SUPPLEMENTARY MATERIAL

Self-assembled nano-micelles for oral delivery of luteolin utilizing intestinal lymphatic pathway targeting pancreatic cancer

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Materials and method

S1. Formulation development and characterization. The formulation preparation began with creating an empty effervescent system by weighing specific excipients (citric acid, SBC, and SDS) and mixing them thoroughly. The efficiency of this effervescent drug delivery system was related to gas bubble generation, so the formulation ratio was chosen based on foam-forming capacity. A defined ratio of Citric acid, SBC, and SDS was mixed, and upon exposure to water, bubbles were immediately formed. A ratio of 1:1.2:0.7 (Citric acid/SBC/SDS) was selected based on bubble formation. Luteolin (LUT) was loaded into this effervescent carrier, and its loading was determined qualitatively using fluorescence intensity.

For particle size distribution and surface charge measurements, the LUT-NG mixture was treated with DI water (1:100). 5 min later, the reaction product (that conversion of gas bubble into micelles) was directly transferred into the cuvette and subsequently, the measurement was recorded at 25°C. For data reproducibility, all experiments were performed in triplicates.

The quantitative measurement of the particle size and morphology was confirmed with a Transmission electron microscope (TEM, JEOL JEM-2100, Tokyo, Japan). For TEM observation, the LUT-NG effervescent formulation was transferred into a 2 ml eppendorf tube then 1 ml of DI water was added and allowed to conversion of gas bubbles into micelles. A small volume of solution was immediately drop-casted onto the copper grid of 300 mesh size (Ted pella Inc.).

Preparation of effervescent enteric coated granules. The process involved the formulation of enteric-coated granules. Subsequent to characterization and analysis, we progressed to the development of the final formulation. Basic trituration was conducted to ensure the uniform mixing of components, and this blend was employed for granule preparation. Given the sensitivity of certain chemicals to moisture and temperature, a non-aqueous wet granulation method was employed. Following the granulation, an enteric-coating was applied to enhance stability in the acidic stomach environment. This coating facilitates the delivery of the formulation to the small intestine by preventing drug absorption in the stomach.

S3Assessment of antitumor activity.

Tumor volume was calculated by the following formula:

Tumor volume = (length × width^2)/2

At the end of the experiment, the animals were sacrificed and tumors were harvested.

Intratumor distribution of nanoparticles- Again LUT accumulation after oral treatment at tumor side was determined IVIS analysis. confirmative study was also performed in PANC-1 xenograft model, in which mice received orally of ICG-labeled NPs. Mice were sacrificed 24 h post injection; the tumor tissue was collected for ex vivo IVIS imaging to detect the NIR fluorescence and overlaid with the bioluminescence signal from the tumor tissue.

Analysis of tumor angiogenesis- The initiation of new vessel formation, facilitating tumor nutrition and metabolite outflow, is a crucial requirement for tumor development. This process commences when the tumor attains a size of approximately 200 mm^3 [1]. Here we analyzed tumor blood vessels formulation by imaging of tumor solid mass. Images were acquired at the end of experiment and the effect was evaluated on the basis of number of blood vessels on tumor mass and branching of blood vessels.

Histological Examinations: The specimens (tumor) were fixed with 4% paraformaldehyde for 24 h, subsequently, embedded in paraffin wax and cut in 5 μm. H&E staining, IHC was performed for expression of VEGF A, Caspase-3, TNF-α and FAK further massons trichome's staining were carried out on the sections of these tissues.

Western Blot: The expression of VEGF A and Caspase-3 was determined by western blot analysis. Protein concentration in tumor tissue was measured by BCA kit (Promega, USA). The protein solution was denatured, loaded to 10% SDS-PAGE electrophoresis gel (running 2 h at 120 V), and transferred to a nitrocellulose membrane (running 2 h at 300 mA). The membrane was blocked with 5% BSA solution for 1 hr, incubated with primary antibody, including Monoclonal Antibody to VEGFA (Cloud-clone), Caspase-3 (Invitrogen) and beta-actin (Cloud-clone) overnight at 4 °C, washed, and incubated with IgG HRP-linked secondary antibody (Invitrogen, USA). Finally, membranes were visualized using Pierce ECL Western Blotting

Substrate (Thermo Scientific, USA). Intensity of western blot bands were determined with Gel doc system (Bio-Red).

RT-PCR: Total RNA was extracted from tumor mass using the Thermofisher RNA isolation kit (USA). mRNA level was measured by TaqMan qRT-PCR. RNA (10 ng) was converted to cDNA by TaqMan miRNA reverse transcription kit. PCR reaction was conducted (Real gene) using TaqMan Universal Master Mix and primers. Expression was quantified using SYBR Green RT-PCR(Promega). The GAPDH primer assay was used following the manufacturer's protocol.

