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Anti-mutagenic effect of citral against Mitomycin-C

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Abstract

Objective : To evaluate the anti-mutagenic activity of citral against the known clastogen mitomycin-C. Materials and methods: Citral was tested at 40 and 80 mg/kg body weight (b.w.) against mitomycin-C (MMC-4 mg/kg, b.w.) using micronucleus test system. Micronucleus (MN) frequency was evaluated in bone marrow and peripheral blood erythrocytes in mice and the sampling was done after 24, 48 and 72 h of clastogen treatment. The antioxidant effect of citral was studied *in vitro* by super oxide scavenging activity. Results: The results showed that citral significantly ($p<0.001$) inhibited the MN frequency induced by MMN, further it had exhibited a good super oxide scavenging activity (EC_{50}=19 mg/ml). Conclusion : The observations indicate that continuous presence of citral was essential in minimizing the genotoxic effects of MMC. Besides, it can be suggested that the antioxidant potential of citral was responsible for the anti-mutagenic effect of citral against MMC.

Key words: Citral, Micronucleus test, Mitomycin-C, Antioxidant.

1. Introduction

The extensive and increasing use of chemical substances in industry, public health, agriculture and personal hygiene, has made society increasingly aware of the acute and chronic injury to human health due to chemical exposure. It is widely recognized that most, if not all the cancers, may be due to these environmental and dietary mutagens. This has resulted in greater emphasis on toxicological studies that mainly include chronic toxicity, carcinogenicity, teratogenicity and mutagenicity [1]. Mutagenicity testing assumes importance since the pollutants can cause deleterious somatic and heritable changes without showing any immediate toxic effects. Many a times these defects occur not only due to the presence of genotoxic agents but also due to the lack of antimutagenic/anticarcinogenic agents in our diet. The best way to minimize the effects is by identifying the antimutagens and anticarcinogens in our diet and increasing their use [2]. Micronucleus assay in mice is a well-established method to study the mutagens and antimutagens [3].

Citral, a monoterpene aldehyde, is a major constituent of *Cymbopogon citratus* (lemongrass oil, Family: Graminea). It was known to posses

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antimicrobial, anti-inflammatory, carminative, diuretic, deodorant, CNS stimulating and other effects [4]. Citral was found to possess anti-cancer activity against prostate gland tumour in various strains of rats [5]. The toxicity studies indicate that citral is devoid of major toxicity and carcinogenic potential in both mice and rats [6]. Previously, we had reported the anti-clastogenic effect of citral against cyclophosphamide and nickel chloride induced mutagenicity [7,8].

Further studies are needed to assess the anti-clastogenic potential of citral and to understand the role of citral in anti-mutagenicity. Hence the present work was undertaken to evaluate the anti-mutagenic activity of citral against a potent cytotoxic agent Mitomycin-C and to study the antioxidant effect of citral *in vitro*.

2. Materials and methods

2.1 Antimutagenic activity

2.1.1 Animals

Eight week-old healthy, laboratory-bred, Swiss Albino mice (Mus musculus) of either sex, weighing 25±3 gm were maintained under conventional laboratory conditions prior to the experiment. The experiments were conducted in CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animals, Chennai, India) approved animal house.

Six mice were randomly grouped for individual treatment and each group consisted of 3 males and 3 females. Different groups of animals received citral suspension for 7 consecutive days and on 7th day mitomycin-C (MMC) was administered after 1 h of last dose of citral. The bone marrow and peripheral blood sampling was done after 24, 48 and 72 h of MMC treatment. The group of animals received solvent (Tween 80 with distilled water) only for 7 days were taken as negative control group and the groups treated with acute dose of MMC were considered as positive control group. To find out the effect of citral *per se* on MN induction, 80 mg/kg of citral was administered for 7 days.

2.1.2 Doses, treatment and sampling

Pure citral sample was obtained from Jagdale Scientific Research Foundation (JSRF), Bangalore. A suspension of citral (wt/ml = 0.8928) was made using 2-3 drops of Tween 80 with distilled water and two doses *viz*, 40 and 80 mg/kg body weight were administered through oral route. MMC-4 mg/kg (B.No: 10899, Kyowa Hakko Kogyo Co Ltd, Japan) was dissolved in distilled water and administered by intra-peritoneal route [9].

2.1.3 Bone marrow MN test and scoring

The same experimental animals were used for both peripheral blood MN and bone marrow MN assays. The animals were sacrificed by cervical dislocation. Animals were cut open to excise femur and tibia. Bone marrow MN slides were prepared by using the modified methods of Schmid [7, 9]. Marrow suspension from femur and tibia bones prepared in 5% bovine serum albumin (BSA), was centrifuged at 1000 r.p.m. for 8 min and the pellet was resuspended in a required quantity of BSA.

A drop of this suspension was taken on a clean glass slide and smear was prepared on the glass slide and air-dried. The slides were fixed in methanol, stained with May-Grunwald-Giemsa and MN were identified in two forms of RBCs (i.e. polychromatic erythrocytes as PCEs and normochromatic erythrocytes as NCEs). About 2000 PCEs and corresponding NCEs per animal were scanned for the presence of MN.

