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Assessment of Anticlastogenic Effect of *Piper longum* Fruit Extract on Cyclophosphamide-Induced Micronucleus Formation in *Swiss albino* Mice

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ABSTRACT

Piper longum, commonly referred as 'Pippali', has found its traditional use in India, Malaysia, Singapore and other South Asian countries as an analgesic, carminative, anti-diarrhoeic, immunostimulant, post childbirth to check postpartum hemorrhage and to treat asthma, insomnia, dementia, epilepsy, diabetes, rheumatoid arthritis, asthma, spleen disorder, puerperal fever, leprosy etc. Plants have been used for medical purposes since the beginning of human history and are the basis of modern medicine. It is usually cultivated for its fruit, which is dried and used as spice. The plant grows into a shrub with large woody roots, numerous creeping and jointed stems that are thickened at the nodes. The leaves are without stipules and spreading in nature. In this study, the protective effect of Piper longum extract has been tested against cyclophosphamide (CP)-induced micronuclei formation in mouse bone marrow cells. The three test doses, namely 100, 150 and 200 mg/kg body weight of Piper longum extract provided protection when given 24 hr prior to the single ip administration of cyclophosphamide (50 mg/kg body weight). A dose dependent inhibition of micronuclei formation was observed which was statistically significant ($p<0.05$) as compared to the cyclophosphamide group. It was observed that Piper longum extract alone could not induced micronuclei formation at the test dose 100 mg/kg body weight. Therefore, it's seemed to have a preventive potential against CP-induced micronuclei formation in Swiss mouse bone marrow cells.

Key Words: *Piper longum, Micronucleus, Mutagenic, Genotoxic, Medicinal Plant, Anticlastogenic.*

INTRODUCTION

Secondary metabolites of various plants have been traditionally utilized for the betterment of human health. Plants belonging to genus Piper are amongst the most important medicinal plants used in various systems of medicine. More than 1,000 species belong to this genus and *P. longum* is one of the most well-known species amongst them, including *Piper nigrum* and *Piper bettle*.

P. longum forms an active constituent of the widely used Ayurvedic poly-herbal formulation "Trikatu" [1]. The widespread use of this herb in different formulations as documented in ancient Ayurvedic manuscripts such as *Charaka samhita* [2], *Susruta samhita* [3] Vagbhata's astanga hrdayam [4] *etc*. suggests its vital importance in traditional Indian medicinal system.

P. longum is an indigenously growing plant in India and is also cultivated in the tropical and subtropical regions of Asia and Pacific islands [5]. It is usually cultivated for its fruit which is dried and

used as a spice. The plant grows into a shrub with large woody roots, numerous creeping and jointed stems that are thickened at the nodes. Leaves are without stipules and spreading in nature. Fruits are small, oval shaped berries and grow as spikes that are collected after maturation. Dried form of these spikes makes "pippali" while the root radix is known as "pippalimula".

The dietary piperine is known for its bioavailability and digestion enhancing properties. *In vitro* studies have shown the role of piperine in relieving oxidative stress by quenching free radicals and reactive oxygen species. While it is known to act as an anti-mutagenic and anti-tumor agent [6], anti-diarrheic and anti-dysenteric properties of this spice enhance its medicinal value [6].

The pharmacological properties of this plant also include anti-oxidant, anti-inflammatory, hepatoprotective, immunomodulatory, anti-microbial, anti-platelet, anti-hyperlipidemic, analgesic, anti-depressant, anti-amoebic, anti-obesity, radioprotective, cardioprotective and anti-fungal [7]. Methanolic extract of this fruit has been reported to be involved in memory repair and improving memory performance by an in vitro model [8]. Clinical studies have revealed the efficacy of this plant in the treatment of bronchial asthma in children [9].

Anti-diabetic activity of the roots has also been reported. It is widely used as an important constituent in various Ayurvedic medicines to cure diseases like leprosy and tuberculosis and is also used in the treatment of cough, dyspnea, cardiac and spleen disorders, chronic-fever, gout, rheumatic pain *etc*. [10].

In recent years, the advancement in chemistry, pharmacology and systems biology has created a new paradigm for the drug discovery known as network pharmacology (Hopkins, 2008). Integration of traditional knowledge of medicines with recent in silico approaches has led to the identification of novel natural drug compounds.

