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Neuroprotective effect of hesperidin on cerebral stroke using cerebral ischemic reperfusion model

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Abstract
Present study was evaluated the neuroprotective effect of hesperidin against ischemia-reperfusion induced cerebral infarction in rats.
Cerebral ischemia was induced by the transient occlusion of bilateral common carotid arteries for 30 min followed by 12 hrs reperfusion (I/C) in rats. Hesperidin (10 and 20 mg/kg orally) was treated for 14 consecutive days before ischemic reperfusion. After 12 hr reperfusion rats were killed, brains were removed and subjected to measurement of percentage of cerebral infarct volume and malondialdehyde, superoxide dismutase, catalase and inflammatory markers (MPO, TNF-α, IL-10) markers in the brain tissue. The results of this study showed that hesperidin significantly reduced ischemic damage in hesperidin treated rats (20 mg/kg orally) when compared with I/C rats group, and also hesperidin treatment significantly reduced malondialdehyde levels, increased superoxide dismutase, catalase, decreased inflammatory mediators like MPO, TNF-α, IL-10 brain tissue in intact group when compared with I/C group.
Hesperidin alleviated neurological function in rats and the mechanism may be related to augmentation in endogenous antioxidant defense and inhibition of oxidative stress in the rat brain.

Keywords: ischemic reperfusion, hesperidin, antioxidants, inflammatory mediators

Introduction
Ischemic cerebrovascular abnormality is a kind of common and severe health-threatening disorder with high rates of morbidity, mortality and permanent disability in the central nervous system [1-3]. Oxidative stress and excessive inflammation responses were key pathological events in ischemia reperfusion injury. Reactive oxygen species production in tissue injury after reperfusion of ischemic brain. Over production of reactive oxygen species during reperfusion could cause high rate of oxidative stress and lower anti oxidative capacity. In response to inflammatory signals from ischemic and reperfused tissue, leukocytes initially accumulate in the vasculature by adhering to the vascular endothelium and plugging capillaries [4-5]. Hesperidin is a potent antioxidant [6], anti inflammatory agent [7], Anti hypercholesterolaemic activity [8] and hepatoprotective. There is increasing evidence that supports that potent antioxidants and anti inflammatory agents can provide protection against neurodegenerative changes associated with cerebral ischemia reperfusion injury. Therefore the present study was aimed to evaluate the cerebroprotective action of hesperidin against ischemia reperfusion induced cerebral infarction. In the present study, bilateral common carotid arteries occlusion for 30 min and 4hr reperfusion used for experimental model of global cerebral ischemia. The study carried to estimate percentage of infarction, oxidative stress markers melanaldehyde (MDA, SOD and CAT) and inflammatory markers (MPO, TNF-α, IL-10) markers in the brain tissue. The procedures are described as follows.

Materials and Methods
Animals
Wistar albino rats of either sex, weighing 200±25 g, were procured from Mahaveer enterprises, Hyderabad. Animals were consumed a commercial diet for 1 week. The experimental protocol was approved by Institutional Animals Ethics Committee (253/IAEC/SICRA/PhD/2017) and animal care was taken as per the guidelines of CPCSEA (1821/PO/RE/S/15/CPCSEA).

Experimental procedure for neuroprotective effect of hesperidin against ischemia-reperfusion induced cerebral infarction in rats [9]
Wistar rats of either sex weighing between 250 to 300 g were used in the study.

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Each group consisted of 6 animals. Experimental protocols was as follows
Group-1 Sham control
Group-2 Ischemic / Reperfusion (I/R) (Rats received 30 min carotid artery occlusion and 12 hr reperfusion)
Group-3 Hesperidin (10 mg/kg) and Vehicle (in 10 % DMSO+ 10% Tween 80 + 80% distilled water)
Group-4 Hesperidin (20 mg/kg bd.wt) and Vehicle (in 10 % DMSO+ 10% Tween 80 + 80% distilled water)
At the end 14 days of experiment, the brains were removed and subjected to measurement of percentage of cerebral infract volume and oxidative stress markers Malondialdehyde, superoxide dismutase, catalase and inflammatory markers Image result for myeloperoxidase, TNF-α, IL-10 markers in the brain tissue.

Measurement of percentage cerebral infarct volume
The cerebral infarct volume was determined by using TTC [10]. In brief, animals were sacrificed at the end of 4 hr reperfusion and brains were removed rapidly by cervical dislocation and frozen at 4^0C for 5 min. Coronal slices were made in to 2 mm thickness and each slice was immersed in a 10% solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) for 30min. The TTC is converted to red formazon pigment by Nicotinamide Adenine Dinucleotide (NAD) and dehydrogenase present in the viable cells. Thus, the viable

cells stained deep red and dead cells (infarcted) remain unstained. Pale necrotic infarcted tissue was separated and weighed. Percent cerebral infarction was calculated.

