Exploring the Therapeutic Potential: Investigating the Synthesis, Characterization and *in vitro* Anti-Diabetic Efficacy of Silver Nanoparticle Ethanolic Extract from *Boerhavia diffusa*

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Abstract
Many studies explore the effects of Boerhavia diffusa (Bd) combined with synthesized silver-nanoparticles extract (AgNPs) for treating a variety of conditions, although rarely for diabetes. To address this, we investigate the inhibitory effect of BdAgNPs in standard in vitro antidiabetic assays using the whole plant extract dissolved in ethanol (EBdAgNPs). EBdAgNPs was characterized by UV-Visible, XRD and FT-IR spectroscopy techniques. The assays consisted of α-amylase, α-glucosidase, protein glycation, non-enzymatic glycosylation of hemoglobin, glucose uptake by yeast and glucose diffusion. The formation of AgNPs was revealed by observing a surface Plasmon resonance peak at 410 nm.

Notably, EBdAgNPs exhibited robust inhibitory effects in comparison to standard control drugs within the assays. These compelling findings strongly indicate the potential of EBdAgNPs as a viable avenue for treating diabetes mellitus. Further clinical investigations are warranted to fully explore EBdAgNPs potential in novel therapeutic approaches for managing diabetes and related conditions.

Keywords: Silver nanoparticles, *Boerhavia diffusa*, XRD, Diabetes Mellitus, Antidiabetic activity.

Introduction

Nanotechnology has brought transformative innovations to science and industry by employing nanoparticles[32]. However, producing silver nanoparticles (AgNPs) through biosynthesis remains a long-standing challenge[10]. Medicinal plant extracts, widely used in green AgNP synthesis, act as reducing and stabilizing agents[35]. In recent years, nanotechnology has significantly advanced diabetes diagnosis and management, addressing various issues[20,27,34]. Notably, certain plants like *Boerhavia diffusa* (*B. diffusa*) have biologically active compounds promoting natural nanoparticle biogenesis[39]. *B. diffusa*, a tropical plant, is valued in traditional medicine[33]. Our study focuses on using whole *B. diffusa* for antidiabetic treatment. We assess the characterization and antidiabetic potential of ethanolic extract of *B. diffusa*synthesized silver nanoparticles (EBdAgNPs).

Material and Methods

Plants belonging to the species *B. diffusa* (Figure 1) were collected from in and around Coimbatore. The entire plant was authenticated by the Botanical Survey of India (BSI), TNAU, Coimbatore (BSI/SRC/5/23/2013-14/Tech/1041). Extraction and synthesis of AgNPs ethanolic extract of the whole *B. diffusa* plant were using Soxhlet equipment. The extract was then subjected with AgNPs, characterized and analyzed for *in vitro* antidiabetic activity[36].

Figure 1: *Boerhavia diffusa* L.

Synthesis of Silver Nanoparticles: 500mg ethanolic extract of *B. diffusa* was dissolved under 100ml of deionized water. From the above solution, 10ml of ethanolic extract was obtained and mixed with 90ml of deionized water containing 1mM silver nitrate (Figure 2a). Once mixed, they were exposed to the sunlight for 5 to 10 minutes. The greenish solution turned brown indicating that the silver nanoparticle was synthesized (Figure 2b). Furthermore, the solution was added to the centrifugation tube and processed at 13,000rpm for 20 minutes. The supernatant was discarded with the remaining pellet being mixed with deionized water for further processing. This was repeated three times[36]. The resultant pellet was lyophilized and the powdered sample was collected.

Characterization of green synthesized AgNPs particles in ethanolic extract of B. diffusa: The characterization of synthesized silver nanoparticle was analyzed by XRD (X'pert Pro X-ray diffractometer), FT-IR (FTIR spectroscopy – miracle 10, SHIMADZU) and UV-Vis (Shimadzu Bio Spec-nano).

