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Synthesis, Characterization and Evaluation of Antioxidant, Anticancer and Toxicity Properties of Silver Nanoparticles Synthesized From *Syzygium Aromaticum*

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ABSTRACT

Background: *Syzygium aromaticum*, also known as clove, and its essential oil has already been proved to have antioxidant, anti-inflammatory and anticancer properties. Clove is used in various foods owing to its potent antimicrobial and antioxidant properties. Essential oil extracted from clove has been used in traditional medicine for treating various ailments.

Methods: *In silico* analyses of phytocompounds of *Syzygium aromaticum* namely eugenol, B-caryophyllene, gallic acid, crategolic acid, kaempferol, quercetin, cinnamaldehyde, and oleanolic acid were docked with three apoptotic proteins involved in breast cancer, namely BCL-2, BAX and APAF-1 using AUTODOCK. In addition, flower bud extract of *Syzygium aromaticum* was used for the synthesis of AgNPs (silver nanoparticles). The synthesized clove-silver nanoparticles were then characterized using various techniques such as Ultraviolet-visible spectrophotometry, FTIR, FESEM-EDX, DLS and zeta potential to determine the particle size, shape, crystalline structure, and stability of CL-AgNPs and tested for its anticancer potential in MCF-7 cell lines.

Results: *In silico* analysis predicted that phytochemicals of clove have good interactions with the apoptosis related proteins of breast cancer. *In vitro* assay confirmed the cytotoxic effect of the synthesized CL-AgNPs on breast cancer cells using the MCF-7 cell line with the IC50 value of 58.64 µg/ml.

Conclusion*: In vitro* analysis of the anticancer activity of CL-AgNPs in MCF-7 cell line supports the *in silico* study by proving active interactions between the phytochemicals of clove and target proteins of the breast cancer and hence *Syzygium aromaticum* has been proved to possess potential anticancer property. Further research is needed to consider clove-silver nanoparticles as a novel drug for treating breast cancer.

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INTRODUCTION

Cancer is reported as one of the leading death causing diseases in the world of which women are more prone to breast cancer. Annually, 25% of all women around the world who tested positive for cancer are affected with breast cancer. Early detection of breast cancer is the key to a good prognosis and increases the survival rate of about 80%.[1] Silver

nanoparticles from various plant extracts were proven to have lethal effects on breast cancer cells.[2-4]

The modern world is now accepting the fact that ancient medicinal plants are one of the effective ways to prevent, control and treat diseases including cancer.[5-8] Around 80% of the world population use traditional medicine extracted from medicinal plants as their primary health care. Pharmaceutical studies of these traditional drugs derived from plants are the basis of many early drugs such as aspirin, digoxin, morphine, quinine, pilocarpine, etc.[9]

Syzygium aromaticum, also commonly known as Clove, is a dried flower bud belonging to Myrtaceae family and is widely grown in different regions of the world. Clove is widely used as preservatives in various foods including meat because of its antimicrobial and antioxidant properties.[10] Clove essential oil (CEO) was widely used in Indian and Chinese medicine for treating external skin injuries and also acted as a great pain reliever in tooth ache. The constituents of CEO were already proven to have antimicrobial, anticancer, antioxidant and anti-inflammatory activities.[11]

In silico investigations are used to virtually screen a large number of components or chemical structures in a short span of time, increasing the likelihood of finding the best therapeutic drug candidates.[12] Molecular docking was performed with the selected eight potential phytocompounds of clove as ligands, namely, eugenol, b-caryophyllene, gallic acid, kaempferol, crategolic acid, quercetin, cinnamaldehyde and oleanolic acid and three biomarker apoptotic target proteins of breast cancer as target macro-molecules namely BCL-2, BAX and APAF-1.[13]

Size, shape, structure and stability of the CL-AgNPs were studied by the characterization analysis like FTIR, UV-Vis spectrophotometry, DLS, XRD, FESEM-EDX, HRTEM, etc.[14] Aqueous extract of clove was used for the preparation of CL-AgNPs and used for *in vitro* investigations. *In vitro* analyses like antioxidant assay using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method, cytotoxicity analysis against breast cancer cells (MCF-7) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and toxicity studies with *Artemia nauplii* were performed to establish the antioxidant, anticancer and toxicity properties of CL-AgNPs.

METHODS

In silico analysis

3D structures of selected apoptotic target proteins of breast cancer namely BCL-2 (PDB - 4AQ3), BAX (PDB - 4BDU) and APAF-1 (PDB - 1CY5) were acquired from Protein data bank (http://www.rcsb.org/). 3D structures of selected phytochemicals of clove namely eugenol, b-caryophyllene, gallic acid, kaempferol, crategolic acid, quercetin, cinnamaldehyde and oleanolic acid were downloaded from PubChem (http://pubchem.ncbi.nlm.nih.gov/) and ChemSpider databases (http://chemspider.com).

