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# Kaffir lime leaf extract mediated synthesis, anticancer activities and antibacterial kinetics of Ag and Ag/AgCl nanoparticles

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ABSTRACT

A facile phytosynthesis of Ag/AgCl and Ag nanoparticles (NPs) was developed using a crude water extract of *Citrus hystrix* DC (kaffir lime) leaves. The phytochemical and chloride contents of the extract, and various synthesis parameters were studied. The obtained Ag/AgCl and Ag NPs were of the cubic phase and mostly spherical in shape. Particle sizes of the yielded NPs were distributed in a narrow range with an average size of 20 and 38 nm for the Ag/AgCl and Ag NPs, respectively. The reducing function of the phytochemicals and the stabilizing ability of gelatin were demonstrated. The NPs showed significant antiproliferative activities against the carcinoma HCT 116 and adenocarcinoma Caco-2 colorectal cell lines, but had a negative effect toward human fibroblasts. The NPs exhibited excellent antibacterial activities against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus cereus*. The inactivation kinetics obtained in the presence of the NPs showed a biphasic behavior that can be explained by the Cerf model.



## Introduction

Thanks to their unique physical, chemical, and biological properties, metal nanoparticles (NPs) have been employed in a broad spectrum of applications ranging from environmental remediation to consumer products (Iravani, Korbekandi, Mirmohammadi, & Zolfaghari, 2014). Among economically valued NPs, Ag NPs have been introduced into numerous commodities (Kumar, Angulo, Smita, Cumbal, & Debut, 2016; Zhang, Tang, & Vlahovic, 2016), resulting in tremendous global usage and production. In turn, this leads to health and environmental issues surrounding the available synthesis processes (Ge et al., 2014; Iravani et al., 2014; Tsuzuki, 2013; Uchihara, 2007). While most of the physical approaches are energy and capital intensive, the chemical approaches commonly include the use of hazardous chemicals such as $NaBH_4$, $N_2H_4$, and dimethylformamide (DMF) (Iravani et al., 2014). The possibility that the hazardous residues may remain on the derived NPs eliminates their use in the biomedical and medicinal fields (Ge et al., 2014; Uchihara, 2007). To avoid the use of hazardous chemicals and extend the therapeutic potential of Ag NPs into the aforementioned domains, the employment of natural reagents as substitutes for potentially hazardous chemicals has been intensively examined during the last few decades (Dauthal & Mukhopadhyay, 2016; Makarov et al., 2014).

Among the potential candidates as sources for natural products, biological systems including those based on fungi, algae, yeast, and plant extracts offer several advantages (Dauthal & Mukhopadhyay, 2016; Elemike, Onwudiwe, Ekennia, Ehiri, & Nnaji, 2017; Kharissova, Dias, Kharisov, Pérez, & Pérez, 2013; Singh, Kim, Zhang, & Yang, 2016). In particular, the use of plant extracts or phytochemicals are superior in terms of their cost effectiveness, environmental benignity, process simplicity and reliability (Elemike et al., 2017; Mata, Bhaskaran, & Sadras, 2016). Phytochemicals such as terpenoids, flavonoids, alkaloids, and phenolic compounds can serve as both reducing and stabilizing agents in the synthesis of NPs (Dauthal & Mukhopadhyay, 2016; Devi & Ahmaruzzaman, 2016; Elemike et al., 2017; Khademi-Azandehi & Moghaddam, 2015; Makarov et al., 2014). The presence of other

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chemical species such as nitrate, sulfate, and chloride anions in plant extracts may also lead to other products, some of which exhibit additional advantages (Cataldi, Margiotta, Del Fiore, & Bufo, 2003). For example, AgCl NPs have been reported as attractive candidates for applications in both the environmental industry and medical field due to their low water solubility and antibacterial activities (Min, Yang, Kim, & Kwon, 2010). In reality, the use of AgCl NPs is restrained by factors similar to those that limit the adoption of Ag NPs, one of which is the use of hazardous reagents and organic solvents in the synthesis (Iravani et al., 2014; Min et al., 2010).

Ag NPs have been reported to possess unique anticancer and antibacterial properties (Jeyaraj et al., 2013; Kovács et al., 2016; Lemire, Harrison, & Turner, 2013). Reports on the anticancer properties of Ag NPs are relatively immature compared with those describing their antibacterial efficacies. Despite a growing number of reports on their antibacterial activities, the mechanisms by which Ag NPs inhibit the growth or even kill the bacteria are still inconclusive. Whilst some suggested the Ag NPs damage the cell membrane structure and function causing growth inhibition or even apoptosis (Pelgrift & Friedman, 2013), some proposed the possibility that the Ag NPs can penetrate the bacterial cells and disrupt cellular metabolism (Hsueh et al., 2015; Pelgrift & Friedman, 2013). Despite these conflicting reports, kinetic studies on bacteria survival behavior under the influence of Ag NPs are very limited. In other systems, several models have been proposed to describe survival behavior among which the Cerf (Cerf, 1977; Xiong, Xie, Edmondson, & Sheard, 1999), Gompertz (Cerf, 1977; Xiong et al., 1999), and Weibull (Albert & Mafart, 2005) models describe nonlinear survival behavior. A kinetic study provides a fundamental and essential body of knowledge useful for the assessment of further practice.

