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Antimicrobial activity and cytotoxicity of active compounds of *Withania somnifera* extracts

A Thesis

Submitted to the council of College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

Waham Saad Atta

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Supervised by

Dr. Nabeel K. Al-Ani

(Professor in Plant Biotechnology)

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بسم الله الرحمن الرحيم
الحمد لله رب العالمين الرحمن الرحيم
مالك يوم الدين اياك نعبد واياك نستعين
اهدنا الصراط المستقيم صراط الذين
انعمت عليهم غير المغضوب عليهم
ولا الضالين

Supervisors Certification

we, certify that this thesis entitled (**Antimicrobial activity and Cytotoxicity of Active Compounds of Withania somnifera extracts**) was prepared by (**Waham Saad Atta**) under my supervision at the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology.

Signature :

Name: Dr. Nabeel K. Al-Ani

Scientific Degree: Professor

Date:

In review of available recommendation, I forward this thesis for debate by examining committee

Signature :

Name: Dr. Hameed M. Jasim

Scientific Degree: Professor

Title: Head of Biotechnology Department

Date:

Committee Certification

We, the examining committee certify that we have read this thesis entitled **(Antimicrobial activity and Cytotoxicity of Active Compounds of *Withania somnifera* extracts)**and examined the student **(Waham Saad Atta)** in its contents and that in our opinion; it is accepted for the degree of **Master of Science in Biotechnology**.

Signature :
Name: Dr. khulood W. Abood
Scientific Degree: Professor
Date:
(Chairman)

Signature :
Name: Dr. Kamil M. Malih
Scientific Degree: Professor
Date:
(Member)

Signature :
Name: Dr. Nedhaal S. Zbar
Scientific Degree: Instructor
Date:
(Member)

Signature :
Name: Dr. Nabeel K. Al-Ani
Scientific Degree: Professor
Date:
(Member and Supervisor)

I, hereby certify upon the decision of the examining committee

Signature :

Name: Dr. Hadi M. A. Abood

Scientific Degree: Assistant Professor

Title: Dean of College of Science

Dedication

To those whom always give me support and encouragement to insist moving toward my glory future

My Father and My Mother

My uncle

Mustafa Ahmed Reshan

Waham.....

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Summary

In this study *Withania somnifera* leaves were collected from gardens of Al-Nahrain University. Then the leaves powder was used to get three type of extracts, each type of *Withania* extracts (water, ethanol and acetone extract) were subjected to chemical analysis to identify the active compounds in each extract which indicated that alkaloids, glycoside, saponins, flavonoids, fixed oil were present in ethanol, acetone and water extracts, while absent of terpene in water extract and protein in acetone extract, Tannins were not found in all extract types. Numbers of active group in Withania extracts were detected by FTIR method. HPLC analysis was done to detect the concentration of important active compounds (alkaloid, flavonoid and saponin) present in the water, acetone and ethanol extracts of Withania. All three types of Withania extracts contained two types of Alkaloids (Withanolide-A and Withaferin-A), seven types of Flavonoids (Naringenin, Catechin, Luteolin, Hesperetin, Kaempfero, Apigenin and Naringin) and two types of Saponins (Sitoindosides VII and Sitoindosides VIII) appeared as different peaks.

All three types of *Withania* extracts (acetone, water and ethanol extracts) showed antibacterial activity on the following bacteria (*Escherichia coli, Enterobacter sakazakii, Klebsiella pneumonia, Staphylocuss aureus, Staphylocuss epidermis, Streptococcus pyogenes, Proteus mirabilis, Pseudomonas aeruginosa*) with probability ($P \leq 0.05$) and only Acetone extract showed activity on fungal isolate (*Trichophyton violaceum*). The cytotoxic activity of the plant extracts was determined by evaluating the effects on the growth of HepG2 cell line after incubation for 72 hour with different concentrations (80, 100 and 120 mg/ml) of plant extracts (water, ethanol and acetone extracts).

W. somnifera extracts have cytotoxic effect on HepG2 cell line with probability ($P \leq 0.01$), higher cytotoxic effect was belonged to ethanol extract at 120 mg/ml concentration followed by the water extract and the acetone extract was the lowest one.

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List of abbreviations

abbreviation	***Full name***
CAT	*Catalase*
D.W.	*Distilled Water*
DF	*Dilution Factor*
DMSO	*Di Methyl Sulpha Oxide*
DPPH	*1-diphenyl-2-picrylhydrazyl)*
EDTA	*Ehylene- diamine-tetra acetic acid*
ELISA	*Enzyme linked immune absorbent assay*
FCS	*Fetal calf serum*
FLC	*Fast Liquid Chromatographic*
FTIR	*Fourier Transformed Infrared*
GC–MS	*Gas chromatography-mass spectroscopy*
GPX	*Glutathione peroxidase*
HeLa	*Human larynx carcinoma cells*
HepG2	*Human liver carcinoma cell line*
HPLC	*High performance liquid chromatography*
HPTLC	*Thin-layer chromatographic*
M.P.	*Manasa and Punjab*
mm	*Millimeter*
nm	*Nanometer*
NMR	*Nuclear magnetic resistance*
PBS	*Phosphate Buffer Saline*
PDA	*Potato dextrose agar*
PH	*Potential hydrogen*
R.p.m.	*Round per minute*
RPIM-medium	*Roswell Park Memorial Institute*
RT	*Retention Time*
SBL	*Sarcoma Black*
SER	*Sensitizer enhancement ratio*
SOD	*Superoxide dismutase*
UV	*Ultra Violet*
W.S.	*Withania somnifera*
WHO	*Worth health Organization*

Chapter one

Introduction

and Literature review

1. Introduction and Literature review

1.1. Introduction

Increase in the usage of antibiotics during past few years due to development of multidrug resistance among the pathogenic bacteria (Owaise *et al.,* 2005). The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. To reduce these problems, it is necessary to develop new drugs, which might be either synthetic or natural. The synthetic drugs are associated with side effects and toxic effects but the natural one could be safer which are produced naturally by plant or microorganism as secondary metabolite and used as drugs according to World Health Organization (WHO) (Santos *et al.,* 1995). These plant products are frequently considered to be less toxic and free from side effects (Brinker, 1998). World information about medicinal plants reports that almost 800 plants could be used to control different microorganism.

Withania somnifera (L.) Dunal, commonly known as Ashwagandha, is a plant belonging to the Solanaceae family. It is an evergreen shrub. It found in Pakistan, African and Asian Tropics, Europe, Bangladesh, Thailand, Sri Lanka and Northern India particularly in Garhwal region. It is used in Ayurvedic system of medicine, for antioxidant, anticancer, anti-inflammatory, leucoderma, antimicrobial acivity. Roots, leaves and bark have a potential role in the cancer therapy for growth inhibitory of human tumor cell line. Roots and leaves are used in tonic, abortifacient, astringent, nervier, mental problem improvement and also used in arthritis, depression, chronic diseases, infertility, memory loss, breathing difficulties and hormonal imbalance. (Nittala and Lavie, 1988; Kandil *et al.,* 2009). A number of withanolide steroidal lactones have been isolated from the leaves of *W.S.* (Glotter *et al.,* 1973) and exhibit antibacterial,

anti-fungal and antitumor properties. In addition to hypotensive, bradicardiac and respiratory stimulant activity (Devi *et al.,* 1993).

1.2. Aim of study

Evaluation *in vitro* and *in vivo* the antimicrobial activity of *Withania somnifera* using different extracts on different concentration. Besides, phytochemical screening of the acetone, water and ethanol extracts to assess the presence of different phytochemical in three extracts by different qualitative and quantitative method.

1.3. Literature Review.

1.3.1. Medicinal Plants

Medicinal plants are plants, plant parts, plant products, plant extracts and/or plant derived products that are employed in the treatment of diseases or used for their therapeutic properties. They are also used in the sense of improving the health status of human beings (NCCAM, 2005). Most of their effects were discovered through the folkloric medicine, in which the populations around the globe have developed their own strategies to remedy their illness (Lima *et al*., 2005). Herbal medicine is based on the premise that plants contain natural substances that can promote health and alleviate illness. Several herbs provide some protection against cancer and stimulate the immune system. Additionally, several commonly used herbs have been identified by the National Cancer Institute as possessing cancer preventive properties (Al-Attar, 2006). The use of herbs as medicines has played an important role in nearly every culture on earth, including Asia, Africa, Europe and America (Wargovich *et al.*, 2001). Most of these plant-derived medicines were originally discovered through the study of traditional cures and folkloric knowledge and some of these could not be substituted despite the enormous advancement in synthetic chemistry (Gilani and Rahman, 2005).

1.3.2. The *Withania genus*

The genus Withania (Family: Solanaceae) is a highly acclaimed genus of medicinal plants in the Indian Ayurveda system of medicine because of its valuable pharmaceutical and nutraceutical properties. Among the twenty-three known species of Withania, only two (*Withania somnifera* and *Withania coagulans*) are economically significant (Negi *et*

al., 2006) *W. somnifera* is the most exploited species of the family (Hemalatha *et al.,* 2008).

1.3.2.1. Common name and Taxonomy

Withania somnifera commonly known as Ashwagandha, winter cherry, Indian ginseng and poison gooseberry (Gurib-Fakim. and Schmelzer, 2012) is a plant belong to the Solanaceae or night-shade family. The species name *Somnifera* means "sleep-inducing" in Latin (stearn, 1995). Several other species in the genus Withania are morphologically similar (Gupta *et al.,* 2011). Taxonomically the plant is classified as the following (Bector *et al.,* 1968; Anwer *et al.,* 2008)

- Kingdom: Plantae (Plants)
- Sub kingdom: Tracheaobionta (Vascular Plant)
- Super division: Spermatophyta (Seed Plants)
- Division: Magnoliophyta (Flowering Plants)
- Class: Magnoliopsida (Dicotyledonous)
- Sub class: Asteridae
- Order: Solanales
- Family: Solanaceae
- Genus: Withania
- Species: Somnifera
- Binomial name: *Withania somnifera* (L.) Dunal

1.3.2.2. Plant morphology

A dense, hairy erect grayish to mentose herb or under shrub (fig1-1). The roots are stout, long tuberous, fleshy, whitish brown and aromatic. The leaves are simple, alternate or sub-opposite, round-oval shaped (fig1-2). The flowers are greenish-yellow and found in few flowered clusters in axils. The fruit is a round orange-red berry, enclosed in green enlarged calyx. The fruit resembles that of red cherries. The seeds are many, yellow kidney shaped and discoid (Bhandari, 1995).

Figure (1-1) *Withania somnifera* aerial part (Photo by author)

Figure (1-2) Morphology of *Withania somnifera* leaves (Photo by author)

1.3.2.3. Plant Distribution

It is a xerophytic plant, found in the drier parts of India, African and Asian Tropics, Europe, Sri Lanka, Afghanistan, Baluchistan and Sind and is distributed in the Mediterranean regions This shrub is common in Bombay and Western India, occasionally met within Bengal. It grows wildly throughout Iraq, Syria, and Jordan particularly in hotter parts, on waste places and on road sides. It is also cultivated for medicinal purposes in fields and open grounds throughout India. (Nadkarni, 1982 and Aphale and chihba, 2007). In Unani system of medicine, roots of *W. somnifera* commonly known as Asgand are used for the medicinal properties. However, leaves of the plant are also reported to be used medicinally (khan, 1982).

1.3.2.4. Chemical Composition

The phytochemistry of Withania species has been studied extensively by several workers and several groups of chemical such as steroidal lactones, alkaloids, flavonoids, tannin etc. have been identified, extracted, characterized and isolated (Atta-ur-Rahman *et al.*, 1993; Kapoor, 2001). At present, more than 13 alkaloids, 138 withanolides, and several sitoindosides (a withanolide containing a glucose molecule at carbon 27) have been isolated and reported from aerial parts, roots and berries of *Withania* species (Choudhary *et al.,* 1995 and Xu *et al.,* 2011).

The major chemical constituents of this plant, withanolides, are mainly localized in the leaves and roots (Kapoor, 2001). The withanolides are a group of C28-steroidal lactones built on an ergostane structure in which C-22 and C-26 are oxidized to form a six-membered lactone ring. (Glotter, 1991 and Alfonso *et al.,* 1993). The withanolide skeleton may be defined as a 22-hydroxyergostan-26-oic acid- 26, 22-lactone.

Modifications of the carbocyclic skeleton or the side chain give rise to many novel structures variants of withanolides. It has been reported that plants accumulating these polyoxygenated compounds possess enzyme ma-chinery capable of oxidizing all carbon atoms in the steroid nucleus. The characteristic feature of withanolides and ergostane-type steroids is one C8 or C9-side chain with a lactone or lacto ring. The lactone ring may be either six-membered or five-membered and fused with the carbocyclic part of the molecule through a carbon-carbon bond or through an oxygen bridge. Appropriate oxygen substituents may lead to bond scission, formation of new bonds, aromatization of rings and many other kinds of rearrangements resulting in novel structures (Glotter, 1991 and Mirjalili *et al.,* 2009).

Though withanolides are the principal bioactive compounds found in species, there are some withanolides specific to each of them. Withaferin A is a major compound found in *W. somnifera* a unique thio-dimer of withanolide named ashwagandhanolide has been found in W. Somnifera (Subaraju, 2006). Zhao *et al.* (2002) isolated five new withanolide derivatives from the roots of *W. somnifera* together with fourteen known compounds, and recently Tong *et al.* (2011) also reported a novel chlorinated withanolide, 6a-chloro-5b,17a-dihydroxy withaferin A , from *W. somnifera.*

1.3.3. Biological activity and pharmaceutical application

1.3.3.1. Anti-oxidant Activity of *Withania*

Administration of active principles of *Withania somnifera*, consisting of equimolar concentrations of sitoindosides VII-X (saponin), flavonoid (Catechin) and Withaferin A was found to increase superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX)

activity in rat brain frontal cortex and striatum. Antioxidant effect of active glycol-withanolides of *Withania somnifera* (W.S.) may explain, at least in part, the reported anti-inflammatory, immunomodulatory, anti-stress, ant-aging and cognition-facilitating effects produced by them in experimental animals, and in clinical situations (Bhattacharaya *et al.*, 1997).

1.3.3.2. Anti-inflammatory Activity of *Withania*

Withaferin A exhibits fairly potent anti-arthritic and anti-inflammatory activities. Anti-inflammatory activity has been attributed to biologically active steroids, of which Withaferin-A is a major component. It is as effective as hydrocortisone sodium succinate dose for dose (Khare, 2007). It was found to suppress effectively arthritic syndrome without any toxic effect. Unlike hydrocortisone-treated animals which lost weight, the animals treated with Withaferin-A showed gain in weight in arthritic syndrome. It is interesting that Withaferin A seems to be more potent than hydrocortisone in adjuvant-induced arthritis in rats, a close experimental approximation to human rheumatoid arthritis. In its oedema inhibiting activity, the compound gave a good dose response in the dose range of 12-25 mg/kg body weight of Albino rats intraperitoneally and a single dose had a good duration of action, as it could effectively suppress the inflammation after 4 hours of its administration (khan, 1982; Rastogi and Mehrotra, 1998). *Withania somnifera* has been shown to possess anti-inflammatory property in many animal models of inflammations like carrageenan-induced inflammation, cotton pellet granuloma and adjuvant-induced arthritis (Sharada *et al.*, 1996). Detailed studies were carried out to investigate the release of serumglobulin during inflammation by two models of inflammations viz. primary phase of adjuvant induced arthritis and formaldehyde-induced arthritis. The

experiments showed interesting results as most of the APR were influenced in a very short duration and also suppressed the degree of inflammation (Anabalagan and Sadique, 1985).

1.3.3.3. ImunomodulImatory activity

A series of animal studies have demonstrated Ashwagandha to have profound effects on healthy production of white blood cells, which means it is an effective immunoregulator and chemoprotective agent (Kuttan, 1996 and Ziauddin *et al.* 1996) In a study using mice, administration of powdered root extract from Ashwagandha was found to enhance total white blood cell count. In addition, this extract inhibited delayed-type hypersensitivity reactions and enhanced phagocytic activity of macrophages when compared to a control group (Davis and Kuttan, 2000) Recent research suggests a possible mechanism behind the increased cytotoxic effect of macrophages exposed to *W. somnifera* extracts (Iuvone *et al.* 2003). Nitric oxide has been determined to have a significant effect on macrophage cytotoxicity against microorganisms and tumor cells. Iuvone *et al*. (2003) demonstrated *W. somnifera* increased no production in mouse macrophages in a concentration-dependent manner.
This effect was attributed to increased production of inducible nitric oxide synthase, an enzyme generated in response to inflammatory mediators and known to inhibit the growth of many pathogens (Bogdan, 2001). Research has also shown Ashwagandha to have stimulatory effects, both *in vitro* and *in vivo*, on the generation of cytotoxic T-lymphocytes, and a demonstrated potential to reduce tumor growth (Davis and Kuttan, 2002). The chemopreventive effect was demonstrated in a study of ashwagandha root extract on induced skin cancer in Swiss albino mice given Ashwagandha before and during exposure to the skin cancer-causing agent (7,12-dimethylbenz[a]anthracene) (Prakash *et al.*

2002). A significant decrease in incidence and average number of skin lesions was demonstrated compared to the control group. Additionally, levels of reduced glutathione, superoxide dismutase, catalase, and glutathione peroxidase in the exposed tissue returned to near normal values following administration of the extract. The chemopreventive activity is thought to be due in part to the antioxidant/free radical scavenging activity of the extract.

1.3.3.4. Other Activities of *Withania*

Other Activities of *W. somnifera* include Anti-ageing effect (Rastogi and Mehrotra, 1998). Morphine tolerance and dependence-Inhibiting effect (Rao *et al.,* 1995), Musculotropic activity (khan, 1982), Macrophage-activating effect (Dhuley, 1997), Neuropharmacological activity (Schliebs *et al.,* 1997), Anti-hyperglycemic effect (Bhattacharaya *et al.,* 1997), Hepatoprotective activity (Khare, 2007; Rastogi and Mehrotra, 1998), Anticonvulsant activity (Kulkarni and George, 1996).

1.3.4. Metabolite profiling

The analysis of total metabolite of a plant is important to extend our understanding of complex biochemical processes within a plant. Significant technological advances in analytical systems like nuclear magnetic resistance (NMR), gas chromatography-mass spectroscopy (GC–MS) and high performance liquid chromatography (HPLC) have opened up new avenues for plant metabolomic research aimed at rapidly identifying a large number of metabolites quantitatively and qualitatively. This has become an important area of investigations in pharmacology and functional genomics of medicinal plants. Comprehensive chemical analysis is required not only to establish correlation between complex chemical mixtures and molecular pharmacology, but also to understand

complex cellular processes and biochemical pathways via metabolite-to-gene network (Nakabayashi *et al.,* 2009). The metabolic constituents, particularly secondary metabolites differ by tissue type and sometimes with growth conditions (Abraham *et al.,* 1968). Such variations often lead to inconsistent therapeutic and health promoting properties of various commercial plant preparations (Sangwan *et* a*l.,* 2004; Dhur *et al.,* 2006) and the compositional standardization of herbal formulation becomes difficult.

1.3.5. Antimicrobial Activity

The antimicrobial activity of the roots as well as leaves has been shown experimentally. Withaferin-A in concentration of 10mg/ml inhibited the growth of various Gram-positive bacteria, acid-fast and aerobic bacilli, and pathogenic fungi. It was active against Micrococcus pyogenic var *aureus* and partially inhibited the activity of Bacillus subtilis glucose-6-phosphatedehydrogenase. Withaferin-A inhibited Ranikhet virus. The shrub's extract is active against Vaccinia virus and Entamoeba histolytica (khan, 1982; Rastogi and Mehrotra, 1998; Khare, 2007). Asgand (*Withania* root) showed the protective action against systemic Aspergillus infection. This protective activity was probably related to the activation of the macrophage function revealed by the observed increases in phagocytosis and intracellular killing of peritoneal macrophages induced by Ashwagandha treatment in mice (Dhuley, 1998).

Antibiotic activity of Withaferin-A is due to the presence of the unsaturated lactone-ring. The lactone showed strong therapeutic activity in experimentally induced abscesses in rabbits, the being somewhat stronger than that of Penicillin. It substantiates the reputation of the

leaves as a cure for ulcers and carbuncles in the indigenous system of medicine (khan, 1982).

1.3.5.1. Bacterial skin infection

There are many types of bacteria; most of them can cause disease. Many species play beneficial roles producing antibiotics and foodstuffs. Soil living bacteria perform much essential function in the biosphere, like nitrogen fixation. Our body is covered with commensally bacteria that make up the normal flora. Bacteria such as *Staphylococcus spp.*, *Corynebacterium spp., Brevibacterium spp., and Acinetobacter* live on normal skin and cause no harm. *Propionibacteria* live in the hair follicle of adult skin and contribute to acne (Daniel *et al.,* 2002).

The classification of bacterial skin infection (pyodermas) is an attempt integrates various clinical entities in an organized manner. It is caused by the presence and growth of microorganisms that damage host tissue. The extent of infection is generally determined by how many organisms are present and the toxins they release (Roberts and Chamber, 2005). Common bacterial skin infection include boils, cellulites, erysipelas, impetigo, folliculitis, furuncles, and hot tube folliculitis. Boils which is skin infection caused by *Staphylococcus* is quite common. Cellulite is an infection of the deeper layers of skin and the connective tissue below the skin's surface that poorly demarcated borders. People with cellulites usually have an area of red, Swollen, tender, warm, and is usually caused by *Streptococcus spp. or Staphylococcus spp.* (Bjornsdottir *et al.,* 2005; Roberts and Chambers, 2005) and *Pseudomonas sp.* which is also found on the skin of the healthy persons and inhabitant of soil, water, and vegetation (Toder, 2004).

