Original Article

Antifungal activity of green sulfur nanoparticles synthesized using *Catharanthus roseus* extract against *Microsporum canis*

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Abstract

Elemental sulfur has been used for a long time to treat superficial mycoses in both medical and veterinary practices, but effective treatment requires high concentrations of sulfur. Encapsulation of sulfur in nanoparticles can potentially allow the delivery of high concentrations of sulfur to infections. Sulfur nanoparticles were synthesized from *Catharanthus roseus* extract and sodium sulfide at various pH conditions (4, 6, and 7). Particle identification and characterizations were performed by X-ray diffraction analysis (XRD), scanning electron microscopy (SEM), and dynamic light scattering analysis. Broth microdilution and time-kill assays were used to determine the antifungal effect against *Microsporum canis* DMST29297. The X-RD analysis showed that the fine yellow powder of sulfur nanoparticles contained α-orthorhombic sulfur and SEM showed a uniform distribution and similarity in size of almost spherical particles. Sulfur nanoparticles synthesized with *C. roseus* extract at pH 7 had the smallest average size (480±39.6 nm) and highest antifungal activity (MIC 1.56 mg/ml). The fungicidal activity of the sulfur nanoparticles was time-dependent, eliminating *M. canis* DMST29297 only after 24 hours. Green sulfur nanoparticles synthesized with *C. roseus* extract have potential to be developed as an antifungal agent against *M. canis*. However, increased antifungal activity should be further developed.

Keywords: *Catharanthus roseus*, Green synthesis, *Microsporum canis*, Sulfur nanoparticles

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Introduction

The fungus *Microsporum canis* is the most common causative pathogen of dermatophytosis in pets, which presents as alopecia, scales, crust, erythema, and papules on the face and skin (Khurana *et al*., 2016; Pasquetti *et al*., 2017). Topical treatment with azole antifungal drugs is used for the elimination of localized fungal infections in the epidermis and hair surface while ketoconazole, griseofulvin, and terbinafine have been shown to be effective in the treatment of generalized dermatophytosis (Moriello *et al*., 2017; Abdalla, 2018). However, there can be side effects from long-term antifungal drug use in the treatment of multifocal or generalized infections such as allergies, liver and kidney toxicity, and skin irritation (Bossche *et al*., 2003). In addition, many dermatophyte species are resistant to azole antifungal drugs leading to unsuccessful treatment outcomes and persistence of symptoms (Debnath *et al*., 2016; Aneke *et al*., 2018). Therefore, there is interest in sulfur topical drugs that are also effective against *M. canis*.

Elemental sulfur is a non-metal element that has been used for a long time to treat skin infections caused by bacteria, fungi, and parasites and is the active antimicrobial ingredient in numerous cosmetics such as soaps, creams, ointments, and lotions. While sulfur is generally non-toxic, exposure to high concentrations of sulfur can cause irritation. The development of nanoparticle formulations of sulfur can potentially increase the concentration of sulfur at the application site and reduce the problems of exposure (Boros *et al*., 2010; Koch *et al*., 2012; Gogoi *et al*., 2013). Nanoparticle-based drug delivery systems are ideal for the treatment of infectious skin diseases due to their quantum-size properties that can enhance the physical, chemical, and pharmacological properties of encapsulated particles (Preprem *et al*., 2012). Indeed, Massalimov *et al.* (2012) demonstrated that 25 nm sulfur nanoparticles were 4-9 times more effective against *Penicillium notatum*, *Aspergillus niger*, and *Candida albicans* than 8 µm micronized sulfur particles.

There are many protocols that have been used to synthesize sulfur nanoparticles such as water-in-oil microemulsion methods, electrochemical methods, liquid-phase precipitation methods, and green synthesis methods (Shamsipur *et al*., 2011; Soleimani *et al*., 2013; Suleiman *et al*., 2015). Green synthesis methods have certain advantages over other methods as they are generally not complicated or costly to perform and do not use toxic reagents that can contaminate the environment. Green synthesis methods use biomolecules such as proteins, enzymes, phenols, amines, and alkaloids from plants and microorganisms to produce stable nanoparticles (Kouzegaran and Farhadi, 2017). A report by Paralikar and Rai (2017) showed that sulfur nanoparticles derived from sodium polysulfide and *Azadirachta indica, Catharanthus roseus, Mangifera indica*, and *Polyalthia longifolia* plant extracts had a particle size of 70–80 nm and were effective against *Escherichia coli* and *Staphylococcus aureus*. Khairan *et al.* (2019) reported that *Allium sativum* extract could be used to synthesize sulfur nanoparticles that had antifungal activity against *C. albicans*. However, as there are no studies of the anti-fungal effect of green sulfur nanoparticles on *M. canis*, we developed and synthesized a green sulfur nanoparticle using *C. roseus* extract, a herb that is readily available in Thailand, and tested it for antifungal activity against *M. canis* DMST29297.

