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Phytoremediation of methotrexate induced genotoxicity in *Heteropneustes fossilis* using extracts of *Centella asiatica*

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Abstract
The extracts of *Centella asiatica* (commonly known as Mandukaparni), are widely used in ethnobotanical. This study has first taken the initiative of assessing their chemo-protective properties against Methotrexate (MTX) insult. *Heteropneustes fossilis* was chosen as the model for this experiment and they were divided into three groups. Group 1 was kept as the control; Group 2 was given sub-lethal concentrations of MTX; Group 3 was administered *Centella asiatica* extract simultaneously with MTX. The extent of genotoxicity was monitored by micronucleus assay. This setup enabled us to clarify whether the extract had any remedial effect on MTX induced genotoxicity. The results indicated that *Centella asiatica* extract can be used to alleviate the damage induced by MTX. This study has opened up new frontiers for preventing any collateral damage done by chemotherapy drugs and directing their effects towards only the carcinogenic cells. Further research is required for practical application of these plant extracts in cancer patients, as an efficient mechanism for delivering the drug to the normal tissues is yet to be invented.

Keywords: *Centella asiatica;* Methotrexate; Micronuclei; post-treatment care for cancer patients

1. Introduction
Methotrexate has an essential role in the treatment of diseases like acute lymphocytic leukemia, non-Hodgkin's lymphoma, osteosarcoma, choriocarcinoma, head and neck cancer, and breast cancer [1]. It has also become an important therapeutic alternative in the treatment of severe psoriasis [2] and in the suppression of graft-versus-host disease after bone-marrow transplantation [3] as well as in the experimental treatment of various rheumatic diseases after primary therapy has failed [4]. Through various research and experiments we have come to know about the basic steps in the action of methotrexate, the biochemical and genetic changes that guide to resistance to the drug. These information's have led scientists in turn to clinical experiments with new combinations of methotrexate and with high-dose regimens overcome resistance [5].

Though the application of MTX is to some extent restricted because of its side effects like nausea, vomiting and ulceration. It has a variety of cutaneous side effects, particularly when administered in high doses. The most common adverse effects include: hepatotoxicity (liver damage), ulcerative stomatitis, leukopenia and thus predisposition to infection, abdominal pain, fatigue, fever, dizziness, acute pneumonitis, rarely pulmonary fibrosis, and kidney failure. These happen due to the formation of dihydrofolate reductase which is important for maintaining the cellular tetrahydrofolate pool during purine and thymidine synthesis [6] [7].MTX induced toxicity results into formation of micronuclei in several cells and tissues. So in terms if we measure changes in number of micronuclei, we will get the extent of toxicity [8].

Nowadays, there is an increasing interest in the biochemical functions of natural antioxidant extracts from vegetables, fruits, and medicinal plants, which can help us to reverse this effect [9]. In this project, we have used extract of *Centella asiatica* to measure whether it has any effect on MTX induced genotoxicity.

C. asiatica (commonly known as Indian Pennywort) is famous for its beneficial role in human health. It is a perennial herb that has been used for centuries in Ayurvedic medicine to treat several disorders such as insanity, asthma, leprosy, ulcers and eczema and for wound healing etc. The antioxidant activity of phenolic compounds present in *C. asiatica* has been attributed to its oxide-reduction properties, which play an important role in the neutralization of free radicals [10].

A number of studies have depicted the noteworthy protective effect of the plant against several diseases. *C. asiatica* has potential as its natural antioxidant extract reflects its capability to become an entrant to prevent oxidative damage, increasing health benefits [11]. Many experiments especially in animal studies and in human interventions have shown its wide pharmacological activities in brain improvement and neuro-protection effect [12].

