Screening of the Desmutagenic Capacity of an Extract from Crude Drug by Spore Rec-Assay

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(Received August 25, 1999; Accepted October 20, 1999)

We investigated the desmutagenic action of extracts from Smilax rhizome, Mulberry bark and Berchemia racemosa on Trp-P-1 and Trp-P-2 by means of spore rec-assay. The results obtained are as follows: (1) 50% ethanol and ethanol extracts from each crude drug showed desmutagenic action on Trp-P-1 and Trp-P-2. (2) Most of the desmutagenic activity in 50% ethanol extract from each crude drug was due to an ethyl acetate soluble fraction. (3) The interaction between the ethyl acetate soluble fraction from each crude drug and Trp-P-1 was much stronger in Mulberry bark and Berchemia racemosa than in Smilax rhizome.

Key words —— spore rec-assay, desmutagenic action, extract from crude drug, promutagen, folk medicine

INTRODUCTION

The *Bacillus subtilis* spore rec-assay, with and without an *in vitro* metabolic activation system,[1] is able to detect DNA-damaging substances with high sensitivity and precision, and this assay procedure is more simple and rapid than Ames salmonella test.[2] Consequently, it is very efficient to use this system to screen modification factors which suppress or inactivate the DNA-damaging activities of mutagens and carcinogens which give positive results in the rec-assay. However, the spore rec-assay has rarely been employed to search for such factors except for a report on the desmutagenic action of foodstuff extracts against Trp-P-1 and mutagenic products generated by sorbic acid with sodium nitrite.[3]

We therefore tried to utilize the spore rec-assay as a screening test for naturally occurring desmutagens. The samples for screening desmutagen were chosen from crude drugs used in folk medicine in Japan, as there are few studies on the antimutagenic effects of such a sample. In this paper, we report the desmutagenic action of extracts from Smilax rhizome (Tuber of *Smilax glabra* ROXBURGH), Mulberry bark (Cortices of *Morus alba* LINNE) and Berchemia racemosa (Stem of *Berchemia racemosa* SIEB. *et* ZUCC.) on the promutagens Trp-P-1 or Trp-P-2.

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MATERIALS AND METHODS

Materials —— Trp-P-1 and Trp-P-2, used as promutagen and hemin, were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Rat liver S9, aroclor 1254-induced, was obtained from ICN Pharmaceuticals, Inc. (Aurora, OH, U.S.A.). NADP monosodium salt and glucose 6-phosphate disodium salt used as cofactor were obtained from CALZYME Laboratories, Inc. (San Luis Obispo, CA, U.S.A.) and Oriental Yeast Co., Ltd. (Japan), respectively. Other reagents and solvents used were of the purest grade commercially available.

Sample —— Smilax rhizome and Berchemia racemosa were purchased from Kinokuniya Pharmacy (Tokyo), and Mulberry bark was obtained from Akira Shiino Co., Ltd. (Tokyo).

Preparation of Extract from Crude Drug —— The crude drug components were extracted with water,[4] 50% ethanol[5,6] and ethanol.[5] The water extract was prepared as follows. About 50 g of commercial crude drug was refluxed with 500 ml water for 60 min. After cooling to room temperature, the content was filtered by suction. The residue was similarly refluxed and filtrated. The filtrates were combined, and the solution was concentrated under reduced pressure at below 50℃ using a rotary evaporator until all the water had evaporated. The residue obtained was used

as a water extract. The 50% ethanol and ethanol extracts were prepared in the same manner as the water extract. Furthermore, 50% ethanol extract was added to 200 ml of water and stirred. This aqueous solution was partitioned with 200 ml of ethyl acetate two times (extraction time, 60 min). The ethyl acetate phase and water phase were evaporated under reduced pressure and the resulting extracts were used as ethyl acetate soluble and insoluble fractions, respectively.

