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In-vitro Antiglycation Activity of Zinc Oxide Nanoparticles Synthesized from the Bioactive Fraction of *Bambusa arundinacea* Leaf Extract.

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ABSTRACT

The present study was conducted to investigate an *invitro* antiglycation capacity of biologically synthesized zinc oxide nanoparticles (ZnO NPs) from zinc acetate and sodium hydroxide utilizing an antioxidant-rich fraction of *Bambusa arundinacea* leaf extract. ZnO NPs were characterized by FTIR, TEM and UV–Vis spectroscopy. The antiglycation potential of ZnO NPs was measure by using the formation of AGEs fluorescence intensity and the level of fructosamine in bovine serum albumin (BSA)-glucose assay. The inhibitory activity of glycosylated Hb was also measured. Four fractions were collected using column chromatography of *Bambusa arundinacea* leaf extract named F1, F2, F3 and F4. The results showed that F3 exhibits the greatest phenolic content (87.36 ± 9.32 mg gallic acid equivalent (GAE)/g) and flavonoid content (29.65 ± 6.52 mg quercetin equivalent (QE)/g). In the DPPH radical scavenging activity, F3 had the lowest IC_{50} values of 46.7 µg/ml indicate the highest antioxidant potential. Based on this observation, F3 was used as a reducing agent for the synthesis of ZnO NPs. The Result of TEM showed that ZnO NPs were spherical in shape with a size range of 60-98 nm. The result showed that ZnO NPs at different time duration (1-4 weeks) have significantly inhibited the formation of AGEs in terms of the fluorescence intensity of glycated BSA of the study. The ZnO NPs also markedly declined the level of fructosamine and formation of glycosylated Hb which are directly associated with the reduction of advanced glycation end products (AGEs) formation. Overall, the study suggests biologically synthesized zinc nanoparticles showed the strong antiglycation potential and considered as a potential source of therapeutic agents for AGEs related disorder.

Keywords: Antiglycation, *Bambusa arundinacea,* Nanoparticles, Zinc oxide

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INTRODUCTION

Zinc is a metallic element that possesses a distinctive physical and chemical property which makes it extremely useful and essential in the biological systems. It is one of the most essential micronutrients that play an important role in maintaining many biological processes [1]. Recently, research on zinc homeostasis receives increasing attention with regard to diabetes in the scientific society. The presence of zinc enhances the transport of glucose into the cells by promoting the secretion of insulin [2], insulin signalling [3] and phosphorylation of insulin receptors [4]. An earlier study demonstrated that zinc increases the stability and bioavailability of insulin makes it an insulin-like effect [5]. It is well established that zinc act as a cofactor of several antioxidant enzymes such as superoxide dismutase involved in regulating oxidative stress. The deficiency of zinc leads to increased oxidative stress and is associated with the development of diabetes and its associated complication [6]. Diabetes mellitus mostly type-2 is characterized by mainly insulin resistance and pancreatic β-cell dysfunction. Persistence of chronic hyperglycemia is one of the most contributing factors in diabetic macro and microvascular complications [7]. Under chronic hyperglycemia, AGEs, a group of heterogeneous compounds that results from protein glycation has been intensively studied [8]. Accumulation of protein glycation products in different tissue is a key contributing factor in the development of various diabetic complications [9, 10]. Inhibition of glycation induced protein cross-linking at a different stage is one of the remedial approaches that prevent the progression of complications associated with diseases. The compound with antiglycation may have to offer therapeutic potential in avoiding the different AGEs related way of life illnesses [11]. Currently, different natural and synthetic agents have been identified with inhibitory effects on AGEs formation, however just a few have effectively entered clinical preliminaries.

Nanomedicine is an emerging field and regarded as the upcoming drug therapy revolution. Nanoparticles synthesized from the natural source have been reported to exhibit a variety of bioactivities like as anti-bacterial, anti-larvicidal, antifungal, anti-inflammatory and antioxidant activities [12]. ZnO NPs have high solubility and biocompatibility which makes it highly mimic for the various biological applications like molecular labeling, drug delivery and nanomedicine [13]. ZnO NPs are biologically safe and due to their large surface area with high catalytic activity have gained significant attention to applying in the biomedical field. In the last few years, nanotechnologists highly utilized biosynthetic routes for the synthesis of nanoparticles compared to physicochemical methods. Natural compounds present in the plant act as a capping agent to stabilize the biological properties of nanoparticles [14]. A previous study reported that the biosynthesis of silver nanoparticles (AgNPs) was significantly reduced the AGEs formation in a concentration-dependent manner [15]. In recent times, it was reported that gold nanoparticles (GNPs) have shown antiglycation activity and prevent the glycation of alpha-crystallin protein [16].

