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Anti-Mutagenic Activity of *Phyllanthus amarus* Schum & Thonn In Vitro as Well as In Vivo

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Methanolic extract of *Phyllanthus amarus* was tested for its anti-mutagenic activity in *Salmonella typhimurium* strains TA1535, TA100, and TA 102 (Ames test). *P. amarus* extract was able to inhibit the activation of 2-acetaminofluorene (2-AAF) and aflatoxin B_1 at concentrations [illegible] per plate. It was also found to inhibit mutagenicity induced by direct acting mutagens sodium azide (NaN_3), N-methyl-N-nitro-N-nitrosoguanidine (MNNG), 4-nitro-O-phenylenediamine (NPD), at concentrations [illegible] mg to 0.25 mg. Urinary mutagenicity produced in rats by benzo[a]pyrene was inhibited by the oral administration of *Phyllanthus* extract. These results indicate significant anti-mutagenicity of the extract in vitro as well as in vivo. *Teratogenesis Carcinogenesis and Mutagenesis* 22:285–291, 2002. © 2002 Wiley-Liss, Inc.

Key words: *Phyllanthus amarus*; mutagenesis; anti-mutagenesis; anti-carcinogen; urinary polyaromatic hydrocarbon; aflatoxin B_1

INTRODUCTION

Phyllanthus amarus (syn. *Phyllanthus niruri*, Euphorbiaceae) is an important plant in the treatment of liver disorders and jaundice in traditional Indian medicine. It has been effective against infective hepatitis [1]. It is also used in many stomach disorders and diseases of the genitourinary system. The extracts were found to have hypoglycemic, antifungal and anticancer activity. The liver protective effect of *Phyllanthus* has been demonstrated in chemical liver toxicity models [3]. Simultaneous administration of *P. amarus* extract has been reported to inhibit the hepatocellular carcinoma development induced by N-nitrosodiethylamine [4]. *P. amarus* administration was also found to increase the life span of rats with hepatocellular carcinoma [5]. We showed recently that

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P. amarus extract has potent free radical scavenging activity and can scavenge superoxides and hydroxyl radicals and could inhibit lipid peroxides [6]. A variety of hydrolysable tannins purified from *amarus* were found to be potent inhibitors of rat liver cyclic AMP-dependent protein kinases. These hydrolisable tannin inhibitors are the most specific and potent plant derived inhibitors of cyclic AMP-dependent protein kinase catalytic subunit yet found (IC 50 0.2 1.7 IM) [7]. Phyllanthin.

Title of *P. (unarms.* exhibited anti-genotox propertie N. In the present study, :rive determined the antimutagenic activity of *P. mums* in vitro and in vivo usine a number of *Salmonella typhymurium* strains.

MA :ERIAL AND METHODS

:went broth, L. histidine. and biotin were purchased from (Bombay, India). 2-Acetaamidoflourine (2AAF). 4-nitro-0-phenylene (kaoline (NPD), N-methyl-N-nitro-nitrosoguanidine (MNNG), aflatoxin B_1 and benzo[*a*] pyrene were procured from Sigma Chemical Co. (St. M(.)1 All mutagens were non-toxic to .1. *typhimurium* at the concentration used study as seen from the background lawns [^{9}]. glucose-6-phosphate. dimethyl sulphoxide (DMSO), and sodium azide (NaN_3) were obtained from Research Laboratories. VI other ts used were of analytical

Bacterial Strains

S. typhimurium strains TA100, TA102, and were originally procured from Professor 13.N. Ames. I niversity of 1: alifornia. Berkeley, CA. and after in nutrient broth were m ials and frozen in presence of dimethyl sulphoxide (9%) aro: k 70 culture was inoculated iii Iresh broth and grown for 37 before each experiment.

