NEURO PROTECTIVE EFFICACY OF PHYTOTHERAPEUTIC METHANOLIC EXTRACT OF POLYHERBAL (TRIPHALA) ON IMIDACLOPRID INDUCED TOXICITY IN WISTAR RATS

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ABSTRACT

Human population continues the possibility of developing neurodegenerative diseases. Two important factors contributing to degenerative brain diseases are oxidative stress and inflammation. Acetylcholine esterase (EC 3.1.1.7) is an enzyme, actively involved in cleaving the ester bond found in the molecule acetylcholine (a neurotransmitter). The enzyme plays an important role in detecting a wide array of neurodegenerative disorders like Parkinson’s disease, Alzheimer’s disease and Huntington’s disorders. Fruits of *Terminalia chebula, Terminalia bellirica, Phyllanthus emblica* are compositions of traditional preparation known as polyherbal drug Triphala which is commonly prescribed by many traditional healthcare practitioners. It is an important healthcare tonic for detoxification, rejuvenation, anti-inflammation and anti-ulcer. In order to elucidate their health related benefits, the neuroprotective and anti-inflammatory effects of methanol extract of fruits were investigated. In addition, underlying mechanisms of the neuroprotective effect and histopathological activities were revealed. The results from the present study not only support traditional uses of the polyherbal formulation Triphala but also provide benefits in treatment and prevention of neurodegenerative diseases or other disorders which oxidative stress and inflammation is implicated.

**KEYWORDS:** Triphala, *Terminalia chebula, Terminalia bellirica, Phyllanthus emblica*, Imidacloprid (IMI), nAChRs.
INTRODUCTION

Fruits and vegetables are an essential part of a nutritious and healthy diet; however, the health benefits are compromised by consistent contamination with pesticide residues. In previous work, pesticide was detected in a range of fresh vegetables. Acetylcholine Esterase is one such enzyme that is found in the central nervous system of animals. The activity of enzyme plays a major role in diagnostic purposes to establish normal cognitive and motor functions in C.N.S related diseases. Human health risks vary with the type of the pesticides and also with the extent of vulnerability. Immediate human health hazards from pesticides include mild headaches, flu, skin rashes, blurred vision and other neurological disorders and rarely, paralysis, blindness and even death. Long run health impacts include cancer, infertility, miscarriage, male sterility, birth defects, and effects on the nervous system. Pesticides can also interfere with drug-metabolizing enzymes, especially Cytochrome P450 leading to drug interactions. Toxicological studies of Imidacloprid are limited, but they have shown mild pathological changes in the brain, kidney and liver of exposed rats at high doses. Studies of the metabolites of neonicotinoids have shown that they can be bioactive and act as nAChR agonists or cause secondary toxicity in mammals. Nicotinoids can be formed as metabolites of neonicotinoids with greater selectivity for vertebrate nAChRs than to insect nAChRs.

Harada is known as "the Tibetan king of medicine," and it act as famous tonic for the heart, brain as well as for long life, which is having the phytoconstituents, alkaloids, flavanoids, carbohydrates, saponins, tannin and polyphenols. Terminalia chebula is one of the Ayurvedic herb used for adaptogenic/antistress potential. Adaptogenic herbs are distinct from other substances in their ability to balance the endocrine hormones and the immune system and they help to maintain optimal homeostasis. Terminalia bellirica and Phyllanthus emblica has OH-alcohol, CH-alkane, CF-alkyl halide, =CH-alkene and other functional groups reported in the previous study. Earlier study revealed that 1,2,3-Benzentriol, Furfural, n-Hexadecanoic acid, 2-Furan Carboxaldehyde, 5-(hydroxy methyl)- and many other compounds were present are responsible for hepatoprotective effect.

