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Newly formulated extract of *Zingiber officinale* as reducing agent for Silver nitrate Nanoparticals

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Abstract
Ginger *(Zingiber officinale*) is a member of a plant kingdom family, and important in consumed dietary and also as a traditional medicine to treat various types of diseases. Ginger is composed of several bioactive compounds curcumene, zingberene, zingereone, gingerol derivatives. The aim of the present study was to evaluate the phytochemical compositions of four ginger extracts (water, ethanol, chloroform and petroleum ether (40-60 ° C) as well as soxhlet and oil extract products. In addition, to estimate total phenol contents, antioxidant activity using DPPH assay methods. The chemical compositions of oil and ethanolic extract were analyzed using UV, FTIR, TLC and GC-MS techniques. The antibacterial activity of ginger extracts against various human pathogenic bacteria was determined by agar diffusion method, minimum inhibitory concentration (MIC), as well as catalytic activity as reducing agent in silver nanoparticles (AgNPs) were determined.

Keywords: *Zingiber officinale*, GC-MS, DPPH, MIC, AgNPs

1. Introduction
Ginger (*Zingiber officinale*) belongs to the Zingiberaceae family, and is one of most widely used species and it is found in several foods and beverages. Ginger has shown a broad pharmacological effects [1] such as gastro protective, anti-bacterial, anti-inflammatory, antioxidant, anti-diabetic agent.
For centuries [2], it has been widely used for the treatment of nausea, vomiting, emetic, arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, fever, infectious diseases and helminthiasis.
Phyto-nanotechnology has drawn significant attention due to its rapid [3, 4], eco-friendly, non-toxic, cost-effective protocol in a single step synthetic process without the use of high pressure, energy, temperature or toxic chemicals. In plant-mediated synthesis, the extract is mixed with a metal precursor solution at room temperature and particular pH for a certain period of time.
Three methodologies through which AgNPs can be synthesized are chemical, physical and green methods [5]. Lately, a number of research groups have adopted the green plant-based reduction methods which are also considered safe, simple and cost effective protocols The main advantage of the green method was the presence of naturally occurring biomolecules, such as proteins, enzymes, tannins, phenols, sugars and flavonoids that can be used safely as reducing and stabilizing agents to form stable nanometals [6-10].
The aim of this study was to quantify the phytochemical constituents of *Zingbiber officinale* in different solvents to estimate total phenol, saponins, flavonoids, carbohydrates and evaluate the antioxidant and anti- bacterial activities. Alcoholic extract of the ginger useful as reducing stabilizing agent (green agent) in the synthesis of silver nitrate nanoparticles AgNPs against different types of targeted bacteria.

2. Experimental
2.1 Materials
Fresh ginger (rhizome) of 12 Kg was purchased from local market [Mosul, Iraq], and sample was authenticated by specialist from the College of Agriculture/University of Mosul. All other chemicals and reagents were of analytical grade and used without further purification.

2.2 Methods
2.2.1 Extraction Process Using Water as Hydrodistillation [11]
The extraction of the essential oil present in fresh ginger sample was done in a Clevenger apparatus. The fresh ginger sample was grinded into mash using a manual blender.

The 500mL round bottom flask of the Clevenger apparatus was filled with about 250mL water, then 100g of the grinded fresh ginger was added into the flask. The quick fit Clevenger apparatus was set on a thermostatic heating mantle. The temperature was set to 80°C. The extraction process was set for about 5h. This contain oil and water mixture and was separated by running off the water and reading the oil in the inbuilt calibrated tube, extraction process repeated several times till reach 2 kg of fresh ginger have been used. The oil was then weighed.

2.2.2 Extraction Process Using Soxhlet [12]

A 15g of powdered ginger root was percolated using 150mL 80% of methanol in the soxhlet apparatus at 60-65°C. This percolate was filtered and then it was evaporated to dryness under reduce pressure, the obtained extract was stored in air tight bottles at temperature lower than 50°C.

