PHARMACOGNOSTIC ASSESSMENT OF SILVER NANOPARTICLES FROM ENDOPHYTIC BACTERIA

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ABSTRACT

The field of nanotechnology is the most promising area of the research, Plant leaves are potential hosts to a plethora of beneficial microorganism like endophytic bacteria. Endophytic bacteria live symbiotically with the plant and in turn helping the plants in number of ways. The present assessment were undertaken to isolate and identify bacterial endophytes in leaf tissues of *Moringa oleifera* plants growing at India also in Asia, Africa ,south America .A total of two endophytic bacteria were isolate from the parts of the plant. On the basis of the morphological and biochemical characterization of the endophytes they are identified as staphylococcus species. Endophytic based silver nanoparticles were found to possess significant antioxidant (DPPH and Phosphomolybdenum) and antidiabetic activity (alpha amylase assay).

Keywords: Moringa oleifera, Endophytic bacteria, nano particles, Phosphomolybdenum, DPPH, alpha amylase activity.

INTRODUCTION

Moringaoleifera, the most widely found species of the Moringaceae family thrives in tropical. Insular climate and is found in abundance nears rivers. The tree ranges from 5- 10 m in height. (Anwar et al., 2007). It can withstand humid and dry lands, deprived soils and tolerate a wide. Range of rainfall (Morton, 1991). Many parts of the Moringa tree are edible and numerous nutritional and medicinal properties have been attributed to its roots, bark, leaves, flowers, fruits, and seeds (Mbikay, 2012). Phytochemical analyses have revealed that Moringa leaves are a rich source of potassium, calcium, phosphorous, iron, vitamins A and D and essential amino acid (Gowrishankar et al., 2010). Antioxidants and flavonoids have also been reported from other parts of the plant such as seeds and fruits (Anjorin et al., 2010).Traditionally Moringa isalso known as Drumstick in countries like India and Nigeria where it is cultivated as a crop. Its pods are cooked and eaten whereas the leaves can be cooked or stored as dried powder both of which are a rich source of proteins (Mbikay, 2012). The leaf extracts of Moringa contain antioxidant properties and inhibit the peroxidation of linoleic acid. Extracts of leaves were also shown to prevent the bleaching of carotene and scavenge radicals in the DPPH radical scavenging assay (Sindhuraju & Becker, 2003). *Moringaoleifera* is a plant that is often called the drumstick tree, themiracle tree, the

ben oil tree, or the horseradish tree. Moringa has been used for centuries due to its medicinal properties and health benefits. It also has antifungal, antiviral, antidepressant, and anti-inflammatory properties. Itcontains a variety of proteins vitamins and minerals. Moringa contains many healthful compounds such as Vitamin A, VitaminB1, VitaminB2, VitaminB3,VitaminB6and ascorbic acid, calcium, potassium, and magnesium Phosphorus zinc. Moringa is also extremely low in fat and contains no harmful cholesterol. The tree is native to India but also grows in Asia, Africa and South American. Protecting on nourishing skin and hair treating edema, protecting the liver, preventing and treating cancer, treating stomach complaint, fighting against bacterial disease, making bones healthy, treating mood disorders, protecting the cardiovascular system, helping wounds to heal, treating diabetes, treating asthma, protecting against Kidney disorder, Reducing high blood pressure, Treatinganemia and sickle cell disease. Moringa (*Moringaoleifera* Lam.) is a multipurpose tropical tree. It is mainly used for food and has numerous industrial, medicinal and agricultural uses, including animal feeding. Nutritious, fast-growing and drought-tolerant, this traditional plant was rediscovered in the 1990s and its cultivation has since become increasingly popular in Asia and Africa, where it is among the most economically valuable crops. It has been dubbed the "miracle tree" or "tree of life" by the media (FAO, 2014; Radovich, 2009; Orwa et al., 2009; Bosch, 2004). Plant inner tissues are colonized by bacterial organisms known as endophytes. The relatively recent application of culture independent and molecular high throughput techniques allowed the description of a large diversity of endophyticbacterial taxa. These microorganisms can be found in any plant organ, including fruits and legume nodules. Nanotechnology as the manipulation of matter with at least one dimension sized from 1 to 100 nanometers. Two main approaches are used in nanotechnology. Inthe "bottom-up" approach, materials and devices are built from molecular components which assemble themselves chemically by principles of molecular recognition. In the "top-down" approach, nano-objects are constructed from larger entities without atomic-level control**.** Nanoparticles (also known as ultrafine particles 225) are defined as having one structural dimension of less than 100nm making them comparable in size to subcellular structures, including cell organelles or biological macromolecules, thereby enabling their ready incorporation into biological systems.

