ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Development of Phytosome – Black Tea Extract Complex By Different Methods And Study Of Cholesterol's Effect On Entrapment Efficiency.

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ABSTRACT

Phytosome is a complex of active ingredient from plants (phytoconstituent) and phospholipids, formulated to improve its absorption orally or topically. Different of preparation methods of phytosome can produce different entrapment efficiency of the phyto constituent. Other factor such as adding cholesterol in the formula can alter effectiveness of the entrapment in phytosome. The aim of these studies were to determine the effect of different preparation methods and the effect of cholesterol on entrapment efficiency of phytosome. Phytosome was formulated in three different methods: solvent evaporation, reflux and mechanical stirring with a comparison of black tea, phosphatidyl choline and cholesterol in the ratio of 1:2:0.2, 1:2:0,4, and 1:2:0.6. The determination of entrapment efficiency was performed using HPLC. Before HPLC assay, formulas were being centrifuged at 12,000 rpm for 45 minutes. Data from the entrapment of formula 1,2, 3, and 4 respectively were 5.6438%±4.28;6.7387%±4.48;-7.0510%±0.00; and 2.8140%±0.69. There was no significant difference between each formula so it could be concluded that there was no effect of cholesterol in freeze-dried black tea brewed powder phytosome formulation to increased entrapment efficiency and the result showed that reflux method giving the highest entrapment efficiency (7,4799±2,8091%).

Keywords: phytosome, phosphatidyl choline, freeze dried of black tea, solvent evaporation, mechanical stirring, eficieny entrapment

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ISSN: 0975-8585

INTRODUCTION

Gout is a metabolic disease characterized by recurrent episodes of acute arthritis due to deposits of monosodium urate in joints and cartage [2] that can lead to severe disability and in which systemic changes occur that can result in shortening of life. Gout occurs in individuals having high level of serum uric acid or hyperuricemiacondition[3]. Uric acid is a poorly soluble substance that is the major end product of purine metabolism.

The cornerstone of the prevention and treatment of gout is antihyperuricemic therapy, either by uricosuric drugs or by Xanthine Oxidase (XO) inhibitors, such as allopurinol[4].Although allopurinol is a very efficient drug, various adverse effects can occur, especially individuals with decreased renal functions. Most common adverse effects of allopurinol are gastrointestinal distress, hypersensitivity reactions and skin rash. Symptoms of allopurinol toxicity include fever, rash, vasculitis, eosinophilia, and worsening of renal function, which can lead to a fatal outcome especially in elderly patients with renal disorder [3].

An increasing number of researches in the XO-inhibition-therapy-area have suggested the use of plant asalternative medicine. Based on Rohdianaet al (2014), black tea (Camellia sinensis(Linnaeus) Kuntze) Broken Orange Pekoe (BOP), Pekoe Fanning (PF), and Broken Pekoe (BP) can inhibit Xanthin Oxidase activity of more than 50% [1]. Black tea is manufactured from fresh tea leaf which is mechanically treated to give rise to enzyme conversion of flavanols [5].Using developed HPLC-MS-MS it was determined the main polyphenol present in black tea are (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-gallocatechin (GC), (−)-gallocatechin gallate (GCG) in a low concentration, (−)-epigallocatechin gallate(EGCG), theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3_-gallate (TF2B), and theaflavin-3, 3_-digallate (TF3) [6]. As result of investigation on xanthine oxidase activity of Rashidinejad et al (2016), it was proved that compound responsible for XO inhibitor activity were (+)-catechin (C) and (-)-epigallocatechin gallate (EGCG) respectively, and catechin seemed to significantly affect xanthine oxidase less than EGCG[7].

Cos et al. (1998) reported that C, EC and EGC did not inhibit xanthine oxidase up to 100µM[8], whereas Tao et al. (2016) increased the concentration of sample up to 10 folds and found that green tea extract could inhibit xanthine oxidase up to 1000 ppm[6]. It was proven that polyphenols activity on xanthine oxidase is concentration dependent. In an experiment, high amount of polyphenol concentration is needed to be an xo inhibitor. An amount that is imposible to be found in dairy product.

