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Biosynthesis of silver nanoparticles using flower extract of *Cassia roxburghii* DC and its synergistic antibacterial efficacy

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Abstract. In the present study, green synthesis of silver nanoparticles (AgNPs) was attempted using flower extract of *Cassia roxburghii* belonging to the family Fabaceae. Synergistic antibacterial activity of AgNPs with 15 commercial antibiotics was evaluated against 2 Gram-positive bacteria (*S. aureus* and *B. cereus*), 2 Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and 4 fungi, which included one clinical isolate (*C. glabrata, C. albicans, C. neoformans* and *1 Candida sp.*, clinical isolate). Characterization of synthesized AgNPs was done by various spectral analysis, including UV-Vis spectroscopy, Fourier transform infrared spectroscopy, X-ray diffraction, Transmission electron microscopy and zeta potential. The UV-Vis spectrum of AgNPs showed absorption maxima at 348 nm. TEM analysis revealed that AgNPs were flower-like and/or triangular in shape, ranging from 5 to 360 nm in size. The antibacterial activity of AgNPs with antibiotics was better than antibiotics alone against the tested bacterial strains. The highest enhancing effects were observed for 7 antibiotics against *S. aureus*. This is a simple, economic, ecofriendly, nontoxic, quick and green synthesis of AgNPs using an aqueous flower extract of *Cassia roxburghii*. It can be definitely used in cosmetics, foods and medical applications.

1. Introduction

Nanoparticles have received considerable attention because of their unique physical and chemical properties, owing to their size, and have wide and multiple applications in biotechnological and pharmaceutical industries. They are used in medicine, diagnostics and drug delivery systems [1], cancer therapy [2], cosmetics, electronics and house hold appliances [3], agriculture [4], and antibacterial and antifungal agents [5]. They also show antioxidant [6] and anti-inflammatory activity [7]. Silver nanoparticles are also used for biological sensing and imaging because of their strong optical features [8].

Infectious diseases are a major public health threat globally. The majority of problematic infections are caused by multi drug resistant pathogens. For example, Gram-positive *Staphylococcus aureus* has evolved from penicillin-resistant phenotypes into a methicillin-resistant strain (MRSA), which has become a global epidemic [9]. *Enterococcus faecium, Staphylococcus aureus, Clostridium difficile, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacteriaceae* (including *Escherichia coli, Klebsiella pneumoniae, Enterobacter* spp.) are all multi drug resistant strains. This severely limit therapeutic options and cause increased morbidity and mortality. The widespread an-

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tibiotic resistance of these pathogens is most alarming in hospital settings (nosocomial infections) [10]. This situation urges the importance of searching for novel and potent therapeutic agents to treat bacterial and fungal infections.

To tackle this burning problem, a ray of hope was the use of natural extracts from medicinal plants and metal particles, like silver. The therapeutic applicability of silver and medicinal plants in treating bacterial infections is already well known [11,12]. A more recent approach is the use of silver nanoparticles as antimicrobial agents, which has given quite successful results, making them an attractive alternative to antibiotics.

Silver nanoparticles can be synthesized by various methods, like ultraviolet irradiation, aerosol technologies, lithography, laser ablation, ultrasonic fields, heating and electrochemical reduction, photochemical reduction and application of reducing chemicals, like hydrazine hydrate and sodium citrate, sodium borohydride, formaldehyde, polyethylene glycol, glucose, etc. [12]. However, these techniques are expensive and sometimes hazardous chemicals are involved in their synthesis, which are harmful to the environment. To circumvent this, many biological systems, like bacteria, fungi, yeast, cyanobacteria, actinomycetes and plants, have been used. The best, however, was the use of plant parts, because they are economic, easily available and ecofriendly [13]. An added advantage is that silver nanoparticles can be synthesized by any part of the plant, like the leaf, flower, stem, seed, peel, etc. [14-19].

The *Cassia* (Family: Fabaceae) species have biological and pharmacological activities, such as being hepatoprotective [20], antimicrobial [21,22], antioxidant [23], immune-modulatory [24], analgesic and anti-inflammatory [25], antidiabetic [26], etc. *Cassia roxburghii* is one of the medicinal plants used in ethnomedicine for the treatment of various liver ailments [27].