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2.2.3 Extraction Process Using Organic Solvent [13]

The fresh ginger wash, peel, pulverize then dry in shade at room temperature for 14 days. A 50g of dried ginger macerated in 500mL of distill water, chloroform, petroleum ether [40-60°C], and ethanol for 72h, filter by Whatman No.1, all extracts were dried and precipitate as gum.

2.2.4 Qualitative Phytochemical Screening Tests [141-5]

Extracts obtained from the previously mentioned extraction methods were subjected separately to the phytochemical screening tests including, Alkaloid, Flavonoid, Carbohydrate, Protein, Phenol &Tannin, Saponine, Steroid, Quinine, Terpenoid & Coumarin as shown in Table 1.

2.2.5 Estimation of Total Phenol Content [16]

The total phenolic concentration of all extracts were estimated, (10 µl) of each extract was taken in a 96 well microplate and 25 µl of Folin reagent and 230 µl of 4.4% of Na_2CO_3 were added and incubated for 30 min in dark place. Then, the absorbance was measured at 630 nm in the ELISA (Rayto, plate reader ELISA. RT, 2100C, China). A calibration curve was prepared using standard gallic acid (100 –600 mg/L, R^2 = 0.9978) and used to express the results as gallic acid equivalents (GAE) as shown in Table 2.

2.2.6 Determination of Antioxidant Activity Using DPPH [17]

Antioxidant activity of ginger extracts based on the scavenging activity of stable DPPH free radical was determined as follows:

Three volumes (50,100&150) µl of ginger extracts were individually added to test tubes then completed volume to 1mL by D.W, 1mLof DPPH solution (0.2 m M in ethanol)was added to each tube, mixed well and incubated at room temperature for 30 min. repeat the same procedure to prepare the control without ginger extract. Ascorbic acid solution (0.03% w/v) was used as a positive control. The absorbance (A) of solution was measured at 517 nm using UV-VIS spectrophotometer (Spectro-UV-VIS double beam, UVD-2950, labomed Inc. USA), inhibition of DPPH free radical in percent I% was calculated from following equation:

$$I\% = [(Ac-As)/Ac]\times 100$$

2.2.7 Chromatographic studies

A. Thin layer chromatography [12]

The oil and ethanol extracts were tested using TLC analytical plates coated with silica gel-G of 0.2 mm thickness. The solvent system used a mixture of Butanol- acetic acid-water (4:1:1 v/v). This mixture migrates on the silica coated plates by the capillary action. Fully developed coated plate was air dried. Iodine vapour used as detecting reagent. These spots were expressed by its retention factor (R_f).

B. Gas Chromatography-Mass Spectrometry GC-MS [18-19-20]

The experimental GC-MS was carried out on a (Agilent technologies GC- MS. CA, USA) are obtained in the form of mass spectra, relative intensity (RI) against mass to charge ratio (m/z) for all the studied compounds using electron ionization (EI). Cinnamic acid, cinnamyl alcohol and methyl cinnamate are used as standard for identification.

Chemical composition of oil and ethanol extracts of ginger was tested by GC–MS) with a quadruple detector and a capillary column (30 m×0.25 mm innerdiameter×0.25µm film thickness). Helium was used as the carrier gas with a constant flow of 1.2 mL/min. The initial temperature of oven temperature program was set at 40°C and continued for 4 min, rising by 5°C/min to 250°C, which continued for 10 min. The injector temperature was 250°C. The volume of injected sample was 1µL. Electron ionization (EI) was used in the MS and standard mass spectra with 70 ev ionization energy were recorded m/z from 0-500 at one hour.

2.2.8 Biosynthesis of Silver Nanoparticles AgNPs by Using the Ginger Extracts [21-22]

Ginger extracts were prepared by adding 19 g of the dried plant powder to 100 mL DW. The mixture was stirred for 15 min at 55◦C. The extract was then cooled and filtered through Whatman No.1 filter paper. The filtrate was stored in a sealed storage tube and solutions of the extracts were kept in refrigerator.

