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Green Synthesis of Silver Nanoparticles from *Caesalpinia gilliesii* (Hook) leaves: antimicrobial activity and *in vitro* cytotoxic effect against BJ-1 and MCF-7 cells

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ABSTRACT

Green synthesis of silver nanoparticles using *Caesalpinia gilliesii* (Hook) leaves extract (70% MeOH) for the first time as a reducing agent were investigated for their antimicrobial and cytotoxic activity (using the MTT assay). After exposing the oxidizing agent of silver ions to *C. gilliesii* leaves extract, the rapid reduction in the solution is observed due to the construction of silver nanoparticles. The prepared nanoparticles were elucidated by using UV-visible spectroscopy, Fourier Transforms infrared spectroscopy (FT-IR) and transmission electron microscope (TEM). The purified silver nanoparticles demonstrated promising antimicrobial activity against tested pathogens than hydroalcoholic extract. Cell viability by using the MTT assay demonstrated cytotoxic activity of the synthesized Ag NPs with C. *gilliesii* against normal skin fibroblast (BJ-1) and human breast cancer cell (MCF-7) with IC_{50}= 80.1 and 36.5 μg/mL at 48 hours incubation, respectively. Depending on the phenolic and flavonoid contents, *C. gilliesii* could be used for simple, nonhazardous, eco-friendly, cost-effective and efficient synthesis of Ag NPs that can be applied into medicinal field.

INTRODUCTION

The scientists all over the world always look for new and useful applications to humanity. Especially, the emergence of drug resistance is a significant problem due to misuse of antibiotics policy inside the countries that encourage us to develop alternative antimicrobial drugs from medicinal plants (Parekh *et al.*, 2005; Bagiu *et al*., 2012). Moreover, the toxicity of natural products has attracted the scientists for preparations as antitumor agents that elevate people's health without detectable adverse effect (Nazarizadeh *et al.*, 2013).

Nanoscience is considered one of the latest sciences which have attracted the attention of scientists. This is a new field that incorporates the fabrication and usage of nanoscale size materials to various applications (Rao *et al*., 2013; Filippo *et al*., 2010; Vivek *et al*., 2012), especially it's clean, non-toxic and eco-friendly (Jayaseelan *et al*., 2013).

Several extracts of plants, marines and microorganisms have been reported for the preparation of silver nanoparticles (Seema *et al.*, 2010). As though, the potential of natural sources as bioactive materials for the preparation of nanoparticles and their compatibility with biological systems is not fully discussed.

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All approaches come up to give hope to be applied in large scale, for commercial applications by managing different physical and chemical parameters or genetically modified microbes' production that overmuch characteristic reducing agents and thereby, controlling biological nanoparticles resulted (Kannan *et al.,* 2010), which reacts as a capping agent and prevent the nanoparticles accumulation. Phytoconstituents of plant extracts such as phenolics, terpenoids, plant enzymes and their derivatives react as reducing agents in the presence of metal sources for the formation of Ag NPs (Jacob *et al.,* 2011; Thakkar *et al.,* 2010). Moreover, some plant extracts have been explored in the production of metallic nanostructures under different environmental conditions. Nevertheless, in some cases, plants can absorb metals from the surrounding environment and accumulate them as nanostructures form inside the tissues (Quester *et al.,* 2013). Leguminosae (Fabaceae) family is the second largest flowering plants that exceed in the number of genera and species. It is widespread in distribution, divided into three sub-families: Mimosaceae; Caesalpinacae and Papilionaceae. Caesalpinacae demonstrates approximately 11% of the identified legume flora (Kirkbride, 1986), mostly equatorial and sub equatorial in allocation. Many species of *Caesalpinia* used in folk medicine, like *Caesalpinia bonducella* seeds (Rao *et al.,* 1994), *C. digyna* and *C. sappan* (Penpun *et al.,* 2005) and were reported as antioxidant (Saenjum *et al.,* 2010; Shukla *et al.,* 2009), anti-inflammatory (Chakraborthy *et al.,* 2009; Sagar *et al.,* 2009), hepatoprotective, antibiotic (Tasleem *et al.,* 2009), antidiabetic (Kannur *et al.,* 2006; Farook *et al.,* 2011), antiviral (Jiang *et al.,* 2002 a, b), and anticancer (Nakamura *et al.,* 2002) activities. As, the plant family is rich in nitrogen compounds, saponins, terpenoids, glycosides derivatives and phenolics (Sivasankari *et al.,* 2010; Banskota *et al.,* 2003; Kalauni *et al.,* 2006; Jadhav *et al.,* 2003). *Caesalpinia gilliesii* (Hook) (Yellow Bird of Paradise) is a fast growing tree with Argentine nationality (South America). It grows in Egypt (Borg El Arab) and cultivated in private farms in Egypt (El-qanater El khairia). Previous preliminary phytochemical screening on leaves extract manifested the existence of carbohydrates, glycosides, saponins, phenolics, fats and terpenoids with different ratios (Osman *et al.,* 2013).The present work aimed to estimate the total content of Phenolics (TPC) and Flavonoids (TFC) in the hydroalcoholic extract of *C. gilliesii* leaves, synthesize, characterize, evaluate antimicrobial activity and cytotoxicity effects on both Skin normal human cell line (BJ-1) and human breast cancer cells (MCF-7) of AgNPs obtained from *C. gilliesii* through various characterization techniques like UV-visible spectroscopy, transmission electron microscope (TEM) and Fourier Transforms infrared spectroscopy (FTIR). To our knowledge, the preparation of Ag NPs by *C. gilliesii* has not been discussed yet.

