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PROTECTIVE EFFECT OF *HYDRILLA VERTICILLATA* (LINN.F) ROYLE AGAINST CEREBRAL ISCHEMIA REPERFUSION INJURY IN RATS

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ABSTRACT

Hydrilla verticillata (HYD) is well reported to modify inflammatory response and oxidative stress which are key pathophysiological finding of cerebral reperfusion injury. Therefore present work was designed to investigate the effect of HYD on cerebral reperfusion injury in rats. Each protocol comprised cerebral ischemia (CI) for 30 min followed by reperfusion(R) for 1 h. Animals were treated with HYD (50mg/kgp.o) for seven days. At the end of the experiment, brain tissue was utilized for the measurement of oxidative stress markers, inflammatory response, infarct size and histopathological findings. HYD treated rats demonstrated a significant reduction in infarct sizes when compared with CI/R group of rats. HYD treatment demonstrated a significant decreased in malondialdehyde, nitric oxide levels and a significant increase inthe level of reduced glutathione, superoxide dismutase and catalase,showed modification in oxidative stress. HYD treatment confirmed a significant decrease in myeloperoxidase, C - reactive protein and TNF-α levels indicated a change in the inflammatory response. Histopathological findings revealed a reversal of damage in HYD treated rats. HYD treatment reduced DNA fragmentation of brain tissuein treated animals. HYD was found to be cerebroprotective against CI/R by anti-inflammatory and antioxidant activities.

Keywords: Cerebral Reperfusion Injury, *Hydrilla verticillata*, Stroke

INTRODUCTION

The ischemia is described as a decrease in oxygen supply or an increase in oxygendemand. With increasing duration and severity of ischemia, greater cell damage candevelop, with a predisposition to a spectrum of reperfusion - associated pathologies collectively called reperfusion injury **[1]**.

It is believed that CI/R injury induces an inflammatory response that elicits tissuedamage in several organs. Reactive oxygen species (ROS) with nitric oxide (NO) play a vital role in the pathophysiology in CI/R injury **[2]**. Inflammation contributes substantially to the pathogenesis of I/R with a central role for particularcells, adhesion molecules, and cytokines **[3]**. Neutrophils are the inflammatory cells that produce high levels of ROS during I/R injury. Myeloperoxidase (MPO) is found in neutrophils catalyse the formation of hypochlorous acid (HOCl), a toxic agent to cellular components, which initiates oxidative injury.

Studies have focused on the possible capacity of natural compounds extracted from fruits, vegetables and beverages to prevent certain age-related neurological disorders. Many active components extracted from traditional herbs have been demonstrated to show neuroprotection against ischemic brain injury in experimental studies. A growing body of research has reported that a burst of ROS is produced during ischemia/reperfusion, which leads to the oxidation of lipids, proteins and DNA and subsequently cellular damage and apoptosis **[4]**. Therefore, much attention has been paid to the rescue of brain injury after ischemia/reperfusion via inhibition of ROS bursts. According to the pharmacological mechanisms elucidated in numerous reports, we evaluated the natural products that possess protective effects on ischemic brain injury. We characterized the promising targets for ischemic brain injury.

Hydrilla Verticillata (HYD) is a potentialsource of secondary metabolites, natural antioxidants, and also, it has antimicrobialactivity. Hydrilla enriched with vitamin B, especially vitamin B12 and the essentialmineral calcium, fatty acid like gamma linoleic acid, antioxidants such as zinc, selenium, SOD, vitamin C, chlorophyll, DNA, RNA and proteins **[5]**. Hence, *Hydrilla verticillata* may be a promising keystone species which can be exploited fortherapeutic uses.

MATERIAL AND METHODS

Plant materials and preparation of the extract

The plant of *Hydrilla verticillata* (Linn.f) Royal was collected from the School of Science Botany Department, Gujarat Universityand Authenticated by the taxonomist. The plant of HYD was clean and dried. The dried powder was extracted in a Soxhlet apparatus at 50 °C with 200 ml of 95% ethanol and allowed to stand for 72 h at room temperature. The dried extract was stored in an airtight container in a cool and dry place **[6]**.

