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Clove Oil Nanoemulsion Versus Alcoholic Extract: Preparation, Characterization, and Comparative Anticancer Efficacy Against Gastric Cancer Cells

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ABSTRACT: ***Background:*** *Gastric cancer is a global health issue, and efforts are being made to discover new compounds that can be used to treat or prevent this disease. Clove (Syzygium aromaticum) contains eugenol and other bioactive compounds that have antioxidant and anticancer effects. The use of nanoemulsion technology can help to improve the solubility, stability, and bioavailability of these natural compounds, which in turn may enhance their therapeutic ability.* ***Objective:*** *This study aimed to compare the chemical composition and anticancer activity of clove oil nanoemulsions and alcoholic clove extract against human gastric cancer cells.* ***Methods:*** *Both clove oil nanoemulsion and alcoholic extract were prepared using standardized methods, with 50% ethanol employed for the extraction of active compounds. The samples were characterized by dynamic light scattering (DLS) for particle size analysis and Fourier-transform infrared spectroscopy (FTIR) for identification of functional groups and structural features. Their anticancer activity was evaluated using the MTT assay on a gastric cancer cell line by testing multiple concentrations and assessing effects on cell viability.* ***Results:*** *FTIR analysis revealed the presence of key functional groups in both the nanoemulsion and the alcoholic extract, such as phenolic hydroxyl groups (O–H) and aromatic bonds (C=C), confirming the presence of eugenol as the main active ingredient in both preparations. However, the nanoemulsion exhibited higher spectral intensity and a better distribution of nonpolar compounds, indicating improved solubility and stability of the active compounds. Both samples demonstrated antioxidant activity and the ability to inhibit the growth of gastric cancer cells. The nanoemulsion was found to have significantly higher potency compared to the alcoholic extract, with an IC_{50} value of 36.30±1.47 µg/mL compared to 41.67±1.53 µg/mL for the extract.* ***Conclusions:*** *This difference is attributable to enhanced solubility, stability and cellular uptake of the active compounds in the nanoemulsion. The results suggest that clove oil nanoemulsions exhibit superior antioxidant properties and anticancer activity compared to conventional alcoholic extracts indicating that nanotechnology and traditional methods together present a unique opportunity for improving bioactivity and bioavailability of natural anticancer agents, which could lead to the development of effective therapies as treatment options for gastric cancer.*

KEYWORDS: *Alcoholic extract; Clove oil; Nanoemulsion; Anticancer activity; Gastric Cancer Cells*

INTRODUCTION

Stomach cancer is a leading cause of cancer-related deaths worldwide, making the ongoing search for anticancer drugs with higher efficacy and lower toxicity compared to conventional treatments essential [1], [2]. In this context, medicinal plants and their components are receiving increasing attention as potential sources of anticancer compounds, given their high concentrations of phenolic compounds and bioactive substances [3], [4].

Clove (*Syzygium aromaticum*) is one of the most widely used plants in traditional medicine. [5], [6] Its biological activities are primarily attributed to eugenol and a range of other phenolic compounds, which have demonstrated antioxidant, anti-inflammatory, and antibacterial properties, along with numerous reports indicating its anticancer efficacy in cellular and animal studies [7], [8]. Research has also focused on the role of clove essential oils and extracts in inhibiting cancer cell growth and inducing apoptosis pathways, as well as their effect on regulating intracellular oxidative stress mechanisms [9], [10]. Extraction and method of extraction are very important components of determining how effective two plant-derived compounds can be when extracted from plants. The ratios of volatile and non-volatile compounds (e.g., eugenol) found within distilled essential oils and alcoholic extracts are different, which will affect their bioavailability [11], [12]. The same applies for the variability between different extraction processes (high heat, ultrasound, and Clevenger apparatus) as they can also change both the chemical composition and yield of essential oils produced; therefore, traditional extraction methods are limited in their ability to maintain the stability and increase the bioavailability of active compounds once the extraction is complete [13], [14]. In response to these limitations, nanoemulsions represent a potential solution by increasing the surface area to volume ratio of the product while simultaneously reducing droplet size, thus improving the stability and bioavailability of volatile oils [13], [15]. These systems also demonstrate superior cell penetration and improved bioactivity compared to traditional systems [16], [17]. This led to the development of clove oil nanoparticles, based on properties such as particle size, stability, and anticancer and antimicrobial activities [18], [19]. To analyze these systems, spectroscopic techniques such as FTIR were used to identify the chemical signatures of the extracts and oils, revealing key functional groups such as OH, C=O, and aromatic rings linked to eugenol [20], [21]. An alcoholic extract of clove oil was prepared using a Clevenger apparatus, then converted into an oil-water nanoemulsion, and tested at different concentrations to investigate its effect on gastric cancer cell viability under controlled cell culture conditions [22], [23]. FTIR was also used to compare the chemical properties of both the alcoholic extract and the nanoemulsion [24], [25].

