RESEARCH ARTICLE

In Vitro Evaluation of Apigenin-Coated Silver Nanoparticles Against *Trichomonas vaginalis*: A Potential Alternative to Metronidazole

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ABSTRACT

Trichomonas vaginalis is a protozoan parasite responsible for trichomoniasis, a prevalent sexually transmitted infection. Metronidazole is the standard treatment; however, concerns regarding side effects and emerging resistance necessitate the exploration of alternative therapies. This study aimed to evaluate the antiparasitic activity of silver nanoparticles coated with apigenin (Api@AgNPs) against T. vaginalis trophozoites in vitro. The nanoparticles were synthesized via a green synthesis method and characterized using UV-Vis spectroscopy and transmission electron microscopy, confirming an average size of 38.3 nm with a quasi-spherical morphology. Trophozoites were exposed to varying concentrations of Api@AgNPs and metronidazole for 24 and 48 hours. Viability was assessed using Trypan blue staining, and CC_{50} values were determined by probit regression. Api@AgNPs exhibited CC_{50} values of 111.211 µg/mL and 81.331 µg/mL at 24 and 48 hours, respectively, compared to 107.09 µg/mL and 76.542 µg/mL for metronidazole. These findings suggest that Api@AgNPs possess promising anti-trichomonal activity and may serve as a potential therapeutic agent alone or in combination with metronidazole, pending further in vivo validation.

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INTRODUCTION

Today, parasitic diseases are considered major global health challenges and cause significant social and psychological burden every year. Trichomoniasis, which is caused by the flagellated protozoan *Trichomonas vaginalis*, can lead to urinary tract infection and is one of the most common parasitic diseases transmitted through sexual contact [1]. Based on reported information and statistics, the prevalence of trichomoniasis in Iran ranges from 38.8% to 0.009, with an average estimated rate of 8% [2]. Infection in women is mostly asymptomatic, and approximately of 25 to 50% of cases have clinical symptoms [3]. This parasite causes vaginitis, urethritis, cervical swelling, burning, itching, and foamy discharge. *T. vaginalis* is believed to increase the risk of HIV infection by destroying the protease secreted by white blood cells that protects is responsible for protecting vaginal mucosal cells from HIV [4, 5]. On the other hand, this parasite is responsible for 11% of all cases of prostatitis, epididymitis, and male infertility [6]. The most common treatment for trichomoniasis is metronidazole, but there are numerous reports from many countries regarding the prevalence of resistance and carcinogenic and teratogenic side effects of the drug (especially in the first trimester of pregnancy), so researchers have been looking for alternative drugs with fewer side effects [7]. Currently, the use of nanoformulations in the field of drug delivery and diagnostic agents has expanded as promising new strategies. By using nanocarriers, therapeutic agents can be

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delivered to cells and increase the intracellular drug concentration to the level required to induce cytotoxicity [8]. Various nanostructures have been developed as imaging contrast agents [9] or for the development of in vitro diagnostic methods [10]. Today, one of the new methods for treating parasitic infections is the use of metallic nanoparticles such as silver, which is currently receiving a lot of attention for the synthesis of its nanostructured particles [11]. This is due to the unique properties of silver, including its antimicrobial properties at the nanoscale [12]. These include increased permeability and surface area-to-volume ratio, which enhance the surface energy and intensify the antimicrobial effect. Additionally, silver nanoparticles alter cell membrane integrity, increasing permeability and disrupting cellular function [13].

The sustainable approach to provide metallic nanoparticles (MNPs) is green synthesis, by applying biological sources such as herbal extracts or their phytochemicals. This method reduces environmental hazards and enhances biocompatibility, making nanoparticles more suitable and in many cases more stable for biomedical and industrial applications [14]. Apigenin is a natural plant flavonoid found in many fruits, vegetables, and herbs. It is a bioactive compound that has various biological effects [15]. In this study, apigenin coated silver nanoparticles (Api@AgNPs) were synthesized and characterized. Then, the performance of Api@AgNPs in inhibiting the *T. vaginalis* trophozoites was evaluated and compared with the drug metronidazole.

