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INVESTIGATION OF THE CYTOTOXIC AND ANTILEISHMANIAL EFFECTS OF *FAGONIA INDICA* L. EXTRACT AND EXTRACT MEDIATED SILVER NANOPARTICLES (AgNPs)

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Abstract

Leishmaniasis is one of the globally neglected tropical disease cause by protozoan parasite of the genus Leishmania. In Pakistan, cutaneous leishmaniasis is more sporadic in the Afghan refugee camps, which is concern for the local villager and Pakistani population. In the current study an approache was made to synthesise biogenic silver nanoparticles using *Fagonia indica* leaf extract. Furthermore, the antileishmanial activity of the nanoparticles was evaluated compared to the crude extracts against *Leishmania tropica* which is the causative agent of cutenious leishmaniasis. MTT cell viability assay was used to determine the non toxicological concentration of the extract and nanoparticle in macrophage cell lines (J774), and the antileishmanial activity. We found that silver nanoparticles are not toxic to macropage cell above 30 µg/ml. where as the IC_{50} against leishmania parsites was calculated as 8.16±0.63 µg/ml and 4.8±0.819 µg/ml for extract and AgNPs respectively. We also determine the infection index of the parasite in the macrophage cell. The infectivity of parasites also decreases as compared to control group after activation of macrophages. We further, evaluate the mechanism of growth inhibition using Griess reagent for the estimation of nitrogen oxide. We found that both the extract and AgNPs produce an elevated level of nitrogen oxide free radical. These radical produce oxidative stress in the cell that lead to the reduced metabolic activities of the parasites and ultimate death. Overall, the results indicate that *Fagonia indica* leves extract and AgNPs are potent antileishmanial agents.

Key word: Biosynthesis, *Fagonia indica*, Cytotoxicity, Antileishmanial, MTT cell viability assay.

Introduction

Leishmaniasis is one of the globally neglected tropical disease cause by protozoan parasite of the genus Leishmania (Das *et al.*, 2010). Leishmaniasis is endemic to 98 countries, affect more than 12 million peoples worldwide and more than 350 million are at high risk (Ngure *et al.*, 2009; Alvar *et al.*, 2012). Leishmaniasis is classified into three categories based on their clinical manifestations. These clinical forms are cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL). The disease is caused by the bite of an infected Phlebotomus and injecting the promastigotes form of the Leishmania. The parasite then complete its life cycle in the host macrophages in the non-flagellated form called as amastigotes (Sousa *et al.*, 2014). The disease is caused by different species of the parasites, such as *Leishmania major, L. tropica, L. infantum L. aethiopica* and *L. donovani*. Since leishmaniasis is a group of diseases, so its sign and symptom depends on the parasite type and type of infection in the host (Alavi-Naini *et al.*, 2012). In Pakistan the disease was confined to northern area, but now a days widespread cases are reported throughout the country. In 2002, WHO reported 5000 cases of cutaneous leishmaniasis in the Kurram agency of Khyber Pakhtunkhwa (KP) (Brooker *et al.*, 2004). Cutaneous leishmaniasis is caused by *L. tropica* specie of the parasite which is characterized by disfiguring skin and social humiliation. In Pakistan, cutaneous leishmaniasis is more sporadic in the Afghan refugee camps, which is concern for the local villager and Pakistani population. Cutaneous leishmaniasis has been reported from all over the Pakistan. The most endemic area are the Swat, Chitral, Gilgit, Mansehra, Skurdu, Mansehra, Abbottabad, Chillas, Rawalpindi, Dir, DG Khan, Azad Kashmir,Khuzdar, Lasbela, Jacobabad, Quetta, Qila Saifullah, DI Khan, Pisheen, and Larkana. The environmental condition are suitable for the growth and development of the sand fly carrying the parasites (Ali and Afrin, 1997; Durrani *et al.*, 2012; Shah *et al.*, 2014; Tariq *et al.*, 2016). Therefore, for long term control of leishmaniasis in Pakistan requires cost effective treatment modalities to combat the disease transmission.

