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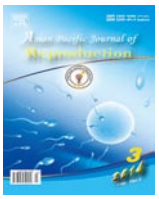

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Effect of different polarities leaves crude extracts of Omani *Juniperus excels* on antioxidant, antimicrobial and cytotoxic activities and their biochemical screening

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ABSTRACT

Objective: To prepare different polarities leave crude extracts of *Juniperus excels* (*J. excels*) and to determine their phytochemical screening, antioxidant, antimicrobial and cytotoxic activities. **Methods:** The phytochemical screening of different crude extracts was determined by well-established methods. The antioxidant activity was determined by free radical scavenging (2,2-diphenyl-1-picrylhydrazyl, DPPH) method. The antimicrobial activity was evaluated by agar disc diffusion method and cytotoxic activity was determined through brine shrimp lethality assay. **Results:** The phytochemical tests showed that alkaloids, flavonoids, saponins, steroide, triterpenoids and tannins are present in all leave crude extracts except chloroform crude extract do not contain tannins. The antioxidant results of crude extracts were found to be in the order of hydro alcoholic > chloroform> ethyl acetate >hexane. All leave crude extracts showed moderate antibacterial against gram positive and gram negative food borne pathogen bacteria. The cytotoxicity results revealed that hexane and chloroform extracts have killed all the shrimp larvae at the concentration of 500 μg/mL. **Conclusion:** More *in-vivo* and *in-vitro* studies along with detailed phytochemical investigations are needed in order to potentially use this plant in the prevention and therapies of some oxidative damage related diseases.

1. Introduction

Medicinal plant species represent a large source of new compounds that help for the preparation of new drugs. The therapeutic activity of plants is due to their biologically active polyphenolic compounds. Mostly flavonoids and phenolic acids which possess antioxidant, anti-lipoxygenase and anticancer activities[1, 2]. All developing countries and Gulf countries, most of the population still depends on folk medicine to treat serious diseases including cancers and various types of inflammations. *J. excels* is distributed in Balkan countries such as Turkey, Syria and Lebanon, Georgia, Armenia, Azerbaijan, Iran and Turkmenia. It is found in the mountain chains in Iran and on Al Jabel Al Akhdar, Al Jabel Al Koor and Jabel Shams in Oman. It is also found in Saudi Arabia. It survives under harsh climatic conditions of mountain rocky, and is considered as a unique precursor tree species in such habitats. Junipers are long-lived trees which sometimes live up to 2 000 years. It is belong to the Cupressaceae family. *J. excels* is a tall shrub or tree up to 20 meter tall with a trunk as large as 2 meter in diameter[1]. *J. excels* is evergreen and has two types of

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leaves. Seedlings develop needle-like leaves 8-10 mm in length, while adult trees grow 0.6-3 mm long scale-shaped leaves[4]. The major chemical components in the *J. excels* leaves were monoterpene hydrocarbons, sesquiterpene hydrocarbons and oxygen-containing sesquiterpenes[5].

The report indicates that the other major phenolic constituents found in extracts of *J. excels* species are lignans, coumarins, sesquiterpenes, abietane, labdane and pimarane diterpenes, flavonoids, biflavonols, flavone glycosides, and tannins[6]. The high concentrations of α-pinen (29.7%), cedrol (25.3%), α-muurolene (4.4%) and 3-carene (3.8%) were found in the leaves essential oil[6]. The authors reported that some diterpenes such as ferruginol (abieta-8, 11, 13-triene-12-ol) and sandaracopimeric acid (isopimara-8, 15-diene-18-oic acid) exhibited significant activity against food borne pathogen bacterial strains[7].

The tree is used as an antiseptic and parasiticide for animals in Turkey[4]. The leave boiled extract is widely used for asthma, dysmenorrhea, cough, bronchitis, common cold, jaundice and tuberculosis[7]. The raw leaves are used as an anti-bacterial and anti-parasit[8]. The therapeutic activity was reported for female the female cones used for Cardiac and nervous problems, diuretic, prostate, urinary problems, to relieve headache and fever[9]. In addition, they are applied for treating skin diseases[9]. Sometimes, it is used as pain reliever by covering the painful part of the body with paste of the leaves for about 4 hours a day until the pain is completely relieved[10]. Some people use it for relieving rheumatism pains, backache and tooth pain[10]. The potential uses of *J. excels* essential oil for aromatherapy, mood scents, scent masks, soaps and candles, cosmetics and fragrances, lotions and remedies[11].

