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Original Article

Antioxidant, anticancer, and apoptosis-inducing effects of *Piper* extracts in HeLa cells

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Key Words
Anticancer; Antioxidant;
Apoptosis; Cervical cancer;
HeLa cell line; Piperaceae

Abstract

Objective: Cervical cancer is the second most common cancer as well as one of leading cause of cancer-related death for women worldwide. In regards to that issue, focus of this paper will be on popularly used Piperaceae members including *Piper betle* L, *Piper* cf *fragile* Benth, *Piper umbellatum* L, *Piper aduncum* L, *Piper pellucidum* L. This research was conducted to elucidate the antioxidant, anticancer and apoptosis inducing activities of Piperaceae extracts on cervical cancer cells, namely HeLa cell line.

Methods: The anticancer activity was determined by inhibiting the proliferation of cells. Apoptosis inducing was determined by inhibiting proliferation cells and by SubG1 flow cytometry. The antioxidant activity is determined by using superoxide dismutase value and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity.

Results: The highest anticancer activity at 24 h incubation was found for *P.pellucidum* extract (IC_{50}: 2.85 µg/ml); The anticancer activity at 48 h incubation was more than at 24 h for all extracts. The highest apoptotic activity was found for *P.betle* (12.5 µg/ml) at both 24 and 48 h incubatio. The highest antioxidant activity was also represented by *P.betle* extract.

Conclusions: All Piperaceae extracts have high anticancer activity; longer incubation increase anticancer activity. *P.betle* extract has the highest antioxidant property.

INTRODUCTION

Oxidative stress is caused by free radicals and induces many chronic and degenerative diseases, including atherosclerosis, ischemic heart disease, aging, diabetes mellitus, cancer, immune suppression, and neurodegenerative diseases [1]. Free radicals can inflict cellular damage by attacking and damaging lipids, proteins, DNA and RNA. Cancer risk is increased by mutations in cancer-related genes or post-translational protein modifications by nitration, nitrosation, phosphorylation, acetylation, poly(ADP-ribosyl)ation by free radicals or lipid peroxidation byproducts such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) which are reactive aldehydes [2]. Free radicals modulate cell growth and tumor promotion by activating signal-transduction pathways and inducing transcription of proto-oncogenes such as c-fos, c-jun and c-myc, which are involved in stimulating growth [2, 3]. The role of free radicals in carcinogenesis has been demonstrated *in vitro*; they damage DNA and modify the structure and function of proteins that maintain cellular integrity and promote angiogenesis. DNA damage by free radicals has been demonstrated by using hydrogen peroxide (H_2O_2) in the presence of peroxidation activator $Fe_2(SO_4)_3$, which induces chromosome fragmentation [4]. Free radicals increase tumorigenesis by causing DNA damage and mutation, inhibiting apoptosis, stimulating cell cycle/proliferation and inhibiting DNA repair [2].

Reduction of unstable and reactive free radicals, induction of apoptosis, and inhibition of cell proliferation can be achieved via antioxidants that

protect cells from free radical attack, reduce apoptosis, and inhibit cell proliferation. We hope to identify natural antioxidants from herbal medicine as sources for replacing synthetic antioxidants, which are limited by their carcinogenicity [5]. Not much data are available concerning the antioxidant, anticancer and apoptosis-inducing activities of natural herbal medicines, especially *Piper*, which is frequently consumed by Indonesian people to prevent and treat many kinds of diseases. *Piper* is a plant belonging to Piperaceae that includes Daun Sirih or piper betel (*Piper betle* L), Seuseurehan or Spanish elder (*Piper aduncum* L), Sasaladahan (*Piper pellucidum* L), Gedebong (*Piper umbellatum* L), and Sirih Merah (*Piper* cf *fragile* Benth). Here, we have characterized the antioxidant, anticancer, and apoptosis-inducing activities of ethanol extracts of *Piper*.

