ORIGINAL ARTICLE

Hepatoprotective Effects and Testicular Toxicity of Centella asiatica L. Aqueous Extract in Diabetic Rats

Aszrin FN[a], Adam SH[b], Abdul Mutalib M[a], Hooi CT[c], Shirley Gee HT[d], Mohamad Shariff MH[e], Mokhtar MH[f]

[a]School of Graduate Studies, Management and Science University, Selangor, Malaysia;
[b]Pre-clinical Department, Faculty of Medicine and Defense Health, Universiti Pertahanan Nasional Malaysia, Malaysia
[c] Department of Microbiology, Faculty of Medicine, Manipal University College Malaysia, Melaka, Malaysia
[d]Center for Toxicology and Health Risk Research (CORE), Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Selangor, Malaysia
[e]Faculty of Health Sciences, University Technology MARA (UiTM) Pulau Pinang, Bertam Campus, Pulau Pinang, Malaysia
[f]Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia

ABSTRACT

INTRODUCTION: Centella asiatica has been widely studied as an herbal substitute for treating diabetes mellitus (DM). This study aimed to investigate the effects of C. asiatica on the liver and testes in a rat model of type 2 diabetes mellitus (T2DM). **MATERIALS AND METHODS:** Forty adult male Sprague-Dawley rats were induced with diabetes using a single intraperitoneal injection of streptozotocin-nicotinamide (STZ-NA). Three days after induction of diabetes, the rats were treated with either 250 or 500 mg/kg body weight/day of C. asiatica aqueous extract (CAAE) for 48 days. Serum, liver, and testes were collected for analysis. Parameters measured included fasting blood glucose (FBG), body and organ weights, gonadosomatic and hepatosomatic index, testicular steroidogenesis activity (HSD17B3, testosterone levels, sperm count), and liver biochemical markers. Additionally, antioxidant and lipid peroxidation levels, together with sperm count were assessed in the liver and testes respectively. **RESULTS:** CAAE treatment improved FBG levels and mitigated weight loss in the STZ-NA group. Oxidative stress markers were ameliorated in both organs after CAAE treatment. Liver serum biochemical markers showed improvement, while testicular steroidogenic function declined. Sperm count decreased compared to the STZ-NA group. **CONCLUSION:** CAAE ameliorates hyperglycaemia and oxidative stress in the liver and testes but may cause testicular dysfunction in DM. In conclusion, the study demonstrated that C. asiatica is able to reverse STZ-NA-induced oxidative stress and hyperglycaemia but exacerbates testicular dysfunction.
evaluate our study outcome.

Keywords
Centella asiatica; antioxidant; antidiabetic; antifertility; liver.

Corresponding Author
Dr. Siti Hajar Adam
Pre-clinical Department,
Faculty of Medicine and Defense Health,
Universiti Pertahanan Nasional Malaysia,
57000 Kuala Lumpur, Malaysia
Email: siti.hajar@upnm.edu.my

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease that affects the world's population. In Malaysia, the prevalence of DM is estimated to be 31.3% and could affect 7 million people aged 18 years and older by 2025.[1] Type 2 diabetes mellitus (T2DM) is mainly associated with a disturbance of glucose homeostasis in the body due to insulin resistance. Insulin resistance exacerbates the development of oxidative stress, leading to impaired cell and organ function, resulting in morbidity and mortality.[2,3] Increased oxidative stress and free radical formation tend to contribute to the development of diabetic complications affecting organs such as the testes, liver, kidneys, and eyes.[4-8]

In the liver, insulin resistance impairs its overall function, resulting in increased glucose production and breakdown, causing elevated blood glucose levels and dysregulation of liver enzymes.[9] Insulin resistance and oxidative stress serve as the key trigger in causing testicular dysfunction, decreased gonadotropin secretion and abnormal sperm parameters, ultimately contributing to infertility.[10-12]

Current T2DM treatments mainly consist of prescribing oral hypoglycaemic agents (OHA) with or without insulin along with lifestyle modification. However, the long-term use of OHAs poses risks such as hypoglycaemia, gastrointestinal discomfort, weight gain, allergic reactions, and vitamin B12 deficiency.[13] These side effects may lead to non-adherence to treatment, necessitating exploration of alternative therapies.

Centella asiatica, or '*pegaga*,' offers diverse pharmacological benefits including antioxidant, anti-inflammatory, antidiabetic, hepatoprotective, wound healing, sedative, and anxiolytic effects.[14-17] These properties stem from phytochemical compounds like triterpene glycosides (madecassic acid, asiatic acid), madecassoside, and asiaticoside,[18] which are beneficial to human health. *C. asiatica* increases insulin secretion,[19] aiding in pancreato-protection and alleviating diabetic nephropathy and neuropathy[20] through compounds like asiaticoside, brahmoside, and brahminoside.[21] However, potential side effects at standard doses remain unclear, including conflicting impacts on male reproductive function.[22,23]

The current study aimed to determine the effects of *C. asiatica* aqueous extract (CAAE) on liver and testicular function in a T2DM rat model. This study could fill the knowledge gap on the role and limitations of *C. asiatica* as an alternative treatment for T2DM.

