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Original Research Article

Evaluation of antioxidant and anticancer effects of *Piper betle* L (Piperaceae) leaf extract on MCF-7 cells, and preparation of transdermal patches of the extract

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Abstract

Purpose: *To determine the antioxidant and anticancer effects of Piper betle (P. betle) leaf extract on human breast cancer MCF-7 cells, and to develop transdermal patches containing the extract.*
Methods: *The leaf extract of P. betle was prepared by maceration method, and its antioxidant activity was evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Cytotoxicity and suppression of cell migration (indices of anticancer activity) were also assessed in MCF-7 cells by sulforhodamine B (SRB) and wound healing assays, respectively. Transdermal patches were developed using the casting method, and the resultant patches were evaluated with regard to their physical appearance and mechanical properties before and after a stability test.*
Results: *The extract exhibited antioxidant activity with half-maximal inhibitory concentration (IC_{50}) of 30.0 ± 0.1 µg/mL. It also showed cytotoxicity with an IC_{50} of 114.3 ± 14.9 µg/mL, and significantly suppressed the migration of MCF-7 cells at a dose of 25 µg/mL. Based on desirable characteristics, patch base formulations containing 4.2 % pectin, 0.4 % hydroxyl propyl methylcellulose (HPMC), 0.4 % polyvinyl pyrrolidine K-90 (PVP-K90) and 3 % propylene glycol (PG) were selected for incorporation into the extract.*
Conclusion: *Leaf extract of P. betle exhibits potential anti-breast cancer properties. A transdermal patch containing 0.03 % of the extract can be successfully developed for treatment of breast cancer.*

Keywords: *Piper betle leaf, Transdermal patches, Breast cancer, Antioxidants*

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INTRODUCTION

Breast cancer is one of the deadliest diseases, and the most common cancer in women worldwide. Currently, treatment failures in breast cancer are associated with drug resistance and drug toxicity [1]. Thus, there is need for development of newer and less toxic chemotherapeutic agents. Recently, many researchers have focused attention on medicinal

plant extracts rich in flavonoids and phenolic compounds as potential sources of alternative chemotherapeutic agents. This is due to reports from many studies showing that these compounds exert anticancer effects [2,3]. Moreover, positive correlations have been established between the antioxidant activities of plant extracts and their proliferative inhibition effects [4].

Piper betle L (*Piperaceae*) are generally named as betel leaf. It is one of many medicinal plants that have become popular in cancer studies [5]. Studies have shown that leaf extract of *P. betle* possess phenolic compounds such as hydroxychavicol, chavibetol and eugenol [5,6]. In addition, it has been noted that leaf extract of *P. betle* exhibits antioxidant activity, as revealed through DPPH, hydroxyl, nitric oxide and superoxide anion radical scavenging activities, as well as ferric reducing antioxidant power (FRAP) assay. Moreover, the anti-proliferative effect of *P. betle* leaf extract has been documented against B lymphocyte cell line (Raji cells) [7], KERATIN-forming tumor cell line (KB cells) [8], and MCF-7 cells [9]. These findings indicate the potential of the extract for use in the breast cancer treatment. However, not much is known about the effect of the leaf extract of *P. betle* on MCF-7 cell migration. Cancer metastasis is a crucial step in the prognosis of cancer patients [10]. The wound healing assay is usually used to determine migration of cancer cells. The inhibitory potential of *P. betle* leaf extract on the migration of MCF-7 cells can be demonstrated though its suppression of relative closure of scratch wound in the wound healing assay. If the extract suppresses healing of the scratch would, it may be reasonably concluded that it reduces cancer cell metastasis, implying that its application may result in good prognosis of cancer patients. Then, the extract could be considered as exerting good chemo-preventive effect.

Transdermal patches are dosage forms formulated for effective delivery of active principles/compounds at controlled rates, and within predictable times [11,12]. Transdermal administration is one of the promising alternative routes for delivery of anticancer drugs due to its safety, ease of administration, reduced frequency of dosing, by passing of hepatic first-pass effect, and reduce incidence of adverse side effects, when compared with the oral route [11].

This present study was examined the determination of the antioxidant and anticancer activities of leaf extract of *P. betle* on human breast cancer (MCF-7) cells, and development of transdermal patches of the extract.

