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Green Synthesis, its Characterization and Antimicrobial Activities of Zinc Oxide Nanoparticles from *Silybum marianum* Seeds Extract

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Abstract

Silybum marianum L. called as milk thistle has the medicinal parcels with a multitude of pharmacological operations. The current study was the green conflation of ZnO-nanoparticles (NPs) from seeds of *Silybum marianum* (L.) ZnO-NPs therefore synthesized were subordinated to characterization using standard ways similar as FTIR and UV Visible spectrophotometer. The presence of phytochemical constituents proves that *Silybum marianum* seed is rich in the secondary metabolites in the three extracts of aqueous, ethanol and chloroform. The conflation of essence and semiconductor nanoparticles is an expanding area due to the implicit operations in the development of new technologies. Especially, biologically synthesized nanomaterial has come an important branch of nanotechnology. The present work, described the conflation of Zinc oxide nanoparticles (ZnO NPs) using seed waterless excerpt of *Silybum marianum* L. and its antimicrobial conditioning. The nanoparticles were gain characterized by UV-visible spectroscopy, and Fourier transfigure infrared spectroscopy (FTIR). In this study we also delved antimicrobial exertion of green synthesized ZnONps and the antioxidant activity in the ethanol extract higher when it compared with the other two extracts. Eventually concluded the zinc oxide nanoparticles displayed intriguing antimicrobial exertion with both gram positive and gram negative bacterial and incentive at micromolar attention.

***Key words*:** *Silybum marianum* L., ZnO NPs, Green synthesis, FT-IR, Antimicrobial activities

Milk thistle (*Silybum marianum*) is a perennial herb with medicinal properties. The seeds contain silymarin, a group of compounds believed to have antioxidant and anti-inflammatory effects. Milk thistle is a plant that gets its name from the white veins on its large, spiky leaves. One of the active compounds in milk thistle called silymarin is extracted from the seeds of the plant. It is believed that silymarin has antioxidant properties. *Silybum marianum* has other common names including cardus marianus, milk thistle, milk thistle, milk thistle, milk thistle, Mediterranean thistle, variegated thistle, and Scottish thistle. This species is an annual or biennial plant of the daisy family (Asteraceae). Milk thistle (*Silybum marianum* L. Gaernt.), sometimes called wild artichoke, is a medicinal plant that has been used for thousands of years as a remedy for various diseases. Milk thistle is an annual or biennial plant in the daisy family (Asteraceae) that blooms with crimson flowers in July and August. Milk thistle needs a warm atmosphere and dry soil and can grow up to 3 m tall and 1 m wide. Most often, however, it reaches a height of 0.9-1.8 m. Milk thistle's native habitats are southern Europe, southern Russia, Asia Minor and northern Africa, and it has been naturalized in North and South America and southern Australia.

The development of effective remediation methods for waterborne pathogens such as B. Shiga toxin-producing Escherichia coli, has aroused great interest among scientists in the development of new biocides or disinfectants to complement conventional antibiotics [1]. Antimicrobial agents can be roughly divided into two types: organic and inorganic. Organic antimicrobial materials are often less stable, especially at elevated temperatures and/or pressures, than inorganic antimicrobial materials [2]. Inorganic materials such as metals and metal oxides have received increasing attention over the past decade due to their ability to withstand harsh processing conditions and are generally considered safe for humans and animals [3]. The inorganic NPs such as silver, gold, copper, CuO, TiO2 and ZnO have profound antibacterial activities.

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Among the inorganic NPs, ZnONps are of particular interest because they can be prepared easily inexpensive and safe material for human beings and animals. They are extensively used in the formulation of health care products [4].

ZnONps has attracted the attention of scientists due to its semiconducting properties, unique antibacterial, antifungal, wound healing and UV filtering properties, and high catalytic and photochemical activity. The goal of nanobiotechnology is to promote the use of economic and ecological reducing and limiting agents from living matter such as fungi, microbes, algae and plant matter (including tissues, seeds and fruits, etc.) for their potential use in medicinal compounds.

