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Original article

Cellular proliferation/cytotoxicity and antimicrobial potentials of green synthesized silver nanoparticles (AgNPs) using *Juniperus procera*

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ABSTRACT

Juniperus spp. are used as medicinal plants in many countries like Bosnia, Lebanon, and Turkey. In folk medicines, these plants have been used for treating skin and respiratory tract diseases, urinary problems, rheumatism and gall bladder stones. The objectives of this work were to synthesize silver nanoparticles (AgNPs) using a coniferous tree, *Juniperus procera* leaf extract and testing the synthesized AgNPs for its antimicrobial potentials, hemolytic activity, toxicity and the proliferative effects against normal and activated rat splenic cells. Leaf extract was prepared using acetone and ethanol as solvents. AgNPs were prepared using the acetone extract. AgNPs were validated using UV–Vis spectroscopy and scanning electron microscopy (SEM). Functional groups in the extract were identified using Fourier Transform Infrared (FT-IR) spectroscopy. SEM images of AgNPs showed spherical and cubic shapes with a uniform size distribution with an average size of 30–90 nm. FT-IR spectroscopy showed the presence of many functional groups in the plant extract. AgNPs showed promising antimicrobial activity against tested bacteria and fungus. AgNPs also expressed a stimulating activity towards the rat splenic cells in a dose dependent manner. Acetone as solvent was safer on cells than ethanol. Green synthesized AgNPs using *J. procera* might be used as a broad-spectrum therapeutic agent against microorganisms and as an immunostimulant agent.

1. Introduction

Plants are naturally bestowed with numerous bioactive compounds that have desirable medicinal and health benefits and are traditionally used for prevention of many chronic diseases (Yogalakshmi et al., 2010). Herbs are used for preventing and curing many diseases such as the fibrosis and injury of liver (Al-Attar and Shawush, 2015; Hsieh et al., 2008; Yuan et al., 2008). Some useful plant species, like the *Juniperus* species, are used to treat some human illness such as ulcers, hyperglycemia, bronchitis, tuberculosis, pneumonia, intestinal worms, cure liver problems and heal wounds (Burits et al., 2001; Loizzo et al., 2007). The medicinal uses of *Juniperus* plants are widespread in many countries such as Saudi Arabia, Bosnia, Lebanon, and Turkey and according to folk medicine was used for treating skin and respiratory tract diseases (Öztürk et al., 2011), urinary problems, rheumatism and gall bladder stones (Šarić-Kundalić et al., 2011).

The uses of silver nanoparticles (AgNPs) in the sector of pharmaceutical industries are wide (Satyavani et al., 2011) as these nanoparticles have a powerful inhibitory potential against many microorganisms (Majeed et al., 2016; Mohanta et al., 2017) and is a good drug carrier in treatment of cancer (Nayak et al., 2016). Nowadays, AgNPs are of great interest. The synthesis of AgNPs can be performed through many chemical methods and physical ways that involve chemical (Bindhu and Umadevi, 2015; Wiley et al., 2005), photochemical (Mallick et al., 2005; Pileni, 2000), and electrochemical reduction (Liu and Lin, 2004; Sandmann

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et al., 2000) as well as vaporization (Bae et al., 2002; Smetana et al., 2005). All of these methods utilize many toxic chemicals to act as reducing agents. So, the presence of alternatives to these toxic materials will be beneficial for the human health and environment.

To overcome the many side effects represented by the toxicity of chemicals used in the synthesis and biological applications of AgNPs, plants or their extracts have been used to replace chemicals in the AgNPs synthesis (biosynthesis process). Plants and plant extracts have many natural organic constituents; phyto-molecules that have protective and reducing properties which are very important for the reduction of silver ions depending on the natural compounds and reductive enzyme complexes. Recently, extracellular AgNPs were synthesized using different plant extracts as a potential reducing agent (Ahmed et al., 2016; Mohanta et al., 2017; Raja et al., 2017).

