Study of Antimutagenic Potential of Phytoconstituents Isolated from *Terminalia arjuna* in the Salmonella/Microsome Assay

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Abstract

Two fractions ET-1 and TW isolated from *Terminalia arjuna* were evaluated for their antimutagenic potential against 4-nitro-o-phenylenediamine (NPD) in TA98, sodium azide in TA100 and 2-aminofluorene, in both TA98 and TA100 tester strains of *Salmonella typhimurium* using bacterial reverse mutation assay. Both the fractions inhibited the mutagenicity of 2AF significantly in both the strains. ET-1 fraction inhibited the mutagenicity of base pair substitution mutagen sodium azide by 77.41% at $1 \times 10^3 \mu\text{g}/0.1\text{ml}$. It did not inhibit the mutagenicity of NPD. TW fraction showed insignificant inhibitory activity against the mutagenicity of NPD and sodium azide in TA100 and TA98 tester strains respectively. ¹H-NMR, ¹³C-NMR and IR-spectroscopic data of the fractions revealed ET-1 fraction as a diglycoside of triterpene and TW fraction as tannin in nature.

Keywords: *Terminalia arjuna*; Antimutagenesis; Ames assay; *Salmonella typhimurium*.

1. Introduction

From thousands of years, plants have been utilized as medicines [1]. Major constituents of more than 50% of all the drugs in clinical use are natural products and their derivatives. One of the potential uses of plant-derived compounds is as antimutagenic agents [2, 3]. As a result of human civilization and global environmental pollution, the rate of mutations has increased and one of the

ways to neutralize the effect of such mutagenic agents is to identify those substances that can antagonize their effect. Plants are the promising source of antimutagens which occur in them as secondary metabolites. These antimutagens may help in strengthening the cell defences against environmental mutagens/stress. Nowadays, there is an increasing interest in natural compounds that can act as protectors against diseases [4]. Several therapeutic properties of medicinal plants are

known in obstetrics and gynecology [5], respiratory disorders [6], skin disorders [7], cardiac diseases [8], and mental health [9]. It has been suggested that halving the rate of mutations would delay the onset of most cancers and might be adequate in the lifetime of many individuals [10]. There are different classes of secondary metabolites present in the plants exhibiting antimutagenic activities. *Terminalia arjuna* is a well known medicinal plant. Its bark particularly is extensively used in ayurvedic medicines. It is popularly used as cardiac tonic. The decoction of the bark of *arjuna*, cane sugar and boiled cow's milk is highly recommended in heart diseases complicated with endocarditis, pericarditis and angina. The bark is also prescribed for biliousness, sores and as an antidote to poisons, and is believed to have the ability to cure hepatic, congenital, venereal and viral diseases [11]. Keeping in mind

the medicinal importance of *T. arjuna*, the present study aimed at isolating potent antimutagenic principles/factors from this important medicinal plant with the potential to combat a variety of mutagens.

2. Materials and methods

2.1 Chemicals and bacterial strains

Salmonella typhimurium strains TA98 and TA100, kindly supplied by Professor B.N. Ames, University of California, Berkeley, USA were used. 4-nitro-o-phenylenediamine (NPD), sodium azide, 2-aminofluorene (2-AF) were procured from Sigma Chemical Co. (St Louis, MO, USA). The bark of *T. arjuna* was collected from a tree growing on the campus of Guru Nanak Dev University, Amritsar.

Fig. 1 Isolation of ET-1 and TW fraction from acetone extract of *Terminalia arjuna*

2.2 Isolation

The bark was made free of dirt and other impurities by washing and then immediately dried in oven at 40-60 °C. The bark was finely

powdered and extracted by Soxhlet apparatus by sequential use of different organic solvents in their increasing order of polarity. Acetone extract obtained, was first made aqueous by adding

distilled water. It was then extracted with diethyl ether. White-coloured precipitates separated out from ether extract. This was referred to as **ET-1 fraction**. The remaining acetone extract solution was then extracted with ethyl acetate to obtain ethyl acetate extract. The remaining aqueous acetone extraction solution was then kept in refrigerator for 4-5 days. White-coloured solid separated out. It was filtered and thoroughly washed with distilled water to remove impurities. This white solid was named as **TW fraction** (Fig. 1). These two fractions were examined for their antimutagenic potential. In order to characterize these fractions chemically, ^1H -NMR, ^{13}C -NMR and IR spectroscopic analysis was carried out.