(a) (b)

Figure 2: (a) Plant extract (*B. diffusa*) + $AgNO_3$ (b) Synthesized AgNPs of *B.diffusa*

***In vitro* antidiabetic activities**

The α-amylase inhibition assay: From 1 mg/ml stock solution, different concentrations of plant extracts were prepared in phosphate buffer. 10, 20, 40, 60, 80 and 100 μg/ml of EBdAgNPs and acarbose were combined with 500μl of α-amylase (0.5 mg/ml) and incubated for 10 minutes at room temperature. Following that, 500μl of 1% starch solution was added and incubated for 10 minutes. The reaction mixture was then treated with 1 ml of dinitro salicylic acid (DNS), a colouring reagent and heated for 15 minutes in a boiling water bath before adding 10 ml of distilled water. A blank was prepared by replacing the enzyme with the phosphate buffer for each concentration of the sample set to quantify the absorbance of the coloured extracts. The absorbance was determined at 540nm. With the help of equation 1, the inhibition percentage was computed as described in Ishwarya et al[12].

$$\% \text{ inhibition} = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} 100 \quad (1)$$

The α-glucosidase Assay: The α-glucosidase inhibitory activity was determined using the spectrophotometer[26]. Yeast α-glucosidase was dissolved at 0.1U/ml in 100mM phosphate buffer, pH 7.0, including 2000 mg/l bovine serum albumin and 200 mg/ml sodium azide as an enzyme source. As a substrate, para-nitro phenyl-α-Dglucopyranoside was utilized. EBdAgNPs (5%) plant extract was weighed, with serial dilutions of 62.5, 31.25, 15.6, 7.8, 3.9 and 1.95 mg/ml dimethyl sulfoxide with distilled water being prepared in equal volume. For 5 minutes, ten microliters of plant extract dilutions were incubated with a 50 microliter enzyme source. Following the incubation, 50 microliters of substrate were added and incubated for 5 minutes at room temperature. A microtitre reader was used to measure the absorbance, with a setting of 405 nm. The inhibition percentage was also calculated using equation 1.

Non-Enzymatic Glycosylation of Haemoglobin Method: In a 0.01 M phosphate buffer at pH 7.4, solutions of glucose (2%), haemoglobin (0.6%) and gentamycin (0.02%) were made. 1.0 ml of each of the above solutions were combined with 1.0 ml of the EBdAgNPs extract of varying concentrations 10, 20, 40, 60, 80 and 100 μg/ml. The reaction mixture was incubated for 72 hours at room temperature in the dark. Subsequently, colorimetric analysis at 520 nm revealed that much of the haemoglobin had been glycosylated. Metformin was used as a standard comparison drug. The inhibition percentage was determined using formula according to Gupta et al[11].

Inhibition *in vitro* protein glycation: Fructose at 1000 mM in 200 mM phosphate buffer at pH 7.4 (4.0 ml) was incubated for 24 hours with 5.0 ml of Bovine serum albumin (BSA) at 20 mg/ml in 200 mM phosphate buffer, pH 7.4. Additionally, 1.0 ml of EBdAgNPs at concentrations of 250, 500, 750 and 1000 μg was added. After incubation, the fluorescence intensities of the reaction mixtures were measured in the emission range of 370-650 nm, with an excitation wavelength of 360 nm. The amount of fluorescent advanced glycation endproducts (AGEs) produced was correlated with the fluorescence intensity. Pioglitazone, a common anti-glycation drug, served as a positive control at final concentrations of 1.25, 0.75 and 0.25 mg/ml. The percentage inhibition of fluorescent AGE formation was calculated using equation 2 as reported by Avwioroko et al[5]:

$$\% \text{ inhibition} = \frac{(FC - FB) - (FS - FSB)}{FC - FB} 100 \quad (2)$$

where FC is the fluorescence intensity of the control, FCB is the fluorescence intensity of the control blank, FS is the fluorescence intensity of the sample and FSB is the fluorescence intensity of the sample blank.

