Research Article

The Comparison of Antioxidative and Proliferation Inhibitor Properties of Fresh and Dried *Piper betle* L. Leave Extracts on MCF-7, Hela, and SK-LU-1 Cancer Cell Lines

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ABSTRACT

This research aimed to identify the bioactive compounds present in dried and fresh betel leaf extracts. The cytotoxicity properties and antioxidant activity of the extracts were assessed *in vitro*. Gas chromatography–mass spectrometry is a method for identifying biologically compounds (GC–MS). The GC-MS analysis identified eugenol as the primary compound in dried betel leaves (37.56%), while 2.5-dimethylbenzoic acid was the primary compound in fresh betel leaves (89.58%). Antioxidant activity is determined by DPPH's ability to neutralize free radicals. The ability of dried betel leaf extract (IC_{50} = 3.21 µg/mL) to capture free radicals is significantly greater than that of fresh betel leaf (IC_{50} = 22.97 µg/mL). On the cell lines of MCF-7, HELA, and SK-LU-1, cytotoxic activities were evaluated using the sulforhodamine B assay (SRB). With IC_{50} values of 21.88 µg/mL and 26.68 µg/mL in MCF-7 and HeLa cell lines, the dried betel leaf extract is more effective than fresh betel leaf extract at causing cytotoxicity in cancer cells. There is a positive correlation between the antioxidant activity of the plant and the antiproliferative effects of the extracts, as demonstrated by these findings. Thus, the fresh and dry states of the leaves are more strongly associated with antioxidant activity and cytotoxicity against breast cancer, cervical cancer, and lung cancer.

INTRODUCTION

Cancer is the leading cause of death worldwide, accounting for approximately 10 million deaths in 2020. Moreover, lung cancer accounted for the highest mortality rate, with an estimated 1.8 million deaths (18%). Breast cancer became the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%). Cervical cancer is the fourth most common cancer in women globally, with an estimated 604.000 new cases and 342.000 deaths. The global cancer burden is expected to be 28.4 million cases by 2040, a 47% increase from 2020 [1]. Currently, failures in cancer treatment are associated with drug resistance and toxicity. Hence, there is necessary to develop new chemotherapeutic agents that are less toxic while inhibiting the drug resistance of cancer cells.

There is growing evidence that natural compounds are highly specific to tumor cells and have fewer side effects on non-cancer 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]. As potential alternative sources of chemotherapeutic agents, researchers have recently focused on medicinal plants rich in natural compounds such as phenolics, flavonoids, alkaloids, taxanes, and phytoalexins, etc. The cytotoxicity properties of these compounds include inhibition of cancer cell proliferation, tumor development, angiogenesis, metastasis, inflammation, and induction of apoptosis. In addition, they can modulate the immune system's response and protect healthy cells from free radical damage [3]. By reducing free radicals and oxidative stress, natural antioxidants contribute to the repair of DNA damage, the reduction of abnormal cell division, and the prevention of mutations. There is a positive correlation between the antioxidant activity of plants and the effects that inhibit the growth of cancer cells, according to studies [4]. Approximately 80% of the population in developing countries is dependent on natural or herbal medicines to treat various diseases. A large number of plants are constantly screened for their chemical and pharmacological properties. By applying modern extraction techniques and pharmacological evaluation, many plant species have been introduced into medicine.

Piper betle L. 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, carbohydrates, 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 biological activities are due to the high antioxidant effect of the compounds contained in this plant. Betel leaf extract exhibits antioxidant potential through DPPH free radical capture, superoxide, and nitric oxide [5]. Studies have shown that the leaf extract of *P. betel* has phenolic compounds such as hydroxychavicol, chavibetol, eugenol… Hydrochavicol in betel leaf induces cell cycle arrest at the S phase or G2/M and apoptosis in pancreatic cancer cells [6]. Water extract from betel leaf activates ATM, p73, and JNK pathway expressions, and inhibits *in vivo* mouse tumor growth via the MAPK-p73 pathway in human hepatocellular carcinoma [7]; hexane extract exhibits significant cytotoxicity to Hela cells [8]. These findings point out the potential of betel leaf in the antioxidant and treatment of various cancers. Suryasnata Das *et al.* (2019) report has shown various results of DPPH free radical depending on the different content of total phenolic and flavonoid, eugenol and eugenol acetate due to 3 extraction technique using acetone as a solvent, namely soxhlet, sonication and maceration [9]. Ann Nazira *et al.* (2012) reported on different solvents (water, methanol, ethyl acetate, and hexane) resulted in total phenolic and flavonoid content so the antioxidant capacity and the ability to inhibit MCF-7 cell proliferation were also different [10]. From these studies, it is found that the presence and activity levels of various phytochemicals are responsible for their different pharmacological properties, which is essential for assessing their potential for human benefits and health. Therefore, different extraction modalities play a vital role in the content, type of phytochemical and toxic effect for each type of cancer cell.

