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Research Article

INHITION OF MICRONUCLEUS FORMATION BY ALCOHOLIC AND ETHYL ACETATE FRACTIONS OF ROOT OF LAWSONIA INERMIS AGAINST CYCLOPHOSPHAMIDE ON MICE BONE MARROW AND PERIPHERAL BLOOD CELLS

Minabasirian[1]*., Manjula S.N[1].,Mruthunjaya K[2] and V.Ch. Jeevani[1]

[1]Department of Pharmacology, JSS College of Pharmacy, JSS University, Mysore-15, India
[2]Department of Pharmacognosy, JSS College of Pharmacy, Must Be mentioned

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ABSTRACT

Anti-clastogenic activity of alcoholic (LIALC) and ethyl acetate (LIEAC) fractions of root of *lawsonia inermis* (LI) on micronucleus (MN) formation on mice bone marrow and blood cell were studied. Mice were treated with two doses of LIALC and LIEAC (200 and 300 mg/kg) for 14 days and challenged with normal (distilled water) and CYCLO (cyclophosphamide) treated group. Mice were scarified; peripheral blood cells and bone marrow were collected. The formation of MN was calculated based on PCE/NCE (indicator of proliferation). PCE/NCE of LIEAC (200 and 300 mg/kg) in bone marrow was observed 0.55±0.27 and 0.625±0.10, and in peripheral blood cells was 0.46±0.09 and 0.5±0.87 respectively, which showed significant increase compared to CYCLO treated group. The CYCLO treated mice, showed fall in PCE/NCE ratio, however both LIALC and LIEAC, at 200 and 300 mg/kg, inhibited micronuclei formation but percentage of inhibition of MN formation was more in LIEAC at 300 mg/kg.

INTRODUCTION

DNA damage, can be due to environments 'factors or cell metabolic processes which occurs at rate of 10^3 to 10^6 per cell per day. (Feigelson. HS, *et al* 1996). UV damage, alkylation/methylation, X-ray damage and oxidative damage are examples of induced damage. Spontaneous damage can causes base loss, deamination of the amino acids, proteins and DNA, alteration in sugar backbone of DNA (deoxy ribose) and tautomeric shift. (Friedberg EC, *et al* 2006). Clastogen is any agent that causes breaks in chromosome and / or alters it's arrangements of sequence. It can damage the genetic material (DNA) by direct attack or by formation of free radicals. Clastogen can be ionizing radiation or any chemical (e.g. pesticides) or any cytotoxic drug. (Hallowell B, 1994)

A micronucleus test is a standard test used in toxicological screening for potential genotoxic compounds (Schmid.W, 1975). The mammalian in vivo micronucleus test is used for the detection of damage induced by any substance to chromosomes and also is a method for analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals, usually rodents. The main purpose of the micronucleus test is to identified the mutagenic and cytogenetic substances which leads to formation of micronuclei lagging chromosomes fragments or whole chromosomes. (Schmid.W, 1975) (Heddle.J.A. *et al*, 1983) in development process of erythroblast to polychromatic erythrocytes, in bone marrow, the main nucleus is extruded, any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. Micronuclei is visualized in these cells because they lack in main nucleus. An increase in frequency of micronuclei formation in polychromatic erythrocytes in treated animals is indicators of chromosomal damage. (Mavournin. K.H *et al*, 1990).

The principal end point of this test is either frequency of micronucleated immature erythrocytes (polychromatic –PCE) or the number of mature erythrocytes (normochromatic –NCE) in peripheral blood cells that contain MN among a total number of mature erythrocytes (MacGregor, J.T *et al,* 1987). The micronucleus test is standard for assessing mutagenic agents both endogenous or exogenous factors, although they are varies among species (Donaldson. M.S, 2004).

Nowadays anticlastogenic compounds have been successfully used in management of mutagenesis (Chi Chen *et al*, 2005), chemoprevention (Sasaki YF *et al*, 1990), radioprotection, diabetic complications, and cardiac stress (Block G, 1992).

