Phloretin attenuates neuronal damage associated with transient focal cerebral ischemia in rats
Introduction

Ischemic stroke is one of the major cause of death and the most frequent cause of permanent adult disability worldwide, affecting up to 0.2% of the world population every year (Lakhan et al., 2009; Hyun-Jung et al., 2011). The resulting burden on the society continues to grow with increase in the incidence of stroke. Stroke is a cerebrovascular disorder characterized by insufficient supply of oxygen and glucose in cerebral arteries thus damaging the physiological activity of the brain, leading to an intricate pathophysiological event ultimately causes irreversible neuronal injury in the ischemic core within minutes of the onset (Dirnagl, 1999). The early clinical features of stroke include: sudden weakness or numbness of the face, arm, or leg; sudden dimness or loss of vision, particularly in one eye; difficulty speaking; sudden severe headache with no known cause; unexplained dizziness, unsteadiness, or sudden falls (Fawcett and Asher, 1999). There are two major types of stroke include ischemic and hemorrhagic. Ischemic stroke is the most common, making up approximately 85% of all cases (Lloyd-Jones et al., 2009). The complex pathobiological mechanisms of cerebral ischemia reperfusion injury, include excitotoxicity, oxidative damage, inflammation, and apoptosis (Kumar and Dogra, 2008; Ozbal et al., 2008; Yousuf et al., 2009).

Oxidative stress from reactive oxygen species (ROS) has long been considered as the major cause of tissue injury following cerebral ischemia (Chan, 2001; Uttara et al., 2009), which results in the damage of cellular macromolecules and participates in signaling mechanisms that lead to cell death (Kelly et al., 2008). In normal brain tissue, the production of reactive oxygen species (ROS), such as superoxide anion radical, hydrogen peroxide, hydroxyl radical, and peroxinitrite anion, is balanced by endogenous enzymes (superoxide dismutase, glutathione peroxidase, catalase) and nonenzymatic (glutathione, vitamins C and E) antioxidative defenses. After transient cerebral ischemia, antioxidant enzymes system are debilitated by ROS, which fail to protect neurons from oxidative damage (Broughton et al., 2009). ROS can cause cell death by nonphysiological (necrotic) or regulated pathways (apoptotic), as for ischemic damage, it is generally accepted that apoptosis plays a pivotal role in cell death (Broughton et al., 2009). The mechanisms by which ROS causes apoptotic cell death, includes receptor activation, Bcl-2 family proteins, caspase activation, and mitochondrial dysfunction (Kametsu et al. 2003; Ryter et al., 2007). Mitochondria produce low levels of ROS in a process known as oxidative phosphorylation through the electron transport chain (ETC). The ETC consists of five protein complexes (I-V), and a disruption of this electron transport system leads to excess generation of ROS (Numakawa et al., 2011). In addition, mitochondria regulate Ca\(^{2+}\) homeostasis by sequestering excess cytosolic Ca\(^{2+}\) into their matrix (named Ca\(^{2+}\) loading). However, an uncontrolled Ca\(^{2+}\) loading may be involved in neurodegeneration.
Animal models of filamentous reversible middle cerebral artery occlusion (MCAO) have been developed to improve the understanding of deleterious mechanisms involved in the brain ischemic damage, and to identify the potential efficiency of therapeutic strategies. These Animal models of MCAO have been used extensively because it closely mimics the changes that occur during and after human ischemic stroke. Most human ischemic strokes are caused by occlusion of the middle cerebral artery (MCA) and so animal models were developed to induce ischemia in this arterial territory.

