Supporting Information

Synergic Effect of *Ocimum Sanctum* and *Trigonella Foenum-graecum L* Water Extract on *In-Situ* Green Synthesis of Silver Nanoparticles and as an Anti-agent for Antibiotic Resistant Food Spoiling Organism

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MATERIALS AND METHOD

Materials. *The Ocimum sanctum* and *Trigonella foenum-graecum L* leaf purchased from local market, silver nitrate ($AgNO_3$), was purchased from Sigma Aldrich, USA. RPMI 1640 and MTT were purchased from Merck, India. Methanol HPLC grade from Merck. Mueller-Hinton Agar (MHA) was obtained from HIMEDIA (India) and rutin were obtained from Sigma Co. (St. Louis, MO, USA). Bacterial strain of *pseudomonas fragi, pseudomonas fluorescens, and salmonella* were bought from MTCC, Chandigarh, India.

Preparation of the Extract. 5g of *Ocimum sanctum* and *Trigonella foenum-graecum L* leaf had been taken, respectively and washed thrice in distilled water for 20 min, dried, cut into very fine pieces and were put in 500 mL Erlenmeyer flask with 100 mL triple distilled water and then boiling the mixture at 75 ^{0}C for 30 min and finally filtered with 450 μm pore size filter paper.

Synthesis of Silver Nanoparticles. Silver nanoparticles were prepared in aqueous solution by reducing silver ions with a mixture of *Ocimum sanctum* and *Trigonella foenum-graecum L leaf* extract (1:1, v/v). The detailed synthetic procedure of silver nanoparticles,[1] with necessary modifications, is as follows. The particles were prepared in aqueous phase by biochemical reduction of silver nitrate solution of various concentrations (1, 2, 3, and 4 weight %) using a 1:1 v/v ratio of *Ocimum sanctum* and *Trigonella foenum-graecum L* extract. 50 mL double distilled water taken in 100 mL round bottom flask and 200 µL (1-4 weight %) of silver nitrate solution was added with constant stirring for 20 min in each separate flask. For each set of experiments, 400 µL solution of *Ocimum sanctum* and *Trigonella foenum-graecum L* leaf extract (1:1, v/v) were added to the stirred mixture at 25 ºC for 4h and it became brownish-yellow in color indicates the formation of silver nanoparticles. A similar procedure applied for the synthesis of Ag NPs in *Ocimum sanctum* and *Trigonella foenum-graecum L* leaf extract, respectively. Synthesized Ag NPs were purified by centrifuged method at 6000 rpm to remove biproducts for $AgNO_3$ reduction.

Synthesis of Citrate capped Silver Nanoparticles

In 50 mL $AgNO_3$ (0.01 M) aqueous solution, tri-sodium citrate was added in molar ratio of $AgNO_3$: tri-sodium citrate of 1:0.05 and stirring for 2h and then 0.2 ml of freshly prepared aqueous $NaBH_4$ (0.01 mM) solution was added for reduction. Synthesized Ag NPs were purified by centrifuged method at 6000 rpm to remove biproducts for $AgNO_3$ reduction.[2]

Characterization of Nanoparticles

UV-Vis Spectroscopy. UV-vis absorption spectra show the characteristic plasma band of the reaction mixture and NPs formations was monitored using UV-1601 Shimadzu UV-VIS spectrophotometer.

Dynamic light scattering (DLS). Dynamic light scattering is a technique in physics which can be used to determine the size distribution profile of small particles in suspension in solution. Light

scattering (Zetasizer. Ver. 6.01, Malvern) technique used to determine the size distribution profile of silver nanoparticle's reaction solution.

X-ray Diffraction Measurements. X-ray diffraction (XRD) analysis of green synthesized silver nanoparticles lyophilized powder was carried out on a Phillips PW1830 instrument operating at 40 kV and a current 30 mA with CuKa radiation.

Total Attenuated Reflectance (ATR)/FT-IR analysis. Agilent infrared spectroscopy (Carry 630) equipped with ZnSe cell was used to obtain spectra of plant extract with /without Ag NPs, untreated and treated bacteria to observe functional interaction on chemical constituents of respective system. [1]

Transmission Electron Microscopy (TEM). Field emission transmission electron microscope (FE-TEM) [JEOL, JEM-2100, 200 kV] was used for analysis of the green synthesized Ag NPs. Samples were prepared by placing small drops of dispersed silver nanoparticles (lyophilized powder) in water on carbon coated copper grids and allowing the solvent to slowly evaporate at room temperature. Samples were analyzed without staining.

MTT Assay. Macrophages were collected by peritoneal lavage from sodium thioglycolate-stimulated mice. The peritoneal cells were collected in RPMI-1640 medium (incomplete), centrifuged at 1400 x g for 10 min at 4 °C, washed twice and finally re-suspended in complete medium. Macrophages at a density of 1 x 10^6 cells mL^{-1} were seeded in 96-well tissue culture plates (0.1 mL per well) and exposed to various size of Ag NPs (10 nm, 20 nm, 30 nm and 40 nm spherical shape at a concentration of 1, 2, 5, 10, 15, 20, 50 and 30 µg/mL for 12 h in a CO_2 incubator (5% CO_2, 37° C) to calculate the LD_{50}. Cell viability was evaluated using a modified MTT assay, where the conversion of 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan by mitochondrial enzymes served as an indicator of cell viability and

amount of dye produced is directly proportional to the number of metabolically active cells. Accordingly, absorbance at 492 nm represented the number of live cells. LD_{50} value was calculated from the dose-response curve and used as measure for drug resistance and the mean percentage viability was calculated as follow [3]

$$\frac{\text{Mean specific absorbance of treated Macrophages}}{\text{Mean specific absorbance of untreated Macrophages.}} \text{ X } 100$$

