the muffle furnace under higher temperature. Then sample was transferred into the muffle furnace and burn for (4.5 hr), at a temperature of 500 ^{o}C and ignited until light gray ash resulted. The sample was then cooled in a desiccator and weighed.

$$\text{The ash content (\%)} = \frac{weight\ of\ residule}{weight\ of\ sample} \times 100 \quad \text{.....(3.2)}$$

3.3.3. Extract Yield of saponin

The extraction yield is a measure of the solvent's efficiency to extract specific components(saponin) from the original material (*Agave sisal* juice) and it was defined as the amount of extract recovered in mass compared with the initial amount of the sisal juice. It is presented in percentage (%).The dry extract obtained after filtration and evaporation of the solvent then weighed to obtain the extraction yield.

$$\text{Extraction yield (\%)} = \frac{\text{driedd weigt of saponin after evaporation by rotary vacuume evaporator}}{\text{total weight of samle (agave sisalana juice)}} \times 100 \text{.. (3.3)}$$

3.4. Qualitative characterization of saponin

Saponin qualitatively characterized based on their foaming and hemolysis activities

3.4.1. Determination of saponin foaming properties

First measured 2g of saponin and 20ml of distilled water, then both saponin and distilled water added into 50ml conical flask. After added, shaken the mixture of water and saponin vigorously for 30sec and it produced foam. Next put the flask on the table and observed the results for 30min.

3.4.2. Leiberman –burchard steroid test

Measured 3ml of acetic anhydride and 0.8 g of saponin, then mixed them. Next measured 3ml of sulfuric acid, and added it into the mixture of acetic anhydride and saponin.

3.5. Characterization of steroidal saponin (sapogenin) by Analytical method

The analytical instruments, such as Nuclear magnetic resonance (NMR), UV-Spectroscopy photo meter and FTIR spectroscopy are used to characterized sapogenin.

Nuclear magnetic resonance13_CNMR: Determines the number of carbon skeletons, and the number of signals that indicates number of carbons content in the materials. UV-spectroscopy is used to identify the compounds by their wave length. And analysis the presence of steroidal saponin based on functional group peaks, which obtained from the result of FTIR Spectroscopy or NMR.

3.6. Experimental Design and Data Analysis

The input variables effect on the yield of extraction (saponin) and hydrolysis (steroidal saponin or sapogenin) were analyzed by design experiment using deign expert 7.o. In this study, Design expert7.0.0, software experimental design method, full factorials design was available to determine the effect of the two operating variables of the extraction of saponin from agave sisalana leaves juice and three operating variables of saponin acid hydrolysis. These operating variables of saponin extraction were, extraction time and solvent to materials ratio, while the operating variables of saponin acid hydrolysis were, hydrolysis time, temperature, and acid concentration.

saponin start to extract and solvent enter into inner part of material at the time of 15 hr and 0.5 (solvent to material ratio) (C. Mosafi et al, 2017).During hydrolysis of saponin, at $60^oC$with 2M start to break down the bond between sapogenin and sugar (F.G.Lonard et al, 2003)

Table 3.1: Factors of saponin extraction, maceration extraction method

Factors	level		
Time(hr)	15	20	25
Solvent to material ratio	0.5	1	1.5

Table 3.2: Factors of saponin acid hydrolysis

Factors	level		
Time(hr)	1:30	2:30	3:30
Acid conce.(M)	2	4	6
Temperature (^{o}C)	60	70	80

- Acid concentration expressed by molarity. M=mole (n)/ volume (L)

Chapter four

4. Results and Discussion

4.1. Moisture content of steroidal saponin

The Moisture content was determined using equation (3.1) and is presented in Table 4.1.

As illustrated in the table 4.1, the moisture content of saponin varieties was conducted in triplicates and the average moisture content was taken as mean . From table 4.1 the result shown, 69.5 %(w/w) of saponin(before dry).

Table 4.1: Moisture content of saponin

No.	Saponin before drying(g)	Saponin after dry(g)	Loss (g)	Moisture content (%)	Moisture content average (%)
1	2	0.62	1.38	69	69.5%
2	2	0.6	1.4	70	
3	2	0.61	1.39	69.5	

4.2. Saponin ash content

The ash content was calculated using the equation (3.2), it was performed in triplicates. Ash content of the saponin shown in the table 4.2.

Table 4.2 : Ash content of saponin

No.	Sample weight(g)	Residue weight(g)	Ash content (%)	Mean (%)
1	2	0.23	88.5	89
2	2	0.21	89.5	
3	2	0.22	89	

4.3. Saponin extracted value

The extracted value of saponin was calculated based on equation (3.3) saponin extracted weighted divided by the sample weighted. Table 4.3, shown the percentage of saponin extracted value.

Table 4.3: Extracted value of saponin (for 200gof agave sisalana leaves)

Run	Amount of Extracted saponin(g)	Percentage of extracted saponin (%)
1	11.48	5.7
2	16.4	8.2
3	14.80	7.4
4	15.20	7.6
5	16.2	8.1
6	13.20	6.6
7	15.60	7.8
8	14.4	7.2
9	17	8.5

4.4. Qualitative characterization result of saponin

4.4.1. Saponin conformation Foam test

After mixing saponin with the distilled water vigorously shacked, then foam formed and it remained for 30 min without changing its amount. The saponin foam was stable for 30 min so, it indicates the sample contains saponin.

4.4.2. Leibermann –burchard steroid test

After mixing acetic acid anhydride, sample of saponin and sulfuric acid, the color changed from violent to blue green. The color change indicates the presence of steroids in the sample (Verma et al., 2013).

4.5. Determination of the amounts of saponin from different *Agave sisalana* leaves species

At least 200 species of agave sisalan plants exist in the world. In Ethiopia there are 3 types of sisal species. Their local names are nechi Algae (one), wende Algae (two) and set Algae (three).Their difference is based on fiber content and strength, degree of skin irritation, leaves size and color. The above information were taken from local farmer and Ethiopian researchers, they study on the growth of different agave species (Asfawu et al, 2011)and(S. Sulaiman et al.,2014). The amount of saponin was determined from each Agave leaves by measuring the content of saponin after extraction by ethanol and concentrating with vacuum rotary evaporator.

Table 4.4: Content of saponin in different sisal plants

No.	Type of agave sisalana	Weight of sisal leaves peels (g)	weight of sisal leaves juice (ml)	Solvent (ethanol ml)	Extraction time (hr)	Concentrating time (hr)	Amout of saponin (ml)
1	Agavesisalana leaves (wende Algae)	250g	115	500	20	50min	7.5
2	Agavesisalna leaves (nechi algae)	250g	150	500	20	1hr -1.5hr	4
3	Agavesisalana leaves (set algae)	250g	100	500	20	1hr-1.25hr	1.5

Agave sisalana one (wende Algae); from the above Table4.4, wende Algae leaves have more saponin content than the other. Agave sisalana two (nechi algae); nechi algae contained more awater but less amount of saponin than wende Algae. Agave sisalana three (set algae); it does not have the ability of irritating human body. It contain water, sugar precipitate and other solid materials.

Generally, from the above three species, agave sisalana one or by its local name" wende algae" contains more saponine from the rest. Therefore, it is recommended for production of active pharmaceutical ingredient (API). Next to agave sisalana one, by its local name nechi algae is good for production of pharmaceutical staring materials.

4.6. Optimazation of extraction condition

Time of extraction, solvent to material(s/material) ratio were the main factors during maceration extraction at constant temperature**.** the levels of time: 15hr, 20hr and 25hr Solvent to material ratio : 0.5, 1, and 1.5. Large ratio of extraction solvent to materia leands to un necessary waste, while small ratio leads to incomplet extrction of saponin (K.Cheng et al, 2017). the exprimental runs are shown in Table 4.5.

Table 4.5 : Experimental results of saponin extraction

	Factor 1	Factor 2	Response 1
Run	A: time(hr)	B:s/material	Saponin(%)
1	15.00	0.50	5.7
2	25.00	1.50	8.2
3	25.00	0.50	7.4
4	20.00	0.5	7.6
5	20.00	1.00	8.1
6	15.00	1.00	6.6
7	25.00	1.00	7.8
8	15.00	1.50	7.2
9	20.00	1.50	8.5

Table 4.6: ANOVA for Response Surface Quadratic ModelAnalysis of variance

Source	Sum of Squares	df	Mean Square	F-Value	P-value Prob > F	Remark
Model	6.20	5	1.24	85.80	0.0020	significant
A- time	2.67	1	2.67	184.62	0.0009	
B-s/material	1.82	1	1.82	125.65	0.0015	
AB	0.090	1	0.090	6.23	0.0880	
A^2	1.62	1	1.62	112.15	0.0018	
B^2	5.000E-003	1	5.000E-003	0.35	0.5977	
Residual	0.043	3	0.014			
Cor Total	6.24	8				

The Model F-value of 85.80 implies the model is significant. There is only a 0.20% 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 (time), B (solvent to material rato), A^2 (quadratic term of time) aresignificant model terms. A^2 indicates Selfi interaction effect of time affects extraction of saponin while the value of B^2 indicates the yield of saponin not affected by the selfi interaction effect of solvent to material ratio.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). From the above table time and solvent to materials has significant effect on extraction of saponin. There p- value 0.0009 and 0.0015 respectively. so, time and solvent to material ratio are directly affects saponin extraction from *agave sisalana* juice.

Table 4.7: ANOVA model effectiveness measurement

Std. Dev.	0.12	R-Squared	0.9931
Mean	7.47	Adj R-Squared	0.9815
C.V. %	1.61	Pred R-Squared	0.9153
PRESS	0.53	Adeq Precision	28.703

The "Pred R-Squared" of 0.9153 was in reasonable agreement with the "Adj R-Squared" of 0.9815. The "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 28.703 indicates an adequate signal. This model can be used to navigate the design space.

R-squred 0.9931 means 99.31% of the variation in the out put (yield of saponin) affected or explained by the input variables (time of extraction and solvent to materials ratio).

Adj-Rsqured and pred R-squred are with in in agreement , because the differene between them is less than 0.2.

Table 4.8: Confidence interval and coefficient estimate of extraction parametrs

Factor	Coefficient estimate	df	Standard Error	95% CI Low	95% CI high	VIF
Intercept	8.10	1	0.090	7.81	8.39	
A- time	0.67	1	0.049	0.51	0.82	1.00
B-s/material	0.55	1	0.049	0.39	0.71	1.00
AB	-0.15	1	0.060	-0.34	0.041	1.00
A^2	-0.90	1	0.085	-1.17	-0.63	1.00

4.6.1. Development of Model equation

Full factorial application offers an empirical relationship between the response function and the independent variables. The mathematical relationships between the response (saponin) and the independent variables time of extraction (A) and solvent to materials ratio (B) in terms of coded and actual factors can be determined by Design Expert software. The model equation that

correlates the response (Y or saponin yield) to the extraction process variables in terms of coded factors after excluding the insignificant terms was given in equation.

Final Equation in Terms of Coded Factors:

Yield of saponin =(8.10+0.67 * A)+(0.55* B) -(0.15 * A * B)–(0.90*$A^{2)}$-(0.050*$B^{2)}$……. (4.1)

Based on this regression model equation which is explained by coded factor terms ,the response yield was affected by linear terms time of extraction (A), and solvent to materials ratio (B) and, quadratic terms ($A^2$and B^2) and interaction quadratic terms (AB) was insignificant. According to the coefficients in equations, it was evident that the percentage of response yield increases with the extraction time (A) until optimum point(20hr) and solvent to material ratio (B). Time and solvent material ratio have positive linear effect on extraction yield. The Interaction effects of time of extraction and solvent materials ratio (AB) has negative effect on the response yield.

Final Equation in Terms of Actual Factors:

Yieldofsaponin=(-11.46667)+(1.63333*time)+(2.70000*s/material)-(0.060000*time*s/material) -(0.036000*$time^{2)}$0.20000*$s/material^2$…….(4.2)

4.6.2. model adequncey

The model was tested for adequacy by analysis of variance (ANOVA). The model of regression was found to be reasonably significant with the correlation coefficients of determination of R-Squared, adjusted R-Squared and predicted R-Squared having a value of 0.9931, 0.9815 and 0.9153 respectively. The quality of the model developed could be evaluated from their coefficients of correlation. The value of R-squared for the developed correlation is 0.9931; this means that 99.31% of the total variation in the yield of saponin was attributed by the experimental variables studied. The “Pred R-Squared” is 0.9153, it is close to the "Adj R-Squared" of 0.9815 the difference between them is less than 0.2 so that is acceptable difference. The “Adeq Precision" measures the signal to disturbance ratio due to random error; a ratio greater than fouris acceptable. The yield of saponin has ratio of 28.703, which indicates an adequate signal. Therefore, this model can be used to navigate the design space.

Table 4.9: Actual and preidcted values of saponin yield

Std. order	Actual value	Predicted value	residual	levera nge	Internally Studentized residaual	Externally Studentized residual	Influenc e Fitted Value DFFITS	Cook's Distance
1	5.70	5.78	-0.083	0.806	-1.572	-3.062	* -6.23	* 1.71
2	7.60	7.50	0.10	0.556	1.248	1.470	1.643	0.325
3	7.40	7.42	-0.017	0.806	-0.314	-0.261	-0.531	0.068
4	6.60	6.53	0.067	0.556	0.832	0.775	0.866	0.144
5	8.10	8.10	0.000	0.556	0.000	0.000	0.000	0.000
6	7.80	7.87	-0.067	0.556	-0.832	-0.775	-0.866	0.144
7	7.20	7.18	0.017	0.806	0.314	0.261	0.531	0.068
8	8.50	8.60	-0.100	0.556	-1.248	-1.470	-1.643	0.325
9	8.30	8.22	0.083	0.806	1.572	3.062	* 6.23	* 1.71

∗Exceeds limits.

- Actul value : actul value is the value that is obtained by observation or by mearuring the available data . It s also called observed value. So, this actual value is measured value from expremental results.
- Predicted values : the predicted value is the value of the variables , predicted based on the regression analysis.
- Residual: the residual value is the difference between the actual or observed value and the predicted value.Residual = Observed – Predicted ,positive values for the residual (on the y-axis) mean the prediction was too low, and negative values mean the prediction was too high; 0 means the guess was exactly correct.
- Leverage: Leverage is a measure of how far an independent variable deviates from its mean. These leverage points can have an effect on the estimate of regression coefficients.

