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In-vitro hepatoprotective activity of *Moringa oleifera* mediated synthesis of gold nanoparticles

T. S. Belliraj, Anima Nanda[@] and *R. Ragunathan

Department of Biomedical Engineering, Sathyabama University, Rajiv Gandhi Salai, Chennai, India
**Centre for Bioscience Research Centre, Eachanari, Coimbatore, India*

ABSTRACT

The present study was designed to investigate in-vitro hepatoprotective activity of Moringa oleifera mediated synthesis of gold nanoparticle. The gold nanoparticles (GNPs) were synthesised from pods of Moringa oleifera. Aqueous, ethanolic and chloroform extracts have been screened for the suitability of nanoparticles synthesis. Phytochemical screening was performed to identify the major secondary metabolites and its antibacterial efficacy towards Bacillus subtilis, Escherichia coli, Klebsiella pneumonia and Staphylococcus aureus were investigated. The pod mediated synthesis of gold nanoparticles were characterised by Ultraviolet-visible spectroscopy, scanning electron microscopy (SEM), X-ray diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR). Further, in-vitro hepatoprotective activity of gold nanoparticleis carried out by HEP G2 cell line (liver hepato-cellular carcinoma). Chloroauric acid when exposed to pod extract was reduced and converted to gold nanoparticles. The phytochemical screening indicated the presence of alkaloids, terpenes, phenol and tannin, saponins, flavonoids, quinines and proteins. The antibacterial efficacy proved that Moringa oleifera could be used in the treatment of infection caused by such pathogens as S. aureus, E. coli, B. Subtilis and K. pneumoniae. The ultraviolet-visible spectrum of the gold nanoparticles showed an absorption peak at around 559 nm, the scanning electron microscopy indicated that the average size of gold nano particle was about 40 - 80nm, X-ray diffraction showed that the particles were crystalline in nature, FTIR showed the presence of functional group involved in the reduction, stabilization and capping of the GNPs. From the tryphan blue assay, gold nano particle showed greater activity on HEP G2 cell line.

Keywords: GNPs, *Moringa oleifera*, Phytochemical analysis, Hepatoprotective activity.

INTRODUCTION

Nanotechnology is an anticipated manufacturing technology that allows the long-established trend towards smaller, faster, cheaper materials and devices also. Gold nanoparticles (GNPs) are the most compatible nanomaterial for preparation of engineered nano-platforms in smart sensing devices [1]. Biomedical applications using gold nanoparticles (GNPs) have become very active research area in the recent years. A large variety of possible biomedical applications has been examined, i.e., drug and gene delivery etc. [2]. Previously it was reported that gold nanoparticles could be synthesized by using yeast, fungi, bacteria, plant extracts which provides an inspiration for studies on green chemistry routes [3]. Present investigation is to synthesize stablized GNPs by reduction of aqueous chloroauric acid solution using *Moringa oleifera* pod extract at normal room temperature. *Moringa oleifera* is one of the 14 species of family Moringaceae, native to India, Africa, Arabia, South Asia, South America and the pacific and Caribbean Islands. Because of *Moringa oleifera* has been naturalized in many tropic and subtropics regions worldwide, the plant is referred to number of names such as horse radish tree, drumstick tree, ben oil tree, miracle tree, and Mothers best friend [4]. In the present work we have utilised the *Moringa oleifera* for the synthesis of gold nanoparticle and to investigate the role of phytochemical compounds for the antibacterial activity and hepato protective activity.

EXPERIMENTAL SECTION

Medicinal plant: *Moringa oleifera*

Moringa oleifera (*M. oleifera*) (Family: Moringaceae, English name: drumstick tree) has been reported to be essentially used as an ingredient of the Indian diet since ages. It is cultivated almost all over India and its leaves and fruits are traditionally used as vegetables [5]. Various parts of this plant such as the leaves, roots, seed, bark, fruit, flowers and immature pods act as cardiac and circulatory stimulants, possess antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepato protective activity [6].

Collection of plant materials

Moringa oleifera (Pods; n=10) were purchased from local market at Coimbatore. They were washed under running water to eliminate dust and other foreign particles and dried at room temperature.

