Antibacterial and anticancer potential of *Brassica oleracea var acephala* using biosynthesised copper nanoparticles

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ABSTRACT

Introduction: *Brassica oleracea var acephala* was studied for preliminary phytochemical screening. The results showed that the ethanolic crude extract of the leaf contain high phytochemical activity hence *B.oleracea var acephala* is rich in flavonoids, phenolic compounds, carbohydrates and phytosterols.

Materials and methods: The ethanolic extract was used to synthesise copper nanoparticles. The copper nanoparticles were successfully synthesised from copper sulphate solution which was identified by the colour change from dark green colour of the extract. Thus the B.oleracea var acephala is a good source to synthesis copper nanoparticles. The synthesised copper nanoparticles were characterised using Scanning Electron Microscope (SEM) analysis. The SEM image displayed the high-density nanoparticles synthesised by leaf extracts and that the nanoparticles were crystals in shape.

Results: The copper nanoparticles (CNP) bind to the leaf extract. *B.oleracea var acephala* also has shown the antimicrobial and antioxidant activity. A comparative study was done between ethanolic its crude extract and nanoparticles. Both extracts exhibited zone of inhibition and better antioxidant potential but the CuNPs shows major zone of inhibition and showed more antioxidant activity. Anticancer activity of *B.oleracea var acephala* against Cervical HeLa cell line was confirmed using ethanolic crude extract and CNP. The results showed that HeLa cells proliferation was inhibited with increasing concentration of ethanolic crude extract and copper nanoparticles. From the results, it was seen that percentage viability of the cancer cells decreased with increased concentration of the samples whereas cytotoxicity against HeLa cell lines increased with the increased concentration of the samples.

Conclusion: Thus *B.oleracea var acephala* possesses anticancer activity against HeLa cell lines.

KEYWORDS:
Brassica oleracea var acephala, Copper Nanoparticles, Ethanolic crude extract, antioxidant and cytotoxicity

INTRODUCTION

Cancer cells destroy normal cells and are derived because of the imbalance in the body. To correct this imbalance, cancer need to be treated. Every year, millions of people are diagnosed with cancer, leading to death.[1] Currently new therapies and techniques have been developed to treat cancers by utilizing nano materials. Carefully synthesized like bio mediated nanoparticles are particularly encouraging as healing drug for the treatment of cancer. Eco friendly synthesized nanoparticles are used as a nano drug to control cancer.[2] The search for anticancer drugs from natural sources began with the discovery of Podophyllotoxin in the late 1960s, further leading to discovery of vincristine, vinblastine, campthothecin and taxol.[3] Nearly 65–80% of people in developing countries still use traditional herbal medicine as treatment. This is because of cultural beliefs. And that herbal treatment is affordable, accessible.[4]

According to the American Cancer Society the annual fatality due to cancer globally accounts for more than 3 million. Chemotherapy for cancers which includes high risk dosage of chemical drugs leading to high toxic cases at times. Medicinal plants ease and cure cancer via using the compounds which have natural anti-oxidant and anti-cancer activities which can inhibit or kill carcinogenic cells. Many herbal plants are anti-oxidants which can be consumed to inhibit cancer or potentiate chemotherapy. Many phytochemicals extracted from herbs could reduce proliferation of cancer cells by apoptosis, stop their metastasis and angiogenesis.[5] The medicinal plants is *Brassica* Spp. *Brassica oleracea* is a plant that is derivate from Europe and currently it is found all over the world. It belongs to the family *Brassicaceae* (Cruciferaceae). Cruciferaceae family has many medicinal plants. It has over 300 genera and over 3000 species distributed worldwide.[15] Generally consumed cruciferous vegetables are broccoli, Brussels sprouts, kale, mustard, cabbage, turnips, cauliflower, boy choy and Chinese cabbage.[6-16] Many research found that consuming high amount of cruciferous vegetables is linked to lower cancer incidence.[17] These vegetables are anti-oxidants, anti-bacterial, anti-cancer and anti-fungal.[18]

Brassica vegetables contain indole-3-carbinol, a chemical which helps in DNA repair in cells and helps to obstruct the

This article was accepted: 30 September 2020
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growth of cancer cells.[19,20] They are also a good source of carotenoids with broccoli containing higher level of carotenoids.[21] Brassica vegetable is strong modulator of the innate immune response system with strong antiviral, antibacterial and anticancer activity.[22] However, it is also an antiandrogen.[23] In this study, the synthesis of copper nanoparticle from *B.oleracea var acephala* was studied. The study also aimed to assess the anti-microbial, cytotoxicity and anti-cancer activity of the synthesised copper nanoparticles.