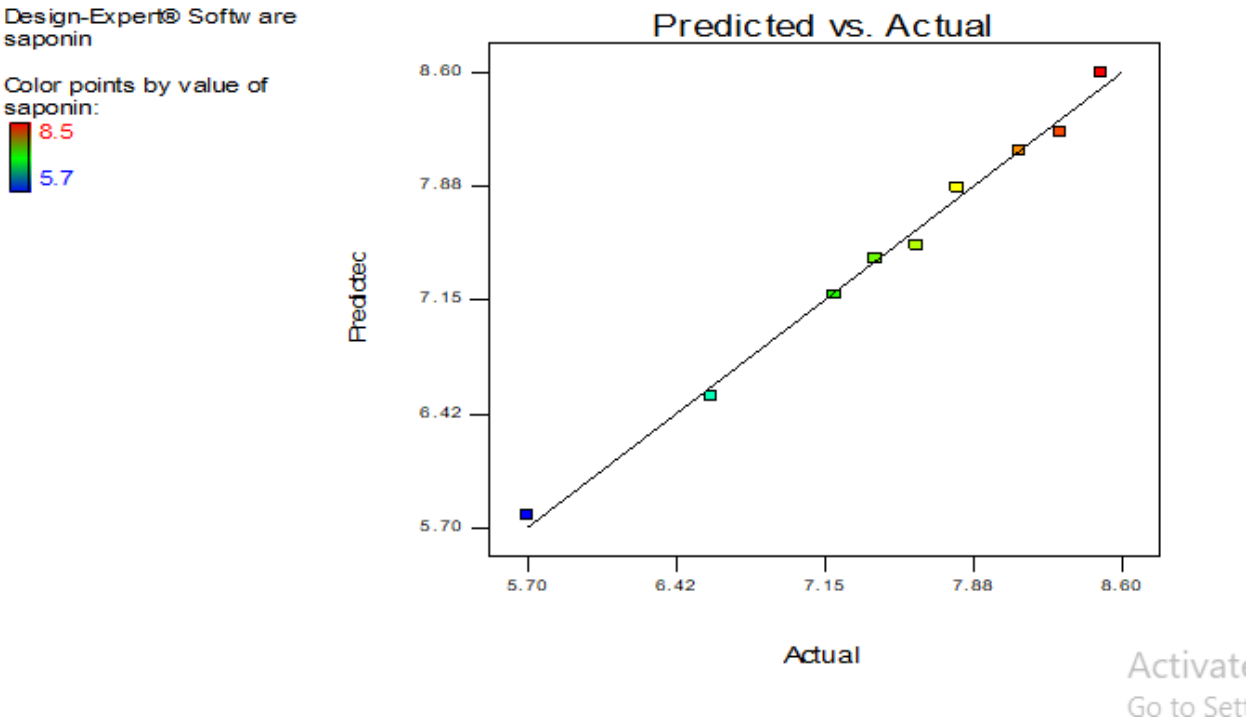

Figure 4-1: Actual vs predictd value of saponin

The graph of the above shows that the model is accurate, as there's a strong correlation between the model's predictions and its actual results.

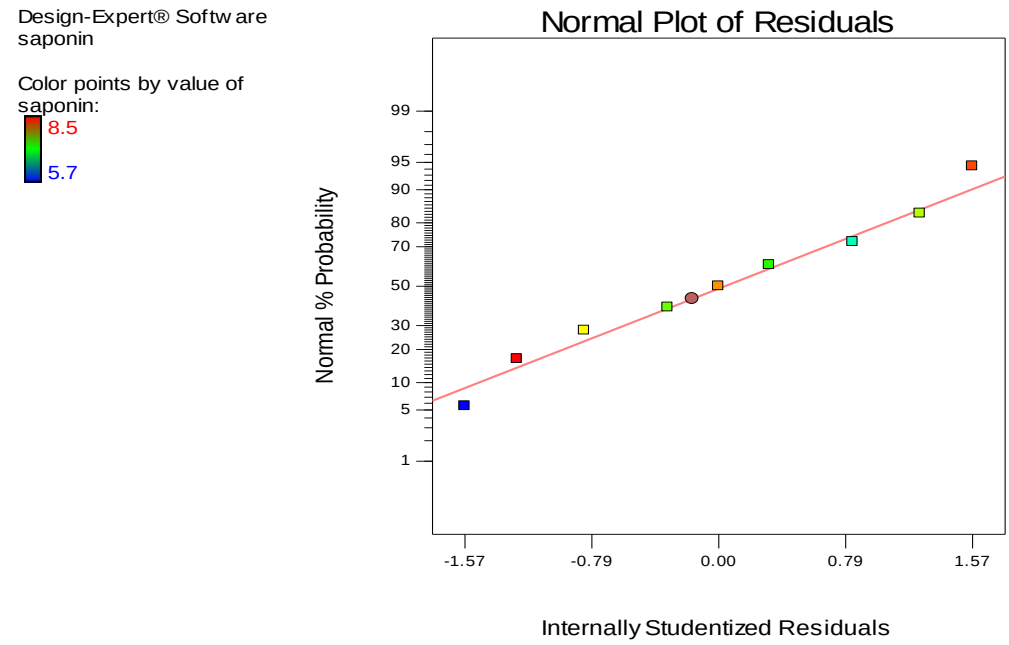

Figure 4-2: Internally studentized residuals vs normal %of probability

A normal probability plot allows us to check that the errors are normally distributed. It plots the residuals against the expected value of the residual as if it had come from a normal distribution.

4.6.3. Effect of extraction time on saponin yield

The effect of extraction time was significant, its p- value from the Table 4.6 is 0.0009 this is less than 0.05.

The extraction of saponin from the sisal leaves juice with organic solvent is liquid liquid extraction type. So, it does not needed extraction time above 25hr. From Figure 4.3 one can see that as the extraction time increased from 15hr up to 25hr, the yield of saponin increased, but gradually the yield decreased with small difference. The reason, why the yield of saponin decreased may be due to the dissolution of other unwanted materials and the saponin returns to the original materials or processed reverse dissolve. At 20hr, I obtained maximum yield, but after 20hr the yield of saponin was decrease (K.Cheng et al, 2017).

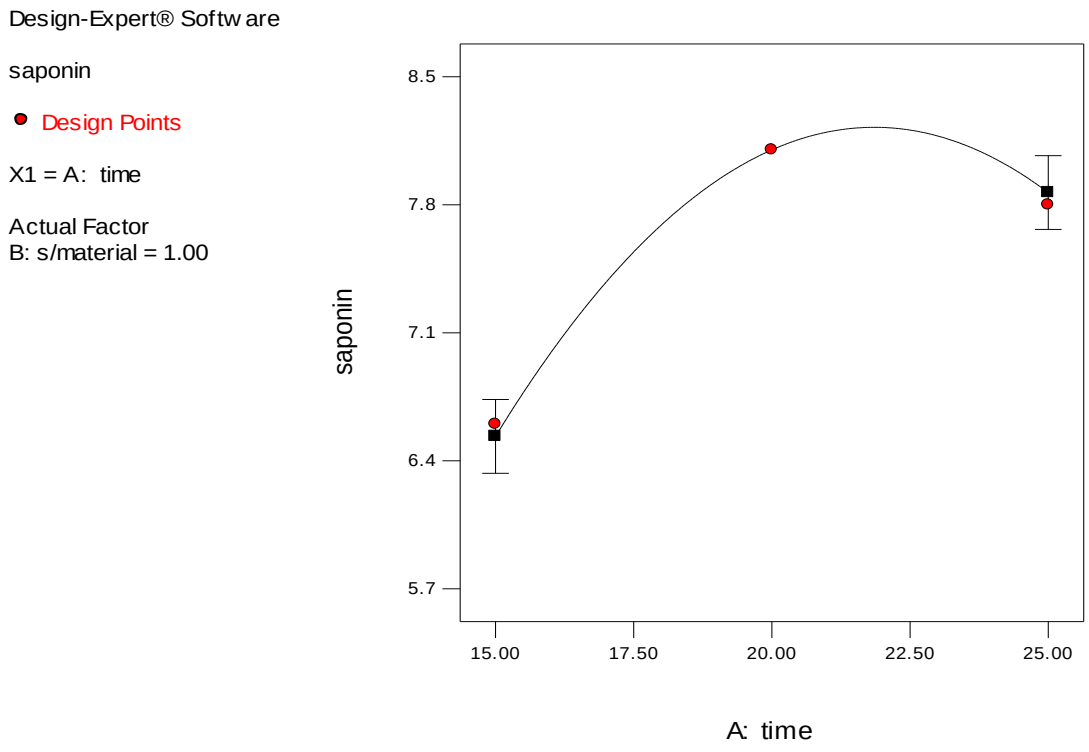

Figure 4-3: Effect of extraction time on saponin yield

4.6.4. Effect of solvent to material ratio on saponin yield

The yield of saponin increased with increased in ratio from 0.5 to 1.5. Higher amount of saponin extracted, when organic solvent amount increased, but it required higher energy for removed solvent by rotary evaporator, it takes more time of evaporation.

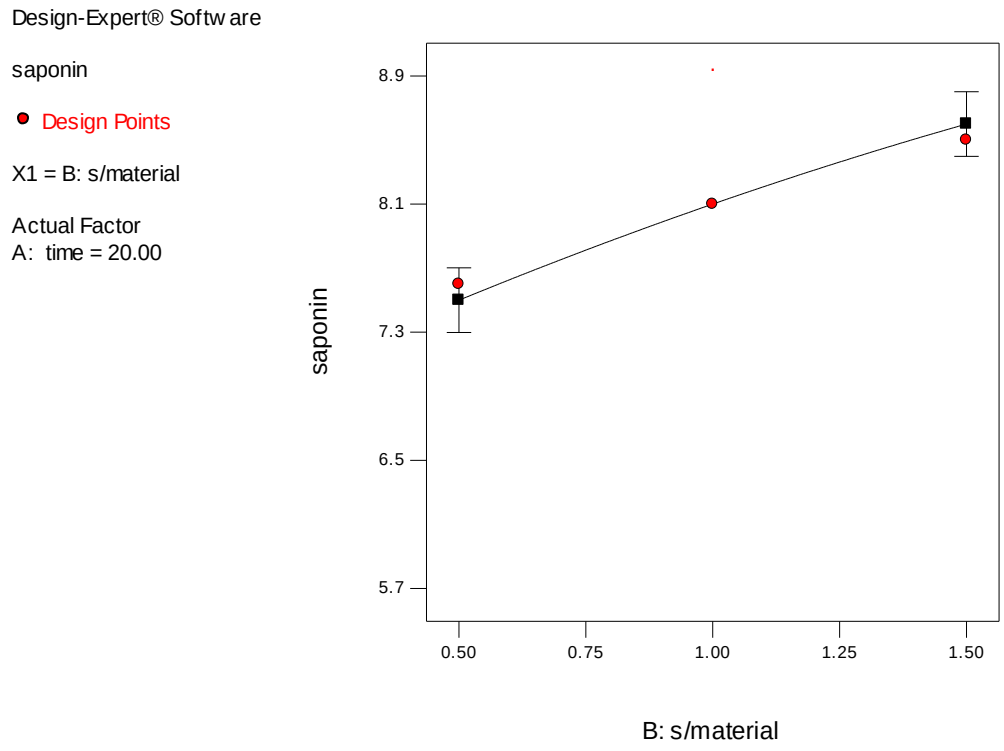

Figure 4-4: Effect of solvent to material ratio on saponine yield

The reason why 1.5 ratios was selective, at this ratio obtained good result because, it has the ability to extract saponin by penetrated the materials and entered into inner part, and then dissolved the solute. Above 1.5 its cost was higher and below 1.5 obtained small amount of saponin yield. Extract by 1.5 ratios, not difficult to pass the outer parts of the materials. According to thus, used higher amount of solvent was not recommended. Optimum amount of Saponin yield obtained at the ratio of 1.5.

4.6.5. Interaction effect of time and solvent to material ratio

The Interaction effects of extraction time and solvent material ratio on saponin extraction rate are very low; its p-value is greater than 0.05, so the interaction was insignificant. Figure 4.5, 4.6 and 4.7 show the interaction effect of time and solvent to materials ratio on the yield of saponine. As shown from the figures, increase both time and solvent to material ratio leads to increase the yield of saponin but gradually decrease the yield.

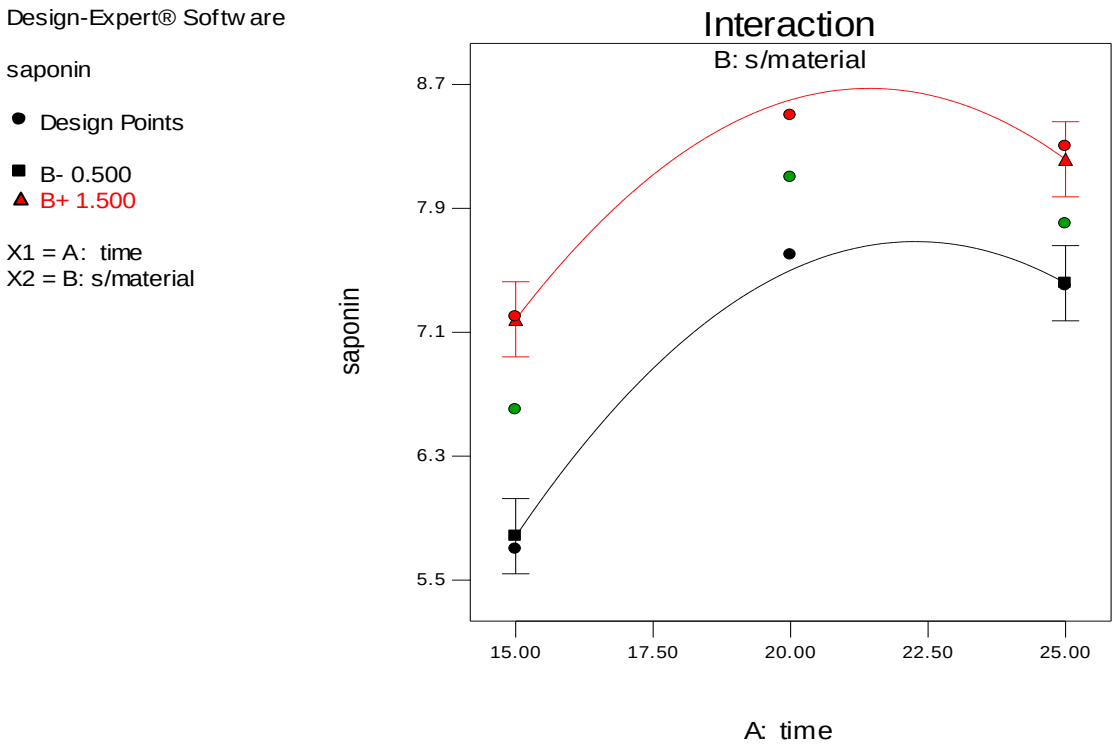

Figure 4-5: The interaction effect of time and solvent to material ratio on saponin

