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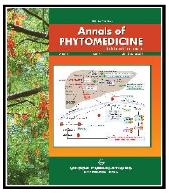

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Synthesis of copper nanoparticles from the leaf extract of *Psidium guajava* L. and its antioxidant, anticancer, antifungal activities

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Abstract

Over the past years, the field of nanotechnology has immensely evolved. It is no longer a single industry but now has developed to become a shared collection of materials and practices as it has created a larger impact on various industries such as chemicals, electronics, agriculture, medicine and space. The usage of nanoparticles in the medical industry has gained a lot of interest lately due to their promising properties making them an excellent focus of interest. Green synthesis of copper nanoparticles has shown to be advantageous as it is highly conductive and inexpensive than silver and gold. However, the downfall is the aggregation and oxidation of copper nanoparticles. The usage and selection of a suitable stabilizer for the capping of copper nanoparticles can overcome these problems. The present study reveals copper nanoparticles synthesized from leaf extract of *Psidium guajava* L., displayed antifungal efficacy against the pathogen *Candida albicans* with a 0.5 cm zone of inhibition. The synthesized copper nanoparticle shows anticancer activity in HeLa cell lines with 1000 µg/ml concentration of the nanoparticle. In future, the copper nanoparticles can be effective in the treatment of cancer and human pathogens.

1. Introduction

Nanoparticles are generated using eco-friendly and non-toxic chemicals in consonance with the principles of green chemistry. Some sources of nanoparticles have been animals, plants and microorganisms. Copper-based nanoparticles synthesized from various plant extracts have several applications; namely, antimicrobial and antifungal activity, DNA nanotechnology, as a catalyst to magnify chemical reactions. There has been a lot of work and research around the synthesis of copper-based nanoparticles. Several plant extracts such as *Magnolia kobu*, *Terminalia arjuna*, *Aloe vera*, *Bifurcaria bifurcate*, *Tabernaemontana divaricata, etc*., have been used to synthesise these nanoparticles (Ghosh *et. al.*, 2020). An evergreen edible fruit-bearing plant *P. guajava* from the family of Myrtaceae contains many phytochemicals (glycosides, terpenes, tannins and triterpenes). The plant leaves have been noted for their antibacterial activity and antimicrobial activity (Patil and Rane 2020). The leaf extracts of this plant are commonly used in traditional medicine. The fruit and leaf are commonly used for treating sicknesses including hypertension, dysentery, diabetes, skin disease and gastroenteritis. The leaf has high secondary metabolites with pharmacologic properties of antitumor, antiviral and anti-inflammatory activities. Flavonoids present in the leaf can inhibit the gram-positive and negative bacteria due to the presence of pinene and terpinene (Sathiyavimal *et al.,* 2021). Leaf extracts of *P. guajava* containing phytochemicals play a pivotal role in the formation of nanoparticles. Both the nanoparticles and extracts possess antimicrobial, antihyperglycemic and antidiabetic activities. The properties of nanoparticles are chemically stable and economically efficient in suitable dosages (Nagaraja *et al*., 2022). The second greatest cause of death is cancer globally. Because of its variability in tissue, it is tough to identify the specific location and thus making a major obstacle in treatment (Hemalatha *et al*., 2023). The WHO global cancer report 2020 mentions that there are more than 10 million mortality reports due to cancer disease. In those, 74% of them is due to prostate, lung, colon and cervical cancers. Even though, there are advanced technology in cancer treatment. It remains a major threat to human health (Swetha and Swamivel, 2023). Many numbers of human disorders and infections are extensively treated by herbal drugs prepared from various biological sources like leaves, roots, flowers, bark, and stem. The foundation for therapy in various time span of mankind is hugely dependent on medicinal plants (Pragyandip *et al.,* 2023)

The oil extracted from the plant leaves also possesses antibacterial and antifungal properties against *Staphylococcus mutants* and *C. albicans (*Alam *et al*., 2023). Lack of knowledge still prevails with the fruit and leaves medical properties of many plants *including P. guajava* and *S. torvum*. Large scale growing units is required to promote the market of increasing the quantity of these plants. Awareness on the health benefits of these plants should be promoted in the market in both domestic and international levels (Sahil *et al.,* 2023). In a study, copper nanoparticles show 50% inhibitory concentration against the HCT-116 cell line. The particles facilitated significant detachment, shrinking, membrane blebbing and distortion of cancer cells. Early apoptosis was also observed due to the dose of copper nanoparticles. *Ex vivo* and *in vivo* models are in need to

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validate these findings (Tabrez *et al*., 2022). Silk fibroin nanoparticles synthesized from guava are potential natural antioxidants for cosmeceuticals areas. The DPPH assay of the extract in the particle was conserved and the particle can significantly protect the plant extract from degradation at high temperatures (Pham *et al*., 2023)

2. Materials and Methods

2.1 Sample collection

The guava leaves were collected from Sattur, Tamil Nadu.

