Food Bioscience 61 (2024) 104950

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Effects of microencapsulated phenethyl isothiocyanate on gastrointestinal cancer cells and pathogenic bacteria

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ARTICLE INFO

Keywords:
Phenethyl isothiocyanate
Gastrointestinal cancer
Microparticles
Cell migration
Chemoprevention
Nutraceutical

ABSTRACT

Gastrointestinal cancers remain a global health burden, demanding more effective prevention and treatments. Phenethyl isothiocyanate (PEITC), a compound derived from cruciferous vegetables, stands out as a promising nutraceutical agent due to its chemopreventive and therapeutic properties. However, its therapeutic translation remains limited mainly due to its poor water solubility and rapid metabolism. Herein, we encapsulated PEITC into biocompatible chitosan-based microparticles with an extra virgin olive oil core to improve its bioavailability and stability. Pure PEITC's biocompatibility and microencapsulated PEITC's stability and antibacterial activity were evaluated. The antibacterial activity analysis showed microencapsulated PEITC as a promising antibacterial agent against gastrointestinal pathogenic bacteria (two Gram-positive and two Gram-negative). The impact of both pure and microencapsulated PEITC was assessed on gastrointestinal cancer cells (MKN45 gastric cancer and SW48 colon cancer cell lines). PEITC exhibited threshold or hormetic dose-dependent toxicity in colon fibroblasts and decreased gastric cancer cells' migration capacity, enhanced upon encapsulation into microparticles. In addition, microencapsulated PEITC induced downregulation of phosphorylated AKT, FAK, and ERK1/2 proteins, disrupting motility signaling pathways and tubulin expression. These findings suggest that the delivery of PEITC via chitosan-based microparticles holds promise as a nutraceutical delivery strategy against gastrointestinal disorders that predispose to cancer.

1. Introduction

Gastrointestinal cancers represent a significant global health challenge, with limited treatment options and less-than-optimal patient outcomes (Alsina et al., 2023; O'Morain and O'Morain, 2019; Sung et al., 2021). Despite the advancements in cancer therapeutics, pursuing more effective treatments remains paramount. Recently, there has been growing interest in exploring the potential of naturally occurring bioactive compounds as complementary nutraceutical therapies for gastrointestinal cancer (Kang et al., 2021).

Isothiocyanates, bioactive compounds derived from cruciferous vegetables like broccoli, brussels sprouts, and watercress, show promise in inhibiting the development and proliferation of cancer cells, both *in vitro* and *in vivo* (Palliyaguru et al., 2018). Among these compounds, phenethyl isothiocyanate (PEITC) has emerged as an encouraging candidate due to its remarkable chemopreventive and therapeutic properties, including antimicrobial effects (Romeo et al., 2018), antioxidant and anti-inflammatory properties (Coscueta et al., 2022; Kala et al., 2018), and anticancer activity (Connolly et al., 2021; Gupta et al., 2014). Over the past decade, research has increasingly focused on the antimicrobial properties of PEITC (Romeo et al., 2018). Studies have demonstrated that PEITC effectively disrupts the membrane properties of several bacteria, triggers bacterial stress responses, and impacts biofilm development by disrupting quorum-sensing systems (Borges et al., 2014, 2015; Kaiser et al., 2017; Krause et al., 2021). Moreover, PEITC has shown promise as an anticancer agent by inhibiting tumor cell

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https://doi.org/10.1016/j.fbio.2024.104950
Received 3 April 2024; Received in revised form 14 August 2024; Accepted 15 August 2024
Available online 21 August 2024

proliferation, migration, invasion, and metastasis, while also inducing apoptosis through various signaling pathways (Dai et al., 2016; Du et al., 2024; Jeong et al., 2017; Lai et al., 2010; Lee et al., 2015; Yang et al., 2010). PEITC can also induce S-phase cell cycle arrest, decrease mitochondrial transmembrane potential, cause DNA damage, and activate Caspase-3, underscoring its broad therapeutic potential in cancer treatment (Du et al., 2024).

Nonetheless, the nutraceutical translation of PEITC faces several challenges resulting from its inherent physicochemical properties, poor water solubility, and rapid metabolism (Kala et al., 2018; Morris & Dave, 2014; Oliviero et al., 2018). These limitations contribute to insufficient systemic bioavailability and suboptimal delivery to the tumor site, compromising its therapeutic efficacy. Researchers have dedicated substantial efforts to address these limitations associated with such promising cancer therapeutic agents (Wang & Bao, 2021). It has been considered essential to incorporate PEITC into biocompatible carriers as a novel and promising approach to improve the pharmacokinetic properties and stability of the encapsulated drug (Batista et al., 2018; Zambrano et al., 2019). This approach aims to protect PEITC from degradation, increase its systemic bioavailability, and ensure its therapeutic efficacy (Batista et al., 2018; Zambrano et al., 2019).

Previously, we successfully developed a microparticle system for gastrointestinal delivery using the ionic gelation methodology with chitosan, a biocompatible, biodegradable, mucoadhesive, and non-toxic polymer, with an extra virgin olive oil core for PEITC encapsulation (Coscueta et al., 2021). Our comprehensive characterization of these microparticles, which included assessments of particle size, encapsulation efficiency, storage stability, Fourier-transform infrared spectroscopy, and *in vitro* biocompatibility, motivated us to further explore the innovative potential of PEITC encapsulated in this chitosan-based delivery system for biomedical and nutraceutical applications.

In this study, we investigated the biocompatibility of pure PEITC and evaluated its antibacterial activity within the previously developed microparticle system against well-established gastrointestinal pathogens. Moreover, microencapsulated PEITC's bioavailability and stability was assessed using gastrointestinal cancer cells. Lastly, we explored the impact of microparticle-encapsulated PEITC on the malignant behavior of these cancer cells.

2. Materials and methods

2.1. PEITC microparticle production

Microparticles (MP) were produced using the ionic gelation methodology, as previously described (Coscueta et al., 2021). Briefly, low molecular weight (107 kDa) chitosan (Sigma-Aldrich, St. Louis, USA) with 75%–85% deacetylation degree was dissolved at 5 mg/mL in acetic acid 1% for 72 h stirring at room temperature (RT, 22 °C) (pH 5.0). PEITC (Santa Cruz Biotechnology Inc., Dallas, Texas, USA) was encapsulated by adding 12 mL of chitosan, extra virgin olive oil (Oliveira da Serra, Algés, Portugal) (olive oil:chitosan mass ratio of 1.45), and PEITC (PEITC:chitosan mass ratio of 0.25), in a final volume of 27 mL. The mixture was submitted to ultrasonication (VCX 130, Sonics & Materials, Newtown, USA) for 1 min at 70% intensity, and 3 mL of Sodium Tripolyphosphate (TPP) (Sigma-Aldrich) was added at RT (22 °C). Finally, the system was ultrasonicated for 1 min at 70% intensity. The obtained MP had a final concentration of 3.063 mM PEITC.