To minimize amount of consuming black tea for hyperuricemia therapy, phyto-active compounds could be complexed with phospholipids in an nanodelivery colloidal system knows as phytosome. Phytosome is a new herbal delivery system formulation which have better absorption and enhanced bioavailability than original plant extracts[9].

Differentpreparationmethodsof phytosome can produce different entrapment efficiency of phytoconstituent. Other factorsuch as adding cholesterol in the formula can alter effectiveness of the entrapment in phytosome.The objective of this study were to determinetheeffectofdifferentpreparationmethodsandtheeffectof cholesterol on entrapment efficiency of black tea extract complexed with phosphatidylcholine with (+)-catechin as marker compound.

MATERIALS

Black Tea (Camellia sinensis (L) (Gambung), (+)-catechin (Sigma-Aldrich Singapore),Phosphatidylcholine (Phospholipon G-60 Lipoid® Ludwigshafen, Germany), cholesterol (Merck Millipore), acetonitrile (Merck Millipore), absolute ethanol (≥ 99.9%, Merck Millipore), dicloromethane (Merck Millipore), and aqua pro injection.

Extraction

Orthodox black tea BOP (Broken Orange Pekoe) grade was bought from Gambung Research Institute for Tea and Cinchona (RITC), Bandung Indonesia. Approximately 20.0 g of dry tea leaves was brewed with 1 L

fresh boiling water for 6 minutes with mild stirring. The cooled down tea infusion was filtered through 150 mesh filter membrane and powdered using freeze dryer. Dry powder extract was used in further investigation.

HPLC Analysis

High performance liquid chromatography coupled with UV-Vis detector was used to analyzed concentration of marker compound in powdered extract. 20 µl sample was injected onto an Inertsil-OD3 C-18 reverse phase column. The flow-rate was 0,5 ml/minutes, with isocratic elusion system and acetonitrile : water (20:80) as mobile phase. Marker compound used for concentration analysis of the extract was (+)-catechin, bought from Sigma Aldrich (Singapore). 5,0 mg catechin was dissolved in 10,0 ml water and diluted to made calibration curve in six different concentrations.

Development of Tea extract-Phytosome

Phytosomes were prepared using thin layer hydration method with three differentapparatus: (1) using rotary evaporator vacuum; (2) reflux; and (3) mechanical stirring to choose the best method to develop phytosome.

Preparation of tea extract-phytosome using rotary evaporator vacuum

The first formulationwas prepared by using rotary evaporator vacuum method. Phosphatidylcholine (PC) was dissolved in dicloromethane/ethanol while tea-extract-powder was dissolved in enough amount of anhydrous ethanol. The mixture was ultrasonicated for 30 minutes to obtained perfect dissolution. The mixture was taken into a round bottom flaskand evaporated in rotary evaporator at 40±2^{o}C until all solvent evaporatedperfectly and dry thin film produced[10]. To obtain a smooth thin and homogeny film, optimization of rotation speedwas conducted. Rotation speed of rotary evaporator investigated in varied rotation per minutes (rpm): 30; 45; 60; 90; and 120.

Moreover, the prepared thin layer had been kept overnight in room temperature prior to hydration. The film was hydrated with distilled water in a rotary at optimized temperature. Hydration parameters further being optimized to obtain optimal phytosomes complex, which were: rotation speed and hydration temperature.

Preparation of tea extract-phytosome using reflux apparatus

PC and tea-extract were dissolved in dicloromethane/ethanol as before procedure explained. After being ultrasonicated, the mixture was refluxed for 3 hours at 70±2^{o}C. After 3 hours of refluxing, the mixture was cooled and poured to petri dish. The dish was kept open overnight at room temperature for evaporation of solvent. The product was then kept in hot air oven at 60±2^{o}C for 2 hours. The dried thin film was stored in desiccators for further use[11].

Preparation of tea extract-phytosome using simple mechanical stirring

PC and tea-extract were dissolved in the same solvent and ultrasonicated for the same time. The mixture was stirred at 250 rpm for 10 minutes. After stirring the mixture was poured to petri dish and being blown with hot air to evaporate the solvent.

Evaluation of Phytosome

Determination of Vesicle Size and Distribution

The vesicle size and polydispersity index were analyzed by dynamic light scattering system spectroscopy using a Beckman Coulter® Delsa™ Nano C particle Analyzer. To perform the measurement, the

sample was introduced into the disposable cell and the both vesicle size and vesicle size distribution were read in light intensity of 3,000 to 30,000[13].