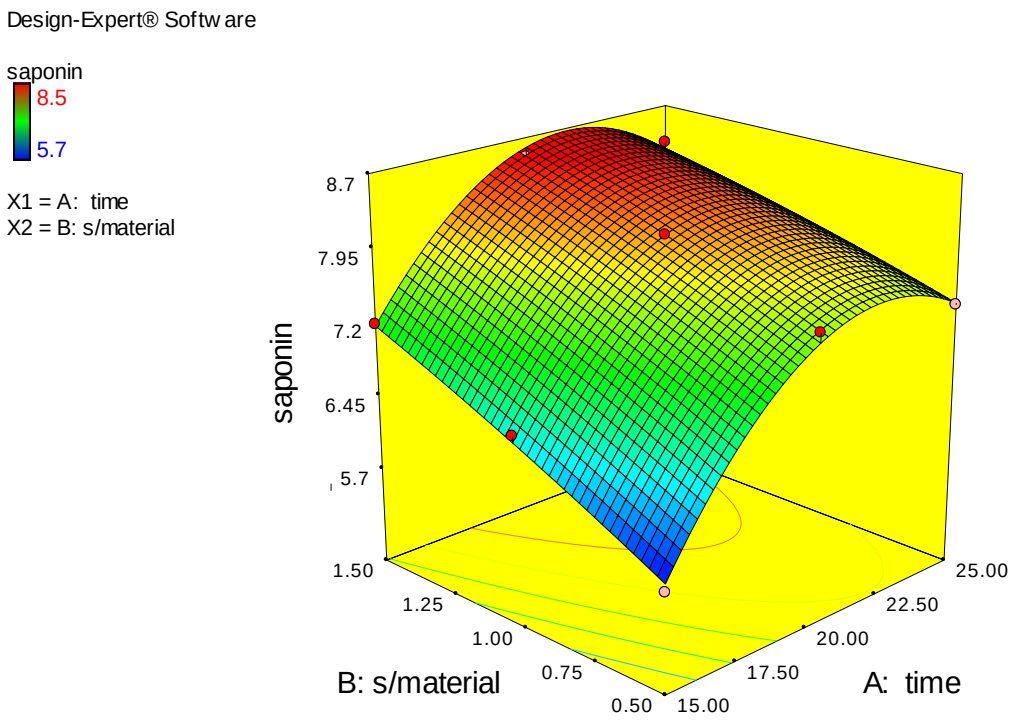

Figure 4-6: solvent to materials ratio and time interaction effect on saponin yield 3D graph

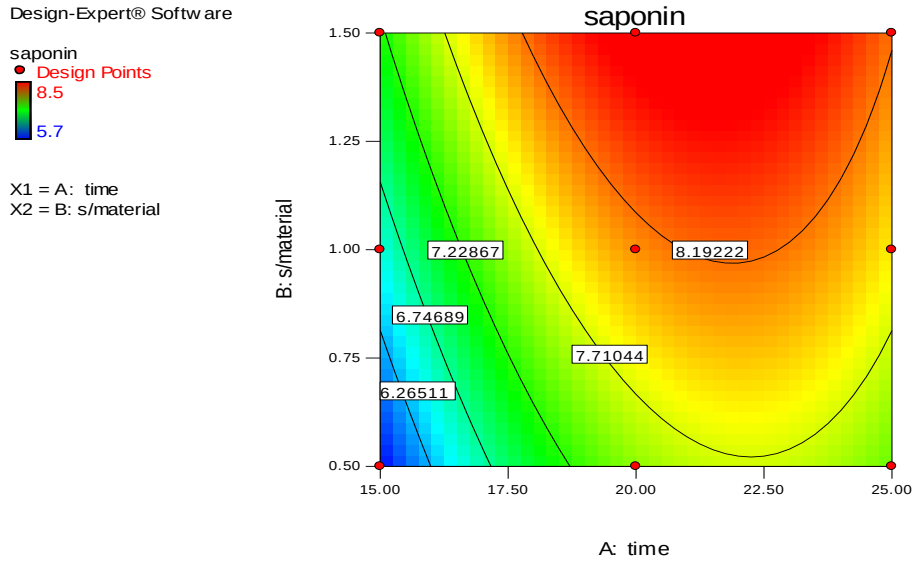

Figure 4-7: interaction effect of time and solvent to materials ratio on saponin yield

4.7. Saponin hydrolysis

The chemical structure of saponins, as shown in chapter two in figure2.2, they are the combination of sugar molecule and sapogenin molecule. The bond between sapogenin(aglycone) and sugar molecule was break down by heating with hydrochloric acid.

4.7.1. Determination of the Effects of Hydrolysis Parameters

Saponin hydrolysis parameters are, time of hydrolysis, temperature and acid concentration. Saponin hydrolysis at Temperature of 60^0C required high acid concentration and longtime of hydrolysis ,on the other hand hydrolysis at the higher temperature of 90^0C and100^0C required low acid concentration and short time(A.S. Cheok et al.,2013). The samples were treated at temperature of 60^0C, 70^0C and 80^0C .It used Acid concentration of 2M, 4M, and 6M and at the time of 1.5hr, 2.5hr, and 3.5 hr.

Table 4.10: Runs of saponin hydrolysis expremental data

Run	A: time (hr)	B:acid conc.(M)	C:temperature (^{0}C)	Steroidal saponin(%) (sapogenin)
1	2.30	4.00	60.00	0.64
2	1.30	2.00	70.00	0.65
3	1.30	2.00	80.00	0.68
4	1.30	2.00	60.00	0.4
5	1.30	4.00	80.00	0.62
6	3.30	2.00	70.00	0.68
7	1.30	4.00	70.00	0.66
8	1.30	6.00	60.00	0.55
9	3.30	2.00	60.00	0.45
10	2.30	2.00	60.00	0.52
11	3.30	4.00	60.00	0.6
12	2.30	4.00	80.00	0.74
13	2.30	6.00	70.00	0.72
14	3.30	2.00	80.00	0.7
15	3.30	4.00	70.00	0.73
16	1.30	4.00	60.00	0.52
17	2.30	2.00	70.00	0.71
18	2.30	6.00	80.00	0.67
19	1.30	6.00	80.00	0.55
20	3.30	6.00	70.00	0.68
21	3.30	6.00	60.00	0.63
22	3.30	4.00	80.00	0.7
23	2.30	6.00	60.00	0.68
24	2.30	4.00	70.00	0.77
25	1.30	6.00	70.00	0.61

Table 4.11 : Analysis of varinece (anova) analysis table

	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	0.21	9	0.023	67.94	< 0.0001	Significant
A- time	0.018	1	0.018	53.76	< 0.0001	
B- acid conc.	2.450E-003	1	2.450E-003	7.30	0.0151	
C- Temperature.	0.060	1	0.060	178.98	< 0.0001	
AB	1.633E-003	1	1.633E-003	4.87	0.0415	
AC	3.333E-005	1	3.333E-005	0.099	0.07565	
BC	0.046	1	0.046	135.92	< 0.0001	
A^2	0.032	1	0.032	94.66	< 0.0001	
B^2	9.335E-003	1	9.335E-003	27.81	< 0.0001	
C^2	0.036	1	0.036	108.11	< 0.0001	
Residual	5.707E-003	17	3.357E-004			
Cor Total	0.21	26				

The Model F-value of 67.94 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, AB, BC, A^2, B^2, $C^2$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).

Table 4.12: ANOVA model effectiveness measurment

Std. Dev.	0.018	R-Squared	0.9730
Mean	0.64	Adj R-Squared	0.9586
C.V. %	2.87	Pred R-Squared	0.9268
PRESS	0.015	Adeq Precision	30.740

The "Pred R-Squared" of 0.9268 is in reasonable agreement with the "Adj R-Squared" of 0.9586."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 30.740 indicates an adequate signal. This model can be used to navigate the design space. As shown on table 4.12, the model is significant because the value of R-squred, Adj R-squared, pred R-squred and Adeq precision are acceptable.

R-squred value of 0.9730 means 97.30 % of the variation in the out put variable was explained by the in put variable (time , temprature and acid concentartion) ; or 97.3% of sapogenin yiled affected by the in put variable of temperature , time and acid concentration.

Table4.13: Confidensal interval and variables coeffcient estimate of saponin hydrolysis

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	0.76	1	9.330E-003	0.75	0.78	
A- time	0.032	1	4.319E-003	0.023	0.041	1.00
B- acid conc.	0.012	1	4.319E-003	2.555E-003	0.021	1.00
C- Temperature.	0.058	1	4.319E-003	0.049	0.067	1.00
AB	0.012	1	5.289E-003	5.071E-004	0.023	1.00
AC	-1.667E-003	1	5.289E-003	-0.013	9.493E-003	1.00
BC	-0.062	1	5.289E-003	-0.073	-0.051	1.00
A^2	-0.073	1	7.480E-003	-0.089	-0.057	1.00
B^2	-0.039	1	7.480E-003	-0.055	-0.024	1.00
C^2	-0.078	1	7.480E-003	-0.094	-0.062	1.00
Coefficient	Standard	95% CI	95% CI			

4.7.2. Development of Model equation

The application of RSM offers an empirical relationship between the response function and the independent variables. The mathematical relationships between the response (steroidal saponin) and the independent variables time of hydrolysis (A), acid concentration (B) and temperature(C) in terms of coded and actual factors can be determined by Design Expert software. The model equation that correlates the response (Y or steroidal saponin or sapogenin) to the hydrolysis reactions variables in terms of coded factors after excluding the insignificant terms was given in equation.

The model equation that correlates the response (yield of steroidal saponin) to the hydrolysis process variables (time, temperature, and acid concentration) in terms of actual value after

excluding the insignificant terms was given below. The predicted model for percentage of yield of steroidal saponin or sapogenin in terms of the coded factors is given in equation (4.1).

Final Equation in Terms of Coded Factors:

steroidal saponin =+0.76+0.032 * A+0.012 * B+0.058 * C+0.012 * A * B

-1.667E-003 * A * C-0.062* B * C-0.073 * A^2-0.039 * B^2-0.078*C^2……...equation 4.3 a

The importance of the model developed equation was evaluated from their coefficients of correlation. As shown in the final equation in terms of coded factors, the response yield was affected by both linear terms (A, B, C) and quadratic terms (A^2, B^2, and C^2) and interaction quadratic terms (AB and BC). All coefficients of linear terms were positive and the response yield was positively affected by linear terms but the coefficients of interaction terms were negative, accepts AB and the response yield were negatively affected by quadratic terms.

Final Equation in Terms of Actual Factors:

Steroidalsaponin=(4.92618)+(0.35478*time)+(0.28714*acidconc.)+(0.12738*temprature).+(5.83333E)- (003*time * acid conc.)- (1.66667E-004* time * temprature.)- (3.08333E-003* acid conc.*temprature).-(0.072778*time$^{2)}$-(9.86111E-003*acidconc.$^{2)}$-(7.77778E)-004*temperature^2……equation 4.3b

4.7.3. Model adequacy

The model was tested for adequacy by analysis of variance (ANOVA). The model of regression was found to be reasonably significant with the correlation coefficients of determination of R-Squared, adjusted R-Squared and predicted R-Squared having a value of 0.9931, 0.9815 and 0.9153 respectively. The quality of the model developed could be evaluated from their coefficients of correlation. The value of R-squared for the developed correlation is 0.9931; this means that 99.31% of the total variation in the yield of saponin was attributed by the experimental variables studied. The “Pred R-Squared” is 0.9153, it is close to the "Adj R-Squared" of 0.9815 the difference between them is less than 0.2 so that is acceptable difference. The “Adeq Precision" measures the signal to disturbance ratio due to random error; a ratio greater than four is acceptable. The yield of saponin has ratio of 28.703, which indicates an adequate signal. Therefore, this model can be used to navigate the design space.

Table 4.14: Actual and Predicted valus of steroidal saponin (sapogenin)

Actual Value	Predicted Value	Residual	Leverage	Internal studentazed residual	Externally Studentized Residual	Run Order
0.40	0.42	-0.022	0.509	-1.717	-1.832	4
0.52	0.52	3.519E-003	0.343	0.237	0.230	10
0.45	0.47	-0.015	0.509	-1.197	-1.214	9
0.52	0.52	-3.148E-003	0.343	-0.212	-0.206	16
0.64	0.63	0.011	0.259	0.681	0.670	1
0.60	0.59	0.010	0.343	0.686	0.674	11
0.55	0.55	4.630E-003	0.509	0.361	0.351	8
0.68	0.66	0.017	0.343	1.134	1.145	23
0.63	0.64	-5.370E-003	0.509	-0.418	-0.408	21
0.65	0.62	0.029	0.343	1.957	2.157	2
0.71	0.71	-3.704E-003	0.259	-0.235	-0.228	17
0.68	0.66	0.019	0.343	1.284	1.311	6
0.66	0.66	-3.704E-004	0.259	-0.023	-0.023	7
0.77	0.76	5.185E-003	0.259	0.329	0.320	24
0.73	0.72	6.296E-	0.259	0.399	0.389	15

		003				
0.61	0.62	-0.011	0.343	-0.735	-0.725	25
0.72	0.74	-0.017	0.259	-1.080	-1.086	13
0.68	0.71	-0.028	0.343	-1.857	-2.018	20
0.68	0.66	0.016	0.509	1.226	1.246	3
0.73	0.76	-0.025	0.343	-1.708	-1.820	27
0.70	0.70	-9.259E-004	0.509	-0.072	-0.070	14
0.62	0.64	-0.022	0.343	-1.483	-1.542	5
0.74	0.74	-4.815E-003	0.259	-0.305	-0.297	12
0.70	0.70	-2.037E-003	0.343	-0.137	-0.133	22
0.55	0.54	9.074E-003	0.509	0.707	0.696	19
0.67	0.66	0.015	0.343	0.985	0.984	18
0.64	0.62	0.016	0.509	1.226	1.246	26