2.2. PEITC microparticles' physical characterization

Microparticles of PEITC were kept in a liquid state, and their physical properties were analysed by dynamic light scattering (DLS), assessing particle size (Dh), polydispersity index (PdI), and zeta-potential (ZP). Dh values reported are representative of the Z-average based on the % intensity of scattered light by each particle fraction or family. Measurements were performed at 25 °C using a disposable folded capillary cell (Malvern Panalytical Ltd., Malvern, UK) employing NIBS (Non-Invasive Back-Scatter) technology at a fixed detection angle of 173°. The Zetasizer NanoZSP photometric correlation spectrometer (Malvern Panalytical Ltd.) was used to measure MP size. Data were acquired and analysed using Zetasizer v. 7.11 software (Malvern Panalytical Ltd.). All measures were performed in triplicate.

2.3. PEITC microparticles' stability analysis

The microbial stability of the MP suspension was determined over 13 weeks at different temperatures: RT (22 °C), 4 °C, and −20 °C. Microbial contamination was quantified by determining mesophilic aerobic bacteria and Enterobacteriaceae. Serial decimal dilutions of the sample were made in sterile peptone water, seeded in triplicate on the respective medium, and subsequently incubated under optimal conditions for each specific microorganism. Total aerobic mesophilic bacteria were quantified on plate count agar (PCA) (Biokar Diagnostics, Allonne, France) and aerobically incubated at 30 °C for 24 h. Enterobacteriaceae were streaked on violet red bile-glucose agar (VRBGA, Lab M, Bury, UK) and incubated aerobically at 37 °C for 24 h. The droplet technique described by Miles et al. (1938) was used for samples incubated in PCA. For samples incubated in VRBGA, the embedding plate technique was used.

2.4. Antibacterial activity

Growth inhibition curves were used to determine the microencapsulated PEITC's antimicrobial activity. Briefly, Gram-positive *Bacillus cereus* (ATCC 2599) and *Listeria monocytogenes* (NCTC 10357), and Gram-negative *Escherichia coli* (ATCC 25922) and *Salmonella enterica* (ATCC 13076) strains were cultured in the presence of 1% (v/v) of an inoculum containing 10^8 CFU/mL. Free PEITC and MP-containing PEITC (MP + PEITC) were added in half-serial dilutions (dilution factors 2–128, i.e., 1500-23 μM PEITC and 1532-24 μM MP + PEITC). Muller-Hinton broth (Sigma-Aldrich) inoculated without PEITC was used as a growth control, while Muller-Hinton broth without inoculum was used as a blank. Optical density (OD) was evaluated at 600 nm with 1 h time intervals over 24 h at 37 °C using a microplate reader (Multiskan GO, Thermo Scientific, Vantaa, Finland). Three independent experiments were performed for each microorganism.

2.5. Cell lines and culture conditions

Non-transformed human colon fibroblasts (CCD-18Co) were obtained from the American Type Culture Collection (ATCC) and cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA, USA) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Gibco, Waltham, MA, USA). The MKN45 gastric cancer and SW48 colon cancer cell lines were obtained from the Japanese Collection of Research Bioresources (Tsukuba, Japan) and the ATCC, respectively. Both MKN45 and SW48 cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco), supplemented with 10% heat-inactivated FBS (Gibco), and maintained at 37 °C in a 5% CO_2 atmosphere.

2.6. Metabolic activity assay

The effect of pure PEITC on the metabolic activity of human normal colon fibroblasts (CCD-18Co) was assessed using a resazurin reduction assay as a security assessment method. CCD-18Co cells were seeded in triplicate in a 96-well plate at a density of 5×10^3 cells/well and treated with increasing concentrations of pure PEITC (0–100 μM). Cells treated with 1 mM hydrogen peroxide (H_2O_2) (Merck, Darmstadt, Germany) or 1% dimethyl sulfoxide (DMSO) (PanReac AppliChem, Darmstadt, Germany) were used as positive and negative controls of metabolic inhibition, respectively. After 24 h, fibroblasts were incubated with 0.02 mg/

mL resazurin (Sigma-Aldrich) for 3 h at 37 °C. The fluorescence intensity of resorufin (excitation 530 nm; emission wavelength 590 nm) was measured using a Synergy™ MX microplate reader (Agilent, Santa Clara, CA, USA).

2.7. Viability assay

The viability effect of pure PEITC and microencapsulated PEITC on gastrointestinal cancer cell lines was determined by plating MKN45 and SW48 cells (1.25×10^5 cells/well) in 12-well plates. After 24 h, cells were incubated with increasing concentrations of pure PEITC, empty polymeric MP, or microencapsulated PEITC (1, 10, and 30 μM). For cell death control, 1 mM H_2O_2 was used as a positive control, while 1% DMSO served as the negative control. After 24, 48, and 72 h of culture, live and dead cells were counted in a Neubauer chamber using trypan blue (Gibco).

2.8. Wound-healing assay

Wound-healing assays were performed using 2-well silicone ibidi culture inserts (ibidi, Gräfelfing, Germany). MKN45 (8×10^4 cells/well) and SW48 cells (12×10^4 cells/well) were plated on each side of the 2-well silicone ibidi culture inserts. After 24 h, the inserts were removed, creating an empty space (wound). Cells were treated with PEITC, empty MP, or MP + PEITC (10 and 30 μM) and incubated at 37 °C in a 5% CO_2 atmosphere. Treatment with 1% DMSO served as the negative control. Wound closure was monitored over 72 h, with the free space between cells measured at 24, 48, and 72-h time points. ImageJ software was used to calculate the % of open area by measuring the free space between the cells at each time point and normalizing it to the initial free area (right after the insert removal).