Phytochemical evaluation

Phytochemical screening for the presence of the alkaloids, phenols, saponins, flavonoids and terpenoids were done **[7]**.

Total phenolic content

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent. The plant extract (0.5 mL) was mixed with 0.5 mL of FC reagent (1:1 diluted with distilled water) and incubated for 5 min at 22°C followed by addition of 2 mL of 20% Na_2CO_3. The mixture was then incubated further at 22°C for 90 min, and the absorbance was measured at 650 nm. The total phenolic content (mg/mL) was calculated using gallic acid as standard.

Total flavonoid content

The total flavonoid content (mg/mL) was determined using aluminium chloride ($AlCl_3$) method. The assay mixture consisting of 0.5 mL of the plant extract, 0.5 mL distilled water, 0.3 mL of 5% $NaNO_2$, which was incubated for 5 min at 25°C. After that, 0.3 mL of 10% $AlCl_3$ and 2 Ml of 1 M NaOH was added to the reaction mixture. Finally, the absorbance was measured at 510 nm by using Quercetinas a standard.

Test for alkaloids

The plant extract (0.5 mL) was added to 2 mL of hexane, shaken well and filtered. After that, 3 mL 2% HCl was added to the plant extract. Moreover, the reaction mixture was heated and filtered. Finally, A drop of picric acid was added to the filtrate to develop yellow precipitate indicative of the presence of alkaloids.

Test for terpenoids

The plant extract in a final volume of 3 mL was mixed with 1 mL of chloroform and 1 mL of conc. H_2SO_4 to observe the intense red-brown colouration indicative of the presence of terpenoids.

Animals

The Wistar ratsof (either sex) (250-300 gm) were acclimatized in polypropylene cage (22 ± 3 °C with a 12 hours light-dark cycle). The protocol described here was approved by the Institutional Animal Ethical Committee (No PIPH16/18 921/PO/ReBi/S/05/CPCSEA)and conducted according to the guidelines of CPCSEA.

Experimental design

The animals were divided into 3 groups (n=6). Group 1 (NSO) Rats underwent all surgical procedures without carotid artery occlusion. Group 2 (CI/R) Rats underwent carotid artery occlusion for 30 min and followed by reperfusion for 1 hr. Groups 3 (HYD) Rats treated with HYD (50mg/kg p.o.) for the one week and on day seven underwent carotid artery occlusion for 30 minfollowed by reperfusion for 1 hr.

Induction of CI/R

Rats were anesthetized by ketamine (60 mg/kg i.p.) and diazepam (5 mg/kg i.p). Bilateral carotid arteries were exposed and occluded with thread for 30 min to induce ischemia. It was followed by reperfusion for 1hr **[8]**.

Determination of infarct size and area of infarction

Evaluation of infarct size carried out by 2, 3, 5-triphenyl tetrazolium chloride (TTC) staining method **[9]**. Following CI/R, animals were decapitated; the brains were removed and placed in cold saline. Coronal brain slices (2mm thick) were made. Then the slices were incubated in phosphate buffer saline (pH 7.4) containing 2% TTC at 37^{o}C for 10 min and then kept overnight in neutral-buffered formalin.A high-resolution scanner acquired the images of the TTC-stained section. The infarct size was measured by using Primostar Trinocular Microscope and compared between treatment groups and CI/R control group of rats **[10]**.

Histological Analysis

For histopathological analysis brain was fixed in 10% formalin and embedded in paraffin. The paraffin-embedded tissues were sectioned and stained with hematoxylin-eosin and analyzed by light microscopy. The histological sections were examined by an observer blinded to the treatment regimen, for the extent of brain tissue injury. The following morphological criteria were used to determine the histopathological damage: score 0, no damage; score 1 (mild), interstitial edema and focal necrosis; score 2 (moderate), diffuse brain cell swelling and necrosis; score 3 (severe), necrosis with the presence of contraction bands, neutrophil infiltration, and the capillaries were compressed; and score 4 (highly severe), widespread necrosis with the presence of contraction bands, neutrophil infiltration, capillaries compressing and haemorrhage **[11]**.