Estimation of oxidative stress and inflammation parameters
Preparation of brain tissue for estimation of oxidative stress and inflammation: Separate animal groups were used for the estimation of oxidative and inflammation parameters. Brain of each animal was removed at predetermined time of reperfusion following decapitation and was washed in cold 0.9% saline, kept on ice and subsequently blotted on filter paper, then weighed and homogenized in cold phosphate buffer (0.1M, pH 7.4) using Remi homogenizer. Homogenizer procedure was performed as quickly as possible under complete standard conditions. The homogenate was centrifuged at 1000 rpm at 40^0C for 3 min and the supernatant divided in two portions, one of which was used for measurement of malondialdehyde (MDA) [11] The remaining supernatant was again centrifuged at 12000 rpm at 4^0C for 15 min and used for the measurement of superoxide dismutase [12], catalase, myeloperoxidase (MPO), tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10). Tissue protein was measured by the method followed by lowery *et al.* method [13].
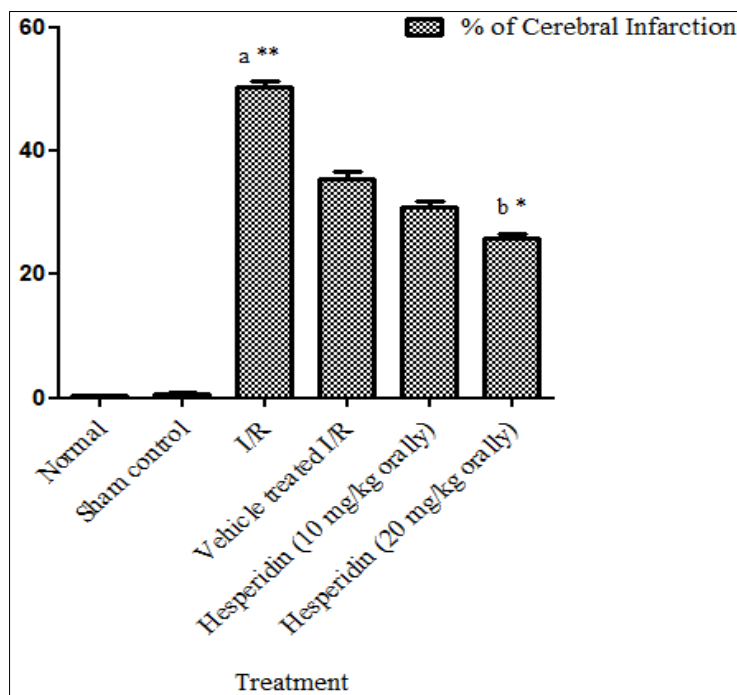
Results

Fig 1: Effect of Hesperidin on percentage infarct size in cerebral ischemia reperfusion injury in rats.

Values are expressed as mean +S.E.M of 6 animals. The percentage Cerebral Infarction size was significantly (P< 0.01[a**]) increased in I/R group rats compared to normal rats

similararly size was significantly (P< 0.05 [b*]) decreased in Hesperidin treated rats.

Fig 2: Effect of Hesperidin infarct size in cerebral ischemia reperfusion injury in rats
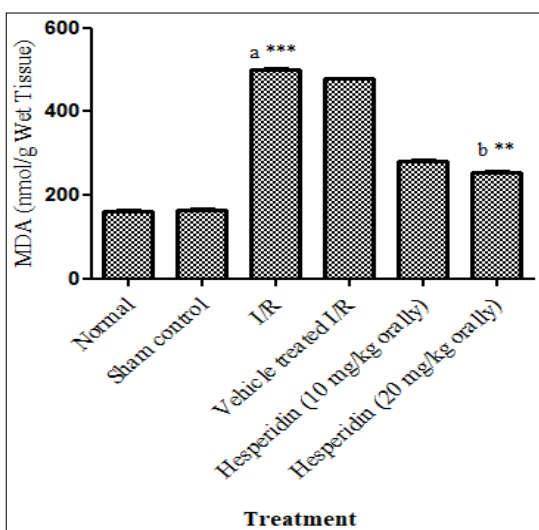

Fig 3: Effect of Hesperidin on MDA (nmol/g Wet Tissue) levels in infarcted tissue in cerebral ischemia reperfusion injury in rats.