2.9. Western blotting

MKN45 and SW48 cells were lysed using RIPA buffer and cell scrappers to obtain total protein cell lysates. Protein was quantified using the Pierce TM BCA Protein Assay kit (Thermo Scientific, Waltham, MA, USA). An amount of 20 μg of protein lysates was then separated based on their molecular weight by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (GE Healthcare, Amersham, UK). Membranes were blocked with 5% non-fat milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (Sigma-Aldrich) and probed overnight with total and phosphorylated AKT, FAK, and ERK1/2 primary antibodies (Table 1) diluted in 5% BSA (Sigma-Aldrich) in PBS containing 0.1% Tween 20. The chemiluminescence signal was obtained using the ECL detection reagent and detection films (GE Healthcare Life Sciences). α-Tubulin and GAPDH (Table 1) were used as loading controls. Two independent batches of protein lysates were used for each cell model.

2.10. Statistics

Data are presented as mean ± SEM, except for the dispersion index size (Z-average, nm), zeta-potential (mV), and growth curves time points, which are shown as mean ± SD. For the cell viability assays, three independent experiments with two technical replicates per condition were performed, and a two-way ANOVA was used for statistical analysis. For the wound-healing assays, two independent experiment were conducted, each with three technical replicates per condition. Statistical analysis of the untreated and treated groups over time was performed using a two-way ANOVA. Normality of the data sets was assessed using the Shapiro-Wilk test, while homoscedasticity was evaluated using the Levene test. Statistical analysis of metabolic activity, viability, and wound-healing assays was performed using Prism software (GraphPad, v9.0), while the other statistical analyses were performed using RStudio 2023.09.1+494. In all statistical tests, p values ≤ 0.05 were considered to be statistically significant (* means $p \leq 0.05$; ** means $p \leq 0.01$; *** means $p \leq 0.001$; **** means $p \leq 0.0001$), with 95% confidence interval.

Table 1
List of antibodies.

Antibody	Clone	Source	Dilution
AKT	#9272	Cell Signaling	1:1000
Phosphorylated AKT	#9271 (S473)	Cell Signaling	1:1000
FAK	#3285	Cell Signaling	1:1000
Phosphorylated FAK	#3283	Cell Signaling	1:1000
ERK1/2	#4695	Cell Signaling	1:1000
Phosphorylated ERK1/2	#9101	Cell Signaling	1:1000
α-Tubulin	DM1A	Sigma Aldrich	1:10000
GAPDH	0411 (SC-47724)	Santa Cruz	1:5000

3. Results

3.1. Biocompatibility of pure PEITC

Pure PEITC's biocompatibility was assessed on fibroblasts isolated from normal colon tissue (CCD-18Co) to evaluate the compound's cytotoxic effects on healthy cells. Different doses relevant to prevention or therapy were tested in a range of concentrations from 0 to 100 μM of PEITC. We observed that CCD-18Co cell morphology was significantly impacted when cells were treated with concentrations above 50 μM PEITC, as evident from the noticeable cellular reduction and apparent compromised membrane integrity (Fig. 1A). These observations were further supported by a significant decrease in cellular metabolic activity at concentrations above 50 μM PEITC (Fig. 1B).

3.2. Evaluation of microparticle stability

The stability of the MP under various storage conditions (RT, 4 °C, and −20 °C) was evaluated to understand the microbiological and physicochemical properties of the developed system. Importantly, no microbial contamination was detected throughout the study (data not shown). Different storage temperatures had distinct effects on the MP, with −20 °C causing destabilization, aggregation, and an increase in particle size, whereas storage at RT and 4 °C maintained their initial size distribution. As shown in Fig. 2A, the system stored at −20 °C in the first week reached a PdI of 1, suggesting possible destabilization and aggregation leading to an extensive size dispersion. Additionally, Fig. 2B illustrates a phenomenon of aggregation and an increase in average particle size when stored at −20 °C. However, the zeta potential (Fig. 2C) did not indicate destabilization of the particles in the suspension, suggesting that once equilibrated at the analysis temperature, the system is stable in the new size dispersion. Fig. 2D reveals that MP stored at RT and 4 °C maintained their initial size distribution, while the MP stored at −20 °C showed a distribution centered on sizes outside the detection limit of the equipment. Based on these findings, the subsequent experiments were performed with MP stored at 4 °C, presenting a PdI of 0.26 ± 0.01, Z-average of 496 ± 9 nm, and zeta-potential of 36 ± 3 mV.

3.3. Evaluation of the antibacterial activity of microencapsulated PEITC

The antibacterial activity of free PEITC (PEITC) and microencapsulated PEITC (MP + PEITC) was evaluated against foodborne pathogens commonly found in the gastrointestinal tract, including *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. The minimum inhibitory concentration (MIC) was determined for each bacterial strain. The results represented in Fig. 3 demonstrate that *B. cereus* and *E. coli* exhibited MIC values of 1500 μM and 383 μM for PEITC and MP + PEITC treatments, respectively. For L. *monocytogenes*, the MIC values were 750 μM for PEITC and 96 μM for MP + PEITC

