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Original Article

Green synthesis, Antioxidant Potential and Hypoglycemic Effect of Silver Nanoparticles using Ethanolic Leaf Extract of *Clausena anisata* (Willd.) Hook. F. Ex Benth. of Rutaceae

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ABSTRACT

The current study was conducted to examine the hypoglycemic effect of Silver Nano particles (SNPs) using ethanolic leaf extract of *Clausena anisata* (Willd.) *Hook f.* ex Benth. A cost effective and eco-friendly technique for the green synthesis of SNPs from 1mM $AgNO_3$ solution through the leaf extract was carried out. The biosynthesized SNPs were characterized using UV spectro photometry, Field Emission Scanning Electron Microscopy (FESEM), X-ray Diffraction analysis (XRD), Energy Dispersive Spectroscopy (EDS) and Fourier transform infra-red (FTIR) spectrometry. DPPH assay was done to determine the antioxidant activity and for *in vitro* hypoglycemic activity glucose uptake by yeast cells, alpha-amylase inhibition assay, adsorption capacity and glucose diffusion assay was carried out. The SNPs of ethanol leaf extract were found to be spherical in shape and up to 60.67 nm average in size. They showed alpha-amylase inhibitory activity of 80.32% at 500 μg/ml and IC_{50} 100 μg/ml. The glucose uptake by yeast cells was found to increase with an increase in concentration. The maximum glucose uptake was found to be 68.29% at 10mM concentration. The molar concentration of glucose was directly proportional to the glucose binding capacity of extracts. The rate of glucose diffusion across the membrane was found to increase from 30 to 180 minutes. The DPPH scavenging activity was found to be potent (71.60%) at 500 μg/ml. The hypoglycemic effect exhibited by the SNPs was revealed by simple *in vitro* model of yeast cells, mediated by glucose absorption, increasing glucose diffusion and glucose transport across the cell membrane.

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INTRODUCTION

Type II Diabetes is a metabolic disorder in which either insulin hormone is not produced in sufficient quantities or the insulin produced is defective and cannot move the glucose into the cells. It is characterized by chronic hyperglycemia that is associated with micro and macro vascular complications that can lead to visual impairment, blindness, kidney disease, nerve damage, amputations, heart disease, and stroke.[1] Glucose control (fasting blood sugar in diabetes - 126 mg/dl and above; random blood sugar in diabetes - 200 mg/dl and above) is the major target in the management of type II diabetes. Therefore, it is necessary to reduce hyperglycemia in order to decrease the onset and progression of micro vascular complications.[2] There are several synthetic drugs like biguanides, sulfonylurea, Meglitinides, Thiazolidinediones, Alpha-Glucosidase Inhibitors, GLP-1 Inhibitors, Pramlintide and DPP-4 Inhibitors are available but due to their high cost and side effects, traditional treatment with plants having medicinal potential becomes an alternative option for health conscious and financially poor populations.[3]

Clausena anisata (Willd.) Hook f. ex Benth deciduous shrubs or small tree found in tropical and South Asia is used traditionally in treating a wide range of diseased conditions, which is indigenous in South Africa. As with other species of Rutaceae family, the leaves, fruits and stem bark are rich in aromatic essential oils. The dried leaves of this plant act as an arthropod repellent,[4] like filling material for mattresses and pillows against lice, fleas and bedbugs,[5] anti-inflammatory and antifungal activity,[6] antibacterial activity,[7] diabetes (hypoglycemia),[8] rheumatism and migraine headache,[9] in management of epilepsy,[10] for cough and in treatment of tuberculosis,[11] it act as mosquito repellant against *Anopheles arabiensis*.[12,13] The leaves or roots decoction are taken for gastro-intestinal disorders, analgesia (headache, toothache) pneumonia and antipyretic,[14] hypotension, venereal diseases, sinusitis and sore throat, as an anthelmintic and aphrodisiac,[15] antioxidant and crushed leaves are used in treating wounds in domestic animals,[16]

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as a tonic to cure breast pain in pregnant women,[17] to control convulsions in infants and as a tonic to prevent rickets.

We report for the first time, the biosynthesis and characterization of SNPs generated by the reduction of *C. anisata* ethanolic leaf extract. The biologically synthesized nano particles were analyzed and tested for its antioxidant and hypoglycemic activity *invitro*.

MATERIALS AND METHODS

Procurement of plant material

Fresh leaves of *C. anisata* (Figure 1) were procured in the months of October to November (2013) from Manamettupatti of Viralimalai Taluk (Pudukottai District, Tamilnadu). The plant was identified by Dr. S. John Britto, Director, The Rapinat Herbarium and Centre for Molecular Systematics of St. Joseph's College, Tiruchirappalli – 620 002 and authenticated as *Clausena anisata* (Willd.) *Hook f.* ex Benth. vouchered herbarium by specimen No: LB NSD 001.

