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Original Research Article

Cytotoxic, colony formation and anti-migratory effects of *Spilanthes acmella* (Asteraceae) aerial extract on MCF-7 cells and its cream formulation

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Abstract

Purpose: *To determine anti-breast cancer activities of Spilanthes acmella (S. acmella) extract.*
Methods: *S. acmella was macerated with 95% ethanol. Phenolic, flavonoid content and antioxidant activity of the extract were assessed using Folin Ciocalteu method, aluminum chloride (AlCl3) colorimetric method and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, respectively. Cytotoxicity, colony formation and cell migration suppression on MCF-7 cells, representing anti-breast cancer effects, were also evaluated by sulforhodamine B (SRB), clonogenic and wound healing assays, respectively. Creams containing the extract were formulated and then characterized in terms of their physical appearance, viscosity and pH, before and after stability testing.*
Results: *The crude extract contained phenolic content of 62.8 ± 5.2 mg gallic acid equivalent/g and flavonoid content of 375.6 ± 20.1 mg rutin equivalent/g. The results showed that the extract exhibits antioxidant effect with half-maximal inhibitory concentration (IC_{50}) of 1.2 ± 0.1 mg/mL. It showed cytotoxicity on MCF-7 cells with IC_{50} of 37.1 ± 1.1 µg/mL in 48 h and inhibited colony formation of cells with IC_{50} of 44.9 ± 1.3 µM. In addition, it demonstrated an anti-migration effect at a concentration of 50 µg/mL. The developed creams displayed good physical appearance and maintained stable physical properties overt a two-month period.*
Conclusion: *S. acmella extract exhibits potential anti-breast cancer activity. The cream containing the extract is promising for the topical treatment of breast cancer.*

Keywords: *Spilanthes acmella, Antioxidant, Anti-breast cancer, Flavonoids, Phenolics, Topical cream*

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INTRODUCTION

Cancer is the second-leading cause of death for humans worldwide after cardiovascular diseases [1]. The estimated number of deaths caused by cancer worldwide will be more than 13.1 million by the year 2030 [2,3]. Among various cancers, breast cancer is considered the most serious health problem worldwide for women. Treatment with drugs and surgery is currently the standard used for breast cancer. However, the failure of breast cancer treatment is generally reported due

to drug resistance and drug toxicity [4,5]. Therefore, a more effective and safer anti-breast cancer agent is needed. Medicinal plants have gained intensive focus for being effective and value-added sources of anticancer agents [6].

Spilanthes acmella (*S. Acmella*) belongs to the *Asteraceae* family. This edible medicinal plant is a well-known treatment for toothaches because of its anesthetic effect [7,8]. In addition, it is used for the treatment of fever, pain, flu, gastric ulcer and malaria in traditional medicine around the world [9,10]. However, there is no data for the anticancer activity of the extract of *S. acmella*. Among the various bioactive compounds found in medicinal plants, flavonoids and phenolic generally respond to antioxidant activity. In addition, it has been reported that plants enriched with antioxidant activity exhibit *in vitro* anticancer activity [11]. This is due to cancer being closely linked to oxidative stress in the human body after exposure to free radicals. Thereafter, gene mutations may occur, resulting in carcinogenesis. For the anti-breast cancer activity of plant extract, cytotoxic effect on breast cancer cells is required. Also, the inhibition of replicative ability and the suppression of metastasis in the cells require necessary investigation. This is due to metastasis and the proliferation of cancer cells after treatment with either surgery or chemotherapy related to the potential of recurrence for breast cancer [12]. Generally, colony formation assay is used to determine the replicative ability of cancer cells, while wound healing assay is used to determine the migration of cancer cells [13,14]. Therefore, the inhibition of colony formation and the suppression of cell migration effects of the extract on human MCF-7 breast cancer cells were evaluated in this study.