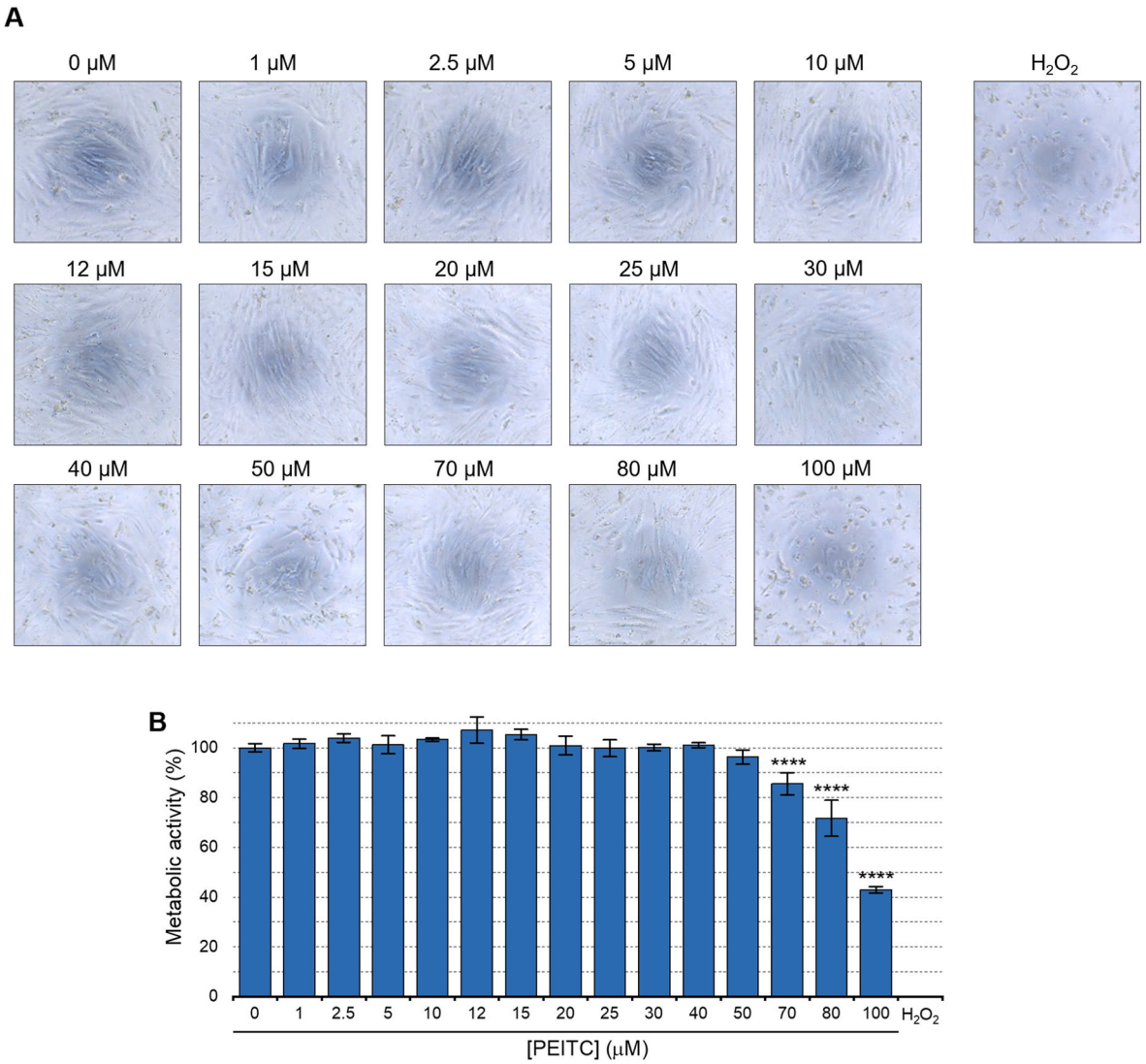

Fig. 1. Impact of different doses of pure PEITC in normal colon fibroblasts (CCD-18Co). (**A**) CCD-18Co cell morphology observed under a light microscope after 24 h of treatment with increasing concentrations of pure PEITC. Magnification 20×. (**B**) Assessment of the metabolic activity, measured by resazurin reduction assay, after 24 h of treatment with increasing concentrations of pure PEITC in CCD-18Co cells. As a positive control of metabolic inhibition, 1 mM H_2O_2 was used. Results are shown as mean ± SEM, and two-way ANOVA was used for statistical analysis. ****$p < 0.0001$ compared with the negative control (0 μM PEITC, 1% DMSO).

treatments. Interestingly, *S. enterica* displayed the highest resistance, with MICs greater than 1500 μM for PEITC and 1532 μM for MP + PEITC treatments.

3.4. Effect of microencapsulated PEITC on morphology and viability of gastrointestinal cancer cells

The effect of the microencapsulated PEITC in gastrointestinal cancer cell lines was assessed by treating MKN45 and SW48 cells with concentrations below 50 μM PEITC, as determined by the biocompatibility assay. 1, 10, and 30 μM concentrations of pure PEITC (PEITC), empty polymeric MP (empty MP), and MP containing PEITC (MP + PEITC) were tested for 72 h, and cell morphology and viability were assessed. Both pure PEITC and encapsulated PEITC induced marked changes in cell morphology in MKN45 and SW48 cells, with loss of cell adhesion and a marked increase in cell aggregates formation (Fig. 4A and C). These morphological changes were significant at 30 μM of pure and microencapsulated PEITC, compared with the lowest PEITC concentrations and empty MP conditions. Regarding cellular cytotoxicity, the results showed that cell viability was affected by high concentrations of pure PEITC (30 μM), as well as MP + PEITC (30 μM) (Fig. 4B and D). This effect on cell viability was even more evident over time (24 h–72 h).

3.5. Effect of microencapsulated PEITC on the migration capacity of gastrointestinal cancer cells

The impact of pure and microencapsulated PEITC on malignant phenotypes of cancer cells, such as their migration capacity, was evaluated. A wound-healing assay using the MKN45 and SW48 cell lines was performed, and 10 and 30 μM of pure PEITC, empty MP, and MP + PEITC were tested for 72 h. The results showed that the migration capacity of MKN45 cells significantly decreased with increasing concentrations of both pure PEITC and PEITC encapsulated in MP (Fig. 5A and B). Furthermore, we observed an accentuated negative effect on the migration of gastric cancer cells when using PEITC encapsulated in MP, compared to the effects of pure PEITC at concentrations of both 10 and 30 μM. Curiously, we also found that empty MP were able to affect the migration capacity of these cells negatively. However, when comparing the same concentrations of empty MP and PEITC-containing MP (10 or 30 μM), the most significant decrease in the migration capacity of gastric cancer cells was found when PEITC was present. Regarding SW48 cells, there were no significant differences in their migration capacity when

