Anticancer Study
15.1 *In vitro* anticancer efficacy testing

*In vitro* anticancer efficacy testing of prepared nanoparticles were evaluated using MTT assay\textsuperscript{142} and sulforhodamine B (SRB) assay.\textsuperscript{143,144}

**MTT Assay**

- Human breast adenocarcinoma (MCF-7) cells were cultured in DMEM supplemented with 10% inactivated FBS, penicillin (100 IU/mL), streptomycin (100 µg/mL) and amphotericin B (5 µg/mL) in an atmosphere of 5% CO\textsubscript{2} at 37°C until confluent. Cultured BRL3A cells were dissociated with trypsin phosphate versing glucose (TPVG) solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm\textsuperscript{2} culture flasks and all experiments were carried out in 96 microtitre plates.

- The monolayer MCF-7 cell culture was trypsinized and the cell count was adjusted to 1.0x10\textsuperscript{5} cells/mL using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 mL of the diluted cell suspension (approximately 10,000 cells) was added.

- Prepared polymeric nanoformulations were diluted with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 5 mg/mL concentration, which was sterilized by filtration and finally centrifuged. Serial dilutions (1000, 500, 250, 125, 62.5 µg/mL) were prepared and Paclitaxel was used as positive control at the same concentrations.

- After 24 hour, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µL of different concentrations of polymeric nanoformulations and positive control were added.

- The plates were then incubated at 37°C for 3 days in 5% CO\textsubscript{2} atmosphere and microscopic examination was performed and observations were noted every 24 hour interval.

- After 72 hours, the drug solutions (Plain polymeric nanoformulation and Paclitaxel) in the wells were discarded and 50 µL of MTT in PBS was added to each well.
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- The plates were gently shaken and incubated for 3 hours at 37°C in 5% CO₂ atmosphere. The supernatant was removed and 100 µL of propanol was added and the plates were gently shaken to solubilize the formed formazan.

- The absorbance was measured using a microplate reader (ELx800, Bio-Tek) at a wavelength of 540 nm.

- The percentage growth inhibition (% GI) was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC₅₀) values is generated from the dose-response curves.

- The experiments were performed in triplicate.

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\text{% GI} = 100 \times \frac{\text{Mean optical density of individual test group}}{\text{Mean optical density of control group}}
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SRB Assay

- Human ovarian cancer cell (Ovkar-3), human hepatoma cell (HEPG2) and human cervix cancer cell (HeLa) were cultured separately in Dulbecco's modified eagle medium (DMEM) supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL) and amphotericin B (5 µg/mL) in atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks and all experiments were carried out in 96 microtitre plates.

- Pure quercetin, rutin, silibinin, quercetin-rutin, and quercetin-silibinin loaded polymeric nanoformulations were diluted with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/mL concentration, which was sterilized by filtration and finally centrifuged. Serial dilutions (10, 20, 40, 80 µg/mL) were prepared. Adriamycin (Doxorubicin) was used as positive control at the same concentrations.

- The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0x10⁵ cells/mL using DMEM containing 10% FBS. To each well of the 96 well
microtitre plate, 0.1 mL of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µL of different concentrations of pure quercetin, rutin, silibinin, doxorubicin, quercetin-rutin, and quercetin-silibinin loaded polymeric nano formulations were added. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, and microscopic examination was performed and observations were noted every 24 hours interval. Fix the cells with ice-cold trichloroacetic acid (TCA) for 1 hour at 4°C. Wash the plates five times in distilled water and allowed to dry in the air. After 72 hours, the drug solutions in the wells were discarded and 50 µL of SRB solution was added to each well of the dry 96-well plates and allow staining at room temperature for 30 min. Remove the SRB solution by washing the plates quickly with 1% v/v acetic acid, five times, to remove unbound dye. Dry the washed plates in the air. Extract the bound SRB by adding 100 µL of 10 mM unbuffered Tris Base (pH 10.5) to each well and shaking for 5 minutes on a shaker platform. The absorbance was measured using a micro plate reader (ELx800, Bio-Tek) at a wavelength of 492 nm. Parameters such as GI₅₀ (Concentration of the drug that produces 50% inhibition of the cells), TGI (Concentration of the drug that produces total inhibition of the cells) and LC₅₀ (Concentration of the drug that kills 50% of the cells) were calculated. The experiments were performed in triplicate.

