RESEARCH ARTICLE

MLC 901 Decreases HSP-70, MMP-9, Cerebral Infarction Volume and Functional Outcome in Acute Ischemic Stroke Rat Model

Ilsa Hunaifi[1,2], Andi Kurnia Bintang[3],*, Jumraini Tammasse[3], Isra' Wahid[4], Mohammad Hatta[5], Andi Asadul Islam[6], Andi Alfian Zainuddin[7], Paulus Sugianto[8]

[1]Department of Neurology, Faculty of Medicine and Health Sciences, Universitas Mataram, Jl. Pendidikan No. 37, Mataram 83125, Indonesia
[2]Doctoral Program, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan No.Km. 10, Makassar 90245, Indonesia
[3]Department of Neurology, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan No.Km. 10, Makassar 90245, Indonesia
[4]Department of Parasitology, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan No.Km. 10, Makassar 90245, Indonesia
[5]Department of Microbiology, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan No.Km. 10, Makassar 90245, Indonesia
[6]Department of Neurosurgery, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan No.Km. 10, Makassar 90245, Indonesia
[7]Department of Public Health, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan No.Km. 10, Makassar 90245, Indonesia
[8]Department of Neurology, Faculty of Medicine, Universitas Airlangga, Jl. Prof. Dr. Moestopo No.47, Surabaya 60132, Indonesia

*Corresponding author. Email: a.kurnia_b@yahoo.co.id

Received date: Mar 4, 2025; Revised date: May 6, 2025; Accepted date: May 9, 2025

Abstract

BACKGROUND: Acute ischemic stroke (AIS) is usually treated with thrombolysis, however the percentage of patients receiving this therapy is not quite low. Therefore, it is necessary to find alternative therapy using neuroprotective agent such as Moleac (MLC) 901. Heat shock proteins (HSP)-70 and matrix metalloproteinase (MMP)-9 are usually related to AIS due to the triggered stroke-induced physiological stress. However, the effect of MLC 901 on *Hsp70* mRNA expression, HSP-70 and MMP-9 remains unclear. This study was conducted to determine the effect of MLC 901 on those three parameters in relation to cerebral infarction volume and functional outcomes in an AIS model.

METHODS: Rats were induced with AIS using unilateral common carotid artery occlusion (UCAO) and received three different treatments: 43.2 mg/200 gBW MLC 901, 21.6 mg/200 gBW MLC 901, and sodium carboxymethyl cellulose (CMC-Na), that were administered orally for 14 days. HSP-70 and MMP-9 protein levels were assessed using enzyme-linked immunosorbent assay (ELISA), and *Hsp70* mRNA expression was assessed using quantitative real-time polymerase chain reaction (qRT-PCR). Foot fault scores for evaluation functional outcome and infarction volume were assessed by ImageJ.

RESULTS: AIS-induction increased HSP-70, MMP-9, and *Hsp70* mRNA expression within 24-48 h. MMP-9, HSP-70 , and *Hsp70* mRNA expression were reduced by MLC 901. MLC 901 at dose of 43.2 mg/200 gBW and 21.6 mg/200 gBW were effective in reducing these levels compared to the control. MLC 901 improved functional outcomes and decreased cerebral infarction volume. Moreover, a dosage of 43.2 mg/200 gBW was more effective in reducing *Hsp70* mRNA expression and HSP-70, improving functional outcomes, and reducing cerebral infarction volume than a dosage of 21.6 mg/200 gBW, but not MMP-9 protein.

CONCLUSION: MLC 901 effectively decreased *Hsp70* mRNA expression, HSP-70 and MMP-9 protein levels, infarct volume, and functional outcomes. MLC 901 could be a potential therapeutic agent for AIS.

KEYWORDS: MLC 901, HSP-70, MMP-9, acute ischemic stroke

Indones Biomed J. 2025; 17(3): 252-62

Introduction

Stroke is a leading contributor to mortality and disability worldwide, with a higher incidence in less-affluent nations. (1) In Indonesia, there are an increase in stroke prevalence to 10.9 cases per 1,000 individuals, up from 7 cases per 1,000 in 2013.(2) The impact of stroke is significant, since almost half of stroke patients are having permanent disability and are unable to return to work.(3,4) Acute ischemic stroke (AIS) is the most common type of stroke, which is a condition caused by a blockage in a blood vessel supplying blood to the brain, resulting in reduced blood flow and potential brain damage.

Intravenous thrombolysis is the standard treatment for AIS, but most of times it cannot be performed and only around 9.1% of Asian patients receive this therapy. (5,6) Therefore it is necessary to have alternative therapy for AIS, and research into neuroprotective strategies such as Moleac (MLC) 901 is particularly promising. The complexity of the cerebral ischemia cascade is such that a single neuroprotective intervention is insufficient to prevent brain damage. This therapy targets multiple molecular and cellular mechanisms, thereby mitigating cerebral ischemia during the acute phase.(7) Herbal remedies such as *Astragali radix* and *Prunus persica* may offer protection against ischemia-reperfusion injury, reduce neuroinflammation and oxidative stress, enhance brain microcirculation, and modify microglial polarization.(7,8) Studies have shown that MLC 901 inhibits the expression of peroxiredoxin-6 (Prx-6), nuclear factor kappa beta (NF-κB), and toll-like receptor (TLR)-4 following middle cerebral artery occlusion (MCAO).(9) Research on MLC 901 for traumatic brain injury has shown that taking 0.8 gram of MLC 901 (two capsules) three times daily for six months lead to enhancement in executive function and attention. (10) However, the optimal oral dose for AIS remains unclear. Intraperitoneal injection of MLC 901 at 40 µg/kg body weight can prevent brain injury, maintain the blood-brain barrier, and reduce cerebral oedema. Moreover, this treatment has been shown to mitigate neurological deficits in cerebral ischemia by suppressing the mRNA expression of inflammatory cytokines.(9)

Stroke-induced physiological stress triggers the production of heat stroke protein (HSP)-70 and other HSPs. (11,12) HSP-70 is reported to suppress NF-κB, thereby reducing matrix metalloproteinase (MMP-9) activity.(11) Additionally, it works in conjunction with mitogen-activated protein kinase (MAPK) to modulate various cellular functions, including migration, apoptosis, differentiation, proliferation, and metabolism. Considering the increased MAPK levels following stroke, HSP-70 is being considered as a potential therapeutic target for ischemic stroke. (13) Moreover, the disparity between intracellular and extracellular HSP-70 has been suggested as a possible factor in the development of chronic diseases. Studies have revealed that extracellular HSP-70 stimulates the release of pro-inflammatory molecules via TLR 2/4 through the action of myeloid differentiation factor 88 (MyD88).(14)

Currently, no studies have investigated the efficacy of MLC 901 on *Hsp70* mRNA expression, HSP-70 and MMP-9 protein expression, functional outcomes, and cerebral infarction volume. Hence it is vital to investigate the efficacy of MLC 901 on these biomarkers in AIS. Research using experimental animal models of AIS should be undertaken to determine its safety and efficacy prior to advancing its therapeutic use in humans.(15) This study was conducted to determine the efficacy of MLC 901 in modulating the expression of *Hsp70* mRNA, HSP-70 and MMP-9 proteins, functional outcomes, and magnitude of cerebral infarction volume.

