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Research Article

AN *IN VITRO* STUDY OF AMELIORATIVE EFFECT OF COMBINATION OF ROOT EXTRACT OF *OROXYLUM INDICUM* WITH COW URINE DISTILLATE ON CYPERMETHRIN INDUCED CYTOTOXICITY ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES USING MTT ASSAY

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ABSTRACT

Cypermethrin is a pesticide which is used by the farmers of Jabalpur as well as throughout the country. Farmers are exposed to it while spreading it on the crops. In the current study, effect of combination of root extract of *Oroxylum indicum* with cow urine distillate on cypermethrin induced cytotoxicity on human peripheral blood lymphocytes using MTT assay was assessed as cow urine and *Oroxylum indicum* has been found life supporting agents. Different concentration of cypermethrin were used to induce toxicity. Aqueous fraction of 10% root extract of *Oroxylum indicum* in combination with 50% cow urine distillate showed ameliorative effect. This combination may be used for the treatment against cypermethrin induced cytotoxicity in the farmers exposed to it.

Keywords: MTT assay, Cytotoxicity, Ameliorative effect, Cow urine, *Oroxylum indicum*

INTRODUCTION

Pyrethrins/ Pyrethroids are a family of compounds either directly derived from a species of Chrysanthemum flowers (pyrethrins) or manufactured to resemble these chemicals (pyrethroids). In general, these compounds are considered relatively safe to humans because of their low-toxicity compared with other classes of pesticides. The pyrethroids are considered to be less toxic than the pyrethrins and are being increasingly used for agricultural purposes and crop protection to control many pests, including pests of cotton, fruit and vegetable crops[1]. However the pyrethroids are highly toxic to aquatic organisms and fish as well as to bees.[2] Pyrethroids are synthetic analogues of pyrethrins, the active substances in the flowers of Chrysanthemum, *Cineraria folium*. Pyrethroids can be classified into two large groups. Type I pyrethroids do not contain a cyano group in their molecules and Include allethrin, tetramethrin, permethrin and phenothrin. Type II pyrethroids contain a cyano group at the α-carbon position and include newer compounds, such as deltamethrin, cyphenothrin, Cypermethrin (CYP) and fenvalerate. The two types of pyrethroids cause somewhat different symptoms of mammalian poisoning. Poisoning with type I pyrethroids is characterized by hyper excitation, ataxia, convulsions and eventual paralysis; poisoning with type II pyrethroids, by, hypersensitivity choreoathetosis, tremors and paralysis. Despite differences in the symptoms, both types of pyrethroids have the same major target site; the sodium channel of nerve membrane, i.e., the channel directly responsible for generating action potentials.[3] Cypermethrin has become one of the most important insecticides in wide scale use. It has wide uses in cotton, cereals, vegetables and fruits, for food storage, in public health and in animal husbandry.

Cypermethrin an analogue of pyrethrins is classified by the World Health Organization (WHO) as moderately hazardous (class II). It interacts with the sodium channels in nerve cells through which sodium enters the cell in order to transmit a nerve signal. These channels can remain open for up to seconds compared to the normal period of a few milliseconds, after a signal has been transmitted.[4] Cypermethrin also interferes with other receptors in the nervous system. The effect is that of long-lasting trains of repetitive impulses in sense organ.[5]

Cow (*Bos indicus*) urine/gomutra has been elaborately explained in Ayurveda and described in "Sushruta Samhita", "Ashtanga Sangraha" and other Ayurvedic texts as an effective medicinal substance/secretion of animal origin with innumerable therapeutic properties.[6] Bhav Prakash Nighantu described gomutra as the best of all types of animal urine (including human) and enumerates its various therapeutic uses.[7] Persons who drink gomutra regularly are said to live a healthy life, remaining unaffected by the vagaries of old age, even at age 90.[8] Gomutra is called "Sanjivani" and "Amrita" in Ayurveda. In addition, it has applications as a biopesticide in organic farming along with cow dung, cow's milk and other herbal ingredients. Gomutra is not a toxic waste material. 95% of it is water, 2.5% consists of urea, and the remaining 2.5% is a mixture of minerals, salts, hormones and enzymes.[9] Traditional medicines, whether from Ayurveda or Siddha are based on classical texts and systems, practices and products handed down over generations going back to Charak, Sushruta, Vagabhatta, the Ashtangahridaya and the Samhitas. Several medicinal properties of cow urine have been mentioned and in Indian tradition, cow urine is believed to have therapeutic properties.[10]