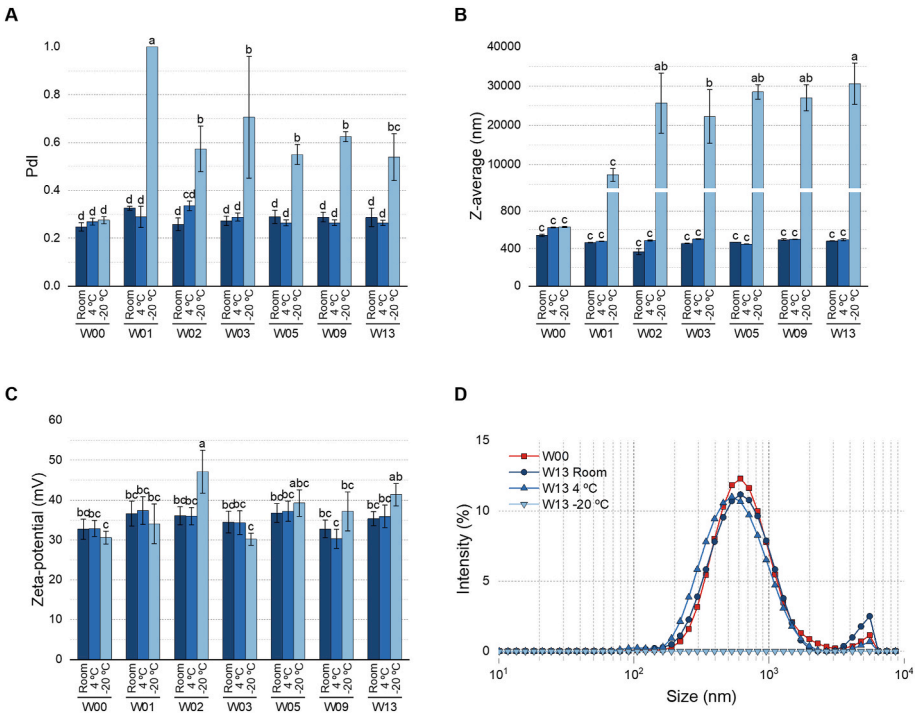

Fig. 2. Physicochemical stability of polymeric microparticles. Results are shown over 13 weeks (W00–W13) of polydispersion index (PdI), mean size of particle distributions (Z-average, nm), zeta-potential (mV), and initial and final profile of particle distributions for microparticles with pure PEITC (**A**, **B**, **C**, and **D**, respectively) with storages of the microparticles at Room Tempertature, 4 °C and −20 °C. The bars at the same week that share the same letter (a, b, c) do not present significant difference between their means ($p > 0.05$), according to the acquired data. Statistical analysis of the variance (One-Way ANOVA) was performed with Tukey's post-hoc test.

treated with 10 μM of pure PEITC, empty MP, or encapsulated PEITC (Fig. 5C and D). At higher concentrations, SW48 cells exhibited higher sensitivity to the PEITC treatment, detaching almost immediately, rendering it impossible to assess their migration capacity under such conditions.

3.6. Effect of microencapsulated PEITC on the activation of key proteins involved in signaling pathways associated to the motility capacity of cancer cells

Pure and microencapsulated PEITC effect on the expression of crucial proteins involved in different motility signaling pathways was examined. Evaluation encompassed the expression levels of total and phosphorylated FAK, AKT, and ERK1/2 (p-FAK, p-AKT, and p-ERK1/2) proteins. Furthermore, tubulin, a major component of microtubules, was assessed, with GAPDH serving as a loading control.

Treatment with 30 μM of pure PEITC and microencapsulated PEITC led to a significant decrease in p-AKT, p-FAK, and p-ERK1/2 expression in MKN45 gastric cancer cells (Fig. 6A). Regarding the SW48 colon cancer cell line, similar results were found with 30 μM of pure and microencapsulated PEITC, showing a reduction in the activation of AKT, FAK, and ERK1/2 (Fig. 6B). Interestingly, we found that the treatment with 30 μM of pure and encapsulated PEITC significantly impacted the expression of the cytoskeletal protein tubulin in both cell lines (Fig. 6A and B).

4. Discussion

PEITC, despite its chemopreventive and therapeutic properties, encounters challenges in clinical use due to poor solubility and rapid metabolism, resulting in restricted availability at tumor sites (Kala et al., 2018; Morris & Dave, 2014; Oliviero et al., 2018). To address these issues, researchers are delving into innovative strategies, including encapsulating PEITC in biocompatible carriers, aiming to enhance its stability, bioavailability, and efficacy against tumors (Batista et al., 2018; Zambrano et al., 2019).

In the present study, we explored the biocompatibility of PEITC and evaluated its stability, antibacterial activity, and bioavailability within chitosan-based MP. This delivery approach aimed to mitigate PEITC degradation, ensuring its therapeutic effectiveness. A preliminary biocompatibility assay revealed that PEITC's toxicity on normal human fibroblasts is concentration-dependent, with a threshold response or even a hormetic (J-shaped) response (Ramaiah et al., 2017). Concentrations above 50 μM compromised cellular integrity and metabolic activity, highlighting the need for dosage optimization to ensure cellular safety. By studying the microbiological and physicochemical properties of the developed chitosan-based MP system, it was possible to demonstrate its microbiological stability, as no microbial contamination was detected throughout the study, which is crucial for its potential use in human consumption. Furthermore, among the different storage temperatures tested, 4 °C was identified as the optimal storage temperature to ensure the stability and functionality of the MP system.

Given the well-established association between bacterial infections and gastrointestinal cancers (Duijster et al., 2021; Fox & Wang, 2007; Mager, 2006; Parsonnet, 1995; Vogelmann & Amieva, 2007), we evaluated whether PEITC encapsulated in our MP system could influence microbial populations within the gastrointestinal tract. Importantly, the observed MIC values, which indicate the PEITC concentration required for bacterial growth inhibition, highlight the varying sensitivity of the different pathogens tested, including *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica* strains. Notably, the MIC values for microencapsulated PEITC were lower compared to those for free PEITC against most of these pathogens. Furthermore, the MIC values obtained for free PEITC align with the literature reports for *E. coli* and *L. monocytogenes*, with a mean value of 614 μM (Borges et al., 2015). Our study suggests that microencapsulated PEITC exhibits promising antibacterial activity against various gastrointestinal pathogens, underscoring its potential to modulate microbial balance within the

