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Phytoremediation of methotrexate induced genotoxicity using polyphenol extracts of *Asteracantha longifolia* Nees. and *Piper betle* Linn

Shirsendu Ghosh, Abhishek Chatterjee, Aniruddha Mukhopadhyay and Pinakiranjan Chakrabarti

Abstract
The extracts of *Asteracantha longifolia* and *Piper betle* are widely used ethnobotanics. 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 six groups. Group 1 was kept as the control; Group 2 was given sublethal concentrations of MTX; Group 3 was administered *A. longifolia* extract prior to as well as simultaneously with MTX; Group 4 was given *A. longifolia* extract only along with MTX; Group 5 was administered *P. betle* extract prior to as well as simultaneously with MTX and Group 6 was given *P. betle* extract only along with MTX. This setup enabled to clarify which extract had either a preventive or a remedial aptitude at dealing with MTX induced genotoxicity. The results indicated that *A. longifolia* extract can be used to remedy the damage induced by MTX and *P. betle* extract is the best at preventing it. 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: *Asteracantha longifolia*; *Piper betle*; Methotrexate; Micronuclei; post-treatment care for cancer patients

1. Introduction
Methotrexate (MTX), a folate antagonist agent, is mainly used in the treatment of malignant tumors and non-neoplastic diseases [1]. It is also used in treatment of autoimmune diseases (like Rheumatoid Arthritis), acute lymphoblastic leukemia and other forms of carcinoma, severe psoriasis and as an abortifacient drug. MTX can be used to manage cases of ectopic pregnancy [2]. Although an important therapeutic agent, the clinical application of MTX is often limited by its side effects like severe nausea and widespread gastrointestinal ulceration. Rather than the direct action on the gastrointestinal tract, these side effects are probably due to the inhibition of the synthesis of dihydrofolate reductase which is essential to maintain the cellular tetrahydrofolate pool during purine and thymidine synthesis [3]. Therefore, MTX affects not only tumor cells, but also rapidly-dividing cells such as crypts of gastrointestinal mucosa. For the same reason, it is also very important as a chemotherapeutic agent [4].
Cyclical high doses of MTX, as used for acute leukemia [5, 6], or the relatively high doses of MTX used to treat severe psoriasis have been associated with liver hepatotoxicity, including progressive hepatic fibrosis and cirrhosis [7, 8]. On the other hand, MTX can cause increased serum creatinine levels, uremia, and hematuria, while its administration in high doses has been reported to cause acute renal failure [9]. MTX-induced toxicity appears to be a consequence of the interaction of many factors: dosing schedule and length of treatment, patients' risk factors, type of disease, presence of genetic and molecular apoptotic factors and many others [10].
It has also been demonstrated that the cytosolic NAD (P)-dependent dehydrogenases [11] and NADP malic enzyme are inhibited by MTX, suggesting that the drug could decrease the availability of NADPH in cells [12]. Under normal conditions, NADPH is used by glutathione reductase to maintain the reduced state of cell glutathione, which is known as an important cytosolic antioxidant, protective against reactive oxygen species (ROS). Thus, the significant reduction in glutathione (GSH) levels promoted by MTX leads to a reduction of effectiveness of the antioxidant enzyme defense system, sensitizing the cells to ROS [13].
The extent of toxicity caused by MTX translates into formation of micronuclei in various tissues [14]. Hence, the micronuclei count is often used to measure the level of toxicity.

In order to delimit the usage of this drug, its side effects must be dealt with in a way that does not further complicate the cellular conditions. At present, Leucovorin (folinic acid) is generally administered with MTX to decrease MTX-induced toxicity [15]. The search for a better alternative has led us into the realm of ethnobotanics, since phytochemicals are always a better option for use as drugs compared to their synthetic alternatives. Green leafy vegetables are rich sources of many nutrients and their beneficial role has partly been attributed to the antioxidant components present in them of which the major portion is formed by the flavonoids, isoflavones, lignans, catechins and isocatechins [16, 17]. Hence, two such plants with previous records of bearing antioxidant activity have been the focus of this study.

In this study, the ameliorative property of methanol-water extract of *Asteracantha longifolia* and *Piper betle* were investigated against the genotoxic effects of MTX by examining the occurrence of micronucleus (MN) in peripheral erythrocytes of *Heteropneustes fossilis*. This is a new approach on its own, since no such attempts at investigating the role of such ethnobotanics in ameliorating the side-effects of anti-cancer drugs have been made before. This study may provide a new direction for research in the field of post-treatment care for cancer patients.

