Chitosan-Coated Cinnamon/Oregano-Loaded Solid Lipid Nanoparticles to Augment 5-Fluorouracil Cytotoxicity for Colorectal Cancer: Extract Standardization, Nanoparticle Optimization, and Cytotoxicity Evaluation

Kamel M. Kamel, Islam A. Khalil, Mostafa E. Rateb, Hosieny Elgendy, and Seham Elhawary

ABSTRACT: This study aimed to coat lipid-based nanocarriers with chitosan to encapsulate nutraceuticals, minimize opsonization, and facilitate passive-targeting. Phase one was concerned with standardization according to the World Health Organization. Qualitative analysis using liquid chromatography—high-resolution mass spectrometry (LC-HRMS/MS) investigated the active constituents, especially reported cytotoxic agents. Cinnamaldehyde and rosmarinic acid were selected to be quantified using high-performance liquid chromatography. Phase two was aimed to encapsulate both extracts in solid lipid nanoparticles (core) and chitosan (shell) to gain the advantages of both materials properties. The developed experimental model suggested an optimum formulation with 2% lipid, 2.3% surfactant, and 0.4% chitosan to achieve a particle size of 254.77 nm, polydispersity index of 0.28, zeta potential of +15.26, and entrapment efficiency percentage of 77.3% and 69.1% for cinnamon and oregano, respectively. Phase three was focused on the evaluation of cytotoxic activity unencapsulated/encapsulated cinnamon and oregano extracts with/without 5-fluorouracil on HCT-116 cells. This study confirmed the success of the suggested combination with 5-fluorouracil for treating human colon carcinoma with a low dose leading to decreasing side effects and allowing uninterrupted therapy.

KEYWORDS: cinnamon, oregano, 5-fluorouracil, chitosan, solid lipid nanoparticles, colorectal cancer

INTRODUCTION
Colorectal cancer (CRC) is considered one of the most fatal diseases throughout the world. It always comes in an advanced order in the list of causes of mortality throughout the world which represents a huge public health problem. Worldwide, it comes third in the most common cancers after lung and breast, and fourth in cancer related mortality cases. Its incidence usually starts in people between 40 and 50 years old and increases with aging. The current widespread cancer therapy consists of surgery combined with chemo- and/or radiotherapy, while gene therapies did not show the expected success. Single constituents, showed the ability to enhance or synergize the anticancer activity of standard chemotherapy drugs. Their mechanisms of actions were proved at the molecular level; this suggests that the scientists to look upon them as alternative or complementary support for the traditional chemotherapy drugs.

Nutraceuticals and natural products, both total extracts and single constituents, showed the ability to enhance or synergize the anticancer activity of standard chemotherapy drugs. Their mechanisms of actions were proved at the molecular level; this suggests that the scientists to look upon them as alternative or complementary support for the traditional chemotherapy drugs.

Cinnamon is one of the most promising anticancer herbs. It is widely used as a spice, flavoring, and preservative in the food industry. It has been used in medieval medicine for the treatment of a variety of diseases including arthritis, coughing, sore throats, and so forth. Cinnamomum cassia is derived from different sources, and it is the most common form of cinnamon. Cassia is the predominant variety found in retail trade and pastry shops. Its aqueous extract proved to have cytotoxic activity against various types of cancer cell lines like human cervix carcinoma. Cinnamaldehyde, which is the main...
active compound isolated from the stem bark of *Cinnamomum cassia*, showed potent cytotoxic activity against human promyelocytic leukemia cells. Its mechanism was revealed via transduction apoptosis via ROS generation followed by mitochondrial permeability transition and cytochrome c release to the cytosol.10 2′-Hydroxy-cinnamaldehyde, isolated from *Cinnamomum cassia* and 2′-benzoyloxy-cinnamaldehyde, was prepared by the reaction of 2′-hydroxy-cinnamaldehyde and benzoyl chloride; both proved to be strong inhibitors for in vitro growth of 29 kinds of human cancer cells and in vivo growth of SW-620 human tumor xenograft without the loss of body weight in nude mice.11

Oregano (*Origanum vulgare*) is also a widely used spice known by its volatile oil. It has been used traditionally for the treatment of respiratory disorders, indigestion, and rheumatoid arthritis.12 Its ethanolic extract arrested growth and killed cells of colon adenocarcinoma Caco2 cells in both a time- and a dose-dependent manner via activation of both intrinsic and extrinsic apoptotic pathways. The more important is that these effects were selective for cancer cells and achieved by whole extract not by a specific component.13 In vivo, the aqueous extract was tested for its efficacy human body absorption. Furthermore, cytotoxic activity evolution on human colon cancer cell line HCT-116 was carried out to explore the synergistic activity with 5-FU (standard chemotherapeutic for CRC) to be used as complementary or alternative to chemotherapy. Furthermore, cytotoxic activity evolution on human colon cancer cell line HCT-116 was carried out to explore the synergistic activity with 5-FU (standard chemotherapeutic for CRC) to be used as complementary or alternative to chemotherapy.

**Figure 1.** Schematic illustration of the different phases of the study.

**MATERIALS AND METHODS**

**Materials.** Cinnamon bark (*Cinnamomum cassia*) and oregano leaves (*Origanum vulgare*) were bought from the local market and authenticated by taxonomy department then were grinded to fine powder. Ethanol (HPLC grade), methanol (HPLC grade), acetonitrile (HPLC grade), dimethyl sulfoxide, 5-fluorouracil, propylene glycol, chitosan (Mw 260 000 Da), cinnamaldehyde, rosmarinic acid, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), acridine orange, and ethidium bromide were purchased from Sigma.
Aldrich (St. Louis, MO, USA). Compritol 888 ATO (gyceryl behenate, a mixture of ~15% mono-, 50% di- and 35% triglycerides of behenic acid) and Gelucire 40/14 (PEG glyceride) were a kind gift from Gattefosse, France. Poloxamer 407 was obtained from BASF (Florham Park, NJ). Human colon carcinoma (HCT 116; ATCC CCL-247) cells were purchased from American Type Culture Collection (ATCC, NY). 3.3′,5′-Tetramethyl benzidine was purchased from Kirkegaard and Perry Lab (Gaithersburg, MD). Rabbit polyclonal to caspase-3 was purchased from Abcam Inc. (Cambridge, MA). Polyclonal goat antirabbit peroxidase conjugate was purchased from Jackson Immunsearch Lab, USA. A MitoTracker mitochondrion-selective probe, chloromethyl-X-rosamine, was purchased from Life Technologies, Carlsbad, USA. All other reagents were of analytical grade and used as received.

Preparation of Cinnamon and Oregano Extracts. Portions of 100 g of commercially available Cinnamomum cassia bark and Origanum vulgare leaves were crushed and extracted by 70% ethanol by maceration aided by sonication three consecutive times. Extract was then evaporated under suction by a rotary evaporator (model Heidolph rotavapor vv 2000/WB 2000, Germany) and then lyophilized (Free-zone, Labconco, USA) to dry powder.