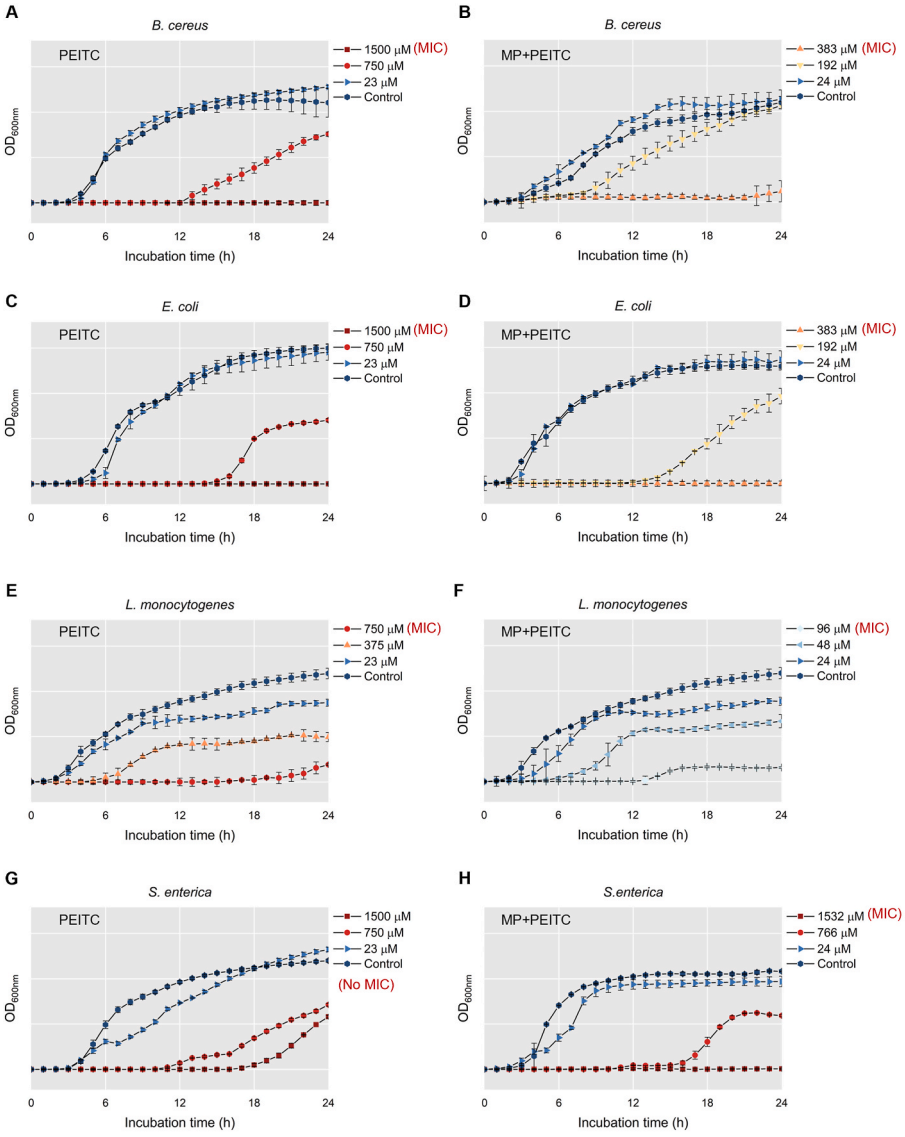

Fig. 3. Growth curves of Gram-positive and Gram-negative bacteria commonly found in the gastrointestinal tract. *Bacillus cereus* (**A** and **B**), *Escherichia coli* (**C** and **D**), *Listeria monocytogenes* (**E** and **F**), and *Salmonella enterica* (**G** and **H**) growth was monitored during 24 h in the presence of free PEITC (PEITC) and microencapsulated PEITC (MP + PEITC). The annotated symbols represent the different concentrations of PEITC (free and microencapsulated) and the control (broth inoculated without PEITC nor MP + PEITC). The concentrations shown in each graph correspond to the lowest concentration tested, the observed MIC, and the first concentration tested at which bacterial growth was observed.

gastrointestinal tract, critical for maintaining gastrointestinal health. However, while our *in vitro* antibacterial efficacy studies provide valuable insights, they may not fully reflect achievable MIC concentrations in complex biological systems. Indeed, the observed MIC values exceeding 90 μM PEITC were beyond the maximum safe concentration previously established for normal fibroblasts (50 μM PEITC). Importantly, achieving MIC concentrations therapeutically may not always be necessary, as lower concentrations of PEITC within biocompatible ranges can still exert significant modulatory effects on pathogens (Lorian, 1993; Romero et al., 2011). However, additional studies are warranted to evaluate cytotoxicity in more advanced cellular models and *in vivo,* and to develop therapeutic strategies that optimize efficacy while ensuring safety.

To understand the potential of our MP system to effectively incorporate and release PEITC, we tested the microencapsulated PEITC on gastrointestinal cancer cells. The notable morphological changes observed in microencapsulated PEITC-treated cancer cells, including loss of cell adhesion and increased cell aggregates formation, suggest that PEITC encapsulated in MP disrupts the structural integrity and organization of cancer cells. This observation aligns with prior findings on the effects of pure PEITC treatment, indicating that our MP system effectively releases the encapsulated drug while maintaining its efficacy (Pawlik et al., 2012; Xue et al., 2014). The more pronounced morphological alterations observed at higher concentrations of microencapsulated PEITC indicated a dose-dependent effect, underscoring the critical role of considering dosage when using PEITC as a therapeutic