The approach has recently gathered much attention by the research community as network pharmacology based studies have been widely used to explore the medicinal activities of herbs like *Withania somnifera* [11] and formulae like Qishenyiqi (Li, *et. al.*, 2014a), Gegen Qinlian decoction [12] *etc*. to understand their molecular level effect in the treatment of syndromes or diseases.

Therefore, we planned to carry out anti-mutagenic activity of *P. longum* extract using micronucleus assay in experimental animals.

MATERIALS AND METHODS

Animal

The study was conducted on random bred, 6-7 weeks old and 24- 28 gm body weight male *Swiss albino* mice. They were maintained under controlled conditions of temperature and light (light: dark, 12 hrs: 12 hrs.).

They were provided standard mice feed and water *ad libitum*. The study Protocol was approved by Institutional Animal Ethical Committee (IAEC), Chhattisgarh Council of Science and Technology, Raipur, India (Ref number 04/IAEC/CCOST/2018).

Chemical

Cyclophosphamide was purchased from Sigma Chemical Co. (St Louis, MO, USA). Other Reagent grades chemical were procured locally.

Extract Preparation

The plant *P. longum* fruits were collected by local market, Raipur Chhattisgarh.

After the drying of fruit powder was defatted with petroleum ether (1000 ml) and the residue was extracted in 50% methanol with the help of soxhlet extraction unit.

The sample was collected and concentrated in water bath at 40-50^{o}C and dried in hot air oven at 40^{o}C. The dried powder was kept in air tied box.

Treatment

P. longum fruits extracts at the different doses were injected 24 hours before the treatment. The positive control group received single i.p. injection of 50 mg/kg cyclophosphamide in 0.9% saline. It is performed as per the method reported by Schmid, (1975) and modified by Aron, *et. al*., (1989).

Micronucleus Assay

For the micronucleus assay, the extract at the volume of 0.2 ml at different doses level such as 500, 1000 and 1500 mg/kg body weight was injected 24 hours before the treatment of cyclophosphamide, to six animals.

The positive control group received single i. p. injection of 50 mg/kg cyclophosphamide in 0.9% saline. The animals were sacrificed by cervical dislocation and bone marrow cells were harvested. The slides were prepared essentially as described by Schmid (1975) and modified by Aron *et al.,* (1980) [13,14].

After staining with May-Gruenwald and Giemsa, a total 1000 cells were scored at the magnification of x1000 (100 x 10x) for each group.

The data are expressed as the average number of micronucleated cells/thousand polychromatied erythrocytes cells (PCE) cells/animals (±SE) for a group of six animals. The results were compared with the vehicle control group using Student's 't' test with significance determined at $p<0.05$.

Experimental Design

The animals were divided into 6 different groups for each extract as follows: Total no. of animals for each group: - 4 mice
P. longum Extract: - 50 and 100 mg/kg body weight.
Cyclophosphamide: - 50mg/kg body wt.

Route of administration: - I.P.

Study Parameter: - Micronucleus count, PCE, NCE count.

Statistical Analysis

The statistical significance was calculated using student's't' test at $p<0.05$.

Groups for Experiment

1) Cyclophosphamide 50 mg/kg (Positive Control)
2) *P. longum* ext. (50 mg/kg) + cyclophosphamide
3) *P. longum* ext. (100 mg/kg) + cyclophosphamide
4) Solvent (water) alone

