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RESEARCH ARTICLE

Green synthesis of multifunctional silver nanoparticles using quercetin and their therapeutic potential

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ABSTRACT

Objective(s): Active species used in bio-chemical for synthesizing nanoparticles is poly phenolic compounds. The ability of flavonoids (e.g. quercetin) to dissolve in water is low and the production of metallic nanoparticles from them in the aqueous medium is hard. Previous studies recommend that quercetin was not capable of reducing Ag^{+} to Ag^{0}. The current research aimed at synthesizing quercetin-mediated silver nanoparticles (Q-AgNPs) and evaluate the antioxidant and anticancer activities of Q-AgNPs in vitro.

Methods: The green synthesis of Q-AgNPs in an aqueous medium has been demonstrated. The resultant nanoparticles were characterized by several analytical techniques of spectroscopy along with modern imaging instruments. The improved radical-neutralizing activity of the Q-AgNPs (Nitric oxide and DPPH) and its ability to chelate iron ions was determined by the colorimetric method. Possible medical applications, including anti-fungal and anti-cancer activities of these nanoparticles, have been assessed.

Results: The nitric oxide and DPPH tests of Q-AgNPs was found to be (IC_{50}=46.47±1.79 and 30.64±3.18µg/mL, respectively). Q-AgNPs exhibited better iron chelating activity than standard EDTA (IC_{50}=3.12 ±0.44µg/mL). Significant anti-cancer potency of Q-AgNPs (IC_{50}=57.42µg/mL) was discovered against HepG2 cell lines after 24-hour exposure. Furthermore, the toxic effects of these nanoparticles (MIC = 4, 8 and >64µg/mL) were determined on Candida krusei, Candida parapsilosis and Aspergillus fumigatus, respectively.

Conclusions: The present method is a competitive option to produce multifunctional nano-scale hybrid materials with higher efficiency and using natural sources for diverse biomedical requests.

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INTRODUCTION

The progressive area of nanotechnology has emerge as a central center of attention of scientific lookup and its impact on various aspects of human life is evident [1]. Nanoparticles have been applied in many areas, from biosensor expansion and medical diagnosis to pharmacy and treatment [2]. Because of unique features higher (surface / volume), ability to absorb in the visible area), they can be used potentially to treat many diseases including cancer [3]. Particle size and hydrophobicity of nanoparticle surfaces are two important factors in the penetration and intra-tumoral accumulation of nanoparticles through enhanced permeability and retention effect. Nanoparticles smaller than 30 nm are subject to kidney purification, whereas nanoparticles >250 nm are a suitable option for phagocytosis [4]. In this process, silver nanoparticles have won plenty interest in the direction of the subject of nano-medicine [5]. Green synthesis approach involving natural biomaterials for preparation of metal nanoparticles have acquired large interest as a viable choice to the present microbial and chemical/physical techniques where toxic, highly expensive chemicals and high maintenance microbes [6] are used [7]. The results of studies in the field of green synthesis recommended that the active and effective species in this practice are natural polymer compounds (e.g. poly phenols) [8]. Simplicity of synthesis procedures of polymeric nanoparticles, their ability to encapsulate a wide load of therapeutic compounds and their bio-compatibility can be considered as their most significant advantages [9]. Current laboratory research have proven that green synthesized Ag^0 nanoparticles have the achievable to set off toxicity in cells derived from a range of cancers. Their activity is possibly to be mediated via proposed mechanisms such as antioxidant safety [10], scavenging of oxidative stress through deactivating of TLR2 (anti-angiogenic property) [11], cell cycle arrest [12] and DNA fragmentation [13]. In the study about of the caffeic acid-capped AgNPs on human hepatoma HepG2 cells confirmed that the nanoparticles had been capable to efficaciously inhibit the growing of HepG2 cells via getting into cells via endocytosis and subsequent induction of apoptosis [14]. In usual physiological conditions, to control active oxygen radicals (AOR) levels, the cells balance the production of AOR by eliminating them through scavenging system but in oxidative stress conditions (e.g. cancer), a large amount of ROS is generated that is highly unstable entities and destroy the cellular machinery entirely [15]. Many cell reinforcement safeguard systems involving non-enzymatic (transferrin and carotenoids) and enzymatic (glutathione reductase and Se-dependent glutathione) mixes are found in our body, but they do not protect us completely from ROS attack in serious oxidative stress conditions during cancer. Several studies have shown that when antioxidants are accompanied with standard therapy, they display improved survival of treated group than the control group [16]. Green synthesis-based nanoparticles approaches can preserve the favorable anti-cancer cell-toxicity of nanoparticles while decreasing their side effects and medical limitations [17]. There is a strong possibility for biological synthesis of metal nanoparticles using macromolecules derived from natural sources as a considerable source and nanoparticles made using them seem to be biocompatible [5]. Consequently, such nanoparticles can be excellent candidates for secure biomedical purposes [7]. Therefore, green synthesis method could be an approach for tackling the issues connected to the toxicity of nanoparticles with the antioxidant properties [6]. V. Kathiravan et al. reported remarkable cytotoxicity activity of *Meliadubia* leaf extract-mediated silver nanoparticles against KB cell line using high therapeutic index value [18]. Considering the issues stated above is based on literature review, we can hypothesize the silver nanoparticles with antioxidant properties display enhanced effectiveness of treated group than the control group. Quercetin (as a representative of flavonoids) is abundant in human food sources. Quercetin is a natural yellow pigment extracted from the outermost rings of red onion. Quercetin is an effective herbal neutralizer of reactive nitrogen and oxygen free radicals in vitro and in vivo. Quercetin boosts the function of antioxidant enzymes in the body through directly controlling free radicals and lipid peroxides and indirectly by using producing non-enzymatic antioxidants. It exerts several biological and wellbeing advancing impacts, including antioxidant, antibacterial and hepatoprotective activities along with preventive effects on cancer, diabetes and inflammatory diseases [19]. Besides, studies on the bioactivity of quercetin has introduced numerous pathways by which this phytochemical may stop or delay the growth of degenerative diseases. In addition to its antioxidant and anti-inflammatory features,

