

ANTI-DIABETIC ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED FROM ASHWAGANDHA ROOT AQUEOUS EXTRACT

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ABSTRACT

According to traditional medicine, *Withania somnifera* also known as Indian ginseng is a plant with a cure for many diseases. This study was conducted to develop an ecofriendly, cheap and effective procedure for the green synthesis of silver nanoparticles (AgNPs) from ashwagandha root aqueous extract (ARAE) and evaluating their *in-vitro* anti diabetic activity. Addition of ARAE to AgNO₃ (1mM) solution kept on stirring at 60°C resulted in the formation of Ashwagandha Root Aqueous Extract Nano particles (ARAENP). Synthesized nanoparticles are subjected to characterization by various procedures. UV-Visible spectroscopy showed absorption peak at 440nm which was absent in ARAE and AgNO₃. FTIR spectroscopy revealed the absorption peaks of different functional groups involved in the formation of ARAENP and further emphasizes role of withanolides in formation of ARAENP. Scanning Electron Microscopy (SEM) revealed average size of ARAENP as 123.23nm. 2 θ peaks at 28.98°, 39.80°, 42.24°, 64.42° and 79.76° were identified by X-ray Diffraction studies (XRD). Stability of ARAENP was determined by zeta potential which was found to be -10mV. Successfully characterized ARAENP were evaluated for their *in-vitro* anti diabetic potential by α -amylase and α -glucosidase inhibitory activities. Acarbose was used as a standard. Inhibitory potential of ARAE and ARAENP against α -amylase and α -glucosidase proves their therapeutic role. Inhibition of enzyme was probably due to the withanolides capped on ARAENP.

Keywords: α -Amylase, α -Glucosidase, Ashwagandha, silver nanoparticles, Diabetes mellitus.

INTRODUCTION

Diabetes mellitus is an epidemic, endocrine disease characterized by increased blood glucose level and glycosuria affecting 6% of the worldwide population. Type 1 and type 2 are the two major types of DM. type 2 accounts for 90% of all cases and may occur due to inadequate secretion of insulin¹ and resistance of insulin receptors². Two important enzymes viz., α -amylase and α -glucosidase are involved in the hydrolysis of starch³. Pancreatic α -amylase converts starch in to disaccharides and oligosaccharides; these are further converted to glucose by intestinal α -glucosidase. These two enzymes have a very important role in the onset of Type 2 DM⁴. Marketed anti-diabetic drugs like Acarbose, Voglibose and miglitol act by inhibiting these enzymes but are associated with serious side effects like diarrhea, bloating

meteorism and distention^{5, 6}. To surmount the drawbacks of available drugs, use of medicinal plants is increasing all over the world because of their low cost, safe and effectiveness.

Withania somnifera commonly known as ashwagandha belonging to family Solanaceae contains withanolides, a group of steroidal lactones in roots⁷. Apart from withanolides other constituents which are present in roots are withaferins⁸ and withanosides⁹. Pharmacological activity of this plant include anxiolytic-anti-depressive¹⁰, antifungal¹¹, antimalarial¹² apoptotic¹³, Hypoglycemic effects¹⁴, anticancer activity^{15, 16}, anti-inflammatory, antitumor, anti-stress, antioxidant, immunomodulatory^{17, 18}, antidiabetic¹⁹. Leaves also exhibit antibacterial, anti-fungal and antitumor properties²⁰.

Distinctive property of nanoparticles is their large surface area to volume ratio. Silver, gold, palladium and platinum are the metals used in the synthesis but silver is superior among all the metals²¹. Physical and chemical methods used for the synthesis are harmful and expensive so there is a need of ecofriendly method for synthesis of nanoparticles²². Green synthesis is another approach for synthesis of nanoparticles increased enormously in current research because of safety, effectiveness and low cost. This innovative technique involves the reduction of Ag⁺ to Ag⁰ and stabilization of AgNPs by biomolecules which are present in plant extract²³. Based on these benefits, present study was designed to use ARAE for the synthesis of silver nanoparticles and further evaluating their *in-vitro* anti-diabetic activity.

