

# Nano *Uncaria gambir* as Chemopreventive Agent Against Breast Cancer

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**Background:** Breast cancer is one of the main causes of death in women. *Uncaria gambir* is an Indonesian herbal plant that can be used as an anti-cancer. However, herbal medicines have low bioavailability, which affects their bioactivity. Nanoencapsulation can increase bioavailability and stability of bioactive compounds in herbal medicines.

**Purpose:** This recent finding tried to unravel anti-cancer and chemopreventive of *U. gambir* nano-encapsulated by Na-alginate.

**Study Design:** *U. gambir* bioactive compounds were isolated and characterized using UV-Vis spectrometer, FTIR, NMR and HR-MS. *U. gambir* extract was nanoencapsulated using Na-alginate. Anti-cancer effect was assessed by MTT assay towards T47D cell. Meanwhile, a chemopreventive analysis was carried out in breast cancer mice-induced benzo[ $\alpha$ ]pyrene. The healthy mice were divided into 8 groups comprising control and treatment.

**Results:** Elucidation of *U. gambir* ethyl acetate extract confirmed high catechin content, 89.34% (w/w). Successful nanoencapsulation of *U. gambir* (*G-NPs*) was indicated. The particle size of *G-NPs* was  $78.40 \pm 12.25$  nm. Loading efficiency (LE) and loading amount (LA) of *G-NPs* were  $97.56 \pm 0.04\%$  and  $32.52 \pm 0.01\%$ , respectively. *G-NPs* had an  $EC_{50}$  value of  $10.39 \pm 3.50$   $\mu\text{g/mL}$ , which was more toxic than the  $EC_{50}$  value of extract towards the T47D cell line. Administration of 200 mg/kg BW *G-NPs* to mice induced by benzo[ $\alpha$ ]pyrene exhibited SOD and GSH levels of 13.69 ng/mL and 455.6 ng/mL. In addition, the lowest TNF- $\alpha$  level was 27.96 ng/mL. A dose of 100 mg/kg BW *G-NPs* could best increase CAT levels by 7.18 ng/mL. There was no damage or histological abnormalities found in histological analysis of the breast tissue in the group given 200 mg/kg BW *G-NPs*.

**Keywords:** breast cancer, chemopreventive, cytotoxicity, nanoencapsulation, *U. gambir*

## Introduction

Cancer is the uncontrolled and abnormal growth of various body cells. Nowadays, 100 different types of cancer pose diversity in the mechanism of action and response to the related treatment.<sup>1</sup> Based on global burden cancer (GLOBOCAN) data reported in 2020, global incidents of new-diagnosed cancer and death-associated cancer death reached about 19.3 million and 10.0 million cases, respectively. With an estimated 2.3 million new cases, female breast cancer is the most frequently diagnosed cancer, followed by lung (11.4%), colorectal (10.0%), prostate (7.3%), and stomach (5.6%) cancers. However, lung cancer is still the most common cause of cancer death.<sup>2</sup> According to RISKESDAS data, Indonesia ranks 8th for cancer cases, increasing from 1.4 cases per 1000 people in 2013 to 1.79 cases per 1000 in 2018.<sup>3</sup>

The interplay between genetic risk and environmental factors causes breast cancer. Both lead to a series of development processes starting from genetically altered epithelial cells, hyperplasia, dysplasia, and in situ cancer till

the end with malignant tumor (cancer).<sup>4-6</sup> This ailment has three major subtypes: luminal, HER2<sup>+</sup>, and Triple-negative.<sup>6</sup> Oxidative stress is essential in early cancer initiation, including breast cancer. Overexposure to ROS (reactive oxygen species) promotes tumor progression and causes an imbalance of antioxidant enzymes. Level of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and paraoxonases (PONs) decrease in bladder cancer compared to control. Moreover, oxidative stress also triggers the production of pro-inflammatory cytokines, TNF- $\alpha$  (tumor necrosis alpha factor), which are involved in cancer development.<sup>7</sup> Therefore, a proper medication that targets oxidative stress, inflammation, and carcinogenesis axis is vital to prevent further cancer development.

Radiotherapy and chemotherapy have so far been the mainstays of breast cancer treatment. Because both methods can harm normal cells while killing cancer cells, their adverse effects are frequently devastating.<sup>8</sup> Several drawbacks of chemotherapy, such as hair loss, bone marrow suppression, drug resistance, gastrointestinal lesions, neurological dysfunctions, and cardiac toxicity, are also inevitable.<sup>9,10</sup> Hence, the need to develop effective cancer treatment has become the main focus of many cancer researches.<sup>11</sup> The use of herbal-based medicines is known to reduce cancer cell proliferation while perceived to have fewer side effects than chemical drugs.<sup>12,13</sup> In respect of abundant herb with anti-cancer potency, *Uncaria gambir*, a member of the Rubiaceae family commonly found in West Sumatra, contains catechin that already proves cytotoxicity against breast cancer cell lines.<sup>14</sup> Evacuasiy et al reported that catechin better exhibits antioxidant and cytotoxicity effects in T47D than in MCF7 cells.<sup>15</sup> The anti-cancer mechanism of catechin is associated with the ability to induce cell apoptosis and inhibit angiogenesis and metastasis of malignant cells.<sup>16</sup> In particular, hydrated catechin suppress MCF7 cell proliferation as well as induces apoptosis by improving the expression of caspase-3, -8, -9, and tumor suppressor p53.<sup>17</sup> Nowadays, successful nanoparticle research as anticancer is reported on epigallocatechin-3-gallate,<sup>18</sup> biosynthesized ZnO NPs, CuO NPs derived from pumpkin seeds extract,<sup>19-21</sup> MnONPs,<sup>22</sup> AuNPs,<sup>23</sup> and AgNPs.<sup>24</sup>

Cancer-nanomedicine (cancer treatment that applied nanotechnology) has been broadly studied and even applied clinically in recent decades.<sup>25</sup> Among numerous nanotechnology tools, biopolymeric nano-capsules offer an up-and-coming alternative design of targeted cancer therapy. Nanocapsules (made from alginate, pullulan, cellulose, polylactic acid, chitosan, and other biopolymers) gain much attention as anti-cancer drug delivery system since nano-size increase the surface area of an active material, exhibiting a high stability and bioavailability but lowering drug toxicity.<sup>26,27</sup> Moreover, biopolymeric nano-capsules are acknowledged for their cost-effective and environmentally friendly drug preparation.<sup>28</sup> There are many technologies to produce nanocapsule, including ultrasound-assisted nanoencapsulation. Bioactive-loaded biopolymeric nanocarriers prepared with sonication are reported to exhibit better colloidal dispersion, gastrointestinal fate and safety in many studies.<sup>29</sup> In addition, ultrasonication owns broad technically benefits comprising simplicity, versatility, environmentally friendly and by-product free, making it preferred in the nanoparticle fabrication.<sup>30</sup> However, excessive immune response as well as production of inflammatory mediators still become common limitation of polymeric-based nanoparticle administration because toxicity of polymeric nanoparticle is influenced by quantum size.<sup>31</sup> Hence proper nanoencapsulation technique must be established to achieve better nanocapsules.

Recent findings elucidate the nanoencapsulation of *U. gambir* using sodium alginate to enhance bioactive compounds' activity with anti-cancer potency. Anti-cancer activity of nano-gambir formulation against breast cancer is assessed in vitro and in vivo. Furthermore, the cytotoxic effect of nano-gambir is evaluated in vitro in the T47D cell line. Meanwhile, model mice suffering from breast cancer are used to evaluate in vivo chemopreventive activity of nano-gambir.

