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The Comparison of Antioxidative and Cytotoxic Activities of Fresh and Dried *Piper betle* L. leave Extracts on MCF-7, HELA and SK-LU-1

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The use of herbal medicines produced from plants and herbs, as one element of complementary and alternative medicine, is still increasing worldwide. In this study, *Piper betle* L. leaf in two states, fresh and dried, was tested to explore its compounds and total phenolic and flavonoid contents. From there evaluated its antioxidant, and cytotoxic activities *In vitro*. Gas chromatography–mass spectrometry is a method for identifying compounds (GC-MS). The total phenolic content was measured by using Folin-Ciocalteu method and the total flavonoid content was based on a complex formation with $AlCl_3$. The antioxidant activity was evaluated by DPPH. The cytotoxic activity was performed against MCF-7, Hela, and SK-LU-1 cancer cell lines using sulforhodamine B assay. Toxicity testing for normal cells was also investigated. Results show the dried betel leaf extract (DBLE) contains eugenol (37.56%) as the primary compound, while 2.5-dimethylbenzoic acid

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(89.58%) was the primary compound in fresh betel leaf extract (FBLE). The extracts are rich polyphenols and flavonoids and have strong antioxidant activity. The polyphenol content in DBLE (240.9 mg GAE/g) is higher than FBLE (165.2 mg GAE/g). Especially, the DBLE has shown significant DPPH scavenging activity (IC_{50} = 3.21 µg/mL) when compared with standard ascorbic acid (IC_{50} = 6.71 µg/mL) and FBLE (IC_{50} = 22.97 µg/mL). The cytotoxic activity of FBLE against MCF-7 and Hela cell lines shows IC_{50} value above 50 µg/mL, while DBLE shows IC_{50} value of 21.88 µg/mL and 26.68 µg/mL, respectively, with no difference for the SK-LU-1 line (IC_{50} values from 35.0 to 38.0 µg/mL). There is a positive correlation between the phenolic content, antioxidant, and cytotoxic activities of the extracts. In conclusion, DBLE exhibits higher antioxidant and cytotoxic activities than FBLE.

Keywords: Betel leaf (Piper betle); fresh and dried leaves; antioxidant; cytotoxicity.

1. INTRODUCTION

"Cancer is the leading cause of death worldwide. Among them, lung cancer, breast cancer, and cervical cancer are common cancer types, especially affecting women the most" [1]. Currently, failures in cancer treatment are associated with drug resistance and toxicity. There is growing evidence that natural compounds are highly specific to tumor cells and have fewer side effects on normal cells; as a result, they offer great promise for the future treatment of cancer. The use of natural compounds as an adjuvant treatment in targeting the apoptotic pathway and inhibiting the self-renewal pathway of cancer cells (Hh, Notch, WNT, and BMI1) inhibits tumor recurrence and chemotherapy drug resistance [2].

Approximately 80% of the population in developing countries is dependent on natural or herbal medicines to treat various diseases. *Piper betle* is a traditional herb of the *Piperaceae* family which is used by many countries due to its wealthy content of macronutrients and micronutrients such as vitamin C, vitamin A, minerals, riboflavin, thiamine, tri-terpenoids, steroids, alkaloids, amino acids, tannins, essential oils, phenolics, flavonoids…There have been reports of biological benefits of betel leaf including platelet suppression, antidiabetic activity, immunomodulatory properties, and antiallergic activity. These activities are due to the high antioxidant effect of the compounds contained in this plant through DPPH, superoxide, and nitric oxide free radical scavenging activities [3]. Studies have shown that the leaf extract of *Piper betle* has phenolic compounds such as hydroxychavicol, chavibetol, eugenol, etc. Hydrochavicol in betel leaf induces cell cycle arrest at the S phase or G2/M and apoptosis in pancreatic cancer cells [4]. Water extract from betel leaf activates ATM, p73, and JNK pathway expressions, and inhibits tumor growth in Hep3B-bearing mice *via* inducing the MAPK-p73 pathway [5]; hexane extract exhibits significant cytotoxicity to Hela cells [6]. Extracting betel leaves with different extraction techniques (soxhlet, sonication, and maceration) and different solvents (water, methanol, ethyl acetate, and hexane) showed various results of total phenolic and flavonoid content, and substance content. Therefore, the antioxidant and cytotoxic activities were also different [7,8]. From the studies, it was found that different extraction methods play an important role in the presence and activity levels of phytochemicals. This is what accounts for their different pharmacological properties, which is essential for assessing their potential for human health benefits.

To study the differences between betel leaf extract extracted from dried leaves and fresh leaves affecting the biological activity, we analyzed the changes of the main phytochemicals, polyphenol and flavonoid content. Then compare the antioxidant and cytotoxic activities *in vitro* of the extracts. The results indicated that the content of phytochemicals, polyphenols and flavonoids, antioxidant and cytotoxic activity against cancer cells have significant differences between fresh and dried extracts of betel leaves.

