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Research article

Oral administration of baicalein-enriched fraction during pre- and post-ischemic stroke alleviated cognitive impairments in rat models

Farah Amna Othman, Nik Nur Hakimah Nik Salleh and Suat Cheng Tan*

School of Health Sciences, Health Campus, University Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

* **Correspondence:** Email: tansc@usm.my; Tel: +609-7677776; Fax: +609-7677515.

Abstract: In recent years, numerous studies have discovered the potential of natural compounds extracted from medicinal plants as a promising alternative treatment for ischemic stroke (IS). *Oroxylum indicum* is among the plants that has been widely proven to contain a beneficial flavonoid compound known as baicalein. However, the therapeutic potential of this compound for IS disease has yet to be demonstrated. In this study, a baicalein-enriched fraction (BEF) was harvested from the leaves of *O. indicum* and tested on neural stem cells (NSCs) to determine its effects on the cell viability and gene expression prior to an in vivo animal study. NSCs were chosen because they are the primitive brain cells that play important roles in neurogenesis after IS injury. It was found that NSCs treated with BEF at concentration of 3.125 µg/mL for 48 hours showed a significant higher cell proliferation rate. Moreover, these cells expressed high NSC markers (Nestin and SOX2) and genes responsible for antioxidant (SOD2) and angiogenesis (ANGPT1) mechanisms, indicating that the BEF treatment could enhance the progenitor brain cells viability and the expression of cytokines that are beneficial to reduce oxidative stress and reinstate blood flow in an ischemic brain. Subsequently, the in vivo therapeutic effects of BEF for IS disease was tested by administering 50 mg/kg b.wt of BEF to male Sprague Dawley (SD) rats via oral gavage before and after induction of IS. Assessments of neurological impairments were performed using a modified neurological severity score (mNSS) and the quantification of the total infarct volume was evaluated as the endpoint of this study. Results showed that an oral administration of BEF improved the behavioral scoring for motor functions (forelimb flexion and forelimb twisting), contralateral sensory functions (paw-whiskers test), motor coordination, balance functions (beam balance test) and reflex functions (pinna, corneal, startle and tail reflex) within 24–72 h; alternatively, the non-treated rats showed continuously lower scores for all the tests throughout the study, indicating that the BEF-treated rats exhibited a faster recovery rate as compared

to the non-treated rats. Such improvements were observed up to two weeks. In addition, histological assessment also revealed a reduction of infarct volume in the BEF-treated rats as compared to the control rats. In summary, the present study demonstrated the potential of BEF extracted from the *O. indicum* as a supplement to improve preclinical IS models.

Keywords: Ischemic stroke; Oral administration; *Oroxylum indicum*; baicalein-enriched fraction; Natural compound

1. Introduction

Ischemic stroke (IS) is a major type of stroke that is caused by occlusion of the cerebral artery, which lead to a permanent loss of neuronal cells and neurological functions. The main pathophysiological events of brain ischemia include a series of complex mechanisms starting from energy failure, ionic imbalance, acidosis, increase intracellular calcium levels, generation of free-radical mediated oxidative stress, disruption of blood brain barrier (BBB), inflammatory cytokine release, and ischemic-reperfusion injury, thereby leading to cell apoptosis and severe neurological damage in the ischemic core region [1]. Currently, early restoration of blood flow to the ischemic areas using recombinant tissue plasminogen activator (rt-PA) remains the standard treatment for IS [2]. However, due to its narrow therapeutic window (<4.5h), high re-incidence rate and short half-life (<5 min) [3], the application of rt-PA is limited. Hence, many researchers are exploring other safer, multi-targeted and more effective therapeutic approaches to tackle this disease, especially using natural products.

Recently, there has been an enormous emphasis on the development of novel neuroprotective agents from natural resources with a higher efficacy and lower side effects to prevent neuronal cell death after IS. Among many of studies conducted on plant-based resources, *Oroxylum indicum* is one of the potential plants that has numerous therapeutic properties. This medium-sized plant originates from the Bignoniaceae family and can be commonly found in the tropical forests of Southeast and Asian region, including Malaysia. *O. indicum* is a medium sized tree, typically reaching heights to 8–15 meters, with branches at the top (Figure 1). The bark of this tree is light brown, soft, and contains green juice, often exhibiting numerous corky lenticles. The leaves of the tree are pinnate, measuring approximately 3–7 cm in length, with opposite pinnae and a very stout, cylindrical rachis. The leaflets are typically in 2 to 4 pairs, measuring 6–12 cm in length and 4–10 cm in width, with an ovate or elliptic shape and acuminate tip (Figure 1A). The tree produces a large number of foetid flowers that are reddish purple on the outside and pinkish yellow on the inside, which grow in erect racemes (Figure 1B and C). The fruit pod of *O. indicum* has an elongated sword-like shape, and its capsules contain yellowish-green seeds that are kidney-shaped and are surrounded by a light-brown papery wing (Figure 1D) [4].

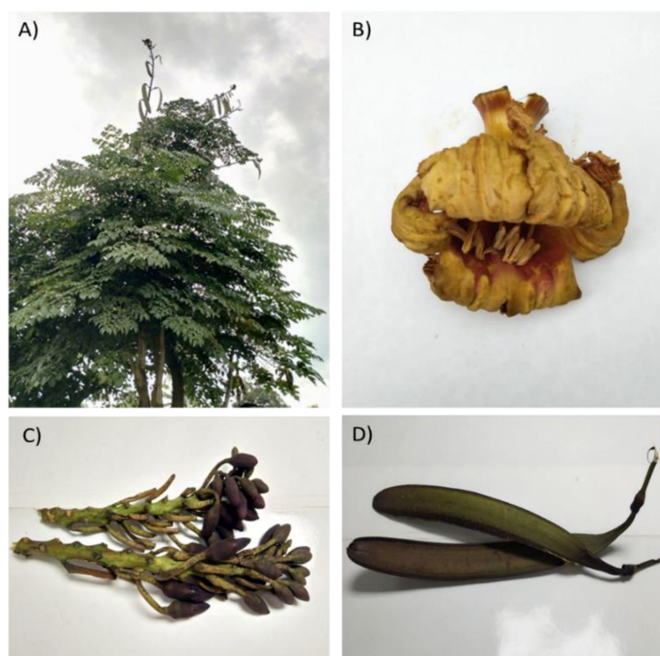

Figure 1. Representative images of A) tree, B) blooming flower, C) efflorescence flower and D) fruit pod of *O.indicum*.