The current treatment of leishmaniasis consists of pentavalent antimonial compounds, amphotericin B, aminoglycosides, miltefosine, pentamidine and paromomycin (Croft & Coombs, 2003). However, these drugs have life threatening side effects, toxicity to normal cells, high cost, resistance and long treatment duration (Croft *et al.*, 2005). Due to these limitation, new and alternative treatment options such as the use of Nanobiotechnology and medicinal plants secondary metabolites could be sort out. The use of Nanobiotechnology for the production of nanomedicine against leishmaniasis is a good alternative approach. Production of metallic nanoparticles using plant secondary metabolites for the bio reduction and capping of metal ions is a cost effective, eco-friendly and biocompatible method for biological applications (Anjum *et al*., 2016; Khalil *et al.*, 2017[a-c]; Riaz *et al.*, 2017). In the present study we synthesised Nano dimension size biocompatible silver nanoparticles against leishmaniasis using *Fagonia indica* L. above ground part extract. Silver nanoparticles have many biotechnological applications, such as antmicrobial agents, biofilters, biosensors, purifying drinking water and many other biological applications. İt has been used as drug delivery system against certain diseases. However, there are limited

studuies on the application of biogenic silver nanopaticles using plant secondary metabolite as a source of bioreduction against leishmaniasis. *Fagonia indica* L. is a deserted herb belong to family Zygophyllaceae, commonly known as Dhamasa. It is found mostly in the arid and deserted sandy area of Pakistan and some other part of the world like India, Africa and USA (Rizvi *et al.*, 1996).Traditionally the dry powder of the plant is used as a decoction for stomach, hepatic and skin problems (wound healing). Chemically it is a rich source of plant secondary metabolites such as phenolic, flavonoids, alkaloids, coumarins, tannins and triterpenoids (Khalik *et al.*, 2000; Shehab *et al.*, 2011; Satpute *et al.*, 2012). Pharmacologically the plant was found to have antibacterial, anti-inflammatory and cancer preventing activities (Jan *et al.*, 2015; Mufti *et al.*, 2015). Moreover the plant has been reported to have no toxic effects on normal cells (Lam *et al.*, 2012).

Recently, researchers studied various molecules and metallic nanoparticles that have apoptotic and potent antiparasitic activities. Several reports shows that plant extracts and biogenic silver nanoparticles triggers apoptosis, DNA fragmentation, production of reactive oxygen and nitrogen species in various biological organisms (Yadegari-Dehkordi *et al.*, 2015). Silver nanoparticles have favourable activities at low concentration and short duration of time against wide range of microbes, including viruses, bacteria, fungi and protozoans (Nilforoushzadeh *et al.*, 2012; Zahir *et al.*, 2015). Silver nanoparticles synthesised from *Dioscorea bulbifera* extract show promising antileishmanial activity with an IC_{50} value of 32 µg/mL(Ghosh *et al.*, 2015). Similarly, gold and silver nanoparticles from *Sargentodoxa cuneata* extract shows an IC_{50} values of 5.29 and 4.37 µg/mL respectively (Ahmad *et al.*, 2015). Biogenic AgNPs of *Euphorbia prostrata* also possess vigorous Leishmanicidal activity with an IC_{50} value of 14.94 µg/ml (promastigotes) and 3.89 µg/ml in amastigotes culture respectively (Zahir *et al.*, 2015). *Isatis tinctoria* mediated synthesis of silver nanoparticles bound to amphoterecine have synergestic effect against Leishmania tropica with an IC_{50} value of 2.43µg/mL (Ahmad *et al.*, 2016). Keeping in mind the immense potential of the *Fagonia indica* leaves extract as cancer preventing and antimicrobial properties, the present study was designed to synthesise silver nanoparticles from Fogonia leaf extract and investigate its biocompatibility in macrophage cell line and evaluate its anti-leishmanial activity against *Leishmania tropica* which is the common entity of cutaneous leishmaniasis in Pakistan. Herein, we found that *Fagonia indica* extracts and extract mediated silver nanoparticles substantially inhibit the growth of the Leishmania parasites by producing nitrogen oxide free radicals.

