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RESEARCH PAPER

Synthesis of silver nanoparticles by *Galega officinalis* and its hypoglycemic effects in type 1 diabetic rats

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ABSTRACT

Objective(s): Diabetes is related with the higher blood levels of liver enzymes and inflammatory factors. *Galega officinalis* is used as a medicinal plant for treatment of diabetes traditionally. In this work, silver nanoparticles (Ag-NPs) were synthesized with green method using *Galega officinalis* extract.

Materials and Methods: The synthesized green Ag-NPs were characterized completely. Intact or diabetic rats receieved intraperitoneal injection of saline or 2/5mg/Kg green synthesized Ag-NPs. Mean serum levels of glucose, hepatic enzymes and hematological parameter were determined. Gene expression of tumor necrotic factor alpha (TNF-α) was done by real-time PCR.

Results: Synthesis of green synthesized Ag-NPs was confirmed by FT-IR, XRD and UV-vis analyses. The FESEM and TEM images showed spherical Ag-NPs with size of 25 nm. The hypoglycemic influence of Ag-NPs using *Galega officinalis* extract is reported for the first time in this study. Blood concentration of liver enzymes, urea, glucose, white blood cells count and TNF-α mRNA levels in visceral adipose tissue significantly declined in diabetic rats receiving Ag-NPs.

Conclusion: The synthesized Ag-NPs using *Galega officinalis* extract may improve complication of diabetes via preventing liver hepatocyte damage and reducing inflammatory factors.

Keywords: *Diabetes; Galega officinalis; Liver enzymes; Silver nanoparticle; TNF-α*

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INTRODUCTION

In addition to high fasting blood sugar (FBS) and defect of insulin signaling pathway, diabetes is accompanied by cardiovascular, kidney and liver dysfunction [1]. Diabetes is related to the oxidative stress, and higher blood levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), creatinine and urea because of liver and kidney damage and increased inflammatory cytokines [1, 2]. Tumor necrosis factor-alpha (TNF-α) is a 157 amino acids peptide which is mainly produced by activated macrophages [2, 3]. TNF-α is one of the most important inflammatory cytokines which is released immediately after trauma and infection. In fact, higher production of TNF-α is a main marker in pathogenesis of tumor development, diabetes, polycystic ovary syndrome and other inflammatory diseases [2, 3].

The *Galega officinalis* belongs to the Fabaceae family and it contains different phytochemical ingredients including alkaloids, flavonoids, tannins, saponins and resins [4]. *Galega officinalis* as an herbal medicine exerts anti-obesity, strong antidiabetic effects and it significantly decreases FBS in diabetes [4]. Several studies demonstrated that the hypoglycemic effects of Galega are mainly due to its alkaloid components named galegine, which rise to the discovery of metformin drug [5[. In diabetic rats, *Galega* stimulates insulin secretion, it counteracts the adverse effects of diabetes on the kidney and liver and it improves dyslipidemia condition [6, 7].

Silver nanoparticles (Ag-NPs) have gained much interest because of their unique properties, such as chemical stability and important

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pharmaceutical effects [8, 9]. Green or chemically methods were usually used for preparation of Ag-NPs [8, 9]. Previous studies which reported anti-inflammatory and antidiabetic effects of chemically synthesized Ag-NPs, however their usage was limited due to some toxic effects. So recent studies are focused on green synthesized Ag-NPs which are more safe, bio-compatible and non-toxic than chemically prepared Ag-NPs [8, 10, 11]. Studies have shown that Ag-NPs can cross the blood-brain barrier, and they could enter different organs, including liver, spleen, kidney, brain and testis following oral, intraperitoneal or intravenous injection [12]. In this work, we synthesized Ag-NPs using *Galega officinalis* aqueous extract and tried to investigate the influences of them on the hematological factors, hepatic enzymes, glucose and gene expression levels of *TNF-α* in the adipose tissue of intact or type 1 diabetic rats.