In order to assess the effectiveness of the drying process on betel leaves, this study carried out the identification of chemical components by analyzing the changes in the main phytochemicals found in the leaves and quantifying the content of polyphenols and flavonoids. Then compares the antioxidant, cancer cytotoxic of fresh and dried betel leaves through MCF-7 breast cancer, HeLa cervical cancer, and SK-LU-1 lung cancer. The results indicated that the content of phytochemicals, polyphenols and flavonoids, antioxidant effects, and cytotoxicity against cancer cells have significant differences between fresh and dried extracts of Betel leaf (*P. betel* L.).

MATERIALS

Plant

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

Cell lines

Three human cancer cell lines including breast cancer cells (MCF-7), cervical cancer cells (HeLa) and lung cancer cells (SK-LU-1) are provided by the Institute of Biotechnology, Academy of Science and Technology Vietnamese technology.MSCs were isolated from Wharton's jelly of human umbilical cords provided by Hue Central Hospital (Hue, Vietnam).

Chemical

Cell culture chemicals: DMEM (Dulbecco's Modified Eagle Medium), L-glutamine, sodium pyruvate, penicillin/streptomycin, 10% FBS (Fetal Bovine Serum), Trypsin-EDTA (0.05%);

Other chemicals: DMSO (Dimethyl sulfoxide), TCA (Trichloroacetic acid), Tris base, PBS (Phosphate Buffered Saline), Ellipticine, SRB (Sulforhodamine B), Acetic acid, quercetin, $AlCl_3$, Folin-Ciocalteu, Na_2CO_3 7.5%, gallic acid, dichloromethane 3%, etc.

Equipment

Inverted microscope (Axiovert 40 CFL), cell counter (Fisher, USA), spectrophotometer (BioTek); GC-MS machine (Agilent GC 7890A), incubator CO_2, deep freezer -80^{0}C, liquid nitrogen tank, analytical balance, pH meter, vortex machine, and common laboratory instruments

METHODS

Extract Preparation

After harvesting, betel leaves were cleaned with distilled water and drained at room temperature. In order 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, 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. For dried betel leaf extract, the fresh leaf samples were dehydrated in an incubator at 40^{0}C and ground into fine powder. The powder (150 g) was soaked with absolute ethanol (500 mL) for seven days; stirring occasionally. The incubated samples were then filtered and evaporated at 40 - 50^{0}C until getting the dark brown concentrated solution. Store samples at 4°C.

Chemical composition analysis of fresh and dried leaf extracts by GCMS

The analysis of extracts 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^{0}C. The column temperature program was as follows: an initial temperature of 40^{0}C, raised to 140^{0}C at a rate of 20^{0}C/min and held for five minutes, the final rate was raised of 4^{0}C/min to 270^{0}C. The mass spectrometry conditions included an ion source temperature of 230^{0}C, 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.

Determination of total polyphenol content

The total phenolic content in fresh and dried leaf extracts was estimated using the Folin Ciocalteau reagent described by Feduraev *et al*. (2019). The approach is based on the reaction of the Folin-Ciocalteu reagent with the phenolic compounds present in the sample, which produces a blue complex that can be measured using visible light spectrometry at 760 nm wavelengths. The reaction occurs in an alkaline medium, therefore the intensity of the blue color that develops depends on the aliquot of phenolic compounds in the test solution.