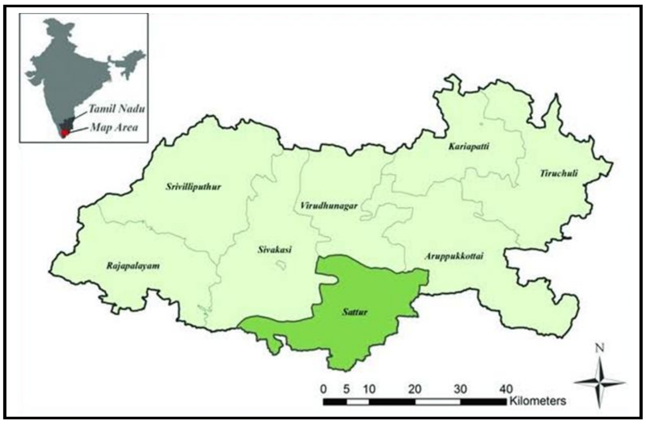


Figure 1: Geographical location of collected plant leaves.

2.2 Synthesis of copper nanoparticles from guava leaves

For synthesis of copper nanoparticles from the plant, guava leaves were collected and washed and cleaned with double distilled water left to air dry. The dried leaves were then crushed using a mortar and piston, and dried again under sunlight.

2.2.1 Preparation of leaf extract from *P. guajava*

For the extraction process, the obtained final dried product of the leaves (100 mg) was treated with 100 ml of ethanol using water bath extraction during the process. It was made sure that the temperature did not exceed the boiling point of the solvent. Whatman No.1 filter paper was used to percolate the extract and then concentrated in a vacuum at 40^0C using Rotary evaporation. The product obtained was then collected and stored in a dried and cleaned conical flask (Biswas *et al.,* 2013).

2.2.2 Preparation of 0.01M $CuSO_4$

0.15 g $CuSO_4$ was weighed and dissolved in 100 ml of sterile water in a sterile conical flask. It was prepared freshly for every time of use.

2.2.3 Synthesis of copper nanoparticles

For the synthesis of copper nanoparticles in a clean and dried conical flask, the reducing agent and the precursor were mixed in 1:1 proportion. 50 ml of filtered plant extract was taken along with 50 ml of freshly made 0.01 M $CuSO_4$ for the reduction of Cu ions. The resultant solution was then incubated for 1 h at room temperature to observe the colour change of the solution indicating the formation of the copper nanoparticle.

2.2.4 Purification of synthesized copper nanoparticles

After 1 h of incubation, the coloured solution thus obtained was centrifuged for 10 min (10,000 rpm). The supernatant was disposed of, and the obtained product was concentrated along with distilled water. The procedure of centrifugation was continued for several times to obtain pure copper nanoparticles.

2.3 Characterization of copper nanoparticles

The synthesized copper nanoparticle was further analyzed for its various properties like size, shape, chemical composition using techniques including UV-Vis spectra study, SEM, EDAX and XRD analysis.

2.3.1 UV-Visible spectra study

The reduction of pure copper to nanoparticles was determined by estimating the UV-Vis spectrum, by diluting a small quality of the sample in distilled water. A UV-Visible spectrophotometer was used for the respective analysis (340-600 nm).

2.3.2 Energy dispersive X-ray analysis (EDAX)

EDAX was used to determine the elemental composition of the sample. The pellet sample was used, and analyses were done at the Department of Nanoscience, Kalasalingam University, Srivilliputhur.

2.3.3 X ray diffraction (XRD) analysis

XRD analysis was carried out on fresh sample to assess the purity of the expected phases and the degree of crystallization, *i.e*., size, composition and crystal structure. The formation and quality of nanoparticles were checked by XRD technique. The colloidal suspension containing metal nanoparticle was dried on a small glass slide and was used for XRD pattern analysis.

2.3.4 Scanning electron microscope (SEM)

The filtrate embedded with copper nanoparticles was dried under a vacuum and subjected to SEM analysis. This study provides information about the surface of the nanoparticle and its internal structure. The SEM study was carried out in the sophisticated instrumentation center, Department of Nanoscience, Kalasalingam University, Srivilliputhur, Tamil Nadu.

