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Molecular study on the effect of dandelion leaf extract and silver nanoparticles in induced diabetic albino rats

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***Abstract*---**The present study investigated the antidiabetic effects of dandelion leaf extract and silver nanoparticles on diabetic albino rats at molecular level, and compared with insulin. Silver nanoparticles were prepared by green chimestry method using dandelion leaf extract as capping and reducing agent, which reduced Silver nitrate (AgNO3) into silver nanoparticles. The morphology, size and crystalline structure, of silver nanoparticles are confirmed by ultraviolet, Scanning electron microscope technique, and X-ray diffraction respectively. The formation and stability of the reduced AgNPs in colloidal solution were detected and monitored by using UV-visible spectroscopy, and the absorption peak appear at 450 nm indicating the specific surface Plasmon resonance of AgNPs. The Scanning Electron Microscope showed that the AgNPs have spherical shape with aparticular size ranging from (13 to 28)nm.The chemical characterization of synthesized AgNPs was tested using the X-ray diffraction technique, which shows that the average crystalline size was 16nm, and the synthesized silver nanoparticles have a polycrystalline structure. Application of nanoparticles in medicine is an attractive proposition. The present study included 35 female wistar albino rats weighting 200±20 grams. Animals were grouped randomly into five groups, (each group = 7) as follows: first group as healthy non diabetic control (control negative) did not receive any type of treatment. The other groups treated intraperitoneally once with 50 mg/kg b.w of streptozotocin, then when these rats became hyperglycemic, they divided into four groups as following: Diabetic group (control positive) did not recieve any type of treatment,

(G3):Diabetic rats treated subcutaneous daily dose of mixed insulin 2 units/kg b.w., (G4):Diabetic rats treated orally daily dose with dandelion leaf extract of 30mg/kg of b.w and, (G5): Diabetic rats treated intraperitoneally daily dose with AgNPs 2.5mg/kg of b.w. for 4 weeks. The molecular study carried out by using Real-Time PCR technique (RT-qPCR) to determine the level of gene expression of insulin -like growth factor 1 and insulin- like growth factor 2 in hippocampal tissues of the studied groups. The results were as follows: the expression of Igf1 decreased significantly in the diabetic group (positive control) compared to the normal group (negative control) ($p < 0.05$), while its expression showed a significant increase in the insulin-treated group compared with the positive control group ($p < 0.05$), while there was a non-significant increase in the expression of Igf 1 in the diabetic groups treated with dandelion leaf extract and AgNPs when compared to the positive control group ($p>0.05$). The expression of Igf 2 was significantly decreased in the control positive group when compared with the control negative group ($p< 0.05$). It was also significantly decreased in the insulin, silver nanoparticles, and dandelion treated groups compared with the control negative group ($p< 0.05$). In Conclusion, dandelion leaf extract and silver nanoparticles have no significant effect on the expression of Igf 1 and Igf 2. However, further research is required to find out the exact mechanisms of dandelion extract and silver nanoparticles responsible for antidiabetic activities and increasing search for improved antidiabetic treatments as an attempt to eliminate the side effects linked with insulin injections.

***Keywords*---**dandelion leaf extract, diabetic albino rats, antidiabetic.

Introduction

Diabetes mellitus is a metabolic syndrome marked by hyperglycemia and abnormal alterations in lipid profiles[1]**.** Chronic hyperglycemia and excessive lipid levels cause oxidative stress and an increase in inflammatory mediators, which damage pancreatic beta cells[2]**.** Hypoglycemia and insulin resistance are two of the most common side effects of traditional diabetes treatment, which includes oral hypoglycemic medications and insulin injections [3]., Nanotechnology is a rapidly developing science that has applications in a wide range of fields, including engineering, biology, chemistry, medicine, and physics, and blurs traditional boundaries between them[4]**.** Silver nanoparticles (AgNPs) have gained a lot of attention among noble metal nanoparticles because of their distinctive properties, such as high electrical conductivity, chemical stability, and catalytic and antibacterial activity[5]. Silver exhibits different characteristics at the nanoscale than it does in bulk[6]**.** The primary ingredients of dandelion root include taraxacin, taraxacerin, inulin gluten, gum, and potash. Taraxacum officinale is also known as Dandelion. Dandelions are one of nature's most plentiful green vegetable sources of beta-carotene, the precursor to vitamin A, and the whole plant can be used for both medicinal and culinary purposes. Dandelion is used medicinally as an anti-diabetic, detoxicant, aperient, and diuretic[7].

