Research Article

Antibacterial hydrogel containing *Piper betle* L. extract for acne treatment, an *ex vivo* investigation

Duyen Thi My Huynh[1], Minh-Ngoc T. Le[1], Van De Tran[2], Duy Toan Pham[3*]

[1] Department of Pharmaceutical and Pharmaceutical Technology, Faculty of Pharmacy, Can Tho University of Medicine and Pharmacy, Can Tho, Vietnam
[2] Department of Drug Administration, Faculty of Pharmacy, Can Tho University of Medicine and Pharmacy, Can Tho, Vietnam
[3] Department of Chemistry, College of Natural Sciences, Can Tho University, Can Tho, Vietnam

ABSTRACT

The current treatments of acne vulgaris and acne-like disorders such as gram-negative folliculitis possess lots of unwanted side effects. Thus, alternative approach of utilizing natural plant extracts, specifically *Piper betle* L., have gained much attention. To this end, this work developed, characterized, and *ex vivo* evaluated novel antibiotics hydrogels containing *P. betle* L. leaf extract for acne treatment. Firstly, the design of experiments (DoE) D-optimal method was successfully developed, optimized, and validated, to investigate the relationship between *P. betle* L. extraction conditions and the extract properties. Secondly, the best extract was encapsulated in the hydrogel formulations composed of carbopol 940, propylene glycol, and cocamidopropyl betaine. Finally, the hydrogel was *ex vivo* determined its antibacterial activity on bacteria isolated from 15 patient acne samples. The optimal extraction condition being an extraction solvent/plant weight ratio of 4.034:1, an extraction time of 2.147 h, and a water extract volume of 91.4 mL. This condition yielded an extract total phenolic content of 3.337±0.034 g GAE/g, and minimum inhibitory concentrations of 32 µg/mL and 128 µg/mL on *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, respectively. The hydrogel possessed suitable properties for a topical medication, including a viscosity of 6800 cps, a pH of 7.0, and a good foaming ability, at both 10°C, 25°C, and 40°C. The hydrogel showed higher antibacterial activity than the positive controls in both gram-positive and gram-negative bacteria. Conclusively, the hydrogel could become a potential pharmaceutical product for acne treatment.

Keywords:
Acne, *Piper betle* L., Extraction, Hydrogel, Antibacterial, Design of experiments

1. INTRODUCTION

Acne vulgaris and acne-like disorders such as gram-negative folliculitis (herein preferred as acne for the entire article), are uncomfortable facial skin problems that possesses a prevalence of 80% in adults[1]. This disease significantly decreases the patient self-confidence and self-esteem, especially in teenagers. There are 4 common causes of acne, namely high sebum production, pilosebaceous follicles keratinization, uncontrolled bacterial growth, and immune-response inflammation. Among them, acne caused by bacteria such as the gram-positive *Staphylococcus aureus (S. aureus)* (for acne vulgaris) and gram-negative *Escherichia coli (E. coli)* (for gram-negative folliculitis), are often serious and demand urgent

treatments[2-5]. Nevertheless, these treatments, including topical benzoyl peroxide, topical/oral antibiotics, topical retinoids, and isotretinoin, although effective, cause lots of unwanted side effects that pose a significant health risks in patients[6-7]. Thus, numerous alternative approaches have been investigated, namely utilizing the natural plant extracts[8-13]. For instance, the green tea polyphenols demonstrates antibacterial action in acne infections and reduce the dermal sebum secretion[11]. To this end, an interesting plant that has gained much attention is the *Piper betle* L.

Belong to the family Piperaceae, *Piper betle* (Betel vine, Betel, Betle, "Trầu không" [*in Vietnamese*]), originated from Central and East Malaysia and has been cultivated for more than 2500 years[14-15], is a popular plant in

