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Original Article

Non-mutagenic and genotoxic effects of water extract of *Piper sarmentosum* using Ames and micronucleus assay

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Abstract

P. sarmentosum possesses a variety of biological activities and has long been used as a food ingredient and traditional medicine to treat many diseases. In this study, its mutagenic and genotoxic activities on *S. typhimurium* TA98 and TA100 and fibroblast (V79) cells were examined. Mediated by S9 activation, the water extract of *P. sarmentosum* at a high dose caused the number of revertant colonies to increase in the tester strains TA98 (20 mg/plate) and TA100 (40 mg/plate), but there was no mutagenic effect in the presence of S9 in mixed condition. The extract (1 - 7.5 mg/ml) showed no significant genotoxic effect based on the frequency of micronucleus formation both with and without S9 in mixed conditions. In conclusion, the water extract of *P. sarmentosum* has neither direct nor indirect genotoxic effects and has no mutagenic effects indirectly. Therefore, the results indicate that *P. sarmentosum* is safe for use as a food supplement.

Keywords: *Piper sarmentosum*, mutagenicity, genotoxicity, Ames assay, micronucleus assay

1. Introduction

Piper sarmentosum (Cha - plu), a plant in the Piperaceae family, is widely distributed in the tropical and subtropical regions, particularly in Southeast Asian countries (Rahman, Sijam & Omar, 2016). It has long been used as a food ingredient and in traditional medicine to cure many diseases (Hussain, Hashmi, Latif, Ismail & Sadikun, 2012). For instance, in the southern part of Thailand, the water extract of the whole plant is used to treat patients with diabetes mellitus to reduce the blood glucose level (Pongmarutai, 1989). In addition, diabetic induced rodents demonstrate reduced blood glucose (Peungvicha *et al.*, 1998) and improved pancreatic islet function as well as increased serum insulin, following treatment with the water extract of *P. sarmentosum* (Luangpirom, Kourchampa & Somsapt, 2014). Moreover,

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recent studies have demonstrated that various parts of the *P. sarmentosum* plant contain many biologically active compounds with pharmacological properties, including antimicrobial (Atiax, Ahmad, Sirat & Arbain, 2011; Masuda *et al.*, 1991), antipsychotic (Ridtitid, Rattanaprom, Thaina, Chit trakarn & Sunbhanich, 1998), antioxidant (Chanwitheesuk, Teerawutgulrag & Rakariyatham, 2005), anti-inflammatory and antinociceptive (Ridtitid Ruangsang, Reanmongkol & Wongnawa, 2007; Rukachaisirikul, Siriwattanakit & Suk charoenphol, 2004), antiangiogenic (Hussain, Ismail, Sadikun, Ibrahim & Malik, 2008), anticancer (Ariffin *et al.*, 2009; Sriwiriyajan, Ninpesh, Sukpondma, Nasomyon & Graidist, 2014), hepatoprotective (Hussain, Ismail & Sadikun, 2010), and anti-atherosclerosis (Amran *et al.*, 2010) activities.

There are many studies indicating toxic effects of *P. sarmentosum*. Previously, the LD_{50} of a whole plant aqueous extract was 10 g/kg in rats (Peungvicha *et al.*, 1998), while the LD_{50} of leaf methanol extract was higher than 5 g/kg (Ridtitid *et al.*, 2007). Interestingly, at a limit dose 2 g/kg, the ethanol extracts of leaf and fruit did not show acute oral toxicity (Hussain *et al.*, 2010) and leaf aqueous extract also did not cause subacute toxicity in the hematological profile, liver, or kidney in rats (Mohd Zainudin, Zakaria, Megat Mohd Nordin & Othman, 2013). However, research on the safety of *P. sarmentosum* involving the genotoxic or mutagenic effects is lacking. In a previous study, the whole plant water extract of *P. sarmentosum* had no effect on chromosomal damage characterized by micronucleus formation in bone marrow cells in the rat (Puchadapirom *et al.*, 2014). Leaf extracts of *P. sarmentosum* showed no mutagenic activity, whereas the dichloromethane extract had the strongest antimutagenic activity in *S. typhimurium* TA98 and TA100 strains (Boonla, Phadungkit, Mahaweerawat & Somdee, 2014).

