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RESEARCH ARTICLE

Formulation of a gambier catechin-loaded nanophytosome and the MTT assay on *HeLa cell lines*

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Abstract

Background: Catechins are good free radical scavengers but exhibit low stability and permeability. Nanophytosomes are currently being developed as the delivery system for phytoconstituents to protect them from decomposition by oxidants or enzymes and increase their permeability. **Objectives:** To formulate gambier catechin-loaded nanophytosomes and perform the MTT assay on HeLa cell lines. **Method:** Five formulations were prepared using soya lecithin at various molar ratios of cholesterol by thin-layer hydration and sonication. The nanophytosomes were characterised by the determination of vesicle size, zeta potential, polydispersity index, morphology with Transmission Electron Microscope, Fourier Transform Infrared Spectroscopy (FT-IR) analysis, freeze and thaw test, entrapment efficiency, and the in vitro cytotoxicity test. **Result:** The optimal formula (F4) with a molar ratio of 1:1:0.8 (catechin:lecithin:cholesterol) resulted in spherical vesicles with an average size (106 ± 0.218) nm, zeta potential -68 mV, polydispersity index 0.412, 93.5% entrapment efficiency and that were stable to temperature changes. FT-IR showed the formation of catechins and lecithin complexes. The activity of catechin-loaded nanophytosomes against HeLa cells showed an IC_{50} of 36.307 g/ml. There was a significant difference in the average percentage of cells undergoing apoptosis in all treatment groups ($p < 0.05$). **Conclusion:** Catechin-loaded nanophytosomes with a molar ratio of 1:1:0.8 (catechin:lecithin:cholesterol) showed moderate cytotoxic activity against HeLa cell lines.

Introduction

Gambier, the dried extract of the leaves of *Uncaria gambir* (Hunter) Roxb, is the prime commodity of West Sumatra. The province has supplied 80% of the total gambier produced in Indonesia and exported to other countries through India and Singapore (Hosen, 2017).

Gambier contains catechin as the main compound which gives its specific taste and odour (Marlinda, 2018). As a polyphenolic compound, catechin shows antioxidant and antibacterial activities. Therefore it has the potential to be formulated as a cancer chemopreventive agent (Cheng *et al*., 2020), anti bactericidal agent (Nakayama *et al.*, 2013), and a drug for diseases associated with free radical formation such as premature ageing, Alzheimer's disease, cardiovascular diseases, and cancer (Kassim *et al.*, 2011). The antiproliferative activity of tea catechins and their metabolites on human cervical cells (SiHa and HeLa cell lines) have been reported (Hera-Terawaki, 2017). The study concluded that oral intake of tea catechins showed chemopreventive effects against cervical intraepithelial neoplasia in humans. Most studies were on the cytotoxic activity of catechin derivatives, such as Epigallocatechin-gallate from green

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tea and other fruits, but the cytotoxicity of pure catechin on HeLa cell lines has not yet been reported.

The Sumatra Biota Laboratory of Andalas University has been able to purify catechin from gambier extract with a purity of 90% or more. This article reports a study on nanophytosome formulation as a delivery system to improve the stability and permeability of catechin. Nanophytosomes are molecular complexes formed between a phospholipid and phytoconstituent, strengthened by cholesterol and thus compatible with lipophilic media (Babazadeh *et al.,* 2017). The independent variable in nanophytosome formation is the proportion of the herbal fraction: phospholipid (1:1) at various cholesterol molar ratios, while the dependent variable is the globule size obtained. This nano-sized vesicle can protect the phytoconstituents and carry them to pass through biological membranes easily (Purnamasari *et al.*, 2020).

Methods

Materials

The following materials were used: pure (+)-catechin (99.99%) obtained from PT Andalas Sitawa Phytolab (Universitas Andalas, Indonesia), soya lecithin (food grade, Shankar soya products, Indonesia), cholesterol (Croda, Indonesia), dichloromethane (Bratachem, Indonesia), and methanol (Bratachem, Indonesia). All the solvents used were of analytical grade. HeLa cell lines and MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were provided by the Laboratory of Biomedical Sciences, Faculty of Medicine, Andalas University.

Design

Catechin-loaded nanophytosomes were made in five formulations (Table I) using thin-layer hydration and sonication methods (Purnamasari *et al.*, 2020). Catechin and lecithin were each dissolved in methanol, cholesterol was dissolved in dichloromethane. The solutions were then mixed and stirred on a magnetic stirrer (IKA C-MAG HS7) at 45°C with a rotation speed of 3,000 rpm for 30 minutes. Solvents were evaporated using a rotary evaporator (Buchi, Rotavapor R-100, Switzerland) at 45°C. The thin film formed was hydrated with 10 ml of water and then sonicated (Elmasonic S 80) for 60 minutes.