A number of medicinal plants have been reported with antiglycation activity in various scientific literatures. *Bambusa arundinacea* (Family: Poaceae) is dispersed throughout India and highly reputed ayurvedic medicinal plants. Survey of scientific literature revealed that the leaves of *Bambusa arundinacea* have hypoglycemic activity, antidiabetic activity [17, 18] and analgesic along with an *invitro* antioxidant activity [19, 20]. Bamboo leaves of all the species contained pharmacological active components such as polyphenols, saponins, general glycosides, glycosylated flavones, betain and cholin that might explain its biological activity [21]. In China, antioxidant of bamboo leaves (AOB) composed mainly of flavone C-glycosides such as orientin, homoorientin, vitexin, and isovitexin is applied as food antioxidants during food preparation [22].

Keeping in view, the present study has been undertaken to investigate the antiglycation potential of biologically synthesized zinc nanoparticles from bioactive-fraction obtained from leaf extract of *Bambusa arundinacea.*

MATERIALS AND METHODS

Preparation of the leaf extract

Bambusa arundinacea was selected for the biosynthesis of ZnO NPs because of its cost-effectiveness, ease of availability and medicinal properties specifically antidiabetic and antioxidant. Fresh leaves from the *Bambusa arundinacea* were collected and authenticated at the Directorate of Medicinal and Aromatic Plants Research, Boriavi during the month of January 2019. The dried leaves of *Bambusa arundinacea* were grinded to a fine coarse powder. The soxhlet extraction technique is a well-accepted method for the extraction

of several bioactive compounds from plants [23]. The powered materials (20g) of the leaves were exhaustively extracted with a 1:1 ratio of water to methanol under the soxhlet extraction process. The collected extract was then evaporated using a vacuum rotary evaporator. The obtained crude extract was weighed and dissolved with appropriate distilled water.

Qualitative phytochemical analysis

The crude extract was analyzed for the presence of various phytochemicals like alkaloids, reducing sugar, saponin, phenolic compounds, flavonoids and glycoside as per standard procedures as described by Harborne [24].

Fractionation of crude leaf extracts by column chromatography

The bamboo leaf extract was subjected to column chromatography over silica gel (230-400 mesh, 40 g) used as stationary phase packed into column (3 cm × 60 cm Genie Bangalore) using methanol. The crude extract was then poured on the bed of silica. The bioactive compounds were then eluted from the charged column with a different mobile phase with a gradual increase in polarity. The elution was carried out by using a gradient solvent system starting with 100% chloroform (F1), chloroform-methanol (1:1, F2), methanol: water (1:1, F3) and 100% water (F4) to give four fractions. All four fractions were subjected to determine total phenolic, total flavonoid content and antioxidant potential.

Determination of total phenolic

The total phenolic content of each fraction was determined using Folin-Ciocalteu method according to Shi *et al*.[25]. The gallic acid was used as standard and prepared a calibration curve. The reaction mixture was prepared by taking 0.3 ml of fraction, 1.5 ml of 10% FCR solution kept for 5 min and further added 1.5 ml of 6% Na_2CO_3 solution. All reaction tube was mixed thoroughly and kept in the dark place for 2 h at room temperature. The absorbance was taken at λ = 760 nm using an ultraviolet–visible spectroscopy (Varian Cary 50). The total phenol present in each fraction was expressed as mg GAE/g.

Determination of total flavonoid

$AlCl_3$ colorimetric method reported previously [26] was used for the determination of total flavonoid present in each fraction. A calibration curve was prepared by using quercetin as a standard. 0.3 ml fraction was mixed with 100 µl of 5% $NaNO_3$ and kept for 5 min at room temperature. A 10% $AlCl_3$·6H2O (0.5 mL) was added followed by 1M NaOH (2 ml) and 2.5 ml of distilled water. After 5 min incubation, absorbance was measured against methanol as blank at 510 nm. The total flavonoid content was expressed in terms of mg QE/g.

