

# Anticancer Activity of Nano Uncaria gambir

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## Article

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# Abstract

Cancer is a disease caused by uncontrolled cell growth. Cancer treatments, such as chemotherapy, have tended to cause adverse effects for patients. An alternative that can be described is the development of natural-based cancer therapies that have relatively low side effects. The development of Na-alginate as a matrix for nanoencapsulation of *Uncaria gambir* has shown growth inhibition activity against HeLa and T47D cell lines. The  $EC_{50} \pm SE$  of *U. gambir* extract on the HeLa cell line was  $1055.14 \pm 42.52 \mu\text{g/mL}$  while on the T47D cell line  $297.15 \pm 15.41 \mu\text{g/mL}$ . On the other hand, the *Ug*-NPs anticancer activity was substantially higher with  $EC_{50} \pm SE$  for HeLa cell line  $28.12 \pm 27.10 \mu\text{g/mL}$ , and T47D cell line  $10.39 \pm 3.50 \mu\text{g/mL}$ . Therefore, the nanoencapsulation of *U. gambir* extracts has increased solubility, selectivity, and activity.

## Introduction

Cancer is an uncontrolled and abnormal proliferation of various cells in the body. Moreover, there are 100 different cancers, which vary substantially in action and response to treatment<sup>1</sup>. The most common cancer diagnosed in both men and women is lung cancer (11.6% of the total cases). The leading cause of cancer death (18.4% of total cancer deaths). This is followed by breast (11.6%), colorectal (9.2%), gastric (8.2%), and liver cancers (8.2%)<sup>2</sup>. In Southeast Asia, Indonesia (136.2 / 100,000 population) ranks 8th in cancer prevalence, while in Asia, it is 23rd. Based on RISKESDAS data, the prevalence of tumors or cancer in Indonesia increased from 1.4 per 1000 population in 2013 to 1.79 per 1000 population in 2018<sup>3</sup>.

Treatment of cancer so far is based on radiotherapy and chemotherapy. The adverse effects of both are often devastating because, in killing cancer cells, these two methods can also harm normal cells<sup>4</sup>. The adverse chemotherapy effects include hair loss, bone marrow suppression, drug resistance, gastrointestinal lesions, neurological dysfunctions, and cardiac toxicity<sup>5</sup>. Besides, Herbal-based medicines are perceived to have fewer side effects than chemical drugs. It has triggered the development of herbal-based drugs increasingly becoming the focus for treatment regimens<sup>6</sup>. The cancer treatment with herbs can inhibit or reduce cancer cell proliferation and help control the adverse effect of conventional<sup>7,8</sup>.

*U. gambir* is a species belonging to the Rubiaceae family. This species is found in Sumatra as a leading commodity from the West Sumatra province<sup>8</sup>. Catechins are among the bioactive components of *U. gambir*<sup>9</sup>. Hence, Catechins have better T47D cell line inhibitory activity than MCF7 cell lines<sup>10</sup>. Anticancer activity of catechins is based on suppressing cell proliferation by inducing apoptosis, inhibiting angiogenesis, and metastasis of cancer cells<sup>11</sup>. Hydrated catechins exhibit anticancer effects by blocking MCF7 cell proliferation and inducing apoptosis by modulating the expression levels of caspase-3, -8, -9, and p53<sup>12</sup>.

The nanotechnology approach was applied for cancer chemotherapy to reduce toxicity, and increase stability, bioavailability, and selectivity<sup>13</sup>. Nanoencapsulation is one of the nanotechnological applications aimed to increase the stability and bioavailability of bioactive compounds because nano-size can increase the surface area of an active ingredient<sup>14,15</sup>. Nanoscale drug delivery systems are formulated from biocompatible and biodegradable polymers. They are being used to develop the administration and targeting strategy for cancer drugs<sup>16</sup>. Nanoencapsulation of *U. gambir* using sodium alginate to increase the plant's anticancer activity.

## Material And Methods

### Material

Sodium alginate food grade (Na-Alginate), distilled water, methanol, ethyl acetate, *n*-hexane, phosphate buffer, ethanol absolute (Merck), phosphate buffer saline (PBS); HCl (Sigma-Aldrich); NaCl (Sigma-Aldrich); NaOH (Sigma-Aldrich); sodium dodecyl sulfate (SDS), and 3-(4,5'-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT). The *U. gambir* sap was obtained from traditional markets in Surabaya, Indonesia.

### Extraction of *U. gambir*

The dried *U. gambir* sap (500 g) was extracted by the maceration method using methanol as a solvent with a ratio of 1: 2 at room temperature for 24 hours. The methanol extract was then concentrated using a rotary vacuum evaporator. This extract was then partitioned with *n*-hexane and ethyl acetate. Ethyl acetate extract was concentrated using a rotary vacuum evaporator.

### Determination of Catechin from *U. gambir*

Methanol, ethyl acetate, and hexane extracts of *U. gambir* were analyzed by UV-Vis spectrometer for catechin content. The extract with the highest catechin content was selected for nanoencapsulation.