Hemolysis Assay. 5 mL blood was collected from an anonymous human donor, drawn directly into K_2-EDTA-coated Vacutainer tubes to prevent coagulation and centrifuged at 3000 rpm for 10 mins and the supernatant was discarded. RBC were washed thrice with 1X PBS, PH 7.4 for 5 min. washed erythrocytes were finally suspended in 1X PBS (1:9 dilution). To study Hemolysis, 950 µl of the above suspension was mixed with 50 µL solution of same concentration (15 µg/mL) of various sizes of silver nanoparticles (10, 20, 30 and 40 nm, respectively to obtain RBC suspension) and also 1:9 diluted RBC suspension was mixed with 1 %Triton X-100 solution for complete lysis of the erytrocytes. RBC suspended in 1mL PBS was used as negative control to assess background lysis, if any. Each suspension was incubated at 37 ^{0}C for 15 min, 30 min, 60 min, 120 min and 180 min. After the predetermined time of incubation, the suspension was centrifuged and the supernatant was analyzed using spectrophotometer at 540 nm. The percentage of hemolysis was determined using the following equation:

$$\%\text{Hemolysis} = [(Abs_s - abs_o)/ (Abs_{100} - Abs_o)] \times 100$$

Where Abs_o is the absorption of the blank sample, Abs_s is the absorption of the sample and Abs_{100} is the absorption of the sample treated with tritonX-100.[4]

Antimicrobial Activity Assessment. The antibacterial activities of Ag nanoparticles were tested against bacterial strains of *pseudomonas fragi, pseudomonas fluorescens and salmonella.* Bacterial strains obtained from MTCC; Chandigarh were maintained in glycerol stock 15 % (vol/vol) at -80 ºC. The cultures were revived in Luria Bertani broth kept in a incubator cum shaker (200 rpm) at 37º C until the desired cell population was obtained. Antibacterial activity was determined on Mueller Hinton agar by disc diffusion method. In this method, bacterial cultures were diluted to 10^5 cells/mL and 0.1mL were plated on Mueller Hinton agar plates, then sterile discs were loaded with required dose of *Ocimum sanctum* and *Trigonella foenum-graecum L* leaf extract alone and in combination. Similar procedure was applied for antibacterial properties evaluation experiments for Ag nanoparticles synthesised in *Ocimum sanctum* and *Trigonella foenum-graecum L* leaf extract in water (1:1, v/v) for each bacterium. Also, similar procedure was repeated with 40 nm Ag nanoparticles synthesised by other method to see the synergic antibacterial effect of Ag NPs and *Ocimum sanctum* and *Trigonella foenum-graecum L* leaf extract in water. For control, other disc was supplemented with solvent (water) for control. The resulting zones of inhibition were measured in millimetres. [5]

Evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The MIC and MBC of 40 nm green synthesized Ag NPs solution in *Ocimum sanctum* and *Trigonella foenum-graecum L* leaf extract in water, without plant extract, and only *Ocimum sanctum* and *Trigonella foenum-graecum L* leaf extract in water were measured

using reduction process of resazurin dye solution (0.02% w/v) in water. The MIC tests were done using standard broth microdilution methods in 96-well round bottom microtiter plate, while MBC test was performed on the MHA plates for each bacteria (10^6 CFU/mL), respectively. For each MIC test, required amount of the synthesized Ag NPs (with or without *Ocimum sanctum* and *Trigonella foenum-graecum L* leaf extract in water), and *Ocimum sanctum* and *Trigonella foenum-graecum L* leaf extract in water solution was added and diluted two-fold with the bacterial inoculums in 100 µL of MHB. Colum 3 to 12 were used for experimental sample whereas, column 1 used for negative control (only media) and column 2 used for positive control (media and bacteria). Concentration of experimental solution (Ag NPs / plant extract) in column gradually increased from 3 to 12^{th} column. In each well of the microtiter plate, 30 µL of the resazurin solution was added and then incubated at 37°C for 24 h. Any color changes were observed. Blue/purple color indicated no bacterial growth while pink/colorless indicated bacterial growth. The MIC value was taken at the lowest concentration of antibacterial agents that inhibits the growth of bacteria (color remained in blue). The MBC was defined as the lowest concentration of the antibacterial agents that completely kill the bacteria. MBC test was performed by plating the suspension from each well of microtiter plates into MHA plate. The plates were incubated at 37°C for 24 h. The lowest concentration with no visible growths on the MHA plate was taken as MBC value.[6,]

