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Research Article

INHIBITION OF 2-AMINOFLUORENE AND 4-NITRO-*O*-PHENYLENEDIAMINE MUTAGENICITY BY NATURAL FOOD COLORANT PLANT *RUBIA CORDIFOLIA* L.

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ABSTRACT

Rubia cordifolia L. (Rubiaceae) is commonly known as Indian Madder. It is used as natural food dye in various countries. In this study, different extracts were isolated from roots of *Rubia cordifolia* L. and their antimutagenic activity was assessed in Ames Histidine reversion assay using *Salmonella typhimurium* against mutations induced by direct-acting mutagen 4-nitro-*o*-phenylenediamine (NPD) and against S9-dependent mutagen 2-Aminofluorene (2-AF) in TA98 tester strain of *S. typhimurium*. Phytoconstituents of roots of *Rubia cordifolia* were fractionated into highly polar methanol extract (RME) and comparatively less polar chloroform extract (RCE). RME was found to be quite effective in inhibiting the mutagenicity of 2AF, i.e. indirect-acting mutagen, in comparison to the mutagenicity induced by direct-acting mutagen i.e. NPD. The inhibitory activity against NPD was 41.69% and 58.21% at the maximum dose tested (2.5×10^3 µg/0.1 ml) during coincubation and preincubation mode of experiments, respectively. RME significantly inhibited 2AF-induced histidine revertants by 70.71% and 71.70% respectively during coincubation and preincubation mode of experiments with IC_{50} of 500 µg/0.1 ml in the preincubation mode of experiment. Chloroform extract (RCE) reduced the mutagenicity of NPD by 59.04% in the preincubation mode of experiment at the maximum tested dose of 2.5×10^3 µg/0.1 ml with IC_{50} of 664 µg/0.1ml. RCE completely inhibited the 2AF mutagenicity, as about 99% inhibitory effect was recorded in both coincubation and preincubation mode of treatments.

Keywords: *Rubia cordifolia L.* Mutagenicity, Antimutagenic activity, 4-nitro-*o*-phenylenediamine, 2-Aminofluorene.

INTRODUCTION

Prolonged exposure of genotoxic chemicals present in environment, in food stuffs and various drugs can cause alterations in genetic makeup leading to diseases like cancer. The damage caused to DNA by different environmental mutagens/genotoxins may be a cause of disability in organisms. The accumulation of these DNA alterations/mutations can be associated with different types of diseases like cancer, various degenerative diseases, aging and genetic abnormalities [1, 2]. In the last few decades, the investigation of medicinal plants to explore their biological effects on human beings has acquired great interest [3]. Furthermore, in modern lifestyle, we cannot avoid exposure to carcinogenic risk factors, e.g. cigarette smoke, chronic infections and inflammations, nutrition and dietary factors and various environmental factors [4]. In order to avoid this risk, it is necessary to minimize human exposure to these genotoxins and maximize the exposure to naturally occurring antimutagenic/antigenotoxic agents such as natural plant products [5, 6, 7]. Such compounds may be helpful in preventing diseases like cancer and several mutation-related diseases by revitalizing body defense mechanisms or by acting as protective agents [8].

The search for antimutagenic agents is an important one, since mutagenic and carcinogenic factors are omnipresent in human environment and elimination of all of them seems to be imposible [9]. The enzymes responsible for the activation of the promutagens are present in different cells of mammals and that activation happens frequently [10] and many cases, even a very low exposure to the mutagenic agent may be enough to induce a genotoxic effect. Dietary components express a wide range of activities that can affect carcinogenesis. Naturally occurring substances in foods have been shown in laboratory experiments to serve as dietary antimutagens [11]. The evaluation of bacterial mutagenicity is of particular importance as an initial test for complex mixtures because of the possibility that one or more compounds can be positive [12, 13]. On the other hand, protective action of plants or their metabolites on genetic material has been reported, leading to its repair or to preserve its integrity [14, 15].

