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Efficacy evaluation of hepato and nephroprotective properties of Ethanolic *Withania sominifera* root extract (EWRE) against rotenone induced toxicity in male Wistar rats

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ABSTRACT

Rotenone is a lipophilic natural compound mostly obtained from the roots and stems of Lonchocarpus and Derris species used as a pesticide and known as mitochondrial respiratory chain complex I inhibitor as a result ATP production stops and activates reactive oxygen and nitrogen species leading to neurodegeneration by apoptosis. Acute exposure to 2mg/kg/b.wt of Rotenone (Rot) to males rats is proven to be cytotoxic and induces histological alternations in soft tissues. *Withania somnifera* (WS) is a versatile multifaceted medicinal herb which exhibits anti-cancer, anti-aging, anti-diabetic, anti-oxidative and anti-inflammatory responses. The present study reports the hepato and nephroprotective properties of Ethanolic *Withania sominifera* root extract (EWRE) and other cytoprotectants against Rotenone induced histological change liver and kidney tissue of male Wistar rats (n=45). The experiment rats were divided into 9 groups (Group I: Control, Group II: 2mg/kg/b.wt Rotenone treated, Group III: 100 mg/kg.bw EWRE, Group IV: 40mg/kg/b.wt Doxycycline; Group V: 40mg/kg/b.wt Ellagic acid; Group VI: 2mg/kg/b.wt Rotenone-100mg/kg/b.wt EWSR; Group VII: 2mg/kg/b.wt Rotenone-40mg/kg/b.wt Doxy; Group VIII: 2mg/kg/b.wt Rotenone-40mg/kg/b.wt Ellagic acid; Group IX: 2mg/kg/b.wt Rotenone-EWSR-Doxy-EA. Rats from group 2, 6-9 received 2mg/kg/b.wt Rotenone (Rot) for 4 weeks and later they were treated with 100 mg/kg./b.wt of EWRE (orally); 40mg/kg/b.wt doxycycline; and 40mg/kg/b.wt Ellagic acid (Intraperitoneally) individually and in combination for 40 days and on day 70 rat were sacrificed by cervical dislocation. Histomorphology of liver and kidney was assessed by H&E stain. Our results suggests that acute exposure of Rot in adult rats caused detrimental effects in the liver and kidney. The Rotenone induced cytotoxicity was ameliorated with EWRE administration, i.e. 100 mg of EWRE showed better reversal efficiency than 40 mg of Doxycycline and Ellagic acid individually and combination.

Keywords: *Withania somnifera, Rotenone, Hepatotoxicity, Nephrotoxicity, Histopathology*

1. INTRODUCTION

Environmental toxicants aggregation into the natural resources due to overuse has become a severe concern to animals and human health due to chronic exposure to them i.e., endotoxins (Lipopolysaccharide), pesticides (Rotenone, DDT (Dichlorodiphenyltrichloroethane)), herbicides (Paraquat and 2,4- dichlorophenoxyacetic acid (2,4-D)), synthetic drugs (MPTP). Over the years researchers have developed both in vitro and in vivo models in order to elucidate the mechanism of toxicants and symptomatic assessment in acute and chronic exposure. Among the toxicants, rotenone is one such toxicant which is known to induce deleterious effects like neurotoxicity, nephrotoxicity, hepatotoxicity and other known effects.

Rotenone is a lipophilic phytochemical abundantly found in the roots and stems of Lonchocarpus and Derris species and it's a known mitochondrial toxin that inhibits respiratory chain complex I, ATP production, mitochondrial dysfunction, and activates reactive species (oxygen and nitrogen) leading to oxidative stress and neurodegeneration. Rotenone has been associated with parkinsons disease, which is a progressive neurological disease-causing selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) region of brain (i.e., Midbrain). Literature review has demonstrated an increased link between rotenone exposure and Parkinson's disease and other neurological disorders.

The chronic exposure to rotenone can root its accumulation in the detoxifying organs (liver and kidney) due to its lipophilic nature and induce mitochondrial dysfunction, inflammation in hepatocytes and nephrocytes (Liu et al 2016; Jiang et al 2017). But, due to scarce of animal studies, there is still a lot of scope for understanding the rotenone induced toxicity in other soft tissues i.e., reproductive tissues, liver, kidney, heart, spleen, and lungs. The blood flow through the liver and kidney is high, as these are main detoxifying and excretory organs. Hence, the focus has been shifted towards the treatment or ameliorative options as the currently available drugs can lessen the PD and other allied symptoms but cannot halt the disease's progression.