Bio-markers of oxidative stress, inflammatory response and DNA fragmentation

The cerebral tissue was assayed for levels of lipid peroxidation (MDA content) and endogenous antioxidant enzymes like reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione

peroxidase (GSHPx), xanthine oxidase (XO) activity, and nitric oxide (NO).C-reactive protein (by using diagnostickits, Nicholas India Pvt. Ltd., India) and TNF-α (Endogen, mouse TNF-α kit, Pierce Biotech Int., Rockford, Illinois, USA)were determined at serum level. Cerebral tissue analyzed for MPO activity as neutrophils infiltration **[12]**. Genomic DNA was extracted from cardiac tissue using a DNA extraction kit (DNeasy kit, Axygen), electrophoresis was carried out at 80 V for 1–2 h **[13]**.

Statistical Analysis

All the values are expressed as mean ± SEM. Statistical significance was tested between more than two groups using one-way ANOVA followed by the Bonferroni multiple comparisons test by using a computer-based fitting program (Prism, GraphPad 8). Differences were considered to be statistically significant when P<0.05,*P < 0.001*.P<0.001.

RESULTS

Phytochemical evaluations.

The percentage yield of HYD was 5.71% w/w. Preliminary phytochemical screening revealed the presence of secondary metabolites shows the presence ofalkaloids, phenol flavonoids and terpenoids was reported to be absent from **Table 1**.

Effect of HYD on Infarct size

HYD treated rats showed a significant reduction in infarct size when compared with CI/R rats. (P<0.001) **(Figure 1)**.

A large infarction area observed mainly in the caudal and rostral side of the hippocampus in the damaged brain of CI/R, whereas the infarction was markedly reduced in the rat brains treated with HYD as compared to CI/R group of rats **(Figure 2)**.

Effect of HYD on histopathological evaluation

Figure 3 represents the photomicrographs of brain sections stained with Haematoxylin and Eosin, 10X from I/R groups demonstrated lymphocytic proliferation and neuronal necrosis (B) as compared to NSO rats. There is a significant reversal of damage observed in HYD treated rats (C)

Effect of HYD on oxidative stress markers

CI/R group of rats established a significant (P<0.001) increase in MDA level when compared with the NSO group. We observed a significant decrease in activities of GSH, Catalase, and SOD in CI/R groups of rats when compared with the NSO group (P < 0.001). Treatment with HYD found to significant (P <0.001) decrease MDA level and significant increase in activities of GSH (P < 0.001), Catalase (P < 0.001) and SOD (P < 0.001) when compared with CI/R group **Figure 4**.

We observed significant (P < 0.001) higher

NO level in CI/R group when compare with NSO group which was significant (P < 0.001) lower in HYD group when compare with CI/R group (**Figure 5)**.

Effect of HYD on Inflammatory mediators in CI/R.

CI/R group of rats established a significant (P < 0.001) increase in MPO level when compared with the NSO Group, which was significantly (P < 0.001) decreased in HYD treated rats.CI/R group of rats established a significant (P < 0.001) increase in CRP level when compared with the NSO group which was significantly (P < 0.001) decreased in HYD treated rats.CI/R group of rats established a significant (P < 0.001) increase in TNF-α level when compared with the NSO group which was significantly (P < 0.001) decreased in HYD treated rats **(Figure 6)**.

Effect of HYD on DNA fragmentation

Apoptosis was evaluated by DNA fragmentation analysis. The typical DNA laddering activity was observed in the CI/R control and in HYD groups, which indicated cell apoptosis. HYD groups of rats demonstrated a decrease in DNA fragmentation and apoptosis in comparison to the CI/R group **(Figure 7)**.