Evaluation of antioxidant potential

The antioxidant potential of each fraction was determined according to the method describe by Adebiyi *et al*.[27]. Fifty microlitre of each fraction at different concentrations was mixed with 1.95 ml of 0.1 mM ethanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The reaction mixture was kept at room temperature for 20 min in a dark place. Absorbance was measured at 515 using Uv-spectroscopy and ascorbic acid was used as a positive control. Antioxidant potentials was calculated as percentage of inhibition as % I = [(Ao - Ae)/Ao]*100 (Ao = absorbance without extract; Ae = absorbance with extract) and determined IC_{50} value.

Green synthesis of ZnO NPs

The chromatographic fraction (F3) contained a higher amount of phenolic and flavonoid content with higher antioxidant capacity was selected for the synthesis of zinc nanoparticles. Green synthesis of ZnO NPs from F3 was carried out by the method of Santoskumar *et al*. [28] with some modification. 50 ml of Zinc acetate (1.0mM) was prepared in distilled water. 10 ml of obtained fraction F3 was added dropwise in the zinc acetate solution along with 20 ml of 2.0M NaOH solution under steady stirring at 60°C for 5 hours to accomplish a complex formation. The synthesis of zinc nanoparticles was confirmed by observing the color

change from whitish to light yellowish color after incubation time. The light yellow solid product obtained by centrifugation at 8000 rpm for 15 min and the pellet was collected. A pellet was washed in double-distilled water, dried and used for further studies.

Characterization of biosynthesized ZnO NPs

The biosynthesized ZnO NPs were characterized for its maximum absorbance using UV-Vis spectrophotometry. The optical properties of zinc oxide nanoparticles were determined by taking the absorbance in the range of 300-700 nm using the UV/Vis spectrophotometer. The dried nanopowder of zinc was mixed with 200 mg of potassium bromide and then pressed into pellets for Fourier transform infrared spectroscopy (FT-IR) analysis carried out using Perkin-Elmer model spectrometer with scanning range was 400–4000 cm^{-1}.

A Morphological study using microscopic methods

Transmission Electron Microscopy (TEM) of ZnO NPs was performed at Sophisticated Instrumentation Centre for Applied Research and Testing (SICART), Vallabh Vidhyanagar, Gujarat. Samples of the biosynthesized ZnO NPs were drop on the carbon-coated copper grids and kept overnight under vacuum desiccation to dry before loading them on to a specimen holder.

Determination of invitro antiglycation activity of ZnO nanoparticles

Glycated albumin was prepared by taking the mixture of BSA (40mg/ml) and glucose (500mM) in potassium phosphate buffer (200 mM with pH 7.4) containing 0.02% sodium azide for 4 weeks in the absence or presence of the ZnO nanoparticles separately. Aminoguanidine (AG) was used as a positive control. The formation of total AGEs was assessed by monitoring the production of these fluorescent products spectrofluorometrically (PerkinElmer) using fluorescence intensity at an excitation wavelength of 370 nm and an emission wavelength of 440 nm.

Determination of Fructosamine

The effect of ZnO nanoparticles on the formation of fructosamine was quantified by a colorimetric method using nitroblue tetrazolium as reported previously [29]. Samples (30 microlitres) were added to 180 microlitre of nitro blue tetrazolium reagent (0.2M carbonate buffer (pH 10.3) containing 0.574mM nitroblue tetrazolium) at 37°C. The absorbance change at 530 nm in the interval 5–15 minutes after the start of the reaction was measured. The standard curve prepared using 1-deoxy-1-morpholinofructose (1-DMF) was used to determine the level of fructosamine expressed in mM/L.

Hemoglobin Glucose Assay

The solution of ZnO nanoparticles was added to a mixture containing glucose (2.0%) and hemoglobin (0.6%) and gentamicin (0.2%) in 10mM phosphate buffer (pH 7.4). Aminoguanidine was used as a positive control. The test samples were then incubated in a dark room at room temperature for 4 weeks with intermittent shaking. The glycosylation of hemoglobin was estimated by the method described by Parker *et al.*[30]. The level of formation of glycosylated Hb was estimated by spectrophotometrically at 520nm. The % inhibition of glycosylated hemoglobin was calculated. Absorption with control was considered as 100% glycosylation.

Statistical analysis

The test results were expressed as the mean ± standard deviation on the triplicate determination. Data were analyzed by one-way ANOVA using MS Excel 2010 software, while a comparison of the mean values was assessed using the Tukey's test, and statistical significance was estimated at $p < 0.05$.

