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Extracellular Bio-synthesis of Bio-active Nano-silver Using Alfalfa Seedling.

Gamal H Rabie[1]*, Hegazy S Hegazy[1], Lamis D Shaban[1], and Diana S Raie[2].

[1] Faculty of Science, Zagazg University, Zagazig City, Sharqya State, Egypt.
[2] Egyptian Petroleum Research Institute, Nasr City, Cairo, Egypt.

ABSTRACT

After exposure of seedling to aqueous solution of silver nitrate, spherical silver nanoparticles are bio-synthesized with a size ranges from 5 to 30 nm. Nanoparticles are characterized using high resolution transmission electron microscope, ultraviolet–visible absorption spectroscopy and FTIR spectroscopy. Adjustment of bulk material pH plays a significant role in metal bio-sorption and nanoparticle morphology. A spherical shape in a size ranges from 2 nm to 7.5 nm occurs at pH 10. The main reducing agent is supposed to be an antioxidant exuded as a result of metal stress. The stabilizing agent could be polyphenols in aliphatic mode. The bio-synthesized spherical silver nanoparticle ranging from 2 nm to 7.5 nm is tested against *Candida albicans*. It is approved its fungicidal effect. So, alfalfa seedling can be used a bio-maker for bio-active nano-silver.

Keywords: Silver Nanoparicles, Alfalfa, Seedling, Anti-fungal, Bio-synthesis, Antioxidants.

Corresponding author

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INTRODUCTION

Communication between plants and metals has been pictured in a new form after introducing living organisms as routes for nano-synthesis [1]. If plants are exposed to such metals, they will receive a stress message. Metallo-tolerant plants reply with a protection mechanism of a bio-sorbtion [2] or bio-complexation through bio-molecules exudates [1]. Studying this interaction, botanists find that metals are bio-converted into nano-items either inside [2] or outside the cells [1]. Consequently, getting useful of self-defense of plants is leading to green style of bio-active nano-items production. Thus bio-molecules are bio-machines [3].

Machines are defined as any connected system to modify, transfer, and direct applied forces in a programmed style to achieve a particular task. In this context, many bio-molecules that can perform specific objectives are machines [3]. For nanoparticles synthesis, biological path uses bio-molecules, for example antioxidants, as reducers and stabilizers [1]. They are characterized by accuracy, availability and cheapness, besides being safe and bio-degradability [3]. Plants are distinguished other biological systems by being available, inexpensive, and easily to be cultivated and harvested [4]. They overcome the problem of purification of strains like microbe. They rise above the risk of contamination or mutation into pathogenic organism. In addition, they are abundant sources for antioxidants for the task of reduction and stabilization [4]. Plant as a whole-cell approves its ability to create nanoparticles outside [1] and inside [5] their cells. Besides, cell free exudates [4] and extracts [6] from different parts can fabricate nanoparticles.

In the present study, we select alfalfa seedling as a nano-silver builder unit. From one hand, seedling is a suitable bio-mass for its growing metabolism. From another hand, the choice of alfalfa depends on its ability to absorb [2] and adsorb silver [7] from agar media [5], Hoagland's solution [2] and de-ionized water [8]. Also, it has aptitude to transform silver nitrate into nano-silver inside its cells using seedling grown hydroponically [2] and on agar medium [9]. Hydroponic grown alfalfa seedling is used for nano-gold extracellular bio-production [8]. From our available literature, it is the first report on extracellular production of nano-silver outside alfalfa seedling cells. In addition, silver in a bulk form has anti-microbial activity [1] but it suffers from precipitation and complex formation outside pathogens which cause losing its efficiency [10]. So, their nanoparticles are regarded as a reservoir for ions [11]. Their activities are depending on several factors such as size [12], shape [13], surface charge [14], and the stabilizing agent [15]. So, studying of its bio-activity is required for each new method of synthesis. Therefore, our focus is to clarify; the mechanism used to synthesize nano-metal, how to control the morphology of biogenic nano-silver and the quality of bio-produced nano-silver through its bio-activity.

