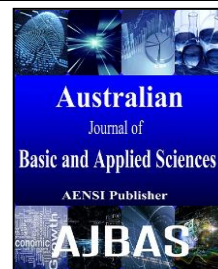




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Biosynthesis of silver and silver chloride nanoparticles by *Parachlorella kessleri* SAG 211-11 and evaluation of its nematocidal potential against the root-knot nematode; *Meloidogyne incognita*

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ABSTRACT

The present study aimed to prepare silver and silver chloride nanoparticles (Ag and AgCl-NPs) using the crude aqueous extract of the green microalga *Parachlorella kessleri* SAG 211-11, study their physicochemical properties and evaluation of their nematocidal proficiency against the 2nd larval instars and egg hatchability of *Meloidogyne incognita*. This process showed a rapid formation of highly stable Ag and AgCl-NPs. UV-Vis absorption spectrum revealed the biosynthesis of Ag and AgCl-NPs with a maximum absorbance at 414 nm. TEM analysis showed formation of spherical Ag and AgCl NPs with an average size of 10.3 to 47 nm. Crystalline nature of the synthesized nanoparticles are also confirmed by their remarkable peaks in the XRD patterns corresponding to 111, 200, 220 and 311 planes for Ag-NPs and 111, 200, 220, 311, 222, 400 and 331 planes for AgCl-NPs. The synthesized Ag/AgCl-NPs exhibited magnetic properties with -33.5 mv surface charge. Fourier Transform Infra Red spectroscopy (FTIR) of purified nanoparticle fractions suggested that proteins are the main molecular entities involved in Ag and AgCl-NP formation and stabilization. The biosynthesized Ag/AgCl-NPs exhibited significant reduction of eggs hatchability at low concentrations (10-20% v/v) as well as, significant increase in larval mortality over the chemical nematocidal at concentrations (50–500 µl/L). LC50 values were 200 and <50 µl/L at 24h and 48h of incubation respectively; 500µl/L completely inhibited larval growth by 100%. The presented study highlights the possibility of large-scale production of Ag/AgCl-NPs by using *P. kessleri* due to its simplicity, accessibility and effectiveness and can be highly recommended as a promising tool for biological control of root knot nematode (*M. incognita*).

INTRODUCTION

Over the past two decades, there has been remarkably increasing interest on biosynthesis of nanomaterials from algae due to their simple and attractive applications in many fields of science, e.g. agriculture (Abdel-Raouf *et al.*, 2017; Hamed *et al.*, 2017; Hussein *et al.*, 2017; Ibraheem *et al.*, 2017), drug delivery systems (Saravanan *et al.*, 2011), and electronics (Warren *et al.*, 2012). Silver nanoparticles including both silver chloride nanoparticles (AgCl-NPs) and metallic silver nanoparticles (Ag-NPs), besides their combinations Ag and AgCl-NPs are more reactive and toxic than their other macroscopic counterparts due to their larger surface areas to volume ratios (Ivask *et al.*, 2013). Ag-NPs have been the most extensively studied silver nanoparticle type for both human health and environmental perspectives (Panyala *et al.*, 2008). Silver chloride is perhaps the most widely recognized and has been extensively used as: photographic material, promising photo catalysis at