MATERIALS AND METHODS:

Glass were soaked in cleaning solution and washed thoroughly withrunning tap water. They were then cleaned with detergent solution and rinsed several times with tap water and finally in distilled water and air dried. The glassware and media were sterilized in autoclave at 15psi for 20 minutes, at120˚c. *Moringo oleifera* leaf samples were collected from healthy trees. Collectedleaves were covered with paper and were placed in dry and clean plastic bags. Debris were removed from the leaf samples by rinsing with tap water, afterwards, all explants were surface sterilized by washing with combination of distilled water and 70%ethanol.The surface sterilized explants were then transferred to plates contain nutrient agar (NA).Four explants were transferred to each plate with a total of two plates per sampled tree. All plates were incubated at 30 c for up to 1week, and were observed for bacterial growth every two days. Emerging mycelia from the end of explants were subculture in freshly prepared agar

(NA) plates to obtain pure cultures. Stock cultures were maintained in nutrient broth (NB) and stored at 30 degree Celsius A 10ml of culture filtrates were mixed with 45ml of 5mM aqueous silver nitrate (AgNo3) in 250ml flask and incubate at 30 c for 2 weeks. Positive synthesis of silver nanoparticles were determined based on the color change to dark brown. The expansion of nanotechnology in various research areas has led to the need to use analytical techniques for the analysis and characterization of nanoparticles. Several characterization techniques are available, including microscopic, separation and spectroscopic techniques. Nanoparticles are usually characterized in the literature by their size distribution, morphology, surface properties, stability and interactions. The main characterization techniques of nanoparticles in general and AgNP in particular Gram staining was carried out by following standard staining procedures and techniques the microscope examination was performed with the oil immersion objective of the bright field microscope Catalase is the enzyme that breaks hydrogen peroxide (H_2O_2) into H_2O and O_2. Hydrogen Peroxide is often used as a topical disinfectant in wounds, and the bubbling that is seen is due to the evolution of O_2 gas. H_2O_2 is a potent oxidizing agent that can wreak havoc in a cell; because Of this, any cell thatuses O_2 or can live in the presence of O_2 must have a way to get rid of the Peroxide. Catalase activity of the isolates was estimates by using H_2O_2 solution onto the microscopic slides containing the culture of the isolates separately observations were taken for immediate bubble formation CO_2 water bubbles Release of O_2 Bubbles indicate positive catalase activity. Basically, this is a test to see if an organism is an aerobe. It is a check for the presence of the electron transport chain that is the final phase of aerobic respiration. Normally, oxygen is the final electron acceptor for this system. In the oxidase test, an artificial final electron acceptor (N,N,N‟,N‟-tetramethylphenylenediaminedihydrochloride) is used in the place of oxygen. This acceptor is a chemical that changes color to a dark blue/purple when ittakes the electron from the last element (cytochrome oxidase) in the electron transport chain. The oxidase tests used to identify bacteria that produce cytochrome oxidase an enzyme of the bacterial electron transport chain when present the cytochrome oxidase oxidizes the reagent tetramethyl-p phenylenediamine to (indophenols) purple colour in product when the enzyme is not present in the reagent reminds reduced and is colourless.

