ORIGINAL ARTICLE

CITRAL, A COMPONENT OF LEMONGRASS OIL INHIBITS THE CLASTOGENIC EFFECT OF NICKEL CHLORIDE IN MOUSE MICRONUCLEUS TEST SYSTEM

SYED IMAM RABBANI, KSHAMA DEVI, SALMA KHANAM* AND NOOR ZAHRA

*Department of Pharmacology, *Department of Pharmacognosy,*
Al-Ameen College of Pharmacy, Bangalore 560 027

ABSTRACT

Citral is a major component of *Cymbopogon citratus* (lemongrass oil). The aqueous suspension of citral (60 mg/kg body weight, per oral) treated for one week was tested for the anti-clastogenic effect using mouse micronucleus test system. A known mutagen nickel (Nickel chloride-10 mg/kg, b.w. intra-peritoneal) was used to induce the nuclear damage measured in polychromatic erythrocytes and normochromatic erythrocytes. The frequency of the micronucleated erythrocytes were studied in peripheral blood and bone marrow after 24, 48 and 72 hours of mutagenic exposure. The antioxidant activity of citral was tested *in vitro* by superoxide scavenging method. The results indicated that citral significantly ($P<0.01$) inhibited the formation of micronuclei induced by nickel. Further, a good superoxide scavenging activity (EC_{50}=19 mcg/ml) was observed in citral treated groups, suggesting that the antioxidant action could be responsible for the anti-clastogenic effect of citral against nickel chloride.

Keywords: Citral, micronucleus test, nickel chloride, superoxide scavenging activity.

INTRODUCTION

Many chemicals used in the industry, agriculture, personal hygiene and health care are known to cause genotoxicity in the human host system. The extensive use of these chemicals and the presence of several hazardous pollutants in the environment have contributed in the genotoxic complication like mutations (Ames, 1983). Mutation although causes various somatic and heritable disorders, in many instances their effects were not immediately observed in the present generation. Hence, it has become inevitable to test the mutagenic potential of entire chemicals used by the human population (Heo *et al*., 1996). Earlier studies suggest that not only the presence of mutagens, even the lack of anti-mutagens in the host system is responsible for the genetic damages. Since it is difficult to avoid all the mutagens from entering in to our system, the best way to minimize their action will be to identify the therapeutically safe anti-mutagens and increase their use (Hayatsu *et al*., 1988). Micronucleus (MN) assay in mice is a commonly used method to study the mutagens and anti-mutagens (Heddle *et al*., 1983).

Nickel (Ni^{2+}) in the body is required in micro quantity for the regulation of homeostasis of blood, functioning of enzymes and bacteria. It can enter the body by various routes like nasal, gastro-intestinal, dermal as well as by surgical or dental implants and hemodialysis (Costa and Mollenhauer, 1980). Prolonged exposure of Ni^{2+} has been reported to show various toxicities such as renal, teratogenic, cardiovascular, pulmonary, carcinogenic and mutagenic effects (Dhir *et al*., 1991).

Citral a monoterpene aldehyde is a major constituent of *Cymbopogon citratus* (Family: Graminea). It is known to possess antimicrobial, anti-inflammatory, carminative, diuretic, deodorant and CNS stimulating activity (Carbajal *et al*., 1989). Citral has also been found to possess anti-cancer activity against prostate gland tumor in various strains of rat (Carlini *et al*., 1986). The toxicity studies indicate that citral is devoid of major toxicity and carcinogenic potential in both mice and rat (Gomes *et al*., 1998 and Ress, 2003). Previously, we had reported the anti-mutagenic effect of citral against cyclophosphamide-induced mutagenicity (Rabbani *et al*., 2004). Moreover, citral (20 mg/kg) had also shown inhibitory effect ($P<0.05$) against nickel chloride (Rabbani *et al*., 2005). However, no mechanism of action was evaluated and the effect of citral at higher dose was studied. Hence the present work was undertaken to evaluate the anti-clastogenic activity of citral against nickel metal and study the antioxidant property of citral *in vitro*.