Qualitative Analysis of Cinnamon and Oregano Extracts Using LC-HRMS/MS. Qualitative analyses for cinnamon and oregano extracts were conducted by gradient separation using a Poroshell EC-C18, RP analytical high-performance liquid chromatography (HPLC) column (2.7 μm, 2.1 × 100 mm, Agilent, USA) with a mobile phase of 0–100% acetonitrile over 25 min followed by 100% acetonitrile over 5 min at a flow rate of 0.5 mL/min. High-resolution mass spectral data (HRMS) was obtained from a Thermo Instruments MS system (Finnigan LTQ/LTQ Orbitrap) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler, and Accela Pump) with the following conditions: capillary voltage 45 V, capillary temperature 260 °C, auxiliary gas flow rate 10–20 arbitrary units, sheath gas flow rate 40–50 arbitrary units, spray voltage 4.5 kV, mass range 100–2000 amu (maximum resolution 60 000). The obtained suspension was stored at 4 °C, 30 min and 15 000 rpm) (model 3K 30, Sigma, Germany). Cinnamaldehyde was measured in the supernatant of centrifuged (4 °C, 30 min and 15 000 rpm) followed by loading cinnamon and oregano extracts in optimum formulation. A Box–Behnken design was applied to optimize the formulation variables using Design Expert 10.0.3.1 software (Stat-Ease, Inc., Minneapolis, MN, USA.). The selected independent variable lipid phase (mixture of Compritol 888ATO and Gelucire 44/14) concentration, poloxamer concentration, and chitosan concentration are presented in Table 1. A total of 17 runs were required (12 factorial and 5 center points). The dependent variables were analyzed using an analysis of variance (ANOVA) to identify the significance of each variable and interaction between variables (P < 0.05). Different mathematical models were generated for dependent variables [mean particle diameter (P.S.), polydispersity index (PDI), and zeta potential (ZP)]. Design Expert software optimization modules were used to predict the optimum combination of different variables that fulfill the predetermined specification (Table 1). The predicted runs were prepared to validate the models using percentage bias equation.25

\[
\text{bias(%) = } \left| \frac{\text{predicted} - \text{actual}}{\text{actual} \times 100} \right|
\]

Characterization of the Prepared SLN-Cs Nanoparticles. Particle Size, Polydispersity Index, and Zeta Potential Measurement. A dynamic light scattering (DLS) technique was used to measure the mean particle size, polydispersity index (PDI), and zeta potential (ZP) at room temperature (Malvern Zetasizer Nano ZS, Malvern Instruments, Malvern, UK).23 The nanoparticle suspension was appropriately diluted in 1 mM sodium chloride.24 Triplicate measurements were done to calculate means and standard deviations.

Entrapment Efficiency Measurement. The indirect technique was used to determine the entrapment efficiency (EE%) of either cinnamaldehyde (for cinnamon extract) or rosmarinic acid (for oregano extract) in SLN-Cs nanoparticles. SLN-Cs formulations were centrifuged (4 °C, 30 min and 15 000 rpm) (model 3K 30, Sigma, Germany). Cinnamaldehyde was measured in the supernatant of cinnamon extract-loaded SLN-Cs formulation using the HPLC method. Rosmarinic acid was measured in the supernatant of oregano extract-loaded SLN-Cs formulation using the HPLC method mentioned in Quantitative Analysis of Cinnamon and Oregano Extracts Using HPLC section. The entrapment efficiency was then calculated using eq 2.

\[
\% \text{entrapment efficiency} = \left( \frac{W_{\text{total}} - W_{\text{free}}}{W_{\text{free}}} \right) \times 100
\]

where \(W_{\text{total}}\) is the initial equivalent weight of cinnamaldehyde and rosmarinic acid and \(W_{\text{free}}\) is unentrapped cinnamaldehyde and rosmarinic acid in the supernatant.

Particle Morphology. Morphological characterization was performed using transmission electron microscope (TEM) (JEM-
Evaluation of Cell Death Mode. The cell death mode of the tested samples was investigated by examining apoptosis and necrosis ratios using double stain with acridine orange/ethidium bromide according to the method developed by Ribble et al.27 Briefly, cells (0.5 × 10^5 cells/well) were seeded in 96-well plates and incubated at 37 °C for 24 h with tested samples in a humidified 5% CO₂ atmosphere. Following incubation, the samples were removed by trypsinization and incubated in 50 μL of phosphate-buffered saline (PBS), 2 μL of acridine orange (100 μg/mL in PBS), and 2 μL of ethidium bromide (100 μg/mL in PBS). A portion of 10 μL of stained cell suspension was visualized and counted using fluorescence microscopy. The live cells have a normal green nucleus; early apoptotic cells have bright green to yellow nucleus with condensed or fragmented chromatin. Late apoptotic cells display fragmented orange chromatin, and cells have died from direct necrosis have a structurally dark orange to red nucleus.28 The percentage of apoptotic or necrotic cells for each sample was determined (at least 500 cells) using the following equation:

\[
\% \text{ cell death mode} = \left( \frac{\text{total no. of apoptotic or necrotic cells}}{\text{total no. of cells counted}} \right) \times 100
\] (6)

Estimation of Apoptosis Mediator (Caspase-3). The level of caspase-3 was measured in cell lysate by a quantitative indirect immunoassay ELISA technique29 using rabbit polyclonal antibodies to caspase-3. 96-well bottom polystyrene microtiter plates (Greiner Labortechnik, Kremsmunster, Austria) were coated with cell lysates (50 μL/well) and incubated 1 h at 37 °C then overnight at 4 °C in humidified chamber. After incubation, the plates were washed three times with washing PBS after removing cell lysate followed by blocking the remaining protein-binding sites using blocking buffer. Then, antibodies “rabbit polyclonal to caspase-3” (Abcam Inc., Cambridge, MA, USA) were added to the plates, and the plates were incubated for 1 h at 37 °C then overnight at 4 °C in humidified chamber. Diluted polyclonal goat anti-rabbit peroxidase conjugates (Jackson Immunsearch Lab, USA) were added to each well; then the plates were incubated for 1 h at 37 °C. After incubation and washing, substrate buffer was added to carry out the enzyme reaction; then 1 M HCl was added to stop color development. The absorbance was measured at 450 nm (yellow color) using a microplate reader (FLUOstar OPTIMA, BMG Labtech GmbH, Offenburg, Germany). Caspase-3 level was expressed as the fold of control optical density values.

Determination of Mitochondrial Transmembrane Potential. The mitochondrial transmembrane potential (ΔΨₘ) was investigated by MitoTracker Red CMX-Ros staining (Life Technologies, Carlsbad, USA).30 Briefly, cells were grown on coverslips inside a Petri dish filled with the appropriate culture medium. After incubation, the media was removed, and cells were stained by 1 mM of MitoTracker Red CMX-Ros. A total of 500–1000 stained cells were visualized by a Carl Zeiss automated fluorescence microscope with Zen 2011 software at 400× magnification. A reduction in CMX-Ros staining is indicative of a cell that has mitochondria with a reduced transmembrane potential (ΔΨₘ⁻).

Statistical Analysis. All data are presented as the mean ± standard deviation and statistically analyzed by either Student’s t test or one-way analysis of variance (ANOVA) using the software GraphPad Prism Software Version 6 (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

To achieve this objective, the current study is divided into three phases as illustrated in Figure 1. The first phase is extraction of nutraceuticals followed by qualitative and quantitative analysis. The second phase is focused in optimizing SLN (core)/chitosan (shell) carriers to encapsulate the nutraceutical extract. The third phase is concerned with the cytotoxicity evaluation of unencapsulated (free) and encapsulated extracts and combination with 5-fluorouracil.