Cosmetics and water disinfection industry. The negative effect of nanoparticles is neutralized by the presence of natural metabolites in plants and other biological materials. Nanoparticles produced through an eco-friendly approach are being considered in various fields including Synthetic chemistry, Biotechnology, Environmental sciences and Biochemistry..Biosynthesized zinc oxide (ZnO) nanocomposites exhibit unique properties. Many researchers have found it attractive for research in many scientific disciplines due to its novel applications. Important properties of ZnO include piezoelectric, catalytic, pyroelectric, optoelectronic, and semiconductor properties. The research focus is on the synthesis of ZnONps from extracts of Silybum marianum (L.) Gaernt. (milk thistle) seeds. Various bioassays, including antioxidants and antibacterial agents, have been identified to test the potency of synthesized NPs.

MATERIALS AND METHODS

Seeds collection

Silybum marianum (L.) Gaernt. (Milkthistle) seeds were collected from Villupuram.

ZnONps biosynthesis

ZnONPs biosynthesis was carried out by adding zinc acetate di-hydrate (molecular weight:219.51 g/mol) to the plant extract in a 100:1 ratio comprising 1 mM of zinc acetate di-hydrate into 100 ml of seed extract. The pH was set at 12 and the three extract was taken kept for 24 h at 37°C. After 24 h, ZnONps gathered at the base. The supernatant was discarded and the solution containing NPs was introduced into 1.5 mL micro centrifuge tubes and with 1 mL of pure ethanol keep in centrifuge for 10 minutes at 12000 rpm. The supernatant was disposed of and the left-over pellets were washed thrice with the distilled water. Calcination (500 °C) of the NPs was done for 2h to increase the crystalline of the NPs.

Collection of samples

The seeds of *Silybium marianum* were collected from a local market of Villupuram, Tamil Nadu, India. The seeds were ground and then stored in an airtight container at room temperature to prevent any gain in moisture.

Synthesis of ZnONps from the milk thistle seed powder

ZnONps biosynthesis was carried out by adding zinc acetate di-hydrate (molecular weight: the plant extract in a 100:1 ratio comprising 1 mM of zinc acetate di-hydrate into 100Ml of seed extract. The pH was set at 12 and the extract was then kept for 24h at 37°C. After 24h, ZnONPs gathered at the base. The supernatant was discarded and the solution containing NPs was introduced into 1.5 mL micro centrifuge tubes and washed with 1 mL of pure ethanol keeping centrifuge for 10min at 12,000 rpm. The supernatant was disposed of and the left-over pellets were washed thrice with the distilled water. Calcination (500 °C) of the NPs was done for 2h to increase the crystallinity of the ZnONPs [12].

Phytochemical screening of extracts

Preliminary phytochemical screening of the extracts was carried out as per the methods and tests given by Day and Raman [11].

Tests for Carbohydrates

Preparation of test solution

The test solution was prepared by dissolving the test extract in powder, aqueous, ethanol and acetone.

a) *Molish's test*

Test solution with few drops of Molish's reagent and concentrated H_2SO_4 added slowly from the sides of the test tube. Formation of violet ring at the junction of two liquids indicates the presence of carbohydrates.

Tests for proteins and amino acids

Preparation of test solution

The test solution was prepared by dissolving the extract in powder, aqueous, ethanol and acetone.

a) *Biuret test*

To the test solution, 4% sodium hydroxide and few drops of 1% copper sulphate solution was added, development of violet or pink color indicates the presence of protein.

Tests for secondary metabolizes

Test for alkaloids

a) *Wagner's test*

To the extract (1ml) add 1ml of Wagner's reagent prepared by mixing 2g of iodine and 6g of potassium iodide in 100ml distilled water. The formation of reddish-brown precipitate was an indication of the presence of alkaloids.

Test for tannins

Modified Prussian blue test

To 1ml of extract, added 1 ml of 0.008M Potassium Ferrocynate and 1ml of 0.02M $FeCl^3$ in 0.1M Hcl. Appearance of blue color indicated the presence of Tannins.

Test for saponins

a) *Froth test*

About 5ml of diluted extracts were taken in a test tube and shaken vigorously and kept for 5min. Formation of foamy layer indicates the presence of saponins.

Test for glycosides

About 2ml of the concentrated extracts taken in a test tube and add a quantity (10ml of 50% H2So4) was added to the mixture was heated in a water bath shaker for 15min. To these mixtures add 2ml of Fehling's solution and then the mixture was boiled. Development of a brick-red precipitate indicated the presence of glycosides in the extract.

Test for flavonoids

A 2ml of each extract were taken in separate test tube add few drops of sodium hydroxide solution. The yellow color was formed and it became turn to colorless while addition of diluted sulfuric acid confirmed the presence of Flavonoids.