MATERIALS AND METHODS

A. Antimutagenic activity

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 Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), Chennai, India approved animal house. The study was conducted according

Corresponding author: e-mail: syedrabbani09@yahoo.com

Table 1: Inhibitory effect of citral on the frequency of MN in bone marrow and peripheral blood erythrocytes induced by nickel chloride

Time interval	Treatment (Dose-mg/kg)	Bone marrow micronucleus test		Peripheral blood micronucleus test	
		% MN in PCEs	% MN in NCEs	% MN in PCEs	% MN in NCEs
24 hr	Control	0.42 ± 0.030	0.61 ± 0.031	0.15 ± 0.039	0.46 ± 0.026
	Citral (60)	0.51 ± 0.055	0.58 ± 0.062	0.19 ± 0.053	0.46 ± 0.017
	$NiCl_2$ (10)	0.83 ± 0.058[c]	0.78 ± 0.016[c]	0.15 ± 0.087	0.52 ± 0.041
	Citral (60) + $NiCl_2$ (10)	0.61 ± 0.029**	0.60 ± 0.035***	0.17 ± 0.040	0.48 ± 0.057
48 hr	Control	0.40 ± 0.036	0.62 ± 0.027	0.13 ± 0.061	0.45 ± 0.052
	Citral (60)	0.42 ± 0.021	0.57 ± 0.041	0.14 ± 0.019	0.47 ± 0.039
	$NiCl_2$ (10)	1.04 ± 0.052[c]	0.82 ± 0.073[a]	0.38 ± 0.041[b]	0.89 ± 0.009[c]
	Citral (60) + $NiCl_2$ (10)	0.68 ± 0.029***	0.61 ± 0.036*	0.21 ± 0.053*	0.64 ± 0.011***
72 hr	Control	0.42 ± 0.014	0.62 ± 0.039	0.15 ± 0.065	0.44 ± 0.021
	Citral (60)	0.44 ± 0.017	0.59 ± 0.081	0.19 ± 0.049	0.48 ± 0.057
	$NiCl_2$ (10)	1.19 ± 0.034[c]	0.85 ± 0.032[c]	0.39 ± 0.037[b]	0.91 ± 0.083[c]
	Citral (60) + $NiCl_2$ (10)	0.72 ± 0.018***	0.67 ± 0.041**	0.28 ± 0.047***	0.68 ± 0.059*

Values are expressed as Mean ± SEM, N=10
Statistics: Unpaired 't' test [a] P<0.05 [b] P<0..01 [c] P<0.001 compared with control,
*P<0.05 ** P<0.01 ***P <0.001 compared with $NiCl_2$
MN-Micronuclei, PCEs-Polychromatic erythrocytes, NCEs-Normochromatic erythrocytes, $NiCl_2$-Nickel chloride.

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, *P<0.001 compared with control

to the guidelines of the U.S Environmental Protection Agency (Movourin *et al*., 1990).

Doses, treatment and sampling

Ten mice were randomly selected for individual treatment group and each group consisted of 5 males and 5 females. Different groups of animals received citral suspension for 7 consecutive days and on 7th day nickel chloride ($NiCl_2$) was administered after 1 hr of last dose of citral. The bone marrow and peripheral blood sampling were done after 24, 48 and 72 hour of $NiCl_2$ treatment. The groups of animals that received solvent only for 7 days were taken as negative control group and the groups treated with acute dose of $NiCl_2$ were considered as positive control group. To find out the effect of citral *per se* on micronuclei induction, 60 mg/kg b.w. of citral was administered for 7 days.

Pure citral sample was obtained from Jagdale Scientific Research Foundation (JSRF), Bangalore, India. A suspension of citral (wt/ml = 0.8928 gm/ml) was made using 2-3 drops of Tween 80 with distilled water and one dose viz: 60 mg/kg b.w. was administered through oral route. $NiCl_2$-10 mg/kg b.w. (B.No: 903161, Nice Chemicals, Cochin, India) was dissolved in distilled water and administered by intra-peritoneal route (Dhir *et al*., 1991).

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 dissected out to excise femur. Bone marrow MN slides were prepared by using the modified method of Schmid (Vijayalaxmi and Venu, 1999). Marrow suspension from femur bone prepared in 5% Bovine Serum Albumin (BSA), was centrifuged at 1000 rpm and the pellet was resuspended in a required quantity of BSA. A drop of this suspension was taken on 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 were identified in two forms of RBCs (i.e. Polychromatic erythrocytes as PCEs and normochromatic erythrocytes as NCEs) using trinocular microscope under oil immersion objective (Plate 1). About 2000 PCEs and corresponding NCEs per animals were observed for the presence of MN.