Preparation of plant extract and SNPs

10g of shade dried leaf powder was macerated at 35^{0}C to 37^{0}C; three to five times for 15 – 20 hrs with 100ml of ethanol in an Erlenmeyer flask (500ml). Then the extract was centrifuged, filtered using Whatmann No. 1 filter paper and safely stored. The biosynthesis of SNP was carried out as outlined by the method.[18]

Optimization and synthesis of SNPs

1ml, 3ml and 5 ml of ethanolic leaf extracts were taken in a separate conical flask and to this 10 ml of 1 mM $AgNO_3$solution was added with constant stirring and were exposed under different conditions like sunlight radiation, direct boiling, microwave irradiation (10s on, 10s off) to prevent overheating and aggregation of metals, UV irradiation and room temperature. The color change of the leaf extract was checked periodically and the color changes from green to dark brown indicate the synthesis of SNPs from the leaves.

Production and recovery of SNPs

For bulk production, 10ml of leaf extract in 100ml of 1mM $AgNO_3$ was prepared. After reduction, the extract consisting of SNPs was subjected to centrifugation at 10,000rpm for 20 minutes, and the supernatant was discarded. To the pellet, 0.1ml of toluene-water was added, air dried, lyophilized and stored for further characterization.

CHARACTERIZATION STUDIES

The biosynthesized SNPs were characterized by the following methods:

Visual Observation

A change of color from green to reddish brown was periodically noticed in the leaf extracts exposed to different conditions as illustrated earlier.

UV Spectro photometric analysis

The formation of SNPs was confirmed and was studied using the spectral analysis. The UV spectra of the biosynthesized SNPs were recorded using Lambda 35, Perkin Elmer Spectrophotometer by continuous scanning from190nm to 1100nm and distilled water was used as the reference.

Fourier Transform Infra Red Spectroscopy Analysis (FTIR)

The functional groups in the biosynthesized SNPs were analyzed by FTIR spectroscopy. These measurements were carried using a Perkin Elmer spectrum RX I FTIR instrument with a wavelength range of 4000cm^{-1} to 400 cm^{-1}.The results were compared for as hift in functional peaks.

Field Emission Scanning Electron Microscopy (FESEM)

FESEM was used to characterize the mean particle size, morphology of the SNPs. A small drop of biosynthesized SNPs solution was placed on a glass slide and allowed to dry. The samples were analyzed by using FEI Quanta 200 FEG machine at a low vacuum in the range 10-20Kv.

Energy Dispersive Spectroscopy (EDS)

The elemental composition of the synthesized SNPs was analyzed with energy dispersive spectroscopy coupled to scanning electron microscope.

X-ray Diffraction Analysis (XRD)

The structure and composition of SNP were studied by XRD (XPERT-PRO Machine). The data was collected in the 2θ range.

Determination of antioxidant activity (DPPH assay)

The antioxidant activity of the SNPs was evaluated by DPPH radical scavenging assay.[19] SNPs of different concentration ranging from 100µg/ml - 500µg/ml was prepared. To this, DPPH at a concentration of 0.1mM was added and incubated at room temperature for 30minutes.The absorbance was recorded at 517nm. The control sample was carried out without adding leaf extract. Ascorbic acid was used as a standard control.

In Vitro Hypoglycemic Activity

Alpha – amylase inhibition assay

The alpha-amylase inhibitory activity was carried out using acarbose as the reference compound[20,21] based on the spectro photometric assay. The SNPs at a concentration of 100 - 500µg/ml was prepared by dissolving in DMSO. 3.246 mg of α-amylase (0.5 U/ml) (EC 3.2.1.1) was dissolved in 100ml of 20mM phosphate buffer (p^H 6.7) containing 6.5mM sodium chloride. Seven duplicate test samples including the blank and control were prepared. In each sample, 250µl of the enzyme was added with 100µl of SNP extracts of different concentrations except blank and were incubated in a water bath at 37^{0}C for 20 minutes with constant stirring in a vortex. After incubation, 250µl of starch (0.5% w/v starch dissolved in 20mM phosphate buffer, p^H 6.7) was transferred into each sample in the test tube to initiate the reaction. It was again incubated at 37^{0}C for 15minutes after vortexing. DNS reagent of 2ml was added and stirred in a vortex. Later it was boiled in a water bath at 100^{0}C for 10minutes, thereafter the mixture was cooled down and the absorbance was measured at 540nm and control sample was carried out without adding the leaf extract.

Glucose uptake by yeast cells

Commercial baker's yeast was prepared in distilled water[22] by repeated centrifugation at 21000rpm for 5 mins until the super natant was clear. The 10% (v/v) concentration of yeast suspension was prepared in distilled water. SNP extracts at concentration of 100µg - 2 mg were added separately to 1ml of glucose solution (5, 10, 25 mm ol/L) and incubated for 10 minutes at 37°C. To this, 100µL of yeast suspension was added to start the reaction and vortexed. Then it was incubated at 37°C for 60 minutes. After 60min, at 3800 r/min for 5 min the tubes were centrifuged and the glucose present in the supernatant was estimated at 540nm.[23]

Glucose Diffusion Assay

1mL of a glucose solution at a concentration of 25mmol/L was prepared by dissolving in 0.15M NaCl[24] and 1% of SNPs were dialyzed in two separate dialysis bags for control and test sample. Then the dialysis membrane was tied at both ends and immersed in a separate beaker containing 10ml of distilled water and 40ml 0.15M NaCl. For control, SNP extract was not added. The beakers were incubated at room temperature. The glucose

content in the dialysate was determined at 30, 60, 120 and 180min by DNS method.[25]

Dinitrosalicylic colorimetric method (DNS)

After incubation, 250µl of the starch (0.5% w/v of starch was dissolved in 20mM phosphate buffer (p^H6.7)) was transferred into each test tube carrying glucose content of dialysate at 30, 60, 120 and 180 mins. The vortexed mixture was incubated at 37˚C for 15mins. DNS reagent of 2ml was added, stirred in a vortex and boiled in a water bath at 100^0C for 10mins. The mixture was cooled and the absorbance was measured at 540nm.