MATERIALS AND METHODS

Materials

Galega officinalis plant was supplied from Dineh Iran Industries Complex. All reagents, silver nitrate ($AgNO_3$ 99%) and sodium hydroxide (NaOH) were supplied from Merck. Alloxan monohydrate was supplied from Sigma Aldrich, U.S.A. Glycosylated hemoglobin (HbA_1C) liver enzymes; albumin, glucose, creatinine and urea kits were supplied from Pars azmoon Co, Iran.

Animals

Twenty male Wistar rats were used. The weight of animals was 180-200 g. Rats were kept under controlled laboratory condition. Animals were maintained under 12 hr light, 12 hr dark cycle. Animals had free access to food and water except determined times which injection of alloxan for induction of type 1 diabetes or blood sampling were done in overnight fasted rats.

Characterization technique

Infrared spectra measured with Bomem MB-Series FT-IR spectrometer. XRD patterns measured on a Rigaku D/Max C III diffractometer using Ni-filtered Cu-Kα radiation (λ =1.5406 Å). Electronic spectra obtained using Shimadzu UV-2100 spectrophotometer. FESEM images prepared with TESCAN mira3 field-emission scanning electron microscope (FE-SEM, Brno, Czech Republic). TEM images prepared using Philips CM30 TEM (Eindhoven, Netherlands) operating at 300 kV.

Fig 1. *Galega officinalis* plant

Preparation of Galega officinalis extract and silver nanoparticles (Ag-NPs)

The *Galega officinalis* plant is shown in Fig 1. Powdered *Galega officinalis* plant (10 g in 100 ml of distilled water) was heated at 50-55 °C for 60 min. The obtained plant extract was filtered and used freshly for preparation of Ag-NPs

For synthesis of Ag-NPs, 50 ml of freshly prepared plant extract was heated at 50 °C, then $AgNO_3$ solution (25 ml, 1×10−2 M) was added dropwise to it. The pH of solution was adjusted on 11 by adding NaOH solution and was stirred at 50 °C [13, 14]. After a short time, the color of solution turned to brown and confirmed successful synthesis of Ag-NPs. It stirred for 2 hr, then the obtained Ag-NPs were purified through centrifugation at 12,000 rpm for 20 min. The formation process and the optical properties of the silver nanoparticles were identified from both the color change and UV-vis spectra.

Experimental design

First, the FBS level was measured in all 16 hr overnight fasted rats. Then, 150 mg/kg alloxan was dissolved in distilled water and it injected intraperitoneally to 15 fasted rats [15, 16]. To prevent severe hypoglycemia, 10% glucose solution was provided for animals for 24 hr. Also, saline was injected to five rats as a control group. The FBS concentration was measured in forth day after injection of alloxan by glucometer in overnight fasted rats.

In 5^{th} day of experiment, intact or diabetic rats received saline or 2.5 mg/kg Ag-NPs for two weeks. The drugs were injected intraperitoneally every morning at 9:00-9:30. Blood samples were collected one day following the last injection in 19^{th} day of experiment. Centrifugation at 3000 rpm for 15 min was used to separate serum specimen. The HbA_1C was measured by

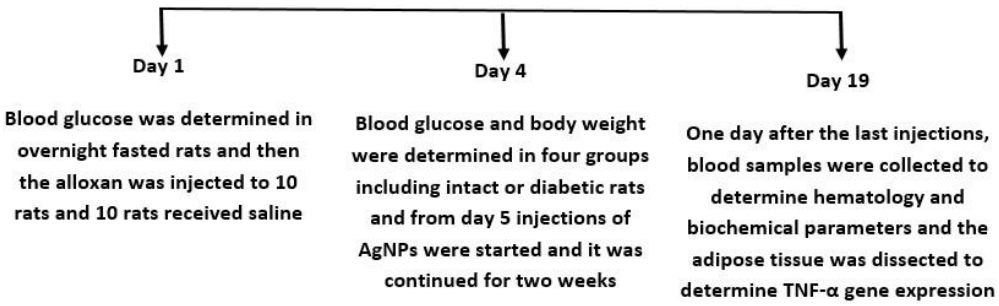

Fig 2. Study design, different treatments and times of sampling

immunoturbidimetric method and ALT, AST, GGT and ALP, albumin, FBS, creatinine, and urea was measured by photometric method. Hematological parameters were determined by using XS800i Sysmex automated hematology analyzer. Study design, different treatments and times of sampling was shown schematically in Fig 2.