Preparation of extracts

1 gm in each of seed and pod samples were mixed with distilled water, ethanol and chloroform (each 10 ml) and incubated at room temperature for 24 hours. After incubation period all the samples were filtered using whatman number 1 filter paper and these extracts were used for qualitative phytochemical analysis and antibacterial studies.

Phytochemical Analysis

Phytochemical screening were performed to assess the qualitative chemical composition of different extracts using commonly employed precipitation and coloration reactions to identify the major secondary metabolites like alkaloids, terpenes, phenol and tannin, sugar, saponins, flavonoids, quinines, proteins and steroids. The phytochemical analyses were carried out using standard procedures [7, 8].

Alkaloids

To 1 ml of extract added mercuric chloride and few drops of iodine solution. Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Terpenoids

2 ml of crude extract was dissolved in 2 ml of chloroform and evaporated to dryness. To this 2 ml of Con. H_2SO_4 was added and heated for about 2 minutes. A grayish colour indicates the presence of terpenoids.

Phenol and Tannin

1 ml of crude extract was mixed with 2 ml of 2% solution of $FeCl_3$. Formation of blue green or black colorization indicates the presence of tannin.

Reducing sugar

The small amount of substance mixed with equal volume of Fehling's A and Fehling's B solution and heated in a water bath. Formation of red colour indicates the presence of sugar.

Saponins

3 ml of extract were diluted with 2 ml distilled water and this was shaken vigorously for 30 seconds in a graduated cylinder. Formation of 1 cm layer of foam indicates the presence of saponin.

Flavonoids

To 1 ml of crude extract was mixed with few fragment of magnesium ribbon and Con HCl was added drop wise. Pink scarlet colour appeared after few minutes which indicate presence of flavonoids.

Quinines

To the 1% test substance 2% sodium hydroxide was added. Formation of blue green or red colour indicates the presence of quinines.

Protein

2 ml of extract was treated with few drop of concentrated nitric acid. Formation of yellow colour indicates the presence of protein.

Test for Sterols

3 ml of crude extract was mixed with 2 ml of chloroform and con H_2So_4 was added sidewise. A red colour is produced in the lower chloroform layer indicates the presence of steroids.

Determination of Antibacterial Activity

The antibacterial activity of the pod extracts was determined using agar well diffusion method. Nutrient agar was inoculated with the given microorganisms viz., *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumonia* and *Staphylococcus aureus* by spreading the bacterial inoculums on the media. Wells were punched in the agar using sterile cork borer and filled with pods extracts. Standard reference antibiotic Methicillin (10 mcg/disc)was used as controls for the tested bacteria. The plates were incubated at 37° C for 24 hours and the antibacterial activity was assessed by measuring the diameter of the zone of inhibition. The antibacterial potential of the different extracts was evaluated by comparing their zones of inhibition.

Synthesis of gold nanoparticles (GNPs)

To prepare gold nanoparticle, 20ml of pod extract was added to 10 ml of triple distilled water in a conical flask. Chloro auric acid solution 1M was added to the mixture with vigorous stirring in a magnetic stirrer at normal room temperature. After 24 hours, colour of the final mixture started to become slightly brown which indicated the formation of GNPs.

Characterisation of gold nanoparticles

UV-Vis Spectroscopy

Ultraviolet-visible spectroscopy or ultraviolet-Visible spectrophotometer (UV-Vis) was used in the present study to find out the absorption of nanoparticles in the UV-Visible spectral region. This means it uses light in the visible and adjacent near-UV and near-infrared (NIR)) ranges. The absorption in the visible range directly affects the perceived color of the chemicals involved. In this region of the electromagnetic spectrum, molecules undergo electronic transitions [6].

Scanning Electron Microscope (SEM) Analysis

Scanning Electron Microscope (SEM) analysis was done using SEM machine. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min[6].

Fourier Transmission Infra Red Spectroscopy (FTIR)

FTIR is a chemical analytical method which measures infrared intensity v/s wavelength or wave number of light. It was used to analyze the bio molecule and also bonding interaction between molecules of the gold nanoparticles. IR spectroscopy detects the vibration characteristics of chemical functional groups of the sample. When an infrared light interacts with matter, chemical bonds will shows stretch, contract and bend form. These chemical functional group tends to adsorb infrared radiation in a specific wave number range of the structure of the rest of the molecule.

