Chitosan-Decorated PLGA-NPs Loaded with Tannic Acid/Vitamin E Mitigate Colon Cancer via the NF-κB/β-Cat/EMT Pathway

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ABSTRACT: Colon cancer is the second highest contributor of cancer-related deaths throughout the world. Treatment strategies with tannic acid and vitamin E are envisaged as desirable and safe owing to their robust antioxidative and anti-inflammatory potential. In the present report, these bioactives have been nanoencapsulated in poly(D,L-lactide-co-glycolic acid) (PLGA) formulations for maintaining sustained release and ensuring enhanced bioavailability. Capping of nanoparticles (NPs) with chitosan was done for enhanced anticancer efficacy and tumor targeting. CS-PLGA-TA-E, administered intraperitoneally, significantly inhibited tumor number and tumor volume and normalized colon histology in the colon cancer. Tissue distribution studies showed that TA/E content from CS-PLGA-TA-E was present in a higher concentration in the tumor tissue than the concentration of TA/E content from PLGA-TA-E or free TA or free E. Also, the TA/E content from all of the treatment groups showed its highest concentration in the tumor compared to other organs. Antioxidant enzymes and proinflammatory cytokines (TNF-α, IL-1β, IL-6) were inhibited by CS-PLGA-TA-E. CS-PLGA-TA-E inhibited markers for tumor growth (EGFR-PI3K-AKT), inflammation (NF-κB/Stat3), β-catenin signaling (β-catenin, c-myc, cyclin D1), EMT (E-cadherin, N-cadherin, vimentin), and apoptosis (Bcl-2) in a significantly greater way as compared with PLGA-TA-E, TA, or E. CS-PLGA-TA-E NPs can be considered promising anticancer drugs for colon cancer.

INTRODUCTION

In terms of cancer-associated death rates, colorectal cancer is among the gravest clinical concerns.1 The five-year survival rate is less than even 10% for most cases.2,3 Prevailing inflammation can lead to tumorigenesis progression4,5 triggered by the presence of proinflammatory cytokines synthesized by immune cells and macrophages in the tumor microenvironment.6,7 Both tannic acid8 and vitamin E9,10 have excellent anti-inflammatory and antioxidant properties.11,12 Previous reports suggest that tannic acid attenuates colon cancer cell proliferation,13 and it also ameliorates 1,2-dimethylhydrazine-induced colon tumor growth.14 There are reports of the anticancer potential of vitamin E in colon cancer prevention.15−17 An animal model of colitis-associated colorectal cancer (CAC) developed with the combination of a carcinogen, azoxymethane (AOM), and colitis inducer-dextran sulfate sodium (DSS), mimics the colon adenocarcinoma in humans.18−20 Ergo, our target was to study the anticancer and anti-inflammatory potential of tannic acid and vitamin E in an AOM/DSS colon cancer model.

Tannic acid absorption through the rat gut wall has been shown to be limited, and its chemical modification in the gut alters the polyphenol to an extent where it becomes physically, biologically, and chemically different from the original form.21,22 Peak blood availability of tannic acid occurs 3 h after its administration.21,23 The low water solubility of vitamin E caused less tissue absorption,24 and complexity of the vitamin E metabolism arises due to its bioavailability being affected by numerous factors.25 Thus, slow and sustained release and targeted delivery of both tannic acid and vitamin E are required for efficacious remedy.

Nanosized carriers, such as poly(D,L-lactide-co-glycolic acid) nanoparticles (PLGA-NPs), in vivo, protect the drug from degradation26 and ensure slow and sustained release.27,28 PLGA polymer NPs are nontoxic, biodegradable, and biocompatible.26,29 Chitosan, a natural biopolymer, has been reported to prevent carcinogenesis by modulating apoptotic, antioxidant, inflammatory, and antiangiogenic pathways.30−33 Chitosan has also been used to enhance drug release, bioavailability, and drug cytotoxicity.34,35 As a result, we decided to coat our PLGA nanoparticles with chitosan for enhanced and targeted anticancer activity.

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Cytokines secreted by the tumor stroma cells lead to an increase in tumor growth and invasiveness by activating oncogenic signaling pathways like nuclear factor kappa B (NF-κB) by tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) and activation of signal transducer and activator of transcription 3 (STAT3) by interleukin 6 (IL-6) −39

Phosphoinositide 3-kinase (PI3k)-protein kinase B (AKT) signaling pathway activation leads to the release of proinflammatory cytokines.40,36 Epidermal growth factor receptor (EGFR) is an upstream regulator of the Wnt/β-catenin pathway in many tumors, whereby it activates c-myc and cyclin D1 that control G1/S cell-cycle progression.41,42

Overexpressed β-catenin and the EGFR-PI3K-AKT pathway lead to epithelial-mesenchymal transition (EMT) progression in colon cancer,43 which promotes metastasis,44

Hitherto, no reports of tannic acid and vitamin E preventing CAC progression in an AOM/DSS murine model are available. Thus, we decided to study the combined effect of both the anti-inflammatory compounds against the CAC disease model. Further, to enhance the bioavailability and bioaccessibility of the target compounds in the murine model of CAC, we formulated them inside chitosan-coated PLGA nanoparticles. We also investigated the regulation of underlying pathways

Figure 1. Characterization of tannic acid- and vitamin E-loaded PLGA-TA-E and CS-PLGA-TA-E nanoparticles. (A) PLGA-TA-E and CS-PLGA-TA-E particle surface topology determination using atomic force microscopy. The NPs were observed by AFM, and the acquired images were analyzed using scanning probe microscopy (SPM) tools for laboratory study. (B) Particle size distribution from differential light scattering (DLS) with PLGA-TA-E and CS-PLGA-TA-E. (C) Fourier transform infrared spectroscopy (FTIR) spectra of tannic acid, vitamin E, PLGA, chitosan, and PLGA-TA-E and CS-PLGA-TA-E nanocomposites. (D) Encapsulation efficiency percentage and drug-loading percentage of tannic acid and vitamin E in PLGA-TA-E and CS-PLGA-TA-E nanocomposites. (E) Percentage of release of tannic acid and vitamin E from PLGA-TA-E and CS-PLGA-TA-E nanocomposites over a time period of 0–96 h. Result is the mean ± standard deviation (SD) from triplicate independent experiments.
that control colon cancer growth and progression, such as EGFR-PI3K-AKT, Wnt/β-catenin, and EMT markers.

### RESULTS

**Characterization of PLGA-TA-E.** Atomic force microscopy (AFM) experiments elucidated that the surface topology of PLGA-TA-E and CS-PLGA-TA-E NPs agreed with spherical geometry, uniformly distributed without aggregation (Figure 1A). AFM data showed that for CS-PLGA-TA-E nanoparticles, the size was between 140 and 165 nm and for PLGA-TA-E it was between 110 and 140 nm. The overall thickness of CS-PLGA-TA-E nanoparticles was between 0.6 and 4.3 nm and that of PLGA-TA-E was between 1.2 and 4.8 nm. Also, the peaks of PLGA-TA-E and CS-PLGA-TA-E nanoparticles showed that the nanoparticles were spherical and smooth in nature.

The DLS data showed that PLGA-TA-E NPs had a diameter of 118 ± 2.5 nm, with a polydispersity index value of 0.43. The diameter of CS-PLGA-TA-E NPs was 152 ± 4.7 nm, with a polydispersity index of 0.251, indicating a lower polydispersity than PLGA-TA-E NPs (Figure 1B).

Fourier transform IR findings demonstrated the compatibility among PLGA, TA, E, chitosan, PLGA-TA-E, and CS-PLGA-TA-E, as presented in Figure 1C. The spectra covered the region between 4000 and 1000 cm⁻¹. The significant peaks attributed to tannic acid and vitamin E confirm the presence of
different functional groups. The strong, broad peak at 3430 cm$^{-1}$ was due to O–H stretching, while the sharp peak at 1650 cm$^{-1}$ corresponded to the presence of carbonyl (C=O) and alkene (C=C) groups in tannic acid. The strong, sharp peak at 3400 cm$^{-1}$ was due to O–H stretching, and the small sharp peak at 1648 cm$^{-1}$ corresponded to the presence of alkene (C=C) groups in vitamin E. PLGA showed one weak, small peak at 3492 cm$^{-1}$ due to O–H stretching and a short sharp peak at 1775 cm$^{-1}$ due to C–H bending and carbonyl (C=O stretching) groups. Chitosan showed small sharp peaks at 3285, 3349, 3411, 3461, and 3544 cm$^{-1}$ due to N–H and O–H stretchings at 1545 cm$^{-1}$ due to amine and amide bonds, and the bands at 1025 and 1080 cm$^{-1}$ due to C–O stretching. The PLGA-TA-E nanocomposite showed a peak at 3444 cm$^{-1}$, which suggested that the O–H groups of tannic acid, vitamin E, and PLGA were conserved. Another small peak at 1634 cm$^{-1}$ denoted the conservation of the alkene (C=C) and carbonyl (C=O) groups of tannic acid and the alkene groups of vitamin E in the PLGA-TA-E nanocomposite. The CS-PLGA-TA-E NPs showed a broad peak at 3350 cm$^{-1}$, which
suggested that the O−H groups of tannic acid, vitamin E, and PLGA and the O−H and N−H groups of chitosan were conserved. Another short sharp peak at 1743 cm$^{-1}$ denoted the conservation of C≡C groups of TA, E, and C≡O group of tannic acid. A short peak at 1615 cm$^{-1}$ and a very short peak at 1537 conserve the amide and amine groups, and a sharp peak at 1083 cm$^{-1}$ conserves the C−O stretching of chitosan.

