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Research Article

EVALUATION OF ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF VERNONIA AMYGDALINA LEAF EXTRACTS AS AN AUXILIARY IN NATURAL HAIR SHAMPOO

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ABSTRACT

Objective: This study evaluated the phytochemical compounds, antioxidant, and antimicrobial activities of *Vernonia amygdalina* (VA) leaf extract and used it as an auxiliary herbal constituent in natural hair shampoo.

Methods: VA leaf was macerated in various solvents. All crude extracts were phytochemical compounds screened and determined the antioxidant and antimicrobial activities before formulating a natural shampoo formula. Natural shampoos were evaluated with physic-chemical properties and sensory satisfaction.

Results: About 95% ethanolic extract was the most suitable substrate for product development. It reveals the inhibition zone from 7.00±0.00 to 15.00±1.00 mm and possesses a broad spectrum of antibacterial agents against both Gram-positive and Gram-negative bacteria. The MICs and minimum bactericidal concentrations value ranged from 6.250 to 100 mg/ml and 25 to >200 mg/ml, respectively. About 95% ethanolic extract also revealed high antioxidant activity. The IC_{50} value of DPPH of 95% ethanolic extract was 1.88±0.02 µg/ml (% inhibition of 85.73±0.01) and the ferric reducing ability power was 23.00±0.50 mg AAE/100 gDW. These high biological activities may be due to a broad range of phytochemical compounds, including saponins, tannins, flavonoids, terpenoids, steroids, cardiac glycosides, and alkaloids.

Conclusions: The VA leaf was suitable to use as an ingredient in natural shampoos with a low detrimental effect on normal skin flora. The shampoo with 0.00188% of 95% ethanolic extract was the most suitable formula in terms of characteristics and stability. This formula also obtained the highest satisfaction level under our sensory evaluation.

Keywords: *Vernonia amygdalina*, Phytochemical compounds, Antioxidant and antimicrobial activities, Natural shampoo formulation.

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INTRODUCTION

Creating new natural products has presently received more attention due to their demand in the cosmeceutical industry. In Asia, topical herbal products containing bioactive ingredients with pharmacologic benefits have been used in creating beauty for thousands of years. However, there was little scientific literature to support the use of such ingredients in the modern cosmeceutical industry. Herbal plants have a natural-synthetic ability to produce bioactive compounds which called phytochemicals or secondary metabolites. These compounds exert bacteriostatic and bactericidal activities against pathogenic microorganisms. The continued research about the bioactivity of phytochemical compounds has the objective to discover novel biomolecules that possess to resist free radicals and pathogenic microorganisms for solving of the treatment failure (disease) [1]. The bioactive substances were not beneficial only in the medical field but also for pharmaceutical or cosmeceutical products. The shampoo is one of the important daily used requisites. The modern shampoo is generally made by combining surfactant, co-surfactant, soften, and conditional agent with the thickener and nourish agent [2]. However, new generation customers are consciously aware of the effects of overmuch synthetic chemical usage that may have a side effect such as hair loss, hair dryness and hair breakage. Therefore, products with natural ingredients received more attention due to their higher extra unique natural active compounds and lower side effects on the body.

Vernonia amygdalina (VA) (Nan Chao Wei), a member of the family Asteraceae, is a small shrubbery that grows throughout tropical Africa. It is called "bitter leaf" due to its bitter taste. Its leaf is served as the vegetable and culinary herb in soup [3]. This plant has presented various pharmacological properties such as antimicrobial [4], antifungal [5], antioxidant [6], anti-inflammatory [7], antitumor [8], anticancer [9], and anti-allergic [10]. Many bioactive compounds had been isolated from the extracts of VA such as flavonoids, saponins, terpenes, sesquiterpenes, edotides, xanthones, and alkaloids [11,12]. Therefore, this experiment will determine the phytochemical composition of VA leaf extracts growing in Thailand and specify the potential fraction that showed the ability of natural antioxidant and antimicrobial agents. The most suitable extracted will be used as an auxiliary compound in natural hair shampoo formulation and evaluated in terms of characteristics and stability. The satisfaction level and sensory evaluation will also be tested.

METHODS

Plant material and preparation of extracts

Fresh leaves of VA were collected from Min Buri District, Bangkok Province, Thailand, from Jan to May 2019. The leaves were properly cleaned with running tap water (2–3 times) and sterile distilled water (1 time), respectively. The plant materials were dried in shade and cut into small pieces in a size of 0.5–1.0 cm. All leaf samples were then separately macerated with 95% ethanol, 75% acetone, and 99.8% chloroform solutions in a ratio of 1:20 (plant:solvent; [w/v]) for 7 days compared with boiled into distilled water (DW:ratio of 1:12.5; [w/v]) at 60°C for 30 min. The mixture was filtered through a filter paper (Whatman No. 1) and centrifuged at 8000 rpm for 15 min. The filtrate obtained was subsequently concentrated under vacuum on a rotary evaporator. The concentrated crude extracts were kept at –20°C under dark condition until further analysis. Approximately 0.1–0.2 mg/ml of crude extract solutions were prepared with each solvent extractions

before further phytochemical screenings, total phenolic and flavonoid contents, and antioxidant activities study.