#### Synthesis of Ug-NPs<sup>17</sup>

The *U. gambir* ethyl acetate extract was added with Na-alginate solution in a ratio of 1 : 2. The solution mixture was homogenized using ultrasonic (JY-9211DN, Ningbo Scientz Biotechnology, China) to obtain Ug-NPs. The Ug-NPs functional group was characterized by FTIR (Shimadzu IRTracer-100). The physicochemical properties of Ug-NPs, including polydispersity index (PDI), particle size, zeta potential ( $\zeta$ ) (Dynamic Light Scattering, Zetasizer Nano ZS, Malvern). Atomic Force Microscope (AFM, Agilent Technologies 5500) was determined for sample morphology, and TGA (Perkin Elmer TGA 4000) was used to observe the decomposition changes of Ug-NPs.

#### The Analysis of Ug-NPs Stability<sup>17</sup>

The stability test was aimed to determine the *Ug*-NPs stability, which is influenced by pH, temperature, and NaCl concentration. The observation was made by changing UV-Vis spectra and the level of turbidity of *Ug*-NPs.

#### Loading and Release of *Ug*-NPs <sup>17,18</sup>

The Calculations of Loading Efficiency (LE) and Loading Amount (LA) were based on equations (1) and (2). The loading value interpreted how much of the bioactive components of *U. gambir* extract were trapped in the *Ug*-NPs.

$$\%LE = \frac{\text{Mass of } U. \text{ gambir extract on } Ug - NPs}{\text{Mass of } U. \text{ gambir extract in feed}} \times 100\% \quad \dots(1)$$

$$\%LA = \frac{\text{Mass of } U. \text{ gambir extract on } Ug - NPs}{\text{Mass of } Ug - NPs} \times 100\% \quad \dots(2)$$

The release of material was determined as the amount of catechins from *U. gambir* extract that could be released at pH 4, 7, and 9.

## Cytotoxicity Test

HeLa and T47D cell lines that have been grown on RPMI 1640 media and have 80% confluence conditions are distributed on 96-well plates with several  $10 \times 10^4$  cells / well. Cells were incubated for 24 hours in a CO<sub>2</sub> incubator, then 100 µL of culture media containing samples (*U. gambir* extract and *Ug*-NPs) were added and incubated again for 24 hours. At the end of incubation, the culture medium in the plate was discarded and then washed with PBS. Each well was added with 100 µL of MTT (5 mg/mL) reagent, incubated again for 4 hours at 37°C in 5% CO<sub>2</sub>. After 4 hours, 100 µL of 10% SDS solution was added in 0.01 N HCl. The absorbance was measured using an ELISA reader (Bio-Rad) at λ 550 nm. The EC<sub>50</sub> values were obtained by using a dose-response calculation.

## Statistical Analysis

One-way ANOVA analysis was used to determine the anticancer effect of *U. gambir* extract with *Ug*-NPs on HeLa and T47D cell lines. Post-hoc analysis was carried out by Tukey's test. If the p-value was < 0.05, then statistically *U. gambir* nanoencapsulation process significantly increased the anticancer activity.

## Results

### Catechins of *U. gambir*

*U. gambir* sap was extracted with methanol and partitioned with n-hexane and ethyl acetate. Catechin levels in the three *U. gambir* extracts are shown in Fig. 1. *U. gambir* ethyl acetate extract has the highest

catechin content, 89.34% (w/w), then processed for nanoencapsulation with Na-alginate. Catechins are bioactive compounds that have been reported for anticancer activity.

## Ug-NPs Characterization

*Ug*-NPs is a nano-capsule of *U. gambir* extract with Na-alginate. Nano-capsule characterization based on physicochemical properties is shown in Table 1. It was injured that *Ug*-NPs have a particle size of  $452.63 \pm 5.29$  nm based on the particle size. The polydispersity index (PDI) measures size heterogeneity that indicates size distribution or agglomeration/aggregation in sample<sup>19</sup>. PDI *Ug*-NPs value  $0.55 \pm 0.01$  indicates that the particle size distribution tends to be homogeneous. The particle size distribution is considered broad if the  $PDI > 0.7$ <sup>20</sup>.

Table 1  
Physicochemical of *Ug*-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>Ug</i> -NPs                                             | $452.63 \pm 5.29$   | $0.55 \pm 0.01$ | $-40.87 \pm 0.90$   |
| <b>Notes:</b> Each data presented as mean $\pm$ SD (n = 3) |                     |                 |                     |

The smaller the polydispersity index number, the more uniform the particle size because if the size difference between particles is bigger, this will affect the particle characteristics<sup>21</sup>. Zeta potential ( $\zeta$ ) in Table 1 shows that Na-alginate, *U. gambir* extract, and *Ug*-NPs are negative. This may be due to the large number of hydroxy groups that are electronegative. The Zeta potential value for *Ug*-NPs is between Na-alginate and *U. gambir* extract. This is presumably because Na-alginate's electropositive group partially covers the electronegative group in the constituent *U. gambir* extract. Nanoencapsulation with a zeta potential value of more than  $\pm 30$  mV is stable in suspension as surface charge prevents aggregation<sup>22</sup>. A large Zeta potential indicates better stability due to the larger electrostatic repulsion between particles. The *Ug*-NPs tend to be stable with a  $-40.87 \pm 0.90$  Mv value. Morphological analysis by AFM showed that the *Ug*-NPs were spherical (Fig. 2).