MATERIALS AND METHODS

Plant material

We collected samples from several locations in Indonesia: *P.betle* from Bogor, *P.aduncum* from Coblong-Bandung, *P.pellucidum* from Ciater-Bandung, *P.umbellatum* from Cibadak-Sukabumi, and *P.fragile* from Puncak-Bogor. The plants were identified by staff at the herbarium, Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. Leaves from each plant were chopped and dried in a dry tunnel (40-45°C) to a stable water level (10% water content), then chopped finely in a blender, producing a 60 mesh size flour.

Preparation of extracts

The dried leaves of each plant (250 g) were ground and immersed in 96% ethanol. After 72 h, the filtrates were collected and the residues were immersed again in 96% ethanol for 72 h. These treatments were repeated until the filtrate remained colorless. The filtrates were evaporated with a rotary evaporator at 40°C. The extracts were stored at 4°C. The ethanol extracts of *P.betle*, *P.fragile*, *P.umbellatum*, *P.aduncum* and *P.pellucidum* were dissolved in 10% dimethylsulfoxide (DMSO; Merck) and diluted to appropriate working concentrations with Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) for the proliferation assay [6]. The extracts were dissolved in HPLC-grade methanol (Merck) to verify antioxidant activities in the context of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenger and superoxide dismutase (SOD) activities.

Cell culture

The human cervical cancer HeLa cell line was obtained from the Stem Cell and Cancer Institute of Jakarta, Indonesia. The cells were grown and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich), and 100 µg/ml streptomycin (Sigma-Aldrich), and incubated at 37°C in a humidified atmosphere with 5% CO_2 [6, 7]

Cell viability assay

To determine cell viability, we used the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, WI, USA) with an optimized reagent containing resazurin converted to fluorescent resorufin by viable cells that absorb light at 490 nm [8]. Briefly, the cells were seeded in a 96-well plate (5×10^4 cells per well) and, after 24-h incubation, were supplemented with *Piper* extracts at various concentrations, and incubated for 24 and 48 h. Untreated cells served as the negative control. MTS was added to each well at a ratio of 1:5. The plate was incubated in 5% CO_2 at 37°C for 2-4 h. Absorbance was measured at 490 nm on a microplate reader. The data are presented as the percentage of viable cells (%) and were analyzed by calculating the median inhibitory concentration (IC_{50}) using Probit Analysis (SPSS 20).

DPPH scavenging activity assay

Briefly, 50 µl extracts and eugenol (Sigma-Aldrich) were added to a microplate followed by 200 µl DPPH (Sigma-Aldrich) solution (0.077 mmol/l in methanol). The mixtures was shaken vigorously and kept in the dark for 30 min at room temperature; DPPH scavenging activity was determined with a microplate reader at 517 nm [9]. The radical scavenging activity of each sample was measured according to following formula:

$$\textbf{Scavenging \%} = (A_c - A_s) / A_c \times 100$$

-A_s; sample absorbance

-A_c; negative control absorbance (without sample)

Superoxide dismutase activity assay

The SOD assay was performed with a SOD assay kit (Cayman) comprising assay buffer, sample buffer, radical detector, SOD standard, and xanthine oxidase. SOD standards were prepared by introducing 200 µl diluted radical detector and 10 µl SOD standard (7-level standard) per well. Sample wells contained 200 µl of the diluted radical detector and 10 µl of the sample. The reaction was initiated by adding 20 µl of the diluted radical detector to all wells. The mixtures were shaken carefully for few seconds, incubated for 20 min at room temperature, and SOD activity was measured on a microplate reader at 440-460 nm. The linearized SOD standard rate and SOD activity were calculated using the equation obtained from the linear regression of the standard. One unit is defined as the amount of enzyme to yield 50% dismutation of the superoxide radical [10]. The *Piper* extracts were tested at 3 concentrations in triplicate.