Tannic acid and vitamin E encapsulation efficiencies in PLGA-TA-E and CS-PLGA-TA-E were 68.5 ± 2.1 and 59 ± 1.2%, respectively. They increased to 80 ± 3.4 and 81 ± 0.5%, respectively, for TA and E loaded into CS-PLGA-TA-E NPs. The PLGA-TA-E drug loadings for tannic acid and vitamin E were 19 ± 2.2 and 9 ± 4.2%, respectively. The CS-PLGA-TA-E drug loading for tannic acid and vitamin E increased to 28 ± 0.8 and 13 ± 4% (Figure 1D).

The in vitro drug release kinetics of TA and vitamin E from PLGA-TA-E and CS-PLGA-TA-E are shown in Figure 1E. At the 12 h time point, TA showed a 9.2% release from PLGA-TA-E and a 14.5% release from CS-PLGA-TA-E, whereas vitamin E showed a 15.3% release from PLGA-TA-E and a 17% release from CS-PLGA-TA-E. At 48 h, TA showed a 38.3% release from PLGA-TA-E and 68.5% release from CS-PLGA-TA-E, whereas E showed a 41% release from PLGA-TA-E and a 58.2% release from CS-PLGA-TA-E. At 84 h, the release of TA enhanced to 69% from PLGA-TA-E and from CS-PLGA-TA-E, whereas E showed a 69% release from PLGA-TA-E and a 86% release from CS-PLGA-TA-E. At 84 h, the release of E was 59% from PLGA-TA-E and 81% from CS-PLGA-TA-E. A steady slow release of TA and E was observed for four days with the highest release occurring at 84 h.

Assessment of Change in Body Weight and Survival Rate in CAC-Induced Mice. As shown in Figure 2A, the body weight had drastically reduced in AOM/DSS-treated CAC model mice compared to the control group. For the other treatment groups, body weight increased up to 5−6 weeks, after which it gradually decreased. However, on the administration of E, TA, PLGA-TA-E, and CS-PLGA-TA-E,
there was an increase in the body weight. After 9 weeks, the CS-PLGA-TA-E therapy showed a substantial improvement in body weight as compared to the other treatment groups. The survival rate in the CAC model had decreased, but it had improved significantly in the treatment groups, as shown in Figure 2B. In addition to the other treatment groups, CS-PLGA-TA-E showed the most substantial improvement in survival rate. The above data suggest that all of the treatment groups had favorable effects in increasing body weight and survivability in the CAC model; however, CS-PLGA-TA-E treatment provided the best results.

Assessment of the Change in Serum Enzyme Levels of Hemoglobin, SGPT, SGOT, SOD, Catalase, and GPx Levels. The biochemical analysis revealed that rectal bleeding reduced hemoglobin levels to 7.5 g/dL in the AOM/DSS CAC model, which increased to 8 and 8.5 g/dL with E and TA treatment and to near-normal levels of 10.5 and 11 g/dL with PLGA-TA-E and CS-PLGA-TA-E treatment (Figure 2C). There is a strong correlation between CAC progression and hepatic biomarkers, and thus, the SGPT and SGOT levels were analyzed. The CAC model showed enhanced SGPT and SGOT levels signifying liver injury. These values were significantly reduced by the treatment with TA and PLGA-TA-E and were the lowest in CS-PLGA-TA-E-treated groups (Figure 2D,E).

Reduced antioxidant levels are also related to colon cancer progression, and its alteration by treatment is of great concern. The SOD, catalase, and GPx levels decreased significantly in the CAC model compared to the control group. This enzyme profile in the PLGA-TA-E and CS-PLGA-TA-E treatment groups was significantly high (Figure 2F).

Therefore, surface modification of PLGA-TA-E with chitosan enhanced the hemoglobin levels, maintained low hepatic biomarker levels, and protected against oxidative stress in the CAC model.

Attenuation of AOM/DSS-Induced Colitis and Tumorigenesis by Chitosan Nanoparticle Administration. In the colitis or CAC model, mice fed on DSS, shortening of the colon length is a marker for evaluating the severity of colon inflammation. Figure 3A shows representative images of the colon (from the cecum to the rectum) in the CAC model and CAC + treatment groups. The colon length had reduced extensively in the CAC model group as compared to that of the control. The colon length in the CAC model was 9.5 cm, and after administration of E, TA, PLGA-TA-E, and CS-PLGA-TA-E, it increased to 10.5, 11, 12, and 13 cm, respectively, and that of the normal colon was 14 cm. Thus, the administration of CS-PLGA-TA-E abolished the DSS-induced shortening of the colon more remarkably (Figure 3B). In this model, the tumor volume decreased by 33.8, 58, 72.5, and 86.5% upon administration of E, TA, PLGA-TA-E, and CS-PLGA-TA-E, respectively (Figure 3C). The total number of tumors in the AOM/DSS model group upon treatment with E, TA, PLGA-TA-E, and CS-PLGA-TA-E decreased by 26.6, 40, 53.5, and 76.6%, respectively (Figure 3D). The above data suggested that all of the treatment groups debilitated the tumor growth induced by AOM/DSS. However, the CS-PLGA-TA-E treatment group exhibited a more pronounced action.

Figure 3E shows the rectal prolapse; this is the point when our treatment was started in the AOM/DSS model. At this point, a tumorous outgrowth was seen in the mice’s colon, and histological analysis showed distortion of crypts.

Figure 3F shows hematoxylin and eosin (H&E)-stained histological sections of the AOM/DSS disease model and the five treatment groups. The colon tissue of the AOM/DSS model animals showed crypt destruction, inflammatory cell infiltration, and a high degree of loss of goblet cells. For the E treatment group, the crypt architecture was better than the AOM/DSS model group. After treatment with TA, the crypt architecture started to improve, with an almost normal number of goblet cells. For PLGA-TA-E and CS-PLGA-TA-E groups, mucosa showed firmly packed glands with a regular quantity of goblet cells, and the architecture of the crypts was standard. In comparison to the other treatment groups, the H/E-stained portions of the CS-PLGA-TA-E treatment group were more similar to the control group.

Thus, CS-PLGA-TA-E could prove to be a more potent anticancer agent against colorectal cancer than the other treatment alternatives.

Tissue Distribution Study of TA and E, in Various Organs, Using High-Performance Liquid Chromatography (HPLC). The distributions of free tannic acid and tannic acid encapsulated in PLGA-TA-E/CS-PLGA-TA-E to the tumor, liver, spleen, lungs, kidneys, and serum are shown in Figure 4A. The TA content from CS-PLGA-TA-E showed its highest concentration in the tumor at all time points (approx. 560 μg/mL at 6 h, 240 μg/mL at 12 h, 180 μg/mL at 24 h, 40 μg/mL at 48 h, and 20 μg/mL at 72 h) as compared to its uptake in the other tissues/organisms (the liver showed its highest uptake of approx. 270 μg/mL at 6 h, spleen 275 μg/mL at 6 h, lungs 90 μg/mL at 6 h, kidney 95 μg/mL at 6 h, and serum 10 μg/mL at 6 h). At 6 h, in the tumor, the TA content from CS-PLGA-TA-E was 2.75 times higher than free TA and 1.45 times greater than the TA content from PLGA-TA-E. In the tumor, TA uptake from CS-PLGA-TA-E showed a gradual decrease from 24 h to 72 h with its availability of 20 μg/mL at 72 h. Free TA was also distributed in all tissues, and its highest concentration was observed at 6 h in the tumor, followed by the kidney, lungs, liver, serum, and spleen. After 6 h, the concentration of free TA in all tissues began to drop sharply, finally reaching negligible levels after 24 h. The TA content from PLGA-TA-E also showed its highest concentration in the tumor at 6 h, followed by the liver, spleen, kidney, lungs, and serum. The concentration of TA from PLGA-TA-E persisted in all tissues for up to 24 h, after which it decreased to negligible levels.