The various mineral and chemical constituents present in cow urine are attributed with different biochemical roles in the body.[11] For example nitrogen remove blood abnormalities, toxins and is a natural stimulant of urinary system; sulphur supports peristaltic movement in large intestine and purified blood; Copper controls unwanted fat; Iron helps in production of haemoglobin and erythropoiesis; Phosphorus helps in removal of stones from

urinary tract: Sodium purifies blood and acts as an antacid; Potassium cures rheumatism and increase appetite, muscular strength, stamina and activeness; Manganese prevents growth of germs, tissue necrosis; Calcium is a blood purifier and strengthens bones; gold is a germicidal and increases immunity. Our body contains many micronutrients that give us strength for life, but these micro-nutrients are flushed out of our body when we urinate. Cow urine meets the deficiency of these micronutrients in the body. It maintains the balance of these substances in our body and cures even the so-called incurable diseases.[12]

Plants have been a source of medicinal products since a long time. To date, approximately 100 species of plants have been examined and some active constituents have been isolated and identified, for instance several of the current chemotherapeutic drugs like vinblastine, methotrexate, taxol, and so forth, were first identified in plants. *Oroxylum indicum (L.) Vent*. tree has been used for the preparation of an Ayurvedic medicine since a long time. Every part of this tree contains metabolites that possess medicinal value with anticancer potential.[13] Hence the present study was designed to observe the ameliorative effect of aqueous fractions of root extract of plant with 50 % cow urine distillate on Cypermethrin induced damage in human lymphocytes using MTT assay.

MATERIALS AND METHODS

Cypermethrin [Cyano-(3-phenoxyphenyl) methyl] 3-(2, 2-dichloroethenyl)-2, 2-dimethylcyclopropane-1-carboxylate used in this study was 25% EC (effective concentration), which was purchased from the market. To prepare desired dilutions, pesticide was dissolved in triple distilled water. The dilutions were made in such a way that the pesticide was added to cell suspensions in the concentration of 1µg/ml, 2.5µg/ml, 5µg/ml, 7.5µg/ml and 10 µg/ml of the culture medium.

Extract preparation: -Root of *Oroxylum indicum* (L.) Vent. was obtained from reserve forest area of Jabalpur (M. P.), India.

Extraction of secondary metabolites from the root of *Oroxylum indicum*

The dried roots were ground into a fine powder form. The root powder (5g) was dissolved in 100 ml ethanol and was incubated in an orbital shaker at 120 rpm for 24 hours at 25±2°C. After incubation period ethanolic crude root extract was filtered and evaporated to dryness. Residue was dissolved in 1 ml distilled water which was then acidified, neutralized again filtered and used as aqueous extract. Now this was again syringe filtered and 10 % aqueous root extract of *Oroxylum indicum* was mixed with 50% of cow urine distillate.

Isolation of lymphocytes from whole blood

Isolation of lymphocytes was performed as per Dayashankar *et al*[14] with some modifications. Blood (3 ml) from healthy female young volunteer donor was collected in sterile EDTA vacutainer. This was diluted with double volume of PBS (1X). Three milliliter of HiSepTM Lymphocyte Separation Medium (LSM) 1077 (Hi Media) was transferred aseptically into a centrifuge tube. This was then carefully overlaid with 9 ml of diluted blood. It was centrifuged at 400 x g at room temperature (RT) for 30 min. Erythrocytes were sedimented and the lymphocytes formed a layer above the Hi Sep layer. Most of the supernatant was aspirated out and then the lymphocyte layer along with half of the Hi Sep layer was carefully aspirated into a separate centrifuge tube. It was then given two washes with isotonic PBS. The cells were counted in a haemocytometer. The cells were appropriately diluted in TC 199 medium (Hi Media) supplemented with fetal bovine serum to give a final concentration of 12.7 x 10^5cells/ml.