Hence, a compound possessing anti-clastogenic activity can improve the quality of life in cancer patients by 'adding life to years than years to life'. Epidemiological studies indicate that

*Corresponding author: **Minabasirian***
Department of Pharmacology, JSS College of Pharmacy, JSS University, Mysore-15, India

susceptibility to cancer is significantly influenced by diet. For instance, frequent consumption of certain fruits, vegetables oils, spices (like ginger, garlic, turmeric etc) are associated with a reduced risk of various types of malignancies (Peto R, *et al*, 1981).

Plants contain a wide variety of free radical scavenging molecules, like flavonoids, anthocyanins, carotenoids, dietry gluthionine, vitamins and endogenous metabolites which posses anti-oxidant activity. These natural anti-oxidant have been shown to function as oxygen quenchers, peroxide decomposers, enzyme activator or inhibitors. (Elliott Middleton *et al*, 2000). Based on these facts several researchers have been pursuing studies on protective effect of the natural compounds against genotoxic and mutogenetic agents like anti-cancer agents and ionizing radiation. (Vipan kumar, 2008). Study by Zhumei Ren was trying to identify the possible protective effect of selected natural antioxidant compounds to minimize the emerging untoward effects in radiotherapy and chemotherapy. (Zhumei Ren *et al*, 2002)

In present study, the role of the natural potent anti-oxidant (Mina Basirian *et al*, 2012) in inhibition of MN formation against CYCLO was studied. CYCLO is a main anti-cancer agent which is used for almost majority of the cancer. CYCLO is a prodrug and inside the body produced the toxic metabolites Metabolites of cyclophosphamide can interact with DNA and proteins, resulting in the formation of adducts. Hence finding the agents which protect the cell against this cytotoxic agent is essential. (H.P.Rang *et al*, 2007).

MATERIALS AND METHODS

Biological material

The roots of lawsonia inermis was collected from the premises of Ayurvedic College, Mysore and was identified by Dr.M.N, Naganandini, the roots were washed, cut and dried. They stored in air-tight container.

Sample preparation

The roots were cleaned and cut into small pieces. It was dried and then milled (1000 g). One portion (300 g) was extracted with 95% ethanol (3:1). Thus was obtained two main extracts (LIALC). The LIALC extract (40 g) was dissolved in 400 ml of distilled water, transferred into a separating funnel and then was fractionated thrice with petroleum ether (400 ml) by vigorous shaking, and was allowed to separate. The upper petroleum ether layer separated and was collected and pooled (LIPET). The lower aqueous layer was further fractionated thrice with ethyl acetate (400 ml) in a similar way as explained above and the upper ethyl acetate (LIEAC) layer was collected and pooled. Ethyl acetate fraction was concentrated and dried under vacuum (Mruthunjaya k, 2008)

Chemicals and Solvents

All reagents used were analytical grade, pH 7.4, Phosphate buffer saline (PBS), citric acid (1.92 % w/v) and Giesma were purchased from Sigma–Aldrich, India. Methanol, alcohol, KCl (0.56%) and ethyl acetate were purchased from Merck, India. Cyclophosphamide was purchased from cipla –India and Bovine Serum albumin was purchased from Hi-media.

Animals

Swiss albino mice, weighing between 25-30 gm., were randomly divided into five groups of 6 animals each and treatment was started as given table 1.

Table 1 classification of mice based on their therapy

GROUPS	TREATMENT
Group I	normal control treated with 0.25% CMC (p.o, for 14 days)
Group II	Positive control treated with CYCLO (100mg/kg, i.p. on 14th days)
Group III	LIALC extract (at 200 mg/kg, p.o. for 14 days) +CYCLO (100mg/kg, i.p on 14 th day)
Group IV	LIALC extract (at 300 mg/kg, p.o. for 14 days). + CYCLO (100mg/kg, i.p on 14th day)
Group V	LIEAC extract (at 200mg/kg, p.o for 14 days) +CYCLO(100mg/kg, i.p on 14th day)
Group VI	LIEAC extract (at 300mg/kg, p.o for 14 days) +CYCLO(100mg/kg, i.p on 14th day)

Treatment

A stock suspension of LIALC and LIEAC at concentrations of 20 mg/ml was prepared by triturating the extract in 0.25% Sodium CMC solution. The suspension was prepared just prior to the dosing. The animals of group III, IV, V, VI were administered orally at a dose of 200 mg/kg and 300 mg/kg of LIALC and LIEAC from the respective stock suspension, for 14 consecutive days along with it freshly prepared CYCLO solution in sterile water was administered on 14th day. On 14th day the animals were sacrificed by cervical dislocation to performed micronucleus test.