Entrapment efficiency

The proportion of encapsulated catechin was determined by centrifuging hydrated thin film at 12,000xg for 45 minutes at room temperature. The supernatant was taken carefully using micropippet. Supernatant was further analyzed using HPLC [13].

$$\% \text{ Entrapment efficiency} = \frac{\text{amount of encapsulated catechin}}{\text{amount of total catechin in the formula}} \text{ x } 100\ \%.$$

Effect of Cholesterol on Phytosome Formulation

Phytosome in three different formulation (table 1) was developed using thin layer hydration method with rotary evaporator apparatus. Different concentration of cholesterol was used to investigate the effect of cholesterol in the entrapment efficiency of phytosome. The three formulas are shown in table 1

Table 1: Formula for investigating effect of cholesterol on phytosome formulation

Formula	F0	F1	F2	F3
Powdered black tea extract (µmol)	5,67	5,67	5,67	5,67
PC (µmol)	11,35	11,35	11,35	11,35
Cholesterol (µmol)	-	1,13	2,27	3,40

RESULT AND DISCUSSION

Extraction

Powdered extract was made by freeze drying method. Dried extract obtained from extraction process was 23,63% b/b from dry black tea leaves extracted.

HPLC Analysis of Extract

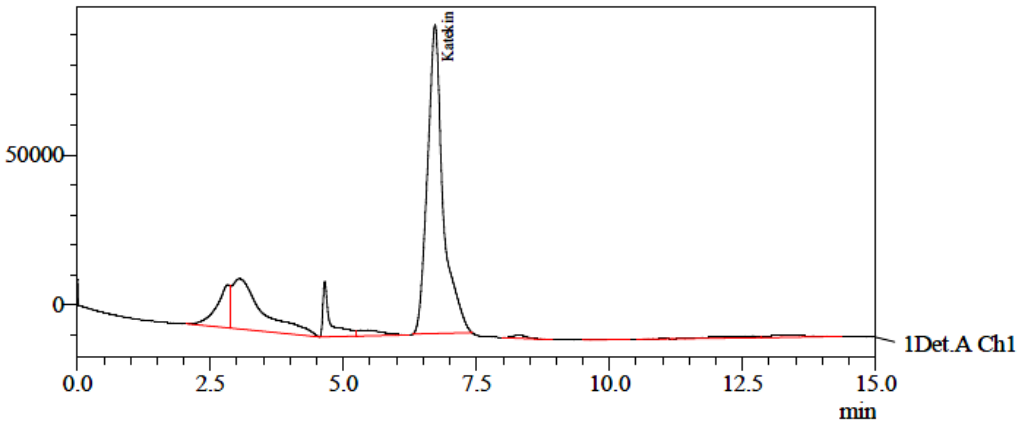
The initial HPLC method for catechin assay was based on Rohdiana et al. (2014) with modification. Before analysis of sample concentration, number of theoretical plates (N), tailing factor, resolution (R) and capacity factor were evaluated. Result concerning the suitability test shown in table 2. All parameters tested were met the criteria, except for tailing factor which was closely exceed 2. Chromatogram of standard catechin is shown in picture 1.

Table 2: System Suitability Test

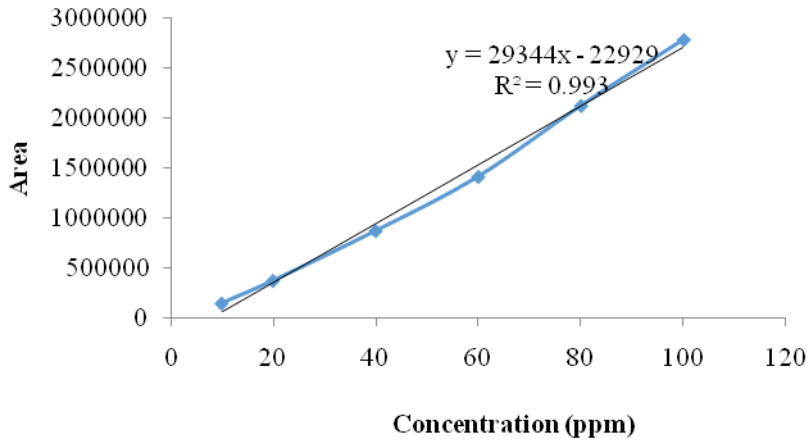
Parameters	Criteria	Result
Theoretical plates	≥ 1000	2981.124
Tailing factor	≤ 2	2.446
Resolusion	≥ 1,5	9.934
Capacity factor	1-10	1.765

