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Efficacy and Safety of Combination Therapy of Zinc and Silver Oxide Nanoparticles in Streptozotocin-Induced Diabetic Rats

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ABSTRACT

In the present study, Zinc oxide nanoparticles andSilver Oxide nanoparticles were evaluated for their in vivo and in vitro antidiabetic activity. The resulting ZnO, Ag_2O were characterized via various methods like UV, XRD, FTIR, PSA and SEM, TEM, and ICP-OES. The synthesized NPs were tested sub-acute oral toxicity model by abiding by the OECD425 guidelines. The male Wistar rats with a weight ranging from 180-200 g were grouped as follows: normal control: those that didn't received any treatment, Diabetic control: who received a single intraperitoneal dose of streptozotocin (45mg kg^{-1}), Zinc Oxide Nanoparticles: received single daily oral dose of (30mg kg^{-1}), Silver Oxide Nanoparticles: received a single daily oral dose of (30mg kg^{-1}) Combine Zinc Oxide and Silver Oxide Nanoparticles (60mg kg^{-1}). Metformin received a single daily oral dose of 100mg kg^{-1}. Synthesized Zn and Ag_2O nanoparticles showed no toxic effect and were considered safe. From the experimental results, it may be concluded that the combined dose of ZnO and Ag_2O had more potent antihyperglycemic activity, further studies are required to establish the mechanism of action.

Key words: *Normal control, Diabetic control, Acute toxicity, Antihyperglycemic activity, Zinc oxide nanoparticles, Silver oxide nanoparticles*

INTRODUCTION

Diabetes can be treated with a variety of methods and doses, but resistance develops over time in diabetic patients. Nanotechnology is developing rapidly and widely used in healthcare. Unique properties of the NPs promote drug development leading to low dose, reduced side effects and convenience of use especially ; Because of their large surface area and unique composition, NPs are finding more effective than traditional methods. Diabetes is defined as a set of metabolic disorders where a person consist of elevated blood sugar level, also known as Diabetes mellitus (DM), either due to insufficient production of insulin by the body or because the cells lack response to the insulin that is released. Classic symptoms of diabetes are polyuria (increased frequency of urination), polydipsia (increased hunger and thirst), and polyphagia [1, 2].

Many research studies have demonstrated the role of metals in glucose metabolism and their association to diabetes deficiency [2, 3]. It was stated that vanadium [4, 5], chromium [5, 6], magnesium [6, 7], silver [8, 9], and zinc [9] play a role in blood sugar management and diabetes therapy [9].

Zinc is also known to improve the structural stability of insulin, which plays a part in insulin production, accumulation, and secretion [10, 11]. Zinc stimulates glucagon secretion and decreases gluconeogenesis and glycogenolysis.

Silver oxide NPs can be used to reduce inadequate diabetes mellitus insulin reaction. They cannot eliminate the lack of insulin (as in diabetes type 1), but they can minimize dependency on exogenous insulin, or maybe eliminate oral hypoglycemic agents in type 2 diabetes [12].

The role of chromium supplementation in improving glucose regulation when eating a regular diet is still debatable. Still, studies in deficiency states seem to have well-validated the role of chromium as an essential trace element needed for average glucose metabolic behavior [13].

MATERIALS AND METHODS

Materials

Silver nitrate, Zinc acetate, ethanol, triethylamine, and double-distilled water are employed in the synthesis process in their pure form. Streptozotocin, Nicotinamide, Metformin, and Citrate buffer were purchased from vendors/suppliers. Instruments used for the study were analytical balance (Schimadzu, ATX 224), UV visible spectrophotometer (JASCO V-730), Homogenizer (Biolab, B L244), cold centrifuge (Remi C-24BL), Ulrasonicator (Spectra Lab UCB- 40D), pH meter (Equip-Tronics, EQ-610), UV semi auto-analyzer (Retina Clinical chemistry analyzer, Delta lab). Analytical grade chemicals were used for synthesis.