RESULTS

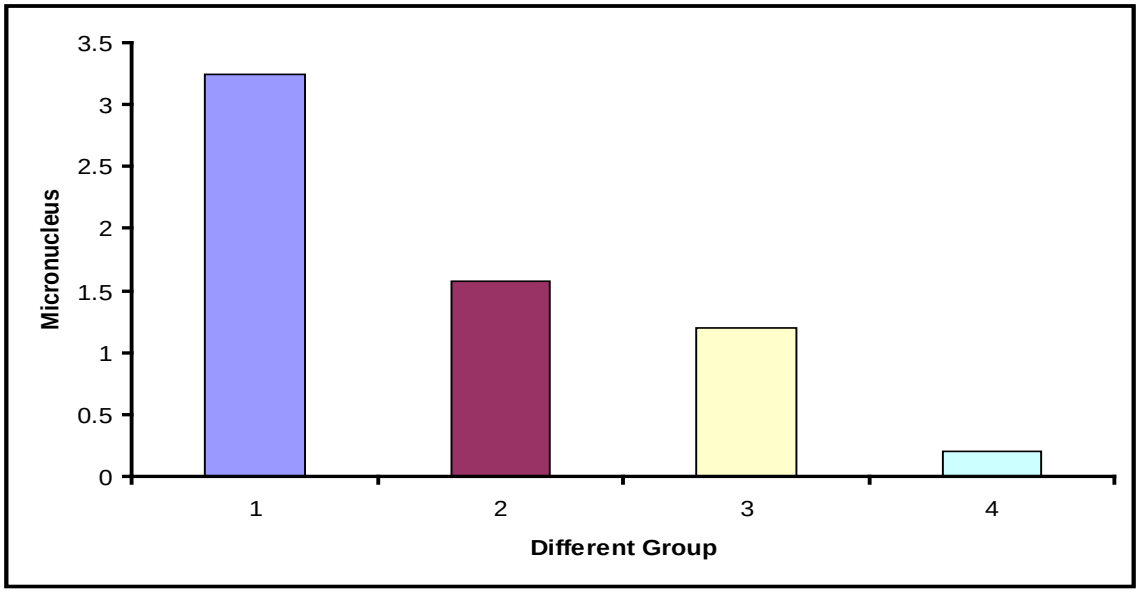
P. longum extract ip at 50 and 100 mg/kg body weight was found to inhibit the micronuclei formation induced by CP given ip at 50 mg/kg body weight. A dose dependent response was remarkable and statistically significant (Table 1).

In the positive control group CP induced micronucleus formation at the dose of 50 mg/kg body weight. It was noteworthy that different doses of *P. longum* and CP used in the present experiments were not cytotoxic for PCE / NCE (normochromatid erythrocytes) ratio in *P. longum* extract treated and positive control as compared to the solvent control group, which remained unchanged.

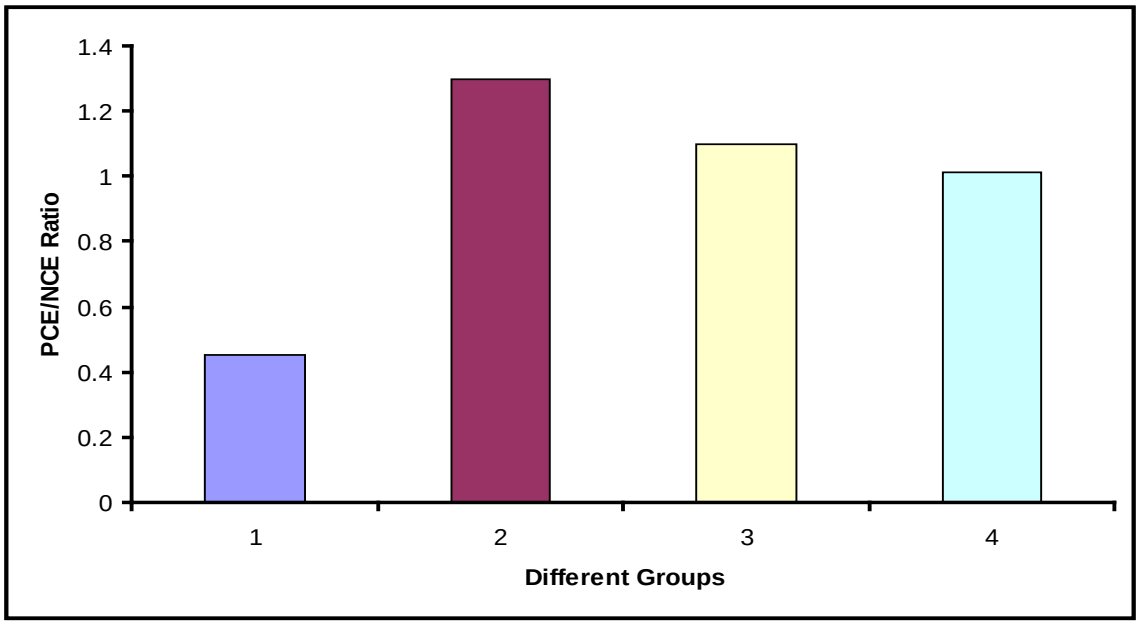
Table 1. Effect of *P. longum* Extract on Micronucleus Formation in Mouse Bone Marrow Cells

