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ORIGINAL

Preparation of a nanoemulsion from clove oil extract containing phenol and studying its effect on a human MCF-7 breast cancer cell line

Preparación de una nanoemulsión a partir de extracto de aceite de clavo que contiene fenol y estudio de su efecto sobre una línea celular humana de cáncer de mama MCF-7

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ABSTRACT

Breast cancer is one of the major health concerns worldwide. Approximately 2,26 million cases of this disease were reported globally in 2020, making it the most dangerous type of cancer. In previous studies, they linked ifosfamide with clove oil based on a nanoemulsion and studied its cytotoxicity. Our research is based on integrating a phenol compound characterized by its insolubility in water, which limits its biological uses, and is considered an antioxidant, with clove oil that contains a high percentage of antioxidants and eugenol. By combining the two in a nanoemulsion system, we successfully prepared it by confirming its composition using FTSEM, FTIR, and UV tests. From a biological perspective, biological surfaces can effectively absorb nanoemulsion droplets to achieve more efficient biological activities. We then calculated the encapsulation efficiency, which was very high. Subsequently, we prepared different concentrations of the nanoemulsion and used the MTT method to test its effect on human breast cancer cell lines and verified cytotoxicity and survival rates for each concentration. We extracted the IC50 value and found that the phenolic nanoemulsion of clove oil successfully killed cancer cells with an increase in concentration.

Keywords: MCF-7 Breast Cancer; Nanoemulsion; Phenol; Clove Oil; Cytotoxicity.

RESUMEN

El cáncer de mama es una de las principales preocupaciones sanitarias en todo el mundo. En 2020 se registraron aproximadamente 2,26 millones de casos de esta enfermedad en todo el mundo, lo que lo convierte en el tipo de cáncer más peligroso. En estudios anteriores, unieron ifosfamida con aceite de clavo a partir de una nanoemulsión y estudiaron su citotoxicidad. Nuestra investigación se basa en integrar un compuesto fenólico caracterizado por su insolubilidad en agua, lo que limita sus usos biológicos, y considerado antioxidante, con aceite de clavo que contiene un alto porcentaje de antioxidantes y eugenol. Combinando ambos en un sistema de nanoemulsión, lo preparamos con éxito confirmando su composición mediante pruebas FTSEM, FTIR y UV. Desde una perspectiva biológica, las superficies biológicas pueden absorber eficazmente las gotas de nanoemulsión para lograr actividades biológicas más eficientes. A continuación, calculamos la eficacia de encapsulación, que fue muy alta. Posteriormente, preparamos diferentes concentraciones de la nanoemulsión y utilizamos el método MTT para probar su efecto en líneas celulares de cáncer de mama humano y verificamos la citotoxicidad y las tasas de supervivencia para cada concentración. Extrajimos

el valor IC50 y descubrimos que la nanoemulsión fenólica de aceite de clavo mataba con éxito las células cancerosas al aumentar la concentración.

Palabras clave: Cáncer de Mama MCF-7; Nanoemulsión; Fenol; Aceite de Clavo; Citotoxicidad.

INTRODUCTION

Tens of millions of people get sick with cancer every year, making it one of the top causes of mortality in the world.[1] This illness develops into a very complicated condition because to a multi-stage carcinogenic process that necessitates several cellular physiological systems, including cell signaling and programmed cell death.[2,3] Although they begin as localized illnesses, cancers are more likely to spread to other parts of the body, rendering them incurable.[4]

One major worldwide health concern is breast cancer. About 2,26 million instances of this illness were reported globally in 2020, making it the most frequent cancer. According to worldwide cancer incidence rates in 2020, this kind of cancer is ranked first, surpassing lung cancer, and is regarded as one of the most common cancer types.[5,6] Unchecked breast cell development is the first step toward breast cancer. Although one in eight women may get a breast cancer diagnosis throughout their lifetime, the disease is treatable if caught early.[7] Proliferation, angiogenesis, hypoxia, programmed cell death, and cell activity are all linked to the underlying causes of cancer. Metastasis and the epithelium-to-mesenchyme transition (EMT) are associated with signaling pathways.[8]

Clove oil is one of the essential oils, derived from clove trees native to Southeast Asia, although it can also grow in other regions. Clove oil is produced by distilling the dried flower buds collected from the clove tree. Other parts of the tree, such as the stem and leaves, can also be used.[9] The uses of clove oil, which ranges in color from nearly clear to light yellow and has a strong, spicy aroma, have varied for centuries across a variety of applications.[10,11]