It is pathogenic only when introduced into areas devoid of normal defense such as disruption of skin and mucous membrane after direct

tissue damage. The bacterium attaches to and colonizes the mucous membranes or skin, invades locally and produces systemic disease. The most serious infections caused by *P. aeruginosa* include infection of wound and burns, malignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia, and septicemia (Bodey *et al.*, 2007).

1.3.5.2. Fungal skin infection

Approximately 90% of fungal skin's infections are caused by dermatophytes, which are parasitic fungi affecting the skin, hair, or nails (john, 1996). There are three groups of dermatophytes, called *Trichophyton* (affects skin, nail and hair), *Microsporum* (a type of fungus that causes ringworm in children) and *Epidermophyton* (A fungal which grows on the outer layer of the skin and cause of tinea). These infections are mostly seen after puberty with exception of tinea capitis, which is a fungal infection; involve scalp hair, seen in children (Muller *et al.*, 1989). Other skin infections are caused by yeast such as *Candida.* Another known as *Malassezia* furfur is a type of fungus that causes brownish patches on skin. This particular yeast resides on skin that has high (oily) sebum content such as the face, scalp and chest. Infection of the skin by *C. albicans* accurse principally in moist, warm part of the body, such as the axilla, intergluteal folds, and groin or inframammary folds (Kovacs and Hruza, 1995). It is most common in obese and diabetic individuals. These areas become red and weeping and many develop vesicles (John, 1996).

1.3.6. Pathogenicity of other studies Microorganism

1.3.6.1. *Enterobacter sakazakii*

E. sakazakii is a member of the family *Enterobacteriaceae,* genus *Enterobacter,* and is a motile peritrichous, gram-negative bacillus (Farmer *et al.*, 1990). *E. sakazakii* is regarded as an emerging

opportunistic human pathogen and the etiological agent of life-threatening bacterial infections(Jaspar *et al.*,1990) The bacterium is ubiquitous being isolated from a range of environments and foods, and the majority of *Enterobacter* cases are in the adult population(Joseph, 2012). It food poising bacteria, in infant can cause bacteraemia, meningitis and necrotising enterocolitis. Some neonatal *C. sakazakii* infections have been associated with the use of powdered infant formula (CDC, 2002; Bowen and Braden, 2006).

1.3.6.2. *Escherichia coli*

E. coli is a Gram-negative, facultatively anaerobic, rod-shaped bacterium of the genus Escherichia that is commonly found in the lower intestine of warm-blooded organisms (endotherms) (Singleton, 1999). Most E. coli strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination (*CDC,* 2012; Vogt and Dippold 2005) The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2 (Bentley and Meganathan 1982) and preventing colonization of the intestine with pathogenic bacteria (Hudault *et al.,* 2001 and Reid *et al.,* Sep 2001).

1.3.6.3. *Proteus mirabilis*

P. mirabilis is a Gram-negative, facultatively anaerobic, rod-shaped bacterium. It shows swarming motility and urease activity. *P. mirabilis* causes 90% of all *Proteus* infections in humans. It is widely distributed in soil and water (Rauprich *et al.*, 1996). This bacteri has the ability to produce high levels of urease, which hydrolyzes urea to ammonia (NH_3), so makes the urine more alkaline. If left untreated, the increased alkalinity can lead to the formation of crystals of struvite, calcium carbonate, and/or apatite. Once the stones develop, over time they may grow large enough to cause obstruction and

renal failure. *Proteus* species can also cause wound infections, septicemia, and pneumonia, mostly in hospitalized patients (Gué *et al.*, 2001).

1.3.6.4. Klebsiella pneumonia

*K.pneumonia i*s a Gram-negative,nonmotile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. It is found in the normal flora of the mouth, skin, and intestines (Ryan and Ray, 2004). Members of the *Klebsiella* genus typically express two types of antigens on their cell surfaces. The first, O antigen, is a component of the lipopolysaccharide (LPS), of which 9 varieties exist. The second is K antigen, a capsular polysaccharide with more than 80 varieties.[4] Both contribute to pathogenicity and form the basis for serogrouping.it can cause a wide range of disease such as pneumonia, urinary tract infection, septicemia, Ankylosing spondylitis and soft tissue infections (Podschum and Ullmann, 1998).

1.3.7. Effect of active compounds on Tumor cell line

In order to study the effect of some active compounds, there are many principle should be taken into consideration to detect inhibitory effect on cancer cells line, numerous cells line should be available (Grafone *et al.*, 2003).

In general, the compound could be cytotoxic or non on cells; therefore, many items have been established for their cytotoxic activity which could be as follows: Identify the anti-tumor effect of compounds, understand the mode of action of these compounds upon cancer cells, detect the effect of compounds on target cells, determine optimal concentration and determine the relation between the concentration and exposure times. (Wilson, 2000).

The main idea over all from determining the cytotoxic assay is cell death or inhibition over growth due to exert cytotoxic effect on these cells. In the last decade, it has become important and essential to determine or identify the cytotoxic assay for these active compounds. It was found the cytotoxic effect of the compound on cancer cells either irreversible or reversible and its effect could be immediate or after weeks (freshney, 2000).

In 1990 the National Cancer institute in USA established a new idea for detect the effects of different compound on cancer cells *in vitro* by providing different cancer cell lines to many tumors in reliable and easy manner ways to get reasonable effect upon these cells(Bodey, 1998).

The cytotoxic assay has several advantages: It can easily analyzed statically, no average could be needed and The relation between time and concentration could be controlled in vivo with viability to control the physical, chemical and physiological effect of the environment beside many experiments could be done in one experiment with little cost by micro-titration system.

On the other hand, cytotoxic assay has some limitation, involves the difficulty of pharmacokinetic action of active compounds *in vivo* and *in vitro*. This depend on regulating the effect of secondary metabolites *in vitro,* while in *vivo* it's depend on distribution the activity over many cells in many ways. Also the log phase for cancer cells *in vitro* is less than that *in vivo* this will affect on the mode of action of the compounds. Furthermore, the permeability of cancer cell *in vivo* is different from that *in vitro,* this mean that the effect of these compound could be differ between *in vivo* and *in vitro*(Freshney, 2001).

1.3.7.1. Cytotoxic activity of *Withania somnifera*

The cytotoxic effect of *Withania* has been studied extensively (Devi, 1996 and Widodo *et al.,* 2007), and it was found that it is the most effective agent in preventing cancer through its ability to reduce the tumor size. Treatment of root extract of *W. somnifera* on induced skin cancer in mice exhibited significant decrease in the incidence and average number of skin lesions compared to control group (Prakash *et al.,* 2002).

Withaferin A, withanolide D & E exhibited significant antitumor activity *in vitro* against cells derived from human epidermis carcinoma of nasopharynx (KB) and *in vivo* against Ehrlich ascites carcinoma, Sarcoma 180, Sarcoma Black (SBL), and E 0771 mammary adenocarcinoma in mice at doses of 10, 12 or 15 mg/kg body-weight(Jayaprakasam *et al.,* 2003).

It also inhibited the growth of roots of *Allium cepa* by arresting the cell division at metaphase, Growth of Ehrlich ascites carcinoma was completely inhibited in more than half of mice which survived for 100 days without the evidence of growth of the tumor. They also acted as a mitotic poison arresting the division of cultured human larynx carcinoma cells at metaphase and in HeLa cultures similar to star metaphase. Withaferin-A caused mitotic arrest in embryonic chicken fibroblast cells. Methylthiodeacetyl colchicine potentiated the effect of Withaferin-A (Palyi *et al.,* 1969).

In another study, *W. somnifera* was evaluated for its antitumor effect in urethane-induced lung adenomas in adult male albino mice. Simultaneous administration of *W. somnifera* extract (200 mg kg-1 body weight daily orally for seven months) and urethane (125 mg kg-1 biweekly for seven months) reduced tumor incidence significantly (Singh *et al.,* 1986).

The presence of an unsaturated lactone in the side-chain to which an allelic primary alcohol group is attached at C25 and the highly oxygenated rings at the other end of the molecule may well suggest specific chemical systems possessing carcinostatic properties (Rastogi and Mehrotra, 1998; Khare, 2007). Withaferin A has been shown to possess Growth inhibitory and radio-sensitizing effects on experimental Mouse tumors (Ganasoundari *et al.*, 1997).

Administration of Withaferin-A in mice inoculated with Ehrlich ascites carcinoma cells was found to inhibit tumor growth and increase tumor-free animal survival in a dose dependent manner (Devi *et al.*, 1995). The alcoholic extract of the dried roots of the plant as well as the active component Withaferin-A isolated from the extract showed significant antitumor and radio-sensitizing effects in experimental tumors *in vivo*, without any noticeable systemic toxicity (Sharada *et* a*l.*, 1996).

One-hour treatment with Withaferin-A in a nontoxic dose of 2.1 M before irradiation significantly enhanced cell killing. Withaferin-A gave a sensitizer enhancement ratio of 1.5 for *in vitro* cell killing of V79 Chinese hamster cells at a nontoxic concentration of approximately 2 M. SER increased with drug dose (Devi *et al.*, 1996).

1.3.7.2. Human liver carcinoma cell line HepG2

HepG2 is perpetual cell line which was derived from the liver tissue with well differentiated hepatocellular carcinoma. These cells are epithelial in morphology (fig 1-3) are not tumorigenic in nude mice (Udeanu *et al.*, 2011).

Figure (1-3) Morphology of HepG2 cell lines (Nguyen, 2012)

The cells secret a variety of major plasma proteins fibrinogen, alpha 2- macroglobulin, alpha 1-antitrypsin, transferrin and plasminogen. HepG2 cells are a suitable *in vitro* model system for the study of polarizedhuman hepatocyte (Ihrke *et al.*, 1993). With the proper culture conditions, HepG2 cells display robust morphology and functional differentiation with a controllable formation of apical and basolateral cell surface domains (Van IJezendoorn and Mostov, 2000) that resemble the bile canalicular and sinusoidal domains (*in vivo*), respectively.

Because of their high degree of morphology and functional differentiation *in vitro,* HepG2 cells are suitable model to study the intracellular trafficking and dynamics of bile canalicular and sinusoidal membrane proteins and lipid in human hepatocyte *in vitro.* HepG2 cells and its derivatives are also used as a model system to studies of liver metabolism and toxicity of xenobiotic, the detection of cytoprotective, anti (environmental and dietary) genotoxic and cogenotoxic agents, understanding hepatocarcinogenesis, and for drug targeting studies. HepG2 cells are also employed in trials with bio-artificial liver devices (Mersh-Sundermann *et al.*, 2009).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Equipment and Instruments

The following Equipment and Instrument were used in this study:

Equipment	**Company (origin)**
Autoclave	Express/Germany
Centrifuge	Beckman/Germany
CO_2 Incubator	Sanyo/Japan
Disposable petri-dish	Sterilin/ England
Disposable micropipette Plastic Tips (Different sizes)	Jippo (Japan)
ELISA Reader	Asays/Austria
Filter paper	Halzfeld(Germany)
FTIR	Shimanduzu/Japan
Gas burner	Grade(England)
Incubator	Memmert/Germany
Inverted Microscope	(MEIJI, Japan)
Laminar flow cabinet	(Heraeus/ Germany)
Lyophilizer	Fisher/ U.K.
Micropipettes (Different sizes)	Witeg (Germany)
pH meter	Metter-Tolled (U.K.)
Refrigerator	Astrin/Japan
Rotary Evaporator	Buchi (Switzerland)
Sensitive balance	Delta Range (Switzerland)
Shaker Incubator	GFL 32221
Spectrophotometer	Cecil(France)
Water bath	GFL (Germany)

2.1.2. Chemicals

The Following chemicals were used in this study:

Chemicals	**Company/ Country**
Absolute ethanol, Absolute acetone, Absolute methanol (0.99%), Trypan blue stain	BDH/England
Di Methyl Sulpha Oxide (DMSO), Fetal calf serum (FCS), RPMI-1640. Hydrochloric acid (HCL), Natural Red Dye, Penicillin, streptomycin, trypsin.	Sigma /U.S.A
Ferric chloride ($FeCL_2$), Potassium Iodide (KI), Mercuric chloride ($HgCl_2$).	Fluke/Switzerland
Sulphric acid	Analar/England

2.1.3. Ready to use media

All media listed below were prepared according to the instructions on container by their manufacturing company.

2.1.3.1. Bacterial and fungi Media

Medium	Company (Origin)
Brain heart infusion agar	Fluka (Switzerland)
Nutrient agar	Fluka
Nutrient broth	Biolife (Italy)
Saboroud Dextrose agar	Oxoid(England)
Saboroud Dextrose broth	Oxoid(England)

The above media were sterilized by autoclaving at 121°C, 15 psi for 15 min (Collee *et al*., 1996).

2.1.3.2. Cell line Media

This medium contained the RPMI-1640 medium base 10g, fetal bovine serum 10%, penicillin 1000000U, Streptomycin 1g and sodium bicarbonate1%.

2.1.4. Standards

The stander was prepared according to Mauricio *et al.* (2007) and Rajaseka and Elango, (2011) preparation method at the ministry of science and biotechnology.

2.1.4.1. Alkaloid Standards

The main components were separated with column under the optimum condition, 3mm particle size, phenomenex C-18 RP (50×4.6mm I.D) column. **Mobile phase**: mixture of acetonitrile: Methanol: Ortho phosphoric acid (55:45:1 v/v), detection UV set at 280 nm, flow rate1.0ml/min, Temperature 25°C.The sequences of the eluted material of the standard were was as follow, each standard was 15µg/ml.

Sec	Subjects	Retention time (min)	Area
1	Withaferin-A	2.22	59730
2	Withanolide A	3.2	17712

2.1.4.2. Flavonoid Standards

The main component were separated with column under the optimum condition Column: phenomenex C-18,3µm size (50×2.0mm ID) column, **Mobile phase:** linear gradient of **solvent A:** 0.1% phosphoric

acid in deionized water **solvent B:**was acetonitrile, gradient program from 0%B to100 for 10 min flow rate 1.2ml/min, Temperature 25°C.

The sequences of the eluted material of the standard were was as follow, each standard was 25µg/ml.

Sec	Subjects	Retention time (min)	Area
1	Naringenin	1.64	10980
2	Catechin	2.78	17167
3	Luteolin	3.78	20622
4	Hesperetin	4.52	26273
5	Kaempfero	5.60	21511
6	Apigenin	6.46	28911
7	Naringin	7.38	24580

2.1.4.3. Saponin Standards

The main component were separated with column under the optimum condition Column: nucleoshellC-18 RP, 2.7 µm particale size (50×4.0mm ID) column, Mobile phase: linear gradient of, **solvent:** was mixture of acetonitrile, **solvent:** was mixture of 20 mmol/L kh2po4, detection UV set at 220 nm, flow rate 2ml/min. Temperature 25°C.

The sequences of the eluted material of the standard were was as follow, each standard was 20µg/ml.

Sec	Subjects	Retention time (min)	Area
1	SitoindosidesVII	2.68	22806
2	SitoindosidesVIII	3.78	52508

2.1.5. Solutions and Dyes Preparation

2.1.5.1. Lead Acetate Solution 1 %(w/v)

Prepared by dissolving 1 g of lead acetate in 100 ml distilled water, it's used for tannins detection (Shihata, 1951)..

2.1.5.2. Ferric Chloride Solution 1 %(w/v)

Prepared by dissolving 1g of ferric chloride in 100ml distilled water, it's used for tannins detection (Shihata, 1951)..

2.1.5.3. Potassium Hydroxide Solution 50 %(w/v)

Prepared by dissolving 50g of potassium hydroxide in 100ml of D.W., it's used for the detection of flavonoid (jaffer, 1983).

2.1.5.4. Fetal Calf Serum (FCS)

An aliquot of 10% FCS added to the media to support the cell growth.

2.1.5.5. Phosphor Buffer Saline (PBS)

One tablet of PBS was discovered in 200ml of distilled water by autoclave then used (Freshnery, 2000).

2.1.5.6. Antibiotic Solution

Two antibiotics were used penicillin and streptomycin. The penicillin, (1000000IU) and streptomycin(1g) each were dissolved in 10 ml of distilled water (D.W.) and stored at -20°C from each of these stocks 0.5 ml was added to one liter of culture media (Freshnery, 2000).

2.1.5.7. Neutral Red Dye

Ten gram of neutral dye was dissolved in 100 ml of PBS, mixed thoroughly and used immediately (Winckler, 1974; Abdul-Majeed, 2000).

2.1.5.8. Elution Buffer

It was freshly prepared by mixing phosphate buffer saline(0.5g) to absolute ethanol (500ml) (w/v) then used directly (Freshnery, 2000).

2.1.5.9. Trypsin blue stain

This stain was prepared by dissolving 0.1g of trypsin blue stain in 100ml of phosphate buffer saline, then filtered using Wattman filter paper. Finally the solution was stored at 4°C until used (Freshney, 2000).

2.1.5.10. Trypsin Solution

Aweigh of 2.5g of trypsin was dissolved in 100ml of PBS, then sterilized by filtrations and stored at 4°C (Freshney, 2000).

2.1.5.11. Versene Solution

Versene solution was prepared by dissolving 1g of ethylene-diamine-tetra acetic acid (EDTA) in 100ml of phosphate buffer saline, then sterilized by autoclaving and stored at 4°C (Freshney, 2000).

2.1.5.12. Trypsin – versene Solution

It was prepared by mixing 20 ml of trypsin solution, 10 ml of versene solution and 370ml phosphate buffer saline and kept at 4°C until used (Freshney, 2000).

. **2.1.5.13. Sodium Bicarbonate Solution**

Sodium Bicarbonate 4.4g was dissolved in 100 D.W. This was stored at 4°C until used (*Allen et al.,* 1977)

2.1.6. Cell Line

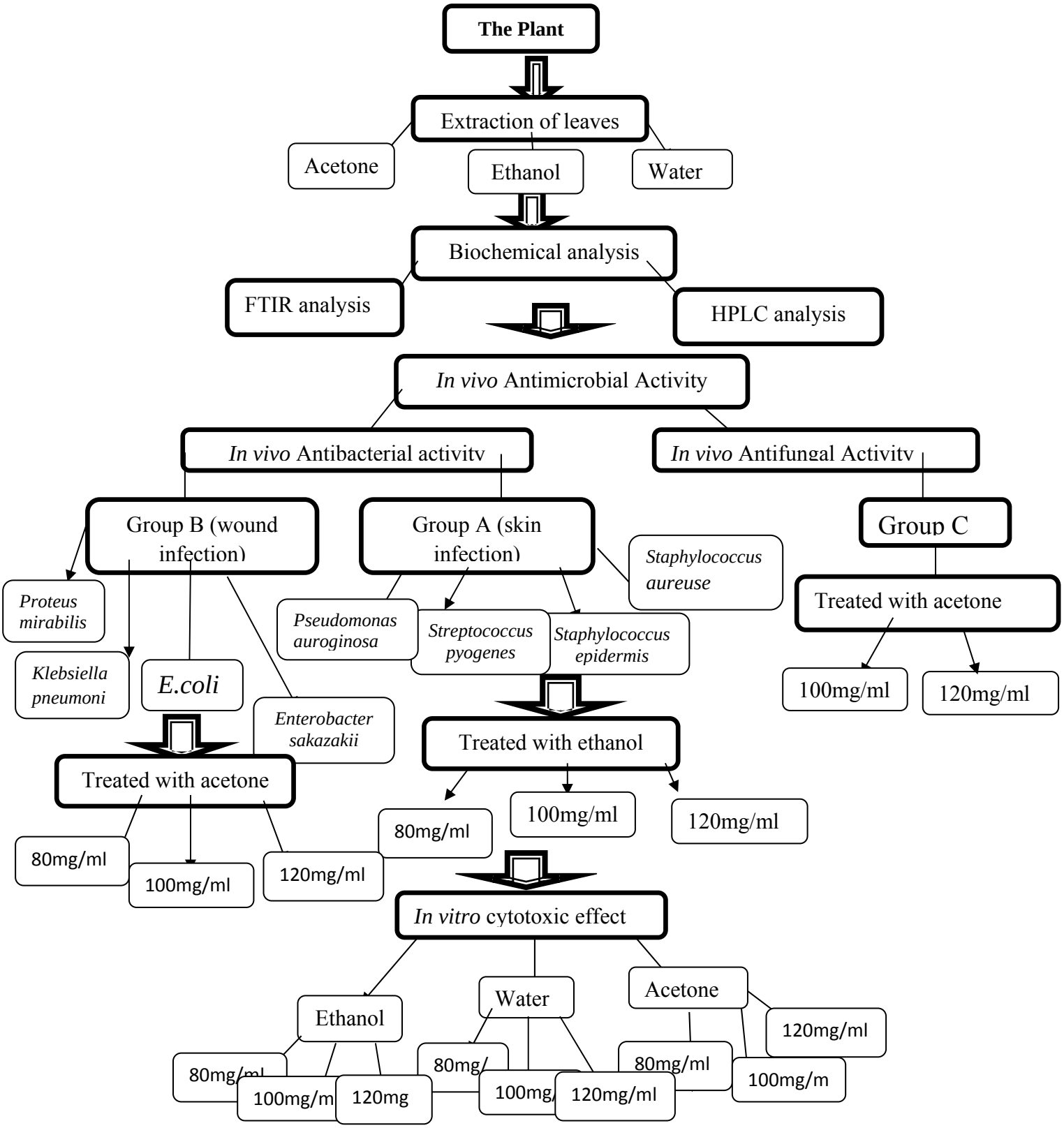
The HepG2 cell line (human liver carcinoma cell line) was kindly provided by Animal cell line culture laboratory, Biotechnology Research Center/ AL-Nahrain University.

2.1.7. Microbial Isolate

Bacterial isolates	**Sources**
pseudomonas aeruginosa	Biotechnology Research Center/ Al- Nahrain University.
Staphylocuss aureus	
Staphylocuss epidermis	
Streptococcus pyogenes	
Proteus mirabilis	
Enterobacter sakazakii	
Escherichia coli	
klebsiella pneumonia	
Fungi isolates	**Source**
Trichophyton mentagrophytes	Biotechnology Research Center/ Al-Nahrain University.
Trichophyton tonsurans	
Trichophyton violaceum	
Microsporum canis	

2.2. Methods

The main steps of research plan were summarized in (2-1).