MATERIALS AND METHODS

Collection of Plants

The plant used in this study was collected from local markets of Kelambakkam, Chennai, Tamil Nadu. The plant authentication number is NISMB4122020. After plants were collected they were cleaned well. The cleaning process involved the following steps. Cleaning, washing, peeling or stripping leaves from the stems. This has to be done by hands in order to get better results. The main purpose of drying is to remove the water from plants so that the plants can be stored. Plants have to be dried immediately as soon as the plants collection or the plant materials will be spoilt. Drying can be done either by natural process or by artificial process. Natural process includes shadow- drying. Plants were placed on drying frames or on stands, to be air-dried in barns or sheds. This may take few weeks for complete drying and the duration depended on the temperature and humidity. After complete drying of plants they were powdered well for further analysis.

Preparation of Crude Extracts

The leaves were rinsed with running tap water and followed by distilled water. The plant sample was air dried, cut with a blade into small pieces. They were covered and shadow-dried under room temperature to remove the residual moisture. The leaves were then pulverized in a mechanical blender. The powdered plant was then used for the preparation of ethanolic extract.

Solvent Extracts

The powder obtained was used for extraction purposes by performing the following the two methods:

Soxhlet Extraction

The powder was extracted by soxhlet apparatus with 80% ethanol (250ml).[24] After extraction, the solvent was evaporated and extracts were preserved at 4˚C. The crude extracts obtained were used for further investigation of Phytochemical screening, and Anti-microbial Evaluation. For phytochemical screening, extracts were dissolved in distilled water.

Cold Maceration

The powdered plant material was kept overnight on a rotary shaker at 100rpm. The extract was filtered with Whattman no.1 filter paper and was stored at 4˚C for further usage.

Qualitative analysis of phytochemicals

Phytochemical analysis was performed for the following components such as alkaloids, amino acids, carbohydrates, phenolic compounds, saponins, phytosterols, flavanoids, and terpenoids.[25]

Synthesis of Copper Nanoparticles

1mM aqueous solution of copper sulphate was prepared and used for the synthesis copper nanoparticles. 10ml of ethanol extract of plant material was added to 90 ml of aqueous solution of 1mM Copper sulphate in a conical flask for reduction to Copper ions and stirred continuously for 15 min at RT (25°c). Here the filtrate acts as stabilising and reducing agent for 1mM of CuSO4. Copper sulphate reduced to copper ions was identified by the colour transformation from light yellow of the extract to dark brown colour. A control setup was also maintained without adding copper sulphate to the plant extract. The formation of copper nanoparticles was further assessed by spectral analysis by UV-Visible spectroscopy in the range between 500-700nm. The copper nanoparticles (CNP) thus obtained was purified by continuous spinning method at 10,000rpm for 30 min followed and pellet dispersion in deionised water to eliminate any unwanted biological materials.[26] Later the CNP were dried and stored for further analysis.

Characterization of Copper Nanoparticles

The formation of CNP was confirmed by UV- Visible spectroscopy using Jasco V-550 spectrophotometer instrument. Size of the CNP was analysed with UV-Spectrometerin the range between 300-700nm. Morphologyand mean particles sizes were determined by SEM analysis.[27,28] The SEM analysis was established by using Hitachi-S 3400N.

Scanning Electron Microscopy (SEM) Analysis

The size and surface morphology of the nanoparticles were obtained by Scanning Electron Microscopy (SEM) analysis. SEM image displayed the high density nanoparticles synthesized by ethanolic crude extract and copper nanoparticles were respectively flakes and spherical in shape. This validated the formation of copper nanostructures. SEM gave assist knowledge into the size and morphology of interest of the Copper Nanoparticles.

The sample was synthesised by placing a drop of colloidal solution of copper sulphate on carbon coated copper grid, excess solution was eliminated using a blot paper and the film on the SEM grid were dried for analysis. The sample was observed under the microscope operated at an accelerated voltage of 130Kv (Hitachi-S 3400N).