Table 1: Preliminary phytochemical analysis of *hydrilla verticillata* (linn.f) royle

Phytochemical Tests	*Hydrilla verticillata* (HYD) Test Results
Phenolic	+
Flavonoid	+
Alkaloids	+
Terpenoids	-

+: indicates the presence of phytochemicals, -: indicates the absence of phytochemical

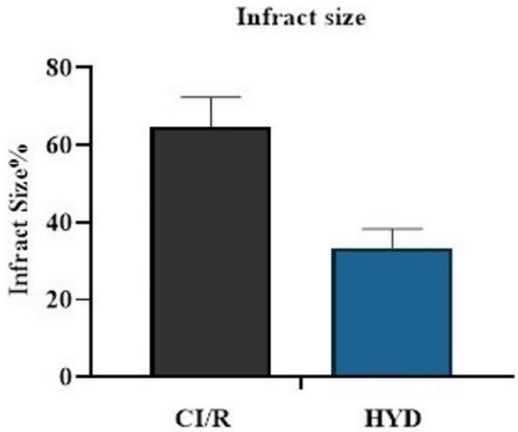

Figure 1: Values are mean ± SEM (n=6), analysed by one-way ANOVA followed by Bonferroni's multiple comparison test. * denotes P < 0.001 for chance differencesvs. I/R rats**

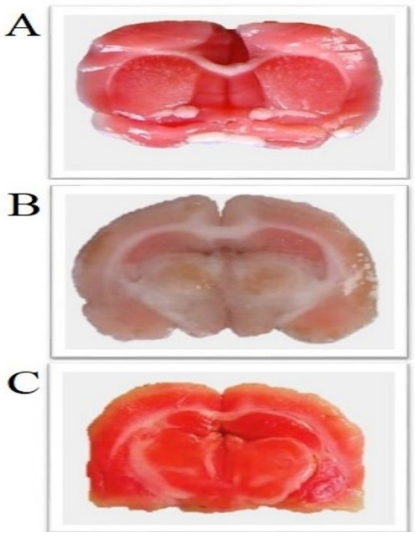

Figure 2: TTC stained transverse section of the brain of; A) Normal sham-operated; B) I/R - Cerebral ischemia/reperfusion injury; and C) HYD treated rats

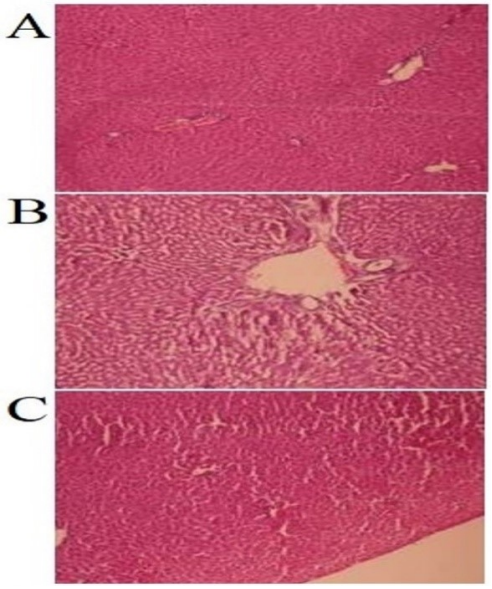

Figure 3: Haematoxylin and eosin stained (10X) brain section of; A) Normal sham-operated group; B) Cerebral ischemia/reperfusion injury; C) HYD pretreated rats respectively

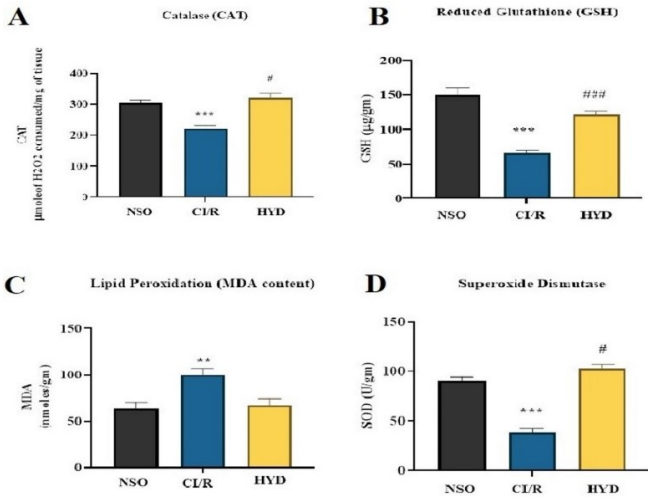

Figure 4: Effect of HYD on oxidative stress markers

Values are mean ± SEM (n = 6), analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests. ### denotes P < 0.001 for chance differences vs NSO rats for CAT, GSH, MDA and SOD level, and * denotes P < 0.001 for chance differences vs I/Rrats**