Analysis of phytochemical constituents

Qualitative screening of the phytochemical compounds of VA leaf extracts was carried out to evaluate the presence of chemical constituents including alkaloids, flavonoids, tannins, steroids, saponins, anthraquinones, cardiac glycosides, and terpenoids, according to the prescribed methods of Harborne (1998) [13].

Estimation of total phenolic content (TPC)

The quantitative of TPC was determined by Folin–Ciocalteu (FC) method according to the procedure of Singleton *et al.* (1999) [14] with slight modification. In brief, 100 µl of extracted solution at an appropriate concentration (0.2 mg/ml) was thoroughly mixed with 750 µl of fresh FC reagent (1:10 dilution with distilled water) and incubated at room temperature for 5 min. Then, 750 µl of 6% (w/v) sodium carbonate (Na_2CO_3) was added and allowed to completely react for 90 min under the dark condition at ambient temperature. The absorbance of the sample solution was measured at 725 nm by spectrophotometer (Hanon instruments, Japan). The standard curve in the range of 0.025-0.500 mg/ml of gallic acid was used for calculating the TPC. The result was expressed as mg of gallic acid equivalent per 100 g of dry weight (mg GAE/100 g DW).

Estimation of total flavonoid content (TFC)

TFC of crude extracts was analyzed using the aluminum chloride colorimetric assay according to the method described by Tinrat and Sila-asna (2017) [15]. In brief, 200 µl of an appropriately diluted solution of the plant extracts (0.2 mg/ml) and 2.3 ml of 30% methanol solution were mixed and followed by the addition of 100 µl of 0.5 M $NaNO_2$ and 100 µl of 0.3 M $AlCl_3$, respectively. The resulting solution was thoroughly mixed with vortex and left to stand for 5 min in the dark at ambient temperature. The absorbance was taken against a reagent blank at 506 nm using UV spectrophotometer. The standard curve in the range of 0.25–5.00 mg/ml of rutin was used for calculating the TFC. The result was expressed as mg of rutin equivalent per 100 g of dry weight (mg RE/100 g DW).

Antioxidant activities

DPPH radical scavenging activity assay

Free radical scavenging activity of crude leaf extracts was evaluated according to the method of Jayalakshmia *et al*. (2015) [16] with slight modification. In brief, 100 µl of sample extracts at an appropriate concentration (0.2 mg/ml) was mixed with 900 µl of 0.1 mM DPPH in methanolic solution under vigorous shaking and incubated under dark condition. The absorbance value of solutions was read after 30 min incubation period at ambient temperature at 517 nm. The antioxidant activity of the sample expressed as IC_{50} value was defined as concentration (µg/ml) of the sample that inhibits the DPPH radicals formation by 50%. The IC_{50} values were calculated using linear regression graphs. The percentage of DPPH scavenging was calculated using the following formulation:

$$\text{DPPH radical scavenging \%} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 is absorbance of the control (DPPH solution)
A_1 is an absorbance of the DPPH mixed with the sample.

Ferric reducing antioxidant power assay (FRAP)

The FRAP was proceeded as previously described by Benzie and Strain (1996) [17]. The freshly prepared FRAP reagent in acetate buffer (1.6 g of sodium acetate and 8.0 ml of acetic acid make up to 500 ml; pH 3.6) was warmed to 37°C in the oven until used. A total of 300 µl of tested extracts at an appropriate concentration (0.2 mg/ml) and 2.7 ml of the FRAP reagent was thoroughly mixed. After 30 min of incubation, the absorbance value of mixture solutions was read at 596 nm. The standard curve in the range of 0.01–0.25 mg/ml of ascorbic acid was prepared. The results were expressed as mg of ascorbic acid equivalents/100 g dry weight (mg AAE/100g DW) of the plant materials.

Determination of antimicrobial activity

Microorganisms and culture condition

The antimicrobial effects of all crude extracts were determined against nine pathogenic strains, including the reference from the American Type Culture Collection and Laboratory collection strains (The Department of Biotechnology, King Mongkut's University of Technology North Bangkok, Thailand) as *Bacillus cereus* DMST 5040, *Enterococcus faecalis* DMST 4736, *Staphylococcus aureus* ATCC 25923, and *Staphylococcus epidermidis* for Gram-positive bacteria group and *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* DMST 8212, and *Salmonella typhimurium* ATCC 13311 for Gram-Negative bacteria group. All tested microorganisms were maintained on brain heart infusion (BHI, Difco) agar medium under 37°C.

Preparation of plant extracts

The stock solutions of plant extracts were dissolved in 5% DMSO and sterile distilled water to a final concentration of 1000 mg/ml for agar diffusion assay and 400 mg/ml for broth microdilution methods (MIC values). The crude extract solutions at different concentrations of 50, 100, 200, 300, and 400 mg/ml were prepared before applying in agar disk diffusion assay.

Agar disk diffusion assay

In vitro antibacterial activity of crude extracts of VA leaf was carried out by agar disk diffusion method at the concentration 50, 100, 200, 300, and 400 mg/ml against tested bacterial strains. In brief, overnight bacterial cultures of tested strains have adjusted the concentration of the $1.0 \times 10^{8-9}$ CFU/ml (OD_{600} = 0.2) by spectrophotometer. The inoculums of tested microorganisms were transferred to solidified agar plates by sterile swab sticks and followed by the mounting of the impregnated sterile paper disk. After that, each paper disk was added with 10 µl of each crude extract at different concentrations. Ampicillin (Amp, 10 µg/ml) and ciprofloxacin (CIP, 10 µg/ml) were used as a positive control. After 1-, 3-, and 5-day incubation at 37°C, all plates were observed the zone of growth inhibition and the diameter of these zones was measured in millimeters (mm) [18].