All values are expressed as Mean± SEM (n=6), The MDA levels were significantly ($P< 0.001^{a***}$) increased in I/R group rats compared to normal rats, similarly MDA was significantly ($P< 0.0^{b**}$) decreased in Hesperidin treated rats compared to I/R group rats.
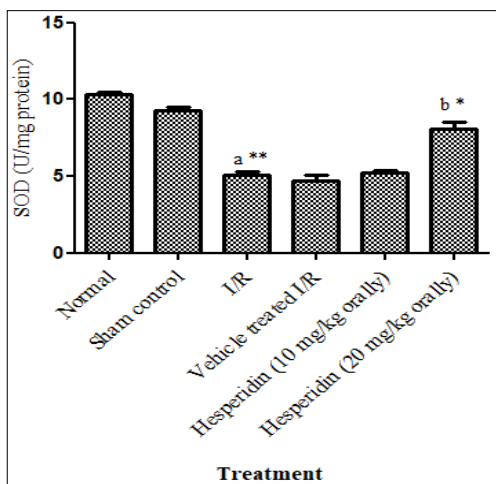

Fig 4: Effect of hesperidin on SOD levels in infracted tissue in cerebral ischemia reperfusion injury in rats.

All values are expressed as Mean± SEM (n=6), The SOD levels were significantly ($P< 0.01^{a***}$) decreased in I/R group rats compared to normal rats, SOD levels are significantly ($P< 0.05^{c*}$) increased in Hesperidin treated rats.
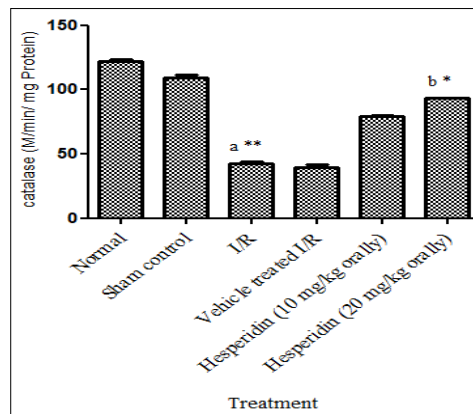

Fig 5: Effect of Hesperidin on catalase (μmoles/min/ mg Protein) levels in infarcted tissue in cerebral ischemia reperfusion injury in rats.

All values are expressed as Mean± SEM (n=6), Catalase levels were significantly ($P< 0.01\ a**$) decreased in I/R group rats compared to normal rats, similararly Catalase levels were significantly increased ($P< 0.05\ b*$) in Hesperidin treated rats.
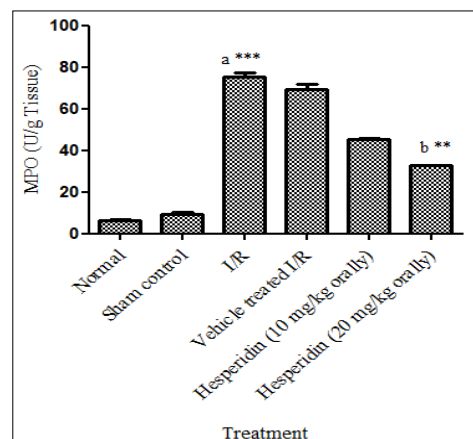

Fig 6: Effect of Hesperidin on Myeloperoxidase (U/g Tissue) levels in cerebral ischemia reperfusion injury in rats

All values are expressed as Mean± SEM (n=6), Myeloperoxidase levels were significantly (P< 0.01 a***) inceecreased in I/R group rats compared to normal rats, Myeloperoxidase levels were significantly (P< 0.01 b** increased in Hesperidin treated rats.

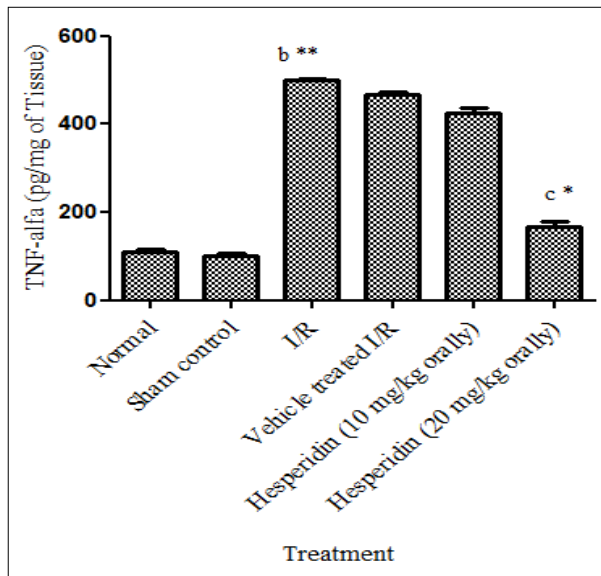**Fig 7:** Effect of Hesperidin on TNF-α (pg/mg of Tissue) levels in cerebral ischemia reperfusion injury in rats