X-Ray Diffraction (XRD) Measurements

The phase formation of bioreduced silver nanoparticles was studied with the help of XRD. The diffraction data of thoroughly dried thin films of nanoparticles on glass slides was recorded on X-ray diffractometer [6].

Hepatoprotective activity

The HEP G2 cell culture was trypsinated and the cell count was adjusted to1.0 x 105 cells/ml using medium containing 10% serum. To each well of the 96 well microliter plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a primary monolayer was formed, the supernatant was flicked off, washed the monolayer once and 100µl of different gold nanoparticle concentrations was added to the cells in microliter plate. The plate was then incubated at 37ºC for 3 days in 5% CO2 atmosphere and microscopic examination was carried out and the observations recorded every 24 hours [9]. After 72 hours, the nanoparticle solutions in the wells were discarded and 50 µl of Tryphan blue was added to each well and it is been viewed in microscope [10].

RESULTS AND DISCUSSION

Phytochemical screening of *Moringa oleifera*

Phytochemical analysis of seeds and pods of *Moringa oleifera* was carried out in aqueous, ethanol and chloroform extracts and the results are given in Table 1.

Table 1: Phytochemical analysis of *Moringa oleifera* with different solvents

Tests	Seed			Pod		
	Aqueous	Ethanol	Chloroform	Aqueous	Ethanol	Chloroform
Alkaloids	+	+	-	+	+	+
Terpenoids	-	+	+	+	+	+
Phenols & Tannins	-	-	-	+	-	-
Sugar	+	-	+	-	-	-
Saponin	+	-	+	+	+	+
Flavonoids	-	-	-	+	-	-
Quinines	-	-	+	+	+	+
Proteins	+	-	+	+	+	+
Sterols	+	+	+	-	+	+

+ indicates presence and – indicates absence with respect to extractive solvents.

Available literature indicated that medicinal plants are the backbone of traditional medicine and the antibacterial activity of plant extract is due to different chemical agent in the extract which was classified as active antimicrobial compounds [11]. Alkaloids are formed as metabolic byproducts and have been reported to be responsible for the antibacterial activity [12]. Phytochemicals such as saponins, terpenoids, flavonoids, tannins, steroids and alkaloids have anti- inflammatory effects [13, 14]. Therefore, majority of the aqueous extract of pod contained the secondary metabolite such as alkaloid, terponoids, flavonoid, phenol and tannins, saponin, quinines and proteins which make the seed of *Moringa oleifera* to posses the biological properties.

Antibacterial efficacy of *Moringa oleifera*

Antimicrobial tests were performed against *B. subtilis, E. coli, S. aureus* and *K. pneumoniae* on nutrient agar plates treated with different concentrations of aqueous pod extracts (Table 2). It was observed that 30 µl concentration of sample shows higher activity. It can be seen that extracts possessed a broad range of activity with the highest zone of 10 and 11 mm for all four microorganism. *Escherichia coli* are the pathogenic organism responsible for the intestinal disorder, gastroenteritis, meningitis. This proves that *Moringa oleifera* could be used in the treatment of infection caused by such pathogens as *S. aureus, E. coli* [15].

Table 2: Antibacterial activity of *Moringa oleifera* at different concentration

Name of the bacteria	Zone of inhibition in mm			
	Pod			Methicillin (10 mcg/disc)
	10 µl	20 µl	30 µl	
E. coli	9	10	11	Nil
S. aureus	8	9	10	Nil
B. subtilis	8	10	11	Nil
K. pneumoniae	8	9	10	Nil

Synthesis of gold nanoparticles

The appearance of slightly brown color following mixing of the plant extract with HAuCl4 solution indicated the formation of GNPs (Fig 1). These particles were characterized for size and bioactive potential.