*Corresponding author:
*Duy Toan Pham Email: pdtoan@ctu.edu.vn

Vietnam. In fact, *P. betle* could be found, either as cultivation or wild plant, in almost every family gardens and farms/fields in the entire three regions of Vietnam (i.e., the North, the Central, and the South), except in the high mountains of above 1500 m. The *P. betle* cultivation in Vietnam and other Asian countries is well associated with the people's custom of consuming *P. betle* in daily life for disease symptom alleviations such as headaches, abdominal pain, gingivitis, colds, and coughs. Recently, *P. betle* has been extensively investigated on its chemical composition and therapeutic effects including antifungal, antibacterial, anthelmintic, antioxidant, wound healing, gastroprotective effects, anti-inflammatory, immunomo-dulatory, and anticancer[16-21]. The main pharmacological components of *Piper betle* are essential oils (0.7-2.6% w/w), of which eugenol, carvacrol, chavicol, catechol, chavibetol, cineol, estragol, caryophyllene, and cadinen are the most dominant compounds[14,16-17,20]. Interestingly, these substances, especially the polyphenolic ones, demonstrate potential antibacterial ability[22-23], which could serve as a safe and effective alternative therapy for the acne treatment, especially for the antibiotics resistant bacteria[18,24]. For instance, tannins could induce bacterial cell membrane damages, phenolic acids could disrupt the membrane integrity, causing leakage of the bacterial intracellular constituents, flavonoids could form complexes with trans-membrane bacterial proteins and inhibit bacterial DNA synthesis[25]. Additionally, *P. betle* leaf extract has shown outstanding antibacterial action in clinical trial in conjunctivitis patient[26]. In the market, the *P. betle* extract appears in numerous stuffs including food, beverage, cosmetics, and supplementary products[17]. Nevertheless, systematic investigations on the influences of the extraction factors such as the solvent/plant ratio, the extraction time, and the extract amount on the *P. betle* antibacterial effect have not been reported. Moreover, research on the pharmaceutical dosage forms containing *P. betle* extract are still limited.

Regarding the pharmaceutical dosage form, hydrogels are one of the most common topical preparations in the skin and mucous membranes for local infectious treatments such as acne. Hydrogels are soft physical formulations that could be formed using suitable gelling excipients including polysaccharide (i.e., starch and sodium alginate), cellulose derivatives, and polymers of fibroin and acrylic acid[27-28]. Due to their outstanding properties of softness, high water content, flexibility, and biocompatibility[29], hydrogels are potential pharmaceutical formulations for delivering *P. betle* extract for the purpose of acne treatments.

Therefore, this study, for the first time, systematically investigated the relationship between various *Piper betle* extraction conditions and the extract total phenolic content as well as their antibacterial effects on *S. aureus* and *E. coli*, using the design of experiments (DoE) method. Moreover, the best extract was then encapsulated in the hydrogel formulations. The *P. betle* loaded hydrogels were then characterized and *ex vivo* determined their antibacterial activity on bacteria isolated from human acne samples.

2. MATERIALS AND METHODS

2.1. Materials

Piper betle leaves were collected at An Giang province, Vietnam, in 2021. The plant samples were analyzed and classified by a botanical specialist. Dichloromethane, methanol, propylene glycol, menthol, sodium carbonate, sodium hydroxide, sodium benzoate, and cocamidopropyl betaine were imported from Xilong Scientific Co., Ltd., Guangdong, China. Folin-Ciocalteu reagent and gallic acid were bought from Sigma-Aldrich, Steinheim, Germany. Carbopol 940 was purchased from CISME, Milan, Italy. All other chemicals for bioassay were of reagent grade or higher.

2.2. *Piper betle* L. extraction and experimental design

Freshly collected *Piper betle* leaves (200 g) were dried at room temperature for approximately 3 days or until the plant moisture (i.e., water content), measured by an infrared moisture analyzer (Kern DAB 100-3, Germany), reached a value of less than 10%. Then, the leaves were ground to pieces, and extracted with water using a reflux extraction method at 70°C. The extracts were then distributed with dichloromethane and this fraction was solvent evaporated by a rotavapor. Finally, the extracts were determined their total phenolic content and anti-bacterial effects on *S. aureus* and *E. coli*. The DoE method, with the D-optimal design (BC-Pharsoft OPT software), using the response surface methodology, was conducted with 3 qualitative factors and 3 corresponding responses. The quadratic model was utilized for the best-fit relationship of the variables and responses. The design aimed to investigate the effects of 3 extraction factors including (1) X_1: extraction solvent/plant weight ratio (3, 4, and 5), (2) X_2: extraction time (1, 2, and 3 h), and X_3: the water extract volume for the distribution with dichloromethane (20, 60, and 100 mL), on the extract antibacterial efficacy on *S. aureus* (Y_1), and *E. coli* (Y_2), as well as the total phenolic content (Y_3). The design was validated with the acceptance criteria of p-values of <0.05, the predicted and adjusted R^2 values differences of <0.2. The optimal condition was set at "maximum" for all three responses. Finally, this condition was used in the wet lab and the real responses were compared with the theoretical parameters, and the model desirability was evaluated.