Materials and Methods

***Microbial strains and culture conditions*:** *M. canis* DMST29297 was obtained from the Department of Medical Sciences, Thailand and maintained at 4ºC on Sabouraud dextrose agar (SDA, Becton Dickinson, France). Before use, *M. canis* colonies were transferred to fresh SDA and incubated at 30ºC for 14 days. Fungal suspensions were prepared by adding 10 ml of Sabouraud dextrose broth (SDB, Becton Dickinson, France) containing 100 µl of polysorbate 20 (Asia Pacific Specialty Chemicals, Australia). Then, fungal hyphae and conidia were collected with a triangle shaped glass rod spreader. The fungal inocula were adjusted to $1x10^4$ CFU/ml and quantified by aerobic plate count technique (Clinical and Laboratory Standard Institute, 2008).

***Plant materials and extraction method*:** Fresh, mature leaves of *C. roseus* were collected from the herb garden of the Pharmacology and Toxicology Laboratory, Faculty of Veterinary Medicine, Khon Kaen University, Thailand. They were washed with water several times to remove dust particles, then dried and finely ground. The *C. roseus* extract was prepared by boiling 10 g of leaf powder in 100 ml of distilled water at 90-100ºC for 20 min and allowed to cool at room temperature before filtering through Whatman filter paper no.1. The filtrate was centrifuged at 170 × g for 5 min and the supernatant was used for the synthesis of sulfur nanoparticles.

***Synthesis of sulfur nanoparticles*:** The synthesis was performed according to the method previously described by Paralikar and Rai (2017), with some modifications. Briefly, finely ground sulfur powder (Daejung chemical & materials Co. Ltd., Korea) was mixed with 1M sodium sulfide (Loba chemie Pvt. Ltd., India) and boiled at 100°C with stirring until the solution became the red/orange color of sodium polysulfide. This solution was mixed with *C. roseus* extract at a ratio of 1:4 and stirred until homogeneous. Then sulfuric acid was added drop by drop to precipitate sulfur nanoparticles with volumes of 2-5 ml giving pH values of 4, 6, and 7. The solutions were centrifuged at 1,860 × g for 45 min. The obtained material was measured by observing precipitate particles separated from the supernatant indicating the formation of sulfur particles. The nanoparticle precipitates were washed with distilled water and absolute alcohol, followed by freeze drying (Coolsafe 110-4 and CryoSafe 18-50, Scanvac, Denmark). Sulfur nanoparticles synthesized without the addition of plant extract (pH 7) were used as control. The macroscopic appearances, color and homogeneity of the obtained powder were recorded.

***Scanning electron microscope (SEM) analysis*:** To investigate the morphology of the synthesized sulfur

nanoparticles, SEM analysis was performed using a JSM-IT200 InTouchScope™ from JEOL, USA set at a power of 15 kV and magnification of 750-10,000 times (Khairan *et al*., 2019).

***X-ray diffraction (XRD) analysis*:** Particle identification by XRD measurements were carried out using a Philips PW 1830 at 2θ angle in the range 20–80° using Cu Kα1 radiation, wavelength (λ) 1.54060 Å, power of 40 kV and 30 mA, angular range 20° ≤ 2θ ≤ 60° step, size 0.02° (counting time one second per step) (Khairan *et al*., 2019).

***Dynamic light scattering (DLS) analysis*:** A zetasizer (Malvern, Zetasizer Nano ZS, England) was used for determining the particle size distribution by DLS technique. The means of DLS was measured in the range of 0.1–1000 µm (Paralikar and Rai, 2017).

***Broth microdilution test*:** Antifungal activity of the sulfur nanoparticles was determined using the broth microdilution method according to Clinical and Laboratory Standard Institute (2008) guidelines, with some modifications. Synthesized sulfur nanoparticles were diluted with 2% v/v xylene in distilled water to achieve a concentration of 200 mg/ml. Serial 2-fold dilution of sulfur nanoparticles was carried out with SDB in 96-well round-bottom microtiter plates (Corning Incorporated, USA) and 50 µl of fungal suspension was added to wells. The well which contains SDB and fungal suspension was considered as positive growth control, while the well containing only SDB was used as negative growth control. The final concentrations of sulfur nanoparticles were 50.000-0.098 mg/ml. The 2% v/v xylene in distilled water was used as diluent control. The plates were incubated at 30ºC for 72-96 h. The minimal inhibitory concentration (MIC) was considered as the lowest concentration of antifungal agents that inhibited visible growth of tested fungus. The mixture of no visible growth wells was transferred to SDA and incubated at 30ºC for 72-96 h. The minimum fungicidal concentration (MFC) was determined from the lowest concentration of antifungal agents that inhibited growth on SDA. Ketoconazole was used as antifungal controls. All tests were performed in triplicate.

