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Research on flavonoids collection, activity assay and initial steps to create tea from *Camellia tamdaoensis* Hakoda et Ninh

Khanh-Hung Nguyen[a], Phuong-Linh Pham[a], Bao-Khanh Lo Thi[a], Xuan-Phong Ong[b], Thi-Thuong Ngo[b], Kim-Dung Le[b], Viet-Hong La[b,*]

[a] *Xuan Hoa High School, Xuan Hoa, Phuc Yen, Vinh Phuc, Vietnam*
[b] *Hanoi Pedagogical University 2, 32 Nguyen Van Linh, Phuc Yen, Vinh Phuc, Vietnam*

Abstract

The *Camellia tamdaoensis* Hakoda et Ninh contains many useful phenolic compounds for human health, among them, flavonoids showed many biological activities such as antibacterial, anti-inflammatory. In this study, we used the recovery extract method to collect concentrated solutions with high flavonoid content from golden tea leaves, in which ethanol served as solvents at the temperature of 70°C for 1 hour . The high golden tea leaves showed good antibacterial properties with Gram (+), Gram (-) and fungi, demonstrate toxicity to HEPG2 liver cancer cell line with IC_{50} 100 ± 2.77 µg/ml. The process of creating tea bags from golden tea leaves with flavonoid supplements was completed. The ground golden tea leaves were sprayed with a solution containing flavonoids from 0.33 - 0.67 mg extract per extract grams of golden leaves, then dry until reaching a constant mass at 50°C. The product finally was packed into filter bags and investigated in terms of the quality of taste and color according to TCVN 3218: 2012. The flavonoid content in this product was 1.6 times higher than that of natural materials. This research's results show the potential to develop golden tea products containing high flavonoids in the future.

Keywords: Extract, flavonoid, activity, antibacterial, yellow flower tea

1. Introduction

Golden tea (Vietnamese name: trà hoa vàng - THV) is the popular name for a group of species in the genus *Camellia*, the family Theaceae with golden tea. According to the International Journal of Camellia, there are 62 species of golden tea all over the world, of which there are 22 species in

* Corresponding author, E-mail: laviethong@hpu2.edu.vn

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Vietnam and 44 species in China. The golden tea species are usually distributed from the South of Guangxi province in China to Vietnam. Golden tea was discovered in Vietnam at the beginning of the 20th century. Most species of golden tea have high economic value and are used as ornamental plants and medicine [1]. Golden tea contains many phenolic compounds such as ellagitannin, taxifolin deoxyhexose, proanthocyanidin, kaempferol derivative, apigenin derivative, glucosyl isorhamnetin, quercetin derivative and platphylloside. Phenolic compounds in golden tea have great medicinal value such as reducing the likelihood of stroke, preventing cancer, supporting the elasticity of blood vessels and regulating blood pressure [2]. Flavonoids are the largest group of natural phenolic compounds, with many biological activities such as antibacterial, inhibiting mitochondrial adhesion, anti-ulcer, anti-arthritic, anti-angiogenic, anti-cancer, and inhibiting protein kinase [3]. In Vietnam, there have been a number of studies on the chemical composition of flowers and leaves of golden tea, such as Nguyen Thi Hong Van et al (2018) [4], N. T. Tuyen et al (2020) [6], Ha Van Huan & Nguyen Van Phong (2015) [7]. The current products from golden tea are still relatively simple, mainly collecting dried flowers for canning, only a few products are made in the form of capsules. However, due to slow growth, and long flowering time, the exploitation of golden tea in the wild takes place indiscriminately, leading to a very limited source of golden tea provided to the people. Based on the above reasons, we proposed the topic "Research on flavonoid collection activity assay and initial steps to create high flavonoids tea in Tam Dao (*Camellia tamdaoensis* Hakoda et Ninh)".

2. Materials and research methods

2.1. Materials

Camellia tamdaoensis Hakoda leaves were collected in Tam Dao National Park in Dai Dinh, Tam Dao, Vinh Phuc. The alcohol chemicals included ethanol (Vietnam), HCl, NaOH, NH_3, $FeCl_3$ solutions (China) provided by the Institute of Scientific and Applied Research, Hanoi Pedagogical University 2.