2.3. Total phenolic content determination

The extract total phenolic content was analyzed utilizing the Folin-Ciocalteu reagent, following the Singleton method with minor modifications[30]. To this end, 0.5 mL of the *Piper betle* extracts were added to a mixture of 2.5 mL Folin-Ciocalteu solution (10% v/v) and 2 mL Na_2CO_3 solution (7.5% w/v), followed by incubation at 37°C in the dark for 1 h, and UV-Vis spectroscopic measured at 765 nm (Jasco V-730, Japan). The total phenolic contents were then calculated based on the gallic acid standard curve (range: 10-60 µg/mL, equation: y=0.0121x+0.0043, R^2=0.9995) and equation (1). The results are expressed in terms of g gallic acid equivalent (GAE) per the total plant weight.

$$\text{Total phenolic content } \left(g\frac{GAE}{g}\right) = \frac{\text{Amount of polyphenol in the extract (mg)}}{1\text{-The plant powder humidity}} \quad (1)$$

2.4. *In vitro* antibacterial efficacy of the extract

The extract antibacterial efficacy on *S. aureus* and *E. coli* were calculated based on equation (2). Regarding the minimum inhibitory concentration (MIC), these values were obtained from the agar dilution method, referenced by the Clinical and Laboratory Standards Institute (CLSI)[31] on *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. Briefly, 0.512 g extracts were diluted in 10 mL DMSO, followed by serial dilution to get a range of different concentrations. These solutions were then mixed with 25 mL sterile Mueller-Hinton agar (MHA) medium to get final concentrations from 8 to 1024 µg/ mL. These mixtures were pour into a petri dish, and allow to solidify to obtain a 4-mm thick agar gel. Finally, 1-2 µL of the test bacterial suspensions were applied to each marked portion with a density of 10^4 CFU/mL. The inoculated agar plates were then incubated at 37°C for 18-24 h. The MIC values were determined as the lowest concentration at which the bacteria are completely inhibited (i.e., cannot grow in the agar). The experiment positive controls were vancomycin for *S. aureus* and colistin for *E. coli*, and the negative control was DMSO.

$$\text{Antibacterial efficacy} = \frac{\text{Amount of the extract}}{\text{Minimum inhibitory concentration}} \quad (2)$$

2.5. Hydrogel formulation and characterizations

The optimal *Piper betle* extract, with highest total phenolic content and antibacterial efficacy, was encapsulated in a hydrogel formulation composing of *Piper betle* extract (3%), menthol (0.2%), carbopol 940 (0.4%, 0.6%, 0.8%, or 1%), propylene glycol (10%, 12%, or 15%), cocamidopropyl betaine (2%, 3%, or 4%), and sodium benzoate (0.1%) (Table 1). Regarding the formulation process, carbopol 940 was dispersed in 20 mL water, followed by mixing with propylene glycol, and pH

adjusted to 7.0 by NaOH 10%. Then, menthol ethanolic solution, sodium benzoate, cocamidopropyl betaine aqueous solution, and *Piper betle* extract, were slowly added to the mixture. The final products were kept at 10°C, room temperature (25°C), and 40°C for further investigations on their properties.

The effects of carbopol, propylene glycol, and cocamidopropyl betaine on the product properties of viscosity, foaming ability, and pH, were then determined. The hydrogel viscosity was measured by a viscometer (Brookfield, USA), with a S64 spindle, and a stirring speed of 6.0 rpm. The pH was determined using a pH meter (C1020, Consort, Belgium) at 25°C. For the foaming ability, 1 mL of the hydrogel was added to 5 mL of water in a 100 mL beaker, followed by stirring at 200 rpm for 30 s. The hydrogel foaming degree was assessed by measuring the height of the foam formed. The gel has good foaming ability, denoted as (+) in the results, when the foam layer height is more than 1 cm.

2.6. *Ex vivo* antibacterial activity test on human acne samples

The optimal *Piper betle* extract loaded hydrogel formulation was then studied its *ex vivo* antibacterial actions utilizing agar diffusion method based on CLSI guidelines[31]. Different bacterial strains were isolated from acne samples collected from 15 patients treated at Can Tho Dermatology Hospital, Vietnam. All sample collecting processes were performed according to the ethical principles for medical research outlined in the Helsinki Declaration[32]. The research ethics were approved by Can Tho University of Medicine and Pharmacy, Vietnam (approval number 1675/DHYDCT. NCKH, December 21, 2018).

After being isolated from the acne samples, the bacteria were concentrated to 10^8 CFU/mL, spread evenly with a sterile cotton swab onto MHA agar plates (3 times, after each time, rotate the plate 90°), followed by incubation at 37°C for 24 h. The dominant colonies with similar characteristic morphology (i.e., 0.5-to-3-mm round/convex/concave colony with similar color) were further sub-cultured in other MHA agar plates. The samples were continued to be incubated at 37°C for 24 h. The bacterial strains were then isolated from the agars and identified by rRNA-sequencing technique with appropriate primers. Then, the agars contained the identified bacteria were punched 6-mm-diameter holes, the holes were 25-mm apart from each other's. Finally, 50 µL of the *Piper betle* extract loaded hydrogel was inserted into each hole, followed by incubation at 37°C for 18-24 h. The zone-of-inhibition diameters were then measured and reported. For the positive controls, vancomycin and colistin was used for gram-positive and gram-negative bacteria, respectively. The negative control was the hydrogel without *Piper betle* extract.