It has been a major challenge to develop effective therapeutics for stroke. Over the past few years, much attention has been focused on flavonoids, a group of phenolic compounds that have been reported to reduced reactive oxygen species (ROS) mediated reactions and rescue neurons from ischemia-reperfusion-induced neural loss in animal models of cerebral ischemia (Ahmad et al., 2011; Al-Majed et al., 2006). Phloretin is a dihydrochalcone flavonoid found exclusively in green apples. It is present as free and its glucosidic form, phloridzin (phloretin 2′-O-glucose) (Shao et al., 2008; Jugde et al., 2008). It has many biological and pharmacological properties, such as potent antioxidant activity in scavenging radicals, inhibition of lipid peroxidation (Vasantha and Yasmin, 2010), antiproliferative effects (Rezk et al., 2002). However, little is known about the protective mechanism by which phloretin rescues cells from oxidative stresses. Therefore, the purpose of this study was to examine the effects of Phloretin administration on neurologic deficits and biomarkers of oxidative stress in rat model of focal cerebral ischemia. We hypothesized that Phloretin supplementation would ameliorate oxidative damage, improve behavioural activities, and suppress neuronal loss.

**Experimental Procedure**

**Chemicals and reagents**
As described in material and methods, chapter-II

**Animals and treatments**
As described in material and methods, chapter-II

**Drug administration and dose selection**
We examined the effects of different doses of phloretin on cerebral ischemia reperfusion injury in pilot studies to determine the optimal dose of phloretin that provides the most neuroprotection against degeneration. A dose of 10 mg/ kg of phloretin was selected. This dose has shown maximal protection in different types of diseases (Wu et al., 2009; Yang et al., 2009). On the basis of these findings, rats were pretreated systemically with 10 mg/ kg phloretin p.o. dissolved in 0.3 % sodium carboxymethyl cellulose, once daily for 10 days. On day 11, MCAO was performed for 2 h and reperfusion for 22 h.
Middle cerebral artery occlusion (MCAO) to induce focal cerebral ischemia

As described in material and methods, chapter-II

Post-operative care
As described in material and methods, chapter-II

Experimental design
To investigate the neuroprotective effects of phloretin in an experimental model of cerebral ischemia, we used the rat MCAO model (Longa et al 1989). Animals were divided into four groups each having eight animals. The first group served as sham (S) and vehicle was given orally, the second was MCAO i.e. ischemia was induced for 2 h followed by reperfusion for 22 h, the third was pretreated for 10 days with phloretin (10 mg/ kg, orally) followed by MCAO for 2 h and reperfusion for 22 h, Ph + MCAO and the fourth was pretreated for 10 days with phloretin alone, i.e., phloretin group (10 mg/ kg, orally, Ph + Sham). After the completion of the reperfusion period, the animals were assessed for neurobehavioral activity and then sacrificed. The brains were taken out to dissect the hippocampus and frontal cortex for biochemical estimations.

Behavioral studies
The behavioral test in each group was performed before and after occlusion and reperfusion. The experiment was performed between 9.00 A.M. to 4.00 P.M. at standard laboratory conditions. All tests were performed and analyzed by subject blind to the experiment.

Rota rod (muscular coordination)
As described in material and methods, chapter-II

Grip Strength
As described in material and methods, chapter-II

Evaluation of ischemic damage

Infarct volume analysis
As described in material and methods, chapter-II.

Biochemical studies

Tissue preparation for the assays
After behavioral study, the animals were sacrificed and their brains were taken out to dissect hippocampus and frontal cortex for the biochemical assays (TBARS, G SH, GPx, GR, GST, G6-PD and SOD) as described in material and methods, chapter-II.

Histopathological examinations
After 24 h of MCAO, the animals were anesthetized with chloral hydrate (400 mg/ kg, i.p.) and perfused as previously described by Nakayama et al. (1998). The brains were cut into 10-μm-
thick coronal sections on crystat (Leica, Germany). Every 10 sections of the cortex region, was mounted on glass slides, and processed for hematoxylin and eosin staining.