quercetin is believed to be capable of modulating mitochondrial function, by changing mitochondrial biogenesis, affecting the membrane potential, and by its impact on electron transport chain and generation of ATP, and eventually inhibiting/ inducing intrinsic-apoptosis. As a poly phenolic compound, quercetin is greatly antioxidant to hinder free radical processes, and its chemistry largely related to this property [20]. Studies have shown that quercetin is a safe compound and no study has shown its carcinogenicity as well as genotoxicity after being orally administered even at high doses (up to 2g/kg) [19]. The normal accessibility, non-harmful nature, and therapeutic estimations of quercetin intrigued us to utilize them for production of silver nanoparticles and assess their effect on colon cancer cells further [7]. In this study, we show that it is possible to prepare AgNPs using quercetin in aqueous solution in alkaline conditions (pH=12). There is no report on the use of quercetin alone in the manufacturing of Ag nanoparticles in aqueous solution. Preceding studies recommend that quercetin was not capable of reducing Ag^{+} to Ag^{0} [8]. This could be ascribed to insolubility in water. Moreover, this paper, for the first time, that quercetin is the reducing agent contributing to the formation of AgNPs with outstanding antioxidant activity and cytotoxicity activity of quercetin-mediated AgNPs (Q-AgNPs) against HepG2 cell line.

MATERIALS AND METHODS

Materials

All reagents in this project had an analytical class certificate and with no additional purifying, and were purchased from reputable companies Merck and Sigma.

Optimized bio-synthesis of Ag nanoparticles

Fifty mL of quercetin solution (0.2mM) at pH=12 was prepared. The clear quercetin solution was added drop wise to a 50 mL 2mM $AgNO_3$ under the temperature of 65°C (200 rpm) away from light. The colorless $AgNO_3$ solution turned brownish showing the creation of AgNPs. The resulting Ag nanoparticles were refined by way of frequent centrifugation at 20,000×g for 20 minutes accompanied by means of re-dispersion of the pellets four times in ethanol and deionized water for removing unreacted quercetin molecules. The decontaminated pellets were re-dispersed in normal saline for further studies.

In vitro antioxidant assays

DPPH examine was measured from the previously published procedure [21] to determine to determine the antioxidant capacity of Q-AgNPs (7-35µg/mL). The DPPH radical-neutralizing capacity of the Q-AgNPs was expressed as (% neutralizing DPPH) calculated utilizing the accompanying formula: $[(A_0–A_S)/A_S] \times 100$, where A_0: the absorbance value of the control (methanol solvent + DPPH free radical) and A_S: the absorbance value of standard or sample. The nitric oxide radical-neutralizing ability of synthesized Q-AgNPs (6.2-62.2µg/mL) was quantified by Eslami et al [22] reaction method. The method introduced by Ebrahimzadeh et al [23] was applied for estimating the chelating of iron (II) ions by Q-AgNPs (1.49-7.46µg/mL), and thus preventing creation of Ferozin-iron (II) complex, which was reported as (% inhibition). The results were determined by the above formula and compared with the corresponding standard in each test. All measurements of the absorbance values were performed with a Perkin Elmer UV-VIS instrument of model EZ201.