Sodium carbonate (7.5% w/v) was prepared in distilled water. The Folin–Ciocalteu reagent was diluted to 1:10 with distilled water just before the experiment (F-C 10%). Different solutions of Gallic acid (50, 100, 150, and 200 µg/mL) and leaf *P.betle* extracts (1 mg/mL) were prepared in methanol. A mixture of 0.5 mL of leaf extract (1 mg/mL) was mixed with 2.5 mL of Folin-Ciocalteu 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.

In order to create the calibration curve, the same method was carried out at various concentrations of gallic acid solutions. The gallic acid calibration curve was used to calculate the phenol content of the fresh and dried leaf extracts. Based on the measured absorbance of these extracts, the flavonoid content was read (mg/mL) on the calibration curve and each sample was calculated using the formula:

$C = c \times V/m$

where C = Total phenolic content compounds in mg GAE/g, c = concentration of gallic acid established from the calibration curve in mg/mL, V = volume of extract in mL and m = weight of plant extract.

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

Determination of total flavonoid content

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

In Methanol, different solutions of quercetin standard (20, 40, 60, 80, and 200 µg/mL) and leaf *P.betle* extracts (1 mg/mL) were produced. Following the addition of 0.3 mL of 5% $NaNO_2$, samples containing 1 mL of fresh or dried leaf extracts (1 mg/mL) were added separately to 4 mL of distilled water. After five minutes, 0.3 mL of 20% aluminum chloride is added and kept for 6 min. Finally, 2 mL of 1 M 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. The same procedure was repeated for the quercetin standard solution at the dilutions. Construct a QE calibration curve based on the absorbance value measured at each concentration to calculate the total flavonoid content in fresh and dried leaf extracts. Similar to the determination of phenolic content, the content of each sample was calculated according to the formula:

$$C = c\ xV/m$$

where C = Total flavonoid content compounds in mg QE/g, c = concentration of quercetin established from the calibration curve in mg/mL, V = volume of extract in mL and m = weight of plant extract.

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

DPPH radical scavenging assay

The antioxidant activity of betel leaf extract was determined through DPPH free radical scavenging according to Jahan *et al* (2010) with some modifications. This method is based on the ability to reduce DPPH in the presence of a hydrogen-donating antioxidant.

2.2-diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical with one unpaired electron delocalized over the whole molecule. The free radical of the DDPH molecule was reduced to 2.2-Diphenyl-1-picrylhydrazine (DPPH-H) by antioxidant compounds in the extract, which reduces the absorbance of DPPH. DPPH possesses a purple color, with maximum absorption at 517 nm in methanol. If compounds have free radical scavenging activity, the solution color will change to brown or orange.

A 100 µM DPPH solution was prepared in methanol solution. Diluting the extract into different concentrations (5 - 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 λmax=517nm and compared to the standard antioxidants (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 lower absorbance of the reaction mixture indicated a higher free radical scavenging activity. The percentage of DPPH inhibition was calculated by using the following formula:

$$\text{DPPH inhibition (\%)} = [(A_0\text{-}A)/A_0] \times 100\%.$$

Where: A_0 = the absorbance of blank sample solution without the compound to be tested

A = the absorbance of the tested sample.

Cytotoxicity on non-cancer cells

MSC was used to test the cytotoxicity of betel leaf extracts on non-cancer cells. The secondary MSCs were cultured on 6-well plates with a density of 1.000 cells/cm^2 in the growth medium of DMEM/F12 medium containing 10% FBS and 1% antibiotic. The cells were incubated at 37°C, under a humidified atmosphere of 5% CO_2. The medium was replaced twice a week, and morphology was examined under inverted optical microscopy. When the cells were approximately 60% confluent, the betel 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.

Determination of cancer cell cytotoxic

The cytotoxic assay *in vitro* has been confirmed by the US National Cancer Institute as a standard cytotoxicity test to screen and detect substances that can inhibit the growth or kill human cancer cells in *in vitro* conditions. The cytotoxic assay of cancer cells was performed according to the method of Skekan *et al*. (1990) by sulforhodamine B (SRB) assay. This method

relies on the property of SRB, which binds stoichiometrically to proteins under mildly acidic conditions and then can be extracted in basic conditions. Therefore, the amount of bound dye can be used as a measure of the number of viable cells.

