EVALUATION OF *IN VITRO* ANTIMUTAGENIC ACTIVITY OF HYPERICUM *PERFORATUM* LINN. IN AMES ASSAY

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Summary

Hypericum perforatum L. is one of the best known and most frequently used medicinal herbs in recent years. It has been used in the treatment of burns, bruises, swelling, anxiety, and more recently, mild to moderate depression. In the present study, the potential antimutagenic effects of the dianthrone rich acetone and aqueous methanol extracts of the aerial flowering parts of *H. perforatum* L. (Guttiferae) were evaluated against 4-nitro-o-phenylenediamine (NPD), sodium azide and 2-aminofluorene induced mutagenicity in the Ames *Salmonella* histidine reversion assay using TA98 and TA100 strains of *Salmonella typhimurium*. Both the extracts showed a remarkable antimutagenic activity (>75%) against the S9 dependent mutagen (2AF). Further, the aqueous methanol extract was fractionated using hexane, chloroform, ethyl acetate and *n*-butanol. Out of all the fractions tested, ethyl acetate fraction turned out to be most potent. The study suggests that *Hypericum perforatum,* owing to its antimutagenic property, can be beneficial for prevention of cancer.

Key Words: Antimutagenicity, 2-aminofluorene, Hypericin, *Hypericum perforatum, Salmonella typhimurium,* Ames assay

Introduction

The role of chemical mutagens in carcinogenesis has been studied extensively, and *in vivo* carcinogenesis has been shown to be related to *in vitro* mutagenesis (1). Chemicals that can induce mutations can also potentially damage the germline leading to fertility problems and mutations in future generations besides causing fatal diseases like cancer (2). Conversely, a large number of antimutagenic factors are also known to exist in nature. In the past few decades, many plant extracts as well as a wide range of plant-derived products such as pigments, vitamins, carotenes, polyphenols, flavonoids and tannins have been identified to be capable of amending the activity of mutagens and carcinogens in various test systems (3, 4, 5, 6). Persistent attempts are being made to screen plants for novel antimutagens. The plant chosen for the present study i.e. *Hypericum perforatum* L. (family Guttiferae), popularly called St. John's wort (SJW) is an evergreen shrub distributed widely in high altitude areas of Himalayas. In the western world (Europe, West Asia and North Africa), it grows as a roadside weed. SJW has been used as an herbal medicine for centuries. Its extract has been proven to be effective in the treatment of mild to moderately severe depression in clinical trials (7, 8) and it is well tolerated even at high doses and has no mutagenic potential (9, 10). In laboratory, promising anticancer effect has been shown by extracts and components of *H. perforatum* (11, 12, 13). In this paper, we report the suppressive effect of *Hypericum* extracts and fractions on his^+ revertants induced by chemical mutagens, NPD, sodium azide and 2-aminofluorene in TA98 and TA100 tester strains of *Salmonella typhimurium.*

Methods

Extraction and fractionation

The flowering aerial parts of *Hypericum perforatum* L. were collected from plants growing in the Chamba region of the northwestern Himalayas from an altitude of 3000 m and authenticated by the herbarium of Institute of Himalayan Bioresource Technology, Palampur. The aerial parts of the plant were shade dried, coarsely powdered and divided into two parts. One part was extracted with aqueous methanol to prepare aqueous methanol extract. The other part was successively extracted with dichloromethane and acetone to yield acetone extract. The aqueous methanol extract was fractionated using solvents of increasing polarity viz. hexane, chloroform, ethyl acetate and *n*-butanol. The solvents in all the cases were evaporated under vacuum and the extracts were further lyophilized and stored at 4˚C for future use.

Antimutagenicity Assay

The *Salmonella* histidine point mutation assay as proposed by Maron and Ames (14) was followed with little modifications (3) to check the inhibitory activity of the extracts. Bacterial strains were kindly provided by Dr. Indu Pal Kaur, University Institute of Pharmaceutical Sciences, Punjab University, Chandigarh. Sodium azide, 4-nitro-o-phenylenediamine (NPD) and 2-aminofluorene (2AF) were procured from M/S Sigma Chemical Co., St. Louis, Missouri (USA). Constant concentrations of two direct-acting mutagens, NPD (20 µg/0.1 ml/plate), sodium azide (2.5 µg/0.1 ml/plate) and one 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 $0.01x10^3$ to $2.5x10^3$ 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. All the test samples and mutagens, i.e. NPD and 2AF, were dissolved in dimethylsulfoxide (DMSO) and sodium azide in distilled water. Two different modes of experimentation were pursued: co-incubation and pre-incubation. In co-incubation technique, 0.1 ml each of bacterial culture, mutagen and extract were added to 2 ml of top agar. In pre-incubation experiments, equal volumes of the mutagens and the extracts were mixed and allowed to stand for 30 minutes at 37°C under continuous 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 the extracts 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 S9 dependent mutagen. The mammalian liver S9 was prepared according to the method of Maron and Ames (14). Soft agar was poured on a minimal glucose agar plate and incubated at 37°C for 48 hours. Concurrently, a positive control (where mutagen but no extract was added) and a negative control (where no mutagen was added) were also set. The activity of each extract was expressed as percentage decrease of reverse mutations

