

ARTICLES

Evaluation of in vitro antimutagenic activity of "seabuckthorn" (Hippophae rhamnoides Linn.) in Ames assay

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Abstract : [Abstract] Objective and Methods Edible medicinal plants are precious antimutagen contenders. Fruits of seabuckthorn (Hippophae rhamnoides Linn.) has a rich source of vitamins and other bioactive substances. In Indian Himalayas, they are being used by the farmers for making jams and pickles and by traditional doctors for making medicines for digestive and lung disorders. Ames Salmonella histidine reversion assay was used in the present investigation to evaluate antimutagenic activity of aqueous methanolic extract of seabuckthorn berries in TA98 and TA100 strains of Salmonella typhimurium using direct (NPD and sodium azide) and indirect (2-Aminofluorene) acting mutagens. Results The extract showed moderate antimutagenicity against NPD (41.84 %) and sodium azide (46.46 %) in TA98 and TA100 tester strains whereas it showed strong antimutagenicity resulting in an inhibition of 56.25 % and 49.63 % of his⁺ revertants induced by 2AF in TA98 and TA100 strains respectively. Conclusion The antimutagenicity of the extract observed in the present study implies chemopreventive pharmacological importance of seabuckthorn and encourages its use as a functional food.

Key words : antimutagenicity ; 2-aminofluorene ; NPD ; sodium azide ; Hippophae rhamnoides ; Salmonella typhimurium ; Ames assay

INTRODUCTION

It has been substantiated by information attained through epidemiological studies that it is realistic to avert cancer and other chronic diseases, some of which share common pathogenic mechanisms, such as DNA damage, oxidative stress, and chronic inflammation. An apparent approach

is forestalling the exposure to well-acknowledged risk factors. As a complementary strategy, it may be possible to render the organism more resistant to mutagens/carcinogens and/or to inhibit progression of the disease by administering chemopreventive agents [1]. In fact, one of the most striking medical practices of the 21st century is the chemoprevention of cancer. Much development has been made in this latest and promising field, yet much needs to be done before prevalent use and implementation of cancer prevention becomes a routine practice [2]. Many plant species are known to elicit antimutagenesis and thus have a full range of prospective applications in human healthcare. Even for populations which use herbs traditionally, encouraging the use of species with chemopreventive actions could be helpful as part of life expectancy improvement strategies: costs are significantly low, herbs have usually little or no toxicity during long-term oral administration and are relatively available at large scale [3]. It has been suggested that regularly consuming anticarcinogens and antimutagens in the diet may be the most effective way of preventing human cancer [4~6] and search for novel antimutagens acting in chemoprevention is a promising field in phytotherapy.

seabuckthorn (*Hippophae rhamnoides* Linn.), also known as 'Leh Berry' in northwestern Himalayas, is a deciduous spiny shrub widely distributed throughout the temperate zone of Asia and Europe [7]. Various reports on its medicinal value have appeared including its anti-inflammatory, antioxidant and wound healing activities [8~10].

The high vitamin concentrations make seabuckthorn fruit highly suitable for the production of nutritious soft drinks. China designated its seabuckthorn sports drinks "Shawikang" and "Jianlibao" as the official beverages for its athletes at the Seoul Olympic Games in 1988, and Russian cosmonauts also were supplied with seabuckthorn beverages, to enhance their health and resistance to stress. It has been claimed that seabuckthorn was the first fruit juice in space [11]. In fact, the plant is predicted as the next major health food fad and is being envisaged as a multipurpose plant with potential applications as nutritious food, medicine, soil enhancer, pollution reducer, soil conservator, landscape management tool and as an important source of firewood [11]. Previously, studies carried out in our laboratory have determined the antimutagenic potential of some food and medicinal plants [12~14]. The present study was planned to evaluate antimutagenic potential of aqueous methanol extract of *Hippophae rhamnoides* employing Ames assay. The results obtained demonstrate the health-promoting potential of the SBT.