2.3 Spectroscopic analysis

2.3.1. Nuclear magnetic resonance spectroscopy

^1H -NMR normal and distortionless enhancement by polarization transfer (DEPT) spectra. ^{13}C -NMR of the fractions were recorded on Bruker Fourier NMR spectrometer ACF-200 (Switzerland). The sample was prepared in dimethyl sulfoxide (DMSO)- d_6 - CDCl_3 mixture. Tetramethylsilane (TMS) was used as an internal standard.

2.3.2 Infra-red spectroscopy

IR of these fractions (ET-1 and TW) were recorded in solid state (KBr) on FTIR Shimadzu DR-8001.

2.4. Antimutagenicity assay

2.4.1 Ames assay

Bacterial short-term assays are useful tools for both genotoxicity and antimutagenesis studies due to their simplicity and flexibility to design experimental procedures that allow clarification of antimutagenesis mechanisms [12]. The Ames test is a widely accepted method for identifying various chemicals and drugs that can cause gene mutation. The plate incorporation assay [13] with little modification [14] was used for the present investigation. To check the antimutagenic potential, two sets of experiments were designed.

2.4.2 Co-incubation

Bacterial culture (0.1 ml), 0.1 ml of direct-acting mutagen (NPD/Sodium azide) and 0.1 ml of non-toxic concentrations of fractions were added in the above order into sterile test tubes containing 2 ml of soft agar and poured onto minimal agar plates. In the case of indirect-acting mutagen, 2-aminofluorene (2AF), 0.1 ml of bacteria, 0.1 ml of 2AF, 0.5 ml of S9 mix and 0.1 ml of fractions were added into 2 ml of soft agar, mixed and poured onto minimal glucose agar plates.

2.4.3 Pre-incubation

NPD/Sodium azide (0.1 ml) and 0.1 ml of non-toxic concentrations of fractions were added into sterile empty test tubes. The mixture was placed in gyratory incubator set at 37°C for 30 minutes (each concentration in triplicate). After incubation, 2 ml of molten agar was added into the test tubes along with 0.1 ml of culture. The contents of the tube were well mixed and poured onto minimal agar plates. In case of 2AF, 0.1 ml of 2AF, 0.5 ml of S9 mix and 0.1 ml of fractions were incubated. Once the soft agar was poured onto minimal agar plates, it was evenly distributed by rotating the dish and then placed on a levelled surface. After solidification, the plates were placed in incubator at 37°C in an inverted position. Different concentrations of the fractions were prepared in DMSO. Non-toxic concentrations were determined to be those where there was no statistically significant difference in the (1) number of spontaneous revertant colonies, (2) size of colonies, and (3) intensity of the background lawn, as compared to the control where no extract/fraction was added. Concurrently, positive control (mutagen but no fraction) was also set. Each concentration was tested in triplicate and the entire experiment was repeated twice.

The antimutagenic activity of each fraction was expressed as percent decrease of reverse mutations as follows:

$$\text{Inhibitory activity (\%)} = \frac{a-b}{a-c} \times 100$$

a = Number of histidine revertants induced by mutagen (NPD/Sodium azide and 2AF)

b = Number of histidine revertants induced by mutagen in the presence of fraction

c = Number of histidine revertants induced in the presence of fraction alone and solvent (negative control)

2.5. Statistical analysis

The results are presented as the mean $\pm$ standard error of two experiments. The data in all the experiments were analyzed for statistical

significance using analysis of variance (one-way ANOVA). The difference among average values was compared by high-range statistical domain (HSD) using Tukey's Test. The significance was checked at $*p < 0.05$.

Table 1. ^{13}C -NMR spectral data of ET-1 fraction (d_6 -DMSO- CDCl_3)

Normal ^{13}C spectrum	DEPT-135		DEPT-90 (CH carbons)	(DEPT-135) - (DEPT-90) (CH ₃ carbons)
	Inphase signal (CH ₃ /CH carbons)	Antiphase signals (CH ₂ carbons)		
15.38	15.38			15.38
15.71	15.71			15.71
15.77	15.77			15.77
16.02		16.02		
16.55		16.55		
22.46		22.46		
23.35	23.35			23.35
23.60	23.60			23.60
25.41		25.41		
26.11		26.11		
26.50		26.50		
26.70		26.70		
27.17				27.17
27.77				27.77
29.22				
30.16				
30.65				
30.90		30.90		
35.66				
36.17				
41.15	41.15		41.15	
42.26	42.26		42.26	
45.61		45.61		
46.39	46.39		46.39	
54.00	54.00		54.00	
59.92		59.92		
66.49	66.49		66.49	
68.59	68.59		68.59	
71.36	71.36		71.36	
75.79	75.79		75.79	
76.44	76.44		76.44	
79.29	79.29		79.29	
81.55	81.55		81.55	
92.48				
93.14	93.14		93.14	
121.51	121.51		121.51	
141.57				
174.36				

