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Research Article

Green Synthesis of Silver Nanoparticles from *Syzygium aromaticum* and Evaluation of its Anti-mycobacterial Activity

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Abstract

With 9 million new cases and 2 million deaths per year, tuberculosis remains a major public health concern. The lack of a viable vaccine, as well as the emergence of novel *Mycobacterium tuberculosis* (MTB), strains that are particularly resistant to treatments, presages a complicated future situation. Biosynthesized nanomaterials are currently proving to be a viable antibacterial therapeutic option, including for MTB infection treatment. The goal of this work is to synthesize silver nanoparticles from *Syzygium aromaticum* and investigate their antimicrobial, anti-tubercular, and cytotoxic properties using zebra fish embryos and *Artemia salina*. The UV spectrophotometer and FTIR measurements were used to characterize the biosynthesized nanoparticles. Antibacterial activity was performed against *S. aureus, P. aeruginosa, E. coli*, and *K. pneumoniae* and exhibited potential inhibitory activity towards the bacterial cultures. MABA assay was used to investigate the anti-mycobacterial activity, the biosynthesized nanoparticle showed the highest percentage of inhibition in both test concentrations (500 and 250 g/ml). The current discovery may also pave the way for the extraction of anti-mycobacterial lead moieties from medicinal plants, which could then be evaluated in preclinical trials.

Keywords: Tuberculosis; Silver Nanoparticles; *Pseudomonas aeruginosa; Escherichia coli; Klebsiella pneumoniae*

Introduction

Tuberculosis is a severe public health problem, with over 2 billion people worldwide afflicted and over 1.3 million deaths expected in 2019 [1]. *Mycobacterium tuberculosis* is the pathogen that causes tuberculosis and is transferred from person to person by aerosols. They are residing and multiply in alveolar macrophages as it has developed several pathways to evade digestion in acidic phagosomes and may persist intracellularly in a dormant phase. In developing countries, including India, tuberculosis remains a

leading cause of mortality [2]. In 2019, rifampicin-resistant MTB produced an estimated 464,000 new cases of human TB, with 78 percent of MDR-TB strains resistant to the two first-line medicines, isoniazid and rifampicin. However, when compared to the annual total of 1.4 million deaths attributable to tuberculosis in general, the number of MDR-TB-related deaths is minimal [1]. The increase in the incidence of drug resistance TB around the world urges the need to develop a new drug derived from natural products such as higher plants to fight against tuberculosis. Medicinal plants usually have the property of curing disease for more than centuries. Usually, plants have been used as traditional medicine for the treatment of various diseases. Only few species of medicinal plants have been investigated for revealing its medicinal properties and others are yet to be discussed. *Syzygium aromaticum L.,* also known as *Eugenia caryophyllata L.,* is a tropical evergreen tree with crimson blooms that belongs to the *Myrtaceae* family [3], endemic to the Maluku Islands in Indonesia, and is mostly used as a spice. They are mostly harvested in India, Pakistan, Indonesia, Madagascar, Zanzibar, Sri Lanka, and Tanzania for commercial use. However, the biggest clove buds oil producers are Indonesia and Madagascar; Buds, leaf, and stem oil are all used to extract oil [4]. A review of previous reports suggests that the *Syzygium aromaticum* (Clove) possess biologically activecomponent which are effective against many types of diseases. Clove buds were chosen as a ligneous, representative substrate in this study because of its importance in cuisine, traditional medicine, pharmaceutics, and cosmetics [5]. Clove and its derivatives of have numerous properties such as antimicrobial activity (Hadidi., *et al*. 2020), anti-viral activity, anti-inflammatory [7], hepatoprotective activity, chemoprotective activity, anti-diabetic and antioxidant activity [6], anti-inflammatory, anti-carcinogenic effects [8], neuroprotective ability, hypolipidemic efficiency and anti-diabetic activity [9-11], etc. Polyphenols present in clove buds have antibacterial and antiviral properties [12-14]. In recent studies, researchers found that Quercetin has been discovered to have a possible role against coronavirus disease 2019 [15]. Along with the plant extract, nanomaterials are currently shown to be a potential alternative to antimicrobial treatments, including the treatment of MTB infection. Nanoparticles have been extensively used for pharmaceutical and industrial purposes. As the nature consists of several metals, only few of them are used for the synthesis of nanoparticles [16]. Among these silver nanoparticles holds a special place because of their expensive properties which is utilized in many industries such as agriculture, textile industry, air filtration, etc., [17]. These silver nanoparticles have the potential to penetrate bacterial cell walls and affect the shape of cell membranes, resulting in cell growth inhibition or even cell death [18]. This is due to their nanoscale size and high specific surface area. Furthermore, biosynthesis of AgNPs using various sources such as microorganisms and plant extracts has been recognized as the green technique and has become an unavoidable trend when compared to other synthetic methods. Without requiring hazardous solvents or generating detrimental byproducts, the reducing agents in these green sources would transfer their electrons to reduce silver ions into silver nanoparticles [19]. These biological molecules would also cover the produced silver nanoparticles and act as capping agents, preventing agglomeration, reducing toxicity, and improving silver nanoparticle antibacterial activity [20]. Probing deep into literature, it also proves that these components flow unique mechanism to provide a potential action against Tuberculosis (TB). Therefore, the nanoparticles of clove will have impact on TB with unique mechanism. Hence the present study was carried out to synthesis silver nanoparticlesfrom *Syzygium aromaticum* (Clove) and exploring its Anti-mycobacterial property.