Synthesis of silver oxide nanoparticles

In a beaker, 0.2M $AgNO_3$ was dispersed in 10 mL double distilled water to form a 1 M suspension. 0.18 M TEA was added to this solution. The solution's colour changed from colourless to black. The precipitates were washed repeatedly with ethanol following stirring of 200 minutes at room temperature, and centrifugation (6000 rpm, 10 minutes) was performed to eliminate unbounded TEA. Particles were vacuum-dried for 24 hours at room temperature. The suspensions of silver nanoparticlesweremade by re-dispersing the dried silver nanoparticles into alpha- terpineol [14].

Synthesis of zinc oxide nanoparticles

Chemical precipitation was utilized to prepare nanoparticles of zinc oxide. A magnetic stirrer was used to vigorously stir a 1M zinc sulfate heptahydrate solution. Drop by drop, 2 M of sodium hydroxide solution was added to this solution. After 18 hours of stirring, a significant amount of white precipitate was produced. This precipitate was collected by filtration and impurities were removed by washing with distilled water and ethanol before drying for 8-hour in a hot air oven at 1000^0 C and converted fine powder with mortar and pestle [15].

Characterization

The optical properties of resulted ZnO and Ag_2O nanoparticles were analyzed by UV-Visible spectroscopy (Jasco 730, Japan). The functional group was determined by Fourier Infra-Red Spectroscopy (FT-IR, Jasco 4100 series, Japan). X-ray diffraction was used to determine the crystalline phase of nanoparticles (XRD, Rigaku, Ultima IV) synthesized at the Central Instrumentation Facility at Savitribai Phule Pune University (SPPU). A nanoparticle size analyzer was used to measure the particle size of the synthesized nanoparticles (NanophoxSympatech, Germany). Scanning electron microscopy was utilized to determine the morphology of nanoparticles (SEM, Carl zeiss, Supra 5, Germany) at Diya Labs, Mumbai. Zeta potential was based on DLVO theory. (Delsa Nano C by Beckmen Counter Inc). ICP-OES analysis was performed to determine the metal concentration in nanoparticles at Adarsh Lab, Navi Mumbai (5110 Agilent Technologies, California).

In vitro activity

Alpha-amylase inhibition assay

The Bernfeid method was used to investigate amylase inhibition in vitro [13, 14]. In summary, 100 µL of test extract (12.5, 25, 50, 100, 200, and 400 µg/ml) was combined with 200 µL of -amylase enzyme (Hi media Rm 638) and 100 µL of 2mM phosphate buffer (pH-6.9). Following incubation of 20 minutes, 100 µL of one percent starch solution was added. Similar procedure was used for controls, except that 200 µL of the enzyme was substituted with buffer. 500 µL reagent of dinitrosalicylic acid was added to both the test and the control. These solutions were immersed in boiling water bath for 5 minutes and the absorbance at 540 nm was measured

using a spectrophotometer, and the inhibition rate of amylase enzyme was calculated using the following formula [15-17].

$$\text{Inhibition (\%)} = \frac{\text{Abs540 (control)} - \text{Abs540 (extract)} \times 100}{\text{Abs540 (control)}} \quad (1)$$

Experimental animal

Male Wistar albino rats weighing 200 to 250 g procured from National Institute of Biosciences in Pune, India were used in the study. The animals were secured in polypropylene cages lined with husk. Food and water are freely available. The animals were fed a standard diet by Nutrivet Life Sciences, Pune, India. The experiment lasted 21 days and was carried out following the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), India. The study protocol was approved by the Sinhgad Institute of Pharmacy, Narhe Institutional Animal Ethics Committee in Pune, India (SIOP/IAEC/2021/01/01) on January 25, 2021.

Acute toxicity studies

Acute toxicity studies were done to determine the in vivo safety of developed ZnO and Ag_2O nanoparticles. Section 4 of OECD Guidelines 422 and 423 [18-20] was followed in these studies. Rats were divided into 3 groups of 5. Each rat group was given an increaseddose of 500, 1000, 1500, and 2000 mg/kg (p.o). Rats were constantly observed for signs of death, abnormal behavior, or dietary changes. A conclusion was reached 14 days later.