Cell-culture medium and cancer cell line

The cancer cell line of human liver cancer (HepG2) was obtained from pasture Institute, Iran. HepG2 were put to grow in RPMI-1640. 10% (v/v) fetal bovine serum (FBS) and a mixture of and penicillin (10,000 IU/mL) / streptomycin (100 µg/mL) were used for enriching all cells-cultured media that were then maintained in a moistened incubator at a temperature of 37 degrees Celsius and Carbon dioxide gas (5%).

Anti-cancer study

The MTT calorimetric assay was applied for measuring cell viability of biological manufactured AgNPs on HepG2 cell lines obtained from the Amol Pasture Institute. The cell lines were sowed in the wells of the plates, with a density of $(5\text{-}10)\times10^3$ cells per well and kept in a moistened Carbon dioxide gas (5%) ecosystem for a full day and night at a temperature of 37 degree Celsius. After 24h, cells exposed to various concentrations (100, 50, 25, and 12.5µg/mL) of Q-AgNPs by considering control. For cell viability, color intensification assayed photometrically at 570 nm by means of a Micro-plate Spectrophotometer. Determination of cell morphology was done using an inverted metallurgical microscope. The following equation

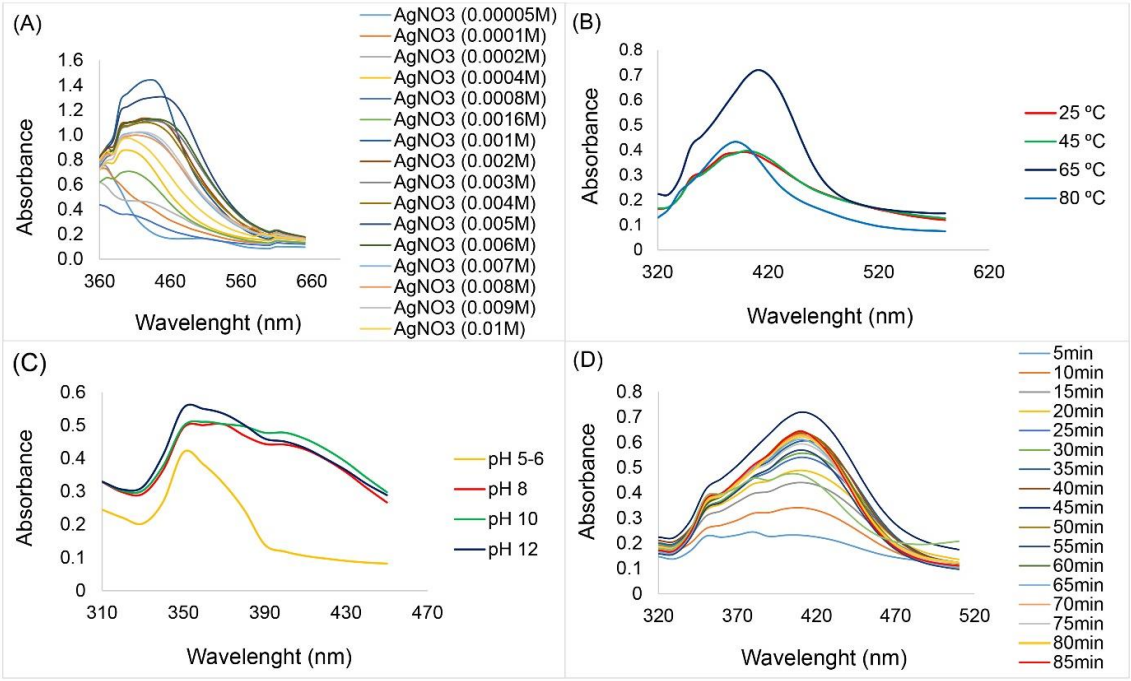

Fig. 1. (A) UV-spectra of Q-AgNPs derived from quercetin with variation of $AgNO_3$concentration. (B) UV–vis spectra at different temperature. (C) UV–vis spectra with variation of solution pH. (D) UV–visible spectra at different time intervals.

was used for calculating cell viability rate: (A sample / A control) × 100; A= Absorbance value. To accurately estimate the effect of Q-AgNPs on inhibiting the growth and proliferation of HepG2 cell category, the half of the maximal inhibition concentration (IC_{50}) of nanoparticles was calculated. To obtain the IC50 value, for treatment (nanoparticles + cells) at each concentration, IC50 values were obtained using linear regression, which was repeated three times and finally expressed as (mean ± SD).

