Green synthesised CuNPs using *Alhagi maurorum* extract and its ability to amelioration of *Mycoplasma pneumoniae* infected pneumonia mice model

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**ABSTRACT**

In this study, copper nanoparticles were synthesised according to green chemistry rules using the aqueous extract of *Alhagi maurorum*. The green-synthesised CuNPs were characterised using different techniques such as EDX, FE-SEM, XRD and FT-IR. The FE-SEM results confirm spherical morphology for the nanoparticles with a size of 42.68–68.87 nm. In vivo, the *Mycoplasma pneumoniae* injection was applied for inducing pneumonia in the BALB/c mice, also the treatment was with CuNPs. The serum parameters such as inflammatory mediators (IL-6, TNF-α, IL-8, TGF and IL-1), total cell counts, and total protein content levels were evaluated. CuNPs regulated the levels of the inflammatory mediators in the infected mice. The cellular arrangements at the histopathological images were ameliorated with the administration of CuNPs. At the antioxidant test, the CuNPs and BHT removed 50% of free radicals at the concentrations of 181 and 110 μg/ml. In conclusion, the results revealed the ameliorative property of CuNPs green-formulated by *A. maurorum* aqueous extract on *M. pneumoniae* infected pneumonia mice model.

1. Introduction

Pneumonia is a lung disease with high clinical signs. Many non-infectious causes, including food aspiration, hydrocarbons, foreign bodies, stomach acid, and allergic reactions, can also cause pneumonia [1]. Pneumonia is the most usual death cause in children around the world, especially in developing countries, and causes about 4 million deaths among children annually. The annual prevalence of pneumonia in developing countries is
0.29% [1–4]. Annually, 151.6 million new cases and 12–20 million (7–13%) of severe cases in need of hospitalisation are reported. The most common bacterial germ of all ages is Streptococcus pneumonia [5–7]. Risk factors such as poverty, low family literacy, low birth weight, malnutrition, and non-breastfeeding in the development of pneumonia in developing countries have been described [7, 8]. Environmental factors that increase the risk of developing pneumonia include going to kindergarten, smoking, exposure to secondhand smoke, and large family populations. One of the bacterial species that causes severe pneumonia is Mycoplasma pneumonia [9–11]. Mycoplasmas are the smallest and simplest release objects known. These bacteria are probably derived from gram-positive bacteria and are phylogenetically related to Clostridia. Their genome is small and consists of a double-stranded cyclic dioxypurinucleic acid molecule containing 500 to 1000 genes [11–13]. M. pneumonia, unlike other species of Mycoplasma, is not present as part of the natural flora, and out of ten accepted human species of the genus Mycoplasma, only this species has been proven to cause disease. This bacterium is one of the causes of lower and upper respiratory tract infections and its clinical picture is progressive as slow tracheobronchitis, with restlessness and dry cough [12–14]. The pathogenicity of this bacterium ranges from mild forms of pharyngitis and tracheobronchitis to cases of acute pneumonia. A wide range of extra-respiratory manifestations such as hematologic, gastrointestinal, renal, musculoskeletal, cardiovascular, immunological, dermatological, and neurological complications have been reported in connection with this bacterium [11–15]. Currently, culture, serological detection methods, and nucleic acid amplification techniques are three available methods for routine diagnosis of M. pneumonia infections. Culture-based methods are time-consuming techniques with relatively low sensitivity and require facilities as well as sufficient experience to interpret the results [11–13]. Nowadays, diagnostic methods based on polymerase chain reaction are considered suitable methods for diagnosing factors such as M. pneumonia due to their sensitivity, specificity, and accuracy [12–14]. Recent studies have reported that the metallic NPs green-mediated has significant antimicrobial effects against pathogenic microbes [15–20].

Camelthorn with the scientific name of Alhagi maurorum is a plant of the Fabaceae family. Camelthorn grows in different places around the world from North Africa to Europe and Asia [21, 22]. The plant is known as an herbal medicine with a wide variety of applications in folklore medicine such as laxative, diuretic, and expectorant properties [23]. Camelthorn is known as an effective remedy for reducing acidity in the gastric problem [24]. A. maurorum is used to heal wounds and also as an agent for diaphoretic and antiseptic [25]. A. maurorum essential oil is an effective remedy for rheumatism [26]. Plants' secondary metabolites play a basic role in herbal medicinal uses, according to earlier studies, Camelthorn is rich in different classes of compounds such as phenolic, alkaloids, flavonoids, fatty acids, and vitamins. Certainly, the existence of various compounds in the plant is the essential factor for the wide variety of pharmaceutical uses of the plant [24, 27, 28].

In this project, copper NPs were formulated according to pharmacology rules by A. maurorum aqueous extract to treat the mouse model of M. pneumonia.

2. Methods and materials

2.1. Preparation and extraction of aqueous extract

At the first step, 100 g of A. maurorum grounded leaves were boiled in 1000 ml for 10 min. Then, the extract was filtrated and concerted using a rotary evaporator. Next, the
concerted extract was dried using a freeze drier. The obtained extract was a powder brown colour and hold in a refrigerator.

