INTRODUCTION
Cerebral ischemia-reperfusion (I/R) injury is the leading cause of death worldwide. The current therapeutic drug of choice is tissue plasminogen activator (tPA). But, its therapeutic approach is limited due to time constraint. Intensive research has been diverted on the development of traditional herbal medicines for effective therapeutic approach. The severity of I/R injury is examined symptomatically by assessing neurobehavioral severity. The severity is measured using a battery of tests like neurological deficit score, grip strength score and adhesive tape removal test. The neuronal damage is quantified by assessing infarcted area, which was measured by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining. SBT treated rats showed substantial improvement in neurobehavioral deficits, significantly inhibited the increased levels in glutamate and calcium, significantly prevented the associated neuronal damage. From our results, it can be concluded that supplementation of SBT significantly mitigated the ischemia-reperfusion induced neuronal damage by inhibiting excitotoxicity.

Keywords: Ischemia/reperfusion, Neurobehavioral deficit, Willow leaved Sea buckthorn, Neuroprotection

MATERIAL AND METHODS
Plant Material and extraction
Willow leaved Sea buckthorn berries powder was obtained from Organic Changsha Herb Inc. China. The berries were subjected to extraction of flavonoid fraction. The percentage yield was found to be 4.57 % w/w.
Measurement of glutamate levels
The glutamate levels were measured according to the method described by Bernt and Bergmeyer\textsuperscript{13} with minor modifications. To 1 ml of supernatant, 2 ml of perchloric acid was added and the pH was adjusted to 9.0 with phosphate buffer. The resulting mixture was subjected to centrifugation at 1500 × g for 15 minutes and was allowed to stand for 10 minutes in an ice bath and then filtered through fluted filter paper. Absorbance was measured at 340 nm. The glutamate levels are expressed as μmol/g tissue.

Measurement of total calcium levels
The total calcium levels were measured according to the manufacturer’s instructions using commercially available kits (Span Diagnostics Ltd., India).

Cerebral infarct area by 2, 3, 5-triphenyltetrazolium chloride staining
After reperfusion for 22 h, rats were decapitated under anesthesia with Ketamine (100 mg/kg) and Xylazine (10 mg/kg) and the brains were kept at ~20°C for 40 minutes. Frozen brain was sliced into uniform coronal sections of about 2 mm thickness each. Brain slices were incubated with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) in 0.2 mol/L phosphate buffer (pH 7.4) at 37°C for 30 minutes and fixed in 10% neutral buffered formalin for overnight. The unstained areas of the fixed brain sections were defined as infarcted. After 24 h of fixation, the slices are photographed. The infarct area was calculated by measuring the unstained and stained areas in each slice, multiplying this by the slice thickness (2 mm).

RESULTS
Effect of SBT on neurobehavioral changes
The animals were scored for neurologic deficit after 22 h of reperfusion using a five point scale. The MCAO model group showed considerably increased (P < 0.001) neurologic scores as compared to the sham group. SBT pretreated animals showed substantial improvement in neurologic deficit score (P < 0.001) in a dose dependent manner as compared with model group (Table 1). The tape removal test is a technique that assesses sensory and motor impairments in forepaw function. After 2 h MCAO/22 h reperfusion, an increase in the time needed to remove adhesive tape from the contra lateral forepaw was observed in the MCAO group as compared with sham group rats (Table 1). Interestingly, dose dependently, SBT pretreated animals showed significant shortened time to remove the adhesive tapes from the forepaws compared with a MCAO group (P < 0.001). The grip strength was found to be significantly decreased (P < 0.001) in the MCAO group as compared to the sham group. Whereas significantly (P < 0.001) improved grip strength was observed in SBT pretreated groups dose dependently as compared to MCAO group (Table 1).

Effect of SBT on glutamate and calcium levels
A significant increase (P < 0.001) in glutamate and calcium levels was observed in MCAO groups as compared to the sham group (Table 2). The SBT pretreated group offered significant restoration of glutamate and calcium levels in comparison to MCAO group (P < 0.001)

Effect of SBT on Infarct area
Figure 1 showed typical pictographs of TTC stained coronal sections of sham, MCAO and SBT pretreated rats. MCAO group showed significant increase in infarct area as compared with Sham group. SBT pretreatment showed a significant reduction in the infarct area as compared with MCAO group. The percentage of infarction is calculated and given in Table 3.
DISCUSSION