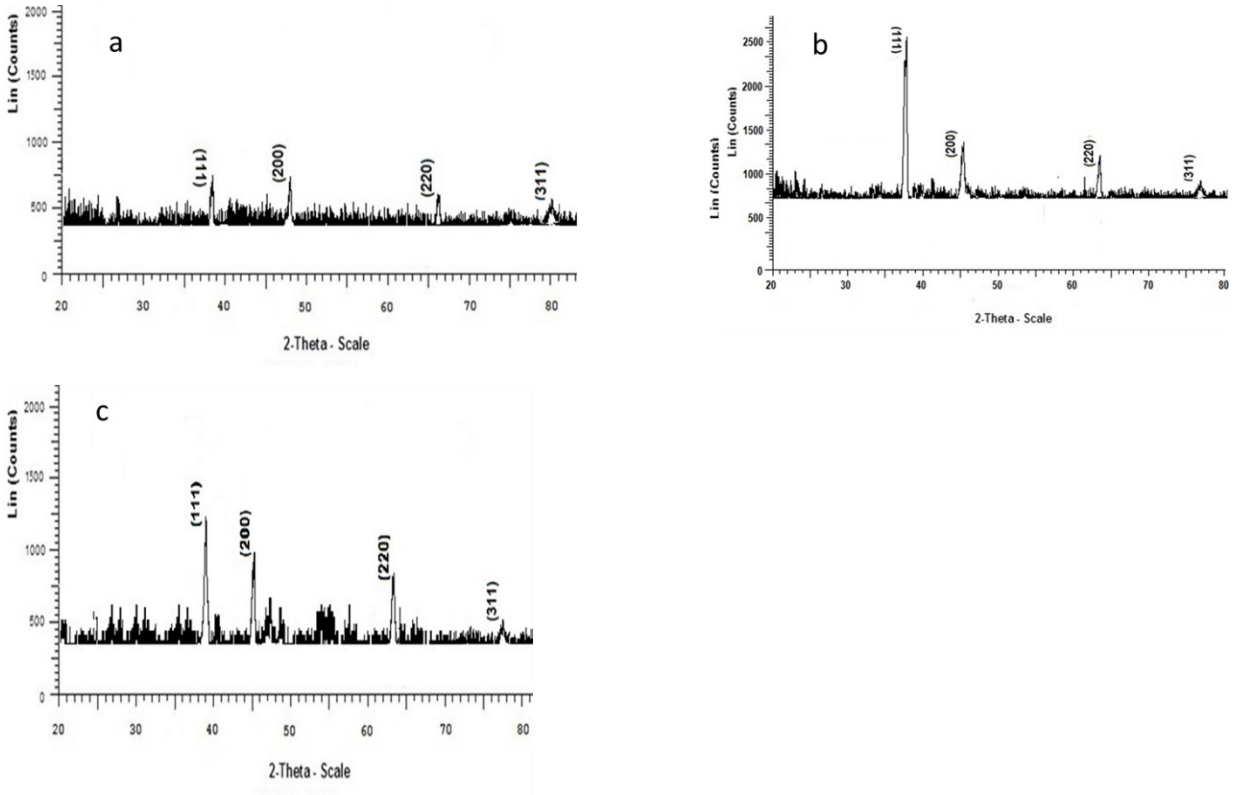

Fig. S1 Powder XRD of lipolyzed sample showing characteristics peak of Ag NPs synthesized in solution of both *Ocimum sanctum* and *Trigonella foenum-graecum L leaf* water extract (1:1, v/v) at (a) 1, (b) 2, and (c) 3 weight % of precursor ($AgNO_3$) concentrations with respect leaf weight, respectively.

