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Biochemical and antimicrobial studies of biosynthesised silver nanoparticles using aqueous extract of *Myrtus communis L.*

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ABSTRACT

Aqueous extract of Myrtus communis L.was mixed with 2 mM $AgNO_3$ solution and heated at 70 °C for 3 min. The fresh suspension of aqueous M. communis was reddish brown in colour, after addition of 2mM $AgNO_3$ and heated at 70 °C for 3 min, the suspension turned t o dark green. The UV-Vis spectral analysis showed silver surface Plasmon resonance band at 370 nm, while High Resolution-TEM investigation reveal that silver nanoparticles of size 100nm was obtained; FTIR was indicated on the active functional groups carboxylic group at 2930 cm^{-1}, metal carboxylic acid and esters groups at1718 cm^{-1}, primary amines at 1623 cm^{-1}, NO_2 group at1380 cm^{-1} and carboxylic group at 1224 cm^{-1}. Also, Total phenolic content of aqueous extract was 318µg/mL, which is more than its sliver nanoparticles solution which was 307 µg/ mL In this work, antimicrobial activity of silver nanoparticles synthesized from aqueous extract of Myrtus communis was investigated on standard bacteria, yeast and clinical MRSA isolate by well diffusion method. Synthesized silver nanoparticles showed good antimicrobial activity against used strains and isolates. Serial dilutions (0.025-50 mg/ml) were made from AgNp solutions; result showed that the MIC of AgNp solutions was 0.0175mg/ml. S.aureus cells were grown in incubator shaker at 37 °C for 24 h. in the presence of 0.0175mg/ml of AgNp solutions and were investigated by TEM to appear vertically more oblong than untreated cells. Some of antioxidant parameters were investigated e.g.: DPPH radical scavenging activity, Ferric reducing power and Hydrogen peroxid e radical scavenging activity, the obtained results indicated that the silver nanoparticles has highest values of these assays. Cytotoxicity was done in vivo via hepatic, kidney enzymes and in vitro via MTT assay. All results were indicated that all tested solutions gave normal ranges, while by using MTT assay; the obtained result showed that Hep-2 cells proliferation was significantly inhibited by aqueous extract with an IC_{50} value of 166.9µg/ml while in case of AgNPs with an IC50value of 40.5µg/ml of the concentration.

Keywords: *Myrtus communis*, Silver Nanoparticles, Antimicrobial activity, Antioxidant activity, Cytotoxicity, Biochemical tests.

INTRODUCTION

Myrtus communis L. Or common Myrtle (Family ¾ Myrtaceae) is one of the important drugs being used in Unani System of Medicine since ancient Greece period. It is recognized as *Aas* and its berries are known by the name of *Habb-ul- Aas*. It is often grown for its attractive foliage, flowers and berries. Its berries, leaves as well as essential oil are frequently used for various ailments like gastric ulcer, diarrhea, dysentery, vomiting, rheumatism, hemorrhages, deep sinuses, leucorrhoea and cosmetic purposes like hair fall control. The leaves, berries and twigs

are used in flavoring of food and wines. In past times, ripe fruits were used as food integrators because of their high vitamin contents.

The antimicrobial activity of the crude preparation of Myrtle on *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, P. vulgaris, P. mirabilis, Klebsiella aerogenes, Salmonella typhi* and *S. shigiella* was determined by Alem *et al* and preliminary study supported its traditional claim of effective anti-infective [1].

A wide range of biologically active compounds such as tannins, flavonoids, coumarins, essential oil, fixed oil, fibres, sugars, citric acid, malic acid and antioxidants are present in the plant. This contribution provides a comprehensive review of its ethno-medical uses, chemical constituents and pharmacological profile as a medicinal plant [2].

The antioxidant activities of the fruit extracts were determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene-linoleic acid assays. The methanol extracts of fruits exhibited a high level of free radical scavenging activity [3]. In another study antioxidant activity of methanol, ethanol, water and ethyl acetate extracts of the leaves and berries were measured. Antioxidant activity was assessed by measuring the ability of the extracts to scavenge the 2, 20-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTSþ) radical. All of the extracts showed significant antioxidant capacity and higher being in leaves[4].Flavonoids and anthocyanins in berries extract were checked for antioxidant activity by TEAC assay and the free radical activity. The myrtle extract showed interesting free radical scavenging activity [5].

Nanosized silver particles have found tremendous applications in the field of high sensitivity biomolecular detection and diagnostics, antimicrobials, antioxidants and therapeutics, catalysis [6] and micro-electronics. Nanoparticles can be synthesized by various approaches like chemical and photochemical reactions in reverse micelles, microwave assisted, thermal decomposition, electrochemical, sonochemical process [7] and also by biological methods. Plant mediated biological synthesis of nanoparticles is gaining importance due to its simplicity, cost effective and ecofriendliness [8] [9].

In this investigation, green synthesis of silver nanoparticles were carried out using aqueous leaf extract of *Myrtus communis L.* and characterized by using UV-visible spectra, Fourier transform infrared spectra, High resolution transmission electron microscope, determination of total phenolic content for both of plant and its silver nanoparticles and evaluated its in-vitro antioxidant properties . The antibacterial activities were investigated against standard Gram negative and Gram positive bacteria, fungi and clinical MRSA. MIC of AgNp solutions was determined. Results showed *that M.communis L.* AgNPs were effective antibacterial agent. To our knowledge green synthesis of silver nanoparticles by this plant leaf extract has not been reported so far.

MATERIALS AND METHODS

Materials:
Rats were kindly obtained from animal house at NODCAR
DPPH radical
Ferric chloride (0.1%)
Folin-Ciocalteau reagent
Gallic acid
Hydrogen peroxide solution (1 mM/L)
Liver and kidney colorimetric enzymatic kits (Diamond Company, Egypt)
sMTT dye (0.5 mg/ml) (Sigma- Aldrich-USA)
Mueller-Hinton agar (MHA)
Nutrient agar
Phosphate buffer (0.2 M, pH 6.6)
Potassium ferricyanide (1%)
Sabouraud Dextrose Agar (SDA) were purchased from Oxoid
Silver nitrate ($AgNO_3$: 99.98%Sigma,Egypt)
Sodium EDTA salt was purchased from Sigma Chemical Co.
Syringe filter 0.22mm (Jet Biofil)
Tri-chloroacetic acid (10%)

Dried plant leaves were obtained from Orman garden Herbarium-Giza-Egypt

Eleven Standard microorganisms and MRSA clinical isolates were kindly supplied from Department of Microbiology in NODCAR
Water Bath (Memmert)
Wattman No.1 filter paper

METHODS

1.Plant preparation:

Dried *M. communis* leaves, Figure 1, were washed with deionized distilled water, Re-dried at 40-45ºC**.**

Figure 1: *Myrtus communis L.* Family: *Myrtaceae*

2.Plant Aqueous extracts preparation

According to Mukunthan *et al.* [10]with modification, 10.0 grams of leaves powder was mixed with 100 ml of sterile distilled water, boiled by using water bath for 10 min, filtered through Wattman no.1 filter paper and then through syringe filter 0.22mm. The filtrate was kept in refrigerator at 4°C for further studies.