Thus, this study was performed to provide more information on its safety for use in medical practice. The present study was carried out to determine the mutagenic and genotoxic activities of *P. sarmentosum* extracts by using two standard tests including the bacterial gene mutation (Ames) assay and a micronucleus test, both with and without S9 liver enzyme metabolic activation conditions.

2. Materials and Methods

2.1 Materials

2.2.1 S9 fraction

A metabolic enzyme (S9 protein fraction) from the liver of phenobarbital-induced rat was obtained from the Department of Biochemistry, Faculty of Medicine, Chiangmai University, Thailand. The preparation of the S9 fraction was performed following Mutsushima, 1976. In the present study, the final concentration of S9 in the treatment medium was 5% (v/v).

2.1.2 Bacterial strains

Salmonella typhimurium strains TA98 (hisD3052, *rfa*, uvrB, pKM101) and TA100 (hisG46, *rfa*, uvrB, pKM101) were firstly prepared and checked for their characteristic genotypes including Histidine dependence (His^-), Biotin dependence (Bio^-), Biotin and histidine dependence (His^-/Bio^-), *rfa* marker, R-factor, and *uvr*B deletion according to standard methods of Maron and Ames, 1983.

2.2 Plant extract preparation

Thai medicinal plant *P. sarmentosum*, voucher No. BK 62472, was collected in the Sireeruckhachati Nature Learning Park, Mahidol University, Thailand and identified by the Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University.

The whole *P. sarmentosum* plant was dried at 50 °C for 24 h, then boiled with 12 fold amount of water (w/v) for 30 min and the supernatant was filtered. The residue was reboiled with water three times. The filtrate was reheated until it reached half volume, and was lyophilized. The final yield was 16.5 % (w/w).

2.3 Mutagenic activity study on *Salmonella typhimurium* by using Ames assay

Evaluation of the mutagenicity of *P. sarmentosum* extract was carried out according to Puchadapirom *et al.* (2012) by using the bacterial reverse mutation (Ames) test both without S9 (- S9) and with S9 (+ S9) metabolic activation conditions (Mortelmans & Zeiger, 2000). In pre-incubation the overnight cultured bacteria (0.1 ml) were incubated with 0.1 ml of the extract at the concentrations of 1, 5, 10, 20, and 40 mg/plate and 0.5 ml of phosphate buffer without and with S9 conditions at 37 ˚C, for 20 min. The molten top agar was added into the mixture that was then poured onto minimal glucose agar plates. The tested plates were incubated at 37 ˚C for 48 h and then the number of revertant colonies was scored. In this study, distilled water was used as the negative control, whereas the mutagens, 4-Nitroquinoline-1-oxide (4-NQO) and sodium azide (NaN_3) at the concentrations of 0.4 and 0.2 µg/plate, were used as positive controls with TA98 (- S9) and TA100 (- S9), respectively; and 0.5 µg/plate of 2-aminoanthracene (2-AA) was used as the positive control with both TA98 (+ S9) and TA100 (+ S9).

2.4 In vitro cytotoxicity screening test on fibroblast (V79) cells

For determining the maximum dose of *P. Sarmentosum* extract, the cytotoxicity of the extract against Chinese hamster lung fibroblast (V79) cells was determined using trypan blue staining technique. Cells were treated with the extract at various concentrations (1 - 10 mg/ml) for 3 h. Then the conditioned medium was removed, and cells were washed with PBS at pH 7.4. New DMEM medium containing 10 % FBS was added and incubated at 37 °C and 5 % CO_2 in an incubator. At 24 h after treatment, cells were harvested and mixed with trypan blue solution. The numbers of dead cells and viable cells were counted, and the percentage of cell viability was calculated.