Materials and Methods

Green synthesis and characterization of AgNPs: Silver Nano powder were produced by reducing silver nitrate ($AgNO_3$) solution with *Fagonia indica* leaves aqueous extracts. The protocol was optimized as 5 mg/ml extract solution mixed with 1 mM $AgNO_3$ solution in the ratio of 1:10 at 60ºC for 2 hours in the dark. The synthesis of the silver nanoparticles was confirmed visually by change in the colour of the mixture into dark brown. Further, the synthesis was confirmed by UV-Vis spectrophotometer in the range of 300-600 nm wavelength. The solution was washed three times by successive centrifugation at 13000 rpm for 20 minutes. The powder obtained was further subject for the investigation of hydrodynamic size using dynamic light scattering spectroscopy (DLS). The crystalline nature and size was determined by X-rays diffraction (XRD) analysis.

Leishmania parasite culture: *Leishmania tropica* (KMU25) promastigotes were cultivated in RPMI-1640 medium added with L-glutamine, Gentamicin (80 µg/mL) and 10% fetal bovine serum (FBS) at 27°C. After four days of incubation the culture was monitored using inverted microscope (Olympus) and passaged for further growth.

Macrophage (J774) cell culture: Mouse macrophage cells (J774) were grown in tissue culture flask (T-25cm) using RPMI-1640 supplemented with Gentamicin (80 µg/mL) and FBS (10%) in a humidified incubator with 5% CO_2 at 37°C. When 80-90% confluent culture obtained, the cells were passaged.

Antileishmanial activities against *Leishmania promastigotes*: The leishmanicidal activities of *Fagonia indica* aqueous extracts and extract mediated silver nanoparticles was investigated using different concentration (5, 10, 20, 25, 50, 100 and 200µg/mL). The viability of the *L. tropica* promastigotes was determined by the metabolic turned over of the purple formazan crystal formed in the living cell by the mitochondrial dehydrogenase enzyme by converting 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) salt. The inhibition of the growth of promastigotes was calculated quantitatively using Microplate ELISA reader (BioTek) at 540 nm.

Biocompatibility assay: Macrophages are the host for the completion of Leishmania life cycle. In order to find out the non-toxic concentrations, a cytotoxicity test of extract and extract mediated silver nanoparticles was performed in macrophage cells (J774). Briefly, macrophages (J774) were cultured as 5×10^4 cells/well in RPMI-1640 medium in a 96 well microplate at 37°C for 24 h. When the cell adhere to the walls, different concentration of extract and nanoparticles (5, 10, 20, 25, 50, 100, 200 and 300 µg/ml) were applied to it. The plate was incubated for 24 hours at 37°C. After, 10 µL MTT solution (5 mg/ml) was added to it followed by 4 hours of incubation. The reaction was stopped by adding100 µl of dimethyl sulfoxide (DMSO). After 30 min the absorbance was measured using microplate reader at 570 nm.

Anti-amastigotes activities: The macrophages were seeded at the rate of 2×10^4 cells per well in a 24 well plate having coverslip put into it and incubated for 24

hours at 37ºC. To the adherent macrophages, *L. tropica* promastigotes were added in the ratio of 10 parasites per one macrophage in each well for 2 hours as per standard culture conditions. The macrophages were washed with phosphate buffer saline (PBS) to remove the free promastigotes and the non-toxic concentration of the extract and nanoparticles were added to each well. The plate were cultured for 24 hours, the macrophages were fixed on the coverslip using methanol for 10 min. The cells were stained with 10% Giemsa stain for 45 minutes. The coverslips were washed and examine under oil emersion microscope. The number of infected macrophages and the average number of amastigotes in each macrophages was estimated in 200 cells. The infection index was calculated as the percentage of infected macrophages multiplied by the average number of amastigotes per macrophage (Elcicek *et al.*, 2013).

Measurement of nitric oxide in cell supernatants: The level of nitrogen oxide produce by macrophages cells against Leishmania parasites was quantified by Griess reagent containing; 1% sulfanilic acid, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 2.5% phosphoric acid. After 24 hours of leishmnaia and macrophage interaction, the cell supernantant was colected and equal volume of Griess reagent was mixed at room temeperature for 10 minutes. The mixture was put in 96 well plate and the absorbance was measured at 540 nm through Microplate ELISA reader.