3.Total phenolic content

Total phenolic content of aqueous solution of *M. communis* leaves was determined [11]. 10 mg of aqueous plant extract was dissolved in methanol to get the appropriate concentration (1 mg/mL). 1mL of aqueous plant extract in a test tube was mixed with 5mL of distilled water. 1mL of Folin-Ciocalteau reagent was added and mixed thoroughly 3min. Then 3mL of saturated sodium carbonate solution was added and the mixture was allowed to stand for 90 min. in dark. The absorbance of the color developed was read at 725 nm using UV–Vis spectrophotometer. The concentration of total phenolic content in the extracts was determined as µg of gallic acid equivalent (GAE) by calibration curve, Figure 2, (r2=0.989). Three replicates were performed for each sample concentration to check the reproducibility of the experimental result and to get a more accurate result. The results were represented as mean ± standard.

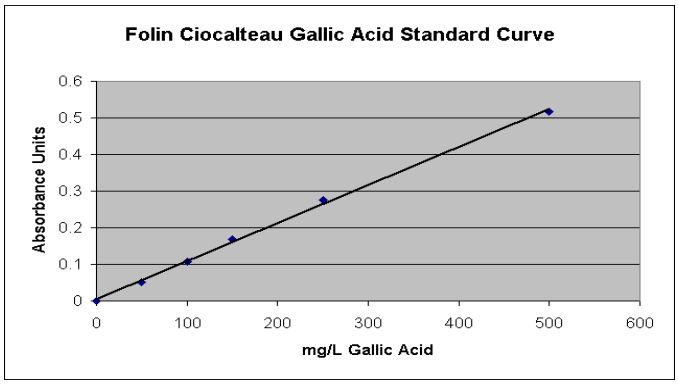


Figure 2: Standard curve of Gallic acid

4.Synthesis of silver nanoparticles using aqueous plant extract:

About 90ml 2mM of $AgNO_3$ solution was mixed with 10 ml of aqueous extract at70 °C, with stirring at 1000 rpm for 3min according to Sulaiman *et al.* [12].

5.Characterization of silver nanoparticles

5.1.UV-VIS spectra analysis

The reduction of pure silver ions (Ag+ ions) of $AgNo_3$ was monitored, after diluting a small aliquot of the sample with distilled water (1:9), then measuring its UV-VIS spectrum after 3 hours of incubation in dark place according to Showmya *et al.* [13].

5.2.FTIR Analysis:

The sediment layers were characterized by FTIR (Model: Spectrum RXI-Micro analytical center-Cairo University). The FTIR spectrum was obtained in the mid IR region of 400-4000 cm^{-1}.The spectrum was recorded using ATR (Attenuated Reflectance Technique). The dried sample was directly placed on the potassium bromide crystals and the spectrum was recorded in transmittance mode [13]**.**

5.3.High resolution Transmission electronic microscopy:

Sample was prepared by drop-coating silver nanoparticles solution onto carbon-coated copper TEM grids. The films on the TEM grids were allowed to stand for 2 min, extra solution was removed using a blotting paper and the grid allowed drying prior to measurement. TEM measurements were performed on a JEOL Model 1200EX instrument operated at an accelerating voltage at 120 kV at National center for researches according to Khadri *et al.* [14].

5.4.Measuring concentration of prepared silver nanoparticles solutions:

The concentration of prepared silver nanoparticles solution of aqueous *M. communis* extracts was measured using TDS (Total Dissolved Salts) device [15], the concentration was recorded as ppm (part per million), each 1 ppm equals to 1 mg/L.

6.Evaluation of antimicrobial activity

The antimicrobial activity of Silver nitrate, aqueous extracts of *M. communis* and silver nanoparticles solutions was determined by well diffusion method according to Irshad *et al.* [16] against 11 Standard microorganisms , where 50 µl of each tested solutions were poured in each well . All microorganisms' inoculum size was adjusted so as to deliver a final inoculum of approximately 10^8 CFU/ml.

M. smegmatis (ATCC 14468),
S. aureus (ATCC 29737),
S. epidermis (ATCC 12228),
M. luteus (ATCC 10240),
B. subtilis (ATCC 23857),
B. pumlius (ATCC 27142),
Bordetella bronchiseptica (ATCC 10580),
E-Coli (ATCC 25922*)*,
P. argeunoasa (ATCC 27853),
MRSA (Clinical isolate)
C.albicans (ATCC10231),
Ampicillin disc (AMP) 10µg used as a positive control in each plate.

7.Determination of Minimum Inhibitory Concentrations (MICs) of silver nanoparticles solution of aqueous *M. communis* extracts

The MIC was determined by the agar dilution method in Mueller Hinton agar medium according to [17].To 19 ml of agar medium, each dilution were added swirled carefully, then poured in Petri dishes and then leave to solidify. Subsequently, 2µl of each bacterial strain (10^4 CFU/ml) were inoculated on the Mueller Hinton agar surface. MIC was defined as the lowest antibiotic concentration, showing no visible bacterial growth after incubation time (37 ◦C for 24h).

8.Transmission electron microscope to compare between control and treated cells

TEM observations were carried out according to [18], using JEOL (jem 1230 EM) Transmission Electron microscopy, in National Research Center, by using *S.aureus* isolate as a model for testing antibacterial action of silver nanoparticles synthesized from *Myrtus communis L.* aqueous extract. *S.aureus* incula were prepared from 12 h age cultures and standardized to10^8 CFU/ml. One milliliter of 10^8 CFU/ml of our MRSA isolate suspensions was incubated with 1 ml of synthesized silver nanoparticles solution for 24h at 37°C. After incubation, the cells were

centrifuged at 6000 rpm for 15 min and washed twice with 0.01 M potassium phosphate buffer (pH 7.0). The samples obtained in the form of pellets after centrifugation at 6000 rpm were fixed with 2% glutaraldehyde for 2 h at 4º C. The pellets thus obtained were dehydrated in a gradient ethanol (10-100%). Up to 40% ethanol centrifugation carried out after which the cells were transferred onto the slide and treated up to 100% ethanol followed by drying the slides in desiccators.