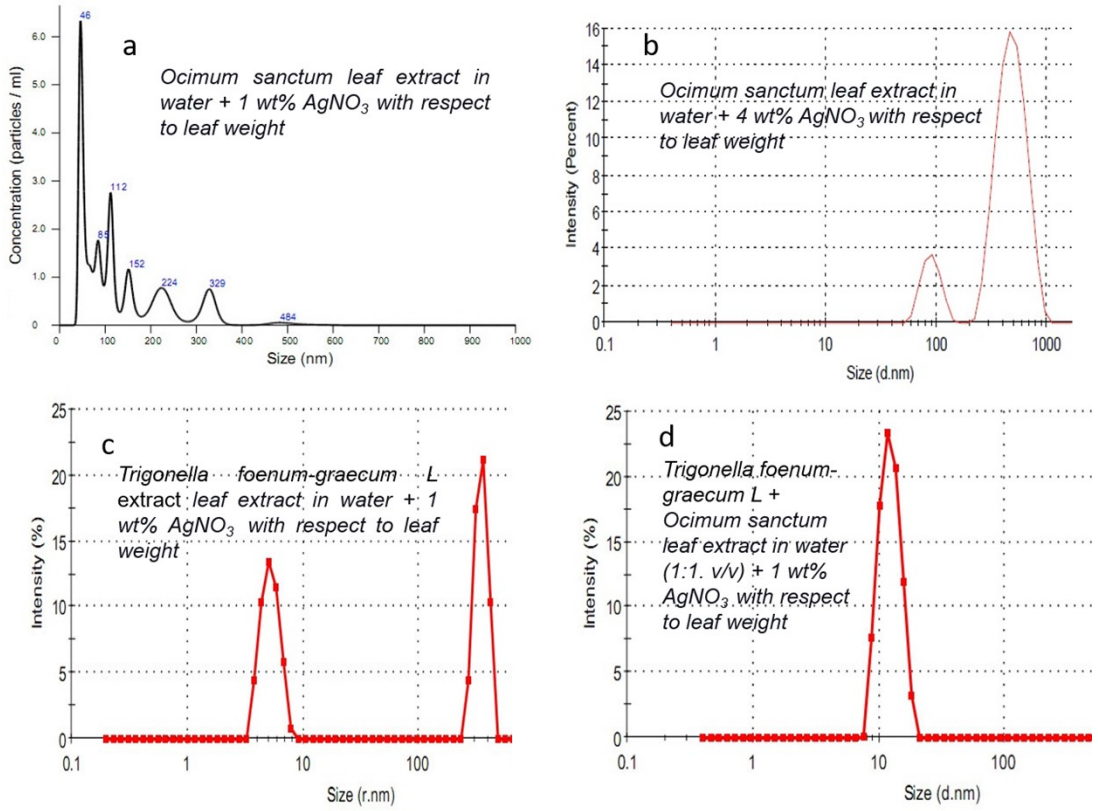

Fig. S2 DLS data for hydrodynamic size of Ag NPs (Poly disperse size of nanoparticles was observed when *Ocimum sanctum or Trigonella foenum-graecum L* water extract was used alone (Fig. S1a and Fig.S1c) as a media for reducing and capping agent, at higher concentration precursor, along with high polydispersity (PD), aggregation was observed (Fig. S1b); However, in combination of *Ocimum sanctum and Trigonella foenum-graecum L* water extract, narrow PD was observed (Fig. S1d)

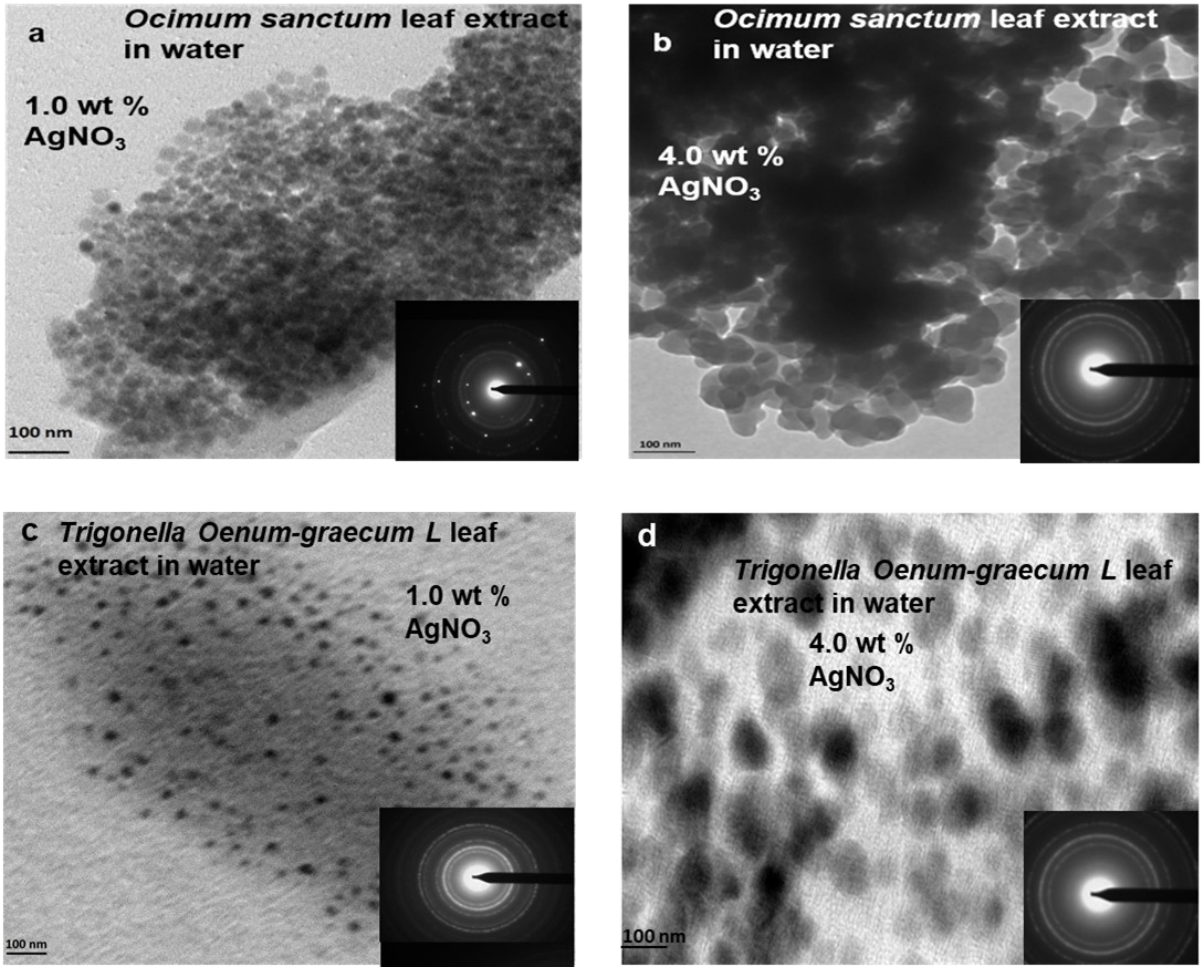

Fig. S3 TEM micrographs of green synthesized Ag nanoparticles in *Ocimum sanctum* (Fig.S3a and Fig.S3b) and *Tigonella foenum-graecum L* (Fig.S3c and Fig.S3d) leaf water extract was used alone as a media for reducing and capping agent. Figures inset image shows selected area electron diffraction pattern of corresponding Ag NPs.