O. indicum plant extracts have been traditionally used as herbal medicines to treat diarrhea, snake bites, smallpox, scabies, rheumatism and as a sex stimulant [5]. The documented medicinal value of this plant lies in some of its bioactive constituents such as alkaloids, polyphenolics, flavonoids, glycosides, tannins and terpenoids [6,7]. Among the various compounds extracted from this plant, flavonoids, particularly baicalein, has been claimed as one of the most prominent components for neurological diseases [8]. The medicinal therapeutic actions of baicalein have been reputed in many neurodegenerative studies, where it was reported to ameliorate behavioral seizures [9], attenuate apoptosis and enhance neurite outgrowth in various cell line and animal studies [9–11]. Previous research suggested that the protective effects of this compound across a wide range of neurodegenerative diseases was due its ability to cross BBB [12]. In addition, owing to its antioxidant property, baicalein also exhibits an ability to reduce oxidative stress. This can directly alleviate the factors that may exacerbate the effects of ischemic injury, which ultimately confers neuroprotection of the ischemic brain [13].

Despite baicalein has been known to be beneficial in treating many diseases, to our knowledge, there is still no study to assess the effectiveness of this compound for IS treatment. Therefore, in this study, the baicalein was extracted from the leaves of *O. indicum* in the form of baicalein-enriched fraction (BEF) and tested on IS rat models to determine its efficiency in conferring protection against ischemic injury. Besides, the present study also presented an in vitro cytotoxicity test prior to the in vivo animal model test in order to ensure that BEF was inherently safe for animal consumption before initiating the animal test. In this study, neural stem cells (NSCs) were chosen as the cell model because the NSCs are the most important primitive brain cells. The cells possess a high potential to mitigate stroke pathology due to their unique ability to differentiate into matured neuronal cells, such as neurons, astrocytes, and oligodendrocytes to repopulate lesion site after the ischemic injury [14]. Recently, there are many studies reported that IS patients undergo natural endogenous neurogenesis at the ischemic

penumbra region of the brain, mostly due to the proliferation of the primitive NSCs [15–17]. Considering that NSCs play significant roles in neurogenesis after the IS injury, as well as they are the progenitor cells of all other neuronal cells, therefore, it is more practical and important to determine the cytotoxicity effects of BEF on NSCs prior to the animal study when compared to other cell types. Last but not least, this study also examined the expression of several useful genes that have potential to regulate/induce recovery from ischemic injury, such as the superoxide dismutase 2 (SOD2), which is a key antioxidant enzyme that can protect the brain tissue against oxidative damage [18], and angiopoietin (ANGPT1), which is best known for its ability to promote the formation of blood vessels [19,20]. In brief, this is a novel study to investigate the therapeutic potential of a neuroprotective compound known as baicalein from the leaves of the *O. indicum* plant to treat the IS disease. The data presented in this study is important to develop BEF as a novel alternative drug to enhance clinical treatment for IS in future.

2. Materials and methods

2.1. Preparation of baicalein-enriched fraction (BEF)

O. indicum leaves were collected from Kampung Pasir Parit, Pasir Mas, Kelantan, Malaysia (LGPS coordinate: latitude 5.905471, longitude 102.1884469). The collected plant material was authenticated and deposited into the herbarium of University Sains Malaysia (USM) with the voucher specimen no. 11751. The crude extract of *O. indicum* was prepared following an established protocol by Kang et al. [13], using a binary solvent procedure. Briefly, a fine powder of *O. indicum* leaves was loaded into a Soxhlet extractor, and gently boiled with petroleum ether until the solvent in distillation flask turned clear. The solvent was discarded and the same extraction procedure was repeated using methanol until the solvent in distillation flask turned clear. The collected solvent was dried using rotary evaporator (Buchi AG, Flavil, Switzerland), weighed and stored at 4 °C until further use. The enrichment of baicalein from the crude extract was prepared based on previous protocols with minor modifications [13]. In brief, a volume of 400 mL mobile phase (10% methanol) was used to equilibrate the Diaion HP20 resin column (Mitsubishi Chemical Corporation, Tokyo, Japan). Then, the slurry suspension from the mixture of crude extract and methanol was loaded onto the top of the resin column. The suspension was then eluted with a gradient mixture of methanol and distilled water to obtain BEF in the final eluted fraction. The collected BEF solvent was dried using rotary evaporator, weighed and stored at 4 °C until further use.

2.2. In vitro BEF treatment on rat fetal neural stem cells (NSCs)

A rat fetal NSC line was purchased (N7744100, Gibco™ USA) and cultured based on the manufacturer protocol. The effects of BEF on the NSC proliferation and viability were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder assay (Invitrogen™, USA). Briefly, rat fetal NSCs were plated at a seeding density of 5×10^4 cells per cm^2 on a CELLStart™ coated culture vessel. The cells were allowed to adhere for at least 24 hours in a CO_2 incubator at 37 °C. Then, during the next day, the medium was replaced with an equal volume of pre-warmed complete StemPro™ NSC SF. The cells were cultured until 70% confluency, before it was preconditioned with BEF at concentrations of 0.75, 1.56, 3.125, 6.25, 12.5, 25, 50, 75 and 100 µg/mL. Three different sets of treatments were performed for 24, 48 and 72 hours. All experiments were carried

