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An *In Vivo* Synergistic Research on the Assessment of the Phytocompound Capped Silver Nanoparticles from *Raphanus sativus* Leaf Extract and *Curcuma longa* Against UTI-Causing *E.coli.*

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Recently, green synthesis of nanoparticles using plant has attracted much attention due to the environmental and economic benefits. The present work demonstrates an eco-friendly and low-cost method to study the antibacterial activity of the silver nanoparticles [AgNPs] biosynthesized from phenolic compounds of plant extracts (R*aphanus sativus* and *Curcuma longa*). Using GCMS and ^{1}H NMR spectroscopy analyses, the presence of phenolic compounds and alcohol groups of long-chain were detected. Polyphenol compound such as catechol and phenolic derivative such as curcumin were capped with silver nanoparticles and proved by SEM analysis. However, the antibacterial potential of these green synthesized phytochemical capped AgNPs was further explained in *in vivo* studies. Albino mouse was induced with multidrug-resistant *E.coli* and was treated with Ciprofloxacin as a conventional drug for the standard groups and with synthesized catechol-curcumin capped silver nanoparticles for the test groups. Histopathology of albino mice kidney and bladder was performed after dosing the mice with specific concentrations of the nanoparticles for 3, 7, and 14 days. Photomicrographs of the kidney and bladder histology revealed cell regeneration, normal renal cortex, and glomerular tufts in the mice exposed to the nanoparticles.

Keywords: *Raphanus sativus, Curcuma longa*, GCMS, Silver nanoparticles, NMR, SEM Histopathology

INTRODUCTION

In the past few decades, nanotechnology has become the most prominent and innovative field in modern and natural science [1]. Green synthesis of nanoparticles from silver, gold, zinc, copper, and platinum have been a promising biological source to provide a platform for the synthesis of nanoparticles by physical and chemical methods [2]. *Raphanus sativus L.,* (radish) belongs to the family Cruciferae is one of the most common ancient cultured plants used for eating; this is due to the presence of potential bioactive compounds, agriculture economy, biological activities such as antimicrobial, antiviral, hypotensive, and antioxidant properties with potential health benefits [3]. *Curcuma longa* (turmeric) belongs to the family Zingiberaceae, displays several biochemical activities such as antioxidant, anticarcinogenic, antifungal, and antimicrobial activities [4]. Urinary tract infection (UTI) is one of the most common bacterial diseases caused by *Escherichia coli, Citrobacter, Enterobacter, Staphylococcus aureus, Staphylococcus saprophyticus, and Streptococcus* [5]. The infectious bacteria typically make up the fecal flora, and the UTI episode begins when an individual's urine flow is blocked by one of the numerous factors, including strictures, calculi, tumors, prostatic hypertrophy, vesicourethral reflux, catheterization, urinogenital surgery,

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and cystoscopy. *Raphanus sativus* and *Curcuma longa* were traditionally used by local ethnic tribes to treat infectious diseases. These plants were tested in a systematic screening with UTI-causing bacteria for being used as a source of non-microbial antimicrobials, so that they can be used as complementary medicine to antibiotics because antibiotics take 3-4 days to arrive. To avoid using of any antibiotic of higher generation during empiric therapy, complementary or synergistic therapy by phytodrugs with any ongoing antibiotic can be prudently used against UTI.

In this present work, we isolate phenol and polyphenol compounds from plant extract of *Raphanus sativus L., Curcuma longa,* and synthesized silver nanoparticle ($AgNO_3$) using combined Curcumin and catechol. The biological activity of the synthesized nanoparticles was studied using antibiofilm and antimicrobial activity against multidrug resistant uropathogenic *Escherichia coli* isolated from urinary tract infections. Furthermore, the phytochemical capped silver nanoparticles were characterized by SEM and NMR methods. In addition, the antimicrobial impact of phytochemical capped silver nanoparticles was evaluated while histopathological and *in vivo* assessments were carried out against pathogenic urinary tract infections in albino mice.

