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PRE-CLINICAL EVALUATION OF EXTRACTS AND ESSENTIAL OILS FROM BETEL-LIKE SCENT *PIPER* SPECIES IDENTIFIED POTENTIAL CANCER TREATMENT

Arisa Sanubol[1], Arunrat Chaveerach[1,2*], Tawatchai Tanee[2,3] and Runglawan Sudmoon[2,4]

[1]Department of Biology, Faculty of Science, Khon Kaen University, Thailand
[2]Genetics and Environmental Toxicology Research Group, Khon Kaen University, Thailand
[3] Faculty of Environment and Resource Studies, Mahasarakham University, Thailand
[4] Faculty of Law, Khon Kaen University, Thailand
***corresponding author E-mail**: raccha@kku.ac.th

Abstract

Background: Nine *Piper* species with betel-like scents are sources of industrial and medicinal aromatic chemicals, but there is lack of information on cytotoxicity and genotoxicity for human safety, including how these plants impact human cervical cancer cell line.
Methods: Plant leaves were extracted with hexane and hydro-distilled for essential oils. The extracts and oils were pre-clinically studied based on cyto - and genotoxicity using microculture tetrazolium (MTT) and comet assays.
Results: The crude extracts showed an IC_{50} in leukocytes and HeLa cells of 58.59 -97.31 mg/ml and 34.91-101.79 mg/ml, the LD_{50} is higher than 5000 mg/kg. With lower values than the crude extracts, the essential oils showed an IC_{50} in leukocytes and HeLa cells of 0.023-0.059 µg/ml and 0.025-0.043 µg/ml, the LD_{50} is less than 50 mg/kg. IC_{50} values showed that the essential oils were highly toxic than the crude extracts. At the level of human genetic materials, the crude extracts of two species, including *P. betloides* and *P. crocatum*, showed a significant toxicity ($p < 0.05$) in leukocytes. The other samples were non-toxic. The crude extracts of all samples showed significant genotoxicity in HeLa cells. The essential oils of all studied *Piper* species showed insignificant toxicity in leukocytes. For HeLa cells, the eight-studied species showed significant toxicity in HeLa cells, whereas only *P. submultinerve* showed insignificant toxicity.
Conclusion: The crude extracts and essential oils should be tested as putative cervical cancer treatments due to less toxicity in human normal cells.

Keywords: betel-like scent *Piper* species; essential oil; crude extracts; cytotoxicity; genotoxicity

Introduction

Piper species have been used as spices, insecticides, medicines and aromatic plants around the world in a variety of human activities (Chaveerach et al., 2006; Janakiraman and Manavalan, 2008; Scott et al., 2008; Fan et al., 2011; Thent et al., 2012). *Piper betle*, the betel plant, is one of the most important and well-known species in the genus.

It contains important chemical substances, such as chavicol, cineol and eugenol, which are used in essential oils, medicines and insecticides (Yusoff et al., 2005; Misra et al., 2009). Eugenol has been reported as having antioxidant and anti-inflammatory properties (Misra et al., 2009). Investigations of the genus *Piper* in Thailand (Chaveerach et al., 2008, 2009) have found that among the 43 *Pip*er species, nine species possess a betel-like scent. These species are wild species that grow well and produce numerous branches and leaves. These species also tolerate and resist disease, which are desirable characteristics in economically important plants. These species are assumed to carry similar useful chemicals as economically grown betel plant. Moreover, some of these species have produced a stronger scent than the betel plant and may contain higher amounts of important chemicals. Therefore, these nine species might be more economically beneficial than the betel plant. The advantage is that there are several plants which could be substituted for betel plant. Sanubol et al. (2014) reported that *P. betle* and the other eight betel-like scented species, including *P. betloides*, *P. crocatum*, *P. maculaphyllum*, *P. rubroglandulosum*, *P. semiimmersum*, *P. submultinerve*, *P. tricolor* and *P. yinkiangense*, which contain eugenol, isoeugenol, chavicol, caryophyllene, sabinene, phellandrene, germacrene A, germacrene D, and sesquiterpenes that could have high potential for industrial purposes.

The properties of major compounds are as follows: isoeugenol, an isomer of eugenol, is a phenylpropene that is synthesized from eugenol and is a constituent of the essential oils of *Piper* plants. Chavibetol, another isomer of eugenol, is an aromatic compound with a spicy odor (Nigam & Purohit 1962). Caryophyllene has a spicy, clove-like aroma (Ghelardini et al., 2001). Phellandrenes are used in fragrances because of their pleasant aromas. The odor of β-phellandrene has been described as peppery-minty and slightly citrusy (Boland et al., 1991). Sabinene is a natural bicyclic monoterpene, which is one of the chemical compounds that contribute to the spiciness of *P. nigrum* or black pepper (Shulgin et al., 1967). Sesquiterpenes are a class of volatile compounds that are typically produced for their antimicrobial and insecticidal properties (Feger et al., 2001; Noge & Becerra 2009).

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Betel oil has been used in modern medicine as an antiseptic component in gels, balms, an anti-inflammatory and as a treatment for many diseases (Information Service Center, Institute of Scientific and Technological Research, Thailand, Available at: http://www.tistr.or.th/callcenter/index.php?option=com_content&task=view&id=97, Accessed 8 June 2013). Because *Piper* species have been used for a variety of human activities, they must be thoroughly tested for toxicity before their use in humans can be approved. Single-cell gel electrophoresis, also known as the comet assay, is a method commonly used to assess genotoxicity (Lorenzo et al., 2013). This assay has been successfully applied to a range of phylogenetically disparate organisms, including mammals and plants (Jha, 2008; Rodrigues et al., 2010). In addition, the comet assay has been increasingly used for genotoxicity testing of industrial chemicals, pharmaceuticals and agrochemicals such as herbicides and pesticides. The comet assay can be used to support drug development as a mechanism predictor, and it is also becoming a common method for genotoxicity testing of potential medicinal plants (Abdelmigid, 2013).

Similar to other *Piper* species, the nine species with betel-like scents should be assessed for both cyto- and genotoxicity levels before they are administered to people. In this study, we analyzed *P. betle*, *P. betloides*, *P. crocatum*, *P. maculaphyllum*, *P. rubroglandulosum*, *P. semiimmersum*, *P. submultinerve*, *P. tricolor* and *P. yinkiangense* which have been used as traditional medicines for remedies of ailments such as antiseptic, anti-inflammatory, herpes simplex and herpes zoster viruses. The results from examining cytotoxicity and genotoxicity of the plant extracts and essential oils are outlined and discussed in the following report.

