

PREPARATION OF *JUNIPERUS* EXTRACT AND DETECTION OF ITS
ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY

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ABSTRACT

This study was aimed to produce silver nanoparticles by fruits extract of *Juniperus phoenicea* and comparison between the ethanolic extract and nano extract through the antioxidant, antibacterial, anti-parasite and cytotoxicity against prostate cancer. The synthesis of silver nanoparticles was shown by many characterizing techniques: UV, FTIR, XRD and SEM. Nano extract exhibited a higher antibacterial, antioxidant, antiparasite compared to ethanolic extract and higher cytotoxicity activity. The nano extract exhibited higher antibacterial activity compared with antibiotics. Finally, we study the toxicity of *J. phoenicea* by Inhibition of RBC hemolysis by H₂O₂, the results exhibited the highest inhibition activity of *J. phoenicea* against H₂O₂. The nanoextract of *J. phoenicea* can be used effectively in the production of potential antioxidant, antiparasite antimicrobial and anticancer.

Keywords: eco-friendly, silver nanoparticles, *Juniperus phoenicea*, anticancer, antioxidant, antimicrobial.

مايخ وآخرون

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تحضير مستخلص العرعر والكشف عن فعاليته ضد مايكروبييه ونشاطه المضاد للاكسدة

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مدرس

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المستخلص

هدفت الدراسة الحالية لتكوين جسيمات الفضة النانوية بواسطة مستخلص ثمار العرعر الفينيقي والمقارنة بين المستخلص الكحولي والنانوي من خلال النشاط المضاد للاكسدة, المضاد للبكتريا, المضاد للطفيليات والسمية الخلوية ضد سرطان البروستات. تم التأكد من تخليق الجسيمات النانوية من خلال استعمال تقنيات تشخيصية متعددة مثل تقنية طيف الامتصاص للاشعة فوق بنفسجية UV, وتقنية التحليل الطيفي للاشعة تحت الحمراء FTIR, تقنية حيود الاشعة السينية XRD والمجهر الالكتروني الماسح SEM. اظهر المستخلص النانوي له فعالية اكثر من المستخلص الكحولي في النشاط المضاد للبكتريا, المضاد للاكسدة, المضاد للطفيليات والسمية الخلوية. اظهر المستخلص النانوي فعالية عالية ضد البكتريا مقارنة بالمضادات الحيوية. اخيرا تم اختبار سمية نبات العرعر وظهر تأثير نبات العرعر في تثبيط عمل بيروكسيد الهيدروجين المحلل لكريات الدم الحمراء. يمكن استخدام المستخلص النانوي لثمار العرعر كعامل قوي مضاد للاكسدة والطفيليات والبكتريا ومضاد للسرطان.

الكلمات المفتاحية: طريقة صديقة للبيئة, جسيمات الفضة النانوية, نبات العرعر الفينيقي, مضاد للسرطان, مضادة للاكسدة, النشاط المضاد مايكروبي.

INTRODUCTION

Plants were used a source of treatment diseases in most parts of the world (6, 9). The genus *Juniperus* followed to Family Cupressaceae (1, 10). *Juniperus* is a great antioxidant fruit, which is used in some popular medicinal systems to treat a diversity of diseases such as gout, arthritis and rheumatism (1). New studies were observed the inhibitory action of *Juniperus* against *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* *Bacillus cereus*, *Cornybacterium* spp and with an extended panel of pathogenic bacteria (11,24). Plant parts such as bark, seed, stem, fruit, and leaf extracts have been successfully used for synthesis of nanoparticles (3). Silver nanoparticles have been used extremely due to their potent, antitumor, antifungal, and antibacterial activity (16). The silver nanoparticles have been widely used in preservation, cosmetics, food packaging and medicine (3, 16). In the group of medicinal plants, the *Juniperus* have medicinal properties and taken into account for the synthesis of silver nanoparticles (1). The synthesized silver nanoparticles are characterized by SEM, XRD, UV and FTIR analysis. Plants have an amount of poly flavonoids and phenols, which used to the reduction of silver ions. These poly flavonoids and phenols are used as antioxidant and antimicrobial agents by the plants to protect themselves from several pathological conditions (11, 24). Many plants are familiar as sources of natural antioxidants and thus play an essential role in the chemoprevention of aging and diseases (1, 10). Between the plants studied to date, one display enormous potential is the *Juniperus phoenicea*. The present study was aimed to produce silver nanoparticles from fruits extract of *Juniperus phoenicea* and comparison between the ethanolic extract and nano extract through the antioxidant , antibacterial ,antiparasite and cytotoxicity against prostate cancer cell line in vitro, also examiend the combined effect of some antibiotics and nanoextract against some pathogenic bacteria. Furthermore, was study the toxicity of *Juniperus phoenicea* by Inhibition of RBC hemolysis caused by H₂O₂.