In the present study, we investigated the antimutagenic activitiy of roots of *Rubia cordifolia* L. (Rubiaceae). It is commonly known as 'Majeeth' used in various gynecological problems, as blood purifier and in skin care products. Not only in Indian Ayurvedic system, but plant is also used in Korean traditional medicines for the treatment of cough, bladder and kidney stones, joint inflammation, uterine haemorrhage and uteritis [16]. Apart from its medicinal value, this plant has also been used as natural food colorants and as natural hair dyes [17]. Keeping in mind various medicinal and food colorant properties of this plant, this study deals with antimutagenic activity of methanol and chloroform extract of *R. cordifolia* L.

MATERIALS AND METHODS

Chemicals and bacterial strains

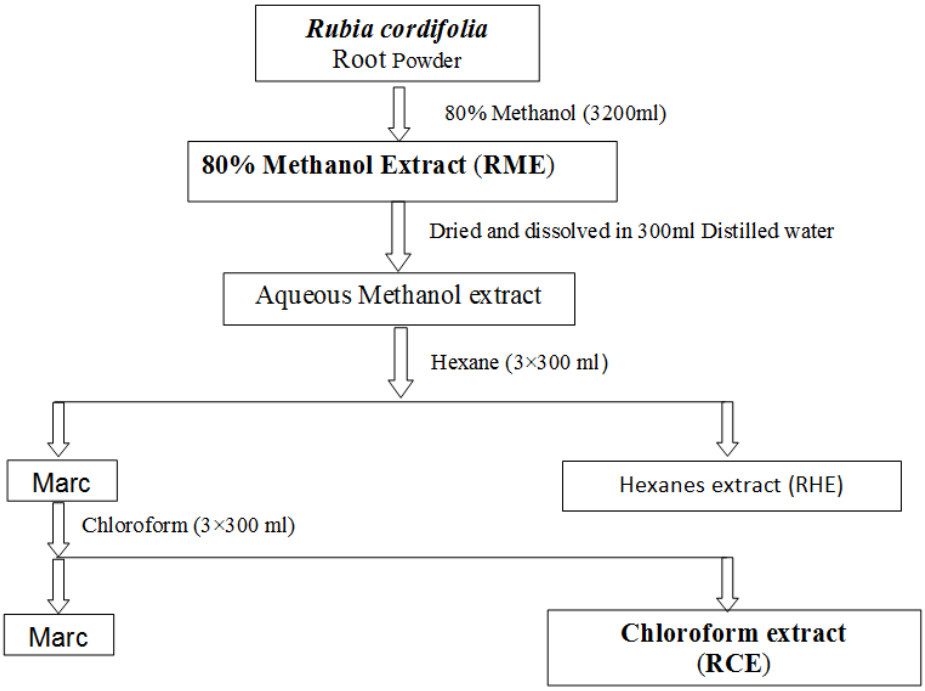
Salmonella typhimurium strain TA98 was purchased form Institut Pasteur, France. 4-Nitro-o-phenylenediamine (NPD) and 2-Aminofluorene (2-AF) were procured from Sigma Chemical Co. (St Louis, MO, USA). Glucose-6-phosphate, l-Histidine, d-Biotin were purchased from Hi-Media, Mumbai, India. All other chemicals employed in the studies were of analytical reagent grade.

Preparation of extracts

Rubia cordifolia L. is a well known medicinal plant. It is commonly known as Indian madder, belonging to the family Rubiaceae. The roots of plant were purchased from local market 'Majith Mandi' in Amritsar. These roots were washed with tap water, dried at room temperature and ground to fine powder. As shown in Flow Chart I the powdered material was extracted with 80% methanol by employing maceration method. The supernatant was filtered using whatman number 1 sheet and concentrated using vacuum rotary evaporator (Buchi Rotavapor R-210). This extract was labelled as *Rubia* methanol extract (RME). RME was made aqueous by dissolving in distilled water. This aqueous methanol extract was partitioned with hexane (3×300ml) for separation of lipophilic compounds. The remaining extract was further fractionated with chloroform (10×300ml). The chloroform soluble portion was collected, pooled and concentrated using vacuum rotary evaporator and labelled as *Rubia* chloroform extract (RCE).