RESULTS

The preliminary phytochemical analysis of leaves of *Bambusa arundinacea* revealed the presence of flavonoids, phenolic compounds, saponins, terpenoids and tannins presented in Table 1. The total polyphenolic and flavonoid content present in crude extract and four fractions obtained after column chromatography is shown in Fig. 1. The total amount of phenolic (182.36 ± 20.26 mg GAE/g) and flavonoid (89.32 ± 7.84 mg QE/g) compounds present in the crude extract indicates that the solvent mixture will be effective in isolating active biological compounds. Of the four fractions**,** F3 had the highest phenolic (87.36 ± 9.32 mg GAE/g) and flavonoids (29.65 ± 6.52 mg QE/g) content followed by F2 and F4 and was statistically significant. F1 had the lowest phenolic and flavonoid content compared to all three fractions. In the present study, DPPH radical scavenging assay has been used which is a widely accepted method to evaluate the antioxidant potential. IC_{50} represents the amount or concentration of extracts needed to scavenge 50% of the free radicals. Antioxidant activity results were summarized by the respective IC_{50} of crude leaf extract with all four fractions and presented in Table 2. The DPPH radical scavenging patterns among the different fractions were shown in Fig. 2. The F3 had the highest scavenging activity with the IC_{50} value of 46.7 µg/ml while the F4 had the lowest scavenging activity (IC_{50} = 200 µg/ml). The optical absorption spectra of green synthesized ZnO NPs were recorded in the range of 200 to 700 nm. UV-Vis spectrum of ZnO NPs produced a surface plasmon absorption band centered at 375 nm has shown in Fig. 3.

Green synthesized ZnO NPs were characterized by FTIR to determine the potential functional groups of biomolecules present on it. The FTIR spectra of ZnO NPs synthesized using fraction (F3) of *Bambusa arundinacea* leaf extract is presented in Fig. 4. A sharp and intense absorption peak at 681.3cm^{-1} was obtained in FTIR spectra confirming the stretching vibration of Zn–O. 1430 cm^{-1} represents the spectrum of C–C stretch (in-ring) which designates the aromatics functional group. It is well known that stretched peak in the range from 1600 to 1800 cm-1 in the present study is attributed to C=O stretch in polyphenols. The peaks at 1191 and 2854 cm-1 indicates the presence of C-O stretching in amino acid and O-H stretch in a carboxylic acid. The intense bands observed at 2043 cm-1 have been assigned to C-O band indicated the presence of carbonyl (CO) species. The surface morphology and structural shape of green synthesized ZnO NPs were observed using TEM presented in Fig. 5.

Most of ZnO NPs visualized in TEM images are spherical and some oval particles are also seen in the micrograph. The average size of the synthesized ZnO nanoparticles was 80 nm. The non-enzymatic BSA/glucose model was adopted to examine the effects of fraction 3 derived from column chromatography of crude *Bambusa arundinacea* leaf extract. The antiglycation capacity of biologically synthesized ZnO NPs was tested for its ability to inhibit the formation of glycated BSA measured by spectrofluorometry and fructosamine assay. The degree of glycation of albumin formed at different time intervals measured relatively by measuring fluorescence intensity presented in Fig. 6. The increased fluorescent intensity in BSA/glucose reaction indicates the level of glycated BSA increased over a period of time. However, in the presence of ZnO NPs, the fluorescent intensity was significantly declined at all time-points studied suggest that lower level of glycated BSA compared to control. However, the inhibitory activity of ZnO NPs (65.8%) on glycation reaction observed is less than that of positive control AG (70.5%) based on the fluorescent intensity.

Fig. 7 shows the inhibitory effects of biologically synthesized ZnO NPs on glycated albumin measured in the form of fructosamine. ZnO NPs treated glycation of albumin and AG treated glycation of albumin showed significant inhibition of glycated albumin (43.41% and 55.19% respectively) when compared to the untreated glycated control. The level of fructosamine formed at the fourth week indicates that the treatment of ZnO NPs significantly inhibited the formation of advanced glycation end-product similar to the positive control AG. Table 3 illustrates the preventive effect of green synthesized ZnO NPs on the formation of hemoglobin glycosylation. Zinc nanoparticles synthesized in the present study can significantly (32.45% $P<0.05$) inhibit the glycosylation of hemoglobin when compared to the control.

