Journal for ReAttach Therapy and Developmental Diversities
eISSN: 2589-7799
2023 July; 6(8s): 1059-1066

# Cytotoxic effect of nutmeg oleoresins assisted zinc oxide nanoparticles using breast cancer cell line

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**ABSTRACT :**

Cancer is a leading cause of death globally. Though there are different treatments available for cancer, the side effects associated are sometimes worse than the disease itself. Hence there is a constant search for new drug candidates to treat the condition and reduce the side effects. Many plant mediated nanoparticles are studied extensively and proved for their anticancer activity.Nutmeg is known for its diuretic, an antidepressant and memory-enhancing properties. It also exhibits great anti-oxidative capacity due to its polyphenolic compounds which may be used for many other uses of this plant. The aim of the present study was to evaluate the cytotoxic activity of nutmeg oleoresin mediated Zinc oxide nanoparticles against breast cancer cell lines. In the present study, nutmeg oleoresin mediated ZnO nanoparticles were prepared initially and confirmed by measuring the absorbance between 350 nm to 700 nm using UV- Visible spectroscopy . It was further evaluated for its anticancer activity on breast cancer cell line . The Cells were treated with different concentrations (10, 20, 40, 60, 80, 100 μg/ml) for 24hrs. The zinc nanoparticles prepared from nutmeg oleoresin showed good cytotoxic activity. The percentage viability was reduced with increase in concentration of nutmeg oleoresin mediated ZnO nanoparticle.The 50% of inhibition was observed at a concentration of 40 μg/ml and was considered as the IC50 value . The present study concluded the dose dependent nutmeg oleoresin mediated zinc oxide nanoparticles on breast cancer cell line. Hence, nutmeg oleoresin mediated zinc nanoparticles may be recommended for its use as an anticancer drug after further invivo studies.

**Keywords**: nutmeg oleoresin; green synthesis; breast cancer cell line; zinc nanoparticles, environment friendly

## INTRODUCTION :

Cancer is a leading cause of death globally. Though there are different treatments available for cancer, the side effects associated are sometimes worse than the disease itself[1]. Hence there is a constant search for new drug candidates to treat the condition and reduce the side effects. Breast cancer is the second most dangerous cancer in the world and the first most prevalent cancer for women[2]. According to the World Health Organization, there were 18.1 million new cases reported and 9.6 million deaths in 2019 and expected it can reach upto 30 million in 2040[3]. Cancer, as one of the most serious illnesses, for a systematic approach for prevention and recovery. Both environmental and lifestyle modifications are also responsible for cancer which include hormonal therapy, ionizing radiation, reproductive behaviours of women, and also alcohol consumption, and various dietary factors, obesity, and mainly lack of physical activity[4]. More than 75% of breast cancer cases are diagnosed in women whose ages range more than 45 years, and the majority of cases are from developed countries[5,6]. Both genetic and environmental factors, those co existing, also can increase the risk of morbidity and reactivation of mammary gland cancer. Histopathology studies breast cancer which is a heterogeneous disease that has both inter and intra tumour variability[7]. A useful model for the investigation of characteristics such as cellular regulation, uncontrollable cell proliferation, limited inhibitor concentration and cell death evasion is breast cancer cell[8].

Plants are being explored for cytotoxic, anti-diabetic, antimicrobial and anti-inflammatory properties[9–11]. Indian spice is explored and also contributes to food as both aroma and taste. Indian spices such as turmeric, nutmeg, ginger, mint, etc. have been shown to treat diseases that range from colds, cough, and also for cancer. Nutmeg is naturally obtained by the solvent extraction of seeds of *Myristica fragrans*. The product gives the characteristic aroma of nutmeg, with a pleasant, warm taste[12]. It is a semi-solid, waxy resin with colours ranging from off white to light orange. Advances in molecular research and in genetic sequencing are further recorded with next-generation DNA sequencing[13,14]. Nanoparticles are the smallest solid particles with a measurement of about 1 - 100 nm. These nanoparticles have a different variety of uses in this modern world in almost every field sector such as in agriculture, medicinal, and technological fields[15,16]. Nanotechnology is one of the evolving fields among other novel technologies in the medical field. Nanotechnology has the property of manipulating materials at the atomic and molecular levels[5,6,17]. Patients with LABC must be treated with combination therapy using systematic and loco-regional methods and involve a well-coordinated clinical program and tight collaboration between medical, surgical and radiation oncologists[18,19]. Nanotechnology is the basis of many main technology innovations in this 21st century[20,21]. Research and its development in the field is growing rapidly throughout the entire world[22]. A major output of this activity is the development of new materials in the manometer scale, particular materials with at least one dimension less than 100 nm. Common uses of nutmeg include its application as a diuretic, an antidepressant and a memory-enhancing compound[23]. Also, it has been shown to exhibit great anti-oxidative capacity due to its polyphenolic compounds and further anti-diabetic and antimutagenic effects[24,25]. Secondary metabolites from different kinds of plants and microbes can be effective cancer drugs[26,27]. Many plant chemical substances are highly toxic to cancer cells which give impacts on living systems as well as on other animals, and are therefore capable of becoming medicines[28,29].

