STUDY ON ROBUSTA GREEN COFFEE BEAN NANOEMULSION EXTRACT

Đến toà soạn 06-07-2023

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TÓM TẮT

NGHIÊN CỨU VI NHŨ TƯƠNG DỊCH CHIẾT CÀ PHÊ XANH ROBUSTA

Nghiên cứu tạo chiết xuất hạt cà phê xanh Robusta bằng phương pháp tạo nhũ tương nano nước-dầu-dung môi thu được nhũ tương nano nước dầu chứa các chất có hoạt tính sinh học là 2,1% caffeine, tổng axit chlorogenic là 40%, 1,29% dầu, ngoài ra còn có quinic, ferulic và caffeic axit. Nhũ tương nano đạt kích thước 100-200 nm, PDI đạt 0,5 và thế zeta 150mV khi có phụ gia chiết chọn lọc là chất hoạt động bề mặt nhũ tương hoá nhỏ hơn 1,5% tạo vi nhũ tương nano ổn định ở pH 4,5 với mật độ 1,305 g/ml và tổng CGA cao hơn. Dịch chiết hạt cà phê xanh dạng nhũ tương nano được thử nghiệm hoạt tính chống oxy hoá với kết quả SC50 là 68,34ppm, có hiệu quả chống lại Gr (-) P. aeruginosa, Gr (+) S. Aureus và mốc F. oxysporum ở nồng độ ức chế tối thiểu MIC 0,012 - 0,025 g/ml và khả năng gây độc tế bào đối với tế bào Hep-G2 ở IC50 là 31,5ppm có CS(%) là 16,11%.

Từ khoá*: Hạt cà phê nhân GCB, axit chlorogenic CGA, caffein, dầu cà phê, hoạt tính kháng khuẩn, vi nhũ tương nanoelmusion, dịch chiết cà phê xanh GCB extract, Tween 80, Propylen glycol PG*

1. INTRODUCTION

Dak Lak province, Vietnam is the largest coffee production center with 204,808 hectares of arable land, the average output is 2.61 tons/ha, mainly growing Robusta coffee. Collecting bioactive compounds from coffee processing in order to create addvalue for the coffee production chain has been studied recently [1-8]. Chongsrimsirisakhol studied polyphenol release and antioxidant activity of the encapsulated green coffee bean (GCB) extract by using food additives [2]. Liza, Pettinato has extracted rich in phenolic compounds from used coffee grounds. The encapsulation process, using liposome as a coating agent that a high encapsulation efficiency leading to the production of polyphenol-rich micro-capsule that have potential industrial applications in the food and cosmetics fields [3,4]. The GCB extract with oil and solvent has antioxidant and antimicrobial effects in nanoemulsion preparations. The nanoemulsion was designed with a spontaneous emulsion method using oil and extract for the preparation nanoemulsion [5]. However, there are not many

studies nano elmusion for pharmaceutical and cosmetic applications, which use both the biological activities of caffeine and chlorogenic acid (CGA) - the main phenolic compound in coffee. Chlorogenic acids are esters of trans-cinnamic acids, such as caffeic acid, ferulic acid, p-coumaric acid, and quinic acid (QA). The main chlorogenic acids in green coffee are caffeoylquinic acid (CQA), dicafeoilquinicos (diCQA) and feruloylquinic (FQA). Therefore, the high content of chlorogenic acid in the products is very valuable because it is beneficial to cell regeneration, prevents collagen breakdown, inhibits nitrogen oxides, anti-aging, increases vascular endothelium, promotes decomposition of lipids and increases insulin. Caffeine is commonly used at less than 3% in topical skin products due to its high antioxidant and antibacterial activity, blocking solar radiation, stimulating blood circulation, helping skin firmness, and supporting skin health, support metabolism of substances, limit fat production [2-8]. Research on nano-emulsions as carriers containing essential oils or phenolics is currently under development because nano-size helps to increase biological effects, protect active ingredients, and increase retention time of biological active ingredients. The emulsification method using different concentrations of vegetable oils and extracts, after testing for antibacterial and fungal activity at different concentrations of nanoemulsions, showed that they have the ability to inhibit free radicals. Due to and good antibacterial activity at 85%-100% against S. *aureus* and S. *cholermidis*, inhibiting 88.79% of E. coli, indicating its potential for therapeutic and cosmetic applications [2-8]. In addition, Vietnam Robusta GCB extracts possessed the highest antioxidant activity compared to others [2]. Therefore, this study was conducted to determine the solvent and additives to obtain high bioactive compounds from Daklak-Vietnam Robusta GCB to evaluate extraction methods, the properties (emulsion formation, particle, stability) and testing the antioxidant, antibiotic and cytotoxicity of the nanoemulsions of Robusta GCB extract.

