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RESEARCH ARTICLE

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Antioxidant and Cytoprotective effect of *Fragaria nubicola* on ischemia reperfusion induced brain injury

Purushottam B. Rakhunde and Syed Ayaz Ali *

Department of Pharmacology, Y.B. Chavan College of Pharmacy, Dr. Rafiq Zakaria Campus, Aurangabad (M.S.) India
Correspondence: ayazpharm@gmail.com

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ABSTRACT

Brain Stroke is one of the prominent causes of death without an efficacious treatment. Fragaria nubicola have potential antioxidant activity due to the presence of phenolic compounds and may be proved as cytoprotective against ischemia-reperfusion induced brain injury. We studied the effect of fresh fruit juice of Fragaria nubicola (10 ml/kg, p.o.) and vitamin E as reference standard drug on 30 min induced ischemia, followed by reperfusion, with the help of evaluation parameters such as neurobehavioral tests consisting of neurodeficit score, beam walk test, rota rod test, hanging wire test and elevated plus maze. The biochemical parameters measured in animals' brain were nitric oxide, malondialdehyde, superoxide dismutase and catalase, in control and treated rats. The fresh fruit juice of Fragaria nubicola treated groups showed a statistically significant improvement in the neurobehavioral parameters such as motor performance [neurological status, significant increase in grasping ability ($P<0.05$), forelimb strength ($P<0.05$) improvement in balance and co-ordination ($P<0.05$)]. The biochemical parameters in the brains of rats showed a significant enhanced activity of enzymatic antioxidants such as catalase ($P<0.01$), superoxide dismutase ($P<0.01$), significant reduction in the total nitrite ($P<0.01$) and lipid peroxidation ($P<0.01$). The above observations suggest that juice of F. nubicola fruits (strawberry) have shown the most pronounced cytoprotective activity.

Key Words: *Fragaria nubicola*, Brain ischemia-reperfusion, Cytoprotection, Oxidative stress.

INTRODUCTION

Stroke is the third most common cause of death in most industrialized countries after cardiovascular disease and cancer, and its incidence is expected to rise with the projected increase in the number of the aging population [1]. Reactive oxygen species have been reported to play an important role in pathophysiology of cerebral ischemia. Vascular reperfusion subsequent to transient occlusion results in worsening of damage if reperfusion is achieved after some critical period of occlusion (i.e. ischemia). Free radicals are believed to be responsible for this so-called reperfusion injury. These oxy-free radicals initiate lipid peroxidation and can cause damage to macromolecular components of cell [2, 3].

During ischemia, cellular adenosine triphosphate (ATP) is degraded to form hypoxanthine. Normally, hypoxanthine is oxidized by xanthine dehydrogenase to xanthine. However, during ischemia, xanthine dehydrogenase is converted to xanthine oxidase. Unlike xanthine dehydrogenase, which uses nicotinamide adenine dinucleotide as its substrate, xanthine oxidase uses oxygen and therefore, during ischemia, is unable to catalyze the conversion of hypoxanthine to xanthine, resulting in a build up of excess tissue levels of hypoxanthine. When oxygen is reintroduced during reperfusion, conversion of the excess hypoxanthine by xanthine oxidase results in the formation of toxic reactive oxygen species (ROS). Reperfusion of ischemic tissues results in the formation of toxic ROS, including superoxide anions (O_2-), hydroxyl radicals (OH^-), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2) and nitric oxide-

derived peroxynitrite. Reactive oxygen species are potent oxidizing and reducing agents that directly damage cellular membranes by lipid peroxidation [4] Peroxynitrite [5] and hydroxyl radical [6] are reported to produce DNA nicking. ROS are also documented to activate lysosomal enzymes, which may contribute to neuronal injury [7]. Additionally, mitochondrial damages due to ROS release may contribute to delayed cell death after cerebral ischemia and reperfusion [8].

Ellagic acid of *Fragaria nubicola* (Rosaceae) is also responsible for antioxidant activity [9]. Antioxidants are associated with reduced risk of cancer and cardiovascular diseases [10] and many other ailments [11]. Antimicrobial and anti-inflammatory properties of *Fragaria* fruit extracts [12, 13] are consistent with the folkloric use as remedy for skin diseases and wounds.

Based on the earlier reports on antioxidant phytoconstituents present in *Fragaria nubicola* fruit. Therefore, in this study it was decided to investigate the effects of *Fragaria nubicola* fruit juice on ischemia reperfusion induced brain injury by studying the antioxidant effect in relationship with neurobehavioural and biochemical parameters of brain.

MATERIALS AND METHODS

Drugs and chemicals used

Fresh fruit of *Fragaria nubicola* (Strawberry) were homogenised using a domestic mixture for 10 min and filtered through eight layers of muslin cloth.