1.1 About the plant extracts used

Hygrophila auriculata (K. Schum.) Heine Syn. *Asteracantha longifolia* Nees. (Acanthaceae) [Plate I] has been shown to possess hypoglycemic activity in human subjects [18]; exhibits hepatoprotective activity against paracetamol and thioacetamide intoxification in rats [19]; pacifies CCl_4-induced liver dysfunctions [20]; antitumor [21] and promotes anabolic and androgenic activities [22]. *A. longifolia* seeds have been reported to ameliorate the activities of antioxidant enzymes Glutathione Peroxidase and Catalase in hepatocarcinoma [23]. The plant is also known to possess antibacterial [24, 25], free radical scavenging and lipid peroxidizing [26] activities.

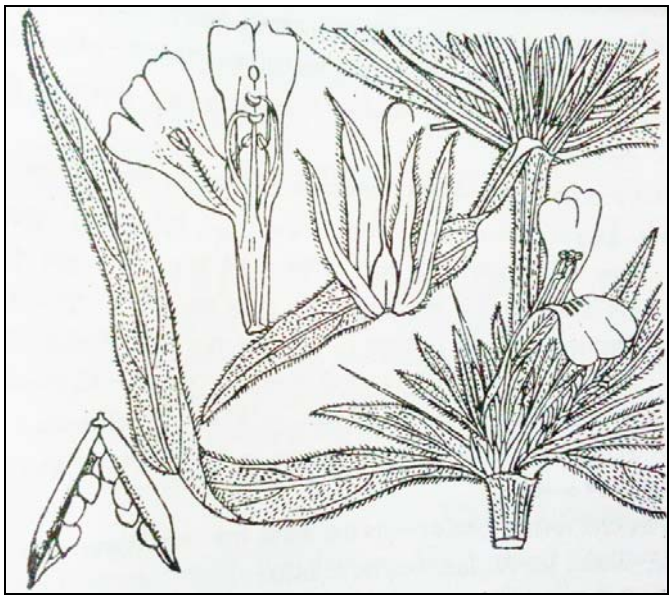

Plate 1: *Asteracantha longifolia*

Experimentally, the leaves of *Piper betle* Linn. (Piperaceae) [Plate II] are shown to possess antimicrobial [27], gastroprotective [28], wound healing [29], hepatoprotective [30], antioxidant [30-32], anti-fertility on male rats [33] and antimotility effects on washed human spermatozoa [34].

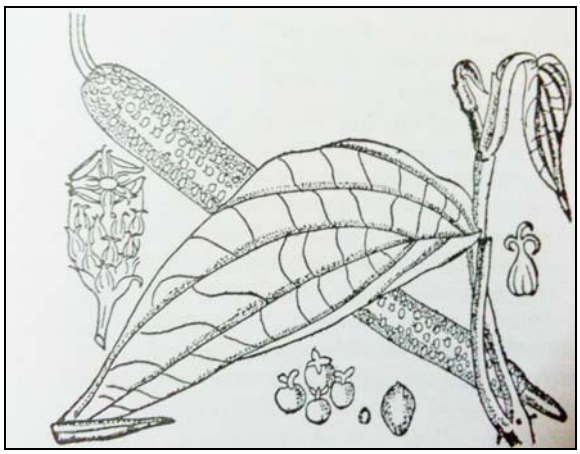

Plate 2: *Piper betle*

The leaf extract has significant stimulatory influence on pancreatic lipase activity in experimental rats [35]. The leaf extract inhibits radiation induced lipid peroxidation. The extract also increased the activity of superoxide dismutase activity in a dose dependent manner, indicating elevation of antioxidant status in Swiss albino mice [31]. *P. betle* leaves also afforded a significant hepato-protective effect and improved the tissue antioxidant status by increasing the levels of non-enzymatic antioxidants (reduced glutathione, vitamin C and vitamin E) and the activities of free radical-detoxifying enzymes in liver and kidney of ethanol-treated rats [30].