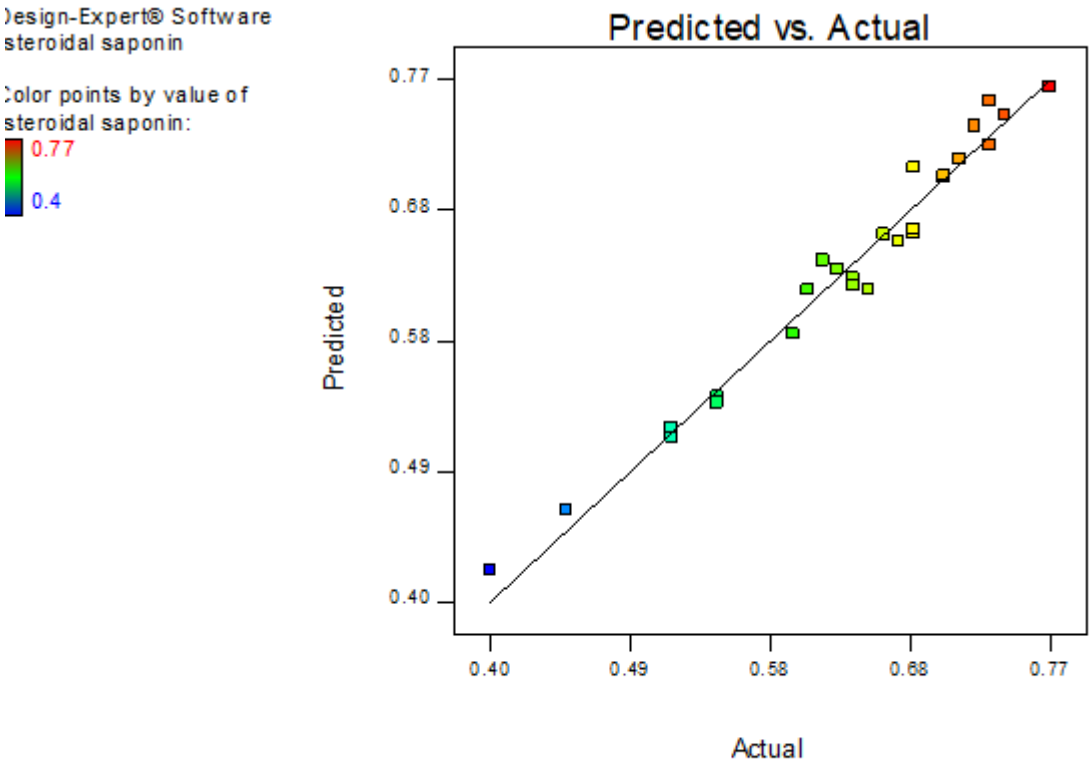

Figure 4-8: comparsion of actual and predicted value of steroidal saponin

The figure 4.8showns that the model is accurate as there's a strong correlation between the model's predictions and its actual results.

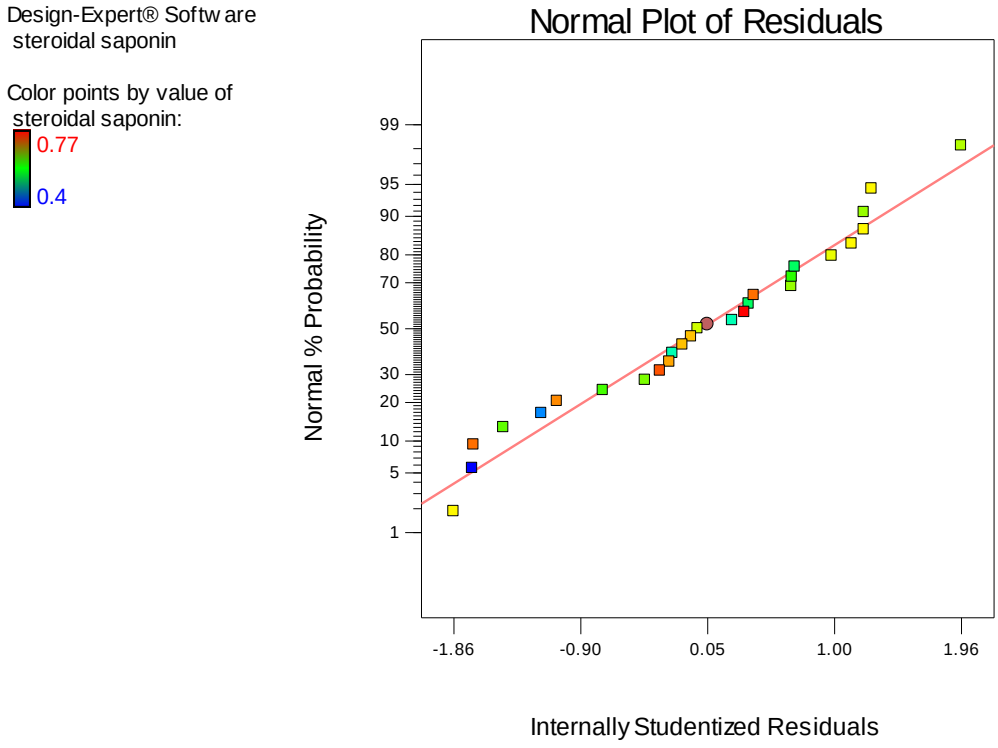

Figure 4-9: Internally student zed residuals and normal % probability graph

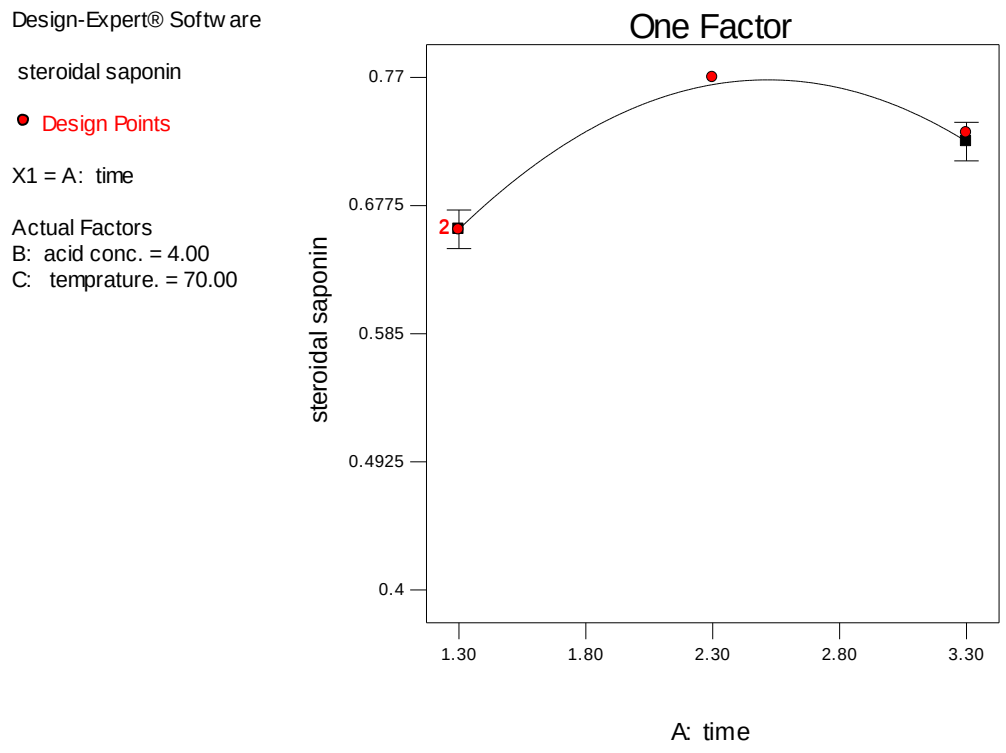

Figure 4-10: Effect of hydrolysis time on steroidal saponin yield

4.7.4. Effect of hydrolysis time on steroidal saponin

The yield of sapogenin increased with increase in time, but decreased after 2:30 hr. For example sapogenin at 1:30hr, 1:80hr, 2:30hr and 2:80hr produced 0.4%, 0.4925%, 0.77% and 0.74%. saponin hydrolysis for long time , caused reduction of sapogenin yield . The sapogenin yield after 2:30hr decreased, but it has small difference from 0.77%; when increased time of hydrolysis, gradually loss high yields of sapogenin. So, the time effect on sapogenin during hydrolysis was significant, its p-value is less than 0.05.

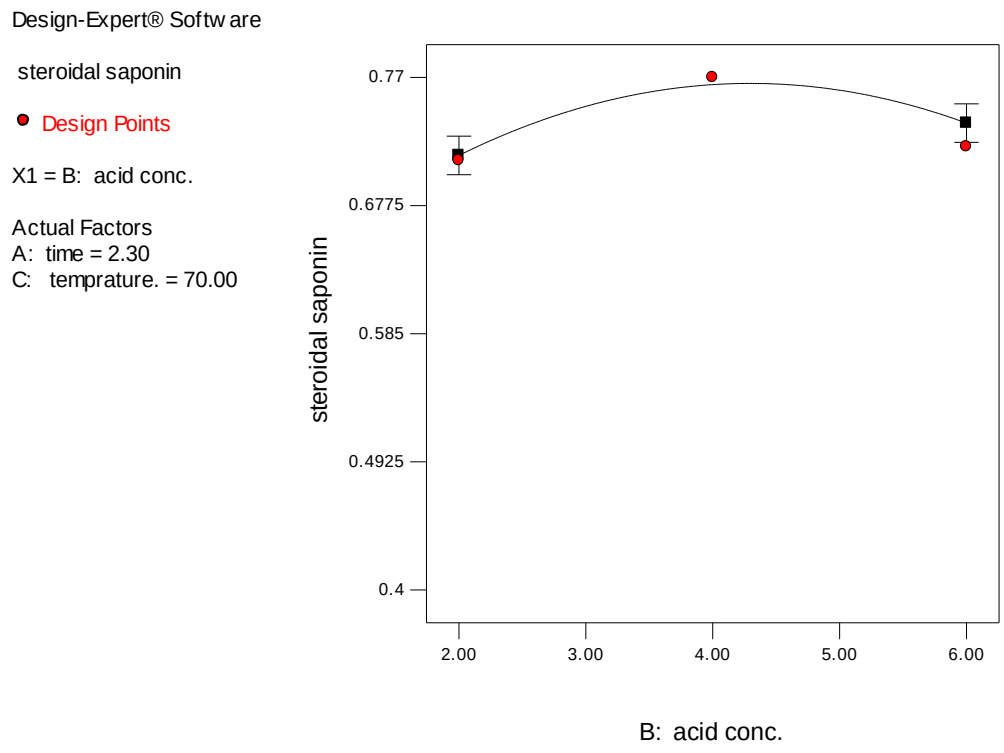

Figure 4-11: Effect of acid concentration on steroidal saponin

4.7.5. Acid concentration effect

The effect of acid concentration on the steroidal saponin(sapogenin) shown in the figure 4.11. Acid concentration increased from 2M to 4M, the yield of steroidal saponin(Sapogenin) increased, but acid concentration exceeded 4M the yield of steroidal saponin decreased due to, increased breakdown the bond of sapogenin ,and then it leads to produced blacken wanted powder (increased by product) (K.Cheng et al, 2017).

Generally Acid concentration significantly affects saponin hydrolysis. Its p- value from table 4.11 is 0.0151, this is less than 0.05.

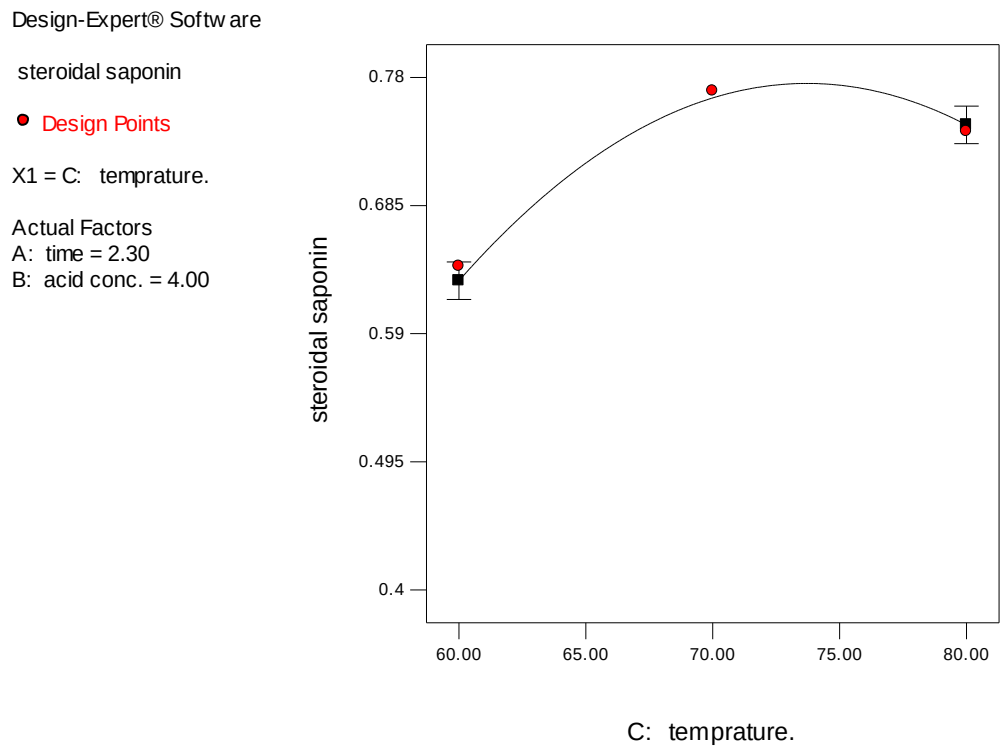

Figure 4-12: Effect of temperature on steroidal saponin

4.7.6. Effect of temperature on steroidal saponin

Figure4.12 shows that, increased temperature from 60^{o}C to70^{o}C the yield of steroidal saponin increased from 0.4 % to 0.77%. But after 70^{o}C, it leads to decrease the yield. Because heating at higher temperature with acid loss saponin and produced unwanted materials (by product) like ashes.The p-value from the table 4.11 is 0.0001 this is less than 0.05; so, the effect of temperature on saponin hydrolysis was significant.

