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Toxicity of Imidacloprid on Peripheral Blood Lymphocytes by MTT Assay and the Ameliorative Effect of Extract of *Tinospora cordifolia (Gilloe) Extract*

Research Article

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Abstract

Imidacloprid (IMI) is a widely used insecticide which has a specific affinity for insect neonicotinoid acetylcholine receptors. Like all insecticides which are used in excess it tends to bioaccumulate in the environment. So it was thought worthwhile to study its cytotoxicity to human peripheral blood lymphocytes in concentrations ranging from 1.5mM to 4mM after 2 hours and 18 hours exposure by MTT method. Trypan blue test was also used to determine the percentage of living cells. The ameliorative effect of an extract of the stem in water and ethanolic extract of leaves of *Tinospora cordifolia* (Thunb.) Miers, was also studied. The viability of the lymphocytes showed a fall with increasing concentrations at an exposure of 2 hours. After 18 hours exposure to the IMI only, the viability showed a significant dose dependent drop. Trypan blue test for viability was also conducted. Addition of *Tinospora* extract raised the viability significantly at 2 hours of incubation. In fact this increase was greatest at 3.5mM and 4mM concentration of drug. The ameliorative effect was maximum at 2 hours. Addition of *Tinospora* leaf extract showed a significant increase in cell viability at 18 hours of incubation as compared to values obtained with only the drug. Thus a considerable loss of viability of lymphocytes was seen after exposure to the drug in the selected concentrations but herbal extracts seem to help to make the damage less marked. The cells showed a significant rise in viability when incubated with *Tinospora* leaf extract only, confirming its supportive action in cell proliferation. However, taking into account the evident fall in cell viability caused by exposure to the considerably dilute concentrations tested, caution is needed to prevent over exposure to the pesticide while spraying.

Key Words: Imidacloprid, Lymphocytes, MTT, Toxicity, *Tinospora cordifolia (Thunb) Miers,* Trypan blue.

Introduction

The use of pesticides has been an established agricultural practice to ensure a good crop yield. This is essential to feed our ever-increasing population. The term pesticide can be replaced with plant protective products (PPP) according to European food safety authority.

Pesticides are one of the very common substances that cause deterioration of the environment

However, indiscriminate use of these pesticides or insecticides leads to pollution of soil and water. Once incorporated in the soil or water they are taken up by the plankton and then gradually reach the higher trophic level and finally man through the food chain.(1,2) Thus study of the toxic effect of pesticides on organisms and cells of the human system is very relevant.

Imidacloprid (IMI) belongs to the neonicotinoid category which includes selective systemic and single mode activity pesticides introduced in the 1990s. It was patented by Bayer and marketed in 1991. It is a compound derived from nicotine and it inactivates insect nicotinoid acetylcholine receptors. It is favoured for use due to its selective toxicity to insects over vertebrates.(3) Neonicotinoids are neurotoxic insecticides that act by binding covalently to nicotinic acetylcholine receptors and as a result they obstruct the acetylcholine to bind to its receptor. Thus it inhibits the post synaptic transmission, so the insect is paralysed and it may lead to death of the organism.(4) IMI is reported to be toxic to non target insects such as honeybees etc, so it may be disturbing the ecosystem.

IMI also known as *N*-[1-[(6-chloropyridin-3-yl)methyl]imidazolidin-2-ylidene]nitramide has molecular formula C9H10ClN5O2 , with a molecular weight of 255.6 g/mol, its melting point is 136.4 to 143.8 °C (277.5 to 290 .8 °F). In appearance, it consists of colourless crystals.

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IMI can be used as a foliar spray, for seed treatment and stem application etc. It has a broad spectrum systemic insecticidal activity against several sucking insects that infest many types of crops. It is considered to be one of the insecticides used in the largest quantity all over the world.(5) Due to its widespread use it was thought that a study of its toxicity to human peripheral lymphocytes may lead to an unveiling of interesting facts regarding the extent of damage as related to exposure. The ameliorative effects of *Tinospora cordifolia* stem and leaf extract were also explored.