***Time-kill test*:** The time-kill assay was performed according to the method previously described by Aiemsaard *et al.* (2020). Briefly, 100 µl of *M. canis* suspension (10^4-10^5 CFU/ml) was mixed with 900 µl of sulfur nanoparticle suspension (in distilled water) to give final concentrations of 1, 5, and 10 times MIC in 1,000 µl. After incubation for 15 and 30 min, 1, 3, 6, 12, and 24 h at 30 ºC, a 100 µl sample was serially 10-fold diluted with 0.89% sodium chloride solution and 100 µl of dilutions 10^{-1} to 10^{-4} was inoculated onto SDA. After incubation at 30ºC for 72-96 h, the colonies of visible growth of tested fungus were counted and recorded. Each experiment was performed in triplicate.

***Statistical analysis*:** The normality of the data was assessed by the Shapiro-Wilk test. The differences in particle size between synthesis condition were tested with one-way ANOVA. The *P*-value < 0.05 indicated statistical significance. All tests employed Statistics Package for the Social Sciences (SPSS) software for windows 10 (version 19.0, SPSS Statistics; USA, KKU licenses).

Results

There was no difference in the macroscopic appearance of green sulfur nanoparticles synthesized using *C. roseus* extract at each pH condition or synthesized without plant extract, all sulfur nanoparticles appeared as a fine yellow powder. The SEM analysis (Fig. 1) shows that green sulfur nanoparticles synthesized with *C. roseus* extract had a uniform size, shape and distribution. In contrast, the sulfur nanoparticles synthesized without plant extract were a range of sizes and had coalesced to form large aggregates.

The X-RD analyses (Fig 2.) of sulfur nanoparticles synthesized under all conditions show the same diffraction peaks, which are well-matched with the standard pattern of orthorhombic phase sulfur from the Joint Committee on Powder Diffraction Standards (JCPDS no. 08-0247). All results exhibit the same maximum of Bragg reflection at 2θ indicating the sulfur nanoparticles were crystalline in nature (Fig. 2). The analyzed particles showed X-RD peaks at 21.31°, 22.44°, 23.12°°, 23.14°, 25.90°, 26.30°, and 27.78°. The DLS analysis revealed that pH affected the size distribution (Fig. 3) and mean particle size (Table 1) of sulfur nanoparticles synthesized using *C. roseus* extract. The control sulfur nanoparticles synthesized at pH 7 in the absence of *C. roseus* extract had a mean particle size of 690±157.9 nm. With the addition of *C. roseus* extract, the mean particle size was 784±78.9 nm at pH 4 and 655±107.0 nm at pH 6. At pH 7 the mean particle size of the nanoparticles synthesized with *C. roseus* extract was 480±39.6 nm, significantly smaller than for the other preparations ($P<0.05$) (Table 1).

The antifungal efficacy of sulfur nanoparticles against *M. canis* DMST29297 is shown in Table 2. The results demonstrated that green sulfur nanoparticles synthesized using *C. roseus* extract at pH 7 had the highest antifungal activity with MIC and MFC values of 1.56 and 6.25 mg/ml, respectively, followed by the nanoparticles synthesized using *C. roseus* extract at pH 6 and 4 (MIC values of 6.25 and 12.50 mg/ml, respectively). The sulfur nanoparticles synthesized without *C. roseus* extract showed no antifungal effects, with MIC and MFC above the range of tested concentrations (MIC>50.00 mg/ml). The internal antifungal control, ketoconazole, had MIC and MFC values of 0.00012 and 0.00025 mg/ml, respectively.

Time-kill tests were performed at 1, 5, and 10-times the MIC for each of the *C. roseus* green sulfur nanoparticle preparations. That is 12.50, 62.50, and 125.00 mg/ml for pH 4, 6.25, 31.25, and 62.50 mg/ml for pH 6, and 1.56, 7.80, and 15.60 mg/ml for pH 7. Fig. 4 shows the time-kill kinetics. No substantial differences in killing efficacy were observed between the different MIC concentrations for each sulfur nanoparticle preparation although the 10-times MIC nanoparticles synthesized at each pH condition had a slightly higher fungal eradication effect compared to

the 1-times and 5-times MIC. From 15 min to 3 h there was a 1.87 to 2.30-$\log_{10}$ reduction in the number of viable fungal cells for all preparations, while only the10-times MIC of sulfur nanoparticles synthesized at pH 7 showed a 3-$\log_{10}$ reduction (99.9% killing), which was at 12 h. After 24 h, all treatments had eradicated more than 99.999% of the fungal cells (5-log10 reduction).