Mammals have two insulin-like growth factors (**IGF-1** and **IGF2**) that are key mediators of somatic growth, tissue differentiation, and cellular responses to stress [8]. the IGF system has an imperative pathophysiological role across a range of metabolic abnormalities, including obesity, insulin resistance (IR), and diabetes [9]. IGF1 enhances the phosphorylation of the pro-apoptosis factor and increases the level of Bcl-2 anti-apoptotic protein via the phosphatidylinositol 3 (PI3) kinase pathway in brain cells [10]. Alterations in circulating IGF-I concentration can also result from hepatic disease, renal dysfunction, and diabetes mellitus [11]. This work used albino rats as a model to produce silver nanoparticles utilizing a green chemistry method using dandelion leaf extract and investigate their antidiabetic effects at the molecular level.

Materials and Methods

Preparation of Aqueous Leaf Extract of Dandelion

Taraxacum officinale leaves were gathered and washed several times with water, then sterilized with 50% alcohol for 5 minutes to eliminate foreign elements including dust, particles, and fungal spores, then washed with distilled water more than 5 times, then sun-dried to remove any remaining moisture. The leaves were cut into small pieces, and 5gm of finely chopped leaves were placed in a cleaned and sanitized 250 ml Erlenmeyer flask with 125ml distilled water, which was then heated to 95°C in a water bath for 20 minutes to prepare the broth solution. The plant extracts (broth) were cooled to room temperature before being filtered with 0.6 size Whitman filter paper[12]. The prepared dandelion leaves extract showed red color, as illustrated in figure 1, the broth stored in refrigerator ($3C^{o}$) for further study.

Synthesis of AgNPs

9ml of 1mM silver nitrate $AgNO_3$ (purchased from Sigma-Aldrich, Germany) solution was combined with 1ml of dandelion leaf extract and stirred for 15 minutes at 60 ^{o}C on a thermal stirrer. When the color shifted from pale yellow to dark brown, it was an excellent indicator of AgNPs synthesis. The suspended particles were centrifuged at 4000 rpm for 1 hour and rinsed three times with distilled water. The pellet of silver nanoparticles was dried in an oven at 40 ^{o}C for three hours, the powder was stored in a dark [13].