Preparation of Cinnamon and Oregano Extracts. In the past decade, there is an increase in the recognition of herbal...
products as alternative or complementary therapy to conventional therapeutics. Therefore, all herbal medicines should fulfill the basic safety requirements. According to World Health Organization, quality control of medicinal plant materials includes (1) macro and microscopic examination, (2) foreign organic matter, (3) ash values, (4) moisture content, (5) extractive values, (6) crude fiber, (7) qualitative chemical evaluation, (8) chromatographic examination, (9) quantitative chemical evaluation, and (10) toxicological studies.

Both plants were subjected to macro and microscopic examination and the removal of matters other than studied plants. Fungal and bacterial count studies, heavy metal analysis, aflatoxin analysis, and total ash analysis were done, and they all meet the permissible limits (data not presented). The most important parameters for standardization and quality control of medicinal plant materials are qualitative chemical evaluation, chromatographic examination, and quantitative chemical evaluation. These parameters will be discussed in the following sections.

Qualitative Analysis of Cinnamon and Oregano Extracts Using LC-HRMS/MS. The LC-HRMS/MS analysis of the ethanolic extract of cinnamon and oregano (Tables S1 and S2; Figure 2) indicated the presence of a range of polyphenolics with different chemical classes ranges. The cytotoxic activities of the identified main active constituents in both cinnamon and oregano extracts were reported against several types of cancer cells. Out of LC-HRMS analysis, 15 compounds identified in cinnamon extract and 13 compounds in oregano extract proved to have significant cytotoxic activity according to the literature (a list of literature mentioned in Supporting Information; Table S3; Figure S1). The identified compounds in cinnamon were cinnamaldehyde, 2-methoxy-cinnamaldehyde, 2′-hydroxycinnamaldehyde, eugenol, myristicin, kaempferol, kaempferol-3-rutinoside, cinnamic acid, coniferaldehyde, apigenin, emodin, quercetin, quercetin-3-O-rhamnoside, procyanidin B2, and in oregano were ursolic acid, rutin, quercetin-3-O-glucoside, salvianolic acid A, 3-O-p-coumaroylquinic acid, caffeic acid, apigenin, naringenin, luteolin, quercetin, chlorogenic acid, rosmarinic acid, and apigenin-7-O-glucoside. These findings support the theory of superiority of using “whole food” extract in some cases rather than individual isolated active constituents in the treatment of chronic complicated diseases like cancer, especially when cancer cells develop resistance against repeated use of conventional treatment with single chemotherapy and metastasis spreading in different organs, which needs a therapy regimen consisting of the combination of different drugs with diverse mechanisms of action. The richness of single extract with many cytotoxic active constituents having different mechanisms of action provides this advantage (Table S3 and Figure S1). This may explain why adding certain plant extract chemosensitizes the resistant cancer cells toward traditional chemotherapy, like the addition of rosemary extract that sensitizes resistant colon cancer cells toward 5-FU through downregulating enzymes related to 5-FU resistance. On the contrary, some anticancer active constituents in food that help fighting cancer, when isolated and concentrated in pills and used by cancer patients, were found to increase the risk of cancer like β-carotene when used as a supplement for smoker and asbestos workers was found to increase the risk of lung cancer.

Quantitative Analysis of Cinnamon and Oregano Extracts Using HPLC. For the quantitative analysis of oregano and cinnamon extracts by HPLC, rosmarinic acid and cinnamaldehyde were used as standards to be the main active constituents responsible for the cytotoxic activity of both extracts. Calibration curves of standards were done (Figure S2), and the percentages of standards in both extracts and formula were determined. Cinnamon extract was found to contain 1.89% of cinnamaldehyde, while oregano extract was found to contain 6.42% of rosmarinic acid. The formula was done by the combination of 55% cinnamon extract and 45% oregano extract according to cytotoxicity study results. This formula was found...
to contain 0.75% cinnamaldehyde and 3.3% rosmarinic acid (Table 2).

### Table 2. Measured Percentage of Cinnamaldehyde and Rosmarinic Acid in Cinnamon Extract, Oregano Extract, and Formula Using HPLC

<table>
<thead>
<tr>
<th></th>
<th>Rosmarinic Acid</th>
<th>Cinnamon</th>
<th>Cinnamaldehyde</th>
<th>Oregano</th>
<th>FLK</th>
<th>LGC</th>
<th>FLK</th>
<th>LGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>Plant extract</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Rosmarinic Acid</td>
<td>6.42</td>
<td>3.5</td>
<td>3.3</td>
<td>6.42</td>
<td>3.5</td>
<td>3.3</td>
<td>6.42</td>
<td>3.5</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>1.89</td>
<td>0.85</td>
<td>0.75</td>
<td>1.89</td>
<td>0.85</td>
<td>0.75</td>
<td>1.89</td>
<td>0.85</td>
</tr>
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</table>

### Development of Extract-Loaded Core/Shell (SLN-Cs) Nanoparticles

Cinnamon and oregano were extracted using 70% ethanol which made them rich with hydrophilic and hydrophobic polyphenolic compounds. The presence of different polyphenolic compounds in herbal extracts could lead to a synergistic therapeutic activity. Therefore, the main objective of encapsulation phase is to improve entrapment of all active compounds in lipid matrix. Lipids with glycerides bonded to fatty acid with different lengths of carbon chains as Compritol 888 ATO and Gelucire 44/14 were used to satisfy an entrapment objective. Compritol 888 ATO is a famous glyceride with a 22 carbon atom chain which is mainly in solid lipid nanoparticles with melting point between 69 and 74°C and HLB around 2. On the other hand, Gelucire 44/14 is a glyceride with a 12 carbon atom chain, a melting point around 44°C, and an HLB around 14. The suggested combination made them a good candidate for the encapsulation of herbal extracts in SLN matrix. SLN as the potential carrier of chemotherapeutic agents have different advantages like controlling drug release, improving drug permeation, and cellular uptake and overcome drug resistance. Nanocarriers can improve drug delivery to the cancer cells by decorating it using appropriate ligands/coating layer on their surface. In the current study, decorating the nanocarriers with chitosan as a cationic polymer could improve the retention of extracts in the tumor. To the best of our knowledge, this is the first study to report the enhancement of cinnamon and oregano bioavailability, stability, and therapeutic activity using SLNs coated with chitosan. In the current study, SLNs coated with chitosan were prepared and optimized using hot melt emulsification homogenization/ultrasoundation method using a mixture of lipid with different melting point. Extract-loaded SLN-Cs were prepared using optimum levels of experimental variables.

### Formulation Considerations

SLN-Cs nanoparticles were prepared by hot melt emulsification homogenization/ultrasoundation method that relies on the emulsification of a blended molten lipid mixture on surfactant aqueous phase to prepare coarse emulsion followed by ultrasonication to control the globule size. The obtained anionic SLNs were coated with a positively charged chitosan layer by molecular attraction forces (electrostatic interactions). Different levels of materials were studied to control the particle size and its corresponding charge.

### Statistical Analysis of Experimental Data by Design Expert Software

Box–Behnken experimental design was used to analyze experimental runs results as demonstrated in Table S4. Different dependent variables were measured and analyzed using an analysis of variance (ANOVA). The suggested model for PS, PDI, and ZP was a quadratic one. Furthermore, the coefficient estimates and p-values for each coefficient between experimental factors and measured responses were estimated (Table 3).

