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Artículos originales

Cytotoxicity and Wound Healing Effect of Camphor Menthol Based Natural Deep Eutectic Solvents Against Human Epidermoid Carcinoma (A431) Cell Line

Citotoxicidad y Efecto Cicatrizante de Disolventes Eutécticos Naturales a Base de Alcanfor y Mentol Contra la Línea Celular de Carcinoma Epidermoide Humano (A431)

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Conflict of interest

The authors declare no conflict of interest.

Abbreviations

CM-NADES: Camphor menthol based natural deep eutectic solvent
A431: Human Epidermoid Carcinoma Cell
MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
IC50: Inhibitory Concentration
DMEM Dulbecco's Modified Eagle Medium
FBS: Fetal Bovine Serum
DMSO: Dimethyl Sulfoxide
OD: Optical Density
Sy.x: standard error of the estimate

Abstract

Introduction: Skin cancer is a serious health issue and finding effective treatments is crucial. This study investigated the effects of camphor-menthol based natural deep eutectic solvent on human epidermoid carcinoma A431-cells, a model for skin cancer.

Method: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to assess cell viability and cytotoxicity by measuring absorbance at 570 nm. The absorbance at 570 nm was measured to determine the optical density values, and cell viability was calculated as a percentage. Additionally, an *in vitro* scratch assay was performed to evaluate the impact of camphor-menthol based natural deep eutectic solvent on cell migration.

Results: The results revealed a concentration-dependent decrease in both optical density values and cell viability, with an inhibitory concentration of 311.7 µg/ml. The treated group exhibited a significant reduction in wound area compared to the control, suggesting potential antimigratory effects. Since cell migration is key to cancer spread, this could be an important finding.

Conclusion: In conclusion, these findings highlight the cytotoxic and migration-inhibiting properties of camphor-menthol based natural deep eutectic solvent, supporting its potential as a therapeutic agent against skin cancer. These findings highlight the potential of natural compounds in cancer treatment. More research is needed to understand how camphor-menthol based natural deep eutectic solvent works and to confirm these results in more complex biological systems.

Keywords: Camphor; menthol; natural deep eutectic solvent; A431 cell line; MTT assay; *in vitro* wound healing activity.

Resumen

Introducción: El cáncer de piel es un problema de salud grave, y encontrar tratamientos eficaces es crucial. Este estudio investigó los efectos de un disolvente eutéctico profundo natural a base de alcanfor y mentol sobre células de carcinoma epidermoide humano A431, un modelo para el cáncer de piel.

Método: Se realizó un ensayo con bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio para evaluar la viabilidad celular y la citotoxicidad mediante la medición de la absorbancia a 570 nm. Se determinaron los valores de densidad óptica y se calculó la viabilidad celular en forma de porcentaje. Además, se llevó a cabo un ensayo de rasguño *in vitro* para evaluar el impacto del disolvente eutéctico profundo natural a base de alcanfor y mentol en la migración celular.

Resultados: Los resultados revelaron una disminución dependiente de la concentración tanto en los valores de densidad óptica como en la viabilidad celular, con una concentración inhibitoria (IC50) de 311,7 µg/ml. El grupo tratado mostró una reducción significativa en el área de la herida en comparación con el control, lo que sugiere posibles efectos anti migratorios. Dado que la migración celular es clave en la propagación del cáncer, este hallazgo podría ser importante.

Conclusión: En conclusión, estos hallazgos destacan las propiedades citotóxicas e inhibidoras de la migración del disolvente eutéctico profundo natural a base de alcanfor y mentol, lo que respalda su potencial como agente terapéutico contra el cáncer de piel. Estos resultados subrayan el potencial de los compuestos naturales en el tratamiento del cáncer. Se necesita más investigación para comprender el mecanismo de acción del disolvente eutéctico profundo natural a base de alcanfor y mentol y confirmar estos resultados en sistemas biológicos más complejos.

Palabras clave: Alcanfor; mentol; disolvente eutéctico profundo natural; línea celular A431; Ensayos de supervivencia celular; Actividad de cicatrización de heridas *in vitro*.

Highlights

Skin cancer is the most common cancer global, with 331,722 new cases and almost 58,667 deaths reported from this disease in 2022. Its incidence and mortality rates have been increasing every year.

A431 cells are considered for their high expression of the Epidermal Growth Factor Receptor (EGFR) and are commonly employed as a model system to investigate EGFR signaling and its role in cancer.

Deep eutectic solvents (DESs) are a novel class of eco-friendly solvent with a wide range of applications. NADESs are an attractive type of DESs. When DES is formed from naturally existing neutral, acidic, or basic components, they are said to be NADES. These solvents have been used to replace organic solvents in a variety of fields.