Phytochemical analysis: Test for Carbohydrates, Tannins, Saponins, Flavonoids, Alkaloids, Quinones, Glycosides, Cardiac glycosides, Terpenoids, Phenols, Coumarins, Steroids, hloba tannins, Anthraquinone were analyzed. A total antioxidant activity of methanol and aqueous extract were determined according to the method. The sample was readed with the stable DPPH radical in a methanol solution, the reaction mixture consisted of different concentration of each sample and add 2ml of DPPH solution (0.4 mM) when DPPH reacts with an antioxidant compound which can donate hydrogen, it is reduced, the reaction mixture was incubated at 20 minutes in dark condition the changes in colour (from deep violet to light yellow), were read absorbance at 517 nm using UV- VIS spectrophotometer. The methanol serves as blank. The scavenging activity percentage (AA %) was determined according to the formula. % inhibition = [Control- Sample] ×100 Control The total antioxidant capacity of the methanol, aqueous extracts was according to the Phosphomolybdenum method different concentration of each sample and add 1ml of phosphor molybdenum (0.049g ammonium

molybdenum, sodium phosphate(0.036g), and Sulphuric acid(588ul) reaction mixture was incubated in water bath at 90˚C for 90 minutes. After the samples were cooled to room temperature, the absorbance of methanol and aqueous solution of each sample was measured at 695 nm using UV spectrophotometer. The methanol serves as blank. The higher absorbance value indicated higher antioxidant activity. % inhibition = [sample – control] × 100.

The alpha-Amylase inhibitory activities of the given plant samples were carried out according to the method reported by Nickavar*et al*. (2009). The starch solution (0.5% w/v) used as the substrate was prepared by boiling potato starch in distilled water for 15 min. The enzyme solution was prepared by dissolving 1 mg of porcine pancreatic α - amylasein 20 mM phosphate buffer (100 mL, pH 6.9).The sample solutions were prepared in DMSO (dimethyl sulfoxide) in different concentrations (10 to 100 mg/mL).The DNS solution (20 mL 96 mM 3,5-dinitrosalicylicacid, 12 g sodium potassium tartrate in 8 mL of 2 M NaOH and 12 mL deionized water) was used as the coloring reagent of reaction. Three sets of experiments were conducted: test, blank and control. A mixture of 1mL of each of the test and enzyme solutions, in a test tube, was incubated at 25°C for 30 min. Then, after taking out 1 mL from this mixture, 1 mL of the above mentioned starch solution was added and the mixture was incubated at 25°C for 3 min. Finally, 1 mL of the DNS solution was added. The tube was then covered and heated in water bath at 85ºC for 15 min. After coolingthe tube, the reaction mixture was diluted with distilled water (9 mL). It was mixed well and the absorbance was recorded at 540nm. In case of blank, the DNS solution was added prior tothe addition of the starch solution, while rest of the method was same as for the test. For control, all procedure was again the same except that plant extract was replaced by 1 mL of DMSO. Acarbose, a well-known anti-diabetic medicine, was used as a positive control. The percentage inhibition was calculated by the formula: %Inhibition = [(Ac – As) /Ac] × 100

RESULTS AND DISCUSSION

The presence of phytochemicals in *Moringo oleifera* was checked and theresults were provided in the **table 5.1.1.** The preliminary phytochemical test werehelpful in finding out the biochemical constituents present in the plant material. The extracts of *Moringooleifera* when test with different solvents showed positive results for important phytochemicals such as carbohydrates, alkaloids, glycosides, cardiacglycosides, terpenoids, steroids.