2.2. Research Methods

2.2.1. Research Diagram

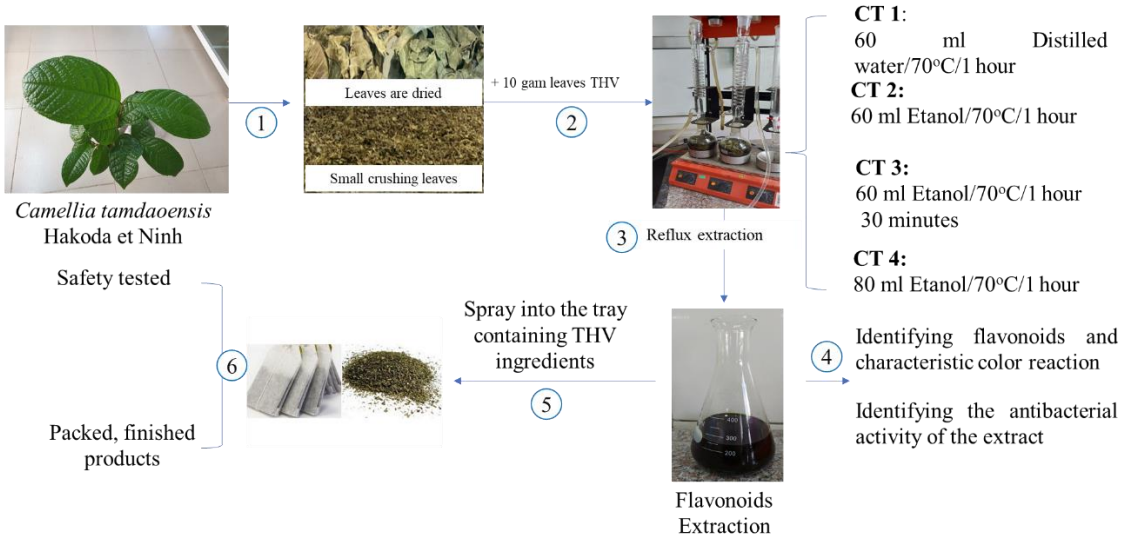

Figure 1. Diagram of the research process

2.2.2. Research Methods

a. Flavonoid extraction method

Preparing flavonoid extract:

Leaves of *Camellia tamdaoensis* Hakoda et Ninh were washed, dried at 50^{o}C and then ground using household blenders. Extracting flavonoids using reflux extraction, in which the solvent was 90% ethanol, and the ratio of sample and solvent was 10 grams of sample and 60 or 80 ml of solvent at 70^{o}C for 1 hour and 1 hour 30 minutes (Figure 1). The formulation using water as the solvent was considered as the reference formulation.

Identifying flavonoids and characteristic color reaction: (1) Cyanidin reaction; (2) Reaction with 10% NaOH; (3) Reaction with alkali NH_3; (4) Complexation reaction with 5% $FeCl_3$ as described by Nguyen Viet Dan and Nguyen Viet Tuu (1980) [8].

b. Identifying the antibacterial activity of the extract

The antimicrobial activity was tested based on the multi-concentration dilution method described by Luu Vu Phuong et al (2021) [9]. The strains of the microorganisms tested were: *Escherichia coli* ATCC25922, *Pseudomonas aeuginosa* ATCC27853, *Salmonella enterica* ATCC12228, *Enterococcus faecalis* ATCC299212, *Staphylococcus aureus* ATCC25923, *Bacillus cereus* ATCC14579, *Candida albicans* ATCC10231.