## Materials and Methods

### Material

The *Uncaria gambir* was taken from Bima, Sumbawa Island, Nusa Tenggara Barat Province, Indonesia. The plant was identified by staff at the Indonesian Biology Generation Foundation ("Yayasan Generasi Biologi Indonesia [YGBI]") with the certificate No. 232/02.Genbinesia/2022, and a voucher specimen had been deposited at the herbarium of YGBI. Methanol, ethanol, n-hexane, ethyl acetate, sodium alginate, distilled water, phosphate buffer, phosphate buffer saline (PBS); benzo[*a*]pyrene (Merck), Na-CMC, SOD kit (BT-Lab), CAT kit (BT-Lab), GSH kit (BT-Lab), TNF- $\alpha$  kit (BT-Lab), Neutral Buffered Formalin (NBF) 10%. The T47D cell line used has been approved by the health research ethical clearance

commission, Faculty of dental medicine, Airlangga University with ethical clearance certificate number: 028/HRECC.FODM/I/2020.

## Extraction and Isolation of *U. gambir*

The powder of *U. gambir* sap ( $\pm 1$  kg) was macerated by methanol for  $1 \times 24$  h with 1:2 ratio and repeated 3 times. A rotary vacuum evaporator then concentrated the methanolic extract at  $50^\circ\text{C}$ . The thick extract already obtained was subsequently partitioned by n-hexane and ethyl acetate at the same ratio. Next, the ethyl acetate extract of *U. gambir* was concentrated by rotary vacuum evaporator until yielded *U. gambir* ethyl acetate extract powder. The ethyl acetate extract of *U. gambir* sap was isolated using vacuum column chromatography followed by gravity column chromatography. The pure isolates were then characterized using a UV-Vis spectrometer, FTIR, NMR, and HR-MS.

## Characterization of G-NPs

The technique reported by Kristanti et al was used to nanoencapsulation an ethyl acetate extract of *U. gambir* with Na-alginate.<sup>32</sup> The nano-capsule product is then stated as *G-NPs*. The physicochemical properties of *G-NPs* were evaluated by a polydispersity index (PDI), zeta potential ( $\zeta$ ), and particle size (Dynamic Light Scattering, Zetasizer Nano ZS, Malvern). Analysis of the functional groups of NPs was assessed by FTIR (Shimadzu IRTracer-100), while analysis decomposition of *G-NPs* was performed by TGA (Perkin Elmer TGA 4000). AFM characterized the topography of G-NPs.

## Stability Analysis of G-NPs

The stability of *G-NPs* in protecting bioactive compounds was assessed against several parameters, comprising temperature, pH, and salt concentration (NaCl). In addition, UV-Vis absorption spectra were performed in each parameter evaluation. Moreover, the degree of turbidity of nanoparticles was evaluated too.<sup>33</sup>

## Loading and Release of G-NPs Calculations

The loading amount (LA) and loading efficiency (LE) of bioactive components were, respectively, calculated by Equations 1 and 2. The release of the *G-NPs* bioactive compound was determined by Equation 3.<sup>33,34</sup>

$$\%LE = \frac{\text{Mass of samples on G - NPs}}{\text{Mass of samples in feed}} \times 100\% \quad (1)$$

$$\%LA = \frac{\text{Mass of samples on G - NPs}}{\text{Mass of G - NPs}} \times 100\% \quad (2)$$

$$Ct' = Ct + \frac{v}{V} \sum_0^{i-t} Ct \quad (3)$$

Where Ct': concentration correction at t time

Ct: measured concentration at t time

V: total volume of buffer used

v: volume of aliquots

## Cytotoxicity Assay

Research using the T47D cell line has been ethically certified by the Health Research Ethical Clearance Commission, Faculty of Dentistry, Universitas Airlangga, Indonesia (Ethical Clearance Certificate, No. 028/HRECC.FODM/I/2020). The research was conducted at the Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada. T47D cell lines were cultured in RPMI 1640 media and further seeded at 96-well plates with a density of  $10 \times 10^4$  cells / well upon reaching 80% confluence. The culture was further incubated for 24 h in an incubator ( $37^\circ\text{C}$ ; 5%  $\text{CO}_2$ ). About 100  $\mu\text{L}$  of culture media containing samples (*U. gambir* extract and *G-NPs*) were added to each well, and incubation was carried out for 24 h. After incubation, all medium was discarded and then washed with PBS. Cytotoxicity assay was done by adding 100  $\mu\text{L}$  of MTT (5 mg/mL) reagent to

each well and then incubated for 4 hours at 37°C in 5% CO<sub>2</sub>. The MTT assay was stopped by giving 100 µL of 10% SDS within 0.01 N HCl. The absorbance was measured using an ELISA reader (Bio-Rad) at λ 550 nm. The EC<sub>50</sub> value of each sample was obtained by using a dose-response calculation.

## Chemopreventive Potency

Current animal research has been ethically certified by the Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga, Indonesia (Ethical Clearance Certificate, No. 351/HRECC.FODM/VI/2021). The healthy mice (*Mus musculus*) Balb/c at the age of 6–8 weeks with 20–25 g of body weight and never experienced pregnancy were placed in the cage with lighting conditions 12 hours per day, temperature 25 °C, and humidity ± 50–60%. All mice were then grouped into 8 cohorts comprising:

- C<sub>0</sub>: Control, mice were treated with water only.
- C<sub>1</sub>: Cancer control, mice were injected with benzo[α]pyrene (0.2 mL / 2 days for 5 times) in the 2nd week and observed for 8 weeks.
- T<sub>1</sub>: Treatment 1, mice were given *U. gambir* extract (50 mg/kg BW) orally every day for 8 weeks. In the 2nd week of treatment, an injection of benzo[α]pyrene was carried out.
- T<sub>2</sub>: Treatment 2, mice were given *U. gambir* extract (100 mg/kg BW) orally every day for 8 weeks. In the 2nd week of treatment, an injection of benzo[α]pyrene was carried out.
- T<sub>3</sub>: Treatment 3, mice were given *U. gambir* extract (200 mg/kg BW) orally every day for 8 weeks. In the 2nd week of treatment, an injection of benzo[α]pyrene was carried out.
- T<sub>4</sub>: Treatment 4, mice were given *G-NPs* (50 mg/kg BW) orally every day for 8 weeks. In the 2nd week of treatment, an injection of benzo[α]pyrene was carried out.
- T<sub>5</sub>: Treatment 5, mice were given *G-NPs* (100 mg/kg BW) orally every day for 8 weeks. In the 2nd week of treatment, an injection of benzo[α]pyrene was carried out.
- T<sub>6</sub>: Treatment 6, mice were given *G-NPs* (200 mg/kg BW) orally every day for 8 weeks. In the 2nd week of treatment, an injection of benzo[α]pyrene was carried out.

All mice cohorts were sacrificed at week 8, and their blood was taken for clinical blood analysis to measure levels of SOD, GSH, CAT, and TNF-α. In addition, each mice group was also taken breast tissue, liver, kidney, spleen, and pancreas for histopathological analysis.

