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Anti-Clastogenic Effects of Citral

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ABSTRACT

Citral is a major constituent of *Cymbopogon citratus* or lemongrass oil. The anti-clastogenic effect of citral (20 mg/kg) was tested against the known mutagens cyclophoshamide, mitomycin-C and nickel metal ($NiCl_2$) in mice. Micronucleus (MN) frequency was evaluated in both bone marrow and peripheral blood erythrocytes. The sampling was done after 24 h, 48 h and 72 h of clastogen treatment. Results show that citral had significantly ($p < 0.01$) decreased the frequency of MN induced by the three clastogens in bone marrow and peripheral blood erythrocytes.

Keywords: *Citral, Antimutagenicity, Micronucleus test, Clastogens*

The consequences of technological progress and industrial revolution had been the invasion by a large number of chemicals of different classes, of the human environment through air, food and water. It is widely recognized that most, if not all cancers, may be due to various environmental and dietary mutagens that has resulted in greater emphasis on toxicological studies that mainly include chronic toxicity, carcinogenicity, teratogenicity and mutagenicity [1]. Among these, mutagenicity testing assumes prime importance since these environmental and dietary factors can cause deleterious somatic and heritable changes without showing any immediate toxic effect. Many times these defects occur not only due to the presence of genotoxic agents but also due to lack of antimutagenic/anticarcinogenic agents in our diet [1, 2]. The best way to minimize these effects is identifying the antimutagens and anticarcinogens in our diet and increasing their use [2]. Micronucleus antimutagenic assay is a well-established method to study the mutagens and antimutagens [3, 4].

Citral a monoterpene aldehydes is a major constituent of lemongrass oil (*Cymbopogon citratus*) which belongs to the family Graminea. It was known to posses antiseptic, antimicrobial, anti-inflammatory, carminative, diuretic and central nervous system stimulating effects [5]. Citral was found to possess anticancer effect against prostate gland tumor in various strains of rats [6]. The toxicity studies indicate that citral is devoid of major toxicity and carcinogenic potential in both mice and rats. It was also reported that citral is devoid of mutagenic effect in *in vitro* models [7]. More over, reports suggested the presence of an anti clastogenic effect of citrus extract against irradiation in mice [8]. Hence the present study was undertaken to evaluate the anti-mutagenic effect of citral against known clastogens: cyclophosphamide (CP), mitomycin-C (MMC) and nickel chloride ($NiCl_2$) in animal mutagenic screening model.

MATERIALS AND METHODS

Animals

Eight week-old healthy, laboratory-bred, Swiss Albino mice (Mus mus'culus), of either sex, weighing 25 ± 3g were maintained under conventional laboratory conditions, at temperature 25±2˚C, and a 12 h natural light period. Commercial pellet diet (Gold Mohur, Lipton India) and tap water were provided *ad libitum*. 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 selected for individual treatment groups 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 clastogens were administered after 1 h of the last dose of citral. The bone marrow and peripheral blood sampling were done after 24, 48 and 72 h of clastogen treatment. The groups of animals received only vehicle for 7 days were taken as negative control group and the group treated with acute dose of clastogen was considered as positive control group. To find out the effect of citral *per se* on MN induction, 50 mg/kg of citral was administered for 7 days and the MN frequency was evaluated.

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 tween 80 and distilled water and 20 mg/kg body weight was administered orally.

The clastogens CP [3], MMC [3] and $NiCl_2$ [9] were dissolved in distilled water and administered by intraperitoneal route.

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 killed by cervical dislocation. The femur and tibia were excised. Bone marrow MN slides were prepared by using the modified method of Schmid [3]. Marrow suspension from femur and tibia bones prepared in 5% bovine serum albumin (BSA), was centrifuged at 1000 rpm and the pellet was resuspended in BSA solution. A drop of this suspension was placed on a clean glass slides and smears were prepared and the slides were air-dried. The slides were fixed in methanol, stained with May-Grunwald-Giemsa and MN was identified in two forms of RBCs (i.e. polychromatic erythrocytes as PCEs and normochromatic erythrocytes as NCEs). About 2000 PCEs and corresponding NCEs per animals were scanned for the presence of MN.

