Upgrading the therapeutic potential of catechin against Ehrlich carcinoma and mitigating oxidative stress in the liver employing chitosan polyaspartate as nanodelivery vehicles

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1. Instrumentation

FT-IR spectra were recorded on a BRUKER Tensor-37 FTIR spectrophotometer in the range 400–4000 cm^{-1} as KBr discs or in the 4000-550 cm^{-1} region with 2 cm^{-1} resolution with an ATR (attenuated total reflection) unit (Platinum ATR-QL, Diamond). For signal intensities the following abbreviations were used: br (broad), sh (sharp), w (weak), m (medium), s (strong), vs (very strong). The particle shape of new materials was examined using transmission electron microscope (TEM). The images were taken by a JEM-2011F microscope (JEOL, Japan) operated at 200 kV. The morphology of the formed micro and nano-composites was investigated using Scanning electron microscopy (SEM, Hitachi S-7400, Hitachi, Japan) supported with energy dispersive –X-ray (EDX) to determine the elemental analysis of the formed products.

1.1. Chemicals and instrumentations

Our earlier study [1] provided the low-molecular weight chitosan (LMC) (MW 12000 Da, deacetylation degree ≥84.9%) from crab shells; Sigma-Aldrich, USA, provided the trans-resveratrol (99%), lecithin (LEC) (97%), cholesterol (CLS) (99%), and pentasodium tripolyphosphate (TPP). Throughout, MilliQ water (MQW) was utilized. All other chemicals and solvents were of the reagent variety.

For the structural characterization of new materials, the spectroscopic methods were employed including FTIR (Fourier transform infrared), UV-Vis, and EDX (energy dispersive X-ray) analyses. The particle size distribution (PSD) was determined utilizing DLS (dynamic light scattering), while the physical and colloidal stability of CAT@CPANVs was investigated using Zeta potential (ZP) measurements. The morphological features of new nanoformulations were inspected using SEM (scanning electron microscope) and TEM (transmission electron microscope) analyses. Specifications for these instrumentations were provided in the supplemental materials (**SM**).

1.2. Preparation of nascent CPANVs

A modified protocol from the previously reported ionotropic gelation method [2] was used to synthesize the nascent CPANVs . Initially, a stock chitosan solution (0.1% w/v) was prepared by dissolving 0.1 g of chitosan in 100 mL of 1% CH_3COOH and adjusted to pH 5.5. Concurrently, the lipid vesicles' dispersion was prepared by dissolving 27 mg LEC and 3 mg CLS in 900 µL of chloroform:methanol (2: 1 v/v) mixture to prepare a lipid solution which subjected to rotary evaporation (50 °C, 100 rpm) under reduced pressure to form a thin lipid film. This film was then hydrated by a freshly prepared TPP aqueous solution (0.1% w/v) while gently vortexing, forming lipid vesicles' dispersion. After that, the LEC-CLS-TPP mixture's dispersion was then gradually added to the 25 mL chitosan solution under strong magnetic stirring at 25°C until a hazy opalescent dispersion, indicative of NPs generation, was visually seen. The formulated NPs were then stirred continuously for an additional 30 min. Centrifugation (7,000 rpm) was then used for 15 min at 10°C and to reduce the volume of NPs dispersion from (about 25 mL) to 0.7 mL. Following this, the NPs were rinsed with 3 mL of phosphate buffer and then centrifuged again under the same conditions to get □0.7 mL of NPs dispersion. Finally, the solid CPANVs product was isolated by freeze-drying the NPs dispersion for 72 h.

1.3. *In vitro* release kinetics of CAT@CPANVs

The was dialysis method was applied to study the in vitro release of RV from CAT@CPANVs

using a dialysis bag of molecular weight cutoff (MWCO) (12–14 kDa) [3]. The receptor phase consisted of an ethanol solution and a phosphate buffer saline (PBS) mixture with a ratio of 7:3 (v/v). In brief, 50 mg of CAT@CPANVs was mixed with 50 mL of PBS buffer (pH 7.4) and then added to a dialysis bag. After that, the bag was submerged in 200 mL of the receptor phase, which was stored at 37°C and agitated at 250 rpm for 24 h. At specific time intervals, 1 mL of the sample was removed and replaced with an equivalent volume of new receptor phase to maintain a consistent volume. The samples were analyzed for the amount of released RV using spectrophotometry at 306 nm. The cumulative RV release from CAT@CPANVs was calculated employing formula (**Eq. 2**):

$$\mathrm{OR\%} = \sum_{t=0}^{t} \frac{\text{released RV amount}}{\text{original RV amount}} \times 100 \quad (\mathbf{Eq.\ 1})$$

After that, numerous kinetic models, such as zero order, first-order, second-order, Higuchi, and Korsmeyer-Peppas equations, were used to examine the in vitro release results. [4]. After fitting the data using Origin2022 Pro software, we identified the model that best captured the dynamics of the RV process.

