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Ministry of Higher Education and Scientific Research
University of Baghdad
College of Pharmacy

Phytochemical Investigation of Steroidal Sapogenin (Tigogenin) of *Yucca aloifolia* plant cultivated in Iraq

A Thesis

Submitted to the Department of Pharmacognosy and the Committee of Postgraduate Studies of the College of Pharmacy-University of Baghdad in Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmacy (Pharmacognosy)

By

Nabaa Mohammed Ibrahem

(B.Sc. Pharmacy 2004)

Supervised By

Asst. Prof. Dr. Zeinab Jaliel Awad.

2012 ***1433***

بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيم

صدق الله العظيم

الأية ٣٢ سورة البقرة

Certificate

I certify that this thesis was prepared under my supervision at the Department of Pharmacognosy, College of Pharmacy-University of Baghdad as a partial fulfillment of the requirements for the degree of Master of Science in Pharmacy (Pharmacognosy).

Signature:

Name: ***Asst. Prof. Dr. Zeinab Jaliel Awad***.

Date: / / 2012

In view of the available recommendation, I forward this thesis for debate by examination committee.

Signature:

Name: ***Dr. Mohammed H. Mohammed***

Chairman of the committee of postgraduate studies in college of pharmacy

Date: / / 2012

Certificate

We, the examining committee, after reading this thesis, (***Phytochemical investigation of steroidal sapogenin (Tigogenin) of Yucca aloifolia plant cultivated in Iraq)*** and examining the student (***Nabaa Mohammed Ibrahem***), in its content, find that it is adequate as a thesis for the degree of Master of Science in Pharmacy (Pharmacognosy).

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Signature:

Name: ***Prof. Alaa A. Abdul Rasool***

Dean of College of Pharmacy-University of Baghdad.

Date:

Dedication

To my family....

To my teachers.....

To my friends....

With respects.

ACKNOWLEDGEMENT

Start with name of Allah Almighty, who bestowed me,his blessings andhave given me courage to complete this task.

I would like to express my deepest gratitude and appreciation to my respectful supervisor ***Dr.ZainbJalilAwad*** *for her scientific guidance, patience, and help during my study.*

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Contents

List of figures

List of tables

List of Abbreviations

SYMBOL	*DESCRIPTION*
HPLC	***High performance liquid chromatography***
TLC	***Thin layer chromatography***
M.p.	***Melting point***
FTIR	***Fourier Transform Infrared***
IR	***Inhibitory rate of growth***
L20B	***Genetically engineered mouse cell line***
amu	***Atomic mass unit***
NP_S	***Natural products***
^0C	***Degree Centigrade***
EPM	***Equine protozoalmyeloencephalitis***
LDL	***Low density lipoprotein***
ATP	***Adenosine triphosphate***
RA	***Rheumatoid arthritis***
PGE_2	***Prostaglandin*** E_2
AUC	***Area under the curve***
FLS	***Fibroblast-like synoviocytes***
fMLP	***N-formyl-methionyl-leucyl-phenylalanine***
PMA	***Phorbol 12-myristate 13-acetate***
PPAR	***Peroxisomeproliferation- activated receptor***
AA	***Arachidonic acid***
ml	***Millilitre***
$BMSC_S$	***Bone marrow stromal cells***
ALP	***Alkaline phosphatase***
R_f	***Retention factor (mobility relative to solvent front)***
R_t	***Retention time***
mRNA	***Messenger ribonuclic acid***
DF	***Dilution factor***
PBS	***Phosphate buffer saline***
FCS	***Fetal calf serum***
ELISA	***Enzym-Linked immunosorbant assay***
RPMI	***Roswell Park Memorial Institute***

ABSTRACT

Yucca aloifolia is a medicinal plant belong to the family Agavaceae.This plant contains several physiologically active compounds, it is rich source of steroidal saponins especially steroidal sapogenin (tigogenin).Due to their chemical structures, these compounds are of great pharmaceutical importance,since they are considered as starting material for the synthesis of steroidal drugs like cortisones.In addition to that, these compounds are used for the prevention and treatment of some diseases especially arthritis and rheumatism. Also, this plant has important biological activities, likeanti-inflammatory,antiviral,antifungal, antiprotozoal,antibacterial,antihypercholesterolemia,antioxidant,antiplatel etandAnticancer activities.

This study detects the presence of the most important steroidal sapogenin (tigogenin) in the ***Yucca aloifolia*** plant which is widely cultivated in Iraq. The absence of any phytochemical study concerning the tigogenin content in Iraq, and the industrial importance of tigogenin depending on its role as precursor in the synthesis of some steroidal drugs, this study acquired its value.

This study concerned with extraction, identification, isolation, and purification of biologically important steroidal sapogenin tigogenin from the leaves, stems and roots of ***Yucca aloifolia.***

Extraction of this steroidal sapogenin was carried out using different extraction methods. The preliminary identification of this steroidal sapogenin was done using thin Layer chromatography (TLC) trying different solvent systems whileLiebermann –Burchardreagentwas used for detection. This identification was further augmented by using high performance Liquid chromatography (HPLC). The most suitable

extraction, isolation and purification procedures ofsteroidal sapogenin were fully described in this study.

The identification of isolated tigogenin was carried out using melting point (**M.P**.), Thin Layer chromatography (**TLC**), infrared spectroscopy (**IR**) and **HPL**C.

In vitro study using cell line had been done and directed towards preliminary evaluation of cytotoxic activity of Yucca ***aloifoli*a** extract and comparing this activity with that exhibited by tigogenin isolated from ***Yucca aloifoli*a**.This cell line study was carried out using L20B cell line.

The results indicate that extraction of 50gm of dried (leaves, stems, roots) of***Yucca aloifoli*a** byextraction method NO.4 which includeextraction of Powdered plant materialsina soxhlet apparatus with 500 ml dichloroethane for 20-24 hours,then refluxed with 500 ml of 4N $H_2SO_4$for 3 hours and finally extracted withpetroleum ether (b.p 60-80 ^{0}C) in soxhlet apparatus for 10 hours, gives higher yields of extract than the other three extraction methods. In addition to that, TLC analysis revealed the presence of tigogenin compared with that of standardeight different solvent systems.HPLC quantitative estimation shows that tigogenin content in the extract obtained from method NO.4 is higher than that obtained from other extraction methods.

The results showed that both plant extract and tigogenin isolated from the plant exhibited significant cytotoxic activityon L20B cell line.

Natural products are products from various natural sources, plants, microbes and animals. Natural products can be an entire organism (e.g. a plant, an animal or a micro-organism), a part of an organism (e.g. leaves or flowers of a plant, an isolated animal organ), an extract of an organism or part of an organism and an exudate, or pure compound (e.g. alkaloids, coumarins, flavonoids, lignans, steroids and terpenoids) isolated from plants, animals or micro-organisms. However, in practice, the term natural product refers to secondary metabolites, small molecules molecular weight < 1500 atomic mass unit(amu), produced by an organism, but not strictly necessary for the survival of the organism.(1)

The use of natural products, especially plants, for healing is as ancient and universal as medicine itself. The therapeutic use of plants certainly goes back to the Sumerian and the Akkadian civilizations in about the third millenium BC. Hippocrates (ca. 460–377 BC), one of the ancient authors who described medicinal natural products of plant and animal origins, listed approximately 400 different plant species for medicinal purposes. (1)

Apart from natural-product-derived modern medicine, natural products are also used directly in the 'natural' pharmaceutical industry that is growing rapidly in Europe and North America, as well as in the traditional medicine programs being incorporated into the primary health care systems of many countries.(2)

There are three goals of using plants as sources of therapeutic agents in modern medicine. First is to isolate bioactive compounds for direct use as drugs, e.g., digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, and vincristine. Second is to use bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon (and other narcotic analgesics), taxotere, teniposide, and amiodarone,

which are based, respectively, on galegine, Δ^9-tetrahydrocannabinol, morphine, taxol, podophyllotoxin, and khellin. Third is to use the whole plant or a part of it as an herbal remedy, e.g., cranberry, echinacea, feverfew, garlic, ginkgo biloba, St. John's wort, and saw palmetto [3].

Natural products (NPs) have played an important role throughout the world in treating and preventing human diseases.

Natural Products have many pharmacological activities like anticholinergic, analgesics, antiparasitics, anti-inflammatory, antihypertensive,antioxidant, cardiotonic, anti-plateletand anticancer activity. [4]

Since the early 1940s, the searches for agents that may treat or ameliorate the scourge of cancer have involved all aspects of chemistry and pharmacology and throughout all these years, NPs have played an extremely important role. Initially, as the major source of drugs used for direct treatment; secondly as scaffolds upon which chemists would practice their skill and currently as modulators of specific cellular pathways in the tumor cell. [5]

In recent years the steroidal sapogenins(Spirostanesaponins) constitute a large group of glycosides, widely occurring in higher plants. They have found application in pharmaceutical industry mainly as precursors of corticosteroids, steroidalhormones, anticancer drugs and other steroidal drugs due to the simple way of degradation of the spiroketal moiety. The most common steroidal sapogenins used as raw materials for industry are diosgenin from *Dioscoreatokoro*,hecogenin and tigogenin from *Agave sisalana*, *yucca* plants species and other plants.[6]

In this study, Yucca*aloifolia* plant related to Agavaceae family contains many steroidal sapogenins as active constituents like tigogenin, hecogenin, sarsasapogenin and samogenin. [7]

The target of this research was tigogenin as one of the important steroidal sapogenin which is used in industrial pharmacy as a precursor For the synthesis of cortisone, sex hormone ,anticancer drugs and other steroidal drugs.[7],[8]

1.1-Agavaceae :

A family of 20 genera and 670species of short-stemmed, often woody plants distributed throughout tropical, subtropical, and temperate areas of the world. Members of the family have narrow, lance-shaped, sometimes fleshy or toothed leaves that are clustered at the base of each plant. Most species have large flower clusters containing many flowers. The fruit is a capsule or berry [9], [10].

1.2-The genus *yucca*:

Yucca is a genus of perennial shrubs belong to the agave family, (Agavaceae). They are notable for their rosettes of evergreen, tough, sword-shaped leaves and large terminal panicles of white or whitish flowers. They are native to the hot and dry (arid) parts of North America, Central America, South America, and the Caribbean[11].

This genus contains about 35–40 species. Many of them as ornamental plants, commonly cultivated in the tropical gardens of the World [12].The most common species are:

1- Y. gloriosa

2- Y. schidigera

3-Y.filamentosa

4-Y.elephantipes

5-Y. aloifolia

1.3-*Yucca aloifolia*

1.3.1-Classification:

Kingdom: plant

Division: Magnloiphyta

Class:Liliopsida

Subclass: Liliidae

Family: Agavacea

Genus: *Yucca*

Species: *aloifolia*

Scientific name: *Yucca aloifolia*

The word yucca comes from the island of Haiti. In addition, this plant is sometimes called Spanish bayonet for its large and sharp leaves. Other nicknames are beagrass, soapgrass and Aloe yucca [13].

1.3.2-Description of the plant:

Yucca aloifolia: is a shrub or tree-like evergreen plant with either single or clumped erect strong stems ranging in height from 4 to 9 meters.

Its leaves form a large rosette. They are stiff, sword-like, and quite long (up to 150 cm) with serrate marginal fibers. They have evergreen persistence, and alternate, simple, parallel arrangement [11].

Yucca is notable for its beautiful erect terminal clusters of white or white cream or purple flowers (up to 130cm tall), individual flower consist of six fleshy creamy or white petals, blossoms in early spring and develops seeds in summer They are usually aromatic during the night .[11]Figure (1-1), (1-2)

Yucca root has the appearance of a long tapered sweet potato.It has a rough brown rind on the outside with an inside that starchy and can range in color from white to brown. [11], [14].

The fruit is a dry cap split when it completes its growth and starts to expel the seeds or does not bloom. The pollination in the Yucca plant occurs with help of female's butterflies of *Yucca* plant. [15]

Figure(1-1): *Yucca aloifolia* flowers [11]

Figure(1-2)*: yucaaaloifolia*[11]

1.3.3-Distribution of *Yucca aloifolia*:

The natural distribution ranges of the *Yucca aloifolia* covers a vast area of North and Central America. From Baja California in the west, northwards into the southwestern United States, through the drier central states as far north as Alberta in Canada, and moving east along the Gulf of Mexico, and then north again, through the Atlantic coastal and inland neighboring states. To the south, the genus is represented throughout Mexico and extends into Guatemala,to North –western Arizona and southern Nevada[(12)].

Yucca has adapted to an equally vast range of climatic and ecological conditions. [(14)]

It is to be found in dry rocky deserts, badlands, prairies, grassland, mountainous regions, light woodland, coastal sands, in subtropical and semi-temperate zones, although this is nearly always arid to semi-aridYucca are widely cultivated in the western US as a landscape plant. In the years from 1897 to 1907, Carl Ludwig Sprenger created and named 122 Yucca hybrids. In Iraq, yucca plant is cultivated in gardens [(16)].

1.4-Folkloric uses of *yucca* plant:

Native American tribes in the southwestern United States and Northern Mexico found numerous uses for the *yucca*, dating back hundreds of years. Several tribes, including the Western Apaches on the Fort Apache Reservation in Arizona, use the plant today. The most common uses seem to be for hygiene. Where the roots of the yucca are pounded to remove extracts that are made into shampoo and soap. In addition also yucca leaf fibers are used to make dental floss and rope. [(17)]

Historically, Western Apaches mixed ground juniper berries with yucca fruit to make gravy. They also made a fermented drink from juniper

berries and yucca fruit pounded to a pulp and soaked in water. Other Native American groups washed their hair with yucca extract to treat dandruff and loss. In addition; they used a mixture of soap made from yucca sap and ground aster to wash newborn babies to stimulate hair growth. [17]

Americans also used the soapy leaves from yucca as poultices or for baths for skin sores and sprains as well as to treat burns and abrasions [18].

The extract of the *Yucca* is also used as an additive in natural pet foods. It is reported to speed up bowel elimination, reduce fecal and urine odor, and improve digestion in dogs and cats. It can also be added to pet food as a spray or drops. Several studies also show that when added to animal feed, *Yucca* extract can reduce noxious ammonia gas in the waste products of poultry, pigs, cows, and horses. A decrease in ammonia levels can increase egg production in chickens and milk production in dairy cattle [19].

Native Americans also used yucca plants for a variety of other non-medical purposes, including making sandals, belts, cloth, baskets, cords, and mats. Such uses can still be found today among some Indians tribes [20].

Navajos would tie a bunch of yucca fibers together and use it as a brush for cleaning metates. A number of commercial uses for yucca extract have been found, including adding it to root beer, alcoholic beer, and cocktail mixers as a foaming agent. The bittersweet dark brown extract is also used as an additive in ice cream and other foods `if added in little amount [20].

In Central and South America, yucca root is traditionally used to control and harvest fish populations. In sufficient quantities, the saponins in yucca are toxic and will stun or kill fish. To accomplish this, fishermen add a large

quantity of mashed yucca to the water. As the saponins affect the fish, they float to the surface, where the fishermen collect them. [21]

Yucca saponins are used for their surfactant activity in a commercial product for tempering grains .Tempering is a chemically facilitated process by which moisture is added to grains prior to further processing. [22]

1.5-Pharmacological activity of *yucca* plant:

1.5.1-Anti-arthritic and Anti-inflammatory activity:

Yucca products have been used for many years for reputed anti-arthritic effects, both by Native Americans and more recently by the pharmaceutical industry, where manystudies on the anti-arthritic effects of yucca were reported that symptoms of pain and swelling in arthritic human patients were relieved by consumption of yucca tablets [23-25].

The *yucca* is rich in steroid-like saponins that elevate the body's production of cortisone; this possibly explaining the herb's ability to help in arthritic pains. [21, 24]

Recent research suggests another possible mode of action of yucca in preventing arthritis by anti-inflammatory activity. Where Yucca contains many anti-inflammatory compounds (steroidal sapogenins)like hecogenin, tigogenin, sarsasapogenin, gitogenin, samogenin,and smilagenin. It also contains polyphenolics such as resveratrol and yuccaols A, B, C, D and E [26,27].In additions yucca saponins have the ability to break up inorganic mineral obstructions and deposits. It also blocks the release of toxins from the intestines, which inhibit normal formation of cartilage. [7]

*Yucca*saponins are also found to be as effective as metronidazole in killing giardia tropozoites in the intestine [28].

1.5.2-Antiviral activity:

Yucca leaf contains protein that found to inhibit the growth of herpes simplex virus types 1 and 2 and human cytomegalovirus [29].

1.5.3-Antifungal activity:

Steroidal saponin glycosides from the stems, leaves, and flowers of Yucca have demonstrated antifungal effects against a number of human pathogens in vitro [30-32].

1.5.4-Antiprotozoal activity:

Yucca Saponins have pronounced antiprotozoal activity. The mechanism of the antiprotozoal effects is that saponins form irreversible complexes with cholesterol in protozoal cell membrane causing damage to the integrity of the membrane and cell lysis ([33].This has been well demonstrated with rumen protozoal in vivo.[33],and in vitro[34].The antiprotozoal activity requires the intact saponin structure with both the nucleus and side chain(s) present.

Yucca saponins are effective in killing the giardia tropozoites in the intestine [35].

An interesting possibility is that yucca saponins may control the protozoa that cause the fatal disease equine protozoal myeloencephalitis (EPM). This disease has been reported from throughout North America [36-38].The protozoal organism causes this disease has been isolated and named *Sarcocystisneurona*[39].

1.5.5-Antibacterial activity:

It was observed that *Yucca* extract (YE) stimulated growth of *Prevotellaruminicola*, whereas the growth of *Streptococcus bovis*wassuppressed. The antibacterial properties were most pronounced against Grampositive bacteria [40, 41].

The mode of action of antibacterial effects of *yucca* saponins seems to involve membranolytic properties, rather than simply altering the surface tension of the extracellular medium. Thus, their inhibitory activity is associated with adsorption to microbes and is, therefore, influenced by microbial population density [42].

It has been observed a concentration-dependent growth response of *E.coli*K-12 to yucca saponins, with growth promoting activity at low concentrations and inhibition at higher levels. Thus, the impact on a mixed bacterial population such as in the rumen is difficult to predict. [43]

1.5.6-Cholesterol-lowering activity:

It has been known for many years that yuccasaponins form insoluble complexes with cholesterol [44].Saponins form micelles with sterols, such as cholesterol and bile acids. The hydrophobic portion of thesaponin (the aglycone or sapogenin) associates (lipophilic bonding) with the hydrophobic sterol nucleus, in a stacked micellar aggregation [45].

Cholesterol-lowering properties of saponins in humans are of obvious interest. There is little clinical trial information. Bingham observed a reduction in serum cholesterol levels in human patients receiving yucca tablets for arthritis relief. This seems to be the only study reported inwhich a saponin product has been given directly to human subjects [46].Dietary yucca extracts lower total and LDL cholesterol level in hypercholesterolemic humans [47].

1.5.7-Antioxidant activity:

In vitro antioxidant activity has been evaluated for extracts of whole plant yucca as well as for phenolic and stilbene constituents. [48-51]. Free radicals scavenging assays and other techniques have demonstrated antioxidant activity greater than the reference quercetin. In addition inhibition of lipid peroxidation and nitric oxide NO generation has been demonstrated. [48 - 51]

In another study yucca extract exhibit a protective effect against nitrate-induced oxidative stress in rats, a decrease in methemoglobin,andtissue nitric oxide levels, as well as increases in glutathione and positive histological findings, were also shown.[52]

1.5.8-Antiplatelet action:

Reservatrol and yuccaols reduced thrombin-induced platelet aggregation in vitro. [53, 54]

1.5.9-Anticancer activity:

Reservatrol-derived gloriosaols have shown anti-proliferative and apoptotic-inducing activity in solid tumor and leukemia cell lines. [55] Aqueous alcoholic extracts of Yucca flowers have exhibited antitumor activity against B16 melanoma in mice. Some studies had revealed that differences in saponin structure including the type and number of sugar moieties attached by a glycosidic bond at C-3 influence biological responses. [121]

Systematic fractionation of the extract by means of solvent extraction and gel permeation chromatography led to separation of two galactose containing

polysaccharide fractions with marked inhibitory activity against B16 melanoma, but ineffective against L1210 and P388 leukemias in mice. [56]

1.6-Active constituents of *Yucca aloifolia*:

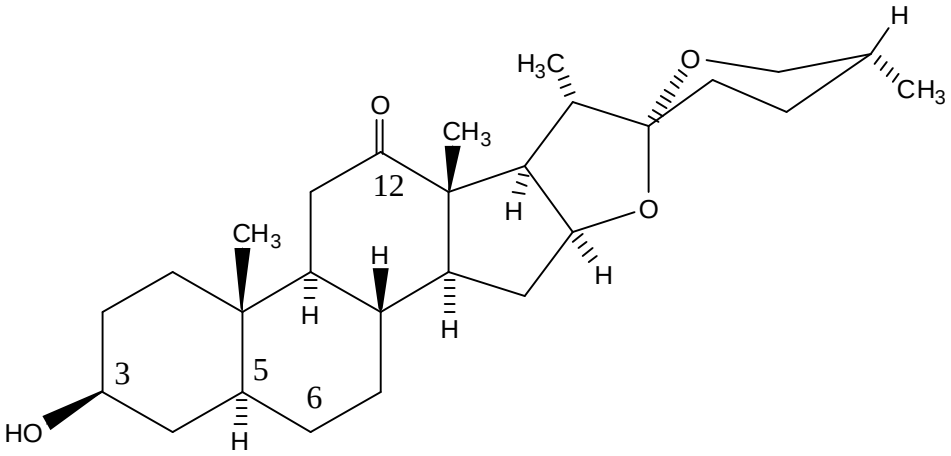
The yucca plant contains several physiologically active phytochemical. It is a rich source of steroidal saponins, and is used commercially as a saponin source [57]. Most species of yucca plant have the same active constituents. [7]

The roots, flowers and leaves of the yucca are rich in steroidal saponin glycosides consisting of a sapogenin and one or more sugars. Most species contain sarsasapogenin, tigogenin, furostanol, and spirostanol [48,49,58-62].

Yucca aloifolia leaves contain up to 1.4%tigogenin (a compound that can be used in the commercial synthesis of steroidal hormones),sarsasapogenin, gitogenin, hecogenin, smilagenin, neotigogenin, neogitogenin and samogenin[7].as in figure (1-3)

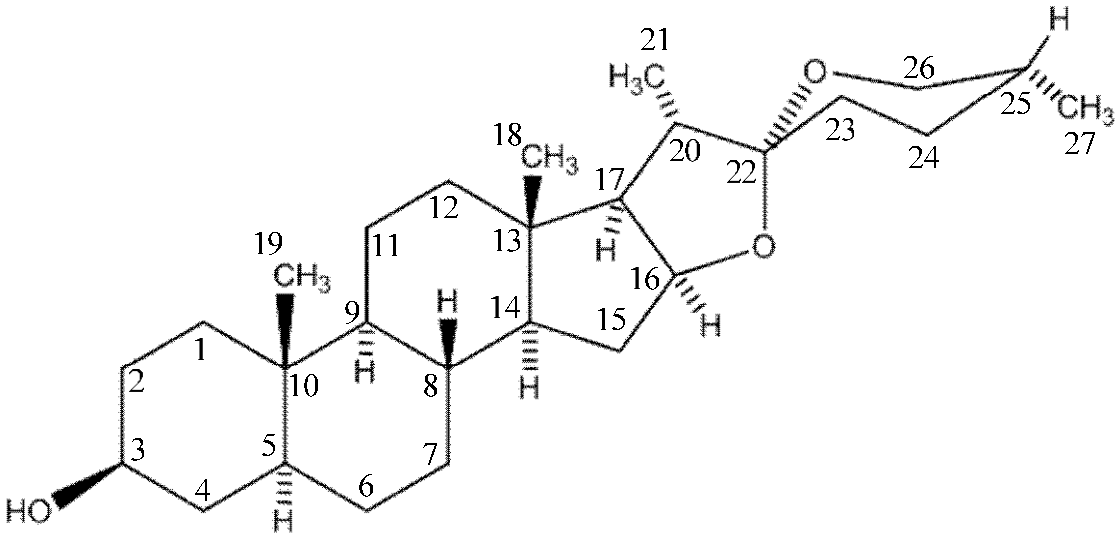
The flowers contain aloifoline is specifically active against Lewis lung – tumour as well as other transplanted mouse neoplasms. The seeds contain indole melanins. [7]

Phenolic compounds (novel yuccaols, gloriosaols and resveratrol) and spirostane aglycones are numerous in yucca species [49,55,57,63].as in figure (1-3).Also this plant has quite a large amount of vitamins A, C and B complex, Considerable amount of iron, potassium, phosphorous, manganese, and copper .[56,64,65]



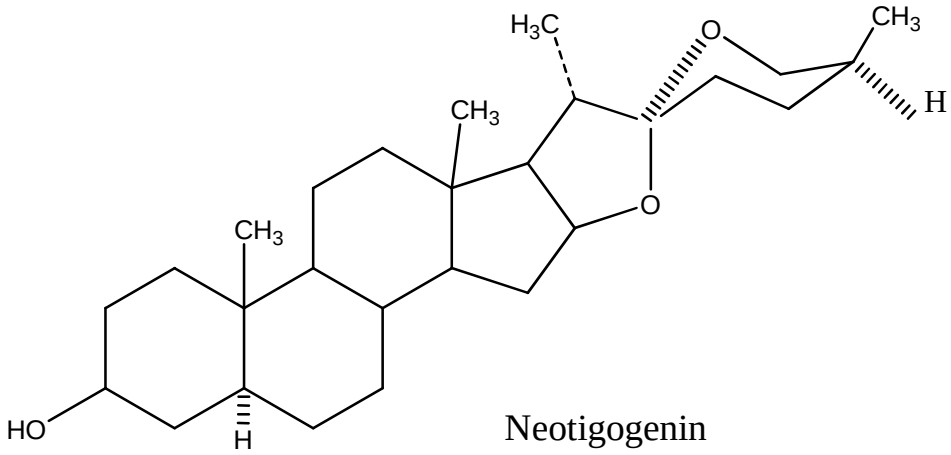
hecogenin

(5α-spirostan-3ß-ol-12-one)[72]