Assessments

Vesicle size analysis

Particle size and particle size distribution, polydispersity index (PDI), and zeta potential were measured by a particle size analyser (Horiba SZ 100). Samples were diluted using distilled water and placed in an electrophoretic cell.

Vesicle morphology

The morphology of catechin-loaded nanophytosomes was analysed by Transmission Electron Microscopy (TEM, JEOL JEM 1400) at 30,000x magnification. The sample was placed on a TEM copper grid coated with a carbon film.

Fourier-transform infrared spectroscopy (FT-IR)

FT-IR spectra of catechin, lecithin, cholesterol, and the nanophytosome were obtained on a Perkin Elmer Spectrum 1000 FT-IR Spectrometer. Scans were done at the 4000-600 cm^{-1} wavenumber range.

Vesicle stability

The physical stability of the nanophytosomes was observed by the freeze-and-thaw cycling test. The formulations were kept in storage at temperature -5°C for 24 hours and then at 25°C for another 24 hours. The test was repeated for three cycles.

Entrapment efficiency (EE)

The nanophytosomes were centrifuged for 45 minutes at a speed of 15,000 rpm at 4°C. The concentration of free catechin in the supernatant was measured using UV-Visible spectrophotometry (Shimadzu UV-1700) at 278 nm. EE was determined according to the equation:

$$\mathrm{EE}\ \% = \frac{\mathrm{W_{(added\ drug)}} - \mathrm{W_{(free\ drug)}}}{\mathrm{W_{(added\ drug)}}} \times 100\%$$

Where, $W_{(added\ drug)}$ is the amount of catechin added to the formulation, $W_{(free\ drug)}$ is the amount of free catechin in the supernatant.

Evaluation of cytotoxic activity

HeLa cell lines were subcultured and maintained at 37°C at 5% CO_2 until 80% confluent. The cells were then treated with 100 µl catechin-loaded nanophytosomes F4 at various concentrations (400, 200, 100, 50, 25, and 12.5 µg/ml, respectively) in quadruplicate and incubated for 24 h. The untreated cells were used as a negative control, and cells incubated only with dimethyl sulfoxide (DMSO) (0.5%, v/v) as vehicle control. After the treatment, 20 µL of MTT solution (0.5 mg/ml) was added to each well and the plates were incubated at 37°C for 2-4 h. DMSO was added once the formazan formed and the absorbance was measured at 595 nm using an ELISA microplate reader (xMark Microplate Reader) (Hara-Terawaki *et al.*, 2017).

Cytotoxicity was expressed as IC_{25}, IC_{50}, and IC_{75} values which were determined from the semilogarithmic plot of concentration and percentage of cell viability.

Cell cultures that had been treated with nanophytosomes F4 at concentrations equal to IC_{25}, IC_{50}, or IC_{75} values were prepared. 10 µL of acridin orange-propidium iodide reagent (0.5 mg/ml) was added to each well and incubated for ten minutes at room temperature (Hussain *et al.*, 2019). Morphological changes in the cell lines were observed under an inverted microscope (Zeiss, Germany).

Statistical analysis

Data are shown as mean ± standard deviation. The MTT assay results were analysed by one-way ANOVA (α = 0.05) to establish the significant differences between treatments.

Results

The mean vesicular size of F1-F5 was in the range of 42 nm - 4.23 µm (Table I), and there was an increase in vesicular size after three cycles of the freeze-and-thaw test. Formula 4 as an optimum formulation showed spherical vesicles (Figure 1) with zeta potential, PDI, and EE values as described in Table I.

Table I: Composition of catechin-loaded nanophytosomes, the mean vesicle size before and after the physical stability test and the Zeta potential, PDI, and EE of optimum formulation

Formulation	Molar ratio of Catechin: Lecithin: Cholesterol	Particle size* (nm)		Physicochemical properties of optimum formulation
		Before	After	
F1	1 : 1: 0.1	42 ± 0.216	1,090 ± 0.317	Optimum formulation: F4
F2	1 : 1: 0.2	609 ± 0.324	1,700 ± 0.324	Zeta potential : -68 mV;
F3	1 : 1: 0.4	4,230 ± 0.355	14,000 ± 0.323	PDI : 0.412;
F4	1 :1 : 0.8	106 ± 0.218	138 ± 0.235	EE : 93.5%
F5	1 : 1 : 1	201 ± 0.251	326 ± 0.272	