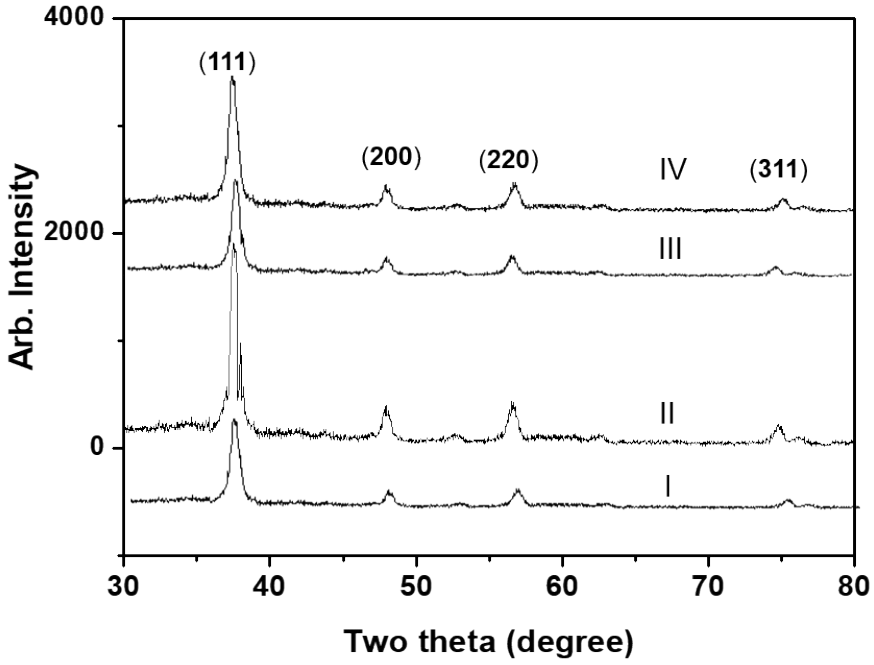

Fig. S4 Powder XRD spectra of lipolyzed sample of Ag NPs synthesized by using (I) 1 wt % $AgNO_3$, (II) 41 wt % $AgNO_3$, concentration with respect the leaf of *Ocimum sanctum* in water; (III) 1 wt % $AgNO_3$, (IV) 4 wt % $AgNO_3$, concentration with respect the *Trigonella foenum-graecum L* leaf in water.

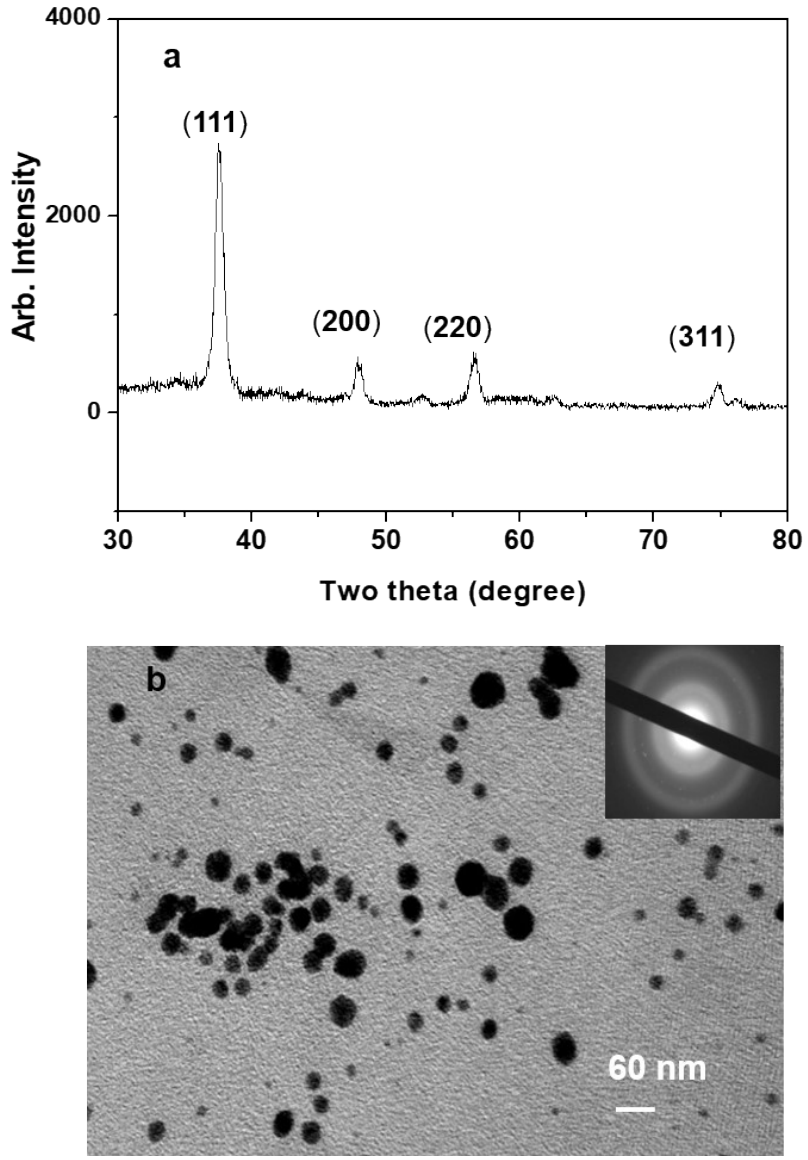

Fig. S5 (a) Powder XRD spectra of lipolyzed sample of citrate capped synthesized Ag NPs and (b) TEM micrograph of citrate capped synthesized Ag NPs (inset image is selected area electron diffraction pattern (SAED).