Micronuclei formation in bone marrow

Bone marrow preparations were made by modified method of Schimid (1973). In this study instead of fetal calf serum, 5% bovine albumin was prepared in buffered saline (pH 7.2) and was used as suspending medium. The animals were sacrificed by cervical dislocation, tibia and femur bones were removed, and bone marrow was flushed with syringe containing 5% bovine albumin solution. The suspension was mixed properly and centrifuged at 1000 rpm for 8 minutes. The supernatant was discarding and thick cell suspension was made in 5% bovine albumin solution. A drop of suspension was placed in clean slide and spread with help of spreader slide. Then slides were air dried, after drying, fixation were done by placing slide in methanol for 10 minutes. Geimsa stain were prepared and diluted with phosphate buffer (1:6) and slides were transferred into Geisma stain and kept for 10 minutes. Then slides were rinsed several times in phosphate buffer .Finally the slides were kept in buffered water for 5 minutes, followed by air dried and observation for MN in polychromatic (PCE) and normochromatic (NCE) erythrocytes under the microscope. The ratio of PCE/NCE was calculated. (Syed Imam Rabbani *et al*, 2004)

Micronuclei formation in peripheral blood

For peripheral blood micronucleus test, blood were collected from the aorta after administration of anesthesia using 1% sodium citrate as anti-coagulant, smears were prepared on clean glass slides. After fixation of slides with methanol for 10 minutes, Giemsa was used as staining agent, freshly prepared Geimsa in phosphate buffer (1:6) and kept for 10 minutes. The slides were rinsed several times with phosphate buffer and kept

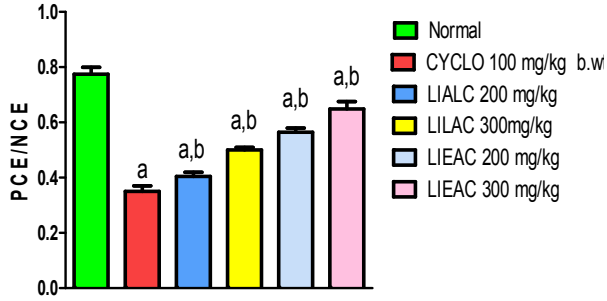
in water buffer for 5 minutes. The slides were air dried and observed for presence of MN in polychromatic (PCE) and normochromic (NCE) erythrocytes under microscope. The ratio of PCE/NCE was calculated. (Syed Imam Rabbani *et al*, 2004)

Statistical Analysis

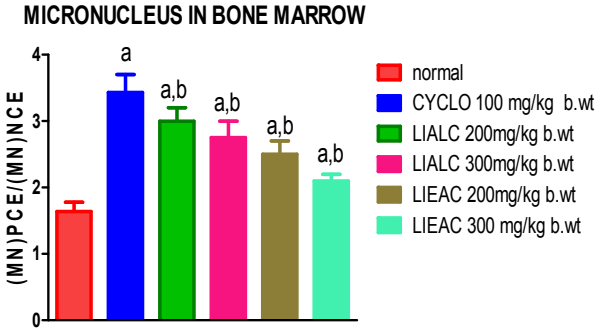
The results were shown mean± standard error of mean (SEM). All data were analyzed by one-way ANOVA followed by tukeys post-hoc test using graph pad prism version 5.0 software. The difference between the control and experimental groups were considered significant if, $p< 0.05$.

