Antimicrobial and Cytotoxic Activity of *Withania somnifera*

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Abstract

Withania somnifera (L) Dunal popularly known as 'Ashwagandha' had been used in folk medicine. Ten grams of leaves powder were used to extract the active substances as water, ethanol, and acetone extracts. The extracts were subjected to chemical analysis to identify the active components, Alkaloids, Glycosides, Saponins, Flavonoids, Fixed oil was present in ethanol, acetone and water extracts while terpene was absent in water extract, Proteins were not found in acetone extract, Tannins were absent in all types of extracts. HPLC analysis revealed that all 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 in same retention times with standard. *Withania* extracts contained numbers of active group detected by FTIR. Antimicrobial activities for all types of extracts werecarried out against different types of bacteria and fungi but only Acetone extract showed antimicrobial activity on the fungus (*Trichophyton violaceum*). *W. somnifera* extracts had cytotoxic effect on HepG2 cell line, higher cytotoxic effect in ethanol extract appeared at 120 mg/ml concentration, Water extract was inbetween and acetone extract was the lowest one.

Keywords: *Withania somniferous*, active compounds, HPLC, FTIR, HepG2 cell line, antimicrobial activities.

Introduction

Increase in the usage of antibiotics during past few years worldwide led to development of multidrug resistance among the pathogenic bacteria [10]. Due to the emergence of multiple drug resistance in human pathogens has necessitated a search for new antimicrobial substances from other sources including plants. 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 produce naturally by plant or microorganism as secondary metabolite and use as drugs according to World Health Organization. These plant products are frequently considered less toxic and free from side effects. Some of these plants have also been pharmacologically tested and shown to be of some value in human diabetes treatment.

Withania somnifera (L.) belong 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 region. It is used in medicine, for antioxidant, anticancer, anti-inflammatory and antimicrobial activity. Roots, leaves and bark have a potential role in the cancer therapy for growth inhibitory of human tumor cell lines: ant carcinogenic activity, anti-granuloma, anti-oxidative and chemo protective activity. 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 [8]. A number of withanolide steroidal lactones have been isolated from the leaves of *W. Somnifera* and exhibit antibacterial, anti-fungal and antitumor properties exhibits hypotensive, bradicardiac and respiratory stimulant activity [2]. Its roots has a great demand in drug and in herbal drug industry and its extract used in uterine construction and recommended in child birth in difficult cases. Seeds powder is used for coagulating milk. Ashwagandha was given to

old people and pregnant women as a nutrient of health restorative.

In the first part of this study the Evaluation of *in vitro* the antimicrobial activity of *Withania somnifera* using different extracts of different concentrations were carried out, Besides, phytochemicals screening of the extracts was also carried out to assess the presence of different phytochemical in different extracts by different qualitative and quantitative methods. The second part investigatesthe cytotoxicity of the extracts on some cell lines.

Materials and Methods

W. somnifera was collected in November 2013 at morning from gardens in Al-Nahrain University. Arial parts of this plant were air dried in shade at room temperature for 15 days, and then the leaves were dissected and grinded into powder by using electric grinder. The powder material was stored in airtight glass bottles protected from sunlight until use.

Preparation of water extract

Ten grams of the powder were mixed with 100ml distilled water during extraction the mixture was kept in water bath at 50°C for 6 hours. Theextracts filtered with 0.2µm filter paper and the filtrate was concentrated by lyophillizer and stored at room temperature [12].

Preparation of alcohol extracts

Ten grams of Withania leaf powder was extracted with 100ml of ethanol for ethanol Extracts and 100ml of acetone for acetone and put in a shaker incubator for 48 hour at room temperature then filtered with 0.2µm filter paper and the filtrate was concentrated by allowing the solvent evaporated and stored at room temperature [12].

Measurement of extract acidity

Magnetic stirrer mixed Ten grams of the leaf powder was used to mix with 250 ml of distilled water, then the suspension filtered with 0.2µm filter paper and the filtrate measured using pH meter [13]. In addition, the acidity of ethanol and acetone extracts measured.

Detection of some active compounds in Withania leaf extracts

The crude water, ethanol and acetone extracts of *Withania* leaf tested for the presence ofphytochemicals using standard qualitative procedures [5].

Sample preparation

One g of leaf sample was weighed and dissolved in 50ml of methanol (0.99%) (HPLC grade). Further dilution 1ml of this solution to 50ml using (0.99%) methanol HPLC grade.