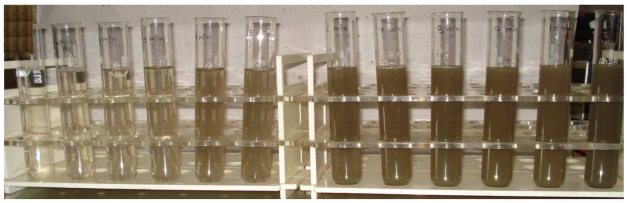
In the present study, for the first time, silver-nanoparticles were synthesized by the reduction of aqueous silver ions and aqueous extract of *Cassia roxburghii* DC flowers (Figure 1(a)). Characterization was done by various spectral analyses and, finally, synergistic antibacterial activity with commercial antibiotics against some pathogenic bacterial and fungal strains was investigated.

2. Materials and methods

2.1. Chemicals

Fresh flowers of *Cassia roxburghii* DC were collected from Saurashtra University Campus, Rajkot, Gujarat, India. All chemicals were obtained from Hi Media Laboratories and Sisco Research Laboratories Pvt. Limited, Mumbai India. Ultra purified water was used for all the experiments.

(a)



(b)

Figure 1. (a) Photograph of *Cassia roxburghii* DC. (b) Color change in the reaction mixture within 10 min.

2.2. Preparation of the extract

Fresh flowers were thoroughly washed with tap water, followed by double distilled water, and then cut into small pieces. 5 g of cut flowers were boiled for 5 min in 100 ml ultra-pure water and filtered through Whatmann No. 1 filter paper. The filtered *C. roxburghii* flower extract was used for the synthesis of silver nanoparticles.

2.3. Synthesis of silver nanoparticles

Silver nanoparticles were synthesized by the addition of 6 ml of flower extract to 40 ml of 1 mM $AgNO_3$ solution. The synthesis of silver nanoparticles was carried out at room temperature (25°C±2°C) for 24 h in the dark. The silver nanoparticles solution thus obtained was purified by repeated centrifugation at 10,000 rpm for 10 min, followed by redispersion of the pellet of silver nanoparticles into acetone. After air drying, the purified silver particles were stored at 4°C for further analysis.

2.4. Characterization of the synthesized silver nanoparticles

The synthesis of silver nanoparticles solution is easily observed by ultraviolet - visible (UV-Vis) spectroscopy. The reduction of the Ag^+ ions in solution was monitored by periodic sampling of the aqueous component and measuring the UV-Vis spectra of the solution. UV-Vis spectra were recorded as a function of time of reaction on a spectrophotometer UV-1601 PC spectrophotometer (Shimadzu, Japan) at a 400-700 nm range, operated at interval of 10 nm.

2.5. FTIR analysis of Ag nanoparticles

Possible functional groups involved in the synthesis and stabilization of silver nanoparticles were studied

by FTIR spectroscopy. The FTIR was recorded in the range of 400-4000 cm^{-1} Nicolet IS10 (Thermo Scientific, USA). The various modes of vibration were identified and assigned to determine the different functional groups present in the *Cassia* flower extract.

2.6. Zeta potential measurement

Zeta potential is an essential parameter for the characterization of stability in aqueous nano suspensions. The zeta potential measurement was performed using a Microtra spectrometer (Zetatra Instruments).

2.7. XRD measurement

The structure and composition of synthesized AgNPs were analyzed by XRD. The formation of silver nanoparticles was determined by an X'Pert Pro X-ray diffractometer (PAN analytical BV), operated at a voltage of 40 kV and a current of 30 mA, with Cu Kα radiation, in $\theta - 2\theta$ configurations. The crystallite domain size was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherrer formula. $D = 0.94\lambda/\beta \text{Cos}\theta$, where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the Full Width at Half Maximum (FWHM), and θ is the diffraction angle.

2.8. TEM analysis of silver nanoparticles

TEM analysis was done to visualize the shape, as well as to measure the diameter of the biologically synthesized silver nanoparticles. The sample was dispersed in double distilled water. A drop of thin dispersion was placed on a "staining mat". A carbon coated copper grid was inserted into the drop with the coated side upwards. After about ten minutes, the grid was removed and air dried, then screened in a JEOL JEM 2100 Transmission Electron Microscope.

2.9. Antimicrobial activity

The antimicrobial activity of AgNPs with 15 commercial antibiotics (6 mm) and antibiotics alone was determined against 2 Gram-positive bacteria (*S. aureus* and *B. cereus*) and 2 Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and 4 fungal (*C. albicans, C. glabrata, C. neoformans* and No. 44, a clinical isolate) strains, using an agar disc diffusion method [28]. A stock solution of 4 mg/ml of AgNPs was prepared and 20 μl per disc was added.