1.1 About the plant extract used

Centella asiatica (Linn.) commonly known as Indian Pennywort (Apiaceae) is one of the chief herbs for treating skin problems, to heal wounds, for revitalizing the nerves and brain cells, hence primarily known as a "Brain food" in India. *C. asiatica* is a reputed medicinal plant for its various pharmacological effects beneficial for human health. Besides its potent wound healing property, a number of studies described the noteworthy protective effect of the plant against several diseases. Antioxidant activity of *Centella asiatica* possesses neuro-protective effect and effects against age related oxidative damage in rat's brain [13]. The anti-oxidant enzymes, like superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHPx) were remarkably increased, and anti-oxidants like glutathione (GSH) and ascorbic acid were decreased in lymphoma-bearing mice after oral treatment of *C. asiatica* extract. [14]. Administration of aqueous extracts of *C. asiatica* also exhibited to counteract lead-induced oxidative stress male rats [15].

Centella asiatica leaves exhibit higher antioxidant activities using boiled aqueous extraction compared to aqueous extract in DPPH and FRAP assays. Total flavonoid content and total phenolic content also respond better in boiled aqueous extraction when compare to aqueous extraction [16, 17].

Preclinical studies have shown that methanolic extract of *C. asiatica* helps in inhibiting breast cancer cells by inducing apoptosis in different cancer cell lines like HeLa, HepG2,SW48 etc. [18]. Some studies have also revealed that extract of *C. asiatica* lowered the blood glucose levels to normal in glucose tolerance test carried out in the alloxan induced diabetic rats [19].

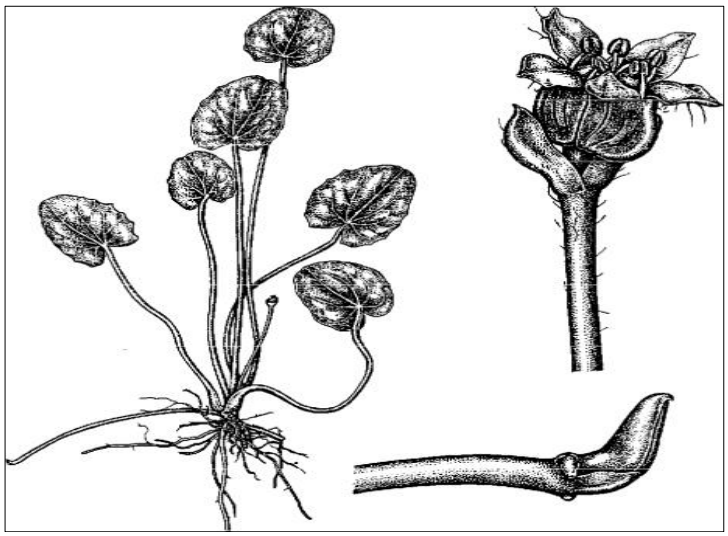

Fig 1: *Centella asiatica*

2. Materials and Methods

2.1 Chemicals used

The chemicals used for the experiment were procured from the Sigma-Aldrich Corporation, USA.

2.2 Fish

Heteropneustes fossilis (50±5g), with average length of 20±5 cm and roughly of same weight, were obtained from local fish market and transported to the laboratory. During acclimatization, for a span of two weeks, the fish were fed *ad libitum* with *Tubifex* sp., once a day.

2.3 Water composition

Water composition was as follows: temperature = 24±2 °C; total hardness = 67.50±3 mg/L $CaCO_3$; pH = 7.82±0.07; dissolved oxygen = 7.30±0.6 mg/L; alkalinity = 180±17 Mg/L $CaCO_3$. Water used was aerated dechlorinated tap water; moreover, temperature, dissolved oxygen, pH were analyzed daily and a 12h light: 12h dark photo period was Maintained. The experimental procedure was approved by the institutional ethics committee. Maintenance of the animals was in accordance with the guidelines of the Government of India for the use of laboratory animals.

