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Invitro Anti-Mutagenic Potential of *Potentilla fulgens:* A Western Himalayan Plant

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Abstract

Invitro anti-mutagenic potential of different extracts/fractions of *Potentilla fulgens* has been made by an Ames histidine reversion assay using TA98 and TA100 tester strains of *Salmonella typhimurium* against the direct-acting mutagens, 4-Nitro-O-phenylenediamine (NPD) and Sodium azide. *P. fulgens* has been used in traditional system of medicine as anti-hyperglycemic, hypoglycemic, anti-hyperlipidemic, anti-oxidant, anti-inflammatory and anti-ulcerogenic agent. Results indicated that n-butanol and water fractions showed strong inhibition activity against the mutagens (NPD, Sodium azide) in both co-incubation as well as pre-incubation treatments in TA98 and TA100 tester strains.

Keywords: Co-incubation, inhibition, mutation, *Potentilla fulgens,* pre-incubation

1. Introduction

DNA is an active molecule that is repetitively damaged and repaired [1]. Factors responsible for DNA damage may be exogenous (e.g. environmental agents, food constituents) or endogenous metabolic processes [2]. As a response to DNA damage, basically all organisms have developed repair mechanisms to preserve the integrity of their genetic material [3, 4]. Unrepaired DNA lesions block replication and transcription which can lead to cell death, miscoding information and generation of mutations [5, 6]. Mutation is an important factor in carcinogenesis and substances causing mutation can also increase the possibility of cancer. The best way to reduce the incidence of cancer is to avoid the exposure to mutagens [7]. It is believed that a diet high in plant derived foods offers a protective effect [8, 9]. Accordingly, as a part of overall strategy geared towards defeat of cancer, it is now apparent that screening of medicinal plants can play an integral role.

Potentilla fulgens Lodd. is an alpine plant of Western Himalayas that is consumed in all parts of the world for its promising medicinal properties [10]. This plant has many pharmacological uses, i.e. anti-hyperglycemic, hypoglycemic, anti-hyperlipidemic, anti-oxidant, anti-inflammatory and anti-ulcerogenic properties [11]. Recently gastroprotective activity of ethanolic root extract of *P. fulgens* has also been reported [12].

In continuation to our work on medicinal and aromatic plants [10, 11, 13] we have decided to explore anti-mutagenic activity of Western Himalayan plant *P.fulgens* as no such study has been reported so far.

2. Materials and Methods

2.1 Chemicals

Salmonella typhimurium strains TA98 (frameshift mutation test) and TA100 (base pair substitution test), were procured from IMTech (Institute of Microbial Technology), Chandigarh. Sodium azide for TA100, 4-Nitro-O-phenylenediamine (NPD) for TA98, were procured from M/S Sigma Chemicals Co. (St Louis, MO, USA).

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2.2 Preparation of Plant Material and Extraction

P. fulgens was collected from Bharmour region of Chamba district during September 2012 at an altitude of 2000 m .The roots and aerial parts of the plant were separated, cleaned, air dried and subjected to chopping and grinding to obtain powder. Dried root powder was extracted with aqueous methanolic (20:80, v/v) at room temperature. The above procedure was repeated for three times to obtain aqueous methanolic extract (340 gm). The aqueous methanolic extract obtained was further fractionated using different solvents i.e. Petroleum ether, Ethyl acetate, n-butanol and water. Solvents of different polarities were used for the separation of different metabolites. All the fractions were dried using rota vapour under reduced pressure yielding petroleum ether (20.01 gm), ethyl acetate (20.83 gm), n-butanol (90.83 gm) and water (15.89 gm) respectively. Crude powder was obtained which was stored at room temperature for further testing.