Preparation of diagnostic mutagens (Positive controls)

4-nitro-o-phenylenediamine (NPD) (20 µg/0.1 ml in DMSO) was used as direct-acting mutagen. In the case of S9-dependent mutagen, 2-aminofluorene (2AF; 20 µg/0.1 ml in DMSO) was used for the antimutagenic studies.



Flow Chart I: Extraction of various extracts from roots of *Rubia cordifolia* L.

Thin Layer Chromatography

TLC of the extracts i.e. RME, RCE was carried out along with the standard Alizarin (anthraquinone) using solvent system, Hexane: Ethyl acetate: Glacial acetic acid (65:30:5) (Fig. 1).

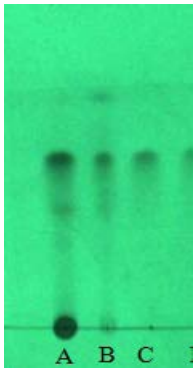


Fig. 1: Thin layer Chromatography of various extracts of *R. cordifolia* L.

A: RME
B: RCE
C: Alizarin(standard)
Solvent System – Hexane : Ethyl acetate: Glacial acetic acid
65 : 30 : 5

Salmonella/Microsome Assay

Antimutagenicity Screening

The antimutagenic potential of extracts from roots of *Rubia cordifolia* was evaluated by using Ames *Salmonella* plate incorporation assay [18] using TA98 tester strain of *S. typhimurium* with slight modification [19]. To check the antimutagenic potential, two sets of experiments were designed.

Co-incubation

Bacterial culture (0.1 ml), 0.1 ml of direct-acting mutagen (NPD) 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.

Pre-incubation

NPD (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 gyrorotary incubator set at 37oC 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 37oC 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:

Inhibitory activity (%) = a-b/a-c × 100

a = Number of histidine revertants induced by mutagen (NPD/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)

Statistical Analysis

Results are presented as the average and standard error of two independent experiments with triplicate plates/dose/experiment. The data were analysed 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.

RESULTS

Antimutagenicity assay

Methanol extract (RME)

RME was found to be quite effective in inhibiting the mutagenicity of 2AF, i.e. indirect-acting mutagen, in comparison to the mutagenicity induced by direct-acting mutagen NPD. The inhibitory activity against NPD was 41.69% and 58.21% at the maximum dose tested (2.5×10^3 µg/0.1 ml) during co-incubation and pre-incubation mode of experiments, respectively (Table-1). RME significantly reduced 2AF-induced histidine revertants as the inhibitory activity of 70.71% and 71.70% was observed during co-incubation and pre-incubation mode of experiments with IC_{50} of 500 µg/0.1 ml in the preincubation mode of experiment (Table-3).

Chloroform extract (RCE)

RCE reduced the mutagenicity of NPD by 59.04% in the pre-incubation mode of experiment at the maximum tested dose of 2.5×10^3 µg/0.1 ml with IC_{50} of 664 µg/0.1ml (Table-2). RCE was quite effective against 2AF-induced mutations. It, in fact, completely inhibited the 2AF mutagenicity, as about 99% inhibitory effect was recorded in both co-incubation and pre-incubation mode of treatments (Table-4).