Because the characteristics of the derived Ag NPs can significantly vary depending on the molecular structures and concentrations of phytochemicals in the plant extracts as well as the synthesis parameters, it is useful to examine alternative sources of phytochemicals such as a crude water extract of *Citrus hystrix* DC (kaffir lime) leaves. Kaffir lime is notably inexpensive and readily available in Asia. Its leaves are reported to be rich in phytochemicals containing phenolic and carboxylic functional groups, which make them potentially useful for the successful synthesis of Ag NPs (Dauthal & Mukhopadhyay, 2016; Devi & Ahmaruzzaman, 2016; Elemike et al., 2017; Makarov et al., 2014; Singh et al., 2016; Waikedre et al., 2010). To increase the stability of the synthesized Ag NPs, the natural stabilizer gelatin has also been employed in the synthesis (Darroudi, Ahmad, Zak, Zamiri, & Hakimi, 2011). The influence of the silver ion and gelatin concentrations on the derived NPs were investigated. The reducing function of the phytochemicals and the stabilizing ability of the gelatin are demonstrated. The anticancer activities against adenocarcinoma and carcinoma colorectal cell lines are reported. The antibacterial activities and their kinetic studies against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus cereus* are presented.

## Materials and methods

### *Raw materials and analytical techniques*

Kaffir lime leaves were collected from Lampang province, Thailand. The crude water extract of the leaves was prepared fresh before every use by boiling 10.0 g of the fresh leaves in 200.0 mL of boiling water for 30 min. The total phenolic content of the crude extract was determined using the Folin–Ciocalteu procedure (Singleton & Rossi, 1965). The organic species in the crude extract were analyzed by gas chromatography–mass spectrometry (GC–MS; Agilent, USA; J&W HP-5MS Ultra Inert GC Column, 30 m length, 0.25 mm internal diameter, 0.25 μm film thickness).

The UV–visible spectra were recorded using a Lambda 25 UV–Vis spectrophotometer (Perkin Elmer, USA). The IR spectra were recorded using a Tensor 27FT-IR spectrometer (Bruker, Germany). Transmission electron microscopy (TEM) was conducted using a TECNAI G2 20 S-twin microscope (FEI, USA) equipped with a selected area electron diffraction (SAED) unit. Powder X-ray diffraction (PXRD) was performed using a Bruker D8 Advance diffractometer (Cu Kα, Ni filter, $\lambda = 1.540598$ Å, 40 kV, 30 mA). Thermogravimetric/differential scanning calorimetric analyses (TGA/DSC) were conducted using a STA 409 PC/PG instrument (Netzsch, Germany).

### *Kaffir lime leaf extract-assisted synthesis*

As a general procedure, 5.0 mL of the water extract of the kaffir lime leaves and 10.0 mL of $AgNO_3$(aq) (99.0%; Aldrich, USA) were added dropwise into 5.0 mL of gelatin (Labchem, USA) solution with vigorous stirring at room temperature. After the addition of both reagents, the mixture was left at ambient temperature and UV–Vis spectroscopy was used to follow the progress of the reaction up to 192 h. Varying the concentrations of $AgNO_3$(aq) (0.20–2.0 mmol/L) and gelatin (0.25–2.0 mg/mL) were investigated.

### *Anti-proliferative tests against human colorectal cells*

The NPs chosen for the anti-proliferative tests included the as-synthesized Ag/AgCl NPs yielded from the reaction using 1.0 mmol/L $AgNO_3$(aq), 0.50 mg/mL gelatin and 72 h of reaction time, and the corresponding Ag NPs yielded after the thermal treatment of the above reaction products at 900 °C for 1 h. 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) was used to evaluate the anti-proliferative activities of these NPs against primary human dermal fibroblasts (ATCC® PCS-201-012TM) and two types of human colorectal cancer cell lines, namely the colorectal carcinoma HCT 116 (ATCC® CCL-247TM) and the colorectal adenocarcinoma Caco-2 (ATCC® HTB-37TM). The tested cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/mL penicillin. Initially, 96-well cell culture microplates were seeded with 200 μL of cell suspension at $1 \times 10^4$ cells per well for the primary human dermal fibroblasts and $5 \times 10^3$ cells per well for the HCT 116 and Caco-2 cells. After 24 h of incubation, cells were treated with the NPs at four different concentrations: 200, 100, 50, and 25 μg/mL. Three wells containing the same number of the cells acted as the negative control on each plate. After 120 h of incubation in 5% $CO_2$ and 37 °C for the primary human dermal fibroblasts, and 72 h for HCT 116 and Caco-2 cells, the medium solution was removed and WST-1 solution was added to each test well and further incubated at 37 °C for 1 to 2 h. The optical density was then measured with a microplate reader at 490 nm to determine cell viability (Jarray et al., 2011).

### *Antimicrobial activities assessment and kinetic study*

The Ag/AgCl and Ag NPs produced using the same synthesis conditions employed for those used for the anti-proliferative tests were used in the assessment of antibacterial activities against Gram-positive *S. aureus* (ATCC 25923) and *B. cereus* (ATCC 11778), and Gram-negative *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853). The antimicrobial effectiveness test was adopted for this assessment (Sutton & Porter, 2002). The initial concentrations of the bacterial stocks were determined based on the apparent turbidity compared with McFarland No. 0.5. For testing, the bacterial stocks were added into a suitable nutrient media of 150–250 million

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