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Protective Effect of Alcoholic Extract of Amla (*Emblica officinalis*) Fruits on Cerebral Reperfusion Injury in Rats

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ABSTRACT

Ischemia-reperfusion injury is a phenomenon whereby cellular damage in a hypoxic organ is accentuated following the restoration of oxygen delivery. Wistar rats of either sex (250-300 g) were treated for 15 days with alcoholic extract of *Emblica officinalis* fruits (250-500mg/kg) prior to ischemic reperfusion. After 15 days ischemia was produced by clamping the right common carotid artery for 25 min and reperfusion for 40 min by unclamping. Brain was dissected out to prepare a homogenate to measure the level of malondialdehyde, superoxide dismutase, catalase, vitamin C and protein estimation. Animals subjected to ischemic reperfusion without any treatment were included as model control and animals treated with vitamin E were included as a standard. Animals subjected to ischemic reperfusion without any treatment showed higher level of oxidative enzymes and lower level of protective enzymes as compared with the animals treated with alcoholic extract of amla fruits. In histopathology, protection against the injury in the animals treated with alcoholic extract of amla fruits was noted.

Key words: *Emblica officinalis*, ischemia-reperfusion injury, malondialdehyde

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INTRODUCTION

When a tissue is subjected to ischemia, a sequence of chemical events is initiated that may ultimately lead to cellular dysfunction and necrosis. If ischemia is ended by the restoration of blood flow, a second series of injurious events ensue producing additional injury. Thus, whenever there is a transient decrease or interruption of blood flow the net injury is the sum of two components—the direct injury occurring during the ischemic interval and the indirect or reperfusion injury that follows. When there is a long duration of ischemia, the "direct" damage resulting from hypoxia alone is the predominant mechanism. For shorter durations of ischemia, the indirect or reperfusion-mediated damage becomes increasingly more important. For example, it has been shown that the intestinal injury induced by 3 h of ischemia (flow reduced to 20% of normal) and 1 h of reperfusion is several times greater than that observed after 4 h of ischemia alone.[1] These results demonstrate that the injury produced by reperfusion can be more severe than the injury induced by ischemia per se. This same pattern of relative contribution of injury from direct and indirect mechanisms has been shown to occur in all organs. Ischemia-reperfusion injury is a phenomenon whereby cellular damage in a hypoxic organ is accentuated following the restoration of oxygen delivery. Reoxygenation of the ischemic tissue may promote the generation of various reactive oxygen metabolites, which are known to have deleterious effects on various cellular functions. There are different mechanisms responsible

for reperfusion injury such as leukocyte-endothelium interactions, reactive oxygen species and involvement of complement system. Herbal medicines are recognized by World Health Organization as an essential building block for primary health care, especially in developing countries such as India, but the herbal and other indigenous sources have not adequately been explored for the presence of safe and effective plants for the treatment of ischemic-reperfusion injury. It is known that consumption of fruits and vegetables is essential for normal health of human beings. Vegetarian diet can reduce the risk of cancer, atherosclerosis, etc. *Phyllanthus emblica*, also known as amla, has been used in Ayurveda, the ancient Indian system of medicine. It has been used for treatment of several disorders such as common cold, scurvy, cancer and heart diseases.[2] It is believed that the major constituent responsible for these activities is vitamin C (ascorbic acid). Ascorbic acid shows antioxidant, anti-inflammatory and antimutagenic properties.[3] It is a very effective free-radical scavenger. However, there are some *in vivo* studies indicating that antioxidant activities of amla cannot be attributed to ascorbic acid alone and that the overall effect is due to other polyphenols such as ellagic acid, gallic acid, tannins, etc.[4]

In the light of above findings the present investigation was undertaken to study the potential of *Emblica officinalis* in the treatment of cerebral ischemic-reperfusion injury.

MATERIALS AND METHODS

Plant material

Shade-dried fruits of *E. officinalis* (Amla) were used for the preparation of plant extract.