Aqueous and oil extracts of plants were used as reducing agent (catalyst) in the green synthesis of AgNPs. The effect of extract, silver nitrate $AgNO_3$ concentration, time and temperature of reaction were standardized to obtain the optimum values for the synthesis of AgNPs. The solutions of the extracts were diluted to 5% (v/v). Two milliliters of (10 mmol/L) of the $AgNO_3$ solution was added to the extract solution drop by drop under heating (55°C) for 25min. with vigorous stirring. The yellowish solution of the diluted plant extract changed to yellowish-brown colloidal dispersion after the addition of the $AgNO_3$ solution, indicating the formation of nanoparticles of silver (AgNPs). After the synthesis of AgNPs, the solution was centrifuged at 8000 rpm for 10 min to separate AgNPs from the solution, and the sediment of AgNPs was ready for analysis. The dried AgNPs were kept in a microtube in the refrigerator for further study. A 0.01mole of ciprofloxacin hydrochloride dissolve in 10 ml D.W, stir until completely dissolve, 1:1 ratio of Ag nanoparticles with ciprofloxacin solution, heat at 95 °C for 15 min. Repeat the same procedure to prepared meropenem AgNPs. Both ciprofloxacin and meropenem nanoparticals are used as standards antibacterial agents.

2.2.9 Antibacterial activity extracts from Zingiber officinale [23]

The antibacterial activity of the extracts was studied on human pathogenic fresh culture of *Escherichia coli* and *Staphylococcus aureus* by using standard disc diffusion method muller hinton medium were plated as a swab culture

bacteria, triplicates were done and plates were incubated at 37°C for 24 h. Fresh overnight cultures were taken and spread on the muller hinton agar plates to cultivate bacteria. Sterile paper discs of 5 mm diameter saturated with (ginger extract, silver nanoparticle) were placed in each plate and incubated again at 37° C for 24 h and the antibacterial activity was measured based on the inhibition zone around the disc impregnated with plant extract and synthesized silver nanoparticle respectively.

2.3 Statistical analysis
Data were analyzed using Microsoft excel.v. 10, study findings were explained in words, table and figure. Tests were carried out in triplicate and the results were calculated as standard means (± SD).

3. Results & Discussion
The water extract of *Zingiber officinale* was containing carbohydrate only. In contrast, the ethanolic and soxhlet extracts are containing most constituents especially flavonoid & phenol products Table 1 [14-15].

The functional groups of extracts were identified using infrared spectra (Bruker spectrometry). The characteristic peaks frequencies ν $_{cm}^{-1}$: 3278 OH(phenolic) ; 2920 C-H(Aromatic); 2850 C-H(Aliphatic); 1707 C=O(keto group); 1670 C-O(bending). The phytochemical screening tests for crude *Zingiber officinale* using different extracts Table 1.

Table 1: Phytochemical screening tests of crude *Zingiber officinale* using different extracts.

Phytoconstituents	Water extract	Ethanol extract	Soxhlet Methanol	Chloroform extract	Petroleum Ether [40-60]
Alkaloid	-ve	+ve	+ve	-ve	-ve
Flavonoid	-ve	+ve	-ve	-ve	+ve
Carbohydrate	+ ve	-ve	+ve	-ve	-ve
Protein	-ve	+ve	+ve	-ve	-ve
Phenol & Tannin	-ve	+ve	+ve	-ve	-ve
Saponine	-ve	+ve	-ve	-ve	-ve
Steroid	-ve	-ve	+ve	+ve	+ve
Quinine	-ve	+ve	+ve	+ve	-ve
Terpenoid	-ve	+ve	+ve	+ve	-ve
Coumarin	-ve	-ve	+ve	+ve	+ve

+ Ve means positive test; - Ve means negative test.

Total phenolic contents were measured using ELISA technique at wavelength 630nm. Oil extract show lowest content of phenolic as compare with gallic acid as standard or other extracts. Chloroform or water extracts were showed higher activity with standard as shown in Table 2 & Fig.1 respectively. Oil extract was showed a significant values with other extracts.