Topical formulations are convenient and minimize side effects when compared to other administration routes. Nowadays, there are topical formulations of 5-fluorouracil (5-FU), diclofenac and imiquimod for skin cancer treatment [15]. Additionally, patients with cutaneous metastasis breast cancer have been treated with chemotherapy combined with topical treatment, either 5-FU cream or imiquimod cream, in order to produce effective outcomes and better quality of life [16,17]. Creams are interesting as a topical formulation in this study because one or more active compounds can be dispersed or dissolved in the formulation [18]. Compared to ointments, creams are more preferable for many patients because of the ease of spreading and removal after application onto the skin. Thus, the current study aimed to determine the phytochemical screening, phenolics, flavonoids, antioxidant activity and anti-breast cancer activities of *S. acmella* extract. Creams containing the extract was further formulated.

EXPERIMENTAL

Preparation of plant sample and *S. acmella* extract

The plant sample (voucher no. 110896) was identified and deposited at the Queen Sirikit Botanic Garden (QBG) Herbarium, Chiang Mai. The aerial part of the plant was cleaned with water, chopped into small pieces and air-dried. It was then dried at 50 ^{o}C using a hot-air oven for 48 h. The dried plant was ground into powder and was macerated with 95 % ethanol for 72 h. Following filtration, the extract was concentrated using a rotary evaporator (Heidolph, Germany) at a controlled temperature of 50 ^{o}C. The yield of the extract was determined using Eq 1.

$$\text{Yield (\%)} = \{(\text{DWCE})/(\text{DWP})\}100 \quad \text{(1)}$$

where DWCE is the dry weight of the crude extract and DWLP is the dry weight of the *S. acmella* plant powder.

Phytochemical screening

Phytochemical screening of the extract was evaluated by specific chemical reaction for secondary metabolites including flavonoids, alkaloids, tannins, cyanogenic glycosides, saponins, antraquinones, terpenes and steroids [19]. The intensity of color or the formation of precipitate was used as positive responses to the screening tests.

Determination of phenolic and flavonoid contents

Phenolic content

In order to determine the phenolic content of the extract, 80 µL of Folin**-**Ciocalteu reagent was added to 40 µL of the extract solution **(**200 µg**/**mL**)**, which was then mixed together**.** After incubation for 5 min, 80 µL of 7 **%** sodium carbonate **(**Na_2CO_3**)** was added to the mixture**.** Thereafter, the reaction mixture was incubated for 30 min. The absorbance was measured at 750 nm using a microplate reader (Synergy H1, Biotek Instruments, Friedrichshall, Germany)**.** The phenolic content was calculated from the standard curve of gallic acid **(**20 **-** 100 µg**/**mL**)** and presented as gallic acid equivalent **(**GAE**)** per gram of crude extract**.**

Flavonoid content

In order to determine the flavonoid content of the extract, 100 µL of 2 **%** $AlCl_3$ solution was added to 100 µL of the extract solution **(**100 µg**/**mL**).** After incubation for 10 min, the absorbance was measured at 415 nm. The flavonoid content was calculated from the standard curve of rutin **(**20 **-** 100 µg**/**mL**)** and presented as rutin equivalent **(**RE**)** per gram of crude extract**.**

DPPH radical scavenging activity

Briefly, 100 µL of each extract solution (0.05 - 2.0 mg/mL) was mixed separately with 100 µL of 5.0 mM DPPH in a 96-well plate. After incubation for 20 min, the absorbances of the blank (without the test sample, Ab_{blank}) and the sample (Ab_{sample}) were determined at 540 nm. Gallic acid was served as a positive control. DPPH radical scavenging ability (S) was calculated using Eq 2.

$$S\ (\%)=((Ab_{blank} - Ab_{sample}))/Ab_{blank}) \times 100 \quad \text{.....(2)}$$

Evaluation of the effects of *S. acmella* extract on cytotoxicity, colony formation and cell migration

Cytotoxicity assay

The MCF-7 breast cancer cell (ATCC #HTB-22; Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), streptomycin (100 µg/mL) and penicillin (100 U/mL). The cytotoxic effect of the extract was assessed by the Sulforhodamine B (SRB) method, as previously described [20,21]. The cells were plated and then treated with the medium containing *S. acmella* extract in a dose of 0 – 500 µg/mL for 48 h. Then, they were stained with 0.4 % SRB dye solution at room temperature. After incubation for 30 min, the free SRB dye was discarded from the cells by washing several times, then solubilized in 10 mM Tris base buffer (pH 7.4). Absorbance was measured at 540 nm.