9.Antioxidant activity assay:

9.1.DPPH radical scavenging activity:

Various concentrations of plant extract and its synthesized Ag-NPs solutions separately were mixed with 3.0 ml of methanol solution containing DPPH radical (6×10^{-5} mol/L). The mixture was shaken vigorously and left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by recording the absorbance at 517 nm using UV–Vis spectrophotometer. DPPH radical-scavenging activity was calculated by the following equation:

DPPH radical - scavenging activity (%) = ((A_{DPPH} - A_s) /A_{DPPH}) x 100

Where A_{DPPH} is the absorbance without samples and A_s the absorbance in the presence of the samples. A lower absorbance of the reaction mixture indicated a higher DPPH radical-scavenging activity [19] [20].

The DPPH radical scavenging activity in the extracts was determined as % of Ascorbic acid equivalent by calibration curve, Figure 3, (R^2=0.98).

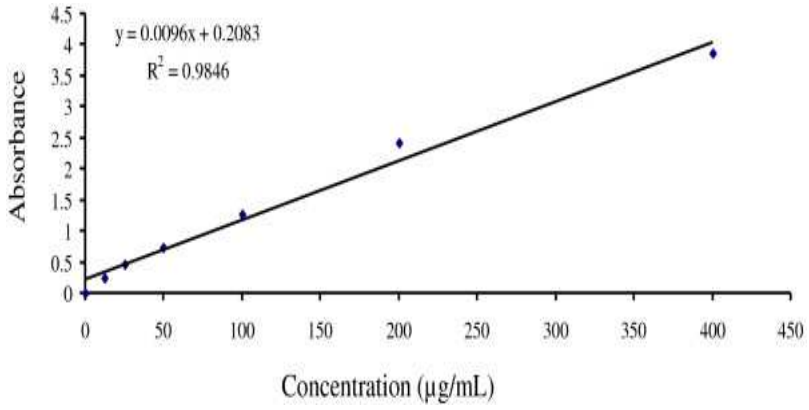


Figure 3 : Ascorbic calibration curve

9.2.Ferric reducing power:

Exactly 1 ml of the plant extract and its synthesized Ag-NPs was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50°C for 30 min. afterwards; 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min.

Finally, 2.5 ml of the upper layer was pipetted out and mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (0.1%) was added. The absorbance was measured at 700 nm using a Perkin Elmer Lambda 35 UV-Visible Spectrophotometer. The intensity of reducing power is directly proportional to the absorbance of the reaction mixture [21].

9.3.Hydrogen peroxide radical scavenging activity

The ability of the extracts to scavenge hydrogen peroxide was determined according to Cetinkaya and Kersten [22]. Hydrogen peroxide solution (1 mM/l) was prepared with 50 mM phosphate buffer (pH 7.4). Different concentrations (20–100 µg) of the extracts (1 ml) were allowed to react with 0.6 ml of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. Hydrogen peroxide scavenging activity was calculated according to the following equation:

Hydrogen peroxide scavenging activity (%) = ((A_c -A_s/ A_c) X 100

Where A_c is the absorbance without samples and A_s is the absorbance in the presence of the samples. The concentration of hydrogen peroxide in the assay medium was determined using a standard curve, Figure 4,(R^2: 0.999).

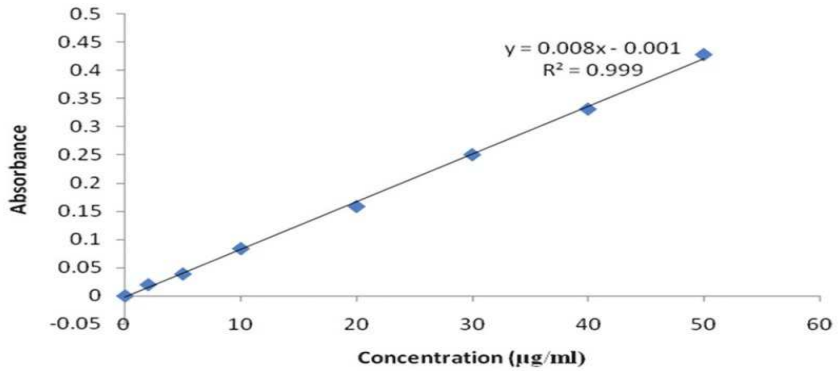


Figure 4 : Hydrogen peroxide Standard curve

10-Evaluation of *in vitro* cytotoxic activity of plant extract and its synthesized silver nanoparticles (MTT assay):

Hepg-2 cells precultured 96-well plates (Nunc-USA) were treated with descending double fold serially diluted each of aqueous *M. communis* extract and its synthezied Ag-NPs at 37°C for the required time interval. Negative cell control was included. Residual living cells were treated with 25 µl of MTT dye (0.5 mg/ml) (Sigma- Aldrich-USA) at 37°C for 4 h. MTT was discarded. Plates were PBS washed three times. Dimethyl sulfoxide (BDH-England) was added as 50 µl/well. Plates were shaked for 30 min to dissolve the produced intracellular blue MTT-Formazan complex. Optical densities (O.Ds) were measured at 570 nm using an ELISA plate reader (Dynatech- England). Data were reported for three independent experiments as mean ± SD [23].

Viability percentage was calculated as follows according to Chen *et al.* [24]:

Cell viability%= (O.D of treated cells / O.D of untreated cells) X100

Statistical significance between treated and untreated cells was determined using one way ANOVA. Differences at P values less than 0.05 were considered significant.

IC50 of test plant extract and its synthesized Ag-NPs was determined using prism program.

11.Evaluation of *in vivo* cytotoxic activity of plant extract and its synthesized silver nanoparticles via Studying of Hepatic and kidney enzymes:

11.1.Animals and diets

Twenty female albino rats (weight of 95- 101 g) were acclimated for one week prior to the experiment. They were housed in seven groups (five per each) in universal polypropylene cages at room temperature (25±2◦C), till the end of the experiment (9 weeks) [25].