3. Results and discussion

The flower extract or $AgNO_3$ solution alone did not show any change in colour, but, as soon as both were mixed, the solution changed to a brownish colour within 2 min, the intensity of which sharply increased. Within 10 min, it turned to black, indicating the synthesis of silver nano particles (Figure 1(b)). Maximum colour intensity was observed within 2 h, after which there was very little change in visual colour. This reaction mixture was kept in the dark for 24 h at room temperature ($25 \pm 2^{\circ}C$) for complete reduction of silver ions. The observed color change in the reaction mixture is in agreement with previous reports by Raja et al. [29]. The colour change occurred because of the phytochemicals present in the flower extract, which reduced the silver metal ions into silver nanoparticles due to the excitation of surface plasmon resonance for AgNPs.

The UV-Vis spectra recorded from the flower extract of *C roxburghii* at different time intervals of reaction are presented in Figure 2(a). The maximum absorption peak was at 348 nm. The peak observed for AgNPs is comparable to that of *Morinda pubescens* [30].

The obtained maximum absorption peaks in the UV spectra are assumed to correspond to silver nanoparticles and are well dispersed in the aqueous solution without any aggregation [31]. Silver nano particles can be synthesized by reducing silver ions using some chemicals. But, in green synthesis, the

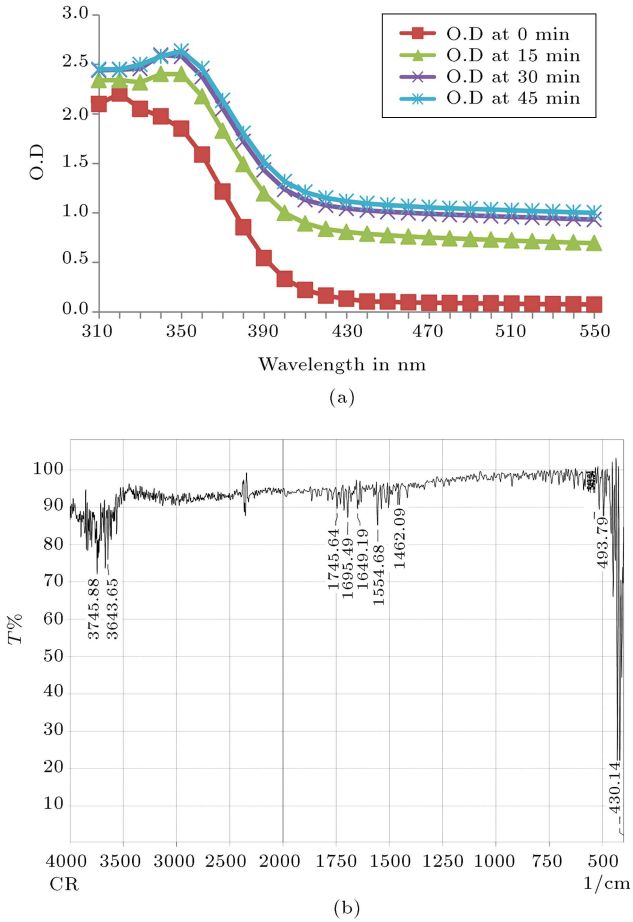


Figure 2. (a) UV-visible spectrum of biosynthesized CR-AgNPs at different time intervals showed peak at 348 nm. (b) FTIR spectrum of biosynthesized CR-AgNPs.

natural plant extract acts as a reducing agent for generating silver nanoparticles.

FT-IR has become an important tool in understanding the involvement of functional groups in the relation between metal particles and biomolecules. In the present study, FT-IR measurements were performed to identify the possible biomolecules responsible for capping and stabilizing the silver nanoparticles synthesized using aqueous flower extract of *C. roxburghii*. The spectra of AgNPs (Figure 2(b)) revealed strong bands at 3745.88, 3643.65, 1745.64, 1695.49, 1649.19, 1554.68, 1462.09, 543.94 and 493.79, respectively. The intense bands at 3745.88, and 3643.65 cm^{-1} are characteristic groups of the primary O-H stretching of alcohols and phenols. The peak 1745.64 cm^{-1} corresponds to the C=O stretch of anhydrides. The peaks of 1695.49, 1649.19, 1554.68 and 1462.09 cm^{-1} are assigned to the stretching of C=O, C-C, C=C and C-I, respectively, of aldehyde, the C=C ring, and the aromatic group. The peak of 543.94 cm^{-1} corresponds to the C-X (fluro alkanes, chloro, bromo, iodo alkenes) compound. The peak 493.79 cm^{-1} corresponds to the C-I stretch of the iodo compound. These functional groups may play an effective role in the green synthesis of silver nanoparticles. But, the possible mechanism is unclear and needs further investigation.