In the past two to three decades, researchers' attention has shifted to phytobased therapy, which uses trace elements, microbial metabolites, essential oils, and extracts from medicinal plants that have beneficial biological effects like anticonvulsant, analgesic, anxiolytic, antidepressant, antioxidant, and anti-inflammatory properties. The present study showed that hepatic and renal tissues are vulnerable to rotenone and cause cellular damage likely because of its high metabolic state, active enzymes, and massive oxygen demand (Meng et al., 2016).

Dietary polyphenols are often present in fruits, vegetables, tea, and cocoa products. They are frequently extracted from phenolic acids and exhibit a wide range of biological properties, including defence against oxidative stress and the prevention of degenerative diseases. Evidence from experiments suggests that these items' and their extracts' antioxidant capacities may be connected to their hepatoprotective qualities. Ellagic acid (EA) is one of the most studied polyphenols because of its potent antioxidant activity, capacity to scavenge free radicals, antiviral and antibacterial properties, and ability to prevent apoptosis. It is well known for its anti-diabetic and anti-carcinogenic properties. The ellagi-tannins found in strawberries, blackberries, cloudberries, and raspberries are the main sources of this substance.

Withania somnifera (WS) L. is a versatile medicinal herb found abundantly in subtropical climates around the world belongs to Solanaceae family. Traditional practitioners have utilised this multi beneficial herb to cure neurological diseases, cancer, asthma, diabetes, ulcers, hepatitis, eyesores, arthritis, heart problems, and haemorrhoids. Secondary metabolites such as steroids, alkaloids, flavonoids, phenolics, saponins, and glycosides are high in WS. Based on the recent scientific reports, various plant parts and Withanolides (Alkoloids) are well-known for its anti-cancer, anti-diabetic, anti-neurodegeneration, cardioprotective, anticancer, antioxidant, antibacterial, antifungal, anti-inflammatory, hepatoprotective, anti-depressant, and hypoglycemic properties. Thus, we designed an in vivo study to study the effects of Rotenone in presence and absence of ethanolic Withania somnifera root extract (EWSR) and other known cytoprotectants (Doxycycline (Doxy) and Ellagic acid (EA) on rat brain, liver and kidney.

2. MATERIAL AND METHODS

Preparation of Ethanolic *Withania somnifera* root extract (EWSR)

Dried *Withania somnifera* (WS) roots were bought from local markets of Hyderabad, Telangana state. The roots were washed thoroughly with distilled water, dried, powdered and later soaked in 50% ethanol (Absolute ethanol diluted with double distilled water) stirred continuously at room temperature for next 7 days using a magnetic stirrer. The extract was later on filtered fine sieves and Whatmann's No1 filter paper and later lyophilized into powder.

Experiment design

Experimental 45 male Wistar rats weighing 160–200 grams and regular pellet diet was procured from National Institute of Nutrition (NIN), Hyderabad, India. Prior to the experimentation, rats were housed in separate polypropylene cages for 7 days. These 45 rats were grouped in 9 groups (5 rats/group). Group one rats were given only sunflower oil (controls), whereas rats from group 2 received Rotenone (2 mg/kg bw) via intraperitoneal route for 4 weeks. The groups from 3rd to 5th were treated with only experimental cytoprotectants individually, whereas groups from 6th to 9th received rotenone (2 mg/kg bw) initially for 4 weeks and post confirmation of symptoms of rotenone toxicity, the rats were treated with 6th to 9th experimental cytoprotectants i.e., individual treatment with EWSR (100 mg/kg bw), Doxycycline (Doxy, 40 mg/kg bw), and Ellagic acid (EA, 40 mg/kg bw) and in combinations (EWSR (100 mg/kg bw)-Doxy, 40 mg/kg bw-EA, 40 mg/kg bw) next for 6 weeks via gavage, the experimental rats were maintained for next 7 days before sacrificing via cervical dislocation, and their organs were removed and immediately stored in 10% paraformaldehyde for histopathological studies. The body weights were recorded at different timepoints before sacrificing experimental animals and later brain weights were also recorded.

Table 1: Time duration of rotenone exposure to male Wistar rats (experimental days).