2. MATERIALS AND METHODS

2.1. Materials

Green coffee beans collected from Cu M'gar - Dak Lak Province of Vietnam. Samples were weighed, dried at 45^{o}C to constant weight, determined the moisture content. The material is ground to a size < 1 mm.

2.2. Chemicals, experimental equipment

Chemicals: Food alcohol 96^{o}, deion water, chloroform, hexane, ethyl acetate, Tween 80, propylene glycol PG.

Green coffee bean (GCB) extraction method: 100 grams of fine GCB powder soaked in ethanol with different alcohol ratios of 50-80^{o}, liquid/solid ratio is 1/10, extracted for 1 hour with ultrasound at 50-60Hz. Extraction temperature range from 25-90^{o}C. Centrifugation of solid and liquid extract phase.

Method for obtaining nanoemulsion GCB extract with high caffeine and chlorogenic acid content:

nanoemulsion GCB extract was obtained by spontaneous oil water emulsion method [6]. GCB extract (v/v), water alcohol (ml) and emulsifier (w/v) mixed with liquid phase containing green coffee oil (w/v), higher concentration ethanol (v/v) and emulsifier (w/v). Then the solvent is removed under low pressure and temperature <60^{o}C. Stop distillation when the solution reaches 40% by weight of extracted green coffee. Measure the pH, density, nano/micro emulsion particle size and chlorogenic and caffeine content.

Laboratory equipment: UV Absorption Spectroscopy vis Shimadzu 1800, D8-Advance 5005, S-4800 FESEM-Hitachi. Nicolet iS10 FTIR Spectrometer IR Spectrometer (Thermo Fisher Scientific USA, HPLC- Agilent 1260, Elemental analyzer model Flash EA 1112 CHNS-O/MAS200 Micromeritics TriStar II 3020 version 3.02. HORIBA SZ-100V2 for size analysis. Analysis methods of TCVN:9294 :2012,TCVN:8557:2010, TCVN:8559:2010, TCVN:8560:2010.

2.3. Analysis method

2.3.1. Caffeine analysis method

Caffeine concentration analysis was based on a caffeine calibration curve constructed at 274 nm with a UV-vis Shimadzu 1800. The solvent used to recover caffeine in the extract was chloroform with a caffeine recovery efficiency as high as 99%. The extract after filtration was aspirated 1mL, added to 10mL with chloroform and compared using chromatograms to compare and quantify with the standard curve [10].

2.3.2. 3-CGA analysis method

3-CGA analysis was based on a standard curve at 325 nm with UV-Vis and on HPLC high-pressure liquid chromatography with Merck 3-CGA standard. After filtering, the extract was decaffeinated with chloroform at a ratio of 1:1. Aspirate 1ml of the decaffeinated solution and make up the volume of 10ml with methanol and perform photometric comparison using chromatograms to compare and quantify with the standard curve or with the 3CGA standard on HPLC [9, 10].

2.3.3. Determination of total CGA

Use 5-CQA, 3-CGA as standards for CGAs, including monomers and dimers, or use the published conversion factors of monomers and dimers at 325 nm. The monomers were calculated based on their ratio to 5-CGA which is 1.00, 4-CGA 0.92 and 3-CGA 0.94. The conversion coefficients of the CGA dime averaged and scaled to 5-CQA are 1.65. The feruloylquinic acid conversion factor is averaged and approximated to 5-CQA is 0.99 [9]

2.3.4. Antimicrobial activity assay Evaluation of the antimicrobial activity of the samples was performed on a 96-well microtiter plate according to the method of Vander Bergher with the control microorganisms [11].

2.3.5. Antioxidant activity assay Determination of antioxidant activity based on the ability to scavenge free radicals generated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) by Brand Williams, the reagent was dissolved in dimethyl sulfoxide (DMSO 100%) and DPPH was mixed in 96% ethanol. Absorption of DPPH at λ = 515 nm using a TECAN reader (Infinite® 200 PRO, Switzerland) after adding DPPH to the sample solution on a 96-well microplate. Results expressed as the mean of at least 3 replicate trials± standard deviation ($p \leq 0.05$) [12]

Cytotoxicity assay was determined by Likhiwitayawuid applied for the cell line of Hep-G2 (Human hepatocellular carcinoma) at the National Cancer Institute and the College of Pharmacy, Chicago [13].

