

Datura Stramonium leaves extract Silver Nanoparticles regulates PINK1 gene in Parkinson's disease model of *Drosophila melanogaster*

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Abstract

In recent years, several eco-friendly processes for the synthesis of silver nanoparticles (AgNPs) are increasing; they have tremendous applications in the field of diagnosis and therapeutic values for various diseases /disorders. In this article the green synthesis of *Datura stramonium* leaves extract silver nanoparticles (DS-AgNPs) was carried out and are characterized for different properties like reduction of Ag^+ to Ag^0 , absorption maxima, functional group of secondary metabolites, crystalline structure, morphology, and size by using UV-Vis spectroscopy, fourier transform infrared spectroscopy (FTIR) X-ray spectroscope (XRD), scanning electron microscopy (SEM) and atomic force microscopy (AFM) respectively. Further the effects of DS-AgNPs on WT Park TM3 SB and WI Park 25 Parkinson's mutant *Drosophila melanogaster* was assessed by the Negative geotaxis assay and PINK 1 gene expression. Treatment with DS-AgNPs exhibited increased geotaxis behavior and with a positive survival percentage, finally PINK1 was found to be down regulated as the treatment concentrations increased in WI Park 25 mutant but up regulated in WT Park TM3 SB mutant PD model. Although more molecular mechanism needs to be understood, however the use of DS-AgNPs can be a promising treatment strategy for the PD.

Introduction

Synthesis of nanoparticles (NPs) becoming revolutionary technique that has a wide range of applications in drug delivery medicine, agriculture, sensors, cosmetics and food industry [1]. Among all type of NPs metal and metal oxide nanoparticles have been thoroughly examined and scientifically evaluated for their excellent properties, such as very good surface to volume ratio, dispersion, incorporation, easy modification, shape and solidity etc. [2], Owing to these properties metal and metal oxide nanoparticles, they display enhanced antioxidant, antimicrobial, and anticancer, anti-inflammatory wound healing, properties [3–5].

Among many metal ions, Silver is extensively used in nano systems and employed for various purposes. Silver nanoparticles (AgNPs) have excellent medical applications when compared with other metal nanoparticles [6] Different approaches for the preparation of AgNPs have been employed, such as sol-gel, hydrothermal, spray pyrolysis, microwave-assisted techniques, chemical vapor deposition, ultrasonic condition, and precipitation method [7, 8]. Compared to all other methods green synthesis is considered to be good method, exhibits no or reduced toxicity, environmentally safe [9]. Plant extracts contain a number of secondary metabolites, such as flavonoids, alkaloids, phenolic compounds, terpenoids, etc., which play a critical role in the synthesis acting as a capping agents and give stability to NPs [10, 11], therefore a number of plants and herbal extracts have been used to green synthesis of nanoparticles [12].

The present work focuses on the green synthesis of AgNPs nanoparticles from the plant *Datura stramonium*, commonly known as “Kaladhatur” or “thorn apple”, which belongs to the *family: Solanaceae, order :Solanales, Kingdom: planta* is an indigenous endangered medical pant seen in the southern part of India, tropical regions of centurial and South America, and warm regions of central Europe, Africa [13], the leaf of the *Datura stramonium* leaves or entire plant is used in the treatment of

various human ailments including ulcers, wounds, inflammation, rheumatism and gout, sciatica, bruises and swellings, fever, asthma, bronchitis and toothache [14]. The plant extract is rich in terpenoids, flavonoids, tannins, phenolic compounds, tropanes, and alkaloids, glycoside, steroids [15]. The crude extract of the plant has a varying degree of anti-microbial, antioxidant, anti-inflammatory, anti-rheumatoid, anti-ulcer, and anti-asthmatic properties [16–18].

Above mentioned advantages prompted to synthesis of AgNPs from *Datura stramonium*, One of the most common neurodegenerative diseases is Parkinson disease (PD) is characterized by dopaminergic (DA) neuron degeneration in the substantia nigra pars compacta, accompanied with locomotors defects [19]. In most cases it occurs sporadically as a result of many different environmental risk factors; and mutations in a number of genes can also cause familial forms of PD [20]. Since the discovery of PINK1 mutations in familial Parkinsonism, there are up to 50 different PD- linked PINK1 mutations identified in diverse populations. The human PINK1 is a ubiquitously expressed 581 amino acid protein with an N – terminal mitochondrial targeting sequence (MTS), a transmembrane domain (TM), and a highly conserved Ser/Thr kinase domain with homology to the Ca²⁺/calmodulin-dependent protein kinases family [21, 22]. *D melanogaster* stands to be widely used to understand many aspects of neurodegenerative disorders particularly PD [23].