	Rotenone treatment	**Post rotenone treatments - administration of cytoprotectants**
Experimental days	1-28 days (Rotenone exposure)	29-70 days (post-rotenone exposure and administration of neuroprotectants)

Histopathology studies

Rats	
Days of collection/ sacrifice and experiments performed	1st, 28th, 70th

Hematoxylin and Eosin staining (Hippocampus and Midbrain)

For one to two days, brain tissues were preserved in 10% formaldehyde. The tissues were successively hydrated for 10–15 minutes with distilled water and alcohol concentrations of 100%, 75%, 50%, and 30%. Different alcohol concentrations were employed to dehydrate the tissue, including 30%, 50%, 70%, 90%, and 100% alcohol for 20 minutes at a time, followed by two changes of xylene for 10 minutes, before the tissue was embedded in wax at a temperature of 55°C (minimum two changes required). Using a rotary microtome, embedded tissue slices of 5-7 microns were created (Leica RM2255 Fully Automated Rotary Microtome). The brain sections were examined with an Olympus microscope after being stained with hematoxylin and eosin (Vemuri et al 2021).

3. RESULTS

Morphometric analysis (Body and brain weights)

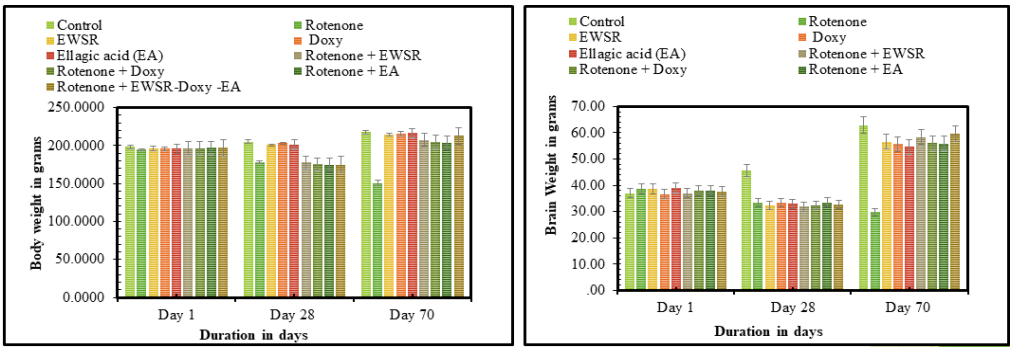

Figure 1: Rotenone effects on the Body and brain weights in rats before and after administration of neuroprotectants.

The above figure 1, present the body and brains weights of the experimental rats recorded at different time points. The control and individual cytoprotectants administered rats gained body weight properly and appeared healthy until end of the experiment and their brains were also appeared corresponding their body weights. Whereas the rats which were treated with rotenone for 28 days showed gradual decrease in their body and brains signifying the cytotoxic effects of rotenone, and observed loss of appetite, frequent urination, loss of body muscle in these rats. Post-rotenone treatment, the rats which received ameliorative treatments individually and in combination showed improvement in body and brain weights and overall health of the rats also improved clearly indicating the cytoprotective properties of cytoprotectants against rotenone induced cytotoxicity.

Hepatoprotective effects of EWSR against Rotenone

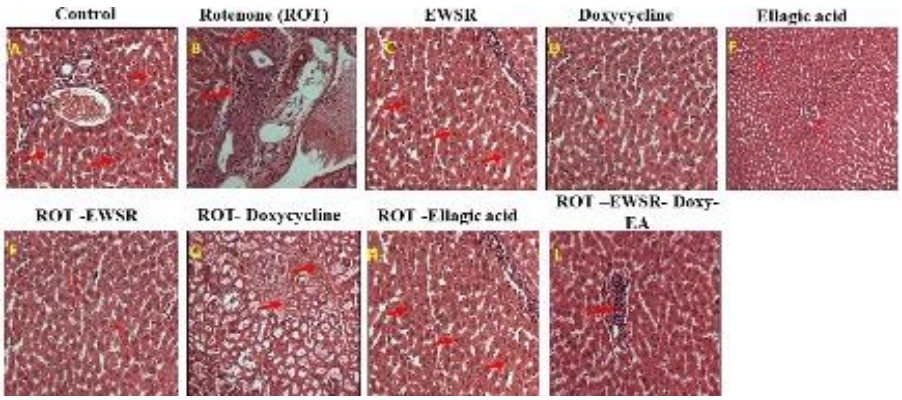

Figure 2: Histopathological assessment of hepatic tissue in rats treated with Rotenone and other cytoprotectants

Above figure 2 illustrates the morphological changes in hepatic tissue when rotenone treated with and without cytoprotectants (EWSR, Doxy, and EA alone and in combination) the control groups showed normal morphology of hepatocytes were observed in the portal, periportal and centri lobular region of liver (Red arrows). The positive control group (rotenone) showed multi focal peri portal and peri biliary inflammation and fibrosis was observed (red arrow) clearly signifying the cell damage or injury. The EWSR, Doxy and EA groups also showed normal morphology of hepatocytes were observed in the portal, periportal and centri lobular region of liver (Red arrow) indicating the cytoprotective efficacy of these cytoprotectants.