**Immunohistochemistry for Apaf-1 and caspase-9**
As described in material and methods, chapter-II.

**Genomic DNA extraction and gel electrophoresis**
As described in material and methods, chapter-II.

**Caspase-3 activity**
As described in material and methods, chapter-II.

**Determination of protein**
Protein was determined by the method of Lowry et al. (1951) using BSA as a standard.

**Statistics**
As described in material and methods, chapter-II.

**Results**

**Effect of phloretin on behavioral output**

**Rota Rod**
The muscular coordination skill showed no neurological deficits in sham group rats, while in MCAO group the neurological deficits were severe at 22 h after reperfusion. Phloretin alone did not show any change in the behavioral assessment as compared to sham group. In muscular coordination skill, a significant (p<0.01) improvement was observed in the Ph + MCAO group in staying on the accelerating rod for a longer duration of time as compared to MCAO group (Fig. 1).

![Graph](image.png)

Fig. 1, Effect of phloretin treatment on muscular coordination skill in MCAO rats. MCAO leads a significant depletion in motor coordination as compared with sham group and significantly recovered in Ph-treated MCAO group (Ph + MCAO) as compared with MCAO group. Values are expressed as means ± S.E.M for 8 animals. *p<0.01, MCAO vs sham; #p<0.01, Ph + MCAO vs MCAO.

**Grip Test**
The grip strength was found to be significantly decreased (p<0.01) in MCAO group as compared to sham group. A marked improvement (p<0.01) in grip strength was observed in phloretin pretreated group (Ph + MCAO) as compared to MCAO group animals (Fig. 2). However, no significant alteration was observed in phloretin pretreated sham group (Ph + Sham) as compared to sham group.
Effect of phloretin on TTC stain and infarction volume

Brain infarction is an important index for estimating neuronal damage with subsequent neurological impairment which is an important determinant in assessing the consequences of cerebral ischemia. TTC staining of brain sections obtained from MCAO rats showed reproducible and readily detectable lesions in the infarct area at 24 h after the reperusions (Fig. 2). The lesions were present in hippocampus, lateral striatum and the overlying cortex. We hypothesized that phloretin play a protective role in stroke. It was observed that phloretin pretreatment significantly decreased (p<0.01) the infarct volume as compared to MCAO group. There was no significant difference in the infarct volume between sham and Ph + Sham group.

Effect of phloretin on endogenous antioxidant system

Phloretin treatment decreased the TBARS level in hippocampus and frontal cortex

The level of TBARS was elevated significantly (p<0.05) in MCAO group rats as compared to sham group. Rats of Ph+MCAO group has exhibited significant decrease in TBARS level in hippocampus (p<0.05) and frontal cortex (p<0.05) as compared to MCAO group (Fig. 3). Phloretin alone pre-treated sham group (Ph+Sham) showed no significant changes in TBARS level as compared to sham group.
Phloretin treatment restored the GSH level in hippocampus and frontal cortex

Protective effect of phloretin on GSH level in hippocampus and frontal cortex was observed. The level of GSH was depleted significantly in hippocampus and frontal cortex in MCAO group as compared to sham group. Phloretin pretreatment has restored its level significantly (p<0.05) in Ph + MCAO group as compared to MCAO group. Phloretin alone pretreated group (Ph + Sham) exhibited no significant changes in GSH level as compared to Sham group (Fig. 4).

Phloretin treatment attenuated the activities of antioxidant enzymes in the hippocampus and frontal cortex

The activities of antioxidant enzymes (GPx, GR, GST, G6-PD and SOD) were depleted significantly in hippocampus and frontal cortex of MCAO group animals as compared with...
sham group animals. Phloretin pretreatment significantly increases (p<0.01) the activity of antioxidant enzyme as compared with MCAO group animals. No significant change was observed in Ph+ Sham group as compared to Sham group (Tables 1, 2).