MATERIALS AND METHODS

Alfalfa seeds are grown hydroponically, from sterilized seed, in de-ionized water, for 4 weeks under artificial light [2]. A known fresh weight of seedling (0.2 mg) is exposed to 2 ml of 0.1 M aqueous $AgNO_3$ [4]. For pH dependent study, the pH of $AgNO_3$ solution is adjusted in the range of pH 2 to pH 11 using diluted nitric acid and aqueous ammonium hydroxide. The reactions proceed in the absence of ambient light, at room temperature and incubated for 24 h [8]. Following the exposure to the metal substrate, plant samples are collected then washed, dried, ashed and acid digested for metal content analysis by atomic absorption spectroscopy. The change of $AgNO_3$ color is studied by ultraviolet–visible spectrophotometer. Nanoparticle shape, size and particle size distribution are determined using TEM [1]. Nano-silver is purified from the exudates by centrifugation. The pellet is washed 3 times with DI then dried and grinded with KBr pellets for stabilizing agent prediction *via* FTIR measurement [6]. The supernatant is processed for reducing agent assay. Total proteins, carbohydrates, phenols, and flavonoids contents are calorimetrically quantified using Lowery, phenol sulfuric acid, Folin–Ciocalteu, aluminum chloride assay methods, respectively.

Effect of Ag^0 and Ag^+ on *Candida albicans*

A known wt (0.01 g) of both Ag^0, with size range from 2-7.5 nm, and $AgNO_3$ are dissolved in 100 ml distilled water. The prepared anti-fungal stocks are filter sterilized by 0.45 µm filter membrane. Disks containing 10 µg of tested agent are prepared by placing 0.01 ml of the prepared stocks on sterile 0.5 cm diameter paper disks [16]. Prepared sterilized disks with tested anti-fungal agents and distilled water, as

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control, are placed on Sabouraud dextrose agar medium swabbed with at a concentration 10^7 spore/ml for *Candida albicans*. The plates are incubated at 37 ºC for overnight. The inhibition zone is measured in cm after the 24 h of incubation and recorded. IC_{50} of Ag^0 and Ag^+ using water as control against *Candida albicans* are determined by turbidimetric method. K^+ in cell free filtrate analysis by atomic absorption spectroscopy **[2]**. The results are expressed as µg of K^+/g of *Candida albicans* fresh wt.

Statistical Analysis

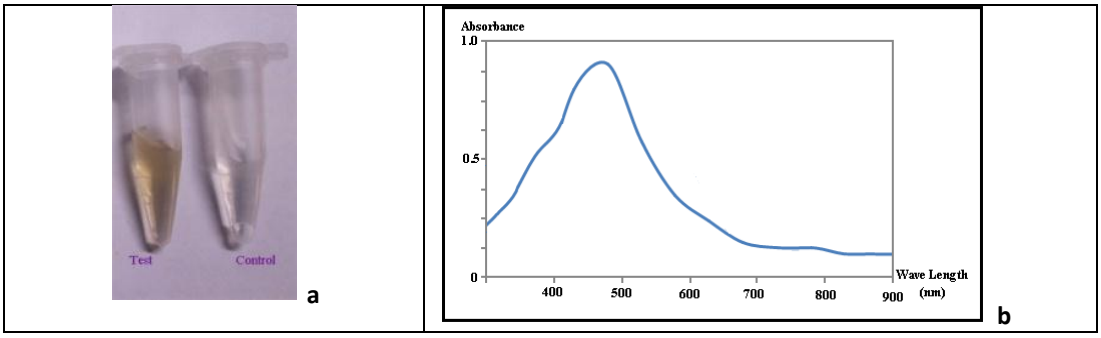
Statistical analysis is done at SPSS 11.0. Samples are analyzed in triplicates. The difference in either shapes or sizes of Ag^0 with and without pH adjusted $AgNO_3$, and the difference in K^+ efflux is calculated by one way ANOVA test. Differences in either IC_{50} between Ag^0 and Ag^+ or sensitivity of *C. albicans* to them are calculated using the t-test for independent samples.