The results showed that both preparations exhibited an inhibitory effect on the growth of gastric cancer cells, but the nanoemulsions demonstrated higher efficacy, attributed to improved delivery of the active compounds and increased bioavailability [26], [27]. Therefore, this study aims to explore the relationship between extraction techniques, chemical characterization properties, and the development of clove oil nanoemulsions and to evaluate their effect on biological response, particularly their anti-gastric cancer activity. The study also explores the potential use of clove nanosystems, such as nanofibers, as a supportive or alternative option to conventional chemotherapy.

MATERIALS AND METHODS

Preparation of Alcoholic Extract

Fifty grams of cloves were weighed, as shown in Figure 1A, and placed in a 500 mL flask. A mixture of 120 mL of water and 120 mL of ethanol was then added to the flask. The mixture was stirred magnetically for one hour to ensure homogeneous mixing. After 24 hours, the mixture was stirred again for an additional hour, as shown in Figure 1B. The mixture was then filtered to obtain the filtrate, which was subsequently dried using a rotary evaporator at 45°C and under reduced pressure to obtain a semi-solid extract. This extract was then transferred to an air-drying dish at room temperature for 24 hours, as shown in Figure 1C. After drying, the resulting extract, weighing 9 grams, was collected and subjected to Fourier transform infrared (FTIR) and UV-Vis spectroscopy to determine its chemical properties and identify the active constituents of the clove extract.

Extraction of Clove Oil

Clove oil was obtained using a Clevenger apparatus. Fifty grams of cloves were weighed, as shown in Figure 1, and placed in a 500 mL flask. 250 mL of distilled water was added to the flask, and the Clevenger apparatus was securely fastened to ensure proper steam distillation. The mixture was heated with continuous stirring using a magnetic stirrer, and the extraction process lasted for 5 hours. After the extraction was complete, the condensed oil was collected in the apparatus's collection tube, as shown in Figure 2A. The clove oil was then separated from the aqueous layer, and any remaining moisture was removed using anhydrous sodium sulfate (Na_2SO_4). The purified oil was then stored in a dark-colored container at 4°C until use. The extraction rate of the clove oil was calculated and found to be 3.5–4%.

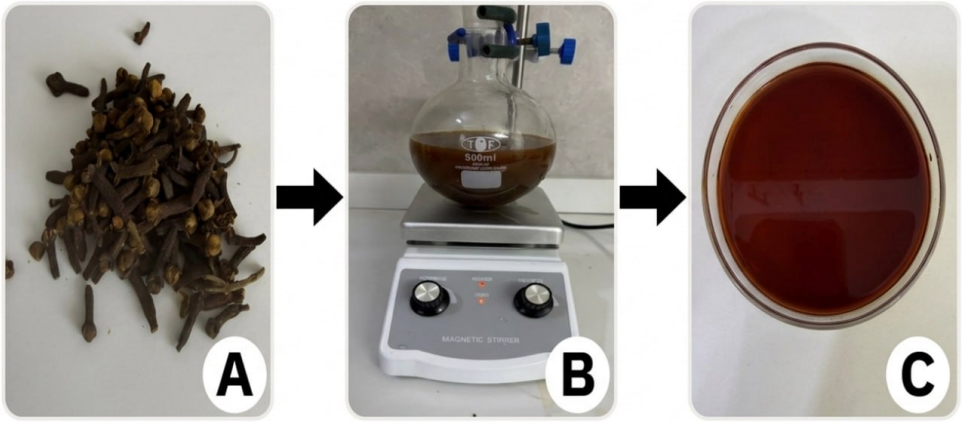

Figure 1. Steps involved in the preparation of the clove extract

Preparation of the Nanoemulsion

Preparation of the oil phase: The oil phase was prepared by adding 2 mL of clove oil to 2 mL of the surfactant Span 80 in a 20 mL glass flask. The mixture was then stirred for 10 minutes using a magnetic stirrer to ensure homogeneity.

Preparation of the aqueous phase: The aqueous phase was prepared by adding 8 mL of the surfactant Tween 80 to 88 mL of deionized distilled water. The mixture was then stirred for 10 minutes using a magnetic stirrer to obtain a homogeneous solution [7].