Percent inhibition of mutagenesis = $[(x - y / x - z) \times 100]$;

where "*x*" = No. of histidine revertants induced by mutagen; "y" = No. of histidine revertants induced by mutagen in the presence of test sample; 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) (15).

Results

The extracts obtained were evaluated for their antimutagenic effect against well established mutagens, 2-aminofluorene, NPD and sodium azide via a renowned genotoxicity assay i.e. Ames assay using TA98 and TA100 tester strains of *Salmonella typhimurium*. The extracts did not show any mutagenicity or marked thinning of the background lawn. The acetone extract from flowering parts of *H. perforatum* showed weak to moderate antimutagenic potential against NPD and sodium azide in TA98 and TA100 strains respectively. However, the same extract showed strong antimutagenic response against S9 dependent mutagen (2AF) in both the tester strains (Table 1). Furthermore, the antimutagenic response was found to be dose-dependent. Table 2 indicates that aqueous methanol extract was relatively more effectual as compared to the acetone extract in inhibiting the mutagenic effect of various mutagens. A strong reversal of sodium azide induced mutagenicity (59.79%) was observed at the highest dose of 2.5 x 10^3 µg / 0.1 ml / plate in the pre-incubated cultures in TA100 strain. When tested against 2AF, the activity was found to be dose-dependent, and at the highest tested concentration, an inhibition of 79.88% was observed in the pre-incubation mode in TA98 strain. In TA100 strain, the diminution of number of revertant colonies was 81.19% in the co-incubated mode at the concentration of 1.0 x 10^3 µg / 0.1 ml / plate (Table 2). Since aqueous methanol extract was found to be more potent, it was fractionated using hexane, chloroform, ethyl acetate and *n*-butanol. Out of all the fractions tested, only ethyl acetate fraction showed significant antimutagenic activity. Table 3 exhibits the antimutagenicity of ethyl acetate fraction in opposition to the mutagenic effect of NPD and sodim azide. The fraction reduced the mutagenicity of NPD and sodium azide in a dose-dependent manner in TA98. It inhibited NPD by 56.24% in pre-incubation mode. In TA100, the maximum effects observed were

49.37% and 50.25% in co- and pre-incubation assays respectively. Upon blending with S9 mix, the effect significantly amplified to 83.21% in the pre-incubation experiment in TA98 strain. However, against the S9 dependent mutagen (2AF) a remarkable modulatory effect of 93.12% was observed upon pre-incubation in TA100. As shown in Table 3, a clear dose-response relationship is seen between the concentration of ethyl acetate fraction and the suppression of mutagenicity. Better inhibitory effect was obtained in pre-incubation mode of treatment in all the experiments. The extracts and the ethyl acetate fraction were shown to cause precipitation of the S9 at their highest concentration tested (Tables 1–3)

Table 1. Effect of acetone extract of *Hypericum perforatum* on the mutagenicity of NPD, sodium azide and 2AF in TA98 and TA100 strains of *Samonella typhimurium.*