1.4. *In vivo* studies

1.4.1. Animals

60 adult male Sprague-Dawley rats, weighing 150–160 g, were purchased from the National Research Centre's animal facility in Dokki, Giza, Egypt. They were maintained in regular laboratory settings (12 h light/dark cycle, 22 ± 2 °C, 50–60% humidity) and had unrestricted access to water and a standard diet. In compliance with the National Institutes of Health's (NIH) guidelines for the care and use of laboratory animals [5], Suez University's Institutional Animal Ethics Committee (IAEC) approved all experimental methods (Approval No.: SUEZ Sci_IRB:19/04/2025/22).

1.4.2. Tumor sample

The EACs came from the Egyptian National Cancer Institute and were intraperitoneally transplanted into the peritoneal cavities of the mice in our laboratory every eight or ten days.

1.4.3. LD_{50} (The median lethal dose) of the tested compound

CAT@CPANVs' median LD_{50} was ascertained by applying the Meier and Theakston technique. At serial doses of 1- 10 mg/kg respectively, the salt of potassium of CAT@CPANVs was administered into 40 mice (four for each dose). Twenty animals remained (four for each dose) and were monitored for an entire day following their administration of 15, 20, 50, 75, and 100 mg/kg, in turn [6].

1.4.4. The dose-response curve

It was created to determine the ideal dosage for in vivo anti-cancer effect. Six groups of five mice each were created. Group 1 received an intraperitoneal injection (IPI) of EACs (2.5×10^6 cells/mice) on day one. Group 2 mice received 2.5 mg/kg of CAT@CPANVs after EACs injection. Group 3 received 5 mg/kg CAT@CPANVs, Group 4 received 10 mg/kg, Group 5 received 15 mg/kg, and Group 6 received 20 mg/kg of CAT@CPANVs after EACs injection. The 10-day study included injections at 2, 4, 6, 8, and 10 days, and afterward, EACs viability was assessed using the trypan blue (TB) exclusion technique [7].

1.4.5. Experimental design

Four groups of sixty mice were used: Group 1 (control) received daily saline IPI for ten days; Group 2 received CAT@CPANVs (15 mg/kg) intraperitoneally daily for ten days; Group 3 (positive group) received EACs injection (2.5×10^6 cells/mice) on day one; Group 4 (therapeutic) received CAT@CPANVs (15 mg/kg) intraperitoneally post-EAC injection for 10 days. On day eleven, mice were collected in heparinised saline. Blood samples were taken from the retro-orbital venous plexus of the rats while they were anaesthetised with halothane inhalation, and they were centrifuged for ten minutes at 1435 g. The serum were kept at -20°C prior to analysis [8]. After the mice were given 120 mg/kg of sodium thiopental to put them to sleep, the liver tissues were removed and divided into two portions. To make tissue homogenate, the initial component was preserved in PBS. The remainder portion was kept for histological and immunohistochemical examination in 10% formalin. To establish life span extension, three animals per group were monitored until they died, except the drug and control groups [9].

1.4.6. The viability and count of EACs

Calculating the total and viable EACs at a magnification of ×40 and counting the number of cells/mL in each group allowed us to evaluate the viability of EACs using the trypan blue exclusion approach.

1.4.7. TB solution (1%)

To make a stock TB solution, one gramme of tuberculosis (TB) was dissolved in one hundred millilitres of distilled water (DW). The working TB solution was made by diluting 1 mL of stock solution in 9 mL of DW. The harvest cells were stained with 1% TB solution and allowed to incubate for ten minutes at 37°C. Afterward, the cells were examined under microscope with hemocytometer to determine whether EACs were living and dead. We counted the EACs by using the formula number of cells/ML = Mean number of viable cells per 5 square × 25 × dilution factor $\times 10^4$ [10].

1.4.8. Life span

Life span was calculated in order to examine the effect of CAT@CPANVs on life span prolongation [11]:

$$\textbf{Life span prolongation} = [(T - C)/C] \times 100.$$

T : days when treated mice remained viable.
C : days when control mice remained viable.

[T/C ratio] : [average existence time of cured/average existence time of control] × [100].

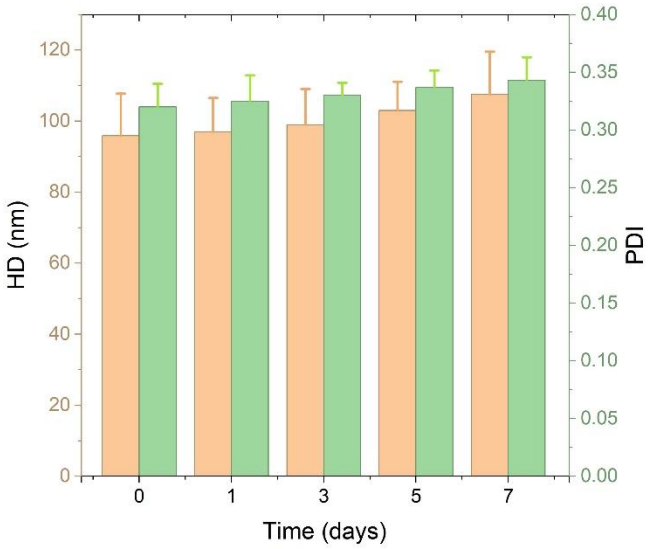

Figure S1: Storage stability of CAT@CPANVs under physiological conditions

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