Extraction of *P. amarus*

The stem and leaves of *P. amarus* from the Thrissur district of amid d ied it 50 of the plant was identified and H hero kept at Anala center. Dried part of *P. amarus wet'* pa powder twice in 5 vol of methanol by stirring ov ro m temperature centrifuged. The natant w evapor ed u reduced pressure rota evaporator. The ield of Initially we tested Me ant icrobial activity of the extract fo *typhimurium* separately and have used the of concentration at P. *amarus* extract. which produce inn. tion of bacterial growl h our

:mination of In vitro Anti-Mutagenicity

experiments were pet formed accordine to the method Markin mind Aries [9]. 1. *typhymurium* strains (TA153, TA100, and TA102) were used far assessing the anti mutagenic activit *P. amarus* extract.

Male Wistar rats (150-180g) were sodium phenobarbitol (0.1 %) in drinking water for 4 days to induce liver microsomal [I' Animals were

killed on the 5th day, livers were removed aseptically, and microsomal fractions from S9 were prepared [9]. The protein content of the S9 fraction was 40±2 mg/ml as determined by the method of Lowry et al. [11]. 2-AAF (50 µg/plate) and aflatoxin-B_1 (5 µg) were pre-incubated with sodium phosphate buffer (0.2 M, pH 7.4, 200 µl), NADP (0.1 M 25 µl), $MgCl_2$-KCl- (1.65 M KCl + 0.4 M $MgCl_2$, 10 µl), glucose-6-phosphate (1 M. 25 µl), S9 (200 µl), and the bacteria TA1535 (100 µl approximately 10^8 cells) for min at *31'C* in the pi esence of various concentrations of P. *amarus* extract. mixture was plated on previously prepared minimal agar plates and incubated, :it 37°C for 48 h, after which the number of revertants was counted. The percentage inhibition of mutagenicity was calculated using the formula x 100/(R_1–SR), where R_1 is the number of revertants in the presence of mutagen alone, R2 is -the number of revertants in the presence mutagen and drug, and SR is the spout: nexus revertants.

1'. amarus extract was also Jested for its inhibitory activity or direct acting mutagens MNNG (1 µg/plate), NPDA (20 µg/plate), and sodium azide (2.5 µg/plate) induced revertant formation using *Salmonella* tester strains TA1535, TA100, and 1A102 (approximately 10^8 cells) by the method of Maron and Ames [9].

Determination of Urinary Mutagenicity in Rats

Male Wistar rats were divided into three groups (six rats/group). The group I animals were given distilled water through oral intubation, which served as control. Group 11 animals were given benzo[*a*]pyrene (10 mg/rat) by i.p injection as a single dose. Group III were given *1'. amarus* extract (500 mg/kg bwt) for 12 day's. On the 13th day benzo[*a*]pyrene (10 mg/rat) in sun flower oil was administered by i.p. injection as single dose. All the rats were put into metabolic cages and urine samples were collected for 24 h after injection. The samples were frozen immediately and kept at −70°C until analysis.

The urine samples were filtered through Whatman number 1 filter paper and was passed through an XAD-2-Amberlite column to concentrate the mutagens [12]. Twenty milliliters of urine that was collected from a rat was loaded onto an XAD column (40 m^2 x 10 m^{-}). The adsorbed components were eluted with 10 ml acetone. --The -eluants were evaporated to dryness at 60°C and stored at reconstituted in 1.5 ml diemethyl sulphoxide just before they were used for the mutagenicity assay. To check the mutagenicity 0.1 ml bacteria and 0.1 ml column eluvate were mixed with 2 ml top agar containing traces of histidine and biotin plated 'm minimal agar medium. The revertants were counted after incubation in the dark at 37'C for 48 it.

RESULTS

Anti-mutagenicity of *P. amarus* Extract Using Direct Acting Mutagens

Sodium azide (NaN_3). *Phyllanthus* extract was found to reduce the number of revertants produced by sodium azide (2.5 µg/plate) to *S. typhymurium* strains TA 1535, TA100, and TA102. Antimutagenicity was found to be concentration dependent and. 50% inhibition was produced at a concentration of 1-2 mg/plate (Table 1).

