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Original Article

Dietary supplementation of *Muntingia calabura* leaves ameliorates reactive oxygen species and malondialdehyde levels: clinical study on alloxan-induced hyperglycemic rats

Nurpudji Astuti Taslim [a, *], Nathania Sutisna [a], Fahrul Nurkolis [b], Faqrizal Ria Qhabibi [c], Rudy Kurniawan [d], Nelly Mayulu [e]

[a] *Division of Clinical Nutrition, Department of Nutrition, Faculty of Medicine, Hasanuddin University, Makassar, 90245, Indonesia*
[b] *Department of Biological Sciences, State Islamic University of Sunan Kalijaga (UIN Sunan Kalijaga), Yogyakarta, Yogyakarta, 55281, Indonesia*
[c] *Medical School Department, Faculty of Medicine, Brawijaya University, 65145, Malang, Indonesia*
[d] *Alumnus of Department of Internal Medicine, Faculty of Medicine, University of Indonesia – Cipto, Mangunkusumo Hospital, Jakarta, 10430, Indonesia*
[e] *Department of Nutrition, Universitas Muhammadiyah Manado, Manado, 95115, Indonesia*

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SUMMARY

Background and aims: Kersen (*Muntingia calabura L.*) leaves are commonly found in Indonesia and are studied for their antidiabetic effect due to their high flavonoid and antioxidative capacity. Our study aimed to determine the effects of the dietary supplementation of kersen leaves extract on reactive oxygen species (ROS) and malondialdehyde (MDA) levels in hyperglycemic Wistar rats.
Method: We used 44 male adult Wistar rats (*Rattus novergicus*) divided into four groups. Alloxan (100 mg/kg body weight) was used to induce hyperglycemia. Kersen leaves (500 mg/kg body weight) were given to two groups; one group was administered before and after the hyperglycemic condition, and only after the hyperglycemic condition was achieved in the other group.
Results: Comparing ROS between groups after administration of kersen leaves extract on the last day after alloxan administration showed significant changes in ROS, $P = 0.047$ ($P < 0.05$). Regarding oxidative stress, the reactive oxygen species was positive correlated with malondialdehyde but was significant in only one group ($r = 0.733$, $P = 0.024$).

* Corresponding author.
E-mail address: pudji_taslim@yahoo.com (N.A. Taslim).

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Conclusion: Giving kersen leaves extract to Wistar rats with alloxan-induced hyperglycemia can downregulate ROS and MDA levels.

Introduction

Hyperglycemia conditions in individuals diagnosed with diabetes mellitus can cause the process of glucose auto-oxidation, protein glycation, and activation of the polyol metabolic pathway, further accelerating the formation of reactive oxygen compounds [1]. Glucose that undergoes an auto-oxidation process will form hydroxyl radicals. In contrast, glucose that reacts with protein will form Amadori products which, if untreated, will be followed by the formation of advanced glycosylation end-products (AGEs). The accumulation of AGEs in various tissues is the main source of Reactive Oxygen Species (ROS) [2,3]. The increase in reactive oxygen compounds can cause further damage to lipids, DNA, and proteins in various tissues, resulting in an imbalance between protective antioxidants and increased production of free radicals, known as oxidative stress [1].

Oxidative stress is considered a state of imbalance between free radicals and antioxidants. Furthermore, the prevalence of oxidative stress during diabetic complications has increased the production of free radicals and weakened antioxidant defenses [1]. As a result of the free radical attack, the glucose auto-oxidation and protein glycosylation mechanisms can potentially cause damage. Diabetes also influences microsome activity and cytochrome P450 patterns. Changes to the cytochrome P450 system may increase free radical production in the microsomal compartment [1]. ROS occur naturally, but their accumulation can be a marker of oxidative stress. As a result of the increase in free radicals, cell membrane lipid peroxidation increases the final product, namely malondialdehyde (MDA) [2,3]. MDA is an extremely toxic carcinogen that causes tissue damage [1].