4.7.7. Interaction effect of acid concentration and hydrolysis time

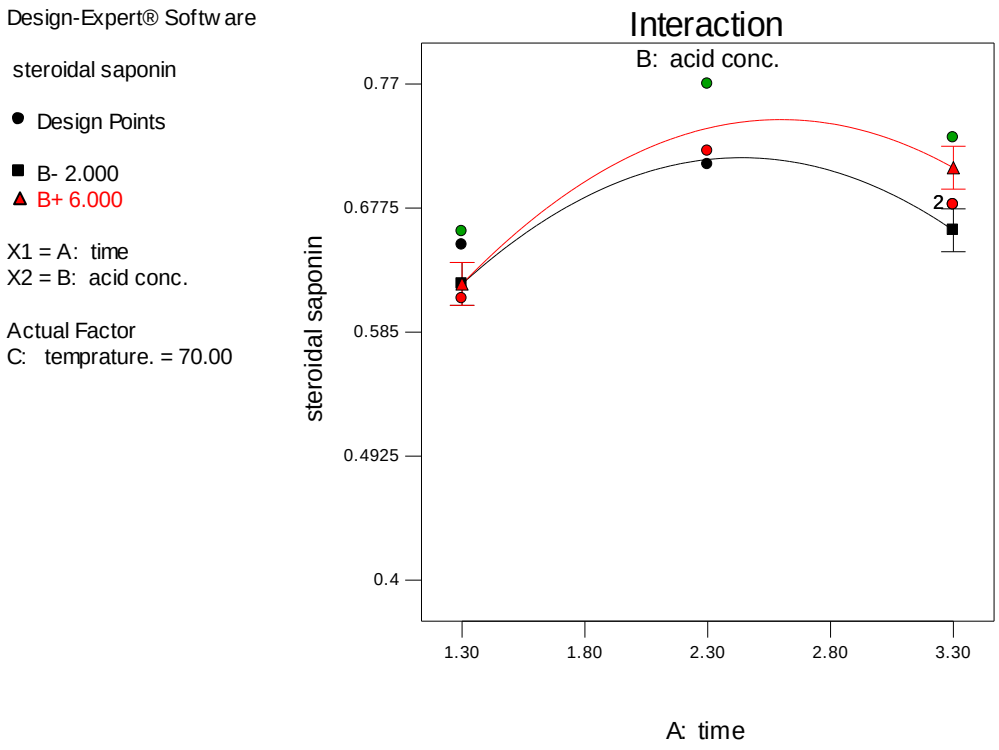

Figure 4-13: Interaction effect of acid concentration and time on steroidal saponin (spogenin) yield

The above figure 4.13 indicates the interaction effect of time and acid concentration on sapogenin yield. Bothe acid concentration and time increased the yield also increased until time of 2.30hr and obtained the maximum amount of yield (0.77%) at 2:30hr and 4M acid concentration. After 2:30hr with increased acid concentration the sapogenin yield decreased, because acid concentration increase with long time, leads to damage yield. The interaction effect of time and acid were significant, the interaction p-value from table 4.11 is 0.0415.

.

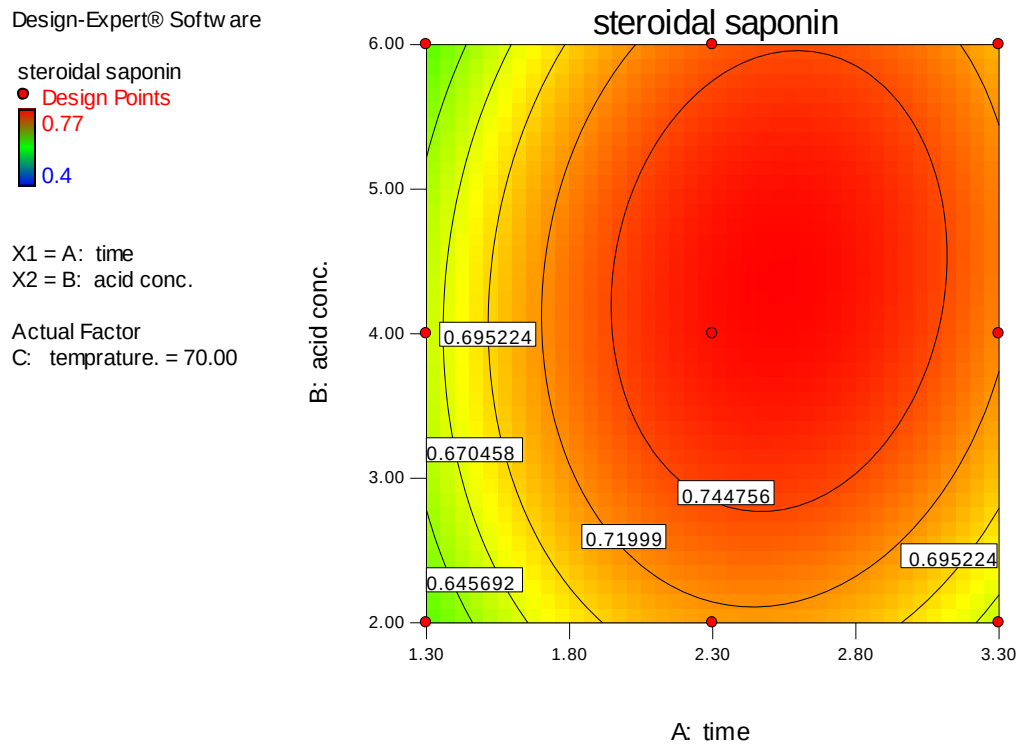

Figure 4-14: Interaction effect of hydrolysis time and acid concentration on sapogenin (steroidal saponin)

4.7.8. Interaction effect of temperature and hydrolysis time on steroidal saponin(sapogenin)

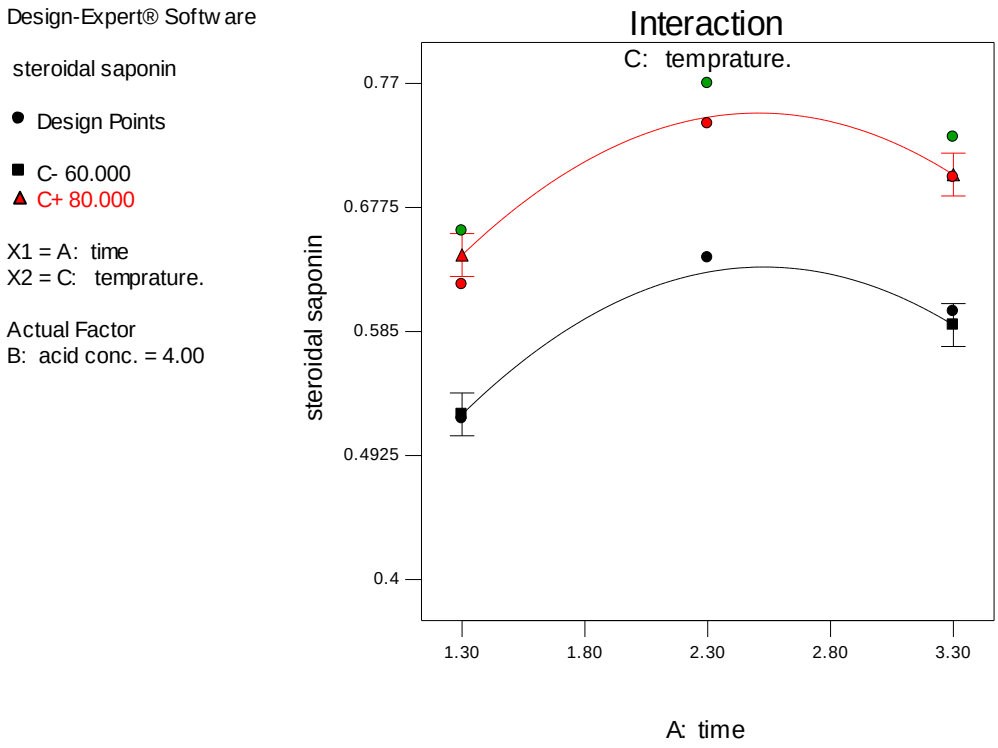

Figure 4-15: Interaction effect of hydrolysis time and temperature on sapogenin

From the above figure 4.15, analysis the interaction effect of both time and temperature; the interaction effect was not significant, its p-value from table 4.11 is 0.07565, this is greater than 0.05. The yield of sapogenin from the above figure at 60^0C with 1:30hr, 2:30hr and 3:3hr was 0.52%, 0.64%, and 0.6% respectively. Next increased the temperature from 60^0C to 70^0C, the result at this temperature with time of hydrolysis 1:30hr, 2:30hr and 3:30 hr was, 0.66%, 0.77% and 0.7% respectively. Increased the temperature to 80 ^{0}C, and the yield of sapogenin was 0.62% at1:30hr, 0.74% at 2:30hr, and 0.7%at 3:30hr. The yield of steroidal saponin increased, when increase the temperature from 60^0C to 70^0C with increase time; after 70^0C increased temperature with also time the yield decreased.

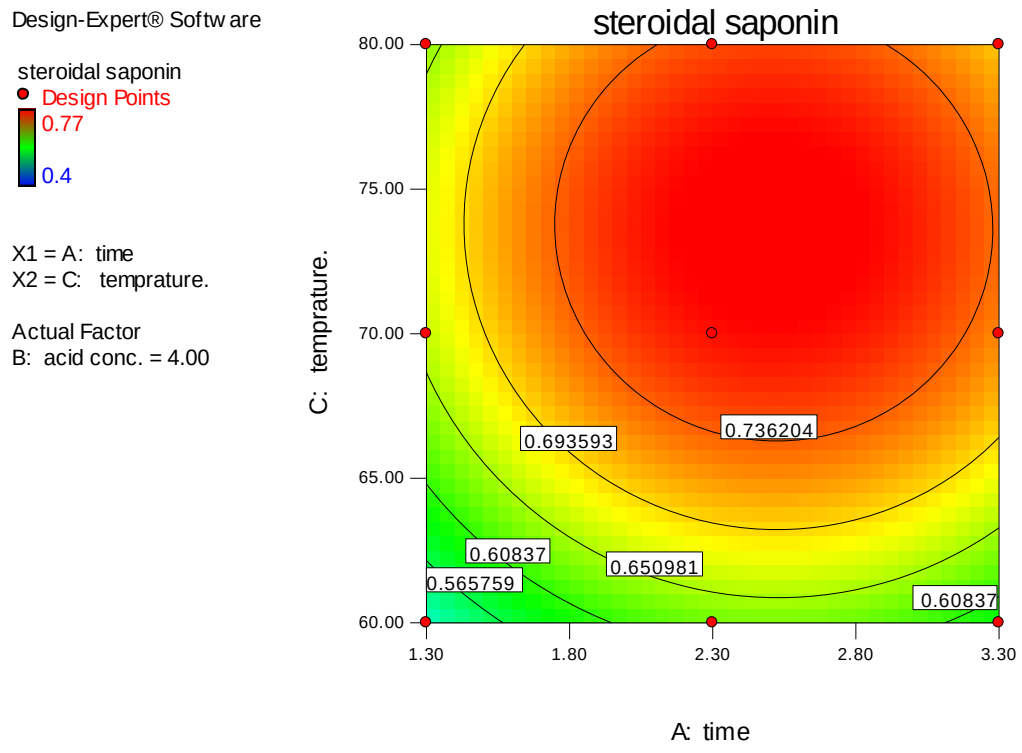

Figure 4-16: Interaction effect of acid concentration and temperature on steroidal saponin

4.7.9. Interaction effect of acid concentration and temperature

Figure 4.16 as shows that, the result of interaction effect of temperature and acid concentration. Both temperature and acid concentration increased, and then the yield of sapogenin was increased; when temperature increased from 60^{o}C to 70^{0}C and acid concentration increased from 2M to 4M. But after 4M Acid concentartion and 70^{0}C, the product of the yield decreased , because heated saponin at higher temperature with higher concentration of acid leands to loss sapogenin and produced higher by produt.

The sapogenin yield, at lower temperature with higher acid concentration, was produced higher sapogenin yield; for example, at 80 ^{0}C with 2 M, 70^{0}C with 3M and 70^{0}C with4M produce 0.68%, 0.72% and 0.77% respectively

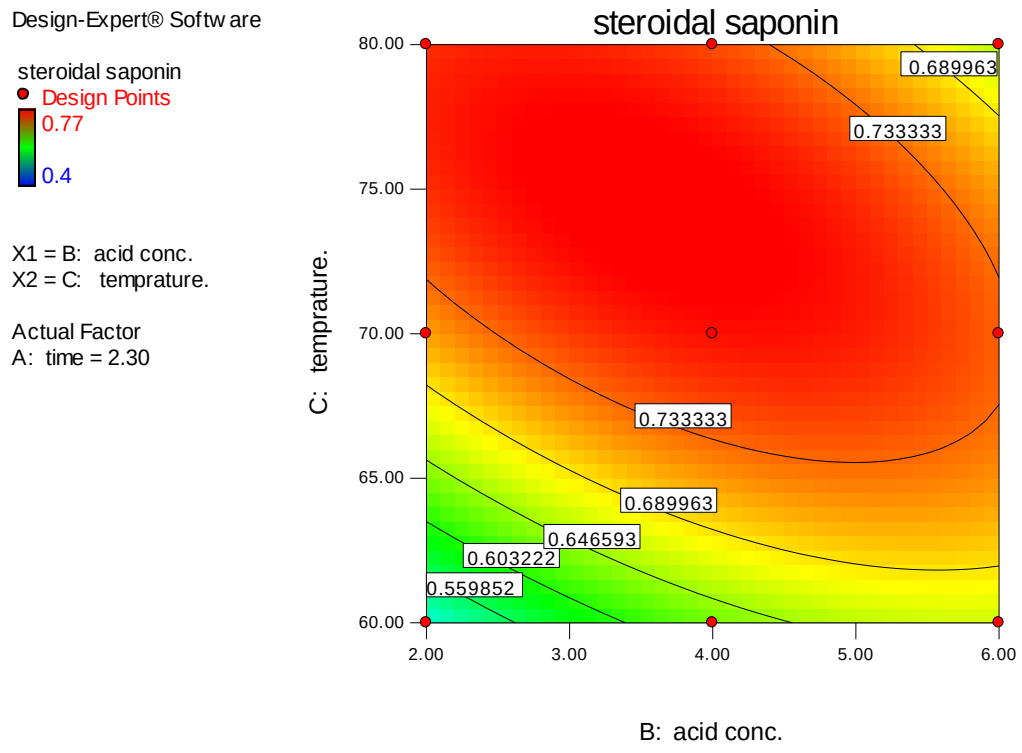

Figure 4-17: acid concentration and temperature vs steroidal saponin , contour graph

At lower temperature with lower acid concentration, leads to minimum removal of sugar. This means, low amount of sapogenin produce. On the other hand, at higher acid concentration and higher temperature obtained low yield of sapogenin because, highly breakdown of the sapogenin bond and caused thermal degradation of sapogenin.

Generaly the interaction effect of acid concentaration and tempearature was significant ,its p-value is 0.0001 ,this is less than 0.05.