### Analysis of Dependent Variables

To develop a suitable nanocarrier for herbal extracts, the effect of different independent variables (lipid phase, poloxamer, and chitosan concentrations) on measured responses was analyzed and illustrated as in Figure 3. A dynamic light scattering (DLS) technique was used to measure the hydrodynamic diameter and zeta potential of both SLNs and SLN-Cs formulations. ZP readings could be used to describe the stability of dispersions where the aggregation of the particles decreased in charged particles due to the degree of repulsion. Furthermore, ZP readings could be used to confirm the deposition of chitosan layer when the total system charge reversed from negative charge (for SLNs) to positive charge (for SLN-Cs). The results indicated that PS was significantly affected by all material attributes (Table 3). According to quadratic experimental model, PS was significantly increased when lipid ($p < 0.0001$), poloxamer ($p = 0.0047$), and chitosan ($p < 0.0001$) levels increased in both SLNs and SLN-Cs formulations (Table 3). Furthermore, a positive correlation between PS and lipid mixture concentration (0.441) and chitosan (0.718) was observed (Table 4). Uncoated SLNs exhibit a PS ranging from ~245 to ~350 nm (Table S4 and Figure 3a) at different levels of other variables which could be attributed to the tendency of lipids to coalesce at a high lipid concentration as explained in Stokes’s law. On the other hand, coating SLNs with chitosan (0.5–1%) showed a significant increase ($p < 0.0001$) in PS ranged from ~275 to ~430 nm (Table S4 and Figure 3b,c). PS rises along with the increase of chitosan, representing 270.9 and 347.8 nm with the concentration of 0.5% and 1% chitosan at middle level of other variables, respectively, compared with uncoated SLNs with an average particle size of 248 nm. ZP was significantly affected only when lipid ($p < 0.0001$) and chitosan ($p < 0.0001$) levels increased in both SLNs and SLN-Cs formulations (Table 3). Furthermore, a negative

### Table 3. Coefficient Estimate and p-Value for That Coefficient between Experimental Factors and Measured Responses

<table>
<thead>
<tr>
<th></th>
<th>Intercept</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>AB</th>
<th>AC</th>
<th>BC</th>
<th>A²</th>
<th>B²</th>
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<td>PS</td>
<td>271.50</td>
<td>31.19</td>
<td>−12.90</td>
<td>50.79</td>
<td>−0.87</td>
<td>−10.00</td>
<td>−0.93</td>
<td>26.30</td>
<td>24.38</td>
<td>26.50</td>
</tr>
<tr>
<td>P-value</td>
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<td>0.0047&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.8502</td>
<td>0.0602</td>
<td>0.8418</td>
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<tr>
<td>PDI</td>
<td>0.34</td>
<td>0.05</td>
<td>−0.01</td>
<td>0.00</td>
<td>0.00</td>
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<td>−0.01</td>
<td>0.03</td>
<td>−0.06</td>
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<tr>
<td>p-value</td>
<td>0.0252&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.4821</td>
<td>0.8862</td>
<td>0.9193</td>
<td>0.6159</td>
<td>0.6159</td>
<td>0.6870</td>
<td>0.3127</td>
<td>0.0269&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>ZP</td>
<td>428.01</td>
<td>−38.59</td>
<td>0.27</td>
<td>241.73</td>
<td>−1.95</td>
<td>−14.10</td>
<td>−2.73</td>
<td>10.26</td>
<td>−2.38</td>
<td>−165.66</td>
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<tr>
<td>p-value</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9454</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.6681</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

<sup>a</sup>AAbbreviations: CE, coefficient estimate; A, lipid; B, poloxamer; C, chitosan; PS, particle size; PDI, polydispersity index; ZP, zeta potential.

<sup>b</sup>A Significant coefficient estimate.
A high positive correlation between ZP and chitosan (0.861) were observed (Table 4). The ZP values of uncoated SLNs ranged from \(-\sim 15\) to \(-\sim 25\) mV (Table S4 and Figure 3g). The negativity of SLNs could be linked to the effect of carbon chains of Compritol and Gelucire.\(^{16}\) On the other hand, coating SLNs with chitosan (0.5\%–1\%) showed a significant shift (\(p < 0.0001\)) in ZP ranged from \(\sim +13\) nm to \(\sim +23\) mV (Table S4 and Figure 3h,i). A positive correlation (0.419) between PS and ZP was observed. When SLNs was mixed with the chitosan-containing solution, the PS of SLN-Cs increased (\(p < 0.0001\)) compared with SLNs and ZP changed.

Table 4. Correlation between Experimental Factors and Measured Responses

<table>
<thead>
<tr>
<th>factors</th>
<th>measured responses</th>
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<tbody>
<tr>
<td></td>
<td>PS</td>
</tr>
<tr>
<td>lipid conc (% w/v)</td>
<td>0.441(^a)</td>
</tr>
<tr>
<td>poloxamer conc. (% w/v)</td>
<td>(-0.148)</td>
</tr>
<tr>
<td>chitosan conc. (% w/v)</td>
<td>(-0.006)</td>
</tr>
<tr>
<td></td>
<td>0.115</td>
</tr>
<tr>
<td></td>
<td>0.419(^b)</td>
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</table>

\(^a\)Appropriate correlation between factor vs response or response vs response. \(^b\)Partial correlation.
reversely from negative (∼ −15 to ∼ −25 mV) to positive value (∼ +13 nm to ∼ +23 mV), providing evidence of chitosan coating. SLNs with a PS of which was 248 nm, possessed a ZP of −19.3 mV. With the increasing amount of chitosan concentration from 0.5% to 1% (v/w), ZP values varied from +15.5 to +19.9 mV, at the middle value of other variables. Due to the high density of amino groups in chitosan, the deposition of chitosan led to the increase in density of positive electron cloud that affected the overall particle charge. This shift in the surface charge could be attributed to hydrogen bonding and complex formation between the polysaccharide (chitosan) and the glyceride head groups (lipid) that could justify the coating mechanism involved in SLN-Cs formation. These results were in agreement with several studies focused on developing a lipid carrier coated with a chitosan layer.\textsuperscript{21,39–41} As previously reported, the nanoparticle surface has a significant effect on drug pharmacokinetic parameters. The positively charged nanoparticles will be valuable due to its interaction with negatively charged cell membrane components could occur. Furthermore, the small particle size will improve cell internalization through endocytosis mechanism.\textsuperscript{42}

Table 5. Validation of the SLN-Cs Nanoparticle Model Generated from a Box–Behnken Design