Antioxidant activity (Hydroxy free radical assay)

The ethanolic extract was taken at different concentrations from S1 to S5. The extract was made up to 1ml with distilled water. The control tube contained only the distilled water. To each test tubes 1ml of 1.5Mm Ferrous Sulphate, 0.7ml of 6Mm Hydrogen peroxide and 0.3ml of 20Mm Sodium Salicylate was added. The same procedure was performed for another set without Sodium Salicylate. The tubes were then incubated for 1 hour at 37˚C. Then the absorbance was measured at 562nm using colorimeter.

Anti-microbial activity (Disc Diffusion method)
Antibacterial activity of the extract was determined on Muller and Hinton Agar using Kirby-Bauer disk diffusion method.[29] Bacterial strains *(Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa)* grown on nutrient agar at 37°C for 24 hours were suspended in a saline solution (0.85% NaCl) and the cell density was adjusted to 0.5 MacFarland turbidity standard to yield a bacterial suspension of 1.5x108CFU/ml. The standardized inoculum was transferred and spreaded on the Muller Hinton Agar (MHA) plates using sterile swabs. Sterile Whatmann No. 1 filter paper discs (approx.5mm diameter) were placed on the inoculated MHA plates and the ethanolic crude extract and nanoparticles was loaded over the discs at different concentrations. A standard antibiotic disc (Ampicillin 10mcg) was placed as control. The plates were incubated at 37˚C for 1 day. Triplicate was done for each strain.

The plates were determined for the bacterial growth inhibition, indicate by the clear zone outside the disc. The zones are measured in milimeter using sliding calipers or a ruler. The diameter of inhibition zone caused by the crude extract and copper nanoparticle was compared to those produced by the commercial control antibiotics Ampicillin (10mcg). The absence of inhibition zone was considered as no activity. The organisms are reported as susceptible, intermediate or resistant to the extracts that have been tested.

Anti-cancer activity
The ethanolic crude extract and CNP is detected with the cell culture. In cell culture technique the extraction and nanoparticles have been used to check the activity. The cell line (HeLa) has been used for cell cytotoxicity test and cell viability.

Preparation of cell suspension
HeLa cells was cultured in flask with 25mL DMEM and 10% FCS by suspending in the media and gentle passaging homogenized cells.

Seeding of cells
A 24 well culture plate was added with several sample concentrations (0 to 200µg/mL) and cell suspension. The culture plate was incubated at 37°C in a humidified CO2 incubator for 48 hrs. After check with inverted microscope to have 80% confluency, cytotoxicity assay was done.

Cytotoxicity assay
The assay done is (3-(4, 5-dimethyl thiazol-2yl) -2, 5-diphenyltetrazolium bromide (MTT). The yielded purple formazan indicate viable cell number which is inversely proportional to the amount of cytotoxicity. MTT was added to the 48h incubated cells for 3h in RT. Then, all the content was discarded and added with 100µl SDS in DMSO to dissolve the formazan crystals, absorbance were detected in Read Well Touch micro plate reader at 570nm.[30]

RESULTS

Extraction yield
The ethanol extract was prepared from the powdered leaves of *B.oleracea var acephala* by using soxhlet apparatus. It has yielded 1.3440g/dL after concentrating under vacuum and dried in desiccator.

Phytoconstituents of Ethanol Extract from *Brassica oleracevaar acephala*
Qualitative phytochemical analysis of B.oleracea var acephala revealed some interesting facts such as secondary metabolites like alkaloids, proteins, tannins, carbohydrates, and phenol. The various phytochemical tests showed positive for phenolic compound, tannins, terpenoids and phytosterol in the case of flavonoid and phytosterol showed weakly positive and saponins showed negative. The result suggested that the various phytoconstituents possess potential antimicrobial and anti-oxidant which leads to the isolation of new compounds (Table I).

Synthesis of Copper ($CuSO_4$) Nanoparticles
The formation of CNP was observed by the change of colour in the solution from dark green to light green for the plant extract. Copper sulphate reduced to copper ions was identified by the colour change from dark green of the extract to light green colour.