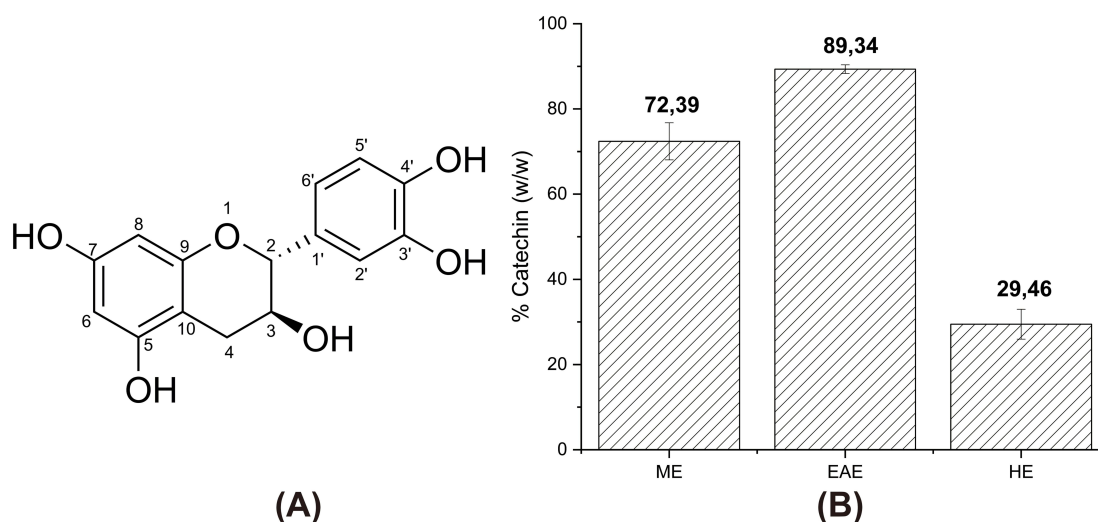
## Statistical Analysis

The software GraphPad Prism 8 was utilized for the statistical analysis. The anti-cancer effects of *G-NPs* and *U. gambir* extract were examined using one-way ANOVA. Tukey's test was also used to conduct post-hoc analysis to identify group differences. In this instance,  $p < 0.05$  was deemed significant.

## Results

### The Elucidation of *U. gambir* Bioactive Compound

Methanol was used to extract the *U. gambir* sap, divided using n-hexane and ethyl acetate. *U. gambir* extract was further determined its main bioactive compound by chromatographic separation and elucidation of the bioactive compound structure was also carried out. Based on the elucidation result, the main bioactive compound of *U. gambir* was (+)-catechin (Figure 1A). This compound was found to be a brownish-white solid with spectra analysis showed as followed, UV-Vis (MeOH) spectra: λ<sub>max</sub> 281 nm; IR spectra ν<sub>max</sub> (cm<sup>-1</sup>): 3650 (OH stretching), 2962 (C-H sp<sup>3</sup> stretching), dan 1598 (C=C in ring); <sup>1</sup>H-NMR (Bruker 600 MHz, DMSO-*d*<sub>6</sub>) δ<sub>H</sub> (ppm): 4.59 (*d*, 1H, *J* = 5.0, H-2), 3.80 (*m*, 1H, H-3), 2.65 dan 2.34 (*dd*, 1H, *J* = 5.0 dan 16.0, H-4), 5.78 (*d*, 1H, *J* = 2.0, H-6), 5.66 (*d*, 1H, *J* = 2.0, H-8), 6.70 (*d*, 1H, *J* = 2.0, H-2'), 6.66 (*d*, 1H, *J* = 8.0, H-5'), 6.58 (*dd*, 1H, *J* = 2.0 dan 8.0, H-6'), 4.83 (*s*, 1H, OH-3), 9.15 (*s*, 1H, OH-7), 8.91 (*s*, 1H, OH-5), 8.83 (*s*, 1H, OH-3'), 8.87 (*s*, 1H, OH-4'); <sup>13</sup>C-NMR (Bruker 150 MHz, DMSO-*d*<sub>6</sub>) δ<sub>C</sub> (ppm): 80.7 (C-H, C-2), 67.5 (C-H, C-3), 28.1 (C-H<sub>2</sub>, C-4), 157.3 (C, C-5), 95.5 (C-H, C-6), 157.8 (C, C-7), 94.8 (C-H, C-8), 157.2 (C, C-9), 99.0 (C, C-10), 131.5 (C, C-1'), 115.2 (C-H, C-2'), 145.8 (C, C-3'), 144.6 (C, C-4'), 116.1 (C-H, C-5'), 121.0 (C-H, C-6'); ESI-MS: *m/z* 291.2 [M+H<sup>+</sup>]; optical rotation +17°. The highest



**Figure 1** (A) Structure of (+)-catechin; (B) Catechin content of *U. gambir* extract.  
**Abbreviations:** ME, methanol extract; EAE, ethyl acetate extract; HE, n-hexane extract.

catechin content, 89.34% (w/w), was found in the *U. gambir* ethyl acetate extract (Figure 1B), which was then processed with Na-alginate for nano encapsulation.

## Characterization of G-NPs

The physicochemical properties of *G-NPs* are shown in Table 1. *G-NPs* were nano-capsule of *U. gambir* extract encapsulated by Na-alginate biopolymer. *G-NPs* had a particle size of  $78.40 \pm 12.25$  nm. The polydispersity index (PDI) measured size heterogeneity of *G-NPs* indicating size distribution or agglomeration/aggregation of sample where  $PDI > 0.7$  was considered broad particle size distribution.<sup>35,36</sup> PDI value of *G-NPs* was  $0.55 \pm 0.01$ , indicating that particle size distribution tended to be homogeneous since the smaller polydispersity index meant more uniform particle size.<sup>37</sup>

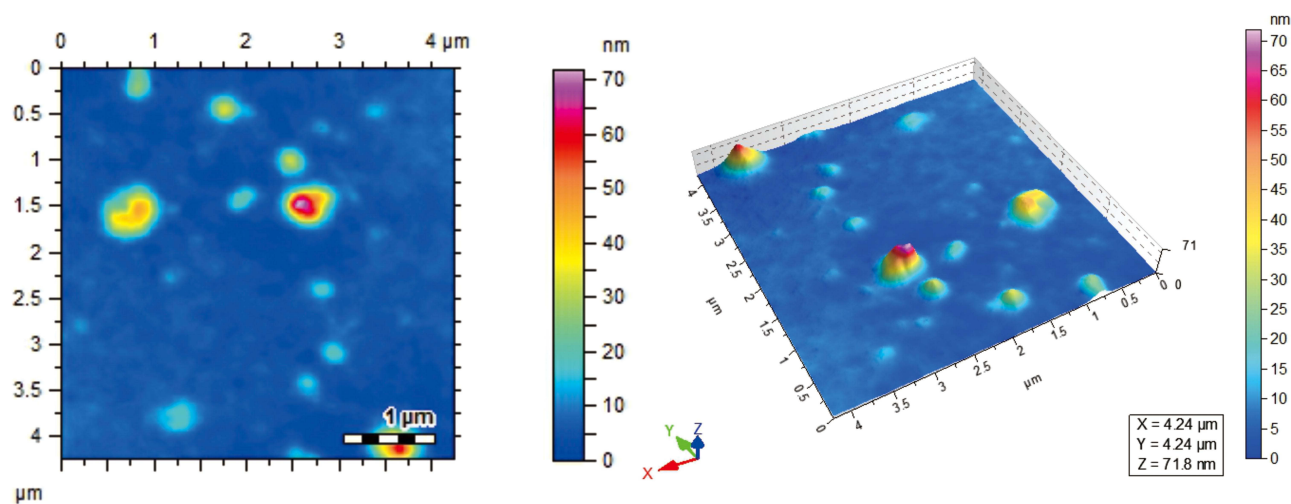
Zeta potential ( $\zeta$ ) analysis validated the successful encapsulation of *G-NPs*. Based on the result shown in Table 1, Na-alginate  $-37.20 \pm 3.06$  mV, *U. gambir* extract  $-44.47 \pm 0.47$  mV, and *G-NPs*  $-40.87 \pm 0.90$  mV had a negative value which was indicated as a low  $\zeta$  value. A large number of electronegative hydroxy groups were to blame for the phenomenon. The Zeta potential value for *G-NPs* was comparable to that of *U. gambir* extract and Na-alginate. This is presumably because an electropositive group of Na-alginate partially compromised the electronegative group within *U. gambir* extract. Since a Zeta potential value more significant than  $\pm 30$  mV was considered stable because surface charge prevented aggregation,<sup>38</sup> this recent nanoencapsulation Zeta potential result of  $-40.87 \pm 0.90$  mV demonstrated stable suspension. Due to the increased electrostatic repulsion between particles, a more significant Zeta potential indicated improved stability. Based on AFM analysis, the size of *G-NPs* was  $\pm 70$  nm. This supported result of the DLS measurement (Figure 2).