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low cost method for the removal of hazardous materials and organic pollutant, antibacterial agent, antifungal agent, antioxidant (Gopinath *et al.*, 2013; Kumar *et al.*, 2015; Kang *et al.*, 2016). Ag-NPs could be used as a strong cell wall disruptor to release carbohydrates and lipids from the green microalga *Parachlorella kessleri* for biofuel production (Abdul Razack *et al.*, 2016). Recent approaching techniques had been established for synthesis of silver chloride nanoparticles by chemical agents (Guzmán *et al.* 2009), host-guest nanocomposite materials (Zhao *et al.*, 2008), radiation (Deekonda *et al.* 2016), photochemical methods (Henglein 1998) and electro-spinning (Nguyena *et al.*, 2010). However, the biogenic (green) synthesis of Ag and AgCl-NPs could provide clean, non-toxic, simple and ecofriendly environmental nanocomposites (Thakkar *et al.*, 2010; Abdel-Raouf *et al.*, 2013). Indeed, a number of algae, fungi, yeast, bacteria, and viruses (Klaus *et al.*, 1999; Nayak *et al.*, 2011; Abdul Razack *et al.* 2016) or plant extracts (Gopinath *et al.*, 2013; Nezamdoost *et al.*, 2014; Kumar *et al.*, 2015) were used in green biosynthesis Ag-NPs. Microalgae are photosynthetic microorganisms which could provide a wide spectrum of sustainable sources characterized by valuable products that can be used in consumer goods and biotechnological applications (Pulz and Gross 2004). The broad spectrum of these highly valuable microalgae-derived products currently include fatty acids, pigments and enzymes as well as the microalgal biomass, which is considered a healthy food supply (Derner *et al.* 2006). Microalgae are considered as nanofactories for nanoparticles production, due to their rapid and high growth rates and high biomass production in a short time. Cyanobacteria, such as *Anabaena* spp., *Calothrix* sp., and *Leptolyngbya* sp., as well as the green microalgae including *Chlamydomonas reinhardtii* and *Chlorella vulgaris* have been reported to biosynthesize intracellular gold, silver, palladium and platinum NPs (Brayner *et al.*, 2007; Barwal *et al.*, 2011; Ferreira *et al.*, 2016). The green microalga *P. kessleri* is mainly characterized by high biomass and lipid productivity (Přibyl *et al.*, 2012; Fernandes *et al.*, 2013), and possessing distinct antioxidant properties. It also could be used as bioremediator for treatment of bio-industrial wastewaters (O'Rourke *et al.*, 2016), as well as for biogas production in large scale (Klassen *et al.*, 2015). However, using *P. kessleri* in green biosynthesis of Ag nanocomposites and screening of their nematicidal proficiency has not addressed before. In Egypt, plant parasitic nematodes especially the root-knot nematode *Meloidogyne* spp. represents one of the most important agricultural pests of many economic fields and vegetable crops (Ibrahim *et al.*, 2010; Ibrahim *et al.*, 2014). *M. incognita* is the most important genera of plant parasitic nematodes which distributed all over the world and infects thousands of plant species (Hassan *et al.*, 2016). Root-knot nematodes can be managed by chemical nematicides but most of them are expensive, pollute the environment or have been withdrawn from use (Greco *et al.*, 1992; Abd-Elgawad, 2008). The main goals of this study were to investigate the biosynthesis and characterization of Ag/AgCl-NPs using the aqueous algal extract of the green microalga *P. kessleri* and to screen their nematicidal effect against eggs hatching and larval mortality of the tomato root knot nematode *M. incognita*.

MATERIALS AND METHODS

Algae species and culture condition:

The fresh-water green microalga *Parachlorella kessleri* SAG 211-11 was obtained from the algal culture collection of Microbiology Department, Soil, Water and Environment Research Institute, Agricultural Research Centre, Giza, Egypt.

Biosynthesis of Ag and AgCl-NPs:

The biosynthesis of Ag and AgCl-NPs by the aqueous algal extract of *P. kessleri* was conducted according to the method described by Morsy *et al.* (2014).

Characterizations of biosynthesized Ag and AgCl-NPs:

Visual characterization:

Biosynthesis of Ag and AgCl-NPs were confirmed by visual observations of the developed colour in the reaction mixture flask including the algal extract with 1mM AgNO₃ solution, as compared to controls. The colour changed from pale yellow to brown was visually checked, which indicated the extracellular synthesis of Ag and AgCl-NPs. The Ag and AgCl-NPs, were obtained by centrifugation the mixture at 12000 rpm for 10 min and washing the pellets thrice with double distilled water. The obtained Ag-NPs pellets were then lyophilized for further studies.

UV-Vis spectral analysis:

The UV-visible spectrum was recorded using UV-visible spectrophotometer (UV-2600 Shimadzu, Germany). Samples were measured between 200–500 nm wavelengths and the maximum range was recorded. All measurements were carried out at room temperature.

Transmission Electron microscopy analysis:

TEM micrographs were obtained from FEI, Netherland, Tecnai G20 Super twin, double tilt TEM, operating at an accelerating voltage of 200 kV. The sample suspension was sonicated for 5 minutes to separate the agglomerated particles and to get homogeneous sample. A drop of the suspension was sampled using a

micropipette and immediately placed on a film on a support grid. The sample examined after the solvent has completely evaporated.

X-ray diffraction analysis:

The crystallinity and elemental composition of the developed NPs were assessed and identified by X-Ray powder diffractometer (202964 Panalytical Empyrean) with CuK α 1 radiation, the voltage and the current of the X-ray source were 40 KV and 30 mA, respectively. The sample was drop-coated onto silica plate by applying many layers of small amount of the sample on the plate with intermittent drying. This leads to a thick coat of the sample.