Scheme (2-1) Methodology of research project

2.2.1. Plant Collection.

W.somnifera was collected in November 2013 at morning from garden in Al-Nahrain University. Arial parts of this plant were air dried in shade at room temperature for 15 days, then the leaves were separated and grinded into powder by using electric grinder.

2.2.2. Lab animals

Healthy adult mice with (4-5) weeks weighting (18-20) g were obtained from animal house of Biotechnology Research Center / Al-Nahrain University.

2.2.3. Preparation of Reagent, Solution, Media, and stain.

2.2.3.1. Reagents

The following reagents were used for the detection of active compounds in plant extracts.

2.2.3.1.1. Wagner's reagent

Two grams of potassium iodide were dissolved in 5ml of distilled water, then 1.27g of iodine was added and stirred until dissolved, the volume was completed to 100ml by add distilled water (Smolensk *et al.*, 1972). This reagent is used for the detection of alkaloids. Appearance of brown precipitation is an indication for the presence of alkaloid (Hussein, 1981).

2.2.3.1.2. Mayer's reagent

Solution A: A quantity of 35g of mercuric chloride was dissolved in 60ml of distilled water.

Solution B: A quantity of 5g of potassium iodide were dissolved in 10ml of distilled water, then equal volume of Solution A and B were mixed and the volume was completed to 100ml by adding distilled water (Smolenski *et al.,* 1972).This reagent was used for the detection of alkaloids. Appearance of white precipitate is an indication for the presence of alkaloid (Hussein, 1981; Treas and Evan, 1987).

2.2.3.1.3. Dragendroff's reagent

Two stock solutions were prepared.

Solution A: A quantity of 0.6 g of bismuth sub nitrate was dissolved in 2ml of concentrated hydrochloric acid and 10ml of distilled water.

Solution B: A quantity of 6g of potassium iodide were dissolved in 10 ml of distilled water, then equal volume of Solution A and B were mixed together with 7ml of concentrated hydrochloric acid and 15 ml of distilled water, and the whole was completed to 400 ml by adding distilled water (harborne, 1973). This reagent was used for the detection of alkaloids. Appearance of orange precipitate is an indication for the presence of alkaloids (fahmy, 1933).

2.2.3.1.4. Fehling reagent

Solution A: A quantity of 35 g of copper sulfate were dissolved 100ml distilled water, then diluted by distilled water to the volume 500ml.

Solution B: A quantity of 7g of sodium hydroxide and 175 g of Roshail salt were dissolved in 100ml of distilled water; the volume was completed to 500ml by adding distilled water, then A and B solutions were mixed in equal volumes (sarkas *et al.,* 1980).This reagent was used for glycosides detection.

2.2.4. Preparation of Plant Extract

2.2.4.1. Water extract

For preparation of water extract, 10g of air dried powder of leaves was added to 100ml distilled water. During extraction the mixture was keep in water bath at 50°C for 6 hour, the suspension was filtered with 0.2µm filter paper(size particles is 80) and the filtrate was concentrated by rotary evaporator and stored at room temperature (Santha and Swiminathan, 2011).

2.2.4.2. Acetone extracts

Ten grams of *Withania* leaf powder was extracted with 100ml of acetone for acetone and put in a shaker incubator for 48 hour at room temperature the suspension was filtered with 0.2µm filter paper and the filtrate was concentrated by allowing the solvent evaporated and stored at room temperature (Santha and Swiminathan, 2011).

2.2.4.3. Ethanol extracts

Ten grams of *Withania* leaf powder was extracted with 100ml of ethanol for ethanol Extracts and put in a shaker incubator for 48 hour at room temperature the suspension was filtered with 0.2µm filter paper and the filtrate was concentrated by allowing the solvent evaporated and stored at room temperature (Santha and Swiminathan, 2011).

2.2.5. Detection of Some Active Compounds in *Withania* Leaf extracts

2.2.5.1. Detection of Alkaloids

Ten ml of the concentrated plant extract acidified by 4% of 13N hydrochloric acid were tested with following reagent to ascertain the presence of alkaloids (Wagner reagent, Mayer's reagent, Drangdroffs

reagent) appearance of yellow to purple spot indicate the presences of alkaloid (Harbone, 1973).

2.2.5.2. Detection of Glycoside

few drop of10%hydrochloric acid was added to5 ml of plant extract, then left in boiling water bath for 20 min, the acidity was neutralized by adding few drop of sodium hydroxide solution, equal volume of fehling reagent was added, the appearance of yellow or red precipitate is an indicated the present of reducing sugar (Shihata, 1951; Harbone, 1973).

2.2.5.3. Detection of Flavonoid (Shinoda test)

The extract of 5ml was treated with concentrate sulfuric acid. Appearance of yellowish pointed the presence of anthocyanin, yellow to orange indicates the presence of flavones, and orange to crimson indicate the presence of flavones (jaffer, 1983).

2.2.5.4. Detection of Tannins

Ten ml of plant extract was divided into two equal parts.

- Few drops of 1% lead acetate solution was added to part one, the appearance of gelatinous white precipitate indicates the presence of tannins.
- Few drops of 0.1%ferric chloride solution (white color) was added to part two, the greenish blue color or a blue-black color indicates the presence of tannins(Shihata, 1951).

2.2.5.5. Detection of Saponins.

- Plant extract was shaken vigorously in a test tube, the formation of foam remaining for few minutes indicate the presence of saponins.

- Five milliliter of the plant extract was added to 3ml of mercuric chloride solution, the appearance of white precipitate of white indicate the presence of saponins (Shihata, 1951; Stahl, 1969).

2.2.5.6. Detection of Terpenoids (Salkowski test)

One gram of the plant extract was precipitated in a 1-2 ml of chloroform, and then a drop of acetic anhydride and a drop of concentrated sulfuric acid were added, the appearance of reddish brown represents the presence of terpenoids (Al-Abid, 1985).

2.2.5.7. Detection of Fixed oil

Spot test was done for the detection of fixed oil. In this test, small quantity of 0.5 g alcoholic extract between two papers. Appearances of oil spot on the paper indicate the present of fixed oil (Harbone, 1973; Santha and Swiminathan, 2011).

2.2.5.8. Detection of Protein compounds (Millon's Test)

quantity of 0.5g of the extract was dissolved in 3 ml of water and subjected to Xantho protein test then 3ml of the extract was added to 1ml of the concentrate Nitric acid then the solution was heated for 1minute and cooled under tap water, a white precipitate was obtained. It was made alkaline by excess of 40% NaOH. The appearances of orange precipitate indicates the presence of protein (Harbone, 1973).

2.2.6. Sterilization Methods

1. Autoclaving

Culture medium, reagent and solutions were sterilized by autoclaving at 121°C, 15psifor15min (Collee *et al*., 1996).

2. Filter Sterilization.

Solution which are liable to heat were sterilized by Filtration through Millipore filter unit with pore size 0.45μm or 0.2 μm, this method was used to sterilized plant extracts(Abeeda,1982).

3. Dry Heat Sterilization

Glassware was sterilized by dry heat at 180°C for 2-3 hours (Cappuccino-shermany, 1987).

2.2.7. FTIR (Fourier Transformed Infrared) Analysis

Nature and chemical structure of the active compound were examined using the Fourier transformed infrared spectroscopy (FTIR) in order to characterized the chemical nature of compounds. FTIR spectrometry, an advanced type of infrared spectrometry, which give the functional groups that are found in the compound in order to propose a chemical structure of the test compound.

2.2.8. Preparation of Standards and Sample for HPLC Analysis

2.2.8.1 Standard Preparation

A weight of 10mg of standards were dissolved in 50ml of methanol (HPLC grade) to get 200ppm which was further diluted by dissolving 1ml of this solution in 50ml methanol (Mauricio et *al*., 2007; Rajaseka and Elango, 2011).

2.2.8.2. Sample Preparation

A weight of 1g of leaf sample was accurately weighed and dissolved in 50ml of methanol (99%) (HPLC grade).Further dilution by adding 1ml of this solution to 50ml using (99%) methanol HPLC grade.

2.2.8.3. Method used for High Performance Liquid Chromatography (HPLC)

A volume of 20ml of the standard and 20ml of the sample were injected to HPLC and record the chromatogram, calculated the content of the sample in comparison with standard. The concentrations were calculated according to the following equation: Concentration = [area of sample/area of standard] × sample concentration× dilution factor (Mahdi *et al.,* 2012).

2.2.9. Propagation of Microorganism.

Nutrient agar /broth were used as the media for the culturing of bacterial isolates. Loops full of the bacteria were inoculated in the nutrient broth to refresh the bacterial isolate and incubated at 37°C for 24hrs then 20µl of cultured media was transfered into nutrient agar and brain heart infusion agar then incubated at 37° C while Potato dextrose agar (PDA)/and potato dextrose broth were used as the media for the culturing of fungal isolate. Loops full of all the fungus cultures were inoculated in the potato dextrose broth and incubated at 28° C for 72hrs and the 20 µl of cultured media was transfered into potato dextrose agar and incubated at 28°C.

2.2.10. Maintenance of Bacterial Isolates.

Maintenance of Bacterial strains was performed according to Johnson *et al*., (1988) as following:

A. Short-term storage.

Bacterial strains were maintained for 2-3 weeks on nutrient agar plates, plates were sealed tightly with parafilm, and stored at 4°C.

B. Medium-Term storage.

Bacterial strains were maintained for 3-4 months by stabbing Nutrient agar slant in a screw tubes containing 5-8ml of nutrient agar and stored at 4°C.

C. Long-Term storage.

Single colony was inoculated in brain heart broth and incubated for 24hrs, and then 8.5ml of cell suspension was mixed with 1.5ml of glycerol, and stored at -20°C.

2.2.11. Spore Suspension Preparation.

Spore suspension were prepared according to faraj method (faraj, 1990), Spores were harvested by adding 5ml/slant of sterilized water containing 0,1% tween 80 to aid wetting and separation of spores, the suspension was filtered through sterile cotton wool, the filter was centrifuged at 3000 r.p.m for 5 minutes. The supernatant was removed and the spore were washed twice by re-suspending in sterile distilled water and further centrifuged. Then 5ml of sterile distilled water was added to precipitate and mixed vigorously by the vortex for 1min.

One drop of the suspension was added to haemocytometer by Pasteur pipette, spores were calculated under high power X40 of light microscope using the following equation:

$$\textit{Concentration of spore} = (Z \times 4 \times 106)/n \text{ spore/ml}$$

Where n: total number of small squares, Z: total No. of spores (faraj, 1990).

2.2.12. Antimicrobial Activity of Plant Extracts

2.2.12.1. *In Vitro* Antimicrobial Activity

2.2.12.1.1. Antibacterial Activity

Withania extracts were screened for their antibacterial activity *in-vitro* by well diffusion method (Bagamboula *et al.,* 2004). The stock solution was prepared by dilution 10g of powder in 1L of D.W. or D.W. with one or two drops70% methanol to help in dissolved alcoholic extract powder (jawad *et al.,* 1988). The stock solution was sterilized by Millipore filter unit under aseptic condition (Ibrahim, 2003).Different concentrations were prepared (0.9, 3, 5, 7, 10, 15, 25, 50,100 mg/ml) according to the equation (George, 1968):

$$C_1V_1=C_2V_2$$

The Nutrient media were mixed and sterilized in autoclave and the poured in Petri-dishes, surface of nutrient media was swabbed with 0.1ml of a suspension contain 1×10^{8} cfu/ml. The agar was left to set and in each of these plates, 5mm in diameter, were cut using a sterile cork borer and the agar discs were removed. Using sterilized micropipettes 25µl of different solvents with selected *Withania somnifera* extracts was added in to the well, allowed to diffuse at room temperature for two hours.

The plates were then incubated in the dark and light incubator at 37ºC for 24 hours. the control well was made in the center of plate swapped with 0.01ml of extract solvent diluted with D.W. the inoculated plate were kept at 37ºC for 24 hours (Jawad *et al.,*1988; NCCLS,2005).The diameters of the growth inhibition zones were measured after 24 hours of incubation averaged and the mean values were tabulated.

2.2.12.1.2. Antifungal Activity

Withania extracts were screened for their antifungal activity *in-vitro* by well diffusion method (Pereze *et al.,* 1999), potato extract agar was used. The potato extract media which contain wells was swabbed with 0.1ml of spore suspension contain 1×10^4 cfu/ml and incubated at 28ºC for 48-72hours.

2.2.12.2. *In Vivo* Antimicrobial Activity

Healthy adult animal with 4-5 weeks weighting 18-20 g were obtained from Al-Nahrain animal house, their type were not recognized. These animals were divided into three group each group were maintained in separated plastic cage at temperature 35-40 ºC, and they had free excess to eat (standard pellets) and water throughout the experiment. First groups contain the animals that infected with bacteria that cause skin infection, second group involve the bacteria can cause wounds contamination by contaminated water and Third group contain fungal that cause skin infection. All of these animals were treated with three different extract concentrations and three replicate for each one, in comparison with negative control which were treated with D.W. these groups were:

- **First Group:** involve the bacteria that do skin infection and these divided into four group each group contains one bacterial isolate which injected freshly under animal skin and treated with three different concentration of ethanol extracts (80, 100,120 mg/ml) and with three replicate which was chosen for treatment because It was the most benefit one in killing these bacteria that cause skin infection (*pseudomonas aeruginosa*, *Staphylocuss aureus, Staphylocuss epidermis, Streptococcus pyogenes)*.
- **Second Group:** These groups involve the bacteria that found in contaminated water, sewage sludge and air and can cause wound

contamination in a careful cases. Which divided into four group each group contain one bacteria isolate which used to contaminated the animal wound and treated with three different concentration of acetone extract (80- 100-120 mg/ml) and with three replica was chosen to treatment these bacteria even water extract was most effective on *Klebsiella pneumonia and Proteus mirabilis* but the water extract was lowest ability to killing another two so we chose the most suitable one for all four bacteria which was the acetone extract. These bacteria which can causes wound contamination and cause inflammation to the wound are (*Proteus mirabilis, Enterobacter sakazakii, Escherichia coli, klebsiella pneumonia).*

- **Third Group:** Involve the fungal that causes skin infection *(Trichophyton mentagrophytes, Trichophyton tonsurans, Trichophyton violaceum, Microsporum canis*). Only one fungus(*Trichophyton violaceum)* that inhibited by acetone extract and treated with different concentrations (100-120) mg/ml of Acetone, The only extract that showed effect on the fungi and with three replicate for each concentrations and three replicate for control which treated with only D.W..
- The skin was shaved with razor for hair removal, cleaned and disinfected with cotton saturated with 99% alcohol. For first groups the needle was used for injected the pathogenic bacteria under their skin, the second groups were contaminated the scarp with swap saturated with bacteria while the third one also injected under skin.

2.2.13 Method of cytotoxicity

2.2.13.1 Maintenance of the cell line

Cancer cell lines were monitored to form a confluent monolayer. Sub culture was established by discarding the old medium. This is followed by washing the cell with sterile PBS under aseptic condition, then 3ml trypsine-versine solution was discarded by washing. Using growth medium followed by addition the new growth medium, redistributed in special falcon and incubated at 37°C (Freshney, 2000).

2.2.13.2 Cell culture and culture conditions

HepG2 cell line was used in this study, the cells were grown as a monolayer, spindle like cells. Cells were cultured in PRMI 1640 medium supplement with 10% FCS, containing 50 mg/ml streptomycin and 1000U/L penicillin. The cell line was grown as a monolayer in humidity atmosphere at 37°C with 5% CO_2.The experiments were performed when cells were healthy and at the logarithmic phase of growth Freshney, (2000). $HepG_2$ cell line at passage (40) used in this study were supplied by animal cell culture laboratory, Biotechnology Research Center / AL-Nahrain University.

2.2.13.3 Cytotoxicity assay

This method was carried out according to Freshny (2000). The cells suspension prepared by detaching cell flask with 2 ml of trypsin solution, when a single cell suspension appeared 20ml of growth medium supplement with 10% fetal calf serum(FCS) added to flask to inactivate the trypsin effect, then the viability counted by trypan blue dye the viability should be more than 95%. The cell suspension was well mixed followed by transforming 200µl/well into each well of the 96 well flat bottom microtiter using automatic micropipette containing (1×10^{5} cell/well).Plastics

were incubated at 37°C until 60-70% confluence of the internal surface area of well for $HepG_2$ cell line. The cells were exposed to different concentration of plant extract (80.100 and 120 mg/ml).

The negative control wells which contained only the cells with culture medium, then the plates were incubated at 37°C in an incubator supplement with (50%) $CO_2$for 72 hours after elapsing the incubation period, 50µl/well of natural red dye were added and incubated again for 2 hr. The content of plate were removed by washing the cells 3 times with PBS then 100µl 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 on 492 nm then inhibition rate was length on 492 nm, then inhibition rate for each concentration were determined according to formula (Gao *et al.*, 2003). Abs= Absorbance.

Inhibition Rate (%) = [(Abs.at492nm of control- Abs.at 492nm of test) ÷Abs.at492nm of control] ×100

1.2.14. Statistical Analytics

Statistical analysis was done using Minitab 15 statistical analysis software. Two way ANOVA test was used to compare different groups among each other and with control. All value were expressed as mean (M±SE) (Steel and Torriem, 1982).

Chapter Three

Results and Discussion

3. Results and Discussion

3.1. Plant Extracts.

3.1.1. Water, Ethanol and Acetone Extracts.

Aliquot of two and half (2.5g) was the weight of the water extract resulted after evaporation of distilled water,which represents 25% of the original leaves sample weight; this extract appeared with brown color. However, ethanol and acetone residue obtained after evaporation of ethanol or acetone solvent was 3g which represent30% of the original leaves sample weight. The appearance of the extract was dark green in color but the ethanol extract with more viscosity than acetone extract and never converted to powder like acetone.

3.2. Detection of Some Active Compounds in the Plant extracts.

The water, ethanol and acetone extract of *Withania* were subjected to chemical analysis to identify the compounds in each extract. Table(3-1) shows the presence of active compounds in *Withania* extracts, which alkaloids, glycoside, saponins, flavonoids, fixed oil were presented in ethanol, acetone and water extracts, while terpenes absent in water extract and protein absent in acetone extract.Tannins were not found in all three extract, even with the method using ($Fecl_2$) which gave the same result. This shows the compounds were extracted in polar solvents rather than non-polar solvents (Owais *et al.* 2005). The presence of these bioactive compounds in the plant has been reported to confer resistance against pathogenic (Srinivasan *et al.,* 2001).

Table (3-1) Secondary Metabolite in *Withania* extracts

Phytochemicals	Test performed	Indicator	Water	Acetone	Ethanol
Alkaloids	Wangers test Mayers test Dragenroff s Test	Brown ppt. White ppt. Orange ppt.	+	+	+
Glycoside	Fehling test	Red Orange ppt.	+	+	+
Saponin	Shaking test Mercuric test	Foam for few min White ppt.	+	+	+
Flavonoids	Shinoda test	Red ppt.	+	+	+
Tannins	Lead acetate test Ferric chloride test	Gelatinous white ppt. Greenish blue color	–	–	–
Terpenes	Salkowski test	Reddish brown	-	+	+
Protein	Millon's test	Orange ppt.	+	–	+
Fixed oil	Spot Test	Oil stain on paper	+	+	+

Note = + indicate the presence, - indicate the absence of the active compound

The results were agreed with Santha and Swiminathan (2011) but disagree with Velu and Baskaran (2012) who report that alkaloids were absent in water extract, while protein were absent in ethanol instead of acetone, these differences in water extracts may be due to alkaloid present in two type: alkaloid with free base cannot be extract with water but they extract with alcohol only, and salt base alkaloid which extract with water extracts. These results disagree with Panchawat (2012) research in which the fixed oil was absent in ethanol and water extracts but it was found in acetone extract, and tannins are present in water and ethanol extract but not found in acetone. Khare (2007) reveled the leaves of the plant are reported to contain 12 withanolides, unidentified alkaloids (yield, 0.09%), many free amino acids, chlorogenic acid, glycosides, glucose, condensed tannins, and flavonoid.

3.3. Fourier Transformed Infrared (FTIR) Analysis

The *FTIR* spectrum used to identify the functional group of the active compound present in the extracts of the plant based on the peaks value in the region of IR radiation. When the plant extract passed into the *FTIR,* the functional groups of components separated based on its peaks ratio. Table (3-2), fig (3-1), fig (3-2) and fig (3-3) shows the active group present in ethanol, acetone and water extracts.