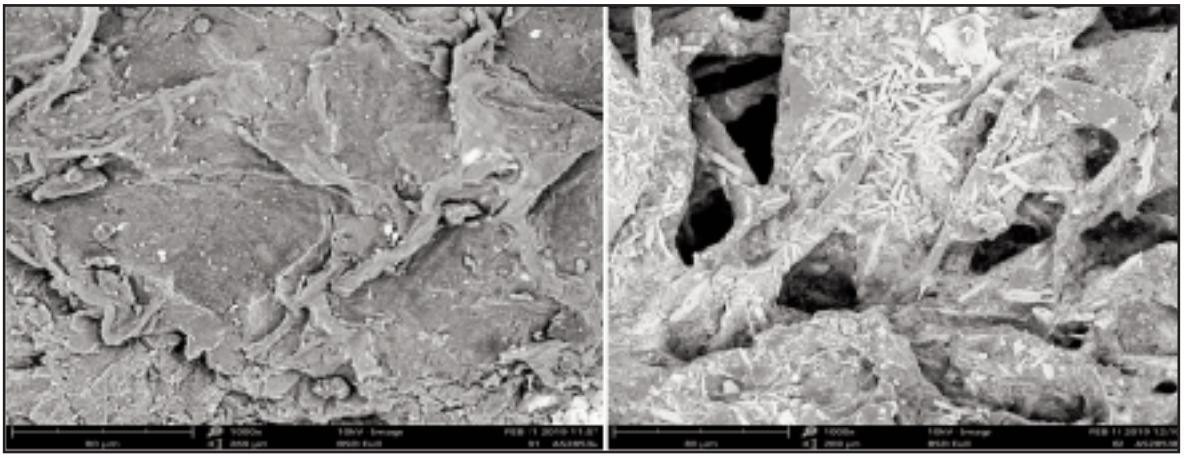
Characterization of copper nanoparticles (SEM analysis)
The SEM images of the crude extract and CuNPs are shown in Figure 1. The size and surface morphology of the nanoparticles were obtained by SEM analysis. The electrostatic interactions and hydrogen bond between the bio-organic capping molecules bond are used to synthesise CNP from plant extract. It was shown that spherical and relatively uniform shape of the CNP was confirmed in the range of 60-100nm. The quantitative and qualitative analysis of elements may be important in the production of copper nanoparticles. They were identified by EDAX analysis. Due to the Surface Plasmon Resonance, the copper nanoparticle shows the absorption peaks of higher counts.

Figure 2 shows that the ethanolic crude extract and synthesized copper nanoparticles from *Brassica oleracea var acephala* exhibit antioxidant potential and they act as best source for natural antioxidant compound. The percentage of inhibition for both the samples increased with increasing concentration of the samples. The result showed that the hydroxyl radical scavenging activity observed was in the range of 81-92% at the concentration of 10µg/ml. Hence it states that while scavenging hydroxyl radicals, the ability of copper nanoparticles (92%) was found to be higher than the ethanolic crude extract.

Antimicrobial activity (Disc diffusion method)
In this study the Agar disc diffusion method was used to evaluate the antimicrobial activity of the ethanolic crude extract and synthesized nanoparticles, by measuring the inhibition zone of the test microorganisms. The results obtained by extract of *B.oleracea var. acephala* showed that ethanolic crude extract and CNP exhibited prominent antibacterial activity against *E.coli*, *S.aureus* and *P.aeruginosa*. It also showed antifungal activity against *Candida albicans*. Comparing both the samples, CNP showed maximum zone of inhibition against test organisms. This was because nanoparticles have more antimicrobial activity compared to the crude extract. Thus, this study suggested that synthesized

Table I: Phytochemical screening of Brassica Oleracevaar acephala

S.No	Constituent	Test	Result
1.	Flavonoids	Alkaline reagent test	+
2.	Tannins	Ferric Chloride test	+
3.	Saponin	Forth foaming test	-
4.	Phytosterol	Libermann-Burchard's test	+
5.	Alkaloids	Mayer's Reagent	-
6.	Terpenoids	Salkowski test	+



Leaf Extract Copper Nanoparticles

Fig. 1: Comparative SEM image of Leaf Extract vs. Silver Nanoparticles at 10000 X.