2. MATERIALS

2.1 Plant

Fresh betel leaves (*Piper betle* L.) in the mature stage were collected from 8 a.m. to 10 a.m. in Thua Thien Hue province, VietNam. Choosing leaves that are intact, fresh, and free from pests.

2.2 Cell lines

Three human cancer cell lines including breast cancer (MCF-7), cervical cancer (HeLa) and lung cancer (SK-LU-1) cells are provided by the

Institute of Biotechnology, Vietnam Academy of Science and Technology.

Mesenchymal stem cells (MSCs) were isolated from Wharton's jelly of human umbilical cords provided by Hue Central Hospital (Hue, Vietnam).

3. METHODS

3.1 Extract Preparation

Betel leaves were cleaned and drained at room temperature. To collect the maximum possible content of bioactive phytochemicals for developing and applying in specific areas, betel leaf extract was examined in two states, fresh and dry.

For fresh betel leaf extract (FBLE), the leaves were crushed and soaked with ethanol 70% v/v in a ratio of 1:10 (w/v) for five hours at 50°C; stirring occasionally [9]. For dried betel leaf extract (DBLE), the fresh leaves were dried in an oven at 40^0C and ground into fine powder. The powder (150 g) was soaked with absolute ethanol (500 mL) for seven days at room temperature; stirring occasionally [10]. The samples were then filtered and evaporated at 40-50^0C until getting the dark brown concentrated solution [11]. Store samples at 4°C.

3.2 Gas Chromatography-mass Spectroscopic (GC-MS) Analysis

The analysis of extract compounds was performed on an Agilent GC-MS instrument equipped with a GC 7890A gas chromatograph and an MS 5975C mass spectrometer detector. The column used in the analysis was the DB-XLB capillary column (60 m x 0.25 mm x 0.25 µm). The extract was diluted with 3% dichloromethane solvent and filtered through a 0.45 µm PTFE filter. Analyses using isocratic modes were carried out using high-purity helium as carrier gas at a column flow rate of 1 mL/min with a ratio of 100:1. The temperature of the injection port was 250^0C. The column temperature program was as follows: an initial temperature of 40^0C, raised to 140^0C at a rate of 20^0C/min and held for five minutes, the final rate was raised from 4^0C/min to 270^0C. The mass spectrometry conditions included an ion source temperature of 230^0C, ionization energy of 70 eV, and a mass scan range of 40–500 amu. Peak areas were used for quantifying the constituent percentage in total betel leaf extracts.

3.3 Determination of Total Polyphenol Content

The total phenolic content (TPC) in FBLE and DBLE were estimated using the Folin–Ciocalteau (F-C) reagent described by Feduraev et al. (2019).

The F-C 10% reagent was diluted with distilled water just before the experiment. Different solutions of gallic acid (50, 100, 150, and 200 µg/mL) and betel leaf extracts (1 mg/mL) were prepared in methanol. A mixture of 0.5 mL of extract or standard solution at each concentration was mixed with 2.5 mL of F-C 10% reagent for several seconds. After 4 min, 1 mL of 7.5% Na_2CO_3 was added and incubated at room temperature in the dark. Absorbance was measured at 760 nm after two hours.

The TPC was calculated based on the gallic acid calibration curve using the formula:

$$C = c \times V/m$$

Where:
C = TPC (mg GAE/g); c = concentration of gallic acid established from the calibration curve (mg/mL); V = volume of extract (mL) and m = weight of plant extract (mg).

Results were expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract). All experiments were performed in triplicate.

3.4 Determination of Total Flavonoid Content

With slight modifications, the total flavonoid content (TFC) in the extracts was estimated by the aluminum chloride ($AlCl_3$) colorimetric method described by Marinova et al. (2005).

In methanol, different solutions of quercetin standard (20, 40, 60, 80, and 200 µg/mL) and betel leaf extracts (1 mg/mL) were produced. Following the addition of 0.3 mL of 5% $NaNO_2$, a sample containing 1 mL of extract or standard solution at each concentration was added separately to 4 mL of distilled water. After five minutes, 0.3 mL of 20% $AlCl_3$ is added and kept for 6 min. Finally, 2 mL of 1M NaOH solution was added, then up to 10 mL with distilled water and mixed well. The optical absorbance of the reaction solution was measured at 510 nm after 10 minutes. Similar to the determination of TPC, TFC was calculated based on the quercetin calibration curve according to the formula:

$$C = c \times V/m$$

Where:
C = TFC (mg QE/g); c = concentration of quercetin established from the calibration curve in (mg/mL); V = volume of extract (mL) and m = weight of plant extract (mg).

Results were expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g extract). All experiments were performed in triplicate.

3.5 DPPH Radical Scavenging Assay

The antioxidant activity of betel leaf extracts was determined through DPPH free radical scavenging according to Jahan et al. (2010) with some modifications.

A 100 µM DPPH solution was prepared in methanol solution. Diluting the extract into different concentrations (5, 10, 15, 20, 25 µg/mL). The mixture consisting of 1.5 mL of 100 µM DPPH and 1.5 mL of extract was shaken well and then incubated in the dark at room temperature for 30 min. The absorbance of the mixture was spectrophotometrically measured at λ_{max} = 517 nm and compared to the standard antioxidant (ascorbic acid).

