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EVALUATION OF THE ANTIOXIDANT ACTIVITY BY THE DPPH RADICAL SCAVENGING METHOD OF FREE AND LIPOSOME-ASSOCIATED COCOA EXTRACTS[1]

AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE PELO MÉTODO DE SEQUESTRO DE RADICAIS - DPPH DE EXTRATOS DE CACAU LIVRE E ASSOCIADO A LIPOSSOMAS

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ABSTRACT

Polyphenols have benefits to health, as a potent antioxidant action, however, such benefits can be committed due to the instability facing the different conditions. In this sense, the nanoencapsulation is a tool that allows imprisoning compounds with activity, protecting them against degradation and improving stability. This way, the aim of this work was to identify and to quantify the cocoa compounds, to nanoencapsulate them and to evaluate the antioxidant activity in comparison to the raw extract. The chromatographic analysis showed that the predominant flavonoids in the cocoa extract were the epicatechin, epigallocatechin, isochercetin and the kaempferol. Liposomes were produced by the reverse phase evaporation method and present adequate nanometric characteristics. The antioxidant activity, EC50, from liposomes containing cocoa extract was superior to the one from the free extract, demonstrating the liposome efficiency in protecting the active and improve its activity.

Keywords: nanotechnology, phenolic compounds, Theobroma cacao.

RESUMO

Os polifenóis possuem benefícios à saúde, como uma potente ação antioxidante, contudo, tais benefícios podem ser comprometidos devido à instabilidade frente a diferentes condições. Neste sentido, a nanoencapsulação é uma ferramenta que permite aprisionar compostos com atividade, protegendo-as contra a degradação e melhorando a estabilidade. Assim, o objetivo neste trabalho foi identificar e quantificar os compostos do cacau, nanoencapsulá-los e avaliar a atividade antioxidante em comparação ao extrato bruto. A análise cromatográfica demonstrou que os flavonoides predominantes no extrato de cacau foram a epicatequina, epigalocatequina, isoquercetina e o caempferol. Os lipossomas foram produzidos pelo método de evaporação em fase reversa e apresentaram características manométricas adequadas. A atividade antioxidante, EC50, dos lipossomas contendo extrato de cacau foi superior ao extrato livre, demonstrando a eficiência do lipossoma em proteger os ativos e potencializar a sua atividade.

Palavras-chave: *nanotecnologia, compostos fenólicos, Theobroma cacao.*

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INTRODUCTION

Cocoa is a fruit abundant in polyphenol, among them, it is highlighted the procianidines, compounds with a protecting effect against several diseases (AFZAL; SAFER; MENON, 2015). Due to its action, as a natural antioxidant, it acts as protecting and preventing some pathologies designed as "diseases of oxidative stress" like as cancer, cerebrovascular diseases, Alzheimer's disease, airway disease (CHOI et al., 2012), associated in the development and progression of diabetes (YOUNG et al., 1995) making important use of food rich in these compounds (VALENZUELA; SANHUEZA; NIETO, 2003). In natural extracts there is antioxidant activity, due to the bioactive substances present in food, however, such action keeps committed because of its physio-chemical instability (ISAAC et al., 2009). Among various conditions that can affect the stability of natural products, we can highlight oxygen, moisture and temperature (THAKUR, 2011). Nanoencapsulation with liposomes can preserve these compounds, protecting them against degradation, being that for environmental reasons as pH, temperature, salt and organic solvents, maintaining its characteristics of stability and solubility (JAFARI et al., 2008), and can also help the hydrophobic components to become soluble in hydrophilic environments and vice versa (BASNET et al., 2012; JAFARI et al., 2008). Because of that, nanotechnology can be applied to different compounds, increasing its action in several biological and technological systems potentializing its effect.

Liposomes carry vesicular characteristics organized by one or more lipidic layers, being able to encapsulate active hydrophilic and/or lipophilic which the aqueous phase is found in the partitioned interior and the lipophilic in the membrane (BENSON, 2005). In this context, the present work aims to identify and to quantify the phenolic compounds present in the cocoa extract, to nanoencapsulate the extract in liposomes and to evaluate the antioxidant activity of free and liposomal cocoa extract.