11.2.Experimental design:

For each group as in Table 1, 1 ml of each Treatment was used as a single dose except for control group, up to the end of experiment.

Table 1: Group of albino rats receiving different treatments

Group(s)	Treatments	Concentration	Intra-peritoneal dose
I	Not treated(Control)	--------	----------
II	$AgNO_3$	13.3 mg/ml	1ml
III	Aqueous *M. communis*	100mg/ml	1ml
IV	AgNP(Aqueous)	0.35mg/ml	1ml

Body weight (BW) was observed weekly during the experimental period (9 weeks). At the end of the experimental period, animals were fasted overnight, blood samples were withdrawn by a fine capillary glass tubes from the orbital plexus vein. The blood was collected in EDTA containing tubes and left at room temperature. Plasma was obtained by centrifugation at 3000 rpm for 15min and then stored at -20◦C until analysis [25].

11.3.Biochemical procedures Using enzymatic colorimetric procedures Kits
11.3.1.Measuring Hepatic enzymatic activity [26]
-Aspartate aminotransferase (AST; E.C.2.6.1.1.)
-Alanine aminotransferase (ALT; E.C.2.6.1.2.)
-Total bilirubin

11.3.2.Measuring Kidney enzymatic activity [27] [28] [29]
-Creatinine
-Urea

12.Statistical analysis
The grouped data were statistically evaluated using one sample test with SPSS/20 software.

RESULTS AND DISCUSSION

1.Total phenol content:
Total phenols present in aqueous extract of *M.communis* and its sliver nanoparticles was determined from the regression equation (y = ax + b) of calibration curve of Gallic acid standard solution and expressed in gallic acid equivalents (Figure 2).

Total phenolic content of aqueous extract was 318µg/mL, which is more than its sliver nanoparticles solution which was 307 µg/ mL.

Phenolics are secondary plant metabolites that are present in every plant and plant product. Many of the phenolics have been shown to contain high levels of antioxidant activities. Phenolic compounds present in the plants acting as antioxidant or free radicals scavengers [30] due to their hydroxyl groups which contribute directly to the antioxidant action. Phenolic compounds are effective hydrogen donors, making them good antioxidants [31].

2.Synthesis of silver nanoparticles
Green synthesized silver nanoparticles aqueous product is shown in Figure 5. The fresh suspension of aqueous *M. communis* was reddish brown in colour, after addition of 2mM $AgNO_3$ and heated at 70 °C for 3 min, the suspension turned to dark green.

Figure 5: Showing colour changing. From left to right: 2mM $AgNO_3$, *M. communis* Aqueous extract, Green synthesityzied silver nanoparticles aqueous product

3.Characterization of silver nanoparticles

3.1.UV-VIS spectra analysis

Formation of silver nanoparticles was confirmed using UV-VIS spectral analysis that showed silver surface plasmon resonance band at 370 nm, Figure 6.

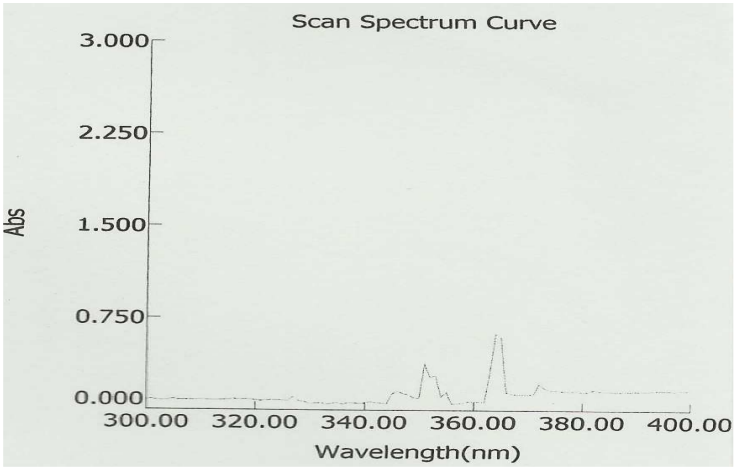


Figure 6: UV/Vis absorption spectra of reduction of silver ions to silver nanoparticles after heating at 70 °C for 3 min

3.2.FTIR Analysis:

FTIR studies were carried out and results of nanoparticles spectrum are showed in Figure 7 and Table 2, where 1718cm^{-1} band refers to presence of metal carboxylic acid and esters groups which support high antimicrobial efficacy of silver nanoparticles. While 1623cm^{-1} band refers to primary amines, the absorption peak at around 1046 cm^{-1} can be assigned as absorption peaks of -C-O-C- or—C-O-. The peak at around 1623 cm^{-1} is assigned to the amide I bonds of proteins. The bonds or functional groups such as -C-O-C-,—C-O- and -C=C- derived from heterocyclic compounds and the amide I bond derived from the proteins which are present in the extract are the capping ligands of the nanoparticles**.** [32],while peaks 1380cm^{-1} indicate on presence of NO_2 group which indicates on the composition of NO_3 to NO_2 group [33]**.**

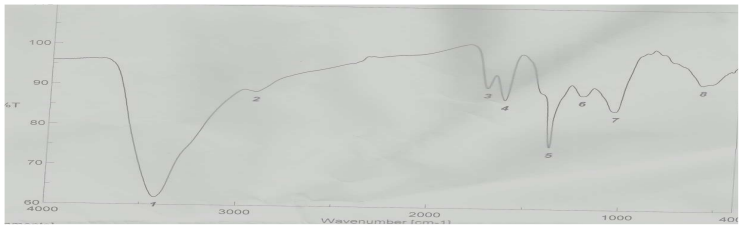


Figure 7: FTIR of aqueous *M. comminus* Nanopaticles synthesized by $AgNO_3$

Table 2: Wavelengths observed in FTIR of aqueous *M. comminus* Nanopaticles synthesized by $AgNO_3$ functional groups

Wavelength(cm^{-1})	Functional Group
2930	carboxylic group
1718	metal carboxylic acid and esters groups
1623	primary amines
1380	NO_2 group
1224	carboxylic group
1046	carboxylic group

3.3.HRTEM:

HRTEM, Figure 8, gives a clear vision of shape and size of the nanoparticles, the silver nanoparticles formed were predominantly of mono- spheres nature with diameter approximately 100 nm.