MTT assay

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed according to Mosmann[15] with some modifications. Aliquots (180 µI) of the prepared lymphocyte suspension (12.7 x 10^5 cells/ml) were seeded into a 96-well polystyrene tissue culture plate in nine replicates. The experiment was performed in two sets. Set 1 was performed for testing the cytotoxicity of Cypermethrin while set 2 was performed to find the ameliorative effect of root extract of *Oroxylum indicum* in combination with 50% Cow urine on CYP induced cytotoxicity on human PBL at 24 Hours exposure.

Set -1: -One row containing only medium and cells served as a control. The dilutions of Cypermethrin were made in such a way that the pesticide was added to cell suspensions in the concentration of 1µg/ml, 2.5µg/ml, 5µg/ml, 7.5µg/ml and 10 µg/ml of the culture medium. Each concentration of the cyperinethrin was tested in five replicates. The plate was incubated for 2 h at 37°C with cyperinethrin at 5% $C0_2$. After incubation, 20 µl aliquots of MTT solution (5 mg/ml in PBS) were added to each well and reincubated for 2 h at 37°C. Then 100 µI of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals followed by overnight incubation at 37°C. The culture plates were then placed in an enzyme-linked immunosorbent assay (ELISA) microplate reader and absorbance was read at 600 nm.

Set- 02: - Extract with cow urine was added after inducing 2hrs cypermethrin exposure on human PBL. The plate was further incubated for next 24 hrs at 37°C at 5% CO_2. After incubation, 20 µl aliquots of MTT solution (5 mg/ml in PBS) were added to each well and re-incubated for 2 h at 37°C. Dimethyl sulfoxide (DMSO) was added to each well (100 µI) to dissolve the formazan crystals followed by overnight incubation at 37°C. The culture plates were then placed in an enzyme-linked immunosorbent assay (ELSA) microplate reader and absorbance was read at 600 nm.

The amount of color produced was directly proportional to the number of viable cells. OD of various concentrations of Cypermethrin was noted and the final OD was calculated after making the due adjustment for these two factors. All assays were performed in nine replicates per dose. Cell viability of controls was taken as 100%. Cell viability rate was calculated as the % of MTT absorption as follows:

Cell survival = (Mean experimental absorbance/Mean Control absorbance) x100

RESULTS AND DISCUSSION

The results indicated drop in cell viability% when the cells were treated with different concentrations of Cypermethrin. Cells treated with 1µg/ml, 2.5µg/ml, 5µg/ml, 7.5µg/ml and 10 µg/ml showed range from 83.01% at 1 µg/ml to 42.80% cell viability% at 10µg/ml) on 2hrs exposure on Human PBL. Cells exposed with different concentrations of CYP for 24 hours showed viability % from 80.40 to 61.60% (Table 1, Figure 2). The result indicated the drop-in cell viability when the concentration of Cypermethrin increased which was found to be significant (Table 2).

Table 1: Cytotoxicity of CYP (Mean)

Concentration of CYP	Effect of CYP toxicity on 2 Hours exposure	Effect of CYP toxicity on 24 Hours exposure
Control	100	100
1µg/ml	79.05	83.01
2.5µg/ml	73.85	62.5
5µg/ml	65.75	54.7
7.5µg/ml	53.76	50.07
10µg/ml	51.18	42.8

Table 2: Two-way ANOVA for analysis for CYP cytotoxicity at 2 hours and 24 hours

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	3908.782	5	781.7563	40.02772	0.000491	5.050329
Columns	77.57167	1	77.57167	3.971848	0.102862	6.607891
Error	97.65188	5	19.53038			
Total	4084.005	11				

Table 3: Effect of 10% root extract with 50% cow urine after CYP induced cytotoxicity

Concentration of CYP	Cell Viability %±SE	
	Effect of CYP toxicity (24 Hours Exposure)	Effect of 10% root extract with 50% cow urine distillate after CYP induced cytotoxicity at 24 Hours exposure
Control	100	100
1µg/ml	83.01±0.04	80.40±0.031
2.5µg/ml	62.50±0.03	78.14±0.03
5µg/ml	54.70±0.04	70.01±0.09
7.5µg/ml	50.07±0.02	64.90±0.03
10µg/ml	42.80±0.01	61.60±0.30