The purpose of this study is to explore the effect of AgNPs developed from *Datura* on PD mutant *Drosophila melanogaster*. Further to examine the effect of dosage and duration of exposure, to understand the negative geotaxis and gene expression profile.

Materials And Method

2.1 Chemicals.

Silver nitrate (AgNPs), TRizol reagent, Red Taq master mix 2X, Chloroform, Isopropanol, Glycogen, Ethanol, Agarose, and Ethidium was purchased from Sigma-Aldrich. cDNA synthesis kit was purchased from Thermo Fischer scientific. *Drosophila* Flies are obtained from NCBS Bangalore.

2.2 Preparations of *Datura stramonium* leaves extract (DSLE).

Fresh leaves of *Datura stramonium* (family: Solanaceae) were collected from the Botanical garden of Karnatak University Dharwad. The leaves were washed and left to dry. Then the dried leaves were powdered by a motor and 20 g of powdered leaves were mixed with 100 ml of deionized water and heated at 80 °C using a heater – stirrer for 10 mins. Then the mixture was filtered by whatman filter paper - 1 and was stored (-4 °C) for the further use [24].

2.3 Biosynthesis of AgNPs using DSLE

Freshly prepared 85 ml of 1mM of silver nitrate (AgNO_3) and 15ml of DSLE solution was constant stirring (100 rpm/min) for 60 mins the reduction process was carried by kept in a dark place at room temperature. The color changes from light orange to dark brown that indicates the formation of *Datura stramonium* capping silver nanoparticles (DS-AgNPs). The complete reduction of Ag^+ into Ag^0 AgNPs occurs within 25-30 mins. Further, the solution was centrifuged at 2000 rpm for 20 min. washed with distilled water repeated twice and the last DS-AgNPs were re-suspended in 5 ml of double distilled water [25].

2.4 Characterization of AgNPs

The reduction property of silver ions in aqueous DSLE is characterized by operating with 1 nm resolution and wavelength 200-800 nm by using UV-1800 Shimadzu Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The functional groups present in the phytoconstituents responsible for the reduction of DS-AgNPs were analyzed with an FTIR spectrophotometer. Dried samples with potassium bromide (1:100) were documented by the FTIR machine (NICOLET 6700, Thermo fisher) using ranges between 500 and 4000 cm^{-1} with a resolution of 4 cm^{-1} . XRD was used to investigate the phase and crystalline structure of the biosynthesized DS-AgNPs. The analysis was done on an X-ray diffractometer (PANalytical Empyrean) using $\text{Cu}_K\alpha$ radiation with a wavelength of 1.540 Å at 40 kV and 30 mA, and a scanning rate of 0.02 cm^{-1} in the region of 2θ ranging between 5 to 100°. Scanning electron microscope (SEM) (JOEL JSM-IT500 LA) at a magnification of 50000 X, the sample subjected by fixing on carbon paper coated with nano sheets were taken in a beaker and sonicated by adding some polar solvent. Once the particles get dispersed in solvent a small drop was put in a Cu grid and observed under SEM, in order to found the surface morphology of DS-AgNPs. In addition the samples were prepared by spin coating the DS-AgNPs solution into the glass slide to analyzing AFM. The prepared slides were air dried at 28°C in room temperature for 12 hrs. The particle size, shape and surface morphology of silver nanoparticles were determined.

2.5 *Drosophila melanogaster* stock

Parkinson's disease model of *Drosophila melanogaster* flies (WI Park 25 and WT Park TM3 SB) were obtained from the National Center for Biological sciences, in Bangalore Karnataka India. Flies were maintained on 12 hrs light/12 dark at 22°C in a BOD incubator (70% humidity), with a standard diet until the end of the experiment.