Radical scavenging activity is expressed as inhibitory concentration (IC_{50}), i.e., extract concentration necessary to decrease the initial concentration of DPPH by 50%. The percentage of DPPH inhibition was calculated by using the following formula:

$$\text{DPPH inhibition (\%)} = [(A0-A)/A0] \times 100\%$$

Where:
A0 = the absorbance of blank sample solution without the compound to be tested;
A = the absorbance of the tested sample.

3.6 Cell Culture

MCF-7 breast cancer, Hela cervical cancer, and SK-LU-1 lung cancer cells were maintained in a DMEM growth medium containing 10% FBS, supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin, and 1 mM sodium pyruvate. Mesenchymal stem cells were cultured in the growth medium of DMEM/F12 medium containing 10% FBS and 1% antibiotic. Cells were grown to confluence in a humidified atmosphere containing 5% CO_2 at 37°C. Morphology was examined under inverted optical microscopy.

3.7 Testing the Effect of *Piper betle* L. Extract on MSCs

Mesenchymal stem cells (MSCs) are multipotent stem cells found in bone marrow that are important for making and repairing skeletal tissues. In addition, MSCs have a migration ability when activated, they can migrate into other injured and inflammatory sites to repair and regenerate. In this study, MSCs were used for toxicity testing of betel leaf extracts on normal cells.

The secondary MSCs were cultured on 6-well plates with a density of 1.000 cells/cm^2 in the growth medium. When the cells were approximately 60% confluent, the betel leaf extracts at various concentrations were added to the dishes. Cell monitoring was extended to 48 hours. Images of the MSCs at each test concentration were captured in three different fields to estimate error bars. MSC numbers were assessed by using the ImageJ software.

3.8 Cytotoxicity Test Using Sulforhodamine B (SRB) assay

The cytotoxicity test in cancer cells was performed according to the method of Skekan et al. (1990) by sulforhodamine B (SRB) assay.

Trypsinization to collect cell pellets. Adjust the cell concentration with medium to obtain the appropriate cell culture density per well of the 96-well plate at a volume of 190 µl. The extracts were dissolved in DMSO and diluted with culture medium (FBS-free) at concentrations of 2000 µg/mL, 400 µg/mL, 80 µg/mL, and 16 µg/mL. Aspirate 10 µL of the diluted extract introduced into wells containing the prepared cancer cell fluid mixture to achieve a final concentration of 100 µg/mL, 20 µg/mL, 4 µg/mL, and 0.8 µg/mL. After culturing for 72 hours, 20% TCA solution was added to immobilize cells for 60 min at 4^0C. DMSO 1% was used as a control at day 0 (the final concentration in the well was 0.05%). Cells in this well were incubated for only one hour. After fixation, cells were stained with 100 µl of 0.4% SRB for 30 min at 37^0C. Remove residual dye by washing it three times with 1% acetic acid and then drying it at room temperature. Then, the SRB was dissolved in 10 mM unbuffered Tris base, gently shaken for 10 min, and read the OD results at 540 nm on an ELISA Plate Reader (Biotek). Ellipticine at final concentrations in each well of 10, 2, 0.4, and 0.08 µg/mL was used as a positive control. The test was repeated 3 times. The IC_{50} value (concentration that inhibits 50% of

growth) was determined using TableCurve 2Dv4 computer software.

The percentage inhibition of cell growth was determined by the following formula:

$$\% \text{ cell inhibition} = 100 - \left(\frac{At - Ab}{Ac - Ab} \times 100\right)$$

Where;
At = Absorbance value of the test compound;
Ab = Absorbance value of blank;
Ac = Absorbance value of the control.

3.9 Statistical Analysis

The collected data were expressed as mean ± standard deviation (mean ± SD). Microsoft Excel software has been used to analyze data and draw graphs. The $p<0.05$ indicates statistically significant differences. The significant difference between the experimental samples was performed by one-way ANOVA.

4. RESULTS

4.1 Obtaining *P. betle* Extract

The distillation method extracted fresh and dry betel leaves on a laboratory scale. After being extracted, both extracts were in a concentrated state with a dark brown color and the characteristic pungent smell of betel. In terms of solubility, DBLE was more soluble in water than FBLE. All extracts were easily soluble in alcohol. The yield extracted from dried betel leaves (8.89%) was higher than that of fresh ones (4.95%) (Table 1).

4.2 Gas Chromatography- Mass Spectroscopic (GC-MS) Analysis

GC-MS results have identified FBLE contained the chemical compositions including 2.5-dimethylbezoic acid (89.58%), 2-methoxy-1-hydroxy-4-allylbenzene (3.18%), eugenol (2.89%), allylguaiacol (2.85%) and chavicol acetate (1.49%). Chemical compositions of DBLE identified were eugenol (37.56%), chavicol acetate (37.34%), caryophyllene (12.71%), Acetyleugenol (10.17%) and humulene (2.22%) (Table 2). The result showed that DBLE's main compounds chavicol acetate and eugeunol were not presented in FBLE. Meanwhile, the main chemical constituent identified in FBLE was 2.5-dimethylbenzoic acid which accounted for 89.58% and was not presented in DBLE.