Preparation of plant extract

The coarse powder (500 g) of the dried fruits of *E. officinalis* was extracted using 50% ethanol (2000 ml) in a soxhlet extractor. The extract was concentrated under reduced pressure to yield a syrupy mass. The extract was stored in an airtight container in a cool place and used throughout the project.

Acute toxicity study

Acute Toxicity Study was carried out as per OECD guidelines.

Preliminary phytochemical screening

Standard phytochemical tests were used to screen the extract for the presence of different constituents. Briefly, $FeCl_3$ test was used to characterize for tannins, Dragendroff's reaction and Mayer's test was used for alkaloids and Fehling's test was used for reducing sugars. Similarly, HCL-magnesium test was used for flavonoids, while frothing test was deployed for saponins and Liebermann–Buchard reaction was used for detection of the presence of triterpenoids and steroids.[5,6]

Experimental protocol

Animals and Drug Treatment Protocol

Albino rats of Wistar strain of either sex (200-250) were housed in cages with free access to standard diet and water. Animals were divided in five groups, each containing six animals.

Drug treatment protocol

Animal are divided into various groups. Normal control group (Group I) received only saline without ischemia reperfusion, whereas animals from model control group (Group II) received only ischemia reperfusion without any treatment. Animals from Group III to Group V received test drugs such as standard drug vitamin E (200 mg/kg) p.o., alcoholic extract of *E. officinalis* fruits, (250mg/kg) p.o., alcoholic extract of *E. officinalis* fruits (500 mg/kg) p.o. once daily for 15 days prior to ischemia reperfusion.

Cerebral ischemia reperfusion[7]

At the end of 14th day food was withdrawn and animals were fasted overnight. On the next day, the last dose of drug was given and after 2 h the animals were anesthetized by diazepam (5 mg/kg, i.m.) and ketamine (50 mg/kg, i.m.). Heparin 4000 units/kg (i.v.) was administered. Ischemia was produced by clamping the right common carotid artery, using bulldog clamp for 25 min followed by reperfusion for 40 min by unclamping the artery.

Preparation of tissue homogenate

Brain was dissected out and immediately stored at -20° C. One gram of tissue was homogenized with 10 ml Tris-hydrochloride (20 mM, pH 5.8-7.2). The prepared homogenates were centrifuged at 10000 rpm for 10 min and supernatant was used for determination of antioxidant parameters. Pieces of tissue were stored in 10 % formalin for light microscopic study.

Estimation of free-radical generation

Malondialdehyde level[8]

Briefly, 1 ml sample was mixed with 0.2 ml 4% (w/v) sodium dodecyl sulfate, 1.5 ml 20% acetic acid in 0.27 M

hydrochloric acid (pH 3.5) and 1.5 ml 0.8% thiobarbituric acid (TBA). The mixture was heated in a hot water bath at 85° C for 1 h. The intensity of pink color developed was read against a reagent blank at 532 nm following centrifugation at 1200 rpm for 10 min. The amount of malondialdehyde was calculated using molar extinction coefficient 1.56×10^5 M^{-1} cm^{-1} and reported as nanomoles of malondialdehyde per milligram of protein.

Preventive antioxidants

Superoxide dismutase[9]

The sample (0.1 ml) was mixed with 0.1 ml EDTA (1×10^{-4} M), 0.5 ml of carbonate buffer (pH 9.7) and 1 ml of epinephrine (3×10^{-3} M). The optical density of formed adrenochrome was read at 480 nm for 3 min at intervals of 30 s and results were expressed as units per minute per milligram of protein.

Catalase[10]

An aliquot of 50 µl of sample was added to buffered substrate (50 mM phosphate buffer pH 9.7 containing 30 mM H_2O_2) to make up the volume to 3 ml. The decrease in absorbance was read at 240 nm for 2.5 min at intervals of 15 s. The results were expressed as mean absorbance of catalase activity. The activity was calculated using extinction coefficient of H_2O_2, 0.041/µmol/cm^2. Results were expressed as µmol of H_2O_2 utilized per minute per milligram of protein.