Clove oil may help stop the growth of many cancer cells, such as breast cancer, cervical cancer, and colon cancer, as clove extracts increase cell death and disrupt the division of cancer cells. Clove oil is used to help improve circulation; it helps relieve muscle pain and contributes to promoting good blood flow to various areas of the body. When used for aromatherapy purposes, clove oil will help stimulate the brain, thereby increasing attention and activity.[12]

Clove oil also has a high content of the antioxidant eugenol, which explains the benefits of clove oil for weight loss due to its effect on blood sugar and cholesterol levels. It also accelerates circulation, metabolism, and digestion when consumed in a drink.[13]

In our study, we used clove oil as a carrier medium for phenol molecules. Carbolic acid is a colorless crystalline solid with a pleasant tar-like odor, often referred to as the smell of hospitals. Its overall chemical formula is C6H6O, and its structure consists of a hydroxyl group attached to a phenyl ring, making it an aromatic compound.[14] It is one of the important compounds on the list of antioxidants. Phenol is characterized by its low solubility in water and good solubility in oil, which reduces its biological activity and limits its use in aquatic environments. Therefore, a nano-emulsion of phenol dissolved in oil was prepared, and to enhance the ability of clove oil as an anti-cancer and antioxidant agent, we combined phenol molecules and clove oil together in a nano-emulsion system.[15,16] From a biological perspective, biological surfaces can effectively absorb nano-emulsion droplets for more efficient biological activities. Nano-emulsions are spherical particles that act as carriers for drug molecules, ranging in size from 10 to 1000 nanometers. Thus, a nano-emulsion of phenol, dissolved in clove oil, which is also an antioxidant, was prepared by converting it into nano-sized droplets through encapsulation in suitable surfactants.[17,18] Due to this encapsulation, phenol becomes physically and chemically stable in the aquatic environment. These carriers are solid particles with an amorphous and lipophilic surface, thereby increasing the therapeutic efficacy of the drug. Due to the effect of phenol and the compounds present in the oil used, it can be utilized as a suitable treatment for breast cancer. We conducted an investigation into cellular toxicity using the MTT assay, where a human breast cancer cell line was treated with different concentrations of nanoemulsion phenol. The results indicated that as the concentration increased, cell viability decreased.[19,20]

METHOD

Extraction of clove oil

The essential oils present in plants are extracted using the Clavenger method.[1] Dried Indian clove was obtained from local markets in Baghdad. A total of 150 grams of the plant was taken, as shown in figure 1, and placed in a 500 ml round-bottom flask. Then, 200 ml of distilled water was added. After assembling the

Clavenger apparatus and placing it on the magnetic heater, the temperature was set to 100 degrees Celsius. After 80 minutes, clove essential oil was obtained, as shown in figure 2, and it was collected for the next step.