MATERIALS AND METHODS

Chemicals and Plant Collection

Standard laboratory grade chemicals, Silver nitrate ($AgNO_3$), and methanol were purchased from Sigma-Aldrich, India, with 99.5% purity. The leaves of *Raphanus sativus* and *Curcuma Loga* were collected from in and around Thiruvannamalai, Tamil Nadu, India. A multi-drug-resistant *Escherichia coli (E.coli)* isolates were collected from urine specimens of Saveetha Dental College and Hospital patients with suspected UTI, who had not received antibiotics.

GC-MS Analysis

The components were separated using Helium as the carrier gas at a constant flow rate of 1 ml min^{-1} using a Clarus 680 GC with a fused silica column packed with Elite5MS (5 percent biphenyl 95 percent dimethylpolysiloxane, 30 m 0.25 mm ID 250m df). During the chromatographic run, the injector temperature was set to 260 °C. The extract (1L) was prepared as follows: pumped into the instrument at 60 °C for 2 min, followed by 300 °C at the end. It was heated to 300 °C for six minutes at a rate of 10 °C per minute 1.The mass detector was set to 240 °C for the transfer line, 240 °C for the ion source, and 70 eV for the ionization mode electron impact, with a scan period of 0.2 s and a scan-interval of 0.1 s. The fragments range from 40 to 600 Da. The components' spectrums were compared using the spectrum database of known components in the GC-MS NIST library.

Phenol and Polyphenol Extraction

Solid-phase extraction [phenols from *Raphanus sativus*] and Filtration [Polyphenols from *Curcuma longa*] was carried out and the filtrates were collected in a Petri plate each of 10 ml quantity and the solvent was evaporated at the atmospheric pressure in a water bath. After drying the residual weight was noted down and subtracted from the initial empty weight of the Petri plate, and the amount of extracted phenolic compounds was calculated.

Biosynthesis of Silver Nanoparticles

Curcumin-Catechol AgNP wassynthesized by reducing silver nitrate ($AgNO_3$) with an aqueous solution of curcumin-catechol (0.19 g) that was prepared by dissolving in deionized water (18 ml). Curcumin-catechol aqueous solution was added drop wise with constant stirring to 1 mM $AgNO_3$ aqueous solution (42 ml) under the boiling condition in conical flasks. The mixed solutions were boiled for 3 h for reduction of Ag ions followed by cooling at room temperature for 30 min. The flask was covered with aluminum foil to maintain dark conditions throughout the reduction process to avoid any photochemical reactions.

NMR Spectroscopy

One-dimensional ^1H NMR spectra were recorded with a Bruker NMR Avance spectrometer operating at 300 MHz for ^1H. The samples were dissolved in dimethyl sulfoxide-d6 (DMSO-d6) as solvent. A volume of 20 µl of tetramethylsilane (TMS) was added as the internal reference. Chemical shifts were reported in parts per million (ppm) relative to tetramethylsilane (TMS) expressed in δ units, and spin multiplicities were given as s (singlet), d(doublet), dd (double doublet), t (triplet), or m (multiplet).

SEM Analysis

The surface morphology and the size of the Phytochemical capped silver nanoparticles were analyzed by Scanning Electron Microscope (SEM). A sample was placed at room temperature; overnight, thin layer of silver nanoparticle placed on the sample holder and was scanned at different magnification.

In Vivo Experimental Design

The mice were maintained in cages and fed with free access to water and feed. The experiments were conducted according to ethical norms approved by the Government of India and Institutional Animal Ethics Committee guidelines. Twelve animals were randomly divided into four groups, three animals in each. Group 1 was control. Group 2 was administered with ciprofloxacin (5 and 10mg/Kg body weight respectively). Groups 3 was the treatment groups, treated with a mixture [Catechol-Curcumin capped silver nanoparticles] containing 12.5 mg Kg^{-1}. Group 4 which was kept for the non-treated group: transurethral catheter for inoculating bacteria in the bladder [10 µl]-Non-treated. The drugs were administered orally. The animals were allowed to have free access to their food and water before and during the period of the study.

Histopathology

The fixed specimens of the Bladder and kidney were processed overnight for dehydration, clearing, and impregnation using an automatic tissue processor (Sakura, Japan). The specimens were embedded in paraffin blocks using an embedding station (Sakura, Japan), and serial sections of 4 µm thickness were cut using a microtome (ModelRM2245, Leica Biosystems, Wetzlar, Germany). We used an autostainer (Model 5020, Leica Biosystems, Wetzlar, Germany) for Hematoxylin & Eosin staining of the sections. The mounted specimens were observed and scored under light microscopy. For a semi-quantitative comparison of the structural changes, the abnormalities in the tissue sections were graded from 0 (normal structure) to 3 (severe pathological changes).