Glucose Adsorption Capacity

SNP at a concentration of 1% was added to 25mL of 5, 10, and 25 mm ol/L glucose solution in four separate experiments. The content was vortexed and incubated in water bath at 37°C for 6hrs and centrifuged at 4800 rpm/mins for 20mins. The glucose content in the supernatant was determined at 540nm.

Statistical analysis

The results were expressed as the mean ± standard deviation from three independent experiments with triplicates. One way analysis of variance (ANOVA) was performed followed by Tukey's test. P< 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Among various concentration and methods used, 1ml of extract exposed under sunlight irradiation was found to be very effective and showed the maximum synthesis of nano particles. In this study, it was observed that there was visible color change when silver nitrate was added to the leaf extract. Initially, the leaf extract was green in color and after the addition of silver nitrate;it was turned into brown in color (Figure 2).The ethanolic leaf extract of *C. anisata* reduced silver ions into SNP was evidenced by the visual color change. This indicated the formation of SNPs due to excitation of surface Plasmon vibration in silver nano particles.

UV – Vis spectral analysis of SNPs

The SNP leaf extract was scanned in the wavelength ranging from 190nm-1100nm by using UV spectrophotometer (model: Lambda 35). The profile showed the peaks at 488.99 nm (Figure 3) with the absorption 0.86693 and it corresponds to the surface plasmon resonance of SNPs. This is in accordance with the earlier findings.[27] Depending on the shape, size, aggregation nature and the adjacent dielectric medium[28] the absorption band of metal nano particles is conquered by the SPR.

FTIR Spectrum

The bio molecules responsible for capping and efficient stabilization of the SNPs synthesized by leaf extract was identified by FTIR measurements and were similar to the earlier report.[29] The SNP plant extract was passed into the FTIR (Model: Perkin Elmer Spectrophotometer system), based on its peak ratio the functional groups of the components were separated. The identification was based on the FTIR peaks attributed to stretching and bending vibrations (Figure 4). Nearly eight areas were identified and produced in Table 1.

FESEM image analysis

The size and shape of the SNP formed were analyzed by FESEM and the images are shown. the hydrogen bond and electro static interactions between the bio-organic capping molecules bound to the SNPs was responsible for the image. The SNPs were not in direct contact that may be due to the stabilization of the nano particles by a capping agent. The larger silver particles may be due to the aggregation of the smaller ones.[30] Synthesized SNPs showed spherical form and with a size distribution ranging from 13 to 61 nm. The average size was found to be 60.67nm (Figure 5).

Table 1: FTIR spectral peak values and functional groups obtained for the SNP leaf extract of *C. anisata*

S.No	Peak Value (cm^{-1})	Bond Type	Functional Groups
1	667.22	-C ≡ C-H	Alkynes
2	1015.41	C-O Stretch	Alcohol, ester, ether
3	1360.46	N-O symmetric stretch	Nitro compounds
4	1631.77	N-H bend	Primary amine
5	2083.51	-C ≡ C- Stretch	Alkynes
6	2833.19	RCO_2H	Carboxylic acid
7	3432.71	O-H stretch/ H-bonded	Phenol, alcohol
8	3464.31	O-H stretch/ H-bonded	Phenol, alcohol

EDS Analysis

The percentage of SNPs in the sample was analyzed by EDS. For this, the synthesized SNPs was characterized by using FEI Quanta 200 FEG HR-SEM equipped with EDAX instrument. The EDS spectra showed the different types of elements like Carbon (18.97%), Chlorine (13.43%), Oxygen (9.61%), Copper (2.76%) and Silver (55.24%) present in the sample (Figure 6).

XRD Characterization

The XRD spectra were used to confirm the crystalline nature of the SNPs. The X-ray diffraction pattern of SNP was confirmed by the characteristic peak observed in XRD image. Four prominent peaks were observed at 2θ = 31.85º, 44.0º, 64.2º, and 77.2º, (Figure 7) which corresponded to (111), (200), (220) and (311) respectively. The diffraction pattern inferred that the synthesized SNPs possessed nano-dimensional state.[27]

Alpha- Amylase inhibition assay

The glucose obtained from the indigestible carbohydrate food which contains starch is readily absorbed from the gastrointestinal tract into the blood stream after the hydrolysis of glycosidic bonds by the enzyme α-amylase. In diabetics, the inhibition of this enzyme reduced the high postprandial blood glucose level. In the present study, an *in vitro* alpha-amylase inhibition model was used to screen the SNPs of *C. anisata* to evaluate the hypoglycemic effects. The alpha-amylase inhibitors obstruct the absorption and the digestion of carbohydrates.[31] Acarbose, a synthetic alpha-amylase inhibitor delays the digestion of carbohydrates and inhibits the action of pancreatic amylase in the break down of starch, which leads to side effects such as abdominal pain, diarrhea and soft faeces in the colon. The SNPs of *C. anisata* inhibited the alpha amylase with IC_{50} value at 100µg/ml similar to the principle as that of Acarbose. The maximum inhibition of 80.32 % was found to be at 500 µg/ml when compared to standard 85.24% (Figure 8).