Figure 1. Shows the dried clove plant used in our research

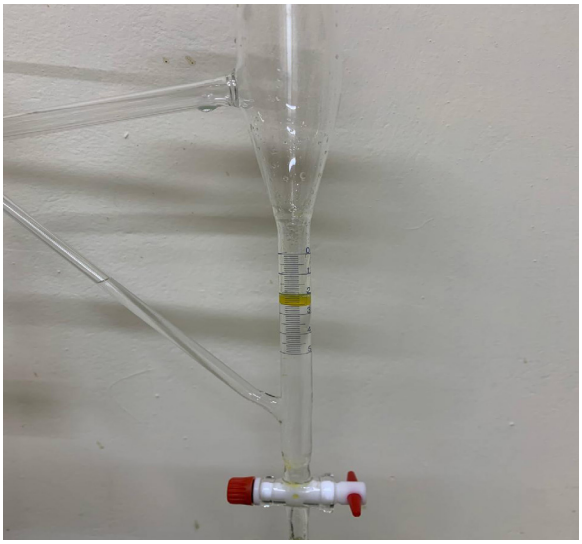

Figure 2. Shows the dried clove plant used in our research

Preparation of the nanoemulsion containing phenol

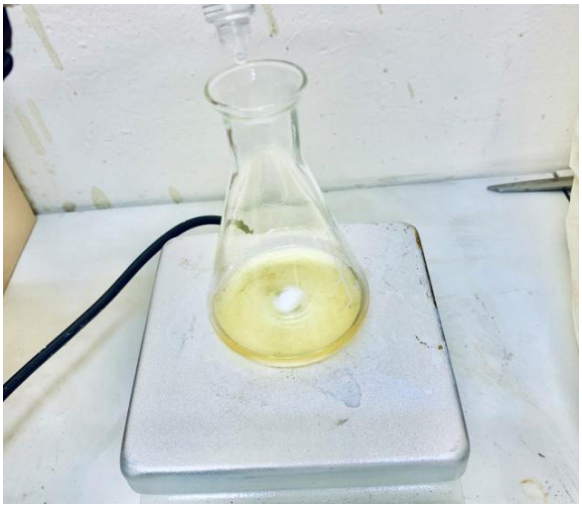

Figure 3. Shows the step of adding distilled water to the emulsion drop by drop with continuous stirring

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1. 0,75 grams of clove oil extracted in the previous step was mixed with 50 mg of phenol, and then it was placed on the magnetic stirrer for 25 minutes.
2. 1,55 grams of SP80 were added to the above components and mixed for 7 minutes.
3. After that, we added 7,45 grams of Tween 80 and stirred for 7 minutes.
4. The next step was to add 2,5 grams of ethanol and mix for 20 minutes.
5. Then, we slowly added 12,75 grams of distilled water dropwise while continuously stirring for 35 minutes, as shown in figure 3.
6. Finally, the nanoemulsion was placed in the ultrasonicator for two hours, as shown in figure 4. nanoemulsion of clove oil reinforced with phenol was obtained, and we studied the shape and size of the oil emulsion droplets using an FESEM. We then conducted FTIR analysis of the phenol used in the first step and the nanomaterial to confirm the presence of phenol within the nanodroplets prepared in the final step.

Figure 4. Shows the final step in preparing the nanomulsion using the ultrasonic water bath device

Measurement of the maximum wavelength

To determine the maximum wavelength of the nanomaterial in methanol, a UV-VIS device from Shimadzu was used. The range from 190 to 800 nanometers was measured at intervals of 0,5 nanometers. The absorption spectrum was then analyzed to determine the maximum absorption point, which was reported as the maximum wavelength of curcumin.

Drawing the calibration curve of the nanoemulsion

Six dilutions with concentrations of 1, 2, 4, 6, 8, and 10 microliters of the prepared emulsion were made in a test tube, and each tube was filled to 3 cubic centimeters with methanol and pumped using a sampling device. First, 100 microliters of the initial stock was taken and dissolved in 10 cubic centimeters of methanol, then the other concentrations were calculated, made from it, and mixed. Absorption of all concentrations using a spectrophotometer in quartz stones at the maximum wavelength was measured for the obtained curcumin. A standard curve was drawn using absorbance values, and the concentration of curcumin was obtained from Excel, resulting in the linear equation.

Determination of the encapsulation efficiency of phenol within nanoemulsion droplets

Encapsulation efficiency is a method to evaluate the preparation of nanoparticles for drug delivery. The ratio of the drug in the nanoparticles compared to the total amount of drug used in the preparation process indicates this and is usually expressed as a percentage. The direct method was used to determine the encapsulation efficiency by centrifuging the emulsion in a refrigerated centrifuge at 4 degrees Celsius at a speed of 13,000 rpm for 30 minutes, and the supernatant is absorbed. It is measured at a wavelength of 287 nanometers. The encapsulation efficiency is then calculated using the following formula:

Encapsulation efficiency (%) = Amount of encapsulated drug / Total drug × 100.

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Evaluation of MCF-7 breast cancer cells cultivation and their survival ability using MTT

The MTT assay is a common method for measuring cell viability and proliferation in biological research.

This assay relies on the ability of living cells to convert the yellow tetrazolium salt known as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) through mitochondrial dehydrogenase activity into the blue formazan product. The formazan product is insoluble in aqueous solutions

and can be dissolved in solutions containing detergents such as dimethyl sulfoxide (DMSO) and can be measured spectrophotometrically. Since the MTT assay is relatively simple, it is quick and sensitive and is widely used. It can be employed to measure the effects of various treatments such as drugs, chemicals, or environmental factors on cell survival and proliferation. It is also used to determine the optimal cell density for experiments, assess the cytotoxicity of compounds, and is useful for establishing dose-response curves. The methodology for investigating the viability of breast cancer cells with this MTT method is as follows:

MCF-7 breast cancer cells were cultured using DMEM media containing 10 % FBS for 48 hours.