Table 1. *Piper betle* extract loaded hydrogel formulations, x and y is the optimal amount of carbopol 940 (in the range 0.4-1%) and propylene glycol (in the range 10-15%), respectively.

Formula	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Carbopol 940 (%)	0.4	0.6	0.8	1	x	x	x	x	x	x
Propylene glycol (%)	10	10	10	10	10	12	15	y	y	y
Cocamidopropyl betaine (%)	3	3	3	3	3	3	3	2	3	4
Piper betle L. extract (%)	3									
Sodium benzoate (%)	0.1									
Menthol (%)	0.2									
NaOH 10%	Adjust pH to 7.0									
Water (%)	Fill up to 100									

Table 2. D-optimal designated combinations of 3 independent variables (X_1: extraction solvent/plant weight ratio (3, 4, and 5), X_2: extraction time (1, 2, and 3 h), and X_3: the water extract volume for the distribution with dichloromethane (20, 60, and 100 mL)) and their corresponding responses of the extract antibacterial efficacy on *S. aureus* (Y_1) and *E. coli* (Y_2), and the total phenolic content (Y_3, g GAE/g).

Formula	X_1	X_2 (h)	X_3 (mL)	Y_1	Y_2	Y_3 (g GAE/g)
1	3	1	20	40625	10156	1.969
2	4	3	20	73750	18438	3.210
3	4	2	60	62188	15547	2.838
4	4	1	100	54063	13516	2.771
5	3	3	20	53438	13359	2.220
6	5	2	20	56250	14063	2.883
7	5	3	100	77188	19297	3.561
8	5	2	60	67188	16797	2.972
9	4	2	20	60313	15078	2.836
10	3	1	60	36875	9190	1.955
11	5	3	60	76563	19141	3.449
12	4	1	60	43750	10938	2.238
13	5	2	100	62500	15625	3.293
14	3	2	100	46875	11719	2.055
15	5	1	100	53438	13359	2.577
16	5	1	20	52188	13047	2.540
17	4	3	100	69688	17422	3.377
18	3	2	60	48438	12109	1.857
19	3	3	60	50938	12734	2.216

2.7. Statistical analysis

All experiments were performed in triplicate. Quantitative results are expressed in terms of mean±SD (standard deviation). Differences between samples, if any, were denoted by Student's t-test and ANOVA test, with *p* values of <0.05 for statistical significances.

3. RESULTS AND DISCUSSION

3.1. *Piper betle* L. extraction and experimental design

Piper betle extract has been proven its potential antibacterial activity, especially for acne treatment. However, systematic investigations on the influences of the extraction factors on the *Piper betle* total phenolic content and its antibacterial effect is absent. Therefore, in this section, we utilized DoE method, focused on D-optimal design, to critically elucidate these relationships. Since an increase in the extraction solvent volume could increase the amount of active compounds in the extract, it also increases the inactive component content, raises

the experimental cost, and harm the environment. Similarly, a longer extraction time could yield higher compound content, yet it might degrade the active agents. Therefore, these factors were chosen to study their effects on the extract properties. To this end, the software generates 19 formulas (Table 2) from 3 independent variables (X_1: extraction solvent/plant weight ratio (3, 4, and 5), X_2: extraction time (1, 2, and 3 h), and X_3: the water extract volume for the distribution with dichloromethane (20, 60, and 100 mL)) and 3 corresponding responses of the extract antibacterial efficacy on *S. aureus* (Y_1) and *E. coli* (Y_2), and the total phenolic content (Y_3, g GAE/g).

Regarding the antibacterial efficacy, on both the *S. aureus* (Y_1) and *E. coli* (Y_2), all three extraction factors proportionally affected the *Piper betle* extract activity (Figure 1 and Figure 2). The *Piper betle* inhibitory effects on *S. aureus* and *E. coli* enhanced significantly with the increases of the extraction solvent/plant weight ratio from 3 to 5, the extraction time from 1 to 3 h, and the water extract volume for the distribution with dichloromethane from 20 to 100 mL. In terms of the extract total phenolic content (Figure 3), the polyphenol concen-

trations increased with an increase in the extraction time from 1 to 3 h and the water extract volume for the distribution with dichloromethane from 20 to 100 mL. Interestingly, the extracts yielded more phenolic content when the extraction solvent/plant weight ratio increased from 3 to 4.5, yet the content slightly decreased when this ratio further increased from 4.5 to 5. This might be because at a higher solvent volume, the polyphenol compounds must compete with the other inactive compounds, in terms of saturated solubility, thus, a less total phenolic content was observed[33].