MCF-7, Hela, and SK-LU-1 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. The cell lines were cultured at 37°C, under a humidified atmosphere of 5% CO_2. Trypsinization causes cell detachment, followed by the inactivation of trypsin by suspension of cells in a growth medium. Cell pellets were formed by centrifugation. Adjust the cell concentration with the growth 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 cell 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 at the concentrations introduced into wells containing 190 µl of the cancer cell fluid mixture prepared above to achieve a final concentration of 100 µg/mL, 20 µg/mL, 4 µg/mL, and 0.8 µg/mL. The samples were incubated at 37^{0}C, 5% CO_2 for 72 hours. 20% TCA solution was added to immobilize cells for 60 min at 4^{0}C. 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^{0}C. 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 to ensure accuracy. The IC_{50} value of (concentration that inhibits 50% of growth) will be determined using TableCurve 2Dv4 computer software.

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

% cell inhibition = 100 – {(At – Ab) / (Ac – Ab)} x 100

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

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.

RESULTS

Obtaining *P. betel* Extract

Figure 1: Betel leaf.

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, the dry betel leaf extract (DBLE) was more soluble in water than the fresh betel leaf extract (FBLE). Both extracts (DBLE and FBLE) 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).

Table 1: The recovery efficiency of two types of extracts.

Types of extract	Weight (g)		Recovery efficiency (%)
	Material	Extract	
Fresh	200	9.90	4.95
Dry	100	8.89	8.89

Figure 2 and Table 2.

GC-MS results have identified that each extract has 5 phytochemical constituents but in different compositions and content. 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%). It can be seen that two major compounds of DBLE identified were Chavicol acetate (37.34%) and Eugenol (37.56%). Caryophyllene (12.71%), Acetyleugenol (10.17%) and Humulene (2.22%) were also found with small contributions. 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-Dimethyl benzoic acid which accounted for 89.58% of the total extract was not presented in DBLE.

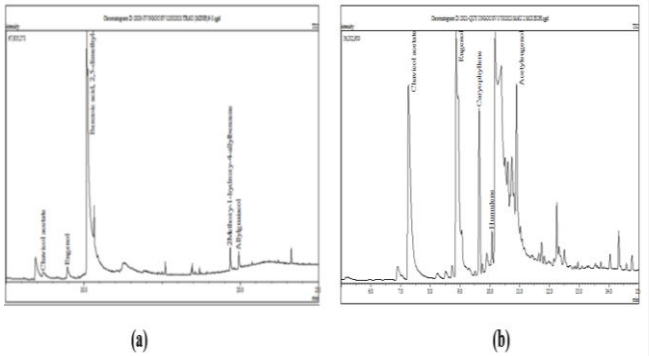

Figure 2: GC-MS chromatogram a) Fresh betel leaf extract; b) Dry betel leaf extract.

Table 2: Chemical compositions of fresh and dry betel leaf extract.

No	Fresh betel leaf extract		No	Dry betel leaf extract	
	Chemical compositions	%		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

The results of GC-MS analysis of both extracts were similar to previous studies in the world, however, there was a difference in the content of compounds. GC-MS analysis of *P.betle* leaf extract collected from Denpasar, Indonesia contained 31 compounds including eugenol (25.03%), and 2.5 -dimethyl benzoic acid (12.08%) which were two main components [10,11]. In the latest study by Selvaraj Ganesh Kumar *et al* (2022), GC-MS analysis of methanol extracts from *P.betle* leaves in India revealed that the main active compounds were 2.5-dimethylbenzoic acid, 3.5-dimethylbenzoic acid, methionine,... [12]. Suliantari *et al* (2008) analyzed components of betel leaf extract by GC-MS and detected components such as chavicol, eugenol, caryophyllene, xylene and chalarene [13]. The presence of caryophyllene, eugenol, and acetyl eugenol in the ethanol extract from the leaves of *P. betle* was also confirmed by HPTLC and GCMS analysis in the study of Annegowda *et al* [14]. 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. [15].

Total phenolic and flavonoid contents

The Total phenolic content (TPC) and Total flavonoid content (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.

Table 3: Total phenolic content (mg GAE/g) and total flavonoid content (mg QE/g) of DBLE and FBLE.

Extract	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
FBLE	165.2 ± 4.6	76.4 ± 2.1
DBLE	240.9 ± 0.11	82.1 ± 2.9

The results in Table 3 revealed that the 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.