MATERIALS AND METHODS

Preparation of a positive sample of the parasite Trichomonas vaginalis

The *Trichomonas vaginalis* isolate used in this study was kindly provided by the Department of Parasitology, Faculty of Medicine, Shahid Beheshti University of Medical Sciences (Tehran, Iran). The sample was originally collected and maintained under appropriate laboratory conditions, ensuring the preservation of its viability and pathogenic characteristics for subsequent experimental analyses.

Synthesis of Apigenin-coated Silver Nanoparticles

The synthesis of apigenin coated silver nanoparticles was performed based on our previous work [16]. Apigenin (Shaanxi Huike Botanical Development Co.) was dissolved in 15 mL of deionized water, and the pH was adjusted to 10 using potassium bicarbonate (K_2CO_3 300 mM, Merck Chemicals Co.). The solution was stirred until complete dissolution. An equal volume (15 mL) of silver nitrate ($AgNO_3$ Dr. Mojalleli Chemicals Co.) stock solution was added dropwise to the stirring apigenin solution. The reaction proceeded under continuous stirring for 15 minutes, during which a color change indicated nanoparticle formation. The nanoparticles were centrifuged at 4000 rpm for 10 minutes, and the supernatant was discarded. The pellet was resuspended in deionized water. This washing step was repeated five times to remove excess apigenin and unreacted silver ions. The purified nanoparticles were redispersed in a smaller volume of deionized water to obtain a concentrated solution. The final nanoparticle concentration was determined using Inductively Coupled Plasma (ICP) spectroscopy.

Characterizations of Api@AgNPs

The shape, and size of the Api@AgNPs were examined using transmission electron microscopy (TEM, Zeiss EM 900). For this purpose, 50 µL of the nanoparticle solution with a concentration of approximately 100 µg/mL was placed on the formvar carbon coated TEM grids, and after the aqueous solvent dried and the nanoparticles were fixed on the grid, investigation was performed. After preparing micrographs of Api@AgNPs, the diameter of at least 100 nanoparticles was calculated using digital micrograph software, and these sizes were used to draw a size distribution diagram using Origin 6 software. A double beam UV-visible absorption spectroscopy (SPEKOL 2000, Analytik Jena, UK) equipped with quartz covet (1 cm of optical path) was applied to evaluate optical properties such as plasmonic peaks and other characteristics.

Preparation of complete culture medium TYI-S-33

To prepare complete culture medium, 88 ml of culture medium, 10 cc of inactivated young calf serum (at 56°C for 30 minutes) and 2 cc of vitamin 18 and antibiotics penicillin and streptomycin were added, then the complete culture medium was distributed under sterile conditions in sterile falcon and stored at -20 until use [7].

In vitro experiments

Following large-scale cultivation of

Trichomonas vaginalis trophozoites in TYI-S-33 medium, a trophozoite suspension was prepared at a concentration of 2.5×10^5 cells/mL, designated as the stock solution. Subsequently, 200 µL of this stock (equivalent to 5×10^4 trophozoites) was aliquoted into each well of the assay plates. The nanoparticle formulation was dissolved in physiological saline to obtain a stock concentration of 10 mg/mL. Serial dilutions of both the nanoparticle extract (ranging from 0.48 to 500 µg/mL) and the reference drug metronidazole (ranging from 1.62 to 250 µg/mL) were prepared. These test solutions were added to the wells containing the trophozoites in three independent replicates, followed by incubation at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 and 48 hours. Post-incubation, parasite viability was assessed using Trypan blue exclusion and quantified with a hemocytometer. The 50% cytotoxic concentration (CC_{50}) was determined via probit regression analysis. Metronidazole was used as the positive control, whereas untreated T. vaginalis trophozoites maintained in TYI-S-33 medium served as the negative control. Statistical significance between groups was assessed, and results with p-values < 0.05 were considered significant [17].