Additionally, the model and these relationships were reliable due to the reasonable differences of less than 0.2 between the model predicted R^2 (0.9239) and adjusted R^2 (0.9741)[34]. Utilizing the model, we obtained the optimal extraction condition for getting the highest
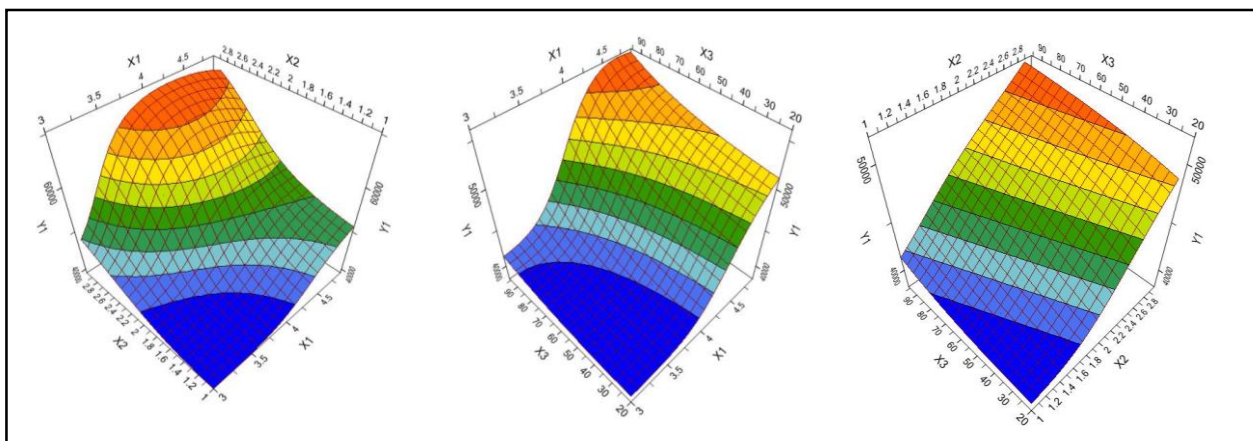

Figure 1. 3D response surface plots demonstrating the effects of X_1: extraction solvent/plant weight ratio, X_2: extraction time, and X_3: the water extract volume for the distribution with dichloromethane, on the extract antibacterial efficacy on *S. aureus* (Y_1).
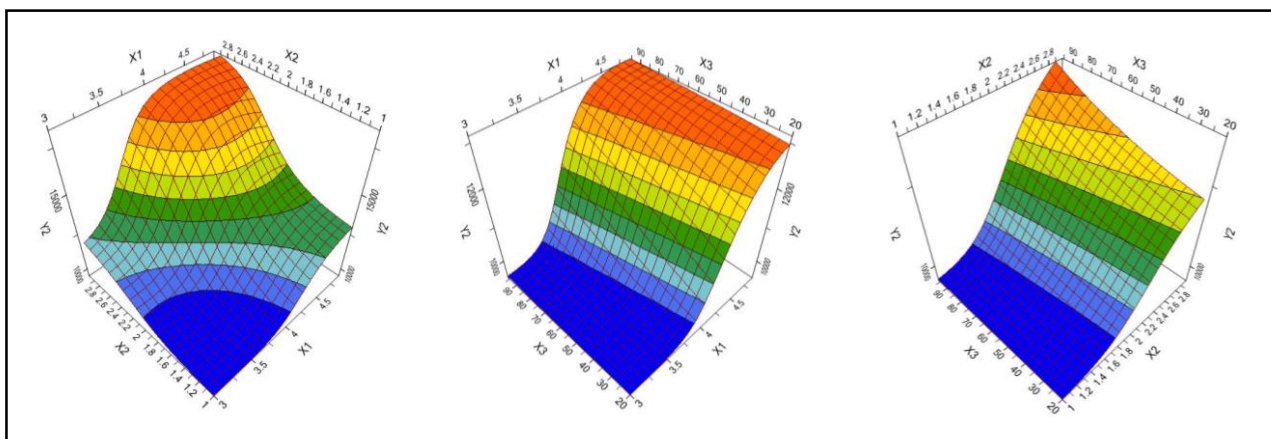