FTIR spectra of *Ug*-NPs showed a blend of Na-alginate and *U. gambir* extract peaks. Absorptions at  $1519\text{ cm}^{-1}$  (C-C in ring) and  $1033\text{ cm}^{-1}$  (C-O-C stretching vibration) were from *U. gambir* extract. Meanwhile, absorption at  $1419\text{ cm}^{-1}$  (vibrations of carboxylate salt ion) and  $1122\text{ cm}^{-1}$  (deformation -OH in -COOH) were from Na-alginate (Fig. 3).

The TGA analysis of *Ug*-NPs was carried out to determine the composition and the interaction between the active compounds in the *U. gambir* extract and the sodium alginate coating material (Fig. 4). The *Ug*-NPs decomposed at a temperature of  $70^{\circ}\text{C}$ , which showed a decrease in sample weight of  $\pm 10.5\%$ . The

*U. gambir* extract also decomposed at a temperature of around 70° C with a decrease in sample weight of  $\pm 12.1\%$ . At a temperature of 178° C, *Ug*-NPs and extracts of *U. gambir* decomposed with a decrease in sample weight of  $\pm 16.2\%$  and  $\pm 33.1\%$ , respectively. The Na-alginate decomposed at a temperature of 290° C with a decrease in sample weight of  $\pm 35.3\%$ . At the same temperature, *Ug*-NPs also decomposed with a decrease in sample weight of  $\pm 25.2\%$ . Based on FTIR and TGA analysis, it is suspected that only physical interactions exist between the constituent of *U. gambir* extract and Na-alginate in the *Ug*-NPs, and apparently, no chemical interaction exists between the two.

## Stability of *Ug*-NPs

The stability of *Ug*-NPs must be maintained so that there is no aggregation and changes in the bioactive components of *Ug*-NPs. The UV-Vis spectra on the *Ug*-NPs showed two absorption bands indicating bioactive catechins in *U. gambir* extract. Change in pH from 3 to 11 tended had to affect no the absorption bands of the *Ug*-NPs, which ranged from 383 to 386 nm. At pH 12, there was a bathochromic shift in the first absorption band to 410 nm. In general, in the second absorption band, changes in pH from 3 to 11 result in a hyperchromic effect. Meanwhile, at pH 12, it causes a hypsochromic and hyperchromic shift. The turbidity level of *Ug*-NPs tends to be higher at acidic pH 3–5 and lower at pH 6–12. It is influenced by the stability of Na-alginate, which tends to be less stable at acidic pH. Consequently, the Na-alginate precipitates. Below pH 5, the free  $-\text{COO}^-$  ions will form protonated  $-\text{COOH}$ . As a result, the electrostatic repulsion between the chains decreases, and they can approach and form hydrogen bonds. This increases in viscosity. Meanwhile, if the pH is more alkaline, the depolymerization is slow. Consequently, the viscosity will decrease<sup>23,24</sup> (Supplementary Fig. 1).

The temperature changes between 30° C – 100° C generally did not affect the UV-Vis absorption pattern and did not significantly affect the turbidity level of *Ug*-NPs. Increasing the temperature will decrease the viscosity of Na-alginate (Supplementary Fig. 2)<sup>24</sup>. Ionic stability of *Ug*-NPs showed that NaCl concentration did not significantly affect the UV-Vis absorption band of *Ug*-NPs, both at 0–0.3 M concentrations. However, increasing NaCl concentration affected the turbidity of *Ug*-NPs. The higher concentration of NaCl is associated with the higher turbidity levels of *Ug*-NPs. It may be due to the stability of Na-alginate in NaCl solution. Monovalent salt (NaCl) can affect the rate of hydration of Na-alginate, which results in precipitation (Supplementary Fig. 3)<sup>25</sup>.

## Loading and Release of *Ug*-NPs

Loading efficiency (LE) showed that  $97.56 \pm 0.04\%$  of the bioactive components of *U. gambir* extract are adsorbed in Na-alginate micelles. Meanwhile,  $32.52 \pm 0.01\%$  is the loading amount (LA) of the bioactive components of *U. gambir* extract in *Ug*-NPs. The release of bioactive components of *U. gambir* extract on the *Ug*-NPs showed that at pH 4 the release tends to be slow. It showed that the *Ug*-NPs stability at pH 4, is less stable due to a decrease in the electrostatic repulsion of Na-alginate. Thus, it will result in the *Ug*-NPs aggregation. Besides, the formation of hydrogen bonds results in the shrinking of the pores of the *Ug*-NPs. So, the release tends to be slower i.e.  $12.10 \pm 0.03\%$  after 24 hours. For pH 7 and 9, the release

tends to be faster i.e.  $27.65 \pm 0.03\%$  and  $31.37 \pm 0.00\%$  after 24 hours, respectively. This is because the repulsion between particles at neutral or alkaline pH causes the pores of the *Ug*-NPs to enlarge, which results in a faster release of bioactive components (Fig. 5).