Preparation of the initial emulsion: The aqueous phase was placed on the magnetic stirrer, and the oil phase was added drop by drop very slowly, while stirring continuously for 3 hours to ensure the formation of the initial emulsion, as shown in Figure 2B.

Droplet Reduction for Nanoemulsification: To obtain nano-sized oil droplets, the initial emulsion was placed in an ultrasonic bath for 30 minutes. This process was repeated five times with a one-hour interval between each cycle, as shown in Figure 2C. The resulting emulsion was collected and subjected to dynamic light scattering (DLS) to determine the size of the oil droplets and evaluate their volumetric distribution. Subsequently, FTIR and UV spectroscopy were performed.

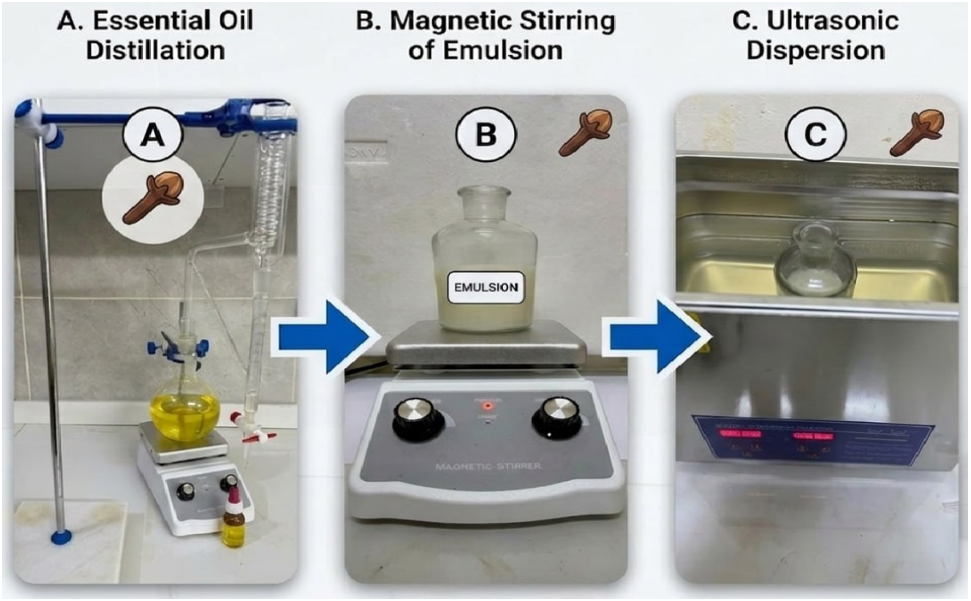

Figure 2. Stages involved in the preparation of the nano-clove oil emulsion

Dynamic Light Scattering (DLS) Analysis of Nanoparticles

The particle size distribution in the oil-in-water nanoemulsion was determined using dynamic light scattering (DLS). This technique relies on the random motion of nanoparticles resulting from collisions with the medium's molecules, leading to variations in the intensity of the scattered light, which is then used to calculate particle size. The nanoemulsion sample was first prepared by sonicating it for 15 minutes to ensure homogeneity and minimize particle agglomeration. Subsequently, 400 μL of the nanoemulsion was diluted with distilled water to a final volume of 3 mL in the measuring tube.

The DLS instrument was set to the appropriate refractive index for oil-in-water systems, ensuring that the sample concentration was within the recommended range for dynamic scattering measurement. Three independent measurements were performed, and the average nanoparticle size was calculated based on the recorded readings to ensure accuracy and reproducibility.

Functional Groups Study Using FTIR

Both the alcoholic extract and nano-emulsion were chemically characterized to identify and compare the chemical groups of each by preparing tablets from their combined potassium bromides through compressing both ingredients in a force compression device, then placing those tablets in a Fourier transform infrared spectrometer (FTIR) from 400 to 5000 cm^{-1} for analysis.

Cytotoxicity Study of Nanoemulsification and Alcoholic Extract of Clove

Cytotoxicity was assessed using the MTT assay according to a standard protocol. The assay relies on the ability of living cells to reduce the MTT salt to formazan crystals, which are then dissolved in a suitable solvent such as DMSO to measure spectral absorbance and determine cell viability.