***Tinospora cordifolia* (Thunb.) Miers**

Tinospora cordifolia (Thunb.) Miers (family Menispermaceae) is a woody climber found commonly in India. It grows very well in tropical regions. Its root, stems, and leaves are used in Ayurvedic medicine(6). It is known as 'Gilloe ,Giloy or Guducchi' in common language and also known as 'Amrita' in Sanskrit because of its medicinal use from ancient period . It is a creation of nature with many medicinal values such as improving the immune system. It is frequently used in Ayurvedic system of medicine and its extracts have proved to be very useful in medical research as they have negligible side effects compared to other medicine of chemical origin. It was not found to be carcinogenetic to human peripheral blood lymphocytes up to a concentration of 3000µg/ml. (7)

Tinospora cordifolia (Thunb) Miers is used to treat peptic ulcers, hepatitis, diabetes, high cholesterol, upset stomach, gout, allergic rhinitis, some types of cancer, rheumatoid arthritis, gonorrhea and other such diseases, used as anti-inflammatory remedy and is known to boost the immune system. (8,9,10)

Materials and Methods

The EC (effective concentration) of IMI used was 17.8%. The composition of its commercially available emulsion is given below:

Chemical composition

- Imidacloprid AI (active ingredient) - 17.8
- Non-ionic emulsifier (ethoxylated alkyl aryl phenol derivative) - 2.5
- Polyvinylpyrrolidone copolymer - 1.0
- Dimethylsulphoxide - 38.4
- N-methyl pyrrolidone - Q.S
- **TOTAL - 100**

Since other workers (4) explored the viability of various forms of IMI in many very dilute concentrations ranging from 1.42 M to 9.5×10^{-6}, it was thought worthwhile to include concentrations ranging from 1.5mM to 4mM for viability testing. As its molecular weight is 255.6 gm, first 4mM solution was made by dissolving 20.4 mg of IMI in 20 ml of distilled water. From this stock solution 1.5 mM, 2.5mM ,3.5mM and 2mM,3mM and 4mM solutions of IMI were made by appropriate dilution of the stock with distilled water. These concentrations were selected because the cytoxicity of IMI to human lymphocytes was found to be 1.7×10^{-3} M to 2.0×10^{-3} M (4) by earlier workers.

Tinospora stem extract

The aqueous extract of the *Tinospora cordifolia* (Thunb.) Miers stem was made according to the technique mentioned by Sharma *et al*, (11), Sengupta *et al.*(12) and Patagiri *et al* (9) ,with some modifications. The stem of *Tinospora cordifolia* (Thunb.) Miers (about 1.5 cm thick) was collected, then wiped with ethanol, then washed with mild detergent and continuously running tap water for 30 min. It was again washed in distilled water. It was then cut it into very small pieces and macerated in mixer to make a paste. Five grams of the paste were taken and further ground in 20 ml of distilled water and the slurry kept for 4 hours inside the laminar air flow chamber with intermittent shaking. Then this mixture was filtered through Whatman no.1 filter paper and the filtrate was collected and used fresh for the experiment. The whole procedure was conducted under aseptic conditions.

***Tinospora cordifolia* (Thunb.) Miers leaf extract**

5 mg of dried leaf powder was extracted with 300 ml of 50 % ethanol in Soxhlet for 48 hrs.

The extract was then evaporated at 50 ˚C on a hot plate to give slurry. 2.5 g leaf powder yielded 1.01 g of extract slurry. This was dissolved in 50 % ethanol to give a concentration of

100 mg/ml and then appropriately diluted with medium to give 200 µg/ml concentration. 10µl of this was loaded in every well of the ELISA Plate.