Test for phenol

Ferric chloride test

To the extracts add 3-4 drops of 5% Ferric chloride solution and observed the formation of dark blue or blackish color which may indicate the presence of phenol in the extracts.

Test for terpenoids

About 5ml of each leaf extract was taken and add 2ml of chloroform and 3ml of concentrated Sulfuric acid notice the formation of layer.

Characterization of synthesized silver nanoparticles

Ultra-violet visible spectroscopy

The freshly prepared *Silybum marianum* seed extract mediated ZnO NPs samples was characterized using (UV-750; Jasco, Tokyo, Japan) UV – VIS Spectrometer. Samples were scanned in the range of 500–200 nm to record its absorbance at room temperature [13].

Fourier transform infrared spectroscopy (FTIR)

FTIR (FT/IR-4600 jasco, Tokyo, Japan) analyses were performed to determine the presence of various functional groups *Silybum marianum* seed extract mediated ZnO NPs samples. The analysis was carried out with over 64cumulative scans in the wavenumber between 4000 and400 cm_1, with 4cm_1 resolution (13].

Antimicrobial assay

In the present study, in vitro antimicrobial activities were carried out by the using of disc-diffusion method. This method followed had the following procedure: First of all, Petri plates were prepared with 20 mL of sterile Muller Hinton Agar for bacteria and 20 mL of Sabourdad Dextrose Agar for fungi. The 24 hr prepared test cultures of inoculums were swabbed on the top of the solidified media and allowed to dry for 10 min. Previously prepared ZnO nanoparticles impregnated discs at the concentrations of 200, 100 and 50 µg/mL for bacteria and fungi were placed aseptically on sensitivity plates with appropriate controls. The tests were conducted with 20µg/disc per disc with three replicates. The loaded discs were placed on the surface of the medium and left for 30 minutes at room temperature for compound diffusion. Negative control was prepared using respective solvent. Ciprofloxacin (5 µg/disc) was used as positive control for bacteria and Amphotercin-B (100 units/discs) was used as positive control for yeast. All the plates were then incubated for 24 hr at 37 °C for bacteria and 28 °C to 35 °C for yeast, respectively. The sensitivity was recorded by measuring the clear zone of growth inhibition of agar surface around the discs in millimeter.

RESULTS AND DISCUSSION

Data in (Table 1) shows the presence and absence of phytochemical constituents in the three extracts of aqueous, ethanol and chloroform in *Silybum marianum* seeds. This shows the milk thistle seed is rich in phytochemical constituents like alkaloids, flavonoids, tannin, terpenoids, etc. and (Table 2) shows the presence of antioxidant activity in the three extracts of Aqueous, ethanol and chloroform in *Silybum marianum* seeds hence it shows the high amount of antioxidant activity in the ethanol extract 48 ± 1.58 when it compares with the other two extracts.

FTIR characterization

The FT-IR spectra ((FT/IR-4600 jasco, Tokyo, Japan)) of *Silybum marianum* (L.) seed extract has shown absorption bands at 3352 and 2924 cm^{-1} representing O−H and C−H stretching of polyols. The absorption peak is located at around 1647 cm^{-1} represented C=C stretching vibrations of aromatic rings. Stretching vibrations present at 1452 and 1251 cm^{-1} are associated with O−H and C−OH vibrations of polyols, respectively. Stretching vibrations are located at 1045 and 663 cm^{-1} represented C−N stretching and N−H was of amines, respectively. Small bands at 1734 and 1386 cm^{-1} are represented C=O stretching vibrations of carboxylic acid. These bands indicate polyols (phenolic acid and flavonoids), terpenoids, and protein compounds are abundant in seed extract. FTIR spectra of green synthesis ZnONps (Fig 1) have shown absorption band at 445 cm^{-1}, 1040 cm^{-1}, 1450 cm^{-1}, 1710 cm^{-1}, 2920 cm^{-1} and 2855 cm^{1}, respectively. The peaks in the region between 600 and 400 cm^{-1} are allotted to M–O(Zn-O).