2.4 *In vitro* antioxidant assay

The ethanolic extract of the plant was taken for the *in vitro* antioxidant assay.

2.4.1 Radical scavenging activity

The total radical scavenging capacity of the solvent extracts of *P. guajava* leaf was determined by using the DPPH assay.

2.4.2 Antifungal activity of copper nanoparticles using agar contact method

The agar contact method was used to analyse the hostile activities of synthesized nanoparticles. To evaluate antifungal activity wells were punctured in an LB plate using Cork barer which was sealed with an overnight culture of *C. albicans* (Chalandar *et al.,* 2017). About 20-100 µl of synthesized copper nanoparticles and ethanol leaf extract (positive control) were added to the well. Incubated at 28^0C for 16 h and antifungal activity was determined by measuring the zone of inhibition (*C. albicans*).

2.5 Anticancer activity of synthesized copper nanoparticles

The synthesized copper nanoparticles was verified for the anticancer activity using MTT assay.

2.5.1 Cell line culture

HeLa cell lines were obtained from Veterinary College, Vepery. Thus, the obtained cells were perpetuated in a suitable growing media (Minimal Essential Medium). The media was amplified with 10% FBS, penicillin (100 U/ml), and streptomycin (100 ìg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37°C.

2.5.2 Reagents

The following reagents were purchased from the respective places: Hi Media Laboratories, Cistron Laboratories, Sigma Aldrich Mumbai.

2.5.3 *In vitro* assay for anticancer activity (MTT assay)

To perform the *in vitro* assay 1×10^5 cells/well were plated in microtiter plated (24-wells) incubated for 24 h at 37°C amidst 5% CO_2 condition. The synthesized copper nanoparticles samples were added to the incubated plate in different concentrations and re-incubated for another 24 h. Post incubation, the respective well containing the sample and the cells were washed with PBS (pH 7.4) or MEM without serum. 100 µl/well (5 mg/ml) of 0.5% MTT was added and incubated for 4 h. Following 4 h of incubation, 1 ml of DMSO was added. UV Spectrophotometer was used for reading the absorbance at 570 nm where DMSO served as the blank.

3. Results

3.1 Synthesis of copper nanoparticles from guava leaves

Excitation of surface plasmon vibration in nanoparticles, lead to the colour change exhibiting light green to bluish colour and the colour change which was observed after one hour of incubation (Figure 2), indicated the reduction of Cu ions. Hence, this confirmed the reduction of copper sulphate using *P. guajava* leaf extract.

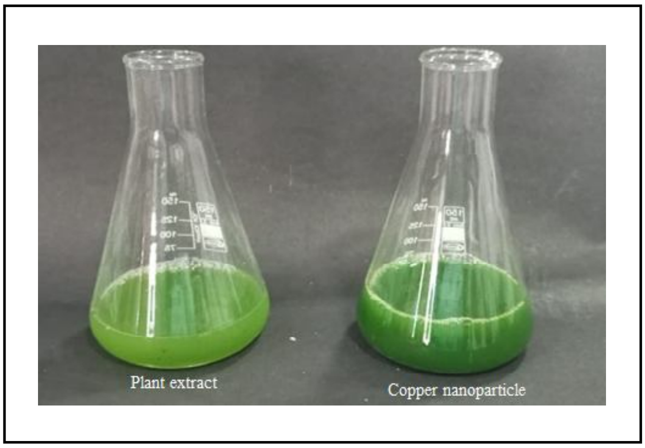


Figure 2: Synthesis of copper nanoparticles from guava leaves.

3.2 Characterization of copper nanoparticles

The results of copper nanoparticles characterized using UV-Vis spectrometer, SEM, EDAX and XRD analysis are found below.

3.2.1 UV-Vis spectra analysis

UV-Vis spectra recorded the reaction solution of reduced copper sulphate in the leaf extract of *P. guajava* (Figure 3). The absorption spectra were recorded at the 340-600 nm wavelengths where a peak was observed at 570 nm corresponding to the SPR of copper nanoparticles.

