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ANTIMUTAGENIC ACTIVITY OF D-LIMONENE

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ABSTRACT

D-Limonene is a monoterpene abundantly present in citrus fruits with excellent medicinal properties. Limonene act on many cellular targets in cancer cells with known chemopreventive activity. Limonene is shown to prevent the growth of many cancer types in preclinical cancer models. The aim is to determine the antimutagenic activity of limonene in bacterial reverse mutation test and *in vivo* micronucleus test. In bacterial reverse mutation test, a combination of limonene with known mutagens exhibited a strong reduction in the number of revertant colonies compared to mutagen alone. Reduction was observed both in the presence and absence of metabolic activation. In micronucleus test a significant reduction in % micronucleated polychromatic erythrocytes was noticed in test animals (limonene + mitomycin C) compared to positive control animals (mitomycin C). Current studies show the significant antimutagenic activity of limonene which might be the rationale for its well known chemopreventive / anticancer activity.

KEY WORDS: Limonene, Antimutagenic, Micronucleus, bacterial reverse mutation test, Mitomycin C

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INTRODUCTION

Mutations and DNA rearrangements are implicated in many important human diseases, including atherosclerosis, autoimmune and neurodegenerative diseases, diabetes and cancer [1]. It is evident that point mutations in oncogenes and tumour suppressor genes of somatic cells are involved in tumour formation in humans and experimental animals. Genotoxicity testing is routinely employed for preclinical screening of new chemical entities and natural compounds. Many naturally occurring compounds with genoprotective activity are known to protect cellular components from genotoxic damage and prevent diseases. There is increasing evidence that plant extracts contain antimutagenic properties. Different prokaryotic and eukaryotic tests, routinely used to detect a variety of mutagens and carcinogens, are suitably adapted for identifying agents with antimutagenic and anticarcinogenic potential, as well as for elucidating their mechanisms of action. In combination with mammalian enzymes, they can provide information about the metabolic activation or detoxification that an agent may undergo in vivo. The bacterial reverse mutation test (BRMT) is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity. It is rapid, inexpensive and relatively easy to perform. Amino-acid requiring strains of *Salmonella typhimurium* are used in the test. These bacterial strains have several features that make them more sensitive for the detection of mutations and provide reliable information on the types of mutations that are induced by genotoxic agents. The principle of bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain [2]. As the tester strains do not posses enzyme systems which are present in mammals to metabolize pro- mutagens to active metabolites capable of reacting with DNA, an external metabolic activation system of post mitochondrial fraction (S9) is added. In vivo test for detection of micronucleus formation is also suitably established to indicate DNA damage caused by carcinogens/mutagens. Micronuclei (MN) are cytoplasmic chromatin containing bodies formed when acentric chromosome fragments or whole chromosomes lag during anaphase and fail to become incorporated into daughter cell nuclei during cell division. Bone marrow polychromatic erythrocytes (PCE) of mice are examined for micronuclei. An increase in the frequency of micronucleated PCE (MNPCE) is an indication of genetic damage [3]. Briefly the animals are treated with limonene prior to the administration of known carcinogen and bone marrow cells were examined for reduction in the MNPCE. D-limonene is a monocyclic monoterpene with a lemon-like odor and is a major constituent in several citrus oils (orange, lemon, mandarin, lime, and grapefruit). It is listed in the Code of Federal Regulation as generally recognized as safe (GRAS) for a flavoring agent [4]. High concentrations of limonene are found in adipose tissue and mammary gland than in less fatty tissues [5]. Limonene has been clinically used to dissolve cholesterol containing gallstones [6] and because of its potential for gastric acid neutralization, it has also been used to relieve heartburn and gastroesophageal reflux disorder (GERD) [7]. Limonene acts on many cellular targets in cancer cells such as; immune modulation, modulating chemical carcinogenesis, anti-oxidant activity and apoptosis but selective inhibition of the prenylation of monomeric G-protein, Ras which regulate cell growth and transformation might be relevant for anticancer effects of limonene [5]. Limonene has well-established chemopreventive activity against many types of cancers. In preclinical cancer models, it has been shown to prevent or delay the growth of a number of cancer types including lymphomas [8], mammary [9], gastric [10], liver [11], lung [12], and prostate cancer [13]. It is also shown to decrease the number of aberrant crypt foci (ACF) in azoxymethane induced putative preneoplastic carcinogenesis in rodents [14]. For many years azoxymethane induced ACF has been used as surrogate biomarkers in the screening of anticancer agents. D-Limonene has been used for many years as a flavor and fragrance additive in the food industry. It is increasingly used as an industrial solvent in the manufacturing of resins, wetting and dispersing agent and in insect control. Occupational exposure to limonene is an inevitable event during its production and use [15]. The average daily dietary intake of limonene has been estimated to be about 0.3 mg/kg b.wt [16]. Therefore the current study is designed to determine antimutagenic activity of D-Limonene in bacterial reverse mutation test [2] and micronucleus test in mice [3] against known mutagens.