Methodology for Evaluating the Effect of Nanoemulsifiers and Alcoholic Extracts on Gastric Cancer Cells Using the MTT Test

Gastric cancer (AGS) cell lines were cultured and expanded in DMEM medium supplemented with 10% fetal bovine serum (FBS) for 48 hours. After 48 hours, the supernatant was removed, the cells were washed with saline, and trypsin was added to lyse the cells (for about 2–3 minutes), and then the complete media was added back to stop the trypsin activity. Afterwards, the cells were centrifuged and resuspended in complete media. For counting the number of cells, a small amount of the cell-resuspended media was placed in a Neubauer counting chamber and viewed with a light microscope to count the total number of cells in four quadrants of a Neubauer counting chamber and calculated with the standard equation (1). A total of 1.5 million cells were counted in this study. All counted cells will be diluted to the appropriate concentration of media and plated in 96-well plates for the MTT assay to determine cytotoxic effects of the nanoemulsifier and alcoholic extract.

RESULTS AND DISCUSSION

Particle Size Analysis of the Nanoemulsion

The nanoemulsion was prepared using self-assembly with clove oil as the oil component and with Tween 80 and Span 80 as surfactants, in addition to water. To ensure a homogeneous emulsion, the product underwent ultrasonic pretreatment before measurements.

Particle size was determined using dynamic light scattering (DLS), one of the most accurate methods for analyzing the size distribution of nanoparticles in suspensions. DLS analysis showed intensity-to-size data. The obtained PDI values indicate the degree of homogeneity of the nanoemulsion system, where lower PDI values reflect a narrow size distribution and better stability of the formulation, as shown in the data in Table 1.

After data analysis and graphing using Excel, the average particle size of D50 was found to be 82.7 nm, as shown in Figure 3, indicating the successful preparation process in achieving the nanoscale size range.

Table 1. Dynamic light scattering (DLS) scan data of the prepared nanoemulsion

Size nm	In (%)	Cum (%)	Size nm	In (%)	Cum (%)	Size nm	In (%)	Cum (%)	Size µm	In (%)	Cum (%)
0.10	0.00	0.00	2.27	0.00	0.00	51.6	7.81	14.89	1.18	0.22	99.21
0.12	0.00	0.00	2.65	0.00	0.00	60.4	12.09	26.97	1.37	0.17	99.38
0.14	0.00	0.00	3.10	0.00	0.00	70.6	15.58	42.56	1.61	0.11	99.49
0.16	0.00	0.00	3.62	0.00	0.00	82.5	16.74	59.30	1.88	0.06	99.55
0.19	0.00	0.00	4.24	0.00	0.00	96.5	14.98	74.27	2.20	0.01	99.58
0.22	0.00	0.00	4.95	0.00	0.00	113	11.16	85.43	2.57	0.01	99.50
0.25	0.00	0.00	5.79	0.00	0.00	132	6.93	92.36	3.50	0.01	99.59
0.30	0.00	0.00	6.77	0.00	0.00	154	3.54	95.95	3.51	0.00	99.60
0.35	0.00	0.00	7.92	0.00	0.00	180	1.54	97.49	4.10	0.01	99.61
0.41	0.00	0.00	9.26	0.00	0.00	211	0.55	98.05	4.79	0.03	99.63
0.48	0.00	0.00	10.8	0.00	0.00	246	0.16	98.21	5.61	0.04	99.68
0.55	0.00	0.00	12.7	0.00	0.00	288	0.04	98.26	6.55	0.06	99.74
0.65	0.00	0.00	14.8	0.00	0.00	337	0.01	98.26	7.66	0.07	99.81
0.76	0.00	0.00	17.3	0.00	0.00	394	0.01	98.27	8.96	0.07	99.88
0.89	0.00	0.00	20.2	0.01	0.01	460	0.02	98.38	12.5	0.05	99.93
1.04	0.00	0.00	23.6	0.06	0.07	538	0.05	98.33	14.2	0.04	99.97
1.21	0.00	0.00	27.6	0.22	0.28	629	0.09	98.24	14.3	0.02	99.99
1.42	0.00	0.00	32.3	0.70	0.99	735	0.15	98.57	16.7	0.01	100.00
1.66	0.00	0.00	37.8	1.88	2.87	860	0.20	98.77	19.6	0.00	100.00