Animals

Eight-ten weeks old Albino Wistar rats of either sex weighing 225-250g were used in the present study. The animals were maintained under controlled conditions of temperature (23 ± 20 °C), humidity (50 ± 5%) and 12-h light-dark cycles. All the animals were acclimatized for ten days before the study. They had free access to standard pellets as basal diet and water ad libitum. Animals were acclimatized to laboratory conditions for 48 h before the start of the experiment to minimize if any of non-specific stress.

Experimental protocol

The complete experiments were carried out in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee (IAEC) the approval no. was CPCSEA/IAEC/P'col-10/2010-11/26, dated 11th December 2010. Effective steps were adopted to minimize algesia and distress with animal experimental procedures to the animals. The animals were anesthetized with ketamine (50 mg/kg) injected intra-peritoneally. A small incision was made on the neck region and the muscles of the neck were retracted for finding the common carotid artery. The carotid artery was further isolated and 30 minutes of ischemia was given to the animal by blocking the left internal branch of the common carotid artery with micro-vascular clip (bulldog clamp). After the complete ischemic period, the neck muscles were stitched and an antimicrobial cream was applied. The juice of *Fragaria nubicola* was administered to the respective group of animals described below for a period of eight days. The animals were divided into four groups, each group comprised 05 animals.

Group1 (sham) animals served as normal control and received vehicle (saline 10ml/kg, p.o.) for 8 days.

The ischemia reperfusion was produced in groups 2 to 4. After the induction of 30 minutes ischemia and reperfusion.

Group 2 animals (Toxic control) were administered with vehicle (saline, 10ml/kg, p.o. for 8 days).
Group 3 animals were administered with standard drug (Vitamin E, 50 mg/kg, p.o. for 8 days).
Group 4 animals were administered with fresh fruit juice of *Fragaria nubicola* (10 ml/kg, p.o. for 8 days). The saline, fruit juice of *Fragaria nubicola* and vitamin E were administered orally with the help of oral gavage needle. Seven additional days of post ischemic survival time were provided. On the 7th and 8th days neurobehavioural studies was carried out. On the 8th day after completion of behavioural tests the animals were sacrificed by an overdose of ketamine (75 mg/kg, i.p.). The brains were isolated and frozen for biochemical tests.

Neurobehavioral Test

Neurodeficit score

The neurological status of the animals was evaluated using the methods described by Bederson et al [14]. Accordingly, four different categories of neurological findings were observed and reported: 0 = no observed neurological deficit; 1 = contralateral forelimb flexion with wrist flexion and shoulder adduction; 2 = reduced resistance to lateral push; and 3 = circling movements towards the ipsilateral side.

Rota rod test
Sensorimotor performance was evaluated using a Rota Rod Test. The animals of all groups were tested for their ability to remain on the rotating bar at a speed of 22 revolutions per minute (rpm) on a Rotarod apparatus. Each animal was trained for a minimum of three trials. After 8 post ischemic days the animals were tested for motor impairment after administration of test drug. Latency to fall off from the rotating rod was noted for each trial with a 5 min maximum to termination of the trials.

Hanging wire
All the animals were suspended by their forelimbs on a wire stretched between 2 poles, 45 (centimetres) cm above a thick sponge sheet. The time in seconds (sec), until the animal fell down, was recorded. 2 min of cut off time was allotted to conduct this test. This task was taken as a measure of grasping ability and forelimb strength.

Beam walk test
Beam walk test was undertaken to evaluate fore and hind limb motor co-ordination. A single animal was individually placed on a beam walk apparatus made up of a wooden bar 60 cm in lenght and 1.5 cm in width and 50 cm in height. The motor performance of all the animals was scored on scale ranging from 0 to 4. The test was a exclusive test for animals subjected to cerebral ischemia and reperfusion. For motor incordination, No. of foot slip; No. of falls; distance travelled along beam was studied and recorded.

Biochemical Estimation
For this part of the study 6 animals per group were used. After completion of the neurobehavioural tests, the animals were sacrificed with an overdose of an anaesthetic and the brain were isolated. All six brains were used for biochemical tests. The individual brain were homogenized and used for various biochemical tests.

Preparation of Post Mitochondrial Supernatant (PMS)
The brain tissues were homogenized in chilled potassium phosphate buffer (50mM, pH 7.4) using a Potter-Elvehjem homogenizer. The homogenate was centrifuged in a refrigerated centrifuge at (10,500 rpm) for 20 minutes at 4°C to obtain the PMS, which was used for several of enzymes such as catalase, superoxide dismutase and nitric oxide.