All human primer were designed as mentioned below: -

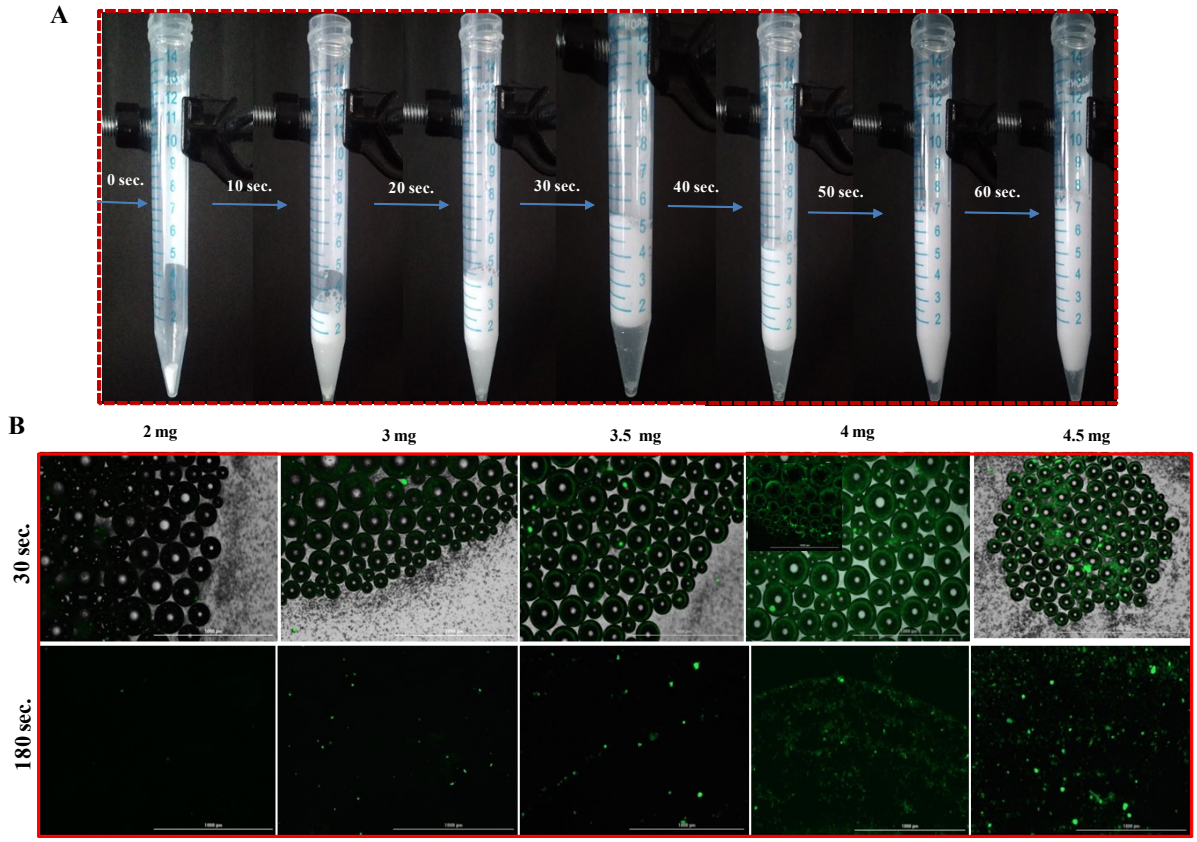
Gene symbol	**Primer sequence from 5'-3'**
Ki67	FP: TAACCATCATTGACCGCTCCTTT RP: CTTGACCTTCCCCATCAGGGTC
TNF-α	FP: GAGTGACAAGCCTGTAGCCC RP: CCCTTCTCCAGCTGGAAGAC
VEGFA	FP: GAGCTTCCTACAGCACAACA RP: CCAGGACTTATACCGGGATTTC
PTK2	FP: CTCCTACCCTCTACAGCCTTAT RP: CTTCTCTTCCTCCAGGATTGTG
Caspase-3	FP: GCTGCCTGTAACTTGAGAGTAG RP: GTATGGAGAAATGGGCTGTAGG
GAPDH	FP: ATGAAGGGGTCATTGATGG RP: AAGGTGAAGGTCGGAGTCAA

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). At the significant levels of $P < 0.5$(*), $P < 0.01$(**), and $P < 0.001$(***), one-way ANOVA was used to test the difference between the experimental group and the control group. The analyses were performed using GraphPad Prism 7.0 Software (La Jolla, CA, USA).

Results.

S1 &S2. Formulation development and characterization.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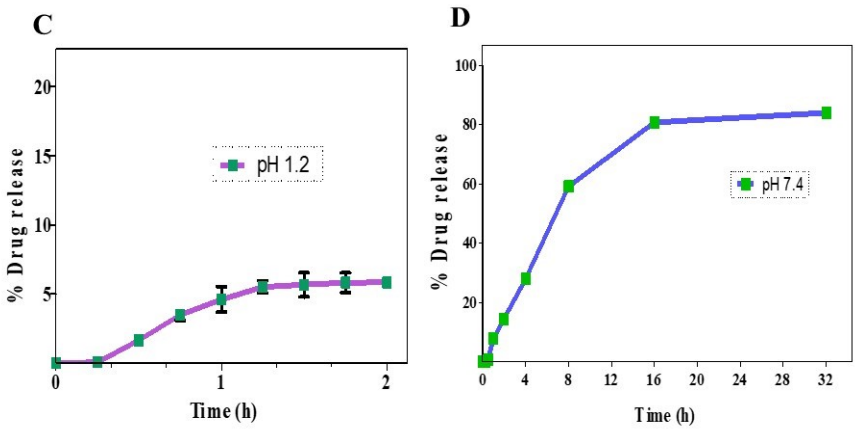

Fig. S1. Formulation development ;**(a)** Demonstration of bubble formation capacity of the 6th formulation based on foam-forming volume. **(b)** Fluorescence microscopy was used to analysis of drug loading in NG system. In-vitro drug release pattern of Enteric-coated effervescent granules of LUT. (c) Drug release estimation at 1.2 pH for 2 hand, (b) Drug release estimation at 7.4 pH for 32 h. This figure reprinted from publication, Vol no. 69, Archana Karole,Shabi Parvez,Richa Singh Thakur,Shyam Lal Mudavath, title 'Effervescent based nano-gas carrier enhanced the bioavailability of poorly aqueous soluble drug: A comprehensive mechanistic understanding' Page No. 103167, 2023, with permission from Elsevier. License Number -5717490248269.

References.

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