The distributions of free vitamin E and vitamin E encapsulated in PLGA-TA-E/CS-PLGA-TA-E to the tumor, liver, spleen, lungs, kidneys, and serum are shown in Figure 4B. Vitamin E content from CS-PLGA-TA-E showed its highest concentration in the tumor, in most of the time points (approx. 95 μg/mL at 6 h, 80 μg/mL at 12 h, 60 μg/mL at 24 h, 20 μg/mL at 48 h, and 10 μg/mL at 72 h) as compared to its uptake in the other tissues/organisms (liver showed its highest uptake of approx. 75 μg/mL at 6 h, spleen 97 μg/mL at 6 h, lungs 45 μg/mL at 6 h, kidney 42 μg/mL at 6 h, and serum 3 μg/mL at 6 h). At 6 h, in the tumor, E uptake from CS-PLGA-TA-E was 1.63 times higher than free E and 1.28 times higher than E uptake from PLGA-TA-E. In the tumor, E content from CS-PLGA-TA-E showed a gradual decrease from 24 h to 72 h with its availability of 10 μg/mL at 72 h. Free E was also distributed in different tissues, and the highest concentration was observed at 6 h in the tumor, followed by the lungs, liver, kidney, spleen, and serum. After 6 h, the concentration of free E in all tissues started to drop drastically, with insignificant levels after 12 h.
At 6 h, E released from PLGA-TA-E showed its highest concentration in the tumor, followed by the liver, spleen, kidney, lungs, and serum. The concentration of E from PLGA-TA-E persisted in all tissues for up to 24 h, after which it decreased to negligible levels.

Therefore, at all of the time points, TA/E from CS-PLGA-TA-E was distributed in the highest concentration in the colon tumor.

**TA and E Dual Administration Suppressed EGFR-Pi3k-AKT Signaling in the CAC Model.** The epidermal

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**Figure 5.** Inhibition of EGFR-Pi3k-AKT signaling pathways in AOM/DSS-induced CAC by tannic acid, vitamin E, PLGA-TA-E, and CS-PLGA-TA-E. (A-i) Immunohistochemical expression levels of EGFR, Pi3K-p85, and AKT1/2/3 in all of the experimental groups (magnification: 60×; scale bar is 20 μm). The nucleus was observed by 4′,6-diamidino-2-phenylindole (DAPI) staining. The experimental groups are the control, the AOM/DSS model, the AOM/DSS+ vitamin E, the AOM/DSS+ tannic acid, the AOM/DSS+ PLGA-TA-E, and the AOM/DSS+ CS-PLGA-TA-E. (A-ii) Bar graph shows % immunopositive cells from the immunohistochemical analysis of the EGFR, Pi3K-p85, and AKT1/2/3 proteins in all of the groups.
Figure 6. Inhibitory effect of tannic acid, vitamin E, PLGA-TA-E, and CS-PLGA-TA-E treatment on NF-κB/STAT3 signaling pathways in colon carcinoma. (A) Levels of inflammatory mediators TNF-α, IL-6, IL-1β, IL-23, and IL-17 in all of the experimental groups. Standard curve of OD versus concentration of cytokines (pg/mL) was plotted according to instructions on the information brochure. (B-i) Immunohistochemical expression levels of mTOR, NF-κB-p65, and p-STAT3 and the nuclear colocalization of NF-κB-p65 and p-STAT3 in all of the experimental groups (magnification: 60×; scale bar is 20 μm). The nucleus was observed by DAPI staining. Light-blue arrows demarcate the nuclear colocalization of NF-κB-p65 and p-STAT3. The experimental groups are the control, the AOM/DSS model, the AOM/DSS+ vitamin E, the AOM/DSS+ tannic acid, the AOM/DSS+ PLGA-TA-E, and the AOM/DSS+ CS-PLGA-TA-E. (B-ii) Bar graph shows % immunopositive cells from the immunohistochemical analysis of the mTOR, p-STAT3, and NF-κB-p65 proteins in all of the groups.
growth factor receptor (EGFR) pathway plays a central role in cancer progression. Downstream proteins of the EGFR pathway, viz., the PI3K/Akt/mammalian target of rapamycin (mTOR), lead to the overexpression of proinflammatory cytokines and cell proliferation stimulating proteins. These pathways are in turn activated by the nuclear translocation of transcription factors, NF-κB and STAT3.46,39 Therefore, inhibiting the EGFR-PI3k-AKT signaling is of prime concern to alleviate CAC. Data obtained from the immunohistochemical analyses of EGFR, PI3k-p85, and AKT1/2/3 show that they were highly overexpressed in the CAC model. Free E and free TA suppressed the expression levels of EGFR, PI3k-p85, and AKT1/2/3. However, treatment with CS-PLGA-TA-E showed a more significant attenuation of their expression (Figure 5A-i). Statistical analyses of the expression levels of EGFR, PI3k-p85, and AKT1/2/3 are shown in Figure 5A-ii.

TA and E Dual Therapy Suppressed AOM/DSS-Induced Proinflammatory Cytokine Activation and Inhibited the NF-κB and STAT3 Signaling Pathways. Cytokines (TNF-α, IL-6, IL-1β) stimulated by the EGFR pathway are known to promote tumor development by promoting angiogenesis and by reducing immune cell-mediated tumor elimination.40,47 IL-17 and IL-23 are also known to promote CAC by enhancing tumor cell survivability.37 Therefore, blockade of inflammatory cytokines reduces the severity of CAC progression. Enzyme-linked immunosorbent assay (ELISA) assay depicted that colonic levels of the proinflammatory TNF-α, IL-6, IL-1β, IL-17, and IL-23 were noticeably elevated in the AOM/DSS model group. Treatment with E, TA, or PLGA-TA-E significantly inhibited these cytokines; however, their levels were more substantially attenuated by CS-PLGA-TA-E treatment (Figure 6A).

Aside from the EGFR pathway, most tumor-promoting cytokines activate NF-κB and STAT3 signaling in premalignant cells, which activates antiapoptotic factors, inhibits apoptotic factors, and promotes cell proliferation and angiogenesis. NF-κB signal transduction in CAC leads to its localization in the nucleus, where it causes gene transcription. STAT3 prolongs the duration of the NF-κB nuclear retention. Phosphorylation of STAT3 by the receptor tyrosine kinase pathway allows its movement in the nucleus and binds to specific DNA sequences to induce cell proliferation factors.48 Data obtained from the immunohistochemical analysis of mTOR, p-STAT3, and NF-κBp65 show that they were highly overexpressed in the tumor...
In the CAC model, there was also colocalization of p-STAT3 and NF-κB into the nucleus (shown by arrows). E and TA treatment decreased mTOR, p-STAT3, and NF-Bp65 expression levels, but PLGA-TA-E and CS-PLGA-TA-E treatment showed a more substantial decrease. Also, the treatment with CS-PLGA-TA-E significantly lowered p-STAT3 and NF-κBp65 colocalization in the nucleus (Figure 6B-i). Statistical analyses of the expression levels of mTOR, p-STAT3, and NF-κBp65 are shown in Figure 6B-ii.

Thus, chitosan-modified PLGA-TA-E inhibited the proinflammatory cytokines and p-STAT3/NF-κB expression levels.

Impact of Nanoformulation of Tannic Acid and Vitamin E on the Wnt/β-Catenin Pathway and EMT (Epithelial-Mesenchymal Transition) Markers in the Colon Adenocarcinoma Model. The Wnt/β-catenin pathway is the central regulator of colon carcinoma. Under normal situations, many proteins phosphorylate β-catenin and lead to its ubiquitination and proteasomal degradation. However, an activated PI3k/AKT or NF-κB signaling in cancer leads to overexpression and nuclear localization of β-catenin. It activates the transcription and the expression of c-Myc and cyclin D1. Data obtained from the immunohistochemical analysis of β-catenin, c-Myc, and Cyclin D1 show that they were highly overexpressed, and β-catenin localized into the nucleus (shown by arrows) in the CAC model. E and TA reduced the overexpressed levels of β-catenin, c-Myc, and Cyclin D1, but treatment with CS-PLGA-TA-E showed a more substantial impact on reducing their expression levels.

Treatment with CS-PLGA-TA-E significantly lowered the translocation of β-catenin to the nucleus (Figure 7A-i). Statistical analysis of the expression levels of β-catenin, c-Myc, and Cyclin D1 are shown in Figure 7A-ii.

β-catenin is known to activate the EMT pathway by transactivation of the EGFR/PI3k/AKT pathway. It causes the loss of E-cadherin and the gain of N-cadherin and vimentin, all of which facilitate the loss of epithelial features.
and the gain of mesenchymal features (EMT), resulting in metastasis.\textsuperscript{53} Immunohistochemistry showed that the CAC model had low levels of E-cadherin and high levels of N-cadherin and vimentin. The expression levels were reversed by treatment with E, TA, PLGA-TA-E, and CS-PLGA-TA-E. Treatment with chitosan nanoparticles resulted in significant E-cadherin overexpression (Figure 8B-i). Statistical analysis of the expression levels of E-cadherin, N-cadherin, and vimentin are shown in Figure 7B-ii.

Thus, chitosan nanoformulation with TA/E can attenuate colon adenocarcinoma by inhibiting the Wnt/β-catenin pathway and by preventing EMT.

