Tropical Journal of Pharmaceutical Research April 2011; 10 (2): 153-159

Available online at http://www.tjpr.org

Research Article

Absence of Mutagenicity in Three Nigerian Medicinal Plants - *Bidens pilosa, Cleistopholis paterns* and *Tetrapleura tetraptera*

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Abstract

Purpose: *Bidens pilosa, Cleistopholis paterns, and Tetrapleura tetraptera are plants that are used traditionally for cancer treatment, as well as anti-bacterial, and anti-inflammatory agents in Africa. We used the Ames test to evaluate the bacterial reverse mutation effects in these plants.*
Methods: *In the in vitro Ames test, Salmonella typhimurium strains TA 98, TA 100, TA 1535, TA 1537 and Escherichia coli WP2uvrA were used with or without metabolic activation by S9 mix. The highest concentration of B. pilosa, C. paterns, and T. tetraptera for the Ames test was established at 5000 µg/plate.*
Results: *B. pilosa, C. paterns, and T. tetraptera did not cause any increase in the number of his$^+$ revertant colonies compared to the negative control values obtained from S. typhimurium and E. coli WP2uvrA strains, with or without the addition of S9 mixtures. The results indicated that these plants are non-mutagenic to all the five S. typhimurium and E. coli test strains in the presence and absence of metabolic activation.*
Conclusion: *B. pilosa, C. paterns, and T. tetraptera showed negative results in the bacterial reverse mutation test, suggesting that it is potentially safe to use them as medicinal plant supplements even at high doses.*

Keywords: *Bidens pilosa; Cleistopholis paterns; Tetrapleura tetraptera; Ames test*

Received: 27 September 2010 *Revised accepted: 11 February 2011*

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INTRODUCTION

Traditional medicine is practised by a large proportion of the Nigerian population for their physical and psychological health needs [1]. As Africa is comparatively far behind in the development and control of its medicinal plant industry, researchers are investigating several aspects required for the development of the medicinal plant trade in the region [2]. One of the basic criteria set by World Health Organization (WHO) for the use of herbs as medicines is that they should be shown to be non-toxic [3,4]. Bacterial reverse mutation assay (Ames test) was used in this work to evaluate the mutagenic potential of the methanolic extracts of one purposely selected and widely used plant (*Bidens pilosa*) and two other randomly selected plants (*Cleistopholis patens* and *Tetrapleura tetraptera*).

B. pilosa was selected due to its wide distribution and popularity in recipes for the management of various diseases. It is distributed in almost all tropical and subtropical countries and is used in a variety of ailments including diabetes [5], inflammation [6], and hepatitis [7]. In contrast to *B. pilosa*, there are very few scientific reports on the effects of *C. patens* and *T. tetraptera*. However, these two plants are commonly found in tropical Africa, including Nigeria, and are widely used as a folk medicine. In a previous study, the extract of *C. patens* was found to have significant antiplasmodial (antimalarial) activity [8]. Also, in Nigeria and some other West African countries, the nutritional, molluscidal, and other ethnomedical properties of extractives from *T. tetraptera*'s fruit have been reported [9,10].

The Ames test is a biological assay to assess the mutagenic potential of chemical compounds. A positive test indicates that the chemical might act as a carcinogen [11,12]. The test is used worldwide as an initial screen to determine the mutagenic potential of new chemicals and drugs because there is a high predictive value for rodent carcinogenicity when a mutagenic response is obtained [13,14]. We considered it necessary to undertake a study to determine whether *B. pilosa*, *C. patens*, and *T. tetraptera* show any mutagenic activity and could be safely used for potential pharmaceutical purposes.

EXPERIMENTAL

The Ames test was performed using the incorporation method of exposure in accordance to the work of Maron and Ames [15].

Sample preparation

B. pilosa (Abere oloko, Yoruba language, Nigeria), *C. paterns* (Apako, Yoruba language, Nigeria), and *T. tetraptera* (Aidan, Yoruba language, Nigeria) extracts were from the International Biological Material Center (IBMC, Korea). The plant materials were completely dried at room temperature for 5 days and were powdered. Each powdered material was extracted with 99.9 % methanol, filtered and dried under reduced pressure at 45 ℃. The stock was stored below -20 ℃ until used.

Test bacterial strains

The tester strains used were histidine-requiring *Salmonella typhimurium* TA 98, TA 100, TA 1535 (The Korea Collection for Type Cultures, KCTC, Korea), and TA 1537 (Moltox, Boone, NC, USA), and tryptophan-requiring *Escherichia coli* WP2*uvrA* (KCTC, Korea). The culture stocks were stored below -80 ℃. The tester strain was freshly prepared by pre-culturing for 12 h at 37 ℃ with agitation at 210 rpm in 2.5 % nutrient broth No. 2 (Oxoid, Hampshire, UK). Dimethyl sulfoxide (90 µl) (DMSO, Generay Biotech, Shanghai, China) was added to 1 ml of the dispersion of the test strain, and the resultant suspension was used for the assay.