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Apoptosis assay

Cells were harvested for apoptotic studies at 80% confluence in T25 flasks. Cells were harvested with trypsin-EDTA (0.25-0.038%) and washed with PBS. HeLa cells were seeded in 12-well plates at 10^4 cells per well and incubated for 24 h with various extract concentrations. After 24 h, cells were rinsed with PBS, fixed with trypsin-EDTA and incubated at 37°C for 5 min. The medium was added in a 3:1 ratio of medium:trypsin-EDTA and centrifuged at 1500 rpm for 5 min. The supernatant was discarded; 70% ethanol was added to the pellet and the mixture was incubated at 4°C for 5 min. The cells were centrifuged again at 1500 rpm for 5 min and the supernatant was discarded. The cells were stained with propidium iodide (PI) solution (in PBS) and placed in the dark by wrapping the tubes in aluminum foil for 15 min prior to flow cytometry. Apoptosis was measured by cell cycle analysis in a flow cytometer. The apoptotic cells were determined by SubG1 area and are presented as a percentage of total cells.

RESULTS

Antioxidant activities of *Piper* extracts

The antioxidant activities of *Piper* extracts were examined in the context of DPPH scavenging and SOD activities. The DPPH free radical scavenging activity of *P.fragile*, *P.umbellatum*, *P.aduncum*, and *P.pellucidum* extracts and eugenol as a control was measured as a representative of antioxidant activity. The IC_{50} is the concentration of antioxidant needed to scavenge 50% of the DPPH free radicals. *P.umbellatum*, and *P.pellucidum* extracts exhibit high levels of DPPH scavenging activity, and *P.fragile* and *P.aduncum* have low DPPH scavenging activity (Table 1, Fig.1).

The SOD activity of *Piper* extracts can be seen in Table 2. SOD activity was found to be concentration-dependent. The highest SOD activity at 500 µg/ml and 125 µg/ml was shown by the *P.betle* extract, while *P.umbellatum* and *P.pellucidum* showed the highest activities at 31.25 µg/ml; the lowest SOD activity at all concentrations was exhibited by eugenol.

Anticancer activity of *Piper* extracts

The viability of HeLa cells treated with extracts of *P.betle*, *P.fragile*, *P.umbellatum*, *P.aduncum*, and *P.pellucidum* decreased in a concentration-dependent manner; higher extract concentrations exhibited stronger anticancer activity (Figs.2&3).

Anticancer activity was also found to be concentration-dependent; higher concentrations more strongly inhibit proliferation (Figs.2&3). The IC_{50} of *Piper* ethanol extracts in HeLa cells after 24 h incubation demonstrated that *P.fragile* extract was the most active and that

Table 1. IC_{50} DPPH scavenging activity of *Piper* extracts. The DPPH scavenging activity test was measured triplicate for each extract. [Linear equations, coefficient of regression (R^2), and IC_{50} were calculated.]

Samples	Linear equation	R^2	IC_{50} (µg/ml)
P.fragile	y=0.843x+5.751	0.945	52.49
P.umbellatum	y=3.311x+0.849	0.991	15.36
P.aduncum	y=0.482x+0.429	0.995	102.84
P.pellucidum	y=4.675x+7.94	0.785	9
Eugenol	y=11.443x+6.5	0.965	3.8

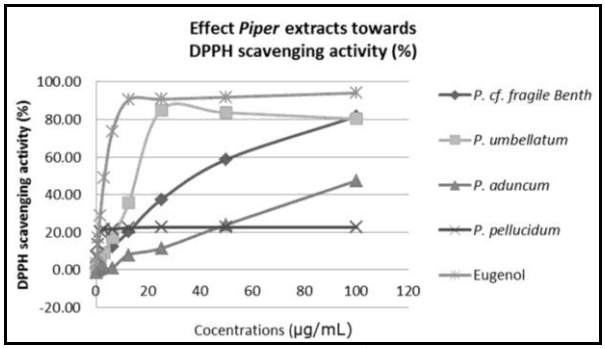

Figure 1. DPPH scavenging activity of *Piper* extracts diluted in methanol to 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, and 0.195 µg/ml.