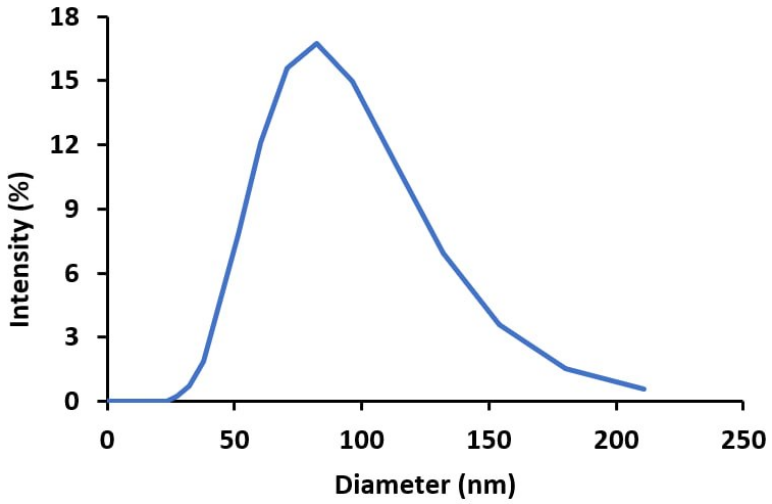

Figure 3. Dynamic light scattering (DLS) scan obtained using VSL analysis, showing D50 particle size extraction

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR analysis of the alcoholic extract of clove revealed a variety of active compounds, the most prominent being eugenol, along with other phenolic and aromatic compounds. The recorded spectra supported the identification of these compounds as follows:

Eugenol—the main active compound in clove: Its presence was confirmed by the appearance of several distinct peaks, including an O–H vibrational band at 3448 cm^{-1}, indicating the presence of a phenolic hydroxyl group; a C–H vibrational band at 2981 cm^{-1}, associated with aliphatic groups;

a C=C aromatic bond vibration at 1639 cm^{-1}; C–O and O–CH_3 bands between 1265–1045 cm^{-1}, which are among the most important indicators of the presence of eugenol; and a signal at 686 cm^{-1}, associated with aromatic compounds. Polyphenols: A wide range of hydroxyl groups (O–H) was observed, indicating the presence of diverse phenolic compounds in the extract, including tannins, other polyphenols, aromatic and hydrocarbon compounds. Some secondary peaks indicated the presence of compounds such as allyl phenols and possible traces of β-caryophyllene, although at a lower concentration compared to clove essential oil, as shown in Figure 4.

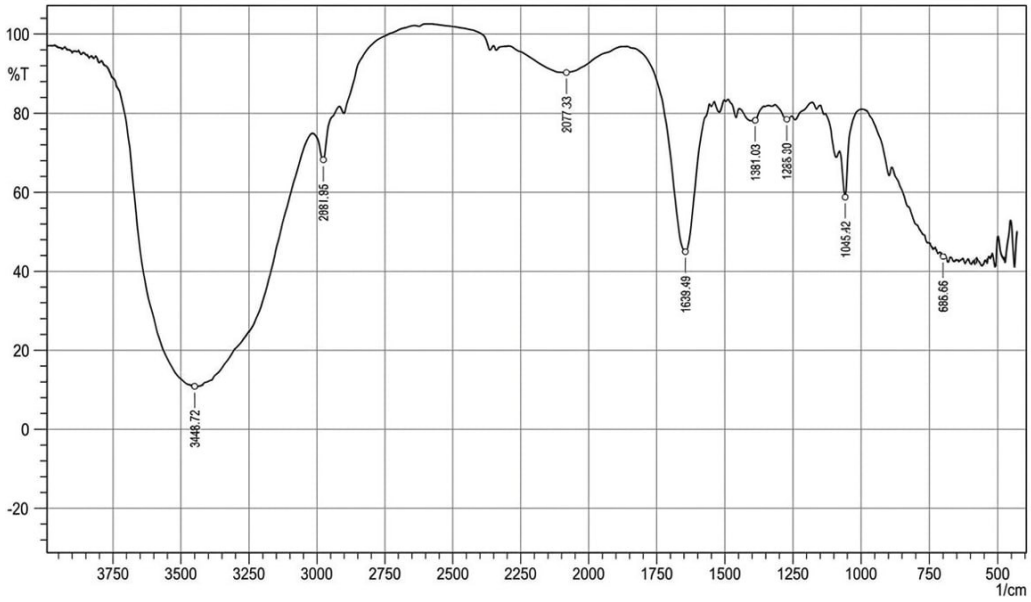

Figure 4. FTIR spectrum of the clove alcoholic extract

FTIR analysis of the oil or emulsion revealed a number of characteristic compounds, most notably: OH groups (3441 cm^{-1}): Indicate the presence of phenols and eugenol with the effect of water. The peak is wider compared to the alcoholic extract. C–H aliphatic (2926 cm^{-1}): Indicate long hydrocarbon chains and a higher oil concentration, confirming the oily nature of the sample. C=C aromatic/H–O–H vibration (1637 cm^{-1}): Confirms the presence of eugenol with the effect of waterAromatic ring vibrations (1508 and 1458 cm^{-1}): Reflect concentrated aromatic compounds such as eugenol and β-caryophyllene. C–O in eugenols and phenols (1354, 1256, and 1097 cm^{-1}): Indicates the presence of phenolic and etheric groups. C–H outside the ring plane (941 cm^{-1}): promotes the presence of aromatic terpenes, as shown in Figure 5.