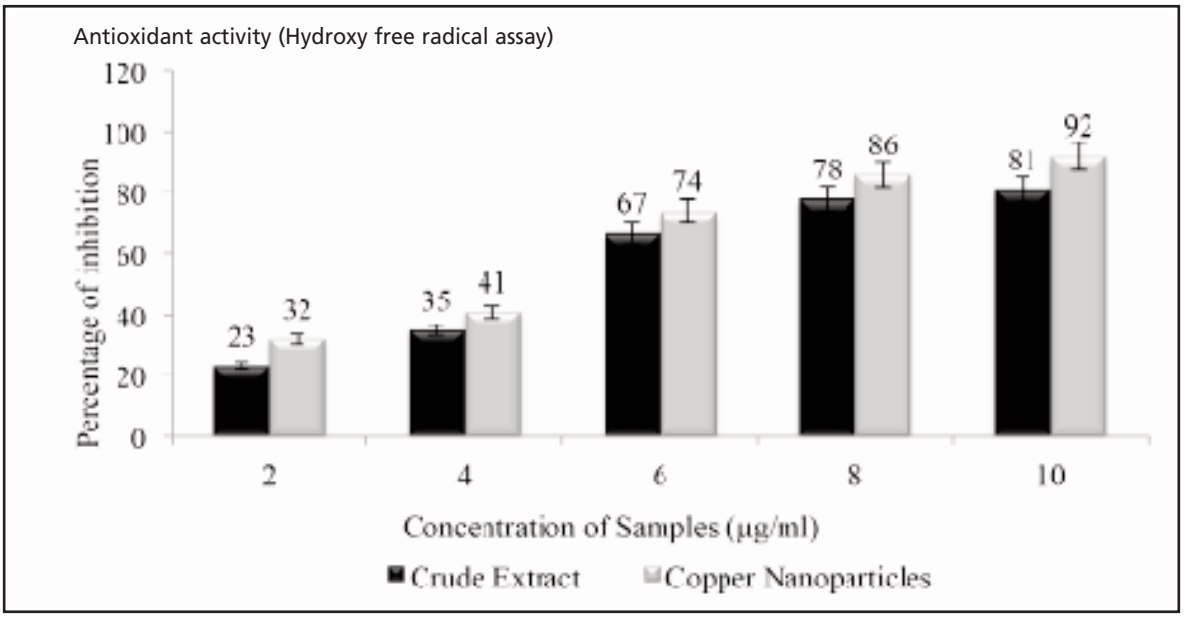


Fig. 2: Antioxidant activity in different concentrations.

copper nanoparticles of the leaf extract provide broad range antimicrobial activity against these pathogenic organisms (Figure 3).

Anti-Cancer activity (Cytotoxicity assay)

The *in vitro* cytotoxicity of the ethanolic crude extract and CNP was assessed against HeLa cervical cancer cell line at different concentrations. The samples demonstrated a high cytotoxicity against the HeLa cell line. The result indicated HeLa cells growth was significantly suppressed by ethanolic crude extract and CuNPs with an IC_{50} value of 170.6622µg/ml of the crude extract and 119.0805µg/ml of the nanoparticles. The percentage toxicity increased with higher concentration of CNP indicates that synthesized CNP