<table>
<thead>
<tr>
<th></th>
<th>run 1</th>
<th>run 2</th>
<th>run 3</th>
<th>average (% bias)</th>
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</thead>
<tbody>
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<td>lipid conc. (% w/v)</td>
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<td>2</td>
<td>2</td>
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<tr>
<td>poloxamer conc. (% w/v)</td>
<td>2.29</td>
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<td>chitosan conc. (% w/v)</td>
<td>0.40</td>
<td>0.41</td>
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<tr>
<td>predicted PS (nm)</td>
<td>mean ± SD</td>
<td>254.7 ± 8.9</td>
<td>255.1 ± 8.9</td>
<td>254.2 ± 8.9</td>
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<tr>
<td>actual PS (nm)</td>
<td>mean ± SD</td>
<td>267.6 ± 5.4</td>
<td>266.0 ± 9.3</td>
<td>270.7 ± 7.9</td>
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<tr>
<td></td>
<td>% bias</td>
<td>5.1</td>
<td>4.3</td>
<td>6.5</td>
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<tr>
<td>predicted PDI</td>
<td>mean ± SD</td>
<td>0.28 ± 0.04</td>
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<tr>
<td>actual PDI</td>
<td>mean ± SD</td>
<td>0.30</td>
<td>0.29</td>
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<td></td>
<td>% bias</td>
<td>9</td>
<td>7</td>
<td>8</td>
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<tr>
<td>predicted ZP (mV)</td>
<td>mean ± SD</td>
<td>+15.26 ± 0.64</td>
<td>+15.3 ± 0.64</td>
<td>+15.0 ± 0.64</td>
</tr>
<tr>
<td>actual ZP (mV)</td>
<td>mean ± SD</td>
<td>+16.40 ± 0.92</td>
<td>+15.98 ± 0.58</td>
<td>+16.39 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>% bias</td>
<td>7.5</td>
<td>4.5</td>
<td>9.3</td>
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Figure 4. (a) Particle morphology using a TEM image of SLN-Cs nanoparticles, (b) DSC thermograms of chitosan, gelucire, compritol, CI-NPs, and OR-NPs, and (c) release profile of cinnamaldehyde for cinnamon extract (CI), rosmarinic acid for oregano extract (OR), cinnamaldehyde from CI-NPs, and rosmarinic acid from OR-NPs.
Table 6. Mathematical Models of the Regression for in Vitro Release Profiles of Cinnamaldehyde for Cinnamon Extract (CI), Rosmarinic Acid for Oregano Extract (OR), Cinnamaldehyde from CI-NPs, and Rosmarinic Acid from OR-NPs

<table>
<thead>
<tr>
<th>model</th>
<th>equation</th>
<th>CI</th>
<th>CI-NPs</th>
<th>OR</th>
<th>OR-NPs</th>
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<tr>
<td>zero-order</td>
<td>( F = k_dt )</td>
<td>0.480</td>
<td>0.191</td>
<td>0.576</td>
<td>-0.290</td>
</tr>
<tr>
<td>first-order</td>
<td>( F = 100[1 - \exp(-k_dt)] )</td>
<td>0.954</td>
<td>0.834</td>
<td>0.969</td>
<td>0.809</td>
</tr>
<tr>
<td>Higuchi</td>
<td>( F = k_t e^{-kt} )</td>
<td>0.927</td>
<td>0.889</td>
<td>0.928</td>
<td>0.720</td>
</tr>
<tr>
<td>Korsmeyer–Peppas</td>
<td>( F = k_{0g} t^a )</td>
<td>0.964</td>
<td>0.993</td>
<td>0.940</td>
<td>0.988</td>
</tr>
<tr>
<td>Hixson–Crowell</td>
<td>( F = 100[1 - (1 - k_{HC} t)^3] )</td>
<td>0.917</td>
<td>0.764</td>
<td>0.961</td>
<td>0.639</td>
</tr>
<tr>
<td>Hopfenberg</td>
<td>( F = 100[1 - (1 - k_{HP} t)^3] )</td>
<td>0.954</td>
<td>0.834</td>
<td>0.968</td>
<td>0.809</td>
</tr>
<tr>
<td>Baker–Lonsdale</td>
<td>( 3/2[1 - (1 - F/100)^{2/3}] - F/100 = k_{BL} t )</td>
<td>0.968</td>
<td>0.978</td>
<td>0.941</td>
<td>0.929</td>
</tr>
<tr>
<td>model parameter</td>
<td>( k_{BL} = 0.061 )</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( k_{HP} = 23.04 )</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>( n = 0.33 )</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>( k_i = 0.967 )</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( k_{HP} = 31.63 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( n = 0.27 )</td>
<td></td>
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</tr>
</tbody>
</table>

*Best fitting model.

The PDI value represents the homogeneity of particle size distribution as the low PDI value refers to monodispersion to moderate dispersion.\(^{43}\) The PDI values of both SLNs and SLN-Cs formulations ranged from \(~0.2 to ~0.4 (Table S4 and Figure 3d–f). PDI was significantly influenced (0.0257) only by lipid concentration (Table 3) with a positive correlation (0.564) with PDI (Table 4) which was observed in F2 (lowest PDI 0.22, 2% lipid conc.) and F4 (highest PDI 0.43, 5% lipid conc.) of formulations with optimum response levels. Three predicted noticeable effects overlaid different model parameters, a desirability function and operating design for SLNs had been extensively studied.\(^{15}\) Various types of nanoparticles have been examined for ability to encapsulate herbal products in SLNs. Compritol and poloxamer have been shown as advantageous in terms of loading capacity, controlling release rate, and consequently improved bioavailability. Furthermore, coating SLNs with chitosan could improve the delivery to the cancer cells. In the present study, cinnamon extract and oregano extract were encapsulated in optimum SLN-Cs formulation (2% lipid phase concentration, 2.3% poloxamer concentration, and 0.4% chitosan concentration). Cinnamon extract-loaded SLN-Cs (CI-NPs) formulation exhibited a PS of 266.27 nm, PDI of 0.31, ZP of +14.7, and EE% of 77.3%, while oregano extract-loaded SLN-Cs (OR-NPs) formulation exhibited a PS of 271.34 nm, PDI of 0.28, ZP of +16.1, and EE% of 69.1%.

Optimization of Core/Shell (SLN-Cs) Nanoparticles. The limits of optimized formulation were based on minimizing PS and PDI and maximizing ZP (Table 1). To achieve the selected limits, a desirability function and operating design space overlaid different measured responses was performed. In Figures 3j and 4k, the contour plot of operability area and 3D surface plots of desirability function visualize the design space of formulations with optimum response levels. Three predicted optimized formulations were suggested as Table 5; each was performed to validate the experimental model. On the basis of the generated data, the suggested optimum formulation with 2% lipid phase concentration, 2.3% poloxamer concentration, and 0.4% chitosan concentration produced a maximum desirability function of 0.801 (Figure 3). The optimized SLN-Cs nanoparticles predicted parameters were PS of 254.77 nm, PDI of 0.28, and ZP of +15.26.

Validation of the Experimental Model. The experimental validation of the model was performed using three suggested runs after selecting model constraints. The predicted outcomes obtained from the model and actual results were compared. Furthermore, the percentage bias was calculated. The results revealed a low % bias for PS, ZP, and EE recording (Table 5). These findings confirm the fitting and validity of the generated mathematical model for different response predictions.

Encapsulation of Cinnamon and Oregano Extract. Lately, herbal products have been studied for the prevention and treatment of different types of cancer due to arising of tumor resistance to traditional therapies. Famous herbal products had been studied due to its chemotherapeutic activity like curcumin, resveratrol, gingerol, and capsaicin. Nevertheless, these herbal products were facing several limitations like instability (essential fatty acids are easily oxidized), low solubility, low permeability, and subsequently limited bioavailability.\(^{14}\) These limitations can be enhanced by the encapsulation in different types of nanocarriers to improve therapeutic activity by passively or actively targeting to specific organs or tissues such as liposomes, solid lipid nanoparticles, micelles, and polymer nanoparticles. Among all nanocarriers, SLNs had been extensively studied.\(^{15}\) Various types of lipids and surfactants have been examined for ability to encapsulate herbal products in SLNs. Compritol and poloxamer have been shown as advantageous in terms of loading capacity, controlling release rate, and consequently improved bioavailability. Furthermore, coating SLNs with chitosan could improve the delivery to the cancer cells. In the present study, cinnamon extract and oregano extract were encapsulated in optimum SLN-Cs formulation (2% lipid phase concentration, 2.3% poloxamer concentration, and 0.4% chitosan concentration). Cinnamon extract-loaded SLN-Cs (CI-NPs) formulation exhibited a PS of 266.27 nm, PDI of 0.31, ZP of +14.7, and EE% of 77.3%, while oregano extract-loaded SLN-Cs (OR-NPs) formulation exhibited a PS of 271.34 nm, PDI of 0.28, ZP of +16.1, and EE% of 69.1%.