Method used for High Performance Liquid Chromatography (HPLC)

Aliquot of 20ml of standardprepared as in [11].and sample injected to HPLC and record the chromatogram, calculated the content of the sample in comparison with standard.

Antibacterial activity

The extracts obtained above were screened for their antibacterial activity *in-vitro* by well diffusion method [1].the stock solution was prepared by dilution 1g of powder in 10 ml of D.W. or D.W. with one or two drops 70% methanol, the stock solution was sterilized by Millipore filter unit under aseptic conditions. Different concentrations used (0.9, 3, 5, 7, 10, 15, 25, 50,100, 125 mg/ml). The Nutrient medium that is a medium that can be using for all bacterial types *(Escherichia coli, Enterobacter sakazakii, Klebsiella pneumonia, Staphylocuss aureus, Staphylocuss epidermis, Streptococcus pyogenes, Proteus mirabilis, Pseudomonas aeruginosa*) was sterilized in autoclave and then poured in petri-dishes, surface of nutrient mediumswabbed with 0.1ml of a suspension contain 1×10^8 cfu/ml. The agar left to solidify and in each of these plates, 5mm in diameter, cut using a sterile cork borer and the agar discs removed. Using sterilized micropipettes 25µl of different solvents with selected *W. somnifera* extracts added into the well, allowed to diffuse for two hrs. at room temperature. The plates then incubated in the dark and light incubator at 37ºC for 24 hrs. The control well was made in the center of plate swapped with 0.01ml of extract solvent diluted with D.W. the inoculated plates kept at 37ºC for 24 hrs. The diameters of the inhibition zones measured

after 24 hours of incubation and the mean values tabulated.

Antifungal activity

The same method as for bacteria was followed instead of nutrient agar media, potato extract agar was used [1]. The potato extract medium was swabbed with 0.1ml of suspension containing 1×10^4cfu/ml then incubated at 28ºC for 48-72hrs.

Cytotoxicity assay

This method was carried out according to [3]. The cell suspension wasprepared by detaching cell flask with 2 ml of trypsin solution when a single cell appeared, 20ml of growth medium supplement with 10% fetal calf serum was added to the cell flask to inactivate the trypsin effect then the viability should be more than 95%. The cell suspension was well mixed followed by transforming 200µl/well into each well of the 45 well flat bottom micro-titer using automatic micropipette containing(1×10^5cell/well). Plastics (which contain cell) were incubated at 37°C until 60-70% confluence of the internal surface area of well for HepG2 cell line. The cells exposed to different concentration of plant extract (80.100.120 mg/ml in 1L). The negative control wells that contained only the cells with culture medium were incubated at 37°C in an incubator supplement with (50%) CO_2 for 72 hours after elapsing the incubation period, 50 µl/ well of natural red dye added and incubated again for 2 hr. The content of plate were removed by washing the cells 3 times with PBS then 100 µl 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 reading by using ELISA reader at a transmitting wave on 492 nm then inhibition rate was length on 492 nm, then inhibition measured according to the formula-by-formula.

Results and Discussion

Water, Ethanol and Acetone Extracts.

A quality of two and half (2.5g) was the weight of the residue resulted after evaporation of distilled water, which represents 25% of the original leaf sample weight; this extract appeared with brown color. However, ethanol and acetone residue obtained after evaporation of ethanol or acetone solvent was 3g that represent 30% of the original leaf sample weight. The appearance of the extract was dark green in color but the ethanol was with more viscosity than acetone extract.

Detection of some active compounds in the plant extracts.

The water, ethanol and acetone extract of *W.somnifera* were subjected to chemical analysis to identify the active compounds in each extracts. Table (1). Reveled that thealkaloids, glycosides, saponins, flavonoids, fixed oil were presented in ethanol, acetone and water extracts while terpenes were absent in water extracts, protein were not found in acetone extract, tannins not found in all types of extracts, even with the method using ($Fecl_2$) which also gave the same result. These result may because of type of organic solvent, each organic solvent has different polarity with different ability to attract the compound with same polarity, therefore when the organic polarity increase their ability to extract active compound increase. The present of these bioactive compounds in the plant has reported to confer resistance against pathogenic [17].

Table (1)
Secondary Metabolite Detected 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	Oily spot on paper	+	+	+

(+) indicate the present, (-) indicate the absent.

The results agreed with [12]. but they disagree with [20] results in which alkaloids were absent in water extract and protein were absent in ethanol extracts, these differences in water extracts may be alkaloids were absent in water extract and protein were absent in ethanol instead of acetone, these differences in water extracts may be due to the 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, While the different in ethanol and acetone might be because of putting the extract in shaker for 48 hrs. Depending on the way of previous reference [12] to get extracts that might cause protein damage.

