

Gallic Acid Alleviates Diabetic Glomerulopathy in Diabetic Mice through Suppression of p47phox and TGF- β 1

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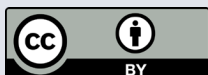
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Received: July 14, 2025;

Revised: October 11, 2025;

Accepted: October 15, 2025

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ABSTRACT

OBJECTIVE This study aims to evaluate whether gallic acid (GA) alleviates diabetic glomerulopathy in a type 2 diabetic mouse model.

METHODS ICR mice were fed a high-fat diet (60% fat) and providing a 25% (w/v) fructose solution for 10 weeks and given multiple intraperitoneal injections of streptozotocin (STZ) (35 mg/kg) to induce type 2 diabetes (T2D) and kidney injury. After diabetes onset, mice were treated with GA at 30 or 60 mg/kg/day for four weeks. Fasting glucose levels, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), renal malondialdehyde (MDA) levels, renal superoxide anion radicals ($O_2^{\cdot-}$) formation, glomerular histology, and the expression of p47phox, a key regulator of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and transforming growth factor beta 1 (TGF- β 1), were assessed.

RESULTS Diabetic mice exhibited marked hyperglycemia, increased HOMA-IR, and elevated renal oxidative stress, as demonstrated by higher MDA levels, enhanced $O_2^{\cdot-}$, and increased p47phox expression ($p < 0.05$ vs. controls). Histological analysis showed glomerular hypertrophy, enlarged glomerular volume, expanded Bowman's space, thickening of the glomerular basement membrane, and raised TGF- β expression ($p < 0.05$ vs. controls). Notably, GA treatment significantly reduced these pathological changes, with the 60 mg/kg/day dose being more effective than the 30 mg/kg/day dose.

CONCLUSION GA effectively reduces oxidative stress and mitigates structural kidney alterations in a diabetic mouse model, highlighting its potential as an adjunct therapy for diabetes-induced kidney injury.

KEYWORDS gallic acid, diabetic glomerulopathy, type 2 diabetic mice, oxidative stress

INTRODUCTION

Diabetic kidney disease (DKD), a primary microvascular complication of type 2 diabetes (T2D), is driven by chronic hyperglycemia (1). This sustained hyperglycemic state accelerates glomerulopathy by promoting glomerular hyperfiltration, oxidative stress, inflammation, and fibrosis within the kidney (2). These pathological processes lead

to structural changes, including glomerular basement membrane (GBM) thickening, expanded Bowman's space, and increased glomerular volume, ultimately impairing glomerular filtration and permeability (3, 4).

At the molecular level, the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and the upregulation of transforming

growth factor-beta 1 (TGF- β 1) are recognized as key mediators of diabetic glomerulopathy (5, 6). The NOX complex, whose activation is critically dependent on its regulatory subunit p47phox, generates an excess of superoxide anions ($O_2^{\cdot-}$). This surge in reactive oxygen species drives oxidative stress and inflammation, disrupting glomerular homeostasis. Concurrently, the cytokine TGF- β 1 promotes fibrosis by recruiting inflammatory cells, inducing epithelial-mesenchymal transition (EMT), and stimulating extracellular matrix accumulation within the glomerulus (7, 8).

Gallic acid (GA; 3,4,5-trihydroxyl-benzoic acid) is a natural phenolic compound found in numerous fruits and vegetables. As a potent anti-oxidant, GA has demonstrated pharmacological effects in animal studies, including blood glucose reduction, decreased albuminuria, and kidney protection under diabetic conditions (9, 10). However, despite these promising results, data on its specific effects in diabetic glomerulopathy remain limited, and the underlying mechanisms of its protective action are still unknown.

To investigate T2D-associated kidney injury, a well-established mouse model combining a high-fat diet (HFD) with multiple low-dose injections of streptozotocin (STZ) was used. This dual approach effectively mimics the human condition: the HFD induces peripheral insulin resistance, while subsequent STZ injections partially destroy pancreatic β -cells, leading to impaired insulin secretion. The model successfully recapitulates the progression of T2D and key pathological alterations of DKD, such as glomerular hypertrophy and renal fibrosis, making it a valuable tool for studying the pharmacological effects of GA.

This study aims to examine the protective effect of GA on glomerulopathy in HFD/STZ-induced T2D mouse model.

METHODS

Animals and treatment protocols

Male ICR mice, aged five weeks and weighing 25 to 30 grams, were obtained from and housed in the Northeastern Laboratory Animal Center, Khon Kaen University, Thailand. The animals were housed in a room with an HVAC (Heating, Ventilation, and Air-Conditioning) system and were maintained on a 12-hour light/dark cycle. The mice had unrestricted (*ad libitum*) access to

standard drinking water and were provided with a standard chow diet from Charoen Pokphand Co. Ltd., Thailand. All animal procedures were approved by the Khon Kaen University Institutional Animal Ethics Committee (IACUC-KKU-25/66).

Forty mice were randomly assigned to one of five groups: a control group (group 1, $n = 8$) that received deionized water (5 mL/kg BW/day, *i.g.*), and four diabetic groups. Diabetes was induced in the diabetic groups by feeding a 60% HFD and providing a 25% (w/v) fructose solution for 10 weeks, with a STZ injection at week 4. Diabetic mice (fasting blood glucose > 200 mg/dL 72 hours post-STZ) were divided into four subgroups at week 7 and received daily oral gavage treatments for 4 weeks: group 2 (DM, $n = 8$) received deionized water (5 mL/kg BW/day), group 3 (DM+ GA30, $n = 8$) received GA (30 mg/kg BW/day), group 4 (DM+ GA60, $n = 8$) received GA (60 mg/kg BW/day), and group 5 (DM+ Met200, $n = 8$) received metformin (200 mg/kg BW/day), with all treatments dissolved in deionized water.

Measurement of body weight and blood glucose profile

Body weight was measured weekly and fasting blood glucose every two weeks. An intraperitoneal glucose tolerance test (IPGTT) was performed in weeks 0, 4, 6, and 10. For the IPGTT, overnight-fasted mice were injected with glucose (2 g/kg, *i.p.*), and blood glucose was monitored at 15-, 30-, 60-, and 120-minutes post-injection using a glucometer (ACCU-CHEK®, Roche, Germany).

At the end of the study, blood was collected via cardiac puncture to obtain plasma. Insulin concentrations were quantified using a commercial sandwich ELISA kit (Millipore Corp., MA, USA) according to the manufacturer's protocol. Insulin resistance was calculated using the homeostasis model assessment (HOMA-IR) formula: $[\text{Fasting Glucose (mmol/L)} \times \text{Fasting Insulin } (\mu\text{IU/mL})]/22.5$.

Assay of production rate of $O_2^{\cdot-}$ in kidney tissues

$O_2^{\cdot-}$ production in kidney tissue was quantified using a lucigenin-enhanced chemiluminescence assay (11). Excised kidney sections were incubated with lucigenin (60 μM), and the resulting signal was measured with a luminometer. Results were normalized to tissue dry weight and expressed as relative light units (RLU)/mg/min.

Measurement of MDA in kidney tissues

Lipid peroxidation was assessed by quantifying malondialdehyde (MDA) levels using a thiobarbituric acid reactive substances (TBARS) assay, following established procedures (11, 12). Briefly, 150 μ L of kidney homogenate was mixed with 10% trichloroacetic acid, 5 mM EDTA, 8% SDS, and 0.5 μ g/mL butylated hydroxytoluene and incubated at room temperature. Subsequently, 0.6% thiobarbituric acid was added, and the mixture was heated at 95°C for 30 minutes. After cooling, the samples were centrifuged to pellet the precipitate, and the absorbance of the supernatant was measured at 532 nm. MDA concentrations were calculated from a standard curve generated with 1,1,3,3-tetraethoxypropane (0.3–10 μ M).

Assessment of histomorphometry of glomeruli

The upper portion of the kidney was fixed in 4% phosphate-buffered paraformaldehyde for 48 hours, then dehydrated in graded ethanol (70%, 80%, 95%, and 100%, 1 hour each). Afterward, the tissue was cleared with xylene (three 1-hour changes) and infiltrated with xylene–paraffin mixtures (2:1, 1:1, and 1:2 ratios, 1 hour each), followed by pure paraffin overnight. Finally, the specimens were embedded in paraffin blocks, sectioned at 2 μ m, and stained with Hematoxylin and Eosin (H&E) for morphological and glomerular volume examination.

Assessment of ultrastructure of glomeruli by transmission electron microscopy

The kidneys were quickly harvested and cut into cubic millimeter sized pieces. The tissues were collected and fixed in 2.5% glutaraldehyde at 4°C, washed with PBS, ethanol, and acetone, and then embedded. Ultrathin sections were prepared and post-stained with a 2% saturated solution of uranyl acetate and lead citrate. Ultrastructural changes in the GBM and podocytes were observed using transmission electron microscopy (TEM) at 15,000 $\times$ magnification. Measurements and calculations were performed to determine GBM and podocyte by using the ImageJ software (NIH).

Western blot analysis of renal p47phox and TGF- β expression

Protein expressions of p47phox and TGF- β 1 in kidney homogenates were quantified by Western

blot, adapted from Sangartit et al. (13). Proteins were separated by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon®, Germany), and probed with p47phox (Cell Signaling Technology, Danvers, MA, USA) and TGF- β 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies and an HRP-conjugated secondary antibody. Bands were visualized using an ECL substrate (Super Signal™ West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA, USA), and their intensities were captured and normalized to β -actin as a loading control.

Statistical analysis

Statistical analysis was performed to compare the results among the different groups. The data are presented as the mean $\pm$ standard error of the mean (SEM). To determine whether there were significant differences among the groups, a one-way analysis of variance (ANOVA) was conducted. If the ANOVA revealed a significant difference, a post-hoc Tukey test was performed to identify which specific groups differed from one another. The level of statistical significance was set at a p -value of less than 0.05, indicating that any observed differences with a p -value below this threshold were considered statistically significant.

RESULTS

Effect of GA on body weight and blood glucose in diabetic mice

Mice were successfully induced with T2D after administration of a HFD combined with multiple doses of STZ injection, as demonstrated by significantly increased body weight and elevated fasting blood glucose levels in the diabetic group compared to controls ($p < 0.05$, Figure 1A, 1B). Treatment with GA at both 30 mg/kg and 60 mg/kg doses effectively reduced blood glucose levels to levels comparable with metformin treatment ($p < 0.05$, Figure 1B), though body weight remained unchanged across all treatment groups (Figure 1A).

The IPGTT revealed impaired glucose clearance in the diabetic mice, with prolonged elevated plasma glucose levels compared to controls ($p < 0.05$, Figure 1C, 1D). Both GA treatment groups and Met significantly improved glucose clearance capacity ($p < 0.05$, Figure 1C, 1D).

Insulin resistance assessment using HOMA-IR showed markedly elevated levels in the diabetic group versus controls ($p < 0.05$; Table 1). GA treatment dose-dependently reduced insulin resistance, with HOMA-IR values of 1.78 for the 30 mg/kg group and 1.19 for the 60 mg/kg group (Table 1). Met treatment achieved similar improvement with a HOMA-IR. These results indicate that GA treatment, particularly at 60 mg/kg/day, effec-

tively alleviates insulin resistance in diabetic mice comparable to Met therapy.

Effect of GA on histomorphological changes of glomeruli in diabetic mice

H&E staining of kidney tissue revealed that persistent hyperglycemia in the DM group led to significant structural damage, indicative of kidney injury. Compared to controls, DM mice exhibited

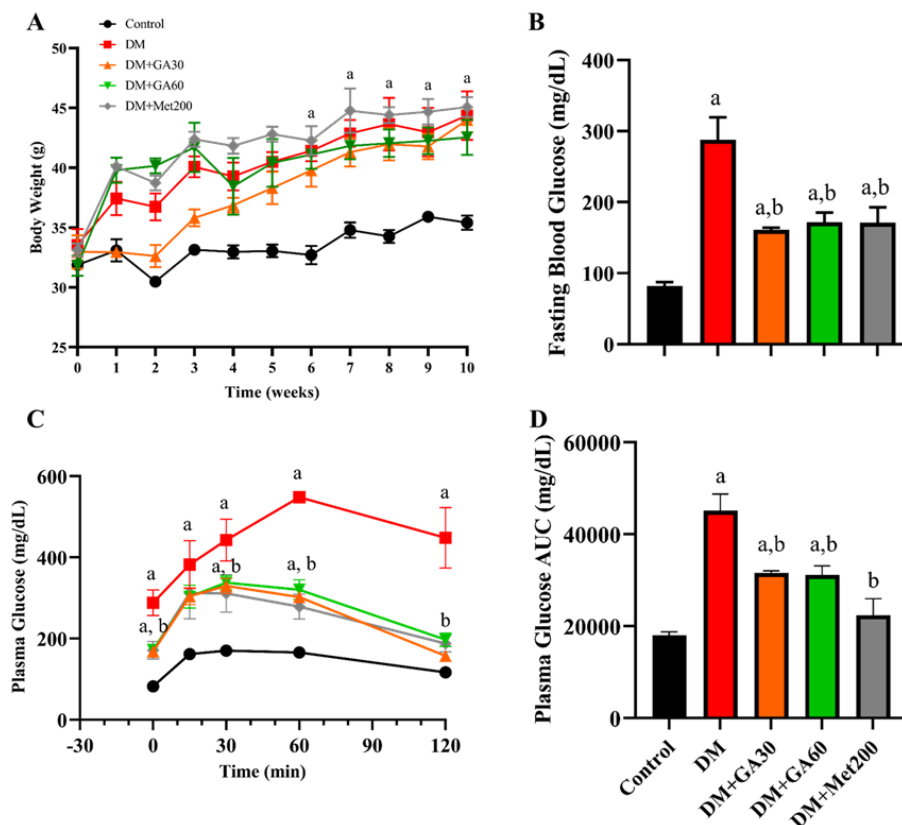


Figure 1. Effect of GA on body weight and blood glucose in diabetic mice. This figure displays changes in body weight over the study duration (A), fasting blood glucose levels at week 10 (B), and glucose progression during an IPGTT at week 10 (C), with the corresponding area under the curve (AUC) depicted in (D). Data are presented as means $\pm$ SEM. Statistical comparisons are indicated as follows: ^a $p < 0.05$ compared to the control group; ^b $p < 0.05$ compared to the DM group.

Table 1. Effect of GA on fasting insulin, fasting glucose and HOMA-IR in diabetic mice at week 10

Group	Fasting insulin (μ U/mL)	Fasting glucose (mg/dL)	HOMA-IR
Control	1.52 $\pm$ 0.23	90.34 $\pm$ 7.30	0.33 $\pm$ 0.08
DM	5.10 $\pm$ 1.93 ^a	251.57 $\pm$ 11.58 ^a	2.47 $\pm$ 0.76 ^a
DM+GA30	4.30 $\pm$ 0.87 ^{a,b}	184.53 $\pm$ 14.22 ^{a,b}	1.78 $\pm$ 0.21 ^{a,b}
DM+GA60	3.21 $\pm$ 0.14 ^{a,b,c}	170.92 $\pm$ 9.68 ^{a,b}	1.19 $\pm$ 0.06 ^{a,b,c}
DM+Met200	3.42 $\pm$ 0.60 ^{a,b,c}	168.56 $\pm$ 8.37 ^{a,b,c}	1.17 $\pm$ 0.05 ^{a,b,c}

Data is presented as means $\pm$ SEM; Statistical significance is indicated as follows; ^a $p < 0.05$ compared to the control group, ^b $p < 0.05$ compared to the DM group, and ^c $p < 0.05$ compared to DM+GA30 group.

marked glomerular hypertrophy, characterized by significant increases in glomerular area, glomerular volume, and Bowman's space area ($p < 0.05$, Figure 2A, 2C-2E). Crucially, these pathological changes were counteracted by therapeutic intervention. GA administration (60 mg/kg/day) effectively normalized the renal morphology (Figure 2C-2E), while Met also provided significant protection by alleviating these structural alterations ($p < 0.05$). This demonstrates that both GA and Met can protect against or reverse the detrimental morphological effects of diabetes on the kidney.

Effect of GA on ultrastructural changes of glomeruli in diabetic mice

TEM at 15,000x magnification was used to examine ultrastructural changes in renal tissues, focusing on the GBM, podocytes, and filtration slit pores (Figure 3A).

The diabetic group demonstrated significant GBM thickening and increased podocyte base width compared to controls ($p < 0.05$, Figure 3C, E). Additionally, filtration slit pore numbers were notably reduced in diabetic mice (Figure 3D, $p < 0.05$ vs. controls), indicating impaired glomerular filtration structure.

Treatment with GA at both 30 mg/kg and 60 mg/kg doses effectively reversed these structural alterations, with improvements comparable to Met treatment ($p < 0.05$). Both GA and Met successfully restored normal GBM thickness, podocyte morphology, and filtration slit pore density in diabetic mice (Figure 3C-3E).

Effect of GA on renal oxidative stress in diabetic mice

The evaluation of oxidative stress through MDA measurement demonstrated significantly elevated levels in renal tissues of diabetic mice compared to controls ($p < 0.05$, Figure 4A). This increase in MDA levels indicates heightened lipid peroxidation and oxidative damage in diabetic conditions.

Treatment with GA at both 30 mg/kg and 60 mg/kg doses effectively reduced elevated MDA levels ($p < 0.05$ vs. DM; Figure 4A). Met treatment produced comparable results, significantly decreasing MDA levels in renal tissues ($p < 0.05$ vs. DM; Figure 4A). These findings demonstrate that both GA and metformin effectively mitigate diabetes-associated oxidative stress.

Analysis of superoxide anion production in kidney tissue revealed significantly increased

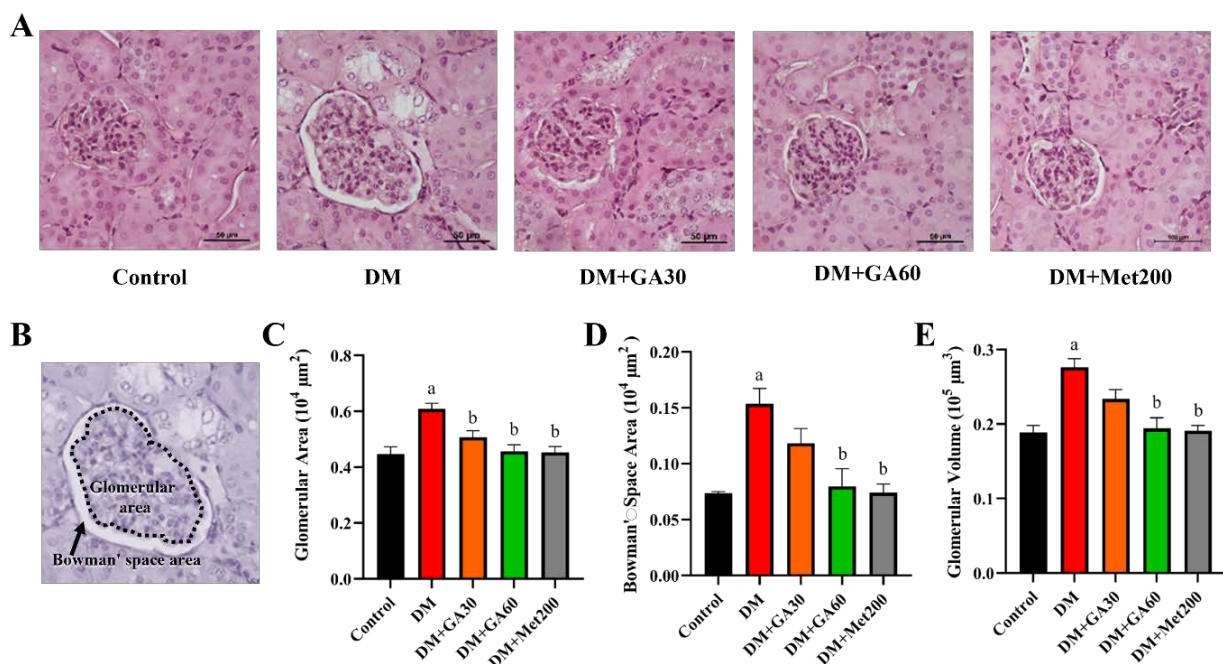


Figure 2. Effect of GA on histomorphological changes of glomeruli in diabetic mice. Representative glomerular sections (H&E) from $n = 3$ animals per group were photographed at $400\times$ (A). Glomerular area is outlined by the dotted line; Bowman's space is the clear area outside the outline and is indicated by the black arrow (B). Quantification of glomerular area (C), Bowman's space area (D), and glomerular volume (E) are shown. Data are means $\pm$ SEM. Statistical significance: ^a $p < 0.05$ vs. control group; ^b $p < 0.05$ vs. DM group.

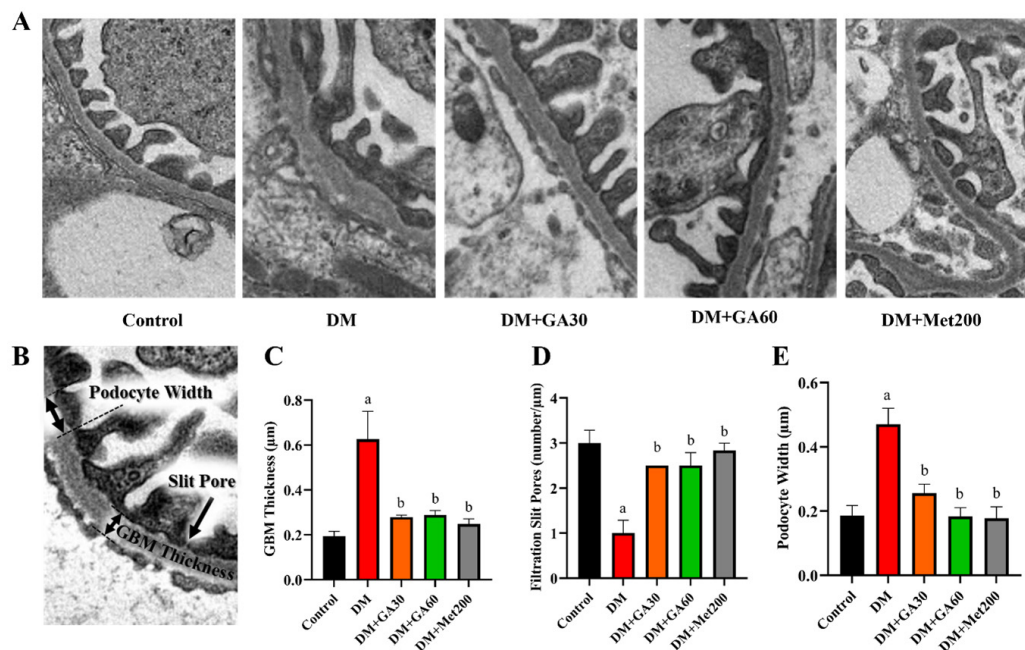


Figure 3. Effect of GA on ultrastructural changes of glomeruli in diabetic mice. The ultrastructure of the glomeruli was examined with TEM at 15,000× magnification (A). In panel (B), GBM thickness, filtration slit pore, and podocyte width are illustrated. Quantitative analyses of GBM thickness (C), filtration slit pore density per area (D), and podocyte width (E) were performed on three samples from each group using ImageJ (NIH). Data are means ± SEM. Statistical significance: ^a $p < 0.05$ vs. control group; ^b $p < 0.05$ vs. DM group.

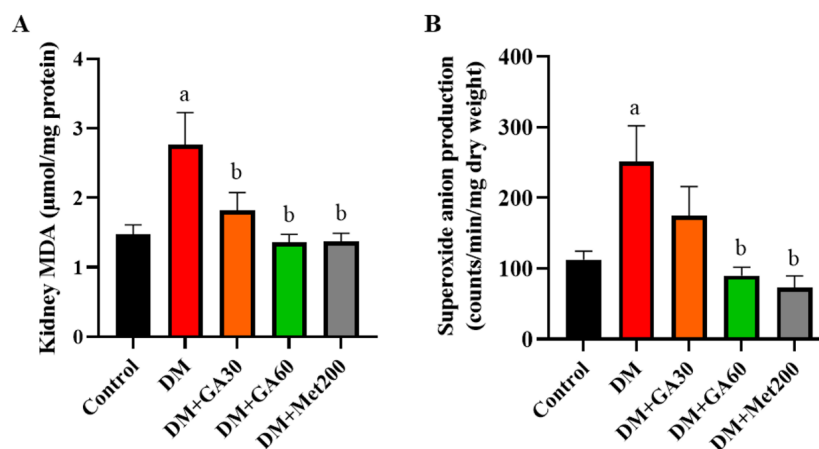


Figure 4. Effect of GA on renal oxidative stress in diabetic mice. Levels of MDA (A) and the rate of superoxide anion production (B) were measured in the kidney tissue of all animals ($n=8$ per group). Data are presented as means ± SEM. Statistical significance: ^a $p < 0.05$ vs. control group; ^b $p < 0.05$ vs. DM group.

generation in the diabetic group compared to controls ($p < 0.05$, Figure 4B). Treatment with GA at 60 mg/kg effectively attenuated superoxide production, while Met treatment similarly contributed to the reduction of oxidative stress in diabetic mice ($p < 0.05$).

Effect of GA on TGF-β1 and p47phox protein expression in kidney of diabetic mice

To further explore the underlying causes of structural changes in the kidneys, we analyzed

the expression of TGF-β1, an inflammatory marker associated with extracellular matrix deposition, using Western blotting (Figure 5A). The DM group exhibited increased TGF-β1 expression compared to the control group ($p < 0.05$). Notably, both treatment with GA and Met significantly inhibited this elevated expression, indicating that both treatments may provide protective effects against renal structural changes ($p < 0.05$).

Additionally, to identify the source of superoxide anion production in the kidney, we assessed

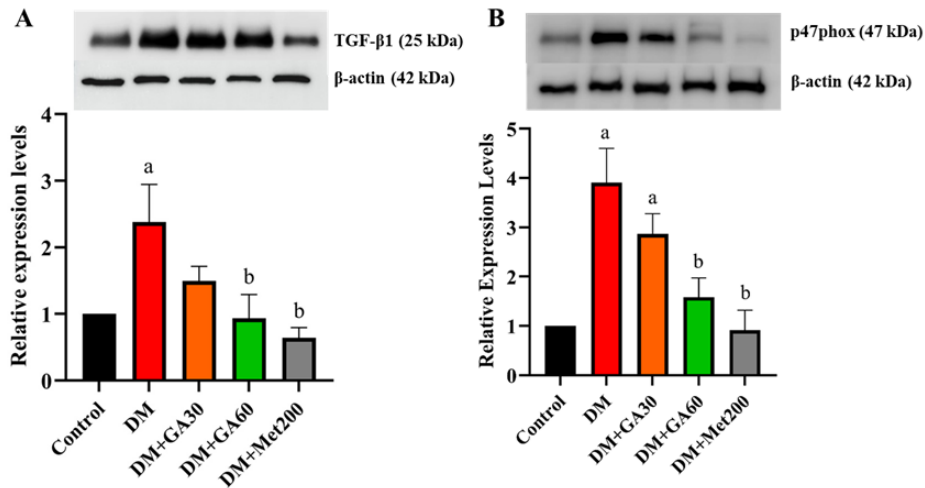


Figure 5. Effect of GA on TGF-β1 and p47phox protein expression in kidneys of diabetic mice. The figure shows the protein expression and quantitative band analysis of TGF-β1 (A) and p47phox (B) in kidney tissue. Representative images were obtained from three specimens per group. Data are presented as means ± SEM. Statistical significance: ^a $p < 0.05$ vs. control group; ^b $p < 0.05$ vs. DM group.

p47phox protein expression in renal tissues. This analysis revealed increased expression levels in the DM group ($p < 0.05$) (Figure 5B). Importantly, administration of GA at a dose of 60 mg/kg/day significantly reduced oxidative stress in the DM group, further supporting its protective role in mitigating kidney injury ($p < 0.05$).

DISCUSSION

This investigation demonstrates that GA effectively prevents diabetic kidney injury through a comprehensive therapeutic approach that addresses both metabolic dysfunction and glomerulopathy. The study utilized a clinically relevant T2D model, combining HFD with multiple low-dose STZ administration to create insulin resistance and pancreatic beta-cell dysfunction that closely mirrors human diabetic pathophysiology.

The experimental model successfully induced characteristic diabetic complications, including hyperglycemia, oxidative stress, and significant kidney damage manifested through glomerular hypertrophy, increased Bowman's space, and thickened basement membranes. These structural alterations were accompanied by markedly elevated MDA levels and overexpression of inflammatory mediators p47phox and TGF-β1, indicating considerable oxidative stress and inflammatory pathway activation within renal tissue (14). These findings are consistent with a well-established body of research demonstrating the central role of oxidative stress and inflammation in the pathogenesis

of DKD. For instance, chronic hyperglycemia is known to trigger the overproduction of ROS through various metabolic pathways, leading to oxidative damage to renal cells. This process, in turn, activates inflammatory cascades, including the upregulation of cytokines like TGF-β1, which promotes fibrosis and extracellular matrix deposition, ultimately causing the structural kidney damage observed in this model (15, 16). Thus, the model effectively replicates key pathological mechanisms of DKD.

GA supplementation at 30 mg/kg for 4 weeks produced significant therapeutic benefits by reducing blood glucose levels, oxidative stress, and inflammation while preventing further progression of renal damage. The 60 mg/kg/day dose demonstrated superior efficacy, suggesting that optimal therapeutic concentration maximizes the compound's antioxidant and anti-inflammatory properties. This dose-dependent response indicates that GA achieves its protective effects through concentration-dependent mechanisms that require adequate bioavailability for maximum therapeutic impact.

GA significantly improved insulin sensitivity in diabetic mice, as indicated by a marked reduction in HOMA-IR. This improvement is likely due to GA's capacity to reduce hepatic lipid deposition and mitigate oxidative stress and inflammation, which disrupt insulin signaling pathways (17). These combined effects lead to enhanced glucose tolerance and metabolic control (17). This result

is consistent with previous findings where GA reversed HFD-induced changes in GLUT-4 and IRS-1 expression, activated key metabolic regulators like AMPK and SIRT1, reduced oxidative stress, and preserved pancreatic β -cell function, thereby improving glucose handling (18).

GA effectively mitigated renal oxidative stress and inflammation, evidenced by suppressed rate of renal $O_2^{\cdot -}$ production and reduced expression of p47phox and TGF- β 1. Protective molecular mechanisms involve sophisticated regulation of inflammatory and fibrotic signaling. GA suppresses p47phox, a key NADPH oxidase subunit, through direct free radical scavenging and by inhibiting NF- κ B activation and its nuclear translocation, thereby reducing NADPH oxidase activity (19, 20). Crucially, this suppression of NADPH oxidase-derived oxidative stress may also contribute to the downregulation of TGF- β 1, as reactive oxygen species are known to activate this pro-fibrotic cytokine. Additionally, GA directly inhibits fibrotic signaling by suppressing the Smad and MAPK pathways downstream of TGF- β 1, preventing the excessive collagen production and extracellular matrix accumulation that drive basement membrane thickening and glomerular hypertrophy (21). The therapeutic actions observed in our study are strongly supported by evidence from other systems. Consistent with our findings, GA has been shown to reduce inflammation and oxidative stress in lung disease by modulating Nrf2-HO-1-NF- κ B signaling (22). Furthermore, its protective role in the central nervous system has been confirmed, where it reduces inflammation and apoptosis in the hippocampus of neurodegenerating rats by targeting TNF- α (21). Collectively, these studies underscore GA's versatile and potent ability to counteract disease pathology across multiple organ systems.

Regarding renal protection, GA demonstrated remarkable efficacy in preserving glomerular structure. The compound successfully mitigated the characteristic glomerulopathy, including increased glomerular area, Bowman's space expansion, basement membrane thickening, podocyte fusion, and reduced filtration slits. These protective effects were achieved through GA's targeted intervention in key pathological pathways that drive diabetic nephropathy progression.

While the present study focused on morphological outcomes, assessment of kidney function is limited in this study. However, previous investigations have shown that GA improves functional parameters by decreasing serum creatinine levels and reducing the urinary albumin-to-creatinine ratio. Moreover, combined treatment with metformin and GA (30 mg/kg daily) yielded superior improvements compared to either monotherapy, although those studies did not assess glomerular morphology (23). The current findings thus establish a crucial mechanistic link, suggesting that GA's documented functional benefits are directly tied to its profound ability to preserve glomerular architecture.

The study's findings confirm GA's dual therapeutic approach, which simultaneously addresses metabolic dysfunction and prevents structural renal damage. This comprehensive mechanism resulted in marked improvements in glomerular architecture, including reduced basement membrane thickening, preserved podocyte integrity, and maintained filtration slit architecture. The compound's ability to normalize oxidative stress markers, including MDA and superoxide anion levels, further demonstrates its potent antioxidant properties and protective role against inflammatory renal injury.

CONCLUSIONS

In conclusion, this research establishes GA as a promising therapeutic candidate for diabetic nephropathy management through its unique ability to target multiple pathological processes simultaneously. The compound's comprehensive approach to addressing both metabolic dysfunction and structural renal damage positions it as a valuable adjunctive therapy for diabetes-related complications. The evidence presented supports advancing GA toward clinical evaluation, with significant potential for improving patient outcomes through targeted prevention and management of DKD.

ACKNOWLEDGEMENTS

P.B. was supported by Scholarship for developing research capabilities at the graduate level, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

FUNDING

This study was supported by Invitation Research Fund, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. (Grant Number IN67046).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding this manuscript.

AUTHOR CONTRIBUTIONS

P.B.: data curation, investigation, methodology, writing – original draft; P.W.: visualization, validation; K.S.: methodology, validation; W.S.: conceptualization, supervision, project administration, funding acquisition, methodology, validation, writing – review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

The animal study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of National Research Council of Thailand. (IACUC-KKU-25/66; approved March 21, 2023).

INFORMED CONSENT STATEMENT:

Not applicable. This study did not involve human subjects.

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Evaluation of Cardiovascular Biomarkers and Lipid Profiles in Diabetic Patients Undergoing Coronary Artery Bypass Grafting (CABG) Surgery Compared to a Control Group

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Received: July 9, 2025;
Revised: September 19, 2025;
Accepted: October 15, 2025

ABSTRACT

OBJECTIVE Cardiovascular diseases (CVDs), particularly coronary artery disease (CAD), remain among the leading causes of morbidity and mortality worldwide. Type 2 diabetes mellitus (T2DM) significantly accelerates the progression of CAD through metabolic disturbances, including dyslipidemia and elevated homocysteine levels. This study aimed to evaluate cardiovascular biomarkers and lipid profiles in diabetic patients undergoing coronary artery bypass grafting (CABG) surgery, compared to a non-diabetic control group.

METHODS A total of 200 participants were included in this study, consisting of 100 patients with type 2 diabetes who had undergone CABG or angioplasty, and 100 age-matched individuals with type 2 diabetes without any history of cardiovascular disease, who served as controls.

RESULTS Homocysteine levels were elevated in 75.0% of diabetic patients versus 58.0% of controls ($p = 0.03$). Low high-density lipoprotein cholesterol (HDL-C) was observed in 93.0% of diabetics compared to 68.0% of controls ($p = 0.01$). No statistically significant differences were found in total cholesterol, low-density lipoprotein cholesterol (LDL-C), troponin, or creatine kinase-MB (CK-Mb) levels between groups ($p > 0.05$). These findings suggest that hyperhomocysteinemia and low HDL-C are significantly associated with CAD in diabetic populations. The presence of elevated homocysteine in a substantial proportion of the control group may reflect subclinical metabolic dysfunction or undiagnosed risk factors, warranting further investigation.

CONCLUSIONS These results underscore the potential utility of homocysteine and HDL-C as biomarkers in cardiovascular risk stratification. Their integration into routine screening protocols may enhance early detection and preventive strategies, particularly in high-risk or resource-limited settings.

KEYWORDS homocysteine, high-density lipoprotein cholesterol, type 2 diabetes, coronary artery disease, biomarkers

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INTRODUCTION

Cardiovascular diseases (CVDs) continue to be the leading cause of morbidity and mortality worldwide, posing a substantial burden on health-care systems and societal resources (1). Among these, coronary artery disease (CAD) is the most prevalent, resulting from the narrowing or blockage of coronary arteries due to atherosclerosis (2). The interplay between metabolic disorders, such as type 2 diabetes mellitus (T2DM), and the progression of CAD has garnered significant attention, with mounting evidence underscoring the role of metabolic abnormalities in exacerbating cardiovascular risks. T2DM, characterized by chronic hyperglycemia and insulin resistance, contributes to vascular dysfunction and amplifies the risk of myocardial infarction and other ischemic events. This intersection of diabetes and cardiovascular health underscores the critical need for understanding the mechanisms that underpin these diseases (3).

In recent years, the role of homocysteine, a sulfur-containing amino acid derived from methionine metabolism, has emerged as a pivotal biomarker in cardiovascular research (4). Hyperhomocysteinemia has been implicated in endothelial dysfunction, oxidative stress, and pro-inflammatory states, all of which contribute to the pathogenesis of atherosclerosis. Studies by Tomić M et al. have shown that elevated plasma homocysteine levels are linked to nonproliferative retinopathy in individuals with T2DM who do not have renal disease (5). Similarly, Smith et al. reported that more than half of patients undergoing angioplasty presented with high plasma homocysteine levels, further emphasizing its relevance in cardiovascular care (6). Despite these insights, the clinical utility of homocysteine as a routine diagnostic marker remains contested, particularly in resource-constrained settings where cost considerations limit its widespread use.

Lipid profile abnormalities are another cornerstone of cardiovascular risk assessment. High-density lipoprotein cholesterol (HDL-C), often termed “good cholesterol,” plays a protective role in cardiovascular health by facilitating reverse cholesterol transport and exerting anti-inflammatory effects (7). Conversely, low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) are commonly associated with an increased risk of atherosclerosis and vascular events (8). M

Georgoulis (2022), explored the link between lipid abnormalities and T2DM, noting that low HDL-C and high LDL-C levels significantly contribute to vascular obstruction and hypertension (9). However, the relative importance of these lipid parameters in CAD progression remains a subject of debate, necessitating further investigation into their individual and combined effects.

Biochemical markers such as troponin and creatine kinase-MB (CK-Mb) enzymes have long been recognized as gold standards for diagnosing acute myocardial infarction (MI). However, their role in predicting chronic atherosclerotic changes is less clear. While elevated troponin and CK-Mb levels are indicative of myocardial injury, these markers do not necessarily reflect the underlying pathophysiological processes leading to CAD. This distinction highlights the need for a multifactorial approach to cardiovascular risk stratification, incorporating both traditional markers and emerging biomarkers like homocysteine (10).

Demographic characteristics also significantly influence cardiovascular risks. Existing literature highlights a higher prevalence of CAD among older adults and males, reflecting the multifaceted nature of cardiovascular risk factors. These differences emphasize the importance of personalized preventive strategies and targeted interventions (11).

The interplay of diabetes, lipid abnormalities, and elevated homocysteine levels offers a compelling area for research, particularly in understanding the mechanisms underlying CAD in diabetic populations. While hyperhomocysteinemia and dyslipidemia are independently linked to increased cardiovascular risk, their combined effects remain underexplored (12). Moreover, confounding factors such as age, gender, and comorbidities further complicate the assessment of these relationships. Conditions including chronic kidney disease, hypothyroidism, and certain medication regimens—such as methotrexate or antiepileptics—may also contribute to elevated homocysteine levels, underscoring the complexity of risk stratification (13).

Despite advances in cardiovascular research, critical gaps remain in elucidating the pathophysiological connections between diabetes, lipid irregularities, and vascular health. For instance, the role of hyperhomocysteinemia as either a causative factor or a consequence of T2DM remains ambiguous (14). Similarly, the protective role of HDL-C in mitigating cardiovascular risks in dia-

betic patients warrants further investigation, especially given the challenges of pharmacologically increasing HDL-C levels. Bridging these knowledge gaps is vital for developing predictive models and effective therapeutic strategies aimed at improving outcomes for patients with diabetes and CAD (15).

METHODS

Rationale and sample size determination

The rationale for this study stems from the need to clarify how metabolic markers—particularly homocysteine and lipid profiles—interact in the development of CAD among patients with T2DM. Although each marker is independently associated with cardiovascular risk, their combined predictive value remains underexplored. By investigating these markers in a comparative framework, this study aims to generate insights that could inform more precise screening and intervention strategies for high-risk populations.

To ensure adequate statistical power and reliable findings, the sample size was calculated using Cochran's formula for cross-sectional studies. The prevalence of CAD in diabetic patients was considered, alongside a 95.0% confidence level and 5.0% margin of error, to determine the minimum number of participants required. This study analyzed 200 samples: 100 patients with type 2 diabetes who had undergone CABG or angioplasty, and 100 diabetic individuals under 50 years of age without any history of cardiac disease, who were selected as the control group. During the 6-month study period, 132 CABG surgeries were performed at Ahvaz Oil Hospital, of which 100 patients (77.8%) had type 2 diabetes. Due to the high prevalence of diabetes among CABG patients, selecting an equal number of non-diabetic controls was not feasible; therefore, diabetic individuals without cardiac disease were used as controls.

Participant selection and eligibility criteria

Eligible participants were selected through purposive sampling of patients receiving care at Naft Hospital, Ahvaz, Iran. The study group included individuals aged 40–70 years with a confirmed diagnosis of T2DM and documented CAD, based on clinical, biochemical, and diagnostic criteria outlined by the American Diabetes Association (ADA) 2016 Guidelines and cardiovascular evaluation protocols. The control group consisted of age-

and gender-matched individuals without a history of diabetes or significant cardiovascular events. All participants were required to provide informed consent. Exclusion criteria for both groups included a history of chronic kidney disease, liver dysfunction, autoimmune disorders, current use of lipid-lowering or homocysteine-modulating medications, acute infections or inflammatory conditions at the time of enrollment, pregnancy or lactation, and incomplete medical records or refusal to undergo laboratory testing.

Fasting blood sugar (FBS)

FBS levels were measured using a glucose oxidase-peroxidase (GOD-POD) enzymatic assay (Azar Tashkhis Azma, Tehran, Iran). Serum samples were collected after 10–12 hours of fasting. Standard quality control procedures were followed using normal and pathological serum controls.

Hemoglobin A1C (HbA1C)

Whole blood samples collected in K3-EDTA tubes were analyzed using an automated immunoassay system (Roche cobas c series).

Total cholesterol

Total cholesterol was measured enzymatically using the Cholesterol oxidase-Peroxidase (CHOD-POD) method (Azar Tashkhis Azma, Tehran, Iran). Serum samples were obtained after overnight fasting. Pre-analytical factors such as posture and tourniquet application were controlled. Samples were stored at 2–8°C prior to analysis.

Triglycerides (TG)

TG levels were determined using the glycerol phosphate oxidase-Peroxidase (GPO-POD) enzymatic method. Serum samples were collected after 10–14 hours of fasting. EDTA plasma was used when applicable. Lifestyle factors (e.g., alcohol intake, exercise) were considered during interpretation.