Glucose uptake by yeast cell: A 10% v/v suspension of commercial baker's yeast was prepared in distilled water. The yeast suspension was repeatedly centrifuged at 3,000 × g for 5 minutes until the supernatant fluids became clear. Next, 1 ml of glucose solution at concentrations of 5, 10 and 25 mM was added to different concentrations of EBdAgNPs at 20, 40, 60, 80 and 100 μg/ml. The mixture was incubated at 37 °C for 10 minutes. Subsequently, 100 μl of the yeast suspension was added to the above mixture, which was then vortexed. The reaction was allowed to proceed for 60 minutes at 37 °C. After 60 minutes, the tubes were centrifuged at 2,500 × g for 5 minutes to separate the supernatant. The amount of glucose in the supernatant was

then determined. Metronidazole was used as the standard drug for comparison. The percentage increase in glucose uptake by yeast cells was calculated using the formula defined by Gaddala and Nataru[9].

Glucose diffusion assay: To assess *in vitro* glucose diffusion, a cellulose ester dialysis tube (CEDT) was filled with 2 mL of a solution containing 0.15 M NaCl and 0.22 mM glucose. This solution was divided into two groups: one with the addition of plant extract (50μg/ml) as the treated group and the other without the extract as the control group. Following this, the CEDT was placed inside a 50 ml centrifuge tube and both ends of the tube were tightly sealed. 45 ml solution of 0.15 M NaCl was added to the centrifuge tube. The entire setup was then placed in an orbital shaker, maintaining a constant temperature of 27°C. At regular intervals of 60 minutes, the concentration of glucose in the external solution surrounding the CEDT was measured to monitor and to track the diffusion of glucose[4].

Results and Discussion

Characterization of EBdAgNPs: In this study, we comprehensively characterize EBdAgNPs using a multi-technique approach. UV-Visible spectroscopy, X-ray diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR) analyzed the nanoparticles' structure and optical properties. This integrated analysis offers insights into EBdAgNPs' composition, crystalline structure and potential applications.

UV Visible spectroscopic measurements: The EBdAgNPs was monitored by recording absorption spectra at wavelengths spanning from 250 to 800nm (Figure 3). In UV Visible spectroscopy the excitation of surface plasmon resonance in EBdAgNPs indicates strong characteristic peak observed around 410nm. This peak corresponds to AgNPs standard surface plasmon resonance absorption band.

One of the most successful strategies for assessing nanoparticles is ultraviolet visible spectroscopy[30]. An identical study was presented reporting that, the synthesis of AgNPs is facilitated by the presence of many phenols, proteins, tannins, flavonoids, alkaloids, quinones, sterols, carbohydrates, amino acids, terpenoids and coumarins in the *Phagnalon niveum* plant extract. Similar to this, AgNPs UV-Vis absorbance spectrum showed a maximum absorbance peak between 360 and 410 nm at varied concentrations and time intervals[42].

X-ray diffraction (XRD): The XRD pattern of EBdAgNPs was examined with the results shown in figure 4. The diffracted intensities varied between 10 and 80. Particularly strong Bragg reflections were observed at 2θ values of 27.99, 32.48, 46.46, 55.00 and 57.60 degrees, corresponding to the lattice planes of (111), (200), (220), (311) and (222) respectively, within the face-centered cubic structure of silver. The expansion of Bragg's peaks demonstrates the organisation of nanoparticles and Debye-Scherrer's[24] condition determines that the average size of AgNPs was 24nm.