Metabolic activation (S9 mix)

Rat liver S9 fraction was purchased from Wako Pure Chemicals (Japan). Cofactor mix was prepared by adding 9 ml of sterile distilled water to Cofactor-Ⅰ (Wako Pure Chemicals, Japan) and the cofactor solution was filtered. S9 mix (1 ml) contained 0.05 ml of S9 fraction, 0.05 ml distilled water, and 0.9 ml of cofactor mix that included 8 µmol $MgCl.6H_2O$, 33 µmol KCl, 5 µmol G-6-P, 4 µmol NADPH, 4 µmol NADH, and 100 µmol 0.1 M sodium phosphate buffer (PBS, pH 7.4).

Mutagenicity test

The mutagenicity test was performed by culturing the test samples with tester strains, with or without metabolic activation (S9 mix). *B. pilosa* was tested at the following concentrations 312, 1250, and 5000 µg/ml. *C. paterns* was used at 312, 2500, and 5000 µg/ml and *T. tetraptera* was used at 630, 2500, and 5000 µg/ml. These doses were determined after the toxicity test had been carried out. For the plate incorporation method, 2 ml of top agar with 0.5 mM histidine/biotin *(S. typhimurium)* or with 0.5 mM tryptophan *(E. coil* WP2*uvrA)* was added to 500 µl of PBS, 100 µl of the test solutions, and 100 µl of strains culture, which was then poured onto a plate of minimal glucose agar. The influence of metabolic activation was tested by adding 500 µl of S9 mix (5 %) in place of PBS. The positive controls for the cultures without S9 mix were 2-(2-furyl)-3-(5-nitro-2-furyl) – acrylamide (0.01 µg/plate) (AF-2, Wako, Japan) for TA 98 and *E. coli* WP2*uvrA* strains, sodium azide (5 µg/plate) (NaN_3, Sigma, St. Louis, MO, USA) for TA 100 and TA 1535 strains, and 9-aminoacridine hydrochloride (50 µg/plate) (9-AA, Sigma, USA) for TA 1537 strain. The positive control used with S9 mix was 2-aminoanthracene (5 µg/plate) (2-AA, sigma, USA) for all tester strains. PBS was used as the negative control. All plates in a given assay were incubated at 37 ℃ for 48 h and scored.

Data analysis

The results were expressed as the mean number of revertants per plate. A sample was considered mutagenic when it induced a 2-fold increase in the number of revertant colonies over negative control. A possible mutagenic potential was assumed if the quotient ranges between 1.7 and 1.9 in combination with a dose effect relationship. No mutagenic potential was assumed if all quotients ranged between 1.0 and 1.6.

RESULTS

In this study, the Ames test [15] along with *S. typhimurium* TA98, TA100, TA1535, TA1537, and *E. coli* WP2*uvr*A was used for the assessment of the mutagenic activity of *B. pilosa, C. paterns,* and *T. tetraptera* in the presence or absence of metabolic activation with S9 fraction. As shown in Table 1, toxicity of *B. pilosa, C. paterns,* and *T. tetraptera* to *S. typhimurium* was preliminarily investigated in tests carried out with TA100 by the plate incorporation method with or without addition of S9 fraction. The plants did not cause any decrease in the number of his^+ revertant colonies compared to the negative control values obtained from tester stains.

Therefore, we proceeded to the next stage which is assessing the mutagenicity using plate incorporation method [15]. The highest concentration of *B. pilosa, C. paterns,* and *T. tetraptera* for the Ames test was established at 5000 µg/plate, and a total of three concentrations were tested. As a result, *B. pilosa, C. paterns,* and *T. tetraptera* did not cause any increase in the number of his^+ revertant colonies compared to the negative control values obtained from *S. typhimurium* and *E. coli* WP2*uvr*A strains, with or without the addition of S9 mixtures (Tables 2 - 4). All the strains used in the study exhibited marked increase in the number of revertants when treated with positive controls. This

Table 1: Toxicity test of *B. pilosa*, *C. paterns*, and *T. tetraptera* extracts using *S. typhimurium* TA 100 with or without S-9 mix (mean ± SD, n = 3)