MATERIALS AND METHODS

COCOA EXTRACT

The cocoa seeds are from the Forastero variety, coming from the CEPLAC/CEPEC (Comissão Executiva do Plano da Lavoura Cacaueira e Centro de Pesquisas do Cacau) located in Ilhéus/BA, Brazil. The seeds were received and previously dried and peeled. The cocoa seeds polyphenols were extracted according to the methodology described by Jonfia-Essien et al. (2008), with some modification. The seeds were previously ground and degreased in soxhlet, using ciclo-hexane as a solvent. After this step, the cocoa powder obtained was put in flow cabinets until the complete solvent evaporation. For the extraction of the cocoa phenolic compounds, 10g of cocoa powder was weighed and added to 100 mL of a hydro alcoholic solution of methanol (MeOH 70%) and then, it was taken

to agitation for 2 hours protected from light. After that, the solution was filtered and dried in rotary evaporator and stored in amber frask. The extract which was not used for liposome preparation is named free extract.

IDENTIFICATION AND QUANTIFICATION OF PHENOLIC COMPOUNDS

All chemical were of analytical grade. Methanol, formic acid, gallic acid, caffeic acid, caffeine and theobromine were purchased from Merck (Darmstadt, Germany). Catechin, epicatechin, epigallocatechin, quercetin, isoquercitrin, rutin and kaempferol were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

Quantification of compounds by HPLC-DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using C18 column (4.6 mm x 150 mm) packed with 5µm diameter particles; the mobile phase was water containing 1% formic acid (A) and acetonitrile (B), and the composition gradient was: 13% of B until10 min and changed to obtain 20%, 30%, 50%, 60%, 70%, 20% and10% B at 20, 30, 40, 50, 60, 70 and 80 min, respectively, following the method described by Kamdem et al. (2013) with slight modifications. Theobroma cacao extract methanol 70% were analyzed at a concentration of 10 mg/mL. The flow rate was 0.6 mL/min, injection volume 50 µl and the wavelength were 257 nm for gallic acid, 280 for catechin, epicatechin, epigallocatechin, theobromine and caffeine, 327 nm for caffeic acid, and 365 nm for quercetin, quercitrin, isoquercitrin, rutin and kaempferol. All the samples and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.025 - 0.350 mg/ml for kaempferol, quercetin, quercitrin, isoquercitrin, rutin, catechin, epicatechin and epigallocatechin; and 0.030 - 0.450 mg/ml for gallic acid, caffeic acid, theobromine and caffeine. Chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (210 to 600 nm).

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve (BOLIGON et al., 2013).

LIPOSOME PREPARATION

Liposomes were prepared in triplicate, using the reverse phase evaporation method according to Szoka and Papahadjopoulos (1978), with adaptation. For the liposome preparation, two phases were previously prepared, as to the quali-quanti composition described in table 1. For comparison, blank liposomes, omitting the extract presence, were also prepared as described in table 1.

Table 1 - Composition of blank liposomes (BL) and liposomes containing cocoa extract (LCE). Final Volume: 100 mL.

Composition	**Liposome**	
Oily Phase (OP)	BL	LCE
Phosphfolipid	0.8 g	0.8 g
Cholesterol	0.15 g	0.15 g
Vitamin E	0.02 g	0.02 g
Ethanol	40 mL	40 mL
Aqueous Phase (AP)		
Polisorbate 80	0.15 g	0.15 g
Ultra pure Water	100 mL	100 mL
Cocoa extract	-	0.1g

The constituents of the aqueous phase (AP), polisorbate 80, cocoa extract and ultra pure water, were added in a beaker of 250 mL, and homogenized with the help of a magnetic agitator, with temperature of 30ºC, until its complete solubility.

For the Oily Phase (OP), the compounds were added in a round-bottom balloon and put in an ultrasound bath during 10 minutes for its homogenization. After the homogenization of both phases, 4 mL of AP was added in the OP and then the mixture was taken again to the ultrasound bath for 10 minutes more for the formation of reverse micelle. After 10 minutes, the formulation was taken to the rotaevaporator (85 rpm, temperature of 35ºC) for the complete solvent evaporation and formation of the organogel. After that, the rest of the AP was added and put again in the rotaevaporator without vacuum, during 30 minutes (120 rpm/ 35ºC) for agitation, and the formation of liposomal vesicles. After this phase, a process of extrusion, to uniform the size of the vesicles, using membranes of 0.45 μm and 0.22 μm. After all these steps the liposomes were characterized.