Induction of diabetes in experimental rats

Diabetes was induced in overnight fasted rats with a single intraperitoneal injection (i.p.) of freshly prepared streptozotocin 45 mg/kg bw in 0.1 M citrate buffer (pH 4.5), 15 min after Nicotinamide (120 mg/kg bw in saline) i.p. All of the experimental rats' blood glucose levels were measured three days after STZ treatment, with a range of 250 mg/dl considered diabetic, and used for the experiment. Treatment began on the fourth day after the STZ injection and lasted 21 days [21-23].

Experimental design

The rats were separated into 6groups (I-VI), with each group consisting of five rats (n=5) [24-26].

Group I: Normal Control Rats: For 30 days, rats were given free access to a standard pellet diet.
Group II: Diabetic control rats: For 30 days, diabetic rats were given free access to a standard pellet diet.
Group III: metformin-treated diabetic rats (positive control) were given 100 mg/kg p. o. for 21 days.
Group IV: Diabetic rats were given 30mg/kg of ZnO, p.o. for 21 days.
Group V: Diabetic rats were given 30mg/kg of Ag_2O, for 21 days **(Table 1)**.
Group VI: Diabetic rats treated with a combination of ZnO and Ag_2O Nps.

Table 1. Experimental Design

Sr. No.	Treatment	Group	Route of administration	No. of animals used.
I	Normal (Saline)	I	i.p.	5 Rats
II	Diabetic control (DC) (Streptozotocin) (45mg/kg)	II	i.p.	5 Rats
III	Standard Metformin (50 mg)	III	p.o.	5 Rats
IV	Zinc oxide Nanoparticles (50mg)	IV	p.o.	5 Rats
V	Silver Oxide nanoparticles (30mg)	V	p.o.	5 Rats
VI	Combine (ZnO+Ag_2O) (30mg)	VI	p.o.	5 Rats
	Total animals			30

Experimental procedure

At the terminal of the research, all animals (rats) were fasted overnight and anesthetized using Ketamine (55mg/kg) via IM injection before being sacrificed. The kidney, liver, and spleen were extracted and ice-cold normal saline was used for washing organs [27-29].

General condition, food and water consumption, body weight, urine volume, pH, and frequency

Each rat group's condition, including coat colour, skin infection, mental state, and psychological activities, will be monitored on a routing basis. Water and food intake, body weight, urine output for volume per hour and pH, and stool quality are all recorded regularly [17, 30].

Estimation of blood glucose

The diabetic status of the rat was determined by checking fasting blood glucose levels, weekly throughout the study period. Blood was collected by pricking the rats' tails with a sharp razor, anda glucometer was used to determine glucose levels (Accu-chek instant Glucometer by Roche Diabetes Care, India) [31-33].

RESULTS AND DISCUSSION

UV Visible spectroscopy

The results of UV-Visible Spectroscopy are shown in **(Figure 1i)**. The absorption spectrum displayed the maximum absorption of ZnO nanoparticles at 374.5nm and Ag_2O nanoparticles at 238 nm.