Phytochemical tests for *Tinospora cordifolia* extract

Test for total phenolic content was done by Folin Coicalteau reagent by the method of Spanos and Wrolstad (13).The determinations were done at 760 nm with the help of a standard curve obtained with different concentrations of Gallic acid from the stock solution containing 1mg/ml, dilutions containing 20, 30, 40, 50 and 100 µg /ml were made. The phenolic content of the extract was determined from the gallic acid standard curve and expressed as mg gallic acid equivalent (GAE) per gram of the extract.

Flavonoid determination

The total flavonoid content of the aqueous extract was determined by the aluminium chloride colorimetric method described by Chang *et al.*(14).The standard Quercetin stock solution was prepared by dissolving 5.0 mg in 1ml of methanol. Standard solutions of quercetin were made by serial dilutions of the stock solution using methanol (containing 5 to 200 µg/ ml). The flavonoid content of the extract was determined from the standard curve and expressed as mgQE/g extract.

Lymphocyte Separation

Separation of lymphocyte from blood was done by standard method explained elsewhere (15). A preinformed, willing female donor in good health was requested to donate 3 ml of the blood for the investigation. It was collected in commercially available EDTA/ heparinised vials and 6 ml of 1×PBS was added to it under aseptic conditions. Meanwhile 3 ml of lymphocyte separating medium was taken in a

centrifuge tube. Then 6 ml of diluted blood was carefully poured over the LSM layer, the tube being held in an inclined position the tube was then centrifuged at 2300 rpm in a REMI R- 4C centrifuge at about 25^{O}C for 30 min. The erythrocytes sedimented at the bottom of the tube and the WBC collected as a layer of just below the plasma layer. These were taken out carefully with a pipette, then washed twice with 1×PBS by centrifugation. The final cell pellet was resuspended in 0.5 ml of PBS and cells were counted in a hemocytometer. They were then diluted in an adequate amount of animal cell culture medium 199(M) supplemented with FBS and PHA to give a final cell count of 8.7× 10^4 cells/ml to 1.5x10^6 cells/ml for the two sets of IMI concentrations.

MTT Assay

The method was originally given by Mossman *et al.*(16).The method is based on the reduction of yellow colour (as it is tetrazolium compound) of MTT dye to purple coloured formazan crystals due to enzymes like succinic dehydrogenase released from the oxidative machinery of the living cells. The purple formazan compound formed is in direction proportion to the number of living cells. The crystals were then solublised by the addition of organic solvents like DMSO. The colour intensity so obtained was read with ELISA micro plate reader at 600nm.

For performing the assay, aliquots of 180 µl of the prepared lymphocyte suspension were seeded into the wells of a 96 well plate in three replicates. One row of these replicates (containing cells and medium) served as a control in each treatment. In the subsequent rows of wells containing cells and medium, aliquots of 20 µl of the IMI solutions to be tested (1.5mM, 2.5mM and 3.5mM) were added. The plate was put in the incubator at 36.5^{O}C to 370C for 2 hours, after which 20 µl aliquots of MTT solution (5mg/ml) were added and the cells were again put in the incubator for 2 hours. Then 100 µl of DMSO was added to each of these wells and incubated overnight, after which their OD was read at 600 nm. The same procedure was followed for exposure to the IMI concentrations for 18 hours. To observe the effects of *Tinospora* stem and extract, 10 µl of the extract and 10 µl of IMI solutions were added to the wells containing cells and medium and incubated for 2hours and 18 hours after which the same experimental procedure was followed. The wells containing only cells and medium for 2 and 18 hours incubation served as controls for both types of experiments. Appropriate corrections were made for background OD and OD of 20 µl aliquots of the insecticide or of Tinospora stem extract and the insecticide. The same experiment was performed with *Tinospora* leaf extract, taking the drug concentrations of 2, 3 and 4mM. The statistical significance of the results was determined by one way ANOVA and student's t test.