Peripheral Blood MN Test and Scoring

Peripheral blood smears were prepared from tail vein within 30 seconds after 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 stains [10]. About 2000 NCEs and the corresponding PCEs per animal were scored for the presence of MN.

Statistical Analysis

The statistical significance of the results was tested using nonpaired t-test and one-way Anova [3].

RESULTS

The bone marrow MN assay indicated that citral had inhibited the MN percentage induced by the clastogens. When citral (50 mg/kg) alone was tested, a non-significant increase in the frequency of micronucleated erythrocytes was observed in bone marrow and peripheral blood MN tests. CP had affected nuclear damage after 48 h while MMC and $NiCl_2$ had produced clastogenicity after 24 h of exposure. Citral prevented nuclear damage, in a time dependent manner, as observed from the decreased MN frequency in PCEs and NCEs. Citral had induced a significant ($p < 0.01$) prevention of MN levels at the three tested time intervals. One way Anova indicated a highly significant ($p < 0.001$) effect at 72 h (F=337.49 for PCEs and F=162.19 for NCEs).

In peripheral blood MN test, the MN induction was observed after 48h of exposure. CP had shown the significant ($p < 0.001$) elevation of MN level from 72 h

Table 1. Inhibitory effect of citral on the frequency of MN in bone marrow and peripheral blood erythrocytes induced by CP, MMC and $NiCl_2$.

Time interval	Treatment (Dose in mg/kg)	Bone Marrow Micronucleus Test		Peripheral Blood Micronucleus Test	
		% MN in PCE (Mean ± SEM)	%MN in NCE (Mean ± SEM)	% MN in PCE (Mean ± SEM)	%MN in NCE (Mean ± SEM)
24h	Control	0.4133 ± 0.030	0.62 ± 0.029	0.14 ± 0.376	0.46 ± 0.024
	Citral (50)	0.505 ± 0.071	0.44 ± 0.020	0.16 ± 0.055	0.51 ± 0.151
	CP (50)	0.56 ± 0.085	0.67 ± 0.060	0.16 ± 0.795	0.55 ±0.077
	MMC (4)	1.55 ± 0.199^c	1.08 ± 0.154^c	0.18 ±1.216	0.61 ± 0.116
	$NiCl_2$ (10)	0.84 ± 0.058^b	0.77 ± 0.058^a	0.15 ± 0.788	0.52 ± 0.039
	Citral (20) + CP (50)	0.59 ± 0.062	0.60 ± 0.103	0.15 ± 0.632	0.49 ± 0.981
	Citral (20) + MMC (4)	1.21 ± 0.650^a	0.78 ± 0.340^a	0.16 ± 0.370	0.52 ± 0.408
	Citral (20) + $NiCl_2$ (10)	0.64 ± 0.140^a	0.62 ± 0.092^a	0.14 ± 0.113	0.48 ± 0.129
48h	Control	0.423 ± 0.038	0.62 ± 0.028	0.14 ± 0.386	0.46 ± 0.034
	Citral (50)	0.53 ± 0.079	0.50 ± 0.017	0.15 ± 0.440	0.48 ± 0.066
	CP (50)	1.75 ± 0.058^c	0.89 ± 0.048^c	0.17 ± 0.956	0.53 ± 0.106
	MMC (4)	4.42 ± 0.224^c	2.55 ± 0.141^c	0.72 ± 1.545^c	2.21 ± 0.120^c
	$NiCl_2$ (10)	1.02 ± 0.073^c	0.84 ± 0.095^b	0.36 ± 0.673^c	0.91 ±0.064^c
	Citral (20) + CP (50)	0.98 ± 0.011^c	0.82 ± 0.057	0.16 ± 0.937	0.49 ± 0.142
	Citral (20) + MMC (4)	2.01 ± 1.033^c	1.43 ± 0.231^c	0.31 ± 0.011^b	0.65 ±0.477^c
	Citral (20) + $NiCl_2$ (10)	0.60 ± 0.002^b	0.61 ± 0.062^a	0.20± 0.841^a	0.59 ± 0.074^b
72h	Control	0.40 ± 0.036	0.62 ± 0.030	0.14 ± 0.396	0.45 ± 0.025
	Citral (50)	0.49 ± 0.023	0.55± 0.017	0.16 ± 0.030	0.43 ± 0.148
	CP (50)	2.17 ± 0.090^c	0.92 ±0.041^b	0.45 ±3.067^c	2.06 ± 0.092^c
	MMC (4)	5.67 ± 0.243^c	2.60 ± 0.104^c	1.04 ±0.328^c	3.46 ±0.155^c
	$NiCl_2$ (10)	1.14 ± 0.067^c	0.86 ± 0.044^b	0.40 ±0.695^c	0.93 ±0.103^c
	Citral (20) + CP (50)	1.29 ± 0.004^c	0.69 ± 0.173^a	0.26 ±0.487^c	0.88 ± 0.132^c
	Citral (20) + MMC (4)	2.78 ± 0.431^c	1.14 ± 0.932^c	0.40 ±0.380^c	1.72 ±0.321^c
	Citral (20) + $NiCl_2$ (10)	0.64 ± 0.189^c	0.59 ± 0.328^a	0.18 ±0.084^c	0.54 ±0.157^b