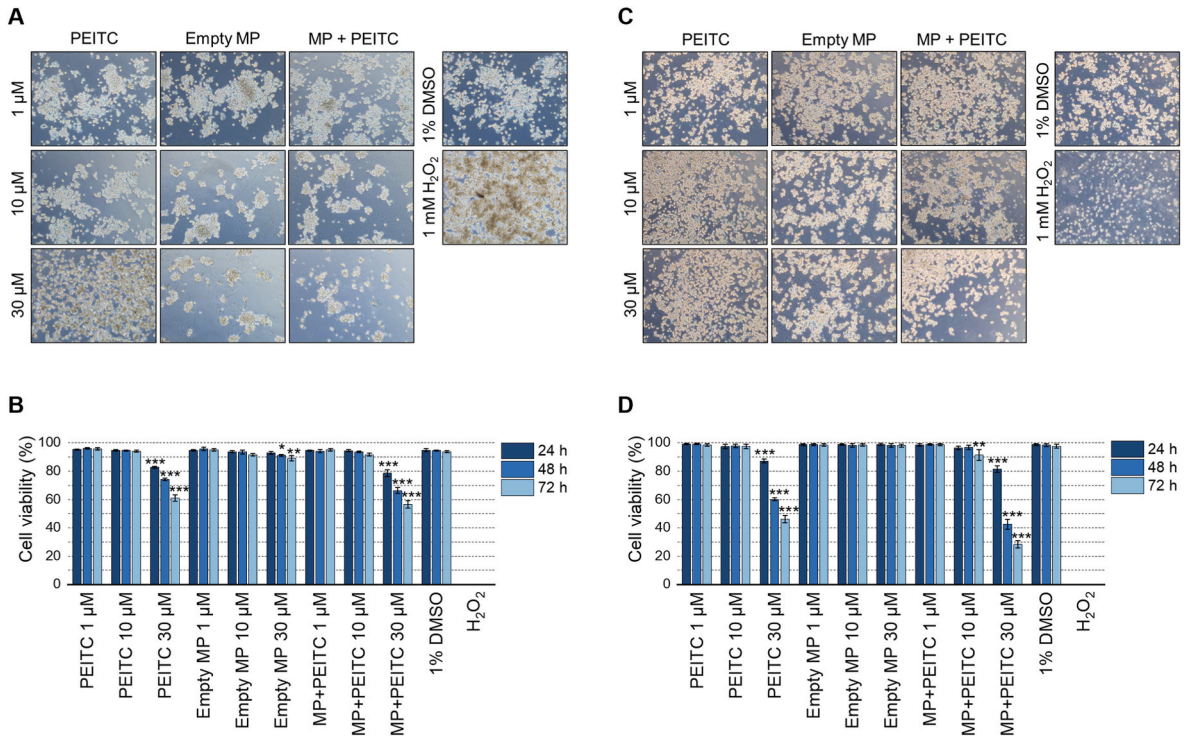

Fig. 4. Effect of pure and microencapsulated PEITC on gastrointestinal cancer cell's morphology and viability. Cell morphology of MKN45 gastric cancer cell line (**A**) and SW48 colon cancer cell line (**B**) after 24 h of culture with different concentrations of PEITC, empty MP or MP + PEITC (1, 10, and 30 μM), showing altered morphology when using 30 μM of both PEITIC and MP + PEITC. Cell viability of MKN45 (**C**) and SW48 (**D**) cell lines after 24, 48, and 72 h of culture with different concentrations of PEITC, empty MP or MP + PEITC (1, 10, and 30 μM), showing the impact of 30 μM of both PEITC and MP + PEITC on the viability of the cells. 1% DMSO and 1 mM H_2O_2 were used as negative and positive controls of toxicity, respectively. Magnification 10×. Three independent experiments were performed, each with two technical replicates per condition. Results are shown as mean ± SEM, and two-way ANOVA was used for statistical analysis. $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the negative control (1% DMSO).

agent. Additionally, we noted differential impacts of microencapsulated PEITC on the morphology and viability of SW48 cells compared to the MKN45 cell line, possibly due to differences in cellular characteristics, such as cell permeability. Moreover, our study unveiled a decrease in cell viability with increasing concentrations of both pure and microencapsulated PEITC, highlighting its potential as an anticancer agent. Importantly, there were almost no differences in cancer cell cytotoxicity observed between free and microencapsulated PEITC, reinforcing that our delivery system enhances the stability of PEITC and potentially improves therapeutic efficacy by enhancing its bioavailability. The time-dependent increase in the impact on cell viability suggests PEITC's potential cumulative effects, leading to increased cell death. We hypothesized that the observed toxic effects of microencapsulated PEITC might be linked to the dysregulation of the cytoskeletal protein tubulin. This hypothesis was supported by the complete disruption of tubulin observed in gastrointestinal cancer cells when treated with higher doses of both pure and microencapsulated PEITC. These results are in line with previous literature documenting the impact of pure PEITC on the dysregulation of tumor cell cytoskeleton (Chen et al., 2012; Øverby et al., 2014; Pawlik et al., 2012; Tang et al., 2011; Visanji et al., 2004; Xue et al., 2014; Zhen et al., 2023), supporting the effective release of PEITC by the MP while preserving its effectiveness.

Additionally, we assessed the microencapsulated PEITC's effect on malignant properties of gastrointestinal cancer cells, notably deciphering their migration capacity, which is key for tumor invasion and metastasis formation. The migration capacity of MKN45 cells decreased as the concentrations of PEITC increased. This effect was particularly prominent when cells were treated with microencapsulated PEITC, highlighting the enhanced potential of this delivery system to boost the therapeutic impact of PEITC. However, an impact on cell migration was also observed when cells were treated with empty MP. Previous works have underscored the potential of chitosan, the primary MP component, to influence the aggressiveness of cancer cells (Chen et al., 2014; Jiang et al., 2015), offering a conceivable rationale for the observed effects. Nevertheless, the significant difference in migration capacity between empty MP and MP with PEITC encapsulated emphasizes the substantial role of PEITC in driving the observed effects.

To better understand the cellular mechanisms underlying encapsulated PEITC's effects on gastrointestinal cancer cells' migration capacity, we explored its impact on key signaling molecules involved in cell motility. As already suggested in the literature, PEITC has the potential to disrupt critical signaling pathways by modulating the expression of key proteins such as protein kinase B (AKT), focal adhesion kinase (FAK), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and nuclear factor kappa beta (NF-kβ) (Dai et al., 2016; Du et al., 2024; Gao et al., 2011; Jeong et al., 2017; Lai et al., 2010; Lee et al., 2015; Loganathan et al., 2012; Yang et al., 2010). We observed a noteworthy reduction in phosphorylated AKT, FAK, and ERK1/2 protein expression in the microencapsulated PEITC-treated cancer cells. This observation suggests the effective release of the encapsulated PEITC while preserving its efficacy. The downregulation of these proteins can disrupt the MAPK/ERK and PI3K/AKT signaling pathways, which are crucial for cancer cell migration, as reviewed by Du et al. (2024), Noorolyai et al. (2019), and Sun et al. (2015).