2.6 Experimental design

The climbing assay, Survival percentage, and PINK1 gene expression, were carried out. The flies of both sexes (WT Park TM3 SB and WI Park 25), from 1 to 5 days of age, were divided into four different groups each group containing 120 flies (60 males and 60 females)

- Group 1: only standard diet WT Park TM3 (untreated)
- Group 2: standard diet with AgNPs (100µg, 200µg/µl) WT Park TM 3 SB (treated)
- Group 3: SB Standard diet WI Park 25 (untreated)
- Group 4: standard diet with AgNPs (100µg, 200µg/µl) WT Park 25 (treated)

The above grouped flies were exposed to dose dependent DS-AgNPs for 5 day, every day once media was changed. After 5 days flies were submitted to negative geotaxis, survival percentage (10days) and gene expression study.

2.7 In Vivo Assay

2.7.1 Climbing assay

Twenty flies were selected from each treated groups and briefly anesthetized with CO₂ and placed with the individual in geotaxis tube on the form pad and plug the open end of the tubes (length 15 cm and diameter 3cm). After 15 min recovery from CO₂ exposure, the flies were gently tapped to the bottom of the tube and allowed to climb for 30 sec and number of flies crossing the 9 cm was recorded, the test were, repeated for 3 times for each group to calculate the percentage of flies out of the total number of flies tested [26].

$$\% \text{ of flies climbed} = \frac{\text{Number of flies reaching the 9 cm mark in 30 sec}}{\text{Total number of live flies in the tubes}} \times 100$$

2.7.2 Survival percentage

The survival rate was evaluated by counting once in a 24 hrs. The number of living flies until 10 days. Around 60 flies per group were included in the survival data and the total number of flies' represents the data were expressed as percentage of surviving flies [27].

2.8 Gene expression study

2.8.1 RNA extraction using TRIzol reagent.

The RNA was isolated from 30 flies form (15 males and 15 females) WT Park TM3 SB and WI Park 25 were taken in a separate micro-centrifuge tube and homogenized with 1000 µl TRIzol reagent, allowed to vortex for 10 mins at room temperature. 200 µl of chloroform was added and the sample was spined for 15 mins at 12000 rpm at 4 . Upper aqueous phase was transferred to a new clean micro centrifuge tube, 500 µl of isopropanol and 1.5 ml of glycogen was added, the resultant mixture was incubated at room temperature for 10 mins. Samples were centrifuged at 12000 rpm for 10 mins at 4 . Supernatant was discarded and the RNA pellet was washed by adding 1 ml of 70% ethanol and stored in 80 for overnight. Sample was centrifuged at 12000 rpm for 5 mins at 4 , 70% of ethanol is removed and the RNA pellet was air dried. The pellet was then re-suspended in 95 µl of DECP-treated water [28, 29]

2.8.2 Real Time PCR for Gene expression analysis

Reverse transcriptase polymerase chain reaction (RT- PCR) was carried out using Techno Prime system to determine the levels of PINK1 and GAPDH mRNA expressions; the cDNA was synthesized from 2 µg of RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific) with oligo dT primer according to the manufacturer's instructions. The reaction volume was set to 20 µl and cDNA synthesis was performed at 42 for 60 min, followed by RT inactivation at 85 for 5 min. The primers were taken to amplify the genes are as shown in Table 1.

2.9 Amplification and Quantification of genes

The PCR mixture contained 1 µl of cDNA, 10 µl of Red Taq Master Mix 2X (Amplicon) and 1 µM of each complementary primer specific for PINK1 and GAPDH (internal control) sequence. The samples were denatured at 94 for 5 min, and amplified using 35 cycles of 94 for 30 sec, 55 for 30 sec, and 72 for 1 min for PINK1 is annealing temperature 47 and for G6PDH the annealing temperature was set to 42 for 30 sec followed by a final elongation at 72 for 10 min. The optimal numbers of cycles have been selected for amplification of these genes. 10 µl of the final amplification product were run on a 2% ethidium- stained agarose gel and photographed.

Results And Discussion

3.1. Synthesis and characterization

The aqueous solution of DSLE with AgNO_3 changes the color of the solution from light orange to dark brown after 4 hr of incubation; it indicates the reduction process of Ag^+ to Ag^0 Nanoparticles (Fig 1a). Further UV-Visible spectroscopy analysis conforms the formation DS-AgNPs by absorption maxima is 459 nm (Fig 1b), and is supported by surface Plasmon resonance pattern for DS-AgNPs [30, 31].

