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In vivo evaluation of luteolin-loaded phytosome

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Abstract
Luteolin exhibits antioxidant, antimicrobial, and antiinflammation activities. However, it shows poor oral bioavailability due to its low lipid-solubility. The aim of this study was to improve absorption of luteolin in the gastro-intestinal tract. Luteolin was formulated into luteolin-loaded phytosome (LLP) by thin film hydration method. *In vivo* evaluations were then performed to see plasma levels of luteolin in rats given oral LLP suspension and compared with those in the control group given pure luteolin suspension. Round-shaped LLPs were confirmed with average particle size 105.3 nm, PDI 0.735, zeta potential -34.4 mV, and entrapment efficiency 91.12%. *In vivo* studies showed a 3.54-fold increase in plasma level (AUC = 5426 µg.min/mL) of luteolin compared with those in control group. Phytosomes formulation successfully increased the absorption of luteolin hence it can serve as a promising delivery system for drugs with low lipids solubility.

Keywords: Absorption, *in vivo*, luteolin, phytosome, plasma concentration

Introduction
Luteolin (5, 7, 3', 4'-tetrahydroxiflavones) is an active compound found in most herbs and food plants. Many researches have reported that luteolin exhibits antioxidant, antimicrobial, antiinflammation, anticancer, and antimutagenic activities, and also has effect on cardiovascular disease by decreasing cholesterol level and blood pressure [1, 2]. However, it shows poor oral bioavailability due to its low lipid-solubility and metabolism in gastrointestinal tract [3, 4]. Many have claimed that phytosome formulation is effective to improve absorption and oral bioavalability of low lipid-solubility active compounds [5, 6, 7]. The objective of this study was to improve absorption of luteolin in the gastro-intestinal tract.

Materials and Methods
Materials
All chemical reagents were of analytical grade and used without further purification unless otherwise stated. Luteolin (purity grade = 98% HPLC) was purchased from Acetar Biotec (Cina); *L-α-phosphatidilcholine* (soybean phosphatidilcholine, P 3644) was purchased from Sigma (United State of America); Methanol (LiChrosolv®), dimethylsulfoxide (DMSO), acetic acid glacial, and dichloromethane were purchased from Merck (Germany). All solutions used in high performance liquid chromatography (HPLC LC-20AD, Shimadzu, Japan) analysis were filtered and degassed using a 0.22 µm membrane filter with a filtration system.
Male Sprague-Dawley rats, weighing 200-250 g, were obtained from Faculty of Veterinary, Bogor Agriculture Institute laboratory animal center (UPHL FKH IPB). The rats were kept in an environmentally controlled room (temperature 25-30 ºC, relative humidity 80-90%) for 14 days prior to experiments. All animal procedures were conducted in accordance with all appropriate regulatory standards according to the protocol approved by the ethics committee of Faculty of Veterinary, Bogor Agriculture.

Preparation and Characterization of LLP (Luteolin-Loaded Phytosome)
The LLPs were prepared by thin film hydration methods [8]. Briefly, 60 mg luteolin (LU) were dissolved in 70 mL of methanol, while 180 mg soybean phosphatidilcholine (SPC) were dissolved in 20 mL dichloromethane. The two solution were transferred to a glass round bottom flask and refluxed (4h, 60 ºC) resulting a clear yellow solution. Solvents then removed by vacuum evaporator (Rotavapor® R II, Buchi, Germany) at 337 mbar, 40ºC, to produce the thin film which was hydrated with 200 mL of distilled water.
The entrapment efficiency and luteolin content in LLP were then determined using UV-visible spectroscopy (350 nm). The average particle size, polidispersity index (PDI) and zeta potential

of the luteolin-loaded phytosomes (LLPs) were determined by DLS using Malvern Zetasizer (Malvern Instruments, UK). The 2D surface morphology of the LLPs was visualized by TEM (JEM 2100, Japan).

***In vivo* Evaluation of Luteolin-loaded Phytosome (LLP)**

In vivo evaluation of LLP was carried out based on methods describe in previous studies with modification [1, 9]. The rats were fasted overnight (8 h) before experiments with free access to water and randomly divided into two groups (n=6). The free luteolin suspension and LLP suspension were administrated to rats by oral gavage at dose of 20 mg/kg. 0.3-0.5 mL of blood was collected into tubes (with EDTA) at 0, 10, 20, 30, 60, 90, 120, 180, and 240 minutes after administration. Then plasma was separated immediately by centrifugation at 10.000 rpm for 10 minutes and stored at -20^{o}C for analysis.

Luteolin was extracted from the plasma by liquid-liquid extraction method for HPLC analysis. Briefly, 0.2 mL 6% perchloric acid was added slowly into 2 mL plasmasample to precipitate protein completely. Luteolin was extracted with 3 mL of ethyl acetate by vortexing for 5 minutes. After centrifugation for 10 min at 3500 rpm, supernatant of organic phase were carefully transferred to another tube and evaporated to dryness in the vacuum desiccators under room temperature. The resulting residue was reconstituted in 250 µL of mobile phase, the mixture was centrifuged for 10 min at 5.000 rpm and supernatant was injected into HPLC apparatus.

