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The Neuroprotective Effect of Hesperidin Combination with Ellagic Acid on Rotenone Induced Rat Model in Parkinson's Disease

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ABSTRACT

The Parkinson's disease is the neurodegenerative condition (PD) most common. Occurs as a result of the gradual loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which reduces dopamine levels in the striatum that disrupts motor function. Neurotransmitter alterations, neuroinflammation, dopaminergic neuron loss, postural instability, and motor loss connected with bradykinesia, tremor, stiffness, and akinesia are its characteristic features. Plant component hesperidin is categorised as a "bioflavonoid" found mostly in Citrus fruits. It has medicinal uses. Hesperidin has antioxidant, anti-inflammatory, anti-diabetic, and neuroprotective qualities. Another naturally occurring secondary metabolite of bioactive polyphenolic chemicals found in numerous plant taxa is ellagic acid (EA). It possesses anti-oxidant, anti-inflammatory, as well as neuroprotective characteristics. Thus, this work assessed the neuroprotective potential of hesperidin in combination with ellagic acid in a model of Parkinson's illness produced in rats using rotenone. From day one to day twenty-eight, rotenone was injected intraperitoneally at a dosage of 1.5 mg per kg. Hesperidin [50 mg per kg orally] ellagic acid [10 mg per kg orally], and hesperidin [25 mg per kg orally] along with levodopa [10 mg per kg orally] as a conventional pretreatment medication. Every week, all behavioural characteristics were evaluated. Then all animals were sacrificed on the 29th day, the striatum was separated for biochemical analysis (SOD, GSH, and Total protein). Combined hesperidin (25 mg per kg po) and ellagic acid (5 mg per kg po) treatment significantly enhanced the neuroprotective impact on the striatum, surpassing the effects observed with individual administration of hesperidin or ellagic acid alone. According to the study's findings, hesperidin and ellagic acid together had a stronger anti-oxidant and neuroprotective impact on rats then individual treatment.

Keywords: Parkinson's disease, Substantia nigra pars compacta (SNpc), Rotenone, Hesperidin, Ellagic acid, Levodopa and Dopaminergic neuron.

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INTRODUCTION

The second major prevalent neurological illness is Parkinson's disease (PD) [1]. Its frequency ranges from 0.5-2 percent in those 65 to 69 years of age, to one to three percent in persons 80 years of age and beyond [2]. Among the often-seen motor symptoms of Parkinson's disease include hypokinesia, shivering, inflexibility, and unstable posture [3]. Non-motor symptoms and indicators of Parkinson's disease include disturbed sleep, emotional disturbances, cognitive difficulties, and autonomic dysfunction, which can further deteriorate a person's quality of life [4]. A single histological feature that are the cause of Parkinson's disease is a gradual degeneration buildup of dopamine-containing neurons in the substantia nigra, which leads to a significant reduction in dopamine levels in the striatum [5]. Parkinson's disease may be caused by oxidative stress, neuroinflammation, toxic substances, genetics, and other reasons [6-8]. The administration of Rotenone into striatum of rat produces well established model of PD [9]. Rotenone produces oxidative stress, which can cause inflammation and eventually cell death, so specifically destroying the dopaminergic nigrostriatal system [10,11].

However, due to the complex processes underlying Parkinson's disease, a number of therapies that affect the course of the illness in several ways are being considered as alternatives [12,13]. Numerous antioxidant substances, including flavonoids obtained from natural sources, have shown neuroprotective properties in models that are either in vitro or in vivo of PD [14]. One particular flavonoid glycoside that is commonly present in oranges and lemons is the bioflavonoid hesperidin [15]. Significant anti-inflammatory, antiviral, anticancer, and antidepressant-like effects have been described for it [16]. Present study appears to suggest that ellagic acid (EA), the most therapeutically effective pomegranate component [17]. Ellagic acid shows anti- oxidative, anti-inflammatory, anti-carcinogenic like properties [18].

One of the most significant defence mechanisms for reducing oxidative stress is the glutathione (GSH) system, which eliminates free radicals and keeps protein thiols in the proper redox equilibrium [19,20]. Another crucial mediator in the decrease of oxidative stress is superoxide dismutase (SOD) [21]. Using a rotenone-induced Parkinson's disease (PD) model in rats, the current study proceeded to evaluate the effects of chronic treatment with hesperidin, ellagic acid and levodopa on behavioural, biochemical, molecular, and histological markers.