Clonogenic assay

In the determination of the colony formation inhibition of the extract, viable cells (500 cells) were plated in a 6-well plate and incubated for 24 h. Then, the *S. acmella* extract (100 µL) with various concentrations were added and further incubated for 24 h. The cells were then washed, and the fresh culture medium was added. After that, the cells were cultured for another 10 days (the cell culture medium was changed every 2 days). Thereafter, 0.5% crystal violet in methanol was used to stain the cultured cells, and the colony number was determined by direct counting method.

Cell migration assay

The cells were plated in a 24-well culture plate for 24 h at 37°C. Then, a straight wound in each well was created by scratching the cell using a sterile pipette tip. The extract solution (0 –100 µg/mL, 100 µL) was added and incubated for 48 h. Thereafter, the area of the uncovered region of the wound was measured using an inverted microscope (TS100, Nikon, Japan) at a magnification of x10. The percentage of relative closure of the scratch, indicating the anti-migratory effect of the extract, was calculated from the area data.

Preparation, characterization and stability study of cream bases

Cream bases were formulated using the cold process. The ingredients of the cream bases are shown in Table 1. The oil phase consisted of light mineral oil and Novemer ^{TM} EC-1, while the water phase consisted of polysorbate 20, Supguard GM-BP and purified water. The ingredients of the oil and water phases were dissolved separately and then mixed together with constant stirring using a stirring rod. In this investigation, the total weight of the oil phase was varied in the range of 3-8 %w/w, named cream bases 1, 2 and 3.

Table 1: Composition of cream base

Composition	Function	Amount (g)		
		Cream base-1	Cream base-2	Cream base-3
Light mineral oil	Oil phase	2	3	4
Novemer ^{TM} EC-1	Rheology modifier	1	3	4
Polysorbate 20	Emulsifier	0.5	0.5	0.5
Supguard GM-BP	Preservative	0.5	0.5	0.5
Purified water	Water phase	91	93	96
Total weight	-	100	100	100

***Note*:** Novemer ^{TM} EC-1 Polymer = Acrylates/Acrylamide copolymer 26 - 28%, mineral oil (NF Grade) 22 - 24%, polysorbate 85 1 - 3% and water 45 - 51%; Supguard GM –BP = Diazolidinyl urea, iodopropynyl butylcarbamate and propylene glycol

Characterization of cream bases

The characteristics of the prepared cream bases were studied as follows:

Physical appearance

The physical appearances of the cream bases in terms of color, smoothness and homogeneity were observed.

Viscosity determination

The viscosity of cream bases was measured at 25 °C using a Brookfield DV-II programmable rheometer equipped with a CP52 cone and plate spindle (Brookfield Engineering laboratories Inc., Massachusetts, USA).

pH determination

The pH of cream bases was measured at 25°C using a pH meter (SevenEasy, Mettler Toledo, USA).

Stability study

The stability of the cream bases was performed using a heat/cool cycling test. In a cycling test, the cream bases were kept in sealed containers. The samples were kept at 4 °C for 24 h, followed by 60 °C for 24 h. This process was carried out for six cycles. Thereafter, the physical appearance, viscosity and pH of the cream bases were evaluated, as mentioned above.

Preparation, characterization and stability study of creams containing the extract

After evaluation of the physical properties and stability test, the cream base with suitable viscosity and pH for topical use was selected and subjected to incorporation of the extract. The extract was added into the selected creams by dissolving the extract in water phase. The stability test of cream containing the extract was carried out at 4°C, room temperature and 45°C for 2 months. The physical appearance, viscosity and pH of the prepared creams were evaluated, before and after the stability test.

Statistical analysis

The data was analyzed using a paired t-test and one-way analysis of variance (ANOVA), followed by Turkey's post hoc test using SigmaStat software version 3.5 (Systat Software Inc., San Jose, CA, USA). p -value < 0.05 was considered a statistically significant difference.