MATERIAL AND METHODS

Materials

Silver nitrate ($AgNO_3$, Merck), methanol (HPLC, SD Fine-Chem Limited), other chemicals and solvents used were of high grade unless mentioned and were covered from Egyptian market.

Collection and Identification of Plant

Caesalpinia gilliesii (bird of paradise) [(Wall. ex Hook.)] were harvested from Egypt (Borg El Arab) in May 2015. The taxonomical characteristics were approved (Osman *et al.,* 2016) and kept in the herbarium of phytochemistry and plant systematics, pharmaceutical and drug industrial research division, National Research Centre, Dokki, Cairo, Egypt (CAIRC) (M-130) 2015.

Plant Extract Preparation

Freshly collected leaves plant materials were washed several times with domestic water then distilled water after that drying in shade for 8 days at room temperature for 7 days. Hydroalcoholic extract (70 %) of leaves by maceration were prepared. The obtained extract was dried then powdered, stored at 4 °C and used for further investigation.

Estimation of Total Phenolic Content

The total phenolic content (TPC) was calculated as gallic acid equivalent (mg GAE) per g of sample according to the Folin-Ciocalteu procedure (Zilic *et al.*, 2012).

Estimation of Total Flavonoid Content

The total flavonoid content (TFC) was quantitated as catechin equivalent (mg CE) per g of sample (Zilic *et al*., 2012) using aluminum chloride ($AlCl_3$) colorimetric assay.

Biosynthesis of Ag NPs

To study the effect of extract quantity on the reduction and the size of the biosynthesized nanoparticles. At room temperature, Ag NPs were synthesized by the reduction of 10 mL of $AgNO_3$ solution of constant concentration (1 mM) with different concentration of *C. gilliesii* extract (100 µl to 300 µl) from the stock solution (0.04 g extract /10 ml solvent). Also, shake the prepared mixture with hand and allowed to stand in the dark (r.t).

The obtained Ag NPs were purified by repeated centrifugation (10,000 rpm / 20 min) followed by redispersion in deionized water. This process was repeated twice to avoid undesirable matter and isolate the pure Ag NPs (Zayed *et al.,* 2015).

Description of the Biosynthesized Ag NPs

The V-630 UV-Vis spectrophotometer (Jasco, Japan) was used to determine the band metal wave length. Transmission electron microscope (TEM) (JEOL-JEM-1011, Japan) was used to determine the shape and sizes of the Ag NPs. FTIR 6100 spectrometer (Jasco, Japan) exhibited the different functional groups of the prepared nanomaterials in the range of 4000–400 cm^{-1} (Zayed *et al.,* 2015).