2. Materials and methods

2.1 Chemicals used

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

2.2 Fish

Heteropneustes fossilis (50±5g), with average length of 20±5 cm and approximately of same weight, were procured from local fish market (Jadavpur Super Market, West Bengal, India) and transported to the laboratory in glass bottles. During acclimatization, for a period 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 ^{0}C; total hardness = 67.50 ±13 mg/L CaCO3; pH = 7.82 ± 0.07; dissolved oxygen = 7.30 ± 0.6 mg/L; alkalinity = 180 ±17 mg/L CaCO3. Water used was aerated dechlorinated tap water; moreover, temperature, dissolved oxygen, pH were analyzed daily and a 12h light: 12h dark photoperiod 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

A. longifolia and *P. betle* plants were collected from the local market. The polyphenolic compounds were then extracted by the methods developed by Mallick *et al.* [36] with slight modifications. At first, stem and leaves of *A. longifolia* and only the leaves of *P. betle* were washed thoroughly with tap water. Test samples were then dried in a hot air oven and ground to powder using a grinder. 80 ml of 80:20 methanol and water mixtures were taken in conical flasks and to them 2 gram dried sample powders added. Mouths of the conical flasks were then covered (to avoid loss of methanolic part by

evaporation). The conical flasks were 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 stored at 4^0C. 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 Determination of polyphenols
Total polyphenol content of the plants were determined by the Folin–Ciocalteau method as described by Matthaus [37].

2.6 Experimental setup and exposure
After two weeks of acclimation, the fish were randomly divided into six groups; each group containing 3 fish. The first group was held in tap water as a control group. Fishes in group 2 were exposed to sub-lethal concentration of 0.5 mg/liter of MTX based on determined 96-h LC50 value for *H. fossilis*. Fishes in group 3 and 5 were first exposed to 1 mg/ml of plant extract for 7 days prior to MTX administration and then simultaneously to 0.5 mg/liter of MTX along with the plant extract. Fishes in group 4 and 6 were exposed only to the simultaneous treatment with 1 mg/ml of plant extract and 0.5 mg/liter of MTX. Groups 3 and 4 were administered with *A. longifolia* plant extract; whereas groups 5 and 6 were given the *P. betle* plant extract. Experimental aquaria were aerated and test media were replaced every day. No fish mortality occurred during these exposures. One fish was sacrificed from each group in 7 days intervals (total 3 times) for micronucleus study. The whole experimental cycle was repeated thrice.

2.7 Micronucleus assay
Blood samples were collected from the fish by standard caudal vein puncture technique using heparinized syringe and proceeded for slide preparation. For each fish, five microscopic slides were prepared. 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 [38]. 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.

2.8 Statistical analysis
All the statistical analyses [39-41] were performed using Sigma Stat® version 3.5 (© Systatsoftwares Inc. USA, 2006) and SPSS® version 10.0 (© SPSS Inc. USA, 1989). The significance of differences among the groups were assessed using an one way repeated measure analysis of variance (ANOVA) test followed by Tukey Test which was selected as the *post hoc* test in order to isolate the group(s) that differs from the others. P values < 0.05 are considered as significant.

3. Results
3.1 LC_{50} of Methotrexate
During the course of the acute toxicity study, behavioral changes were observed in the *H. fossilis* fishes after they were introduced into different concentrations of MTX. They showed quick, abrupt and irregular 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, Fig. 1).

Table 1: Micronucleus pattern of different treatment groups.

Treatment group	Days of treatment	Mean of MN frequencies/1000 erythrocytes	Std Deviation	Std Error
C	7	2.060	0.122	0.0702
	14	1.953	0.0351	0.0203
	21	1.323	0.0874	0.0504
MTX	7	5.643	0.0802	0.0463
	14	5.807	0.0473	0.0273
	21	18.657	0.0651	0.0376
A+MTX	7	5.593	0.0651	0.0376
	14	4.910	0.0900	0.0520
	21	4.007	0.0802	0.0463
MTX+A	7	4.280	0.0954	0.0551
	14	3.980	0.105	0.0608
	21	3.250	0.101	0.0586
P+MTX	7	3.893	0.0850	0.0491
	14	1.930	0.105	0.0608
	21	0.973	0.0651	0.0376
MTX+P	7	5.580	0.0700	0.0404
	14	4.927	0.0950	0.0549
	21	3.963	0.0814	0.0470

KEY: C=control, MTX= methotrexate treated group, a+mtx= methotrexate treated (along with prior and simultaneous application of *A. longifolia* plant extract) group, mtx+a= methotrexate treated (along with simultaneous application of *A. longifolia* plant extract) group, p+mtx= MTX treated (along with prior and simultaneous application of *P. betle* plant extract) group, mtx+p= MTX treated (along with simultaneous application of *P. betle* plant extract) group.

The number of micronuclei induced by MTX is greatest after 21 days of treatment (Fig. 1); hence the effects of the two plant extracts given in two different sets of conditions can be best understood by measuring deviations of this value produced by the respective treatments. All the remaining data obtained have not been considered for further analysis.