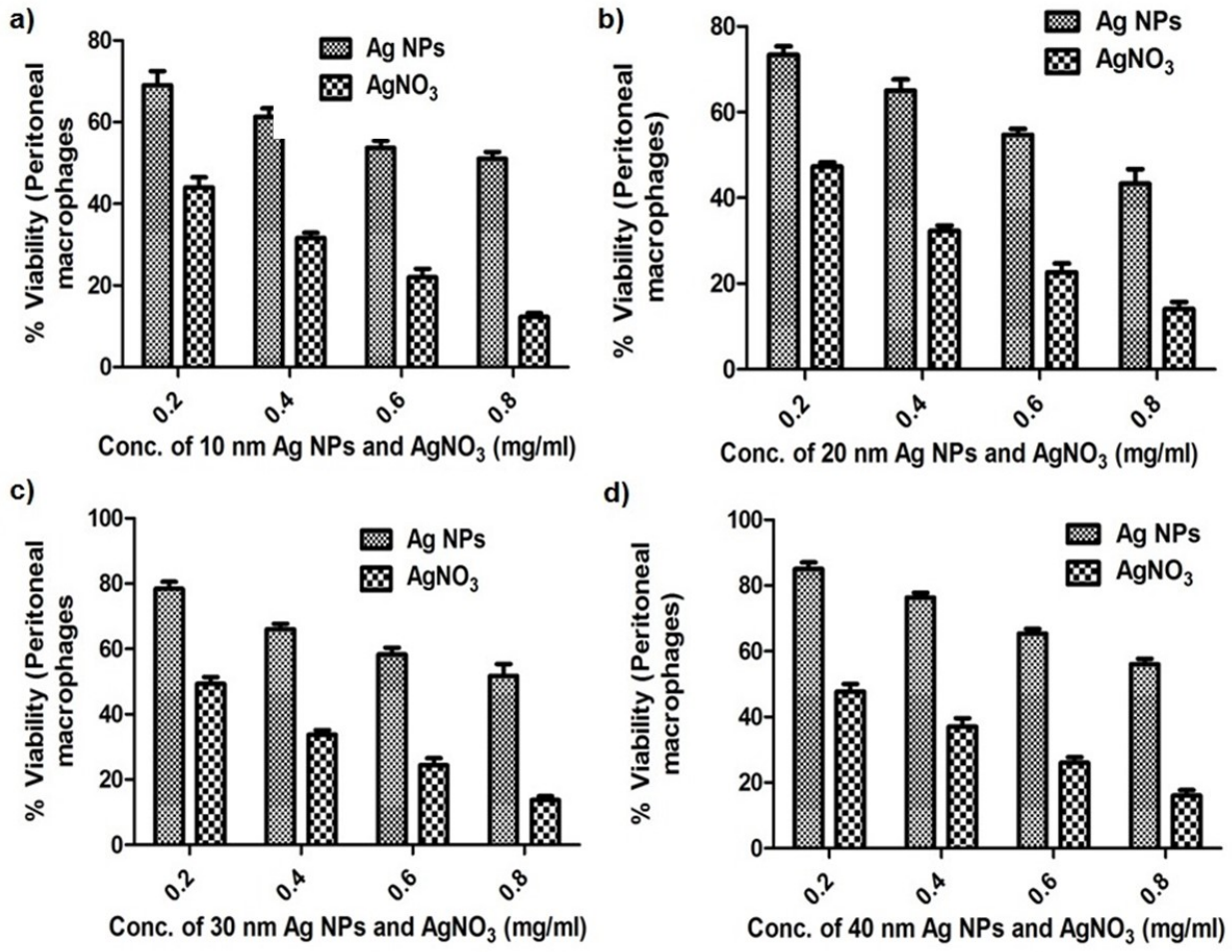

Fig.S6 Plots of cell viability MTT assay studied for various sizes of Ag NPs (Ag NPs) synthesized using mixture of *Ocimum sanctum* and *Trigonella foenum-graecum L leaf* water extract and $AgNO_3$ compared with negative control (Triton X-100) after 24h incubation period at same concentration 0.5g/mL (10 µg/mL (confidence interval ± 1.09), 15 µg/mL 20 µg/mL 25 µg/mL and 30 µg/mL (confidence interval ± 1.09) for 10 nm, 20 nm, 30 nm and 40 spherical shape Ag NPs and $AgNO_3$, respectively. This shows that the Ag NPs are less cytotoxic than $AgNO_3$ and as the size of Ag NPs is increases cytotoxicity decreases.