MATERIAL AND METHODS

Collection/Extraction of Plant Material

The plant material was collected from Lahul Spiti in north-western Himalayas at an altitude of 3,500 m and authenticated by the herbarium of Institute of Himalayan Bioresource Technology (IHBT), Palampur. Drying of berries, pulverisation and extraction was carried out at IHBT, Palampur. The berries were shade dried, coarsely powdered and extracted with aqueous methanol (80 % MeOH). The solvent was evaporated under vacuum and the extract was further lyophilized and stored at 4 °C until future use.

Antimutagenicity Assay

The Salmonella histidine point mutation assay proposed by Maron and Ames [15] was followed with little amendments as suggested by Bala and Grover [16] to verify the inhibitory activity of the extract. Bacterial strains were kindly provided by Dr. Indu Pal, University Institute of Pharmaceutical Sciences, Punjab University, Chandigarh, India. Sodium azide, 4-nitro-o-phenylenediamine (NPD) and 2-aminofluorene (2AF) were procured from M/S Sigma Chemicals Co., St. Louis, Missouri (USA). Constant concentrations of two direct-acting mutagens, NPD (20 µg/0.1 ml/plate) and sodium azide (2.5 µg/0.1 ml/plate) and S9-dependent mutagen, 2AF (20 µg/0.1 ml/plate) were used as positive controls. Non-toxic concentrations of the test sample used for investigating the antimutagenicity ranged from 0.01×10³ to 2.5×10³ µg/0.1 ml/plate. These concentrations were categorized as non-toxic because they showed a well-developed lawn, almost similar size of colonies and no statistical difference in the number of spontaneous revertants in test and control plates. In the course of checking antimutagenicity, two modes of treatment were followed: co-incubation and pre-incubation. In co-incubation process, 0.1 ml each of bacterial culture (about 1~2×10⁸ cells/ml), mutagen and extract were added to 2 ml of top agar. In pre-incubation system, equal quantity of the mutagen and the extract were blended and allowed to stand for 30 min at 37 °C under incessant shaking and 0.2 ml of this was added to 2 ml of soft agar with 0.1 ml of fresh bacterial culture.

The impact of aqueous methanol extract on the mutagenicity of indirect-acting mutagen (2AF) was studied by mixing 0.5 ml of S9 mix directly into soft agar containing 0.1 ml of bacterial culture and 0.1 ml of promutagen. Soft agar was poured on a minimal glucose agar plate at 37 °C for 48 h. Concurrently, a positive control (where mutagen but no extract was added) and a negative control (where no mutagen was added) were also set. All the test samples and mutagens, i.e. NPD and 2AF, were dissolved in dimethylsulfoxide (DMSO) and sodium azide in distilled water. The activity of each extract was expressed as percentage decrease of reverse mutations

$$\text{Percent inhibition of mutagenesis} = \left[\frac{x-y}{x-z} \times 100 \right] ;$$

$$\text{Percent control of mutagenic activity} = \left[\frac{y}{x} \times 100 \right] ;$$

where "x" = No. of histidine revertants induced by mutagen; "y" = No. of histidine revertants induced by mutagen in the presence of extract; and "z" = No. of revertants in the negative control. The antimutagenic potency was categorized as 'strong' (>50 % inhibition of mutagenic activity), 'moderate' (between 25% and 50 % inhibition of mutagenic activity) and 'weak' (<25 % inhibition of mutagenic activity) [17].

Statistical Analysis

The results are presented as the mean and standard error of three independent series of experiments with three plates/dose/experiment. Mean of nine plates was taken for analysis. The data were analyzed for statistical significance using analysis of variance (one-way and two-way ANOVA). Linear relation between dose and percent control of mutagenic activity was obtained by a simple regression and correlation analysis.