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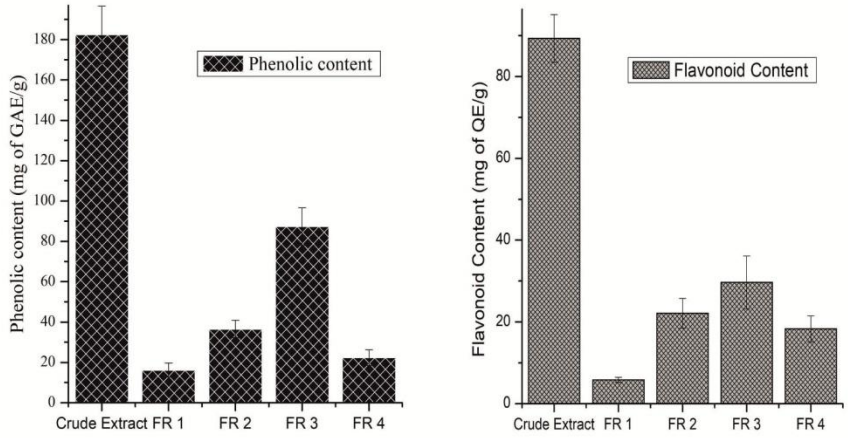

Figure 1: Total phenolic and flavonoid content present in the crude *Bambusa arundinacea* leaf extract and all four fractions collected from column chromatography.
(*) Indicates significantly *P*<0.05 differ from F1, F2 and F4.

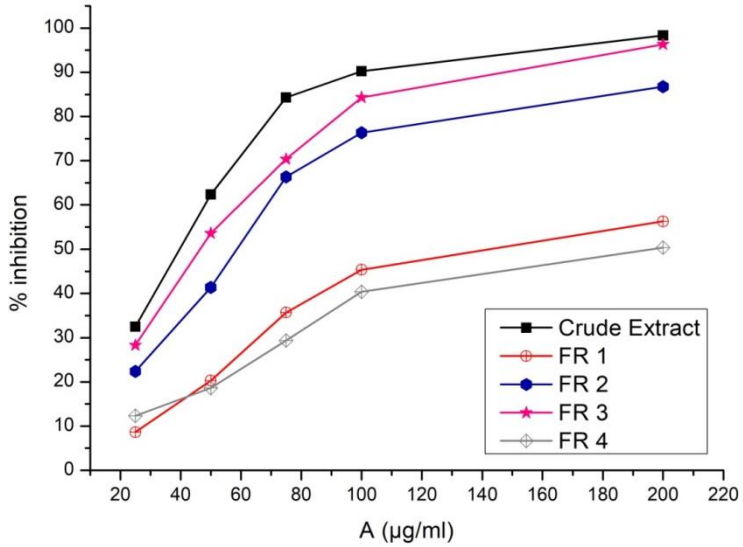

Figure 2: Antioxidant potential in the form of % inhibition of bioactive fraction F1-F4 and crude *Bambusa arundinacea* leaf extract (DPPH radical scavenging assay).

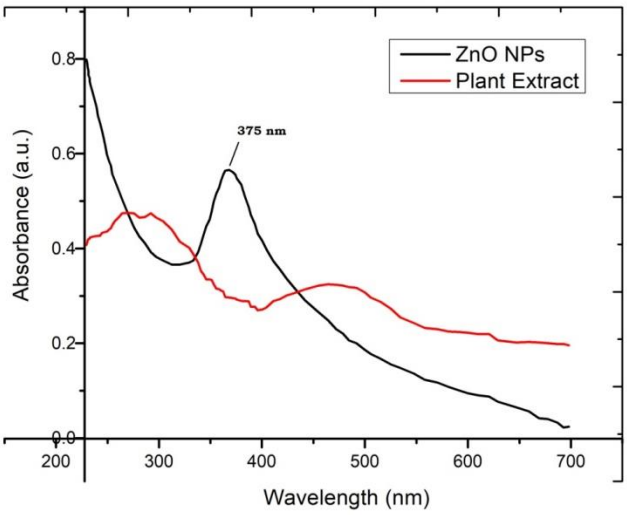

Figure 3: UV-VIS spectroscopic analysis for biologically synthesized ZnO NPs and most bioactive fraction F3 obtained from crude *Bambusa arundinacea* leaf extract.