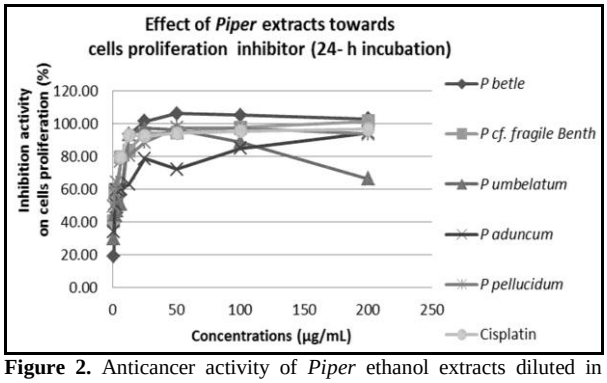

Figure 2. Anticancer activity of *Piper* ethanol extracts diluted in DMSO to 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, and 0.781 µg/ml and incubated for 24 h. Inhibition of cell proliferation was interpreted as anticancer activity.

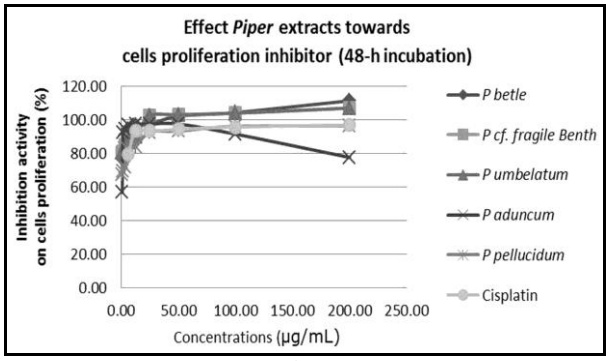

Figure 3. Anticancer activity of *Piper* ethanol extracts diluted in DMSO to 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, and 0.781 µg/ml and incubated for 48 h. Inhibition of cell proliferation was interpreted as anticancer activity.

cisplatin was more active than all the extracts. At 48 h of incubation, the *P.fragile* extract was more active than cisplatin and all other extracts (Table 3).

Apoptosis- inducing effect of *Piper* extracts

P.betle, P.fragile, P.umbellatum., P.aduncum, and *P.pellucidum* ethanol extracts induced apoptosis in HeLa cells after 24 and 48 h incubation (Table 4); increased incubation of contact between the cancer cells and anticancer agent increased apoptotic induction. The strongest apoptosis inducers after 24 h incubation were *P.betle* at 12.5 µg/ml (80.9%), *P.umbellatum* at 25 µg/ml (85.41%), *P.aduncum* at 100 µg/ml (80.72%), and cisplatin at 100 µg/ml (75.21%). The strongest apoptosis inducers at 48 h incubation were *P.betle* at 12.5 and 25 µg/ml (95.35 and 95.87%, respectively), *P.fragile* at 50 µg/ml (87.17%), *P.aduncum* at 100 µg/ml (81.52%), and cisplatin at 100 µg/ml (95.53%).

Table 2. Mean and Tukey's HSD post hoc test of SOD activity of *Piper* extracts. SOD activity was measured in triplicate for each extract. [Linear equation, coefficient of regression (R^2) of SOD standard and SOD activity of *Piper* extracts and eugenol were calculated.]