EXPERIMENTAL

Preparation of *P. betle* leaf extract

The *P. betle* leaves were collected from the botanical garden of School of Pharmaceutical Sciences, University of Phayao, from October to December, 2018. The plant sample was identified by Dr. Prachaya Srisanga, Herbarium Curator at The Botanical Garden Organization, Queen Sirikit Botanic Garden (QBG). A voucher specimen was deposited at the QBG Herbarium, Chiang Mai (QBG voucher number 110895). The fresh leaves were washed, chopped into small pieces and air-dried. They were further dried at 50 ^{o}C using a hot-air oven for 48 h. The dried leaves were ground into powder, and were extracted with 95 % ethanol for 72 h. Following filtration, the extract was concentrated using a rotary evaporator (Heidolph, Germany) at controlled temperature of 50 ^{o}C. The percentage yield of the extract was determined using Eq 1.

Yield (%) = (WCE)/(WLP)100…….(1)

where WCE is dried weight of the crude extract, and WLP is dried weight of the *P. betle* leaf powder.

Evaluation of total phenolic and flavonoid contents, and antioxidant activity

*Phenolic cont*ent

In the evaluation of total phenolic content (TPC) of the extract, 40 µL of the extract (200 µg/mL) was mixed with 80 µL of Folin-Ciocalteu reagent for 5 min. Thereafter, 7 % sodium carbonate (Na_2Co_3) was added to the mixture, and the reaction mixture was incubated for 30 min. The solution absorbance was determined at 750 nm using a microplate reader (Synergy H1, Biotek Instruments, Friedrichshall, Germany). The TPC was determined using a gallic acid standard curve prepared using gallic acid with a serial concentrations of 20 - 100 µg/mL. The TPC was presented as gallic acid equivalent (GAE) per gram of crude extract.

Flavonoid content

In the evaluation of total flavonoid content (TFC) of the extract, 100 µL of 2 % aluminum chloride ($AlCl_3$) solution was added to the sample solution (100 µg/mL, 100 µL). After incubation for 10 min, the absorbance intensity was measured at 415 nm. The TFC was calculated from the standard

curve of rutin (20 - 100 µg/mL) and presented as rutin equivalent (RE) per g of the crude extract.

Antioxidant activity

In the evaluation of antioxidant activity, the extract solution was prepared by dissolving the extract in 95 % ethanol to obtain concentrations of 0.05 - 2.0 mg/mL. Then, 100 µL of each extract solution was separately mixed with 100 µL of 5.0 mM DPPH in a 96-well plate. Following incubation at room temperature for 20 min, absorbance of the control (without the test sample, A_{blank}) and that of the sample (A_{sample}) were determined at 540 nm. Gallic acid served as a positive control. The DPPH radical scavenging ability (D) was determined using Eq 2.

$$D\% = \{(A_{blank} - A_{sample})/A_{blank}\}100 \ldots\ldots\ldots (2)$$

Evaluation of effects of *P. betle* extract on cytotoxicity and cell migration

Cell culture and cytotoxicity assay

In the determination of cytotoxic activity of *P. betle* extract, the human breast cancer cell, MCF-7, (ATCC #HTB-22, Manassas, USA) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplementing of 10 % fetal bovine serum (FBS), 100 µg/mL streptomycin and 100 U/mL penicillin. The SRB method was used to determine the cytotoxic activity as previously described [12,13]. Cells were plated, exposed to the medium containing *P. betle* extract (0 – 250 µg/mL) for 48 h and then cells were stained with 0.4 % SRB for 30 min at room temperature. After incubation time, cells were discarded SRB dye, washed several times to remove excess SRB dye, and solubilized in Tris base buffer (10 mM, pH 7.4). The absorbance were measured at 540 nm using a microplate reader (Opsys MR™, Dynex Technologies, USA).

Suppression of cell migration

In the determination of anti-migratory effect of the extract, the cells were plated onto 24-wells culture plate for 24 h at 37 °C. Then, cell was created a straight wound by scratching the cell with a sterile pipette tip, and the cells were separately incubated with various doses of the extract (0 –100 µg/mL) 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 [14,15]. The percentage of relative closure of the scratch was calculated from the area data.

Formulation of transdermal patch base

The transdermal patch base was formulated using a film casting method. Three types of polymers i.e. pectin, HPMC and PVP-K 90 were used as film-formers, while propylene glycol (PG) or PEG-400 act as plasticizer, and paraben served as a preservative. Based on the best physical appearance of the preliminary trial batch (data not shown), the three types of polymers were combined at various ratios to generate a fixed polymer amount at 5 % (w/w) in each patch formulation. The amount of plasticizer used was varied from 1 to 5% (w/w). The compositions and formulation code of the preliminary trial batches are shown in Table 1.

