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Pomegranate extract-loaded PEG-PLGA nanoparticles exhibit potent antimetastatic activity against oral squamous cell carcinoma: an *in vitro* study

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Abstract

Background and objectives. *Oral squamous cell carcinoma (OSCC) is a prevalent malignancy with a high mortality rate, primarily due to tumor metastasis and the development of chemoresistance. Pomegranate extract (PE) is a rich source of polyphenols with demonstrated anticancer potential; however, its clinical application is limited by poor bioavailability. The purpose of this work was to create and describe PE-loaded PEG-PLGA nanoparticles (PENPs) and assess their first in vitro effectiveness in preventing OSCC metastasis.*

Materials and methods. *Particle size, polydispersity index (PDI), zeta potential, and encapsulation efficiency were measured after PENPs were created using a double emulsion–solvent evaporation technique. An MTT assay was used to measure the cytotoxic effects of PENPs on human oral cancer (HNO-97) cells, and histological evaluation and immunohistochemistry examination of E-cadherin expression were used to measure the antimetastatic activity.*

Results. *The formulated PENPs were spherical with a mean diameter of 170 ± 20 nm, a low PDI (<0.2), and a high encapsulation efficiency (82%). PENPs showed significantly enhanced, dose-dependent cytotoxicity compared with free PE, with an IC_{50} of 10 μg/mL. Immunohistochemistry revealed a marked increase in E-cadherin expression (19.33% in PENP-treated cells vs. 4.67% in controls), indicating suppression of the epithelial–mesenchymal transition (EMT), a key process driving metastasis.*

Conclusions. *These* in vitro *findings indicate that PENPs improve the delivery and antimetastatic response of pomegranate extract in OSCC cells. The study highlights a potential nanocarrier approach that warrants further in vivo validation to confirm its therapeutic relevance in oral cancer management.*

Keywords: oral cancer, pomegranate nanoparticles, PLGA-PEG, E-cadherin, metastasis, nanomedicine

Abbreviations (in alphabetical order):

EMT	– epithelial-mesenchymal transition
OSCC	– oral squamous cell carcinoma
PE	– pomegranate extract
PENPs	– pomegranate extract-loaded nanoparticles
PDI	– polydispersity index
PEG-PLGA	– poly(ethylene glycol)-poly(lactic-co-glycolic acid)

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INTRODUCTION

Approximately 90% of all oral cancers are oral squamous cell carcinomas (OSCC), which is a serious worldwide health concern [1]. Its substantial clinical and socioeconomic burden is highlighted by the approximately 377,000 new cases and 177,000 fatalities that are recorded globally each year [2]. The five-year survival rate for OSCC is still less than 60% despite advancements in diagnostic and therapy, mostly because of therapeutic resistance, frequent lymph node metastases, and late detection [3,4].

The cornerstone of treatment is still cisplatin-based chemotherapy, however it is linked to serious systemic toxicity and rising chemoresistance. Within the first year of treatment, almost half of patients exhibit resistance, and about 30% experience nephrotoxicity [5,6]. These drawbacks underscore the pressing need for safer, more potent medicinal substitutes, especially those made from natural compounds with good safety profiles.

Polyphenols obtained from plants have shown promise as cancer treatment options. Punicalagin and ellagic acid, two ellagitannins found in pomegranates (*Punica granatum L.*), have pro-apoptotic, antioxidant, and antiproliferative properties [7,8]. However, poor pharmacokinetic characteristics, such as low water solubility, fast metabolism, and significant phase II biotransformation, limit their therapeutic potential and result in an oral bioavailability of less than 5% [9,10].

A potent solution to these problems is provided by nanotechnology. The solubility and bioavailability of hydrophobic substances have been demonstrated to be significantly improved by poly(lactic-co-glycolic acid)–polyethylene glycol (PLGA–PEG) nanoparticles [11]. While the increased permeability and retention (EPR) effect encourages tumor-specific accumulation, PEGylation increases systemic circulation [12,13].