Molecular docking was performed using AUTODOCK v4.6.2 software. Visualization of docked molecules was carried out using the Discovery studio visualizer application. Polar hydrogen was exposed for each target molecule and the torsion regions were determined for each ligand. The docking was executed using the Lamarckian Genetic Algorithm with default parameters and 10 Genetic Algorithm runs were executed. The grid box spacing for X-, Y- and Z- axis was set between 60 Å to 90 Å so that the major part of the molecule was embedded facilitating blind molecular docking.[15,16] The drug likeness and pharmacokinetic properties of the selected phytochemicals of clove were obtained from SWISSADME (http://www.swissadme.ch/).[17] The phytocompounds were screened for drug likeness by Lipinski's rule of five.[18]

In vitro analysis

Preparation of plant extract

Dried flowers of Clove (*Syzygium aromaticum*) were cleaned to remove unwanted impurities and dried further. Fully dried clove was finely powdered. Then, 15g of powdered clove was weighed and added to 250 ml of water at 70-80°C for about 15minutes. Boiled solution was then allowed to cool and centrifuged at 5000 rpm for 15mins. It was then filtered thrice using Whatman filter paper. The clear filtrate obtained was the aqueous plant extract of the clove (Clove-extract). The Clove-extract was then stored at 4 °C until further use.

Synthesis of clove-silver nanoparticles (CL-AgNPs)

First, 200ml of Clove-extract was added to 1litre of 1mM silver nitrate ($AgNO_3$) solution in the ratio of 1:5. The reduction of silver ions by the metabolites of clove extract was confirmed by the immediate color change observed upon addition of $AgNO_3$.[19-21] The content was placed under dark condition at room temperature for 24 hours and then centrifuged thrice at 10000 rpm for 10 mins to obtain Clove-silver nanoparticles (CL-AgNPs). CL-AgNPs found in the pellet was then washed using ethanol and transferred to eppendorf tubes. The tubes were then centrifuged at 13000rpm for 15mins using a mini-centrifuge machine. The supernatant was discarded and then the eppendorf tubes containing the pellet were dried using a thermostat at 100°C for 45mins. Dried CL-AgNPs were scraped and stored for further use.

Characterization of synthesized CL-AgNPs

The first characterization study of synthesized NPs was the color change of solution mixture that was observed visually.[20,21] Conventionally, various characterization techniques such as UV–Visible spectrophotometry, FTIR (Fourier transform infrared spectroscopy), FESEM (Field emission scanning electron microscopy), DLS (Dynamic light scattering), zeta potential and XRD (X-ray diffraction) were used to study the size, shape, surface, stability and dispersion of the nanoparticles.[14]

Toxicity study

Initially, *Artemia nauplii* cysts were incubated for 48 hrs in saline water with vigorous aeration. This experiment was performed in 24 well microtiter plate added with five healthy 48hr old *Artemia nauplii* in each well and treating them with various concentrations of CL-AgNPs (1ppm, 5ppm and 10ppm) in triplicates. Then, incubation was done at room temperature under dark conditions and the number of dead and alive shrimps were counted at 24 hrs and 48 hrs.[22]

Antioxidant assay (DPPH method)

DPPH assay was used to study the radical scavenging activity of synthesized CL-AgNPs. DPPH was prepared using methanol solution at 0.2mM concentration. Different concentrations of CL-AgNPs were added as triplicates in the microtiter plate along with 0.2mM methanolic DPPH solution and incubated under dark condition for 30mins. The absorbance was taken at 517nm, using a Multimode plate reader. Ascorbic acid was used as the Standard and 0.2mM methanolic DPPH remained as the Control.[2] The percentage of free radical scavenging activity was determined using the formula,

[(Absorbance of Control-Absorbance of CL-AgNPs)/Absorbance of Control]*100

MTT assay

The cytotoxicity of the CL-AgNPs against breast cancer cells (MCF-7) was analyzed using MTT assay. MCF-7 cells were seeded at a density of $0.1x10^5$ cells into each of the wells in a 96 well plate as triplicates and incubated for 24 hrs in a humidified 5% CO_2 incubator at 37ºC. After 24 hrs, the old media was discarded and fresh media was added. The cells were then treated with different concentrations of CL-AgNPs (10, 20, 40, 60, 80 and 100µg/ml) dissolved in DMSO and further incubated for 24hrs in a humidified 5% CO_2 incubator at 37ºC. Cells that were left untreated were used as negative control. Media was aspirated from the wells and then treated with 100µl of 0.5mg/ml of MTT, followed by incubation for 3hrs in a humidified 5% CO_2 incubator at 37ºC. MTT solvent was then completely removed from the wells and then 100µl of DMSO was added to each of the wells to dissolve the purple formazan crystals. The plate was then incubated for 15-30mins under dark conditions after which the absorbance was measured at 570nm (test wavelength) and 620nm (reference wavelength).[23] The percentage of cell viability was determined by,