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay

The MIC values of the extracts were carried out by a two-fold serial dilution method in 96 well microtiter to determine the susceptibilities of pathogenic strains and extracts [19]. The concentration of crude extracts range from 200 to 0.195 mg/ml was used. 200 µl of plant extracts (400 mg/ml of concentration) was added to each 96-well microplate well which contained 200 µl of BHI broth. Then, 5 µl of bacterial suspension with the concentration of 10^{8-9} CFU/ml (OD_{600} = 0.2) was added to each well. The 96-well microtiter plates were incubated at 37°C for 24 h. Similar tests were performed simultaneously for growth controls (BHI + inoculums) and sterility controls (BHI + test sample). The first wells showing no visible disorder were considered as MIC values. Then, a small medium from MICs determination was transferred to the agar plates and re-culture to evaluate the MBC values.

Development of natural hair shampoo

Formulations of natural shampoos were prepared using different proportions (%) of ingredients and concentration of VA leaf extracts (Table 1). The ingredient phases were prepared and mixed until an ideal homogeneous dispersion. The mixture was then mixed using slow stirring on a magnetic stirrer until no precipitate remained at the bottom.

Physical appearance/visual inspection

Five grams of each formulated shampoo were investigated for the physical appearance by organoleptic inspections in terms of external appearance, color, and odor [20].

Table 1: Ingredients of natural shampoo formulations in this study

Ingredients	Function	Formulation (%w/w)		
		A	B	C
Phase A				
Sodium Laureth Sulfate 2EO (Texapon N 8000; 28%)	Cleaning Agent	12.50	12.50	12.50
Coco-glucoside and Glyceryl oleate (Lamesoft PO 65)	Soften and conditional agent	3.65	3.65	3.65
Coconut Diethanoiamide Glycerine (Aminon C-025)	Thickener/Viscosity adjuster	2.95	2.95	2.95
Cocamidopropyl betaine (CAPB)	Surfactant/Cleaning Agent	2.95	2.95	2.95
Phase B				
Deionized Water	Diluent	72.8	68.799	68.782
Polyquaternium – 7 (PQ 7)	Conditioning agent	2.45	2.45	2.45
Sodium lauryl sulfate (EMAL 10G)	Surfactant (Anionic)	2.45	2.45	2.45
Phase C				
Vitamin B5	Nourish hair	-	1.00	1.00
Keratin	Nourish hair	-	1.00	1.00
Phase D				
Vernonia amygdalina (bitter leaf) extracts	Active ingredient	-	0.00188	0.0188
Phase E				
P10	Preservative	0.25	0.25	0.25
Fragrance	Fragrance	-	2.00	2.00
Total		100	100	100

Determination of wetting time

The wetting time of the formulated shampoo was determined. One-inch square paper was placed on the aqueous solution of 1% (w/w) shampoo. The time has been recorded until a paper was becoming wet [21].

Dirt dispersion

Two drops of formulated shampoos were added to 10 ml of distilled water in the tested tube. One drop of India ink was added, then stops and shakes for 10 times [22].

Sensory evaluation of formulated natural shampoo

All formulated shampoos were evaluated the sensory, including the appearance, odor, color, foaming quantity, feelings after use, packaging, and overall acceptability by 5-point hedonic scales compared with a commercialize shampoos (5 = like extremely to 1 = dislike extremely; 30 persons).

Determination of solid content (%)

The solid content of the formulated shampoos was investigated. In brief, 4 g of the sample was placed in an evaporating dish for a total weight recorded. The evaporating dish was placed in a water bath to dryness. After complete evaporation, the evaporating dish was weighed again after cooling down to room temperature. The percentage of solid content was then calculated after the sample was completely dry.

pH measurement

The formula shampoos were diluted in distilled water to achieve the final concentration of 10% (v/v). The pH was measured using a pH meter at room temperature [22].

Foaming ability and foam stability

Foaming ability was determined by the cylinder shake method with slight modification. Fifty milliliters of the 1% shampoo solution were put into a 250 ml graduated cylinder. The cylinder was covered and shakes. The total volume of the foam content every 1 min was recorded and continuously done until 5 min [22].

Statistical analysis

Results were performed in triplicate and expressed as mean±SD. Significance differences for multiple comparisons were assessed by the one-way ANOVA and followed by t-tests. Statistical significance was taken at 95% confidence ($p \leq 0.05$).

RESULTS AND DISCUSSION

Phytochemical screening

The percent yield of VA crude extraction was ranged from 7.5% to 30% as in distilled water extract (DWE; 30.00%), 95% ethanol (EE; 22.50% [w/v]), 99.8% chloroform (CE; 17.50% [w/v]), and 75% acetone extract (AE; 7.50% [w/v]). The difference in yield percentage was depended on the influence of different polarities of solvent solutions. The qualitative phytochemical screening of VA leaf extract in different solvents was showed in Table 2. The crude extracts of VA leaf revealed some secondary metabolites, including saponins, tannin, flavonoids, steroids, terpenoids, and alkaloids. In this experiment, acetone extracts (AE) of VA leaf represented the highest amount of the phytoconstituents, including saponins, tannin, steroids, terpenoids, cardiac glycosides, and alkaloids. Steroids and terpenoids which were major phytochemical components of VA leaf were found in all solvent.