The active group different in their present from one extract to another in which the acetone and ethanol extracts were different from water by containing carboxylic-a (3400) groups, alkene(3000), acid-chloride (1800-1700), Ester(1735), amide(1651-1631), alcohol(1076), acetone extract and water extract different from ethanol by alkyne (3300). While the water was different by Sulfoxide (1050), ethanol extract different by containing Nitro(R-no_2)(1550) and acetone extract different from other by containing anhydride(1843-1793)

The results were agreed with Nema *et al.* (2012) who reported that number of peaks indicated that *Withania* extracts contained number of active groups, the results were also agreed with Bashir *et al.* (2013) results which also indicated that *Withania* extracts were rich in the active group that appeared as peak

Table (3-2) FTIR Peaks Values and Functional of *Withania* extracts

Extracts present	**Functional groups**	**Peaks value**
Water, ethanol, acetone	Iodide, Bromide	667
Water, ethanol, acetone	Chloride	709
Water, ethanol, acetone	Fluoride	1400-1000
Water, ethanol, acetone	Alkanes	2900
Water, ethanol, acetone	Ch2	1450
Water, ethanol, acetone	Imines	1690
Water, ethanol, acetone	Aromatic(C=C)	1435
Water, ethanol, acetone	Aldehyde	2900-2800
Water, ethanol, acetone	Alkenes	1600
Water, ethanol, acetone	Amines	1350
Ethanol, acetone	carboxylic-a groups	3400
Ethanol, acetone	Alkene	3000
Ethanol, acetone	acid-chloride	1800-1700
Ethanol, acetone	Ester	1735
Ethanol, acetone	Amide	1651-1631
Ethanol, acetone	Alcohol	1076
Water, acetone	alkyne	3300
Water	Sulfoxide	1050
Ethanol	Nitro(R-no_2)	1550
Acetone	Anhydride	1843-1793

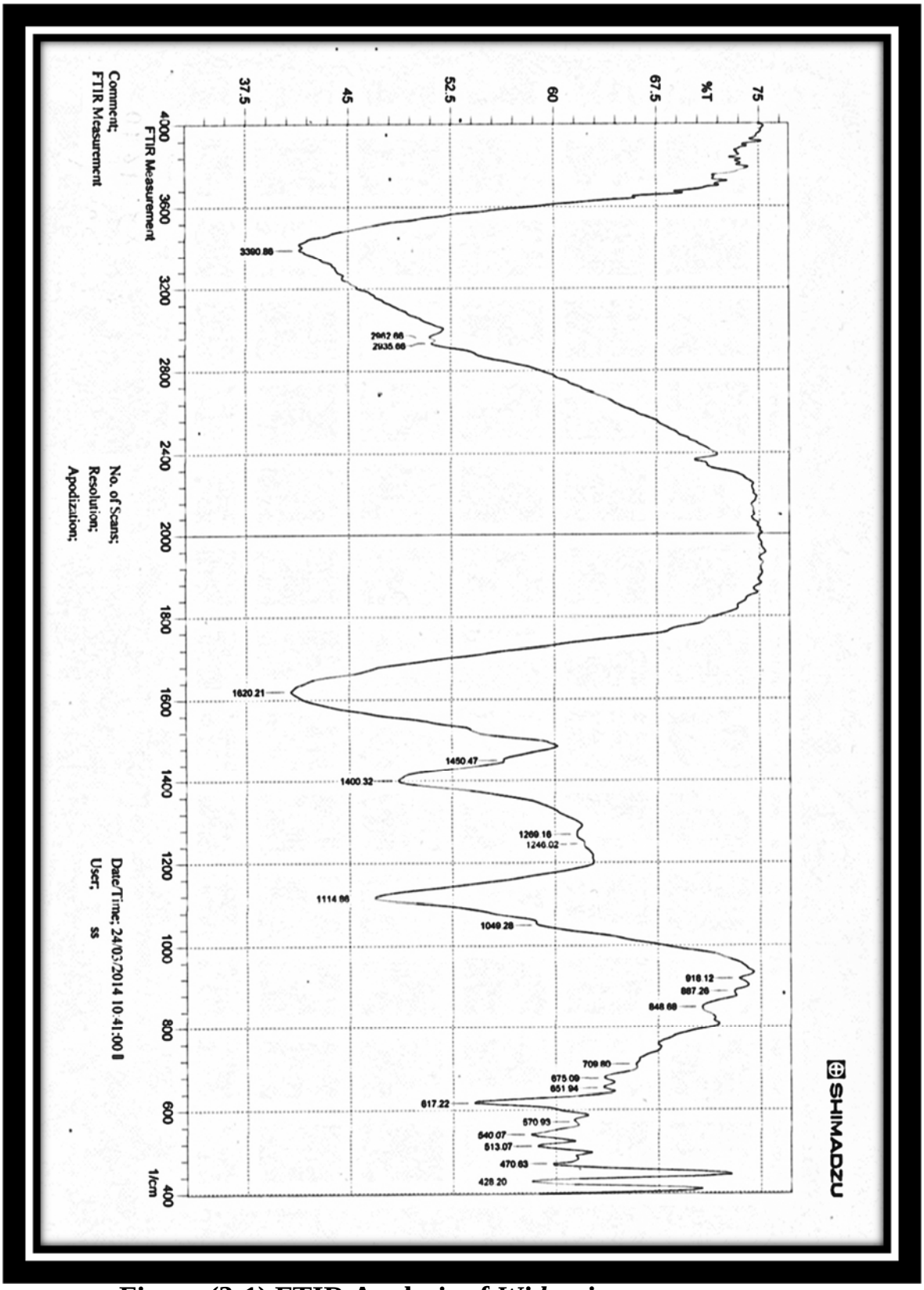


Figure (3-1) FTIR Analysis of *Withania* water extract.

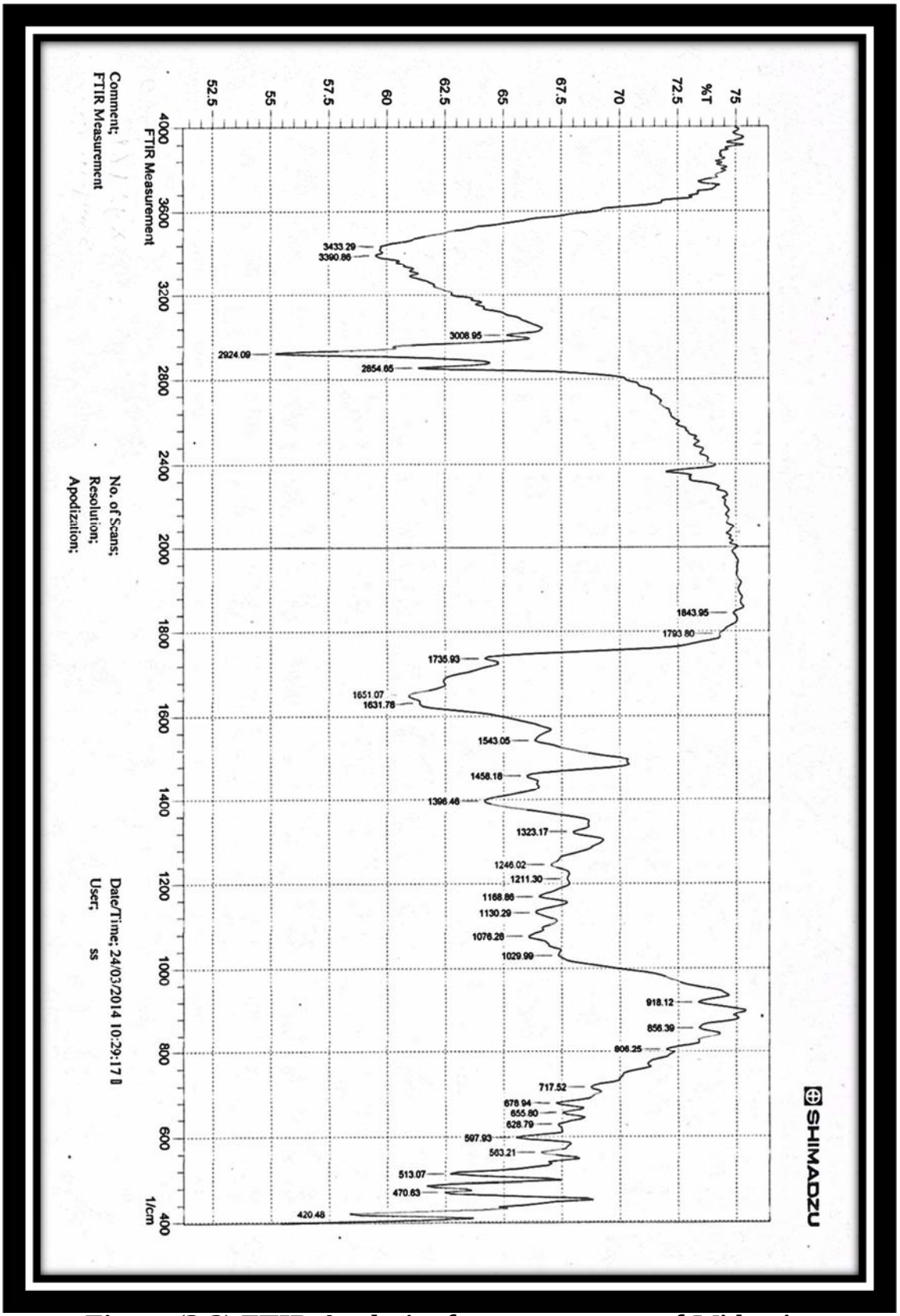


Figure (3-2) FTIR Analysis of acetone extract of *Withania*.

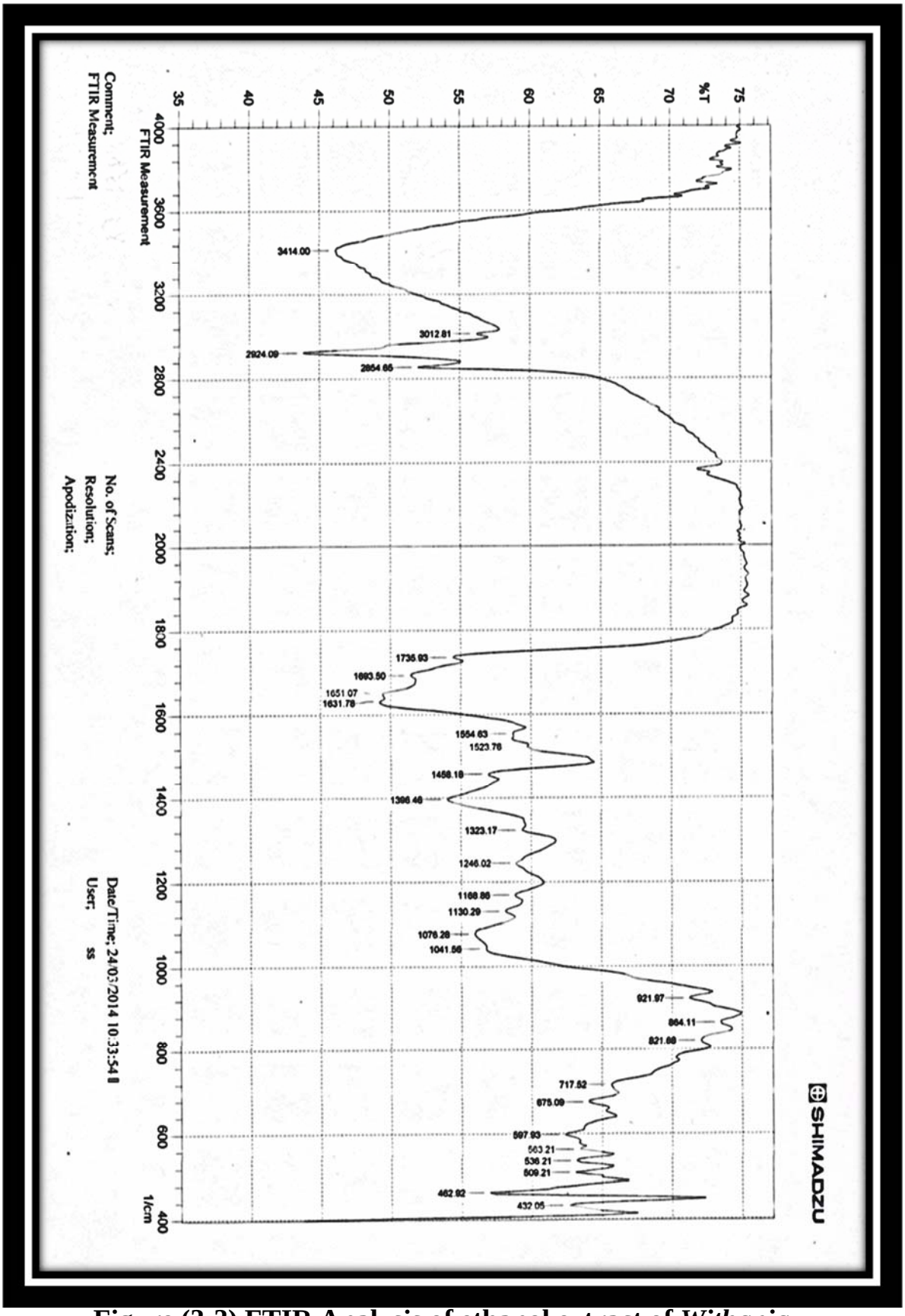
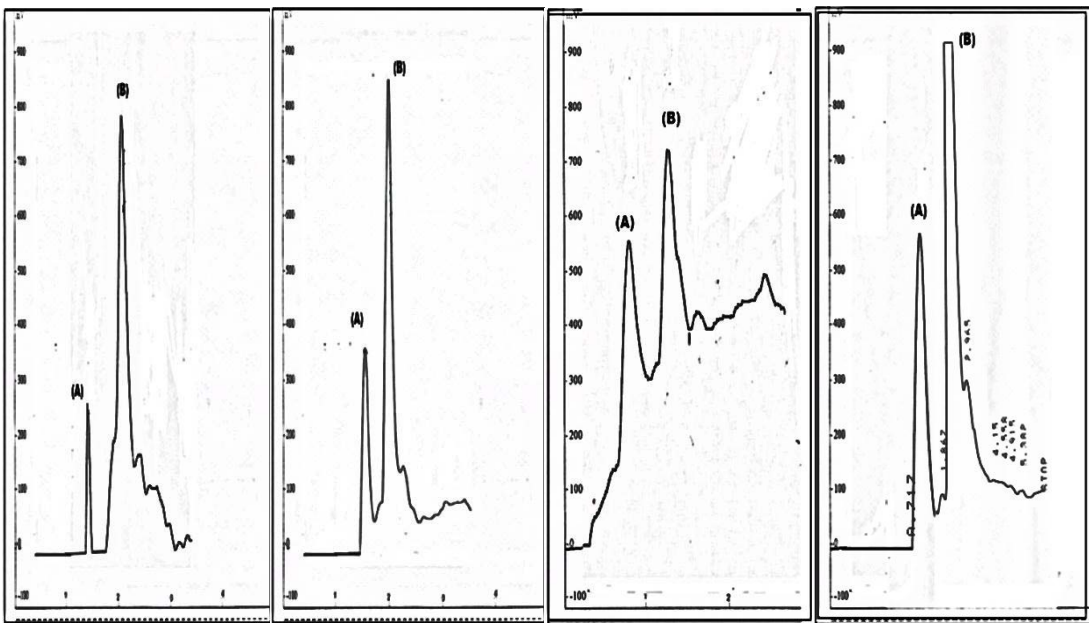


Figure (3-3) FTIR Analysis of ethanol extract of *Withania*

3.4. HPLC Analysis of *Withania* Extracts

HPLC analysis was done to detect the concentration of important active compounds present in the Water, Acetone and Ethanol extract of *Withania* including: Alkaloid (Low molecular weight Nitrogenous compounds), Flavonoid (polyphenolic compounds) and Saponin (steroid or triterpenoid glycosides). Figure (3-4) revealed a different peaks of alkaloids present in *Withani*a ethanol, acetone and water extracts in same retention time in compare with a stander but with different area. In figure (3-4) the first peak was belong to Withaferin-A which in all extracts present in concentration lower than the second peak which belong to Withanolide-A that found in higher concentration in all three extracts.(X-axis= min, Y-axis=mV).



Stander **Ethanol** **Water** **Acetone**

Figure (3-4) Alkaloids of *Withania* extracts

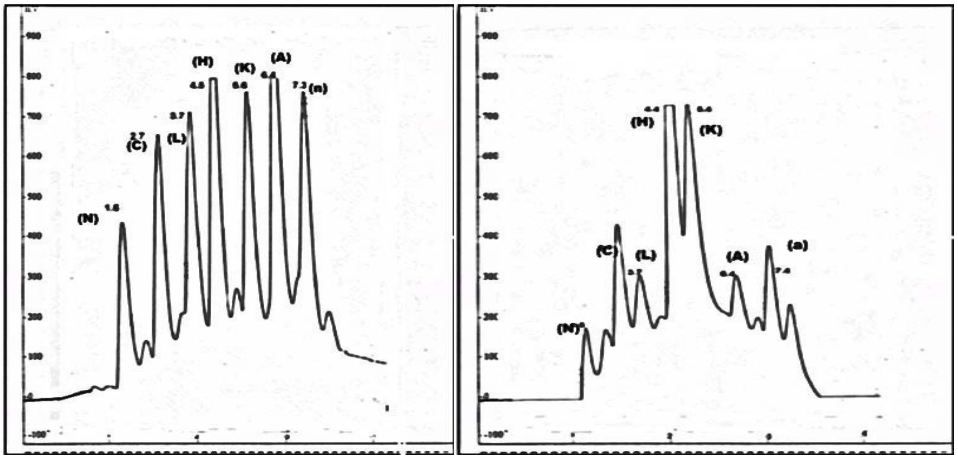
Table (3-3) shows the higher concentration of Withaferin-A was present in water extract followed by the ethanol extract and the lowest one was acetone extract, while the higher concentration of Withanolide-A was in

the acetone extract and the ethanol was close to acetone extract whereas, the water extract was less one according to the peaks that appear in same retention time but in different area in each extract. These results were agreed with Sharma (2013) and the higher content of alkaloid in *withania extracts* exhibit their higher antibacterial and antioxident activity siriwardane *et al* (2013) who provided that *Withania* leaves contain withaferin-A in higher concentration than other parts of plant. It was also agree with Jain *et al.*, (2012) who also found the leaves contain Withaferin-A and Withanolide-A. The results were also agreed with Rastogi and Mehrotra, (1998) who found that *Withania* extract was rich in these two types of alkaloid.

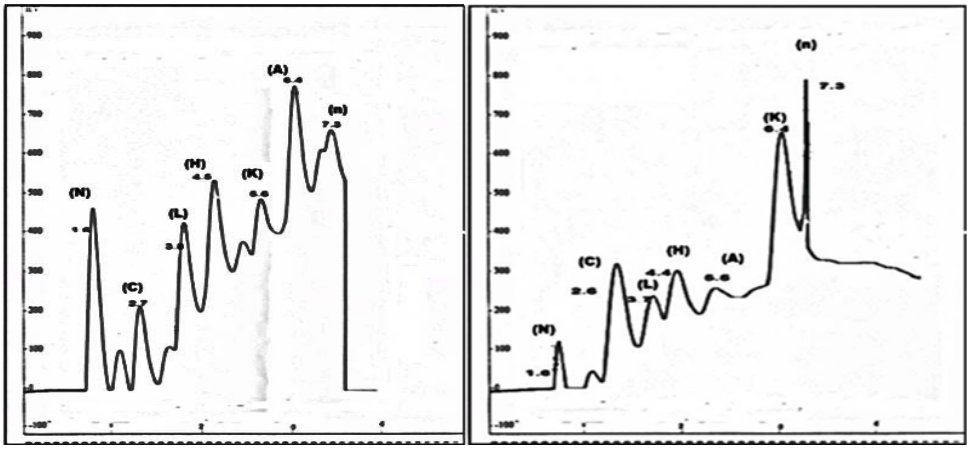
Table (3-3) Alkaloids contents of *Withania* extracts

Alkaloid contents of Ethanol extract				
Sec	Subjects	Retention time(min)	Area	Concentration(µg/ml)
1	Withaferin-A	2.2	6379	24.09
2	Withanolide-A	3.2	10133	63.4
Alkaloid contents of Water extract				
Sec	Subjects	Retention time(min)	Area	Concentration(µg/ml)
1	Withaferin-A	2.16	7300	48.8
2	Withanolide-A	3.18	6968	51.1
Alkaloid contents of Acetone extract				
Sec	Subjects	Retention time(min)	Area	Concentration(µg/ml)
1	Withaferin-A	2.28	11429	15.5
2	Withanolide-A	3.25	50024	67.9

Different peaks of Naringenin(N), Catechin(C), Luteolin(L), Hesperidin(H), Kaempfero(K), Apigenin(A) and Naringin(n), present in Acetone extract, Ethanol extract and water extract of Withania which appeared as different peak in same retention time with stander but in different area. Naringenin appeared as first peak(N), second peak (C) belong to Catechin, third one(L) was Luteolin, fourth peak(H), was Hesperetin, (K) peak refer to Kaempfero, (A) peak refer to Apigenin and (n) peak refer to Naringin in Figure (3-5).



Stander **Ethanol**



Acetone **Water**

Figure (3-5) Flavonoids of *Withania* extracts

Table (3-4) showed that higher concentration Naringenin was in ethanol extract followed by acetone extract and water extract, higher

concentration of Catechin was in water extract followed by Acetone and ethanol extract. While, Luteolin, Hesperidin higher concentration was in acetone extract, followed by water extract and ethanol extract. Kaempfero present in high quantity in water extract followed by ethanol extract and acetone extract was the lowest one. Apigenin present in higher concentration in both ethanol extract and water extract which followed by acetone extract. Water extract had a higher concentration of Naringin then acetone extract, while the ethanol showed lowest concentration. Flavonoid content of Withania exhibit their anti-inflammation activity Naringenin, Luteolin, Hesperidin, Kaempfero, Apigenin and Naringin well known as anti-inflammation compound while Catechin as antioxidant. These results were agreed with Sivamani *et al.,* (2014) and Bashir *et al.,* (2013*)* who's also found more than five types of flavonoids in plant but in different concentration. Also agreed with Nema *et al*., (2012) results which revealed that *Withania* also had nine type of flavonoid appeared as different peaks seven peaks of them was resemble to ours peak but in different concentration.