MATERIALS AND METHODS

Sample Collection and Authentication

Fresh fruits of Terminalia chebula Retz., Terminalia bellirica(Gaertn)Roxb. and Phyllanthus emblica L. were collected from hill areas, Atthipattu (Thiruvannamalai), Therambattu (Vellore) and Sirumalai (Dindugal). The samples were identified and authenticated by Dr.
Extraction

500gm fruits of *Terminalia chebula, Terminalia bellirica* and *Phyllanthus emblica* were shade dried, pericarp and mesocarp of fruits were pulverized into fine powder individually and formulated in 1:1:1 ratio using a stainless steel blender. Extracts were prepared by using Soxhlet extractor and 95% Methanol were used as solvent, the residue was filtered and concentrated under reduced pressure by rotary evaporator. The final extracts were stored in closed containers until further analysis.[14,15] Methanolic extracts of flowers was subjected to preliminary phytochemical screening of various constituents.[16,17]

Drugs and Chemicals

Imidacloprid 70% (W/G) was procured from Mercury Agro Agency at Kumbakonam, which was manufactured and marketed by Bayer Crop Science Limited, Mumbai. The diagnostic chemicals were obtained from Biomarketting, Thanjavur and followed standard operating procedures.

Experimental Animals

Albino Wistar rats were selected as experimental animals between 6-8 weeks weighing and 160-180 grams were procured from Central Animal Facility, SASTRA University, Thanjavur, Tamilnadu, India. The rats were housed in solid bottom polypropylene cages, three rats per cage. Autoclaved rice husk was used as the bedding material and it was changed once in 3 days. The animals were maintained in the animal house sustained temperature at 22 ± 2°C and humidity 30-70% with light/dark cycle for 12 hours. The food provided for the animals were standard diet containing pelleted food and water *ad libitum* (Nutrilab Rodent Feed, Bangalore, India). The experiments were carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC), SASTRA University (Approval Number: 302/SASTRA/IAEC/RPP dated 29.04.2014). All the hygienic practice was followed during the maintenance of animals according to the guiding principles in the use and care of animals. Induction and treatment were given by ball tipped stainless steel feeding needle orally.
Experimental Design
Experimental animals after the adaptation of 2 weeks, rats were randomly assorted and allocated into 11 groups of six rat (n=6) in each group. Group I: These animals were maintained on normal diet and served as control, Group II: Imidacloprid (Disease Control - 40mg/kg/b.w), Group III: Imidacloprid (Disease Control - 80mg/kg/b.w), Group IV:Imidacloprid 40mg/kg/b.w + *Terminalia chebula* 500mg/kg/b.w, Group V: Imidacloprid 80mg/kg/b.w + *Terminalia chebula* 500mg/kg/b.w, Group VI: Imidacloprid 40mg/kg/b.w + *Terminalia bellirica* 500mg/kg/b.w, Group VII:Imidacloprid 80mg/kg/b.w + *Terminalia bellirica* 500mg/kg/b.w, Group VIII: Imidacloprid 40mg/kg/b.w + *Phyllanthus emblica* 500mg/kg/b.w, Group IX: Imidacloprid 80mg/kg/b.w + *Phyllanthus emblica* 500mg/kg/b.w, Group X: Imidacloprid 40mg/kg/b.w + Triphala 500mg/kg/b.w and Group XI: Imidacloprid 80mg/kg/b.w + Triphala 500mg/kg/b.w orally for 4 weeks. Before necropsy blood was collected by retro orbital puncture in plain and heparinized tubes, centrifuged at 3000rpm for 10 minutes and the serum was separated, after sacrifice brain was removed and washed with saline, stored at 4°C for further biochemical and histopathological investigations. Acetylcholine Esterase was estimated by Ellmans method.$^{[18,19]}$

Histopathological Studies
For histopathological study the fresh liver tissues were collected and immediately fixed in 10% formalin, dehydrated in gradual ethanol (50-100% v/v) cleared in Xylene by tissue processor (Leica TP 1020, made in Jerman) and embedded in paraffin. The paraffin embedding technique (Leica EG 1150C) was carried out; sections were done with microtome (Leica RM2125 RTS) at 5µm thickness. Sections were prepared and then stained with hematoxylin eosin dye by automatic staining (Leica ST 4040) for photographic microscopical studies were observed by Trinocular microscope (Nikon, Digital Sight DS-Fi2, Made in Japan).