Morphological Study. TEM was used to investigate the morphology of SLN-Cs nanoparticles (Figure 4a). It was observed that SLN-Cs nanoparticles were spherical in shape. Furthermore, a well-defined spherical core–shell structure was observed which confirming chitosan coating of SLNs. The observed particle size using TEM image was in agreement with the obtained size by a Zetasizer (Table S5) which was around 250 nm with a moderate dispersed size distribution.

Thermal Analysis. DSC is a well-known technique to observe the effect of combining material on its thermal behavior as a physicochemical property. DSC was conducted to confirm the assembly of SLN-Cs in case of entrapping either cinnamon or oregano extracts as demonstrated in Figure 4b. The melting points of lipid compartment (Gelucire 40/14, Compritol 888ATO) were an endothermic peaks at 54.20 and 76.23 °C, respectively.\(^{34,36}\) On the other hand, chitosan as a shell layer exhibited a broad endothermic peak centered at about 100 °C, due to the removal of absorbed moisture, and a sharp exothermic peak at ~300 °C.\(^{25}\) Thermograms of extract-loaded SLN-Cs formulations exhibited two elongated endothermic peak similar to lipid compartment ingredients and one exothermic peak for the chitosan characteristic peak. The first peak showed a slight shift and increase in elongation which could be attributed to the presence of essential oils in cinnamon and oregano.\(^{35,46}\) Furthermore, extract-loaded SLN-Cs formulations did not show any new peaks that could indicate extract components. These results proved the assembly of unloaded and loaded SLN-Cs formulations.

In Vitro Drug Release. The release profile of cinnamaldehyde and rosmarinic acid from cinnamon extract (CI) and
Cinnamaldehyde and rosmarinic acid release pro
than 0.5 according to classi
Cs formulations exhibited a Fick di
faster than cinnamaldehyde from CI-NPs. After 24 h, 79.06
be noticed that the release of rosmarinic acid from OR-NPs was
Cinnamaldehyde and rosmarinic acid release (Figure 4c). It can
from OR-NPs, while 85.0 ± 4.9% of cinnamaldehyde was released from CI-NPs.
Furthermore, 89.3 ± 5.1% of rosmarinic acid was released from OR-NPs, while 63.06
coated lipid matrix resulted in a signi
ificant delay in the cinnamaldehyde and rosmarinic acid release (Figure 4c). It can
be noticed that the release of rosmarinic acid from OR-NPs was
faster than cinnamaldehyde from CI-NPs. After 24 h, 79.06 ± 5.1% of rosmarinic acid was released from OR-NPs, while 63.06 ± 4.9% of cinnamaldehyde was released from CI-NPs.
Furthermore, 89.3 ± 3.2% of rosmarinic acid was released from OR-NPs, while 85.0 ± 5.2% of cinnamaldehyde was released from CI-NPs after 48 h. Eventually, around 100% of both rosmarinic acid and cinnamaldehyde were released from OR-NPs and CI-NPs after 72 h.

The release profiles of free extract and extract-loaded SLN-
Cs formulations were fitted to different kinetic models: zero
order, first order, Higuchi, Korsmeyer–Peppas, Hixcon–Crowell, Hopfenberg, and Baker–Lonsdale models. The best
fitting models for release cinnamaldehyde and rosmarinic acid from extracts were Baker–Lonsdale and first-order model with the highest \( r^2 \), respectively (Table 6). On the other hand, the best fitting model for release cinnamaldehyde and rosmarinic acid from SLN-Cs formulations were the Korsmeyer–Peppas model with the highest \( r^2 \). Korsmeyer–Peppas is a model that
described drug release from a carrier system. The diffusional exponent (\( n \)) is used to describe the drug-release mechanism. Cinnamaldehyde and rosmarinic acid release profile from SLN-
Cs formulations exhibited a Fick diffusion model as \( n \) value less than 0.5 according to classification previously described.47

**Evaluation of Cytotoxic Activity against Human Colon
Carcinoma.** Colorectal cancer (CRC) is one of major causes
of cancer death worldwide. Over 70% of CRC cases are
sporadic and related to lifestyle. Epidemiological studies
inversely correlate CRC incidence with the intake of fruits
and vegetables but not with their phenolic content. Preclinical
studies using in vitro (cell lines) and animal models of CRC
have reported anticancer effects for dietary phenolics through
the regulation of different markers and signaling pathways. To
date, polyphenols of different herbs especially cinnamon and
oregano were used as an adjuvant in CRC management.
Overall, the clinical evidence of dietary phenolics against CRC
is still weak, and the amounts needed to exert some effects
largely exceed common dietary doses.48

Different research efforts investigated the effect polyphen-
olics, such as curcumin, resveratrol, isoflavones, green tea
extracts (epigallocatechin gallate), black raspberry powder
(anthocyanins and ellagitannins), bilberry extract (anthocya-
nins), ginger extracts (gingerol derivatives), and pomegranate
extracts (ellagitannins and ellagic acid), as possible future
adjuvant in CRC management.48 For example, the chemopre-
ventive activity of black raspberry seeds as a source of
ellagitannins, ellagic acid, urolithin A, and urolithin B was
evaluated on HT-29 colon cancer cells by Cho et al.49 Black
raspberry seeds contents inhibited the proliferation of the cancer cells cell cycle arrest at the G1 phase for ellagic acid, and
urolithins caused cell cycle arrest at the G2/M phase and
upregulated p21 expression.50 In breast cancer, 10% dietary flax
seed enhanced the inhibitory effect of tamoxifen on estrogen-
dependent human breast cancer (MCF-7) in nude mice with or
without 17-estradiol supplementation.51

To narrow this gap of using phenolics against CRC,
nanotechnology is introduced to deliver a specific amount of
polyphenolic which could improve the therapeutic activity. A
study reported using nanoencapsulation of propolis extract to
enhance the oral delivery of and improve its aqueous solubility
and bioavailability and allow its controlled release with
enhanced anticancer activity against human liver cancer
(HepG2) and human colorectal cancer (HCT 116) cells. A
propolis-loaded nanosystem induced a more cytotoxic e
fect on HepG2 cells than HCT-116 cells and mediated a 3-fold higher
therapeutic e
fficiency than free propolis. The apoptosis assay
indicated that the propolis-loaded NMs induce apoptosis of
HepG2 cells and significantly decrease their number in the
proliferative G0/G1, S, and G2/M phases.55