Phytochemical analysis:

S. No	Phytochemical constituents	Results
1.	Carbohydrates	Positive
2.	Tannins	Negative
3.	Saponins	Negative
4.	Flavonoids	Negative
5.	Alkaloids	Positive
6.	Quinones	Negative
7.	Glycosides	Positive
8.	Cardiac glycosides	Positive
9.	Terpenoids	Positive
10.	Triterpenoids	Negative
11.	Phenols	Negative
12.	Coumarins	Negative
13.	Steroids	Positive
14.	Phlobatannins	Negative

Isolation of bacterial endophytes: The isolated endophytic bacteria were identified as *Staphylococcus aureus* based on microbial observation colony growth, morphological characteristics was identified shown in the figure. Staphylococcus species were used in the synthesis of silver nanoparticles.

Mixed culture of endophytes

Pure isolation of bacterial endophytes

Identification of bacterial endophytes

The clear zone of haemolysis –beta haemolytic shows that staphylococcus aureus because hemolysin is one of the important virulence factors for staphylococcus aureus and causes the typical beta haemolytic phenotype which is called complete haemolytic phenotype as well.

Table Identification of bacteria

S. No.	Test	Results
1.	Gram Staining	Gram positive – cocci
2.	Blood agar	Clear zone of haemolysis-beta haemolytic
3.	Catalase	Positive
4.	Oxidase	Negative
5.	Nutrient agar	Golden yellow colonies

Green synthesis of endophytic bacteria:

Silver ions were reduced to silver nanoparticles when added endophytic extractsand it as observed that the color of the mixed solution turned from white solution to dark brown which indicated the formation of silver nanoparticles dueto surface plasmon resonance after 24 h of incubation (fig 5.4). The formation and stability of silver nanoparticles in the solution are confirmed by UV-Visspectrophotometer studies.

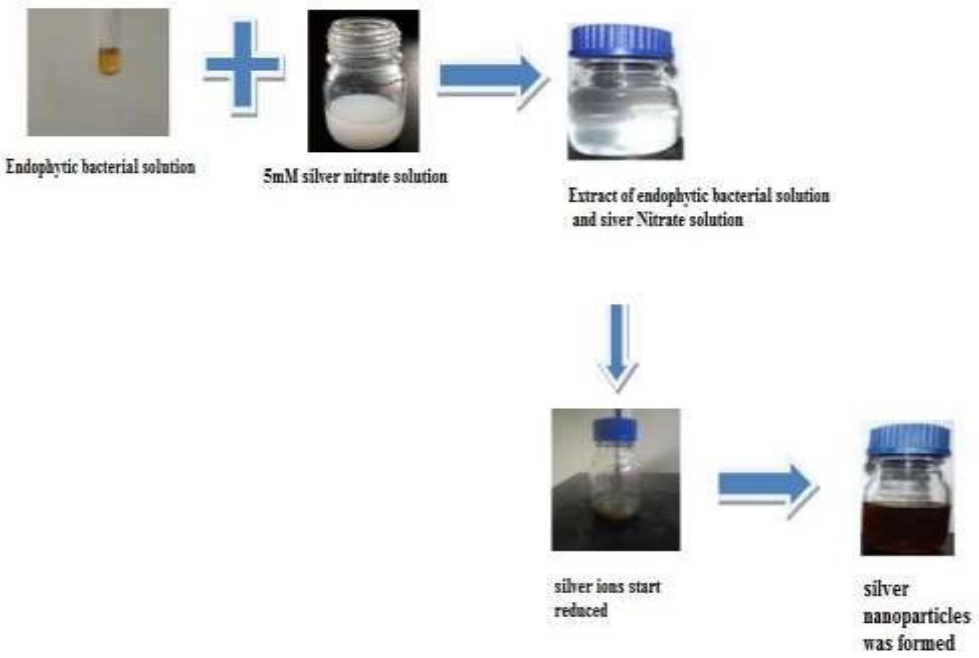


Fig 5.4 shows the schematic representation of the silver Nano particles

UV-Vis Spectrophotometer: The formation and stability of silver nanoparticles in the solution are confirmedby uv-vis spectrophotometer studies shown in fig (5.5).