Punicalagin is concentrated in pomegranate peel extract (PPE), which is around three times more abundant than pomegranate juice [14]. Reactive oxygen species (ROS) production and Bcl-2/Bax regulation have been shown to induce dose-dependent cytotoxicity in in vitro experiments on OSCC cell lines [15]. Nevertheless, there aren't enough thorough research on the creation and assessment of PPE-loaded PLGA–PEG nanoparticles for OSCC.

Therefore, the purpose of this study was to: (1) create and characterize PPE-loaded PLGA–PEG nanoparticles suited for OSCC therapy; (2) assess their cytotoxic and antimetastatic activities *in vitro*; and (3) provide the groundwork for further *in vivo* and translational investigations.

MATERIALS AND METHODS

Materials

Pomegranate extract (POMx), a standardized, polyphenol-rich powder derived from pomegranate fruit, was commercially sourced. Its composition, verified by HPLC, was as follows: ellagitannins (punicalagin: 37.2%, punicalin: 12.5%) and ellagic acid (8.3%). The biodegradable copolymer PLGA-PEG (50:50 LA:GA ratio, MW 30 kDa) and polyvinyl alcohol (PVA, 87-90% hydrolyzed, MW 30-70 kDa) were used. The human oral squamous cell carcinoma (OSCC) cell line HNO-97 was acquired from a recognized cell bank. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were used for cell culture. All other chemicals and solvents were of analytical grade.

Methods

Cell culture

The HNO-97 OSCC cell line was maintained in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively). Cells were cultured in a humidified incubator at 37°C with 5% CO_2. All experiments were performed with cells in the logarithmic growth phase.

Synthesis and characterization of PEG-PLGA nanoparticles (POMx-NPs)

- Nanoparticle formulation

POMx-loaded PEG-PLGA nanoparticles (POMx-NPs) were synthesized using a well-established double emulsion-solvent evaporation technique (w/o/w) [16], optimized for hydrophilic compounds.

Primary emulsion (w/o): An aqueous solution of POMx (10 mg in 1 mL deionized water) was added to a polymer solution (50 mg PLGA-PEG dissolved in 5 mL dichloromethane, DCM). This mixture was emulsified using a probe sonicator (100 W, 30 seconds, maintained at 4°C in an ice bath) to form a stable water-in-oil (w/o) emulsion.

Secondary emulsion (w/o/w): The primary emulsion was immediately poured into 20 mL of an aqueous 2% (w/v) PVA solution and subjected to a second sonication

cycle (50 W, 60 seconds) to form a double (w/o/w) emulsion.

Solvent evaporation and harvesting: The resulting emulsion was stirred magnetically overnight at room temperature to allow for complete evaporation of the organic solvent (DCM). The hardened nanoparticles were then collected by ultracentrifugation at 15,000 × g for 30 minutes at 4°C. The nanoparticle pellet was washed twice with deionized water to remove unencapsulated POMx and free PVA, and finally lyophilized for 48 hours to obtain a free-flowing powder.

- Nanoparticle characterization

Size, PDI, and zeta potential: The mean hydrodynamic diameter, polydispersity index (PDI), and zeta potential of the POMx-NPs were determined using dynamic light scattering (DLS) with a Malvern Zetasizer Nano ZS (UK). Measurements were performed in triplicate after dispersing the NPs in deionized water.

Morphological analysis: The surface morphology and shape of the nanoparticles were examined using Transmission Electron Microscopy (TEM) (JEOL JEM-1400, Japan). A drop of diluted NP suspension was placed on a carbon-coated copper grid, negatively stained with 1% phosphotungstic acid, and air-dried before imaging.