The CM-NADES was evaluated for *in vitro* cytotoxicity on A431 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay is a widely used method to measure cell viability and growth. The IC50 values were measured at two different time points (0 and 24 h) in A431 cells. Cell migration was tested using a scratch wound healing assay.

The results indicate that CM-NADES has a concentration-dependent cytotoxic effect on A431 cells. As the concentration of CM-NADES increases, the OD values at 570 nm decrease, suggesting reduced cell viability. This trend is also reflected in the % of Cell Viability, which declines with increasing CM-NADES concentration. At 500 µg/ml, a significant reduction in both OD and % Cell Viability confirms a strong cytotoxic effect.

Overall, these findings suggest that CM-NADES reduces A431 cell viability in a dose-dependent manner. Treatment with CM-NADES at a concentration of 311.7 µg/ml significantly enhances wound healing compared to the control group. The CM-NADES treated group shows a higher percentage of wound closure, suggesting it is more effective in promoting healing.

Introduction

Skin cancer is the most common cancer global, with 331,722 new cases reported and almost 58,667 deaths from this disease in 2022. Its occurrence and death rates have been increasing every year. It is divided into two primary categories: melanoma skin cancer (MSC) and non-melanoma skin cancer (N-MSC). While MSC originates from pigment-producing melanocytes, and N-MSC, which develops from keratinocytes. Melanoma was ranked as the 17th most widespread cancer according to Global Cancer 2022 data. N-MSC, is the 5th most frequent type of cancer[1].

Melanoma multiplies swiftly, is extremely aggressive, and is challenging to cure. N-MSC divided into basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCC divide slowly and rarely spreads, while some types of SCC can be extremely aggressive and advanced. N-MSC is the most common malignant cancer in white people, and its occurrence is on rise. It is a significant worldwide health concern and significantly more prevalent than melanoma[1].

A431 is a N-MSC cell line, exactly derived from epidermoid carcinoma (a type of SCC) of the skin, exactly from an 85-year-old female patient[2]. A431 cells are considered for their high expression of the Epidermal Growth Factor Receptor (EGFR) and are commonly employed as a model system to investigate EGFR signaling and its part in cancer[3].

In modern science, immortalized cells are commonly employed in research instead of primary cells. Compared to primary cells, they are easier to breed in huge quantities, persist longer, and can be genetically modified, are fewer advantages. Unlike primary cells, they also provide consistent results since they do not vary between different donors[4]. Additionally, cell lines offer a pure number of cells, which is beneficial since it guarantees a consistent specimen and repeatable outcomes[5].

Deep eutectic solvents (DESs) are a novel class of eco-friendly neoteric solvent with a wide range of uses[6]. DES is formed when two or more solid or liquid components mixed ratios, results in the formation of liquid having a lower melting point than any of the individual components[7]. Among the two components one is hydrogen bond donor (HBD) and another one hydrogen bond acceptor (HBA)[8]. The

HBDs and HBAs, interact through hydrogen bonding to form a stable eutectic system[9]. Combining HBA with HBD results in charge delocalization, which creates DESs with large nonsymmetric ions that have lower lattice energies and melting temperatures than the individual substances[10].

NADESs are an attractive type of DESs. When DES is formed from naturally existing neutral, acidic, or basic components, they are said to be NADES. In recent years, natural deep eutectic solvents (NADESs) have gained attention as green solvents for pharmaceutical and biomedical applications. It has been used to replace organic solvents in a variety of fields[11]. Because NADES follow the principles of green chemistry, making them environmentally friendly and efficient[12].

Terpenoids, often referred to as terpenes or isoprenoids, are present in a variety of plants and living things. Terpenoids, which were first utilized in flavorings and fragrances, are today utilized in a variety of fields, especially medicine. Some terpenoids have recently been found to have promising medicinal uses in both the prevention and treatment of N-MSC. Terpenoids' astringent and antimicrobial qualities are largely responsible for their acknowledged function in accelerating wound healing. These characteristics promote wound constriction and accelerated epithelialization[13].

The anticancer actions of natural materials, specifically two terpenoids menthol and camphor, have been studied using several cancer cell lines. The results show that menthol and camphor have strong cytotoxic effects on cancer cells[14]. Camphor and menthol in combination form NADES. However, no research has explored a CM-NADES against the A431 human epidermoid carcinoma cell line. Given the growing interest in NADESs and the therapeutic potential of terpenoids, this study aims to explore the cytotoxic effect (using the MTT assay) and wound healing potential (via *in vitro* scratch assay) of CM-NADES on A431 cells, thereby identifying a possible natural and green therapeutic approach for N-MSC.