2.2. Green synthesis and chemical characterisation of CuNPs

The CuNPs was synthesised using a reported assay [29, 30]. First, 50 ml of the plant extract (with a concentration of 0.01 g/L) was added to 50 ml of Cu(NO₃)₂·3H₂O (0.3 M). Next, the mixture was stirred at 70 °C over a night. A dark brown participation (CuNPs) was formed during the time. Deionised water and ethanol were used to wash CuNPs three times. The final residue was centrifuged at 14,000 rpm for 12 min. In the end, the precipitate was put in an oven at 55 °C to dry.

2.3. Antioxidant activities determination of CuNPs

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) was performed to assess the nanoparticles’ antioxidant activities. 2 ml of 0.2 mM DPPH solution was mixed by 5 ml of NPs suspension at dilutions of 1–1000 µg/ml. The incubation was done for 30 min at room temperature and absorbed at 517 nm in the spectrophotometer. The free radical adsorption percentage was calculated by the below formula so that A⁰ was the control sample adsorption and A¹ was the desired sample adsorbent [19, 20]:

\[
\text{DPPH scavenging effect (\%)} = \frac{\text{A₀} - \text{A¹}}{\text{A₀}} \times 100
\]

2.4. In vivo design

The animals (n = 24) were divided following groups:

I. Normal control.
II. Pneumonia infection control: Infected with M. pneumonia solution.
III. T1: Infected with M. pneumonia solution + Cu NPs (50 µg/kg).
IV. T2: Infected with M. pneumonia solution + Azithromycin (AZM), 200 mg/kg.

After 3 days, the chloroform was used for anaesthetising the mice. By tracheal cannulation of PBS into the right side lingual or middle lobe thrice, the BALF was gained, and immediately it was centrifuged at 8000 rpm for 15 min to remove the debris. The total cell numbers of BALF were gained by hemocytometer. The total protein level was determined by the Bradford technique and protein assay kit. The BALF inflammatory cytokines levels such as IL-6, TGF, IL-8, TNF-α and IL-1 were inspected by the ELISA technique. The eosin and hematoxylin staining was performed to stain the lung sections, and then the histopathological changes were investigated under light microscopy [31].

The data were assessed by SPSS ver22 statistical software and a one-way ANOVA test. p < 0.01 was considered remarkable.

3. Results and discussion

The XRD diagram of CuNPs is presented in Figure 1. XRD is an effective method to study nanoparticle crystallinity. The results approve the formation of CuNPs in small size and well crystallising. The different signals at 2θ values were compared with the standard database of PDF card No. 04-012-7238. The signals at 32.40, 35.48, 38.65, 48.75, 58.19,
61.27, 68.07 and 74.88 are indexed as (110), (11-1), (111), (20-2), (202), (−113), (221) and (22-2). The data are matched to those of copper oxide signals [30]. The crystal size of CuNPs was obtained using Scherer’s equation with an amount of 31.21 nm. According to previous reports on the green synthesis of CuNPs, the nanoparticles were in the range size of 12–40 nm [32–36].

The elemental study of CuNPs was evaluated using EDX analysis. The EDX diagram of CuNPs is presented in Figure 2. The successful synthesis of copper nanoparticles is approved by the signals that belong to the copper element (0.95 Kev, CuL\(\alpha\); 8.1 Kev, Cu K\(\alpha\), 8.8 Kev, Cu K\(\beta\)) [37]. The presence of oxygen is confirmed by the peak around
0.5 keV (OL\textgreek{a}) which corroborates the formation of copper oxide, this is in agreement with previous studies on the formation of copper oxide in the green synthesis of CuNPs [38]. Another signal at 0.20 keV belongs to CL\textgreek{a} that exhibits the presence of carbon in CuNPs. The carbon and oxygen signals are correlated to the secondary metabolites of \textit{A. maurorum} that are bound to the CuNPs surface.

The morphology investigation of CuNPs was carried out using the FE-SEM technique. Figure 3 presents the FE-SEM images of CuNPs. The result shows that CuNPs are formed in spherical morphology with 42.68–68.87 nm for particle size. CuNPs are also aggregated which is a common characteristic for synthetic metallic nanoparticles using green approaches [32, 39–43]. In the literature, the range size of 5–100 nm has been reported for the green-synthesised copper nanoparticles [44–47].

The FT-IR spectra of \textit{A. maurorum} extract and CuNPs are exhibited in Figure 4(a, b). The bands at the wavenumbers of 444 and 609 cm\(^{-1}\) belong to Cu–O approving the formation of CuNPs as copper oxide. This is in agreement with EDX and XRD results. Cheirmadurai et al have reported the bands for green synthetic of CuNPs with a little difference in wavenumber [30, 48]. Furthermore, the peaks at 3423 and 2928 cm\(^{-1}\) for O–H and C–H, 1453 to 1679 cm\(^{-1}\) for C=C and C=O, and 1057 cm\(^{-1}\) for –C–O belong to the different functional groups of \textit{A. maurorum} secondary metabolites that play the reducing and capping role to CuNPs synthesis. These constituents include different classes
of compounds including phenols, flavonoids, and triterpenes, which were found as *A. maurorum* secondary metabolites previously [49, 50]. The FT-IR spectra of the plant extract and CuNPs are similar together approves the linkage of secondary metabolites of the plant extract to the CuNPs surface.