Sl	GROUPS	MN PCE ± SE	PCE/NCE ± SE
1.	Positive control Cyclophosphamide alone (50mg/kg)	3.24 ± 0.47	0.451 ± 0.10
2.	*P. longum extract* (50 mg/kg) + cyclophosphamide (50mg/kg)	1.58 ± 0.52*	1.30 ± 0.74
3.	*P. longum* ext. (100 mg/kg) + cyclophosphamide (50mg/kg)	1.20 ± 0.78*	1.10 ± 0.57
4.	Solvent (water)	0.20 ± 0.25	1.01 ± 0.23

** denotes statistically significant as compared to cyclophosphamide group at p<0.005*

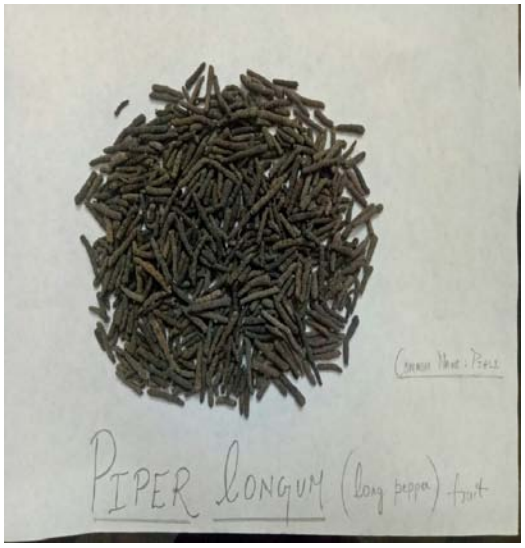


Grp – 1 Effects of P. longum extract on micronucleus formation in mouse bone marrow cells