2.3 Anti-mutagenic Assay

Anti-Mutagenic studies for different fractions of *P.fulgens* were carried out by employing Ames assay as proposed by Maron and Ames (1983) [14] with minor variations as recommended by Bala and Grover (1989) [15]. TA98 and 100 tester strains of *Salmonella typhimurium* were used for the present study. The fresh cultures of tester strains, having density of 1-2 × 10^9 CFU/ml were used to test the anti-mutagenic activity of different fractions of *P.fulgens*. The Minimal Agar plates were prepared one day before use. Top agar was autoclaved and stored at 4˚C. Before the initiation of experiment, it was melted and kept at 45˚C. Two sets of experiments i.e. co-incubation and pre-incubation were carried out. The different concentrations of fractions of *P.fulgens* were used. The concentrations used were: 100 µg/0.1ml, 250 µg/0.1ml, 500 µg/0.1ml, 1000 µg/0.1ml and 2500 µg/0.1ml. All these concentrations were prepared in DMSO in sterile conditions. The negative control was run with different concentrations of fractions to verify the toxicity of test sample. The concentrations were considered non-toxic if the number and size of revertant colonies in negative control were equivalent to that of spontaneous revertant colonies. Also for non-toxic effect of fractions, the intensity of background lawn should be equivalent to the control having only bacterial culture. For determining the toxicity of test sample, 0.1ml of extract or fractions of different concentrations along with 0.1ml of freshly grown culture was added to top agar maintained at 45°C. The mixture was then plated on the Minimal Glucose Agar plates which were then incubated at 37°C for 48 hours. The anti-mutagenicity of different fractions was determined against the known mutagens that are characteristic for each strain depending on the reversion event. Before checking the anti-mutagenicity, the effect of mutagen of known concentration was also checked on the tester strains. This was done in order to ensure responsiveness of tester strains to mutagens as well as the efficacy of pro-mutagen. To confirm the effect of mutagen on tester strains, 0.1ml of freshly grown culture along with 0.1ml of mutagen of known concentration was added to soft agar. The contents were then poured on to the Minimal Glucose Agar plates after thorough mixing. The plates were then incubated at 37˚C for 48 hours and effect of mutagen was determined by counting the revertant colonies. In order to determine the anti-mutagenicity potential of different fractions, co-incubation mode of experiment was designed which involves the addition of 0.1ml of bacterial cultures, 0.1ml of direct-acting mutagen (NPD and Sodium azide) and 0.1ml of non-toxic concentrations of extract and fractions into the 2ml of soft agar. The soft agar was then poured onto minimal agar plates. The plates were then incubated at 37°C for 48 hours and efficacy of extracts was determined by counting the revertant colonies. The anti-mutagenicity potential of fractions was also determined in pre-incubation mode of experiment. It involves the mixing of 0.1ml of mutagen (NPD or Sodium azide), 0.1ml of non-toxic concentrations of extract and fractions in sterile test tubes. The mixture was then incubated at 37°C for 30 minutes. After incubation, the mixture along with 0.1ml of freshly grown culture was added to top agar which was then poured onto the minimal plates and incubated at 37°C for 48 hours. All samples were assayed using triplicate plates per run and each experiment was conducted twice in order to make estimation of variation.

2.4 Percent Inhibition

The anti-mutagenic activity of each fraction was expressed as percent decrease of reverse mutations as follows:

$$\text{Inhibition (\%)} = (a - b)/(a - c) \times 100$$

where,

a = Number of histidine revertants induced by mutagen (Sodium azide, NPD)

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

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

2.5 Percent of Control

$$\text{Percent of Control} = (b/a) \times 100$$

Where, a = number of histidine revertants induced by mutagen alone and b = number of mutagens in the presence of extract/fractions.

2.6 Statistical Analysis

Results are presented as the Mean ± SE of two independent experiments with triplicate plates/dose/ experiment. The data was analyzed for statistical significance using analysis of variance (one-way and two-way ANOVA) and the difference among means was compared by High-range Statistical Domain (HSD) using Tukey's test [16].

3. Results

Present study indicates that aqueous methanolic extract of plant exhibited 46.8% and 41.8% inhibitory activity at the maximum dose tested (2500 µg/0.1ml) in co-incubation and pre incubation mode of treatment respectively against NPD a direct acting mutagen in TA98 strain of *S. typhimurium*. It reduced his$^+$ revertants induced by Sodium azide in TA100 strain by 50.8% and 35.8% in co-incubation and pre-incubation mode of treatment respectively as shown in Table 1.