Evaluation of antimicrobial activity

Gram-positive, gram-negative bacterial pathogens and yeast were used to test the antimicrobial activity of *C. gilliesii* extract and Ag NPs by the agar well diffusion method (Perez *et al*., 1990). After cooling and solidifying the media, 100 µL of the tested compound solution prepared by dissolving (40 mg/ml) hydroalcoholic extract of *C. gilliesii* and (4 mg/ml) of *C. gilliesii* Ag NPs as stock solutions were loaded per well, then incubated for 24 h at 37 °C. Distilled water (DW) were used to dissolve negative control. Also, 50 µg/ml of both Vancomycine and ketoconazole were prepared as standard. After incubation, the calculated average zone of inhibition in millimeters (mm) is recorded in **Table 2**.

Minimum Inhibitory Concentration (MIC) determination

The bacteriostatic activity of tested samples (inhibition zones (IZ) ≥ 16 mm) was then evaluated using the two-fold serial dilution technique (Scott, 1998). The final concentrations of the solution were 500, 250, 125 and 65 µg/ml. The concentration which showed no growth it considered the minimum inhibitory concentration (MIC).

Evaluation of Cytotoxic activity

Cell culture

Human Caucasian breast adenocarcinoma (MCF-7) and Skin normal human cell line (BJ-1) were maintained in RPMI and DMEM F12 medium, respectively. All media was supplemented with 10% fetal bovine serum, incubated at 37 ^{o}C in 5 % CO_2 and 95% humidity. Cells were sub-cultured using trypsin 0.15 %.

Cell viability assay

Cell viability was estimated by MTT assay (Mosmann, 1983). Briefly, 10000 cells per well of MCF-7 and BJ-1 cell lines (in 96 well plates) were seeded. After 24 h, the medium was changed to serum-free medium containing a final concentration of the samples of 100 µg/ml in triplicates. The cells were treated for 48 h. Doxorubicin was used as positive control (100 µg/ml) and 0.5 % DMSO was used as negative control (Thabrew *et al.*, 1997; Menshawi *et al.*, 2010). The calculation of the cytotoxicity % = [(1- (av(x) / (av(NC))]* 100

(Where; Av: average, X: absorbance of sample & NC: absorbance of negative control)

The absorbance was calculated at 595 nm and a reference wavelength of 620 nm. Moreover, IC_{50} was calculated by using SPSS 11 program (Bassyouni *et al.*, 2014).

RESULTS AND DISCUSSION

Estimation of Total Phenolic Content

The TPC for hydroalcoholic extract was estimated by using gallic acid as standard. The gallic acid concentration (5-50 µg) conformed to Beer's Law at 725 nm with a regression coefficient (R^2) = 0.9985. The plot has a slope (m) = 0.0242 and intercept = 0.0211. The equation of standard curve is Y = 0.0242X + 0.0211 (**Figure 1 and Table 1**).

Estimation of Total Flavonoid Content

The TFC of the hydroalcoholic extract was measured with the aluminium chloride colorimetric assay using Catechin as standard. The Catechin solution of concentration (5-100 µg) conformed to Beer's Law at 510 nm with a regression co-efficient (R^2) = 0.9989. The plot has a slope (m) = 0.0048 and intercept = 0.0091. The equation of standard curve is y = 0.0048x + 0.0091 (**Figure 2 and Table 1**).

TPC and TFC calculated from the previous standard curves as showed at **Table 1** that confirm the existence of phenolics and flavonoids structures.

Table 1: Results of total phenolic and flavonoid content for *C. gilliesii* leaves extract.

Concentration of extract	Phenolic content (mg of gallic acid equivalent/ g dry material)	Flavonoid content (mg of catechin equivalent/ g dry material)
Hydroalcoholic extract, 20mg/2ml	80.07438 mg-GAE/gm	35.375 mg-CE/gm

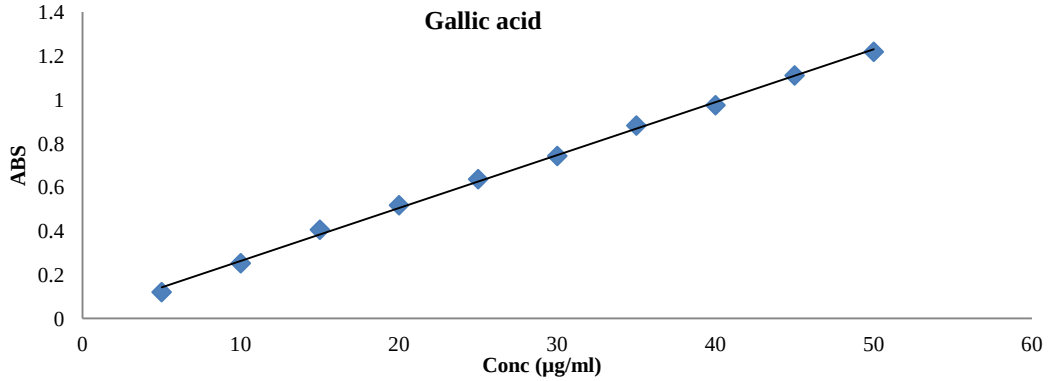

Fig. 1: Total phenolic content for standard gallic acid.