3. Results

3.1. Analysis of fractions

3.1.1. Analysis of ET-1 fraction

The fraction shows 38 signals in its normal ^{13}C -NMR spectrum (Table 1). Out of these, 35

signals appear in the range δ 0-100 and only three signals appear downfield, i.e., at δ 121.51, 141.57, 174.36. The signal at δ 174.36 could be due to aldehyde, ketone or carboxylic acid groups, but normally carbonyl groups appear in the range δ 180-200. Therefore, the signal at δ 174.36 points

towards the presence of carboxylic group. The signals at 121.51 and 141.57 show the presence of one double bond. The spectrum in the region of δ 0-100 is so complex that its off-resonance proton decoupled ^{13}C spectrum can not be used for differentiating CH_3 , CH_2 , CH and quaternary carbons. Thus various distortionless enhancement by polarization transfer (DEPT) techniques have been used. DEPT-135 spectrum shows 10 antiphase signals at δ 16.02, 16.55, 22.46, 25.41, 26.11, 26.50, 26.70, 30.90, 45.61 and 59.92 which confirms the presence of 10 CH_2 carbons. Also the presence of eight signals (δ 29.22, 30.16, 30.65, 35.66, 36.17, 92.48, 141.57 and 174.36) in DEPT-135 spectrum in comparison with normal ^{13}C -NMR spectrum shows the presence of eight quaternary carbons. So, there are six sp^3 hybridized quaternary carbons (δ 29.22, 30.16, 30.65, 35.66, 36.17 and 92.48), one olefinic (δ 141.57) and one carboxylic carbon (δ 174.36). Further, the presence of one signal at δ 121.51 and the absence of δ 141.57 signal show that the double bond presents in the fraction is a trisubstituted one. DEPT-135 spectrum also shows 20 inphase signals corresponding to CH_3 and CH carbons. Seven signals appearing from δ 15-30 could be due to CH_3 carbons, and 12 signals downfield δ 40-100 and one at δ 121.51 could be due to CH . In order to differentiate these signals, DEPT-90 spectrum, which shows only signals due to CH carbons, has been recorded. DEPT-90 spectrum shows 13 signals at δ 41.15, 42.26, 46.39, 54.00, 66.49, 68.59, 71.36, 75.79, 76.44, 79.29, 81.55, 93.14 and 121.51. The signals at δ 121.51 correspond to olefinic carbon and the remaining 12 signals shows the presence of 12 CH carbons in this fraction. Seven signals at δ 15.38, 15.71, 15.77, 23.35, 23.60, 27.17 and 27.77 show the presence of seven CH_3 units. Therefore the combination of normal ^{13}C -NMR, DEPT-135 and DEPT-90 spectra shows that this fraction possesses 7 CH_3 , 10 sp^3 CH_2 , 12 sp^3 CH and 6 sp^3 quaternary carbons along with one trisubstituted double bond and one COOH units. In infrared (IR) spectrum of this fraction, the presence of carbonyl absorption band at 1725 cm^{-1} shows the presence of ester group. But it is not a simple Me ester, as its ^{13}C -NMR shows the clear absence of CH_3 group around 60 ppm. In UV spectrum, this

fraction shows λ_{max} at 255 nm and a valley 207 nm. The observation that this fraction on hydrolysis and carbohydrate test shows the presence of glucose unit and the presence of CH signal at δ 93.14 and CH_2 signal at δ 59.92 along with other lowfield OCH signals at δ 66.49, 68.59, 71.36, 76.44, 79.29 and 81.55, conspicuously shows the presence of two glucose units. Therefore, this fraction is a diglycoside of triterpene. One of these glucose units forms a glycoside bond with carboxylic acid, but the position of other glucose unit could not be determined.