Table (3-4) Flavonoid contents of *Withania* extracts

	Flavonoid contents of Acetone extract			
Sec	Subjects	Retention time (min)	Area	Concentration µg/ml
1	Naringenin	1.63	17367	7.4
2	Catechin	2.75	11806	5.0
3	Luteolin	3.80	21216	9.0
4	Hesperidin	4.54	30364	13.0
5	Kaempfero	5.64	27408	11.7
6	Apigenin	6.45	45455	19.4

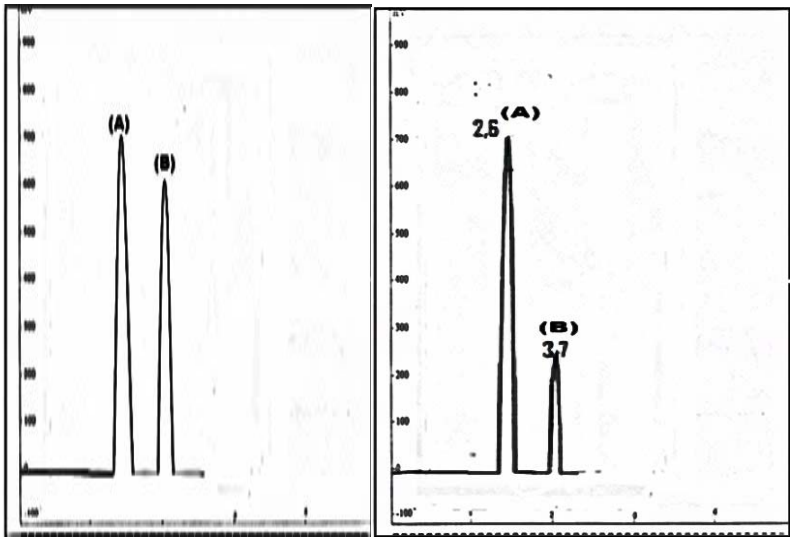
7	Naringin	7.34	30043	12.8

Flavonoid contents of Water extract				
Sec	Subjects	Retention time (min)	Area	Concentration µg/ml
1	Naringenin	1.62	8493	4.8
2	Catechin	2.69	14150	8.1
3	Luteolin	3.75	12875	7.3
4	Hesperidin	4.53	11415	6.5
5	Kaempfero	5.58	30920	17.7
6	Apigenin	6.46	35078	20.1
7	Naringin	7.33	29281	16.7

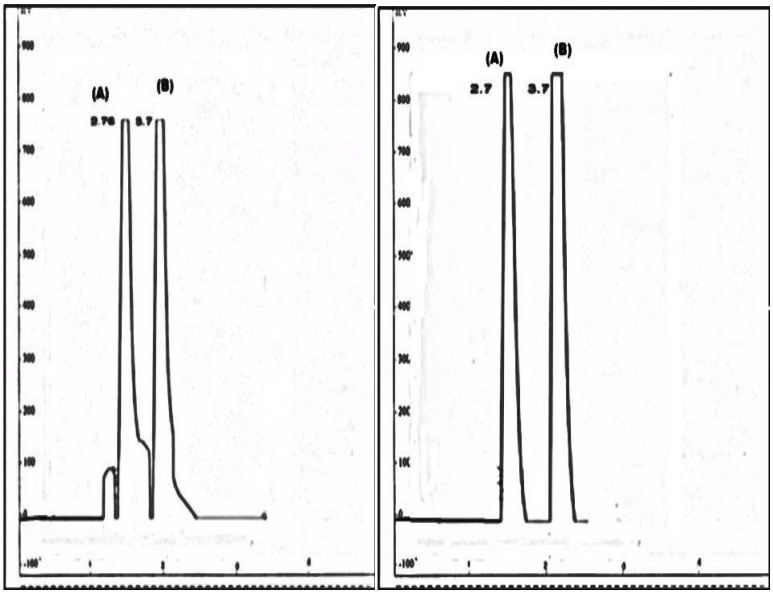
Flavonoid contents of Ethanol extract				
Sec	Subjects	Retention time (min)	Area	Concentration µg/ml
1	Naringenin	1.57	5000	7.8
2	Catechin	2.7	14183	4.5
3	Luteolin	3.7	27211	8.8
4	Hesperidin	4.4	25254	8.06
5	Kaempfero	5.4	49676	15.8
6	Apigenin	6.04	67599	21.6
7	Naringin	7.4	25000	7.9

Figure (3-6) Revealed different peaks with different retention times and area of saponins present in water extract, ethanol extract and acetone extracts of *Withania.* These compounds were: Sitoindosides VII(A) which appeared as first peak and Sitoindosides VIII(B) as second peak. These

peaks were already appeared and identified in Saponins Standar (S), which used for comparing to identify the peaks in extracts.



Stander **Water**



Acetone **Ethanol**

Figure (3-6) Saponin of *Withania* extracts

Table (3-4) Showed different concentrations of Saponin compounds, The highest concentration of Sitoindosides VII was in acetone extract then ethanol extract but the concentration of Sitoindosides VIII was in acetone

extract followed by ethanol extract, whereas water extract showed lowest concentration of Sitoindosides VII and Sitoindosides VIII. These (Sitoindosides VII and Sitoindosides VIII) compounds known as antioxidant. The results were agreed with Mirjalili *et al.,* (2009) and Betsy *et al.* (2000) who reported that several Sitoindosides have been isolated from aerial parts which include: Sitoindosides VII and Sitoindosides VIII. Singh *et al.* (2010) study also found that *Withania*'s leaves extracts had two types of saponins (Sitoindosides VII and Sitoindosides VIII).

Table (3-5) Saponin contents of *Withania* extracts

Saponin contents of Acetone extract				
Sec	Subjects	Retention time(min)	Area	Concentration µg/ml
1	Sitoindosides VII	2.76	90801	39.9
2	Sitoindosides VIII	3.76	12204	53.6
Saponin contents of Ethanol extract				
Sec	Subjects	Retention time (min)	Area	Concentration µg/ml
1	Sitoindosides VII	2.7	43742	40
2	Sitoindosides VIII	3.76	52597	48
Saponins contents of Water extract				
Sec	Subject	Retention time(min)	Area	Concentration µg/ml
1	Sitoindosides VII	2.68	19543	34
2	Sitoindosides VIII	3.76	20274	21.5

3.5. *In vitro* Antimicrobial Activity of *Withania* Extracts

3.5.1. Antibacterial Activity

The antibacterial activity of *Withania* extracts was test on the following bacteria (*p. aeruginosa, S. aureus, S. epidermis, S. pyogenes, P. mirabilis, E. sakazakii, E. coli, k. pneumonia)* by different concentration of extracts range from 0.9 to 100 *mg/ml* (0.9, 3, 5, 7, 10, 15, 25, 50, 100mg/ml) of water, ethanol and acetone extract with probability ($P \leq 0.05$). Figure (3-7) and fig (3-8) revealed diameter inhibition zone of *Withania* water extracts on bacteria in dark and under light, respectively in which that *Withania* water extract higher effect was *on P. mirabilis* and *K. pneumonia* followed by *S. pyogenes, E. coli*, *Staphylocuss and P. aeruginosa* but the lowest water extract effect was with *E. sakazakii.* The figures also showed that dark and light had no effect on the diameter of inhibition effect of the extract that mean the extract in dark and light had the same antibacterial activity with probability ($P>0.01$).

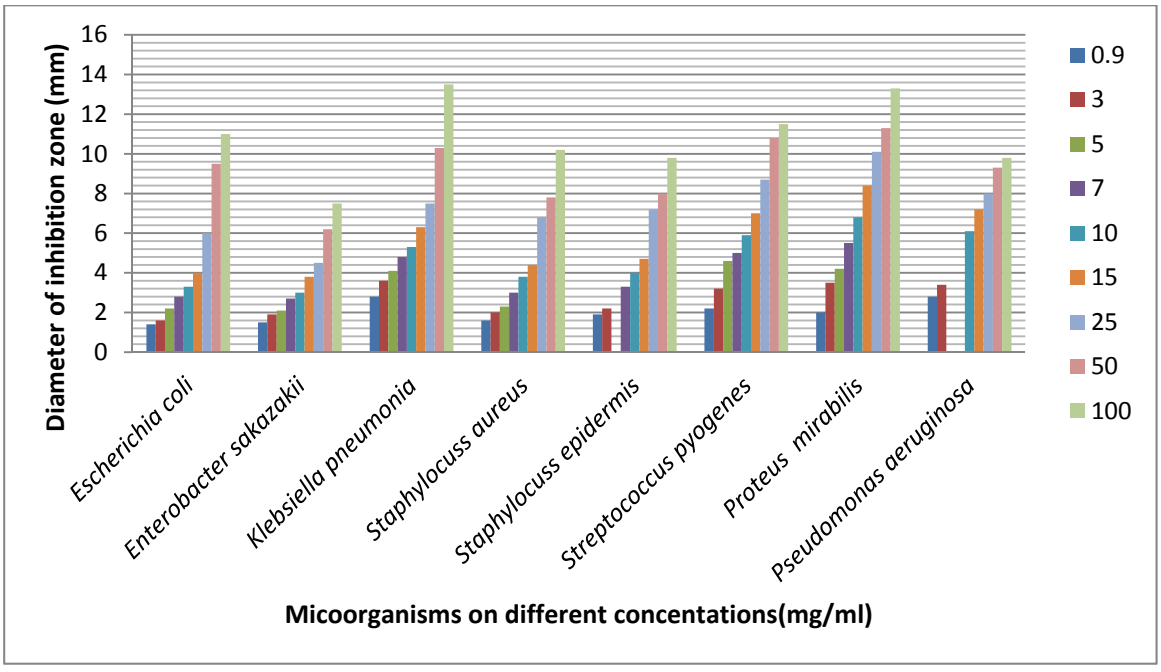


Figure (3-7) Diameter inhibition zone of *Withania* water extracts on bacteria in dark.

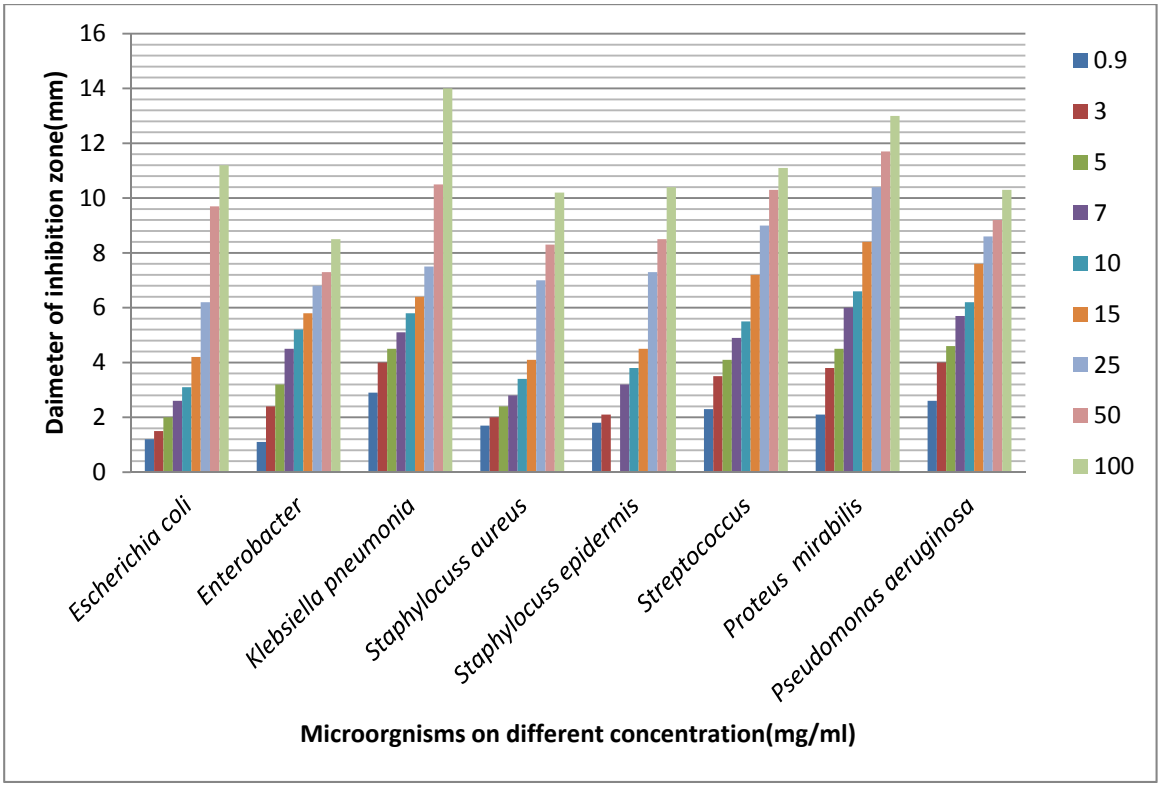


Figure (3-8) Diameter inhibition zone of *Withania* water extracts on bacteria under light.

Figure (3-9) and (3-10) of *Withania* ethanol extract Diameter of bacteria inhibition zone revealed that *Withania* ethanol extract on dark and light higher effect was with *S. epidermis, S. aureus, E. coli and S. pyogenes* followed by *P. aeruginosa, E. sakazakii and K. pneumonia* but *P. mirabilis* showed the lowest inhibition diameter with *Withania* ethanol extract in dark and light.

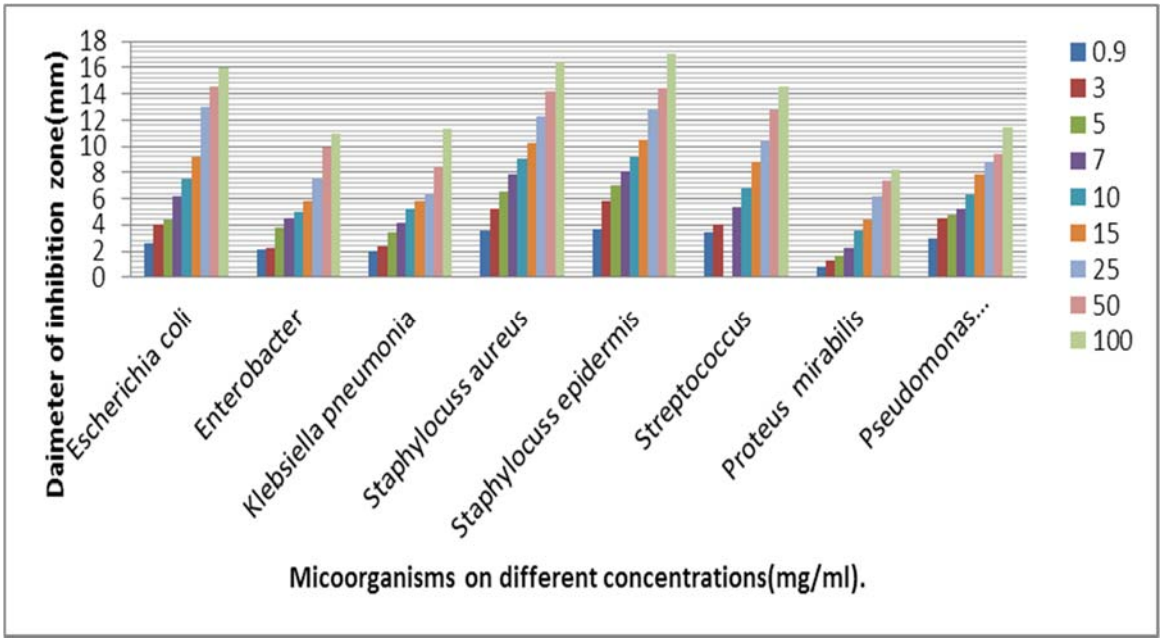


Figure (3-9) Diameter inhibition zone of *Withania* ethanol extracts on bacteria in dark.

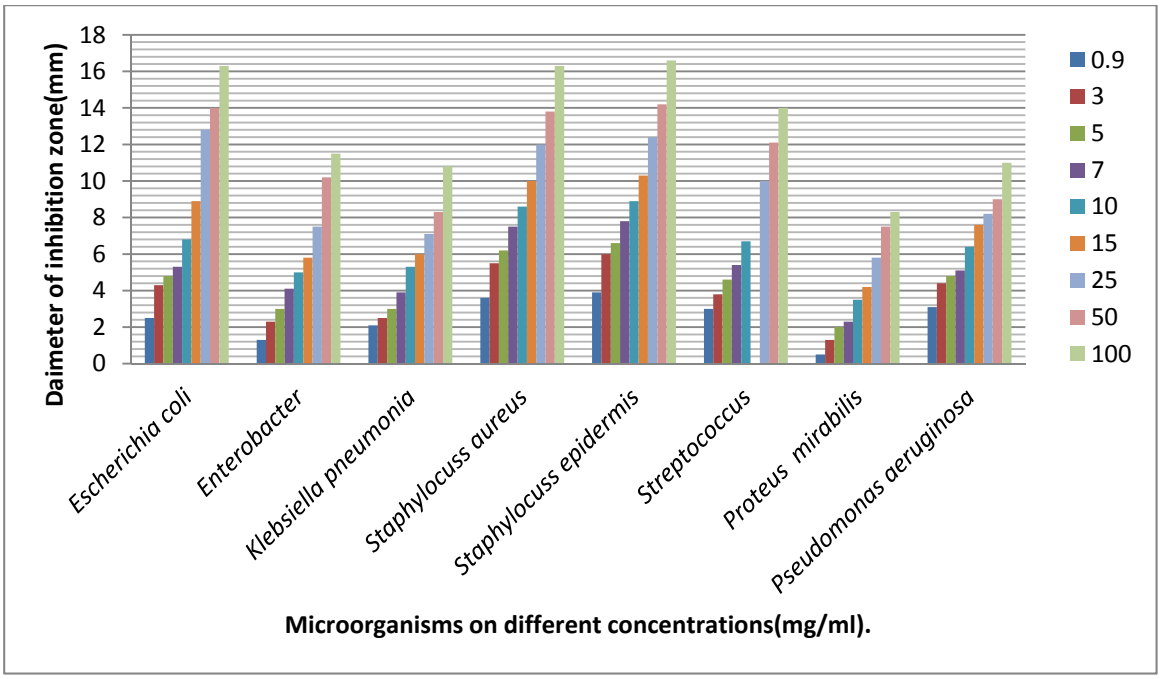


Figure (3-10) Diameter inhibition zone of *Withania* ethanol extracts on bacteria under light.

Figures (3-11) and (3-12) of *Withania* acetone extracts bacterial inhibition zones in dark and light shows that the higher acetone activity was

with *S. pyogenes*, *S. aureus*, *S. epidermis and E. coli* followed by *P. aeruginosa and E. sakazakii* while the lowest acetone effect was with *P. mirabilis and k. pneumonia*.

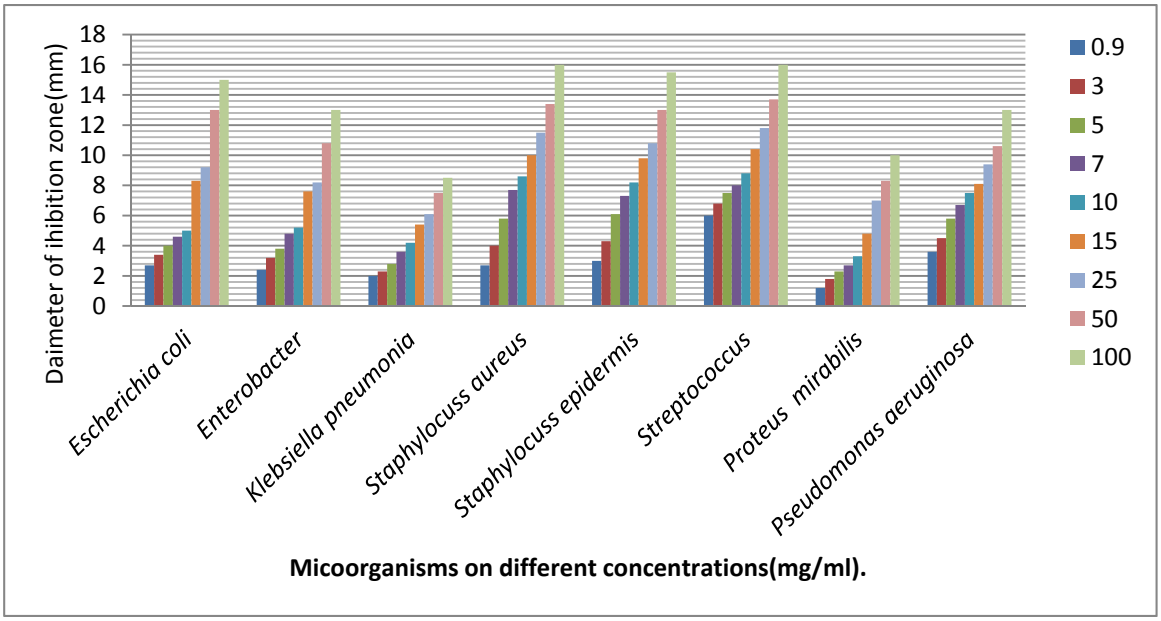


Figure (3-11) Diameter inhibition zone of *Withania* acetone extracts on bacteria in dark.