Figure 2. 3D response surface plots demonstrating the effects of X_1: extraction solvent/plant weight ratio, X_2: extraction time, and X_3: the water extract volume for the distribution with dichloromethane, on the extract antibacterial efficacy on *E. coli* (Y_2).
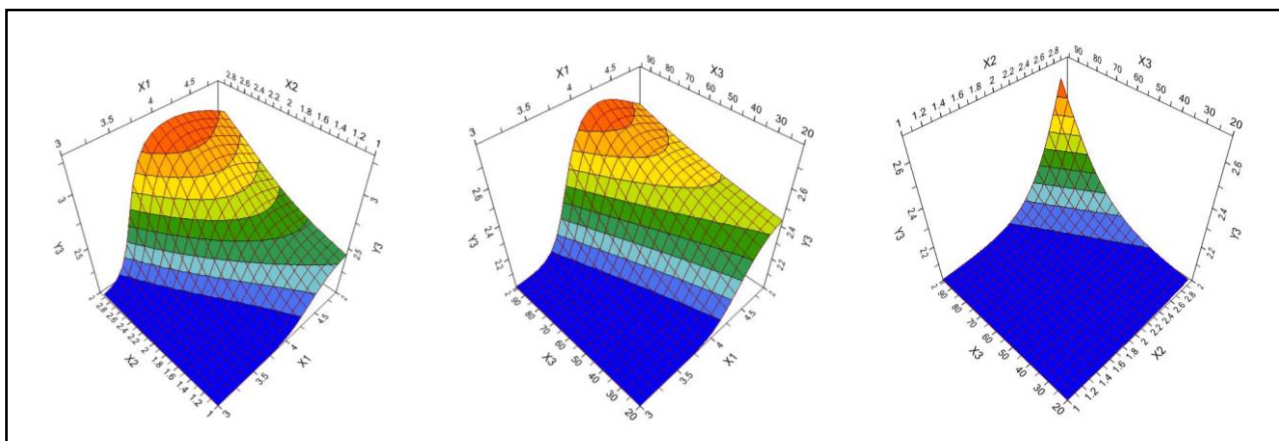

Figure 3. 3D response surface plots demonstrating the effects of X_1: extraction solvent/plant weight ratio, X_2: extraction time, and X_3: the water extract volume for the distribution with dichloromethane, on the extract total phenolic content (Y_3).

Table 3. D-optimal design validation of the optimal extraction condition for *Piper betle* L. (actual and predicted) (n=3). The acceptable criteria were set at a confidence level of 95% (α=0.05).

Optimal extraction condition:
- Extraction solvent/plant weight ratio: 4.034:1
- Extraction time: 2.147 h
- Water extract volume for the distribution with dichloromethane: 91.4 mL

Extract property	Antibacterial efficacy on *S. aureus*	Antibacterial efficacy on *E. coli*	Total phenolic content (g GAE/g)
Actual	70688.000±330.719	17505.000±272.923	3.337±0.034
Predicted	70161.177	17378.379	3.261

Table 4. *Piper betle* L. extract loaded hydrogel characteristics of viscosity, foaming ability, pH. Results are expressed in terms of mean±SD (n=3).

Formula	Viscosity (cps)			Foaming ability			pH		
	10°C	25°C	40°C	10°C	25°C	40°C	10°C	25°C	40°C
F1	2231±145	2217±140	2209±189	+	+	+	6.86±0.11	6.85±0.12	6.85±0.13
F2	6540±333	6512±305	6521±201	+	+	+	6.92±0.08	6.92±0.07	6.91±0.08
F3	7813±321	7834±274	5824±220	+	+	+	7.12±0.09	7.13±0.06	7.12±0.11
F4	11313±529	11234±510	11224±478	+	+	+	7.11±0.07	7.10±0.08	7.12±0.10
F5	6545±236	6518±280	6529±262	+	+	+	6.91±0.10	6.92±0.08	6.91±0.08
F6	6842±301	6821±263	6854±307	+	+	+	6.98±0.11	6.99±0.10	6.98±0.12
F7	7332±248	7324±311	7354±340	+	+	+	7.12±0.06	7.13±0.05	7.12±0.11
F8	6843±229	6825±256	6851±219	-	-	-	6.95±0.05	6.93±0.12	6.94±0.09
F9	6842±210	6821±249	6854±238	+	+	+	6.99±0.06	6.98±0.09	6.98±0.10
F10	6842±310	6821±323	6854±240	+	+	+	7.05±0.07	7.04±0.06	7.03±0.13

total phenolic content and antibacterial activity, with an extraction solvent/plant weight ratio of 4.034:1, an extraction time of 2.147 h, and a water extract volume for the distribution with dichloromethane of 91.4 mL. This condition was then wet-lab tested, and the actual responses results, together with the theoretical values, are shown in Table 3. No significant difference was noted between the theoretical and actual values ($F=2.26<F_{crit}=18.51$), indicating that the model was fit and reliable.