Material and Method

Materials

Camphor was obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, and menthol from Reachem Laboratory Chemicals Pvt. Ltd., Chennai. DMEM medium, Fetal Bovine Serum (FBS), and antibiotic solution were sourced from Gibco (USA). DMSO (Dimethyl sulfoxide) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/ml) were procured from Sigma (USA). 1X PBS was supplied by Himedia (India), while 96-well tissue culture plates and wash beakers were obtained from Tarsons (India).

Cell culture

A431 human skin cancer cells were obtained from the National Center for Cell Science (NCCS), Pune. They were cultured in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS), 100 µg/mL penicillin, and 100 µg/ml streptomycin, and maintained and maintained at 37°C in a 5% CO_2 incubator[15].

MTT assay for cell cytotoxicity

The CM-NADES was evaluated for *in vitro* cytotoxicity on A431 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cultured A431 cells were harvested through trypsinization, collected in a 15 ml tube and plated at a density of 1×10^5 cells/ml (200 µl/well) in a 96-well tissue culture plate. The cells were maintained in DMEM medium supplement with 10 % FBS and 1% antibiotic solution incubate for 24-48 h at 37°C. After incubation, the wells were washed with sterile PBS and treated with different concentrations of the CM-NADES a in serum-free DMEM medium. Each sample was tested in triplicate, and the cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. After the incubation period, MTT solution (20 µl of 5 mg/ml) was added to each well and the cells were incubated for another 2-4 h until purple formazan crystals were clearly visible under an inverted microscope. The medium, along with MTT (220 µl), was then removed, and the wells

were washed with 1X PBS (200 µl). To dissolve the formazan crystals, 100 µl of DMSO was added, and the plate was shaken for 5 minutes. The absorbance of each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA). The percentage of cell viability and IC_{50} values were determined using GraphPad Prism 6.0 software (USA)[16].

Determination of IC50 value for CM-NADES using dose-response curve analysis

Based on Khan[16] the IC50 values were measured at two different time points (0 and 24 h) in A431 cells. To determine the dose-response relationship, the cells were exposed to increasing concentrations of CM-NADES (10, 40, 80, 200, 400, and 500 µg/ml) and notice the difference in both 0 and 24 h. A best-fit dose-inhibition curve was created, and the final IC50 and log-IC50 values were calculated using a non-linear dose-response curve with a 95% confidence interval. The goodness-of-fit test is one of the oldest and most studied problems in statistics[17].

Scratch wound healing migration assay

Cell migration was tested using a scratch wound healing assay described by Bahar and Yoon[18]. A431 cells (2×10^5) were grown in six-well plates until they formed a monolayer covering 90% of the surface. A sterile pipette tip (1 mm) was used to create a scratch in the cell layer. The wells were washed four times to remove loose cells and then incubated in a medium with 1% FBS. Pictures were taken at 0 and 24 h after making the scratch. The cells were treated with CM-NADES and incubated for 24 h. Cell migration was observed using an inverted microscope with a camera, and the wound area was measured using ImageJ software (NIH, Bethesda, MD, USA). The percentage of wound closure was calculated by comparing the untreated and treated CM-NADES.

Results

MTT assay for cell cytotoxicity for CM-NADES

The MTT assay is a widely used method to measure cell viability and growth. It works by converting yellow, water-soluble substance (MTT) into a purple, water-insoluble formazan through enzymatic reactions in living cells. This essay is popular because it allows testing multiple samples at once using 96-well plates and automated systems. The process has two steps: First, active cells change MTT into purple formazan, which clumps together due to its low water solubility, causing inaccurate results. For accurate spectrophotometric detection of formazan, the crystals must be completely dissolved in a suitable solvent. DMSO is the preferred solvent because it fully dissolves bacterial cells and prevents clumping[19].

Optical density (OD) value at 570 nm

The control mean OD value is 0.480. The Table 1 shows the raw absorbance values from the MTT assay, which are the basis for calculating cell viability. Highlight that OD values decrease as concentration increases, indicating dose-dependent cytotoxicity. Figure 1 presents the visual decline in OD with increasing CM-NADES concentration to the findings in Table 1.

Table 1. OD value for CM-NADES at different concentrations.