Materials and Methods

Plant materials

Leaves of nine *Piper* species, including two species with male (♂) and female (♀) individuals, led to a total of 11 individual samples were collected, namely *P. betle* ♂ (A. Chaveerach 341), *P. betle* ♀ (A. Chaveerach 16, BK63494), *P. betloides* (A. Chaveerach 171, BK63720), *P. crocatum* (A. Chaveerach 12), *P. maculaphyllum* (A. Chaveerach 126, BK63815), *P. rubroglandulosum* ♂ (A. Chaveerach 314, BK63816), *P. rubroglandulosum* ♀ (A. Chaveerach 340), *P. semiimmersum* (A. Chaveerach 115), *P. submultinerve* (A. Chaveerach 223), *P. tricolor* (A. Chaveerach 64) and *P. yinkiangense* (A. Chaveerach 133). The leaves were used for crude extraction and essential oil distillation for addressing their toxicity.

Crude extract preparation

The leaves were rinsed with water and air-dried until the water evaporated. Twenty-five grams of each sample were ground into powder and added to 120 ml hexane (analytical grade) solvent, the samples were left for three days at room temperature and then filtered through a filter paper. Ninety ml of the filtrate (crude extract) was collected and maintained at -20°C. The hexane was evaporated with a vacuum concentrator (ScanVac LaboGene, Denmark) at -20°C, 200 rpm for 2 h. A dark green, thick, viscous crude extract was obtained at concentration of 208.33 mg/ml. Ninety ml of dimethyl sulfoxide (DMSO) was added to the extract and maintained at -20°C until the cytotoxicity and genotoxicity experiments were conducted. Upon testing, the crude extracts were serial-diluted to obtain a final concentrations of 208.33, 104.17, 52.08 and 26.04 mg/ml as working concentrations.

Steam distillation for essential oil preparation

Fresh, matured leaves (1 kg) of each of the 11 studied samples were subjected to steam distillation for 3 h with 3.8 L water using WHM12017 Heating Mantles with a condensation set (Daihan Scientific Co. Ltd., Korea). After distillation, the oils were collected from two parts. The first part floating on the water surface in a collecting flask was transferred to a new bottle by an auto pipette and maintained at -20°C. The second part, which was mixed with water in the flask, was transferred to a separation funnel and dichloromethane (25:500 mL) was added, mixed well and left for 1 h at room temperature. The dichloromethane-oil remaining at the bottom of the funnel was collected, and the dichloromethane was evaporated using a Rotary Evaporator (Buchi Rotavapor R-210, Switzerland). Finally, oil from the second part was combined with the first part and maintained at -20°C until the cytotoxicity and genotoxicity experiments were conducted. Upon testing, the original viscous concentration was diluted with water to a 1% (v/v) concentration and then was serial-diluted to obtain final concentrations of 0.1, 0.01, 0.001 and 0.0001 µl/ml as working concentrations.

Cell preparations and plant extract treatments

Buffy coat samples were received from a blood bank. Leukocytes were isolated using a Ficoll-Paque Plus (GE Healthcare, Sweden) following the company's protocol. The isolated cells were re-suspended in PBS. A cell suspension was prepared for toxicity tests at a concentration of 6×10^5 viable cells/ml in modified RPMI-1640 medium (Gibco, Life Technologies, USA), with 2.05 mM L-glutamine and 25 mM HEPES, supplemented with 10% FBS, 5 µg/mL phytohemagglutinin (PHA), 100 µg/ml streptomycin, and 100 U/ml penicillin. The cell suspension was divided into a 96-well microplate (Thermo Scientific, China) at 100 µl per well. Then, 10 µl of the prepared crude extracts at various

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concentrations was applied to the cells in each well and incubated at 37°C for 4 h in a humidified atmosphere of 95% air and 5% CO_2. For HeLa cells, exponentially growing cells were prepared at a concentration of 6×10^5 cells/ml in DMEM (Gibco, Life Technologies, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (AppliChem, An ITW, Germany). The cell suspension was added to a 96-well microplate at 125 µl per well and incubated for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2 before the addition of the test extracts at 12.5 µl per well. Then, the plate was incubated for 24 h before the MTT assay.

MTT assay for cytotoxicity testing

Cellular reduction of tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA) formed a violet crystal formazan through mitochondrial succinate dehydrogenase activity of the viable cells, and the violet crystal formazan was quantified following the methods of Freshney (2010).

At the end of the treatment, the medium was removed by autopipette for the HeLa cells and by centrifuge plates at 1,500 rpm for 10 min for the leukocytes. The MTT was added to a final concentration of 0.5 mg/mL. Then, the plates were wrapped with aluminum foil and incubated for 4 h at 37°C. After the formazan crystals were solubilized by adding 100 µl DMSO to each well, the plates were left in the dark for 2–4 h. The absorbance was read at 570 nm with a microtiter plate spectrophotometer (Fluorescence microplate reader; SpectraMax M5 series, Molecular Devices). Wells containing medium and MTT without cells were used as blanks. Each concentration treatment was performed in triplicate.

Each concentration treatment was performed in triplicate. Doses inducing 50% inhibition of cell viability (the IC_{50} value) were determined by plotting graph between extract concentration and cell viability, % of control and calculated using the following equation: cell viability (%) = average viable of treated cells/average viable negative control cells x 100 to reveal the cytotoxicity of the plant extracts. The IC_{50} values were used for LD_{50} calculation (Walum, 1998) to release hazardous levels (World Health Organization, 2009). Essential oils were in liquid form, therefore calculation of their IC_{50} and LD_{50} was based on a density of *P. nigrum* oil which is 0.878 (Graham, 1843).