Materials and Methods

Sample collection and extract preparation

The seeds of Syzygium aromaticum (cloves) were purchased from nearby shop in Sholinganallur, Chennai, Tamilnadu. Seeds were washed with tween 20 detergent followed by distiller water for 2 to 3 times to remove the impurities. Then they were dried for 24 hours under shade. The dried materials were ground to fine powder using mixer grinder. Take 1 gram of ground clove powder; add 50 ml of double distilled water. Boil the mixture using water bath at 60°C for 10-15 minutes method proposed by [21]. After cool down filter the extract using Whatman No.1 filter paper. Then the filtrate was stored in 4°C for further use.

Biological synthesis of silver nanoparticle

Two ml of clove extract was added in to 98 ml of 0.1 mM aqueous $AgNO_3$ solutions and kept in a dark condition at room temperature for about 24 hours. The bio reduction of Ag + ions in aqueous solution was monitored by color change from pale yellow to brown

[22]. The solution is centrifuged at 4000rpm for 15 minutes and the pellet was transferred into Petri plates and allowed to dry. The powder was scrapped and stored in sterile Eppendorf tubes and used for UV analysis.

UV-visible absorbance spectroscopy

UV-Visible spectra of silver nanoparticles in aqueous solution with different wavelengths in nanometers from 340 to 800 nm were used to evaluate the progress of the reaction between metal ions and the leaf extract. Within an hour of starting the reaction, silver ions were reduced and silver nanoparticles were formed. AgNO3 was used to maintain control [23].

Fourier transforms infrared spectroscopy (FTIR)

The solution of produced silver nanoparticles was centrifuged for 30 minutes at 10000 rpm for FTIR measurements. To remove any unbound proteins or enzymes that aren't encapsulating the silver nanoparticles, the pellet was washed three times with 5 mL deionized water. A vacuum drier was used to dry the pellet. FTIR was used to examine it.

Antimicrobial activity

The agar well diffusion method is used to determine the antibacterial activity of the extracts prepared from two different plant *Syzygium aromaticum* and *Coffea arabica* using different solvents such as Hexane, Ethyl acetate and Methanol. The test microorganism used was *Escherichia coli, Pseudomonas aeruginosa Klebsiella pneumonia*, *Staphylococcus aureus*. The Nano synthesized samples of *Syzygium aromaticum* and *Coffea arabica* were tested for antibacterial activity. The plates were incubated at 37^0C for 16-18 hours. The zone of inhibition (mm) was measured and the results were tabulated [24].