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. FTIR results revealed a number of chemicals such as Bromide, Iodide, Chloride, Fluoride, Alkanes, Ch2, Imines, Oxine, Aromatic(C=C), Aldehyde, Alkenes, Amines which all was found in all Withania extracts (Ethanol, Acetone, and Water) which were presented in the next figures expect 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). While the water was different by Sulfoxide (1050). The acetone extract and water extract different from ethanol by alkyne (3300) and ethanol extract different by containing Nitro(R-no_2)(1550). the acetone different from other by containing anhydride (1843-1793).

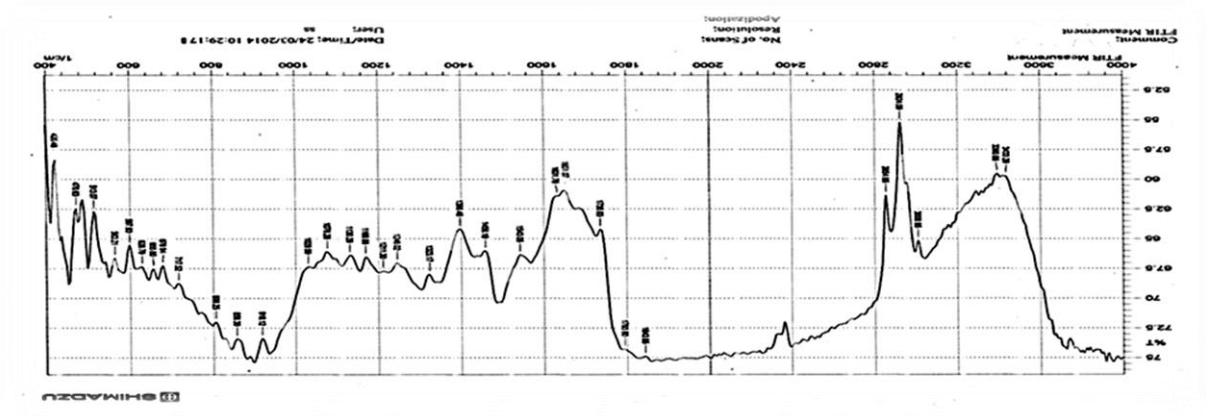


Fig.(5) FTIR Analysis of Withania Acetone extract.

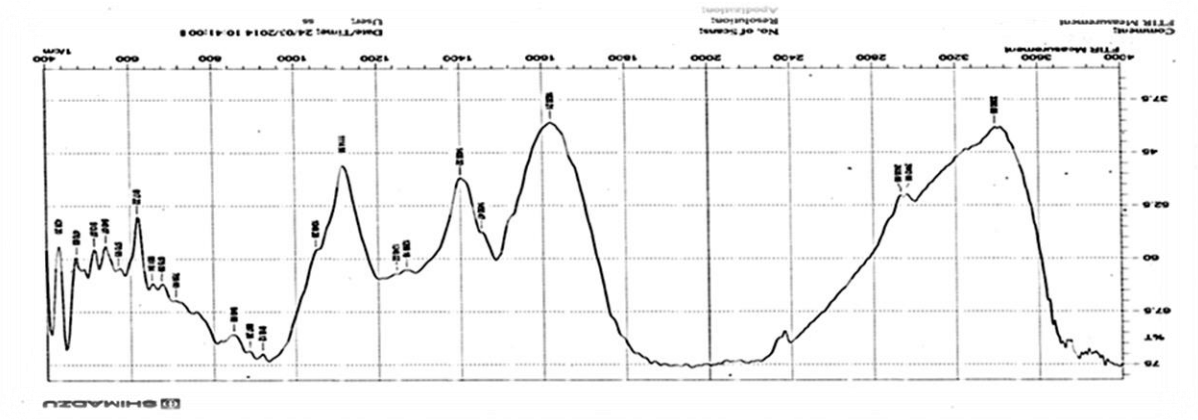


Fig.(4) FTIR Analysis of Withania water extract.