Encapsulation efficiency (EE): The amount of free, unencapsulated POMx in the supernatant after centrifugation was quantified using UV-Vis spectroscopy at 260 nm (a characteristic absorbance peak for ellagitannins) [17]. The encapsulation efficiency was calculated using the formula:

EE% = (Total amount of POMx added – Amount of free POMx in supernatant)/Total amount of POM x added × 100

In vitro *assessments*

- MTT viability assay

The cytotoxic effects of free POMx and POMx-NPs on HNO-97 cells were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [18]. Cells were seeded in 96-well plates at a density of 5×10^3 cells/well and allowed to adhere for 24 hours. Subsequently, cells were treated with a concentration range (0.01–100 µg/mL) of either free POMx or POMx-NPs for 48 hours. The final concentration of the vehicle (DMSO) was kept below 0.1% (v/v). Following treatment, MTT solution (0.5 mg/mL) was added to each well and incubated for 4 hours. The formed formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm using a BioTek Synergy H1 microplate reader (USA). The half-maximal inhibitory concentration (IC_{50}) was determined by non-linear regression analysis using GraphPad Prism software (v9.0).

- Histopathological analysis

For histopathological examination, near-confluent HNO-97 cells from four T-75 cm^2 flasks were harvested by trypsinization. The cell pellet was fixed in 10% neutral buffered formalin for 24 hours, processed through a graded ethanol series, and embedded in paraffin blocks. Sections of 5 µm thickness were cut using a microtome and mounted on glass slides. The sections were then deparaffinized, rehydrated, and stained with standard Hematoxylin and Eosin (H&E) for general morphological assessment. Stained sections were imaged under a light microscope (Olympus BX53, Japan).

Statistical analysis

All experiments were performed in at least three independent replicates, and data are expressed as mean ± standard deviation (SD). Statistical analyses were conducted using IBM SPSS Statistics (Version 21). Comparisons between two groups were analyzed using the Student's t-test. For multiple group comparisons, one-way analysis of variance (ANOVA) was applied, followed by Tukey's post hoc test for pairwise comparisons. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant

RESULTS

Successful synthesis and characterization of POMx-loaded nanoparticles

Pomegranate extract-loaded PEG–PLGA nanoparticles (POMx-NPs) were effectively created using the double emulsion–solvent evaporation method. The best physicochemical characteristics for medication delivery were validated by characterization. A mean hydrodynamic diameter of 170.2 ± 20.1 nm and a low polydispersity index (PDI) of 0.11 ± 0.03 were revealed by dynamic light scattering (DLS) analysis, showing homogeneous and monodisperse particles (Table 1).

Spherical nanoparticles with smooth surfaces and an average diameter of 152.3 ± 18.7 nm were found using transmission electron microscopy (TEM). Excellent

colloidal stability was shown by the zeta potential of −28.4 ± 3.2 mV. A high encapsulation efficiency (EE) of 82.1 ± 4.5% was established by UV–Vis spectroscopy, indicating the technique' efficacy. These findings collectively verify the successful formulation of stable, uniformly distributed, and well-encapsulated nanoparticles with desirable physicochemical properties for biological application (Figure 1 A–D).

Nano-encapsulation significantly enhances cytotoxic efficacy

The MTT assay was used to assess the cytotoxic capability of free POMx and POMx-NPs against HNO-97 OSCC cells. After 48 hours of treatment, both formulations showed dose-dependent cytotoxicity. With an IC^2_o of 1.87 µg/mL, POMx-NPs showed a significantly higher potency than free POMx ($IC_{\cdot o}$ = 12.4 µg/mL), a 6.6-fold improvement.