[Absorbance of CL-AgNPs/Absorbance of Control]*100

Statistical Data Analysis

The values obtained from the experimental data were expressed in terms of Standard Deviation. Statistical significance was determined using Student T-test and probability values *P≤0.05 were considered to be statistically significant and ***P≤0.001 were considered to be of high statistical significance.

RESULTS AND DISCUSSION

In silico analysis

Molecular docking was performed using AUTODOCK v4.6.2 software with the eight selected phytochemicals of *Syzygium aromaticum* (clove) namely, eugenol, b-caryophellene, gallic acid, kaempferol, crategolic acid, quercetin, cinnamaldehyde and oleanolic acid[11] with three apoptotic biomarker proteins of breast cancer such as BCL-2 (PDB ID- 4AQ3), BAX (PDB ID- 4BDU) and APAF-1 (PDB ID- 1CY5). Visualization of docked molecules was carried out using Discovery studio visualizer webtool. Based on ADME analysis of the selected phytochemicals, all the ligands can be considered for docking except oleanolic acid since it violates one rule of Lipinski's rule of five.

Table 1. Interaction of phytocompounds from *S. Aromaticum* with the apoptotic target proteins of breast cancer.

Binding energy (kcal/mol)	Target Proteins		
Ligands	BCL-2	BAX	APAF-1
Eugenol	-6.2	-5.2	-4.7
B-caryophyllene	-5.8	-6.2	-4.6
Gallic acid	-5.6	-6.4	-5
Crategolic acid	-8.2	-7.7	-6.2
Kaempferol	-7.3	-7.3	-5.6
Quercetin	-8.7	-7.4	-5.6
Cinnamaladehyde	-6.5	-4.9	-3.9
Oleanolicacid	-8.2	-7.8	-5.8

All the selected ligands were interacted well with all the three apoptotic biomarkers of breast cancer by

exhibiting low binding energy values as mentioned in Table 1.

Specifically, the interactions between BCL-2 with crategolic acid and quercetin as shown in Figure 1a have low binding energy values of -8.2kcal/mol and -8.7kcal/mol, respectively, suggesting that these phytochemicals have greater capability to interact with BCL-2 (anti-apoptotic protein) and thus reduce the chances of tumor cell proliferation. Interactions of crategolic acid, quercetin and kaempferol with BAX as shown in Figure 1b have binding energy value of -7.7kcal/mol, -7.4kcal/mol and -7.3kcal/mol, respectively, indicating lesser possibility of upregulation process and hence may induce apoptosis. APAF-1 is an apoptosis-inducing protein like BAX and its interaction with crategolic acid, kaempferol and quercetin show that they can also induce apoptosis as they exhibit binding energy values of -6.2kcal/mol, -5.6kcal/mol and -5.6kcal/mol, respectively, as shown in Figure 1c. These significant interactions of three of the phytocompounds of clove with the important apoptosis-oriented proteins like BCL-2, BAX, APAF-1 exhibit a promising outcome to formulate a drug using these phytochemicals to treat breast cancer.