CM-NADES concentration (µg/ml)	OD Value at 570 nm (in triplicates)			Average
Control	0.48	0.47	0.49	0.4807±0.0081
10	0.47	0.47	0.48	0.4737±0.0070
20	0.45	0.46	0.46	0.4577±0.0055
40	0.45	0.45	0.46	0.4490±0.0061
60	0.45	0.44	0.44	0.4423±0.0047
80	0.44	0.43	0.44	0.4353±0.0021
100	0.42	0.43	0.44	0.4283±0.0078
200	0.42	0.42	0.41	0.4160±0.0036
300	0.40	0.41	0.40	0.4027±0.0060
400	0.38	0.39	0.39	0.3847±0.0061
500	0.23	0.28	0.28	0.2603±0.0272

CM-NADES: camphor-menthol (CM) based natural deep eutectic solvent; OD: optical density values

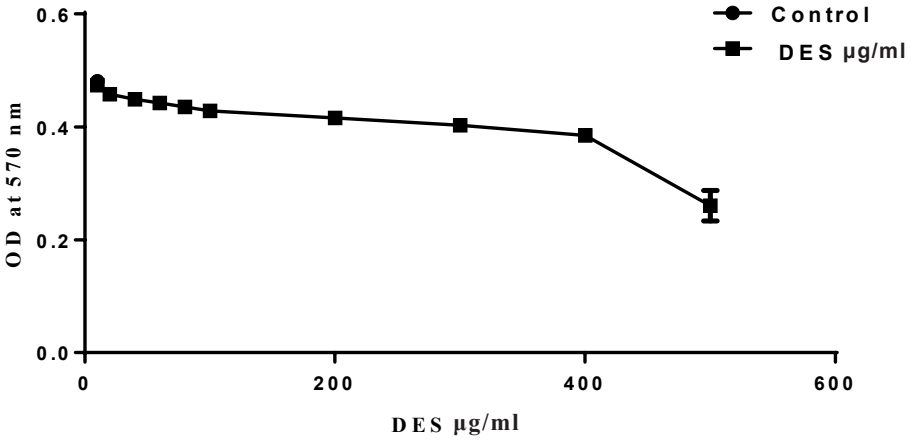

Figure 1. Effect of camphor-menthol (CM) based natural deep eutectic solvent concentration (µg/ml) on Optical Density (OD) at 570 nm.

Cell viability (%)

The cell viability for CM-NADES at different concentrations (µg/ml) is shown in Table 2. This Table 2 presents the cell viability of A431 cells treated with various concentrations of CM-NADES, demonstrating its concentration-dependent cytotoxicity. A marked decline in viability is observed at higher concentrations, particularly at 500 µg/ml. This trend is further illustrated in Figure 2, where the graph confirms the dose-dependent cytotoxic effect. Figure 3 provides visual evidence, showing morphological changes and damage in cells exposed to higher CM-NADES concentrations, supporting the quantitative viability data.

Table 2. Cell viability for CM-NADES at different concentrations

CM-NADES concentration (µg/ml)	Cell viability (%) (in triplicates)			Mean Value (%)
Control	100	100	100	100.000±0.000
10	97.29	98.54	100.20	98.677±1.460
20	94.58	94.79	96.66	95.343±1.145
40	92.70	92.91	95.00	93.537±1.272
60	92.91	91.04	92.50	92.150±0.983
80	91.04	90.2	90.83	90.690±0.437
100	87.91	88.75	91.04	89.233±1.620
200	86.45	87.5	86.04	86.663±0.753
300	82.70	85.2	83.75	83.883±1.255
400	78.75	81.25	80.41	80.137±1.272
500	47.70	57.29	57.70	54.230±5.659