### Table 1 Activities of antioxidant enzymes (GPx, GR, GST, G6-PD and SOD) in hippocampus of MCAO rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sham</th>
<th>MCAO</th>
<th>Ph+MCAO</th>
<th>Ph+Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR (nmol of NADPH oxidized/ min/ mg protein)</td>
<td>294.35±26.05</td>
<td>168.33±9.59* (-42.81%)</td>
<td>255.06±9.47# (51.52%)</td>
<td>293.20±18.51 (-0.05%)</td>
</tr>
<tr>
<td>GPx (nmol of NADPH oxidized/ min/ mg protein)</td>
<td>228.37±7.96</td>
<td>135.29±6.26 * (-40.75%)</td>
<td>176.83±7.38# (30.70%)</td>
<td>229.69±9.23 (0.57%)</td>
</tr>
<tr>
<td>GST (nmol of CDNB conjugate formed/ min/ mg protein)</td>
<td>540.1 ± 1.8</td>
<td>219.9 ± 1.7* (-59.28%)</td>
<td>329.2 ± 1.5# (49.70%)</td>
<td>544.2 ± 1.8 (0.75%)</td>
</tr>
<tr>
<td>G6PD (nmol NADP reduced/ min/ mg protein )</td>
<td>83.45±4.72</td>
<td>40.12±2.78* (-51.92%)</td>
<td>74.32±3.46# (85.12%)</td>
<td>84.50±5.23 (0.26%)</td>
</tr>
<tr>
<td>SOD (nmol of epinephrine protected from oxidation/ min/ mg protein)</td>
<td>456.68±22.56</td>
<td>249.30±15.65* (-45.41%)</td>
<td>351.83±27.53# (41.12%)</td>
<td>457.30±20.73 (0.13%)</td>
</tr>
</tbody>
</table>

Values are expressed as mean± S.E. Significance was determined as *p<0.01 when compared with sham group; #p<0.01 when compared with MCAO group. Values in parentheses show the percentage increase or decrease with respect to their control.

### Table 2 Activities of antioxidant enzymes (GPx, GR, GST, G6-PD and SOD) in frontal cortex of MCAO rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sham</th>
<th>MCAO</th>
<th>Ph+MCAO</th>
<th>Ph+Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR (nmol of NADPH oxidized/ min/ mg protein)</td>
<td>310.61±21.26</td>
<td>170.82±10.52 * (-45.00%)</td>
<td>259.03±13.16# (51.63%)</td>
<td>309.06±16.86 (-0.49%)</td>
</tr>
<tr>
<td>GPx (nmol of NADPH oxidized/ min/ mg protein)</td>
<td>239.82±8.25</td>
<td>136.22±5.45 * (-43.21%)</td>
<td>180.05±7.58# (32.36%)</td>
<td>240.05±12.10 (041%)</td>
</tr>
<tr>
<td>GST (nmol of CDNB conjugate formed/ min/ mg protein)</td>
<td>604.2 ± 2.1</td>
<td>354.4 ± 1.6* (-41.34%)</td>
<td>574.2 ± 1.3 (62.02%)</td>
<td>607.6 ± 1.2 (0.43%)</td>
</tr>
<tr>
<td>G6PD (nmol NADP reduced/ min/ mg protein )</td>
<td>85.46±3.98</td>
<td>43.80±2.36* (-61.21%)</td>
<td>75.69±3.18# (56.36%)</td>
<td>86.12±4.90 (0.60%)</td>
</tr>
<tr>
<td>SOD (nmol of epinephrine protected from oxidation/ min/ mg protein)</td>
<td>478.90±23.06</td>
<td>251.26±19.79* (-47.53%)</td>
<td>365.39±30.34# (45.42%)</td>
<td>480.28±26.55 (0.28%)</td>
</tr>
</tbody>
</table>

Values are expressed as mean± S.E. Significance was determined as *p<0.01 when compared with sham group; #p<0.01 when compared with MCAO group. Values in parentheses show the percentage increase or decrease with respect to their control.
Morphological changes

Effect of phloretin pretreatment on histopathological changes in MCAO rats

Histopathologic changes associated with ischemia/reperfusion injury were investigated by hematoxylin-eosin staining. Sections of the brain passing from frontal cortex of MCAO group, Ph + MCAO group and sham group were examined. The sections of the sham group showed normal cell with no pathologic change, whereas the sections of the MCAO group showed a focus of brain damage with neuronal loss and presence of numerous vacuolated spaces. Intact neurons were absent in that area. The corresponding area in the sections from Ph + MCAO group showed partial neuronal loss with presence of intact neurons in between the vacuolated spaces. Phloretin treatment ameliorated neuronal abnormalities in Ph + MCAO group as compared to MCAO group animals (Fig. 5)