out in triplicate. Meanwhile, non-preconditioned rat NSCs were cultured in normal NSC SFM complete medium without the addition of BEF for the same duration. After 24 hours, 48 hours and 72 hours, 20 µL of 5 mg/mL MTT was added to each well and the 96-well plates were wrapped with aluminium foil before incubated in the humidified incubator at 37 °C for 4 hours. The medium was then discarded and 100 µL of absolute DMSO was added into each well to dissolve the blue formazan crystals formed by viable cells. The plate was shaken on an orbital shaker for 15 minutes. The optical density (OD) of the suspension was measured at 570 nm using microplate reader (Bio-Rad, USA). Half-maximal inhibitory concentration (IC50) of each solvent was determined using graph of viable cells percentage against the log10 concentration (mg/mL) of the extract. The percentage of viable cells was determined according to the following formula:

$$Cell\ viability\ (\%) = \left(\frac{OD\ of\ sample}{OD\ of\ negative\ control}\right) \times 100\%$$

2.3. RNA extraction and real-time PCR

Total RNA was isolated from a harvested cell pellet using the RNeasy mini extraction kit (Qiagen, Germany). The RNA concentration was determined using a Nanodrop^{TM} UV-visible spectrophotometer (ND-1000; Thermo Fisher Scientific, USA). Subsequently, the RevertAid^{TM} H Minus Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was used to initiate the synthesis of first-strand cDNA. Quantitative real-time PCR was conducted with the CFX96 Real-Time PCR detection system (Bio-Rad) using qPCRBIO SyGreen Mix (PCR Biosystem, USA), following the manufacturer's instructions. For gene-specific amplification, the following primer pairs were designed using NCBI Primer BLAST: Nestin, 5'-TGG AGC AGG AGA AGC AAG GT -3' and 3'-CCT CCA GCA GAG TCC TGT ATG T -5', SRY-box transcription factor 2 (SOX2), 5'-CAC ATG AAG GAG CAC CCG GA -3' and 3'-CCT CCG GGA AGC GTG TAC TT -5', Angiopoietin 1 (ANGPT1), 5'- TCA GTG GCT GCA AAA ACT TGA GA -3' and 3'-ATG GTG GCC GTG TGG TTT TG -5', Superoxide dismutase 2 (SOD2), 5'-TGG CGG GGG CCA TAT CAA TC -3' and 3'- CTC CAG CAA CTC TCC TTT GGG T -5', Hypoxanthine phosphoribosyltransferase 1 (HRPT1), 5'- AGT CCC AGC GTC GTG ATT AGT-3' and 3'- CGA GCA AGT CTT TCA GTC CTG TC-5', and Ribosomal protein L13A (RPL13A), 5'-GCT GCC GCA CAA GAC CAA AA-3'. The PCR protocol involved an initial heat activation step at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s and an extension at 65 °C for 20 s. Data analysis was performed using the comparative threshold cycle (Ct) method, and the results were expressed as fold differences, which were normalized to the housekeeping genes HRPT1 and RPL13A [21].

2.4. Animals sample size calculation

The animal experiment was performed using 9–10 weeks old healthy adult male Sprague Dawley (SD) rats, obtained from the Animal Research and Service Centre (ARASC) with body weight range between 250–300 g (n = 5/group), USM Health Campus. The animal sample size per group was calculated using the PS Power Software (version 3.0) based on reliable data obtained from a related previous study [22]. All the experimental procedures were performed in accordance to the guidelines

and approval by the USM Institutional Care and Use Committee (IACUC). [Ethical number: USM/IACUC/2019/(120(1019)].

2.5. In vivo BEF treatment on SD rats

Ten male SD rats were randomly assigned into two groups: control group and treated group (n = 5 per group), in accordance with the previously established methods for IS treatment, where the rats given oral administration of plant extract [23]. Rats in the control group were given an oral administration of 10% DMSO diluted with saline (10 mL/kg), while treated group received 50 mg/kg of BEF extracted from *O. indicum*. The vehicle solvent and BEF were administered 1 hour before and after the induction of IS. Throughout the experimental period, the general behavior and body weights of the rats were recorded daily, with special attention given on the days where ISs were induced.

2.6. Ischemic stroke (IS) induction

All the experimental rats were anesthetized with 100 mg/kg intraperitoneal (i.p.) ketamine and 5 mg/kg i.p. xylazine. A 21-gauge stainless steel guide cannula was stereotaxically implanted into right middle cerebral artery (1.6 mm anterior and 5.2 mm lateral to bregma) according to the surgical protocol developed by Ansari et al. [24]. The body temperature of the rats was maintained at 37 ± 0.5 ℃, 1 and 2 h after surgery using a heating pad. Then, the rats were individually housed after the surgery on a 12 hour day/night cycle at a temperature of 18–22 ℃ and allowed to recover for 24 h before the induction of IS. After 24 hours, recovered conscious rats were subjected to IS induction using perivascular administration of endothelin-1 (ET-1) (American Peptide Company, Inc. CA, USA) (60 ρmol in 3 µL of saline) via the implanted cannula guide. Then, the rats were placed in a clear box for behavioral observation to determine their stroke severity for 60 minutes.

2.7. Assessment of neurological behaviours

The behavioral evaluation of neurological deficit scores was conducted after induction of IS (Day 0), followed by evaluations on Day 1, 2, 3, 7 and 14 (n = 5 for each group). The detailed procedures for each of the tests are listed as follows.

2.7.1. Motor test

Motor tests were conducted using forelimb flexion and forelimb twisting [25]. For forelimb flexion, rats were suspended 10 cm above the bench top by catching the tail for approximately 5 s. Rats that extended both forepaws towards the ground was considered normal (score 0). Contralateral forepaws of rats that had damaged brains may not reach the ground; on other hand, flexion to the contralateral side could be observed with a certain degree: slight flexion (score 1), 45° flexion (score 2), 90° flexion (score 3). Score 3 usually accompanied with obvious and severe twisting of thorax. For forelimb flexion test, rats were also suspended 10 cm above the bench top by catching the tail for approximately 5 seconds, where any twisting of thorax (defined as rats reaching up toward the tail) was scored. Rats that reached for the ground were considered normal with no neurological deficit (score 0). Rats that had brain damage on their striatum or cortex may show a slight wobble to

contralateral side (score 1), the occasional twisting up towards the tail (score 2) or twisting all the way up to the tail (score 3).