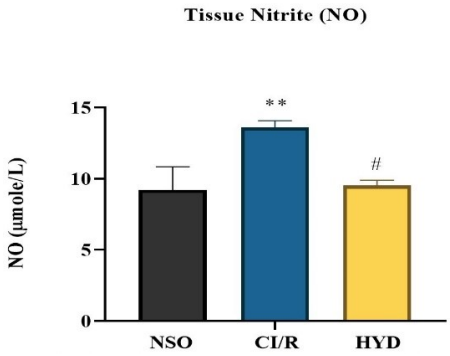

Figure 5: Effect of HYD on level of NO in brain tissue

Values are mean ± SEM (n=6), analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests. # denotes P < 0.001 for chance differences vs NSO rats for NO level, and * denotes P < 0.001 for chance differences vs I/R rats for NO level**

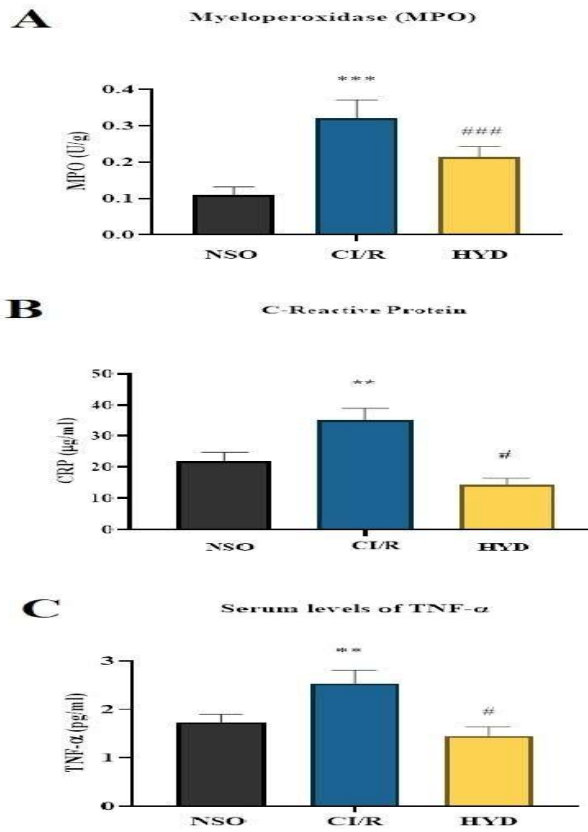

Figure 6: Effect of HYD on Inflammatory mediators in CI/R

Values are mean ± SEM (n = 6), analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests. ### denotes P < 0.001 for chance differences vs NSO rats for MPO, CRP and TNF-α level, and * denotes P < 0.001 for chance differences vs I/Rrats**

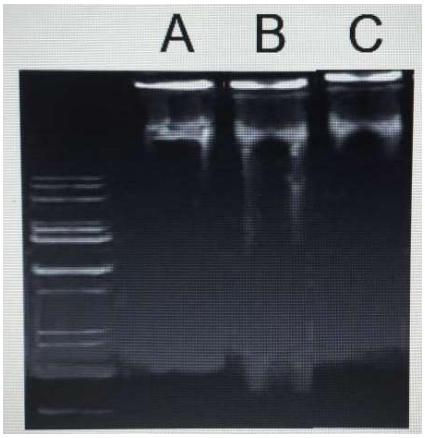

Figure 7: Effect of HYD on DNA fragmentation

A) Normal sham-operated group; B) Cerebral ischemia/reperfusion injury; C) HYD pretreated rats, respectively

DISCUSSION

Several existing drugs with potential neuroprotective activity have been used or are being tested in the treatment of stroke. However, most of them do not exhibit satisfactory clinical outcomes due to a variety of patients and drug-associated factors. A series of novel drugs targeted at interfering with distinct processes of stroke pathogenesis such as neuron apoptosis, excitotoxicity, inflammation, and angiogenesis are under development **[14]**.