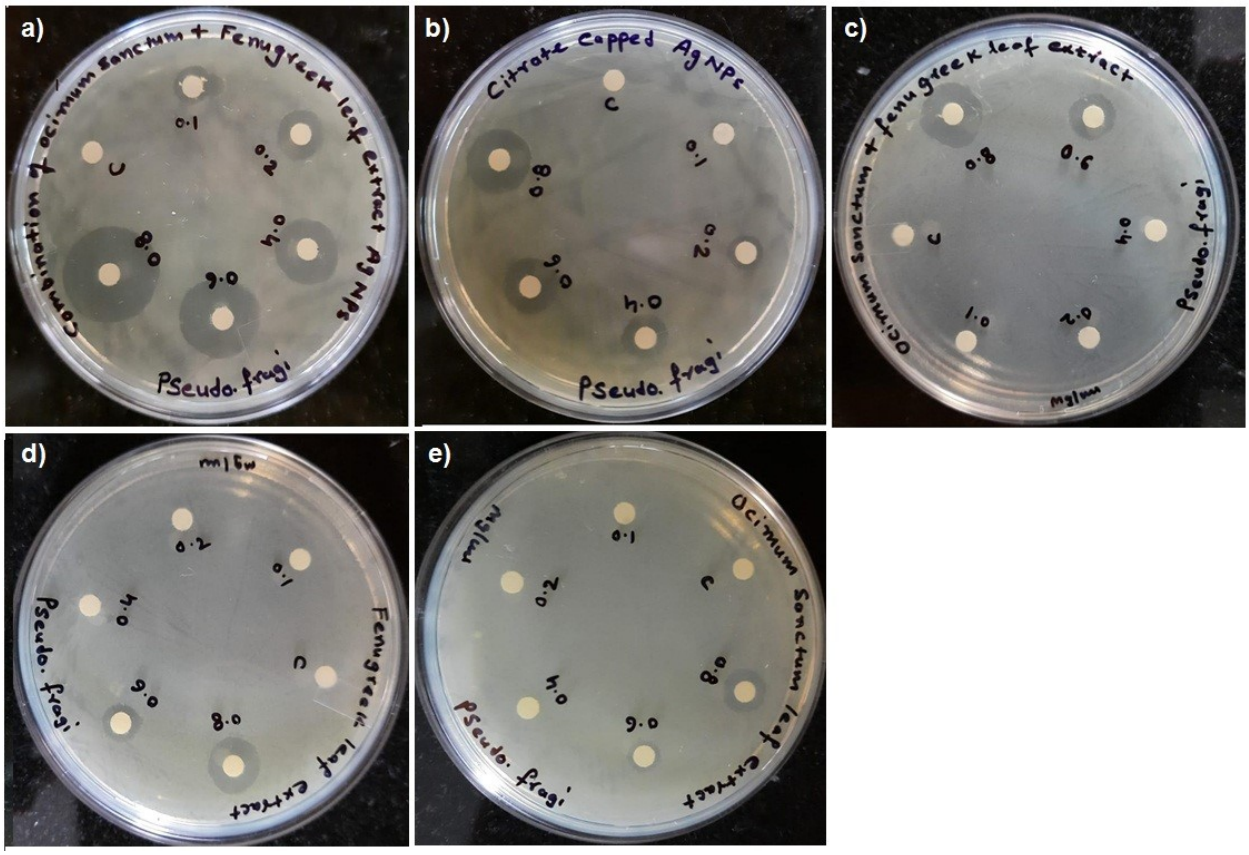

Fig. S7A Effect of concentration of Green synthesized 40 nm Ag NPs, Citrate capped Ag NPs, combined effect of *Ocimum sanctum* and *Trigonella foenum-graecum L* water and individual effect of *Ocimum sanctum* and *Trigonella foenum-graecum L* water on inhibitory effects for *Pseudomonas fragi*, and comparison with water as a control.

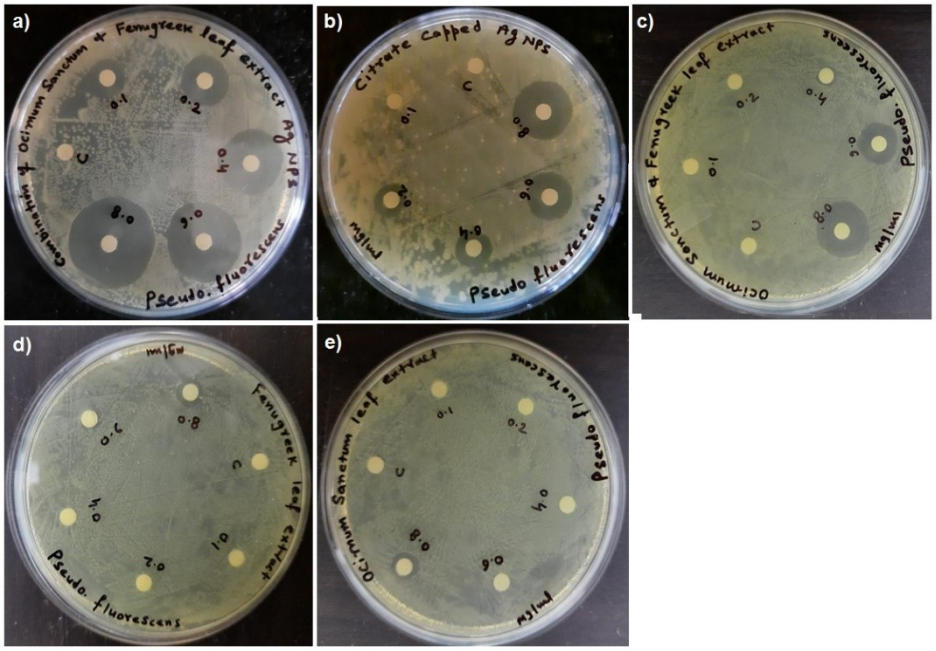

Fig. S7B Effect of concentration of Green synthesized 40 nm size Ag NPs, Citrate capped Ag NPs, combined effect of Osmium *sanctum* and *Trigonella Oenum-graecum L* water, and individual effect of *Ocimum sanctum* and *Trigonella foenum-graecum L* water on inhibitory effects for *Pseudomonas fluorescens*, and comparison with water as a control.