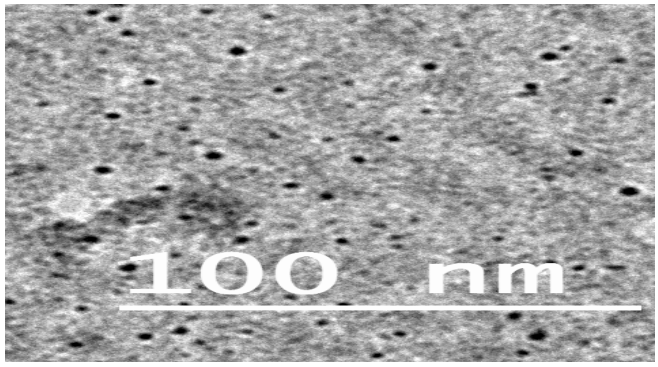


Figure 8: TEM micrographs of AgNP of aqueous *M. communis* extract

3.4.Total Dissolved Salts (TDS):

Concentrations of prepared solutions were measured using TDS = 350ppm(0.35mg/ml) [15] shown in Figure 9:

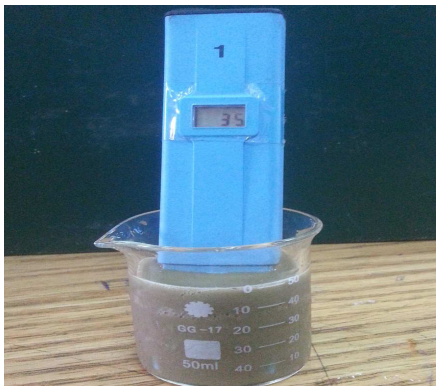


Figure 9: Measuring Concentrations of prepared nanoparticles solutions

4.Evaluation of antimicrobial activity:

In this study, silver nanoparticles solution antimicrobial activity was investigated and demonstrated via the zone of inhibition as in Table 3 and Figure 10. Aqueous product and 2 mM Silver nanoparticles, both of *M. leutus* and *S. epidermis* gave the highest inhibitory zone at 33 mm. It was noticed that silver nanoparticles solution produced from *M. communis* also exhibited potent antifungal activity against *C. albicans* which gave 21.6mm as zone of inhibition.

Thus, silver nanoparticles could be considered as excellent broad-spectrum antibacterial agents. Since the biosynthesized silver nanoparticles showed considerable antifungal activity, they could be encourged to be widely used in clinical applications. This observation is in agreement with earlier studies [34] [35] [36] [37].

The mechanism of inhibitory action of silver nanoparticles on microorganisms, is not very clearly understood, it could be by their adhesion to the cell membrane and further penetration inside or by interaction with phosphorus containing compounds like DNA disturbing the replication process or preferably by their attack on the respiratory chain. It has also been suggested that a strong reaction takes place between the silver ions and –SH (thiol) groups of vital enzymes, such as NADH dehydrogenase II in the respiratory system, which is implicated as a candidate for the site of production of reactive oxygen species in vivo [38].

Therefore, inhibition of this enzyme results in an increase in the free radical production. The increase in catalase production in the presence of ROS (Reactive Oxygen Species) could be explained by the necessity for cells to reduce the concentration of H_2O_2, which is the source of the free radicals. It is proposed that reactive oxygen species can induce apoptotic pathways in bacteria which could ultimately lead to their death [38] [39] silver nanoparticles showed excellent antimicrobial activity.

Table 3: Antibacterial and Antifungal activity of Aqueous *M.communis*, $AgNo_3$ and AgNp solutions

Test organisms	Aqueous *M.communis*		
	$AgNO_3$	Extract	AgNP
M. smegmatis (1)	16.66± 0.577	23.67± 1.154	26.67± 0.577
S. aureus(2)	16.33± 1.154	28.67± 1.527	32.67± 0.577
S. epidermis(3)	14.67± 0.577	30.00± 000	33.33± 1.528
M. luteus(4)	21.33± 1.1547	29.33± 1.155	33.00± 1.732
B. subtilis(5)	11.00± 1.000	18.67± 1.155	23.33± 1.155
B.pumlius(6)	12.67± 0.577	21.33± 1.155	23.67± 1.155
Bordetella(7)	17.33± 0.577	23.67± 3.055	29.67± 0.577
E-Coli(8)	16.33± 0.577	18.33± 1.527	20.00± 1.000
P. argeunoasa(9)	16.67± 0.577	21.67± 2.081	29.00± 1.732
MRSA(10)	21.00± 1.000	19.33± 0.577	23.67± 0.577
C.albicans(11)	14.00± 1.000	13.67± 0.577	21.67± 0.577

*Ag^+: silver ions **Extract: Aqueous ***AgNP: silver nanopatricles ****

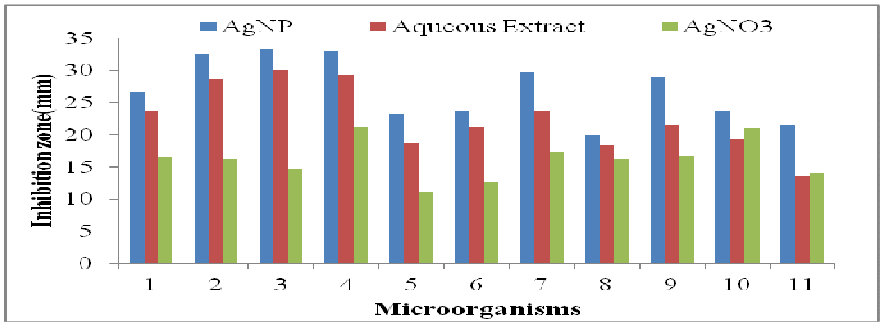


Figure 10: Antibacterial and Antifungal activity of Aqueous *M.communis*, $AgNo_3$ and AgNp solution

5.Minimum Inhibitory Concentration (MIC):

Serial dilutions (0.025-50 mg/ml) were made from AgNp solutions; results recorded in showed that the MIC of AgNp solutions was 0.0175mg/ml,
Table **4**.