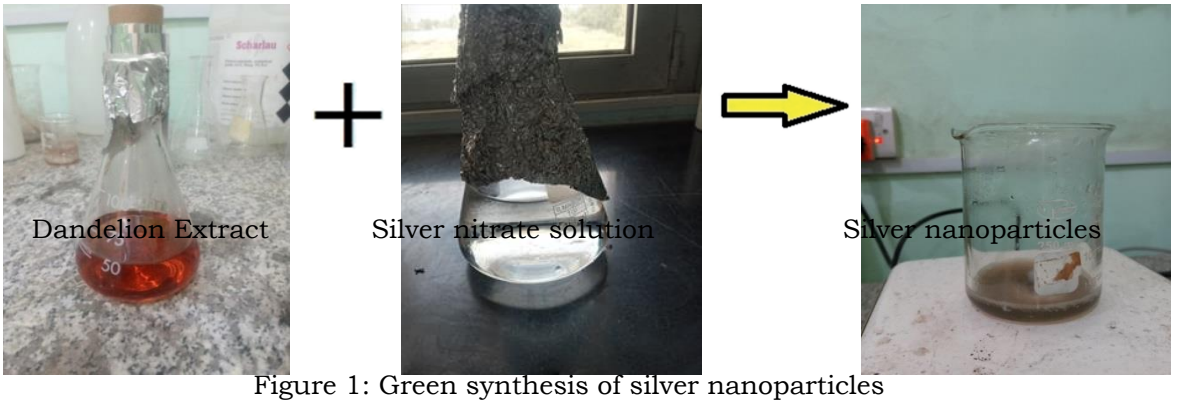

Figure 1: Green synthesis of silver nanoparticles

The synthesized AgNPs were tested using UV-visible spectroscopy, Scanning electron microscope (SEM), and X-ray diffraction (XRD). The UV-visible spectra were investigated over a 300–900 nm range with a UV-1600 series UV-visible spectrophotometer (Shimadzu Corporation, Japan). Scanning Electron Microscope (SEM) analysis was done to characterize the morphology of nanoparticles by using (JEOLJSM 5700 SEM machine, Holland). The structure of the synthesized AgNPs was examined via XRD (XRD-6000; Shimadzu Corporation, Japan). The XRD patterns were recorded at a scan speed of 4°/min.

Animals Experiment

35 healthy female wistar albino rats weighting 200±20 g were used in the experiment. All animals were maintained under a constant 12-hrs-light/12-hrs-dark exposure cycle at temperature around 25◦C and were fed with standard diet pellet and water *ad libitum*. The experiment was achieved under the supervision and approval of the Animals Ethical Committee at the University of Kufa.

Induction of Diabetes

According to the procedure of [14], prior to diabetes induction, the rats were restrained from food for 14 hours; they were only access to water. Then, diabetes was induced by intraperitoneal injection of 0.6 ml of freshly prepared solution of streptozotocin (STZ) (50 mg/kg, i.p) dissolved in distilled cold normal saline. In addition, equivalent volume of normal saline was injected to rats in the control group. After STZ injection, the rats freely had an access to water and food. In order to counter any possible development of hypoglycemic shock, they were given 5% glucose solution for 48 hours instead of water to reduce the severity after initial hypoglycemic stage in the body of rats, as STZ damaged the pancreatic B-cells and caused releasing of insulin in large amounts. Five days post induction, Accu-Chek Instant S glucometer (Roche, Mannheim, Germany) was used to determine the fasting blood glucose level of the rats. Rats with fasting glycemia more than 250 mg/dl were used as diabetic.

Experimental Design

The rats were divided randomly into five groups, each group contain seven animals and treated as the folowing:
Control negative: Healthy non diabetic rats did not receive any type of treatment.
Control positive: Diabetic rats did not receive any type of treatment.
G3: Diabetic rats treated subcutaneous daily dose of mixed insulin
2 units/kg b.w [15]
G4: Diabetic rats treated orally daily dose with dandelion leaf extract of 30mg/kg of b.w [12]
G5: Diabetic rats intraperitonially daily dose with AgNPs 2.5mg/kg of b.w. [16]
The period of experiment was 4 weeks. At the end of the experiment, the rats were fasted overnight, than euthanized under general anesthesia and sacrified. Sample from hippocampus were taken in order to measure the expression levels of insulin like growth factor 1 gene (Igf1) and insulin like growth factor2 gene (Igf2) by using real- time PCR technique.

Molecular Study

In this study, real-time qPCR technique was used to evaluate the levels of the expression of *Igf* 1 and *Igf* 2 in the hippocampus of rats.

Total RNA extraction by use Easy-spin™ (DNA free) total RNA extraction Kit

1. 100mg fresh hippocampal tissues were prepared.
2. 1ml of Lysis Buffer was add and homogenized tissue sample using a homogenizer.
3. the sample was vigorouslied by vortex for 10 sec. in room temperature.
4. 200µl of Chloroform was add and mixed by vortex very well.
5. the sample was centrifuged for 10 min. at 13,000 rpm (4°C), then transferal 400µl of the top liquid to an empty 1.5ml eppendorf tube.
6. 400 µl of Obligatory Buffer was add and gently mixed with a pipette, then set aside for 1 minute at room temperature.
7. After that, the top solution was transfered to the spin pilaster and centrifuged for 30 seconds at 13,000rpm. The spin column was replaced in the same 2 mL collection tube as the flow-through. Then it was repeated again.
8. The spin column was filled with 700 µl of wash buffer A. Then the tubes was covered gently and centrifuged at 13,000 rpm for 30 s. Then the spin column was putted into the same 2 mL collection tube as the flow-through and discard the flow
9. The spin column was washed by adding 700 µl of buffer B to it and centrifuged for 30 sec. at 13,000 rpm. Discard the flow by placing the spin column back into the same 2 ml collection tube.
10. Next, the pillar membrane was centrifuged for 1-2 minutes at 13,000 rpm to dry it.
11. the spin column was placed in a clean eppendrof tube (1.5ml) and pour 50µl of Elution Buffer straight over the membrane. Nurture for 1 min. at ambient temperature, after that, centrifuge for 1 min. at 13,000rpm to extract RNA.