NANOPARTICLES CHARACTERIZATION

The liposome characterization was done through the polydispersity index and particle diameter, zeta potential and pH determination.

MEDIUM DIAMETER AND POLYDISPERSITY INDEX DETERMINATION

The determination of the medium diameter and polydispersity index of the formulations was done through a dynamic light scattering (Zetasizer nano-ZS model ZEN 3600, Malvern Instruments), after its dilution (500 times, v/v) in ultrapure water.

The results were determined through the values of the average from the three different samples.

ZETA POTENTIAL DETERMINATION

The zeta potential was evaluated through electrophoresis in the Zetasizer nano-ZS equipment, model ZEN 3600, after the dispersion dilution in NaCl 10 mM solution (500 times, v/v) previously filtered through a 0.45 μm membrane. The results were expressed in millivolts (mV) from an average from three determinations of different batches.

ANALISYS OF THE ANTIOXIDANT ACTIVITY

The analysis of the antioxidant activity was done by the methodology of DPPH radical scavenger. For this 1 mg of the extract was weighed and diluted in 10 mL of methanol (main solution - MS), and after that being added in tubes of 500 μL of the sample (MS) and 500μl of methanol, following a serial dilution in different concentrations (250, 125, 62.5, 31.3, 15.6, 7.8, 3.9 μl) using methanol as a diluent for a final volume of 500 μL. After that, one part with 500 μL of each dilutions was transferred to tubes containing 2500 μL of the DPPH radical.

For liposomes containing the extract, 1000 μL of the formulation was diluted in 10 mL of methanol and after that, one part with 120 μL of this solution was transferred to 880 μL of methanol. From this one, one part with 500 μL was taken and the same amount of methanol was added. So, a serial dilution followed for tubes containing methanol, to obtain different concentrations (120, 60, 30, 15, 7.5, 3.8, 1.9, 0.9, 0.2 μl) and after that, 2500 μL of the DPPH radical (0.004% v/v) was added.

Both mixtures were let in the dark for 30 minutes, in room temperature. The control was prepared in the same way, using methanol for the correction of the base line. The DPPH solution was daily prepared, stored in amber flask and at 4ºC among the measurements. The absorbance was read at 517 nm. With the values, the inhibition percentage was calculated according to the following formula (YEN; DUH, 1994): % Inhibition = [(ADPPH-AExtr) /ADPPH] × 100, where ADPPH is the absorbance value of the DPPH blank sample, and AExtr is the absorbance value of the test solution. AExtr was evaluated as the difference between the absorbance value of the test solution and the absorbance value of its blank. The IC50 values are reported as final concentration of extract in the cuvettes defined as μg/mL of extracts required decreasing the initial DPPH concentration by 50%.

STATISTICAL ANALISYS

All determinations were done in triplicate, and the results were expressed in average and standard deviation. The average comparison was done by the variance analysis (ANOVA) and the Tukey test ($p<0,05$), using Assistat 7.7 beta. To compare the antioxidant activity was used Student's t, test employing Graphpad prism 5.0®.