Desmutagenic Assay —— A dimethyl sulfoxide (DMSO) solution of Trp-P-1 (0.4 mg/ml) or Trp-P-2 (1.6 mg/ml) and that of the test sample (40—400 mg/ml) were mixed in equal quantities, and a 25 μl portion was used for the assay. The DNA-damaging activities of promutagens before and after the addition of test sample were measured by spore rec-assay with metabolic activation, and their desmutagenic capacities were estimated. The DNA-damaging activity was shown as the difference between two lengths of growth inhibition zones for H17Rec$^+$ and M45Rec$^-$ strains. The length of the inhibition zone was measured by slide calipers and was calculated using the following equation.

Inhibition zone (mm)$=(a-b)/2$

a : Diameter of inhibition zone of test sample

b : Diameter of paper disk (9 mm)

The experiments were repeated three times for each case, and the mean value was calculated.

Interaction between Ethyl Acetate Soluble Fraction and Trp-P-1 —— One ml of aqueous solution of Trp-P-1 (0.2 mg/ml) was put in a stoppered test tube, and 2 ml of propyl acetate solution of ethyl acetate soluble fraction (100 mg/ml) was added and mixed well. After the mixture was shaken for 15 min and centrifuged at 3000 rpm for 10 min, the propyl acetate layer was separated. Two ml of propyl acetate was added to the remaining aqueous layer and the above procedure was repeated. The combined propyl acetate solution was evaporated under reduced pressure at below 50℃ and the residue obtained was dissolved in 1 ml DMSO. The DNA-damaging activity induced by Trp-P-1 in the aqueous layer and organic layer were measured in the spore rec-assay with metabolic activation.

RESULTS AND DISCUSSION

Yield and DNA-Damaging Activity of Extract from Crude Drug

The yield of each crude drug extract is shown in Table 1. A higher yield is obtained in the order of water extract, 50% ethanol extract, ethanol extract.

The results of spore rec-assay with and without metabolic activation on the extracts from the crude drug are also shown in Table 1. The killing effects towards M45Rec$^-$ and H17Rec$^+$ strains are observed in the 50% ethanol extract from Mulberry bark and ethanol extracts from Smilax rhizome, Mulberry bark and Berchemia racemosa in the assay without metabolic activation. However, these extracts were not judged positive in the spore rec-assay, because the value subtracted the length of the inhibition zone for H17 from that for M45 is less than 2 mm.[7] In the case of the assay with metabolic activation, all extracts gave clearly negative results. These results indicate that none of the extracts from crude drug possess DNA-damaging activity.

The Desmutagenic Action of Extracts from Crude Drug

At first, to validate the method, a preliminary experiment was performed using hemin[8] as the standard antimutagen and Trp-P-1 as the promutagen. As a result, hemin inhibited the DNA-damaging activity of Trp-P-1 with a dose-response relationship, which agreed with the report by the Ames Salmonella test.[9]

Next, the desmutagenic action of each crude drug extract on the promutagens Trp-P-1 (5 μg/disk) and Trp-P-2 (20 μg/disk) was investigated at the dose of 0.5—5 mg/disk. As shown in Fig. 1—3, the water extracts from each crude drug did not exhibit much inhibitory effect against Trp-P-1 and Trp-P-2, even at the maximum dose of 5 mg/disk. In contrast, 50% ethanol and ethanol extracts from each crude drug showed obvious desmutagenic activity against these promutagens. That is, Smilax rhizome completely inhibited the DNA damagenicities of Trp-P-1 and Trp-P-2 at doses of 1 and 5 mg/disk, respectively (Fig. 1). The inhibitory effect of Mulberry bark was similar to that observed for Smilax rhizome (Fig. 2). Berchemia racemosa also suppressed the DNA-damaging activities induced by these promutagens at the dose of 5 mg/disk (Fig. 3).