**Codelivery of TA and vitE with Chitosan Nanoparticles in the CAC Model Promotes Apoptosis.** As evident from our CAC model, activation of the proinflammatory mediators, NF-κB/STAT3 pathways, EGFR/P13k/AKT, or the β-catenin signaling leads to cell survival.\textsuperscript{54} Immunohistochemistry (Figure 8A-i) showed that expressions of proapoptotic proteins Bax and Bad decreased in the AOM/DSS model. However, they were increased by treatment with TA, E, and, more significantly, by PLGA-TA-E and CS-PLGA-TA-E. Expression of the antiapoptotic protein Bcl-2 increased in the model system but decreased significantly after treatment with CS-PLGA-TA-E. Statistical analyses of the expression levels of Bax, Bad, and Bcl-2 are shown in Figure 8A-ii. The AOM/DSS model reduced caspase 3, caspase 9, and cytochrome C levels, but the combined effect of TA, E, and their chitosan nanoformulation significantly increased them (Figure 8B,C).

Hence, the codelivery of TA and E promote apoptosis in colorectal tumor.

**DISCUSSION**

Systemic inflammation is a marker of the severity of cancer progression in at least 20–40% of colorectal cancer patients. According to numerous studies, inflammation has a wide range of effects on CAC pathogenesis, including promoting tumor cell proliferation and tumor growth, blocking antitumor immunity by increasing proinflammatory cell types, and promoting subsequent metastasis.\textsuperscript{50} Tannic acid\textsuperscript{11,12} and vitamin E\textsuperscript{51,52} have anti-inflammatory and antitumor properties. These compounds have been encapsulated within PLGA-NPs to provide enhanced bioavailability and sustained release in the tumor. To improve cellular drug delivery, nanoparticle surfaces have recently been decorated with ligands, and we have improved our PLGA-NPs with chitosan. Chitosan imparts cationic charge. Since CS-coated nanoparticles have a positive charge, they attract the negatively charged cell membrane, increasing the permeability of poorly permeable drugs like tannic acid\textsuperscript{53,54} and drugs with low solubility like vitamin E.\textsuperscript{55} Chitosan also affects the tumor cell metabolism, slowing cancer progression.\textsuperscript{54,56} Furthermore, as demonstrated by our *in vitro* drug release research, chitosan’s mucoadhesive nature supports the sustained release of drugs.\textsuperscript{56} Around 20–400 nm-sized nanoparticles have been employed for chemotherapy\textsuperscript{57,58} and for PLGA-TA-E NPs, the mean particle size was 118 ± 2.5 nm with PDI 0.43 and for CS-capped NPs, the mean particle size was 152 ± 4.7 nm with a PDI value of 0.251. The successful trapping of a CS layer to the PLGA NP surface contributes to its increased size. The PDI values were 0.43 and 0.25, suggesting a particle size distribution that was relatively narrow. Spherical nanoparticles are the best form for facilitating a longer circulation time and better absorption by cancer cells,\textsuperscript{58} and our AFM data shows that both PLGA-NPs and CS-PLGA-NPs are spherical. All of the relevant functional groups of tannic acid, vitamin E, and chitosan were conserved in their PLGA-encapsulated and chitosan nanoparticle forms. The addition of chitosan to the surface of PLGA-TA-E improved the encapsulation efficiency and drug loading of TA and E. At all time frames, E and TA release from CS-PLGA-TA-E was greater than the release from PLGA-TA-E, according to drug release kinetics.

Body weight and survivability of BALB/C mice increased with E, TA, PLGA-TA-E, and CS-PLGA-TA-E treatment as compared to the AOM/DSS model but more significantly with CS–NPs. Chitosan decoration of PLGA-TA-E enhanced the hemoglobin and antioxidant levels and maintained low hepatic enzyme levels. Although the administration of E, TA, and PLGA-NPs abolished the DSS-induced shortening of the colon, reduced the numbers of tumors or tumor volume, and maintained the crypt architecture and the average number of goblet cells, all of the above parameters were controlled more remarkably by the CS-PLGA-TA-E treatment.

CS–NPs have been reported to remain for longer times in blood circulation due to their low uptake by the reticuloendothelial system (RES), i.e., a system where phagocytes clear the circulation of drug particles. They can easily evade the disorganized and disoriented tumor vasculature sites by the enhanced permeability and retention (EPR) effect owing to their small size.\textsuperscript{59} These are the probable reasons why TA/E released from CS–NPs showed the highest uptake in the tumor compared to the other tissues at all time points and remained for 72 h. Free TA/E started diminishing in the tumor after 6 h, and TA/E from PLGA-TA-E cleared after 48 h. Free TA or E or TA/E content from PLGA-TA-E and CS-PLGA-TA-E was more concentrated in the organ tissues than the plasma, suggesting that both TA and E rapidly penetrate various target organs, following intra-peritoneal administration. A significant amount of TA or E from PLGA-TA-E and CS-PLGA-TA-E in the liver at all time points is indicative of intrahepatic circulation and drug detoxification. Also, a negligible amount of TA or E from PLGA-TA-E or CS-PLGA-TA-E in the kidney, spleen, lungs, and serum at 72 h reflected higher metabolic rates. The administration of CS-PLGA-TA-E formulation compared to PLGA-TA-E, free E, or free TA led to a higher accumulation of TA/E within the tumorous tissues.

EGFR is known to be overexpressed in solid tumors, and resistance to EGFR targeting drugs like cetuximab has shown an increase in proinflammatory cytokines, especially IL-1β, NF-κB, and STAT3. Resistance to EGFR targeting drugs has also led to the induction of EMT. TA inhibits breast cancer via the EGFR/Stat3 pathway,\textsuperscript{60} and thus, we wanted to find out if TA has the potential to inhibit CAC progression by inhibiting the EGFR/P13k/AKT/mTOR pathway. Similarly, although there are reports of vitamin E controlling lung tumorigenesis by the EGFR-ERK pathway,\textsuperscript{61,62} there are no reports that show that vitamin E inhibits CAC by the EGFR pathway. In our research, E and TA individually reduced the expression levels of EGFR and its downstream PI3k-pathway,\textsuperscript{61,62} and mTOR, which were elevated in the CAC model. The expression of these proteins were significantly decreased when they were treated with PLGA-NPs and, in particular, CS–NPs.

Tumor stroma cells secrete proinflammatory cytokines to increase tumor growth by activating tumorigenic signaling
mediators like activation of NF-κβ by TNFα and IL-1β and activation of STAT3 by IL-6. NF-κβ activation promotes tumor development by increasing cell proliferation, angiogenesis, tumor invasion, and metastasis and activating the antiapoptotic Bcl-2, which inhibits apoptosis.53 STAT3 activation promotes gastrointestinal epithelial regeneration and proliferation by inhibiting the proapoptotic Bax and Bad and upregulating antiapoptotic Bcl-2, Bcl-xL, and proliferative genes such as cyclin D1.63,64 Activated Stat3 leads to prolonged activation of NF-κβ.66,67 IL-23 and IL-17 also play significant roles in colon cancer progression by activating STAT3 and IL-6, henceforth controlling cell proliferation.68 TNFα antagonists (infliximab and adalimumab)69 and IL-6 antagonists (tocilizumab, siltuximab) are currently used to treat cancer. Antagonists of IL-1β (canakinumab) are also under trial for cancer treatment.70 Thus, inhibition of TNFα, IL-1β, IL-6, NF-κβ, and Stat3 plays a pivotal role in controlling colorectal cancer. The elevated proinflammatory cytokine (TNF-α, IL-1β, IL-6, IL-17, and IL-23) profile in the adenocarcinoma model was reduced significantly by our chitosan nanoparticles. Tannic acid has been reported to inhibit breast cancer and gingival cancer by modulating the NF-κβ/Stat3 pathway. Vitamin E has been reported to block the NF-κβ/Stat3 pathway in macrophages and some cancer cells.71 In the colon cancer model, TA and vitamin E both decreased NF-κβ and Stat3 expressions and nuclear localization. CS-PLGA-TA-E substantially decreased NF-p65 and pStat3 expressions and nuclear localization compared to TA or E alone.

The aberrant Wnt/β-catenin pathway is the central regulator of colon carcinogenesis. It functions by controlling the β-catenin translocation to the nucleus, where it controls downstream transcription factors. In normal conditions, axin, tumor suppressor adenomatous polyposis coli (APC), CK1β, and GSK-3β phosphorylate β-catenin, leading to its ubiquitination and its proteasomal degradation. When activated PI3K-AKT or NF-κβ signaling inhibits GSK-3β, β-catenin leads to C-Myc and cyclin D1 activation, allowing the cell cycle to enter the G1/S process.72 Also, c-Myc has been reported to control chromosomal aberrations and increase resistance to chemotherapy in cancer cells.73 There are quite a few existing drugs like Genistein and Sulindac, which target β-catenin for inhibition of cancer.74 Thus, targeting the inhibition of β-catenin, cyclin D1, and C-Myc becomes a crucial factor in controlling CAC. A tannin compound from Sanguisorba officinalis caused apoptosis of HT-29 colon cancer cells by blocking the Wnt/β-catenin signaling75 and γ-tocopherol attenuated moderate colon tumorigenesis in mice via the Wnt/β-catenin pathway.17 In our colitis-associated carcinoma (AOM/DSS) model, we found that intraperitoneal administration of only TA and only E suppressed β-catenin, c-myc, and cyclin D1 expressions and reduced β-catenin nuclear localization. The combined effect of TA and E, as well as their chitosan capping, decreased the expressions of β-catenin, c-myc, and cyclin D1 and reduced the β-catenin nuclear localization.