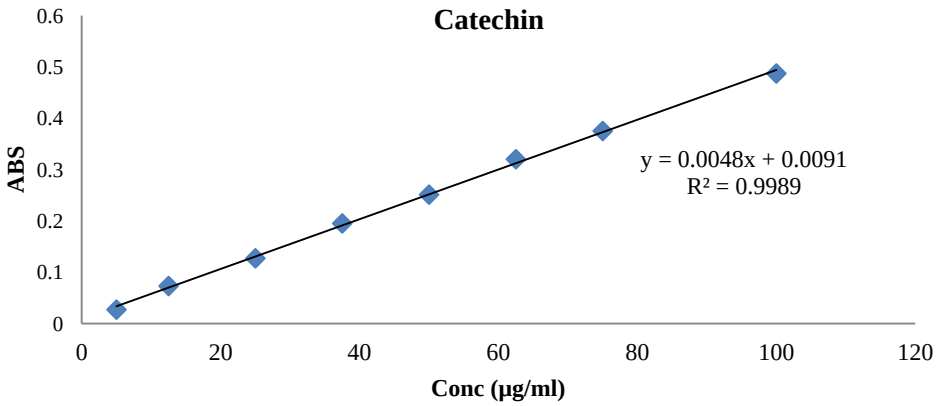

Fig. 2: Total flavonoid content for standard Catechin.

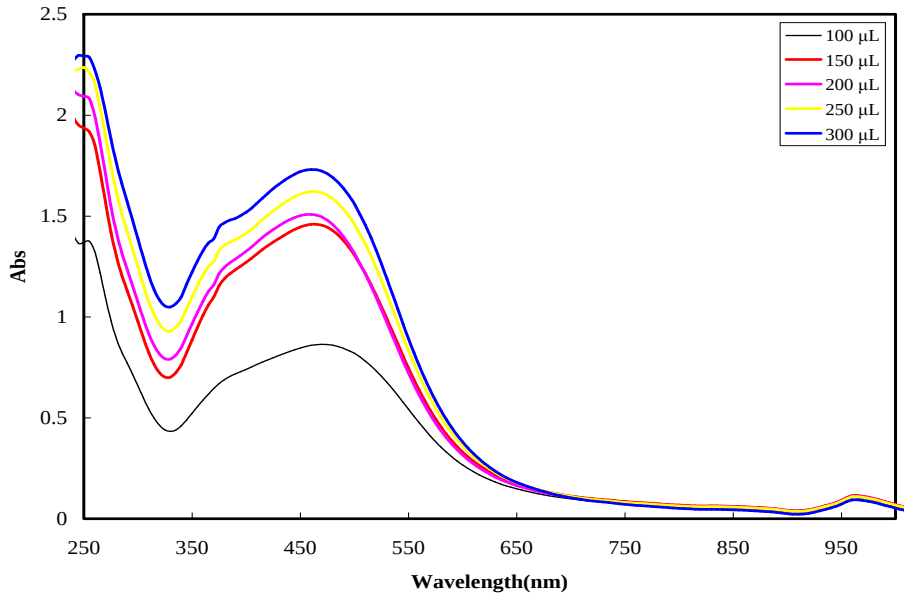

Fig. 3: the SPR band of Ag nanoparticles recorded by UV–vis spectra as a function of varying addition of *C. gilliesii.*