MATERIALS AND METHODS

The study was conducted in Sugen Life Sciences Pvt. Ltd, Tirupati and Palamoor Biosciences Pvt Ltd, SVS Medical college campus, Mahaboob nagar, India from January 2015 to May, 2015.

(i) Bacterial reverse mutation test

Chemicals

Magnesium sulphate ($MgSO_4.7H_2O$), Citric acid monohydrate, Potassium phosphate dibasic (K_2HPO_4), Sodium ammonium phosphate, ($NaHNH_4PO_4.4H_2O$), Dextrose, L- Histidine, D- biotin, Sodium chloride, Sodium dihydrogen phosphate (NaH_2PO_4), Disodium hydrogen phosphate (Na_2HPO_4), D- Glucose –6-phosphate, β NADP, Magnesium chloride, Dimethyl sulphoxide, Potassium chloride, Sodium azide (26628-22-8), Mitomycin-C (50-07-7), 2-aminoanthracene (613-13-8), were obtained from sigma. Nutrient broth (oxoid), Rat liver S9(moltox).

Positive controls

2-aminoanthracene (10µg/plate) was used for all tester strains (TA 1535, TA 1537, TA 98, TA 100 and TA 102) in the presence of metabolic activation system, whereas Sodium azide (10µg/plate) and Mitomycin-C (0.5µg/plate) was used for tester strains TA 1535 and TA 102

respectively in the absence of metabolic activation system.

Test strains

The test strains, *Salmonella typhimurium* TA 1535, TA 1537, TA 98, TA 100 and TA 102 were procured form Molecular Toxicology. INC, Boone. NC 28607, USA. Strains were inoculated separately in Oxoid nutrient broth no2 and incubated at 37± 1°C for 14 hours and were then diluted to yield 1-2 x 10^9 cfu/ml by comparing with standard optical density graphs (Maron DM and Ames BN, 1983). Genotyping of the strains was performed prior to study initiation to ensure the genetic integrity.

Treatment

Pre-incubation method both in the presence and absence of metabolic activation system (with and without S9 at 5%v/v) was followed for the evaluation anti-mutagenic properties of D-limonene. Limonene was dissolved in DMSO and used at a concentration of 1.25 mg per plate. External metabolic activation system that is post mitochondrial fraction (S9) was used for the treatment in the presence of metabolic activation system. S9 fraction (5%v/v) is supplemented with cofactor mix (D- Glucose – 6- phosphate, β NADP, and salts) and used. Tester strains were treated with 100 µl of DMSO as a negative control and as well as with known mutagens (Sodium azide, Mitomycin-C & 2-aminoanthracene) which serves as a positive control. On the other hand, limonene at a concentration of 1.25 mg/plate was mixed with the positive control for evaluation of antimutagenic activity. A tube containing 100µl of test culture, 500µl of S9 mix (for test in the presence of metabolic activation) or 500 µl of 0.1 M sodium phosphate buffer (for test in the absence of metabolic activation) and 100µl of limonene and test mutagen was mixed and pre-incubated for 30 minutes at 37± 2°C. The mixture was then added to 2ml top agar and poured on minimal glucose agar (MGA) plates and was incubated for 48 hours at 37± 2°C. Colonies were counted manually. Reduction in colony number was calculated by formula: A – B/A x 100. (A- represents number of colonies with known mutagen; B- represents the number of colonies with limonene and mutagen combination).[2]

(ii) In vivo micronucleus test

Chemicals

Foetal bovine serum (FBS) were purchased from Sigma-Aldrich (St Louis, MO, USA), potassium dihydrogen orthophosphate (KH2PO4), sodium hydroxide (NaOH), Giemsa stain, methanol and carboxy methyl cellulose were purchased from Qualigens Fine Chemicals Pvt. Ltd. (Mumbai, India).