Chain breaking antioxidants

Vitamin C[11]

Sample (0.5 ml crude homogenate) was precipitated with 4.5 ml of 4% chilled TCA and left for 30 min on an ice bath. Sample was centrifuged after 30 min at 1000 rpm for 10 min at 4°C. About 1.0 ml of supernatant was mixed with 0.25 ml of coloring reagent and heated at 80°C for 30 min. Coloring reagent was prepared by mixing 5 ml thiourea (5% w/v) in triple distilled water), 5.0 ml copper sulfate (0.6% w/v) and 90 ml of 2,4, dinitrophenhydrazine (2.2% w/v in 1.0N H_2SO_4). The reaction was terminated by addition of 1.75 ml 65% H_2SO_4 and tubes were left at room temperature for 15 min. Absorbance of yellow color was read at 540 nm against blank reagent. A standard curve of ascorbic acid (5-30 µg) was also run simultaneously. The vitamin C content was then calculated, which have been reported as microgram ascorbic acid per milligram of protein.

Protein estimation[12]

About 100 mg of tissue was weighed and homogenized in 5 ml of distilled water. The homogenate (0.2 ml) was added to 4 ml of solution C (Solution A: 2 g sodium hydroxide, 10 g sodium carbonate, 0.1 g sodium-potassium tartrate in 500 ml of distilled water, Solution B: 0.5 g cuprous sulfate in 100 ml distilled water, Solution C: 10 g Sol A and 0.2 ml Sol B) and 0.6 ml of distilled water is added and allow to stand for 15 min at 37°C. Folin-phenol reagent 0.4 ml was added and incubated for 30 min. Absorbance was read at 540 nm. Amount of protein in 100 g of tissue was calculated from the graph of standard albumin.

Histopathology

Briefly, the procedure used included fixation of the tissue with formalin, embedding in paraffin blocks, sectioning with microtome (0.7-µ thickness) and finally staining by Hematoxylin and Eosin stain technique.

Principle

Hematoxylin stains nucleus light blue, which turns red in the presence of acid. The differentiation is achieved by treating the tissue with acid solution the counter staining is performed by using eosin, which imparts pink color to cytoplasm.

Statistical analysis

All values were expressed as mean ± SEM of six observations. The statistical analysis was performed using Student's unpaired *t* test. The value of *P* less than 5% ($P < 0.05$) was considered statistically significant.

RESULTS

Acute toxicity study

Alcoholic extract of *E. officinalis* fruits was found to be safe up to 5000 mg/kg p.o. given to mice. No any signs for behavioral as well as any physical changes were found.

Preliminary phytochemical screening

The alcoholic extract of *E. officinalis* was found to be rich in vitamin C, gallic acid, polyphenols, phyllemblin.

Malondialdehyde level

Malondialdehyde is an indicator of level of lipid peroxidation [Figure 1]. Animals treated as a model control group shows higher level of malondialdehyde as compared with normal control group. Alcoholic extract of *E. officinalis* fruits shows significantly ($P < 0.05$) lower level of malondialdehyde at a dose of 250 and 500 mg/kg as compared with animals treated as model control group and comparable to animals treated as standard.

Catalase level

Catalase is an antioxidant enzyme liberated during the state of oxidative stress and reperfusion injury. Animals treated as a model control group shows lower level of catalase as compared with normal control group. Alcoholic extract of *E. officinalis* fruits shows significantly ($P < 0.05$) higher level of catalase at a dose of 250 and 500 mg/kg as compared with animals treated as model control group and comparable to animals treated as standard [Figure 2].

Vitamin C level

Similar to catalase, vitamin C is an antioxidant vitamin. Animals treated as a model control group shows lower level of vitamin C as compared with normal control group. Alcoholic extract of *E. officinalis* fruits shows significantly ($P < 0.05$) higher level of vitamin C at doses of 250 and 500 mg/kg as compared with animals treated as model control group and comparable to animals treated as standard [Figure 3].