Methods

Animal Preparation

Twenty-four adult male rats, weighing between 200 and 300 grams, aged 10–12 weeks, were procured from the Department of Agriculture and Food Security in Maros Regency, South Sulawesi. The rats were housed in a controlled environment, maintained at a temperature of 25±2°C and a humidity level of 60±10%, with unrestricted access to water and food. All experimental protocols were conducted at the Molecular Biology and Immunology Laboratory, Universitas Hasanuddin, Makassar. The experiment protocol was approved by The Ethical Committee of Universitas Hasanuddin/Universitas Hasanuddin Teaching Hospital/Wahidin Sudirohusodo General Hospital (No. 629/UN4.6.4.5.31/PP36/2023). Every attempt was made to reduce the quantity of animals used in each group and to minimize suffering of the animals.

Drug Administration

Neuroaid II MLC 901 (Moleac Singapore, Singapore) comprises of nine herbal extracts, including *Radix salvia* miltiorrhizae, *Radix astragali*, *Radix paeoniae* rubra, *Radix angelicae* sinensis, *Radix polygalae*, *Rhizoma chuanxiong*, *Carthamus tinctorius*, *Prunus persica*, and *Acori* tatarinowii

rhizoma. Tetramethylpyrazine (TMP), ligustilide, ferulic acid, hydroxyl safflower yellow A (HSYA), β-Asarone, total paeony glycoside (TPG), astragaloside IV (AST), presenegenin, salvianolic acid B (SAB), and tanshinone IIB (TSB) are among its active ingredients. MLC 901 was administered with rat oral gavage 90 minutes after stroke induction and continuing once daily for 14 days. The dosage for rats was calculated based on previously established human equivalent doses.(10,16)

AIS Model and Treatment Procedure

Following an acclimatization period, three groups of rats were randomly selected (n=8 per group): 1) AIS rats treated with 43.2 mg/200 gBW MLC 901; 2) AIS rats treated with 21.6 mg/200 gBW MLC 901; and 3) AIS rats treated with 0.25% sodium carboxymethyl cellulose (CMC-Na) as a negative control (NC). The unilateral common carotid artery occlusion (UCAO) technique was used to generate AIS. MLC 901 and CMC-Na was administered 90 minutes post-induction. Given that this model closely replicates the clinical presentation of AIS in humans, a normal control group was not included. Treatment was initiated post-acute ischemic stroke critical phase to enhance neuroprotection. The experimental design and the study timeline was illustrated in Figure 1.

The UCAO technique, as described in a previous study, was used to establish the AIS model.(17) An intraperitoneal injection of a mixture of 10 mg/kgBW xylazine and 80 mg/kgBW ketamine was used to anesthetize the rats. While under anesthesia, the rats were positioned supine on a surgical table. A small incision, 1–2 cm in length, was made to reveal the left common carotid artery along the midline of the neck. A bulldog clamp was then applied to occlude the artery for 180 minutes. Following the occlusion period, the clamp was removed to allow reperfusion (Figure 2).

Measurement of *Hsp70* mRNA Expression Using Quantitative Real Time-PCR (qRT-PCR)

Samples of blood were taken at baseline (prior stroke), 24 hours (day 1), 48 hours (day 2), and 336 hours (day 14) post-AIS. Nucleic acid extraction and measurement of *Hsp70* mRNA expression were performed according to established protocols.(18) The target gene's expression profile was identified using qRT-PCR, with expression levels represented as the ratio of the specific gene of interest to that of the housekeeping gene GAPDH. *Hsp70* mRNA was detected using specific forward (5′ ACGAGGGTCTCAAGGGCAAG-3′) and reverse (5′ CTCTTTCTCAGCCAGCGTGTTAG-3′) primers, at 63°C for a 107 bp product (GenBank ID: NM_031971). GAPDH was detected using forward (5′ TGCACCACCAACTGCTTAGC -3′) and reverse (5′ GGCATGGACTGTGGTCATGAG-3′) primers, also at 63°C for an 87 bp product (GenBank ID: NM_017008) (Macrogen, Seoul, Korea). The CFX Connect real-time PCR system was used to carry out the PCR amplification (Bio-Rad Laboratories, Hercules, CA, USA) in a 96-well format with a 0.1 mL reaction volume. Each sample was analysed in triplicate to ensure reliability and accuracy. This study did not investigate MMP-9 mRNA expression, as an increase in MMP-9 is definitively linked to the deterioration of AIS.(19)

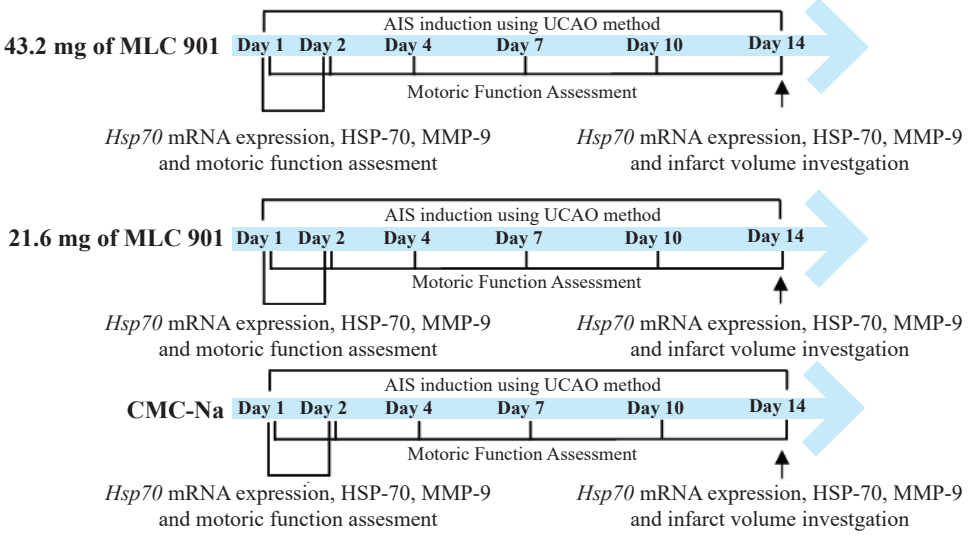

Figure 1. The experimental design and study timeline.