Na-alginate and *U. gambir* extract shared a peak in the *G-NPs*' FTIR spectra. *U. gambir* extract was responsible for absorptions at  $1033\text{ cm}^{-1}$  (C-O-C stretching vibration) and  $1519\text{ cm}^{-1}$  (C-C in ring). In contrast, Na-alginate was absorbed in  $1419\text{ cm}^{-1}$  (vibrations of the carboxylate salt ion) and  $1122\text{ cm}^{-1}$  (deformation of -OH in -COOH) (Figure 3). The composition of the active compounds in the *U. gambir* extract and their interactions with the sodium alginate coating material were determined through TGA analysis of *G-NPs* (Figure 4). *G-NPs* and *U. gambir* extract deteriorated at a temperature of  $70^\circ\text{C}$ , which showed a decreasing sample weight of 10.5% and 12.1%, respectively. At  $178^\circ\text{C}$ , *G-NPs* and

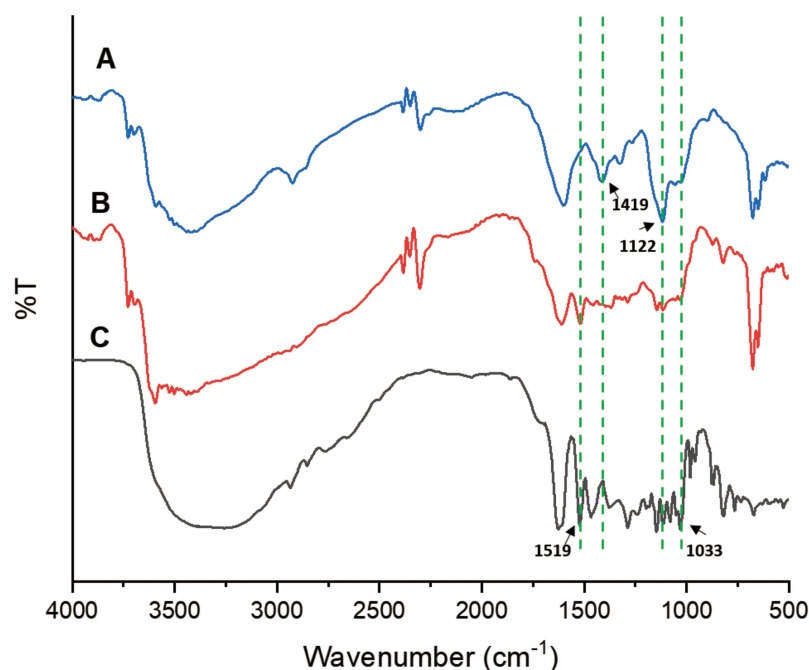
**Table 1** Physicochemical of *G-NPs*

Type	Size $\pm$ SD (nm)	PDI $\pm$ SD	$\zeta \pm$ SD (mV)
Na-Alginate	$600.20 \pm 105.02$	$1.00 \pm 0.00$	$-37.20 \pm 3.06$
<i>U. gambir</i> extract	$586.00 \pm 13.86$	$0.47 \pm 0.05$	$-44.47 \pm 0.47$
<i>G-NPs</i>	$452.63 \pm 5.29$	$0.55 \pm 0.01$	$-40.87 \pm 0.90$