Glucose uptake by yeast cells

Inhibition of glucose uptake in 5mM, 10mM, and 25mM glucose concentration: The rate of glucose transport across the yeast cell membrane was studied and SNP leaf showed a percentage inhibition of 50 while Acarbose showed a percentage inhibition of 62.82 at a concentration of 2000µg/ml (Figure 9) for 5mM.The graphical representation of the inhibition of glucose uptake in 10mM glucose concentration was presented (Figure 10). It showed a percentage inhibition of 68.29 while

Figure 1: Leaves of Clausena anisata (Willd.) Hook. f. ex Benth. from Manamettupatti, Viralimalai Taluk (Pudukottai District, Tamilnadu)

Figure 2: Synthesis of SNP a) 1 mM AgNO3 b) C. anisata ethanolic leaf extract c) Visible color change from green to brown under sunlightx

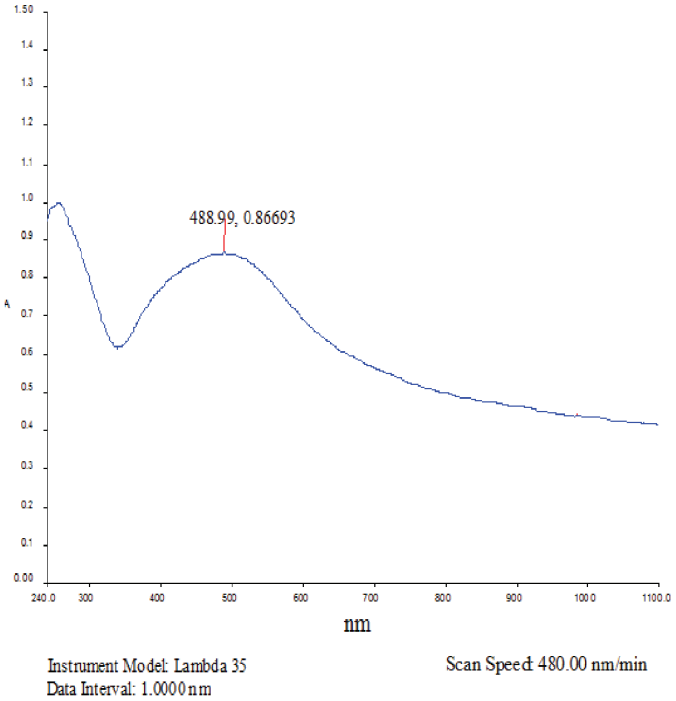


Figure 3: UV-Vis Spectra of synthesized SNP from ethanolic C. anisata leaf extract

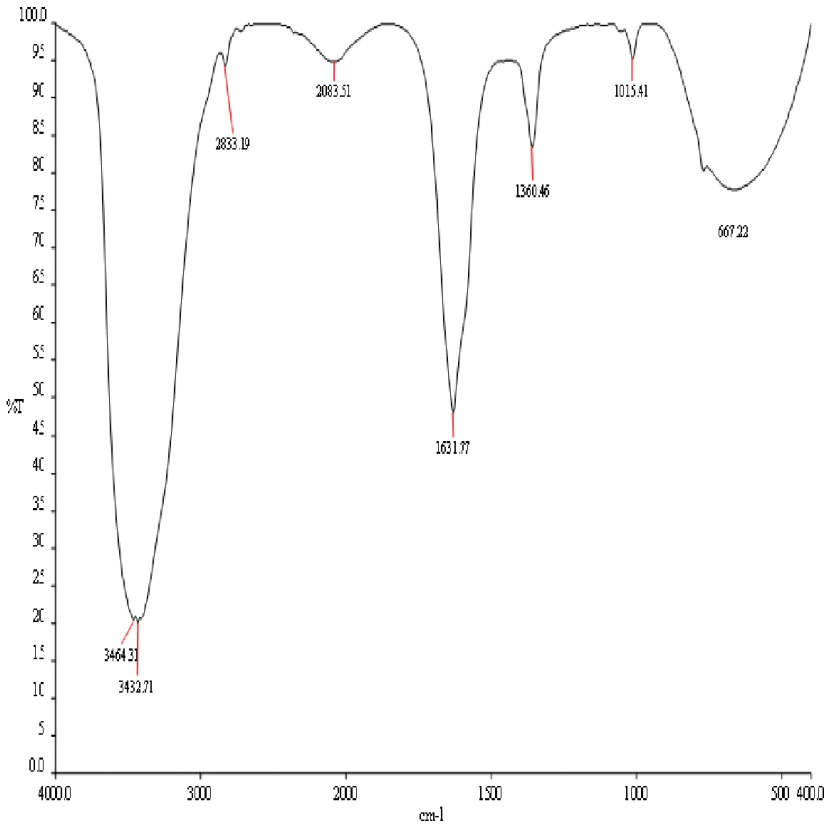


Figure 4: FTIR Spectrum of synthesized SNP from ethanolic C. anisata leaf extract