Antifungal testing

Toxic effect of Q-AgNPs and fluconazole on pathogenic fungi, used to be examined in opposition to various species of pathogenic fungi isolated from the Invasive Fungi Research Center of Mazandaran University of Medical Sciences. The test was performed according to the standard clinical and laboratory protocol [24]. The solution of therapeutic nanoparticles (100µL) RPMI culture-medium (90 µL) were located in the wells of the plates. Inoculation of the plates was done by transferring 10 µl of fungal culture to each well until the final concentration of 3×103 colony forming units (CFU)/ml was reached. This was done at a temperature of 35 degree Celsius by 72 hours. After incubation, the adsorption values were logged to determine the growth of the wells separately at 530 nm by a micro-plate reader. The test was repeated twice and the minimal inhibitory concentration (MIC) was computed in accordance with the CLSI M27-A3 document [25].

RESULTS AND DISCUSSION

In this section, the green synthesized AgNPs were first described based on the results obtained from several modern techniques. Afterwards, the antioxidant, antifungal and anticancer activities of the nanoparticles have been assessed.

Optimization of nanoparticle synthesis

Based on UV-Vis spectroscopy using a Perkin Elmer UV-VIS instrument of model EZ201, different chemical/physical parameters were optimized for increasing the yield of nanoparticle synthesis. Effect of $AgNO_3$ concentration was studied in different concentrations of $AgNO_3$ (0.05-4 mM) using 0.1mM of quercetin (Fig. 1A). The highest concentration of AgNPs can be produced from a one mM solution of $AgNO_3$ based on the maximum absorption at the wavelength of 420 nm.

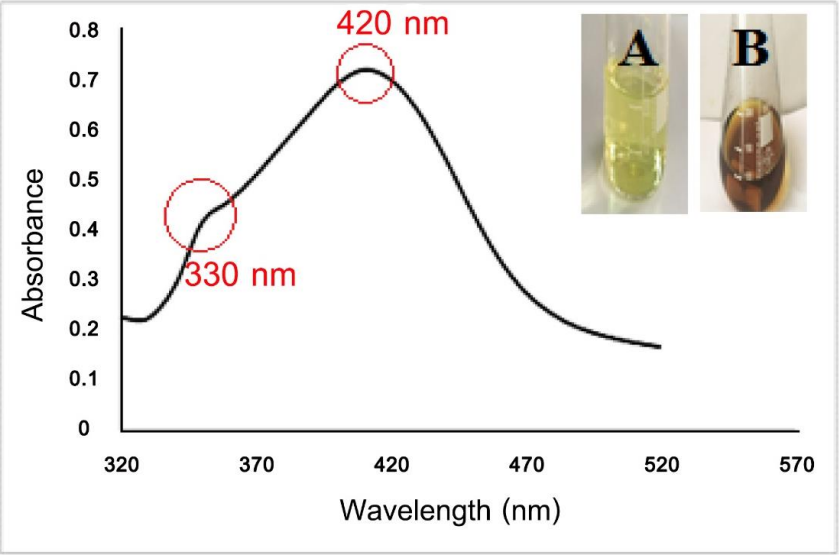

Fig. 2. UV–vis spectrum of a 0.1 mM aqueous solution of quercetin (pH 12) in presence of [Ag^+] = 1mM at 65 °C, inset showing the change in color of the materials involved in synthesis of stabilized silver nanoparticles, (A) silver nitrate, (B) quercetin, (C) colloidal solution of the resultant silver nanoparticles.