RESULTS AND DISCUSSION

The aim of the present study is the development of catechol synthesized silver nanoparticles mediated by *Raphanus sativus* L. extracts, curcumin from *Curcuma Loga*, obtained through solid-phase extraction method, and to test their potential application for controlling Uropathogenic *E.coli.*

GC-MS Analysis

Methanolic leaf extract of *Raphanus sativus* and *Curcuma longa* were subjected to gas chromatography-mass spectrometry (GC-MS) analysis to identify the bioactive compounds. The methanolic leaf extract showed the presence of ten major bioactive compounds in *Raphanus sativus* and nine major bioactive compounds in *Curcuma longa,* shown in Table 1 and Fig. 1. From the results, Urea,N,N'-bis (2-hydroxyethyl), 2,4-pentadienenitrile, furfural, dimethyltrisulfide, propylparaben, 1,4-dihydrothujopsene-(l1), 4(2,5-dihydromethoxyphenyl) butylamine, 1-butene, 4-isothiocyanato-1(methylthio), 2(3H)-naphthalenone , 4,4a,5,6,7,8-hexahydro-1-methoxy, and 5-hydroxymethylfurfural were the bioactive compound found in *Raphanus sativus*. Waheed A *et al.* in 2019 stated that *Raphanus sativus* extract were rich in compounds such as oleic acid (1), oleic acid (2), and erucic acid, representing 30.011%, 5.464% and 16.411% of the total oil, respectively [6]. Bicyclo [4.1.0]-3-heptene, 2-isopropenyl-5-isopropyl-7,7-dimethyl, xylopropamine, 1-(3,3-dimethyl-but-1-ynyl)-1,2-dimethyl-3-methylene cyclopropane, 6-octen-1-yn-3-ol, 3,7-dimethyl, ethyl iso-allocholate, curcumin, α-curcumene, β-curcumene, and Isocurcumenol were the major bioactive compounds found in *curcuma longa*, shown in Fig. 2. Similar to our study, Xu LL *et al.* stated that turmeric powder contained high concentrations of a potent biological active phytochemical compound curcumin [7].

Solid-phase Extraction

Phenolic chemicals were separated from the crude extract of *Raphanus sativus*. However, various chemicals such as alkaloids, flavonoids, terphenoids were present. As a result, the crude extract was shaken with chloroform and placed in solid-phase extraction, alkaloids dissolved in chloroform, and phenolic-rich fraction was obtained. The quantity of extracted curcumin was estimated, shown in Table 2. Then, the residual weight of the Petri plate is recorded down and deducted from the original empty weight of the Petri plate.

Table 1. The Major Phytochemical Compounds in *Raphanus Sativus* and *Curcuma Longa*

Raphanus sativus		*Curcuma longa*	
Phytochemical	Molecular weight	Phytochemical	Molecular weight
Urea,N,N'-bis (2-hydroxyethyl)	148	Bicyclo[4.1.0]-3-heptene,2-isopropenyl-5-isopropyl-7,7-dimethyl	204.35
2,4-Pentadienenitrile	79	Xylopropamine	163.26
Furfural	96	1-(3,3-Dimethyl-but-1-ynyl)-1,2-dimethyl-3-methylene cyclopropane	162.7
Dimethyltrisulfide	125	6-Octen-1-yn-3-ol, 3,7-dimethyl	152.2
Propylparaben	180	Ethyl iso-allocholate	436.6
1,4-Dihydrothujopsene-(l1)	206	Curcumin	368.4
4(2,5-Dihydromethoxyphenyl)butylamine	181	α-Curcumene	202.3
1-Butene ,4-isothiocyanato-1 (methylthio)	159	β-Curcumene	204.7
2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-1-methoxy-	180	Isocurcumenol	234.3
5-Hydroxymethylfurfural	126		