Statistical Analysis
The results were expressed as mean ± SD of six rats per group and statistical significance was evaluated by ANOVA using SPSS (Version 20.0) program, values are considered statistically significant when p<0.05.
## RESULTS AND DISCUSSION

Table 1: Effect of Imidacloprid Treatment on Brain Weight and Acetyl Choline Esterase activity of Albino Wistar Rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Brain Weight (gm)</th>
<th>Acetyl Choline Esterase (μM/min)</th>
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<tr>
<td>Control</td>
<td>1.73±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.43±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>IMI (40mg/kg)</td>
<td>1.70±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.21±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMI (80mg/kg)</td>
<td>1.66±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMI (40mg/kg) + <em>Terminalia chebula</em></td>
<td>1.72±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.33±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMI (80mg/kg) + <em>Terminalia chebula</em></td>
<td>1.74±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.38±0.04&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMI (40mg/kg) + <em>Terminalia bellirica</em></td>
<td>1.69±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31±0.01&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMI (80mg/kg) + <em>Terminalia bellirica</em></td>
<td>1.74±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.43±0.01&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMI (40mg/kg) + <em>Phyllanthus emblica</em></td>
<td>1.72±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.36±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMI (80mg/kg) + <em>Phyllanthus emblica</em></td>
<td>1.72±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.41±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMI (40mg/kg) + Triphala</td>
<td>1.71±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.43±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMI (80mg/kg) + Triphala</td>
<td>1.67±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.44±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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</table>

Values are expressed as Mean ± SD for six rats

Mean values within the column followed by different letters (Superscript) are significantly (<i>P</i> < 0.05) different from each other and same letter are non-significant were comparison by Duncan’s multiple range test (DMRT).
Figure 1: Histopathological Examination of Brain tissue section in control and Experimental rats (Hematoxylin & Eosin, 10X)
A. Control, B. IMI – Disease Control (40mg/kg), C. IMI – Disease Control (80mg/kg), D. IMI (40mg/kg) + T. chebula, E. IMI (80mg/kg) + T. chebula, F. IMI (40mg/kg) + T. bellirica, G. IMI (80mg/kg) + T. bellirica, H. IMI (40mg/kg) + T. emblica, I. IMI (80mg/kg) + T. emblica, J. IMI (40mg/kg) + Triphala, K. IMI (80mg/kg) + T. Triphala

Pesticide is associated with the structural damage to organs and tissues along with pathological and inflammatory changes resulting into altered morphology of affected organs in terms of weight. No significant difference was observed in the values of body weight and organ weight of Imidacloprid treated animals compared to the control group of the current study. The changes in body weight and organ weight relates with the toxic properties of insecticide Imidacloprid. Acute toxicity study has been described earlier in terms of changes in certain morphometric parameters viz. surface volume and organ volume.\(^{[20]}\)

The level of Acetylcholine Esterase in the brain of Imidacloprid treated groups were significantly decreased in disease control group II (0.21 ± 0.01) and III (0.28 ± 0.02) was shown in table-1 affected rats when compared with normal control (0.43 ± 0.01) rats. Terminalia bellirica 80mg/kg/bw (0.43 ±0.01), Phyllanthus emblica 40mg/kg/bw (0.41 ± 0.01) and triphala 40mg/kg/bw (0.43 ± 0.02) and 80mg/kg/bw (0.44 ± 0.02) treated groups resulted significant increase in the activity of the enzyme in brain.

Salivation and vomiting have been reported following oral exposure. Very high oral exposures may lead to lethargy, vomiting, diarrhoea, salivation, muscle weakness and ataxia which are all indicative of Imidacloprid’s action on nicotinic receptors. Other signs of exposure at high doses are uncoordinated gait, tremors, and reduced activity.\(^{[21]}\) Imidacloprid induced histological and biochemical alterations in liver also found in female albino rats. Chronic exposure to Imidacloprid also induces inflammation and oxidative stress in the liver and Central Nervous System of rats.\(^{[22]}\)

As per the present study Imidacloprid produced degeneration in brain such as Severe inflammation, Severe focal, Degeneration in Horizontal cells, Degeneration in betz cells, Neural damage in in low dose (40mg/kg b.w) and Mild focal inflammation in high dose imidacloprid administration (80mg/kg b.w) exhibited in Figure 1. Oral administration of Imidacloprid leads to histopathological changes in the brain regions. The neonicotinoid has also been observed to damage the developing vasculature in cerebellum.\(^{[23]}\) Significant ACHe inhibition was observed at higher dose of Imidacloprid as compared to control rats.
Imidacloprid produced similar inhibition in Serum Acetyl Choline Esterase Activity in quails.\cite{24}