Table 2. ^{13}C -NMR spectral data of TW fraction

Normal ^{13}C -NMR spectrum	DEPT-90
16.09	
59.20	
68.16	68.16
68.83	68.83
70.30	70.30
91.84	91.84
98.33	98.33
105.81	
106.49	
108.95	108.95
109.84	109.84
110.86	110.86
112.69	
127.33	
134.56	
134.92	
137.86	
139.44	
144.74	
146.93	
150.94	
156.81	
157.15	
157.28	
184.72	

3.1.2 Analysis of TW fraction

^1H -NMR of this fraction (d_6 -DMSO- CDCl_3) shows a multiplet in the region δ 3.00-4.20, a typical for the presence OCH_2 and OCH of carbohydrates, and the signals in aromatic region between δ 7.20-8.20 indicate the presence of aromatic CH units. In its IR spectrum, the absorption bands at 1500 , 1560 and 1630 cm^{-1} also

show the presence of aromatic rings which is further confirmed by the absorption band at 750 cm^{-1} due to out of plane C-H bending. The presence of ArOH (O-H stretching) is indicated by a broad band at 3400 cm^{-1} . The absorption band at 1740 cm^{-1} points towards the presence of carboxyl group. Its normal ^{13}C -NMR spectrum exhibits 6 signals at δ 59.20, 68.16, 68.33, 70.30, 91.84 and 98.33 in aliphatic region and 18 signals at δ 105.81, 106.49, 108.95, 109.84, 110.86, 112.69, 127.33, 134.56, 134.92, 137.86, 139.44, 144.74, 146.93, 150.94, 156.81, 157.15, 157.28 and 184.72 in aromatic region along with one very downfield signal at δ 184.72 indicating the

presence of carboxylic group (Table 2). Its DEPT-90 spectrum shows eight signals at δ 68.16, 68.83, 70.30, 91.84, 98.33, 108.95, 109.84 and 110.86. The signals at 68.16, 68.83, 70.30, 91.84 and 98.33 in aliphatic region show the presence of five CHOH carbons of carbohydrate units. The signals at δ 108.95, 109.84 and 110.86, which are positive signals in DEPT-90, show the presence of ArCH carbons which are quite upfield, indicating that these are ortho to hydroxyl group. The signals in the range δ 127-140 could be due to ArC attached with other carbons. The above characteristics indicate that the isolated fraction is tannin in nature.

Fig 2. Effect of ET-1 fraction from *Terminalia arjuna* on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *Salmonella typhimurium*.

3.2 Antimutagenicity

3.2.1. ET-1 fraction

ET-1 fraction was tested for its antimutagenic potential against two direct-acting mutagens NPD and sodium azide. From Table 3 and Fig. 2, it is evident that ET-1 fraction had negligible

modulatory effect on the mutagenicity of NPD in TA98 strain of *Salmonella typhimurium*. Maximum inhibitory effect observed was just 16.10% at the highest dose of $1 \times 10^3 \mu\text{g}/0.1\text{ ml}/\text{plate}$ in pre-incubation mode of experiments. ET-1 fraction significantly inhibited the mutagenicity of sodium azide. An inhibitory effect of 68.35 % was

observed in co-incubation experiments at the concentration of 1×10^3 $\mu\text{g}/0.1$ ml which enhanced to 77.41% when ET-1 fraction was incubated with sodium azide at 37°C for 30 minutes. ET-1 fraction was also tested for its ability to inhibit the mutagenicity of indirect-acting mutagen 2AF in both the tester strains. In TA98 and TA100 tester

strains, the ET-1 fraction inhibited the 2-AF induced histidine revertant colonies by 77.08 and 70.80% respectively at the highest test dose of 1×10^3 $\mu\text{g}/0.1$ ml/ plate in pre-incubation mode of experiments (Table 4, Fig. 3).

Table 3. Effect of 'ET-1' fraction from *Terminalia arjuna* on the mutagenicity of NPD in TA 98 and sodium azide in TA100 tester strains of *Salmonella typhimurium*