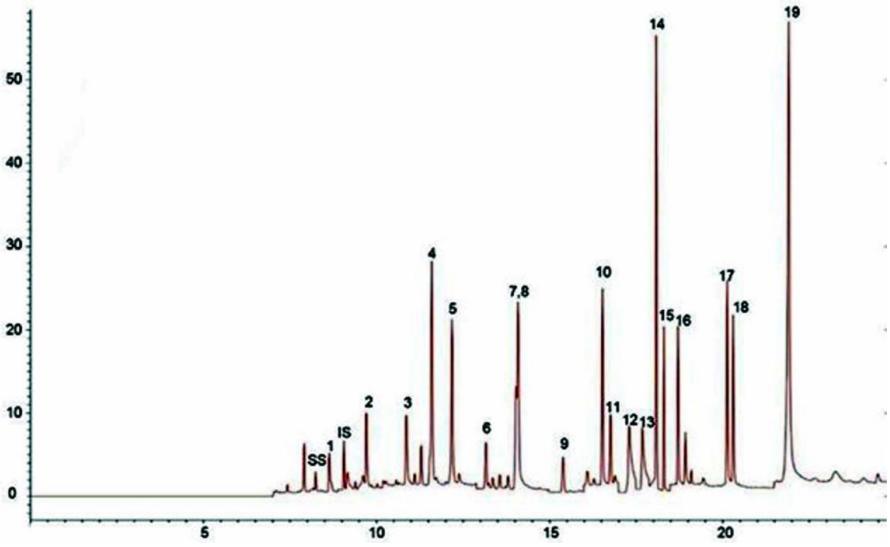

Fig. 1. GC-MS analysis of *Raphanus sativus.*

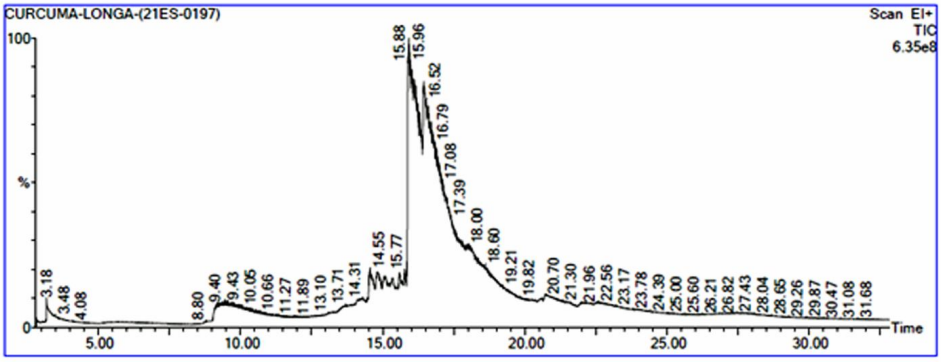

Fig. 2. GC-MS analysis of curcuma longa**.**

Table 2. Solvent Extraction of Curcumin

Particle size	Curcumin content g/100 g
	Solvent-methanol
250 μ	0.01238
44 mesh	0.01337
30 mesh	0.01239
Above 30 mesh	0.01139

Table 3. Experimental ^{1}H NMR of Curcumin

H-Position	^{1}H NMR		
	δ (ppm)	J (Hz)	Number of H
1	5.8 (s)	-	1
2	16.1 (bs)	-	1
3,3’	6.5 (d)	15.6	2
4,4’	7.6 (s)	15.6	2
5,5’	-	-	-
6,6’	7.1 (dd)	8.4	2
7,7’	6.9 (d)	8.4	2
8,8’	-	-	2
9,9’	-	-	-
10,10’	7.3 (s)	-	2
OMe	3.9 (s)	-	6