S-9mix	Dose (mg/ml)	Revertants/plate									
		5000	***2500***	***1250***	***630***	***315***	***160***	***80***	***40***	***0***	****PC***
Without	*B. pilosa*	90 ± 3	102 ± 2	116 ± 1	110 ± 2	104 ± 3	115 ± 4	109 ± 2	102 ± 3	83 ± 1	823 ± 8
	C. paterns	165 ± 3	131 ± 4	121 ± 5	135 ± 2	113 ± 3	107 ± 1	109 ± 3	100 ± 2	101 ± 3	845 ± 7
	T. tetraptera	157 ± 4	149 ± 2	153 ± 2	142 ± 4	132 ± 3	127 ± 1	128 ± 6	116 ± 5	106 ± 2	836 ± 9
With	*B. pilosa*	180 ± 1	160 ± 5	165 ± 4	154 ± 4	166 ± 3	173 ± 5	170 ± 1	172 ± 2	180 ± 3	904 ± 9
	C. paterns	197 ± 3	185 ± 3	168 ± 3	170 ± 2	167 ± 3	160 ± 1	159 ± 3	151 ± 3	172 ± 4	912 ± 8
	T. tetraptera	175 ± 2	173 ± 2	168 ± 3	171 ± 7	153 ± 6	162 ± 5	160 ± 3	148 ± 3	158 ± 4	875 ± 9

**Positive control (100 µl/plate): NaN_3 for TA 100/-S9 and 2-AA for TA 100/+S9.*

Table 2: Reverse mutation assay using *S. typhimurium* and *E. coli* WP2 treated with *B. pilosa* (mean ± SD, n = 3)

S-9 mix	Test substance	Dose (µg/plate)	Colonies/plate (mean ± S.D.)				
			TA 98	***TA 100***	***TA 1535***	***TA 1537***	***WP2 uvrA***
Without	*NC	100 (µl/plate)	15 ± 2	106 ± 4	9 ± 3	10 ± 2	21 ± 1
	B. pilosa	5000	10 ± 1	93 ± 5	9 ± 1	13 ± 2	20 ± 1
		1250	13 ± 2	114 ± 2	9 ± 1	5 ± 1	13 ± 1
		312	7 ± 1	104 ± 3	11 ± 1	5 ± 1	12 ± 1
	**PC		370 ± 7	890 ± 10	403 ± 10	320 ± 6	798 ± 8
With	*NC	100 (µl/plate)	15 ± 3	105 ± 4	112 ± 1	4 ± 1	13 ± 1
	B. pilosa.	5000	10 ± 2	140 ± 5	13 ± 1	4 ± 1	18 ± 2
		1250	13 ± 3	118 ± 6	13 ± 1	5 ± 1	19 ± 2
		312	7 ± 2	106 ± 3	11 ± 3	7 ± 1	17 ± 1
	**PC	5	312 ± 8	954 ± 15	432 ± 12	380 ± 9	834 ± 18

**The negative control was PBS; **the positive control (100 µl/plate) used during -S9 mix was AF-2 (0.01 µg/plate) for TA98 and WP2uvrA strains, NaN_3 (5 µg/plate) for TA100 and TA 1535 strains, and 9-AA (50 µg/plate) for TA1537 strain; during +S9 mix, 2-AA (5 µg/plate) was used for all tester strains.*

Table 3: Reverse mutation assay using *S. typhimurium* and *E. coli* WP2 treated with *C. paterns* (mean ± SD, n = 3)

S-9 mix	Test substance	Dose (µg/plate)	Colonies/plate (mean ± S.D.)				
			TA 98	***TA 100***	***TA 1535***	***TA 1537***	***WP2 uvrA***
Without	*NC	100 (µl/plate)	32 ± 3	134 ± 5	21 ± 3	6 ± 1	30 ± 1
	C. paterns	5000	19 ± 2	170 ± 5	13 ± 2	5 ± 2	20 ± 2
		2500	21 ± 1	142 ± 4	14 ± 1	7 ± 1	26 ± 3
		312	20 ± 2	131 ± 2	14 ± 2	10 ± 1	21 ± 4
	**PC		387 ± 10	915 ± 9	391 ± 5	379 ± 8	739 ± 9
With	*NC	100 (µl/plate)	29 ± 2	163 ± 6	13 ± 1	7 ± 1	25 ± 3
	C. paterns	5000	18 ± 1	205 ± 4	16 ± 1	4 ± 1	32 ± 4
		2500	18 ± 2	157 ± 3	16 ± 1	8 ± 2	23 ± 1
		312	17 ± 1	143 ± 6	12 ± 1	4 ± 1	25 ± 3
	**PC	5	311 ± 9	976 ± 11	420 ± 9	402 ± 10	814 ± 13

**The negative control was PBS; **the positive control (100 µl/plate) used during -S9 mix was AF-2 (0.01 µg/plate) for TA98 and WP2uvrA strains, NaN_3 (5 µg/plate) for TA100 and TA 1535 strains, and 9-AA (50 µg/plate) for TA1537 strain; during +S9 mix, 2-AA (5 µg/plate) was used for all tester strains.*

Table 4: Reverse mutation assay using *S. typhimurium* and *E. coli* WP2 treated with *T. tetraptera* (mean ± SD, n = 3)