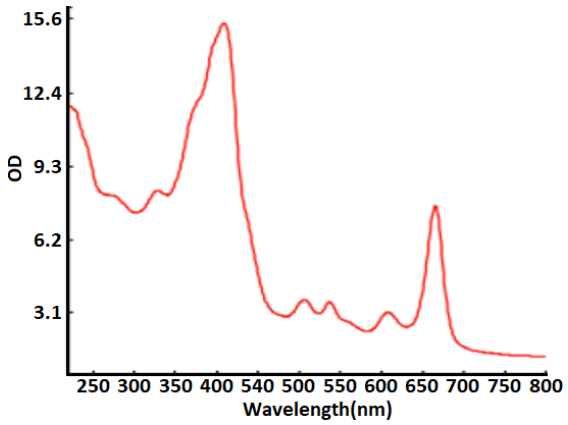

Figure 3: UV – Visible Spectrum of EBdAgNPs

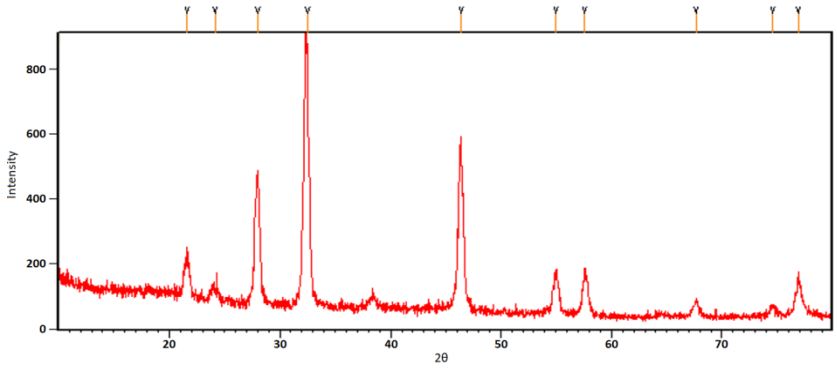

Figure 4: X-Ray Diffraction of EBdAgNPs

XRD is often used to analyse the composition or crystalline structure of the sample at specific conditions as shown by Ahmed et al[3]. The absorption spectra obtained at a higher pH (10) revealed that quercetin was around 19nm[25] and the average crystalline nature of the AgNPs of *Capparis zeylanica* L, leaf of extract was shown to be 35nm[13].

Fourier Transform Infrared Spectroscopy (FTIR) analysis: FTIR was performed in the range 1000 to 4000 cm^{-1} to determine which characteristic functional groups were present as shown in figure 3. The peaks at 1566 cm^{-1} and 1666 cm^{-1} reveal the C=C stretching of alkene[8]. The 1975 cm^{-1} and 2090 cm^{-1} peaks show the N=C=S stretching of isothiocyanate[46]. The 2399cm^{-1} peak shows the O=C=O stretching of carbon dioxide[37] whereas 2677 cm^{-1} indicates the C-H stretching of aldehydeade[1]. The distinct peak at 2877 cm^{-1} is attributed to C-H stretching vibrations of alkane[29]. The peak 3116 cm^{-1} is of O-H carboxylic stretch[43]. Avwioroko et al[5] showed that FTIR spectroscopy can be used to indicate when EBdAgNPs have reduced and stabilised silver nanoparticles (Figure 5).

Furthermore, similar analyses were performed on other natural extracts, such as fresh leaf Lemongrass[9], Mulberry leaves[6] and *Tabernaemontana ventricosa*[19]. The functional biomolecules containing carboxylic group have been found to be involved in the reduction of silver ions. This was confirmed by analyzing the FTIR spectrum[2].