Anti-mycobacterial activity Microplate alamar blue assay (MABA)

Mycobacterium smegmatis were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC) India. They were maintained on Lowenstein-Jensen (LJ) slopes and cultured on Middlebrook 7H9 broth. Cultures were grown aerobically on 7H9 broth at 37°C adjusted spectrophotometrically to a no. 1 McFarland tube standard, and further diluted 1:10 in 7H9 broth for the test [25]. Briefly, 100 µl of 7H9 broth was dispensed in each well of a sterile flat-bottom 96-well plate, and serial twofold dilutions of the crude extracts and each positive control drug were prepared directly in the plate. In growth control wells add 100 µl of Middlebrook 7H9 broth. In DMSO control wells, add 100µl of 10% DMSO containing M7H9 broth. In drug control wells, add 100µl Rifampicin containing M7H9 broth (2 µg/ml). In test wells, add 100 µl of desired concentration of test samples dissolved in M7H9 broth. In negative control wells, add 200 µl of M7H9 broth. Add 100µl of *M. smegmatis* suspension in all the above wells except the negative control well. Incubate the plate at 37 ◦ c for 24 hours. After incubation, add 32.5µl of prepared dye to all the other remaining wells. If there is no colour change observed, further incubate the plates for 24 hours. All the experiments were carried out in triplicate and were conducted in a Biosafety Level Three (BSL-3) laboratory.

Cytotoxicity assay using zebra fish embryos

Zebra fish embryos were purchased from the zebra fish aquarium in Kanchipuram district. For toxicity studies, 20 healthy post hatched zebra fish were transferred to the wells of a 24-well plate along with 1 ml of embryo water (60 mg of sea salt/liter of ultrapure water). Different concentrations of nano synthesized plant extracts at (5, 10, 25, 50 and100 µg ml^{-1} concentration) were added to the wells and incubated for 72 hours at 28.5°C. Tests were performed in duplicate and repeated thrice. Mortality of the zebra fish was noted after 24, 48 and 72 h. The embryos that appeared opaque and white in colour. The dead embryos were degraded soon, whereas the structures of intact embryos were more visible by 48 hours which allowed a clear distinction between the dead and alive. The mortality rate is calculated. At the end of the incubation period, the embryos were photographed using a light contrast microscope [26].

Cytotoxicity assay using *Artemia salina*

Artemia salina eggs were purchased from the nearby aquarium in Kanchipuram district. Dried cysts were placed in a bottle containing artificial sea water which was prepared by dissolving 35 g of sodium chloride in 1 L of distilled water. After 36–48 h incubation at room temperature (28–30°C) under conditions of strong aeration and continuous illuminations, the larvae hatched within 48 h. The evaluation of cytotoxicity of NPs in *A. salina* was performed according to the previous methods. The assay was carried out on larvae of brine shrimp. Around 200µl of plant

extract from each concentration was taken and added in test wells at 5,25,50,75 and 100 concentration and artificial sea water serves as control. 20 nauplii per well was added in all the wells including control wells. The plate was incubated at room temperature under strong aeration. After 24 hours of incubation, the survival rate of nauplii and the percentage of lethality was determined using following. The numbers of surviving nauplii in each well were counted under a stereoscopic microscope after 24 h. The experiments were conducted in triplicate for each concentration [27].

Percentage of lethality = [(Test – control)/control] x 100.

Results

Synthesis of silver nanoparticles

The silver nanoparticles were synthesized using a green synthesis approach in this work. Silver nitrate was mixed with the plant extract and incubated at room temperature. The color change was tracked and recorded. Triplicate reactions were used in the tests. Formation of nanoparticle production described in figure 1, it was validated by the yellowish-brown color change. No color change was observed in the Control silver nitrate solution.