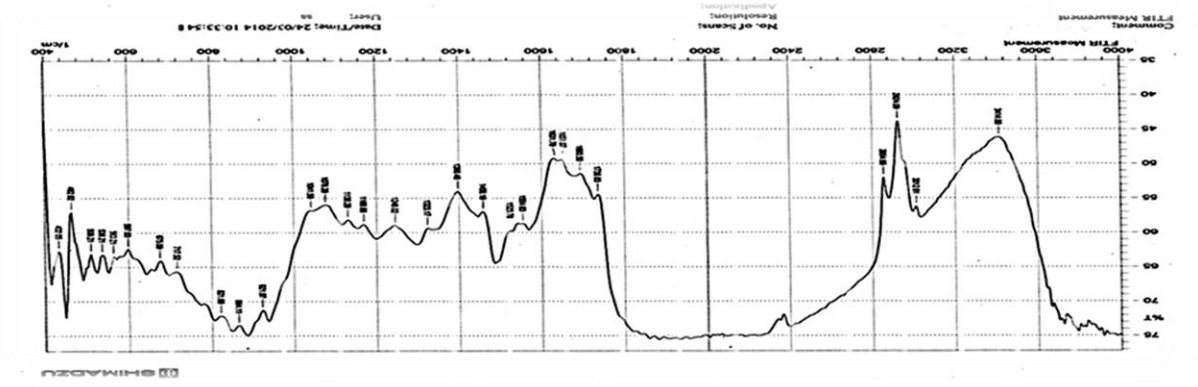


Fig.(6) FTIR Analysis of ethanol extract of Withania.

These results agreed with [9] in which the number of peaks, which indicated that *Withania* extracts rich with numbers of active groups.

present in all extract of Withania includingalkaloids, flavonoids and saponins. X-axis= min, Y-axis=mV.

HPLC Analysis of *Withania* Extracts

HPLC analysis was done for detect the concentration of important active compounds

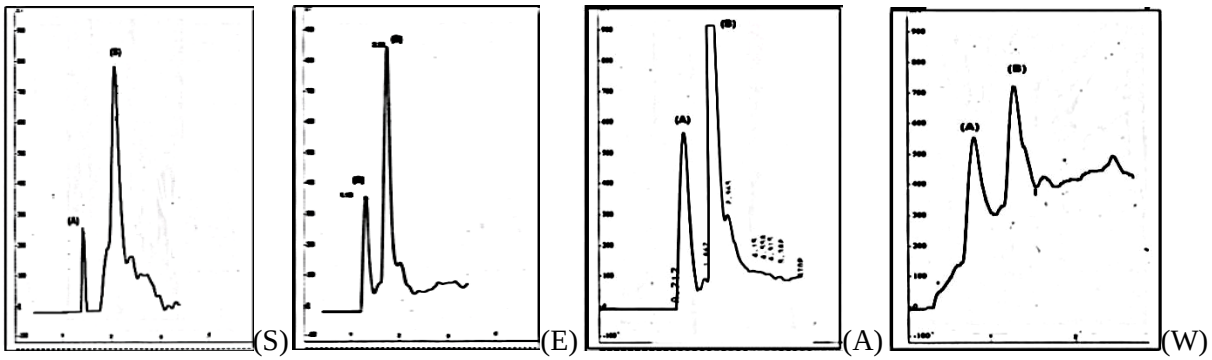


Fig.(1) Alkaloid found in Withania extracts by using HPLC.

Showed major peaks with different retention times and area of Alkaloid present in alkaloid Standard (S) and in Water (W), Ethanol (E) and Acetone (A) extracts of Withania withthreedilutions factor include Withaferin-A (A) and Withanolide-A (B).

The first peak was belong to Withaferin-A(A) which in all extracts present in concentration lower than the second peak

which belong withanolide-A that found in higher concentration in all three types according to Standard peaks which previously known and used to compare with extracts peaks[11] Results agreed with [7] which report that the leaves contain Withaferin-A and Withanolide-A. The concentrations calculated according to the following equation: Concentration = [area of sample/area of standard] × sample concentration × dilution factor.

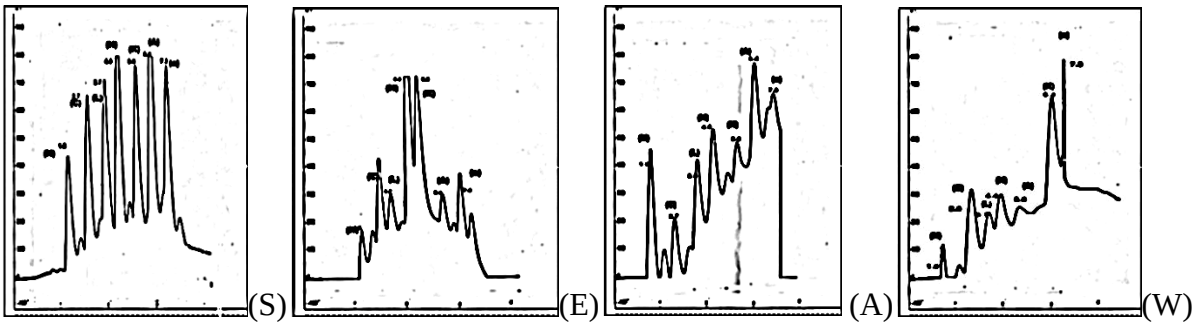


Fig.(2) Flavonoid in Withania extracts by using HPLC.