All values are expressed as Mean± SEM (n=6), TNF alfa levels were significantly (P< 0.01 b**) inceecreased in I/R group rats compared to normal rats, similararly TNF alfa levels were significantly decreased (P< 0.05 c*) increased in Hesperidin (20 mg/kg bd.wt.) treated rats.
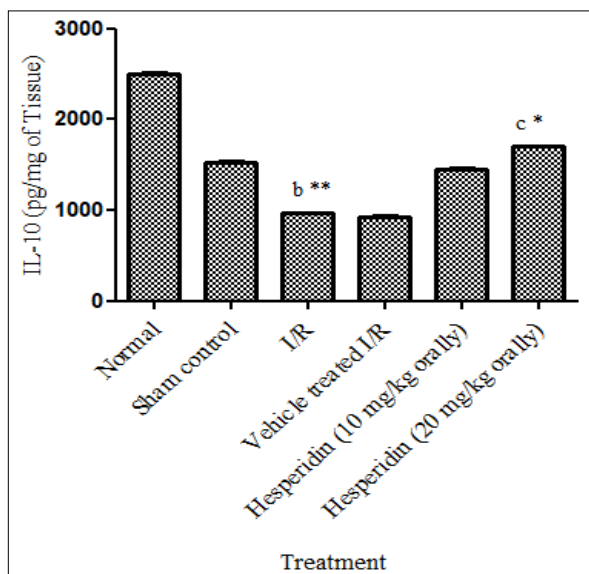

Fig 8: Effect of Hesperidin on IL-10 (pg/mg of Tissue) levels in cerebral ischemia reperfusion injury in rat

All values are expressed as Mean± SEM (n=6), IL 10 levels are significantly (P< 0.01 b**) decreased in I/R group rats compared to normal rats, IL 10 levels are significantly (P<0.05c*) decreased in Hesperidin treated rats.
The ischemic/reperfusion groups (I/R) rats were exhibited significant increase % of cerebral infarction from 0.2 ± 0.01 to 48.7 ± 1.2 (P < 0.01a**), similararly size was significantly decreased 48.7 ± 1.2 to 17.9 ± 0.7 (P < 0.01 b*) in Hesperidin

(20 mg/kg) treated rats (fig 1, 2). The MDA levels were significantly increased 162 ± 0.98 to 499 ± 2.8 (P < 0.001a***) in I/R group rats compared to normal rats, similarly MDA was significantly (P < 0.01b**) decreased from 499 ± 2.8 to 276 ± 1.9 in Hesperidin (20 mg/kg) treated rats compared to I/R group rats (Fig 3). The SOD levels were significantly (P < 0.01b**) decreased from 10.3 ± 0.21 to 5.1 ± 0.21 in I/R group rats compared to normal rats, SOD levels were significantly 5.1 ± 0.21 to 7.8 ± 0.32 (P < 0.05c*) increased in Hesperidin (20 mg/kg) treated rats (Fig 4). Catalase levels were significantly (P < 0.01 b**) decreased from 121.76 ± 1.54 to 42.43 ± 1.9 in I/R group rats compared to normal rats, similararly Catalase levels were significantly increased 42.43 ± 1.9 to 93.54 ± 1.3 (P< 0.05c*) in Hesperidin treated rats (Fig 5). Myeloperoxidase levels were significantly (P< 0.01b**) inceecreased from 6.5 ± 0.21 to 75.4 ± 2.1 in I/R group rats compared to normal rats, similarly myeloperoxidase levels were significantly (P < 0.01b**) decreased from 75.4 ± 2.1 to 29.6 ± 1.2 in Hesperidin (20 mg/kg) treated rats (Fig 6). TNF alfa levels were significantly (P < 0.01 b**) inceecreased from 110.78 ± 5.4 to 498.95 ± 4.8 in I/R group rats compared to normal rats, similararly TNF alfa levels were significantly decreased from 498.95 ± 4.8 to 298.43 ± 2.7 (P < 0.05 c*) increased in Hesperidin (20 mg/kg) treated rats (Fig 7). IL 10 levels are significantly decreased from 2500.67 ± 12.87 to 965.65 ± 6.9 (P < 0.01 b**) in I/R group rats compared to normal rats, Similararly IL 10 levels are significantly (P <0.05c*) decreased from 965.65 ± 6.9 to 1698.87 ± 3.5 in Hesperidin treated rats (Fig 8).