This work aimed to study the cell proliferative/anti-proliferative potentials as well as the anti-microbial activities found in *J. procera* leaf acetone extract and silver nanoparticles prepared by the extract.

2. Materials and methods

2.1. Plant material and preparation of extract

The leaves of *J. procera* were collected in January 2017. The plant sample was kindly identified by an expert taxonomist at the Biology Department, of the University. The active ingredients in the latex of the plant were extracted according to Ghramh et al. (2018) with some modifications. The leaves, after mild washing, were air-dried under shade at room temperature and ground into powder using an electric grinder and 300 g of the resulted powder was added to 1.5 L of either acetone or ethanol and stirred for 72 h. The resultant plant extracts were separately filtered and dried at reduced temperature using a rotary evaporator, and stored at 22 °C. Stock solution, at 1% concentration, was prepared by dissolving 1 g dried acetone extract materials in 100 mL acetone or 1 g dried ethanol extract materials in 100 mL ethanol and stored at 4 °C in the dark until used. The acetone stock is the most suitable when used in culture media (Vandhana et al., 2010).

2.2. Green biosynthesis of silver nanoparticles

Silver nanoparticles were prepared according to Ndikau et al. (2017) with little modifications. One mL of acetone plant extract stock and 0.5 mL Triton X-100 were added to 98.5 mL 1 mM $AgNO_3$ solution in an Erlenmeyer flask and incubated for 24 h at room temperature until the color of the mixture changed.

2.3. Characterizations

UV–visible spectrophotometer (Lambda 25, PerkinElmer), at the wavelengths from 200 nm to 600 nm, was used to analyse and monitor the process of AgNPs synthesis at 1 nm resolution. The surface morphology of the prepared AgNPs was studied by using a scanning electron microscopy (SEM; Hitachi S4800) at an accelerating voltage of 90 kV. Fourier Transform infrared spectroscopy (FT-IR, JASCO 460 plus) was used at room temperature to identify the functional groups of bioreductant in the extract using the KBr pellet technique within the range 600–4000 cm^{-1} at a rate of 16 times and the clarity of 4 cm^{-1}.

2.4. Antimicrobial activity

The antimicrobial potential of prepared silver nanoparticles was tested using microorganisms. The extract stock was diluted 100 times by adding 1 mL stock to 99 mL of distilled water containing 0.5 mL of Triton X-100 to match the concentration of the extract found with the nanoparticles.

2.4.1. Test microorganisms

The microorganisms utilized in this study were representative of Gram positive (*Bacillus subtilis* and *Micrococcus luteus*), Gram negative (*Proteus mirabilis* and *Klebsiella pneumoniae*) pathogenic bacteria and *Candida albicans* as a model of yeast. The isolates were streak plated on a nutrient agar and maintained at 4 °C.

2.4.2. Antimicrobial susceptibility testing (AST)

Well diffusion assay was adopted according to Daud et al. (2005). Into the 6 mm wells a 40 μL of each diluted plant extract and prepared nanoparticles were aseptically pipetted into the wells in separate plates. The plates were incubated at 4 °C for 3 h and then at 32 °C for an additional 18 h. Penicillin (10 μg) was used as positive control. Diameter of the zone of inhibition, in triplicate, around each of the 6-mm well was measured and the means were calculated.

2.5. Effects of plant extract and biosynthesized AgNPs on splenic cells proliferation

Adult male Sprague Dawley rats, whose weight was approximately 250–300 g, were kindly supplied by the animal rearing house at the University. This study was performed under the protocol and approval # 2017-03-38 of Research Ethics Committee, Faculty of Medicine. The animals were killed by cervical dislocation and the splenic cells were aseptically prepared in serum-free RPMI-1640 medium (SFM) [consisting of RPMI-1640 medium, augmented by 100 U/100 μg/ml penicillin/streptomycin, 2 mM L-glutamine (all from Gibco), 2 mM sodium pyruvate, 2% sodium bicarbonate, pH 7.2 (both from Seromed) and HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ehtanesulfonic acid) buffer solution (Sigma, Aldrich)] according to Gu et al. (2013). The viability of prepared cells was monitored through the exclusion of trypan blue by live cells (Böyum, 1968). Cells were enumerated to 5×10^4/mL in SFM including 10% fetal calf serum (Gibco) (culture medium). The cell culture was performed in a microwell tissue culture plate (TPP, Merk) by adding 100 μL of cell suspension (5000 cells/well) and acetone extract (10 μg/mL, 50 μg/mL, 100 μg/mL, and 200 μg/mL).