**Notes:** Each data presented as mean  $\pm$  SD (n=3).



**Figure 2** AFM 2D topography images of *G-NPs*.

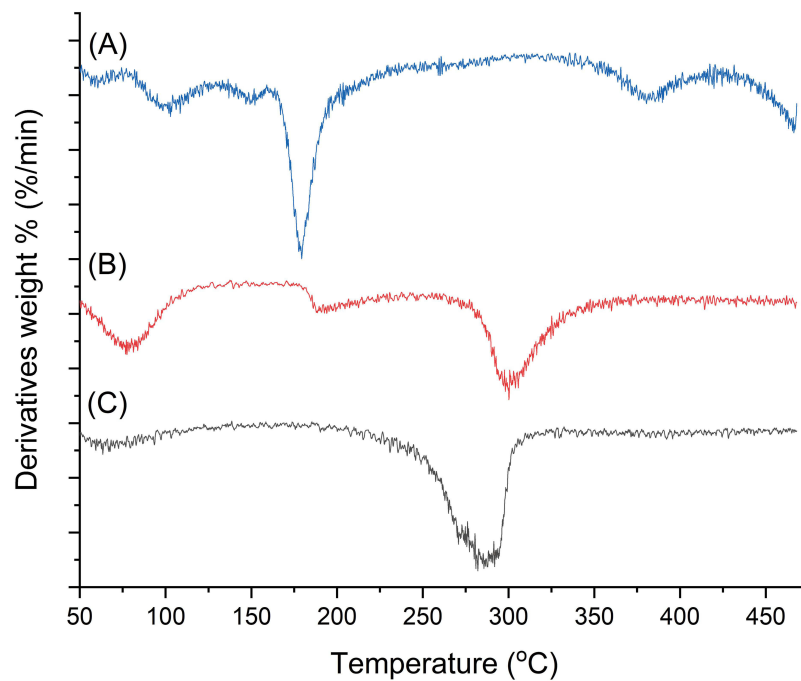


**Figure 3** FTIR spectra of (A) Na-alginate, (B) *G-NPs*, and (C) *U. gambir* extract.

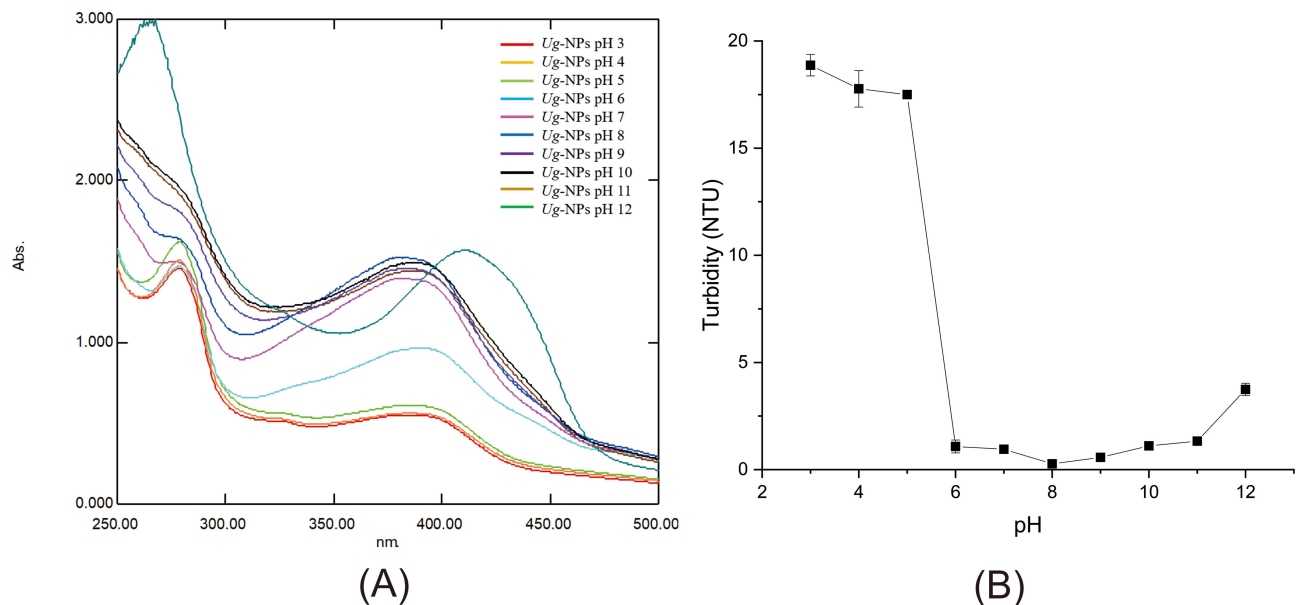
extracts of *U. gambir* decomposed with a decrease in sample weight of 16.2% and 33.1%, respectively, while Na-alginate decomposed at a temperature of 290 °C with a decrease in sample weight of 35.3%. *G-NPs* also decomposed with a decrease in sample weight of 25.2% at 290 °C. FTIR and TGA analysis indicated there was no chemical interaction between the constituent of *U. gambir* extract and Na-alginate in the *G-NPs* but only physical interactions.

## Stability of *G-NPs*

Previously, (+)-catechin was confirmed in the *U. gambir* extract. Nanoencapsulation was opposed as a means of safeguarding this bioactive compound against degradation. *G-NPs*' stability could be maintained without causing aggregation or changes to their bioactive components. Absorption band of *G-NPs* did not change at 383–386 nm after exposure to different pH 3 to 11 (Figure 5). At pH 12, first absorption band experience bathochromic shift that appeared in wave length 410 nm. A hyperchromic effect was usually observed in the second absorption band when pH was



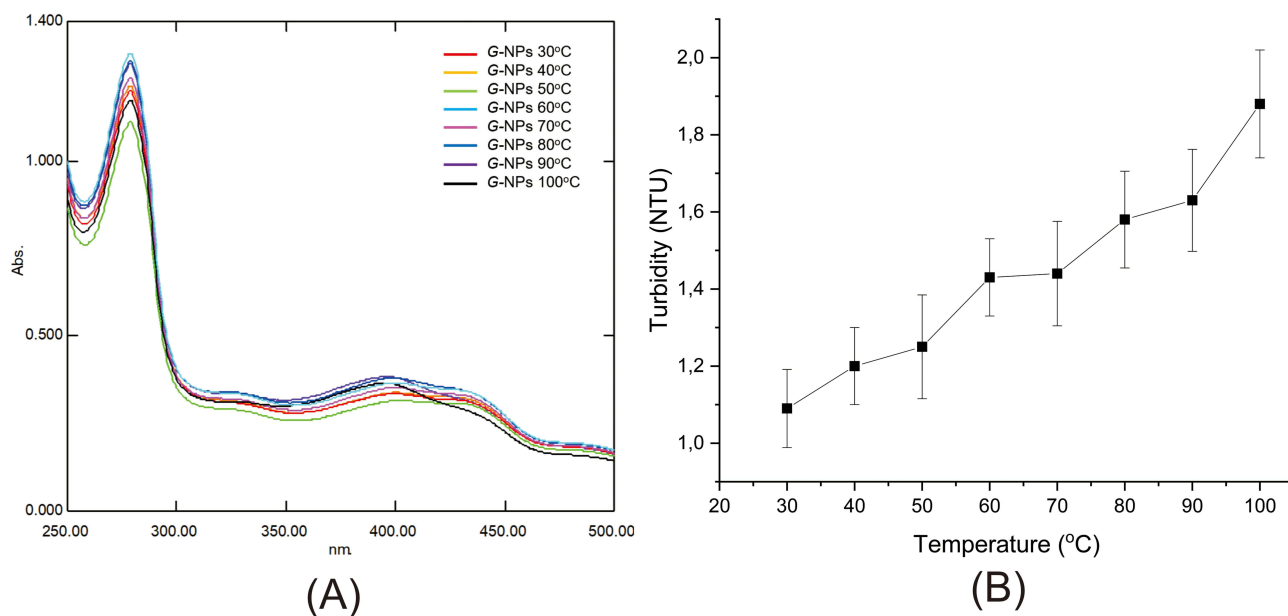
**Figure 4** TGA analysis of (A) Na-alginate, (B) *G-NPs*, and (C) *U. gambir* extract.



**Figure 5** Stability of *G-NPs* against pH. (A) UV-Vis spectra, and (B) Turbidity.

changed from 3 to 11. In the meantime, it caused a shift between hypochromic and hyperchromic at pH 12. *G-NPs* typically had a turbidity level higher in an acidic (pH 3–5) environment and lower in a neutral to a basic one (pH 6–12). *G-NPs* tended to be less stable and precipitated at acidic pH because of the stabilizing effect of Na-alginate. Below pH 5, the free  $\text{-COO}^-$  ions would form protonated  $\text{-COOH}$ . As a result, the electrostatic repulsion between the chains decreased enabling hydrogen bonds formation which further caused viscosity increment. However, depolymerization retardation occurred in an alkaline environment, resulting in a decrease of viscosity.<sup>39,40</sup>

*G-NPs*' turbidity level and UV-Vis absorption pattern were largely unaffected by temperature changes between 30°C and 100°C. The viscosity of Na-alginate will decrease as the temperature rises (Figure 6).<sup>41</sup> *G-NPs*' ionic stability indicated that the UV-Vis absorption band of *G-NPs* was unaffected by NaCl at concentrations between 0 and 0.3 M. However, *G-NP* turbidity was