Preparation of the primers

According to instruction of the primer synthesizer company, the primers (originally lyophilized), were dissolved in the free ddH2O to obtain a final concentration of 100 pM/µl which served as a stock solution that stored at -20°C. Aconcentration of 10 pM/µl was prepared as work solution working.

Primers of gene expression experiment:
Primers were used in this project are shown in Table 1.

Table 1: Characteristics of primers of IGF1, IGF2 and CYPA genes.

Target gene	Primer name	5'-3'	Pcr product	Reference
IGF1	F	CTGGTGGACGCTCTTCAGTTC	112 bp	Designed
	R	ACTCATCCACAATGCCCGT		
IGF2	F	GCTTGTTGACACGCTTCAGT	153 bp	Designed
	R	GGGGTGGCACAGTATGTCTC		
CYPA	F	TATCTGCACTGCCAAGACTGAGG	127 bp	[17]
	R	CTTCTTGCTGGTCTTGCCATTCC		

Protocol of GoTaq® 1-Step RT-qPCR System for Real-Time PCR (Gene expression assay):

1- A real-time instrument for one-step RT-PCR was Set up in standard or fast mode. (Table 3).
2- The GoTaq® 1-Step RT-PCR Scheme components, including RNA masters and primer pairs, was Melted in an ice box at room temperature. The thawed component tubes was Mixed thoroughly right away. All reagents was kept in an ice box.
3- The dilution RNA samples were prepared by nuclease free water to get final concentration {mRNA (500fg–100 ng)} in water or other quantitative PCR a diluent that is compatible.
4- All reaction components which show in table 2 were mixed in tube and put in ice box. After each step, the mixture was mixed gently by pipetting, than collected all reaction volumes and transfer on plate.
5- The plate was transferred from the ice box to the instrument that has been pre-programmed. Begin running right away.
6- After the run was finished, the data was collected and the data was analyzed.

Table 2: Preparation of Real-Time PCR solutions

Components	Concentration	Volume (20µl)
GoTaqTM qPCR master mix, 2x	1x	10 µl
Forward primer	10 µM/µl	2µl
Reverse primer	10 µM/µl	2 µl
GoScriptTM RT mix for 1-step RT-PCR	1x	0.4 µl
ddH_2O	-	3.6 µl
RNA template	250 ng	2µl

Table 3: Real-Time PCR conditions (According to the instruction of GoTaq® 1-Step RT-qPCR System)

Stage	Temp. (°C)	time	cycles
Reverse transcription	42	15min	1
RT inactivation/Hot-start activation	95	10min	1x
Denaturation	95	10sec.	40x
Annealing/data collection	60	30sec.	
Extension	72	30sec.	
Dissociation	72	2min	1x

Statistical Examination

The results were reported as mean± SEM and analyzed by using the GraphPad Prism software package (GraphPad Software Inc., San Diego, CA, USA). Groups were compared by one-way ANOVA followed by Tukey's multiple comparison tests. Value of $p < 0.05$ was considered to be statistically significant.

Results

Characterization of the prepared silver nanoparticles

The Ag nanoparticles' UV–visible absorption spectra were recorded and presented in figure 2. Metal NPs have free electrons, which yield a surface plasmon resonance (SPR) absorption band, due to the mutual vibration of electrons of metal nanoparticles in resonance with light wave. It is also observed that the surface plasmon peak that occurs at 450 nm. The appearances of the peaks show the characteristics of surface plasmon resonance of silver nanoparticles.
The produced nanoparticles have a spherical shape, as shown in Figure 3, and an excellent distribution together with the sample, according to SEM images. The particle size ranged from 13 to 28 nanometers, with an average particle size of 19.5 nanometers.