Calibration curve is determined and presented in picture 2 to access linierity. From the curve, the correlation coefficients obtained was 0,9969.

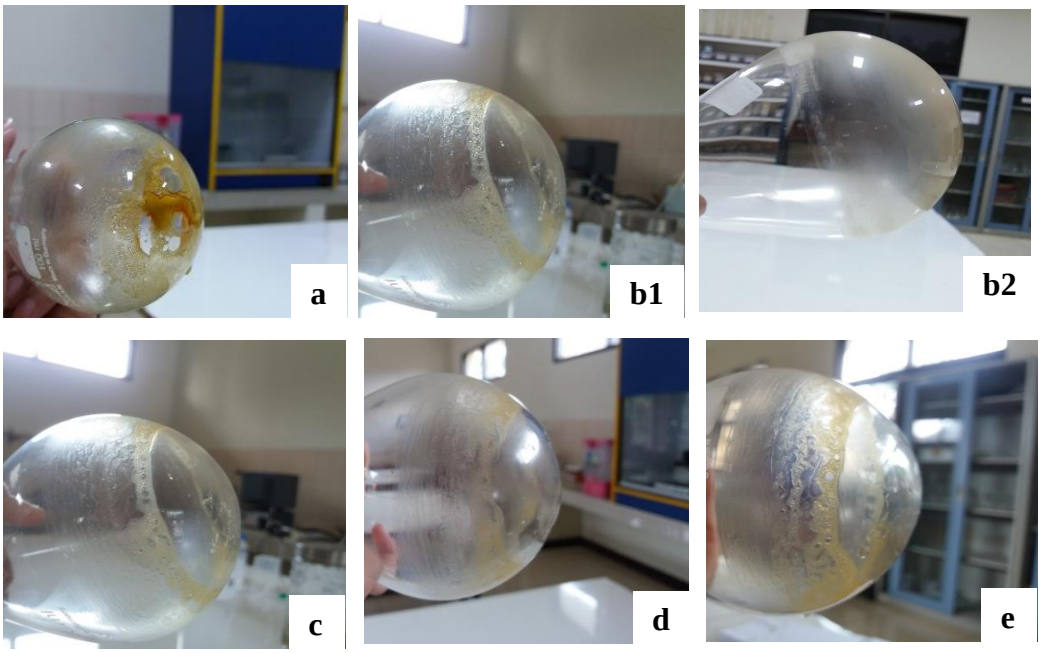
ISSN: 0975-8585



Picture 1: Chromatogram of standard tea catechin



Picture 2: Calibration curve of standard tea catechin



Picture 3: Dry thin layer produced after evaporation in various rotation speed: a) 30rpm; b1) 45rpm (dichloromethane was used as solvent); b2) 45rpm (ethanol was used as solvent); c) 60rpm; d) 90rpm; e) 120rpm.

Development of Phytosome Using Different Methods

Preparation of tea extract-phytosome using rotary evaporator vacuum

Thin layer and hydration processes were done using three different apparatus to obtain the best method to prepare tea extract-PC complex of phytosome. The first method applied was based on Rasaie et al. (2014).

There are several parameters that affecting the formation of thin layer, therefore optimization were done. First parameter investigated was rotation speed of rotary evaporator. The result of the optimizationare shown in table 3 and picture 3. As can be seen in table 3 and picture 3, the best result of thin layer (smooth and homogeny layer) was obtained by 45 rpm. Rotation speed faster than 45 rpm result in a bubble formation at the bottom of the flask. Best thin layer was obtained using ethanol as solvent. Ethanol has boiling point higher than dichloromethane, so evaporation process runs longer than when dichloromethane was used. Too quick evaporation process can result in an non-homogeny layer and too slow speed of rotation result in a non-homogeny film.