RESULTS AND DISCUSSION

HPLC ANALYSIS

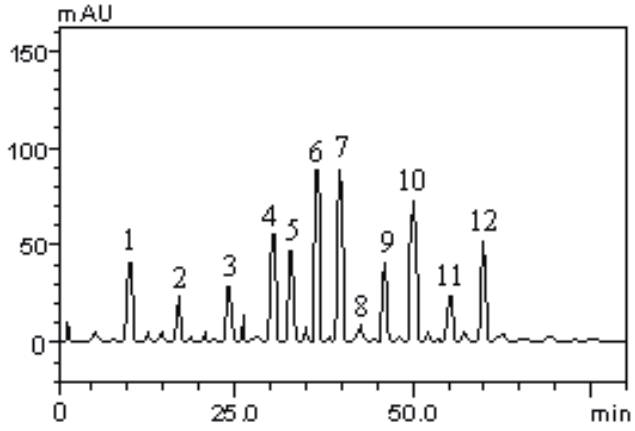
After HPLC analysis we can get calibration curves for following compounds: Gallic acid: Y = 12593x + 1308.5 (r = 0.9998); caffeic acid: Y = 11746x + 1265.1 (r= 0.9996); catechin: Y = 12790x + 1345.9 (r= 0.9995); epicatechin: Y = 12649x + 1189.4 (r = 0.9998); epigallocatechin: Y = 11579x + 1304.9 (r= 0.9997); theobromine: Y = 13517x + 1187.2 (r= 0.9991); caffeine: Y = 11825x + 1318.7 (r= 0.9999); quercitrin: Y = 13165x + 1205.7 (r= 0.9999); isoquercitrin: Y = 12560x + 1357.3 (r= 0.9999); rutin: Y = 13983x + 1171.3 (r= 0.9998); quercetin: Y = 13165x + 1292.5 (r= 0.9996) and kaempferol: Y = 12539x + 1183.0 (r= 0.9997).

HPLC fingerprinting of *Theobroma cacao* extract revealed the presence of the gallic acid (t_R = 11.46 min; peak 1), catechin (t_R = 17.35 min; peak 2), caffeic acid (t_R = 24.87; peak 3), caffeine (t_R = 30.68; peak 4), epicatechin (t_R = 33.17 min; peak 5), theobromine (t_R = 37.09 min; peak 6), epigallocatechin (t_R = 38.91 min; peak 7), rutin (t_R = 44.15 min; peak 8), quercitrin (t_R = 46.28 min; peak 9), isoquercitrin (t_R = 50.03 min; peak 10), quercetin (t_R = 53.79 min; peak 11) and kaempferol (t_R = 58.64 min; peak 12) As figure 1 shows.

According to Zumbé (1998) the main phenolic compounds found in cocoa are inside the tanine and flavonoid categories. Phenolic from cocoa are stored in the seeds cotyledons, rich in polyphenols mainly in catechins (flavan-3-ols) and procyanidins, compounds found as predominant in this study.

For Senter, Robertson and Meredith (1989) the techniques of HPLC are largely used, even to separate or quantify different phenolic compounds. Many are the mobile phases available for the analysis of anthocyanins, procyanidins, flavanones, flavonols, flavones and phenolic acid. Ali et al. (2015) quantified seventeen phenolic compounds presente in the cocoa, by HPLC-UV being that: the gallic acid, caffeic acid, vanilic acid, epicatechin, rutin, catechin, coumaric acid, sinapic acid, chlorogenic acid, kaempferol, genistein, protochatecoic acid, naringin, apigenin, quercitin, morin and siringic acid, presenting values for gallic acid (0.84 ± 0.45 mg/g) with retention time of 5.9 9min, epicatechin (1.39 ± 0.14mg/g) with retention time of 13.5 min and catechin (1.32 ± 0.47mg/g) with retention time of 11.03 min.

Figure 1 - Representative high performance liquid chromatography profile of *Theobroma cacao*. Gallic acid (peak 1), catechin (peak 2), caffeic acid (peak 3), caffeine (peak 4), epicatechin (peak 5), theobromine (peak 6), epigallocatechin (peak 7), rutin (peak 8), quercitrin (peak 9), isoquercitrin (peak 10), quercetin (peak 11) and kaempferol (peak 12).



The table 2 shows the phenolic compounds content and metilxantins found in the methanol extract. The extract showed 10 phenolic compounds and 2 metilxantins. Jalil and Ismail (2008), described that the amount of phenolic compounds present in the *Theobroma cacao* depends on the plant genotype, temperature, humidity, region and the fermentation process, among others.

Table 2 - Content of phenolic compounds and metilxantins found in the methanol extract of the cocoa seed.