2.5 Genotoxic activity study on fibroblast (V79) cells by using micronucleus test

V79 cells (5×10^4 cells) were cultured and treated with *P. sarmentosum* extract at the doses 1, 2.5, 5 and 7.5 mg/

ml in DMEM medium containing 10 % FBS without (– S9) and with (+ S9) conditions at 37 ˚C for 3 hours. Distilled water was used as a negative control, while MMC (1 µg/ml) and CP (20 µg/ml) were used as positive controls in – S9 and + S9 conditions, respectively. Then the conditioned media were removed, and the cultures were maintained in a fresh DMEM media for 3 h. Then 3 µg/ml of cytochalasin B was added as a mitotic division blocker. At 24 h of the experiment, the treated cells were harvested and stained with Giemsa. Micronuclei (MN) of each treatment group were assessed for the number of micronuclei per 1000 binucleated cells under a light microscope (1000x magnification). In addition, the numbers of mononucleated cells, binucleated cells and trinucleated cells (Figure 1) were counted for the cytokinesis-block proliferation index (CBPI) and percent cytostasis of the extract calculations as described below (Himakoun, Tuchinda, Puchadapirom, Tammasakchai and Leardkamolkarn, 2011).

CBPI = [(No. of mononucleated cell) + (2 x No. of binucleated cell) + (3 x No. of trinucleated cell)] / Total number of cells

(%) Cytostasis = 100 – 100 [(CBPIT - 1) / (CBPIC - 1)] :

CBPIT = CBPI of the treated group

CBPIC = CBPI of the negative control group

2.6 Statistical analyses

All experiments were performed in triplicate. The results are presented as mean ± standard deviation (SD). Statistical comparisons between the numbers of revertant colonies with control (spontaneous revertant colonies) were performed using the independent samples t - test. The differences in MN frequencies among groups were obtained by using one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. Statistical significance was called at $p < 0.05$, using SPSS version 16 statistical software.

3. Results and Discussion

3.1 Mutagenic activity of *P. sarmentosum* water extract

Substances used in foods or as medicines need to undergo non-clinical safety testing. In the present study, the bacterial reverse mutation (Ames) assay was used to screen for possible mutagenicity of the water extract of *P. Sarmentosum* according to standard guidelines, using *S. typhimurium* TA strains (Maron & Ames, 1983; Mortelmans & Zeiger, 2000; Organisation for Economic Co-operation and Development [OECD], 1997). The two strains used in this study were TA98 and TA100 as they have plasmid pKM101, a mutagenesis-enhancing plasmid, and the combination of TA98 and TA100 is known to be sensitive and can detect a large proportion of known bacterial mutagens (Maron & Ames, 1983). In addition, since a new compound for drugs or food supplements can produce mutagenic metabolites from metabolism by liver enzymes (Attia, 2010), studying the *P. sarmentosum* extract under S9 metabolic activation conditions was also employed.

In this study, none of the doses from 1 to 40 mg/plate exhibited toxicity to the tester strains, either with or without S9 metabolic activation conditions. The mutagenicity

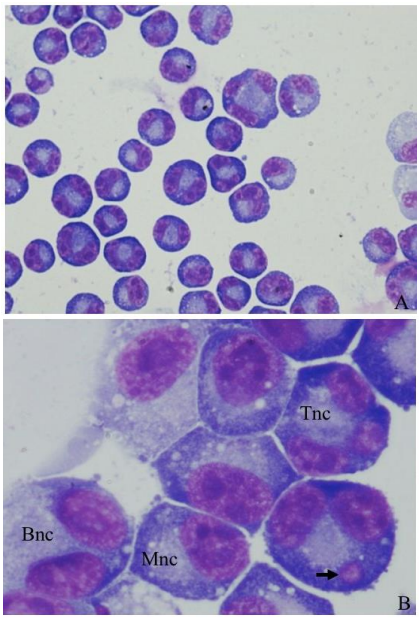

Figure 1. Effects of *P. sarmentosum* extract on micronucleus formation in V79 cells. (A) V79 cell line after staining with Giemsa (x200); (B) V79 cell line with mononucleated cell (Mnc), binucleated cell (Bnc), trinucleated cell (Tnc), and a micronucleus (MN) in a binucleated cell (arrow) (x1000). The MN can be distinguished as having a clearly defined membrane, separate from the main nucleus, and with a diameter less than 1/3 the diameter of the main nucleus.