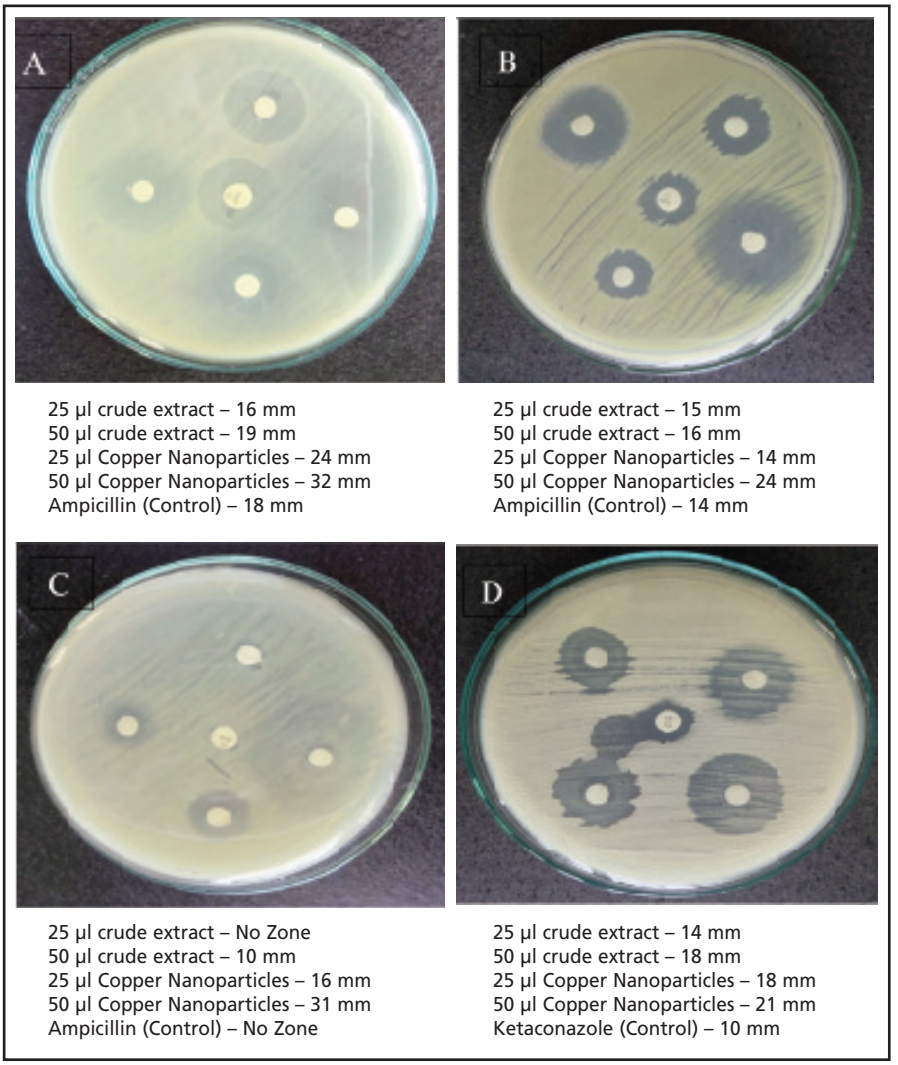


Fig. 3: Antimicrobial activity of crude extract and Copper nanoparticles against A) *Escherichia coli*, B) *Staphylococcus aureu*, C) Pseudomonas aeruginosa and D) *Candida albicans*.

could be of important use in medicine as anticancer agent. It is observed that viability of the cancer cells reduces with increased concentration of the samples whereas cytotoxicity against HeLa cell lines increases with the increased concentration of the samples (Figure 4 and 5).

DISCUSSION

Brassica oleracea var acephala has been studied for preliminary Phytochemical screening. The results of the present research finding showed that the ethanolic crude extract of the leaf was found to contain High Phytochemical activity hence its it is rich in Flavonoids, Phenolic compounds, Carbohydrates and Phytosterols. Plant-mediated produce of nanoparticles attracted very high interest because they are eco-friendly and low time consuming properties[9] Biological ways to synthesize nanoparticles with microorganisms, plants, and enzymes have many benefits than conventional physical and chemical methods.[10]

Nanoparticles are the basic element of Nanotechnology. The ethanolic extract was used to synthesise copper nanoparticles and the result showed that the copper nanoparticles was successfully synthesized from copper sulphate solution which was identified by the colour change from dark green colour of the extract. Thus the Brassica oleracea var acephala can be a good source for production of copper nanoparticles. Moreover, green synthesis of nanoparticles is another excellent technique, using methods of oxidation and reduction during the generation of nanoparticles.[12] Hence there is scope to develop new methods for the production of Copper nanoparticles. Nanoparticles were produced from all the parts of the plant separately like seed, stem, flower, leaf and skin of the fruits. The nanoparticles produced from plant extract were found to have medicinal benefits of plant extract and can be served as drug and cosmetic applications.[13]

The produced copper nanoparticles were characterized further using Scanning Electron Microscope (SEM) analysis. The SEM image displayed the high density nanoparticles produced from leaf extract and that the nanoparticles were crystals in shape. Nanotechnology is the study and development of materials at atomic, molecular and macromolecular scales that leads to manipulate the