MATERIALS AND METHODS

Juniperus Fruits and the extraction

Dry fruits of *Juniperus* were collected, washed in distilled water and dried by air-drying. Fifty gms of powdered fruits were extracted with ,ethanol 300 ml (70%) for 7-14 hrs by Soxhlet apparatus method. Then the extract was evaporated to dryness and stored in a freeze until used (3).

Microbial strains

The pathogens strains were provided by microbiology lab, science department, university of technology in Baghdad these: *Pseudo. aeruginosa*, *staph. aureus*, and *Candida albicans*.

Silver nanoparticles

Ten milliliters of the extract were mixed with 90 mL of 0.01 mmol/mL aqueous AgNO₃ and display to sunlight for 1 h. A modification from yellowish to reddish brown color was noticed (3).

Antibacterial activity

The ethanolic and nano extract of *Juniperus* were established through, agar well diffusion technique against *S. aureus*(G+), *C. albicans* (yeast), and *P. aeruginosa* (G-). the clean cultures of the microbes were subculture on MHA .the quantity of each bacterial suspension was balanced to that of 10⁶ CFU/ml standardized by 0.5 McFarland standard and used as the inoculums for performing agar well diffusion test, Then wells were formed on plates in gel puncture. 50 µl, of sample 20,40,60 and 80mg, concentration, one-to-one was transferred on to each well on all plates then, incubation {at 37 °c}for 24 hours, and the diameter of effective zone was estimated in mm then,The tests plates were done in triplicate (24).

Antioxidant activity

Estimation of antioxidant activity (radical scavenging activity) was showed by D.P.P.H:(1,1-Diphenyl-2-picrylhydrazyl). D.P.P.H 4.3mg has thawed 3.3 ml in ethanol then, its saved safely from light with cover the test tubes using aluminum foil. The next concentrations of *Juniperus* extracts were prepared 40,80,160 µg/mL with three replicates per concentration 25 µL of each concentration was full with an amount of ethanol until it became 500 µL and then over with 500 µL of DPPH and incubated at 37 ° C

for 30 min. The absorbance at 517 nm was then measured through the next calculation (15).
Scavenging effect % = [(A_{OD} – BOD)/ A_{OD}] * 100

A_{OD} = Optical density control, bod= optical density sample

Accounts of nanoparticles Created nanoparticles were checked by UV, FTIR, SEM, and XRD. UV-Vis spectral analysis and tested by using spectrophotometer (TechcompUV2300). FTIR, an analysis showed the possible naturally appear organic molecules. XRD sizes of the Ag nanoparticles solution drop-coated on glass and prepared on a Shimadzu XRD-6000 model with (40 kV 30 mA with Cu k α radiation). The crystallite domain size was intended from the width of the XRD peaks and by the Scherer formula : ($D = 0.94 \lambda / \beta \cos \theta$) (14).

Maintenance of cell cultures

The prostate cancer cell line was got by the Iraqbiotechcellbank unit then, kept in RPMI-1640 improved by 100 μ g/mL streptomycin, 100 units/mL penicillin, and 10% Fetal bovine. We passaged the cell by, Trypsin EDTA ,reseeded in 50%, convergence double in a week then, incubated on 37 °C(20).