		TA98		TA100	
Treatment	Dose ($\mu\text{g}/0.1\text{ml}$)	Mean $\pm$ SE	Percent Inhibition	Mean $\pm$ SE	Percent Inhibition
Spontaneous	-	57.33 $\pm$ 3.03	-	171.00 $\pm$ 3.29	-
Positive Control					
NPD	20	959.00 $\pm$ 33.02 ^a	-		-
Sodium azide	2.5		-	1948.33 $\pm$ 31.37 ^a	-
Negative control					
	1.00×10^3	65.00 $\pm$ 2.16	-	167.33 $\pm$ 0.98	-
	0.50×10^3	61.00 $\pm$ 2.94	-	164.66 $\pm$ 2.12	-
	0.25×10^3	61.00 $\pm$ 2.05	-	162.00 $\pm$ 1.88	-
	0.10×10^3	64.33 $\pm$ 2.22	-	166.33 $\pm$ 2.62	-
	0.01×10^3	67.00 $\pm$ 1.69	-	167.66 $\pm$ 1.18	-
Co-incubation					
	1.00×10^3	851.00 $\pm$ 4.26 ^b	12.08	731.00 $\pm$ 22.81 ^b	68.35
	0.50×10^3	910.50 $\pm$ 15.88 ^{ab}	5.40	749.50 $\pm$ 56.57 ^c	67.21
	0.25×10^3	934.00 $\pm$ 4.48 ^a	2.78	911.50 $\pm$ 17.67 ^d	58.04
	0.10×10^3	940.00 $\pm$ 2.95 ^a	2.12	974.00 $\pm$ 4.48 ^{de}	54.67
	0.01×10^3	948.50 $\pm$ 12.30 ^a	1.17	1076.50 $\pm$ 4.71 ^{ef}	48.96
Pre-incubation					
	1.00×10^3	815.00 $\pm$ 5.20 ^b	16.10	569.50 $\pm$ 14.99 ^b	77.41
	0.50×10^3	848.50 $\pm$ 19.90 ^{bc}	12.30	863.50 $\pm$ 10.52 ^c	60.82
	0.25×10^3	876.00 $\pm$ 7.61 ^{bcd}	9.24	992.00 $\pm$ 8.058 ^d	53.53
	0.10×10^3	915.00 $\pm$ 4.48 ^{acd}	4.91	1001.50 $\pm$ 39.13 ^{de}	53.13
	0.01×10^3	958.00 $\pm$ 3.33 ^a	0.11	1060.00 $\pm$ 21.34 ^{def}	49.88

NPD: 4-nitro-o-phenylenediamine

ONE-WAY ANOVA

Positive control and co-incubation $F(5, 30) = 5.93^*$; HSD = 65.70

$F(5, 30) = 242.70^*$; HSD = 119.06

Positive control and pre-incubation $F(5, 30) = 13.02^*$; HSD = 67.01

$F(5, 30) = 382.80^*$; HSD = 97.18

Data shown are mean $\pm$ SE of two repeated experiments;

a-g, within the column with the same letter means does not differ at $*p < 0.05$

3.2.2. TW fraction

TW fraction was also tested for its antimutagenic potential against two direct-acting mutagens NPD and sodium azide. TW fraction isolated from *T. arjuna* did not significantly

inhibit the his⁺ revertants induced by NPD in TA98 strain in both co-incubation and pre-incubation experiments (Table 5, Fig. 4). Against sodium azide as well inhibitory effect was insignificant and the maximum inhibition was

only 29.84 % induced at the dose of 1×10^3 $\mu\text{g}/0.1$ ml in pre-incubation experiments whereas, in co-incubation experiments, the effect was just 21.52 % at the same dose. However, in TA98 tester strain a remarkable inhibitory effect was observed against 2AF-induced his⁺ revertants in pre-incubation mode of experiments, where the fraction was incubated with mutagen along with S9 mix at 37° C for 30 minutes. TW fraction completely inhibited the mutagenicity of 2AF and, even at the lowest dose of 0.01×10^3 $\mu\text{g}/0.1$ ml, 95 % inhibition of histidine revertants was observed.

In co-incubation experiments, the maximum inhibitory effect observed was 48.45 % at the highest test dose of 1×10^3 $\mu\text{g}/0.1$ ml/ plate (Table 6, Fig. 5). In TA100 tester strain, TW fraction inhibited the 2AF-induced mutagenic effect maximally by 67.18 % at 1×10^3 $\mu\text{g}/0.1$ ml/ plate in pre-incubation studies (Table 6, Fig. 5). The effect was found to be more in pre-incubation mode of experiments. In co-incubation mode, 61.14 % inhibition was observed at the highest test dose.

