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IN-VITRO NEUROPROTECTIVE ACTIVITY OF SILIBININ AGAINST MPTP (1-METHYL-4-PHENYL-1, 2, 3, 6-TETRAHYDROPYRIDINE) INDUCED NEUROTOXICITY MODEL

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ABSTRACT: In the present study, we examined the effect of Silibinin on the accumulation of oxidative stress induced by MPTP by *in-vitro*. After decapitation, healthy rat brain was removed rapidly from the skull and rinsed with cold artificial cerebrospinal fluid (ACSF) which has been equilibrated with 95% O_2 / 5% CO_2 gas mixture. Group I brain was incubated in CSF serve as normal, Group II lenses brain was incubated in CSF and DMSO (10%) serve as disease, Group III brain was incubated with MPTP (1 ng/mL) Group IV brain was incubated in MPTP with bromocriptine (10 µg/mL), Group V brain was incubated in MPTP with Silibinin (10 µg/mL) Group VI brain was incubated in MPTP with Silibinin (20 µg/mL). After 1 h of incubation brain slices were homogenized in PBS buffer, pH 7.4 and estimated for protein content, LPO* and GSH as per the procedures. MPTP incubated brain LPO* levels were increased, and GSH activity decreased compared to normal group. Silibinin treated group brain LPO* levels were significantly decreased and GSH activity was increased. The research results were concluded that the Silibinin exhibited significant neuroprotective effect against MPP + free radicals due to their antioxidant activity.

INTRODUCTION: Glials, a specialized type of non-neuronal cell, regulate the neuronal microenvironment and provide support to the nervous system [1, 2]. Amongst the glial cells, astrocytes are abundantly present with a close connection to neurons in the brain and spinal cord [3] regulating various physiological and pathological conditions. Astrocyte metabolism is a key feature on which the neurons are functionally dependent, including its role in energy metabolism and synthesis of neurotransmitters by maintaining the amino acid homeostasis [4].

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Astrocytes play a dynamic role in the brain [5] and are associated with apoptosis, ischemia and various neurodegenerative disorders [6, 7]. The brain's vulnerability towards oxidative stress is highly dependent on astrocytes, and thus astrogliosis may critically impair the survival of neurons [8, 9]. Astrocyte activation releases neuroinflammatory molecules like the proinflammatory cytokines tumor necrosis factor α (TNFα); interleukin (IL-1β and IL-6) [10].

These neuroinflammatory cytokines modulate the astroglia dependent apoptosis resulting in malignant glioma development. Reactive astrocytes are a key feature for formation of the 'glial scar' expressing the glial fibrillary acidic protein and ultimate consequence of neuronal death [11]. Silibinin, a flavonolignan obtained from the fruit and seed extracts of 'milk thistle', (*Silybum marianum*, Asteraceae) is one of the most

biologically active component among the three isomers (Silibinin, silychristin and silidianin) which collectively form 'Silymarin.' It is well known for its excellent hepatoprotective effect and has been reported to act as cardioprotective, having anti-cancer activity, immunomodulatory effect and an excellent antioxidant, inhibiting lipid peroxidation and scavenging free radicals [12, 13, 14, 15]. Clinical studies assessing the effect of Silibinin and Silymarin on the human liver cirrhosis have provided promising results in normalizing the hepatic markers.

Moreover, the ongoing clinical trials with a positive outcome include prostate cancer treatment and death cap (*Amanita phalloides*) poisoning [17]. It has been shown to decrease the microglia and astrocyte activation in the brain of Parkinson's and Alzheimer's patients [18]. Moreover, Silibinin has shown a protective mechanism on heavy metal-induced- neurotoxicity [19], oxaliplatin-induced oxidative stress [20] and diabetes-associated neuronal injury. In the present study was evaluated the potential neuroprotective potential of Silymarin on MPTP induced neurotoxicity using *in-vitro* model.

MATERIAL AND METHODS:

Experimental Procedure for Neuroprotective Effect of Silibinin by in *In-vitro*:

Materials: MPTP (Sigma Aldrich) Silibinin (Yarrow Chem Pvt.Ltd) sucrose (Loba Chem Pvt. Ltd.,) Bromocriptine (Para Chem Pvt. Ltd.,).