Statistical analysis

Probit regression was employed to determine the CC_{50} values, with statistical computations performed using SPSS software, version 23. All experiments were conducted in triplicate, and data are expressed as mean ± standard deviation (SD). Statistical comparisons between treated and control groups were evaluated using appropriate statistical tests. A p-value of less than 0.05 was considered statistically significant. Data were presented with Origin 6 software.

RESULTS

Investigating the characteristics of Api@AgNPs

Api@AgNPs were synthesized by apigenin in an ecofriendly one-pot synthesis process. Using UV-Vis spectroscopy, the presence of a characteristic plasmonic peak at 413 nm was revealed, confirming the nature of silver nanoparticles. UV-Vis spectroscopy was also used to investigate the stability of nanoparticles in physiological environments, which is necessary for their function. Api@AgNPs were stable in the presence of PBS buffer, but a slight red shift in the plasmon peak was observed, and a new peak at 415 nm was observed. With the addition of 10% serum protein (FBS), the plasmonic peak was shifted to 420 nm (Figure 1).

Transmission electron microscopy (TEM) was used to measure shape, size and size distribution of Api@AgNPs (Figure 2). The synthesized nanoparticles are almost spherical, but their crystalline facets are visible in the micrographs. The average size analysis showed that their size is 38.3 ± 9.6 nm. In the higher magnification micrograph, a distinct organic coating is visible around the nanoparticles (Figure 2B). Since no other organic compound other than apigenin was used for synthesis, this coating is derived from apigenin.

Study of the effect of Api@AgNPs and metronidazole on T. vaginalis in vitro

In this study, the efficacy of Api@AgNPs

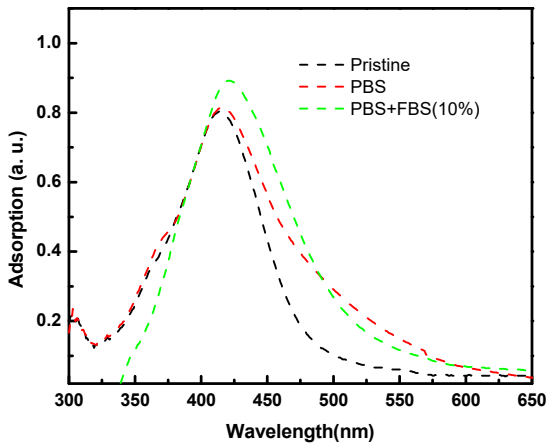

Fig-1. Absorption spectra of pristine Api@AgNPs, Api@AgNPs dissolved in PBS, nanoparticles dissolved in PBS plus 10% FBS.

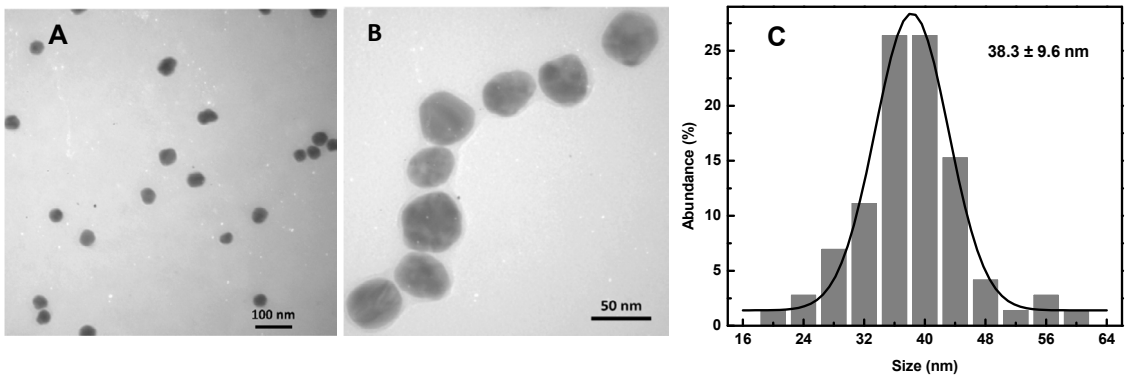

Fig-2. TEM micrographs of Api@AgNPs (A, B) And Particle size distribution diagram of Api@AgNPs (C)