c. Determining in vitro cytotoxicity against the liver cancer cell line Hepg2

MTT (3-[4,5-dimetylthiazol-2-yl]-2,5-diphenyltetrazol brom) assay for *in vitro* cytotoxic activity against the liver cancer cell line HepG2. Cells were grown overnight for plate stabilization. Next, the compound to be studied was added with different concentrations from low to high and repeated 3 times. Dimethyl Sulfoxide (DMSO) was used as a negative control and performed similarly. Samples were incubated for 24 or 48 hours. Next, DTT [(4,5-dimethylthiazol -2-yl)-2,5-diphenyl tetrazolium bromide] 5 mg/mL (Sigma-Aldrich) was added to the wells (10% volume) and incubated for 4 hours at 37°C. Then all the environment was removed. Cells were washed twice with phosphate buffer. Cell survival was measured by the MTT assay. Formazan crystals were dissolved in DMSO and the absorbance was measured at 562 nm. The data were analyzed and compared with the control group. Toxicity was determined by the formula: % toxic = ((control - sample)/control) x 100. The survival cells after treatment were observed by microscope at 40X magnification.

d. Creating golden tea products

The steps to create tea bags were as described previously [10], [11], [12] with some improvements: Leaves were dried at 40^{o}C. Then, dried leaves were ground using blenders. The flavonoid extract from THV leaves was used to spray into the tray containing THV ingredients, which were then dried at 50oC for 3 hours. Two grams of THV were put into a filter bag and packed according to the procedure described previously. The quality assurance of the product formulation (CT1: 100 THV leaf materials, CT 2: Spraying 0.33 ml of THV leaf extract/gram, CT 3: Spraying 0.67 ml of THV leaf extract/gram, CT 2: Spray 1.0 ml of extract/gram of THV leaves) was conducted using the scoring method according to TCVN 3218:2012. Accordingly, each product formula was surveyed in 20 people, aged from 15 to 50 years old. The average scores of all surveys were calculated. Then, the importance of each criterion was calculated and evaluated with the total score from 18.2 to 20 being Good, from 15.2 to 18.1 being Fair, from 11.2 to 15, 1 being Moderate, from 7.2 to 11.1 being Poor, and ≤ 7.1 being Bad. Determination of total flavonoid content at the Institute for Food Safety and Hygiene.

3. Results and discussion

3.1. Extraction of flavonoids

The flavonoid extracts in different solvents have different characteristic colors. In this study, the ethanol extracts were yellow-green (test tube #1, Fig. 2c, e, g), while the aqueous extracts were yellow (test tube number 1, Figure 2a).

Table 1. Characterization of THV leaf extracts containing flavonoids by characteristic color reaction

No.	Qualitative reaction	Test results of extracts from the formulas			
		1	2	3	4
1	Cyanidin reaction	+	+++	++	++
2	Reaction with NaOH 10%	+	+++	++	++
3	Reaction with $FeCl_3$	+	+++	++	+++
4	Reaction with NH_3	+	+++	+++	+++

Notes: (+) see the characteristic color of the reaction; (++) characteristic color is evident; (+++) featured color is very clear/bold