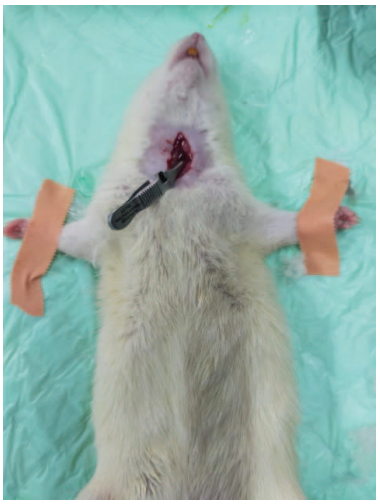

Figure 2. Preparation of AIS rat model using UCAO technique for 180 minutes.

Measurement of Plasma Biomarker (HSP-70 and MMP-9) Using Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to quantify soluble protein levels of HSP-70 and MMP-9 in tail blood samples collected at baseline (prior to stroke induction), and at 24 hours (day 1), 48 hours (day 2), and 336 hours (day 14) post-AIS.(20) Blood samples were processed using the Rat HSP-70 kit (Cat. No. LS-F37346; LS Bio, Seattle, WA, USA) and the Rat MMP-9 kit (Cat. No. LS-F32423; LS Bio). Samples were analyzed in duplicate to ensure data reliability. ELISA Procedure accordance with previous study. The ELISA Reader 270 (Biomerieux, Marcy-l'Étoile, France) was used to measure the absorbance values in less than 30 minutes at a wavelength of 450 nm.(18) The concentrations of the target soluble proteins were expressed in ng/ml for HSP-70 and pg/mL for MMP-9.

Measurement of Functional Outcome (Motoric Score) Using Ladder Rung Walking Test

The Ladder Rung Walking Test apparatus (Figure 3) was utilized to evaluate neurological deficits (motor scores) in rats at baseline (prior to surgery) and on day-1, -2, -4, -7, -10, and -14 following AIS induction.(21) Each rat navigated a cylindrical staircase comprising a 1-meter pathway with rungs spaced at varying distances. Hindlimb movements were carefully observed and recorded on video for analysis. Rats that experienced slips were indicative of reduced or impaired motor function. The evaluation of foot placement on the rungs was conducted using a seven-category scale, based on the nature of the errors and the positioning of the hindlimbs.(22) Each animal underwent five rounds of training and testing during each session. The average error score was subsequently analyzed. The percentage of errors for each trial, as well as the quality of placement for both the right forelimb and hindlimb, were assessed. Errors were classified with scores of 0, 1, and 2 (representing paw slips or falls), and the average was calculated over five trials using the formula: (number of errors/number of steps) × 100, yielding the Foot Fault Score. Error score data were presented as a percentage out of 100.

Measurement of Cerebral Infarction Volume Using Image J

On day 14, all rats were euthanized. The brains were extracted and sectioned into coronal slices using a rat brain slicer, and then immersed in a 2% solution of 2,3,5-Triphenyltetrazolium Chloride (TTC) at 37 °C for 30 minutes. Areas of infarction were identified as white regions and quantified using ImageJ software (National Institutes of Health (NIH), Bethesda, MD, USA).(23)

Statistical Analysis

All data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 26 (IBM Corporation, Armonk, NY, USA). The normality of the data distribution was assessed using the Shapiro–Wilk test. Data are presented as mean±standard deviation (SD). The relationships among *Hsp70* mRNA expression, HSP-70 and MMP-9 levels, functional outcomes, and cerebral infarction volume were evaluated using the Spearman correlation test. The efficacy of MLC 901 on *Hsp70* mRNA expression, HSP-70 levels, functional outcomes, and cerebral infarction volume were analyzed using a One-way ANOVA, followed by the Least Significant Difference (LSD) post hoc test. The impact of MLC 901 on MMP-9 levels was assessed using the Kruskal-Wallis test then Mann-Whitney post hoc test. The result was statistically significant if $p<0.05$.

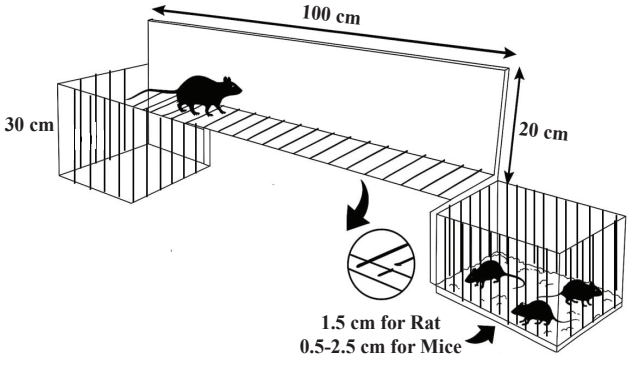

Figure 3. Ladder rung walking test apparatus for measurement of motoric score.

Results

Efficacy of MLC 901 in HSP-70 and MMP-9 Protein

AIS resulted in an increase in HSP-70 and MMP-9 protein levels from 24 to 336 h post-stroke compared to baseline (Figure 4A and 4B). At 24, 48, and 336 h after MLC 901 was administered, there was a significant difference of HSP-70 levels between the three groups (p<0.001). Post hoc analysis results indicated that MLC 901 treatment at doses of 43.2 and 21.6 mg/200 gBW significantly reduced HSP-70 levels compared to the negative control. Furthermore, the 43.2 mg/200 gBW dose of MLC 901 demonstrated greater efficacy in reducing HSP-70 protein levels compared to the 21.6 mg/200 gBW dose within the 48 to 336 h post-treatment period (Table 1). MMP-9 levels also exhibited significant differences between the three groups (p<0.001). Administration of MLC 901 significantly reduced MMP-9 protein levels compared to the negative control. However, MLC 901's efficacy on lowering MMP-9 protein levels at 24, 48, and 336 h did not differ significantly between dosages of 43.2 and 21.6 mg/200 gBW (Table 1).