of *P. sarmentosum* water extract towards the reverse mutation of *S. typhimurium* is shown in Table 1 and Figure 2. In the condition without S9, the numbers of spontaneous revertant colonies of TA98 and TA100 were 22.70 ± 3.0 and 36.83 ± 5.17 colonies/plate, respectively (negative control). All the positive control groups greatly exceeded the negative control with statistically significant increases ($p < 0.05$). The treatment of *S. typhimurium* with the extract at doses of 10, 20, and 40 mg/plate dose dependently and significantly increased the numbers of revertant colonies (32.67 ± 4.11, 46.00 ± 5.33, 169.00 ± 37.33 colonies for TA98 and 166.33 ± 9.44, 203.50 ± 23.00 and 416.17 ± 76.50 colonies for TA100, respectively) when compared to the negative control ($p < 0.05$). Interestingly, the number of revertant colonies of both strains was more than twice the number of spontaneous revertant colonies at the high dose (TA98 was 20 mg and TA100 was 40 mg). According to guidelines, a compound is classified as a mutagenic if the mean number of revertant colonies in one or more treatment groups is at least 2 - fold greater than that of the negative control group (Brusick, 1987; Cariello, 1996; Samiei *et al.*, 2015). Thus, without S9, *P. sarmentosum* at high dose induced a mutagenic effect against both TA98 and TA100, whereas at low doses (1 - 10 mg) it lacked mutagenicity. The results are consistent with the study of Boonla *et al.* 2014, who found that the leaf extract of *P. sarmentosum* had no mutagenic activity at the doses tested (0.5, 1, 2 and 4 mg) which are lower than in our study.

In the presence of metabolic activation (+ S9), the number of revertant colonies of TA98 treated with the *P. sarmentosum* extract showed a dose dependent and significant

Table 1. Mutagenic effect of *P. sarmentosum* extract on *S. typhimurium* TA98 and TA100

Test group	Dose/plate	Number of revertant colonies per plate (mean ± SD)			
		- S9		+ S9	
		TA98	TA100	TA98	TA100
DW	-	22.70 ± 3.00	136.17 ± 11.78	36.83 ± 5.17	166.00 ± 13.33
PS	1 mg	24.17 ± 3.50	140.17 ± 22.17	35.50 ± 3.67	169.50 ± 16.00
	5 mg	29.50 ± 6.00	147.33 ± 13.00	43.67 ± 6.22	165.83 ± 7.22
	10 mg	32.67 ± 4.11*	166.33 ± 9.44*	44.50 ± 3.83*	167.33 ± 8.00
	20 mg	46.00 ± 5.33*,**	203.50 ± 23.00*	49.67 ± 7.22*	166.83 ± 31.83
	40 mg	169.00 ± 37.33*,**	416.17 ± 76.50*,**	50.17 ± 10.17*	179.83 ± 10.17
NaN_3	0.2 µg	ND	792.50 ± 11.50*,**	ND	ND
4-NQO	0.4 µg	186.00 ± 14.17*,**	ND	ND	ND
2AA	2.5 µg	ND	ND	1546.83 ± 81.11*,**	1433.67 ± 81.11*,**

Note: *Significant difference in comparison with negative control (distilled water: DW) at $p < 0.05$; **number of revertant colonies > 2x spontaneous revertant colonies; PS: *P. sarmentosum* extract, NaN_3: sodium azide; 4-NQO: 4-nitroquinolene-1-oxide; 2AA: 2-aminoanthracene; ND: not done; -S9: without S9 metabolic activation condition; +S9: with S9 metabolic activation condition