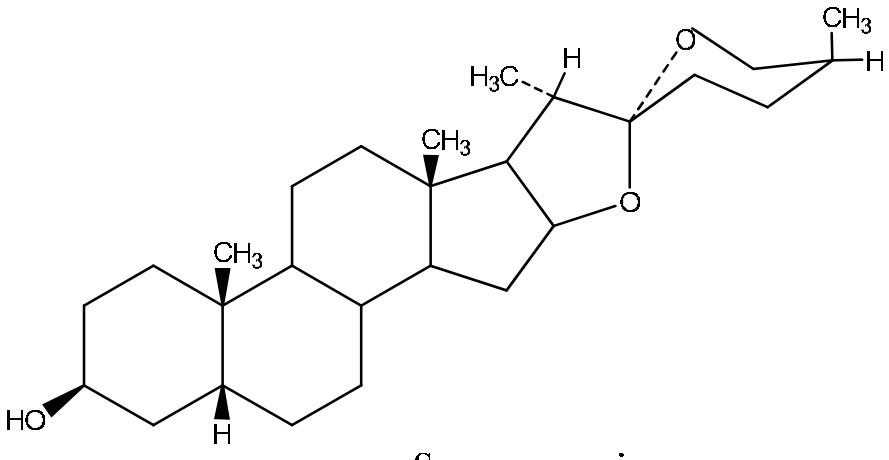
tigogenin

(25R)-5alpha-Spirostan-3beta-ol [(94)]



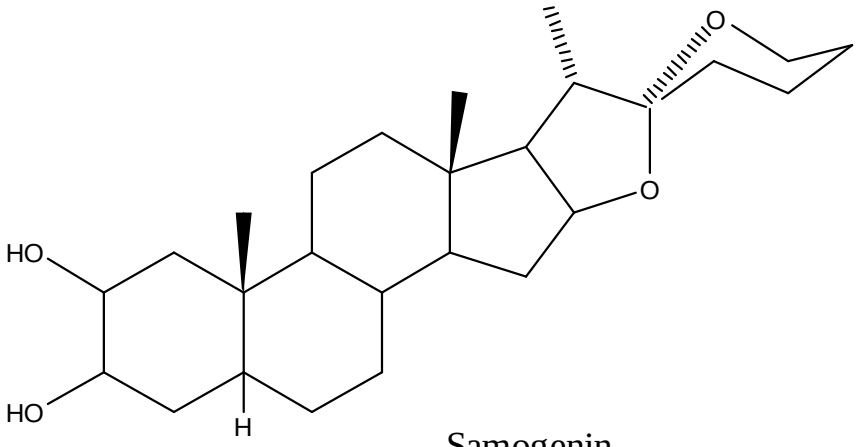
Neotigogenin

(3**β**,5**α**,25*S*)-spirostane-3-Ol[(66)]



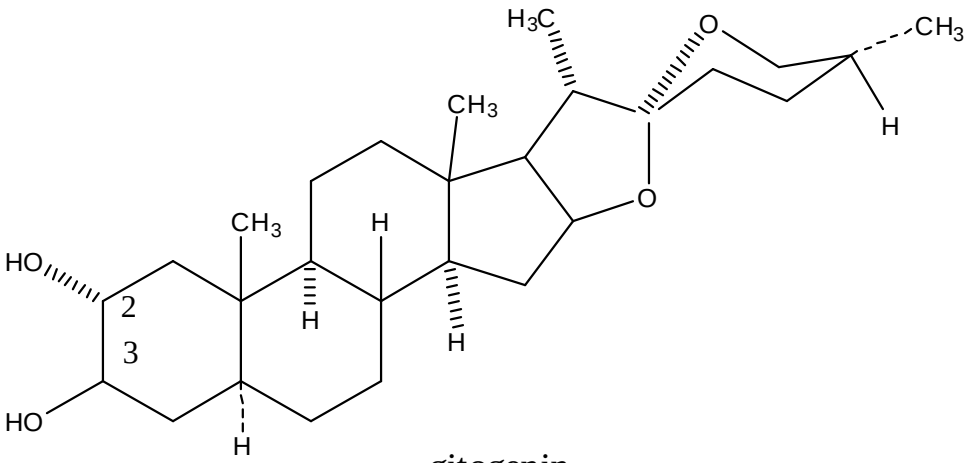
Sarsasapogenin

(3β,5β,25*S*)-spirostan-3-ol[(94)]



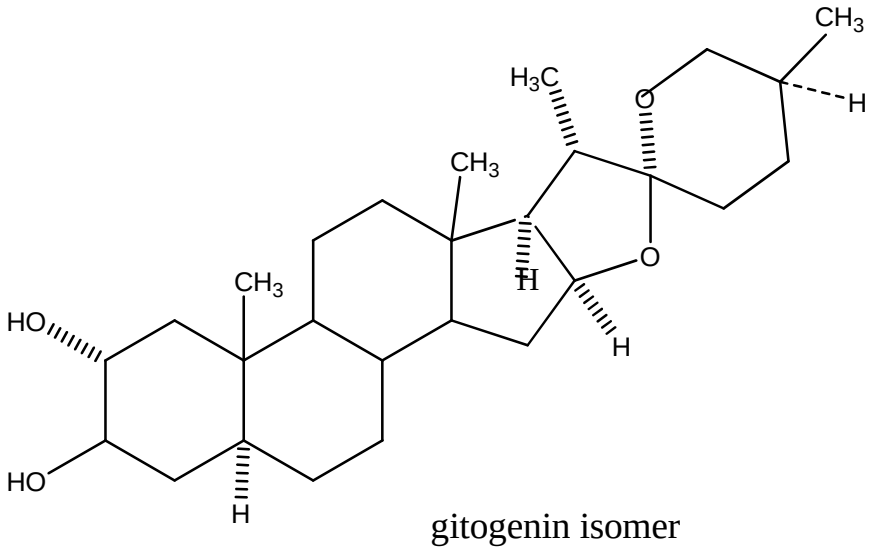
Samogenin

(25R)-5β-Spirostane-2β,3β-diol[67]



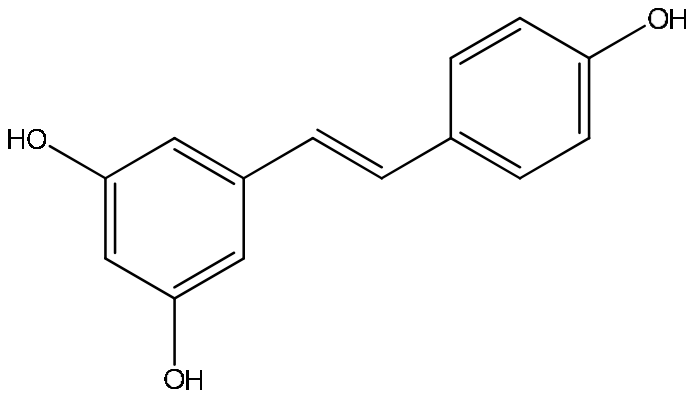
gitogenin

(3β,5α,25R)-spirostane-,3-diol[68]



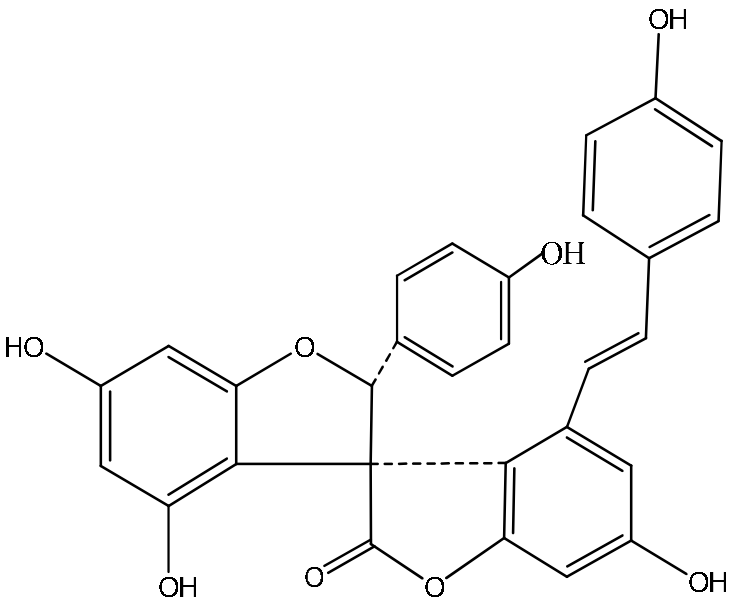
gitogenin isomer

(2α, 3β,5α,25S)-spirostane- 2,3-diol[68]

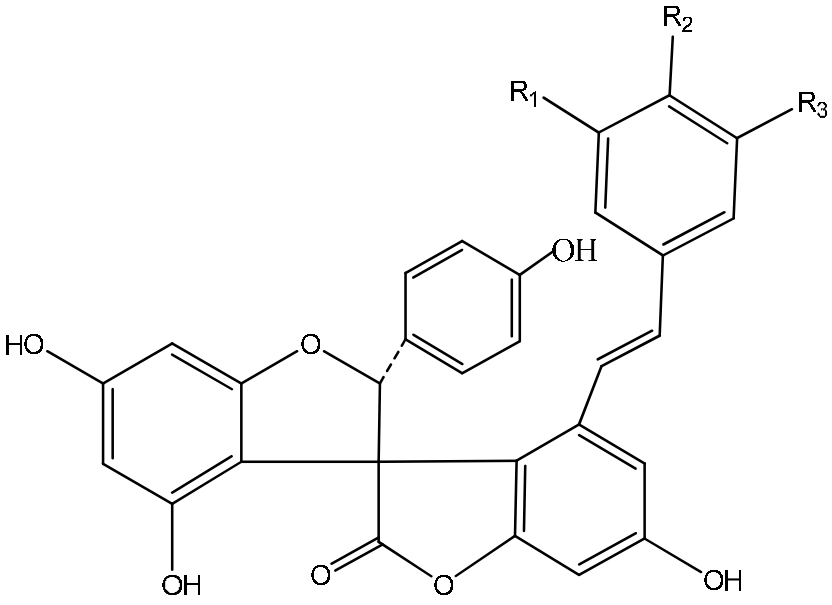


Resveratrol

3,4',5-trihydroxystilbene[69]



Yuccaol A



Yuccaol B R_1=H R_2=OH R_3=H
Yuccaol C R_1=OH R_2=OMe R_3=OH (69)

Figure(1-3): chemical structures of some active constituents of *Yucaaaloifolia*.

1.7-Saponins:

Saponins are generally known as non-volatile, surface active compounds that are widely distributed in nature, occurring primarily in the plant kingdom. The name ‘saponin’ is derived from the Latin word sapo, which means ‘soap’, because saponin molecules form soap-like foams when shaken with water. They are structurally diverse molecules that are chemically referred to as triterpene and steroid glycosides. They consist of non polaraglycones coupled with one or more sugar moieties [(70)]. This combination of polar and non-polar structural elements in their molecules explains their soap-like behavior in aqueous solutions, which shows itself as milk like exudates in some plants [(71)].

Saponins have a diverse range of properties such as sweetness and bitterness [(72)], foaming and emulsifying properties, in addition to the biological and pharmacological properties like: Haemolytic, Molluscicidal, Insecticidal, Antifungal, Antibacterial, Antiparasitic, Antiviral, Cytotoxic, Anti-inflammatory, Antioxidant, Antispasmodic, Anti-ulcerogenic, Adaptogenic, Vaccine adjuvant, Hepatoprotective, Neuroprotective, Anti-hyperlipidemic, Cognition enhancing, Aphrodisiac, and Woundhealing activities. Saponins have found wide applications inbeverages,confectionery, in cosmetics as well as pharmaceutical products [(73)].

Saponin content in the plants depends upon many factors such as the cultivar, the age, physiological state, and geographical location. In fact, the same species may vary in saponin composition and quantity when it is grown in different places [(74, 75)].

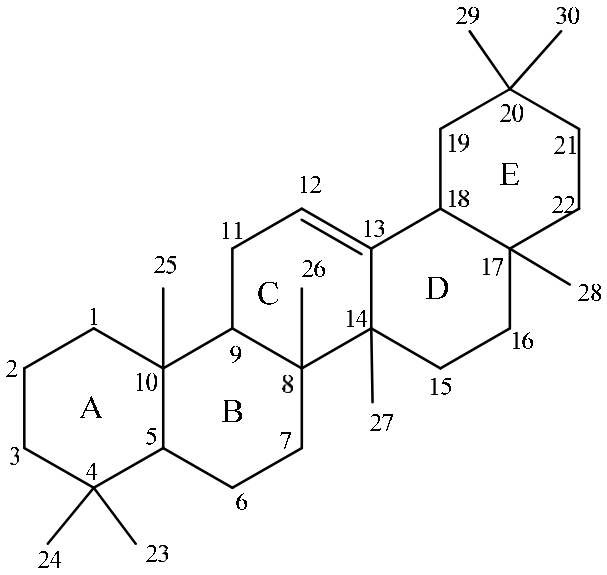
Saponins are localized in plant organelles that have a high turnover rate, which implies that they are not only metabolically active but they may be important regulatory substances in the development of an organism. It seems

that the advantage of saponins to the plant producing them is that they may function as protecting agents, growth regulators, and allele chemicals [74, 75].

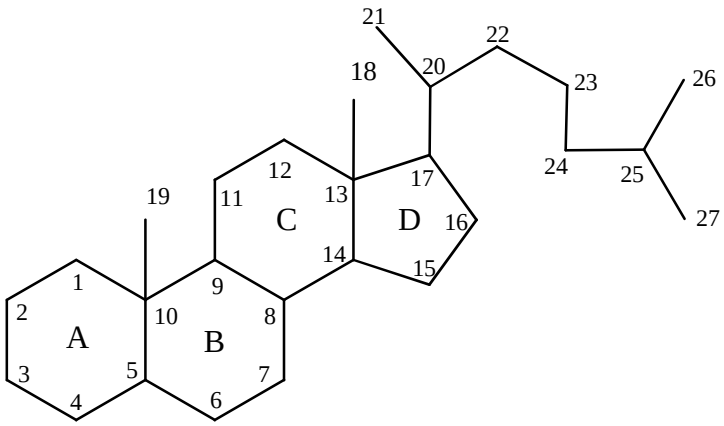
Saponins glycosides have a high molecular weight and are often occur as complex mixtures with components differing only slightly from one another in the nature of the sugars present, or in the structure of the aglycone. Saponins glycosides are hydrolyzed by acids to yield free aglycone (sapogenin), and various sugars and related uronic acids [9].

The saccharide chains commonly contain glucose, arabinose, rhamnose, xylose and glucuronic acid. These sugars can be attached as one; two or three sugar chains and the term monodesmoside, bidesmoside and tridesmoside have been given to these saponins, respectively [74].

According to the structure of the aglycone or sapogenin, two types of saponin are recognized these are; the steroidal and pentacyclic triterpenoid types (figure1-4). Both of the two kinds have a glycosidal linkage at C-3 and a common biogenic origin via mevalonic and isoprenoid units [9].



Pentacyclic triterpenoid skeleton



Steroid skeleton

Figure(1-4):type of saponins[9].

The steroidal saponins are less widely distributed in nature than the pentacyclic triterpenoids type. The phytochemical surveys have shown their presence in many monocotyledonous families ,particularly the Dioscoreaceae,Agavaceae, and smilaceae. In the dicotyledons, steroidal saponins occur in leguminosae and solanaceae families [9].

Steroidal saponins are of great importance and interest, owing to their relationship with such compound as sex hormones, cortisone, diuretics,

steroids, vitamin D and the cardiac glycoside. Some are used as starting material for synthesis of these compounds [9].

All steroidal saponins have the same configuration at the spiro center C-22, but stereoisomer at C-25 exist, e.g. tigognin and neotigognin and mixtures of these two sterioisomers co-occur in the plant [76].

1.8-Biosynthesis of Steroidal saponine:

Sterols and tigogenin biosynthesis has been achieved via mevalonic acid pathway.

Firstly, the acetate converted to acetyl COA via COA-SH ,then acetyl COA condensed with another molecule of acetylCOA to give acetoacetyl COA, the last one condensed with another molecule of acetyl COA to obtain 3-Hydroxy-3-methyl glutaryl-COA ,then this compound converted via 2NADPH to mevalonic acid , after that the mevalonic acid undergoes phosphorylation, decarboxylation with aid of ATP to create Isopentyl pyrophosphate then to Geranyl pyrophosphate , Geranyl pyrophosphate coupled with Isopentyl pyrophosphate to give Farnesyl pyrophosphate [77].

Farnesyl pyrophosphate is coupled with itself to create squalene, this catalyzed by squalene synthase, which is considered a potential requlatory point.

For sterol biosynthesis so that the suppression of sterol biosynthesis has been correlated with a reduction in squalene synthase enzyme activity [78].

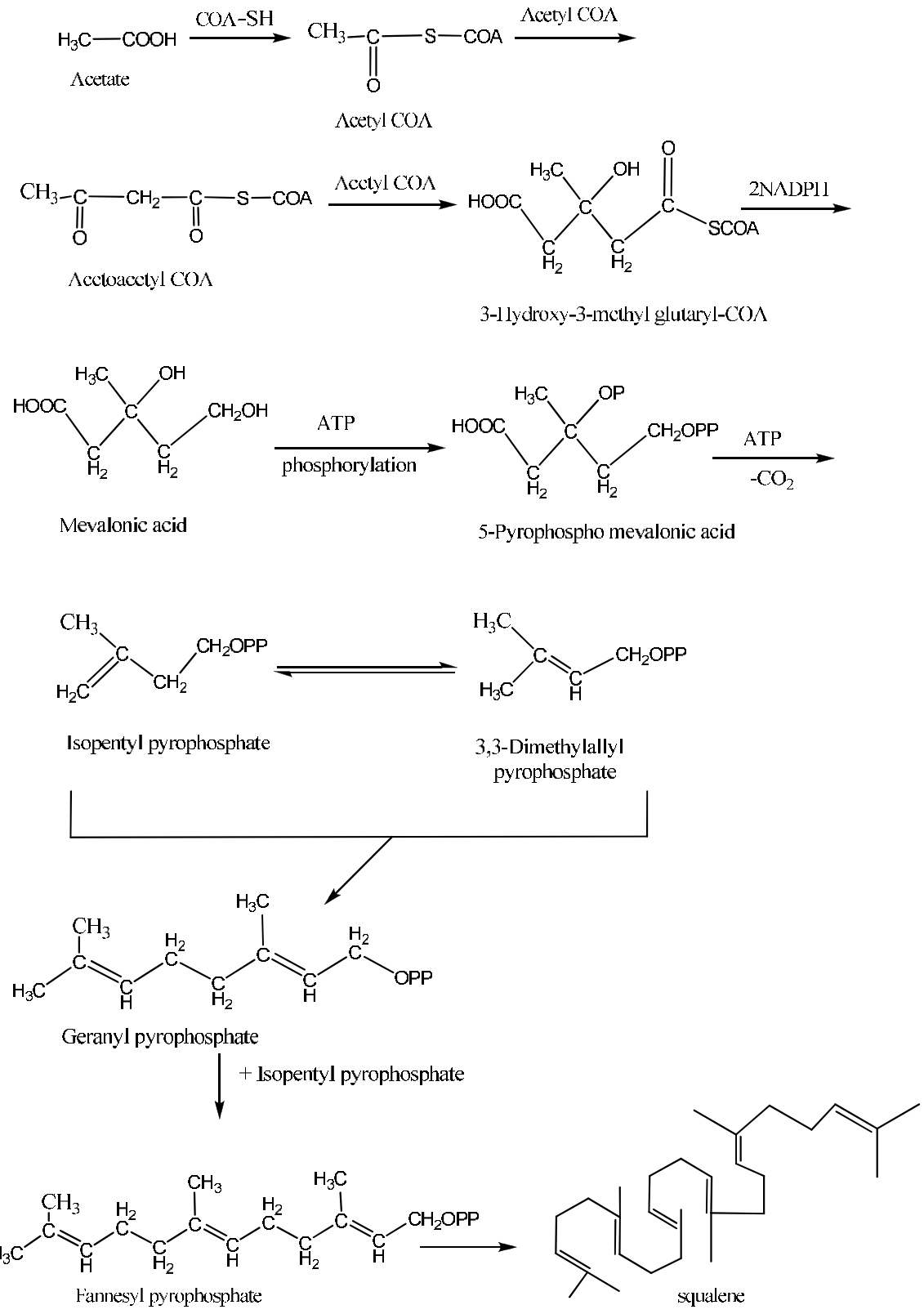
Squalene is converted by squaleneepoxidase, a mixed function oxygenase requiring O2 and NADPH, into squalene -2, 3-oxide.

The next step is cyclization of squalene-2, 3-oxide. This is a reaction which, part form photosynthesis itself, distinguishes photosynthetic organism from non-photosynthetic organisms.

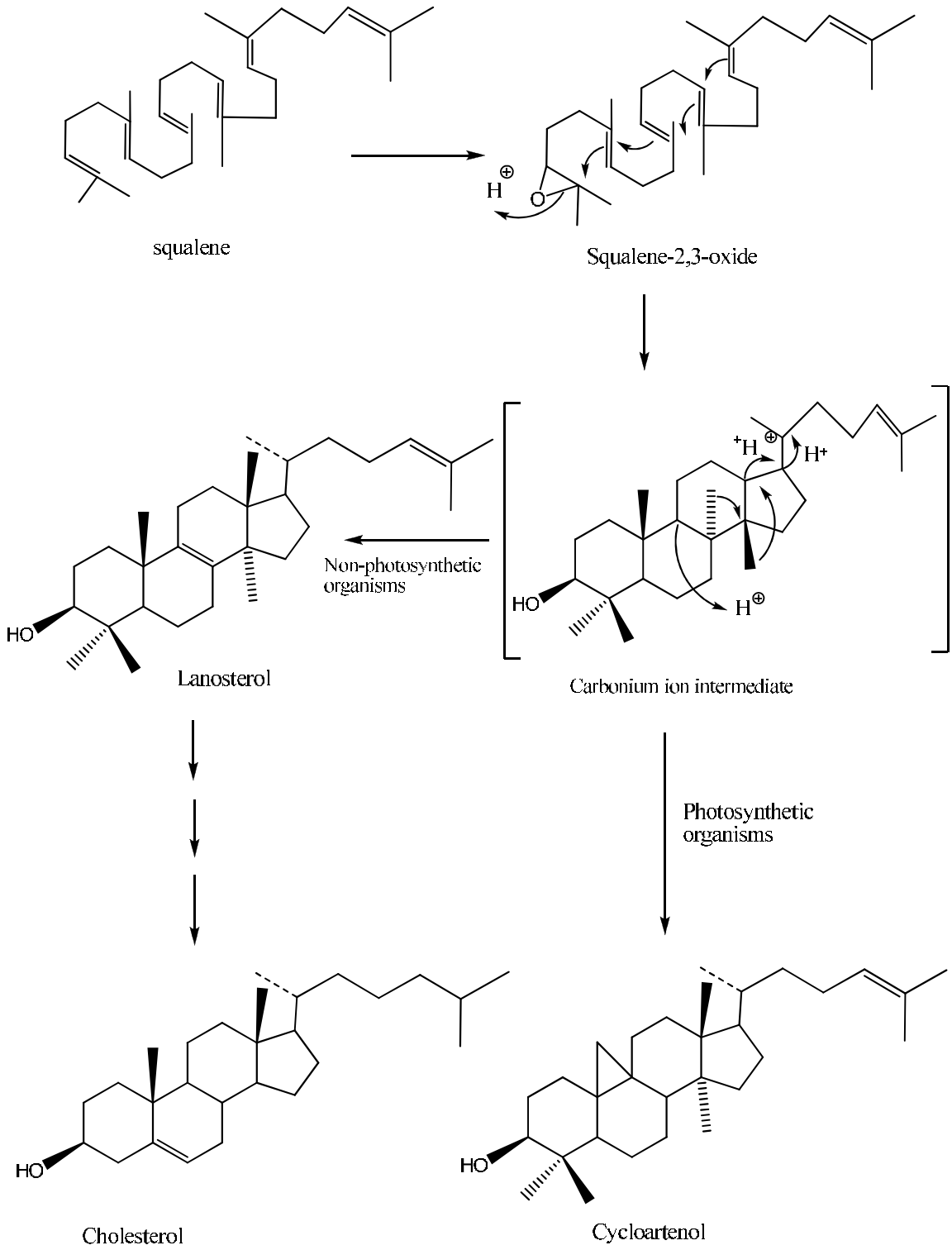
In photosynthetic organisms (e.g. higher plants, algae) the cylcization product is cylcoartenol whilst in non-photosynthetic organisms (e.g. fungi, animals) the product is lanosterol. The cyclization of squalene-2,3-oxide to cycloartenol is catalyzed by squalene -2,3-oxide: cycloartenolcyclase [74,79].

The widely publicized view that cholesterol is synthesized via cycloartenol in plants and vial lanosterol in animals [77](figure1-6).

Cycloartenol I the precursor of higher plant steroids. Through its conversion to 24-methylene cycloartenol or cholesterol, cycloartenol is the precursor of phytosteroids(figure1-5 and 1-6).



Figure(1-5):Formationofsqualeneinsterolsbiosynthesis[9,77]



Figure(1-6): Biosynthesis of cholesterol [9, 80]

Cholesterol is the precursor of the C_{27}sapogenins which have spiroketal structure [79,81].

1.9-Toxicity and side effects:

Little is known about the toxicity of yucca saponins, and the effect of long-term ingestion is not well defined. Saponins are generally considered to be poisonous to lower forms of life, but are nearly nontoxic to humans when taken orally. However, their injection into the bloodstream causes hemolysis, even at extreme dilutions [58]. A 12-week feeding study in rats found Mohave yucca extract to be essentially nontoxic [82]. Studies evaluating the hepatoxicity and nephrotoxicity of steroidal saponins in sheep found major adverse renal effects, including tubular necrosis and hemorrhage, with increases in serum creatinine and urea. On histological examination, biliary crystals attributed to unhydrolyzedsaponins were found in the liver and bile ducts [83].

The standard dosage of concentrated yucca saponins is two to four tablets or capsules a day. Yucca concentrate is also available as a tea, with the usual dosage being 3–5 cups a day. Capsules and tablets are commonly sold in doses of 500 milligrams. A bottle of 30, 60, 90, or 100 units costs $6–10 and can usually be found in health food stores [84].

Saponins extracted from yucca plants are generally considered safe when used in traditional doses. In recent years, the only reported minor problems are rare cases of **diarrhea** and **nausea**. Some people who are sensitive to plant allergens may develop a mild skin rash from contact with yucca sap [84].