Data analysis: The experiments were performed in triplicates. The results were expressed asmean±SD.IC_{50} values was calculated using Table curve linear fit model. Viability of the cells was calculated as;

$$\%\ Viability = \left(\frac{OD of\ test\ sample}{OD\ of\ the\ Control}\right) * 100$$

Results and Discussions

Green synthesis and characterization: Biomimetic silver nanoparticles was prepared by combining 5 mg/ml extract with 1 mM silver nitrate solution in the ratio of 1:10 at 60ºC. A dark brown color change was observed which is the first indication of nanoparticle synthesis. After 2 hours of incubation, the synthesis was confirmed by measuring the surface plasmon resonance of AgNPs through UV-Vis spectrophotometer in the range of 300-600 nm. A surface plasmon resonance peak at 415 nm specific for silver nanoparticles was observered in the visible range (Fig. 1).

Dynamic light scattering (DLS): Thee hydrodynamic size of the colloidal solution of silver nanoparticle was determined by Zeta sizer. Figure 2A show the average size of the synthesised nanoparticles as 23.68 nm and width as 18.45 nm. The number based distribution of of nanoparticles indicate that the nanoparticles are monodisperesed and uniform in size (Fig. 2B).

X-rays diffraction (XRD): Figure 3 demonstarte the XRD pattern of the synthesised nanoparticles. The brag reflection at 2 theta degree 28.28º (001), 32.06º (101), 37.93º (111), 46.68º (200), 50.99º (222) and 65.48º (220) indicate the sphericalsymmetry of the nanoparticle with an average size 18.98 nm. Clear peaks of silver nanoparticles at the respective angles corresponds to the (JCPDS No. 03-0921) which reflect that nanoparticles are crystalline in nature.

Antleishmanial activities (Anti-promastigote assay): Figure 4A shows the antileishmanial activities of the *Fagonia indica* leaf extract anf leaf extract mediated silver nanoparticles. The figur show that the extract and nanoparticles have concenteration dependent inhibitory effect on the growth of the parasites.with the increasing in concenteration of the nanoparticles the growth become retarded. The MTT viability assay shows that only 15% and 9% of the cells were viable in the extract and nanoparticles treated cells respectively at 200 µg/ml concenteration. The lowest concentration 5 µg/ml inhibit the growth at the rate of 44.65% and 51.13% both in extract and nanoparticle exposed cells respectively. The IC_{50} value were calculated as 8.16±0.637µg/ml and 4.8±0.819 µg/ml respectively.

Biocompatibity assay using J774 machrophage cells: The cytotoxicity of extract and silver nanoparticles were evaluated through MTT cell viability assay (Fig. 5). Extract and biogenic silver nanoparticles were applied in the range of 5-300 µg/ml concenteration to macrophage cells J774 in 96 well microplate. After 24 hrs of exposure cell were treated with MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) solution. The mitochondrial dehydrogenase enzyme produce by living cells react with MTT that produce purple formazon crystal. we found a linear relationship of the formazan crystal produced and the number of viable cells. The results shows concentration dependent inhibition of the growth of J774 cell related to control macrophage cells. The IC_{50} value for both the extract and nanoparticles were calculated as above 500 µg/ml. The non toxicological concentration were above 30 µg/ml both for the fagonia leaves extract and silver nanoparticles, which is very less for leishmania toxicity, i.e., 8 µg/ml and 4 µg/ml respectively. The results show that the fagonia extract and naoparticles are non toxic to macrophages cells.

Determination of infection index of *L. tropica*: The infectivity index was estimated after leishmania-macrophage interaction. Table 1 shows the infection percentage in macrophages by *L. tropica* amastigotes exposed to 8 and 4 µg/ml extract and silver nanoparticles respectively for 2 hours. The infectivity was calculated after 24 hours and 48 hours of incubation. The data shows that the parasites lose its infectivity and its growth property. Intracellular promastigotes in macrophages (aveage parasites in a macrophage cell) were detected as 2 amastigotes in control group, 1 each in extract and nanoparticles treated parasites after 48 hours of infection. From the data it is also cleared that both the extract and nanoparticles are effective in reducing infection percentage of *L. tropica* promastigotes. However, the AgNPs were found to be more effective than plant extract.