Low-density lipoprotein (LDL)

LDL cholesterol was calculated using the Friedewald formula: $LDL = \text{total cholesterol} - HDL - (TG/5)$. This method was applied only when fasting triglyceride levels were < 400 mg/dL. Indirect estimation was avoided in cases of chylomicronemia or familial dysbetalipoproteinemia.

Homocysteine

Homocysteine levels were measured using an enzymatic immunoassay (Bio-Rad, USA). Plasma (EDTA or heparinized), serum, and urine samples were collected after 10–12 hours of fasting. Samples were processed and frozen within one hour to preserve stability.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics version 26.0. Categorical variables, including HCN, HDL-C, LDL-C, FBS, troponin (TN.L), HbA1C, CK-MB, triglycerides (TG), and cholesterol (CHO), were summarized as frequency and percentage. Group differences in categorical variables were assessed using the Chi-square test, and Fisher's exact test was applied when expected cell counts were below 5.

Continuous variables were expressed as mean $\pm$ standard deviation (SD), and comparisons between patients and controls were performed using the independent samples t-test after confirming normality with the Kolmogorov–Smirnov test.

All statistical tests were two-tailed, and a $p < 0.05$ was considered statistically significant.

RESULTS

A total of 200 participants were included in

the analysis, consisting of 100 diabetic patients with cardiovascular involvement (CABG or angioplasty) and 100 diabetic controls without any history of cardiac disease. The distribution of biochemical and metabolic parameters between the two groups is presented in Table 1. Homocysteine (HCN) levels were significantly higher in patients compared with controls (75.0% vs 56.0%, $p = 0.004$). HDL-C levels also differed markedly, with 93.0% of patients classified as “Low” compared with 68.0% of controls, while 28.0% of controls demonstrated “High” HDL-C values ($p < 0.001$). LDL-C distribution showed a significant difference, with controls exhibiting a higher proportion of “High” LDL-C (36.0% vs 17.0%, $p = 0.001$).

FBS levels were elevated in both groups, with no statistically significant difference (97.0% vs 96.0%, $p = 0.700$). Troponin (TN.L) positivity was observed only in patients (4.0%), resulting in a significant difference ($p = 0.043$). HbA1C levels were uniformly high in both groups ($p = 0.316$). CK-MB elevation was substantially more frequent in patients (51.0% vs 0%), indicating a strong association with cardiovascular involvement ($p < 0.001$). Significant differences were also observed in triglyceride and cholesterol (CHO) distributions between groups ($p = 0.048$ and $p = 0.002$, respectively). These findings highlight the metabolic and biochemical disturbances associated with cardiovascular disease in individuals with type 2 diabetes.

Table 1. Biochemical and metabolic parameter distribution among diabetic cardiac patients and diabetic controls, with corresponding P-values calculated using Chi-square or Fisher's exact test.

Parameter	Category	Patients n (%)	Controls n (%)	p-value
HCN	High/Normal	75 (75.0%) / 25 (25.0%)	56 (56.0%)/44 (44.0%)	0.004
HDL-C	Low/Normal/High	93 (93.0%) / 7 (7.0%)/ 0 (0.0%)	68 (68.0%)/4 (4.0%)/ 28 (28.0%)	< 0.001
LDL-C	Normal/Borderline/High	62 (62.0%)/21 (21.0%)/ 17 (17.0%)	64 (64.0%)/0 (0.0%)/ 36 (36.0%)	0.001
FBS	High/Normal	97 (97.0%)/3 (3.0%)	96 (96.0%)/4 (4.0%)	0.700
Troponin (TN.L)	Positive/Negative	4 (4.0%)/96 (96.0%)	0 (0.0%)/100 (100.0%)	0.043*
HbA1C	High/Normal	99 (99.0%)/1 (1.0%)	100 (100.0%)/0 (0.0%)	0.316*
CK-MB	High/Normal	51 (51.0%) / 49 (49.0%)	0 (0.0%)/100 (100.0%)	< 0.001*
TG	Normal/Borderline/High	85 (85.0%)/5 (5.0%)/ 10 (10.0%)	77 (77.0%)/4 (4.0%)/ 19 (19.0%)	0.048
CHO	Normal/Borderline/High	84 (84.0%)/8 (8.0%)/ 8 (8.0%)	61 (61.0%)/21 (21.0%)/ 18 (18.0%)	0.002

HCN, homocysteine; HDL-C; high-density lipoprotein; LDL-C, low-density lipoprotein, FBS, fasting blood sugar; TN.L, troponin; HbA1C; hemoglobin A1c, CK-MB; creatine kinase-MB, TG; triglyceride, CHO; cholesterol

Analysis of comparison

A statistically significant difference was observed in homocysteine levels, with a higher proportion of “High” HCN among patients compared to controls ($p = 0.004$). HDL-C levels also differed markedly, as 93.0% of patients exhibited “Low” HDL-C compared with 68.0% of controls, while 28.0% of controls demonstrated “High” HDL-C ($p < 0.001$). LDL-C distribution showed a significant shift toward the “High” category in controls (36.0% vs 17.0%, $p = 0.001$). FBS levels were elevated in both groups without a significant difference ($p = 0.700$). Troponin positivity was detected only in patients ($p = 0.043$). HbA1C levels were uniformly high in both groups ($p = 0.316$). CK-MB elevation was significantly more frequent in patients (51.0% vs 0%, $p < 0.001$). Significant differences were also observed in triglyceride ($p = 0.048$) and cholesterol ($p = 0.002$) distributions, with controls showing higher proportions of borderline and high values. These findings collectively demonstrate substantial biochemical and metabolic disparities between diabetic patients with cardiovascular involvement and diabetic controls.

DISCUSSION

This study investigated the relationship between T2DM, metabolic markers, and cardiovascular risk factors, focusing on patients undergoing CABG at Ahvaz Naft Hospital. During the 6-month period, 132 patients underwent CABG at Ahvaz Oil Hospital, among whom 102 individuals (77.8%) had type 2 diabetes. The final study sample consisted of 200 participants: 100 diabetic patients with cardiovascular involvement (CABG or angioplasty) and 100 diabetic individuals without any history of cardiac disease who served as controls. The patient group had a mean age of 62.4 ± 13.0 years, with 74.0% males and 26.0% females, while the control group had a mean age of 43 ± 11 years, comprising 59.0% males and 41.0% females. These demographic differences reflect the age- and gender-related susceptibility to cardiovascular disease in diabetic populations. Our findings align with prior research. Yeromenko and Lavie identified diabetes as the most prevalent metabolic disorder in the Western world and emphasized its significant role in CAD and mortality (17). In the present study, elevated homocysteine levels were observed in 75.0% of diabetic

patients, consistent with Schnyder and Roffi, who reported high plasma homocysteine in over 50.0% of angioplasty patients (18).

This supports its role as a vascular risk factor. However, the prevalence observed in this study was notably higher, possibly due to differences in patient selection or regional dietary patterns. Lipid profile abnormalities were also pronounced, particularly low HDL-C levels. A striking 93.6% of diabetic patients demonstrated HDL-C levels below the normal range, compared to 68.0% in the control group (19). This is significant, as HDL-C is a protective lipoprotein that facilitates cholesterol removal and reduces cardiovascular risk (20). In contrast, LDL-C levels were less concerning, with 59.1% of patients having normal LDL values, suggesting that while LDL contributes to atherosclerosis, its role in this cohort appears secondary to other factors such as homocysteine and HDL-C (21). Further analysis revealed that total cholesterol was normal in 84.0% of patients, while triglycerides were within normal limits for 85% of the control group. These findings suggest that low HDL-C is the primary lipid abnormality in this population, consistent with XC Jiang, who demonstrated a strong correlation between high homocysteine, low HDL-C, and vascular complications in diabetic patients (22). Interestingly, the utility of certain biomarkers like troponin and CK-Mb for predicting atherosclerosis was limited. While these markers are critical for diagnosing MI, they did not demonstrate significant predictive value for CAD susceptibility in this cohort. The elevated CK-Mb levels in 49.1% of patients were likely attributable to trauma from surgical procedures, such as angioplasty or CABG, rather than underlying disease (23, 24).

From a clinical perspective, the combined assessment of FBS, A1C, HDL-C, LDL-C, and homocysteine offers a robust strategy for identifying individuals at high risk of CAD (25). Notably, FBS and A1C remain critical for diagnosing and monitoring diabetes, while lipid profiles and homocysteine levels provide additional insights into cardiovascular risk. Interventions such as improving HDL-C through dietary modifications, pharmacological therapies, and the administration of B vitamins (B12 and folic acid) to reduce homocysteine levels are essential components of comprehensive care for diabetic patients (26).

Study limitations

Several limitations should be acknowledged. First, the age and gender distribution between groups was not fully matched, which may have introduced bias in interpreting cardiovascular risk. Second, the sample size, while adequate for preliminary analysis, limits generalizability and statistical power for subgroup comparisons. Third, the inclusion of patients undergoing CABG introduces procedural variability, as surgical stress may influence biomarker levels such as CK-Mb. Fourth, while standard laboratory assays were used, inter-assay variability and lack of centralized calibration may have affected measurement consistency. Finally, lifestyle factors such as diet, physical activity, and medication adherence were not controlled, which could have confounded metabolic marker interpretation.

Future studies should aim for larger, matched cohorts and incorporate longitudinal designs to better assess causality and biomarker dynamics over time. Comparative analysis across geographic regions and inclusion of genetic and lifestyle variables may further refine cardiovascular risk models in diabetic populations.

CONCLUSIONS

In conclusion, diabetic patients with elevated homocysteine levels, low HDL, and high LDL are at the greatest risk for coronary artery disease. A multifactorial approach, including stringent diabetes control, HDL enhancement, and LDL and homocysteine reduction, is essential for effective management and prevention of cardiovascular complications.

ACKNOWLEDGMENTS

None

FUNDING

No funding was received for conducting this study.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

H.M: formal analysis, resources, conceptualization, software, validation, methodology; M.G: writing — review & editing, writing — original

draft, A.M: preparation, visualization, investigation, supervision, project administration, funding acquisition.

All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The original findings of this study are provided in the article and the supplementary material.

INSTITUTIONAL REVIEW BOARD STATEMENT

All experimental procedures were reviewed and approved by the Ethics Committee of Islamic Azad University, Dezful Branch, under the approval code 16530520962002. The project was conducted in August 2018 and fully complied with the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

INFORMED CONSENT STATEMENT

Informed consent was obtained from all individual participants included in the study.

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Efficacy of Task-Oriented Mirror Therapy on Enhancing Functional Recovery for Unilateral Neglect Post Stroke

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Received: May 27, 2025;

Revised: October 22, 2025;

Accepted: November 14, 2025

ABSTRACT

OBJECTIVE Stroke is a neurological condition with multiple complications, one of the most common being Unilateral Spatial Neglect (USN), where patients lose awareness of one side of their body. Both task-oriented mirror therapy and task-oriented therapy can aid in improving functional performance. The objective of the study was to compare the efficacy of task-oriented mirror therapy with task-oriented therapy in improving functional recovery among patients with USN.

METHODS This was a comparative study of 30 individuals. Using the sealed envelope method, participants were randomly assigned into group A or group B with 15 individuals in each group as per the inclusion and exclusion criteria. Group A was administered task-oriented mirror therapy and group B was administered task-oriented therapy. The therapy sessions were conducted for 45 minutes daily, 5 times a week, over a span of 4 weeks. The outcome measures were evaluated using the Catherine Bergego Scale (CBS) and the Line Bisection Test.

RESULTS Task-oriented mirror therapy had a greater impact on unilateral spatial neglect compared to task-oriented therapy without a mirror. A notable difference was found in the post-test mean and standard deviation values for CBS in group A [8.20 ± 2.624] and Group B [15.07 ± 3.105], as well as for line bisection test in group A [7.27 ± 1.534] and Group B [14.53 ± 2.973], both with a $p < 0.001$.

CONCLUSION Task-oriented mirror therapy showed better improvement in functional outcomes for patients with unilateral spatial neglect compared to task-oriented therapy.

KEYWORDS Catherine Bergego Scale, Line bisection test, post-stroke, rehabilitation

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INTRODUCTION

Stroke is a neurological condition resulting from vascular damage to the central nervous system, with hypertension being a key risk factor (1). Stroke is prevalent among patient populations and can cause significant morbidity and mortality.

Strokes are classified as ischemic and haemorrhagic strokes (2). The Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification system divides cerebral ischemia patients into five distinct categories: major artery atherosclerosis, cardioembolism, small vessel occlusion, stroke

of other determined causes, and stroke of undetermined origin (3). Globally, approximately 11.6 million ischemic strokes (IS) and 5.3 million hemorrhagic strokes (HS) occur annually. Notably, low- and middle-income countries contribute to 63% of IS cases and 80% of HS cases (4) although they represent only 54% of the world population.

Unilateral spatial neglect (USN) is a condition characterized by impaired awareness of the contralesional space. Individuals with USN fail to respond to various stimuli or situations originating from the side opposite to their brain lesion, despite having intact sensory and motor functions (5).

USN occurs frequently, affecting approximately 25–30% of stroke victims, with over 90% of cases involving lesions in the right hemisphere. In the acute phase, USN is observed in 20% of individuals with left-hemisphere lesions (LHL) and 43% of those with right-hemisphere lesions (RHL). After three months, USN persists in 17% of RHL patients and 5% of LHL patients (6).

Spatial neglect can be classified into egocentric and allocentric types using a spatial representation framework. Individuals with egocentric (body-centered) neglect tend to overlook their own body or personal space, while those with allocentric (stimulus-centered) neglect fail to attend to the contra-lesional side of external stimuli (7).

Rehabilitation strategies for USN include diverse methods such as robotics, virtual reality, and prism adaptation training. Robotic-assisted therapy, in particular, has shown potential in enhancing midline orientation and spatial awareness; however, it has not shown significant effects on functional performance (8). Virtual reality interventions hold promise for treating USN, but there is a lack of high-quality randomized trials to substantiate their efficacy (9). Prism adaptation training has been shown to reduce symptoms of USN, though its beneficial effects tend to be short-lived (10).

The fundamental concept of mirror therapy (MT), a low-cost patient-centered technique, is based on creating a visual illusion using the mirror reflection of the unaffected limb. Additionally, MT may activate mirror neurons and/or the primary motor, sensorimotor, and premotor cortex. A previous study using functional MRI analysis demonstrated that human mirror neurons are more strongly activated during functional move-

ment than during normal movement (11, 12). This approach gives patients the impression that their paralysed limbs are still intact by emphasising their healthy, unaffected limbs (13). Mirror therapy can enhance performance of activities of daily living (ADL), reduce pain and symptoms of unilateral neglect, and improve physical function in the paretic upper and lower limbs (14). Task-oriented Training for treating upper extremity impairment in chronic stroke patients appears to be a promising intervention strategy. This approach has shown improvements in motor function (15).

While numerous articles compare MT with other interventions, there is a lack of research specifically comparing task-oriented mirror therapy with task-oriented therapy without a mirror. Additionally, many therapies primarily focus on the upper limb activities, but there is a lack of specific research that includes lower limb activities. The aim of this study is to compare the effectiveness of task-oriented MT with task-oriented therapy in enhancing functional recovery.

METHODS

This study was conducted in a private setting on patients with unilateral neglect. Sample size determination was performed using G*power software with alpha 0.05 and power beta 0.95 and an effect size of 0.5. The calculated sample size was 42 (Figure 1). Due to dropouts and elimination of some potential participants, the final sample size was 30. The participants were selected according to predefined inclusion and exclusion criteria. Using the sealed envelope method, participants were randomly assigned into one of two groups, group A and group B, with 15 individuals in each group. Group A received task-oriented MT, while group B received task-oriented therapy without a mirror. Allocation concealment was maintained using opaque sealed envelopes which were opened after participant details were written. After receiving an explanation of the techniques, all participants provided written informed consent. The blinding of assessors was done primarily to avoid an assessment bias, ensuring that assessors measuring the functional recovery outcomes were unaware of the group assignments. The subjects in both groups underwent therapy for 45 minutes daily, 5 times a week, over a span of 4 weeks. Outcome measures were evaluated

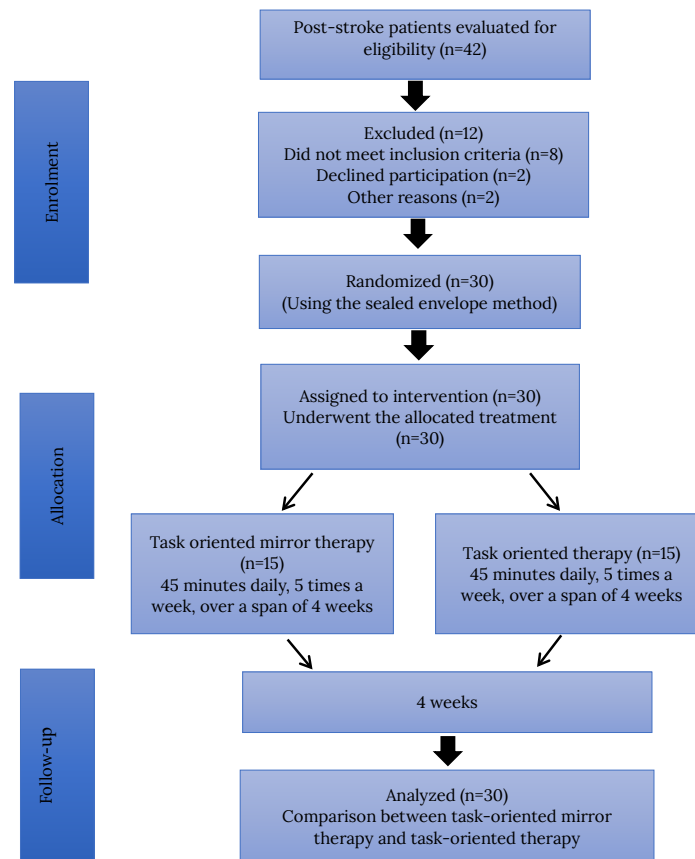


Figure 1. Study selection flow chart

using the Catherine Bergego Scale (CBS) and the Line Bisection Test (LBT) both before and after the treatment.

Ethical clearance

The study was conducted in accordance with the ethical committee on human experimentation of institution ISRB on 376/07/2024/ISRB/UGSR/SCPT.

Inclusion criteria

The study included subjects with unilateral neglect following a stroke, specifically those who exhibited a positive sign on the line bisection test and had a score of more than 10 on CBS, indicating a significant level of neglect. Participants aged between 40 and 70 of both genders were considered eligible for inclusion.

Exclusion criteria

Subjects who had undergone recent surgery or had fractures were excluded from the study to avoid any potential health risks and complications. Additionally, individuals diagnosed with depression, limb weakness not caused by stroke,

other perceptual disorders, or significant visual deficits were also excluded. Furthermore, participants deemed unsuitable by the attending physician were excluded.

Treatment procedure

Group A

Group A underwent task-oriented MT. The mirror was placed vertically in front of the unimpaired limb, with the impaired limb positioned behind the mirror. The subjects performed tasks using the unimpaired limb while focusing on the mirror's reflection which showed the movement of the impaired limb. For the upper limb, a standard mirror was placed on the table, while for the lower limb a larger mirror was positioned vertically between the legs, with the impaired leg placed behind the mirror. A one-minute break was given after every two exercises.

Group B

Group B underwent task-oriented training without a mirror. The subjects performed tasks using the impaired limb with additional cues and manual assistance. A one minute break was given after every two exercises.

Upper limb tasks: (25 minutes per session)*Grasping and releasing balls*

This task involved squeezing and releasing sponge balls, repeated 10 times (16).

Using a spray bottle

In this task, the bottle was filled with water, and the subject was asked to spray the water by squeezing the bottle. The subject was instructed to spray water 10 times (16).

Kneading putty

In this task, the subject was asked to create clay models using clay or putty. They were instructed to make 2 or 3 models (16).

Pinching coins

The task involved pinching a coin between the thumb and index finger. The subject was asked to hold the coin for 2-5 minutes (16).

Using a spoon

In this task, the subject was asked to transfer marbles or beans from one bowl to another using a spoon. They were instructed to fill the entire bowl (16).

Lifting a heavy can (shown in Figure 2).

For this task, the subject was asked to lift the cans or water bottles filled with water and to repeat the action 10 times (16).

Wiping a table with a cloth

The subject was asked to wipe a table using either a wet cloth or a dry cloth. They were instructed to wipe the entire table (16).

Picking up objects with tongs

In this task, the subject was asked to pick up different objects using a pair of tongs (16).

Lower limb tasks: (20 minutes per session)*Rocker or wobble board*

In this task, the subject was seated with their feet on a rocker board. They were instructed to rock the board back and forth, side to side, and in circular motions, repeating each movement 10 times (17).

Foot roller

The subject was asked to sit with one foot placed on a foot roller. They were instructed to roll their foot back and forth over the roller while applying gentle pressure, repeating the action 10 times (17).

Picking up sticks/curling cloth with toes

In this task, the subject sat with objects (such as sticks or cloths) on the floor in front of them. They were instructed to pick up the objects using

their toes, either one at a time or several at once, and to attempt to pick up all the objects (17).

Kicking the ball

In this task, the subject sat with the ball in front of them. They were instructed to kick the ball by performing knee extension, repeating the action 10 times (18).

Rolling the ball

In this task, the subject sat with the ball in front of them. They were instructed to roll the ball forward and back using their leg, repeating the action 10 times (18).

Outcome measure*Catherine Bergego Scale*

The CBS includes 10 components, each comprising a set of questions related to activities of daily living. Each component is scored from 0 to 3, where 0 signifies no neglect, 1 indicates mild neglect, 2 represents moderate neglect, and 3 denotes severe neglect. The total score ranges from 0 to 30, with 0 indicating no neglect, 1-10 suggesting mild behavioural neglect, 10-20 reflecting moderate behavioural neglect, 21-30 signifying severe behavioural neglect, and a score of 30 representing a more severe form of neglect.

The internal validity of the Catherine Bergego Scale found unidimensionality of items by Rasch analysis. All categories of the Korean Kessler Foundation Neglect Assessment Process (KF-NAP) showed high internal consistency (19). The CBS is an adequate and validated scale for assessing patients with USN after stroke. The CBS is more sensitive than pen-and-paper tests for USN detection (20).



Figure 2. Lifting a heavy can

Line Bisection Test (LBT)

The LBT is used to identify the presence of unilateral spatial neglect. During the test, the participant is instructed to mark the midpoint of a series of horizontal lines using a pencil. Typically, the mark deviates towards the side of the brain lesion.

Studies have shown excellent test-retest reliability, with values ranging from $r = 0.84$ to $r = 0.93$. Another study reported adequate test-retest reliability (Pearson $r = 0.64$) (21). The LBT has construct validity for determining the presence of Unilateral Neglect (ULN). One study showed a correlation between the LBT and the Star Cancellation Test ($r = 0.48$) (21).

Data analysis

The statistical analysis in this study was conducted using IBM SPSS software version 30.0.0.0. A paired t-test was used to compare task-oriented MT and task-oriented therapy, assessing the differences between pre-test and post-test scores from the CBS and the LBT. Additionally, an unpaired t-test was conducted to compare post-test results between the two groups.

RESULTS

The result showed significant improvements in functional outcome in both groups, with a $p < 0.001$. Baseline characteristics of patients are summarized in Table 1. In group A (mirror), the CBS scores (mean $\pm$ SD) decreased from 17.20 ± 3.590 to 8.20 ± 2.624 (Cohen's $d = 2.87$), while in Group B (no mirror), they decreased from 17.40 ± 3.019 to 15.07 ± 3.105 (Cohen's $d = 0.98$).

Similarly, in the LBT, group A improved from 14.87 ± 3.091 to 7.27 ± 1.534 (Cohen's $d = 2.09$), whereas Group B improved from 15.87 ± 2.997 to 14.53 ± 2.973 (Cohen's $d = 0.49$). Statistical analyses are shown in (Table 2-5).

Post-test comparisons confirmed that Group A showed better reductions in scores compared to group B, indicating a more significant enhancement in functional outcomes. The results demonstrate that task-oriented MT led to significant improvements in functional rehabilitation compared to task-oriented therapy without a mirror.

Table 1. Baseline comparison between group A and group B

Variable	Group A	Group B
Age (Mean $\pm$ SD)	54 $\pm$ 8.15	55.4 $\pm$ 9.14
Mini Mental Scale Examination (MMSE)	25 $\pm$ 1.30	25.06 $\pm$ 1.16
BMI (Mean $\pm$ SD)	24.06 $\pm$ 1.06	24.3 $\pm$ 1.42
Gender (M/F)	7/8	8/7
Comorbidities		
Diabetes	8	7
Hypertension	3	5
Dyslipidemia	4	3

*SD, standard deviation, *BMI, body mass index

Table 2. Pre and post-test values of CBS in group A and group B

Groups	Pre-test Mean $\pm$ SD	Post-test Mean $\pm$ SD	t-value
Group A (N = 15)	17.20 $\pm$ 3.590	8.20 $\pm$ 2.624	18.825
Group B (N = 15)	17.40 $\pm$ 3.019	15.07 $\pm$ 3.105	9.260

SD, standard deviation; CBS, Catherine Bergego Scale

Table 3. Pre and post-test values of Line Bisection Test in group A and group B

Groups	Pre-test Mean $\pm$ SD	Post-test Mean $\pm$ SD	t-value
Group A (N = 15)	14.87 $\pm$ 3.091	7.27 $\pm$ 1.534	14.032
Group B (N = 15)	15.87 $\pm$ 2.997	14.53 $\pm$ 2.973	10.583

SD, standard deviation

Table 4. Post-test values of CBS and Line Bisection Test in group A

Test	Mean	SD value	t-value
Post-test (Line bi-section test)	7.27	1.534	-8.413
Post-test (CBS)	8.20	2.624	-6.542

SD, standard deviation; CBS, Catherine Bergego Scale

Table 5. Post-test values of CBS and Line bisection test in group B

Test	Mean	SD value	t-value
Post-test (Line bisection test)	14.53	2.973	-8.413
Post-test (CBS)	15.07	3.105	-6.542

SD, standard deviation; CBS, Catherine Bergego Scale

DISCUSSION

In adults, stroke is the most common cause of long-term impairment. Stroke is the second most common cause of death and disability globally, and the highest illness burden is found in low- and middle-income nations (22). In our study, we compared task-oriented MT (group A) and task-oriented therapy without a mirror (group B) for USN in post-stroke patients. By comparing the mean values and SD for Group A and Group B, it was evident that task-oriented MT had a better impact compared to task-oriented therapy without a mirror.

Task-oriented MT significantly enhanced functional recovery by utilizing visual feedback to reduce the symptoms of USN. This is consistent with Sim & Kwon and Pandian et al. who noted that rehabilitation techniques such as MT were effective in the treatment of USN. This form of therapy works by using a mirror to provide visual feedback to the brain, tricking it into thinking that there is movement or sensation on the neglected side. This process may provide support for neural circuitry, allowing for better motor functioning and diminishing symptoms of neglect (14, 17). Utilisation of visual input, particularly using MT, has been identified as enhancing functional outcomes better than conventional task-orientated therapies without visual feedback. Fong et al. provided initial evidence of the therapeutic advantages of MT for treating patients with stroke and spatial neglect. Their observations revealed how mirror-induced visual feedback assists in mitigating symptoms of neglect by prompting attention to the neglected side. This process triggers cortical reorganisation and facilitates neuroplasticity, ultimately improving spatial awareness and

functional recovery. Mirror therapy is therefore a worthwhile adjunct to traditional rehabilitation techniques in patients with spatial neglect (7).

Task-oriented mirror treatment improves spatial awareness and visual-spatial integration and reduces symptoms of spatial neglect. This seems to be beneficial to the patient and is consistent with Klinkwan et al.'s physiological findings from 2021. Even though motor rehabilitation played a significant role in their study, they also suggested that mirror treatment would be able to reduce neglect by activating the neglected side through visual input (23).

Although psychology was not the primary focus of our research, the research literature recommends that MT can also provide some significant psychological and motivational advantages. Mirror therapy has been proven to enhance patients' moods and confidence, which can, in turn, have a positive effect on their level of rehabilitation engagement. The process of performing normal movement can help overcome the feeling of helplessness and enhance emotional well-being. However, the psychological effects have been suggested to be limited in patients with more significant impairments and those who find it hard to stay motivated during therapy. This highlights the need for further studies to improve the psychological effects of MT in various populations (7, 24).

In our study, we found that task-orientated therapy had beneficial effects on the participants similar to previous studies. For example, Pathan demonstrated that task-orientated training contributed to improvements in motor ability and functional outcomes in adults with chronic stroke, but noted that there were inconsistencies with improvements in activity-based outcomes.

This may mean that while task-orientated approaches can have a substantial effect on stroke rehabilitation, there could be variability based on the utility of the task and the individual characteristics of the challenge or goal, demonstrating a need for further individualised rehabilitation approaches (25).

Even though our findings are positive, some research has indicated that not all stroke patients respond favourably to MT. Their response often depends on personal expectations, which can reduce their motivation if outcomes do not meet their hopes. Only some stroke patients respond positively to MT; for others, it may cause frustration or decrease their motivation if no progress is shown (24). In our study, providing participants with consistent feedback and clearly defined goals contributed to positive outcomes.

In summary, our research showed that compared to task-oriented therapy without a mirror, task-oriented MT greatly improves functional recovery and spatial awareness in USN patients. The effect of visual feedback as provided with the use of MT in combination with the extremity task was vital in facilitating reactivation of pathways in the brain, reduction of symptoms of neglect, and enhancement of ADL. This supports the use of the task-oriented MT, a promising therapeutic approach that utilises the mirror neurones to improve functions and the quality of life of stroke patients with USN syndrome.

Limitations and future hope of the study

This study has some limitations that need to be explained. Due to the nature of the intervention, participants were aware of the type of treatment provided; double-blinding was not possible. The study included only a limited number of participants. Even though the sample size calculation was done using G* power software to determine whether the sample size was efficient, a larger sample size is needed to increase the reliability of the findings. Additionally, the course of treatment was only four weeks. A longer follow-up is needed to evaluate the durability of the therapeutic effects.

Further advances in technology, interdisciplinary approaches, and personalised treatment strategies might enhance the use of task-orient-

tated MT for unilateral neglect post stroke. Incorporating neuroimaging techniques like fMRI or EEG could potentially provide insights into cortical changes associated with MT. Comparing recent innovations such as virtual reality and non-invasive brain stimulation could potentially improve effectiveness and should be incorporated in future studies.

CONCLUSIONS

The research demonstrated that task-oriented MT is more effective than task-oriented therapy without a mirror. Mirror therapy enhances the functional outcome and spatial awareness of patients suffering from unilateral spatial neglect. The results point to the fact that both mirror neurons and visual stimuli greatly aid in enhancing the functional outcome and symptoms of unilateral neglect in the individual.

ACKNOWLEDGEMENTS

The authors would like to express their sincere gratitude to the department and all those who provided guidance, support, and encouragement throughout this research journey. We also extend our deepest appreciation to all the participants who contributed their time and effort to this research.

FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

AUTHOR CONTRIBUTIONS

D.S.: conceptualization, methodology, formal analysis, and manuscript drafting, assistance in data acquisition and formatting of the final manuscript; K.D.: data collection, investigation, and literature review, project administration and coordination of research activities; P.S., P.K.: supervision, validation, and critical revision of the manuscript; R.V., V.J.: statistical analysis and data interpretation; V.S.: visualization, graphical representation, and proofreading.

DATA AVAILABILITY

The data can be given by the corresponding author with reasonable requests.

INSTITUTIONAL REVIEW BOARD STATEMENT

This study was approved by the Institutional Scientific Review Board of Saveetha College of Physiotherapy (Approval Number: 376/07/2024/ISRB/UGSR/SCPT, approval date July 10, 2024). All procedures were conducted in accordance with institutional ethical standards and the Declaration of Helsinki.

INFORMED CONSENT STATEMENT

Written informed consent was obtained from all individual participants involved in the study.

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Comparative Assessment of Serum Vitamin D and Calcium Levels in Hypothyroid Patients in Albayda, Libya

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Received: September 23, 2025;

Revised: October 26, 2025;

Accepted: November 14, 2025

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ABSTRACT

OBJECTIVE Vitamin D is essential for the proper functioning of numerous organs, including the thyroid gland. For that reason, a deficiency in vitamin D is considered a potential risk factor for developing various thyroid disorders, particularly hypothyroidism. The present study aimed to evaluate the serum concentrations of vitamin D and calcium in individuals who have been diagnosed with primary hypothyroidism.

METHODS The research was conducted on a cohort of 100 hypothyroid patients at the Department of Internal Medicine, Albayda Medical Center, in Albayda, Libya. Serum levels of vitamin D, calcium, and thyroid profiles (thyroid stimulating hormone (TSH), T3, and T4) were measured in all participants. A control group consisting of 100 healthy individuals was used for comparison. Statistical analysis was performed using SPSS software version 20.0 with independent t-tests and Pearson correlation analysis.

RESULTS Our findings indicate that the serum levels of vitamin D and calcium were significantly lower among the hypothyroid group compared to the control group (12.21 ± 2.02 ng/mL and 4.40 ± 1.24 mg/dL vs 45.53 ± 10.51 ng/mL and 10.88 ± 1.45 mg/dL, $p = 0.008$ and 0.006 , respectively). However, we noticed non-significant differences in changes in the serum levels of vitamin D and calcium between males and females in the hypothyroid group ($p = 0.578$ and 0.410 , respectively). Pearson correlation analysis indicated that the TSH levels of the hypothyroid group had statistically significant negative correlations with the serum vitamin D and calcium levels ($r = -0.144$, $p = 0.030$ and $r = -0.145$, $p = 0.029$, respectively).

CONCLUSIONS Based on these results, it is strongly advised that individuals with hypothyroidism be screened for vitamin D and calcium deficiencies and, if necessary, receive appropriate supplementation.

KEYWORDS vitamin D, calcium, hypothyroidism, vitamin d deficiency, Albayda

INTRODUCTION

Vitamin D is a fat-soluble vitamin that is crucial for regulating several minerals, especially calcium. It is synthesized in the skin upon exposure to sunlight, especially ultraviolet B rays (1). Vitamin D is also present in a variety of dietary sources,

including liver, fortified dairy products, fatty fish, and nutritional supplements (2). There are two main types of vitamin D: D2 (ergocalciferol), which is present in certain plants and fortified food products, and D3 (cholecalciferol), which is synthesized in the skin and is found in animal-

derived foods (2). Once in the body, vitamin D is converted in the liver to 25-hydroxyvitamin D, its primary circulating form. It is then further converted into its active form, 1,25-dihydroxyvitamin D (calcitriol), in the kidneys (3). The thyroid gland, a butterfly-shaped gland located anteriorly in the lower part of the neck, is essential for regulating the body's metabolism through the production of thyroid hormones, mainly triiodothyronine (T_3) and thyroxine (T_4) (4). Additionally, the parafollicular cells (C cells) of the thyroid gland produce and secrete calcitonin, a hormone involved in maintaining calcium homeostasis in the bloodstream (5). Vitamin D plays an important role in maintaining bone health and proper bone mineralization. Recent studies have indicated a correlation between low vitamin D levels and various health issues, including cardiovascular disease, diabetes, cancer, obesity, and osteoporosis (6). A growing body of evidence also supports a significant association between vitamin D deficiency and hypothyroidism. For example, previous studies have shown that the prevalence of vitamin D insufficiency in individuals with Hashimoto's thyroiditis is markedly higher (92.0%) compared to healthy controls (63.0%) (7, 8). Additionally, studies have reported that patients with Graves' disease also often present with low levels of Vitamin D (9, 10). Furthermore, studies have identified a negative correlation between serum vitamin D levels and both calcium and thyroid-stimulating hormone (TSH) levels (11). Consequently, our current study was undertaken to evaluate serum vitamin D and calcium levels in Libyan individuals with hypothyroidism due to high prevalence of both vitamin D deficiency and hypothyroidism in Libya, which are linked to negative health outcomes.

METHODS

This study was conducted at the Department of Internal Medicine at Albayda Medical Center, Albayda, Libya, from January to December 2024, involving 100 patients diagnosed with hypothyroidism and 100 healthy individuals. The hypothyroid group was recruited from the Department of Internal Medicine of Albayda Medical Center, while the control group was recruited from Albayda Medical Center staff members. The inclusion criteria for the hypothyroid group were patients diagnosed with primary hypothyroidism

who were taking anti-hypothyroidism medications (e.g., levothyroxine) for almost 5 years. We excluded from the study any individual with hepatic or renal disorders, malignancy, pregnancy, lactation, or having medications altering bone metabolism. The controls included individuals with no history of hypothyroidism and who were not taking vitamin D supplements during the study period. Blood samples were collected from all participants after an 8-hour fasting period. The levels of T_3 , T_4 , and TSH were measured using the Cobas c111 ECLIA analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The normal reference ranges for serum thyroid profiles are 1.4-4.4 pg/dL for T_3 , 0.7-2.1 ng/dL for T_4 , and 0.3-4.0 mU/L for TSH (12). The serum levels of vitamin D and calcium were measured using a Cobas e411 ECLIA analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Data are presented as mean $\pm$ standard deviation (mean $\pm$ SD) and were analyzed using SPSS software, version 20.0. Serum vitamin D and calcium levels of the control and hypothyroid groups were compared using independent t-tests, while Pearson correlation analysis was applied to assess the association between vitamin D/calcium levels and TSH levels in the hypothyroid group. We also used independent t-tests to compare serum vitamin D and calcium levels of males and females of the hypothyroid group because females made up the vast majority of that group (70.0%) in our study. Results were considered significant when $p < 0.05$.

RESULTS

Our study included 100 hypothyroid patients (30 males and 70 females) and 100 healthy controls (45 males and 55 females). Most participants were aged between 35 and 55 years, with no significant differences in age or gender distribution between the two groups ($p = 0.568$ and 0.436 , respectively). The majority of the patient group had also been diagnosed with type 2 diabetes mellitus (T2DM) and had been on hypoglycemic medications (e.g., metformin and sitagliptin) for over three years. Our findings indicate that the serum levels of vitamin D and calcium were significantly lower in the hypothyroid group compared to the control group (12.21 ± 2.02 ng/mL and 4.40 ± 1.24 mg/dL vs 45.53 ± 10.51 ng/mL and 10.88 ± 1.45 mg/dL, $p = 0.008$ and 0.006 , respectively). Moreover,

the serum TSH levels were significantly high in the hypothyroid group (10.98 ± 1.94 vs. 3.63 ± 0.30 mU/L, $p = 0.009$) while the serum levels of T3 (1.02 ± 0.30 vs. 2.94 ± 1.45 pg/dL, $p = 0.001$) and T4 (0.59 ± 0.20 vs. 1.57 ± 0.70 ng/dL, $p = 0.002$) were significantly lower as shown in Table 1.

We observed non-significant differences in the serum levels of vitamin D, calcium, and thyroid profiles between male and female hypothyroid patients ($p > 0.05$; Table 2).

Pearson correlation analysis indicated that the TSH levels of the hypothyroid patients had statistically significant negative correlations with the serum vitamin D and calcium levels ($r = -0.144$, $p = 0.030$ and $r = -0.145$, $p = 0.029$, respectively) as presented in Table 3.

DISCUSSION

Vitamin D deficiency in individuals with hypothyroidism is primarily attributed to either inadequate dietary intake or poor intestinal absorption of vitamin D. To the best of our knowledge, this research is among the few that explore and evaluate the relationship between vitamin D and calcium levels in the context of hypothyroidism in Albayda, Libya. This study was conducted to assess and compare the serum levels of vitamin D and calcium in patients diagnosed with primary hypothyroidism versus healthy individuals without any reported symptoms or history of hypothyroidism or other thyroid-related conditions. The study found that the serum levels of both vitamin D and calcium were significantly lower in the

Table 1. Comparison of thyroid profiles and vitamin D/calcium levels of study participants

Variables	Control group (Mean $\pm$ SD)	Hypothyroid group (Mean $\pm$ SD)	p-value
Age (years)	43.37 $\pm$ 8.47	44.60 $\pm$ 8.90	0.568
Gender, n (%)			
Males	45 (45.0)	30 (30.0)	0.436
Females	55 (55.0)	70 (70.0)	
Serum T3 (pg/dL)	2.94 $\pm$ 1.45	1.02 $\pm$ 0.30	0.001*
Serum T4 (ng/dL)	1.57 $\pm$ 0.70	0.59 $\pm$ 0.20	0.002*
Serum TSH (mU/L)	3.63 $\pm$ 0.30	10.98 $\pm$ 1.94	0.009*
Serum Vit D (ng/mL)	45.53 $\pm$ 10.51	12.21 $\pm$ 2.02	0.008*
Serum Ca ²⁺ (mg/dL)	10.88 $\pm$ 1.45	4.40 $\pm$ 1.24	0.006*

TSH, thyroid stimulating hormone; Vit, vitamin; Ca²⁺, calcium ion

*, means statistically significant differences ($p < 0.05$)

Table 2. Comparison of thyroid profiles and vitamin D/calcium levels of the hypothyroid group

Variables	Hypothyroid group (Mean $\pm$ SD)		p-value
	Males (n = 30)	Females (n = 70)	
Serum T3 (pg/dL)	0.79 $\pm$ 0.32	0.81 $\pm$ 0.42	0.286
Serum T4 (ng/dL)	0.64 $\pm$ 0.34	0.52 $\pm$ 0.26	0.208
Serum TSH (mU/L)	10.98 $\pm$ 1.94	10.92 $\pm$ 1.87	0.362
Serum Vit D (ng/mL)	11.63 $\pm$ 1.87	12.79 $\pm$ 2.17	0.578
Serum Ca ²⁺ (mg/dL)	4.40 $\pm$ 1.24	4.02 $\pm$ 1.15	0.410

TSH, thyroid stimulating hormone; Vit, vitamin; Ca²⁺, calcium ion

Table 3. Correlation coefficients between vitamin D/calcium and TSH of the hypothyroid group

Correlations	Vitamin D (r, p-value)	Calcium (r, p-value)
TSH (mU/L)	$r = -0.144$, $p = 0.030$	$r = -0.145$, $p = 0.029$

TSH, thyroid stimulating hormone

hypothyroid group than in the control group ($p = 0.008$ and 0.006 , respectively). These findings align with those of Ashok et al., who investigated the impact of vitamin D deficiency on the thyroid gland and reported that inadequate vitamin D levels may lead to reduced thyroid hormone production (13). Additionally, vitamin D and calcium serum levels showed significant strong negative correlations with TSH levels in the hypothyroid group ($r = -0.144$, $p = 0.030$ and $r = -0.145$, $p = 0.029$, respectively), suggesting a potential significant correlation between vitamin D/calcium deficiencies and hypothyroidism. Similarly, Li et al. reported that the serum TSH levels of the patient group had significant high negative correlations with the serum vitamin D levels ($r = -0.150$, $p = 0.024$) (14). Consistent with earlier studies, our research found no significant differences in serum vitamin D or calcium levels between males and females in the hypothyroid group ($p = 0.578$ and 0.410 , respectively). For instance, Madkhali et al. reported similar findings in their study on vitamin D status in Saudi Arabia, observing no gender-based variation (15). Similarly, Naseri et al. concluded that vitamin D levels did not differ significantly between men and women in a large-scale study conducted in Iran (16). In line with our results, a study conducted by Qanbar et al. also identified hypovitaminosis D and hypocalcemia in individuals with hypothyroidism, reinforcing the link between these deficiencies and thyroid dysfunction (17). Appunni et al., using a nationally representative sample of 7,943 participants, found that 25.6% of individuals with hypothyroidism had vitamin D deficiency, compared to 20.6% among healthy controls (11). While it is commonly observed in many Muslim-majority countries that women tend to have lower vitamin D levels compared to men—mainly due to clothing practices (e.g., wearing a hijab) and social-religious customs—some studies challenge this assumption. For example, Aghapour et al., in a comprehensive study conducted in Iran, found no significant differences in vitamin D levels between men and women (18). Our own findings align with this conclusion, revealing a high prevalence of severe vitamin D deficiency in both male and female participants. This is supported by earlier research showing an overall vitamin D insufficiency rate of 83.5%, with no significant corre-

lation with gender (19). In addition, our study's findings corroborate those of a study by Faid et al., who also reported a widespread and severe vitamin D deficiency among both sexes (20).