Statistics: Unpaired 't' test. ^{a} p <0.05. ^{b} p <0.01. ^{c} p <0.001. n=6.
MN = Micronuclei, PCEs = Polychromatic erythrocytes, NCEs = Normochromatic erythrocytes, CP = Cyclophosphamide, MMC = Mitomycin-C, $NiCl_2$ = Nickel chloride.

whereas MMC and $NiCl_2$ had exerted the effect after 48 hr. Citral exhibited anti-mutagenic effect against the three tested clastogens. A significant ($p < 0.01$) prevention of MN level was observed in CP, MMC and $NiCl_2$ administered groups. Anova analysis showed highly significant ($p < 0.001$) group difference at 72 h ($F=107.23$ for PCEs and $F=203.38$ for NCEs) (Table 1).

Discussion

In the present investigation, the anti-clastogenic potential of citral (20 mg/kg) was evaluated against three known mutagens *viz*; cyclophosphamide (CP), mitomycin-C (MMC) and nickle chloride ($NiCl_2$). The results showed a time-dependent inhibitory effect of citral in the frequency of micro-nucleus (MN) in the poly-chromatic erythrocytes (PCEs) and Normo-chromatic erthrocytes (NCEs). Two types of erythrocytes (PCEs and NCEs) were selected to evaluate the percentage level of MN, as they were easy to differentiate depending on staining characteristic and to observe the extent of nuclear damage during the erythropoiesis [3].

The micronucleus test developed by Schmid and coworkers has become a widely used method to evaluate mutagens and antimutagens [11]. Presence of more than 6% of MN in the erythrocytic population indicates genotoxicity [12]. The other commonly employed methods to evaluate the mutagens or antimutagens include mammalian chromosomal aberration test, specific locus mutation induction in mice, sister-chromatid exchange assays, unscheduled DNA synthesis assay, salmonella mutation assay, E.coli test system and drosophila test system [13].

Citral is an essential oil of *Cymbopogon citratus* (lemon grass oil) and contains the mixture of geometric isomers geraniol and neral. Citral when applied externally was found to exert anti-inflammatory, anti-septic, anti-rheumatism, deodorant and granulation-promoting effect [2, 5]. When administered orally, citral was reported to produce expectorant, appetite stimulant (digestant), choleretic, carminative, spasmolytic, anti-inflammatory, diuretic and sedative action [14]. Apart from citral, lemon grass oil also contains geraniol, myrcene, citronellal, limonene, linalool and dipentene and none of them were reported to be mutagenic [5]. However, to confirm the mutagenic potential, citral was tested at 50 mg/kg. The higher dose was selected as the earlier reports suggested that drugs above the therapeutic concentration might induce nuclear damages responsible for mutagenicity [1, 2]. The results indicated that there was no significant increase in the frequencies of MN in erythrocytes (Table 1). Hence citral *per se* lacks the mutagenic potential in the tested doses [15].