2. Materials and methods

2.1. Chemicals

All organic solvents and acid were used in this experiment obtained Sigma-Aldrich Company, UK. The other necessary chemicals such as 2-diphenyl-1-picrylhydrazyl, (DPPH), sodium sulphate, sodium hydroxide, hydrochloric acid, sulphuric acid, acetic anhydride and ferric chloride for different test were obtained from BDH, Germany. Gram positive bacteria *Staphylococcus aureus* (*S. aureus*) and gram negative bacteria *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) were from Biological Department, College of Art and Science, University of Nizwa. Filter paper discs of diameter 6 mm were obtained from Whatmann Company. Nutrient ager and plastic Petri dishes were from Ghalau Chemie Company. Brine shrimp eggs (ARTEMIA CYSTS) were purchased from GOAQUA, Taiwan. Sea salt was obtained from Al-Qurum Muscat. All the glassware used in this present experiment was from Borosil, India.

2.2. Instruments

UV spectra were recorded on Thermo Spectronic spectrophotometer (Great Britain, UK, Model No. Biomate) Ultrospeck in methanol (λ_{max} in nm). Rotatory evaporator was used Yamato Rotary Evaporator, Model RE 801, Japan. Incubator used in this experiment obtained from Gen Lab Model: MINO/75F, Serial number: Y5K041.

2.3. Plant material

The leaves of *J. excels* sample was collected from Al Jabel Al Akhdar in Bahla, Nizwa, Oman, on 12th of November 2012 at 3.00-4.00 pm. The collected leaves were dried under shade at room temperature. The dried leaves were pulverized by using heavy duty blander machine.

2.4. Preparation of crude extracts

The powdered leaves of *J. excels* (296.92 g) were macerated in absolute ethanol solvent (2 500 mL) at room temperature for one week. The macerate was filtered using buncher funnel apparatus to give clear solution. The ethanol solvent was evaporated by using rotatory evaporator to give semi solid mass. The semi-solid mass was suspended in ethanol-water (1:1) and then finally extracted successively with hexane, chloroform and ethyl acetate. All solvents were removed under vacuum using rotatory evaporator.

2.5. Phytochemical screening

2.5.1 Identification of alkaloids

Small amount of each crude extract of *J. excels* (0.05) was dissolved in chloroform (10 mL). Fresh Mayers reagent (2 mL) was added to it. A creamy precipitate was obtained immediately that shows the presence of alkaloids.

2.5.2. Identification of flavonoids

The stock solution of each extract (1 mL) and few drops of dilute sodium hydroxide solution were added. An intense yellow color appeared. It becomes colorless on addition of a few drops of dilute hydrochloric acid. This indicate the presence of flavonoids.

2.5.3. Identification of saponins

1 mL of the stock solution was diluted with (10 mL) distilled water and shaken by hand for 15 minutes. The formation of

foam layer in the test tube showed the presence of saponins.

2.5.4. Identification of steroid

1 mL of the stock solution was dissolved in chloroform (10 mL) and an equal volume of concentrated sulphuric acid was added carefully to the test tube by sides. The upper layer turn into red color and the lower layer showed yellow with green fluorescence, means the presence of steroids.

2.5.5. Identification of triterpenoids

Small amount of the crude extract (5 mg) was dissolved in chloroform (2 mL) and then acetic anhydride solution (1 mL) was added to it. One milliliter of concentrated sulphuric acid was added carefully to the solution. The formation of reddish violet colour shows the presence of triterpenoids.

2.5.6. Identification of glycosides

2 mL of the stock solution and hydrochloric acid was added to it. The excess of HCL was neutralized with NaOH solution. Few drops of Fehlings solution A and B added. The occurrence of red precipitate shows the presence of glycoside.

2.5.7. Identification of tannins

About 1 mL of each stock solution was mixed with water and heated on water bath, filtered and ferric chloride added to the filtrate. The dark green solution indicates the presence of tannins.