MATERIALS AND METHODS

Centella asiatica Aqueous Extract (CAAE) Preparation

Fresh vacuum-packed *C. asiatica* powder was obtained from Secret Barn Sdn. Bhd., Sungai Petani, Kedah, and verified by a taxonomist at the Institute of Bioscience, Universiti Putra Malaysia (Voucher No: MFI 0212/21). The extraction method was adapted from Kumari et al. 2016 with slight modification involved mixing the powder with distilled water (1:6 ratio), immersing in a 95°C water bath for 20 minutes, macerating in an orbital shaker (Thermo Fisher Scientific, SHKE4000) for 48 hours at room temperature, and filtering using Whatman No. 1 filter paper. The filtered solution was freeze-dried and stored at -20°C.[22]

Experimental Animals

Forty healthy male adult Sprague-Dawley rats (150-250 g) were housed at 25±3°C with good ventilation and a 12-hour light-dark cycle. They had access to standard pellets and water *ad libitum* throughout the experiment. The MSU Ethics Committee guidelines (MSU-RMC-02/FR01/11/L3/004) were followed.

Induction of Diabetes

To induce diabetes, rats were injected intraperitoneally with 65 mg/kg streptozotocin-nicotinamide (STZ-NA) in cold 0.1M sodium citrate buffer (pH 4.5) after 16 hours of fasting with water provided *ad libitum*.[23] Nicotinamide (100 mg/kg) was administered before the STZ injection to minimize β-cell destruction. After 72 hours, blood glucose levels were measured from the tail vein using an ACCU-CHECK Active Glucose Monitor (Roche, Germany). Rats with blood glucose levels >16.7 mmol/L on day 7 were considered diabetic and used for further experiments.[23]

Experimental Design

Forty rats were divided into 5 groups of 8 (Figure 1). After confirming diabetes, each group received a fixed daily ration, pre- and post-weighed after 24 hours to determine food consumption. Daily consumptions were recorded and fresh provisions replenished. Treatment doses were based on Deshpande et al. (2015).[24]

Oral Glucose Tolerance Test (OGTT) and Intraperitoneal Insulin Tolerance Test (IPITT)

OGTT and IPITT were performed 48 days post-treatment. Rats were fasted overnight before each test. For OGTT, rats received glucose (2 g/kg), and blood glucose levels were measured from the tail vein at 0, 30, 60, 90, 150, and 210 minutes. For IPITT, conducted 48 hours later for recovery, same set of rats received insulin (0.75 units/kg), and blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 minutes.

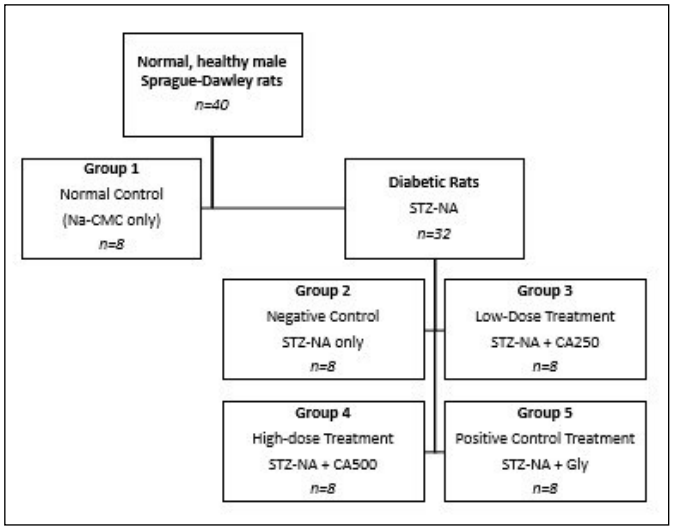

Figure 1. Animal grouping for study design

Sample collection

Blood samples were collected from overnight-fasted rats via cardiac puncture 48 days post-treatment. The samples were centrifuged at 2200 rpm for 10 minutes (Hettich EBA20, Germany) for plasma and serum analyses. Liver and testes were excised, weighed, washed in ice-cold saline, and stored at −80 °C. Tissue homogenates were prepared by homogenizing 0.1 g of tissue in 1 ml of phosphate-buffered saline (pH 7.4) and centrifuged at 5,000 g for 5 minutes at 4 °C to obtain the clear supernatant for specific assays.

Assessment of Serum Parameters

Testosterone levels were assessed using an ELISA kit (CSB-E05100r). 50µL each of supernatant, HRP-conjugate, and antibody solution were added to each well in duplicates, mixed, and incubated for 1 hour at 37°C. Wells were aspirated, washed with 200µL of wash buffer, and left to stand for 10 seconds. Then, 50µL each of Substrate A and Substrate B were added, incubated for 15 minutes at 37°C, and mixed with 50µL of stop solution. Optical density was measured at 450nm.