Table 1: Effect of aqueous methanolic extract on the mutagenicity of NPD in TA98 and Sodium azide in TA100 tester strains of *S. typhimurium*

Treatment	Dose(µg/0.1ml)	TA98 (Mean ± SE)	% inhibition	TA100 (Mean ± SE)	% inhibition
Spontaneous		24.33 ± 6.50	_	63.66 ± 17.01	_
Positive control					
NPD	20	983 ± 262.71	_		
Sodium azide	2.5			1028.66 ± 274.92	_
Negative control	2500	13 ± 3.47	_	52.66 ± 14.07	_
	1000	11.33 ± 3.02	_	57 ± 15.23	_
	500	18 ± 4.81	_	59.66 ± 15.94	_
	250	23 ± 6.14	_	49.66 ± 13.27	_
	100	24.33 ± 6.50	_	64 ± 17.01	_
Co-incubation	2500	528.33 ± 141.20	46.8	532.66 ± 142.36	50.8
	1000	460 ± 122.94	53.8	537.66 ± 143.69	50.5
	500	508.66 ± 135.94	49.1	622.33 ± 166.32	41.9
	250	472.20 ± 126.20	53.2	556.70 ± 148.78	48.2
	100	468.53 ± 125.22	53.6	541.85 ± 144.81	50.4
Pre-incubation	2500	576.66 ± 154.12	41.8	678.33 ± 181.29	35.8
	1000	539.66 ± 144.23	45.6	695 ± 185.74	34.3
	500	521 ± 139.24	47.8	764.66 ± 204.36	27.2
	250	573.66 ± 153.31	42.6	966.66 ± 258.35	6.3
	100	595 ± 159.02	40.4	804.66 ± 215.05	23.2
One way ANOVA					
Positive control and coincubation		F(5, 12) = 28.6994* HSD = 215.26		F(5, 12) = 3333.873* HSD = 19.33908	
Positive control and preincubation		F(5, 12) = 30.91869* HSD = 188.06		F(5, 12) = 1719.9* HSD = 20.86464	
Two way ANOVA					
Coincubation and Preincubation					
Treatment		F(1, 20) = 3.912		F(1, 20) = 7.299*	
Dose		F(4, 20) = 2.16019		F(4, 20) = 1.899	
Treatment x Dose		F(4, 20) = 0.32		F(4, 20) = 1.853	

NPD: 4-nitro-*O*-phenylenediamine. *P≤0.05

These results showed that aqueous methanolic extract showed good inhibitory activity against NPD as well as Sodium azide. The relationship of percent of control with dose and effect of extract on the mutagenicity of NPD in TA98 and Sodium azide in TA100 tester strains of *S.typhimurium* is depicted in (Fig. 1). Moreover, extract shows highest inhibitory activity at dose of 1000 µg/0.1ml in co-incubation i.e. 53.8% while for pre-incubation method highest inhibitory activity was observed at dose of 2500 µg/0.1ml i.e. 50.8%. Extract was quite effective against the mutagens and showed good anti-mutagenic activity.

The inhibitory effect of different fractions is categorized mainly into four groups depending upon their activity. These groups are 'weak' (<25%), 'moderate' (25%–50%), 'strong' (50%–75%) and 'very strong' (>75%). AVP shows moderate to strong activity against NPD as well as Sodium azide.