3. RESULTS AND DISCUSSION

3.1. Chemical composition of GCB

Dak Lak Robusta green coffee beans (GCB) are substandard seeds that are eliminated from the harvest of red ripe coffee, the beans are ground to a density of 0.85g/ml to increase extraction efficiency, the beans have a caffeine ratio of 2,1-2,7%, 3-CGA from 6.7-10%, coffee bean oil 0.1-0.7%.

3.2. Solvents for green coffee bean extract

The high temperature is beneficial for the extraction. However, in order not to affect the quality of biological active substances, the extraction temperature is carried out at less than 60^0C. The alcohol concentration affected the extraction efficiency and composition of substances. Sample M_1 extracted with 50^0 ethanol at 60^oC got high extraction efficiency of both caffeine and 3-CGA compared to the M_2 extracted with 80^0 ethanol or M_3 water extracted at the sample temperature. The amount of amount of 3-CGA reached 6.83 %, caffeine obtained 2.1%. Table 1, Table 2 and Fig.1, Fig.2 and chlorogenic acid is most soluble in dilute alcohol of 50% ethanol.

Table 1. Absorption of caffeine at 274 nm and 3-CGA at 325 nm

ABS of the extract	50^0 ethanol (M_1)	80^0 ethanol (M_2)	H_2O (M_3)
Caffeine	0.974	0.853	0.541
3-CGA	0.936	0.638	0.339

Table 2 is the results of analysis of active substances of Dak Lak Robusta coffee extract with alcohol 80^o and alcohol 50^o. Chlorogenic acid content is 6.7% and 6.83%, caffeine is 2.07% and 2.1%, caffeic acid 0.06% and 0.07%, Ferulic 0.03% and 0.035%. Alcohol extract 50^o has higher CGA and total CGA content is used to obtain nanoemulsion of bioactive compounds. The pH was 4-4,5 to prevent from the degradation of CGA.

Table 2. Components of GCB extract

	Alcohol solvent	**50^0 (M_1)**	**80^0 (M_2)**
1	pH	4.5	4.3
2	Density, g/ml	1.305	1.325
3	Chlorogenic acid, %g/100g	6.83	6.7
4	Caffeine, %	2.1	2.07
5	Caffeic acid, %	0.07	0.06
6	Ferulic acid, %	0.035	0.03
7	Coffee bean oil, %	1.29	1.05
8	Total CGA, %	40	39.2

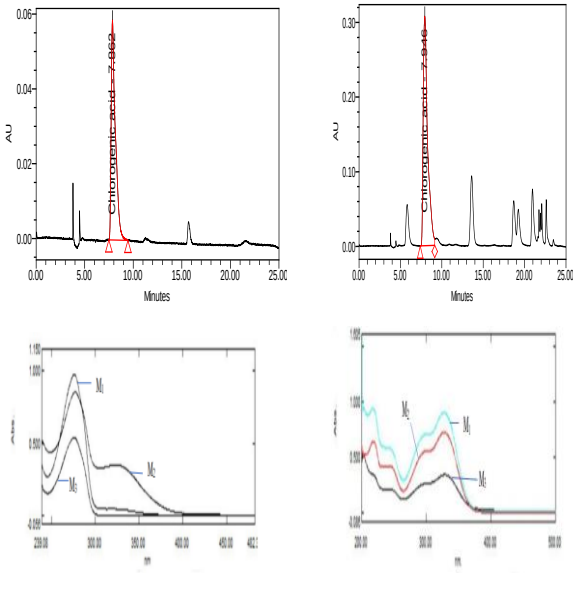


Fig. 1 a. HPLC of 3-CGA and of M_1

b. Caffeine and CGA UV-Vis of M_1,M_2, M_3

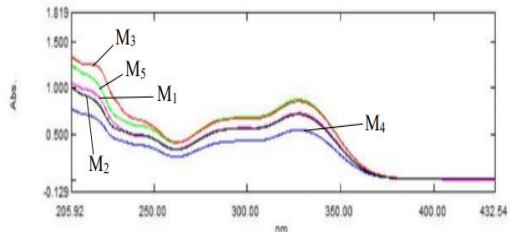
Most of the chlorogenic acid (CGA) is lost when roasting coffee (over 50%) [9], CGA in Dak Lak Robusta extract with 50^{o}C alcohol accounts for nearly 6.9% as a source of targeted antioxidants. Absorption of CGA in the stomach, converted to caffeic acid, quinic acid, converted to glucuronide, and sulphate reduces blood glucose-related diseases. CGA helps metabolize sugar for patients with diabetes, high blood fat. Green coffee extract is used for obesity type 2 diabetes, Alzheimer's disease, stroke, hypertensive endothelial function, no side effects. In particular, CGA helps skin regeneration and fights collagen-degrading enzymes [2-4].