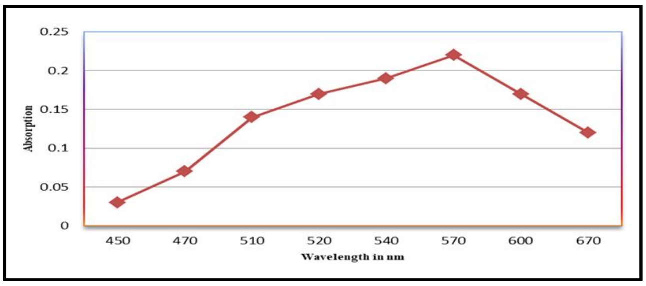


Figure 3: UV-Vis absorption spectrum of nanoparticles synthe-sized by using *P. guajava* leaf extract.

3.2.2 SEM analysis

SEM analysis was employed to visualize the size and shape of copper nanoparticles. The typical SEM image (Figure 4) displays the product mainly comprised of particle-like Cu nanoclusters with all-around sizes ranging from 150 to 200 nm. However, further observation with high magnification revealed that smaller nanoparticles were responsible for the assembly of these Cu nanoclusters, which exhibit good uniformity and the average diameter is about 40 nm. The average size of these nanoparticles is about 40-45 nm.

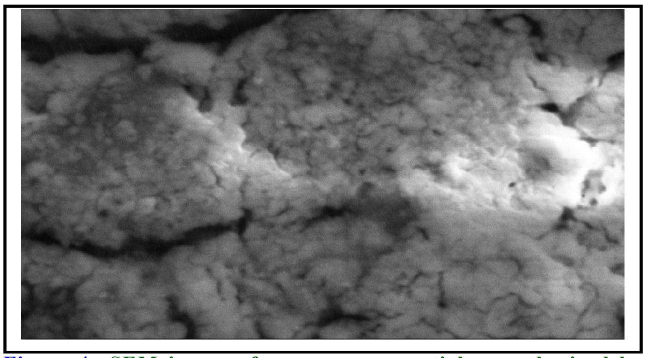


Figure 4: SEM image of copper nanoparticles synthesized by using *P. guajava* leaf extract.

3.2.3 EDAX study

The peak generated by the EDAX analysis when used to analyse the sample confirmed the presence of cu nanoparticle. Figure 5 depicts a graphical representation of the peaks generated, where the y-axis exhibits X-ray counts and the x-axis exhibits energy in Kev.

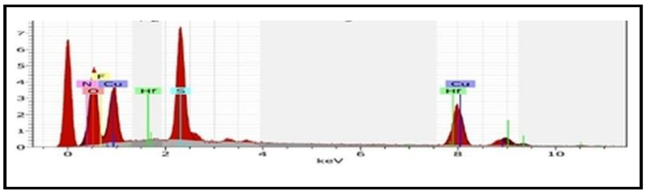


Figure 5: EDAX analyses of copper nanoparticles synthesized by using *P. guajava* leaf extract.

3.2.4 XRD analysis

The presence of these cu nanoparticles was validated by the presence of characteristic pinnacle observed in the XRD image (Figure 6). The whole spectrum in the image displayed intense peaks ranging from 14 to 16 nm. This revealed and confirmed the presence of copper nanoparticles in the sample.

