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Anti-mutagenic effects of compounds obtained from *Eclipta alba* linn. against strains of *Salmonella typhimurium*

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Abstract: In this experimental study, antimutagenic activity of compounds obtained from *Eclipta alba* Linn was screened by using Ames assay for detecting direct mutagenic activity and those requiring the metabolic activation. The crude compound extracted from *Eclipta alba* was considered as antimutagen and analyzed in this experiment. Two strains of *Salmonella typhimurium,* TA163 and TA96 were used to analyze the test. These two strains are confirmed as histidine requiring mutant strains. When the mutagen is added to the culture, the strain is mutated back, thereby losing the histidine dependence for its growth. By this study, the crude compound of *Eclipta alba* prevents the strain to be mutated back to the non-dependence for the genotyping of the *Salmonella* strains were performed by histidine requirement, rfa mutation analysis, UVrB mutation, R-factor analysis toxicity tests and antimutagenicity assay. The antimutagens obtained from the plant extract were determined for antimutagenic activity against direct acting mutagens and mutagen needing activation. For direct acting mutagens, NPD (N- nitro-o-phenyl diamine), MNNG (N- methyl-N-nitro-N-nitro soguanidine) and NaN_3 (sodium azide) with 1mg of the plant extract gives 98%, 95.2% and 90.7% inhibition the reverted colonies were observed whereas the mutagen needing activation 2-AAf (2-acetyl aminofluorine) gives 96.6% inhibition was observed. These above results indicated that the extract could inhibit the mutagenicity induced by direct acting mutagens as well as mutagens needing activation. Thus the extracts isolated from the test plant have possibility of antimutagenic activity of compound and further biochemicals extracted from the test plant will be analyzed.

Key words: *Eclipta alba*; Antimutagenicity; Strains of *Salmonella typhimurium;* NPD; MNNG; NaN_3; 2-AAf.

Introduction

Mutations occur in somatic cells play an important role in cancer initiation and other subsequent stages of the carcinogenesis.[1] Human begins are constantly exposed to the environment which is highly polluted with chemical and biological contaminants. Many of the pollutants are mutagenic and carcinogenic among which chemical mutagens cause cancer.[1-4] Mutagens induce changes in DNA, produces oncogenic activation at times lead to carcinogenesis. Prevention of the harmful effect of Mutagens in human system is one of the major strategies for cancer control; but not all Mutagens are carcinogens.[5,6]

Large number of plant species act as great source of biologically active compounds whose effect on human health or genetic material is mostly unknown. The usage of plant extracts to heal different types of diseases is very common in traditional medicines[7] that are frequently substituted for modern medicines. In recent years there has been a greater interest in investigating bioactive compounds extracted from plants and their effects on DNA.

This investigation performing with various assays employing different model organisms. Compounds present in plants could act as protective agents with respect to human carcinogenesis, acting against the initiation, promotion, progression stages of this process[8] consequently, the identification of bio active compounds that may reduce Mutations and there by provide benefits as chemo prophylactive agents for cancers are gaining importance. It has been suggested that the use of antimutagen and anticarcinogen in daily life in herbal form will be the most effective procedure for preventing human cancer and genetic diseases. These compounds interfere with Mutagen metabolism or they may act as Mutagen scavenger.[8-10]

Many of the traditional natural compounds like herbs are reported to be antimutagenic and anticarcinogenic. Among them, turmeric has been confirmed that contains curcumin an antioxidant, anticarcinogenic and antimutagenic in nature. Other phytochemicals such as flavanoides, polyphenols are also reported to possess antimutagenicity. Carotenoids are a major group of phytocompounds which are abundantly present in many deep colored fruits and vegetables.

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Epidemiological studies have suggested that carotenoids may decrease the incidence of major clinical disease such as cancer[10] cardiovascular diseases[8] and age related macular degeneration[11] consumption of carotenoid rich diets from fruits and vegetables have been associated with a decreased risk diets from lungs, stomach and prostate cancer.[8,12,13] Lutein is one of the major carotenoid which has been shown to inhibit carcinogenesis.[2,12-17]

In this study we critically evaluated the antimutagenic activities of aqueous extract of *Eclipta alba* against *Salmonella typhimurium* strains (Ames test).

Materials and Methods

The aqueous extract of *Eclipta alba* was done by traditional procedure.[7] The extract was concentrated by petroleum ether evaporation method and it was subjected for further antimutagenic analysis.