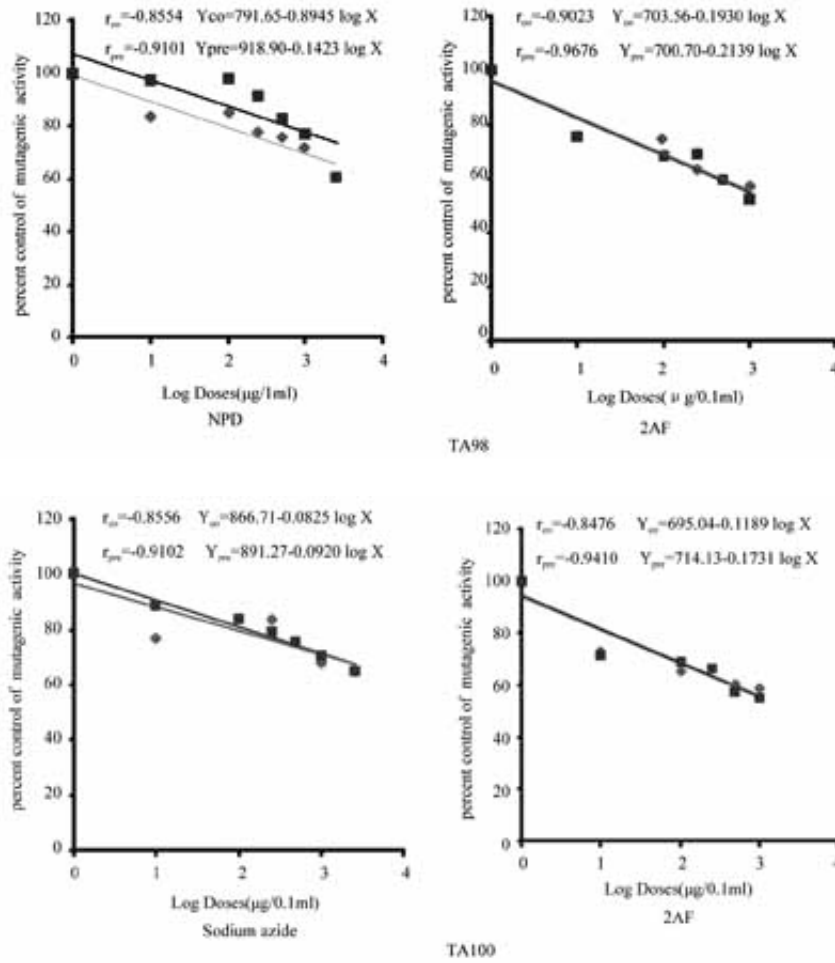


Figure 1 Relationship between different concentrations of aqueous methanol extract of H.rhamnoides and percent control of mutagenic activity of NPD,sodium azide and 2AF in TA98 and TA100 tester strains of S.typhimurium

■Pre-incubation ◆Co-incubation

Table 1 Effect of Aqueous Methanol Extract of Seabuckthorn (Hippophae rhamnoides) on the Mutagenicity of NPD and Sodium Azide in TA98 and TA100 strains of Salmonella typhimurium

Treatment	Dose (g/0.1 ml)	npd		Sodium Azide	
		TA98		TA100	
		His ⁺ revertants/plate Mean ± SE	Percent Inhibition	His ⁺ revertants/plate Mean ± SE	Percent Inhibition
Spontaneous		31.11 ± 2.36	-	240.22 ± 17.25	-
Positive Control	20	967.78 ± 52.46	-	-	-
	2.5	-	-	1071.78 ± 36.50	-
Negative Control	0.01 × 10 ³	31.67 ± 1.65	-	229.22 ± 21.94	-
	0.10 × 10 ³	29.00 ± 2.20	-	214.56 ± 17.01	-
	0.25 × 10 ³	26.56 ± 2.04	-	235.78 ± 18.81	-
	0.50 × 10 ³	33.22 ± 2.00	-	226.22 ± 17.88	-
	1.00 × 10 ³	30.22 ± 2.41	-	242.44 ± 20.35	-
	2.50 × 10 ³	31.44 ± 2.65	-	216.56 ± 17.76	-
Co-Incubation	0.01 × 10 ³	804.78 ± 14.40	17.41	822.67 ± 127.54	29.13
	0.10 × 10 ³	810.22 ± 11.47	16.80	911.22 ± 21.50	19.36
	0.25 × 10 ³	747.00 ± 7.96	23.62	894.33 ± 16.91	20.99
	0.50 × 10 ³	727.33 ± 13.28	25.55	803.56 ± 27.91	32.08
	1.00 × 10 ³	694.44 ± 26.65	29.12	728.33 ± 7.30	40.06
	2.50 × 10 ³	576.11 ± 20.17	41.84	680.33 ± 26.32	46.46
Pre-Incubation	0.01 × 10 ³	939.33 ± 14.17	3.04	945.56 ± 10.48	14.76
	0.10 × 10 ³	946.00 ± 19.74	2.32	901.22 ± 16.41	20.57
	0.25 × 10 ³	880.78 ± 26.46	9.31	849.44 ± 6.16	26.29
	0.50 × 10 ³	799.78 ± 8.90	17.85	806.44 ± 14.81	31.74
	1.00 × 10 ³	741.89 ± 4.70	24.06	757.00 ± 24.15	36.72
	2.50 × 10 ³	585.00 ± 20.07	40.89	686.78 ± 19.61	45.69
One Way ANOVA					
Positive Control and co-incubation		F(6,56) = 39.5*		F(6,56) = 3.9791*	
Positive Control and pre-incubation		F(6,56) = 95.712*		F(6,56) = 50.514*	
Two way ANOVA					
Co-incubation and pre-incubation					
Treatment		F(1,96) = 122.06*		F(1,96) = 0.8368*	
Dose		F(5,96) = 129.24*		F(5,96) = 13.921*	
Treatment × dose		F(5,96) = 7.5753*		F(5,96) = 1.4492*	