MATERIAL AND METHODS

Animals

Wistar male rats weighing between 150 and 200 grams, were obtained from Vyas Labs, Under IAEC Protocol number: (I/IAEC/AU/07/2023/WR ♂) NIN, Hyderabad, India. The Animal Ethics Committee gave its Consent to the animal experiments. Animals were housed in a controlled setting possessing limitless water availability and a standard laboratory pellet chow diet (22-24 degrees Celsius, 45 percent– A 12-hour light/dark cycle with 50% humidity). The investigations were conducted between the hours of 9 am and 6 pm whenever possible.

Experimental design

Male Wister rats (150-200g) will be used as experimental animal. Animals are separated into six categories, with each group allocated six animals.

Group-1: Control group treated with normal saline for 28 days.

Group-2: Parkinson's model group rats were administered for 28 days' rotenone [1.5mg/kg i.p.] which was dissolved in 0.1 percent DMSO.

Group-3: Rats treated with rotenone [1.5mg/kg i.p] + Ellagic acid [10mg/kg oral] [Test drug 2] for 28 days, they received 0.1 percent dimethyl sulfoxide (DMSO) dissolved rotenone and 0.1 percent DMSO dissolved ellagic acid before the administration of rotenone.

Group-4: Rats treated with rotenone [1.5mg/kg i.p] + Hesperidin [50mg/kg oral] [Test drug 1]. Rotenone and hesperidin was administered dissolved in 0.1 percent DMSO for a period of 28 days.

Group-5: Rats treated with rotenone [1.5mg/kg i.p] + Hesperidin [25mg/kg oral] +Ellagic acid [5mg/kg oral]. All the treatments were administered dissloved in 0.1 percent DMSO for a period of 28 days.

Group-6: Rats treated with Rotenone [1.5mg/kg i.p] + levodopa [10mg/kg oral] [Standard drug] Rotenone was administered dissolved in 0.1 % DMSO followed by levodopa dissolved in 3mg/ml water for 28 days.

Behavioural studies

Narrow Beam Walk Test

The evaluation of foot slip counts and gait irregularities involved the utilization of beam crossing challenge and narrow beam walk test. To prevent intentional falls, the setup comprised a horizontal thin beam suspended 100 cm above the ground. The beam, measuring 130 cm in length and 1 cm in width, had a black box with nesting materials at the end serving as a platform or completion point. Each animal received training on the little beam. Every attempt included a recording of their foot slippage and delay to cross the beam [22].

Rotarod

Every animal's motor performance and coordination were evaluated using a rotarod device. It is made up of a spinning rod with four compartments that is 40 cm in diameter with 75 mm of height (Rustay et al. 2003). All animal had undergone seven days of preparatory training before the treatment. At 25 rpm, the animals were now placed on the spinning rod on the day of the experiment in order to measure their ability to coordinate their movements. The average fall-off time was recorded and the on the rotarod, with 180s of cutoff time [23,24].

Homogenization and dissection

On day 29, cervical dislocation was used to sacrifice the animals, and the brains were extracted and immediately chilled to 80 degree Celsius. The striatum was separated from the brains and placed them on

dry ice. Following that, the tissue samples were homogenised using an ice-cold 0.1 M phosphate buffer (pH = 7.4), ten times (w/v) the weight of the tissue. Centrifuging for 15 minutes at 4 °C at 10,000g was employed to separate the striatal homogenates [25]. The measurement of bio-chemical parameters was subsequently performed using the homogenised striatal suspension solution.

Bio-chemical parameters

Estimation of total protein and total soluble protein

The brain homogenates are diluted using freshly prepared bovine serum albumin (0.5 mg/ml). To 0.2 ml of brain supernatant, 1 ml of Lowry reagent (0.5 percent copper sulphate (CuSO4)) was added, mixed and incubated for 10 mins at room temperature. Followed by further addition of 0.1 ml of Folin-Ciocalteu reagent and incubation in dark for 30 minutes at room temperature. The absorbance for each sample is measured at 660 nm using a spectrophotometer [26].

Estimation of Superoxide Dismutase

By pipetting-tipping out 20 to 8 µl of the chemical and increasing the volume to 100 µl with 0.01 N HCl, the rate of auto-oxidation of pyrogallol was evaluated. A solution of 100 µl each of Tris EDTA and distilled water was added to 600 ml of Tris HCl buffer. 50 µl of pyrogallol and 50 µl of the sample were added to a reaction vessel, the temperature was adjusted to 25 degrees Celsius, and the sample was evaluated for three minutes by looking for a spectral shift at 420 nm using a 0.5 nm bandwidth. The rate at which pyrogallol auto-oxidizes may be halved for each unit of SOD activity [27].