Trypan Blue Viability Test

The effect of IMI on lymphocyte survival was also assayed by the Trypan Blue dye exclusion test. The procedure followed was basically given by Strober. (17) The test is based on the fact that living cells with undamaged cell membranes have the capacity to exclude certain dyes such as Trypan blue, Propidium etc. so that they cannot enter the cells. To conduct the test, to

30 µl of the cell suspensions in serum free media, 10 µl each of 1mM, 2mM, 3mM and 4mM dilutions of IMI were added. To 10 µl of these suspensions, equal amounts of 0.4% trypan blue solution was added and the mixture was allowed to incubate for about 3 minutes at room temperature. Then 10 µl of this suspension was taken and the cells were counted in a haemocytometer. The total number of living cells can be calculated by the formula:

$$AxBxCxDx10^4,$$

Where A= Volume of the cell suspension (ml), B= Dilution factor in Trypan blue, C= Mean number of unstained (living) cells and D= Mean number of stained (dead) cells and % viability is given by viable cell count/total cell count ×100

Results

Phytochemical analysis of the *Tinospora* stem aqueous extract

The aqueous stem extract of *Tinospora* had a phenolic content of 0.425 mg GAE/g of the extract. Phytochemical analysis of ethanolic extract of *Tinospora cordifolia* leaves regarding phenolics showed the presence of 2.19µg/ml of gallic acid equivalent and regarding flavonoids, the same extract showed the presence of 631.57µg/ml of quercitin equivalent in solution containing 200 µg/ml of extract (the concentration which was actually used for treatment of the cells).

Results of the MTT assay

Treatment with different concentrations of IMI only (1.5mM, 2.5mM and 3.5 mM) for 2hours showed a dose dependent decrease in %viability which was statistically non-significant (as determined by one way ANOVA). However, when the incubation period of the cells with the insecticide was increased to 18 hours, a significant dose dependent decrease in viability was found.

Table 1: Viability % with different concentrations of IMI after 2 hours and 18 hours treatments

Concentrations of Imidacloprid (in mM/ml)	Average % viability ± SD 2 hr Treatment	Average % viability ± SD 18 hr Treatment
CONTROL	100 ±0.01	100±0.009
1.5mM	94.5±3.89	99.9±0.005*
2.5mM	93.46±1.22	99.7±0.087*
3.5mM	90.96±3.15	85.2±0.044*

* significant at $p<0.05$

When the viability of the cells treated with drug only was compared to those to which *Tinospora* stem extract was added and incubated for 2 hours, it was evident that the viability increased significantly at all concentrations but the greatest was found when the

concentration was highest (3.5mM) [Figure 1, Table 2]. After 18 hours it was highest at 1.5mM) [Figure 2].

Figure 1: Comparative viability of cells and medium with drug only (C+M+IMI) and with *Tinospora* stem extract added (C+M+IMI+T), at 2 hours of incubation ,* significant at p<0.05.

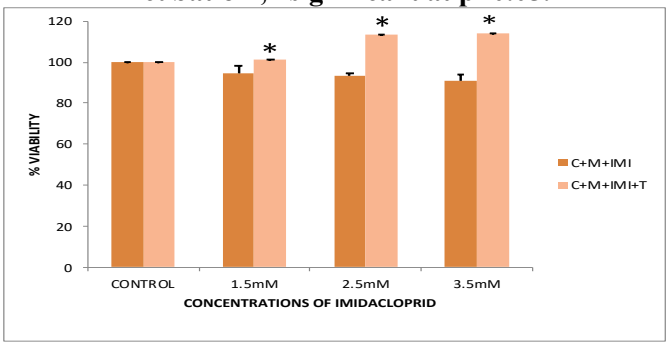
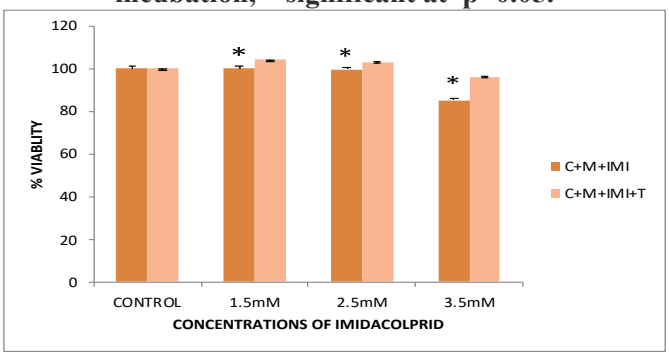