Table 4 : Minimum Inhibitory Concentration

Con.(mg/ml)	0.035	0.0175	0.008	0.004	0.002	0.001
G/NG	NG	NG	G	G	G	G

*G, Growth, *NG, No Growth

6.Effect of AgNp solutions on morphogenesis of *S.aureus* Cells

The morphology of *S.aureus* treated cells was examined using Transmission Electron Microscope (TEM). *S.aureus* cells were grown in incubator shaker at 37 °C for 24 h. in the presence of 0.0175mg/ml of AgNp solutions. It was found that the untreated (control) of *S.aureus* cells, **Figure 11**, typically had a structured nucleus and a cytoplasm with several elements of endomembrane system enveloped by a regular, intact cell wall plasma membrane lying closely to the cell wall. While after being exposed to AgNp solutions, cells appeared vertically more oblonged, Figure 12,and after being exposed to plant extract, there is an obvious shrinking in cell size with deformation in the cell wall, Figure 13.

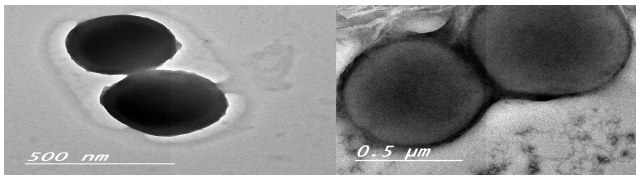


Figure 11: TEM of *S.aureus* control cells

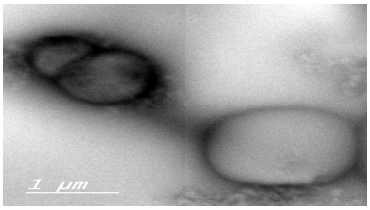


Figure 12: TEM of *S.aureus* after treatment with AgNp solutions

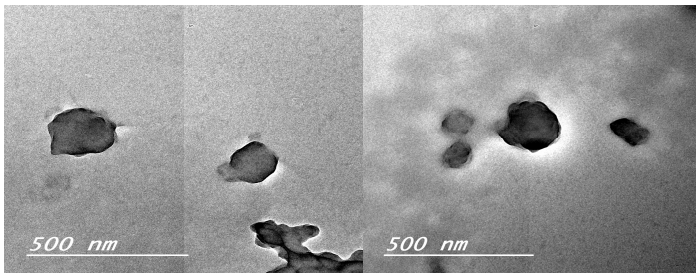


Figure 13: TEM of *S.aureus* after treatment with plant extract

7.Antioxidant activity assay:

7.1.DPPH radical scavenging activity

Plants contain specific metabolites that are acknowledged to perform a range of purposeful activities. It is well known and also reported in literature that plant mediated nanoparticles synthesis involves sequential reduction followed by capping with these constituents of plants [40]. However, interactions of these components with metals are less well studied and kind of activity they may confer to nanoparticles hasn't been reported so far. We are reporting for the first time antioxidant activity of silver nanoparticles capped with plant constituents possessing free radical scavenging activity. Antioxidant activity of AgNPs is shown in terms scavenging capacity and % anti-oxidant activity in Table 5.

Capped silver nanoparticles were found to be potent free radical scavenger.. An antioxidant works in stopping the oxidation by neutralizing the free radicals produced. In order to neutralize the free radicals, the antioxidant itself undergoes oxidation. The activity and stability of the silver nanoparticles are affected during anti- oxidation process and also they are oxidized in the presence of air. This antioxidant activity may be due to the capping constituents present in plant extract and present on metal surface [41].

7.2.Hydrogen peroxide radical scavenging activity

The hydrogen peroxide radical-scavenging activity of aqueous extract of M. communis and its AgNPs was estimated by comparing the percentage inhibition of formation of peroxyl radicals with that of vitamin C. Hydrogen peroxide scavenging activity of aqueous extract and its AgNPs are presented in Table 5. Both aqueous extract and its AgNPs showed moderate inhibition against peroxyl radical which was less in comparison with vitamin C. These results showed that AgNPs is more potent than aqueous extract in neutralizing hydrogen peroxide radicals. Most of the hydrogen peroxide was scavenged by the extracts. Sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells.

7.3. Ferric reducing power:

In the reducing power assay, the presence of antioxidants in the extract of *M. communis* and its AgNPs would result in the reduction of Fe3+/ferricyanide complex to its form. The reducing power of compound may serve as a significant indicator of its potential antioxidant activity (Meir et al. 1995). The ferric reducing power of extract of *M. communis* and its AgNPs was determined by comparing with that of vitamin C. Absorbance values of *M. communis* extract and its AgNPs are presented in Table 5.

The reducing power of ascorbic acid was found to be significantly higher than those of *M. communis* extract and its AgNPs. The reducing power increased with increasing the phenolic content of the extract. This data imply that *M. communis* extract and its AgNPs have significant ability to react with free radicals to convert them into more stable nonreactive species and to terminate radical chain reaction.

Table 5 : Measuring Antioxidant activity

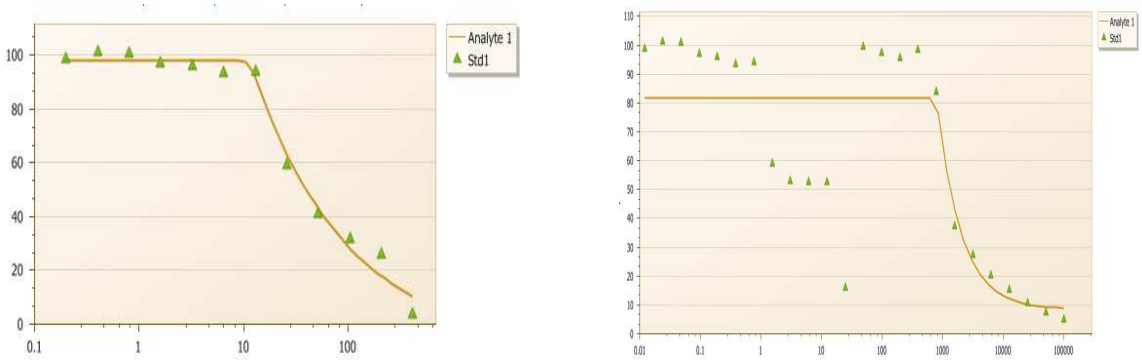
Name of Measured Assay	Aqueous *M .communis* extract	Sliver Nanoparticles of *M. communis*
DPPH (%)	58±0.252	75±0.234
Hydrogen peroxide Scavenging activity (%)	68±0.097	87±0.005
Ferric reducing power (OD)	0.0947±0.008	0.0489±0.001

8.Measurements of in Vitro cell toxicity via Studying of Hepatic and kidney enzymes

The *in vitro* cytotoxicity of the aqueous extract of *M. communis* and its silver nanoparticles was evaluated against normal human cell line at different concentrations. The samples demonstrated a considerable cytotoxicity against the Hep-2 cell line. Results shown in

(a) **Silver IC50 40.5 µgm/ ml** **(b) plant extract IC50= 166.9 ugm / ml**

Figure 14 ,that Hep-2 cells proliferation was significantly inhibited by aqueous extract (a) with an IC_{50}value of 166.9µg/ml while in case of AgNPs (b) with an IC50value of 40.5µg/ml of the concentration. Cyclo-phosphamide is used as standard control. The % toxicity increases with increase in concentration of silver nano particles suggests that biosynthesized silver nanoparticles could be of immense use in medical field to certain extent as anticancer agent. From the results indicated it is seen that percentage viability decreases with concentration whereas cytotoxicity increases with concentration demonstrating a direct dose dependent relationship [42].