Herein, a preclinical study on human colon carcinoma cell
line is presented. First, a dose response curve was conducted
to calculate the IC50 of 5-FU and both free and encapsulated
extracts. Then, 50% of IC50 of extract when combined with 5-
FU was tested to evaluate the synergistic activity. A cell death
mode of single and combination was studied to understand the
impact of combining extract with 5-FU on the death
mechanism. Apoptosis mediator and mitochondrial trans-
membrane potential were measured to complete the bigger

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>Concentration used</th>
<th>5-Fluorouracil (5-FU) (µg/mL)</th>
<th>Cinnamon extract (Cl) (µg/mL)</th>
<th>Encapsulated cinnamon extract (Cl-NPs) (µg/mL)</th>
<th>Oregano extract (OR) (µg/mL)</th>
<th>Encapsulated oregano extract (OR-NPs) (µg/mL)</th>
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<tr>
<td>T1</td>
<td>12.17 ± 1.2</td>
<td>19.90 ± 1.9</td>
<td>29.80 ± 2.9</td>
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<td>37.22 ± 3.7</td>
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<tr>
<td>T2</td>
<td>6.08</td>
<td>9.95</td>
<td>14.90</td>
<td>13.24</td>
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<td>T3</td>
<td>50% IC50</td>
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<td>14.90</td>
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<tr>
<td>T5</td>
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<td>14.90</td>
<td>13.24</td>
<td>18.61</td>
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</table>

Table 7. List of Trial Compositions in the Cytotoxic Activity Study against Human Colon Carcinoma
Cytotoxic Activity of 5-FU, Free, and Encapsulated Extracts against HCT-116 Cells. The cytotoxicity of 5-fluorouracil (5-FU), cinnamon extract, oregano extract, and encapsulated extracts was assessed using an MTT assay on HCT 116 cells after 24 h incubation. The cell viability was inhibited by unencapsulated (free) and encapsulated extracts to <50% of the cells with concentrations less than 100 μg/mL, while the cell viability was inhibited by 5-FU to ~20% of the cells with concentrations ~40 μg/mL (Figure 5). An effective cytotoxicity activity in a dose-dependent manner was observed (Figure 5a). A single treatment with each sample exhibited a gradual decrease in cell viability as their concentration was increased reaching an IC$_{50}$ of 12.16, 19.9, 29.8, 26.4, and 37.2 μg/mL for 5-FU, cinnamon extract (CI), oregano extract (OR), encapsulated cinnamon (CI-NPs) and encapsulated oregano (OR-NPs), respectively (Figure 5b). There was a significant difference (P < 0.05) between 5-FU cytotoxicity and other samples using Tukey’s multiple comparison post hoc test where significance difference from T1 to T5 represented as letters a, b, c, d, and e. Furthermore, a significant difference between free and encapsulated extracts was observed (Figure 5b). However, cinnamon extract showed more potent cytotoxic activity on HCT-116 cells growth in comparison to oregano extract. Furthermore, encapsulated extracts (CI-NPs and OR-NPs) exhibited higher IC$_{50}$ than free extracts (CI and OR). This could be attributed to only 63.06 ± 4.9% and 79.06 ± 5.1% was released from CI-NPs and OR-NPs after 24 h, respectively.

Cytotoxic Activity of Combining 5-FU with Extracts and Encapsulated Extracts against HCT 116 Cells. This study was conducted to screen the synergistic/antagonistic effect of extracts when combined with 5-FU or with themselves. First, half IC$_{50}$ of 5-FU as a standard drug showed 25% cell inhibition (Figure 5c). Co-administration of half IC$_{50}$ of each extract with half IC$_{50}$ of 5-FU exhibited higher inhibition percentage of cancer cells reaching 61.3% (Figure 5c, T7) and 56.4% (Figure 5c, T9) for CI and OR, respectively, which is higher than the inhibition percentage of each individual extract or theoretical added inhibition percentage (50%). This confirm the synergistic effect when coadministered free extracts with 5-FU. Oregano and cinnamon were with different nature and from different parts (leaves for Oregano’s extract and bark for cinnamon’s extract). Each extract has its own exclusive diversity of special active constituents that are different from the other. The diversity of active constituents could be the main reason.
for the difference in mechanisms of action of killing cancer cells. Therefore, the coadministration of the combined extracts (CI+OR) with 5-FU was tested (Figure 5c, T13). Half of IC_{50} of 5-FU with CI and OR achieved the highest inhibition percentage reaching 71.1% cell inhibition. This confirms the benefit of combining different active constituents from different plants due to synergistic activity. Furthermore, the major concept of using treatment regimen is to treat cancer with a minimum concentration of chemotherapeutic agents especially when severe side effects are probable. This golden rule was achieved when combining both extracts with 5-FU as a standard chemotherapeutic agent with a well-known side effects. On the other hand, similar patterns were observed in encapsulated extracts where combining CI-NPs, OR-NPs and CI-NPs + OR-NPs with 5-FU exhibited lower inhibitory effect than free extracts 57.7%, 50.8%, and 62.8%, respectively (Figure 5c, T8, T10, T14). This could be correlated to the percentage released after 24 h incubation of formulation with cancer cells.

**Evaluation of Cell Death Mode.** The cell death mode was analyzed using two different DNA dyes, where vital cells were stained in green, early apoptotic cells were stained in bright green to yellow, late apoptotic cells were stained in orange, and necrotic cells were stained in red (Figure 6c). The cells were counted in each color population. There was a significant difference (P < 0.05) in A/N ratio between all groups except 5-FU (T1) and OR-NPs (T5) using Tukey’s multiple comparison post hoc test (Figure 6b). As shown in Figure 6a, the incidence of death mechanism in control treated cells was apoptosis.
(2.2%) by nearly half the necrosis cell percentage (4.2%) with 0.52 apoptosis/necrosis relative ratios (A/N) (Figure 6b). The treatment with 5-FU or cinnamon extract shifted the mechanism to apoptosis (38.6% and 31.2%, respectively) rather than necrosis (12.4% and 17.2%, respectively), while oregano extract kept the necrosis mechanism (26.7%) nearly similar to apoptosis (24.9%). The A/N ratios were 3.11, 1.81, and 0.93 for 5-FU, CI, and OR (Figure 6b). To the same extent, encapsulated extracts exhibited higher apoptosis mechanism for encapsulated cinnamon extract (29.2%) and higher necrosis mechanism for encapsulated oregano extract (35.5%). The A/N ratios were 1.24 and 0.42 for CI-NPs and OR-NPs (Figure 6b). The combination of half of IC₅₀ of cinnamon and oregano extracts with or without 5-FU showed that the superiority of apoptosis mechanism with 33.1% for CI + OR and 39.7% for 5-FU + CI + OR. The A/N ratios were 1.97 and 3.06 for CI + OR, 5-FU + CI + OR samples (Figure 6b). If the data correlated with % cell inhibition (Figure 5), coadministration of CI + OR reached 71.1% cell inhibition that mainly occurred by the apoptosis mechanism. On the other hand, combining encapsulated cinnamon and oregano extracts shifted the mechanism toward necrosis. The A/N ratio of CI-NPs + OR-NPs was 0.79 (Figure 6b). This could be attributed to the faster release rate of OR-NPs than CI-NPs (Figure 4c) as 79.06 ± 5.1% and 63.06 ± 4.9% were released from OR-NPs and CI-NPs after 24 h, respectively. In contrary, encapsulated extracts, when coadministered with 5-FU, preserved the apoptosis mechanism as the major mechanism (28.9%). The A/N ratio of 5-FU + CI-NPs + OR-NPs was 1.2 (Figure 6b). However, the apoptosis mechanism was reduced than free extracts (39.7%) due to the faster release of oregano than cinnamon from encapsulated formulation.