Synthesis of zinc nanoparticles using nutmeg oleoresin suggests that it is a better alternative to chemical synthesis as it is simple and eco-friendly[30]. The stable zinc nanoparticles are synthesised from nutmeg oleoresin with an average size ranging from 20-50 nm[31]. There is a wide use of eugenol for different kinds of purposes such as household products, fragrance in soaps and cosmetics, skin care products, flavouring substances for food, and also dental and pharmaceutical products[32,33]. The assessment of the antimicrobial activity was based on the measurement of the diameter of the inhibition zone formed around the well and the mean values are recorded[34][35,36]. It is used for the treatment of measles, haemorrhoids, toothaches, worms, and joint pain, as well as infections caused by bacteria and fungus[37,38]. Some breast-feeding women use nutmeg to increase milk flow[39,40]. In foods, nutmeg is used as a culinary spice and to prevent food poisoning[41,42]. Previously, our team had conducted numerous studies which included other oleoresins and plant extracts[43,44]. Our team has extensive knowledge and research experience that has translate into high quality publications[45]

The present study was to evaluate the cytotoxic effect of nutmeg oleoresin mediated Zinc oxide nanoparticles against breast cancer cell line

## MATERIALS AND METHODS :

**Study Setting:** The study was conducted in the Cancer and Stem Cell Laboratory , Saveetha Dental College, Chennai, India. Ethical clearance number IHEC/SDC/UG-1969/21/123
The pros of this study were that the nutmeg oleoresin and Zinc sulphate was used as a powder which can make it easier for measuring and for its applications. The cons of this study were that it can have minor errors when a micropipette was used which can not be easily corrected. It also can be done on many culture test tubes and there may be the presence of more bacterial and fungal organisms which can give negative results. The sampling method used here is random. Internal validity criteria for this research is a validation of procedure done by nano researchers and External validity criteria can be the validation.

**Preparation of nutmeg oleoresin:** The nutmeg oleoresin extract was collected from Synthetic Industries Pvt Limited ,Kerala , with a product code - 4010000484. 1g of nutmeg oleoresin was dissolved in 100ml of distilled water and was taken in a conical flask. The temperature of the heating mantle was set to about 50 - 60 degree celsius and the time taken to heat is about 5 - 10 minutes.

**Preparation of nanoparticles:** 0.477g of 20mM of zinc sulphate was measured and was dissolved in 100ml of distilled water. 99.5ml of the solution was taken in a conical flask and mixed with 0. 5ml of nutmeg oleoresin solution. This new solution was kept in an orbital shaker, a magnetic stirrer for the formation of nanoparticles. The change in colour was visualised and photographs were taken. The synthesised nutmeg oleoresin mediated zinc oxide nanoparticles were confirmed by UV-Visible spectroscopy. The peak value of UV spectroscopy of the extract sample was found to be 290 nm.

The solution was then centrifuged using a Lark refrigerated centrifuge at 8000rpm for 15 minutes and the pellets were collected and washed with distilled water. The final purified pellets were collected and dried at 60 degree Celsius and stored in an airtight Eppendorf tube.