Positive control

Mitomycin C (Sigma-Aldrich) was used as positive control to induce DNA damage.

Animals

The experiments were performed on male Swiss albino mice (8 weeks old) obtained from CPCSEA approved vendor (Mahaveer enterprises, Hyderabad, India). All experimental procedures were approved by the Institutional Animal Ethics Committee. The mice were housed in separate polypropylene cages in experimental room. All the animals were maintained at 24 ± 2° C and the relative humidity set at 30-70% with a 12:12-hours light-dark cycle. They were fed with standard pellet feed purchased from Nutri lab (Hyderabad, India) and UV sterilized water *ad libitum*. Mice were acclimatized in the experimental room for five days before beginning the experiment.

Grouping and treatment

Swiss albino mice (n=20) were divided into four groups viz., G1 (vehicle control), G2 (positive control; mitomycin C only), G3 and G4 (test groups; limonene + mitomycin C). Each group comprises of five animals (n=5). G1 mice were administered orally with 1.0 % carboxy methyl cellulose (CMC). G2 mice were administered intraperitoneally (i.p) with mitomycin C at 2mg/kg b.wt to induce micronuclei formation; whereas G3 and G4 mice were administered orally with a single dose of limonene (in CMC) at 1000 and 2000 mg/kg b.wt respectively followed by mitomycin C (i.p) at 2 mg/kg b.wt. For animals in test groups the limonene was administered 2 hours prior to the mitomycin C injection. The compounds were administered at a volume of 10 ml/kg b.wt. [3]

Preparation of bone marrow smears

All the animals were sacrificed by CO_2 asphyxiation at 24 hours after mitomycin C administration. Both femoral bones were excised and bone marrow was aspirated into fetal bovine serum (FBS) and centrifuged (Hettich-Universal 320R) at 425g for ten minutes. The supernatant was removed leaving 0.5 ml of FBS along with cell pellet for smear preparation. Smears were air dried, fixed in methanol and stained with 5% Giemsa stain (in phosphate buffer; pH 6.8) for 15-20 minutes and mounted with DPX mountant. The slides were observed under light microscope (Olympus CX21, Japan) with 1000x magnification for the presence of micronucleus. All the slides were given code numbers prior to the examination. [3]

Examination of slides

For each mouse 400 normochromatic erythrocytes (NCE) to its corresponding polychromatic erythrocytes (PCE) were counted and P/E ratios (PCE/PCE+NCE) were calculated to determine the cytotoxicity. To determine the genoprotective activity a minimum of 4000 polychromatic erythrocytes per animal was screened and the % micronucleated polychromatic erythrocytes (%MNPCE) were calculated [3].

Statistical analysis

The data of micronucleus test were analyzed by one way ANOVA followed by Dunnett pair wise comparison t test incase the ANOVA result is significant. Analysis was done using Graph pad prism 5. The genoprotective activity of limonene was determined by comparing the %MNPCE of test groups vs. positive control. $p<0.05$ is considered as significant.

RESULTS

All the strains used in this assay retained their genetic characters, as confirmed by genotyping; that shows suitability and validity of the test system used. Test strains were treated with known mutagens with and without limonene and the number of revertant colonies and % reduction were shown in Table1. Test strains (TA 1535, TA 98, TA 100 and TA 102) treated with a combination of positive control (2-aminoanthracene;10µg/plate) and limonene in the presence of metabolic activation system were shown reduction in colony number ranging from 30-67% compared to the positive control, whereas in TA 1537 strain such reduction was not observed. However TA 1535 strain in the absence of metabolic activation shows 32% reduction in colony number when treated with sodium azide (10µg/plate) and limonene combination. A similar finding was observed in TA 102 strain also without metabolic activation. It showed a reduction of 69% with a combination of positive control (mitomycin C; 0.5µg/plates) and limonene.