Thus, the observed peak was to be longer and sharper than the peaks of other AgNO3 concentrations (Fig. 1A), which shows the formation of relatively monodispersed nanoparticles. Moreover, there was again a decrease in absorbance measures; hence, 1mM concentration of $AgNO_3$ was chosen for further tests. As the incubation temperature increased, the adsorption of the reaction mixture increased, and this was accompanied by an increase in the amount of synthesized AgNPs [26]. This feature applied to temperatures up to 65°C, and from then on, as the temperature increased, the absorption process decreased (Fig. 1B). The effect of pH on this reaction was investigated with a change from 5 to 12 (Fig. 1C). As the pH increased, the size of the nanoparticles formed became smaller, which was consistent with the report of Veerasamy et al [27]. Under alkaline conditions, the nucleation process seems to prevail over the agglomeration of nanoparticles, resulting in a larger number of smaller nanoparticles being synthesized. This indicates that the pH parameter of the synthesis medium performs a key function in regulating the morphology and diameter of the synthesized Ag nanoparticles. At the end of the reaction, the medium final pH, has been decreased and changed toward the acidic range (from 10 to 7). Pattanayak et al. [28] have reported similar results. The absorption rate of the colloidal solution of the reaction medium increases so that over time it reaches its maximum value after 45 minutes (Fig. 1D). Accordingly, optimization process performed a fundamental position in increasing product yield, stability, and aggregation. The overall optimized reaction condition was temperature: 65°C, reaction time: 45 min, [$AgNO_3$]: 1mM, pH=12, and [Quercetin]: 0.1mM. All UV-vis spectra were performed with a Perkin Elmer UV-VIS instrument of model EZ201.

UV–vis spectral analysis

The alteration in colour of the reaction medium from light green to clear brown was a clear sign that quercetin-mediated nanoparticles were forming (Fig. 2) after adding quercetin at pH=12. This was confirmed by observing the absorption band at 420 nm, which is a unique feature for silver nanoparticles (Fig. 2). This band is characterized by the oscillation of the electrons in the outer layer of Ag atoms due to collisions with light waves, termed Resonance Surface Plasmon (SPR). For verifying the SPR band due to the formation of AgNPs, quercetin and/or $AgNO_3$ solution spectra logged and no absorption peak appeared around the 420 nm region (Fig. 2). Quercetin aqueous solution has strong absorption peaks in the 250, 330 and 380 nm regions (Fig. 3). Nevertheless, the combination of silver nitrate and quercetin

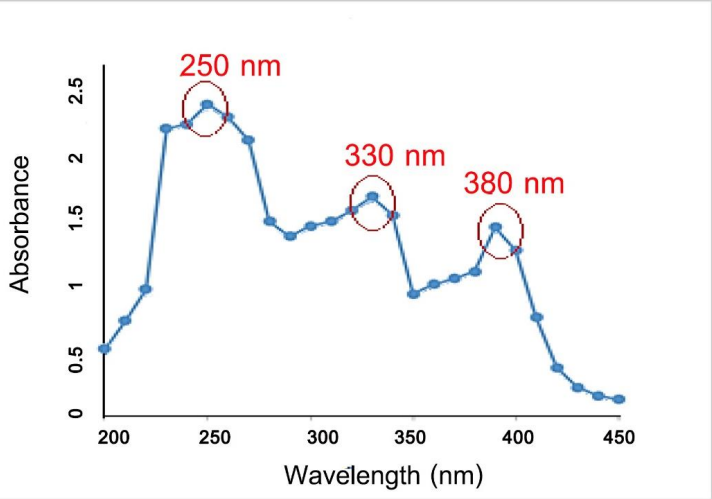

Fig. 3. UV–vis spectrum of a 0.1 mM aqueous solution of quercetin.

aqueous solutions, in the process of producing AgNPs led to a distinct absorption absorption peak of about 420 nm and any other at 330 nm, which suggests an attachment of quercetin with Ag^+, which recommends a ligand-metal bond between quercetin and Ag by way of carbonyl functional group (C═O) [29, 30]. A comparable absorption-peak has also been appeared in the course of the interplay of curcumin with its synthesized silver nanoparticles [31]. During the reaction progress, it is possible to observe the creation of AgNPs by detecting the increase in absorption rate at 420 nm. The absorbance increased over time and it was maximized within 45 minutes. Thus, it can be considered as a function of biosynthesized AgNPs concentration. According to the Mie's theory [32], the spherical metal nanoparticle shows lone plasmonic absorbance band, while the anisotropic nanoparticles display more than one absorption band [33]. Accordingly, the appearance a peak about 330 nm can represent the quercetin molecules located on the AgNP shell. The UV-vis spectrum also revealed that the obtained Q-AgNPs were relatively monodispersed. According to the literature, the narrowness of the SPR peak indicates that the particle size distribution is smaller [34]. In an alkaline environment, quercetin molecule, by losing hydrogen atom in hydroxyl functional group, become a stronger reducing mediator to transform Ag^+ to Ag^0 and eventually nano Ag, on the other hand, it become a stronger complexing mediator for Ag^+ ions. Therefore, quercetin molecule can simultaneously produce silver nanoparticle and can also adhere to its surface and make it stable, which is in line with recent research in this field [35, 36].