Zeta Potential measurements:

Surface charge of synthesized Ag and AgCl-NPs were measured using Malvern Zeta sizer instruments (MAL1121994)

FTIR analysis:

FTIR spectra data is commonly used for identifying biomolecules responsible for the reduction of Ag ions and capping of the biosynthesized Ag and AgCl-NPs. The samples of the freeze-dried synthesized nanoparticles were grinded with potassium bromide (KBr) and the spectrum was recorded on FTIR spectroscopy Vertex 70.

Preparation of the root-knot nematode (*M. incognita*) culture:

Individual egg-masses of distinct root-knot nematode were collected from diseased tomato plants by the aid of a special needle. A stock culture of the 2nd larvae instars of *M. incognita* (the infective phase) were obtained from the collected mature egg-masses after immersion in sterilized water for 7-10 days. The newly obtained healthy second stage larvae were reared on tomato seedling planted in pots filled with sterilized soil under greenhouse conditions for more than 45 days to obtain egg and larval suspensions from infected roots with obvious galls.

Nematicidal activity of Ag/AgCl-NPs against larvae of *M. incognita*:

The efficiency of the aqueous extract *P. kessleri* as well as their biosynthesized nanoformulations (Ag/AgCl-NPs) against the 2nd larval instars of *M. incognita* were conducted by testing five of different concentrations (v/v); 50, 100, 200, 500 and 1000 (μ L) from the original stocks using sterilized distilled water as diluents. Ten ml from each concentration was mixed with 1000 juveniles of *M. incognita* in 25 ml vials. The vials incubated at 27°C for 24 and 48h. All treatments were conducted in three biological replicates and the average results were compared to Vydate (chemical nematicide) and distilled water as negative control. The number of active and inactive second stages larvae was counted and the percentages of inactive larvae were calculated to evaluate the percentage of larval mortality along with the incubation periods.

Nematicidal activity of Ag/AgCl-NPs against eggs hatchability of *M. incognita*:

The efficiency of the aqueous extract of *P. kessleri* and its nanoformulations against eggs hatching of *M. incognita* were tested by applying 10 ml of serial concentrations; 10, 20, 40, 60 and 80% (v/v) from the original stock of each treatment using sterilized distilled water as diluents to 1 ml of egg suspension (1000-1500 eggs) in 25 ml vials. The vials were incubated at 27°C for 14 days. Treatments were conducted in three biological replicates and the average results were compared to Vydate (chemical nematicide) and distilled water as negative control. After incubation, hatching percentages were evaluated using a special microscopic slide for counting nematodes followed by evaluation of the reduction percentages of eggs hatchability. The following equations were used for calculation of hatching percentage and reduction percentage:

$$\text{Egg hatching \%} = (\text{number of juveniles} / \text{number of juveniles} + \text{number of eggs}) \times 100$$

$$\text{Reduction \%} = (\text{egg hatching \% of control} - \text{egg hatching \% of treatment}) / \text{egg hatching \% of control}$$

3.7. Statistical analysis:

Data collected were statistically analyzed using the completely randomized design. Averages were compared according to Duncan's multiple range test (Duncan 1955). Analysis was performed using the computer program (ASSISTAT version 7.7 en).

RESULT AND DISCUSSION

Visual characterization:

There are distinctly clear changes in colors of the algal extract-inoculated flasks before and after (5 min) autoclaving process (Fig.1).The colours were changed from light yellow (Fig. 1C) to dark brown (Fig. 1D) in

the flask including *P. kessleri* aqueous extract and 1mM AgNO₃. This may be attributed to excitation of surface plasmon resonance (SPR) of the synthesized Ag and AgCl-NPs (Mulvaney, 1996). No change was recorded in the control flasks (Figs 1 A-B). Similar observations were recorded in some recent studies (Shivaji *et al.* 2011; Gopinath and Velusamy 2013). The brown colour produced indicates to synthesis of Ag and AgCl-NPs and reduction of Ag⁺ ions.