The results indicate that the alcoholic extract of clove is rich in polyphenolic compounds and flavonoids, giving it strong antioxidant activity, while the clove oil emulsion contains a higher proportion of nonpolar compounds and aromatic terpenes such as β-caryophyllene, in addition to a high concentration of eugenol. Comparison of FTIR spectra demonstrates the effectiveness of each extraction method in isolating different classes of compounds, with alcoholic extraction being preferred for polar compounds, while oily extraction is preferred for terpene compounds and volatile oils, as shown in Table 2.

The comparison results indicate that the alcoholic extract of cloves contains a higher amount of antioxidants compared to the clove oil emulsion. This is because alcohol extraction is able to extract phenolic compounds and highly effective free radical-scavenging substances more effectively than oil extraction [28], [29]. Therefore, the alcoholic extract is richer and more potent in terms of its antioxidant content.

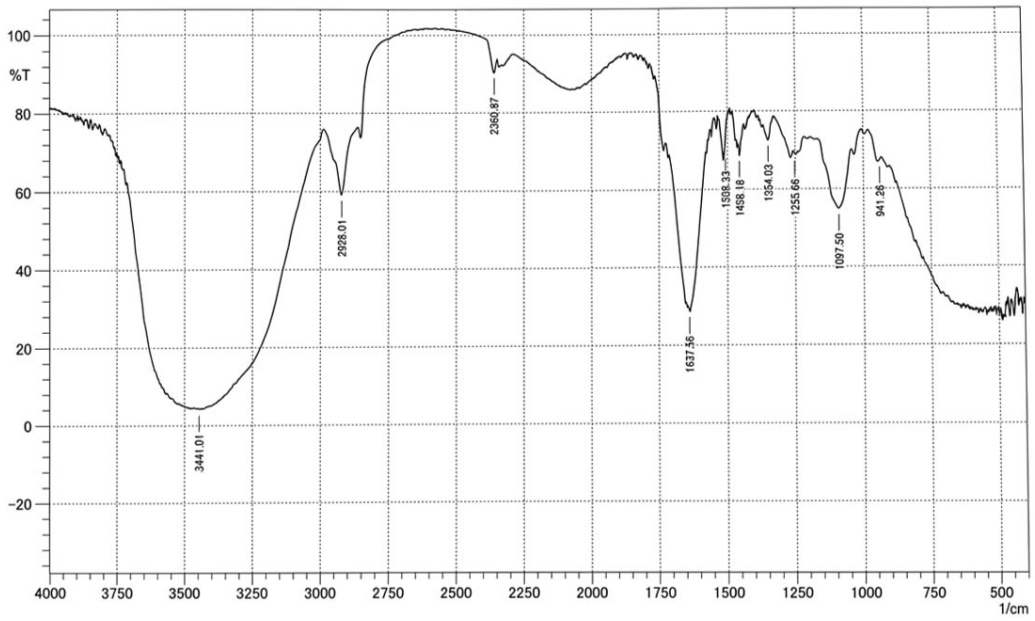

Figure 5. FTIR spectrum of the clove oil emulsion

Table 2. Comparative FTIR spectral analysis of the clove alcoholic extract and clove oil emulsion

Peak No.	Ethanolic Extract (cm^{-1})	Oil Emulsion (cm^{-1})	Functional Group	Interpretation
1	3448.72	3441.01	O–H stretching	Phenolic OH; extract richer in polyphenols, emulsion influenced by water
2	2981.95	2926.01	C–H stretching (aliphatic)	Stronger in emulsion due to terpenes; weaker in extract
3	2077.33	2360.87	Overtone / CO_2	Non-structural; aromatic overtone in extract, CO_2 interference in emulsion
4	1639.49	1637.56	C=C aromatic / H–O–H bending	Eugenol aromatic C=C; in emulsion partially overlapped with water bending
5	1381.03	1354.03	C–H bending	Aromatic bending; more intense in emulsion
6	1265.30	1255.66	C–O stretching (phenolic/O–CH_3)	Signature peak of eugenol; strong in both samples
7	1045.42	1097.50	C–O (ether / phenolic)	Extract shows strong polyphenolic signals; emulsion shows typical eugenol ether peak
8	686.66	941.26	Aromatic out-of-plane bending	Extract shows deeper aromatic bending; emulsion shows terpene-associated aromatic bending