Compounds	Extract MeOH 70% (mg/g)	LOD μg/mL	LOQ μg/mL
Gallic acid	10.15 ± 0.02 a	0.008	0.025
Catechin	7.09 ± 0.01 b	0.019	0.063
Caffeic acid	7.23 ± 0.01 b	0.027	0.089
Caffeine	13.68 ± 0.01 c	0.013	0.043
Epicatechin	10.56 ± 0.03 a	0.032	0.105
Theobromine	19.85 ± 0.01 d	0.018	0.060
Epigallocatechin	19.87 ± 0.02 d	0.024	0.081
Rutin	2.69 ± 0.01 e	0.031	0.102
Quercitrin	10.23 ± 0.01 a	0.007	0.023
Isoquercitrin	17.41 ± 0.02 f	0.025	0.082
Quercetin	7.16 ± 0.01 b	0.011	0.034
Kaempferol	13.28 ± 0.03 c	0.036	0.118

Extract MeOH 70% - methanolic extract; LOD - Detection Limit; LOQ - Quantification limit.

After the characterization, the extract was encapsulated in liposomes and the vesicles were characterized. Table 3 shows the results of the characterization of blank liposomes and containing cocoa extract. In relation to the average size it can be observed that the presence of the extract influenced in the size of the vesicles, which was inferior for the formulations containing the extract, presenting statistic difference between the same column.

Studies from Tronnes (2012) with liposome encapsulated polyphenols from the green tea showed the size closer to the one found in this study, inferior to 200 nm, for blank liposomes and liposomes containing actives. As well as, from De Assis et al. (2014) who developed liposomes with *Chlorella* extract, and obtained a diameter of 239± 4.21 and polydispersity index of 0.261± 0,00.

Table 3 - Results of particle size, polydispersity index, zeta potential and pH of blank liposomes and cocoa extract liposomes.

Parameter	Blank Liposome (BL)	Liposome with Cocoa Extract (LCE)
Size (nm)	195.7 ± 4.05[a]	167.5 ± 2.31[b]
PDI	0.258 ± 0.01[a]	0.262 ± 0.01[a]
Zeta (mV)	-14.67 ± 4.5[a]	-13.01 ± 5.2[a]
pH	4.25 ± 0.1[b]	5.34 ± 0.5[a]

Size values, pdi, zeta and pH, in the same column, followed by the same letter do not differ among them. Tukey Test at 5% probability level was applied ($p<0,05$).

In relation to the polydispersity index (PDI) of the formulations proposed in our work, the results showed values lower than 0,3, indicating the homogeneity in the size of the vesicles, not showing any statistic difference among the liposomes. For Cadeo et al. (2008) a PDI superior to 0.7 indicates that the sample has a wide distribution of size, and that inferior results indicate homogeneity in the particle distribution in the formulation.

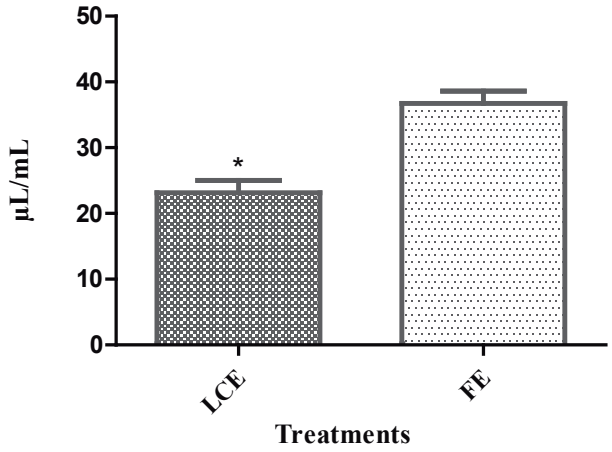
Yet, the zeta potential of both liposomes showed values closer to -14 mV, and the pH around 5.0, showing statistic difference among the formulations. For Ferreira et al. (2005) the zeta potential is used for characterizing different disperse systems. The method applied is based in the electrophoretic mobility evaluation of vesicles, using a cell containing two electrodes, what allows the movement of particles in suspension. For Van de Ven et al. (2011) the attraction speed depends on the surface charge, so, helping in the knowledge of its stability. Fang et al. (2006) prepared liposomes containing catechin, epicatechin and epigallatocatechin, and obtained zeta potential of -5mV, values under the ones found in this study, as table 3 shows. For Schaffazick et al., (2003) and Muller, Ghola and Keck (2011) the size of the particle, the zeta potential, the active content and the pH are physico-chemical parameters used for monitoring the nanoparticles stability.