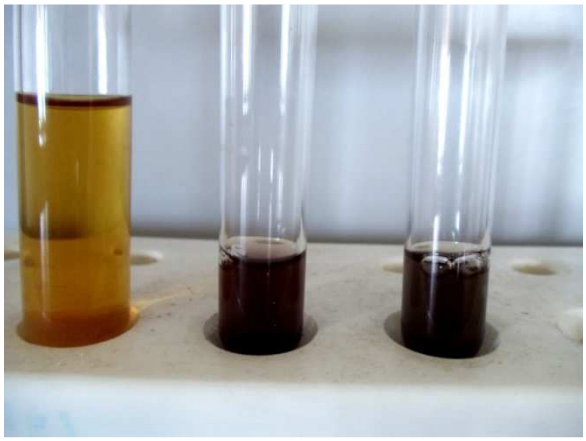

Fig 1: Synthesis of gold nanoparticles

Characterisation of gold nanoparticles

UV-Vis spectrophotometry

The absorption of gold nanoparticle was measured and plotted using the means of all peaks mean by single beam spectrophotometer and absorption maxima was noted at different wavelength (400-600 nm) (Fig 2). The synthesized colloidal gold showed the maximum absorption at 559 nm. This absorption peak primarily depends on morphology; inter particle distance, stabilizer and chemical surroundings [3].

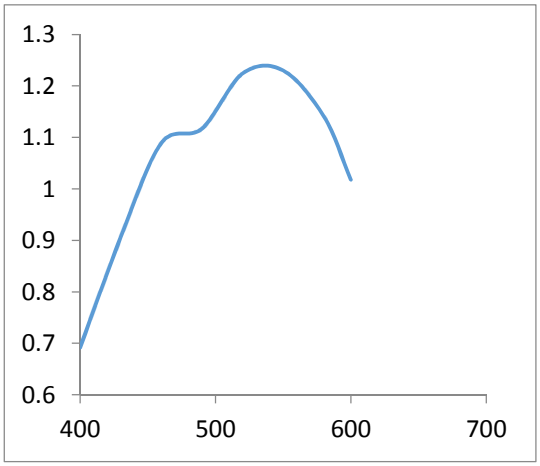

Fig 2: UV-vis spec. analysis of gold nanoparticle synthesised from *Moringa oleifera*

Scanning Electron Microscope (SEM) Analysis

SEM (scanning electron microscopy) studies were carried out to determine the size and morphology of the synthesized GNPs. The gold nanoparticles are manifestly three-dimensional structures and while most of them may be described as irregular, some possess various distinct shapes. The average size of gold nano particle is about 40 - 80 nm (Fig 3). In the image the nanoparticles are aggregate into larger conglomerates. The factor responsible for difficulties connected with getting higher magnification was high susceptibility of nanoparticles to aggregate into larger conglomerates due to the presence of proteins and metabolites present in the extract [16].

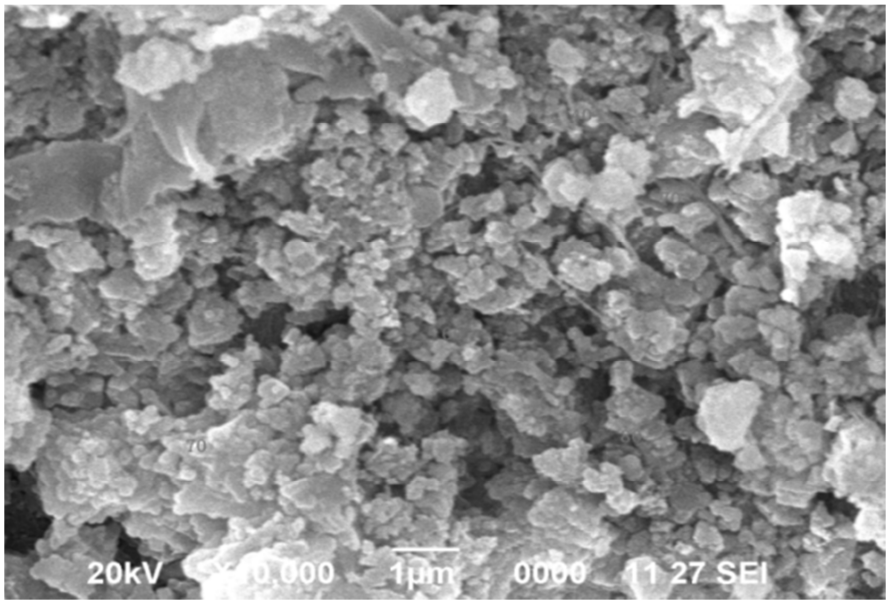

Fig 3: Scanning Electron Microscopic image of gold nanoparticles

Fourier Transform Infrared Spectroscopic

FTIR spectroscopy was done to identify the functional groups involved in the reduction, stabilization and capping of the GNPs and the results is given in Table 3 and Fig 4.