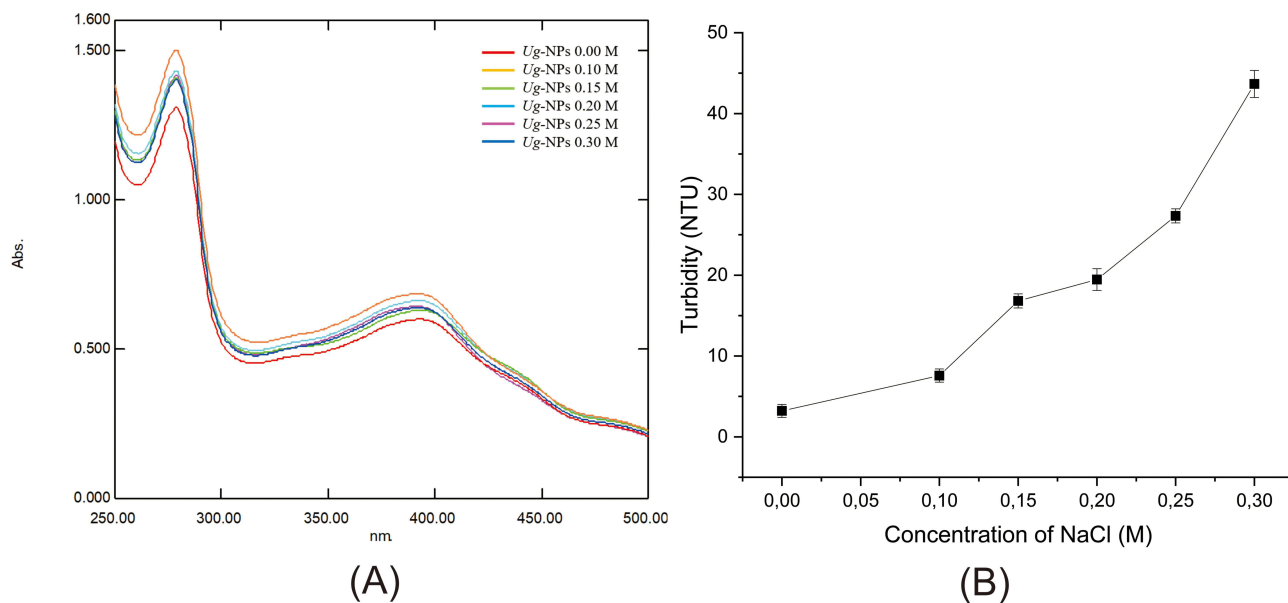


**Figure 6** Stability of G-NPs against temperature. (A) UV-Vis spectra, and (B) Turbidity.

affected by an increased NaCl concentration. This phenomenon is probably because Na-alginate is stable in NaCl solution. The monovalent salt NaCl could affect the precipitation rate of Na-alginate (Figure 7).<sup>42</sup>

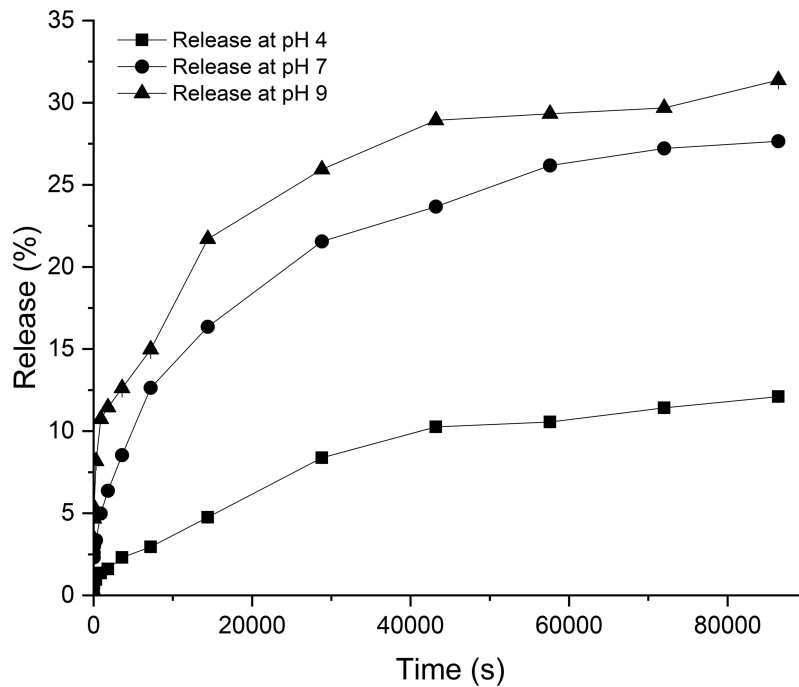
## Loading and Release of G-NPs

Both loading efficiency (LE) and loading amount (LA) analysis indicated successful adsorption of *U. gambir* bioactive compound in Na-alginate micelles with percentages  $97.56 \pm 0.04\%$  and  $32.52 \pm 0.01\%$ , respectively. Release of bioactive compound tended to slow at pH 4 since the electrostatic repulsion of Na-alginate decreased, which was further ended by G-NPs aggregation (Figure 8). Besides that, the formation of hydrogen bonds caused the shrinking of G-NPs pores. This is exemplified by the percentage release of  $12.10 \pm 0.03\%$  after 24 h exposure to acidic pH (Figure 8). As for pH 7 and 9, the release got to be faster, with percentages of  $27.65 \pm 0.03\%$  and  $31.37 \pm 0.00\%$  after 24 h, respectively. Repulsion between particles at neutral or alkaline pH caused G-NPs pores enlargement.



**Figure 7** Stability of G-NPs against NaCl concentration. (A) UV-Vis spectra, and (B) Turbidity.

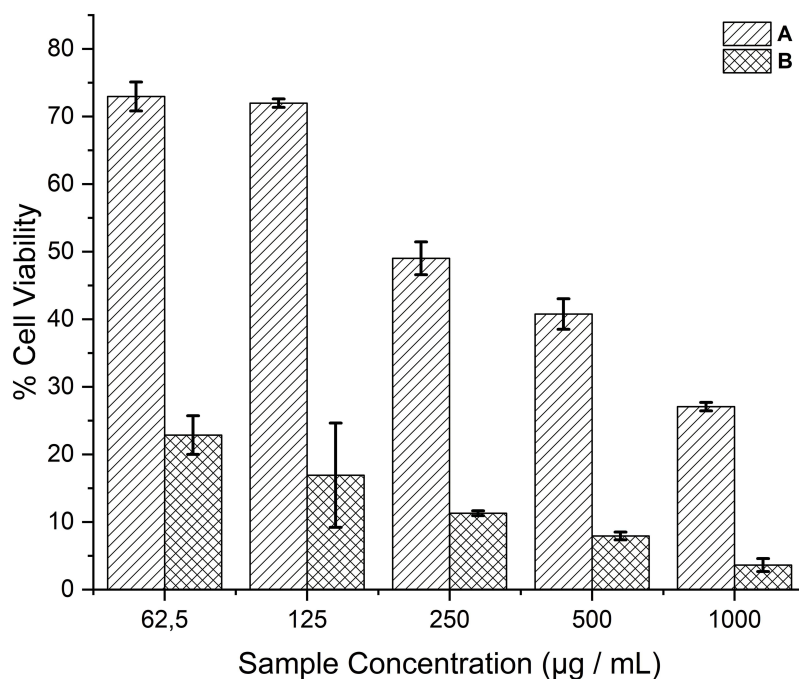




**Figure 8** Effect of pH on the release of bioactive components of *U. gambir* from *G-NPs*.

### Cytotoxic Activity of *G-NPs*

The results of an evaluation of the anti-cancer activity of *U. gambir* extract and *G-NPs* against T47D cell lines were presented as  $EC_{50} \pm SE$ . The cytotoxicity effect of both extract and *G-NPs* was followed in dose-dependent manner. However, a decrease in % cell viability of *G-NPs* was better than extract (Figure 9). *G-NPs* formulation had  $EC_{50} 10.39 \pm 3.50 \mu\text{g/mL}$ , which was better toxic than the  $EC_{50}$  value of extract  $297.15 \pm 15.41 \mu\text{g/mL}$ . This result demonstrated that nanoencapsulation increased *U. gambir* extract's growth inhibitory activity against the T47D cell line. Measurable