3.2. Hydrogel formulation and characterizations

The optimal *Piper betle* extract was then encapsulated in hydrogel formulations, followed by being characterized their viscosity, foaming ability, and pH. Since the *Piper betle* extract possessed a MIC of 32 µg/mL and 128 µg/mL on *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, respectively. Thus, to ensure the hydrogel antibacterial activity, we chose the *Piper betle* extract percentage in the formula to be 3%, which had a concentration of more than 20 times higher than the extract MIC.

Ten formulas (F1-F10, Table 1) were investigated to denote the effects of the gel-forming agent carbopol 940, the viscosity-control emollient propylene glycol, and the surfactant cocamidopropyl betaine, on the hydrogel properties (Table 4). These agents, at the selected percentages, have been proved to be safe and suitable for cosmetics and pharmaceutical products such as anti-acne hydrogel[28,35-36]. The results indicate that an increase of carbopol 940 from 0.4 to 1% significantly enhances the hydrogel viscosity from approximately 2000 cps to 10000 cps, in agreement with a previous study[37]. The F3

formula (0.8% carbopol 940) was not stable at high temperature of 40°C for 1 week; and the F4 formula (1% carbopol 940) was too viscous. Thus, the carbopol 940 at a ratio of 0.6% was selected. Similarly, an increase in the propylene glycol amount slightly increases the hydrogel viscosity, yet did not affect the product pH and foaming ability[38]. Since the F7 formula (15% propylene glycol) was thick and viscous, the F6 formula (12% propylene glycol) was chosen. Finally, the surfactant cocamidopropyl betaine contributed to the hydrogel foaming ability, as the F8 formula (2% cocamidopropyl betaine) did not foam, yet the F9 and F10 formulas, with 3% and 4% cocamidopropyl betaine, respectively, gave visible foam. Conclusively, the formula with 0.6% carbopol 940, 12% propylene glycol, 3% cocamidopropyl betaine, and 3% *Piper betle* extract was chosen for the *ex vivo* antibacterial study. It is worth to note that although cocamidopropyl betaine is a surfactant and could cause skin irritation in the leave-on cosmetic products, its concentration of 3% has been confirmed to be safe and suitable for these types of formulations[39].

3.3. *Ex vivo* antibacterial activity test on human acne samples

To determine the *Piper betle* extract loaded hydrogel potency as a pharmaceutical product for acne treatment, 15 acne samples from 15 patients were collected and isolated the acne-causing bacteria. These bacteria belonged to 2 main groups of gram-positive cocci (*Staphyllococci*) and gram-negative bacilli (Enterobacteriaceae, *Pseudomonas spp.*) (Table 5). Since vancomycin and colistin are two of the most sensitive

antibiotics for these two groups, especially in the antibiotic resistance cases[31], therefore, in this study, these antibiotics were selected as positive controls for the experiments.

Firstly, the negative control (hydrogel without *Piper betle* extract) showed no effects on the bacteria, suggesting that the hydrogel compositions did not affect the product antibacterial ability. Secondly, the results demonstrate that the *Piper betle* extract loaded hydrogel possessed high antibacterial activity, with larger zone of inhibitions (i.e., diameters of >15 mm), as compared to the positive controls, in most samples (Table 5). This indicates that the product was effective on both gram-positive and gram-negative bacteria, with stronger activities on gram-positive bacteria (zone-of-inhibition diameter of about 20 mm) than on gram-negative ones (zone-of-inhibition diameter of about 8 mm) (Figure 4). These data corresponded well with the antibacterial

efficacy of the pure *Piper betle* extract on *S. aureus* (gram-positive bacteria) and *E. coli* (gram-negative bacteria) (Table 2), thus indicating that the *Piper betle* extract loaded hydrogel could preserve the antibacterial efficacy of the extract. Interestingly, the product effects on the standard bacterial strains were much higher than the acne-sample isolated ones. This is due to the fact that bacteria in acne samples have gained antibacterial resistant property (i.e., less sensitive to the antibacterial agents) as a results of irrational uses of antibiotics in Vietnam[40-41]. Lastly, the fact that the hydrogels possessed better antibacterial effects than the positive controls (vancomycin and colistin for gram-positive and gram-negative bacteria, respectively) might, again, be explained by the resistance of the tested bacteria against these commercial drugs. Conclusively, this proved that the *Piper betle* extract loaded hydrogel had much potential to become a novel acne treatment in the future.