Salmonella mutagenicity test (Ames test): The strains used for antimutagenicity studies were *Salmonella typhimurium* TA163 and TA96 TA 163 were kindly supplied from national salmonella phage center Lady Hardinge Medical College, New Delhi. Detected the mutagens that caused base pair substitutions while TA96 detected and involved in various frame shift mutagens. Frozen cultures of the salmonella strains were stored at 20°C.[13] A fresh nutrient broth culture was grown to a density of 2 X 10^9 cells/ml and for each 1 ml of culture 0.09ml of dimethyl sulphoxide was added as a cryoprotective agent; the bacterial culture *Salmonella typhimurium* was inoculated in fresh nutrient broth and grown for 12 hrs at 35°C before each experiment. For this test, Spizizen's salt solution (10x) and histidine 1 biotin solution (0.5 mm) histidine biotin plates, nutrient broth, animal agar plates, ampicillin plate and top agar were prepared and autoclaved.[13,14]

Preparation of mammalian liver s9 fraction: In order to detect the efficiency of the carcinogens requiring metabolic activation were pre-incubated with rat liver fraction (s9 fraction) in presence of *Salmonella typhimurium* before placing to minimal agar plate. The preparation comprises 1.5g of agar and 8.5 ml of distilled water autoclaving for 20 min at 121°C and adding 10 ml of sterile 10 x Spizizen's salt solution ($MGSO_4$ - 0.02g, sodium citrate - 1g, K_2HPO_4 - 14g, KH_3PO_4 - 6g, $(NH4)2SO_4$ - 2g, warm distilled water -100 ml) and 5 ml of sterile 40 % glucose. The liver microsomal enzymes of male wistar rats (150-200g) were induced by giving 0.1% and sodium phenolbarbital in drinking water for 4 days.[6] On the 5th day, animals were scarified liver was removed and washed several times in 0.15M KCl and the homogenate was prepared aseptically. The homogenate was centrifuged at 10000rpm for 10 min at 4°C and the supernatant was filtered using Millipore (0.2 mm) and was used as s9 fraction.[4]

Confirming genotypes of *Salmonella* strains:
Histidine requirement: Cotton swab was dipped in 12 hrs broth culture and a single sweep was made across biotin control plate and histidine / biotin plate, which was prepared by autoclaving 1.5g of agar and 83.4ml of distilled water for 20 min at 121°C and adding 10 ml of sterile spizizen's salt solution along with 5ml of sterile 40% glucose and 1 ml of sterile histidine to the hot agar solution. The solution was cooled slightly and the sterile biotin was added before pouring into petridishes. The plates were incubated overnight at 37°C and the growth was examined on the next day and the histidine character of the test strain was confirmed.[8,13,17]

Rfa mutation: Strains having the deep rough (rfa) character were tested for crystal violet sensitivity. Fresh overnight culture (0.1ml) of the test strains TA163 and TA96 was added to the tube containing 2 ml of molten agar heated at 48°C the top agar tubes containing 0.6 gm of agar and 0.5 g of sodium chloride in 100 ml distilled water were sterilized and vortexed for 30 seconds at low speed and poured on nutrient agar plates without histidine and biotin. The plates were tilted and rotated for the even distribution of top agar on the plates and it was left undisturbed for solidification. 10µl of 1 mg/ ml solution of crystal violet was pipetted to the center of sterile paper disc (1/4inch) and disc were transferred to each of the inoculated plates and slightly pressed using sterile forceps. The plates were incubated at 37°C and observed for crystal violet sensitivity.[13.17]

UVrB Mutation: The UVrB mutation was confirmed by demonstrating uv sensitivity in strains that contain mutation.[4,6] The R- factor strain TA163 and non R-factor strain TA96 were streaked in parallel stripes with sterile swabs in nutrient agar plate. The piece of cardboard was placed over the uncovered plate so that half of the bacterial streak was covered. The plates were irradiated with a 15 W/ germicidal lamp approximately at a distance of 35cm and were irradiated for 8 seconds and incubated at 37°C for 12-24hrs.[13.17]

R-factor: The R-factor of the *Salmonella typhimurium* strain TA163 was tested for the presence of ampicillin resistance factor by streaking the culture across on ampicillin plate, prepared by autoclaving 1.5g of agar and 8ml of distilled water for 20min at 121°C and adding 10ml of sterile spizizen's salt solution along with 5ml of sterile 40% glucose and 1ml of sterile histidine were added. After the solution was cooled slightly

0.6ml of sterile biotin and 0.4ml of sterile ampicillin solution (8mg/ml 0.02N NAOH) were added and poured into sterile petridishes. The non R-factor strain TA96 was tested on the same plate as a control for ampicillin activity.[13,17]