Table 3: FTIR absorption range and functional groups

Absorption ranges(cm-1)	Functional group	Type of vibration
3453.04	Alcohols & phenols	O-H Stretch
1643.04	Alkenes	C-C=C Symmetric Stretch
1560.52	Amines—Primary	N-H Stretch
1415.03	Amides	N-H Stretch
1026.20	Ethers	C-O Stretch

These carboxyl and amide group indicated the presence of secondary amines which is a signature marker of proteins confirming the biofabrication of the nanoparticles by the action of the protein or phytochemicals [17].

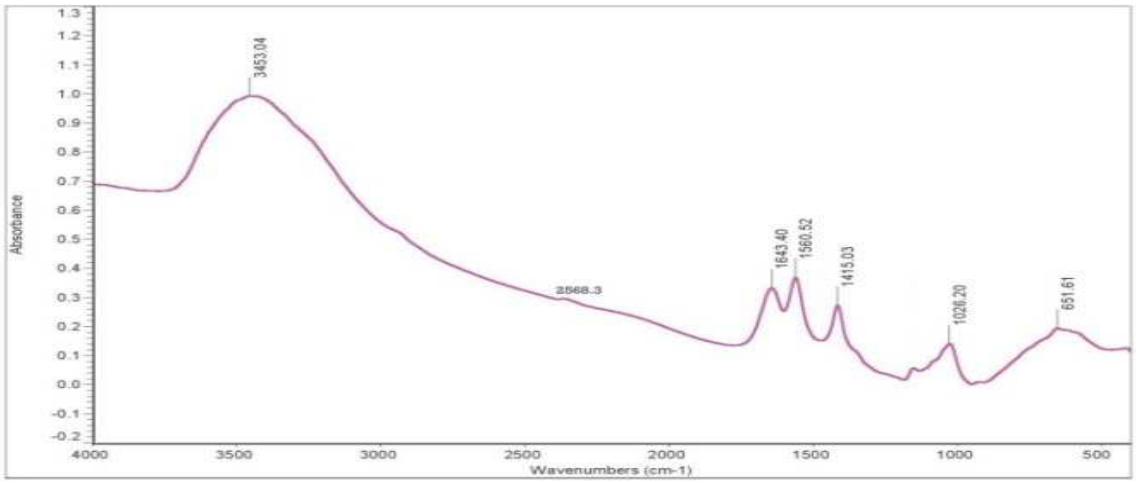

Fig 4: FTIR of gold nanoparticle synthesised from *Moringa oleifera*

X-ray diffraction

Moringa oleifera extract-mediated synthesises of gold nanoparticle was confirmed by the characteristic peaks observed in the XRD image (Fig 5). All diffraction diffraction lines observed at 2θ angle 38.2°, 44.29°, 64.68° respectively clearly show that the particle composition was crystalline in nature. With reference to the JCPDS data file No.01-1174, it was concluded that the nanoparticles were crystalline in nature with no impurities [18].