4.6. Characterization of steroidal saponin (sapogenin) by Nuclear Magnetic Resonance (NMR) and Fourier Transform infrared (FTIR) Spectroscopy

4.6.1. 13_CNMR

13_CNMR determined the number of carbon by its number of signals. The sapogenin has 27-30 numbers of carbons. So, as the result of NMR in the figur4.18 or figure 4.19 identified at least 27 carbon atoms, this indicates, the presence of hecogenin. Its chemical shift from the below table4.15 determine, hybridization of each carbon.

Table 4.15: Chemical shift range and kind of compounds (M. Lasha et al 2008) and(K.D. Balerzak et al, 1996)

ppm	hybridization	Kinds of compounds
0-70	Sp^3	CH3 (alkane)
70-100	Sp^3 and sp	C-O and C-N
100-160	Sp^2	Aromatic C and c=c
160-210	Sp^2	Aldehyde and ketone carbonyl(c=o)

The chemical structure of sapogenin contains alkane, aromatic carbons, aldehyde, ketone, carbonyl and C-O and C-N (they bounded with sugar).

Table 4.16: Chemical shift of sapogenin

Number of carbon	13_CNMR result Chemical shift of sapogenin	Reference of Chemical shift 0f sapogenin
C1	38.58	39.7
C2	128	127.7
C3	71.3	71
C4	37.73	37
C5	131.82	132
C6	115	115.6
C7	38.15	39
C8	38.36	37.9
C9	57.2	56
C10	37.94	37
C11	20.5	20.6
C12	39.00	39.2
C13	62.94	62.7
C14	38.79	38
C15	69.45	70
C16	81.02	81
C17	62.71	62
C18	154	154.5
C19	21.9	21.5
C20	44.21	43.1
C21	17.67	17.5
C22	101.72	100.9
C23	180	179.8
C24	176	175.8
C25	64.1	64
C26	67.63	67.
C27	17.81	17.2

The above Table 4.1-15 indicates the chemical shift of 13_CNMR result and sapogenin chemical shift reverence. Based on the above comparison of chemical shift, it approved the experiment product is mostly approaches to hecogenin.

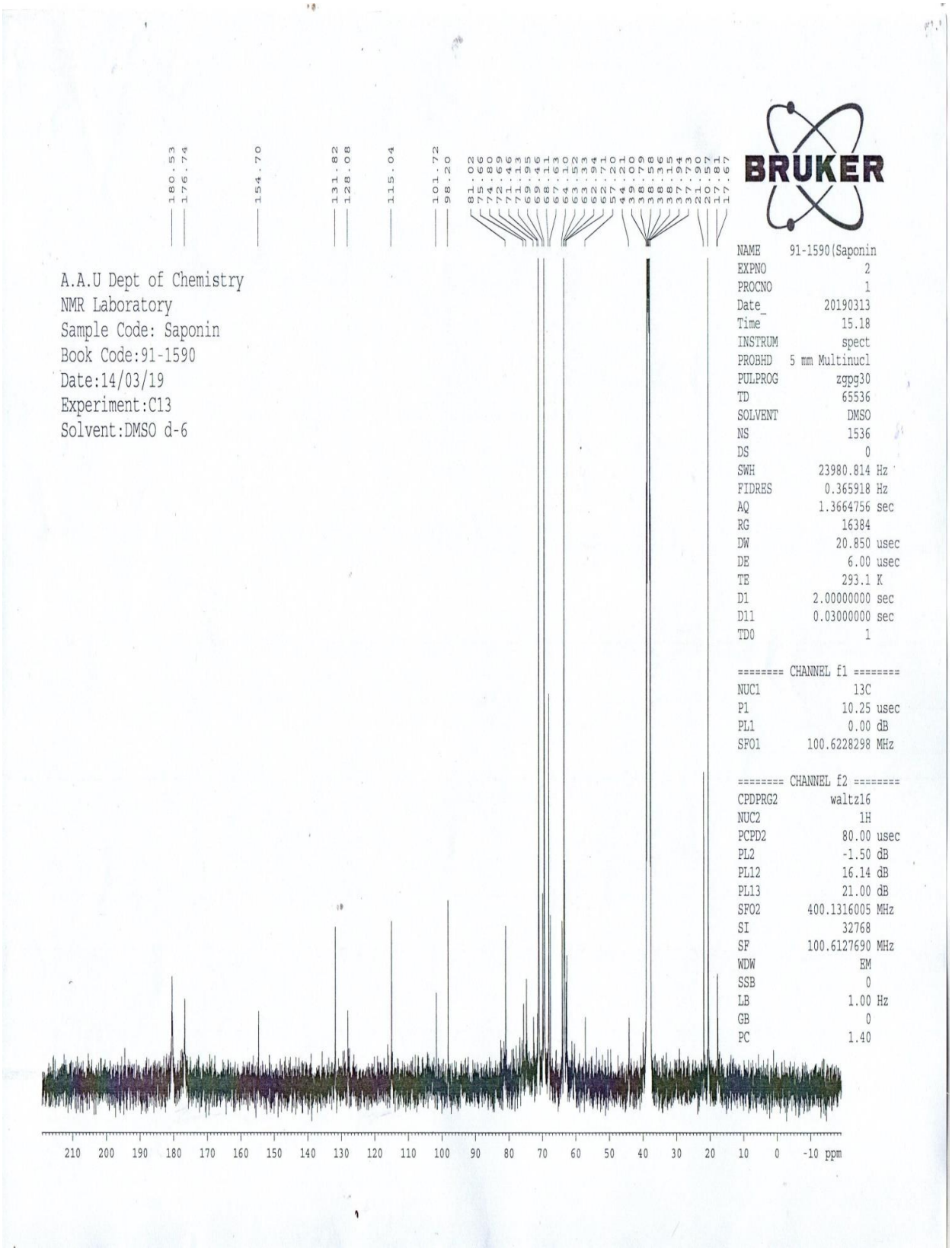

Figure 4-18: 13CNMR results of steroidal saponin

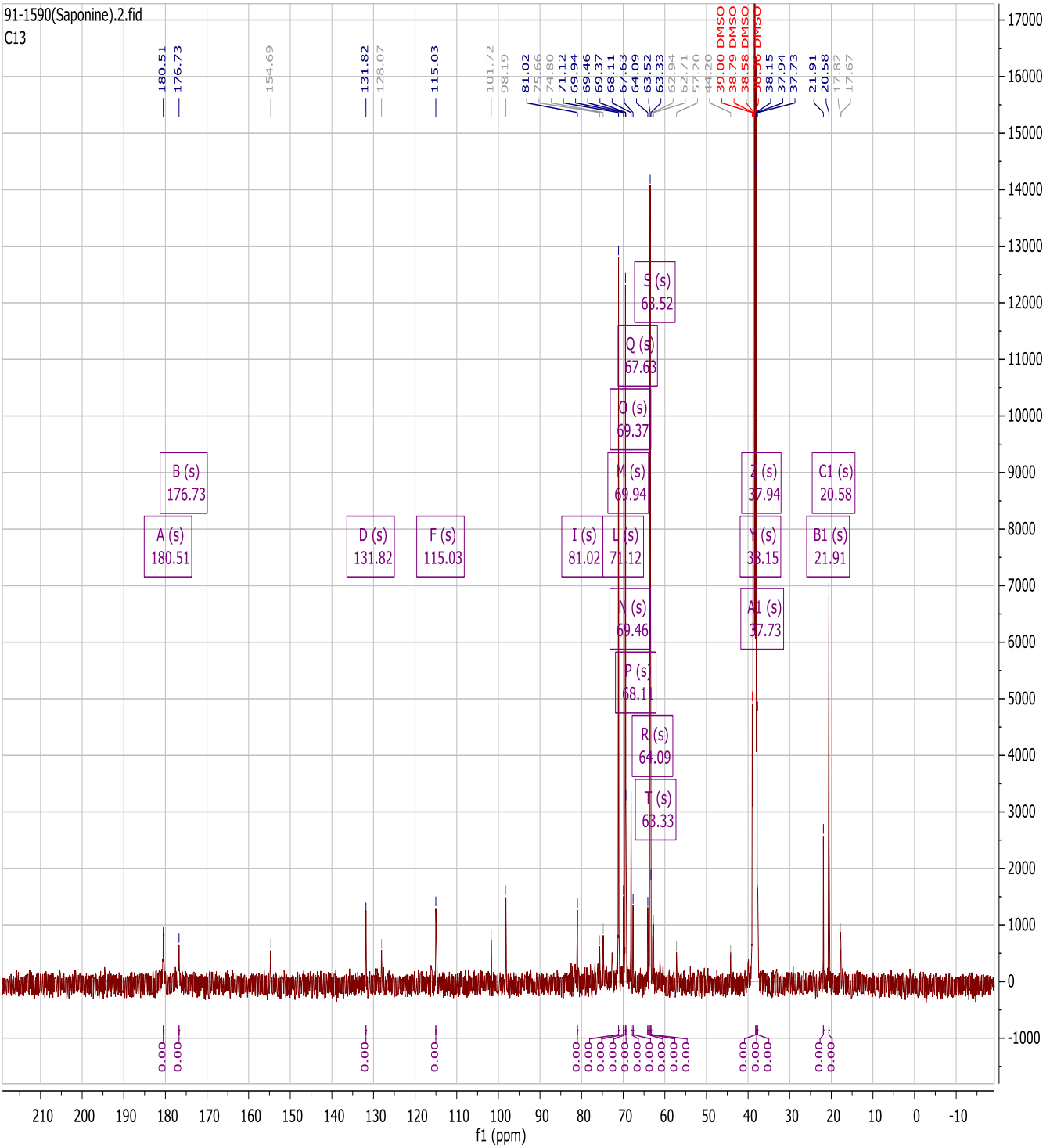

Figure 4-19: 13_CNMR result of steroidal saponin(sapogenin)

4.6.2. HNMR Result

Table 4.17 chemical shift of proton of sapogenin

NO.	HNMR result and chemical shift (ppm)	Standard chemical shift (ppm)	Description of proton
1	0.797,0.966	0.7-0.9	methyl
2	1.189	1-1.1.3	Methylene(CH_2-R1R2)
3		1.5-2	methine
4	2.370 &2.502	1.8-2.7	Methyl(CH_3-CO-)
5	2.8	2.03	alpha to nitrogen(c is attached to N)
6	3.319& 3.471	3-3.5	N-methyl
7	3.536,3.576 &3.573	3.5-3.6	alkoxy
8	3.634,3.752, 3.874 &3.994	3.6-4.7	Methylene(CH_2-O-)
9	4.651	4.5-6.1	alkene
10	6.595 & 6.963	6.2-8.2	aromatic

The above table 4.17 is the HNMR results and proton standard chemical shift of sapogenin; the results of HNMR of sapogenin include within the range of standard or acceptable proton chemical shift. So, the chemical shifts of proton NMR results (experimental results) are the chemical shift of sapogenin.