Different peaks of Naringenin (N), Catechin (C), Luteolin (L), Hesperetin (H), Kaempfero (K), Apigenin (A) and Naringin (n), present in Acetone extract(A), Ethanol extract (E) and water extract (W) present peaks of water at 1.5 dilution factor.

Water extract at 4 dilution factor show very low concentration of compound which appear as only base line correction which mean that the extract contain the compounds but in low concentrations. Therefore, we repeated HPLC for water but with 1.5dilution factor to allow the active compound appear as peaks in same retention time of Standard but in different area. These results agreed with [18] who's also found more than five types of flavonoids but in different concentrations. The concentrations of these flavonoids depend on the growth culture condition and the active compound extraction method.

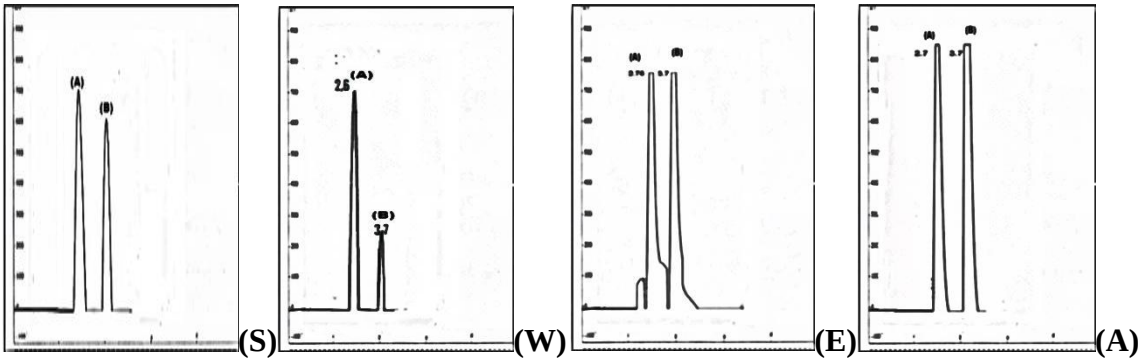


Fig.(3) Saponins in Withania extracts by using HPLC.

Fig.(3) Revealed different peaks with different retention times and area of saponins present in the water (W), ethanol (E) and acetone (A) extracts of Withania with two dilution factor these compounds were: Sitoindosides VII (A), Sitoindosides VIII(B).

These peaks in Fig.(3) already appeared and identified in Saponins Standard (S). They used to compareand identify the peaks in extracts which appeared in retention time resemble to Standard retention time [11]. Sitoindosides have been isolated and reported from aerial parts whichinclude: Sitoindosides VII and Sitoindosides VIII that we indicated its present in the extract according to the peaks appeared in retention same to the Standards. [14] result was also found *Withania* leaf extracts had two types of saponins (Sitoindosides VII and Sitoindosides VIII) resemble to our results because they used same method of extraction.

In vitro Antibacterial activity

The antibacterial activity of *W.somnifera* extracts was tested on the following bacteria (*Pseudomonas aeruginosa, Staphylocuss aureus, Staphylocuss epidermis, Streptococcus pyogenes, Proteus mirabilis, Enterococcus, Escherichia coli, Klebsiella pneumonia*) by different concentrations of extracts range from (0.9,3,5,7,10,15,25,50,100mg/ml) of water, ethanol and acetone extract in the dark and light. The figures revealed that Withania water extract range from (7.5mm) for *Pseudomonas aeruginosa* to (9.8mm) for *Enterobacter* which was presenting the lowest inhibition zone detected by water extract, while *Klebsiell*a (13mm) and *Proteus* (14mm) showed the highest inhibition diameterby water extract on dark and light. However, figures for ethanol extract revealed that *Withania* extracts inhibition zone range from (0.5-0.8mm) for*proteus* on dark and light which presented the lowest inhibition diameter while (2.0-2.1mm) for *Klebsiella*, (3.5-3.6mm) for *Staph. aureus* and (3.7-3.9mm) for *Staphlycoccus Epidermis* which presented the highest inhibition diameter followed by *Pseudomonas aeruginosa*at 0.9 mg/ml *Withania* ethanol extracts concentration. However, the highest *Withania* ethanol extract at 100mg/ml was to *Staphylocuss aureus* (16.3-16.5mm), *Staphylocuss epidermis* (16.6–17mm) and for *Escherichia coli (*16-16.3mm).