DISCUSSION

Gene or chromosomal somatic mutations may contribute to acquired disorders such as cancer and germinal mutations and are likely to contribute to inherited defects in the offspring of individuals exposed to mutagenic agents[20]. Mutagenicity and carcinogenicity are toxicological endpoints which pose a great concern being the major determinants of cancers and tumors [21]. The Ames mutagenicity test in *Salmonella typhimurium* is a bacterial short term *in vitro* assay aimed at detecting the mutagenicity caused by chemicals. Mutagenicity is considered as an early alert for carcinogenicity [22]. Over 200 tests that detect chromosomal effects and gene mutation (and related effects) have been developed [23] since the first such test was devised [24]. The original purpose of these early tests, which often are referred to as short-term tests for genetic toxicity, was simply to identify potential carcinogen, an application of the tests that remains controversial [25, 26]. Since the report of Novick and Szilard [27], many of the short-term tests for genetic toxicity also have been used to identify antimutagens. By extention, it appears to be widely assumed that these tests can be used to identify potential anticarcinogens.

Table1: Effect of RME from *Rubia cordifolia* on the mutagenicity of NPD in TA98 tester strain of *Salmonella typhimurium*

Treatment	Dose (µg/0.1 ml)	TA98	
		Mean±SE	Percent Inhibition
Spontaneous		35±2.906	
Positive control			
NPD	20	1192±44.27[a]	
Negative control	2.50×10^3	23±1.358	
	1.00×10^3	22±2.376	
	0.50×10^3	24±1.579	
	0.25×10^3	22±1.174	
	0.10×10^3	26±2.007	
	0.01×10^3	28±2.092	
Coincubation	2.50×10^3	513±26.28[b]	41.69
	1.00×10^3	583±22.45[bc]	39.96
	0.50×10^3	659±47.24[bcd]	37.54
	0.25×10^3	762±68.31[cde]	36.50
	0.10×10^3	809±46.58d[ef]	34.77
	0.01×10^3	916±46.58[efg]	20.93
Preincubation	2.50×10^3	519±30.06[b]	58.21
	1.00×10^3	621±31.01[bc]	49.39
	0.50×10^3	765±6.353[d]	36.93
	0.25×10^3	801±23.10[de]	33.82
	0.10×10^3	903±18.32[ef]	25.00
	0.01×10^3	963±20.76[fg]	19.80

One-way ANOVA:
Positive control and coincubation: F(6, 35)=27.396*: HSD=193.216
Positive control and preincubation: F(6,35)=67.569*: HSD=120.206
Two-way ANOVA:
Coincubation and preincubation
Treatment F(1, 60)=7.49*
Dose F(5,60)=41.005*
treatment×Dose F(5,60)=0.588*

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

Table 2: Effect of RCE from *Rubia cordifolia* on the mutagenicity of NPD in TA98 tester strain of *Salmonella typhimurium*

		Mean±SE	Percent Inhibition
Spontaneous		28±3.591	
Positive control			
NPD	20	1610±137.4[a]	
Negative control	2.50×10^3	31±3.658	
	1.00×10^3	30±2.798	
	0.50×10^3	33±2.565	
	0.25×10^3	27±2.79	
	0.10×10^3	32±1.99	
	0.01×10^3	31±0.966	
Coincubation	2.50×10^3	666±39.20[b]	59.67
	1.00×10^3	843±35.11[bc]	48.48
	0.50×10^3	965±38.53[bcd]	40.77
	0.25×10^3	1018±51.43[cde]	37.42
	0.10×10^3	1247±66.29[def]	22.94
	0.01×10^3	1464±93.53[af]	08.91
Preincubation	2.50×10^3	676±32.56[b]	59.04
	1.00×10^3	763±21.97[bc]	53.54
	0.50×10^3	808±28.57[bcd]	50.69
	0.25×10^3	1003±61.60[cde]	38.37
	0.10×10^3	1009±29.26[cdef]	37.98
	0.01×10^3	1235±66.06[efg]	23.70

One-way ANOVA:
Positive control and coincubation: F(6, 35)=20.72*: HSD=329.98
Positive control and preincubation: F(6,35)=24.03*: HSD=290.94
Two-way ANOVA:
Coincubation and preincubation
Treatment F(1, 60)=16.091*
Dose F(5,60)=45.005*
treatment×Dose F(5,60)=2.182