Ethylacetate fraction exhibited 49.5% and 49.2% inhibitory activity at the maximum dose tested (2500 µg/0.1ml) in co-incubation and pre-incubation mode of treatment respectively against NPD in TA98 strain of *S. typhimurium*. It inhibited his^+ revertants induced by Sodium azide in TA100 strain by 9.5% and 51.9% in co-incubation and pre-incubation mode of treatment respectively (Fig. 2). Fraction showed weak inhibitory activity at every dose of treatment for co-incubation in TA100 strain while it showed moderate inhibitory

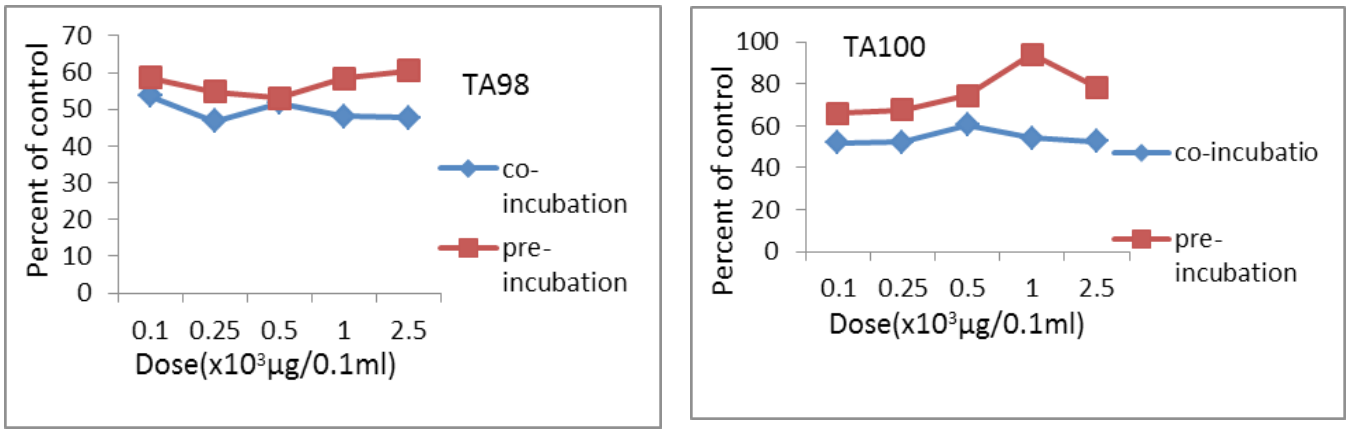

Fig. 1. Effect of aqueous methanolic fraction on the mutagenicity of NPD in TA98 and Sodium azide in TA100 tester strains of *S.typhimurium*.

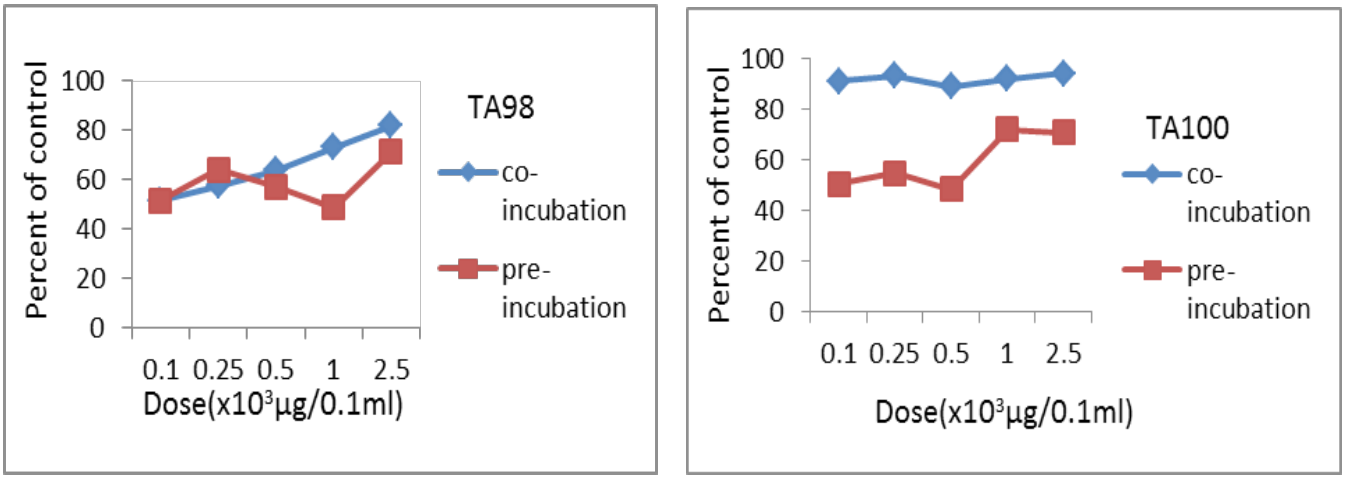

Fig. 2. Effect of ethyl acetate fraction on the mutagenicity of NPD in TA98 and Sodium azide in TA100 tester strains of *S.typhimurium*.