Estimation of Reduced Glutathione (GSH)

GSH levels were determined by measuring a yellow chromophore that resulted from the reaction of GSH with 2,5-dithiobis (2-nitrobenzoic acid). The brain homogenate was mixed with 10 percent trichloroacetic acid (TCA) and centrifuged at 2000×g for 10 minutes to extract the constituent parts [28].

Histopathological studies

Hematoxylin and eosin staining was the method used for the histological study of brain sections. The mid-brain transverse segment (Substantia nigra) was embedded in paraffin wax after being fixed in a calcium formal solution. Sections with a thickness of 7–9 mm were produced, Hematoxylin and eosin staining, then studied under a microscope to determine the total estimated number of lesions per hemisphere [29,30].

Statistical analysis

Mean ± SEM (Standard Error of Mean) is the format used for all data. The groups' differences were analysed to evaluate their significance. For the statistical analysis, the Graph Pad Prism 10 programme was utilised along with Tukey, Bonferroni, and one- and two-way analysis of variance (ANOVA) tests [31-33].

RESULTS

Behavioural studies

Measurement of Latency time (sec) in Narrow Beam Walk Test

The results indicate that on day 1, the latency time (in seconds) for crossing the beam with no significant difference observed. However, as the rats were exposed to rotenone over the course of treatment, there was a notable increase in the amount of time needed to cross the beam. By the end of day 28 of rotenone exposure, a significant decrease ($p<0.01$) in crossing time was recorded compared to the control group.

On Day 1, all treatment groups exposed to rotenone exhibited substantial differences. On Day 28, rats treated with ellagic acid showed significant increase ($p<0.01$), hesperidin showed ($p<0.01$), and the combination group hesperidin+ellagic acid showed significant increase ($p<0.001$). These times were significantly lower than the rotenone group, indicating an attenuation of the increased in time to move across the beam. By contrast, the standard group treated with levodopa showed an increase time to get across the beam on day 28, and this increase was statistically significant ($p<0.001$). It has been represented in **(Figure1)**.

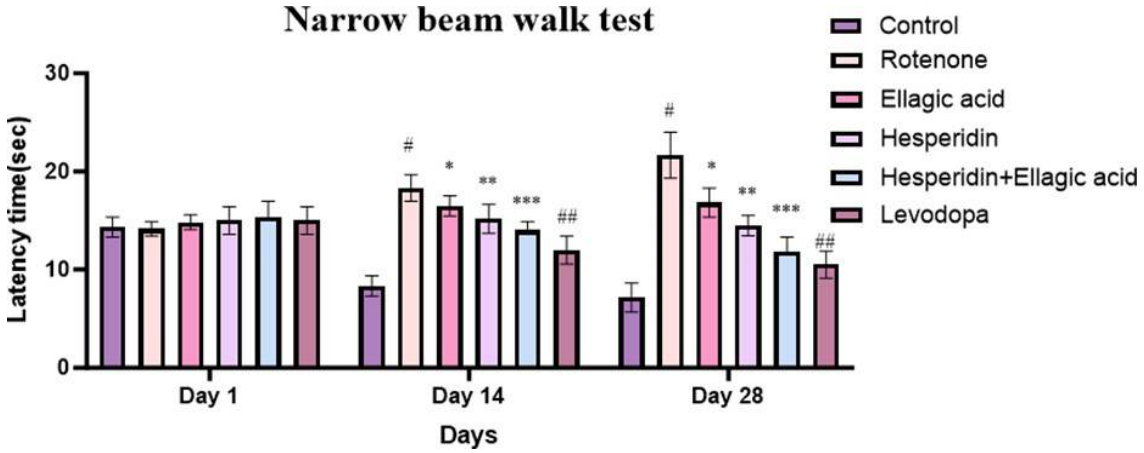

Figure 1: Measurement of latency time (sec) in narrow beam walk test.

Measurement of Foot slips count in Narrow Beam Walk Test

The results indicate that on day 1, how frequently a foot slips when traversing a beam with no significant difference observed. However, as the rats were exposed to rotenone over the course of treatment, there was a notable increase in the number of foot slips. By the end of day 28 of rotenone exposure, a significant decrease ($p<0.01$) in comparison to the typical control group, the number of foot slips was noted.

On Day 1, all treatment groups exposed to rotenone exhibited considerable differences. On day 28, rats treated with ellagic acid showed significant increase ($p<0.01$), hesperidin showed ($p<0.01$), and the combination group hesperidin+ellagic acid showed ($p<0.001$). These numbers were significantly lower than the rotenone group, indicating an attenuation of the increased number of foot slips. In comparison, the standard group treated with levodopa on day 28, and this increase was significant statistically ($p<0.001$). It has been represented in **Figure 2**.