3.3. Nanoemulsion GCB extract with selective extraction additive Tween 80 and Propylene Glycol PG

Prepare five nano emulsion GCB extracts samples denoted M_1-M_5. Samples denoted M_1- no selective extraction additive; M_2 -1% Tween 80; M_3 - 2 % Tween 80; M_4 - 3% Tween 80; M_5 -3% (Tween 80: PG is 1:1) by creating the nano/micro emulsion by spontaneous emulsion method with low pressure alcohol solvent vacuum evaporation at 60°C and get the extraction efficiency was 40%, the density of the extract was 1.305g/ml and the pH was 4-4.5. UV- Vis spectra of GCB extraction of M_1-M_5 listed in Table 3. The results showed that M_5 used 3% selective extraction emulsion (Tween 80: PG was 1:1) had the highest 3-CGA content with the absorption of 0.876nm compared to the others and having nanoemulsions size of 176.9, 199.7, 200.04 nm at 3 replicate trials (Fig. 2).

Table 3 Abs at 325 nm of CGA and its UV vis spectrum of extracts with additives

	M_1	**M_2**	**M_3**	**M_4**	**M_5**
Abs	0.727	0.714	0.857	0.545	0.876



M_3 used Tween 80 surfactant at 2% with Abs reaching 0.857. M_5 (1.5% Tween and 1.5% PG) gave the highest CGA extraction efficiency. The additives and surfactants have played the role of selective extraction additives for the efficiency of extraction and also the nanoemulsion stabiliser in water oil nanoemulsion method.

1% surfactant Tween 80 was added to M_1 and sonicated for 20 min more at 50-60Hz and analysed particle size by HORIBA SZ-100V2 machine. The nanoemulsion size of M_1 reached 100 nm, PID was 0.5, zeta potential 150 mV and 2.1% caffeine, 6,7% CGA was 6.7% and total CGA of 40%, Table 2, Fig 2.

The surfactants could play the role of selective extraction additive and nano emulsion stabilizer for green coffee bean extraction at the rate of 1-2%.

Nanoparticle size is seemed to related to the concentration of caffeine and chlorogenic acid. Tween 80 can be combined with PG at a rate of 1.5% in which the emulsion obtained a rich caffeine and chlorogenic acid nano emulsion of 100- 200 nm in size.

All nano/micro emulsions are stable when tested at 100 times dilution, 5000 rpm centrifugation and reached stable UV-Vis absorbance of nanoemulsions over time

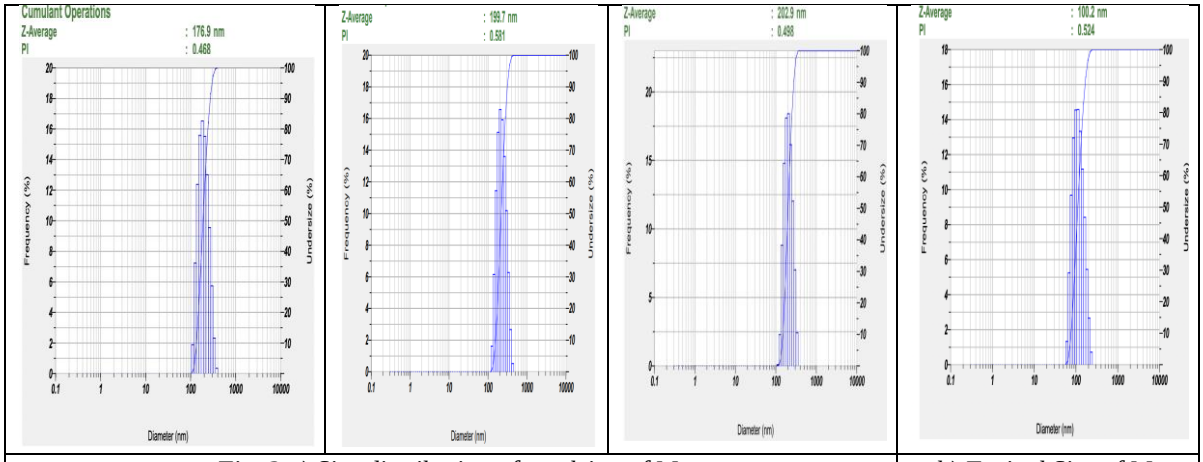


Fig. 2 a) Size distribution of emulsion of M_5 | *b) Typical Size of M_1*

Table 4. Antibiotic, antioxidant activity and cytotoxicity assay of nanoelmusion GCB extract