Discussion
The brain is highly susceptible to cerebral ischemia-reperfusion injury, it produces cerebral damage [15]. Cerebral infarction is considered one of the important components of cerebral ischemia, it can be atherothrombotic or embolic [16]. Cerebral infarctions fluctuate in their severity with one third of the cases resulting in death. Cerebral ischemia-reperfusion injury produces cerebral infarction via a complex cascade of patho biological events that progresses over a short period of time [15, 17]. The cerebral infarction has been associated with ROS, which react with cellular macromolecules such as proteins, lipids and nucleic acids leading to damage of the neuron [18, 19].
Ischemia and reperfusion cause brain injury via multiple pathways. Previous studies demonstrate that reactive oxygen species are elevated during cerebral ischemia and reperfusion, which plays a major role in the pathophysiology of ischemic stroke or cerebral I/R related injury.
MDA is end product of lipid peroxidation, the results clearly indicates the cytotoxic effect of free radical by peroxidation on brain tissue, because it contains large amount of phospholipids that are rich in polyunsaturated fatty acids leading to neuronal death [20]. Hesperidin treatment significantly reduced the elevated tissue MDA levels contributing to partial protection. This may be because of involvement of multiple pathways in global cerebral ischemia-reperfusion injury. Present study demonstrated that superoxide dismutase, catalase levels were significantly reduced in ischemia reperfusion (I/R) control group when compared to sham control. To prevent oxidative damage, mammalian cells have developed a complex antioxidant defense system that include enzymatic activities superoxide dismutase, catalse and free radical scavengers such as glutathione, vitamin C and E [21, 22]. Hesperidin treatment in

our present investigation increased the endogenous antioxidant enzymes superoxide dismutase, catalase indicating enhanced biochemical defenses to scavenge the overproduced free radicals. The present study explored the cerebral damage in terms of infarction observed in ischemia reperfusion injured brain tissue as evidenced by the increased percentage of infarct volume in I/R group rats when compared to Sham control. Infarction volume in the brain is an important determinant in assessing the consequences of cerebral ischemia which leads to neurological impairment.

Myeloperoxidase (MPO) is the most abundant component in azurophilic granules in neutrophils and has often been used as a histopathological marker for neutrophils. It is also expressed in the myeloid line, especially in monocytes and macrophages/microglia. MPO interacts with hydrogen peroxide to generate highly reactive species including hypochlorite (OC1–) and radicalized oxygen species (O2•–, ONOO•–). MPO mediated radicalization of molecules induces apoptosis and nitrotyrosination of proteins. MPO is also key component of inflammation and has been shown to play a major role in animal models of stroke in the post hypoxic inflammatory response during cerebral I/R injury [23, 24, 25, 26]. Hesperidin treatment significantly reduced the inflammation characterized by decrease in myeloperoxidase activity in animal subjected to cerebral I/R injury.

The inhibitory role on the release of cytokines, such as IL-lβ and TNF-α, from activated glia, inhibitory action against iNOS induction and subsequent NO• production in response to glial activation, ability to inhibit the activation of NADPH oxidase and subsequent ROS generation in activated glia, capacity to down regulate the activity of pro-inflammatory transcription factors such as NF-KB. The potential to modulate signaling pathways such as MAPK cascade. Similarly, Hesperidin treatment significantly reduced the inflammation characterized by decrease in tumor necrosis factor-α (TNF-α) in animal subjected to cerebral I/R injury. In the present study, significant increase in cerebral infarction in I/R group rats and significant decrease in cerebral infarction in Hesperidin treated rats was observed. These results were in accordance with earlier reports [27, 28]. Therefore, the results in the present study propose that Hesperidin has significant cerebroprotective action as evidenced by significant decrease in cerebral infarct volume. The evidence supports the antiinflammatory role of Hesperidin in cerebral I/R period. Interleukin-10 activity was considerably increased in animals treated with Hesperidin as compared with nontreated group. These results suggest the anti-inflammatory role of Hesperidin in cerebral I/R period.

Conclusion
The present investigation demonstrates the antioxidant effects of hesperidin tested in cerebral ischemia and reperfusion induced oxidative stress. The results suggest that the hesperidin is protective against ischemia-induced oxidative stress by mechanisms involving inhibition of free radical generation, inflammatory mediators generations against ischemic reperfusion induced decreases and that this extract may have potential as a therapy for the oxidative stress-related disorders.

Conflict of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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