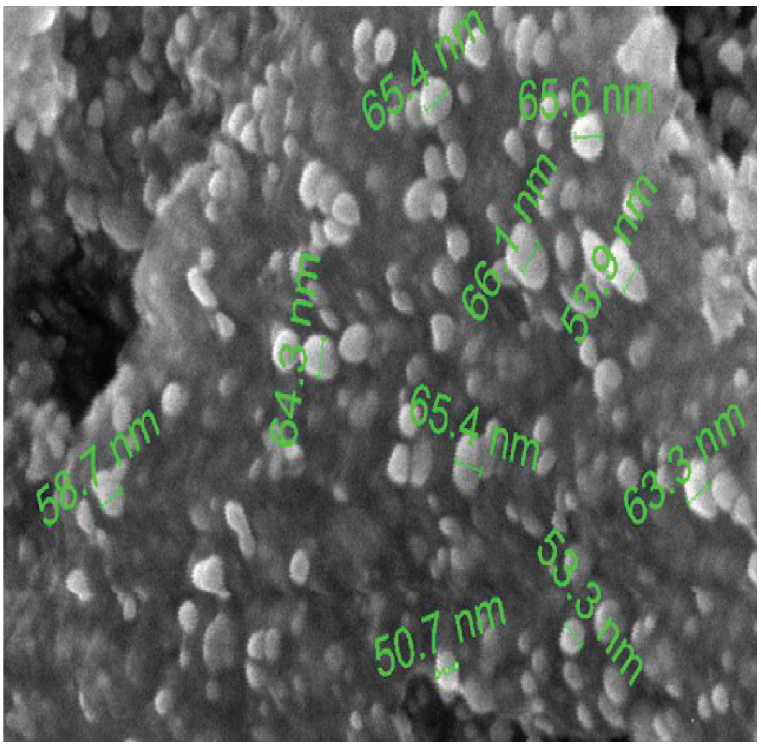
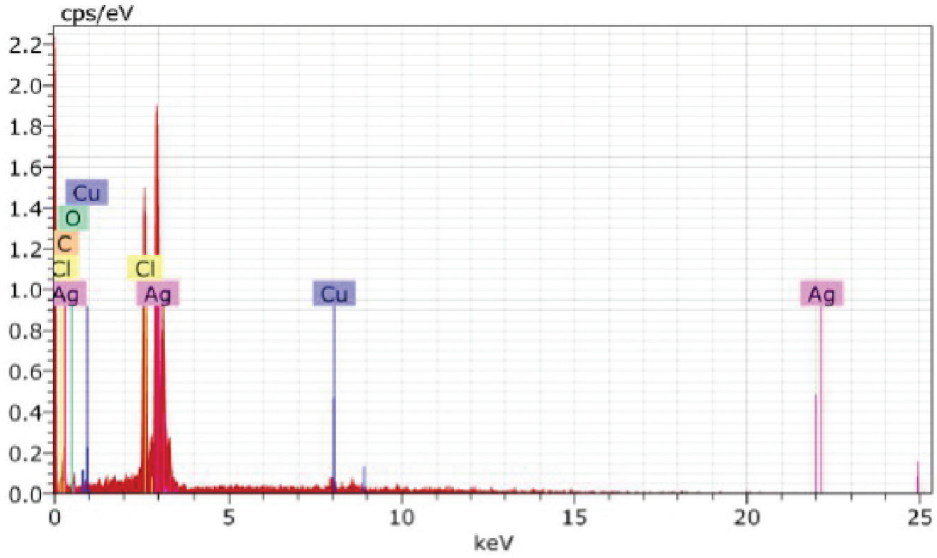


Figure 5: SEM image of synthesized SNP from ethanolic C. anisata leaf extract



Spectrum: training

El	AN	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
Ag	47	L-series	48.03	55.24	16.45	1.72
C	6	K-series	16.49	18.97	50.71	5.86
Cl	17	K-series	11.67	13.43	12.16	0.52
O	8	K-series	8.36	9.61	19.29	4.08
Cu	29	K-series	2.40	2.76	1.40	0.23
		Total:	86.95	100.00	100.00	