Efficacy of MLC 901 in *Hsp70* mRNA Expression

Within 24 h of AIS, all groups exhibited a significant increase in *Hsp70* mRNA expression. Significant differences in *Hsp70* mRNA expression levels were observed among the groups at 24, 48, and 336 h, attributed to MLC 901 administration (p<0.001) (Figure 4C). Both dosages of MLC 901 (43.2 and 21.6 mg/200 gBW) substantially decreased *Hsp70* mRNA expression at 24, 48, and 336 h as compared to the negative control. Furthermore, after 48 to 336 h, the 43.2 mg/200 gBW dosage considerably reduced *Hsp70* mRNA expression in comparison to the 21.6 mg/200 gBW dose (Table 2).

The Efficacy of MLC 901 on Functional Outcome

Motor performance scores declined following the induction of AIS. The administration of MLC 901 significantly influenced motor performance scores among the groups from 96 h to 336 h (p<0.001) (Figure 4D). Treatment with MLC 901 at doses of 43.2 and 21.6 mg/200 gBW resulted in substantial improvements in motor performance scores compared to the negative control (Figure 5D). Furthermore, on the fourth day of treatment, the 43.2 mg/200 gBW dose demonstrated significantly greater improvement in motor performance scores compared to the 21.6 mg/200 gBW dose (Table 3).

Efficacy of MLC 901 on Cerebral Infarction Volume

Cerebral infarction volume serves as a measure of brain damage resulting from acute ischemic stroke. Table 4

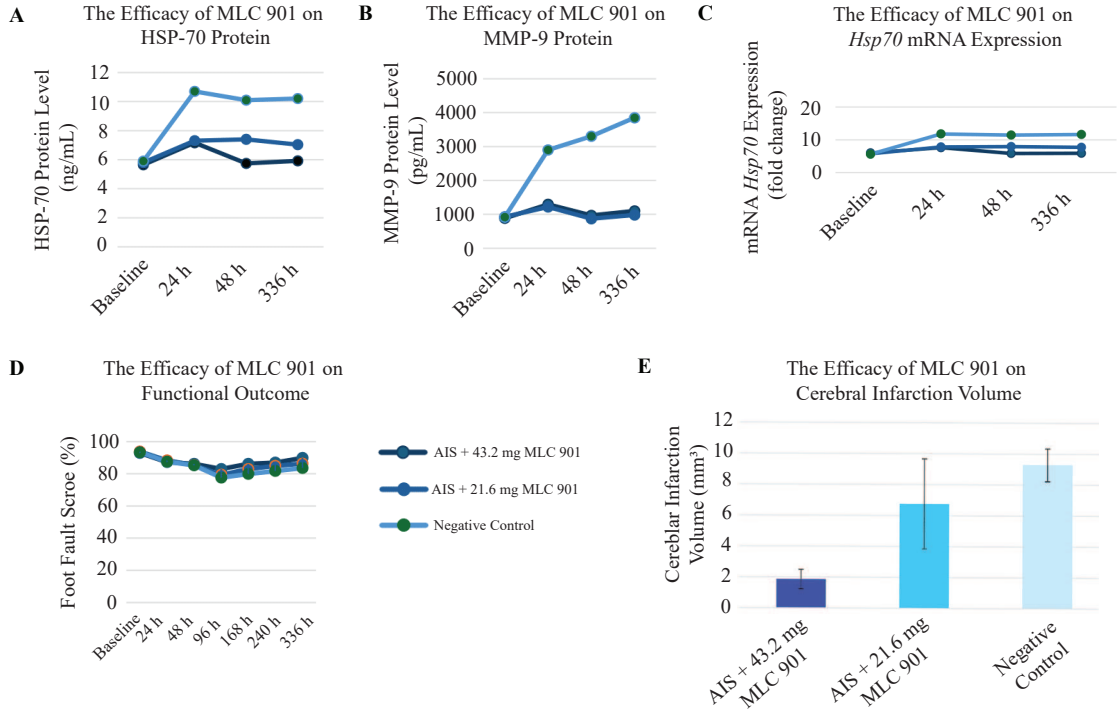

Figure 4. The efficacy of MLC 901 based on the time after treatment. A: HSP-70 protein; B: MMP-9 protein; C: *Hsp70* mRNA expression; D: functional outcome (motoric score); E: cerebral infarction volume.

Table 1. Comparison of efficacy of MLC 901 on HSP-70 and MMP-9 protein between groups.

Time	Group	HSP-70		Post Hoc Test (*p*-value)[1]	MMP-9		Post Hoc Test (*p*-value)[2]
		Mean±SD (ng/mL)	*p*-value[1]		Median (Min-Max) (pg/mL)	*p*-value[2]	
Baseline	AIS + 43.2 mg MLC 901	5.66±0.30	0.480		883.08 (662.40–1247.27)	0.830	
	AIS + 21.6 mg MLC 901	5.80±0.51			927.70 (644.34–1296.58)		
	Negative Control	5.90±0.35			916.25 (670.62–1166.94)		
24 h	AIS + 43.2 mg MLC 901	7.17±0.43	0.000*	0.580[a]	1294.54 (957.66–1528.83)	0.000	0.600[a]
	AIS + 21.6 mg MLC 901	7.31±0.53		0.000*[,b]	1218.35 (1006.19–1408.33)		0.000*[,b]
	Negative Control	10.70±0.54		0.000*[,b]	2896.92 (2606.57–3793.52)		0.000*[,b]
48 h	AIS + 43.2 mg MLC 901	5.74±0.36	0.000*	0.000*[,a]	965.53 (899.36–1247.70)	0.000	0.000*[,a]
	AIS + 21.6 mg MLC 901	7.40±0.44		0.000*[,b]	872.23 (650.90–1263.96)		0.000*[,b]
	Negative Control	10.10±0.38		0.000*[,b]	3302.99 (3111.57–4206.09)		0.000*[,b]
336 h	AIS + 43.2 mg MLC 901	5.92±0.54	0.000*	0.000*[,a]	1101.12 (1020.06–1263.96)	0.000	0.000*[,a]
	AIS + 21.6 mg MLC 901	7.04±0.35		0.000*[,b]	979.40 (872.52–1215.30)		0.000*[,b]
	Negative Control	10.21±0.27		0.000*[,b]	3850.38 (3361.294969.55)		0.000*[,b]

*Significant if $p<0.05$, [1]Analyzed with One-way ANOVA and LSD Post Hoc test, [2]Analyzed with Kruskal Wallist Test and Mann Whitney Pos Hoc Test. [a]compared to the AIS + 21.6 mg MLC 901 group, [b]compared to the negative control group.

and Figure 4E and 5E showed that there was a significant difference between the groups in the efficacy of MLC 901 administration on cerebral infarction volume ($p<0.001$). In comparison to the negative control, the administration of MLC 901 at dosages of 43.2 and 21.6 mg/200 gBW significantly decreased the cerebral infarction volume (Figure 6). Furthermore, the reduction in infarction volume was significantly greater with the 43.2 mg/200 gBW dose than with the 21.6 mg/200 gBW dose.