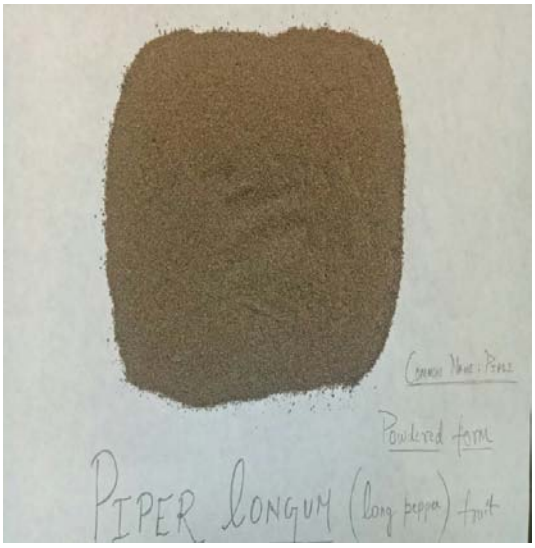


Grp – 2 Effects of P. longum extract on PCE/NCE ratio in mouse bone marrow cells

PHOTO PLATES

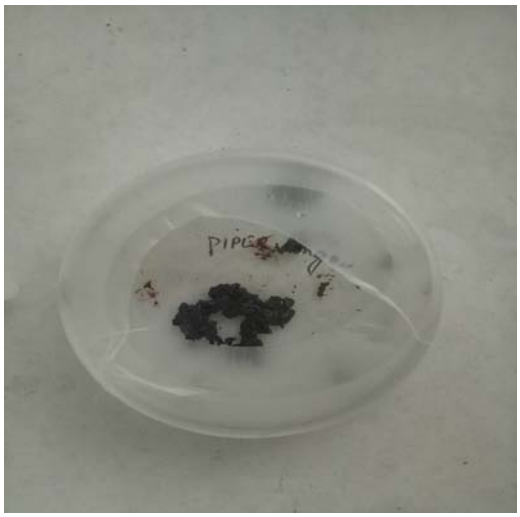


P. longum

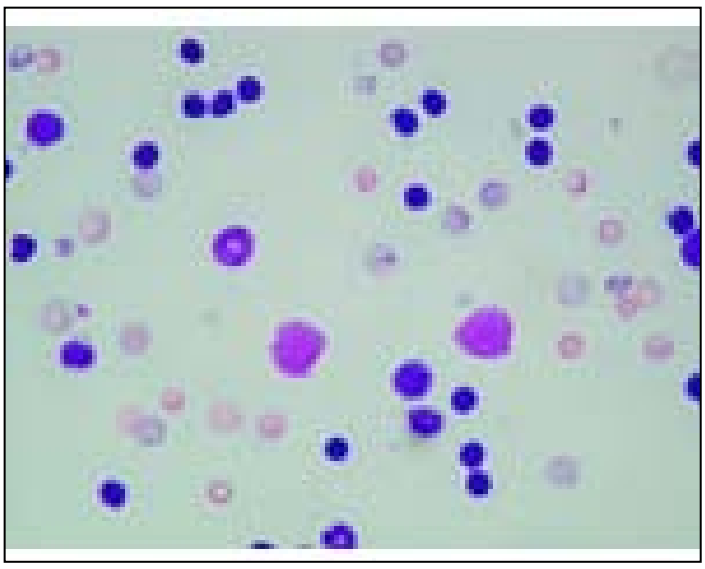


Powder of P. longum

Extract of P. longum



Dried Extract of P. longum



Micronucleus

DISCUSSION

Herbs and spices are processed in foods from early times for seasoning as well as to increase shelf life of food and to restore health. Coriander is one of miraculous herb that functions as both, spice as well as herbal medicine. Although plant can be grown throughout the year, it is processed to increase its palatability, profitability and facilitate international trade. The leaves and fruits are highly fragrant and contain nutrients like fat, proteins, vitamins minerals etc. Its health benefits activities ranging from antibacterial to anticancer activities. Most important and well characterized property of coriander is its use as antioxidant. Due to its multifunctional uses and protective and preventive action against various chronic diseases, this herb is righty called as “herb of happiness”. Moreover, processing of fruits and leaves of coriander is the best way to preserve this herb.

The medicinal value of plants lies in some chemical substances that have a definite physiological function in the human body. Different phytochemicals have been found to possess a wide range of medicinal properties, which may help in protection against various diseases. For example, alkaloids protect against chronic diseases; saponins protect against hypercholesterolemia and steroids and triterpenoides show the analgesic properties. The mutagenic effects of *P. longum* extract were evaluated by *In-vitro* micronucleus assay. Mutagenicity was present when the *P. longum* extract caused mutagenicity when used in high concentrations in both Salmonella typhimurium TA97 and TA102 strains [15]. The antimutagenic activity was also observed in coriander juice against the mutagenic activity of 4-nitro-ophenylenediamine, m-phenylenediamine and 2-aminofluorene was investigated using the Ames reversion mutagenicity assay (his- to his+) with the S. typhimurium TA98 strain as indicator organism [16].

The relationship between food, nutrition and cancer, and the knowledge that cancer may be a preventable disease has resulted in an increased interest in studying the mutagenic or antimutagenic potential of some dietary constituents [17]. Considerable emphasis has been laid down on the use of dietary constituents to prevent the mutagen induced mutation and/or chromosomal damage due to their relative non-toxic effects. CP (indirect-acting mutagen), a chemotherapeutic drug, damages chromosomes through generate of free radicals and alkylating DNA thereby producing mutation [18]. CP was often used as positive control in genotoxic test, both in laboratory animals or in cell cultures in the presence of liver S-9 fraction. The types of chromosomal aberrations induced by CP as a positive control were reported to be chromosome break, chromatid break, chromatid exchange, chromosomal exchange and ring chromosome [19]. In the present study, the types of chromosomal aberrations (except ring chromosome) reports [20]. CP generates alkylating metabolites following biological activation, resulting in formation of mutant cells [21]. Antigenotoxic agents especially those present in natural substances act through different cellular pathways involving endogenous sequestration of mutagens by various enzymes. Preventing the formation of carcinogens from precursors, blocking the metabolic activation of carcinogens by increasing the activation of detoxification enzymes might inhibit initiation of cancer [22]. The previous study demonstrated that alkaloids and flavonoids have chemopreventive effects against most of the carcinogens [23].