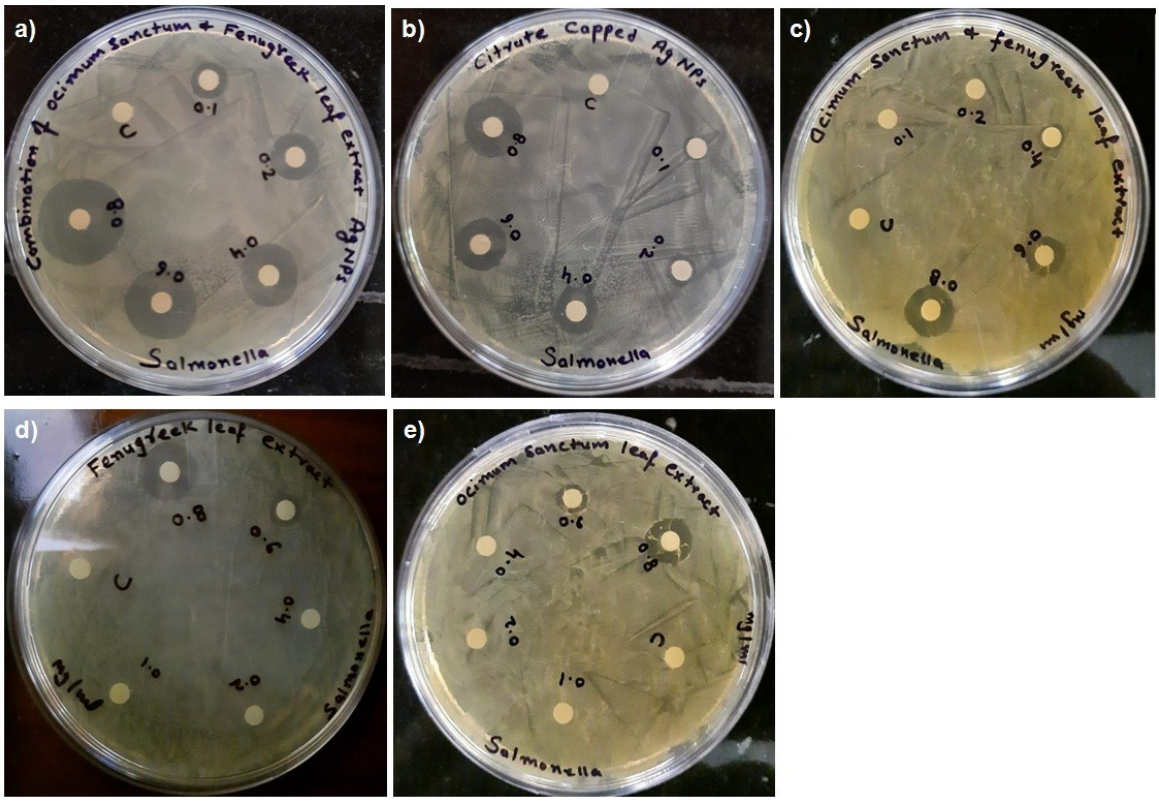

Fig. S7C Effect of concentration of Green synthesized 40 nm size Ag NPs, Citrate capped Ag NPs, combined effect of Ocimum *sanctum* and *Trigonella Oenum-graecum L* water, and individual effect of *Ocimum sanctum* and *Trigonella foenum-graecum L* water on inhibitory effects for *Salmonella*, and comparison with water as a control.

The result showed that, combination of *Ocimum sanctum* and *Trigonella Oenum-graecum L* water Ag NPs Ag NPs (0.8 mg/ml) exhibited maximum antibacterial activity as compared to citrate capped Ag NPs, combination of *Ocimum sanctum* and *Trigonella Oenum-graecum L* water *fenugreek leaf* extract and which showed the minimum against all three bacterial. The result of the inhibitory zone against *Pseudomonas fragi, Pseudomonas fluorescens and Salmonella* is determined from Figs. S4A, S4B, and S4C, respectively.

The competitive observation of the inhibitory effect of green synthesized Ag NPs on *Pseudomonas fragi, Pseudomonas fluorescens and Salmonella* and its comparison with citrate capped Ag NPs, *Ocimum sanctum* and *Trigonella foenum-graecum L* water, *Trigonella foenum-graecum L* water and *Ocimum sanctum* leaf water extract shown in Table S1 (data is an average zone of inhibition). With increasing the concentration of Ag NPs and leaf extracts, zone of inhibition increases for the *Pseudomonas fragi, Pseudomonas fluorescens and Salmonella.* However, maximum antibacterial activity of green synthesized Ag NPs was observed against the *Pseudomonas fluorescens*. In comparison to the citrate capped Ag NPs, *Ocimum sanctum* and *Trigonella foenum-graecum L* water Ag NPs (0.6 mg/ml) shows statistically significant antibacterial properties against the all three bacteria. The green synthesized silver nanoparticles showing efficient for antibacterial application due to enhanced bacterial cell-wall permeability, cellular respiration of the bacteria, and interacting with phosphorus, nitrogen or sulfur containing group in DNA and protein of the bacteria to penetrate inside.[7]

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