Table 1. The recovery efficiency of two types of extracts

Extracts	Weight (g)		Recovery efficiency (%)
	Material	Extract	
FBLE	200	9.90	4.95
DBLE	100	8.89	8.89

Fig. 1. Betel leaf

Table 2. Chemical compositions of FBLE and DBLE

FBLE			DBLE		
No	**Chemical compositions**	**%**	**No**	**Chemical Compositions**	**%**
1	2.5-Dimethylbenzoic acid	89.58	1	Eugenol	37.56
2	2-Methoxy-1-hydroxy-4- allylbenzene	3.18	2	Chavicol acetate	37.34
3	Eugenol	2.89	3	Caryophyllene	12.71
4	Allylguaiacol	2.85	4	Acetyleugenol	10.17
5	Chavicol acetate	1.49	5	Humulene	2.22

Some studies also reported compounds in betel leaves similar to our study. GC-MS analysis of *P.betle* leaf extract collected from Denpasar, Indonesia contained 31 compounds including eugenol (25.03%), and 2.5-dimethylbenzoic acid (12.08%) which were two main components [12]. Methanol extracts from *P.betle* leaves in India revealed that the main active compounds were 2.5-dimethylbenzoic acid, 3.5-dimethylbenzoic acid, methionine [13]. Betel leaf extract from Indonesia analyzed components such as chavicol, eugenol, caryophyllene, xylene and chalarene [14]. The presence of caryophyllene, eugenol, and acetyl eugenol in the ethanol extract from the leaves of *P.betle* from Malaysia was also confirmed by HPTLC and GCMS analysis [15]. The chemical composition of betel leaves in different areas of Odisha also contained components such as eugenol, caryophyllene, acetyl eugenol, humulene, cadinene, 2-aminocarbonylbenzoic acid, etc [16].

4.3 Total Phenolic and Flavonoid Contents

TPC and TFC were calculated based on the respective standard linear of gallic acid ($y = 0.009x + 0.1222$, $R^2 = 0.9931$) and quercetin ($y = 0.0134x + 0.0078$, $R^2 = 0.9995$). The results are presented in Table 3.

TPC and TFC of DBLE were higher than that of FBLE, especially the TPC of both extracts was considerably different, ranging from 165 to 241 mg GAE/g. The respective TPC and TFC found in FBLE were 165.2 ± 4.6 mg GAE/g and 76.4 ± 2.1 mg QE/g. Meanwhile, TPC and TFC recorded in DBLE were 240.9 ± 0.11 mg GAE/g and 82.1 ± 2.9 mg QE/g.

Our study shows that dried betel leaf extract has similar TPC and higher TFC than the betel leaf study in Ho Chi Minh City (249.96 mg GAE/g and 27.82 mg RE/g) and Binh Duong province (386.343 mg GAE/g and 55.073 mg QE/g) in Vietnam [17,18]. The dried betel leaf from Osdisa extracted by sonication, soxhlet, and maceration methods using acetone as solvents found TPC from 50.0 to 57.6 mg GAE/10mg and TFC from 32.1 to 49.79 mg QE/mg [7]. The extract from fresh betel leaves in Indian regions had TPC from 95.0 to 128.0 mg GAE/g and TFC from 51.0 to 62.0 mg catechin which was lower than in our study [19]. The difference in total phenolic and flavonoid contents of betel leaf extract in distinct studies depends on geographical location as well as an extraction method.

4.4 Antioxidant activity

The antioxidant effect of FBLE and DBLE was determined based on DPPH radical scavenging activity. With the aim of comparison, ascorbic acid was selected to be a positive control. The DPPH assay results are shown in Table 4. The DPPH scavenging ability is proportional to the concentration of extract. The higher the concentration is, the better the free radical scavenging capacity is. The inhibition of DPPH of DBLE was considerably higher than that of FBLE with IC_{50} values of 3.21 µg/mL and 22.97 µg/mL respectively. It can be seen that the antioxidant activity of DBLE is 7 times higher than that of FBLE. Furthermore, the IC_{50} value of DBLE was lower than that of the positive control - ascorbic acid (3.21 µg/mL and 6.71 µg/mL respectively), which indicates that the crude extract from the dried betel leaf has a strong antioxidant capacity.