2.7.2. Contralateral sensory test

A contralateral sensory test was conducted using the vibrissae-evoked forelimb placing test, also known as the paw-whiskers test [26]. This test was originally developed by Barth' and Stanfield, where they used modified Neurological Severity Scores (mNSS) with a slight alteration. The animals were held by the trunk above the hindquarters to allow their forelimbs to move freely. The rats were then oriented on the left side of the body (contralateral side) towards the wall, with ipsilateral forepaw restrained, and slowly moved until the vibrissae brushed against the wall. A successful placement of the contralateral forepaw for every 10 trials was recorded.

2.7.3. Motor coordination and balance test

Motor coordination and integration of motor movement for balance was assessed using beam balance test developed by Doeppner et al. [27] with minor adjustments. Before the test was conducted, all the experimental rats were trained for at least three days to traverse the beam successfully. The beam walking apparatus was made with the measurement of 3 cm wide, 70 cm long, 5 mm thick and 20 cm height. The rats performance was rated as follows: traversed the beam without any slips of the hindlimb (score 0); only one slip of the hindlimb (score 1); traversed the beam with more than one foot slip, but less than 50% (score 2); traversed the beam with more than 50% foot slips of the affected hindlimb (score 3); tried to traverse the beam, but fell (score 4); did not move, but was able to stay on the beam (score 5); and was not able to stay on the beam without falling (score 6).

2.7.4. Reflex test

The reflex test was evaluated using ear, eye, sound and tail reflex developed by Yarnell et al. using the Revised Neurobehavioral Severity Scale (NSS-R) with slight modifications [28]. For ear reflex, each auditory meatus (ear) was lightly touched with the cotton end of a long Q-tip and the rats reaction was observed as follows: quick flattening of the ear flap for both ears (score 0); twitching of ear flap or movement away from the stimulus of either ear (score 1); and no response of both ears (score 2). For eye reflex, each eye was lightly touched with the cotton end of a Q-tip. The response of each ear was recorded as follows: a complete and immediate eye blink of both eyes (score 0); a delayed eye blink of either eye (score 1); and no response of both eyes (score 2). For sound reflex, a sharp, short and strong clap of the hands was performed at least 10 to 15 cm away from the rats. The reflex of rats was recorded as follows: A quick jumpy movement followed by freezing (score 0); a slow movement (score 1); and no response (score 2). Lastly, for tail reflex, a strong pinch using the experimenter's fingers was applied between the middle area and base of the rat's tail. To ensure the same force was applied with each pinch, the tail was placed between the knuckles of the index finger and pressed down with the tip of the thumb. The rats response was recorded as follows: a sharp and immediate squeak (score 0); a delayed or weak squeak or turn around (score 1); and response (score 2).

2.8. Measurement of cerebral infarct volume

The rats were decapitated after 14 days of the experimental period, where their brain was carefully removed, cleaned and weighted for next procedure. The measurement of the infarct size of experimental animals was conducted using a 2, 3, 5-triphenyltetrazolium chloride (TTC) stain. The collected brain from each rat was coronally sectioned with a brain slicer (2 mm interval starting from 1 mm of the frontal lobe). Each sliced brain tissue was incubated in a 1% TTC solution for 30 minutes. Then, all tissue sections were transferred and incubated in 10% formalin for 20 minutes. The TTC-stained tissues were observed and measured using Image J program (National Institute of Health, Bethesda, MD) to obtain infarct volume according to the following formula:

$$\text{Percentage of infarct volume of whole brain} = \left(\frac{\text{Total absolute infarct volume}}{2 \times \text{Total contralateral volume}}\right) \times 100\%$$

where:

1) $\text{Total absolute infarct volume} =$

$$\text{Sum of} \left(\begin{matrix}\text{Absolute unadjusted} \\ \text{infarct area}\end{matrix} \times \left(\frac{\text{Left (contralateral)hemisphere area}}{\text{Right (ipsilateral)hemisphere area}}\right) \times 100\%\right) \times 2\ \text{mm}$$

2) $\text{Total contralateral volume} = \text{Sum of contralateral area} \times 2\ \text{mm}.$

2.9. Statistical analyses

All values collected were analyzed using the SPSS software and expressed as mean ± standard error mean (S.E.M). For comparisons between the control and treated groups, the data were analyzed using a T-test. For comparisons of the behavioral scoring within the group, the data were analyzed using a one way analysis of variance (ANOVA). Multiple comparisons were analyzed using the post-hoc Tukey's test. All data with a p value of 0.05 or less ($p < 0.05$) were considered as significant.

3. Results

3.1. Effects of BEF preconditioning on the proliferation and viability of NSCs

Rat fetal NSCs were treated with BEF at serial concentrations ranging from 0 to 100 µg/mL for 24, 48 and 72 hours, to determine the effects of BEF on NSC proliferation and viability. NSCs displayed healthy spindle-like cell morphology, which attached to the culture vessel surface as a monolayer when treated with BEF (Figure 2). NSCs treated with either 3.125, 6.25 or 12.5 µg/mL of BEF for 48 hours showed healthy and confluent cells (Figure 2, indicated by yellow box). Considering that there was no significant difference between the NSCs treated with these three concentrations, 3.125 µg/mL of BEF for 48 hours was chosen as the optimal condition for NSC treatment (Figure 3).