2.4 Preparation of plant extract

C. asiatica plants were collected from the local market. The polyphenolic compounds were then extracted by the methods proposed in a study with some slight changes [20]. At the beginning, stem and leaves of *C. asiatica* were washed thoroughly with tap water. Test samples were then dried in a hot air oven and ground to powder with the help of a grinder. 80 ml of 80:20 methanol and water mixtures were taken in conical flasks and to them 2 gram dried sample powders were added. Mouths of the flasks were then covered (to avoid loss of methanolic part by evaporation). The conical flasks were then kept overnight in shaking for extraction. The extracts were then filtered using Whatman® Grade 1 qualitative filtration paper and the filtrates were concentrated in a rotary evaporator. The concentrated methanolic fractions obtained were then pooled and kept at 4 °C. The required amounts for the doses (1 mg polyphenolic compounds expressed as gallic acid equivalents/ml) were then dissolved in water to obtain the water extracts.

2.5 Experimental setup and exposure

The fishes were randomly divided into three groups; each group containing 4 fish. The first group was held in tap water

as a control group 1. Fishes in group 2 were exposed to sub-lethal concentration of 0.5 mg/litre of MTX based on determined 96-h LC50 value for *H. fossilis*. Fishes in group 3 was exposed to 1 mg/ml of plant extract for along with 0.5 mg/litre of MTX. One fish was sacrificed from each group in 7 days intervals (total 3 times) for micronucleus study. The whole experimental cycle was repeated thrice.

2.6 Micronucleus assay
Blood samples were collected from the fish by standard caudal vein puncture technique using heparinized syringe and proceeded for slide preparation. Clean slides were taken and the peripheral blood from each fish was smeared onto the slides with proper coding. The coded slides were air dried for 12 h and then fixed in absolute methanol for 10 min. After fixing, the same slides were stained in aqueous Giemsa (5%) for 10 minutes [21]. 1000 erythrocytes were counted separately in each slide to ascertain the frequencies of micronuclei present. The frequencies of micronuclei in erythrocytes were counted under the compound light microscope with 45× objective and 15× eye pieces. The frequencies of MN were expressed per 1000 cells.

3. Results

3.1 LC50 of Methotrexate
During the course of the toxicity study, behavioural changes were observed in the *H. fossilis* fishes after they were introduced into different concentrations of MTX. They showed quick and rapid movement for some time in the beginning and later settled down. These tendencies were found to be directly proportional to the concentrations of MTX. The fishes were considered dead once their opercular movement ceased and they fell to the bottom of the tank with their body upturned. The fishes in the control did not show any sign of stress up to 96 hours study period. The percent mortality was found to be increasing in all the different concentrations with increase in the time period of exposure up to 96 hours.
The percent mortality data was used in calculation of LC50 value at 96 hours' time interval. Calculations revealed the LC50 value at 96 hours to be 2.13 mg/L.

3.2 Micronucleus Pattern
Sex-related differences were not observed with respect to either micronuclei induction; therefore, data from males and females were collapsed into one group. Micronucleus pattern of all *H. fossilis* treatment groups were studied for every 7 days and are tabulated below.

Table 1: Micronucleus pattern of different treatment groups

Treatment Group	Days of Treatment	Mean MN Frequencies/1000Erythrocytes	Std. Deviation	Std. Error
Control	7	2.963	0.162	0.061
	14	2.321	0.421	0.046
	21	1.981	0.231	0.032
MTX	7	5.662	0.721	0.021
	14	8.506	0.523	0.042
	21	17.821	0.291	0.039
MTX+ PE	7	4.851	0.652	0.048
	14	4.462	0.731	0.056
	21	3.664	0.105	0.061

Key: MTX= Methotrexate Treated Group, MTX+PE= Methotrexate along with simultaneous application of *C. asiatica plant* extract

During the course of 21 days, behavioural changes of fishes were studied into different concentrations of MTX. Micronuclear assay were done for every 7days interval & the data shows significant changes in MN patterns after 21 days.
With respect to the control group, in the 1st 7days the MN numbers increased about 91 % group 2 administered with MTX and 63.7 % in group 3 which had MTX & plant extract. But with respect to MTX administered group 2, MN numbers dropped 14.3% in group 3 after 7days.