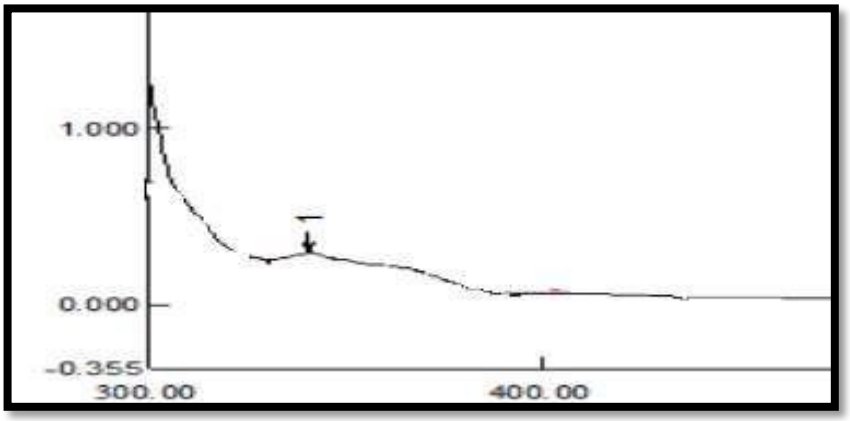


Figure 5.5 UV –visible spectrum of a silver nanoparticles solution is shown.

The absorbance of the visible or ultraviolet light is presented in a calibrationgraph,this is measured by many standard solutions . This absorbance of lightmeasurement of an unknown solutions indicates us to determine its concentration by reading off the graph.

Antioxidant activity

DPPH assay

The antioxidant of the methanol and aqueous endophytic bacterial extractwas measured on the basis of the scavenging activity of the stable DPPH free radical. It shows active site of antioxidant in methanol extract by increasing of inhibition by increasing of concentration and the result was shown in the figure 5.6 (a), (b), (c).

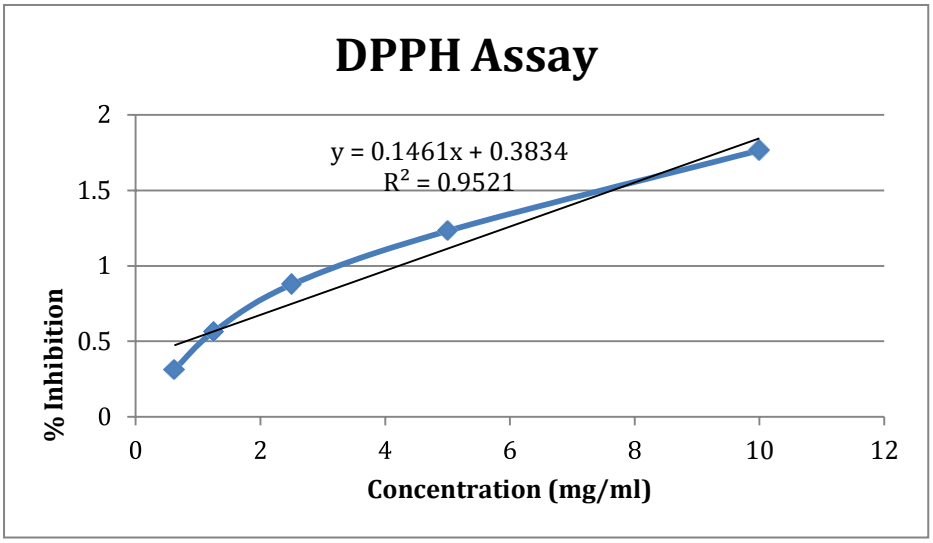


Figure 1.6.1 antioxidant activity by DPPH

Table 1.2.1(a)