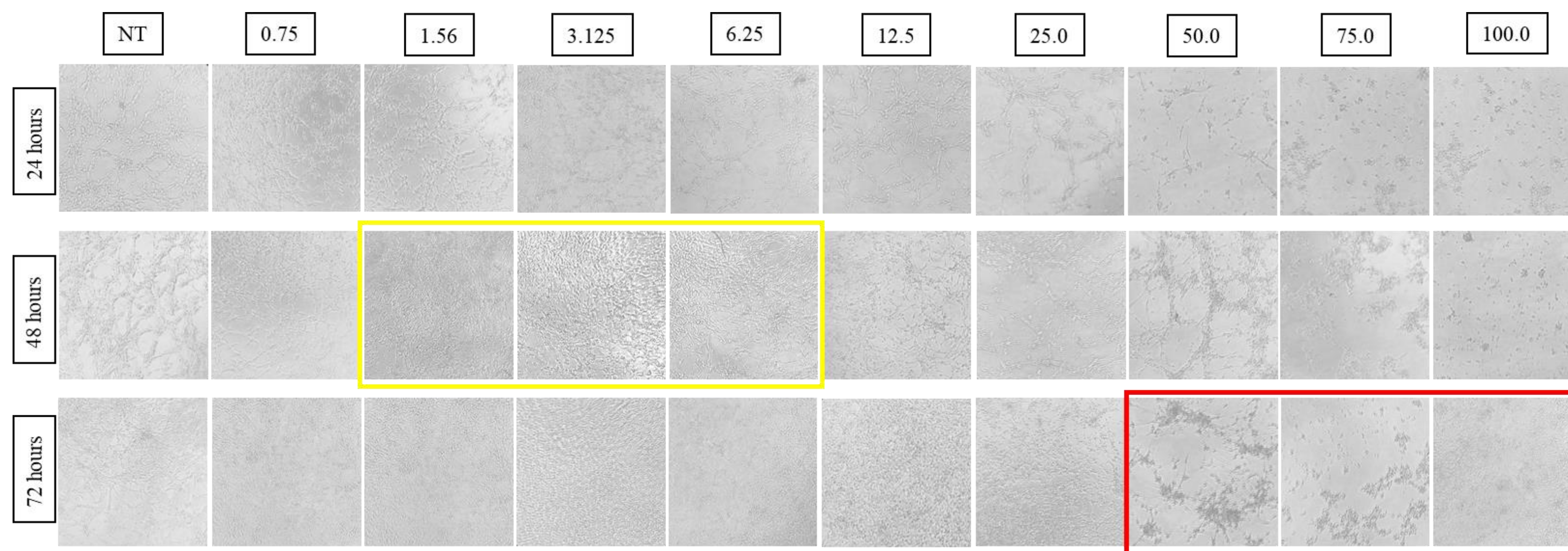

Figure 2. Representative images showing the morphology of NSCs treated with BEF at different concentrations ranging from 0–100 mg/mL at 24, 48 and 72 hours, respectively. For negative control, NSCs were treated with 1% DMSO. The images were captured at 10× magnification. All cells treated with BEF showed healthy except those treated with high concentrations for prolonged duration (as indicated by red box). NSCs treated with either 3.125, 6.25 or 12.5 µg/mL of BEF for 48 hours showed the densest cell morphology compared to other groups (as indicated by yellow box).

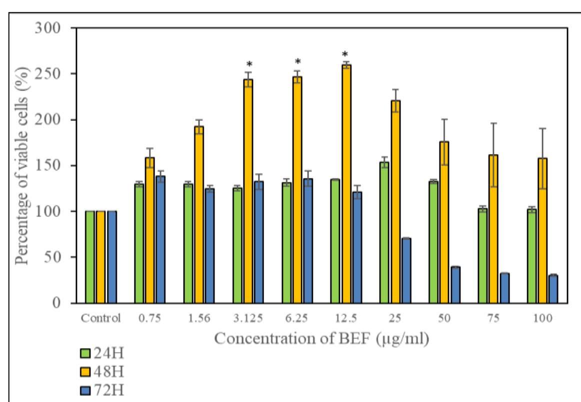

Figure 3. Percentage of viable rat fetal NSC cell line after BEF treatment for 24, 48 and 72 hours. The optimal proliferation rate was achieved with 3.125 µg/mL of BEF within 48 hours. Values represented mean ± SEM. (* indicates $p < 0.05$ vs. other groups).

3.2. Effects of BEF preconditioning on the gene expression of NSCs

The mRNA expression level of NSCs treated with 3.125 µg/mL of BEF for 48 hours was investigated using real time PCR. Results showed that the expression level of NSCs markers (Nestin and SOX2) were not significantly affected by the treatment, thus indicating that the BEF treatment did not alter the stemness of NSCs (Figure 4A and B). Meanwhile, the expression levels of the antioxidant gene (SOD2) of BEF-treated NSCs were significantly upregulated by 3.55-fold as compared to the non-treated cells (Figure 4C). It was also found that the expression level of the angiogenic gene (ANGPT1) was significantly upregulated by 6.28-fold in the BEF-treated NSCs as compared to non-treated NSCs (Figure 4D). Taken together, these data indicate that the BEF treatment could significantly enhance the antioxidant and angiogenesis potential of brain cells without compromising their natural characteristics.

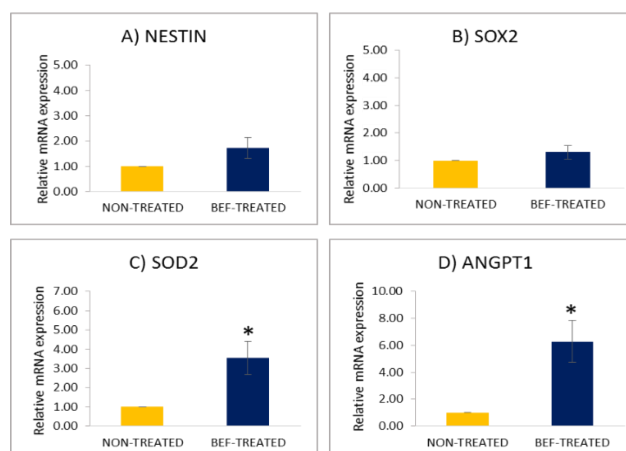

Figure 4. Relative mRNA expression for (a) Nestin, (b) SOX2 and (c) SOD2 and d) ANGPT1 after BEF-treatment. Relative mRNA expression of genes of interest were normalized to HRPT1 and RPL13A as housekeeping genes. (* $p < 0.05$ vs. non-treated group).