2.5.1. Study of anti-proliferative effects

To a sufficient volume of prepared cell culture, Phytohaemagglutinin (PHA, Sigma) at a final concentration of 5 μg/mL was added to stimulate cell proliferation. The anti-proliferative capability which may be found in the extract were tested by adding different concentrations of *J. procera* leaf extract and biosynthesized AgNPs separately at final concentrations of 10 μg/mL, 50 μg/mL, 100 μg/mL, and 200 μg/mL to wells containing cells. Cells without extract were included representing fast dividing cell control. Plates containing the cells were incubated at 37 °C for 72 h in 5% CO_2 (Memmert, Gmbh) (Yashi et al., 1993). The MTT technique was used to measure the changes in cell numbers of different treated cells (Mosmann, 1983) using Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific) according to manufacturer instructions.

2.5.2. Study of cytotoxic/proliferative effects

Cytotoxic (cell killing) or stimulating (induction of normal cell's division) potentials which may be found in the extract were examined by incubating different concentrations of *J. procera* leaf extract and biosynthesized AgNPs separately with final concentrations of 10 μg/mL, 50 μg/mL, 100 μg/mL, and 200 μg/mL with normal

splenic cells separately. Cells without any treatment were included as normal cell control. Culture plates were incubated and the increase or decrease in cell number was monitored as mentioned above.

The results were expressed as a percentage of increase/decrease in growth, according to Oves et al. (2013).

2.6. Red blood cell lysis test

The cytotoxicity was also determined by testing the hemolytic activity of the *J. procera* leaf extracts and biosynthesized AgNPs separately at final concentrations 200 μg/mL using the method defined by Oves et al. (2013) with few amendment. Fresh cow blood (50 mL) was placed in 50 mL Falcone tube containing EDTA to evade blood coagulation, slightly mixed and then divided into several sterile 15 mL Falcon tubes and placed in centrifugation for 10 min at 1500*g*. The blood supernatant was decanted and RBCs were washed four times with chilled (4 °C) PBS. The washed RBCs were resuspended in chilled PBS to get 10% hematocrit. A 100 μL of prepared ethanol and acetone *J. procera* leaf extract and biosynthesized AgNPs were added to 900 μL of prepared RBCs in 1.6 mL Eppendorf tubes separately and incubated for 60 min at 37 °C. 1.5% Triton X-100 (positive control) and phosphate buffer saline (PBS, negative control) were included in the test. All tubes were centrifuged for 10 min at 2000 RPM. After centrifugation supernatants were taken from the tubes, and the absorbance was measured at 576 nm.

2.7. Statistical analysis

All data were expressed as the mean of three triplicates. Different concentrations of the extract and extract generated AgNPs differences were analyzed with one-way analysis of variance (ANOVA) using SPSS (version 17). Differences of $p \leq 0.05$ were considered to be statistically significant.

3. Results and discussion

Juniperus is considered as one of the important genera of the family Cupressaceae. It includes about 70 species of *Juniperus* that are distributed throughout the world (Al-Attar et al., 2016; Moein et al., 2010; Topçu et al., 1999). It is established that the *Juniperus* species are a source of natural products with antibacterial, antifungal and insecticidal potentials (Barrero et al., 2005; El-Sawi et al., 2007; Tumen et al., 2012). *J. procera* is also used as a local treatment for tuberculosis and jaundice (Samoylenko et al., 2008), intestinal worms and eye infections (Klauss and Adala, 1994). Pankaj et al. (2010) investigated several fractions isolated from *Juniperus* bark and leaves and found that it could inhibit aflatoxigenic *Aspergillus flavus* and *A. niger* growth. Hexane and ethanolic extracts prepared from *J. lucayana* aerial parts showed antifungal activities (Nuñez et al., 2007).