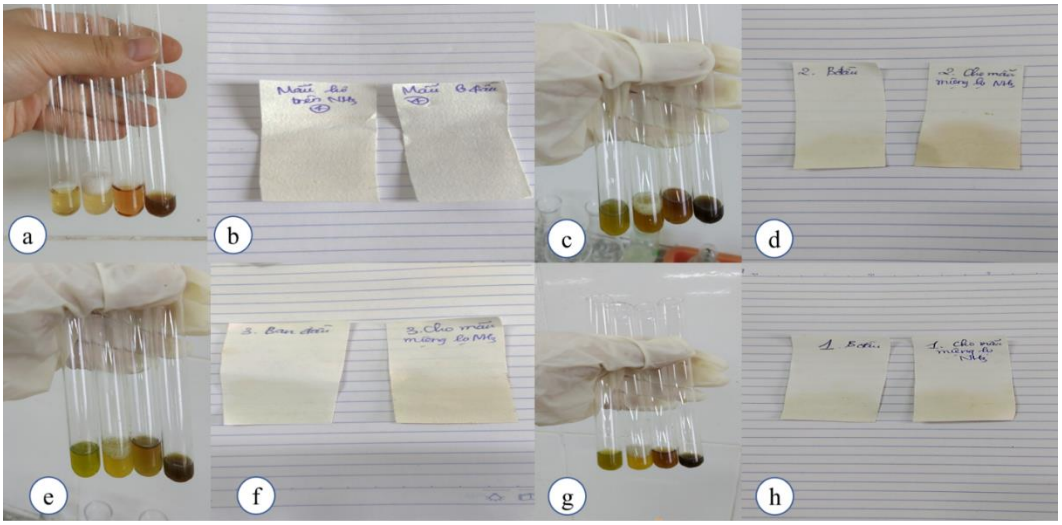

Figure 2. Flavonoid qualitative reaction based on characteristic color reaction

(a), (b) formula 1, (c), (d) formula 2, (e), (f) formula 3, (g), (h) formula 4; For each formula, test tubes from left to right: Cyanidin reaction- 10% NaOH reaction -FeCl3 reaction- NH3 reaction

The results show that, in all 4 CTs, there was a characteristic color reaction of flavonoids, the Cyanidin reaction (test tube No. 2, Figure 2a, c, e, g) had a color shifting from yellow-green to darker yellow. The reaction with 10% NaOH (tube number 3, Figure 2a, c, e, g) resulted in a yellow-brown precipitate. When adding 1ml of water, the color turned darker brown. Reaction with alkaline NH_3 (Figures 2b, d, f, h) included: Putting a drop of the extract on filter paper, drying and placing it on top

of a vial containing concentrated NH_3. It was observed that the dot had a darker color (the color indicated that the medicinal product contained flavones, flavonols, flavanols). The complexation reaction with 5% $FeCl_3$ (test tube No. 4, Figure 2a, c, e, g) resulted in a greenish-brown precipitate.

Based on the color intensity of the characteristic reaction (Table 1), it can be concluded that formula 2: flavonide was extracted most efficiently among the four studied formulations, namely: 10 g of crushed THV leaves, supplemented with 60 ml of ethanol refluxed at 70ºC for 1 hour. The extract was obtained by vacuum distillation to a high yield, for further research. The flavonoid extract from THV was determined by a characteristic reaction, shown in Figure 2 and Table 1.

3.2. Results of determining the antibacterial ability of extracts containing flavonoids

In some previous experiments, polyphenols from golden tea had an inhibitory effect on the plant pathogenic fungi *Bipolaris maydis*, *Colletotrichum musae* and *Fusarium oxysporum* by inhibiting the growth of mycelium, the germination of spores [13]. In this study, the antibacterial ability of the extracts from golden tea leaves is shown in Table 2.

Table 2. Tested antibacterial ability of golden tea extract

Samples	**Gram +**			**Gram -**			**Yeast**
	E. faecalis	*S.aureus*	*B. cereus*	*E. coli*	*P. aeruginosa*	*S. enterica*	*C. albicans*
MIC (µg/ml)							
THV extract	128	256	128	128	64	256	32
Steptomycin	256	256	128	32	256	128	-
Cyclohexamide							32

The analysis showed that: For Gram (+): THV has equivalent resistance to Steptomycin in strains of *Staphylococcus aureus* ATCC25923 and *Bacillus cereus* ATCC14579; better resistance to Enterococcus faecalis strain ATCC299212. For Gram (-) bacteria strains, the resistance of THV extract was lower than that of the control agent Steptomycin in *Escherichia coli* strain ATCC25922 and *Salmonella enterica* strain ATCC13076, while for *Pseudomonas aeruginosa* strain ATCC27853, golden tea extract had good resistance. than Steptomycin control. For the yeast: THV showed resistance comparable to that of the control cyclohexamide in Candida albicans strain ATCC10231.

Thus, through the above results, it can be seen that the resistance to bacterial and yeast strains of golden tea was relatively good, which was the basis for us to propose the plan to use golden tea to make tea products. Filter bags enhance the body's resistance and immunity.