To determine the gene expression of *TNFα*, animals were anesthetized by intraperitoneal injection of ketamine and xylezine. Visceral adipose tissue was dissected and stored at -80°C. Total RNA was isolated using PureZOL kit (Bio RAD, U.S.A). The cDNA was synthesized using 1µg of total RNA according to the manufacture of cDNA synthesis Kit (Thermo Scientific Co., U.S.A). Real-time PCR was done using corbett rotor gene 6000 detection system and SYBR Green I kit (Takara Bio Inc., Japan). The PCR cycling conditions were as following: first denaturation 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 secs, annealing at 6o °C for 20 secs (*TNFα* and *GAPDH*) and extension at 60 °C for 25 sec. Specific oligo nucleotide sequences for forward and reverse primers used were: *TNFα* forward 5′- ACTACGATGCTCAGAAACACACG -3′ and reverse 5′- AGACATCTTCAGCAGCCTTGTG -3′, and *GAPDH* forward: 5′-AAGTTCAACGGCACAGTCAAG-3′ and reverse: 5′- CATACTCAGCACCAGCATCAC-3′. Amplified products for *TNFα* and *GAPDH* were 198 and 120 base pairs respectively. The $2^{-\Delta\Delta CT}$ was used to calculate the changes in mRNA levels.

Statistical analysis

SPSS software version 16, one-way ANOVA and post hoc Tukey test were used for analysing the results. The results are shown as mean ± SEM. Significance was defined by $P \leq 0.05$.

RESULTS

Characterization of the Ag-NPs

FT-IR spectra of *Galega officinalis* extract and and Ag-NPs are inserted in Fig 3. The FT-IR spectrum of extract shows bands at 3365, 1620 and 1064 cm^{-1} corresponding respectively to O–H, C=O, and C–OH stretching bands. The observed bands at 2936 and 1411 cm^{-1} are related to the C–H stretching bands in *Galega officinalis* extract. For Ag-NPs, the characterization bands were observed at 1080, 1419, 1604, and 3380 cm^{-1}. According to Fig 3 (b), reduction of $AgNO_3$ solution with extract, cause a decrease in the intensity of bands at 3365 and 1064 cm^{-1}, also cause a redshift of these bands in compare with extract, both of these, confirm the involvement of hydroxy groups in reduction process. Also shift of observed sharp band at 1620 for extract to 1604 cm^{-1} (in FT-IR spectra of Ag-NPs) is correspond to the bonding of C=O groups with Ag-NPs [13, 14]. Also bands corresponding to stretching and deformation vibration of Ag−O observed respectively at 1610 and 650 cm^{-1}.

The crystalline nature of Ag-NPs was calculated by powder XRD analysis (Fig 3). The diffraction