Samples	Concentrations (µg/ml)		
	500	125	31.25
P. betle	5.21 ± 0.49^{c}	4.47 ± 0.17^{d}	0.64 ± 0.14^{b}
P.fragile	2.19 ± 0.41^{b}	0.63 ± 0.15^{a}	0.09 ± 0.06^{a}
P.umbelatum	2.72 ± 0.32^{b}	2.44 ± 0.04^{c}	1.89 ± 0.11^{c}
P.aduncum	2.58 ± 0.25^{b}	1.82 ± 0.19^{b}	0.95 ± 0.09^{b}
P.pellucidum	2.56 ± 0.12^{b}	2.21 ± 0.1^{c}	1.68 ± 0.12^{c}
Eugenol	0.87 ± 0.05^{a}	0.67 ± 0.15^{a}	0.2 ± 0.02^{a}

Data are presented as mean ± standard deviation. Different letters in the same column (among extracts) are significant at $P < 0.05$ (Tukey's HSD post hoc test).

Table 3. The IC_{50} of *Piper* ethanol extracts in HeLa cells after 24 and 48 h incubation. [Each extract was measured in triplicate and growth inhibition was analyzed using probit.]

Samples	IC_{50} (µg/ml)	
	24 hours	48 hours
***P.betle* extract**	7.13	0.136
***P.fragile* extract**	2.93	0.005
***P.umbellatum*extract**	6.71	0.439
***P.aduncum* extract**	3.91	0.53
***P.pellucidum* extract**	2.85	0.12
Cisplatin	0.07	0.01

DISCUSSION

The data in Table 1 shows that *P.pellucidum* extract and eugenol, a component of *P.betle* [11, 12], exhibited the most active DPPH scavenging activity, consistent with previous indications that the essential oil of *P.betle* is a strong antioxidant [13] and that the ethanol extract of *P.betle* exhibits good DPPH scavenging activity [14]. Essential oil, methanol and aqueous extracts of *P.betle* exhibit antioxidant activities, including DPPH scavenging, iron chelation and reducing power [11]. This result is consistent with previous findings that *P.betle* extract exhibits antioxidant activity [15]. In the present study, *P.betle* extract exhibited the strongest SOD activity compared to other samples (Table 2). Eugenol was the poorest antioxidant among the tested *Piper* extracts. These data were not consistent with the DPPH scavenging activity (Table 1) and also with previous reports that eugenol can improve the antioxidant status of the rat intestine after short- and long-term (15 days and 90 days, respectively) oral administration of 1000 mg/kg, a dosage reported to be highly hepatoprotective; thus, eugenol seem to be nontoxic and protective [16]. In another study, however, eugenol exhibited potential benefits in the management of isoproterenol-induced cardiac hypertrophy in rats [17].

Table 4. Effect of various *Piper* extracts in HeLa cells by SubG1 (%) after 24 and 48 h incubation. [The apoptosis assay was performed with a flow cytometer. The apoptotic cells were determined on the basis of the SubG1 area from cell cycle analysis and are presented as a percentage of all cells.]

µg/ml	Cisplatin		*P.betle*		*P.fragile*		*P.umbelatum*		*P.aduncum*		*P.pellucidum*	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
1.56	19.45	34.62	11.03	36.81	6.77	10.79	10.96	10.8	5.91	9.99	7.32	10.8
3.12	20.65	29.84	16.72	16.06	7.9	12.92	7.43	8.94	8.57	8.86	7.26	8.94
6.25	28.54	39.19	33.77	32.75	8.79	10.95	13.14	10.59	11.3	5.89	8.62	10.59
12.5	38.57	64.58	80.9	95.87	10.81	10.99	16.1	15.07	12.82	7.65	8.74	15.07
25	51.33	79.2	16.82	95.35	33.43	35.59	85.41	70.9	12.09	7.54	14.46	70.9
50	59.89	86.44	13.89	8.37	63.55	87.17	50.17	55.11	19.66	11.94	40.85	55.11
100	75.21	95.53	17.41	15.5	23.64	30.38	47.04	36.39	80.72	81.52	27.51	36.39