In the present study, we investigated the potential neuroprotective activity effect of HYD against a global model of CI/R in rats. In line with the above mentioned neurological disorders, the rationale for the use HYD is to decrease oxidative stress, inflammation, and reduced infarction after focal ischemiaand that is why in present work we investigated the effect of HYD with above said bio-markers **[15, 16]**. We estimated LPO, SOD, CAT, GSH and NO levels in the brain tissue as an index to assess the severity of oxidative damage and subsequent protection by HYD.

Experimental models of stroke have been developed in animals in an attempt to mimic the events of human cerebral ischemia. It is well documented that transient global cerebral ischemia results in neurological abnormality **[17]**. Results indicated neuroprotection against HYD BCCA occlusion-induced CI/R brain damage. TTC is converted to red formazan pigment by nicotinamide adenine dinucleotide (NAD) and dehydrogenase present in living cells. Hence viable cells were stained deep red. The infarcted cells lose these enzymes and thus, remained unstained dull yellow **[18]**. The present study demonstrated a deep red section of the brain in HYD treated rat to confirm the protective effect against CI/R. Histopathological study showed marked congestion of blood vessels, neutrophil infiltration and neuronal necrosis in the CI/R group. The HYD treated group revealed that there was a significant decrease in neutrophil infiltration and brain damage as compared to CI/R group. Earlier histopathological studies on ischemic rat brain demonstrated that free radical scavengers mightreverse cerebral damage caused by reperfusion injury **[19]**. Our study reports the same and is following these studies. The data of the study showed that LPO and NO activity were significantly increased. In contrast, SOD, CAT and GSH activity significantly decreased in the CI/R groups confirms the brain damage due to oxidative stress. These results are inconsistent with the earlier study reported that in the presence of excess oxygen species, inactivation of detoxification systems and degradation of antioxidants lead to an increase

in LPO and NO while a decrease in SOD, CAT and GSH **[20, 21]**.

There are substantial experimental pieces of evidence that ROSs are produced in the brain during ischemia and reperfusion (I/R) injury. One of the oxygen-free radical that elevates in cerebral ischemia is NO; nevertheless, its precise role in this neuropathology remains controversial. NO is beneficial as a messenger or modulator, but in conditions such as oxidative stress, it is potentially toxic **[22]**. There are several sources for NO overproduction caused by cerebral ischemia. The highly reactive ONOO− provided a mechanistic basis for oxidative stress derived from increased NO production caused by CI/R. The data from the present study showed that HYD treatment significantly reduced NO levels as compared to the CI/R group of rats. Ischemia also results in the activation of endogenous microglia in the first hour or two following the insult. The peripheral leukocytes and microglia mount a robust inflammatory response with the induction of cytokine and chemokine expression as well as elevated expression of adhesion molecules, iNOS, COX2 and other inflammatory mediators which act to exacerbate the tissue damage **[23, 24]**. Treatment with HYD decreased in NO level might be due to inhibition of the expression of iNOS that generally expressed in I/R. Our results are following earlier reported protective effect on short-term CI/R in rats. ROS leads to neuronal death by the various oxidizing component of the cellular system such as lipids, proteins, and nucleotides. ROS attacks on biological membrane, leading to oxidative destruction of polyunsaturated fatty acids (PUFAs). It has been suggested LPO plays an important role in the pathogenesis of delayed neuronal damage in global ischemia showed that reperfusion injury in rat brain led to a significant increase in LPO levels **[25, 26]**. The results of the present study, where HYD ameliorated LPO, an effect that could be attributed to its capacity to scavenge free radicals, as showed by the observed restoration of the antioxidant enzyme activities. Our findings are by the study reported the anti-oxidant activity of HYD. The brain has its own endogenous ant oxidative defence mechanisms to counteract oxidative burst following ischemia-reperfusion injury. Enzymatic antioxidants such as SOD and CAT safeguard the brain against reperfusion injury. Superoxide anion (O2-) is known to produce highly toxic hydroxyl radicals mainly through its reaction with H_2O_2 (Haber–Weiss reaction) **[27]**. The superoxide adduct is disputed by SOD into H_2O_2, which is converted to water and oxygen by catalase. In ischemic conditions, these defence mechanisms fail to