Administration of Imidacloprid at the rate of 80mg/kg/b.w/day through oral gavage for 28 days resulted in neurotoxicity which was evident from histopathological changes in brain like marked congestion in cerebellum, degeneration of purkinje cells with loss of dentrites, vacuolation around neurons, shrunken neurons, chromatolysis and ultrastructural alterations like vacuolar mitochondria, apoptotic nuclei with disrupted and margination of chromatin material.\cite{25}

Neuronal loss could be observed in parietal cortex, dedifferentiation was found in hypothalamus and straitum and monoaminergic, cholinergic and amino acidergic deficits were shown in several brain regions.\cite{26} Administration of Imidacloprid produced liver degeneration such as cytoplasmic vacuolation, mild vacuolar degeneration, mild hepatic damage in low dose (40 mg/kg b.w) and appearance of blood streaks, hepatic damage and severe vacuolar degenerations were observed in high dose of induction (80 mg/kg b.w).\cite{27}

Biological stress is a response to physical, chemical, biological and emotional changes consisting of a pattern of metabolic and behavioural reactions that helps strengthen the organism.\cite{28} During stressful condition the energy requirement of the organism is increased, resulting in enhanced generation of free radicals.\cite{29} *Terminalia chebula* showing antistress activity against stress induced models. Flavanoids, tannins and phenolic glycosides possess a variety of biological activities including adaptogenic activity.\cite{30} Triphala has been reported to possess anti-aging properties and improves the mental faculties.\cite{31} *Emblica officinalis* is one of the ingredients of a drug MENTAT which facilitates learning the memory.\cite{32} Brain is particularly vulnerable to oxidative stress because of its high rate of oxidative metabolic activity, intense production of reactive oxygen species (ROS) metabolites. ROS namely superoxide and hydroxyl free radicals, together with hydrogen peroxide have been proposed to cause neurotoxic effect and initiate a free radical-mediated chain reaction causing additional damage to diverse areas in the brain.\cite{33} It is generally agreed that plant is major source of natural bioactive compounds. Nowadays, variety of plant products which have potent antioxidative and anti-inflammatory properties have been proposed to use for prevent and treatment of neurodegenerative disorders.\cite{34,35}
Triphala may be a potent and novel therapeutic agent for scavenging of NO, and thereby inhibit the pathological conditions caused by excessive generation of NO and its oxidation product and peroxynitrite. These findings may also help to explain, at least in part, the pharmacological activities like rejuvenating, adaptogenic, anti-infection, anti-inflammatory, cardioprotective and neuroprotective activities of this traditional and clinically used nontoxic drug.\[36\] Acute toxicity of Imidacloprid following occupational, accidental or suicidal ingestion indicated mild clinical effects such as tachycardia, nausea, vomiting to severe respiratory failure, seizures and even death in human.\[37\] Acute intoxication by imidacloprid or its metabolites resulted in the rapid appearance of neurotoxicity symptoms such as hyper-responsiveness, hyperactivity and trembling and leads to hypo-responsiveness and hypo-activity.\[38\] The metabolites of Imidacloprid are found in the liver and kidneys of rats after a single dose. Toxic signs like salivation, vomiting, lethargy, diarrhoea, muscle weakness and atoxia are indicative of Imidacloprid action on nicotinic receptors.\[39\]

CONCLUSION
This study reveals neuroprotective effect of the methanol extracts polyherbal fruits, it can be suggested that the neuroprotective effect may be due to their phytocompounds and their pharmacological properties. Results from this study not only support the traditional uses of *Terminalia chebula, Terminalia bellirica, Phyllanthus emblica* as well as Triphala but also provide benefit in neurodegenerative diseases or other disorders which oxidative stress and inflammation is implicated. Further pharmacological evidences at molecular level are required to establish the mechanism of the action of polyherbal medicine. This study focuses on the efficacy and safety of Triphala in medicines, with many outcomes in humans and animals; and described some of the mechanisms responsible for many effects of this traditional medicine and phytochemical analysis. Triphala may possibly be used to alleviate the brain functions and its mechanism of action to distinguish the bioactive and ameliorative potentiality of the drug and helpful to the scientific world.

REFERENCES