activity for TA98 strain with maximum inhibition activity of 49.5% at dose of 2500 µg/0.1ml. This shows that co-incubation method was ineffective in TA100 strain at all doses for ethylacetate fraction while for TA98 strain was quite effective. Moderate to strong inhibitory activity was observed in both co-incubation and pre-incubation methods of treatment at a dose of 1000 µg/0.1ml in TA98 strains i.e. 43.5% and 36.6% respectively (Table 2). For TA100 strain inhibitory activity for co-incubation treatment was less i.e. 7.2%, but for pre-incubation treatment good inhibitory activity was observed i.e. 47.6%.

n-Butanol showed strong inhibitory activity in both tester strains in co-incubation as well as pre-incubation method. Inhibitory activity was 60.4% and 35.4% in co-incubation and pre-incubation respectively in TA98 tester strains while 55.6% and 62.0% inhibition was observed in TA100 tester strains respectively (Table 3). The relationship between percent of control and dose showed that at highest dose of 2500 µg/0.1ml. n-Butanol fraction exhibited high inhibition of mutagens in co-incubation as well as pre-incubation method while as the concentration was decreased to 100 µg/0.1ml, there was decrease in the inhibition of the mutagens (Fig. 3).

This shows that with decrease in concentration the effect of n-butanol fraction on mutagens decreased. Water fraction showed moderate inhibitory activity as shown against both tester strains. It was observed that water fraction exhibited 56.6% and 60.7% inhibitory activity in co-incubation and pre-incubation mode

Table 2: Effect of ethyl acetate fraction on the mutagenicity of NPD in TA98 and Sodium azide in TA100 tester strains of *S. typhimurium*.

Treatment	Dose(µg/0.1ml)	TA98		TA100	
		(Mean ± SE)	% inhibition	(Mean ± SE)	% inhibition
Spontaneous		21.33 ± 5.70	_	63.66 ± 17.01	_
Positive control					
NPD	20	682.66 ± 182.45	_		
Sodium azide	2.5			1028.66 ± 274.92	
Negative control	2500	16 ± 4.27	_	51.33 ± 13.71	_
	1000	14 ± 3.74	_	54.33 ± 14.52	_
	500	11.33 ± 3.02	_	63.66 ± 17.01	_
	250	15.33 ± 4.09	_	54.33 ± 14.52	_
	100	19 ± 5.07	_	49.33 ± 13.18	_
Co-incubation	2500	352.66 ± 94.25	49.5	935 ± 249.88	9.5
	1000	391.66 ± 104.67	43.5	957.66 ± 255.94	7.2
	500	434.33 ± 116.08	36.9	915.66 ± 244.72	11.7
	250	498.33 ± 133.18	27.6	946.66 ± 253.00	8.4
	100	558.66 ± 149.30	18.6	969 ± 258.97	6.0
Pre-incubation	2500	352.66 ± 94.25	49.2	520.66 ± 139.15	51.9
	1000	437.66 ± 116.97	36.6	564.33 ± 150.82	47.6
	500	390.33 ± 104.32	43.5	497.33 ± 132.91	55.0
	250	333.66 ± 89.17	52.2	739.33 ± 197.59	29.6
	100	485.66 ± 129.79	29.6	729.33 ± 194.92	30.5
One way ANOVA					
Positive control and coincubation		F(5, 12) = 11.83439* HSD = 211.40		F(5, 12) = 6.365017* HSD = 92.48	
Positive control and preincubation		F(5, 12) = 18.81142* HSD = 177.24		F(5, 12) = 49.02198* HSD = 171.24	
Two way ANOVA					
Coincubation and Preincubation					
Treatment		F(1, 20) = 7.181*		F(1, 20) = 451.448*	
Dose		F(4, 20) = 9.680*		F(4, 20) = 13.876*	
Treatment x Dose		F(4, 20) = 4.082*		F(4, 20) = 8.473*	