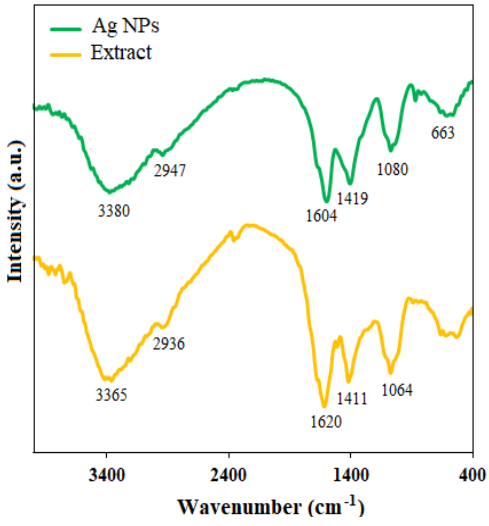

Fig 3. FT-IR spectra of extract and Ag-NPs

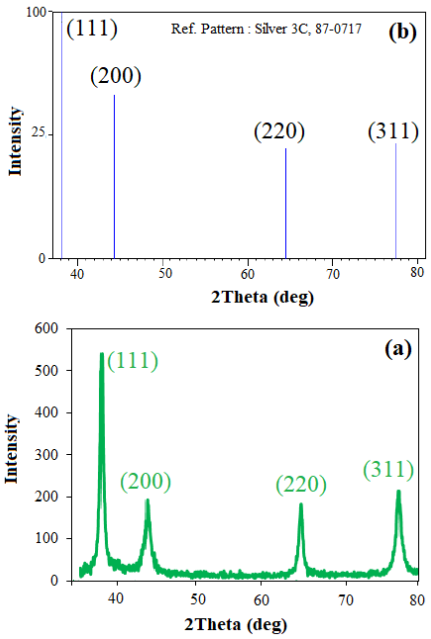

Fig 4. XRD patterns of (a) biosynthesized Ag-NPs and (b) JCPDS card of Ag-NPs

peaks observed at 2θ = 38.32 °, 44.37 °, 64.67 ° and 77.48 ° indexed to (111), (200), (220) and (311) reflection planes of cubic structure of Ag-NPs [17, 18]. Broadening of diffraction peaks confirm nanoscale of the synthesized Ag-NPs [13, 18]. The average crystallite size of the synthesized Ag-NPs calculated as 32 nm using Debye-Scherrer equation as following:

$$XRD = 0.9\,\lambda / (\beta\cos\theta) \qquad \text{Eq. 1}$$

Where XRD refers to average crystal size, λ is the wavelength of Cu Kα radiation, β is the corrected full-width at half-maximum of the main diffraction peak of (111), and θ is the Bragg angle.

According to Fig 5, UV-vis spectrum of extract demonstrated bands at 220 and 265 nm. For Ag-NPs, the bands of extract appeared at 212 and 270 nm as well as a sharp extinction band at 405

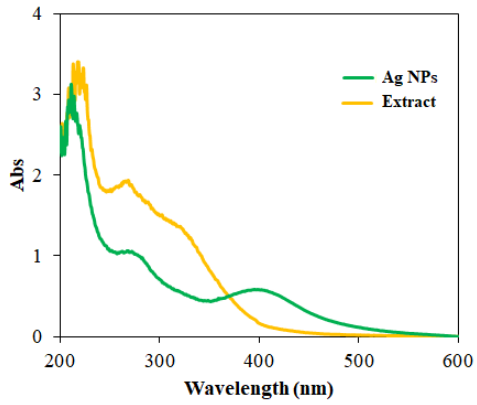

Fig 5. UV-vis spectra of plant extract and silver NPs

nm; this band is characteristic band of Ag-NPs and confirm the successful synthesis [14, 17].

FESEM and TEM images were used for studding the morphology and particle size. The FESEM images (Fig 6) revealed spherical morphologies for the Ag-NPs with partial aggregation.

TEM images of the silver nanoparticles revealed spherical morphology with small size and good disparity (Fig 7). TEM images revealed average particle size of 25 nm for Ag-NPs with homogeneous distribution.

Hematology and biochemical tests

Serum FBS concentration was determined in fasted overnight animals on 4th day. From 5th day of experiment, the animals received Ag-NPs for 14 days. One day after last injection in 19th day, the FBS was determined. According to the results in 19th day, the decline of mean serum FBS concentrations did not changed significantly in intact rats receiving Ag-NPs (group 2) in comparison to intact control rats (Fig 8). While in diabetic animals (group 3), the FBS level was significantly increased in comparison to intact control rats ($P \leq 0.05$, Fig 8). The serum FBS significantly declined in diabetic rats treated