Liver enzymes (Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), and Alkaline Phosphatase (ALP)) were determined using diagnostic kits. Serum samples were analysed at the UPM Veterinary Centre (Case No.: R-2164). Serum was separated by centrifugation, and liver function parameters namely total bilirubin, and total protein were measured using established biochemical assays. Quality control measures were implemented, and data were analysed statistically.

Tissue Somatic Index

Before sacrifice, initial and final rat weights were recorded to calculate the gonadosomatic index (GSI) and hepatosomatic index (HSI). GSI and HSI assesses organs' size and weight. After sacrifice, testes and liver were weighed using an electronic scale (Shimadzu, BL2200H, Japan), fixed in 10% formalin, snap-frozen in liquid nitrogen, and stored at -20°C for future analysis. The tissue somatic index was calculated using the following formula:

$$Tissue\ Somatic\ Index\ (\%) = \frac{Organ\ Weight\ (g)}{Body\ Weight\ (g)} \times 100$$

Assessment of Antioxidant Activities

Lipid peroxidation in organs' tissue was measured using the ElabScience® Malondialdehyde (MDA) Colorimetric Assay Kit (E-BC-K025-S), with the TBARS method used to quantify MDA levels. Tissue homogenate in 10% phosphate buffer (pH 7.4) was prepared using an ultrasonic homogeniser (Ross, EQX-WT500-P2, USA), and post-mitochondrial supernatant (PMS) was obtained by centrifugation. Antioxidant activities were determined using the Catalase (CAT) Activity Assay Kit (E-BC-K031-S), Total Superoxide Dismutase (T-SOD) Activity Assay Kit (WST-1 Method) (E-BC-K025-M), and Glutathione Peroxidase (GSH-Px) Activity Assay Kit (E-BC-K096-S), following the manufacturers' instructions.

Assessment of Testicular Steroidogenic Enzymes Assay

Testicular tissue was homogenized (10%) in phosphate buffer (pH 7.4) using an ultrasonic homogenizer, followed by centrifugation at 10,000 rpm for 20 minutes at 4°C to obtain PMS. Enzyme activity of 17β-hydroxysteroid dehydrogenase (HSD17B3) was measured using the Human HSD17B3 ELISA Kit (EH14680) from FineTest. Results were expressed as enzyme activity per protein unit or as a percentage of control for the tissue samples.

Determination of Sperm Count

Epididymal sperm was diluted 1:20 to achieve optimal density for counting. Sperm count was determined using a haemocytometer under a Labomed LX-500 LED digital microscope (USA).

Statistical Analysis

Overall results were analysed using SPSS for Windows version 29.0 and GraphPad Prism 9 software. Values were expressed as Mean ± SD and analysed by one-way ANOVA. Tukey's post hoc test was used to detect differences between experimental groups. Statistical significance was set at $p<0.05$ and $p<0.001$, indicating different levels of significance. Results with $p<0.001$ were considered highly significant, while those with $p<0.05$ were considered statistically significant.

RESULTS

Effects of CAAE on Metabolic Parameters

Table 1 shows the body weight, food, and water intake data for the experimental animals. On Day 1 of treatment, the STZ-NA group exhibited significant changes ($p < 0.001$) in all three parameters compared to the normal group. Low-dose CAAE treatment increased weight by 20%, while high-dose CAAE and Glybenclamide (Gly) treatments showed a more substantial increase by 33.2% and 57% respectively. However, both food and water intake decreased by an average percentage of 13.86% in the CAAE group and 26.14% in the Gly-treated group when compared to the STZ-NA group.

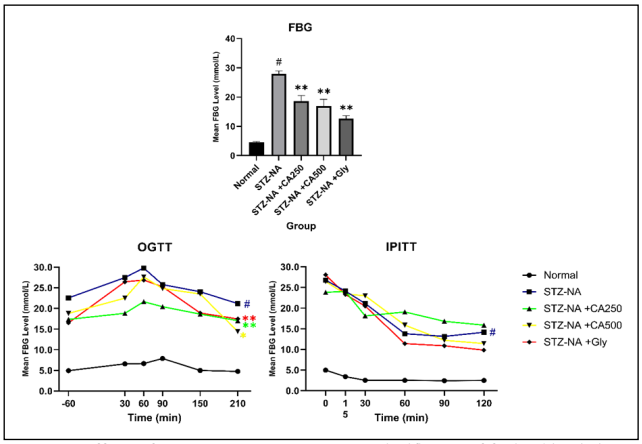

Figure 2. Effects of CAAE on serum parameters. Significance of fasting blood glucose (FBG); oral glucose tolerance test (OGTT), and intraperitoneal insulin tolerance test (IPITT) are presented as #p<0.001 vs Normal, **p<0.001 vs STZ-NA group; #p<0.001 vs Normal, *p<0.05 **p<0.001 vs STZ-NA group; #p<0.001 vs Normal, respectively.