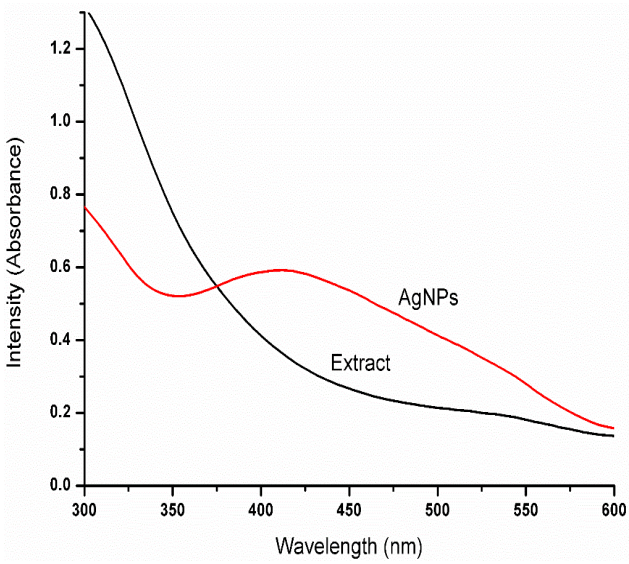

Fig. 1. UV-Vis. Spectra of silver nanoparticles mediated by *Fagonia indica* leaf extract.

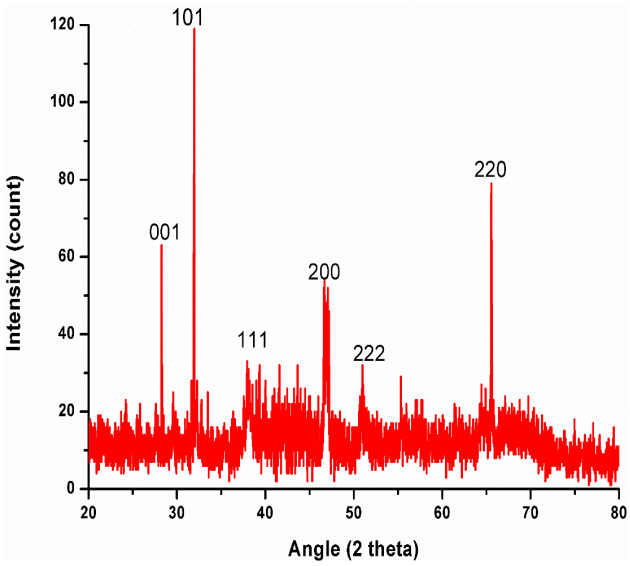

Fig. 3. X-Rays diffraction (XRD) pattern of AgNPs.