After removing the supernatant and washing the cells using a washing solution, trypsin was added and incubated for 2-3 minutes before neutralization with complete medium. After that, the cells were centrifuged and resuspended in complete medium. To count the cells, a small amount of the primary cell suspension was added to the medium and placed under a microscope on a Neubauer slide. Cells were counted in four square areas of the slide, each measuring 16, and calculated using the formula. In this study, 1,5 million cells were counted and diluted in the medium. Then, the diluted cells were placed in a 96-well plate with an appropriate amount.

RESULTS AND DISCUSSION

Results of FESEM

The examination was conducted in the Republic of Iran, and the structure and size of the prepared nanoemulsion were studied using a scanning electron microscope. The results showed the presence of spherical droplets with similar dispersion and sizes that were somewhat comparable, within the nanoscale of less than 100 nanometers, as shown in figure 5.

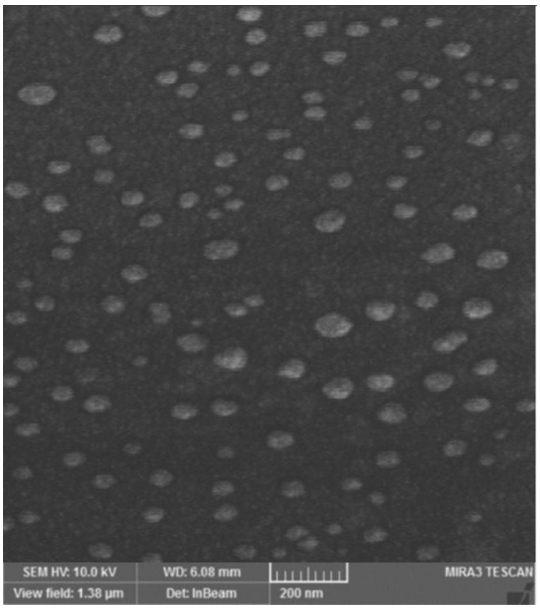

Figure 5. The form shows the result of the examination FESEM

FTIR Test

1. We have confirmed the use of pure phenol through FTIR spectroscopy using a device from Shimadzu, and the examination was conducted at the University of Baghdad. A peak was found at 3000-3020, corresponding to the aromatic C-H group, and a peak was found at 1475-1600, corresponding to aromatic C=C. Additionally, a broad peak was found at 3600-3000, corresponding to phenolic OH, with an exact match to the spectrum of free phenol. as illustrated in figure 6.
2. It was confirmed that the nano-emulsion of clove oil contains phenol through FTIR spectral analysis, where a peak was found at 3000-3020 corresponding to the aromatic C-H group. Peaks at 1460,11 and 1641 were found, corresponding to aromatic C=C, and a broad peak at 3479,58 was also found, corresponding to phenolic OH, which showed similarity to the spectrum of the used phenol. Thus, we confirmed that the prepared emulsions contain phenol within them, as shown in figure 7.