In this study the *P. longum* extract ip at 50 and 100 mg/kg body weight was found to inhibit the micronuclei formation induced

by CP given ip at 50 mg/kg body weight. A dose dependent response was remarkable and statistically significant (Table 1). In the positive control group CP induced micronucleus formation at the dose of 50 mg/kg body weight. It was noteworthy that different doses of *P. longum* and CP used in the present experiments were not cytotoxic for PCE / NCE (normochromatid erythrocytes) ratio in *P. longum* extract treated and positive control as compared to the solvent control group, which remained unchanged.

Experimental data revealed that there might be correlation between total phenolic and antioxidant capacity of different extracts of lemon grass. However, some literature demonstrated that antioxidant was not solely dependent on phenolic content but it may be due to other phytoconstituents as tannins, triterpenoid or combine effect of them. Another set of experiment the genotoxic study was also done which shows a better effect against cyclophosphamide as positive control and shown antimutagenic agent. So, in future it can be used as an alternate to synthetic antioxidant and antimutagenic agent.

Much effort has needed to increase *P. longum as* a dietary supplement in food so as to acquire antioxidant potential in our body naturally to fight against oxidative stress and other harm generated by free radicals and to protect against mutagenic effect induced by cyclophasphomide. Further more, detailed studies on the isolation and characterization of the plant extract as well as *in vivo* and *in vitro* assays will be necessary in discovering new biological agent for various disease.

CONCLUSION

Piper longum (*P. longum*, also called as long pepper) is one of the common culinary herbs that has been extensively used as a crucial constituent in various indigenous medicines, specifically in traditional Indian medicinal system known as Ayurveda. The fruits of *Piper longum* Linn are very well-known medicine for diseases of the respiratory tract, viz. cough, bronchitis, asthma, etc.; as counter-irritant, analgesic when applied locally for muscular pains and inflammation and as general tonic and hematinic.

They are carminative and known to enhance the bioavailability of food and drugs. *Piper longum (P. longum*, also called as long pepper) is one of the common culinary herbs that has been extensively used as a crucial constituent in various indigenous medicines, specifically in traditional Indian medicinal system known as Ayurveda.

The Indian traditional medicine (ITM) system commonly known as Ayurveda is a more than four thousand years old heritage of the Indian subcontinent and is a huge repository of information about multiple natural medicines for their therapeutic potential. *P. longum* is an important constituent of many Ayurvedic formulations and is most widely used as a part of “Trikatu”. However, the multi-targeting potential of this herb and underlying mechanism of its cellular-level action are still unexplored.

Micronucleus is also the name given to the small nucleus that forms whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei during cell division. In newly formed red blood cell in humans, these are known as Howell-jolly bodies. In normal people and many other mammals, which do not have nuclei in their red blood cells, the micronuclei are removed rapidly by the spleen.

Hence, high frequencies of micronuclei in human peripheral blood indicate a ruptured or absent spleen. In mice, these are not

removed, which is the basis for the vivo micronucleus test. In the current study, we targeted the antimutagenic potential of *P. longum* extract was evaluated using CP induced micronucleus formation and PCE / NCE ratio in bone marrow cells of *Swiss albino* mice. The P. *longum* extract at 50 and 100 mg/kg body weight was found to inhibit the micronuclei formation induced by CP given ip at 50 mg/kg body weight. A dose dependent response was remarkable and statistically significant (Table 1). In the positive control group CP induced micronucleus formation at the dose of 50 mg/kg body weight. It was noteworthy that different doses of *P. longum* and CP used in the present experiments were not cytotoxic for PCE / NCE (normochromatid erythrocytes) ratio in *P. longum* extract treated and positive control as compared to the solvent control group, which remained unchanged. Furthermore, detailed studies on the isolation and characterization of the active plant compound will be necessary for discovering the new biological agent for Anti mutagenic property. It is therefore concluded that *P. longum* and its bioactive compound exhibited micronucleus formation and also protect the CP induced cell damages in bone marrow cells of Swiss albino mice. Further detailed research studies are needed to obtain more scientific data on this miraculous King of spices.

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