Concentration (mg/ml)	Mean OD
10	1.765
5	1.231
2.5	0.876
1.25	0.564
0.62	0.312

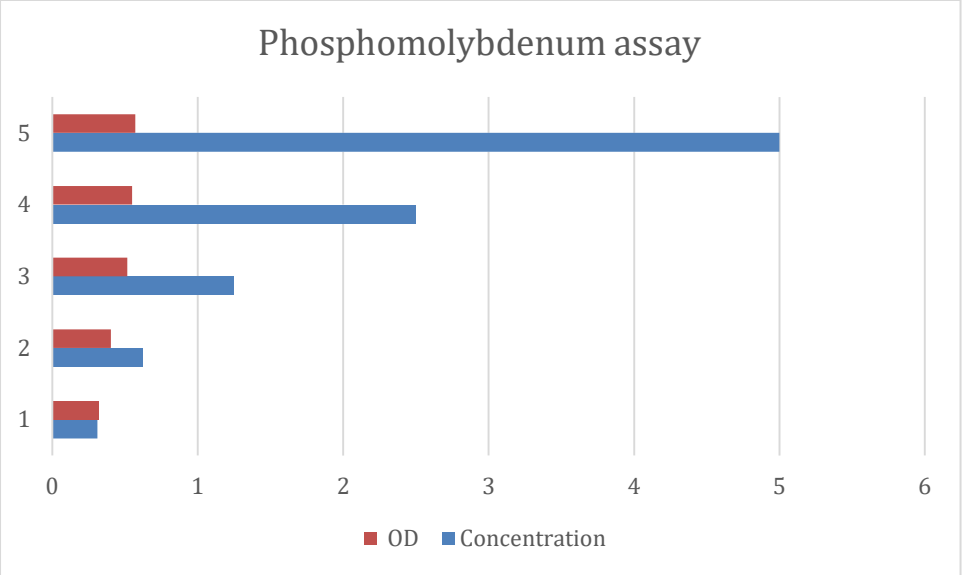
Table 1.2.2 (b)

Concentration (mg/ml)	Mean OD	% Inhibition
5	0.039	86.46
2.5	0.100	65.28
1.25	0.106	63.19
0.62	0.193	32.99
0.31	0.203	29.51
Control	0.288	0.00

The active site of antioxidant in methanol extract by increasing of inhibition by increasing of concentration was determined by silver nanoparticle wasdetermined

Antioxidant activity by phosphomolybdenum Assay:

Preliminary test of antioxidant using phosphomolybdenum assay shows presence of scavenging activities in sample shown in the fig 5.5**,**Alpha amylase inhibitory assay.



Anti diabetic activity: Alpha amylase inhibitory assay: Result:

Table 1.3

CONCENTRATION (mg/ml)	(OD)	% Inhibition
5	0.318	62.188
2.5	0.403	52.081
1.25	0.515	38.763
0.62	0.547	34.958
0.31	0.57	32.224
Control	0.841	

Table 1.4

Metformin Standard Concentration (µg/ml)	(OD)	% Inhibition
10	0.978	12.13
20	0.864	22.37
40	0.558	49.87
80	0.441	60.38
100	0.253	77.27
Control	1.113	

Figure 1.7.1 showing alpha amylase inhibitory activity by metformin stdconc.