Figure 6: EDX plot of synthesized SNP from ethanolic C. anisata leaf extract

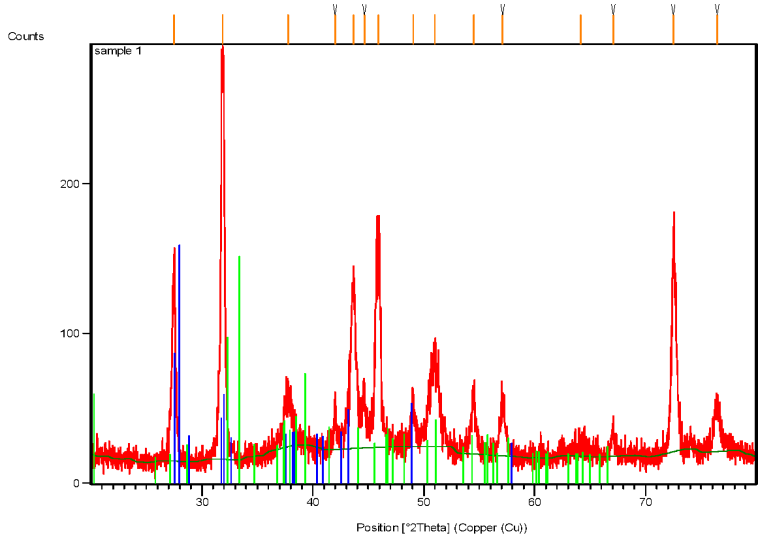


Figure 7: XRD pattern of synthesized SNP from ethanolic C. anisata leaf extract

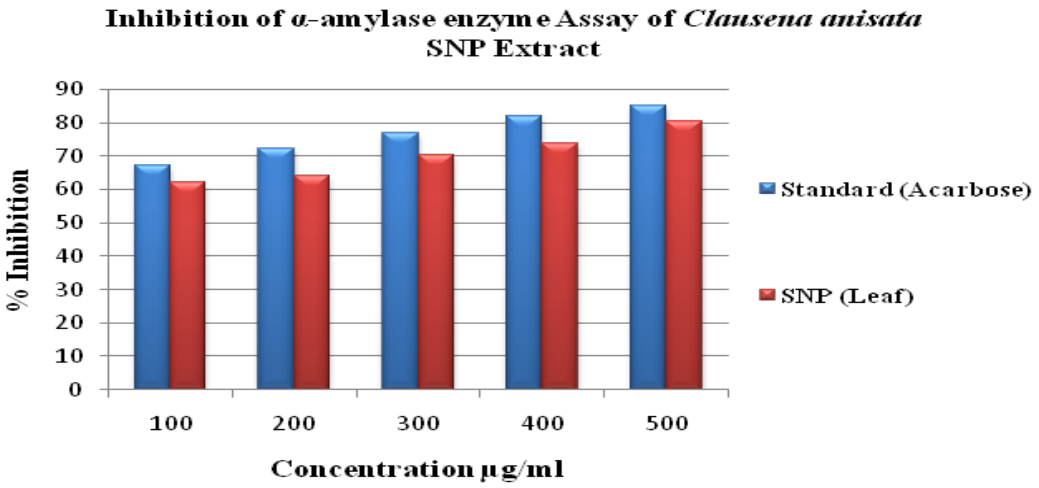


Figure 8: Alpha amylase inhibitory activity of synthesized SNP from ethanolic C. anisata leaf extract

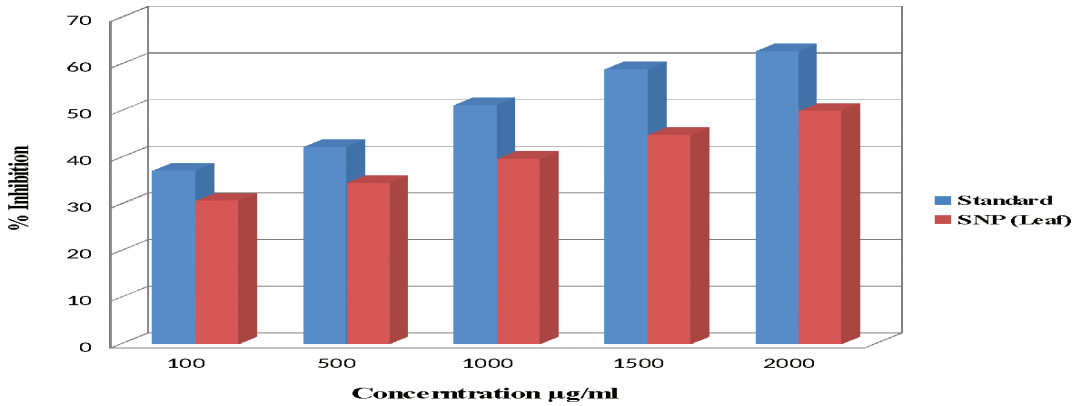


Figure 9: Effect of synthesized SNP from ethanolic C. anisata leaf extract on the uptake of glucose by yeast cells at 5mM concentration

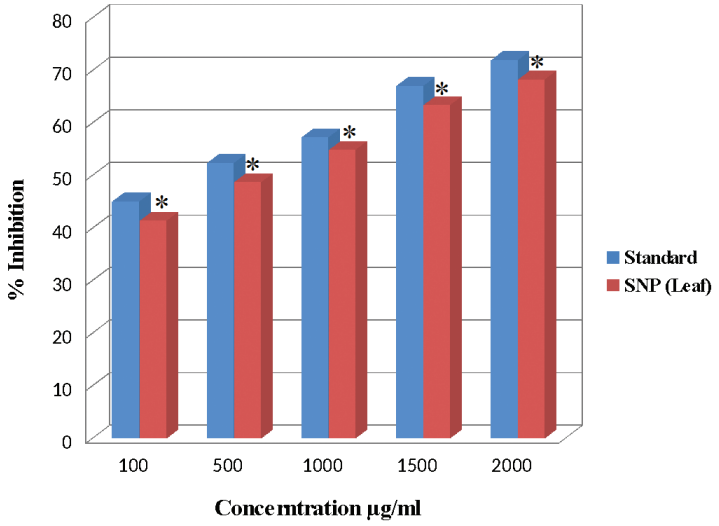


Figure 10: Effect of synthesized SNP from ethanolic C. anisata leaf extract on the uptake of glucose by yeast cells at 10mM concentration