RESULTS

Effect of LIALC and LIEAC on micronuclei (MN) formation in mice bone marrow



Graph1 Effect of LIALC and LIEAC on MN formation in mice bone marrow (PCE/NCE)



Graph2 Effect of LIALC and LIEAC on MN formation in mice bone marrow [(MN) PCE/(MN)NCE]

a, b: statistical significance ($P < 0.05$); a:statistical significant difference ($P< 0.05$) to normal, b: statiscal significant $P< 0.05$) To CYCLO. PCE: number of polychromatic erythrocytes screened; (MN) PCE: number of micronucleited polychromatic erythrocytes, NCE: number of normochromatic erythrocytes screenrd (n=2000), (MN)NCE: number of micronucleated normochromatic erythrocytes: PCE/NCE: ration of polychromatic to normochromatic (indicator of proliferation rate)

A significant decrease in PCE/NCE ratio was observed in CYCLO treated group as compare to normal treated mice. His indicated that rate of proliferation was reduced in CYCLO treated group. A significant decrease in MN formation was observed in both LIALC and LIEAC treated groups at two doses 200 and 300 mg/kg when compare to CYCLO treated group. Whereas the significant increase in PCE/NCE was observed in LIEA (0.55±0.27 and 0.625±0.10 respectively) when compared to CYCLO group (PCE/NCE values of0.33±0.290.45) and ratio of (MN) PCE/ (MN)NCE for both doses of LIEAC(200 and 300 mg/kg) was 2.3±0.27 and 2.0±0.10 respectively which had been shown significant increase decrease as compared to CYCLO treated group. (3.16±0.29). (graph1, table1 & figure1). These results revealed that although the LIALC was showed inhibition in MN formation but LIEAC was more protective agent than LIALC.

a,b: statistical significance ($P < 0.05$); PCE: number of polychromatic erythrocytes screened;(MN)PCE: number of micronucleited polychromatic erythrocytes, NCE: number of normochromatic erythrocytes screenrd (n=2000), (MN)NCE: number of micronucleated normochromatic erythrocytes: PCE/NCE: ration of polychromatic to normochromatic (indicator of proliferation rate)

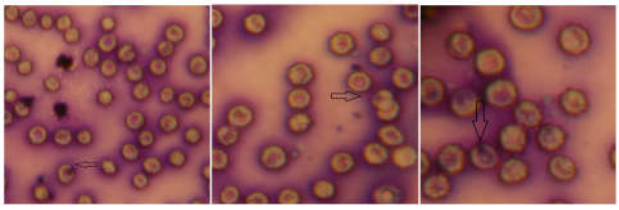
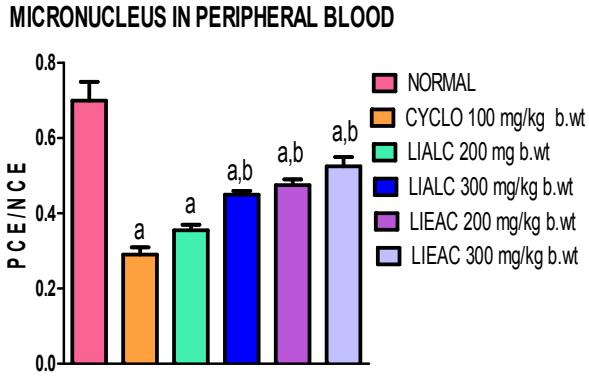


Figure1 Micronuclei formations in mice bone marrow

The MN formation was showed by arrow marks in mice bone marrow, the freshly prepared Giemsa in phosphate buffer (1:6) was used as staining agents. The MN was observed as a dark blue dot in a bone marrow cells.