NMR Spectroscopy

NMR is the most common technique used to identify the magnetic property of biological compounds [8]. The experimental chemical shift of ^{1}H of curcumin and cathechol were observed in dimethyl sulfoxide-d6 (DMSO-d6) as a solvent. Two multiplets were present regardless of the solution, while the addition of NaOH with 1:1 ratio did not cause any changes in the ^{1}H NMR spectra shape. However, with increasing the molar ratio of the NaOH, the spectra were shifted toward the more negative values. The above changes clearly indicated that the chemical structure of the catechol did not change significantly, while the shifts might indicate the formation of sodium salts of catechol (Fig. 3). The chemical structures in dried turmeric extract were identified by comparison of the ^{1}H NMR spectra shown in Fig. 4, and are presented in Table 3. The chemical shift of aromatic protons was in the range of 6.5-8.0 [9,10]. From the present investigations, the chemical shift of the hydrogen ions was in the range of 5.8-7.3 ppm. The chemical shift of O-H proton was observed at 5.8 ppm, representing the aromatic C-OH. Chemical shift at 7.6, 7.1 ppm denotes the hydrogen atom in benzene ring and ethylene group. The proton shift at 6.5, 6.9 ppm shows the presence of hydrogen ions in ethylene group.

SEM Analysis

To identify the morphology and the size of the bioreduced silver nanoparticles, Scanning Electron microscope (SEM) analysis was used. From the current

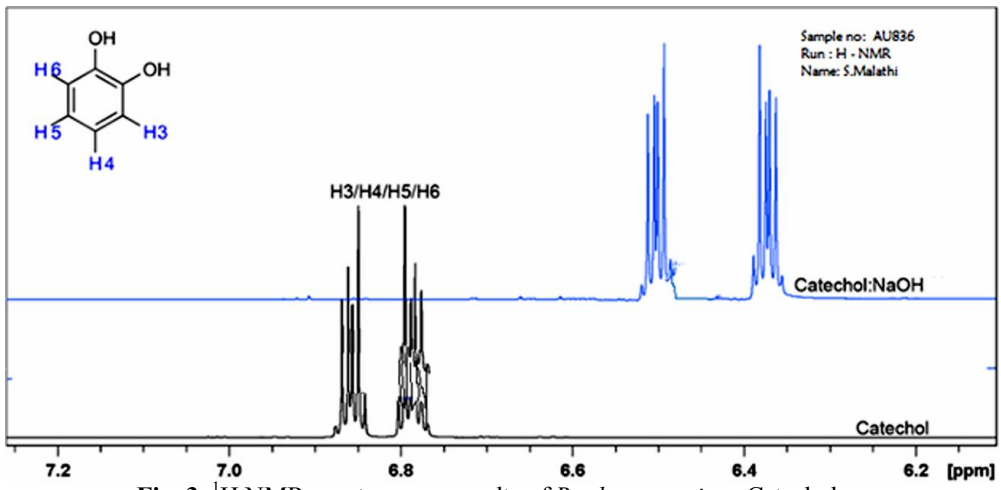

Fig. 3. ^{1}H NMR spectroscopy results of *Raphanus sativus*-Catechol.

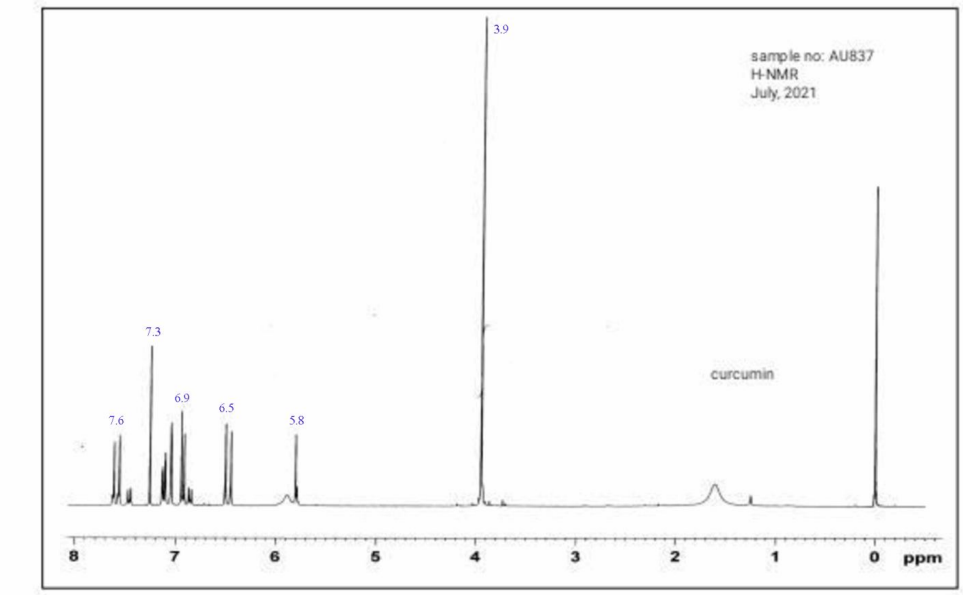

Fig. 4. [1]H NMR spectroscopy results of *Curcuma longa*-Curcumin.