Effects of CAAE on the Liver

Figure 3 illustrates the impact of CAAE on liver enzymes (AST, ALP, ALT), hepatic lipid peroxidation, antioxidant enzymes, and HSI. In the STZ-NA group, liver enzymes increased significantly by 73.9% compared to the normal group ($p<0.001$). CAAE treatment reduced liver enzyme levels, with the STZ-NA+Gly group approaching near-normal levels. Malondialdehyde MDA increased in the STZ-NA group by 147.2% and decreased significantly with CAAE or Gly treatment. GPx activity improved by 54.5% post-treatment, along with increased CAT, and T-SOD activities. HSI values in the untreated diabetic group were significantly higher (73.7%) compared to the Normal group, with all treatment groups showing a significant descent ($p<0.001$). The STZ-NA+Gly group approached the normal group's HSI. Higher CAAE doses correlated with a gradual decrease in HSI.

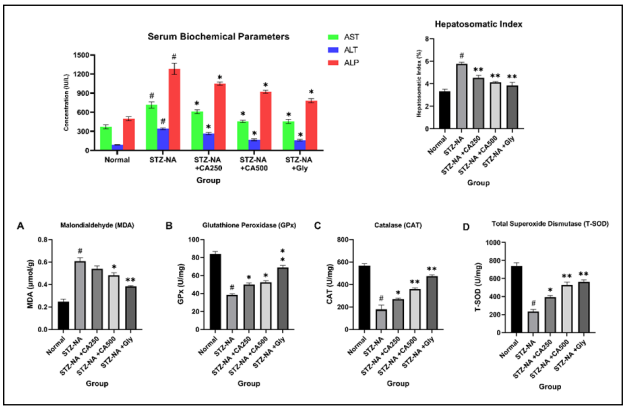

Figure 3. Effect of CAAE towards the liver. Significance of AST, ALT and ALP; Lipid peroxidation and antioxidative enzymes activity levels; HSI are presented as #p<0.001 vs Normal, *p<0.05 vs STZ-NA group; *p<0.05 **p<0.001 vs STZ-NA group; #p<0.001 vs Normal, **p<0.001 vs STZ-NA group, respectively.

Effects of CAAE Towards on Testes

Figure 4 shows the outcomes of lipid peroxidation, antioxidant enzymes, testicular steroidogenesis activity, and GSI levels. In the STZ-NA group, MDA levels increased fourfold in value compared to the normal group, indicating oxidative stress, whereas CAAE treatments approached normal levels. Our study showed a significant increase in antioxidant enzyme activity observed in the CAAE-treated and STZ-NA+Gly groups compared to the negative control group. Testicular HSD17B3 levels significantly decreased by 38.3% in the STZ-NA group, improving slightly ($p<0.001$) with CAAE treatment. Testosterone levels decreased by 50.6%

(p<0.001) in STZ-NA, further reduced with CAAE, while Gly-treated group increased by 11.86% relative to STZ-NA group. Sperm count decreased (p<0.01) in STZ-NA, with CAAE reducing it further, while Gly increased it. GSI decreased (p<0.01) in the STZ-NA group, with both CAAE treatments showing a similar decreasing trend.

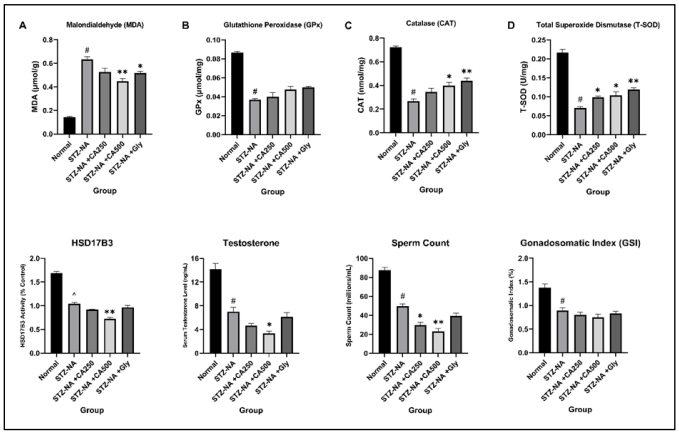

Figure 4. Effects of CAAE Towards the Testes. Significance of lipid peroxidation and antioxidative enzymes activity levels; HSD17B3; testosterone levels; Sperm count; GSI are presented as #p<0.001 vs Normal, *p<0.05 **p<0.001 vs STZ-NA group; ^p<0.05 vs Normal, **p<0.001 vs STZ-NA group; #p<0.001 vs Normal, *p<0.05 vs STZ-NA group; #p<0.001 vs Normal, *p<0.05 **p<0.001 vs STZ-NA group; #p<0.001 vs Normal, respectively.