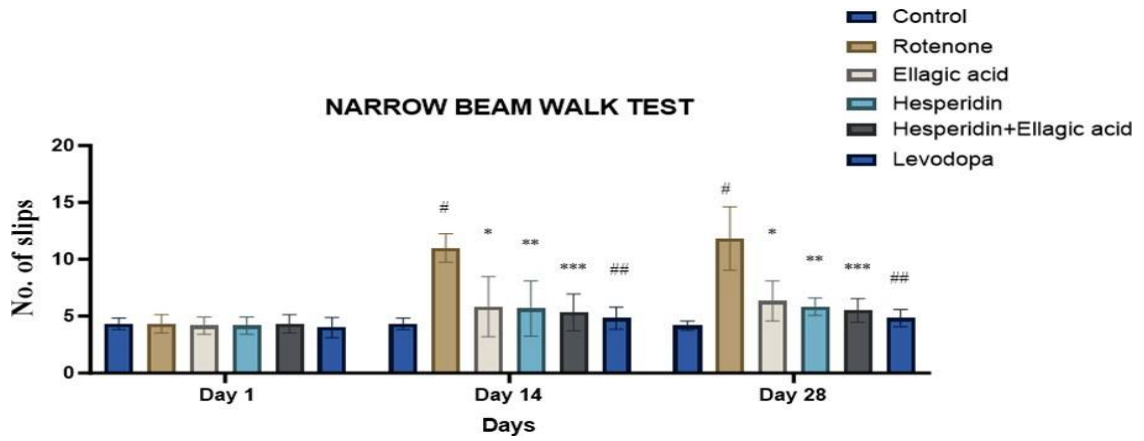

Figure 2: Measurement of foot slips count in narrow beam walk test.

Measurement of Fall of time in Rota-rod

On day 28, the rotenone group exhibited a fall-off time which was found to be significant ($p<0.01$). Ellagic acid demonstrated a dose-dependent increase in fall-off when contrasted with the rotenone group, with significant results ($p<0.01$). Hesperidin also showed an increase in fall-off time when contrasted with the rotenone group, with significant results ($p<0.01$). The combination of hesperidin and ellagic acid exhibited a more pronounced alteration in fall-off time compared to the rotenone group, with a significant value ($p<0.001$). The standard group (levodopa) showed having a significant value when compared to the rotenone group ($p<0.001$). It has been represented in **Figure 3**.

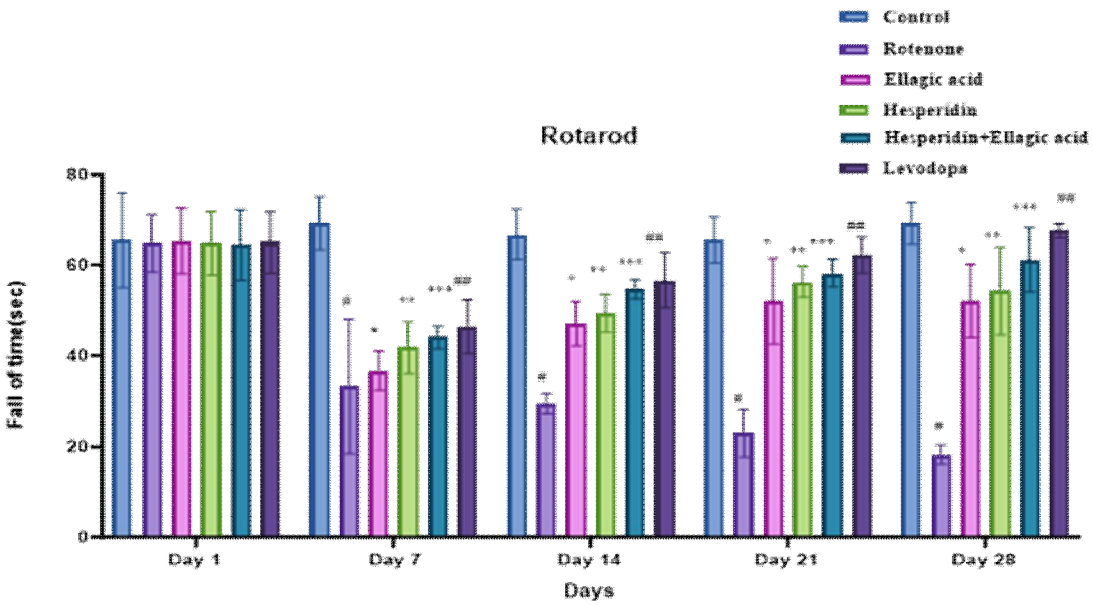

Figure 3: Measurement of fall of time in Rota-rod apparatus.