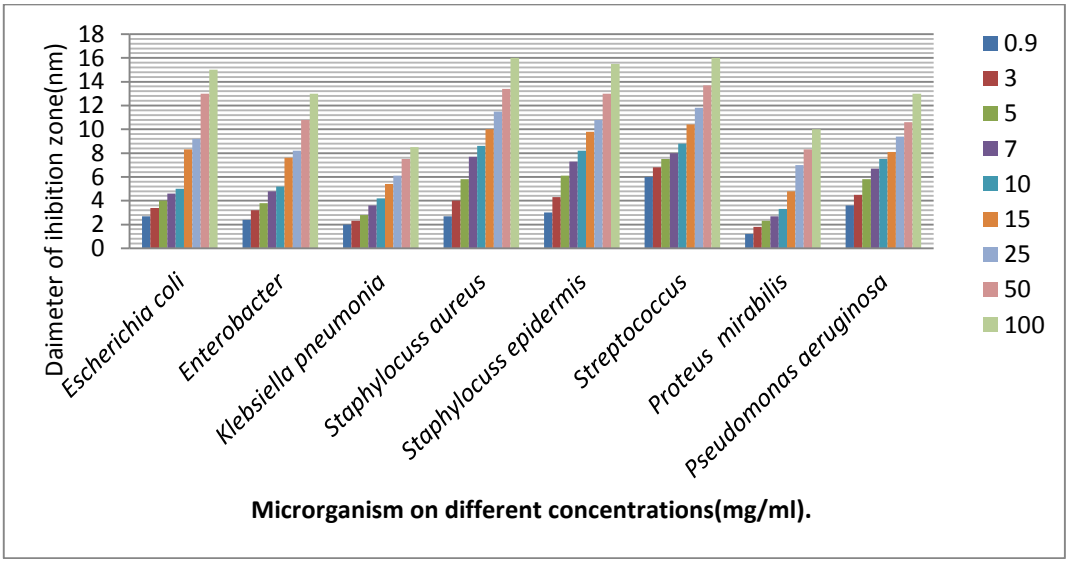


Fig.(7) Bacterial inhibition on dark of Withania Acetone extracts.

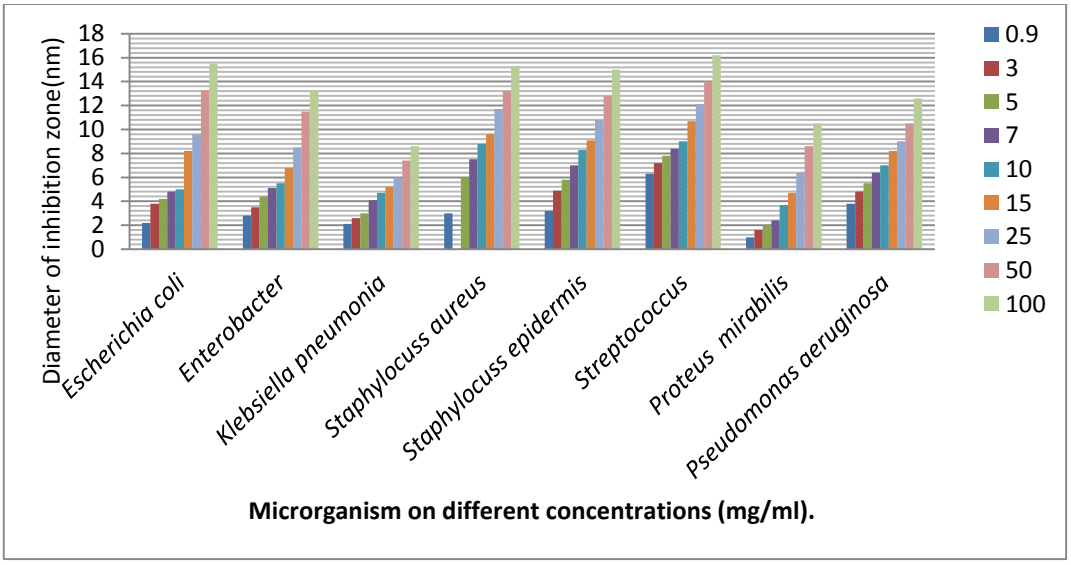


Fig.(8) Bacterial inhibition of Withania Acetone extract on light.