Cytotoxicity Assays

Cytotoxic effect was determined by MTT assay. MTT, cell activity evaluation was regulated on 96well plates. We seeded cell lines in, 1×10^4 cells/well. After 24, cells were held with the proved complex. Cell activity was tested after 72 hour by discard the medium, addition 28 μ L of 2 mg/mL from MTT, and incubation for 1.5 hour at 37 °C. After discard the M.T.T solution, the crystals often residual in the wells, were solubilized with 130 μ L of Dimethyl Sulphoxide by 37 °C incubation for 15 min with shaky (12). The absorbance was estimated on a micro plate reader at 492 nm. The test was worked in triplicate. The effective level was determined by the following calculation:-

Inhibition rate = [A-B/A] *100

A and B are the optical density of control and test,

Antiparasite activity

MTT assay was used to estimate the anti-parasitic effects of ethanolic and nano extract of *Juniperus* against amastigote forms of *Leishmania tropica*. In the beginning, 100 μ L

of the amastigote (106 cells/mL) harvested from a logarithmic growth phase were additional to 96-well tissue culture plate by RPMI medium 1640. After that, 100 μ L of tested material was added to every well and incubated at $25 \pm 1^\circ\text{C}$ for 48 hrs then, 10 μ L of MTT solution was added per well and the microtiter plate was incubated for 4 hrs at 25°C. The media was then detached and 100 μ L of DMSO solution was added to solubilize the formazan crystals. Amastigote was cultured at whole medium with no drug as the positive control and whole medium with no amastigote and drugs as blank. The absorbance was measured for all William 492 nm using an ELISA reader (18). The cytotoxicity can be designed by the following parameters. Inhibition rate % = [(A - B/ A)]X 100 Where A is the optical density of control and B is the optical density of treated sampl.

Inhibition of erythrocyte hemolysis by hydrogen peroxide

Venous blood was collected from a healthy person and then supplied into heparinized tubes. the blood was centrifuged in 1000 rpm to 15 min, then washed 3 times by phosphate buffered saline (0.2 M, pH 7.4), then suspended in the similar buffer to the desired hematocrit level. A portion of erythrocyte 200 μ L was transported into a test tube with 100 μ L of hydrogen peroxide (100 μ M) for inducing hemolysis. The tested compounds 200 μ L were added with softly swirled and incubated for 3hr at 37 °C. PBS 8mL was added then the solution was centrifuged on a speed 3000 rpm for 10 min. The absorbance of the contents was tested at 540 nm (23). The inhibition of hemolysis was calculated to the following equation:

$Scavenging = [\% \text{ Absorbance of control} - \text{Absorbance of sample} \setminus \text{Absorbance of control}] \times 100\%$

RESULTS AND DISCUSSION

UV analysis

The silver nanoparticles biosynthesis was checked using UV- visible spectroscopy. The fruit *Juniperus* extract was yellowish in next color, adding of AgNO₃ solution and display to the sun for one hour, then turned brown (Fig. 1). the Ag external Plasmon resonance band at 413 nm. (Fig. 2).

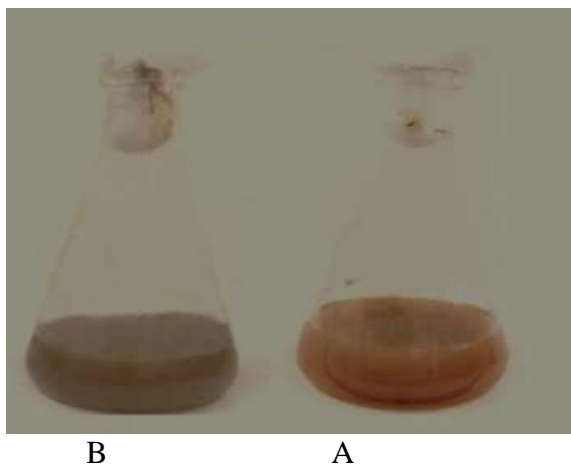


Figure 1. formation of silver nanoparticles by *Juniperus* A- $AgNO_3$ with *Juniperus* B- Color altered by sunlight