Figure 2: Comparitive viability of cells and medium with drug only (C+M+IMI) and with *Tinospora* stem extract added (C+M+IMI+T), at 18 hours of incubation, * significant at p<0.05.



Treatment with IMI solutions and *Tinospora* stem extract

Treatment of the cells with the different concentrations of IMI solutions and the aqueous extract of *Tinospora cordifolia* (thunb.) Miers stem (10µl of each) showed that when the cells were incubated for 2 hours in the mixture, there was a significant increase in the viability % .

Table 2: Viability % with different concentrations of IMI and *Tinospora* stem extract after 2 hours and 18 hours treatments

Concentrations of Imidacloprid (in mM/ml)	Average % viability ± SD 2hr treatment	Average % viability ± SD 18 hr Treatment
CONTROL	100±0.01	100±0.009
1.5mM	101.3±0.017*	104±0.035
2.5mM	113.1±0.018*	103.2±0.017
3.5mM	114.1±0.064*	96±0.069

*= significant at p<0.05

When the cells were treated with 2mM, 3mM, and 4mM of the drug and incubated with and without *Tinospora cordifolia* leaf extract, the results are shown in tables and figure 3 & 4.

Figure 3: Comparitive study of Imidacloprid toxicity and effect of Tinospora cordifolia leaf extract at 2 hours exposure.[C= cells,M=medium, IMI= Imidacloprid , T= Tinospora leaf extract, *significant(p<0.01)].

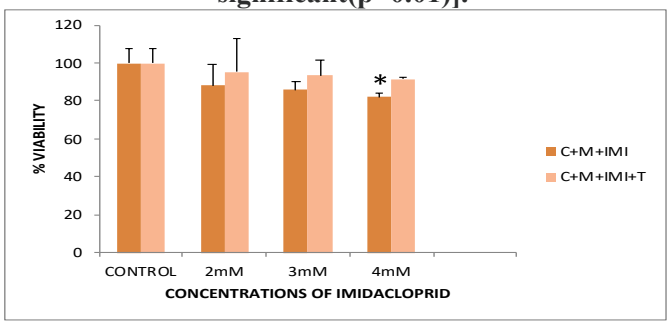


Figure 4: Comparitive study of Imidacloprid toxicity and effect of *Tinospora cordifolia* leaf extract at 18 hours exposure. [C= cells,M=medium, D= drug (imidacloprid),T= Tinospora leaf extract, *significant(p<0.05)].

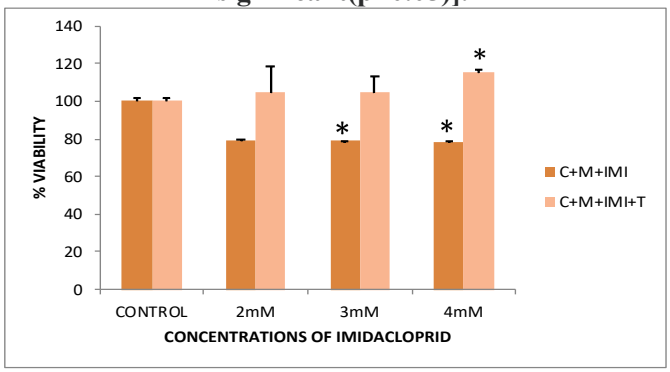


Table 3: Average Viability % of cells, medium and various concentration of IMI after exposure of 2 hrs and 18 hrs.