Although antimutagens in crude drugs have been investigated in recent years,[10,11] there are no reports so far on the desmutagenic action of Smilax rhizome or Berchemia racemosa. As for

Table 1. Results of Spore Rec-Assay with and without Metabolic Activation on Extract from Crude Drug

Sample	Dose (mg/disk)	Inhibition zone (mm)				DNA-damaging activity[c]
		−S9[a]		+S9[b]		
		H17	M45	H17	M45	
Smilax rhizome						
H_2O ext. (28.0%)[d]	5.0	0.0	0.0	0.0	0.0	−
50%EtOH ext. (14.9%)	5.0	0.0	0.0	0.0	0.0	−
EtOH ext. (4.1%)	5.0	2.9	4.0	0.0	0.0	−
Mulberry bark						
H_2O ext. (16.0%)	5.0	0.0	0.0	0.0	0.0	−
50%EtOH ext. (14.0%)	5.0	3.0	3.8	0.0	0.0	−
EtOH ext. (5.7%)	5.0	4.5	5.6	0.0	0.0	−
Berchemia racemosa						
H_2O ext. (22.0%)	5.0	0.0	0.0	0.0	0.0	−
50%EtOH ext. (20.5%)	5.0	0.0	0.0	0.0	0.0	−
EtOH ext. (7.9%)	5.0	0.0	1.3	0.0	0.0	−
	Dose (μg/disk)					
Positive control						
Mitomycin C	0.1	6.2	15.5	−	−	+
Trp-P-1	5.0	−	−	0.0	6.0	+
Negative control						
Kanamycin	50.0	10.5	11.0	−	−	−

a) Without metabolic activation. *b*) With metabolic activation. *c*) Difference in inhibition zone between M45 and H17 : − ; Less than 2 mm, + ; more than 2 mm. *d*) The numbers in parentheses represent the yield of extract from crude drug.