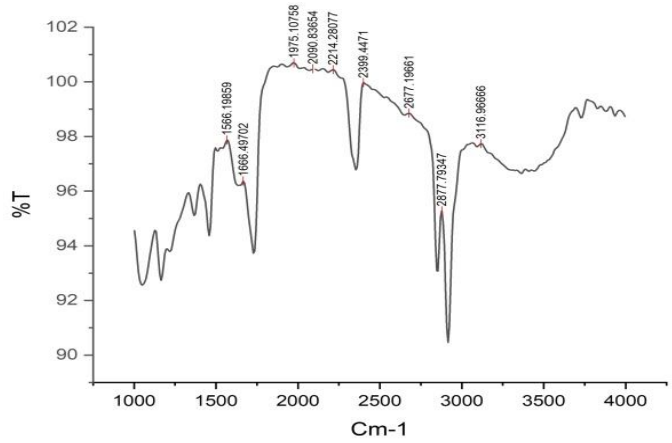

Figure 5: Fourier Transform Infrared Spectrum of EBdAgNPs

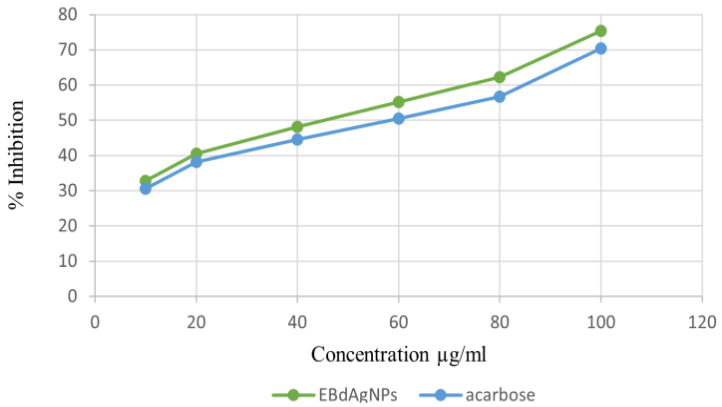

Figure 6: Alpha Amylase Activity of EBdAgNPs

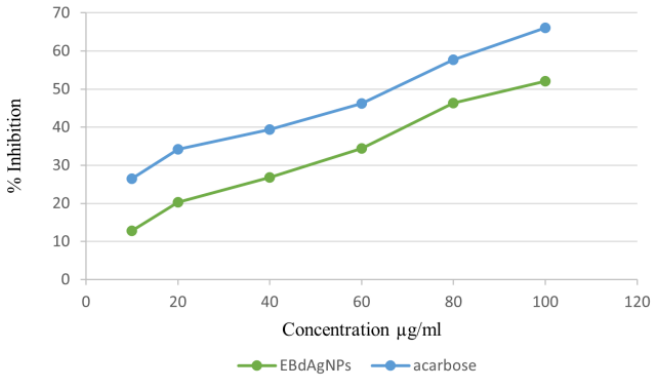

Figure 7: Alpha Glucosidase Inhibitory Activity of EBdAgNPs

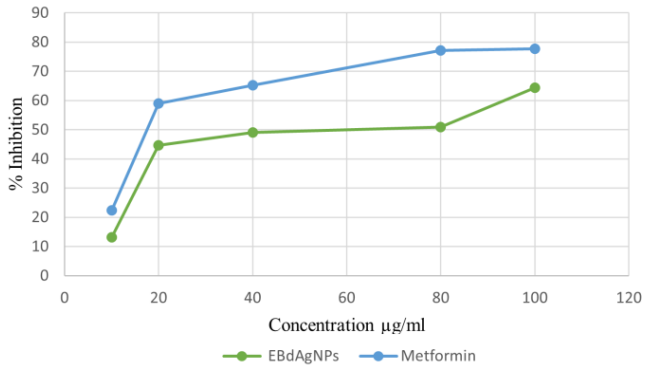

Figure 8: Non-enzymatic Glycosylation of EBdAgNPs

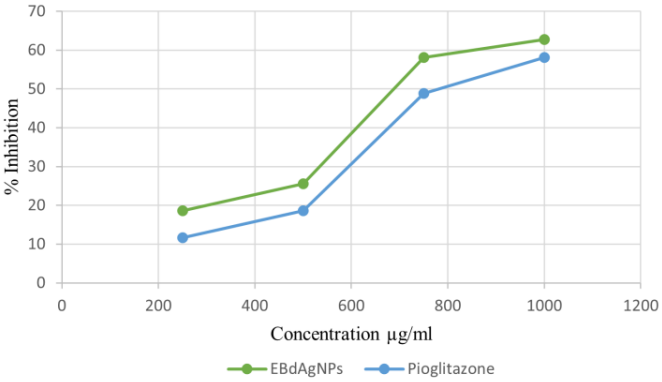

Figure 9: Protein Glycation in EBdAgNPs

Antidiabetic Study: We introduce comprehensive anti-diabetic assays that gauge EBdAgNPs' potential as a diabetes management agent. These assays target vital aspects of glucose metabolism. We analyze EBdAgNPs' inhibitory effects on α-amylase, starch hydrolysis enzyme and α-glucosidase, carbohydrate breakdown enzyme. Additionally, we study their impact on non-enzymatic glycosylation, protein glycation, yeast cell glucose uptake and inhibition of glucose diffusion through cell membranes. These assays collectively reveal EBdAgNPs' capacity to influence critical diabetes pathways, yielding insights for future therapeutics.

Inhibition of α-amylase activity of EBdAgNPs: Hyperglycemia defines diabetes mellitus, a group of metabolic disorders. Blood sugar is managed using insulin, oral hypoglycemic drugs, or carbohydrate enzyme inhibitors. Due to insulin and hypoglycemic side effects, researchers seek novel anti-diabetic sources[38]. α-amylase, vital for carbohydrate metabolism, is targeted for natural inhibitors[21]. For instance, Kifle and Enyew[17] found *Bersama abyssinica fresen* inhibiting α-amylase concentration-dependently (100-1000 μg/ml). *Bersama abyssinica fresen*'s crude extract showed lower IC_{50} compared to acarbose, suggesting its potential as an anti-diabetic α-amylase inhibitor source.