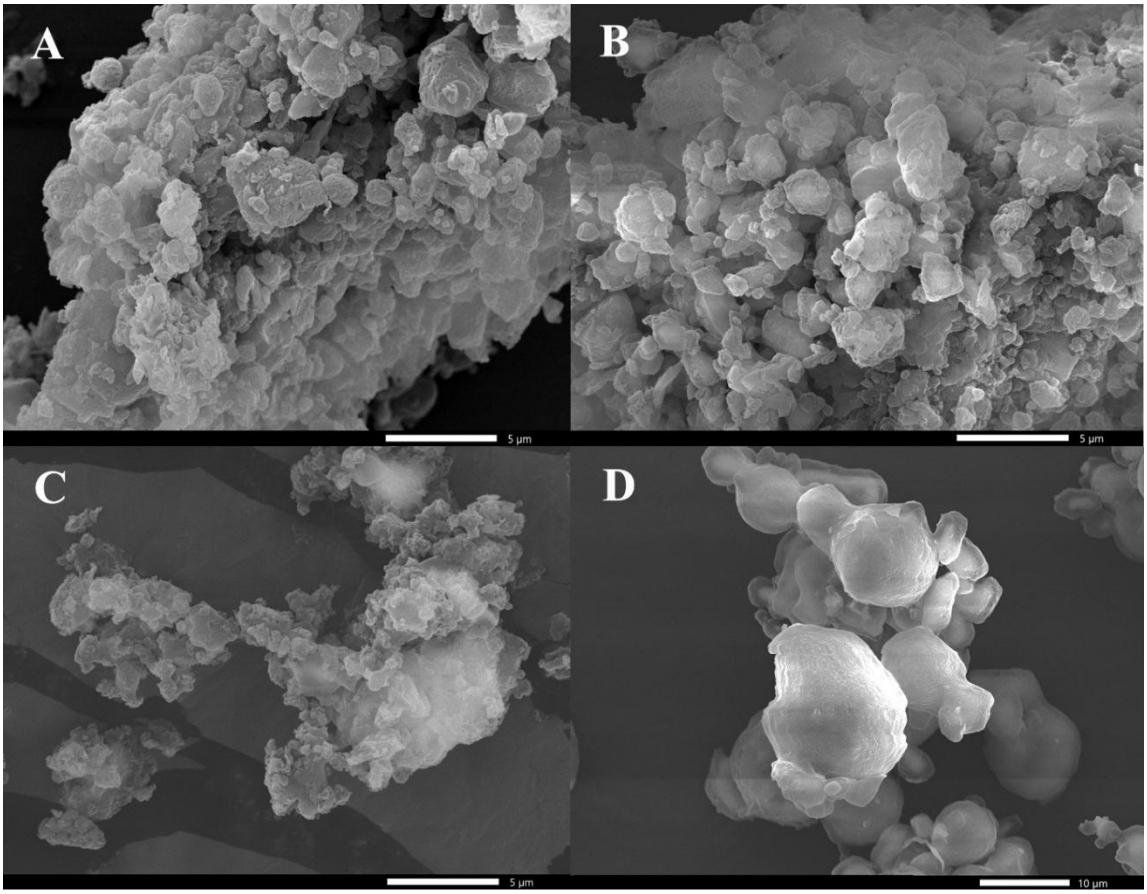

Figure 1 SEM images show the difference in size distribution and homogeneity sulfur nanoparticles synthesized using sodium sulfide with *C. roseus* extract at various pH conditions; pH 4 (A) and pH 6 (B) the most particles were larger than pH 7 (C) (5,000X) but smaller than the particle synthesized without *C. roseus* extract (D) (2,000X).