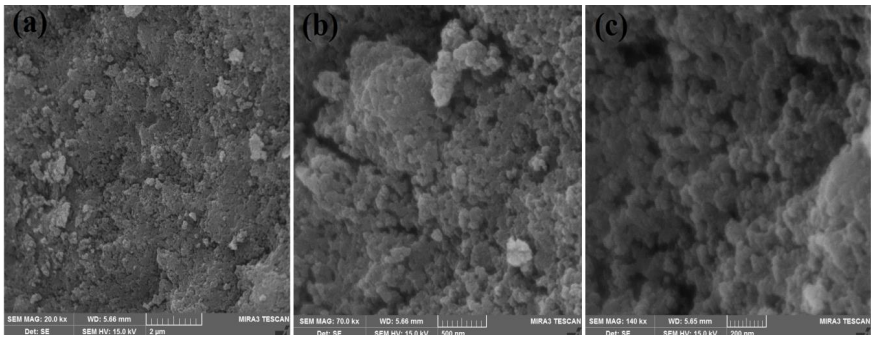

Fig 6. FESEM images of Ag-NPs

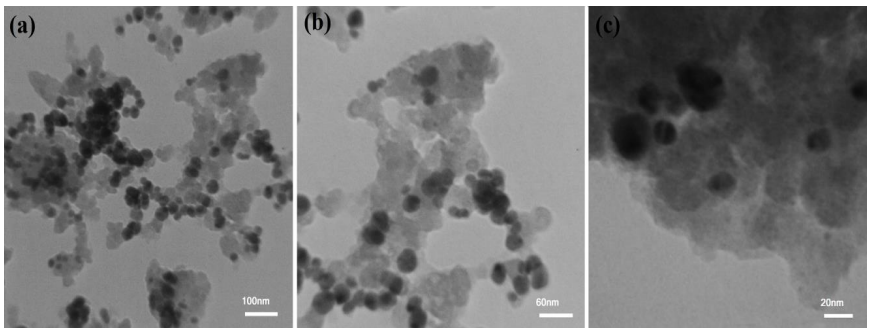

Fig 7. TEM images of Ag-NPs

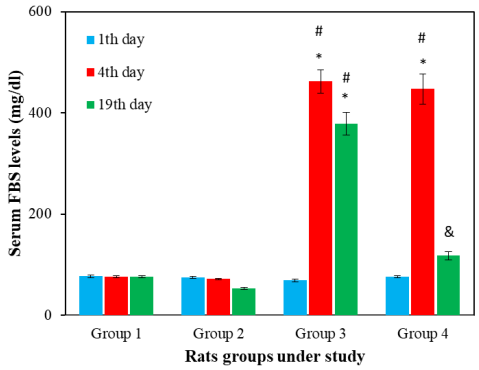

Fig 8. Influences of synthesized Ag-NPs using *Galega officinalis* on FBS in intact or diabetic rats. Comparison has been reported between different groups in 1th day, 4th day or 19th day (following the injections of drug from 5th day of experiment for 14 days). Group 1: intact control rats, group 2: intact rats receiving Ag-NPs, group 3: diabetic rats and group 4: diabetic rats receiving Ag-NPs. *: compared to intact control, #: compared to Ag-NPs, &: compared to diabetic rats

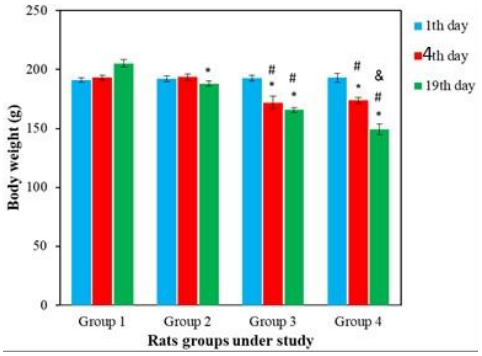

Fig 9. Effects of synthesized Ag-NPs using *Galega officinalis* on body weight in intact or diabetic rats. Comparison has been reported between different groups in 1th, 4th or 19th day (following the injections of drug from 5th day of experiment for 14 days). Group 1: intact control rats, group 2: intact rats receiving Ag-NPs, group 3: diabetic rats and group 4: diabetic rats receiving Ag-NPs. *: compared to intact control, #: compared to Ag-NPs, &: compared to diabetic rats

with the Ag-NPs (group 4) compared to diabetic control rats ($P \leq 0.05$, Fig 8).