Table 1 Phytochemical screening of *silybum marianum* [seed]

Phytochemical constituent	Aqueous extract of *Silybum marianum*	Ethanol extract of *Silybum marianum*	Chloroform extract of *Silybum marianum*
Alkaloids	+	+	+
Tannin	+	+	+
Terpenoids	+	+	+
Flavonoids	+	+	−
Reducing sugar	+	+	+
Glycosides	+	−	−
Phenol	+	+	+
Protein	+	+	+
Ferric chloride	+	+	+

+ Positive. - Negative

The band at 445 cm^{-1} confirms stretching vibrations of zinc oxide NPs. The shift observed in FT-IR spectra of ZnONps after bioreduction band indicate the participation of polyols, terpenoids, and proteins having functional groups of amines, alcohols, ketones, and carboxylic acid in bioreduction reactions. Terpenoids are poorly water-soluble and hence may not be among prime moieties involved in the bioreduction reaction. However, proteins seem to exhibit little importance in biosynthesis of nanoparticles as reported earlier. Therefore, water-soluble phenolic acid and flavonoid compounds are believed to play a major role in bioreduction reaction. But the possible mechanism is still unclear and needs further investigation.

Estimation of antioxidant activity

Table 2 Mean and standard deviation value of antioxidant activity

Solvent	*Silybum marianum* [Seed]
Aqueous extract	37 ± 3.42
Ethanol extract	48 ±1.58
Chloroform extract	40 ± 2.06

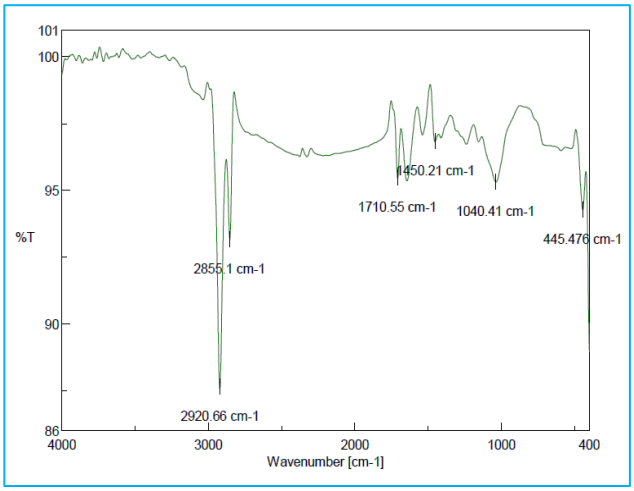

Fig 1 FTIR analysis of ZnO nanoparticles extracted from *Silybum marianum* seed

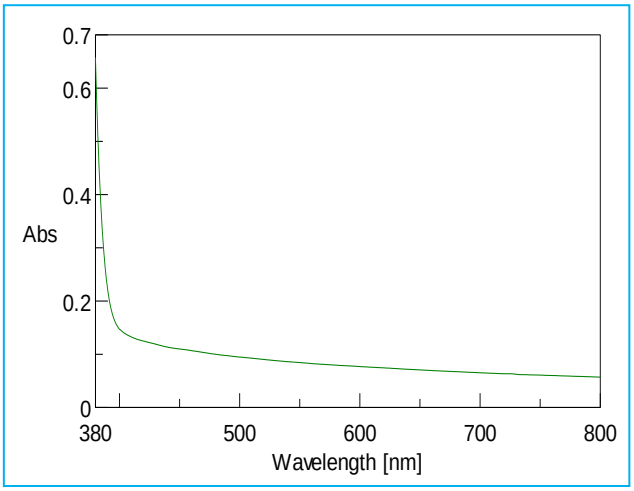

Fig 2 Characterization of UV-VIS spectrophotometer

Table 3 Antimicrobial property of ZnONps extracted from *silybum marianum* seeds

Name of the organisms	Mean Zone of Inhibitiona (mm)b Concentration 200 µg/mL			
	Bare *Silybum marianum* seed extract mediated ZnO NPs	Control	Control Cip	(5µg/disc)/ Amp-B (100 units/disc)
Staphylococcus aureus	15.4 = 0.56	18.5 ± 0	2 5.3 ±0.28	32.0 ± 0.50
Bacillus subtilis	8.6 ± 0.76	12.8 ± 0.76	21.1 ± 0.57	31.1 ± 0.78
Pseudomonas aeruginosa	7.6 ± 0.76	11.1 ± 0.28	21.3 ± 0.58	29.5 ± 0.50
Proteus mirabilis	9.3 ± 0.50	11.1 ± 0.28	20.0 ± 0.50	31.3 ± 0.50
Escherichia coli	9.8 ± 0.76	13.5 ± 0.50	21.6 ± 0.57	30.0 ± 0.50
Candida albicans	8.6 ± 0.70	11.3 ± 0.57	19.8 ± 0.76	13.6 ± 0.50
Candida tropicalis	7.5 ± 0.57	9.3 ± 0.57	17.1 ± 0.28	15.3 ± 0.50

a Diameter zone of inhibition (mm) including the disc diameter of 6mm; b Mean of three assays
± Standard deviation; Ciprofloxacin (5µg/disc) for bacteria: Amphotercin –B (100 units/disc) for *Silybum marianum*