The present study employed preliminary screening of neuroprotective effect of flavonoid fraction of willow leaved sea buckthorn in transient focal cerebral ischemia-reperfusion induced by employing MCAO model. Since the focal cerebral ischemia model with transient MCAO followed by reperfusion in experimental animals is generally accepted as the most appropriate model for human stroke$, we used the MCAO reperfusion model to induce ischemic injury. SBT exhibited neuroprotection, as evident from improvement in the neurobehavioral deficit, reduction in infarct volume, calcium and glutamate levels. Cerebral ischemia reperfusion injury produced significant impairments in the neurological function and coordination, impairing the sensory-motor system. Motor sensory deficits are evaluated based on a neurological scale. It is observed that flavonoid fraction of SBT showed significant improvement in the neurological scoring compared to the ischemic control group suggesting the efficacy of the SBT flavonoid fraction in reversing the MCAO induced cerebral ischemia reperfusion injury. Ischemia leads to the release of cellular toxic mediators and increases the permeability of the blood brain barrier. Hyper permeability of the BBB leads to brain cellular swelling and causes brain infarction and edema$. Triphenyltetrazolium chloride (TTC) staining has been employed in the present study to determine the area of infarction in brain tissue. TTC is a water soluble dye that is reduced to formazone by the enzyme succinate dehydrogenase and cofactor NAD, present in mitochondria and stain viable tissue deep red in color. Ischemic tissue with damaged mitochondria remains unstained. Present investigations revealed that pre-treatment with SBT flavonoid fraction offered effective protection dose dependently compared to neuronal damage induced by MCAO, which could be the one of the protective mechanism of SBT against I/R injury. Excitotoxicity through over activation of NMDA receptors by the extensive release of glutamate is well established as an important trigger to the tissue damage in focal cerebral ischemia. Activation of NMDA receptors elevates the influx of Ca$^{2+}$ and that of non-NMDA glutamate receptors promotes the influx of Na$, both of which can lead to membrane depolarisation. In turn, depolarisation can activate plasma membrane voltage-dependent Ca$^{2+}$ channels, leading to additional Ca$^{2+}$ influx. Furthermore increased calcium activates enzymes, such as xanthine oxidase and NOS that are involved in the ROS generation leading to lipid peroxidation and neuronal damage$. In the present study, the glutamate levels were estimated, pre-treatment with flavonoid fraction of SBT effectively reduced the excitotoxicity by decreasing the glutamate levels thereby reducing the tissue damage. This ability of flavonoid fraction of SBT to reduce the glutamate induced tissue damage has contributed to the neuroprotection from the cerebral ischemia reperfusion injury. Calcium released due to excitotoxicity and depolarized neurons and glia due to energy depletion, enters into damaged neurons through voltage-gated calcium channels, via NMDA and AMPA receptor operated channels, and also from intracellular stores. Cytoplasmic calcium activates enzymes and second messenger cascades that contribute to cell death. Activated proteolytic enzymes break down elements of the

| Table 1: Effect of SBT on neurobehavioral changes after I/R injury |
|-----------------|-----------------|-----------------|
| Groups | Neurological deficit score | Grip strength score | Adhesive tape removal time (sec) |
| Sham | 0.02 ± 0.00*** | 3.57 ± 0.03*** | 19.12 ± 6.28*** |
| MCAO | 3.03 ± 0.04*** | 1.42 ± 0.10*** | 126.11 ± 20.33*** |
| SBT 250 mg/kg | 1.13 ± 0.06*** | 1.83 ± 0.06*** | 60.27 ± 7.45*** |
| SBT 500 mg/kg | 1.29 ± 0.09*** | 2.99 ± 0.04*** | 41.39 ± 5.52*** |

Values are expressed as mean ± SEM [n = 6]; *(P < 0.05), **(P < 0.01), ****(P < 0.001) vs sham group; ++*(P < 0.05), ++*(P < 0.01), +++*(P < 0.001) vs MCAO group

| Table 2: Effect of SBT on glutamate and calcium levels |
|-----------------|-----------------|-----------------|
| Groups | Glutamate levels (µg/mg protein) | Calcium levels (µg/mg protein) |
| Sham | 18.26 ± 0.03*** | 0.818 ± 0.02*** |
| MCAO | 38.99 ± 0.08*** | 2.147 ± 0.10*** |
| SBT 250 mg/kg | 30.97 ± 0.09*** | 1.363 ± 0.06*** |
| SBT 500 mg/kg | 21.47 ± 0.06*** | 1.098 ± 0.04*** |

Values are expressed as Mean ± SEM [n = 6]; *(P < 0.05), ***(P < 0.001) Vs Sham group; ++*(P < 0.01), +++*(P < 0.001) vs MCAO group

| Table 3: Effect of SBT on percentage of infarcted area |
|-----------------|-----------------|-----------------|
| Groups | Percentage of infarcted volume |
| Sham | 0.36 ± 0.02 |
| MCAO | 43.62 ± 0.07*** |
| SBT 250 mg/kg | 24.82 ± 0.04** |
| SBT 500 mg/kg | 2.24 ± 0.01*** |

Values are expressed as Mean ± SEM [n = 3]; *P < 0.05, ***P < 0.001 Vs Sham group; ++P < 0.01, +++P < 0.001 vs MCAO group

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cytoskeleton, leading to protein aggregation. Calcium-mediated lipolysis damages membranes and along with nitric oxide synthase activation provides nitric oxide and fatty acid substrates for free radical production. Apoptotic cascades are stimulated by the rise in calcium through mitochondrial permeability. Glutamate release is stimulated by calcium-dependent excitotoxicity and the released glutamate in turn causes Ca\(^{2+}\) channels to open, leading to further Ca\(^{2+}\) overload forming a vicious cycle. Thus calcium plays a unique role in the ischemic pathophysiology. In the present study results demonstrated that calcium levels were elevated in Ischemic control group compared to sham group rats which is in consonance with the earlier reports. A prominent reversal of MCAO induced elevation in calcium levels by SBT flavonoid fraction may also be attributed to its protective effects. It can be concluded that flavonoid rich fraction of SBT significantly ameliorated the ischemia-reperfusion induced neurobehavioral deficit, excitotoxicity. This preliminary report provides the scientific basis for the in-depth evaluation of neuroprotective mechanism of willow leaved sea buckthorn berries.

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