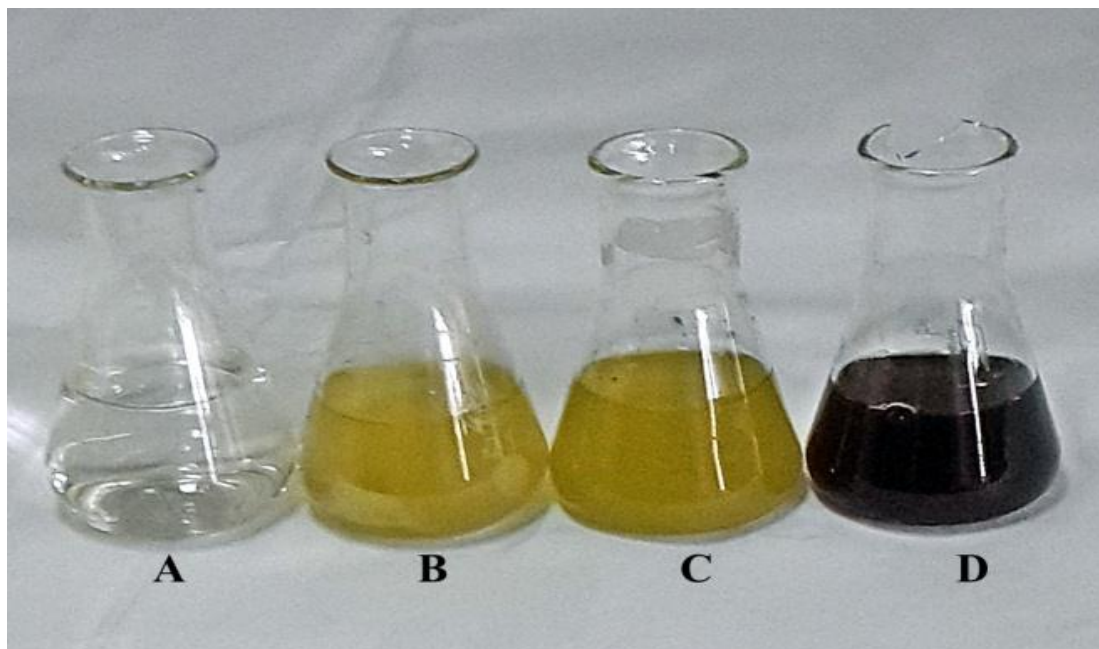


Fig. 1: Direct visualization of Ag/AgCl-NPs bio-production using aqueous extract of *P. kessleri* SAG 211-11; (A) negative control (1mM AgNO₃), (B) positive control (aqueous algal extract), (C) the algal extract with 1mM AgNO₃ before autoclaving, (D) the algal extract and 1mM AgNO₃ after 5 min autoclaving.

UV-Vis spectroscopy:

The biosynthesis of Ag and AgCl-NPs were confirmed by UV-vis spectroscopy (Fig. 2). This technique is a familiar, unique and simple and analyses the materials based on their optical properties. The UV-visible absorption spectrum showed that there is a single surface plasmon resonance (SPR) absorption band at 414 nm. This result confirms the presence and biosynthesis of Ag and AgCl-NPs. In agreement with this observation, many investigators pointed out that silver nanoparticles exhibit UV-Vis absorption maximum ranged between 410 and 440 nm and it is also assigned to other different metal nanoparticles (Sharma *et al.* 2009). The recent contribution of Ferreira *et al.*, (2016) indicated to biosynthesis of Ag/AgCl-NPs using *Chlorella vulgaris* with UV-Vis absorption maximum 415 nm. The negatively-charged polysaccharides of the algal extract tightly bonded with Ag and AgCl-NPs and these confines free electrons of the nanoparticles in a smaller volume. This result in a high free electron density leading to a high plasmon frequency. Therefore, a sharp peak was observed at a lower wavelength (Dallas *et al.*, 2011). Mie's theory (2014) stated that "only a single SPR band demonstrates small and spherical nanoparticles, while anisotropic particles show two or more SPR bands. Accordingly, the presence of a single band in this study (Fig. 2) indicates formation of spherical and small-sized of Ag and AgCl-NPs biosynthesized nanoparticles (Saifuddin *et al.*, 2009; Mie *et al.*, 2014).

Transmission electron microscopy (TEM):

TEM micrograph analyses revealed that shape of Ag and AgCl-NPs were predominantly cubic and evenly distributed without significant agglomeration. The size was ranged from 10.3±0.6–47±0.6 nm (Fig.3). Same results were obtained by Sahayaraj *et al.*, (2012) and Chaturvedi and Verma, (2015).