Sliver or silver nanoparticles were prepared by adding the plant extract to the $AgNO_3$ solution. The mixture was incubated for 24 h at room temperature. Synthesis of AgNPs was indicated by the change of the mixture into the brown-yellow color (Fig. 1). The characteristic brown color is attributed to the excitation of Surface Plasmon Response (SPR) with the silver nanoparticles (Ranganathan et al., 2012). This color change indicates that the changes that happened in the metal oxidation state from Ag^+ to the reduced form Ag^0 were by biomolecules in plant extract through the nitrate reductase enzyme. The mentioned enzymes secreted into the solution may reduce the silver nitrate to silver nanoparticles by capping agents like proteins (Manivasagan et al., 2013). The power of *Juniperus* may be attributed in inclusion of a

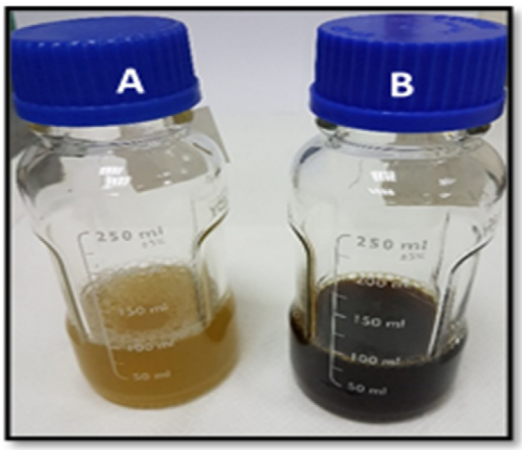

Fig. 1. Acetone extract of *Juniperus procera*. A: Acetone extract without $AgNO_3$ and B: Acetone extract after adding $AgNo_3$ for 24 h.

wide variety of phenolic acids, flavonoids, chlorogenic acid, caffeoylquinic acids, etc, which could act as reducing agents to react with silver ions and as scaffolds to direct the formation of AgNP in solution (Al-Attar, 2011; Lai et al., 2007).

Formation of the AgNPs by the extract was monitored by the UV–visible spectrum (Fig. 2). Pure extract showed two absorption peaks at 230 and 275 nm. These two peaks may be responsible for the reduction of silver ion to silver metal due to the presence of polyphenol and other functional groups (Ranganathan et al., 2012). Testing the extract after the formation AgNPs resulted in a characteristic absorption spectrum at 417 nm. This may be due to SPR which proves the synthesis of silver nanoparticles at room temperature by using the extract (Parikh et al., 2008).

Biosynthesis of the AgNPs at room temperature was confirmed by FT-IR spectrum (Fig. 3). The broad band appeared at ~3442 cm^{-1} is due to the presence of O—H stretching vibration (Li et al., 2017; Liu et al., 2006). The strong band appeared at 1026 cm^{-1} is ascribed to the C—O stretching vibration of OH group, which also confirms the presence of the phenolic group in the extract and interacted with the silver ion. Scissoring vibration of the NH_2 group in amines and the stretching mode of the C=C bonds in aromatic rings seem to be responsible for the band at 1616 cm^{-1}. In addition to the referred modes, the vibration bending of the N—H bonds in