DISCUSSION

Findings from this study demonstrated the protective effect of CAAE on liver function and potential toxic effects on the testes in STZ-NA-induced male diabetic rats. Dosages of 250 mg/kg/day and 500 mg/kg/day were chosen based on effective doses reported in previous study.[24] The treatment duration of 48 days was selected to evaluate the sub-acute progression of diabetes and observe diabetic complications such as polyphagia and polydipsia.[24]

Administering CAAE for 48 days improved fasting blood glucose levels, consistent with previous observations.[25] The hypoglycaemic effect of *C. asiatica* is attributed to active compounds such as asiatic acid,[26] asiaticoside,[27] and madecassoside,[28] known for their glucose-lowering activities. This was supported by the OGTT and IPITT results. The OGTT in the final week showed a rapid decline in hyperglycaemic peaks in the CAAE-treated group, indicating enhanced peripheral disposal of glucose load.[29] Similarly, IPITT results demonstrated reduced blood glucose levels compared to the diabetic control group, suggesting improved insulin sensitivity possibly through enhancements in insulin receptors, glucose transporters, or enzymes involved in glucose phosphorylation.[30]

Furthermore, *C. asiatica* stimulates insulin secretion from pancreatic β-cells,[31] enhancing insulin availability and alleviating symptoms of diabetes mellitus like polydipsia and polyphagia. Additionally, improved blood glucose levels contribute to reduced fat and muscle catabolism for energy,[30] thereby preventing excessive weight loss.

In this study, diabetic rats showed decreased liver weight with increased serum liver enzymes. Elevated AST and ALT levels may result from increased demand for gluconeogenic substrate and compromised hepatocyte membrane integrity, that led to substrate leakage.[32,33] Administering CAAE for 48 days reduced serum AST and ALT levels. Given hyperglycaemia-induced liver injury,[34] the hepatoprotective effect of CAAE likely stems from improved glucose control, as observed in this study. Choi et al. (2016) also reported a similar hepatoprotective effect of *C. asiatica*, showing improvement in liver histoarchitecture and fibrosis in dimethyl nitrosamine-induced liver injury in rats.[35]

In T2DM, overproduction of reactive oxygen species (ROS) contributes to liver disease progression by depleting antioxidant enzyme activities such as glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD). CAAE has proved to mitigate the disruption of oxidative redox balance that caused liver damage in T2DM by enhancing liver antioxidant enzymes.[35-37] Administering CAAE reverses the depletion of antioxidant defence enzymes (CAT, GPx, and SOD) in the liver and reduces lipid peroxidation. *C. asiatica* possess potent antioxidant compounds that can prevent the free radical-induced peroxidation of liver damage by its scavenging ability.[36]

Despite the hepatoprotective effect of CAAE, its effect on the testes remains controversial. Some studies have revealed the fertility-inhibiting effect of *C. asiatica,*[38-40] while other studies have reached opposite conclusions.[41]

In this study, administration of CAAE able to restored OS and lipid peroxidation in the testes. CAAE inhibits lipid peroxidation by scavenging free radicals,[42] while preserving the integrity of the testicular cell membrane,[41,43] and protecting against oxidative damage. It also restores depleted antioxidant enzymes such as SOD and GPx, which are critical for neutralizing free radicals and reducing oxidative stress.[44]

Interestingly, CAAE caused greater testicular dysfunction despite restoration of oxidative status, as evidenced by the reduction in sperm count, testosterone, and HSD17B3 enzymes in this study. These results suggest that, despite its strong antioxidant potential, CAAE may have a direct toxic effect on the male reproductive system. This reinforces the damage caused by the lack of insulin in DM, which blocks the secretion of gonadotropins, leading to a deficiency of testosterone.[45] In addition, the key enzyme required for the conversion of androstenedione to testosterone, HSD17B3 was decreased, which may indicate a direct toxic effect of CAAE on Leydig cells.

The Leydig cell is the primary androgen source that can be directly affected by the toxic effects of CAAE which disrupts spermatogenesis, reduces sperm count and overall sperm quality. *C. asiatica* caused decrement in these parameters, possibly due to lipocalin enzyme and sorbitol dehydrogenase activity.[46-48] Analysis expression of the lipocalin members, Lcn8 and Lcn9, also showed disappearance of lipocalin protein in the *C. asiatica*-treated group, indicating possible effects on sperm maturation and epididymal function.[46] Lipocalin proteins are significant in reproductive functions and sperm development in the male reproductive system.[46,47] Likewise, four weeks of treatment with *C. asiatica*, possibly due to damage to the Leydig and Sertoli cells involved in spermatogenesis.[49]

The toxic effect of CAAE on testicular tissue aligns with Yunianto et al. (2017), who reported that the ethanol extract of *C. asiatica* induced apoptosis of spermatogenic cells in normal rats. This was evidenced by increased apoptotic germ cells per testicular tubule and significant decreases in FSH, LH, and testosterone levels.[48] The observed toxicity may result from synergistic effects of the active substances in *C. asiatica*. Interestingly, asiatic acid has been shown to enhance spermatogenesis by inhibiting apoptosis in rats fed a high-fat diet.[43] However, other compounds in *C. asiatica* may counteract asiatic acid's beneficial effects on spermatogenic function.