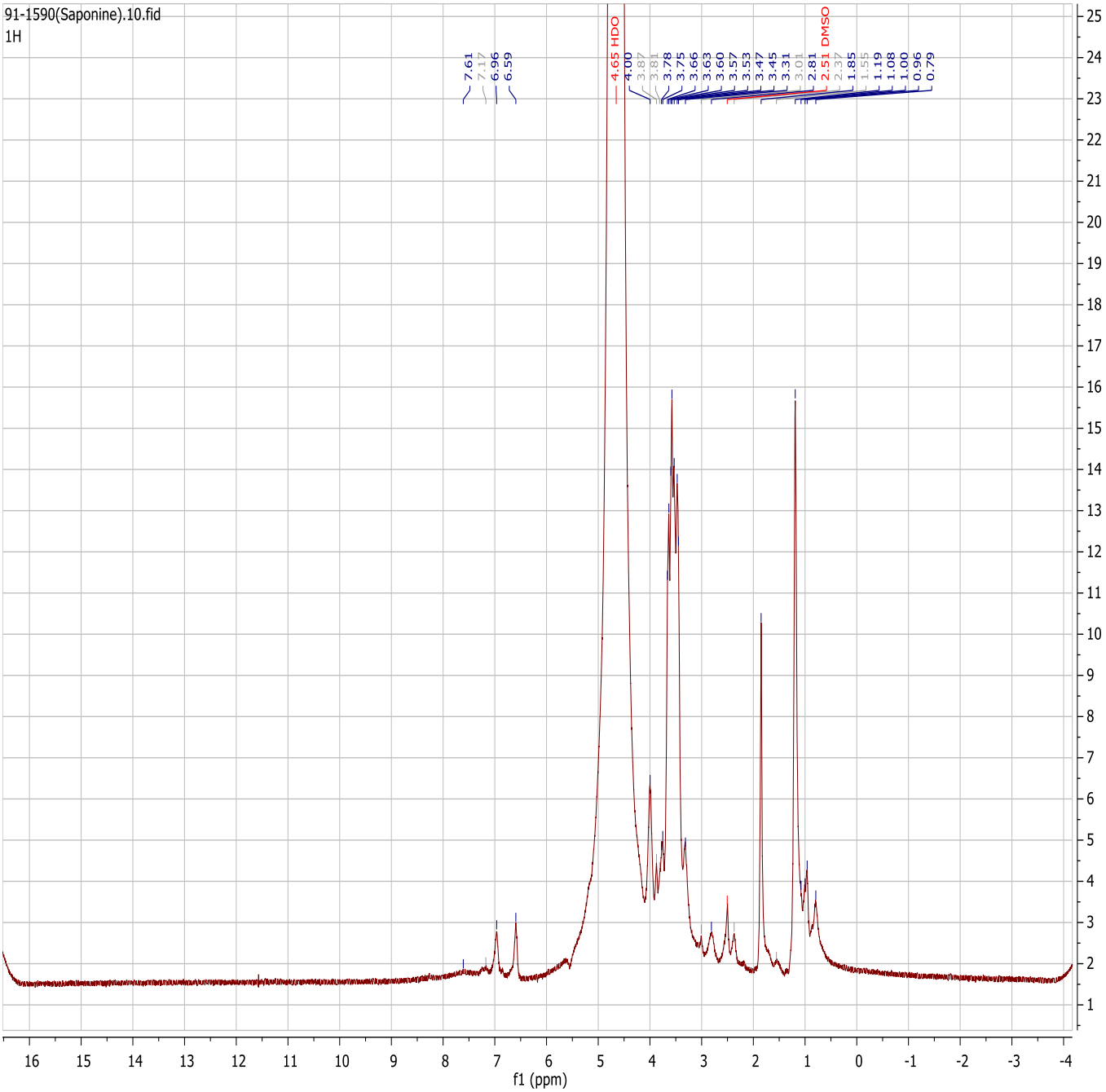

Figure 4-20: HNMR result of sapogenin

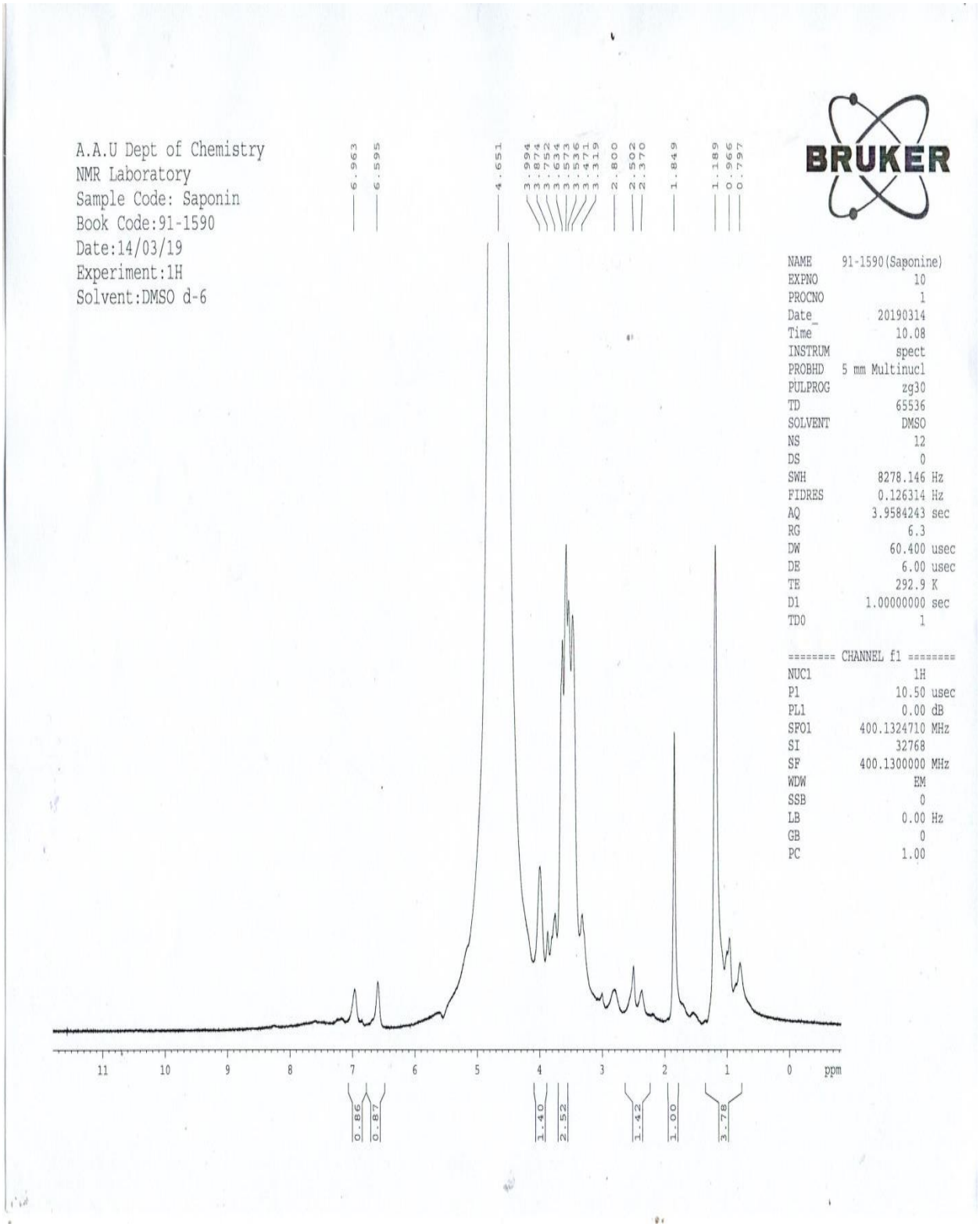

Figure 4-21: 1HNMR RESULT of sapogenin

4.6.3. FTIR SPECTOROSCOPY

The functional group, in the sample of steroidal saponin is identified by FTIR Spectroscopy. The data in the table 4.22 has shown its compounding functional group. The characteristic observation band, at, 3531.67cm^{-1} for hydroxyl group (-OH), 2942.09 cm^{-1}(C-H, Stretch methylene), 2820.08cm^{-1}(C-H, stretch methyl), 1742.37cm^{-1}(C=O, carbonyl stretch), 1649.48cm^{-1} (C=C, stretch), 1415.28cm^{-1}(C-H, bend), and1223.72cm^{-1} (c-o, stretch carbonyl) .All data below indicates, the functional group, which include in the sample (A.Ade-Ajay et al., 2011).

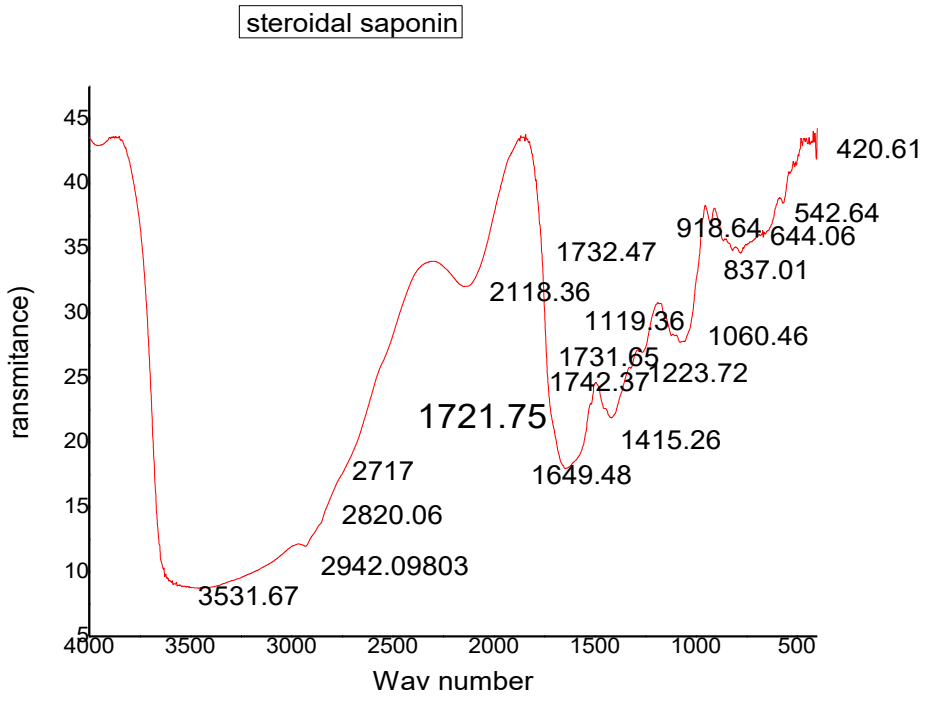

Figure 4-22: FTIR spectroscopy of hecogenin at room temperature

Table 4.18: FTIR peaks and its functional group

No.	peaks	Functional group
1	3531.67	OH, alcohol group
2	2942.09	c-H, stretch methylene
3	2820.08	C-Stretch methyl
3	1742.37	C=o, carbonyl stretch
4	1649.48	C=c , stretch
5	1415.28	c-H , bend
6	1223.72	c-o, stretch carbonyl
7	1119.36	c-H , Bend
8	1060.46	c-o, stretch
9	918	c-H, bend

4.6.4. UV-Spectroscopy photo meter

Steroidal saponin can be identified at the maximum peaks of 430 nm (V. Singh et al,2013) and (Mendhulkar et al. 2015). According to the previous researchers, the result of UV –Spectroscopy analysis of hecogenin at room temperature is indicated on figure 4.23. As shown in the figure 4.23 confirms the presence of steroidal saponin (sapogenin) in the sample. From the Figure 4.23, the maximum peak at 430 nm indicates steroidal saponin (S. Sulaiman et al, 2014). So, the sample that was extracted from agave sisal leaves contained steroidal saponin.

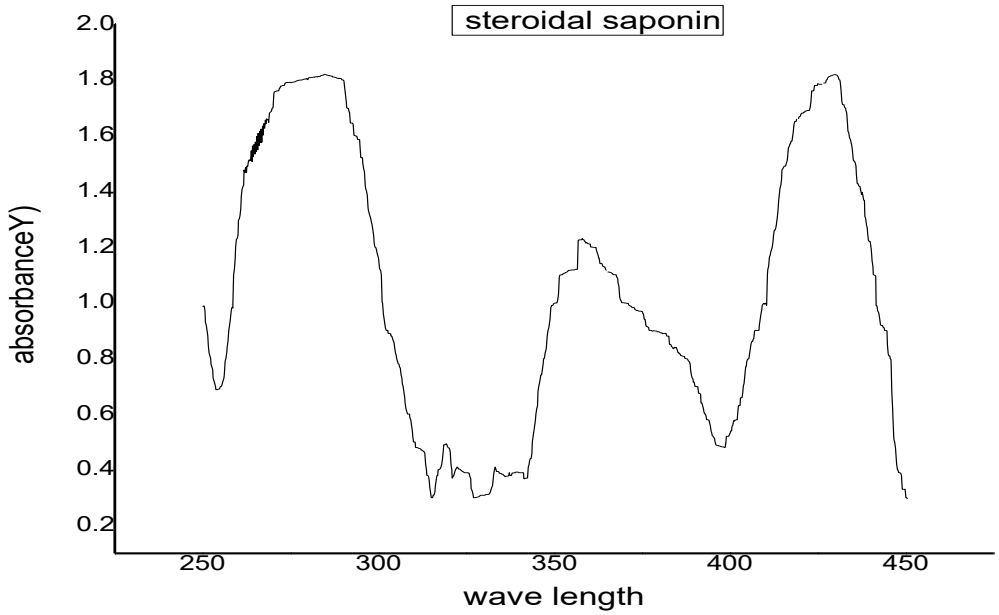

Figure 4-23: UV-Spectroscopy photo meter result of sapogenin

Generally, NMR, FTIR and UV- Spectroscopy clearly, identified, steroidal saponin (sapogenin), that was separated from saponin.

Chapter five

5. Conclusion and Recommendation

5.1. Conclusion

This study aimed at producing a pharmaceutical starting material, sapogenin, for partial substitution of the imported raw materials for pharmaceutical production. In this study, sapogenine (active pharmaceutical ingredients) has been successfully extracted from saponin by acid hydrolysis method. The saponin was first extracated from agave sisalana leaves by using ethanol as a solvent. NMR, FTIR and UV- spectroscopy were used to identify the isolate, sapogenin. These results clearly indicate that the isolated material was sapogenin. Foam test result on saponin showed that the extracted material was saponin. The optimum parameters in extraction and hydrolysis were determined by using ANOVA. Maximum saponin yield was extracted at 20 hr and 1.5 solvent to material ratio and the optimum parameters for separation of sapogenin from the saponine were 70 ^{0}C, 4M and 2.5 hr.

5.2. Recommendation

Ethiopia has the shortage of active pharmaceutical ingredients; so problem can be solved by investing on the *Agave sisalana* plants. Costs incurred by importing the active ingriedients can be minimized if the government invests on the sisal plants and use it for production of active pharmaceutical ingredients for local pharmaceutical industry.

Investors, if they invest on production of sapogenin active pharmaceutical ingredients, from *Agave sisalana* plant, they will be profitable because, in Ethiopia we have excess raw materials and high demand. In addition, Ethiopia does not have any active pharmaceutical ingredient factory.

In addition to study on the preparation active pharmaceutical ingredient of sapogenin; will investigate on the production of pharmaceutical drug for, anti-cancer, anti-cardiovascular, sexual dis order treatment and blood pressure from the *Agave sisalana* plant extract.

Agave sisalana plants also available for biofuel, food and beverage, so, researchers study on this area, it has great advantage for country.

Chapter six

6. References

Abdulkadir, Ahmed. &Hussen, Endris.(2013). Systematic review of plant steroids as potential anti-fungal and anti -cancer agents: The Journal of Phyto pharmacology 2013; 4(2): 121-125.

Ade-Ajayi, A. F., et al. "Preliminary phytochemical and antimicrobial screening of Agave sisalana Perrine juice (waste)." *Journal of Environmental Chemistry and Ecotoxicology* 3.7 (2011): 180-183.

Al Jasem, Yosef, et al. "Preparation of steroidal hormones with an emphasis on transformations of phytosterols and cholesterol-a review." *Mediterranean Journal of Chemistry* 3.2 (2014): 796-830.

Almaraz-Abarca, Norma, et al. "The phenols of the genus Agave (Agavaceae)." *Journal of Biomaterials and Nanobiotechnology* 4.03 (2013): 9.

Anwar, Z., & Hussain, F. (2017). Steroidal Saponins : An Overview of Medicinal Uses,and study on the pants used to tradetional and moderine *11*, 20–24.

Asfaw, Kinfemichael Geressu. "Investigation of the reasons for the unique growth and development of Agave species (Agave sisalana and Agave americana) crop plants at the southern, central, north western and eastern parts of tigray, Ethiopia." *Curr. Res. J. Biol. Sci* 3 (2011): 273-281.

Beedemariam, Gebremedhin, and T. Gedif. "Assessment of pharmacy work force in ethiopia." *Ethiopian pharmacy association and Addis Ababa Univerity* 5 (2011).

Biesalski, H., Dragsted, L. O., Elmadfa, I., D, M. (2009). Bioactive compounds : Definition and assessment of activity, 1–4. https://doi.org/10.1016/j.nut.2009.04.023.

Biesalski & Goel.(2009). Research Article STUDIES ON PHYSICAL PROPERTIES OF SISAL (Agave sisalana) PLANT LEAVES, *8*(48), 2004–2007.

Cheng, Kun., Gao, H., Wang, R. R., Liu, Y., Hou, Y. X., Liu, X. H., … Wang, W. (2017). Evaluation of extraction and degradation methods to obtain chickpeasaponin B1 from chickpea (CicerarietinumL.). *Molecules*, *22*(2). https://doi.org/10.3390/molecules22020332.

chigodi, m. o., samoei, d. k., & muthangya, m. (2013). phytochemical screening of agavesisalana perrine leaves (waste) department of chemistry and biochemistry , pwani university , p . o . box 195 - 80108 , kilifi , kenya department of biological sciences , pwani university , p . o . box 195 - 80108 , kili, 200–204.

Cushman, John C., et al. "Development and use of bioenergy feedstocks for semi-arid and arid lands." *Journal of Experimental Botany* 66.14 (2015): 4177-4193.

Debnath, Mousumi, et al. "Biotechnological intervention of Agave sisalana: a unique fiber yielding plant with medicinal property." *Journal of Medicinal Plants Research* 4.3 (2010): 177-187.