Toxicity: Toxicity was tested using minimal glucose agar medium prepared by autoclaving 1.5g of agar and 85 ml / of distilled water for 20min at 121°C and to which 10ml of sterile spizizen's salt solution along with 5 ml of sterile 40% glucose were added. Different concentration of the plant extract 1 mg, 500 µg, 250 µg and 100µg was added to the medium, mixed well and poured into sterile petridishes and overnight culture of the strains TA163 and TA96 were streaked on to it and the plates incubated for 24hrs.[13,17]

Antimutagenicity assay: Ames test was performed to analyze anti-mutagenicity of plant extract in *S. typhimurium* strains TA163 and TA96 using direct mutagens and those needing activation. All the experiment was done in triplicates.[10,12,13]

Determination of antimutagenicity against mutagens: Antimutagenicity assay without microsomal activation was done by plate incorporation method. Fresh bacterial cultures of *S. typhmurium* strains TA163 and TA 96 (2 x 10^9 cells/ml) were mixed with 2ml of molten agar containing 0.5mM histidine/biotin solution prepared by heating 12.4mg of D-biotin in 100ml of distilled water to which 9.6gm of histidine was added and autoclaved for 20min at 121°C and stored in a glass bottle at 4°C. Different concentration of the plant extract (0.1- 1 mg /plate) and direct acting mutagens such as NaN_3 (2.5µg/plate), N-Nitro-o-phenylene diamine (20µg/plate) or N- methyl N-nitro N-nitroso guanidine (1µg /plate) were added to the molten agar and spread over minimal agar plates. The plates were incubated for 48 hrs at 37°C and the revertant colonies were counted.[5,10,13,17]

Determination of antimutagenicity against mutagens needing activation: Antimutagenicity of *Eclipta alba* extract was tested in *S. typhmurium* strain Ta163 against the mutagen 2- Acetyl-Amino-Fluorine (20 µg / plate) which needs microsomal activation according to the method of plate pre-incubation. 0.5 ml of s9 mix (0.2 M phosphate buffer - 2.5ml, 0.1 M NADP solution - 0.25ml, 1 M glucose-6-phosphate solution - 0.025 ml, 1.6 M KCl - 0.4 ml, $MgCl_2$ solution - 0.1ml, rat liver s9 - 0.5ml, sterile distilled water – 1.675ml) was incubated with mutagens, 0.1 ml of bacterial culture (2 x 10^9 cells/ml) concentration of plant extract and incubated for 30 min at 37°C. Further, it was overlaid on minimal glucose agar plates and incubated for 48 hrs at 37°C and the revertant colonies were counted.[5,10,13,17]

Results and Discussion

The test for histidine requirement showed growth only on histidine plates for the test strains TA163 and TA96 after incubation at 37°C indicating the absolute requirement of histidine /biotin for the strains to grow. A clear zone on inhibition around the crystal violet disc confirmed the rfa mutation of strains TA163 and TA 96. The UV sensitivity of the organism due to UVrB deletion was observed by the growth of TA163 and TA96 only on non-irradiated side of the plates. On the ampicillin plates, growth was observed only along the streaks made with TA 163 which indicated the presence of R-factor.

The addition of plant extract did not inhibit the growth of the *Salmonella* strains which indicated that they are nontoxic to these organisms. At a concentration of 1 mg / plate, plant extract showed 90.7% inhibition in the revertent colony formed by sodium azide (25µg / plate) for 500µg, 250µg, 100µg the salmonella stain TA96 was inhibited at the percentage of 78.5, 57.0 and 29.5 respectively. In case of N-methyl N nitro N-nitroso guanidine (1µg / plate) TA96 revertant, the inhibition rate was 95.2% at a concentration of 1mg plant extract, where as 83.4%, 71.5% and 45.3 % inhibition rate at 500µg, 250µg and 100µg respectively.

Salmonella strain TA96 revertents produced by N-nitro-O-phenylene diamine (20µg / plate) was observed to be inhibited upon 98.0% for 1 mg / plate plant extract and 68.7%, 39.2% and 13% for 500µg, 250µg and 100µg of plant extract respectively. Mutagenicity produced by 2-Acetyl-Amino-Flourine (20µg/plate) to salmonella attain TA163 after its activation by S9 fraction was found to be inhibited in the range of 96.6, 79.2, 63.4 and 42.0% at a concentration of 1 mg, 500µg, 250µg and 100µg respectively. Table.1 highlighted the antimutagenicity of plant extract (*Eclipta alba*) against NaN_3, MNNG, NPD and 2–AAF on *Salmonella typhimurium* strain TA163.