Catalase activity was assayed in the post mitochondrial supernatant by the method of Clairborne (1985) [15] Superoxide dismutase (SOD) level was measured by the method of Marklund (1985) [16]. The total protein was estimated by Lowry's et al (1959) method [17]. Nitric oxide was estimated by an indirect measurement of nitrite, nitrate and total nitrite in rat brain extract supernatants obtained after centrifugation. The total nitrite contents of the sample were measured from the calibration curve for nitrite. The total nitrite was estimated by the method of Green et al (1982) [18]. The marker of lipid peroxidation i.e. malondialdehyde (MDA) in the homogenate was determined by the method of Ohkawa et al (1979) [19].

Statistical Analysis
Neurobehavioral data was analysed by analysis of variance (ANOVA) followed by post Dunnett's test. With the help of Graph Pad Instat followed by Dunnett's test at level of significance $P < 0.05$ value. All data were shown as the mean ±SEM. Statistical analysis was performed using Instat Graph Pad version 3 statistical software (USA).

RESULTS

Behavioral result
The animal model of ischemia was established by temporary blocking of the carotid artery for 30 min (ischemia) followed by reperfusion and it leads to sudden loss of vision, memory, coordination and balance. The cognitive and neurological status of the animals was assessed by neurobehavioral test.

Fragaria nubicola improves neurodeficit score and motor performance in ischemic rats
Neurobehavioural tests such as neurodeficit scoring, pole fall and hanging wire results showed impairment in neurobehavioural scale in group 2 ischemic rats (Table 1). The neurobehavioural outcomes [such as neurological deficit score, grasping ability ($P < 0.05$), forelimb strength ($P<0.05$), and motor function ($P < 0.05$) were significantly improved by administration of fresh fruit juice of *Fragaria nubicola* and vitamin E as compared to vehicle treated group 2 animals. Statistical non significant improvement was observed in rotarod test by *Fragaria nubicola* and vitamin E treatment as compared to group 2 (Table. 1).

Fragaria nubicola improves biochemical changes in ischemic rats
The Catalase activity was significantly increased by *Fragaria nubicola* ($P<0.01$) and vitamin E ($P<0.01$) treatment as compared to group 2. The superoxide dismutase activity was decreased in vehicle treated group 2 as compared to

sham control (P<0.01). Whereas treatment with *Fragaria nubicola* significantly increased the superoxide dismutase activity (P<0.01) in the brain tissues. The total nitrite level was elevated in group 2 ischemic rats. *Fragaria nubicola* treatment significantly reduced (P<0.01) the increased total nitrite level as compared to group 2. *Fragaria nubicola* treatment also reduced the lipid peroxidation as compared to group 2 rats (P<0.01) (Table. 2)

Table. 1. Effect of fresh fruit juice of *Fragaria nubicola* and Vitamin E on neurobehavioral tests in ischemia-reperfusion injury in rats.

Group no.	Group name	Neurodeficit mean score	Rotarod (mean fall in time in secs)	Hanging wire (mean fall time in secs)	Beam walk test (secs)
1	Sham (control)	0	6.298 ±0.996	86.3 ± 2.977	1.3±0.2000
2	I/R + Vehicle	2.7	4.006±0.4141**	49.3 ± 2.155**	3.7±0.2440**
3	I/R + Vitamin E	1.5	4.732±0.3461$^{\#}$	62.1 ± 1.289$^{\$}$	1.8±0.2469$^{\$}$
4	I/R + *Fragaria nubicola*	1.4	4.598±0.3238$^{\#}$	74.0 ± 1.789†	1.6±0.2449$^{\$}$

Fragaria nubicola and Vitamin E improves the neurobehavioural outcomes in ischemic rats. Neurobehavioural tests such as neurodeficit scoring, pole fall and hanging wire results showed impairment of neurobehavioural deficit in ischemic rat. The neurobehavioural outcomes (such as neurological deficit score, grasping ability, forelimb strength, and motor function) of these rats showed a significant improvement by *Fragaria nubicola* and vitamin E treatment. The data mentioned is of n=5 in each group and the Values are mean ± S.E.M. Data were analyzed by ANOVA followed by Dunnetts test. (**) indicates a significant difference for Ischemia group vs sham control group at p<0.01. '$' and '†' indicates a significant difference for treated group vs. ischemic group at p<0.05 and p<0.01 respectively. And '#' indicates non-significance.

Table. 2. Effects of fresh fruit juice of *Fragaria nubicola* and Vitamin E on Catalase, SOD, Nitric oxide and MDA in brain tissue of ischemia-reperfusion injury in rats.