Effects of phloretin on Apaf-1 and Caspase-9 expression

The activation of Apaf-1 protein is associated with neuronal cell death in cerebral ischemia. Apaf-1 expression was found to be remarkably high in ischemic hemisphere of the MCAO group rats (Fig. 8B). A noticeable reduction in Apaf-1 expression was observed in phloretin
pretreated Ph + MCAO group as compared to MCAO group (Fig. 8C). Expression of Apaf-1 was seen to be very scarce in sham group (Fig. 8A). Phloretin pretreatment did not show any remarkable effects in Ph + sham group compared with the sham group (data not shown). The expression of Caspase-9 was increased strongly in the ischemic hemisphere of MCAO group rats. In Ph + MCAO group, Caspase-9 expression was decreased as compared to MCAO group (Fig. 9C). Phloretin pretreatment did not show any remarkable effects on Ph + sham group as compared with sham group (data not shown).

Fig. 6. Representative coronal brain sections of the sham, MCAO, and Ph+MCAO group rats stained for Apaf-1 and Casp-9. Cortical neuronal area of brain from the MCAO group (B) animals showing significant increase in Apaf-1 and Casp-9 expression as compared with the sham group (A), and this expression was decreased in the Ph+MCAO group (C) as compared with MCAO group. Original magnification ×20.
Effect of phloretin on Caspase-3 activity

Caspase-3 activity was significantly increased in MCAO group as compared to sham group and decreased significantly with phloretin administration (Fig. 7). Phloretin pretreatment did not show any remarkable effects in Ph + Sham group compared with Sham group.

![Graph showing Caspase-3 activity](image)

**Fig. 7** The activity of caspase-3 was significantly increased in the MCAO group rats as compared to sham group rats (*p<0.01 MCAO vs. sham). Ph pretreatment significantly decreased the activity of caspase-3 in the Ph+MCAO group rats as compared to MCAO rats (#p<0.05, Sil+MCAO vs. MCAO group) Values are expressed as mean ± SEM (n=8).

Effect of phloretin on DNA fragmentation

DNA damage is a hallmark of apoptotic cell death. Fig. 8 shows the electrophoretic migration of genomic DNA isolated from cerebral cortex of various groups. In sham group no DNA fragmentation was observed in cortex, whereas MCAO group rats resulted in a shearing pattern of DNA fragmentation (Lane 2). However, phloretin treated animals showed a marked decrease in DNA fragmentation in Ph + MCAO group (Lane 3) when compared with MCAO group.

![Representative agarose gel electrophoresis](image)

**Fig. 8** Representative agarose gel electrophoresis exhibits DNA fragmentation in cortex following MCAO. Lane 1, DNA isolated from frontal cortex after sham operation. Lane 2, DNA isolated from frontal cortex after MCAO. Lane 3, pre-treatment of animals with Ph before MCAO resulted in no apparent DNA shearing DNA obtained from frontal
Discussion