UV-vis Spectroscopic Studies

Ag nanoparticle dispersions are characterized by their brilliant colors. The appearance of the yellowish brown color is because of the surface plasmon resonance (SPR) in the reaction mixture has been taken as an evident for the formation of Ag nanoparticles (Rivero *et al.*, 2013). **Figure 3** illustrates the UV-vis spectra of Ag nanoparticles formed by adding different concentrations of *C. gilliesii* to 10 mL of 10^{-3} M $AgNO_3$ solution. The as-prepared samples show an absorption in the visible region at 465-475 nm due to the SPR band. The intensity of the SPR band grows with the incremental addition of *C. gilliesii*. The increasing intensity of the SPR band indicates that more Ag^+ ions are reduced to Ag nanoparticles. The increased amounts of the extract mean that there are large numbers of functional groups available for the reduction and capping of the Ag nanoparticles. Furthermore, the SPR band exhibits a blue shift (from 470 to 455 nm) as the extract quantity increases from 100 to 200 µL and then it moves toward the longer wavelengths (from 455 nm to 460 nm) as the extract quantity increases from 200 to 300 µL. this behavior could be discussed in the light of the law of mass action i.e. the reaction rate relates directly to the reactants concentration. Hence, it could be concluded that with increasing *C. gilliesii* concentration, the reaction rate increases. Increasing the reaction rate resulted in a faster reduction of Ag^{1+} ions which in turn enhances the nucleation rate. Thus, the blue shift of the SPR is a consequence of the formation of smaller Ag nanoparticles. Further addition of *C. gilliesii* extract enhances the growth rate to produce bigger particles which are reflected in the red shift of the SPR (Zayed *et al.*, 2015) as it showed in **Figure 3**.

FTIR Spectroscopic Studies

The phytochemical results show that the leaves extract of *C. gilliesii* is consisting of a complex mixture of phytochemicals such as saponines, coumarin derivatives, flavonoids, plant sterols,

carbohydrates or glycosides, tannins, cardiac glycosides and cyanogenic glycosides (Osman *et al.*, 2013). These phytochemical species are rich in hydroxyl and amino groups. Several reports attributed the reduction of the metal nanoparticles to the presence of such functional groups (Rai *et al.*, 2013; Thakkar *et al.*, 2010; Iravani *et al.*, 2013; Dauthal *et al.*, 2016).

Hence, the FTIR is a sensitive tool for determining the functional groups responsible for the Ag nanoparticles reduction. Figure 4 showed the FTIR spectra of the *C. gilliesii*-stabilized Ag nanoparticles as compared with that of the naked extract. The spectrum of *C. gilliesii* extract exhibit a broad IR peak spread over the spectral region (3600−3000 cm^{-1}). The IR bands in this region are attributed to the stretching vibrations of the –OH, N−H and C−H groups (Samfira *et al.*, 2015). The IR signal at 3395 cm^{-1} was assigned to the (–OH) group whereas that at 3190 cm^{-1} was due to the stretching vibration of amino groups (–N−H) present in proteins (Zayed *et al.*, 2015). The bending vibrational peak of (–NH) group was remarked at 1616 cm^{-1} while a broad IR peak was found at 1408 cm^{-1} was due to in-plane bending of (–OH) of Phenol or tertiary alcohol. The IR band located at 1062 cm^{-1} is revealed to the stretching vibration of (–C–N) aromatic and aliphatic amines (Barth 2000). Upon interaction with $AgNO_3$, four more remarkable changes on FTIR spectra were remarked. First, the (–OH) band was sharpened and shifted to 3424 cm^{-1} while that of amino group was shifted to 3236 cm^{-1}. Second, the (–NH) bending was shifted to the higher energy side at 1624 cm^{-1}. Third, the appearance of the new peak at 1245 cm^{-1} was attributed to the (–C–O) bending (Butnariu and Giuchici, 2011). Fourth, the (–C–N) stretching vibration was intensified and slightly shifted to 1076 cm^{-1}. These spectroscopic results ascribed the reducing potential of the *C. gilliesii* extract to the presence of hydroxyl and amino groups present within its phytochemicals.

TEM Studies

The shape and size of the as-prepared Ag nanoparticles are evaluated using the HRTEM technique. Figure 5 (a, b) displays the TEM image and the histogram of the particle distribution of the prepared Ag NPs. It can be seen that the as-prepared nanoparticles are mainly spherical in shape with particle size varies between 3-6 nm. The particles are separated from each other which reflect the capping action of the plant extract in the preparation process.