Traditional medicine includes a diverse range of indigenous traditions from around the world. Over 80% of the world's population still uses traditional medicine for primary healthcare, demonstrating its tenacity [4]. Plants are the most important source of traditional medicines [5]. They account for over a quarter of all drug candidates trialed for clinical use, globally [6]. According to Newman and Cragg, nearly 80% of new chemical substances observed were derived from natural sources or semisynthetic modifications in cancer research [7]. Bioactive plant products used in drugs are derived from secondary metabolites. These substances are classified as phenolic, including polyphenols, tannin, and quinone, and flavonoids are well-known for their antioxidant, cytotoxic, and antimicrobial properties [8–10]. Nevertheless, the underutilization of plants continues to hinder drug development. Plant-derived substances and their derivative products are widely used in other clinical uses, most notably as chemotherapeutic agents [11].

Muntingia calabura L., locally known as Kersen (Makassar), Talok (Java), or Kerukup Siam (Malaysia), is a medicinal herb that is currently being studied extensively for its antidiabetic, antioxidant, antinociceptive, antiulcer, and anti-inflammatory effects [12]. This plant originated in Southern Mexico but is also found in Southeast Asia [12]. As one of the many plants easily found in Indonesia, *Muntingia calabura L.* (Kersen) has the potential to provide an alternative treatment for hyperglycemia and could be used as an antioxidant for preventing complications related to oxidative stress.

Therefore, we aimed to examine the potential antihyperglycemic and antioxidant effects of *Muntingia calabura L.* (Kersen) extracts by measuring Reactive Oxygen Species (ROS) and malondialdehyde (MDA) in alloxan-induced hyperglycemic Wistar rats.

Materials and methods

Clinical study design for rats

Rats were housed in cages and kept in a climate-controlled environment (27 °C, 50–60% relative humidity) with a balanced light-dark cycle. All rats were acclimated in the lab for 10 days before the experiment. During the research, rats had unrestricted access to conventional animal feed or pellets from Citra Ina Feedmill, PT, and drinking water. The rats were randomly divided into four treatment groups after the 10-day acclimatization period. This study used a laboratory experimental method. A total of 44 adult male Wistar (*Rattus novergicus*) rats aged 8–10 weeks were randomly selected and divided into four groups. Determination of sample size in experimental animals refers to the method/protocol and sample size formula in similar published studies using experimental animals [32–34].

An intraperitoneal injection of 100 mg/kgBW alloxan was administered to induce hyperglycemia. We obtained *Muntingia calabura* L. leaves extract using vacuum oven methods from established protocol [31], which were authenticated by Hasanuddin University Research Centre for Clinical Nutrition (#CLIN2023). The division of the animal groups was.

1. Group 1 (K1): aquadest was given through a feeding tube during the research period. This group did not get an injection of alloxan 100 mg/kgBW.
2. Group 2 (K2): alloxan 100 mg/kgBW/day was administered on the 10^{th} day. Then aquadest was given through a feeding tube.
3. Group 3 (K3): 500 mg/kgBW/day of kersen leaves extract (via feeding tube) was administered 7 days before alloxan administration (day 0) until the 17^{th} day.
4. Group 4 (K4): 500 mg/kgBW/day of kersen leaves extract (via feeding tube) was administered from the 3^{rd} day post alloxan induction (10^{th} day) until the 17^{th} day.

In each group, intra-cardiac blood samples were taken to examine Random Blood Glucose, ROS, and MDA.

This research was conducted in the animal laboratory at the Faculty of Medicine, Hasanuddin University, Makassar. MDA examination was carried out at the Hasanuddin University Medical Research Center laboratory. The research was carried out from September 2021 to October 2021.

Inclusion and exclusion criteria

The inclusion criteria in this study included: (1) Wistar rats (*Rattus novergicus*), (2) male gender, (3) body weight around 150–200 grams, (4) 8–10 weeks old, (5) normal behavior and activities, (6) no visible anatomic abnormalities, (7) no visible appearance of dull hair, loss, or baldness also active movement, and (8) blood glucose > 200 mg/dl 3 days after alloxan administration. The exclusion criteria included: (1) abnormal exudate discharge from the eyes, mouth, anus, and genitals and (2) death during the adaptation period.

Biomedical analysis of blood sampling procedure

The sample was put into a serum separator tube and agglomerated for 30 minutes before being centrifuged for 15 minutes with approximately 1000 x g. Blood samples were stored in the Hasanuddin University Research Medical Center Laboratory. Blood glucose was measured using the glucometer Accu-Check. ROS was measured using the ROS Elisa kit, and blood MDA levels were measured using the MDA Elisa kit. Among the collected sample, only 34 samples were able to be analyzed for statistical analysis (Figure 1).