CONCLUSIONS

Our research findings indicate that patients diagnosed with primary hypothyroidism commonly exhibit vitamin D deficiency and hypocalcemia. The significant negative correlations between vitamin D/calcium and TSH levels suggest that the degree and severity of hypothyroidism is significantly associated with serum vitamin D and calcium deficiencies.

Limitations

The small sample size of participants, being a single-center study, the need to measure parathyroid hormone levels (PTH) and to determine its impact on vitamin D and hypothyroidism, and the fact that only one blood sample from each participant was examined, possibly leading to measurement error, are the four main limitations of this study.

Recommendations and future studies

We advise physicians to be aware of potential vitamin D deficiency and hypocalcemia when TSH levels exceed 8 mU/L. Additionally, we recommend regular evaluation of hypothyroid patients' serum levels of calcium and vitamin D and prescription of the proper calcium and vitamin D supplements as part of their treatment plan.

Additional large sample size studies are required to determine whether vitamin D deficiency is a causal factor in the pathogenesis of hypothyroidism or a consequence of the disease. Moreover, further prospective clinical studies should examine the effect of PTH on vitamin D levels and hypothyroidism. Multicenter studies in other regions are needed before our results are widely used. Finally, hypoglycemic medications may lead to vitamin D deficiency in hypothyroid patients, a possible relationship which was not considered in this study. Further studies are required to confirm our results.

ACKNOWLEDGMENTS

We would like to convey our heartfelt thanks to all the participants for their generous assis-

tance and collaboration in making this study possible. Our sincere gratitude is also extended to everyone who enabled us to complete this research. We also wish to acknowledge with great appreciation the essential contribution of the Laboratory Staff at Albayda Medical Center, who allowed us to use the lab equipment and all the required tools in the laboratory.

FUNDING

No funding was received for conducting this research study.

CONFLICTS OF INTEREST

There were no conflicts of interest related to this research article.

AUTHOR CONTRIBUTIONS

N.I.: conceptualization, literature review, data analysis, methodology, data curation, and writing original draft preparation; Y.M.: method validation, data curation, data analysis, software, review & editing, and supervision. Both authors have reviewed and approved the final version of this manuscript submitted for publication.

DATA AVAILABILITY STATEMENT

The data that support the results of this study can be obtained from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

The Medical Ethics Board of Albayda Medical Center granted approval for the study protocol in accordance with the Declaration of Helsinki (protocol code: AMC765/11 and date of approval: November 10th, 2023) prior to study initiation.

INFORMED CONSENT STATEMENT

All participants gave written informed consent for their data to be published.

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Repeatability and Reproducibility of an Automated Image-Processing Software for Quantitative Conjunctival Hyperemia Analysis: A Pilot Study

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Received: May 29, 2025;

Revised: August 30, 2025;

Accepted: November 5, 2025

ABSTRACT

OBJECTIVE Conjunctival hyperemia reflects the severity of various ocular conditions and is a common side effect of glaucoma medications, posing a significant concern for affected patients. Most existing clinical grading systems are subjective and rely heavily on individual physician interpretation, making interobserver comparison difficult. We developed an automated grading software to objectively and quantitatively assess conjunctival hyperemia. This study aimed to evaluate the repeatability and reproducibility of a newly developed automated software for quantitative assessment of conjunctival hyperemia in glaucoma patients in a pilot validation study.

METHODS In this pilot study, glaucoma patients using at least one topical anti-glaucoma medication and exhibiting conjunctival hyperemia were enrolled. Each patient's conjunctival hyperemia was assessed using the automated software, which segmented images into four regions: blood vessel area, strong red area, weak red area, and non-red area. An automatic region of interest (ROI) registration function was incorporated to enhance measurement consistency. Repeatability was assessed using two measurements from the same image, and reproducibility was tested using separate images of the same eye.

RESULTS Eighty images from twenty glaucoma patients were analyzed (collected between December 1, 2016 to December 31, 2018). Following administration of 10% phenylephrine ophthalmic solution, the percentage of conjunctival hyperemia decreased significantly in all regions ($p < 0.001$). Spearman correlation coefficients for repeated measurements on the same image were 0.884 (blood vessel), 0.982 (strong red), and 0.932 (weak red). Corresponding values for repeated images of the same eye were 0.988, 0.992, and 0.995, respectively. The intraclass correlation coefficient (ICC) for clinical grading by ten ophthalmologists was 0.969 (95% CI: 0.952–0.981).

CONCLUSION This pilot study demonstrates that the proposed automated software provides an objective and quantitative assessment of conjunctival hyperemia with excellent repeatability and reproducibility. The system shows high correlation with clinical grading and may serve as a reliable tool for monitoring conjunctival hyperemia changes in clinical practice.

KEYWORDS repeatability, reproducibility, conjunctival hyperemia, automated, software

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INTRODUCTION

Conjunctival hyperemia occurs in various ocular diseases, including inflammatory conditions (e.g., allergic conjunctivitis, scleritis, uveitis), infections (e.g., conjunctivitis, keratitis), dry eye syndrome, and drug-induced ocular surface disorders (1). The degree of hyperemia correlates with disease severity, making it a key indicator in clinical monitoring. In glaucoma patients, conjunctival hyperemia is a common side effect of topical medications, affecting treatment adherence and quality of life.

Several subjective grading scales, such as the Efron scale, the original Cornea and Contact Lens Research Unit (CCLRU) scale, and the Brien Holden Vision Institute scale, are commonly used in clinical practice (2–5). However, these rely on physician interpretation and suffer from interobserver variability. Currently, many objective conjunctival grading systems have been developed to improve accuracy in clinical assessment, but they are still not widely available in routine clinical practice (6–23).

We developed an automated software tool to objectively evaluate conjunctival hyperemia in glaucoma patients. This software quantitatively assesses hyperemia using pixel coverage in pre-defined regions of interest (ROIs), categorized into blood vessel area, strong red area, weak red area, and non-red area. This pilot study aimed to evaluate the software's repeatability and reproducibility, as well as its correlation with clinical grading.

METHODS

Design

This cross-sectional pilot study was conducted in the Department of Ophthalmology, Faculty of Medicine, Chiang Mai University, from December 1, 2016 to December 31, 2018. The software was created and developed in collaboration with the Department of Computer Engineering.

Participants

We recruited 20 open angle glaucoma patients who were using at least one anti-glaucoma medication and complained of conjunctival hyperemia. The severity of conjunctival hyperemia was evaluated in all patients using both the automated conjunctival hyperemia grading software and assessments by ten ophthalmologists.

The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Chiang Mai University (approval number: 452/2015; approved October 26, 2015). The study followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants.

Image collecting and processing

The temporal and nasal bulbar conjunctival images were taken using a Topcon SL-D7 digital-ready slit lamp. We used diffuse illumination, a 30-degree angle between the lamp and microscope arm, brightest light level and 175 Watt/second flashlight. The objective magnification used was 10. All images were saved in red (R), green (G), and blue (B) JPEG format with a resolution of 3264×2448 pixels. All images were taken twice with a 10-second interval. After finishing taking the first image, the patients were moved away from the slit lamp and their head position was readjusted before taking the second image. Two images were acquired before and after repositioning to evaluate intra-observer repeatability. The next step was to apply the topical 10% phenylephrine ophthalmic solution which is a strong vasoconstrictor and waited for 30 minutes. We then took another two images using the same protocol. These images were processed using our newly developed conjunctival grading software to evaluate repeatability and reproducibility.

Software algorithm

According to the direction of the lighting sources, there was sometimes reflected light or glare on eye surface inside ROI. The system first identified the glare area by considering R, G, and B values of each pixel in the image. For 24-bit color images which have the maximum R, G, and B values of 255, if R, G, and B were greater than 250, the system marked that pixel as glare pixel and removed it from the image. (Figure 1) Note that, in this step, median filter was applied to refine the shape of glare regions.

The software transformed the input image from RGB Color Space to YCbCr Color Space where Y is a luminance of the pixel, Cb is a blue-difference chroma of the pixel and Cr is a red-difference chroma of the pixel.

Y and Cr of pixels are used to identify blood vessel pixels. Multi-scale morphological top-hat operators were applied to Y Channel to remove

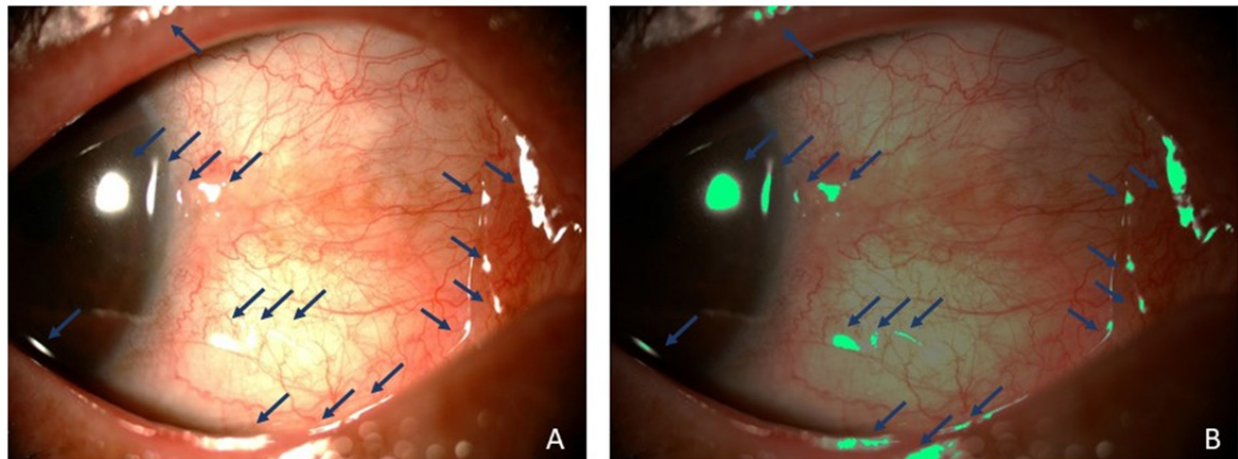


Figure 1. Anterior segment images show (A) light reflection or glare area is identified by pixel detection. (blue arrow) (B) all light reflection or glare area will be removed from the image before starting conjunctival hyperemia grading process.

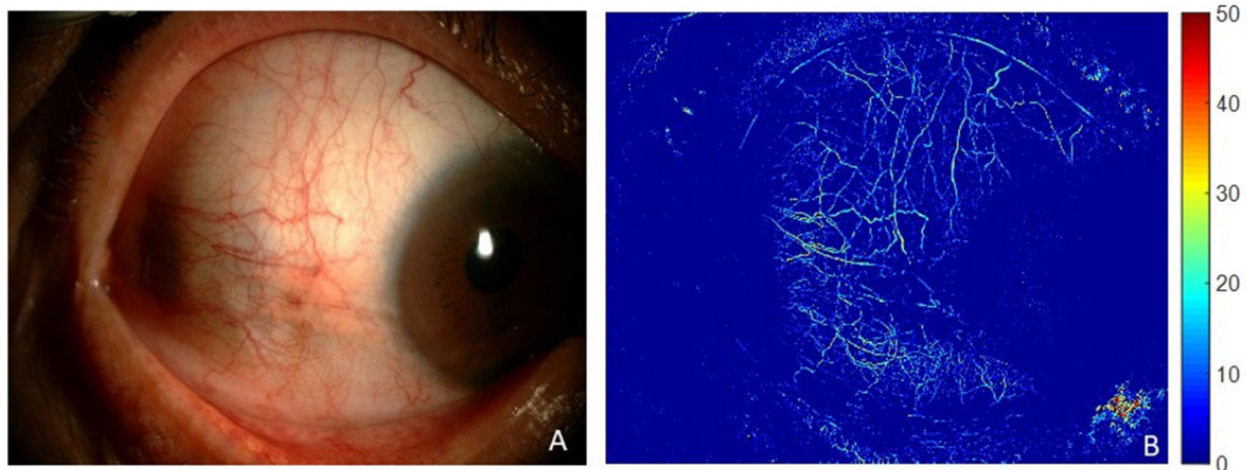


Figure 2. Anterior segment images show before (A) and after (B) the system transforms the input image from RGB Color Space to YCbCr Color Space and compute a blood vessel enhanced image V.

a nonuniform lighting condition (24). Then, the software computed a blood vessel enhanced image V which is defined by this following equation (see also Figure 2)

$$V = \begin{cases} 255 - Y & ; Cr > 0, \\ 0 & ; Cr \leq 0. \end{cases}$$

To obtain blood vessel pixels, an adaptive thresholding was applied to V. Based on assumption that real blood vessel pixels should be connected to other pixels, tiny connected components were considered as random noise and removed. Finally, a morphological thinning operator is applied to the result to improve the shape of blood vessel. (Figure 3)

After the software identified blood vessel, the other pixels were classified according to three redness classes (i.e, strong red pixels, weak red

pixels, and normal pixels). The input image was transformed from RGB Color Space to HSV (hue, saturation, value) Color Space. H channel (hue of pixel in range of [0,1]) was used to classify redness of pixels as

$$Class = \begin{cases} Strong\ Red & ; |0.5 - H| \geq Th_1, \\ Weak\ Red & ; Th_2 \leq |0.5 - H| < Th_1, \\ Normal & ; otherwise. \end{cases}$$

In this work, we set the threshold values as $Th_1 = 0.45$ and $Th_2 = 0.42$. Based on the color scale shown in Figure 4, the hue value of the pure red color is equal to 0 or 1. If we consider the colors with the hue value between [0, 0.05] and [0.95, 1.00], those colors are very close to pure red based on human perception. Colors with hue values near 0.00 or 1.00 correspond to red on the HSV scale; values between 0–0.05 and 0.95–1.00 are

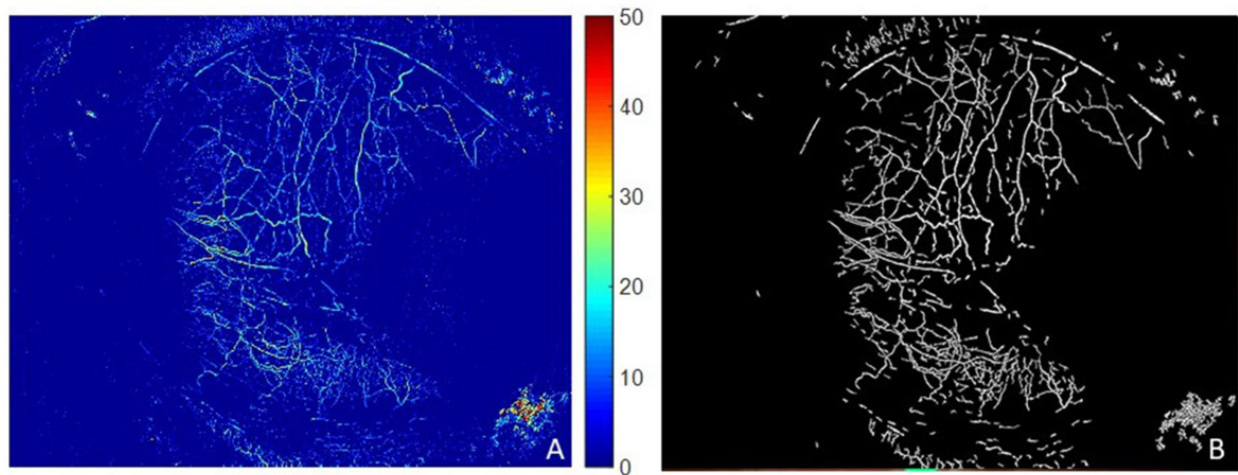


Figure 3. Images demonstrate before (A) and after (B) use of the system, an adaptive thresholding and a morphological thinning operator to improve the blood vessel shape.

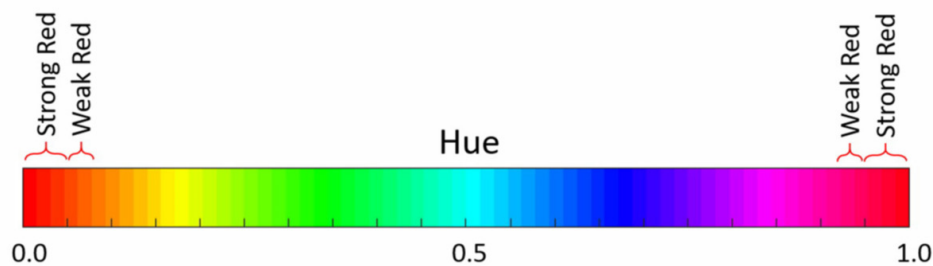


Figure 4. Color scale that illustrates H values and their corresponding colors.

perceptually indistinguishable from pure red. An average just identifiable hue value of pure red is around 0.04, with a standard deviation of around 0.01 (25). As a result, we set the value of Th_1 as 0.45. The visible blood vessels on the white part of the eye (sclera) are bright red or reddish-pink because they are near the surface, allowing us to see the blood's color directly. Depending on the lighting conditions and the camera's white balance, their hue can sometimes appear red-orange. The red-orange and reddish-pink colors have hues of around 0.08 and 0.92, respectively. That's why we set the value of Th_2 as 0.42.

The number of pixels in each area is counted and compared to the number of pixels in ROI, resulting in the area ratio in percentage.

The ROIs on the bulbar conjunctiva were manually chosen in the nasal and temporal areas, which are easily observable by others. We evaluated the percentage of blood vessels and degree of conjunctival hyperemia in the four classes (blood vessel area, strong red area, weak red area and non-red area).

A local feature matching is used for the automatic ROI registration between two images. (26, 27) The algorithm can be described as follows: (Figure 5)

A. Key-points were extracted from both images. For a reference image for which the user has manually selected ROI, the key-points outside ROI will be removed. The key-points are typically defined as corner pixels in the image; therefore, most of the key-points in the reference image are located along blood vessels. However, the key-points in the target image were typically distributed over the whole image (including blood vessels).

B. The system determined the key-points in the target image that most likely matched the key-points in the reference image by comparing Histogram of Oriented Gradients (HoG) (28) of the pixels surrounding the key-points.

C. Based on matched key-point pairs, the system identified a geometric transformation matrix between coordinates in the two images. To find an optimal parameter of the transformation matrix, Random Sample Consensus (RANSAC) was applied (29). RANSAC can determine the optimal

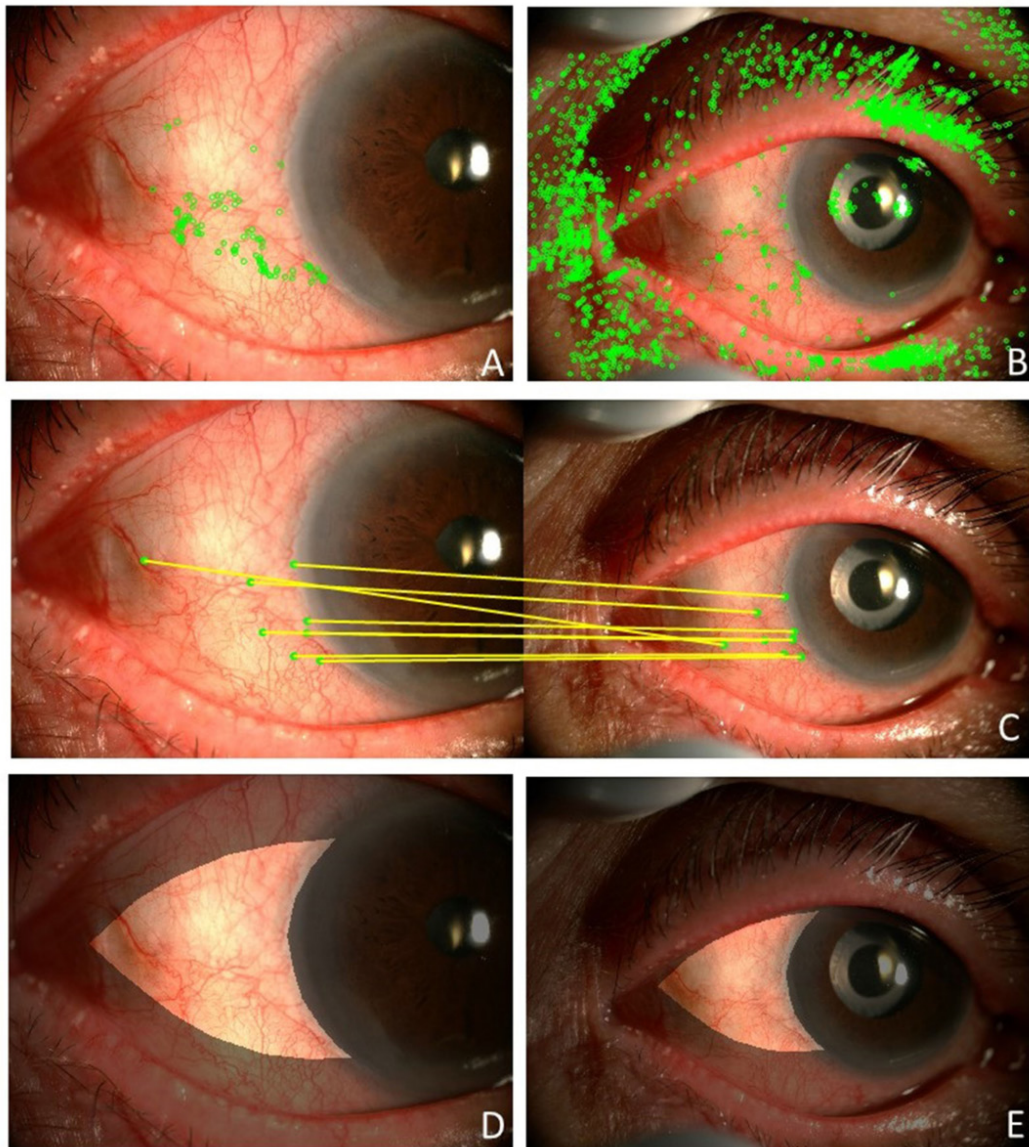


Figure 5. Key-points (green dots) along blood vessel are extracted from the reference image. (A) Key-points (green dots) over the whole image are extracted from the target image. (B) The system determines the key-points in the target image match to the key-points in the reference image by comparing the pixels surrounding the key-points. (C) The coordinates of the pixels in region of interest in the reference image transform to the coordinates in the target image by using the transformation matrix (D, E).

parameters with robustness to any outliers (i.e., incorrectly matched pairs).

D, E. Finally, the coordinates of the pixels in ROI in the reference image are then transformed to the coordinates in the target image by using the transformation matrix.

Clinical grading

Ten ophthalmologists independently viewed and graded each image on a 0 to 4-point scale based on the Efron grading scale (2, 3) without any time limitation. The reference grading scale was shown together with the image being graded on the same page to eliminate the inconsistencies

between computer screen's display.

Statistical analysis

All statistical analyses were performed using SPSS version 23.0 (SPSS Inc., Chicago, Illinois, USA). Normality of data was tested using Shapiro-Wilk test, and it is noted that all data sets showed non-normal distributions. As such, the percentage of conjunctival hyperemia before and after the application of 10% phenylephrine ophthalmic solutions were compared using the Wilcoxon matched pairs test. P value < 0.05 was considered significant.

Repeatability of the software was assessed by comparing two hyperemia percentage measurements from the same image. Statistical tests included the Wilcoxon signed-rank test, the Spearman correlation coefficient (rs), and the Bland-Altman method.

Reproducibility was evaluated by comparing hyperemia percentages from two separate images taken 10 seconds apart, using the same set of statistical tools: the Wilcoxon signed-rank test, Spearman's rs, and Bland-Altman analysis.

Reliability of the clinical grading from ten ophthalmologists was shown in intraclass correlation coefficient: The correlation of clinical grading and automated grading was tested by the Spearman correlation coefficient (rs).

The strength of correlation for Spearman's (rs) was interpreted as follows: 0.00–0.19: very weak, 0.20–0.39: weak, 0.40–0.59: moderate, 0.60–0.79: strong and 0.80–1.0: very strong.

RESULTS

A total of 80 images were analyzed using our newly developed automated grading software. The average of the median, lower quartile, and upper quartile of the percentage of conjunctival hyperemia before applying 10% phenylephrine ophthalmic solution for each image were 10.21 (6.70, 14.64), 3.20 (0.71, 11.87) and 60.38 (42.47, 72.02) for blood vessel area, strong red area and weak red area, respectively. The average of the median, lower quartile, and upper quartile of the percentage of conjunctival hyperemia before applying 10% phenylephrine ophthalmic solution for each image were shown in Table 1. The percentage of conjunctival hyperemia after the application of 10% phenylephrine ophthalmic solutions was significantly reduced in all areas ($p < 0.001$). (Figure 6)

Software repeatability

The repeatability of software was tested by calculating the percentage of conjunctival hyperemia twice of each image. The median, lower quartile, and upper quartile of the percentage of conjunctival hyperemia for the first measurement were 12.31 (9.50, 15.73), 8.03 (3.21, 28.09) and 51.20 (39.14, 63.93) for blood vessel area, strong red area and weak red area, respectively. The median lower, quartile, and upper quartile of the percentage of conjunctival hyperemia for the second measurement of the same image was 12.38 (9.47, 15.60), 7.75 (3.52, 27.01) and 48.67 (38.88, 62.23) for blood vessel area, strong red area and weak red area, respectively. The Spearman correlation coefficients were 0.884, 0.982 and 0.932 for blood vessel area, strong red area and weak red area, respectively. (Table 2)

Bland-Altman plots of the mean difference conjunctival hyperemia percentage in each area of two measurements on each eye were shown in Figure 7. The mean percentage of conjunctival blood vessel area differences were -0.439% ; 95% confidence interval (CI): -1.359% – -0.420% ; 95% limits of agreement (LoA): -3.329% – -2.451% . The mean percentage of conjunctival strong red area differences were 0.196% ; 95% CI: -0.808% – 1.201% ; 95% LoA: -2.956% – 3.313% . The mean percentage of conjunctival weak red area differences were -0.918% ; 95% CI: -3.269% – 1.434% ; 95% LoA: -8.268% – 6.432% .

Software reproducibility

The reproducibility of software was tested by calculating the percentage of conjunctival hyperemia of two images taken 10 seconds apart. The ROI was manually drawn in the first image by user and was automatically registered into the second image by software. The median and IQR of the percentage of conjunctival hyperemia of

Table 1. The median and interquartile range of the percentage of conjunctival hyperemia before and after applying 10% phenylephrine ophthalmic solutions.

The percentage of conjunctival hyperemia in each area			
	Before ^a median (Q1, Q3)	After ^b median (Q1, Q3)	p-value
Blood vessel area (%)	10.21 (6.70, 14.64)	4.21 (2.86, 10.58)	< 0.001
Strong red area (%)	3.20 (0.71, 11.87)	0.27 (0.02, 1.60)	< 0.001
Weak red area (%)	60.38 (42.47, 72.02)	28.93 (15.65, 44.89)	< 0.001

Before^a, before applying 10% phenylephrine ophthalmic solutions; after^b, after applying 10% phenylephrine ophthalmic solutions; Q1, lower quartile; Q3, upper quartile; %, percentage

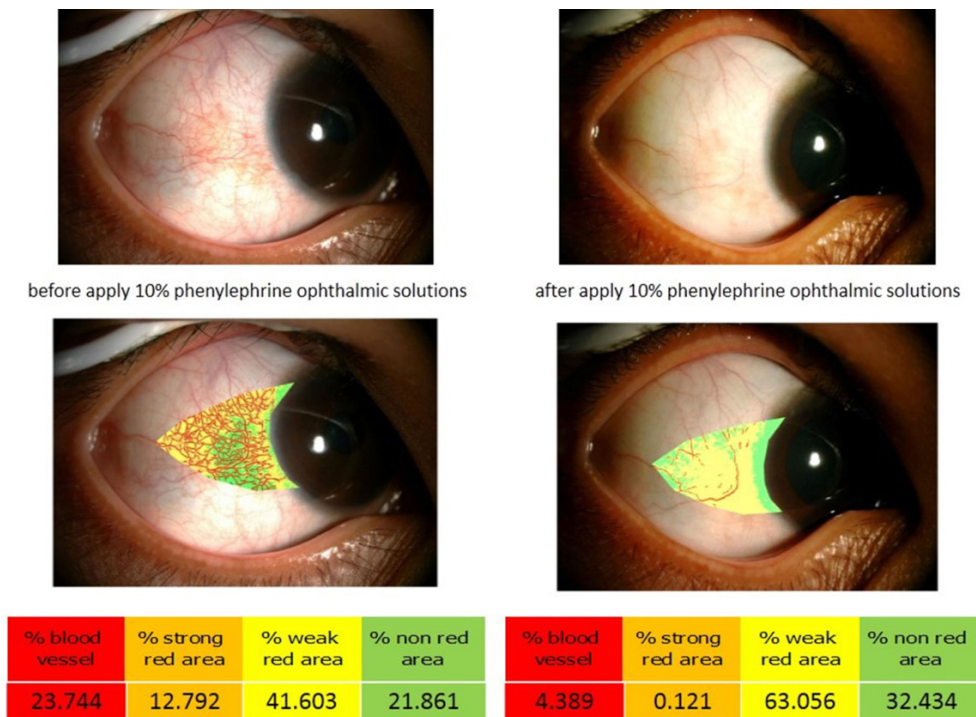


Figure 6. Anterior segment images reveal region of interest of temporal bulbar conjunctiva of right eye. The system evaluates the percentage of blood vessels and degree of conjunctival hyperemia in the four classes (blood vessel area, strong red area, weak red area and non-red area) before (A) and after (B) applying 10% phenylephrine ophthalmic solutions.

Table 2. The median and interquartile range of the percentage of conjunctival hyperemia in each area of twice measurement of the same image.

The percentage of conjunctival hyperemia in each area of twice measurement of the same image			
	1 st measurement median (Q1, Q3)	2 nd measurement median (Q1, Q3)	rs
Blood vessel area (%)	12.31 (9.50, 15.73)	12.38 (9.47, 15.60)	0.884
Strong red area (%)	8.03 (3.21, 28.09)	7.75 (3.52, 27.01)	0.982
Weak red area (%)	51.20 (39.14, 63.93)	48.67 (38.88, 62.23)	0.932

Q1, lower quartile; Q3, upper quartile; rs, Spearman correlation coefficient

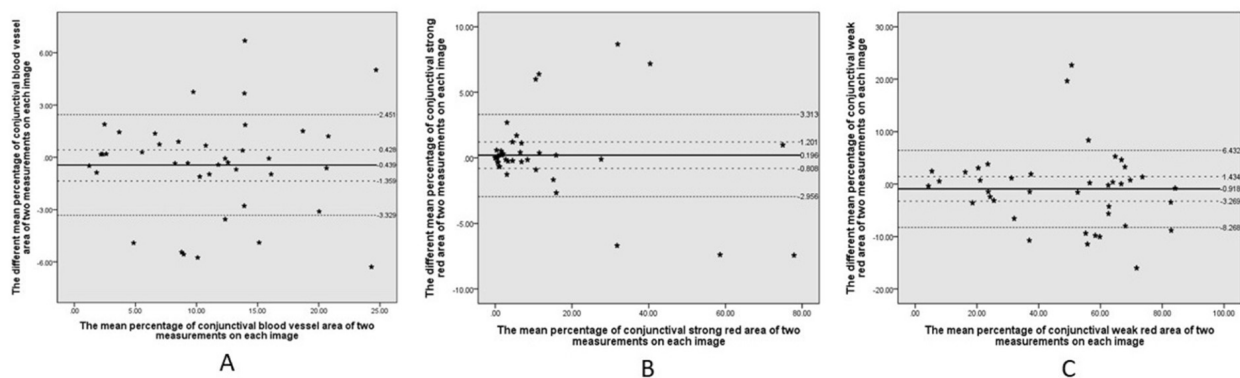


Figure 7. Bland-Altman plots of the mean difference conjunctival hyperemia percentage in each area of two measurements on each image.

- The mean difference conjunctival hyperemia percentage in each area of two measurements
- — — — The 95% confidence limits of mean difference in conjunctival hyperemia measurement
- - - - - The 95% limit of agreement of conjunctival hyperemia measurement

Table 3. The median and interquartile range of the percentage of conjunctival hyperemia in each area of two images of the same eye.

The percentage of conjunctival hyperemia in each area of two images of the same eye			
	1 st image median (Q1, Q3)	2 nd image median (Q1, Q3)	rs
Blood vessel area (%)	10.83 (6.09, 15.44)	11.63 (6.78, 15.00)	0.988
Strong red area (%)	4.76 (0.72, 14.50)	4.256 (1.24, 14.65)	0.992
Weak red area (%)	54.09 (24.29, 64.14)	54.88 (25.88, 65.25)	0.995

Q1, lower quartile; Q3, upper quartile; rs, Spearman correlation coefficient

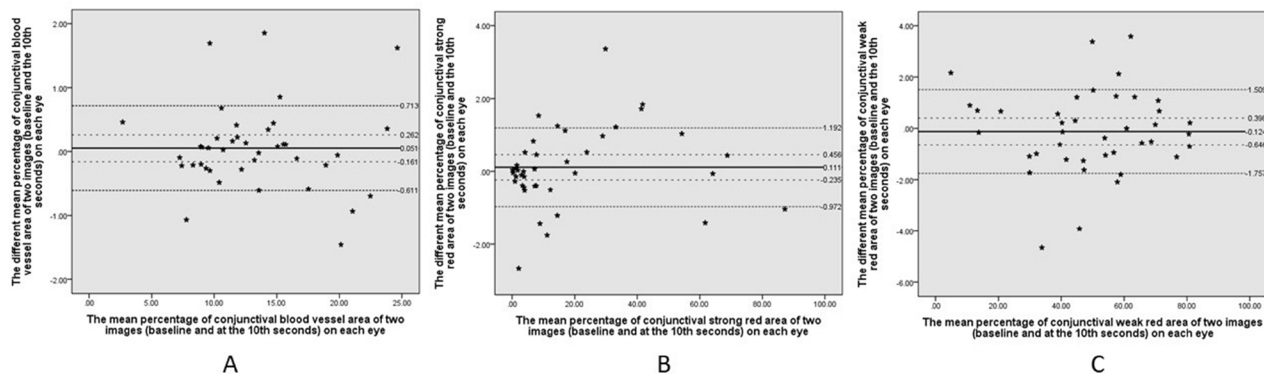


Figure 8. Bland-Altman plots of the mean difference conjunctival hyperemia percentage in each area of two images on each eye.

- The mean difference conjunctival hyperemia percentage in each area of two images
- — — — The 95% confidence limits of mean difference in conjunctival hyperemia measurement
- - - - - The 95% limit of agreement of conjunctival hyperemia measurement

the two images of the same eye were shown in Table 3. The Spearman correlation coefficient (rs) values of 0.988, 0.992 and 0.995 for blood vessel area, strong red area and weak red area, respectively. (Table 3)

In comparison with the Spearman correlation coefficients obtained from the software repeatability testing, we found that the Spearman correlation coefficients obtained from the software reproducibility were higher in all three areas.

Bland-Altman plots of the mean difference conjunctival hyperemia percentage in each area of two images on each eye was shown in Figure 8. The mean percentage of conjunctival blood vessel area differences were 0.051%; 95% CI: -0.161-0.262%; 95% LoA: -0.611-0.713%. The mean percentage of conjunctival strong red area differences were 0.111%; 95% CI: -0.235-0.456%; 95% LoA: -0.972-1.192%. The mean percentage of conjunctival weak red area differences were -0.124%; 95% CI: -0.646%-0.398% and 95% LoA: -1.757% -1.509%.

The intraclass correlation coefficient of clinical grading from ten ophthalmologists was 0.969 (95% CI: 0.952-0.981). The correlation of ten clinical graders and automated grading in each area was tested the Spearman correlation coefficient (rs). (Table 4). The correlation of ten clinical graders and automated grading in the summation of blood vessel and strong red area, as well as in the strong red area alone, as depicted in the graph. (Figure 9)

Table 4. The correlation of clinical grading and various automated grading

	rs
Clinical grading & blood vessel area	0.462
Clinical grading & strong red area	0.752
Clinical grading & weak red area	0.374
Clinical grading & vessel + strong red area	0.824
Clinical grading & vessel + weak red area	0.273
Clinical grading & strong + weak red area	0.401
Clinical grading & vessel + strong red + weak red area	0.574

rs, Spearman correlation coefficient

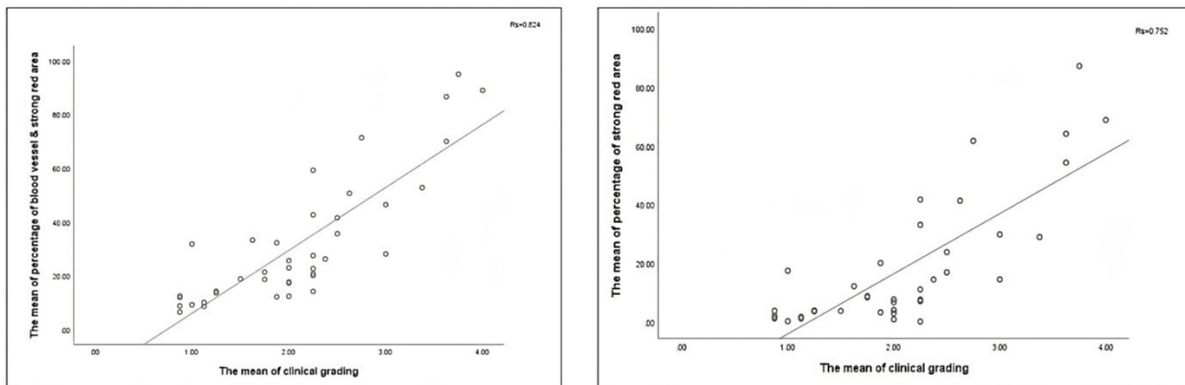


Figure 9. The correlation of ten clinical graders and automated grading.

DISCUSSION

The results of the present study show that our newly developed conjunctival hyperemia analysis software can automatically perform quantitative assessment of conjunctival hyperemia in the percentage of three areas in ROI: blood vessel area, strong red area and weak red area. The percentage of conjunctival hyperemia after application of 10% phenylephrine ophthalmic solutions was significantly reduced in all area which resulted from vasoconstriction effect of phenylephrine. While a self-controlled design comparing pre- and post-medication outcomes could have provided additional strength, we instead employed phenylephrine blanching as a standardized reference to account for vascular variability. This served as an internal benchmark, though it may not fully reflect the long-term effects of anti-glaucoma therapy. The results showed very high repeatability and reproducibility. The clinical grading had the best correlation with the summation of blood vessel and strong red area, followed by the strong red area. The previous studies only focused on blood vessel (6-17, 30) but we here have extended this to blood vessel, strong red area and weak red area. Currently, several objective conjunctival grading systems were developed to improve accuracy in clinical assessment. These grading systems were developed using different software algorithm, such as edge detection, smoothing, color extraction, morphometry and thresholding (6-18, 30). The summary of software algorithm is shown in Table 5.

A primary objective of this study was to determine the repeatability and reproducibility of conjunctival hyperemia analysis software. The results

showed very high repeatability and reproducibility. To the best of our knowledge, there has been no grading software using the same software algorithm as ours. Local feature matching was used for the automatic ROI registration between two images. This algorithm provides precise results in comparing the conjunctival hyperemic change in clinical practice, especially in patients with difficulty maintaining the eye in the same position during taking anterior segment image at every visit. The automatic ROI registration also improves software repeatability and reproducibility.

We found the correlation between clinical grading by ophthalmologist and automated grading scale by our software show the best result with the summation of blood vessel and strong red area, which we can apply for our clinical practice.

Our method incorporates ROI registration and classification into multiple redness categories (blood vessel, strong red, weak red). This approach enhances precision but depends on high image quality and adequate computational resources. The limitations of our study include the fact that no controls were included, so a range of percentage of conjunctival hyperemia in normal subjects was not established. These limitations indicate the need for further investigation in a normal population. A limitation is the use of 10% phenylephrine as a reference, as it produces an unnaturally white appearance that may not represent physiologic baseline conjunctiva. This factor should be considered in interpreting the findings.

To improve the repeatability of the system, the automatic ROI selection should be developed. This work is ongoing. There are some failure cases of ROI registration such as blurred images. We

Table 5. Summary of software processing and algorithm in previous studies

Authors	Summary of software processing and algorithm					
	Image collection tool	Resolution of image (pixel)	Software algorithm	Region of interest	Grading scale	Automatic registration
Schulze MM, et al. (6)	NikonFS-3V	Not mentioned	Luminance/ chromaticity	Average redness	0-100	No
Yoneda T, et al. (8)	Canon	3888 × 2592	Thresholding	Blood vessel	10-point step	No
Wolffsohn JS (15)	Not mentioned	700 × 525	Edge detection	Average redness	%	No
Fieguth P, et al. (7)	Not mentioned	Not mentioned	Edge detection	Average redness	%	No
Peterson RC, et al. (30)	NaCV-53200, Yokohama	Not mentioned	Edge detection	Blood vessel, Average redness	0-100	No
Sumi T, et al. (19)	Topcon	3216 × 2136	Thresholding	Pixel coverage	%	No
Li M, et al. (23)	Haag-streit BX900, Topcon SLD2, Kanghua SL-7E	512 × 768	Deep learning-based segmentation/ classification (mask distillation)	Blood vessel, Average redness	1-4	No
Ostheimer P, et al. (22)	Occyo GmbH	Not mentioned	color-intensity-based formula	Average redness	-0.5-1.0	No
The present study	Topcon	3264 × 2448	Thresholding/ Morphological top-hat transformation/Local features	Blood vessel, Average redness in 3 scale	%	Yes

suggest that the quality of the image should be assessed before input to the grading system.