Comet assay for genotoxicity testing

The comet assay was performed using the IC_{50} value of each species extracts and essential oil according to a method described by Singh et al. (1988) with slight modifications. Briefly, after incubation with extracts and essential oil, cell pellets were obtained by centrifugation and re-suspended in PBS. One hundred µl of 0.5% low melting point agarose (LMA) was mixed with 40 µl of the cell suspension. The mixture was dropped onto glass slides that were pre-coated with 1% normal melting point agarose. Next, cover slips (22 mm × 50 mm) were placed on top of the slides and stored at 4°C. After 10 min, the cover slips were removed, and the slides were submerged in a lysis solution (8 M NaCl, 0.6 M EDTA pH 8, 0.2 M Tris, 0.1% Triton X-100) for at least 1 h. The slides were then soaked in electrophoresis buffer (6 mM EDTA pH 10, 0.75 M NaOH) for 40 min. Next, electrophoresis was performed for 25 min at 26 volts and 300 milliamps at 4°C. After electrophoresis, the slides were immediately neutralized with 0.4 M Tris buffer (pH 7.5) three times for 5 min each. The slides were then stained with 1 µg/ml ethidium bromide (60 µl per slide) overnight at 4°C in the dark, and images were obtained using an image analysis system (Isis) attached to a fluorescence microscope (Nikon, Japan). Images of 150 cells were randomly captured and analyzed using ImageJ software and statistically analyzed using GraphPad Prism software. The negative control was untreated cells, and the positive control was cells with broken DNA from UV exposure.

Results

Cytotoxicity of the crude extracts and the essential oils

MTT assays for the crude extract and the essential oil of 11 *Piper* samples, including *P. betle* ♀-♂, *P. betloides*, *P. crocatum*, *P. maculaphyllum*, *P. rubrograndulosum* ♀-♂, *P. semiimmersum*, *P. submultinerve*, *P. tricolor* and *P. yinkiangense*, on leukocytes and HeLa cells were conducted. The cell viability percentage is shown in Figs. 1-2 for leukocytes and Figs. 3-4 for HeLa cells. Tables 1-2 display the relationship between the concentrations and cell viability, including IC_{50} and LD_{50} values.

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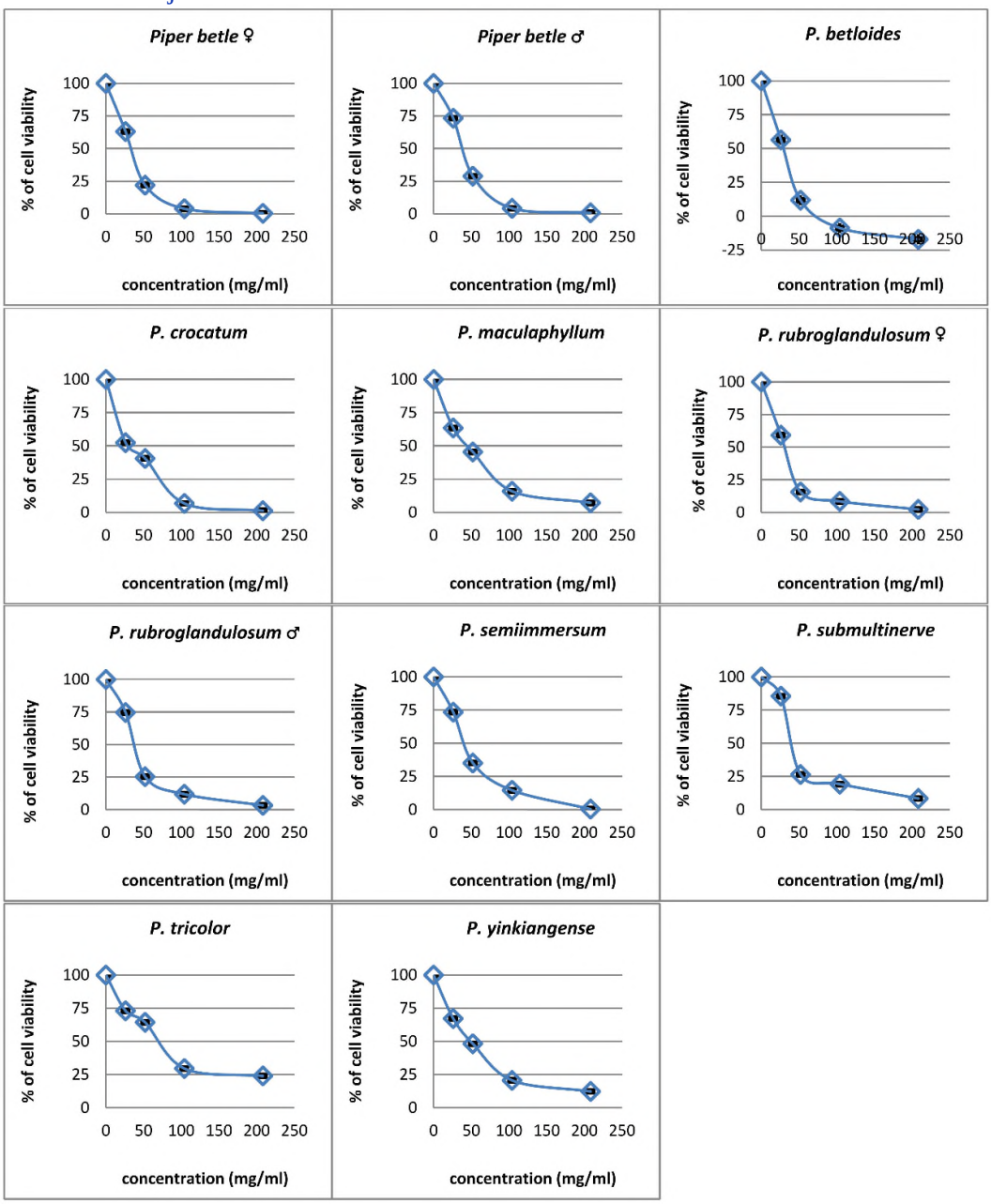

Figure 1:The cytotoxicity in leukocytes following treatment with 11 crude extracts from *Piper* species.