**Estimation of Apoptosis Mediator (Caspase-3).** To confirm the apoptosis mechanism of cell death, caspase-3 was selected as an apoptosis mediator to be measured. Generally, all samples exhibited to some extend a good ability for activation of caspase-3 with a significant difference ($P < 0.05$) when compared to control group T15 (Figure 7a). Furthermore, 5-FU (T1) showed no significant difference ($P < 0.05$) when compared to free extract groups (T2, T4, T11, and T13) but showed a significant difference ($P < 0.05$) when compared to encapsulated extract groups (T3, T5, T12, and T14). Half IC₅₀ of 5-FU, CI, and OR showed a high expression of caspase-3 reached to 4.3, 3.9, and 3.5 fold of control, respectively. These data completed the bigger picture when correlated with cell death mode experiments as the apoptosis percentage was 38.6% for 5-FU, 31.2% for CI, and 24.9% for OR. The encapsulated
extracts expressed caspase-3 by 2.36 and 1.41 fold of control for half IC\textsubscript{50} of CI-NPs and OR-NPs, respectively. This was in the same context of decreasing percentage of apoptotic cells when encapsulating extracts as mentioned in previous experiment. The combination of half of IC\textsubscript{50} of cinnamon and oregano extracts with or without 5-FU showed the superiority of caspase-3 expression with 3.97 for CI + OR and 4.46 for 5-FU + CI + OR. Combing 5-FU with both extracts was the highest caspase-3 expression which is similar to cell death mode experiment with highest apoptosis percentage 39.69%. On the other hand, combining encapsulated cinnamon and oregano extracts with and without 5-FU expressed caspase-3 by 2.94 and 2.53 for 5-FU + CI-NPs + OR-NPs and CI-NPs + OR-NPs.

**Determination of Mitochondrial Transmembrane Potential.** The mitochondrial function was estimated through ΔΨ\textsubscript{m}−dependent uptake and the CMX-Ros retention in the mitochondria. The fluorescence intensity for each cell was analyzed by an image analyzer, and the mean intensity for each experiment was calculated, as presented in Figure 1b. One of major targets in cancer therapy is mitochondria due to its energy production. The mitochondrial membrane potential suppressed during apoptosis. In general, all samples exhibited to some extent an ability to reduce the mitochondrial membrane potential (Figure 1b) with a significant difference (P < 0.05) when compared to control group T15 (Figure 1b). Furthermore, 5-FU (T1) showed no significant difference (P < 0.05) when compared to T2 and T4 groups but showed significant difference (P < 0.05) when compared to T3, T5, T11, T12, T13, and T14. The mitochondrial membrane potential loss and the activation of caspase-3 suggests the apoptosis pathway as the main cell death mode.\textsuperscript{50} Moreover, half IC\textsubscript{50} of 5-FU, CI, and OR showed low intensity with 26.3, 30.41, and 32.54, respectively. These data confirmed the results of caspase-3 activation experiment for the apoptosis pathway. The encapsulated extracts suppressed the mitochondrial membrane potential (ΔΨ\textsubscript{m}) by 52.2 and 74.5 for half IC\textsubscript{50} of CI-NPs and OR-NPs, respectively. This was in the same context of decreasing caspase-3 activation when encapsulate the extracts as mention in previous experiment. The combination of half of IC\textsubscript{50} of cinnamon and oregano extracts with or without 5-FU showed the superiority of ΔΨ\textsubscript{m} suppression with 39.1 for CI + OR and 21.8 for 5-FU + CI + OR. Combing 5-FU with both extracts was the highest ΔΨ\textsubscript{m} suppression which is similar to caspase-3 estimation experiment with the highest caspase-3 expression 4.46. On the other hand, combining encapsulated cinnamon and oregano extracts with and without 5-FU suppressed ΔΨ\textsubscript{m} by 58.1 and 66.2 for 5-FU + CI-NPs + OR-NPs and CI-NPs + OR-NPs.

Previous studies showed similar results relating synergistic activity of whole food extracts with traditional chemotherapy. Cotreatment of single dose of Gelam honey with different concentrations of 5-FU showed a significant suppressive effect in comparison with of 5-FU alone at the same concentrations against HCT 116 colorectal cancer cells. It caused 3-fold cell growth reduction percentage higher than 5-FU alone.\textsuperscript{52}

In conclusion, cinnamon and oregano extracts were encapsulated in core/shell nanocarrier with desired physico-chemical properties. Furthermore, both extracts inhibited HCT 116 cells which confirmed their cytotoxic activity against colon cancer. From all cytotoxic evaluation experiments, combining 5-FU with both extracts either free or encapsulated exhibited highest percentage cell inhibition (71.1% and 62.8%), high percentage apoptotic cells (39.7% and 28.9%), high caspase-3 activation (4.46 and 2.94), and high suppression of mitochondrial membrane potential (21.76 and 58.1) for unencapsulated extracts with 5-FU and encapsulated extracts with 5-FU, respectively. The slight decrease of encapsulated extracts in their activity than free extracts could be a result of sustain release profile of extracts from nanoparticle matrix. These data are confirming the success of the suggested combination with one of standard drugs (5-FU) for treating human colon carcinoma with low dose of 5-FU, consequently decreasing side effects. Further animal studies are recommended to confirm the generated data in the current study.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b03093.

(1) Tentative compounds identified in cinnamon extract by quasimolecular ion [M+H]\textsuperscript{+} using LC-HRMS/MS (original data for Figure 2), (2) tentative compounds identified in oregano extract by quasimolecular ion [M+H]\textsuperscript{+} using LC-HRMS/MS (original data for Figure 2), (3) reported cytotoxic active constituents identified in cinnamon and oregano extracts by LC-HRMS/MS (original literature survey for Figure 2), (4) values of the three experimental factors and measured responses according to the matrix designed by Box–Behnken design (original results of experimental design), (5) chemical structure of reported cytotoxic active constituents identified in cinnamon extract and oregano extract by LC-HRMS/MS (chemical structure of identified compound in Figure 2), and (6) chromatograms and calibration curves of cinnamaldehyde and rosmarinic acid using HPLC (curve used to calculate data in Table 2) (PDF)

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**Author Contributions**

L.A.K. conducted and analyzed data of the nanoparticle section and wrote and reviewed the manuscript.

**Notes**

The authors declare no competing financial interest.

**ABBREVIATIONS USED**

5-FU, 5-fluorouracil; ANOVA, analysis of variance; CI, cinnamon; CI-NPs, cinnamon extract loaded solid lipid nanoparticles coated with chitosan; CRC, colorectal cancer; DLS, dynamic light scattering; DSC, differential scanning calorimeter; EE, entrapment efficiency; HCT-116 cells, human colon carcinoma ATCC CCL-247; HPLC, high-performance liquid chromatography; LC-HRMS/MS, high-resolution mass/mass spectrometry liquid chromatography; OR, oregano; OR-NPs, oregano extract loaded solid lipid nanoparticles coated with chitosan; PDI, polydispersity index; PEG, poly(ethylene glycol); PS, particle size; ROS, reactive oxygen species; SLN-Cs, solid lipid nanoparticles coated with chitosan; SLNs, solid