3.3. General behaviour after stroke induction and treatment with BEF

In this study, there were no observable deaths or abnormal behaviours such as seizures, myoclonus or epilepsy observed following treatment with BEF. All experimental rats showed a stable weight increment (<20% of initial weight) throughout the 14-day study period, thus indicating the normal growth pattern of the experimental rats (Table 1). Moreover, the body weight changes of the BEF-treated rats were not significantly different than the non-treated control group.

Table 1. Body weight of control and treated rats.

Groups (n=5)	Body weight (g) at Day 0	Body weight (g) at Day 7	Body weight (g) at Day 14	Body weight changes between D0 and D7*	Body weight changes between D7 and D14*
Control	336.00 ± 7.66	338.60 ± 7.95	359.80 ± 4.45	0 to 5 g (0.00 to 1.38%)	6 to 33 g (1.73 to 10.21%)
Treated	342.20 ± 6.27	312.40 ± 19.08	359.80 ± 15.04	-20 to -11 g (3.22 to 5.98%)	32 to 56 g (9.69 to 16.13%)

* All the weekly body weight changes recorded were within normal increment range (< 20% of initial weight).

3.4. Relative organs weight (ROW)

All collected vital organs did not show any morphological and colour changes during the macroscopic examination. Moreover, the ROW values of the brain, heart, lung, liver, kidney and reproductive organs also showed no significant difference between the treated and control groups (Table 2).

Table 2. Relative organs weight of control and treated rats (n = 5).

Organ	**Control group**	**Treated group**
Brain	0.56 ± 0.01	0.55 ± 0.02
Heart	0.37 ± 0.01	0.37 ± 0.01
Lung	0.68 ± 0.07	0.64 ± 0.04
Liver	3.99 ± 0.18	4.36 ± 0.04
Kidney	0.72 ± 0.01	0.70 ± 0.01
Testis	1.00 ± 0.02	0.88 ± 0.05
Epidydemis	0.16 ± 0.01	0.15 ± 0.01

3.5. Neurological deficit score

In this study, behavioral scoring of experimental rats treated with a single administration of BEF pre- and post-stroke induction demonstrated a noteworthy improvement in all the behavioral tests conducted (Figure 5). Firstly, the motor function deficit score was the highest for both groups, with severe forelimb flexion and twisting in both groups of rats on Day 0 (Figure 4A). Notably, the BEF-treated rats started to show an improvement in motor function on Day 2 (0.45 ± 0.05), as compared to the non-treated rats (0.63 ± 0.03). As the experimental period extended over 14 days, the BEF-treated

group displayed a significantly lower deficit score (0.34 ± 0.04), as compared to the control group (0.54 ± 0.06). This indicated that the BEF was effective in reducing the motor function deficit after the IS induction, compared to non-treated group.

On the other hand, motor coordination and balance deficit scores (Figure 5B) were designed to assess the hindlimb function and balance of rats by ability to successfully transverse a beam. The results showed a significant improvement in the BEF-treated rats as early as Day 3; the improvement remained persistent until the end of the treatment period (Day 14), as compared to the control rats, which consistently had a lower performance than the treated rats throughout the experimental period of two weeks.

A contralateral sensory test (Figure 5C) was conducted to assess the successful forepaw placement in rats when their whiskers were brushed against the wall. Notably, the BEF-treated rats exhibited significantly higher contralateral sensory score starting from Day 2 (4.20 ± 0.37 vs. 2.38 ± 0.44); this trend was maintained until Day 7 and 14, as compared to the control rats. Conversely, the control rats had a consistently low contralateral sensory function score throughout the study period.

In this study, the reflex functions were significantly affected after IS induction; however, the affected reflex functions did not exhibit statistically significant improvements in either the control or the BEF-treated rats over the entire experimental period (Figure 5D).

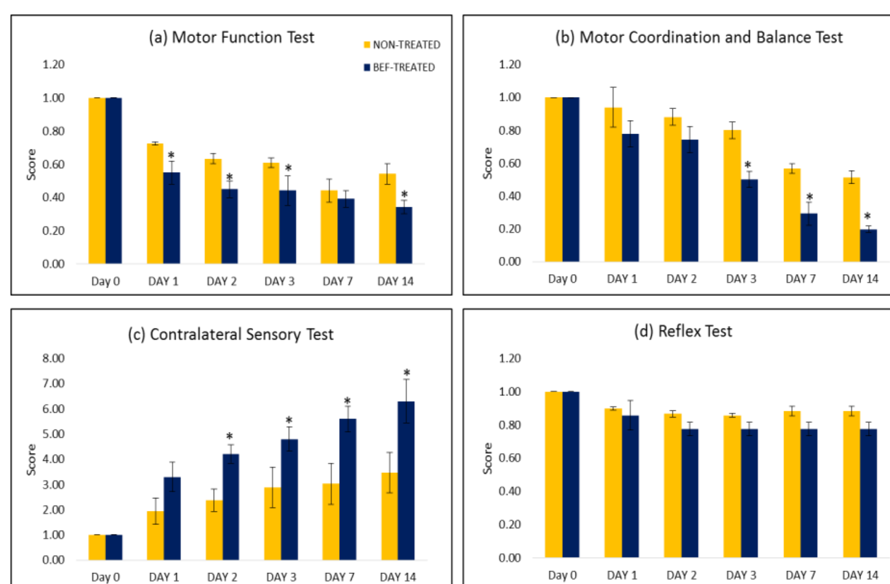

Figure 5. Behavioral scoring of (a) motor function, (b) motor coordination and balance, (c) contralateral sensory and (d) reflex tests of non-treated (control) and BEF-treated IS rats BEF. * $p < 0.05$ vs. control group.