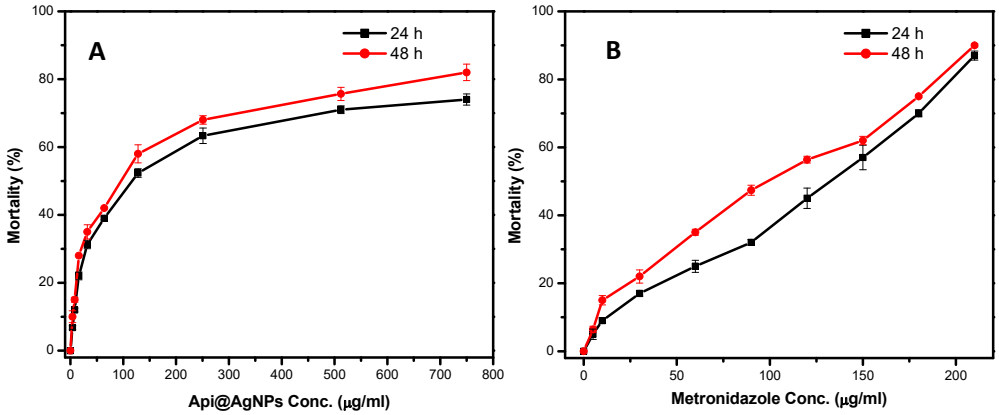

Figure 3: Mortality rate of Api@AgNPs (A) and metronidazole (B) on the *T. vaginalis* trophozoites

in treating *T. vaginalis* trophozoites was investigated and compared with the standard drug metronidazole. The results of the efficacy of both treatments on the mortality of the *T. vaginalis* trophozoites are demonstrated in Figure 3. Our study showed that metronidazole eradicates parasites in vitro at much lower concentrations. A very small number of parasites survive at concentrations of up to 750 µg/mL of nanoparticles. However, at a concentration of 250 µg of the drug, the parasites were eradicated.

To better compare the results at different times, the CC50 of both treatments was calculated (Figure 3). The average dose of 50% lethality of the nanoparticles was calculated to be 111.211 µg/mL (991/96-128.225) at 24 hours (Figure 3A) and 81.331 µg/mL (92.715-71.503) at 48 hours (Figure 3B). The effect of metronidazole on *T. vaginalis* trophozoites was studied. The CC_{50} dose of metronidazole was calculated to be 107.09 µg/mL (89.089-131.323) at 24 hours and 76.542 µg/mL (64.879-91.230) at 48 hours. Statistical analysis indicated significant differences in parasite viability between treated and control groups at both 24 and 48 hours ($p < 0.05$)in concentration higher than 128 µg/ml.

DISCUSSION

Trichomoniasis is a sexually transmitted parasitic disease of particular importance according to the World Health Organization [18]. Humans are the only known host of *Trichomonas vaginalis*. Trichomoniasis is not only an important cause of vulvovaginitis in women [19], but it can also be a possible cause of pelvic inflammatory disease, cervical malignancy, and a factor facilitating HIV transmission. In addition, *T. vaginalis* can cause adverse effects in pregnancy [20]; by secreting cell detachment factor, it can cause rupture of fetal membranes and cause premature births, low birth weight infants, and fetal death.

Antimicrobial effects of various plant extracts such as Allium sativum and Melaleuca alternifolia against *T. vaginalis* supports phytochemicals

potential as alternative treatments [21, 22]. Recently, the antimicrobial activity of various separated flavonoids and their derivatives against *T. vaginalis* was demonstrated [23, 24]. While apigenin has been studied for its antimicrobial effects against a range of pathogens, including bacteria and other protozoa [25], its activity against *T. vaginalis* has not been specifically documented in the available literature. Therefore, it's potential as a treatment for trichomoniasis remains unexplored. Here silver nanoparticles was synthesized with apigenin and an apigenin derivate coating on the surface of silver nanoparticles has been demonstrated. Recent studies have explored the use of silver nanoparticles as potential treatments for *Trichomonas vaginalis* infections. One notable study utilized an eco-friendly synthesis method to produce AgNPs using Juglans regia (walnut) leaf extract. These biosynthesized nanoparticles demonstrated significant anti-*T. Vaginalis* activity, comparable to the standard treatment with metronidazole [26].