Biochemical parameters

Effect of Hesperidin and Ellagic acid on protein levels

The group that was in comparison with the usual control group was received rotenone after 28 days had a considerably lower total protein level with significant ($p<0.01$). Nonetheless, the treatment groups that received only ellagic acid and hesperidin saw a notable rise in total protein levels ($p<0.001$) compared to the rotenone group. The prominent increase in protein level in combination group Hesperidin+Ellagic acid ($p<0.001$) has shown compared to rotenone. Animal treated with levodopa ($p<0.001$) has risen in the level of protein when compared to rotenone. It has been represented in **Figure 4**.

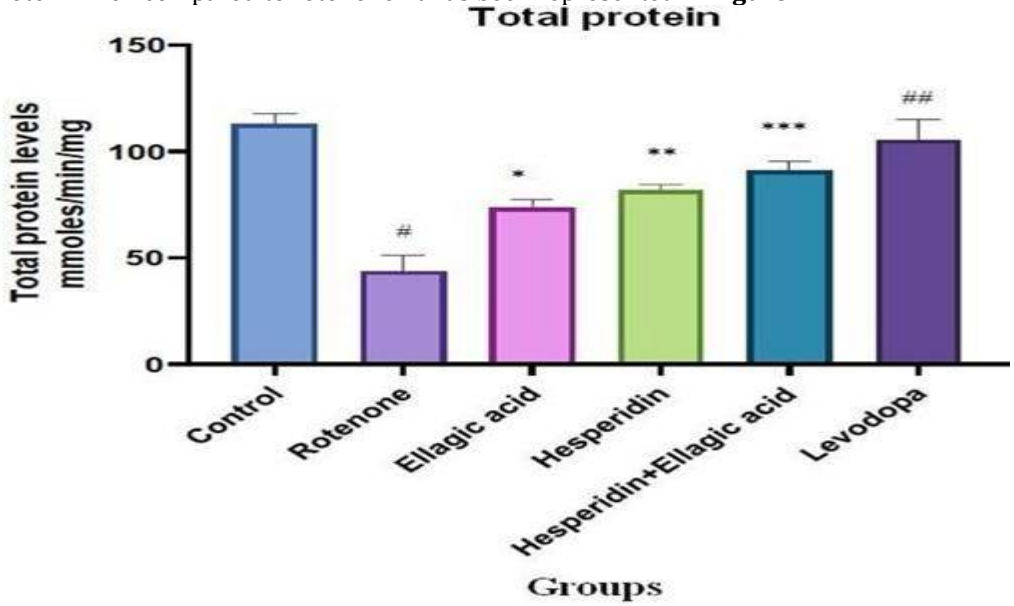

Figure 4: Effect of hesperidin and ellagic acid on protein levels.

Hesperidin and Ellagic acid's effects on Superoxide dismutase

Rats treated with rotenone had a considerably lower level of superoxide dismutase (SOD) than the normal control group ($P<0.01$). In contrast to the normal control group, the treatment groups that received just ellagic acid and hesperidin had higher SOD with significant value ($P<0.01$). The amount of SOD ($P<0.001$) significant increased when hesperidin and ellagic acid were combined. The levodopa treated animals also showed a considerable rise in their level of SOD, with a significant value ($p<0.001$) in comparison to the negative control group and a lower level in comparison to the normal control group. It has been represented in **Figure 5**.