CP and MMC are alkylating anti-tumor agents. These agents after biochemical activation react with electron rich areas of susceptible molecules such as nucleic acid and proteins [3]. The nuclear damage is responsible for the mutagenicity while the effect on proteins will further aggravate the malfunctioning of the host cell [3]. CP has been used to evaluate the mutagenic as well as anti-mutagenic agents and was reported to induce chromosomal damage and MN formation in rats, mice, Chinese hamsters and even in transgenic mouse [16]. Several agents of plant origin like vitamins, eugenol, tannic acid, green tea etc., were reported to possess anti mutagenic effect against CP induced clastogenicity [2, 5]. In our study, the appearance of MN after 48 h in bone marrow and 72 h in peripheral blood indicated that the pro-drug of cyclophosphamide may require completion of one cell cycle to induce the cytotoxic effects in the host cell. The prior treatment of citral showed a decrease in the MN frequency in PCEs, NCEs and the significant inhibition ($p < 0.01$) was observed in bone marrow as well as in peripheral blood tests. The study carried on ascorbic acid further indicated a significant anti mutagenic effect against CP and the response was reported to be mediated through the scavenging action of ascorbic acid against CP [3]. Ascorbic acid being an strong reducing agent was thus reported to inhibit the genotoxicity of CP [3]. Besides, the antioxidant activity of citral was tested in human intestinal homogenates *in vitro* and reports show that citral inhibited the conversion of beta-carotene to retinoic acid by preventing the oxidation process [17]. As one of the mechanisms for the scavenging action is antioxidant activity, in our study also citral might have acted in the similar pattern to decrease the MN percentage.

MMC is an antibiotic isolated from *Streptomyces caespitosis* and considered to be one of the most toxic drugs available clinically [3]. MMC was known to produce the nuclear damage in mouse bone marrow, mouse lymphoma cells and eukaryotic and prokaryotic cells[3]. The anti-clastogenic effect against MMC was reported for galangin, vitamins A and E, carotenoids, tannic acid and asafetida [2, 14]. Among them carotenoids commonly present in carrots, tomatoes etc were found to posses antioxidant activity by the scavenging action[2]. In our investigation citral significantly ($p < 0.001$) inhibited the MMC induced MN formation at 24, 48 and 72 h in both the assays. Moreover, eugenol and ginger oil were found to exert anti-inflammatory and anti-rheumatic activity against mycobacterium tuberculosis induced arthritis in Sprague-Dawley rats. An earlier study indicated a potent anti-inflammatory effect of citral in rats [5]. As one of the mechanism suggested for anti-inflammatory and anti-rheumatism was due to scavenging effect [18], in the present study citral might had exerted the scavenging activity in preventing the mutagenic effects of MMC.

Nickel, long stood out among the six metallic elements (manganese, cobalt, iron, copper, nickel and zinc) as being apparently without any biological function. However recent evidence suggests its importance in physiological regulation of homeostasis of blood. Identification of nickel containing enzymes in plants and also the existence of dietary induced nickel deficiency symptoms in animals including rats, chicken, swine and goats [19, 20] also suggests a role for Ni^{2+} in physiological functions. The clastogenic and carcinogenic potential of Ni^{2+} have been attributed to its affinity towards certain membrane structure, including the poly nuclear membranes, the vacuole wall and numerous lipid structures [20]. The present study showed an anticlastogenic effect of citral against $NiCl_2$. Citral decreased the MN percent significantly (p <0.01) in both PCEs and NCEs tested in bone marrow and peripheral blood erythrocytes. As reported earlier, the aqueous extract of edible dried fruits of *Phyllantus emblica* limited the clastogenicity of nickel chloride. The results further indicated that the anticlastogenic effect was due to ascorbic acid, a major component of the *Phyllantus emblica* fruit [21]. More recent work showed that pre-treatment with vitamin-E had decreased the mutagenic response to nickel chloride in Chinese hamster cell lines [22]. Antioxidant actions of ascorbic acid and vitamin-E have already been shown [3], since citral was found to possess anti oxidant activity [17], it can be suggested that in the present research citral could have exhibited an antioxidant activity to attenuate the nuclear damage induced by the clastogens.

CONCLUSION

In the present investigation, citral had produced a mild anti-clastogenic effect evident from the decreased micronuclei frequency observed in polychromatic and normochromatic erythrocytes. Citral prevented the nuclear damage induced by cyclophosphamide, mitomycin-c and nickel chloride in both bone and peripheral blood micronucleus tests. Further study can be considered to evaluate precisely the role of citral in anti-mutagenicity.

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