Fourier transform infrared (FTIR) spectroscopy was provide information on secondary metabolites, generally providing functional groups that involved in the reduction and capping of DS-AgNPs (Fig 1c), The FTIR Spectrum of AgNPs shoed major absorbance peak at 1112.57, and 1322.68, 1348.21, 1454.80, 1633.05, 2360.68, 2960.92, and 3381.49, cm^{-1} . (Table-2) That signifies the presence of phytoconstituents and act as the capping agents for green synthesized DS-AgNPs [32].

The patterns of the face centered free and crystalline structure of Biosynthesized DS-AgNPs using DSLE was done by XRD. Was recorded in the 2θ angle in between $5-100^\circ$, the data XRD data shows $2\theta = 111, 200, 220, 311, 222$, and 400 planes (fig 1d) [33]. .

The morphology of DS-AgNPs synthesized using DSLE was examined by using SEM (Fig 1e). The DS-AgNPs were uniformly distributed and exhibits spherical in shape, moreover, the high agglomeration of biosynthesized DS-AgNPs were possibly induced by dehydration exerted during the preparation of sample.

Size and shape of DS-AgNPs were determined by using atomic force microscopy (AFM) to monitor DS-AgNPs dissolution and agglomeration pattern. AFM data reveals that the particles are monodispersed and spherical in shape size range was 39.06 nm, 43.67 nm and 58.24 nm without any agglomerations in topography structure, 3D nanoparticles with a distance of 62.69 nm from each other. A statistical treatment of AFM images was performed to explore the 3D nanostructure of DS-AgNPs (fig 1f).

3.2 In Vivo assay

3.2.1 Claiming assay and survival assay.

The exposure of AgNPs (100 μg and 200 μg) for five days on climbing time crossing number in the geotaxis and survival percentage in WI Park 25 and WT Park TM3, mutant *Drosophila melangaster* (Figure 2a and 2b), significantly decrease the climbing time and increase the crossing number compared to the untreated (control) group and AgNPs treating group. The effect of the exposure to AgNPs for 5

days significantly increase the survival rate, the comparison demonstrated that AgNPs for a 10 days at the concentration of 100 µg and 200 µg, improve the life time compare to the control group (Fig 2c and 2d).

3.3. Expression studies of PINK1 gene.

Effect of biosynthesized AgNPs by using DSLE on PINK1 gene expression was studied in WI Park 25 and WT Park TM3 SB by semi quantitative on PINK1 gene internal control GAPDH was used to normalize the gene expression. The study revealed the expression of PINK1 was down regulated as the treatment concentrations increased WI Park 25 *Drosophila melangaster* and PINK1 is up regulated as the treatment concentration in WT Park TM3 SB the amplified GAPDH and PINK1 genes in both the group of files respectively (fig 3a and 3b).

The relative expression of PINK1 gene in WI Park 25 and WT Park TM3 SB *Drosophila melangaster*, similarly relative expression of PINK1 gene in WT Park TM3 SB *Drosophila melangaster* is duplicated (Table 3a and 3b) respectively. The result show that the expression levels of PINK1 at WT Park 25, 100 µl treated has shown 0.4 and 200 µl treated has shown 0.63 fold expression compare to control (fig 4a). And the expression levels of PINK1 of WT Park TM3 SB at *Drosophila* at 100 µl treated has shown 1.26 and 200 µl treated has shown 2.64 fold expression compare to control (fig 4b).

Conclusion

The DS-AgNPs are exploited for the diagnosis and treatment of various disorders. In the current study we have used *Datura stramonium* for the green synthesis of DS-AgNPs and are characterized further used to find out the possibility of developing a treatment strategy for PD. The PD variant of WT Park TM3 SB and WI park 25 were fed with DS-AgNPs has shown decreased longevity and increase dosage has resulted with decrease in climbing activity and increase the crossing numbers. Effect of DS-AgNPs on PINK1 gene expression its revealed that expression of PINK 1 was down regulated as the treatment concentration increase with WI Park 25 variant *Drosophila melangaster* and PINK1 was up regulated as the treatment in WT Park TM3 SB variant *Drosophila melangaster*.

Overall, the Negative geotaxis study indicates DS-AgNPs has positive effect on the PD treatment strategy; however the mechanism of up and down regulation of PINK1 of two different variant *Drosophila melangaster* needs addressed.