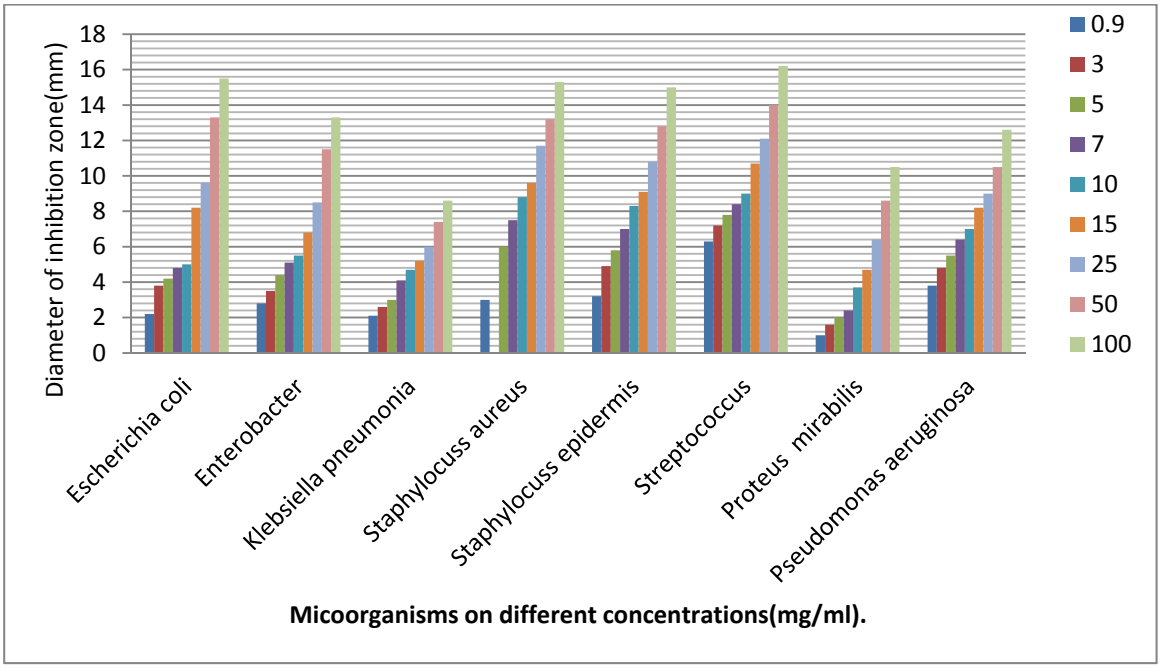
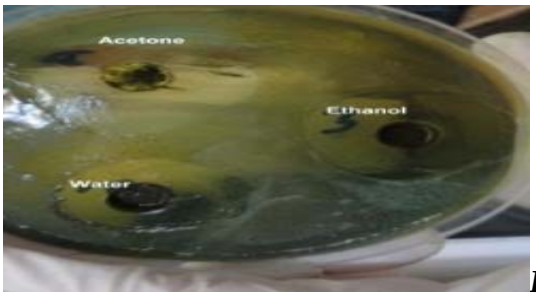
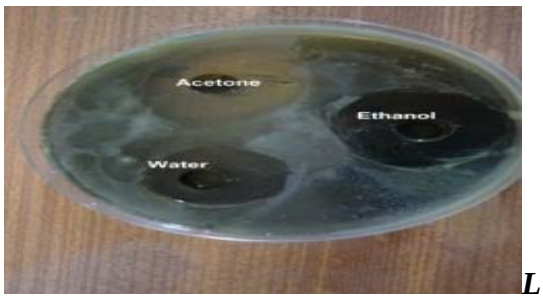


Figure (3-12) Diameter inhibition zone of *Withania acetone* extracts on bacteria under light

All figures showed that the highest inhibition diameter were in *S. aureus, S. epidermis* and *E. coli caused* by ethanol extract followed by inhibition diameter of acetone extract and then water extract. On other hand water extract showed highest inhibition on *P. mirabilis, K. pneumonia* which followed by acetone and then ethanol. The acetone had highest inhibition on *P. aeruginosa, E. sakazakii, S. pyogenes* which followed by ethanol and finally water extract. That means the acetone extract was the best for inhibition of most studies bacterial sample. These results were agreed with Humaira *et al.* (2012) *for P. aeruginosa, E. sakazakii, S. pyogenes* but disagree for *E.coli, P. aeruginosa, K. pneumonia, and* the result was also agreed with Velu. And Baskaran, (2012). *For S. pyogenes, P aeruginosa, K. pneumonia,* but dis-agreed in the *E.Coli* which had the highest killing ability by acetone followed by water and the ethanol been the lowest The differences might be because they used different method to extract the active compound because they found difference in the active compound present. The beneficial effect of the extract typically resulted from secondary metabolite present in plant (Wink, 1999).

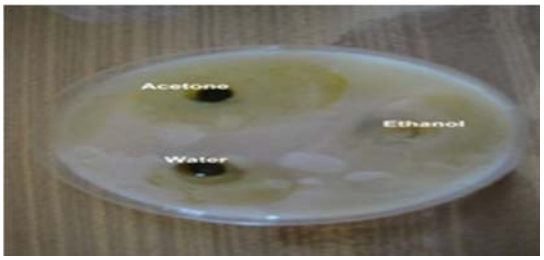


D

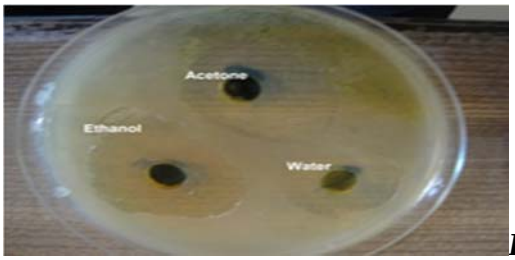


L

Pseudomonas aeruginosa

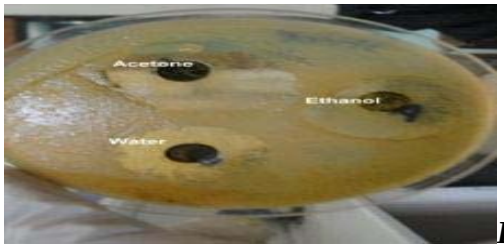


L



D

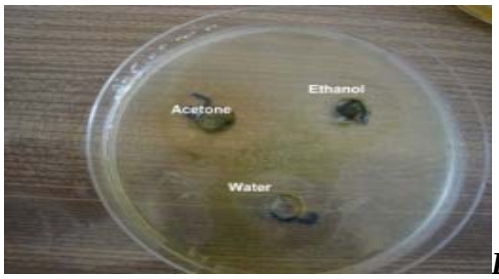
Streptococcus pyogenes



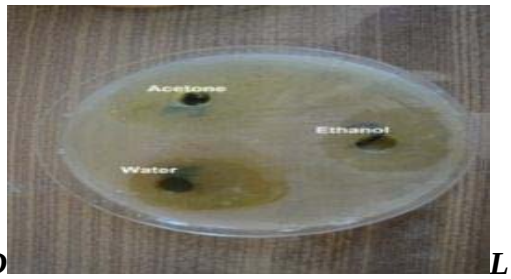
D


L

Enterobacter sakazakii

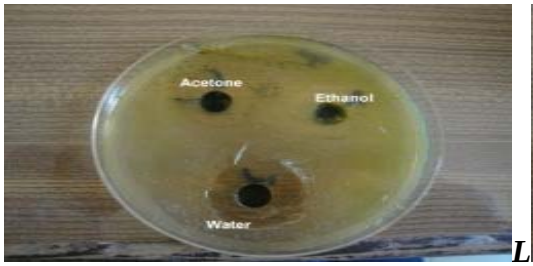


D

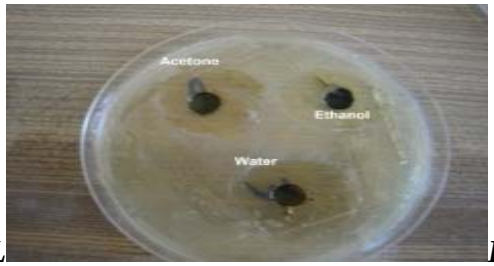


L

Proteus mirabilis

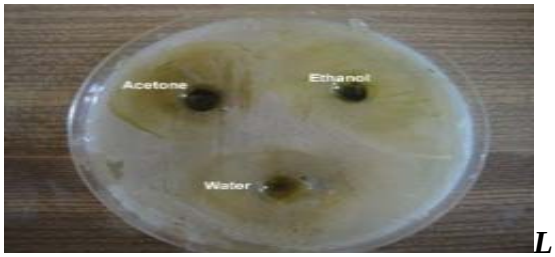


L



D

Klebsiella pneumonia



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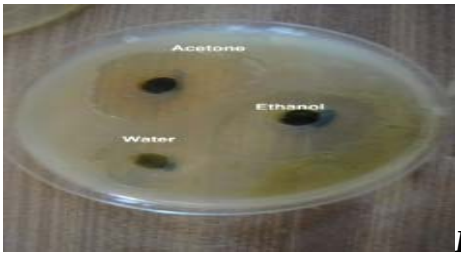
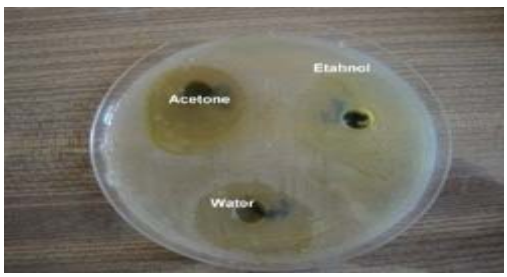
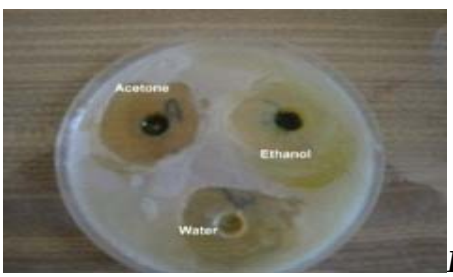


D

Staphylocuss aureus



L



D

Staphylocuss epidermis

Figure (3-13) Inhibition zone by 100(mg/ml) of Withania extracts in light and dark

It is clear from the data present in previous figures that *S. aurous* and *S. epidermis* was the most susceptible one to two kind of extracts followed by E. coli and *S. pyogenes.* While *P. mirabilis* was the most resistance one. The finding that staph is susceptible to a variety of extracts was recording by other (Madamombe and Afolayan,2003). Variation in susceptibility and resistance of bacteria species may due to the genetic variation and cellular structural (Frazier and Westhoff, 1987). Furthermore, the results were disagree with Santhi and Swaminathan, (2011) results in which acetone had higher inhibition on *S, aureus, K, pneumonia* followed by ethanol extract and water extract. While *P. aeruginosa* and *E. coli* were affected by acetone and water extract, ethanol extract had no effect for *E.coli,* but it was agree in *P. mirabilis*.

The antibacterial activity of *Withania* Extract may be because they had high quantity of alkaloid and flavonoid well known by their antibacterial according to Singh and Kumar, (2012) and Bashir *et al.,* (2013). The effect of alkaloid on microorganism may be attributed to plasma membrane destruction, the interaction with metabolic reactions, and in enzymes vital to growth and reproduction (Abdul-Rahman, 1995). While Flavonoid which is highly oxidized polyphenolic their activity depend on sit and number of hydroxyl group are inhibitory to the growth of microorganism (Taiz and Zeiger, 2002).Their activity is probability due to their ability to form a complex with bacterial membranes. (Cowan, 1999).

3.5.2. *In vitro* Antifungal activity of Withania Extracts

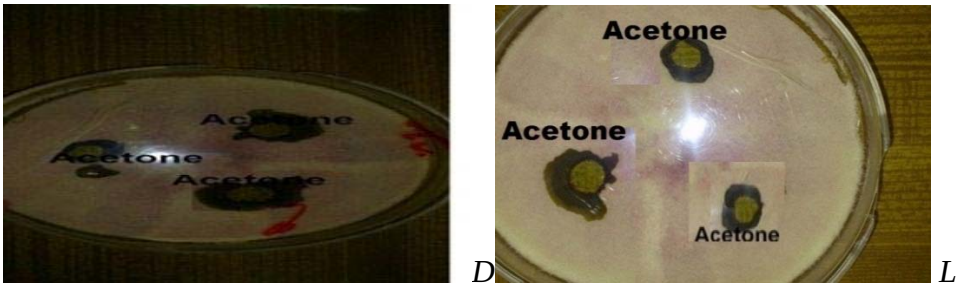
The antifungal activities of *Withania* extracts (acetone, ethanol and water extract) were done on different concentrations starting from 0.9 to 100 and 120mg/ml but had no activity on these fungal with different type including: *Trichophyton mentagrophytes, Trichophyton tonsurans*,

Microsporum canis. However, acetone extract showed activity against *Trichophyton violaceum* by 100 and 120 mg/ml concentration of extract whereas, water and ethanol show no activity even at high concentrations of the extracts.

This may be due to the strength of fungus cell wall, as it is formed form three separate layers, the lower, middle glucans layers and outer glycoprotein layers are interferon fibrillar polymers held together by covalent bonds and chitin polymer chains are present throughout the cell wall and make fungi more protected from extra-cellular stress (Gow and Goody, 1983). There are three mode of action of plant extracts:

1) Inhibition of microbial cell wall formation or bio-synthesis of essential protein.
2) Disruptions of deoxyribonucleic acid (DNA) metabolism.
3) Alternative of normal function of cellular membrane (Tayler *et al.*, 1996).

Table (3-5) and fig (3-14) shows that the ethanol, water extract and acetone extract at different concentration had no activity on *Trichophyton mentagrophytes, Trichophyton tonsurans*, *Microsporum canis.* However, only the acetone extract showed activity against *Trichophyton violaceum* in light and in dark which done from beginning to see if the extract have different action on light and if the extract have compound which is oxides under light and effect on extract action.



D *L*

Figure (3-14) T. violaceum inhibition zone by Acetone extract.

Table (3-6) *Withania* extracts as Antifungal

Diameter of Inhibition zone (0.9-120) mg/ml			
Organism	***Acetone***	Ethanol	Water
T. mentagrophytes	—	—	—
T. tonsurans	—	—	—
M. canis	—	—	—

Diameter of Inhibition zone				
Organism	Acetone(100-120)		Ethanol	Water
T. violaceum	**(100-120)L**	**(100-120)D**	—	—
	1.3-3.0	**1.5-3.4**		

Not = 3replicateL= light D=dark**.**

These antimicrobial activities of *Withania* extracts may due to Flavonoid action (Bashir *et al.* 2013). Jawetz *et al.* describe the mechanism of action of flavonoid (phenolic hydroxylated compound) against microorganism (membrane disruption, binding or make complex with cell wall, inactivation of enzyme, and binding to proteins). The results were agreed with Peter *et al., (2013*) in which *T. mentagrophytes* and *Microsporum* showed no effect for all kinds of extract at low concentrations and no effect with methanol extract even at high concentration which had same active compound of water and ethanol extracts.

3.6. *In-Vivo* Antimicrobials activity

3.6.1. Antibacterial activity

In these test the animal divided into two group, the first one contain the animal injection with bacteria have ability to do some type of skin infection (*P. aeruginosa, S. pyogenes, S. aureus, S. epidermis)* and second

one involve the bacteria can cause wound contamination (*Escherichia coli, Enterobacter sakazakii, Klebsiella pneumonia, Proteus mirabilis).*

Figure (3-15) show the morphological change(a) after injection with fresh live *Staph aureus* these change may, due to many virulence factor of staph such as exfoliative toxin which is an exotoxin produced by *S. aureus*, causing blisters in human and animal skin, detachment with in the epidermal layer and the loss of keratinocyte cell-cell adhesion in the superficial epidermis (Nishifuji *et al.*,2008) and the change after daily treatment with 80, 100 and 120 mg/ml concentration of ethanol extract in pictures (b) and (c), these pictures was for one of thirty nine animal was used in these test involve three different concentration for curing with three replicate and three control which treatment with D.W.

.

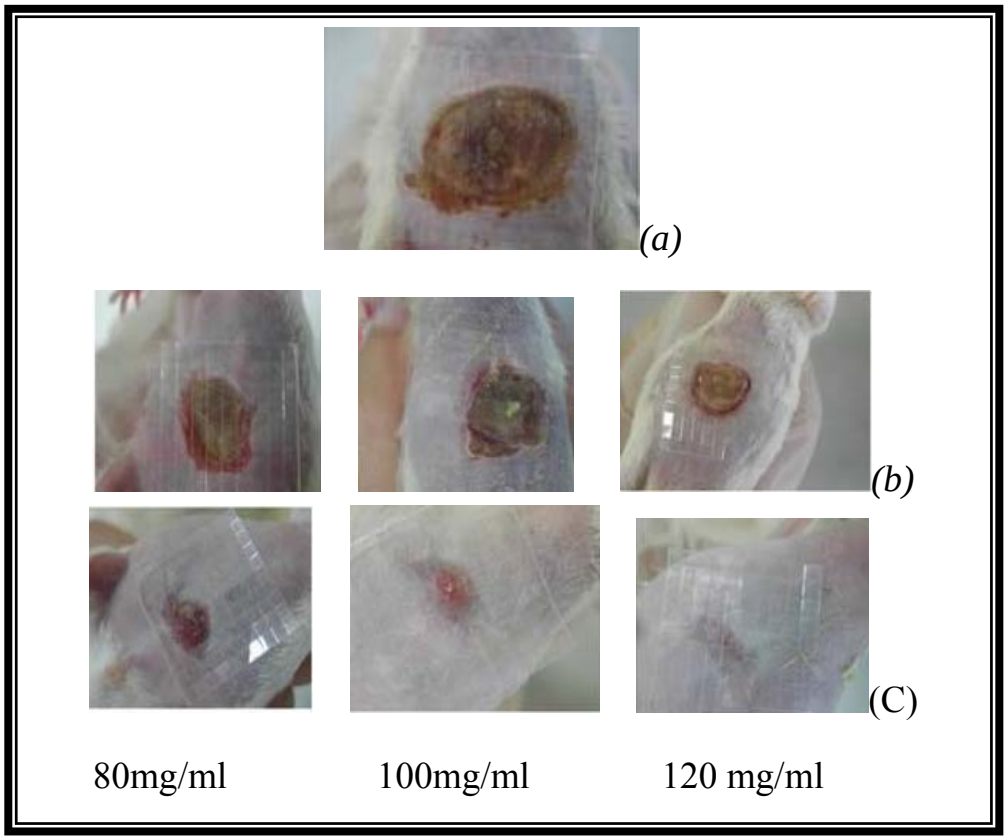


Figure (3-15) Morphological change and repair of group-A which caused by S. aureus

Figure (3-16) reveled the morphological change caused by *Proteus mirabili*s and actone extract. First picture(a) showed the damge caused by wound countamnination with *Proteus mirabilis* due to the bacteria virulence factor which cause infalmmation in the wound of mice, Others(b) and (c) was the change after daily treatment with acetone extract of 80mg/ml, 100mg/ml and 120mg/ml concentration.

These results was agreed with Humaira *et al.*, (2012) research result which also found the acetone and ethanol extact had activity on killing these bactria.

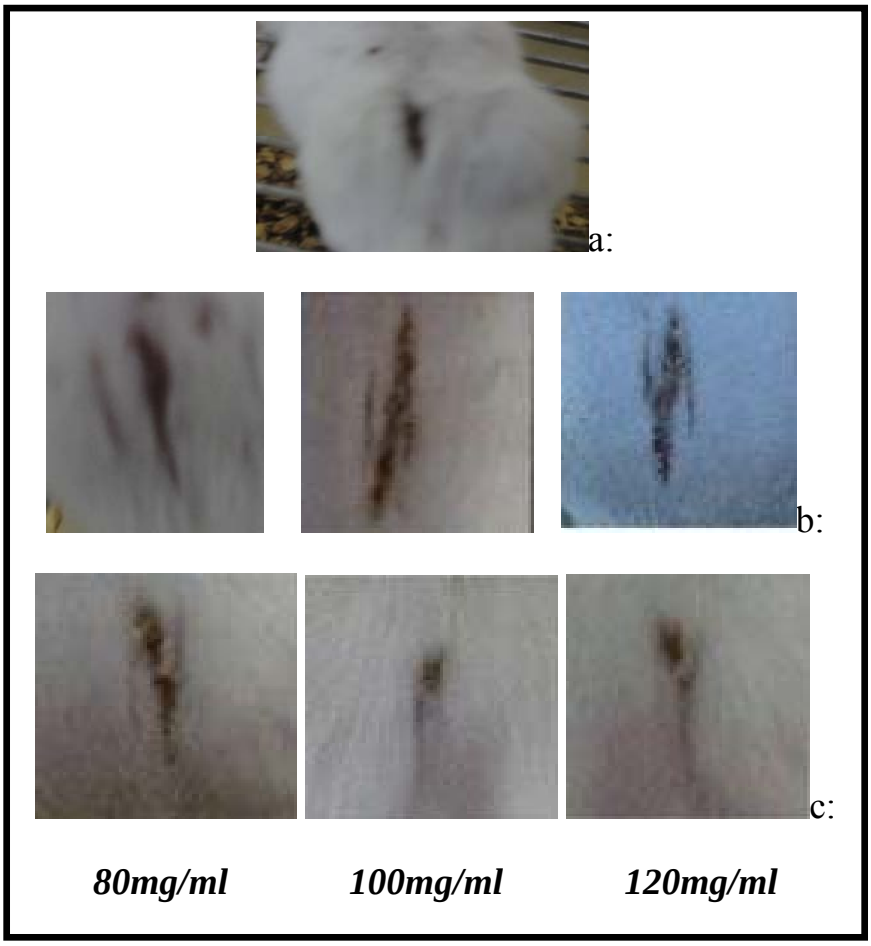
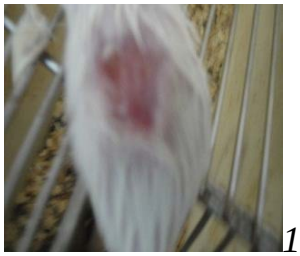


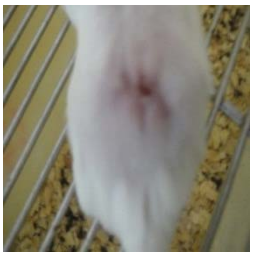
Figure (3-16) Morphological change and repair of group B which caused by P. mirabilis.

3.6.2. Antifungal Activity

Only one fungus (*Trichophyton violaceum)* was affected by acetone extract different concentrations (100-120) mg/ml of acetone, the only extract that showed effect on the fungi. Figure(3-17) showed the damage caused by the fungus after injection under skin due to the adherence of the fungal spores into the keratinophilic layer of the skin producing skin infection, Regular margin were seen , redness, flat, erythematous plaques with a raised border .Scaly plaques may be studded with papules or crusts. Hairs are broken close to the skin; they may plug the hair follicle. This represent patches can remain stable for years or may enlarge if not treated (Lin *et al.*, 2004) and the change after 11 days of daily treatment with 100mg/ml concentration of acetone extract. This was chosen for used to treatment because it was the extract working on *Trichophyton violaceum* fungus.