The Cytotoxic Effect of Clove Alcohol Extract on Gastric Cancer Cells Using the MTT Assay

Gastric cancer cells were cultured in 96-well plates. Eight different concentrations of clove oil nanoemulsion (10, 20, 30, 40, 50, 60, 70, and 80 μg/mL) were prepared from a 10 mg/mL base solution, and 100 μL of each concentration was added to the cells in triplicate. After 24 hours of incubation, 20 μL of the prepared MTT solution was added to each well. Three hours later, 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorption was measured at 570 and 630 nm using an ELISA reader.

Cell viability was calculated using the following formula:

$$Cell\ Viability(\%) = \frac{Sample\ Absorption}{Control\ Sample\ Absorption} \times 100 \quad (1)$$

The resulting value was 41.67±1.53 μg/mL; the results showed that the alcoholic extract of cloves has a concentration-dependent cytotoxic effect, with increasing concentrations significantly inhibiting the growth of human gastric cancer cells, as shown in Figure 6.

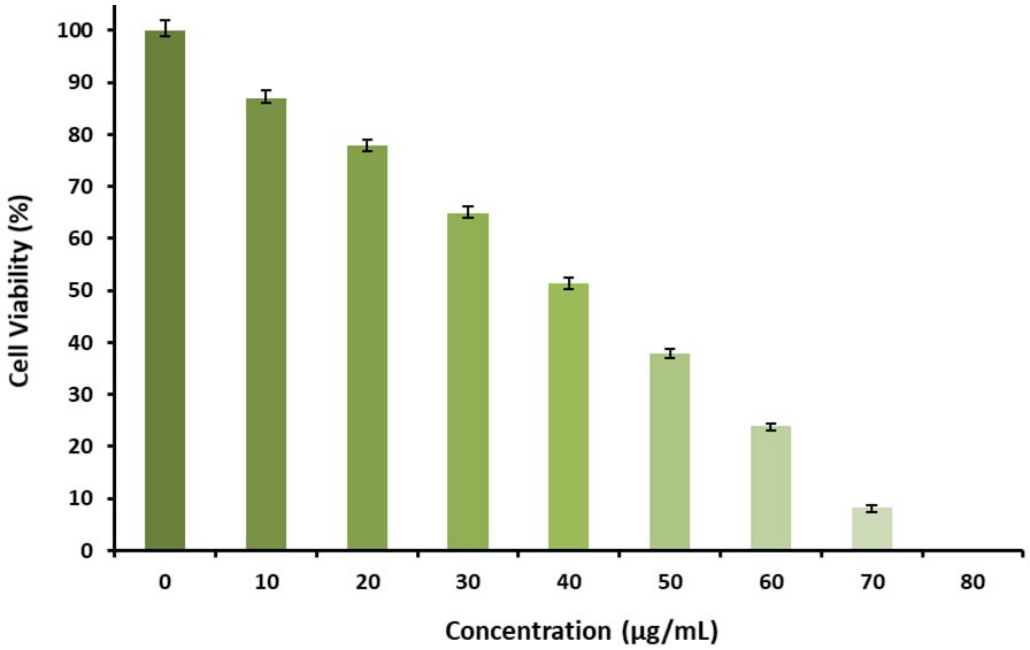

Figure 6. Concentration-dependent effects of the clove alcoholic extract on cancer cell viability, with an IC_{50} equal to 41.67±1.53 μg/mL

The Cytotoxicity of Clove Oil Nanoemulsion on Gastric Cancer Cells Using the MTT Assay

Gastric cancer cells were cultured in 96-well plates. Eight different concentrations of clove oil nanoemulsion (10, 20, 30, 40, 50, 60, 70, and 80 μg/mL) were prepared, and 100 μL of each concentration was added to the cells in triplicate. After 24 hours of incubation, 20 μL of the prepared MTT solution was added to each well. Three hours later, 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorption was measured at 570 and 630 nm using an ELISA reader.

The resulting IC_{50} value was 36.30±1.47 μg/mL; the results showed that the clove oil nano-emulsion has a concentration-dependent cytotoxic effect and a greater effect than the alcoholic extract of clove, as increasing the concentration significantly inhibits the growth of human gastric cancer cells, as shown in Figure 7.