From all extractions, seven phytochemical compounds were found in VA leaf extracts, whereas anthraquinones have not been found in any solvent in this experiment. Saponins, steroids, terpenoids, and cardiac glycosides were found in three solvent extracts (aqueous, 95% ethanol, and 75% acetone) which was consistent with the study of Alara *et al.* (2019) [4]. These seven phytochemical compounds found can exert antimicrobial and antioxidant activities [23-25]. In the previous studies of VA, different main constituents have been reported, including flavonoids [26], saponins [27], terpenoids [26, 27], anthraquinones [27], and alkaloids [27], steroids [27], tannins [26], and cardiac glycosides [27]. This experiment has found the different amounts and components of phytochemical compound compare with the previous studies. The reason of this difference might be because of the difference in plantation area, whether condition, water level, and soil condition, for example. Different plantation conditions might influence the appearance of phytochemical composition in VA.

Quantitative analysis of the total phenolic and flavonoid contents

Phenolic and flavonoids are plant secondary metabolites that possess several pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, and among biological activities [28]. In this experiment, all VA leaf extracts for each solvent were assayed for total phenolic and flavonoid content (TPC and TFC). Quantitative analysis of TPC and TFC is shown in Table 3. The highest TPC was significantly obtained for aqueous extract (DWE) followed by 75% acetonic and 95% ethanolic extracts as 374.10±1.40, 69.30±0.70, and 772.80±0.7 mg GAE/100 g FW ($p \leq 0.05$), respectively. The TFC of VA leaf extracts were range from 69.30±0.70 to 772.8±0.70 mg RE/100 g FW. The 75% acetonic extract with 772.8±0.70 mg RE/100 g FW of TFC showed

Table 2: Preliminary phytochemical analysis of *Vernonia amygdalina* (bitter leaf) extracts

Phytochemical compounds	Distilled water	95% Ethanol	75% Acetone	99.8% Chloroform
Saponin				
Froth test	++	+	+	–
Tannin				
10% $FeCl_3$	–	–	+++	–
Flavonoid				
Shinoda test	–	+	–	+
10% lead (IV) acetate	+	–	–	–
Anthraquinone	–	–	–	–
Steroid				
Liebermann test	–	+	+	++
Keller–Kiliani test	+++	+	+	+
Terpenoid				
Salkowski test	+	++	++++	+++
Cardiac glycosides				
Kedde reagent	–	–	+++	+
Keller-Kiliani Test + 10% $FeCl_3$ test	–	–	–	+
Alkaloid				
Dragendorff's reagent	–	–	+++	++
28% NH_4OH	–	–	–	–
Wagner's reagent	–	–	+++	++

+: Presence, –: Absence

Table 3: Total phenolic and flavonoid contents and antioxidant activities of *V. amygdalina* leaf extracts

Tests	Solvent extraction			
	Distilled water	95% Ethanol	75% Acetone	99.8% Chloroform
Total phenolic content (mg GAE/100g FW;±SD)	374.10±1.40*	158.50±0.50	315.7±1.40	88.2±1.40
Total flavonoids content (mg RE/100g DW; ±SD)	69.30±0.70	77.00±0.50	772.8±0.70*	258.30±0.70
Antioxidant capacity				
FRAP assay (mg AAE/100 g DW; ±SD)	179.20±0.70	23.00±0.50	448.00±2.80*	112.00±0.70
DPPH assay (mg AAE/100g DW±SD)	160.30±0.50	283.50±0.50*	173.60±0.70	217.00±0.70
% Inhibition	34.64±0.01	85.730±0.01*	37.540±0.01	48.820±0.01
$IC_{50\ Sample}$; μ g/ml	16.40±0.03	1.88±0.02*	8.32±0.04	3.52±0.04
$IC_{50\ Ascorbic\ acid}$; μ g/ml	0.166±0.001			

GAE: Gallic acid equivalent, RE: Rutin equivalent, AAE: Ascorbic acid equivalent, IC50 : 50% Inhibitory concentration, *: p≤0.05, Ascorbic: Standard control

significantly higher antioxidant activity (p≤0.05) compare with other extracts (Table 3). However, fraction with higher phenolic and flavonoid contents had not always been represented strong antioxidant activity. The different solvent type has a significant influence on quantitative analysis and the concentration of phenolic and flavonoid compounds but not the antioxidant activity.

Antioxidant activities of VA leaf extracts

Oxidative stress is an imbalance of free radicals and antioxidant agents in the body. It has been reported to be associated with various human diseases and aging [29]. In this study, VA leaf extracts expressed an *in vitro* antioxidant activity when examined with DPPH radical scavenging assay and radical scavenging properties (FRAP assay) as IC_{50} in Table 3.