MATERIALS AND METHODS

Plant Material Collection and Extraction:

Roots of Ashwagandha plant were collected from in and around areas of Sangareddy Dist. in Dec 2017. Plant material was authenticated at Botanical Survey of India, Hyderabad, and Telangana, India. PRIP/PCOG/17-18/001 is the reference number given to herbarium and it is stored in dept. of Pharmacognosy, Pulla Reddy Institute of Pharmacy, Hyderabad, India. Freshly collected roots were washed under running tap water, dried in shade for 15 days, sliced in to small pieces and grinded to coarse powder. 20gm of powder was boiled with 200ml of double distilled water (DDW) for 1hr. After cooling to room temperature, mixture was filtered with whatman filter paper no.1 and filtrate is refrigerated (4°C) for future use.

Synthesis of ARAENP:

190ml of 1mM AgNO₃ solution was prepared by using DDW, 10ml of aqueous extract was added slowly to it, reaction was carried out in a conical flask kept on stirring at a temp. of 60°C for 8hrs. Change in the

color of solution specifies the formation of ARAENP. Solution was centrifuged at 4000rpm to obtain the pellet of nanoparticles which was stored for future use²⁴.

Characterization of ARAENP:

UV-Visible spectroscopy and Visual identification of ARAENP:

Color of ARAE and AgNO₃ solution was taken as control and change in the color of reaction mixture after addition of ARAE to AgNO₃ solution is the first indication for the formation of ARAENP. UV-visible spectroscopy was conducted in a range of 200-800nm by using UV-VIS Spectrophotometer (UV3000, LBINDIA) model No. 18-1885-01-0115.

FTIR spectroscopy of ARAE and ARAENP:

In order to determine the involvement of functional groups in the formation of ARAENP FTIR spectroscopy (BRUKER, ALPHA) was conducted in the range of 4000 to 500 cm⁻¹. ARAENP synthesized also contains biomolecules which are not capped on nanoparticles; they are removed by dissolving in DDW and centrifuged at 5000rpm for 10 min, procedure is repeated for 3 times and final pellet obtained is dried in at 60⁰C in hot air oven and used for characterization.

SEM studies of ARAENP:

Size, shape and morphology of ARAENP was determined by using SEM (ZEISS) operated at accelerating voltage 10.00kV, magnification 50.00 KX, working distance 8.5mm. Minute quantity of ARAENP was dropped on copper grid coated with carbon, extra solution was removed by using blotting paper and grid was used for study.

XRD studies of ARAENP:

Crystallographic structure of ARAENP was determined by using XRD (SHIMADZU, XRD7000) operated at scan speed – 5.0 deg/min, sampling pitch – 0.02 deg, preset time is 0.24 sec, 2 θ range from 10⁰ to 80⁰, 30mA current, 40kV voltage with K α 1 Cu radiation, λ = 1.54 Å. Crystallographic structure of ARAENP was calculated by using peaks of XRD. Average size of ARAENP was assessed by using the Debye-Scherrer equation²⁵.

$$D = \frac{k\lambda}{\beta \cos\theta}$$

Where D = average size of ARAENP, k = constant (0.94), λ = wavelength of X-rays (1.546Å), β = full width at half maximum (FWHM), θ = diffraction angle in degrees.

Zeta potential and Dynamic Light Scattering (DLS) studies of ARAENP:

Stability of ARAENP was determined by using light scattering method for measuring zeta potential (HORIBA SCIENTIFIC, SZ100) operated at electrode Voltage - 3.4V, Conductivity - 0.168mS/cm, temperature (25⁰ C) and Viscosity of dispersion medium (0.894mPa.s). Particle size distribution was estimated by DLS measurements.