Methods: Preparation of artificial cerebrospinal fluid (ACSF), pH 7.4 contains sodium chloride (122 mM), potassium chloride (3.1 mM), calcium chloride (1.3 mM), magnesium sulphate (1.2 mM), glucose (10 mM), and glycylglycine (30 mM). Sodium chloride, 122 mM: 1.425 g of NaCl was weighed potassium chloride (3.1 mM): 46.22 mg of KCl was weighed calcium chloride (1.3 mM): 28.85 mg of $CaCl_2$ was weighed magnesium sulfate (1.2 mM): 59.136 of Mg sulfate was weighed glucose (10 mM): 0.36 g of glucose was weighed glycylglycine (30 mM): 0.792 g of glycylglycine was weighed. All the above chemicals (in their specified amounts) were dissolved in 200 ml of distilled water. The solution of salts can be prepared and kept in refrigerator and glucose with glycylglycine can be added later on the day of the experiment.

Methodology: After decapitation, the brain was removed rapidly from the skull and rinsed with cold artificial cerebrospinal fluid (ACSF) which has been equilibrated with 95% O_2 / 5% CO_2 gas mixture.

Treatment and Evaluation of Selected Bio-markers: *In-vitro* studies were performed using sagittal slices of male mouse brain of 1 mm thickness. The slices were incubated at 37 °C in ACSF of pH 7.4, in an oxygen-rich atmosphere as described by Mcllwain with or without MPTP (1 nM) for 1 h [21]. In one group of experiments, slices were pretreated with Silibinin (10 and 20 µg/mL) for 0.5 h before incubation with MPTP (1 nM) for 1 h). The brain slices were homogenized in PBS buffer, pH 7.4 and estimated for protein content, LPO* and GSH as per the procedures.

Statistical Analysis: All data are expressed as means ± SEM. Statistical differences among the experimental groups were tested by using a one-way analysis of variance (ANOVA) and Dunnet test was employed for multiple comparisons. P-values less than 0.05 were accepted as significant.

RESULTS:

Effects of Silibinin on Protein Content, GSH, and LPO on Brain Slices: The effects of Silibinin on biochemical parameters in the sagittal brain slices are tabulated as follows.

TABLE 1: EFFECT OF SILIBININ ON SELECTED BIOMARKERS TESTED ON SAGITTAL BRAIN SLICES

Groups	Protein content [µg/mg tissue]	LPO [nmoles TBARS/mg protein]	GSH [µmoles/ mg protein]
Normal control	15.43 ± 2.3	3.65 ± 0.9	0.036 ± 0.007
DMSO control	14.12 ± 1.2	3.21 ± 1.3	0.032 ± 0.004
MPTP (1 ng/ml)	4.56 ± 1.3***	13.4 ± 1.7***	0.0098 ± 0.0006**
Bromocriptine (10 µg/mL)	8.43 ± 1.8	9.25 ± 1.1	0.027 ± 0.008
Silibinin (10 µg/mL)	6.45 ± 1.3	78 ± 1.2**	0.018 ± 0.006
Silibinin (20 µg/mL)	8.26 ± 1.3**	7.32 ± 1.4	0.021 ± 0.007**

All values are expressed in Mean ± SEM. Statistical analysis determined by ANOVA followed by Dunnet's method of comparison. b denotes treated groups were compared against MPTP group. while the a denotes, MPTP control group was compared against the DMSO control.