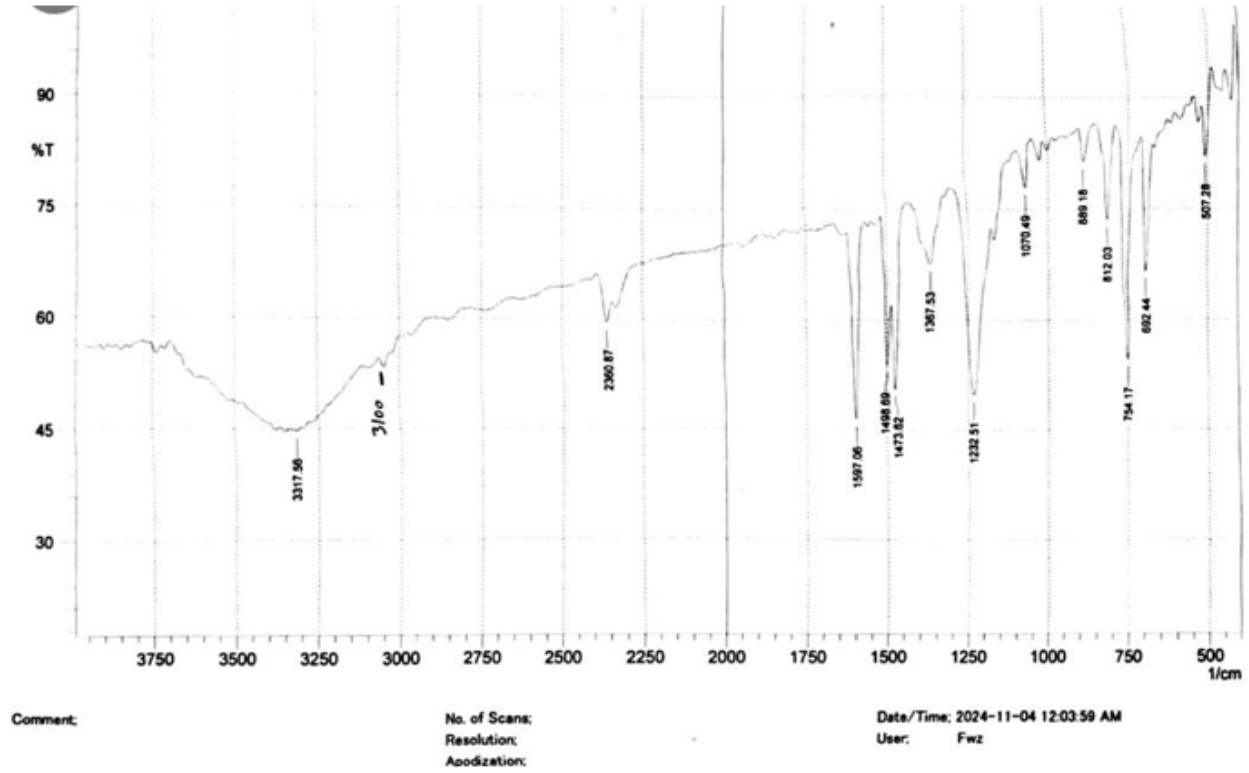

Figure 6. Shows the FTIR spectrum of pure Phenol

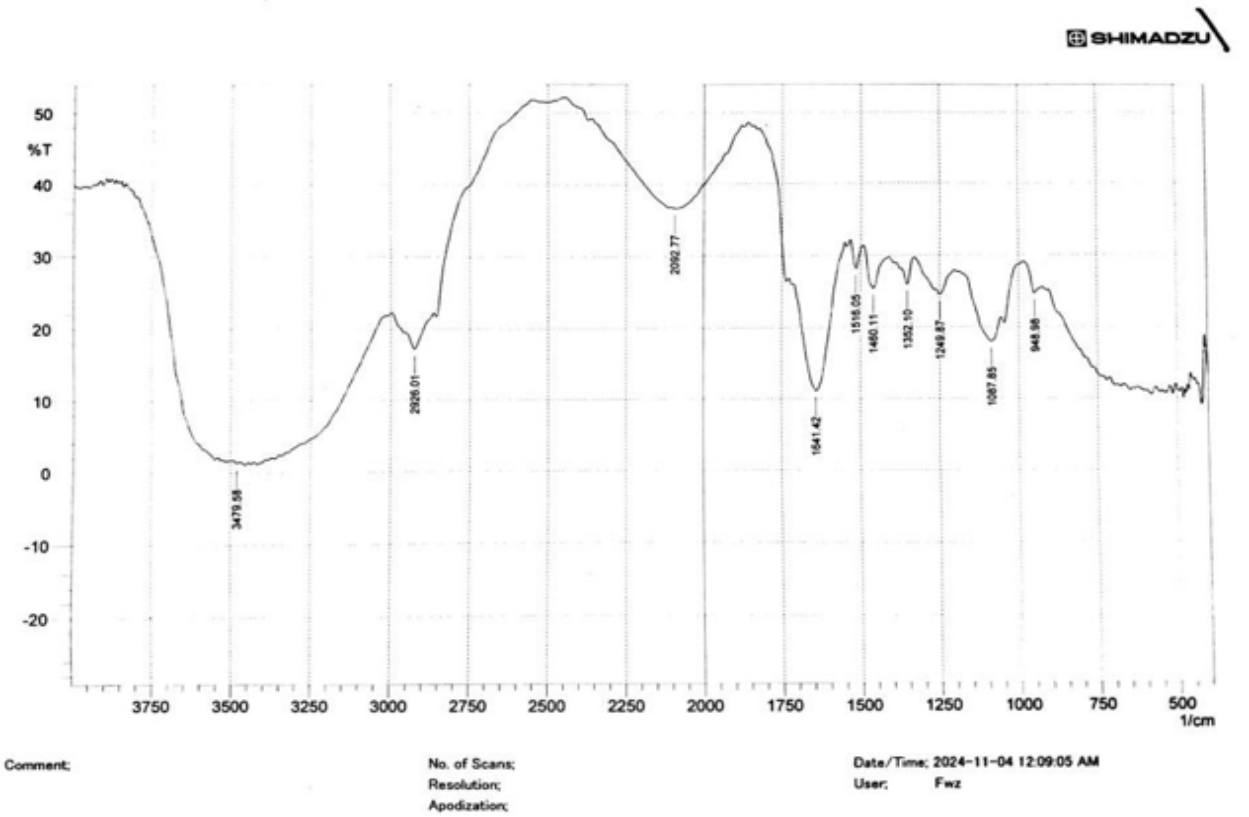

Figure 7. Shows the FTIR spectrum of the phenol-containing nanoemulsion

Determination of the absorption wavelength

To determine the wavelength, ultraviolet technique was used to obtain the absorption spectrum (10 mg/ml) dissolved in methanol, with a maximum wavelength of 287 nanometers. Excel software was used to plot the peak as shown in figure 8.

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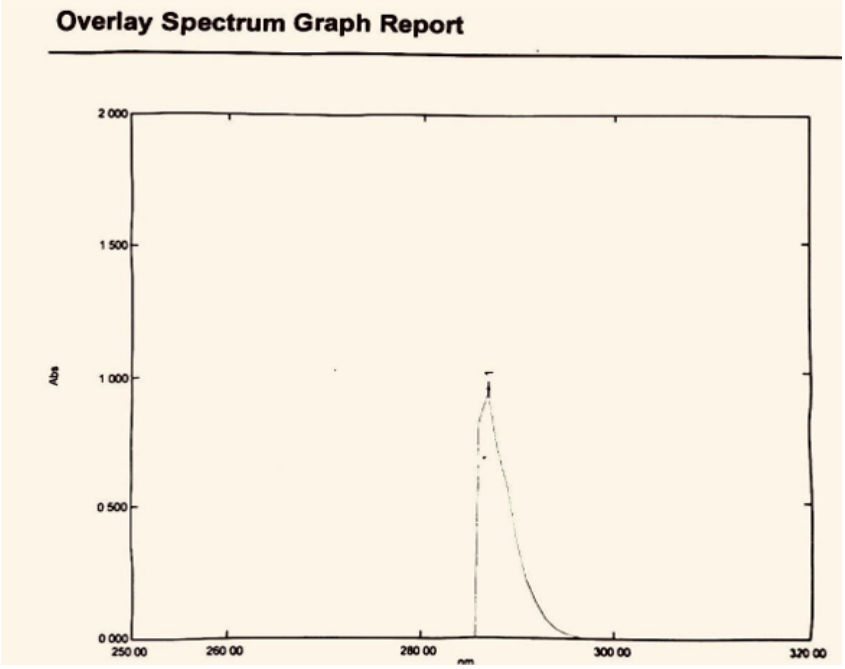

Figure 8. Shows the result of the UV-VIS examination and the determination of the wavelength of the nanoemulsion

Calibration Curve Determination

The maximum wavelength was measured using reliable and accredited sources. To correlate between the drug concentration and the spectrophotometric absorbance, emulsions prepared at concentrations of 1, 2, 4, 6, 8, and 10 were diluted in methanol, and their absorbance was measured at a wavelength of 287 nanometers. As shown in table 1, the calibration curve was plotted, and the equation of the straight line and its R value were calculated. As illustrated in figure 9, in addition, the spectrophotometer used methanol as a blank solution at a wavelength of 287 nanometers.

Table 1. Shows the concentration values in micrograms/ml versus the absorbance values for each concentration

Concentration (micrograms/ml)	Absorbance nm
1	0,679
2	0,724
4	0,789
6	0,831
8	0,879
10	0,954