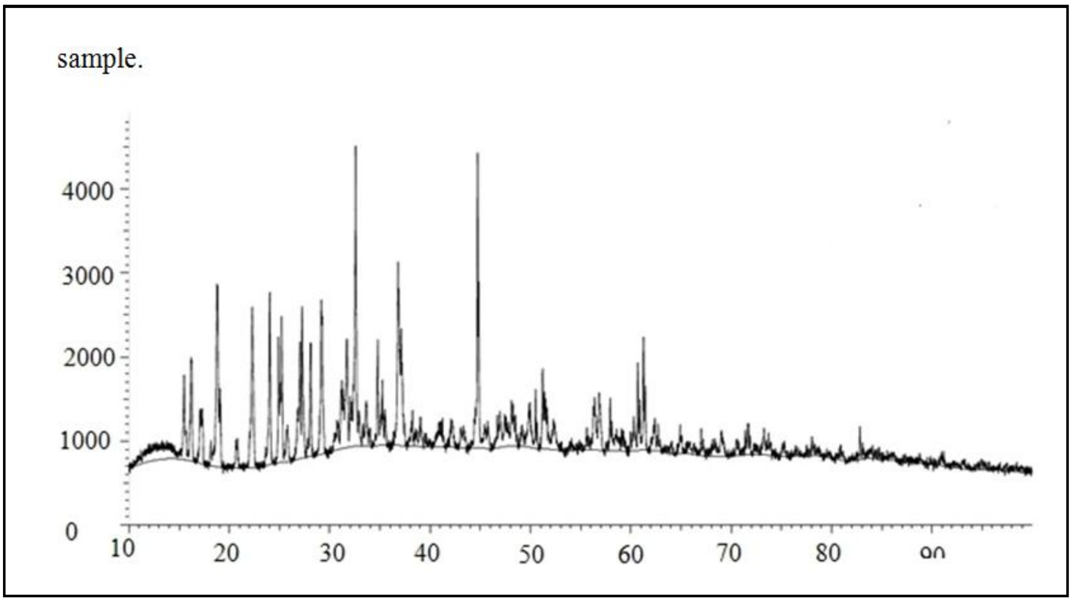


Figure 6: XRD analyses of copper nanoparticles synthesized by using *P. guajava* extract.

3.2.5 Antioxidant activity

The antioxidant activity was carried out, and the ethanolic extracts of *P. guajava* leaf were evaluated by DPPH assay. 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 mg/ml concentrations then were subjected to each plant extract. The average value and standard deviation were calculated by using triplicate values (Figure 7).

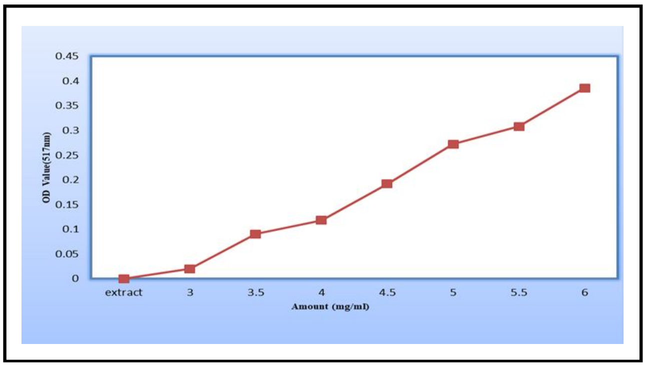


Figure 7: Antioxidant activities of synthesized copper nanoparticles.

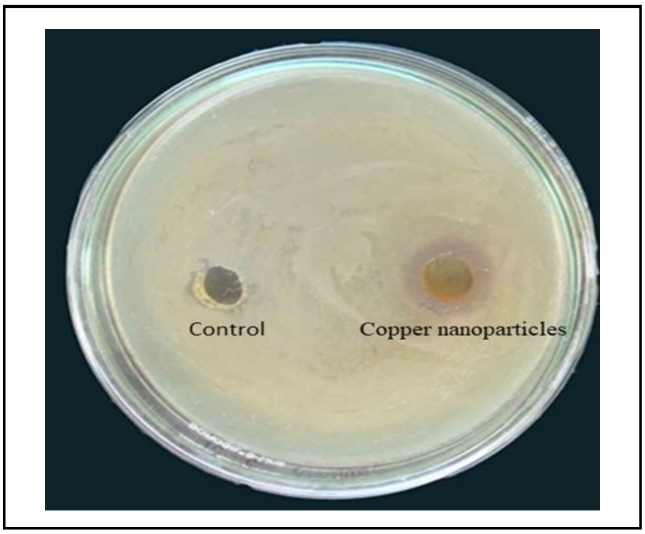


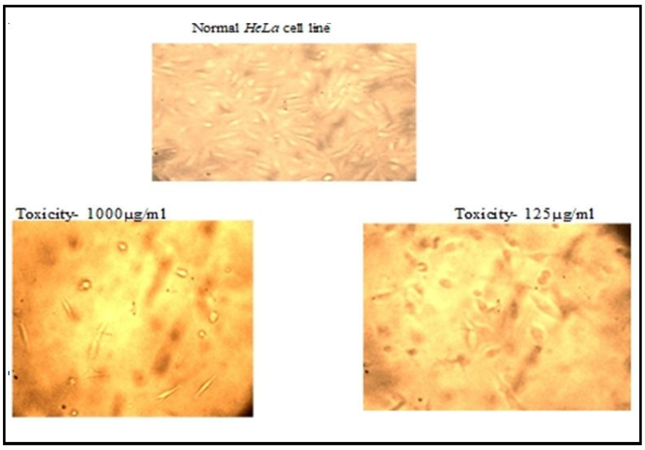
Figure 8: Antifungal activities of copper nanoparticles synthesized in the extract of *P. guajava* against *C. albicans* in agar well diffusion assay.

3.3 Antifungal activity of copper nanoparticle

In vitro assay was performed on the fungal isolates using synthesized copper nanoparticles to identify the antifungal effects of copper nanoparticles. The green synthesized copper nanoparticles showed the inhibitory effects against *C. albicans* with a zone of inhibition of 0.5 cm after 24 h of incubation (Figure 8).