TPC of DBLE in this investigation (240.9 ± 0.11 mg GAE/g) was similar to the results of the study on betel leaves collected in Ho Chi Minh, VietNam by Lam Thi Truc Nguyen *et al.* (2020) (249.96 ± 6.42 mg GAE/g) [16]. According to the study by Hoang Thuy Duong *et al.* (2021) in Binh Duong, VietNam, various solvent extracts of dried leaves obtained by using ultrasonic waves contained higher TPC (386.343 mg GAE/g) and lower TFC (55.073 mg QE/g) compared with the equivalent figures in our study [16,17]. The dried betel leaf extracts by sonication, soxhlet, and maceration methods using acetone as solvents found TPC from 50 - 57.6 mg GAE/10 mg

and TFC from 32.1 - 49.79 mg QE/mg [9]. The extract from fresh betel leaves in Indian regions had TPC from 95 - 128 mg GAE/g and TFC from 51 - 62 mg catechin which was lower than in our study [18]. The difference in phenolic and flavonoid contents of betel leaf extract in distinct studies depends on geographical location as well as an extraction method.

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. Ascorbic acid is a significant antioxidant. Its deficiency can lead to a delay in wound healing and failure of bone healing, the healing power of ascorbic acid is attributed to its free radical scavenging activity [19].

The DPPH assay results are shown in Table 4 and Figure 3. The results show that the DPPH scavenging ability is proportional to the concentration of extract. The higher the concentration is, the better the free radical scavenging capacity is. However, the IC_{50} values for the inhibition of DPPH of DBLE were considerably lower than that of FBLE (3.21 µg/mL and 22.97 µg/mL respectively). It can be seen that the antioxidant capacity of DBLE is 7 times higher than that of FBLE. This is completely consistent with the result that the percentage inhibition of free radical scavenging of DBLE is always higher at all concentrations. 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 ethanol extract from the dried betel leaf has a strong antioxidant capacity.

Table 4: DPPH radical scavenging activity of both extract 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	69.9 ± 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

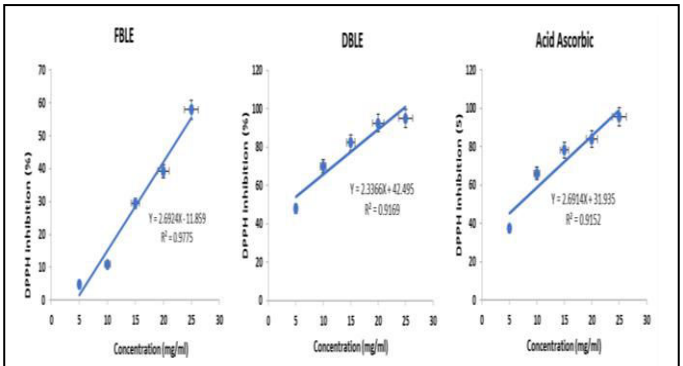

Figure 3: Graphical representation of DPPH radical scavenging assay of betel leaf extract and ascorbic acid. Data represent the mean ± SE of 3 independent experiments.

Cytotoxicity of betel extracts toward non-cancer cells

Non-cancer cells can respond to signals sent from nearby neighboring cells or by foreign factors added to the culture medium. For the above reasons, it is necessary to investigate. Cytotoxicity 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 were exposed to different concentrations (25 µg/mL - 125 µg/mL) of FBLE and DBLE, monitoring for cytotoxicity for 48 hours at concentrations of FBLE and DBLE (Figure 4).

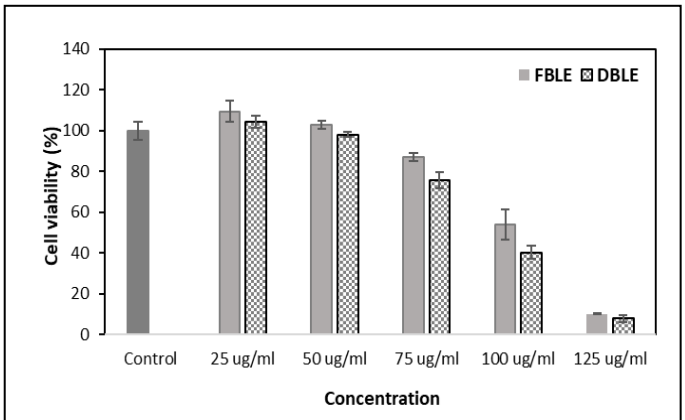

Figure 4: Evaluation of the cytotoxicity of betel leaf extract on non-cancer cells.