β-catenin is known to activate the EMT pathway by transactivation of the EGFR/Pi3k/AKT pathway. Cadherin-dependent cell adhesion necessitates the development of a complex between E-cadherin (a tumor suppressor) and cytosolic-β catenin for cytoskeleton stabilization, cell growth, and epithelial phenotype maintenance.76 Formation of N-cadherin–catenin adhesive complexes leads to β-catenin translocation to the nucleus and activation of the PI3K-AKT pathway. Similarly, activation of EGFR-Pi3K-AKT can also upregulate N-cadherin.79,80 Therefore, loss of E-cadherin or cytosolic β-catenin and N-cadherin overexpression promote tumorigenesis, better migration, and invasive abilities in tumor cells.81 Tannic acid prevented EMT in breast cancer cells,71 and γ-tocotrienol inhibited EMT in human breast cancer cells.82 We ventured if inhibiting EMT with TA and E could delay colon cancer progression in the AOM/DSS model. E-cadherin levels were low in our AOM/DSS model, but N-cadherin and vimentin levels were high. These levels were significantly altered by treatment with TA and E alone. The combined effect and chitosan capping more significantly enhanced the E-cadherin and lowered the N-cadherin and vimentin levels. A gain of E-cadherin and loss of N-cadherin and vimentin inhibit EMT by lowering migration and invasiveness of tumor cells.83

Our treatment groups activated the apoptotic pathway, which is a downstream pathway of the above pathways that contributes to tumor cells’ programmed cell death. Proapoptotic markers were increased and antiapoptotic markers were decreased strongly by the chitosan nanoparticles.

### CONCLUSIONS

Our results show that tannic acid showed better anticancer activity than vitamin E in the colorectal adenocarcinoma model. The anticancer activity of the PLGA nanoformulation containing both tannic acid and vitamin E was even stronger. However, the chitosan modification of the PLGA nanoformulation allowed a much better and higher retention period within the tumorous tissues. Of all of the treatment categories, chitosan-modified PLGA nanoparticles had the strongest anticancer ability, and it accomplished so by inhibiting proinflammatory cytokines, the EGFR-Pi3k-Akt pathway, Wnt/B-cat signaling, EMT transition, and promoting apoptosis. Thus, chitosan-modified PLGA-TA-E could provide a beneficial milieu in preventing CAC progression.

### EXPERIMENTAL SECTION

**Chemicals.** Tannic acid (purity ≥99.0%), vitamin E-TPGS (D-α-TPGS), PLGA (85:15), and azoxymethane (13.4 M, purity ≥98%) were bought from Sigma-Aldrich, MO. Chitosan (low MW, extrapure), dichloromethane (DCM) and poly(vinyl alcohol) (PVA) were purchased from Sisco Research Laboratories (Gurugram, India). DSS colitis-grade (36 000–50 000 Da) was purchased from MP Biomedicals (Costa Mesa, CA). Serum ALT, AST, SOD, catalase, and GPx assay kits were bought from ARKRAY Healthcare Pvt. Ltd (Surat, India), and the mouse hemoglobin assay kit was from Abcam. IL-1β, IL-6, TNF-α, IL-17, and IL-23 levels were calculated using ELISA kits from R&D (MN). All antibodies were purchased from Sigma-Aldrich, Abcam, and Cell Signaling Technologies (MA). Primary antibodies were EGFR (goat antimonoclonal IgG), PI3K-p85 (rabbit antigoal polyclonal IgG), AKT1/2/3 (mouse antirabbit monoclonal IgG1), mTOR (human antimonoclonal IgG2a), NF-κB-p65 (goat antirabbit polyclonal IgG), p-STAT3 (mouse antigoal polyclonal IgG), β-catenin (goat antimonoclonal IgG1), cyclin D1 (goat antirabbit polyclonal IgG), c-myc (rabbit antigoal polyclonal IgG), E-cadherin (human antimonoclonal IgG1), N-cadherin (human antirabbit monoclonal IgG1), vimentin (donkey antigoal polyclonal IgG), Bax (mouse antigoal polyclonal IgG), Bad (mouse...
antirabbit polyclonal IgG), and Bcl-2 (human antimouse monoclonal IgG1). Secondary antibodies were used in different combinations using anti-IgG goat antimouse-FITC/Alexa Fluor 555/Alexa Fluor 647 or anti-IgG goat antirabbit-FITC/Alexa Fluor 555/Alexa Fluor 647 or anti-IgG donkey antigoat-FITC/Alexa Fluor 555/Alexa Fluor 647. All other chemicals and solvents used were available commercially and of a high degree of purity.

**Preparation of PLGA-TA-E and Chitosan-Capped PLGA-TA-E Nanoparticles.** Tannic acid/vitamin E-loaded PLGA-NPs (PLGA-TA-E) were prepared using the emulsion solvent evaporation method.83 Briefly, 150 mg of PLGA was solubilized in 6 mL of dichloromethane, 40 mg of tannic acid in 2 mL of ethanol, and 40 mg of vitamin E TPGS in 2 mL of ethanol. For 2 min at 70% amplitude, the probe sonicator combined and emulsified the above solutions in ice. The PLGA mixture solution was then given a dropwise application of 0.3% poly(vinyl alcohol) (PVA 10 mL) through the tube walls. Further emulsification was done at 0 °C using the probe sonicator for another 2 min. Finally, the above mixture was stirred for 4 h at room temperature to evaporate dichloromethane. The PLGA-TA-E NPs were centrifuged at 20 000 rpm for 1 h and washed three times to remove the unloaded TA, E, and PVA.

For preparing chitosan-coated PLGA nanoparticles, the above PLGA-TA-E nanoparticle pellets were suspended in 10 mL of double-distilled water and then mixed with an equal volume of CS solution (2 mg/mL dissolved in 1% w/v acetic acid). The mixture was stirred for 2 h at room temperature to form CS-capped PLGA-TA-E NPs. After three washes, the NPs were extracted by centrifugation at 18 000 rpm for 1 h. To obtain the respective NP powder, the PLGA-TA-E and CS-PLGA-TA-E pellets were lyophilized for three days and preserved at −20 °C.35

**Characterization of PLGA-TA-E and CS-PLGA-TA-E.** The PLGA-TA-E NPs and CS-PLGA-TA-E NPs were examined for characterization in terms of Fourier transform IR, particle size, surface morphology, shape, the efficacy of drug encapsulation, percentage of drug loading, and release of in vitro products. The procedures are listed next.

**Encapsulation Efficiency and Drug Loading.** NPs (5 mg) were soaked in 5 mL of phosphate buffer for 30 min, and it was centrifuged at 20 000 rpm at 4 °C for 1 h. To eliminate the unbound drug, the precipitate was washed twice with a fresh solvent. The transparent supernatant solution was analyzed for unbound TA and E contents using a UV spectrophotometer (JASCO V-730, spectrophotometer, Tokyo, Japan) at maximum absorbance values of 271 and 295 nm, respectively. TA and E standard curves were obtained by plotting the concentrations (10−50 mg/mL) against their respective absorbances.

The percentage of drug loading and entrapment efficiency were calculated using the following formula

\[
\text{encapsulation efficiency} = \left( \frac{\text{the total amount of drug released from the lyophilized NPs}}{\text{amount of drug initially taken to synthesize the NPs}} \right) \times 100
\]

\[
\text{drug loading} = \left( \frac{\text{amount of drug found in the lyophilized NPs}}{\text{amount of lyophilized NPs}} \right) \times 100
\]

**Particle Size Measurement.** A Zetasizer 3000 HSA (Malvern Instruments, U.K.) was used to measure particle size and distribution. Using 12 mm cells at 90 degrees and a temperature of 25 °C, differential light scattering (DLS) was used to determine the mean NP diameter. Before the tests, the NPs were diluted with double-distilled water, and 500 μL was loaded into the cuvette for DLS and polydispersity index readings.

**Fourier Transform Infrared Spectroscopy.** To obtain various functional groups, the IR spectra of tannic acid, vitamin E, PLGA, chitosan, PLGA-TA-E, and CS-PLGA-TA-E were obtained. The NP powder was put inside the IR machine after centrifuging and lyophilizing the NP formulations. Powder forms of TA, E, PLGA, and pure chitosan were also placed inside the IR machine. Various peaks in the IR spectrum were

| Table 1. Experimental Work Plan for the Development of Colorectal Tumors Using AOM/DSS and Its Amelioration by Treatment with TA, E, PLGA-TA-E, and CS-PLGA-TA-E |
|---------------------------------------------------------------|
| **Animals Sacrificed** | **Treatment** | **2% DSS** | **DW water** | **2% DSS** | **DW water** | **2% DSS** | **DW water** | **2% DSS** | **DW water** | **2% DSS** | **DW water** |
| Acclimatization | n=10 | n=10 | n=10 | n=10 | n=10 | n=10 | n=10 | n=10 | n=10 | n=10 | n=10 |
| Control | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| AOM/DSS | AOM given intraperitoneally and DW water gavaged along with DSS, at the respective time points |
| AOM/DSS+vitE | AOM given intraperitoneally and DW water gavaged along with DSS, at the respective time points. Vitamin E-TPGS (40 mg/kg) given every alternate day intraperitoneally, beginning from treatment point. |
| AOM/DSS+TA | AOM given intraperitoneally and DW water gavaged along with DSS, at the respective time points. Tannic acid (40 mg/kg) given every alternate day intraperitoneally, beginning from treatment point. |
| AOM/DSS +PLGA-TA-E | AOM given intraperitoneally and DW water gavaged along with DSS, at the respective time points. PLGA-TA-E (40 mg/kg) given every alternate day intraperitoneally, beginning from treatment point. |
| AOM/DSS +CS-PLGA-TA-E | AOM given intraperitoneally and DW water gavaged along with DSS, at the respective time points. Chitosan-PLGA-TA-E (40 mg/kg) given every alternate day intraperitoneally, beginning from treatment point. |
interpreted for different functional groups using the Bruker Tensor-27 FTIR spectrometer, in the attenuated total reflectance (ATR) mode.