The XRD investigation revealed peaks with orientations of (111), (200), (220), and (311) at 37.90°, 44.30°, 64.50°, and 76.80°, respectively (figure4), which is typical of AgNPs structure. The low intensity line widening of the diffraction peaks indicated that the produced materials were in the nanometre range. Using the Scherrer equation, the average crystallite size was determined based on the full width half maximum of the diffraction peaks, it found around 16nm.

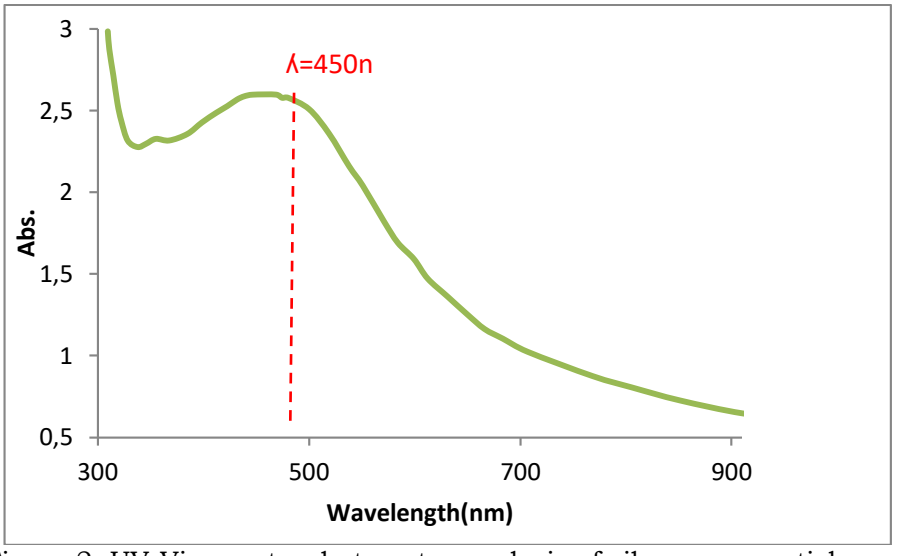

Figure 2: UV-Vis spectrophotometer analysis of silver nanoparticles.

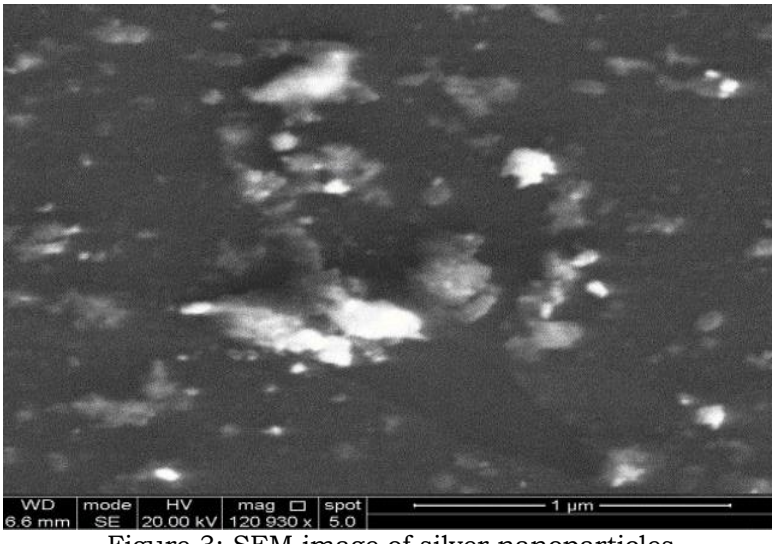

Figure 3: SEM image of silver nanoparticles.