***In-vitro* anti diabetic activity:**

Effect of Acarbose, ARAE and ARAENP on α -amylase activity:

Various concentrations (2 to 10mg/L) of Acarbose, ARAE and ARAENP were prepared by using DDW. 100 μ L of each of the concentrations was taken in a test tube; to this add 500 μ L of 20mM sodium phosphate buffer with 6mM NaCl pH 6.9 containing porcine pancreatic α -amylase (0.5 mg/mL). Mixture was incubated for 10min at 25 $^{\circ}$ C. 500 μ L of 1% starch solution prepared in 20mM sodium phosphate buffer with 6mM NaCl pH 6.9 was added to each test tube. Reaction mixture was again incubated at 25 $^{\circ}$ C for 10 minutes. 1ml of 3, 5 dinitro salicylic acid was added to stop the reaction; mixture was further incubated for 5 min in boiling water bath. Cooled to room temperature and 10ml of DDW was added and absorbance was measured by using UV-Visible spectrophotometer at 540 nm. Without Acarbose, ARAE and ARAENP, a complete reaction mixture was taken as control. All the tests are carried out in triplicates. Inhibitory activity of α -amylase was calculated by using the formula²⁶:

$$\% \text{ inhibition} = (\text{Acontrol} - \text{Asample} / \text{Acontrol}) \times 100.$$

Effect of Acarbose, ARAE and ARAENP on α -glucosidase activity:

Various concentrations (2 to 10mg/L) of Acarbose, ARAE and ARAENP were prepared by using DDW. 20 μ L of each of the concentration was taken in a test tube. To this add 100 μ L of α -glucosidase (16.9 U/ml) solution in 0.1M phosphate buffer pH 6.9. Incubate at 25⁰ C for 10 minutes and then add 50 μ L of 5mM p-nitrophenyl- α -D-glucopyranoside solution prepared in 0.1M phosphate buffer pH 6.9. Reaction mixture was incubated at 25⁰ C for 5 minutes and absorbance was measured by using UV-Visible spectrophotometer at 405 nm. Control contains complete reaction mixture without Acarbose, ARAE or ARAENP²⁷. All the tests are carried out in triplicates. Inhibitory activity was expressed as percentage inhibition and determined by using the formula:

$$\% \text{ inhibition} = (\text{Acontrol} - \text{Asample} / \text{Acontrol}) \times 100$$

RESULTS AND DISCUSSION

UV-Visible spectroscopy and Visual identification of ARAENP:

Visual proof for the formation of ARAENP is change of color of AgNO_3 solution from colorless to dark brown. Excitation of surface Plasmon resonance and reduction of Ag^+ to Ag^0 are the two major causes for the change of color of solution (Figure 1). Temperature, time and stirring accelerated the reaction. Our results are in accordance with the results of Rashid *et al.*²⁸ where they also showed change in color solution. UV spectra of ARAE, silver nitrate and ARAENP are presented in figure 2. Many studies reported the characteristic peak of SNPs in the range of 400-450nm which was absent in ARAE and AgNO_3 , whereas a peak at 440nm was successfully appeared in ARAENP spectrum. This is very close to the peak at 443nm obtained by Haytham M.M.Ibrahim²⁹ with the AgNPs synthesized using banana peel extract.