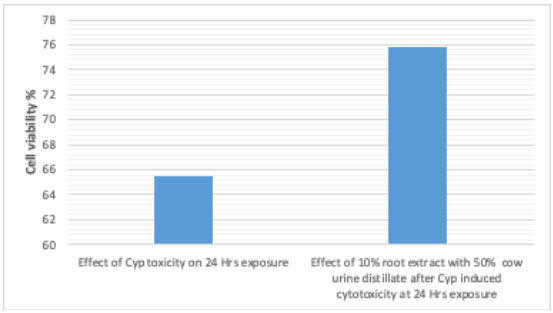

Figure 1: Ameliorative effect of aqueous root extract of *Oroxylum indicum*

Table 4: Two-way ANOVA for the analysis of the effect of 10% root extract with 50% cow urine distillate after CYP induced cytotoxicity at 24 hours exposure

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	3132.533	5	626.506655	14.94744	0.004994	5.050329
Columns	320.0234	1	320.0234083	7.635241	0.039684	6.607891
Error	209.5699	5	41.91398833			
Total	3662.127	11				

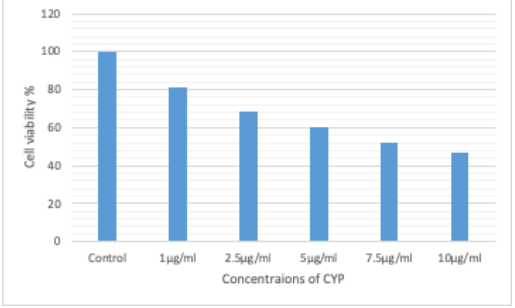

Figure 2: Effect of Concentrations of CYP on viability % of Human PBL after ANOVA

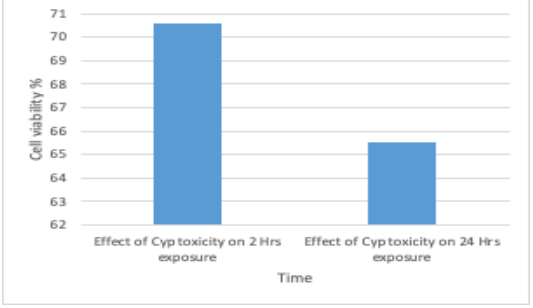

Figure 3: Effect of time on viability % of Human PBL after ANOVA analysis

The effect of exposure time was also directly proportional to the cytotoxicity (Figure 3). Treatment of Root extract (10%) in combination with 50% cow urine on CYP induced 2 hrs cytotoxicity on human PBL showed increase in viability % (Figure 1, Table 3). The difference was found to be significant also (Table 4).

Survival rate of damaged lymphocytes was enhanced after root extract treatment (Figure 1). Aqueous fraction of 10% root extract of *Oroxylum indicum* in combination of 50% cow urine distillate showed ameliorative effect as a treatment against cypermethrin Induced cytotoxicity on human PBL. It showed increase in cell viability % which ranged from 80.4% at 1 µg/ml to 61.6% at 10µg/ml.

In vitro studies on human blood samples can give information of the toxicological effects of the cypemnethrin in human blood samples. So far there are few reports of the impact of cypermethrin on lymphocyte cultures of human.[16] The indiscriminate use of pesticides and herbicides to increase crop productivity has aroused a great concern among the environmental and health scientists due to their adverse effect in both targets as well as non-target species. Although substantial information is available regarding their environmental and ecological impact not much is known in regard to its toxicity in the mammalian system.[17]

Patel *et al*[17] studied in vitro induction of cytotoxicity and DNA strand breaks in Chinese hamster ovary (CHO) cells exposed to cypermethrin, pendimethalin and dichlorvos. They exposed CHO cells to 1 µM, 10 µM, 100 µM, 1000 µM and 10,000 µM cypermethnn, pendimethalin and dichlorvos for 3 h and cytotoxicity was assessed by MTT assay. They found Cypermethrin induced a significant ($p< 0.05$) DNA damage only at higher concentrations (1000 and 5000 µM), while dichlorvos and pendimethalin exhibited higher extent of cytotoxicity as compared to cypennethrin.