To improve the correlation of the system to the clinical grading by ophthalmologist, more experiments should be conducted to identify the optimal threshold for area classification.

The experiments presented in this paper demonstrated the robustness of the system to variation of the eye gazing directions, scales of the eye in the image, orientation of the eyes, the directions of light sources. Further investigation should be done for evaluating the robustness across different light sources or image resolution.

CONCLUSION

This pilot study validates an automated conjunctival hyperemia analysis software that delivers objective, repeatable, and reproducible results. The proposed software provides an automated, quantitative, and objective assessment of conjunctival hyperemia with excellent repeatability and reproducibility, including strong correlation with clinical grading by ophthalmologists.

This pilot validation also demonstrates that the algorithm's use of automatic ROI registration between two images gives precise results for tracking conjunctival hyperemic changes in clinical practice. The use of our software may help improve disease monitoring in various ocular conditions.

ACKNOWLEDGMENTS

We thank Kittika Kanjanarattanakorn and Rochana Phuackchantuck, staff members of the research administration unit, Faculty of Medicine, Chiang Mai University Hospital, for their statistical assistance.

FUNDING

The authors received no financial support for this study

CONFLICTS OF INTEREST

The authors have no conflicts of interest to report

AUTHOR CONTRIBUTIONS

L.H.: conceptualization, methodology, investigation, data curation, formal analysis, and writing – original draft and writing – review & editing; K.Path.: supervision and writing – review & editing; N.T.: supervision and writing – review & editing; W.C.: supervision and writing – review & editing; S.A.: supervision and writing – review & editing; P.K.: data curation, investigation, and writing – review & editing; N.P.: data curation and writing – review & editing; T.E. o'brien: supervision, formal analysis, and writing – review & editing; K.Pat.: conceptualization, methodology, investigation, supervision, writing – original draft, and writing – review & editing.

All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request

INSTITUTIONAL REVIEW BOARD STATEMENT:

The study protocol was approved by Research Ethics Committee, Faculty of Medicine, Chiang Mai University (Certificate of Approval No. 452/2015; Study Code: OPT-2558-03328; Research ID: 3328; approval date: October 26, 2015). The study was conducted in accordance with the Declaration of Helsinki and applicable institutional ethical guidelines.

INFORMED CONSENT STATEMENT:

Informed consent was obtained from all subjects involved in the study.

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Clinical and Imaging Features of Melioidosis: A Study at Maharaj Nakorn Chiang Mai Hospital

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Received: March 16, 2025;

Revised: October 26, 2025;

Accepted: December 19, 2025

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ABSTRACT

OBJECTIVE Melioidosis is a life-threatening infectious disease caused by *Burkholderia pseudomallei*. The clinical presentations are diverse with high mortality. The disease requires timely and appropriate antibiotics. Imaging studies play an important role in the diagnosis. This study evaluated clinical manifestations, risk factors, and imaging patterns of melioidosis.

METHODS This retrospective study reviewed medical records and imaging studies of patients with melioidosis conclusively identified by cultures at Maharaj Nakorn Chiang Mai Hospital during 2017–2023.

RESULTS Of 33 melioidosis patients, most were male (75.76%) and the mean age was 57. Fever (66.67%) and localized pain (48.48%) were the common symptoms, while septicemia occurred in 33.33% of cases. Diabetes mellitus (48.48%) was the predominant risk factor. Imaging studies, including chest radiographs, computed tomography (CT), magnetic resonance imaging (MRI), and ultrasonography, detected infections in 90.90% of the patients, with lung involvement being the most frequent (39.39%). CT was particularly effective in identifying pulmonary and visceral abscesses, while MRI aided in diagnosing osteoarticular infections. Imaging successfully guided specimen collection in 78.57% of hemoculture-negative cases.

CONCLUSIONS This study underscores the importance of imaging in diagnosing melioidosis, particularly in patients with risk factors, and highlights the need for tailored imaging strategies based on clinical features.

KEYWORDS melioidosis, sepsis, pneumonia, diagnostic imaging, multi-detector computed tomography

INTRODUCTION

Melioidosis, caused by the Gram-negative bacilli *Burkholderia pseudomallei*, is a significant infectious disease often associated with environmental exposure. Individuals with certain conditions such as diabetes mellitus, chronic kidney disease, chronic lung disease, thalassemia, corticosteroid use, and immunosuppressive therapy

are at higher risk (1). The disease is particularly prevalent in Southeast Asia and Northern Australia (2). The global burden of melioidosis in 2015 was approximately 165,000 new cases and 89,000 deaths (3), prompting the World Health Organization (WHO) to develop comprehensive strategies for a better understanding of the disease (4). Melioidosis can be misdiagnosed due to its diverse

clinical presentations and lack of awareness of the disease. Variations in prevalence across different areas can be attributed to underreporting. The management of melioidosis is a world-wide public health challenge, not only in the endemic regions (5, 6).

Diagnosing melioidosis is challenging due to its wide range of clinical symptoms, the severity of the disease, and diverse organ involvement. In the mildest form, patients may experience low-grade fever, fatigue, and general malaise, which can be easily mistaken for a common viral illness. Localized symptoms may develop when certain organs are involved, such as cough and chest pain in lung infection, or abdominal pain when the liver or spleen is involved. Patients might also present with localized pain and swelling in the skin or with soft tissue infection. In severe cases, melioidosis can lead to pneumonia, abscesses in multiple organs, and bacteremia, manifesting as high fever and septicemia. In the most critical form, the infection causes septic shock, multiorgan failure, and a high risk of mortality (1, 3, 6, 7). These nonspecific clinical presentations complicate differentiation from other diseases, necessitating a high level of clinical suspicion. A definite and reliable diagnosis can be made only by positive cultures of *B. pseudomallei* from clinical specimens (8). Cultures take at least 48 hours, which may cause treatment delays (1). Serology tests alone cannot reliably make the diagnosis or exclude melioidosis (8). Collecting specimens from the appropriate sites is crucial for an accurate diagnosis. Appropriate treatment begins with 4 to 6 weeks of intravenous ceftazidime or meropenem during the acute phase, followed by 3 to 6 months of oral antibiotics such as doxycycline, trimethoprim-sulfamethoxazole, or amoxicillin-clavulanate during the eradication phase, as well as eradication of the infected sites (1, 7, 9).

Imaging studies have been used as an initial evaluation of several diseases, including melioidosis. Radiography, ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) are also commonly used (10–15). Pulmonary infection, the most common infected organ in melioidosis, can be detected by chest radiographs or CT (16, 17). The presence of abscesses, which is a sign of infection in any organ, can be detected by ultrasonography (US), CT, or MRI.

Knowing the clinical and imaging patterns of the disease assists understanding the manifestation of melioidosis, leading to clinical suspicion, appropriate use of imaging modalities, and timely correct diagnosis (3, 5). This study aims to explore the clinical manifestation and imaging patterns of patients with melioidosis at Maharaj Nakorn Chiang Mai Hospital, with emphasis on the usefulness of imaging studies.

METHODS

After the Institutional Research Ethics Committee approval (RAD-2567-0186), this retrospective observational cohort study was conducted using data of melioidosis patients at Maharaj Nakorn Chiang Mai Hospital from January 2017 to December 2023. Patients with cultures positive for *B. pseudomallei* were included in the study. Exclusion criteria were patients who did not receive imaging investigation, had poor image quality or had only positive titer without positive culture. From the database of the Microbiology Laboratory, 40 patients had positive cultures of *B. pseudomallei*. We excluded seven of those patients who did not receive imaging studies at the initial presentation. Thirty-three patients met the inclusion criteria.

The patients' medical records were reviewed for demographic data, clinical symptoms, type of positive culture specimens, and risk factors, which include diabetes mellitus, alcohol intake, chronic lung disease, chronic renal disease, thalassemia, soil contact, smoking, immunosuppression, rheumatic heart disease or cardiac failure, and malignancy (2, 6, 7, 9, 18, 19).

Imaging studies were reviewed by four medical students and consensus was reached. The interpretation was performed by reviewing radiology reports and re-evaluating the images, and the final diagnosis was confirmed by a radiologist sub-specialized in advanced body imaging and musculoskeletal radiology. Data are presented as means or proportions. All analyses were performed using STATA software version 16 (Stata Corp., College Station, TX, USA).

RESULTS

Of 33 patients with melioidosis, 25 patients (75.76%) were male and the mean age of the patients was 57 years (range 21–89 years). The clinical

presentations of these patients were diverse with fever being the most common presentation ($n = 22$, 66.67%). Eighteen patients (54.55%) had an acute fever, while four (12.12%) exhibited intermittent fever. Localized pain in the infected organs was reported by 16 patients (48.48%). Septicemia with unstable vital signs (septic shock), a fatal condition, occurred in 33.33% ($n = 11$) of the cases. Risk factors were identified in 29 patients (87.88%), with diabetes mellitus the most common ($n = 16$, 48.48%). Twenty-three patients (69.70%) had more than one risk factor. The list of risk factors and number of patients with each factor is shown in Table 1.

Of the 33 patients, nineteen cases (57.58%) were confirmed by positive blood cultures with *B. pseudomallei*. Among these, the organism was detected from blood culture alone in seven patients (36.84%), while for the other 12 patients (63.16%) it was detected in blood and other specimens. In addition, of the 33 patients, 14 (42.42%) showed positive only from other clinical specimens. No serological testing for melioidosis was performed in any of the patients in this study. Details of the clinical presentations, laboratory investigations, and imaging results are presented in Table 2 and 3. Imaging studies detected infected sites in 30 patients (90.90%). Multiple organ involvement was detected in 16 patients (48.48%). Among 14 patients who were hemoculture negative but had positive clinical specimens, imaging helped to target the sites of specimen collection in 11 patients

Table 1. Risk factors

Risk factors	n (%)
Diabetes mellitus	16 (48.48)
Smoking	11 (33.33)
Alcohol drinking	8 (24.24)
Chronic kidney disease	6 (18.18)
Soil contact	5 (15.15)
Chronic lung disease	4 (12.12)
Immunosuppression	4 (12.12)
Malignancy	3 (9.09)
Thalassemia	3 (9.09)
Rheumatic heart disease	3 (9.09)

(78.57%), while in 3 patients, imaging studies did not allow detection of the infection site, but these patients did have chest radiographs for screening of pulmonary infection. Details of where cultures were obtained is shown in Supplementary Table 1 and 2.

The lung was the most common infected site (13 patients, 39.39%) (Figure 1), and was more frequent in patients with positive hemoculture (11 of 19 patients, 57.89%) than those who had negative hemoculture (2 of 14 patients, 14.29%). Other involved organs were spleen (5 patients, 15.15%) (Figure 2), liver (3 patients, 9.00%) (Figure 3), and prostate (3 patients, 9.00%) (Figure 4). One patient each had brain and adrenal gland abscesses. Of three patients with prostatic abscesses, two had disease in other urogenital organs: one had associated epididymitis, and the other had a renal abscess. Bone and soft tissue structures were

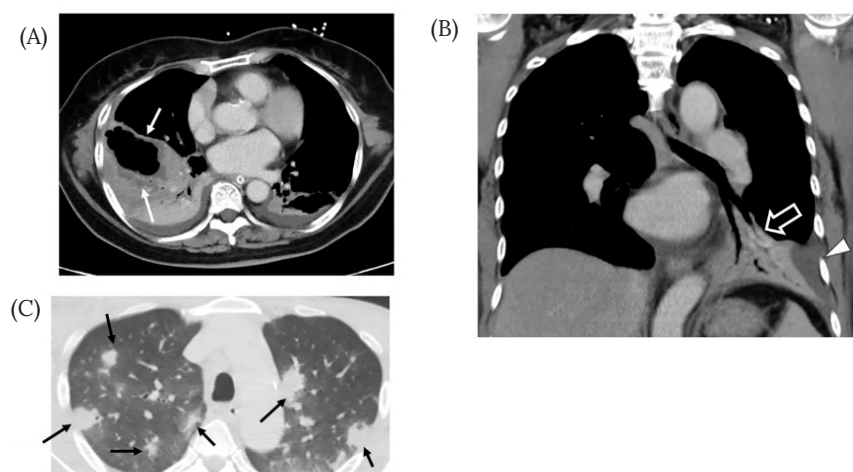


Figure 1. Patterns of lung involvement in melioidosis in 3 patients. (A) Axial CT of a lung abscess in a 65-year-old woman shows a cavity in the right lung (arrows). (B) Coronal CT displayed in soft tissue windows of a 59-year-old woman shows lung consolidation in the left lower lobe (open arrow) and pleural effusion (arrowhead). (C) Axial CT of a 45-year-old man shows multiple nodules, representing septic emboli (black arrows).

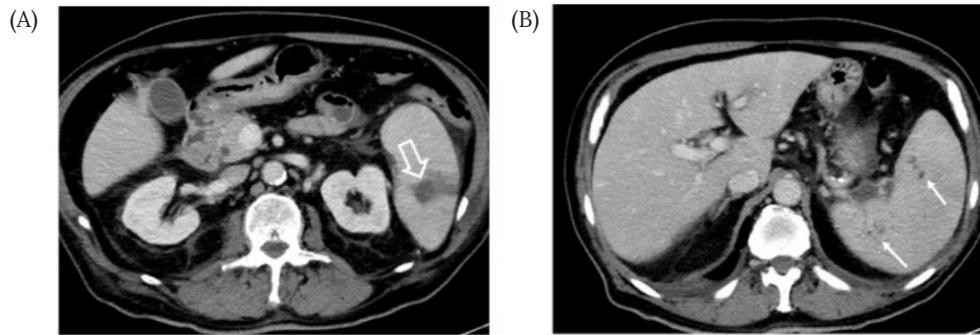


Figure 2. Splenic abscesses in a 70-year-old man. Axial CT shows (A) a large abscess (open arrow) and (B) clusters of microabscesses (arrows).

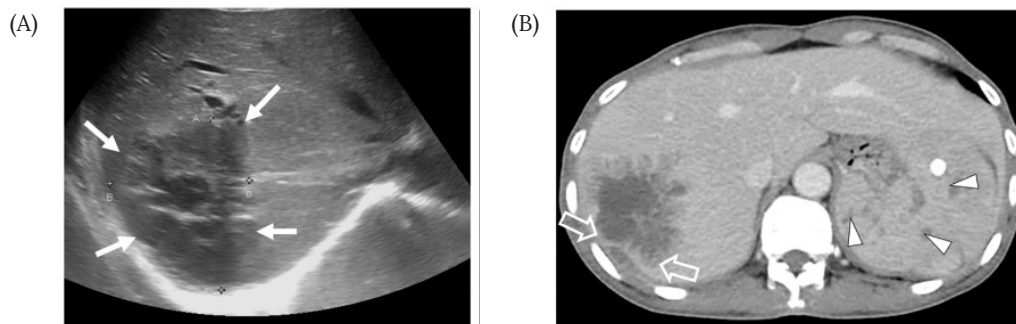


Figure 3. Liver abscesses in a 52-year-old man. (A) The US of the liver shows a multiloculated liver abscess (arrow). (B) Axial CT shows a liver abscess with a ruptured wall (open arrows) and multiple microabscesses in the spleen (arrowheads).



Figure 4. Prostatic abscess in a 50-year-old man. Coronal CT shows an abscess (arrow) [UB, urinary bladder].

the infected sites in eight patients (24.24%), and MRI was performed in 5 patients for diagnosis or treatment planning.

The patients were initially treated by antibiotics, either ceftazidime or meropenem, except for one asymptomatic patient whose melioidosis was incidentally detected from a dissected lymph node as part of a treatment for thyroid cancer (Table 2, case 14). Among the 33 patients, 16 (48.48%) completed the course of treatment at our hospital with favorable outcomes, while nine (27.27%)

were referred to local hospitals for treatment. Six patients had poor outcomes (18.18%): four died, and two were discharged from the hospital while in unstable clinical status. These six patients all had positive blood cultures.

DISCUSSION

This case series provides a comprehensive overview of melioidosis, highlighting the need for a combination of clinical presentations, identification of patient risk factors, and the utility

Table 2. Clinical and imaging findings of 19 patients with hemoculture positive

Case	Sex/ age	Symptom	Risk factor	Positive other specimens	Chest X-ray	US	CT	MRI	Outcome
1	M/44	Fever	DM, Smoking, Alc, HIV	Sputum	Y patchy RLL/LLL	N/A	Y liver & spleen microabscesses	N/A	C
2	M/45	Fever Facial and abdominal pain	Smoking, Alc	-	N/A	N/A	Y lung septic emboli, abscesses in neck muscles and adrenal gland	N/A	C
3	M/75	Fever	Smoking, Alc	-	N/A	N/A	Y spleen large & micro-ab- scesses	N/A	C
4	M/50	Pyuria	DM, Smoking, CKD, Thalas- semia, RHD	Urine	N	N/A	Y prostate abscess epididymorchitis	N/A	C
5	M/68	Fever Respirato- ry distress	DM, Lung cancer	-	N	N/A	N/A	N/A	C
6	F/65	Fever Abdominal pain	DM, Smoking, CLD	Pus (lung ab- scess)	Y lung abscess RUL	N/A	Y septic emboli lung abscess pleural effusion	N/A	C
7	M/28	Gluteal and sacral pain	DM	Pus (bone and gluteal soft tissue)	Y patchy RUL	N abdo- men	Y patchy RML	Y liver micro- abscesses, sacrum os- teomyelitis	C
8	M/67	Fever Respirato- ry distress	DM, Thal- assemia	Sputum	N/A	N/A	Y spleen microabscess	N/A	C
9	M/69	Fever Productive cough Diarrhea Pyuria	Smoking, CKD, CLD	Urine	N	N/A	N/A	N/A	C
10	M/63	Fever Pyuria	-	Urine	N/A	N/A	Y peribronchial infiltra- tion RUL kidney& prostate abscesses	N/A	C
11	M/63	Fever Productive cough	Smoking, Alc	-	Y patchy LUL	N/A	N/A	N/A	R
12	M/58	Fever Lower limb pain	CKD	-	N	N	N/A	N	R

Table 2. Clinical and imaging findings of 19 patients with hemoculture positive (continued)

Case	Sex/ age	Symptom	Risk factor	Positive other specimens	Chest X-ray	US	CT	MRI	Outcome
13	F/59	Fever Chest pain	DM, CKD, Soil contact	Pleural fluid	Y patchy LLL pleural fluid	Y pleu- ral effu- sion	Y patchy LLL pleural effusion	N/A	R
14	M/62	SVC syn- drome	DM, Smoking, CKD	Mediastinal lymph node	Y right paratra- cheal mass	N/A	Y necrotic lymph node	N/A	D 3 days
15	M/48	Fever Respirato- ry distress Abdominal pain	DM Hepato- cellular carcinoma	Ascitic fluid	N	N/A	Y ascites	N/A	D 13 days
16	F/58	Fever Respirato- ry distress Diarrhea	-	-	Y patchy RLL, LLL	N/A	Y septic emboli patchy RLL/LLL	N/A	D 2 days
17	F/71	Fever	DM, CLD, RHD	-	Y septic emboli	N/A	N/A	N/A	D 6 days
18	M/89	Respirato- ry distress Pyuria	DM, Smoking, CLD	Urine	Y patchy RLL, LLL	N/A	Y patchy RLL, LLL pleural effusion prostate abscess	N/A	U
19	M/80	Fever Respirato- ry distress Diarrhea	Alc, Soil contact	Sputum	Y patchy RLL	N/A	Y patchy RLL pleural effusion	N/A	U

Y, positive; N, negative; N/A, the study was not performed; DM, diabetes mellitus; Alc, alcohol drinking; CKD, chronic kidney disease; CLD, chronic lung disease; RHD, rheumatic heart disease; SLE, systemic lupus erythematosus; HIV, human immunodeficiency virus infection; SVC syndrome, superior vena cava syndrome; RML, right middle lobe of the lung; RLL, right lower lobe of the lung; LUL, left upper lobe of the lung; LLL, left lower lobe of the lung; C, Complete treatment at our site; R, referred to local hospital; D, death; U, patient-initiated discharge with unstable condition - patient refused further treatment and chose to continue care at home

of imaging studies in the diagnosis. Findings indicate that patients with positive blood cultures have a higher risk of death. The clinical presentations were varied, and a majority of the patients had risk factors. In terms of diagnostic imaging, a chest radiograph proved beneficial in identifying infection in the lung, which is the most common infected site in melioidosis (20). Additionally, in this study lung involvement suggested septicemia and a severe form of the disease. This supports the value of chest radiographs as an effective initial investigation, which should be performed in all patients when there is suspicion of melioidosis (6). CT, MRI, and US provide a more comprehen-

sive assessment and help target sites for clinical specimens.

Each imaging modality has distinct strengths and limitations. US is a radiation-free method that is particularly useful for detecting intra-abdominal infections such as hepatic, splenic, and renal abscesses, as well as musculoskeletal conditions like muscle abscesses or joint effusions. However, its utility is limited in evaluating deep-seated infections, and accuracy may be affected by operator experience or obscuration from bowel gas. CT is highly effective in identifying multiple lesions in the chest, abdominal organs, and deep musculoskeletal structures such as the spine. Contrast-

enhanced CT is especially valuable for delineating abscesses, although the use of iodinated contrast carries risks of adverse reactions and renal function deterioration. Nevertheless, CT remains a preferred modality in emergency settings and for critically ill patients who cannot tolerate the longer scan times required for MRI. MRI is superior for assessing osteoarticular involvement due to its excellent ability to demonstrate both bone lesions and soft tissue extension. In addition, MRI provides high sensitivity for central nervous system disease, detecting small brain abscesses, meningitis, cerebral edema, and spinal cord compression.

CT is a highly effective imaging modality for detecting subtle lung lesions and other visceral organ infections. It helps define the extent and severity of the disease, as multiple organ involvement is common (approximately 49% in our series) (20). CT can detect pulmonary septic emboli that may not be visible on chest radiographs (Figure 5, Table 3 case 1). Imaging findings of melioidosis in the lung are variable, depending on the severity and stages of the disease. During bacteremia, the presence of septic emboli in both lungs is the most common pattern (16). Melioidosis can present as acute pneumonia with solitary or multifocal patchy infiltration. In the chronic stage, pulmonary melioidosis is seen as fibronodular infiltration, resembling pulmonary tuberculosis (10, 16, 17). CT is more sensitive than chest radiography for detecting subtle pulmonary lesions, especially in the early stages of fatal disseminated melioidosis (21). Therefore, chest CT should be considered in patients with high clinical suspicion despite negative chest radiographs.

This study highlights the role of advanced imaging studies like CT, US, and MRI. CT is particularly valuable in detecting localized infections in visceral organs. However, the features of melioidosis in visceral organs are variable and are not specific to melioidosis. Patterns of lesions can be multiple small microabscesses or large uni- or multi-locular abscesses (22). The US can help detect abscesses in the liver and spleen as well as soft tissue lesions (13). MRI, on the other hand, is useful in evaluating osteoarticular infections (five patients in our series) (13, 23). In this study, it was shown to be more effective than CT and US in detecting liver microabscess (Figure 6, Table 2 case 7).

Four patients in this study presented with pyuria, of whom three had CT scans revealing prostatic abscesses. Transrectal ultrasound is recommended for prostatic abscesses as the first-line investigation (24), and drainage can be performed via transrectal ultrasound guidance. However, due to the need for a special ultrasound probe and trained operators, as well as the invasiveness of this procedure, it may not be accessible for all cases. CT is a useful alternative tool and offers the additional advantage of measuring the abscess size, which is crucial when drainage is recommended for abscesses larger than 2 cm (24). CT also helps detect other urogenital organ involvements, such as the kidney or testis (24, 25). Melioidotic prostatic abscesses have imaging features that overlap with other pyogenic abscesses (12). Gas-forming (emphysematous) abscesses, however, are most often due to *Klebsiella pneumoniae* (26).

Spondylitis was found in one patient who presented with febrile illness and liver abscesses. Melioidotic spondylitis demonstrates highly variable patterns, and imaging alone cannot reliably distinguish it from other etiologies. Rapid structural destruction may resemble pyogenic infection, whereas a more indolent course with gradual bone loss, large abscesses, and minimal inflammatory response can mimic granulomatous infections such as tuberculosis, brucellosis, or fungal spondylitis. The presence of disseminated



Figure 5. Subtle septic emboli in a 52-year-old man with septic arthritis and osteomyelitis of the shoulder who underwent a CT examination to search for visceral organ involvement as part of an evaluation of persistent fever. Axial CT in the lung window shows septic emboli (arrows) which were undetectable by chest radiographs (not shown)

Table 3. Clinical and imaging findings of 14 patients with positive cultures from specific organs

Case	Sex/ age	Symptom	Risk factor	Specimens	Chest X-ray	XR	US	CT	MRI	Outcome
1	M/52	Fever Right shoulder pain Leg pain	DM, Smok- ing, Alc	Pus Joint fluid	N	N shoul- der	Y pretibial soft tissue abscess	Y lung septic emboli necrotic lymph nodes	Y shoulder osteomy- elitis septic arthritis	C
2	M/30	Right facial pain	Alc	Pus	N	N/A	N/A	Y preorbital cellulitis	N/A	C
3	M/56	Fever Epigastri- um pain	DM, Alc, Thalas- semia	Pus liver	N	N/A	Y liver multi- loculated & micro- abscesses spleen micro- abscesses	Y liver spleen abscesses	N/A	C
4	M/49	Knee pain	Soil contact, HIV	Pus	N	Y osteo- myeli- tis	N/A	N/A	Y osteomy- elitis septic arthritis	C
5	F/74	Lower limb pain	DM	Pus	N/A	N/A	Y necrotizing fasciitis	N/A	Y necrotizing fasciitis	C
6	F/72	Enlarge cervical neck with sinus tract	Soil contact	Pus	N	N/A	N/A	N/A	N/A	C
7	M/60	Fever	DM, Smok- ing	Bronchoal- veolar lavage Mediastinal lymph node	Y patchy RML	N/A	N/A	Y bronchoalveolar infiltration (tree-in-bud) necrotic medi- astinal lymph node spleen micro- abscesses	N/A	C
8	M/53	Fever Back pain	-	Pus psoas muscle and liver	N/A	Y spon- dylitis	N/A	Y liver micro- abscesses spondylitis	Y spondylitis	R
9	M/61	Fever Draining pus from chest wall defect related to aspergil- loma	Smok- ing, Soil contact, CLD	Pus chest wall	Y chronic lung disease & fistula	N/A	N/A	Y chest wall fistula and abscesses	N/A	R
10	M/49	Fever Abdominal pain	DM	Pus	N/A	N/A	Y spleen abscess	Y spleen abscess	N/A	R
11	F/40	Headache	SLE	Pus	N/A	N/A	N/A	Y Brain abscess	N/A	R

Table 3. Clinical and imaging findings of 14 patients with positive cultures from specific organs (continued)

Case	Sex/ age	Symptom	Risk factor	Specimens	Chest X-ray	XR	US	CT	MRI	Outcome
12	M/63	Fever	-	Pus	N	N/A	N/A	Y necrotic medi- astinal lymph- node	N/A	R
13	M/45	Fever	Alc	Urine	N	N/A	N/A	N/A	N/A	R
14	F/21	Incidental finding from neck node dis- section	Thyroid cancer	Lymph node	N	N/A	N	N/A	N/A	No treatment

Y, positive, N, negative; N/A, the study was not performed; DM, diabetes mellitus; Alc, alcohol drinking; CLD, chronic lung disease; SLE, systemic lupus erythematosus; HIV, human immunodeficiency virus infection; RML, right middle lobe of the lung; XR, radiograph of the location with pain; C, complete treatment at our site; R, referred to local hospitals

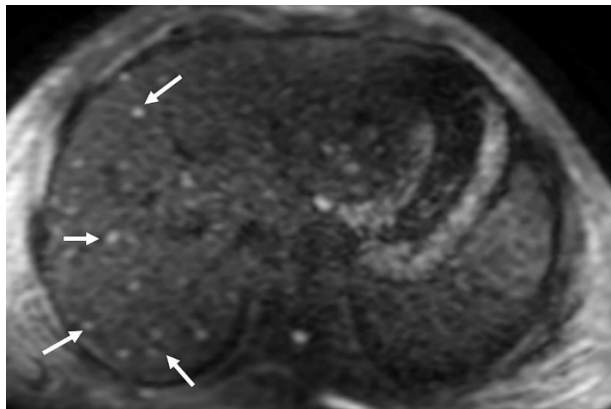


Figure 6. A 28-year-old man with diabetes mellitus initially presented with gluteal and sacral pain, and later complained of abdominal discomfort. US and CT of the abdomen were unremarkable (not shown). Axial MRI on diffusion-weighted image (b value 800) shows bright signal intensity of multiple microabscesses in the liver (arrows)

disease and additional organ lesions in patients with risk factors who are living in or travelling to endemic regions can help narrow the differential diagnosis toward melioidosis (27).

Adrenalitis is a rare manifestation of melioidosis with only a few reported cases (28, 29). The CT findings of the case in the present study (Figure 7, Table 2 case 2) revealed an enlarged adrenal gland with peripheral rim enhancement and central low attenuation, indicating a suppurative abscess. Other infectious diseases such as adrenal tuberculosis and histoplasmosis in the early stage can present similar features. In the later stages, adrenal tuberculosis and histoplasmosis show gland atrophy and calcification (30, 31). Hemor-

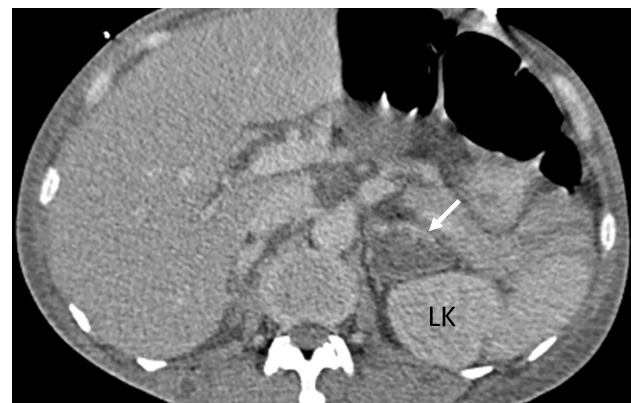


Figure 7. A 45-year-old male smoker with chronic alcohol consumption presented with facial and abdominal pain. CT of the abdomen shows a left adrenal abscess with thin peripheral rim enhancement and central low attenuation (arrow). [LK, left kidney]

rhagic tumors of the adrenal gland can mimic adrenal abscesses. Primary adrenal gland tumors associated with hemorrhage include pseudocyst, myelolipoma, adrenal hemangioma, pheochromocytoma, and adrenocortical carcinomas. Common hemorrhagic metastasis tumors are bronchogenic carcinoma and malignant melanoma. The presence of a thickened irregular rim of a tumor component or tumoral fat can help distinguish tumors from adrenal abscesses (31).

Surprisingly, one patient diagnosed with thyroid cancer presented with a lymph node culture positive for melioidosis as an incidental finding. This case aligns with the pathophysiology of melioidosis, where *B. pseudomallei* can evade immune detection by invading various cell types, such as

macrophages. This mechanism enables the bacterium to persist in a latent form that may reactivate when the host's immune system is compromised (1, 6). To the best of our knowledge, there are no specific guidelines for managing asymptomatic individuals with positive *B. pseudomallei* cultures. Recent recommendations for patients with lymphadenopathy or culture-positive resected specimens from deep-seated infections suggest medical treatment consisting of a 4-week intensive phase with intravenous therapy, followed by a 3-month eradication phase with oral antibiotics (32).

The present study has several limitations. First, it was conducted within a single institution in Chiang Mai, Thailand, which may limit the generalizability of its findings to other regions where melioidosis is endemic. Additionally, the study's small sample size restricted its ability to capture the full spectrum of clinical and imaging features of the disease. Many patients had multiple comorbidities, making it difficult to distinguish clinical and imaging features directly attributable to melioidosis from those arising from other underlying conditions. This issue was compounded by the presence of nonspecific symptoms in some patients, which complicated efforts to confirm melioidosis as the primary diagnosis. Additionally, the study was affected by the structure of Maharaj Nakorn Chiang Mai Hospital as a tertiary referral center. Some patients were discharged to primary care facilities for further treatment, which limited assessment of treatment outcomes. Variations in imaging procedures performed during the disease episode complicated comparing findings across cases.

CONCLUSIONS

In conclusion, melioidosis has diverse clinical and imaging patterns. Awareness should be raised in the case of patients with febrile illness or signs of infection, particularly those with risk factors for melioidosis. The selection of imaging modalities should be tailored based on the fact that the lung is the most commonly affected site, and many patients have multiple organ involvement. Chest radiographs should be performed in all cases at the initial stage. CT of the chest should be performed in severely ill patients with bacteremia, even those with negative chest radiographs. CT of the abdomen is useful when

intra-abdominal organ involvement is suspected and in males with pyuria.

ACKNOWLEDGEMENTS

We would like to extend our appreciation to the Microbiology Laboratory for providing data.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

AUTHOR CONTRIBUTIONS

N.P.: conceptualization, data curation, investigation, formal analysis, writing – review and editing; N.W.: writing – original draft, investigation, formal analysis; W.W.: writing—original draft, investigation, formal analysis; S.K.: writing – original draft, investigation, formal analysis; S.S.: writing—original draft, investigation, formal analysis; P.K.: data providing, writing—review and editing.

DATA AVAILABILITY STATEMENT

The datasets used in the current study are available from the corresponding author on reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

Ethical approval for this study was obtained from the Institutional Review Board (RAD-2567-0186, approval date April 17, 2024)

INFORMED CONSENT STATEMENT

Informed consent was waived due to the retrospective design of the study and the use of anonymized data.

SUPPLEMENTARY MATERIALS

The following supporting information can be downloaded at: [Supplementary appendix](#)

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Supplementary Table 1 Specimens obtained for cultures and where *B. pseudomallei* was isolated in 19 patients with hemoculture positive.

Case	Sex/Age	Obtained specimens	Positive specimens
1	M/44	Blood, liver, spleen, sputum, urine	Blood, sputum
2	M/45	Blood kidney muscle	Blood
3	M/75	Blood, spleen	Blood
4	M/50	Blood, urine	Blood, urine
5	M/68	Blood, sputum, urine	Blood
6	F/65	Blood, lung (lung abscess), sputum, stool	Blood, pus (lung abscess)
7	M/28	Blood, bone, muscle, sputum, urine	Blood, pus (bone and gluteal soft tissue)
8	M/67	Blood, sputum, urine	Blood, sputum
9	M/69	Blood, sputum, urine	Blood, urine
10	M/63	Blood, urine, stool	Blood, urine
11	M/63	Blood, lymphnode, lung (pleural eff)	Blood
12	M/58	Blood	Blood
13	F/59	Blood, lung (pleural effusion)	Blood, pleural fluid
14	M/62	Blood, lymphnode	Blood, mediastinal lymph node
15	M/48	Blood, ascites fluid	Blood, ascitic fluid
16	F/58	Blood	Blood
17	F/71	Blood	Blood
18	M/89	Blood, sputum, urine	Blood, urine
19	M/80	Blood, sputum, urine	Blood, sputum

Supplementary Table 2 Specimens obtained for cultures and where *B. pseudomallei* was isolated in 14 patients with positive culture from specific organs.

Case	Sex/Age	Obtained specimens	Positive specimens
1	M/52	Blood, sputum, pus and joint fluid (shoulder)	Pus and joint fluid (shoulder)
2	M/30	Pus (right cheek)	Pus (right cheek)
3	M/56	Sputum, liver, spleen, urine	Pus (liver)
4	M/49	Synovial fluid	Synovial fluid
5	F/74	Pus (lower limb muscle)	Pus (lower limb muscle)
6	F/72	Blood, lymph node	Pus (lymphnode)
7	M/60	Blood, lung, lymph node	Bronchoalveolar lavage, Mediastinal lymph node
8	M/53	Psoas muscle, liver, bone, urine	Pus (psoas muscle and liver)
9	M/61	Blood, lung (chest wall fistula and abscesses)	Pus (chest wall)
10	M/49	Spleen	Pus (spleen)
11	F/40	Brain	Pus (brain)
12	M/63	Lung, lymphnode, sputum, urine	Pus (Necrotic mediastinal node)
13	M/45	Blood, urine	Urine
14	F/21	Lymph node	Lymph node

The Brain's Gendered Architecture: An MRI Study of Cortical and Subcortical Volume Differences

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Received: September 10, 2025;

Revised: December 2, 2025;

Accepted: December 22, 2025

ABSTRACT

OBJECTIVE For the advancement of neuroscience and therapeutic applications, it is crucial to understand how cerebral and subcortical volumes change across sexes. This study utilized magnetic resonance imaging (MRI) to investigate volumetric variations in various brain areas across different sexes.

METHODS A cross-sectional study was conducted on 200 healthy participants (100 males and 100 females). High-resolution MRI scans were acquired using a 1.5T General Electric Signa scanner, and cortical segmentation was performed using a standardized semi-automated method. Statistical analyses were conducted using IBM SPSS, with independent sample t-tests employed to compare volumetric differences between sexes. Effect sizes were computed using Cohen's d, and multiple comparisons were adjusted via the Bonferroni method.

RESULTS There were considerably greater volumes of total cerebral white matter ($p < 0.001$, $d = -1.1$), total cortex ($p < 0.001$, $d = -0.635$), and total cerebrum ($p < 0.001$, $d = -1.352$) in males. Males also had considerably bigger ventricular structures, such as the lateral ($p < 0.001$, $d = -0.71$) and third ($p < 0.001$, $d = -1.229$) ventricles, although there was no significant difference in the fourth ventricle ($p = 0.171$). Males had substantially greater volumes in the inferior temporal gyrus ($p < 0.001$, $d = -0.58$), middle temporal gyrus ($p < 0.001$, $d = -0.55$), and superior temporal gyrus ($p < 0.001$, $d = -0.73$) among cortical areas with low-density sex hormone receptors. Significant differences were seen in the occipital pole ($p < 0.001$, $d = -1.342$), thalamus ($p < 0.001$, $d = -1.282$), and postcentral gyrus ($p < 0.001$, $d = -1.536$) in areas with high-density sex hormone receptors.

CONCLUSIONS This study confirms significant sex-based differences in brain volume, particularly in cortical and subcortical structures. These findings highlight the role of sex hormones in shaping neuroanatomy and provide insights for future research on sex-specific neurological and psychiatric conditions.

KEYWORDS sex differences, cortical and subcortical volumes, MRI, brain morphometry, neuroanatomy

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INTRODUCTION

Many studies have examined sex-based differences in brain morphology, especially with regard to volumetric variances in various brain areas (1, 2). Examining neurodevelopmental patterns, cognitive processes, and possible ramifications in neurological and psychiatric illnesses requires an understanding of these distinctions. The goal of the current study is to thoroughly examine the volumetric differences between males and females in a variety of brain regions, such as the whole cerebrum, cortical structures, ventricular gaps, and areas with different densities of androgen and estrogen receptors (3). This study sheds light on anatomical differences that might be responsible for sex-specific behavioral patterns, neurological susceptibilities, and cognitive capacities (4).

Using a variety of neuroimaging methods, recent research has investigated sex-based variations in brain shape. Males often have bigger regional and overall brain sizes (5, 6), while there are fewer noticeable sex variations in cortical thickness (7). Fronto-parietal, subcortical, and cerebellar areas have been found to have sex-specific differences in gray matter volume. Regional volumetric differences have been observed, with some areas showing higher volumes in males and others in females (7). These differences persist throughout the lifespan and are not solely attributable to body size variations (8). Sex chromosomes and hormones may have contributed to the creation of these disparities, which last throughout life (6). Multivariate sex disparities in gray matter volume, however, diminish when total cerebral volume is taken into account. Crucially, Sanchis-Segura et al. point out that these variations do not create unique “male/female brain” characteristics (9). It has been suggested that sex-specific brain templates be created in order to enhance neuroimaging investigations and more accurately depict population-specific neuroanatomy (5).

The findings reveal significant sex-based differences in total brain volume, with males exhibiting larger total cerebrum and cortical volumes compared to females. Specifically, males demonstrated significantly greater total cerebral white matter and lateral and third ventricle volumes, with large effect sizes (10). However, no significant

sex-based difference was observed in the fourth ventricle volume, indicating that not all ventricular structures follow the same pattern of volumetric variation (2). Further analysis of brain regions with low Estrogen and Androgen receptor densities indicated that males generally had larger volumes across multiple structures, including the superior temporal gyrus, middle temporal gyrus, inferior temporal gyrus, and subcallosal cortex (1). However, some regions, such as the superior occipital lateral gyri and temporal pole, did not exhibit significant sex-based differences, suggesting that receptor density alone does not fully account for volumetric variations (3).

In contrast, analysis of regions with high-density estrogen and androgen receptors revealed a more complex pattern of volumetric differences. Males had significantly larger volumes in key structures, including the postcentral gyrus, occipital pole, hippocampus, insula, and thalamus (4). Additionally, males exhibited larger volumes in the anterior cingulate gyrus, paracingulate cortex, and middle frontal gyrus, which are regions implicated in cognitive control and emotional regulation (10).

Interestingly, the fronto-orbital cortex was significantly larger in females, suggesting potential functional and structural distinctions in this region, which may relate to differences in social cognition and affective processing (2). These results align with existing literature highlighting sex-based variations in brain morphology and emphasizing the importance of considering biological sex in neuroimaging studies. The observed differences may be attributed to a combination of genetic, hormonal, and environmental factors that influence brain development and function (3). Future research should explore the functional implications of these volumetric differences, particularly in relation to cognitive performance, susceptibility to neuropsychiatric disorders, and brain plasticity across the lifespan (4). A deeper understanding of these structural distinctions will enhance our knowledge of sex-specific neurobiological processes and their relevance to health and disease. Hence, this study utilizes magnetic resonance imaging (MRI) to comprehensively analyze sex-based variations in the volumetric measurements of both cortical and subcortical brain regions, aiming to enhance our

understanding of neuroanatomical differences influenced by biological sex.

METHODS

Study design and participants

This study utilized a cross-sectional design to investigate sex differences in cerebral cortical volumes using MRI. A total of 200 healthy participants (100 males and 100 females) were included in the study. Ethical approval (Approval/Reference Number: [MMC/IEC/2022/2945/03/2025, approve April 4, 2025]) was obtained from the institutional review board Mamata Medical College, and informed consent was secured from all participants prior to data collection.

MRI Acquisition parameters and segmentation procedures

MRI scans were obtained at the Department of Radiology, Mamata Medical College, using a 1.5 T General Electric Signa scanner. Coronal spoiled-gradient echo images of the entire brain were acquired with a slice thickness of 3.1 mm, using the following parameters: repetition time (TR) = 40 ms, echo time (TE) = 8 ms, flip angle = 50°, the field of view = 30 cm, matrix resolution = 256 × 256, and one average per scan. The images were subsequently processed and analyzed in the Department of Radiology, Mamata Medical College Khammam, Telangana.