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2023 July; 6(8s): 1059-1066

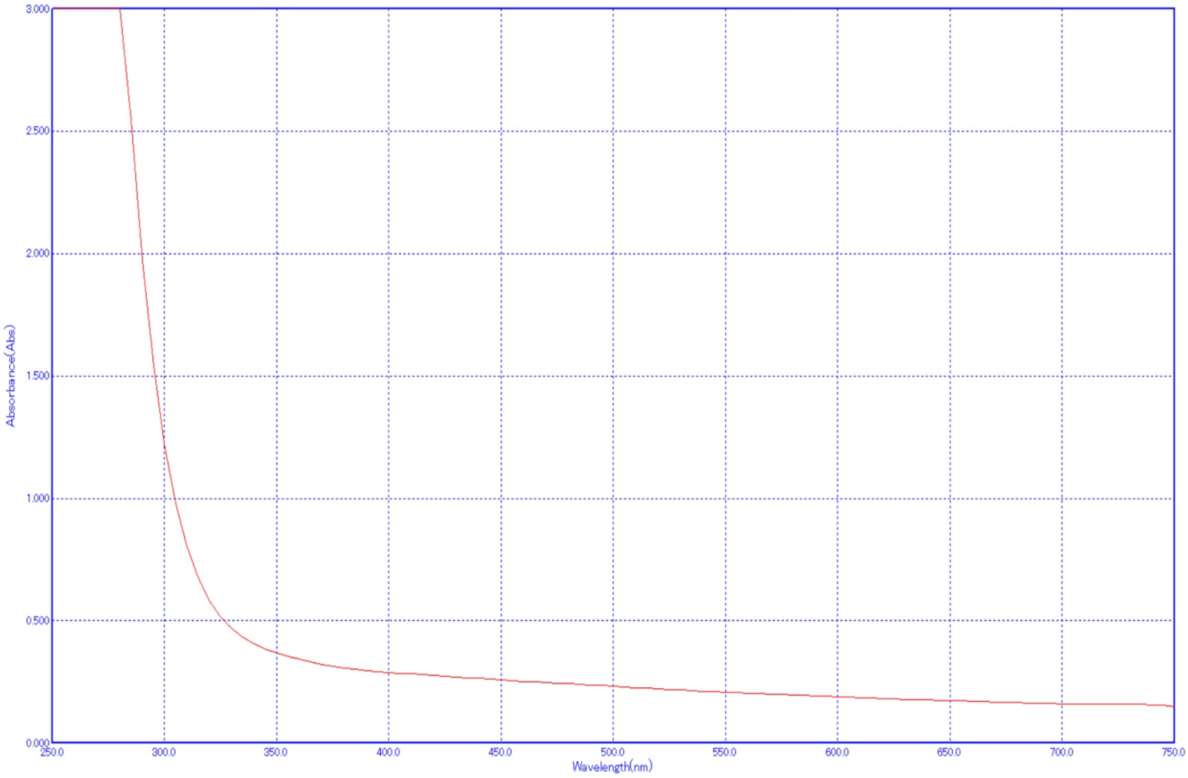


**Figure 1: The graph represents the UV spectroscopy of the sample. The peak value was found to be 290 nm. X axis denotes wavelength (nm). Y axis denotes Absorbance(Abs)**

**Chemicals:** DMEM medium, 0.25% Trypsin-EDTA solution, sodium bicarbonate solution, bovine serum albumin (BSA), low melting agarose, MTT from Sigma Chemicals Co., St. Louis, USA. fetal bovine serum (FBS) and antibiotic/antimycotic solution, DMSO were from Himedia, Sodium phosphate monobasic and dibasic, sodium chloride, sodium hydroxide, sodium carbonate, hydrochloric acid and methanol were purchased from Sisco Research Laboratories (SRL) India.

**Cell culture reagents:**
**DMEM:**
Commercially available DMEM contains 7.5% sodium bicarbonate solution. To 500ml of DMEM, 5ml of penicillin/streptomycin solution and 0.5ml of amphotericin B solution was added. Then the medium was sterile filtered (0.22µ) inside the hood. The medium was then dispensed into sterile containers and stored at 4°C.

**Growth Medium (DMEM with 10% FBS):**10ml of FBS was made up to 100ml using sterile DMEM. It was stored in a sterile container in cool and aseptic condition.

**Phosphate Buffered Saline (PBS; pH 7.4):** 0.63 g of sodium phosphate mono basic (NaH2PO4), 0.17 g of sodium phosphate dibasic (Na2HPO4) and 4.5 g of sodium chloride (NaCl) were dissolved in 500 ml of double autoclaved milliQ water. The pH was then adjusted to 7.4 using 1 N HCl and 1 N NaOH, sterile filtered (0.22 µ) and then stored in a sterile container.

**Trypsin-EDTA versus Glucose Solution:** Trypsin was purchased as 1 x with EDTA (0.5% trypsin, 5.3 mM EDTA sodium salt). . 0.89% Physiological Saline, 890 mg of sodium chloride was dissolved in 100 ml of double autoclaved milliQ water.

**Cell line:** Human breast carcinoma-MCF-7 and MDA-MB-231 cell line was procured from the National Centre for Cell Science (NCCS, Pune), India. The cells were grown in T255 culture flasks containing DMEM medium supplemented with 10% FBS.