The analysis was performed on Diamonsil ODS C_{18} reversed-phase column (5 µm, 250 x 4.6 mm). The optimum separation of HPLC was carried out with a mobile phase composed of methanol and 0.3% acetic acid solution (60:40, v/v) at a flow rate of 0.8 mL/min. The volume of sample injection was 50 µL and the detective wavelength was set at 350 nm. The entire samples were filtrated through a 0.22 µm milipore membrane prior to injection.

Luteolin plasma concentration was calculated based on luteolin standard curve. Area under the concentration-time curve (AUC_{0-t}) was calculated by the trapezoidal method, whereas the peak concentration (C_{max}) and the time to reach C_{max} (T_{max}) were directly recorded from the experimental profiles. The data were presented as mean ± SD, and the Student's t test was used to analyze differences between both groups. Results were considered statistically significant from the control when $P < 0.05$ and very significant when $P < 0.01$.

Result and Discussion

Preparation and Characterization of LLP (Luteolin-Loaded Phytosome)

In phytosome preparation, obtaining a clear solution of drug and PC in the reaction complex is a prerequisite since the formation was due to hydrogen bonding interaction between drugs and PCs. Percentage of entrapment efficiency, luteolin content, and physicohemical properties of LLPs were shown in Table 1 while the 2D surface morphology of the LLPs was shown in Figure 1.

Table 1: Physicochemical properties of luteolin-loaded phytosome (LLP)

Luteolin : Phosphatidylcholine Molar Ratio	1:1
Entrapment Efficiency (%)	91.12
Luteolin Content in 25 mg LLP (%)	22.22
Average Dn (d.nm)	105.3
Polidispersity Index (PDI)	0.735
Zeta Potential (mV)	-34.4

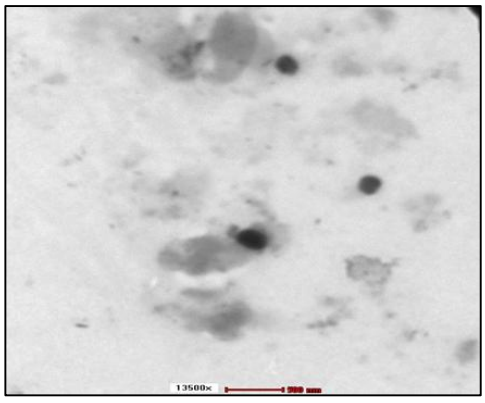

Fig 1: Morphology of LLPs

***In vivo* Evaluation of Luteolin-loaded Phytosome (LLP)**

In vivo evaluation of luteolin-loaded phytosome (LLP) was carried out to examine whether the produced LLPs can significantly improve the absorption of luteolin in the gastrointestinal, by determining luteolin plasma concentration after oral administration. Luteolin plasma concentration in the group administered with oral LLP suspension was higher than those in control group that administered with oral free luteolin suspension (Figure 2). As shown in Table 2, prepared LLPs showed 3.54 times more bioavailability(AUC = 5426 µg.menit/mL) and 11.61 times C_{max} compared to luteolin suspension. As naked luteolin concentration in plasma was significantly low due to low-lipid solubility and glucuronidation by UDP-glucuronosil transferase, the use of nanomaterial helps increase luteolin absorption [3, 4, 11, 12]. Thus it can be suggested that phytosome formulation protects luteolin from glucuronidation so that it can be found in higher level of plasma concentration. These data make a clue for supporting phytosome as a promising delivery system for the enhancement of oral administration of a poorly lipid-soluble drug.

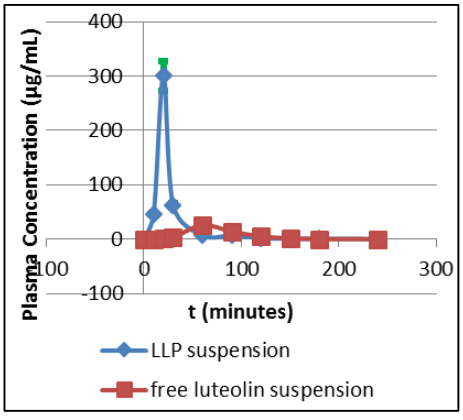

Fig 2: Luteolin plasma concentration after oral administration

Table 2: Pharmacokinetic parameters

Parameters	Luteolin Phytosome	Free Luteolin (Control)
Tmax (minutes)	20	60
Cmax (µg/mL)	300.831	25.918
AUC (µg.min/mL)	5426	1480.309

However, as shown in Figure 2, orally administered of luteolin-loaded phytosome was rapidly absorbed as well as rapidly eliminated. Luteolin concentration in plasma is decreased significantly to 62.39 µg/mL in 30 min after administration. This showed that phytosome formulation has short time duration of action. This phenomenon is corresponds with the negatively charged of LLPs as can be seen in Table 1. Previous studies stated that negatively charged nanoemulsion will rapidly eliminated and have higher reticuloendothelial uptake than those with neutral or positively charged [13].

Conclusion

A novel work in this study is to synthesize LLPs by thin film hydration methods. Round-shaped LLPs were confirmed with average particle size 105.3 nm, PDI 0.735, zeta potential -34.4 mV, and entrapment efficiency 91.12%. The prepared LLPs were rapidly absorbed and significantly increased luteolin plasma concentration and bioavailability after oral administration (Cmax = 300,831 µg/mL, T_{max} 20 minutes, and AUC 5426 µg.menit/mL), hence it can serve as a promising delivery system for drugs with low lipids solubility.

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