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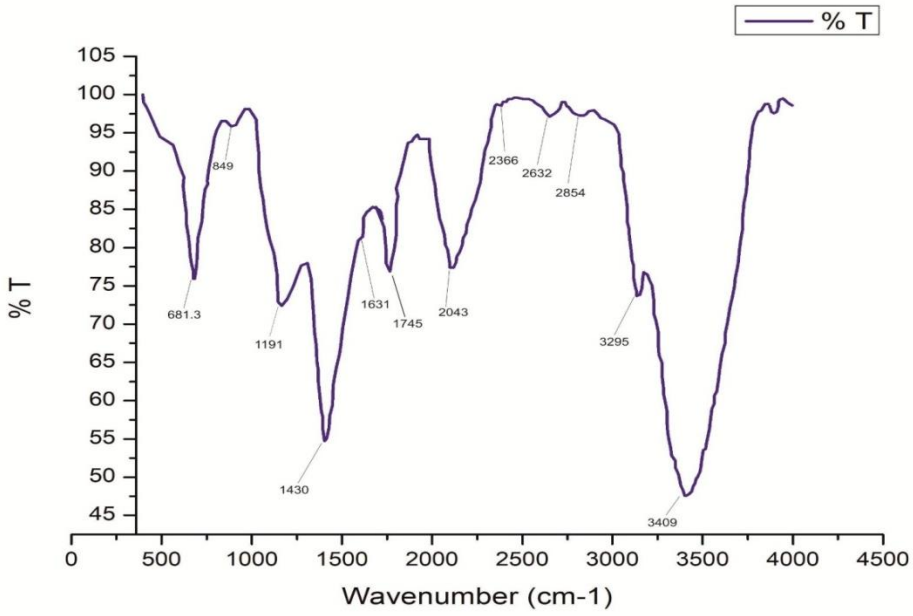

Figure 4: FTIR spectrum of biologically synthesized ZnO NPs

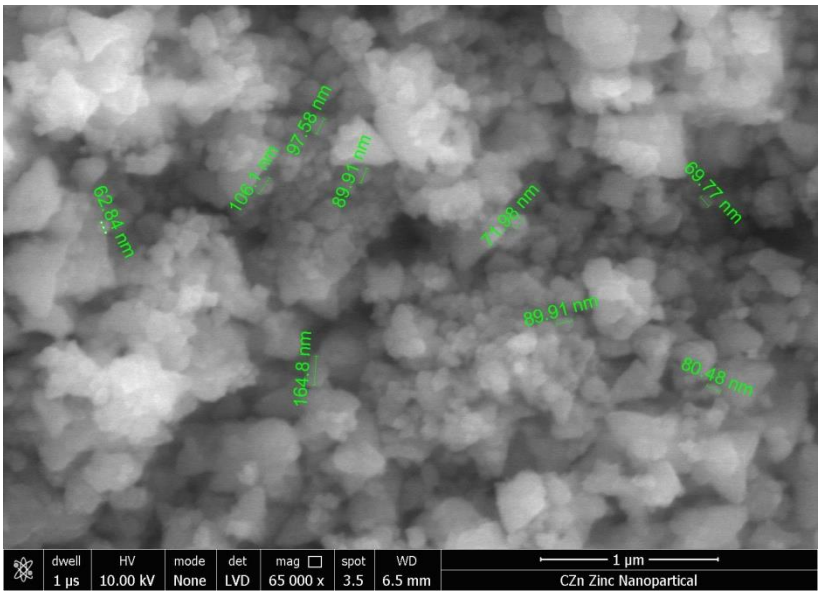

Figure 5: Transmission Electron Microscopy (TEM) showing spherical and oval shape of ZnO NPs synthesized by bioactive fraction F3.