Table 5. *Piper betle* L. extract loaded hydrogel *ex vivo* antibacterial activity on bacteria isolated from acne samples of 15 patients. *, standard bacterial strains. Negative control: blank hydrogel; positive controls: vancomycin for gram-positive bacteria and colistin for gram-negative bacteria. Results are expressed in terms of mean±SD (n=3). Different letters (a, b, c) indicate significant differences between samples in the same row ($p<0.05$).

| Sample	Isolated bacteria	Zone-of-inhibition diameters (mm)		
		Negative control	Positive control	*Piper betle* loaded hydrogel
	Staphylococcus aureus ATCC 25923*	6^a±1	17^b±1	34^c±2
	Escherichia coli ATCC 25922*	6^a±1	8^b±1	24^c±2
1	*Escherichia coli*	6^a±1	8^b±1	8^b±1
2	*Staphylococus aureus*	6^a±1	17^b±1	32^c±2
3	*Staphylococus spp*	6^a±1	17^b±1	39^c±2
4	*Staphylococus spp*	6^a±1	17^b±1	50^c±3
5	*Staphylococus aureus*	6^a±1	17^b±2	28^c±2
6	*Staphylococus spp*	6^a±1	17^b±1	11^c±1
7	*Staphylococus spp*	6^a±1	17^b±2	18^b±1
8	*Klebsiella spp*	6^a±1	8^b±1	36^c±2
9	*Staphylococus aureus*	6^a±1	17^b±2	34^c±2
10	*Staphylococus aureus*	6^a±1	17^b±1	40^c±2
11	*Staphylococus spp*	6^a±1	8^b±1	8^b±1
12	*Staphylococus aureus*	6^a±1	17^b±2	21^c±1
13	*Klebsiella spp*	6^a±1	12^b±1	15^c±1
14	*Staphylococus spp*	6^a±1	17^b±1	31^c±2
15	Staphylococus spp	6^a±1	17^b±1	25^c±2
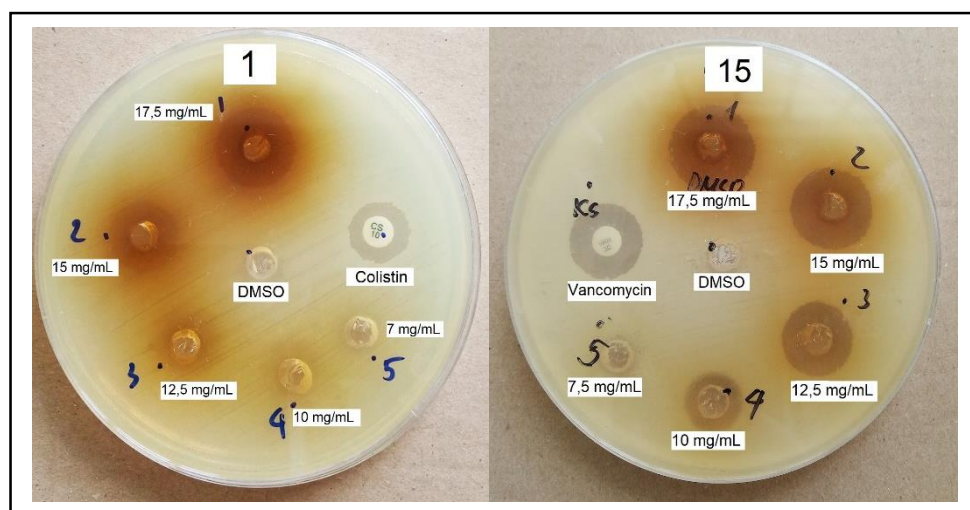
Figure 4. *Ex-vivo* antibacterial tests of the *Piper betle* L. extract loaded hydrogel on the bacteria isolated from acne samples of patient 1 and 15.

4. CONCLUSION

This study, for the first time, investigated the effects of extraction factors on the *Piper betle* L. total phenolic content and antibacterial activities on *S. aureus* and *E. coli* using the design of experiment approach. Moreover, the optimal extract was loaded into hydrogel formulations and characterized their physicochemical properties as well as therapeutic antibacterial actions on bacteria isolated from 15 acne-patient samples. The product possessed great activity on both gram-positive and gram-negative bacteria, with zone-of-inhibition diameters' of more than 15 mm, which were larger than the positive control diameters. Conclusively, the hydrogel could be further researched and developed to become a potential pharmaceutical product for acne treatment.

Author contribution
Conceptualization: D.T.M.H., D.T.P.; methodology: D.T.M.H., M.T.L., V.D.T.; validation: D.T.P., investigation: D.T.M.H., M.T.L., V.D.T.; resource: D.T.P.; writing-original draft: D.T.M.H., D.T.P.; writing-review and editing: D.T.M.H., M.T.L., V.D.T., D.T.P.

Conflict of interest
None to declare.

Ethics approval
The research ethics were approved by Can Tho University of Medicine and Pharmacy, Vietnam (approval number 1675/DHYDCT.NCKH, December 21, 2018).

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