1.10-Tigogenin:

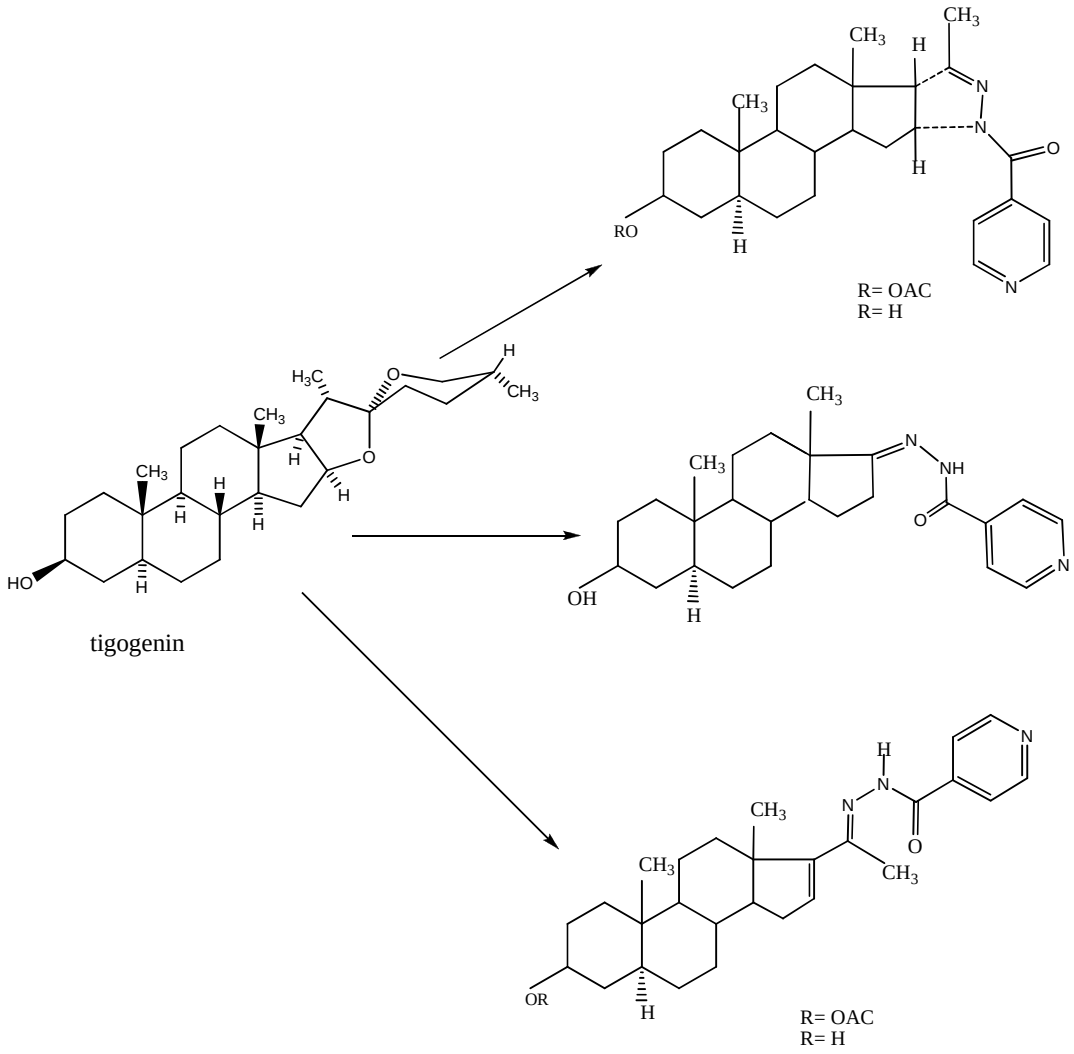
Tigogenin is one of the important steroidal sapogeninused in pharmaceutical industry [73].its chemical structure consist of steroid framework as its key organic feature that has 5trans -fused rings A/D with 5-membered saturated lactone moiety at C-17 position that has important structural feature. The steroid nucleus of tigogeninhas hydroxyl group at C-3 position which is the sugar attachment site [85].According to the industrial importance of tigogenin was choosing for this study.

1.11-The synthesis of some steroidal drugs from Tigogenin:

Steroidal hormones have a variety of pharmaceutical activities, and the demand for steroidal medicines increases 6-10% every year worldwide; of these compounds, estrin amounts to 20%, androgen and anabolic steroids amount to 5-10%, and corticosteroids amount to more than 60%.The raw materials used in the production of these steroids come from natural plants and animals, for example, mainly hecogenin and successively tigogenin [86].

1.11.1-Synthesis of 5α-KetosteroidHydrazones derivatives which have Antituberculosis Activity:

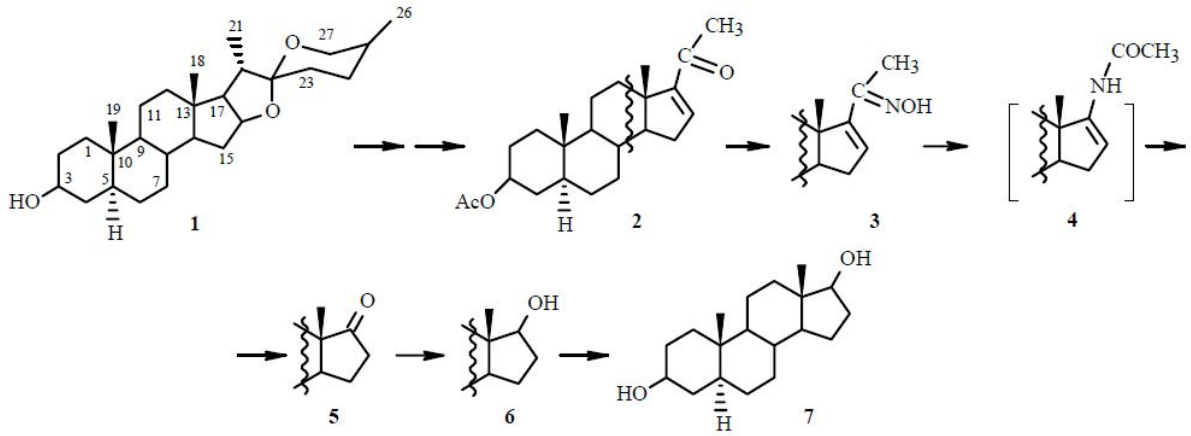
Isonicotinoylhydrazones and thiosemicarbazones of some 5α-ketosteroids were synthesized from tigogenin, theirantimycobacterial activities were studied against *Mycobacterium tuberculosis* and it was shown that some of the synthesized isonicotinoylhydrazones exhibit high antituberculosisactivityas in scheme (1-7). [87]



Scheme(1-7): Synthesis of 5α-ketosteroidhydrazones from tigogenin

1.11.2- Synthesis of 5α-androstan-3β,17β-diolfromtigogenin.

5α-Androstan-3β, 17β-diol (3β-adiol), a known inhibitor of prostate cancer cell growth, was synthesized from tigogenin as in scheme (1-8) [8, 88, 89].

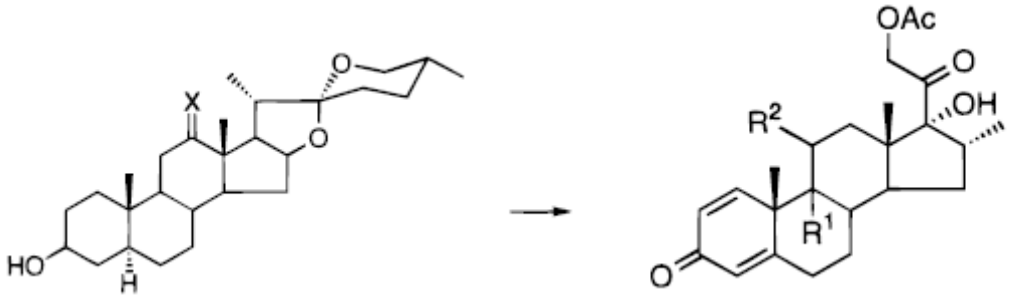


Scheme (1-8): **Synthesis of 5α-androstan-3β, 17β-diol from tigogenin**

1-Tigogenin,2-pregnenolone acetate,3-epiandrosterone acetate,6 -3β-acetoxy-5α-androstan-17β-ol,7-5α-androstan-3β,17β-diol.

1.11.3-The synthesis of Dexamethasone Acetate from tigogenin:

Investigation for synthesizing dexamethasone acetate from tigogeninbegan in 1988, dexamethasone acetate being the most widely used as an anti-inflammatory and antiallergic high-effect corticosteroid and the annual demand for it being about 5 tons worldwide,as in scheme(1-9) [86].



R^1=α-F,

R^2=β-OH

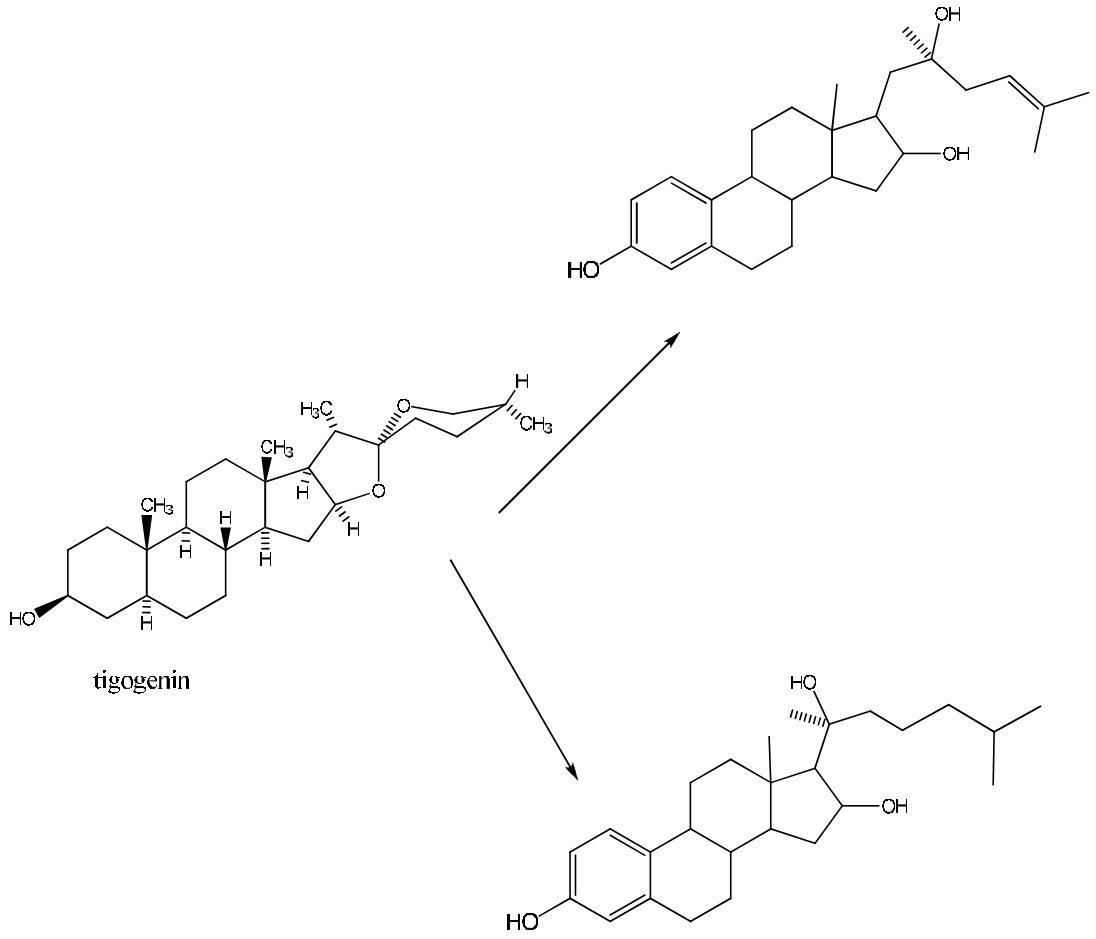
X=H_2

Tigogenin

Scheme (1-9): **The synthesis of Dexamethasone Acetate from tigogenin**

1.11.4-Synthesis of 13,16,20-Polyoxygenated Steroids of Marine Origin and Their Analogs From tigogenin.

These unnatural polyoxygenated steroids analogs and have antitumor activity against three tumor cell lines (breast cancer, MCF 7, lung cancer NCIandoral cancer KB) as in scheme (1-10) .[90]



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Scheme (1-10):Synthesisof 13,16,20-polyoxygenated steroids from tigogenin.

1.12-Pharmacological activity of tigogenin:

1.12.1-Antiarthritic activity:

Tigogenin is found to have inhibiting effect on human rheumatoid arthritis synovial cell survival by increasing apoptosis, P38 mitogen-activated protein kinase activity and up regulation of cyclooxygenase-2.Apoptosis is considered to be one of the mechanisms that regulate autoimmune diseases such as rheumatoid arthritis (RA) [(91)].

Therefore, stimulation of apoptosis with inhibition of the growth of human RA fibroblast-like synoviocytes (FLS) might be useful for the treatment of RA [(92, 93)].

It has been demonstrated that tigogenininhibites proliferation and induced apoptosis of human RA FLS. Moreover, the rate of apoptosis induced by tigogenin was associated with overexpression of COX-2 correlated with overproduction of endogenous PGE2 [(94)].

1.12.2-Effect of tigogenin on the stimulus coupled responses of human neutrophils.

Tigogenin was found to dose-dependently inhibit N-formyl-methionyl-leucyl-phenylalanine (`fMLP), phorbol 12-myristate 13-acetate (PMA), and arachidonic acid (AA)- -induced superoxide generation in human neutrophils by suppressing the receptor-mediated activation and protein kinase C-mediated activation of superoxide generation in neutrophils and the mechanism may involve suppression of the tyrosyl phosphorylation of 45 kDa protein[(95)].

1.12.3- Anti-obesity effect:

Tigogenin inhibits adipocytic differentiation and induces osteoblastic differentiation in mouse bone marrow stromal cells (BMSCs).

It markedly inhibited BMSCs to differentiate into adipocyte and decreased the visfatin secretion and expression levels of peroxisome proliferation- activated receptor (PPAR) and ap2. Since tigogenin can inhibit the expression of (PPAR) an early key transcription factor in the signaling cascade during adipogenesis, it possibly prevents adipogenesis of BMSCs in the early phase[96,97].

Other mechanism showed that alkaline phosphatase (ALP) activitywas significantly elevated in the presence of tigogenin. Similarly, the mRNA expression of other osteoblasticphenotypes,such as Cbfa1(identified as essential transcription-gene factor),collagen type 1 andosteocalcin, also increased following the addition of tigogenin. These results in osteoblastic differentiation suggest that tigogenin is able to differentiate BMSCs into an osteoblastic lineage without adding any osteogenic factors [98,99].

Such conclusion is further strengthened by the evidence that tigogenin increased the matrix calcium deposition in BMSCs slightly but significantly. Accordingly tigogenin might have protective effect on bone and be helpful in preventing the development of osteoporosis by inhibiting adipocyte formation and stimulating osteoblast formation from BMSCs [96].

1.12.4-Hypoglycemic effect:

It has been found that tigogenin in high dose (0.45g/kg produced significant decrease in blood sugar, but in small dose (0.23g/kg) such effect is slightly demonstrated [100].

1.13- Aims of the work:

1- Extraction of the plant saponin glycosides by different methods: in order to estimate the feasibility of each method.

2- Quantitative estimation of tigogenin in Iraqi ***Yuccaaloifolia***.

3- Isolation, purification and identification of steroidal sapogenin(Tigogenin).

4- Confirmation of the isolated compounds using physicochemical and spectral analysis.

5-In vitro evaluation the cytotoxic activity of ***Yucca aloifolia*** plant extract and pure isolated tigogenin on L20B cell line.

2.1- Experimental notes

2.1.1- Instruments and materials:-

1- Electrical sensitive balance: Sartorius / Germany.

2-Ultraviolet light of 254 nm and 366 nm wave lengths(Desaga,Heidelberg,Germany) .

3-Buchi rotatory evaporator attached to vacuum pump.(Swiss)

4- Chiller: Ultratemp 2000, Julabbo F30.(Italy)

5- Oven: Memmert 854 / Germany.

6- Melting point was determined by electro–thermal Melting point (Stuart / UK).

7- IR Analysis was carried out in department of chemistry, college of science ,Al-MustansiriyaUniversity. FT-IR spectra were recorded on Shimadzu FT-IR-84005 Infrared Spectrometer.

8- HPLC Analysis was carried outusingShimadzu 2007 instrument.(Japan)

, in the IBN-SINA statecompany,Repuplic of Iraq,Ministary of Industry and Minerals,Iraq,Bahgdad.

9- chromatographic materials:

A- Analytical TLC:

Silica gel GF 60; layer thickness 0.25mm; 20x20cm aluminum cards; made by MERCK, Germany.

B- Preparative TLC:

Preparative TLCwas performed on 20x20cmprecoated glass sheets of 1mm silica gel containg fluorescent indicator UV 254,madebyMERCK, Germany.

2.1.2- Chemicals:

The chemicals used in this study are listed with their suppliers in table (2-1).

Table (2-1): chemicals used with their suppliers

Chemicals	supplier
Acetic anhydride	Synchemica
Acetonitrile (HPLC grade)	Sigma-Aldrich, Germany
Ammonia 25%	BDH,Ltd.Poole,England
Benzene	GCC. UK
Chloroform	BDH, Ltd. Poole ,England
Dichloroethane	GCC. UK
Ethanol 99.8%	GCC. UK
Ethyl acetate	GCC. UK
Hydrochloric acid 37 %	GCC. UK
Methanol	GCC. UK
Petroleum ether (60 – 80 ^{o}C)	BDH, Ltd. Poole , England
Sulfuric acid	BDH, Ltd. Poole , England
Toluene	BDH, Ltd. Poole , England
Acetone	BDH, Ltd. Poole, England
2-Propanol	BDH, Ltd.poole, England
n-Hexane	BDH, Ltd.Poole, England
Activated charcoal	E.MERCK, Darmstadt, U.S.A.
1,2-dichloroethane	ANOVCHEM
Tigogenin standard	Amplachemicals,China
Sodium bicarbonate	SEELZE-HANOVER,Germany

2.2- Plant material:

The leaves, stems and roots of ***Yucca aloifolia***plant (Agavaceae) were collected from the garden of ***Asst. Prof. Dr. ZeinabJalielAwad's***house (Bahgdad,Plastine street) during September and October (2010).Plant parts cleaned and dried in oven at a temperature between (30-40) ^{0}C for (4-5) hours. The driedplant material was coarsely powdered using mechanical grinder.

2.3-Expeimental work

The experimental work is divided into:

2.3.1 -Extraction of steroidal sapogenin.

2.3.2 -Preliminary identification of steroidal sapogenin.

2.3.3 -Fractionation by column chromatography.

2.3.4 -Isolation and purification of steroidal sapogenin.

2.3.5 -Identification and characterization of the isolated steroidal sapogenin.

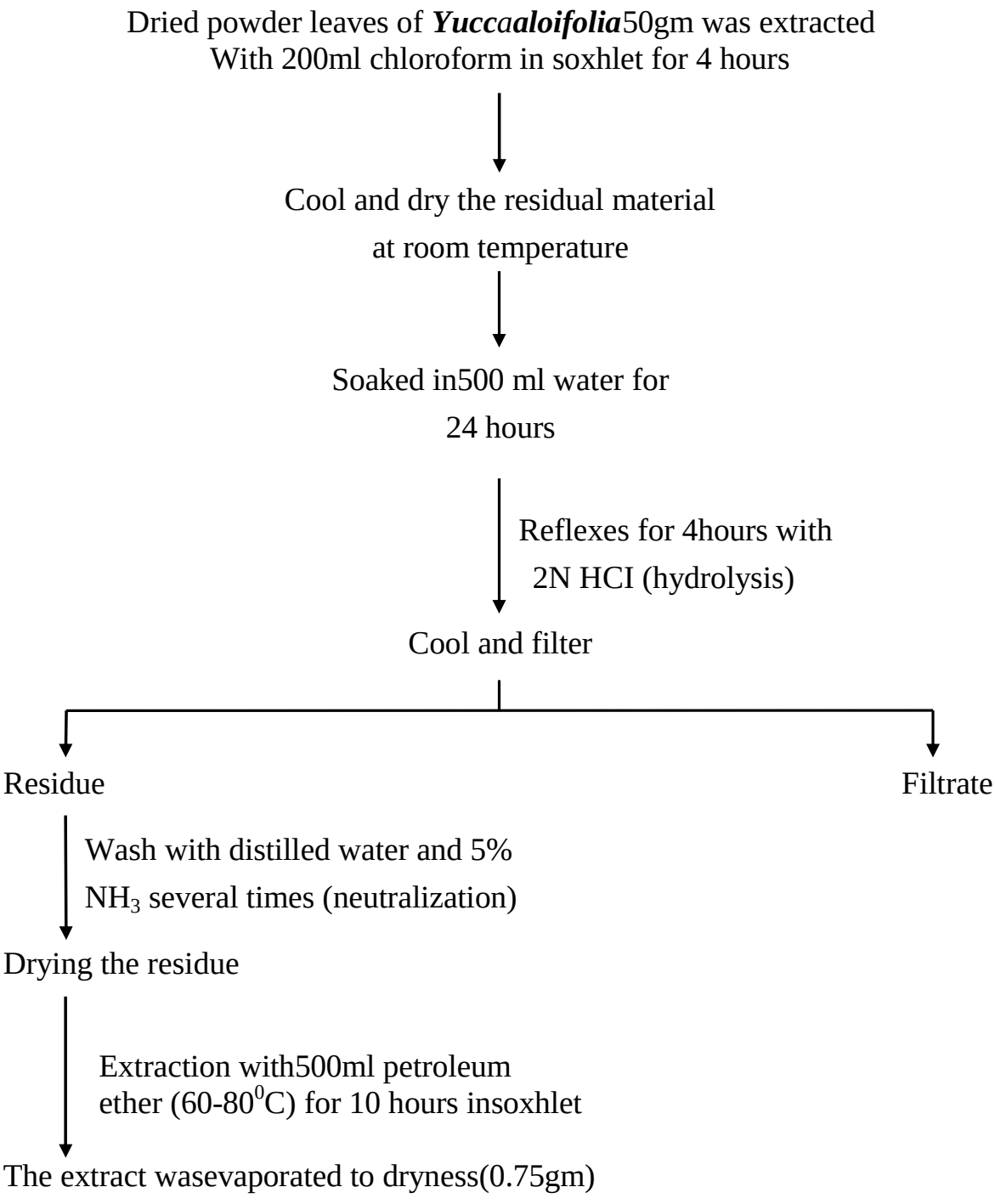
2.3.1-Extraction of steroidal sapogenin:

Extraction of steroidal sapogenin(tigogenin) was carried out using four different methods:

2.3.1.1-Extraction method NO.1:[101,102]

A 50gm of the dried powdered leaves of *Yucca aloifolia* was extracted with 200ml chloroform in soxhletapparatus for 4 hours.The residual plant material was dried at room temperature, and then soaked in water500ml for 24 hours at room temperature, then the plant material was filtered and the residue was acidified with 2 N HCl 500ml reflexed for 4 hours. The mixture was cooled and filtered.The residue was washed with distilled water and 5% NH_3 until neutralization.