**MTT ASSAY:** The cytotoxic effect of nutmeg oleoresin on zinc oxide nanoparticles, were carried out by MTT (3-(4, 5-dimethyl thiazol-2 yl )-2, 5-diphenyl tetrazolium bromide) assay. Further, the viability of breast cancer cells upon drug treatment was assessed by a trypan blue exclusion test. Then the cells were seeded in 96-well plates at the density of

5X103/100µl, after 24hrs cells were treated with different concentrations (10, 20, 40, 60, 80, 100 µg/ml) of nutmeg oleoresin . After incubation, 40 µl of 5 mg/ml MTT stock solution was added to each well and incubated for 3h at 39 °C. The obtained formazan crystals were then solubilized with DMSO and the cell viability was measured at 570 nm using a microplate reader. Cell viability (%) has been shown as a ratio of absorbance in treated cells to absorbance in control cells (0.1 % DMSO). The IC50 was found to be 40µl as the concentration of the sample needed to reduce 50 % of the absorbance in comparison to the control.

Based on MTT assay we selected the IC50 value of nutmeg oleoresin for further studies. The characterisation of morphological changes in breast cancer cells before and after treatment with nutmeg oleoresin were observed under phase contrast microscope. All data obtained were analyzed and computed statistically (SPSS/17 Software Package; SPSS Inc., Chicago, IL, USA) using one-way ANOVA. Post-hoc testing was performed for inter comparisons using the LSD. In each and every test, the level of statistical significance was set at $p<0.05$.

**RESULT AND DISCUSSION:**

The percentage of cell viability and concentration of nutmeg oleoresin Zinc oxide nanoparticles ( Figure 2). The cytotoxic effects of nutmeg oleoresin on MCF-7 cells was determined by MTT assay. The Cells were treated with different concentrations (10, 20, 40, 60, 80, 100 µg/ml) for 24hrs. The 50% of inhibition observed in concentration of 40 µg/ml, which has been taken as IC50 value.Data are shown as means ± SD (n = 3). * compared with the control-blank group, $p < 0.001$.