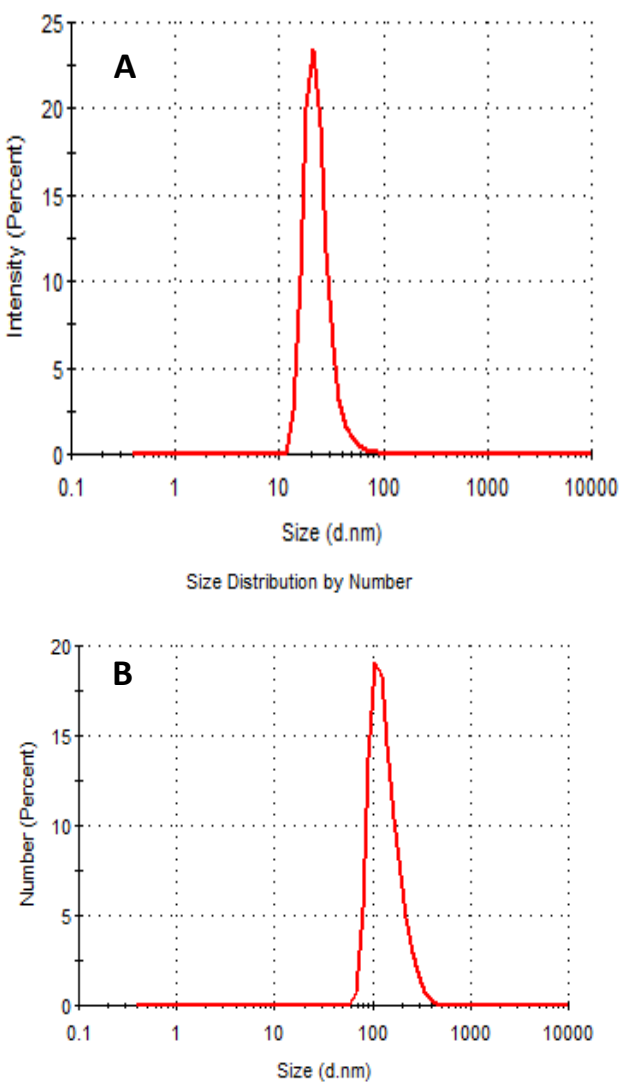

Fig. 2. Size determination of AgNPs by dynamic light scatering (DLS) (A) Size distribution by intensity (B) Size distribution by number.

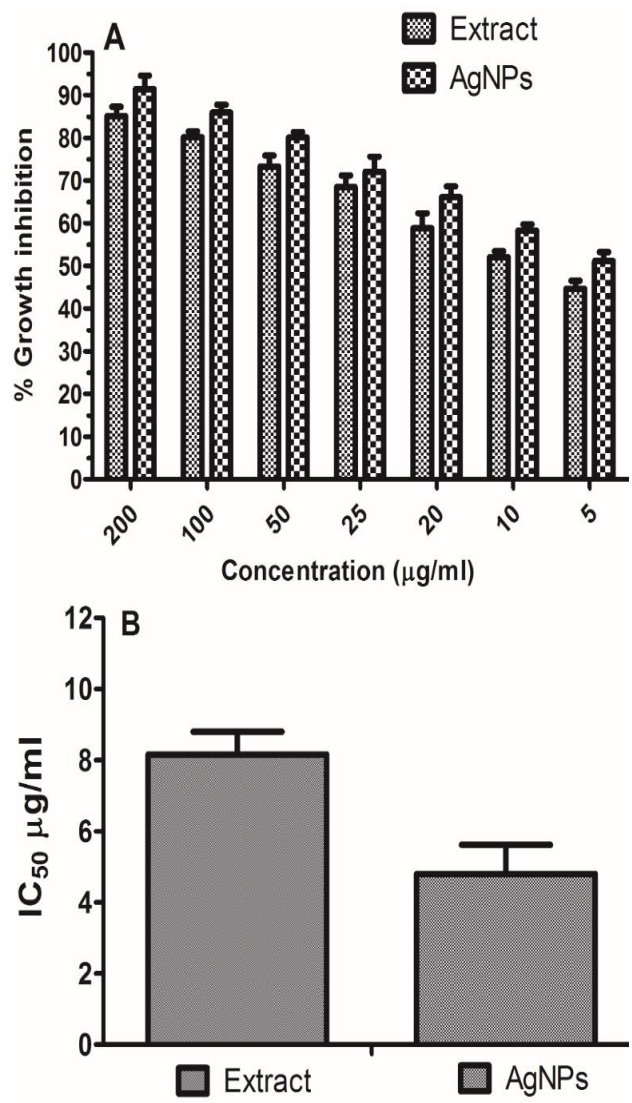

Fig. 4. MTT cell viability assay showing (a) the concentration dependent inhibition of he growth of *L. tropica* promastigotes and (b) IC_{50} concentration inhibit the growth of the parasites at 50%.

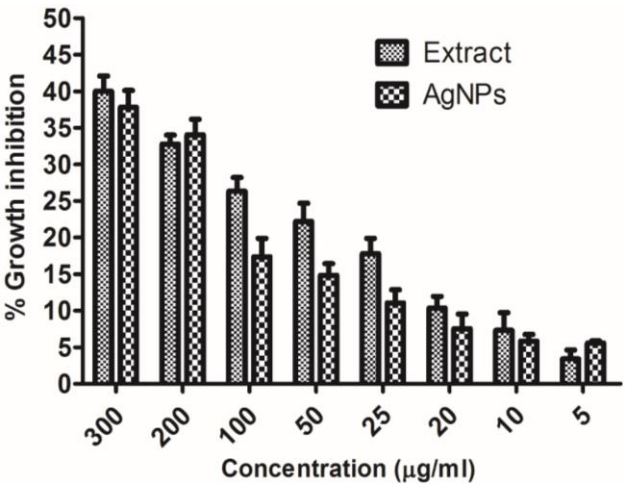

Fig. 5. Cytotoxicity testing of extract and nanoparticles in J774 macrophage cells using MTT cell viability assay.