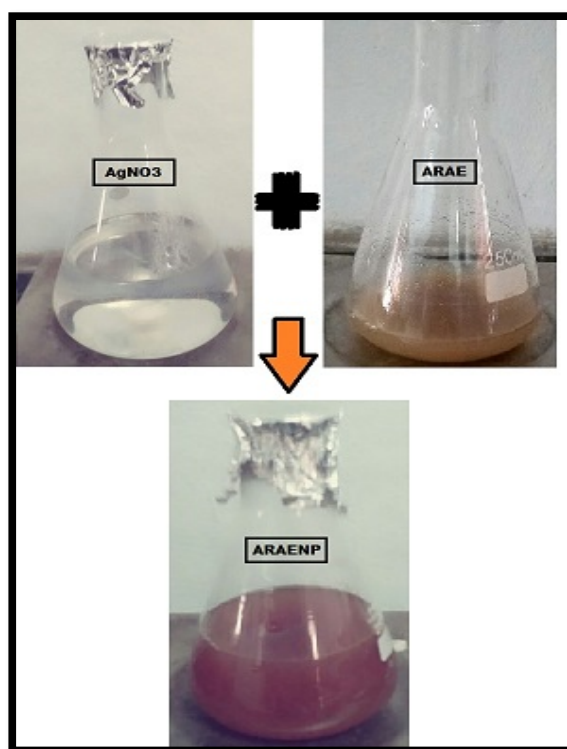


Figure 1: ARAE which is light brown in color when added to AgNO_3 solution 1mM it forms dark brown coloured ARAENP. change of color indicates formation of nanoparticles.

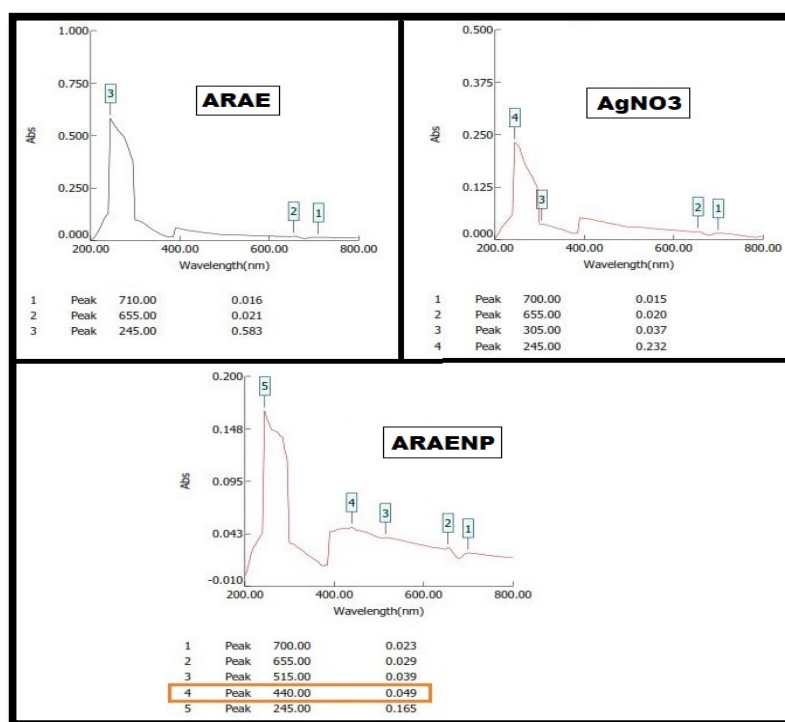


Figure 2: UV spectra of AgNO₃, ARAE and ARAENP.

The melting points of all synthesized compounds were found in open capillary tubes and readings were uncorrected. The structures of the synthesized compounds were supported by physical data (Table-1) and following spectral analysis.

FTIR spectroscopy of ARAE and ARAENP:

FTIR spectroscopy revealed the role of different functional groups involved in the formation of AgNPs depicted in figure 3. Different absorption peaks of ARAE and their shift in ARAENP and their corresponding functional groups are discussed in table 1. Proteins, polysaccharides and enzymes present in extract contains –OH group which undergoes stretching vibrations and produces peak at 3422.4 in ARAE, which is shifted to 3399.1 in ARAENP indicating their role in the formation of SNPs. FTIR spectroscopy proved the capping of functional groups of different chemical constituents present in ARAE on AgNPs. Our results are in accordance with the study of Gole et al where they presented the role of free amino acids and carboxyl groups of proteins present in extract for the formation of AgNPs³⁰. ARAE peaks (1242, 1408 and 1634) and ARAENP peaks (1269.6, 1244.1 and 1629) corresponds to carbon skeleton of withanolides. ARAE peaks (1078.1, 1054.4) and ARAENP peaks (1029) corresponds to withanolides ring band and flavonoids like structure. Thus from these results we can report that withanolides which are main constituents of ARAE are involved in formation of ARAENP. Our results are in

accordance with the results of Anil Kumar et al., where they reported the peaks in the range of 1001-1076 cm^{-1} corresponding to withanolides ring band and flavonoids and peaks at 1236 cm^{-1} , 1400 cm^{-1} and 1614 cm^{-1} representing the carbon skeleton of withanolides³¹.