As shown in Figure 4, 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 cells. DBLE exhibited higher cytotoxicity to MSCs than FBLE.

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. The results of cytotoxicity against cancer cells *in vitro* of extracts from fresh and dried betel leaves are shown in Table 5 and Figure 5.

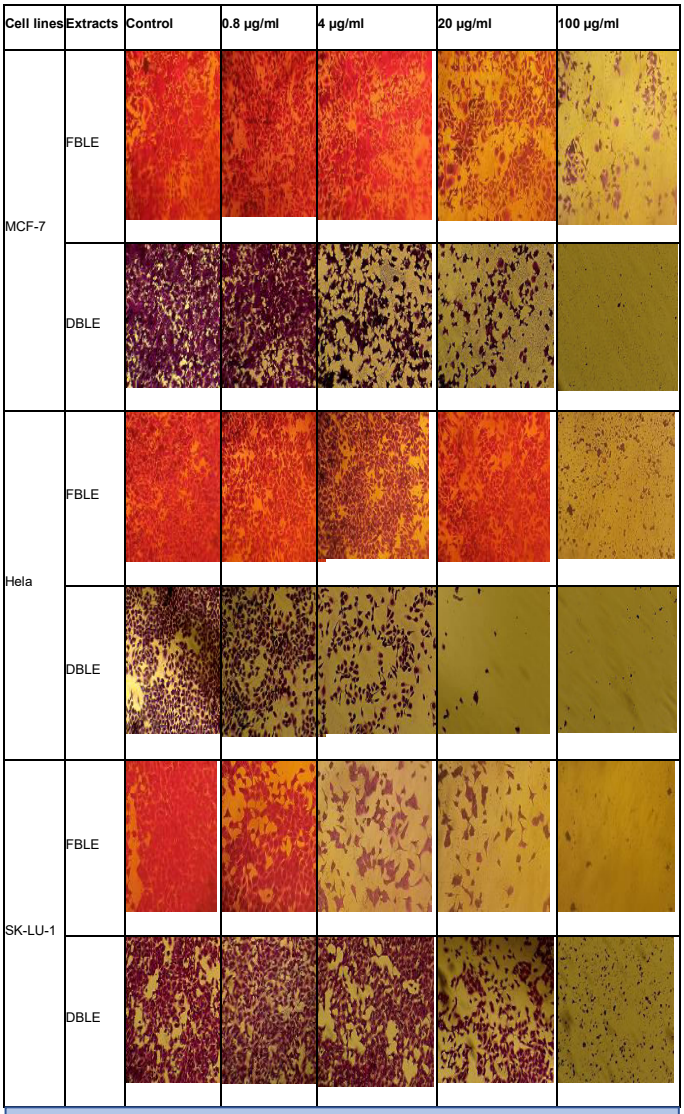

Figure 5: Pictures of MCF-7, Hela, and SK-LU-1 cells that were treated with FBLE and DBLE at different concentrations after 72 hours.

After 72 hours of extract treatment, compared with the control samples, cells shrank and turned into a round shape without adhesion which reduces the density of cells and cell clusters (Figure 5). This result showed that the extract stopped the growth and development of 3 cell lines compared with the control groups.

Table 5: Cytotoxic activity of both extracts on MCF-7, Hela, and SK-LU-1 cells.

Extracts	Cell lines	% inhibition at concentration (µg/ml)				IC_{50} (µg/ml)
		0.8	4	20	100	
FBLE	MCF-7	8.03±0.16	19.03±0.86	25.09±1.44	75.27±1.92	56.05±5.46
	Hela	2.67±0.27	11.52±0.71	19.27±0.74	85.50±1.19	56.7±4.16
	SK-LU-1	7.85±0.43	18.82±0.55	36.38±1.95	87.71±1.74	35.07±1.39
DBLE	MCF-7	5.82±0.53	18.15±0.62	49.47±1.73	96.35±2.29	21.88±1.13
	Hela	11.69±0.29	25.84±1.09	41.57±1.47	99.29±1.71	26.68±1.55
	SK-LU-1	1.16±0.11	9.65±0.61	31.02±2.81	97.56±1.42	38.45±2.94

Table 6: Cytotoxicity results of Ellipticine (positive control) on cancer cell lines.