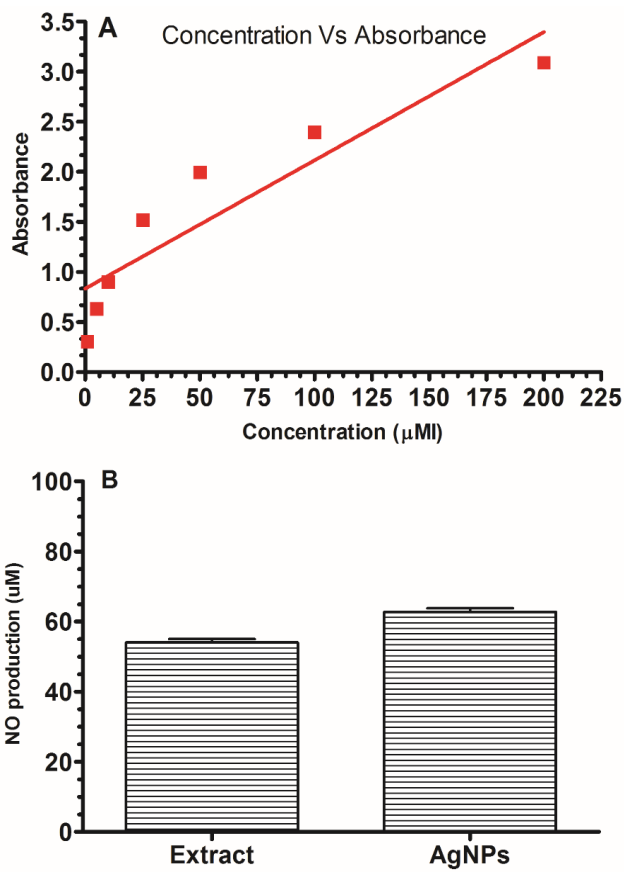

Fig. 6. Production of Nitrogen Oxide by activated macrophage cells (J774) against Leishmania tropica (A) trendline of $NaNO_2$ standard(B) Nitrogen Oxide produced.

Table 1. Infectivity index of *L. tropica* amastigotes in macrophage cells (J774).

Assay	% Infected	Parasite load	Infection index
Control	71 ± 1.04	3.02	*214.42
	62 ± 1.28	2.39	**148.18
Extract (8.16 µg/ml)	39 ± 1.33	1.24	*48.36
	28 ± 0.76	1.13	**31.64
AgNPs (4.8 µg/ml)	36 ± 1.03	1.38	*49.68
	23 ± 0.98	1.07	**24.61

*24 hrs of incubation, ** 48 hrs of incubation

Determination of nitrogen oxide level in macrophage cells: Nitrogen oxide concentration was estimated in the macrophages cells after interation of drug exposed leishmania promastigotes with macrophages cells. A standard curve of $NaNO_2$ was produced using concentration versus abosorbance (Fig. 6a). The unknown amount of nitorgen oxide (NO) produced by macrophages was estimated using the trendline. The results shows that the leaves extract produced 54.06±0.98 µM and nanoparticles produced 62.77±1.03 uM concentartion of nitrogen oxide. This high amount of nitrogen oxide free radical produced by macrophage cells indicate that it kills the leishmania parasites by producing nitrogen free radicals that are toxic to leishmania parasites.