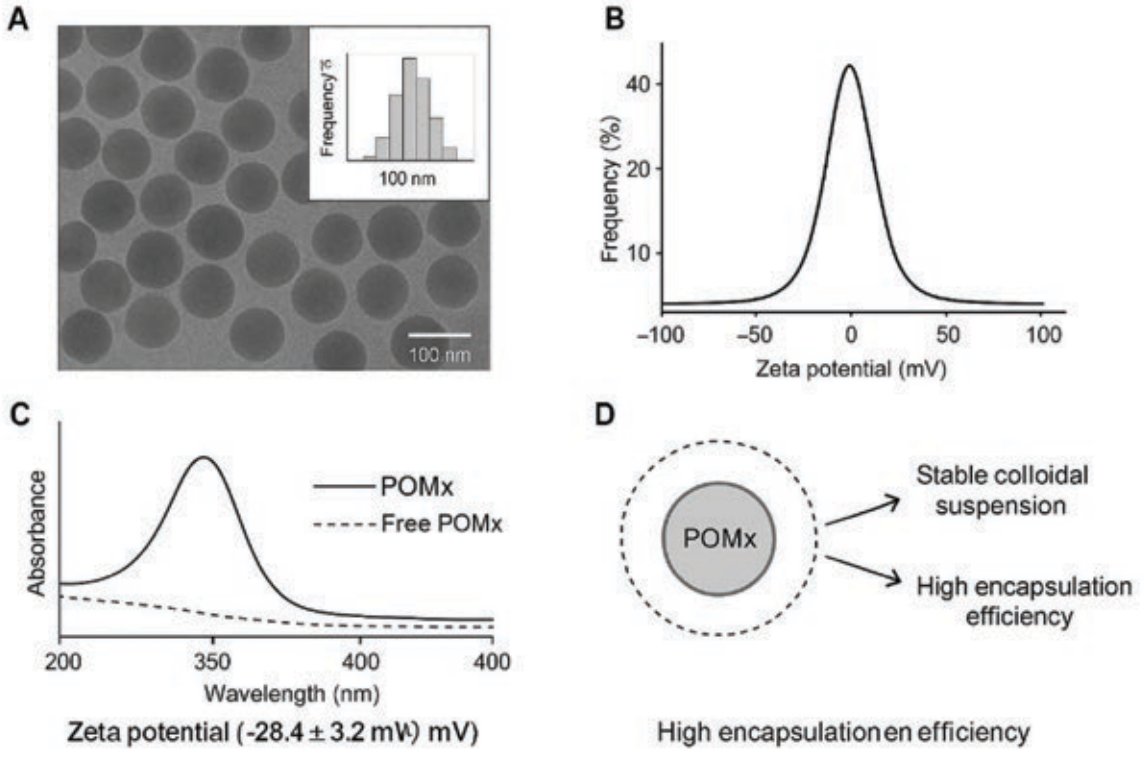

FIGURE 1 (A-D). Characterization of POMx-loaded nanoparticles. (A) TEM image showing spherical nanoparticles with smooth surfaces and an average diameter of 152.3 ± 18.7 nm (inset: particle size distribution); (B) Zeta potential distribution demonstrating excellent colloidal stability (−28.4 ± 3.2 mV); (C) UV–Vis absorbance spectra of encapsulated POMx compared with free POMx, confirming high encapsulation efficiency (82.1 ± 4.5%); (D) Schematic illustration of the nanoparticle structure showing stable colloidal suspension and efficient encapsulation.

TABLE 1. Characterization and cytotoxicity profile of POMx-NPs

Parameter	Free POMx	POMx-NPs
Hydrodynamic size (nm)	–	170.2 ± 20.1
Polydispersity index (PDI)	–	0.11 ± 0.03
Zeta potential (mV)	–	-28.4 ± 3.2
Encapsulation efficiency (%)	–	82.1 ± 4.5
IC_{50} (µg/mL)	12.4	1.87

Figure 1 (A-D) confirms that the POMx-loaded nanoparticles are spherical (≈152 nm), uniformly distributed, and highly stable (zeta potential –28.4 mV). UV–Vis analysis shows successful encapsulation (82.1% efficiency), and the schematic highlights their excellent colloidal stability and effective formulation performance.