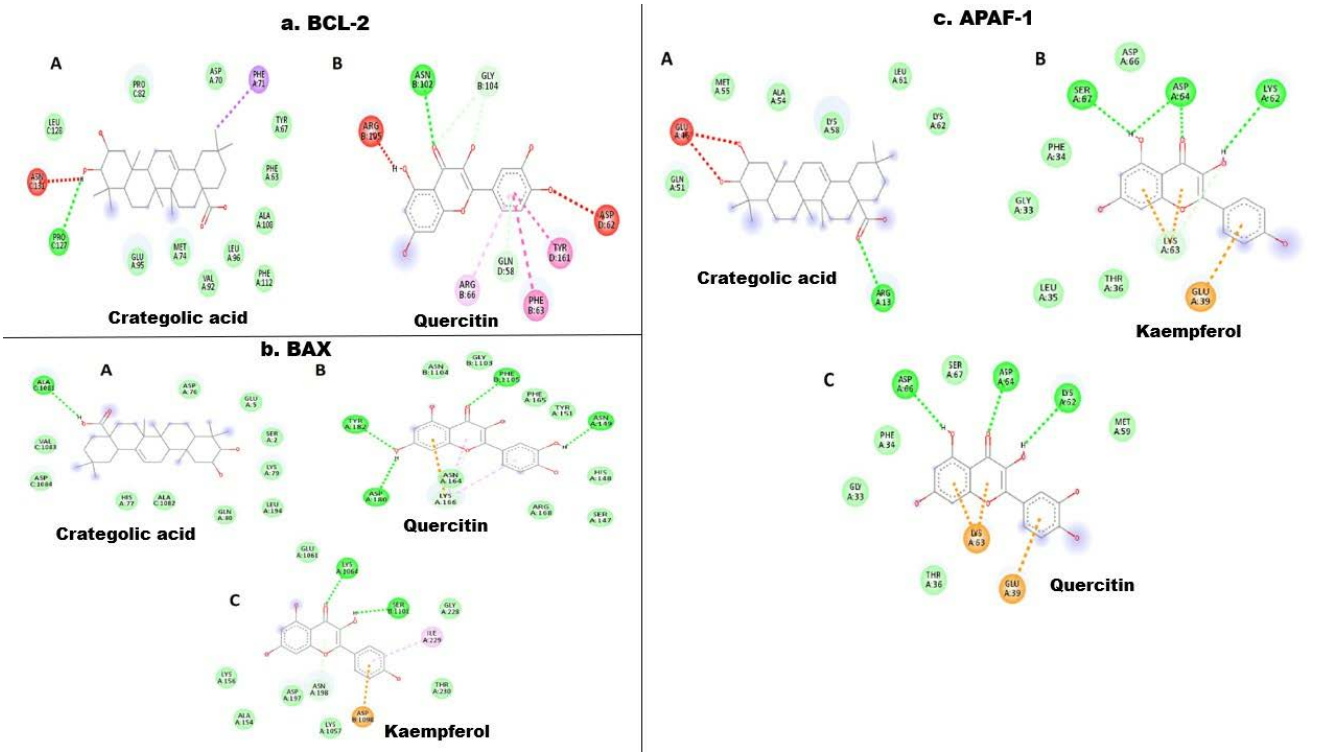

Figure 1. a. Interaction of target protein BCL-2 with crategolic acid and quercetin. **b.** Interaction of target protein BAX with crategolic acid, quercetin and kaempferol. **c.** Interaction of target protein APAF-1 with crategolic acid, kaempferol and quercetin

Therapeutic potential of significant phytocompounds present in Clove

Studies have reported that Crategolic acid, also referred to as Maslinic acid was found to cause caspase independent programmed cell death by altering the ROS (reactive oxygen species) levels and electrochemical potential across mitochondrial membrane. Immunoblotting analyses also revealed the involvement of MAPK signalling pathway responsible for the variance in cell cycle progression. Cyclin D1, Cyclin B1, CDK2 and CDK4 expression were found to be significantly downregulated in MDA-MB-231 and MCF-7 cells after treatment with Crategolic acid and hence facilitating apoptosis of breast cancer cells. [24] Kaempferol was found to exhibit antagonistic activity in ER (Estrogen Receptor) signalling pathway by downregulating the expression of pMEK1/2, pAkt, pIRS-1 induced by E2 (17-β-Estradiol), hence proving its potential as an effective anti-cancer drug.[25] It was also found to downregulate CDK1 and hence arrests cell cycle at G2/M stage inhibiting the proliferation of breast cancer cells.[26] Quercetin, another significant phytocompound of clove was reported to inhibit breast cancer cell proliferation by apoptosis through induction of cytochrome C production from mitochondria. It was also found to reverse Epithelial-Mesenchymal transition (EMT) and hence inhibit metastasis by downregulating the expression of TGFB1, MMPs and mTOR/c-Myc. The antiproliferative activity of Quercetin was also confirmed by reduction in PI3K, P38MAPK and

through stabilization of telomeric DNA structure.[27] Thus, we could infer the therapeutic potential of phytocompounds of *Syzygium aromaticum* in treating breast cancer.

UV-Vis spectrophotometry

UV-Vis spectrophotometry was used to characterize the synthesized CL-AgNPs and it was performed between 200-800nm wavelength range. The formation of CL-AgNPs can be confirmed by UV-Visible spectrophotometry, as it exhibits surface plasmon resonance (SPR), i.e., strong absorbance band in the range between 400nm and 500nm. The formation of SPR is due to the interaction between the light and the mobile surface electrons of AgNPs.[14] Generally, surface plasmon resonance (SPR) peak for silver nanoparticles occurs in the range between 400nm and 500nm. The SPR peak for the synthesized CL-AgNPs dissolved in DMSO was found to occur between 400-450nm. From Figure 2a, it was observed that the exact peak occurred at 425nm confirming both the quality and quantity of CL-AgNPs synthesized by the reduction of $AgNO_3$ to metallic clove nanoparticles.[28]