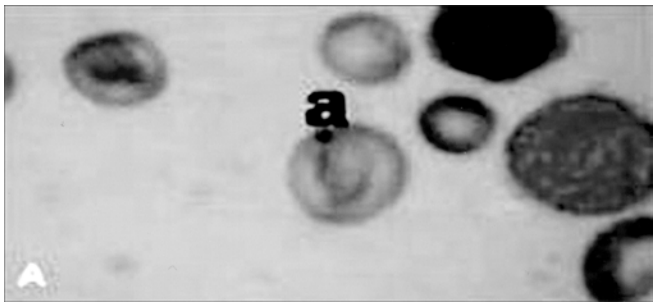

Plate 1: Photography showing micronucleated polychromatic erythrocyte in the bone marrow micronucleus test.

Peripheral blood MN test and scoring

Peripheral blood smears were prepared from tail vein within 30 seconds 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 stains (Schlegel and MacGregor, 1982). About 2000 NCEs and the corresponding PCEs per animal were scored for the presence of MN (Plate 2).

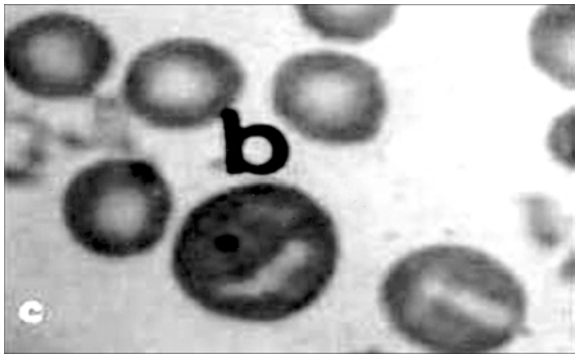

Plate 2: Photography showing the micronucleated normochromatic erythrocytes in peripheral blood micronucleus test.

B. 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, India.

Super oxide anion ($O_2^{\cdot-}$) was generated from the photo reduction of riboflavin and was detected by Nitro Blue Tetrazolium (NBT) reduction method (Jose and Janardhanan, 2000). 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 minutes. 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 a standard antioxidant agent.

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 the significance.

RESULTS

A. Micronucleus assay

The micronucleus test indicated an inhibitory effect of citral on the frequency of micronucleus (MN) induced by $NiCl_2$. In bone marrow MN assay, a significant increase in the percentage of MN was observed in both PCEs and NCEs after the administration of $NiCl_2$. At 48 hr of $NiCl_2$ exposure, the increase in the MN was found to be maximum however, after 72 hr, the increase in the MN was marginal in comparison to the extent of increase observed from 24 hr to 48 hour interval. Citral (60 mg/kg b.w.) had shown a significant ($P<0.01$) inhibitory effect in the micronucleated erythrocytes (i.e, PCEs and NCEs) in all the tested time intervals. The percentage inhibition in the micronucleated cells was found to be; PCEs (27% after 24 hr, 35% after 48 hr and 40% after 72 hr) and NCEs (23% after 24 hr, 26% after 48 hr and 22% after 72 hr). However, citral (60 mg/kg b.w.) administered alone had not exerted a significant variation in the level of MN compared with the control in both bone marrow as well in peripheral blood MN tests (figs. 1-3).

The peripheral blood MN test showed that $NiCl_2$ had induced the clastogenic effect from 48 hr onwards. A significant ($P<0.01$) rise in the MN was found in the polychromatic and normochromatic erythrocytes. Citral (60 mg/kg b.w.) pre-treated groups had shown a significant ($P<0.05$) suppression in the number of micronucleated erythrocytes. The administration of citral had reduced the frequency of micronuclei in PCEs (45% after 48 hr and 29% after 72 hr) and NCEs (28% after 48 hr and 25% after 72 hr) (table 1).

B. Antioxidant activity

The antioxidant activity of citral and catechin were evaluated at four different concentrations (10, 30, 100 and 300 mcg/ml). Citral at 10 and 30 mcg showed 42% and 60% of scavenging effect respectively, while at 100 and 300 mcg,