Deshpande, H. A., and S. R. Bhalsing. "Plant derived novel biomedicinal: diosgenin." *Int. J. Pharmacogn. Phytochem. Res* 6 (2014): 780-784.

Ewen, Margaret, et al. "Prices and availability of locally produced and imported medicines in Ethiopia and Tanzania." *Journal of pharmaceutical policy and practice* 10.1 (2017): 7.ls and cholesterol-a review." *Mediterranean Journal of Chemistry* 3.2 (2014): 796-.

Faizal, Ahmad, and Danny Geelen. "Saponins and their role in biological processes in plants." *Phytochemistry reviews* 12.4 (2013): 877-893.

Fernandes & Mvandos (2013). Role of Plant Containing Saponin on Livestock Production ; A Review, *10*(5), 309–314. https://doi.org/10.5829/idosi.abr.2016.309.314.

Garai,Suntaz.(2016).Journals Advances in Triterpenoid Saponins, 1–23. https://doi.org/10.21767/2472-0151.100020.

Gupta, P. K., et al. "Clinical pharmacokinetics and metabolism of berberine and hydrastine following an oral dose of goldenseal supplement." *Planta Medica* 76.05 (2010): P110.

Hidalgo-Reyes, Martin, et al. "Chemical and morphological characterization of Agave angustifolia bagasse fibers." *Botanical Sciences* (2015): 807-817.

Hostettmann, Kurt, et al. *Phytochemistry of plants used in traditional medicine*. 1995.

Pengelly, Andrew. "Dodonea viscosa." *Australian Journal of Medical Herbalism* 11.1 (1999): 11.

Kimaro, Didas, Balthazar M. Msanya, and Yasuo Takamura. "Review of sisal production and research in Tanzania." (1994).

Kofler, L., and Ph A. Adam. "Die Wertbestimmung der Saponindrogen." *Archiv der Pharmazie* 265.38-42 (1927): 624-652.

Kregiel, Dorota, et al. "Saponin-based, biological-active surfactants from plants." *Application and characterization of surfactants* (2017): 183-205.

Lasha,Murnati., Nkwocha, P. & Nkwocha, P. F C, N. O., (2008). Qualitative and Quantitative Phytochemical Screening of the Aqueous Leaf Extract of Senna mimosoides : Its Effect in in vivo Leukocyte mobilization induced by inflammatory stimulus. *Int.J.Curr.Microbiol.App.Sci*, *4*(5), 1176–1188.

Lorent,Jolfer H., Quetin-Leclercq, J., & Mingeot-Leclercq, M. P. (2014). The amphiphilic nature of saponins and their effects on artificial and biological membranes and potential consequences for red blood and cancer cells. *Organic and Biomolecular Chemistry*, *12*(44), 8803–8822. https://doi.org/10.1039/c4ob01652a.

Lornard FrankG,. (2013). A New Bioactive Steroidal Saponin from Agave attenuata, 3–8.

Marrelli, Mariangela, et al. "Effects of saponins on lipid metabolism: A review of potential health benefits in the treatment of obesity." *Molecules* 21.10 (2016): 1404.

Meragiaw, Misganaw, and Zemede Asfaw. "Review of antimalarial, pesticidal and repellent plants in the Ethiopian traditional herbal medicine." *Res. Rev. J. Herbal. Sci* 3.3 (2014): 21-45.

Mielenz, Jonathan R., et al. "Development of Agave as a dedicated biomass source: production of biofuels from whole plants." *Biotechnology for biofuels* 8.1 (2015): 79.

Mimaki,Yinands., Masika, P. J., & Francis, J. (2003). Anti-inflammatory and analgesic activities of the aqueous leaf extract of Agave sisalana in rats, *7*(14), 1477–1484. https://doi.org/10.5897/SRE11.660

Mohammed, Mona S., et al. "Secondary metabolites as anti-inflammatory agents." *J Phytopharmacol* 3 (2014): 275-85.

Mohamed, Musa., Ashour, A. S., Sadek, A., & Melad, G. (2019). A review on saponins from medicinalplants chemistry,isolation,anddetermination,*8*(1),612.https://doi.org/10.15406/jnmr.2019.08.00199.

Moghimipour, Eskandar, and Somayeh Handali."Saponin: properties, methods of evaluation nd applications." *Annual Research & Review in Biology* (2015): 207-220.

Moghimipour, E. S. K. A. N. D. A. R., et al. "In vitro screening of anti-candida activity of saponins extracted from Glycyrrhiza glabra and Quillaja saponaria." *Asian J Pharm Clin Res* 7.1 (2014): 160-162.

Monterrosas-Brisson, Nayeli, et al. "Anti-inflammatory activity of different Agave plants and the compound Cantalasaponin-1." *Molecules* 18.7 (2013): 8136-8146.

Mosafi,Curnde. (2017). National strategy and plan of action for pharmaceutical manufacturing development in Ethiopia (2015 – 2025) Developing the pharmaceutical industry and improving access, (July).

Morrissey &Gsbourn.(1999). Screening for Triterpenoid Saponins in Plants UsingHyphenated Analytical Platforms. Department of Food Science, Faculty of Science, University of Copenhagen. Published: 24 November 1999.

Muradin, Magdalena, and Zenon Foltynowicz. "Potential for producing biogas from agricultural waste in rural plants in Poland." *Sustainability* 6.8 (2014): 5065-5074.

Muthangya, Mutemi., Mwandogo ,O. Chigodi, and David K. Samoei. "Phytochemical screening of agave sisalana perrine leaves (waste)." (2013).

Mwale, Marizvikuru, and Patrick Julius Masika. "In vivo anthelmintic efficacy of Aloe ferox, Agave sisalana, and Gunnera perpensa in village chickens naturally infected with Heterakis gallinarum." *Tropical animal health and production* 47.1 (2015): 131-138.

Negi Jamsen S, (2013). Naturally occurring saponins: Chemistry and biology. *Journal of Poisonous and Medicinal Plant Research, 1*(1), 1–6.

Osman, Musa., Mohammed, Nuri., Anwar, Hussen.,& Saliman sultan.(2014). Study of Phytochemical Screening, Physicochemical Analysis and Antimicrobial Activity of *Bacopa monnieri* (L) Extracts. International Journal of Pharmaceutical and Clinical Research 2014;

Pereira&Mendhulkar.(2005). Abutilon indicum (Linn .) Sweet leaves , a Natural source of Saponin : a Specrtophotometric assay, *8*(4), 725–729.

Petre,Grmdaz., Jetiyanon, K., Wittaya-areekul, S., & Putalun,W. (2017). Influence of seasons , different plant parts , and plant growth stages on saponin quantity and distribution in Bacopa monnieri, *33*(2), 193–199.8(8): 1222-1229.

Rami, E., Sipai, S., & Patel, I. (2013). Studies on qualitative and quantitative phytochemical analysis of piper longum linn. *International Journal of Pharma and Bio Sciences, 4*(3), 11–17.

Rejinold &Nolando.(2011). Application of foam column as green technology for concentration ofsaponins from sisal (*Agave sisalana*) AND JUÁ(*Ziziphus joazeiro*). Vol. 30, No. 04, pp. 701 - 709, October - December, 2011.

Salum, Abbas, and Glenn Stuart Hodes. "Leveraging CDM to scale-up sustainable biogas production from sisal waste." *17th European Biomass Conference and Exhibition: From Research to Industry and Markets*. 2009.

Santos, Jenter & Branco, A. (2014). GC-MS Characterisation of Sapogenins from Sisal Waste and a Method to Isolate Pure Hecogenin, *9*(1), 1325–1333.

Santos, Jener, et al. "Chemicals from Agave sisalana biomass: isolation and identification." *International journal of molecular sciences* 16.4 (2015): 8761-8771.

Santos, Jenter., Branco, A., Silva, A. F., Pinheiro, C. S. R., Neto, G.,& Uetanabaro, A. P. T., … Osuna, J. T. A. (2009). Antimicrobial activity of Agave sisalana. *African Journal of Biothecnology*, *8*(22), 6181–6184. https://doi.org/10.5897/AJB09.862.

Sarkar, Sitangshu, et al. "Influence of drip irrigation, manure and fertilizers on production of planting materials in sisal (Agave sisalana Perr. Ex Engelm.)." (2018)

Saxena, Mamta, et al. "Phytochemistry of medicinal plants." *Journal of pharmacognosy and phytochemistry* 1.6 (2013).

Savjin,Pendely., Oliveira, B. A. De, Olavo, H., Souza, E. B. De, Márcio, R., & Camargo, C. C. (2015). Toxicity of the extracts of sisal waste , obtained from decortications of the Agave sisalana, (6), 160–165.

Shallangwa, G. A., Hammuel, C., Yebpella, G. G.,Magomya, A. M., & Agbaji, A. S. (2011). Phytochemical and antimicrobial screening of methanol and aqueous extracts of Agave sisalana. *Acta Poloniae Pharmaceutica - Drug Research*, *68*(4), 535–539.

Shimoyama&Bokaldu.(1996). Hemolytic and cytotoxic effects of saponin like compounds isolated from Persian Gulf brittle star (Ophiocoma erinaceus). *Journalo Coastal Life Medicine*, *2*(10), 762–768.

Singh,Vesicarium.(2013). Characterization, Isolation, purification, and structural characterization of the saponins from glottidium vesicarium.

Snhel,Smfozi.,Damon,Gturn.,&Francis, Ardusak.(2015). Phytochemicals: Extraction, Isolation,and Identification of Bioactive Compounds from Plant Extracts.Department of Food Science, College of Agriculture, University of Al-Basrah, Basrah 61004, Iraq. Published: 22 September 2015.

Su,Haung & Zu, kun. (2011). Agave (*Agave spp*.) and its Traditional Products as a Source of BioactiveCompounds. *Departamento de Biotecnología e Ingeniería de Alimentos. Centro de Biotecnología. Tecnológico de Monterrey-CampusMonterrey. Av. Eugenio Garza Sada 2501 Sur, C.P. 64849 Monterrey, N.L. Mexico.Current Bioactive Compounds* 2011, *8,* 000-000.

Sulaiman ,Salman & Cheok, C. (2014). Extraction and quantification of saponins: A review. *Food Research International*, *59*, 16–40. https://doi.org/10.1016/j.foodres.2014.01.057.

Szakiel, Andros., Burton, Trmandel ., Mrona,Glos.,& ztarin , Walsu.(2011). Solubilisation of Hydrophobic DrugsbySaponins. Department of Chemical and Pharmaceutical Engineering, Faculty of Chemistry and Pharmacy, Sofia University, 1164 Sofia,Bulgaria.

Tewari,David., Tripathi, Y. C., & Anjum, N. (2014). World Journal of Pharmaceutical ReseaRch AGAVE sislana : a plant with high chemical diversity, *3*(8), 238–249.

Tewari, Devesh, et al. "Targeting activator protein 1 signaling pathway by bioactive natural agents: Possible therapeutic strategy for cancer prevention and intervention." *Pharmacological research* 128 (2018): 366-375.

Thakur, Mayank, et al. "Chemistry and pharmacology of saponins: special focus on cytotoxic properties." *Botanics: Targets and Therapy* 1 (2011): 19-29.

Trejo-Torres, Jorge C., George D. Gann, and Maarten JM Christenhusz. "The Yucatan Peninsula is the place of origin of sisal (Agave sisalana, Asparagaceae): historical accounts, phytogeography and current populations." *Botanical Sciences* 96.2 (2018): 366-379.

Vaghasiya, Y., R. Dave, and S. Chanda. "Phytochemical analysis of some medicinal plants from western region of India." *Res J Med Plant* 5.5 (2011): 567-576.

Verma, A., Dwivedi, S., & Singh, N. (2013). Spectral analysis of steroidal saponin isolated and purified from leaves extract of *Asparagus racemosus* (Family – Asparagaceae). *American Journal of Advance Drug Delivery*.

Vincken, Hundros., Mofral, Lornad., Gumz,Andro.,&Mahol Ramon. Rendemen Analysis and Phytochemical Screening of *Perinereis aibuhitensis* Extracts. *Department of Biology, Faculty of Mathematics and Natural Sciences, University of Hasanuddin, Tamalanrea 90245, Makassar, Indonesia(2007).* International journal of current research and academic revie, volume 5 number 11 (november-2007).

Xiau Huang XU., Ting, Li., & Chiman, Vivienne.(2016).Anti-Inflammatory Activity of Different Agave Plants and the Compound *Cantalasaponin-1*.

Yan, Kun& Chuin,xu.(2009).Cytotoxic Steroidal Saponins from the Flowers of *Alliumleucanthum. Molecules* 2009, *13*, 2925-2934; DOI: 10.3390/molecules13122925.

Yang and kun.(2006). Anti inffilmatory and anti cancer tretments steriodal saponin extractfrom leaves of *Agave sisalana45-49.*

Yokosuka, Akihito, Yoshihiro Mimaki, and Yutaka Sashida. "Steroidal Saponins from Dracaena s urculosa." *Journal of natural products* 63.9 (2000): 1239-1243.

Youn Kim C, Qing-Ming Che, A. A. Leslie Gunatilaka and David G.I.Kingston(1996).Bioactive Steroidal Alkaloids from *Solanum umbelliferum*. Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0212, 1996.

Appendix

Appendix A

Figure A1: Agave sisalana plant (its, local name nechi Algai)

Figure A2: *Agave sisalana* (from left to right wende algai and set algai)

FigureA3: Saponin extraction from three different agave sisalana plant leaves

FigureA4: Samples from three different agave sisalana species, before evaporation

Appendix B: Saponin foam test

Figure B1: Saponin conformation test

Figure B2: Saponin conformation, foam stability test photo after 30 min

Appendix C: Sapogenin separation

FigureD1: Sapogenin separation after hydrolysis

Figure C2: Sapogenin after removal ofsugar

Appendix D: Calculation of acid and base solution concentration

Acid and base solution preparation

1. Prepare 4M Hydrochloric acid solution with in 500ml of distilled water

Molality = $\frac{mole}{volume}$

$$4M = \frac{m/M}{V}$$

Where, m= mass (g), M= molecular weight (g/mole) , and V= volume (L)

4M=n/M/v

$$4M = \frac{\frac{m}{36}}{500ml/1000ml}$$

Mass of HCl=2 × 36=72g

2. Prepare 4M sodium hydroxide solution within 1000ml distilled water

$$4M = \frac{m/M}{V}$$

4M= m/40

M= 160 g of sodium hydro oxide used for prepared 3M sodium hydroxide solution