Correlation between *Hsp70* mRNA Expression, HSP-70 and MMP-9 Protein with Functional Outcome and Cerebral Infarction Volume

The correlations between motoric score, cerebral infarction volume, *Hsp70* mRNA expression, and HSP-70 and MMP-9 protein levels are shown in Table 5. *Hsp70* mRNA expression demonstrated a significant negative correlation with motor performance scores (r=-0.915, $p<0.001$) and a positive correlation with cerebral infarction

Table 2. Comparison of the efficacy of MLC 901 on *Hsp70* mRNA expression (n=8).

Time	Group	Mean±SD (Fold Change)	*p*-value[1]	Post Hoc Test (*p*-value)[2]
Baseline	AIS + 43.2 mg MLC 901	5.98±0.50	0.470	
	AIS + 21.6 mg MLC 901	5.74±0.78		
	Negative Control	5.61±0.47		
24 h	AIS + 43.2 mg MLC 901	7.60±0.46	0.000*	0.430[a]
	AIS + 21.6 mg MLC 901	7.83±0.64		0.000*[,b]
	Negative Control	11.77±0.60		0.000*[,b]
48 h	AIS + 43.2 mg MLC 901	5.83±0.55	0.000*	0.000*[,a]
	AIS + 21.6 mg MLC 901	7.91±0.43		0.000*[,b]
	Negative Control	11.42±0.32		0.000*[,b]
336 h	AIS + 43.2 mg MLC 901	5.87±0.70	0.000*	0.000*[,a]
	AIS + 21.6 mg MLC 901	7.69±0.49		0.000*[,b]
	Negative Control	11.61±0.28		0.000*[,b]

*Significant if $p<0.05$, [1]Analyzed with One-way ANOVA and LSD Post Hoc test, [2]Analyzed with Kruskal Wallist Test and Mann Whitney Pos Hoc Test. [a]compared to the AIS + 21.6 mg MLC 901 group, [b]compared to the negative control group.

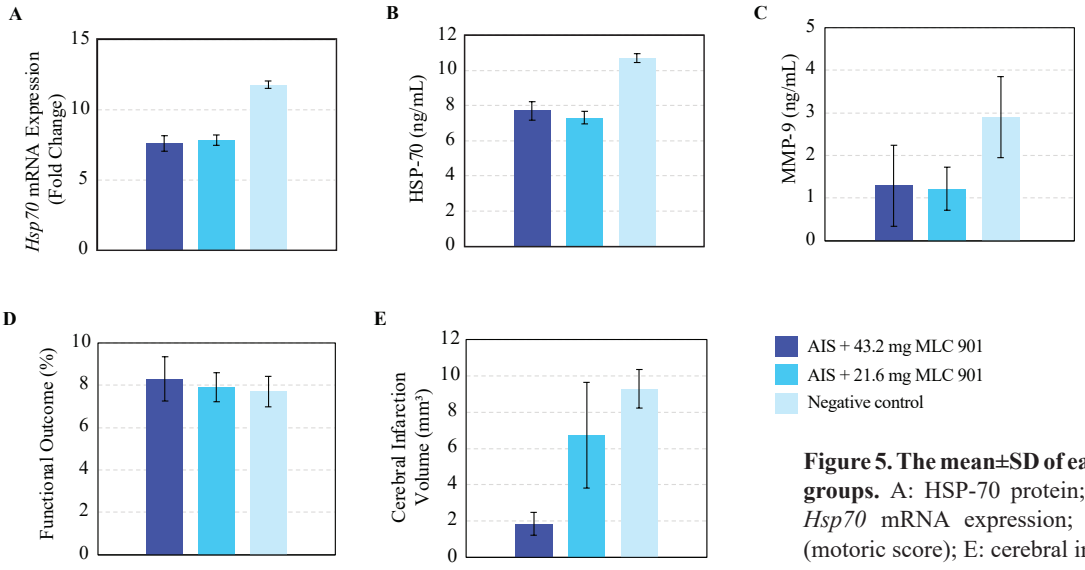

Figure 5. The mean±SD of each parameters between groups. A: HSP-70 protein; B: MMP-9 protein; C: *Hsp70* mRNA expression; D: functional outcome (motoric score); E: cerebral infarction volume.

volume (r=0.803, p<0.001). Additionally, HSP-70 and MMP-9 protein levels showed strong negative correlations with motor performance scores (r=-0.926, p<0.001 and r=-0.631, p=0.001, respectively) and positive correlations with cerebral infarction volume (r=0.717, p<0.001 and r= 0.473, p<0.001, respectively). These findings indicate that

Table 3. Comparison of efficacy of MLC 901 on functional outcome (motoric score) between groups.

Time	Group	Mean±SD (Percentage)	p-value[1]	Post Hoc Test (p- value)[2]
Baseline	AIS + 43.2 mg MLC 901	92.95±1.37	0.330	
	AIS + 21.6 mg MLC 901	93.81±0.81		
	Negative Control	93.41±1.13		
24 h	AIS + 43.2 mg MLC 901	87.27±1.82	0.310	
	AIS + 21.6 mg MLC 901	88.38±1.58		
	Negative Control	87.48±0.84		
48 h	AIS + 43.2 mg MLC 901	86.19±3.09	0.560	
	AIS + 21.6 mg MLC 901	85.32±1.16		
	Negative Control	85.23±0.65		
96 h	AIS + 43.2 mg MLC 901	83.00±0.85	0.000*	0.000*,a
	AIS + 21.6 mg MLC 901	79.38±0.96		0.000*,b
	Negative Control	77.61±2.30		0.030*,b
168 h	AIS + 43.2 mg MLC 901	86.15±1.71	0.000*	0.000*,a
	AIS + 21.6 mg MLC 901	82.92±1.25		0.000*,b
	Negative Control	79.92±1.70		0.001*,b
240 h	AIS + 43.2 mg MLC 901	86.89±0.97	0.000*	0.001*,a
	AIS + 21.6 mg MLC 901	84.97±0.56		0.000*,b
	Negative Control	81.68±1.29		0.001*,b
336 h	AIS + 43.2 mg MLC 901	89.69±1.04	0.000*	0.000*,a
	AIS + 21.6 mg MLC 901	86.33±0.69		0.000*,b
	Negative Control	83.59±0.73		0.000*,b

*Significant if p<0.05, [1]Analyzed with One-way ANOVA, [2]Analyzed with LSD Post Hoc test.