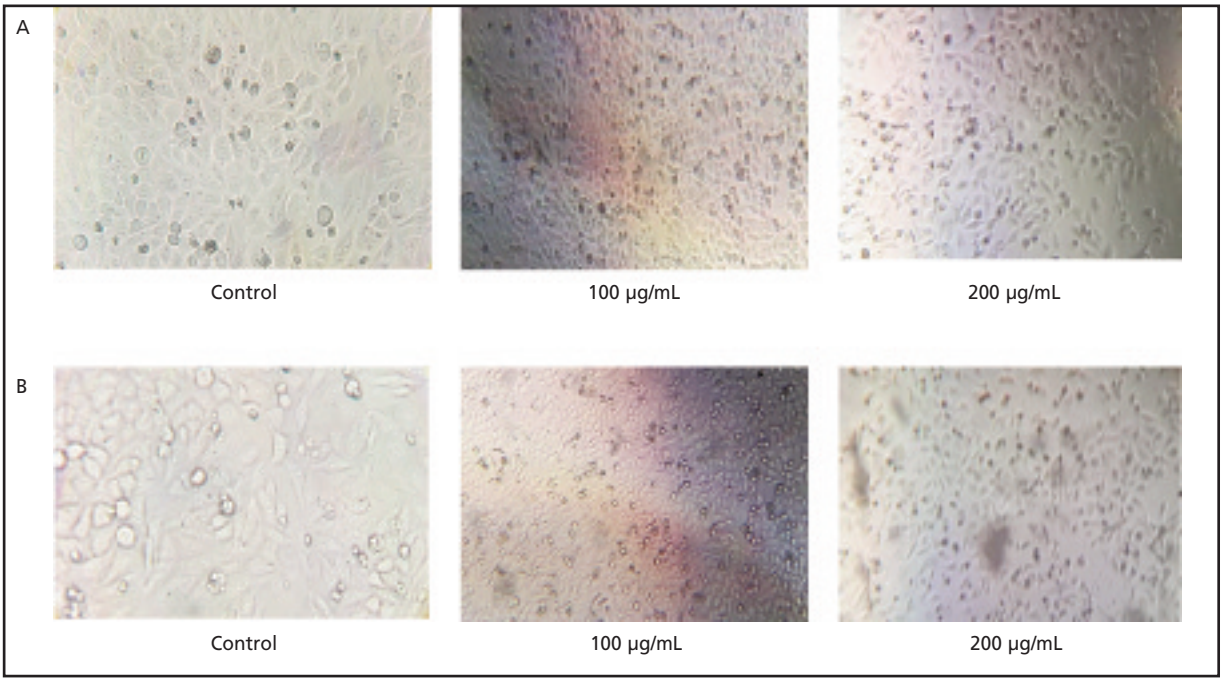


Fig. 4: Anti-cancer activity of A) ethanolic crude extract and B) Copper nanoparticles against the HeLa cell lines.