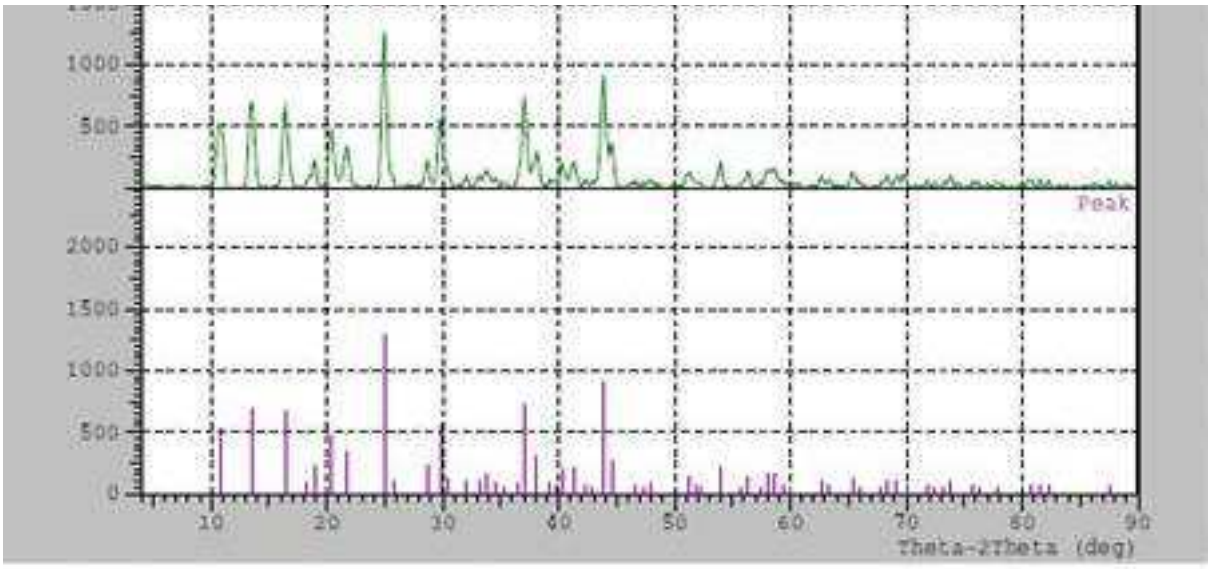

Fig 5: XRD of gold nanoparticle synthesised from *Moringa oleifera*

***Invitro* hepatoprotective activity**

In-vitro hepatoprotective image of *Moringa oleifera* is give in Fig 6. Tryphan blue assay method was used to differentiate viable and non-viable cells. Dead cells were stained as blue. *Moringa oleifera* mediated synthesis of gold nanoparticle were able to protect the liver against different hepatotoxic drugs and toxins such as

acetaminophen, antitubercular drugs and carbon tetrachloride. Moreover, extracts of *Moringa oleifera* leaf, seeds, pods and roots are effective against cyclophosphamide induced toxicity, ulceration, fluoride toxicity and in the improvement of hepato renal function [19].

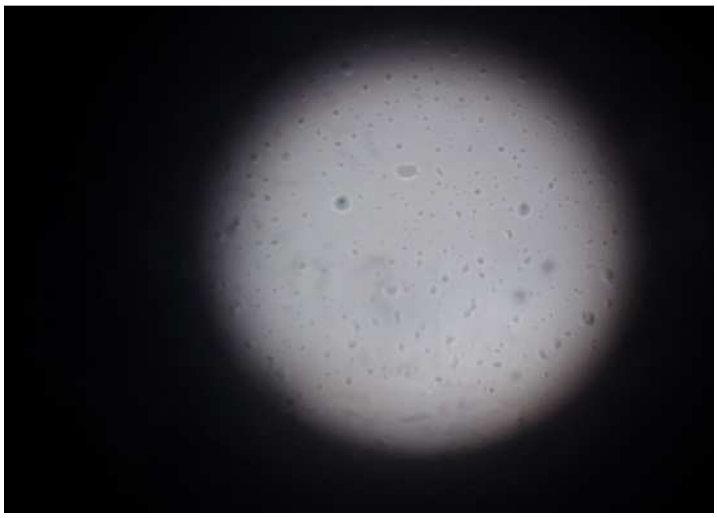

Fig 6: In-vitro hepatoprotective image of *Moringa oleifera*

CONCLUSION

The findings of the present study suggest a new pathway for alternative natural medicine for the treatment of various diseases including the prevention of pathogenic microorganisms. Pods of *Moringa oleifera* having more secondary metabolites will be good for human consumption. The present study has shown the potentials of *M. Oleifera* pods on aqueous extract were quite better than ethanol and chloroform extracts. Similarly antibacterial efficiency of aqueous pod extract was also comparably high than. Therefore *M. oleifera* would be promising natural antimicrobial agent for controlling pathogenic bacteria in pharmaceutical industries. Thus the natural medicinal plant, *Moringa oleifera* might provide protection to humans against infections and degenerative diseases. *M. oleifera* can also be used for hepatoprotective activity.

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