Superoxide dismutase

It is also an antioxidant enzyme liberated during the state of oxidative stress. Animals treated as a model control group shows lower level of superoxide dismutase as compared with normal control group. Alcoholic extract of

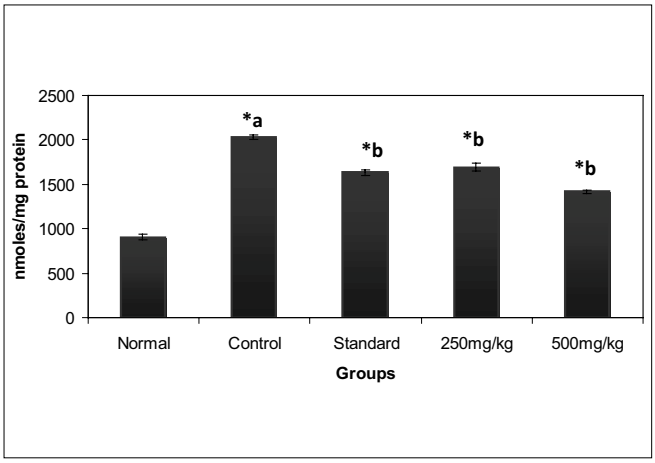

Figure 1: Effect of amla fruits extract on MDA levels after cerebral I/R injury. Each bar represents the mean ± SEM of six observations. *$P < 0.05$, a. Control group compared with normal group, b. Test groups compared with control group I/R injury resulted into significant increase in liver MDA levels. Both AAFE and Vitamin-E treated animals had a significantly lower MDA levels as compared to untreated animals.

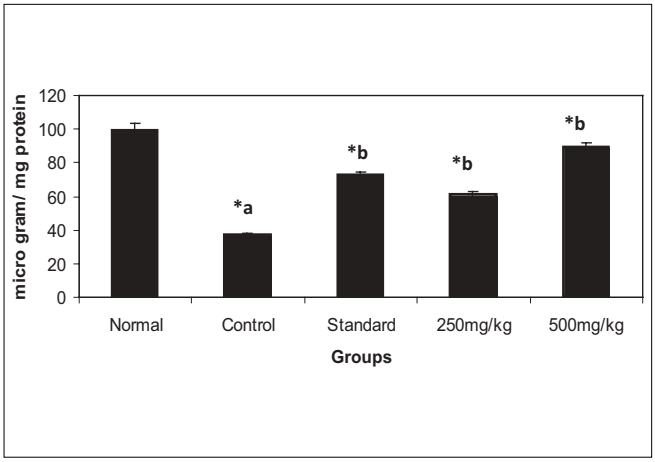

Figure 3: Effect of amla fruits extract on Vitamin-C levels after Cerebral I/R injury. Each bar represents the mean ± SEM of six observations. *$P < 0.05$, a. Control group compared with normal group, b. Test groups compared with control group, I/R injury resulted into significant decrease in liver Vitamin-C levels. Both AAFE and Vitamin-E treated animals had a significantly higher Vitamin-C levels as compared to untreated animals

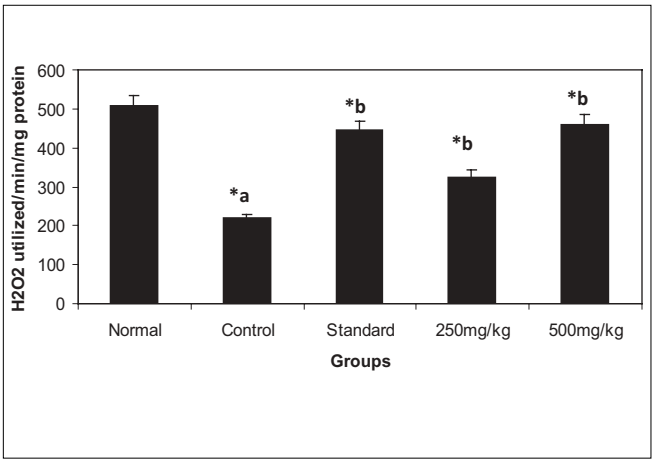

Figure 2: Effect of amla fruits extract on catalase levels after cerebral I/R injury. Each bar represents the mean ± SEM of six observations. *$P < 0.05$, a. Control group compared with normal group, b. Test groups compared with control group, I/R injury resulted into significant decrease in liver catalase levels. Both AAFE and Vitamin-E treated animals had a significantly higher catalase levels as compared to untreated animals.