CM-NADES - camphor-menthol (CM) based natural deep eutectic solvent

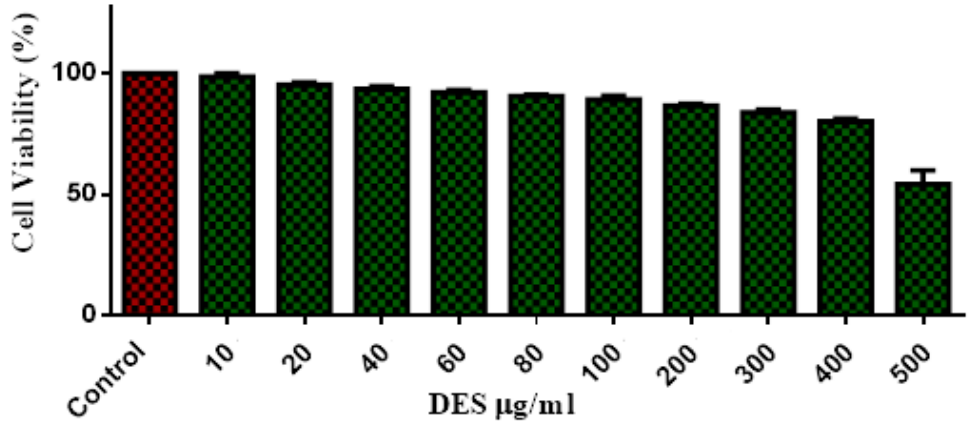

Figure 2. Effect of CM-NADES different concentrations (µg/ml) on cell viability

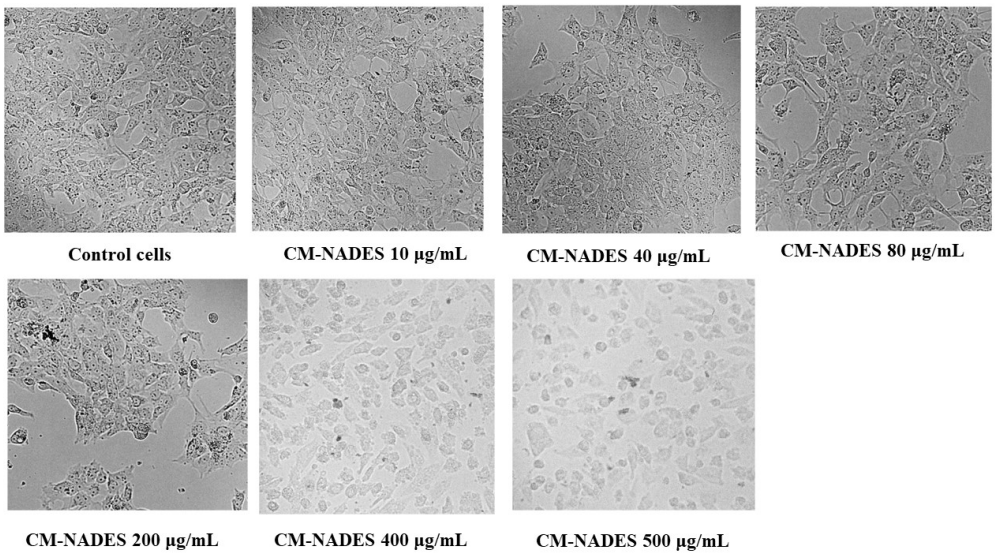

Figure 3. Images of control cells and different concentrations of CM-NADES treated cells.

Determination of IC50 value for CM-NADES using dose-response curve analysis

The IC50 (Inhibitory Concentration-50) is the concentration at which half of the cells stop growing. The percentage of cell growth, measured using the MTT assay, was analyzed by creating a non-linear dose-response curve. A graph was made showing CM-NADES concentration against the normalized response to estimate the IC50 values at 0 and 24 h. The IC50 and log-IC50 values were calculated using a specific equation with a 95 % confidence level. Table 3 presents the IC50 value of CM-NADES, determined as 311.7 µg/ml using dose-response curve analysis, indicating the concentration required to inhibit 50 % of A431 cell viability.

Table 3. IC50 value for CM-NADES using dose-response curve analysis

log (inhibitor) vs. normalized response-variable slope	CM-NADES
Best-fit values	
LogIC50	2.494
HillSlope	-1.459
IC50	311.7 µg/ml
Standard error	
LogIC50	0.05137
HillSlope	0.2832
95% Confidence intervals	
LogIC50	2.389 to 2.599
HillSlope	-2.039 to -0.8793
IC50	244.7 to 397.2

log (inhibitor) vs. normalized response-variable slope	CM-NADES
Goodness of fit	
Degrees of freedom	28
R square	0.7228
Absolute sum of squares	6110
Sy.x	14.77
Number of points analyzed	30

CM-NADES - Camphor-menthol based natural deep eutectic solvent. LogIC50 - Logarithm of the half maximal inhibitory concentration. IC50 - Half maximal inhibitory concentration; Sy.x - Standard error of the estimate.

Wound healing assay for CM-NADES

Wound healing is an essential process to restore tissue function after injury. However, replicating this process in the lab is challenging because it lacks cell debris and complex interactions between different cell types[20]. Figure 4 show wound healing assay for control and CM-NADES–treated cells (311.7 µg/ml) at 0 h and 24 h. The images illustrate cell migration, with the treated group showing greater wound closure, indicating that CM-NADES enhances wound healing. Table 4 show comparison of the densitometry analysis of wound healing assay for control and CM-NADES (311.7 µg/ml) treated. It highlights that treated cells showed higher closure (98.88%) compared to control (84.48%), indicating enhanced wound healing ability of CM-NADES at IC50 concentration. Figure 5 presents the comparative percentage of wound healing at 24 h between control cells and those treated with 311.7 µg/ml of CM-NADES. The treated group shows a marked improvement in wound closure, supporting the findings shown in Table 4 and Figure 5.