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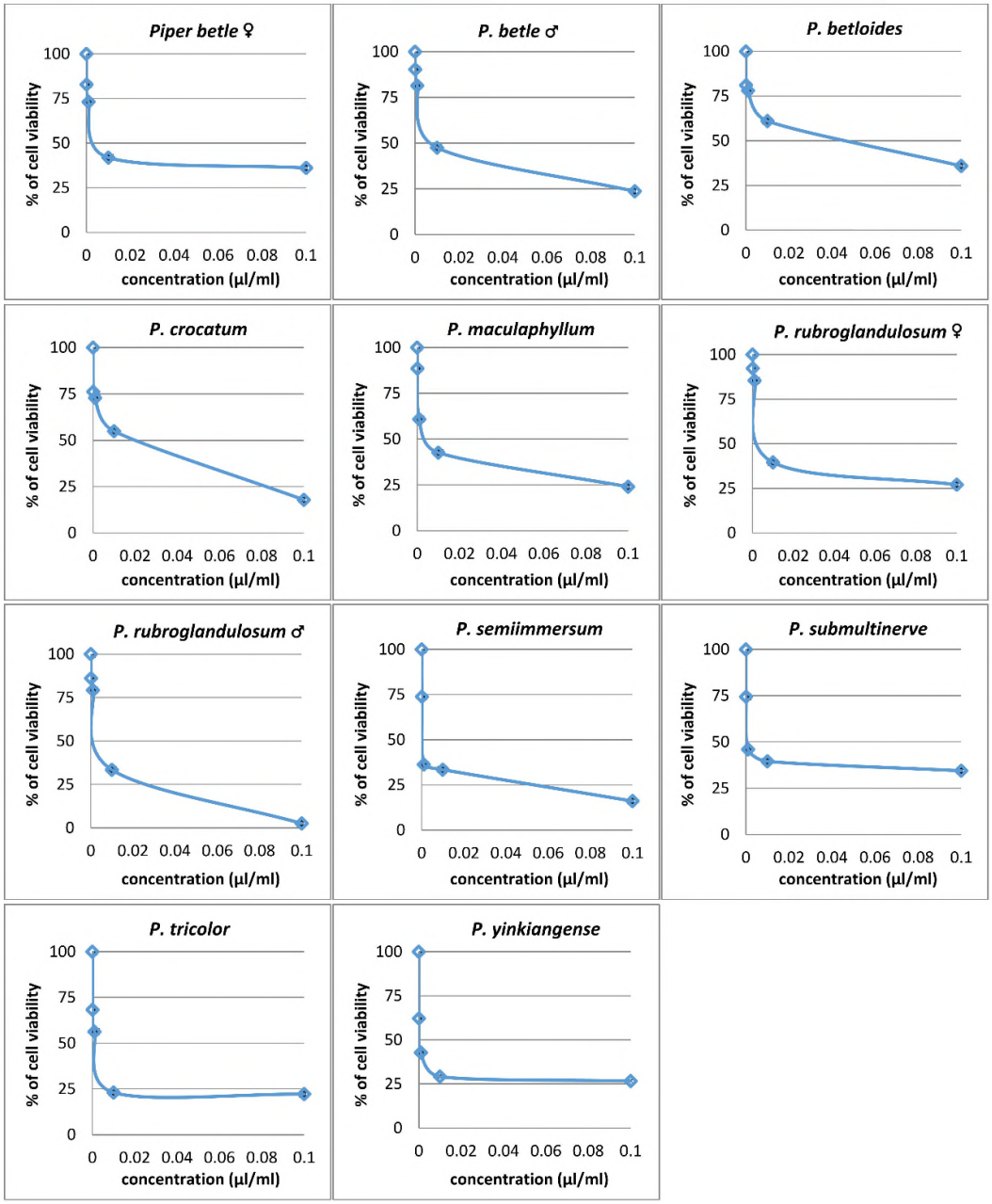

Figure 2: The cytotoxicity in leukocytes following treatment with essential oils from 11 *Piper* samples.

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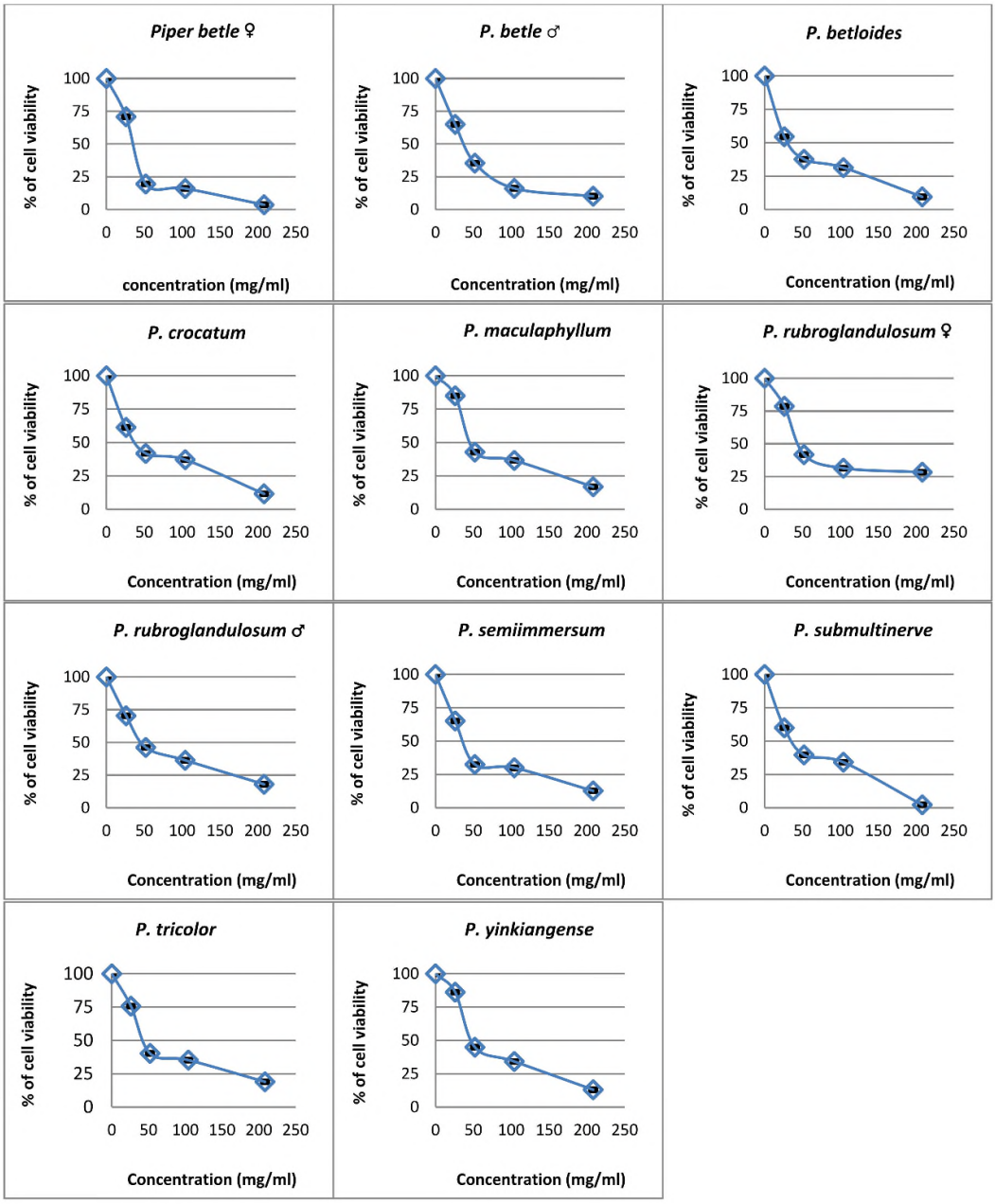

Figure 3: The cytotoxicity in HeLa cells following treatment with 11 crude extracts from *Piper* species.