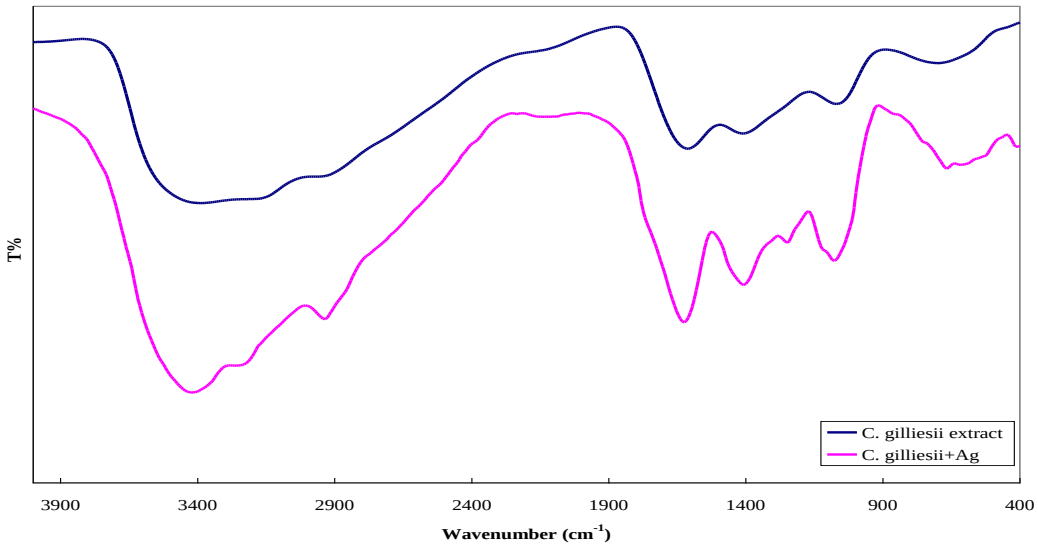

Fig. 4: FTIR spectra of extract stabilized Ag nanoparticles as compared with that of naked plant extract.

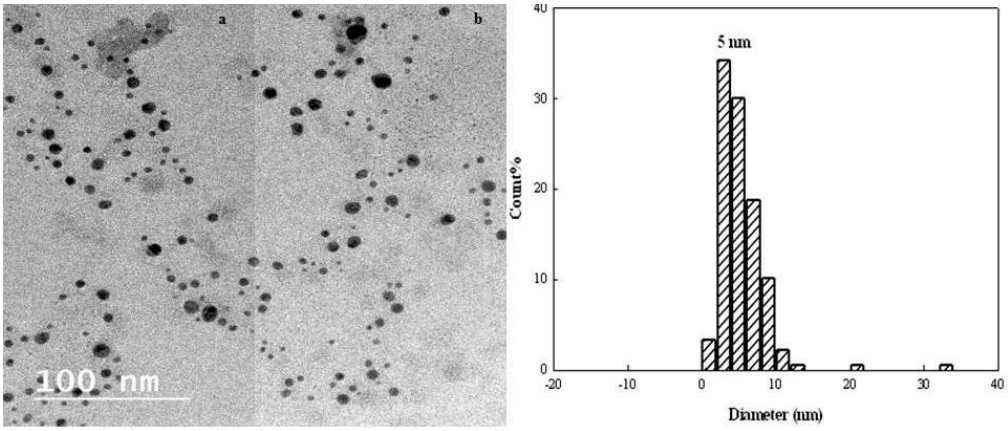

Fig. 5: The TEM images of *C. gilliesii*.-stabilized Ag nanoparticles (a) 250 µL and (b) 300 µL.

Antimicrobial Activity

Challenges in antibiotic resistance of human pathogens encourages us to find new natural alternates to beat this tackling. The antimicrobial activity of hydroalcoholic extract of *C. gilliesii* inhibited the growth of bacteria and yeast in varying degree of inhibition (Table 2).

But, synthesized Ag NPs of *C. gilliesii* extract showed the highest activity against all tested pathogens compared with hydroalcoholic extract as presented at Table 2 and Table 3 that showed the MIC values of Ag NPs for different pathogens in (µg/ml).

Bioactive compound capping, such as redox system play an important roles in Ag NPs formation. The concentration and the small size of the prepared Ag NPs may be play an important role for increasing its antimicrobial activity by easily diffusion or penetration of microorganism cell membrane and inhibited the growth (Manivasagan and Kim, 2015).