3.4 Anticancer activity

The anticancerous activity of ethanol extract of *P. guajava* is represented in the table and images. The tables showed the minimum concentration (62.5 µg) of *P. guajava* has effective toxicity against cervical cancer. In the image analysis, 1000 ug/ml concentrations show an effective result compared with other concentrations of *P. guajava*.



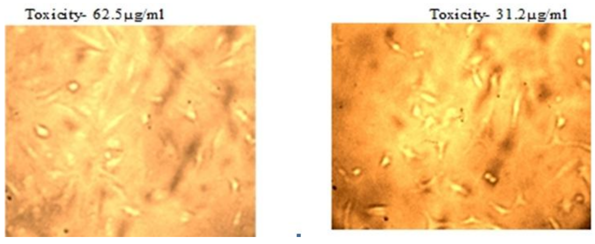


Figure 9: Anticancer effect of copper nanoparticles in HeLa cell line.

Table 1: Anticancer effect of copper nanoparticle *in* HeLa cell line.

Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1000	Neat	0.11	20.00
500	1:1	0.15	27.27
250	1:2	0.20	36.36
125	1:4	0.24	43.63
62.5	1:8	0.29	52.72
31.2	1:16	0.35	63.63
15.6	1:32	0.41	74.54
7.8	1:64	0.47	85.45
Cell control	-	0.55	100

4. Discussion

The change in the developed pigment from pale green to dark brown is considered the prefatory confirmation for the synthesis of nanoparticles. The change in the colour is due to the surface plasmon resonance induced by reducing agent (Nagaraja *et al.,* 2022). EDAX analysis was recorded from the synthesized nanoparticles with the existence of copper with peak absorption range and the presence of other elements might be due to the radiation of the X-ray from the enzyme and protein of the ethanolic leaf extract. The synthesis of nanoparticles was also confirmed using UV Vis spectroscopy with a peak at 570 nm in the present study (Shanmugapriya *et al*., 2022). Reduction in copper ions to copper nanoparticles was indicated due to enhanced surface plasmon absorption peak between 560 nm to 600 nm. The maximum absorption was at 574 nm (Muhammad *et al.,* 2023). The present study confirms the surface plasmon absorption peak at 570 nm.

The synthesized copper nanoparticles have shown to have greater antioxidant potential in accordance with DPPH free radical scavenging assay. The potential of antioxidant activity has increased in 62% concentration-dependent of copper nanoparticles (Ssekatawa *et al*., 2022). In the present study, increased plant extract concentration has increased antioxidant activity.

In a study by Melkamu and Feleke (2022), leaf extract and copper nanoparticles alone were studied for antibacterial activity against some gram-negative and gram-positive bacteria. In the present study also, the leaf extract and copper nanoparticles were verified for antifungal activity against *C. albicans*. Antifungal activity was evaluated using the agar contact method against *C. albicans*. 10 µl of copper nanoparticles were added to the well and a zone of inhibition was observed after incubation. In the present study, 100 µl of copper nanoparticles showed inhibition against *C. albicans* with a zone of inhibition of 0.5 cm (Tahir *et al.,* 2022). There were many studies in which the cell viability decreased with a dose of copper nanoparticles in the colorectal cancer cell lines. The cell viability percentage has decreased with the increased dose of copper nanoparticles (Aboeita, *et al*., 2022). These studies suggest that the increasing concentration of our ethanolic leaf extract synthesized nanoparticles has a detrimental effect on the viability of cervical cancer cell lines.

5. Conclusion

A well known tropic tree *P. guajava* is a common plant prescribed even by doctors and physicians due to its effectiveness against gastroenteritis, diabetes, diarrhea and oral ulcers. Antibacterial and antifungal agents are commonly seen in the leaf of *P. guajava*. The plant also contains many phenolic compounds that are effective in treating cancerous cells and ageing. The ethanolic leaf extract of the plant has high levels of anticancer, antioxidant and hypoglycemic activities. Even though, there are numerous anticancer studies from the leaf extract of the plant. The present study, investigates the anticancer activity of nanoparticles synthesized from the leaf extract of *P. guajava*. The present study concludes that both the leaf extract and the nanoparticles possess multiple medicinal properties which need to be developed further for wide range of applications in the field of medicine. Identification and isolation of new chemical components from the plant will be the key research area in future studies.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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