The data were analyzed using SPSS for Windows version 23 (SPSS Inc., Chicago, III., USA). The data obtained in this study were analyzed using the normality test first. In this case, the Shapiro–Wilk test was the normality test. If the data were normally distributed, then the next tests used the independent t-test, ANOVA test, and Pearson's test. However, if the data were not normally distributed, the

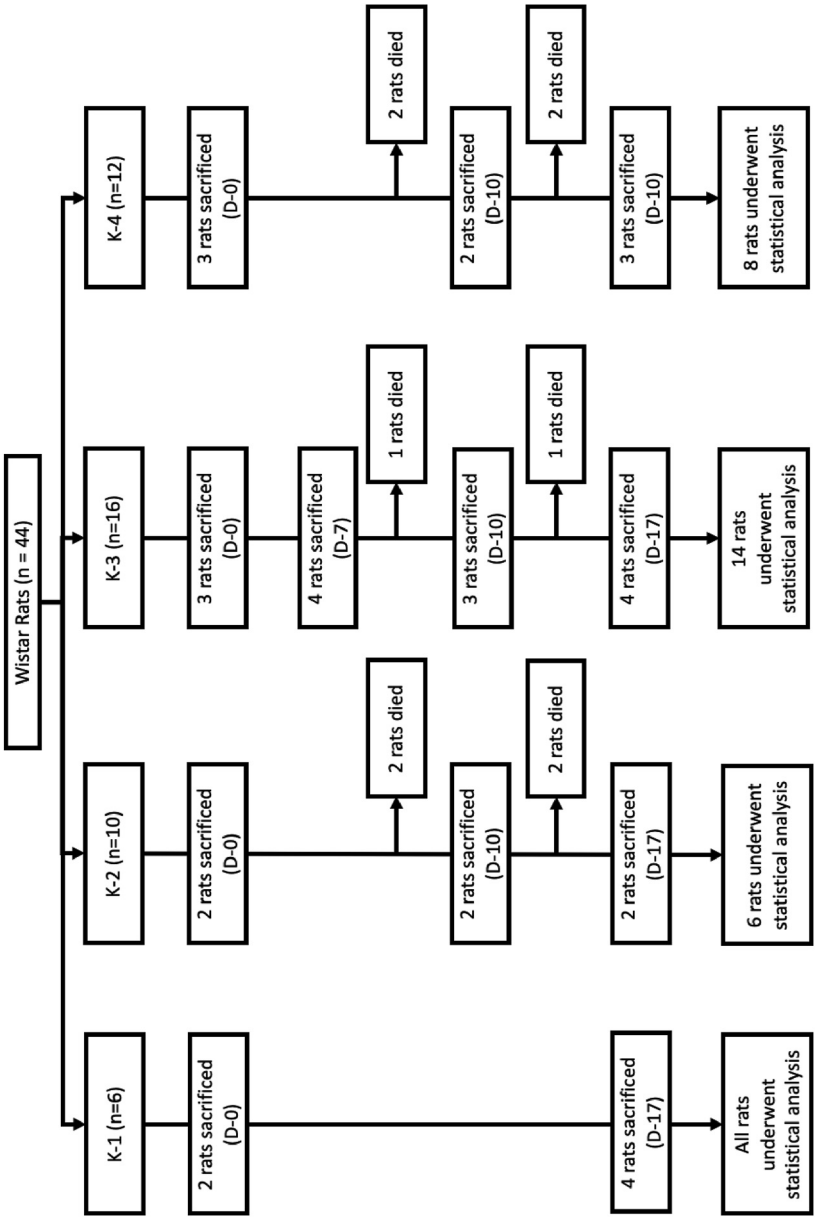

Figure 1. Flowchart of The Study.

Mann–Whitney U test, the Kruskal–Wallis test, and the Spearman test were used. A p-value below 0.05 represented statistical significance (95%CI).

Ethical clearance

The research received ethical approval from the Research Ethics Commission of the Faculty of Medicine, Hasanuddin University, with ethics number 578/UN4.6.4.5.31/PP36/2021. This experimental research was conducted by applying the 3R principle in the research protocol: replacement, reduction, and refinement. The animal use research protocol makes mention of the Declaration of Helsinki and the Council of International Organizations of Medical Sciences (CIOMS). Additionally, all procedures involving animal research adhered to the Guidelines for Reporting *In Vivo* Experiments (ARRIVE).