FTIR

FTIR analysis was carried out for the identification of functional groups present in the synthesized CL-AgNPs. The dried CL-AgNPs were analysed by FTIR spectrometer, using transmittance mode at $4cm^{-1}$ resolution. The resulting spectrum was recorded between 400-$4000cm^{-1}$. [29] The presence of different functional groups of the synthesized CL-AgNPs was determined using FTIR analysis. Figure 2b represents the FTIR result of clove-extract with major peaks observed at $3353.6cm^{-1}$, $2926.45cm^{-1}$, $1727.91cm^{-1}$, $1609.31cm^{-1}$, $1200.47cm^{-1}$ and $1030.77cm^{-1}$ which denotes the presence of alcohol (O-H), alkane (C-H), aldehyde (C-H-O), unsaturated compound(C=C), alkyl amine(C-N), alkoxide (C-O) groups respectively.[30] Comparative analysis of the FTIR result of clove-extract with that of synthesized CL-AgNPs as shown in Figure 2c, revealed a shift in peaks at $3224.4cm^{-1}$ and $1316.18cm^{-1}$ which denotes the shift in the above-mentioned bonds to (=C-H) stretch and (C-O) stretch respectively. The peak at $1609.31cm^{-1}$ remains undisturbed and hence considered to be a strong peak. These shifts in the FTIR analysis of CL-AgNPs confirms the reduction process of the phytochemicals of clove.

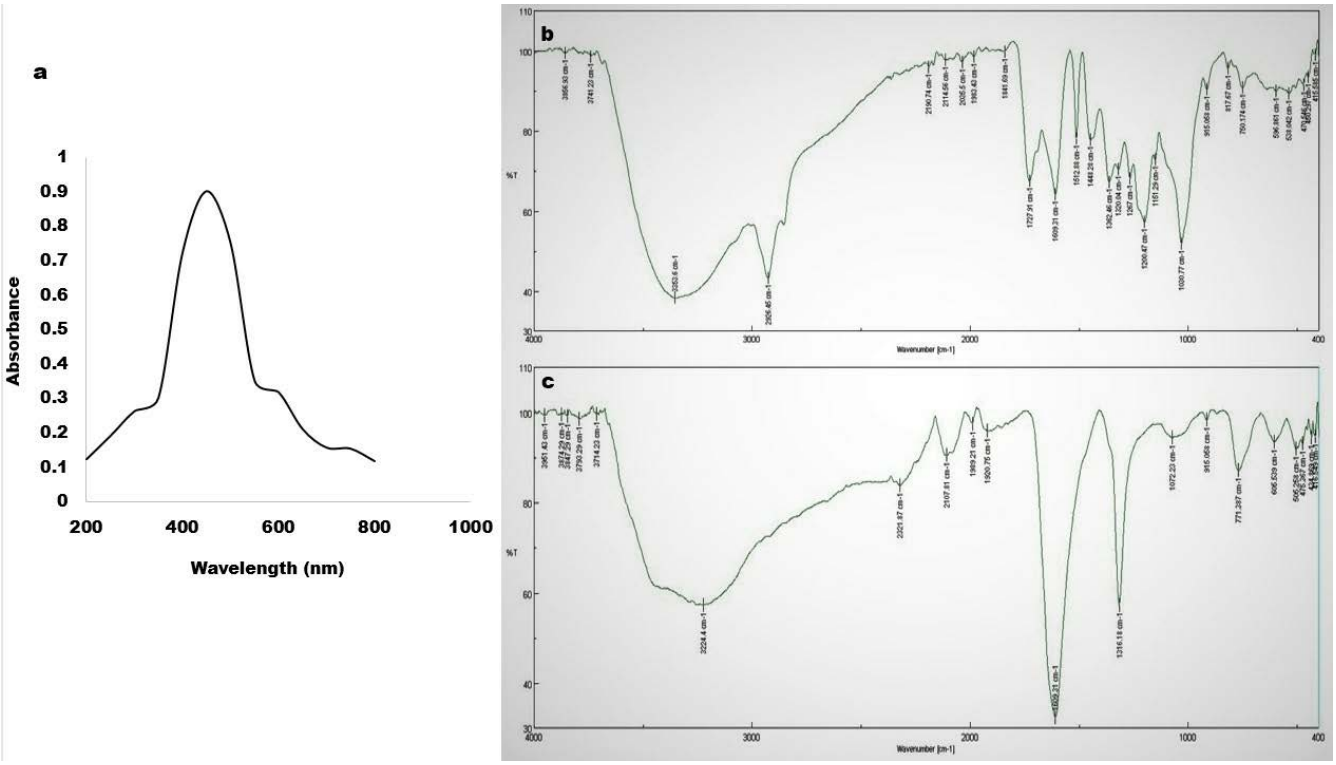

Figure 2. a. UV-Vis spectrophotometry analysis of CL-NPs. **b.** FTIR analysis of Clove extract. **c.** FTIR analysis of CL-AgNPs