Note: Data shown are MEAN±SE of three repeated experiments, *Significant at $P \leq 0.05$

Table 2 Effect of Aqueous Methanol Extract of Seabuckthorn (Hippophae rhamnoides) on the Mutagenicity of 2AF in TA98 and TA100 Tester Strains of Salmonella typhimurium

Treatment	Dose (g/0.1 ml)	TA98		TA100	
		His ⁺ revertants/plate Mean ± SE	Percent Inhibition	His ⁺ revertants/plate Mean ± SE	Percent Inhibition
Spontaneous	-	33.22 ± 1.47	-	231.56 ± 15.82	-
Positive Control	20	956.89 ± 35.14	-	1016 ± 33.75	-
Negative Control	0.01 × 10 ³	32.89 ± 1.71	-	222.11 ± 15.54	-
	0.10 × 10 ³	32.11 ± 2.49	-	213.56 ± 17.03	-
	0.25 × 10 ³	33.11 ± 1.92	-	196.00 ± 12.97	-
	0.50 × 10 ³	32.56 ± 2.04	-	214.33 ± 16.95	-
	1.00 × 10 ³	32.56 ± 1.91	-	206.67 ± 18.70	-
Co-Incubation	0.01 × 10 ³	730.44 ± 36.70	24.51	736.56 ± 36.40	35.20
	0.10 × 10 ³	709.89 ± 51.19	26.71	663.89 ± 49.80	43.88
	0.25 × 10 ³	606.78 ± 36.74	37.90	649.67 ± 30.01	44.67
	0.50 × 10 ³	576.67 ± 36.17	41.13	609.00 ± 42.65	50.77
	1.00 × 10 ³	535.00 ± 25.41	45.64	594.78 ± 37.25	52.05
Pre-Incubation	0.01 × 10 ³	720.78 ± 40.37	25.55	726.56 ± 47.36	36.46
	0.10 × 10 ³	657.44 ± 28.24	32.38	702.33 ± 35.53	39.09
	0.25 × 10 ³	661.33 ± 43.43	31.99	673.89 ± 45.44	41.72
	0.50 × 10 ³	568.00 ± 22.71	42.07	585.00 ± 26.68	53.76
	1.00 × 10 ³	498.11 ± 23.62	49.63	560.78 ± 25.76	56.25
One Way ANOVA					
Positive Control and co-incubation		F(5,48) = 7.44269 *		F(5,48) = 2.94167 *	
Positive Control and pre-incubation		F(5,48) = 10.7283 *		F(5,48) = 5.76566 *	
Two way ANOVA					
Co-incubation and pre-incubation					
Treatment		F(1,80) = 0.33426 *		F(1,80) = 0.00287 *	
Dose		F(4,80) = 16.6904 *		F(4,80) = 8.03248 *	
Treatment × dose		F(4,80) = 0.99123 *		F(4,80) = 0.49108 *	