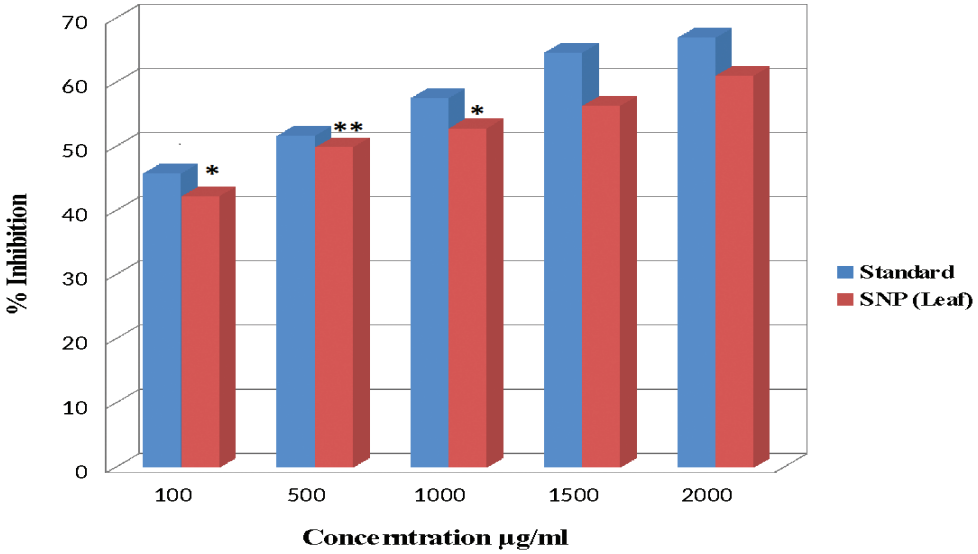


Figure 11: Effect of synthesized SNP from ethanolic C. anisata leaf extract on the uptake of glucose by yeast cells at 25mM concentration

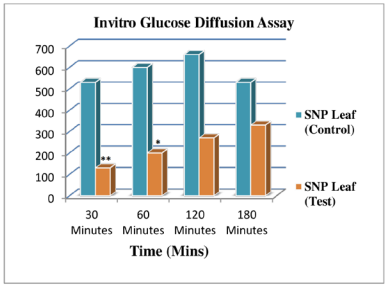


Figure 12: Effect of synthesized SNP from ethanolic C. anisata leaf extract on invitro glucose diffusion

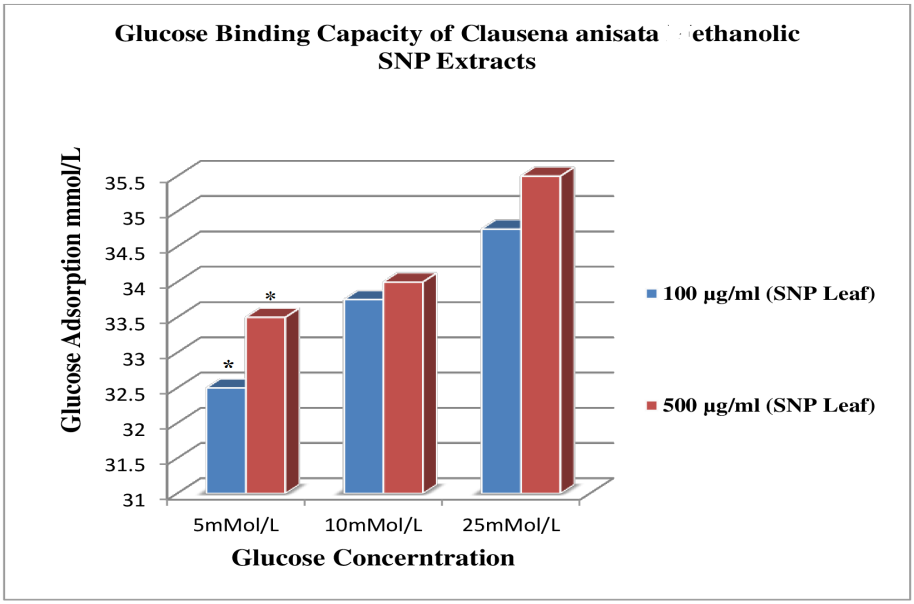


Figure 13: Invitro glucose binding capacity of synthesized SNP from ethanolic C. anisata leaf