Whereas, the EWSR-Rot, and Doxy-Rot groups (post -rotenone treatment) showed normal morphology of hepatocytes were observed in the portal, periportal and centri lobular region of liver (Red arrow) indicating the ameliorative efficacy of these cytoprotectants against Rotenone. But, EA-Rot group tissue appeared to have multiple Foci of tubular necrosis, coagulative type was observed in tubules (arrow), demonstrating partial amelioration against rotenone induced damage. Combination treatment in rats, post-rotenone exposure the hepatic tissue showed mild peri portal inflammation and fibrosis was observed [arrow] demonstrating the protective mechanism against rotenone induced cytotoxicity.

Nephroprotective effects of EWSR against Rotenone

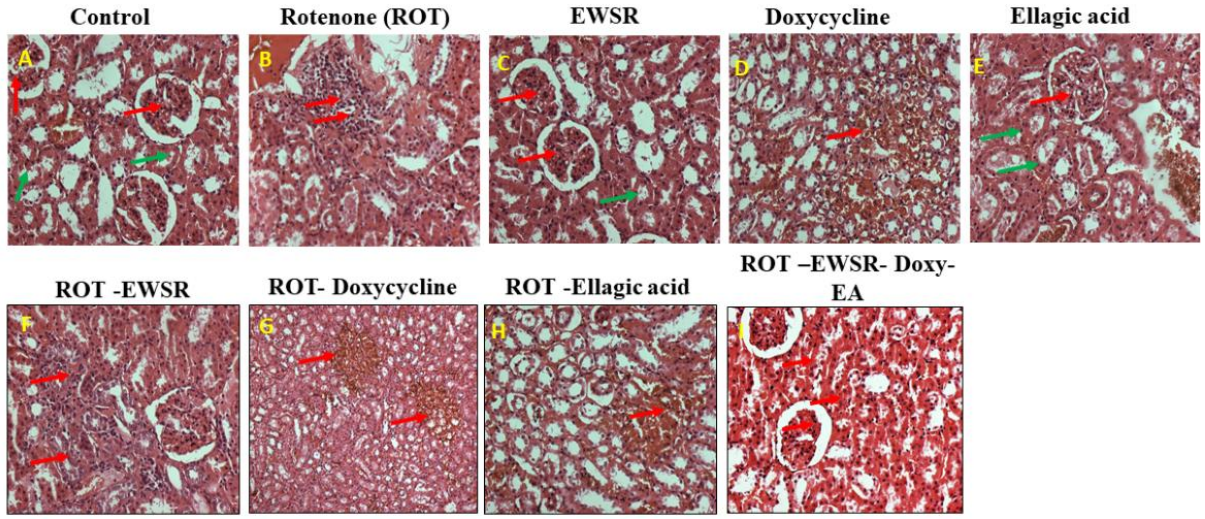

Figure 3: Histopathological assessment of Nephrotic (kidney) tissue in rats treated with Rotenone and other cytoprotectants

Above figure 3 illustrates, the observations observed in the experimental rats. Control tissues appear normal morphology of glomerulus [red arrow] and tubules of kidneys in cortex region [Green Arrows]. Rotenone tissues shows signs of injury, Foci of tubular/interstitial and peri vascular inflammation [red arrow]. EWSR treated group rats tissue showed normal morphology of glomerulus [red arrow] and tubules of kidneys in cortex region [green Arrows]. Doxy treated group rats tissue showed signs of moderate tubular hemorrhage in collecting ducts of kidney [arrow]. EA treated rats tissue appears normal morphology of glomerulus [red arrow] and tubules of kidneys in cortex region [green Arrows]. Tissues of rats treated with ROT and EWSR showed signs of multi focal tubular regeneration in cortex region of kidney [arrow]. Moderate tubular hemorrhage was observed in collecting ducts of kidney [arrow] of the tissues treated with ROT-Doxy and ROT-EA, but the normal morphology of glomerulus and tubules of kidneys [Arrows] was in the tissue treated with ROT-Combination (EWSR-Doxy-EA) signifying the cytoprotective property against ROT induced cytotoxicity.