Results

Table 1 shows the basic characteristics of all Wistar rats included in the study. The ANOVA test was carried out to determine statistical differences. It was found that all groups were not statistically significant in blood glucose ($p = 0.077$). The Kruskal–Wallis test was carried out to determine statistical differences, revealing that all groups were statistically significant in ROS ($p < 0.001$) and MDA ($p = 0.019$).

Table 2 shows the ROS ratio of mice in each group (K1, K2, K3, and K4). Data are presented in terms of mean ± standard deviation and median. Table 3 shows the comparison between groups post alloxan injection in D10 and D17. Data ROS values (mean) in each subgroup are found in Figure 2.

Table 3 shows that kersen leaves extract supplementation had a good antioxidant effect, as observed in groups K3 and K4. Significance was observed on Day-17 (D17); K4 significantly reduced ROS (Table 3 and Figure 2).

Table 4 compares MDA in each group (K1, K2, K3, and K4). Data are presented in terms of mean ± standard deviation and median. Table 5 compares groups post-alloxan injection in D10 and D17. MDA values (mean) in each subgroup are found in Figure 2.

Table 6 shows the results of the correlation test. Based on Sugiyono's (2014) work, the interpretation of the correlation coefficient consists of (1) 0.00–0.199 (very low), (2) 0.200–0.399 (low), (3)

Table 1
Basic characteristics of ROS and MDA.

Group	Blood glucose value (mean ± SD) ng/L	ROS value (mean ± SD) ng/L	MDA value (mean ± SD) nmol/ml
K1	112.20 ± 19.90	210.27 ± 22.84	0.83 ± 0.19
K2	187.20 ± 58.90	198.62 ± 23.74	2.92 ± 1.78
K3	183.00 ± 104.00	39.87 ± 94.00	1.44 ± 0.91
K4	196.30 ± 70.20	52.04 ± 29.24	1.84 ± 1.40
	$p = 0.077$[a]	$p<0.001$[b]	$p = 0.019$[b]

[a] ANOVA test.
[b] Kruskal–Wallis test.

Table 2
ROS levels in each subgroup.

Group	Reactive oxygen species (ROS) (ng/L)					
	D0		D10		D17	
	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median
K1	196.26 ± 15.56	196.26	-	-	217.28 ± 24.33	223.71
K2	216.18 ± 29.09	216.18	198.69 ± 18.85	198.69	180.98 ± 19.40	180.98
K3	137.8 ± 37.53	140.87	213.15 ± 17.46	221.21	216.63 ± 21.35	222.34
K4	34.67 ± 7.35	33.77	91.07 ± 21.92	91.07	43.40 ± 24.70	53.6

D0: day 0 or without intervention; D10: day ten of intervention; D17: day seventeen of intervention.

Table 3
Comparison of ROS between groups at D10 and D17 after alloxan injection.

Day	Group	Difference mean ROS	Difference median ROS	*p*-value	*p*-value
Day-10	K1 vs K3	14.46	22.52	0.248[a]	0.107[c]
	K2 vs K4	107.62	107.62	0.034[b]	
	K3 vs K4	122.08	130.14	0.083[a]	
Day-17	K1 vs K2	36.3	42.73	0.145[b]	0.047[c]
	K1 vs K3	0.65	1.37	0.773[a]	
	K1 vs K3	173.88	170.11	0.000[b]	
	K2 vs K3	35.65	41.36	0.165[a]	
	K2 vs K4	137.58	127.38	0.007[b]	
	K3 vs K4	173.23	170.9	0.034[a]	

[a] Independent t-test.
[b] Mann–Whitney U test.
[c] Kruskal–Wallis test.

0.400–0.599 (moderate), (5) 0.600–0.799 (strong), and (5) 0.800–1.00 (very strong). In the K4 group, we found the correlation was statistically significant with *p*-value = 0.024 ($p < 0.05$), and the magnitude of the coefficient of ROS with MDA was 0.733 (strong positive correlation).