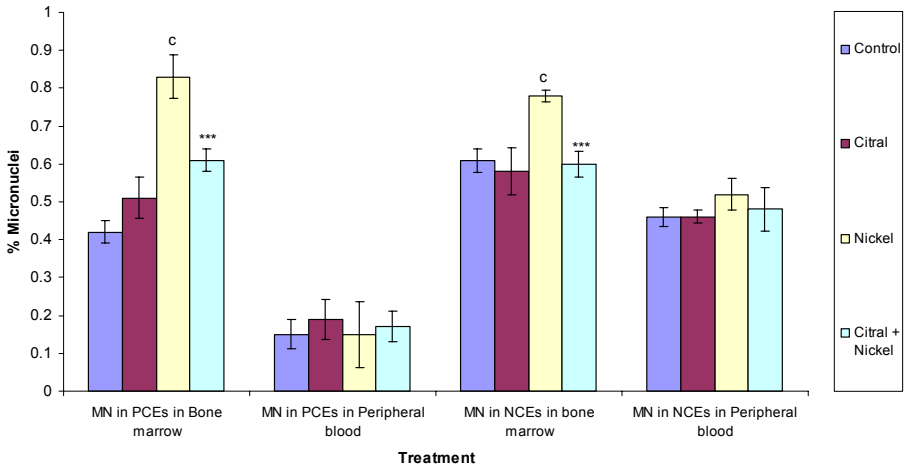

Fig. 1: Effect of citral on the micronuclei frequency after 24 hr of nickel chloride treatment.
[c] P<0.001 compared with control, ***P <0.001 compared with $NiCl_2$

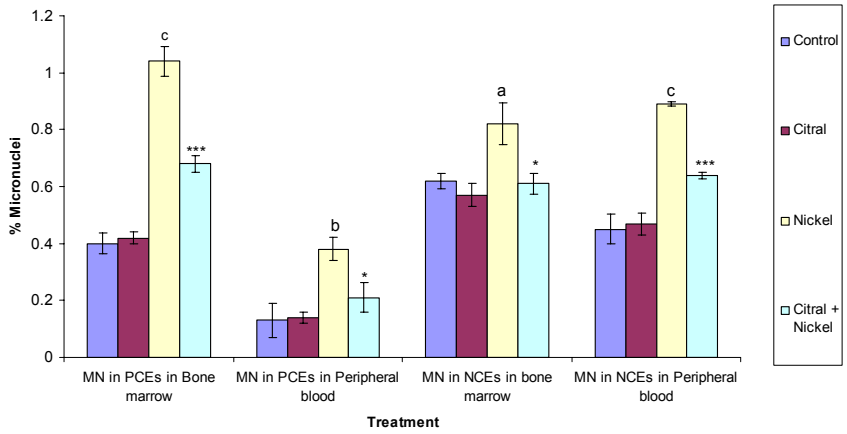

Fig. 2: Effect of citral on the micronuclei frequency after 48 hr of nickel chloride treatment.
[a] P<0.05 [b] P<0..01, [c] P<0.001 compared with control, *P<0.05, ***P <0.001 compared with $NiCl_2$

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 300 mcg (90% scavenging activity). The doses tested for citral and catechin had shown significant variation (P<0.001) compared with the control. The EC_{50} values for citral and catechin were found to be 19 mcg and 7.9 mcg/ml respectively (table 2).

DISCUSSION

In the present study, citral exerted a significant (P<0.01) inhibition in the number of micronucleated erythrocyte induced by $NiCl_2$. The prior administration of citral was found to be effective in limiting the genotoxic effect both in bone marrow and peripheral blood MN assays (table 1).
Micronucleus assay developed by Schmid is a well-established test to evaluate mutagens and anti-mutagens (Schmid, 1975). Two forms of erythrocytes viz., polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) are preferably selected to evaluate the extent of nuclear damage during erythropoeisis and to minimize the errors while counting the micronuclei (Vijayalaxmi and Venu, 1999). Presence of more than 6 % of MN in the erythrocytic population indicates genotoxicity (Heddle *et al*., 1983).

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, granulation-promoting effect (Carbajal *et al*., 1989). When orally administered, citral was reported to produce expectorant, appetite stimulant (digestant), choleretic, carminative, spasmolytic, anti-inflammatory, diuretic, sedative action (Carbajal *et al*., 1989 and Carlini *et al*., 1986). In addition to citral, lemon grass oil contains geraniol, myrcene, citronellal, limonene, linalool and