DPPH free radical is widely used to determine the radical scavenging activity of natural substances because of its stability. The radical scavenging activity in the presence of a hydrogen donating antioxidant can be monitored by reducing the adsorption of DPPH solution. IC_{50} is the concentration of the samples required to inhibit 50% of free radical. Thus, lower in IC_{50} indicates the higher of antioxidant activity. The 95% ethanolic extract of VA leaf showed highest scavenging activity of DPPH radical (IC_{50}=1.88±0.02 μg/ml; % inhibition=85.73±0.01) that indicates approximately eight, four, and two-fold higher antiradical activity when compared with aqueous (IC_{50}=16.40±0.03 μg/ml), 75% acetone (8.32±0.04 μg/ml) and 99.8% chloroform (3.52±0.04 μg/ml) extracts, respectively (Table 3). The IC_{50} values of the standard ascorbic acid were 0.166±0.001 μg/ml. The antioxidant activity of VA leaf was accordingly estimated by FRAP assay, which is presenting of the sample reduction of Fe (III) /tripyridyltriazine (TPTZ) complex to the ferrous form (blue color) (Table 3). In this study, the 75% acetonic extract of VA leaf showed the highest amount of ferric reducing power expressed as ascorbic acid (448.00±2.80 AAE/100 g DW) followed by aqueous extracts (179.20±0.70 mg AAE/100 g DW). There was no correlation between antioxidant activity by DPPH assay and total phenolic and flavonoid content among the various crude extracts. In spite of the lower phenolics and flavonoids content, 95% ethanolic extract showed higher antioxidant activity by DPPH assay (% Inhibition and IC_{50}), but not in FRAP assay. In the other hand, 75% acetonic extract showed the highest total phenolic and flavonoid content and ferric reducing antioxidant (FRAP) assay, but it was not a correlation with DPPH free radical scavenging assay. These results clearly show that the amounts of phenolic and flavonoid in the crude VA leaf extracts are not an indicator for antioxidant power. However, different antioxidant power might be due to other secondary metabolites contained in this plant extracts such as phytol (56.30%) [4,6] which was found to present a strong antioxidant, anti-microbial, anti-inflammatory, and anti-proliferative activity. This phytol was also found to be cytotoxic against breast cancer cell lines MCF7 [30]. Furthermore, synergistic or antagonistic effects among the different phytochemical are also possible in terms of antioxidant. The antioxidant capacities of the crude extracts have a strong relationship with the solvent employed, mainly due to the different antioxidant potential of compounds with different polarities [31].

Antimicrobial activity

The antibacterial activity of aqueous, 95% ethanol, 75% acetone, and 998% chloroform extracts of VA leaf was investigated against nine pathogenic strains using by agar disk diffusion method. The

Table 4: Antibacterial activities of *V. amygdalina* leaf extracts against some bacterial pathogens

Concentration (mg/ml)	Zone of growth inhibition (∅ = 6 mm); mm±SD								
	Gram-positive strains				Gram-negative strains				
	BC	EF	SA	SE	EC	KP	PA	PM	ST
Distilled water									
50	R	R	R	R	R	R	8.33±0.58^{h}	9.00±1.00^{gh}	R
100	R	R	R	R	R	R	7.67±0.58^{ij}	7.00±0.00^{j}	R
200	R	R	R	R	7.00±0.00^{j}	R	7.00±0.00^{j}	8.00±1.00^{hi}	R
300	7.00±0.00^{j}	R	R	R	12.00±1.00^{de}	9.33±0.58^{g}	8.67±0.58^{h}	9.00±1.00^{gh}	R
400	7.00±0.00^{j}	R	R	R	12.33±0.58^{d}	11.33±0.58^{e}	8.00±0.00^{hi}	8.67±0.58^{h}	R
95% Ethanol									
50	7.00±0.00^{j}	R	R	R	R	7.00±0.00^{j}	7.00±0.00^{j}	9.67±0.58^{g}	R
100	8.33±0.58^{h}	R	8.67±0.58^{h}	R	R	7.00±0.00^{j}	7.00±0.00^{j}	10.67±0.58^{f}	R
200	15.00±1.00^{b}	7.00±0.00^{j}	R	R	R		7.00±0.00^{j}	10.00±0.00	R
300	12.00±1.00^{de}	R	7.00±0.58^{j}	R	7.00±0.00^{j}	8.33±0.58^{h}	8.00±0.00^{hi}	9.00±0.00^{gh}	R
400	11.67±0.58^{e}	R	R	R	R	7.00±0.00^{j}	7.00±0.00^{j}	7.00±0.00^{j}	R
75% Acetone									
50	7.00±0.00^{j}	R	12.67±0.58^{d}	R	7.00±0.00^{j}	R	7.00±0.00^{j}	R	R
100	10.33±0.58^{f}	R	12.33±0.58^{d}	R	7.00±0.00^{j}	R	7.00±0.00^{j}	R	R
200	10.67±0.58^{f}	R	15.00±1.00^{b}	R	7.00±0.00^{j}	9.33±0.58^{g}	9.00±0.00^{gh}	7.00±0.00^{j}	R
300	11.67±0.58^{e}	7.00±0.00^{j}	16.00±1.00^{a}	7.00±0.00^{j}	7.00±0.00^{j}	9.67±0.58^{g}	8.33±0.58^{h}	R	R
400	12.33±0.58^{d}	8.00±0.00^{hi}	16.33±0.58^{a}	R	7.00±0.00^{j}	9.33±0.58^{g}	9.33±0.58^{g}	7.00±0.00^{j}	R
99.8% Chloroform									
50	7.00±0.00^{j}	R	11.33±0.58^{e}	10.67±0.58^{f}	R	R	7.00±0.00^{j}	7.00±0.00^{j}	R
100	7.00±0.00^{j}	R	10.00±1.00^{fg}	8.33±0.58^{h}	7.67±0.58^{i}	R	7.00±0.00^{j}	7.00±0.00^{j}	R
200	11.67±0.58^{e}	R	8.67±0.58^{h}	8.00±0.00^{hi}	R	R	8.00±0.00^{hi}	R	R
300	12.00±0.00^{de}	7.00±0.00^{j}	11.33±0.58^{e}	8.00±0.00^{hi}	R	R	7.33±0.58^{ij}	R	R
400	12.33±0.58^{d}	7.00±0.00^{j}	13.67±0.58^{c}	9.00±0.00^{gh}	7.00±0.00^{j}	R	7.00±0.00^{j}	R	R