Table 1: FTIR peak values and corresponding functional groups of ARAE and ARAENP

S No.	Peak in ARAE (cm^{-1})	Peak in ARAENP (cm^{-1})	Corresponding functional group
1.	3422.4	3399.1	Stretching vibrations of –OH group of phenolic acid compounds and carbohydrates with –H bonding.
2.	1634.6	1629.2	Stretching vibrations of C=C of alkenes
3.	1242.8	1269.6 & 1244.1	Stretching vibrations of C-O group of Carboxylic acids
4.	1078.1 & 1054.4	1029.0	Stretching vibrations of C-N group of amines
5.	778.4	781.3 & 715.8	Stretching vibrations of C-H group of alkenes
6.	2070.5	---	Stretching vibrations of N=C=S of isothiocyanate
7.	1408.0	---	Bending vibration of O-H group of polyphenol
8.	600.2 & 576.7	---	Stretching vibrations of C-Br
9.	---	2917.5 & 2849.6	Stretching vibrations of C-H group of aldehydes
10.	---	1384.2	Bending vibrations of C-H group of alkanes

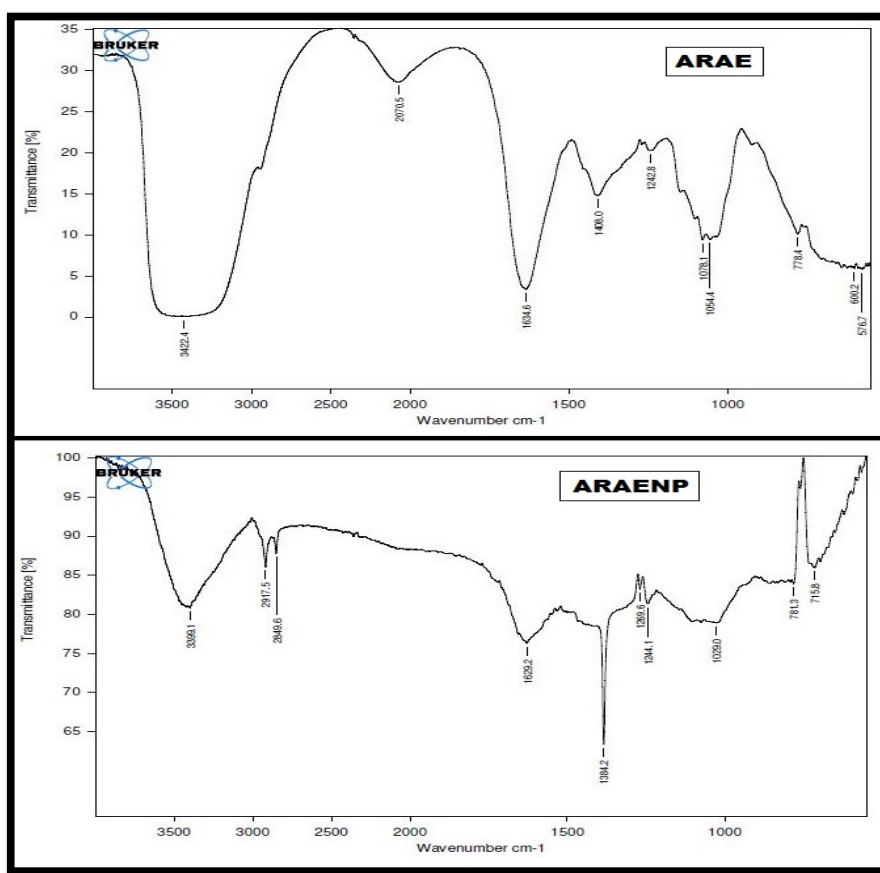


Figure 3: FTIR spectra of ARAE and ARAENP.