Table 3: Optimization of different parameters affect formulation of phytosome developed using rotary evaporator method: rotation speed of rotary evaporator and solvent used

Formula	O1	O2-A	O2-B	O3	O4	O5
PC (µmol)	250	250	250	500	500	500
Dichloromethane (ml)	5	5	-	5	5	5
Ethanol p.a (ml)	-	-	5	-	-	-
Rotation speed (rpm)	30	45	45	60	90	120
Result of thin layer	Not homogeny (Pic. 3-a)	Bubbled, homogeny (Pic. 3-b1)	Thin layer was smooth and homogeny (Pic. 3-b2)	Bubbled, not homogeny (Pic. 3-c)	Bubbled, not homogeny (Pic. 3-d)	Bubbled, not homogeny (Pic. 3-e)

Thin layer was cooled and stored at desiccator overnight to remove remaining organic solvent. Further optimization was hydration parameter. Hydration process conducted by using the same apparatus, rotary evaporator. By these step the tea extract-PC complexswelled and peeled off from the flask wall and forming vasiculate vesicles. To obtained the best condition, rotation speed was done at different rpm. The best hydration condition obtained from experiment were 90rpm rotation speed at 45^{o}C temperature for 20 minutes of hydration. All thin layer was completely suspended in water used for hydration process and translucent liquid was obtained. The phytosomal suspension was finaly sonicated and being analyzed for vesicle size and polydispersity index.

Preparation of tea extract-phytosome using reflux apparatus

Reflux method was the second method that was being optimized. Tea extract and PC dissolved in dichloromethane/ethanolwas reflux for 3 hours using two different drying methods. First drying method was done by putting petri dish in a room temperature to let solvent evaporate naturally, and later was done by using hot air, blown manually using hair dryer. The best drying method to produce thin layer complex was using hair dryer. Chosen method was more rapid than the other and produce the more homogeny layer.

Preparation of tea extract-phytosome using simple mechanical stirring

Phytosomeswere also developed by stirring method. All the three methods produced smooth and homogeny thin layers in round flask or petri dish. All are shown in picture 4.

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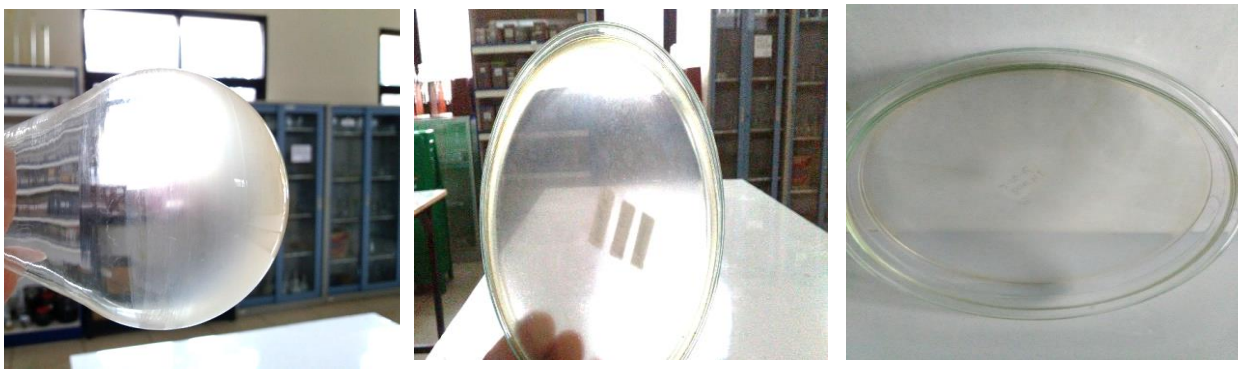
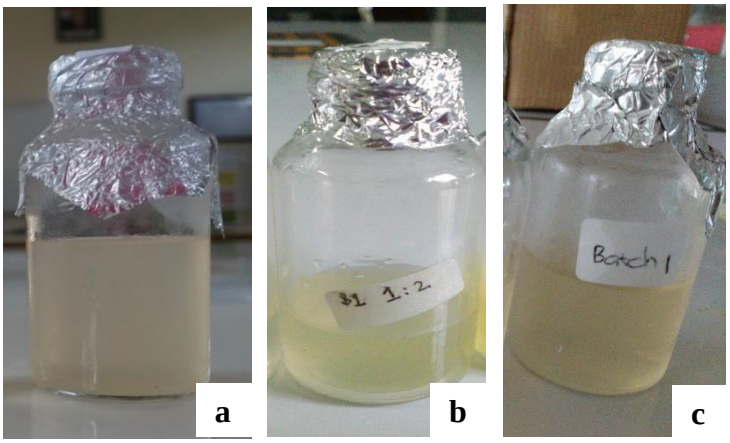
Thin layer obtained from three different methods were hydrated using parameters before optimized: 90rpm; 45^{o}C temperature for 20 minutes in a rotary evaporator. The result can be seen at picture 5.