Table1

Antimutagenic activity of limonene in bacterial reverse mutation test

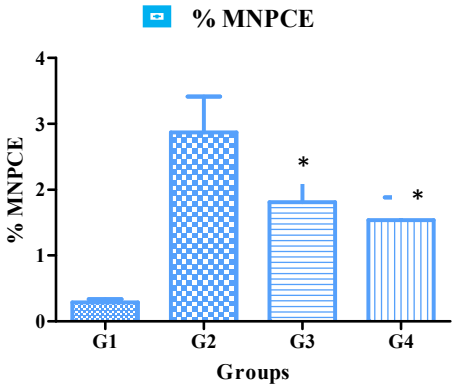
S.No	Bacterial Strains	With S9				Without S9			
		NC	PC	PC+LIM	%Reduction	NC	PC	PC+LIM	%Reduction
1	TA98	28±5.00	471±50.47	158.5±7.77	66.35	NT	NT	NT	NT
2	TA100	125±15.23	920±120.02	298.5±70.00	67.55	NT	NT	NT	NT
3	TA102	276±45.67	1150±250.27	696.5±55.86	39.43	250±41.28	1200±300.91	371±41.01	69
4	TA1535	15±3.29	259±35.52	179.5±64.34	30.69	14±2.80	240±35.18	162±56.56	32.5
5	TA1537	8±2.19	148±26.78	356±73.53	NR	NT	NT	NT	NT

Limonene was used at a concentration of 1.25mg/plate. Combination of limonene with known mutagens exhibited a strong reduction in colony counts compared to mutagen alone. Reduction was observed both in the presence and absence of metabolic activation. NC- negative control; PC- positive control; LIM- limonene; NR- no reduction; NT- not tested. Data were shown as mean ± SD of revertant colonies.

Bone marrow polychromatic erythrocytes were examined for the frequency of micronuclei. Results of % MNPCE were expressed as group mean ± SD in Fig-1. A decrease in % MNPCE in test groups (limonene + Mitomycin C) when compared to positive control (Mitomycin C) group indicates antimutagenic efficacy of limonene. Statistically significant ($p<0.05$) reduction in % MNPCE was observed with oral administration of limonene at 1000 mg/kg b.wt (1.811±0.55) and 2000 mg/kg b.wt (1.540±0.34) compared to positive control group (2.869±0.54). When G3 vs. G4 were compared a dose dependent decrease in % MNPCE was observed, however the difference was not significant. The frequency of % MNPCE in vehicle control group (0.288±0.05) was found to be within the normal range of available historical control data.

Figure 1

Antimutagenic activity of limonene in micronucleus test in mice



G1- Vehicle control; G2- Mitomycin C; G3- Mitomycin C + limonene 1000 mg/kg b.wt; G4- Mitomycin C + limonene 2000 mg/kg b.wt. A significant reduction was observed in % MNPCE of test groups (G3 & G4) compared to positive control (G2). *p<0.05.

DISCUSSION

Since mutations cause many human diseases, identifying natural compounds with antimutagenic activity could be beneficial. Current study shows that limonene is having strong antimutagenic activity both *in vitro* and *in vivo* experimental systems. The antimutagenic mechanism of limonene may be multifactorial. Lipid peroxidation is a natural process of biological system by the effect of reactive oxygen species (ROS) and has been implicated in the pathogenesis of several diseases including cancer. The end product of lipid peroxidation such as malondialdehyde is capable of forming DNA adducts [17] and potential carcinogen. A strong experimental evidence exists that limonene is an excellent cholesterol lowering agent and known to prevent lipid peroxidation

possibly by virtue of its antioxidant activity. It is also well known that limonene deposits in high levels in adipose tissue and has demonstrated lipolytic effects [18]. Free radicals that are generated by cellular process may result in the production of many base adducts, as well as DNA strand breaks and cross links; therefore removal of ROS represents an important strategy in the process of antimutagenesis. It is proved in many experiments that limonene is a free radical scavenger and enhances endogenous antioxidant systems known to protect cellular components from oxidative stress [19]. Another possible mechanism that strongly corroborate with limonene's antimutagenic effect is that, it induces phase I and phase II carcinogen-metabolizing enzymes (cytochrome p450), which metabolize carcinogens to less toxic forms and prevent their interaction with DNA [20].

CONCLUSION

Limonene is shown to have diverse cellular and molecular effects both *in vitro* and *in vivo*, but it is not yet apparent which of these activities are practically related to its anticancer effects. However in the current studies it is demonstrated that limonene possess strong antimutagenic activity, which might be the rationale for its well established chemopreventive / anticancer activity.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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