Cell lines	% inhibition at concentration (µg/ml)				IC_{50} (µg/ml)
	0.08	0.4	2	10	
MCF-7	25.17±1.03	51.22±1.11	88.51±2.29	92.13±2.06	0.34±0.03
Hela	24.04±1.11	50.26±1.03	82.32±1.48	98.63±2.24	0.37±0.02
SK-LU-1	25.34±1.09	49.71±1.72	78.57±2.14	97.94±3.79	0.39±0.002

The results in Table 5 showed that the cytotoxicity properties of fresh and dried betel leaf extracts were high on all three cancer cell lines: MCF-7, Hela, and SK-LU-1. In particular, for FBLE, at a concentration of 100 µg/mL, it 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. When the concentration of extract increased, the inhibitory ability toward cancer cells increased.

The cytotoxicity capacity of the DBLE doubled that of the FBLE on both MCF-7 cells and Hela cells (MCF-7, IC_{50} = 21.88±1.13 µg/mL and IC_{50} = 56.05 ± 5.46 µg/mL; Hela, IC_{50} = 26.68±1.55 µg/mL and IC_{50} = 56.7±4.16 µg/mL, respectively). However, for the SK-LU-1 cell line, the toxic effects of FBLE and DBLE were almost the same (IC_{50} = 35.07±1.39 and IC_{50} = 38.45 ± 2.94 µg/mL respectively).

We use Ellipticine as the positive control to compare the cytotoxic activity of the extract (Table 6). Elippticine was

stable in the experiment and 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,… In this research, DBLE at the concentration of 100 µg/mL caused inhibition on MCF-7 (96.35%) and Hela (99.29%) cells that were more than Ellipticine at 10 µg/mL (92.13% for MCF-7 cells and 98.63% for Hela cells).

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 extract from ethyl acetate exhibited the highest inhibitory effect on the proliferation of MCF-7 cells (IC_{50} = 65 µg/ mL) by the MTT assay [10]. The extract of fresh *Piper betle* leaf only effectively inhibited the proliferation of the KB cell line without affecting the proliferation of Hela cells [21]. In the study of Arisa Sanubol *et al*. (2017) 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 [8]. Thus, with the ability of cytotoxic activity in our study, dried betel leaves can be a potential therapeutic agent in cancer treatment.

DISCUSSION

Scientists are constantly looking for natural drugs to replace chemical drugs in cancer therapy with the goal of less toxicity and side effects. Cancer cytotoxic and antioxidant activities *in vitro* of *P. betel* 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 through inhibition of Nuclear Factor- Kappa B (NF-κB) activation, reduction of Cyclooxygenase-2 (Cox-2) activity, regulation of prostaglandin synthesis, S-phase cell cycle arrest, increasing the generation of reactive oxygen species (ROS), decreasing of B-cell lymphoma-2 (Bcl-2) and inflammatory cytokines, resulting in apoptotic cell death. Moustafa Fathy *et al*. (2019) reported EUG increased the expression of caspase-3, caspase-9, Bax, cytochrome (Cyt-c) and decreased the expression of Bcl-2, cyclooxygenase-2 and interleukin-1 beta in human cervical cancer cells (Hela) [22]. According to Paulo *et al*. (2016), EUG induced apoptosis in cancer cells via promoting ROS production leads to the elimination of G2/M of the phase of the cell cycle and consecutively, clastogenesis *in vitro*; induces Proliferation Cell Nuclear Antigen (PCNA) downregulation and decreases in mitochondrial potential (ΔΨm) while increasing Bax [23]. These series of findings suggest that EUG may induce apoptosis in cancer cells, e.g., breast cancer cells (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),…[23-25]. 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. It also exhibited high antioxidant activity for nitric oxide and hydroxyl radicals with the IC_{50} value 114.34± 0.46 and 306.44 ± 5.28 µg/mL, respectively [26].