1days (a)



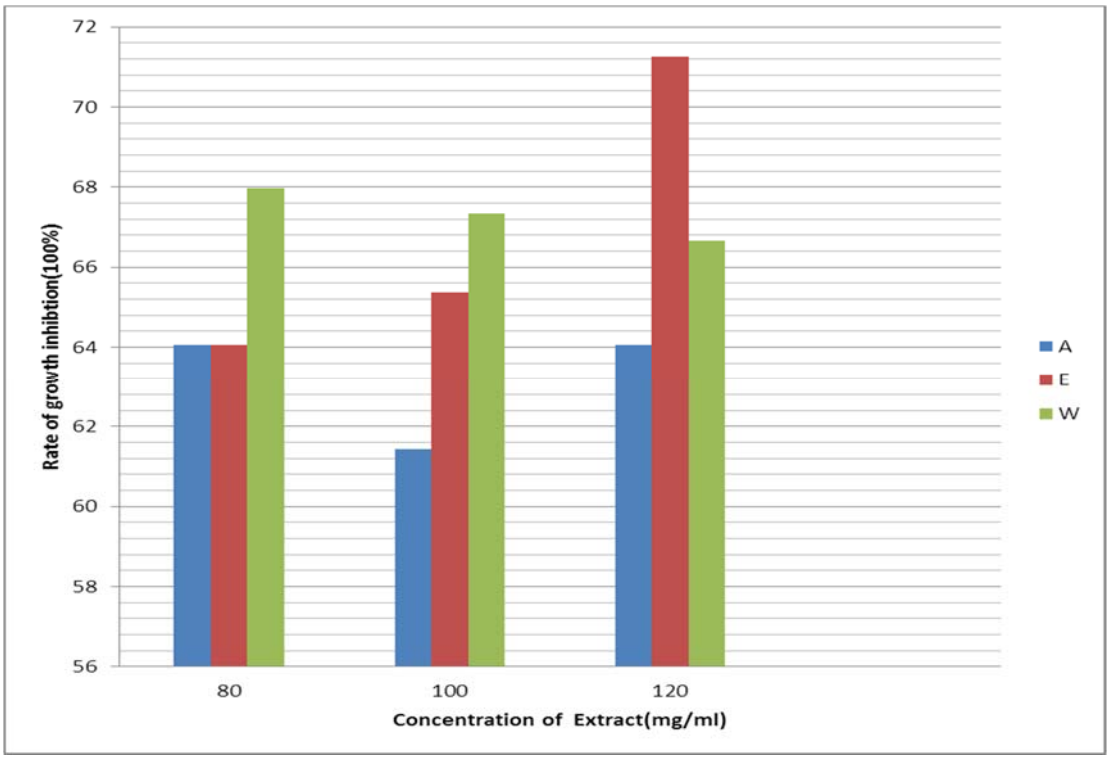
6days(b) *9days(c*) 11days €

Figure (3-17) Morphological change and repair of animal which caused by T. violaceum.

The curative properties of the leaves are attributed to Withaferin A (Khan, 1982). Withaferin A exhibits fairly potent anti-inflammatory activities. Anti-inflammatory activity has been attributed to biologically active steroids, of which Withaferin A is a major component (Khare, 2007).

3.7. Cytotoxic effect of Plant extracts on Tumor cell line

The cytotoxic effect expressed by percentage of inhibition growth rate which represent the cytotoxicity of plant extracts. Optical density of tumor cell line culture was measured at transmitting wave length of 492nm. Figure (3-18**)** showed the inhibition of HepG2 cell lines growth after 72 hr. of incubation by *Withania* extracts (ethanol, acetone and water extract) at different concentrations (80.100.120 mg/ml). The higher cytotoxic effect with probability ($P \leq 0.01$) was belong to ethanol extract at 120 mg/ml concentration, then water extract, while acetone extract was the lowest, with a viability ranging between (32-83%).



Note= (A) acetone, (E) ethanol, (W) water

Figure (3-18) Cytotoxicity effect of *Withania* extracts

The results were agreed with *Widodo et al.,* (2007) who reported *Withania* water extract selective cancer cell killing activity, similar to the alcoholic extract of *Withania* leaves. These result was also agreed with Devi, (1996) who provide that *Withania* as potent anticancer.

Withania extracts may had the same effect on normal cells, may have less on normal cells Zychlinsky, (1999). Indicated the tumor cells differ in morphology than normal one, and one of most important difference in that tumor cells highly express receptors on their membranes than normal one which allow attachment of different compounds. In addition, tumor cell DNA found in relaxant shape, and DNA molecule was found in unstable shape because H-bond connect both strand of DNA.

This makes it easy for component to interfere or to be associated with both strand of DNA, while normal cells DNA has strong H-bond connect both strands to each other and make it more stable so components cannot interfere or to be associated with both strand of DNA. (Belijanski, 2002).The anti-cancer activity of *Withania* may due to Withaferin-A which had been proved as potent anti-cancer (Uma *et al*., 1996) The presence of an unsaturated lactone in the side-chain to which an allelic primary alcohol group is attached at C25 and the highly oxygenated rings at the other end of the molecule may well suggest specific chemical systems possessing carcinostatic properties which may effect on metaphase of the cell division (Khare, 2007).

Conclusions and Recommendations

Conclusions

I. Different groups of the active compound have been detected in *Withania* ethanol extract, acetone extract and water extracts involve: Alkaloids, Glycoside, Saponin, Flavonoid, Fixed oil are present in ethanol extract, acetone extract and water extracts while terpenes were not found in water extract, protein were not found in acetone extract, Tannins were not found in all three extracts.

II. Withania extracts contained numbers of active group were detected by FTIR method.

III. All three types of Withania extracts contained two types of Alkaloids (Withanolide-A and Withaferin-A), seven types of Flavonoids (Naringenin, Catechin, Luteolin, Hesperetin, Kaempfero, Apigenin and Naringin) and two types of Saponins (Sitoindosides VII and Sitoindosides VIII) appeared as different peaks.

IV. All three types of *Withania* extracts (acetone extract, water extract and ethanol extract) showed activity on the following bacteria (*E. coli, E. sakazakii, K. pneumonia, S. aureus, S. epidermis, S. pyogenes, P. mirabilis, P. aeruginosa)* on light and dark and Only acetone extract showed activity on fungus (*Trichophyton violaceum),*

V. Withania extracts have cytotoxic effect on HepG2 cell line.

Recommendation

- Study Withania antiviral activity using different extract.
- Further Quantitative studies of different active compounds present in different *Withania* extracts.
- Extraction different active compound from *Withania* extracts and study their antimicrobial and anticancer, separately and study mode of action of each active compound.

References

Reference

(A)

- **Abdul Majeed, M.R. (2000).** Induction and Characterization of SU99 *plasmacytoma* cell line and its effect on mice immune response.Ph. D. Thesis, Collage of Science, Al-Nahrian University.
- **Abdul-Rahman, G. Y. (1995).** Effect of some medical plants and chemicals on the growth of *pathogenic bacteria*. J. Vet. Sci., 8(20):101-108.
- **Abeeda, S. Y. (1982).**The molecular effect of some plant Extracts on the growth and metabolism of *Some Gram –negative and Gram-positive bacteria*, M.Sc. thesis, Dept. of Microbiology, Collage of Science, University of Babylon, Iraq.
- **Abraham, A.; Kirson. I.; Glotter, E.and Lavie, D. (1968).** A chemotaxonomic study of *Withania somnifera (L.) Dunal*. Phytochemistry,7:957-962.
- **Al-Abid, M. R. (1985).** Zur Zurammen der Absuchlu B memtrame in phoenix dactily Frawwuzburg University, Wurzburg F. R. of Germany.
- **Al-Attar, A. M. (2006).** Comparative physiological study on the effects of rosemary, tarragon and bay leaves extract on serum lipid profile of quail, coturnix coturnix. S. J. Bio. Sci. 13: 90-97.
- **Alfonso, D.; Bernardinelli, G. and Kapetanidis, I. (1993).** Withanolides from Lochroma coccineum. Phytochemistry, 34:517-521.
- **Ali, S. S. (1997).** National Council forPromotion of Urdu Language (NCPUL), New Delhi, Unani Advia-e-Mufradah. 8th ed. 33.
- **Allen, J. W.; Suhuler, C. F.; Mendes, R.W. and Latt, S. A. (1977).** A simplified technique for *in vivo* analysis of sister chromatic

exchange using 5-bromodeoxy uridine tablets. Cytogenetics, 18:231-237.

- **Anabalagan, K. and Sadique, J . (1973**).Withania Somnifera and rejuvenating, herbal drug which controls alpha-2 macroglobulin synthesies during inflammation. Intl, J. crude Drugs Res.23:177-183.
- **Anwer, T.; Sharma, M.; Pillai. K. K. and Iqbal, M.(2008**). Effect of *Withania Somnifera* on insulin sensitivity in non-insulin-dependent diabetes mellitus rats. Basic Clin Pharmacol.Toxicol., 102 (6):498-503.
- **Aphale, A.A. and Chhibba, A.(2007).**Standardizations of Single Drugs of Unani Medicine. Part III, 1st ed. Central Council for Research in Unani Medicine (CCRUM), New Delhi, 9-14.
- **Atta-ur-Rahman, A.; Abbas, S.; Dur-e-Shawar, N. A.; Jamal, A. S. and Choudhary, M. I. (1993).** New withanolides from Withania spp. J. Nat. Prod. 56:1000-1006.

(B)

- **Bagamboula, C.F.; Uyttendaela, M. and Devere, J. (2004).**Food Microbial., 21, 33-42.
- **Bashir, H. S.1.; Mohammed, A. M.; Magsoud, A. S. and Shaoub, A. M. (2013).**Isolation of Three Flavonoids *from Withania Somnifera Leaves (Solanaceae)* and their Antimicrobial Activities.J.of Forest Products and Industries., 2(5). 39-45.
- **Bector, N. P.; Puri, A. S. and Sharma, D. (1968).** Role of *Withania somnifera (Ashwagandla)* in various type of Arthropathies, Indian, J. med. Res., 56:1581-1583.
- **Belijanski, M. (2002).** The anticancer agent *PB* inhibits multiplication of sixteen malignant cell lines, even multi-drug resistant. Genet. Mol. Biol., 23: 224-235.

- **Betsy, B.; Singh, PhD. and Simon, D. (2000).**Scientific Basis for the Therapeutic Use of *Withania Somnifera (Ashwagandha*): A Review. Alternative Medicine Review, 5(4), 334-346.
- **Bhandari, M. M. (1995).** Flora of the Indian desert. MPS Repros Jodhpur, India.
- **Bhattacharya, S. K.; Satyan, K. S. and Ghosal, S. (1997).** Antioxidant activity of glycowithanolides from *Withania Somnifera.* Indian J Exp Biol., 35 (3): 236-239.
- **Bjornsdottir, S.; Gottfredsson, M.; Thorisdottir, A. S.; Gunnarsson, G. B. and Kristjansson, M. (2005).** Risk factor for acute cellulites of the lower limb; prospective case-control study.Clin.Infect. Dis., 41(10):1416-1422.
- **Bodey, G.P.; Boliver, R.; Fainstein, V. and Jedeja, L. (2007).** Infection caused by *Pseudomonas aeruginosa.* Rev. Infect. Dis., 5(2): 276-313.
- **Body, M. R. (1998).** The Ncl *in vitro* anticancer drug discovery screen from: anticancer development guide: Preclinical screening, Clinical trials and approval. B. Tricher Humana press Inc. Totowa, N.J., U.S.A.
- **Brinker, F. (1998**). Herb contraindications and drug interactions, 2nd Ed.; Eclectic Medical Publications: Sandy, OR, USA. 36-82.
- **Budhiraja, R. D.; Sudhir, S. and Garg, K. N. (1983**). Cardiovascular effects of a withanolide from *Withania Coagulans, Dunal* fruits. Indian J. Physiol. Pharmacol., 27:129-134.
- **Bogdan, C. (2001).** J. Nature Immunol, 2, 907-916.
- **Bentley, R. and Meganathan, R. (1982).** "Biosynthesis of vitamin K (menaquinone) in bacteria". Microbiological Reviews 46 (3): 241–80.

- **Bowen, A. B. and Braden, C.R. (2006).** "Invasive Enterobacter sakazakii disease in infants". Emerging Infect Dis 12 (8): 1185–9. doi:10.3201/eid1208.051509.PMC 3291213. PMID 16965695.

(C)

- **Cappuccina, J. and Shermany, N. (1987**). Microbiology, a Laboratory Manual. 2nded. Benjamin, Cummings publishing. Inc. California, pp.53-171.
- **Chaurasiya, N. D. and Sangwan, R. S. (2007).** Leaf ontogenic phase-related dynamics of withaferin A and withanone biogenesis in *Ashwagandha (Withania Somnifera Dunal.)*- An important medicinal herb. J. Plant Biol., 50:508-513.
- **Choudhary, M. I.; Dur-e-Shahwar. N. A.; Parveen. Z.; Jabbar. A.; Ali, I. and Atta-ur-Rahman, A. (1995).** Antifungal steroidal lactones from *Withania Coagulans.* Phytochemistry, 40:1243-1246.
- **Cordell, G. A. (2011).** Phytochemistry and traditional medicine – A revolution in process. Phytochem, Lett., 4: 391-398.
- **Cowan, M. (1999).** Plant product as antimicrobial agents. Americ. Soc. For microbial., 12: 564-582.
- **Centers for Disease Control and Prevention (CDC) (2002).** "*Enterobacter sakazakii* infections associated with the use of powdered infant formula--Tennessee, 2001". *MMWR Morb Mortal Wkly Rep*., 51 (14): 297–300.

(D)

- **Danial, L.; Stuberg, M. D.; Mare, A.; Penrod, M. D.; Richard, A. and Blantny, M. D. (2002).**Common *bacterial skin* infections. America. Family Physicians, 66:119-124.

- **Davis, L. and Kuttan, G. (2002).** Effect of *Withania somnifera* on cell mediated immune responses in mice. J. Exp. Clin. Cancer Res., 21:585-590.
- **Devi, P. U. (1996).***Withania Somnifera* Dunal (Ashwagandha): potential plant source of a promising drug for cancer chemotherapy and radio sensitization. Indian J. Exp. Biol., 34:927-932.
- **Devi, P. U.; Sharada, A. C. and Soloman, F. E. (1995).** In vivo growth inhibitory and radio-sensitizing *effects of Withaferin A* on mouse *Ehrlich ascites carcinoma. Cancer Letters, 95 (1-2): 189-193.*
- **Devi, P. U.; Sharada, A. C. and Solomon, F. E. (1993).** Indian J. Exp. Biol., 31: 607-611.
- **Devi, P.U.; Akagi, K.; Ostapenko, V.; Tanaka, Y. and Sugahara, T. (1996).***Withaferin A:* A new radio sensitizer from Indian medicinal plant *Withania Somnifera.* Intl. J. Radiation Biol., 69 (2): 193-197.
- **Dhuley, J. N. (1997).**Effect of some Indian herbs on *macrophage* functions in Ochratoxin A treated mice. J. Ethnopharmacol., 58 (1):15-20.
- **Dhur, R. S.; Verma, V.; Suri, K. A.; Sangwan, R. S.; Satti. N. K.; Kumar, A. et al., (2006).** Phytochemical and genetic analysis in selected chemotypes of Withania Somnifera. Phytochemistry, 67:2269-2276.
- **Davis, L.; Kuttan, G. (2000).** J. Ethnopharmacol ,71, 193-200.
- **Davis, L.and Kuttan, G.(2002)**. J. Exp Clin Cancer Res,21, 115-118.

(F)

- **Fahmy, I. R. (1933).** Constituents of plant and crude drugs, 1st edition, Paul Berbey, Cairo.

- **Faraj, M.K. (1990).**Regulation of *mycotoxin* formation in Zea mays. Ph.D. Thesis. Dept. of bioscience and biotechnology. University of strathelyde. Glascow.U.K.
- **Frazer, W. c. and Westhoff, D. C. (1987).** Food Microbiology. 3rd ed. Mc Graw. Hill Book Co. New York.
- **Freshney, R. I. (2001).** Application of cell culture to toxicology. Cell Boil. Toxically, 17:213-230.
- **Freshney, R.I. (2000).** Culture of animal cells. Manual for basic Technique (4thed.).Wiley-liss, Ajohnwiley and Sons, Inc. publication, New York.
- **Farmer, J. J.; Asbury, M. A.; Hickmann, F. W. and Brenner, D. J. (1980).** *Enterobacter sakazakii:* a new species of *"Enterobacteriaceae"* isolated from clinical specimens. Int J Syst Bacteriol,. 30:569-84

(G)

- **Ganasoundari, A.; Zare, S. M. and Devi, P.U. (1997).** Modification of *bone marrow* radiosensitivity by medicinal plant extracts. British J. Radiology, 70 (834): 599-602.
- **Gao, S.; Yu, B.; Li, Y.; Dond, W. and Luo, H. (2003**). Anti-proliferative effect of Octereotide on *gastric cells* mediated by inhibition of AKT/ PKB telomerase . World J. Gastroenterol, 9:2362-2365.
- **George, J. D.(1968).**New clinical method for measuring the rate of gastric emptying in the double sampling test meal, From the department of surgery, Institute of clinical science, Royal Victoria hospital, Belfast, 9. 237-242.
- **Glotter, E, (1991).** *Withanolides and related ergostane-type steroids.* Nat. Prod. Rep. 8:415-440.

- **Glotter, E.; Kirson, I.; Abraham, A. and Lavie, D. (1973**). Tetrahedron, 29, 1353-1364.
- **Gow, N. A. R. and Goody, G. W. (1983).** Ultrastructure of chitin in hyphae of *candida albicans and dimorphic and mycelial fungi. Protoplasma*,115:52.
- **Grafone, T.; Marhinelli, C.; Gianning, B.; Terragna, C.; Soverini. S.; Amabile, M. et al., (2003).** *In Vitro* study with FTIs in CML ph.-positive cell lines. Proc. Am. Soc. Clin. Oncol.22:902-908.
- **Gupta, A.; Mittal, A.; Jha, K. K. and Kumar, A. (2011).** "Nature's treasurer: plants acting on colon cancer" (pdf). Journal of Stress Physiology & Biochemistry, 7 (4): 217–231.
- **Gurib-Fakim, A. and Schmelzer, (2012).**"Withania somnifera (L.) Dunal". PROTA (Plant Resources of Tropical Africa / Ressources végétales de l'Afrique tropicale) [Online Database]. Wageningen, Netherlands.
- **Gilani, A. H. and Atta-ur-Rahman (2005).** Trends in ethnopharmacology. Journal of Ethnopharmacology. **100**:43-49.
- **Gué, M.; Dupont, V.; Dufour, A. and Sire, O. (2001).** "Bacterial swarming: *A biological time-resolved FTIR-ATR study of Proteus mirabilis swarm-cell* differentiation".Biochemistry 40 (39): 11938–45.

(H)

- ***Harbone, J. B. (1973).*** *Phytochemical methods. Chapman and Hall, London.*
- ***Hemalatha, S.; Kumar, R. and Kumar, M. (2008).*** *Withania Coagulans Dunal: A review. Phcog Rev., 2:351-358.*

- **Hudault, S., Guignot, J. and Servin, A. L. (2001).** "*Escherichia coli* strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection". *Gut* 49 (1): 47–55.
- ***Humaira, R.; Al Hazzani, A. A.; Shehata, A. I. and Safouh Moubayed, N.M. (2012).****Antibacterial potential of Withania somnifera L. against human pathogenic bacteria form kingdom of Saudia Arabia.African Journal of Microbiology Research, 6(22).4810-4815.*
- **Hussein, F. T. K. (1981).** Agriculture and composition of medicinal plants. Mars house for publication, Al-Reyad (in Arabic).

(I)

- **Ibrahim, N. A. (2003).** *In vivo and In vitro i*mmunological and cytogenetic studies of calendula officinalis effects on albino male mice and acute myeloid leukemia cells, M.Sc. Thesis, Collage of Science, University of Al- Nahrain, Iraq.
- **Ihrke, G.; Neufeld, E. B.; Meads, T.; Shanks, M. R.; Cassio, D.; Laurets, M. et al., (1993).** *WIF-B cells:* an *in vitro* model for studies of hepatocyte polarity. Journal of cell Biology, 123(6): 1761-1775.
- **Iuvone ,T.; Esposito, G.; Capasso, F.; Izzo, A.(2003).** Life Sci .,72, 1617-1625.

(J)

- **Jaffer, H. J.; Mohmood, M. J., Jawad, A. M., Naji, A. and Al-Naib, A. (1983).**Phytochemical and biological screening of some Iraq plant. Fitoterapia.
- **Jawad, A.M.; Jeffery. H. J.; Al-naib, A. and Naji, A. (1988).** Antimicrobial activity of sesquiterpene *lactone and alkaloid* fractions from Iraqi plant .Int. J. Crude plant Drug. Res., 26(4):185-188.

- **Jawatz, M. D.; Melinich, J. L. and Adelberg, E. A. (1998).**Medical Microbiology.2st ed.,Prentice-Hall, USA,PP.2587-2591.
- **Jayaprakasam, B.; Zhang, Y.; Seeram, N. P. and Nair, M. G. (2003).** Growth inhibition of *human tumor cell lines by withanolides from Withania somnifera* leaves. Life Sci., 74:125-132.
- **John, W. (1996).** Principle and Practical of clinical Mycology. Edited by Kibbler,C.C.; Mackenzei, D. W. R. and Odds, F. C.
- **Jaspar, A. H.; Muytjens, H. L. and Kollee, L. A. (1990).** Neonatal meningitis caused by *Enterobacter sakazakii:* milk powder is not sterile and bacteria like milk too [in Dutch]! Tijdschr Kindergeneeskd. 58:151-155.
- **Joseph, E, (2012).** "Diversity of the *Cronobacter* genus as revealed by multi locus sequence typing". J Clin Microbiol 50 (9): 3031–3039.
- **Johnson, J.R.; Moseley, S.L.; Robert, P.L. and Staman, W.E. (1988).** Aerobactin and other virulence factor genes, among strains of *Escherichia coli* causing urosepsis: association with patient characteristics. Infact, Imunn.,56:405-412.