Picture 4: Dryed thin layer produced from different methods: a) solvent evaporation using rotary evaporator; b) reflux method; c) mechanical stirring



Picture 5: Hydration result of thin layer developed by three different methods: a) solvent evaporation using rotary evaporator; b) reflux method; c) mechanical stirring

Determination of Vesicle Size and Distribution

Hydrated thin layer obtained from previous step were being analyzed using Delsa^{TM} Nano C particle size analyzer (Beckman coulter) at 25^{o}C. Average number distribution of phytosome obtained from experiment were in the range of 42,3-499,0 nm with polydispersity index 0,309-0,227. Each of average vesicle are shown in table 4.

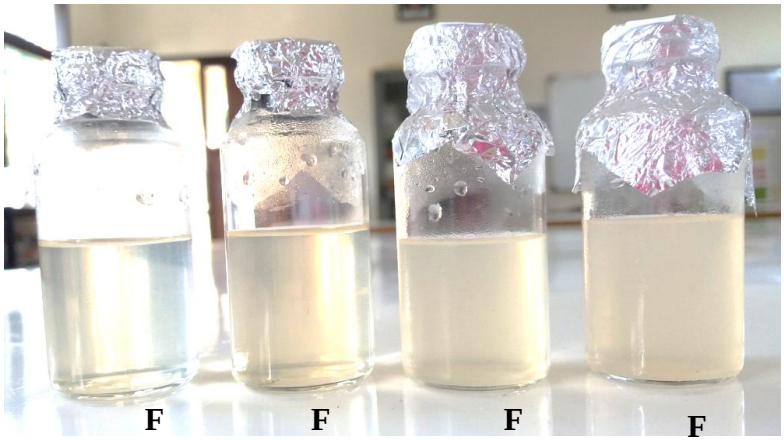
Table 4: Vesicle size of phytosome, developed in three different methods

Experiment	Average Number Distribution ±SD (nm)*	Index Polydispercity
F0 (Control)	-	-
F1 (Solvent Evaporation Method)	42,3 ± 12,0	0,372
F2 (Reflux method)	101,9 ± 27,9	0,309
F3 (Mechanical Stirring Method)	499,0 ± 67,9	0,227

From table 4, we could see all preparation methods can produce low polydispersity index values of less than 0.5. This indicates the uniformity and homogeneity distribution of vesicle size in the samples [12]. Formula prepared using solvent evaporation produce vesicle with the smallest size. This might be because the thin film prepared with this method was formed more evenly than the other methods. While formula prepared

ISSN: 0975-8585

by mechanical stirring metods, produce vesicle size that was the largest (499.0 ± 67.9 nm). This could be because the particle size reduction method used (stirring method) was unable to reduce the size of phytosome, compared with ultrasonication used in F1 and F2, using: ultrasonication 20,000 rpm for 30 minutes.