## Cytotoxic Activity of *Ug*-NPs

The anticancer activity of *U. gambir* extract, and *Ug*-NPs was evaluated against HeLa and T47D cell lines and are presented as  $EC_{50} \pm SE$ . In the HeLa cell lines, *U. gambir* extract was less active in inhibiting the cell growth with  $EC_{50}$   $1055.14 \pm 42.52$   $\mu\text{g/mL}$ . Meanwhile, the *Ug*-NPs significant  $EC_{50}$   $28.12 \pm 27.10$   $\mu\text{g/mL}$  (Fig. 6A). It was supported by statistical analysis, which showed a significant  $p = 0.000$ . It indicated a substantial increase in the activity of *U. gambir* extracts after nanoencapsulation.

On the T47D cell line, the *U. gambir* extract showed  $EC_{50}$   $297.15 \pm 15.41$   $\mu\text{g/mL}$ , while *Ug*-NPs was  $EC_{50}$   $10.39 \pm 3.50$   $\mu\text{g/mL}$  (Fig. 6B). It indicates that the *U. gambir* extract nanoencapsulation has increased the growth inhibitory activity of the T47D cell line. The statistical test showed that *U. gambir* extract nanoencapsulation significantly increased the growth inhibitory activity with a  $p\text{-value} = 0.003$ .

## Discussion

*U. gambir* is among the plants with high catechin content. The catechin content of *U. gambir* ethyl acetate extract was 89.34%. One of the bioactivities of catechins is as an anticancer. The mechanism of anticancer activity of catechins can be through diverse mechanisms such as antioxidants<sup>26</sup>, regulation of drug-metabolizing enzymes, induction of apoptosis<sup>27</sup>, inhibition of cell proliferation<sup>28</sup>, anti-inflammatory<sup>29</sup>, inhibiting cancer metastasis<sup>30</sup>, and regulation of gut microbiota<sup>31,32</sup>. Even though they have high bioactivity, natural ingredients tend to have low solubility and are non-specifically. Such factors affect their overall bioactivity<sup>33</sup>.

*U. gambir* nanoencapsulation process can enhance its anticancer bioactivity. Nanoencapsulation can increase the bioavailability of active plant compounds. The absorption surface area is large in smaller particle sizes and can effectively participate in a paracellular pathway and enter the systemic channel<sup>34</sup>. This process can maximize the bioactivity of *U. gambir* extract. The advantages of Na-alginate as a coating material for *U. gambir* nano-capsules are biodegradable, non-toxic, biocompatible, and non-mutagenic<sup>35</sup>. Na-alginate also plays an essential role in drug delivery of chemotherapy drug components, so it is appropriate for use in the nanoencapsulation of *U. gambir* as chemotherapy<sup>36,37</sup>.

Nanoencapsulation of *U. gambir* extract using Na-alginate was proven to prevent damage or degradation of bioactive components. It was inferred from the UV-Vis spectra of the *Ug*-NPs, which tend to be stable at various pH, temperature, and salt concentrations. Additionally, the *Ug*-NPs can control the release of bioactive components, increase the dissolution rate and permeability of bioactive components of *U. gambir*, prolong plasma half-life, and improve pharmacokinetic profile<sup>38</sup>.

*Ug*-NPs proved to be successful in increasing the anticancer activity of *U. gambir*. The growth inhibitory activity against HeLa cells increased by 97.33%, while in the case of T47D cells, it is 96.50%. The increase in the anticancer activity of the *Ug*-NPs against the HeLa and T47D cell lines was due to the ability of the *Ug*-NPs to deliver bioactive components of *U. gambir* across the plasma membrane. The plasma membrane is an effective barrier in protecting all living cells from the surrounding environment. Additionally, it firmly controls the entry and exit of macromolecular substances. Therefore, a nano-capsule system is needed to overcome this barrier to enter into living cells <sup>39</sup>.

When reaching the cell exterior membrane, *Ug*-NPs can interact with the plasma membrane or the extracellular matrix components and enter the cell, mainly through the process of endocytosis <sup>40</sup>. Cellular uptake of the *Ug*-NPs is measuring more than 200 nm. It is through one of the endocytosis pathways, called macro-pinocytosis <sup>41</sup>. In the process of macro-pinocytosis, all particles and molecules dissolved in the extracellular fluid are captured by a membrane extension or ruffles. Then, it forms macro-pinosomes with a diameter of 0.2-5  $\mu\text{m}$  [40]. After internalization of *Ug*-NPs into cells, macro-pinosomes fuse with lysosomes <sup>42</sup> and the Na-alginate matrix of the *Ug*-NPs is degraded by lysozyme, resulting in a release of bioactive components *U. gambir* extract into cells <sup>43</sup>.