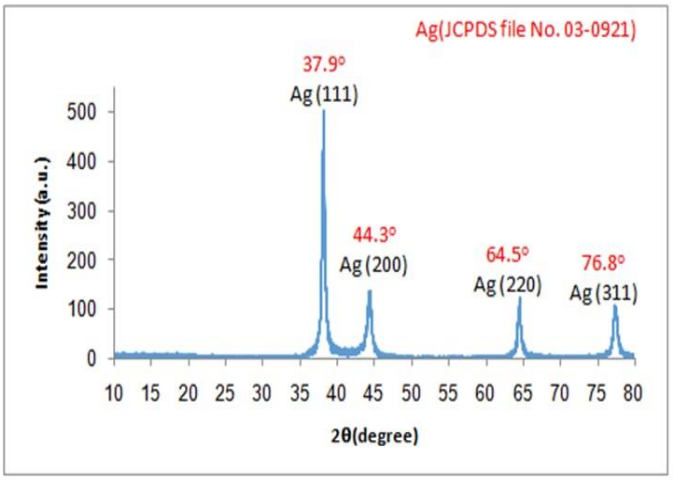

Figure 4: XRD patterns of silver nanoparticles

Molecular Examination

In order to assess the effect of the dandelion leaf extract and silver nanoparticles compared with insulin on the DM-associated neuropathy in diabetic albino rats, the hippocampal expression of Igf 1 and Igf 2 were assessed using qRT-PCR. The ΔΔCt values of genes in the studied groups were obtained from the difference of ΔCt in the treatment groups compared with the control group. Moreover, the formula $2^{-\Delta\Delta Ct}$ was used for the expression of genes, which provides comparative values in the statistical standpoint.

Expression of Igf 1 in the hippocampus of the studied groups:

(Figure 5) shows expression of *Igf1* in the hippocampus of studied groups. It was noticed that mRNA of *Igf 1* was significantly downregulated in the control positive group compared with the control negative group ($p<0.05$), while mRNA of *Igf 1* was significantly upregulated in the insulin treated group compared with the control positive group ($p<0.05$). In the diabetic groups treated with silver nanoparticles and dandelion leaf extract, show nonsignificant increase in the expression of mRNA of *Igf 1* compared with the control positive group ($p>0.05$).

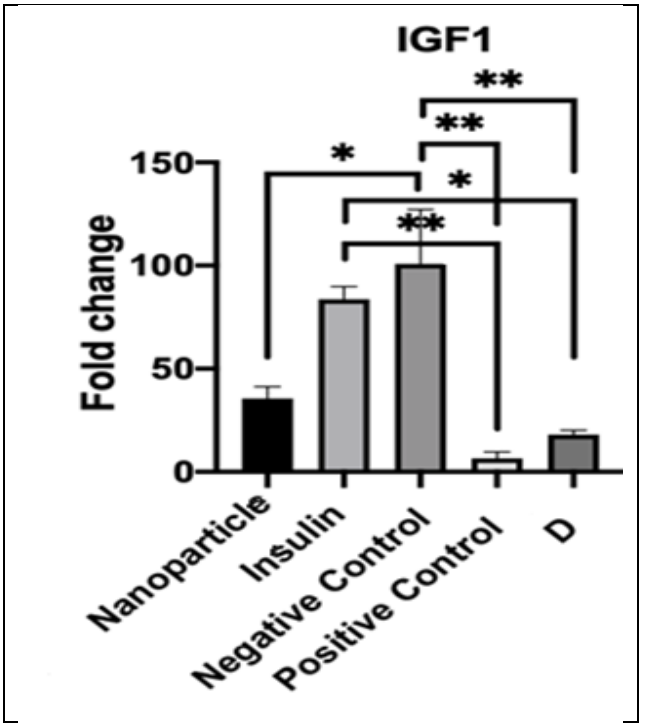

Figure 5: Relative expression of *Igf* 1 mRNA in hippocampus of studied groups; Results were presented in the form of mean± standard deviation (SD). Comparison between groups was done using one-way ANOVA test followed by Tukey's multiple comparison test. Significance was considered at $p < 0.05$, *Cypa* used as house keeping gene.
* significant, ** more significant D= dandelion extract

Expression of Igf 2 in the hippocampus of the studied groups:

(Figure 6) shows expression of *Igf 2* in the hippocampus, it was noticed that mRNA of *Igf2* was significantly down regulated in control positive group compared with control negative group ($p<0.05$). While there was a nonsignificant increase in the expression of mRNA of *Igf 2* in insulin, silver nanoparticles and dandelion treated groups compared with control positive group ($p>0.05$).