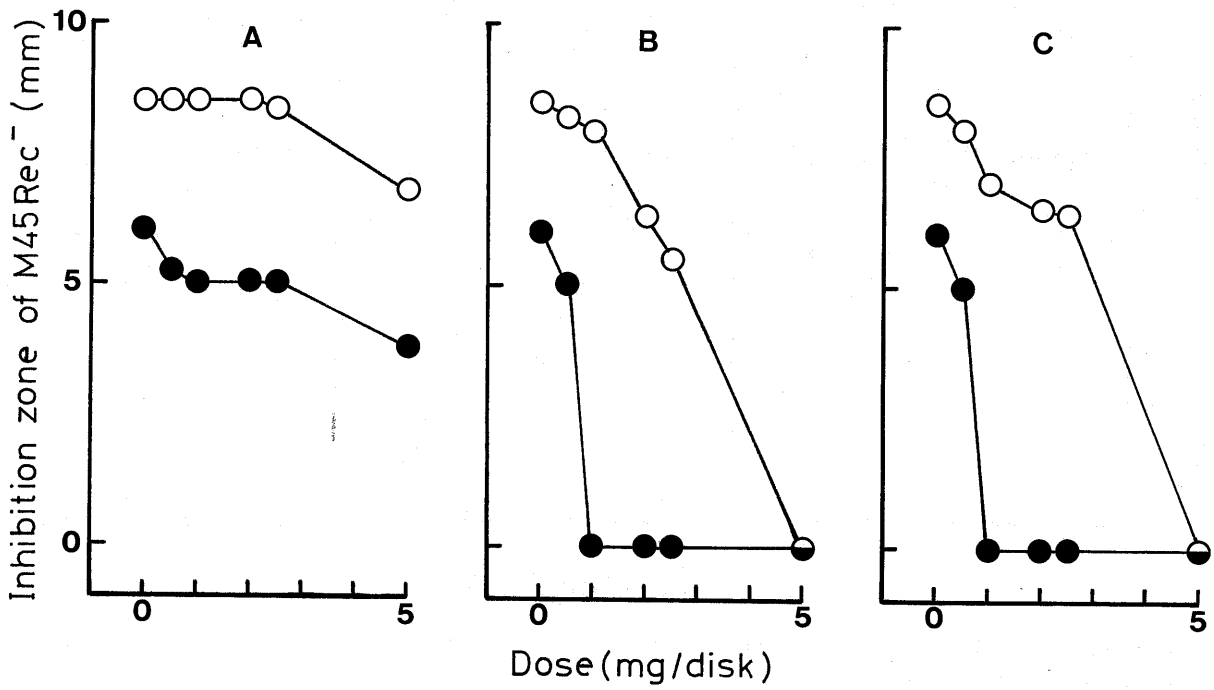

Fig. 1. Desmutagenic Activities of Water Extract (A), 50% Ethanol Extract (B) and Ethanol Extract (C) from Smilax Rhizome on the Promutagen

—●—, Trp-P-1; —○—, Trp-P-2. The inhibition zone of H17Rec$^+$ strain did not appear on the plates of sample and promutagen.

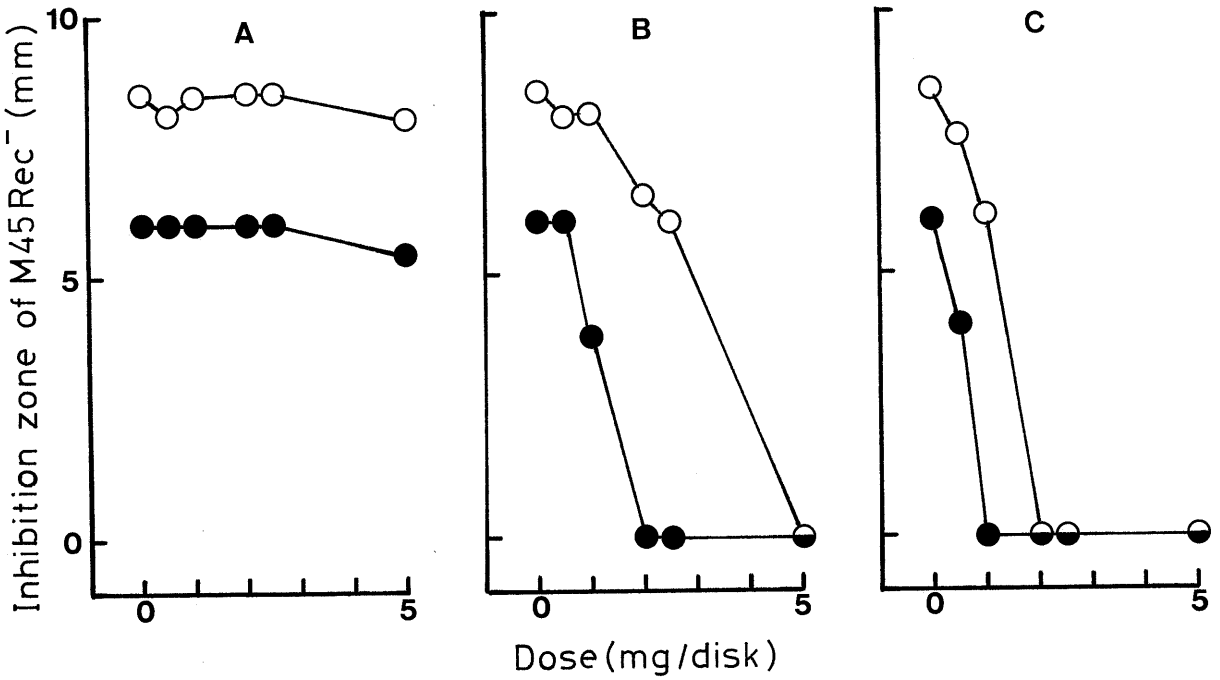

Fig. 2. Desmutagenic Activities of Water Extract (A), 50% Ethanol Extract (B) and Ethanol Extract (C) from Mulberry Bark on the Promutagen
Symbols and other details correspond to Fig. 1