FESEM – EDX

Surface morphology of synthesized CL-AgNPs was determined using FESEM and EDX (Energy dispersive X-ray). Analysis was also performed to obtain information about the elemental composition of CL-AgNPs.[31] FESEM results proved that the synthesized CL-AgNPs have significant surface morphology and size as shown in Figure 3a. The results displayed a good overall outlook of the synthesized CL-AgNPs. The surface composition of

elements of the synthesized CL-AgNPs was analysed using EDX. From the peak as shown in Figure 3b, we could evaluate the composition of synthesized CL-AgNPs as 16.5% of Ag, 49.7% of O and 33.6% of C, confirming the presence of higher quantity of silver ions on the surface of the synthesized CL-AgNPs.

DLS and zeta potential

CL-AgNPs dissolved in DMSO solution was used for DLS analysis to determine the average size distribution of CL-AgNPs in the sub-micron range.[32] The stability of CL-AgNPs and its surface charge were characterized by zeta potential analysis. For this purpose, the nanoparticles were mixed with distilled water and then inserted into zeta potential cell for measurement at parameters, 20V/cm field strength and 25°C temperature.

Average particle size of the CL-AgNPs that were synthesized was analysed using Dynamic light scattering (DLS) analysis.[33] The result of DLS showed the major peak was at 508.2nm diameter as shown in Figure 3c. The zeta potential value of synthesized CL-AgNPs was found to be -19.9mV as observed in Figure 3d. The higher negative zeta potential value of CL-AgNPs revealed that they possessed a great repulsive force towards the charged particles and hence have enhanced stability by reducing the aggregation potential.[34]

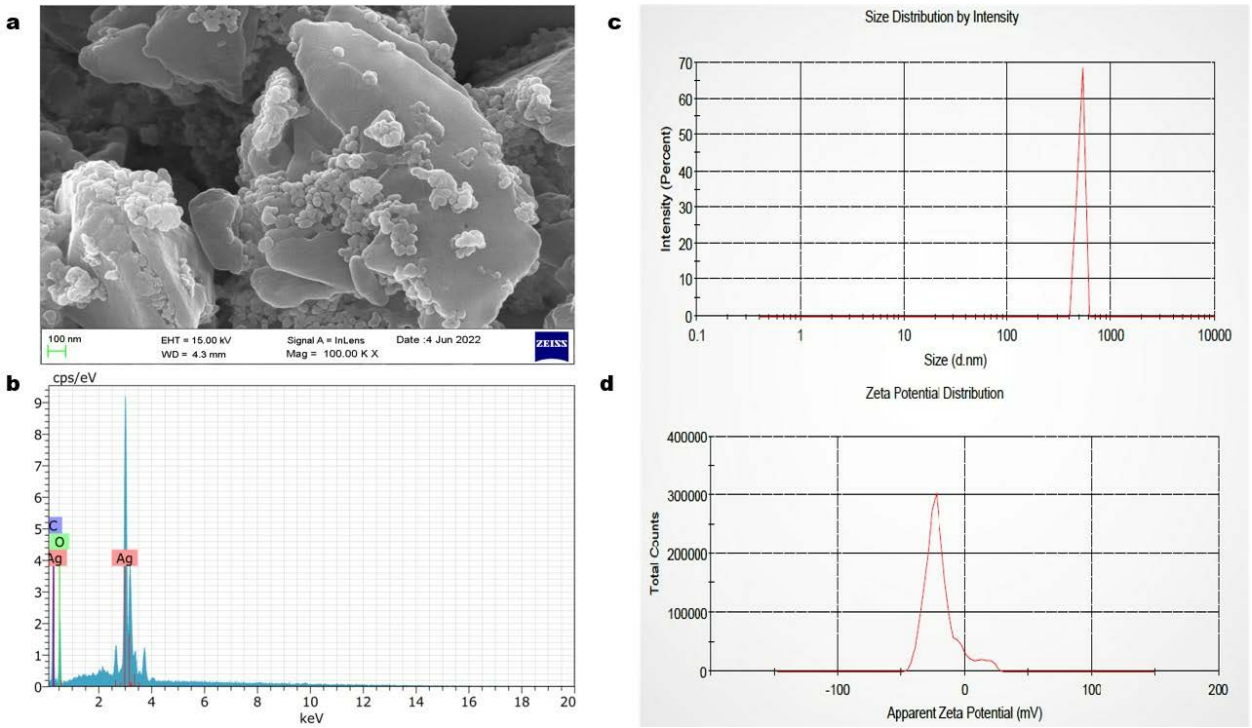

Figure 3. a. FESEM analysis of CL-AgNPs. **b.** EDX analysis of CL-AgNPs. **c.** DLS analysis of CL-AgNPs. **d.** EDX analysis of CL-AgNPs