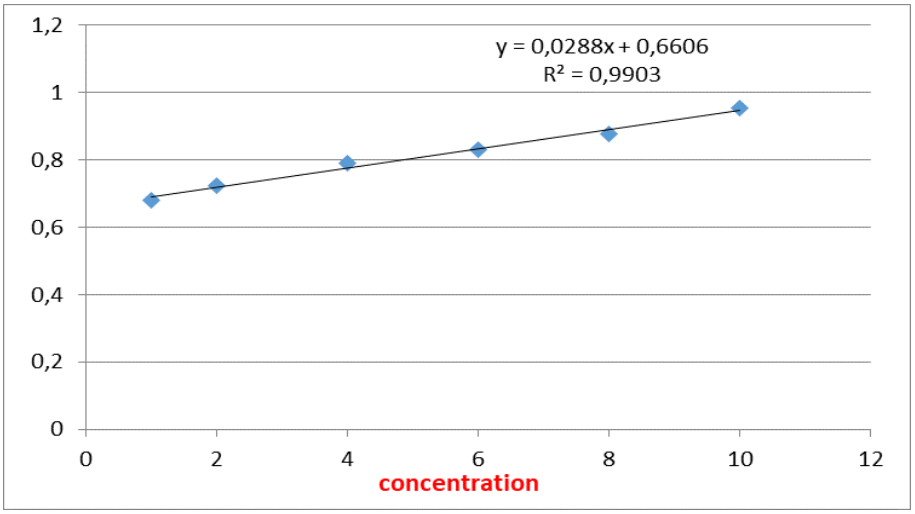

Figure 9. Shows the calibration curve for calculating the equation of the straight line and the value of R

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Efficiency calculation of phenol encapsulation in nanoemulsion

To determine the amount of phenol present in the nanoemulsions, a centrifugation method was used. For this purpose, the nanoemulsion of phenol was diluted with methanol at a ratio of 1:2. The nanoemulsion was placed in a refrigerated centrifuge at 4 degrees Celsius for 30 minutes at a speed of 13 000 RPM. The supernatant was collected and the absorbance was measured at 287 nanometers. The encapsulation efficiency expresses the ratio of the amount of drug in the particles to the total amount of active drug used in the manufacturing process, expressed as a percentage. Using the encapsulation equation, the encapsulation efficiency ratio for the phenol-containing nanoemulsion was obtained, and the percentage was 89 %.

Results of the study on the effect of cellular toxicity of the nano-emulsion of clove oil containing phenol on MCF-7 breast cancer cells using MTT

MCF-7 breast cancer cells were cultured in 96-well plates. Six different concentrations of the nanoemulsion of clove oil containing phenol were prepared: 7, 14, 30, 60, 90, and 120 micrograms/ml from a stock of 10 mg/ml, and 100 microliters of each concentration were added to the cells in triplicate. After two hours, 20 microliters of the prepared MTT solution were added to each well. After 3 hours, 100 microliters of DMSO were added to each well. The absorbance was then read at wavelengths of 570 and 630 nanometers using an ELISA reader. The percentage of cell viability and the percentage of cytotoxicity were calculated using the following formulas:

% Cell viability = Sample absorbance / Control absorbance * 100
% Cell viability for control sample = Control absorbance / Control absorbance * 100
% Cytotoxicity = 100 - Cell viability

The resulting IC50 value was 54,56 micromoles, as shown in table 2 and figure 10.

The conclusion is that with an increase in concentration, the growth of human breast cancer cells is inhibited.

Table 2. Shows the values of absorbance, cellular toxicity, and cell viability for each concentration used

Concentration	Control	120 Micromoles	90 Micromoles	60 Micromoles	30 Micromoles	14 Micromoles	7 Micromoles
Absorbance	0,354	0,083	0,01	0,09	0,285	0,309	0,306
	0,38	0,034	0,075	0,101	0,149	0,314	0,376
	0,33	0,002	0,042	0,112	0,248	0,302	0,334
Mean	0,362	0,039666667	0,042333333	0,101	0,22566667	0,30833333	0,35
%cell viability	100	10,9576428176	11,694290883	27,9005524861	62,338859116	85,1749530386	96,6850828718
%Toxicity	0	89,0423571824	88,305709117	72,0994475139	37,661140884	14,8250469614	3,3149171282