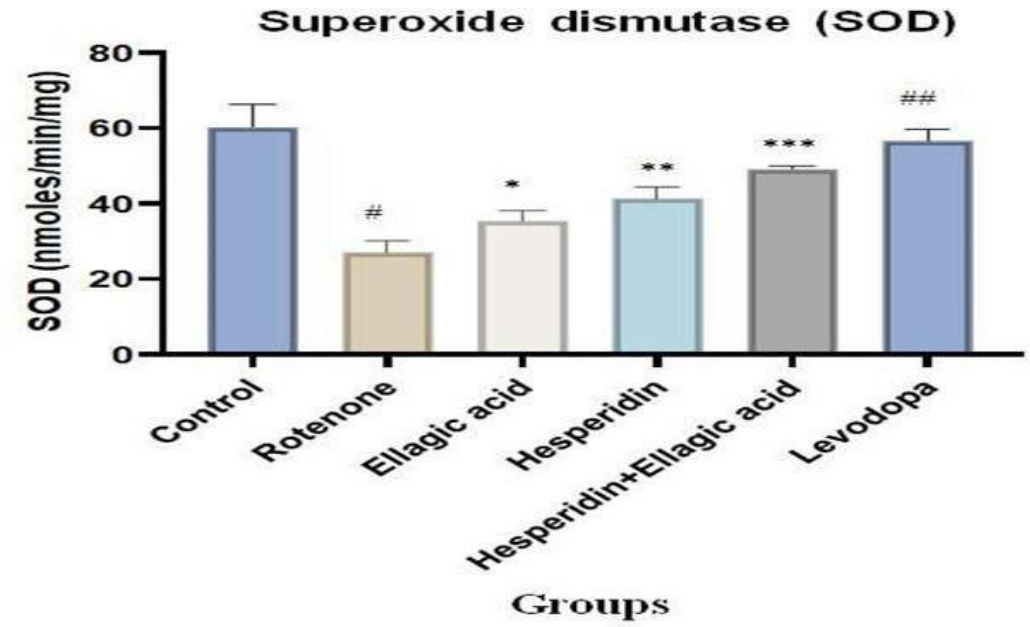

Figure 5: Hesperidin and ellagic acid's effects on superoxide dismutase.

Effect of Hesperidin and Ellagic acid on GSH (reduced glutathione)

GSH is essential in that it is a major antioxidant, aiding in the prevention and repair of oxidative modifications. In the rotenone alone treated group, there was a notable reduction in reduced glutathione levels ($P<0.01$) (GSH) compared to the normal control. However, the treatment groups with Ellagic acid and Hesperidin showed an increase in GSH levels with significant value ($P<0.01$). The combination group, Hesperidin+Ellagic acid, exhibited a significant increase in GSH level ($P<0.001$). Additionally, the group treated with the standard drug also demonstrated a significant increase in GSH levels ($P<0.001$) when compared to the negative control group. It has been represented in **Figure 6**.

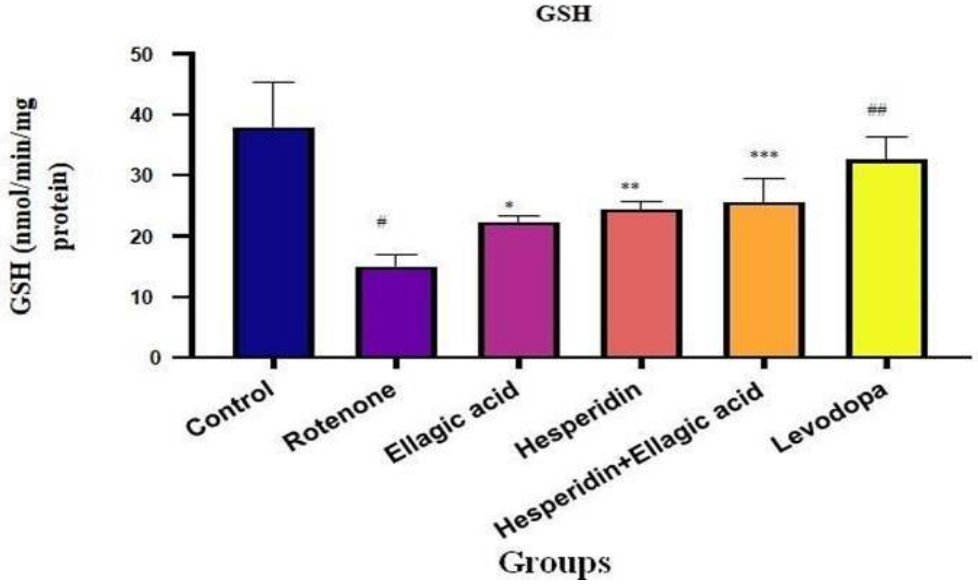

Figure 6: Effect of hesperidin and ellagic acid on GSH (reduced glutathione).

Histopathology

Neuronal damage and loss induced by rotenone treatment (negative control) resulted in inflammation, defined by the neutrophil infiltration seen in the substantia nigra of the midbrain. Normal morphology of dopaminergic neurons was observed in normal control group **(Fig. 7A)**. Moderate amount of necrotic dopaminergic neurons edema with infiltration in neutrophils was observed in ellagic acid treated group **(Fig. 7C)**. Multi foci of mild necrotic dopaminergic damage and inflammation cells with infiltration was observed in Hesperidin treated group **(Fig. 7D)**. Normal morphology of most of the dopaminergic

neurons in the midbrain's substantia nigra was observed in combination Hesperidin+Ellagic acid treated group **(Fig. 7E)**. Normal morphology of the midbrain's substantia nigra contains dopaminergic neurons was observed in levodopa treated group **(Fig. 7F)**.