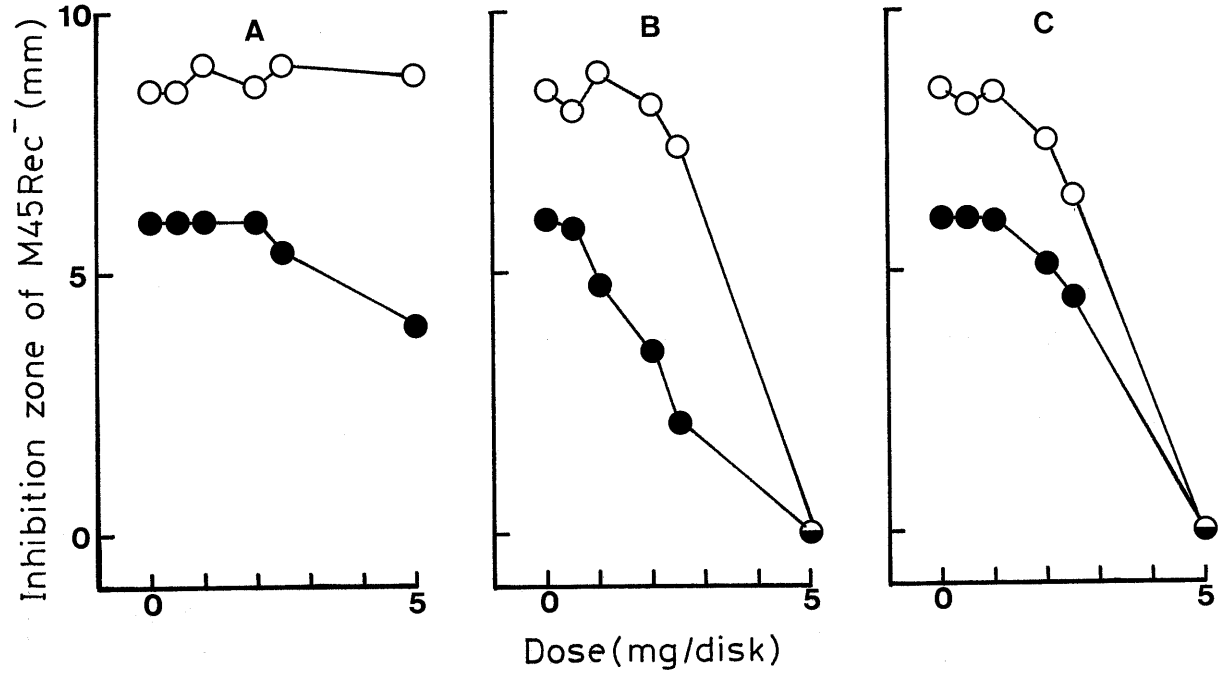

Fig. 3. Desmutagenic Activities of Water Extract (A), 50% Ethanol Extract (B) and Ethanol Extract (C) from Berchemia Racemosa on the Promutagen
Symbols and other details correspond to Fig. 1.

Table 2. Desmutagenic Action of 50% EtOH Extract from Each Crude Drug on Trp-P-1 and Trp-P-2

Sample	Dose (mg/disk)	Added Trp-P-1 (μg/disk)	Added Trp-P-2 (μg/disk)	Inhibition zone (mm)	
				H17	M45
Smilax rhizome					
EA sol. (6.2%)[a]	5.0	0.0	0.0	0.5	1.5
	5.0	5.0	0.0	0.5	1.5
	5.0	0.0	20.0	0.5	1.6
EA insol.	5.0	0.0	0.0	0.0	0.0
	5.0	5.0	0.0	0.0	4.7
	5.0	0.0	20.0	0.0	6.7
Mulberry bark					
EA sol. (9.8%)	5.0	0.0	0.0	1.0	1.7
	5.0	5.0	0.0	1.4	2.1
	5.0	0.0	20.0	1.2	1.9
EA insol.	5.0	0.0	0.0	0.0	0.0
	5.0	5.0	0.0	0.0	6.5
	5.0	0.0	20.0	0.0	8.8
Berchemia racemosa					
EA sol. (11.0%)	5.0	0.0	0.0	0.5	1.1
	5.0	5.0	0.0	0.5	1.2
	5.0	0.0	20.0	0.5	1.2
EA insol.	5.0	0.0	0.0	0.0	0.0
	5.0	5.0	0.0	0.0	5.3
	5.0	0.0	20.0	0.0	7.9
Trp-P-1	—	5.0	—	0.0	6.0
Trp-P-2	—	—	20.0	0.0	8.5

a) The numbers in parentheses represent the yield from 50% ethanol extract.