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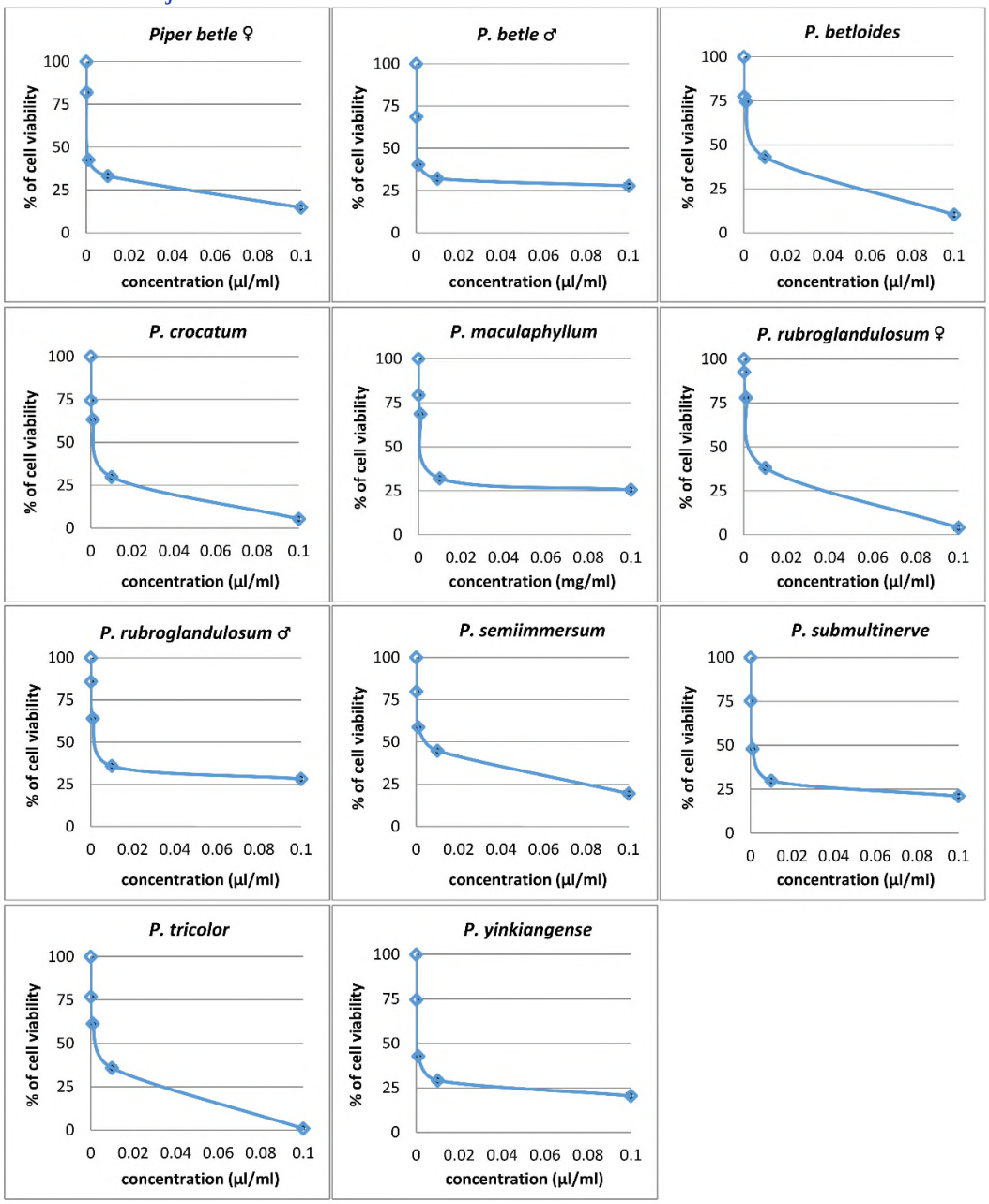

Figure 4: The cytotoxicity in HeLa cells following treatment with essential oils from 11 *Piper* samples.

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Table 1: Percentage of cell viability, IC_{50} and LD_{50} values of crude extracts of nine *Piper* species, 11 samples tested in leukocytes and HeLa cells.

***Piper* species**	**Final conc. (mg/ml)**	**Leukocytes**			**HeLa cells**		
		% cell viability	**IC_{50} (mg/ml)**	**LD_{50} (mg/kg)**	**% cell viability**	**IC_{50} (mg/ml)**	**LD_{50} (mg/kg)**
Piper betle ♀	208.33	3.62	58.59	6275.24	0.47	49.66	5900.85
	104.17	16.04			3.94		
	52.08	19.50			22.03		
	26.04	70.81			63.15		
P. betle ♂	208.33	10.12	65.86	6554.31	0.80	59.06	6293.92
	104.17	16.14			4.23		
	52.08	35.38			28.90		
	26.04	64.99			73.31		
P. betloides	208.33	9.49	68.47	6649.76	-17.11	34.91	5175.80
	104.17	31.34			-8.72		
	52.08	37.63			11.83		
	26.04	54.51			56.38		
P. crocatum	208.33	11.58	79.44	7027.72	1.27	54.58	6111.90
	104.17	37.10			6.76		
	52.08	41.82			40.61		
	26.04	61.32			52.48		
P. maculaphyllum	208.33	16.82	94.72	7503.02	7.23	69.27	6678.56
	104.17	36.58			15.91		
	52.08	42.87			45.46		
	26.04	84.96			63.54		
P. rubrograndulosum ♀	208.33	28.30	97.31	7578.70	2.31	45.70	5721.22
	104.17	31.34			8.43		
	52.08	41.77			15.66		
	26.04	78.62			59.17		
P. rubrograndulosum ♂	208.33	17.92	90.03	7362.61	3.29	61.89	6404.46
	104.17	36.27			11.90		
	52.08	46.12			25.35		
	26.04	70.34			74.68		
P. semiimmersum	208.33	12.68	72.63	6797.28	0.43	66.32	6571.31
	104.17	29.98			14.61		
	52.08	32.49			35.15		
	26.04	65.09			73.45		
P. submultinerve	208.33	2.20	71.06	6742.25	8.46	73.25	6818.81
	104.17	34.17			19.28		
	52.08	39.62			26.51		
	26.04	59.96			85.53		
P. tricolor	208.33	18.97	88.74	7323.19	23.99	101.79	7706.66
	104.17	35.22			29.60		
	52.08	40.19			64.35		
	26.04	75.68			73.09		
P. yinkiangense	208.33	13.00	92.18	7427.54	12.33	77.32	6957.36
	104.17	34.17			20.63		
	52.08	44.86			48.21		
	26.04	86.16			67.26		