Table 4. Effect of ‘ET-1’ fraction from *Terminalia arjuna* on the mutagenicity of 2AF in TA 98 and TA100 tester strains of *Salmonella typhimurium*

Treatment	Dose ($\mu\text{g}/0.1\text{ml}$)	TA98		TA100	
		Mean $\pm$ SE	Percent Inhibition	Mean $\pm$ SE	Percent Inhibition
Spontaneous	-		-		-
Positive Control					
2AF	20	3034 $\pm$ 21.14 ^a	-	762.00 $\pm$ 9.52 ^a	-
Negative control	1.00×10^3	62.00 $\pm$ 3.39	-	133.50 $\pm$ 1.06	-
	0.50×10^3	69.66 $\pm$ 2.37	-	133.50 $\pm$ 1.06	-
	0.25×10^3	64.66 $\pm$ 2.12	-	138.00 $\pm$ 1.41	-
	0.10×10^3	63.33 $\pm$ 3.13	-	135.33 $\pm$ 2.37	-
	0.01×10^3	64.33 $\pm$ 2.68	-	141.66 $\pm$ 1.65	-
Co-incubation	1.00×10^3	935.00 $\pm$ 44.28 ^b	70.62	399.00 $\pm$ 12.97 ^b	57.75
	0.50×10^3	977.00 $\pm$ 2.708 ^{bc}	69.39	517.66 $\pm$ 10.19 ^c	38.87
	0.25×10^3	1236.00 $\pm$ 59.48 ^d	60.55	550.50 $\pm$ 18.18 ^{cd}	33.89
	0.10×10^3	1327.00 $\pm$ 13.42 ^{de}	57.46	619.50 $\pm$ 18.56 ^e	22.73
	0.01×10^3	1362.00 $\pm$ 12.08 ^{def}	56.30	635.00 $\pm$ 11.54 ^{ef}	20.47
Pre-incubation	1.00×10^3	743.00 $\pm$ 5.89 ^b	77.08	317.00 $\pm$ 5.54 ^b	70.80
	0.50×10^3	734.50 $\pm$ 5.85 ^{bc}	77.57	331.50 $\pm$ 28.40 ^{bc}	68.49
	0.25×10^3	747.50 $\pm$ 50.76 ^{bcd}	77.00	355.00 $\pm$ 5.37 ^{bcd}	65.22
	0.10×10^3	864.00 $\pm$ 4.48 ^e	73.04	372.00 $\pm$ 21.47 ^{bcd}	62.23
	0.01×10^3	1140.00 $\pm$ 5.61 ^f	63.77	387.50 $\pm$ 1.60 ^{cdef}	60.37

2AF: 2-aminofluorene

ONE-WAY ANOVA

Positive control and co-incubation: F (5, 30) = 585.28*; HSD = 132.63
and F (5, 30) = 77.08*; HSD= 57.28

Positive control and pre-incubation: F (5, 30) = 1568.71*; HSD = 93.85
and F (5, 30) = 123.00*; HSD= 62.51

Data shown are mean $\pm$ SE of two repeated experiments

a-g within the column with the same letter means does not differ at * p<0.05

Fig 3. Effect of ET-1 fraction from *T. arjuna* on the mutagenicity of 2 AF in TA98 and TA100 tester strains of *Salmonella typhimurium*.

Fig 4. Effect of TW fraction from *T. arjuna* on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *Salmonella typhimurium*.

Table 5. Effect of ‘TW’ fraction from *Terminalia arjuna* on the mutagenicity of NPD in TA 98 and sodium azide in TA100 tester strains of *Salmonella typhimurium*.