SEM studies of ARAENP:

Morphology of surface of ARAENP is presented in figure 4. AgNPs identified by SEM analysis have a size ranging from 95.33 nm to 176.1 nm with an average size of 123.23nm and are spherical in shape. Thus SEM results strongly confirm the role of ARAE as reducing and capping agent in the synthesis of ARAENP.

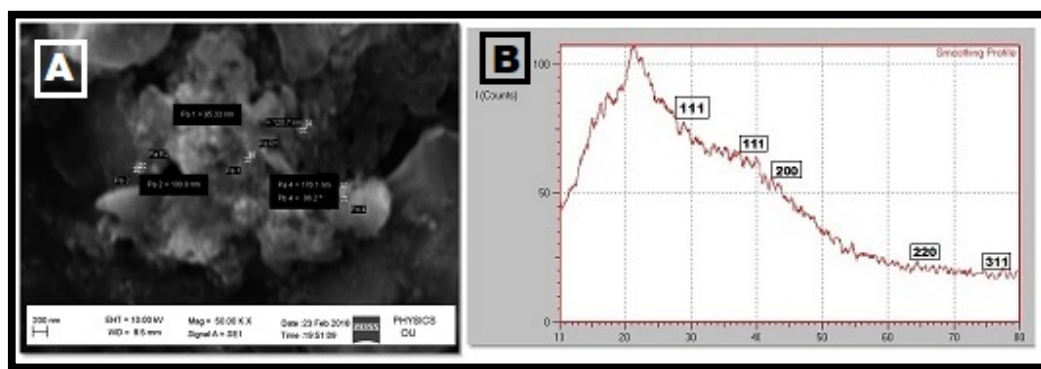


Figure 4: A: SEM micrograph of ARAENP at 200nm; B: XRD pattern of ARAENP.

XRD studies of ARAENP:

2 θ peaks at 28.98°, 39.80°, 42.24°, 64.42° and 79.76° corresponds to 111, 200, 220 and 311 planes of Bragg's reflection of silver. Figure 4 and Table 2 represent the data of XRD analysis. These planes clearly indicate the face centered cubic structure of silver. 12.02nm is the average size of ARAENPs. Our results are in accordance with the study of Selvaraj Raja *et al.*, where they reported the average size of nanoparticles by XRD as 13.07nm³².

Table 2: Size of ARAENP by using Debye-Scherrer equation:

S No.	2 θ (deg)	D (angle)	FWHM (deg)	Int. I (cps deg)	Size (nm)
1.	28.98	3.07860	0.0800	51	18.91
2.	39.80	2.26306	0.1200	46	12.88
3.	42.24	2.13781	0.1600	41	9.81
4.	64.42	1.44515	0.1200	48	14.53
5.	79.76	1.20138	0.4800	60	3.99
Average size of ARAENP					12.02

Zeta Potential and DLS studies of ARAENP:

Zeta potential was conducted to determine the stability of ARAENP. Mean Zeta potential was found to be -10mV. Negative value indicates the capping of constituents present in ARAE on surface of ARAENP. Moreover, negative charge also proves the stability and thus preventing them from agglomeration³³. Average particle size measured by DLS was found to be 418.7nm (Figure 5). Size detected by DLS was higher than SEM analysis because SEM measures physical size of particle without capping agent where as DLS measures size of particle along with capped biomolecules³⁴.