FTIR

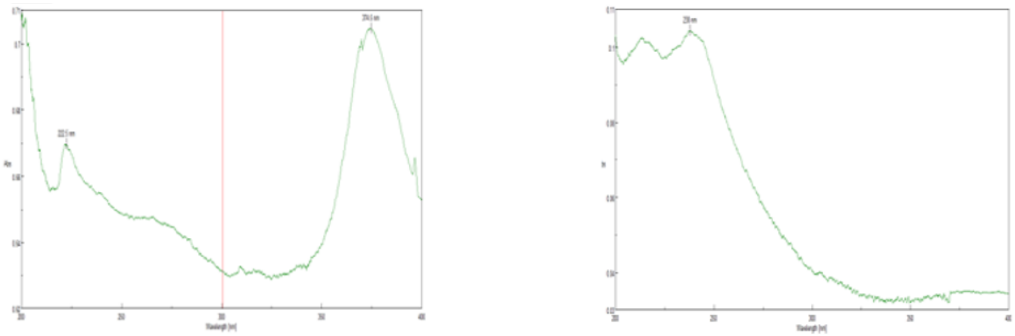
The spectra of FT-IR of synthesized nanoparticles are presented in **(Figure 1ii)**. Because of an adsorbed water molecule on ZnO nanoparticles, the band in ZnO spectra was observed in the 3444.24cm^{-1} region which corresponds to O-H bonds for stretching vibration. The band at 1114.65cm^{-1} peaks ascribed to symmetric C=O bond vibration. The presence of hydroxyl group in the sample shows band at 620cm^{-1} and the band observed at 493.68cm^{-1} conforms to the Zn-O stretching vibrations. In spectra of Ag_2O, peak at 3590.81cm^{-1} correlated with H-O-H bending vibration due to adsorbed water molecule in the sample. 1697.05cm^{-1} band attributed to H-O stretching vibrations which are assigned in the Ag-OH bond. The peaks at 1446.35cm^{-1} and 1298.82cm^{-1} correspond to stretching vibrations of Ag-O and Ag-OH respectively. The band at 873.59cm^{-1} is associated with Ag-O-Ag stretching vibrations.

X-ray diffraction

The XRD diffractogram of ZnO and Ag_2O synthesized nanoparticles is shown in **(Figure 1iii)**. The narrow sharp peaks demonstrate that both ZnO and Ag_2O NPs are highly crystalline. XRD spectra of ZnO, **Figure 3a** displays the diffraction peaks occurring at 2Ɵ 12.3, 21.18, 26.0, 26.56, 32.48, and 35.08 have a highest intensity 690, 258, 371, 305, 315, 317 respectively. XRD spectra of Ag_2O **Figure 3b** reveals the strongest peaks at 2Ɵ values of 13.47, 14.4, 25.08, 27.3, 27.52, 35.18, corresponding the intensities 1680, 3360, 1186, 1362, 2280, 1034 respectively.