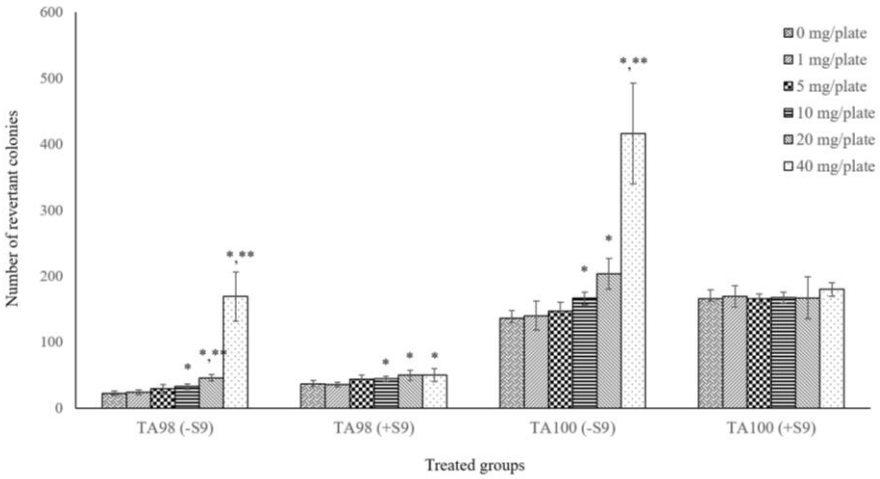

Figure 2. Effect of *P. sarmentosum* extract on the revertant colony formation in *S.typhimurium* TA98 and TA100: *Significant difference in comparison with negative control (0 mg/plate) at $p < 0.05$; **number of revertant colonies > 2x spontaneous revertant colonies

($p < 0.05$) increase when compared to the negative control (36.83 ± 5.17 colonies), but this was not more than 2-fold. In addition, there were no effects on increasing the number of revertant colonies of TA100.

From these results, the water extract of *P. Sarmentosum* at a high concentration exhibited a direct mutagenic effect against *S. typhimurium* strain TA98, more than that against TA100, but did not have an indirect mutagenic effect (via metabolites) against these special strains of *S. Typhimurium* at any dose. It is possible that the mutagenic properties of *P. sarmentosum* extract decrease during liver enzyme metabolism.

3.2 Genotoxic activity of *P. sarmentosum* water extract on V79 cells

Before the genotoxic evaluation, the cytotoxicity of *P. sarmentosum* on the Chinese Hamster lung fibroblast V79 cell line was first checked to select non-toxic doses of the extract for further genotoxic study. The extract showed cytotoxic effects on V79 cells at a concentration higher than 7.5 mg/ml, which caused cell death in more than 50 % (data not shown). Therefore, a concentration of 7.5 mg/ml was used as the highest dose in this study. Evaluation of the possible genotoxicity of different concentrations of *P. sarmentosum* was performed using the *in vitro* micronucleus assay in a V79 cell line according to OECD, 2014. The assay assesses chemical blocking of cell division (cytochalasin B) after treatment of cells with the test compounds. Micronucleus formation is due to acentric chromosomal fragments or whole chromosomes not migrating to the poles during anaphase, which renders it possible to detect clastogenic effects in cells having undergone cell division after being exposed to the mutagens. Thus, the micronucleus rate was used as an indicator for the DNA damaging capacity of the tested compounds (OECD, 2014). From the assay, the cell population consisted of a mixture of cells with one (mononucleates), two (binucleates), or three or more (multinucleates) nuclei. The micro-nucleus was only scored in binucleated cells. The results of our study are summarized in Table 2, where the frequency of micronuclei in any treatment group (dose ranging of 1 - 7.5 mg/ml of the *P. sarmentosum* extract) was not statistically significantly different from the negative controls, either with or without S9 mixed conditions. The

Table 2. Cytotoxic and genotoxic effects of *P. sarmentosum* extract in V79 cell lines