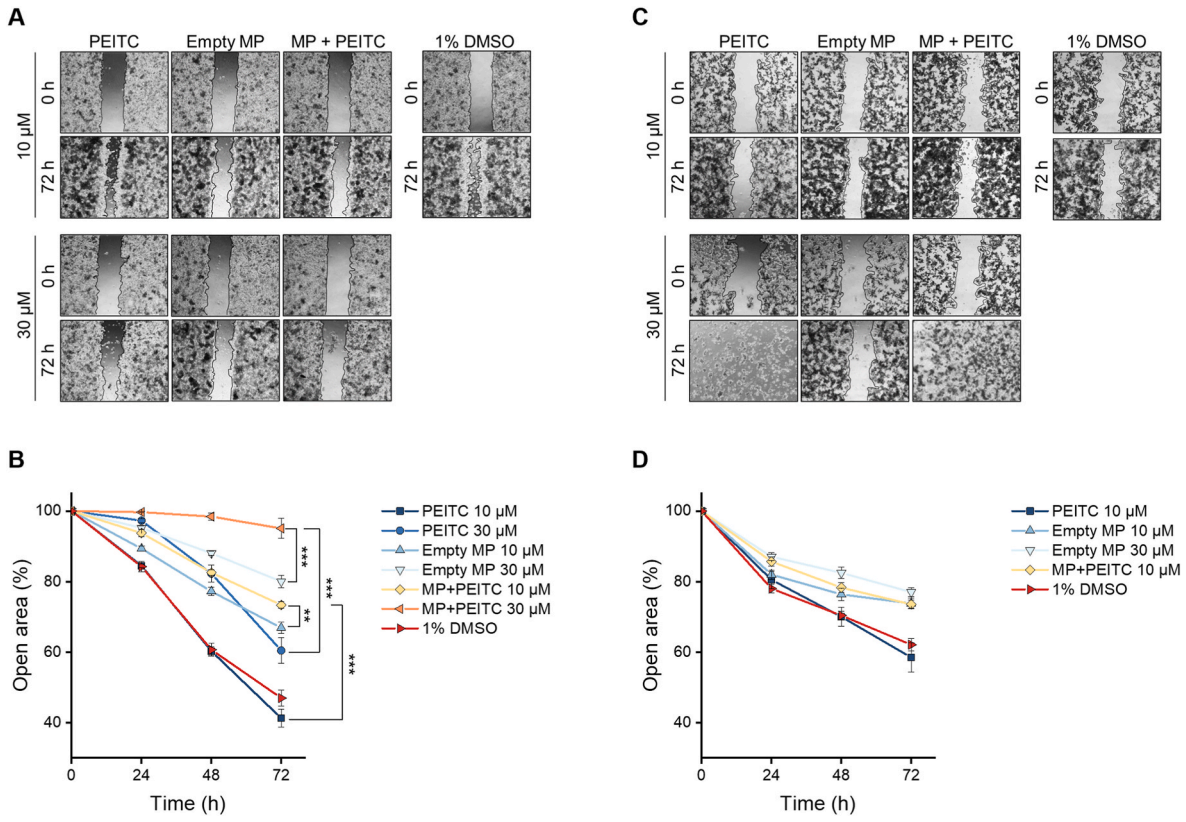

Fig. 5. Effect of pure and microencapsulated PEITC on gastrointestinal cancer cell migration capacity. (**A**, **B**) Wound-healing assay, showing the migration capacity of gastric cancer MKN45 cell line after treatment with various concentrations of PEITC, empty MP, or MP + PEITC (10 and 30 μM) during 72 h. (**C**, **D**) Migration capacity of colon cancer SW48 cell model after treatment with different concentrations of PEITC, empty MP, or MP + PEITC (10 and 30 μM) during 72 h. 1% DMSO was used as negative control. Magnification 10×. Two independent experiments were performed, each with three technical replicates per condition. Results are shown as mean ± SEM, and two-way ANOVA was used for statistical analysis. $^{**}p < 0.01$; $^{***}p < 0.001$.

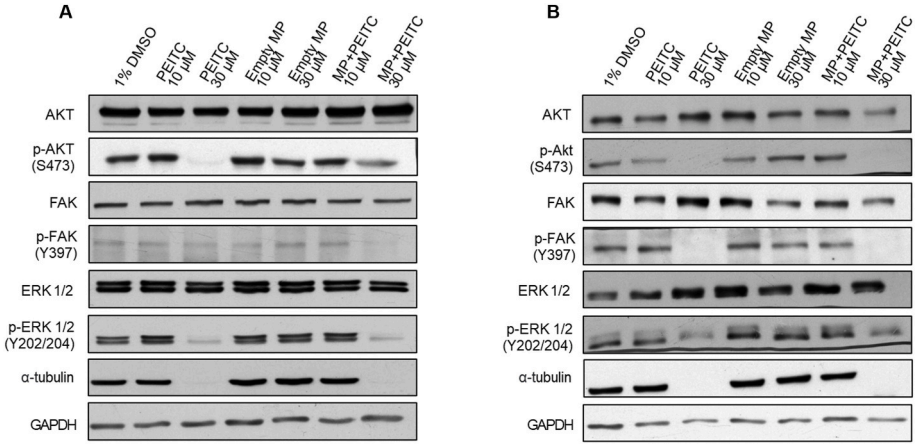

Fig. 6. Pure and microencapsulated PEITC impact on the activation of AKT, FAK, and ERK1/2 and tubulin expression in gastrointestinal cancer cells. Western blotting of total and phosphorylated AKT, FAK and ERK1/2, and tubulin in MKN45 gastric cancer cells (**A**) and SW48 colon cancer cells (**B**) after treatment with 10 and 30 μM of pure PEITC, empty MP or MP + PEITC for 72 h. 1% DMSO was used as control of the assay, and GAPDH as a protein loading control.

5. Conclusions

In conclusion, our study demonstrates that microencapsulated PEITC, delivered via chitosan-based microparticles, offers significant advancements in gastrointestinal health management. Microencapsulation successfully improves the stability and bioavailability of PEITC, resulting in potent antibacterial efficacy at lower concentrations compared to its free form, and highlights its potential to modulate bacterial populations within the gastrointestinal tract. Additionally, microencapsulated PEITC surpasses pure PEITC in impacting gastrointestinal cancer cell integrity, viability, and motility. Overall, these findings underscore the promise of PEITC encapsulated in a chitosan-based delivery system in promoting gastrointestinal health and managing disease progression.

Funding

This work was supported by the project Norte-01-0145-FEDER-000051 - "Cancer Research on Therapy Resistance: From Basic Mechanisms to Novel Targets," supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF); the project POCI-01-0145-FEDER-032094 - "GastroCure: Bioactive Soybean and Cruciferous extracts towards application in gastrointestinal disorders: development, characterization and delivery," supported by the Portuguese Foundation for Science and Technology (FCT), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF); and by National Funds through FCT: 2022.02926.PTDC – "gBIOT: Nutraceutical biopolymeric-biocatalytic microbot against gut inflammatory disorders" (DOI: 10.54499/2022.02926.PTDC); doctoral fellowships 2020.05483.BD to RA and UI/BD/152090–2021 to CCR; and Assistant Researcher contract (2022.04678.CEECIND) to CG.

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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