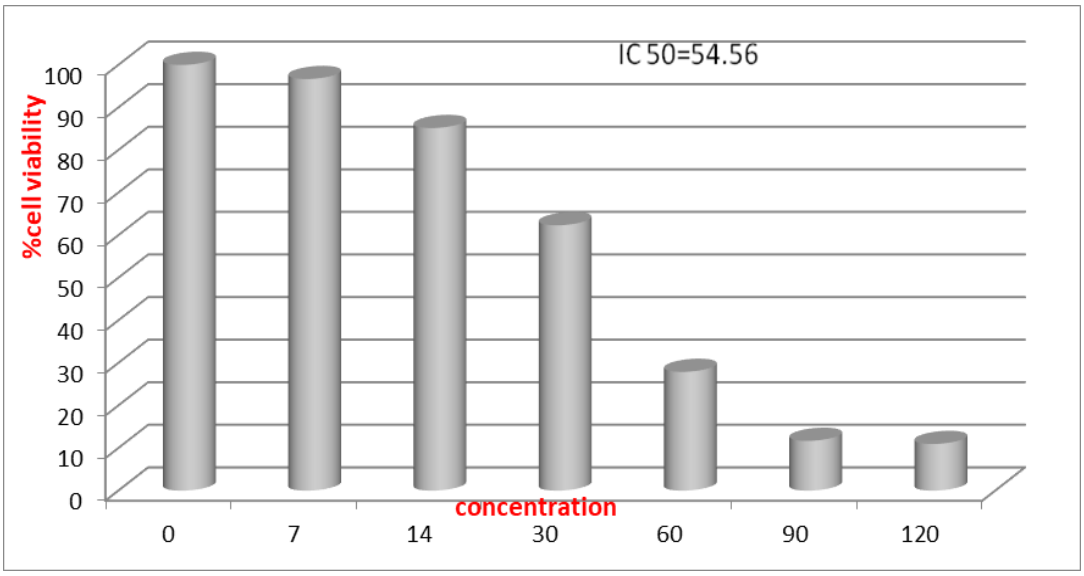

Figure 10. Shows the effect of each concentration on the viability of the cells with the determination of the IC50 value

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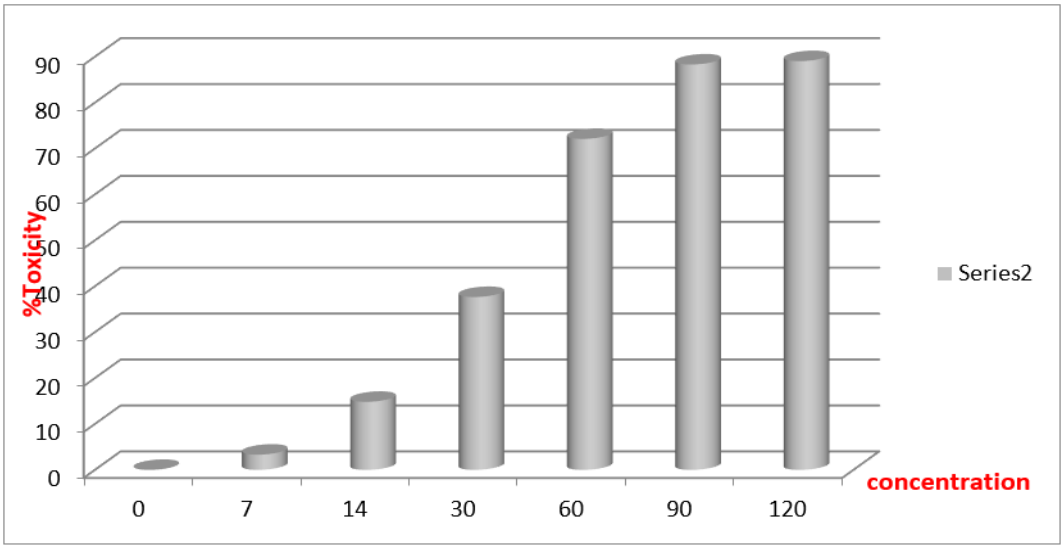

Figure 11. Shows the effect of each concentration on %Toxicity

CONCLUSIONS

In this study, we indicate our success in extracting essential clove oil through the steam distillation method. We then dissolved free phenol in clove oil and successfully prepared a nano-emulsion containing phenol. We verified the encapsulation efficiency using the encapsulation efficiency equation, which reached 89 %. By studying the shape of the nano-emulsion droplets, we conducted FESEM analysis, confirming that all emulsion particles fall within the nanotechnology scale. To ensure the presence of phenol, we performed FTIR and UV tests, confirming its presence. Finally, we cultured breast cancer cell lines and used the MTT method to assess the effects of different concentrations in micromolar units of the prepared nano-emulsion. The results showed that as the concentration increased, the rate of cellular toxicity increased, and the survival rate of the cells decreased, with an IC50 value of 54,56.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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