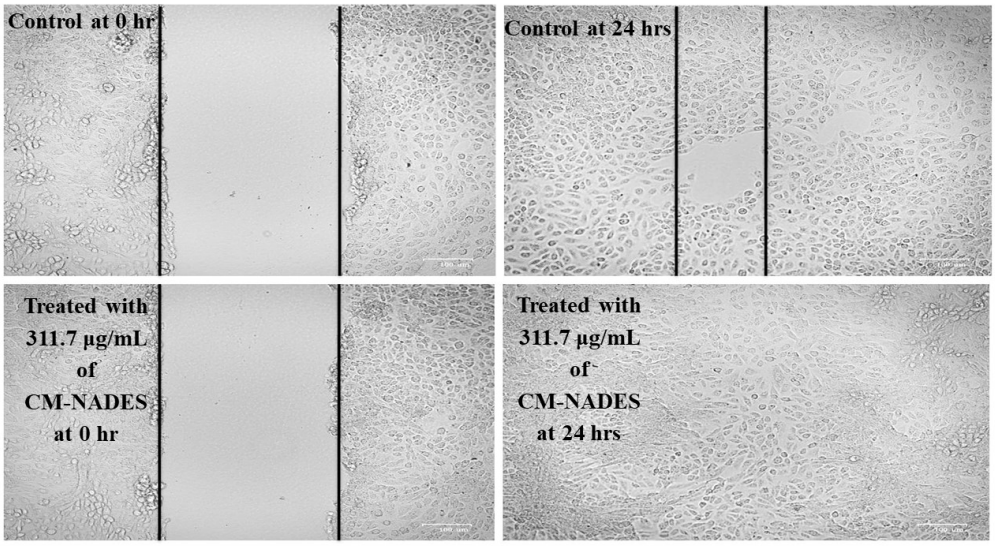

Figure 4. Wound healing assay for control cells and cells treated with 311.7 µg/ml of CM-NADES sample at 0 h and 24 h

Table 4. Comparison of the densitometry analysis of wound healing assay for control and CM-NADES (311.7 µg/ml) treated.

Sample	Time (h)	Area		Average area	Percent (%)		Average percentage
		1st time	2nd time		1st time	2nd time	
Control	0	14396.79	14570.81	14483.80± 123.05	-	-	-
	24	135661.80	125203.28	130432.54±7395.29	85.45	83.51	84.48±1.37
Treated with 311.7 µg/ml of CM-NADES	0	15052.55	16176.96	15614.76± 795.08	-	-	-
	24	217551.13	32653.27	125102.20±130742.53	99.84	97.92	98.88±1.36

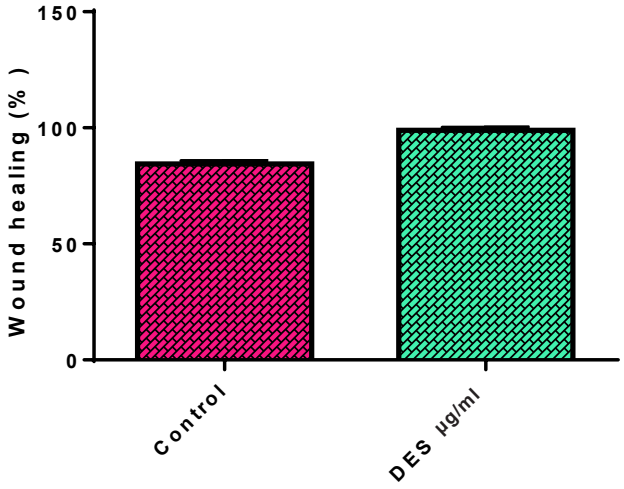

Figure 5. Percentage wound healing for control cells and cells treated with 311.7 µg/ml of CM-NADES at 24 h.

Table 4 shows the results of the wound healing assay analyzed using ImageJ software, comparing the control and the CM-NADES (311.7 µg/ml) treated group. The percentage wound healing for control cells and cells treated with 311.7 µg/ml of CM-NADES at 24 h is shown in Figure 5. Percentage wound healing for control cells and cells treated with 311.7 µg/ml of CM-NADES at 24 h. It evaluates the effect of CM-NADES on wound healing. At 0 h, the initial wound area was measured for both groups. The average wound area for the control group was 14,483.80 ± 123.05, while for the CM-NADES treated group, it was 15,614.76 ± 795.08.