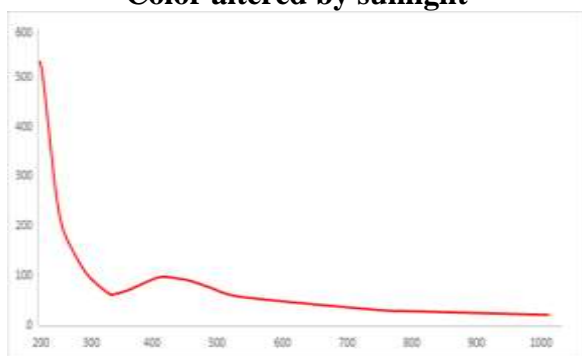


Figure 2. UV-visible spectrums of *Juniperus* nano extract

Character of nanoparticles by SEM

The SEM, explanation the shape of nanoparticles, The Fig.3, displays spherical form.

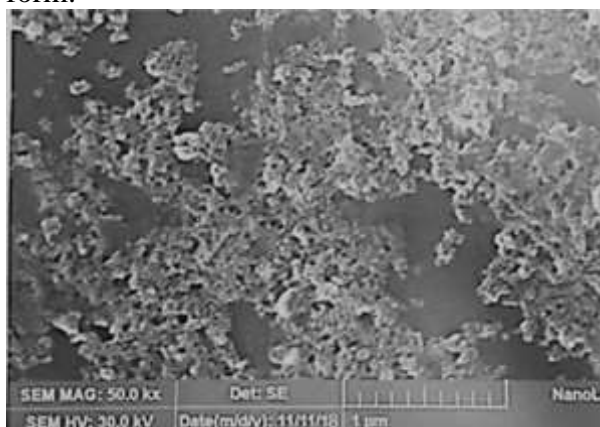


Figure 3. SEM image for *Juniperus* nano extract

X-Ray diffraction XRD analysis

X-ray diffraction (XRD) is a popular analytical technique which has been used for the analysis of both molecular and crystal structures. The XRD shape showed three intense peaks (33, 38 and 40) in the whole spectrum of 2 θ value ranging from 20 to 70 and indicated that the

structure of silver nanoparticles is face-centered cubic (FCC).These are corresponding to 111 and 200 planes for silver, respectively. The average grain size of the silver nanoparticles formed in the bio reduction process was determined using Scherer’s formula and was estimated at 38nm.

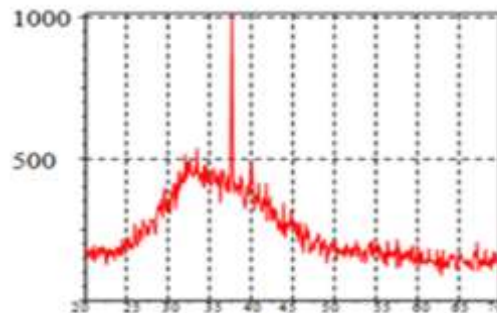


Figure 4. X-ray diffraction analysis for *Juniperus* nano extract

FTIR for ethanolic and nanoextract

The FTIR is give the active groups. The ethanolic extract of *juniperus* displayed the bands at 3390.91 cm^{-1} due to of phenolic OH. The band at 2928.11 cm^{-1} as a result of C-H. The band at 1708.90 cm^{-1} as a result of C=O. The band at 1619.61 due to C=C. The band at,1516.19, 1454.28,1342.51 cm^{-1} as a result of C-H .the band at 1147.60,1033.80, 1232.52 as a result of C-O (4), while the FTIR for nano extract exhibited the same result except for absence the bands at 1147.60 because of reduction of silver ions(4)as shows in Fig(5,6).