**Atomic Force Microscopy.** Atomic force microscopy (AFM) used Agilent Technologies 5500 (CA), operated in the contact mode, to obtain surface topography. The scanning tip was a 220 μm long silicon microcantilever. A test drop of 5 μL (1–50 μg/mL NPs in distilled water) was placed on the freshly cleaved mica sheet and distributed to about 10 mm in diameter. The sample solution was allowed to dry for 15–20 min on the mica sheet and immediately observed by AFM.

The acquired images were analyzed with the aid of scanning probe microscopy (SPM) tools for laboratory study.

**In Vitro Drug Release Studies.** PLGA-TA-E NPs and CS-PLGA-TA-E NPs were solubilized in phosphate-buffered saline (PBS) buffer (1 M, NaCl = 8 g, KCl = 0.2 g, Na₂HPO₄ = 1.44 g, KH₂PO₄ = 0.24 g) were dissolved in 1 L of double-distilled water and the pH was adjusted to 7.2–7.4) to study the *in vitro* release kinetics of tannic acid and vitamin E from the formulation. Each formulation (10 mg) was placed in a dialysis bag with a cutoff size of 5 kDa, placed in 200 mL of PBS, and stirred at 90 rpm for four days. Then, 2 mL of the buffer solution was removed after a fixed time interval and replaced with a fresh buffer. Using a UV–visible spectrophotometer (JASCO V-730 spectrophotometer, Tokyo, Japan) at 271 and 295 nm, the drugs’ release was determined. The experiments were repeated three times, and the average values were plotted in the graph.

**Animals.** The experimental mice were randomized. We worked with a total of 60 mice (*n* = 10), which were randomly selected from 300 inbred male mice of age 4–6 weeks and body weight 28–30 g. All of the male mice were procured from CSIR-Indian Institute of Chemical Biology, Kolkata (animal house division). The mice were fed a certified regular diet and were given autoclaved normal drinking water. The animals were kept at 22–24 °C temperature, 50–60% humidity, and subject to light and dark cycles of 12:12 h. The research procedure conducted on animals was as per the recommendations of the CSIR-Indian Institute of Chemical Biology Animal Ethics Committee.

**Induction of Colitis-Induced Colorectal Carcinoma and Experimental Design.** After acclimatization for 10 days, the mice were arbitrarily divided into six groups of 10 animals and were fed with standard chow diets. The experimental plan of this study is depicted in Table 1. For inducing CAC, all of the mice were intraperitoneally injected with 10 mg/kg body weight of AOM once. One 7 day cycle of DSS (2% in drinking water) was followed by 2 weeks of drinking water in between the cycles. This cycle was continued four times in all of the groups. For the treatment groups with TA (40 mg/kg), E (40 mg/kg), PLGA-TA-E (TA—40 mg/kg + vitamin E—40 mg/kg), and CS-PLGA-TA-E (TA—40 mg/kg + vitamin E—40 mg/kg), the drugs were suspended in PBS and administered intraperitoneally every alternate day. The treatment (as denoted by treatment point arrow) was started after two cycles of 2% DSS intake when the animals developed diarrhea and rectal prolapse.

**Measurement of Serum ALT, AST, Hb, SOD, GPx, and Catalase.** Blood samples were obtained by tail-vein puncture and kept at 4 °C undisturbed overnight. To obtain the serum, samples were centrifuged the next day at 2500 rpm for 10 min at 4 °C. Serum AST (aspartate aminotransferase), alanine transaminase (ALT), hemoglobin (Hb), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase activities were calculated according to the instruction brochure provided with the commercial assay kits.

**Chromatographic Conditions.** The analysis was carried out using a Symmetry C18 chromatographic column (Waters Corporation, MA) (4.6 mm × 250 mm) and particle size 5 μm. The column was maintained at a temperature of 38.0 ± 0.5 °C throughout the analysis. The samples were analyzed with the gradient methodology using methanol (HPLC grade) and water (HPLC grade). The gradient used was as follows

| time (min) | % H₂O | % MeOH |
|-----------|-------|--------|
| 0         | 100   | 0      |
| 2         | 100   | 0      |
| 2.5       | 80    | 20     |
| 6         | 80    | 20     |
| 6.5       | 70    | 30     |
| 15        | 70    | 30     |
| 15.5      | 80    | 20     |
| 20        | 80    | 20     |
| 20.5      | 100   | 0      |
| 30        | 100   | 0      |

The vacuum filtration of the mobile phase was done using a 0.22 μm nylon membrane and degassed by ultrasonication for 15 min before use. The mobile phase was passed through a chromatographic column at a flow rate of 1 mL/min. The injection volume was 100 μL, and UV detection was performed at 271 and 295 nm for TA and E. Each test required 30 min.

**Tissue Distribution Study.** All mice were fasted overnight and were fed only water before the experiments. Standard stock solutions of TA (1 mg/mL) and E (1 mg/mL) were prepared daily by dissolving the specific amounts of the drug in ethanol.

After intraperitoneal administration of free TA, free E, PLGA-TA-E, and CS-PLGA-TA-E, mice were sacrificed at 0, 6, 12, 24, 48, and 72 h. Various tissues (colorectal regions containing the tumor, liver, spleen, lung, kidney) were collected and washed with 0.9% NaCl to remove the extra blood and contents. After blotting them with filter paper, 1 mg equivalent from the tissues was weighed and homogenized in 1
mL of 0.9% NaCl. Then, 100 μL of it was used as the tissue sample. Blood samples were drawn from the tail vein and coagulated for half an hour in an MCT tube. The blood samples were centrifuged at 2000 rpm for 10 min (4 °C), and serum was obtained from the supernatant. Then, 100 μL of the serum was used as the sample. Tissues were stored at −80 °C for further use.86

**Histological Evaluation.** The colorectal tumor tissues in 10% neutral buffered formalin (NBF) were paraffin-embedded, thinly microtome, deparaffinized, and rehydrated using the standard histology procedure. Various pathological changes were assessed using hematoxylin and eosin (H/E) stain.

**Assessment of Serum Cytokines.** Blood samples were extracted at different time points. The serum levels of TNF-α, interleukin 1β (IL-1β), interleukin-23 (IL-23), interleukin-17 (IL-17), and interleukin 6 (IL-6) were determined using the commercially available ELISA kit according to the company’s instruction and guidelines (R&D Systems, MN).

**Immunohistochemistry.** Blocks of tumor tissues that were paraffin-embedded were cut into thin sections using a microtome and mounted on slides. Xylene was used to deparaffinize tumor tissues. The antigen retrieval process was performed for 15 min in a water bath at 100 °C using sodium citrate buffer (sodium citrate-10 mM, 0.05% tween 20, pH = 6.0). Blocking was done in Tris-buffered saline (TBS) for 2 h with a 5% solution of bovine serum albumin (BSA). The tumor tissue sections were mildly rinsed with TBST (TBS, 0.1% Tween 20) for permeabilization. Finally, the sections were incubated in a moist chamber overnight with a 1:500 dilution of the primary antibody. The tissue sections were rinsed with 1 × TBS and incubated with 1:400 dilution of Alexa Fluor 555, FITC, and Alexa Fluor 647 conjugated secondary antibodies at room temperature for 2 h. The nucleus was observed by DAPI (Invitrogen, CA) staining. All of the images were observed using an automated laser scanning confocal microscope (Olympus FV10i, Shinjuku, Tokyo, Japan).

**Statistical Analysis.** All data from minimum three experiments with replicates were represented as mean ± standard deviation (SD). Using the software GraphPad Prism (CA), statistical significance and differences between control, disease, and four other treatment groups were calculated using one-way analysis of variance (ANOVA).

**Future Perspectives.** A number of studies are required in human models for alleviating colon carcinoma using chitosan-capped nanoparticles. Chitosan-modified PLGA-TA-E could then prove to be beneficial in preventing CAC progression and would prevent CAC in the near future.

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**Notes**

The authors declare no competing financial interest.