(a) Silver IC50 40.5 µgm/ ml **(b) plant extract IC50= 166.9 ugm / ml**

Figure 14: Cytotoxicity for (a) AgNPs (b) Plan

8.2. Measurements of *in Vivo* cell toxicity

All mentioned groups were intra-peritoneal injected (Dose =1 ml) of each mentioned solutions, Table 1, except the control group. Rats under experiments were all healthy and gained body weights expect Ahmad *et al.* [32] stated that the green synthesis of AgNPs fulfills all the three main steps, which must be evaluated based on green chemistry perspectives, including (1) selection of solvent medium, (2) selection of environmentally benign reducing agent, and (3) selection of nontoxic substances for the AgNPs stability. So, it is recommended to use silver nanoparticles of aqueous *M. communis* extract.

All groups under study were normal in their hepatic and kidney functions i.e., they had normal levels as shown in Table 6 and Figure 15. Park *et al.* [43]stated that toxicity of nanoparticles depends on many factors including size, shape, chemical composition, surface area, surface charge, and others and that comply with our results. Tang *et al.* [44] recorded that when rats were treated with AgNPs solutions by injection, AgNPs translocated to the blood circulation and distributed throughout the main organs, especially in the kidney, liver, spleen, brain and lung in the form of particles.

Carlson *et al.* [45] and Piao *et al.* [46] suggested that oxidative stress is responsible for the toxicity of Ag NPs , also Ag toxicity has often been associated with ion release and induction of oxidative stress [47]. Ag NPs have been shown to be highly toxic not only to bacteria and fungi, but also to a number of animal species and cultured cells [48] [47].

Table 6: Measurements of in Vivo cell toxicity via Studying of Hepatic and kidney enzymes

Name of Group	Name of test				
	Creatinine (mg/dl)	Urea (mg/dl)	Total bilurbin (mg/dl)	ALT (IU/l)	AST (IU/l)
Group(I) control	0.80± 0.006	26.67± 0.006	0.77± 0.006	10.00± 000	9.67± 0.577
Group(II) AgNO3	1.00± 000	33.33± 000	0.97± 000	11.67± 0.577	12.33±0.577
Group(III) Aqueous Extract	0.90± 0.01	38.67± 0.01	0.688± 0.01	9.33± 0.577	8.33± 0.577
Group(IV) AgNP(Aq.)	0.64± 0.006	38.66± 0.006	0.858± 0.006	8.33± 0.577	8.00± 000
Normal values	1.0	50	1.0	12	12

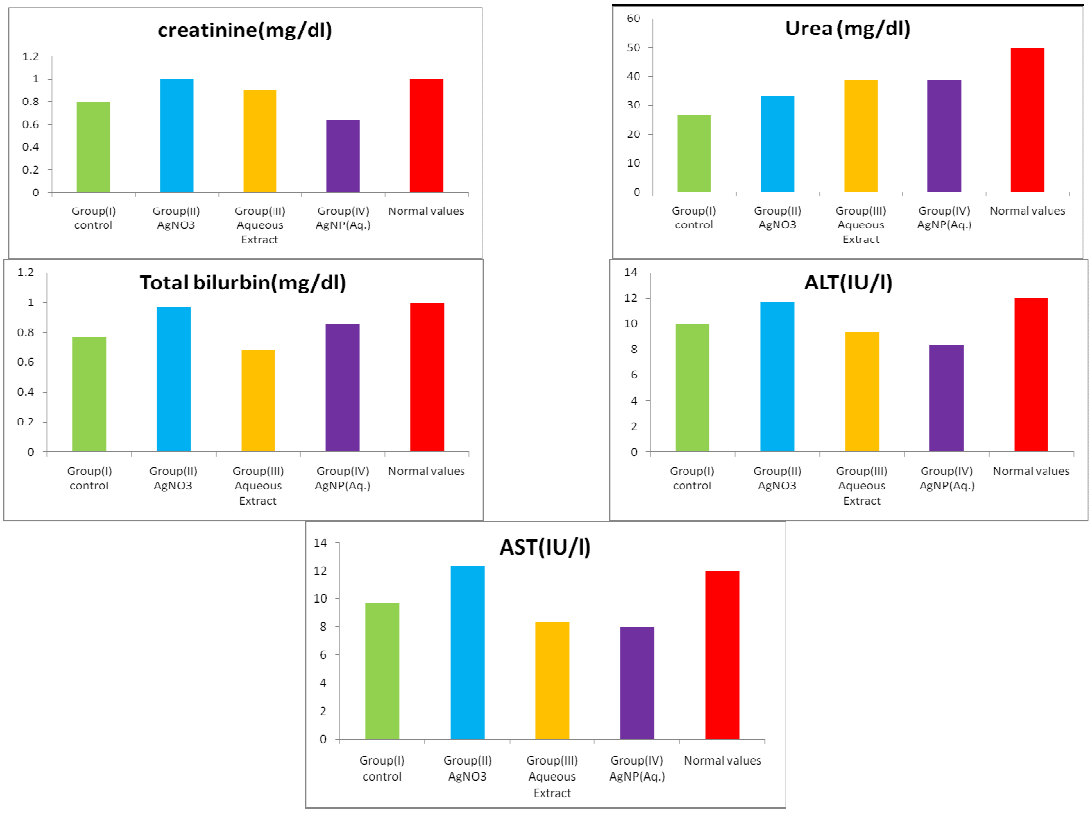


Figure 15:Blood biochemical parameters in albino femal rats by 1 ml dose after anti-protenia injuction of AgNPs (100 nm) after 9 weeks

CONCLUSION

In conclusion, The biosynthesized silver nanoparticles of aqueous *M. communis* extract showed excellent antimicrobial and antioxidant activities and the aqueous extract was recommended for body weight, hepatic and kidney functions and These obtained silver nanoparticles have potential applications in the biomedical field and this simple procedure has several advantages such as cost effective, compatibility for medical and pharmaceutical applications, and safe for biological systems and blood stream. The results of synthesized nanoparticle leads towards the clinical use as antimicrobial agent.