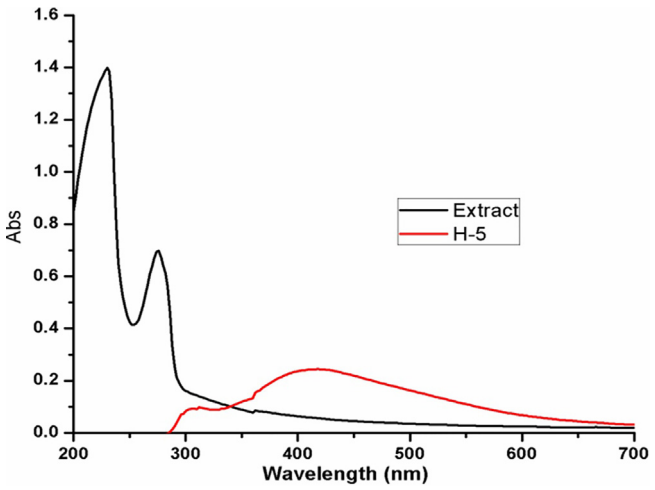

Fig. 2. UV–Vis spectra of silver nanoparticles synthesized by the *Juniperus procera* leaves extract. (A) Acetone plant extract without silver nitrate; and (B) acetone plant extract-reduced silver nanoparticles.

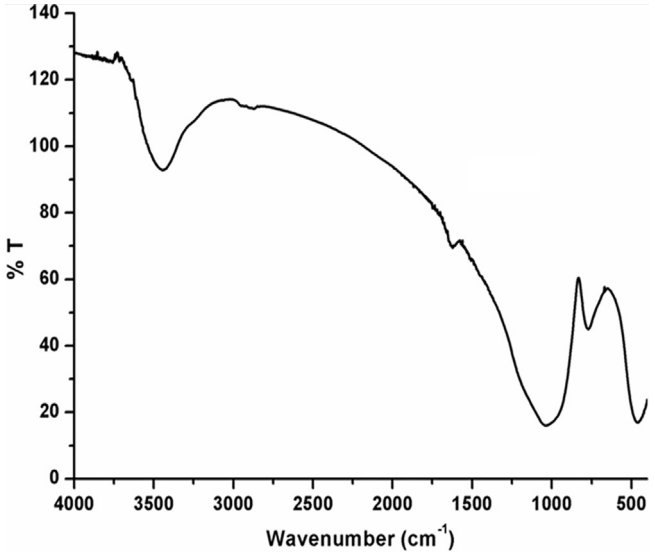

Fig 3. FT-IR spectrum of biosynthesized silver nanoparticles by the *Juniperus procera* leaves extract.

carboxylic acids and derivatives could also contribute to the neighbouring band at 1620 cm^{-1}. A band with stretching vibration appears at ~452 cm^{-1} perhaps assigned to the interaction or adsorption of O—H on the surface of silver nanoparticles. The FT-IR results revealed that the biomolecules of the extract present on the surface of silver nanoparticles (Li et al., 2017; Prathna et al., 2011). The FT-IR results revealed that the production of AgNPs might be stabilized by the existence of polyphenols materials in the extract, which may play a role as powerful bio-reducer and good capping agent; therefore, they help in the formation of stabilized silver nanoparticles.

The shape and size of the biosynthesized AgNPs were monitored by SEM analysis (Fig. 4). SEM images of AgNPs showed spherical and cubic shapes with uniform size distribution. The particles have an average size of 30–90 nm with inter-particle distances, which are magnified at ×13,000 times. The small degree of agglomeration could be seen in SEM investigations may be due to the effect of the bio extract and surfactant during synthesis. The SEM studies clearly depict that the bioorganic extract and surfactant play a momentous role for producing the spherical AgNPs materials.

The antimicrobial activities found in the *J. procera* leaves extract were tested. *J. procera* leaves extract showed inhibition potentials

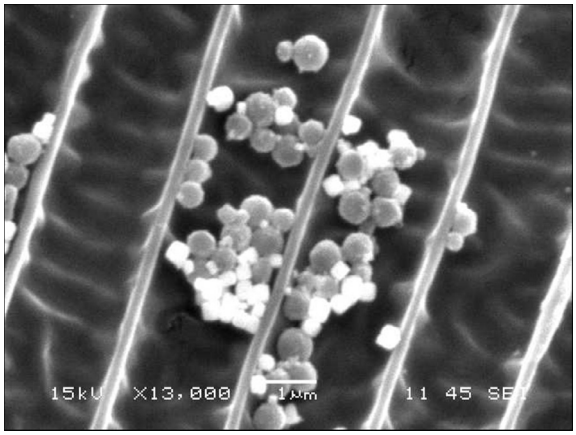

Fig 4. SEM micrograph of the *Juniperus procera* leaves extract prepared AgNPs.