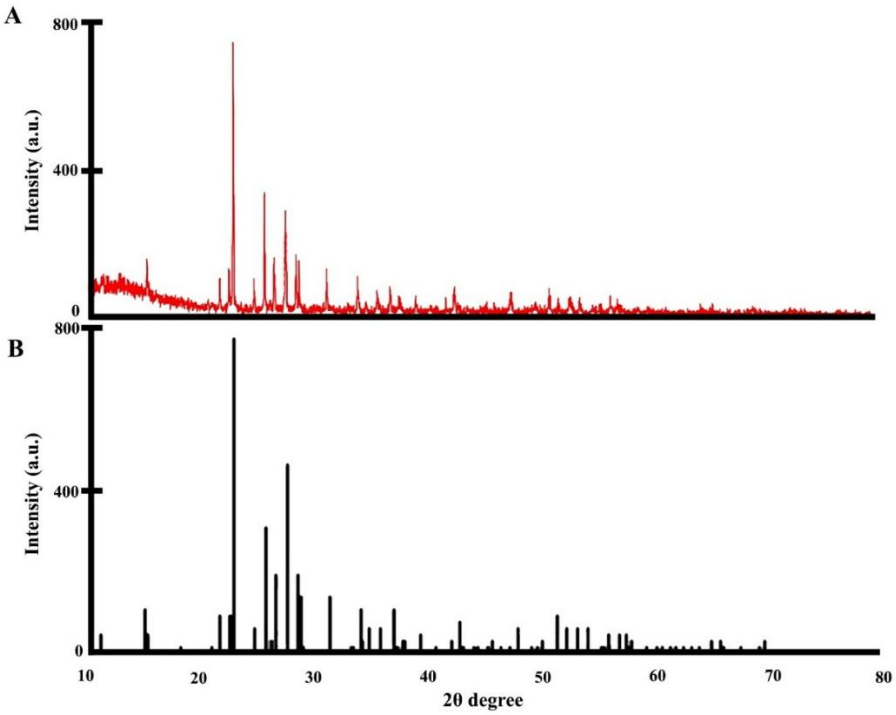

Figure 2 The X-RD pattern of sulfur nanoparticles synthesized from sodium sulfide with *C. roseus* extract at pH 7 (A) compared with orthorhombic phase sulfur (JCPDS no. 08-0247) (B).

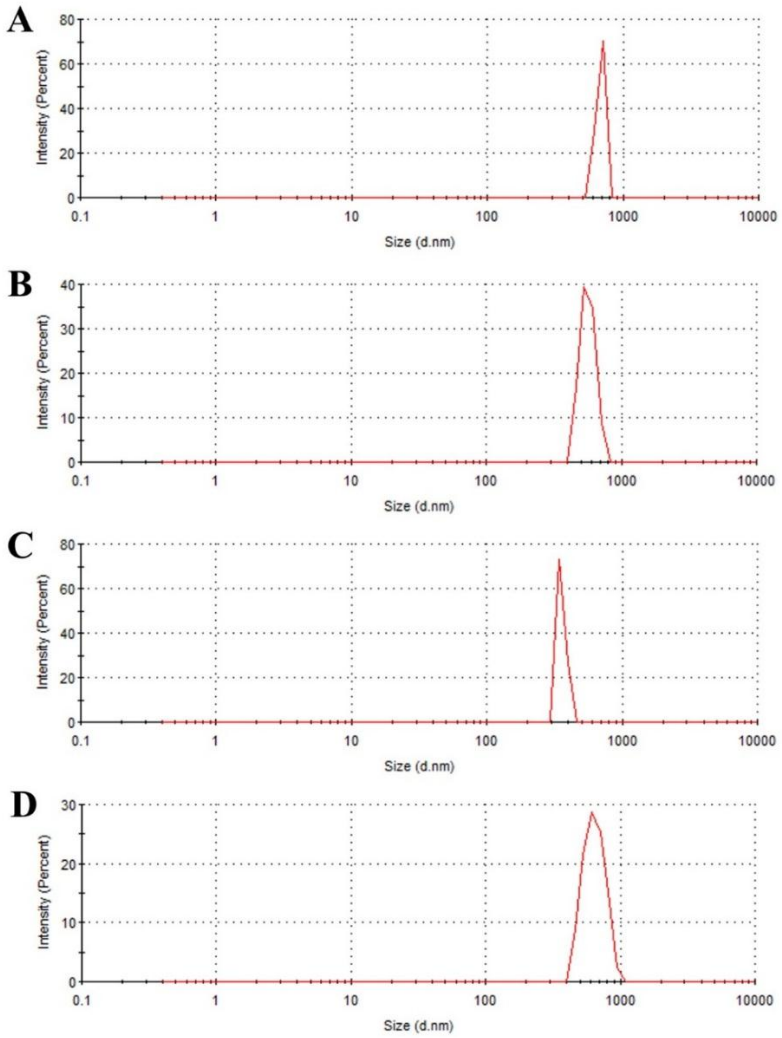

Figure 3 Particle size distribution analysis obtained from DLS. Sulfur synthesized from sodium sulfide with *C. roseus* extract at pH 4 (A), pH 6 (B), pH 7 (C), and without *C. roseus* extract (D).