The zeta potential of the synthesized AgNPs is determined in water as a dispersant. The zeta potential was found to be +29.20 mv (Figure 3); a zeta potential higher than 30 mV or less than −30 mV is indicative of a stable system [32]. It has been stated that secondary metabolites present in the plant are responsible for stabilizing the synthesized nanoparticles [33].

X-Ray Diffraction (XRD) patterns of silver nanoparticles synthesized using the *C. roxburghii* flower at room temperature indicate that the structure of silver nanoparticles is face-centered cubic (fcc) (Figure 4). In addition, the XRD peaks at 2h of 23.36, 23.84, 22.98, 19.22 and 17.73 could be attributed to 111, 200, 220, 311 and 222 crystallographic planes. The lattice constant calculated from this pattern was 4.0865 A, a value in agreement with the literature report (a - 4.086 A, JCPDS File No. 04-0783). This suggests that the silver nanoparticles were crystalline in nature. Sukirtha et al. [34] also reported a similar type of results. The sharpness of the peaks clearly indicated the crystalline nature of the synthesized AgNPs [35].

The elemental composition of powdered samples was determined using TEM equipped with an EDAX detector. Morphological and structural studies were investigated using Transmission Electron Microscopy (TEM). The synthesized Ag particles were spherical, flower-like and/or triangular in shape, ranging from 5 to 360 nm in size (Figure 5(a), (b), (c)). The energy dispersive X-ray analysis (EDAX) shown in Figure 5(e) revealed the strong signal in the silver region and confirmed the formation of AgNPs. Metallic silver nanocrystals generally show typical optical absorption peaks approximately at 2.983 keV, due to surface plasmon resonance. There were spectral signals for C and Cu because of the TEM grid used. From the EDAX spectrum, it is clear that *C. roxburghii* has a percent yield of 61.46% of Ag-NPs. The results indicate that the synthesized nanoparticles are composed of high purity Ag-NPs. In order to verify the crystalline nature of the nanoparticles, the Selected Area Electron Diffraction (SAED) patterns were obtained for the sample containing 1 mmol L^{-1} of $AgNO_3$, for different

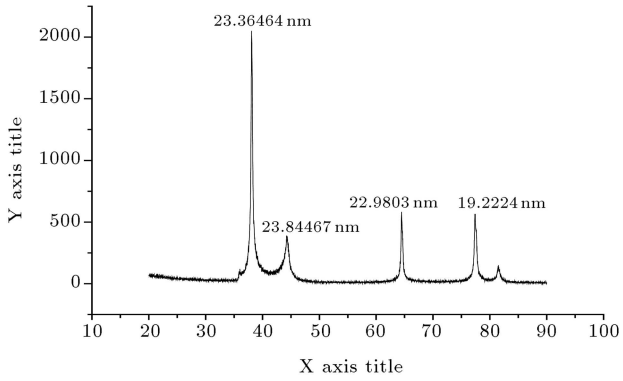
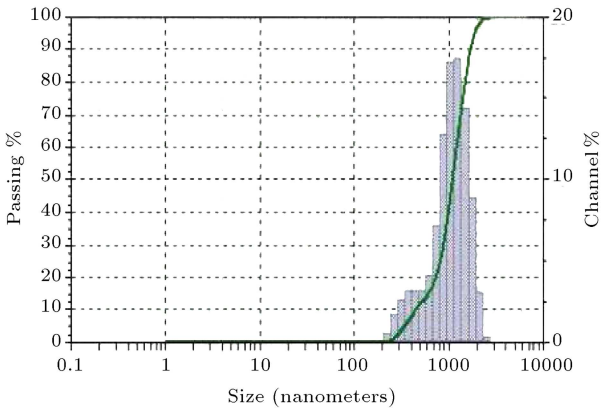


Figure 4. XRD spectrum of biosynthesized CR AgNPs.



Zeta info

Mobility	2.28 u/s/V/cm
Zeta potential	29.20 mv
Charge	0.197 fC
Polarity	Positive
Conductivity	10 uS/cm

Figure 3. Zeta potential measurements of biosynthesized CR-AgNPs.