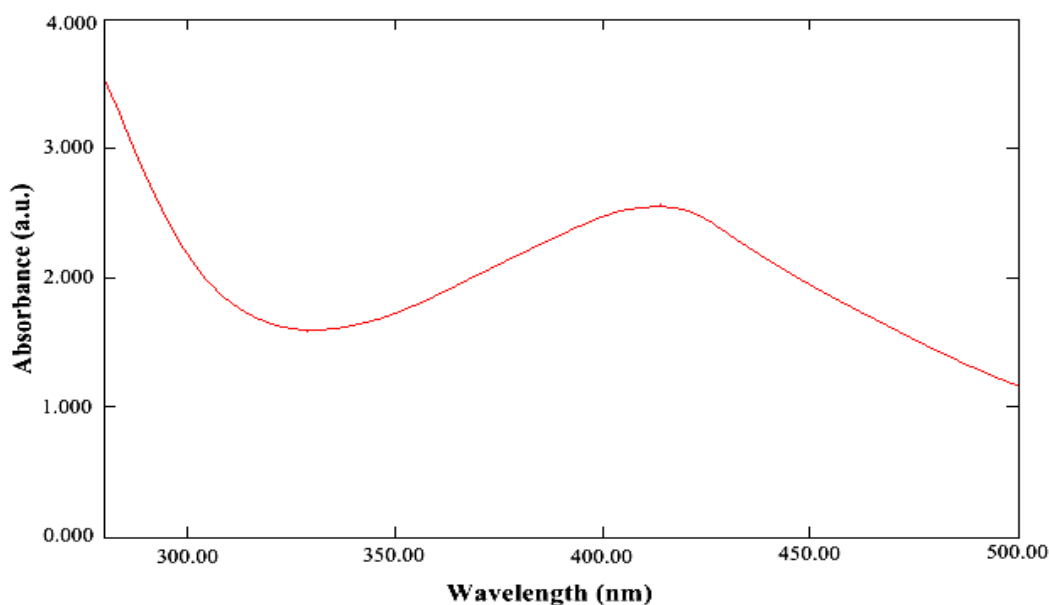


Fig. 2: UV-visible spectroscopy analysis of biosynthesized Ag and AgCl-NPs using the aqueous extract of the green microalga; *Parachlorella kessleri* SAG 211-11.

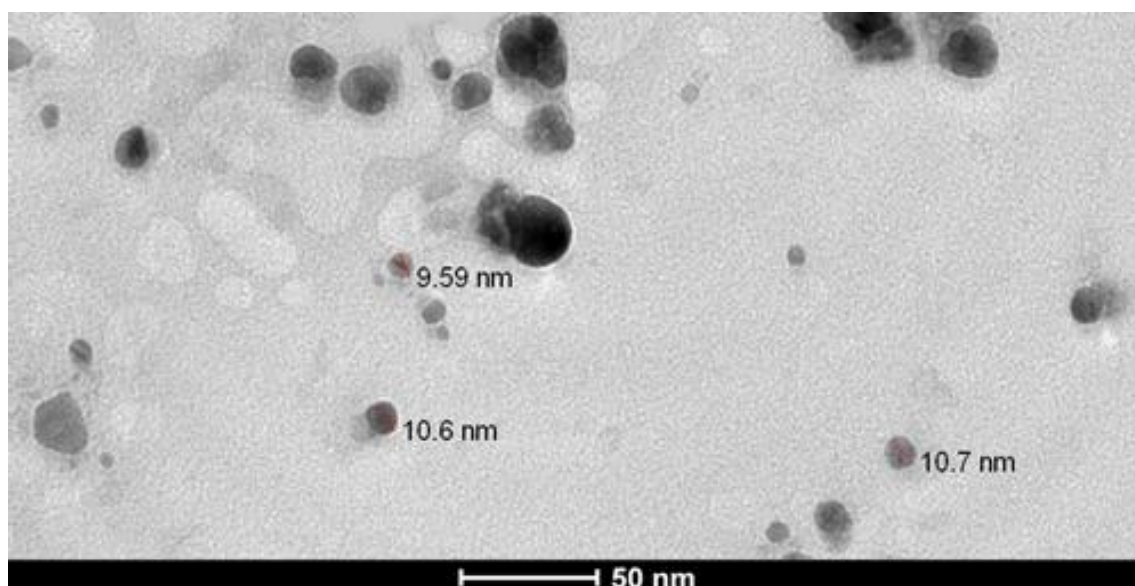


Fig. 3: Transmission electron microscopy (TEM) micrograph of biosynthesized Ag and AgCl-NPs formed by reduction of 10^{-3} M Ag^+ ions by the aqueous extract of *P. kessleri* SAG 211-11.