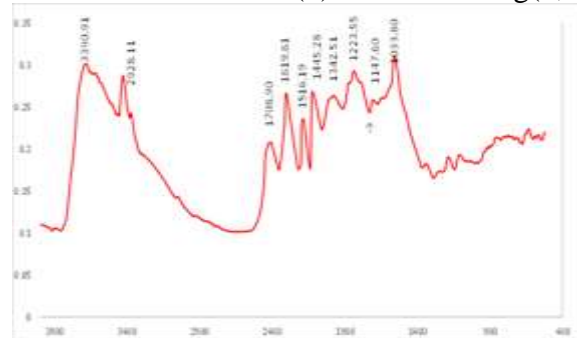


Figure 5. FTIR spectrum for *Juniperus* ethanolic extract

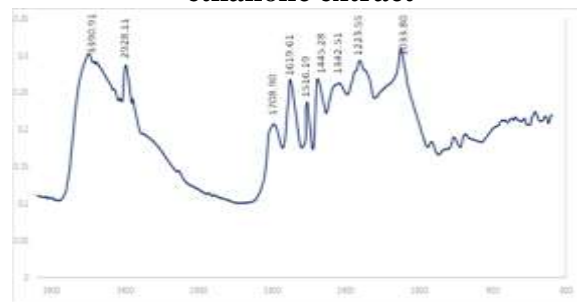


Figure 6. FTIR spectrum for *Juniperus* nano extract

Antimicrobial test of an ethanolic and nano fruit extract of *Juniperus*

Antimicrobial activity of *Juniperus* fruit extract (ethanolic and nanoextract) against three kinds of microorganisms was estimated. The nanoextract showed larger antimicrobial activity compared to ethanolic extract, (Fig,7, 8). The ethanolic extract effect on the growth of *p. aeruginosa*, *S. aureus*, and *C. albicans*, by 14mm ,13mm and 10mm, respectively , While the nano extract 20mm, 19mm and 11mm respectively (Fig. 7,8).

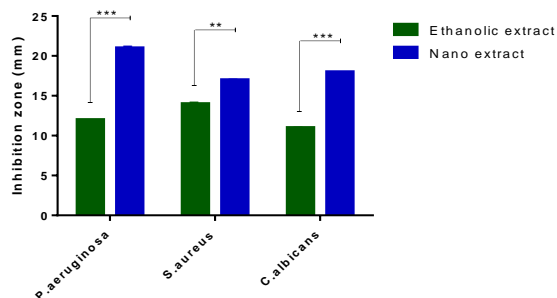


Figure 7. Activity of ethanolic and nanoextract on the growth of bacterial strains, the values represents the Mean \pm S.E **P<0.01, *P< 0.001**

The antimicrobial activity of the silver nanoparticles could be credited to the existence of specific phytochemical components of the ethanolic extract as shown by the FTIR spectra, such as flavonoids, phenolics, tannins, and alkaloids are known to have an anti-inflammatory, antifungal, and antimicrobial effect (19). The nano extract showed larger antimicrobial activity compared to ethanolic extract and the effect of nano extract was more signal against bacteria G-ve than bacteria G+ve, could be due to the changes of the cell wall structure between G-ve and G+ve bacteria(11,24). The studies recommend that the silver ion can effect on phosphorus and sulfur, thus Ag⁺ can relate with phosphorus moieties in DNA and rises DNA modification furthermore, Microbial cell membrane having an abundance of sulfur proteins, silver ion can rejoin by sulfur having. amino acids and affects microbial cell viability (13,25).

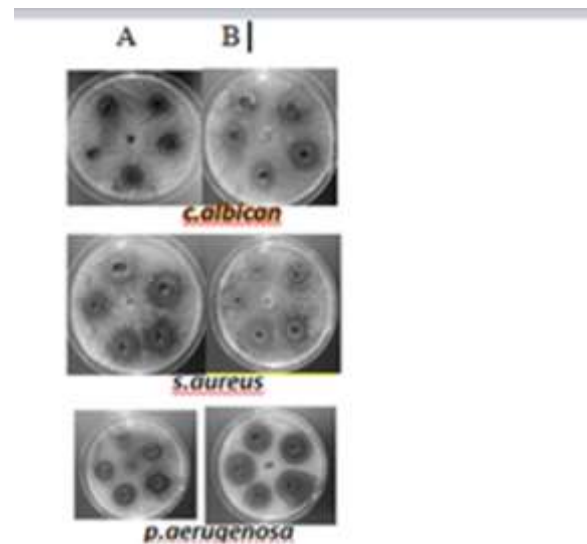


Figure 8. Inhibition zone effects of *Juniperus*. A- ethanolic extract and nano extract. 10, 20, 40, 60, and 80 mg per ml, concentration respectively, A: ethanolic extract B: nano extract