Picture 6: Hydration of thin film of the formula using different amount of cholesterol: F0) without cholesterol; F1) 1,13 µmol; F2) 2,27 µmol; F3) 3,40 µmol

Entrapment efficiency of phytosome

Quantitative analysis was determined to investigate the proportion of encapsulated catechin in phytosome vesicle, all done by triplicates. The entrapment efficiency evaluation was determined by centrifuging phytosome formulae at 12,000 rpm for 45 minutes. The purpose of this step was to precipitate phosphatidylcholine bonded phytoconstituent. The supernatant was taken carefully and analyzed before optimized HPLC condition. The result of entrapment efficiency of the formulae (F1, F2, F3) varied from -34,8300 to 10,7960% (table 5). Other reseachs about phytosome, as Khan et al., 2013, entrapment efficiencies resulting from complex between phyto-phospholipid in several other studies showed varying results entrapment efficiency of about 25-≥90% [14]. In this reseach, efficiency entrapment of black tea extract-PC was small, this might be because the sample preparation method prior to entrapment efficiency measurement unable to precipitate phosphatidylcholine.This was proved by further investigation by centrifuging only phosphatidylcholine dissolved in water to 12,000xg for 45 minutes. The result showed that only 9% of phosphatidylcholine was precipitated.

Table 5: Entrapment efficiency analysis

Formulae	Batchs	Catechin Concentration of the supernatant (%)	Entrapment efficiency (%)
F1 (Control)	1	32,9325	0,0000
	2	32,7216	0,0064
	3	33,0919	-0,0048
F2 (Solvent Evaporation Method)	1	29,3771	10,7960
	2	32,2952	1,9352
	3	30,4675	7,4850
F3 (Reflux method)	1	30,3604	7,8102
	2	29.6033	10,1092
	3	31,4439	4,5202
F4(Mechanical Stirring method)	1	43,9790	-33,5428
	2	44,4028	-34,8300
	3	32,7988	0,4060

Effect of Cholesterol on Phytosome Formulation

To determined effect of cholesterol in the entrapment efficiency of phytosome, freeze dried tea extract powder was made in 3 formulas with one formula as control. Cholesterol used in each formulas had a varied in concentration, which were: 1.13 mol, 2.27 mol and 3.40 mol respectively(table 1). All formulation producedevenly distribute thin layer film. After being hydrated, formula 3 had the clearest physical appearance. The more cholesterol used in the formulae, the more clear the liquid hydration that was got.

Vesicle size analysis results was conducted based on the number distribution diameter, which is the diameter of the largest number of particles. The result showed that formula 1 with smallest amount of cholesterol, had an average diameter the smallest above all sample tested. The result could be seen at table 6. After calculation of the percentage of adsorption using the formula, the result of the entrapment fitosom very small. Average percentage entrapment of F0, F1, F2 and F3 were 5.6438%, 6.7387% -7.0510%, and 2.8140% respectively.

Tabel 6: Average number distribution of cholesterol loaded phytosomeand Entrapment Efficiency (%)

Formula	Average number distribution ± SD (nm)	Polydispercity index (%)	Entrapment Efficiency ± SD (%)
F0	50,8 ± 14,4	0,374	5,6438 ± 4,28
F1	42,3 ± 12,0	0,372	6,7387 ± 4,48
F2	81,6 ± 22,8	0,390	-7,0510 ± 0,00
F3	45,6 ± 13,1	0,328	2,8140 ± 0,69

Based on statistically analysis usingOne-Way ANOVA. From the test results of One-Way Anova concluded that the alpha of 0.05, there was no significant effect of adding the addition of cholesterol to increase adsorption of the formulaphytosome percent (p-value> 0.05).

The results of this study were linier with theory. Phytoconstituent complexed with PC by hydrogen bonding to choline group. Whether or notrigidity of the double layer of lipids in the vesicle will not affect the bond between active with choline group, hence the addition of cholesterol does not affect the adsorption efficiency improvement phytosome. In contrast to the efficiency of niosomsystem, the entrapment efficiency will strongly influenced by rigidity of double layer of lipids in the vesicle as in niosom, bonding of active to PC is not in the choline group, but inside the vesicle. So in case of leaking on a double layer of lipids, then entrapment efficiency will decreases. Therefore in niosom system, cholesterol serves as a stabilizer and a filler layer of lipid vesicles empty so as to prevent leakage and improve entrapment efficiencies.

CONCLUSION

Thin layer film that was developed using rotary evaporator was the best method to develop black tea extract-phosphatydilcholine complex. . There was no significant difference between each formula so it could be concluded that there was no effect of cholesterol in freeze-dried black tea brewed powder phytosome formulation to increased entrapment efficiency and the result showed that reflux method giving the highest entrapment efficiency (7,4799±2,8091%).

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