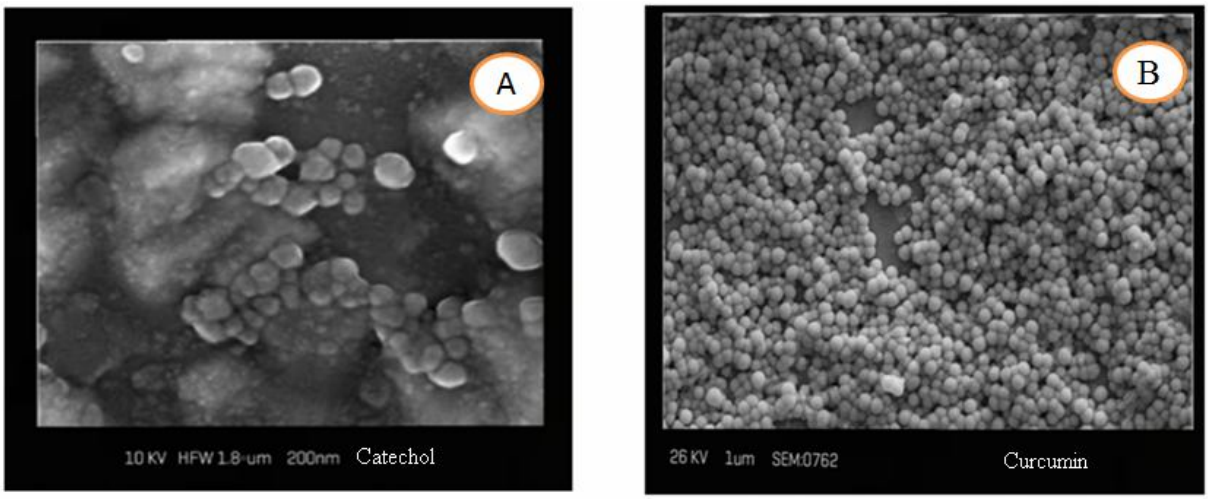

Fig. 5. SEM analysis of catechol and curcumin capped silver nanoparticles.

study, the SEM images of catechol-AgNPs illustrated the particles with defined morphology, and aggregation might be due to the presence of secondary metabolites catechol size in the range of 200 nm, shown in Fig. 5A. The morphology of the curcumin-Ag NPs was spherical. The mean diameter of the crystalline C-Ag NPs was 12.6 ± 3.8 nm, crystalline size less than 50 nm surrounded by the capping agent. The results are shown in Fig. 5B.

Histopathology Findings

A. Urinary bladder of albino mouse in control group showed normal histological structure of the lining mucosal epithelium underlying lamina propria and muscularis, Fig. 6A.

B. Urinary bladder of albino mouse in standard group treated with ciprofloxin showed normal histological structure with connective tissue and muscle layer,

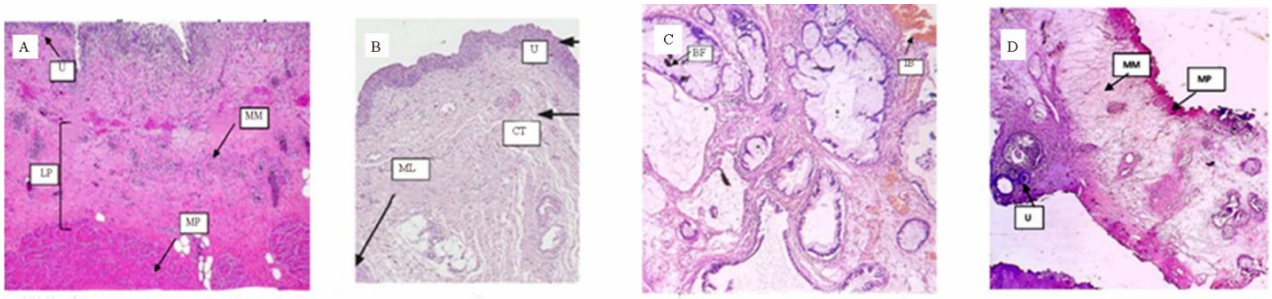

Fig. 6. Histopathology finding of urinary balder A. Control mouse, B. Ciprofloxin treated, C. Test group, D. Treated with phytochemical capped silver nanoparticles.