To ensure consistency in spatial orientation across subjects, each MRI scan was standardized using a three-dimensional coordinate system. This was achieved by aligning the scans based on anatomical landmarks, specifically the midpoints of the decussations of the anterior and posterior commissures and the midsagittal plane at the level of the posterior commissure, following the methodology described by Filipek et al. and Caviness et al. (11, 12). This positional normalization technique minimizes discrepancies arising from variations in head positioning during scanning. After alignment, the scans were resliced into 3.1 mm coronal sections.

Segmentation of the T1-weighted, positionally normalized coronal images involved differentiating gray matter, white matter, and ventricular structures. A semi-automated intensity contour mapping algorithm, in conjunction with signal intensity histogram distributions, was employed

to delineate these structures (11–13). This method partitions the brain into distinct compartments, including the neocortex, subcortical gray nuclei, white matter, and ventricular system subdivisions, which correspond to the natural variations in signal intensity observed in T1-weighted images.

The neocortex, as defined by the gray–white matter segmentation approach, was further subdivided into bilateral regions using the parcellation framework originally established by Caviness et al. and subsequently applied by Goldstein et al. This comprehensive system facilitates the division of the neocortex into anatomically and functionally relevant subdivisions based on well-defined topographical landmarks. Detailed anatomical definitions of these parcellation units can be found in prior studies (12, 13).

MRI segmentation and cortical parcellation were conducted by extensively trained MR technicians with a background in Neuroanatomy or Behavioral Neuroscience. These technicians were supervised continuously by an experienced neuroanatomist (N.M.) to ensure accuracy and reliability. In addition, a blinded specialist radiologist at Mamata Medical College independently reviewed and validated the segmentation and volumetric analysis, ensuring objectivity in the measurement process. Importantly, both the technicians and the radiologist were blinded to all socio-demographic and clinical information of the participants, including sex.

The reliability of these segmentation and parcellation procedures has been established in multiple previous studies, including those focusing on the dataset used in this study (12, 14, 15). Brain region volumes, measured in cubic centimeters (cm³), were computed by multiplying the area of each region on a given slice by the slice thickness and summing the values across all slices in which the region was present. This method ensures precise volume estimation and has been widely used in neuroimaging research.

Statistical methods

All statistical analyses were conducted using IBM SPSS Statistics (version 27.0). The volumetric measurements of different brain regions were compared between males and females using independent sample *t*-tests. The normality of the data was assessed using the Shapiro-Wilk test,

and the homogeneity of variance was evaluated with Levene's test. For each brain region, mean volume ($\pm$ standard deviation) was calculated separately for males and females. Independent *t*-tests were applied to determine whether there were statistically significant differences in regional brain volumes between the two sexes. The *t*-values, degrees of freedom, and *p*-values were reported for each comparison. Effect sizes were computed using Cohen's *d* to assess the magnitude of differences. Effect size interpretation followed the conventional guidelines: small ($d = 0.2$), moderate ($d = 0.5$), and large ($d \geq 0.8$). A *p*-value of < 0.05 was considered statistically significant, and multiple comparisons were corrected using the Bonferroni method to minimize type I errors. The brain regions were categorized based on their developmental sensitivity to sex hormones, distinguishing between regions with low-density and high-density estrogen and androgen receptors. Statistical comparisons were made separately for each category to evaluate sex-related volumetric differences.

RESULTS

Total cerebrum and cortex volume

Males exhibited significantly larger total cerebrum volume compared to females ($t(198) = -9.56$, $p < 0.001$, $d = -1.352$), indicating a large effect size. Similarly, the total cortex volume was greater in males than in females ($t(198) = -4.487$, $p < 0.001$, $d = -0.635$), with a moderate effect size (Figure 1A).

Total cerebral white matter

A significant difference was observed in total cerebral white matter volume, with males having larger volumes than females ($t(198) = -7.781$, $p < 0.001$, $d = -1.1$), suggesting a strong effect size (Figure 1A).

Comparison of brain region volumes between males and females

Ventricular volumes

The lateral ventricles were significantly larger in males than in females ($t(198) = -5.022$, $p < 0.001$, $d = -0.71$), representing a moderate effect. The third ventricle volume also showed a significant sex-based difference, with males having larger volumes than females ($t(198) = -8.689$, $p < 0.001$, $d = -1.229$), indicating a large effect size. However, the fourth ventricle did not show a statistically significant difference between sexes ($t(198) = -1.374$, $p = 0.171$, $d = -0.194$), suggesting minimal variation (Figure 1B). Overall, the findings indicate that males have significantly larger volumes in most brain regions, with the most pronounced differences observed in total cerebrum, total cerebral white matter, and third ventricle volumes. The observed effect sizes highlight substantial sex-based variations in brain morphology.

Comparison of brain region volumes in developmentally low estrogen and androgen receptor regions

Occipital lobe regions

The superior occipital lateral gyri did not show a significant difference between males and females

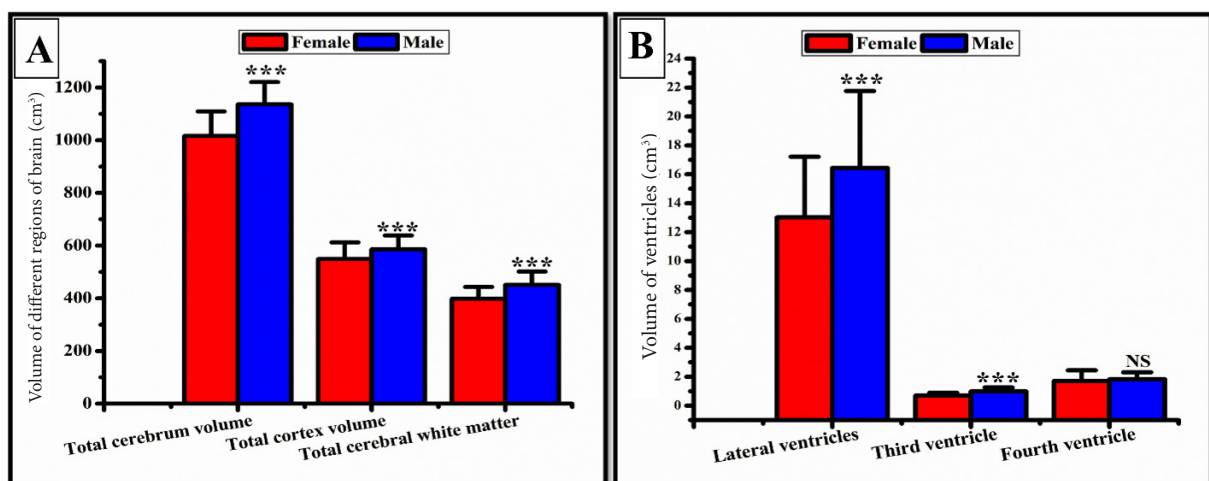


Figure 1. Volume (cm³) of different regions compared between females and males. (A) Volume of different regions of the brain, (B) Volume of the ventricle. ****p* < 0.001.

Table 1. Volume (cm³) of different regions in developmentally low estrogen and androgen receptor regions compared between females and males

Brain region	Females Mean±SD	Males Mean±SD	t-value	p-value	Effect size (Cohen's d)
Superior occipital lateral gyri	37.58±7.11	38.35±8.02	-0.723	0.471	-0.10
Planum polare	3.19±0.61	3.44±0.81	-2.472	0.014	-0.35
Central operculum	7.56±1.03	8.17±1.39	-3.504	0.001	-0.50
Superior parietal lobule	10.85±2.00	12.39±2.70	-4.567	0.000	-0.65
Inferior occipital lateral gyri	17.73±4.04	18.62±4.18	-1.520	0.130	-0.22
Temporal pole	18.30±2.93	18.85±3.59	-1.181	0.239	-0.17
Temporooccipital fusiform gyrus	6.51±1.57	6.93±1.45	-1.933	0.055	-0.27
Frontal pole	55.04±9.51	61.08±9.41	-4.519	0.000	-0.64
Middle temporal gyrus	14.55±2.23	15.77±2.23	-3.857	0.000	-0.55
Precuneus	20.25±3.78	22.42±3.15	-4.428	0.000	-0.63
Superior temporal gyrus	10.47±1.45	11.64±1.74	-5.168	0.000	-0.73
Temporal fusiform gyrus	9.97±1.89	11.70±1.94	-6.364	0.000	-0.90
Inferior temporal gyrus	10.85±2.55	12.37±2.76	-4.065	0.000	-0.58
Subcallosal cortex	4.15±0.87	4.97±0.83	-6.763	0.000	-0.96

($t(198) = -0.723$, $p = 0.471$, $d = -0.1$). Similarly, the inferior occipital lateral gyri also showed no significant difference ($t(198) = -1.52$, $p = 0.13$, $d = -0.22$), indicating minimal sex-based variation in these regions (Table 1).

Temporal lobe regions

The planum polare exhibited a small but significant difference, with males having a larger volume ($t(198) = -2.472$, $p = 0.014$, $d = -0.35$). The superior temporal gyrus ($t(198) = -5.168$, $p < 0.001$, $d = -0.73$), middle temporal gyrus ($t(198) = -3.857$, $p < 0.001$, $d = -0.55$), inferior temporal gyrus ($t(198) = -4.065$, $p < 0.001$, $d = -0.58$), and temporal fusiform gyrus ($t(198) = -6.364$, $p < 0.001$, $d = -0.9$) were all significantly larger in males, with effect sizes ranging from moderate to large. The temporal pole, however, did not show a statistically significant difference ($t(198) = -1.181$, $p = 0.239$, $d = -0.17$) (Table 1).

Parietal lobe regions

The superior parietal lobule showed a moderate sex difference, with males exhibiting larger volumes ($t(198) = -4.567$, $p < 0.001$, $d = -0.65$). The precuneus was also significantly larger in males ($t(198) = -4.428$, $p < 0.001$, $d = -0.63$), indicating consistent sex-related volumetric variation in parietal structures (Table 1).

Frontal lobe regions

The frontal pole was significantly larger in males ($t(198) = -4.519$, $p < 0.001$, $d = -0.64$). The central operculum also showed a notable sex difference, favoring males ($t(198) = -3.504$, $p = 0.001$, $d = -0.5$) (Table 1).

Subcortical and limbic regions

The subcallosal cortex exhibited one of the largest sex differences, with males having significantly larger volumes ($t(198) = -6.763$, $p < 0.001$, $d = -0.96$) (Table 1). Overall, males exhibited larger volumes in most examined brain regions, with particularly strong differences in the subcallosal cortex, temporal fusiform gyrus, and superior temporal gyrus. In contrast, some regions, such as the superior occipital lateral gyri and temporal pole, did not show significant sex-based differences. These findings suggest that sex-related differences in brain volume may be more pronounced in certain cortical and subcortical structures.

Comparison of brain region volumes in developmentally high-density estrogen and androgen receptor regions

Frontal lobe regions

The precentral gyrus showed no significant difference between males and females ($t(198) = 0.082$, $p = 0.935$, $d = 1.0132$). However, the front orbital cortex was significantly larger in females ($t(198) = 2.98$, $p = 0.003$, $d = 0.8539$), while the superior frontal gyrus showed a trend toward a difference but did not reach statistical significance ($t(198) = 1.877$, $p = 0.062$, $d = 1.3961$). The middle frontal gyrus was significantly larger in males ($t(198) = -5.146$, $p < 0.001$, $d = 1.1409$), as was the frontomedial cortex ($t(198) = -6.134$, $p < 0.001$, $d = 0.971$) (Table 2).

Table 2. Volume (cm³) of different regions developmentally high-density estrogen and androgen receptor regions comparing in females and males

Bain region	Female Mean±SD	Male Mean±SD	t-value	p-value	Effect size (Cohen's d)
Precentral gyrus	35.94±0.95	35.93±1.08	0.082	0.935	1.0132
Frontoorbital cortex	13.73±0.83	13.37±0.88	2.980	0.003	0.8539
Superior frontal gyrus	25.20±1.37	24.83±1.42	1.877	0.062	1.3961
Lingual gyrus	14.12±1.36	13.63±1.21	2.704	0.007	1.2866
Anterior cingulate gyrus	12.63±1.17	13.03±1.14	-2.482	0.014	1.1566
Posterior cingulate gyrus	11.24±1.25	11.59±1.11	-2.093	0.038	1.1827
Posterior supramarginal gyrus	11.31±1.18	10.44±1.20	5.140	0.000	1.1871
Middle frontal gyrus	23.22±1.20	24.05±1.08	-5.146	0.000	1.1409
Frontal operculum	3.07±0.68	3.06±0.62	0.064	0.949	0.6512
Caudate nucleus	6.60±0.98	6.85±0.98	-1.786	0.076	0.9770
Superior calcarine sulcus	2.89±0.82	2.84±0.72	0.487	0.627	0.7712
Hippocampus	7.82±1.02	8.50±1.03	-4.691	0.000	1.0276
Putamen	9.37±1.16	9.85±1.08	-2.991	0.003	1.1204
Basal forebrain	2.81±0.78	2.80±0.91	0.044	0.965	0.8505
Insula	14.30±1.14	15.34±1.08	-6.600	0.000	1.1130
Posterior parahippocampal gyrus	3.29±0.97	3.37±0.94	-0.555	0.579	0.9575
Nucleus accumbens	1.14±0.76	1.27±0.80	-1.173	0.242	0.7807
Anterior parahippocampal gyrus	5.55±1.04	5.80±1.10	-1.607	0.110	1.0703
Thalamus	13.35±1.26	14.52±1.31	-6.433	0.000	1.2821
Heschl's gyrus	2.97±0.90	3.32±0.70	-2.999	0.003	0.8025
Supplementary parietal cortex	10.99±0.94	12.15±1.01	-8.381	0.000	0.9731
Paracingulate cortex	11.80±1.06	12.36±1.19	-3.525	0.001	1.1271
Pallidum	3.34±0.70	3.45±0.74	-1.058	0.292	0.7171
Anterior supramarginal gyrus	7.66±1.17	8.26±1.34	-3.377	0.001	1.2569
Postcentral gyrus	26.05±1.22	28.80±1.80	-12.631	0.000	1.5361
Occipital pole	18.02±1.20	21.29±1.47	-17.263	0.000	1.3423
Amygdala	3.12±0.90	4.32±0.86	-9.667	0.000	0.8815
Angular gyrus	10.13±1.20	12.65±1.17	-15.088	0.000	1.1827
Hypothalamus	0.80±0.63	0.97±0.70	-1.792	0.075	0.669
Frontomedial cortex	4.26±0.90	5.10±1.04	-6.134	0.000	0.9710

Cingulate and paracingulate cortex

The anterior cingulate gyrus was significantly larger in males ($t(198) = -2.482$, $p = 0.014$, $d = 1.1566$), as was the posterior cingulate gyrus ($t(198) = -2.093$, $p = 0.038$, $d = 1.1827$). The paracingulate cortex also showed a significant difference, with males having larger volumes ($t(198) = -3.525$, $p = 0.001$, $d = 1.1271$) (Table 2).

Parietal lobe regions

The postcentral gyrus exhibited a large effect size, with males having significantly greater volumes ($t(198) = -12.631$, $p < 0.001$, $d = 1.5361$). The supplementary parietal cortex ($t(198) = -8.381$, $p < 0.001$, $d = 0.9731$) and the anterior supramarginal gyrus ($t(198) = -3.377$, $p = 0.001$, $d = 1.2569$) were also significantly larger in males. The angular gyrus showed a substantial difference, with males having significantly greater volumes ($t(198) = -15.088$, $p < 0.001$, $d = 1.1827$) (Table 2).

Temporal lobe regions

Heschl's gyrus was significantly larger in males ($t(198) = -2.999$, $p = 0.003$, $d = 0.8025$). The hippocampus also showed a notable sex difference, with males exhibiting larger volumes ($t(198) = -4.691$, $p < 0.001$, $d = 1.0276$), as did the anterior and posterior parahippocampal gyrus, though the latter did not reach statistical significance ($t(198) = -0.555$, $p = 0.579$, $d = 0.9575$). The insula was significantly larger in males ($t(198) = -6.6$, $p < 0.001$, $d = 1.113$) (Table 2).

Occipital regions

The occipital pole was significantly larger in males ($t(198) = -17.263$, $p < 0.001$, $d = 1.3423$), while the superior calcarine sulcus showed no significant difference ($t(198) = 0.487$, $p = 0.627$, $d = 0.7712$). The lingual gyrus was significantly larger in females ($t(198) = 2.704$, $p = 0.007$, $d = 1.2866$) (Table 2).

Subcortical structures

The thalamus was significantly larger in males ($t(198) = -6.433$, $p < 0.001$, $d = 1.2821$), as were the putamen ($t(198) = -2.991$, $p = 0.003$, $d = 1.1204$), caudate nucleus ($t(198) = -1.786$, $p = 0.076$, $d = 0.977$), and nucleus accumbens, though the latter did not reach statistical significance ($t(198) = -1.173$, $p = 0.242$, $d = 0.7807$). The amygdala was also significantly larger in males ($t(198) = -9.667$, $p < 0.001$, $d = 0.8815$). The hypothalamus showed a trend toward a difference but did not reach statistical significance ($t(198) = -1.792$, $p = 0.075$, $d = 0.669$) (Table 2).

Basal ganglia and limbic system

The pallidum did not show a significant difference ($t(198) = -1.058$, $p = 0.292$, $d = 0.7171$), nor did the basal forebrain ($t(198) = 0.044$, $p = 0.965$, $d = 0.8505$) (Table 2). Overall, males exhibited significantly larger volumes in most high-density estrogen and androgen receptor regions, particularly in the postcentral gyrus, occipital pole, angular gyrus, thalamus, and amygdala. However, some regions, such as the front orbital cortex and lingual gyrus, were larger in females. These findings suggest that sex differences in brain volume may be more pronounced in specific cortical and subcortical structures related to sensorimotor and limbic functions.

DISCUSSION

The present study provides a comprehensive analysis of sex differences in brain region volumes, comparing male and female cohorts across multiple cortical and subcortical structures. Our findings align with previous literature highlighting consistent sex-based morphological differences, with males generally exhibiting larger brain volumes across most regions. However, the extent of these differences varies across specific brain structures, which may reflect underlying neurobiological mechanisms and functional implications.

Our results indicate that males have significantly larger total cerebrum and total cortex volumes compared to females, consistent with prior neuroimaging studies (2, 3). The effect sizes for these differences were large, underscoring the robust nature of these findings. The greater total cerebral white matter volume in males compared to females is also well-documented and may re-

flect sex differences in axonal connectivity and myelination (16). These differences in white matter organization may have implications for cognitive and functional lateralization between sexes.

Significant sex differences were observed in ventricular volumes, with males having larger lateral and third ventricles. This is consistent with prior research indicating greater cerebrospinal fluid (CSF) volume in males (17). Enlarged ventricular volumes have been associated with neurodevelopmental conditions, and the observed sex differences could have implications for susceptibility to neurological disorders. Notably, the fourth ventricle did not exhibit a significant difference, suggesting that not all ventricular structures are affected by sex-based morphological variations. Recent studies have revealed significant sex differences in brain structure volumes during adolescence and adulthood. Males generally exhibit larger volumes in white matter, gray matter, total brain, and CSF compared to females (18).

However, females show larger accumbens volumes (18). Deep learning analysis of pre-adolescent brains identified sex-specific patterns in cerebellar and subcortical structures, which accurately predicted sex and correlated with working memory performance (19). Ventricular indices, particularly the bicaudate ratio, demonstrate age-related changes and sex differences, with males showing higher values (20). Longitudinal studies of subcortical volumes in adolescents and young adults consistently found larger volumes in males across all seven subcortical regions examined, with sex differences in age-related trajectories observed in both European cohorts studied (21). These findings contribute to our understanding of sex-specific neurodevelopment and may have implications for neuropsychiatric disorders.

In regions characterized by low receptor density, males exhibited significantly larger volumes in several areas, including the superior parietal lobule, precuneus, and superior temporal gyrus. These findings support previous studies demonstrating male-biased volumetric differences in these regions (4). These regions play key roles in visuospatial processing, sensory integration, and auditory functions, suggesting that structural sex differences may contribute to variations in cognitive abilities. However, no significant differences

were noted in the superior occipital lateral gyri and inferior occipital lateral gyri, suggesting that occipital regions may be less influenced by sex-related neurodevelopmental factors.

Research has consistently shown sexual dimorphism in brain structure and development. Males typically have 10% larger total brain volume across all ages (22). Specific regions, including the amygdala, hippocampus, and cerebellum, show sex differences and have high sex steroid receptor density (4, 22). Cortical gray matter development follows an inverted U-shaped trajectory, peaking earlier in females (22). Androgen receptors are present in developing rat visual and prefrontal cortex, with higher expression in males (23). In humans, androgen receptor genotype influences cortical maturation, with more efficient alleles associated with “masculinization” of development (24). These findings suggest that sex and sex steroids shape brain development in a spatiotemporally specific manner, potentially contributing to cognitive and behavioral differences between males and females (4, 24).

Recent studies have explored sex differences in brain structure and development, particularly in relation to sex hormones and their receptors. While males generally exhibit larger brain volumes across many regions (25, 26), sex-specific developmental trajectories in early childhood are less pronounced (25). Androgen receptor (AR) expression shows sex-specific patterns in certain brain areas during prepubertal development (27), and testosterone levels may influence amygdala subregion volumes in adolescents, moderated by AR sensitivity (28). Sex differences in cortical and hippocampal microstructure have been observed, with variations related to hormone levels and receptor gene expression (26). These structural differences are most evident in areas of low laminar cortical complexity. Overall, these findings suggest complex interactions between sex hormones, their receptors, and brain structure throughout development, contributing to our understanding of sex-specific neurodevelopmental processes.

Regions with high estrogen and androgen receptor densities showed mixed sex differences. While males had significantly larger postcentral gyrus, occipital pole, and thalamus volumes, females exhibited larger volumes in the front

orbital cortex and lingual gyrus. These results align with studies suggesting that hormonal influences shape regional brain development differently across sexes (29). The larger front orbital cortex in females has been associated with increased emotional regulation and social cognition, while the greater male thalamic volume may be linked to sensory processing and attentional control. The amygdala and hippocampus were significantly larger in males, consistent with prior findings on sex differences in emotional and memory processing regions (22). These structural differences may contribute to known sex differences in emotional reactivity, memory performance, and vulnerability to psychiatric disorders.

Recent studies have revealed significant sexual dimorphism in brain structure and microstructure. Küchenhoff et al. found sex differences in cortical and hippocampal microstructure, which varied with hormone levels and overlapped with sex hormone receptor gene expression (26). Tan et al. demonstrated that microsatellite polymorphisms in sex steroid receptor genes influence gray and white matter volumes differently between sexes (30). Eijk et al. identified region-specific sex differences in hippocampal subfields, with males showing larger volumes in some areas after controlling for total hippocampal volume (31). López-Ojeda & Hurley discussed how these structural differences may contribute to sex-specific vulnerabilities in affective disorders, particularly noting the role of the amygdala in anxiety (32). These findings collectively suggest that sex hormones and their receptors play a crucial role in shaping brain structure and potentially influencing sex-specific behavioral and psychiatric outcomes.

The observed volumetric differences likely reflect a combination of genetic, hormonal, and environmental influences. Testosterone exposure during neurodevelopment is associated with larger brain volumes in males, while estrogen-related mechanisms may contribute to regional differences favoring females (4). These morphological variations may underlie sex-based cognitive and behavioral differences reported in the literature. Additionally, these differences may have implications for sex-specific risks for neurological and psychiatric disorders, such as autism spectrum disorder, schizophrenia, and major depressive

disorder, which exhibit distinct prevalence rates across sexes.

While our study provides valuable insights, certain limitations should be acknowledged. First, we did not control for potential confounding variables such as total intracranial volume (TIV), which could influence volumetric comparisons. Future studies should incorporate TIV-adjusted analyses to ensure more precise sex comparisons. Second, our findings are based on cross-sectional data; thus, longitudinal studies are needed to establish developmental trajectories of these sex differences. Third, environmental and lifestyle factors, such as education and socioeconomic status, were not examined but may contribute to observed structural variations. Future research should also explore the functional implications of these structural variations, particularly in relation to cognitive performance and neuropsychiatric conditions.

CONCLUSIONS

This study confirms and extends previous findings on sex differences in brain morphology. Males exhibit larger total brain and regional volumes across most areas, with notable exceptions in certain frontal and occipital regions where female-biased differences emerge. These findings contribute to our understanding of sex-based neuroanatomical diversity and highlight the importance of considering sex as a biological variable in neuroscience research. Further research integrating multimodal neuroimaging and longitudinal designs will be crucial in elucidating the developmental and functional significance of these structural differences.

ACKNOWLEDGMENTS

The authors express their sincere gratitude to all study participants for their cooperation throughout the research process. The authors also thank the technical staff of the Department of Radiology, Mamata Medical College, for their assistance with MRI acquisition and processing. Appreciation is extended to the neuroanatomy and MRI technicians for their support during segmentation and volumetric analysis.

FUNDING

This research received no external funding.

CONFLICTS OF INTEREST

The authors declare no conflict of interest related to this study.

AUTHOR CONTRIBUTIONS

S.N.: conceptualization, methodology, data acquisition, statistical analysis, writing – original draft preparation, writing – review and editing; Z.B.: conceptualization, methodology, statistical analysis, writing – review and editing, supervision; K.T.: methodology, data acquisition, statistical analysis, writing – original draft preparation, writing – review and editing, supervision; R.P.J.: data acquisition, writing – review and editing; U.K.: writing – original draft preparation, writing – review and editing, supervision; Trained MRI technicians under the supervision of U.K., K.T., S.N.: MRI segmentation and parcellation.

All authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Mamata Medical College (Approval/Reference Number: [MMC/IEC/2022/2945/03/2025; approved April 4, 2025])

INFORMED CONSENT STATEMENT

Written informed consent was obtained from all participants prior to inclusion in the study.

DECLARATION OF GENERATIVE AI IN SCIENTIFIC WRITING

No generative AI tools were used in the writing, analysis, interpretation, or preparation of this manuscript. All scientific content and writing were completed exclusively by the authors.

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Molecular Docking, Synthesis, Characterization of Novel Pyridine/Piperidine N-Mannich Base Derivatives and their Bactericidal Studies

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Received: August 18, 2025;

Revised: September 28, 2025;

Accepted: December 22, 2025

ABSTRACT

OBJECTIVE This study aims to synthesize and estimate the anti-bacterial, anti-fungal and anti-tubercular activity of novel N-pyridine/piperidine Mannich base derivatives (M1-M6).

METHODS The novel N-pyridine/piperidine Mannich base derivatives were prepared through condensation reactions between pyridine/piperidine, furfuraldehyde and various aromatic/ aliphatic amines. The structures of the synthesised compounds were characterized using FTIR, ¹³C-NMR, ¹H-NMR, and mass spectroscopy. These derivatives were assessed for anti- bacterial, anti-fungal and anti-tubercular activity using in vitro methods such as paper disc diffusion and agar well diffusion methods.

RESULTS The antimicrobial and antitubercular activities of the Schiff base Mannich derivatives (M1-M6) were systematically evaluated. Antimicrobial screening against bacterial strains (*S. aureus*, *E. coli*, *S. epidermidis*, and *P. aeruginosa*) and fungal strains (*Aspergillus fumigatus* and *A. niger*) demonstrated clear concentration-dependent activity. At a concentration of 100 µg/disc, compound M2 exhibited notable inhibition zones of 10.25 mm (*S. aureus*), 9.76 mm (*E. coli*), and 10.43 mm (*A. fumigatus*), while M4 showed comparable activity with 10.03 mm, 8.56 mm, and 9.87 mm, respectively. In contrast, M3 displayed the lowest activity, especially against *P. aeruginosa* and fungal strains, whereas M5 and M6 showed moderate effects. Standard drugs, ciprofloxacin and fluconazole, exhibited superior potency. In antitubercular assays, M2 and M4 showed dose-dependent inhibition of *mycobacterium*, with M2 demonstrating higher efficacy. Docking studies further supported these findings, as M2 and M4 showed strong binding affinities toward InhA and EthR proteins, correlating well with their biological activity.

CONCLUSION These findings emphasize the therapeutic promise of Mannich base derivatives, particularly piperidine-containing compounds (M2, M4), as potential antimicrobial and antitubercular agents. Their higher basicity enhances molecular interactions with microbial targets. Further structural optimization of these scaffolds could improve potency, selectivity, and effectiveness against resistant bacteria, fungi, and *Mycobacterium tuberculosis*.

KEYWORDS pyridine, piperidine, Mannich base, in vitro anti-microbial activity, in vitro anti-tubercular activity

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INTRODUCTION

Pyridine derivatives have been extensively studied for their potential diverse biological activities (1). Novel pyridine derivatives are being designed and synthesized to combat microbial infections, particularly those caused by multi-drug-resistant bacteria (2). They have emerged as promising anti-microbial agents due to their versatile chemical structure and ability to interact with various biological targets (3). The nitrogen atom in the pyridine ring enhances binding affinity with microbial enzymes and proteins, contributing to their potent anti-bacterial and anti-fungal activities (4). In addition to that, pyridine derivatives have shown promise as potential anti-tubercular agents, exhibiting inhibitory activity against *Mycobacterium tuberculosis* enzymes and pathways (5). Tuberculosis (TB) is a bacterial illness caused by *Mycobacterium tuberculosis*, mainly targeting the lungs (6, 7). It remains a major global health issue, resulting in millions of new infections and deaths annually (8). TB is transmitted through airborne droplets released by an infected individual (9). Common symptoms include a constant fever, cough and unintentional loss of body weight (10). Without proper treatment, the disease can be fatal. While TB is curable with antibiotics, the rise of drug-resistant strains creates a significant challenge (11). Efforts to combat TB include developing new treatments, improving diagnosis, and increasing access to healthcare, particularly in high-burden countries (12). Early detection and treatment are crucial (13). The development of novel anti-tubercular agents is essential to combat this disease (14). Pyridine derivatives can disrupt essential cellular processes such as DNA replication, protein synthesis, and cell wall formation (15, 16). Pyridine derivatives have garnered attention for their potential therapeutic applications, including anti-tubercular activity (17). Structural modifications of the pyridine ring have led to the development of compounds with broad-spectrum anti-microbial activity, including efficacy against resistant strains (18). Recent studies have highlighted the promise of pyridine-based compounds as anti-microbial and anti-tubercular agents, demonstrating their potential to target specific bacterial enzymes and pathways (19, 20). As a result of a continuous search for the above-mentioned area, we synthe-

sized and evaluated novel N-Mannich base derivatives as potential anti-bacterial, anti-fungal and anti-tubercular agents. The results reveal that the biological activities of N-Mannich derivatives are similar to that of standard drugs.

METHODS

Chemicals and reagents

The chemicals employed in this study were purchased from reputable pharmaceutical companies, including Sigma, Aldrich, Ranbaxy, and Dr. Reddy's, and were of laboratory reagent grades. The synthesized Mannich base derivatives were characterized using various analytical techniques. A Perkin Elmer FT-IR spectrophotometer (spectrum RX1) was employed to identify functional groups while examining from 4000 cm^{-1} to 400 cm^{-1} . A Bruker 400 MHz spectrometer (Bruker Avance DRX 400) was employed to determine the environmental protons, using deuterated chloroform as a solvent. A Shimadzu mass spectrometer (Q-TOF) was used to determine the molecular weight of the compounds. A Perkin Elmer analyzer (2400 CHNS) was used to identify the atoms present in the synthesized Mannich base derivatives (21). All the instruments were in proper working condition during the study.

Molecular docking

CB-Dock2 is a user-friendly molecular docking tool used to predict the binding interaction between ligands and bacterial enzymes such as *Mycobacterium tuberculosis* InhA/ EthR regulatory proteins. It automatically identifies potential binding sites on the enzyme and docks the ligand without requiring prior knowledge of the active site. The most stable 3D structure of each drug was selected and docked with the target enzyme. Binding affinity assessed using a docking score, with lower values indicating stronger interactions. This helps evaluate and compare the potency of different drug candidates (22).

Preparation of pyridine mannich base derivatives (M1, M3, M5)

A mixture of 0.79 g of pyridine (0.01 mole), 1.69 g of diphenyl amine (0.01 mole)/diethyl amine (0.01 mole)/ethyl methyl amine (0.01 mole) and 0.30 mL of furfuraldehyde was placed in 30 mL of ethyl alcohol. The obtained solution was blended

was refluxed for five hours, after which the warm solution was allowed to cool and then poured into ice water. Precipitated mass was filtered and crystallized from ethyl alcohol (Figure 1).

Preparation of piperidine mannich base derivatives (M2, M4-M6)

A mixture of 0.85 g of piperidine (0.01 mol), 1.69 g of diphenyl amine (0.01 mole)/0.59 g of diethyl amine (0.01 mole)/ethyl methyl amine (0.01 mole) and 0.30 mL of furfuraldehyde was placed in 30 mL of ethyl alcohol. The resulting solution was mixed thoroughly and refluxed for five hours. It was then allowed to cool and poured into cold water. The precipitated product was collected by filtration and recrystallized from ethyl alcohol (23, 24). (Figure 2).

Biological activities

Anti-bacterial activity:

The anti-microbial effects of Mannich base derivatives were estimated by using the paper

disc diffusion method. Anti-microbial efficacy was estimated by measuring the zones of inhibition on Mueller–Hinton agar (anti-bacterial study) and Sabouraud dextrose agar (anti-fungal study). Sterilized culture media filled into plates of 4 mm width, allowed to harden, and then inoculated with various microorganisms: *S. epidermidis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *A. fumigatus*, and *A. niger*. Mannich base derivatives were prepared in dimethylformamide at concentrations of 50 µg and 100 µg. Paper discs soaked in these solutions were placed on the inoculated media and incubated at 37°C for 24 and 48 hours for bacterial and fungal organisms, respectively. Ciprofloxacin (100 µg/disc) and fluconazole (100 µg/disc) served as standard references for anti-bacterial and anti-fungal activity, respectively. A solvent control (dimethylformamide alone) was also included. Antimicrobial activity was assessed by measuring the zones of inhibition produced by the Mannich base derivatives and comparing them with those produced by the corresponding standard drugs (25).

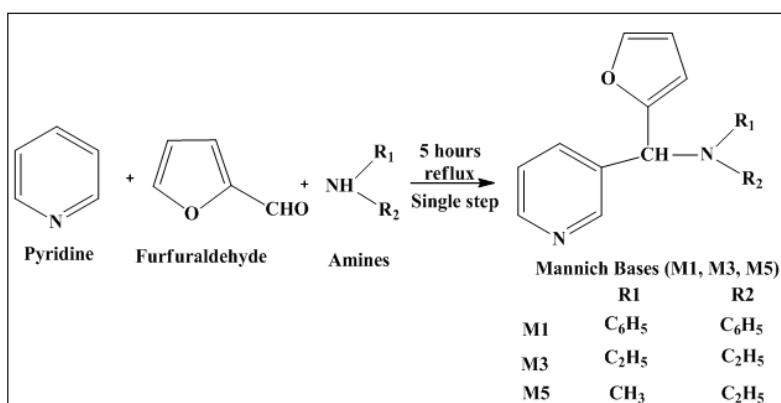


Figure 1. Synthetic scheme of Mannich base derivatives (M1, M3, M5)

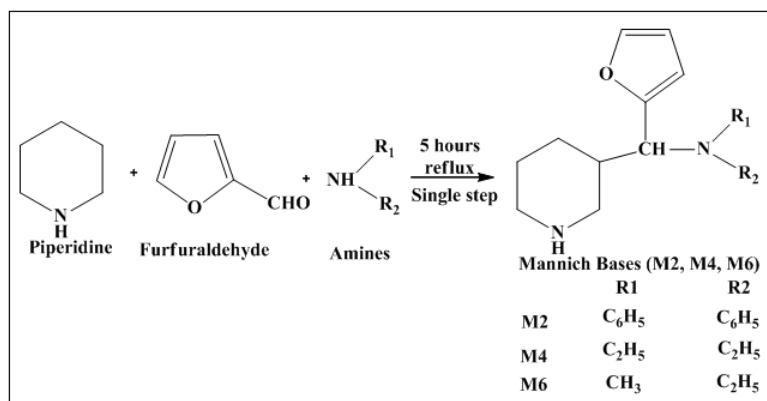


Figure 2. Synthetic scheme of Mannich base derivatives (M2, M4, M6)

In vitro anti-tubercular activity

Preparation of Lowenstein-Jensen medium: 37.24 g of LJ medium base was dissolved in 600 mL of distilled water containing 12 mL of glycerol, with gentle heating applied if necessary to ensure complete dissolution. The solution was then sterilized by autoclaving at 121°C under 15 lb pressure for 15 minutes. Meanwhile, 1,000 mL of whole egg emulsion was prepared under aseptic conditions. After the base medium cooled slightly, the egg emulsion was aseptically added and mixed thoroughly. The combined medium was then sterilized by autoclaving at 85°C for 45 minutes. After sterilization, the medium was inoculated with a culture of *Mycobacterium tuberculosis* obtained from Rontgen's Medical Centre, Thanjavur, Tamil Nadu. The inoculated LJ medium was incubated at 37°C for seven days to facilitate bacterial growth (26).

Agar well diffusion technique

The plates filled with Mueller-Hinton Agar (MHA) were inoculated with a *Mycobacterium tuberculosis* culture adjusted to a 0.5 McFarland standard. Bore wells of 6 mm diameter were produced in the medium using a sterile cork borer. Different concentrations of Mannich base derivatives (100, 500, and 1,000 µg/mL) were prepared for the assay. A volume of 0.3 mL of each concentration was carefully introduced into the wells and allowed to diffuse for 5–10 minutes at room temperature before incubation. The plates were then incubated at 37°C for 48 hours. Each concentration was tested in triplicate to ensure reproducibility and reduce experimental error. After incubation, the diameters of the zones of inhibition (IZD) were measured and recorded for further analysis. Rifampicin was used as the standard control (27).

RESULTS**Molecular docking**

The binding affinities of six N-Mannich base derivatives (M1–M6) were evaluated against *Mycobacterium tuberculosis* InhA and EthR regulatory proteins (Table 1). The results indicate that all derivatives exhibited moderate to strong interactions with both protein targets. M2 showed the highest binding affinity, with –8.30 kcal/mol for InhA and –8.91 kcal/mol for EthR, suggesting its potential as the most effective inhibitor among

the tested compounds. M4 also demonstrated significant binding (–8.02 kcal/mol for InhA and –8.54 kcal/mol for EthR). These results highlight the influence of structural variations among the derivatives on their protein-binding capabilities, which may guide further optimization for anti-tuberculosis activity. Rifampicin exhibited the highest binding affinities toward both *M. tuberculosis* InhA (–8.92 kcal/mol) and EthR (–9.34 kcal/mol) proteins (Table 1 and Figure 3–4).

Chemistry

N-(furan-2-yl)(pyridin-3-yl) methyl-N-phenylaniline-(M1): Yield (%): 78; MP.: 293–295°C; Shade: off white; FTIR($\nu_{\max}$, cm^{-1}): 3408 (str., ArH), 1518 (str., ArC), 1596 (CN), 1774 (CO); ^1H NMR (CDCl_3 , δ ppm): 5.43 (s, 1H, CH-H), 6.45 (d, 1H, J = 10.2 Hz, ArH), 6.52–6.71 (m, 1H, ArH), 6.82–6.89 (m, 2H, ArH), 7.34–7.51 (m, 9H, ArH), 7.74–7.79 (m, 1H, ArH); 7.91–7.96 (m, 1H, ArH), 8.51 (d, 1H, J = 9.6 Hz, ArH), 8.64 (s, 1H, ArH); ^{13}C -NMR (DMSO- d_6 , δ ppm): 80.6 (C-6), 107.5 (C-8), 112.1 (C-9), 119.3 (C-12,16, 18,22), 121.4 (C-14,20), 123.7 (C-4), 129.6 (C-13,15, 19,21), 132.2 (C-4), 134.2 (C-2), 142.3 (C-10), 147.5 (C-5), 149.6 (C-11,17), 150.9 (C-1), 153.4 (C-7); MS (m/z , %): 326 (42) [M^+], 259 (45), 168 (100), 78 (32), 53 (28), 40 (55), 26 (27); Anal. calcd. (found) % for $\text{C}_{22}\text{H}_{18}\text{N}_2\text{O}$ (M.W326): C, 80.96 (81.03); H, 5.56 (5.62); O, 4.90 (4.92); N, 8.58 (8.43).