Assessment of cytotoxicity and response to external factors like pesticides were evaluated by using MTT assay by Suman *et al*[18], which measured mitochondrial metabolism in the entire cell culture and provides information about the percentage of cell survival. Utilizing the MTT assay, the cytotoxicity of cypemethrin was determined on lymphocyte cultures from human peripheral blood samples. The short-term lymphocyte cultures were incubated with various aliquots of the cypermethrin and the LC_{50} was found to be 33.6 µM. In our study also the viability dropped with increasing concentration of Cypermethrin (ranging from 83.0 1% at 1 µg/ml to 42.80% at 10 µg/ml) on 2hrs exposure on human PBL. So in our case also we agree with Suman *et al*[18] because at 10µg/ml the viability was 42.80%. The LC_{50} was found to be slightly less than that of 7.5µg/ml, which brought us very close to Suman *et al*[18] results.

Preventive and curative effect of various plant extracts on embryonic human cells intoxicated with the herbicide was observed and it was seen that defined plant extracts can protect human cells against combined xenobiotic effects. It was observed by the workers that plant extracts can prevent intracellular effects caused by environmental pollutants in two human cell lines. Ameliorative effects of ethanolic Neem plant extract on dietary aflatoxin induced heamatological damage were also reported previously. Antioxidant and hepatoprotective activity or leaf or *Oroxylum indicum* in several *in vitro* models have also been observed which were due to the presence of polar phenolic compounds- flavonoid, tannin etc. in leaf of this plant. In the present work root extract of this tree shows presence of phenolic compounds. Two of its extracts are not only capable to show antioxidant activity but also the aqueous extract is capable to enhance cell viability in the cells damaged after tobacco extract treatment.[13]

Presence of flavonoids baicalein, wogonin and chrysin has been confirmed by GC-MS analysis in both fractions. The work shows the ameliorative effect or root extract fractions on TE damaged lymphocytes which may be due to the activity of flavonoids. Flavonoid baicalein is well known natural antioxidant inhibiting the replication of human viruses and suppressing proliferation of human cancer cells. These factors may have contributed to the ameliorative effects on TE treated lymphocytes.[13]

Effect of various concentrations of tobacco extract on mean % viability of lymphocytes was observed. The shape of the curve shows significant inhibition of cell viability in the normal human lymphocytes treated with different concentrations (6%, 9% & 12.5%) of tobacco extract after 2 hrs treatment. It is clear from Fig.1 that if the viability of the controls (lymphocytes without treatment with TE) was taken to be 100%. The viability dropped as the TE concentration increased. At 6% the mean viability was 46.68%. It decreased gradually as the TE concentrations increased. It reached a minimum viability of 37.07% at 12.5% TE. To overcome the toxic effect of different concentrations of TE after 2 hrs lymphocyte cells were treated with two extracts (aqueous & n-butanol) of root of *Oroxylum indicum* separately for 24 hrs by Mamta *et al*[13]. Cell viability of lymphocytes treated for 24 hours with aqueous & n-butanol extracts showed an Increase in cell viability when TE damaged cells were exposed to plant root extracts. Aqueous extract was capable to enhance cell viability significantly on all concentrations of TE. Increase in cell viability was 16.7, 24.72 & 19.69 folds with 6, 9 & 12.5% TE respectively. The cell viability was enhanced significantly when the cells were treated with n-butanol extract after treatment of 9% of TE, whereas the data obtained on 6 & 12.5 % of TE regarding increase in cell viability were not significant for this extract. The ameliorative capacity of aqueous extract was proved to be better than that of n-butanol extract.

The present study was designed to observe the ameliorative effect of aqueous fractions of root extract of plant with 50 % cow urine distillate on Cypermethrin induced damage in human lymphocytes. We also found that root extract of *Oroxylum indicum* with 50% cow urine distillate showed ameliorative effect against cypermethrin induced 2hrs cytotoxicity on Human PBL.

CONCLUSION

We found that root extract of *Oroxylum indicum* with 50% COW urine distillate showed positive synergistic effect as an ameliorative agent against cypermethrin induced cytotoxicity on Human PBL. This was also found that cypermethrin treatment on human lymphocytes *in vitro* was harmful, as the survival rate of lymphocytes decreased after exposure. Survival rate of damaged lymphocytes was enhanced after root extract treatment. Aqueous fraction of 10% root extract of *Oroxylum indicum* in combination with 50% cow urine distillate showed ameliorative effect as a treatment against cypermethrin induced cytotoxicity on human PBL. This combination may be used for the treatment of CYP induced cytotoxicity in the farmers exposed to it.

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