Table 1: Antimutagenicity of plant extract (*Eclipta alba*) against mutagenic agents on *Salmonella typhimurium* strain TA163

Mutagenic agent	Average number of colonies				
	Control	1mg	500µg	250µg	100µg
NaN_3	260	24 (90.7)	56 (78.5)	112 (57)	186 (29.5)
MNNG	273	13 (95.2)	56 (83.4)	87 (71.5)	155 (45.3)
NPD	240	5 (98)	75 (68.7)	246 (39.2)	208 (13)
2-AAF	298	10 (96.6)	62 (79.2)	109 (63.4)	173 (42)

[Figures in parentheses indicate percentages]
NaN_3 – Sodium azide; MNNG – N-methyl-N-nitro-N-nitro Soguandine;
NPD – N-nitro-o-phenylene diamine; 2-AAF – 2-acetyl aminofluorine

It has been suggested that the use of antimutagen in daily life will be the most effective procedure for preventing human cancer and generic disease. These compounds interfere with mutagen metabolism or they may act as mutagen scavengers.[8] It may also inhibit either the initiation or promotion phase of the carcinogenic.[4-10] Same antimutagens occur naturally in sources such as fruits and vegetables.[10] Non – nutritive dietary constituents such as flavonoids, chlorophyllin, dietary fibers, retinoids etc are some of the dietary antimutagens that have a potential role in cancer chemoprevention.[1,13,17]

A varity of short term tests are currently available for the testing of antimutagenic agents. These tests are much less expensive and less lime consuming which makes them attractive for a screening programme. Some these methods include Ames *Salmonella* / microsome assay / sister chromatid exchange and micronucleus tests.[16,17]

Lutein and β-carotene quench preoxy radicals and demonstrate antioxidant properties against oxidative damage.[16] Lutein may be anti-carcinogenic as well because they can interact with mutagens 1- nitropyrene and aflatoxin B1. A lutein may protect against the development of erythema in human skin.[16,17] Studies suggest that the oral supplementation of lutein may protect the skin from UV- damage and reduce the risk factors attributed to the development of skin cancer.[10-12] When supplemented with other carotenoid lutein and zeaxanthin may reduce the rate of growth in cancerous conditions of the breast.[12-14] Lutein was effective in reducing the adhesion molecules on the cell surface of confluent human endothelial cells a necessary modulation of the vasculature in the pathogenesis of artherosclerosis[15,16]. A higher rate of lutein levels in serum lower the risk of coronary heart in inhibiting the inflammation of skin, following intense exposure to uv light.[13]

The above results indicate that plant extract of *Eclipta alba* mutagenicity induced by direct acting mutagens as well as mutagens needing activation in the *S. typhimurium* strains. This plant extract showed most significant inhibition to the mutagencity induced to TA96 by which the direct acting mutagens such as N-methyl N-nitro N-nitroso guanidine and NaN_3 are observed. The plant extract showed most significant inhibition to the mutagenicity induced to TA163 by the direct acting mutagens N-Nitro-o-phenylene diamine and 2-Acetyl-Amino-flourine requiring activities with to S9 preparation. These results indicate to the antimutagenic activity of plant extract. However, antimutagenic activity of plant extract seemed to be dependent on the concentration of extract nature of mutagen, type of *Salmonella* stain and concentration of mutagen used the extract showed most significant inhibition to the mutagenicity induced to TA96 by the direct acting mutagens such as MNNG and NAN_3.[12-16]

Lutein an antimutagenic compound is present in several colored material and has been reported that upto 5 gm/ kg body weight of lutein did not produced toxicity to rats. Lutein can be one of the best chemopreventive agents to fight against cancer as it is readily available, less cost and nontoxic. More research work is needed to find out the potential of this carotenoid as a chemopreventive agent to fight against cancer.[9,10]

Mutagens involved in the physico-biological changes in DNA and leach to carcinogenesis (increase in tumour incidence). Exposure of ionizing radiations and ultra violet may leads to serve biologic effects that induce a cute symptoms like sun burn to chronic squamous cell skin carcinoma and malignant melanoma,[6-9,12-16] the molecules of chemical carcinogens (mutagens) are DNA, RNA & proteins which leads to multistage process consisting, initiation, promotion and progression earlier studies have been[12,13] reported that infection may be responsible for 15% of all malignancies worldwide, the common mechanisms by infection leads to formation of chronic inflammation, transformation of cells by insertion of oncogenes, inhibition of tumor suppressers and induction of immumosuppression,[13,17] thus this experimentation requires further research on finding the biocompound responsible for antimutagenic in nature.

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