15.2 In-vivo anticancer efficacy testing

- *In-vivo* anticancer efficacy testing was performed to evaluate the efficacy of prepared quercetin, rutin, silibinin, paclitaxel, quercetin-rutin, and quercetin-silibinin loaded polymeric nanoparticles in comparison with pure drug.¹⁴⁴

- Healthy adult female sprague dawley rats (120-200 gram body weight) were procured and randomly assigned to 10 groups, each containing 6 animals in polypropylene cages layered with husk and maintained in a controlled room at a temperature (22±3°C) and light (12 hours light/dark cycle). Animals were allowed free access to water and standard pellet diet. Animals were cared in accordance with the “Guide for the care and use of laboratory animals” and study was conducted in accordance with CPCSEA.
The oestrous cycle was monitored daily by vaginal cytology assay. Briefly, a glass rod was inserted into the vagina and gently touched against the vaginal wall. The vaginal cells were then smeared on to the drop of 0.9% normal saline placed on the glass slide. The smear was observed under light microscope. The 7,12-Dimethylbenz(a)anthracene (DMBA) solution was freshly prepared at a dose of 25 mg/kg of DMBA in 0.75 ml of sunflower oil and 0.25 ml of physiological saline. Animals (except animals in control group) received a single dose of DMBA 25 mg/kg through i.p. route. Animals were allowed to develop tumor, which was identified by palpitation. Mammary tumor induced animals were selected for 60 days treatment (Table 15.1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>1</td>
<td>Control</td>
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<tr>
<td>2</td>
<td>25 mg/kg of DMBA in 0.75 ml of sunflower oil and 0.25 ml of physiological saline to induce breast cancer and received no other treatment</td>
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<tr>
<td>3</td>
<td>25 mg/kg of DMBA in 0.75 ml of sunflower oil and 0.25 ml of physiological saline. After tumor induction, animals were treated orally with pure quercetin (100 mg/kg of body weight)</td>
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<tr>
<td>4</td>
<td>25 mg/kg of DMBA in 0.75 ml of sunflower oil and 0.25 ml of physiological saline. After tumor induction, animals were treated orally with pure rutin (100 mg/kg of body weight)</td>
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<tr>
<td>5</td>
<td>25 mg/kg of DMBA in 0.75 ml of sunflower oil and 0.25 ml of physiological saline. After tumor induction, animals were treated orally with pure Silibinin (100 mg/kg of body weight)</td>
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<tr>
<td>6</td>
<td>25 mg/kg of DMBA in 0.75 ml of sunflower oil and 0.25 ml of physiological saline. After tumor induction, animals were treated orally with pure rutin (100 mg/kg of body weight)</td>
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<th>orally with quercetin nano (100 mg/kg of body weight)</th>
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<tr>
<td>7</td>
<td>25 mg/kg of DMBA in 0.75 ml of sunflower oil and 0.25 ml of physiological saline. After tumor induction, animals were treated orally with rutin nano (100 mg/kg of body weight)</td>
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<tr>
<td>8</td>
<td>25 mg/kg of DMBA in 0.75 ml of sunflower oil and 0.25 ml of physiological saline. After tumor induction, animals were treated orally with silibinin nano (100 mg/kg of body weight)</td>
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<tr>
<td>9</td>
<td>25 mg/kg of DMBA in 0.75 ml of sunflower oil and 0.25 ml of physiological saline. After tumor induction, animals were treated orally with prepared quercetin-rutin dual loaded polymeric nanoformulation (equivalent to 100 mg/kg of body weight)</td>
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<tr>
<td>10</td>
<td>25 mg/kg of DMBA in 0.75 ml of sunflower oil and 0.25 ml of physiological saline. After tumor induction, animals were treated orally with prepared quercetin-silibinin dual loaded polymeric nanoformulation (equivalent to 100 mg/kg of body weight)</td>
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Evaluation of anticancer efficacy

Efficacy of the prepared nanoformulation was assessed using tumorigenesis parameter such as body weight, tumor burden and tumor volume.

Body Weight

Animal body weight was measured weekly once from the starting day to end of the study.

Tumor burden

Number of tumors formed in each animal is called tumor burden. It was found by palpitation and dissection of animal on final day of study.

Tumor volume
The resultant solid tumor was considered to be prelate ellipsoid with one long axis and two short axis. The two short axis were measured with vernier calliper. The tumor weight was calculated by multiplying the length of the tumor with the square of the width and dividing the product by two.

**Statistical analysis**

Statistical analysis was performed using SPSS 18.0 (SPSS, Inc., Chicago, IL) statistical package. Data were expressed as mean ± standard deviation. One way analysis of variance (ANOVA) followed by Duncan multiple comparison method was used to correlate the difference between the variables. Data were considered statistically significant if P value was < 0.001, < 0.01 and < 0.05.