TABLE 1. Anti-Mutagenicity of *Phyllanthus amarus* Extract to *Salmonella typhimurim* Strains Against Sodium Azide as Mutagen

Concentration of the extract(mg/plate)	Average number. of revertants/plate Sodium Azide(2.5µg/plate) TA1535	TA100	TA102
Nil	1039.3±85.6	535.5+40.3	667.5±32:9
	571.7±49.2*	349.0+15.1*	
0.5	693.3+34.6**	403.0+11..4	524.0± 193"
0.25	824.7±67.6	494.5±24.7	614.0±20.5
Spontaneous revertants	25.33±3.1	150.5+15.	273.5±18.9

*$P<0.001$.
$P<0.005$.

TABLE **II. Anti-Mutagenicity of *Phyllanthus amarus* Extract to *Salmonella typhimurim*** Strains **Against N-methyl-N-Nitro-Nitrosoguanidine as Mutagen**

Concentration of the extract(mg/plate)	Average number of revertants per plate MNNG (1 µg plate) TA1535	TA 100	TA102
Nil	495.0+21.2	487.5+17.7	566.0+28.3
1	297.0±16.9*	367.5±26.1**	412.0+22.9**
U.5	358.0± 18.4*	409.04 12.7*	497.5±18.5
0.25	416.5± 12.0**	442.0419.1	538.3±14.8
Spontaneous revertants	22.6±3.0	173.54 24.S	292.5+26.0

•P<0.001.
$P<0.005$.

N-methyl-N-nitro-N-nitrosoguanidine (MNNG). *P. amarus* extract was also found to inhibit mutagenicity produced by MNNG (I µg/plate) in a dose dependent manner in different *Salmonella* strains described earlier. Concentration needed for 50% inhibition was found to be 1–2 mg/plate, which was similar to that of sodium azide (Table

4-nitro-0-phenylenediamine (4-NPD). *P. amarus* extras was found to significantly inhibit the mutagenicity induced by NPD (20 µg/plate) to *Salmonella* strains. Concentration needed for 50% inhibition was similar in the other cases which was between I and 2 mg/plate (Table 111).

Anti-Mutagenicity *of P. amarus* Extract Using Mutagens Which Needed Activation

Mutagenicity produced by 2-AAF and AFB_1 after their activation using S9 fractions was found to be significantly inhibited by *P. amarus* extract. The concentration needed for 50% inhibition of mutagenicity to *Su/mond/a* strain 1535 was found to be only 2 mg/plate in the case of 2-AAF and between 2 and 3 mg/plate in the case of AFB_1 (Table IV).

TABLE III. Anti-Mutagenicity of *Phyllanthus amarus* Extract to *Salmonella typhimurim* Strains .Against 4-Nitro-o-PhenyleneDiamine as Mutagen

	Average number of revertants per plate NPD (20 µg/plate)		
Concentration of the extract(mg/plate)	TA1535	TA100	TA 102
Nil	392.5 + 10.6	528.6± 17.6	635.0 ± 28.9
	196.0 + 15.7*	304.0 ± 15.0*	416.5 ± 24.7*
0.5	253.0 + 23.3*	358.6 ± 22.5	53.1 0 ± 19.8*
0.25	292.0 +12.8	47[illegible].6 + 14.2	590.0 · 13.2
Spontaneous revertants	26.3+7.2	167.6± 17.2	305.5+13.1

*P<0.001.

TABLE IV. Anti Mutagenicity of Extracts of *P. amarus* to *Salmonella typhinurium* TA1535 against 2-Acetamidofluorene(2-AAF) and Aflatoxin B_1 as Mutagens

	Average number of revertants per plate	
Concentration of extract(mg plate)	2-AAF (50 µg/plate)	Aflatoxin (5 µg plate)
Nil	376.0 ± 12.7	261.1±13.5
	190.0 + 14.5*	168.0 ± 12.5*
1	219.5+ 17.8'	181.6+20.4
0	254.0 ± 8.5**	205.3 118.5
	308.5 ± 16.3	223.0 ± 17.0
Spontaneous revertants	3 3.6 ± 7.7	37.0111.1

AAF and AFB_1 were activated with S3 fraction containing NADP and Mg^{++}.
:0.001.
•P 0.005.