In the next 14 days, MN numbers increased 22.56 times in group 2 and this number as gradually decreased 92% in group 3 with respect to the control group. But with respect to MTX administered group 2, MN numbers dropped about 47.5% in group 3(MTX+PE) after 14days.
After 21 days, it is observed that MN numbers increased 7.995 times in group 2 and this number as decreased 84% in group 3 with respect to the control group. But with respect to MTX administered group 2, MN numbers dropped about 79.4% in group 3(MTX+PE) after 21 days.

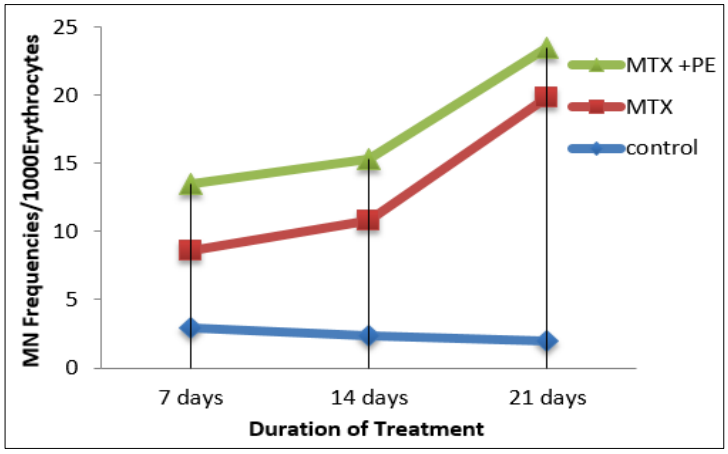

Fig 1: Mean frequency of induced micronuclei in various experimental groups of fishes after 7, 14 & 21 days of treatment [KEY: MTX= methotrexate treated group, MTX+PE= methotrexate along with simultaneous application of *C. asiatica* plant extract]

Table 2: showing for 7 days treatment against control with MTX & MTX+PE as well as with respect to MTX with MTX+PE

7 days treatment	Control	MTX	MTX+PE
Control	-	0.91	0.637
MTX	-	-	-0.143
MTX+PE	-	-	-

Table 3: showing for 14 days treatment against control with MTX & MTX+PE as well as with respect to MTX with MTX+PE

14 days treatment	Control	MTX	MTX+PE
Control	-	22.565	0.922
MTX	-	-	-0.475
MTX+PE	-	-	-

Table 4: showing for 21 days treatment against control with MTX & MTX+PE as well as with respect to MTX with MTX+PE

21 days treatment	Control	MTX	MTX+PE
Control	-	7.995	0.84
MTX	-	-	-0.794
MTX+PE	-	-	-

4. Discussion

So, from the data it can be said that the number of micronuclei induced by MTX is greatest after 21 days of treatment (Table 1) and this number is also decreased 79.4% when administered with MTX & plant extract simultaneously in group 3. MN formations is a common side effect of methotrexate toxicity & in the current study it is exhibited that the MN frequency in the peripheral erythrocytes jumped about 22.56 times that observed in the control specimens. In a previous study, it has been found that extract of *Piper betle* & *Asteracantha longifolia* are capable of significantly reducing the MN frequencies from that observed in the MTX treated group but they could not bring the micronuclei level down to its control value [22]. There the plant extracts were given in two different ways-first, the extract was given prior to MTX administration and also simultaneously with the proper dose of MTX [this is the preventive treatment]; and secondly, the extract was only given simultaneously with MTX [this one is the ameliorative treatment].

In the present study, we have just gone for the ameliorative treatment giving *C. asiatica* extract only simultaneously with MTX and as a result the number of micronucleus is reduced in group 3 after 21 days. This proves that the extract of *C. asiatica* is effective in reducing the MN frequencies in the treatment groups. This means that further research is needed in order to find a suitable ethno-botanic ameliorative measure against MTX induced genotoxicity.

5. Conclusion

In conclusion, the results indicate that the use of *C. asiatica* extract is effective as a preventive measure best used as a remedial manoeuvre step against MTX induced genotoxicity. Repetition of the experiment needs to be done in a suitable mammalian test subject, before the confirmation of the results have any impact on modern medicinal practices.

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