Group no.	Group name	Catalase nmol of H_2O_2 consumed/min/mg protein	SOD U/mg protein	Nitric oxide (µmols)	MDA (nmol of MDA gm^{-1} tissue)
1	Sham (control)	109.69± 3.09	22.37± 1.58	29.54 ± 0.451	6.06 ± 0.135
2	I/R + Vehicle	87.67 ± 2.89**	12.30± 0.68**	65.42 ± 2.658**	14.09 ± 0.307**
3	I/R + Vitamin E	198.21 ± 3.18†	17.27± 0.43$^{\#}$	25.79 ± 0.371†	7.71± 0.174†
4	I/R + *Fragaria nubicola*	162.96±3.35†	19.04± 0.65†	21.46 ± 0.402†	8.18± 0.237†

The Catalase activity was significantly increased by *Fragaria n* and vitamin E treatment as compared to group 2. The superoxide dismutase activity was decreased in vehicle treated group 2 as compared to sham control. Whereas treatment with *Fragaria n* (10 ml kg^{-1} orally) significantly increased the superoxide dismutase activity. The total nitrite was elevated in group 2 ischemic rats. *Fragaria n* treatment reduced the increased total nitrite as compared to group 2. *Fragaria n* treatment also reduced the lipid peroxidation as compared to group 2 rats. The data mentioned is of n=5 in each group and the values are mean ± S.E.M. Data were analyzed by ANOVA followed by Dunnett's test. (**) indicates a significant difference for Ischemia group vs. sham control group at p<0.01.'$' and '†' indicates a significant difference for treated group vs. ischemic group at p<0.05 and p<0.01 respectively. And '#' indicates non-significance.

DISCUSSION

Ischemia is the deficiency of blood in a part, usually due to functional constriction or actual obstruction of blood vessel and reperfusion injury refers to tissue damage caused when blood supply returns to the tissue after a period of ischemia. Brain stroke is a sudden loss of brain function usually caused by a blockade or leakage of a blood vessel. It develops from a complex cascade of cellular events that ultimately leads to cerebral infarction [20] and causes sudden loss of vision, balance, co-ordination, speech and memory [21].

The present study was performed to assess the cytoprotective effect of fresh fruit juice of *Fragaria nubicola* on ischemia reperfusion brain injury. The damage was produced by blocking carotid artery for 30 minutes with the help of microvascular clip followed by reperfusion. Reperfusion of ischemic tissue result in the formation of toxic ROS including, superoxide anions (O_2^-), hydroxyl radicals (OH^-), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2) and nitric oxide-derived peroxynitrite radicals. These toxic ROS induces oxidative stress [4]. Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the capability of antioxidant defences, such as catalase and SOD enzymes. Severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis. ROS can cause cellular damage by oxidizing membrane lipids, essential cellular proteins and DNA [22].

Fragaria nubicola (strawberry) is a fruit grown in India also. *Fragaria nubicola* is rich in ellagic acid and phenolic compounds. The earlier reports supports that the strawberry has potent antioxidant activity [10, 11] it may be proved as neuroprotective against ischemia-reperfusion induced brain injury, so this fruit was chosen for present study. The neuroprotective effect of *Fragaria nubicola* fruit juice and vitamin E as reference was tested in ischemic rats. In this study, we confirm that the fruit juice dose of *Fragaria nubicola* significantly improved the ischemia induced neurological status, forelimb strength, balance and co-ordination i.e. motor performance, neurodeficit score, rotarod test, hanging wire test, beam walk test. The results obtained in this study are supported by a recently published study where *F.limonia* produced the positive effects in ischemia reperfusion brain injury due to its antioxidant potential [23].

The observation of this study clearly shows a protective role of *Fragaria nubicola.* Several other herbal drugs obtained from medicinal plants have also shown the potential neuroprotective effect in rodents [24-25].

In the present study the biochemical investigations reveals that *Fragaria nubicola* significantly increased the catalase and superoxide dismutase enzyme activities, which indicates that *Fragaria nubicola* may decrease the formation of free radicals. Also *Fragaria nubicola* reduced the total nitrite and malondialdehyde which is the marker of lipid peroxidation, suggesting that the antioxidant potential of *Fragaria nubicola* may have decreased the formation of oxygen radicals and further prevented the vicious chain reaction. The finding in this study is supported by the previous studies where curcumin, resveratrol, silymarin, green tea extract containing antioxidant potential have counteracted the ROS generation during ischemia-reperfusion induced brain injury. The phytoconstituents present in *Fragaria nubicola* are ellagic acid and phenolic compounds, which have been already proved to be potent antioxidant. Hence, in this study the neuroprotective effects of *Fragaria nubicola* fruit were observed.

Hence, we suggest that *Fragaria nubicola* fruit juice may be useful in the treatment of stroke and may prove to be neuroprotective. On the basis of our findings, we conclude that *Fragaria nubicola* have improved the ischemia-reperfusion induced neurological deficit, decrease in motor performance on hanging wire and beam walk test and by exerting the antioxidant effects.

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