Concentration of Imidacloprid	Average % Viability ± SD (2hours)	Average% Viability ± SD (18hours)
	C+M+D	C+M+D
Control	100±7.8	100±1.83
2mM	88.27±10.9	79.18±0.1872
3mM	85.69±4.37	78.99±0.049*
4mM	82.12±2.35*	78.22±0.075*

Table 4 : Average Viability % of cells, medium, various concentrations of Imidacloprid and *Tinospora cordifolia* Leaves extract after exposure of 2 hrs. and 18 hrs.

Concentration of Imidacloprid	Average% Viability ± SD (2hours)	Average% Viability ± SD (18hours)
	C+M+D+T	C+M+D+T
Control	100±7.8	100±1.83
2mM	95.15±17.89	104.38±14.42
3mM	93.53±7.66	104.55±8.5
4mM	91.49±0.951	114.99±1.779*

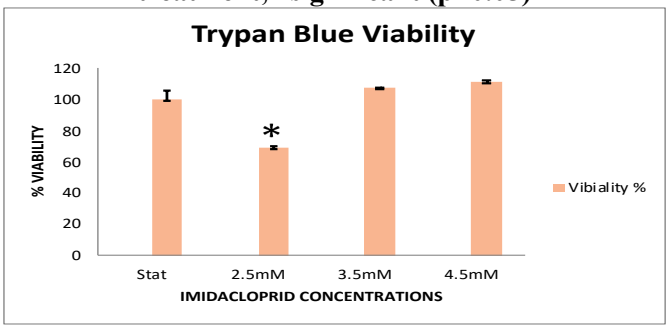
Testing the% viability of the cells by adding only *Tinospora* leaf extract showed a significant increase when incubated for 2 hours and 18 hours as compared to controls to which no extract was added. The viability

obtained after 2 hours incubation was 142.31±14.10 and at 18 hours the value obtained was 143.29±17.82 (the controls values for 2 and 18 hours were 99.99 and 100.01 % respectively).

Results of the Trypan Blue viability test

The trypan blue viability test with 2.5 mM, 3.5 mM and 4.5 mM dose exposure of the cells for 30 minutes revealed an initial decrease at viability at 2.5 mM but at higher concentrations the value showed an increasing trend [figure 5].

Figure 5: Trypan blue viability at 30 minutes of treatment,* significant (p<0.05)



Discussion

The present investigation revealed that a 2 hour *in vitro* treatment of IMI to peripheral blood lymphocytes causes an insignificant dose dependent decrease in viability % at the concentrations tested (1.5mM to 4mM). When the incubation period was extended to 18 hours, a significant decrease in viability was found. Maria et al(4)also reported that1.8x10^{-3}M Jade and 2.0x10^{-3}M Gaucho (both are forms of IMI) reduced the human lymphocyte viability significantly at an exposure of 2 hours, and concentrations of >3.0x10^{-3}M of both lead to cell death obviously causing DNA damage.

The aqueous stem extract of *Tinospora* had a phenolic content of 0.425 mg GAE/g of the extract. It was found to be 12.8 mg of GAE/g of dried aqueous extract by Yadav and Aggarwala (18), but in methanolic extract, Upadhayay,G *et al.* (19) reported it to be 17.48mgGAE/g. Its flavonoid content was found to be 0.28 mgof QE/g of the extract. This is higher than 10.8µg/g reported by Gagandeep kaur *et al*(20)but lower than 6mg/g of dried aqueous extract as found out by Yadav and Aggarwala (18) .

In case of the this investigation, 2, 3, 4mM concentrations, treatment of the cells of the drug only for 2 hours showed a dose dependent decreased in viability. The drop was the highest at 4 mM where it fell to 82.12 %. At 18 hours incubation the drop in viability was more marked and it fell to it 78.22 % at 4mM (significant at p<0.05). The same trend was evident in the treatment with 1.5. 2.5 and 3.5 mM concentrations of the drug, where the increase in viability was highest at 3.5mM.