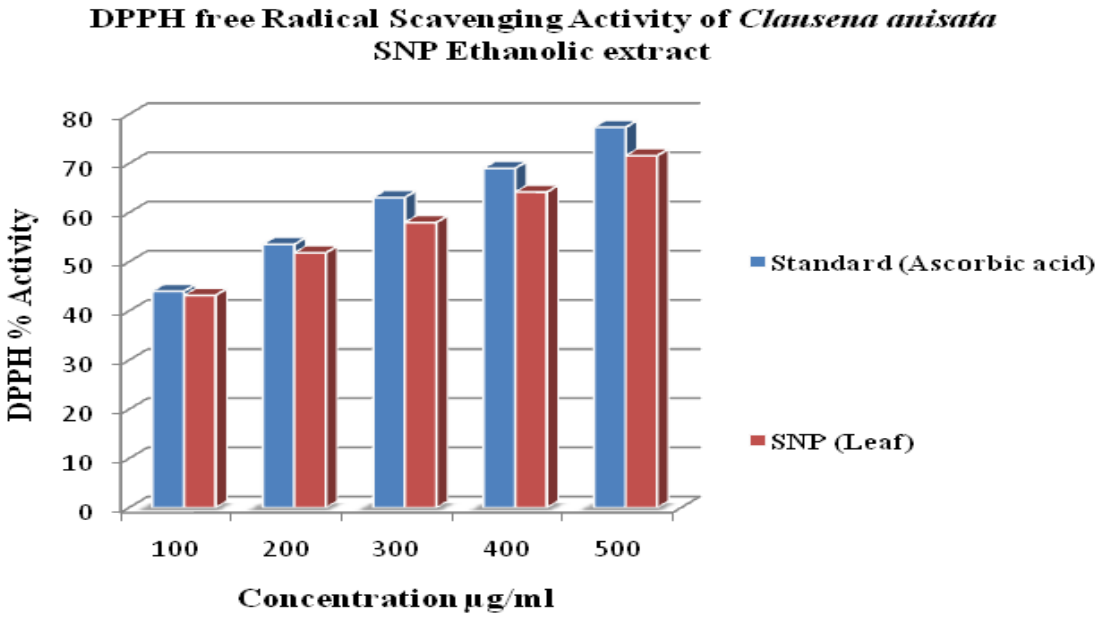


Figure 14: DPPH free radical scavenging activity of synthesized SNP from ethanolic C. anisata leaf

A carbose showed a percentage inhibition of 71.95 at 2000µg/ml. The graphical representation of the inhibition of glucose uptake in 25mM glucose concentration was presented. It showed a percentage inhibition of 61.17 while A carbose showed a percentage inhibition of 67.05 at a concentration of 2000µg/ml (Figure 11).

The glucose uptake in the SNP extract was found to increase in a dose-dependent manner. The amount of glucose remaining in the medium was estimated, which served as a marker for the glucose uptake by the yeast cells. And it was found to be linear in 5mM, 10mM, and 25mM glucose concentrations. From the results, it was clear that like standard SNPs had greater efficiency in increasing the glucose uptake by yeast cells in 5mM, 10mM and 25mM glucose concentrations.[32]

Glucose Diffusion Assay

Most efficient plants phytoconstituents have antihyperglycemic behaviors to increase glucose transport and metabolism in muscle and to stimulate insulin secretion.[33] Glucose concentrations inside the dialysis tubing in the absence or presence of SNPs leaf extract were inversely related to the glucose concentrations in the external solution. In control, the glucose movement out of dialysis had reached a plateau with a mean glucose concentration in the external solution. This confirms that the SNPs of *C. anisata* signified an inhibitory potential of glucose diffusion. GDRI (%) increased with decreasing leaf extract concentration. SNP was found to exhibited greater GDRI percent of 75.47, 66.66, 59.09, 37.73 at 30, 60, 120 and 180 minutes respectively (Figure 12).

Glucose Adsorption Capacity

In the presence of different levels of glucose, the SNP showed the high capacity of glucose adsorption. The glucose adsorption rate was found to persist from higher level to even low level of glucose present in the solution. As the concentration of glucose increased, the glucose adsorption capacity of SNP leaf extract also increased at a rate of 34.75mmol for 100 µg/ml in 25mM concentration of glucose and 35.5mmol for 500 µg/ml in 25mM concentration of glucose (Figure 13).

Determination of antioxidant activity (DPPH assay)

The one of the most widely used methods for screening antioxidant activity of plant extract is DPPH assay. The stable free radical was used to determine antioxidant activity of natural compounds.[34] This assay detected the antioxidant activity of SNP extract and compared with the standard, which showed the IC_{50} values for SNP and ascorbic acid as 200µg/ml. The SNPs showed 71.60% inhibition at 500µg/ml when compared with standard showed 77.38% inhibition at 500µg/ml (Figure 14).

CONCLUSION

The results of the present study made on *Clausena anisata* to study the green synthesis, antioxidant potential and hypoglycemic effect of silver nano particles using ethanolic leaf extract revealed that the green synthesized SNPs showed antioxidant potential and the maximum *in vitro* hypoglycemic activity intervened by decreasing the glucose diffusion rate, increasing the glucose adsorption rate and by glucose transport across the cell membrane. Furthermore, to date no such study has been conducted to evaluate the hypoglycemic property of these SNP extract in this plant. However, these results should be confirmed by *in vivo* models, which is currently underway to confirm these observations for their effective utilization as therapeutic agents.

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CONFLICT OF INTEREST

All authors disclose no conflict of interest.

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