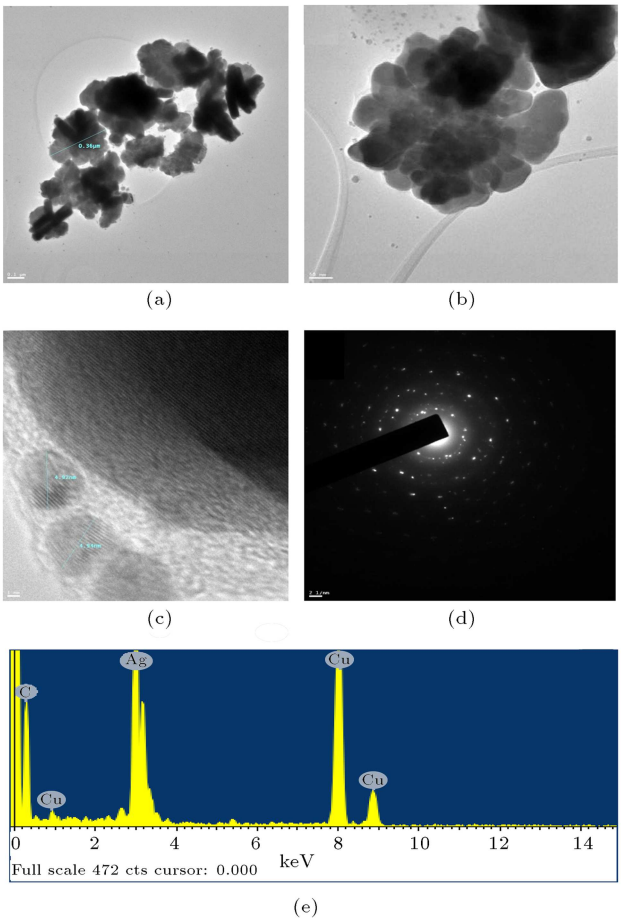


Figure 5. (a),(b),(c) TEM images of Ag nanoparticles in low and high magnification. (d) SAED patterns of the silver nanoparticles. (e) EDX spectrum showed higher percentage of silver signal.

regions and particles, as shown in Figure 5(d). The inset in each pattern shows the respective selected region. The presence of bright circular rings in the SAED patterns confirms the crystalline nature of the silver nanoparticles. The spots, corresponding to various orientations appearing inside the concentric rings, also show that the obtained silver nanoparticles have good crystallinity.

3.1. Antimicrobial activity of AgNPs

The burning health problem facing the world today is the increase in multidrug resistant microorganisms. Existing antibiotics are not able to tackle this problem. Hence, there is an urgent need to find novel strategies to overcome this problem [12,36]. In the present study, 11 commercial antibiotics, which are widely used against bacterial and fungal infections, were tested against 4 bacteria and 4 fungi, individually, and with synthesized AgNPs. The diameter of the zone of inhibition and increase in the fold area for all bacterial and fungal strains was measured. The antibacterial and antifungal activity increased significantly in the presence of AgNPs. AgNPs showed synergistic antibacterial activity with all 11 antibiotics against all 4 bacterial strains with varied levels of increase in the fold area (Table 1). Maximum antibacterial activity was observed against *S. aureus* with the highest increase in fold area (1.0), followed by *B. cereus* (0.91) (Table 1). AgNPs with 7 antibiotics showed a significant increase in fold area against *S. aureus*. The enhanced antibacterial activity with antibiotics is also reported by other researchers [37-41].

Maximum antifungal activity was observed

Table 1. Synergistic activity of CR -AgNPs with different standard antibiotics against Gram-negative and Gram-positive bacteria.

Anti.[a]	*Escherichia coli* (NCIM NO 2931)			*Pseudomonas aeruginosa* (ATCC NO 27853)			*Bacillus cereus* (ATCC NO 11778)			*Staphylococcus aureus* (ATCC NO 29737)		
	Anti. (A)	Anti. + AgNPs (B)	Increase in fold area	Anti. (A)	Anti. + AgNPs (B)	Increase in fold area	Anti. (A)	Anti. + AgNPs (B)	Increase in fold area	Anti. (A)	Anti. + AgNPs (B)	Increase in fold area
AMP	9	9	0	23	27	0.37	-	-	-	33	35	0.1
PB^{100}	9	10	0.23	13.5	15	0.23	11	11	0	9	11.5	0.38
Gen^{10}	13.5	16.5	0.49	22	26	0.39	19.5	20.5	0.10	16	20.5	0.64
C^{30}	25.5	26.5	0.07	14	13.5	0	33	34	0.06	24	32	0.77
P^{10}	-	-	-	14	15.5	0.22	-	-	-	33	41.5	0.58
Ak^{10}	17.5	17.5	0	24.5	29	0.41	22	24.5	0.24	17	21.5	0.59
TE^{30}	22	25	0.22	26.5	33.5	0.59	27.5	29	0.09	28	36.5	0.69
CEP^{30}	10	11	0.17	11	11	0	13	18	0.91	37	40.5	0.19
AMC^{10}	9	9	0	20.5	24.7	0.45	-	-	-	31.5	34.5	0.19
CFP^{30}	9	12	0.7	34	37	0.18	13.5	13.5	-	24	31	0.66
CC^{10}	12	15.5	0.6	-	-	-	14	18	0.65	11.5	16.5	1.0