However, when the cells were incubated with both the drug and *Tinospora cordifolia* (Thunb.) Miers leaves extrac, at 2 hours the viability showed a gradual fall but it was less than that seen with the drug alone [Figure3]. At 18 hours incubation the addition of *Tinospora* leaf extract improved the viability so that in all cases it was higher than controls. The cells treated with 4mM showed the highest increase reaching 104.99% (p<0.005).

It seems a bit odd that cells exposed to higher drug concentrations show a greater recovery.

Perhaps under these conditions the expression of genes controlling the cytochrome P450 system of detoxification is stepped up. This was worked out by Fajun Tian *et al*. (21). Parallel findings were reported by Kapoor et al, (22) who found out that the effect of IMI on elevation of antioxidant enzymes is significant only when the rats are fed the drug at 20mg/kg/day for 90 days. Thus IMI generates oxidative stress and induces changes at higher concentrations and not at 5 and 10 mg. The concentration and time dependent elevation in the activity of CYP dependent enzymes was recorded in rat brain neural and glial cells *in vitro* following exposure to monocrotophos (Tripathi et al, 2013) (23). This is reflected in our findings of increase in viability in response to high drug concentration exposure.

When the exposure time of cells to the drug and *Tinospora* extract was increased to 18 hours, a non significant increase in viability was observed at 1.5mM and 2.5mM, but at 3.5mM the viability fell to 96%. Apparently the cells were not able to recover from the proliferation depression caused by the drug at this concentration even with the help of *Tinospora* extract.

Conclusion

The present investigation revealed that a 2 hour *in vitro* treatment of IMI to peripheral blood lymphocytes causes an insignificant dose dependent decrease in percent viability at the concentrations tested (1.5mM to 4mM). When the incubation period was extended to 18 hours, a significant decrease in viability was found. On the other hand when the lymphocytes were incubated with IMI along with *Tinospora* extract for 2 hours, a significant rise in viability is seen. The rise was maximum at 3.5mM & 4mM concentrations, probably because the CYP dependent enzymes are activated at this concentration. However, when the exposure of cells to imidacoprid and *Tinospora* extract was extended to 18 hours, there was a slight increase in viability at 1.5 to 4 mM compared to 2 hours of incubation. In this context, toxicity studies of low level exposure to human lymphocytes can lead to the formulation of safe guidelines so that they may not become genetic hazards.