RESULTS AND DISCUSSION

In the present study, we expose alfalfa seedling to $AgNO_3$ with and without pH adjustment, then separate the exposed silver to test its anti-candidal activity.

Alfalfa seedling exudes can bio-synthesize Ag^0 through a mechanism supposed to be bottom-up [4]. Fig. (1.a, b, c) is a primitive, supportive and confirmative outputs of nano formation, respectively. It shows the change of silver solution from colorless into yellow, absorption in the visible region and TEM image. These characteristic yellowish look and SPAB (surface plasmon absorption band) are due the excitation of SPR (surface plasmon resonance) in the Ag^0 [1]. In response to the metal stress, seedling exudes antioxidants Fig. (1.d). They may participate in reduction of bulk silver into Ag^0 [6], [1]. The generated excess surface free energy can be minimized by adsorption of organic materials [6]. The outcomes in Fig. (1.e) suspect the stabilizer to be polyphenols with aliphatic mode and bound amide region. However the bio-synthesized Ag^0 is mono-shaped spherical Ag^0, it has size ranges from 5 nm to 30 nm. So, other factors should be studied to control the size.

Our findings approve that pH adjustment plays a significant role in metal bio-sorption and nanoparticle morphology Fig. (2). The ability of alfalfa seedling to absorb [2] or adsorb silver [7] is reported. However the pH dependent bio-sorption affects on the final amount of bio-produced Ag^0 represented by Fig. (2.c), it influences on the size of nanoparticle. In Fig. (2.b), SPAB predicts that the optimum condition to have a uniform size occurs at pH 10. This is enhanced by TEM Fig. (2.d); as spherical shape in a size ranges from 2 nm to 7.5 nm. The increase in size mono-dispersity, at pH 10, may be due to a suitable concentration of bio-molecules for the metal. Also, the electrostatic repulsion force may take part [4]. Thus, we can conclude that our bio-fabricated Ag^0 are negatively charged [4].



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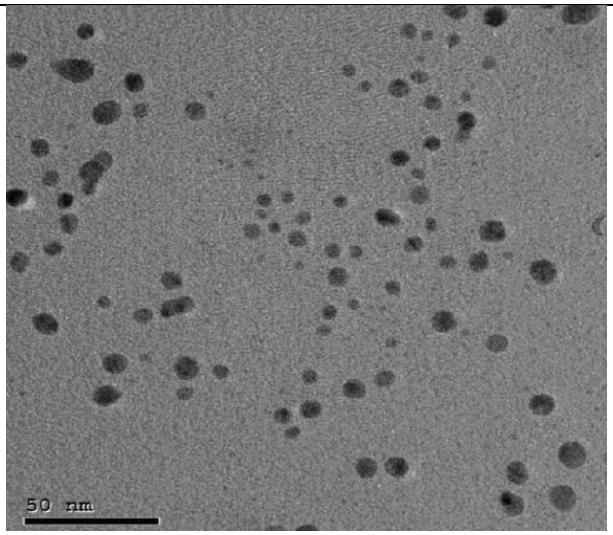

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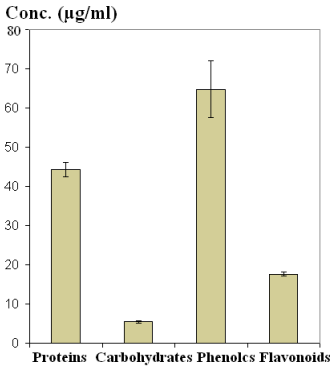