The charge on the surface determines the cellular internalization of the nano-capsule. Moreover, the positive charged nano-capsules are rapidly internalized into cells via the clathrin-dependent endocytosis pathway <sup>41</sup>. The *Ug*-NPs with a positive charge can form clathrin vesicles. The vesicles separate from the plasma membrane and enter into the cytoplasm. Then, they diffuse into the endosome/lysosomal compartment. The environmental acidity increases the breakdown of bonds, and the chemical structures of the *Ug*-NPs by enzymes, causing rapid release <sup>39</sup>. Additionally, positive charge nano-capsules induce membrane depolarization in several different cell types. Membrane depolarization produces an increased concentration of  $\text{Ca}^{2+}$  in the cell that can induce modulation of the intracellular pathway <sup>40</sup>.

## Conclusion

*U. gambir* contains catechin compounds that are known for anticancer activity. Nanoencapsulation of *U. gambir* extract can increase its anticancer activity. The *Ug*-NPs formulation has exhibited a better growth inhibitory activity than *U. gambir* extract against HeLa and T47D cell lines. The nanoencapsulation process of *U. gambir* extract with Na-alginate can also increase the drug delivery in cells, increasing their bioactivity.

## Declarations

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## Availability of data and materials

Not applicable

## Author's contributions

APW, NSA, HIZ, MZF and SHL synthesized Ug-NPs. ALF, and AY tested the anticancer activity. MIC helped in the characterizations of Ug-NPs. The NSA wrote the manuscript, assisted by APW, SHL, and HIZ. ALF, AY, MZF, and MIC kept records and revise manuscripts. The NSA finalized the manuscript. All authors have approved the final manuscript.

## Consent of publication

Not applicable

## Competing interests

In this study, the author has no competing interest.