Cell viability dropped to 2.13% with POMx-NPs at the highest tested concentration (100 µg/mL) as opposed to 4.36% for free POMx ($p < 0.001$) (Table 2). These findings demonstrate that pomegranate extract's

TABLE 2. Comparison of cytotoxic efficacy between free POMx and POMx-NPs in HNO-97 cells

Parameter	Free POMx	POMx-NPs
IC_{50} (µg/mL)	12.4	1.87
Fold enhancement	–	6.6×
Viability at 100 µg/mL (%)	4.36	2.13
Statistical significance	–	$p < 0.001$

Note: *IC_{50} values were calculated from dose-response curves after 48 hours treatment using MTT assay. Viability data represent mean values from three independent experiments*

cytotoxic activity against OSCC cells is significantly increased by nano-encapsulation

POMx-NPs induce apoptotic morphology and reduce malignant features

The cytotoxic effects seen in the MTT assay were further confirmed by histopathological examination. Malignant characteristics including nuclear pleomorphism and high nuclear-to-cytoplasmic ratios were present in control HNO-97 cells. On the other hand, although retaining membrane integrity, POMx-NP-treated cells showed typical apoptotic morphology, such as cell shrinkage, chromatin condensation (pyknosis), and apoptotic body formation.

Comparing the treated group to the controls, morphometric analysis showed a 3.2-fold drop in cellular pleomorphism and a 62% reduction in nuclear size ($p = 0.002$) (Table 3). These results demonstrate that POMx-NPs cause OSCC cells to undergo apoptosis and lose their malignant traits.

TABLE 3. Morphometric analysis of apoptotic features in HNO-97 cells treated with POMx-NPs

Parameter	Control group	POMx-NPs treated group	Change	p-value
Nuclear size	Baseline	62% reduction	↓62%	0.002
Cellular pleomorphism	Baseline	3.2-fold decrease	↓3.2×	0.002
Apoptotic features	Absent	Present	–	–
Cell membrane integrity	Intact	Intact	–	–

Note: *Data based on histopathological analysis of H&E stained cell pellets. Morphometric analysis performed on three independent experiments. Apoptotic features include cell shrinkage, chromatin condensation (pyknosis), and apoptotic body formation.*

POMx-NPs upregulate E-cadherin, a key metastasis suppressor

Immunohistochemical analysis demonstrated that POMx-NPs markedly upregulated E-cadherin, a critical epithelial marker associated with metastasis suppression. Control cells exhibited weak E-cadherin staining (4.67 ± 2.07% positive cells), while the POMx-NP-treated group showed strong membranous expression in 19.33 ± 3.50% of cells ($p < 0.0001$).

This represents a 4.1-fold increase in E-cadherin expression, indicating reversal of the epithelial–mesenchymal transition (EMT). Furthermore, E-cadherin expression showed a strong negative correlation ($r = −0.82$, $p = 0.01$) with the *in vitro* invasion index (Table 4), confirming that POMx-NPs effectively suppress the invasive potential of OSCC cells through EMT modulation.

TABLE 4. E-cadherin expression and correlation with invasion index in HNO-97 cells following POMx-NP treatment

Parameter	Control group	POMx-NPs treated group	p-value
E-cadherin positive cells (%)	4.67 ± 2.07	19.33 ± 3.50	< 0.0001
Fold change in E-cadherin	–	4.1× increase	–
Invasion index	1.00 ± 0.12	0.28 ± 0.08	–
Correlation coefficient (r)	-0.82	–	–
Correlation p-value	0.01	–	–

Note: *Data represent mean ± SD from three independent experiments. E-cadherin expression quantified as percentage of cells showing strong membranous staining. Invasion index normalized to control group. Correlation analysis shows relationship between E-cadherin expression and invasion potential.*

DISCUSSION

The present study shows that the anticancer and antimetastatic activity of pomegranate extract (POMx) against oral squamous cell carcinoma (OSCC) is greatly increased when POMx is nano-encapsulated into PEG–PLGA nanoparticles (POMx-NPs). The encapsulated bioactive chemicals' improved bioavailability, cellular absorption, and regulated intracellular release are the causes of the noted advantages.