Data shown are mean±SE of two repeated experiments
a-g within the column with the same letter means does not differ at

Table 3: Effect of RME from *Rubia cordifolia* on the mutagenicity of 2-AF in TA98 tester strain of *Salmonella typhimurium*

		Mean±SE	Percent Inhibition
Spontaneous		37±2.74	
Positive control			
2-AF	20	4854±611.7[a]	
Negative control	2.50×10^3	34±1.58	
	1.00×10^3	38±0.88	
	0.50×10^3	41±1.80	
	0.25×10^3	34±1.12	
	0.10×10^3	38±1.41	
	0.01×10^3	35±1.43	
Coincubation	2.50×10^3	1458±170[b]	70.71
	1.00×10^3	1973±137.8[c]	59.81
	0.50×10^3	2497±157.1[d]	48.93
	0.25×10^3	2912±8.00[e]	40.32
	0.10×10^3	3450±50[f]	18.73
	0.01×10^3	3952±184[g]	29.15
Preincubation	2.50×10^3	1400±100[b]	71.70
	1.00×10^3	2074±168.5[c]	57.73
	0.50×10^3	2220±120[cd]	54.68
	0.25×10^3	3052±0.5[e]	37.41
	0.10×10^3	3844±16[f]	20.97
	0.01×10^3	4157±111.1[g]	14.47

One-way ANOVA:
Positive control and coincubation: F(6, 35)=273.84*: HSD=297.14
Positive control and preincubation: F(6,35)=357.829*: HSD=281.21
Two-way ANOVA:
Coincubation and preincubation
Treatment F(1, 60)=5.574*
Dose F(1,60)=510.837*
treatment×Dose F(5,60)=19.195*

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

Table 4: Effect of RCE from *Rubia cordifolia* on the mutagenicity of 2-AF in TA98 tester strain of *Salmonella typhimurium*

Treatment	Dose (µg/0.1 ml)	TA98	
		Mean±SE	Percent Inhibition
Spontaneous		36±2.963	
Positive control			
2-AF	20	4764±996.8[a]	
Negative control	2.50×10^3	33±1.195	
	1.00×10^3	36±1.701	
	0.50×10^3	42±0.992	
	0.25×10^3	33±0.764	
	0.10×10^3	39±2.778	
	0.01×10^3	39±1.606	
Coincubation	2.50×10^3	44±6.839[b]	99.83
	1.00×10^3	67±0.882[bc]	99.34
	0.50×10^3	376±90.07[bcd]	92.81
	0.25×10^3	1195±74.75[e]	75.49
	0.10×10^3	2519±99.88[f]	47.48
	0.01×10^3	3215±66.23[fg]	32.76
Preincubation	2.50×10^3	56±9.493[b]	99.58
	1.00×10^3	49±11.61[bc]	99.73
	0.50×10^3	216±81.21[bcd]	96.19
	0.25×10^3	1301±51.39[e]	73.24
	0.10×10^3	3451±168.8[f]	23.54
	0.01×10^3	4056±30.2[fg]	14.97

One-way ANOVA:
Positive control and coincubation: F(6, 35)=112.79*: HSD=756.07
Positive control and preincubation: F(6,35)=142.48*: HSD=760.116
Two-way ANOVA:
Coincubation and preincubation
Treatment F(1, 60)=109.165*
Dose F(1,60)=2171.932*
treatment×Dose F(5,60)=87.863*

Data shown are mean±SE of two repeated experiments
a-g within the column with the same letter means does not differ at