2.6. Radical scavenging activity using DPPH method

The evaluation of antioxidant activity by free radical scavenging method was determined of different crude extracts described by Blois[12] with minor modification. Five concentrations (12.5, 25, 50, 100 and 200 μg/mL) were prepared for each crude extract (hexane, chloroform, ethyl acetate and hydro-alcoholic). Four milliliter of each concentration was placed in a test tube. One milliliter of 0.1 mM methanol solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) was added to the test tube and shaken vigorously. All the test tubes were kept in a dark place at room temperature for 45 min. The same procedure was followed for blank sample but without adding extract. The samples absorbance were measured at fixed wavelength 517 nm by using UV spectrophotometer. The activity of the crude extracts samples was estimated as the inhibition percentage and was calculated by using the following formula.

2.7. Antibacterial activity assay

The antibacterial activity was carried out by disc diffusion method[13]. The prepared crude extracts such as hexane, ethyl acetate, chloroform and hydro alcoholic extracts from *J. excels* leave samples were subjected to serial dilution technique by using dimethyl sulphoxide (DMSO) solvent to give the final concentration of 2 mg/mL, 1 mg/mL, 0.5 mg/mL and 0.25 mg/mL solutions. Then the different concentration crude extracts solutions were filtered by using 0.45 μm Millipore filter paper for sterilization to avoid any contamination. The controls were prepared for this experiment by using the same solvent to dissolve the crude extracts from guava leaves. The prepare each concentration of crude extracts of guava samples was tested for antimicrobial activity against *S. aureus*, *E. coli* and *P. aeruginosa* on nutrient agar plates using disc diffusion method. Whatman filter paper discs (6 mm diameter) were prepared in the laboratory and the sterile paper discs were impregnated with methanol or ethanol. The paper discs were put on different concentration of crude extracts of guava samples individually and placed on the inoculated agar plates. Finally the prepared agar plates were incubated using incubator at 37 ℃ for 27 hours. After incubation, the determination of antibacterial activity was measure by the scale in the diameter of the zones of inhibition against the three tested bacteria. Repeated each method in this experiment was three times.

2.8. Cytotoxicity activity

The cytotoxicity activity of different crude extracts from *J. excels* was determine by brine shrimp lethality method. The brine shrimp eggs were hatched at covered chamber of duo compartment plastic container with sea water for 24 hours. After hatching, the active nauplii were separated from the eggs and used for cytotoxicity activity. Four grams of different polarities plant crude extract samples were placed in a vial and dissolved in 100 μL of dimethyl sulfoxide (DMSO). Series of concentrations were prepared by serial dilution technique with DMSO. From each of these test solutions 50 μL were added to pre-marked test tubes containing 5 mL of sea water and 10 nauplii. The final concentration of the samples in the test tubes were 10, 100, 250, 500, 750 and 1000 μg/mL for six dilutions. Five millilitres of seawater was added to each test tube containing 10 brine shrimp nauplii. After 24 hours, the number of surviving nauplii in each test tube were counted using magnifying glass and recorded. The percentage of lethality of brine shrimps was calculated for each concentration of sample.

2.9. Data analysis

LC_{50} values and 95% confidence intervals of each sample were generated by Finney probit analysis of the percent mortality data using a computer program, EPA probit Analysis Version 1.5 obtained from US Enviromrntal

Protection Agency Website[15].

3. Results

The crude extract was obtained by maceration method with absolute methanol. The ethanol crude extract was defatted with equal volume of water and ethanol and successively extracted with hexane, chloroform and ethyl acetate. The percentage of extraction yield were listed as following: Absolute ethanol: 33.04%, Hexane: 5.12 %; Chloroform: 10.57 %; Ethyl acetate: 13.71 %; Hydro-alcoholic: 21.27 %. The phytochemical screening results are shown in Table 1. The hexane, ethyl acetate and hydro-alcoholic crude extracts *J. excels* showed the presence of alkaloids, flavonoids, saponins, steroids, tannins and triterpenoids. The chloroform extract revealed the presence of all above mentioned chemicals except tannins. However, all extracts did not show any positive test for glycosides.

Table 1
Phytochemical screening of hexane, chloroform, ethyl acetate, and hydro alcoholic crude extracts of *J. excels*.