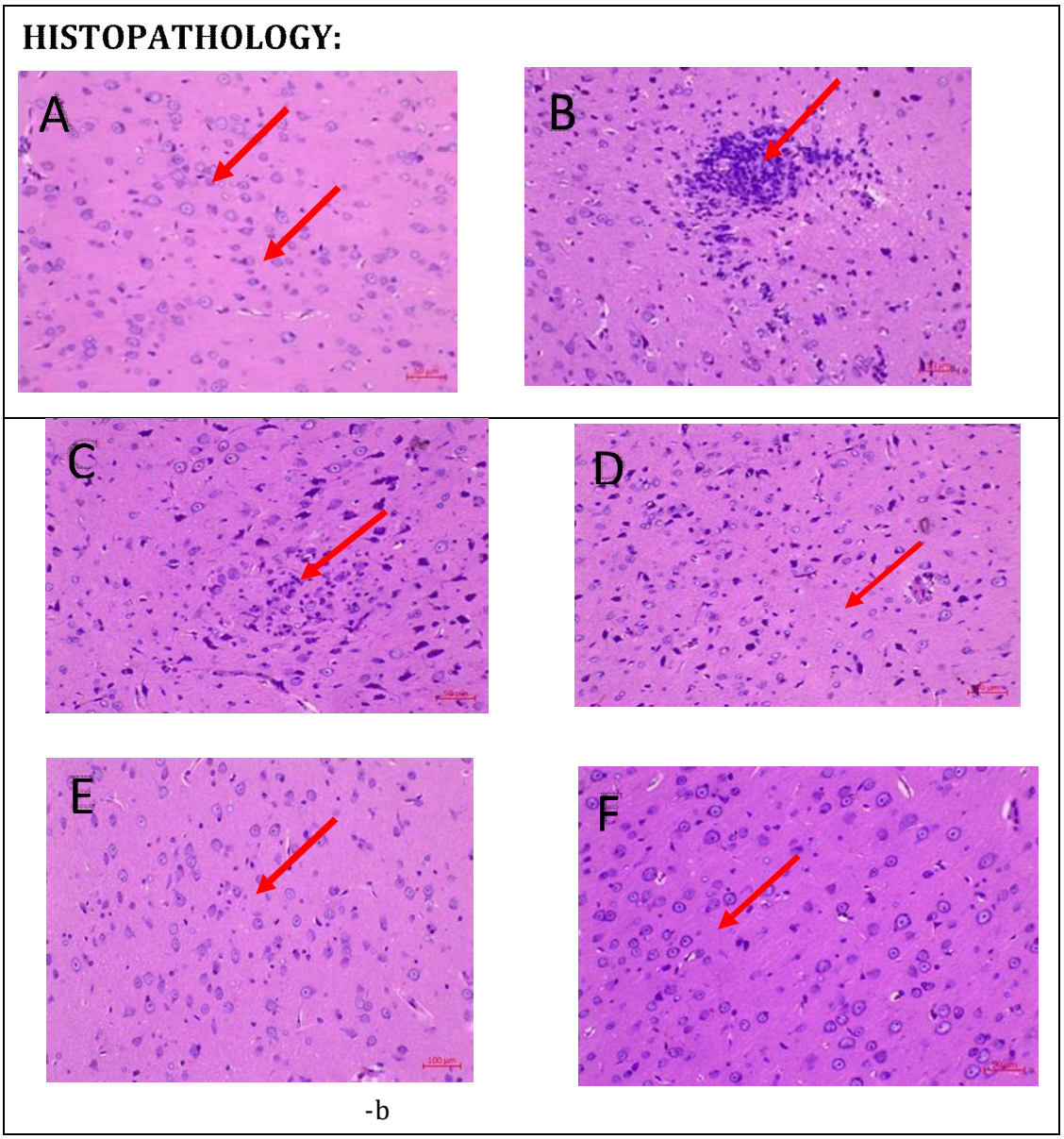

Figure 7: Histopathology study of mid-brain of rats. The figures represent: A-Normal control, B-Rotenone(1.5mg/kg), C-Ellagicacid(10mg/kg), D-Hesperidin(50mg/kg), E- Hesperidin + Ellagicacid (25mg/kg+5mg/kg), F-Levodopa.

DISCUSSION

The primary objective of this study was to look into the neuroprotective properties of a combination of hesperidin and ellagic acid in a rotenone-induced Parkinson's disease model in rats. Parkinson's disease is a neurologically based movement sickness. Low dopamine levels in the brain produce its symptoms. A loss of smell, tremor, bradykinesia postural imbalance and issues with coordination are some of the early symptoms. The primary neuropathology PD is the localised death of dopaminergic neurons in certain brain areas, such as the striatum. The most recent method employed to replicate PD in rats involves the use of rotenone, a mitochondrial toxin with uncertain relevance to the development of Parkinson's illness. Rotenone consistently induces the loss of substantia nigra dopaminergic cells in vivo, a characteristic feature of PD neuropathology. Rotenone, being highly lipophilic, penetrates neurons, builds up in mitochondria and prevents the electron transport chain's complex I from working. It passes the blood-

brain barrier with ease independently of any transporter. Due to their higher fatty acid content, increased ROS levels, and reduced levels of endogenous enzymatic and non-enzymatic antioxidant defense components, brain tissues are considerably more susceptible to oxidative damage.