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Figures

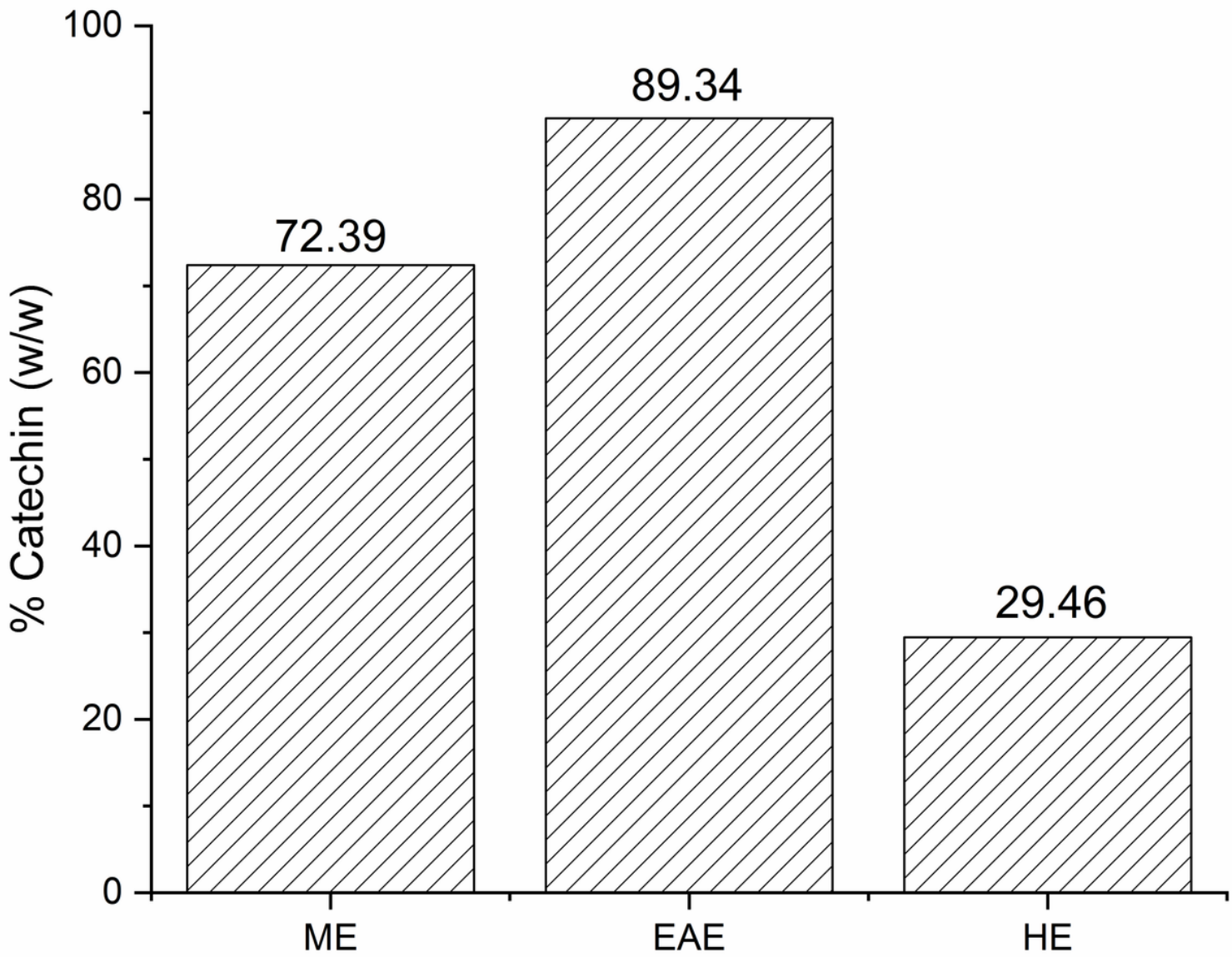


Figure 1

Catechin content of *U. gambir* extract

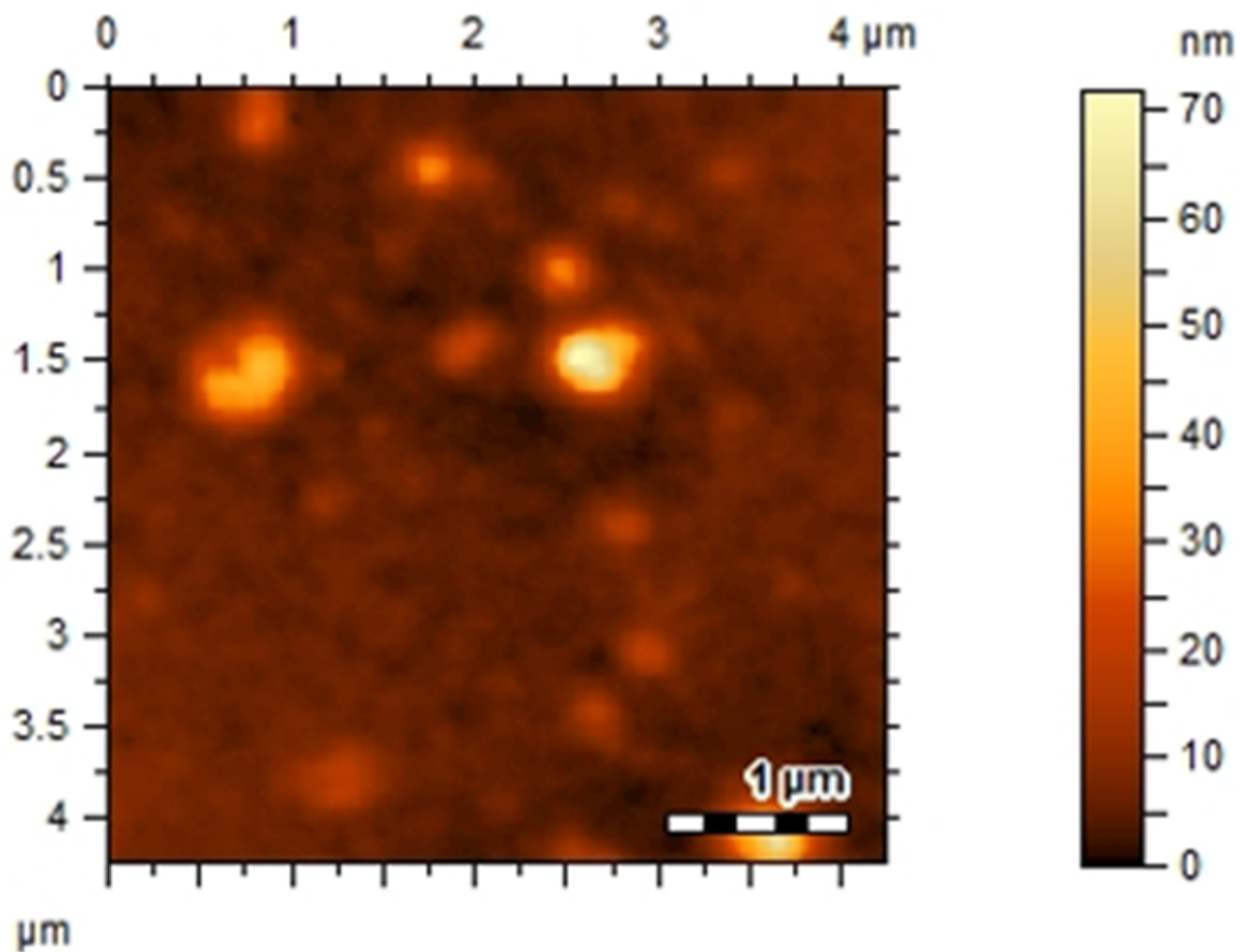


Figure 2

AFM 2D topography images of *Ug*-NPs

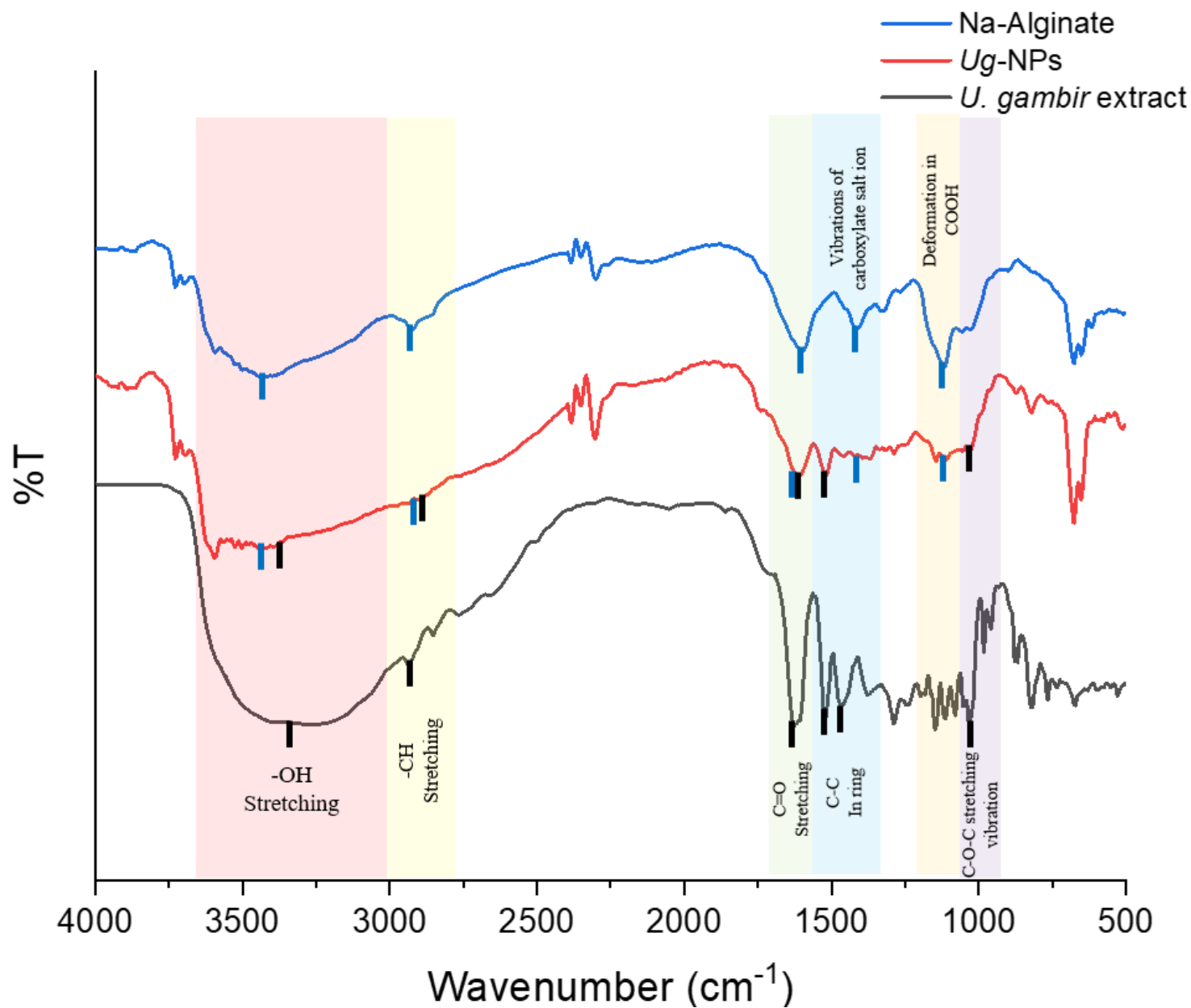