Treatment	Dose (µg/0.1ml)	TA98						TA100					
		-S9			+S9			-S9			+S9		
		His$^+$ revertants/plate Mean ± S.E.		Percent inhibition	His$^+$ revertants/plate Mean ± S.E.		Percent inhibition	His$^+$ revertants/plate Mean ± S.E.		Percent inhibition	His$^+$ revertants/plate Mean ± S.E.		Percent Inhibition
Spontaneous		16.89 ± 6.75			33.33 ± 2.05			205.78 ± 18.97			229.22 ± 13.64		
NPD	20	1085.56 ± 63.19											
Sodium azide	2.5							1063.67 ± 37.39					
2AF	20				1012.00 ± 36.64						1063.67 ± 34.02		
Co-Incubation	0.01x10^3	916.67 ± 29.99		15.56	586.78 ± 38.16		43.45	957.56 ± 10.04		12.72	629.89 ± 58.84		50.94
	0.10x10^3	928.00 ± 16.10		14.66	472.89 ± 48.09		54.94	940.44 ± 10.12		14.24	547.33 ± 35.17		59.37
	0.25x10^3	918.11 ± 7.51		15.86	395.67 ± 35.73		62.86	882.78 ± 6.10		21.86	514.44 ± 56.13		63.19
	0.50x10^3	792.33 ± 24.80		27.83	361.33 ± 38.71		66.47	842.67 ± 11.75		26.98	477.33 ± 42.31		71.02
	1.00x10^3	669.78 ± 104.99		39.42	314.11 ± 43.46		71.16	797.44 ± 11.15		32.35	442.67 ± 57.13		73.79
	2.50x10^3	721.67 ± 28.11		34.46	a			746.89 ± 13.77		37.42	a		
Pre-Incubation	0.01x10^3	959.89 ± 13.51		11.58	421.00 ± 26.34		60.39	970.22 ± 10.35		11.20	591.89 ± 37.33		55.40
	0.10x10^3	964.11 ± 14.99		11.30	400.89 ± 44.14		62.28	951.44 ± 11.40		12.97	546.56 ± 39.51		59.46
	0.25x10^3	919.89 ± 15.07		15.69	359.44 ± 37.95		66.55	888.78 ± 12.87		21.14	530.56 ± 48.67		63.19
	0.50x10^3	913.67 ± 21.18		16.31	326.78 ± 62.81		70.00	848.56 ± 15.65		26.26	471.89 ± 67.79		71.02
	1.00x10^3	799.56 ± 19.26		27.12	268.11 ± 51.72		75.86	878.56 ± 4.57		22.50	409.33 ± 52.38		77.75
	2.50x10^3	643.11 ± 2.34		41.90	a			787.11 ± 11.01		32.67	a		

Data shown are MEAN ± SE of three repeated experiments.
a: Extract caused precipitation of S9 at this concentration

Table 2. Effect of aqueous methanol extract of *Hypericum perforatum* on the mutagenicity of NPD, sodium azide and 2AF in TA98 and TA100 strains of *Salmonella typhimurium.*

Treatment	Dose (µg/0.1ml)	TA98				TA100			
		-S9		+S9		-S9		+S9	
		His$^+$ revertants/plate Mean ± S.E.	Percent inhibition	His$^+$ revertants/plate Mean ± S.E.	Percent inhibition	His$^+$ revertants/plate Mean ± S.E.	Percent inhibition	His$^+$ revertants/plate Mean ± S.E.	Percent Inhibition
Spontaneous		32.11 ± 2.38		29.00 ± 2.06		228.11 ± 1.20		193.44 ± 15.02	
NPD	20	1292.67 ± 12.14							
Sodium azide	2.5					959.11 ± 33.47			
2AF	20			936.44 ± 43.72				1089.00 ± 35.85	
Co-Incubation	0.01x10^3	1237.56 ± 18.68	4.37	473.33 ± 45.74	51.31	986.89 ± 36.97	-3.81	482.67 ± 18.35	69.96
	0.10x10^3	1083.56 ± 13.91	16.58	453.33 ± 35.62	53.38	914.78 ± 27.72	6.07	469.67 ± 37.93	71.59
	0.25x10^3	972.11 ± 24.76	25.40	376.33 ± 63.83	61.78	846.44 ± 24.69	15.39	430.44 ± 43.01	73.33
	0.50x10^3	913.22 ± 24.67	30.13	316.89 ± 36.27	68.59	792.22 ± 23.56	22.81	409.56 ± 37.18	78.82
	1.00x10^3	784.67 ± 23.51	40.31	235.89 ± 40.26	77.43	665.00 ± 17.65	40.24	368.33 ± 52.86	81.19
	2.50x10^3	690.11 ± 21.57	47.93	a		536.00 ± 23.50	57.78	a	
Pre-Incubation	0.01x10^3	1241.67 ± 25.89	4.04	469.78 ± 41.66	51.71	891.56 ± 43.05	9.26	517.33 ± 28.92	65.96
	0.10x10^3	1113.11 ± 19.61	14.24	434.11 ± 43.61	55.50	872.89 ± 25.52	11.80	477.67 ± 34.50	70.67
	0.25x10^3	874.11 ± 28.76	33.17	358.67 ± 51.58	63.73	807.67 ± 20.98	20.68	436.00 ± 36.64	72.71
	0.50x10^3	765.33 ± 29.17	41.87	357.89 ± 59.76	64.05	679.00 ± 24.34	38.28	406.44 ± 43.93	79.08
	1.00x10^3	666.33 ± 18.16	49.70	213.67 ± 35.70	79.88	588.89 ± 21.55	50.65	374.44 ± 41.01	80.50
	2.50x10^3	590.44 ± 18.64	55.86	a		521.22 ± 20.61	59.79	a	

Data shown are MEAN ± SE of three repeated experiments.
a: Extract caused precipitation of S9 at this concentration