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Table 2: The cell viability percentages and IC_{50} values from essential oils of nine *Piper* species, including two species with sex-specific plants, for a total of 11 treatments in leukocytes and HeLa cells.

***Piper* species**	**Final conc. (µl/ml)**	**Leukocyte**			**HeLa cell**		
		% cell viability	**IC_{50} (µg/ml)**	**LD_{50} (mg/kg)**	**% cell viability**	**IC_{50} (µg/ml)**	**LD_{50} (mg/kg)**
Piper betle ♀	0.1	36.13	0.054	35.57	14.80	0.026	27.32
	0.01	41.90			33.18		
	0.001	73.13			42.60		
	0.0001	82.88			82.06		
P. betle ♂	0.1	23.67	0.047	33.76	27.80	0.028	27.98
	0.01	47.55			32.06		
	0.001	81.41			40.36		
	0.0001	90.31			68.61		
P. betloides	0.1	35.94	0.059	36.84	10.31	0.033	29.83
	0.01	61.04			43.05		
	0.001	78.04			74.44		
	0.0001	81.10			77.58		
P. crocatum	0.1	17.90	0.040	31.77	5.38	0.025	26.98
	0.01	54.91			29.82		
	0.001	72.88			63.23		
	0.0001	76.20			74.44		
P. maculaphyllum	0.1	24.04	0.041	32.28	25.56	0.039	31.50
	0.01	42.64			32.06		
	0.001	60.86			68.61		
	0.0001	88.59			79.37		
P. rubrograndulosum ♀	0.1	27.10	0.048	34.23	4.04	0.033	29.83
	0.01	39.57			38.12		
	0.001	85.40			78.03		
	0.0001	92.27			92.60		
P. rubrograndulosum ♂	0.1	2.56	0.031	28.93	28.25	0.043	32.79
	0.01	33.44			35.87		
	0.001	79.26			64.13		
	0.0001	86.13			85.87		
P. semiimmersum	0.1	16.06	0.023	25.90	19.51	0.036	30.69
	0.01	33.44			44.84		
	0.001	36.32			58.74		
	0.0001	73.87			79.82		
P. submultinerve	0.1	34.47	0.042	32.54	21.08	0.028	27.98
	0.01	39.57			29.82		
	0.001	46.01			47.98		
	0.0001	74.48			75.34		
P. tricolor	0.1	22.20	0.027	27.65	0.90	0.025	26.98
	0.01	23.07			35.87		
	0.001	56.32			61.43		
	0.0001	68.34			76.91		
P. yinkiangense	0.1	26.69	0.025	26.63	20.55	0.026	27.32
	0.01	29.20			29.20		
	0.001	42.82			42.82		
	0.0001	62.21			74.48		

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Genotoxicity of the crude extracts and the essential oils

The median olive tail moment values for leukocytes and HeLa cells after treatment with the crude extracts and the essential oil are shown in the Tables 3-4.

Table 3: The median olive tail moment values of leukocytes and HeLa cells after treatment with crude extracts of nine *Piper* species, including two species with sex-specific plants, for a total of 11 extracts.

***Piper* species**	**Leukocyte**		**HeLa cell**	
	Median olive tail moment	*p* value	Median olive tail moment	*p* value
Negative control	1.36		0.11	
Positive control	8.09	< 0.0001	33.31	< 0.0001
Piper betle ♀	2.16	0.4971	1.50	< 0.0001
P. betle ♂	1.85	0.7336	1.40	< 0.0001
P. betloides	4.92	< 0.0023	2.53	< 0.0001
P. crocatum	4.53	< 0.0004	3.60	< 0.0001
P. maculaphyllum	2.72	0.7051	29.97	< 0.0001
P. rubrograndulosum ♀	2.54	0.9053	28.91	< 0.0001
P. rubrograndulosum ♂	2.52	0.7980	32.35	< 0.0001
P. semiimmersum	1.97	0.5914	12.01	< 0.0001
P. submultinerve	2.51	0.3951	0.85	< 0.0001
P. tricolor	2.51	0.5146	19.22	< 0.0001
P. yinkiangense	1.94	0.8967	3.36	< 0.0001

Table 4: The median olive tail moment values of leukocytes and HeLa cells after treatment with essential oils of the 11 *Piper* samples.

***Piper* species**	**Leukocyte**		**HeLa cell**	
	Median olive tail moment	*p* value	Median olive tail moment	*p* value
Negative control	0.90		0.88	
Positive control	8.13	< 0.0001	27.63	< 0.0001
Piper betle ♀	1.49	0.1356	3.76	0.0340
P. betle ♂	0.94	0.6856	1.41	< 0.0001
P. betloides	1.59	0.1335	2.63	< 0.0001
P. crocatum	1.78	0.1637	3.98	< 0.0001
P. maculaphyllum	1.81	0.2610	2.62	< 0.0001
P. rubrograndulosum ♀	1.03	0.3320	5.74	< 0.0001
P. rubrograndulosum ♂	0.98	0.1223	9.14	< 0.0001
P. semiimmersum	1.44	0.2507	6.95	< 0.0001
P. submultinerve	1.25	0.0950	1.30	0.0838
P. tricolor	1.67	0.4365	7.44	< 0.0001
P. yinkiangense	1.53	0.1035	5.57	< 0.0001

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Statistically, the tail moments of leukocytes treated with crude extracts of *P. betle* ♀-♂, *P. maculaphyllum*, *P. rubrograndulosum* ♀-♂, *P. semiimmersum*, *P. submultinerve*, *P. tricolor* and *P. yinkiangense* showed insignificant differences ($p > 0.05$). Therefore, the crude extracts are non-toxic to normal human DNA. Treatments with the extracts of *P. betloides* and *P. crocatum* had significant different ($p < 0.05$) tail moments (Tables 3), which indicates their toxicity to normal human DNA as shown in Figure 5.