On the other hand, the use of plant extracts and oils is one of the methods for manufacturing metal nanoparticles, including silver and gold, which is referred to as biological methods [27]. Nanoparticles prepared by this method typically have a broad size distribution compared to nanoparticles prepared from chemicals such as sodium bromide or sodium citrate. This is due to the presence of a wide range of compounds in the extracts or oils used to make the nanoparticles [28]. Many researchers have used compounds isolated from plants to make nanoparticles. Among these, apigenin can be mentioned, which has antioxidant, cancer prevention, and antimicrobial and antiparasitic effects [29]. In this study, silver nanoparticles coated with the apigenin were investigated in vitro compared to metronidazole on *T. vaginalis* trophozoites.

To evaluate the structural and functional characteristics of nanoparticles synthesized using apigenin as a reducing and stabilizing agent, citrate-capped nanoparticles were employed as a chemical control. Citrate is commonly utilized in the synthesis of metal nanoparticles due to its effective stabilizing properties and significantly lower toxicity relative to other chemical agents such as sodium borohydride and cetyltrimethylammonium bromide (CTAB), which are known to pose higher cytotoxic risks [30]. The colloidal stability of apigenin-coated silver nanoparticles was assessed in phosphate-buffered saline (PBS) solution. Inadequately coated nanoparticles tend to undergo rapid aggregation and precipitation in salt-containing environments, such as buffer solutions, primarily due to the formation of salt bridges between particles. Plasmonic nanoparticles like silver exhibit a characteristic surface plasmon resonance (SPR) peak in the visible region of the electromagnetic spectrum. Aggregation or sedimentation of these nanoparticles leads to noticeable shifts or broadening in their SPR absorption maxima, reflecting changes in particle size distribution and interparticle interactions [16].

In this study, silver apigenin nanoparticles were tested on *T. vaginalis* trophozoites in vitro. According to the results, the average 50% lethality of silver apigenin nanoparticles was calculated to be 111.211 µg/mL at 24 hours and 81.331 µg/mL at 48 hours. In many studies conducted on *Trichomonas vaginalis*, many medicinal plants with high phenolic and flavonoid contents have been used. In the study of Jafari et al., Eugenia caryophylata, Camellia sinensis, and Terminalia chebula Retz had the highest lethality [17]. Apigenin is a natural compound and has been used in anti-inflammatory, vasodilator, anticoagulant, antidiabetic, anticancer, and antimalarial drugs. Apigenin induces ABCC1 transporters, inhibits protein kinase (Pf RIO-2 kinase), and acts as an antioxidant [31]. Silver nanoparticles are approximately 1 to 100 nanometers in size and are manufactured in various shapes. Nanotechnology is an emerging technology that is expected to create new opportunities for the destruction and control of microorganisms using materials and systems at the atomic scale. Silver nanoparticles have attracted the most attention as antiparasitic drugs in the last few decades, because current antiparasitic drugs have side effects and parasites may develop resistance to the drug used. Silver nanoparticles can be used alone or in combination with current antiparasitic drugs or with coated flavonoid compounds, in this study, they were coated with the flavonoid compound apigenin. Apigenin-coated silver nanoparticles are a type of nanomaterial that has attracted attention in recent years due to their potential applications in various fields. These nanoparticles consist of a core of silver atoms surrounded by a layer of apigenin, a flavonoid compound found in many plants. The unique properties of apigenin-coated silver nanoparticles arise from the combination of the properties of silver and apigenin. Silver nanoparticles are known for their antimicrobial,