Test group	Dose	CBPI (mean ± SD)		Cytostasis (%)		MN frequency (mean ± SD)	
		- S9	+ S9	- S9	+ S9	- S9	+ S9
DW	-	1.47 ± 0.07	1.59 ± 0.09	0.00	0.00	4.33 ± 0.67	3.67 ± 0.67
PS	1 mg/ml	1.41 ± 0.02	1.55 ± 0.02	12.77	5.18	4.00 ± 0.58	3.67 ± 0.88
	2.5 mg/ml	1.42 ± 0.01	1.44 ± 0.02	10.64	24.14	4.33 ± 1.33	4.33 ± 0.88
	5 mg/ml	1.40 ± 0.04	1.39 ± 0.07	14.89	32.76	4.67 ± 2.19	4.33 ± 0.67
	7.5 mg/ml	1.39 ± 0.01	1.24 ± 0.11*	17.02	58.63*	4.00 ± 0.58	6.0 ± 1.73
MMC	1 µg/ml	1.17 ± 0.01*	ND	63.83*	ND	121.33 ± 13.35*	ND
CP	10 µg/ml	ND	1.22 ± 0.02*	ND	62.07*	ND	72.0 ± 1.53*

Note: *Significant difference in comparison with negative control (distilled water: DW) at $p < 0.05$; PS: *P. sarmentosum* extract, CBPI: Cytokinesis – block proliferation index; MN frequency: Micronucleated cells per 1000 binucleated cells; MMC: Mitomycin c; CP: Cyclophosphamide; ND: not done; -S9: without S9 metabolic activation condition; +S9: with S9 metabolic activation condition

positive control groups showed significantly increased numbers of micronuclei when compared to the negative control ($p < 0.05$). This result is related to a prior finding, namely that the treatment with *P. sarmentosum* extract for various times at various doses did not increase the frequency of micronucleus formation in bone marrow cells of the rat (Puchadapirom *et al.*, 2004).

In addition, the impact of the water extract of *P. sarmentosum* on V79 cell proliferation from the micronucleus assay was also quantified to assess the cytotoxicity (OECD, 2014). In the assay, cytotoxicity is expressed by the Cytokinesis-Block Proliferation Index (CBPI), which is a ratio computed from three sub-populations of cells with different numbers of nuclei, and by percent cytostasis (Himakoun *et al.*, 2011). As shown in Table 2, the *P. sarmentosum* extract at the highest dose with S9 mixed conditions showed a cytotoxic effect on the treated cells, indicated by significantly decreased CBPI (1.24 ± 0.11) compared to the negative control (1.59 ± 0.09) and significantly increased percent cytostasis (58.63) compared to the negative control (0.00), at $p < 0.05$. Without S9, no *P. sarmentosum* treated group showed cytotoxic effects on the cells, whereas the positive control groups (MMC and CP treatments) showed statistical differences when compared to the negative control group. From the results, although the extract of *P. sarmentosum* at a high dose exhibited a cytotoxic effect on the V79 cells, at concentrations ranging from 1.0 to 7.5 µg/ml it did not cause micronucleus formation with or without S9 metabolic activation systems. Therefore, the water extract of *P. sarmentosum* had neither direct nor indirect genotoxic effects on the V79 cell line.

Our findings suggest that the metabolic products of *P. sarmentosum* water extract did not cause DNA or chromosome damage, as they did not exhibit mutagenic or genotoxic effects in two standard tests, the Ames test and the micronucleus assay. Therefore, the water extract of *P. sarmentosum* as a food supplement or oral drug might be safer than its application in other forms unaffected by liver metabolism, such as by external use in a skin cream, as the extract showed direct DNA damage in the Ames test. However, for safety of the compound in human use, consideration of concentration, method of application and exposure time are all important. Thus, the active compound of *P. sarmentosum*, its chemical structure, pharmacokinetic properties, and also longer periods of toxicity evaluation in animals should be further investigated.

4. Conclusions

The water extract of *P. sarmentosum* exhibited neither direct nor indirect genotoxic effects. A direct mutagenic effect was observed with high dose treatment, whereas the metabolite form of the extract did not exhibit mutagenic properties at any dose level tested. Thus, these results corroborate that *P. sarmentosum* is safe for application as a food dietary supplement or in an oral drug. However, for safety in human use, the concentration of the extract should be considered and further studies are needed over longer periods, including investigations in animal models, to confirm the findings.

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