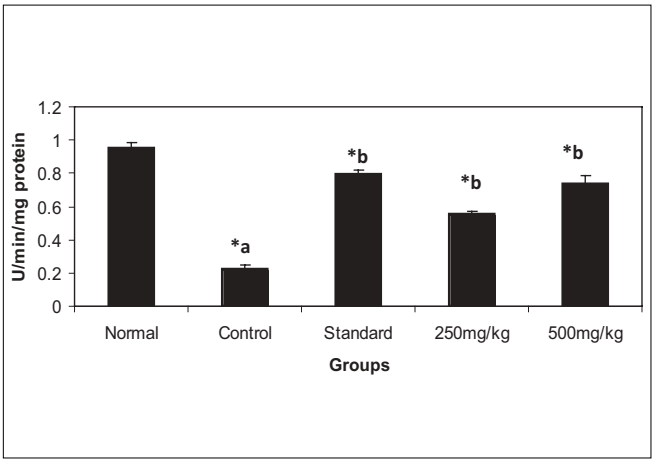

Figure 4: Effect of amla fruits extract on SOD levels after Cerebral I/R injury. Each bar represents the mean ± SEM of six observations. *$P < 0.05$, a. Control group compared with normal group, b. Test groups compared with control group, I/R injury resulted into significant decrease in liver SOD levels. Both AAFE and Vitamin-E treated animals had a significantly higher SOD levels as compared to untreated animals

E. officinalis fruits shows significantly ($P < 0.05$) higher level of superoxide dismutase at doses of 250 and 500 mg/kg as compared with animals treated as model control group and comparable to animals treated as standard [Figure 4].

Histopathology

Animals in normal control groups show normal structure of brain, whereas animals from model control group means without any treatment but with ischemic reperfusion showing brain with multiple foci of necrosis. Vitamin E–treated animals show significant restoration of brain tissue without any necrosis. Animals treated with amla fruit extract (250 mg/kg) plus ischemic reperfusion, showing lesser foci of necrosis as compared with model control group, whereas animals treated with amla fruit extract (500 mg/kg) plus ischemic reperfusion, showing almost absence of necrosis [Figure 5].

DISCUSSION

The present study aimed to study the effect of alcoholic amla fruits extract on cerebral ischemic-reperfusion injury in rats. Cerebral ischemia exists in case of reduced blood flow, which results in a decreased supply of oxygen and nutrients to the brain. Reperfusion injury occurs when there is re-establishment of blood flow after prolonged ischemia that aggravates the irreversible tissue damage due to free-radical generation. Reperfusion injury is thought to be primarily due to free oxygen radicals. During reperfusion hypoxanthine gets accumulated, which is converted to xanthine. In this process, superoxide radicals (O_2) are