Treatment	Dose ($\mu\text{g}/0.1\text{ml}$)	TA98		TA100	
		Mean $\pm$ SE	Percent Inhibition	Mean $\pm$ SE	Percent Inhibition
Spontaneous	-	-	-	-	-
Positive Control	-	-	-	-	-
NPD	20	780.00 $\pm$ 37.87 ^a	-	-	-
Sodium azide	2.5	-	-	1350.00 $\pm$ 5.566 ^a	-
Negative control	1.00 $\times 10^3$	53.00 $\pm$ 3.53	-	161.00 $\pm$ 4.54	-
	0.50 $\times 10^3$	57.00 $\pm$ 1.41	-	167.00 $\pm$ 1.24	-
	0.25 $\times 10^3$	58.00 $\pm$ 1.41	-	167.66 $\pm$ 1.18	-
	0.10 $\times 10^3$	52.00 $\pm$ 5.65	-	162.66 $\pm$ 3.20	-
	0.01 $\times 10^3$	55.00 $\pm$ 3.53	-	165.00 $\pm$ 1.41	-
Co-incubation	1.00 $\times 10^3$	530.50 $\pm$ 15.49 ^b	34.31	1095.00 $\pm$ 12.18 ^b	21.52
	0.50 $\times 10^3$	581.50 $\pm$ 17.31 ^{bc}	27.45	1102.00 $\pm$ 10.64 ^{bc}	20.94
	0.25 $\times 10^3$	582.50 $\pm$ 32.07 ^{bcd}	27.35	1103.50 $\pm$ 10.06 ^{bcd}	20.83
	0.10 $\times 10^3$	596.00 $\pm$ 28.39 ^{bcd}	25.27	1195.00 $\pm$ 17.03 ^e	13.03
	0.01 $\times 10^3$	677.33 $\pm$ 17.09 ^{acde}	14.16	1211.00 $\pm$ 14.12 ^{ef}	11.68
Pre-incubation	1.00 $\times 10^3$	528.50 $\pm$ 19.96 ^b	34.59	995.00 $\pm$ 20.96 ^b	29.84
	0.50 $\times 10^3$	546.00 $\pm$ 32.42 ^{bc}	32.36	1032.50 $\pm$ 7.383 ^{bc}	26.82
	0.25 $\times 10^3$	581.50 $\pm$ 40.18 ^{bcd}	27.41	1212.50 $\pm$ 2.846 ^d	11.61
	0.10 $\times 10^3$	589.50 $\pm$ 26.16 ^{bcd}	26.16	1296.50 $\pm$ 16.27 ^a	4.52
	0.01 $\times 10^3$	687.66 $\pm$ 15.72 ^{ade}	12.73	1280.50 $\pm$ 15.70 ^{ae}	5.84

NPD: 4-nitro-o-phnylenediamine

ONE-WAY ANOVA

Positive control and co-incubation F (5, 30) = 11.79*; HSD = 112.40

and F (5, 30) = 65.94*; HSD= 52.40

Positive control and pre-incubation F (5, 30) = 11.53*; HSD = 129.35

and F (5, 30) = 123.85*; HSD =56.70

Data shown are mean $\pm$ SE of two repeated experiments

a-g within the column with the same letter means does not differ at * p<0.05

Table 6. Effect of ‘TW’ fraction from *Terminalia arjuna* on the mutagenicity of 2AF in TA 98 and TA100 tester strains of *Salmonella typhimurium*.

Treatment	Dose (µg/0.1ml)	TA98		TA100	
		Mean±SE	Percent Inhibition	Mean±SE	Percent Inhibition
Spontaneous	-	-	-	-	-
Positive Control	-	-	-	-	-
2AF	20	1203.00±53.91 ^a	-	762.00±8.81 ^a	-
Negative control	1.00×10 ³	52.50±1.76	-	125.00±3.53	-
	0.50×10 ³	57.50±1.76	-	129.00±0.70	-
	0.25×10 ³	59.00±0.70	-	128.50±1.06	-
	0.10×10 ³	51.50±2.47	-	126.50±1.06	-
	0.01×10 ³	52.00±2.12	-	130.00±1.41	-
Co-incubation	1.00×10 ³	645.33±32.36 ^b	48.45	372.00±43.09 ^b	61.14
	0.50×10 ³	874.33±17.60 ^c	28.66	471.00±24.30 ^{bc}	45.97
	0.25×10 ³	903.50±34.27 ^{cd}	26.15	479.00±2.74 ^{bcd}	44.59
	0.10×10 ³	929.50±16.50 ^{cde}	23.72	483.00±7.21 ^{cde}	43.82
	0.01×10 ³	1044.00±22.17 ^{ef}	13.78	524.00±36.05 ^{cdef}	37.65
Pre-incubation	1.00×10 ³	58.00±5.79 ^b	99.52	334.00±1.98 ^b	67.18
	0.50×10 ³	62.50±6.59 ^{bc}	99.56	340.00±7.39 ^{bc}	66.66
	0.25×10 ³	75.00±2.42 ^{bcd}	98.60	378.00±4.86 ^d	60.61
	0.10×10 ³	63.50±3.25 ^{bcd}	98.95	389.00±12.02 ^{de}	58.69
	0.01×10 ³	109.33±10.37 ^{bcd}	95.00	390.00±5.30 ^{def}	58.86