Table 3. TPC (mg GAE/g) and TFC (mg QE/g) of DBLE and FBLE

Extracts	TPC (mg GAE/g)	TFC (mg QE/g)
FBLE	165.2 ± 4.6	76.4 ± 2.1
DBLE	240.9 ± 0.11	82.1 ± 2.9

Table 4. DPPH radical scavenging activity of both extracts and ascorbic acid

Concentration (µg/ml)	Inhibition (%)		
	FBLE	**DBLE**	**Ascorbic acid**
5	4.83 ± 0.59	47.92 ± 1.74	37.38 ± 0.56
10	10.99 ± 0.47	69.90 ± 1.55	66.15 ± 0.85
15	29.51 ± 0.88	82.37 ± 0.84	78.23 ± 0.99
20	39.31 ± 1.24	92.47 ± 0.75	84.06 ± 0.72
25	57.99 ± 1.10	95.04 ± 1.05	95.71 ± 0.87
IC_{50}	**22.97 µg/mL**	**3.21 µg/mL**	**6.71 µg/mL**

Several studies have also confirmed the high antioxidant activity of betel leaf extract. For instance, the dried *Piper betle* leaf extract with ethanol collected in Phayao Province in Thailand exhibited antioxidant activity with IC_{50} of 30.0 ± 0.1 µg/mL [20]; the ethyl acetate extract from Malaysia dried leaf betel showed the radical scavenging activities against DPPH with IC_{50} of 40.0 µg/mL [8]; and the fresh betel leaf extract from Indonesian with ethanol was found to exhibit antioxidant activity with the IC_{50} of 17.4 ppm against DPPH [21]; fresh betel leaves in Kuala Lumpur demonstrated the DPPH scavenging activity with an IC_{50} value of 179.5 ± 93.1 µg/mL [22].

4.5 Testing the Effect of *Piper betle* L. Extract on MSCs

The toxicity of betel leaf extracts on MSCs by adding dilutions of betel leaf extract to the culture medium to determine cytotoxic concentrations. MSCs with 60% confluency was exposed to different concentrations (25, 50, 75, 100, 125 µg/mL) of FBLE and DBLE, monitoring for cytotoxicity for 48 hours at concentrations of FBLE and DBLE (Fig. 2).

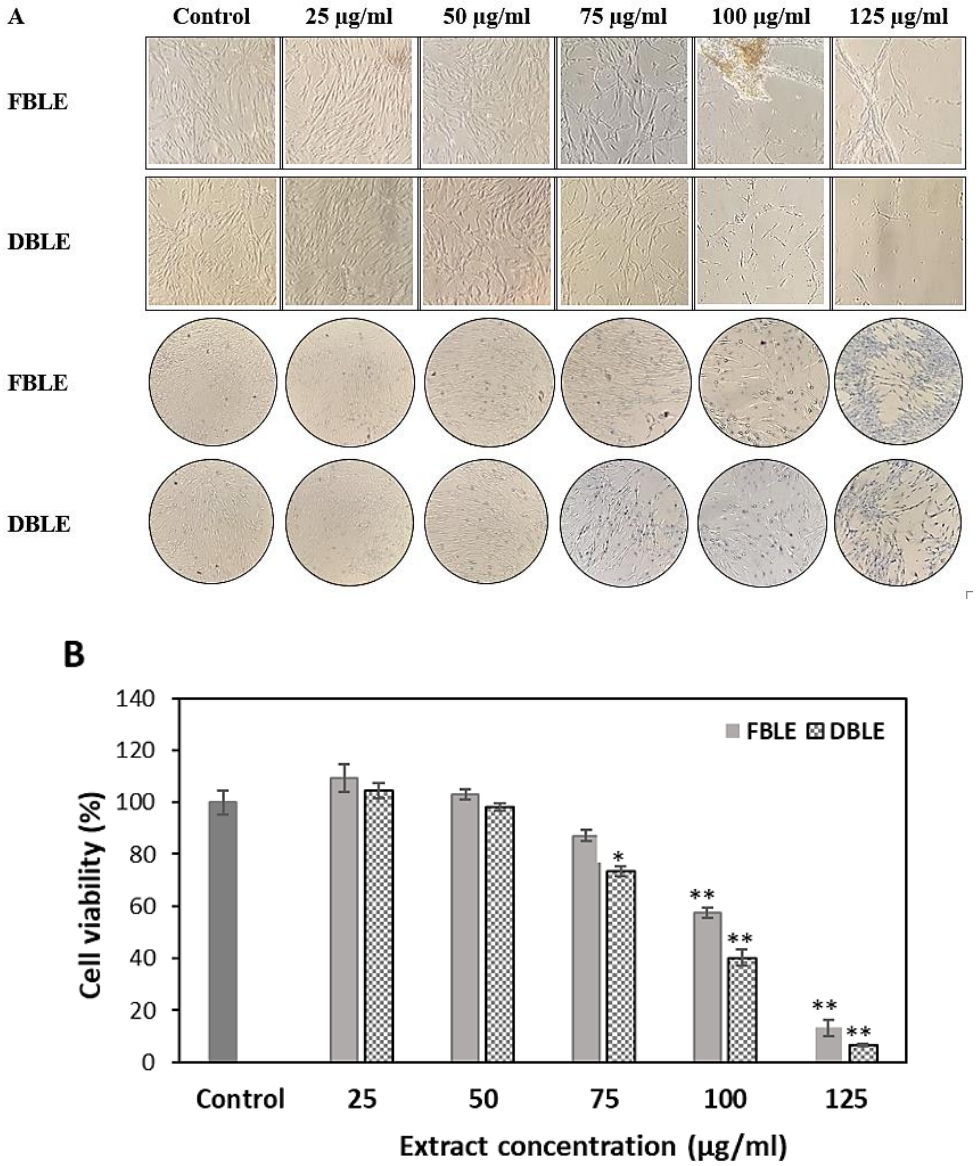

Fig. 2. Effect of *Piper betle* L. extracts at different concentrations on MSCs was presented in photos (A) and a graph (B). Percentage of cell viability at significant concentrations compared to control (*p<0.005; **p<0.001)