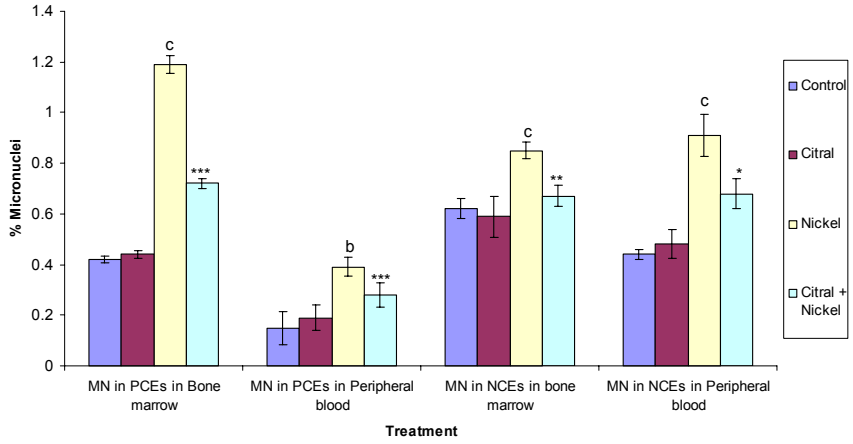

Fig. 3: Effect of citral on the micronuclei frequency after 72 hr of nickel chloride treatment.
[b] P<0..01, [c] P<0.001compared with control, *P<0.05, **P<0.01, ***P <0.001 compared with $NiCl_2$

dipentene and none of the constituents were reported to be mutagenic with salmonella assay (Gomes *et al*., 1998). In the present work, the mutagenic potential of citral was evaluated at 60 mg/kg b.w. (p.o) and the result indicated that there was no significant increase in the frequency of MN in the erythrocytes (table 1). Hence citral *per se* lacks the mutagenic potential in the tested doses (Gomes *et al*., 1998).

Nickel, long stood out among the six metallic elements (Manganese, Iron, Cobalt, Nickel, Cupper and Zinc) as being apparently without any biological function however, the recent evidences suggests the importance of $NiCl_2$ in various body activities as described earlier. The toxicities of nickel are dependent on the route of exposure and the solubility of the nickel compound (Danadevi *et al*., 2004). Nickel salts which are taken up by phagocytosis gets accumulate in vacuoles and appear to have affinity for certain membrane structures, including the polynuclear membranes, the vacuole wall and lipid structures. Kasprzak (1995) reported that larger amounts of $NiCl_2$ bound to nucleoli could be the reason for its mutagenicity and carcinogenicity. Nickel-DNA interaction takes place at DNA phosphate oxygens and the N-positions of adenine and guanine (Doreswamy *et al*., 2004).

The present study indicated that $NiCl_2$ (10 mg/kg b.w.) had significantly increased the incidence of MN in both bone marrow and peripheral blood MN tests. Appearance of MN in peripheral blood after 48 hr of exposure suggested a minimum requirement of 24 hr for the RBCs to cross the bone marrow and appear in the general circulation (Schlegel and MacGregor, 1982) (figs. 1 and 2). Micronucleus frequency was observed to be peak after 48 hr of $NiCl_2$, however, after 72 hr, the percentage MN levels in PCEs and NCEs were found to be almost same as that of 48 hr interval (figs. 1-3). This suggests that $NiCl_2$ exhibits maximum genotoxic effect between 24 and 48 hr and after 72 of exposure, the metal might had either detoxified or had undergone renal clearance (Costa and Mollenhauer, 1980).

Earlier reports suggest that some of the antioxidants having scavenging action has the ability to prevent the chromosomal damages of mutagens (Chen and Yen, 1997). These agents act as exogenous nucleophile, compete with the DNA bases for the ultimate mutagen and thus serve as a possible preventive agent against mutagenesis and carcinogenesis (Hayatsu *et al*., 1988).

Several naturally occurring antioxidants have been reported to be anti-mutagenic / anticarcinogenic in the literature such as, reservertrol (Sgambato *et al*., 2001), black tea (Gupta *et al*., 2002), green teas (Amantana *et al*., 2002), cashew apple juice (Melo Cavalcante *et al*., 2003), phyllantus emblica (Dhir *et al*., 1991) and ascorbic acid (Vijayalaxmi and Venu, 1991 and Perminova *et al*., 2001). Considering the above-cited information, citral was tested for the antioxidant potential by superoxide scavenging method. The super oxide radicals (O_2^-) were generated from the reduction of riboflavin and detected by the extent of formation of blue formazan from NBT reduction (Jose and Janardhanan, 2000). The study revealed that citral in a dose dependent manner inhibited the oxidative process involved in the formation of free radicals (table 2). Besides, citral was reported to prevent the oxidative conversion of beta-carotene to retinoic acid *in vitro* in the human intestinal homogenate (Wang *et al*., 1992). Hence based on these findings, we speculate that citral in the present work might had shown a scavenging effect, prevented the $NiCl_2$-nuclear interaction in the host cell to decrease the frequency of micronucleated cells in the erythrocyte population.

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 (Ames,

1983 and Chen and Yen, 1997). 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 (Carbajal *et al*., 1989 and Carlini *et al*., 1986). 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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