3.6. Cerebral infarct volume

TTC was used to reveal infarcted brain tissue. It was observed that there was a reduction of the infarcted area (whitish area) in BEF-oral treated rats, as compared to the control rats (Figure 6). Then the infracted volume in the TTC-stained brain image was quantified using the Image J software, where the infarct area of BEF-oral treated group showed a reduction (9.4%), as compared to control group (14.5%) (Figure 7).

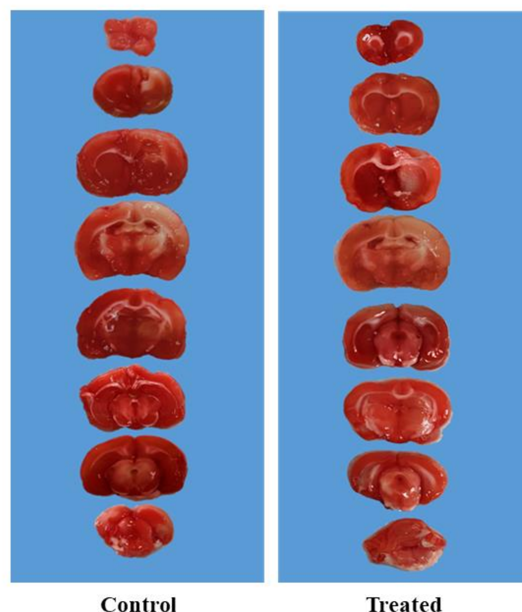

Figure 6. Coronal sections of brains with TTC staining of (a) control and (b) BEF-treated experimental rats 14 days after ET-1 induced IS. Whitish area indicates the infarct area while the deep red staining area indicate normal area of brain.

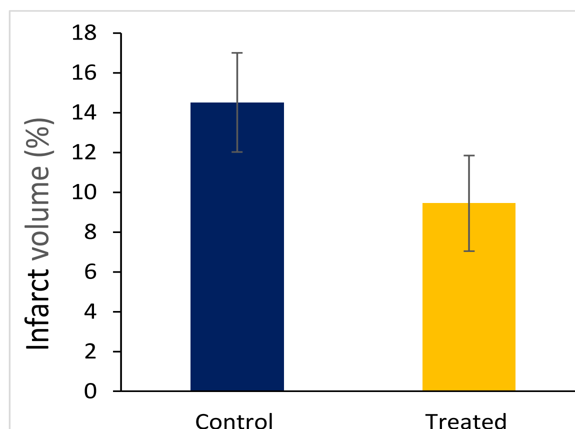

Figure 7. Quantification of infarct volume of control and BEF-treated experimental rats 14 days after ET-1 induced IS. Data showed reduction (despite not significant) in treated group, as compared to control group.

4. Discussion

This study is the first to report the beneficial effects of BEF treatment to improve brain primitive cell proliferation and the expression of genes that could induce recovery from IS injury. Besides, this study also is the first to report that oral administration of BEF before and after the induction of IS could successfully alleviate the cognitive impairments on IS rat models. The data presented in this study is important because, up to date, there is only one clinical drug approved for IS treatment [17].

Furthermore, this one and only approved clinical drug is associated with many limitations, including its inability to regenerate dead neurons after a stroke attack and the limited time window of administration within 3–4.5 hours after a stroke onset to be effective [29]. The short therapeutic window has greatly limited its utilization rates in routine clinical practice because hospitalization delays for stroke occurrence is very common [30]. Considering many limitations of this clinical drug, and that there is no other alternative drug available for IS treatment yet, it is urgently imperative to discover a potential novel neuroprotective drug with potential to treat the disease.

The rationale of utilizing baicalein extracted from *O. indicum* leaves as a drug candidate to treat IS in this study was based on multiple previous studies that have suggested the promising potential of this compound as a neuroprotective agent to treat other neurological diseases such as Parkinson's [31] and Alzheimer's diseases [32]. The neuroprotective effects of baicalein also have been supported by an *in vitro* study using human neuroblastoma-derived neuronal cell line (SHSY5Y) induced with hydrogen peroxide (H_2O_2) to cause neuronal cell damage, where the baicalein was found to successfully confer the strongest protection to the SHSY5Y cells against the H_2O_2 cytotoxicity [33]. Moreover, similar promising results also were obtained from a study involved glutamate stimulation and glucose deprivation in primary cultured rat brain neurons, where the baicalein displayed significant protective effects in neurotoxicity prevention in the brain cells [34].

In the current study, *in vitro* investigation of BEF preconditioning on NSCs revealed that BEF plays a pivotal role in preserving the stemness of NSCs markers, Nestin and SOX2. This property is crucial as it reflects the capability of NSCs for self-renewal and survival [35]. Following IS, blockage in blood vessels supplying blood to the brain may lead to a decrease in oxygen supply, resulting in an upsurge in the production of reactive oxygen species (ROS). These ROS molecules can induce oxidative stress, thereby causing damage to cellular structures, including lipids, protein and DNA [36]. Moreover, ROS also can activate various signaling pathways that contribute to inflammation, blood brain barrier disruption and cell apoptosis [37]. Such cascade events can exacerbate the damage caused by IS and further cause brain injury. Therefore, reducing ROS levels has emerged as a potential therapeutic strategy for IS disease. Baicalein has been known to have potent antioxidant properties, where it can effectively neutralize harmful ROS molecules and provide protection to cells against oxidative damage. This property is in agreement with the findings obtained from this this study, in which the expression levels of an antioxidant gene, SOD2, was significantly upregulated in BEF-preconditioned NSCs.