AIS + 43.2 mg MLC 901 | AIS + 21.6 mg MLC 901 | Negative Control

Figure 6. Brain coronal slice using TTC staining. Administration of MCL 901 indicate reduce cerebral infarction volume compared to the negative control. White arrow: cerebral infarction.

in Wistar rats subjected to an AIS model, elevated *Hsp70* mRNA expression and increased levels of HSP-70 and MMP-9 proteins were associated with the larger cerebral infarction volumes and more severe neurological deficits.

Discussion

Cerebral ischemia triggers an rise in HSP-70 levels through the activation of heat shock proteins and their associated transcription factors (HSP-HSFs), which boost HSP mRNA transcription and translation.(24) This research revealed a notable increase in *Hsp70* mRNA expression and protein concentrations 24 to 48 h after acute ischemic stroke. HSP-70 serves dual purposes: within cells, it inhibits apoptosis, while outside cells, it promotes the release of inflammatory mediators. An imbalance between these intracellular and extracellular forms may contribute to insulin resistance in patients with type 2 diabetes mellitus and other chronic conditions. The extracellular form acts as a damage-associated molecular pattern (DAMP), stimulating the release of pro-inflammatory mediators via TLR 2/4 mediated by MyD88.(14) The outcomes of patients with brain injuries are affected by variations in their elevated HSP-70 levels.(25)

Table 4. Comparison of efficacy of MLC 901 on cerebral infarction volume between groups.

Group	Mean±SD (mm^3)	*p*-value[1]	Post Hoc Test (*p*-value)[2]
AIS + 43.2 mg MLC 901	1.86±0.63	0.000	0.000*,a
AIS + 21.6 mg MLC 901	6.75±2.91		0.000*,b
Negative Control	9.28±1.06		0.000*,b

*Significant if $p<0.05$, [1]Analyzed with One-way ANOVA, [2]Analyzed with LSD Post Hoc test.

The atherosclerosis is significantly affected by inflammation. Within the vascular wall, inflammatory processes initiate the release of proinflammatory mediators and increase the expression of intercellular adhesion molecules (ICAM-1) and vascular cell adhesion molecules (VCAM)-1 and VCAM-3, ultimately inducing HSP-70 expression.(26) While low levels of HSP-70 hasten atherosclerosis progression, elevated levels are linked to arterial fat accumulation, promoting the formation of atherosclerotic lesions and increasing the risk of acute coronary syndrome.(13) The current study demonstrated that MLC 901 administration markedly reduced HSP-70 levels in comparison with the negative control. Similarly, AST-IV inhibits HSP expression. Sustained elevation of HSP-70 levels beyond 24 h is associated with unfavorable outcomes in patients with severe traumatic brain injury or cardiac arrest.(27,28) The higher dose of 43.2 mg/200 gBW of MLC 901 indicated a high level of AST-IV, which could reduce the HSP-70 better than the lower dose of 21.6 mg/200 gBW.(29)

Within 24 to 48 h following ischemia, rats that have experienced a stroke show considerable expression of HSP-70 in brain neurons, the penumbra, and circulation. (20) Overexpression of extracellular HSP-70 activates the NF-κB pathway, leading to the generation of pro-inflammatory cytokines such as IL-6, inducible nitric oxide synthase (iNOS), and IL-1β through interactions with CD14 molecules and TLR 2/4. Neural cells may be damaged by the excessive and sustained production of inflammatory mediators. Moreover, oxidative stress causes an increase in reactive oxygen species (ROS) concentrations, which damages the mitochondria.(30,31) MLC 901 contains quercetin, an HSP-70 inhibitor that suppresses Heat shock factor protein (HSF)1 expression, reduces HSP70 accumulation, and promotes cancer cells.(32) Chronic stressors, such as hypertension, increase HSP-70 expression and trigger the release of inflammatory markers, including

Table 5. Correlation between *Hsp70* mRNA expression, HSP-70 and MMP-9 protein with cerebral infarction volume and functional outcome.

Parameters	Cerebral Infaction Volume		Functional Outcome	
	r	*p*- value	*r*	*p*- value
Hsp70 mRNA Expression	0.803	0.000*	-0.915	0.000*
HSP-70 Protein	0.717	0.000*	-0.926	0.000*
MMP-9 Protein	0.473	0.002*	-0.631	0.001*

*Significant if $p<0.05$, Analyzed with Spearman's Correlation test.

interleukin-6. This leads to elevated C-reactive protein (CRP) levels, proliferation of vascular smooth muscle cells, and advancement of atherosclerosis.(9)

MMP-9 is an indicator of blood-brain barrier damage. High levels of MMP-9 result in severe neurovascular tissue damage, causing hemorrhagic transformation and brain swelling. MMP-9 concentrations exceeding 100 ng/mL are associated with severe cerebral oedema and haemorrhagic transformation. Furthermore, in patients with a modified Rankin Scale (mRS) score >3, elevated MMP-9 levels were notably associated with poor outcomes three months after stroke.(32) Milder stroke consequences are observed with lower MMP-9 levels and are related to the stroke scale.(33) In this study, MMP-9 concentrations peaked at 24 h post-stroke onset and were considerably reduced by MLC 901 treatment. The TMP component in MLC 901 suppressed TNF-α levels, caspase-3 expression, TLR-4/NF-κB expression, and MMP-9 and AQP4 expression. HSYA enhances tissue inhibitor of metalloproteinases (TIMP)-1, which decreases MMP-9 expression 1.(9,34) Seneginin of MLC 901 further minimizes cellular damage by lowering MMP levels, ROS, and apoptosis.(35) AST-IV also inhibits MMP-9 and Aquaporin 4 levels, thereby diminishing post-ischemic cerebral oedema.(36) Treatment with MLC 901 led to notable improvements in neurological deficits from the 14th to the 14th day of administration when juxtaposed with the negative control group. Further analysis revealed that a dosage of 43.2 mg/200 gBW of MLC 901 yielded better neurological outcomes compared to a 21.6 mg/200 gBW dose. Research on ischemic stroke models has demonstrated that MLC 901 constituents, including ferulic acid, AST-IV, β-asarone, and TSB, can alleviate neurological impairment, reduce cerebral infarct size, decrease blood-brain barrier permeability, and mitigate neurological damage.(37)