NPD: 4-nitro-*O*-phenylenediamine. *P ≤ 0.05

Table 3: Effect of *n*-butanol fraction on the mutagenicity of NPD in TA98 and Sodium azide in TA100 tester strains of *S. typhimurium*.

Treatment	Dose(µg/0.1ml)	TA98 (Mean ± SE)	% inhibition	TA100 (Mean ± SE)	% inhibition
Spontaneous		22 ± 5.87	_	50.33 ± 13.45	_
Positive control					
NPD	20	902.33 ± 241.15	_		
Sodium azide	2.5			869 ± 232.25	_
Negative control	1000	20 ± 5.34	_	159 ± 42.49	_
	500	11.33 ± 3.02	_	155.66 ± 41.60	_
	250	14.66 ± 3.91	_	188.66 ± 50.42	_
	100	17.33 ± 4.62	_	179 ± 47.83	_
Co-incubation	1000	369 ± 98.61	60.4	474 ± 126.68	55.6
	500	508.33 ± 135.85	44.2	479.66 ± 128.19	54.5
	250	391.66 ± 104.67	57.5	562.33 ± 150.28	45.0
	100	572.33 ± 152.96	37.2	735 ± 196.43	19.4
Pre-incubation	1000	589.66 ± 157.59	35.4	428.33 ± 114.47	62.0
	500	555.33 ± 148.41	38.9	522.33 ± 139.59	48.5
	250	641 ± 171.31	29.4	555 ± 148.33	46.1
	100	635 ± 169.71	30.2	675 ± 180.40	28.1
One way ANOVA					
Positive control and coincubation		F(5, 12) = 65.07723* HSD = 219.4098		F(5, 12) = 363.2682* HSD = 93.8074	
Positive control and preincubation		F(5, 12) = 85.2183* HSD = 193.6079		F(5, 12) = 1135.388* HSD = 51.9166	
Two way ANOVA					
Coincubation and Preincubation					
Treatment		F(1, 20) = 30.487*		F(1, 20) = 3.109	
Dose		F(4, 20) = 106.724*		F(4, 20) = 885.237*	
Treatment x Dose		F(4, 20) = 5.647*		F(4, 20) = 5.161*	