Table 2: Estimation of Total Phenol compounds using ELISA

Conc. g/l	(A) Gallic Acid	Absorbance (A) Oil Extract	Absorb. Ethanol Extract	Absorb. Water Extract	Absorb. Chloroform Extract
0.1	2.425	0.304	2.442	2.468	2.487
0.2	2.59	0.455	2.603	2.582	2.668
0.3	2.574	0.328	2.535	2.489	2.798
0.4	2.567	0.425	2.516	2.664	2.658
0.5	2.578	0.340	2.557	2.549	2.614
0.6	2.547	0.299	2.518	2.582	2.702

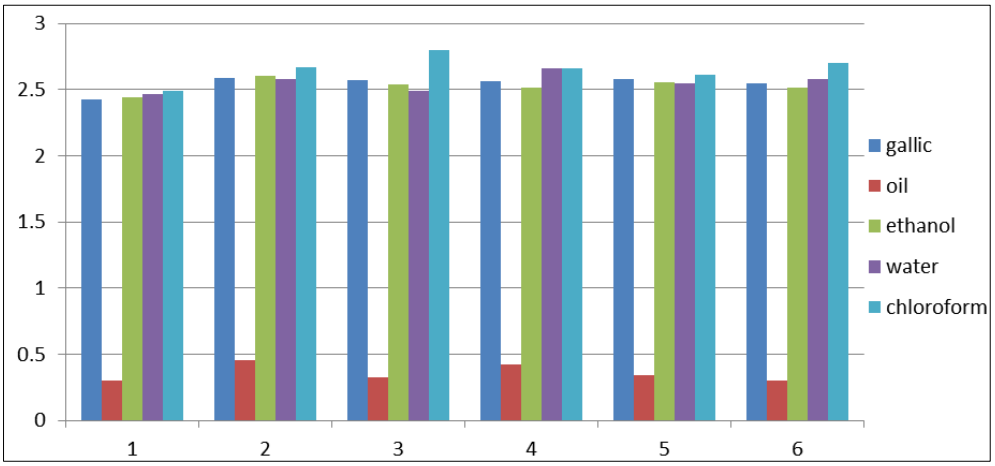

Fig. 1: Histogram of Total Phenolic Contents

To estimate the antioxidant activity of ginger extracts using (1,1-diphenil-2-picrylhydrazyl) DPPH as free radical scavenging mechanism [17]. The absorption was calculated spectrophotometric ally at wavelength 517 nm and ascorbic acid was used as a positive control. The petroleum ether [40-60 °C] extract was giving highest inhibition zones as compared with other extracts as shown in Fig. 2.

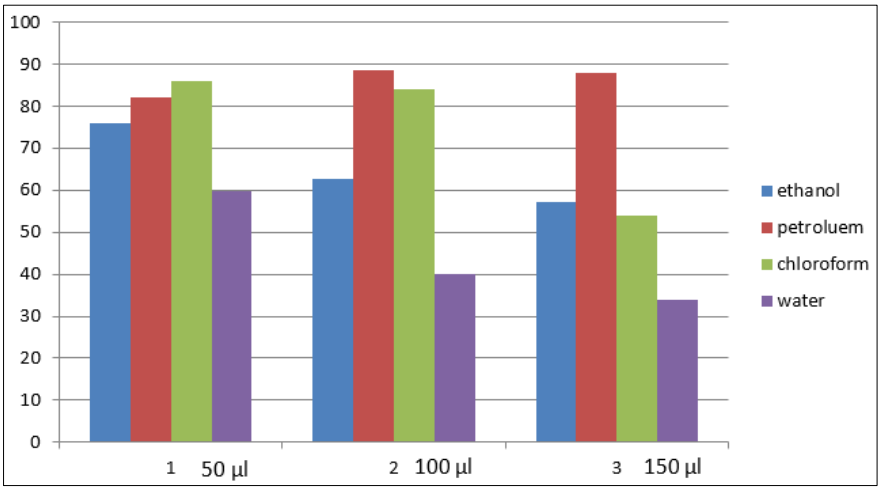

Fig. 2: Histogram of Comparative average free radical scavenging activities of *Zingiber officinale* extracts using DPPH assay.