Table 1
Antimicrobial potentials of *Juniperus procera* leaves extract and extract prepared AgNPs.

Test organisms	Inhibition zone (mm)		
	Juniperus extract	*Juniperus* extract + AgNPs	Penicillin (10 μg)
Micrococcus luteus	9 ± 0.7	28 ± 1.1	8 ± 0.7
Bacillus subtilis	11 ± 0.8	28 ± 1.2	10 ± 0.9
Proteus mirabilis	10 ± 0.9	29 ± 1.3	9 ± 0.9
Klebsiella pneumoniae	9 ± 0.6	18 ± 0.9	10 ± 0.7
Candida albicans	NI	24 ± 0.1.2	10 ± 0.8

NB: Diameter of zones of inhibition are expressed as means of three replicates ± SD ($p < 0.05$).

against bacterial and fungal growth (Table 1). Plant extract only showed mild activity against bacteria and fungus while silver nanoparticles generated by the plant extract showed more activities against Gram positive and negative bacteria as well as yeast. These results are in agreement with others. Some investigators (Pankaj et al., 2010) studied the different fractions isolated from *Juniperus* bark and leaves and found that it can inhibit *Aspergillus flavus* and *A. niger* growth (Nuñez et al., 2007). Other researchers demonstrated that *J. procera*; found at high altitude has antibacterial activities and antifungal potentials (Gherbawy and Elhariry, 2016).

Muhammad et al. (1995), (1996) isolated some components, the (+)-8α-acetoxyelemol, β-peltatin A methyl ether and deoxypodophyllotoxin, that have antibacterial activities.

It was demonstrated that the active biomolecules in *J. procera* leaves included flavonoids and polyphenol (Ali and Elgimabi, 2015); which confer the antibiotic potential of the plant against many species of bacterial pathogens (Jung, 2009). Hence, preliminary screening revealed that the biologically synthesized AgNPs showed significant antimicrobial activity, as compared to other researches (Arokiyaraj et al., 2017; He et al., 2013). This antibacterial activity of the AgNPs that were green synthesized by *Juniperus* may be due the very small size leading to the large surface area of the AgNPs, resulting in binding to the bacterial cell wall and neutralizing the cellular enzymes initiating the disruption of the membrane permeability (Su et al., 2009). Coupled to this, antibacterial activity may be due to the interaction of AgNPs with groups like the thiol group of the L-cysteine protein residues leading to enzymatic dysfunction (Gordon et al., 2010). In addition, the AgNPs may induce the release of reactive oxygen species resulting in the damage to proteins and nucleic acids which cause the cell death (Abdal Dayem et al., 2017).

The immunomodulatory effects of *J. procera* leaf extract and extract prepared nanoparticles using the cell proliferation as a model are used for the search of new therapeutic agents with natural origin (Al-Attar et al., 2016; Arokiyaraj et al., 2007).

Both plant extract alone and with AgNPs showed no inhibitory effects on PHA-stimulated splenic cells, in contrary the extracts at all different concentrations showed stimulatory effects (Table 2). Extract containing AgNPs showed more stimulatory potential than the extract alone. Maximal stimulation was at higher concentration of extract containing nanoparticles (200 μg/mL).

Both plant extract alone and with AgNPs showed no cytotoxic effects on normal splenic cells. Both extracts at all different concentrations used showed stimulatory effects (Table 2).

The activation of the immune response in this model boosts cell proliferation/inhibition through an increase/decrease in the number of cells found in the culture for specific duration (Rocha et al., 2007) and this change can be observed with MTT assay (Mosmann, 1983). The acetone extract of *J. procera* leaves used in this study, induced an increase in cell proliferation both in un-induced or PHA-induced cultures in a dose-dependent response.