^{abcdefghij}is significant at the 0.05 level (p<0.05). BC: *B. cereus* DMST 5040, EF: *E. faecalis* DMST 4736, SA: *S. aureus* ATCC 25923, SE: *S. epidermidis*, EC: *E. coli* ATCC 25922, KP: *K. pneumoniae*, PA: *P. aeruginosa* ATCC 27853, PM: *P. mirabilis* DMST 8212, and ST: *S. typhimurium* ATCC 13311, R: Resistance

antibacterial activity was measured in terms of a diameter of the inhibition zone shown in Table 4. The antibacterial activity of crude VA leaf extracts was depending on the solvent nature used for extraction and the concentration of the plant extract. The 95% acetonic extract was presenting more antibacterial activities when compared with the aqueous, 95% ethanol and 99.8% chloroform extract. This result attributed to the previous results that acetonic extract contains more of the bioactive component when compared with other solvent extracts. Gram-positive strains, including *B. cereus* DMST 5040 (7.00±0.00–15.00±1.00 mm) and *S. aureus* ATCC 245923 (7.00±0.00–16.33±0.58 mm) and Gram-negative strains, including *P. aeruginosa* ATCC 27853 (7.00±0.00–9.33±0.58 mm) and *P. mirabilis* DMST 8212 (7.00±0.00–10.67±0.58) were more sensitive to all crude extracts while *S. typhimurium* ATCC 13311 was the most resistance to all concentrations of crude extracts and showing no inhibition zones.

In this study, the aqueous extract was not found to inhibit all gram-positive pathogens and three Gram-negative pathogens (*E. coli* ATCC 25922, *K. pneumoniae*, and *S. typhimurium* ATCC 13311). The acetonic and ethanolic extracts of VA leaf showed potential activity against seven out of nine tested organisms with the inhibition zone ranged between 7.00±0.00–16.33±0.58 mm and 7.00±0.00–15.00±1.00 mm, respectively. All crude extracts displayed weak antibacterial activity against Gram-positive bacterial, especially aqueous extracts (IZ=7.00±0.00 mm). The 99.8% chloroform displayed weak antibacterial activity against Gram-negative bacterial (IZ=7.00±0.00–8.00±0.00 mm). The zone of inhibition of ampicillin control (10 µg) and CIP control (10 µg) ranges from 7.00±0.00–41.33±0.58 mm to 7.00±0.00–37.67±0.58 mm, respectively (Data not shown).

The result of this study was consistent with Arekemase *et al.* (2013) [32]. They found that the ethanolic extract of VA leaf showed effective resistance against *E. coli*, *S. aureus*, *P. aeruginosa,* and *K. pneumoniae*. This result also correlated to the results of Lyumugabe *et al.* (2017) [33] that the crude leaf extract of VA can resist against *E. coli*, *S. aureus,* and *S. typhimurium*. On the other hand, this finding contradicts the result of Ogundare (2011) [34] which found no antimicrobial properties of ethanolic extract of VA leaf against *E. coli* and *P. aeruginosa*.

The *in vitro* the antibacterial activity of crude VA leaf extracts in broth micro-dilution has been shown in Table 5. The MIC analysis of VA leaf extracts showed the optimum bacteriostatic concentration for aqueous, acetone, and chloroform extracts, while MBC analysis of ethanolic extract represented the optimum bactericidal concentration in all tested bacterial strains. The MIC value of aqueous, 95% ethanol, and 75% acetone crude extracts against all tested pathogenic bacterial showed the interval from 25 to 200 mg/ml. Most of the Gram-positive bacteria tested in this experiment were more susceptible to all extracts than Gram-negative, especially *Bacillus cereus* DMST 5040 (MIC = 25–100 mg/ml and MBC = 50–>200 mg/ml). *E. coli* ATCC 25922 showed more resistant to all crude extracts (MIC = 100–>200 mg/ ml and MBC = 200–>200 mg/ml). The 95% ethanolic extract *of* VA leaf *showed* the strongest *inhibitory activity* against all *bacterial strains growth*, followed by 75% acetone, 99.8% chloroform, and distilled water (aqueous), respectively. All tested bacterial strains were susceptible to ampicillin and CIP. In the study of Habtom and Gebrehiwo (2019) [35], the MIC values of ethanolic extract of VA leaf against *S. aureus* and *E. coli* were 25 and 100 mg/ml that was similar with this study. However, our result was controversy to the findings of Evbuomwan *et al*. (2018) [27] which showed the MICs of *S. aureus* and *E. coli* equal to 100 and 25 mg/ml, respectively.