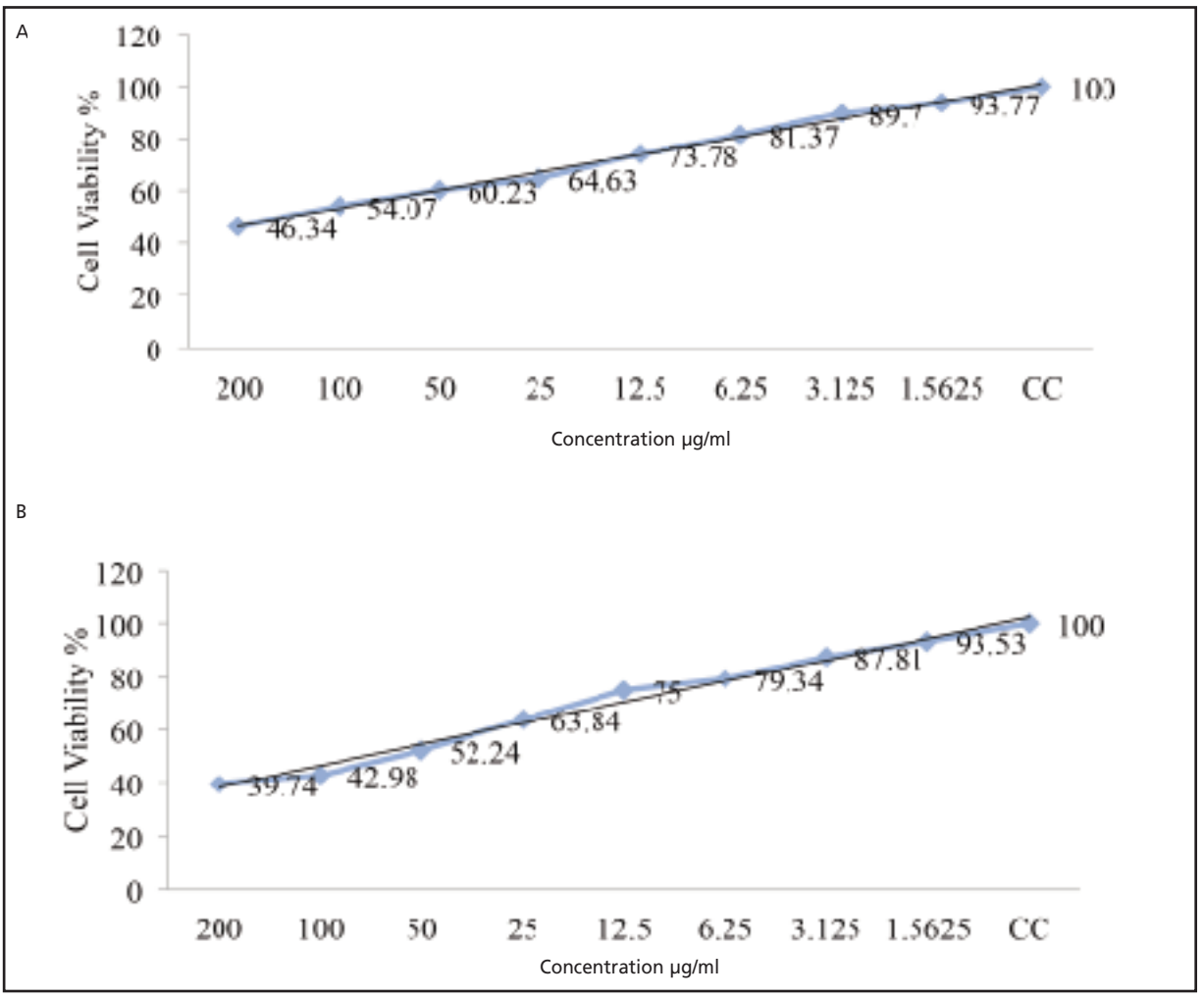


Fig. 5: Cell Viability of A) ethanolic crude extract and B) Copper nanoparticles against the HeLa cell lines.

structures and convert them to 1-100nm scales.[7] This technology deals with the synthesis of nanoparticles with controlled size, shape and disparity of materials at the nanometer scale length.[8] The copper nanoparticles bind to the leaf extract. *Brassica oleracea var acephala* also has shown the antimicrobial and antioxidant activity. This is in line with a recent finding of Singh et al., 2017, and Sikora et al., 2012.[31,32] A comparative study was done between ethanolic crude extract and nanoparticles. Both extracts exhibit zone of inhibition and better antioxidant potential. Copper has been applied since the ancient time. Its anti-bactericidal properties have been found to be higher compared to that of the highly expensive noble metals gold and silver hence, it provides good substitutes over Au and Ag in various chemical and metallurgical processes. Copper nanoparticles have lower potential for redox, and it is likely to oxidize when it is exposed to air. Hence, the synthesis of copper nanoparticles is difficult. They are normally produced via microwave assisted pylol method, hydrothermal method, thermal reduction, etc. But these ways are not cheap and use harsh organic solvents.[14] Therefore, environmentally good synthetic methodologies are preferable. Copper nanoparticles have many applications in heat transfer systems, durable materials, antimicrobial, sensors and catalysts. Because copper nanoparticles are very stable on a matrix and have disinfecting properties, they are used as a bactericide agent to coat clinical equipment. But in final the CuNPs shows major zone of inhibition and also showed more antioxidant activity. Fernandez et al., 2000 also found that copper nanoparticles synthesised from *Brassica oleracea var capitata* has antibacterial activity.[33]

Anticancer activity of Brassica oleracea var acephala against Cervical HeLa cell line was confirmed using ethanolic crude extract and copper nanoparticles. The results showed that HeLa cells proliferation was inhibited with increasing concentration of Ethanolic crude extract and Copper nanoparticles. From the results, it was seen that percentage viability of the cancer cells decreases with increased concentration of the samples whereas cytotoxicity against HeLa cell lines increases with the increased concentration of the samples. Thus Brassica oleracea var acephala possesses anticancer activity against HeLa cell lines. Another study by Hallmann et al., 2017 also proven anticancer activity of Brassica oleracea var capitata extract against gastric adenocarcinoma cells.[34]

CONCLUSION

B.oleracea var acephala has been studied for preliminary phytochemical screening. The results showed that the plant is rich in flavonoids, phenolic compounds, carbohydrates and phytosterols and is a good source for production of copper nanoparticles. The produced CNP from *B.oleracea var acephala* has shown antimicrobial, antioxidant, and anticancer activities.

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