**Figure 3**

FTIR spectra of Na-alginate, *Ug*-NPs, and *U. gambir* extract

**Figure 4**

Thermogram of Na-alginate, *Ug*-NPs, and *U. gambir* extract

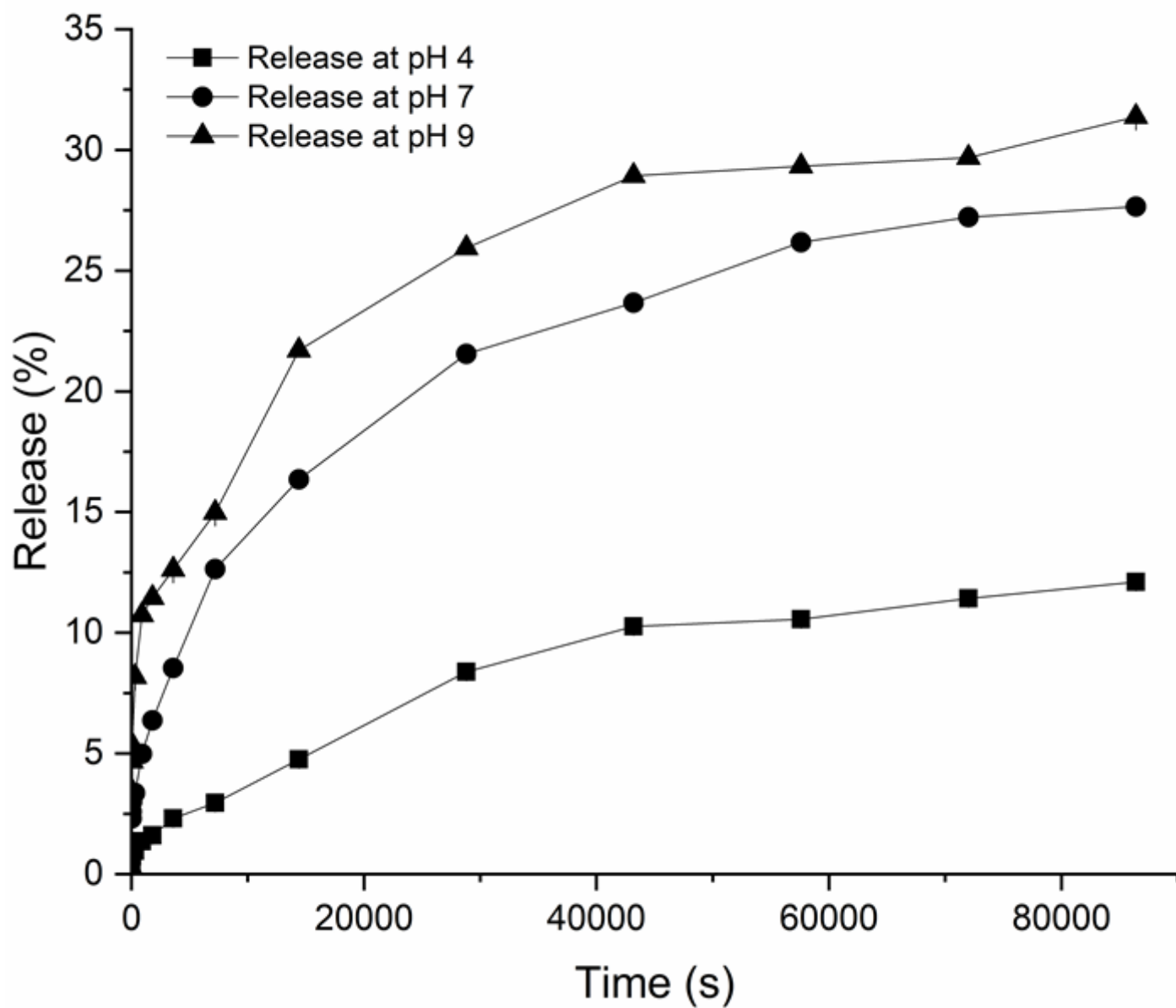


Figure 5

Effect of pH on the release of bioactive components of *U. gambir* from *Ug*-NPs