The purity of the oil and ethanol ginger extracts were checked using chromatographic TLC technique [12], the oil extract showed two spots with different retention times(R_f) are 0.692 and 0.923 respectively, while the ethanol extract only one spot was shown at (R_f) 0.764 using a standard solvent mixture of (butanol- acetic acid-water) (4:1:1 v/v).

The gas chromatography-mass spectrometry (GC-MS) studies, the validity of proposed structures for various (GC-MS)-derived peaks in *Zingiber officinale* extracts fragmentation patterns [18-19-20]. Different organic compounds were determined in oil extract and soxhlet extract of ginger as showed in Tables 3& 4 and Figs 3&4 respectively. The main organic products are alpha-curcumene are present high quantity in both oil and sohexlet extracts, beside others (Zingiberene, beta Sesquiphellandrene, Zingerone) are found in small quantities.

Table 3: Gas chromatography-Mass spectroscopy (GC-MS) of Oil extract ginger (*Zingiber officinale*).

Peak	Compound	m/z	RT (min)	Amount %
1	Citral	165.9	9,820	0.48
2	Neryl acetal	166	11.420	2.97
3	Geranyl acetal	167	12.279	6.00
4	(-)-Isoledene	204	13.242	0.38
5	alpha.-Cubebene	204	13.598	0.67
6	beta.-elemene	204	14.168	0.51
7	(-)-(4S,7R)-Bisabola-1(6),2,10-triene	204	14.635	0.34
8	(Z,E)-.alpha.-Farnesene	204	16.516	0.81
9	Alloaromadendrene	204	16.716	0.42
10	1H-Cyclopropa[a]naphthalene	204	17.264	0.70
11	alpha.-Curcumene	202	17.679	21.83
12	Zingiberene	204	18.027	4.43
13	gamma.-Cadinene	217.9	18.168	3.24
14	beta.-Sesquiphellandrene	220	18.553	6.56
15	7-Amino-3-methylpyrimido(4,5-c)pyridazin-5(6H)-one	220	18.590	3.59
16	Elemol	220	18.879	0.52
17	7-epi-cis-sesquisabinene hydrate	218.1	19.153	9.43
18	+/-.-trans-Nerolidol	204	19.590	0.28
19	m-Cymol	162	19.960	0.31
20	cis-Sesquisabinene hydrate	220.2	20.123	0.51
21	trans-2-(Oct-1-enyl)pyridine	202	20.493	1.10
22	trans-Caryophyllene	222	21.071	0.22
23	delta.-Selinene	204	21.486	1.05
24	Germacrene B	236	21.760	0.36
25	beta.-Eudesmol	222	22.486	2.17
26	(+)-Aromadendrene	222	22.693	0.34
27	Cadinene	204	23.012	0.85
28	Farnesyl alcohol	222	23.219	1.46
29	(-)-Isolongifolol	236	24.093	0.73
30	(E,S)-(+)-Nuciferal	250	24.256	0.56
31	Thymene	216	26.137	3.51
32	m-Cymene	134	26.463	1.34
33	5-Silaspiro [4.4]nona-2,7-diene	136	27.396	1.86
34	(3E)-5-Isopropylidene-2,7-dimethyl-6-oxa-1,3,7,10-undecatetraene	236	28.011	0.42
35	4,7-Methano-1H-indene	250	29.322	0.87
36	Carone	222	29.574	0.59
37	Farnesyl alcohol	166	29.670	0.37

38	1-Formyl-2,2,6-trimethyl-3-cis-(3-methylbut-2-enyl)-5-cyclohexene	166	30.640	0.36
39	(E)-5-Methylcyclonon-5-en-1-one	236	31.574	3.27
40	(1R)-Spiro[camphor-5,2'-1'-oxacyclopropane]	220	31.759	0.47
41	Spiro[4.5]dec-6-en-8-one	238	32.026	0.27
42	3-Methyl-2-(1',1',5'-trimethyl-5'-hexenyl)-2-cyclopropenyl methyl ketone	177	32.344	5.84
43	Viridiflorol	252	33.085	0.31
44	(Z,E)-7-Methyl-4-(1-methylethylidene)-1,7-cyclodecadienemethano	238	34.299	0.37
45	p-Aminosalicylic acid	351	35.262	2.46
46	1-(1-Butyny)cyclopentanol	178	35.773	0.97
47	(-)-10-Camphorsulfonyl chloride	268	36.507	1.26
48	(+)-8(15)-edren-9-ol	234	36.692	0.37
49	(-)-10-Camphorsulfonyl chloride	236	37.410	1.85
50	Geranyl-p-cymene	270	40.077	0.44
Standard 1	Cinnamic acid	147	17.020	99.89
Standard 2	Cinnamyl alcohol	134	11.339	98.85
Standard 3	Methyl cinnamate	176	13.902	100.00