As shown in Fig 9, in the 4th day, a significant decline was observed in mean body weight of diabetic animals compared to intact control rats. In 19th day (following the injections of drug from 5th day of experiment for 14 days), body weight declined significantly in intact rats receiving Ag-NPs, diabetic rats or diabetic rats injected with Ag-NPs compared to intact control group. Also, body weight of diabetic rats injected with Ag-NPs decreased significantly in comparison to diabetic control rats (Fig 9).

The effects of Ag-NPs on hematological parameters of rats were investigated and results are presented in Table 1. In 19th day of experiment (after receiving the drugs for 14 days), the mean red blood cells (RBC) count, mean hemoglobin concentration (HGB), hematocrit percentage (HCT), mean corposcular volume (MCV), mean corposcular hemoglobin (MCH), mean corposcular hemoglobin concentration (MCHC), red blood cell distribution width (RDW-CV) did not indicated any significant change in intact or diabetic rats treated with Ag-NPs. Injection of Ag-NPs into intact rats did not change mean white blood cells (WBC) count in comparison to intact control group. The WBC count decreased significantly diabetic animals treated with Ag-NPs in comparison to diabetic rats ($P \leq 0.05$, Table 1).

Mean platelests count did not changed after injection of Ag-NPs in comparison to intact control rats. Also diabetes causes a significant decline in platelests count compared to control group ($P \leq 0.05$, Table 1). In diabetic rats treated with Ag-NPs, the mean platelests count increased compared to diabetic rats (Table 1).

Effects of Ag-NPs on serum levels of hepatic enzymes, HA1C, albumin, urea and creatinine of rats were studied. As results show in Table 2, mean serum levels of ALT, AST, GGT, ALP, HbA_1C percentage, urea, and creatinine did not change

Table 1. Influences of Ag-NPs using *Galega officinalis* on hematological factors

Hematological factors	Group 1	Group 2 Intact	Group 3	Group 4
RBC (10^6/µL)	7.61± 0.21	8.15± 0.13	9.11± 0.16	8.42± 0.8
HGB (g/dL)	15.54± 0.17	15.8± 0.19	17.57± 0.37	15.83± 1.03
HCT (%)	48.5± 0.54	47.07± 0.68	56.1± 0.46	51.86± 2.87
MCV (fL or 10^{-15}L)	63.78± 1.5	57.8±0.98	61.8±2.19	58.31± 0.83
MCH (pg)	20.35±0.67	19.4±0.18	19±0.17	19.25±0.31
MCHC (g/dL)	31.85± 0.32	33.57±0.3	30.77±0.29	30.96±0.54
RDW-CV (%)	12.65±0.24	13.67±0.41	14.87±0.8	14.25±0.7
WBC (10^3/µL)	12/4± 0.74	10.16± 2	21.86± 2 $^{*,\#}$	10± 1.48$^{\&}$
Platelets (10^3/µL)	963.75± 55.25	1066.5±67.5	520.5± 85.35$^{*,\#}$	689±50.86$^{*,\#}$

Group 1: intact control rats, group 2: intact rats receiving Ag-NPs, group 3: diabetic rats and group 4: diabetic rats receiving Ag-NPs. *; compared to intact control, #; compared to Ag-NPs and &; compared to diabetic rats

in intact rats receiving Ag-NPs (group 2) in comparison with intact control group.

The albumin of diabetic rats treated with Ag-NPs increased significantly compared to the control rats ($P \leq 0.05$, Table 2). Blood ALT, AST, GGT, ALP, HbA_1C percentage and urea concentration of diabetic rats substantially increased compared to the intact control rats ($P \leq 0.05$, Table 2), while it has no effect on albumin and creatinine levels. Interestingly, injection of Ag-NPs to diabetic rats augments albumin and they declined ALT, AST, GGT, ALP, HbA_1C percentage and urea compared to the diabetic rats ($P \leq 0.05$, Table 2). Concentration of creatinine did not substantially alter in diabetic rats treated with Ag-NPs compared to the diabetic animals.