Table 1 Particle size analysis of sulfur nanoparticles synthesized from sodium sulfide and *Catharanthus roseus* extract at various pH conditions.

Synthesis conditions	Particle size (nm)	
	Range	Average
pH 4 with *C. roseus* extract	510-820	784.7±97.8[a]
pH 6 with *C. roseus* extract	400-815	655.0±107.1[a]
pH 7 with *C. roseus* extract	295-490	480.3±39.6[b]
pH 7	400-1,100	690.3±157.9[a]

The averages represent the mean±SD of particles within the size range of 0.1-1,100 µm obtained from 3 replicates with 10 measurements each. Different superscript letters within a column indicate statistically significant differences (P<0.05).

Table 2 Susceptibility of *Microsporum canis* DMST29297 to sulfur nanoparticles synthesized from sodium sulfide and *Catharanthus roseus* extract at various pH conditions.

Sulfur nanoparticles	MIC (mg/ml)	MFC (mg/ml)
pH 4 with *C. roseus* extract	12.50	12.50
pH 6 with *C. roseus* extract	6.25	6.25
pH 7 with *C. roseus* extract	1.56	6.25
pH 7	>50.00	>50.00
Ketoconazole	0.00012	0.00025

Values represent the statistical mode of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) collected from triplicate experiments.