XRD analysis

The structure of the biosynthesized AgNPs from quercetin was also determined with the aid of XRD after washing the sample several times with ethanol to minimize the quantity of NaCl crystals. An X'Pert MPD type diffractometer equipped with cobalt anode as a source of radiation ($\lambda_{Co\text{-}K\alpha 1}$ = 1.78901 Å) used to be used to decide the crystalline shape of the nanoparticle. The generator was set to status (40 kV and 40 mA). Scans were carried out in a range of (start position=10 and end position=100 °2-theta) with a step size of 0.02 [37]. Fig. 4 suggests the XRD spectra of the biosynthesized AgNPs from quercetin. The data document obtained from XRD spectroscopy manifested three easily distinguishable diffraction peaks at 44.58°, 76.47° and 98.64° which are indexed as (200), (311) and (222) of the cubic crystal structure contains silver in every face which is in agreement with the JCPDS data No. 04-0783. The emergence of as yet unidentified peaks (*) = (51.79°, 93.20°) is probably related to the crystallization of bio-molecules in the nanoparticle structure. Our results are similar to the results of silver nanoparticles synthesized using *Ocimum sanctum* [38] and bacterial spore extract [39].

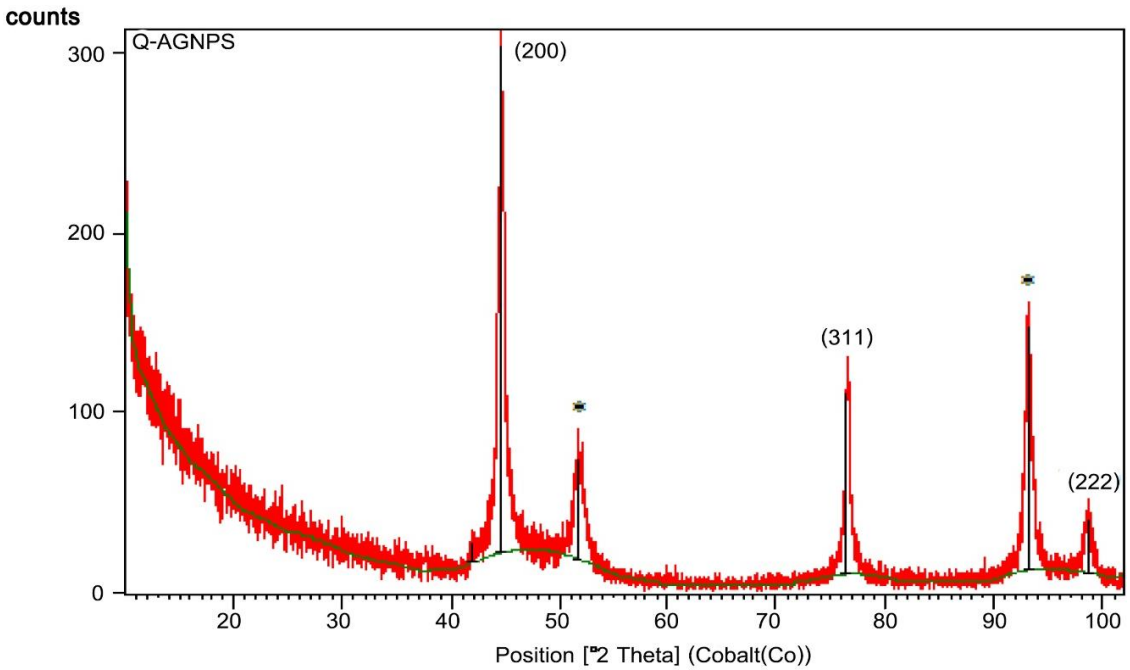

Fig. 4. XRD pattern of biosynthesized silver nanoparticles using quercetin.

Morphology and composition analysis

The field emission scanning electron microscope (FE-SEM) of model MIRA3TESCAN-XMU joined with a Low Vacuum Secondary Tescan Detector equipped with and an energy-dispersive X-ray microanalysis (EDXMA) was applied to characterize biosynthesized AgNPs in terms of size, morphology, and composition [37]. FE-SEM analysis indicates that the synthesized nanoparticles are mainly identical with an almost uniform size distribution showing their stabilization is probably due to the binding of surface quercetin bio-molecules (Fig. 5A). The AgNPs were sphere-shaped with a particle size of below 30 nm. As shown in Fig. 5B, EDXMA was used for the elemental composition determination of the Q-AgNPs. The high-intensity emission peaks at 3keV shows distinctive and powerful signals for the nanoparticles and indicates that the silver element has a greater share in the nanoparticle structure than other nanoparticle constituents. Also, the less intense peak of carbon is indicative of the presence of quercetin molecules in the nanoparticle structure displayed in Fig. 5B (inset). The EDXMA analysis supported of XRD data, represents the conversion of $Ag^{+} \rightarrow Ag^{0}$ and the nature of the quercetin that were loaded on the nanoparticles. As shown in our preceding work, we demonstrated that the hydrodynamic diameter of poly phenol-based metallic nanoparticles measured by the dynamic light scattering technique (>100 nm) is larger than the core diameter of these nanoparticles measured by FE-SEM (~30 nm). It can be concluded that the size of the nanoparticles produced is within acceptable range, both in terms of effectiveness and toxicity [40].