The TNF-α mRNA levels in adipose tissue

Effects of Ag-NPs on TNF-α mRNA levels was evaluated. According to Fig 10, the mean

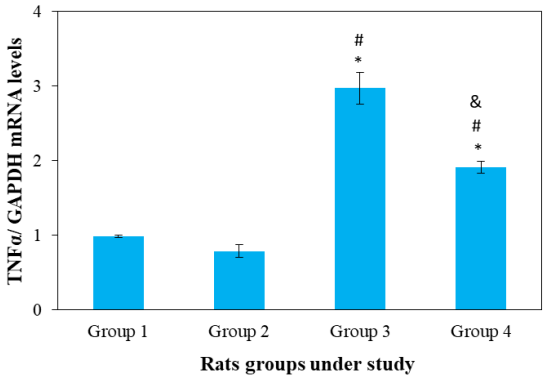

Fig 10. Influences of Ag-NPs using *Galega officinalis* on *TNF-α* gene expresion in four groups of rats under study. Group 1: intact control rats, group 2: intact rats receiving Ag-NPs, group 3: diabetic rats and group 4: diabetic rats receiving Ag-NPs. *; compared to intact control, #; compared to Ag-NPs and &; compared to diabetic rats

Table 2. Influences of Ag-NPs using *Galega officinalis* on serum levels of hepatic enzymes, HA1C, albumin, urea and creatinine

Serum biochemical factors	Group 1	Group 2	Group 3	Group 4
ALT(IU/L)	124.67±8.19	150± 9	1544.3±150.8$^{*,\#}$	244± 53$^{\&}$
AST(IU/L)	53± 1.73	66.33± 0.33	723± 50.8$^{*,\#}$	187± 46.76$^{\&}$
ALP(IU/L)	849± 53	505.33±7.85	3204±339.4$^{*,\#}$	2060±255.3$^{*,\#,\&}$
GGT(IU/L)	7.33±0.33	6.5±0.5	17±1.15$^{*,\#}$	12.33± 1.45$^{*,\#,\&}$
HA_1C (%)	4.78±0.41	4.84±0.1	9.2±0.35$^{*,\#}$	7.09±0.17$^{*,\#,\&}$
Albumin(g/dL)	2.83±0.33	3.4±0.11*	2.84±0.07	3.3±0.47$^{*,\&}$
Urea (mg/dL)	50.66±1.2	73.66±3.71	285.5±9.5$^{*,\#}$	156.33±17.2$^{*,\#,\&}$
Creatinine(mg/dL)	0.68±0.02	0.7±0.01	0.69±0.03	0.74±0.07

Group 1: intact control rats, group 2: intact rats receiving Ag-NPs, group 3: diabetic rats and group 4: diabetic rats receiving Ag-NPs. *; compared to intact control, #; compared to Ag-NPs and &; compared to diabetic rat

mRNA levels of *TNF-α* increased in diabetic rats compared to control group. While it decreased significantly in diabetic animals treated with Ag-NPs in comparison to diabetic control rats.

DISCUSSION

The results indicated a significant hypoglycemic effect of the synthesized Ag-NPs using *Galega officinalis*. Herein, Ag-NPs used dose was chosen based on their ability to decline glucose levels after two weeks of injection. The time duration and used dose of Ag-NP in the present study, was chosen based on previous study which demonstrated chemically synthesized Ag-NPs are able to decline FBS [18]. Different possible mechanisms may be supposed for the hypoglycemic action of Ag-NPs. The Galega officianalis is rich in polyphenolic components [19]. The hypoglycemic influence of Ag-NPs using *Galega officinalis* extract is reported for the first time in this study. Also, the polyphenol-rich diets have potent ability to decline blood FBS due to increasing insulin production or inhibiting the activity of carbohydrate digestive enzymes, such as salivary and pancreatic α-amylase or the membrane-bound enzyme of small intestine epithelium including α-glucosidase and disaccharidases [20, 21]. As previously demonstrated Ag-NPs or consuming the extract of polyphenol-enriched plants suppress the activity of these enzymes [21]. So, the inhibitory effects of Ag-NPs on α-amylase and α-glucosidase may contribute to the management of blood glucose of diabetic rats. The present research concentrates on activity of biosynthesized Ag-NPs to decrease the blood glucose level. However, further studies are necessary to check out the influences of Ag-NPs on carbohydrate digestive enzymes, gluconeogenesis, and glycogenolysis pathways.