This study indicates that the MLC 901 treatment improved neurological outcomes in ischemic stroke models from 4th day (96 h) of administration. In AIS, the extent of cerebral infarction is indicative of neuronal damage, and plays a crucial role in evaluating the effectiveness of therapeutic interventions. Results of this study revealed that MLC 901 substantially reduced cerebral infarction volume compared to that in the negative control group. MLC 901 comprises active ingredients, such as ligustilide, 3-n-butylphthalide, and ferulic acid, which exhibit anti-inflammatory, neurogenic, angiogenic, and anti-atherosclerotic properties, thus improving neurological deficits and diminishing cerebral infarction volume.(9) Moreover, AST-IV, TSB, and β-asarone also contribute to the amelioration of neurological deficits and reduction of cerebral infarction volume.(37)

This research revealed a significant link between *Hsp70* mRNA expression, HSP-70 protein levels, and MMP-9 with the volume of cerebral infarction and neurological impairments. Elevated *Hsp70* mRNA expression was associated with the intensity of head trauma.(25) MMP-9 exhibits a strong connection with cerebral infarction size in ischemic stroke models. Both HSP-70 and MMP-9 play crucial roles in various pathological mechanisms involved in cerebral ischaemia.(38)

The strength of this study lies in its pioneering investigation of the efficacy of MLC 901 on *Hsp70* mRNA expression and HSP-70 protein levels. Moreover, MLC 901 enhances neurological function, elevates MMP-9 protein levels, and diminishes cerebral infarction volume in AIS models. The study also identified the appropriate MLC 901 dosages that could benefit patients with AIS. Nevertheless, the research is constrained by its failure to analyze Hsp70 mRNA in brain tissue and the omission of neural regeneration markers, such as synaptophysin and brain-derived neurotrophic factor (BDNF), Calcineurin (CaN).(36-38) Future studies should focus on analyzing mRNA expression in brain tissue and evaluating neural regeneration markers through histopathological analysis and neuronal regeneration biomarkers.

Conclusion

In this rat model of AIS, MLC 901 treatment significantly reduced *Hsp70* mRNA expression, HSP-70 and MMP-9 protein levels, and cerebral infarction volume, while also significantly improving functional outcomes (motor scores) compared to the control group. The 43.2 mg/200 gBW dose of MLC 901 was generally more effective than the 21.6 mg/200 g dose in reducing *Hsp70* mRNA and protein levels, decreasing infarct volume, and improving functional recovery. These findings suggest that MLC 901 in concentration-dependent manner may have potential neuroprotective effects in AIS.

Acknowledgments

We thank to all staff at the Molecular Biology and Immunology Laboratory Department of Microbiology, Faculty of Medicine, Universitas Hasanuddin University, as well as staff at Faculty of Medicine and Health Sciences, Universitas Mataram for all their support during the study.

Authors Contribution

All authors were involved in concepting and planning the research. IH, AKB, JT, and IW performed the data acquisition/collection and calculated the experimental data and all authors performed the analysis. All authors took parts in drafted the manuscript and designed the figures, aided in interpreting the results, and giving critical revision of the manuscript.

References

1. Feigin VL, Stark BA, Johnson CO, Roth GA, Bisignano C, Abady GG, *et al*. Global, regional, and national burden of stroke and its risk factors, 1990-2019: A systematic analysis for the Global Burden of Disease Study 2019. Lancet Neurol. 2021; 20(10): 1–26.
2. Fadhilah H, Permanasari VY. Economic burden bore by patients and families because of stroke: Policy assessment. J Indones Heal Policy Adm. 2020; 5(3): 91–5.
3. Yang Y, Shi YZ, Zhang N, Wang S, Ungvari GS, Ng CH, *et al*. The disability rate of 5-year post-stroke and its correlation factors: A national survey in China. PLoS One. 2016; 11(11): e0165341. doi: 10.1371/journal.pone.0165341.
4. Ramos-Lima MJM, Brasileiro I de C, de Lima TL, Braga-Neto P. Quality of life after stroke: Impact of clinical and sociodemographic factors. Clinics. 2018; 73: e418. doi: 10.6061/clinics/2017/e418.
5. Gajurel BP, Nepal G, Jaiswal V, Ang SP, Nain P, Shama N, *et al.* Utilization rates of intravenous thrombolysis for acute ischemic stroke in Asian countries: A systematic review and meta-analysis. Med (United States). 2023; 102(42): E35560. doi: 10.1097/MD.0000000000035560.
6. Kembuan MAHN, Mawuntu AHP, Yohanna, Feliana, Tumboimbela MJ. Lower GCS is related to poor outcome among acute stroke patients with COVID-19 in a tertiary referral hospital in Indonesia. Indones Biomed J. 2021; 13(4): 409–17.
7. Xiong XY, Liu L, Yang QW. Refocusing neuroprotection in cerebral reperfusion era: New challenges and strategies. Front Neurol. 2018; 9: 249. doi: 10.3389/fneur.2018.00249
8. Li Y, Wang D, Guo R, Ma B, Miao L, Sun M, *et al.* Neuroprotective effect of Astragali Radix on cerebral infarction based on proteomics. Front Pharmacol 2023; 14: 1162134. doi: 10.3389/fphar.2023.1162134.
9. Widmann C, Gandin C, Petit-Paitel A, Lazdunski M, Heurteaux C. The traditional Chinese medicine MLC901 inhibits inflammation processes after focal cerebral ischemia. Sci Rep. 2018; 8(1): 18062. doi: 10.1038/s41598-018-36138-0.
10. Theadom A, Barker-Collo S, Jones KM, Parmar P, Bhattacharjee R, Feigin VL. MLC901 (NeuroAiD IITM) for cognition after traumatic brain injury: A pilot randomized clinical trial. Eur J Neurol. 2018; 25(8): 1055–e82.
11. Kim JY, Kim JW, Yenari MA. Heat shock protein signaling in brain ischemia and injury. Neurosci Lett. 2020; 715: 134642. doi: 10.1016/j.neulet.2019.134642
12. Nagai M, Kaji H. Thermal Effect on Heat Shock Protein 70 Family to Prevent Atherosclerotic Cardiovascular Disease. Biomolecules. 2023; 13(5): 867. doi: 10.3390/biom13050867.
13. Gugliandolo A, Silvestro S, Sindona C, Bramanti P, Mazzon E. Mirna: Involvement of the mapk pathway in ischemic stroke. a promising therapeutic target. Med. 2021; 57(10): 1053. doi: 10.3390/medicina57101053.
14. Hulina A, Grdić Rajković M, Jakšić Despot D, Jelić D, Dojder A, Čepelak I, *et al.* Extracellular Hsp70 induces inflammation and modulates LPS/LTA-stimulated inflammatory response in THP-1 cells. Cell Stress Chaperones. 2018; 23(3): 373–84.
15. Aulia MA, Lestari K, Faried A, Sartika CR, Wijaya A, Adam A, *et al.* Mesenchymal stem cell therapy for ischemic stroke in animal model: A systematic eeview. Indones Biomed J. 2023; 15(6): 351–7.
16. Widiyani T, Listyawati S. Handbook Penggunaan Hewan Laboratorium dalam Uji in Vivo. 1st ed. Makassar: Nas Media Indonesia; 2022.
17. Indra MR, Gasmara CP. Ucao (unilateral cerebral artery occlussion) method increases the level of MMP- 9 brain tissue in rats model of ischemic stroke. Malang Neurol Journal. 2016; 2(2): 46–50.
18. Fachri M, Hatta M, Massi MN, Santoso A, Wikanningtyas TA, Dwiyanti R, *et al.* The strong correlation between ADAM33 expression and airway inflammation in chronic obstructive pulmonary disease and candidate for biomarker and treatment of COPD. Sci Rep. 2021; 11(1): 23162. doi: 10.1038/s41598-021-02615-2.
19. Saleem S, Wang D, Zhao T, Sullivan RD, Reed GL, Matrix metalloproteinase-9 expression is enhanced by ischemia and tissue plasminogen activator and induces hemorrhage, disability and mortality in experimental stroke. Neuroscience. 2021; 460: 120–9.
20. De La Rosa X, Santalucía T, Fortin PY, Purroy J, Calvo M, Salas-Perdomo A, *et al.* In vivo imaging of induction of heat-shock protein-70 gene expression with fluorescence reflectance imaging and intravital confocal microscopy following brain ischaemia in reporter mice. Eur J Nucl Med Mol Imaging. 2013; 40(3): 426–38.