N-(furan-2-yl) (piperidin-3-yl)methyl-N-phenylaniline (M2): Yield (%): 83; MP.: 301–303°C; Shade: Slight Yellow; FTIR: 3476 (str., Ar-proton), 1518 (str., Ar-C), 1594 (CN), 1772 (CO); ^1H NMR: 2.16 (s, 2H, NH-proton), 5.67 (s, 1H, CH-H), 5.75 (s, 1H, OH-proton), 6.31–6.59 (m, 1H, ArH), 6.76–6.89

Table 1. Binding affinity of N-Mannich base derivatives against *Mycobacterium tuberculosis* InhA/ EthR regulatory proteins

Compounds	<i>Mycobacterium tuberculosis</i> InhA protein (kcal/mol)	EthR regulatory protein (kcal/mol)
M1	-7.46	-7.52
M2	-8.30	-8.91
M3	-7.83	-8.10
M4	-8.02	-8.54
M5	-7.03	-7.22
M6	-7.59	-7.76
Rifampicin	-8.92	-9.34

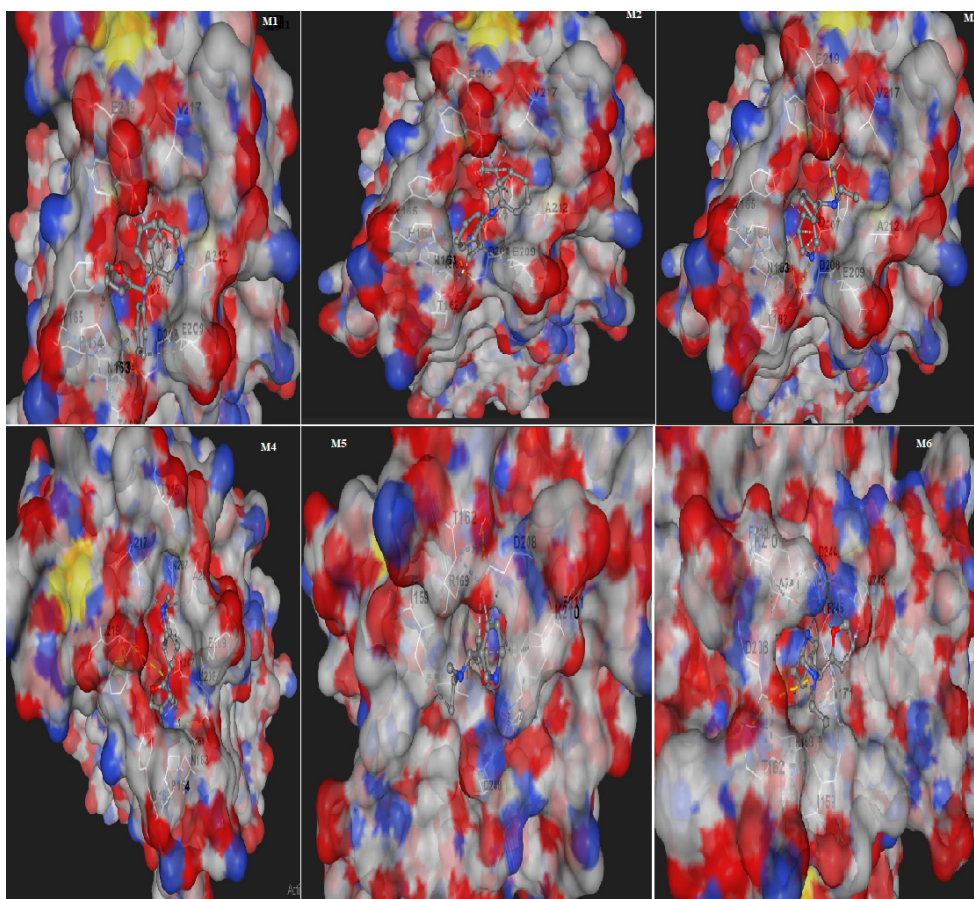


Figure 3. Docking images of Mannich base derivatives against *Mycobacterium tuberculosis* InhA complex

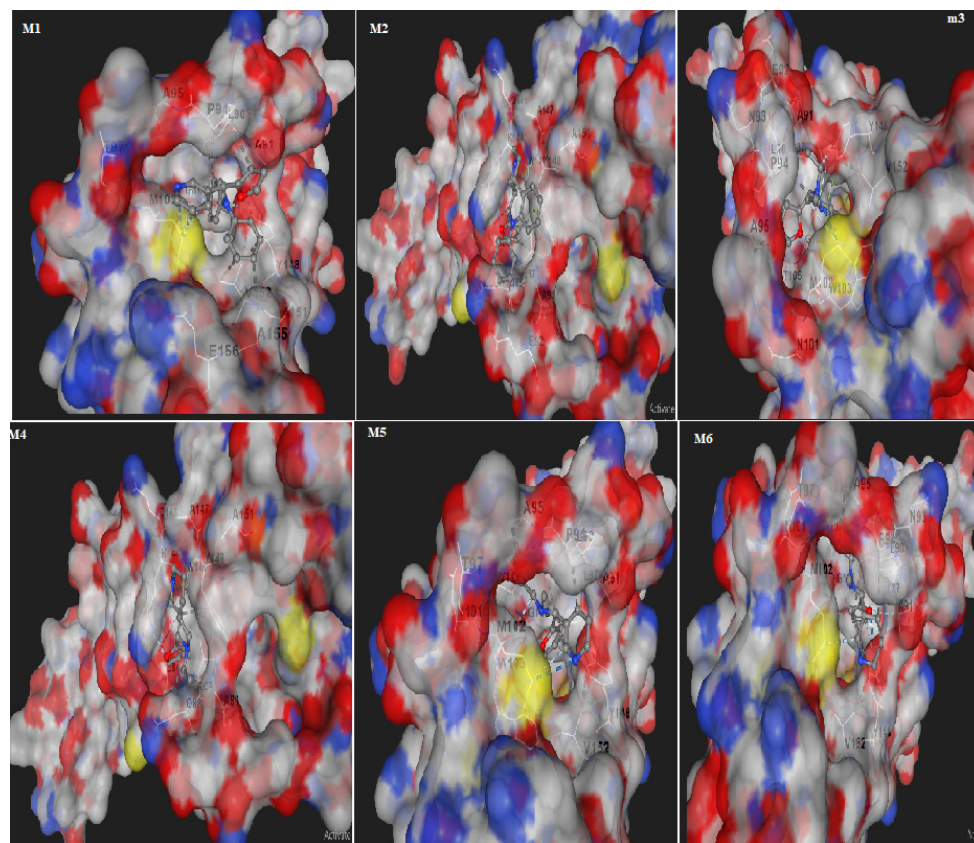


Figure 4. Docking images of Mannich base derivatives against *Mycobacterium tuberculosis* EthR complex

(m, 1H, Ar-proton), 6.82-7.05 (m, 2H, ArH), 7.14-7.39 (m, 1H, Ar-proton), 7.44-7.69 (m, 1H, Ar-proton), 7.72-7.84 (m, 1H, ArH), 8.64 (s, 2H, NH₂-H); ¹³C-NMR (DMSO-*d*₆, δ ppm): 24.3 (C-3), 25.8 (C-4), 34.6 (C-2), 46.7 (C-8), 48.2 (C-1), 73.9 (C-5), 108.4 (C-6), 111.2 (C-12), 119.2 (C-9,16, 18,22), 121.7 (C-14,20), 129.2 (C-13,15, 19,21), 141.7 (C-10), 149.1 (C-11,17), 150.7 (C-7); MS (*m/z*, %): 336 (46) [M⁺], 249 (100), 158(52), 91(48), 78 (26), 40 (37), 38 (25), 27 (28); Anal. calcd. (found) % for C₂₂H₂₄N₂O, (*m.w.* 332): C, 79.48 (79.15); H, 7.28 (7.53); O, 4.81 (4.76); N, 8.43 (8.57).

N-ethyl-N-(furan-2-yl(pyridin-3-yl)-methyl)ethanamine (M3): Yield (%): 87; MP.: 214-216 °C; Shade: light brown; FT-IR: 3452 (*str.*, Ar-H), 1521 (*str.*, ArC), 1586 (CN), 1656 (CO); ¹H NMR: 1.16 (m, 6H, CH₃-proton), 2.48 (d, 4H, *J* = 8.7 Hz, CH₂-H), 5.53 (s, 1H, CH-H), 6.35 (d, 1H, *J* = 9.6 Hz, ArH), 6.55-6.67 (m, 1H, Ar-proton), 7.49-7.59 (m, 1H, Ar-proton); 7.71-7.78 (m, 1H, ArH), 7.92-7.97 (m, 1H, ArH), 8.63-8.69 (m, 1H, Ar-proton), 8.72 (s, 1H, Ar-H); ¹³C-NMR: 13.5 (C-12,14), 46.5 (C-11,13), 72.8 (C-6), 106.3 (C-8), 110.5 (C-9), 123.7 (C-4), 132.5 (C-3), 134.3 (C-2), 142.8 (C-10), 147.3 (C-5), 150.6 (C-1), 152.4 (C-7); MS (*m/z*, %): 230 (37) [M⁺], 201 (65), 172 (100), 105 (38), 78 (42), 40 (29), 29 (22); Anal. calcd. (found) % for C₁₄H₁₈N₂O, (*m.w.* 230): C, 73.01 (73.14); H, 7.88 (7.79); O, 6.95 (6.75); N, 12.16 (12.32).

N-ethyl-N-(furan-2-yl(piperidin-3-yl)-methyl)ethanamine (M4): Yield (%): 69; MP.: 223-225°C; Shade: Off white; FTIR: 3438 (*str.*, ArH), 1535 (*str.*, ArC), 1572 (CN), 1614 (CO); ¹H NMR: 1.12-1.23 (m, 6H, CH₃-proton), 1.69-1.78 (m, 4H, CH₂-proton), 2.10-2.15 (m, 1H, NH-proton), 2.21-2.30 (m, 1H, CH-proton), 2.45-2.52 (m, 4H, CH₂-proton), 2.67-2.74 (m, 2H, CH₂-proton), 2.79-2.85 (m, 4H, CH₂-proton), 4.25 (d, 1H, CH-H), 6.62-6.73 (m, 2H, ArH), 7.74-7.81 (m, 1H, ArH); ¹³C-NMR: 13.8 (C-12,14), 24.2 (C-3), 25.5 (C-4), 35.3 (C-2), 46.3 (C-1), 47.8 (C-11,5), 48.3 (C-13), 68.1 (C-6), 108.7 (C-8), 110.4 (C-9), 141.3 (C-10), 150.2 (C-7); MS (*m/z*, %): 236 (54) [M⁺], 207 (52), 178 (100), 164 (45), 138 (32), 84 (27), 29 (22); Anal. calcd. (found) % for C₁₄H₂₄N₂O, (*m.w.* 236): C, 71.14 (72.02); H, 10.23 (9.92); O, 6.77 (6.89); N, 11.85 (11.17).

N-(furan-2-yl(pyridin-3-yl)-methyl)-N-methyl ethanamine (M5): Yield (%): 77; MP.: 207-209°C; Shade: colourless; FTIR:(3452 (*str.*, ArH), 1526 (*str.*, ArC), 1513 (CN), 1647 (CO); ¹H NMR: 1.08 (m, 3H, CH₃-proton), 2.35 (s, 3H, CH₃-proton), 2.53-2.64 (m, 2H, CH₂-H), 5.40 (s, 1H, CH-H), 6.39 (d, 1H,

J = 9.8 Hz, Ar-H), 6.43-6.51 (m, 1H, ArH), 7.26-7.38 (m, 1H, ArH); 7.68-7.75 (m, 1H, ArH), 7.90-7.98 (m, 1H, ArH), 8.43-8.51 (m, 1H, ArH), 8.69 (s, 1H, Ar-H); ¹³C-NMR: 12.5 (C-13), 37.9 (C-11), 49.4 (C-6), 75.6 (C-12), 106.3 (C-8), 110.1 (C-2), 123.5 (C-3), 132.1 (C-4), 134.4 (C-9), 142.8 (C-10), 147.3 (C-5), 150.2 (C-1), 152.7 (C-7); MS (*m/z*, %): 216 (38) [M⁺], 201 (37), 172 (100), 105 (42), 78 (18), 52 (56), 29 (34); Anal. calcd. (found) % for C₁₃H₁₆N₂O, (MW. 216): C, 72.19 (71.84); H, 7.46 (7.65); O, 7.40 (7.67); N, 12.95 (12.84).

N-(furan-2-yl(piperidin-3-yl)-methyl)-N-methyl ethanamine (M6): Yield (%): 79; MP.: 216-218 °C; Shade: colourless; FT-IR: 3429 (*str.*, ArH), 1540 (*str.*, ArC), 1518 (CN), 1635 (CO); ¹H NMR: 1.05 (t, 3H, CH₃-proton), 1.62-1.76 (m, 4H, CH₂-proton), 2.02-2.10 (m, 1H, NH-proton), 2.15-2.26 (m, 1H, CH-proton), 2.32 (s, 3H, CH₃-proton), 2.45-2.53 (m, 2H, CH₂-proton), 2.61-2.74 (m, 2H, CH₂-proton), 2.84-2.91 (m, 2H, CH₂-proton), 4.19 (d, 1H, CH-H), 6.51-6.58 (m, 2H, ArH), 7.48-7.55 (m, 1H, ArH); ¹³C-NMR: 12.8 (C-13), 24.6 (C-3), 25.5 (C-4), 35.8 (C-2), 41.7 (C-11), 46.2 (C-1), 48.7 (C-5), 49.2 (C-10), 71.8 (C-8), 106.8 (C-6), 110.4 (C-9), 141.6 (C-6), 150.2 (C-12); MS (*m/z*, %): 222 (48) [M⁺], 207 (37), 178 (100), 111 (32), 84 (52), 56 (32), 28 (38); Anal. calcd. (found) % for C₁₃H₂₂N₂O, (*m.w.* 222): C, 70.23 (69.24); H, 9.97 (10.56); O, 7.20 (7.89); N, 12.60 (12.31).

Anti-microbial activity

The anti-microbial evaluation of Schiff base derivatives (M1-M6) was conducted against a range of bacterial (*S. aureus*, *E. Coli*, *S. epidermidis* and *P. aeruginosa*,) and fungal (*A. fumigatus* and *Aspergillus niger*) strains. The study revealed a concentration-dependent increase in activity for all tested compounds, with larger zones of inhibition observed at 100 µg compared to 50 µg. Among the tested compounds, M2 and M4 consistently exhibited the highest anti-microbial activity across both bacterial and fungal strains. At 100 µg, M2 demonstrated strong inhibition zones, particularly against *E. coli* (9.76 mm), *S. aureus* (10.25 mm), and *A. fumigatus* (10.43 mm), approaching the efficacy of standard drugs. Similarly, M4 showed comparable inhibition, with notable activity against *E. coli* (8.56 mm), *S. aureus* (10.03 mm), and *A. fumigatus* (9.87 mm). In contrast, M3 exhibited the weakest activity, especially against *P. aeruginosa* and fungal strains, with

Table 2. Zone of inhibition of Mannich base derivatives (M1-M6) against different microorganism (mm)

Compounds	<i>S. aureus</i>		<i>S. epidermidis</i>		<i>E. coli</i>		<i>P. aeruginosa</i>		<i>A. niger</i>		<i>A. fumigatus</i>	
	50	100	50	100	50	100	50	100	50	100	50	100
M1 (µg/disc)	7.94	9.57	7.23	8.98	8.98	8.45	5.23	8.03	5.98	8.45	5.76	9.54
M2 (µg/disc)	8.79	10.25	7.42	9.56	9.56	9.76	5.68	8.43	6.34	9.76	6.89	10.43
M3 (µg/disc)	6.82	8.63	6.03	8.11	8.11	7.90	4.23	7.41	4.90	7.90	4.34	7.65
M4 (µg/disc)	8.61	10.03	7.30	9.30	9.30	8.56	5.59	8.35	6.21	8.56	6.56	9.87
M5 (µg/disc)	7.50	9.23	6.56	8.76	8.76	8.17	4.13	7.56	5.35	8.17	5.28	8.82
M6 (µg/disc)	7.65	9.42	6.97	8.91	8.91	8.31	4.89	7.87	5.72	8.31	5.63	9.23
Ciprofloxacin (100 µg/disc)	13.85		10.30		9.60		10.35		-			
Fluconazole (100 µg/disc)	-		-		-		-		10.65		10.90	

zones of inhibition significantly lower than those of the reference antibiotics. Compounds M5 and M6 demonstrated moderate anti-microbial activity, with improved performance at higher concentrations but not reaching the levels of M2 and M4. Standard antibiotics Ciprofloxacin and Fluconazole served as positive controls. Ciprofloxacin showed superior anti-bacterial activity with zones ranging from 9.60 to 13.85 mm, indicating higher potency than the Mannich base derivatives at both test concentrations. Fluconazole showed strong anti-fungal activity against *A. fumigatus* (10.90 mm) and *A. niger* (10.65 mm), again outperforming the test compounds. Overall, the data suggest that compounds M2 and M4 possess promising broad-spectrum anti-microbial properties, particularly at higher concentrations. Their activity, while not exceeding that of standard drugs, highlights their potential as scaffolds for further structural optimization and development into more potent anti-microbial agents (Table 2).

Anti-tubercular activity

The compounds M2 and M4 exhibited the highest anti-tubercular activity in the in vitro study. The compounds M2 and M4 showed a significant zone of inhibition on *Mycobacterium* at different concentrations, as shown in Table 3. Piperidine nucleus-based Mannich base derivatives demonstrated greater anti-tubercular activity than pyridine nucleus-based Mannich base derivatives. The anti-TB activity was directly proportional to the concentration. The highest concentration (1,000 µg/mL) of piperidine activities nearest to the standard. M2 Mannich base de-

rivative was more potent than M4 in all the concentrations, with larger inhibition zones ranging from 5.10 mm to 10.35 mm, while M4 ranged from 4.55 mm to 9.20 mm. Rifampicin produced the largest inhibition (12.90 mm), indicating higher potency.

DISCUSSION

The novel Mannich base derivatives (M1-M6) successfully synthesised through a reflux method using ethyl alcohol as the solvent. The compounds were characterized using ¹HNMR, ¹³CNMR, FTIR and mass spectrometry. The FTIR spectra of the compounds demonstrated characteristic absorption bands for aromatic C-H stretching, CN stretching, and CO stretching vibrations. The ¹H NMR spectra revealed the existence of aromatic protons, CH-protons, and NH-protons, while the ¹³CNMR spectra confirmed the presence of aromatic carbons, CH-carbons, and C = O carbons (22). The bactericidal activity of M1-M6 was evaluated

Table 3. In vitro anti-tubercular activity against *Mycobacterium tuberculosis*

Compounds	Inhibition zone diameters (mm)		
	100 µg/mL	500 µg/mL	1,000 µg/mL
M1	3.41	6.53	7.03
M2	5.10	9.80	10.35
M3	3.26	6.02	6.55
M4	4.55	7.30	9.20
M5	3.24	5.93	6.17
M6	3.92	7.14	8.38
Rifampicin (30 µg/mL)	12.90		

against various microorganisms, including bacteria (*S. aureus*, *E. coli*, *P. Aeruginosa*, *S. epidermidis*) and fungi (*A. fumigatus* and *A. niger*). The zone of inhibition (mm) was measured at two concentrations (50 µg and 100 µg) (22). All compounds showed increased activity at the higher concentration (100 µg). The microbicidal evaluation discovered that derivatives M2 and M4 exhibited the highest activity against both bacterial and fungal strains. M2 showed strong inhibition zones against *S. aureus* (10.25 mm), *E. coli* (9.76 mm), and *A. fumigatus* (10.43 mm), while M4 demonstrated comparable inhibition. These findings suggest that the piperidine nucleus in M2 and M4 contributes to their enhanced anti-microbial activity (19, 22). Ciprofloxacin exhibited superior bactericidal activity, with zones of inhibition ranging from 9.60 to 13.85 mm. Fluconazole exhibited strong anti-fungal activity, with zones of inhibition of 10.90 mm and 10.65 mm against *A. fumigatus* and *A. niger*, respectively. M2 and M4 showed significant activity, with zones of inhibition comparable to standard drugs (19, 22). In addition to that, the Mannich base derivatives M1-M6 exhibited significant anti-tubercular activity against *Mycobacterium tuberculosis*. The study revealed piperidine-containing Mannich base derivatives (M2, M4, M6) showed higher anti-TB activity compared to pyridine-containing derivatives (M1, M3 and M5). The anti-tubercular activity assessment showed that M2 and M4 exhibited significant activity against *Mycobacterium tuberculosis*. M2 displayed higher potency, with inhibition zones ranging from 5.10 mm to 10.35 mm, while M4 ranged from 4.55 mm to 9.20 mm. The standard drug, Rifampicin, showed higher potency than both compounds. The observed antitubercular activity is consistent with previous studies demonstrating that nitrogen-containing heterocyclic scaffolds, including pyridine-containing compounds, can serve as promising frameworks for the development of antitubercular agents (5, 19). The study suggests that the piperidine nucleus in Mannich base derivatives enhances their anti-microbial and anti-tubercular activity. The existence of specific functional groups, such as the furan-2-yl group, may also contribute to the observed activity. The synthesised Mannich base derivatives, particularly M2 and M4, showed promising anti-microbial and anti-tubercular activity. The binding

affinities of the N-Mannich base derivatives (Table 1) were compared with their in vitro antitubercular activity against various microorganisms (Table 3). Compounds exhibiting stronger binding affinities, particularly M2 (InhA: -8.30 kcal/mol; EthR: -8.91 kcal/mol), showed higher zones of inhibition against *M. tuberculosis*, especially at 1000 µg concentrations. Similarly, M4 demonstrated both favorable docking scores and notable anti-tubercular activity. Conversely, derivatives with weaker binding affinities, such as M5 and M3, displayed comparatively lower inhibitory zones. Molecular docking has been widely used to investigate the predicted interactions and binding modes of heterocyclic antimicrobial compounds with potential biological targets (19, 22). This correlation indicates that the computational docking results align well with experimental anti-tubercular activity, validating docking as a predictive tool for assessing the potential efficacy of N-Mannich base derivatives against *M. tuberculosis*. It also highlights M2 as the most promising compound for further optimization. These findings provide valuable insights into the development of novel anti-bacterial, anti-fungal and anti-TB therapies. Further structural optimization and development of these compounds may lead to more potent agents against microbial infections.

CONCLUSIONS

The novel Mannich base derivatives (M1-M6) were successfully synthesized via a reflux method and thoroughly characterized using FTIR, ¹H NMR, ¹³C NMR, and mass spectrometry. Anti-microbial evaluation revealed that all compounds exhibited enhanced activity at higher concentrations, with M2 and M4 demonstrating the most potent bactericidal and fungicidal effects. Notably, M2 showed strong inhibition against *S. aureus*, *E. coli*, and *A. fumigatus*, suggesting a crucial role of the piperidine nucleus in biological activity. Anti-tubercular studies further confirmed the superior efficacy of piperidine-containing derivatives (M2, M4, M6) compared to pyridine analogues, with M2 showing the highest potency against *Mycobacterium tuberculosis*. These findings highlight the therapeutic potential of Mannich base derivatives, especially those containing the piperidine moiety, as promising candidates for future anti-microbial and anti-TB drug development.

Structural optimization of the piperidine-based scaffolds (M2, M4) may enhance potency and selectivity against resistant bacterial and fungal strains, as well as *Mycobacterium tuberculosis*. In vivo evaluations, pharmacokinetic profiling, and eventually clinical trials, are essential to validate the therapeutic potential of these Mannich base derivatives. This highlights the translational relevance of the work and underscores their promise as leads for future anti-microbial and anti-tubercular drug development.

ACKNOWLEDGMENTS

The authors would like to thank the GIET School of Pharmacy for extending their support to complete our work from time to time.

FUNDING

The author(s) received no financial support for the research, authorship, and/or publication of this article.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

C.G.: conceptualization, methodology, investigation, data curation, data analysis, writing – original draft; M.D.: validation, data curation, visualization; V.M.: writing – original draft; P.C.: methodology and investigation; L.K.: writing – review & editing.

All authors have read and approved the final version of this manuscript that was submitted for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable. This study did not involve human or animals.

INFORMED CONSENT STATEMENT

Not applicable. This study did not involve human or animals.

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Inhibitory Effect of Virgin Coconut Oil (VCO) on Leukemic Cell Proliferation in Human Promyelocytic Leukemia HL-60 Cells

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Received: September 16, 2025;

Revised: December 15, 2025;

Accepted: December 25, 2025

ABSTRACT

OBJECTIVE Leukemia is characterized by the uncontrolled proliferation of abnormal white blood cells, and although chemotherapy remains a standard treatment, toxicity, relapse, and drug resistance continue to pose major clinical challenges. These limitations draw attention to the need for safer and more effective therapeutic alternatives. Natural products have attracted considerable interest due to their diverse bioactive constituents and promising anti-cancer effects. Virgin coconut oil (VCO) has been reported to offer several health benefits, including antioxidant, anti-stress, anti-inflammatory, weight-reducing, and anti-cancer properties. This study was designed to investigate the capability of VCO to inhibit HL-60 (human promyelocytic leukemia) cell growth by inducing cell cycle arrest and apoptosis.

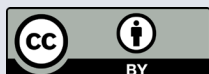
METHODS VCO was analyzed using gas chromatography-mass spectrometry (GC-MS) analysis. Cell viability was assessed using the MTT assay; HL-60 cells were treated with various VCO concentrations for 48 hours. Additionally, cell cycle analysis was conducted by means of staining with propidium iodide, and an apoptosis assay was performed with Annexin V-FITC.

RESULTS GC-MS analysis revealed that VCO contains lauric acid (LA), caprylic acid, capric acid, myristic acid, palmitic acid, linoleic acid, oleic acid, and stearic acid. After treatment, VCO exhibited cytotoxic effects in HL-60 cells with IC₅₀ values between 269 and 300 µg/mL, and its activity was compared to commercial lauric acid (C-LA). Additionally, VCO was found to inhibit cell proliferation in a dose-dependent manner. Cell cycle analysis confirmed that VCO induced cell cycle arrest at the G1-phase. Furthermore, an apoptosis assay demonstrated that treatment with VCO induced apoptotic cell death in HL-60 cells.

CONCLUSION These findings suggest that the anti-leukemic effect of VCO is associated with cell cycle arrest and reduced cell viability through apoptosis.

KEYWORDS virgin coconut oil, human promyelocytic leukemia, HL-60 cells, anti-leukemic activity, cell cycle arrest, apoptosis

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INTRODUCTION

Cancer remains one of the leading causes of death among both males and females. In 2022, there were approximately 20 million new cases and 9.7 million cancer-associated deaths worldwide (1). Over the next two decades, the number of individuals diagnosed with cancer is expected to rise significantly. Leukemia is a type of cancerous neoplasm that is associated with the organs that produce blood cells and is characterized by an increase in the number of abnormal white blood cells (2). Leukemia has been linked to exposure to radiation, toxic chemicals, chemotherapy, viruses and other microorganisms, genetic disorders associated with chromosomal instability, cigarette smoking, and other environmental factors. These factors lead to changes in the genetic structure of leukemia cells, which, in turn, affect the function of other genes, including tumor suppressors and oncogenes. The disease spreads rapidly and can be classified as acute or chronic, myeloid or lymphoid (3). The forms of leukemia are those that impact the myeloid and lymphoid systems, including acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and chronic lymphocytic leukemia (CLL) (4).

This situation highlights the ongoing challenges in cancer management, particularly the lack of effective new drugs to reduce cancer incidence. Recently, plant-derived natural products have attracted considerable interest as potential agents for cancer treatment. Medicinal plants have been used therapeutically for thousands of years and represent a fundamental source of pharmacologically active compounds. The appeal of plant-derived compounds lies in their ability to enhance cancer treatment, often exhibiting synergistic effects when used together with conventional therapies. For example, green tea has been shown to modulate molecular pathways involved in inflammation, oxidative stress, and apoptosis in cancer cells (5). Similarly, curcumin, a bioactive compound from turmeric, has been widely reported to have diverse biological activities, including anti-inflammatory, antioxidant, and anti-cancer properties (6). In this study, we investigated virgin coconut oil (VCO), which is obtained from fresh coconut by a controlled temperature process without the use of chemical additives. This

method retains the bioactive compounds such as polyphenols, fatty acids, sterols, and tocopherols (7). VCO has been reported to exhibit antioxidant, anti-stress (8), anti-inflammatory (9), weight-reducing (10), anti-hypercholesterolemic (11), anti-dermatitic (12) and anti-cancer properties. In addition, VCO consumption during chemotherapy has been shown to improve the quality of life of breast cancer patients (13). However, few studies have examined the anti-cancer effects of VCO. Interestingly, Sheela et al. reported that lauric acid (LA), a major component of VCO, exhibited dose-dependent cytotoxicity in HCT-15 (human colon cancer), HepG2 (human hepatocellular carcinoma), and RAW 264.7 (murine macrophage) cells by inducing apoptotic morphological changes (14). Additionally, VCO has demonstrated anti-liver and oral cancer activity (15).

LA, a saturated medium-chain fatty acid (MCFA) which contains 12 carbon atoms and is the predominant fatty acid in coconut oil, has been linked to several reported health benefits associated with coconut oil consumption (16, 17). Fatty acids are acyclic carboxylic acids with aliphatic chains of varying lengths. Based on carbon chain length, they are categorized into three groups: short-chain fatty acids (approximately 6 carbon atoms), MCFAs (6-12 carbon atoms), and long-chain fatty acids (more than 12 carbon atoms) (18). Fatty acids serve as fundamental components of triacylglycerols, phospholipids, and other complex lipids, and constitute major contributors to dietary fat in humans (19). Previous studies have shown that fatty acids participate in multiple cellular processes, including signal transduction, regulation of gene transcription, metabolism, inflammation, apoptosis, and the synthesis of bioactive lipid mediators (20).

Apoptosis, or programmed cell death, is a specific set of morphological and biochemical changes including cell shrinkage, cytoplasmic condensation, chromatin condensation, DNA fragmentation, and plasma membrane blebbing, that are involved in maintaining tissue homeostasis by controlling cell populations, eliminating aged or damaged cells, and allowing the proliferation of new cells (21). However, carcinogens can interfere with apoptotic pathways, allowing uncontrolled cancer cell proliferation. In general, cell division is regulated by cell cycle control

mechanisms to produce an identical cell. The cell cycle contains 4 stages: gap 1 (G1) phase, synthesis (S) phase, gap 2 (G2) phase, and mitosis (M) phase, during which the cell grows, replicates its DNA, and prepares for mitosis, respectively. The mitotic cell cycle is typically divided into two major phases: the interphase and the M-phase. During the interphase, there will be duplicates of cellular content within the cells which are separated into two genetically identical daughter cells during mitosis. DNA replication occurs during the interphase, specifically in the S-phase. The interphase period separating the S-phase from the M-phase is called the gap phase, with the G1-phase preceding the S-phase and the G2-phase following the S-phase. Evidence suggests that there is a gap between the two main events, duplication and DNA dissociation. However, the steps occurring in the gap are important in the regulation of the cell cycle and involve important decisions, e.g., to enter the cell cycle in G1 and to initiate the process of chromosome division in G2. In unicellular and multicellular eukaryotes, cell division is regulated by a complex network of control, monitoring, and compensation mechanisms that ensure that no errors occur before cells can enter and complete the cell cycle for division. Check-points may delay the progression of the cell cycle or induce the end of the cell cycle or cell death in response to irreversible DNA damage (22). To date, no studies have investigated the antiproliferative, cell cycle arrest, or pro-apoptotic effects of VCO in HL-60 (human promyelocytic leukemia) cells. The present study is designed to evaluate the inhibitory effects of VCO on HL-60 leukemic cell proliferation through the induction of cell cycle arrest and apoptosis.

METHOD

Chemical materials

RPMI 1640 medium supplemented with 25 mM HEPES and L-glutamine, fetal bovine serum (FBS), penicillin/streptomycin, phosphate-buffered saline (PBS), and trypan blue dye solution were purchased from Cytiva HyClone™ (Logan, UT, USA). MTT dye and commercial lauric acid (C-LA) ($\geq 99\%$, GC/titration) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Test materials

Five 100% pure VCO samples were used. Sample No. 1 was a handmade VCO produced using traditional methods by local people. Mature coconuts, aged more than 12 months, were obtained from a coconut farm in Mae Taeng District, Chiang Mai Province, Thailand. Only coconuts that had not sprouted were selected, and their milk was removed from the coconut fruits. The traditional extraction process began with grating mature coconuts, followed by pressing the grated flesh in warm water to obtain coconut milk. The coconut milk was then placed in a clean bag and subsequently frozen at -29°C for 48 hours. After freezing, the solidified coconut milk was melted in sunlight at an ambient temperature of approximately 50°C for 1 hour and 30 minutes to facilitate phase separation into three layers: a water layer, a cream layer, and an oil layer. The oil layer was carefully collected in a clean container and subjected to mild steaming for 5 minutes to remove residual moisture, a step intended to prevent rancidity and improve storage stability. The VCO was then allowed to cool at room temperature before being transferred into sterilized bottles for storage (23). Samples No. 2-5 were obtained from a drugstore (Chiang Mai University Pharmacy, Chiang Mai, Thailand).

Cells and cell culture conditions

The human promyelocytic leukemia (HL-60) cell line (ATCC® CCL-240™) was purchased from ATCC. HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 25 mM HEPES, L-glutamine, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. The cells were grown in a 5% CO_2 humidified atmosphere in incubators maintained at 37°C .

Chemical fingerprinting and fatty acid analysis using gas chromatography-mass spectrometry (GC-MS)

The VCO samples (No. 1-5) were pre-processed as described below. Twenty milligrams of the VCO samples were mixed with 1 mL of methanolic NaOH. The samples were heated at 65°C for 15 minutes with intermediate shaking. After heating and shaking, the samples were cooled to room temperature for 5 minutes. Then, 1 mL of MilliQ water was added to the mixture, followed by 1 mL

of distilled hexane. The mixture was vortexed for phase separation. The clear upper hexane layer was gently withdrawn using a micropipette without disturbing the bottom layer. The hexane layer was transferred to a fresh GC vial and subjected to GC-MS analysis. The fatty acids were separated in a VF-5ms column using a Trace GC Ultra gas chromatograph under the following conditions. The samples were injected at an injection port maintained at 250°C. The samples were then transferred to the column through a split injection having a 50:1 split ratio. The initial oven temperature was set at 50°C, ramped up to 120°C, and then held for 5 minutes after which the temperature was increased to 170°C at a rate of 5°C/min and held for an additional 5 minutes. Subsequently, the temperature was ramped up to 230°C at the same rate and held there for 5 minutes. Finally, the oven temperature was raised to 250°C at 5°C/min and held for 2 minutes. The separated compounds were then injected into a TSQ Quantum XLS Mass spectrometer. The total ion chromatogram was recorded in a scan mode. Ions ranging from m/z 50 to 500 were recorded.

MTT cytotoxicity assay

The cytotoxicity of VCO No. 1 (handmade) and VCO No. 2-5 (commercially manufactured) was evaluated using the MTT assay. Briefly, HL-60 cells in the exponential phase of growth (5.0×10^4 cells/well) were cultured in 96-well plates containing 100 μ L of medium for 24 hours prior to treatment. After that, 100 μ L of fresh medium containing various concentrations (0-3,200 μ g/mL) of the VCO were added to each well and incubated for 48 hours at 37°C. The MTT dye solution was added (15 μ L/100 μ L medium), and the plates were then incubated at 37°C for 4 hours in a humidified 5% CO₂ atmosphere. Following that, 200 μ L of DMSO was added to each well and mixed thoroughly to dissolve the dye crystals. The absorbance was measured using an ELISA plate reader (Thermo Scientific, Multiskan SkyHigh, Singapore) at 540 nm with a reference wavelength of 630 nm. Untreated cells under similar conditions were used as a negative control. High optical density readings corresponded to a high intensity of dye color, indicating a high number of viable cells able to metabolize MTT salts. The fractional absorbance was calculated by the following formula:

$$\% \text{ Cell survival} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

The average cell survival obtained from triplicate determinations at each concentration was plotted as a dose-response curve. The experiment was performed in three independent replicates. The 50% inhibitory concentration (IC₅₀) of VCO was defined as the lowest concentration that reduced cell growth by 50% compared to untreated or vehicle control cultures (0.2% DMSO in culture medium). IC₅₀ values are presented as the mean $\pm$ standard deviation (SD) and were compared to assess their activities (24, 25). The IC₅₀ values (cytotoxic doses) of the VCOs were compared with those of C-LA in relation to their effects on cell cycle analysis and apoptosis assay.

Cell cycle analysis

HL-60 cells (7.5×10^5 cells/well) were seeded into 6-well plates and then treated with a vehicle control or varying concentrations (close to the IC₅₀ values) of VCO No. 1 (representing handmade VCO) and VCO No. 3 (representing commercial VCO, as indicated on the bottle label: "100% virgin coconut oil, high lauric acid, premium grade, and chemical-free"), compared to (close to the IC₅₀ value) C-LA for 24 hours (26). Following treatment, the cells were collected, washed twice with PBS, and fixed in cold (-20°C) 70% ethanol overnight. Ethanol was then carefully removed by centrifugation at $1,500 \times g$ for 3 minutes. The cells were resuspended in 1 mL of staining reagent containing 25 μ g/mL RNase (United States Biological, Salem, MA, USA) and 50 μ g/mL propidium iodide (PI) (Merck Millipore, Darmstadt, Germany) and incubated in the dark at room temperature for 30 minutes. Cell cycle distribution was subsequently analyzed by flow cytometry (Beckman Coulter DxFLEX, Brea, CA, USA).

Apoptosis detection via Annexin V-FITC/PI assay

Phosphatidylserine exposure on the outer membrane of apoptotic cells was assessed using an Annexin V-FITC apoptosis detection kit (Elabscience, Houston, TX, USA). Briefly, HL-60 cells (5×10^5 cells/well) were plated in 6-well plates and treated with vehicle control, VCO (No. 1 and No. 3), or C-LA at the indicated concentrations for 48 hours (27). The treated cells were harvested

by centrifugation at $1,500 \times g$ for 3 minutes at room temperature and washed twice with ice-cold PBS. Then, the cells were stained with Annexin V-FITC/PI according to the manufacturer's instructions and analyzed using flow cytometry.

Statistical analysis

All data are expressed as the mean $\pm$ SD from at least three independent experiments. The comparison between the treated and control groups was performed using Student's t-test. Differences were considered significant when the probability value obtained was found to be less than 0.05 ($p < 0.05$), highly significant if $^{**}p < 0.01$, and extremely significant if $^{***}p < 0.001$.

RESULTS

Chemical parameters of VCO fatty acid profiling of VCO samples

The GC-MS analysis revealed that VCO No. 1-5 contained several saturated fatty acids, along with detectable amounts of unsaturated fatty acids. Notably, all samples shared an identical composition, consisting of LA, caprylic acid, capric acid, myristic acid, palmitic acid, linoleic acid, oleic acid, and stearic acid. The corresponding total ion chromatograms (TICs) for each sample are presented in a previous study (28).

Cytotoxicity of VCO in HL-60 cell line

HL-60 cells were treated with various concentrations of VCO No. 1-5 for 48 hours, resulting

in a pronounced, dose-dependent reduction in cell viability, as shown in Figure 1A-E. The corresponding IC_{50} values for VCO No. 1-5 were 300.0, 285.7, 281.8, 269.6, and 276.2 $\mu g/mL$, respectively. In contrast, C-LA demonstrated substantially greater cytotoxicity at 48 hours, with an IC_{50} value of 20.8 $\mu g/mL$, as shown in Figure 1F. Collectively, these results indicate that although all VCO samples decreased HL-60 cell viability in a concentration-dependent manner, C-LA exhibited markedly higher potency under the experimental conditions.

VCO induces G1 phase arrest in HL-60 cells

To determine whether the anti-proliferative effect of VCO is associated with cell cycle regulation, HL-60 cells were treated with VCO No. 1 (handmade VCO), VCO No. 3 (commercial VCO), C-LA, or a control vehicle. Cell cycle distribution was analyzed by flow cytometry following PI staining. As shown in Figure 2A, treatment with 75, 150, and 250 $\mu g/mL$ VCO No. 1 for 24 hours resulted in a dose-dependent accumulation of cells in the G1 phase, increasing the G1 population from 46.07% in the control group to 47.88%, 49.15%, and 49.86%, respectively. These findings are supported by the quantification in Figure 2B. Similarly, VCO No. 3 enhanced G1-phase accumulation (Figure 3A), with G1 content increasing to 50.33%, 50.12%, and 50.57% at the corresponding concentrations, as shown in Figure 3B. In contrast, treatment with 20 $\mu g/mL$ C-LA significantly

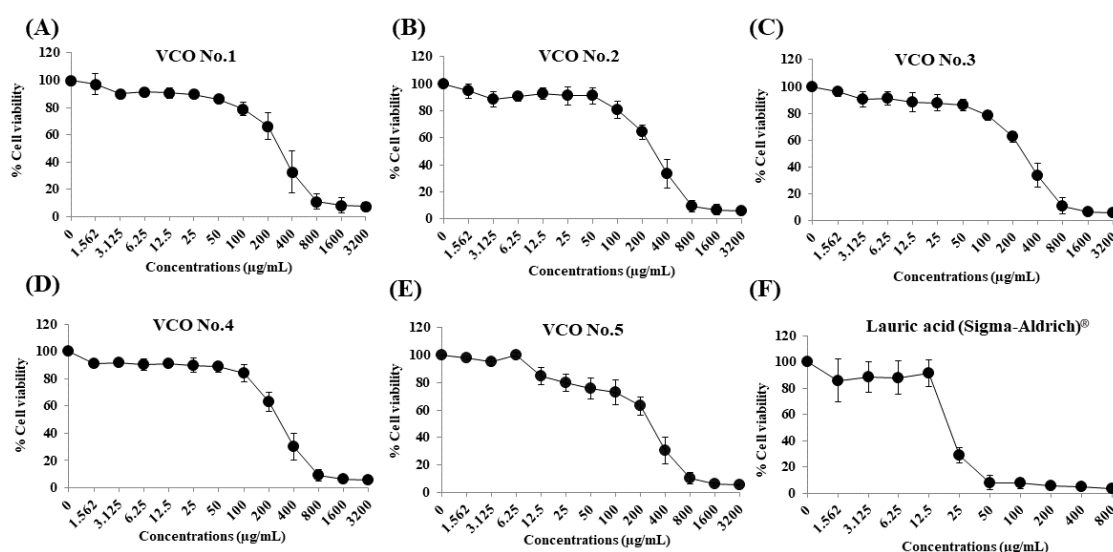


Figure 1. Cytotoxic effects of VCO No. 1-5 (A-E) and C-LA (F) on HL-60 cells. Cells were treated with various concentrations of each sample for 48 hours (VCO) or (C-LA), and cell viability was assessed using the MTT assay. Data are expressed as mean $\pm$ SD from three independent experiments.

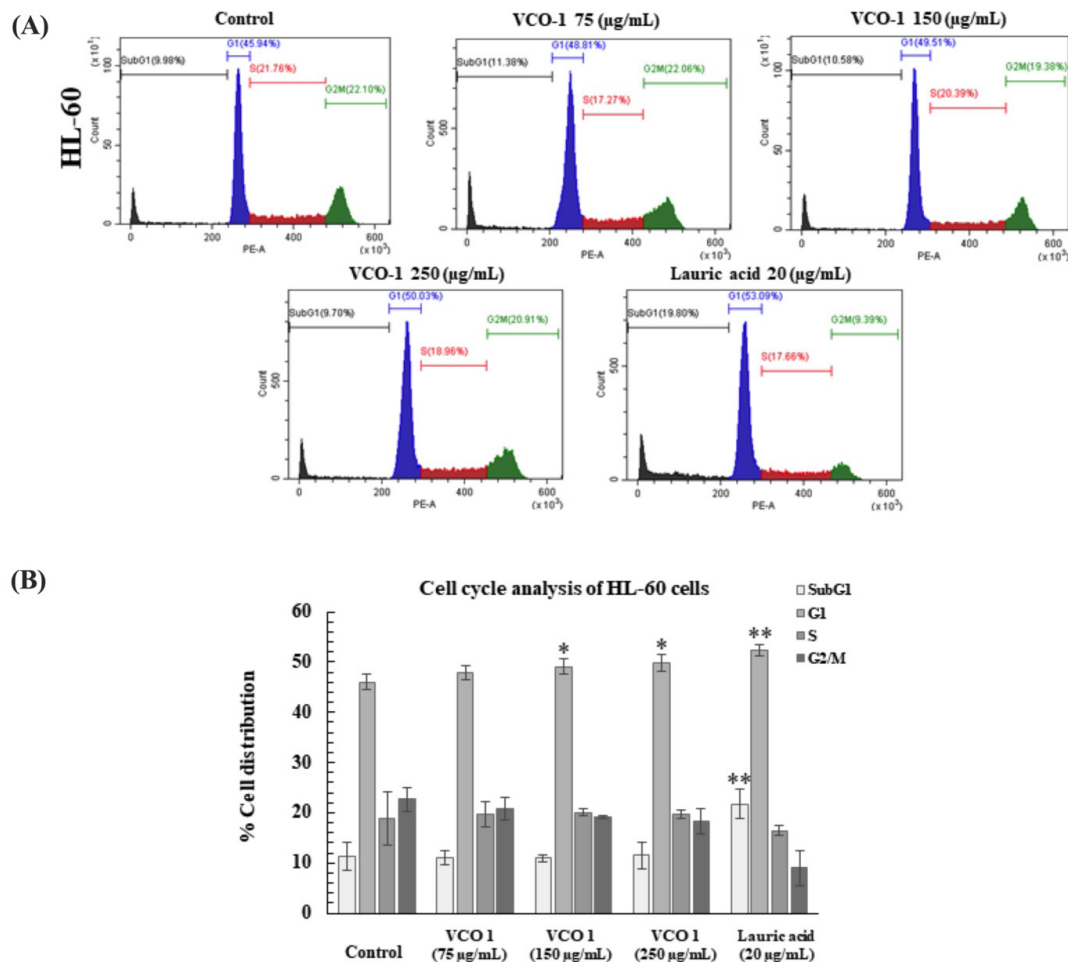


Figure 2. VCO No. 1 inhibits cell cycle progression in HL-60 cells. (A) Representative histograms of cell cycle distribution following 24 hours of treatment with VCO No. 1 or C-LA, stained with PI and analyzed by flow cytometry. (B) Quantitative analysis of cell cycle phases. Data are presented as mean $\pm$ SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. control.

increased the Sub-G1 population from 11.30% to 21.70% and the G1-phase content from 46.07% to 52.37%, suggesting apoptotic DNA fragmentation. Collectively, these findings demonstrate that both handmade (VCO No. 1) and commercial (VCO No. 3) VCO samples similarly induce G1-phase arrest in HL-60 cells, suggesting comparable cell cycle-modulating effects despite differences in preparation.