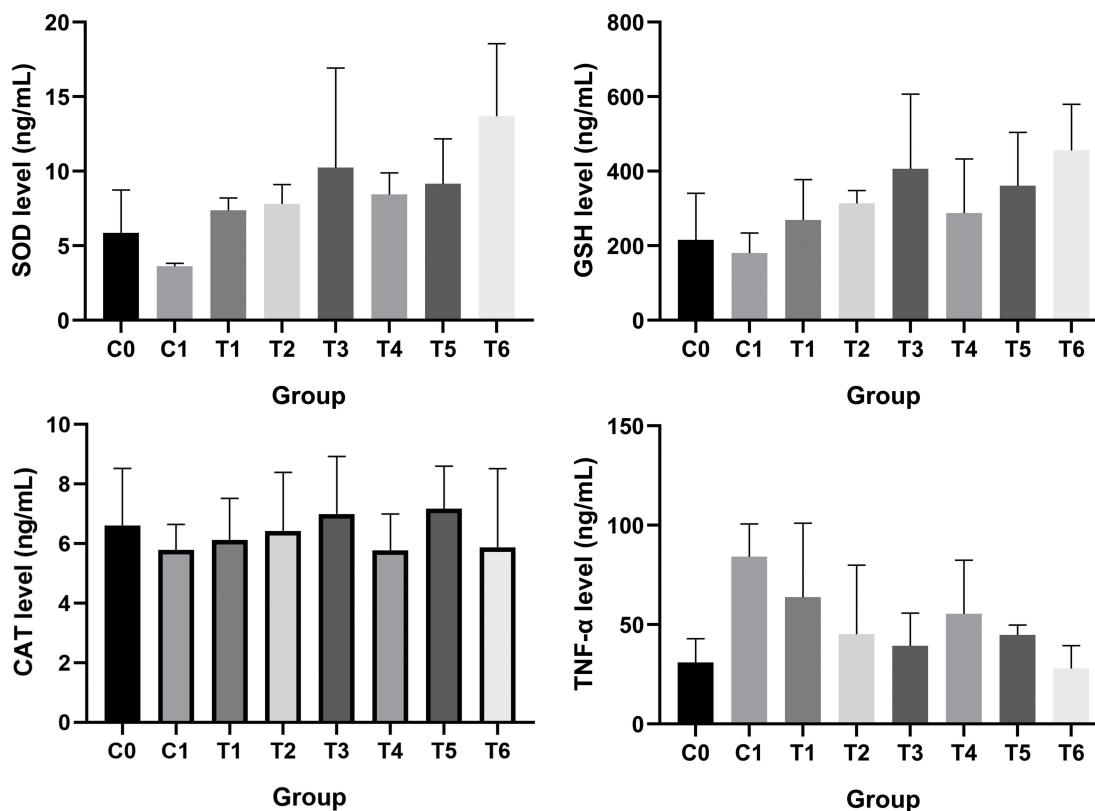


**Figure 9** Cell viability vs concentration of *U. gambir* extract and *G-NPs* towards T47D cell line. (A) *U. gambir* extract and (B) *G-NPs*.

examination affirmed that nanoencapsulation of *U. gambir* removed essentially expanded inhibitory development impact with p-value = 0.003.

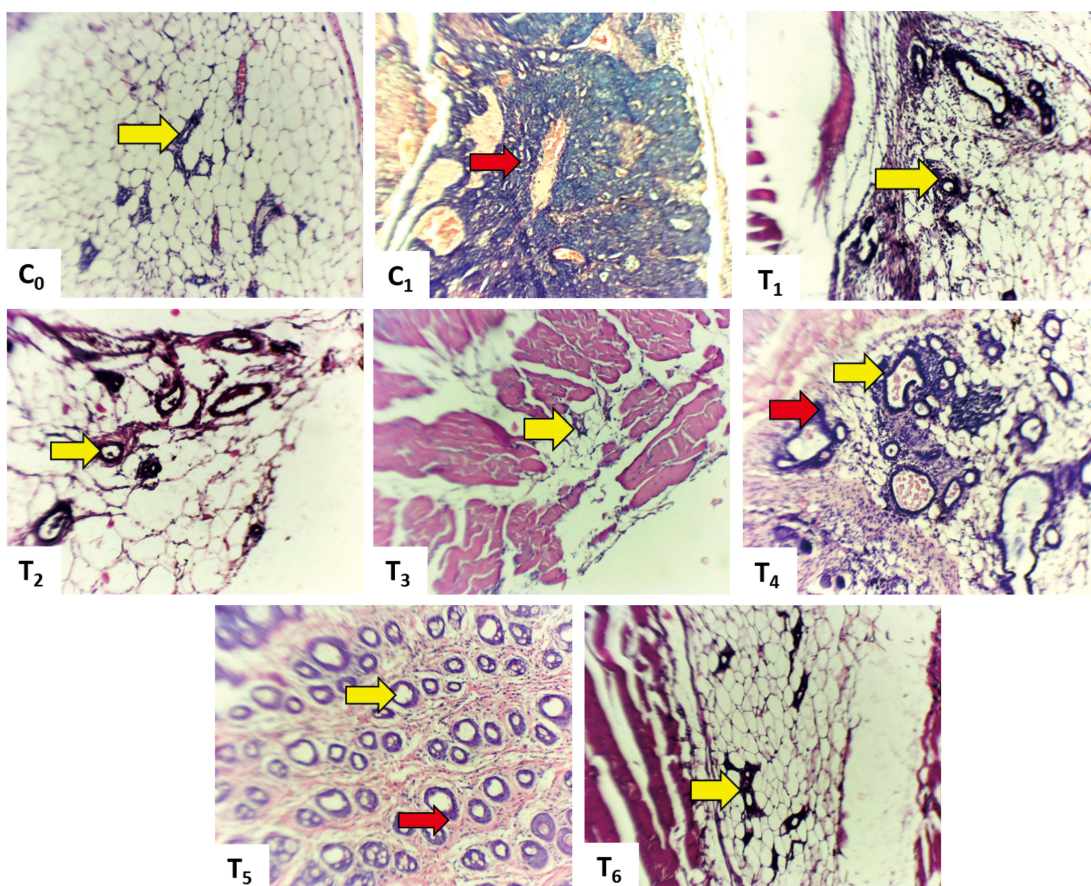
## Chemopreventive Potency

The chemopreventive potency of both *U. gambir* extract and *G-NPs* was assessed in the mice-induced breast cancer by benzo[ $\alpha$ ]pyrene. Benzo[ $\alpha$ ]pyrene was known to induce oxidative stress and affect the level of antioxidant enzyme (SOD and CAT), GST metabolizing enzyme, and inflammatory mediator TNF- $\alpha$ .<sup>43,44</sup> The enzyme superoxide dismutase (SOD) played an essential role in mitigating cancer progression by catalyzing the breakdown of toxic components, superoxide radicals, into harmless components consisting of oxygen and hydrogen peroxide.<sup>45</sup> Based on Figure 10, the lowest level of SOD was found in the cancer control group, 3.62 ng/mL, while the highest level of SOD was found in 200 mg/kg BW *G-NPs* treatment, 13.69 ng/mL. Catalase (CAT) enzyme was needed to detoxify hydrogen peroxide into water and oxygen. This enzyme was aberrantly regulated in cancer.<sup>46</sup> The highest level of CAT was owned by a group of 100 mg/kg BW *G-NPs* treatment at 7.18 ng/mL, as cancer control had the lowest CAT level, 5.79 ng/mL. Like other antioxidant enzymes, glutathione (GSH) was indispensable to scavenging excessive radicals and detoxifying xenobiotics, further preventing oxidative stress in cells.<sup>47</sup> Here, treating both *U. gambir* extract and *G-NPs* could maintain a high level of GSH but 200 mg/kg BW *G-NPs* showed the best one, 455.6 ng/mL. The cancer control cohort had the lowest level of GSH, 180.0 ng/mL. Tumor necrosis factor alpha (TNF- $\alpha$ ) was broadly known as a pro-inflammatory cytokine that was up-regulated in breast cancer. Exposure to a carcinogenic agent like benzo[ $\alpha$ ]pyrene could promote TNF- $\alpha$ .<sup>44</sup> It was proved in the result that the cancer control group had a high level of TNF- $\alpha$  84.24 ng/mL while treatment of *U. gambir* and *G-NPs* could alleviate modulation of TNF- $\alpha$ . Treatment of *G-NPs* maintained a high level of antioxidant enzyme and suppressed pro-inflammatory mediators better than extract. This indicated that nanoencapsulation could enhance the chemopreventive activity of *U. gambir*.