It has been reported that diabetes-induced atrophy of male reproductive organs results from oxidative stress and apoptosis.[50] In our study, the gonadosomatic index (GSI) was initially low in the diabetic group and further decreased with CAAE administration. Additionally, the reduced testosterone levels could contribute to negative effects on somatic and germ-forming cells in the testes, ultimately leading to decreased testicular weight.

LIMITATION OF STUDY

Phytochemical analysis is essential to identify active components and assess potential hepatoprotective and testicular effects. Including spermogram and histology observations will enhance study findings. Future investigations should incorporate electron microscopy to examine *C. asiatica*'s impact on liver histo-architecture, offering insights into liver diseases like inflammation or cirrhosis. Advances in electron microscopy techniques, such as immunoelectron microscopy could elucidate complex molecular mechanisms and guide targeted therapeutic interventions.

CONCLUSION

In conclusion, CAAE alleviate hyperglycaemia in diabetic rats by improving insulin resistance and has a hepatoprotective effect by reducing oxidative stress and maintaining near-normal levels of liver enzymes. However, CAAE administration has toxic effects on the testes, worsening the outcome in male diabetic rats. Further studies should be conducted to identify the compounds responsible for these adverse effects so that safe administration of CAAE is possible without causing harmful side effects in the male population.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1. Institute for Public Health (IPH) NIoH, Ministry of Health Malaysia. Volume I: NCDs – Non-Communicable Diseases: Risk Factors and other Health Problems: Institute for Public Health, National Institutes of Health (NIH), Ministry of Health, Malaysia; 2020.
2. Govender N, Khaliq OP, Moodley J, Naicker T. Insulin resistance in COVID-19 and diabetes. Prim Care Diabetes. 2021;15(4):629-34.
3. Singh A, Kukreti R, Saso L, Kukreti S. Mechanistic Insight into Oxidative Stress-Triggered Signaling Pathways and Type 2 Diabetes. Molecules. 2022;27 (3).
4. Barkabi-Zanjani S, Ghorbanzadeh V, Aslani M, Ghalibafsabbaghi A, Chodari L. Diabetes mellitus and the impairment of male reproductive function: Possible signaling pathways. Diabetes Metab Syndr. 2020;14(5):1307-14.
5. Liu L, Shu A, Zhu Y, Chen Y. Cornuside Alleviates Diabetes Mellitus-Induced Testicular Damage by Modulating the Gut Microbiota. Evid Based Complement Alternat Med. 2021;2021:5301942.
6. Mathur S, Mehta DK, Kapoor S, Yadav S. Liver function in type-2 diabetes mellitus patients. Int J Sci Stud. 2016;3(10):43-7.
7. Pearce I, Simo R, Lovestam-Adrian M, Wong DT, Evans M. Association between diabetic eye disease and other complications of diabetes: Implications for care. A systematic review. Diabetes Obes Metab. 2019;21(3):467-78.
8. Unsal V, Dalkiran T, Cicek M, Kolukcu E. The Role of Natural Antioxidants Against Reactive Oxygen Species Produced by Cadmium Toxicity: A Review. Adv Pharm Bull. 2020;10(2):184-202.
9. Sheng X, Che H, Ji Q, Yang F, Lv J, Wang Y, et al. The relationship between liver enzymes and insulin resistance in type 2 diabetes patients with nonalcoholic fatty liver disease. Hormone and Metabolic Research. 2018;50(05):397-402.
10. Abbasi-Oshaghi E, Khodadadi I, Mirzaei F, Ahmadi M, Tayebinia H, Goodarzi MT. *Anethum graveolens L.* Alleviates Sperm Damage by Limiting Oxidative Stress and Insulin Resistance in Diabetic Rats. The Open Medicinal Chemistry Journal. 2020;14(1).
11. Maresch CC, Stute DC, Alves MG, Oliveira PF, de Kretser DM, Linn T. Diabetes-induced hyperglycemia impairs male reproductive function: a systematic review. Hum Reprod Update. 2018;24 (1):86-105.
12. He Z, Yin G, Li QQ, Zeng Q, Duan J. Diabetes Mellitus Causes Male Reproductive Dysfunction: A Review of the Evidence and Mechanisms. In Vivo. 2021;35(5):2503-11.
13. Ganesan K, Rana MBM, Sultan S. Oral Hypoglycemic Medications2023.
14. Mairuae N, Cheepsunthorn P, Buranrat B. Anti-inflammatory and anti-oxidative effects of *Centella asiatica* extract in lipopolysaccharide-stimulated BV2 microglial cells. Pharmacognosy Magazine. 2019;15 (60).
15. Park DW, Jeon H, Kwon JE, Lee YG, So R, Choe TH, et al. Hepatoprotective effect of *Centella asiatica* 50% ethanol extract against acetaminophen-induced acute liver injury in BALB/c mice. Toxicol Res. 2021;37(2):261-75.
16. Azis HA, Taher M, Ahmed AS, Sulaiman WMAW, Susanti D, Chowdhury SR, Zakaria ZA. In vitro and In vivo Wound Healing Studies of Methanolic Fraction of *Centella asiatica* Extract. South African Journal of Botany. 2017;108:163-74.
17. Wijeweera P, Arnason JT, Koszycki D, Merali Z. Evaluation of anxiolytic properties of Gotukola--(*Centella asiatica*) extracts and asiaticoside in rat behavioral models. Phytomedicine. 2006;13(9-10):668-76.