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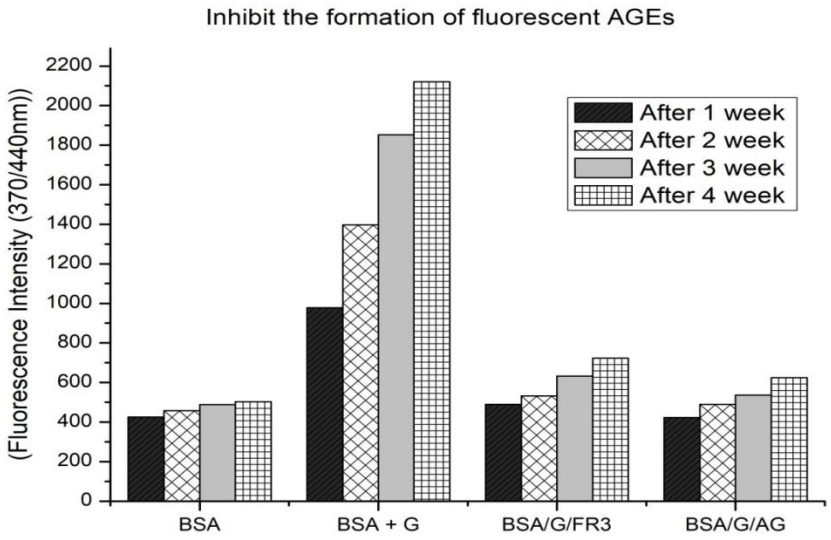

Figure 6: Degree of glycation of albumin formed at different time interval measured relatively by measuring fluorescence intensity by using spectrofluorometry.

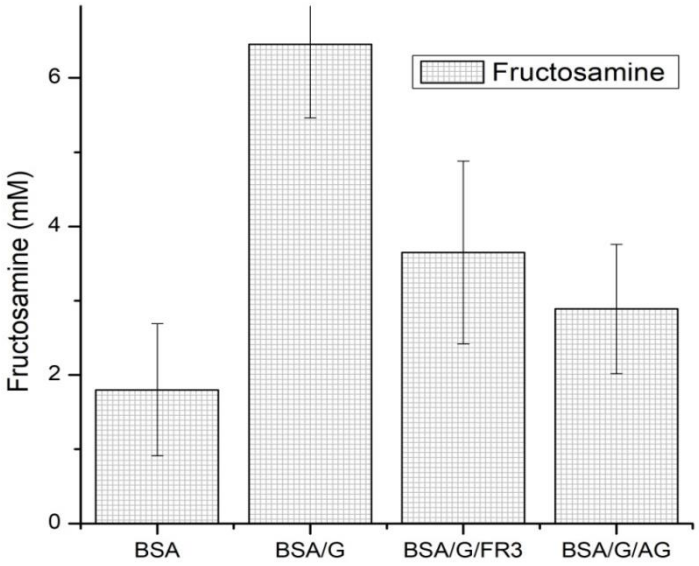

Figure 7: Inhibitory effect of biosynthesized ZnO-NPs from bioactive fraction F3 obtained from crude *Bambusa arundinacea* leaf extract on glycosylated Hb.

Table 1: Phytochemical screening of *Bambusa arundinacea* leaf extract

No.	Test	Test for conformation	Results
1.	Alkaloid	Dragendorff's test	-
2.	Anthraquinone	Borntrager's test	+
3.	Flavonoids	Shindo's test	+++
4.	Tannins	Ferric chloride method	++
5.	Terpenoids	Knollar's test	+
6.	Glycosides	Liebermann's Test.	+
7.	Saponins	lead acetate method	+++
8.	Sterols	H2SO4 test	+

Highly positive '++', Moderate '+', Negative '–'.

Table 2: Antioxidant potential of crude extract and fraction collected after column chromatography with IC_{50} value

Sample	IC_{50} value (µg/ml)
Crude extract	37.5
F1	195
F2	62.3
F3	46.7
F4	200.0

Table 3: Inhibitory effect of biologically synthesized ZnO NPs on the formation of glycosylated Hemoglobin expressed in percentage

No.	Absorbance	Percentage of Inhibition
Control (Hb + glucose)	0.723 ± 0.051	0
In presence of ZnO NPs with Hb + glucose	0.448 ± 0.082	39.73%