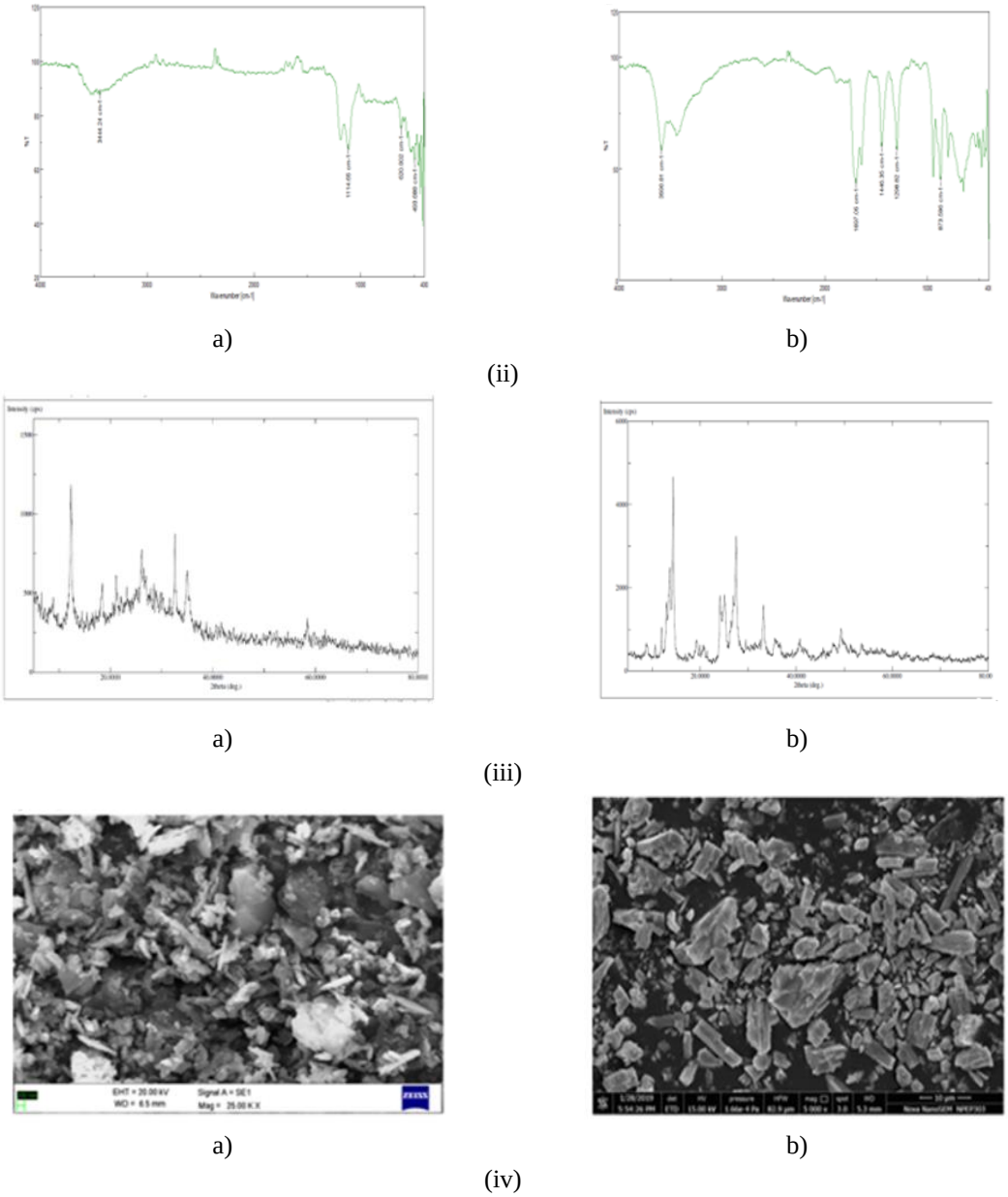
SEM analysis

SEM images of prepared ZnO and Ag_2O nanoparticles are presented in **(Figure 1iv)**. ZnO nanoparticles exhibita needle-like shape **(Figure 4a)**. Whereas SEM of Ag_2O nanoparticles shown in **(Figure 4b)** illustrates rod-like morphology.



a) b)

(i)



a) b)

(ii)

a) b)

(iii)

a) b)

(iv)

Figure 1. ZnO(A) and Ag_2O(B), (i) UV-Visible spectra, (ii) FT-IR spectra, (iii) XRD spectra, (iv) Scanning Electron Microscopy of nanoparticles.

Nanoparticles size analysis

The histogram of nanoparticle size analysis of ZnO and Ag_2O nanoparticles is shown in **(Figure 2i)**. The size of ZnO and Ag_2O nanoparticles from this analysis resulted in 17.06nm and 19.73nm respectively which has good compliance with particle size distribution results from Image J software.

Zeta potential

Zeta potential of ZnO and Ag_2O nanoparticles is shown in **(Figure 2ii)**. The zeta potential of ZnO and Ag_2O nanoparticles was found to be 49.19 mV and -19 mV. Indicating the stability of nanoparticles.

Inductive couple plasma – optical emission spectroscopy (ICP-OES)

Inductively coupled plasma elemental analysis (ICP –OES) was used to determine the concentrations of Zn and Ag. Zn yielded approximately 22.11 percent, while silver yielded approximately 0.08 percent. The results show that Ag NPs were well coupled with Zn NPs and that elution was minimal **(Figure 2iii)**.