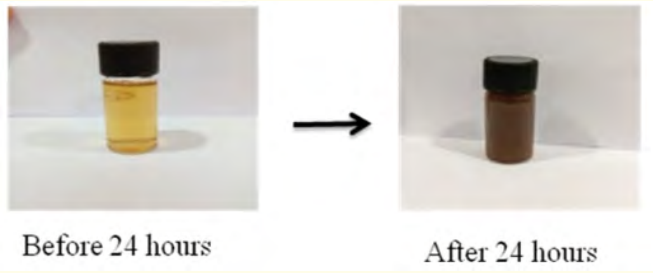

Figure 1: Plant extract with $AgNO_3$.

Analysis of the silver nanoparticles using a UV-spectrophotometer

The UV-spectrophotometer was used to confirm the produced silver nanoparticles. With the use of a UV-vis spectrophotometer, the formation of the silver nanoparticles was further confirmed. Figure 2 depicted the UV-Vis spectrum. At 450 nm, Syzygium aromaticum displayed substantial absorption. Surface plasmon resonance is the name given to this absorption band (SRP).

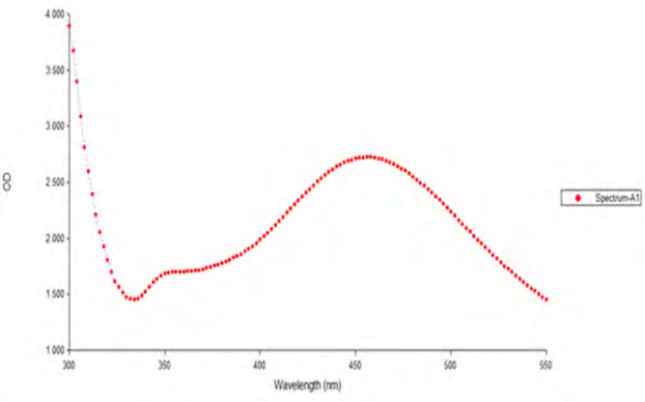

Figure 2: UV-visible spectrum of silver nanoparticles showed plasmon excitation at 450 nm.

Fourier transform infrared spectroscopy analysis (FT-IR)

FT-IR spectroscopic analysis of *Syzygium aromaticum* silver nanoparticles showed in figure 3. The results of FTIR analysis of this study show different stretches of bonds shown at different peaks; 3608.86/cm showed O-H stretch which has medium intensity, 2883.13 (-C-H alkane stretch), 2812.12 (-C-H aldehyde weak intensity), 2746.02, 2674.15, 2571.39/cm (-O-H carboxylic acids with strong intensity), 1906.94/cm(=C-H aromatics), 1797.78/cm (=C-H), 1707.94 (-C=O ketone), 1422.95, 1029.24(C-H alkene strong bend). The analysis was evaluated and confirmed the attachment of the functional group of the silver nanoparticles.

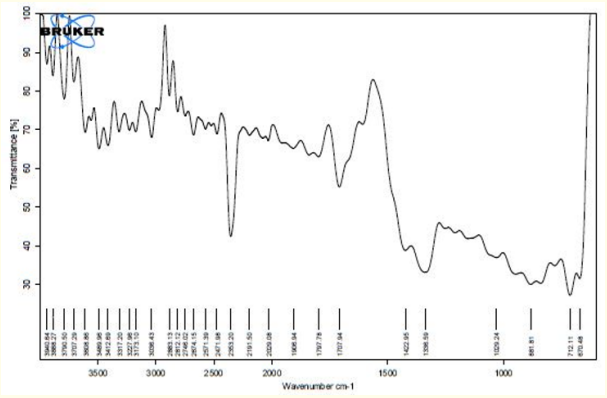

Figure 3: FTIR analysis of silver nanoparticles from Syzygium aromaticum.