Antimicrobial activity of antibiotics

Disc diffusion method was used for estimating the synergistic effect of nano extract of *Juniperus* with antibiotics against, bacteria, the *Juniperus* nanoextract was diverted with antibiotics Amoxicillin and Gentamycin concentration at 10 μ g/ml. Results in table (1) shows a combination of nanoextract with antibiotics exhibited synergistic effect against tested bacteria, A,A amoxicillin, with nano extract displayed a rise in its activity: *P.aeruginosa* before (R, resistance) to (8mm), while *S.aureus*. before (R) to (12mm), G. Gentamycin with nano extract showed an increase in its antimicrobial against, *P.aeruginosa* from (R) to (9mm). While with *S.aureus* from (R) to (10mm). This synergistic result, due to the reaction between antibiotics and nanoparticles, The differences in the cell wall building between gram positive and gram negative bacteria could be due to describe why gram positive bacteria extra sensitive than gram negative bacteria (28). The gram negative have an outside polysaccharide sheath holding the building all polysaccharide mixtures this indications the sheath to high resistible for lipophilic solutes, but gram positive, have one an outer peptidoglycan layer (28). There are several hypotheses could be describe the apparatuses of the synergistic effect, Gentamycin and amoxicillin can destroy bacteria. If bacteria have resistance, another antibacterial agent would destroy the

bacteria. The synergistic effect affected by a rejoinder among antibiotic and nanoparticles (17, 21). Antibiotic particles have numerous effective groups like hydroxy and amido collections. These collections rejoin by nanoparticles through chelation. Antibiotic particles bind each other by van der Waals communication, also further weak bonds(17) The antimicrobial collections come into being, which is join of a nanoparticles core and the antibiotic particles ,When antimicrobial particles effect on the surface of bacteria they reason damage. Therefore, the process of antimicrobial particles making is really that of, increasing the antimicrobial agent's concentration (17, 21, 28)

Table 1. Antimicrobial activity of antibiotics and combination of nano extract with antibiotics

Microorganizime	Inhibition Zone (mm)			
	A	A+N	G	G+N
<i>Staph. aureus</i>	R	12	R	10
<i>Pseudo aeruginos</i>	R	8	R	9

a
N,nanoextract:A,A moxiciline:G,GentamycinR. Resistance

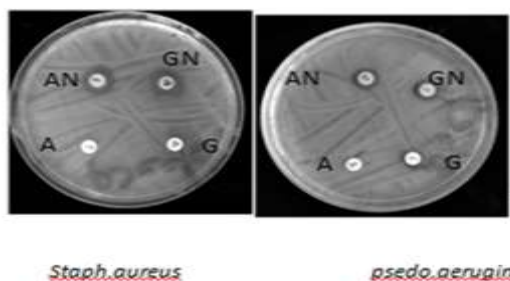


Figure 9. Combined effect of antibiotics and nano extract of *Juniperus* against. Some pathogens. N, nano extract, A, A moxiciline, G, Gentamycin

Antioxidant activity of silver nanoparticles

Antioxidant activity of silver nanoparticles was evaluated through DPPH, free radical scavenging test with Vitamine C as positive control. DPPH (1, 1-Dpicryl- hydrozyl) is a stable complex, takes hydrogen or electrons from Ag nanoparticle (25).The outcomes exhibited effective free radical scavenging by Ag nanoparticles and its found that the activity increased with increasing concentrations of AgNPs Fig.(10). AgNPs are recommended to act as electron donors rejoining with free radicals to change them to new stable

products, which can terminate radical chain reaction. Furthermore, the reducing power of AgNPs connected well with the radical scavenging activity (2, 22). the antioxidant activity of the silver nanoparticles could also be credited to the existence of specific phytochemical components of the ethanolic extract such as phenols, tannin and flavonoids are used for antioxidant(5).