Crude extracts	Hexane	Chloroform	Ethyl acetate acetate	Hydro alcoholic
Alkaloids	+ve	+ve	+ve	+ve
Flavonoids	+ve	+ve	+ve	+ve
Saponins	+ve	+ve	+ve	+ve
Steroide	+ve	+ve	+ve	+ve
Triterpenoids	+ve	+ve	+ve	+ve
Glycosides	−ve	−ve	−ve	−ve
Tannins	+ve	−ve	+ve	+ve

Free radical scavenging activity of different polarities leaves crude extracts of *J. excels* was tested using DPPH method and the results are presented in the Figure 1. In this study, the four leaves crude extracts of *J. excels* were able to decolourise DPPH. The free radical scavenging potentials of the leaves crude extracts were found to be in order of hydro-alcoholic extract>chloroform extract> ethyl acetate extract >hexane extract (Figure 1).

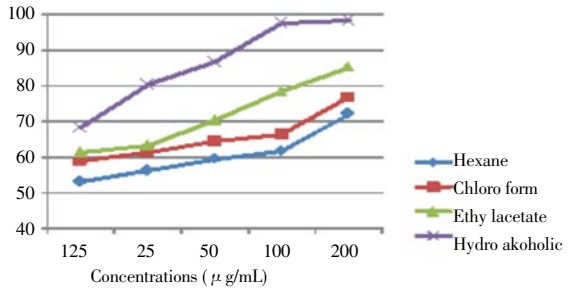

Figure 1. Antioxidant activities of crude extracts of the leaves of *J. excels*.

The *in vitro* antimicrobial activity test of the ethanol crude extract and ethanol derived subtraction of *J. excels* resulted in a range of growth inhibition patterns against food borne pathogenic microorganisms. The leave crude extract exhibited antibacterial activity against one gram positive (*S. aureus*) and two gram negative (*E. coli* and *P. aeruginosa*) bacteria at the concentration of 2 000 μg/mL, 1 000 μg/mL, 500 μg/mL and 250 μg/mL dilution with DMSO. Amoxicillin was used as positive control. All the four extracts of *J. excels*

Table 2
Antimicrobial activity of different leaves crude extracts of *J. excels* against *E. coli*, *P. aeruginosa* and *S. aureus*.

Crude extracts	Concentration (mg/mL)	*E. coli* (mm)	*S. aureus* (mm)	*P. aeruginosa* (mm)
Hexane	2	nd	13.00±0.11	8.00±0.32
	1	nd	8.00±0.07	7.00±0.29
	0.5	nd	7.00±0.34	6.00±0.11
	0.25	nd	6.00±0.27	6.00±0.14
	Standard	24.00±0.10	29.00±0.21	nd
Chloroform	2	8.00±0.15	8.00±0.22	10.00±0.14
	1	9.00±0.22	8.00±0.15	9.00±0.45
	0.5	7.00±0.17	7.00±0.14	7.00±0.19
	0.25	7.00±0.23	nd	7.00±0.22
	Standard	24.00±0.10	29.00±0.21	nd
Ethyl acetate	2	7.00±0.41	11.00±0.14	10.00±0.17
	1	7.00±0.21	8.00±0.21	7.00±0.14
	0.5	6.00±0.26	8.00±0.23	7.00±0.44
	0.25	nd	7.00±0.11	6.00±0.22
	Standard	24.00±0.10	29.00±0.21	nd
Hydroalcoholic	2	nd	9.00±0.32	7.00±0.21
	1	nd	6.00±0.34	7.00±0.13
	0.5	nd	6.00±0.21	6.00±0.27
	0.25	nd	nd	nd
	Standard	24.00±0.10	29.00±0.21	nd

have shown antibacterial activity against all three bacteria within the range 0-13% as shown in Table 2.

Hexane, chloroform and ethyl acetate extracts have displayed activity against the brine shrimp larvae. Mean percent mortalities of shrimp larvae exposed to different leaves crude extracts of *J. excels* are shown in Table 3. Chloroform extract killed all shrimp larvae (percent mortality 100%) at the highest concentration of 500 μg/mL. Percent mortality for hexane extract at the same concentration was 95% (Table 3). The highest cytotoxic activities were shown by chloroform extract. The order of activity was chloroform > hexane > ethyl acetate.

Table 3
Mean percent mortality of different leaves crude extract of *J. excels* by brine shrimp larvae.