RESULTS

Appearance, yield and phytochemical constituents

The *S. acmella* extract was obtained as a dark-green semi-solid with yield of 1.6 % (w/w). The phytochemical constituents of the extract are shown in Table 2. The extract showed positive results for flavonoids and alkaloids. It had been reported that flavonoids possessed anticancer activity, whereas alkaloids exhibited anti-proliferative activity on many different cancer cells [22, 23].

Table 2: Phytochemical profile of *S. acmella* extract

Constituent	Test	*S. acmella*
Flavonoids	Shinoda	+
Alkaloids	Dragendorff	+
Tannins	Gelatin	-
	Ferric chloride	-
Cyanogenic glycosides	Grignard	-
Saponins	Frothing	-
Antraquinones	Brontragen	-
Terpenes & Steroids	Liebermann burchard	-
	Keller kilani	-

+ = Positive; - = Negative

Phenolic and flavonoid contents, and antioxidant activity of extract

The crude extract contained phenolic content of 62.8 ± 5.2 mg GAE/g and flavonoid content of 375.6 ± 20.1 mg RE/g. It exhibited antioxidant activity with IC_{50} of 1.2 ± 0.1 mg/mL, relative to that of gallic acid (IC_{50} of 8.1 ± 0.4 µg/mL).

Cytotoxic effect of the extract

The effect of the *S. acmella* extract on the cell death of breast cancer MCF-7 was explored by the SRB method. This study indicated that the extract increased the cell death of cancer cells in a dose- and time-dependent manner (Figure 1) with IC_{50} values of 80.2 ± 6.8 µg/mL for 24 h (Figure 1 A) and 37.1 ± 1.1 µg/mL for 48 h (Figure 1 B).

Anti-colony formation effect of the extract

The effect of the extract on the replicative capability of breast cancer MCF-7 cells was performed by the clonogenic assay. This study showed that treatment with the extract caused decrease in the colony forming ability of MCF-7 cancer cells in dose-dependent manner (Figure 2 A). The extract strongly inhibited the ability of

colony formation in MCF-7 cancer cells with IC_{50} value of 44.9 ± 1.3 µM (Figure 2 B).

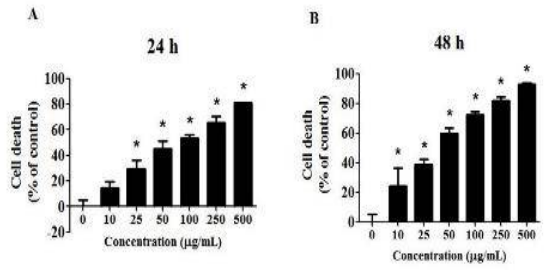


Figure 1: Effect of *S. acmella* extract on MCF-7 cancer cell death. Cells were treated with the extract (0 – 500 µg/mL) for 24 h (A) and for 48 h (B); * $p < 0.05$ when compared with control.

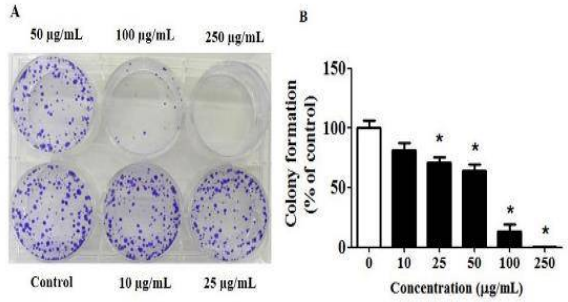


Figure 2: Effect of *S. acmella* extract on colony formation of MCF-7 cancer cell. The cells were exposed to extract (0 – 250 µg/mL) for 24 h, cultured for 10-days and the cells were then stained with crystal violet and photographed (A). The graph showed the colony formation percentage relative to the control (B); *$p < 0.05$ when compared with the control

Anti-migratory effect of the extract

The effect of the extract on MCF-7 cell migration was determined by the wound healing method. The results revealed that the extract inhibited MCF-7 cancer cell migration and was significant at 50 - 100 µg/mL with an IC_{50} value of 53.5 ± 8.9 µg/mL (Figure 3 A and B).