VCO induces apoptosis in HL-60 cells

To evaluate whether the anti-proliferative activity of VCO is associated with the induction of apoptosis, HL-60 cells were treated with VCO No. 1 and VCO No. 3 at concentrations of 150 and 250 $\mu\text{g/mL}$ for 48 hours. C-LA (20 $\mu\text{g/mL}$) and doxorubicin (0.5 μM), a chemotherapeutic agent known to induce apoptosis, were included as positive controls. Apoptotic cells were quantified

using Annexin V-FITC/PI staining followed by flow cytometric analysis. As shown in Figures 4A and 5A, treatment with both VCO No. 1 and No. 3 resulted in a modest, dose-dependent increase in total apoptotic cells, reaching 7.55% and 7.50%, respectively, at 250 $\mu\text{g/mL}$. In contrast, C-LA and doxorubicin markedly induced apoptosis, increasing the apoptotic population to 20.44% and 19.26%, respectively (Figures 4B and 5B). Taken together, these findings indicate that VCO No. 1 and VCO No. 3 can induce apoptosis in HL-60 cells, although to a lesser extent than established pro-apoptotic agents.

DISCUSSION

The purpose of this study was to investigate the effects of VCO on the human promyelocytic leukemia cell line HL-60. VCO is known to contain a high proportion of medium-chain saturated

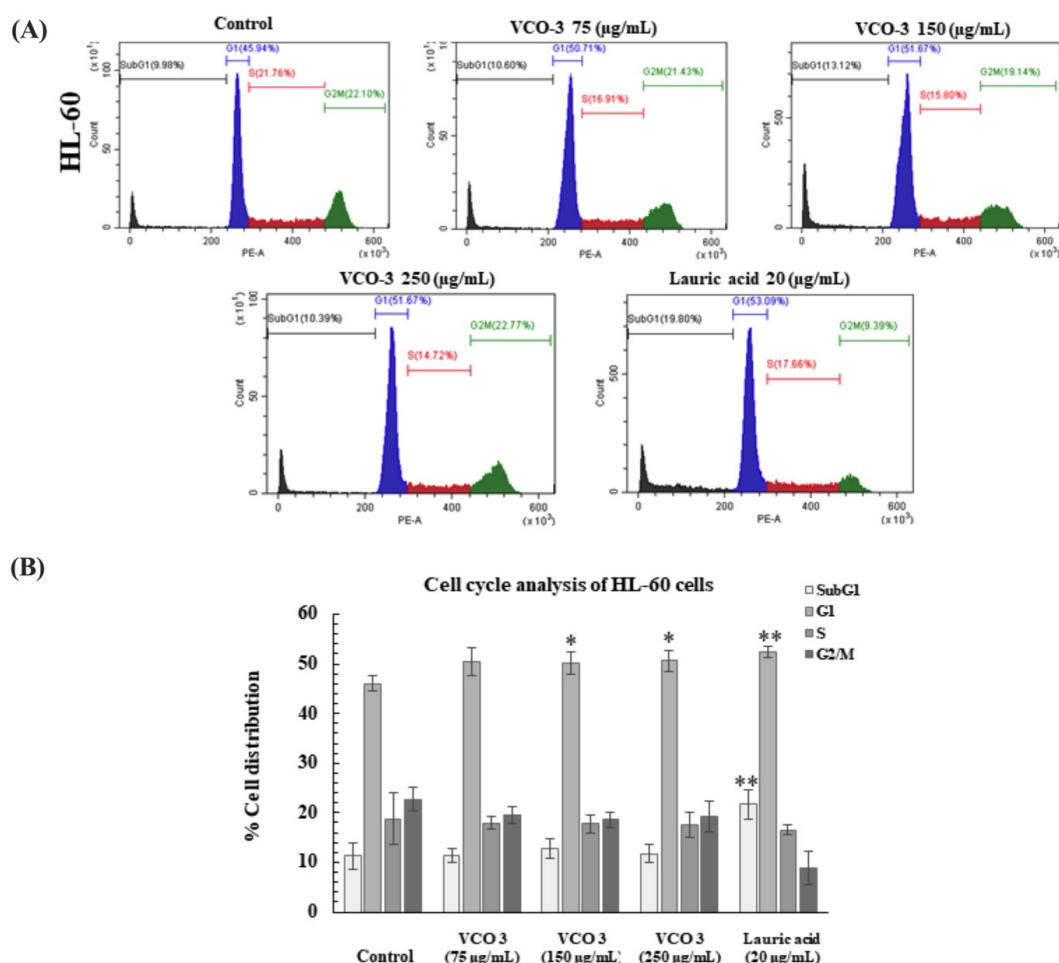


Figure 3. VCO No. 3 inhibits cell cycle progression in HL-60 cells. (A) Representative histograms of cell cycle distribution following 24 hours treatment with VCO No. 3 or C-LA, stained with PI and analyzed by flow cytometry. (B) Quantitative analysis of cell cycle phases. Data are presented as mean $\pm$ SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. control.

fatty acids (MCFAs), particularly LA, which typically accounts for a substantial portion of its fatty acid content (17). In the present study, GC-MS analysis revealed that all five VCO samples (VCO No. 1, handmade; VCO No. 2-5, commercial) contained LA, caprylic acid, capric acid, myristic acid, palmitic acid, linoleic acid, oleic acid, and stearic acid. To evaluate their anti-leukemic potential, HL-60 cells were treated with the VCO samples and subsequently assessed for changes in cell viability, cell cycle distribution, and apoptosis. Treatment with VCO (No. 1-5) significantly reduced cell viability compared with the untreated control, indicating cytotoxic activity against leukemic cells. These results are consistent with previous studies reporting that crude *Houttuynia cordata* (Plu-Kao) extract exhibits cytotoxic effects in HL-60 cells (29), and that LA induces dose-dependent cytotoxicity in various cancer cell lines, including colorectal (HCT-15), hepatocellular (HepG2), and

murine macrophage (RAW 264.7) cells (14). Based on the identical fatty acid profiles determined by chemical fingerprinting and gas chromatography-mass spectrometry, together with the comparable cytotoxic concentrations observed in the MTT assay, VCO No. 1 was selected as the representative handmade VCO. In contrast, VCO No. 3 was chosen as the representative commercial VCO, as indicated by its product labeling stating “100% virgin coconut oil, high lauric acid, premium grade, and chemical-free”. When comparing VCO No. 1 and VCO No. 3, the viability assay demonstrated that VCO No. 3 exhibited slightly higher cytotoxic activity than VCO No. 1. Collectively, these findings support the idea that the bioactive components of VCO, particularly LA, may contribute to its observed anti-proliferative effects in leukemia cells.

Leukemia treatment continues to face major challenges due to the adverse side effects, relapse,

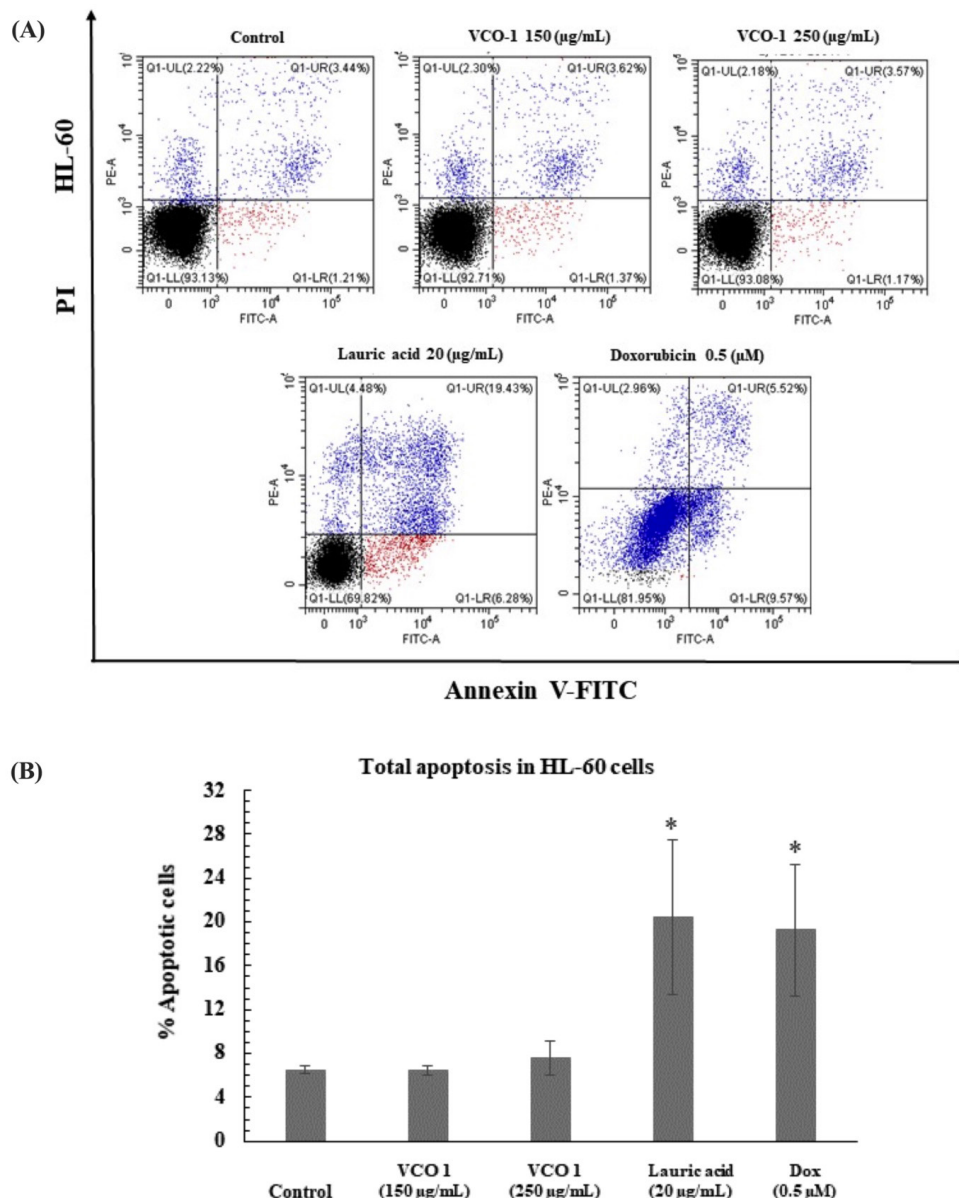


Figure 4. VCO No. 1 induces apoptosis in HL-60 cells. (A) Flow cytometric analysis of apoptosis in HL-60 cells treated with VCO No. 1 (150 and 250 µg/mL) for 48 hours, assessed using Annexin V-FITC/PI staining. Cells treated with 20 µg/mL C-LA and 0.5 µM doxorubicin were served as positive controls. (B) Quantification of total apoptotic cells. Data are presented as mean $\pm$ SD from at least three independent experiments. * $p < 0.05$ vs. control.

and drug resistance associated with conventional chemotherapeutic agents. Therefore, identification of alternative compounds with anti-leukemic potential remains an important objective in cancer therapy. In drug development, cell growth and viability assays are essential for determining the minimum effective concentration required to suppress cancer cell proliferation. In the present study, VCO demonstrated growth-inhibitory effects against HL-60 cells, as revealed by viability assays. Based on these findings, VCO No. 1 and No. 3 were selected for further analysis of cell cycle arrest and apoptosis. Both VCO No. 1 and

No. 3 induced G1-phase accumulation in HL-60 cells, with VCO No. 3 producing a slightly greater effect than VCO No. 1. C-LA also increased cell accumulation in both the Sub-G1 and G1-phases, indicative of apoptotic initiation. These results suggest that VCO inhibits leukemia cell proliferation, at least in part, by inducing G1-phase arrest and promoting apoptotic cell death. This conclusion is consistent with previous studies in colon cancer cells (Caco-2), where LA was reported to induce apoptosis and cell cycle arrest at multiple checkpoints, including G0/G1 and G2/M phases. Moreover, LA has been shown to elevate intra-

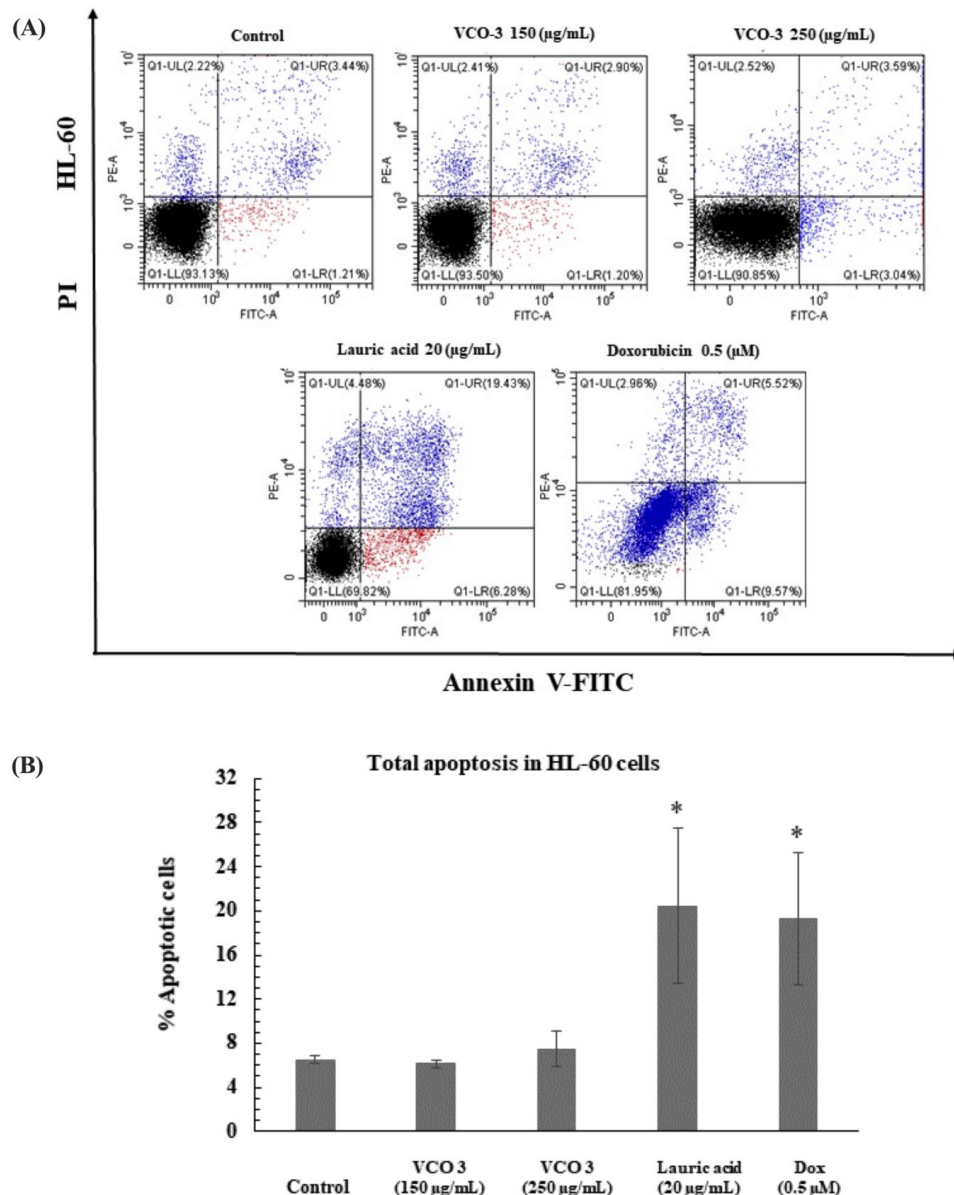


Figure 5. VCO No. 3 induces apoptosis in HL-60 cells. (A) Flow cytometric analysis of apoptosis in HL-60 cells treated with VCO No. 3 (150 and 250 µg/mL) for 48 hours, assessed using Annexin V-FITC/PI staining. Cells treated with 20 µg/mL C-LA and 0.5 µM doxorubicin were served as positive controls. (B) Quantification of total apoptotic cells. Data are presented as mean ± SD from at least three independent experiments. * $p < 0.05$ vs. control

cellular reactive oxygen species (ROS) and reduce glutathione levels, further contributing to its cytotoxic mechanisms (30, 31).

Apoptosis is a programmed cell death mechanism that plays a crucial role in maintaining tissue homeostasis. Through this process, damaged or abnormal cells are eliminated, allowing healthy cells to develop and maintain proper tissue architecture (21). Dysregulation of apoptotic pathways can lead to the persistence of defective cells, thereby contributing to malignant transformation, metastasis, and resistance to anti-cancer therapies (32). In the present study, the ability of VCO to induce

apoptosis in HL-60 cells is supported by its induction of G1-phase cell cycle arrest. Although Lappano et al. previously reported that LA can induce apoptosis in breast cancer cells, this study is the first to demonstrate the pro-apoptotic effects of VCO in HL-60 cells. In the apoptosis assay, treatment with VCO No. 1 and No. 3 led to a modest, dose-dependent increase in apoptotic cells. Both samples induced comparable levels of apoptosis, with VCO No. 1 exhibiting only a slightly higher apoptotic effect than VCO No. 3. In contrast, C-LA and doxorubicin significantly increased apoptotic cell populations in HL-60 cells. Taken together,

these findings indicate that VCO may function as an apoptosis inducer, as evidenced by the elevated apoptotic ratios observed following treatment in HL-60 cells.

These findings highlight the cytotoxic effects of VCO on HL-60 cells through the induction of cell cycle arrest and apoptosis, potentially attributable to their LA content. However, the effective concentrations of VCO (269–300 µg/mL) are substantially higher than in C-LA (20 µg/mL). Consequently, in vivo and clinical applications would necessitate administering VCO predominantly composed of medium-chain triglycerides (MCTs; C6–C12) at relatively high doses. This requirement is consistent with a previous study demonstrating the feasibility of high-dose MCT supplementation. For example, a modified diet containing 25% 8- and 10-carbon MCTs was shown to enhance the efficacy of anti-cancer therapy in a CD-1 Nu mouse model of neuroblastoma (33). Moreover, the clinical application of MCTs for their anti-cancer and therapeutic potential has been documented in several clinical studies. Notably, Schmidt et al. conducted a pilot clinical trial in patients with advanced metastatic tumors to evaluate a ketogenic diet enriched with MCTs. Patients who adhered to the three-month dietary regimen, consisting of an oil-protein mixture containing 21 g of MCT-rich fat, 5 g of carbohydrates, and 14 g of protein, exhibited marked improvements in physical well-being and evidence of tumor reduction, without adverse effects on cholesterol or blood lipid profiles (34).

The present study, together with previous evidence, indicates that VCO exerts inhibitory effects across the three major leukemia subtypes prevalent in Thailand, including chronic myeloid leukemia (CML; represented by K562), acute lymphoblastic leukemia (ALL; represented by MOLT-4) (28), and acute myeloid leukemia (AML; represented by HL-60). The consistent growth suppression observed among these cell lines suggests that VCO may target fundamental cellular processes shared across both myeloid and lymphoid leukemias. Mechanistically, the anti-proliferative effects appear to be mediated, at least in part, through the induction of cell cycle arrest and the activation of apoptotic pathways. Moreover, based on the viability assay, VCO exhibited the greatest cytotoxic potency against the

AML cell line (HL-60), followed by the CML cell line (K562) and the ALL cell line (MOLT-4), respectively (28). These results collectively support the potential of VCO or its bioactive constituents as a complementary therapeutic candidate for leukemia. Nonetheless, further comprehensive investigations are needed to elucidate the specific molecular mechanisms underlying these effects.

CONCLUSIONS

VCO contains both saturated and unsaturated fatty acids, including LA, caprylic, capric, myristic, palmitic, linoleic, oleic, and stearic acids. This study is the first to demonstrate that VCO exerts inhibitory effects on human promyelocytic leukemia HL-60 cells through the induction of G1-phase arrest and apoptosis. Altogether, these results indicate that VCO may possess anti-leukemic potential by modulating cell cycle progression and triggering apoptotic cell death. Moreover, due to its natural origin, VCO holds promise for integration into complementary or alternative therapeutic strategies. However, further in vivo studies and mechanistic investigations are required to confirm its efficacy and therapeutic potential.

ACKNOWLEDGEMENTS

We are grateful to Asst. Prof. Dr. Supachai Yodkeeree, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, for proof-reading the manuscript and providing valuable suggestions for its improvement.

FUNDING

This research received a grant from the Department of Medical Sciences, Ministry of Public Health, Thailand.

CONFLICTS OF INTEREST

There are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

AUTHOR CONTRIBUTIONS

S.S.: project administration, conceptualization, methodology, formal analysis, software, writing — original draft preparation, writing — review and editing; A.W.: formal analysis; N.D.: investigation; N.P.: funding acquisition.

DATA AVAILABILITY STATEMENT

The data that support the finding of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable, as this study did not involve human participants.

INFORMED CONSENT STATEMENT

Not applicable, as this study did not involve human participants.

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Exclusive Breastfeeding, Knowledge, and Attitudes Toward Breastfeeding of Mothers at a Tertiary Hospital in Thailand: A Cross-Sectional Study

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Received: September 11, 2024;

Revised: December 2, 2025;

Accepted: January 7, 2026

ABSTRACT

OBJECTIVE Breastfeeding offers numerous benefits for both mother and child. While many mothers attending follow-up visits at the Pediatrics outpatient center of a tertiary hospital in Thailand are still breastfeeding, the precise rate of exclusive breastfeeding (EBF) up to six months remains unclear. This study aims to assess the rate of 6-month EBF, maternal knowledge and attitudes toward breastfeeding, and risk factors for failure of EBF.

METHODS This cross-sectional study included Thai mothers with babies over six months old who had consulted with our breastfeeding center between September 1, 2020, and December 31, 2022. Participating mothers completed a questionnaire covering personal and obstetric information, past and current breastfeeding patterns, breastfeeding problems, breastfeeding knowledge (good score ≥ 12 out of 15), and attitudes (good score ≥ 40 out of 50). EBF rates and factors related to failure of non-exclusive breastfeeding were studied.

RESULTS The mean age of the 683 mothers in the study was 35.7 ± 3.5 years, with 62.8% being primiparous. Most babies were delivered full-term (98.2%). EBF was achieved in 87.7% of cases. The mean scores of knowledge and attitudes related to breast milk and breastfeeding were 14.5 ± 1.1 and 47.3 ± 2.5 , respectively. The majority were assessed as having good knowledge (97.4%) and positive attitudes toward breastfeeding (98.8%). Significant risk factors for failure of EBF included age ≥ 40 years, separated/divorced status, multiparity, working more than 8 hours/day, contraception use, breastfeeding problems, failure of EBF with a previous child as well as low EBF knowledge and poor EBF attitude scores. Multiparity, contraception use, and breastfeeding problems were significantly associated with EBF in multivariable analysis.

CONCLUSIONS This study showed a high rate of 6-month EBF. Most participating mothers had good knowledge and positive attitudes toward breastfeeding. Multiparity, contraceptive use, and breastfeeding problems were independent risk factors for failure to achieve EBF.

KEYWORDS attitude, breastfeeding, contraception, knowledge, parity

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INTRODUCTION

Breastfeeding has several benefits for both mother and child. Mothers who breastfeed have been found to have a lower incidence of many illnesses such as breast cancer, ovarian cancer, and diabetes mellitus (1). Infants and children who are breastfed and receive proper nutrition also have lower rates of sudden infant death syndrome, necrotizing enterocolitis, diarrhea, respiratory tract infections, otitis media, malnutrition, and demonstrate better growth and development (1, 2). Long-term benefits among children who were breastfed include better intelligence quotient (IQ), a higher likelihood of better education and income in adulthood, and a lower risk of obesity and diabetes mellitus (3).

The World Health Organization (WHO) and the United Nations International Children's Emergency Fund (UNICEF) recommend initiating breastfeeding within the first hour of life and practicing exclusive breastfeeding (EBF) for the first six months. Continued breastfeeding up to two years of age or beyond, with the addition of nutritious and age-appropriate complementary foods, is also recommended (4). However, the WHO reported in 2021 that the global rate of EBF was only 44.0% (1). Rates of EBF vary by country in different years, e.g., 44.9% in Laos in 2017, 62.1% in Nepal and 63.0% in Bangladesh in 2019, and 45.4% in Vietnam in 2021 (5-8).

In Thailand, the national breastfeeding promotion scheme was established in 1991 through the Baby-Friendly Hospital Initiative (BFHI) program (9). This program follows the "WHO Ten Steps to Successful Breastfeeding" (9). However, the Multiple Indicator Cluster Survey (MICS) in Thailand reported EBF rates of only 5.4% in 2006, 12.3% in 2012, 23.1% in 2016, 14.0% in 2021, and 29.0% in 2023 (10-14). These EBF rates are below the national target of 50.0% set by the 12th National Economic and Social Development Plan 2017-2021 of Thailand (15), as well as the global targets of 50.0% by 2025 and 70.0% by 2030 (4).

Many factors may influence the success of EBF in each country, including national policy, economic status, culture, and support from family members and healthcare providers. Even within the same country, such as Thailand, the specific areas where studies are conducted and the characteristic features of mothers and family

members in each study, e.g., working status, financial status and education as well as breastfeeding beliefs, knowledge, and attitudes, may also influence the success of EBF (16, 17).

Apart from these factors, the practices of health personnel in each hospital or region are another crucial foundation for successful breastfeeding. The hospital in this study adheres strictly to the "WHO Ten Steps to Successful Breastfeeding." It begins with one or more educational sessions about breastfeeding, one-to-one or small group classes, and video presentations for pregnant women. Close and individualized support is provided postpartum, with immediate and uninterrupted skin-to-skin contact after birth. Initiation of breastfeeding within one hour of delivery and every three hours thereafter is encouraged. Rooming-in is also standard practice unless contraindicated. These practices are followed regardless of the route of delivery. With a hospital policy supporting EBF, no formula milk products are displayed in-house. Formula feeding is allowed only if the mother or her infant has medical conditions that prohibit breastfeeding, or if the mother insists on this option after thorough counseling. Continual counseling about breastfeeding during follow-up visits is routinely practiced in the pediatric outpatient center. Any problems related to breastfeeding are identified and corrected appropriately.

Although most mothers who brought their babies for follow-up visits in the pediatric outpatient center at this hospital were still breastfeeding, their exact rate of EBF up to six months was uncertain. This study aimed to assess the rate of EBF, maternal knowledge about breast milk and breastfeeding, and attitudes toward breastfeeding. Maternal characteristics and related problems potentially associated with EBF failure were also studied.

METHODS

This study was approved by the Institutional Review Board (IRB) of MedPark Hospital, Bangkok (No. COA 002-2022E, approval date 30/03/2022). The cross-sectional study assessed the rate of 6-month EBF, maternal knowledge and attitudes toward breastfeeding, and risk factors for EBF failure. The study followed the Strengthening the Reporting of Observational Studies in

Epidemiology (STROBE) guidelines, which included the study design, setting, participants, variables, measurement, study size, and statistical methods (18). The study participants were Thai mothers who had delivered either at this hospital or elsewhere but who had consulted with the breastfeeding center at MedPark Hospital between September 1, 2020, and December 31, 2022. Consultations must have commenced within one month after delivery, and their babies must have been at least six months old at the time of the survey. We excluded mothers with health problems, those with babies who had health conditions prohibiting breastfeeding, and mothers who could not recall their breastfeeding patterns for the first six months after delivery.

Prior to the study, the questionnaires for parts three (knowledge) and four (attitudes) in the Thai version were constructed to fit with Thai culture and reviewed, discussed, and revised until consensus was reached among the researchers. The final set of questionnaires was then validated by three experts not involved in the study (one obstetrician and two neonatologists). Reliability was tested with 30 mothers having the same characteristics as the study participants. The reliability of the knowledge and attitude questionnaires was analyzed, yielding Cronbach's α of 0.82 and 0.91, respectively. Personal and obstetric data collected included age, marital status, parity, education, occupation, workplace, duration of daily work (hours), monthly family income, birthplace of the current child, route of delivery, gestational age, maternity leave, plans for the next child, contraception within six months postpartum, and breastfeeding patterns within six months after delivery of a previous child (if any) and the current child. Problems related to breastfeeding the current child were also recorded.

The maternal knowledge section comprised 15 statements about breastfeeding, breast milk, and formula milk, with answers categorized as "correct," "incorrect," or "not sure" (the latter was considered "incorrect"). The total knowledge score ranged from 0 (all incorrect) to 15 points (all correct). Maternal attitudes towards breastfeeding were assessed with ten statements, with each item scored from 1 (strongly disagree) to 5 (strongly agree). The total attitude score ranged from 10 (all strongly disagree) to 50 (all strongly agree).

Mothers who met the inclusion criteria were informed about the study through an information sheet and a verbal explanation provided by a research assistant. Participation in the study was voluntary, and mothers were assured that their decision would not affect their access to medical services. Mothers who consented to participate self-completed the questionnaire. Informed consent was waived as the questionnaire was self-administered. The questionnaire comprised four parts: personal and obstetrical data; breastfeeding patterns and related problems; knowledge about breastfeeding, breast milk, and formula milk; and attitudes toward breastfeeding. Participants could withdraw from the study at any point during the survey. Data collected from the questionnaire were processed in a de-identified and anonymous manner during analysis and presentation. All data were scheduled to be securely retained for a minimum of 5 years following the conclusion of the study, stored on a personal computer protected by an access code.

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 28.0 (IBM Corporation, Armonk, NY, USA). Descriptive statistics were used to investigate the characteristics of the study variables. Continuous variables are presented as mean $\pm$ standard deviation (SD) for normally distributed data or as median and range for non-normally distributed data. Categorical variables are presented as frequency and percentage. For comparison, data are grouped as follows: monthly family income $\geq$ 2,930 USD (the average familial monthly income in Thailand) or less; workplace as "mainly work from home" or "mainly office work"; duration of daily work as $\leq$ eight hours (usual daily work hours) or $>$ eight hours; route of delivery as "cesarean section" or "vaginal delivery"; birthplace of the current child as "in this hospital" or "elsewhere"; maternity leave as "yes" if mothers could leave work for at least one month (minimum duration of permission); and contraception within six months postpartum as "yes" or "no." For the primary outcomes, the breastfeeding pattern for either the previous or current child was grouped as "EBF" (only breast milk without other food, liquid, or water) or "non-EBF" (formula milk or mixed formula and breast milk); both knowledge and attitude scores were categorized as either "good" or "higher" based on

a total knowledge score ≥ 12 and a total attitude score ≥ 40 , respectively (approximately 80.0% of the total score of each, based on Bloom's cutoff point (19)).

A univariable analysis was performed to identify risk factors for EBF failure. Data were compared using the Chi-square test or using Fisher's exact test if 20.0% of expected cell counts were less than 5. Variables that were statistically significant in the univariable analysis were used to identify independent factors of EBF in the multivariable analysis. A p -value < 0.05 was considered statistically significant.

RESULTS

Basic characteristics

Mothers had to be able to recall their feeding patterns over the six-month period following the birth of the child. During the study period, 811 mothers consulted with the pediatric outpatient center. Of those, a total of 128 mothers were excluded because they had delivered elsewhere and did not consult with a breastfeeding center within one month postpartum ($n = 11$), were unable to breastfeed due to health problems ($n = 5$), or had babies less than six months old at the time of the study ($n = 112$).

A total of 683 mothers who met the inclusion criteria agreed to participate in this survey study. The mean age $\pm$ SD was 35.7 ± 3.48 years, with the majority (87.6%) aged under 40. Answers to some questions were incomplete, leading to varying numbers of respondents for each question. The basic characteristics of the mothers and the percentages of their responses to each question are shown in Table 1. Almost all participants (99.3%) were married and had a bachelor's degree or higher (97.7%). Slightly more than half were self-employed or owned a business (60.4%), and more than two-thirds had a monthly family income between 2,930 and 8,800 USD (72.9%). Nearly two-thirds (62.8%) had only one child. Less than half (45.4%) either worked or stayed at home. The mean $\pm$ SD daily work hours were 7.8 ± 2.5 hours. After excluding the mothers who had undergone tubal sterilization, 71.3% of the remainder did not practice contraception. The most common contraceptive method used was condoms (22.9%). We found that 13.8% planned to have their next child within an interval of 2.40 ± 0.85 years (mean $\pm$ SD). Among those who had a previous child, 74.3% had a history of EBF.

Obstetrical history and breastfeeding pattern

Respondents provided obstetrical data, birthplace, and postpartum maternal leave for the current child (Table 2). The mean $\pm$ SD gestational age was 38.1 ± 0.61 weeks, with only 1.8% having

Table 1. General characteristics of the mothers in a tertiary hospital in Thailand (N = 683)

Variables	n (%)
General characteristics	
Age (year)	
< 40	598 (87.6)
≥ 40	85 (12.4)
Marital status	
Married	678 (99.3)
Separated/divorced	5 (0.7)
Parity	
Primipara	429 (62.8)
Multipara	254 (37.2)
Education	
Higher than bachelor	130 (19.0)
Bachelor's degree	537 (78.7)
Lower than bachelor's degree	16 (2.3)
Occupation (n = 666)	
Employee	195 (29.3)
Self employed	402 (60.3)
Others (housewife or unemployed)	69 (10.4)
Monthly family income, USD ^a (n = 674)	
More than 8,800	158 (23.4)
Between 2,930-8,800	491 (72.9)
Less than 2,930	25 (3.7)
Workplace (n = 674)	
Work from home	306 (45.4)
Office	357 (53.0)
Both	11 (1.6)
Daily work hours (n = 610)	
≤ 8 hours	535 (87.7)
> 8 hours	75 (12.3)
Contraception (n = 618)	
No contraception or natural method	141 (22.9)
Condom	21 (3.4)
Pills	2 (0.3)
Condom & pills	10 (1.6)
Calendar method	3 (0.5)
Abstinence	11 (1.7)
Plan next child (n = 652)	
Tubal sterilization	90 (13.8)
Yes planned	551 (84.5)
No plan yet	162 (74.3)
History of breast feeding until 6 months in prior child (n = 218)	
Exclusive breastfeeding	4 (1.8)
Mixed breastfeeding and formula feeding	162 (74.3)
Formula feeding, with or without food supplements	52 (23.9)

^a1 USD approximate to 34.12 THB (20).

Table 2. Obstetrical history and pattern of breastfeeding at six months and problems related to breastfeeding of current child among mothers in a tertiary hospital, Thailand (N = 683)

Variables	n (%)
Gestational age at delivery (n = 569)	
< 37 weeks	10 (1.8)
≥ 37 weeks	559 (98.2)
Mode of delivery (n = 677)	
Vaginal delivery	57 (8.4)
Cesarean section	620 (91.6)
Birthplace (n = 682)	
The hospital of this study	606 (88.9)
Elsewhere	76 (11.1)
Maternity leave of at least 1 month (n = 120)	
Could	106 (88.3)
Could not	14 (11.7)
Pattern of breastfeeding	
Exclusive breastfeeding	599 (87.7)
Mixed breastfeeding plus formula feeding	74 (10.8)
Formula feeding, with or without food supplements	10 (1.5)
Breastfeeding problems	
No	569 (83.3)
Yes	114 (16.7)
Yes, Breastfeeding problems (n = 123) ^a	
No assistance/support	10 (8.1)
Breast issues	56 (45.5)
Inadequate breastmilk	48 (39.0)
Lump forming	3 (2.4)
Mastitis	3 (2.4)
Cracked nipple	1 (1.8)
Breast pain	1 (1.8)
Busy work	23 (18.7)
Fatigue	14 (11.4)
Others	20 (16.3)
Covid-19 infection	8 (6.5)
Food allergy	1 (0.8)
Twins	5 (4.1)
Pregnancy	5 (4.1)
Premature birth	1 (0.8)

^aMultiple responses

delivered preterm (< 37 weeks of gestational age). The majority who had delivery at this hospital (88.9%), had a cesarean section (91.6%), and had maternal leave for more than one month (88.3%).

We found that 87.7% of the mothers in the study had EBF up to six months, 10.8% used a combination of breastfeeding and formula feeding, and 1.5% used only formula feeding. Unfortunately, data regarding the duration of breastfeeding among those who did not exclusively breastfeed were unavailable for most participants. Regardless of their breastfeeding pattern, 113 out of 683

mothers (16.5%) reported experiencing more than one problem with breastfeeding. The two most common problems were breast issues (45.5%) and busy work schedules (18.7%). Notably, nine mothers stopped breastfeeding without providing any reasons. Table 2 shows the breastfeeding patterns in the first six months after delivery and the breastfeeding-related problems.

Knowledge and attitudes toward breastfeeding

Out of 15 questions assessing knowledge of breast milk and breastfeeding, the mean $\pm$ SD score was 14.5 ± 1.1 . Up to 97.4% of participants were classified as having good knowledge (score ≥ 12). More than 90.0% gave correct answers for almost all questions (Table 3). The only statement with a lower rate of correct answers (81.1%) was regarding the effect of breastfeeding during pregnancy.

For the ten items assessing attitude toward EBF, the mean $\pm$ SD score was 47.3 ± 2.5 . Most respondents (98.8%) were categorized as having a good attitude (score ≥ 40). Nearly 90% or more of the responses to most statements were “strongly agree.” The three statements with lower percentages of “strongly agree” (ranging from 40.0% to 70.0%) were related to the impact of breastfeeding on the couple’s sexual relationship, working outside the home, and the mother’s daily living (Table 4).

Factors with a negative impact on EBF for six months

We explored maternal characteristic features that could potentially impact breastfeeding patterns (Table 5). Univariate analysis identified significant factors associated with or risk factors for failure of EBF during the first six-months postpartum including age ≥ 40 years, separated/divorced status, multiparity, working over eight hours/day, using contraception, breastfeeding problems with the current child, a history of failure of EBF with a previous child, low EBF knowledge score, and low EBF attitude score. Multivariable analyses using the logistic regression method were performed using five factors: age, parity, daily working hours, contraception, and breastfeeding problems. The independent risk factors for non-EBF with the current child were multiparity, use of contraception, and breastfeeding problems. Of note, four statistically significant

Table 3. Mothers' knowledge of breast milk and breastfeeding in a tertiary hospital, Thailand (N = 683)

Variables	Correct n (%)	Incorrect n (%)
1. Breastmilk has benefits over infant formula regardless of time*	681 (99.7)	2 (0.3)
2. Breastmilk reduces chance of diabetes mellitus in mother and her child*	674 (98.7)	9 (1.3)
3. Breastmilk is less nutritious than infant formula	655 (95.9)	28 (4.1)
4. Water should be fed after breastfeeding to reduce infant oral candidiasis	674 (98.7)	9 (1.3)
5. Infant fed with fortified-infant formula has higher IQ than BF infant	678 (99.3)	5 (0.7)
6. The risk of contamination in infant formula is higher than that of breast milk*	670 (98.1)	13 (1.9)
7. Preserved breastmilk has lower value than infant formula	674 (98.7)	9 (1.3)
8. Breastmilk reduces risk of respiratory tract infection in infant*	677 (99.1)	6 (0.9)
9. Pregnant mother should stop breastfeeding to avoid uterine contraction	554 (81.1)	129 (18.9)
10. Prolonged breastfeed increases risk of osteoporosis	648 (94.9)	35 (5.1)
11. Mother with health problems should avoid breastfeeding	668 (97.8)	15 (2.2)
12. Infant who had breastfeeding at night has similar risk of dental caries as those with formula milk	669 (98.0)	14 (2.0)
13. Mother who resumes her work should start breast pumping after 1 month	637 (93.3)	46 (6.7)
14. Food supplement for infant should be given after 6 months of age*	678 (99.3)	5 (0.7)
15. Infant should be exclusively breastfed for the first 6 months*	676 (99.0)	7 (1.0)

*Referred to true statements[#]**Table 4.** Attitudes of mothers towards breastfeeding in a tertiary hospital, Thailand (N = 683)

Variables	Level of agreement				
	Strongly disagree n (%)	Disagree n (%)	Neutral n (%)	Agree n (%)	Strongly agree n (%)
1. It is the responsibility of mother to breastfeed	8 (1.2)	5 (0.7)	15 (2.2)	62 (9.1)	593 (86.8)
2. Breastfeeding is a means of bonding between mother and child	1 (0.1)	-	-	5 (0.7)	677 (99.1)
3. Breastfeeding enhances the confidence in potentiality and ability of a mother	2 (0.3)	2 (0.3)	11 (1.6)	41 (6.0)	627 (91.8)
4. Breastfeeding does not bother sexual relationship of the couples	4 (0.6)	25 (3.7)	193 (28.2)	165 (24.2)	296 (43.3)
5. Breastfeeding has no negative impact on working outside home	1 (0.1)	13 (1.9)	152 (22.2)	232 (34.0)	285 (41.7)
6. Breastfeeding does not bother daily living of mother	2 (0.3)	11 (1.6)	48 (7.1)	145 (21.2)	477 (69.8)
7. Breastfeeding has health benefits to both mother and child	-	1 (0.1)	2 (0.3)	49 (7.2)	631 (92.4)
8. Breastmilk has complete nutrition for infant	-	1 (0.1)	1 (0.1)	11 (1.6)	670 (98.1)
9. Breastmilk enhances brain growth and intellectual development of infant	-	1 (0.1)	1 (0.1)	14 (2.0)	667 (97.7)
10. Breastfeeding saves the family expenses	-	1 (0.1)	3 (0.4)	10 (1.5)	669 (98.0)

*Referred to true statements[#]

features from the univariable analysis were not included in the multivariable analysis because of statistical issues: small numbers in the comparative subgroups (marital status, history of EBF for six months with a previous child, knowledge, attitude toward EBF) and correlation with parity (history of EBF for six months with a previous child).