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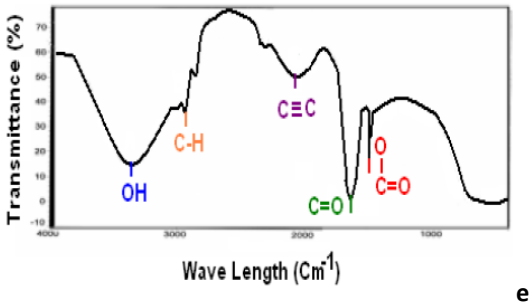

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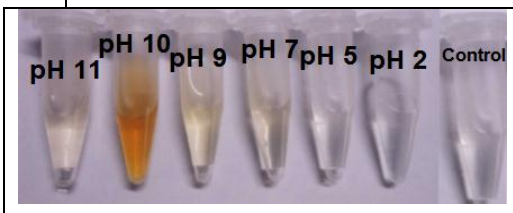
Figure 1: Ag^0 bio-synthesized by seedling exudates reacted with $AgNO_3$ (a) Yellowish color (b) UV-Vis Spectrum (c) TEM Micrograph (d) Quantitative assay of possible reducing agents (e) FTIR Spectrum of possible stabilizing agent



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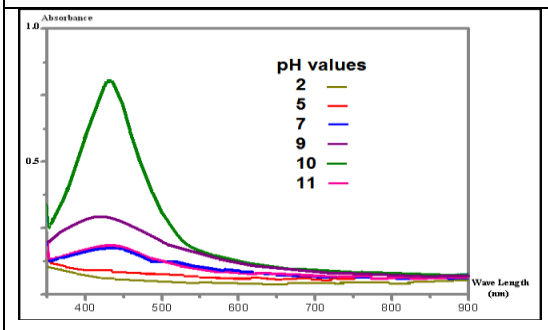

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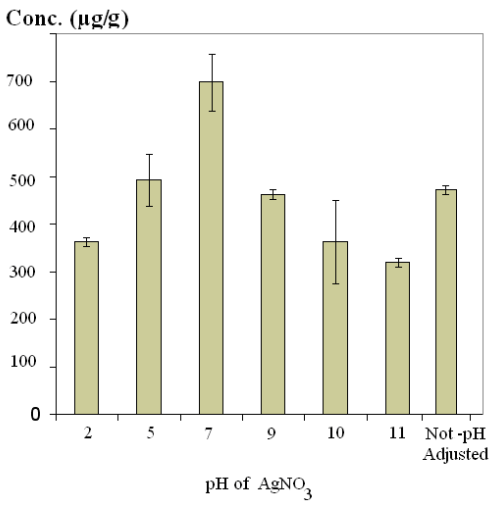