3.3. In vitro cytotoxicity with the liver cancer cell line Hepg2

In particular, flavonoids acted as an antioxidant and protected against cardiovascular diseases, some forms of cancer, and age-related degeneration of cellular components. Their polyphenolic nature allowed them to scavenge harmful free radicals such as super oxides and free hydroxyl radicals [14]. The flavonoid extract from THV leaves was evaluated for its cytotoxic activity against the liver cancer cell line Hepg2. The results are shown in Figure 3 and Figure 4.

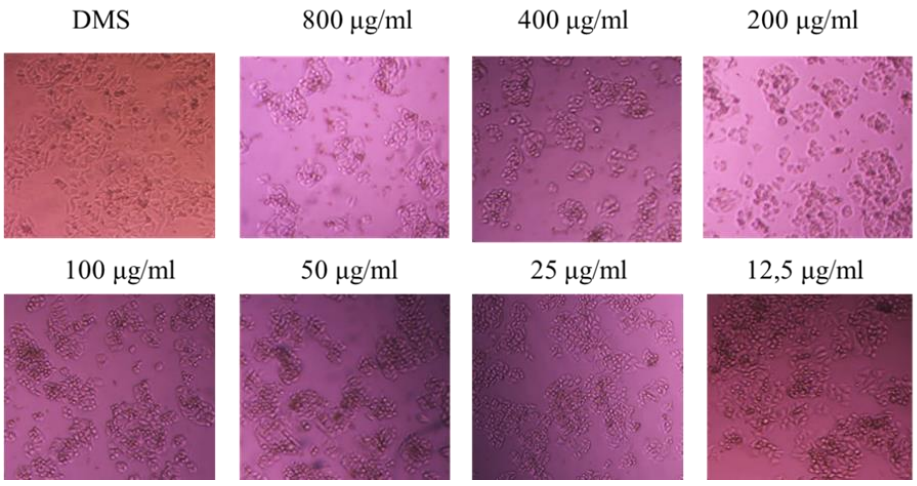

Figure 3. Density and morphology of HepG2 cells when treated with samples at different concentrations.

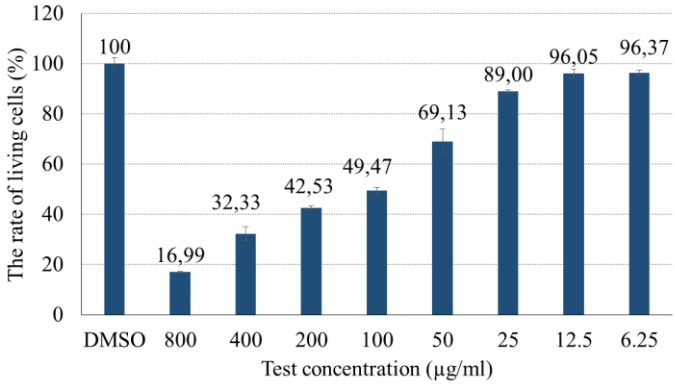

Figure 4. Cytotoxic activity of golden tea extract

The results showed that the density of HepG2 cells decreased significantly when treated with golden tea extract and was dependent on the treatment concentration (Figure 3). The growth inhibitory concentration with HepG2 cells, when treated with THV samples, was recorded with IC_{50} value = 100±2.77 µg/ml.

3.4. Creating products with golden tea with flavonoid content

Tea bags are a very convenient product for users, many products have been developed such as chlorophyll tea bags [10], from pomelo peel [11]. In this study, the steps to create tea bags from golden tea with added flavonoids are described in Figure 5.