Table 4: Gas Chromatography-Mass Spectrometry (GC-MS) of Ginger soxhlet extract.

Peak	Compound	m/z	RT (min)	Amount
1	alpha.-Curcumene	202	17.464	35.56
2	Zingiberene	204	17.931	22.87
3	beta.-Bisabolene	204	18.420	12.24
4	beta.-Sesquiphellandrene	204	18.983	21.06
5	Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha.,4-trimethyl-3-(1-methylethenyl)-	208	19.931	1.34
6	13-Amino-[11]-metacyclophane-1,11-dione	430	21.471	1.46
7	alpha.-Ylangene	204	22.427	2.26
8	Zingerone	194	26.056	3.04
9	Iso Jasmone	166	53.979	0.17

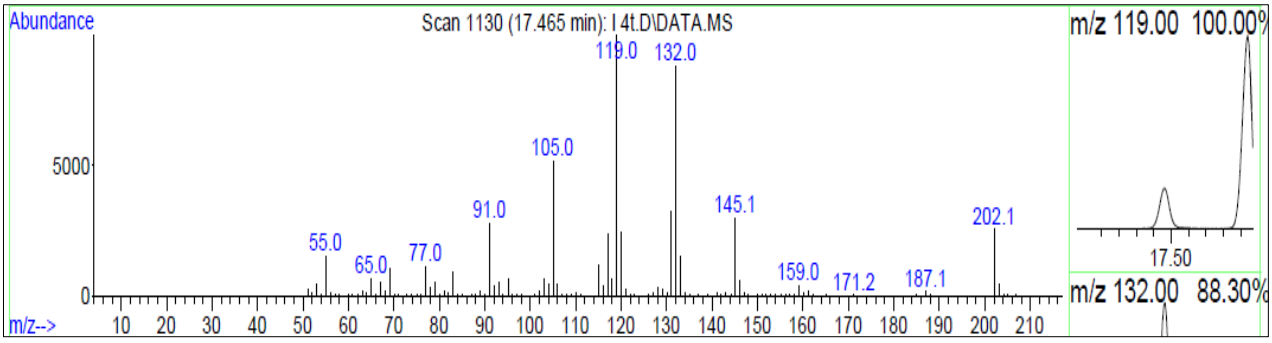

Fig 3: GC-MS of alpha-Curcumene, base peak 119 m/z.

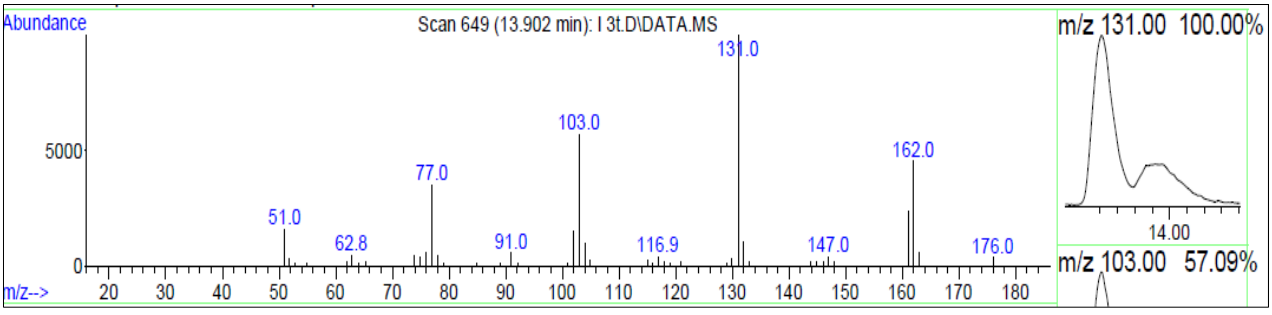

Fig 4: GC-MS of Methyl cinnamate (Standard), base peak 131 m/z.