REFERENCES

[1]Alem, G. , *et al.Ethiop Med J*, **2008**. **46**(1): p. 63-69.
[2]Sumbul, S. , *et al.Hum Exp Toxicol*, **2010**. **29**(11): p. 935-44.
[3]Serce, S. , *et al.Pharmacogn Mag*, **2010**. **6**(21): p. 9-12.
[4]Amensour, M. , *et al.J Food*, **2010**. **8**(2): p. 95-101.

[5]Montoro, P. , *et al.J Pharm Biomed Anal*, **2006**. **41**(5): p. 1614-9.
[6]Tada, H. , *et al.Langmuir*, **2000**. **16**(7): p. 3304-3309.
[7]Maribel, G.G. , *et al.International Journal of Chemical and Biological Engineering*, **2009**. **2**(3): p. 104-111.
[8]Yogeswari, R. , *et al.Journal of Microbiology and Antimicrobials*, **2012**. **4**(6): p. 103-109.
[9]Farooqui, M.A. , *et al.Digest Journal of Nanomaterials and Biostructures*, **2010**. **5**: p. 43-49.
[10]Mukunthan, K.S. , *et al.Asian Pac J Trop Biomed*, **2011**. **1**(4): p. 270-4.
[11]Tshivhandekano, I. , *et al.Asian Pac J Trop Med.* , **1965**.
[12]Sulaiman, G.M. , *et al.Eng. & Tech. Journal*, **2013**. **31**(3).
[13]Showmya, J. , *et al.Plant Cell Biotechnology and Molecular Biology* **2012**. **13(1-2)**: p. 31-38.
[14]Khadri, H. , *et al.Advances in Nanoparticles, 2013, 2, 241-246*, **2013**. **2**: p. 241-246.
[15]Assar, N.H. and H.M. Hamuoda.*IJMR*, **2010**: p. 1-4.
[16]Irshad, S. , *et al.Research Journal of Biology*, **2012**. **2**(1): p. 1-8.
[17]*Clin Microbiol Infect*, **2000**. **6**(9): p. 509-15.
[18]Hafidh, R.R. , *et al.Open Microbiol J*, **2011**. **5**: p. 96-106.
[19]Gulcin, I. , *et al.J Pineal Res*, **2003**. **34**(4): p. 278-281.
[20]Gulcin, I.*J Med Food*, **2011**. **14**(9): p. 975-85.
[21]Yildirim, A. , *et al.J Agric Food Chem*, **2001**. **49**(8): p. 4083-9.
[22]Cetinkaya, A. and R.C. Kersten.*Ophthalmology*, **2012**. **119**(2): p. 376-81.
[23]Berridge, M.V. , *et al.Biotechnol Annu Rev*, **2005**. **11**: p. 127-52.
[24]Chen, X. , *et al.J Biol Chem*, **2010**. **285**(10): p. 7197-207.
[25]Moussa, A.M. , *et al.International Food Research Journal* **2011**. **18**: p. 535-542.
[26]Reitman, S. and S. Frankel.*Am J Clin Pathol*, **1957**. **28**(1): p. 56-63.
[27]Gornall, A.G. , *et al.J Biol Chem*, **1949**. **177**(2): p. 751-66.
[28]Doumas, B.T. , *et al.Clin Chim Acta*, **1971**. **31**(1): p. 87-96.
[29]Fawcett, J.K. and J.E. Soctt.*Journal of Clinical Pathology* **1960**. **13**: p. 156-162.
[30]Kouri, T.T. , *et al.Am J Clin Pathol*, **1999**. **112**(1): p. 25-35.
[31]Rice-Evans, C. and N. Miller.*Prostaglandins Leukot Essent Fatty Acids*, **1997**. **57**(4-5): p. 499-505.
[32]Ahmad, N. , *et al.Biotechnol Res Int*, **2011**. **2011**: p. 454090.
[33]Augustine, R. and K. Rajarathinam.*Int.J.Nano Dim.* , **2012**. **2**(3): p. 205-212.
[34]Dipankar, C. and S. Murugan.*Colloids Surf B Biointerfaces*, **2012**. **98**: p. 112-9.
[35]Sulaiman, G.M. , *et al.Asian Pac J Trop Biomed*, **2013**. **3**(1): p. 58-63.
[36]Suriya, J. , *et al.African Journal of Biotechnology*, **2012**. **11**(58): p. 12192-12198.
[37]Li, G. , *et al.Int J Mol Sci*, **2012**. **13**(1): p. 466-76.
[38]Matsumura, Y. , *et al.Appl Environ Microbiol*, **2003**. **69**(7): p. 4278-81.
[39]Kumar Pandian, S.R. , *et al.Braz J Microbiol*, **2010**. **41**(3): p. 805-9.
[40]Khalil, M.I. , *et al.Food Chem Toxicol*, **2010**. **48**(8-9): p. 2388-92.
[41]Mittal, A.K. , *et al.Nano Biomed Eng*, **2010**. **4**(3): p. 118-124.
[42]CAROLING, G. , *et al.Asian J Pharm Clin Res*, **2013**. **6**(4): p. 165-172.
[43]Park, E.J. , *et al.Environ Toxicol Pharmacol*, **2010**. **30**(2): p. 162-8.
[44]Tang, J. , *et al.J Nanosci Nanotechnol*, **2009**. **9**(8): p. 4924-32.
[45]Carlson, C. , *et al.J Phys Chem B*, **2008**. **112**(43): p. 13608-19.
[46]Piao, M.J. , *et al.Toxicol Lett*, **2011**. **201**(1): p. 92-100.
[47]Johnston, H.J. , *et al.Crit Rev Toxicol*, **2010**. **40**(4): p. 328-46.
[48]Gaiser, B.K. , *et al.Environ Toxicol Chem*, **2012**. **31**(1): p. 144-54.