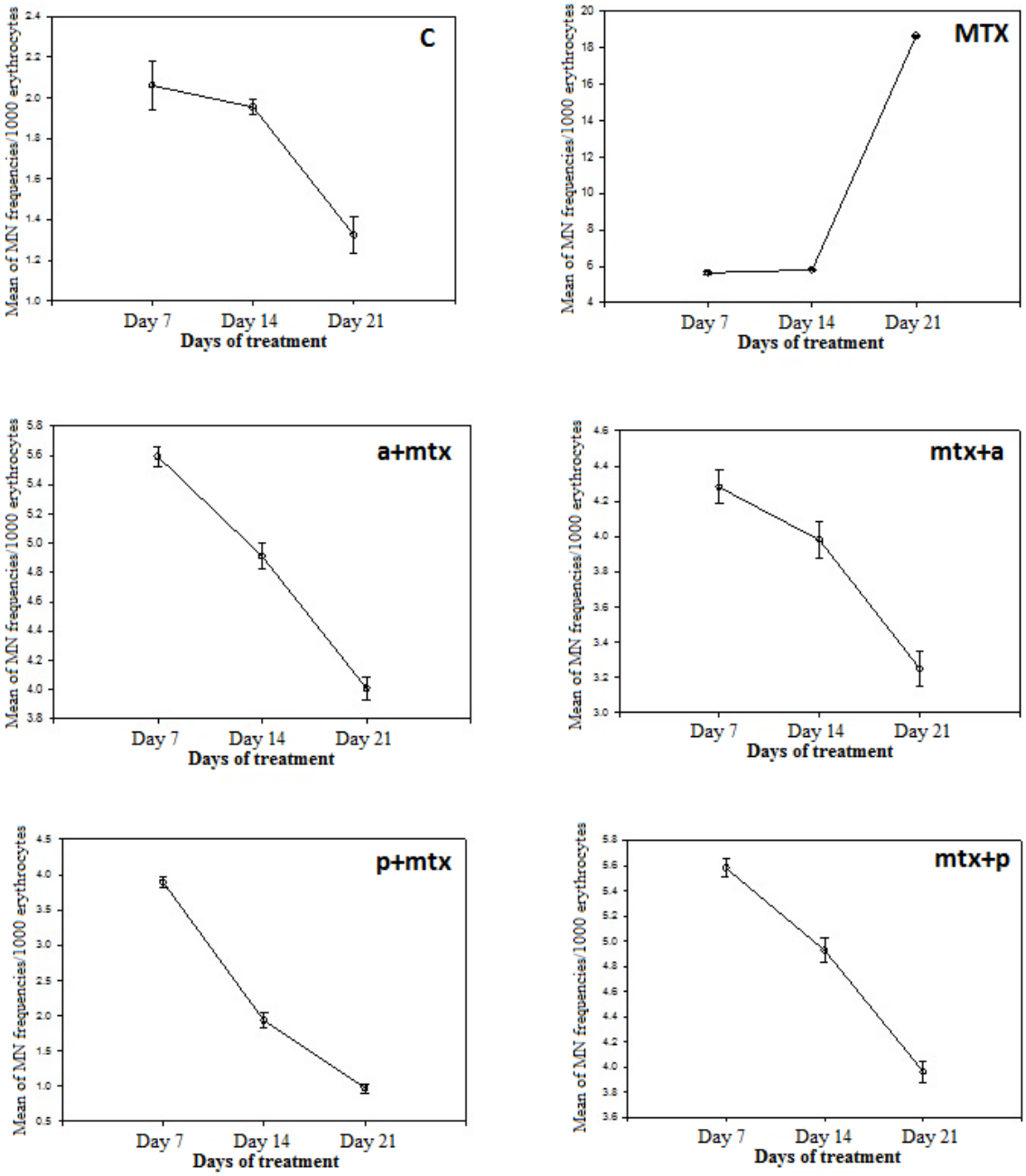

Fig 1: Graphical representation of the data obtained for all the six treatment groups. Refer table 1 for abbreviations of the different treatment groups.

Methotrexate induced a significantly greater ($p<0.001$) number of micronuclei when compared to the control (Group 1). The micronuclei were significantly reduced ($p<0.001$) in all the four treatment combinations, when compared to the methotrexate (Group 2)-treated rats. Within the different treatment group combinations, there was no significant difference ($p>0.05$) only in the case of a+mtx (Group 3) and mtx+p (Group 6) when they were compared with one another. All the other treatment group combinations yielded significant differences ($p<0.05$) among them. These results indicate that, in case of simultaneous treatment with MTX, application of the plant extract of *A. longifolia* has better effects than using *P. betle* extract. The results also indicate that, in case of both prior and simultaneous treatment, the use of *P. betle* extract is more advantageous than using the extract of *A. longifolia*.