The CSIR-IICB, Animal Ethics Committee, permitted all animal studies and experimental protocols (IICB-AEC; registration no.: 147/1999/CPCSEA) that is registered under (CPCSEA-Social Justice and Empowerment Committee for the Purpose of Control and Supervision of Experiments on Animals), Government of India, New Delhi. The authors declare that they received proper review board approval from the Institute and that all animal experiments were performed according to the guidelines outlined in the Declaration of Helsinki.

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**ABBREVIATIONS**

AKT:protein kinase B; AOM:azoxymethane; CAC:colitis-associated colorectal cancer; CS:chitosan; CS-PLGA-TA-E:vanadium acid and vitamin E-loaded PLGA nanoparticles with chitosan capping; DSS:dextran sulfate sodium; EVitamin E TPGS; EGFRepidermal growth factor receptor; EMT:epithelial-mesenchymal transition; IL-1β:interleukin 1β; IL-6:interleukin 6; IL-17:interleukin-17; IL-23:interleukin-23; mTOR:mammalian target of rapamycin; NF-κB:nuclear factor kappa B; PLGA-NPs:poly(D,l)-lactide-co-glycolic acid) nanoparticles; PLGA-TA-E:vanadium acid and vitamin E-loaded PLGA nanoparticle; PI3k:phosphoinositide 3-kinase; STAT3:signal transducer and activator of transcription 3; TA:vanadium acid; TNFα:tumor necrosis factor-α

**REFERENCES**

(1) Chapuis, P. H.; Bokey, E.; Chan, C.; Keshava, A.; Rickard, M. J. F.; Stewart, P.; et al. Recurrence and cancer-specific death after adjuvant chemotherapy for Stage III colon cancer. *Colorectal Dis.* 2019, 21, 164–173.

(2) Rajpal, S.; Venook, A. P. Targeted therapy in colorectal cancer. *Clin. Adv. Hematol. Oncol.* 2006, 4, 124–132.

(3) Akinkuotu, A. C.; Maduekwe, U. N.; Hayes-Jordan, A. Surgical outcomes and survival rates of colon cancer in children and young adults. *Am. J. Surg.* 2021, 221, 718–724.

(4) Eaden, J. A.; Abrams, K. R.; Mayberry, J. F. The risk of colorectal cancer in ulcerative colitis: A meta-analysis. *Gut.* 2001, 48, 526–535.

(5) Yang, Y.; Yang, L.; Jiang, S.; Yang, T.; Lan, J.; Lei, Y.; et al. HMGB1 mediates lipopolysaccharide-induced inflammation via interacting with GPX4 in colon cancer cells. *Cancer Cell Int.* 2020, 20, No. 205.

(6) Grivennikov, S. I. Inflammation and colorectal cancer: Colitis-associated neoplasia. *Semin. Immunopathol.* 2013, 35, 229–244.

(7) Tanaka, H.; Gunasekaran, S.; Saleh, D. M.; Alexander, W. T.; Alexander, D. B.; Ohara, H.; et al. Effects of oral bovine lactoferrin on...
a mouse model of inflammation-associated colon cancer. Biochem. Cell Biol. 2021, 99, 159–165.

(8) Sun, W.; Jiang, H.; Wu, X.; Xu, Z.; Yao, C.; Wang, J.; et al. Strong dual-crosslinked hydrogels for ultrasound-triggered drug delivery. Nano Res. 2019, 12, 115–119.

(9) Chung, K. T.; Wong, T. Y.; Wei, C. I.; Huang, Y. W.; Lin, Y. Tannins and human health: A review. Crit. Rev. Food Sci. Nutr. 1998, 38, 421–464.

(10) Baharuddin, N. S.; Nasir, N. S.; Kamal, N. N.; Saifuddin, S. N.; Mohamad, S. B. Vitamin E: An antioxidant with anticancer properties. Malays. J. Med. Health Sci. 2011, 15, 183–193.

(11) Zhang, J.; Cui, L.; Han, X.; Zhang, Y.; Zhang, X.; Chu, X.; et al. Protective effects of tannic acid on acute doxorubicin-induced cardiotoxicity: Involvement of suppression in oxidative stress, inflammation, and apoptosis. Biomed. Pharmacother. 2017, 93, 1253–1260.

(12) Traber, M. G.; Atkinson, J. Vitamin E, antioxidant and nothing more. Free Radicals Biol. Med. 2007, 43, 4–15.

(13) Yang, P.; Ding, G. B.; Liu, W.; Fu, R.; Sajid, A.; Li, Z. Tannic acid directly targets pyruvate kinase isozyme M2 to attenuate colon cancer cell proliferation. Food Funct. 2018, 9, 5547–5559.

(14) Hamiza, O. O.; Rehman, M. U.; Tahir, M.; Khan, R.; Khan, A. Q.; Lateef, A.; et al. Amelioration of 1,2 dimethylhydrazine (DMH) induced colon oxidative stress, inflammation and tumor promotion response by tannic acid in wistar rats. Asian Pac. J. Cancer Prev. 2012, 13, 4393–4402.

(15) Bazzaz, R.; Bijanpour, H.; Pirouzpanah, S. M. B.; Yaghmaei, P.; Rashhtchizadeh, N. Adjuvant therapy with γ-tocopherol-induced apoptosis in HT-29 colon cancer via cyclin-dependent cell cycle arrest mechanism. J. Biochem. Mol. Toxicol. 2019, 33, No. e22399.

(16) Chen, J. X.; Liu, A.; Lee, M. J.; Wang, H.; Yu, S.; Chi, E.; et al. δ- and γ-tocopherols inhibit pH/Pt/DSS-induced colon carcinogenesis by protection against early cellular and DNA damages. Mol. Carcinog. 2017, 56, 172–183.

(17) Husain, K.; Zhang, A.; Shivers, S.; Davis-Yadley, A.; Coppola, D.; Yang, C. S.; et al. Chemoprevention of oxazepamethane-induced colon carcinogenesis by delta-tocotrienol. Cancer Prev. Res. 2019, 12, 3576–366.

(18) Barrett, C. W.; Fingleton, B.; Williams, A.; Ning, W.; Fischer, M. A.; Washington, M. K.; et al. MTGR1 is required for tumorigenesis in the murine AOM/DSS colitis-associated carcinoma model. Cancer Res. 2011, 71, 1302–1312.

(19) Neufert, C.; Becker, C.; Neurath, M. F. An inducible mouse model of colon carcinogenesis for the analysis of sporadic and drug delivery systems for efficacy improvement; a comprehensive review. Molecules 2021, 25, No. 1486.

(20) Kawabata, K.; Yoshioka, Y.; Terao, J. Role of intestinal microbiota in the bioavailability and physiological functions of dietary polyphenols. Molecules 2019, 24, No. 370.

(21) Dubbs, M. D.; Gupta, R. B. Solubility of vitamin E (α-tocopherol) and vitamin K3 (menadione) in ethanol-water mixture. J. Chem. Eng. Data 1998, 43, 590–591.

(22) Schmolz, L. Complexity of vitamin E metabolism. World J. Biol. Chem. 2016, 7, 14–43.

(23) Danhier, F.; Ansorena, E.; Silva, J. M.; Coco, R.; Le Breton, A.; Prétat, V. PLGA-based nanoparticles: An overview of biomedical applications. J. Controlled Release 2012, 161, 505–522.

(24) Hines, D. J.; Kaplan, D. L. Poly(lactic-co-glycolic) acid-controlled-release systems: Experimental and modeling insights. Crit. Rev. Ther. Drug Carrier Syst. 2013, 30, 257–276.

(25) Roberts, R.; Smyth, J. W.; Will, J.; Roberts, P.; Grek, C. L.; Ghatnerek, G. S.; et al. Development of PLGA nanoparticles for sustained release of a connexin43 mimetic peptide to target glioblastoma cells. Mater. Sci. Eng. C 2020, 108, No. 110191.

(26) Zhang, H.; Li, J.; Patel, S. K.; Palmer, K. E.; Devlin, B.; Rohan, L. C. Design of poly(Lactic-co-glycolic acid) (plga) nanoparticles for vaginal co-delivery of gatifloxin and dapivirine and their synergistic effect for hiv prophylaxis. Pharmaceutics 2019, 11, No. 184.

(27) Pabla, B.; Bissonnette, M.; Konda, V. J. Colon cancer and the α-β-γ-δ catenin pathway and Wnt target genes by RoR2 and ATF2/ATF4 in colon cancer cells. Archivio, M.; Filesi, C.; Varì, R.; Scazzocchio, B.; Masella, R. Biochim. Biophys. Acta Biomater. 2012, 1826, 366–376.

(28) Danhier, F.; Ansorena, E.; Silva, J. M.; Coco, R.; Le Breton, A.; Prétat, V. PLGA-based nanoparticles: An overview of biomedical applications. J. Control. Release 2012, 161, 505–522.

(29) Yang, H.; Li, J.; Patel, S. K.; Palmer, K. E.; Devlin, B.; Rohan, L. C. Design of poly(Lactic-co-glycolic acid) (plga) nanoparticles for vaginal co-delivery of gatifloxin and dapivirine and their synergistic effect for hiv prophylaxis. Pharmaceutics 2019, 11, No. 184.