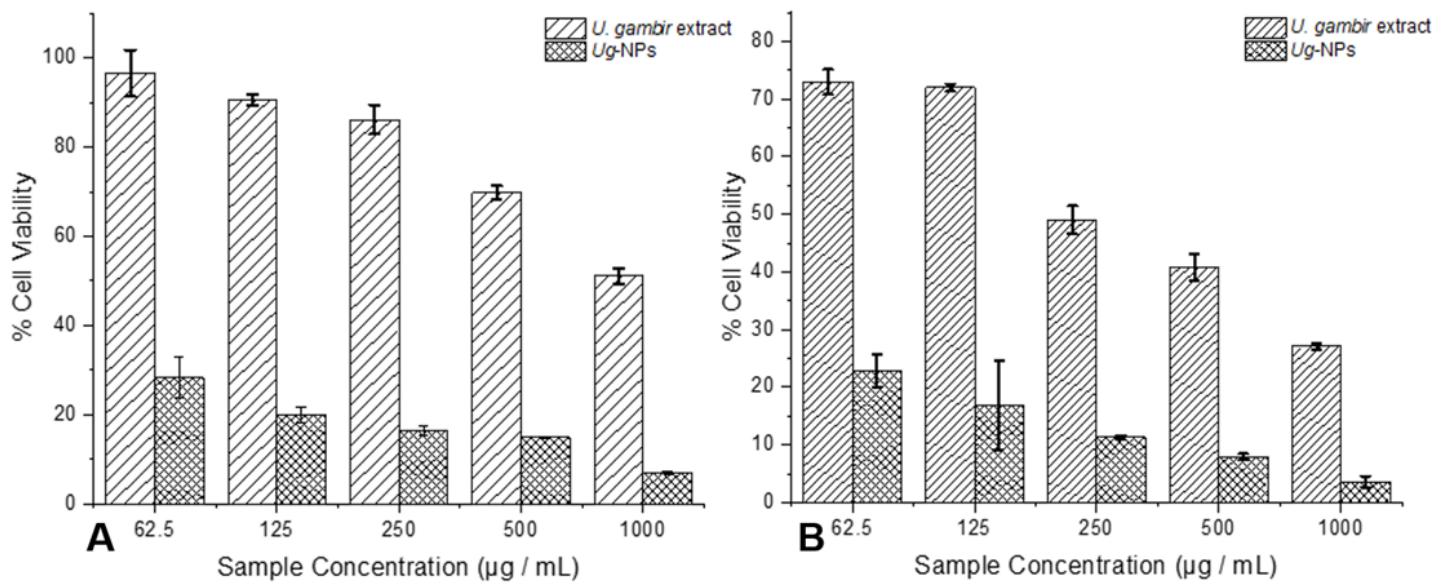


**Figure 6**

Cell viability Vs concentration of *U. gambir* extract and Ug-NPs. **A)** HeLa cell line, and **B)** T47D cell line

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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