The obtained results regarding to investigate the toxicity of chosen dose of injected Ag-NPs, demonstrate the harmless effects of it on hematological factors in control rats and they are in good agreement with previous literatures [22, 23]. Furthermore, the results revealed that the mean WBC count significantly increased in diabetic condition. The obtained data are in consistent with reported results which demonstrated the contribution of increased levels of inflammatory factors and WBC to diabetes [24-26].

There are contradictory results about the harmless hypoglycemic induced dose of chemically synthesized Ag-NPs on liver and kidney functions. Some studies have reported their effective influences on improving liver or kidney damage to diabetes [22, 23]. However, others have demonstrated that chemically synthesized Ag-NPs may exert harmful influences on biological functions, including different gene expression profiles, liver injury because of absorbance of toxic chemical materials [27]. Our results confirmed the harmless and improving effects of the green synthesized Ag-NPs on liver and kidney function in diabetes. Also, the data indicated inhibitory influences of Ag-NPs on TNF-α mRNA levels in diabetes. As previous studies reported the free radicals, higher inflammatory parameters and increased WBC are involved in pathogenesis of diabetes [28, 29[. *In vivo* and *in vitro* studies revealed that lower doses of chemically synthesized Ag-NPs could inhibit inflammatory adipokines such as interleukin-1 (IL-1), interleukin-12 (IL-12), interleukin-6 (IL-6), TNF-α and other ones [30, 31]. The improving effects of green synthesized Ag-NPs using *Galega officinalis* on WBC and liver damage could mediated via decreasing pro-inflammatory cytokines including TNF-α. To find some other effective mechanisms it is suggested that further studies try to investigate the effects of Ag-NPs using *Galega officinalis* aqueous extract on anti-inflammatory and antioxidant mediators.

CONCLUSIONS

In this work, Ag-NPs were synthesized with green method using *Galega officinalis* aqueous extract. The synthesized Ag-NPs have high crystallinity and phase purity with spherical morphologies and particle size of 25 nm. The synthesized Ag-NPs using *Galega officinalis* exert hypoglycemic influences and they exert improving effects on the kidney function and hematological parameters associated with alloxan- induced diabetes. Also the synthesized Ag-NPs show inhibitory effect on increased serum levels of liver enzymes and gene expression of *TNF-α* in diabetic rats. Due to simplicity, safe, eco-friendly, cheap and non-toxic consideration, the synthesized Ag-NPs using *Galega officinalis* may be a suitable replacement for chemically synthesized Ag-NPs to improve the complications of diabetes. They may improve insulin resistance via preventing liver hepatocyte damage and reducing inflammatory factors.

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University of Mohaghegh Ardabili. The authors are grateful to University of Mohaghegh Ardabili and Shahid Beheshti University for supplying the apparatus. Also the authors are thankful to Dineh Iran Industries Complex for providing *Galega officinalis* plant.

ETHICS APPROVAL

This study was approved by the Research and Ethics Committee of Ardabil University of Medical Sciences (IR.ARUMS.REC.1399.355).

CONFLICT OF INTEREST

The authors have nothing to disclose. The authors declare no competing interests.

AUTHOR'S CONTRIBUTIONS

Fariba Mahmoudi: Project definition, statistical analysis, interpretation of results, Farzaneh Mahmoudi: synthesis, characterization and validation of Ag-NPs; Fariba Azimi: Injections of drugs, blood and tissue sampling, statistical analysis and interpretation of results. Mostafa M. Amini: Validation of synthesized Ag-NPs. All authors contributed to the preparation of the present manuscript.

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