In addition, the components of dried betel leaf extract possessed powerful antioxidant compounds such as acetyl eugenol, caryophyllene and humulene [27-29]. Caryophyllene inhibited the proliferation of glioblastoma cells, non-small-cell lung cancer cells, breast cancer cells, and myeloma cells [30-33]. α-Humulene inhibited hepatocellular carcinoma cell proliferation [34]. Especially, the combination of humulene and caryophyllene was more effective in reducing the proliferation of MCF-7 breast cancer cells [35]. Therefore, with the anti-

cancer effect of each substance, simultaneously, there may be a synergism of the substances in DBLE causing apoptosis on breast and cervical cancer cells, which doubles that of FBLE.

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 multiple 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 [36]. 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 (IC_{50} = 3.21 µg/mL compared to 22.97 µg/mL). From our results, it can be seen that the cytotoxicity against cancer cells potential of betel leaf extracts was correlated with their polyphenol content and antioxidant activity.

In *in vitro* culture, cancer cells do not have a mechanism to stop dividing. The continual unregulated proliferation often leads to the formation of clusters of cancer cells. Proteins that act as "growth factors" include membrane proteins that are cell growth and division substrates. When protein is produced in large quantities and non-stop, it is a factor that results in continuous stimulation of cell proliferation. However, when adding betel leaf extract, it was shown that the new cells stopped dividing. Thus, it is possible that the chemical components in the betel leaf extract, after being added to the culture medium, have stopped the cell reproduction or affected the cell membrane protein causing a deficiency of factors to stimulate cell growth.

At 100 µg/ml concentration of betel leaf extract induced cytotoxicity up to 99%. According to the International Standard ISO 10993-5, a concentration of 100 µg/ml is considered a cytotoxic effect (Reduction of cell viability by more than 30%) and belongs to the category 4 toxicity in the table of Qualitative morphological grading of cytotoxicity of extracts (severe reactivity, nearly complete or complete destruction of the cell layers). These proved that 100 µg/ml concentration of Piper leaf extract induced apoptosis in monolayer cultured cancer cells.

Apoptosis is characterized by cell morphological changes, chromatin condensation, DNA cleavage, and nuclear fragmentation [37]. Typical apoptotic features such as rounding, shrinkage and losing contact with adjacent cells. The apoptotic cells produced a loss of cellular adhesion to the substrate. Most cells even detached from the surface of the tissue culture dishes plate and appeared floating in the culture medium. Relatively early detachment from their basal membrane is characteristic of apoptosis of monolayer adherent cells and is called anoikis [38].

Although there was a difference in cytotoxic effect on MCF-7 breast cancer cells 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 dimethyl benzoic compound in high content in FBLE affected the apoptosis of SK-LU-1 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.

CONCLUSION

The results revealed that the drying temperature that makes the state of leaves dry or fresh affected the chemical composition, the content of polyphenols and flavonoids, antioxidant properties, and cytotoxic activity against cancer cells in betel leaves. Dried betel leaf extract indicated that DPPH free radical scavenging activity (IC_{50} = 3.21 µg/mL) was seven times higher than the fresh betel leaf extract (IC_{50} = 22.97 µg/mL). Leaves extracts can inhibit the growth of HeLa cervical cancer cells, MCF-7 breast cancer cells, and SK-LU-1 cancer cells. In particular, the dried leaf extract showed cytotoxicity two times more effectively than the fresh leaf extract on two cell lines MCF-7 (IC_{50} = 21.88 µg/mL) and Hela (IC_{50} = 26.68 µg/mL). At a 100 ug/mL concentration of dried betel leaf extract, the percentage of apoptotic cells was

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96.38% in MCF-7 cells and 99.29% in Hela cells (almost destroyed). Our results of cytotoxicity on cancer cell lines provided a scientific basis for using dried betel leaves as a potential source of chemotherapeutic agents for the treatment of breast cancer and cervical cancer. Researchers can perform more detailed analyses to examine these biological effects, especially in vivo studies. At the same time, it also serves as a premise for further studies about the *in vitro* cancer inhibition mechanism of dimethyl benzoic compounds in the fresh betel leaf extract.

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