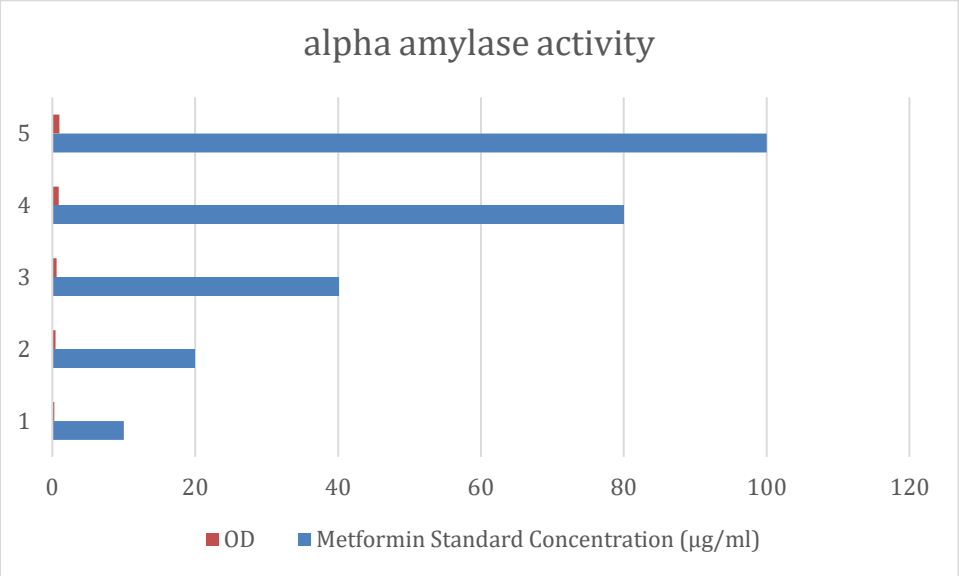
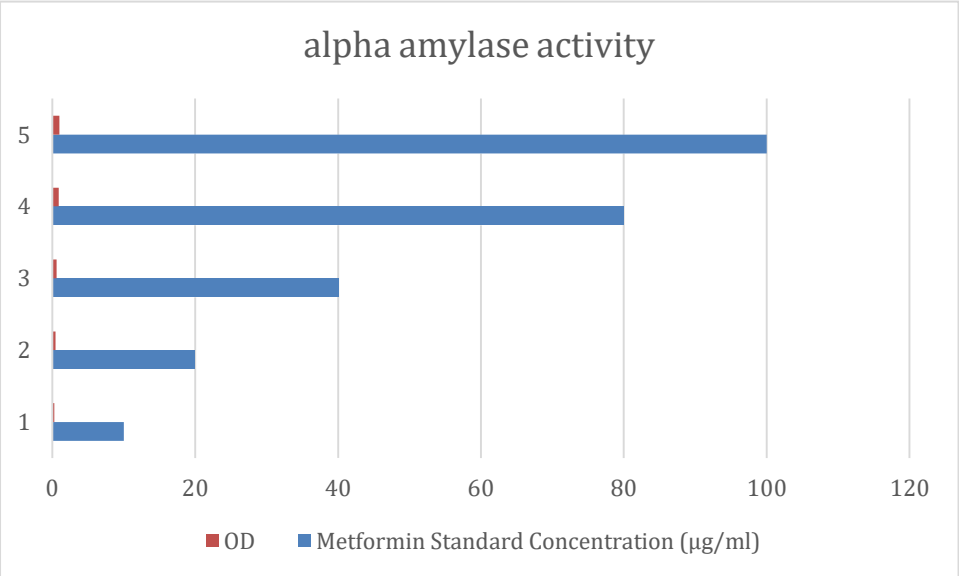


TABLE 1.4 (b)

Nanoparticle Concentration (µg/ml)	(OD)	% Inhibition
10	0.995	10.60
20	0.812	27.04
40	0.703	36.84
80	0.523	53.01
100	0.425	61.81
Control	1.113	

Figure 1.7.2 showing alpha amylase inhibitory activity by nanoparticleconcentration.



DISCUSSION

Silver ions were reduced to silver nanoparticles when added endophytic extracts and its antioxidant properties was checked and it showing the best response when compare to the other method. The silver Nanomaterial‟s prepared by the green synthesis a good physical and chemical property. The biological method of silver nanomaterial's will paved a new pathway for the preparation of nanomaterial and its importance in material science was understood from the study.

CONCLUSION

A critical need in the field of nanotechnology is the development of reliable and eco-friendly processes for synthesis of silver nanoparticles .Here, we have reported a simple biological and low – cost approach for preparation of stable silver nanoparticles using *Moringooleifera***.**

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