EBdAgNPs was assessed for their inhibitory effect on α-amylase in a dose-dependent manner, ranging from 10 μg/ml to 100 μg/ml (Figure 6). EBdAgNPs displayed significant inhibitory efficacy against the enzyme α-amylase, with a 30–70% inhibition range when synthesized from the entire plant. At the maximum doses of 100 μg/ml, acarbose and EBdAgNPs inhibited 70.4% and 75.31% of the enzyme activity respectively with IC_{50} values of 55.4 μg/ml and 46.2 μg/ml. Overall, EBdAgNPs showed higher dose-dependant inhibitory effects on α-amylase when compared with arcobose. These findings demonstrate that EBdAgNPs effectively suppressed the activity of the α-amylase enzyme under *in vitro* conditions.

Inhibition of α-glucosidase enzyme activity of EBdAgNPs: In this study, we explored plant-based α-glucosidase inhibitors. We examined inhibitory effects using EBdAgNPs-derived α-glucosidase enzymes. Blocking carbohydrate-hydrolyzing enzymes like α-glucosidase is a strategy to mitigate glucose absorption in diabetes. However, the common α-glucosidase inhibitor, acarbose, triggers gastrointestinal issues. Recognizing plants' therapeutic potential, recent research has focused on α-glucosidase inhibitors from plants[7,33].

In comparison to acarbose, the EBdAgNPs showed weaker α-glucosidase inhibitory activity, as illustrated in figure 7. The IC_{50} value for acarbose, the standard drug, was determined to be 63.4μg/ml, whereas EBdAgNPs yielded 93.0μg/ml. Despite the higher IC_{50} value for EBdAgNPs compared to the the standard drug, it still exhibited notable inhibitory effects as evidenced by increasing inhibition at higher concentrations. Further enhancement of these effects could potentially be achieved through the isolation and purification of their active constituents.

Inhibition of non-enzymatic glycosylation of EBdAgNPs: Non-enzymatic bonding between hemoglobin (Hb) and glucose causes glycated Hb formation, heightened in hyperglycemic conditions[31]. Plant extracts mitigate glucose-Hb complex, elevating free Hb levels. This was observed in *O. forskolei* leaves and stems[15] and *C. viscosa*[45]. Ukwuani-Kwaja et al[41] noted that plant extracts can surpass standard drugs in inhibiting hemoglobin glycosylation.