Acetone extract figures showed that the lowest *Withania* acetone extract inhibition zones were *Proteuse* (1.0-1.2mm*)* under light and dark condition, *Klebsiella pneumonia* (2.0-2.1mm) and *Enterobacter sakazakii* at 0.9 mg/ml concentration of acetone extract. The highest inhibition zones were in *Streptococcus* (6.0-6.3mm) and *Pseudomonas aeruginosa* (3.6-3.8mm) which showed the inhibition diameter at all concentrations up to 100mg/ml.

All figures showed that the highest inhibition diameter were in *Staphylocuss aureus, Staphylocuss epidermis* and *E.coli* by ethanol extract followed by inhibition diameter of acetone extract and then water extract. Nevertheless, water extract showed highest inhibition for *Proteus mirabilis, Klebsiella pneumonia* which followed by acetone and then ethanol. The acetone extract had highest inhibition for *Pseudomonas aeruginosa, Enterobacter, Streptococcus* that followed by ethanol and finally water extract. These results were agreed with [6] *for Pseudomonas aeruginosa, Enterobacter, Streptococcus for* acetone extractbut it's disagree for *E.coli, P. aeruginosa, K. pneumonia*. The results disagree with [12] results in which *Staphylocuss aureus, Klebsiella pneumonia*highest inhibition was to Acetone extract, ethanol and finally water extract. While, *Pseudomonas aeruginosa* and *E.coli* was to acetone, water and the ethanol had no effect for *E.coli*, but it was agree in *Proteus mirabilis*. The antibacterial activity of *Withania* Extract may be due they had high quantity of alkaloid which well known by their antibacterial according to [15] researchin which they used alkaloid as antibacterial.

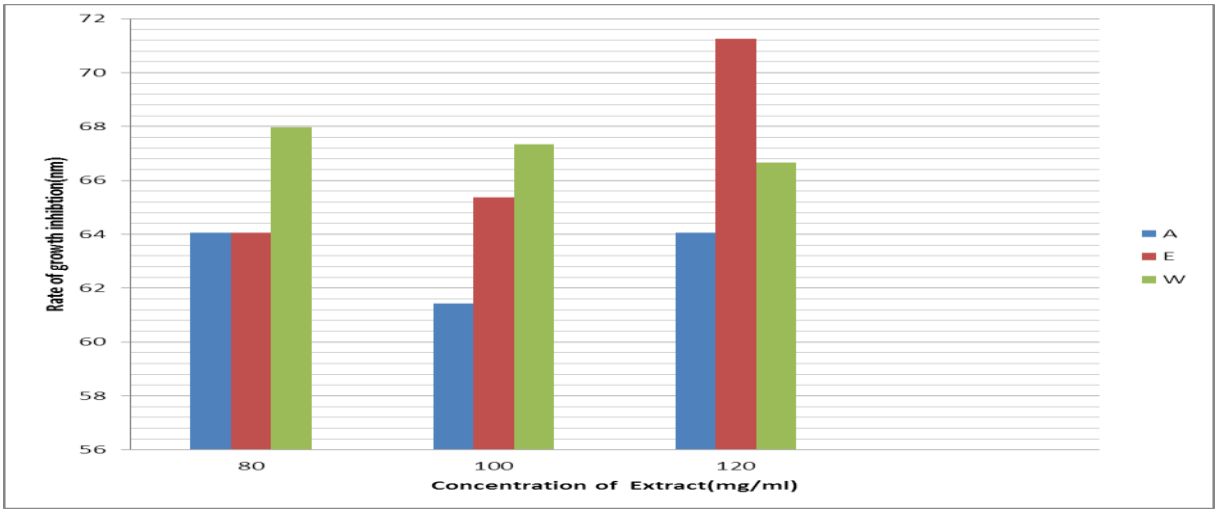
In vitro antifungal activity of Withania extracts

The acetone extract showed activity against *Trichophyton violaceum* by 100 and 120 mg/mlconcentration of extract but the Water and Ethanol show no activity even at higher concentration on the extract. 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 [4].