the 50% ethanol extract from Mulberry bark, its desmutagenic effect against *tert*-butyl hydroperoxide was observed by monitoring its suppressive effect on the SOS-function-inducing activity in *Escherichia coli* PQ 37 strain.[6]

The 50% ethanol extracts from each crude drug, which showed desmutagenic activity against Trp-P-1 and Trp-P-2, were fractionated by ethyl acetate to estimate the properties of the active components. The DNA-damaging activities of ethyl acetate soluble and insoluble fractions are shown in Table 2. Desmutagenic activities were observed in the ethyl acetate soluble fraction. From these results, it is presumed that active factors were soluble in ethyl acetate, but only slightly soluble in water.

Then, the interaction between the ethyl acetate soluble fraction from each crude drug and Trp-P-1 was studied. These experiments were carried out using the same doses as in Table 2. The solvent for the dissolution of the ethyl acetate soluble fraction used propyl acetate instead of ethyl acetate because some promutagens pass over into the ethyl acetate phase when an aqueous solution of Trp-P-1 is shaken with ethyl acetate. After the aqueous solution of Trp-P-1 and the propyl acetate solution of the ethyl acetate soluble fraction were mixed and shaken for 15 min, aqueous and organic layers were separated by centrifugation, and the DNA-damaging activities induced by the promutagen in these layers were measured. The results are shown in Table 3. If the interaction between Trp-P-1 and the ethyl acetate soluble fraction is strong, the DNA damagenicity of the promutagen ought not to be observed in either the aqueous or organic layers. In the case of Smilax rhizome, the aqueous layer showed almost no recognizable decrease in DNA-damaging activity induced by Trp-P-1. On the other hand, Mulberry bark and Berchemia racemosa exhibited no DNA-damaging activity of the promutagen in the aqueous layer. Therefore, it is considered that the interaction between the ethyl acetate soluble fraction from each crude drug and Trp-P-1 was much stronger in Mulberry bark and Berchemia racemosa than in Smilax rhizome.

In the present study, we demonstrated the

Table 3. Interaction between Ethyl Acetate Soluble Fraction from Each Crude Drug and Trp-P-1

Sample	Dose (mg/disk)	Added Trp-P-1 (μg/disk)	Inhibition zone (mm)			
			Aqueous layer		Organic layer	
			H17	M45	H17	M45
Smilax rhizome	5.0	5.0	0.0	6.0	0.7	1.8
Mulberry bark	5.0	5.0	0.0	0.0	1.0	1.5
Berchemia racemosa	5.0	5.0	0.0	0.0	0.7	1.3
Trp-P-1	—	5.0	0.0	6.0	0.0	0.0

Experimental procedures are described in the text.
Inhibition zone of H17 and M45 in organic layers of Smilax rhizome, Mulberry bark and Berchemia racemosa originated from test samples themselves (see Table 2).

desmutagenic action of extracts from Smilax rhizome, Mulberry bark and Berchemia racemosa, used as folk medicine in Japan, on Trp-P-1 and Trp-P-2 by spore rec-assay. While this method is a simple, inexpensive and efficient procedure for screening environmental DNA-damaging and mutagenic chemicals,[12] it is also convenient as a primary screening test to determine naturally occurring desmutagens.

Acknowledgements We thank Dr. M. Doi, director, Ibaraki Prefectural Hygienic Laboratory, for his useful advice.

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