2.1.4 Peripheral blood MN test and scoring

Peripheral blood smears were prepared from tail vein within 30 sec followed by cervical dislocation of the animals. The tails of the animals were cut about 2 cm from the tip so as to allow free flow of blood. Then smears were made

on clean glass slides and air-dried. Blood was diluted using BSA suspending medium, if necessary. The slides were fixed in methanol and stained using Wright-Giemsa stain [7,10]. About 2000 NCEs and the corresponding PCEs per animal were scored for the presence of MN.

2.2 Antioxidant activity

A gift sample of riboflavin was obtained from Astra-Zeneca (P) Ltd, Bangalore. Nitro blue tetrazolium (NBT) was purchased from Loba chemie, Mumbai.

Super oxide anion (O_2^-) was generated from the photo reduction of riboflavin and was detected by nitro blue tetrazolium (NBT) reduction method [11]. The reaction mixture contained 6 mM EDTA, 3 µg NaCN, 2 mM riboflavin, 50 mM NBT, KH_2PO_4-Na_2HPO_4 (buffer, pH 7.8) and different concentrations of citral (10, 30, 100 and 300 µg/ml) suspension to make up the final volume to 3 ml. The tubes were illuminated under incandescent lamp for 15 min. The absorbance was recorded at 530 nm and percentage inhibition was determined by comparing the absorbance of treated groups with the control. Catechin was used as the standard antioxidant agent.

2.4 Statistical analysis

The statistical significance of the results was carried out using unpaired *t* - test and one-way Anova. $p<0.05$ was considered to indicate significance.

3. Results

3.1 Micronucleus assay

In bone marrow MN test, citral showed a time and dose dependent inhibition in the MN frequency. MMC when compared with the control had induced a significant ($p<0.001$) nuclear damage from 24 h of exposure. Citral (40 mg/kg) pretreated animals showed a significant ($p<0.01$) inhibition in the MN frequency observed in PCEs and NCEs at 48 h. When citral was tested at a dose of 80 mg/kg, a significant ($p<0.01$) suppression in the micronucleated cells was observed at all the tested time intervals. However, when citral (80 mg/kg) was administered alone, a non-significant alteration in the MN frequency was observed in both bone marrow and peripheral blood MN tests.

The peripheral blood MN assay showed that MMC had induced a significant elevation in MN level from 48 h of treatment. The prior treatment of citral at 40 mg/kg had decreased the MN level in PCEs at 48 h and 72 h of MMC exposure. Further, citral (80 mg/kg) had exhibited a significant ($p<0.001$) protective effect in both PCEs and NCEs at 48 h and 72 h of intervals (Table 1).

3.2 Super oxide Scavenging activity

The super oxide scavenging activity of citral was evaluated at four different concentrations. Citral at 10 and 30 µg showed 42% and 60% super oxide scavenging effect respectively, while at 100 and 300 µg, the inhibition was found to the extent of more than 70% compared with the control. Similar concentrations were tested for catechin and maximum inhibition was observed at 300µg (90% scavenging activity). The EC_{50} values for citral and catechin was found to be 19 mg and 7.9 mg/ml respectively (Table 2).

4. Discussion

The present research indicated that citral exerted a significant ($p<0.01$) inhibition in the number of micronucleated erythrocytes induced by MMC. The continuous availability of citral was found to be effective in limiting the genotoxic effect observed in both bone marrow and peripheral blood MN assays (Table 1).

Poly-chromatic erythrocytes (PCEs) and normo-chromatic erythrocytes (NCEs) were selected to evaluate the percentage level of MN, as they were easy to differentiate depending on the staining

Table 1.
Inhibitory effect of citral on frequency of MN in bone marrow and peripheral blood erythrocytes induced by MMC