These results indicated that *P. amarus* extract could inhibit the mutagenicity induced by direct acting mutagens and mutagens that needed activation in several *S. typhimurium* strains at low concentrations.

.affect of *P. amarus* Extract on Urinary Mutagenicity Induced by Benzo[a]pyrene in Rats

P. amarus extract was found to inhibit the urinary mutagenicity induced by benzo[*a*]pyrene in rats. In this experiment, urine collected from animals treated with benzo[*a*]pyrene in presence and absence of *P. amarus* extract was tested for mutagenicity three different *Salmonella* strains. Urine from benzo[*a*]pyrene-treated rats was found to exhibit high mutagenic potential as seen from the increase in number of revertants. Administration of *P. amarus* extract was found to inhibit urinary mutagenicity significantly, which was 46% in the case of TA1535, 73% in the case of TA100, and 100% in the case of TA102 (Table V).

TABLE V. The Effect of Extract of *Phyllanthus amarus* on Urinary Mutagenicity Induced by Benzol[*a*]pyrene in Rate

Group NO	Treatment	Average number of revertants per plate		
		TA1535	TA100	TA102
I	•Normal untreated	56.0 + 11.3	164.4±14.5	187.5±9.2
II	Control B[*a*]P	336.7 + 45.1	298.3 + 12.6	392.3 + 29.3
III	Drug 500 mg + B[*a*]P	193.7 + 7.1***	198.3 + 10.4*	247.7 ± 24.4**
	Spontaneous revertants	23.9±2.8	162.6±20.5	283.5+21.7

'Values are without reducing spontaneous revertants.
*P <0.001.
¯P<0.005.

DISCUSSION

In the present study. we have evaluated the antimutagenicity of *P. amarus* extract that is being used in hepatic diseases including acute viral hepatitis [13]. Studies on the anti-mutagenic activity of the extract indicated that the extract inhibited the mutagenicity produced by direct acting mutagens such as NaN_3, MNNG, and 4-NI D and also inhibited the activation of the mutagens such as 2-AAF and AFB_1 by rat liver S9 fraction and thereby inhibited the mutagenesis and possibly carcinogenic potential. Inhibition of mutagenicity of 2-AAF and AFB_1 may be mainly due to inhibition of their activation mediated by P-450 enzymes. Activated metabolites form adducts with DNA and produces mutagenesis and carcinogenesis. In fact we have seen that *P. amarus* extract could inhibit the activation of P-450 enzyme in vitro. *P. amarus* extract also decreased the levels of benzo[*a*]pyrene-induced mutagenesis in urine. This may be due to the inhibition of Phase I enzymes by the extract.

P. amarus is extensively is used in viral hepatitis. Unander et al. have shown that extract could inhibit the viral polymerase [14] and down-regulate hepatitis p virus mRNA transcription and replication [15]. The *P. =ants* extract has been shown to reverse the chromosomal alteration induced by genotoxic agents [8]. *P. amarus* extract has been reported to possess anti oxidant activities [6] and inhibited aniline hydroxylase a P-450 enzyme responsible for the activation of carcinogens. The extract was reported to inhibit the activity of cdc 25 tyrosine phosphatase. a key enzyme involved in cell cycle regulation [16]. Several active compounds have been identified in *P. (Imams* extract. Lignans such as phyllanthin and hypophyllanthin [17], flavanoids such as quercetin and astragaline [IS], ellagitannin such as amarinic acid [19], and hydrolysable tannins such as phyllanthisiin I) [20] and amarin [21] were reported from P. *amarus*. Some of the hydrolysable tannins have been shown to inhibit signal transduction. The extract was also found to inhibit the activity of topoisomerase I and II in Saccharomyces cereviceae mutant cell cultures [5). indicating that other than the inhibition of the activation of carcinogen the extract has multifocal activity to brin about a reduction in the neoplastic condition in animals and humans.

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