Based on our antimicrobial activity, the crude extracts of VA leaf showed inhibitory activity against all nine pathogenic strain, including Gram-positive and Gram-negative strains which indicate their broad-spectrum activity. The difference in antimicrobial effects of VA leaf with the previous studies may due to the age of the plant used, freshness of plant materials, physical factors for growth (light and water), and microbial contamination in the field [36]. The difference sensitivity in susceptibility between Gram-positive and -negative strains to various crude extracts could be due to the structural difference in the bacterial cell wall, composition of outer membrane, and the amount of peptidoglycan. Gram-positive cell wall contains high peptidoglycan content with teichoic acids and showed a thicker cell wall than the Gram-negative cell wall which has a thin peptidoglycan layer without teichoic acids [37].

Table 5: The MICs and MBCs of *V. amygdalina* leaf extracts against some pathogenic strains by broth microdilution method

Solvents extraction	Microorganisms								
	Gram-positive strains				Gram-negative strains				
	BC	EF	SA	SE	EC	KP	PA	PM	ST
Distilled water									
MIC	100	100	100	200	200	100	100	200	200
MBC	>200	>200	>200	>200	>200	>200	>200	>200	>200
95% Ethanol									
MIC	25	25	25	100	100	50	50	100	50
MBC	50	100	100	200	200	200	200	200	200
75% Acetone									
MIC	50	100	6.25	200	200	100	100	200	200
MBC	50	100	25	200	>200	100	100	200	>200
99.8% Chloroform									
MIC	100	100	50	200	200	50	200	100	>200
MBC	200	>200	200	>200	>200	200	>200	>200	>200
Antibiotics									
Ampicil-lin									
MIC	0.039	0.009	0.156	0.019	0.156	0.009	0.019	0.019	0.078
MBC	0.039	0.009	0.156	0.019	0.156	0.009	0.019	0.039	0.078
Cipro-floxacin									
MIC	0.156	0.313	0.019	0.002	0.019	0.313	0.009	0.004	0.078
MBC	0.156	0.625	0.039	0.002	0.019	0.625	0.019	0.078	0.156

BC: *B. cereus* DMST 5040, EF: *E. faecalis* DMST 4736, SA: *S. aureus* ATCC 25923, SE: *S. epidermidis*, EC: *E. coli* ATCC 25922, KP: *K. pneumoniae*, PA: *P. aeruginosa* ATCC 27853, PM: *P. mirabilis* DMST 8212, and ST: *S. typhimurium* ATCC 13311

Natural hair shampoo formulation

Based on the previous results, crude ethanolic extracts of VA leaf were the most suitable to apply in our formulation of natural hair shampoo as an auxiliary ingredient based on the grounds of its higher antioxidant (IC_{50} of 1.88±0.02 µg/ml) and antimicrobial activities. In this study, the natural hair shampoo was formulated by the concentration of 10 and 100 times of IC_{50} value of ethanolic extract shown in Table 1 and named as Formula 1 and Formula 2, respectively. The ethanolic extract of VA leaf was rich in antioxidant and antimicrobial compounds. This valuable point can be used in terms of marketing strategies and the value added of natural shampoo compare with the synthetic active ingredient used shampoo.

Evaluation of natural shampoos

The physicochemical properties of our formulated natural hair shampoo were evaluated compared with the commercial shampoos, including color, odor, and transparency (Table 6). The formulated shampoo (1) and (2) showed opaque, light yellow-brown in color, and have a pleasant odor (Fig. 1). There was no significant difference ($p>0.05$) between our formulated shampoo and commercial shampoo in terms of odor characteristic by 30 volunteer testing. However, the color and transparency levels were significantly different.

The pH level of shampoo is correlated to hair damage. Shampoo with neutral pH (pH 6.5-6.9) can reduce eye irritation between use, improves hair quality, and maintains the ecological balance of the scalp [38]. The pH of formulated and commercial shampoos has shown in Table 6. The acid balanced values were observed with commercial shampoos (6.8±0.02). The pH of formulated shampoo (1) and (2) was found to be nearly commercial with a pH of 6.7±0.02 and 6.5 ±0.02, respectively. High quality shampoo should contain only 20–30% of solid content [22,39]. The high solid content shampoo will be difficult in the washing out process. In this study, both formulated shampoo (1) and (2) showed higher solid content than commercial shampoo as 26.23± 0.30% and 26.45±0.50% compared with 25.53±0.20%. However, the solid content of both shampoos was still lower than 30%. Thus, our formulated shampoos can be considered as easy to rinse shampoo. Another important point of quality shampoo is the dispersion and cleaning performance on the dirty hair. The dispersion of shampoo is an important criterion in shampoo evaluation in terms of cleaning action. If ink or dirt was still appearing inside the shampoo foam, the shampoo will be considered as poor quality shampoo [22]. Our experiment found that both formulated shampoo (1) and (2) can stay over and did not rapidly slip out of the hair. Both formulated shampoo and commercial shampoo showed similar absorption of hair dirt into the foam during the coverage time over the hair. Therefore, both formulated and commercial shampoos were classified as good in the dirt dispersion parameter (Table 6).