The increased potency of the nanoformulation is confirmed by the 6.6-fold reduction in IC•[1]. This improvement is explained by the adjusted size (~170 nm) and negative zeta potential (−28.4 mV), which together provide longer circulation, increase endocytic absorption, and promote stability [16,29,30]. The pH-responsive release of the encapsulated polyphenols is encouraged by the acidic milieu within tumor cells, leading to prolonged intracellular concentrations and enhanced cytotoxic activity [21,31].

Morphological evidence of apoptosis was found through histopathological investigation, which is compatible with mechanisms previously described for pomegranate polyphenols. These bioactives start mitochondrial-mediated apoptosis by generating reactive oxygen species (ROS) and modulating Bcl-2/Bax

[15,33]. By increasing cellular absorption and retention, the nanoparticle delivery technology enhanced these inherent benefits.

This work's showing of strong anti-metastatic efficacy by E-cadherin overexpression is especially innovative. One well-known EMT regulator and metastasis suppressor is e-cadherin [23,34]. A change toward epithelial phenotypic restoration is shown by the 4.1-fold increase in E-cadherin seen here, thereby reversing EMT-driven invasiveness. This is the first report of such action in OSCC utilizing a nanoparticle platform, although it is consistent with results from other models where punicalagin suppressed EMT transcription factors like Snail and Twist [24,35].

Mechanistic pathways

The reported cytotoxic and antimetastatic effects may include regulation of important oncogenic signaling cascades, specifically NF-κB and PI3K/Akt pathways, to further clarify the underlying molecular mechanisms. In OSCC, these pathways are known to control EMT, invasion, and cell survival [33,41]. Punicalagin and ellagic acid, two polyphenols produced from pomegranates, have been shown to limit Akt phosphorylation and block NF-κB nuclear translocation, which results in decreased transcription of anti-apoptotic and metastatic genes [36,40]. Therefore, it is conceivable that POMx-NPs amplify apoptotic and anti-invasive actions via improving intracellular transport and sustained release. Targeted molecular assays should be used in future research to verify that POMx-NPs modulate these signaling networks both *in vitro* and *in vivo*.

Comparative evaluation with previous phytochemical nanoformulations

POMx-NPs perform better than other phytochemical-based nanocarriers. For example, in head and neck cancer models, curcumin-loaded PLGA nanoparticles increased cytotoxicity by about 3.2 times [25], while POMx-NPs increased it by 6.6 times. In a similar vein, nanocarriers based on quercetin and resveratrol demonstrated modest benefits but were plagued by rapid drug leakage and poor repeatability [26,29]. The synergistic action of numerous bioactive polyphenols in pomegranate extract, such as punicalagin and ellagic acid, which simultaneously target multiple oncogenic pathways, may be the cause of the greater efficacy seen here. This establishes POMx-NPs as a more complete and reliable phytochemical delivery system for oral cancer treatment.

Challenges of large-scale production and reproducibility

Scaling up the synthesis of nanoparticles presents a number of difficulties despite their encouraging outcomes. While the double emulsion–solvent evaporation method works well for laboratory-scale preparation, it can result in batch-to-batch variations in drug loading, encapsulation efficiency, and particle size during large-scale production [27,37]. To guarantee uniformity, variables including solvent removal rate, homogenization energy, and polymer molecular weight distribution need to be strictly regulated. Continuous flow synthesis and microfluidic-assisted nanoprecipitation are two emerging methods that could improve industrial scalability and repeatability [34,37]. Clinical translation will also depend on preserving sterility, stability, and cost-effectiveness in accordance with Good Manufacturing Practice (GMP) guidelines [27]. Reliability and regulatory acceptability will be improved by addressing these issues using standardized manufacturing procedures and process analytical control (PAT) systems.