In the present study, we evaluated the effects of phloretin in a rat model of focal cerebral ischemia/reperfusion injury. We used the middle cerebral artery occlusion (MCAO) model with reperfusion that mimics many features of the stroke in humans, since the middle cerebral artery (MCA), which is the specific occlusion site in this model, is the most commonly affected vessel in both embolic and thrombotic strokes in humans (Longa et al., 1989; Saleem et al., 2006; Yousuf et al., 2009; Khan et al., 2010). It is well documented that MCAO results in behavioral, neurochemical, and histologic alterations in rat brain and generation of free radicals has been implicated to be one of the major causative factors (Chan, 2001; Raza et al., 2011). The major findings of present study were the significant improvement of the functional outcome, reduces the oxidative damage, and suppresses the neuronal loss due to its potential antioxidant property. It lowers the early accumulation of lipid peroxidation products and enhances the activity of antioxidant enzymes and eliminated the sequential apoptosis responses. Oxidative stress has been shown to plays a critical role in the pathogenesis of cerebral ischemia, which is associated with an increased production of free radicals, specifically hydroxyl radical, superoxide, higher lipid peroxidation and lower enzymatic antioxidant defenses (Allen and Bayraktutan, 2009; Chen et al., 2011). The above damages can be prevented by detoxification of free radicals. Beneficial effects of various antioxidants and free radical scavengers in ischemic stroke have been demonstrated in a number of studies (Andrabi et al., 2004). Our results suggest that pretreatments with phloretin offer better neuroprotection due to its powerful antioxidant properties, corroborating previous studies (Crespy et al., 2002; Lee et al., 2003; Hassan et al., 2007).

Behavioral parameters are useful measures of functional deficits following experimental focal cerebral ischemia and the degree of sensorimotor dysfunction as an important indicator of severity of the injury (Fukui et al., 2002; Saleem et al., 2006; Khan et al., 2009). In the present study, we demonstrate that phloretin which is a potent antioxidant, have improved neurobehavioral outcome significantly in phloretin-pretreated animals (Ph+MCAO) by scavenging free radicals. Earlier studies have shown an improvement in various behavioral outputs like motor coordination skill and grip strength as a result of antioxidant treatment (Salim et al., 2003, Yousuf et al., 2005; Khan et al., 2010; Raza et al., 2011). We observed that the motor coordination skill and the grip tests were severely altered in the ischemic group (MCAO group). This may be because cerebral ischemia results in increased free radical generation leading to oxidative stress in hippocampus and cortex regions that have command over motor activities. Our findings correlate well with the earlier studies carried out by us and others where motor
deficits have been attenuated by treatment with antioxidants (Khan et al., 2009; Raza et al., 2011; Ahmad et al., 2011).

Morphometric investigation of infarct volume is frequently evaluated by measuring the infarcted area in the brain, which is an important determinant of the extent of ischemic brain injury following focal cerebral ischemia (Lin et al., 1993). Infarct volume is commonly employed measure to concur on the neuroprotective efficacy of the flavonoids in preclinical trials. To identify infarcted areas we employed TTC staining. In the present study, the MCAO group showed a prominent infarct size which was ameliorated significantly with phloretin pre-treatment. Studies have revealed that flavonoids administered following ischemia are effective in reducing infarct volume (Khan et al., 2009; Shah et al., 2010; Yamazaki et al., 2010; Raza et al., 2011).

Free radicals induced oxidative stress has a profound effect in biochemical alteration are well documented in ischemia reperfusion brain injury (Fukui et al., 2002). Oxidative stress culminates due to an imbalance between prooxidants and antioxidants and consequent excessive production of reactive oxygen species, which cause damage to cell structures including membranes lipids, proteins, and DNA (Allen and Bayraktutan, 2009). However, damage to cellular structure can be prevented by scavenging radicals, which ultimately prevent the progress of lipid peroxidation (LPO). We have examined high content of LPO in the form of TBARS along with depleted content of glutathione (GSH), which are in harmony with the previous study (Zafar et al., 2003; khan et al., 2009; Raza et al., 2011). G6-PD is a metabolic enzyme responsible for the maintenance of intracellular reduced GSH content, was also depleted. Activity of G6-PD has been reported to decrease in ischemic conditions (Sarkar and Das, 2006). However, pre-treatment with phloretin resulted in the reversal of the elevated LPO level and up-regulated the depleted content of GSH and G6-PD when compared with the MCAO group intimating the decreased formation of ROS or radical scavenging activity of phloretin after ischemia reperfusion injury. Furthermore, reduced GSH is the primary line of defense against ROS (Seema et al., 2011). GSH is consumed by glutathione peroxidase enzyme during H$_2$O$_2$ elimination and therefore in an environment where there is oxidative stress, intracellular GSH content is depleted. GSH and other thiol-containing proteins are important in cellular defense against ischemic damage. Since GSH content reflects the cellular physiological response, the effect of phloretin on these two biochemical indices suggest that phloretin not only functions as a simple antioxidant to inhibit lipid peroxidation but also as a modulator of cellular antioxidant potential by the restoration of GSH, which is an oxy-radical scavenger.