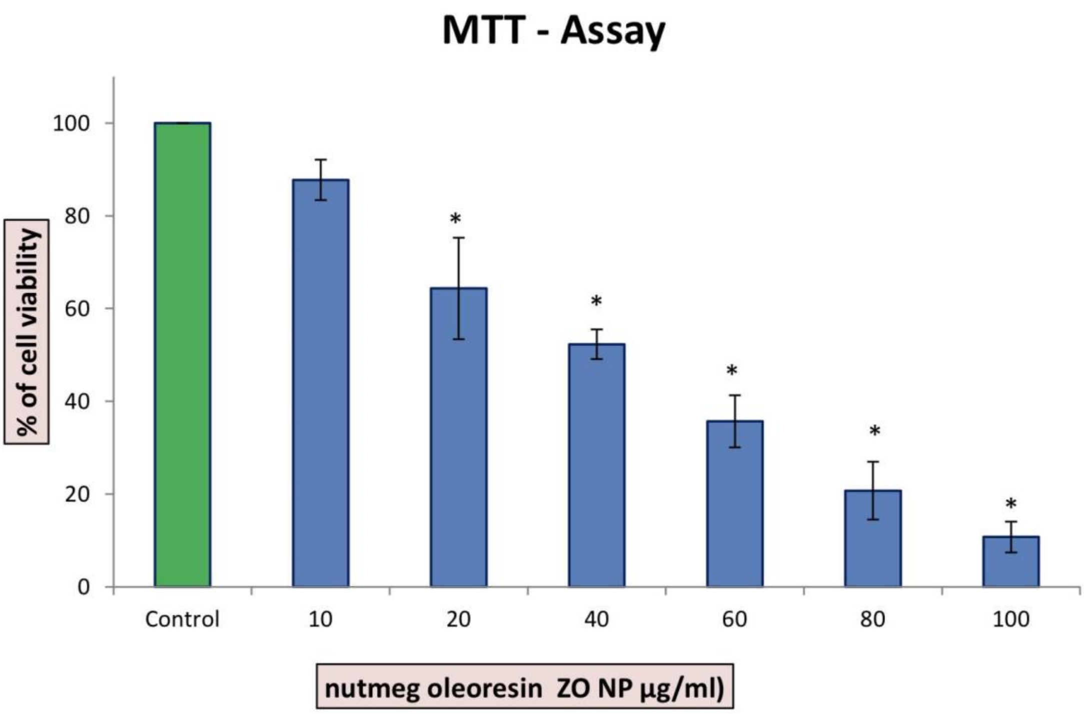


**Figure 2: Anticancer activity of nutmeg oleoresin Zinc oxide nanoparticles.**

X -axis shows the concentration of nutmeg oleoresin mediated Zinc oxide nanoparticles where green color is the control and blue color is the nanoparticles used in the study and Y-axis represents the percentage of cell viability . The cytotoxic effects of nutmeg oleoresin on MCF-7 cells was determined by MTT assay. The Cells were treated with different concentrations (10, 20, 40, 60, 80, 100 µg/ml) for 24hrs. The 50% of inhibition observed in concentration of 40 µg/ml, which has been taken as IC50 value.Data are shown as means ± SD (n = 3). * compared with the control-blank group, $p < 0.001$.

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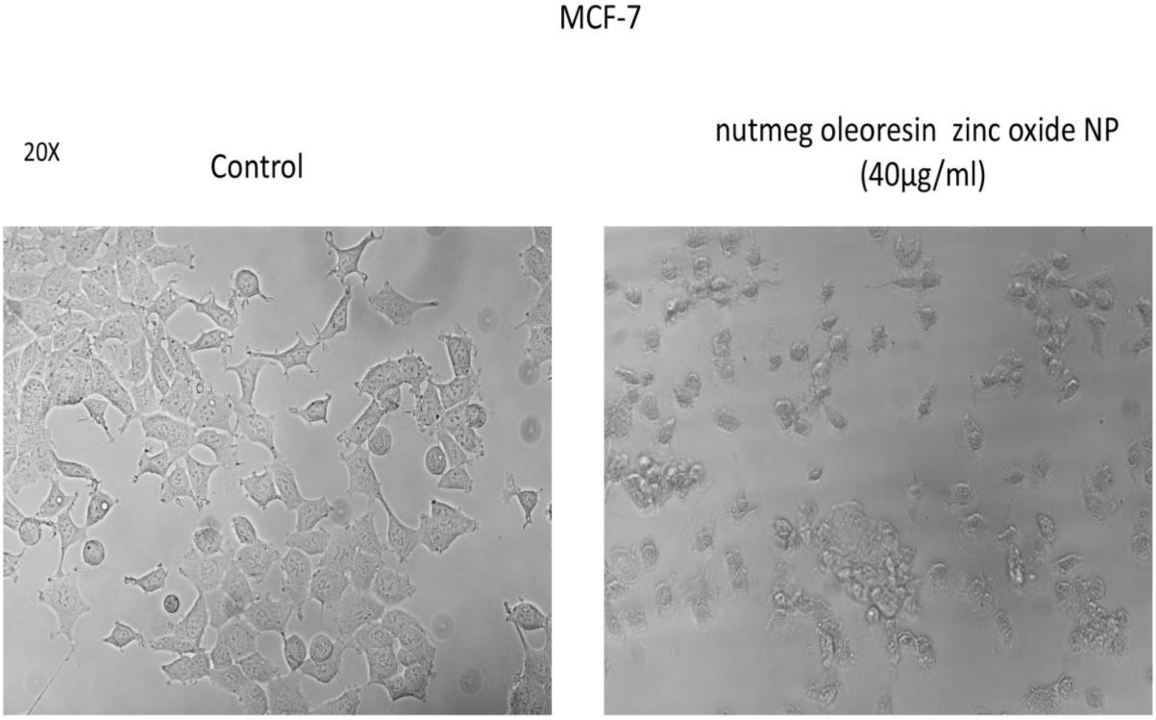


**Figure 3: Anticancer activity of zinc oxide nanoparticles**

Represents the morphological changes in breast cancer cell line upon treatment with nutmeg oleoresin mediated zinc oxide nanoparticles at 40µg/mL for 24hrs under phase contrast microscope at 20x magnification.

The nutmeg oleoresin caused a dose dependent increase in cytotoxicity in the MCF-7 breast cancer cell line. The morphological representation of breast cancer cells upon treatment with nutmeg oleoresin extract for 24 hrs compared to control cells is seen in Figure 3. The results clearly indicate that the findings were clearly cytotoxic to breast cancer cells at a specific concentration and incubation time. It shows that nearly half of the viable cells were killed at the concentration ($IC_{50}$) of 40 µg/ml. At 40 µg/ml, only 50% of the cells are viable . Thus, it can be said that as the drug concentration increases, the viable cells decrease. It proves that nutmeg oleoresin possesses a strong cytotoxic effect against MCF-7 assisted zinc oxide nanoparticles using breast cancer cells.