In this study, we observed a dose-dependent increase in the percentage inhibitory effect on hemoglobin glycosylation as the concentrations of EBdAgNPs increased (Figure 8). This suggests that the plant extracts enhance the amount of free hemoglobin by reducing the formation of the glucose-hemoglobin complex. Among the plant extracts tested, the most significant inhibitions were observed in EBdAgNPs (64.32%) at the highest concentration (100μg/ml), which was comparable to the inhibitory effect of Metformin (77.72%). EBdAgNPs demonstrate a robust dose-dependent response akin to the standard drug, albeit with slightly lower inhibition values, as evidenced by the IC_{50} values of 63.97 μg/ml and 28.64 μg/ml for EBdAgNPs and metformin, respectively. There is potential to enhance the inhibitory response of EBdAgNPs through additional extraction techniques and processing.

Inhibition of protein glycation of EBdAgNPs: Diabetes complications stem from advanced glycation end product (AGE) formation via protein glycation, posing health risks. This assay seeks inhibitors to curb protein glycation, yielding insights for diabetes research and care. Identifying effective inhibitors may open avenues for future investigations in diabetes management. In this study, the *in vitro* inhibitory efficacy of EBdAgNPs extracts on protein glycation was assessed using the model system of bovine serum albumin and fructose. As depicted in figure 9, the inhibitory effect improved with increases in concentration from 250 to 1000 μg/ml.

At a concentration of 1000 μg/ml, Pioglitazone exhibited 58.14% inhibitory effects on protein glycation activity, with an IC_{50} value of 856.25μg/ml. Meanwhile, the EBdAgNPs showed 62.79% inhibitory activity, with an IC_{50} value of 756.35μg/ml. In comparison to the widely used anti-glycation medication, aminoguanidine (IC_{50} = 138 mg/L), the etnanolic extracts of dandelion, roseroot and water extract of Myrica gale demonstrated significant suppression of AGE production with IC_{50} values of 69.4, 74.0 and 70.4 mg/ml respectively[32].

The leaf extracts of *C. bullatus*, *C. zabelii* and *C. integerrimus* were identified as the most potent inhibitors of protein glycation, with IC_{50} values ranging from 32.6 to 36.5 μg/ml. These extracts displayed a capacity twice as high as that of aminoguanidine (IC_{50} = 71.1 μg/ml), a synthetic drug used to treat diabetic complications and known to inhibit the formation of AGEs. Furthermore, conventional polyphenols, particularly procyanidin B2 and (-) -epicatechin, exhibited substantial anti-AGE activity in addition to the extracts, outperforming aminoguanidine significantly in this regard[16].

Glucose uptake assay by yeast cells of EBdAgNPs: Testing yeast cell glucose uptake informs diabetes research. Yeast cells, akin to humans, offer insulin-independent glucose transport insights. It reveals drug targets, clarifies glucose control and enables cost-effective screening. Manipulable yeast strains aid gene and protein studies, enhancing diabetes care. *Centella asiatica* was assessed for glucose absorption in yeast cells at 5mM and 10mM concentrations[44]. Comparing 10mM to 5mM, inhibition increase was lower. *L. hastata* leaves exhibited rising glucose uptake with 25mM, 10mM, 5mM fractions[41]. The impact of the EBdAgNPs samples on glucose transport across yeast cell membranes was investigated in an *in vitro* method that included yeast cells suspended in variable amounts of glucose solution (5, 10 and 25mM) in the presence of extracts at various concentrations (Table 1).

Table 1
Antidiabetic activity of Glucose uptake assay by yeast cells

Glucose conc. (mM)	Sample conc. (μg/ml)	Standard (%)	EBdAgNPs (%)
5	20	5.17±0.294	14.2±0.274
	40	19.5±0.312	32.4±0.49
	60	38.2±0.664	43.4±0.262
	80	60.2±0.64	56.5±0.0987
	100	65.2±2.43	66.5±0.0867
10	20	15.5±0.0917	22.2±0.63
	40	32.3±1.01	36.7±2.35
	60	49.3±1.97	53.1±1.79
	80	50.4±4.98	55.7±2.54
	100	62.3±2.67	64.5±1.13
25	20	32±0.947	21.3±0.643
	40	44.5±1.33	36.6±1.04
	60	49.9±1.98	33.8±2.15
	80	62.3±1.87	41.9±0.898
	100	63±1.76	52.4±1.1