Organism	*Acetone (100-120)*		*Ethanol*	*Water*
Trichophyton violaceum	(100-120)L	(100-120)D	–	–
	1.3-3.0	1.5-3.4		

Cytotoxic effect of plant extracts on tumor cell line

The cytotoxic effect expressed by percentage of inhibition growth rates thatrepresent the cytotoxicity of plant extracts. Optical density of tumor cell line culture measured at transmitting wave's length of 492nm.



N=3, (A) acetone, (E) ethanol, (W) water.

Fig.(9) Cytotoxicity effect of Withania extracts.

Fig.(9) 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).Higher cytotoxic effect was to ethanol extract at 120 mg/ml concentration but the acetone extract was the lowest one and water extract was in the middle with probability ($P \leq 0.01$), Results agreed with [21] in which *Withania* water extract selective cancer cell killing activity, similar to the alcoholic extract of *Withania* leaves o the anticancer activity in water extract could be extremely beneficial for human consumption, *Withania* viability ranging between (32-83%).*Withania* extracts may had the same effect on normal cells, may have less on normal cells because [22] indicated the tumor cells differ in morphology than normal one and one of most important differences in that tumor cells highly express receptors on their membranes than normal one which allow attachment of different compounds. In addition, the DNA of tumor cell found in relaxant shape, and the DNA molecule was found in unstable figure because the H-bond which connect both strand of DNA. This makes it easy for component to interfere or to be associated with both strand of DNA while DNA of normal cells 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. The anti-cancer activity of *Withania* may due to Withaferin-A which had proved as potent anti-cancer [19].

Conclusions

Different groups of the active compounds have detected in *Withania* ethanol, acetone and water extracts involve: alkaloids, glycosides, saponins, flavonoids, fixed oil are present in ethanol, acetone and water extracts while terpenes was absent in water, protein was absent in acetone, Tannins were not found in all types of extract. All types of *Withania* extracts contained two type of Alkaloids (Withanolide-A and Withaferin-A), seven types of Flavonoid (Naringenin, Catechin, Luteolin, Hesperetin, Kaempfero, Apigenin and Naringin) and two types of Saponins (Sitoindosides VII and Sitoindosides VIII) appeared as different peaks. *Withania* extracts contained numbers of active group detected by FTIR. All three types of *Withania* extracts (Acetone, Water and Ethanol extract) showed activity on the following bacteria (*Escherichia coli, Enterobacter sakazakii, Klebsiella pneumonia, Staphylocuss aureus, Staphylocuss epidermis, Streptococcus pyogenes, Proteus mirabilis, Pseudomonas aeruginosa*). Only Acetone extract showed activity on fungus (*Trichophyton violaceum*). *Withania* extracts had cytotoxic effect onHepG2 cell line.

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الخلاصة

نبات سم الفراخ المستعمل بالطب الشعبي. تم استعمال عشرة غرامات من مسحوق الاوراق للحصول علي المستخلص المائي والكحولي (الاستون والايثانول). مستخلصات سم الفراخ (المائي، الإيثانولي والاسيتون) أخضعت للتحليلات الكيميائيه لتحديد المواد الفعالة الموجوده فيها والتي اثبت علي ان قلويدات، غليكوزيد، صابونين، فلافونيدات، الدهون الثابتة وجدت في الجميع لكن لم نعثى علي التربين في المستخلص المائي, ولاعلي البروتين في الأسيتوني ولم يتم العثور على التانين في الأنواع الثلاثة. تحليل [HPLC] أثبتت علي ان كل انواع المستخلصات (الماثي والكحولي) تحتوتي علي نوعين من القوليدات (Withanolide-A and) Withaferin-A, سبعة أنواع من مركبات الفلافونويد (Catechin, Luteolin, Hesperetin, Kaempfero, ApigeninNaringenin, وNaringin) وهناك نوعان من الصابونين (Sitoindosides VII, Sitoindosides VIII) ظهرت كقمم مختلفة. كذالك تم الكشف عن الكروبات الفعاله الموجده في مستخلصات سم الفراخ عن طريق (FTIR). الفعاليه ضد المايكروبيه للمستخلصات تم دراستها علي عدد من البكتريا. المستخلص الاسيتوني كان المستخلص الوحيد الذي له تأثير في الفطريات (*Trichophytonviolaceum*). مستخلصات سم الفراخ كان لها أثر سمي علي خلايا HepG2 أعلى الآثار السامة للخلايا تنتمي للمستخلص الإيثانولي بتركيز 120 ملغ/ مل لكن المستخلص الأسيتوني هو الأدنى اما بالنسبة للمستخلص المائي كان في الوسط.