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All ethanol extracts of *Piper* exhibited potential anticancer activities (Figs.2&3, Table 3), consistent with a previous study in which the aqueous extract of *P.betle* exhibited anticancer activity in cancerous oral epidermal lesions [18]. This result is also consistent with a previous study in which *P.betle* extract inhibited T47D cell (human ductal breast epithelial tumor cell line) proliferation [15]. Ethanol extract of *P.betle* leaves exhibit cytotoxic activity against larvae of *Artemia salina* Leach. Therefore, based on the brine shrimp lethality test (BLT), the ethanol extract of *P.betle* exhibits anticancer activity [19]. The aqueous extract of *P.betle* leaves exhibits cytotoxicity in Hep-2 cells in microculture tetrazolium assays and sulforhodamine B (SRB) assays [20]. The anticancer activity of *Piper* extracts varies by its content; for example, eugenol exhibits dose-dependent cytotoxicity in U2OS (human osteosarcoma) cells [21]. Allylpyrocatechol exhibited anti-inflammatory effects in an animal model of inflammation, and mechanistic studies suggest that allylpyrocatechol targets the inflammatory response of macrophages via inhibition of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and interleukin (IL)-12 p40 through downregulation of the nuclear factor (NF)-κB pathway [22, 23]. Hydroxychavicol is a component of *P.betle* leaves that possesses antioxidant and anti-inflammatory activities [23, 24], inhibits ATCC 25175 (carcinogenic bacteria), and has anticancer properties [24].

Piper extracts were able to induce apoptosis in HeLa cells after 24 and 48 h incubation (Table 4). The most active apoptosis inducer was *P.betle* extract. These data are consistent with those from a previous study in which an alcoholic extract of betel leaves induced apoptosis of chronic myelogenous leukemia (CML) cells expressing wild-type and mutated Bcr-Abl, with imatinib resistant phenotype (STI571 or Gleevec) [25], induced apoptosis in imatinib-resistant cells [25, 26], and exhibited activity against T315I tumor xenografts [25, 26]. The plant extract NPB001-05 from *P.betle* exhibited anti-tumor activity in T315I tumor xenografts, where imatinib failed to exhibit antitumor activity [27]. Hydroxychavicol induces apoptosis in KB (human oral carcinoma) cells through induction of reactive oxygen species (ROS) [28].

The inhibitory effects of *Piper* extracts as anticancer agents and apoptosis inducers are associated with antioxidant glutathione (GSH) levels [21]. The antioxidant property is correlated with anticancer properties, since free radicals are involved in all diseases that involve carcinogenesis [20]. DNA is highly susceptible to free radical attacks. Free radicals can react with cell membrane fatty acids and form lipid peroxides, accumulation of which leads to production of carcinogenic agents such as MDA [29]. Carcinogenesis may be mediated by ROS and reactive nitrogen species (RNS) directly by chronic inflammation (oxidation, nitration of nuclear DNA/RNA or lipids) or indirectly by the products of ROS/RNS, proteins, lipids, and carbohydrates that are capable of forming DNA adducts [29, 30]. Chronic inflammation leads to excessive production of free radicals and reduces antioxidant levels [29]. Tumor cells have higher levels of intracellular ROS than normal cells and ROS is associated with cell proliferation [25, 31]. Hydroxychavicol induces apoptosis in CML cells expressing wild-type and mutated Bcr-Abl, including the untreatable T315I mutation, and acts through the JNK pathway in a ROS-dependent manner, which in turn activates endothelial nitric oxide synthase (eNOS) to kill CML cells [25].

In conclusion, *P.betle* extract has the highest antioxidant activities as demonstrated by DPPH scavenging and SOD activities, and is the strongest inducer of apoptosis at 24 and 48 h incubation in HeLa cells. *P.betle* extract has low anticancer activity in HeLa cells; however, the strongest anticancer activity was observed with *P.fragile* and *P.pellucidum* extracts. *Piper* extracts have great therapeutic potential due to their antioxidant, anticancer, and apoptosis inducing activities.

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