DISCUSSION

In the present study, the leaves of *Bambusa arundinacea* were selected for the synthesis of ZnO NPs due to its antidiabetic and hypoglycemic properties well reported in a previous study [17, 18]. The presence of different phytochemicals in the species of *Bambusa arundinacea* is well documented in an earlier study [31]. Crude leaf extract of *Bambusa arundinacea* was chromatographed on silica gel column chromatography and the total four fractions (F1, F2, F3, and F4) were collected by increasing polarity of the solvent system. It is well established that phenolic and flavonoid compounds are more solubilize in the polar organic solvent [32] therefore, F3 (methanol; water) contained a higher amount of phenolic and flavonoid compounds in the present results. An earlier report has been demonstrated that the methanolic extract of bamboo leaves contained a higher amount of phenols and flavonoids [20]. The result of the DPPH assay showed that F3 contained high amounts of radical scavenging compounds and it perhaps attributed to the higher total phenolics and flavonoids content amongst all four fractions. Those plants extract with a greater content of both phenols and flavonoids also showed higher antioxidant activity. The linear correlation between total phenolic and flavonoid content with antioxidant capacity are well reported [33]. In the present investigation, we have synthesized ZnO NPs by using F3 obtained after column chromatography as capping agents due to the presence of the highest amount of phenolic, flavonoid and antioxidant potential. The electron transitions from the valence band (O2p) to the conduction band (Zn3d) produce intrinsic band-gap absorption at 375 nm which is a characteristic peak of ZnO NPs [34] confirm the formation of zinc oxide nanoparticles. Metal nanoparticles are known to display unique optical absorption spectra due to the property of Surface Plasmon Resonance (SPR) [35]. Literature also suggests that characteristic vibration bands of ZnO are between 400 to 700 cm−1. Attachment of H_2O groups on the surface of particles is further identified from the characteristic peak at 3409 cm−1 [36]. The presence of various functional groups such as –C-O-, -OH, -C=O are derived from phenolic and flavonoid compounds present in the fraction of leaf extract and act as capping agents of the nanoparticles[37]. It is believed that the presence of phenolic and flavonoid compounds in fraction obtained from leaf extract may involve in stabilizing nanoparticles. The size and shape of ZnO NPs synthesized in the present study is similar to zinc oxide nanoparticles synthesized from a green approach [38]. Our results demonstrated that ZnO NPs efficiently inhibited AGEs formation. AG is widely used as AGEs inhibitor, prevents the formation of AGEs by interacting with reactive carbonyls [39]. The severity of diabetic progression could also predict from the measurement of glycated Hb (HbA1c) [40]. Zinc is an important micronutrient with an antioxidative effect. *Invitro* studies have demonstrated that zinc supplementation prevents the AGEs formation of albumin and ultimately reduced the AGEs mediated diabetic complication [41]. Furthermore, recent studies demonstrated the antidiabetic effect of zinc nanoparticles on streptozotocin-induced diabetic rats [42]. In addition to this, administered ZnO NPs significantly diminished the level of serum fructosamine and glycated hemoglobin in the diabetic rats reported recently which supports our results [43]. The level of phenolic content is highly correlated with the antiglycation activity demonstrated in many studies. It is well reported that the presence of phenolic and flavonoids in the aqueous extract of *Bambusa arundinacea* leaf exhibited hypoglycemic activity [18]. The proper mechanism of antiglycation activity exhibited by ZnO NPs synthesized from the isolated bioactive compounds of bamboo leaf extract is currently unknown and was not explored in the present study.

The antioxidative nature of zinc and constituents of bamboo leaf extract [20, 44] could reduce oxidative stress by scavenging free radicals and also reduced the generation of reactive carbonyl compounds. However, the antiglycation activity appears to correlate only partially with the antioxidative property**.** Moreover, a plant-derived phenolic and flavonoid compounds are well-reviewed for its inhibitory activity [45]. The phytochemical constitute of *Bambusa arundinacea* leaf revealed the presence of phenolic compounds quercetin, luteolin, rutin, tricin, caffeic acid, chlorogenic acid and flavonoid compounds like orientin, homoorientin, vitexin and isovitexin [46] which may be present as capping agent on ZnO NPs and showed strong inhibitory effects on the glycation of albumin. The mechanism of ZnO NPs antiglycation activity will be the subject of future studies.

CONCLUSION

The present investigation revealed that the biosynthesized ZnO NPs exhibiting good optical properties. Biologically synthesized ZnO nanoparticle was demonstrated to possess an *invitro* antiglycation activity based on the glucose-BSA assay. Its antiglycation potential was moderately less than that of aminoguanidine in this assay but can be further developed for application as an effective antidiabetic agent.

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Conflict Of Interest Statement

We declare that there is no conflict of interest.

Authors' Contributions

HVP, DBK, DM and HSB involved in designing work plan and edited manuscript. DM and AVM performed whole laboratory. HSB performed the data analysis. DM and DBK wrote the manuscript. HVP and HSB performed critical revisions of the manuscript.

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