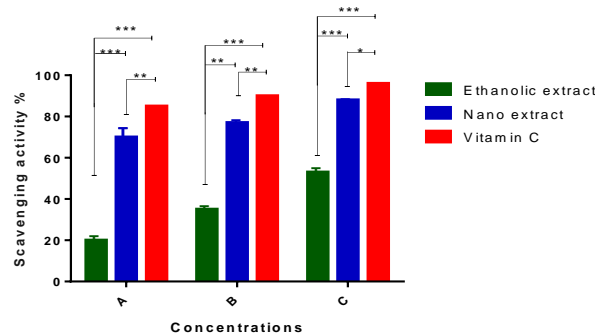


Figure 10. DPPH scavenging activity of ethanolic and nano extract of *juniperus*. (A) 40 µg/ml (B) 80µg/ml (C) 160µg/ml. The Mean ± S.E **P<0.01, *P< 0.001**

Anti parasitic activity

Investigation of the cytotoxic potential of ethanolic and nano extract of *Juniperus* was showed on amastigote phase of *Leishmania tropica*. In Fig.11 exhibite the result of cytotoxicity activity. The lowest concentration that causes cytotoxic could be in the 1.25 µgmL. Cytotoxic activity of *Juniperus* increased with increasing concentration. The ethanolic extract of *Juniperus* presented little inhibiting effects compare with nanoextract of *Juniperus* displayed highly effective on the parasite, that the cytotoxicity rate reached to 85.65%

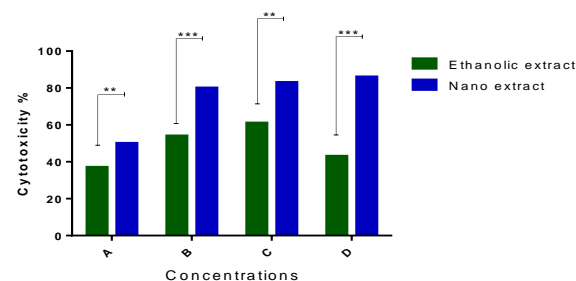


Figure 11. Cytotoxicity effect of *Juniperus* on *L.tropica* . (A) 1.25 µg/ml (B) 2.5 µg/ml (C) 5 µg/ml (D) 10 µg/ml. the Mean ± S.E **P<0.01, *P< 0.001**

Antileishmanial properties could also be credited to the existence of phenols, flavonoids, alkaloids diterpenes and saponins

in the *Juniperus* plant(20).The results confirmed that the nanoextract of *Juniperus* had inhibitory effects on *Leishmania* parasites. Several mechanisms have been suggested that nanoparticles are able to induce reactive oxygen species and can destroy pathogenic microbes through a method called respiratory burst mechanism(13,25).The oxidative damage affected by ROS producing and inhibiting the enzyme that is involved in the process of ROS making and affection on *Leishmania* persistence. The use of nanoparticles as antiparasite agents will act as a big reservoir then will be responsible for an non-enzymatic source of ROS and destroy the invaded parasite(19,29)

A study of anticancer activity of *Juniperus* by MTT assay

The cytotoxic effect of *Juniperus* nano extract against prostate cancer cell line (VCaP) were examined by MTT assay. The nanoextract exhibited the great level of cytotoxic activity with the IC₅₀ values 40.38 in concentration 50 and 100 µg/mL, compared to the concentration of 6.25,12.5,25 µg/ml respectively (Fig12,13). Several phytochemicals, e.g., alkaloids, lignans, phenols, flavonoids, terpenes and steroids have been established to possess prominent cytotoxic properties against cancer cells , detailed the anticancer properties of the flavonoids, which were presented to inactivate carcinogen, arrest the cell cycle, inhibit proliferation, induce apoptosis and differentiate, Prevent oxidation then reversal multidrug resistance (7,26). Earlier readings revealed that various polyphenolic and flavonoids complexes were existing in several *Juniperus* species Especially, these collections of phytochemicals have been extracted from the berries and leaves of *J. phoenicea* and other *Juniperus* species grown up in altered countries (9,28,30). Podophyllotoxin, which is furthermore existing in *J. phoenicea* is a cytotoxic complex against cancer cell lines (1, 31). The cytotoxicity is facilitated through inhibition of microtubule development and this complex helps as a unique starting compound for the semi synthesis of anticancer treatments that are recognized to inhibit topoisomerase II such as teniposide etoposide, or etopophos. (7,26,27). The nano extracts of *J.* showed