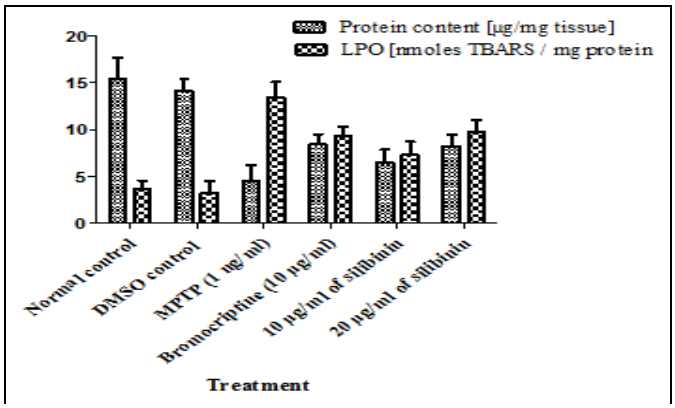

FIG. 1: EFFECT OF SILIBININ ON PROTEIN AND LPO ON SAGITTAL BRAIN SLICES

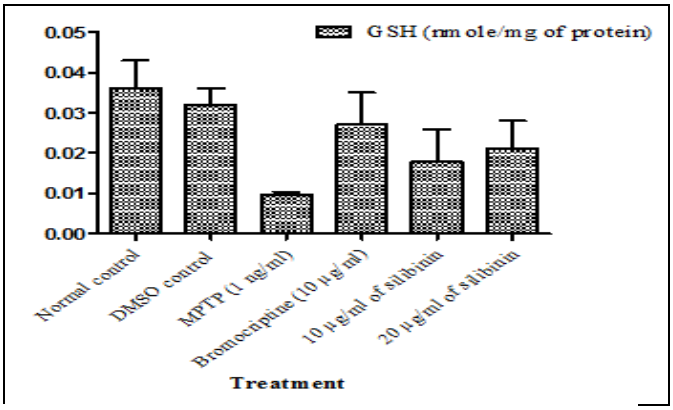

FIG. 2: EFFECT OF SILIBININ ON GSH ON SAGITTAL BRAIN SLICES

There was a considerable decrease in the protein content (4.56 ± 1.2 µg/mg tissue, P<0.001***) and GSH content (0.0098 ± 0.0006) µmoles/mg protein, P<0.001***) while increase in the lipid peroxidation products was observed (123.4 ± 1.7 nmoles TBARS/mg protein, P<0.001***) in the MPTP group. Silibinin at 20 µg/mL showed considerable neuroprotective properties in term of restored GSH levels of 0.021 ± 0.007 µmoles/mg protein (P<0.01**) and decreased LPO levels of 7.32 ± 1.4 nmoles TBARS/mg protein (P<0.01**) and improved protein content 8.26 ± 1.3*. Thus, Silibinin showed better results than bromocriptine in terms of GSH and LPO, while it improved **Table 1** and **Fig 1**, **2**.

DISCUSSION AND CONCLUSION: In human and nonhuman primates MPTP produces clinical, biochemical, and neuropathologic changes analogous to those observed in idiopathic Parkinson's disease. The neurotoxic effects of MPTP are thought to be initiated by MPP+, which is a metabolite formed by the monoamine oxidase (MAO) B-mediated oxidation of MPTP [22]. MPP+ is selectively taken up by high-affinity dopamine and noradrenaline uptake systems and is subsequently accumulated within mitochondria of dopaminergic neurons. There it disrupts oxidative phosphorylation by inhibiting complex I of the mitochondrial electron transport chain [23].

The interruption of oxidative phosphorylation results in decreased levels of ATP [24], which may lead to partial neuronal depolarization and secondary activation of voltage-dependent NMDA receptors, resulting in excitotoxic neuronal cell death [25]. Although excitotoxic neuronal damage has been linked to Ca" influxes, the subsequent crucial steps that lead to cell death remain unknown. Recent evidence has implicated both oxygen free radicals and nitric oxide (NO'). The entry of calcium through NMDA receptor channels into cells stimulates nitric oxide synthase (NOS) activity by binding to calmodulin, a cofactor for NOS. Studies in dissociated cell cultures showed that NOS inhibitors effectively blocked NMDA-induced cell death [26]. Furthermore, NO' may react with superoxide (OZ) to generate peroxynitrite [27], which may promote nitration of tyrosine [26] and produce hydroxyl radicals [28].

In the present study, results revealed that Silibinin exhibited significant neuroprotection against MPP+ free radicals due to the neutralization of LPO* free radicals and enhanced GSH activity. Although the mechanism by which Silibinin regulates MPTP induced oxidative stress remains to be determined, there are several possible explanations. Firstly, as a polyphenolic flavonoid, Silibinin has strong free radical-scavenging activity [29]. Silibinin reacts with a damaging free radical and forms a flavonoid radical, which has greater stability and then breaks the free radical chain reaction [30].

It is possible that Silibinin prevents oxidative damage directly by scavenging free radicals. The results from the present study confirm, for the first time, that Silibinin could alleviate the neurotoxicity induced by MPTP *in-vitro* method. The effect of Silibinin may be attributed to the prevention of oxidative damage, measured in terms of the amount of peroxidized lipid and the level of GSH. Therefore, Silibinin is a potential candidate for a further preclinical study aimed at the treatment of neurotoxicity.

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