As shown in Fig. 2, both FBLE and DBLE did not induce any significant cytotoxicity at concentrations up to 50 µg/mL after 48 hours. The results also indicated that the cell viability at concentrations greater than 75 µg/mL significantly decreased with increasing the exposure concentration, indicating their less efficiency for the growth of the cells. At a 125 µg/mL concentration, betel leaf extract was dramatically toxic to MSCs. DBLE exhibited higher cytotoxicity to MSC than FBLE.

4.5 Cytotoxicity of Extracts Toward Cancer Cell Lines

According to the standards of the US National Cancer Institute, the extract is considered to have cytotoxic activity on cancer cells with $IC_{50} \leq 20$ µg/mL, while the purified substance is $IC_{50} \leq 5$ µM. After 72 hours of extract treatment, compared with the control samples, cells shrank, rounded and lost cellular adhesion to the substrate. Most cells were even detached from the surface of the tissue culture dishes plate and appeared floating in the culture medium which reduces the density of cells and cell clusters (Fig. 3). These are characteristics of apoptosis. At a concentration of 100 µg/mL, FBLE was able to inhibit and kill 75.27% of MCF-7 cells, 85.5% of Hela cells and 87.71% of SK-LU-1 cells. Meanwhile, DBLE at 100 µg/mL can inhibit and kill 96.35% of MCF-7 cells, 99.29% of Hela cells (almost completely) and 97.56% of SK-LU-1 cells (Table 5).