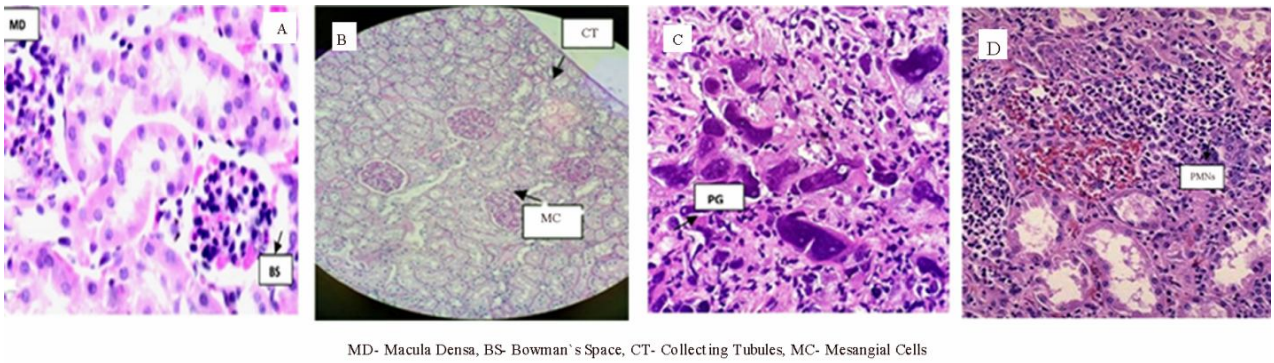

Fig. 7. Histopathology finding of kidney, A. Control mouse, B. Treated with phytochemical capped silver nanoparticles, C. Test group, D. Ciprofloxin treated.

Fig. 6B.

C. Urinary bladder of the mouse in non-treated group with induced *E.coli* showed dysplasia in epithelium, and also mucus production was seen in infiltrates of lamina propria. Intracellular bacterial communities were seen. Biofilm was also obtained, Fig. 6C.
D. Urinary bladder of the mouse in treated group with phytochemical capped silver nanoparticles [synergistically 1:1 ratio (v/v)] Showed that there were no significant changes in organ weight and cell alterations of bladders between mice for each group ($p = 0.19$, $p = 0.68$, and $p = 0.30$, respectively), Fig. 6D.

Kidney Interpretation

A. Renal histology of both control and extract treated animals exhibited normal features: intact glomerular and tubular structures, Fig. 7A and B.
B. Significant ectopic alteration in the structure of kidney tissue in PCNPs-treated mice was related to drastic changes in glomerular attributes and tubular conformation.
C. Pleomorphism obtained in the non-treated group of kidney cells, Fig. 7C.
D. Acute pyelonephritis obtained as of bacterial infections, numerous polymorphonucelar cells, and Leukocytes form cast within the tubules.
E. Ciprofloxacin treated mouse: Kidney section showed normal renal cortex and glomerular tufts, Fig. 7D.

CONCLUSIONS

The current study showed that synthesis of AgNPs using catechol (*Raphanus sativus)* and Curcumin (*Curcuma longa)* was easy, rapid, inexpensive, and environmentally-friendly. The mix treatment using phytochemicals such as

catechol and curcumin mixed with silver nanoparticles (C-CAgNPs) affected the biofilm development and multidrug resistant *E.coli*. The combination therapy (C-C AgNPs) was the most potent therapy to eradicate the biofilm compared to the monodrug therapy. These agents were also nontoxic to ensure the health of human epithelial cells. In addition, the significant antibacterial potential of these green synthesized (C-C AgNPs) was explored in *in vivo* studies. Based on all these results, we concluded that green phytochemical capped silver nanoparticles fabricated in our study showed much greater antibacterial activity than the plant extracts.

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