[a]Anti.: Antibiotic; AMP: Ampicillin; PB^{100}: Polymyxin-B; Gen^{10}: Gentamicin; C^{30}: Chloramphenicol; P^{10}: Penicilli-G; AK^{10}: Amikacin; TE^{30}: Tetracycline; CEP^{30}: Cephalothin; AMC^{10}: Amoxyclav; CFP^{30}: Cefpirome; CC^{10}: Clotrimazole.

Table 2. Synergistic activity of CR-AgNPs with different standard antibiotics against fungi.

Anti.[a]	*Candida glabrata* (NCIM NO 3448)			44 (Clinical fungus isolate)			*Candida albicans* (NCIM NO 3102)			*Cryptococcus neoformans* (NCIM NO 3542)		
	Anti. (A)	Anti. + AgNPs (B)	Increase in fold area	Anti. (A)	Anti. + AgNPs (B)	Increase in fold area	Anti. (A)	Anti. + AgNPs (B)	Increase in fold area	Anti. (A)	Anti. + AgNPs (B)	Increase in fold area
NS^{100}	29	33.5	0.33	21	23	0.19	17	17.5	0.05	23.5	25.5	0.17
KT^{30}	24.5	32.5	0.75	-	-		15	16	0.13	15.5	20	0.66
FLC^{10}	21.5	27	0.57	-	-		20.5	22	0.15	13.5	16.5	0.49
AP^{100}	15	17	0.28	12	12.5	0.08	10.5	11	0.09	12	12	0

[a] Anti.: Antifungal; NS^{100}: Nystatin; KT^{30}: Ketoconazole; FLC^{10}: Fluconazole; AP^{10}: Ampotericin.

against *C. glabrata* with ketoconazole, followed by *C. neoformans*. The highest increase in fold area was 0.75 and 0.66, respectively (Table 2). 44 is a clinical isolate, which also showed some activity against nystatin and amphotericin B, though no activity was observed with ketoconazole and fluconazole. The antifungal activity of AgNPs with commercial antibiotics has been also reported in [42,43]. However, they have been reported against only fungi and only with 2 antibiotics, i.e. fluconazole and amphotericin B, respectively. To date, synthesis of AgNPs with flower extracts is rare and synthesis of AgNPs with *C. roxburghii* flower extract is reported for the first time. Moreover, the combination or synergistic effect of 15 antibiotics with AgNPs against pathogenic bacteria and fungi is a new finding. The reduction of silver ions occurred due to water-soluble phytochemicals, like flavonoids, saponins, alkaloids, triterpenes and phlobatanins present in the flower sample of *C. roxburghii* (Table 3). The results clearly demonstrated that AgNPs synthesized by the green route can definitely compete with commercial antibiotics used for the treatment of microbial infections and, sometimes, are even better.

Table 3. Phytochemical test of CR flower powder.

Test	Result
Flavanoids	++++
Tannins	+
Phlobatanins	+++
Triterpenes	+++
Steroids	-
Saponins	++++
Cardiac glycosides	-
Meyer's	-
Dragondroff	+++
Wagners	-
Legal's	-

Phytochemicals present in less (+), moderate (++), high (+++), and very high (++++) amounts; absent (-).

4. Conclusions

Silver nanoparticles were synthesized using an aqueous flower extract of *Cassia roxburghii*. The method is simple, efficient and ecofriendly. FTIR, XRD and TEM, characterized by UV-Vis spectroscopy, evidenced the formation of nanoparticles. The synthesized *Cassia roxburghii*-AgNPs showed very promising antimicrobial activity against some pathogenic bacterial strains, and the activity was even better than some of the commercial antibiotics. Thus, these ecofriendly silver nanoparticles can be used as an excellent antimicrobial agent against multi drug resistant pathogenic microorganisms. However, more research work, especially on animal models, needs to be done before they can be used as antimicrobial agents.

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