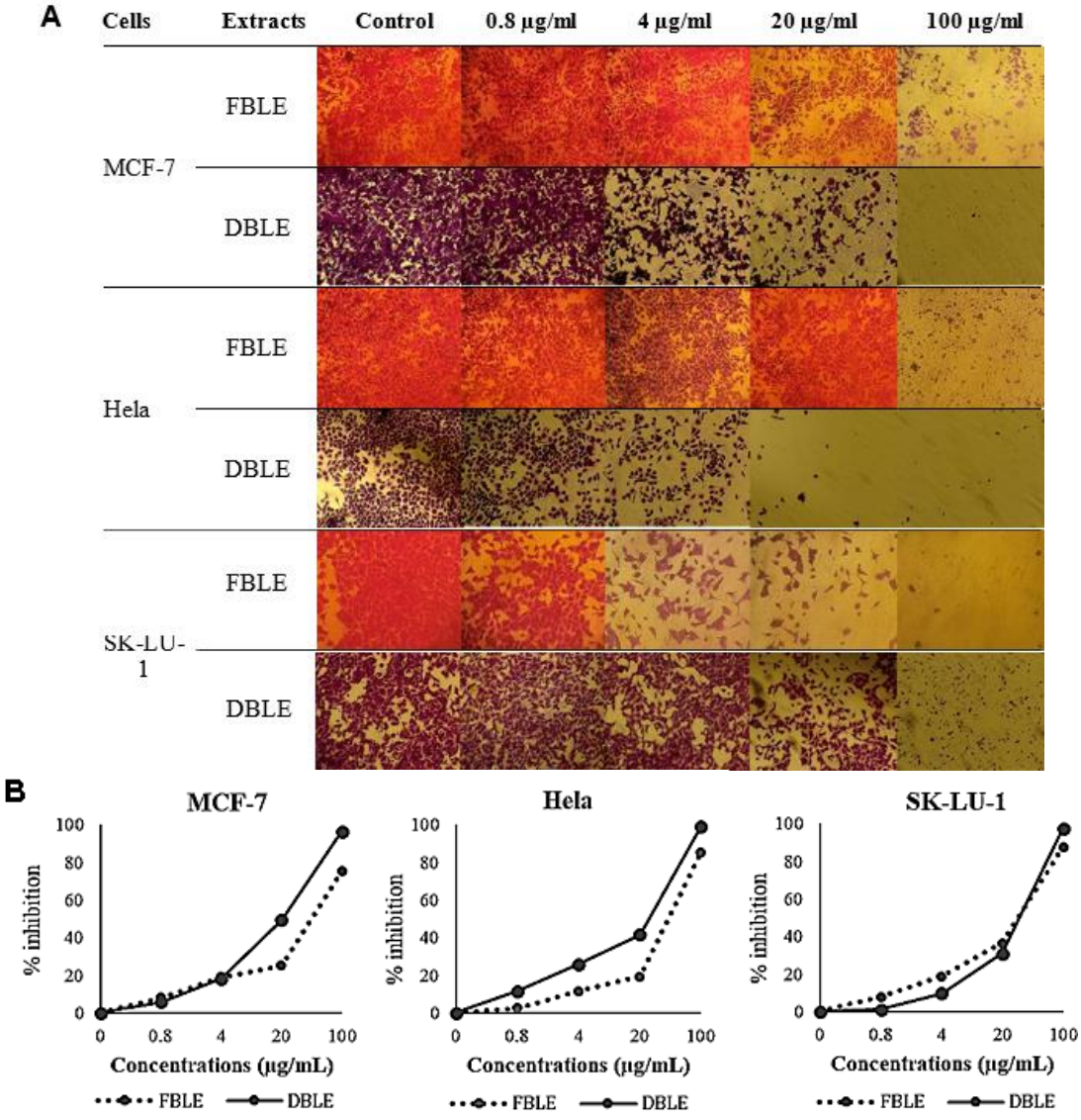

Fig. 3. Cell proliferation inhibitory effect of FBLE and DBLE on MCF-7, Hela and SK-LU-1 cells at different concentrations after 72 hours in photos (A) and a graph (B)

Table 5. Inhibitory growth effects against different cancer cells of FBLE and DBLE compared with Elipticine

Samples	Concentration (µg/ml)	% Inhibition		
		MCF-7	Hela	SK-LU-1
FBLE	**100**	75.27 ± 1.92	85.50 ± 1.19	87.71 ± 1.74
DBLE	**100**	96.35 ± 2.29	99.29 ± 1.71	97.56 ± 1.42
Ellipticine	**10**	92.13 ± 2.06	98.63 ± 2.24	97.94 ± 3.79

Table 6. IC_{50} value of extracts in different cancer cell lines compared with Elipticine

Samples	IC_{50} (µg/ml)		
	MCF-7	Hela	SK-LU-1
FBLE	56.05 ± 5.46	56.70 ± 4.16	35.07 ± 1.39
DBLE	21.88 ± 1.13	26.68 ± 1.55	38.45 ± 2.94
Ellipticine	0.34 ± 0.03	0.37 ± 0.02	0.39 ± 0.02

FBLE and DBLE show high cytotoxic activity against MCF-7 and Hela cell lines, in which the cytotoxicity of DBLE is twice that of FBLE. Specifically, the cytotoxicity of DBLE and FBLE with IC_{50} were 21.88 µg/mL and 56.05 µg/mL, respectively on MCF-7 cell line. Toward Hela cell line, the IC_{50} of DBLE and FBLE were 26.68 µg/mL and 56.7 µg/mL, respectively. However, the IC_{50} for cytotoxicity against SK-LU-1 of two extracts were almost the same at 38.45 µg/mL and 35.07 µg/mL, respectively (Table 6).

In this experiment, Ellipticine was the positive control to compare the cytotoxic activity of the extract. Elippticine is a potent cancer cell inhibitor. However, it is not used for medical purposes due to its high cytotoxicity causing side effects such as nausea, high blood pressure, dry mouth, and fungal infections in the tongue and esophagus, etc. In our research, DBLE at 100 µg/mL concentration caused inhibition on MCF-7 (96.35%) and Hela (99.29%) cells similar to Ellipticine at 10 µg/mL (92.13% for MCF-7 cells and 98.63% for Hela cells) (Table 5).

The results of cancer cell cytotoxicity in our study were higher than some studies in the world. The result of cytotoxicity on breast cancer by SRB revealed that ethanol extract from Thai betel leaves exhibited a cytotoxic effect with IC_{50} = 114 µg/mL [20]. Among the four distinct extracts (water, methanol, ethyl acetate and hexane) of *Piper betle* leaves, the ethyl acetate extract exhibited the highest inhibitory effect on the proliferation of MCF-7 cells (IC_{50} = 65 µg/mL) by the MTT assay [8]. The extract of fresh *Piper betle* leaf only effectively inhibited the proliferation of the KB cell line without affecting the proliferation of Hela cells [23]. Pre-clinical evaluation in cancer treatment of extracts from *Piper* species based on their cytotoxicity by MTT assay, *Piper betle* exhibited antitumor activities on Hela cells with IC_{50} = 49.66 mg/mL [6]. Thus, with the ability of cytotoxic activity in our study, dried betel leaves can be a potential therapeutic agent in cancer treatment.

5. DISCUSSION

Scientists are constantly looking for natural drugs to replace chemical drugs in cancer therapy with the goal of less toxicity and side effects. Cytotoxic and antioxidant activities *in vitro* of *P. betle* have been reported in many studies but mainly focused on the aqueous extracts or polar solvents. However, variations in composition and bioactivity can still occur depending on cultivar, location, growth conditions, and fresh or dried state, so data about antioxidant and cytotoxic activities remain relevant and crucial. In this study, we used ethanol and distilled water to extract fresh and dried leaves of *P.betle* in Vietnam to provide further insight into the antioxidant properties and cytotoxic effects on breast cancer (MCF-7), cervical cancer (Hela), lung cancer (SK-LU-1) cells *In vitro*.