Discussion

Alloxan (2,4,5,6-tetraoxypyrimidine; 2,4,5,6-pyrimidinetetrone) is a derivative of oxygenated pyrimidine that is easily discovered in an aqueous solution as an alloxan hydrate. Alloxan acts as a selective inhibitor of glucose-stimulated insulin secretion (with specific inhibition of glucokinase) and selective necrosis of pancreatic beta cells due to ROS formation; thus, it is commonly used as a diabetes-inducing agent for experimental mice [13,14]. Alloxan can trigger ROS formation through a cyclic reaction with its reduction product, namely dialuric acid [15]. The formation of ROS triggers oxidative stress in experimental animals.

Increasing oxidative stress and various pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β) are induced by the presence of excessive ROS, causing diabetic nephropathy, which leads to end-stage renal disease [16]. Furthermore, insulin resistance due to increasing ROS in the hyperglycemic state contributes to cardiovascular disease (CVD)

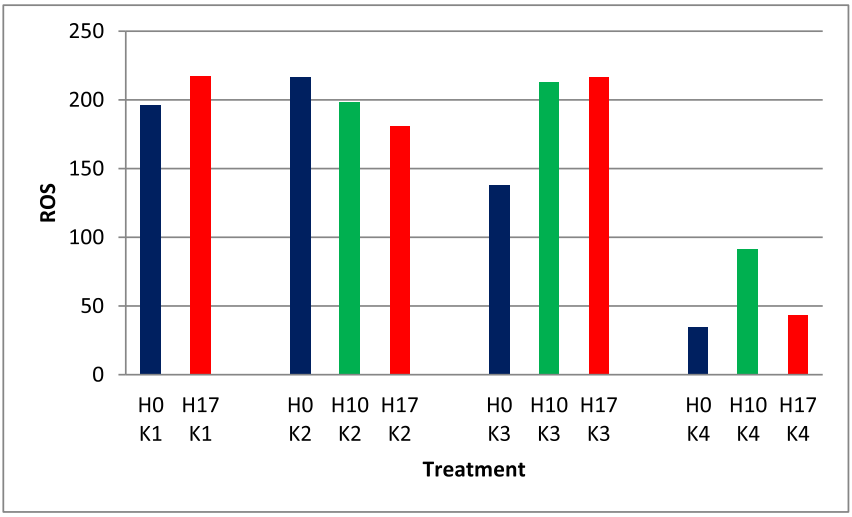

Figure 2. Bar chart showing the ROS values of each group. H0: day 0 or without intervention; H10: day ten of intervention; H17: day seventeen of intervention.

Table 4
MDA level in each group.

Group	Malondialdehyde (MDA)					
	D0		D10		D17	
	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median
K1	1.04 ± 0.11	1.04	-	-	0.73 ± 0.10	0.71
K2	1.00 ± 0.06	1.00	3.73 ± 2.13	3.75	4.03 ± 0.30	4.03
K3	1.30 ± 0.57	1.12	2.97 ± 0.64	2.84	0.93 ± 0.10	0.94
K4	1.63 ± 0.37	1.67	3.95 ± 0.92	3.95	0.79 ± 0.33	0.90

D0: day 0 or without intervention; D10: day ten of intervention; D17: day seventeen of intervention.
In MDA (both D10 and D17), there was no significant difference between K3 and K4, meaning that both had MDA-reducing activity in alloxan-induced rats (Table 5).

Table 5
Comparison of MDA between groups at D10 and D17 after alloxan injection.

Day	Group	Difference mean ROS	Difference median ROS	*p*-value	*p*-value
Day-10	K2 vs K3	0.77	0.91	1.000[a]	0.700[c]
	K2 vs K4	0.21	0.21	0.912[b]	
	K3 vs K4	0.98	1.11	0.248[a]	
Day-17	K1 vs K2	3.3	3.32	0.000[b]	0.070[c]
	K1 vs K3	0.2	0.23	0.043[a]	
	K1 vs K3	0.06	0.19	0.727[b]	
	K1 vs K3	3.1	3.09	0.064[a]	
	K2 vs K4	3.24	3.13	0.002[b]	
	K3 vs K4	0.14	0.04	0.858[a]	

[a] Independent test.
[b] Mann–Whitney U test.
[c] Kruskal–Wallis test.

Table 6
Correlation of ROS and MDA.