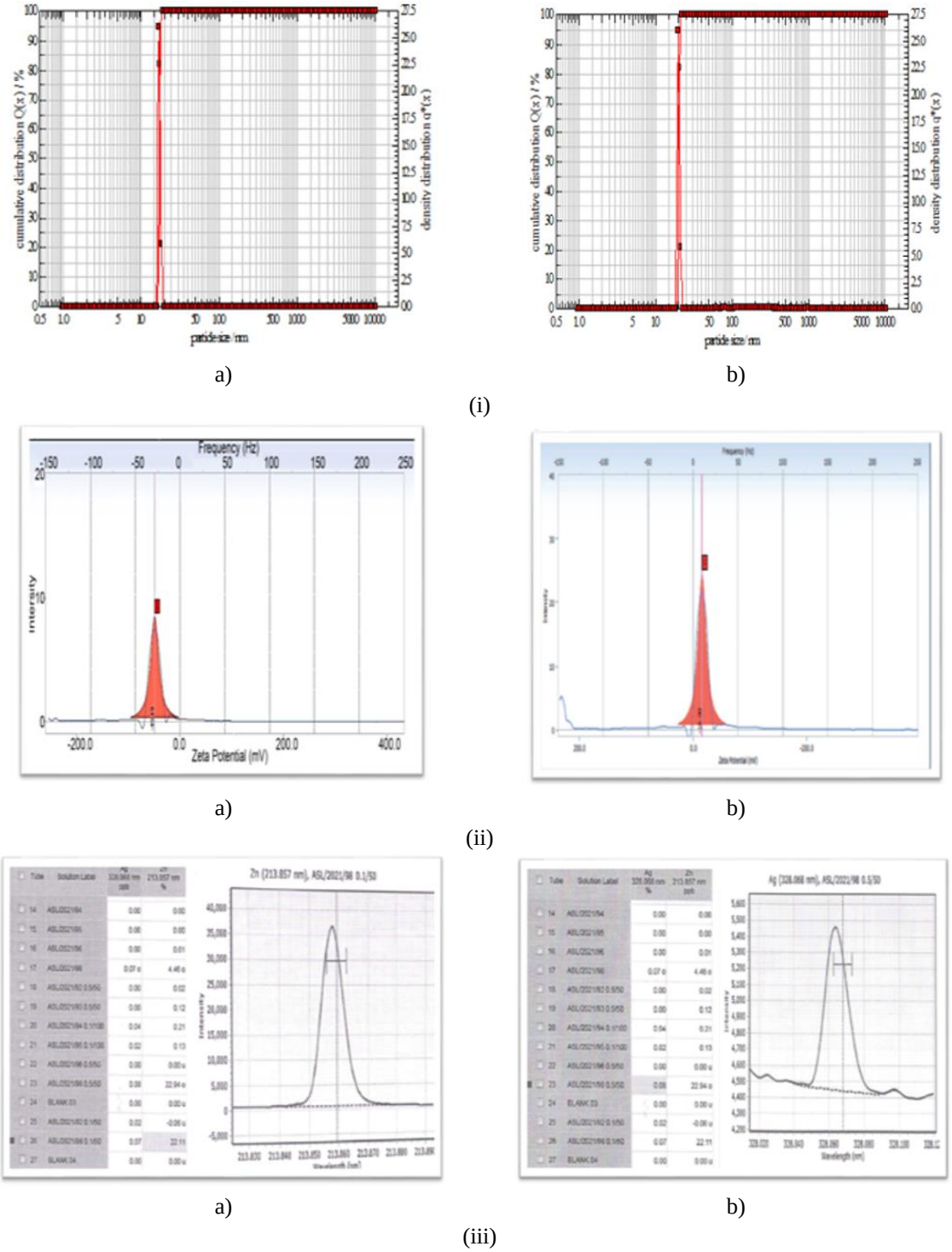


Figure 2. ZnO (A) and Ag_2O (B), (i)Particle size analyzer of nanoparticles, (ii) Zeta potential, (iii) ICP-OES graph

Acute toxicity studies

Prescription of ZnO, Ag2O, and ZnO/Ag2O NPs Orally was seen to have safe effect till a p.o. dose level of 1500 mg/kg, including lack of toxicity signs. Because there was no mortality after fourteendays, the Medial Lethal dose of ZnO, Ag2O, and combined ZnO and Ag2O nanoparticles was calculated to be greater than 1500 mg/kg body weight

Alpha-amylase inhibition assay

The alpha amylase inhibitory activity was expressed as percent inhibition, The positive control sample was prepared using Acarbose (1mg/ml). **Table 2** shows the results of assay.