Cytotoxicity of the Hydroalcoholic Extract and Ag NPs of *C. gilliesii*

Recently, searching about antitumor drug derived from plant materials is increasing, because of their low adverse effects. So, silver nitrate ($AgNO_3$), hydroalcoholic extract of *C. gilliesii* and Ag NPs were investigated to evaluate their cytotoxicity effect against both normal skin fibroblast (BJ-1) and breast cancer cell line (MCF-7). Hydroalcoholic *C. gilliesii* extract and Ag NPs showed cytotoxicity against MCF-7 with 4.2 % and 96.5 % at 100 µg/ml, respectively. Also, hydroalcoholic *C. gilliesii* extract and AgNPs exhibited cytotoxicity against BJ-1 with 33.3% and 70.5 % at 100 µg/ml, respectively. But, $AgNO_3$ showed high cytotoxicity ≥ 90 % at 12.5 µg/ml. By ignoring the physical parameters of the Ag NPs, under aerobic conditions their toxicity only relied on the concentration of the Ag^+ released. So, we must repeat washing of Ag NPs prepared as much as possible to avoid toxicity of silver ion released. For the most active samples, a dose response study was made to calculate their IC_{50} values (**Table 4**).

Although Ag NPs have demonstrated effective antimicrobial and cytotoxic activities, the mechanisms of action of microorganisms and cell death have not been obviously confirmed yet. It has been suggested that Ag^+ released from Ag NPs can react with (-SH) groups which upsetting their respiration mode and the reaction of Ag+ with bases and phosphorus groups of DNA inhibited the DNA replication and thus cell death (Matsumura *et al.*, 2003; Prabhu and Poulose, 2012).

Table 2: Antimicrobial activity expressed as inhibition diameter zones in millimeters (mm) of chemical compounds against the pathological strains based on well diffusion assay.

Chemical compound	*Gram positive bacteria*			*Gram negative bacteria*			*Yeast*
	***Staphelococcus aureus* ATCC 43300**	***Streptococcus pyogenes ATCC19615***	***Proteus spp***	***Salmonella typhimurim* ATCC14028**	***Pseudomonas. aeroginosa* ATCC278223**	***E. coli ATCC 25922***	***Candida Albicans* NRRL Y-477**
Hydroalcoholic C. gilliesii ext.	18	17	13	N.A.	13	12	18
AgNPs of *C. gilliesii*	23	19	16	17	19	21	20
Vancomycine	28	30	24	25	24	22	N.A.
Ketoconazole	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	22

N.A. (No activity).

Table 3: Minimum inhibitory concentration (µg/ml) against the pathological strains based on two fold serial dilution technique

Chemical compound	*Gram positive bacteria*		*Gram negative bacteria*				*Yeast*
	***Staphelococcus aureus* ATCC 43300**	***Streptococcus pyogenes ATCC19615***	***Proteus spp***	***Salmonella typhimurim* ATCC14028**	***Pseudomonas. aeroginosa* ATCC278223**	***E. coli ATCC 25922***	***Candida Albicans* NRRL Y-477**
Hydroalcoholic C. gilliesii ext.	250	250	-	-	-	-	500
AgNPs of C. gilliesii	65	125	500	500	250	125	65
Vancomycine	65	65	65	65	65	65	N.A.
Ketoconazole	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	65

Table 4: IC_{50} (µg/ml) against BJ1 and MCF-7.

IC_{50} (µg/ml) of silver nano particle of *C. gilliesii* extract against Doxorubicin		
	Ag NPs	**Doxorubicin**
BJ-1	80.1	31.6
MCF-7	36.5	26.1

CONCLUSION

Due to the presence of phenolic and flavonoid structures that can be used as both reducing and capping agents into the hydroalcoholic extract of *C. gilliesii* leaves revealed that the silver nanoparticles formed, that possesses potent antimicrobial activity and promising anticancer activity against breast cancer cell lines and safe against normal skin fibroblast cell lines. This study has opened up the possible way for synthesizing multi-drug resistant antimicrobial Ag NPs using natural biomolecules which could be used in pharmaceutical industry. To the best of our knowledge, this is the first article on the green synthesis of metallic Ag NPs using *C. gilliesii* leaves extract.

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