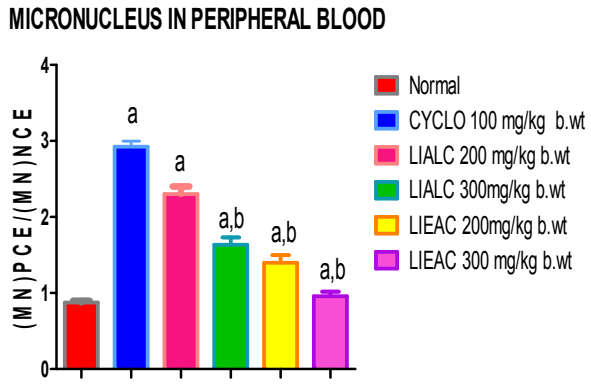
Effect of LIALC and LIEAC on micronuclei (MN) formation in peripheral blood cells of mice



Graph3 Effect of LIALC and LIEAC on MN formation in peripheral blood (PCE/NCE)

Table1 Effect of LIALC and LIEAC on MN formation in mice bone marrow

Groups	(MN)PCE	(MN)NCE	PCE/NCE (MN)	PCE	NCE	PCE/NCE
Group.I-NORMAL	7	4	1.78±0.34	1500	2000	0.75±0.34
Group.II-CYCLO-100 mg/kg	38	12	3.16±0.29[a]	670	2000	0.33±0.290.45[a]
Group.III-LIALC-200 mg/kg	28	10	2.8±0.35[a,b]	789	2000	0.39±0.35[a,b]
Group.IV-LIALC-300 mg/kg	25	10	2.5±0.25[a,b]	990	2000	0.49±0.25[a,b]
Group.V-LIEAC- 200 mg/kg	19	8	2.3±0.27[a,b]	1100	2000	0.55±0.27[a,b]
Group.VI-LIEAC-300 mg/kg	14	7	2.0±0.[10a,b]	1250	2000	0.625±0.[10a,b]



Graph3 Effect of LIALC and LIEAC on MN formation in peripheral blood [(MN) PCE/(MN)NCE]

a,b: statistical significance (P < 0.05); a:statistical significant difference (P< 0.05) to normal, b: statiscal significant P< 0.05) To CYCLO. PCE: number of polychromatic erythrocytes screened; (MN) PCE: number of micronucleited polychromatic erythrocytes, NCE: number of normochromatic erythrocytes screenrd (n=2000), (MN) NCE: number of micronucleated normochromatic erythrocytes: PCE/NCE: ration of polychromatic to normochromatic (indicator of proliferation rate)

A significant decrease in PCE/NCE ratio was observed in CYCLO treated mice as compare to normal group. A significant decreased in MN was observed in both LIEAC at dose of 200 and 300 mg/kg. LIEAC fractions of roots of LI at dose of 200 and 300 mg/kg was showed significant increase in PCE/NCE0.46±0.09 and 0.5±0.87 when compared to CYCLO treated group(0.27±0.34) The CYCLO treated mice, showed increased MN formation and (MN)PCE/(MN) NCE ratio was2.85±0.012 which indicate effect on cell cycle and suppression of erythropoiesis. Whereas the MN formation was significantly decreased for LIEAC at 200 and 300 mg/kg and the ratio of (MN) PCE/(MN)NCE was 1.3±0.43 and 1.02±0.21 respectively. (Graph2, table & figure 2)

The MN formation was showed by arrow marks in peripheral blood of mice, the freshly prepared Giemsa in phosphate buffer (1:6) was used as staining agents. The MN was observed as a dark blue dot in a blood cells. Observation of MN was easier then bone marrow.

DISCUSSION

Micronuclei are cytoplasmic chromatin containing bodies that present as small nucleus around the cell nucleus, due to fragmentation of chromosomes or cancer cell line that are not incorporated into main nucleus after cell division. The presence of MN in cell is consider as DNA damage. (Sonam Pandey *et al*, 2010). Application of antioxidant in clastogenesity has not been studied extensively, although it is generally accepted that endogenous antioxidants provide some degree of protection. Previous studies support that antioxidant nutrients such as vitamin E and citral compounds are protective against clastogenic agents (L.W.Anderson *et al*, 1996). Some antioxidants have the advantage with low toxicity, exhibiting protective action when administered at pharmacological doses. Naturally occurring antioxidants also provide an extended protection against irradiation, including therapeutic potential when administered after irradiation. Enhancement of micronuclei frequency in the bone marrow of CYCLO treated mice has been reported earlier (Deepika Mathur *et al*, 2011) and the same was observed in our present study.