antifungal, and antiviral properties [32], and are effective against a wide range of microorganisms. On the other hand, apigenin has antioxidant, anti-inflammatory and anticancer properties. Considering these properties, apigenin-coated silver nanoparticles offer better antimicrobial activity and biocompatibility compared to traditional silver nanoparticles [33]. Some studies have shown that gold nanoparticles, oxidized metals, silver, chitosan, etc. have growth inhibitory or cytotoxic effects on various parasites including Giardia, Leishmania, Plasmodium, Toxoplasma and worms including Echinococcus multilocularis, Trichinella spiralis and Fasciola hepatica [34]. The antiparasitic activity of silver nanoparticles (AgNPs) is mediated through multiple mechanisms, including disruption of the parasite cell membrane, suppression of metabolic activity, and inhibition of reproductive capacity. Upon release, silver ions (Ag^+) can interact with the parasite's cell membrane, compromising its structural integrity and fluidity. This interaction leads to increased membrane permeability, resulting in the leakage of vital intracellular components, impairment of essential cellular functions, and ultimately, cell death. These multifaceted effects highlight the potential of AgNPs as a potent antiparasitic agent [35]. Silver nanoparticles (AgNPs) exert their antiparasitic effects primarily by inducing programmed cell death (apoptosis), a process largely mediated by the overproduction of reactive oxygen species (ROS). The excessive generation of ROS leads to oxidative stress, resulting in damage to cellular components such as lipids, proteins, and nucleic acids, ultimately triggering apoptosis and parasite cell death [36]. The majority of intracellular stress responses induced by silver nanoparticles (AgNPs) are mediated through the generation of reactive oxygen species (ROS), with oxidative stress being the primary mechanism underlying AgNP-induced cytotoxicity. The release of silver ions (Ag^+) further contributes to ROS production within the parasite, exacerbating oxidative stress. This excessive ROS generation results in damage to critical cellular components, including lipids, proteins, and DNA, ultimately leading to the disruption of cellular functions and the induction of parasite cell death, thereby eliminating the parasite [37]. Besides in this study, the effect of metronidazole on trophozoites of *T. vaginalis* was investigated. The mean 50% lethal dose of metronidazole was calculated to be 107.09 µg/mL (89.089-131.323) at 24 hours and 76.542 µg/mL (64.879-91.230) at 48 hours. In the study of Altemmi et al., the effect of nanoparticle-loaded metronidazole on *T. vaginalis* was found to inhibit the growth of *T. vaginalis* at 95 g/mLµ [7]. In study of Altemmi et al., the effect of nanoparticle-loaded metronidazole on *T. vaginalis* was found to inhibit the growth of *T. vaginalis* by 95 g/mLµ [7]. the study of Jafari et al., the inhibition rate of metronidazole on *T. vaginalis* was 100 g/mLµ which present study is in agreement with the aforementioned studies [17].

The antiparasitic activity of Api@AgNPs is likely mediated through multiple mechanisms. Silver nanoparticles disrupt the parasite's cell membrane, increasing permeability and causing leakage of intracellular components. They also induce oxidative stress by generating reactive oxygen species (ROS), which damage lipids, proteins, and nucleic acids, ultimately triggering apoptosis [35]. Apigenin, as a flavonoid, may enhance these effects through its antioxidant and anti-inflammatory properties, interference with parasite enzyme systems, and modulation of intracellular signaling [38]. The combination of apigenin and silver nanoparticles may thus act synergistically, resulting in the significant reduction in parasite viability observed in both 24- and 48-hour treatments.

A major limitation of the present study is the restricted scope of experimentation, focusing solely on the in vitro antiparasitic effect of Api@AgNPs against *T. vaginalis*. Future research should include in vivo studies, host cytotoxicity profiling, mechanistic investigations, and comparative assays with other nanoparticle formulations to validate and expand upon these findings

CONCLUSION

In this study, silver nanoparticles coated with apigenin were shown to be effective on *T. vaginalis* trophozoites. Considering the results of the effects of the nanoparticles on Trichomonas vaginalis, this nanoparticle can be used as a drug candidate to help treat trichomoniasis in combination with metronidazole or alone, after animal experiments in the form of Vaginal cream or oral human studies can be initiated.

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CONFLICT OF INTERESTS

The author(s) declare that they have no competing interests.

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