Antibacterial activity of nano synthesized compounds and plant extract

The antibacterial effect of nano synthesized compounds and plant extracts on four microorganisms viz., *Staphylococcus aureus, Klebsiella pneumonia, Escherichia coli and Pseudomonas aeruginosa* were determined using agar well diffusion method represented in figure 4. The zone of inhibition representing the antibacterial activity for nano synthesized compounds on green synthesized silver nanoparticles of *Syzygium aromaticum and Coffea Arabica* are reported in table 1. In that all the organisms were inhibited at a maximal level. Whereas in crude extracts of *Syzygium aromaticum* and *Coffea Arabica* showed minimal activity against all the pathogens were described in figure 5 and table 2. Penicillin streptomycin would be the control for all the pathogens.

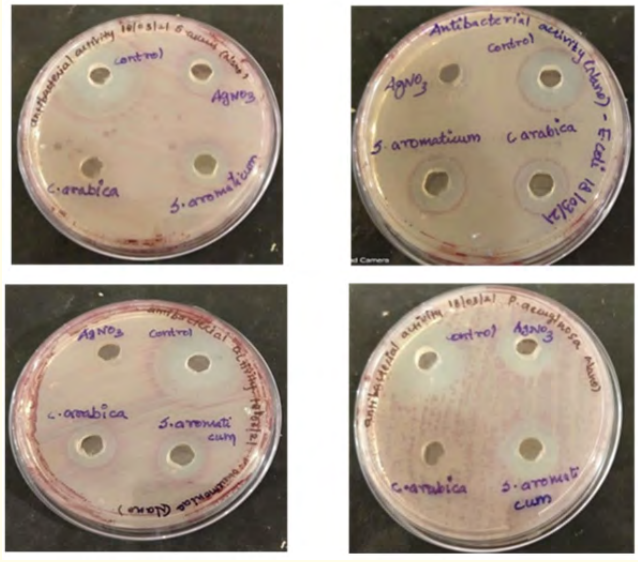

Figure 4: Antibacterial activity of biosynthesized nanoparticles of plant extract.

S.no	Plant name	Zone of inhibition			
		Staphylococcus aureus	***Escherichia coli***	***Pseudomonas aeruginosa***	***Klebsiella pneumoniae***
1	*Syzygium aromaticum*	7	5	10	6
2	*Coffea arabica*	4	4	6	4

Table 1: Antibacterial activity of nano synthesized compounds.

S.no	Plant name	Solvent	ZONE OF INHIBITION			
			Staphylococcus aureus	***Escherichia coli***	***Pseudomonas aeruginosa***	***Klebsiella pneumoniae***
1	*Syzygium aromaticum*	Hexane	4	-	8	6
		Ethanol	-	2	1	4
		Methanol	9	7	8	11
2	*Coffea arabica*	Hexane	-	-	-	-
		Ethanol	-	-	-	-
		Methanol	-	-	-	-

Table 2: Antibacterial activity of plant extracts.

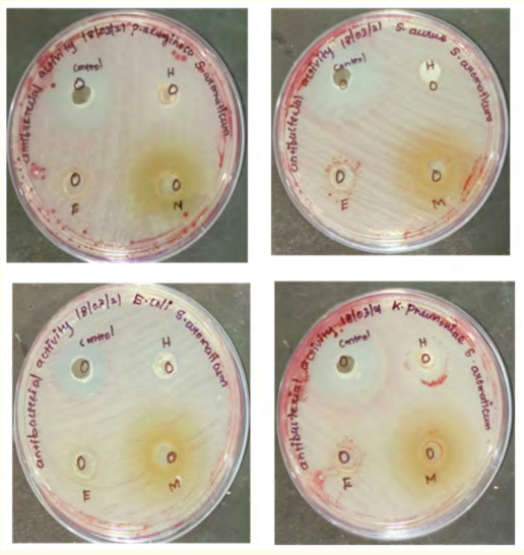

Figure 5: Antibacterial activity of plant extract with different solvent and microorganisms.