CYCLO is the cytotoxic drug and major side effect is myelo-suppression and decrease the erythrocytes drastically, which may affect the quality life of cancer patients, hence finding the protective agent is essential while chemotherapy. The PCE/NCE ratio, is an indicator of proliferation rate of cells (Spencer.CM *et al*, 1995), and was decreased with CYCLO, suggesting its cytotoxic effect and suppression of erythropoiesis As the LI was reported as potent anti-oxidant ,the most fractions of LI namely LIEAC and LIALC at 200 mg/kg and 300 mg/kg were used and results revealed, significant increase in proliferation rate and decreased the CYCLO induced micronuclei formation.

Table2 Effect of LIALC and LIEAC on MN formation in mice peripheral blood

Groups	(MN)PCE	(MN)NCE	PCE/NCE (MN)	PCE	NCE	PCE/NCE
Group.I-NORMAL	6	7	0.85±0.09	1200	2000	0.6±0.65
Group.II-CYCLO-100 mg/kg	40	14	2.85±0.012[a]	550	2000	0.27±0.34[a]
Group.III-LIALC-200 mg/kg	27	12	2.2±0.78[a]	698	2000	0.34±0.23[a]
Group.IV-LIALC-300 mg/kg	17	11	1.54±0.23[a,b]	881	2000	0.44±0.01[a,b]
Group.V-LIEAC- 200 mg/kg	13	10	1.3±0.43[a,b]	935	2000	0.46±0.09[a,b]
Group.VI-LIEAC-300 mg/kg	9	8	1.02±0.21[a,b]	1010	2000	0.5±0.87[a,b]

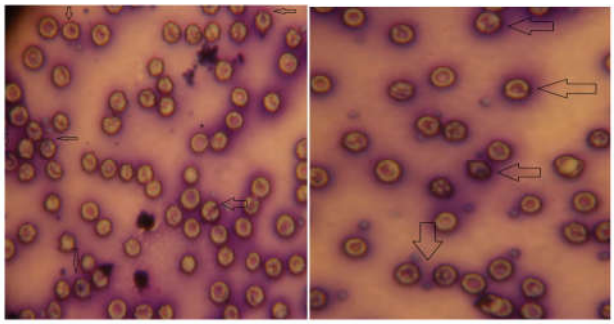


Figure2 Micronuclei formation in peripheral blood cells

This result was indicating their protective role. Many antioxidant nutrients have anti-mutagenic properties and their effects on cancer, needs further examination.

CONCLUSION

In conclusion, both LIALC and LIEAC, at 200 and 300 mg/kg, inhibited micronuclei formation (MN) in mice bone marrow and peripheral blood cells. And significantly increase the proliferation rate of the cells. But percentage of MN formation inhibition was more in LIEAC at 300 mg/kg which make this molecule as a candidate for further study.

Future Scope

Identification of the molecule in biochemistry laboratory and try to isolate the potent molecule, convert it to lead molecule and find the target, although it requires years of journey.

Abbreviations

LI: Lawsonia inermis, LIALC:LI alcoholic extract, LIEAC: LI ethyl acetate extract, MN: micronuclei, CYCLO: cyclophosphamide, PCE/NCE: polychromatic / normochromatic, rpm: resolution per minute PCE:number of polychromatic erythrocytes screened;(MN)PCE: number of micronucleited polychromatic erythrocytes, NCE: number of normochromatic erythrocytes screenrd (n=2000), (MN) NCE:number of micronucleated normochromatic erythrocytes: PCE/NCE :ration of polychromatic to normochromatic (indicator of proliferation rate).

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Mina Basirian was responsible for a conception design, data collection, data analysis, interpretation, and write-up and in the preparation of the draft manuscript. V.Ch.Jeevani, S.N.Manjula and K. Mruthunjaya were involved in the design, data analysis, interpretation, write-up and revision of the paper. All authors read and approved the final manuscript.

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