Discussion

The optical density (OD) values at 570 nm are reported for different concentrations of the CM-NADES. The control group (0 µg/ml) shows an OD value of 0.4807±0.0081, serving as the baseline for comparison. At lower concentrations (10–100 µg/ml), the decrease in OD values is minimal, indicating a mild effect on cell viability. However, at higher concentrations (200–500 µg/ml), the reduction in OD becomes more significant, with the lowest OD value observed at 500 µg/ml, showing a strong cytotoxic effect. The study demonstrates that CM-NADES exhibits cytotoxic activity against A431 cells in a dose-dependent manner. Higher concentrations significantly reduce cell viability, suggesting its potential as an anticancer agent.

The results show that increasing the concentration of DES leads to a gradual reduction in cell viability, indicating a dose-dependent cytotoxic effect. At lower concentrations (10–100 µg/ml), the decrease in cell viability is minimal, suggesting a mild impact on the cells. However, at higher concentrations (200–500 µg/ml), a significant decline in cell viability is observed. Notably, at 500 µg/ml, cell viability drops sharply to 54.23%, showing a strong cytotoxic effect. The findings suggest that DES has a dose-dependent cytotoxic effect on cells. While lower concentrations have a mild impact, higher concentrations significantly reduce cell viability. This indicates its potential use in anticancer applications, but further studies are required to understand its mechanism and therapeutic potential.

IC50 values determined for CM-NADES at two time points (i.e., 0 and 24 h) in the A431 cells. Cells were exposed to increasing concentrations of CM-NADES from 10, 40, 80, 200, 400 and 500 µg/ml at 0 h and 24 h exposure to obtain the best-fit dose-inhibition curve. Final IC50 and log-IC50 values were computed through the non-linear dose-response curve with 95% confidence interval (CI).

The best-fit values provide important insights into the potency and effectiveness of CM-NADES as an inhibitor. The LogIC50 value of 2.494 represents the logarithm of the half-maximal inhibitory concentration (IC50), which is 311.7 µg/ml. A lower IC50 value indicates higher potency, meaning that a smaller amount of CM-NADES is required to achieve a 50% reduction in cell viability. The HillSlope value of -1.459 describes the shape of the dose-response curve, with the negative slope indicating that as the concentration of CM-NADES increases, the inhibitory effect also increases. In general, a steeper HillSlope suggests a more sensitive response to the compound. The results from this curve fitting analysis confirm that increasing CM-NADES concentration leads to a significant reduction in cell viability, demonstrating its potential as an effective inhibitor.

The standard error values show how accurate the estimates for IC50 and HillSlope are. A smaller standard error means a more precise estimate. For CM-NADES, the standard error for IC50 is 0.05137, which means the actual IC50 value is likely very close to the estimated 311.7 µg/ml. Similarly, the standard error for HillSlope is 0.2832, meaning the true HillSlope value is expected to be within 0.2832 of the estimated -1.459.

The 95 % confidence interval shows the range where the true value is likely to be, with 95 % certainty. It is calculated based on the standard error. For LogIC50, this interval represents the possible range of values for the logarithm of the IC50 (half-maximal inhibitory concentration). A lower LogIC50 means a stronger inhibitor. For CM-NADES, the 95% confidence interval for LogIC50 is 2.389 to 2.599, meaning we can be 95% sure that the true LogIC50 value falls within this range.

The HillSlope value shows how steep the dose-response curve is, meaning how sensitive the response is to changes in inhibitor concentration. A steeper slope means a stronger effect with small concentration changes. For CM-NADES, the HillSlope is -1.459 with a standard error of 0.2832. The 95% confidence interval for this value ranges from -2.039 to -0.8793, indicating some uncertainty in the estimate. The negative HillSlope means the dose-response curve slopes downward, showing that as the CM-NADES concentration increases, the response decreases same was confirmed by Ikegame[21]. The size of the HillSlope value helps understand how fast the response drops when the inhibitor concentration rises.

The IC50 value shows how much CM-NADES is needed to reduce the response by 50%. For CM-NADES, the IC50 is 311.7 µg/ml, meaning this concentration leads to half-maximal inhibition. The 95% confidence interval ranges from 244.7 to 397.2 µg/ml, meaning the actual IC50 value is likely within this range. IC50 helps determine potency-a lower IC50 means higher potency because less inhibitor is needed, while a higher IC50 means lower potency, requiring more inhibitor for the same effect.