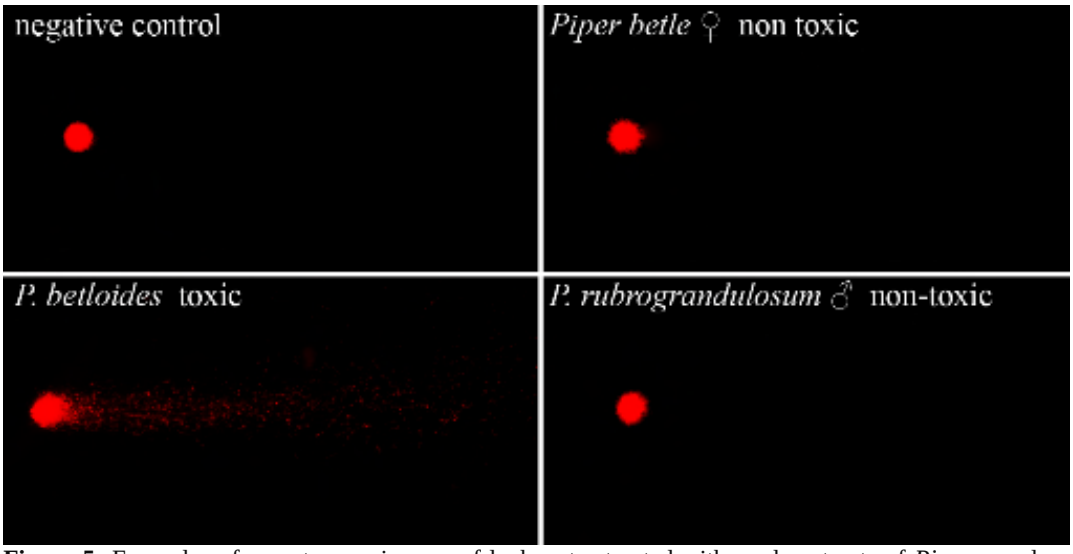

Figure 5: Examples of comet assay images of leukocytes treated with crude extracts of *Piper* samples showing non-toxic and toxic cases.

For HeLa cells treated with all of the crude extracts, the tail moments showed a significant difference ($p < 0.05$). Therefore, the crude extracts were toxic to the human cancer cells as shown in Figure 6.

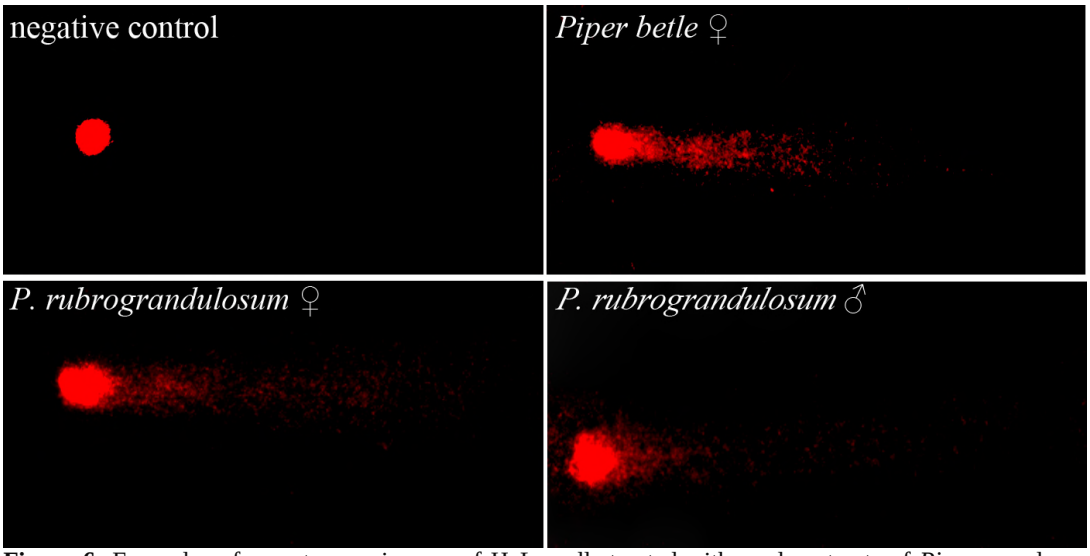

Figure 6: Examples of comet assay images of HeLa cells treated with crude extracts of *Piper* samples which are all toxic.

The median olive tail moment values of leukocytes and HeLa cells treated with essential oils for the 11 samples are shown in Table 4. The tail moments of leukocytes treated with the samples showed insignificant difference ($p > 0.05$), which demonstrates the oils are non-toxic for normal human DNA (Figure 7).

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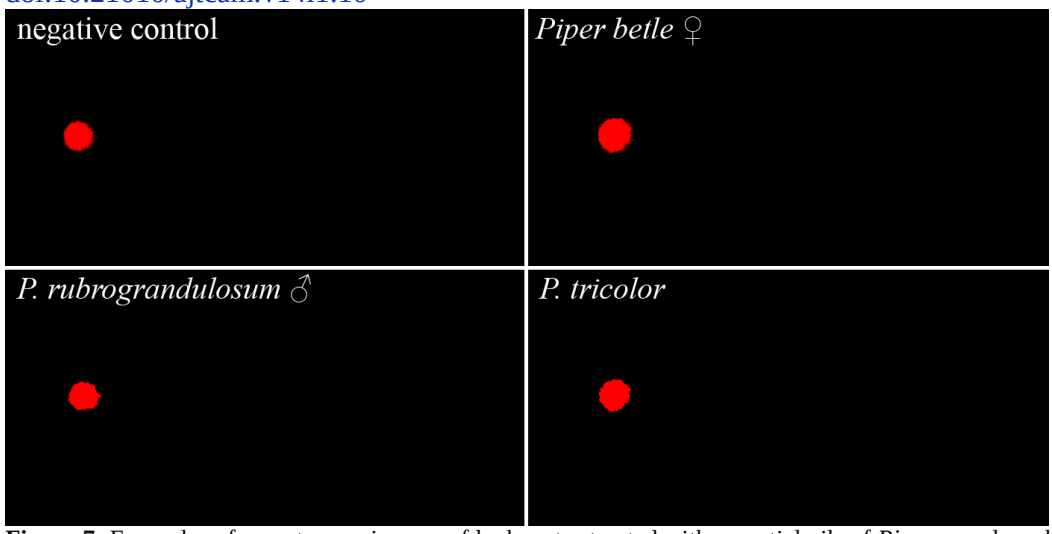

Figure 7: Examples of comet assay images of leukocytes treated with essential oils of *Piper* samples which are all non-toxic.