18. Vinolina NS, Sigalingging R. Phytochemical screening and biomass production of *Centella asiatica (L.)* Urb of Samosir – Indonesia accession cultivated on acid soil with different phosphorus treatments. IOP Conf Series: Earth and Environmental Science. 2021;782(3).
19. Swapna K, Sathibabu Uddandrao VV, Parim B, Ravindarnaik R, Suresh P, Ponnusamy P, et al. Effects of asiatic acid, an active constituent in *Centella asiatica (L.)*: restorative perspectives of streptozotocin-nicotinamide induced changes on lipid profile and lipid metabolic enzymes in diabetic rats. Comparative Clinical Pathology. 2019;28 (5):1321-9.
20. Lou JS, Dimitrova DM, Murchison C, Arnold GC, Belding H, Seifer N, et al. *Centella asiatica* triterpenes for diabetic neuropathy: a randomized, double-blind, placebo-controlled, pilot clinical study. Esper Dermatol. 2018;20(2 Suppl 1):12-22.
21. Ramaswamy AS. Pharmacological studies on *Centella asiatica* Linn.(Brahma manduki)(no Umbelliferae). J Res Industrial Med. 1970.
22. Kumari S, Deori M, Elancheran R, Kotoky J, Devi R. In vitro and In vivo Antioxidant, Anti-hyperlipidemic Properties and Chemical Characterization of Centella asiatica (L.) Extract. Front Pharmacol. 2016;7:400.
23. Adam SH, Giribabu N, Bakar NMA, Salleh N. Marantodes pumilum (Kacip fatimah) enhances in-vitro glucose uptake in 3T3-L1 adipocyte cells and reduces pancreatic complications in streptozotocin-nicotinamide induced male diabetic rats. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2017;96:716-26.
24. Deshpande P, Mohan V, Thakurdesai P. Preclinical Safety Assessment of Standardized Extract of *Centella asiatica* (L.) Urban Leaves. Toxicology International. 2015;22:10.
25. Muhlishoh A, Wasita B, Patriado Nuhriawangsa AM. Antidiabetic effect of *Centella asiatica* extract (whole plant) in streptozotocin nicotinamide-induced diabetic rats. Jurnal Gizi dan Dietetik Indonesia (Indonesian Journal of Nutrition and Dietetics). 2019;6(1):14-22.
26. Swapna K, Uddandrao VS, Vadivukkarasi S, Saravanan G. Asiatic Acid Attenuate Type 2 Diabetes Mellitus Induced Alterations in Acetylcholinesterase and Antioxidant System of Brain in Rats. Bioscience Biotechnology Research Communications. 2020;13(4):2193-9.
27. Fu Q, Wang P, Zhang Y, Wu T, Huang J, Song Z. Effects of dietary inclusion of asiaticoside on growth performance, lipid metabolism, and gut microbiota in Yellow-Feathered Chickens. Animals. 2023;13(16):2653.
28. Tan SC, Rajendran R, Bhattamisra SK, Krishnappa P, Davamani F, Chitra E, et al. Effect of madecassoside in reducing oxidative stress and blood glucose in streptozotocin–nicotinamide-induced diabetes in rats. Journal of Pharmacy and Pharmacology. 2023;75(8):1034-45.
29. Nagy C, Einwallner E. Study of In Vivo Glucose Metabolism in High-fat Diet-fed Mice Using Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT). J Vis Exp. 2018(131).
30. Kusumastuti SA, Nugrahaningsih DAA, Wahyuningsih MSH. *Centella asiatica (L.)* extract attenuates inflammation and improve insulin sensitivity in a coculture of lipopolysaccharide (LPS) -induced 3T3-L1 adipocytes and RAW 264.7 macrophages. Drug Discov Ther. 2019;13(5):261-7.
31. Fitrianda E, Sukandar EY, Elfahmi E, Adnyana IK. Antidiabetic Activity of Extract, Fractions, and Asiaticoside Compound Isolated from *Centella asiatica* Linn. Leaves in Alloxan-Induced Diabetic Mice. Asian Journal of Pharmaceutical and Clinical Research. 2017;10(10).
32. Cheng D, Zhang X, Xu L, Li X, Hou L, Wang C. Protective and prophylactic effects of chlorogenic acid on aluminum-induced acute hepatotoxicity and hematotoxicity in mice. Chem Biol Interact. 2017;273:125-32.
33. Azimi M, Mehrzad J, Ahmadi E, Orafei M, Aghaie F, Ahmadi A, et al. The Effect of Thymus vulgaris on Hepatic Enzymes Activity and Apoptosis-Related Gene Expression in Streptozotocin-Induced Diabetic Rats. Evid Based Complement Alternat Med. 2022;2022:2948966.