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References

1. Preston B. L,Indirect effects in aquatic ecotoxicology assessment.. Implications for ecological risk. Environ. Manage. 2002; 29; 311-323.
2. Hanazato T, Pesticide effects on freshwater zooplankton: an ecological perspective. Environ .Pollut. 2001; 112, 1-10.
3. Tomizawa M , Casida J.E, Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. Annu. Rev. Entomol. 2003; 48; 339–364.
4. María Elena Calderón-Segura , Sandra Gómez-Arroyo , Rafael Villalobos-Pietrini, Carmen Martínez-Valenzuela , Yolanda Carbajal-López , María del Carmen Calderón-Ezquerro , Josefina Cortés-Eslava , Rocío García-Martínez , Diana Flores-Ramírez , María Isabel Rodríguez-Romero , Patricia Méndez-Pérez , and Enrique Bañuelos-Ruiz . Evaluation of Genotoxic and Cytotoxic Effects in Human Peripheral Blood Lymphocytes Exposed *In Vitro* to Neonicotinoid Insecticides . Journal of Toxicology .2012;Article ID 612647
5. Cox L , Koskinen W.C , Yen P.Y,Sorption−Desorption of Imidacloprid and Its Metabolites in Soils**.** J. Agric. Food Chem. 1997; 45(4); 1468–1472
6. Sinha K , Mishra N.P , Singh J , Khanuj S.P.S , Tinospora cordifolia (Guduchi): A reservoir plant for therapeutic applications: A review. IJTK. 2004; 3(3);257-270
7. Chandrasekaran C.V, Mathuram L.N, Daivasigamani P, Bhatnagar U, *Tinospora cordifolia*, a safety evaluation .Toxicology in vitro.2009;23(7);1220-6.
8. Saha S, Ghosh S, *Tinosopora cordifolia* : One plant, many roles .Ancient Science of Life.2012;31(4);151-9.
9. Patgiri B, Bharti L, Umretia , Ravishankar B ,Anti –inflammatory activity of Guduchi Ghana (aqueous extract of *Tinospora cordifolia* Miers.).Ayu.Jan-Mar 2014;35(1);108-110.
10. Kaur G, Singh A.K , Singh C.S, Alam F , Gautam G.K , Phytochemical Investigation of *Tinospora cordifolia* . International Journal of Pharmacy and Natural Medicines .2014 ;2(1); 98-101.
11. Sharma V, Pandey D, Beneficial Effects of *Tinospora cordifolia* on Blood Profiles in Male Mice Exposed to Lead. Toxicol Int. Jan-Jun 2010 ;17 (1);8-11.
12. Sengupta M, Sharma G.D, Chakarborty B, Effect of aqueous extract of *Tinospora cordifolia* on functions of peritoneal macrophages isolated from CCl_4 intoxicated male albino mice. BMC Complement Altern Med. 2011;11;102.
13. Spanos G.A , Wrolstad R.E ,Influence of processing and storage on the phenolic composition of Thompson Seedless grape juice. J.Agric . Food Chem. 1990;38(7);1565-1571.
14. Chang C.C , Yang M.H , Wen H.M , Chern J.C ,Estimation of Total Flavonoid Content in Propolis by Two Complementary Colorimetric Methods. Journal of Food and Drug Analysis . Sep 2002 ;10(3);178-182.
15. Khanna A , Gautam D.S , Jain S.K ,Tobacco Dust Induced Genotoxicity as an Occupational Hazard in Workers of Bidi Making Cottage Industry of Central India . Toxicol Int. Jan-Apr2014; 21(1); 18-23.
16. Mosmann T , Rapid colorimetric assay for cellular growth and survival application to proliferation and cytotoxicity assay. J Immunol Methods . 1983.
17. Strober W . Trypan Blue Exclusion Test of Cell Viability . Curr Protoc Immunol . Nov 2015; 2 (11);A3.B.1-A3.B.3.
18. Yadav R.N.S and Agarwala M ,Pytochemical analysis of some medicinal plants . Journal of Phytology. 2011; 3(12); 10 -14 .
19. Upadhyay G , Tewari L.M , Tewari G , Chopra N ,Pandey N.C ,Upadhyay S.K and Gahtori R ,Evaluation of Antioxidant Potential of Stem and Leaf Extracts of Himalayan *Tinospora cordifolia* Hook .f. & Thomson . The Open Bioactive Compounds Journal .2020 ; 21;1874
20. Kaur G , Prabhakar P.K , Lal U.R , Suttee A ,Phytochemical and Biological Analysis of *Tinospora cordifolia* . IJTPR . Aug –Sep2016 ;8(4);297-305.
21. Tian F , Li C, Wang Z ,Zeng X and Liu J , Identification of detoxification genes in imidacloprid – resistant Asian citrus psyllid (Hemiptera : Lividae) and their expression patterns under stress of eight incescticides. Pest Manag Sci. 2018.
22. Kapoor U , Srivastava M.K , Bhardwaj S and Srivastava L.P , Effect of Imidacloprid on antioxidant enzymes and lipid peroxidation in female rats to derived its No Observed Effect Level (NOEL). J. Toxicol. Sci. 2010 ;35 (4); 577-581.
23. Tripathi V.K ,Kumar V ,Singh A.K ,Kashyap M.P , Jahan S , Kumar D and Lohani M ,Differences in the Expression and Sensitivity of Culutured Rat Brain Neuronal Glial Cells Towards the Monocrotophos . Toxicol Int. May – Aug 2013;20 (2); 177-185.

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