XRD

XRD analysis was done to obtain information regarding the crystalline structure of CL-AgNPs, such as nature of phase, lattice parameter and crystalline grain structure[34], XRD pattern of CL-AgNPs which were analysed in the 2θ range between 10º to 80º using powder diffractometer.[35] From Figure 4a, it was observed that the XRD pattern showed a major peak between 2θ range of 25º to 30º. From this, we could predict that the synthesized CL-AgNPs possess good crystalline structure.

Toxicity study

To study the toxicity level of the synthesized CL-AgNPs on *Artemia nauplii*, different concentrations of CL-AgNPs (1 ppm, 5 ppm and 10 ppm) were used. Based on the number of *Artemia nauplii* dead count, the mortality percentage was calculated after incubation periods of 24 hrs and 48 hrs. From Figure 4b, it was concluded the toxicity level of CL-AgNPs against *Artemia nauplii* showed that the mortality percentage increased significantly as the concentration of CL-AgNPs increased in a dose-dependent manner, proving the fact that the synthesized CL-AgNPs were quite toxic to the *Artemia nauplii* body system when treated with higher concentrations of CL-AgNPs.[36]

Antioxidant activity of CL-AgNPs (DPPH method)

The presence of free radicals in the body interacts with and damages the tissues. Tumor cells often release different types of free radicals which causes tissue damage. It is essential for an anticancer drug to

possess the antioxidant property to scavenge the free radicals released by the tumour cells. In this respect, the antioxidant property of the synthesized CL-AgNPs was analysed using DPPH radical scavenging method. The percentage of DPPH radical scavenging property of different concentrations of CL-AgNPs was found to increase in a dose-dependent manner as shown in Figure 5a. The highest percentage of DPPH radical scavenging activity, 78.2% was observed at 100 µg/ml and the lowest activity, 16.8% was observed at 6.25µg/ml concentration of CL-AgNPs, concluding that synthesized CL-AgNPs have good radical scavenging property. This study also demonstrated that the greater ability of CL-AgNPs to scavenge free radicals was due to the presence of silver ion bound to the phytochemicals of clove as the phytochemicals of clove were already proved to have good antioxidant property by Han & Parker, 2017.[10]

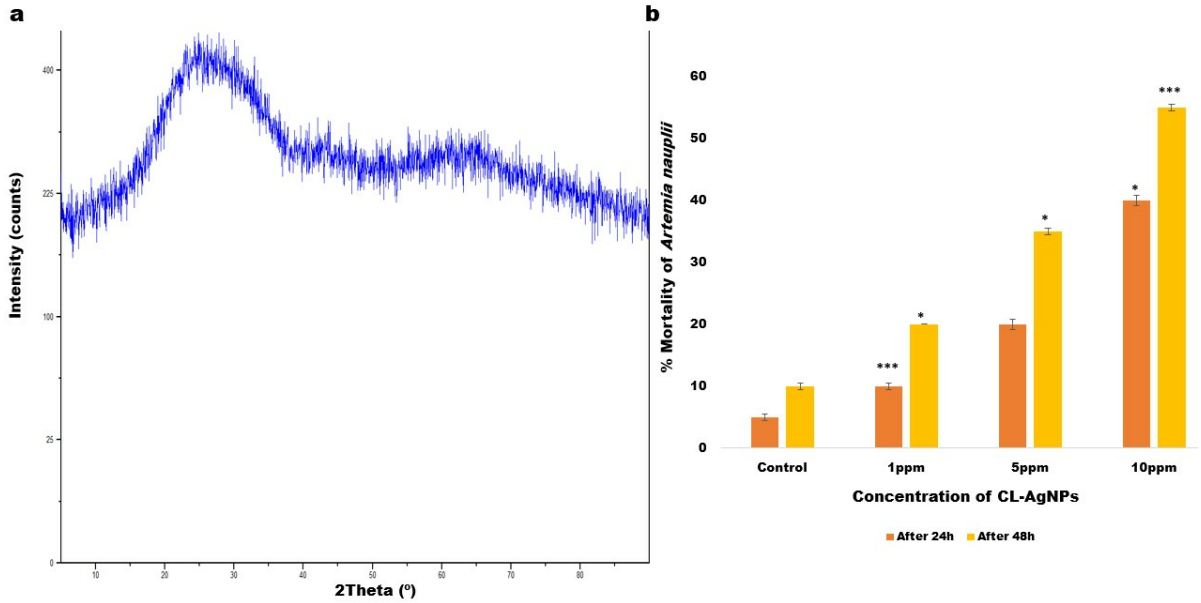

Figure 4. a. XRD analysis of CL-AgNP. **b.** Toxicity effect of CL-AgNPs on *Artemia nauplii* after 24 hrs and 48 hrs