Concentrations (μg/mL)	Hexane	Chloroform	Ethyl acetate
5	0	0	0
50	10	30	30
500	100	95	65

4. Discussion

The hexane, ethyl acetate, chloroform and hydro-alcoholic crude extracts *J. excels* showed the presence of alkaloids, flavonoids, saponins, steroids, tannins and triterpenoids. The chloroform extract revealed the presence of all above mentioned chemicals except tannins. However, all extracts did not show any positive test for glycosides. The comparison results of this study did not entirely correspond with the published data[16]. The ingredients in the leaves of *J. excels* grown in different parts of the world indicated that the composition of individual plants may vary widely due to the climate, growing area and time of collection. Nabi *et al* [16] reported that alkaloids, flavonoids, saponins, phenols and diterpenes are present in the leave crude extracts. However, no terpenoids and cardiac glycoside were present in the leaves crude extracts[16]. Another study by Emami *et al*[17] reported the presence of flavonoids, saponins, and tannins in the crude extracts but the alkaloids was absent in the crude extracts[17].

Free radical scavenging activity of the leaves crude extracts of *J. excels* were tested using DPPH method and the results are presented in Figure 1. The antioxidant is depends on their interaction with oxidative free radicals. The assumption of DPPH method is that the antioxidants react with the stable free radical and gradually discolouration. The degree of discolouration indicates the scavenging potentials of the samples. In this study, the four organic extracts from *J. excels* were capable to decolourise DPPH. The free radical scavenging potentials of the crude extracts were found to be in the order of hydro-alcoholic extract > chloroform extract > ethyl acetate extract >hexane extract. Moein *et al*[18] reported that the radical scavenging activity order of ethyl acetate fraction > butanolic fraction > gallic acid > butyl-hydroxyl toluene (BHT). The results showed that the polar fractions from *J. excels* especially ethyl acetate had strong antioxidant activity[13]. Another study by Emami *et al*[17] reported that the antioxidant activity of leaves crude extracts of *J. excels*. According to their report the antioxidant activity of methanol crude extract was measured using two different tests of the ferric thiocyanate method and thiobarbituric acid. The results indicated that they showed strong antioxidant activity as compared with those of α-tocopherol and butylated hydroxytoluene[14-15].

The antimicrobial activity of hexane and hydroalcoholic extracts showed strong inhibition against *P. aeruginosa* and *S. aureus*. However, the crude extracts did not showed any activity against *E. coli*. The inhibition zones for the hexane and hydroalcoholic extracts against *P. aeruginosa* and *S. aureus* within ranged between 6-13 mm. The results from the present study are in agreement with what has been reported elsewhere on antimicrobial activity of extracts from *J. excels*[14-15]. However, most of the previous studies involved only essential oil. Ehsani *et al* reported the antibacterial activity against pathogenic bacteria of the essential oil of leaf of *J. excels*[19]. The results showed that the essential oil of leaves has a strong effect against gram-positive bacteria like *S. aureus* and has a weak antibacterial activity against gram-negative bacteria like *E. coli*[13]. Finally antimicrobial investigation of the extracts from whole leaves of *J. excels* against various bacteria showed medium antimicrobial activity against *S. aureus*. The polar leaves extract exhibited inhibitory effect against *C. albicansas* reported by Albar *et al*[20].

As shown in Table 4 all leaves plant extracts displayed moderate toxicity against the brine shrimp larvae. Chloroform extract was the most active exhibiting LC_{50} value of 74.89 μg/mL. This results are not matching to the reported for cytotoxic activity of organic crude extracts of *J. excels* by Aliabadi *et al*[21]. Hydroalcoholic extracts was the most active of all tested parts of *J. excels* at concentrations ≥ 20 μg/mL[22]. This difference in LC_{50} value could be due to difference in methodology, while the present study used BST assay their investigation used *in vitro* based assay.

Finally, the researcher has found in the first study which was about antioxidant activity that hydro alcoholic has high antioxidant activity. On the second study which was about phytochemical activity the researcher found that alkaloids, flavonoids, saponins, steroid, triterpenoids and tannins are present in all extracts except that chloroform extract does not contain tannins or glycosides. More *in-vivo* and *in-vitro*

studies along with detailed phytochemical investigations are needed in order to potentially use this plant in the prevention and therapies of some oxidative damage related diseases. In short, the present study provides the biochemical foundation for further chemical analysis. Some animal as well as clinical studies are underway in our labs.

Conflict of interest statement

We declare that we have no conflict of interest.

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