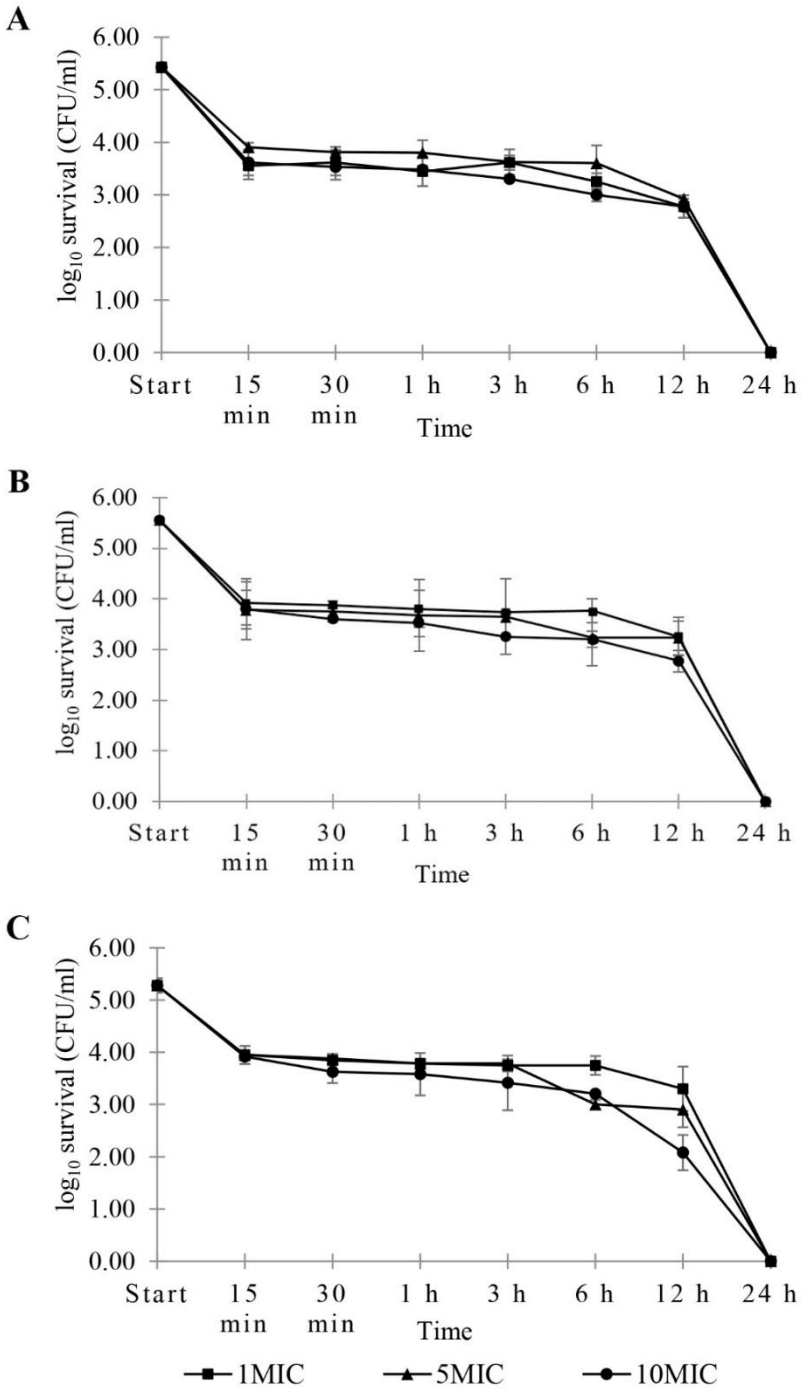

Figure 4 Time-kill kinetics of green sulfur nanoparticles synthesized using sodium sulfide with *C. roseus* extract at various pH conditions against *M. canis* DMST29297. Particles from pH 4; 1xMIC=12.50 mg/ml, 5xMIC=62.50 mg/ml, and 10xMIC=125.00 mg/ml (A). Particles from pH 6; 1xMIC=6.25 mg/ml, 5xMIC=31.25 mg/ml, and 10xMIC=62.50 mg/ml (B). Particles from pH 7; 1xMIC=1.56 mg/ml, 5xMIC=7.80 mg/ml, and 10xMIC=15.60 mg/ml (C). Values represent the means of triplicate experiments with error bars (SD).

Discussion

The green synthesis of sulfur nanoparticles from sodium sulfide with an aqueous extract of *C. roseus* leaves yielded a fine yellow powder made up of a homogenous mixture of spherical particles when observed under SEM. This is consistent with a recent report that used a different leaf extract (from *Rosmarinus officinalis*) and precursor (sodium thiosulphate) to synthesize sulfur nanoparticles (Al Banna *et al.*, 2020). In the current study, the size distribution of the green sulfur nanoparticles was pH dependent, with a smaller average particle size and a narrower range of sizes for sulfur nanoparticles synthesized at pH 7 compared to pH 4 and 6. However, sulfur nanoparticles synthesized without the addition of *C. roseus* extract at pH 7 tended to be larger on average and clumped together to generate a heterogenous mixture of particle sizes. The average particle sizes in this study were higher than the 129.6±15.2 nm reported in a recent study using the same sodium sulfide precursor with a chitosan bioreactor (Saedi *et al.*, 2020) and the 78 nm reported by Khairan *et al.* (2019) who used sodium thiosulfate with

garlic extract (*A. sativum*). According to a study by Soni and Prakash (2011), the pH, temperature, reaction time, reagent concentration, and reagents used can all have an effect on the size and shape of the particles generated, with pH and temperature being most important. The smaller size distribution ranges seen with the addition of *C. roseus* extract suggests that *C. roseus* constituents support the synthetic process to decrease average particle size and improve size distribution. *C. roseus* leaves are rich in phytochemicals such as alkaloids, phenolic compounds, flavonoids, saponins, and tannins that can act as a bioreactor to reduce sulfur ions and then act together with inorganic acids for nucleation with sulfur ions to form stable nanoparticles with a narrow size distribution (Al Banna *et al*., 2020).

X-RD analysis was performed to identify the structure of sulfur nanoparticles based on the comparison of the interatomic-distance characteristics of the condensed matter to the patterns of reference databases. The diffraction peak positions of the synthetic green sulfur nanoparticles were compatible with standardized α-orthorhombic sulfur phase diffraction (α-S_8) according to JCPDS no. 08247. This phase of sulfur is stable at room pressure and temperature. In the orthorhombic allotrope, the S8 rings are arranged in two layers, each perpendicular to the crystal c axis forming a so-called "crankshaft structure". Another common sulfur structure, β-monoclinic sulfur, consists of S8 rings in two kinds of positions that form the ordered skeleton of the crystal. Both the α-orthorhombic and β-monoclinic sulfur are based on S8 molecular units and α-S8 can transform to monoclinic reversibly (Crapanzano, 2008). The S8 α-orthorhombic sulfur structure obtained in our study is consistent with orthorhombic α-sulfur structures generated in previous research by Tripathi *et al.* (2018) using sodium thiosulphate and *Ficus bengalensis* extract, Paralikar and Rai (2017) who used sodium sulfide with four plant extracts (*A. indica*, *C. roseus*, *M. indica*, and *P. longifolia*) and Suleiman *et al.* (2015) who used sodium thiosulphate and tetraoctylammonium bromide (an ionic stabilizer). The α-orthorhombic sulfur is the most common form of sulfur in nature since it is stable at normal temperature and atmospheric pressure conditions. (Crapanzano, 2008). As this sulfur is more stable than other forms, it is widely used in many industries especially agriculture and medicine. It has the advantage in antimicrobial efficacy over micron-sized elemental sulfur and against, which effective against both the conventionally elemental sulfur-resistant and elemental sulfur-susceptible fungi and bacteria (Roy Choudhury *et al*., 2013).