**Figure 10** Level of SOD, GSH, CAT, and TNF- $\alpha$  in each treatment group.

**Abbreviations:** C<sub>0</sub>, control; C<sub>1</sub>, cancer control; T<sub>1</sub>, *U. gambir* extract (50 mg/kg BW); T<sub>2</sub>, *U. gambir* extract (100 mg/kg BW); T<sub>3</sub>, *U. gambir* extract (200 mg/kg BW); T<sub>4</sub>, *G-NPs* (50 mg/kg BW); T<sub>5</sub>, *G-NPs* (100 mg/kg BW); T<sub>6</sub>, *G-NPs* (200 mg/kg BW).



**Figure 11** Breast histology imaging (100 × magnification).

**Abbreviations:** C<sub>0</sub>, control; C<sub>1</sub>, cancer control; T<sub>1</sub>, *U. gambir* extract (50 mg/kg BW); T<sub>2</sub>, *U. gambir* extract (100 mg/kg BW); T<sub>3</sub>, *U. gambir* extract (200 mg/kg BW); T<sub>4</sub>, G-NPs (50 mg/kg BW); T<sub>5</sub>, G-NPs (100 mg/kg BW); T<sub>6</sub>, G-NPs (200 mg/kg BW); yellow arrow (→), ductus mammary; red arrow (→), hyperplasia.

Histological analysis of breast tissue confirmed the carcinogenicity of benzo[ $\alpha$ ]pyrene exposure and the ability of both *U. gambir* extract and G-NPs to prevent alteration of breast cells caused by benzo[ $\alpha$ ]pyrene. According to Figure 11, there was no histopathological changes in C<sub>0</sub>, but the ductal mammary gland in C<sub>1</sub> experience hyperplasia (red arrow). Inflammation was indicated in all treatment groups except T<sub>6</sub>. Inflammatory cell infiltration was shown within adipocytes in T<sub>1</sub> and T<sub>3</sub>. The T<sub>3</sub> treatment group suffered an abscess where inflammatory cells infiltrated, mainly composed of neutrophils.<sup>48</sup> However, hyperplasia was not indicated in both cohort. Fibrosis presented in T<sub>2</sub> was indicated by increasing in dense connective tissue,<sup>49</sup> and cell debris and apocrine metaplasia also indicated in T<sub>2</sub>. Ductal hyperplasia and apocrine metaplasia were indicated in T<sub>4</sub> and T<sub>5</sub>. Meanwhile, T<sub>6</sub> was indicated normal with no histopathological changes.

## Discussion

*U. gambir* was one of the plants with many catechins. Recent research revealed that *U. gambir* ethyl acetate extract contained 89.34% catechins. It was known that catechin had anti-cancer properties. Antioxidants,<sup>50</sup> regulation of drug-metabolizing enzymes, induction of apoptosis,<sup>51</sup> inhibition of cell proliferation<sup>52</sup> and metastasis,<sup>53</sup> anti-inflammatory,<sup>54</sup> and regulation of the microbiota in the gut may all play a role in catechins' anti-cancer activity.<sup>55,56</sup> However, the preparation of natural ingredients such as catechin with no modification showed several drawbacks comprising low solubility and off-target. Such factors affected overall bioactivity.<sup>57</sup>

By increasing the bioavailability of the extract's bioactive compound, the *U. gambir* nanoencapsulation process may enhance its anti-cancer activity. By effectively participating in a paracellular pathway and entering systemic channel,<sup>46</sup> the small G-NPs could increase surface area. In addition, Na-alginate was widely regarded as a coating

material that was biodegradable, biocompatible, and non-mutagenic, making it suitable for drug delivery.<sup>58–60</sup> As indicated in this research, *G-NPs* could prevent the degradation of the bioactive component against pH, temperature, and salt concentrations. Additionally, the *G-NPs* could control release, increase dissolution rate and permeability, prolong plasma half-life, and improve the pharmacokinetic profile of the bioactive compound of *U. gambir* compared to extract alone.<sup>51</sup> Previously, alginate in curcumin–casein–alginate–chitosan nanocomplexes was also proven to improve pharmacokinetics (enhanced bioavailability and cancer therapeutic efficacy against Ehrlich carcinoma) in per-oral treatment.<sup>61</sup>

Regarding cytotoxicity towards the T47D cell line, *G-NPs* showed high toxicity with low EC<sub>50</sub> value compared to *U. gambir* extract. This result is supported by Syarifah et al, by whom the IC<sub>50</sub> of *U. gambir* extract with no modification against the T47D cell line was 1000 µg/mL.<sup>62</sup> Moreover, *G-NPs* also exhibited effective chemoprevention in a model of breast cancer mice-induced benzo[*a*]pyrene (B[a]P). Internalization of B[a]P would attract cytochrome P450 to detoxify. However, the metabolism of B[a]P resulted in the production of a lot of reactive oxygen species (ROS), a variety of unstable and reactive intermediates of B[a]P that have the potential to harm DNA and cause cell transformation and toxicity.<sup>63</sup> Treatment of *G-NPs* maintained a high level of antioxidant enzyme (SOD, CAT, and GSH) and suppressed pro-inflammatory (TNF- $\alpha$ ) mediators better than extract. This further reduced prolonged oxidative stress and inflammation, which are known to contribute to malignancy, including breast cancer.<sup>64,65</sup>

The chemopreventive ability of *G-NPs* is also proven in the histological analysis. No histological changes, such as hyperplasia, were reported in treating *G-NPs* containing 200 mg/kg BW of *U. gambir* extract. Conversely, treatment of T<sub>3</sub> (200 mg/kg BW of *U. gambir* extract only) causes an abscess. Another *G-NPs* group, T<sub>4</sub>, experienced ductal hyperplasia, while T<sub>1</sub> and T<sub>2</sub> also experienced inflammation disorders. This presumably extracts concentration was not enough to compromise the toxicity of B[a]P. Hyperplasia was defined as increasing cell proliferation.<sup>57</sup> These were a form of adaptation in responding to injury caused by B[a]P.

## Conclusion

*U. gambir* contained 89.34% brownish-white solid (+)-catechin. Nanoencapsulation of *U. gambir* extract using Na-alginate was successfully established and has spherical form with the size of 78.40 ± 12.25 nm. It was proven to prevent bioactive compound deterioration against pH, temperature, and salinity as well as control drug release. Moreover, nanoencapsulation of *U. gambir* method increased bioactive compound effectiveness against breast cancer at in vitro level as well as improve its chemopreventive effect at in vivo level compared to free extracts.

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## Disclosure

The authors report no conflicts of interest in this work.

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