Table 3. Effect of ethyl acetate fraction of *Hypericum perforatum* on the mutagenicity of NPD, sodium azide and 2AF in TA98 and TA100 strains of *Salmonella typhimurium.*

Treatment	Dose (µg/0.1ml)	TA98						TA100					
		-S9			+S9			-S9			+S9		
		His$^+$ revertants/plate Mean ± S.E.		Percent inhibition	His$^+$ revertants/plate Mean ± S.E.		Percent inhibition	His$^+$ revertants/plate Mean ± S.E.		Percent inhibition	His$^+$ revertants/plate Mean ± S.E.		Percent Inhibition
Spontaneous		29.11 ± 1.71			38.44 ± 1.49			220.33 ± 34.74			247.89 ± 5.51		
NPD	20	1203.11 ± 25.47											
Sodium azide	2.5							1055.44 ± 33.44					
2AF	20				1107.22 ± 28.03						847.33 ± 14.62		
Co-Incubation	0.01x10^3	1057.78	± 32.23	12.39	1014.56	± 18.99	8.67	1017.67	± 39.09	4.79	775.78	± 25.36	11.51
	0.10x10^3	1026.00	± 16.21	15.13	724.00	± 7.68	35.77	923.33	± 46.59	16.63	727.22	± 9.88	19.75
	0.25x10^3	930.44	± 19.17	23.27	567.11	± 14.42	50.39	860.67	± 20.24	24.51	574.89	± 13.33	43.75
	0.50x10^3	770.67	± 18.51	36.90	415.22	± 9.81	64.65	800.44	± 22.79	31.73	517.44	± 14.13	54.46
	1.00x10^3	731.67	± 24.09	40.22	303.56	± 19.39	74.91	726.56	± 14.57	40.66	337.78	± 9.74	84.19
	2.50x10^3	578.11	± 26.78	53.35	a			666.00	± 20.81	49.37	a		
Pre-Incubation	0.01x10^3	1028.78	± 15.33	14.86	1038.33	± 28.44	6.44	984.33	± 24.54	9.02	820.33	± 30.19	4.34
	0.10x10^3	891.22	± 18.18	26.64	803.11	± 19.13	28.39	966.11	± 26.20	11.24	656.78	± 26.32	31.34
	0.25x10^3	817.78	± 25.91	32.88	605.66	± 25.73	46.80	780.11	± 17.54	34.65	568.44	± 25.15	44.79
	0.50x10^3	720.89	± 23.49	41.15	449.00	± 20.76	61.49	790.67	± 19.48	32.95	447.22	± 22.84	66.05
	1.00x10^3	594.89	± 23.82	51.89	214.44	± 22.11	83.21	719.11	± 21.91	41.58	283.78	± 19.30	93.12
	2.50x10^3	544.33	± 27.89	56.24	a			659.00	± 19.91	50.25	a		

Data shown are MEAN ± SE of three repeated experiments.
a: Fraction caused precipitation of S9 at this concentration

Discussion

A critical analysis of our results confirmed that the extracts suppressed the S9-dependent mutagenicity more competently than the direct-acting mutagenicity. The defensive effect of both the extracts and ethyl acetate fraction on mutagens in the Ames assay may be appropriated to the synergistic interaction of phenolic constituents present in them. It is extensively acknowledged that *H. perforatum* is a rich source of polyphenols viz. hyperforin, hypericin, pseudohypericin, protopseudohypericin, chlorogenic acid, neochlorogenic acid, isoquercitrin, kaempferol, amentoflavone etc. (16). In many earlier studies, polyphenols have been found to show antimutagenicity (17, 18). A number of reports have described phenolics to possess antitumor and anticarcinogenic activities. The mechanisms include interference with the metabolic activation of promutagens, acting as blocking agents and forming adducts with ultimate mutagens and scavenging of free radicals. Against direct acting mutagens, the antimutagenic activity occurs by interacting with the active groups on mutagens or possibly protecting the sites on DNA to be acted upon by mutagen (19). In an earlier study, Silva *et al.* (17) concluded that total ethanolic extract of *H. perforatum* (TE) possesses high antioxidant activity. They suggested that the consumption of TE and, certain fractions, either as pharmaceutical preparations or included in food products, have the potential to confer antioxidant properties with benefit to health. Further, Hunt *et al*. (20) appraised the antioxidant effect of various extracts of *H. perforatum* against the superoxide anions and noted a free radical-scavenging effect for most of the concentrations tested and assumed that this effect could be associated to hypericin, among other compounds. The antimutagenic effect shown by *H. perforatum* is of merit and warrants further investigations to elucidate the molecules and mechanisms responsible for antimutagenicity. Studies are in progress to confirm the antimutagenic principle/s.

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