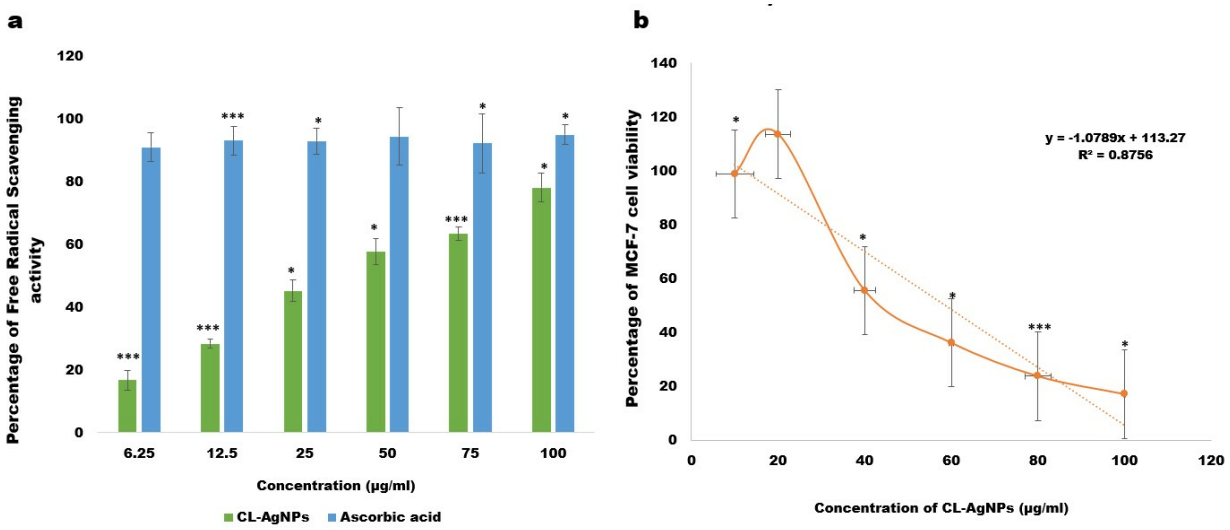

Figure 5. a. Antioxidant assay of CL-AgNPs. **b.** Cytotoxicity of CL-AgNPs against MCF-7 Breast Cancer cells

Anticancer activity of CL-AgNPs (MTT assay)

In silico analysis predicted that phytochemicals of clove have good interactions with the apoptosis related proteins of breast cancer, thereby increasing the possibility of inducing apoptosis in the breast cancer cells. *In vitro* MTT assay was performed in 96 well microtiter plates to observe the cytotoxic effect of the synthesized CL-AgNPs on breast cancer cells using the MCF-7 cell line. It was indicated that CL-AgNPs were lethal against the breast cancer cells. From the concentration-viability graph (Figure 5b), the 50% growth inhibitory concentration (IC_{50}) value of CL-AgNPs was calculated to be IC_{50}=58.64µg/ml. *In vitro* analysis of the anticancer activity of CL-

AgNPs using MCF-7 cell line supports the *in silico* study of active interactions between the phytochemicals of clove and target proteins of the breast cancer and hence *Syzygium aromaticum* has been proved to possess potential anticancer property.

CONCLUSION

Characterization results of UV-Vis, FTIR, FESEM-EDX, DLS and zeta potential and XRD analyses of *Syzygium aromaticum* suggested that CL-AgNPs had been synthesized successfully and also proved to have good size, shape, crystalline structure and stability. Toxicity study suggested that CL-AgNPs have good toxic effect in *Artemia nauplii* system if treated with higher concentrations for a longer duration. The antioxidant assay showed that the synthesized CL-AgNPs possess free radical scavenging activity. MTT assay and *in silico* analyses proved that the synthesized CL-AgNPs have a specific anticancer property especially against breast cancer cells. In addition, this is the first study in which molecular docking of phytocompounds of *Syzygium aromaticum* with apoptotic proteins identified three potential phytocompounds with anti-cancer activity. Further research is needed to consider the silver nanoparticles synthesized from clove extract as an effective drug for treating various types of cancers including breast cancer.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY

Data will be available upon request.

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