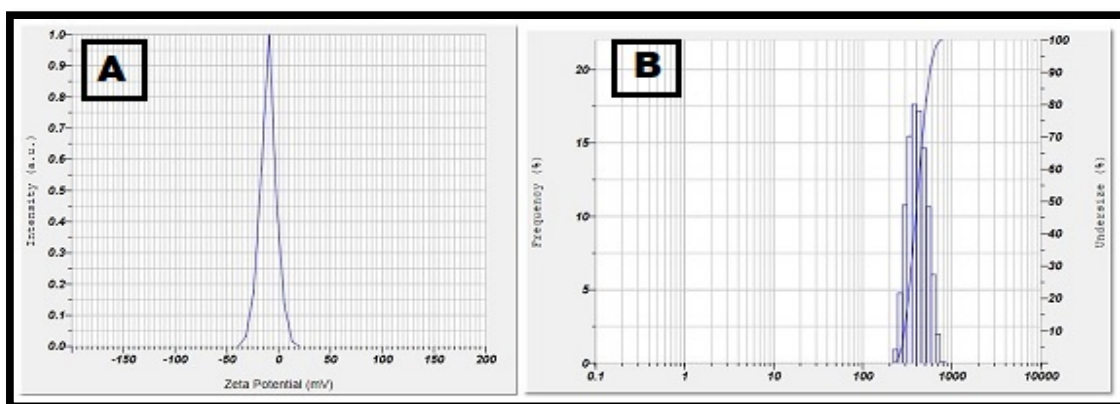


Figure 5: A: zeta potential of ARAENP; B: Size distribution of ARAENP with maximum intensity at 418.7 nm.

***In-vitro* anti diabetic activity:**

Effect of Acarbose, ARAE and ARAENP on α -amylase activity:

Inhibitory effect of Acarbose, ARAE and ARAENP on pancreatic alpha amylase is represented in figure 6. % inhibition increased dose dependently in all three treatments. At 10mg/L concentration of each of Acarbose, ARAE and ARAENP % inhibition was found as 58.16%, 29.08% and 81.67% respectively. % inhibition produced by Acarbose is more than ARAE at all concentrations whereas % inhibition produced by ARAENP is more than both Acarbose and ARAE at all concentrations indicating its effectiveness. α -amylase is an important enzyme in the human body responsible for metabolism of starch i.e., it converts complex polysaccharides in to disaccharides and oligosaccharides. Acarbose is a well known inhibitor of alpha amylase enzyme controlling postprandial blood glucose level in types 2 diabetes mellitus^{35, 36, 37}. Inhibition of these enzymes provided prophylaxis against incidence of serious vascular complications in type-2 diabetes mellitus patients³⁸. Thus according to results of our study ARAE and ARAENP inhibited the enzyme at the same pace as that of Acarbose and inhibited the hydrolysis of complex polysaccharides.

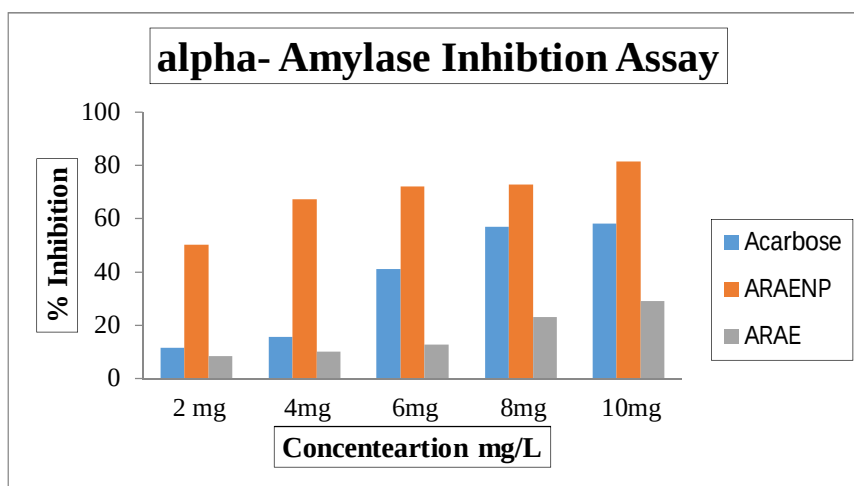


Figure 6: *in-vitro* inhibitory effect of Acarbose, ARAE and ARAENP on α -amylase activity.