In relation to the antioxidant activity evaluation, it was used the DPPH kidnapping method, which is based in the electron displacement through antioxidant action for a free radical, in which its purple coloring is reduced and evaluated by the absorbance reduction (CIEŚLA et al., 2012). The EC50 value for free extract is 40.64µl ± 5.51 and for the liposome containing cocoa extract is 23.14µl ± 0.71. Considering that EC_{50} refers to the concentration in which an active presents a response in the middle between the initial and maximum rates after a specified time of exposition, we can conclude that liposomes potentiated the antioxidant activity of cocoa extract. The data for the two treatments were

statistically analyzed by Student's test which showed a significant difference between LCE and FE (p-value = 0.0067, p <0.05). Moreover, it is important to highlight that the blank liposomes were also put under the same conditions and do not show antioxidant activity.

Regarding to the use of liposomal vesicles for improve antioxidant activity of natural compounds, our results are in accordance, for example, to the ones found by Soares, Graça and Machado (2014) that tested liposomes containing spirulina and obtained results that emphasizing the good performance of liposomes as an important tool to potentialize the antioxidant action. Figure 2 shows the results obtained for the liposome containing cocoa extract and free extract.

Figure 2 - Results of the antioxidant activity of liposomes containing cocoa (LCE) extract in relation to the free cocoa extract (FE).



CONCLUSION

The main phenolic compounds found in the cocoa extract are classified into tannins, metilxantine and flavonoids as the epicatechin, epigallocatechin, isochercetin and kaempferol. The polyphenol content found in the cocoa extract are related to the origin, and the cocoa variety. Liposomes containing cocoa extract with concentration of 1 mg/mL, obtained adequate nanotechnological characteristics and a better antioxidant performance in relation to the free extract. The antioxidant activity was higher in the liposome containing cocoa extract than the free extract. This results confirm the possibility of potentializing the antioxidant activity when using nanotechnology.

REFERENCES

AFZAL, M.; SAFER, A. M.; MENON, M. Green tea polyphenols and their potential role in health and disease. **Inflammo pharmacology,** v. 23, n. 4, p. 151-161, 2015.

ALI, F. et al. Identification of phenolic compounds in polyphenols-rich extract of Malaysian cocoa powder using the HPLC-UV-ESI -MS/MS and probing their antioxidant properties. **Journal of food science and technology**, v. 52, n. 4, p. 2103-2111, 2015.

BASNET, P. et al. Liposomal delivery system enhances anti-inflammatory properties of curcumin. **Journal of pharmaceutical sciences**, v. 101, n. 2, p. 598-609, 2012.

BENSON, H. A. Transdermal Drug Delivery: Penetration Enhancement Techniques. **Current Drug Delivery**, v. 2, n. 1, p. 23-33, 2005.

BOLIGON, A. A. et al. Antimicrobial and antiviral activity-guided fractionation from Scutia buxifolia Reissek extracts. **Acta Physiologiae Plantarum**, v. 35, n. 7, p. 2229-2239, 2013.

CADDEO, C. et al. Effect of resveratrol incorporated in liposomes on proliferation and UV-B protection of cells. **International Journal of Pharmaceutics,** v. 363, n. 1-2, p. 183-191, 2008.

CIEŚLA, Ł. et al. Approach to develop a standardized TLC-DPPH test for assessing free radical scavenging properties of selected phenolic compounds. **Journal of Pharmaceutical and Biomedical Analysis,** v. 70, n. 1, p. 126-135, 2012.

CHOI, D. Y. et al. Antioxidant properties of natural polyphenols and their therapeutic potentials for Alzheimer's disease. **Brain research bulletin**, v. 87, n. 2, p. 144-153, 2012.

DE ASSIS, L. M. et al. Development and characterization of nanovesicles containing phenolic compounds of microalgae Spirulina strain LEB-18 and Chlorella pyrenoidosa. **Advances in Materials Physics and Chemistry**, v. 4, n. 1, p. 6-12, 2014.