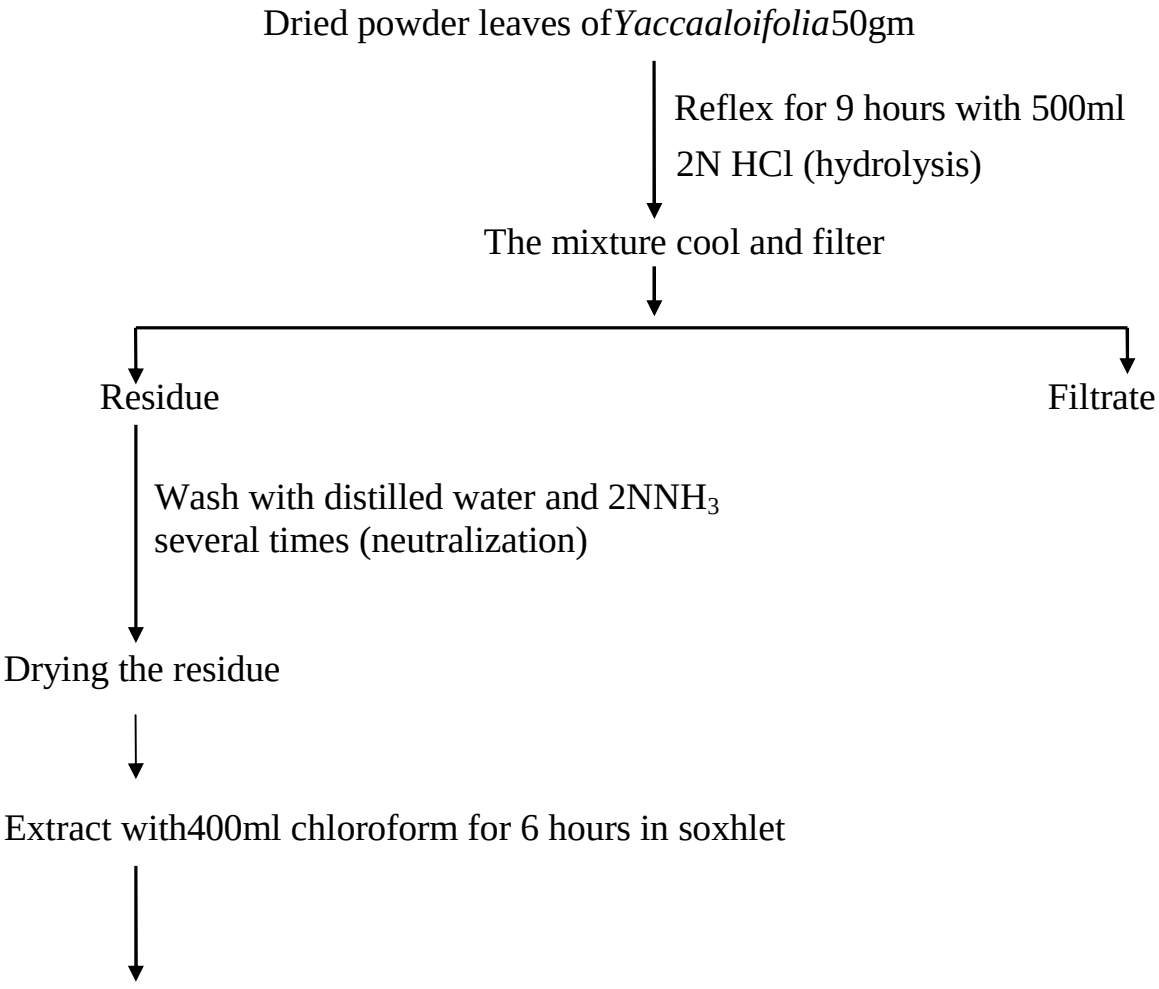
The residue was dried at room temperature over night and then in a hot air oven (at 60 ^{0}C) until complete dryness.The dried residue was extracted with 500ml petroleum ether (60-80 ^{0}C) in a soxhlet extractor for 10 hours and theextract was evaporated to dryness under vacuum, and then subjected to identification, as shown in Scheme2-1:



Scheme (2-1): **General scheme for method No.1 for extraction of steroidal sapogenin from the leaves of *Yucca aloifolia*.**

2-3-1-2. Extraction method No.2 :[103]

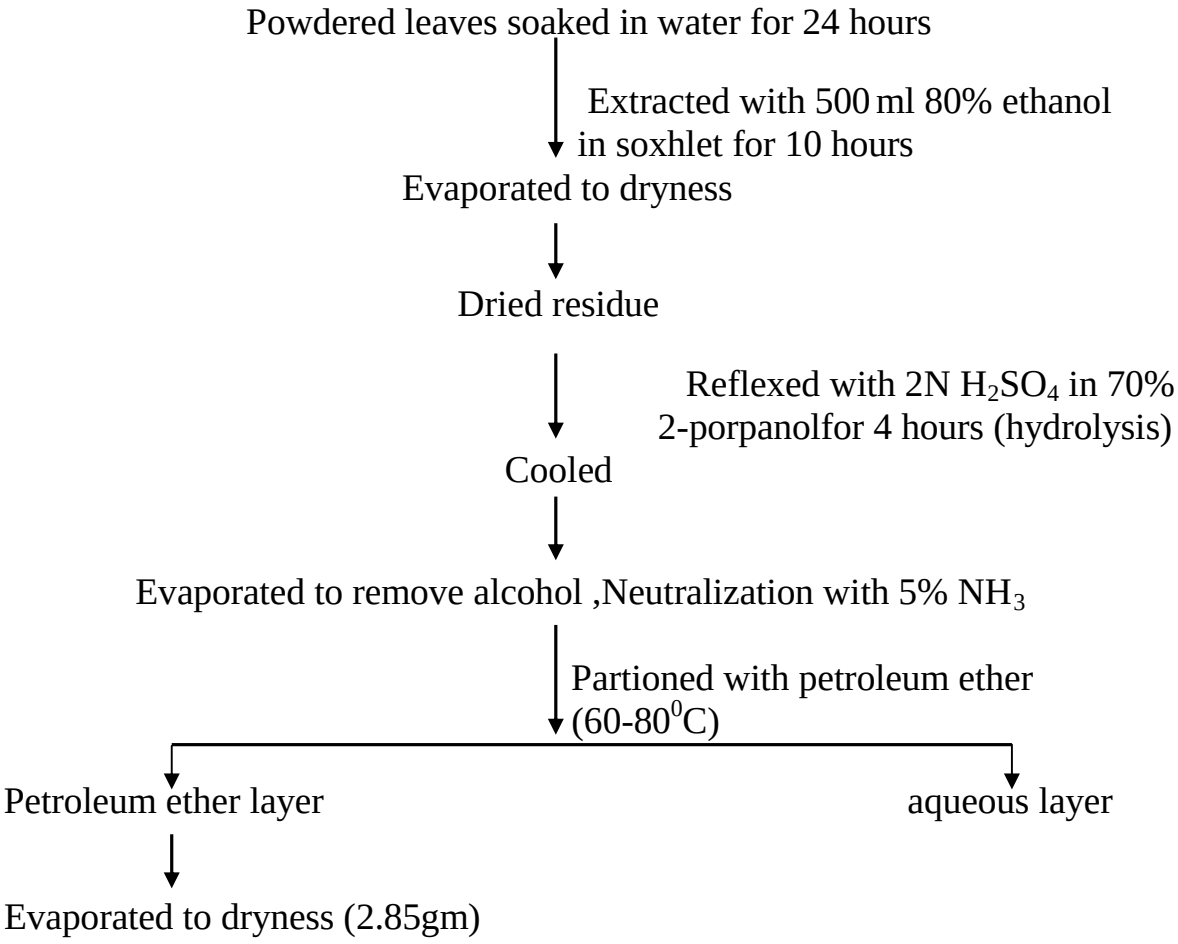
A 50 gm of the dried powdered plants leaves were hydrolyzed under reflex for 9 hours with 500 ml 2N HCl. The mixture was cooled to room temperature andfiltered.The residue was washed with distilled water and 2N NH_3 solution respectively until neutralization. Then the residue was dried over night at room temperature.The dried residue was extracted with the 400 ml chloroform usingsoxhlet apparatus for 6 hours. The extract was evaporated to dryness under vacuum, and then subjected to identification as shown in Scheme(2-2):



Scheme (2-2): General scheme for method N0.2 for extraction of steroidal sapogenin from the leaves of *Yucca aloifolia*.

2-3-1-3. Extraction method No.3: [104,105]

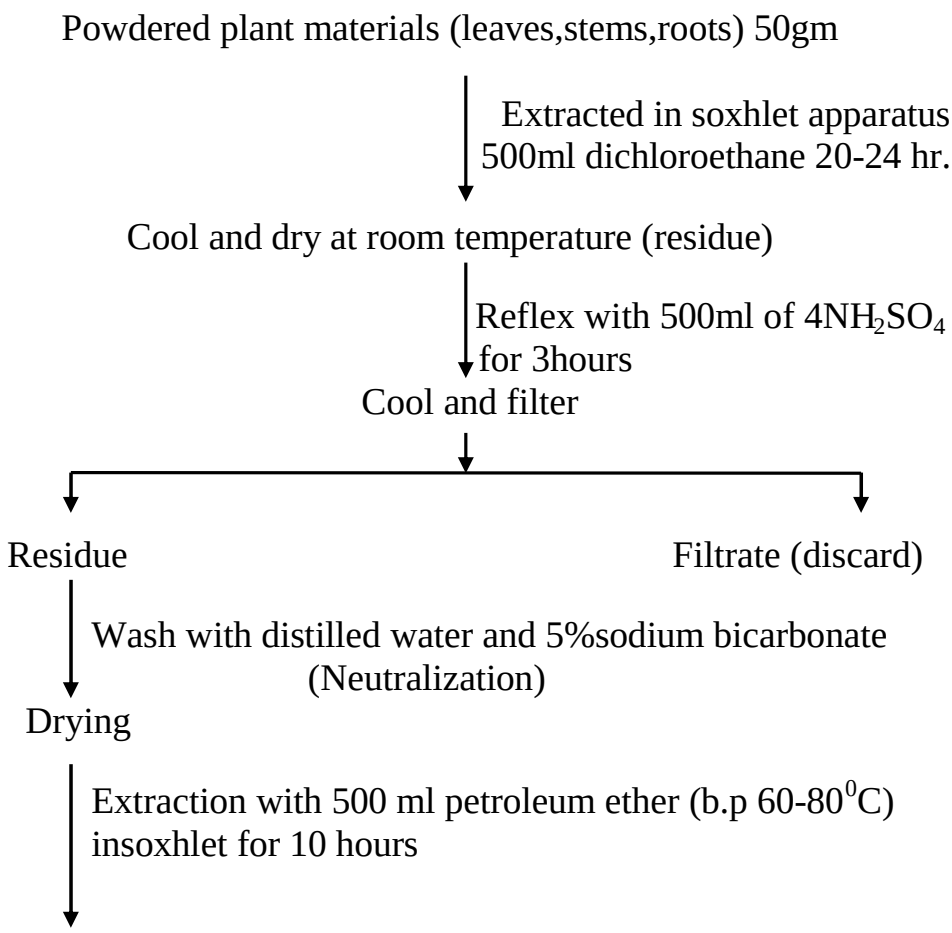
A 50 gm of dried plant material (leaves) was soaked in water for 24 hours and then extracted with 500ml 80% ethanol in soxhlet extractor for 10 hours. The residue was evaporated to dryness under vacuum. The dried residue was reflexed with500ml 2N H_2SO_4 in water containing 70% 2-propanol at 100 ^{0}C for 4 hours. The mixture was filtered and the filtrate was evaporated under vacuum to remove the2-propanol completely.Theaqueous residue was neutralized with 5%NH_3.Theneutralizedsolution was extracted with equal volume of petroleum ether (b.p.60-80^{0}C) using sepratary funnel to give two layers, aqueous layer and the petroleum ether layer.The petroleum ether layer wasseparated,dried with anhydrous Na_2So_4 and evaporated to dryness under vacuum.as shown in Scheme(2-3):



Scheme (2-3):General scheme for method NO.3 for extraction of steroidal sapogenin from the leaves of *Yucca aloifolia*

2-3-1-4. Extraction method No.4:[106]

A 50 gm of dried powdered plant materialsleaves,roots,and stems were extracted in a soxhlet apparatus with 500 ml dichloroethane for 20-24 hours. themarc was dried, with then refluxed 500 ml of 4N $H_2SO_4$for 3 hours. After cooling the mixture was filtered, the marc was washed with water, neutralized with 250ml 5% sodium bicarbonate solution to pH 7.5.The water was removedby evaporation at 100-105C^0. The dry hydrolysate(the residue) was extracted with petroleum ether (b.p 60-80^{0}C) in soxhlet apparatus for 10 hours. The extract was evaporated todryness under vacuum.as shown in Scheme(2-4):



Scheme(2-4):General scheme for method NO.4 for extraction of steroidal sapogenin from the leaves,stems and root of *Yucca aloifolia*.

2.3.2-Preliminary identification of steroidal sapogenin:

The preliminary identification ofsteroidalsapogenin of crude extracts of powdered leaves obtained from the extraction methods No.1, 2, 3 and 4 and the crude extracts of powdered stems and roots obtained from extraction method No.4 was performed by using:

2.3.2.1- Thin Layer chromatography (TLC):

The thin Layer chromatography (TLC) was carried out using the following requirements:

2.3.2.1.1- Silica gel :[107]

Ready made plates of silica gel GF 254 (20×20 cm) of 0.25 mm thickness (MERCK) were used, and then the plates were activated at 110^{o}C for 10 min. before used.

2.3.2.1.2- Developing solvant systems:

A 100 ml volume of the solvent system was placed in a glass tank (22.5 cm × 22 cm× 7cm), and covered with glass lid and allowed to stand for 45 min.for saturation before use.

Different solvent systems were used for detection of steroidalsapogenin (Tegogenin).[108-112]

S_1=Hexane:Acetone (90:10)

S_2 = Chloroform:Methanol(95:5)

S_3= Petroleum ether (b.p.60-80^{o}C):Ethylacetate (75:25)

S_4=Benzene:Acetone (90:10)

S_5= Chloroform:Petroleum ether (b.p.60-80C^{0}):Methanol (85:10:5)*

S_6= Chloroform:Acetone (80:20)

$S_{7=}$ Hexane:Ethylacetate (90:10)

S_8= Hexane:ethanol(98:2)

*This solvent system was prepared by trial not depend on certain reference.

2.3.2.1.3-Reagent usedand It'smethod of preparation:

*** Liebermann–Burchard reagent is used in this study andprepared as follow**:[113]

5 mL of acetic anhydride and 5 mL ofconcentrated sulfuric acid was addedcarefullyto 50 mL of absolute ethanol, while cooling in ice. Spray the developed plate and heat it at 100^{o}C for 5–10 minuts.

Freshly prepared chloroform solution of the standared reference (tigogenin), andchloroform solution of the final products obtained from extraction methods were applied to the TLC plates manually,using capillary tubes,in form of spots and allowed them to dry ,then developed by the ascending technique.The solvent migration limit is being 13-15 cm from the base line .

After development, the plates were dried and detection was done by the above reagent.

2.3.2.2-HPLC Analysis :[114]

Qualitative and quantitative estimation of steroidal sapogenin :

Qualitative and quantitative estimations of Tigogenin component in the crude extract obtained by all extraction methods was carried out using high performance liquid chromatography (HPLC) .The identifications was made by comparism the retention time of sapogenin obtained from crude extract with that ofauthenticstandardat identical chromatographic conditions .

The following equation was used to calculate the percentage of the compound in the plant: -

$$\text{Percentage of compound in the plant} = \frac{(\text{AUC of plant sample / AUC of the standard}) \times \text{Conc. St} \times \text{DF} \times 100}{\text{Weight of the dried plant used in the extraction}}$$

Where:-

The weight of the dried plant used in the extraction = 50 gm

AUC = Area under the curve. DF = Dilution factor.

Conc. St. = Concentration of the standard used in HPLC.

2.3.2.3- HPLC conditions ofTigogenin:

1- Mobile phase : Acetonitrile 100%
2- Column:PhenomenexODS250 mm x 4.6 mm, 5 µm particle size.
3- Column temperature: Ambient
4- Flow rate : 1 ml / min
5- Injection volume : 5 µL
6- Injection concentration: 1 mg /ml

Detection: UVDetector at λ 209 nm.

2.3.3-Isolation and purification of steroidal sapogenin:

2.3.3.1-Fractionation by column chromatography:

The final residue obtained from the leaves by extraction method NO.4(best method) was subjected to column chromatography by using glass column (80 cm x 5 cm) packed with silica gel (0.063-0.200 mm) slurry in (250)ml $CHCl_3$,in a ratio of 20 gm of silica gel to each 1 gm of the residue. A dry loading of the sample (residue) was used by dissolving it in small volume of chloroform and adsorbing it on small amount of silica gel of the same grade used for packing the column, then dried, grinded and applied to the column in order to prevent clogging.The column was eluted by gradient elution technique using $CHCl_3$:methanol with an increasing percentage of methanol from zero to 100% (the ratios of $CHCl_3$:methanol used were 100:0, 95: 5,90:10,85:15,80:20,70: 30,and 60:40, $CHCl_3$:methanol).The column developed by adding 50 ml of each eluent with collecting 5 ml fractions,then monitored by TLC usingS2as mobile phase . A total number of 100 fractions were obtained. Those consecutive fractions, which have the same number of spots with the same R_f values, were combined and evaporated to dryness to get major fractions.

2.3.3.2-Using preparative TLC plates:

Isolation of steroidal sapogenin (tigogenin) is carried out by using preparative TLC which was performed by using ready made plates of 20x20cm, which are coated by silica gel GF 254 layers of 1mm thickness. (Merck).

The major fraction (F_5) obtained by column chromatography was applied as a concentrated solution in a row of spots using capillary tube four times on each plate (the spots should dry before the next application).The solvent systems (S2, S5, S6) was each placed in a glass tank (22.5 cm X 22 cm X 7 cm),and covered with a glass lid and allowed to stand for 45 minutes before

use for saturation.Butthe best solvent used from these three solvent is the S_5 since it givegood distance between bands in preparative TLC plate.

The detection was done usingLiebermann-burchared reagent in one side of the plate. The band corresponding to the tigogenin standard was scraped out and collected in a beaker, mixed with chloroform: methanol (95:5), stirred and left a side for one hour, then filtered. After evaporation of the solvent, the obtained residue was subjected to co-chromatography with the available reference standard of Tigogeninusing different mobile phases foridentification.

2.3.3.3-Purification of steroidal sapogenin:

The isolated compound obtained fromPreparative TLC was dissolved by heating with sufficient quntity of methanol and a small amount of decolorizing charcoal was added to the hot methanol solution until the supernatant liquid was almost colorless. Then the hot solution was filtered. the solvent was evaporated to give solid product [115].

2.4.-IdentificationandCharacterization of the Isolated steroidal saponins:

2.4.1- TLC:-

Analytical TLC was performed by using ready made plates. The purified sapogenin was applied on silica gel plate as one spot of the compound by using capillary tube along with it 's standard, using different mobile phases.

2.4.2- Melting point:-The melting points of the purified sapogenin were done and compared with that of the available standard tigogenin.

2.4.3 – FT-IR:-

Infrared spectra were recorded inKBr disc.

2.4.4 – HPLC analysis.

HPLC conditions ofTigogenin:

1- Mobile phase : Acetonitrile 100%
2- Column:PhenomenexODS250 mm x 4.6 mm, 5 µm particle size.
3- Column temperature: Ambient
4- Flow rate : 1 ml / min
5- Injection volume : 5 µL
6- Injection concentration: 1 mg /ml

Detection: UVDetector at λ 209 nm.

2.5 -The cell line

Cell line study of the *Yucca aloifolia*plantwas carried out in Biotechnology Research Centre/ AL-NahrainUniversity.In this study the preliminary screening on growth inhibition or cytotoxic activities of isolated tigogenin and crude extract from *Yucca aloifolia*were carried out.The study of cytotoxicity was examined on L20B cell line.the investigation included the comparative study between the cytotoxic activity of plant crude extract and that of pure tigogenin isolated from*Yuccaaloifolia*.

2.5.1- Instruments

Instruments used in this study and their manufacturers and suppliers arelisted in table (2-2).

Table (2-2): Instruments used in this study and their manufacturers.

	Instruments	**manufacturers**
1.	Autoclave	Gallenkamp (England)
2.	Balance	Mettler (Switzerland)
3.	Centrifuge	Universal 16A (Germany)
4.	Cooling centrifuge	Chilpsin (England)
5.	ELISA Multi-Well Plate Reader	ASYS, (Austria)
6.	Incubator	Gallenkamp (England)
7.	Inverted phase Microscope	Opton (Germany)
8.	Laminar air flow cabinet	NAPCO,France
9.	Light Microscope	Olympus (Japan)
10.	Microtiter pipettes	Brand (Germany)
11.	Microtitration multi-well plates	Sterilin (England)
12.	Microtome	Leitz,Germany
13.	Mixer	Retsch.Germany
14.	Nalgene filter units(0.22µM, 0.45µM)	Nalge (USA)
15.	Oven	Memmert (Germany)
16.	pH-Meter	Orient Research (USA)
17.	Plastic flask for tissue culture 25cm^2	Falcon (USA)
18.	Plastic tissue culture flask (25C.C)	Nune, Gibeo, England

Materials and Methods

2.5.2 -Materials

2.5.2.1- Chemicals

The chemicals used in this study are of the highest available purity.

Table (2-3): Chemicals used in cell line study and their suppliers.

	Chemicals	**Supplier**
1.	Antibiotics (PenicillinG, streptomycin)	Sigma(USA)
2.	Trypsin solution	BDH (England)
3.	Minimum essential media	Sigma (USA)
4.	Phosphate bufferd saline	Sigma (USA)
5.	Ethanol absolute	BDH (England)
6.	Fetal Calf serum (FCS)	Sigma(USA)
7.	Roswell Park Medium Institute –RPMI-1640	Sigma(USA)
8.	Trypan blue stain	BDH (England)
9.	Neutral red dye	Sigma (USA)
10.	Sodium phosphate Na_2HPO_4	BDH (England)
11.	Sodium carbonate $NaHCO_3$	BDH (England)
12.	Hepes buffer N-2-Hydroxy ethyl piperazine-N-2 ethansulphonic acid ($C_8H_{18}N_2O_4S$)	BDH (England)

2.5.2.2-Preperation of tissue culture solutions:

2.5.2.2.1-Phosphate buffer saline (PBS) :

Phosphate buffer salinewas prepared by dissolving the following materials in 1 liter of D.W:

substance	**Weight**
KH_2PO_4	0.2
Na_2HPO_4	0.9
NaCl	8
KCl	0.2

After control the pH 7.2 the solution put in covering bottle and then sterilized by autoclave, cooling and keep at 4°C Prior to any usage, PBS was warmed at 37°C[(116)].

2.5.2.2.2 -Sodium Bicarbonate Solution:

Sodium bicarbonate (4.4 g) was dissolved in 100 ml of Distilled water and stored at $4^{o}C$[117].

2.5.2.2.3 -Trypsin solution:

One gram of trypsin powder was dissolve in 100ml of (PBS) and stirred constantly using a magnetic stirrer at room temperature. Then the solution was sterilized by filtration using 0.22µm Millipore sterile filter. The solution thendispensed into 20 ml aliquotsuniversal containers, and stored at-20 °C[117].

2.5.2.2.4 -Tissue culture media:

Roswell Park Memorial Institute (RPMI)-1640 medium was prepared as follows:

- 10.4 g of RPMI-1640 medium powder (withHepes buffer and L-glutamine) the powder was dissolved in approximately 600 ml of double distilled water (DDW) and the following components were added to the mixture.
- 100 ml of fetal calf serum (FCS) 10%
- 0.5 ml of penicillinG solution.
- 0.5 ml of Streptomycin.
- 0.25 ml Nystatin
- 5-10 ml of Sodium bicarbonate (4.4%) with a final pH of 6.8-7.2.
- The volume was completed to one liter with D.D.W and the medium was sterilized using0.45 µm filter unit [118].

2.5.2.2.5- Fetal Calf Serum (FCS):

The serum was already thermally inactivated and added in 10% directly for tissue culture media. sterilized by millipore 0.22 μm pore size filter and stored at -20°C until use.[118]

2.5.2.2.6 -Neutral red:

Ten milligrams of neutral red dye were dissolved in 100 ml of PBS and filtered (Whatman filter paper No. 1) it should be used immediatly.[118]

2.5.2.2.7 -Trypan blue stain:

Trypan blue powder (1 gram) was dissolved in 100 ml PBS. The solution was filtered (Whatman filter paper No. 1)and stored at 4ºC (stock solution), and then it was diluted (1:10) in PBS to prepare working solution .[118]

2.5.2.2.8 -Elution Buffer:

It was prepared immediately by mixing PBS to absolute ethanol (v/v) then used directly.[118]

2.5.2.2.9- Antibiotics Solutions:

Each of Benzyl penicillinsodium salt (1000000 IU) and1g streptomycin were dissolved in 5 ml of distilled water and stored at -20°C. From each of these stocks, 0.5 ml was added to one liter of the culture medium under preparation. Dissolving one tablet of Nystatincontain 0.5ml in 50ml distilled water to produce concentration of10mg/ml and then kept the suspension in the refrigerator. [118]

2.6 Assay of growth inhibition:

2.6.1 Cell Culture and Culture Conditions:

L20B cell line was used in this study is a mouse cell line (L-cells), genetically engineered to express the human poliovirus receptor. (N.B. Some laboratories may need to declare L20B cells as genetically modified materials to local authorities in order to comply with national regulations.), the cells were grown as a monolayer, spindle like cells. Cells were cultured in RPMI 1640 media supplemented with 10% FCS, 50 mg/ml streptomycin and 1000U/L penicillin. Cell line was grown as a monolayer in humidified atmosphere at 37°C with 5% CO_2.

The experiments were performed when cells were healthy and at logarithmic phase of growth. L20B cell line at passage (40) which used in this study they were supplied by Animal cell culture laboratory, Biotechnology Research Center / AL- Nahrain University.

2.6.2-Cell Line Preparation for Cytotoxicity Study:

The method used according to Freshney 2000. Cell suspension was prepared by treating 25 cm^3 cell culture flask with 2 ml of trypsin solution .when a single cell suspension appeared ,20 ml of growth medium supplemented with 10% fetal calf serum added to the flask to inactivate the trypsin effect then the viability of the cells counted by using trypan blue dye, the viability should be more than 95% . Cell suspension was well mixed followed by transferring 200 µl/well into each well of the 96 well flat bottom micro titer plate using automatic micropipette each containing ($1* 10^5$ cell/well). Plates were incubated at $37C^0$ until 60 -70% confluence of theinternal surface area of the well for L20B cell line.[118]

Cell cultures in microtiteration plate (96wells) were exposed to range of different concentrationof pure tigogenin and crude extract(12 concentrations from the lowest to the highest starting from 0.039 to 80 µg/ml) prepared by serial two fold dilutions using maintenance media from stock solution of test

sample in triplicate form of each concentration. The negative control wells which contained only the cells with culture media, then the plates were incubated at $37C^0$ in an incubator supplemented with (5%) CO_2 for 72 hrs. After elapsing the incubation period, 50 µl/well of neutral red dye were added and incubated again for 2 hrs. The contents of the plate were removed by washing the cells 3 timeswithPBS then 100 µl of elution buffer added to each well (PBS and absolute ethanol 1:1) to remove the excess dye from viable cells. Optical density of each well was read by using ELISA reader at a transmitting wave Length on 492 nm, then growth inhibition rate were determined for each concentration according to the formula: [118,119]

$$IR\% = \frac{A - B}{A} \times 100$$

IR=inhibition rate, A= the optical density of control, B= the optical density of test.

3.1- Extraction methods of steroidal saponins:

Four extraction methods of steroidal sapogenin were tried to select the best one. The Results revealed that the extraction method NO.4 which involved extraction in a soxhlet apparatus with 500 ml dichloroethane for 24 hours was better, because the percentage yield of crude extract was higher than that obtained from other methods,, as shown in table (3-1). In addition, quantitative estimation by HPLC showed that plant extract obtained from method NO.4 contain higher percentage of tigogenin than that obtained from the other methods, as shown in table (3-6) page 96.

The reason behind the difference in the percentage yield of crude extract between method NO.1 and method NO.4 is due to the variation in the polarity index between chloroform and dichlorehtane.

Table (3-1): Yield and Percentages of crude extracts obtained from extraction methods.

Extraction method	Yield of crude extract(g)	% yield of crude extract	Part used
Method No.1	**0.75gm**	**1.5%**	**leaves**
Method No.2	**2.46gm**	**4.92%**	**Leaves**
Method No.3	**2.85gm**	**5.7%**	**Leaves**
Method No.4	**3.54gm**	**7.08%**	**Leaves**
	1.34gm	**2.68%**	**stems**
	2.23gm	**4.46%**	**roots**

3.2-Preliminaryidentification ofTigogeninbyThin layer chromatography:

Thin layer chromatography (**TLC**) of the plant extract obtained from extraction methods(E) of leaves, stems and roots parts of the plant, confirms the presence of **Tigogenin**.

Tigogenin appeared as a single spot in eight different developing solvent systems (S1, S2, S3, S4, S5, S6, S7and S_8) against**Tigogenin** standard as in figures (3-1 to 3-16). The spots of **Tigogenin** has the same color and R_f values as that of **Tigogenin** standard on the TLC plates after visualization by Liebermann-Burchard spray reagent, table (3-2).

Table (3-2): R_f values of Tigogenin and It's standard in eight different developing solvent systems in TLC from *yucca aloifolia* leaves.

Solvent system	$R_f$values				
	E_1	E_2	E_3	E_4	S
S_1	0.11	0.10	0.13	0.15	0.10
S_2	0.56	0.56	0.54	0.55	0.55
S_3	0.44	0.44	0.43	0.45	0.42
S_4	0.46	0.46	0.46	0.47	0.45
S_5	0.73	0.75	0.75	0.78	0.74
S_6	0.79	0.81	0.81	0.81	0.81
S_7	0.10	0.09	0.10	0.10	0.09
S_8	0.27	0.25	0.24	0.26	0.23

Table (3-3): R_f values of Tigogenin and It's standard in different developing solvent systems in TLC. (Stems and roots parts).