(30) Adhikari, H. S.; Yadav, P. N. Anticancer Activity of Chitosan, Chitosan Derivatives, and Their Mechanism of Action. J. Biomater. 2018, 2018, No. 2952085.

(31) Ta, H.; Dunstan, D.; Coss, C. Anticancer Activity and Therapeutic Applications of Chitosan Nanoparticles. In Chitin, Chitosan, Oligosaccharides and Their Derivatives; Kim, S. K., Ed.; CRC Press LLC, 2010; pp 271–366.

(32) De Simone, V.; Fränzê, E.; Ronchetti, G.; Colantonii, A.; Fantini, M. C.; Di, F. D.; et al. Th17-type cytokines, IL-6 and TNF-α synergistically activate STAT3 and NF-κB to promote colorectal cancer cell growth. Oncogene 2015, 34, 3493–503.

(33) Grivennikov, S. I.; Karin, M. Inflammation and Colon Cancer. Gastroenterology 2010, 138, 2101–2114.

(34) Kanehara, K.; Óhnuma, S.; Kanazawa, Y.; Sato, K.; Kokubo, S.; Suzuki, H.; et al. The indole compound MA-35 attenuates tumorigenesis in an inflammation-induced colon cancer model. Sci. Rep. 2019, 9, No. 12739.

(35) De Simone, V.; Fränzê, E.; Ronchetti, G.; Colantonii, A.; Fantini, M. C.; Di, F. D.; et al. Th17-type cytokines, IL-6 and TNF-α synergistically activate STAT3 and NF-κB to promote colorectal cancer cell growth. Oncogene 2015, 34, 3493–503.

(36) Grivennikov, S. I.; Karin, M. Inflammation and oncogenesis: a vicious connection. Curr. Opin. Genet. Dev. 2010, 20, 65–71.

(37) De Simone, V.; Fränzê, E.; Ronchetti, G.; Colantonii, A.; Fantini, M. C.; Di, F. D.; et al. Th17-type cytokines, IL-6 and TNF-α synergistically activate STAT3 and NF-κB to promote colorectal cancer cell growth. Oncogene 2015, 34, 3493–503.

(38) Grivennikov, S. I.; Karin, M. Inflammation and oncogenesis: a vicious connection. Curr. Opin. Genet. Dev. 2010, 20, 65–71.
(45) Strzaleczky, J. K.; Wielkowszynski, T.; Krakowczyk, L.; Adamek, B.; Zalewska-Zieb, M.; Gawron, K.; et al. The activity of antioxidant enzymes in colorectal adenocarcinoma and corresponding normal mucosa. Acta Biochim. Pol. 2012, 59, 549–556.

(46) Ramu, A.; Kathiresan, S.; Ramadoss, H.; Nallu, A.; Kaliyan, R.; Azamuthu, T. Gramine attenuates EGFR-mediated inflammation and cell proliferation in oral carcinogenesis via regulation of NF-κB and STAT3 signaling. Biomed. Pharmacother. 2018, 98, 523–530.

(47) Paredes, J.; Zabaleta, J.; Garai, J.; Ji, P.; Intizai, S.; Spagnardi, M.; et al. Immune-Related Gene Expression and Cytokine Secretion Is Reduced Among African American Colon Cancer Patients. Front. Oncol. 2020, 10, No. 1498.

(48) Konjevic, G.; Radenkovic, S.; Vuletic, A.; Mirjacic, K.; Jurisic, V.; Srdic, T. STAT Transcription Factors in Tumor Development and Targeted Therapy of Malignancies. In Oncogene and Cancer—From Bench to Clinic; IntechOpen, 2013.

(49) Vallée, A.; Lecarpentier, Y.; Vallée, J. N. Targeting the Canonical WNT/β-Catenin Pathway in Cancer Treatment Using Non-Sterooidal Anti-Inflammatory Drugs. Cells 2019, 8, No. 726.

(50) Tuomisto, A. E.; Mäkinen, M. J.; Väyrynen, J. P. Systemic inflammation in colorectal cancer: Underlying factors, effects, and prognostic significance. World J. Gastroenterol. 2019, 25, 4383–4404.

(51) Cosan, D.; Soyocak, A.; Basaran, A.; Degermenci, I.; Gunes, H. V. The effects of resveratrol and tannic acid on apoptosis in colon adenocarcinoma cell line. Saudi Med. J. 2009, 30, 191–195.

(52) Carbonaro, M.; Grant, G.; Pusztai, A. Evaluation of polyphenol bioavailability in rat small intestine. Eur. J. Nutr. 2001, 40, 84–90.

(53) Fu, S.; Xia, J.; Wu, J. Functional chitosan nanoparticles in cancer treatment. J. Biomat. Nanobiotech. 2016, 12, 1585–1603.

(54) Zhou, S. H.; Hong, Y.; Fang, G. J.; Jiang, Y. Y.; Bi, Y.; Yang, L. H.; et al. Preparation, characterization and anticancer effect of chitosan nanoparticles. J. Clin. Rehabil. Tissue Eng. Res. 2007, 11, 9688–9691.

(55) Thagele, R.; Bagre, A.; Kori, M. L. Chitosan anchored nanoparticles for breast cancer: preparation and evaluation: part-I. J. Drug Delivery Ther. 2019, 9, 1–5.

(56) Chopra, S.; Mahdi, S.; Kaur, J.; Iqbal, Z.; Talegaonkar, S.; Ahmad, F. J. Advances and potential applications of chitosan derivatives as mucosadhesive biomaterials in modern drug delivery. J. Pharm. Pharmacol. 2006, 58, 1021–1032.

(57) Cisterna, B. A.; Kamaly, N.; Choi, W.; Tavakkoli, A.; Farokhzad, O. C.; Vilos, C. Targeted nanoparticles for colorectal cancer. Nanomedicine 2016, 11, 2443–2456.

(58) Li, Y.; Kröger, M.; Liu, W. K. Shape effect in cellular uptake of PEGLyated nanoparticles: Comparison between sphere, rod, cube and disk. Nanoscale 2015, 7, 16631–16646.

(59) Prabaharan, M. Chitosan-based nanoparticles for tumor-targeted drug delivery. Int. J. Biol. Macromol. 2015, 72, 1313–1322.

(60) Darvin, P.; Baeg, S. J.; Joung, Y. H.; Nipin, S. P.; Kang, D. Y.; Byun, H. J.; et al. Tannic acid inhibits the Jak2/STAT3 pathway and induces G1/S arrest and mitochondrial apoptosis in YD-38 gingival cancer cells. Int. J. Oncol. 2015, 47, 1111–1120.

(61) Jiang, Q. Natural forms of vitamin E as effective agents for cancer prevention and therapy. Adv. Nutr. 2017, 8, 850–867.

(62) Darvin, P.; Baeg, S. J.; Joung, Y. H.; Nipin, S. P.; Kang, D. Y.; Byun, H. J.; et al. Tannic acid inhibits the Jak2/STAT3 pathway and induces G1/S arrest and mitochondrial apoptosis in YD-38 gingival cancer cells. Int. J. Oncol. 2015, 47, 1111–1120.

(63) Li, W.; Yang, C. J.; Wang, L. Q.; Wu, J.; Dai, C.; Yuan, Y. M.; et al. A tannin compound from Sanguisorba officinalis blocks Wnt/β-catenin signaling pathway and induces apoptosis of colorectal cancer cells. Chin. Med. 2019, 14, No. 22.

(64) Kourtidis, A.; Lu, R.; Pence, L. J.; Anastasiadis, P. Z. A central role for cadherin signaling in cancer metastasis, its emerging role in haematological malignancies and potential as a therapeutic target in cancer. BMC Cancer 2018, 18, No. 939.

(65) Cao, Z. Q.; Wang, Z.; Leng, P. aberrant N-cadherin expression in cancer. Biomed. Pharmacother. 2019, 118, No. 109320.

(66) Bose, D.; Hsu, C. W.; Bajaj, M.; Cui, H.; et al. Doxorubicin and resveratrol co-delivery nanoparticle to overcome doxorubicin resistance. Sci. Rep. 2016, 6, No. 35267.

(67) Jeon, H. J.; Yeom, Y.; Kim, Y. S.; Kim, E.; Shin, J. H.; Seok, P. J.; et al. Effect of vitamin C on azoxymethane (AOM)/dextran sulfate sodium (DSS)-induced colitis-associated early colon cancer in mice. Nutr. Res. Pract. 2018, 12, 101–109.

(68) Chen, C. W.; Hsiao, C. W.; Wu, C. C.; Jao, S. W. Rectal prolapse as initial clinical manifestation of colon cancer. Z. Gastroenterol. 2008, 46, 348–350.
(86) Fu, Y.; Sun, X.; Wang, L.; Chen, S. Pharmacokinetics and Tissue Distribution Study of Pinosylv in Rats by Ultra-High-Performance Liquid Chromatography Coupled with Linear Trap Quadrupole Orbitrap Mass Spectrometry. *Evidence-Based Complementary Altern. Med.* **2018**, **2018**, No. 4181084.