Extraction from biological organisms can be done both as capping and reducing agents in the metal nanoparticle synthesis. Metal ions reduction combined with biomolecules present in cellular extracts such as vitamins, polysaccharides, amino acids, proteins, and enzymes is environmentally friendly, whereas synthesis with chemicals is not [16–19]. Extensive volumes of reports indicate that the synthesis of metal nanoparticles using bio-organic compounds is successful. Various proteins, enzymes, secondary metabolites, or other capping and reducing agents have a unique role in nanoparticle production by plants [19–21, 42]. Recovery of nanoparticles from plant tissue is expensive and tedious and needs enzymes to destroy plant cellulose tissue [44, 45]. Therefore, it is so easier to use extracts of plants in large and small-scale processing to prepare several metal nanoparticles. Recently, researchers used plant extracts rather than physical and chemical methods to synthesise the metal nanoparticles [42–45]. The extract proteins make the dual function of shape control and metal reduction in the metal nanoparticle synthesis [17–19]. Carboxyl groups in aspartic residues, glutamine, and hydroxy groups in tyrosine residues in proteins have been suggested as metal ion reducing agents [42, 45]. The reduction process is performed by a simple dual-function tripeptide that is mostly identified in the amino acid residue. This synthesis process produces small metal nanoplates with very low dispersion [44, 45]. Green-synthesizing the nanoparticles by plants increase their antioxidant properties of them in comparison to other methods [16–20].

**Figure 4.** FT-IR Spectra of CuNPs and *A. maurorum*.  
![FT-IR Spectra](image)
In the current experiment, the effects of BHT and Cu NPs on the common free radicals (DPPH) were evaluated (Figure 5). In the free radical examination, the IC$_{50}$ of Cu NPs and BHT against DPPH were 181 and 110 $\mu$g/ml, respectively.

Today, bacterial resistance has caused problems in the treatment of patients suffering from *Mycoplasma* infections. So that the antibiotics used in the common treatments of urinary infection gradually lose their effectiveness [51–53]. Nanoparticles have unique physical, chemical, optical and biological properties, which are good candidates for use
inside the body and in laboratory research due to their small size [54–56]. Among their uses, it can be mentioned as a substitute for antibiotics or together with them to treat drug-resistant bacteria. Copper nanoparticles have a spherical and crystalline appearance and are used in various industries. The shape and size of nanoparticles are important for their activity so that smaller particles have stronger and more antimicrobial activity than larger particles [53–57]. These nanoparticles have antibacterial activity against drug-resistant isolated clinical samples, including gram-positive bacteria. The size of these particles is approximately three to one hundred nanometres and they have been used to detect proteins and microorganisms and design biosensors [51–53]. Due to the wide application of copper nanoparticles in the treatment of diseases, few reports have been reported on their side effects on living cells and tissues in the body and cases with contradictory results [52–56]. In general, today, excessive use of common antibiotics has increased antibiotic resistance among Mycoplasma pathogens. Therefore, due to the wide application of

Figure 7. The effect of CuNPs on BALF inflammatory cytokines level (IL-1).

Figure 8. The effect of CuNPs on BALF inflammatory cytokines level (TGF).
copper nanoparticles in the treatment of diseases, little information is available on their effect on gram-negative bacilli that cause *Mycoplasma* infections [54–57].

In a recent study, the levels of inflammatory cytokines and cells and total protein increased due to the *M. pneumonia* infection. The histopathological findings indicated increasing the levels of the lymphocytes and plasmocytes of the lung due to the immune responses against the infection (Figures 6–11).

Administration of the CuNPs regulated the levels of inflammatory cytokines and cells and total protein in serum and reduced the levels of the lymphocytes and plasmocytes of the lung in the lung (Figures 6–11).

### 4. Conclusion

In conclusion, the aqueous extract of *A. maurorum* was used as the reducing agent to synthesise CuNPs. The common chemical characterisation methods such as FT-IR, XRD,
FE-SEM and EDX were used to chemical and physical properties of copper nanoparticles. The obtained results confirmed the synthesis of copper oxide nanoparticles with spherical morphology and with a particle range size of 42.68–68.87 nm. At the free radical examination, CuNPs and BHT removed 50% of free radicals at concentrations of 181 and 110 μg/ml. The cellular arrangements at the histopathological images were ameliorated with the administration of CuNPs. CuNPs regulated the levels of the inflammatory mediators in the infected mice.

Disclosure Statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Data is available on request from the authors.

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