However, Curcumene, zingerene, alpha sequlene, Zingerone have many double bonds and phenolic (OH group) gingerone in their structures, are conjugation, in turn, can change the electron distribution within the molecule [11-13], significantly affecting the antioxidant properties of the products. From the perspective of chemistry, ginger products are tested as catalyst in a redox reaction. For example, the silver ion Ag^{1+} can be reduced to silver metal Ag^{0} by gaining an electron from another molecule Fig. 5. Accordingly, the Ag^{1+}-reducing capacity assay as nanoparticles (AgNPs), because this postulation is supported by the experimental results from the DPPH radical-inhibition assay and showed a weak catalyst antioxidant activities for nanoparticles [18,22] low inhibitory effect except for chloroform extract.

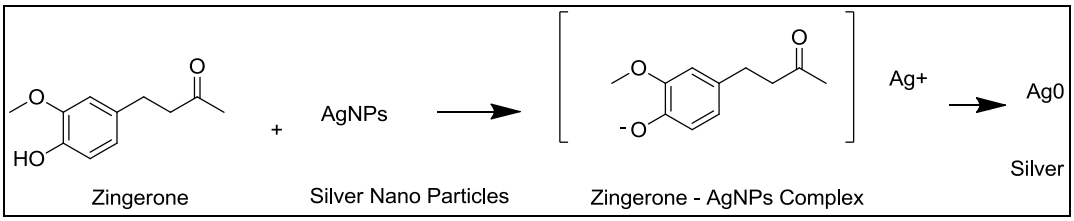

Fig 5: Proposed mechanism as reducing agent (catalyst) for AgNPs

Antibacterial test against two types of bacteria G(-)ve and G(+)ve using ciprofloxacin AgNPs & meropenem AgNPs as a references[21-23] was proved as weak inhibition zones as compared with references. In short, the rank of extract activities as oil > water > ethanol > petroleum ether > against *Staphylococcus aureus* chloroform extract show activity against *E.Coli* as shown in Table 5.

Table 5: Antibacterial activity (zone of inhibition) ginger extracts AgNPs

Type of Extract	*S.aureus*	*E.coli*
Ethanol (AgNPs)	7 mm	8 mm
Water 10 mM (AgNPs)	7 mm	0 mm
Oil AgNPs	20 mm	7 mm
Ethanol Extract (AgNPs) +ciprofloxacin	30 mm	40 mm
Ethanol Extract (AgNPs)+meropenem	35 mm	35 mm
Water Extract (10 mM AgNPs)+ciprofloxacin	32 mm	35 mm
Water Extract (AgNPs)10 mM +meropenem	38 mm	22 mm
Ciprofloxacin alone (Reference)	45 mm	42 mm
Meropenem alone (Reference)	40 mm	43 mm

Minimum Inhibitory Concentration (MIC)[23] of different *Zingiber officinale* extracts (free from AgNPs) on four different bacteria, *E.coli, Bacillus, Serretia, Staphylococcus aureus*. all are isolates at 12.5 mg/ml. Oil extract exhibit inhibitory effect in broth even at concentration of 3.12 mg/ml. *Bacillus* only. While MIC for chloroform extract at concentration 3.12 for *E.coli* and 1.6 for *Bacillus* respectively.

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5. Conclusion
In the present study, four different extracts of *Zingiber officinale* were prepared, phytochemical and chromatographic studies, and stable silver nanocolloids were successfully prepared by a novel green route using aqueous extract of *Zingiber officinale*. The procedure was relatively easy, rapid, inexpensive, eco-friendly and did not require any organic solvents or other toxic reagents. Furthermore, this study demonstrated a reducing agent (catalyst) of the prepared AgNPs by their low antibacterial inhibitory activity.

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