The polymers were completely dissolved in distilled water. Then, the plasticizer and preservative were added under continuous stirring for 60 min. The polymer solutions were left at room temperature overnight to remove air bubbles. Then, 50 g of the polymer solution was poured on a petri dish and dried at 40 ^{o}C using a hot-air oven (Memmert, Schwabach, Germany). The dried patches were removed from the petri dish, wrapped in aluminum foil, and preserved in a desiccator prior to use.

Characterization of transdermal patch bases

Physical appearance

The patches were visually observed for color, homogeneity and flexibility.

Mechanical properties

The tensile strength of each patch was evaluated by a texture analyzer (TA.XT. plus, Stable Micro System, Surrey, UK). The patch was cut into shape of rectangular of 7 cm in length, and 1 cm in width. The patch was fixed between two cell grips of the instrument, with one grip fixed, and the other movable. A force was gradually applied to pull the patch until it was torn at the center. The tensile strength was calculated from Eq 3.

Tensile strength= (force at breaking point)/(area of the sample) …….. (3)

Elongation (E) was determined by comparing the lengths of each patch strip after the break point (FL) was reached, to the initial length of each patch strip before the break point (IL) was reached, as shown in Eq 4.

$$E\ (\%) = \{(FL-IL)/(IL)\}100 \ldots\ldots\ldots (4)$$

Preparation and characterization of patches containing *P. betle* leaf extract

Patch base formulations with good physical appearance and mechanical properties were selected for incorporation of the extract. The extract was dissolved in the blended polymer solution before mixing with other ingredients. The patches containing the extract were evaluated for their physical appearance and mechanical properties as described above. Weight variation, uniformity of thickness, and water uptake of the *P. betle* leaf extract containing patches were determined as indicated.

Weight variation

A set of three patches from each patch base, and patches containing the extract formulation with a diameter of 1 cm^2 were weighed on a digital balance (New Classic MF, ML802, Mettler Toledo, Switzerland) and the mean values were calculated.

Thickness uniformity

The thicknesses of the patches were measured using a vernier caliper (Macoh, Thailand) at five different places, and the mean values were determined.

Water uptake

Patches of each formulation were cut into 1 x 1 cm squares. The squares were accurately weighed and soaked in distilled water (2 mL) at 37 ^{o}C for 5 min. The patches were removed from the water and hung in the air to remove excess free water on their surfaces. They were then weighed again, and water uptake (W) was calculated as shown in Eq 5.

$$W (\%) = \{(FPW-IPW)/(IPW)\}100 \quad \ldots\ldots\ldots (5)$$

where FPW is final patch weight, and IPW is initial patch weight.

Stability studies

Stability studies on the prepared patches were carried out using heat/cool cycling test. In heat/cool cycling test, the patches were packed into aluminum foil and kept at 4 °C for 24 h, followed by kept at 60 °C for 24 h. This process was repeated in five cycles. The physical appearance, tensile strength, elongation, weight variation, thickness uniformity and water uptake of the prepared patch formulations were evaluated when first prepared, and after heat/cool cycling test.

Statistical analysis

The data values are shown in term of means ± standard deviation (SD). Statistical data analysis was done by a one-way analysis of variance (ANOVA) (Sigma Stat software version 3.5). Set value of $p < 0.05$ was considered statistical significance.

RESULTS

Physical appearance, TPC, TFC and antioxidant activity of extract

The leaf extract of *P. betle* was obtained as a dark-green paste at a percentage yield of approximately 1.4 % (w/w). The crude extract had TPC of 626.4 ± 60.5 mg GAE/g, and TFC of 138.5 ± 20.2 mg RE/g. It exhibited antioxidant activity with IC_{50} of 30.0 ± 0.1 µg/mL, relative to that of gallic acid (IC_{50} of 8.2 ± 0.5 µg/mL).

Cytotoxic effect of *P. betle* extract on breast cancer cells

The extract exerted cytotoxic activity on MCF-7 cells. The MCF-7 cell viability was decreased when increasing dose of the extract and it decreased the MCF-7 cell viability with an IC_{50} of 114.3 ± 14.9 µg/mL (Figure 1). In addition, it showed a significant impact at a concentration of 50 µg/mL.