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Declarations

Contributions

Designed the concept and supervised the experiments. Gopinath G and Dr. Kuramkote Shivanna Devaraju carried out experimental analysis, along with Yarajarla Ramesh Babu and Megha Basavaraj Abbigeri interpreted and analysis the PINK1 gene expression in mutant PD module of *Drosophila melanogaster*. Ramesha Hanumanthappa and Manjunath Ajanal carried out synthesis and characterization of DS-AgNPs. All the authors read and approved the manuscript.

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Declaration

Ethical approval not applicable, the research does not involve either human participants, and / or animals

Conflict of interest

The authors declare that there are no competing interests

Tables

Gene	Primer pair	Sequence	T _m	Product size (bp)
GAPDH	FP	GACTTCTGGAAAAACCATTTG	46.6	230
	RP	TGATTTGTGCATATTTTGTGA	46.8	
PINK 1	FP	AAAATGATGTTCAACTACGAC	47.7	174
	RP	CATACAGACAATGTTCGGA	44.6	

Table 1: Primer details used for amplification of PINK1 gene expression

SI No	Absorption peak (cm ⁻¹)	Functional groups
1	1112.57	Ester
2	1322.68	Ester
3	1384.21	Ester
4	1454.80	Thycorbonyl
5	1633.05	Phosphate group
6	2360.43	Sulphate
7	2852.68	Aromatic alkenes
8	2922.77	Carboxylic acids
9	2960.92	Alkyl group
10	3381.49	Alcohol amine

Table 2: The functional groups involved in the biosynthesis of silver nanoparticles

Samples	Band Intensity of PCR Amplicon of Genes		Normalized	Relative Expression	Gene
	G6PDH	PINK 1			
control WT	48167.42	3735.86	0.08	1.00	
100µl WT	50083.23	4912.52	0.10	1.26	
200µl WT	47182.37	9676.40	0.21	2.64	

Table 3a: Relative expression PINK1 gene in WT Park TM3 SB mutant *Drosophila melanogaster*

Samples	Band intensity of PCR Amplicon of Genes		Normalized	Relative Expression	Gene
	G6PDH	PINK 1			
control WI	52913.29	16610.25	0.31	1.00	
100µl WI	39987.13	564.51	0.01	0.04	
200µl WI	43447.94	8628.13	0.20	0.63	

Table 3b: Relative expression PINK1 gene in WI Park 25 mutant *Drosophila melanogaster*

Figures

Figure 1

See above image for figure legend.

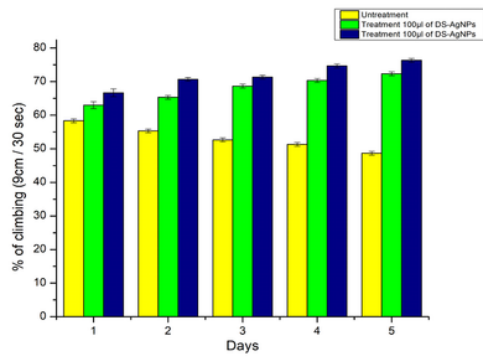


Fig 2a: The Climbing percentage of WT Park-TM3 CR mutant *Drosophila melanogaster*

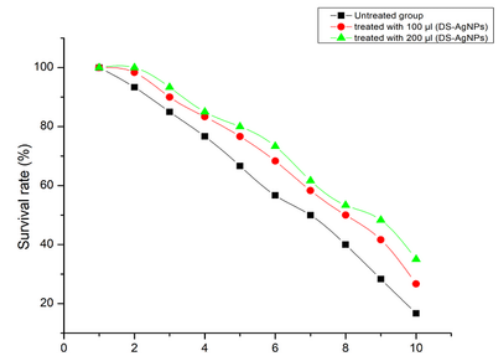


Figure 2

See above image for figure legend.