Antimycobacterial activity - microplate alamar blue assay

Crude plant extracts did not show enhanced reduction of Alamar Blue as compared to the medium control. We observed that the nano synthesized plant extracts of *Syzygium aromaticum* and *Coffea arabica* 250/500 µg/ml showed activity against *M. smegmatis*. It was denoted by the color of the dye that remained blue indicating their capacity to inhibit the growth of *M. smegmatis*. Whereas, the change of dye colour from blue to pink colour indicates the growth of *M. smegmatis* which means no inhibition.

Cytotoxicity assay using zebra fish embryo

In this study five different concentrations of green synthesized silver nanoparticle were used from 5,25,50,75 and 100 mg/ml concentrations. There is no significant mortality at 5 and 25 mg/ml than the other concentrations. Whereas the 50 µg/ml shows moderate and 75 and 100 µg/ml shows high toxicity level. Where the Silver nanoparticles of *Syzygium aromaticum showed* less toxicity in zebra fish larvae at minimal concentration represented in the figure 7 and table 3.

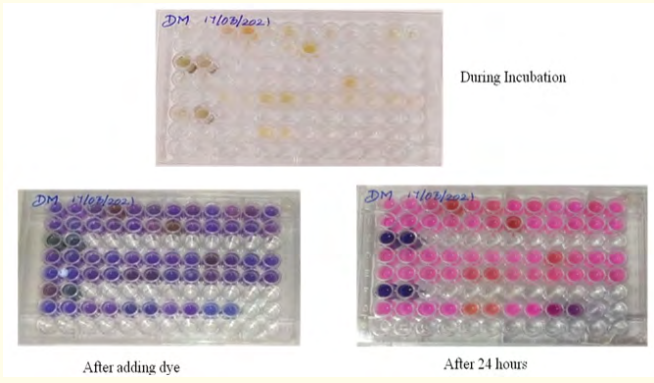

Figure 6: Antimycobacterial activity – microplate alamar blue assay.

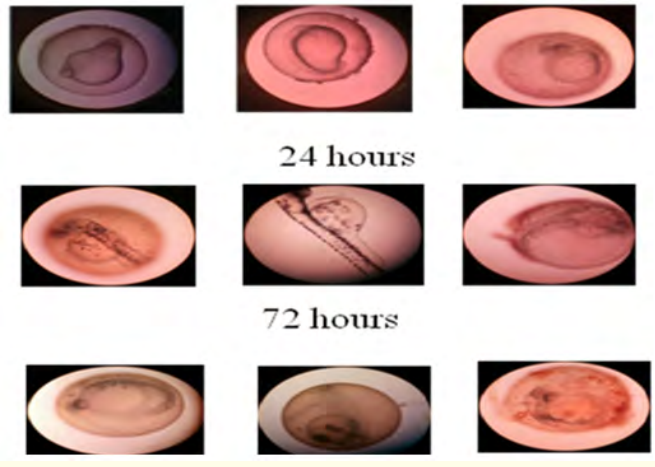

Figure 7: Cytotoxicity test for green synthesized silver nanoparticle using zebra fish embryo. Morphological change observed with high concentration of nano drug.

Plant name	Concentration	No. of survival	No. of Death	% of Lethality
Syzygium aromaticum	5	9	1	10
		9	1	
	25	8	2	30
		6	4	
	50	5	5	45
		6	4	
	75	3	7	70
		3	7	
	100	2	8	85
		1	9	
	Control	10	0	-

Table 3: The cytotoxicity assay of Syzygium aromaticum was assessed using zebra fish Embryos.