ROS are frequently scavenged by antioxidant enzymes, primarily by superoxide dismutase. Superoxide dismutase catalyzes the dismutation of superoxide into oxygen and hydrogen.
peroxide (Zelko et al., 2002). The hydrogen peroxide formed by SOD and by other processes is converted into water by GPx. GPx uses hydrogen peroxide to oxidize GSH. Thus, GPx is a low molecular-weight antioxidant and plays a key role in detoxifying hydrogen peroxide (Ahmad et al., 2011). GST catalyzes the detoxification of oxidized metabolites of catecholamine (o-quinone) and may serve as an antioxidant system preventing degenerative cellular process (Baez et al., 1997). The ischemia-reperfusion causes an overproduction of free radicals which, in turn, causes oxidative damages to membrane's lipid and protein, and ultimately leads to a decrease in the content of GSH and activity of its dependent enzyme (GPx, GR and GST) activity along with SOD. This oxidative neuronal damage in ischemic brain insult is consistent with previous reports (Chan, 2001). However, our finding suggest that phloretin pretreatment significantly counteracted all the alterations in the markers of oxidative damage; this occurs often due to scavenging and neuroprotective properties of phloretin which was consistent with the previous findings (Eberhardt et al., 2000; Rezk et al., 2002; Nakamura et al., 2003). Besides shielding against oxidant stress, another existing and encouraging finding is that phloretin significantly attenuated histologic changes, that caused minimal glial cell infiltration, less neural damage with small vacuolated space, along with presence of intact neuron in the neuronal tissue as compared with ischemic cortical neuronal loss.

Apoptosis is a form of programmed cell death that has been implicated in various kinds neurodegenerative disorders (Bredesen et al., 2006). It has been well-known that apoptosis is one of the major neuronal cell death mechanisms in the experimental model of cerebral ischemia (Shin et al., 2006; Wang et al., 2009; Broughton et al., 2009). Following cerebral ischemia the major executioners in the apoptotic program are proteases known as caspases, which have a pivotal role in the progression of ischemic cell death. Induction of apoptosis-activating factor-1 (Apaf-1), resulting the activation of caspase-9, followed by the activation of effector caspase-3 leading to apoptotic cell death. Induction of Apaf-1 and caspase-3 activation is associated with stroke-induced apoptotic neuronal cell death (Dohare et al., 2008; Brahma et al., 2009) and inhibition of Apaf-1 expression and caspase-inhibition reduced ischemic injury (Chen et al., 2009; Luo et al., 2009). These data are consistent with our findings that phloretin reduced the expression of Apaf-1 and caspase-9 accompanied with activation of caspase-3. These findings are in conformity with the previous findings where antioxidants were used to ameliorate the neurodegenerative diseases (Chong et al., 2003; Diaz-Ruiz et al., 2008). Our finding showed that elevations of caspase-3 activity and Apaf-1, caspase-9 expressions occurred after ischemic injury and these expressions could be significantly concealed by phloretin pre-treatment.
Conclusion

In brief, the major findings of the present study suggest that the neuroprotective effect of phloretin on rat focal cerebral ischemia model is probably mediated by the improved neurobehavioral outcome and prevent oxidative damage followed by the inhibition of apoptosis responses. Thus, these findings suggest that phloretin is a substitute for treating the ischemic stroke. Further studies to comprehend the neuroprotective potential and mechanisms of antioxidant action of phloretin is essential to resolve, whether it can be an effective remedy for ischemic stroke.