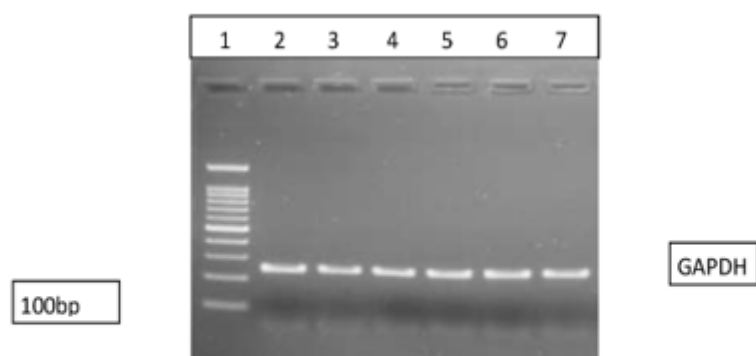


Fig 3a: Amplification of GAPDH gene in WI Park 25 and WT park TM3 SB. Lane 1- Ladder; lane2- control WI; lane 3-100µl WI; Lane 4-200µl WI; Lane 5- control WT; lane 6-250µl WT; lane 7 -500µl

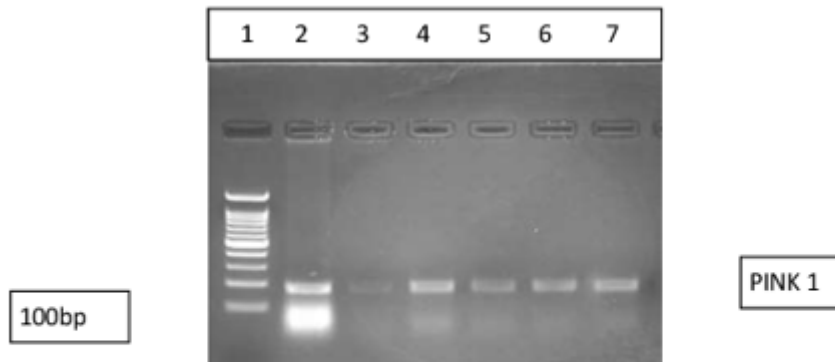


Fig 3b: Amplification of PINK1 gene in WI Park 25 and WT park TM3 SB. Lane 1- Ladder; lane2- control WI; lane 3-100µl WI; Lane 4-200µl WI; Lane 5- control WT; lane 6-250µl WT; lane 7 - 500µl WT

Figure 3

See above image for figure legend.

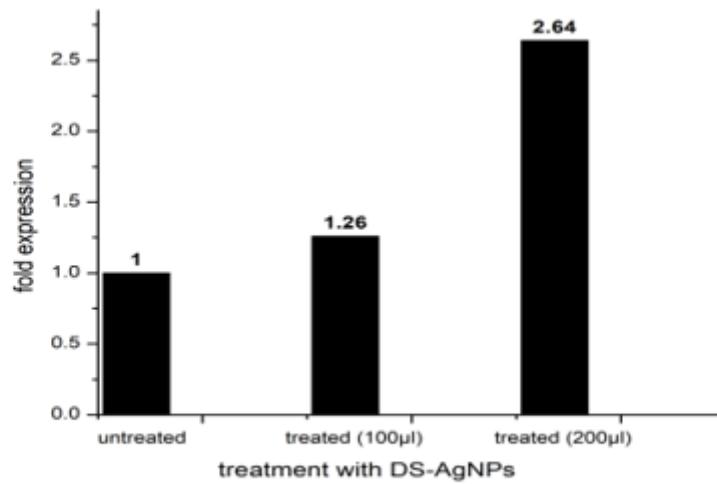


Fig 4a: Relative fold expression PINK1 gene in WI Park 25 mutant *Drosophila melanogaster*

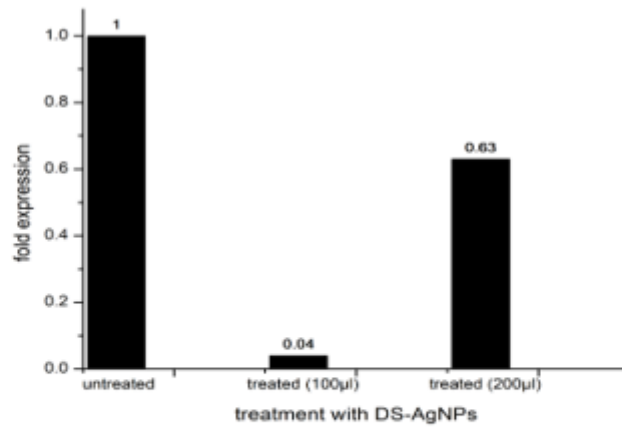


Fig 4b: Relative fold expression PINK1 gene in WT Park TM3 SB mutant *Drosophila melanogaster*.

Figure 4

See above image for figure legend.