Solvent system	Stems	roots	S
S_1	0.13	0.14	0.13
S_2	0.71	0.71	0.69
S_3	0.38	0.40	0.37
S_4	0.41	0.41	0.38
S_5	0.69	0.71	o.69
S_6	0.71	0.68	0.68
S_7	0.08	0.08	0.08
S_8	0.09	0.09	0.09

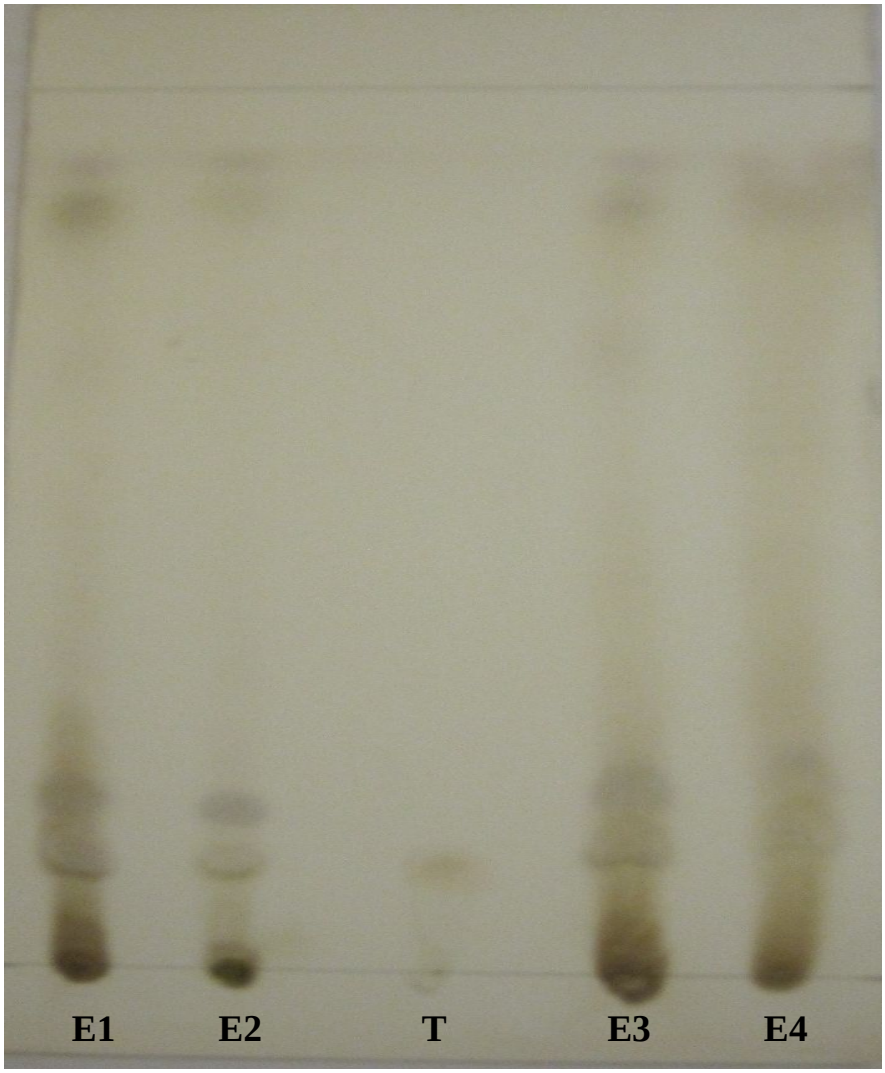


Figure (3-1): TLC for *Yucca aloifolia* leaves extract obtained by extraction methods using silica gel G 60 as adsorbent and (S1) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

E1: extraction method No.1

E2: extraction method No.2

T: Tigogenin standard

E3: extraction method No.3

E4: extraction method No. 4

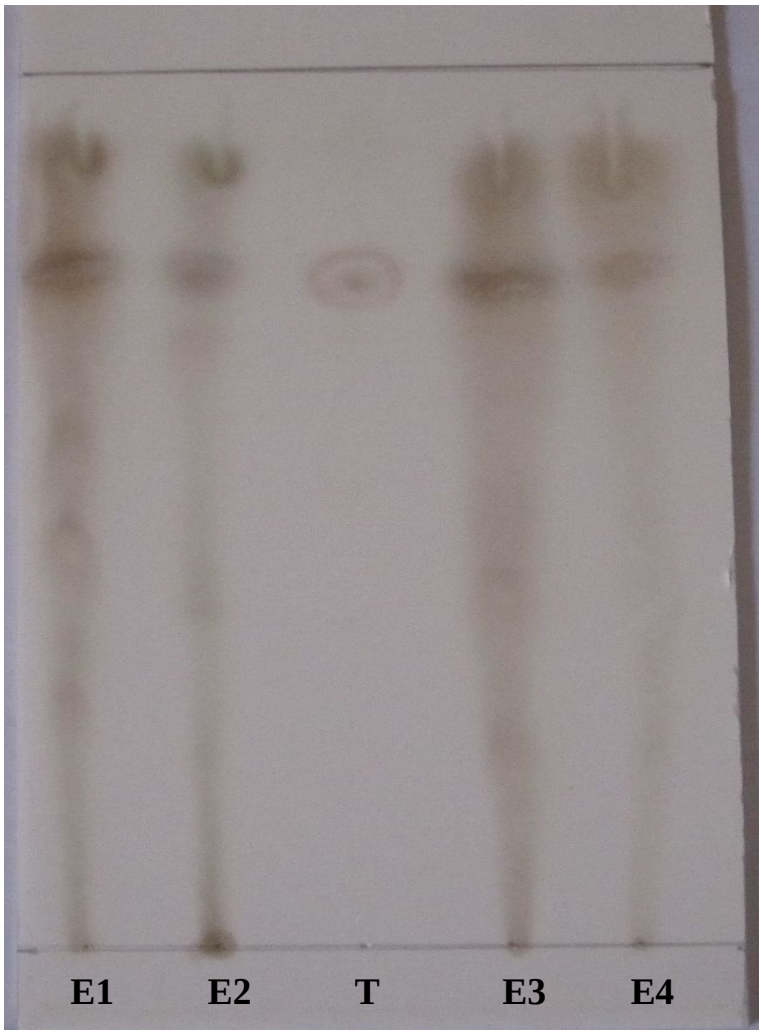


Figure (3-2): TLC for *Yucca aloifolia* leaves extract obtained by extraction methods using silica gel G 60 as adsorbent and (S2) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

E1: extraction method No.1

E2: extraction method No.2

T: Tigogenin standard

E3: extraction method No.3

E4: extraction method No. 4

Figure (3-3):TLC for *Yucca aloifolia* leaves extract obtained by extraction methods using silica gel G 60 as adsorbent and (S3) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

E1: extraction method No.1

E2: extraction method No.2

T: Tigogenin standard

E3: extraction method No.3

E4: extraction method No. 4

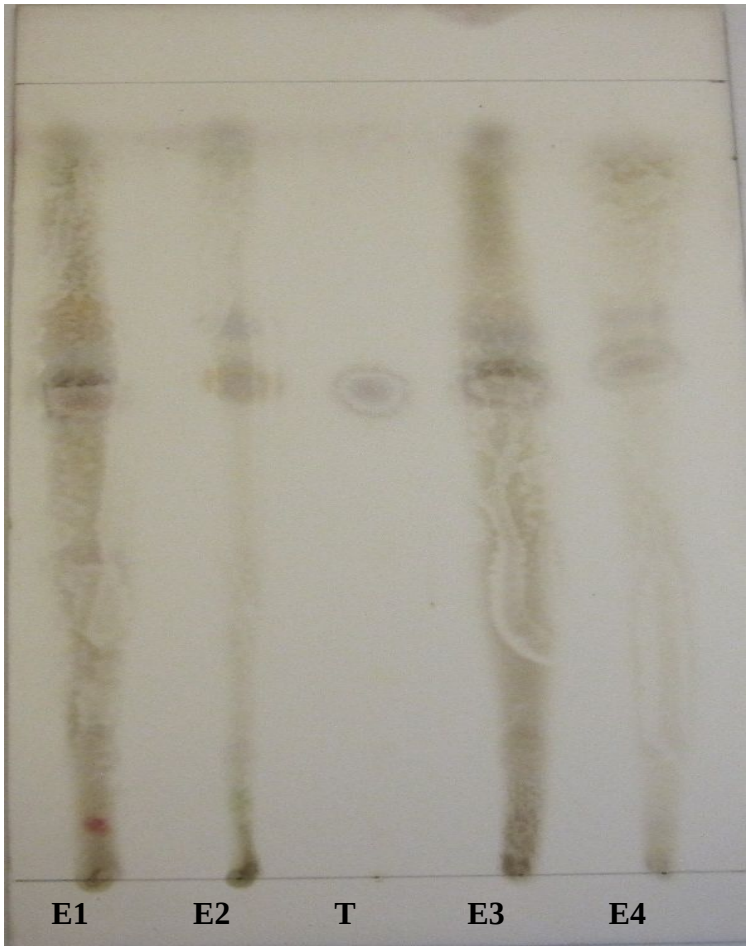


Figure (3-4):TLC for *Yucca aloifolia* leaves extract obtained by extraction methods using silica gel G 60 as adsorbent and (S4) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

E1: extraction method No.1

E2: extraction method No.2

T: Tigogenin standard

E3: extraction method No.3

E4: extraction method No. 4

Figure (3-5):TLC for *Yucca aloifolia* leaves extract obtained by extraction methods using silica gel G 60 as adsorbent and (S5) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

E1: extraction method No.1

E2: extraction method No.2

T: Tigogenin standard

E3: extraction method No.3

E4: extraction method No. 4

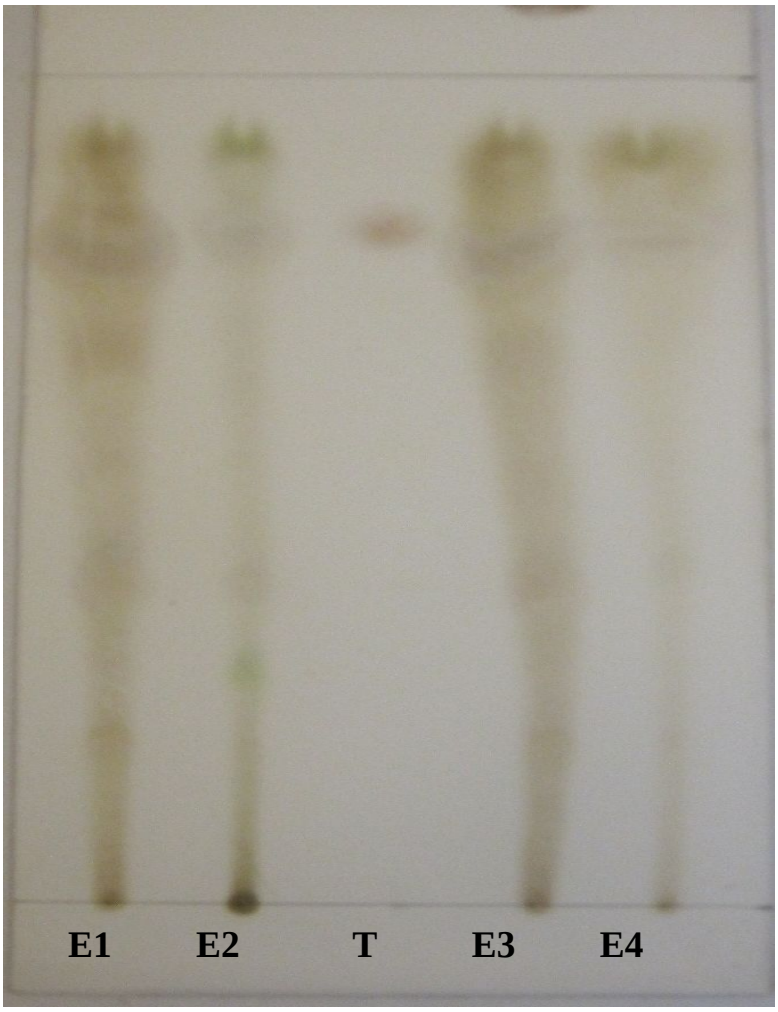


Figure (3-6):TLC for *Yucca aloifolia* leaves extract obtained by extraction methods using silica gel G 60 as adsorbent and (S6) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

E1: extraction method No.1

E2: extraction method No.2

T: Tigogenin standard

E3: extraction method No.

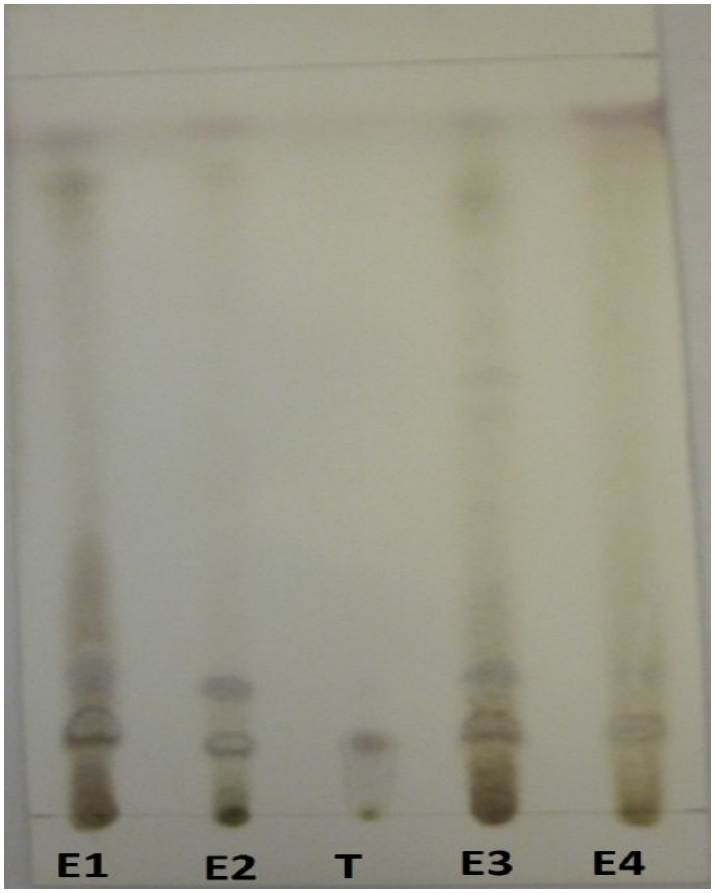


Figure (3-7):TLC for *Yucca aloifolia* leaves extract obtained by extraction methods using silica gel G 60 as adsorbent and (S7) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

E1: extraction method No.1

E2: extraction method No.2

T: Tigogenin standard

E3: extraction method No.3

E4: extraction method No. 4

Figure (3-8):TLC for *Yucca aloifolia* leaves extract obtained by extraction methods using silica gel G 60 as adsorbent and (S8) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

E1: extraction method No.1

E2: extraction method No.2

T: Tigogenin standard

E3: extraction method No.3

E4: extraction method No. 4

Figure (3-9):TLC for *Yucca aloifolia* (stems and roots) extracts obtained by extraction method No.4 using silica gel G 60 as adsorbent and (S1) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

S: stems extract

T: Tigogenin standard

R: roots extract

Figure (3-10):TLC for *Yucca aloifolia* (stems and roots) extracts obtained by extraction method No.4 using silica gel G 60 as adsorbent and (S2) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

S: stems extract

T: Tigogenin standard

R: roots extract

Figure (3-11):TLC for *Yucca aloifolia* (stems and roots) extracts obtained by extraction method No.4 using silica gel G 60 as adsorbent and (S3) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

S: stems extract

T: Tigogenin standard

R: roots extract

Figure (3-12):TLC for *Yucca aloifolia* (stems and roots) extracts obtained by extraction method No.4 using silica gel G 60 as adsorbent and (S4) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

S: stems extract

T: Tigogenin standard

R: roots extract

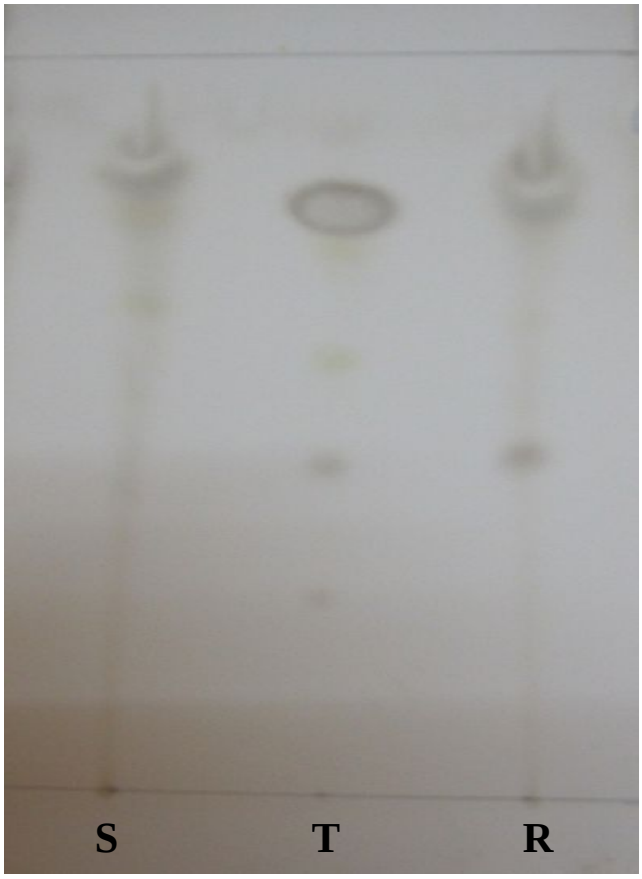


Figure (3-13):TLC for *Yucca aloifolia* (stems and roots) extracts obtained by extraction method No.4 using silica gel G 60 as adsorbent and (S5) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

S: stems extract

T: Tigogenin standard

R: roots extract

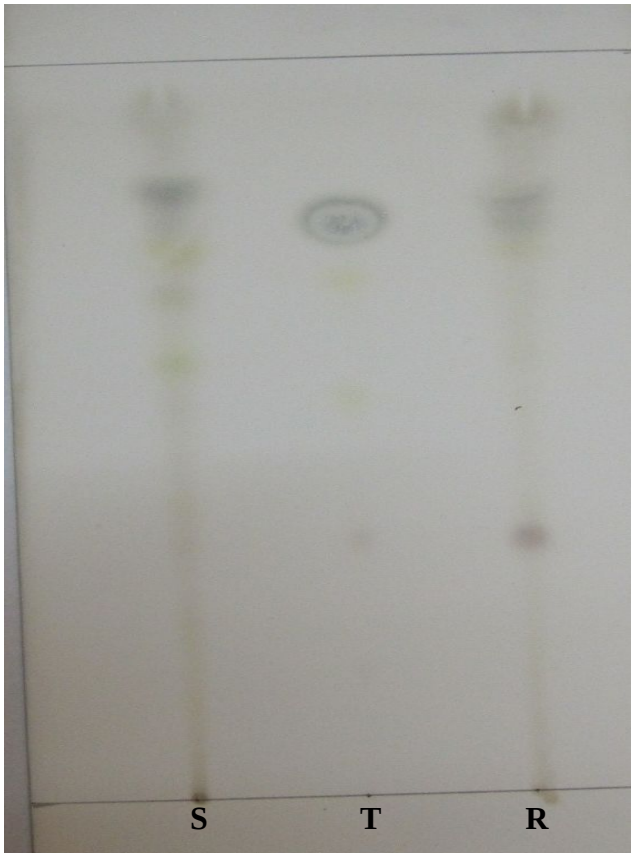


Figure (3-14):TLC for *Yucca aloifolia* (stems and roots) obtained by extraction method No.4 using silica gel G 60 as adsorbent and (S6) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

S: stems extract

T: Tigogenin standard

R: roots extract

Figure (3-15):TLC for *Yucca aloifolia* (stems and roots) extracts obtained by extraction method No.4 using silica gel G 60 as adsorbent and (S7) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

S: stems extract

T: Tigogenin standard

R: roots extract

Figure (3-16):TLC for *Yucca aloifolia* (stems and roots) extracts obtained by extraction method No.4 using silica gel G 60 as adsorbent and (S8) as a mobile face. Visualization by Liebermann-Burcrhard spray reagent.

S: stems extract

T: Tigogenin standard

R: roots extract

3.3- Fractionation by column chromatography.

One hundred fractions obtained from column chromatography were monitored by TLC. The consecutive fractions that have the same number of spots of the same $\mathbf{R_f}$ values were combined to get 8 major fractions, which were concentrated to dryness and subjected to identification, as listed in table (3-4).

In the first 70 fractions, fractions (1–10) gave one spot in TLC and were collected to give fraction one (F1). Fractions (11–20) gave one spot were collected to give fraction two (F2), while fractions (21–30) gave one spot and were collected to give fractions three (F3), fractions (31-40) gave one spot and were collected to give the fraction Four (F4). Fractions (41–52) gave two spots and were collected to give the major fraction five (F5). Fractions (53–54) gave one spot and were collected to give the fraction six (F6). Fractions (55–57) gave two spots and were collected to give the fraction seven (F7).Fractions (58–70) gave four spots and were collected to give the fraction eight (F8). Selected chromatograms for the separated fractions (F1 –F8) are shown in figures (3-17 to 3-24).

In the last30 fractions no spots were appeared in TLC examination.

Table (3-4): Major fractions obtained from column chromatography.

MajorFractions	No. of collections 5 ml each	No. of spots
F1	1-10	1
F2	11-20	1
F3	21-30	1
F4	31-40	1
F5	41-52	2
F6	53-54	1
F7	55-57	2
F8	58-70	4

Figure (3-17): TLC of fraction One (F1) using silica gel G60 as adsorbent and S2 as mobile phase visualization by Liebermann-Burchard spray reagent.

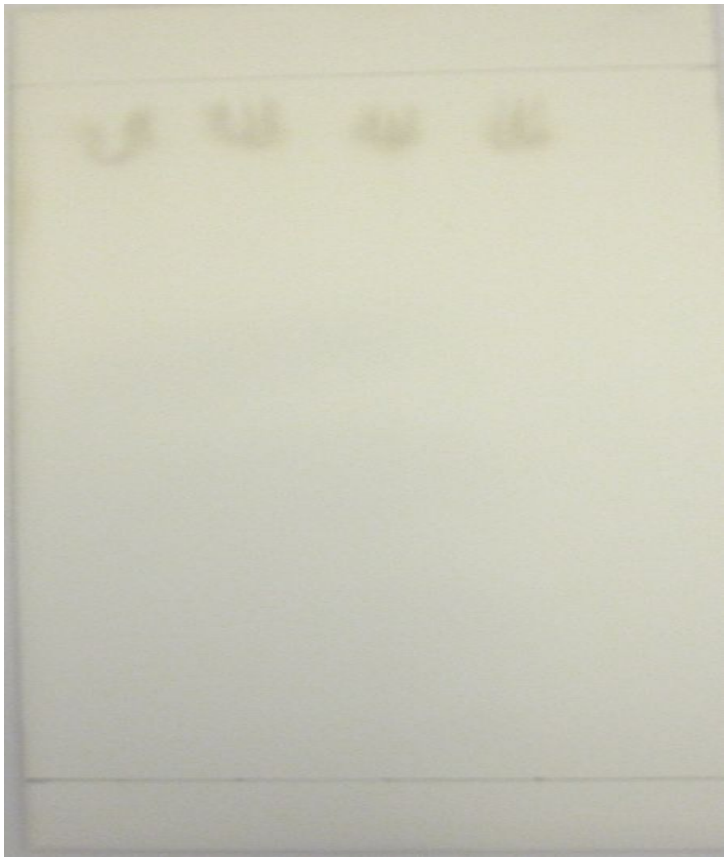


Figure (3-18): TLC of fraction two (F2) using silica gel G60 as adsorbent and S2 as mobile phase visualization by Liebermann-Burchard spray reagent.

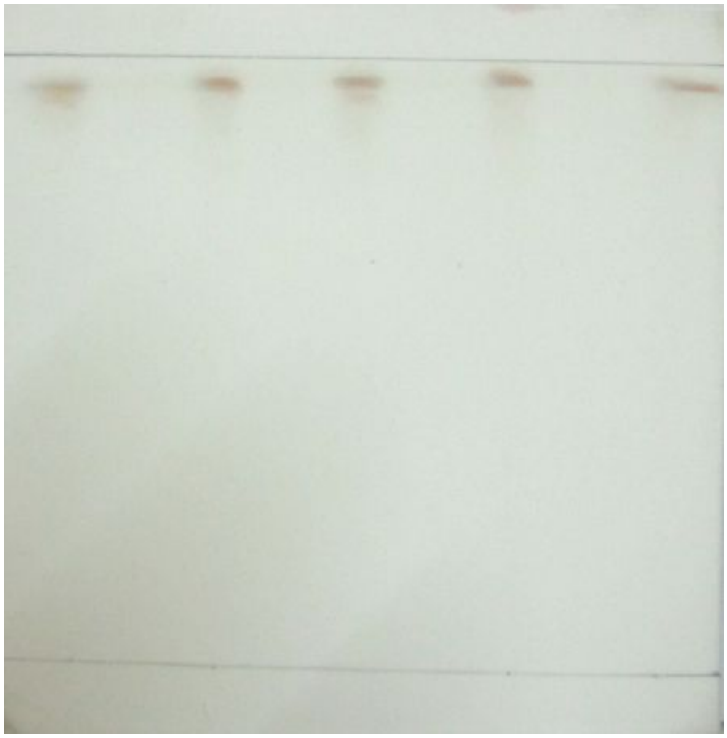


Figure (3-19): TLC of fraction Three (F3) using silica gel G60 as adsorbent and S2 as mobile phase visualization by Liebermann-Burchard spray reagent.

Figure (3-20): TLC of fraction four (F4) using silica gel G60 as adsorbent and S2 as mobile phase visualization by Liebermann-Burchard spray reagent.

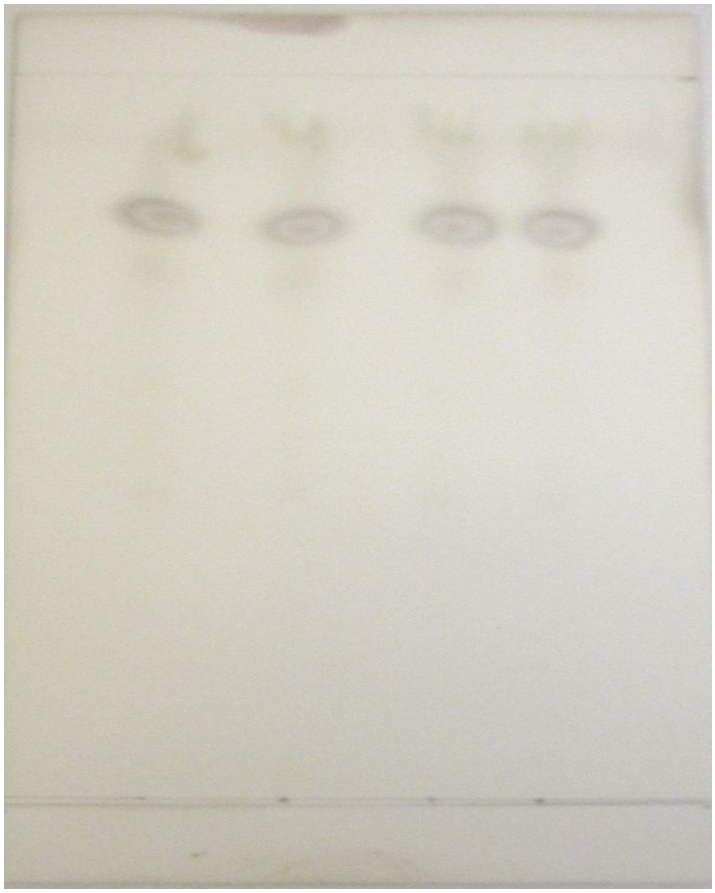


Figure (3-21): TLC of fraction five (F5) using silica gel G60 as adsorbent and S2 as mobile phase visualization by Liebermann-Burchard spray reagent.