X-ray diffraction (XRD):

The XRD pattern of Ag and AgCl-NPs is shown in Fig.4. The crystallinity of the nanoparticles was confirmed by the existence of 4 peaks at 2θ values (37.99° , 42.79° , 64.13° and 76.86°) corresponding to 111, 200, 220 and 311 crystal planes, respectively, of face centered cubic Ag (JCPDS card file no:04-003-1659) which co-exist with other 7 peaks at 2θ values (27.75° , 32.21° , 46.15° , 54.79° , 57.39° , 67.22° , and 74.42°) that were assigned to 111, 200, 220, 311, 222, 400 and 331 crystal planes, respectively, of face centered cubic AgCl (JCPDS card file no: 04-007-3906). The intensity of the peaks denotes to intensity of crystallinity of silver and silver chloride nanoparticles produced. Chlorine contents in the algal extract, previously obtained from BBM components CaCl_2 and NaCl , could be elucidated as the main source for the formation of AgCl-NPs in this study. This observation is considered in a consistent accordance with contribution of Gophinath *et al.* (2013) who biosynthesized AgCl-NPs using *Cissus quadrangularis* leaf extract, and suggested that Cl^- ions present in the leaf extract might be responsible for formation of AgCl-NPs. Paulkumar *et al.*, (2013), in their experimental model on biosynthesis of AgCl-NPs in *Bacillus subtilis*, supposed binding of Cl^- ions already existed in the culture medium to Ag^+ ions.

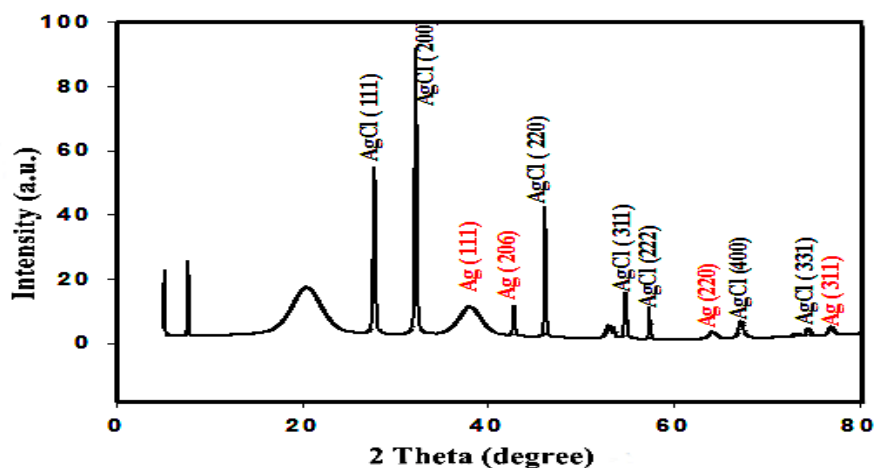


Fig. 4: X-ray diffraction pattern of purified Ag and AgCl nanoparticles biosynthesized by reduction of 10^{-3} M Ag^+ ions by the aqueous extract of *P. kessleri* SAG 211-11.

Zeta potential measurements:

The zeta potential of Ag and AgCl-NPs biosynthesized in this study exhibited -33.5 mV as shown in Fig. 5. Similar result was reported by (Roychoudhury *et al.*, 2016). This high potential value supports well long-term stability, good colloidal nature, and high dispersity of these Ag and AgCl-NPs. These characteristic features might be explained due to the high negative-negative repulsion (Fen *et al.* 2013; Dobre *et al.*, 2014; Roychoudhury *et al.*, 2016).

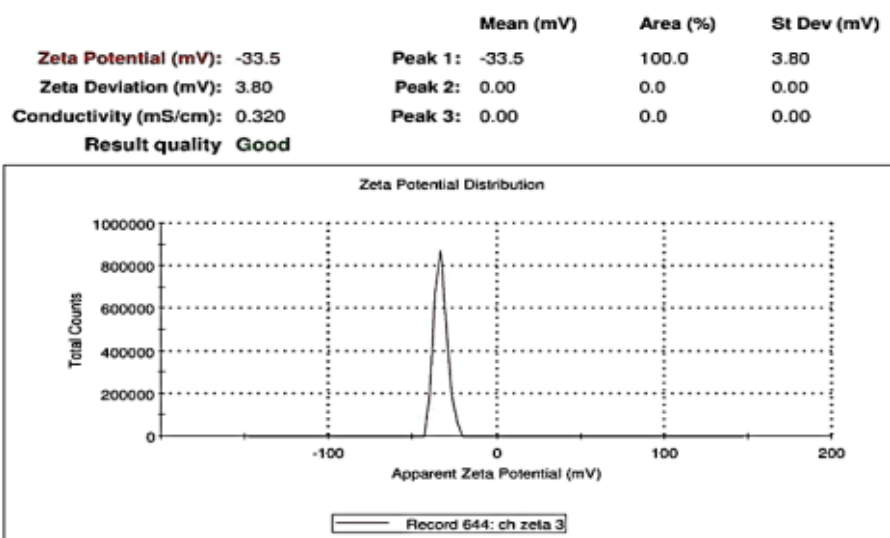


Fig. 5: Zeta potential analysis of Ag and AgCl-NPs biosynthesized using aqueous extract of *P. kessleri* SAG 211-11.