Table 2
Antidiabetic activity of Glucose Diffusion Assay

Sample	Glucose in external solution (mM)				
	30mins	60mins	90mins	120mins	180mins
Absence of drug (Control)	51.9±0.638	61.5±0.57	61.6±0.521	107±0.831	135±0.944
Metformin	18.6±0.309	19.3±0.483	19.8±0.823	19.3±0.248	20.7±0.36
EBdAgNPs	19±0.28	20.4±0.527	21.2±0.351	21.6±0.416	26.5±0.343

The inhibition caused by metronidazole was marginally higher compared to EBdAgNPs, except at 25mM where EBdAgNPs showed higher inhibition. EBdAgNPs showed dose dependent inhibitory behaviour at all glucose concetrations. These findings suggest that the plant extract may effectively enhance glucose absorption, thereby potentially improving glucose utilization and managing blood glucose levels, as supported by previous research[29].

Inhibition of glucose diffusion of EBdAgNPs: Inhibiting glucose diffusion manages diabetes, controlling blood glucose, reducing hyperglycemia risk, enhancing insulin sensitivity and supporting heart health. It assists in postprandial glucose management and potential weight control. This study investigated the impact of EBdAgNPs extract incorporated with synthesized silver nanoparticles, on inhibiting glucose diffusion across a dialysis membrane (Table 2). Glucose diffusion inhibition was measured at intervals of 30, 60, 90, 120 and 180 minutes.

In comparison to EBdAgNPs, the effect of conventional metformin on glucose diffusion showed a less pronounced significance over different time intervals. Notably, the inhibitory level increased progressively from the initial to the final minutes in the sample.

The glucose diffusion inhibition measurements were conducted at intervals of 30, 60, 90, 120 and 180 minutes. Comparatively, the inhibitory effect of conventional acarbose was slightly lower than that of EBdAgNPs in the glucose diffusion assays. To explore the influence of different leaf extract fractions on glucose retardation within the dialysis tube, a glucose diffusion experiment was performed. Notably, significant differences were observed between the fractions in glucose diffusion at various time points. The glucose diffusion in *A. nilgiricum* leaves exhibited an increase from 30 to 180 minutes, as shown by Konappa et al[18]. This experiment provides valuable insights into the impact of leaf extract fractions on glucose diffusion inhibition, offering potential avenues for future research and development of antidiabetic agents.

Conclusion

The current study explores the activity of the whole plant extract of EBdAgNPs through three characterization studies. UV-Vis analysis revealed the rapid synthesis of AgNPs with a characteristic peak at 410nm, indicating successful nanoparticle formation due to the reaction of phytochemicals with silver nitrate solution. XRD analysis demonstrated the presence of lattice planes (111), (200), (220), (311) and (222) in the face-centered cubic structure of silver. FTIR evaluation confirmed the adsorption of an aldehyde group on the nanoparticle surface.

The EBdAgNPs exhibited inhibitory effects on several key activities related to diabetes mellitus including α-amylase, α-glucosidase, non-enzymatic glycosylation of hemoglobin, glucose absorption by yeast cells, glucose diffusion and protein glycation. These findings suggest the potential of EBdAgNPs for the treatment of diabetes mellitus. The method of synthesizing AgNPs through this approach proves to be safe, eco-friendly, cost-effective and efficient, with promising biological characteristics in in vitro tests. The presence of antidiabetic qualities in EBdAgNPs is evident.

However, further research through *in vivo* assays and clinical trials is required to validate the complete curative potential of diabetic illness. Overall, this study presents a promising avenue for the development and application of EBdAgNPs in diabetes treatment and it opens up new possibilities for future investigations in this field.

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