Cytotoxicity assay on *Artemia salina*

The brine shrimp lethality assay was also used to determine the toxicity of nano particles. Five different concentrations of nano synthesized plant extract of *Syzygium aromaticum* was screened. A substance with a lower LC50 value is more toxic than one which has higher LC50 value. According to the results, the 5 µg and 25 µg concentration have low toxicity whereas 50 µg concentration have moderate toxicity and 75 µg and 100 µg concentration have higher toxicity level. The graph was plotted for calculating LC50 value of nano synthesized plant extract of *Syzygium aromaticum* represented in figure 8.

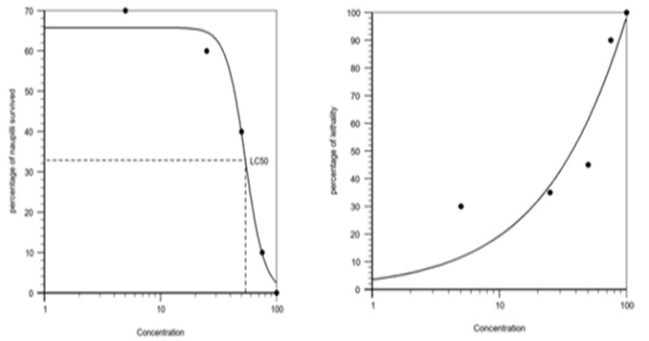

Figure 8: Survival rate and LC50 concentration of artemia for nano synthesized plant extract.

Discussion

Our investigation of *Syzygium aromaticum* aimed to explore the significance of anti-mycobacterial activity. *Syzygium aromaticum* constitute the taxonomically most extensive group of plants. Bioreduction of silver particle was measured using UV-Visible spectroscopy based on the surface Plasmon resonance leading to the increase in color intensity. UV- visible spectrum of nanoparticles synthesized from *Coffea arabica* shows at 460 nm and *Syzygium aromaticum* at 450 nm. The similar study was done by [28] evaluated that the nanoparticles synthesized from *Syzygium aromaticum* showed Plasmon excitation at 430 nm. The SPR is highly influenced by shape and size of the nanoparticles [29]. And the antimicrobial activity of nano synthesized and crude extracts of *Syzygium aromaticum* and *Coffea Arabica* showed inhibition ranges from 4 mm to 11 mm with different solvent extracts. The maximum inhibitory activity was observed in methanolic extracts of *Syzygium aromaticum* against *K. Pneumonia* (11 mm) was reported by [30,31] have found the anti-mycobacterial activity of *Syzygium aromaticum* along with many other medicinal with an indicator organism of *M. tuberculosis* H37RV. In our study the inhibitory activity is at 250 µg/ml concentration by MABA. The anti-mycobacterial activity *Coffea arabica* with an indicator organism as *M. smegmatis,* the MIC of *Coffea arabica* is at 250 ug/ml by MABA which is similar to the study done by [32]. Till now, a few studies have been reported the toxicity effect of nanomaterials on *Artemia salina* [33]. In our study we have analyzed the cytotoxity of the plant *Syzygium aromaticum.* Five different concentrations were used for both test 5 and 25 µg/ml concentration shows low cytotoxicity. Whereas the 50 µg/ml shows moderate concentration and 75 and 100 µg/ml shows high cytotoxicity level. The study done by [33] used 16 nanoparticles to screen the lethality. Only two nanoparticles showed strong toxicity which is LC50 <100 ug/ml).

Conclusion

The flower buds of *Syzygium aromaticum* have a potent antimycobacterial and pharmacological activity**.** It is recommended to be taken because it has many beneficial effects in human health such as it used to as carminative, to cure dental problems and fungal infections and to strengthen the immune system and used to cure respiratory illness. Further studies can be carried out to separate potential and pharmacological compounds using column chromatography, HPLC and NMR spectroscopy. The present study also could pave the way towards possibility to obtain anti-mycobacterial drugs against other mycobacterial species.

Declaration of Competing Interest

All the authors declare that they have no conflict of interest

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Ethical Statement

In this study, no animal has been used.

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