Antioxidant tests in vitro

The antioxidant assays of Q-AgNPs in vitro is displayed in Table 1. The DPPH radical-neutralizing activity of Q-AgNPs increased dose-dependently. The DPPH radical-neutralizing assay exhibited an effective inhibition capacity of Q-AgNPs compared with vitamin C and quercetin standards. Nitric oxide radical quenching ability of the Q-AgNPs was discovered to elevate with increasing concentration, which is comparable to quercetin standard. Q-AgNPs comparatively exhibited better iron chelating activity than standard EDTA. These activities may be attributed to the hydroxyl (-OH) and carbonyl (C=O) functional groups present in quercetin attached to Q-AgNPs. It is noteworthy that the antioxidant capacity of biosynthesized-AgNPs is not only based on the bioactive

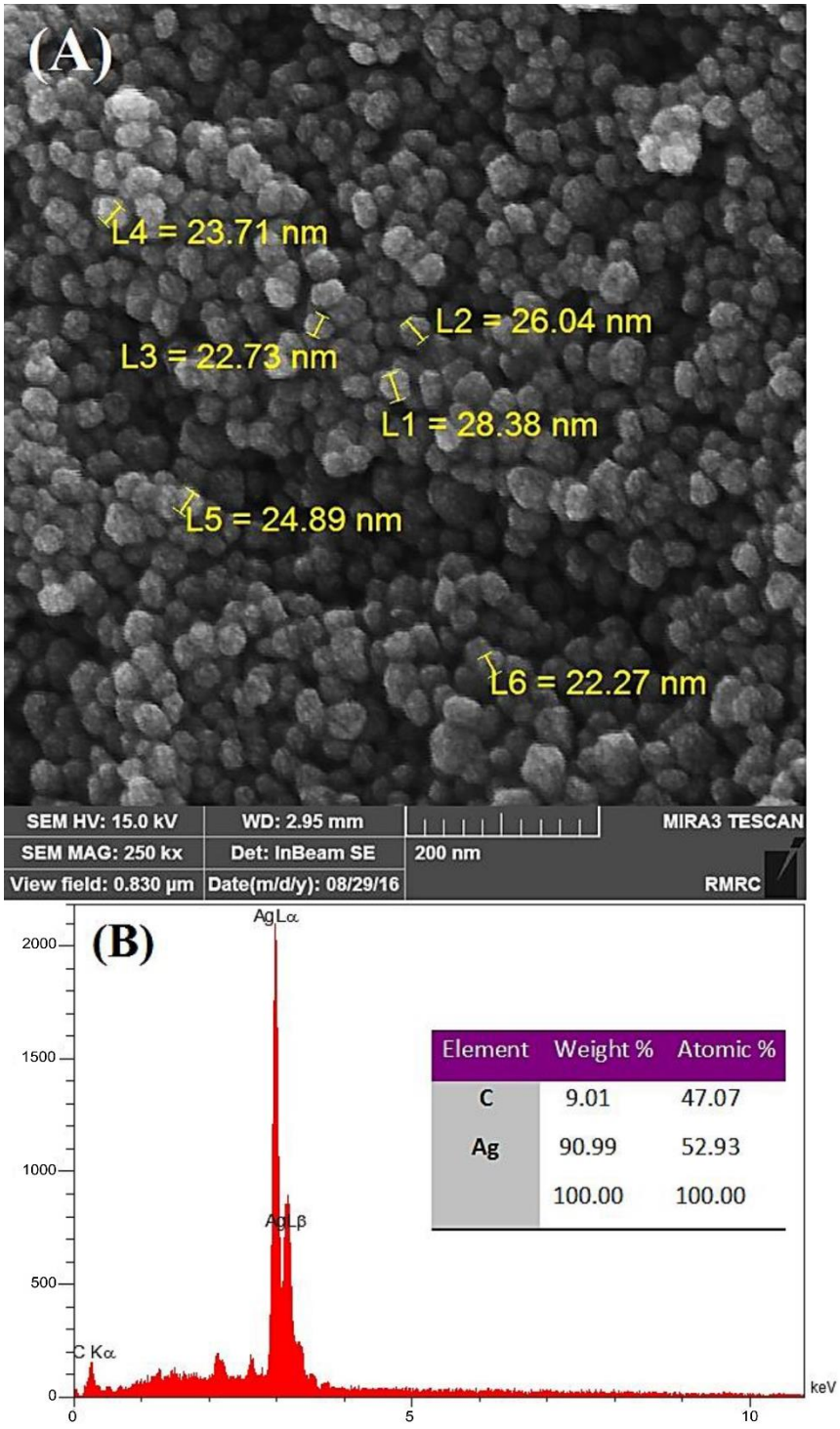

Fig. 5. (A) SEM images, (B) EDS spectra and inset showing the presence of silver nanoparticles and quercetin.

Table 1. Antioxidant activities of Q-AgNPs.

Sample	DPPH free radical scavenging IC_{50} (µg ml^{-1})	Nitric oxide scavenging IC_{50} (µg ml^{-1})	Fe^{2+} chelating ability IC_{50} (µg ml^{-1})
Q-AgNPs	46.47 ± 1.79	30.64 ± 3.18	3.12 ± 0.44
Quercetin	5.28 ± 0.2	20 ± 0.1	—
EDTA	—	—	18 ± 1.5
Vitamin C	5.05 ± 0.1	—	—

All values (mean ± standard deviation, n = 3)

Table 2. *In vitro* susceptibilities of antifungal drug (Fluconazole) and a novel green synthesized nanoparticle compound (Q-AgNPs) against *Aspergillus fumigatus* and two *Candida* isolates from two different species.

Species and isolate	MIC (µg/ml)	
	MC-AgNPs	Fluconazole
A. fumigatus (IFRC 1676)	>64	>64
C. krusei (IFRC 1625)	4	4
C. parapsilosis (IFRC 1626)	8	8

compounds capping on the surface of AgNPs, but also on the nature of bioactive compounds.

Toxicity of Q-AgNPs on the pathogenic fungi in vitro

Toxic effects of the Q-AgNPs against *Aspergillus fumigatus* and two *Candida* isolates from different species was investigated. For exploring the development inhibition impact of Q-AgNPs on the isolates, MIC was estimated and the consequences are shown in Table 2. Q-AgNPs displayed potent antifungal activity against the strain used, which is comparable with standard fluconazole.