(K)

- **Kandil, F. E.; Elsayeh, N. H.; Abou-Douh, A. M.; Ishak, M. S. and Mabry, T. (1994).** J. Phytochemistry, 37, 1215-1216. Int. J. Mol. Sci. 2009, 10.
- **Kapoor, L. D. (2001).** Handbook of Ayurvedic medicinal plants. CRC Press.
- **Khan, P.U. (1982).** The Wealth of India. Vol. X (Sp-W), Publications and Information Directorate, Council of Scientific and Industrial Research (CSIR), New Delhi. 580-585.

- **Khare, C. P. (2007).** Indian Medicinal Plants–An Illustrated Dictionary, First Indian Reprint, Springer (India) Pvt. Ltd., New Delhi, PP. 717-718.
- **Kirson, I.; Glotter, E.; Lavie, D. and Abraham, A. (1971**). Constituents of *Withania Somnifera* Dun. XII. The withanolides of an Indian chemotype. J. Chem. Soc., 11:2032-2044.
- **Kirtikar, K. R. and Basu, B. D.(1980).**Indian Medicinal Plants. 2nd ed. Vol.III, Lalit Mohan Basu, Allahabad, India.1774-1777.
- **Kovacs, S.O. and Hurza, L.L. (1995).** Supper *fecal fungal* infections. Getting rid of lesions that don't go away. Postgrad. Med., 98(6):61-75.
- **Kulkarni, S. K. and George, B. (1996).** Anticonvulsant action of *Withania Somnifera* root extract against pentylene tetrazole (PTZ)-induced convulsions in mice. Phytotherapy Res., 95 (10): 447-449.
- ***Kuttan, G. (1996).*** Indian J. Exp Biol, 34, 854-856.

(L)

- **Liu, D.; Coloe, S.; Baird, R. and Pedersen, J. (2000).**Application of *PCR* to the identification of *dermatophyte fungi* .J Med Microbiol.49:493-497.
- **Lin, R.L.; Szepietowski, J.G. and Schwartz, R. A. (2004).** Tineafaciei an often deceptive facial eruption. Int. J. Dermatol. 43.
- **Lima, C. F.; Andrade, P. B.; Seabra, R. M.; Fernandes-Ferreira, M. and Pereira-Walson, C. (2005).** The drinking of Salvia officinalis infusion improves liver antioxidant status in mice and rats. J. of Ethno. 97: 383-389.

(M)

- **Madamombe, L. and Afolayan, A. (2003).** Evaluation of antimicrobial of extract from South African, Pharmaceut. Biol., 41:199-202.

- **Mauricio, P. J.; Alvaradano, A.; Akessonb, B. and Bergenstahlc,B. (2007).** *Separation of Phenolic and Flavonoid* Compound from food by Reversed-*Phase high performed liquid Chromatography,* Revista Boliviana DE Quimica., 24(1):142-149.
- **Mersh-Sundermann, V.; Knasmuller, S.; Wu, X. J.; Darroudi, F. and Kassie, F. (2009).**Use of a *human-derived liver cell line* for detection of *cytoprotective, antigentotoxic and cogenotoxxic* agents. Toxicology198 (1-3): 329-340.
- **Mirjalili. M. H.; Moyano, E.; Bonfill. M.; Cusido,R. M. and Palazón, P. (2009).**Steroidal Lactones from *Withania Somnifera*, an Ancient Plant for Novel Medicine from novel medicine. Molecules, 14:2373-2393.
- **Muller, G.; Krik, R. and Scott, D. (1989).** Small Animal Dermatology. 4th ed. Philadelphia: WB Saunders. Nail (claw) Disease; P820-825.

(N)

- **Nadkarni, K. M.(1982**). Indian Materia Medica. 3rd ed. Vol. I, Popular Prakashan Pvt. Ltd., Bombay. 1292-1294.
- **Nakabayashi, R.; Kusano, M.; Kobayashi, M.; Tohge, T.; Yonekura-Sakakibara, K. et al., (2009).** Metabolomics-oriented isolation and structure elucidation of 37 compounds including two anthocyanins from Arabidopsis thaliana. Phytochemistry, 70:1017-1029.
- **National Center for Complementary and Alternative Medicine (NCCA)(2005).**National Institutes of Health; Office of Dietary supplements. Available at: http:// ods.od.gov/factsheets/Botanical Backgrounds.

- **National Center for Complementary and Alternative Medicine. (2005).** National Institutes of Health; Office of Dietary supplements. Available at: http:// ods.od.gov/factsheets/Botanical Backgrounds.
- **National Committee for Clinical laboratory Standards (NCCLS) (2005).** Performance Standards for Antimicrobial Susceptibility Testing; MIC testing, Dec, M., 25:87-162.
- **Negi, M. S.; Sabharwal, V.; Wilson, N. and Lakshmikumaran, M. S. (2006).** Comparative analysis of the efficiency of SAMPL and AFLP in assessing genetic relationships among *Withania Somnifera* genotypes. Curr. Sci., 91:464-471.
- **Nema, R.; Jain, P.; Khare, S.; Pradhan, A.; Gupta,A. and Singh, D. (2012).**Study of *Withania Somnifera* with the spatial reference phytochemical, *FTIR and flavonoids* quantification.Int. J. of Pharm. & Life Sci., (IJPLS), 3.3: 1530-1532.
- **Nguyen, H. N. (2012).** Apoptosis induced by paclitaxel-loaded copolymer PLA-TPGS *in HepG2 cells* Adv. Nat. Sci.: Nanosci. Nanotcchnol, (6pp).
- **Nishifuji, k.; Sugai, M. and Amagai, M. (2008).** *Staphylococcal exfoliative* toxin:" Molecular scissors" of bacteria that attack the cutaneneous defence barer in mammals. J.of Dermatol. Sci.,49(1):21-31.
- **Nittala, S.S. and Lavie, S. (1988).** J.Phytochemistry.20.2741–2748.
- **Nur-e-Alam, M.; Yousaf, M.; Qureshi, S.; Baig, I. and Nasim, S. (2003).** A novel dimeric podophyllotoxin-type lignan and a new withanolide from *Withania Coagulans.* Helv. Chim. Acta 86:607-614.

(O)

- **Owais, M.; Sharad, K.S.; Shehbaz, A. and Saleemuddin, M. (2005).** Phytomedicine, 12:229-235.

(P)

- **Palyi, I.; Tyihak, E. and Palyi, V. (1969).** Cytological effects of compounds isolated from *Withania somnifera* Dunal. Herba Hung, 8:73-77.
- **Panchawat, S. (2012).**Pharmacognostical Evaluation of *Withania Somnifera* Leaf form India.Asian Journal of Biochemical and Pharmaceutical Research, (AJBPAD), 1(2).
- **Perez, C.; Agnese, A. M. and Cabrera, J. l. (1999**). J. Ethnopharmacol, 66: 91-96.
- **Peter, G. M.; Peter, A. A.; Joyce, O.; Esther, N. M. and Christine, C. B. (2013).**Antimicrobial Activity and Probable Mechanisms of Action of Medicinal Plants of Kenya*: Withania somnifera*, Warbugiaugandensis, Prunusafricana and Plectrunthusbarbatus. J. DOI.10.1371/journal.pone.0065619.
- ***Podschun, R. and Ullmann, U. (1998).*** *"Klebsiella spp.* as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods, and Pathogenicity Factors". Clinical Microbiology Reviews 11 (4): 589–603.
- **Prakash, J.; Gupta, S. K. and Dinda, A. K. (2002).** Nutr Cancer, 42, 91-97.
- **Prakash, J.; Gupta, S. K. and Dinda, A. K. (2002*).*** *Withania somnifera* root extract prevents DMBA-induced squamous cell carcinoma of skin in Swiss albino mice. Nutr. Cancer, 42:91-97.

(R)

- **Rajaseka, S. and Elango, R. (2011).** Estimate of alkaloid content of *Ashwagandle (Withania Somnifera)* with, HPLC Methods Journal of Experimental science, 2(5):39-41.

- **Rao, P. R.; Rao, K. T.; Srivastava, R. S. and Ghosal, S. (1995**).Effect ofglycowithanolides from *Withania Somnifera* on morphine-induced inhibition of intestinal motility and tolerance of analgesia in mice. Phytotherapy Res., 9 (1): 66-68.
- **Rastogi, R. P. and Mehrotra, B. N.(1998**). Compendium of Indian Medicinal Plants. 2nd Reprint, Central Drug Research Institute, Lucknow and National Institute of Science Communication, Council of Scientific and Industrial Research, New Delhi Vol. 1: 434-436; Vol. 2: 708-710;Vol. 3: 682-684; Vol. 4: 765-766; Vol. 5: 889-891; Vol. 6: 148.Vol.6. Central Drug Research Institute, New Delhi.
- **Rauprich, O.; Matsushita, M.; Weijer, C. J.; Siegert, F.; Esipov, S. E. and Shapiro, J. A. (1996). "**Periodic *phenomena in Proteus mirabilis* swarm colony development". Journal of Bacteriology 178 (22): 6525–38. PMC 178539. PMID 8932309.
- **Reid, G.; Howard, J. and Gan, B. S. (2001).** "Can bacterial interference prevent infection?". Trends in Microbiology 9 (9): 424–428.
- **Roberta, S. and Chambers, S. (2005**). Diagnosis and management of *Staphylococcus aureus i*nfections of skin and soft tissue. Intern. Med.J., 2:97-105.
- **Ryan, K. J. and Ray, C. G. (2004).** Sherris Medical Microbiology (4th ed.). McGraw Hill., ISBN, 0-8385-8529-9.

(S)

- **Sangwan, R. S.; Chaurasiya, N. D.; Lal, P.; Misra, L.; Tuli. R. and Sangwan, N. S. et al., (2004).** Phytochemical variability in commercial herbal products and preparations of *Withania Somnifera*. Curr. Sci., 86:461-465.

- **Santha, M. and Swiaminthan. C. (2011).**Evaluation of antibacterial activity and phytochemical analysis of leaves *of Withania somnifera (L.) Dunal.* International Journal of Current Research.,3(33):010-012.
- **Santos, P. R. V.; Oliveria, A. C. X. and Tomassini, T. C. B. (1995).** Rev. Farm Bioquim.31: 35-38.
- **Sarkas, G. Y.; Al-Rawi, K. M. and Katii, J. M. (1980).** Diagnosis of organic compound (chemical methods).Baghdad University (in Arabic).
- **Schliebs, R.; Liebmann, A.; Bhattacharya, S. K.; Kumar, A.; Ghosal, S. and Bigal, V. (1997).** Systemic administration of defined extracts from *Withania Somnifera (Indian ginseng)* and Shilajit differentially affect cholinergic but not glutamergic and GABAergic markers in rat brain.
- **Sharada , M.; Ahuja, A.; Suri, K. A.; Vij, S. P.; Khajuria; R. K.; Verma, V. *et al.*, (2007).** Withanolide production by in vitro cultures of *Withania Somnifera* and its association with differentiation. Biol. Plant.,51:161-164.
- **Sharada, A.C.; Solomon, F. E.; Devi, P.U.; Udupa, N. and Srinivasan, K.K. (1996**). Antitumor and radio sensitizing effects of withaferin A on mouse Ehrlich ascites carcinoma in vivo. Acta. Oncology, 35 (1): 95-100.
- **Sharma, V. (2013).**HPLC-PDA Method for Quantification of *Withaferin-A and Withanolide-A in Diploid (n=12) and Tetraploid (n=24)* Cytotypes of *"Indian Ginseng" Withania Somnifera (L.) Dunal* from North India.International Journal of Indigenous Medicinal Plants., 46(2):1245 –1250.
- **Shihata, I. M. (1951).** A pharmacological study of Anagolis arvensis. M. D.Vet. Thesis, Cairo University.

- **Sing, G.; Sharma,P. K.; Dudhe,R. and Singh,S. (2010).**Biological activities of *Withania Somnifera.*Annals of Biological Research.1 (3):56-63.
- **Singh, G. and Kumar, P. (2012).**Antibacterial potential of Alkaloids of *Withania Somnifera (L.)* form EUPHORBIA HIRTA L., 4(1):77-81.
- **Singh, N.; Singh, S.P.; Nath, R.; Singh, D. R.; Gupta, M. L.; Kohli, R. P. et al., (1986).** Prevention of urethane induced lung *adenomas by Withania somnifera (L.) Dunal in albino* mice. Pharm. Biol., 24:90-100.
- Singleton, P. (1999). Bacteria in Biology, Biotechnology and Medicine (5th ed.). Wiley. pp. 444–454.
- **Siriwardane, A. S.; Dharmadasa, R. M. and Kosala, S. (2013).**Distribution of *withaferin –A,* An anti-cancer Agent, in different parts of two varieties *of Withania Somnifera (*L.)Grown in Sri-lanka. Pakistan Journal of Biology Sciences, 16(3):141-144.
- **Sivamani, S.; Joseph, B. and Bibhas, Kar. (2014**).Anti-inflammatory activity of *Withania Somnifera* leaf extract in stainless steel implant induced inflammation in adult zebrafish. J. of Genetic Engineering and Biotechnology, 12:1-6.
- **Smolenski, S. J.; Silinis, H. and Farnworth, N. R. (1972).** *Alkaloid* screening I. Lloydia, 35(1): 1-34.
- **Srinivasan, D.; Perumalsamy, L.P.; athan and Suresh, T. (2001).** Antimicrobial activity of certain Indian medicinal plant used in folkoric medicine. J. Ethnopharum., 94:217-212.
- **Stahl, E. (1969).** *Thin layer-chromatoghraphy,* A laboratory hand book, 2th ed. Translated by Ashoworth, M. R. F. Spring. Verage, Berlin, Heidelberg, New York, U.S.A.

- **Stearn, W. T. (1995).** Botanical Latin: History, Grammar, Syntax, Terminology and Vocabulary (4th ed.). Timber Press. ISBN., 321-4.
- **Steel, R. and Torrie J. (1982).** Principles and Procedures of Statistics, 2nd Edition. McGraw-Hill International Book Company. Auckland, London. pp. 198-235.
- **Subaraju, G. V. (2006).** *Ashwagandhanolide*, a bioactive dimeric thiowithanolide isolated from the roots *of Withania Somnifera.* J. Nat. Prod., 69:1790-1792.

(T)

- **Taiz, L. and Zeiger, E. (2002).** Plant Physiology. Sinauer Associates, Inc. Publishers. Stunderland.
- **Tayler, R. S. L.; Edel, F.; Manandher, N. P. and Towers, G. N. (1996).**Antimicrobial activity of Southern Nepal medicinal plants. J. Ethnopharmaco., 50:97-102.
- **Todar, K. (2004).***Pseudomonas aeruginosa*, University of Wisconisin, Med. Dept. of Bacteriol., PP.1-15.
- **Tong, X.; Zhang, H. and Timmermann. B. N. (2011).** Chlorinated withanolides from *Withania Somnifera.* Phytochem. Lett, 4(4):411-414.
- **Trease, J. E. and Evans, W. C. (1987).** Pharmacology. 3thed. Balliere. Tindall, pp.62-68.

(U)

- **Udeanu, D. I.; Corina, I.; Elena, D.; Denisa, M.; Florea, C.; Virginia, V. *et al.*, (2011).** Researches on the Antioxidant Role of Two New Synthesized Prostamindes on Human Hepatocellular Carcinoma Cell Line ($HepG_2$). Journal of framacia, (59); 611.

- **Uma, D. P.; Akagi,K. *et al.* (1996*).*** *Withaferin -A: A* new radio sensitizer from the Indian medicinal plant *Withania Somnifera.* International Journal of Radiation Biology 69(2): 193-197.

(V)

- **Van Ijzendoorn, S. C. and Mostov, K. E. (2000).** Connecting apical endocytosis to the intracellular Traffic infrastructure in polarized hepatocytes. Gastroenterology, 119(6):1791-4.
- **Velu. S and Baskaran, C. (2012).** Phytochemical analysis and in-vitro antimicrobial activity *of Withania somnifera (Ashwagandha).* J. Nat. Prod. Plant Resour., 2 (6): 711-716.
- **Vogt, R. L, and Dippold, L. (2005).** *"Escherichia coli* O157:H7 outbreak associated with consumption of ground beef, June-July 2002". *Public Health Reports (Washington, D.C.: 1974)* **120** (2): 174–8.

(W)

- **Widodo, N.; Kaur, K.; Shrestha, B. G.; Takagi, Y. and Ishii, T. et al. (2007).** Selective killing of cancer cells by leaf extract of *Ashwagandha:* identification of a tumor-inhibitory factor and the first molecular insights to its effect. Clin, Cancer Res., 13: 2298-2306.
- **Wilson, A. P. (2000).** Cytotoxicity and Viability assay. In masters, J.R.W., Anomal cell culture: apractical approach (3rded.). Published by United States by Oxford University Inc. New York.
- **Winckler, J. (1974).** Vital staining of lysosomes and other cell organelles of the rat with neutral Red. Prog. Histochem. Cytochem., 6:1–89.
- **Wargovich, M.; Woods, C.; Hollis, D.M. and Zander, M.E. (2001).** Herbals, cancer prevention and health. J. Nutr., 131: 3034-3036.

(X)

- **Xu, Y. M.; Gao, S.; Bunting, D. P. and Gunatilaka, A.A.L. (2011**). Unusual *withanolides* from aeroponically grown *Withania Somnifera.* Phytochemistry,72:518-522.

(Y)

- **Yu, P.; El-Olemy, M. M. and Stohs, S. J. (1974**). A phytochemical investigation of *Withania Somnifera* tissue cultures. Lloydia, 37:593-597.

(Z)

- **Zhao, J.; Nakamura, N.; Hattori, M.; Kuboyama, T.; Tohda, C. and Komatsu, K. (2002).** Withanolide derivatives from the roots of *Withania Somnifera* and their neurite outgrowth activities. Chem. Pharm. Bull., 50:760-765.
- **Ziauddin, M.; Phansalkar, N. and Patki, P. (1996).** J. Ethnopharmacol, 50, 69-76.
- **Zynchlinsky, L. (1999).** The induction of apoptosis by *bacterial* pathogens. Rev. *Microbial.*, 53: 155- 187.

الملخص

تم جمع اوراق نبات سم الفراخ من حدائق جامعة النهرين. ثم تم استعمال مسحوق الاوراق للحصول علي ثلاثة انواع من المستخلصات. اخضعت مستخلصات *سم الفراخ* (المائي، الإيثانولي والاسيتون) للتحليلات الكيميائيه لتحديد المواد الفعالة الحاوية عليها وأظهرت النتائج أحتواء المستخلصات على القلويدات، الغليكوزيد، الصابونين، الفلافونيدات، الدهون الثابتة. الا ان التربين كان غير موجود في المستخلص المائي، و البروتين كان غير موجود في المستخلص الأسيتوني. تم الكشف عن المجاميع الفعالة الموجودة في مستخلصات *سم الفراخ* عن طريق(FTIR) والذي اظهرت وجود عدد منها ظهرت كقمم مختلفه في جميع انواع المستخلصات.اما تحليل [HPLC] والمستعمل للكشف عن تراكيزالمركبات الفعاله الهامة الموجودة في مستخلصات سم الفراخ المائي والاسيتوني والايثانولي بما في ذلك: القلويدات، الفلافونويد والصابونين والتي اثبت انها تحتوتي علي نوعين من القوليدات هما (Withanolide-A and Withaferin-A)، سبعة أنواع من مركبات الفلافونويد (Catechin, Luteolin, Hesperetin, Kaempfero, Naringenin, Apigenin وNaringin) وهناك نوعان من الصابونينات هما (Sitoindosides VII و Sitoindosides VIII) ظهرت كقمم مختلفة. اظهرت مستخلصات اوراق *سم الفراخ* فعاليتها مايكروبية ضد سلالات البكتريا (*E. coli, E. sakazakii, K. pneumonia, S. aureus, S. epidermis, Streptococcus pyogenes, P. mirabilis, P. aeruginosa*) في الضوء والظلام وبلتاثير معنوي ($P\leq 0.05$) اما المستخلص الاستوني فقط كان له تاثيرا في مثبطا في الفطريات (*Trichophyton violaceum*). كذلك تم تحديد الفعالية السمية للمستخلصات النباتية (المائي، الإيثانول والاسيتون) وتقييم تاثيرها في نمو خط االخلايا السرطانيهHepG2 بعد حضانة لمدة 72 ساعة مع تراكيز مختلفة (80. 100.120 ملغ/مل) من المستخلصات النباتية المائي و الإيثانول والاسيتون. اذ اضهرت تاثيرا سامي علي خط الخلايا السرطانيه HepG2، اعلي تأثيرسمي في خط الخلايا السرطاني كان تعود لمستخلص الإيثانول بتركيز 120 ملغ/مل لكن المستخلص الأسيتون كان له اقل تاثير اما بالنسة للمستخلص المائي كان متوسط مع التاثير المعنوي ($P\leq 0.01$).

جمهورية العراق
وزاره التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانه الاحيائية

الفعالية ضد المايكروبية والسمية للمواد الفعاله لمستخلصات نبات سم الفراخ

رساله

مقدمه الى مجلس كلية العلوم/جامعه النهرين وهي جزء من متطلبات نيل درجه الماجستير في علوم التقانه الاحيائيه

من قبل

وهام سعد عطا
بكالوريوس تقانه احيائية (2011)

باشراف
أ. د. نبيل خلف العاني

ربيع الاول 1453 كانون الثاني 2015