Table 2
Percent of splenic cells growth stimulation after treatment with plant extract and its silver nitrate generated nanoparticles.

Extract concentration (μg/mL)	% of splenic cells growth stimulation			
	Normal		PHA treated	
	Extract	AgNPs	Extract	AgNPs
10	110 ± 0.71	118 ± 0.66	115 ± 0.97	130 ± 1.1
50	115 ± 0.82	122 ± 0.90	125 ± 1.3	140 ± 1.6
100	115 ± 0.91	129 ± 1.1	130 ± 1.4	145 ± 1.8
200	120 ± 0.87	140 ± 1.3	135 ± 1.6	165 ± 2.2

NB: Percentage of splenic cell growth stimulation was expressed as average of three replicates ± SD ($p < 0.05$).

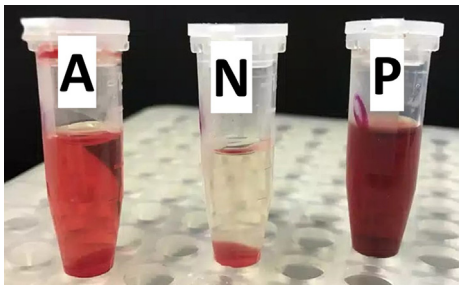

Fig 5. The effect of *Juniperus procera* leaves acetone extract containing AgNPs on cow RBCs. Where A: Acetone extract containing AgNPs; N: Negative control and P: positive control.

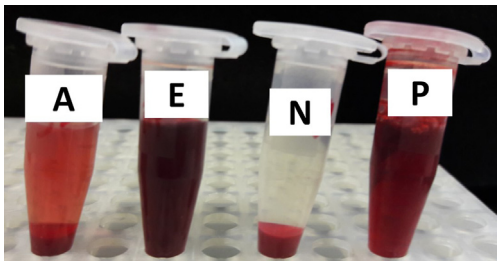

Fig 6. The effect of *Juniperus procera* leaves acetone and ethanol extracts on cow RBCs. Where A: Acetone extract; E: ethanol extract; N: Negative control and P: positive control.

PHA is the most frequently used lectin to activate T lymphocytes *in vitro* to evaluate immune response mediated by this type of cells (Rocha et al., 2007). Thus, the results obtained with the acetone extract of *J. procera* leaves suggest that the component(s) present in the plant could stimulate T lymphocyte proliferation, suggesting the ability of that extract to activate the cellular immune response. The phytochemical picture of these herbs demonstrates the presence of constituents *J. procera* flavonoids and phenol (Al-Attar et al., 2016). Although flavonoids have been described in many works as inhibitors of lymphocytes proliferation (Arokiyaraj et al., 2007; Moiteiro et al., 2001), the action of the flavonoids seems to be inhibited by other compounds with immunostimulatory effects, such as alkaloids (Sheng et al., 2000), that's why the results of this study revealed substantial rise in cell proliferation that can be due to the stimulation of T lymphocytes.

The cytotoxicity was measured by hemolytic activity against cow RBCs with Triton X-100 (positive control). The percentage of lysis was calculated by relating the absorbance of sample to the positive control. The positive control (1.5% Triton X-100) showed about 100% RBCs lysis, while the negative control (PBS) showed no lysis effects on the RBCs. The ethanolic plant extract showed 100% RBCs lysis (Fig. 5) while acetone extract alone or extract with AgNPs showed insignificant lysis effect on RBCs (about 1.75%, Fig. 6). These results supported our idea to use acetone extract rather than ethanol extract in cell culture experiments.

4. Conclusions

Acetone extract of *J. procera* leaves could be used for the green biosynthesis of AgNPs. The extract contained functional groups and metabolite that enabled the synthesis and stabilizing of AgNPs from silver ions. The extract could stimulate normal and PHA-stimulated splenocytes so it can be used as immunostimulant.

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