FTIR analysis:

FTIR spectroscopy measurements was commonly used to identify the possible biomolecules present in aqueous extract of *P. kessleri* responsible for the reduction of Ag^+ ions and capping of the biosynthesized Ag and AgCl-NPs. The FTIR spectrum of the Ag and AgCl-NPs of *P. kessleri* is shown in (Fig 6) number of peaks have been recorded in the spectra thus reflecting a complex nature of the *P. kessleri* aqueous extract. The major infrared absorption peaks detected in the purified Ag and AgCl-NPs were 3277 cm^{-1} which are attributed to primary amide linkage of protein (Rajeshkumar *et al.*, 2014), 2925 cm^{-1} ; indicated stretching vibrations modes of secondary amines (Ahmed *et al.*, 2015). While, peaks at 1639 cm^{-1} and 1548 cm^{-1} ; could be assigned to amide I and amide II respectively (Rajeshkumar *et al.*, 2014; Castro *et al.*, 2013). Peak at 1394 cm^{-1} ; suggested the asymmetric deformation of CH_3 and CH_2 in proteins (Tripathy *et al.*, 2010; Rajeshkumar *et al.*, 2012), 1247 cm^{-1} ; indicated C-O stretching of carboxylic acid group (Gole *et al.*, 2001). Meanwhile, the peak at 1032 cm^{-1} could be assigned to C-N stretching vibrations of aliphatic amines of proteins (Gole *et al.*, 2001). While, 517 cm^{-1} peak suggested the possible involvement of alkyl halides (Rajeshkumar *et al.*, 2014). From the previous

data, it can be suggested that protein molecules may be responsible for the biosynthesis and stabilization of Ag and AgCl-NPs synthesized by *P. kessleri*. This result is in agreement with (Ajitha *et al.*, 2014; Chaturvedi and Verma, 2015) who suggested the involvement of proteins in the reduction of silver nanoparticles.

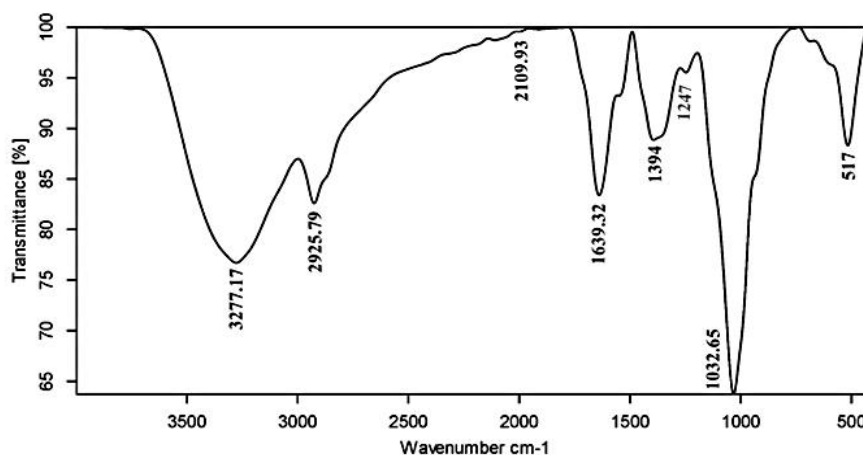


Fig. 6: FT-IR spectrum of Ag & AgCl-NPs synthesized by *P. kessleri* aqueous extract.

Nematicidal activity of Ag and AgCl-NPs against 2nd larval instars of *M. incognita*:

Data in Table.1 revealed that larvae of *M. incognita* were susceptible to the algal extract, Ag/AgCl-NPs and vydate compared to control which reached to 1.7% after 48h. Moreover, the percentage of larval mortality in all treatments increased with increasing concentration from 50-1000 μ L and exposure periods from 24-48h. The algal extract recorded the lowest percentage of larval mortality at both 24h and 48h of incubation period. However, the biosynthesized Ag and AgCl-NPs showed significant increase of larval mortality of *M. incognita* over the chemical nematicide at all tested concentrations. The detected LC₅₀ values of Ag/AgCl-NPs were 200 and < 50 μ L at 24h and 48h of incubation respectively. 500 μ L of Ag/AgCl-NPs completely inhibited larval growth by 100%, similar result was achieved by using 1000 μ L of vydate indicating superiority of Ag/AgCl-NPs over the traditional nematicide. This finding is in agreement with reports of Cromwell *et al.*, (2014) and Hassan *et al.*, (2015) through using Ag-NPs against root-knot nematode in Bermuda grass and *M. incognita*. The nematicidal efficacy of Ag-NPs against root-knot is attributed to its mode of action which is not specific but associated with disrupting and malfunctioning of multiple cellular mechanisms such as membrane permeability, ATP synthesis, and response to oxidative stress in both eukaryotic (Ahamed *et al.*, 2010; Lim *et al.*, 2012).

Table 1: Effect of different concentrations of *Parachlorella kessleri* aqueous extract, the biosynthesized Ag/AgCl-NPs and Vydate nematicide on the percentage of mortality of 2nd larvae instars of *M. incognita* at two different incubation periods under laboratory conditions

Treatments	Concentrations (μ L/L.)	Mortality %	
		24 h	48 h
Ag/AgCl-NPs of <i>Parachlorella kessleri</i>	50	15 h	61.81 c
	100	43.50 d	57.57 d
	200	50 c	78.38 b
	500	100 a	100 a
	1000	100 a	100 a
Algal extract of <i>Parachlorella kessler</i>	50	1.98 l	22.45 j
	100	4.88 j	25.91 i
	200	8.28 i	33.57 g
	500	10.12 i	43.92 f
	1000	20.78 g	55.50 d
Vydate	50	20.11 g	31.38 h
	100	23.50 f	51.00 e
	200	26.50 e	57.57 d
	500	90.45 b	98.51 a
	1000	100 a	100 a
Control		0.01 l	1.73 l

(Means followed by a common letter(s) are not significantly different at the 5% level by DMRT)

Nematicidal activity of Ag/AgCl-NPs against egg hatching of *M. incognita*:

Table.(2) showed the nematicidal efficacy of the aqueous algal extract of *P. kessleri* as well the biosynthesized Ag/AgCl-NPs, Vydate (reference nematicide) and control (distilled water) against the percentage of egg hatching of the root knot nematode; *M. incognita* under laboratory conditions. Data revealed that, the

percentage of egg hatching reduction of *M. incognita* increased significantly with increasing concentration of all treatments from (10–80%) compared to control which, had no effect on egg hatchability, recording 0% at 14 days of incubation. Interestingly, low concentrations of biosynthesized Ag/AgCl-NPs (10% and 20%) had significant toxic effect compared to chemical nematicide (Vydate) ($p \leq 0.05$) where, concentration at 10% showed 16 fold of reduction of egg hatching compared to chemical nematicide. This finding is in agreement with report of Nassar *et al.*, 2016.

Table 2: Effect of different concentrations of the aqueous extract of *Parachlorella kessleri* and the biosynthesized Ag/AgCl-NPs compared to Vydate nematicide on the percentage of egg hatching of *Meloidogyne incognita* under laboratory conditions.

Treatments	Concentrations	Egg hatching %	Reduction %
Ag/AgCl-NPs of <i>Parachlorella kessleri</i> .	10%	14.89 gh	78.98
	20%	12.55 hi	82.29
	40%	11.32 hi	84.02
	60%	10.74 ij	84.84
	80%	6.83 mn	90.36
<i>Parachlorella kessleri</i> aqueous extract	10%	70.42 a	0.63
	20%	66.76 b	5.79
	40%	49.30 c	30.43
	60%	35.57 d	49.80
	80%	19.16 f	72.96
Vydate	10%	67.35 ab	4.96
	20%	23.52 e	66.81
	40%	9.76 jl	86.22
	60%	3.77 no	94.68
	80%	1.79 op	97.47
Control (distilled water)		70.87 a	0.00

Conclusion:

The presented study highlights the possibility of large-scale production of Ag and AgCl-NPs by using the aqueous algal extract of the green microalga *Parachlorella kessleri* SAG 211-11 due to its simplicity, accessibility and effectiveness. The biosynthesized Ag and AgCl-NPs were visually followed throughout distinctly clear changes in colours and experimentally checked using UV-Vis, TEM, FTIR, Zeta sizer and XRD characterization. Ag/AgCl-NPs showed highly stable properties at room temperature. This study also, showed a novel approaching technique for biological control of root knot nematode, *M. incognita* and it can be highly recommended as a promising and cheap nematicide compound against the infective larval phase and eggs hatchability of *M. incognita*.

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