strong antioxidant activity, determined by measuring the radical scavenging effect on DPPH which may be responsible for its cytotoxic effects.

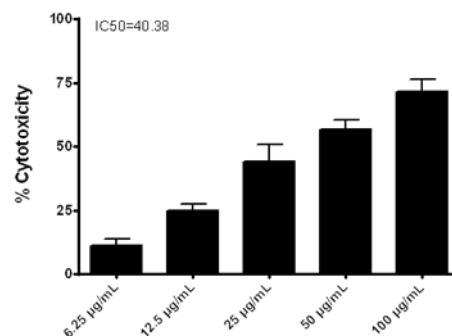


Figure12. Cytotoxic effect of *Juniperus* Nano extract on prostate cancer cell line



Figure 13. Cytotoxic effect of *Juniperus* nano extract on prostate cancer cell line

Inhibition of erythrocyte hemolysis by Hydrogen peroxide

Erythrocytes are a main target for the free radicals due to the high membrane concentration of polyunsaturated fatty acids and the oxygen transport related with redox active hemoglobin molecules, which are strong promoters of activated oxygen species (8). The principle of this test was determined by inhibited the act of hydrogen peroxide which crosses RBC membrane and acts on intracellular moiety and forming ferryl radical or hydroxyl radical through interacting with hemoglobin and initiates a series of reactions resulted in RBC lysis (haemolysis) and discharge hemoglobin in to the supernatant(30). Fig. 14 indicated that ethanolic extract of *Juniperus* exhibited slightly scavenging activity on H₂O₂ at concentration 1.25 mgmL⁻¹ that reached to 14%. The scavenging activity at concentration 10 µgmL⁻¹ exhibited better inhibition activity that the percentage reached to 68%. Because of the ability of *Juniperus* to trap ROS. H₂O₂ to cause damage in

erythrocyte membranes, thereby protecting them from hemolysis

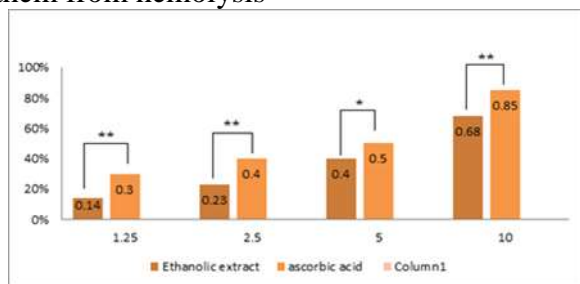


Figure 14. Scavenging activity of juniperus extract on H₂O₂

This study was exposed that silver nanoparticles can be synthesized in a simple method using *J. phoenicea* fruit extract. The XRD and SEM analysis exhibited the sizes and shape of the synthesized AgNPs. FTIR was used to recognize the biomolecules present in *J. phoenicea*. UV was examined to recognize the bioreduction of AgNO₃ to AgNPs. Nanoextract of *J. phoenicea* showed a higher antioxidant, antimicrobial, antiparasite activity compared to ethanolic extract alone. The combined effect of antibiotics and nanoextract exhibit synergism effect in most kinds of bacteria. The *J. phoenicea* nanoextract exhibited strong cytotoxic effects against prostate cancer cell line. *J. phoenicea* had no toxic effect on blood components. It could be concluded that *J. phoenicea* fruit extract can be used effectively in the production of potential antioxidant, antiparasite antimicrobial and anticancer for commercial application.

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