Table 6: Characterizations of formulated and commercial shampoos

Evaluation parameters	Formulated shampoos		Commercial shampoo (Naturals Argan)
	1	2	
Color	Light yellow	Light brown	Light
Odor	Pleasant	Pleasant	Pleasant
Transparency	Opaque	Opaque	Transparent
pH	6.70±0.02	6.50±0.02	6.80±0.02
% Solid content	22.23±0.30	22.45±0.50	25.53±0.20
Foam volume (ml)	95±5	97±5	114±5
Wetting time (s)	157	152	155
Dirt dispersion	Good	Good	Good

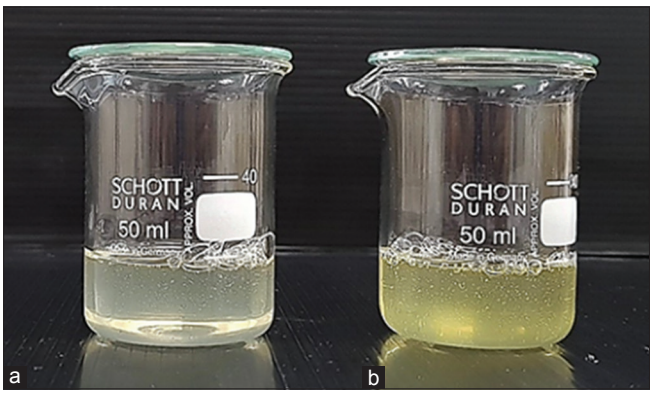

Fig. 1: Physical appearance of formulated natural shampoos (1; a) and (2; b)

The foam quality and foam stability formulations have been shown in Table 6. Our formulation (1) and (2) showed good foam quality and stability. The remaining time of foam forming was about 5 min. Both formulated shampoo (1) and (2) generated the foam volume of 95±5 and 97±5 ml, respectively, while commercial shampoo produced a foam volume of 114±5 ml (Table 6). The formation of foam of our formulated shampoo showed similar sizes, uniform, denser, and stability compared with the commercial samples. In the wetting time test, the wetting times of our formulated shampoos (1) and (2) were 157 and 152 sec, while the commercial shampoo showed the wetting times of 155 s (Table 6). The good quality shampoo should express high wetting times to demonstrate the diffusion ability. Long wetting time can represent a penetration performance of shampoo to the hair shaft [21].

The product satisfaction testing of formulated shampoo (1) and (2) was evaluated in terms of appearance, odor, color, foaming quantity, feelings after use, packaging, and overall acceptability by 5-point hedonic scales (n=30 persons). The acceptability scores of formulated shampoo (1) showed a higher score in all terms compared with formulated shampoo (2). The overall acceptability scores of formulated shampoo (1) and (2) were 4.33±0.55 (like modulately) and 3.63±0.49 (like modulately). In our experiment, the commercial shampoo obtained a customer satisfaction score as 3.57±0.63 (like modulately).

CONCLUSIONS

The crude extracts of VA leaf represent some potential bioactive phytochemical compounds, including saponins, flavonoids, tannins, steroids, terpenoids, cardiac glycosides, and alkaloids which responsible for the antioxidant and antibacterial activities of the plant. These phytochemical compounds exhibited as a broad-spectrum agent in antibacterial activity against both Gram-positive (four tested strains) and Gram-negative (five tested strains) bacteria. The phytochemical in different solvent extraction (distilled water, 95% ethanol, 75% acetone, and 99.8% chloroform) showed different efficiency of bioactive activities (antioxidant and antimicrobial activities) which could be due to their difference in polarity and the ability in phytochemical extraction in the plant samples. The ethanolic and acetonic extracts of VA leaf provided more consistent antimicrobial activity when compared with aqueous and chloroform extracts. Although the ethanolic crude extract expressed lower TPC and TFC than other solvents, it showed the highest antioxidant activity by DPPH assay as 85.730±0.001 of inhibition percentage and IC_{50} value of 1.88±0.02 µg/ml. Thus, the ethanolic crude extract of VA leaf is a rich antioxidant and antimicrobial source and suitable to apply in the natural hair shampoo development. From our experiment, formulation 1 of natural hair shampoo was the most suitable for further commercial development. This formula showed good characteristics, stability, and obtained the highest satisfaction score (4.33±0.55) (evaluated by 30 volunteers). Therefore, VA is a potential source of natural antioxidant and antimicrobial agents to use in natural cosmetic products. Natural hair shampoo from VA extract was not only clean the hair but also contains bioactive substances that protect the hair from oxidative substances or pathogenic bacteria with low effect to the normal skin bacterial flora (*S. epidermidis*). This herbal product can help to reduce the skin irritation of users in terms of antioxidant. Thus, further development of this herbal is noticeable in terms of natural product development.

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AUTHORS' CONTRIBUTIONS

Sirikhwan Tinrat analyzed the laboratory work, analyzed the data, and wrote the manuscript. Both authors read and approved the manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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