Characteristics and stability of cream bases

All prepared cream base formulations showed homogeneous and smoothness texture with white color. The mean viscosity and pH of the prepared cream bases (Cream b-1, b-2 and b-3) are shown in Figure 4. The viscosity of three cream formulations was in the range of 1,200 – 5,100 cps. The viscosity of cream base formulations increased with the increasing total weight of oil phase. The pH of all prepared cream bases was in the range of 6.8 - 7.4. After the stability test, all cream bases remained homogeneous (i.e. no phase separation). The viscosity and the pH values of the creams decreased. Cream base-2 was selected and subjected to incorporate the extract (0.01 % w/w) based on appropriate viscosity and pH following the stability test.

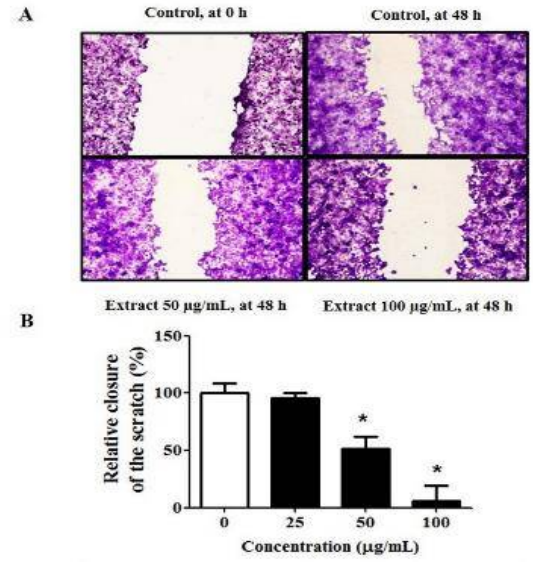


Figure 3: Effect of *S. acmella* extract on migration of MCF-7 cancer cell. The cells were exposed to extract (0 – 100 µg/mL) for 48 h and migration was captured using inverted microscopy (*x10*) (A). All results are expressed as percentage of control groups with three independent experiments and represent mean ± SEM values (B); * $p < 0.05$ when compared with control

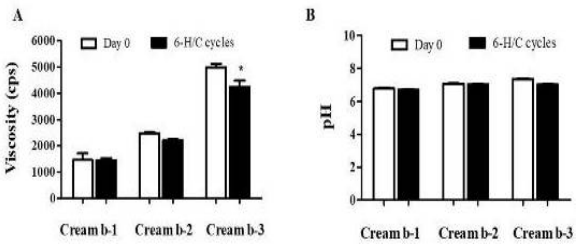
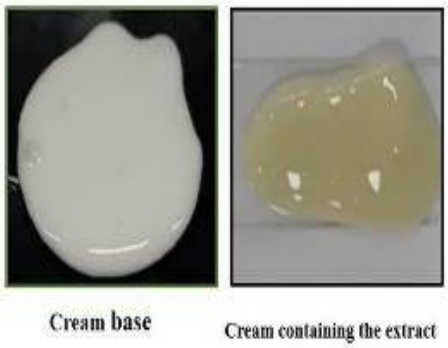


Figure 4: The viscosity (A) and pH (B) of prepared cream bases (Cream b) before and after heating-cooling (H/C) cycling test; * $p < 0.05$ when compared with control

Characteristics and stability of creams containing extract

The physical appearance, mean viscosity and pH of prepared cream base and cream containing the extract are illustrated in Figure 5. The cream containing the extract appeared light brown in color due to the color of the extract (Figure 5 A). Compared with the cream base, the viscosity of the cream containing the extract was lower, while its pH was slightly higher (Figure 5B). Following a heating-cooling cycling test, the cream containing the extract showed no phase

A



B

Characteristic	Formulation	
	Cream base	Cream containing the extract
Homogeneity	Homogeneous	Homogeneous
Viscosity*	2,468.7 ± 42.4	2,145.7 ± 65.8
pH	6.74 ± 0.01	6.93 ± 0.01

* The viscosity was measured at 40 rpm speed with a CP52 cone and plate spindle

Figure 5: Physical appearance (A) and (B) of prepared cream base (Cream b-2) and cream containing the extract

separation. It also showed homogeneity following storage at 4°C, room temperature and 45°C for two-month period (Figure 6). Under all conditions, the viscosities and pH of the creams decreased**.**