In the current investigation, pretreatment with hesperidin and ellagic acid alone and combination with hesperidin and ellagic acid considerably reduced the motor impairments caused by rotenone, demonstrating the neuroprotective potential of these medications. Benefits of hesperidin against rotenone-induced nigrostriatal dopaminergic neuronal damage in rats. The affective and cognitive impairments associated with oxidative damage were enhanced by rotenone when DA was lost [12]. Rats were employed in the experiment with rotenone serving as the negative control (rotenone 1.5mg/kg i.p alone), rats receiving levodopa [10mg/kg orally] as the positive control received both ellagic acid [10mg/kg orally] and hesperidin [50mg/kg orally] alone and in combination of hesperidin+ellagic acid [25mg/kg + 5mg/kg] with rotenone. A range of assessments have been studied in present research to assess motor impairments in rat models of Parkinson's disease, Rotarod tests, walk tests with a narrow beam.

The test of the narrow beam walk is a highly accurate and sensitive method for detecting neural activity loss inside the nigra substantia. Postural instability is a common behavioural abnormality observed in popular models of Parkinson's disease in animals. Rats with Parkinson's illness exhibit a prolonged latency longer to cross the beam and more instances of foot slippage. However, treatment with a combination of hesperidin+ellagic acid was shown to decreased the effects of rotenone in the treated rats. Levodopa was shown to counteract the considerable behavioural alterations that rotenone-induced Parkinson diseased rats displayed in the current investigation.

A rotarod apparatus is typically used for the rotational behavioural test. Its foundation is motor asymmetry brought on by rotenone administration, which causes sensitization, conditioning, and priming issues. Because rotenone destroys nigrostriatal dopamine neurons, the group receiving rotenone in this study had a variety of PD-related behavioural traits. Increased motor coordination was observed in treatment groups.

In this current study protein estimation in the rats treated with rotenone showed decreased significant response compared to control. The hesperidin and ellagic acid alone showed increase in significant value. The combination Hesperidin+Ellagic acid and standard treatment group levodopa showed better significant increase compared to rotenone group.

An accurate indicator of the oxidative stress of the animal brain would be the amounts of both antioxidants, both enzymatic and non-enzymatic, in brain tissue. Because antioxidants are depleted in the process of scavenging free radicals, the quantities of reduced Rats given glutathione (GSH) and superoxide dismutase (SOD) treatment had significantly lower levels of rotenone. The effects of the rotenone were reversed in the rats administered with hesperidin and ellagic acid alone, the combination Hesperidin+Ellagic acid and standard treatment group levodopa showed significant increase.

The midbrain has been identified as the brain region most susceptible to the toxicity of rotenone. The histopathological findings of the study revealed neuronal damage and loss induced by rotenone, accompanied by inflammation and neutrophil infiltration in the midbrain region. Rotenone caused considerable harm to the midbrain of the animals. However, the combination treatment with Hesperidin+Ellagic acid and the standard drug levodopa demonstrated a protective effect, attenuating the normal morphology of dopaminergic neurons in the substantia nigra region of the midbrain. As these results align with the findings from behavioural and tissue parameter assessments.

CONCLUSION

The study explored the ellagic acid and hesperidin's neuroprotective qualities in a rotenone induced Parkinson's disease rat model. The conclusion drawn was that the combination of hesperidin and ellagic acid exhibited beneficial effects in the context of Parkinson's illness, due to its neuroprotective agent potential, which is connected to its antioxidant properties, it may have an impact on motor functioning. The experimental administration of the test compounds, hesperidin and ellagic acid, on rats induced with rotenone demonstrated a notable anti-parkinsonism effect in the study. According to current research, hesperidin and ellagic acid protect dopaminergic neurons from degenerating due to their ability to regulate the redox potential and the process of restoring mitochondrial energy. Consequently, these antioxidants may be used as potential therapeutic targets for the treatment of neurological diseases like Parkinson's disease, either by themselves or in combination.

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Ethical Approval: The study was approved by the Institutional Animal Ethics Committee of Anurag university bearing the research protocol number: (I/IAEC/AU/07/2023/WR).

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