NPD: 4-nitro-*O*-phenylenediamine. *P ≤ 0.05

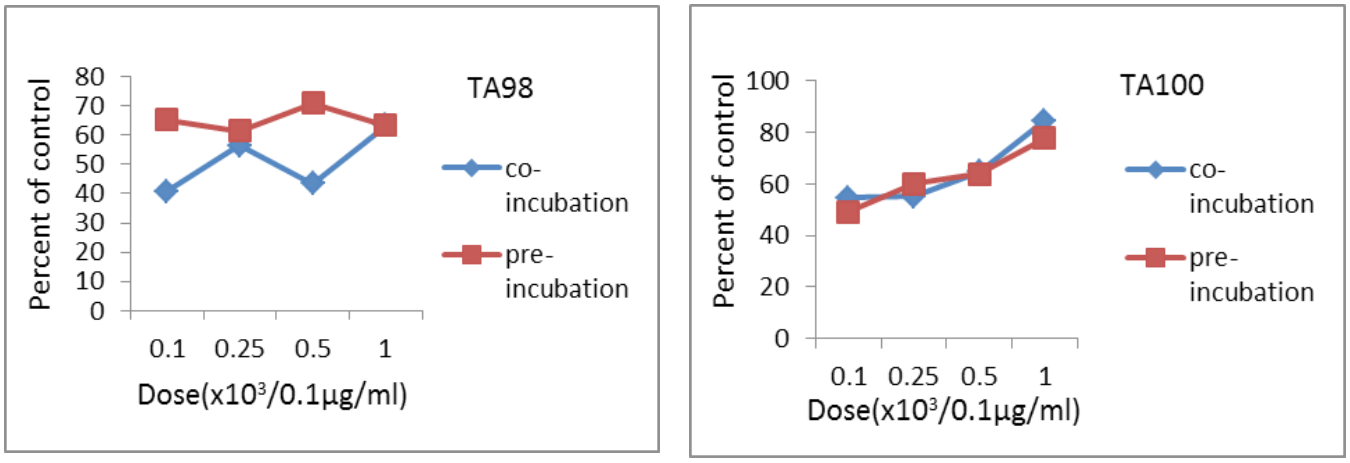

Fig. 3. Effect of *n*-butanol fraction on the mutagenicity of NPD in TA98 and Sodium azide in TA100 tester strains of *S.typhimurium*.

Table 4: Effect of water fraction on the mutagenicity of NPD in TA98 and Sodium azide in TA100 tester strains of *S. typhimurium*

Treatment	Dose(µg/0.1ml)	TA98 (Mean ± SE)	% inhibition	TA100 (Mean ± SE)	% inhibition
Spontaneous		21.66 ± 5.79	_	70 ± 18.70	_
Positive control					
NPD	20	927.66 ± 247.92	_		
Sodium azide	2.5			1051 ± 280.89	_
Negative control	1000	15.66 ± 4.18	_	51.66 ± 13.80	_
	500	8.66 ± 2.31	_	52 ± 13.89	_
	250	11 ± 2.93	_	51.33 ± 13.71	_
	100	16.33 ± 4.36	_	56.33 ± 15.05	_
Co-incubation	1000	356.66 ± 95.32	56.6	705.66 ± 188.59	34.5
	500	422.66 ± 112.96	54.9	628.66 ± 168.01	42.2
	250	430 ± 114.92	54.2	681.66 ± 182.18	36.9
	100	479.66 ± 128.19	49.1	718 ± 191.89	33.4
Pre-incubation	1000	373.33 ± 99.77	60.7	545.33 ± 145.74	50.6
	500	388 ± 103.69	58.7	633.66 ± 169.35	41.7
	250	445 ± 118.93	52.9	704 ± 188.15	34.7
	100	427 ± 114.12	54.9	748.33 ± 200.00	30.4
One way ANOVA					
Positive control and coincubation		F(5, 12) = 267.6119* HSD = 108.8332		F(5, 12) = 167.5206* HSD = 159.2796	
Positive control and preincubation		F(5, 12) = 142.83* HSD = 148.544		F(5, 12) = 224.8215* HSD = 138.528	
Two way ANOVA					
Coincubation and Preincubation					
Treatment		F(1, 20) = 1.571		F(1, 20) = 3.612	
Dose		F(4, 20) = 361.518*		F(4, 20) = 361.518*	
Treatment x Dose		F(4, 20) = 2.446		F(4, 20) = 2.446	