Anti-cancer activity

AgNPs have been used appreciably as anti-microbial compounds, but few studies investigated the toxicity of phyto-compound-modified silver nanoparticles against cancer cells. Nevertheless, studies report that quercetin show cytotoxic activity against various cancer cellular categories including HepG2 [41], thyroid cancer cell lines [42], SW480 (Human colon cell adenocarcinoma) [43], HeLa (human cervical cancer cells) [44]. Therefore, for showing versatile biomedical activities of Q-AgNPs, its cellular toxicity against HepG2 (human liver cell carcinoma) was assessed in concentrated range (12.5–75µg/ml). Q-AgNPs exhibited the impact of cellular toxicity in the HepG2 cell category as dose-dependent (IC50= 57.42µg/ml). The mechanism of this action remains unclear. Further studies should explore the cellular toxicity of these nanoparticles in other cancer cell categories.

CONCLUSIONS

The production of silver nanoparticles with the help of quercetin in an aqueous medium was introduced as a biological method. Seemingly, the green route was efficient and rapid for synthesizing AgNPs. The antioxidant flavonoids (quercetin) simultaneously reduce silver ions and stabilize the resulting nanoparticles as a capping agent. Since the ability of flavonoids to dissolve in water is low, the production of metallic nanoparticles from them in the aqueous medium, which also has their therapeutic properties, is hard. As far as we know, this is the first report of any flavonoid used for the biosynthesis of AgNPs in an aqueous environment. Quercetin-hybridized silver nanoparticles showed substantial iron chelating ability, antifungal and cytotoxic effects against liver cancer cells. This promises a potential therapeutic compound in related diseases. Moreover, the present method will be a reasonable option to produce multifunctional nano-scale hybrid nanoparticles using natural sources with proven therapeutic features, which can be used in diverse biomedical applications.

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CONFLICT OF INTEREST

Interests are declared without conflict.

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