2AF: 2-aminofluorene

ONE-WAY ANOVA

Positive control and co-incubation F (5, 30) = 34.67*; HSD = 138.25
and F (5, 30) = 26.50*; HSD= 109.40

Positive control and pre-incubation F (5, 30) = 410.82*; HSD = 97.86
and F (5, 30) = 481.64*; HSD= 32.01

Data shown are mean ± SE of two repeated experiments

a-g within the column with the same letter means does not differ at * p<0.05

4. Discussion

Both the fractions ET-1 and TW quite effectively suppressed the mutagenic effect of the aromatic amine i.e. 2AF. In both the cases as seen from the results, the antimutagenic activity was marked at the pre-incubation mode of experiments where the respective fractions were incubated with S9 and 2AF for 30 minutes at 37°C. The metabolic

activation of 2AF involves N-oxidation by cytochrome P4501A2 and subsequent activation is by N-acetyltransferase [15]. The inhibitory activity against the mutagenicity of 2AF exhibited by ET-1 is supported by similar studies carried out by Usia et al. [16] in which triterpenes from MeOH-soluble fraction of water extract of *Catharanthus roseus* inhibited cytochrome P450. β-Ionone, a cyclic terpenoid has been shown to be

potent inhibitor of CYP isoforms preventing the conversion of cyclophosphamide, aflatoxin B1 to its genotoxic metabolite [17]. Similarly alpha-bisabolol, a sesquiterpene alcohol from *Matricaria chamomilla* markedly suppressed the mutagenicity of indirect acting mutagens by inhibiting their activation to the mutagenic forms [18]. The tannin fraction 'TW' showed marked antimutagenic activity against frame shift mutations in the pre-incubation mode of experiments wherein even at the lowest test dose of 0.01×10^3 /ml, the his⁺ revertants were inhibited by 95%. The tannin

fraction 'TW' only inhibits the metabolic activation of promutagen 2AF and acts as blocking agents [19]. The additional effect may also be attributed in part to the protection of DNA by the interaction of the phenolic groups of the tannin. Barch and Fox [20] have also suggested the binding of ellagic acid to DNA to be one of the mechanisms involved in blocking of methylation of the O⁶ position of guanine by methylbenzyl nitrosamine. Tannin fraction from *Terminalia arjuna* was shown to effectively inhibit the mutagenicity of 2AF in Ames assay [21].

Fig 5. Effect of TW fraction from *T. arjuna* on the mutagenicity of 2AF in TA98 and TA100 tester strains of *Salmonella typhimurium*.

Against the direct-acting mutagens, ET-1 fraction shows very promising antimutagenic activity against base pair substitution mutagen sodium azide. Sodium azide (NaN₃) induced G: C→A: T transitions [22]. Various studies have shown the potential of triterpenes as antimutagenic agents. Barre et al. [23] reported 22-beta-acetoxylantic acid, a triterpene from *Lantana*

camara to possess antimutagenic activity. Triterpenoid cynarasaponins from *Cynara cardunculus* L. reduced chemically induced mutagenesis *in vitro* [24]. The tannin fraction TW exhibited insignificant activity in inhibiting the mutagenicity of both NPD and Sodium azide in TA98 and TA100 tester strains respectively. Polyphenols have been proposed to act via a

number of mechanisms. Hour et al. [25] suggested that gallic acid can be bound or inserted into the transporters of the outer membrane of the cell and block the mutagen from being transferred into the cytosol. Both intracellular and extracellular mechanisms are involved in the action of phenolics against mutagens [26-28]. Inhibition of the protein transporters, P450 activity preventing 2AF activation, interaction with the active metabolite may be grouped into the extracellular mechanisms. A part of the antimutagenic effect may be due to the direct protection of DNA from electrophilic mutagens or their metabolites and/or by formation of adducts that may result in the prevention of genotoxic damage [29]. The antioxidant activity of the polyphenols may also attribute to its protective effect. Caffeic acid and chlorogenic acid have been reported to suppress the mutagenic activity of N-methyl-N-nitro-N-nitrosoguanidine in *Salmonella typhimurium* strain TA 1535 by scavenging electrophilic degradation products of the carcinogens [30]. The isolation of phytochemicals from the important medicinal plant *Terminalia arjuna* showing antimutagenic activity against specific mutagens warrants further in vivo studies for their probable use as chemopreventive/chemotherapeutic agents.

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