The LogIC50 values for CM-NADES range from 2.389 to 2.599. The HillSlope values indicate the sensitivity of the response to changes in inhibitor concentration. The IC50 values provide insights into the potency of CM-NADES as an inhibitor. In the provided Table 3, the confidence intervals for LogIC50, HillSlope, and IC50 values are relatively narrow, suggesting that the estimates are precise.

Degrees of freedom (DF) help measure how accurately a model fits the data. They are calculated by subtracting the number of estimated parameters from the total number of observations. In this study, there are 30 observations (one for each inhibitor concentration) and 2 estimated parameters (logIC50

and HillSlope). So, the degrees of freedom are 30 - 2 = 28. DF is used to calculate the standard error, which shows how precise the model's predictions are.

DF are important for making statistical analysis accurate and reliable. They affect how precise the results are and help determine if findings are statistically significant. DF plays a key role in hypothesis testing, confidence intervals, and model fitting. Having the right number of DF ensures that the analysis is strong and trustworthy. It is important to consider DF when interpreting results and making conclusions.

The R square value shows how well the regression model fits the data. It tells us how much of the variation in the response variable is explained by the independent variable (log(inhibitor)) and the model. A higher R square value means a better fit. For CM-NADES, the R square value is 0.7228, which suggests a moderate fit of the model to the data.

The absolute sum of squares (ASS) measures how much error exists between the actual data and the model's predictions. It is found by adding up the squared differences between the observed values and the predicted values. For CM-NADES, the ASS value is 6110, which shows how much error is in the model fit. A lower ASS value means the model fits the data better.

The Sy.x (standard error of the estimate) shows how much the actual data points differ from the predicted values in a regression model. It measures the average distance between the observed values and the regression line. A lower Sy.x value means the predicted values are closer to the actual data, indicating a better model fit.

The Sy.x value helps measure how well the model fits the data and how much variation exists in its predictions. It is also used to calculate R-squared, which shows the model's accuracy. For CM-NADES, the Sy.x value is 14.77, indicating the level of variation in the predictions.

The 'Analyzed' column shows the number of data points used in the analysis. In this case, three different concentrations were tested, with each concentration repeated 30 times, leading to a total of 30 data points. More repetitions improve the reliability and accuracy of the results. The model fit suggests that the data is high quality, helping create a more accurate regression model.

The percentage of wound closure at the 0 h time point is not provided. However, at 24 h, the wound area and percentage of closure are reported for both the Control and CM-NADES treated groups. The Control group had a wound area of 125,203.28, with a closure percentage of 83.51 %. In contrast, the CM-NADES treated group had a smaller wound area of 32,653.27, corresponding to a closure percentage of 97.92%. These results indicate that wounds in the CM-NADES treated group healed more effectively than those in the Control group after 24 h.

The average percentage of wound closure was calculated for each group and time point based on multiple replicates. At 24 h, the Control group had an average wound closure of 84.48±1.37%, meaning the wound was about 84.48% closed. In comparison, the CM-NADES treated group showed a higher average wound closure of 98.88±1.36%, indicating nearly complete healing at this time point.

The results indicate that treatment with CM-NADES at a concentration of 311.7 µg/ml significantly improves wound healing compared to the Control group. The CM-NADES treated group shows a higher percentage of wound closure, suggesting it is more effective in promoting healing. This difference may be due to the beneficial properties of CM-NADES. The study suggests that the densitometry analysis confirms CM-NADES treatment effectively promotes wound healing. This is evident from the higher wound closure observed in the CM-NADES treated group compared to the Control group.

Conclusion

This study demonstrates that CM-NADES exhibits a concentration-dependent cytotoxic effect on A431 cells, as evidenced by reduced OD values (570 nm) and decreased cell viability percentages with increasing CM-NADES concentrations. The IC50 was determined to be 311.7 µg/ml, indicating moderate potency. At this concentration, CM-NADES also significantly enhanced wound healing *in vitro*, as shown by greater wound closure compared to the control group. At 500 µg/ml, a significant reduction in both OD and cell viability percentages confirms a strong cytotoxic effect. Additionally, the small standard deviations indicate that the data is precise and reliable. These findings suggest that the treatment with CM-NADES at a concentration of 311.7 µg/ml significantly improves wound healing compared to the Control group. The CM-NADES treated group shows a higher percentage of wound closure, suggesting it is more effective in promoting healing. Hence CM-NADES (311.7 µg/ml) holds potential as a dual-action therapeutic agent with both anticancer and wound healing properties. However, the study is limited to *in vitro* conditions using a single cell line. Further studies, including mechanistic investigations and *in vivo* validation, are necessary to confirm its therapeutic applicability and safety profile.

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