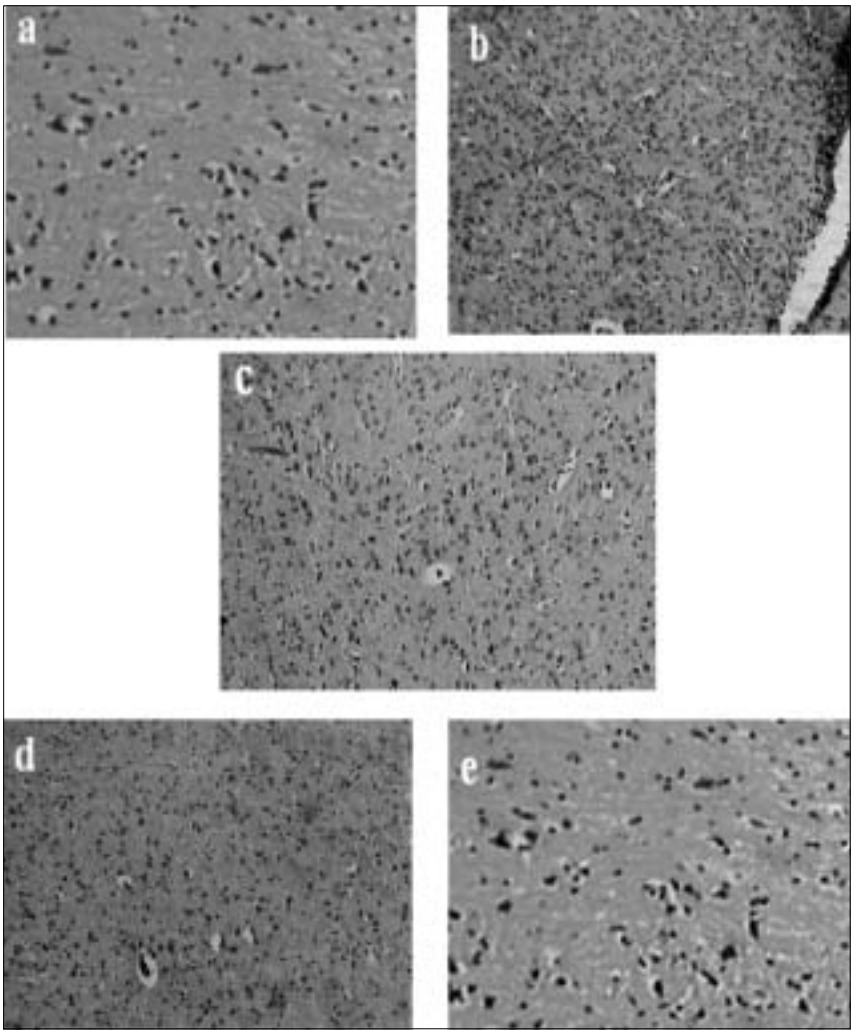

Figure 5: Photomicrographs of brain sec tions of rat stained with haemotoxylin and eosin (x100). a) normal rats (Group I) showing normal structure; b) Ischemic Reperfusion (Group II) showing brain with multiple foci of necrosis; c) Vitamin E- treated rats (Group III) showing significant restoration of brain tissue without any necrosis; d) Ischemic Reperfusion plus extract (250 mg/kg) treated rats (Group IV) showing lesser foci of necrosis; e) Ischemic Reperfusion plus extract (500 mg/kg) treated rats (Group V) showing almost absence of necrosis

generated and converted to hydrogen peroxide (H_2O_2) or hydroxyl radical (OH^-).[13] The free radicals generated are scavenged by endogenous antioxidant mechanism that involves superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT).[13,14] In case of excessive free-radical generation the endogenous protective system may not prove sufficient, resulting in damage. Exogenous antioxidant such as vitamin C, vitamin E, β-carotene can also scavenge potent radicals and prove useful in preventing damage.[15] A microcirculatory disturbance seems to be involved in the mediation of ischemic-reperfusion injury.[16] Large numbers of herbal drugs have been reported to possess antioxidant properties. However, none has been proved to be effective in acute ischemic-reperfusion injuries. In the present study, we have attempted to evaluate the efficacy of pretreatment with alcoholic amla fruit extract. Because in the present study, the brain is subjected to more acute injury, a 15-day pretreatment with alcoholic extracts of amla fruit was considered.