4. DISCUSSION

A significant component of Asian cultural history, medicinal plants are well-known and highly respected throughout the world as a major source for the discovery of new pharmaceuticals. Asia is a continent with a vast abundance of plant resources, which create a wide variety of bioactive compounds, making it a rich source of many kinds of medicines. In Asia, a sizable portion of basic medical care is still obtained from plants, and both the formal and commercial sectors of the economy see heavy sales of plant material or extracts. A member of the Solanaceae family with the common names winter cherry, ashwagandha, and Indian ginseng, *Withania somnifera* (L.) Dunal has been used medicinally for over 3000 years [2]. Astringent, liver tonic, anti-inflammatory, and aphrodisiac were some of the previous uses for the herb. Recently, it has also been used to treat senile dementia, ulcers, emaciation, bronchitis, asthma, and ulcers. Clinical trials and animal studies support the use of ashwagandha for Parkinson's disease, inflammation, anxiety, and neurological disorders. Ashwagandha may be a beneficial supplement for people enduring radiation and chemotherapy due to its chemopreventive effects.

Literature study showed existing evidence on mechanism related to Rotenone (ROT) induced toxicity in brain and other

tissues functions in the rats. Latest research has indicated that the infants can get exposed to several environmental chemicals through breast milk (Human and Rodents). The foetuses are vulnerable to toxins during gestation as there is only a partial protection is given by blood brain barrier against them from entering the central nervous system. New insights have been reported about the neurotoxicants and the neurodevelopmental consequences due to early exposure to these industrial chemicals. ROT exposure leads to neurodegenerative changes in cerebral cortex which is evidenced by learning and memory deficits and impairment of motor activities and behavioural alterations. But there are still a gaps and scope to understand the ROT toxicity profile in small rodents, humans on chronic or acute exposure. The present study was conducted in rats to evaluate the protective efficiency of EWRE against ROT induced alterations in hepatocytes and nephrocytes in male Wistar rats.

Figure 1 describes the cytotoxic effects of ROT i.e., leading to loss of body and brain weight in adult male rats when treated for 4 weeks. Ameliorative (i.e., neuroprotective and cytoprotective) effects of EWSR and other cytoprotectants against ROT cytotoxicity was measurable in time dependent manner and it was comparable to that of control and individual cytoprotectants treated rats.

Observations from the histological assessment of hepato and nephrotic tissue of the experimental rats, clearly indicate the hepato and nephrotoxic effects of Rotenone in male Wistar rats (Figure 2 and 3), when treated for 4 weeks intraperitoneally. Rotenone treatment has clearly initiated inflammatory and oxidative responses in hepatocytes and nephrocytes causing loss of cells, injury, and signs of haemorrhage. Similarly, the figure 2 and 3 also elucidates the mitigative properties of experimental cytoprotectants when administrated individually or in combination. But, the EWSR individual and combination treatment has shown significant mitigative effects against ROT induced toxicity.

Although the precise mechanism underlying this is unknown, our study's findings on choosing the effective dose of EWSR (i.e., 300 mg/kg) on ROT-induced hepato- and nephrotoxicity indicated that 300 mg of EWSR was more prominent in protecting liver and kidney against ROT-induced hepato- and nephrotoxicity. The fact that EWSR exhibits both hepatoprotective and nephroprotective effects may be attributable to its ability to scavenge free radicals after first fostering improved antioxidant activity using natural antioxidants. Based on the histopathological findings it can determined the hepato and nephroprotective mechanism of EWSR against ROT, but the precise mechanism is still elusive and needs further studies.

5. CONCLUSION

Rotenone (ROT) is a cytotoxin which not only causes neurodegeneration but also causes loss of hepatocytes and nephrocytes on prolonged treatment. The treatment with EWSR and other cytoprotectants show signs of better or decreased cytotoxicity and regenerative properties. Among all experimental cytoprotectants, EWSR showed significant hepato and nephroprotective compared to other cytoprotectants (Doxy and EA) when administrated individually and also exerted similar effects when administered in combination. Hence, it is safe to mention that EWSR can serve as beneficial supplement for regular consumption and to reduced the oxidative and inflammatory stress.

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