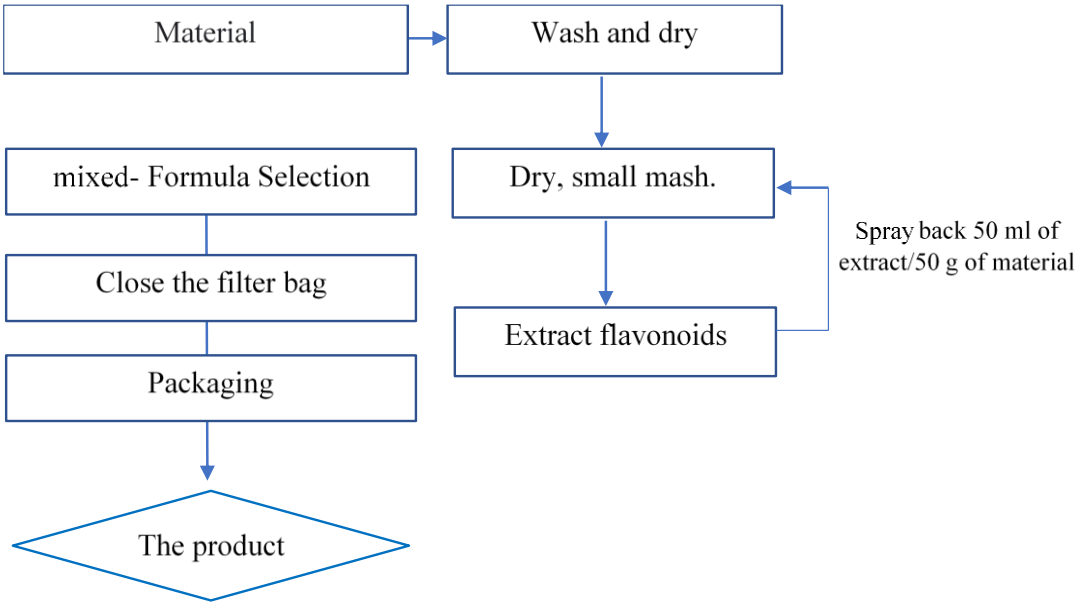

Figure 5. The proposed technological process to create flavonoid golden tea in the form of filter bags

Figure 6. Several steps in the process of making tea bags with flavo+ golden tea

(a) the material was dried and crushed; (b) sprayed the flavonoid-containing solution back into the material; (c) dried the materials; (d) packing; (e) finished the product

Table 3. Determination of sensory criteria by scoring method for golden tea products (according to TCVN 3218:2012)

For mula	**Average score for each evaluation criterion**				**The importance of each criterion**				**Total scores**
	Clarity	Color	Smell	Taste	Clarity	Color	Smell	Taste	
1	3,35	3,00	3,25	3,35	3,35	1,80	3,90	4,02	13,07
2	4,45	4,70	4,30	4,35	4,45	2,82	5,16	5,22	17,65
3	4,05	4,05	4,35	4,40	4,05	2,43	5,22	5,28	16,98

3	3,10	3,10	3,50	3,70	3,10	1,86	4,20	4,44	13,60

Analysis of Table 3 shows that tea samples in different formulations had different composite scores, ranging from 13.07 to 17.65 (points). Among those formulas, formulas 2 and 3 achieved the quality level of Good (according to TCVN 3218:2012), and the remaining two formulas reached the quality level of Medium. Thus, the initial determination in the spray formula of 0.33 - 0.67 ml of THV leaf extract/g gave the product a good level, which is a potential formula for carrying out further studies.

Next, formula 2 was used to test the total flavonoid content, the control to determine the flavonoid content was golden tea leaves collected from the wild. The results showed that the flavonoid content in these two formulas reached 4.43 (mg/g) and 2.76 (mg/g), respectively, meaning that the flavonide content in tea products produced in this study was 1.6 times higher than natural.

4. Conclusion

- Extracts containing flavonoids were effectively extracted from *Camellia tamdaoensis* Hakoda leaves by reflux extraction with ethanol solvent at 70^{o}C for 1 hour.

- Extracts from THV leaves showed relatively good activity against Gram (+), Gram (-) and *Candida albicans* ATCC10231 fungi. The extract exhibited toxicity to the liver cancer cell line HepG2 with IC_{50} value = 100±2.77 µg/ml.

- The process of creating THV tea products in the form of filter bags was completed. The additional spray formula of 0.33 - 0.67 ml of extract/g of THV leaves was suitable. The flavonoid content in the product was 1.6 times higher than that of natural materials. This is a potential recipe for commercial product development.

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