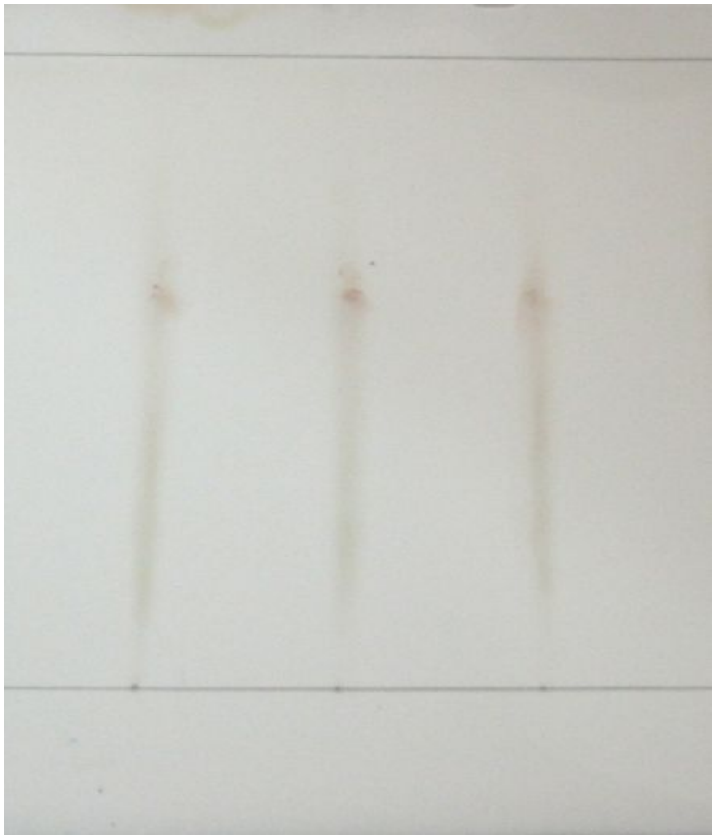


Figure (3-22): TLC of fraction six (F6) using silica gel G60 as adsorbent and S2 as mobile phase visualization by Liebermann-Burchard spray reagent.

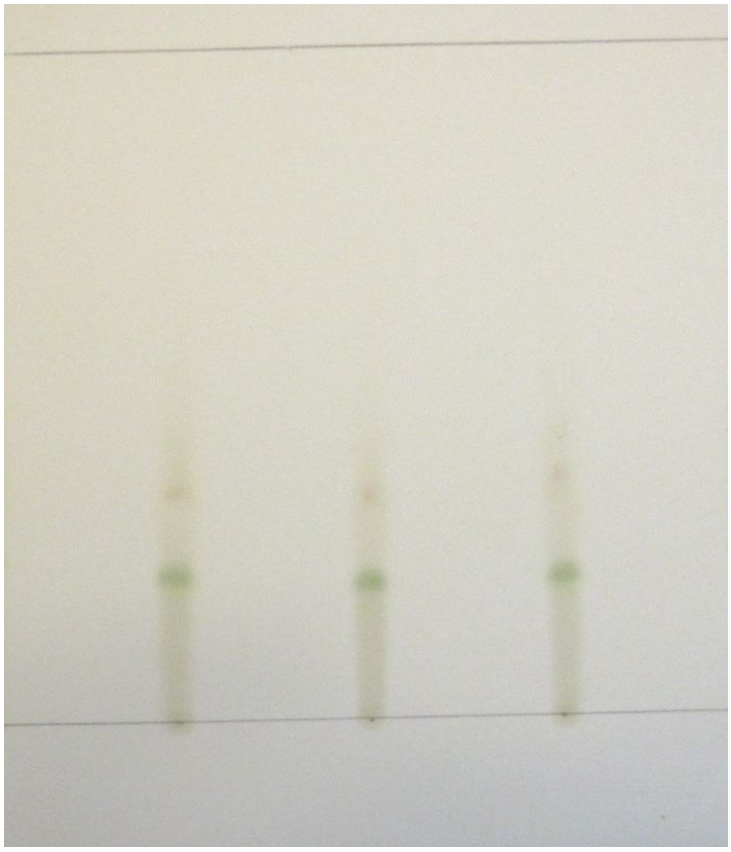


Figure (3-23): TLC of fraction seven (F7) using silica gel G60 as adsorbent and S2 as mobile phase visualization by Liebermann-Burchard spray reagent.

Figure (3-24): TLC of fraction eight (F8) using silica gel G60 as adsorbent and S2 as mobile phase visualization by liebermann-Burchard spray reagent.

3.4- Selection of the best developing solvent systems.

For ***Y. aloifolia*** saponins the developing solvent systems S_2 which is formed from chloroform: methanol (95: 5), S5= Chloroform:Petroleum ether (b.p.60-80C^0):Methanol (85:10:5) and
S_6= Chloroform:Acetone (80:20) was found to be the best developing solvent systems and they were more efficient for qualitative and quantitative analysis, as in figures (3-2, 3-5, 3-6).

3.5- Isolation of steroidal sapogenin by preparative layer chromatography:

The fraction(F5)was subjected to preparative TLC using S_2:chloroform: methanol (95:5),S_5:Chloroform:Petroleum ether (b.p.60-80C^0):Methanol (85:10:5) and S_6= Chloroform:Acetone (80:20),as shown in figures(3-25 to 3-27).

The residues of the isolated bands from all fractions were subjected to co-thin layer chromatography with tigogenin reference standard, in order to check if tigogenin present in any of these fractions as shown in figures (3-28 to 3-31).

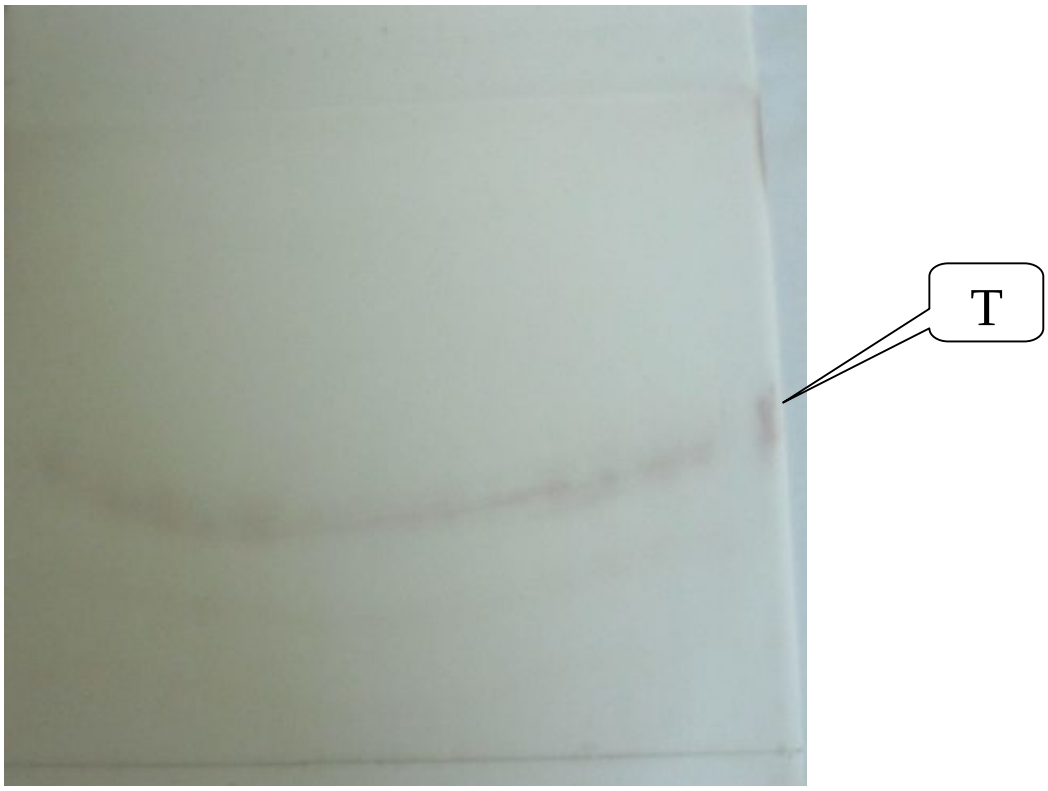


Figure (3-25): Chromatogram of preparative TLC for fraction five, using silica gel G60 as adsorbent and (S2) as a mobile phase. Detection by Liebermann-Burchard reagent.

T: Tigogenin standard

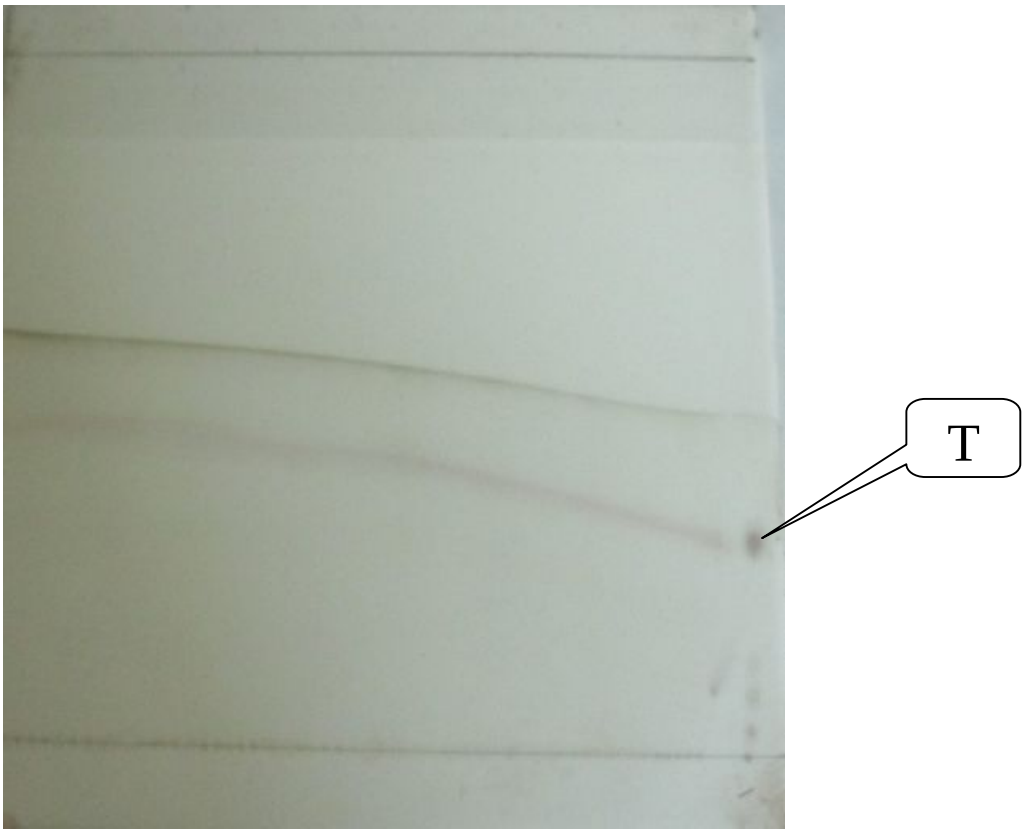


Figure (3-26): Chromatogram of preparative TLC for fraction five, using silica gel GF as adsorbent and (S5) as a mobile phase. Detection by Liebermann-Burchard reagent.

T: Tigogenin standard

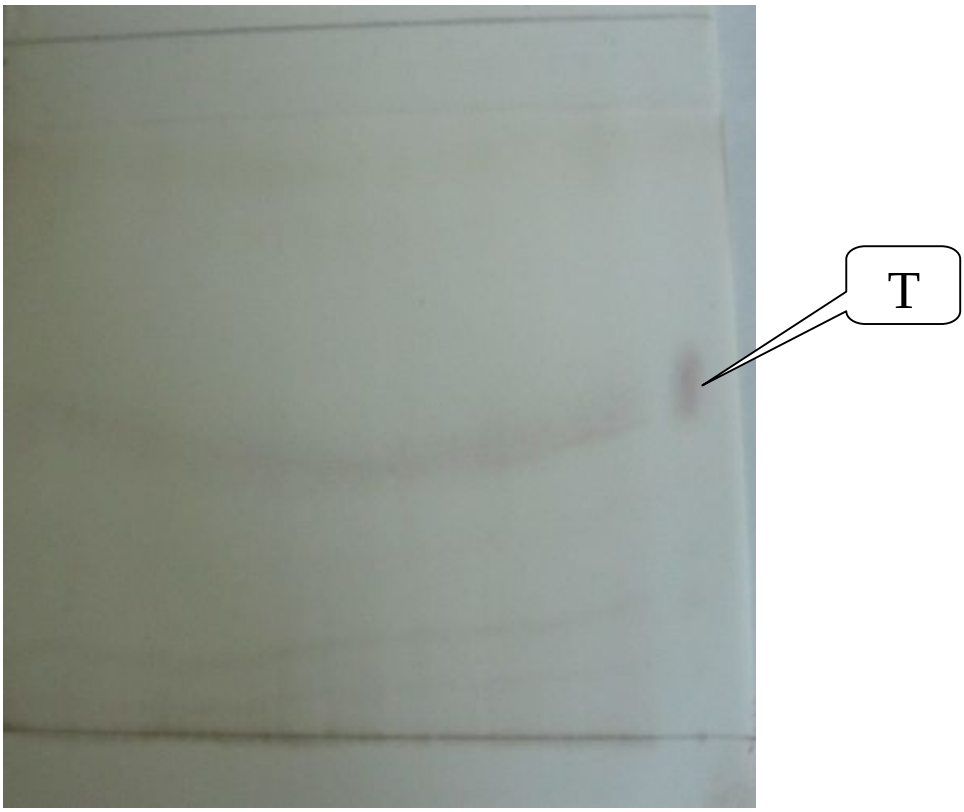


Figure (3-27): Chromatogram of preparative TLC for fraction five, using silica gel G60 as adsorbent and (S6) as a mobile phase. Detection by Liebermann-Burchard reagent.

T: Tigogenin standard

Figure (3-28): Co-TLC of all bands isolated from fractions F1, F2 with Tigognin reference standard using silica gel G60 as adsorbent and (S2) as mobile phase. Visualization by Liebermann-Burchard spray reagent.

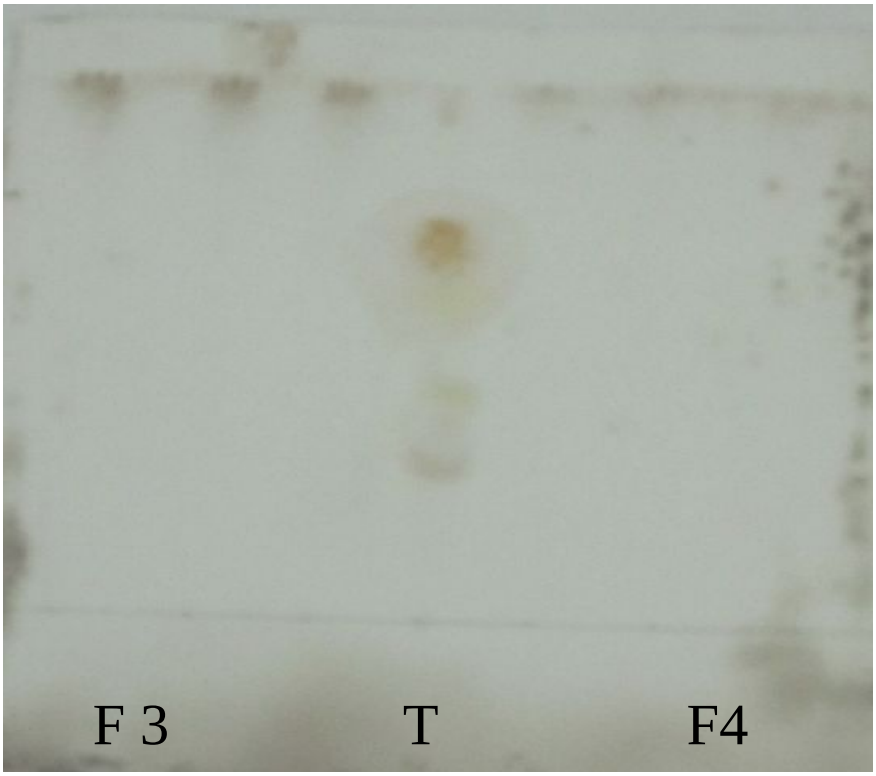
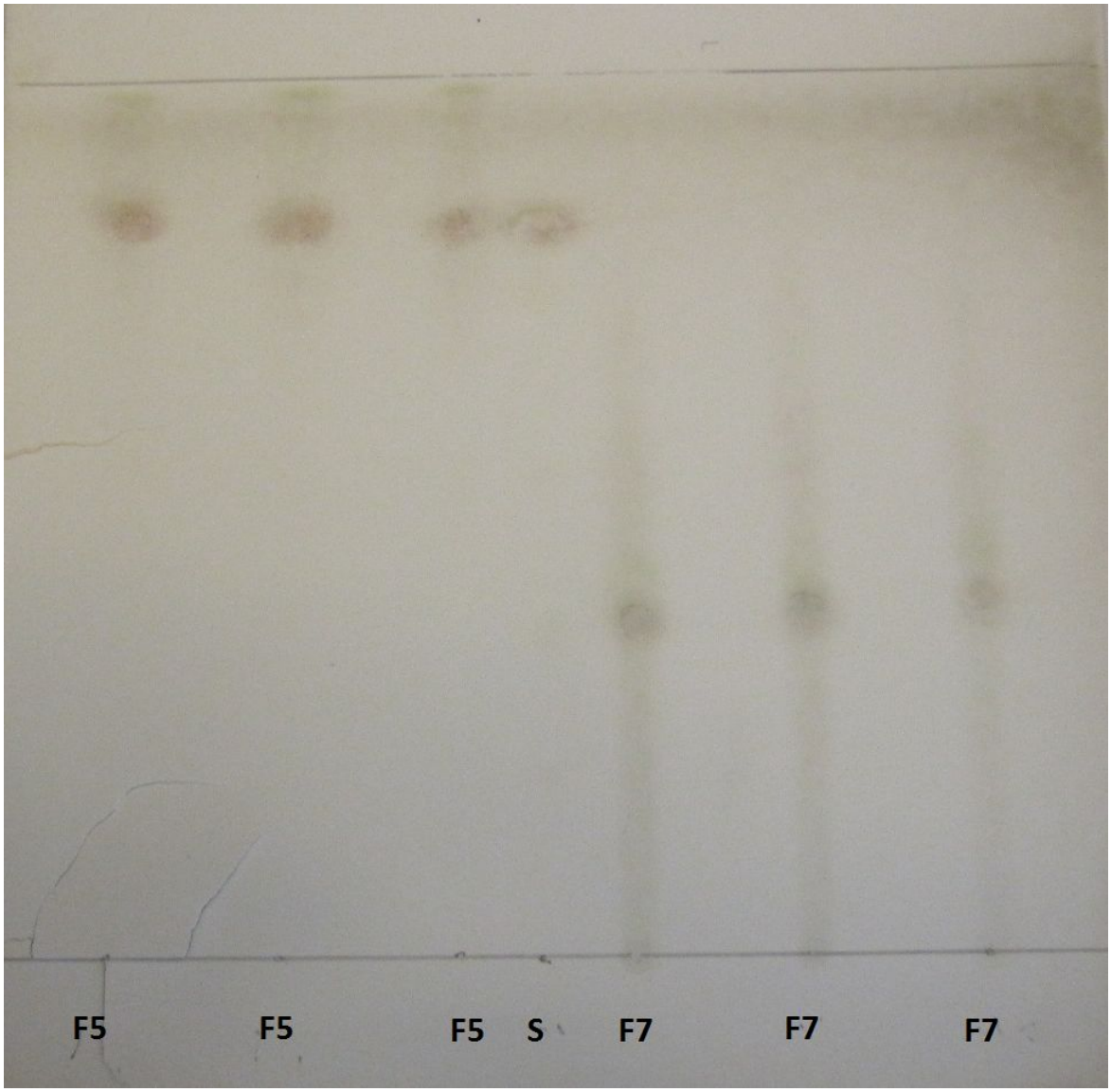


Figure (3-29): Co-TLC of all bands isolated from fractions F3, F4 with Tigognin reference standard using silica gel G60 as adsorbent and (S2) as mobile phase. Visualization by Liebermann-Burchard spray reagent.



Figure(3-30):Co-TLC of all bands isolated from fraction F5 and F7 with Tigognin reference standard using silica gel G60 as adsorbent and (S2) as mobile phase. Visualization by Liebermann-Burchard spray reagent.

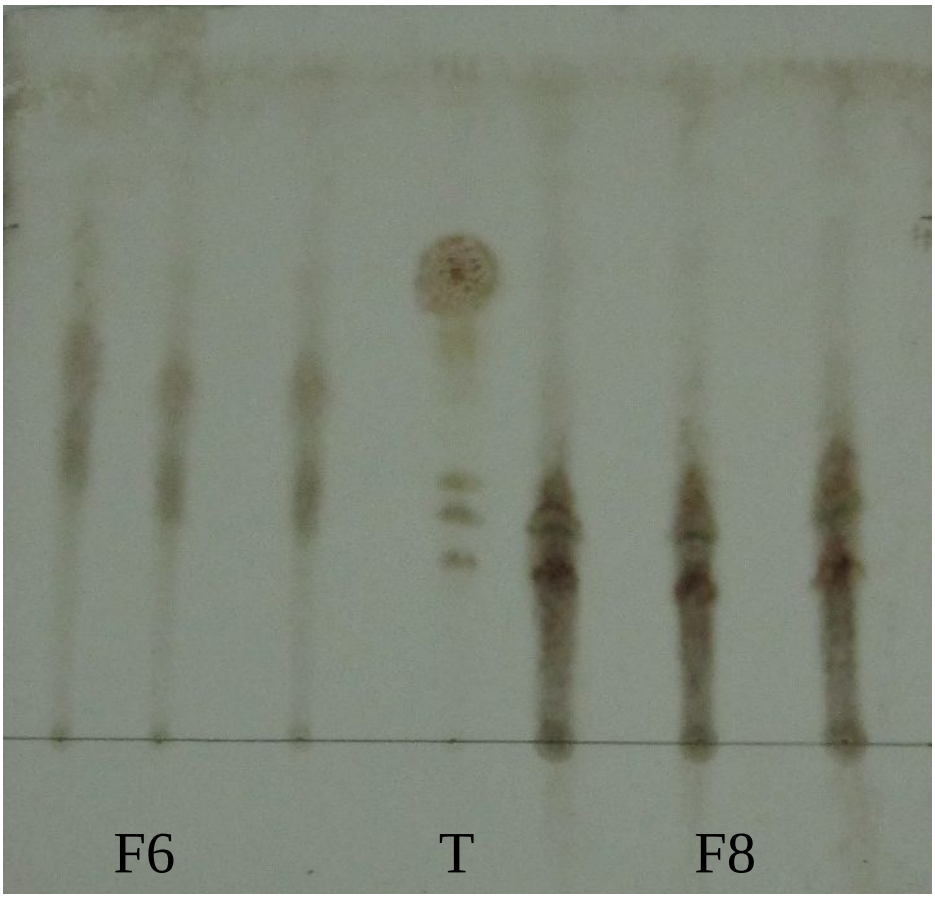


Figure (3-31): Co-TLC of all bands isolated from F6, F8 with Tigognin reference standard using silica gel G60 as adsorbent and (S2) as mobile phase. Visualization by Liebermann-Burchard spray reagent.

3.6- Characterization and identification of the isolated steroidal sapogenin:

3.6.1- TLC:-

In analytical TLC using spiking technique in the best three mobile phases (S2,S5,S6), **tigogenin** appeared as a single spot with the same color and R_f values as that of tigogenin reference standard after spraying with Liebermann-Burchard reagent and heating, as shown in figures (3-32 to 3-34).

3.6.2 - Melting point:-

The isolated tigogenin was identified from its sharp melting point of 200 – 201 ^{o}C (uncorrected) compared to standard tigogenin melting point 202-204 ^{o}C (uncorrected).

Figure (3-32): TLC chromatogram of qualitative analysis of isolated Tigogenin using silica gel G60 as adsorbent and (S2) as mobile phase.

Visualization by Liebermann-Burchard spray reagent.

A : Tigogenin reference standard

a : isolated Tigogenin

M : mixed spots of isolated compound and reference standard

Figure (3-٣٣): TLC chromatogram of qualitative analysis of isolated Tigogenin using silica gel G 60 as adsorbent and (S_5) as mobile phase. Visualization by Liebermann-Burchard spray reagent.