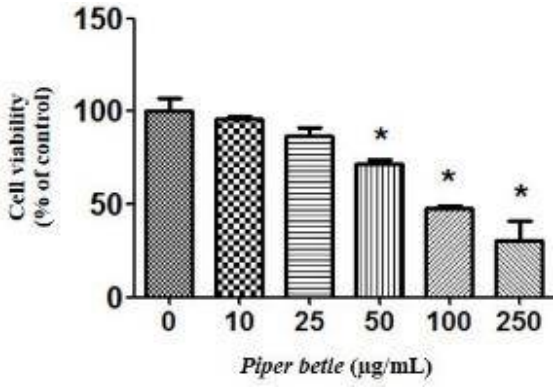

Figure 1: Cytotoxic activity of leaf extract of *P. betle* on MCF-7 cells, relative to control after 24 h

Anti-migratory effect of extract of *P. betle* on breast cancer cells

The results showed that the *P. betle* extract suppressed migration of the cancer cells in a concentration-dependent manner, with a significant effect at a concentration of 25 µg/mL (Figure 2 a). At this concentration, the extract suppressed the migration of breast cancer cells by approximately 30 %, relative to the control group (Figure 2 b).

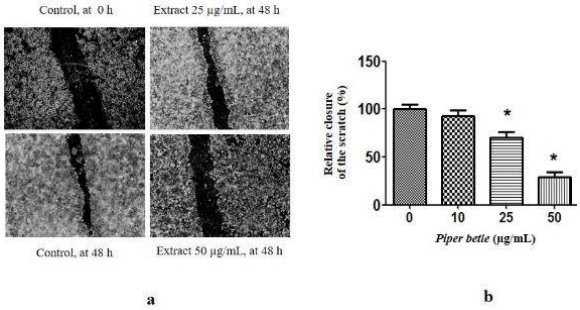

Figure 2: Pictures of migration of MCF-7 cancer cells incubated with the leaf extract of *P. betle*, and control for 48 h (a). Effect of the extract on percentage relative closure of scratch wound after treatment for 48 h, relative to control (b)

Characteristics of patch base formulations

Ten patch base formulations were given product codes F-1 to F-10 (Table 1). All formulations were yellowish, homogeneous, flexible and smooth films (data not shown). There were no problems removing the prepared patch base formulations from the petri dishes after oven-drying. The tensile strength and percentage elongation indicated the strength and elasticity of the patches, respectively [16]. The mechanical properties of the patch bases are shown in Figure 3. All patches of the prepared formulations exhibited percentage elongation at break values in the range of 29.88 – 72.88 %, and they showed tensile strength values in the range of 0.35 - 3.49 N/mm^2. In general, preferable patches for transdermal application should be flexible enough to follow the movements of the skin without breaking and the films should be strong enough to prevent abrasion of the films during contact with clothing [17]. Patch base formulation F-3 was selected for use in the preparation of transdermal patch containing the extract because it exhibited a high percentage elongation at break of 76.13 ± 12.68 % with a tensile strength of 1.19 ± 0.31 N/mm^2 (Figure 3). The extract was added to the patch formulation at the level of 0.03 % (w/w).

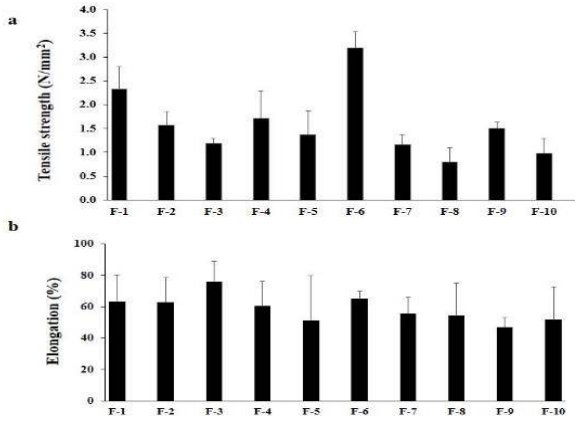

Figure 3: Tensile strength (a) and elongation (b) of patches

Characteristics of patch base and patch containing leaf extract of *P. betle*

The patch base was light yellow in color, while the patch containing the extract appeared dark green in color due to the color of the extract (Figure 4). The mechanical properties, weight, thickness and water uptake of the patches are shown in Table 2. Compared with the transdermal patch base, the tensile strength of patch containing the extract was increased, whereas the elongation was decreased.

a b

Figure 4: Physical appearance of (a) patch base (F-3) and (b) patch containing the extract

Table 1: Composition and formulation code given of patch base prepared

Composition	Function	Amount (mg)									
		F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8	F-9	F-10
Pectin	Film-former	4.2	4.2	4.2	4.2	1.67	0.4	3.0	3.6	4.2	1.0
HPMC	Film-former	0.4	0.4	0.4	0.4	1.67	0.4	1.0	0.7	0.4	1.0
PVP K-**90**	Film-former	0.4	0.4	0.4	0.4	1.67	0.4	1.0	0.7	0.4	3.0
PG	Plasticizer	1.0	2.0	3.0	4.0	5.0	-	-	-	-	-
PEG-400	Plasticizer	-	-	-	-	-	1.0	2.0	3.0	4.0	5.0
Paraben	Preservative	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Water	Solvent	93	92	91	90	89	93	92	91	90	89
Total weight	-	100	100	100	100	100	100	100	100	100	100