Uv-vis spectrophotometer characterization

The room temperature UV–vis absorption spectrum of the ZnO-NPs is shown in (Fig 1). The ZnO-NPs are dispersed in water with concentration of 0.1 wt% and then the solution is used to perform the UV–vis measurement. The spectrum reveals a characteristic absorption peak of ZnO at wavelength of 370 nm which can be assigned to the intrinsic band-gap absorption of ZnO due to the electron transitions from the valence band to the conduction band. The band gap energy of zinc oxide nanoparticles is calculated by using formula [13].

E = hc/λWhere h (6.626x10-34 Js) is planks constant, c (3 x 108 m/s) is the velocity of light and λ (370 nm) is the wave length. The band gap energy of zinc oxide was found to be 3.3eV.

Antimicrobial studies

The antimicrobial activities of ZnONps towards bacteria and fungi depend on particle size, powder concentration, morphology, specific surface area, etc. The concentration of ZnONps increase (50, 100 and 200 µg/mL) and also increase in antimicrobial activities is due to the increase of H_2O_2 concentration from the surface of ZnO. The generated H_2O_2 molecules can penetrate the cell membrane and kill the bacteria. Generally, a smaller particle size of metal oxide nanoparticles is correlated with a larger band gap and consequently unfavorable conditions for recombination of excitations. Consequently, more available excitations will lead to the generation of a higher concentration of reactive oxygen species (ROS) and subsequently to enhance antimicrobial activities.

Moreover, green synthesized ZnONps (capped with seed extract) are compared with Bare ZnO (uncapped seed extract) and leaf of *Silybum marianum* (L.) at concentration of 200µg/mL. The changes in the zones diameter (mm) among them are statistically significant for the five bacterial and two fungi strains that are revealed by using One-way ANOVA analysis as shown in the (Table 3). Interestingly, the sensitivity to antimicrobial substances may be different among strains even of the same species. The control does not produce any zone of inhibition. Result shows that green synthesized ZnONps (capped seed extract) have enhanced antimicrobial activity than Bare ZnO (uncapped seed extract) and seed of *Silybum marianum* L).

CONCLUSION

The phytochemical analysis of milk thistle seed extract shows that it is rich in phytochemicals such as alkaloids, phenols, terpenoids, flavonoids, tannins, etc. in three different extracts such as water, ethanol and chloroform. The antioxidant activity of *Silybum marianum* seeds shows that the high antioxidant capacity in the ethanolic extract is higher than the other two extracts. Water-soluble phenolic acid and flavonoid compounds are believed to play an important role in the bioreduction reaction confirmed by FT-IR spectrum. The UV-Vis spectrophotometer shows that the spectrum shows a characteristic ZnO absorption peak at 370 nm, which is due to the intrinsic absorption of the ZnO band gap due to electronic transitions from the valence band to the conduction band.

ZnONps increased with increasing concentrations (50, 100 and 200 µg/ml) due to the increasing H2O2 concentration on the ZnO surface. Furthermore, the green synthesized ZnONps are more potent than the mere seeds of ZnO and *Silybum marianum* seed extract mediated ZnONPs have become an indispensable research tool in modern times with a wide range of applications in almost all fields. However, the most studied applications are in the medical sciences; Current research involves the use of nanoparticles derived from different parts of *Silybum marianum* (L.) gart. (Milk thistle) as an antimicrobial and antioxidant agent. ZnONps were chosen for the present study because of their biocompatible nature and efficient synthesis protocols. All NPs synthesized in this study showed potent biological activity. ZnONps proved to be the most effective antimicrobial agent against the species tested. It has also been found that *Silybum marianum* seed extract mediated ZnO NPs possesses the most potent antioxidant properties. These results indicated that green ZnONps biosynthesized with *S. marianum* seed extracts could be suitable candidates for various future applications in biomedical research.

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