A: Tigogenin reference standard

a :isolated Tigogenin

M: mixed spot of the isolated compound and the reference standard

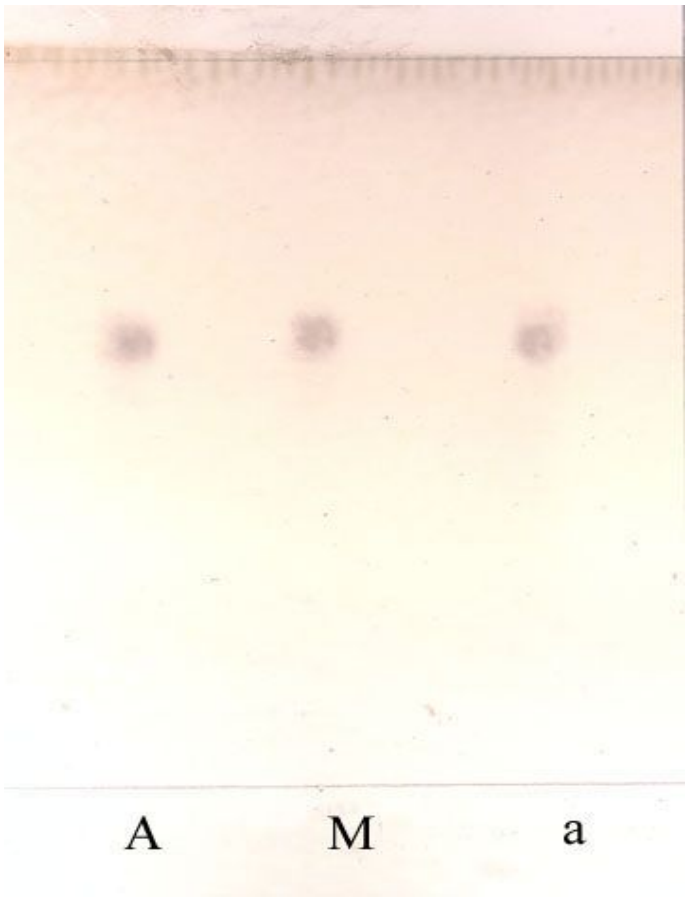


Figure (3- 34): TLC chromatogram of qualitative analysis of isolated tigogenin using silica gel G 60 as adsorbent and (S_6) as a mobile phase. Visualization by Liebermann-Burchard spray reagent.

A: Tigogenin reference standard

a: isolated tigogenin

M: mixed spot of the isolated compound and the reference standard

3.6.3-FT-IR:

Further characterization of isolated tigogenin with infrared – spectroscopic analysis was done, using tigogenin standard as a reference.

The IR spectrum (KBr) of isolated tigogenin showed υ_{max} absorptions in cm^{-1} at:3524,3390-3263,2929,2848,1456, and 1373,1242–1049 which are identical with that of tigogenin standard, as shown in figures (3-26 & 3-27).The characteristic IR absorption bands showed by isolated tigogenin and tigogenin standard and their assignment are listed in table (3-5).

Table (3-5): The characteristic IR absorption bands (in cm^{-1}) of the isolated tigogenin in comparison with that of tigogenin as reference standard [(120)]

Functional group	Isolated tigogenin	Tigogenin standard	Assignment
Free O-H	3524	3522	Free O-H stretching of alcohol
O-H	Broad band (3390-3263)	Broad band (3389-3269)	Broad O-H stretching band indicate hydrogen bonding
C–H	2929,2848	2941,2874	Asymmetric and symmetric stretching of CH_3 and CH_2 groups
C-H	1456,1373	1456,1373	C-H bending of CH_2 and CH_3
C-O	1242-1049	1242-1049	C-O stretching of aliphatic ether

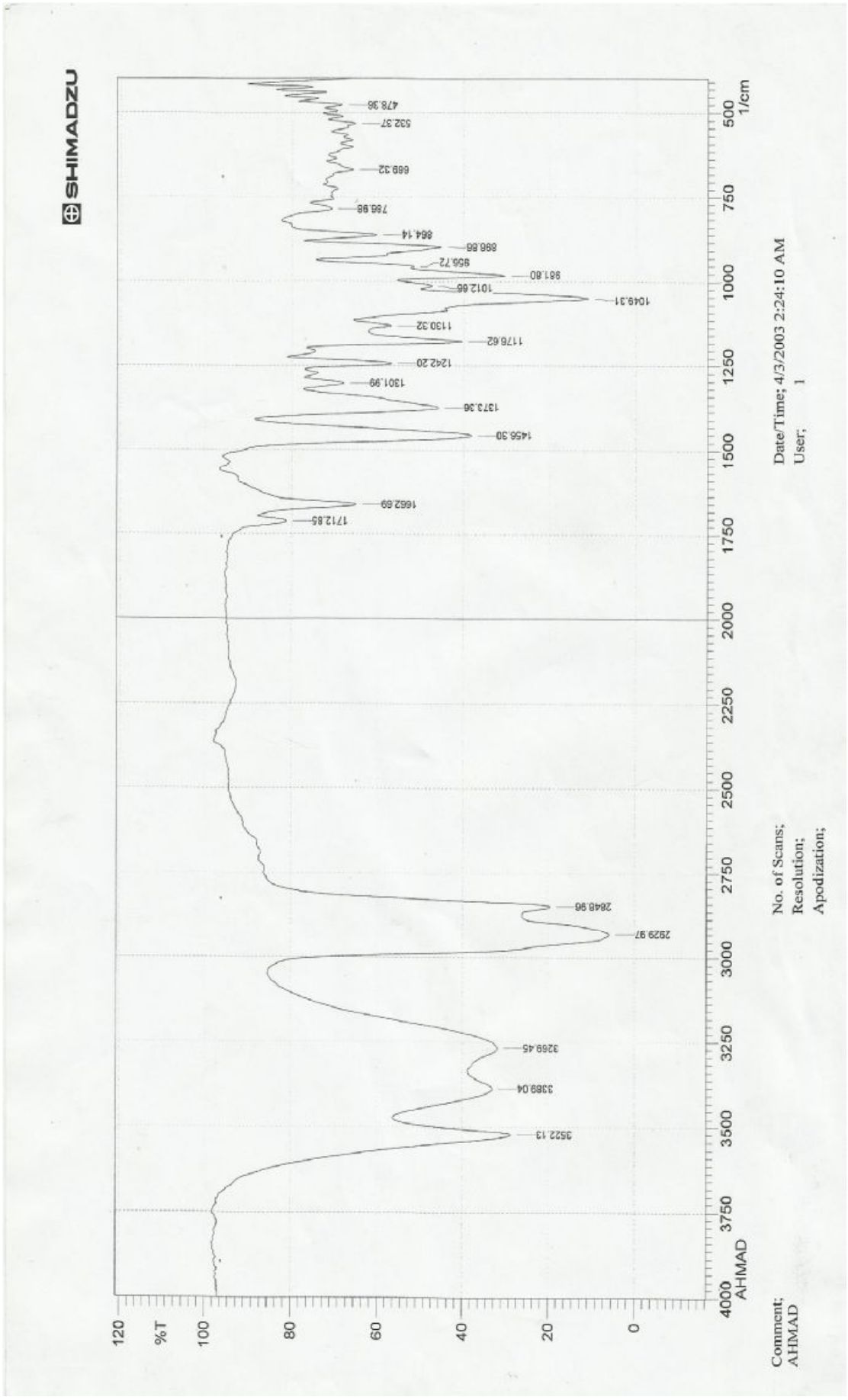


Figure (3-35): IR spectrum of tigogenin standard

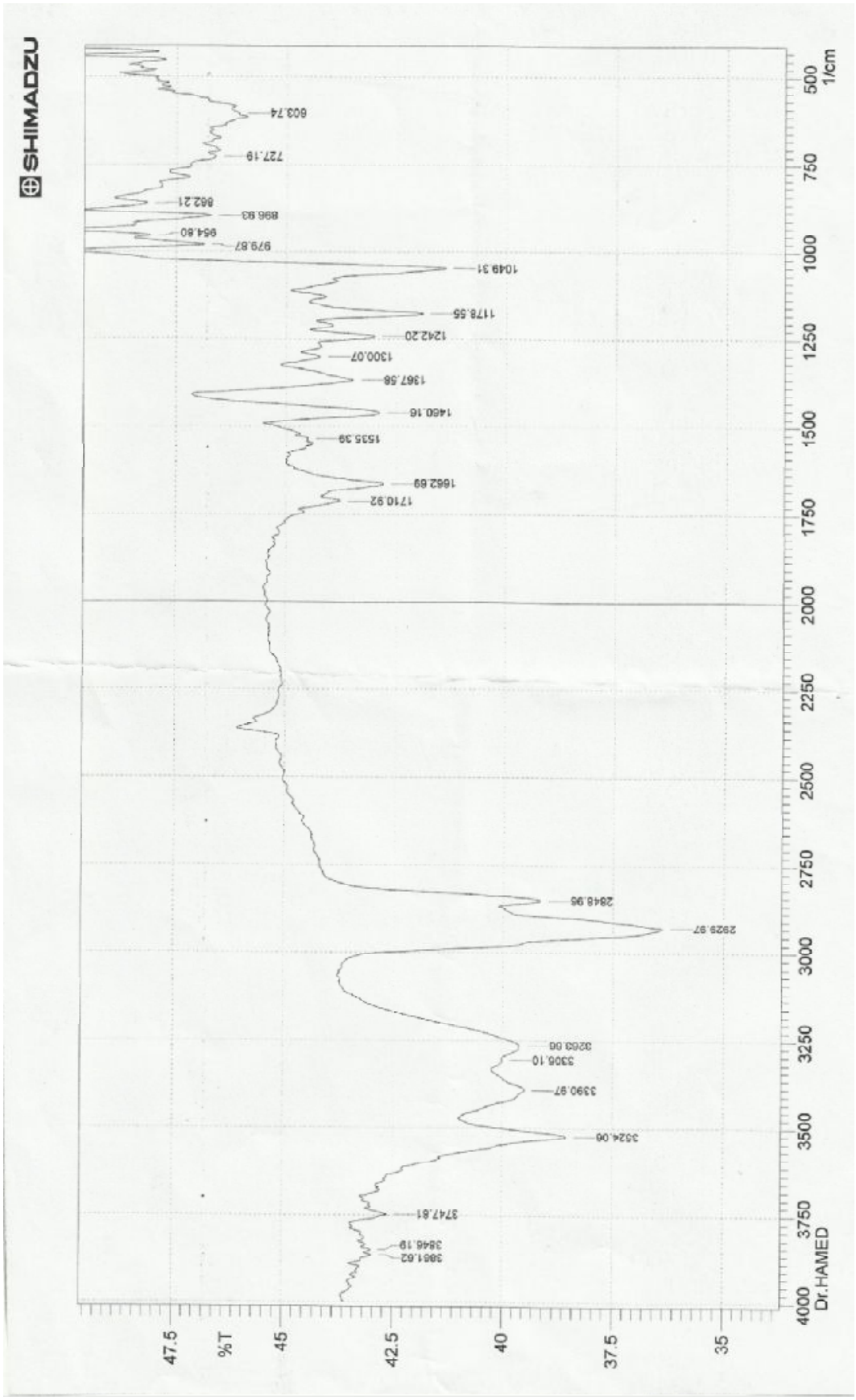


Figure (3-36): IR spectrum of isolated tigogenin

3.7- HPLC analysis.

The identification of tigogenin was further confirmed using HPLC analysis. In HPLC, qualitative identifications were made by comparing of retention time obtained at identical chromatographic conditions of analyzed samples and authentic standard.

The information obtained from HPLC method of analysis reveal that tigogenin present in all parts of the plant with higher percentage in plant leaves in comparison with other parts of the plant. In addition, HPLC analysis of leaves extract reveals that leaves extract obtained from extraction method NO.4 contain higher percentage of tigogenin than leaves extracts obtained from other extraction methods. As shown in table (3-6):

The result indicates that the HPLC method was efficient for qualitative identification and quantitative determination of tigogenin.

Table (3-6): The percentage of tigogenin in all parts of plant extracts by HPLC analysis:

Extraction methods	%Yield of tigogenin	Parts used
E1	2.6%	leaves
E2	3.66%	leaves
E3	3.86%	leaves
E4	5%	Leaves
	3.14%	Stems
	4.2%	Roots

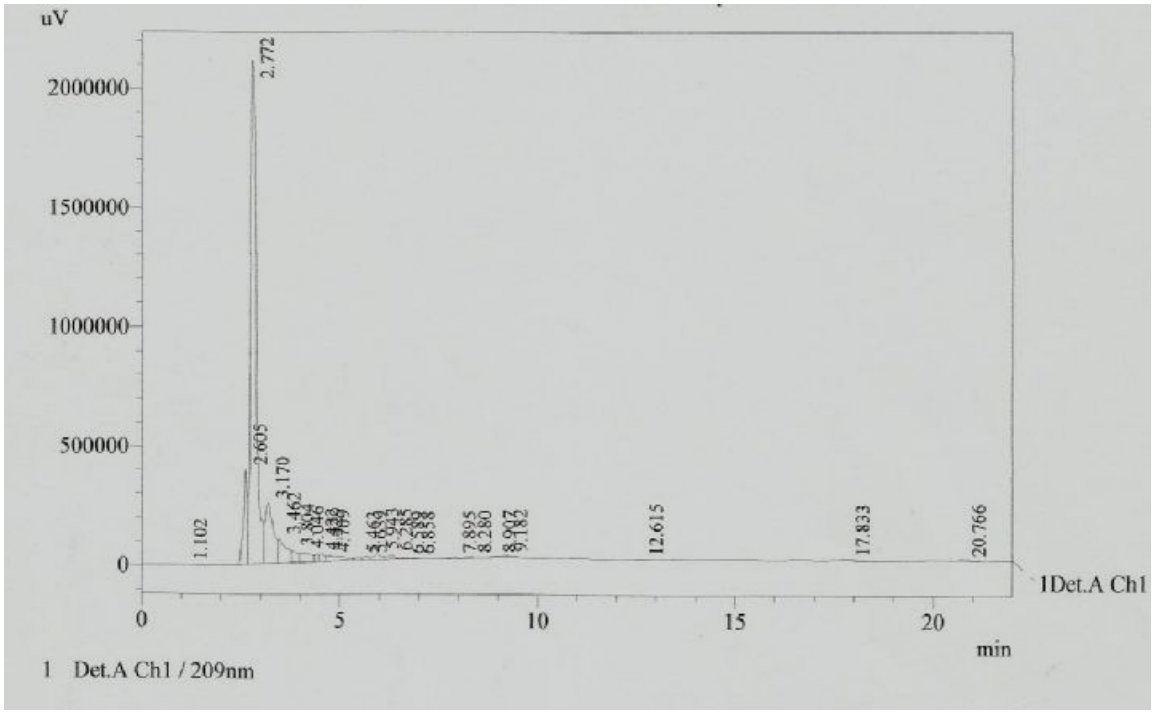


Figure (3-37): HPLC analysis of *Yucca aloifolia* extract obtained by extraction method No. 1.

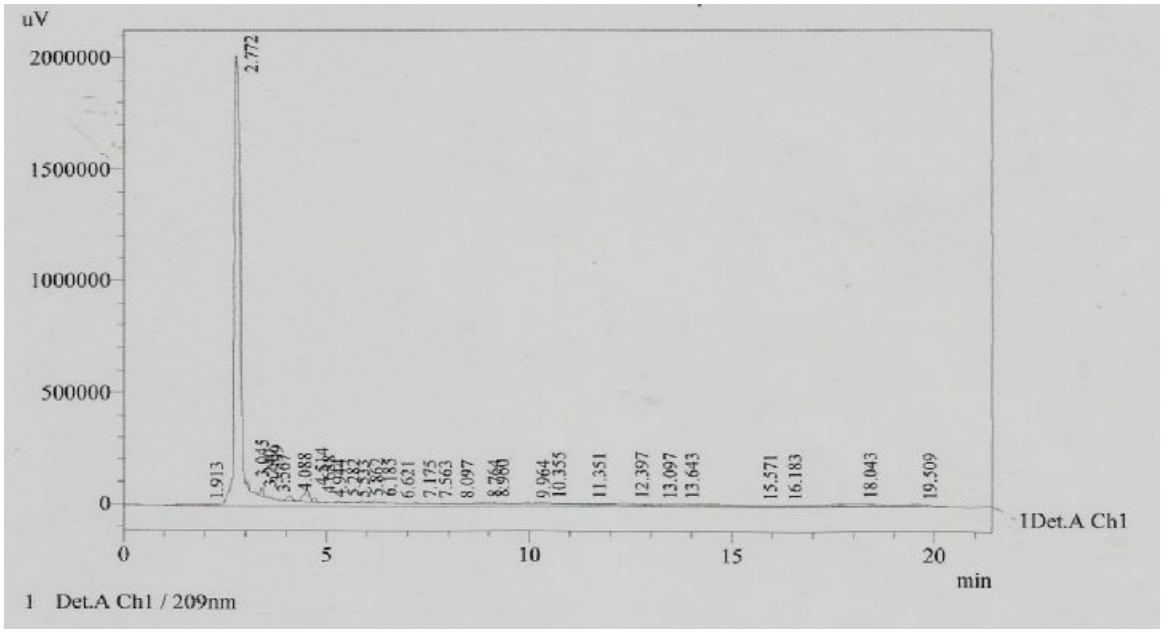


Figure (3-38): HPLC analysis of *Yucca aloifolia* extract obtained by extraction method No. 2.

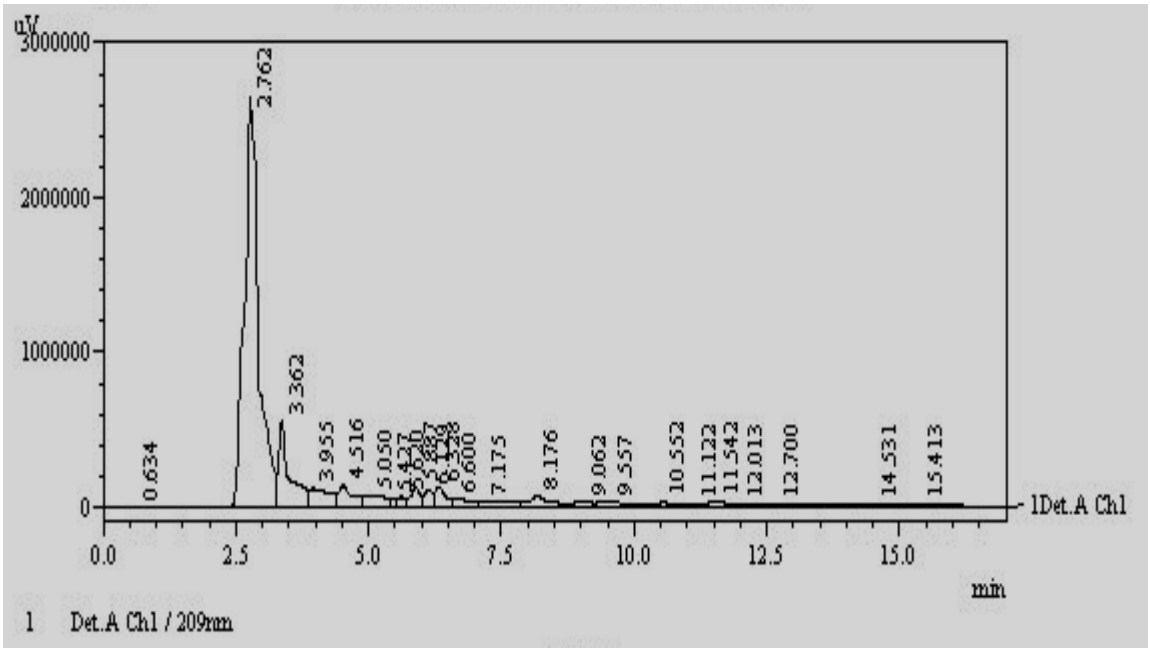


Figure (3-39): HPLC analysis of *Yucca aloifolia* extract obtained by extraction method No. 3. (Leaves).

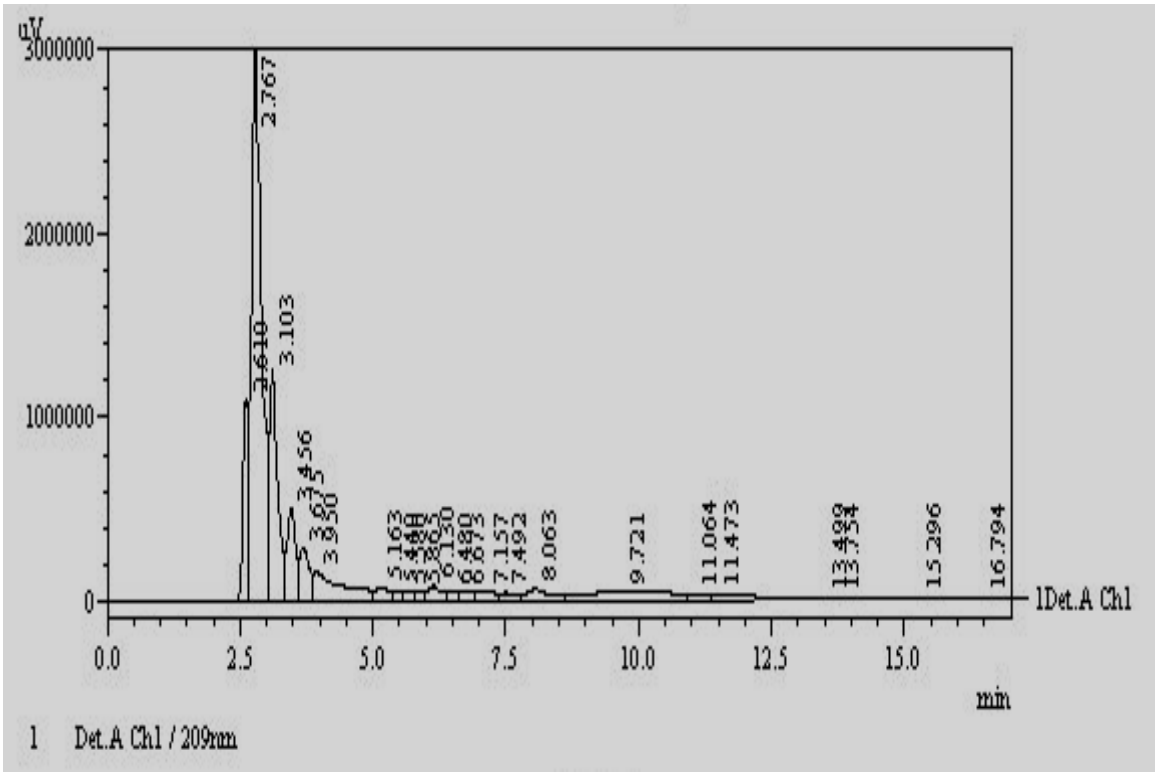


Figure (3-40): HPLC analysis of *Yucca aloifolia* extract obtained by extraction method No. 4(leaves).

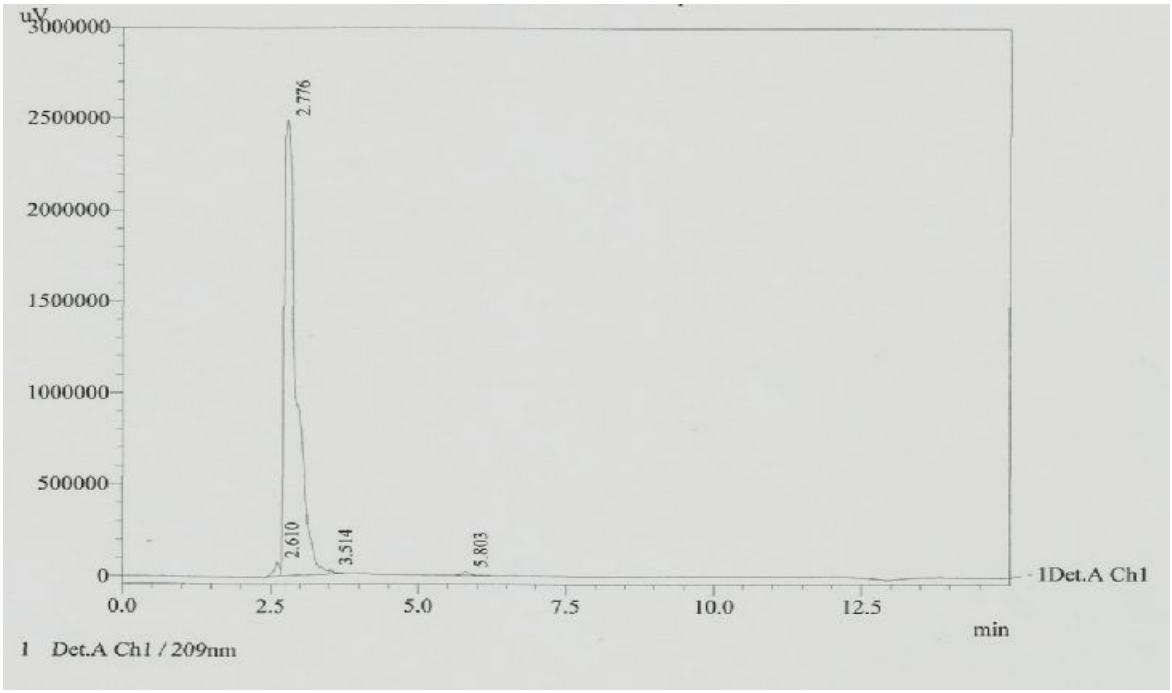


Figure (3-41): HPLC analysis of *Yucca aloifolia* extract obtained by extraction method No. 4 (Roots).

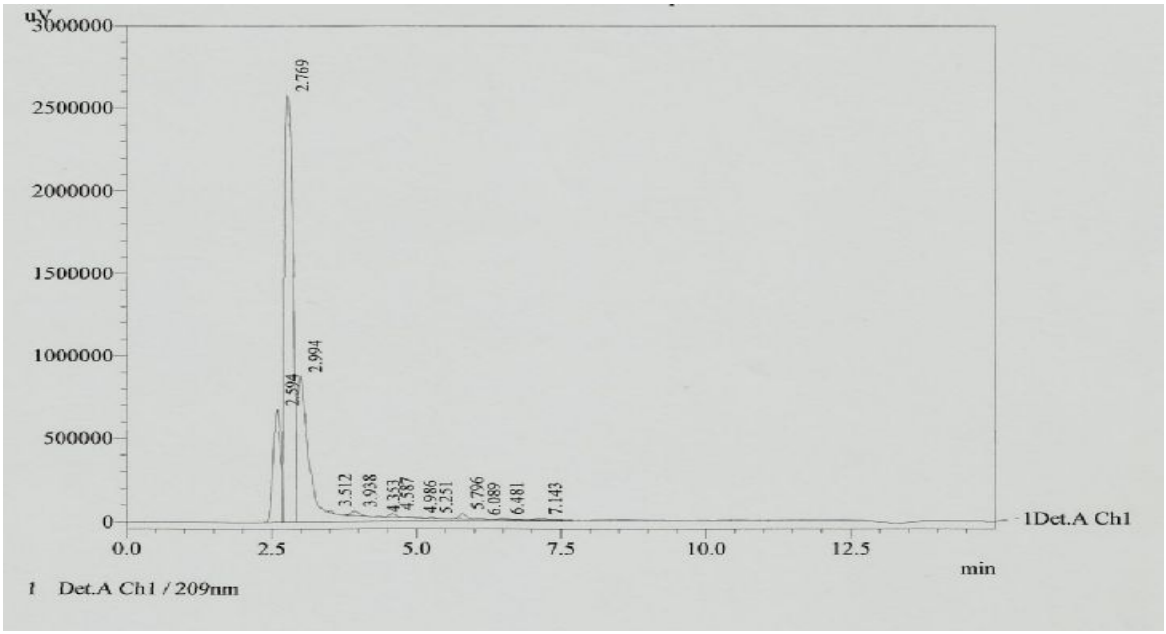


Figure (3-42): HPLC analysis of *Yucca aloifolia* extract obtained by extraction method No. 4 (stems).