Table 2. Comparative data of % inhibition of *Alpha- amylase* by ZnO, Ag_2O and combine ZnO and Ag_2O NPs

Concentration (µg/ml)	Silver oxide nanoparticles	Zinc oxide nanoparticles	Combine nanoparticles	% inhibition activity of ZnO NPs	% inhibition activity of Ag_2O	% inhibition activity of Combine NPs
12.5	0.22	0.42	0.46	26.31	61.40	19.29
25	0.22	0.41	0.42	28.07	61.40	26.31
50	0.22	0.37	0.40	35.08	61.40	29.82
100	0.21	0.37	0.33	35.05	63.15	42.10
200	0.21	0.34	0.29	40.35	64.91	49.12
400	0.16	0.21	0.24	63.15	71.92	57.89
Std Acarbose (1mg/ml)	0.22	0.22	0.22	63.25	61.4	62.69

Antidiabetic study

Changes in body weight

In the present study, the initial body weights of all experimental groups revealed no discernible difference among the groups. Diabetic control rats (Group II) gained less weight after 21 days (P0.01) than normal control rats (Group I). In comparison to diabetic control rats, the body weights of ZnO and combined ZnOand Ag2O NPs (Group III and VI) (p0.01) **(Table 3 and Figure 3i)**.

Table 3. Bodyweight Changes : Once weekly the body weights were recorded for all the groups of experimental rats.

Body weight(gram)	Quarantine week	Induction week	1st week treatment	2nd week treatment	3rd week treatment
Normal	266.6	266	268.3	267.33	265.33
Diabetic control	254	183.66	179.66	174.33	169.66
Standard	257.33	184	189.33	197	202.66
ZnO NPs	158.66	170.33	178	186	190.33
AgO NPs	232.33	173.33	177.33	183.33	186.33
Combine NPs	252	192	201	205.66	206.33

Blood glucose level variation in diabetic rats after ZnO, Ag_2O and Combine ZnO and Ag_2O Npstreatment

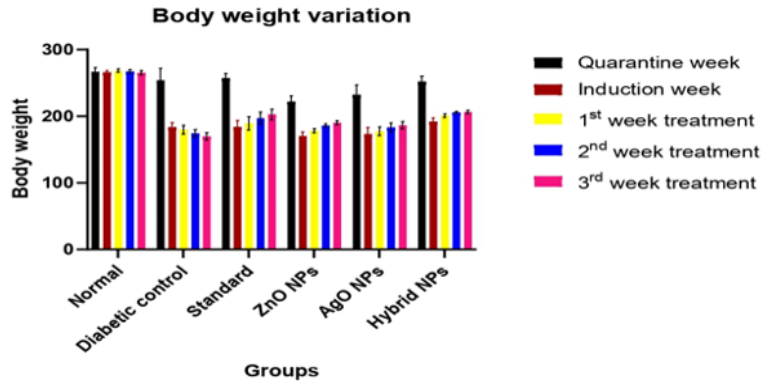
The diabetes-induced groups of rats (II, IV, V, and VI) were found to have higher blood glucose levels on the first day of the experiment when relatedto the control rats (Group I) and Standard (Group III). After 21 days of the experiment, diabetic STZ (Control Gr. II 347) rats had higher blood glucose levels than normal control rats (Group I). ZnO and ZnO/Ag2O rats had lower blood glucose concentrations when compared to diabetic control (Group II) rats. Furthermore, blood glucose concentrations in rats given the standard drug Metformin decreased significantly (<P0.0001) (Group III). After 21 days of testing, which was the same for Groups III and VI **(Table 4; Figure 3ii)**.

Table 4. Blood glucose estimation and urine output

Blood Glucose (mg/dl)	Normal	Control	ZnO	AgO	Combine NPs	Standard
Quarantine week	104.66	109	107	95	87.66	110.6
Induction week	102	184.66	351.66	403.33	381.66	449.33
1 week treatment	109.66	348.66	299	382	337.66	320.66
2 week treatment	110	423.66	284.66	356.66	262.33	190
3 week treatment	122	415	234.33	284.33	190.66	151
Urine(ml) (Output)	6	0.86	14.7	03	2.33	2.33

Urine output

All six groups' urine output volume was monitored from the quarantine week to the third week of treatment. The outcomes were presented in **(Table 5 and Figure 3iii)**.