Cytotoxicity studies mainly indicate safety and benefits of nanoparticles to medicine are numerous. Since nutmeg is a very commonly used herb almost everywhere, this study can be carried out with low cost and more benefits. It can be also used in the field of Nano medicine for the upcoming future generations. As far as the concentration of the nanoparticles, the toxicity remained the same apwhich didn't affect the nauplius. Hence from the current study, it can be seen that the moderate concentration for biomedical applications. A similar study conducted previously by Apurba Sarker Apu, et al, reported that it was aimed to synthesise copper nanoparticles from the aqueous extracts of the aerial part of Boerhavia diffusa Linn. (B. diffusa) and to assess their cytotoxicity, antioxidants, and to investigate the bioactivities of crude n-hexane. , Gruener et al, reported a study that was aimed to synthesise copper nanoparticles from the aqueous extract of Fulcaria vulgaris leaf and to assess their cytotoxicity, antioxidant, antifungal, antibacterial, and cutaneous wound healing properties. Study by Subramanian Raghunandhakumar shows that uncontrolled cell proliferation was assessed by specific cell proliferative markers. Another study by Raghunandhakumar, Subramanian suggests that thymoquinone could suppress cell growth and cell survival via arresting the cell-cycle in the G2/M phase and inducing apoptosis of neuroblastoma cells.

In the present study, the ZnO-NPs that were synthesized using nutmeg oleoresins displayed a clear change in colour from light blue to greenish blue within 24 hours and did not undergo any further change in colour indicating the synthesis process is complete.This visual observation of change in colour indicates the complete formation of nanoparticles. Previous studies have reported the change in colour during the synthesis process of metal nanoparticles synthesized using Zingiber officinale. Medicinal plants are contested as an alternative drug for cancer prevention and/curation in many nations around the world. A greater therapeutic effect of the above plant derived active compounds will be found to be an effective anti cancer drug against cancer cells. Plants also have the opportunity to supply newer therapies, making them a potentially rich source of novel anticancer substances. Recently, numerous treatments have been proposed for cancer, which use plant-derived materials. Any herbs and plants include chemicals that influence human physiology, and these bioactive compounds include tannins, sugars, hormones, and flavonoids. Another study by Selvamani Asokkumar concludes that overall findings suggested that the ION effectively ameliorated lung carcinogenesis, which is attributed to the anti proliferative and antioxidant potential through free radical scavenging property.

Limitations of this study is that, since it is an in vitro analysis, only the laboratory and therapeutic use of the herb was analysed but not about its effects and interaction inside the body. Further studies should concentrate more on the assessment of the interaction of the nutmeg Oleoresin inside the body. Environmental error should be avoided for getting the correct results. Recently, green nanotechnology has been gaining popularity because of its exclusion of damaging reagents and cost-effectiveness. Some of such research includes evaluation of cytotoxic properties of nutmeg oleoresin which are prepared from cumin oil. The synthesised nutmeg oleoresin mediated nanoparticles were confirmed by UV-Visible spectroscopy.

The limitation is that this is only an *in vitro* study and needs many more evaluations to use in humans . Hence, the future scope for this study can lead to the development of commercial products that are safe, effective, and are economical after conducting studies in *invivo* models .

**CONCLUSION** :

In the present study, it was observed that nutmeg oleoresin mediated zinc nanoparticles have cytotoxic properties on breast cancer cell lines. Green synthesis of Zinc nanoparticles using nutmeg oleoresin was of low cost and convenient to carry out. Hence, it is a promising candidate for anticancer therapy. The nanoparticles demonstrated enhanced anti-cancer properties, likely due to the synergistic interaction between the bioactive compounds in nutmeg and the ZnO-NPs. The results reveal their potential to inhibit cancer cell growth and induce cell death through mechanisms such as oxidative stress and apoptosis. These findings underscore the potential of nutmeg-derived ZnO-NPs as an eco-friendly and promising approach for alternative breast cancer therapies. Further research is needed to explore their mechanisms of action and clinical applications.

**ACKNOWLEDGEMENT**:
The authors thank The Synthetic Industries Pvt Ltd Kerala , for providing nutmeg oleoresin for the study. The authors also extend their sincere gratitude to the Saveetha Dental College for their constant support to carry out this research.

**CONFLICT OF INTEREST**:
All the authors declare no conflict of interest in the study.

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