Effect of Acarbose, ARAE and ARAENP on α -glucosidase activity:

At 10mg/L % inhibition produced by Acarbose, ARAE and ARAENP was found to be 94.90%, 85.83% and 94.11% respectively. There was a dose dependent increase in % inhibition of all three treatments (Figure 7). Moreover, Acarbose and ARAENP produced more inhibition than ARAE. α -glucosidase is responsible for conversion of oligosaccharides and disaccharides in to monosaccharides which are then absorbed in to blood circulation causing elevated postprandial blood glucose level which is one of the

most important cause of Type-2 Diabetes Mellitus^{39, 40}. Based on the results, ARAE, ARAENP and Acarbose inhibited the enzyme at the same pace and thus prevent the further hydrolysis of oligosaccharides and disaccharides, and control elevation of postprandial blood glucose level in type -2 diabetes patients.

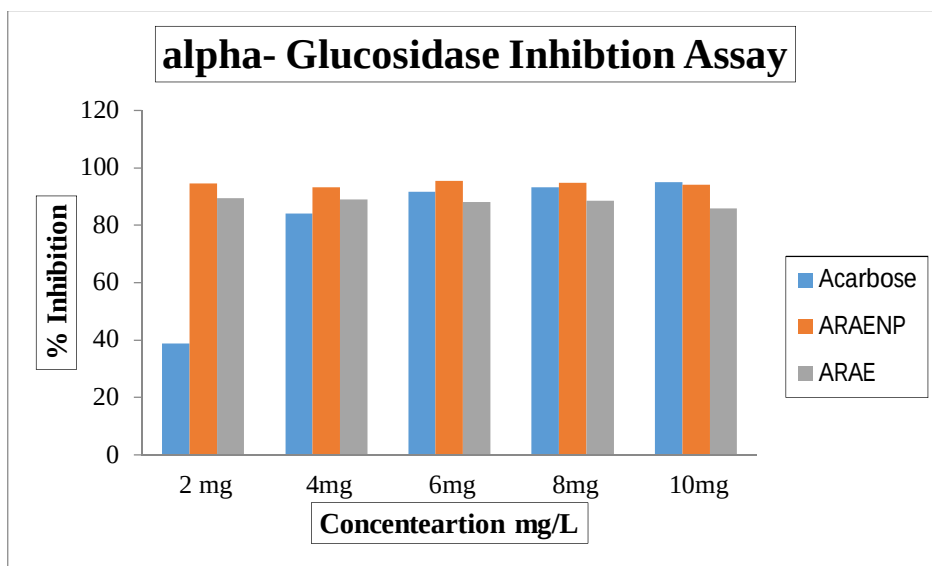


Figure 7: *in-vitro* inhibitory effect of Acarbose, ARAE and ARAENP on α -Glucosidase activity.

Acarbose was reported with many side effects and also increase in the levels of liver enzymes⁴¹. Because of these adverse effects there is a need of discovering an alternative treatment with high therapeutic efficacy and fewer side effects. Medicinal plants are marvelous source for treatment of diabetes with minimum side effects. In this context our ARAENP synthesized from ARAE proved to be an excellent candidate for the treatment of type-2 diabetes mellitus with fewer side effects. Effect produced by ARAENP is due to the withanolides which are capped on them.

CONCLUSION

This study presents, green synthesis as safe, cheaper and eminent tool for the production of nanoparticles. Different characterization methods prove the formation of ARAENP. UV- Visible spectroscopy shows peak at 440nm, functional groups and withanolides as capping material on ARAENP are identified by FTIR, particle size confirmed by SEM, XRD presents the crystalline structure and stability was proved by zeta potential. In addition, data obtained in this study also proves the inhibitory potential against α -Amylase, α -Glucosidase enzymes of ARAENP which is probably due to withanolides which are capped on it and thus establishes their effectiveness in treatment of diabetes mellitus. However further in depth studies are required to be conducted in terms of *in-vitro* and *in-vivo* procedures to develop ARAENP as

potent contender with high therapeutic efficacy and low side effects for the treatment of type 2 diabetes mellitus.

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