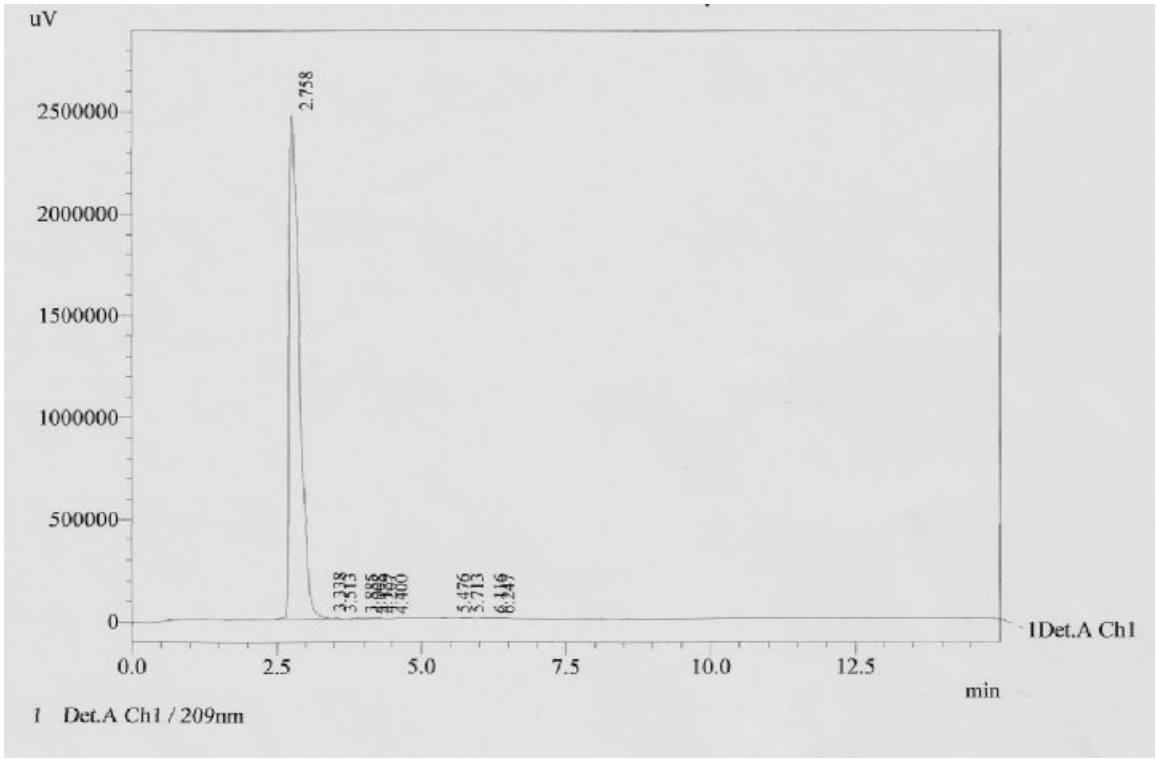


Figure (3-43): HPLC analysis of Tigogenin isolated from the leaves of *Yucca aloifolia* plant.

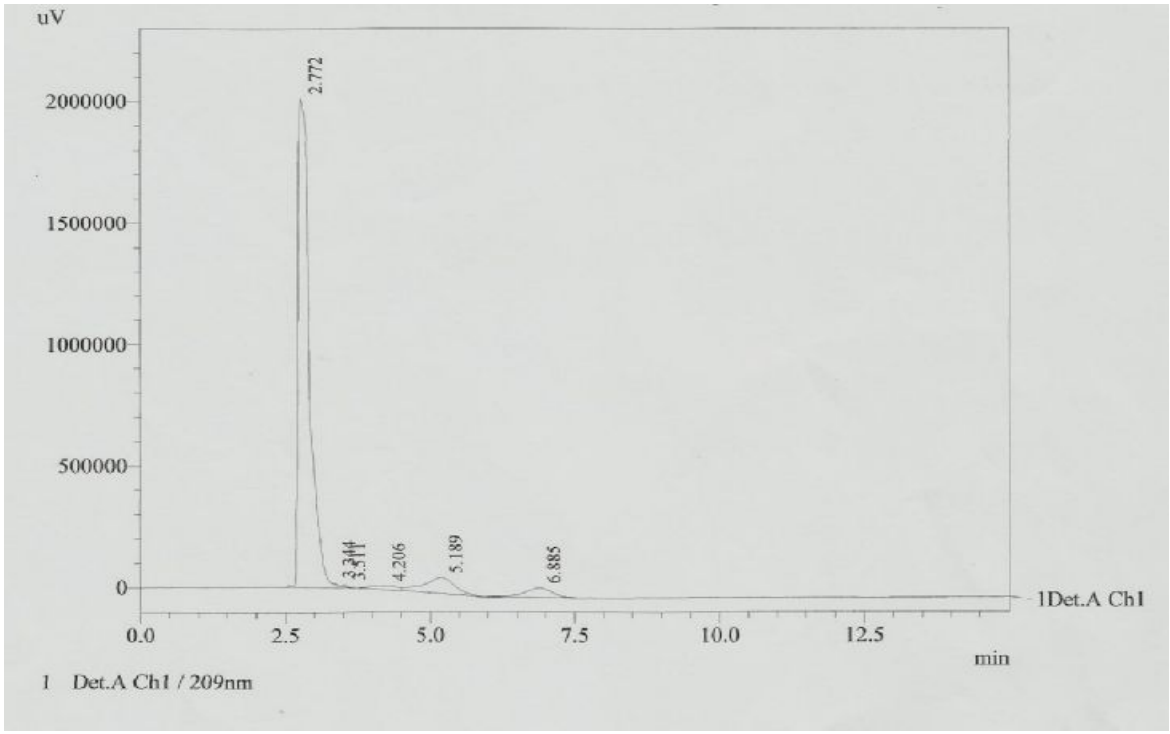


Figure (3-44): HPLC analysis of Tigogenin standard.

3.8-Cell line:

Table (3-7) and Figure (3-45) demonstrate the cytotoxic activity of twelve concentrations of two fold-dilutions of isolated tigogenin and plant extract on L20B cell line after 72 hours exposure time.

Statistical analysis revealed that only high concentrations of isolated tigogenin (80, 40, 20, 10 and 5µg/ml) showed significant cytotoxic effect on L20 B cell line ($P<0.05$) as compared with control group (0.0µg/ml). While L20B cell line culture treated with low concentrations (2.5, 1.25, 0.625, 0.312, 0.156, 0.078 and 0.039µg/ml) showed no significant cytotoxic effect when compared with negative control group (0.0µg/ml), ($p>0.05$).

The analysis data indicated also that there are significant differences between means of cell viability of L20B cell line culture treated with plant extract at concentrations of 80, 40, 20,10,5 and 2.5µg/ml and those of negative control (0.0µg/ml),($P<0.05$) as shown in table (3-7) and figure (3-45). Again, no significant differences observed between means of cell viability of L20B cell line treated with low concentrations of plant extract (1.25, 0.312, 0.156, 0.078 and 0.039µg/ml) comparing to negative control culture (0.0µg/ml).

The results showed that cell survival in both plant extract and isolated tigogenin treated cultures was progressively decreased with increasing the concentration, data analysis demonstrated significant decrease in cell viability with the increase in concentration of each plant extract and isolated tigogenin ($P<0.05$), figure(3-45).

The result in figure (3-45) exhibits the greatest effect of isolated tigogenin and plant extract at higher concentration during 72 hours of exposure. The cell line manifested loss their feature and highly decreased in viability cell number of L20B cell line after exposure to high and low concentration of isolated tigogenin, as seen in figures (3-47) and (3-48) comparing with confluent monolayer and cohesive cell of control cell, figure (3-46).

Table (3-7): **Cytotoxic effect of different concentrations of isolated tigogenin and plant extract on L20B cell line for 72 hour**.

Concentrations µg/ml	Optical density, (cell viability)	
	Isolated Tigogenin	**Plant Extract**
80	A,a 0.253±0.008	A,a 0.279±0.011
40	A,a 0.227±0.02	A,a 0.337±0.0189
20	A,a 0.214±0.018	A,a 0.333±0.0489
10	A,a 0.206±0.013	A,a 0.259±0.1245
5	A,a 0.456±0.02	B,b 0.257±0.0074
2.5	B,a 00.719±0.018	C ,b 0.276±0.0708
1.25	C,a 0.776±0.022	C,b 0.995±0.1248
0.625	D,a 0.686±0.003	C,b 1.208±0.0906
0.312	E,a 0.7527±0.037	C,b 1.426 0.0828
0.156	E,a 0.789±0.01	C,b 1.449±0.0905
0.078	E,a 0.714±0.019	C,b 1.424±0.0455
0.039	E,a 0.758±0.021	C,b 1.371±0.0729
0.0 (control)	0.792	1.595

L20B cell line:-

* Different small letters vertically significant at levels ($P< 0.05$)

* Different capital letters horizontally significant at levels ($P<0.05$).

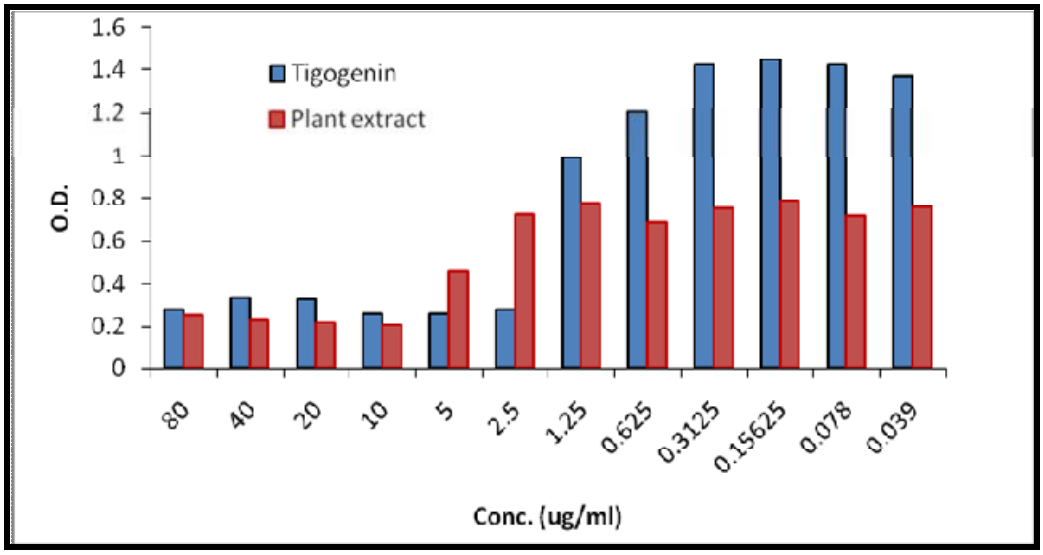


Figure (3-45): The cell viability (optical density or absorbance), of different concentrations of isolated tigogenin and plant extract using L20B cell line.

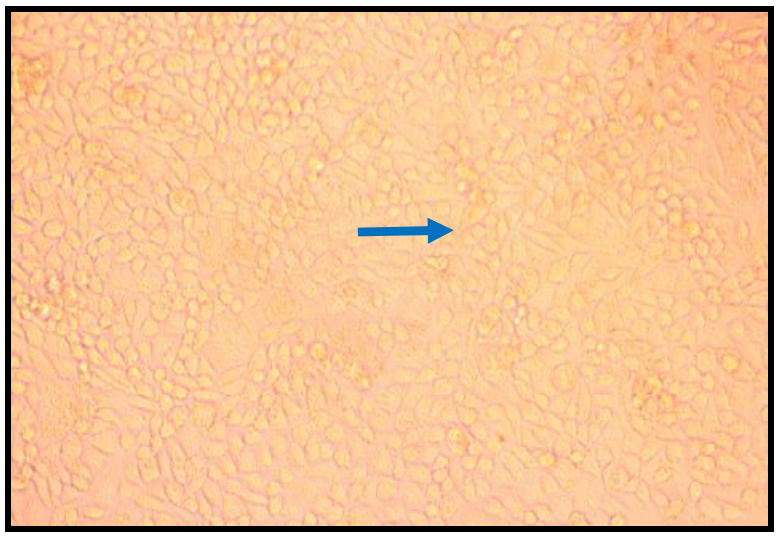


Figure (3-46): L20B cell line shows confluent monolayer (→), no empty spaces, (control) 100X.

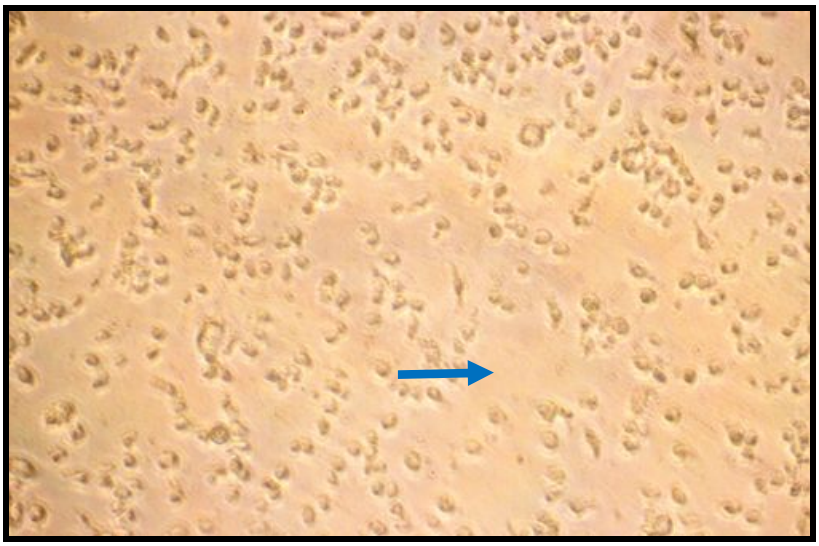


Figure (3-47):L20B cell line shows reduced in the viability cell number (➡) after exposure to high concentration (40 µg/ml) of tigogenin after 72 hours, 100X.

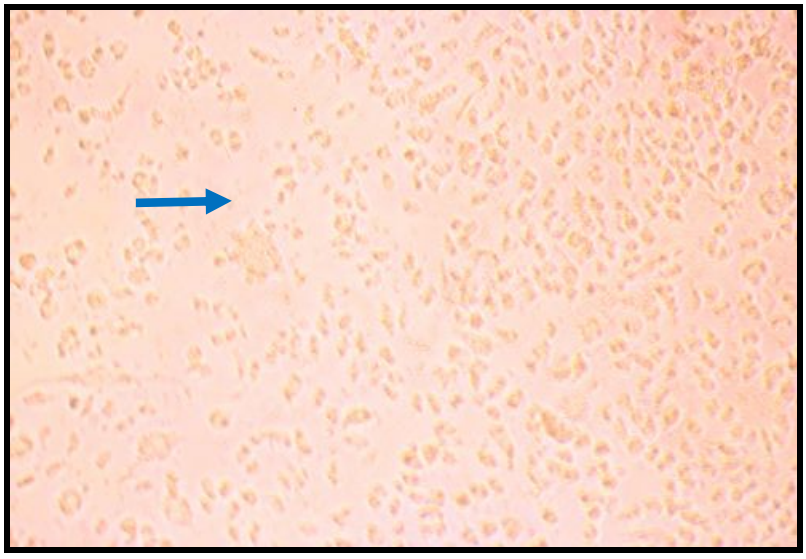


Figure (3-48):L20B cell line shows reduced in the viability cell number (➡) after exposure to low concentration (1.25µg/ml) of plant extract after 72 hours, 100X.

3.9-Growth inhibitory effect:

Figure (3-49) and table (3-8) show the effect of both tigogenin and plant extract on proliferation of L20B cell line after 72 hour of exposure. The antiproliferative effect of tigogenin on L20B cell line revealed that tigogenin had highest inhibitory growth on proliferation of L20B cell line culture at concentrations (80, 40, 20, 10 and 5µg/ml) range from (71.53% ,76.8%, 75.867%, 74.433%, and 48.73%) respectively, compared with control cells which have consider to give 0% rate. While the growth inhibition rate of treating L20B cell line with low concentrations (2.5, 1.25, 0.625, 0.312, 0.156 and 0.078µg/ml) of isolated tigogenin was reached to (19.13%, 12.66%, 22.83%, 15.26%, 19.7% and 11.3%) respectively.

On the other hand, the plant extract also has antiproliferative effect on L20B cell line with higher inhibitory growth on proliferation of L20B cell line at concentrations (80, 40, 20, 10, 5 and2.5 µg/ml) range from (81.83%, 78.87%, 78.68%. 83.13%, 80.54% and 72.21%) respectively.

In fact, highest inhibitory rate (IR) (83.13%) occurs at concentration 10µg/ml. This is may be due to other active constituents found in the plant extract which has also antiprolifrative effect. While the isolated tigogenin, exhibited highest IR (76.80%) at concentration 40µg/ml. This may be due to simple differences in technical preparation of cultures and reading of optical density because no significant differences in the inhibition rate observed between first four concentration ((80, 40, 20and 10 5µg/ml).

By comparing between the cytotoxic effects exhibited by plant extract and isolated tigogenin using L20B cell line, the general trend of this cell line study demonstrated that plant extract had greater IR on cell survival than pure tigogenin when they are used in higher concentrations with significant differences exerts at concentrations (5, 2.5, 1.25µg/ml), (P<0.05). While at low concentrations (0.312, 0.156, 0.078 and 0.039µg/ml), tigogenin had greater IR on L20B cell line than plant extract, however statistically there is

no significant differences between the cytotoxic effect of plant extract and that of isolated tigogenin when they are used in higher concentrations (80, 40, 20, and 10µg/ml), ($P > 0.05$). Percent of IR was measured by using the following equation (IR %=[(A-B)/A] × 100, A is optical density of negative control and B is optical density of treated cells).

Table (3-8): Percent of inhibition rate of different concentrations of isolated tigogenin and plant extract on L20B cell line for 72 hour.

Concentration (µg/ml)	% of inhibition rate	
	Isolated Tigogenin	**Plant extract**
80	A,a 71.533	A,a 81.833
40	A,a 76.8	A,a 78.877
20	A,a 75.867	A,a 78.687
10	A,a 74.433	A,a 83.13
5	B,a 48.733	A,b 80.547
2.5	C,a 19.133	A,b 72.217
1.25	C,a 12.667	B,b 38.033
0.625	C,a 22.833	C,a 23.667
0.312	C,a 15.267	C,a 10.433
0.156	C,a 19.7	C,b 8.933
0.078	C,a 11.3	C,a 10.4
0.039	C,a 14.7	C,a 13.6
0.0 (control)	D,a 0	D,a 0

L20B cell line:-

* Different small letters vertically significant alt levels ($P< 0.05$)

* Different capital letters horizontally significant at levels ($P<0.05$).

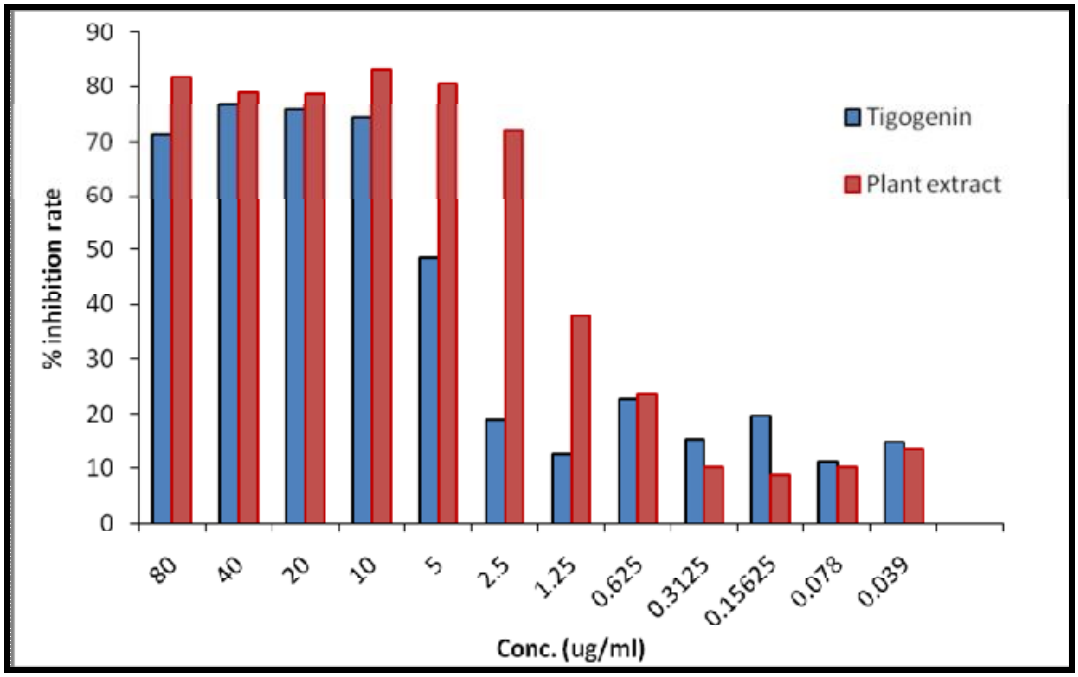


Figure (3-49): Percent of inhibition rate of different concentrations of tigogenin and plant extract at 72 hour exposure period.

Conclusions:-

1- Phytochemical investigation of ***Yucca aloifolia*** cultivated in Iraq revealed the presence of tigogenin as major steroidal saponin.

2- Iraqi ***Yucca aloifolia*** contain tigogenin in a goodpercentage relative to those reported in literature for***Yucca aloifolia*** grown outside Iraq. This need to perform other pharmacological studies to highlight their actions as therapeutic agents.

3- It is found that Extraction in soxhlet apparatus with 500mldichloroethane is better method for extraction of saponin glycosides from leaves and roots of ***Yucca aloifolia*** in comparison with other extraction method.

4- HPLC analysis of ***yucca*** extract showed the presence of tigogenin in Iraqi ***Yucca aloifolia*** .

5- In vitro study proved that ***Yucca aloifolia*** extract has significant cytotoxic activity on L20B cell line greater than that of the pure tigogenin. This maybe explained on the basis of that ***Yucca aloifolia*** extract (plant extract) may contain other active constituents that may potentiate its cytotoxic activity.

Recommendations :-

1- Qualitative and quantitative study of other steroidal sapogenins present in *yucca aloifolia*.

2- In vitro and in vivo study of the activity of ***Yucca aloifolia*** active ingredients including antibacterial, antifungal and antioxidant activity.

3- Screening the plant for other phytophores from other chemical classes such as phenolic compounds that are present as important phytophores in the plant under study.

4- Studying parameters affecting on the production of saponin glycosides including season, environmental conditions ,chemical agents as well as genetic modification that when achieved it will enhance their production if possible.

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الخلاصة

يعتبر نبات اليوكا (*yucca aloifolia*) نبات طبي ينتمي إلى العائلة (Agavaceae) وينمو بريا في منطقة أمريكا الجنوبية كما يستزرع في معظم مناطق العالم و منها العراق.

يحتوي نبات اليوكا على العديد من المركبات ذات الفعالية الحيوية حيث أنه غني جدا بالمركبات الصابونية الستيرويدية وخاصة مادة التيكوجنين, وتعتبر هذه المركبات مهمة من الناحية الصيدلانية لكونها تستخدم كبوادئ في صناعة بعض الأدوية الستيرويدية المهمةمثل الكورتزون واستخدامها في معالجة العديد من الأمراض خاصة التهاب المفاصل و الروماتزم, إضافة إلى استخدمتها الطبية الأخرى كمضاد للبكتريا و الفطريات و خافض للدهون و مضاد للأكسدة والسرطان.

أثبتت هذه الدراسة وجود احدى المواد الستيرويدية المهمة الموجودة في نبات اليوكا المستزرع بكثرة في العراق وهي مادة التيكوجنين . ونظرا لعدم وجود دراسة كيموحيوية سابقة في العراق على هذه المادة ولاستخدامها في صناعة بعض الأدوية الستيرويدية المهمة , اكتسبت هذه الدراسة أهميتها.

في هذه الدراسة تم استخلاص و كشف وفصل وتنقية مادة التيكوجنين من أوراق وسيقان جذور نبات اليوكا. استخلصت هذه المادة الستيرويدية من خلال استخدام اربع طرق مختلفة للأستخلاص. ولقد تم الكشف عن وجود التيكوجنين في الأربع المستخلصات باستخدام تقنية كروماتوكرافيا الطبقة الرقيقة وباستخدام ثمان مذيبات مختلفة كوسيط ناقل بعدها تمت عملية الفصل و التنقية. للتحقق من نوعية المركب المفصول و درجة نقائه استخدمت مجموعة تقنيات و التي شملت كروماتوكرافيا الأداء العالي السائلة(HPLC), درجة الانصهار للمركب المفصول و مطياف الأشعة تحت الحمراء (IR). كما تمت دراسة الفعالية السمية لكل من مستخلص نبات اليوكا و مادة التيكوجنين في نبات اليوكا على خط الخلايا السريطانية نوع (L20B).

أثبتت النتائج ان استخلاص خمسون غرام من أوراق وسيقان وجذور نبات اليوكا الجافة بواسطة الطريقة الرابعة للاستخلاص أعطت منتج أعلى من المستخلص بالمقارنة مع بقية الطرق الثلاثة للاستخلاص. إضافة إلى أن تقنية كروماتو كرافيا الطبقة الرقيقة أثبتت وجود مادة التيكوجنين بالمقارنة مع المادة القياسية لهذه المادة في ثمانية مذيبات مختلفة.

كما أثبتت تقنية كروماتوكرافيا الأداء العالي السائلة بان كمية التيكوجنين المستخلص الذي تم الحصول عليه بالطريقة الرابعة للاستخلاص كان أعلى من الذي تم الحصول عليه من الطرق الأخرى. كما أظهرت نتيجة خط الخلايا أن كل من مستخلص النبات و التيكوجنين له فعالية سمية واضحة.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بغداد
كلية الصيدلة

دراسة كيميائية للستيرويدات الصابونية (تيكوجنين) في نبات اليوكاالمستزرع في العراق

رسالة مقدمة إلى فرع العقاقير والى لجنة الدراسات العليا في كلية الصيدلة - جامعة بغداد كجزء من متطلبات الحصول على شهادة ماجستير علوم في الصيدلة
(العقاقير الطبية)

من قبل
نبأ محمد إبراهيم

(بكالوريوس صيدلة ٢٠٠٤)

بأشراف
الدكتورة زينب جليل عواد