Antibiotic activity assay Minimum inhibitory concentration MIC,g/mL with initial concentration of sample 0,025g/mL	*Bacteria Gr (-)*		*Bacteria Gr (+)*		*Mushrooms*		*Yeast*	
	E. *coli*	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*A. niger*	*F. oxysporum*	*S. cerevisiae*	*C. albicans*
	(-)	**0.0125**		**0.025**		**0.0125**		(-)
Antioxidant activity assay	*Initial concentration of the sample (ppm)*		*Scavenging capacity (SC,%)*		*SC_{50} (ppm)*		*Results*	
DPPH/EtOH+ascorbic	**50**		**81.56 ±0.23**		**17.49**		Positive	
DPPH/EtOH + DMSO	-		0		-		Negative	
Green coffee extract M_1	**500**		**73.16±0.31**		**68.34**		Positive	
Cytotoxicity assay	*Highest test concentration*		*CS (%) Hep-G2 cell line*		*IC_{50} Hep-G2 cell line*		*Results*	
DMSO	-		100		-			
(+) control	5 µg/mL		**1.18±0.34**		**1.12 µg/mL**			
Green coffee extract M_1	100 ppm		**16.11±1.82**		**31.25** ppm		Positive	

3.4 Antibiotic, antioxidant and cytotoxicity assays of nanoemulsion GCB extract

GCB extract nanoemulsion has activity against 3 tested microorganisms *P. Aeruginosa*, *S. Aureus* and *F. Oxysporum* at 0.012-0.025g/mL of the Minimum Inhibitory Concentration. The DPPH-induced free radical scavenging efficiency of each sample was measured and calculated based on the percentage of free radical neutralization relative to the blank and negative controls. Table 4 shows that the antioxidant activity on the DPPH system at SC_{50} was 68.34ppm and also cytotoxic to Hep-G2 cells at IC_{50} 31.5ppm having CS(%) is 16,11%, Table 4.

Besides, CGA plays a role in regulating glucose and lipid metabolism, improving insulin resistance, and reducing the risk of type 2 diabetes and cardiovascular diseases. The daily oral doses of CGA at 13.5mg to 1200 mg can reduce fasting blood glucose (FBG), improve glucose tolerance, enable weight loss /prevent weight gain, and improve blood pressure in hypertensive patients. Daily intake of 200 mg or more may reduce FBG, with a dose-effect relationship in the range 13.5-500 mg/d. The U.S. Food and Drug Administration considers 400 milligrams (4 cups brewed coffee) a safe amount of caffeine for healthy adults to consume daily and reduce 50% for pregnant women and children under age 12 should not consume caffeine [8].

4. CONCLUSION

Using the food additive and surfactant Tween 80 and propylene glycol of low doze (less than 1.5%) could increase the effective of caffeine and chlorogenic extraction and making the stable nanoemulsion. The nanoelmusion GCB extract with high caffeine and chlorogenic is stabilized at pH of 4.5 with density of 1.305 g/mL and get the nanoemulsion particle size from 100nm -200nm. The nanoemulsion GCB extract against DPPH free radicals reached SC_{50} of 68.34ppm. The bioactive nanoemulsion extract from green coffee was effective against Gr (-) P *Aeruginosa*, Gr (+) S. *aureus* and F. *oxysporum* molds at minimum inhibitory concentrations MIC is 0.012-0.025g/mL and cytotoxic to Hep-G2 cells at IC_{50} 31.5ppm having CS (%) is 16.11%. The results of the analysis show that the nanoelmusion green coffee bean extract could be applied in pharmaceutical and cosmetic sector. Robusta nanoelmusion green coffee beans extract is having high bio active compounds was obtained by oil-water aqueous alcohol-solvent with surfactant assistant obtained an nano-emulsion of size of 100-200nm with high bioactive compound of caffeine and chlorogenic acid, high content of coffee oil, quinic, ferulic, caffeic acid, amino acid, nutrients and micro elements should be studied more.

Acknowledgments: The studies carried out with the fund from the Department of Science and Technology- DakLak PPC according to HD 14 dated 6 January 2023 and the supportive fund from VAST coded NCVCC07.02/22-23.

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