FANG, J. Y. et al. Enhancement of the transdermal delivery of catechins by liposomes incorporating anionic surfactants and ethanol. **International journal of pharmaceutics**, v. 310, n. 1, p. 131-138, 2006.

FERREIRA, H.; LÚCIO, M.; SIQUET, C.; REIS, S. Utilização de modelos membranares na avaliação da atividade de fármacos. **Química nova**, 39, 41-50, 2005.

ISAAC, V. L. B. et al. Protocolo para ensaios físico-químicos de estabilidade de fitocosméticos. **Revista de Ciências Farmacêuticas básica e aplicada**, v. 29, n. 1, p. 81-96, 2009.

JAFARI, S. M. et al. Nanoparticle encapsulation of fish oil by spray drying. **Food Research International**, v. 41, p. 172-183, 2008.

JALIL, A. M. M.; ISMAIL, A. Polyphenols in cocoa and cocoa products: is there a link between antioxidant properties and health? **Molecules**, v. 13, n. 9, p. 2190-2219, 2008.

JONFIA-ESSIEN, W. A. et al. Phenolic content and antioxidant capacity of hybrid variety cocoa beans. **Food Chemistry**, v. 108, p. 1155-1159, 2008.

KAMDEM, J. P. et al. Trichilia catigua (Catuaba) bark extract exerts neuroprotection against oxidative stress induced by different neurotoxic agents in rat hippocampal slices. **Industrial Crops and Products**, v. 50, p. 625-632, 2013.

MÜLLER, R. H.; GOHLA, S.; KECK, C. M. State of the art of nanocrystals-special features, production, nanotoxicology aspects and intracellular delivery. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 78, n. 1, p. 1-9, 2011.

SCHAFFAZICK, S. R. et al Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos. **Química Nova,** v. 26, p. 726-737, 2003.

SENTER, S. D.; ROBERTSON, J. A.; MEREDITH, F. I. Phenolic compounds of the mesocarp of cresthaven peaches during storage and ripening. **Journal of Food Science**, v. 54, n. 5, p. 1259-1268, 1989.

SOARES, L. A. S.; GRAÇA, C. S.; MACHADO, C. S. Avaliação da capacidade antioxidante pelo método de sequestro de radicais -dpph de extratos fenólicos de *spirulina* leb -18 encapsulados com lecitina de arroz. In: XXVI CONGRESSO REGIONAL DE INICIAÇÃO CIENTÍFICA E TECNOLÓGICA EM ENGENHARIA, 26., 2014. Alegrete - RS. **Anais...** Universidade do Pampa/UNIPAMPA, 2014. p. 2.

SZOKA, F.; PAPAHADJOPOULOS, D. "Procedure for Preparation of Liposomes with Large Internal Aqueous Space and High Capture by Reverse-Phase Evaporation." **Proceedings of the National Academy of Sciences of the United States of America**, v. 75, n. 9, p. 4194-4198, 1978.

THAKUR, L. et al. Novel approaches for stability improvement in natural medicines. **Pharmacognosy reviews**, v. 5, n. 9, p. 48, 2011.

TRONNES, J. N. Development of liposomal formulation for green tea catechins targeted for the treatment of vaginal inflammation. **Advances in Materials Physics and Chemistry,** v. 4, n. 1, p. 6-12, 2012.

VALENZUELA, A.; SANHUEZA, J.; NIETO, S. Cholesterol oxidation: Health hazard and the role of antioxidants in prevention. **Biological Research,** v. 36, p. 291-302, 2003.

VAN DE VEN, H. et al. Nanoparticles loaded with the antileishmanial saponina escin: Factor influence study and in vitro efficacy evaluation. **International Journal of Pharmaceutics,** v. 420, p. 122-132, 2011.

YEN, G. C.; DUH, P. D. Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species**. Journal Agricultural Food Chemistry,** v. 42, p. 629-632, 1994.

YOUNG, I. S. et al. The effects of desferrioxamine and ascorbate on oxidative stress in the streptozotocin diabetic rat. **Free Radical Biology and Medicine**, v. 18, n. 5, p. 833-840, 1995.

ZUMBÉ, A. Polyphenols in cocoa: are there health benefits? **BNF Nutrition Bulletin**, v. 23, p. 94-102, 1998.