In this study, it was also found that the expression of ANGPT1 was significantly upregulated in BEF-preconditioned NSCs group. ANGPT1 is an important signaling molecule that plays a role in the process of vasculogenesis (the formation of blood vessels) and early angiogenesis [38]. In response to ischemic injury, these signaling molecules exert their effects through paracrine signaling, that is, they act locally in the immediate vicinity of the cells that produce them. Thus, the upregulation of ANGPT1 is important for the stabilization and maturation of blood vessels, the increment of vascular integrity and reduction of permeability, which helps to maintain blood flow and oxygen supply to the affected area [39]. In agreement with this, Moxon et al. (2019) also reported that baicalein can significantly reduce the ischemic infarct area through a revascularization mechanism [19].

In this study, it was proven that the BEF treatment did not cause any negative compound-induced toxicity, as well as any abnormal alteration in the behavior and physical appearance of the treated rats throughout the experimental period, thus suggesting the negative side effect of BEF treatment on the experimental rats. The safety profile of natural product is important as not all natural products are

inherently safe for long term consumption for health maintenance and disease prevention [40]. Unfortunately, some natural products on the market have been found to contain toxic compounds, such as heavy metals and microbes, as well as banned ingredients such as aristolochic acids [40]. In Malaysia, the National Pharmaceutical Regulatory Agency is responsible for the regulatory control of medicinal products and cosmetics, including natural products. For registration purpose, the safety of natural products is primarily determined through the review of documents, including monographs, research articles and scientific reports [41]. One of the main factors hampering safety evaluations of natural products is the lack of toxicological data from animal studies.

In terms of the therapeutic effects of BEF treatment, this study provided a comprehensive assessment of neurological deficit tests, which was based on the individual scoring of different aspects of neurological functions. Unlike previous behavioral scoring systems, which examined the overall indicator of neurological deficit to produce an average score of behavioral function such as the modified Neurological Severity Score (mNSS), Bedearson and Garcia scores, in the current study, our profiling of neurological deficits were divided into deficits were distinct behavioral components, where the outcomes of each test were individually analyzed to specifically assess the sensitivity of each test. The behavioral scoring from this study revealed a significant improvement of the treated rats in all of the test conducted, as early as day 1 after the BEF administration when compared to non-treated rats. The motor coordination and balance test resulted in a lower motor score, alongside a higher score for the contralateral sensory test, which taken together, indicated an improvement in neurological impairment. Among all of the individual neurological deficit test, the motor, contralateral sensory, motor coordination and balance exhibited higher sensitivities, as these tests have broader ranges of scores (max score: 3/test for motor test, 10/test for contralateral sensory test, 6/test for motor coordination and balance) as compared to the reflex test (max score: 2/test), which has a large gap between the experimental groups.

A feasible mechanism that might explain the fast recovery of treated rats might be attributed to baicalein's broad spectrum of biological functions that includes antioxidant [42], anti-inflammatory [43], neuroprotective [11] and angiogenic [44] properties, as reported across numerous studies. As an antioxidant, baicalein could scavenge free radicals and reactive oxygen species (ROS) generated during IS, effectively countering the oxidative stress and mitigating its detrimental impact on the affected neurons [44]. Furthermore, its anti-inflammatory capabilities inhibit the activation of pro-inflammatory pathways and reduce the production of inflammatory cytokines, thereby preserving the neuronal integrity and function while suppressing brain inflammation associated with IS [45]. Aside from that, numerous findings reported that baicalein also possesses neuroprotective properties, where it can modulate various signaling pathways crucial for neuronal survival, growth and maintenance [46,47]. For example, baicalein has been shown to promote the expression of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) [48], which are essential for supporting neuronal growth and survival. It can also protect neurons from excitotoxicity and apoptosis (cell death), further contributing to neuroprotection [49]. Moreover, as a compound that also exhibits angiogenic properties, baicalein could enhance the blood flow to the affected ischemic areas, thus providing essential nutrients and oxygen for tissue repair and regeneration [50].

Additionally, it was suggested that the protective effects of baicalein against the neurological diseases is associated to its unique ability to cross the blood–brain barrier (BBB) [51]. The BBB is a highly selective semipermeable border of endothelial cells that prevents non-selected solutes in the circulating blood from crossing into the extracellular fluid of the central nervous system (CNS) where

neurons reside [52]. The BBB confers protection to the brain cells, but it also has been a great hurdle for therapeutic brain drug delivery. It was documented that the physiological hurdle of the BBB has stopped 95% of molecules for drug development [53]. Thus, it was extremely important and beneficial that baicalein possesses an ability to cross the BBB, offering a great opportunity for this active compound with anti-oxidant and anti-inflammatory potential as a novel drug for IS treatment.

The positive outcomes observed in the behavioral scoring was then supported with the measurement of the infarct volume, with a reduced whitish area in the right brain hemisphere of treated rats as compared to the control rats. This supports the notion that baicalein's multifaceted mechanisms contribute to the improved neurological behavior after IS treatment. Nonetheless, to ensure the reliability of behavioral scoring and infarct volume measurement using TTC, additional tests assessing the degree of histological changes should also be conducted.

5. Conclusions

In this study, BEF was extracted from the leaves of *O. indicum* and administered through an oral route to SD rats before and after IS induction. The stud findings clearly revealed the effectiveness of this compound to alleviate cognitive impairments of the IS rat models via a comprehensive assessment of neurological deficit tests, based on a significant improvement of individual scoring of motor function, motor coordination and balance, and contralateral functions. In brief, this is the first study that reports the therapeutic potential of BEF extracted from *O. indicum* for IS disease. The data presented here is important as a fundamental reference for further studies of BEF in a longer duration setting, its mechanism of toxicity and how it affects the animal metabolism. Most importantly, this data is also crucial for the future development of BEF for therapeutic usage in pre-clinical and clinical studies.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no conflict of interest.

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