Note: Data shown are MEAN ± SE of three repeated experiments, *Significant at $P \leq 0.05$

RESULTS

The extract exhibited moderate inhibitory effect at certain concentrations on the mutagenicity of direct acting mutagens, NPD and sodium azide. As is clear from Figure 1, a dose-dependent response was observed with the maximum percent inhibition obtainable at the maximum non-toxic dose tested. The co- and pre-incubation studies did not illustrate any sizeable variation and were quite comparable with each other. As is comprehensible from Table 1, the inhibitory activity of aqueous methanol extract against NPD in TA98 was observed to be 41.84% and 40.89% in the co and pre-incubation modes of experimentation respectively. However, the maximum inhibition induced by the aqueous methanol extract against 2AF in TA 98 was 49.63% in the pre-incubation mode (Table 2).

It can be observed from Table 1 that at the maximum dose tested, antimutagenesis against sodium azide was slightly more pronounced than NPD. In TA100, the inhibition exerted by the

extract against sodium azide in the co and pre-incubation schemes was observed to be 46.46% and 45.69%, respectively. The extract showed a significant modulatory effect with inhibition of 56.25% and 52.05%, respectively, in the co-incubation and pre-incubation experiments against 2AF in TA 100, at the highest dose of the extract tested (Table 2).

DISCUSSION

Seabuckthorn is normally consumed by locals of northwestern Himalayas and contiguous areas in forms of juices, jams, jellies, pickles, etc. The local healers make extensive use of the medicinal properties of the plant. It is being promoted as a functional food and the fruits are among the most nutritious of all berries. They are rich in a variety of components formerly confirmed to exhibit antimutagenic activity, namely flavonoids and other antioxidants viz. vitamins C and E; several carotenoids; including beta-carotene (pro-vitamin A); certain enzymes; and other substances [11]. The imminent use of seabuckthorn as a functional food is also supported by a number of in vivo and in vitro studies which revealed anticarcinogenic effects of seabuckthorn in laboratory animals and against various cell lines [18~21]. Inhibitory effects in carcinogenesis are important in respect of control and spread of disease. It has been shown that *Hippophae* inhibits benzo (a) pyrene-induced forestomach and DMBA-induced skin papillomagenesis in mouse [22]. It was of interest to verify whether seabuckthorn was capable of antimutagenic action against known mutagens.

A serious examination of the results revealed that in the current work, the antimutagenic activity displayed by *Hippophae rhamnoides* increased when the culture media was supplemented with S9 mix, which is a mammalian metabolic activation system. The cytochrome-based P450 metabolic oxidation system is capable of metabolizing a large number of chemicals to DNA-reactive, electrophilic forms [23]. It is also known that antimutagens are classified into two groups, desmutagens and bioantimutagens [24]. Desmutagens neutralize mutagens before or during attack of DNA, and bioantimutagens activate DNA repair processes. If the test samples were bioantimutagens, the inhibitory effect would be detected when direct-acting mutagens are used in Ames test. However, in the present study, we found that the antimutagenicity was better defined against indirect-acting mutagens.

In many previous studies, the antioxidant and anticarcinogenic nature of SBT has been well established. An earlier study has reported the cytoprotective and antioxidant properties of alcoholic leaf extract of seabuckthorn against hypoxia induced oxidative stress in C-6 glioma cells [25]. A recent report has described the protective effect of REC-1001, a fraction isolated from berries of *Hippophae rhamnoides* against radiation-induced mitochondrial and genomic DNA damage [26]. Antioxidant properties of flavonoids of SBT and the radical scavenging effect of *Hippophae rhamnoides* have also been described lately [27,28]. Isolation of antioxidant proanthocyanidins from the SBT has also been reported [29]. Keeping in prospect the number of previous investigations about the antioxidative and anticarcinogenic nature of *Hippophae rhamnoides*, it can be anticipated that the antimutagenic activity observed in the present endeavor may be via the antioxidative mechanism. Studies are underway to identify the compounds responsible for the antimutagenic activity.

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