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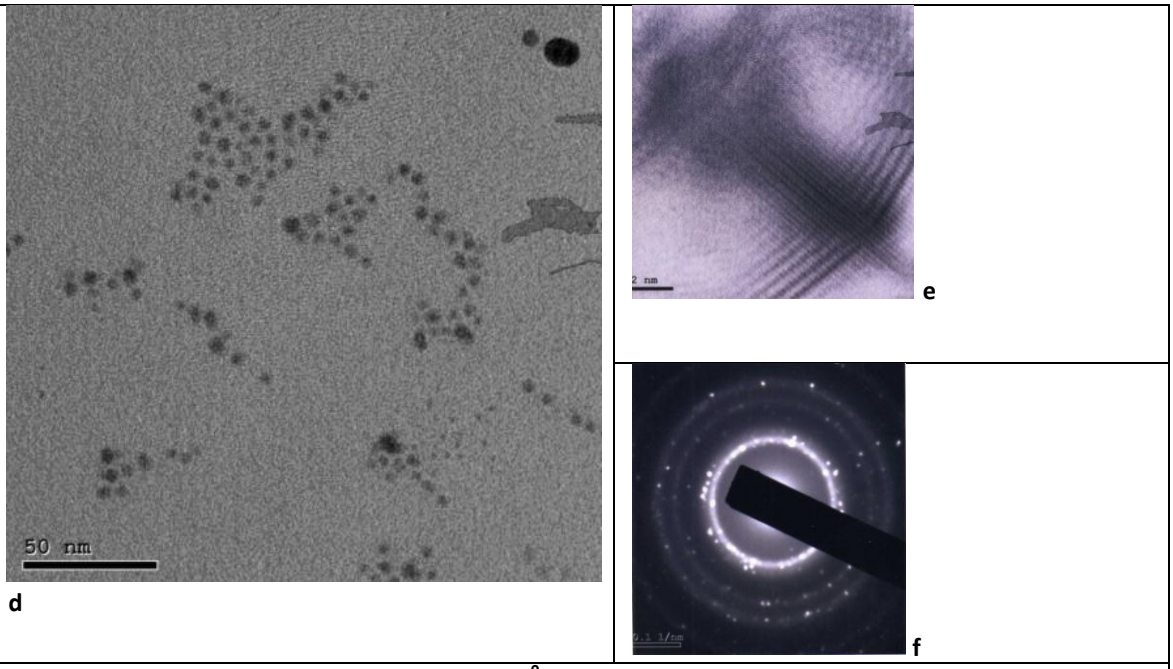
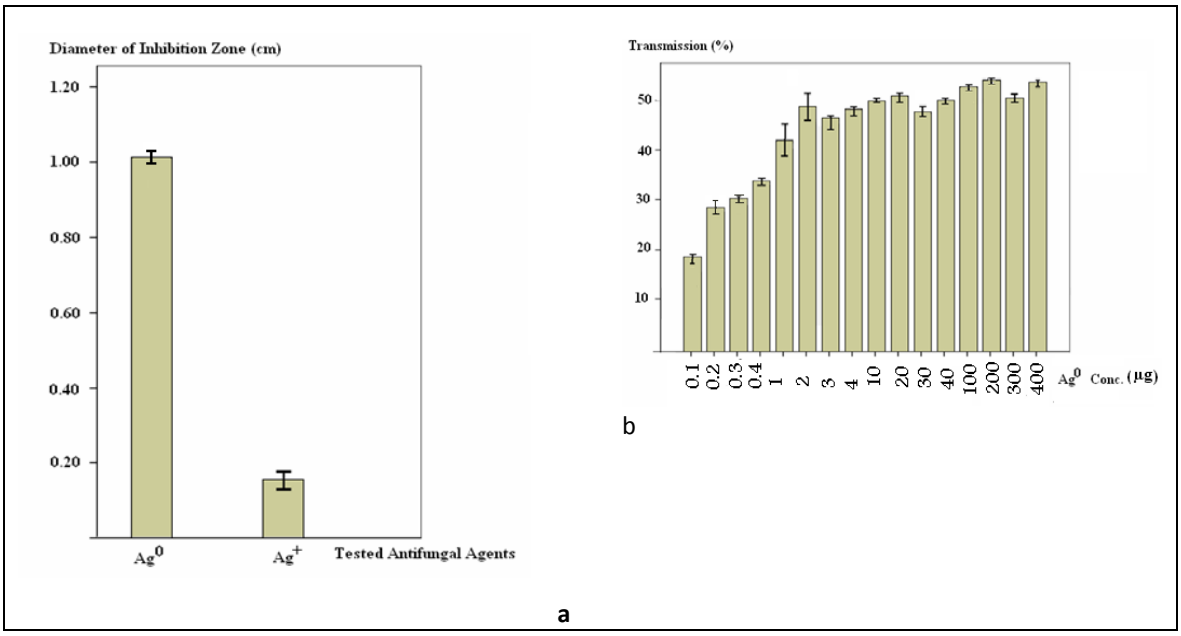


Figure 2: Effect of pH on bio-synthesis of Ag^0 by alfalfa seedling exudates (a) Different degree of yellowish color at different pH (b) UV-Vis Spectra (c) pH-dependent Ag bio-sorption by Seedling (d) TEM micrograph of bio-synthesized Ag^0 at pH 10 (e) HRTEM micrograph of bio-synthesized Ag^0 at pH 10 (f) SAED pattern bio-synthesized Ag^0 at pH 10