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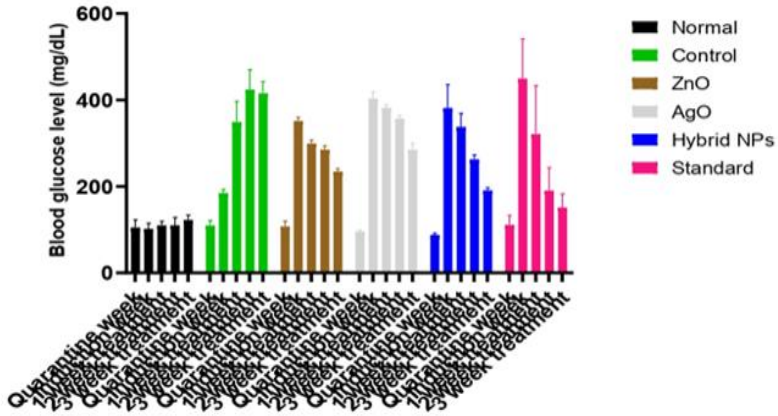

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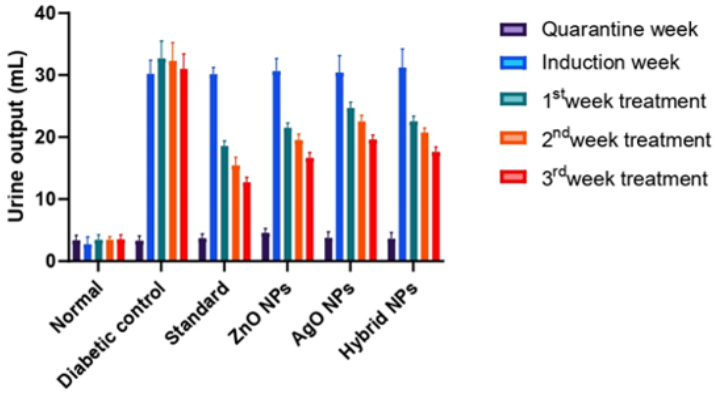

(iii)

Figure 3. (i) Body weight variation of rats, (ii) Blood glucose level variation of rats, (iii) Urine output of rats

In the present study, STZ administration of 45 mg/kg to rats csan destroy pancreatic beta cells which can lead togrowthof constant hyperglycaemicconditionsimilar to type II diabetes in human beings. In the current evaluation prescriptionof STZ at 45 mg/kg to rats was seento increase the blood glucose levels on the third day of the experimental period, this may be due to islet destruction and beta cell death. In diabetes, body cells cannot use glucose as an energy source, instead protein is used as an energy source. This reduces protein storage and weight loss. In this study, streptozotocin-induced diabetic rats showed weight loss throughout the experimental period. Oral treatment of ZnO NPs and combination ZnO & Ag_2ONPs to diabetic rats for 21 days yieldedconsiderable increment in body weight in comparisonto the Ag_2O Nps and control rats. The % inhibition values of alpha-amylase inhibitory activity of ZnO, Ag_2O, and Combine NPs were similar to that of Acarbose. Thus, the study suggested that NPs metal oxide Npscan be used as an Antidiabetic agent due to its significant effects to reverse blood glucose concentration. They exhibit remarkable alpha-amylase inhibitory activity, further investigation is needed to prove the antidiabetic mechanism.

CONCLUSION

The present study suggested that ZnO and Ag_2O NPs can be used as an Antidiabetic agent because of their significant role into reversingblood glucose concentration. The combined dose of ZnO and Ag_2O NPs exhibits remarkable alpha-amylase inhibitory activity, further investigation is needed to prove the antidiabetic mechanism.

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CONFLICT OF INTEREST : None

FINANCIAL SUPPORT : None

ETHICS STATEMENT : The experiment was carried out according to the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institute of Animal Ethics Committee (IAEC) (SIOP/IAEC/2021/01/01; dated 25/01/2021).

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