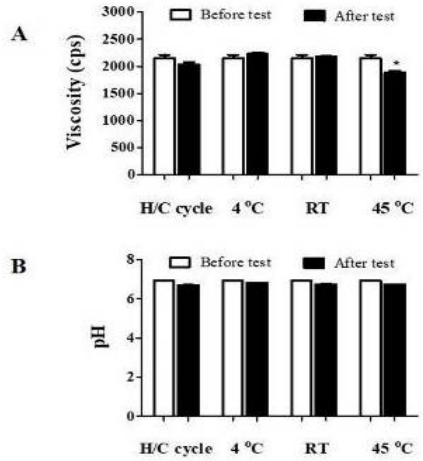


Figure 6: The viscosity (A) and pH (B) of prepared cream containing the extract before and after heating-cooling (H/C) cycling test as well as at 4 °C, room temperature (RT) and 45 °C for 2 months; * $p < 0.05$ when compared with control

DISCUSSION

At present, the natural source of chemotherapeutic agent for cancer treatment is gaining interest. This study is the first report to study the anticancer effect of *S. acmella* extract. In this study, *S. acmella* extract was evaluated for its chemotherapeutic effects in terms of antioxidant activity, cytotoxicity, colony formation inhibition and cell migration suppression on MCF- 7 cells. SRB method, the clonogenic assay and the wound scratch healing assay are usually used to determine cell cytotoxicity, cell replicative ability and cell metastasis, respectively.

The results showed that *S. acmella* extract exhibited anti-breast cancer activities because the extract showed cytotoxicity and colony formation inhibition effects on human breast MCF-7 cancer cells. It also had an anti-migration effect, indicating inhibited metastasis on the MCF-7 cells [13,14]. This study showed agreement with previous studies showing that ethanolic extract of *S. acmella* possessed high antioxidant activity [24]. This is due to the extract consisting of phenolics and flavonoids content. The antioxidant effect of the *S. acmella* extract may be responsible for its cytotoxic effect since previous reports showed that tumor cell line treated with a high dose of antioxidants resulted in cytotoxic effect [11].

Spilanthol, an alkylamide, is the main active compound that can be found in *S. acmella*. It can be extracted from *S. acmella* using ethanol based on its amphiphilic structure [25]. Spilanthol is usually used for the treatment of toothaches due to its anesthetic and analgesic effects. However, there is no data concerning spilanthol used for cancer treatment or anticancer activity. Besides spilanthol, scopoletin, phenolic compound, vanillic acid and *trans*-ferulic acid have been found in the aerial part of *S. acmella* [18]. Scopoletin and vanillic acid have been reported to have a cytotoxic effect on cancer cells [26,27]. Thus, scopoletin and vanillic acid will be determined and used as a marker for anti-breast cancer activity of the *S. acmella* extract. The mechanism of action will be identified in the future.

This study suggests that *S. acmella* has potential anticancer effects and is therapeutic value added in medicinal plant. The application of the extract on breast skin as a local treatment for breast cancer is safer and might produce effective outcomes. Cream formulations are preferable for topical use. Thereafter, cream containing the extract was prepared. After stability testing, the physical appearance and pH of the prepared cream remained the same, while its viscosity decreased significantly. However, viscosity remained within the working range for topical use.

CONCLUSION

The results show that *S. acmella* extract exhibits anticancer effects against human breast cancer cells. The anticancer activity of the extract may be directly related to the induction of cytotoxicity, inhibition of colony formation and suppression of cell migration on MCF-7 cells. The cream containing the extract has been successfully formulated and shown to have potentials for use in topical breast cancer treatment.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by them. Supavadee Boontha designed the study and the experiments, and prepared the manuscript. Tasana Pitaksuteepong assisted in experimental work and appraised the manuscript. Benjaporn Buranrat was responsible for MCF-7 cell study. Thatcha Thoedyotin, Thanaphon Saengtabtim, Pathitta Im-erb and Natthamon Chaniad prepared the extract and participated in formulation of the cream.

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