The present study indicates the susceptibility of *Candida albicans* to negatively charge spherical Ag^0 and Ag^+. In Fig. (3.a), Ag^0 in both *in vitro* disc diffusion assays has a significant higher anti-fungal activity. In IC_{50}experiments Fig. (3.b, c); the lower activity of ionic silver may be due to complex formation and extra-cellular precipitation [10]. The superior activity of Ag^0 is due to being a delivery system. It may degrade over time resulting in the release of Ag^+ when it get touch with the dissolved oxygen in water [11]. The larger surface area enables it to be higher fungicide reactivity.



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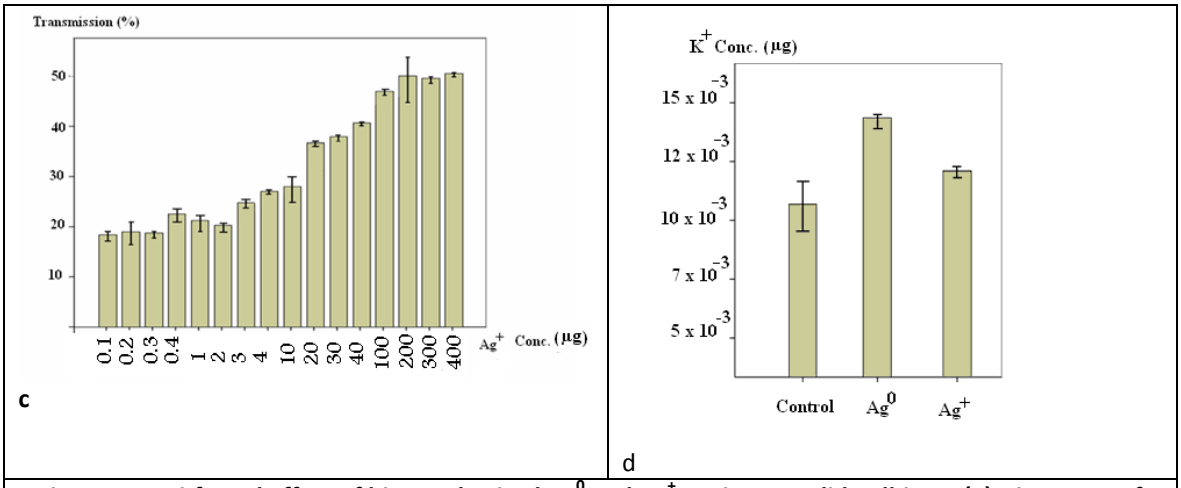

Figure 3: Anti-fungal Effect of bio-synthesized Ag^0 and Ag^+ Against *Candida albicans* (a) Diameters of Inhibition Zones (b) IC_{50} of Ag^0 (C) IC_{50} of Ag^+ (D) Concentration K^+ (g) Efflux Induced by 0.1 µg of Ag^0 and Ag^+

To date, several mechanisms have been postulated for the anti-microbial property of Ag^0. It interacts with yeast cell wall components [10] and disrupts cell membrane [17] leading to a disruption in the ion efflux system occurs. Ag^+ inhibits the activity of plasma membrane H^+ ATPase during the treatment [18]; as it forms complexes with the sulfur-, nitrogen- or oxygen-containing functional groups of microbial enzymes [11]. This may explain our outcome indicating Ag^0 and Ag^+as inducers for K^+efflux Fig. (3.d). The metal depletion may cause the formation of irregular-shaped pits in the outer membrane. Consequently, the progressive release of lipo-polysaccharide molecules and membrane proteins causes a change in membrane permeability [19]. So, the bio- Ag^0 is bio-active particle.

CONCLUSION

From this study, we present Ag^0 biological formation, pH as a significant effect, silver bio-sorption as a challenge, and positive anti-fungal activity. So, the opportunity of using alfalfa seedling as a green maker for bio-active nanoparticle is growing.

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