HPMC = hydroxyl propyl methylcellulose, PVP K-90 = polyvinyl pyrrolidine K-90; PG = propylene glycol, PEG-400 = polyethylene glycol 400, Paraben = paraben conc

Table 2: Physical appearance and mechanical properties of patch before and after stability test

Formulation	Tensile strength (N/mm^2, ± SD)	Elongation (%) ± SD	Weight (mg ± SD)	Thickness (mm, ± SD)	Water uptake (%) ± SD
Patch base					
Before test	1.19 ± 0.31	76.13 ± 12.68	81.7 ± 0.6	0.73 ± 0.10	46.76 ± 5.84
After test	2.33 ± 0.58$^{\#}$	56.73 ± 15.80$^{\#}$	58.0 ± 0.9$^{\#}$	0.60 ± 0.07	203.98 ± 14.70$^{\#}$
P. betle patch*					
Before test	1.45 ± 0.11	65.18 ± 2.92	65.4 ± 2.8	0.67 ± 0.10	98.17 ± 26.66
After test	1.59 ± 0.07	47.02 ± 2.88$^{\#}$	59.8 ± 0.1	0.61 ± 0.01	206.40 ± 7.63$^{\#}$

**P. betle* patch = patch containing *P. betle* extract; $^{\#}p < 0.05$, compared with the patch base

Stability

The results of stability test showed that the weight, thickness and percentage elongation of the patch base were decreased, whereas percentage water uptake and the tensile strength of the patch were increased. Similar results were obtained with the patch containing the extract (Table 2).

DISCUSSION

Nowadays, there are attempts to discover potent anticancer agents from herbal medicine, especially anti-cancer agents with low toxicity. A few studies have reported that medicinal plants with high antioxidant activities suppress the proliferation of cancer cells [2, 3]. The present study was assessed the antioxidant and anticancer effects of *P. betle* leaf extract on the human breast cancer cells, MCF-7. The results indicated that the *P. betle* leaf extract of had high antioxidant activity, most likely due to the presence of phenolic and flavonoid compounds in the extract. In addition, it has been reported that phenolic compounds and flavonoids have both chemotherapeutic and chemo-preventive effects [2-4]. The results revealed that the leaf extract of *P. betle* exerted dose-dependent anticancer activity against breast cancer cells. These findings are in agreement with previous reports [9]. Moreover, the extract inhibited metastasis of MCF-7 cells. Thus, the leaf extract of *P. betle* inhibits cancer metastasis and exerts chemo-preventive effects.

Reactive oxygen species (ROS) have been related with cancer pathogenesis. The anticancer activity of the *P. betle* extract may be due to its high antioxidant activity. In a study, it was revealed that hydroxychavicol, a crucial phenolic in *P. betle* leaf extract, was metabolized to an electrophile which conjugated with reduced glutathione; this increases the ROS sensitivity of cancer cells, resulting in enhanced apoptosis [18]. The cytotoxic and anti-migratory effects of the extract on MCF-7 cells suggest that local treatment i.e. application of the extract on the breast skin might produce effective outcomes. Consequently, the *P. betle* leaf extract was converted into transdermal patches, based on the finding that it was preserved in the patch formulation, and it exerted prolonged anticancer effect after the patch was applied on the skin.

To develop transdermal patch formulations, pectin was selected as a major component of the polymer blend due its good gelling property, high stability, biocompatibility and affordability [19,20]. The tensile strength of the prepared patch base decreased with increasing plasticizer concentration, due to the fact that plasticizers reduced the intermolecular forces between the chains of adjacent macromolecules [21]. Stability tests revealed that the prepared patch was stable throughout the study period. However, it is important to investigate the long-term stability and release profiles of the prepared patches in further studies.

CONCLUSION

The results obtained in present study indicate that the leaf extract *of P. betle* exerts antioxidant activity, also inhibits the viability and migration of MCF-7 cells. Thus, the extract has promising potential for development into an anticancer agent for breast cancer. This study is the first to develop *P. betle* patch against breast cancer. A transdermal patch containing the extract was successfully developed. The developed patch has potential uses for local treatment of breast cancer.

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. Jirapon Taowkaen, Thanaporn Phakwan, Teerapong Worauaichai and Piyarat Kamonnate prepared the extract and participated in formulation of the patch.

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