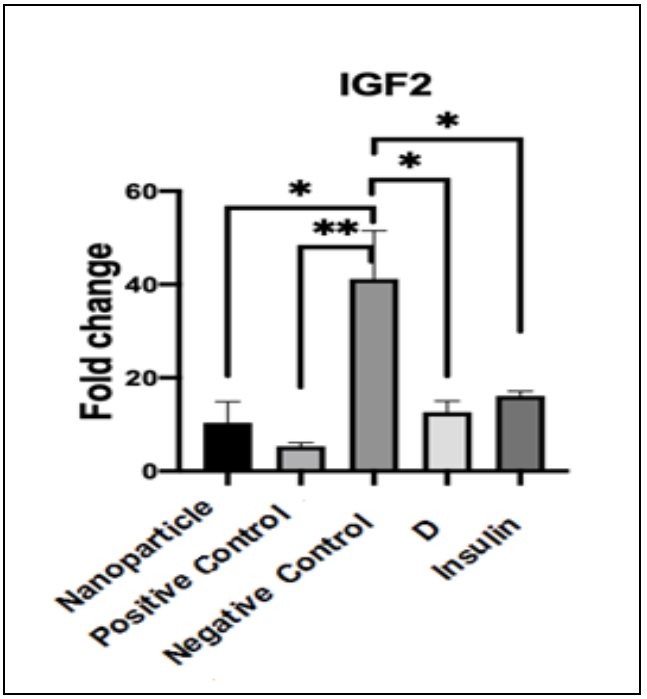

Figure 6: Relative expression of *Igf* 2 mRNA in the hippocampus of studied groups; Results were presented in the form of mean± standard deviation (SD). Comparison between groups was done using one-way ANOVA test followed by Tukey's multiple comparison test. Significance was considered at $p < 0.05$, *Cypa* used as house keeping gene.
* significant, ** more significant
D= dandelion extract

Discussion

Plants extracts contain biomolecules including polyphenols, ascorbic acid, flavonoids, sterols, triterpenes, alkaloids, alcoholic compounds, polysaccharides, saponins, β-phenylethylamines, glucose and fructose, and proteins/enzymes which could be used as reductant to react with silver ions and therefore used as scaffolds to direct the formation of AgNPs in the solution [18]. Reduction of Ag+ resulted in the color change from colorless to intense brown due to the formation of AgNPs which reflected bioreduction of Ag+ to Ag^0. The change in appearance seemed due to excitation of surface plasmon vibrations/ resonance in the AgNPs, depicted on the concentration of Ag ion revealed attachment of biomolecules forming the NPs and finally change in color [19]. The progress of reduction of silver nitrate to AgNPs can be easily evaluated using a UV-Visible spectrophotometer. The result showed strong and characteristic surface Plasmon resonance centered at 450 nm, this is because AgNPs can absorb light in the visible region due to the surface Plasmon resonance phenomenon based on their size and shape [20]. Scanning electron microscopic analysis (SEM) of the synthesized AgNPs show nanoparticles with spherical shape and homogenous distribution through the glass substrate. The white big shape of particle refers to aggregation due to Van der Wals bond and this means that the colloidal needs

more sonication time [21]. The X-Ray Diffraction (XRD) Analysis results of the present study revealed that AgNPs crystalline with face-centred cubic (fcc) structure. In both cases, the peak corresponding to the (111) plane are more intense than the peaks of other planes. Interestingly, most of researchers[16],[22],[23], that synthesized the nanoparticles using plant extracts seem to obtained a similar crystal structure.