In order to unravel the presence of such antimutagenic constituents in the present investigating plant, the extracts of *Rubia cordifolia* were tested against the direct acting mutagen, NPD and S9-dependent mutagen, 2-aminofluorene (2AF), in an attempt to simulate the *in vivo* condition. Both the extracts were quite effective in inhibiting the mutagenicity of 2-AF (Table 3&4). The extracts were also tested against direct-acting mutagen, 4-nitro-o-phenylenediamine (NPD), a frameshift mutagen in TA98 tester strain of *S. typhimurium*. On comparing the inhibitory potency of extracts of *Rubia cordifolia* against 2AF in TA98 tester strain of *S. typhimurium*, chloroform extract (IC_{50} of RCE was 98.6 µg/0.1 ml/plate) was found to be more active than RME (500 µg/0.1 ml/plate) (Table 3&4). Takahashi et al. [28] studied the antigenotoxic activity of purpurin (1,2,4-trihydroxyanthraquinone) produced from madder root (*Rubia tinctorium*).

On comparing the effect of extracts of *Rubia cordifolia* against direct and indirect-acting mutagens, it is evident that they are quite effective in combating the mutagenicity of polyaromatic hydrocarbons (PAH) (2AF in this case) which require metabolic activation to chemically reactive species catalyzed by cytochrome-P450 which, in turn, are believed to exert mutagenic and carcinogenic effects in the biological systems. The mutagenicity/carcinogenicity of PAH may, in part, be due to in situ generation of PAH cation free radicals. Reactive oxygen intermediates, including superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radicals, are known to induce biological damage by reacting with cellular macromolecules, such as, nucleic acids, proteins as well as various membrane components. Against the direct acting mutagen NPD, the extracts exhibited medium to high activity as shown in Table 1&2. Kaur et al. [29] determined the antimutagenicity of phenolic fractions of *Terminalia arjuna* against two direct-acting mutagens, NPD and sodium azide, and against the S9-dependent mutagen 2-aminofluorene (2AF), in TA98 and TA100 tester strains of *Salmonella typhimurium* and found that the phenolic fractions of *T. arjuna* inhibited revertants induced by the S9-dependent mutagen more remarkably than the direct-acting mutagens. There are several reports of polyphenolic substances including anthraquinones exhibiting antimutagenic activities.

One of the mechanisms by which extracts of *R. cordifolia* exhibit antimutagenicity may be due to their antioxidant activity, i.e. scavenging oxygen radicals. Kaur et al. [30] studied free radical scavenging activity of *Rubia cordifolia* and found that extracts were rich in anthraquinones and their glycosides. Earlier, we reported the isolation of an anthraquinone alizarin from *Rubia* cordifolia with very good antimutagenic and antigenotoxic potential [31]. TLC of the RME and RCE of *Rubia cordifolia* shows that it contains alizarin which may account for its present antimutagenic activity. Rao et al. [32] studied the hepatoprotective effects of rubiadin, a major constituent isolated from *Rubia cordifolia* against carbon tetrachloride (CCl_4)-induced hepatic damage in rats. Cai et al. [33] isolated hydroxyanthraquinones from the roots of *Rubia cordifolia* and studied them for their antioxidant activities. Phapale and Thakur, 2010 [33] studied the antimutagenic potential of *Feronia limonia* and *Lagerstroemia speciosa* and also found out the phenolic contents in both the plants. The phenolic contents in *Feronia limonia* was lesser than *Lagerstroemia speciosa*, however it showed significant antimutagenic activity that may be attributed to the other bioactive phytoconstituents present in the plant. Patel *et al*., 2011 [34] evaluated the anticancer activity of root extracts of *Rubia cordifolia* and observed IC_{50} values of 11.92 µg/ml, 21.44 µg/ml, 29.02 µg/ml for methanol extract, petroleum ether extract and dichloromethane extract respectively against Hep-2 cell lines and Hela cell lines.

CONCLUSIONS

The present study suggests that the extracts from the roots of *Rubia cordifolia* possesses antimutagenic properties. As this plant exerts antimutagenic activity, its future pharmacological application is of particular importance because it may be consequential in the

prevention of cancer and develop their application for food and cosmetic industry.

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