protect the tissue from oxidative damage because of the overproduction of oxygen radicals, inactivation of antioxidant enzymes, and the consumption of antioxidants in the ischemic tissue **[28]**. The current study demonstrated that the SOD and CAT activities in the hippocampal region were decreased dramatically after ischemic insult. These results are consistent with the studies reported a reduction in the SOD and CAT levels in the ischemia group **[29]**. Furthermore, prior treatment with HYD enhanced the levels of SOD and CAT. This is under the study reported enhancement in enzymatic SOD and CAT levels conferred by German chamomile. GSH considered being the most prevalent and important intracellular non-protein thiol, has a crucial role as a free radical scavenger observed that a decline in GSH could reflect oxidative stress **[30]**. In the current study, GSH content was moderately reduced due to CI/R insult. Results obtained from the present study agree well with the other studies reported a reduction in GSH levels in I/R group. The overproduction of oxygen free radicals can be detoxified by the endogenous antioxidants causing their cellular stores to be reduced **[21]**. The results of the study showed a significant increase in the GSH levels with HYD treated groups as compared to CI/R which are in accordance with the previously reported study where GSH levels increased confirmed by a neuroprotective effect.

MPO is the most abundant component in neutrophils myeloid line, especially in monocytes and macrophages/microglia **[31]**. MPO interacts with hydrogen peroxide to generate highly reactive species including hypochlorite (ClO) and radicalized oxygen species. The MPO-mediated radicalization of molecules induces apoptosis and nitro-tyrosination of proteins **[32]**. An increased MPO level was observed in the ischemia-induced group. The data from the present study showed that HYD treatment significantly reduced MPO levels as compared to CI/R group.

CRP is a protein synthesized by the liver. CRP is a marker of inflammation. Increased CRP levels can prompt a significant increase in the content and activity of serum pro-inflammatory cytokines. This marker thoroughly and sensitively reflects the presence of inflammation. This phenomenon is probably caused by the intense inflammatory response resulting from large-artery atherosclerosis. Another scholar compared the correlation between CRP levels and infarcted volume and found a positive relationship. The majority of clinical scholars use CRP as an indicator for assessing stroke progress, prognosis, and recurrence **[33]**. In

the present study, the trend of serum CRP levels shortly after cerebral ischemia was found to be increased. The results showed that HYD treatment significantly reduced CRP levels as compared to CI/R group.

TNF-α is a cytokine with activated macrophages and polypeptide hormone has extensive biological functions, mainly related to inflammatory and immune reaction. TNF- α secretion in the early cerebral ischemia or synthetic is the leading cause of brain infarction. It has increased the permeability of vascular endothelial cells. It induces cell adhesion factor expression, and so on.TNF-α induced leukocyte infiltration after cerebral ischemia and plays a vital role in tissue damage, which can cause a multi-core white blood cell aggregation and activation and release of inflammatory mediators **[33]**. The data indicate that HYD treatment significantly reduced TNF-α levels as compared to CI/R group.

The release of cytochrome C into the cytosol leads to the formation of the apoptosome, which stimulates the activation of procaspase-9 and procaspase-3. Active caspase-3 activates caspase-activated DNAase, leading to DNA fragmentation.Here we confirmed the occurrence of apoptosis in rats brain is greater in CI/R with than NSO **[34]**. Our findings that HYD treatment lowers DNA fragmentation which indicated a reduction of nuclear oxidative stress in cardiac tissue followed by CI/R in diabetic rats.

CONCLUSION

In conclusion, the present study provided, to the best of our knowledge, the first experimental evidence suggesting a potential benefit of Hydrilla Verticillata treatment in the management of acute ischemic stroke. We recommend that antioxidant and membrane stabilizing activity of the Hydrilla Verticillata might be responsible for the cerebroprotective action in the experimental animals. Further studies are required to pursue the new lead emerging from the present results to exploit the full therapeutic potential of Hydrilla Verticillata in cerebrovascular diseases.

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