In our study, the cytotoxicity of DPLE was 2 times higher than that of the FBLE for MCF-7 and Hela. This result may be due to the effect of the eugenol compound (EUG) with higher concentrations. Studies of eugenol showed its different effects on cancer cells; firstly, the effect of preventing cancer through its antioxidant effect, and secondly, the effect of killing cancer

cells by affecting several signaling pathways. Eugenol regulated multiple molecular targets to mediate cytotoxicity against cancer types through inhibition of Nuclear factor- kappa B (NF-κB) activation, regulation of prostaglandin synthesis, reduction of Cyclooxygenase-2 (Cox-2), B-cell lymphoma–2 (Bcl-2), interleukin-1 beta, and Proliferation Cell Nuclear Antigen (PCNA) activity; decreases in mitochondrial potential (ΔΨm) and inflammatory cytokines; increasing the generation of reactive oxygen species (ROS) and the expression of caspase-3, caspase-9, Bax, cytochrome (Cyt-c); S-phase cell cycle arrest; resulting in apoptotic cell death [24,25]. These series of findings suggest that EUG may induce apoptosis in cancer cells, e.g., breast cancer (MDA-MB-231, MCF-7), cervical cancer (SiHa, Hela), glioblastoma (DBTRG-05MG), lung cancer (A459), colon cancer (NCM-460), other melanoma cells (SK-Mel-28, A2058) [26,27]. Eugenol, one of the main components of betel leaf, has also been shown to possess anti-inflammatory effects in various animal models with different inflammatory agents [28].

In addition, the components of DBLE possess powerful antioxidant compounds such as acetyl eugenol, caryophyllene and humulene [29,30]. Caryophyllene inhibited the proliferation of glioblastoma, non-small-cell lung cancer, breast cancer, and myeloma cells [31,32]. Humulene inhibited hepatocellular carcinoma cell proliferation [33]. Especially, the combination of humulene and caryophyllene was more effective in reducing the proliferation of MCF-7 cells [34]. Therefore, we propose that the active constituents of the extract may interact additively or synergistically to protect against cancer.

Furthermore, the polyphenol content in DBLE (240.9 mg GAE/g) was higher than in FBLE (165.2 mg GAE/g). Polyphenols have been reported to have preventive effects against tumor initiation through numerous mechanisms such as the avoidance of genotoxic molecule formation and the blockade of mutagenic transforming enzyme activity; regulation of heme-containing phase I metabolic enzymes such as cytochrome P450s (CYPs); regulation of phase II metabolic enzymes to detoxify carcinogens, such as NADPH-quinone oxidoreductase-1 (NQO1), quinone reductase (QR), glutathione S-transferase (GST), as well as preventing DNA damage [35]. These implied that the polyphenol content may contribute to the cytotoxicity of breast and cervical cancer in this study at the tested concentrations of betel leaf. The DPPH free radical scavenging ability of DBLE was also higher than FBLE (IC_{50} = 3.21 µg/mL compared to 22.97 µg/mL). It can be seen that the cytotoxicity against cancer cell potential of betel leaf extracts was correlated with their polyphenol content and antioxidant activity.

Although there was a difference in cytotoxic activity on MCF-7 breast cancer and Hela cervical cancer cells of both extracts, their cytotoxic effect was similar on SK-LU-1 lung cancer cells. That may be due to the dimethylbenzoic compound with high content in FBLE affected the apoptosis of SK-LU-1 cells by a mechanism that has not yet been discovered. The resulting difference between fresh and dried betel leaves may be due to the thermal effect of the drying process on the product, which leads to the degradation of some phytochemicals, namely 2.5-dimethylbenzoic acid, and optimal conditions for the formation of eugenol compounds in dried leaves. These results can serve as a premise for further studies on the molecular mechanisms and signaling pathways in anti-cancer of fresh and dried betel leaves.

6. CONCLUSION

The results revealed that the different states of betel leaf, dry or fresh, affected the chemical composition, the polyphenols and flavonoids content, antioxidant and cytotoxic activities. Dried betel leaf extract showed significantly better antioxidant DPPH radical scavenging activity than fresh betel leaf extract. The cytotoxic activity of fresh leaf extract and dried leaf extract against lung cancer line SK-LU-1 was the same. However, this activity on MCF-7 breast cancer and Hela cervical cancer cell lines of dried betel leaf extract was two times higher than that of fresh betel leaf extract. These results may be due to differences in polyphenol and flavonoid content and main components in each extract.

Our results of cytotoxicity on cancer cell lines provided a scientific basis for using betel leaf as a potential source of chemotherapeutic agents for the treatment of breast cancer, cervical cancer and lung cancer *in vivo*, especially using dried betel leaf. At the same time, it also serves as a premise for further studies about the cancer inhibition mechanism of dimethylbenzoic compounds present in the fresh betel leaf extract.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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