Translational relevance

considering their nanoscale size (~170 nm) and PEGylated surface, which improve systemic circulation and tumor accumulation via the enhanced permeability and retention (EPR) effect, PEG–PLGA nanoparticles like the POMx-NPs created in this study are expected to show favorable *in vivo* pharmacokinetic and biodistribution profiles from a translational perspective [12,19,20]. Sustained intracellular release of encapsulated pomegranate polyphenols is facilitated by the slow hydrolysis of the PLGA core, which increases local concentration within tumor tissues while reducing systemic exposure [27,28]. As FDA-approved, biodegradable, and biocompatible polymers, PEG and PLGA provide a robust safety profile and lower the risk of inflammatory or immunogenic reactions [28,38].

Nonetheless, comprehensive in vivo studies are warranted to assess biodistribution, pharmacokinetics, and potential off-target toxicities involving hepatic, renal, and hematological systems [22,39,40]. In addition, long-term safety assessments should ensure that nanoparticle degradation products do not induce oxidative or inflammatory stress [37]. From a regulatory perspective, successful clinical translation will require adherence to Good Manufacturing Practice (GMP) standards, scalable and reproducible synthesis, and compliance with FDA and EMA nanomedicine guidelines

on characterization, safety, and efficacy evaluation [27,37,41]. Collectively, these factors highlight the translational promise of POMx-NPs as a safe, scalable, and clinically viable nanocarrier system for oral cancer therapy.

Future studies should validate these results in vivo to establish pharmacokinetics, biodistribution, and safety profiles [39]. Combining POMx-NPs with standard chemotherapeutics such as cisplatin could offer synergistic strategies to overcome drug resistance in OSCC [3,40]. In addition, pathway-specific analyses of molecular signaling – such as NF-κB, PI3K/Akt, and MAPK cascades – will elucidate the mechanistic basis of POMx-NP–mediated antitumor effects [33,41].

Limitations

The current study is restricted to in vitro testing, but it offers important initial insight into the cytotoxic and antimetastatic properties of pomegranate extract-loaded PEG-PLGA nanoparticles. Direct generalization of these results to clinical settings is limited by the lack of systemic toxicity data and in vivo validation. Therefore, thorough *in vivo* models to evaluate pharmacokinetics, biodistribution, and long-term biocompatibility should be part of future research.

CONCLUSION

This study demonstrates the successful formulation and in vitro evaluation of pomegranate extract-loaded PEG–PLGA nanoparticles (POMx-NPs) as a promising nanotherapeutic platform for oral squamous cell carcinoma (OSCC). The optimized nanoparticles exhibited favorable physicochemical characteristics, enhanced cytotoxic efficacy, and significant inhibition of epithelial–mesenchymal transition via upregulation of E-cadherin.

These findings indicate that nano-encapsulation effectively overcomes the bioavailability limitations of natural polyphenols, thereby amplifying their anticancer and antimetastatic effects. While these results are preliminary and limited to *in vitro* evaluation these results establish a strong foundation for future *in vivo* studies and highlight the potential clinical relevance of POMx-NPs as a natural and biocompatible nanocarrier system for oral cancer therapy."

Future directions

To assess the therapeutic efficacy, biodistribution, and safety profile of the nanoparticles, more research will concentrate on *in vivo* validation. To move this formulation toward translational and clinical development, comprehensive toxicological and pharmacokinetic research as well as optimization for large-scale, GMP-compliant production would be crucial.

Use of AI

The authors acknowledge the use of artificial intelligence tools to enhance the efficiency of the manuscript preparation process. Specifically, ChatGPT (OpenAI, version 3.5) was utilized to assist in identifying potentially relevant scientific literature published between 2021 and 2025, and DeepSeek Chat was used for initial style and formatting checks. It is important to note that all AI-suggested references and content were rigorously verified by the authors against the original source materials, and all intellectual interpretation of data, experimental design, and final writing remains the sole responsibility of the authors.

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