Group	Coefficient	*p*-value	Description
K1	−0.393	0.441[a]	No correlation
K2	−0.349	0.497[a]	No correlation
K3	−0.046	0.876[b]	No correlation
K4	0.733	0.024[a]	Positive correlation

[a] Pearson test.
[b] Spearman test.

through endothelial vascular dysfunction, atheroma plaque formation, diastolic abnormality, and ventricular hypertrophy [16,17]. Based on the various clinical problems mentioned, it is important to discover potential drugs that have a mechanism of action in reducing ROS formation or stored ROS. In particular, these drugs should come from natural sources considering nearly 80% of new chemical substances observed were derived from natural sources.

MDA is a metabolite resulting from lipid peroxidation by free radicals with the formula $C_3H_4O_2$ [18]. MDA is one of the most frequently used indicators of fat peroxidation. Sanchez (2020) strengthens this statement, stating that the MDA mediator is an end product of fat peroxidation used as a biological biomarker of fat peroxidation and can determine the degree of oxidative stress [19]. Damage to organ cells occurs in the phospholipid unsaturated fatty acids (PUFA) of cell membranes, forming lipid peroxides [20]. MDA can be used to indicate increased lipid peroxide formed due to oxidative stress [21].

The decrease in MDA levels after administering kersen leaves extract in hyperglycemic rats was related to the antioxidant ability of kersen leaves extract. Kersen leaves extract contains compounds such as flavonoids, saponins, alkaloids, triterpenoids, tannins, and glycosides [22–24]. This finding is in

accordance with the research of Karita *et al.* (2021), who suspected that kersen leaves extract could reduce MDA levels due to the activity of flavonoids (2.4-dihydroxy-3-methoxydihydrochalcone, 8-hydroxy-6-methoxyflavone, quercetin, rutin, fisetin) and saponins contained in kersen leaves. These compounds have been validated by previous studies [25,31].

Flavonoids can prevent injury caused by free radicals in several ways [26]— by scavenging free radicals directly. Flavonoids are oxidized by free radicals, thus making the radicals more stable and less reactive [27]. In other words, flavonoids will stabilize ROS and react with reactive radicals [28]. Flavonoids act as scavengers of hydroxy and super hydroxy radicals, thereby protecting pancreatic cell lipid membranes against damaging reactions. Flavonoids donate an atom (H) of the phenolic hydroxyl (OH) group when reacting with free radicals [29]. Flavonoids play a role in suppressing pro-inflammatory cytokines, modulating transcription factors and inflammatory pathways, and reducing ROS levels by acting as scavengers against free radicals [30]. In addition, flavonoids are also known to reduce lipid peroxidation and restore insulin receptor sensitivity in cells. This condition causes a decrease in blood glucose levels.

The current study revealed significant results in the K4 group compared to the K3 group. However, it remains unclear how the mechanism of protection against damage due to fat peroxidation is better in the administration of kersen (*Muntingia calabura L.*) leaves extract after pancreatic damage occurs. This experiment demonstrates and confirms that kersen (*Mautingia calabura L.*) leaves extract contains numerous bioactive substances that are capable of repairing ROS and MDA conditions.

To the best of the author's knowledge, this study was the first to assess the preventive effect of kersen leaves extract before pancreatic cell damage occurs. This study is a clinical study on experimental animals; it does not yet represent efficacy in humans. However, because this is the first study to test the efficacy of kersen leaves extract on ROS and MDA amelioration, this dose can be used as an adjuvant to human clinical trials in the future.

Conclusion

Dietary supplementation of *Muntingia calabura L.* ameliorates the reactive oxygen species and malondialdehyde levels in alloxan-induced hyperglycemic rats. This shows the potential of *Muntingia calabura* leaves as a functional food candidate for improving diabetes and oxidative stress condition. Of course, clinical trials in humans are urgently needed and are being planned.

Author contributions

NAT and NS: conduct experiments, analyzed data, write the manuscript, design research, and conceptualize ideas; while FN, NAT, FRQ, RK, and NM: contribute to data analysis, critiquing manuscript, interpret manuscript results, assisting in the processing of data, as well as helping to revise and visualization or illustration editing. Authors and contributors have read and also approved this final manuscript.

Data availability statement

The data presented in this study are available on request from the corresponding author.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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