*Before and after the freeze-and-thaw test at 4 and 25°C for three cycles, expressed as mean vesicle size ± standard deviation

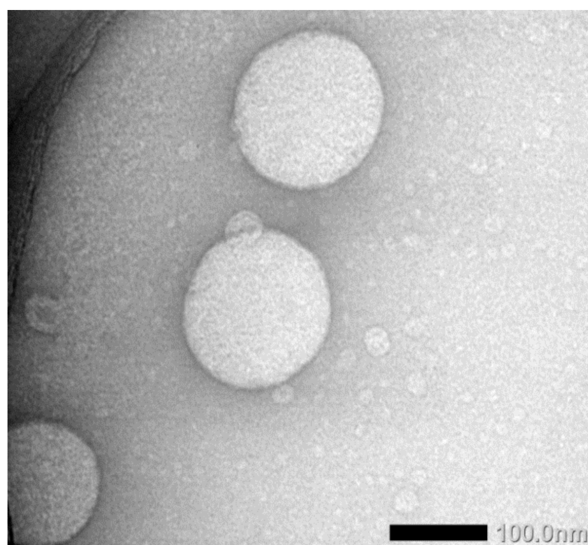

Figure 1: TEM image of nanophytosome F4 (scale bar = 100.0 nm)

FT-IR analysis was carried out to identify an interaction between catechin and lecithin to form a molecular complex in the nanophytosome. The FT-IR spectra of nanophytosome F4 (Figure 2B) showed a disappearance of the phenolic OH stretching of catechin either at 3405.08 or 3209.72 cm^{-1}, suggesting an interaction of catechin and lecithin through this functional group.

Results from the cytotoxic activity of F4 at concentrations 400, 200, 100, 50, 25, and 12.5 µg/ml showed cell viability of 3.41; 16.41; 44.55; 49.23; 61.40; and 69.15% respectively. A semilogarithmic plot of concentrations against cell viability obtained a linear regression equation (y = -36.422x + 106.76; r = 0.9739) producing the IC_{25}, IC_{50} and IC_{75} values of 173.78 µg/ml; 36.307 µg/ml, and 7.41 µg/ml, respectively.

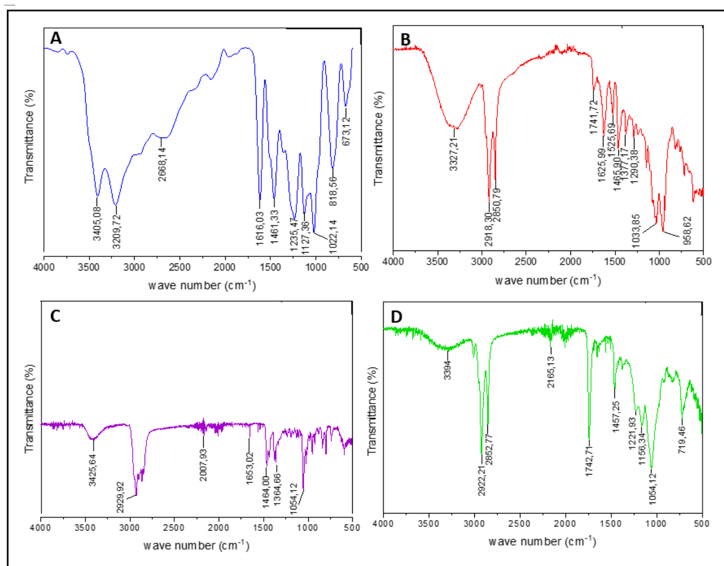

Figure 2. FT-IR spectra of pure catechin (A); nanophytosome F4 (B); cholesterol (C); and lecithin (D)

Figure 3 shows morphologic changes in the cell lines treated with F4 at concentrations equal to IC25, IC50 and IC75 values in comparison to control cells. The percentage of apoptotic cells was (28.61 ± 1.99)% at IC25; (50.55 ± 4.87)% at IC50; (69.80 ± 4.34)% at IC75 compared with (10.94 ± 2.16)% of control. There was a significant difference in the percentage of cells undergoing apoptosis in all study groups ($p < 0.05$).