The thiobarbituric acid reacting substance (TBARS) assay is used as an indicator of lipid peroxidation and levels of free radicals. The assay is based on the reactions of thiobarbituric acid with malondialdehyde produced during lipid peroxidation.[17] As observed in our study, the increase in malondialdehyde in brain affected by ischemic-reperfusion injury suggested enhanced lipid peroxidation. In our study, alcoholic amla fruit extract pretreated animals showed significantly less lipid peroxides due to ischemic-reperfusion injury than untreated animals. Vitamin E–treated animals also showed lesser degree of lipid peroxidation than control group. Hence, it is possible that the mechanism of protection of brain by alcoholic amla fruit extract might be due to its antioxidant effect. Catalase decomposes hydrogen peroxidase and converts it to water and diatomic oxygen, whereas superoxide dismutase generates H_2O_2 from free radicals. An increase in the production of superoxide dismutase without a subsequent elevation of catalase leads to the accumulation of hydrogen peroxidase, which is converted to hydroxyl radicals that produced deleterious effect on brain. In the present study, catalase levels were found to be less in animals subjected to ischemic-reperfusion injury. The reactive-oxygen-species–induced lipid peroxidation causes more production of hydroxyl radicals, which then inactivates the catalase.[18] In alcoholic amla fruit extract–treated animals, increase in the catalase activity was observed. These suggest generation of hydroxyl radicals. Thus, catalase activity can be more in alcoholic amla fruit extract–treated animals. These findings are in agreement with another experiment.[19] Vitamin E–treated animals also showed a similar effect.

Superoxide dismutase is an important endogenous antioxidant and prevents the production of free radicals. Superoxide dismutase levels were significantly higher in alcoholic amla fruit extract–treated animals when compared to the control animals. Non-enzymatic antioxidant tocopherol acts as free-radical quencher. Vitamin C helps in the maintenance of tocopherol level in membranes.[20] As an antioxidant, it scavenges free radicals and reactive oxygen molecules, which are produced during metabolic pathways of detoxification. A high dose of vitamin C (14 mg/kg) is necessary to detoxify large amounts of oxygen radicals.[21] It was demonstrated that the high dose of vitamin C along with the pretreatment with vitamin E is very effective in preventing catalase exhaustion and protection against ischemic reperfusion injury. In alcoholic amla fruit extract–treated animals vitamin C concentration was higher as compared with control group, suggesting an increase in the endogenous antioxidant content by alcoholic amla fruit extract. Vitamin E also showed similar effect on vitamin C level.

Generation of reactive oxygen species is a central event in the reperfusion period.[22] Reperfusion injury is thought to be primarily due to attack by the generated oxygen radicals. Neutrophil activation and metabolism of xanthine by xanthine oxidase are the major source of these detrimental mediators. Extracellular fluids, such as plasma, contain little antioxidant capacity as compared with brain. Therefore, administration of antioxidants during the early phase of reperfusion was suggested to have beneficial effects. Various antioxidants have been demonstrated to possess protective potential. Glutathione (GSH),[23] superoxide dismutase, allopurinol[24] and alpha-tocopherol[25] have all been shown to attenuate cerebral ischemic-reperfusion injury. Imbalance between cellular production of reactive oxygen species and the inability of cells to defend against them is called oxidative stress (OS).[26-28] OS is involved in acute and chronic CNS injury and is a major factor in the pathogenesis of neuronal cell damage.[29] Free radicals and related reactive oxygen species mediate much of the damage that occurs after brain ischemia finally leads to cell damage.[30] Ischemic-reperfusion injury is known to produce necrosis of brain, which can be directly visualized by histological study; biopsy of the rat brain subjected to ischemic-reperfusion injury showed significant necrosis. The brain tissue obtained from alcoholic amla fruit extract–treated animals showed only mild congestion and no necrosis. This suggests that amla fruits have significant protective action against ischemic-reperfusion injury. From the above discussion it can be concluded that amla has antioxidant property, which is responsible for beneficial effects in cerebral ischemia-reperfusion injury.

CONCLUSION

From the results of the present investigation, it can be concluded that alcoholic extract of *E. officinalis* fruits provide significant protection against cerebral ischemic-reperfusion injury in a rat model.

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