Discussions

In the last few decades, noble metal nanoparticles particularly silver manifest significant role as compared to their other counterparts. Currently the nano particles attract the attention of many scientist in a wide arrays of applications. Recently biosynthetic methods using natural bioreducing agent from plants and other organism emerged as a new, simple and cost effective method for obtaining silver nanoparticles (AgNPs). Medicinal plnts are rich source of plant secondary metabolites that have various biological application (Rehman *et al.*, 2017; Aziz *et al.*, 2017), and coud be used for the reduction of metal ions into metal nanoparticles. Biogenic silver nanoparticles are extensively used as antibacterial, anticancer, antiparasiticand water purification of pathogenic microbes (Li *et al.*, 2008). It is cost effctive and easy to synthesised. İn the present study *Fagonia indica* which is an important anticancerous medicinal plant were used to synthesise the silver nanoparticles. The synthesised nanoparticles was characterized using UV-Vis spectoscopy, dynamic light scatering (DLS) and X-rays diffraction (XRD) methods. Characterization of the nanoparticles is significant in order to determine the size, shape, and delivery of the nanoparticle in a controlled way (Zhang *et al.*, 2007; Vilchis-Nestor *et al.*, 2008). The characterization data shows surface plasmon resonance at 415 nm which is specific for silver nanoparticles. the same was observed by Song and Kim (Song and Kim, 2009) in leaf extracts of Persimmon, Pine, Magnolia, Platanus and Ginkgo.The DLS data shows that the hydrodynamic size of the silver nanoparticle was in the nanoscale range which were further confirmed by the XRD pattern. The average size calculated was 56 nm with spherical symmetry.Our results are in close agreement with that of the earlier studies reported by many scientist using various plant extracts, such as, *Murraya koenigii* leaf (Philip *et al.*, 2011), *Cinnamomum zeylanicum* leaf (Smitha *et al.*, 2009), *Aloe vera* (Chandran *et al.*, 2006), *Camellia sinensis* (Loo *et al.*, 2012), *Mangifera indica* leaf (Philip, 2011). It has been reported that secondary metabolites in plants such as phenolics, flavonoid, saponon, tananis, and alkaloid are responsible for the bioreduction of metal nanoparticles (Li *et al.*, 2007).

Silver nanoparticles have been reported to have various biological application. Earlier reports shows AgNPs as antimicrobial (Dibrov *et al.*, 2002; Kaviya *et al.*, 2011; Hsueh *et al.*, 2015), antiparasitic (Allahverdiyev *et al.*, 2011; Gherbawy *et al.*, 2013; Saad *et al.*, 2015), anti tumour (Sriram *et al.*, 2010; Khalifa *et al.*, 2016; Mussa Farkhani *et al.*, 2016; Gomaa, 2017), antioxidant and anti-inflammatory properties (Abdel-Aziz *et al.*, 2014; El-Rafie and Hamed, 2014; Nagaich *et al.*, 2016). In our study we for the first time reported the antileishamnaila activities of green synthesised silver nanoparticles using Fagonia leaf extract. Our results shows that the synthesised nanoparticles and *Fagonia indica* leaf extract are not toxic to the macrophage cells, which is the host for the completion of life cycle of the leishmania parasites. However, we found that the nanoparticles is very effective (IC_{50}, 4.8 µg/ml) as compared to the crude extract (IC_{50}, 8 µg/ml). our results are in close agreement to that of Ahmad *et al*. (Ahmad *et al.*, 2015; Zahir *et al.*, 2015). The results also demonstarte that the leishmania parasites lose their ability to infect the macrophage cells after exposure to the nanoparticles for a short time period. It is suggested that the nanoparticles and extract interfere the surface proteins of the parasites that are responsible for infection. Further more, the amastigote form of the parasite lose its ability to enter the macrophage cells. This may be attributed that the macrophage cells release free radical on activation with non toxic concentration of the extract and or nanoparticles (Allahverdiyev *et al.*, 2011). For this nitrogen oxide level was estimated using griess reagent assay. The results show that macrophage produce elevated level of nitrogen oxide as indicated in the figure 6b. These eleveated level of nitrogen oxide free radical may induce oxidative stress that ultimately lead to the death of the parsites(Elcicek *et al.*, 2013). Overall, the results indicate that the antileishmanial activites of the extract and extract mediated silver nanoparticles was comparable. However, the nanoparticles showed an enhanced activity at low concentration compared to the crude extract as depicted by Ahmad *et al*. (Ahmad *et al.*, 2015). Further more, the silver nanoparticles synthesised from *Fagonia indica* leaf extrcat could be an alternative safe and effective antileishmanial agent. Since, this is the first report to investigate the antileishmanial effect of the AgNPs from *Fagonia indica.*So it is further suggested to identify the lead compound in *Fagonia ndica* extract and the molecules responsible for the bioreduction of silve nanoparticles.

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Competing interests: The authors declare that they have no competing interests.

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