21. Ulya T, Ardianto C, Anggreini P, Budiatin AS, Setyawan D, Khotib J. Quercetin promotes behavioral recovery and biomolecular changes of melanocortin-4 receptor in mice with ischemic stroke. J Basic Clin Physiol Pharmacol. 2021; 32(4): 349–55.
22. Metz GA, Whishaw IQ. Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: A new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. J Neurosci Methods. 2002; 115(2): 169–79.
23. Weber RZ, Bernardoni D, Rentsch NH, Buil BA, Halliday S, Augath MA, *et al.* A toolkit for stroke infarct volume estimation in rodents. NeuroImage. 2024; 287: 120518. doi: 10.1016/j.neuroimage.2024.120518.
24. Hu C, Yang J, Qi Z, Wu H, Wang B, Zou F, *et al.* Heat shock proteins: Biological functions, pathological roles, and therapeutic opportunities. MedComm. 2022; 3(3): e161. doi: 10.1002/mco2.161.
25. Zhao J, Wang T, Lv Q, Zhou N. Expression of heat shock protein 70 and Annexin A1 in serum of patients with acutely severe traumatic brain injury. Exp Ther Med. 2019; 19(3): 1896–902.
26. Popa-Fotea NM, Ferdoschi CE, Micheu MM. Molecular and cellular mechanisms of inflammation in atherosclerosis. Front Cardiovasc Med. 2023; 10: 1200341. doi: 10.3389/fcvm.2023.1200341.
27. Jenei ZM, Széplaki G, Merkely B, Karádi I, Zima E, Prohászka Z. Persistently elevated extracellular HSP70 (HSPA1A) level as an independent prognostic marker in post-cardiac-arrest patients. Cell Stress Chaperones. 2013; 18(4): 447–54.
28. Da Rocha AB, Zanoni C, De Freitas GR, André C, Himelfarb S, Schneider RF, *et al.* Serum Hsp70 as an early predictor of fatal outcome after severe traumatic brain injury in males. J Neurotrauma. 2005; 22(9): 966–77.
29. Wang Y, Wu J, Wang D, Yang R, Liu Q. Traditional Chinese medicine targeting heat shock proteins as therapeutic strategy for heart failure. Front Pharmacol. 2022; 12: 814243. doi: 10.3389/fphar.2021.814243.
30. Alberti G, Paladino L, Vitale AM, Caruso Bavisotto C, Conway de Macario E, Campanella C, *et al.* Functions and therapeutic potential of extracellular hsp60, hsp70, and hsp90 in neuroinflammatory disorders. Appl Sci. 2021; 11(2): 736. doi: 10.3390/app11020736.
31. Pinzon R, Wijaya VO, Paramitha D. Vitamin D status and cognitive performance of post stroke patients. Mol Cell Biomed Sci. 2021; 5(1): 22–6.
32. Wang L, Deng L, Yuan R, Liu J, Li Y, Liu M. Association of matrix metalloproteinase 9 and cellular fibronectin and outcome in acute ischemic stroke: A systematic review and meta-analysis. Front Neurol. 2020; 11: 523506. doi: 10.3389/fneur.2020.523506.
33. Surjawan Y, As'ad S, Ranakusuma TAS, Wijaya A. C-reactive protein and matrix metalloproteinase-9 are associated with outcome of ischemic stroke. Indones Biomed J. 2012; 4(3): 157–63.
34. Chang LL, Li C, Li ZL, Wei ZL, Jia XB, Pang ST, *et al.* Carthamus tinctorius L. Extract ameliorates cerebral ischemia-reperfusion injury in rats by regulating matrix metalloproteinases and apoptosis. Indian J Pharmacol. 2020; 52(2): 108–16.
35. Ao H, Feng W, Peng C. Hydroxysafflor yellow a: A promising therapeutic agent for a broad spectrum of diseases. Evidence-based Complement Altern Med. 2018; 2018: 8259280. doi: 10.1155/2018/8259280.
36. Chen Z, Yang Y, Han Y, Wang X. Neuroprotective effects and mechanisms of senegenin, an effective compound originated from the roots of polygala tenuifolia. Front Pharmacol. 2022; 13: 937333. doi: 10.3389/fphar.2022.937333.
37. Huang S, Lin M, Pan X, Tan Q, Tan KL. The potential of mlc901 (Neuroaid iiTM), a traditional chinese medicine. Neurosci Res Notes. 2019; 2(2): 18–24.
38. Choi JI Ha SK, Lim DJ, Kim SD, Kim SH. S100ß, matrix metalloproteinase-9, D-dimer, and heat shock protein 70 are serologic biomarkers of acute cerebral infarction in a mouse model of transient MCA occlusion. J Korean Neurosurg Soc. 2018; 61(5): 548–58.