Time interval	Treatment (Dose)	Bone marrow micronucleus test		Peripheral blood micronucleus test	
		% MN in PCE (Mean ± S.E)	% MN in NCE (Mean ± S.E)	% MN in PCE (Mean ± S.E)	% MN in NCE (Mean ± S.E)
24 h	Control	0.413 ± 0.030	0.62 ± 0.029	0.14 ± 0.376	0.46 ± 0.024
	Citral (80mg/kg)	0.525 ± 0.055	0.57 ± 0.076	0.24 ± 0.759	0.44 ± 0.049
	MMC (4mg/kg)	1.53 ± 0.199[a]	1.08 ± 0.154[a]	0.18 ±0.216	0.61 ± 0.116
	Citral (40mg/kg)+ MMC 4mg/kg)	1.42 ± 0.096	0.82 ± 0.084*	0.15 ± 0.158	0.51 ± 0.091
	Citral (80mg/kg)+ MMC (4mg/kg)	0.91 ± 0.117***	0.77 ± 0.088**	0.14 ± 0.328	0.56 ± 0.065
48 h	Control	0.42 ± 0.038	0.62 ± 0.028	0.14 ± 0.386	0.46 ± 0.034
	Citral (80mg/kg)	0.52 ± 0.038	0.52 ± 0.034	0.18 ± 0.345	0.45 ± 0.027
	MMC (4mg/kg)	4.42 ± 0.224[a]	2.55 ± 0.141[a]	0.72 ± 0.545[a]	2.21 ± 0.120[a]
	Citral (40mg/kg)+ MMC 4mg/kg)	2.52 ± 0.237***	1.75 ± 0.110**	0.55 ± 0.952	1.56 ± 0.124**
	Citral (80mg/kg)+ MMC (4mg/kg)	1.65 ± 0.179***	1.33 ± 0.146***	0.40 ± 0.154*	1.06 ± 0.122***
72 h	Control	0.40 ± 0.036	0.62 ± 0.030	0.14 ± 0.396	0.45 ± 0.025
	Citral (80mg/kg)	0.52 ± 0.041	0.56± 0.0278	0.16 ± 0.243	0.45 ± 0.031
	MMC (4mg/kg)	5.67 ± 0.243[a]	2.60 ± 0.104[a]	1.04 ± 0.328[a]	3.46 ± 0.155[a]
	Citral (40mg/kg)+ MMC 4mg/kg)	4.45 ± 0.271**	2.19 ± 0.163	0.53 ± 0.641	2.26 ± 0.157***
	Citral (80mg/kg)+ MMC (4mg/kg)	3.44 ± 0.161***	1.82 ± 0.101***	0.30 ± 0.216***	1.72 ± 0.088***

Statistics: Unpaired *t* test a p<0.001 compared with control, n=6; * p<0.05 ** p<0.01 ***p <0.001 compared with MMC
MN- Micronuclei, PCEs-Polychromatic erythrocytes, NCEs-Normochromatic erythrocytes, MMC-Mitomycin-C.

characteristics and to observe the extent of nuclear damage during erythropoiesis [9].

Presence of more than 6% of MN in the erythrocytic population indicates genotoxicity [10]. The other commonly employed methods to evaluate the mutagens or antimutagens include Mammalian chromosomal aberration test, Specific locus mutation induction, Sister-chromatid exchange assays, Unscheduled DNA synthesis assays, Salmonella mutation assay, *E. coli* test system, Drosophila test system, etc. [12].

In the present work, the mutagenic potential of citral was evaluated at a dose of 80 mg/kg (p.o) and the result indicated that there was no significant increase in the frequency of MN in erythrocytes (Table I). Hence citral *per se* lacks mutagenic potential in the tested doses [13].

MMC is an alkylating anti-tumor antibiotic, isolated from *Streptomyces caespotosis*. MMC, considered to be one of the most toxic drugs available clinically, reacts with electron rich areas of susceptible molecules such as nucleic

Table 2.
Percentage super oxide radical scavenging activity

Drug	Concentration (µg)			
	10	30	100	300
Citral	42.31± 1.34	60.01 ± 0.75	73.67 ± 4.38	79.08 ± 2.61
Catechin	62.59 ± 3.71	77.84 ± 3.69	86.37 ± 3.21	91.22 ± 2.97

Values were compared with control and expressed as Mean ± SE n=6

acid and proteins [9]. The clastogenic effect of MMC was reported from mouse bone marrow, mouse lymphoma cells, eukaryotic and prokaryotic cells [14].

Various agents of plant origin like galangin, vitamins, carotenoid, tannic acid were reported to possess anti-clastogenic effect against MMC [14-16]. Earlier reports suggest that antioxidants having scavenging action might prevent the chromosomal damage of mutagens [17]. Carotenoids, vitamins etc. being antioxidant, were also found to possess anti-clastogenic effect in mice [9,18].

In the earlier study, we had suggested that the anti-clastogenic effect of citral against Cyclophosphamide and nickel chloride was due to the antioxidant property of citral [7,8]. Precisely to study the role of citral, the antioxidant activity was evaluated in the present study. The superoxide radicals (O_2^-) were generated from the reduction of riboflavin and detected by the extent of formation of blue formazan from NBT reduction [11].

The study revealed that citral in a dose dependent manner inhibited the oxidative process involved in the formation of free radicals (Table 2). Moreover, citral was reported to prevent the oxidative conversion of beta-carotene to retinoic acid *in vitro* in the human intestinal homogenate [19]. Based on this information, we speculate that citral in the present work might have acted as an antioxidant to induce a scavenging effect in preventing the nucleophilic attack of mitomycin-C.

There are reports in the literature regarding the necessity to include therapeutically safe antimutagens in our diet. Abafado (herbal tea) is popular among folk tradition [1,17]. It has been reported earlier that herbal tea of lemongrass oil was used by the Brazilian folks in the treatment of nervous disturbances like insomnia, anxiety, irritability [4,20]. Naturally occurring antimutagens can be studied as abafado since these agents, being less toxic, could limit the clastogen-related genetic abnormalities in the host cell.

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