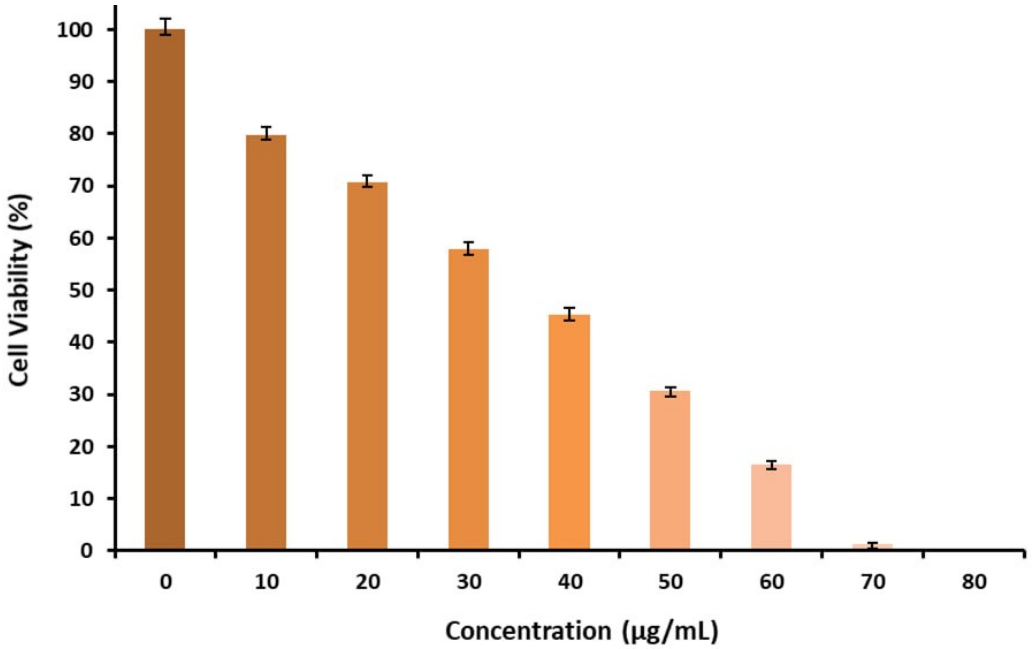

Figure 7. Concentration-dependent effects of the clove oil nanoemulsion on cancer cell viability, with an IC_{50} equal to 36.30±1.47 μg/mL

CONCLUSION

Results from this study (a) demonstrate that the chemical and biological production and evaluation of the alcoholic clove extract and clove oil nanoemulsion have been successful. Using the Clavinger method, the alcoholic clove oil was produced, and after the removal of the active components of the alcoholic extract, the clove oil became a stable nanoemulsion. The particle size of the nanoemulsion was measured by DLS analysis to have an average nanosize of 82.7 nm, which contributes to the increases in their biological activity. In addition to DLS, the chemical groups present in both the alcoholic extract and clove oil nanoemulsion were identified using FTIR. A direct comparison of the active groups can be made between the alcoholic extract and nanoemulsion to show how the chemical composition of each impacted their biological activity. After creating both substances at eight different concentrations and testing both against a stomach cancer cell line, both products show the ability to inhibit the growth of cancer cells. The IC_{50} values obtained from the study indicate that the alcoholic extract has an IC_{50} value of 41.67±1.53 μg/mL; the IC_{50} value for the clove oil nanoemulsion is 36.30±1.47 μg/mL, where the concentration required to cause cancer cell death in 50% of the cells was determined. This illustrates the superiority of the nanoemulsion and its ability to produce a more potent inhibitory effect at a lower dose. This superiority demonstrates how important nanotechnology is to enhancing the transport of the active ingredient and increasing its ability to affect target cells. Given the aforementioned, the study recommends investing in nanotechnology to produce plant extracts and improve their therapeutic efficacy. It also encourages further research into cellular mechanisms of action, alternative cell lines, and the potential application of these findings in innovative therapeutic fields.

SUPPLEMENTARY MATERIAL

None.

AUTHOR CONTRIBUTIONS

Zahraa Ibrahim Hameed: Investigation, Visualization, and Writing – original draft. Amer Hamied Hussein: Methodology, Validation, Writing – review & editing, and Formal analysis.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF GENERATIVE AI USE

The authors declare that no generative AI or AI-assisted technologies were used in the preparation of this manuscript.

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