The broth microdilution results revealed that the MIC of ketoconazole (control) on the tested fungal strain was in accordance with the Clinical and Laboratory Standard Institute (2008) which recommended MIC rang of azole antifungal drugs for dermatophytes for broth dilution procedures were 0.00003-0.00025 mg/ml. Green sulfur nanoparticles generated with *C. roseus* extract had at least 4-32 times more anti-*M. canis* DMST29297 activity than sulfur nanoparticles synthesized without *C. roseus* extract. Thus, the antifungal activity was related to particle size in which green sulfur nanoparticles synthesized at pH 7 had the lowest particle size and showed the highest antifungal efficacy. These results substantiate that appropriate control of pH during the synthetic processes is necessary for good particle characteristics and efficacy. No previous study has investigated the activity of sulfur nanoparticles against *M. canis*, but some reports have shown antimicrobial effects of sulfur nanoparticles against other fungi and some bacterial strains. Saedi *et al.* (2020) reported that sulfur nanoparticles synthesized from sodium sulfide with chitosan had antifungal activity against *Aspergillus flavus* ATCC22546 and *C. albicans* ATCC18804, and antibacterial activity against *S. aureus* ATCC13565 and *Listeria monocytogenes* ATCC15313. Paralikar and Rai (2017) reported that sulfur nanoparticles from sodium thiosulfate and *C. roseus* extract had activity against *E. coli* ATCC14948 and *S. aureus* ATCC33591.

The time-kill assay of sulfur nanoparticles synthesized using *C. roseus* extract showed time-dependent fungicidal activity. The increase of sulfur nanoparticle concentration to 10 times its MIC had a minimal effect on eradication rate. These results are in accordance with a recent study that showed a time-dependent antimicrobial effect of sulfur nanoparticles synthesized from sodium thiosulfate with chitosan against *S. aureus* ATCC13565, *E. coli* ATCC11234, *C. albicans* ATCC18804, and *A. flavus* ATCC22546 (Kim *et al.*, 2020). In contrast, Paralikar and Rai (2017) reported that the antibacterial activity of sulfur nanoparticles synthesized with *C. roseus* leaf extract was time- and dose-dependent against *S. aureus* ATCC33591 and *E. coli* ATCC14948. Previously, plant extracts have been shown to improve the dispersion and antimicrobial activity of sulfur nanoparticles (Preprem *et al.*, 2012). The antifungal effects of the sulfur nanoparticles used in the current study may have been enhanced by the *C. roseus* extract supporting an increase in the surface/volume ratio of the sulfur nanoparticle providing an increased contact area between the surface of the sulfur nanoparticles and the organelles of fungal cells. This report did not study the antimicrobial activity of the herbal extract alone as a control group, as at the final process of the synthesis, the obtained particles were washed several times with distilled water and absolute alcohol to remove any residual biomolecules and herbal extracts. Therefore, the antifungal effect of the particles may not be directly derived from the herbal extracts, but only from synthetic green sulfur nanoparticles. Several previous studies were indicated that green sulfur nanoparticles affect the biological molecules of microbial cells, especially membrane bound organelles by causing membrane rupture and lysis. Roy Choudhury *et al.* (2012) reported that one fungicidal mechanism of sulfur nanoparticles was to target cell membranes by reducing the total lipid content in the cell, and Paralikar and Rai (2017) demonstrated that sulfur nanoparticles destabilized the microbial membrane by changing the membrane zeta potential. In addition, a report of Roy Choudhury *et al.* (2013) also demonstrated that the antifungal activity of sulfur nanoparticles correlated to the small particle size and their ability to pervade the microbial cell wall. Fungal cells treated with the particles showed significant

deformities on the cell surfaces and suppressed mitochondrial enzymes involved in cellular respiration and oxidative phosphorylation.

In conclusion, nanoscale sulfur particles were successfully obtained from a green synthetic method using sodium sulfide, sulfuric acid and an aqueous extract of *C. roseus* leaves and conducting the synthesis at pH 7 generated sulfur nanoparticles with the smallest average particle size and highest anti-*M. canis* DMST29297 activity. The time-kill kinetics demonstrated that the fungicidal activity was time-dependent. These effects should be further investigated *in vivo* for development as a drug formulation for treating dermatophyte infections in dogs.

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