S-9 mix	Test substance	Dose (µg/plate)	Colonies/plate (mean ± S.D.)				
			TA 98	***TA 100***	***TA 1535***	***TA 1537***	***WP2 uvrA***
Without	*NC	100 (µl/plate)	28 ± 2	185 ± 3	17 ± 2	5 ± 1	55 ± 1
	T. tetraptera	5000	25 ± 1	164 ± 5	16 ± 2	5 ± 1	50 ± 2
		2500	24 ± 2	169 ± 5	22 ± 1	4 ± 1	50 ± 2
		630	30 ± 3	149 ± 5	17 ± 2	7 ± 2	56 ± 3
	**PC		871 ± 14	819 ± 9	424 ± 11	406 ± 5	834 ± 6
With	*NC	100 (µl/plate)	19 ± 3	177 ± 6	15 ± 2	6 ± 2	60 ± 2
	T. tetraptera	5000	24 ± 3	170 ± 5	21 ± 1	5 ± 2	42 ± 2
		2500	24 ± 2	173 ± 4	19 ± 3	7 ± 1	35 ± 1
		630	24 ± 4	172 ± 5	17 ± 1	5 ± 1	47 ± 3
	**PC	5	501 ± 18	987 ± 10	450 ± 5	370 ± 3	751 ± 12

**The negative control was PBS; **the positive control (100 µl/plate) used during -S9 mix was AF-2 (0.01 µg/plate) for TA98 and WP2uvrA strains, NaN_3 (5 µg/plate) for the TA100 and TA 1535 strains, and 9-AA (50 µg/plate) for TA1537 strain; during +S9 mix, 2-AA (5 µg/plate) was used for all tester strains.*

confirmed the sensitivity of the tester strains to mutagens and thus the validity of the assay.

The results indicate that *B. pilosa, C. paterns,* and *T. tetraptera* were non-mutagenic to all the five *S. typhimurium* and *E. coli* test strains in the presence and absence of metabolic activation.

DISCUSSION

The use of traditional herbal remedies as alternative medicine play a significant role in Africa, since they form part of the culture and beliefs of the indigenous population and also feature significantly in primary healthcare. However, objective evidence that these remedies are effective and not harmful is lacking [16,17]. Little information is available on the toxicological properties of African herbal preparations. In this study, we evaluated the mutagenic activities of *B. pilosa*, *C. patens*, and *T. tetraptera* to determine whether these plants can be safely used for potential pharmaceutical purposes. *B. pilosa* is an annual tropical plant used as a folk medicine for various therapies and as a popular ingredient in herb teas in China. Despite its popularity and wide application, there are no available genotoxicity data on this plant. Methanol extracts of *C. patens* was found to have *in vitro* activity, at less than 25 µg ml^{-1}, against *Pleiocarpa mutica*, which is the commonest species in the highly malaria endemic areas of Africa [8]. However, many plant preparations including *C. patens* are used in Africa to treat fevers/malaria without any scientific evidence of efficacy or toxicity [8]. *T. tetraptera* (known in Yoruba as Aridan) is the most studied of all plant molluscicides, but lacks much of the required toxicological data [18]. The high cost of synthetic molluscicides and drugs puts them beyond the reach of the majority of the people in developing countries who need them [19]. A cheap and efficient way to solve this problem is to use potent plant molluscicides including *T. tetraptera*, which can be collected freely from the tropical forests of West Africa and Nigeria. This study should aid the investigation of the underlying mechanisms of *B. pilosa*, *C. paterns*, and *T. tetraptera* by providing the toxicological data of these plants.

The Ames test is a widely accepted method for identifying various chemicals and drug that can cause gene mutations [14], and has a high predictive value for *in vitro* carcinogenicity. Therefore in this study,

incorporation Ames test using *S. typhimurium* TA98, TA100, TA1535, TA1537, and *E. coli* WP2*uvr*A was employed to assess the mutagenic activity of *B. pilosa, C. paterns,* and *T. tetraptera* in the presence or absence of metabolic activation with S9 fraction. The results obtained show that *B. pilosa, C. paterns,* and *T. tetraptera* are non-mutagenic up to the dose of 5000 µg/plate both in the presence and absence of S9. Since not all herbal products yield negative results for mutagenicity [20], it is encouraging to find that these three commonly used plants in Nigeria are not mutagenic.

CONCLUSION

The results indicate that *B. pilosa, C. paterns*, and *T. tetraptera* are not mutagenic. This implies that in the absence or presence of S9 fractions, these plants can protect the bacterial DNA from attacks by mutagens. Thus, *B. pilosa, C. paterns,* and *T.* tetraptera showed negative results in the bacterial reverse mutation test, suggesting that it is potentially safe to use them as medicinal plant supplements, even at high doses.

AUTHORSHIP STATEMENT

All the authors ***contributed equally*** to this project and should be considered co-first authors

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