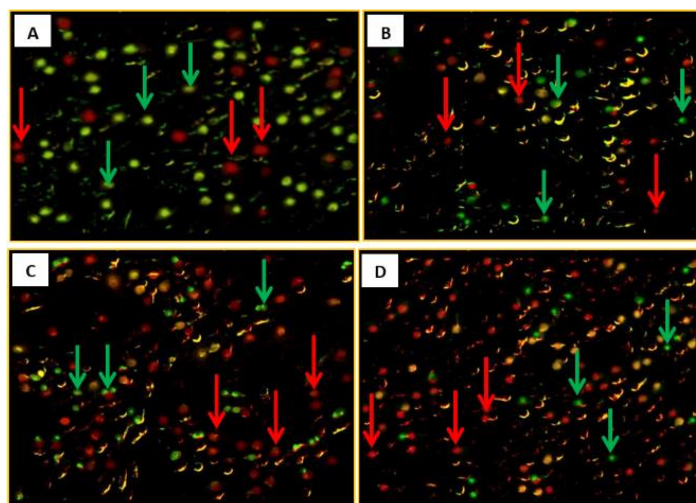

Figure 3: Morphological changes in the HeLa cell lines: control (A); after treatment of F4 at IC_{25},(B); IC_{50} (C) and IC_{75} (D) (green colour shows viable cells; red colour shows apoptotic cells)

Discussion

Lecithin has lipophilic (phosphatidyl moiety) and hydrophilic groups (choline moiety) so catechin can interact with the phosphate group of the choline head to produce molecular complexes that are compatible with the physiological membranes (Babazadeh *et al.*, 2017). The complexes will form micellar mixtures with the addition of water (Hebbar & Mathias, 2018). A molar ratio of 1:1 (phytoconstituent : phosphatidylcholine) is preferable. Cholesterol is added to make the structure of the phospholipid bilayer flexible and physically stable (Purnamasari *et al.*, 2020).

The resulting micellar mixtures (F1 – F5) were in the form of a transparent to cloudy white solution which

remained stable after the freeze-and-thaw tests. These results indicate that the entrapment of catechin into nanophytosomes can protect the compound from the oxidation-induced discolouration that often occurs in free catechins. The optimum formula is F4 with a molar ratio of 1:1:0.8 (catechin:lecithin:cholesterol) which produced homogeneously distributed spherical vesicles (PDI < 0.5) of size (106 ± 0.218 nm) and zeta potential value of < -60 mV, indicating excellent stability of the nanovesicle in solution (Kumar & Dixit, 2017).

Complex formation and interaction between catechin and lecithin were approved by the FT-IR spectra. The spectra of catechin presented characteristic peaks at 3405.08 and 3209.72 cm^{-1} (O-H stretching vibration), and several sharp peaks below 1800 cm^{-1}. The loss of one peak and a shift to 3327.21 cm^{-1} in the nanophytosome spectra indicated that several phenolic OH groups have formed hydrogen bonds. These results are in agreement with the previous report about hydrogen bonding formation in rutin-loaded nanophytosomes (Babazadeh *et al.,* 2017).

Catechin-loaded nanophytosome F4 showed an inhibitory effect against HeLa cell lines and exhibited a moderately active cytotoxic activity IC_{50} = 36.307 µg/ml). The IC_{50} of tea catechins (epigallocatechin-3-gallate) against the HeLa cell line was 54 µg/ml, as reported by Chakrabarty *et al* (2015). Studies have shown that herbal anti-cancer-loaded nanophytosomes improve oral bioavailability and anti-cancer activities (Babazadeh *et al.,* 2017). For example, the in vitro cytotoxic test against liver cancer of silibinin-loaded nanophytosomes was three times greater than that of silibinin alone. The silibinin-loaded nanophytosome showed an IC_{50} of 48.68 µg/ml while silibinin alone produced an IC_{50} value of 485.45µg/ml (Ochi *et al.*, 2016).

Visual observation of cells undergoing apoptosis using the double staining method showed an increase in the number of apoptotic cells (red or orange colour, Figure 3) with an increase in F4 concentration compared with the control. There was a significant difference in the mean percentage of cells undergoing apoptosis in all treatment and control groups ($p < 0.05$). This is in agreement with a study on the effect of catechin hydrate on MCF-7 breast cancer cells. The study reported the ability of catechins to induce apoptosis by modulating the expression levels of pro-apoptotic caspase-3, caspase-8, caspase-9, and TP53 (Alshatwi, 2010). Another study reports that tea catechin (epigallocatechin gallate) affects the induction of apoptosis by modulating P53, caspase-3, and caspase-7 in lung cancer cells A549 (Yamauchi *et al*., 2009).

Conclusion

Catechin-loaded nanophytosomes with a molar ratio of 1 : 1 : 0.8 (catechin : lecithin : cholesterol) produced homogeneously distributed spherical vesicles (PDI = 0.412) with size (106 ± 0.218 nm) and zeta potential -68 mV, which exhibited a moderately active cytotoxic activity with IC_{50} of 36.307 µg/ml, and an apoptotic cell percentage of(50.55 ± 4.87)%.

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