34. Mohamed J, Nazratun Nafizah AH, Zariyantey AH, Budin SB. Mechanisms of Diabetes-Induced Liver Damage: The role of oxidative stress and inflammation. Sultan Qaboos Univ Med J. 2016;16 (2):e132-41.
35. Choi MJ, Zheng HM, Kim JM, Lee KW, Park YH, Lee DH. Protective effects of *Centella asiatica* leaf extract on dimethylnitrosamine-induced liver injury in rats. Mol Med Rep. 2016;14(5):4521-8.
36. Shi C, Wang Q, Rao Z, Shi Y, Wei S, Wang H, et al. Diabetes induces hepatocyte pyroptosis by promoting oxidative stress-mediated NLRP3 inflammasome activation during liver ischaemia and reperfusion injury. Annals of translational medicine. 2020;8(12).
37. Hong W, Hwang-Bo J, Jeon H, Ko M, Choi J, Jeong YJ, et al. A Comparative Study of the Hepatoprotective Effect of *Centella asiatica* Extract (CA-HE50) on Lipopolysaccharide/d-galactosamine -Induced Acute Liver Injury in C57BL/6 Mice. Nutrients. 2021;13(11).
38. Akbar B, Susilo, Nissa RA, Ritonga RF, Lestari S, Astuti Y, Parwito. Antifertility Effect of the Ethanol Extract of *Centella asiatica L.* Urban Against the White Rat (*Rattus norvegicus L.*) in the Early Post-Implantation. IOP Conf Series: Journal of Physics: Conference Series. 2018;1114.
39. Solihati N, Purwantara B, Supriatna I, Winarto A. Histomorphological changes, sperm quality and testosterone level after administration and cessation of Pegagan (*Centella asiatica*) extract in rats. Journal of the Indonesian Tropical Animal Agriculture. 2018;43 (3).
40. Sukarjati EW, Pramusinta IA, Rahayu A. Potential Extract of Lerak Fruit (Sapindus Rarak), Pegagan (*Centella asiatica*,) Mimba Seed (*Azadirachta Indica* A. Juss) and Mixing of Third Extract to Motility and Viability of Spermatozoa Guinea Pigs (*Cavia Porcellus*). Int J Heal Med Res. 2023;2(06):141-6.
41. Oyenihi AB, Opperman M, Alabi TD, Mpahleni B, Masola B. *Centella asiatica* alleviates diabetes-induced changes in fatty acid profile and oxidative damage in rat testis. Andrologia. 2020;52(10):e13751.
42. Intararuchikul T, Teerapattarakan N, Rodsiri R, Tantisira M, Wohlgemuth G, Fiehn O, Tansawat R. Effects of *Centella asiatica* extract on antioxidant status and liver metabolome of rotenone-treated rats using GC-MS. Biomed Chromatogr. 2019;33 (2):e4395.
43. Miao XL, Gao GM, Jiang L, Xu R, Wan DP. Asiatic acid attenuates high-fat diet-induced impaired spermatogenesis. Exp Ther Med. 2018;15(3):2397-403.
44. Jatayu D, Nursyam H, Hertika AMS. Antioxidant effect of *Centella asiatica* ethanolic extract to superoxide dismutase (SOD) level on Cyprinus carpio liver. Research Journal of Life Science. 2018;5(3):163-72.
45. Pittella F, Dutra RC, Junior DD, Lopes MTP, Barbosa NR. Antioxidant and cytotoxic activities of *Centella asiatica* (L) Urb. Int J Mol Sci. 2009;10 (9):3713-21.
46. Wen Z, Liu D, Zhu H, Sun X, Xiao Y, Lin Z, et al. Deficiency for Lcn8 causes epididymal sperm maturation defects in mice. Biochem Biophys Res Commun. 2021;548:7-13.
47. Stopkova R, Otcenaskova T, Matejkova T, Kuntova B, Stopka P. Biological Roles of Lipocalins in Chemical Communication, Reproduction, and Regulation of Microbiota. Front Physiol. 2021;12:740006.
48. Yunianto I, Das, S., Mat Noor, M. Antifertility properties of *Centella asiatica* ethanolic extract as a contraceptive agent: Preliminary study of sperm proteomic. Asian Pacific Journal of Reproduction. 2017;6(5).
49. Heidari M, Heidari-Vala H, Sadeghi MR, Akhondi MM. The inductive effects of *Centella asiatica* on rat spermatogenic cell apoptosis in vivo. J Nat Med. 2012;66(2):271-8.
50. Aly HA. Mitochondria-mediated apoptosis induced testicular dysfunction in diabetic rats: ameliorative effect of resveratrol. Endocrinology. 2021;162 (4):bqab018.