4. Discussion

Methotrexate is a very versatile drug, which can be used in remedying many diseases among which is also cancer, the king of all maladies. But, the practical use of this drug is greatly limited by its side effects. One of such side effects is the induction of micronuclei formation by MTX, which has also been exhibited in this current study where the MN frequency in the peripheral erythrocytes jumped about 14 times that observed in the control specimens. In a previous study done by Bonassi *et al.* [42], it has been found that a higher MN frequency is a clear indicator for cancer risk. For this reason, this phenomenon has become a dilemma as, in the process of ameliorating cancer with MTX, the patient becomes prone to carcinogenic development in other healthy tissues.

In the present study, methanolic extracts of two plants (viz., *Asteracantha longifolia* and *Piper betle*) have been administered in two different ways— in the first method, 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]. This temporal difference in using the extract with respect to MTX administration was done to ascertain the effectiveness of each of the two extracts at either preventing or remedying the side-effect of micronuclei induction. Since, the objective of this study is to come up with a way to reduce the genotoxic effects of MTX, it is only obvious to check for preventive measures as well.

Coming back to the results, this study clearly demonstrates that both of the plant extracts are capable of significantly reducing the MN frequencies from that observed in the MTX treated group (refer Figure 2 for better understanding). But, here, it is to be noted that most of these treatment combinations could not bring the micronuclei level down to its control value. Only in the preventive treatment using *P. betle* extract, the levels of induced micronuclei could be kept under that of the control. Therefore, among the two plant extracts used in this experiment, *P. betle* is the best choice in terms of a preventive measure.

As far as the ameliorative treatments go, neither of the two plant extracts could produce results at par with the previous treatment, just discussed. Although these extracts were not nearly as potent as ameliorative measures, but still *A. longifolia* produced a better result of the two. After 21 days of treatment, the MN levels in this treatment group were about 2.5 times that observed in the control (Table 1). This means that further research is needed in order to find a suitable ethno-botanic ameliorative measure against MTX induced genotoxicity.

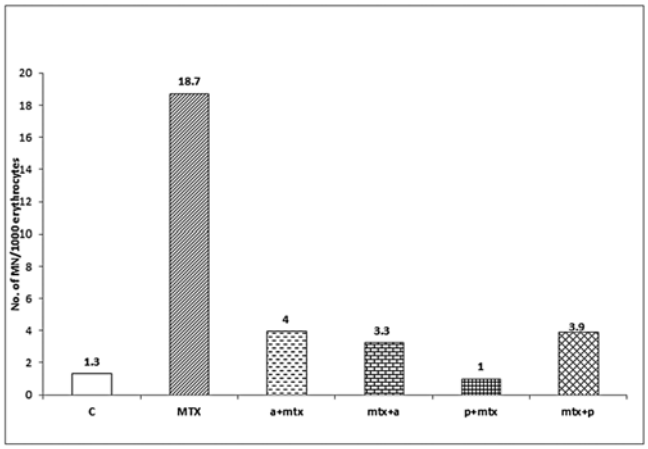

Fig 2: Mean frequency of induced micronuclei /1000 erythrocytes in various experimental groups of fishes as recorded after 21 days of treatment

The study is the first one of its kind, as the anti-genotoxic property of these two plants have not been investigated before. Hereby, this study claims that the plant extracts of *Asteracantha longifolia* and *Piper betle* possess a protective effect against genotoxicity and the ill-effects of cancer chemoprevention. The one and only drawback of this study is the use of a fish model in the experiment. For the said reason, repetition of the experiment needs to be done in a suitable mammalian model, before a confirmation of the results to have any impact on modern medicinal practices can be given. There is need of further investigation for finding the plant extracts' principal active component and thereby using it in treating actual patients.

5. Conclusion

In conclusion, the results indicate that the use of *P. betle* extract is most fruitful as a preventive measure and that of *A. longifolia* is best used as a remedial measure against MTX induced genotoxicity. The reason behind this is that *P. betle* cannot remediate and *A. longifolia* fails to prevent micronuclei induction by MTX. But, the specialties of both the extracts can be exploited to further reduce the genotoxic effects. Therefore, we suggest to perform another experiment where *P. betle* extract will be administered to the specimen before MTX is given and *A. longifolia* extract will be provided simultaneously with MTX, in the same treatment group. The suggested experiment will surely help to clarify the extent of affectivity of these ethnobotanics in the field of post-chemotherapy care.

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