In HeLa cells, the tail moments for cells treated with the oils from eight species, showed significant differences ($p < 0.05$) regarding their toxicity as examples showed in Figure 8. However, the oil of *P. submultinerve* showed insignificant toxicity ($p > 0.05$).

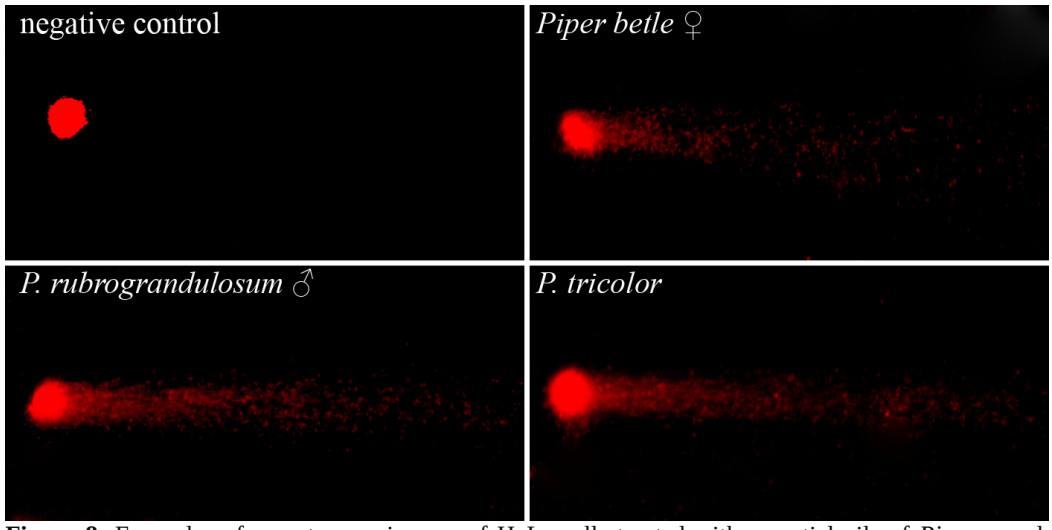

Figure 8: Examples of comet assay images of HeLa cells treated with essential oils of *Piper* samples which are all toxic.

Discussion

Many *Piper* species are well-known medicinal plants, aromatic plants and spices popularly used worldwide for a variety of purposes, including the production of substances and essential oils (Chaveerach et al. 2008; Misra et al, 2009; Rekha et al., 2014; Ghosh et al., 2014; Rintu et al., 2015). In addition to *P. betle*, there were newly reported eight betel-like-scented *Piper* species, which contained such potential substances as *P. betle*. All the nine betel-like-scented *Piper* plants were reported as diverse sources of industrial and medicinal aromatic chemicals such as eugenol, chavicol, isoeugenol, chavicol and etc. (Sanubol et al., 2014). Unfortunately, the previous reports on the various usages exactly in human lacked of toxicity testing. Therefore, this is the first disclosing results on the toxicity of nine beneficial *Piper* species continually from the previous work on their usages (Sanubol et al., 2014).

With the notion that, before these plants are used in a variety of human activities, they must be thoroughly tested for toxicity. In this study, we investigated toxicity by examining cytotoxicity levels with MTT assays and studying genotoxicity with comet assays. All of the studied crude extracts showed IC_{50} values in leukocytes and HeLa cells starting from low values of 58.59 mg/ml for *P. betle* ♀ to 97.31 mg/ml for *P. rubrograndulosum* ♀ and from 34.91 mg/ml for *P. betloides* to 101.79 mg/ml for *P. tricolor*. These values suggest that the leaves crude extracts may be toxic to normal human cells, such as leukocytes, and cervical human cancer cells, such as HeLa cells. Compared to

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crude extracts, the essential oils had lower IC_{50} values in leukocytes and HeLa cells starting from low values of 0.023 µg/ml for *P. semiimmersum* to 0.059 µg/ml for *P. betloides* and 0.025 µg/ml of *P. crocatum* and *P. tricolor* to 0.043 µg/ml of *P. rubrograndulosum* ♂. These values indicate that the essential oils may be toxic both in normal human cells, such as leukocytes, and cervical human cancer cells line, such as HeLa cells, with a higher toxicity than the crude extracts supporting by LD_{50} values. The LD_{50} of all the crude extracts is higher than 5,000 mg/kg classified in class III slightly hazardous where the LD_{50} of the essential oils is less than 50 mg/kg classified in class I b highly hazardous (World Health Organization, 2009).

However, with in-depth examination of human genetic materials, only two crude extracts, *P. betloides* and *P. crocatum*, showed significant toxicity ($p < 0.05$) for leukocyte DNA. The other samples showed insignificant toxicity ($p > 0.05$). It is interesting that all of the crude extracts showed significant toxicity ($p < 0.05$) in HeLa cells. The essential oils were similarly toxic in HeLa cells, but they lacked significant toxicity in leukocytes. Interestingly, all of the studied species except for *P. submultinerve* showed significant toxicity ($p < 0.05$) in HeLa cells. *P. submultinerve* showed insignificant toxicity ($p > 0.05$).

Conclusion

Piper betle and other eight betel-like-scented *Piper* species previously reported for their potential chemical constituents were tested for their toxicity on human cancer cell line, HeLa cells and normal cells, leukocytes. The essential oils were highly toxic than the crude extracts at cytotoxicity level on leukocytes. At the level of genetic materials, except for *P. betloides* and *P. crocatum*, the crude extracts were non-toxic to leukocytes. Interestingly, the crude extracts of all samples showed a significant genotoxicity in HeLa cells. All the essential oils showed insignificant toxicity in leukocytes. For HeLa cells, except for *P. submultinerve* all of the oils showed significant toxicity in HeLa cells. It is obvious that these data supported usages of the plants for many purposes in the past and present. Therefore, some of the crude extracts and essential oils of the studied species should be tested as a putative cervical cancer treatment due to the lack of toxicity against human normal cells.

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