NPD: 4-nitro-*O*-phenylenediamine. *P ≤ 0.05

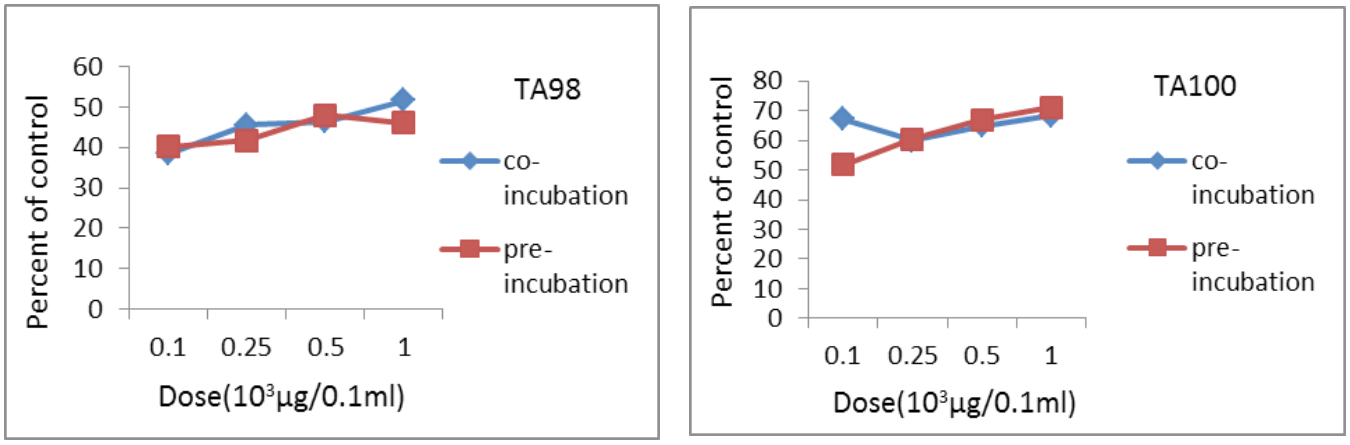

Fig. 4. Effect of water fraction on the mutagenicity of NPD in TA98 and Sodium azide in TA100 tester strains of *S.typhimurium*.

of treatment respectively against NPD a direct acting mutagen in TA98 strain of *S. typhimurium* whereas it reduced his$^+$ revertants induced by Sodium azide in TA100 strain by 34.5% and 50.6% in co-incubation and pre-incubation treatment respectively (Table 4).

Water fraction showed strong inhibitory activity at all concentrations in co-incubation as well as pre-incubation treatments (Fig. 4). Inhibitory activity was satisfactory even when the concentration of dose was decreased to 100 µg/0.1ml.

4. Discussion

The anti-mutagenic activity of extracts appears to be linked to their scavenging property as well as due to their ability to affect the process of enzymatic activation, on the other hand there are many ways in which environmental factors, such as polyphenols, can inhibit or alter the sequence of events leading to mutations. Thus the modifying action of extracts may not be limited to interaction at the level of cytochrome P450-mediated metabolism of promutagen/ carcinogens. The mechanism of protection of these structurally very diverse compounds may be multifactorial [17]. The consumption of the drugs, fruits and vegetables, however, often results in enhancement of enzymes involved in the metabolism of xenobiotic and carcinogens thereby accelerating their metabolic disposal. Of particular interest, in this respect, are the Glutathione S-Transferases (GST), a family of phase II transformation enzymes [18]. Owing to the varied nature of the compounds, a combination of antimutagens will probably be necessary and, moreover, it is essential to confirm putative anti-mutagenic activity observed *in vitro* through the use of animal models. The net effect is that we currently know very little about what may well prove to be an interesting and exciting area of anti-mutagenic and anti-carcinogenic research.

Previous study revealed that the methanolic extract of the roots of *P.fulgens* was found to be active against certain tumors in a dose-dependent manner. The herb and the underground parts of the same plant were also used to treat various ailments, including neoplastic diseases [19]. It has also been reported that the aqueous root extracts of the herb of *P.fulgens* are active against neoplastic tumours murine ascites Dalton's lymphoma (DL), depending on the method of administration [20]. The anti-mutagenic activity of the fractions may be due to their free radical scavenging abilities. Reactive oxygen species and other free radicals cause oxidative stress which in turn causes DNA, protein, and lipid damage which leads to change in chromosome instability, genetic mutation, and modulation of cell growth that result in cancer [21]. Also a part of the anti-mutagenic 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 [22]. This study reveals that *P.fulgens* can be potentially used as an anti-mutagenic plant.

5. Conclusion

There is need of development of anti-mutagenic agents that are able to reduce the occurrence of mutations. Till now no anti-mutagenic activity has been reported on any of *Potentilla* species, more work needs to done so as to explore the anti-mutagenic use of these species. Plants like *P.fulgens* are quite effective in acting as anti-mutagenic agents. This report supports the argument that traditional medicinal plants like *P.fulgens* are valuable sources that are effective in fighting against variety of ailments including cancer.

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