IGFs are neurotrophic factors capable of supporting neurite outgrowth and endurance in peripheral and central neurons [24]. IGF-1 has similar structures and functions like those of insulin particularly for peripheral uptake of glucose and fatty acids [25]. IGF-1 has multiple effects in the CNS, regulating early brain development, myelination, synapse formation, adult neurogenesis, production of neurotransmitters and cognition [26].

Furthermore, it is usually considered that IGF-1 is a potent neuroprotective compound, and that this is, at least partially, due to inhibition of neuroinflammation [27]. IGF-2 actions have been poorly characterised, however relevant roles have been determined for foetus development and cerebral protection [28]. Other study found that IGF-II plays an important role, especially in prenatal growth [29]. Growth hormone is produced by the anterior part of the pituitary, GH-releasing hormone (GHRH) and somatostatin, which are secreted by hypothalamic neurons into the hypophyseal portal circulation, are important peptides regulating the synthesis and pulsatile release of pituitary GH [30]. GH crosses the blood-brain barrier and IGF-I expressed in brain may be regulated by GH in a region-specific manner [31]. Insulin-like growth factor inhibits GH secretion through endocrine negative feedback [32]. Several lines of evidence suggest that regional deficiency of insulin in the portal circulation, a condition that persists in all conventionally treated patients with type 1 diabetes, produces dysregulation of the GH-IGF-IGFBP axis [33]. Decreased insulin production can lead to hyperglycemia, hyperglycemia causes oxidative stress conditions and may be cause damage in the pituitary growth hormone- releasing hormone receptors that results in the decreased secretion of GH, when GH secretion decreases, so does the IGF-1 production [34].

In diabetes, IGFs activity is reduced [35]. Other study found that T1D is characterized by both a decrease in IGF-1 levels and an increase in sequestration of IGFs by higher levels of IGFBP-1 resulting in further decrease in bioavailable free IGFs [33]. Huangs et al; found the level of IGF1 in rats with streptozotocin-induced diabetes decreases [36]. All these studies agree with the results of the present study, in which the level of expression of IGF1 and IGF2 in hippocamus of diabetic rats was significant decreases as compared with control negative group.

GH resistance with low IGF-I as is frequently seen in patients with T1D is often related to portal hypoinsulization, and down regulation of growth hormone receptors [37]. IGFBP-1 is regulated mainly by insulin, it interacts with IGF-1 and IGF-2, and is used as a shuttle for IGFs to target tissues and regulate the action of free IGF-1,IGFPB-1 is regarded as the primary regulator of IGF-1 bioactivity, portal hypoinsulinization of T1D leads to higher amounts of IGFBP-1, which decreases accessible bioactive IGF-1, these effects are the concomitant chronic inflammation accompanying T1D and has an important part in the progression of

diabetes and diabetes-related complications [38]. Insulin and IGF-1 exercise their effects by interacting with their corresponding receptors, namely the insulin receptor and IGF-1 receptor (IR and IGF-1R), the receptor-ligand interactions induce intracellular signaling cascades resulting in metabolic or mitogenic effects and are involved in the regulation of metabolism [39].

IGF-1 is necessary for normal insulin sensitivity, impairment in IGF-1 synthesis results in insulin resistance [38]. All these mechanisms may explain the significant upregulation of IGF1 gene expression in diabetic rats treated with insulin compared with the control positive group. The level of IGF1 and IGF2 expression was a non-significant increase in the AgNPs treated group as compared with the control positive group. According to Afifi and Abdelazim (2015), it was possible because AgNPs treatment of diabetic rats prevented the decline in SOD, CAT, GRD, and GPx activity and mRNA expression levels. The high activity and expression levels of antioxidant enzymes in the brains of AgNPs-administered rats may be due to the effect of these nanoparticles on improving insulin secretion and ameliorating the oxidative stress induced by impairment of glucose homeostasis [40].

Extract from the leaves of dandelion exhibited significant antioxidant activity according to [41]. The antioxidant activity of these extracts seems to be related to the presence of phenolic compounds in the leaves of dandelion [42]. The presence of antioxidants in silver nanoparticles and dandelion extract can reduce oxidative stress, so that the pituitary GHRH receptor synthesis may be returned to normal and the production of GH and IGF-1 will be improved.

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