DISCUSSION

The achievement of an 88.0% EBF rate for six months in this study is notably high compared with the Thailand's national target goal of 50% by 2021 (15) and the WHO global target goal of 70% by 2030 (4). This rate also surpasses EBF figures reported from various countries (5-8, 21-24). The success of this hospital-based program in meeting and exceeding these targets can be

Table 5. Features associated with exclusive breastfeeding or as risk factors for non-exclusive breastfeeding in a tertiary hospital, Thailand (N = 683)

Variables	n	Exclusive breastfeeding		Crude OR (95% CI)	p	Adjusted OR (95% CI)	p
		Yes n (%)	No n (%)				
Age (year)							
< 40	599	530 (88.6)	68 (11.4)	1.00	-	1.00	-
≥ 40	85	69 (81.2)	16 (18.8)	1.81 (0.99-3.29)	0.050*	1.58 (0.78-3.21)	0.202
Marital status							
Married	678	598 (88.2)	80 (11.8)	1.00	-	-	-
Separate/divorced	5	1 (20.0)	4 (80.0)	29.90 (3.30-270.85)	< 0.001**	-	-
Occupation (N = 666)							
Self-employed	471	415 (88.1)	56 (11.9)	1.00	-	-	-
Employee	195	170 (87.2)	25 (12.8)	1.09 (0.66-1.80)	0.738*	-	-
Parity							
Primipara	429	393 (91.6)	36 (8.4)	1.00	-	1.00	-
Multipara	254	206 (81.1)	48 (18.9)	2.54 (1.60-4.05)	< 0.001*	2.08 (1.23-3.53)	0.006
Education							
> Bachelor	667	585 (87.7)	82 (12.3)	1.00	-	-	-
< Lower	16	14 (87.5)	2 (12.5)	1.02 (0.23-4.57)	1.000**	-	-
Family income, USD ^b (n = 674)							
≥ 2,930 USD	649	569 (87.7)	80 (12.3)	1.00	-	-	-
< 2,930 USD	25	21 (84.0)	4 (16.0)	1.36 (0.45-4.05)	0.538**	-	-
Workplace (n = 674)							
Work at home	306	270 (88.2)	36 (11.8)	1.00	-	-	-
Office or both	368	320 (87.0)	48 (13.0)	1.13 (0.71-1.79)	0.617*	-	-
Workhours/day (n = 610)							
≤ 8 hours	535	478 (89.3)	57 (10.7)	1.00	-	1.00	-
> 8 hours	75	59 (78.7)	16 (21.3)	2.27 (1.23-4.22)	0.008*	1.89 (0.97-3.71)	0.063
Birthplace (n = 682)							
MedPark hospital	606	534 (88.1)	72 (11.9)	1.00	-	-	-
Elsewhere	76	65 (85.5)	11 (14.5)	1.26 (0.63-2.49)	0.515*	-	-
Route of delivery (n = 677)							
Normal labor	57	46 (80.7)	11 (19.3)	1.00	-	-	-
Cesarean section	620	547 (88.2)	73 (11.8)	0.56 (0.28-1.13)	0.099*	-	-
Maternal leave (n = 120)							
Cannot	14	12 (85.7)	2 (14.3)	1.00	-	-	-
Can	106	84 (79.2)	22 (20.8)	1.57 (0.33-7.54)	0.733**	-	-
Contraception (n = 652)							
No	454	408 (89.9)	46 (10.1)	1.00	-	1.00	-
Yes	198	163 (82.3)	35 (17.7)	1.91 (1.18-3.06)	0.007*	1.76 (1.01-3.09)	0.048
Plan for next child (n = 652)							
Yes	90	83 (92.2)	7 (7.8)	1.00	-	-	-
No	562	488 (86.8)	74 (13.2)	1.80 (0.80 - 4.04)	0.150*	-	-
Breast feeding problems							
No	569	520 (91.4)	49 (8.6)	1.00	-	1.00	-
Yes	114	79 (69.3)	35 (30.7)	4.70 (2.87-7.71)	< 0.001*	4.13 (2.35-7.28)	< 0.001
Pattern of breast feeding in prior child (n = 218)							
Exclusive	162	153 (94.4)	9 (5.6)	1.00	-	NA ^a	-
Non-exclusive	56	25 (44.6)	31 (55.4)	21.08 (8.97-49.52)	< 0.001*	-	-
Knowledge							
Score ≥ 12	665	587 (88.3)	78 (11.7)	1.00	-	NA ^a	-
Less than 12	18	12 (66.7)	6 (33.3)	3.76 (1.37-10.31)	0.016**	-	-
Attitude							
Score ≥ 40	675	595 (88.1)	80 (11.9)	1.00	-	NA ^a	-
Less than 40	8	4 (50.0)	4 (50.0)	7.44 (1.82-30.32)	0.010*	-	-

^aNA, not appropriate for multivariable analysis due to statistical issues (small number of certain subgroups); ^b1 USD approximate to 34.12 THB (20); *p-value by Chi-square test; **p-value by Fisher's exact test

attributed to several key factors, including effective hospital practices, maternal characteristics, and support systems.

The rates of EBF vary depending on several factors across different levels and aspects. National or cultural backgrounds, as well as overall economic status, are important considerations when setting targets, policies, and achievement rates for EBF in each country. In developing countries, EBF rates range from 45.0% to 63.0% (5-8), whereas in developed countries, the rates are much lower, ranging from 1% to 24.9% (25, 26). In developing countries, a lower economic status often makes breast milk a crucial source of nutrition for children. Even within the same country, factors such as geographic location, population size, and characteristics of the population can also impact the rate of EBF.

For example, a study from Jiaying, Zhejiang province, China, found a 37.0% rate of EBF among 494 mothers (23). Another national cross-sectional survey in the same country, which included over 5,000 infants, reported a 30.0% EBF rate (21). Another community-based study among mothers working in the informal sector in Kampala, Uganda reported an EBF prevalence of 42.8% (27). In Thailand, two studies reported six-month EBF rates of 37.4% among women recruited from three public provincial hospitals during the COVID-19 pandemic (28) and 66.0% among 65 employees in the hospital in Bangkok (29). The differences in these rates are likely due to variations in participant characteristics and their living and/or working environments. For instance, a study conducted in three public hospitals in areas affected by COVID-19 outside major cities which collected data from unemployed mothers or those working from home reported a 37.0% EBF rate (28). This was considerably lower than the 66.0% rate found among 65 health workers in a hospital located in the Bangkok Metropolitan area (29).

The high rate of EBF for six months observed in this study can likely be attributed to a combination of factors, including clinical practices related to breastfeeding and the characteristics of the mothers themselves. Several studies have shown that support from healthcare professionals can significantly influence a mother's decision to breastfeed or bottle-feed (30, 31). Various hospital

features and healthcare providers' health-related practices likely contributed to the high EBF rate in this study. Healthcare professionals' support and attitudes can influence maternal infant-feeding decisions (32). Our hospital's breastfeeding policy (30) aligns with the WHO Ten Steps to Successful Breastfeeding (9). The advocacy and practice of our health personnel, including the perinatologist, neonatologist, pediatrician, and nurses, also play a crucial role. Furthermore, being a private hospital with an optimal ratio of physicians to patients and an adequate number of health personnel at all levels allows provision of a range of services, such as counseling and education in various settings, immediate postpartum care for both mothers and babies and ongoing support in the pediatric wards and outpatient centers.

Maternal characteristics and socioeconomic features are also key factors in the success of EBF. Many previous studies have reported the mothers' high socioeconomic status as one favorable factor for EBF achievement (21, 23, 29). Most mothers in this study had a high socioeconomic status (Table 1) and should have had greater resources, contributing to the high rate of EBF in this study. Other favorable features of the mothers identified from univariate analysis were younger age, being married, primiparity, working $\leq$ eight hours a day, not using contraception, a history of successful EBF with a previous child, no breastfeeding problems, good knowledge of and a good attitude toward EBF.

On the other hand, we would like to emphasize significant unfavorable or risk factors for non-achievement of EBF. Using univariate analysis, separated/divorced status showed the highest risk for non-EBF (odds ratio (OR) = 29.90) followed by a history of non-EBF with a previous child (OR = 21.08), low attitude score (OR = 7.44), breastfeeding problems (OR = 4.70), low knowledge score (OR = 3.76), multiparity (OR = 2.54), working > 8 hours/day (OR = 2.27), using contraception (OR = 1.91), and age $\geq$ 40 years (OR = 1.81). These risk factors for non-achievement identified in this study have also been reported in many previous studies (21-23, 33-37). Separated/divorced status was found to have the highest risk for non-EBF in our study. These women may struggle to raise their baby by themselves without physical and/or psychological support from

their spouse, leading to an earlier stop of EBF (23, 33). However, this could not be confirmed as an independent risk factor due to the small number of separated/divorced women in this study which precluded multivariable analysis.

Other risk factors that were statistically significant for non-EBF in our univariate analysis were a history of non-EBF with a previous child as well as low scores on attitude toward and knowledge of EBF. These factors may be linked to the mother's personality, which can influence her attitudes and knowledge regarding breastfeeding practices. Previous studies have reported that the history of EBF with a previous child, as well as good knowledge of and good attitude toward EBF, have a positive influence on successful EBF (21, 22, 36). Again, in this study the limited number of participants having non-EBF with a previous child and the low number displaying poor attitudes or low knowledge scores hindered our ability to conduct further in-depth analysis to establish these as independent risk factors for non-EBF.

The associations between non-EBF and long working hours (> 8 hours), which was statistically significant ($p = 0.008$) and with mothers aged 40 years or older, which was only marginally significant ($p = 0.050$), can likely be attributed to the fact that mothers may experience exhaustion and fatigue due to long working hours, as well as potential health issues or fatigue among older mothers. However, other influencing factors may have rendered these two features not statistically significant in multi-variate analyses.

This study, using multivariable analysis, identified only 3 features as independent risk factors for non-EBF: breastfeeding problems (aOR = 4.13), multiparity (aOR = 2.08), and use of contraception (aOR = 1.76). The risk of breastfeeding problems and multiparity for non-EBF identified in this study are consistent with many previous reports (29, 34, 36, 38, 39). Breast issues were the most common problem reported in this study. Some of these issues are likely to have been detected early by immediate self-awareness and could be remedied by medical care, so the mothers could continue breastfeeding (35, 38, 39). However, other issues were likely more difficult to overcome, discouraging the mothers from continuing breastfeeding. This study did not explore in detail whether the reported problems could poten-

tially have been resolved. The second important problem for breastfeeding in this study was 'being busy with work.' Other studies also reported being busy with work or unfavorable workplace environments, e.g., inconvenient uniforms, inadequate private location or equipment, and workload, were barriers to successful EBF (31, 36). Although most mothers in this study may not have had problems similar to those reported in previous studies, their relatively high socioeconomic background could have been associated with high responsibility and being busy, especially business owners.

Multiparity was found to be an independent risk factor in this study, probably due to the time spent caring for the elder siblings. Previous studies have had inconsistent findings regarding the influence of parity (21, 23). One found multiparity tended to be a risk for non-EBF (21), whereas the others reported primiparous status was associated with non-EBF (23). The impact of multiparity remains to be confirmed in future studies. Regarding the use of contraception, some authors have reported that combined hormonal oral contraceptives could reduce the volume of breast milk (40). We attempted to assess the possible effect of the hormonal contraceptive method (among 23 mothers), but we did not find any association with non-EBF (data not shown).

Although not significant risk factors for non-EBF, we would like to relate two features of interest: the site (hospital) and the delivery route. The mothers who had delivered elsewhere had higher non-EBF, 14.5%, compared to 11.9% of mothers who delivered in the hospital of this study. We propose that our continual promotion and support in the pediatrics outpatient center may have bolstered the use of EBF by mothers who had delivered elsewhere and had received inadequate antepartum and immediate postpartum coaching, making this feature not statistically significant. Regarding the negative impact of cesarean section, previous studies have reported a lower rate of EBF among mothers who had cesarean section compared to normal delivery due to postoperative pain, stress, and the use of general anesthesia which could hinder maternal-newborn contact and delay the initiation of breastfeeding (21, 41, 42). Our study did not encounter this problem, probably because the study

hospital used regional block instead of general anesthesia, so immediate latching could be promoted.

We are aware of some limitations in this study. First, the study population was mothers who had high socioeconomic status. Second, the participants were mothers who had received medical services in a breastfeeding clinic. These may have introduced a selective bias due to their higher knowledge and better attitudes toward EBF than other mothers, and may limit the generalization of the findings to other populations. Third, our survey study was conducted during the COVID-19 pandemic. There were many unpredictable factors which could potentially have affected each individual and their surrounding situations. For example, the policy of “working from home” instead of going to the office should positively affect the rate of EBF. On the other hand, an extra precaution against COVID-19 transmission, especially among family members in the same household, may have had a negative impact on the rate of EBF due to a lack of support for the mother. Fourth, all statements assessing the attitude of mothers towards breastfeeding were positive. This was intended to estimate the extent of agreement of the respondents with those statements. This might, however, have led to a response bias. Future studies should be cautious regarding this issue. Fifth, the duration of EBF among those who could not achieve EBF was unavailable. Knowledge of this time period is certainly important for problem-solving and/or intervention. Future studies may focus on this issue.

Our study had notable strengths. First, the number of participants was quite large compared to previous reports. Although the majority of the participants had high socioeconomic status and good knowledge of and attitudes toward EBF (partly from having received medical service in a breastfeeding clinic), other characteristic features, especially the ones that were associated with non-EBF, could be informative to the other health practitioners in this field. Second, we designed the questionnaire to capture various aspects of both the mothers and their babies, with the aim of exploring potential obstacles to successful EBF. The knowledge and attitude components and potential factors influencing breastfeeding were carefully designed and validated by experts in the field before the study commenced.

Although conducted in a single hospital where the participants may have had different characteristics than other populations, our hospital practices which contributed to a high rate of EBF as well as other factors that helped reduce the risk of failure for EBF, should be a lesson-learned for other hospitals and health units in the area. For example, it is crucial to identify mothers at risk of EBF failure during pregnancy and the early postpartum period. In those cases, various supportive activities should be implemented, such as encouraging mothers and their families, promoting group support led by successful breastfeeding mothers, providing continuing support from physicians and other healthcare providers, and creating “breastfeeding-friendly” environments.

CONCLUSIONS

This study demonstrated that 88% of mothers used EBF for the first six months. Significant risk factors for the non-achievement of EBF were a history of non-EBF for the first six months with a previous child, separated/divorced status, low EBF attitude scores, problems with breastfeeding the current child, low knowledge scores, long working hours, use of contraception, and older age. The independent risk factors were multiparity, use of contraception, and breastfeeding problems.

ACKNOWLEDGMENTS

The study was supported by the MedPark Hospital Research Center. The authors thank the colleagues in the Pediatrics and Women Health Centers for providing general administrative support.

FUNDING

MedPark Hospital Research Funds supported the study.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

S.U.: conceptualization, methodology, data curation, investigation, and writing – review & editing; K.L.: conceptualization, methodology, investigation, and writing – review & editing; S.T.: conceptualization, methodology, formal analysis,

data curation, funding acquisition, writing – original draft, and writing – review & editing; W.K.: conceptualization, methodology, data curation, writing – original draft, and writing – review & editing; N.A.: formal analysis, data curation, and writing – review & editing; C.P.: conceptualization, methodology, investigation, and writing – review & editing.

All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

DATA AVAILABILITY

Data are available upon a request to corresponding author.

INSTITUTIONAL REVIEW BOARD STATEMENT

This study was approved by the Institutional Review Board of MedPark Hospital (No. COA 002-2022E, approval date 30/03/2022).

INFORMED CONSENT STATEMENT

Informed consent was waived by the institutional policy and nature of the study wherein data were obtained from the self-administered questionnaire.

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Prevalence of Neurological Abnormalities from Neuroimaging in Acute Acquired Comitant Esotropia

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Received: December 10, 2025;
Revised: January 18, 2026;
Accepted: January 26, 2026

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ABSTRACT

OBJECTIVE This study aimed to determine the prevalence and characteristics of patients with acute acquired comitant esotropia (AACE) who had abnormal findings on neuroimaging.

METHODS A retrospective review of data from patients diagnosed with AACE who had an onset age of greater than six months, cycloplegic refraction of less than +3.00 spherical diopters, and had undergone neuroimaging between 2014 and 2024 was conducted.

RESULTS A total of 45 patients, 28 males (62.2%) were included in the study. The overall mean age was 10.2 years, and the mean angle of deviation was 36.11 prism diopters (PD) for distance and 36.1 PD for near. Of the 45 patients, 43 (95.6%) had normal neuroimaging and 2 (4.4%) had abnormal neuroimaging. Abnormal findings consisted of a small vascular anomaly in one patient and an arachnoid cyst in another. The mean age at onset was 5.5 years in the abnormal neuroimaging group and 8.8 years in the normal neuroimaging group ($p = 0.46$). The mean near angle of deviation was 27.5 PD versus 36.8 PD ($p = 0.21$) in patients with abnormal and normal neuroimaging, respectively. The mean distance deviation was 25 PD and 36.9 PD ($p = 0.19$) in the abnormal and the normal neuroimaging group, respectively. No patients demonstrated associated abnormal neurological symptoms or signs.

CONCLUSIONS No predictive factors associated with abnormal neurological findings were identified as statistically significant. Despite the infrequency of abnormal neuroimaging findings in AACE and the absence of identified predictive factors, clinicians should remain aware of the possibility of underlying brain abnormalities.

KEYWORDS strabismus, esotropia, acute acquired comitant esotropia, neurologic manifestations, neuroimaging

INTRODUCTION

Acute acquired comitant esotropia (AACE) is characterized by an acute onset of esodeviation without any limitation in ocular motility. It is essential to distinguish AACE from infantile and

accommodative esotropia (1). AACE is a relatively rare condition, affecting approximately 0.3% of pediatric strabismus cases (2, 3). The etiologies of AACE have been reported to include functional, systemic, and intracranial diseases. Recently,

an increased prevalence of functional AACE has been noted, which is believed to be linked to excessive near work associated with the use of digital devices (4), particularly during the COVID-19 pandemic (5-7).

AACE is classified into three types (8). Type I (Swan type) is attributed to loss of fusion commonly triggered by monocular occlusion. Type II (Burian-Franceschetti type) refers to idiopathic AACE, often precipitated by physical or psychological stress with minimal hyperopia of less than +3.00 diopters (D). Type III (Bielschowsky type) is associated with myopia. Some researchers classify AACE into seven types (2), which includes a neurological AACE subtype associated with intracranial pathologies.

Additionally, many studies have found that AACE is associated with neurological pathologies that require neuroimaging. Some reports suggest that the risk of neurological disease is comparable in both comitant and incomitant esotropia (9).

Various neurological diseases have been associated with AACE, including medulloblastoma, astrocytoma of the corpus callosum and cerebellum, pontine glioma, hydrocephalus, cerebellar ataxia, Arnold-Chiari malformation, idiopathic intracranial hypertension, myasthenia gravis, and thalamic lesions (10). Therefore, some researchers emphasize that intracranial issues may contribute to AACE and recommend using MRI as a diagnostic screening tool (6, 11, 12).

Some studies have suggested the characteristics of AACE that may indicate underlying neurological diseases may assist physicians in determining when to prioritize neurological evaluations and consider brain magnetic resonance imaging (MRI). Intracranial disease associated with AACE has been linked to factors such as A or V pattern esotropia, greater esodeviation at distance, recurrence of AACE, nystagmus, and abnormal ocular or neurological signs (e.g., headache, cerebellar imbalance, weakness, or papilledema) (2, 10, 13, 14). However, many studies report that coexisting or underlying neurological diseases were not found in patients with isolated AACE, suggesting that neuroimaging may not be necessary (1, 15, 16). This has resulted in a controversial debate on whether or not MRI is necessary for children with AACE. In light of this uncertainty, some clinicians routinely obtain neuroimaging in all

patients diagnosed with AACE to assure patient safety, whereas others reserve imaging for high-risk patients or those with abnormal neurological symptoms or signs.

Currently, there is no consensus on guidelines for considering further neuroimaging in AACE. Physicians must weigh the likelihood of detecting abnormal results against the risks of radiation exposure, the potential adverse effects of general anesthesia in uncooperative patients, and cost-effectiveness, particularly in public health settings.

Although many studies have been performed in Caucasian populations (17-20), the number of studies has increased in Asian populations (5, 21-24). Racial differences may affect the prevalence and characteristics of patients with abnormal neurological conditions associated with AACE. The higher prevalence of myopia in Asian populations may also be a relevant factor.

Considering the limited data on the prevalence and characteristics of neurological AACE in Asian populations and the lack of consensus on neuroimaging indications, understanding the prevalence of abnormal neurological findings and the characteristics of ocular manifestations, along with associated systemic signs and symptoms in patients in Asian populations with AACE who have neurological pathologies could guide physicians in making decisions regarding further investigation.

METHODS

The data were collected using International Classification of Diseases (ICD)-10 codes for patients diagnosed with convergent comitant strabismus (H50.0), intermittent heterotropia (H50.3), unspecified strabismus (H50.9), and diplopia (H53.2) at Chiang Mai University (CMU) hospital between June 2014 and June 2024.

The inclusion criteria were age at onset greater than six months, cycloplegic refraction of less than +3.00 spherical equivalent, and having undergone brain MRI or computed tomography (CT) interpreted by radiologists. Exclusion criteria were any abnormalities of the ocular structures, a history of ocular surgery or trauma, a reduction in esotropia with hyperopic spectacle correction, pre-existing neurological diseases, congenital neurological pathologies, and cases of AACE without brain CT or MRI evaluation.

Data collection included general demographic information, ocular findings (best-corrected visual acuity, age at and duration of onset, presence of diplopia, angle of deviation for near and distance, anterior and posterior segment examination, ocular motility, cycloplegic refraction, sensory test results, presence of nystagmus, presence of an A or V pattern, and systemic findings (history of systemic involvement such as papilledema, nausea, vomiting, developmental regression, reduced coordination, dizziness, headache, and tinnitus). Associated neurological signs were also recorded, including impairment of other cranial nerves and ataxia. Neuroimaging findings were obtained from brain CT with or without contrast and brain MRI.

Data analysis and statistical methods included descriptive data presented as mean or median. Categorical variables are reported as numbers and percentages. To compare continuous variables between patients with normal and abnormal neuroimaging, an independent t-test was used for normally distributed data, whereas a Mann-Whitney U test was applied for non-normally distributed data. Chi-square or Fisher's exact test was used to analyze categorical variables, depending on the distribution. Predictive factors for abnormal neuroimaging were assessed using odds ratios and 95% confidence intervals (CI). A $p < 0.05$ was considered statistically significant, and all analyses were performed using Jamovi version 2.3.28.0 (The Jamovi Project, Sydney, Australia).

Institutional ethics approval was obtained from the Faculty of Medicine, CMU. All data were fully anonymized.

RESULTS

A total of 2,835 patients were identified based on these ICD-10 codes. Among these, 236 patients met the criteria for AACE of whom 45 patients (19.1%) had undergone neuroimaging, as shown in Figure 1.

Of the 45 patients who underwent neuroimaging, 23 patients (51.1%) underwent a brain CT without contrast, 18 patients (40.0%) underwent a brain CT with contrast, and 4 patients (8.9%) underwent brain MRI, as shown in Figure 2.

Many of the neuroimaging procedures (81.0%) were not from our center. Among the 45 patients who underwent neuroimaging, neurological abnormalities were identified in 2 patients (4.4%). All abnormal findings were detected through brain CT, as shown in Figure 3.

Among the 45 patients with AACE who underwent neuroimaging, the mean age was 10.2 years, with a median age of 8 years and a maximum age of 32 years. The median ages for the normal and abnormal neuroimaging groups were 8 years and 6 years, respectively. The patients were divided into three age groups: < 10, 10–18, and > 18. The majority of cases (31 patients, 68.8%) occurred in those under 10 years old. Additionally, there were more male patients, including 28 boys (62.2%). The underlying diseases identified included G6PD deficiency, anemia, and sinusitis. No medication was used to treat those underlying diseases. No significant differences in baseline characteristics were observed between the abnormal and normal neuroimaging groups, as shown in Table 1.

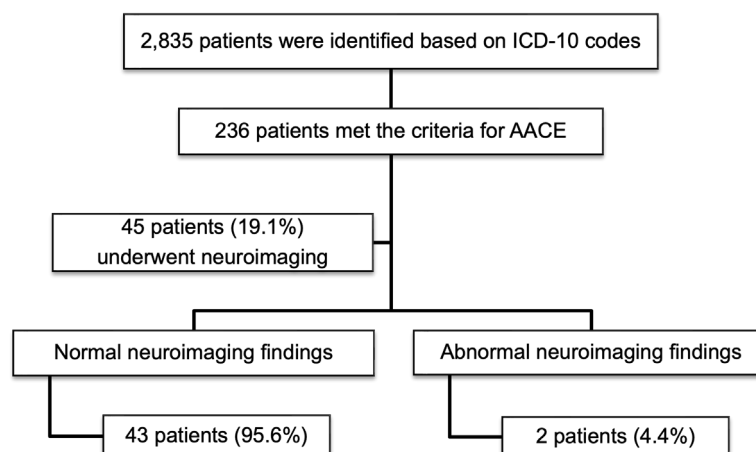
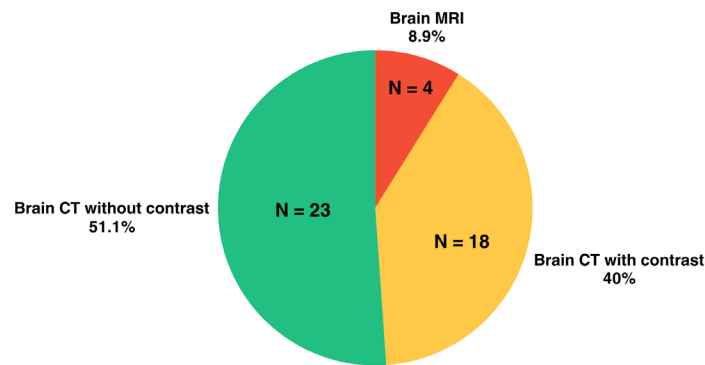


Figure 1. Patient selection flowchart for AACE identified from ICD-10 codes: of 236 patients who met the AACE criteria, 45 underwent neuroimaging. Of those, 2 (4.4%) had abnormal neuroimaging findings.



CT, computed tomography; MRI, magnetic resonance imaging, N, numbers

Figure 2. Distribution of neuroimaging procedures among 45 patients with AACE: brain CT without contrast was the most common modality (23 patients, 51.1%), followed by brain CT with contrast (18 patients, 40.0%), and brain MRI (4 patients, 8.9%).

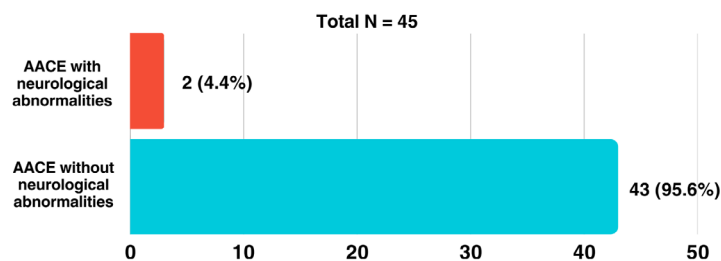


Figure 3. Prevalence of abnormal neuroimaging findings in AACE, identified in 2 of 45 patients (4.4%) who underwent neuroimaging.

Table 1. Demographic data of AACE patients with abnormal and normal neuroimaging

Characteristics	Total (N, %)	Abnormal neuroimaging (N, %)	Normal neuroimaging (N, %)	p-value
Age (years)				0.38
Mean	10.2	6	10.4	
Median	8.0	6	8	
Min, max	2, 32	6	2, 32	
Age group				1.00
< 10 years	31 (68.8)	2 (100.0)	29 (67.4)	
10–18 years	7 (15.6)	0 (0.0)	7 (16.3)	
> 18 years	7 (15.6)	0 (0.0)	7 (16.3)	
Gender				0.52
Male	28 (62.2)	2 (100.0)	26 (60.5)	
Female	17 (37.8)	0 (0.0)	17 (39.5)	
Underlying diseases				1.00
Present	2 (4.4)	0 (0.0)	2 (4.7)	
Absent	43 (95.6)	2 (100.0)	41 (95.3)	
Medication used				N/A
Present	0 (0.0)	0 (0.0)	0 (0.0)	
Absent	45 (100.0)	2 (100.0)	43 (100.0)	

N, number; N/A, not applicable

For ocular findings, the overall mean and median ages at onset were 8.7 and 7 years, respectively. The mean age at onset was 5.5 years in the abnormal neuroimaging group and 8.8 years in the nor-

mal neuroimaging group. The median duration of esotropia prior to the first outpatient visit was approximately 5 months. Symptoms of diplopia were reported in 25 patients (55.6%). A history

of intense near work was recorded in 7 patients (15.6%), all in the normal neuroimaging group. No patients had nystagmus or abnormalities in the anterior or posterior segment upon ocular examination. The overall mean angle of deviation at near was 36.1 prism diopters (PD). The overall mean near deviation was 36.8 PD in the normal neuroimaging group and 27.5 PD in the abnormal neuroimaging group. The overall mean distance deviation was 36.9 PD in the normal group and 25 PD in the abnormal neuroimaging group. Neither A nor V patterns were observed. A total of 27 out of 45 patients (60.0%) underwent sensory

testing (Worth 4-Dot). Diplopia was observed at near in 18 patients (66.7%) and at distance in 17 patients (63.0%), all of whom were in the normal neuroimaging group. The remaining patients in both groups reported suppression of conflicting information from one eye or the other. The overall spherical equivalent cycloplegic refraction demonstrated a mean of -0.04 D in the right eye and +0.21 D in the left eye. No significant differences in ocular findings were found between the two groups, as shown in Table 2.

No associated abnormal neurological symptoms or signs were observed among the patients

Table 2. Ocular findings in AACE patients with abnormal and normal neuroimaging

Characteristics	Total (N = 45)	Abnormal neuroimaging (N = 2)	Normal neuroimaging (N = 43)	p-value
Age at onset (years)				0.46
Mean	8.7	5.5	8.8	
Median	7	5.5	7	
Min, max	1, 32	5, 6	1, 32	
Duration of onset (months)				0.71
Mean	17.6	6.3	18.2	
Median	5	6.3	5	
Min, max	0.1, 216	0.5, 12	0.1, 216	
Diplopia, n (%)				0.58
Present	25 (55.6)	1 (50.0)	24 (55.8)	
Absent	20 (44.4)	1 (50.0)	19 (44.2)	
Near work history, n (%)				1.00
Present	7 (15.6)	0 (0.0)	7 (16.3)	
Absent	38 (84.4)	2 (100.0)	36 (83.7)	
Presence of amblyopia	7 (15.6)	0 (0.0)	7 (16.3)	1.00
Near deviation (PD)				0.21
Mean	36.10	27.5	36.8	
Median	35	27.5	35	
Min, max	7, 70	25, 30	7, 70	
Distance deviation (PD)				0.19
Mean	36.11	25	36.9	
Median	35	25	35	
Min, max	15, 70	25	15, 70	
Sensory tests at near (N=27), n (%)				0.33
Diplopia	18 (66.7)	0 (0.0)	18 (69.2)	
Suppression	9 (33.3)	1 (100.0)	8 (30.8)	
Sensory tests at a distance (N=27), n (%)				0.37
Diplopia	17 (63.0)	0 (0.0)	17 (65.4)	
Suppression	10 (37.0)	1 (100.0)	9 (34.6)	
Spherical equivalent (OD) (D)				0.90
Mean	-0.04	+0.13	-0.05	
Median	+0.38	+0.13	+0.38	
Min, max	-9.63, +2.00	-0.13, +0.38	-9.63, +2.00	
Spherical equivalent (OS) (D)				0.49
Mean	+0.21	+1.19	+0.17	
Median	+0.63	+1.19	+0.50	
Min, max	-10.75, +2.25	+0.63, +1.75	-10.80, +2.25	

D, diopters; N, number; N/A, not applicable; OD, oculus dexter (right eye); OS, oculus sinister (left eye); PD, prism diopters

in this study, including dizziness, tinnitus, ataxia, other cranial nerve impairments, abnormal cerebellar signs, developmental regression, reduced coordination, and there were no symptoms or signs of increased intracranial pressure such as nausea, vomiting, headache, or papilledema.

The analysis found no significant differences in any characteristics considered as potential predictive factors, including gender and age at onset, between the abnormal and normal neuroimaging groups ($p > 0.05$), as shown in Table 3.

Among the patients with AACE who underwent neuroimaging, abnormal neurological imaging findings were identified in 2 patients as described below.

Case #1

A 6-year-old Thai boy was referred from a provincial hospital with a history of horizontal diplopia two weeks prior. There was no history of previous trauma. At presentation, he had an esotropia of 25 PD at both distance and near by the alternate prism cover test. His Snellen visual acuity was 6/9, OU. The Worth 4-Dot test demonstrated alternate suppression, predominantly in the right eye. His cycloplegic refraction was +1.25 -2.75 x 20, OD and +2.50 -1.50 x 170, OS. A brain CT conducted at a private hospital revealed a small vascular anomaly, with no mass detected. The anomaly was observed and no further management was required. The patient was scheduled for bilateral medial rectus recession.

Case #2

A 6-year-old Thai boy presented with a history of esotropia for one year. He was generally healthy with normal growth and development and had no underlying medical conditions. He reported no symptoms such as headache, vomiting, seizure, or weakness. His Snellen visual acuity was 6/9, OU. Cycloplegic refraction was +0.50 -0.25 x 175 OD and +0.75 -0.25 x 5 OS. His eye examination showed esotropia of 30 PD by the Krimsky test. Grade I inferior oblique overaction was observed in both eyes. A brain CT without contrast performed at Chiang Mai University hospital revealed an extra-axial cerebrospinal fluid density lesion in the right middle cranial fossa, most likely an arachnoid cyst. Neurosurgery and pediatric specialists were consulted, and a brain MRI was performed. The MRI revealed a stable-sized arachnoid cyst

Table 3. Predictive factors for abnormal neuroimaging in patients with AACE who underwent neuroimaging

Predictive factors	Adjusted odds ratios	95% confidence interval	p-value
Gender	NE	NE	NE
Age at onset	0.799	(0.799, 1.414)	0.44

AACE, acute acquired comitant esotropia; NE, not estimable

measuring 5.5 x 5.0 x 5.1 cm located at the right anterior temporal and inferior frontal regions. The mass effect caused posterior displacement of the right anterior temporal lobe and anterosuperior displacement of the right inferior frontal lobe, resulting in compression of the frontal horn of the right lateral ventricle and a 0.4-cm leftward bowing of the midline structures. No brain herniation was observed. The patient underwent cyst excision, and the postoperative diagnosis was an arachnoid cyst at the right sylvian cistern (type I). Bilateral medial rectus recession is planned for the patient when the angle of deviation stabilizes.

DISCUSSION

Since the COVID-19 pandemic, the prevalence of AACE has increased significantly, likely due to increased screen time and intense near work prompted by lifestyle changes in the COVID era (5-7). Older children and adults have been reported to be more susceptible to AACE than younger children (25). In our study, the mean and median ages of the patients were relatively young, at 10.2 years and 8 years, respectively. The patients were classified into three age groups (16): under 10 years, 10 to 18 years and over 18 years old. In this study, 68.8% were in the group under 10 years of age. This finding is consistent with previous studies which reported mean ages of 4.7 years (2), 5.5 years (1), 9.8 years (15), and 10.8 ± 8.6 years (16). However, several other studies have reported AACE in older age groups with a mean ages of 32.35 ± 24.40 years (13) and 22.9 ± 16.1 years (5). Notably, all patients with abnormal neuroimaging in our study were younger than 10 years old, although this difference did not reach statistical significance. The mean age in the abnormal neuroimaging group was 6 years, which is younger than the mean age of 7.1 years (range 3-13 years) reported in previous studies (9, 20, 23, 26).

The overall mean age at onset in our study was 8.7 years. In the group with neurological abnor-

malities, the mean age was 5.5 years, which was lower than the overall average but not statistically significant. This is comparable to a previous study that reported a mean age of 4.7 years (2, 3).

The duration of esotropia prior to the outpatient visit in this study varied widely, ranging from 1 day to 216 days. This is consistent with a study that reported a mean duration of 15 ± 3.8 days and a range of 1–180 days (2).

Intense near work is associated with AACE. A history of prolonged near work was reported in only 7 patients, all with normal neuroimaging findings. Due to the retrospective nature of the study, however, information extracted from patient charts may have been incomplete, especially in regard to specific subjective details.

In all cases, both anterior and posterior segment examinations were unremarkable. Additionally, no abnormal neurological signs, nystagmus, or A or V pattern which have been reported in previous studies were noted (2, 10, 13, 14). The degree of esotropia varies greatly among different studies and populations. In this study, the mean angles of deviation at near and distance were 36.10 PD and 36.11 PD respectively, which aligns with a study by Buch et al. that reported a similar moderate degree of deviation (37 and 35 PD) (2). However, these findings contradict several previous studies that reported lower angles of deviation (5, 13, 15, 27), with one study suggesting age differences as a possible explanation (13). On the contrary, a study by Montriwet reported a greater angle of esotropia in younger individuals, with a distance deviation of 43.57 ± 9.77 , 51.54 ± 8.75 , and 30.14 ± 12.39 PD observed in younger children, older children, and adults, respectively (16). The mean angles of esotropia at near fixation in the three age groups were 43.57 ± 9.37 , 51.15 ± 9.39 , and 31.43 ± 12.15 PD.

In our study, we found that the abnormal neuroimaging group demonstrated smaller deviations at near and distance compared to the normal neuroimaging group, with values of 27.5 PD and 25 PD at near and distance. In contrast, the normal neuroimaging group showed larger deviations of 36.8 PD and 36.9 PD at near and distance, although this difference was not statistically significant. This may be due to the smaller sample size in the abnormal neuroimaging group. These findings are in contrast to a study that reported a greater

angle of deviation at distance ($> 40\%$) as a considerable risk factor for the presence of intracranial pathology (2).

The prevalence of intracranial abnormalities varies in different studies and currently there is no consensus on neuroimaging for AACE. Among the 45 patients in this study who underwent neuroimaging, neurological abnormalities were identified in 2 patients (4.4%). All abnormal findings were detected through brain CT. This prevalence is similar to that reported in several studies e.g., a study by Buch et al. that found a prevalence of 6% among 48 patients (2), Dotan et al. which reported a prevalence of 5% among 20 cases (15), and a study by Mittendorf et al. which found one out of 21 patients (4.7%) with abnormal but insignificant MRI findings (1). In contrast, no abnormal neuroimaging findings were identified in patients with AACE in any of the three series (28–30). Montriwet also reported no neurological abnormalities in 36 patients with AACE (16). This lack of findings is consistent with Iimori et al., who reported that intracranial diseases were entirely excluded through brain MRI or CT for all 41 patients included in their study (31). In contrast, a study by Surachatkumtonekul et al. on acquired esotropia found abnormalities in a total of 64 out of 117 cases (36.2%) although their study included all cases of acquired esotropia, not exclusive to AACE (13).

Various factors influence the selection of imaging modalities, including the limited availability of MRI, extended wait times for MRI appointments, the timing of MRI interventions, and the necessity for pediatric deep sedation. In settings where these factors are a concern, brain CT may be a more viable option for screening neurological abnormalities. While CT may not offer the fine, detailed information provided by MRI, it remains highly effective in detecting significant lesions that can play a critical role in guiding appropriate primary management decisions, especially in regions with limited resources, such as Thailand.

It can be observed that the majority of patients in this study were imaged at the referring hospital (81.0%), primarily to rule out life-threatening conditions, with CT being the most frequently used due to its greater availability prior to referral. The imaging was done prior to obtaining cycloplegic refraction results. Among the patients in the present study, some with normal neuroimaging

were excluded because subsequent cycloplegic refraction revealed hyperopia greater than +3.00 D spherical equivalent.

Concomitancy in acute-onset esotropia does not rule out an underlying serious neurological condition; therefore, the decision whether to perform neuroimaging or not should not be based only on concomitancy (12, 28). Notably, even rare conditions such as Lhermitte–Duclos disease have been reported to manifest as typical AACE, characterized by strict comitancy, even when accompanied by other focal neurological symptoms (32).

Abnormal neuroimaging findings in this study included one case of a small vascular anomaly and an arachnoid cyst in the right anterior temporal and inferior frontal regions, findings which had not been reported in previous studies. Other studies with a similar mean age of patients with AACE have identified conditions such as hydrocephalus, pontine glioma, and thalamic glioma (2).

Previous studies have reported associations between AACE and neurological disorders, including Arnold–Chiari syndrome, hydrocephalus, thalamic lesions, myasthenia gravis, and certain types of seizures (3, 5, 9, 10, 12, 33, 34), brain tumors (19), medulloblastoma, brainstem or cerebellar tumors, medullary pilocytic astrocytoma, cerebellar astrocytoma, Chiari I malformation, idiopathic intracranial hypertension, cerebellar ataxia (9, 10, 12, 23, 28, 33–35), inflammation, degenerative conditions, and vascular lesions (13).

An important observation from this study is that the absence of any signs of increased intracranial pressure such as headache, vomiting, diplopia and blurry vision does not rule out neurological abnormalities. This was demonstrated in case #2 who presented as a generally healthy young child without any abnormal symptoms other than acute esotropia but who had a large arachnoid cyst causing ventricular compression and bowing of midline structures, requiring urgent neurosurgery. Thus, it is important for physicians and ophthalmologists to be aware of the possibility of significant intracranial lesions despite the absence of symptoms or signs of increased intracranial pressure in AACE patients, especially young children who may not be able to completely express their symptoms.

No characteristics observed in the two patients with abnormal neuroimaging findings were sig-

nificantly different from those in patients with normal neuroimaging findings, and no predictive factors were identified that could suggest a higher likelihood of neuroimaging abnormalities. This is consistent with reports of neural abnormalities without abnormal neurological signs, such as several brain tumors, including cerebellar astrocytomas, cerebellar medulloblastomas, pontine gliomas, and astrocytoma of the corpus callosum with hydrocephalus (2, 9, 10, 12, 13, 15, 34, 36).

Since none of the neuroimaging abnormalities in this study offered a convincing explanation for AACE, these results were most probably incidental findings. This observation is consistent with research conducted by Sriraman (37), which reported abnormal neuroimaging findings in 4 out of 99 cases (4.04%), including an old intracranial hemorrhage, a cavernoma, a suspected meningioma, and a saccular aneurysm; however, none of these pathologies provided a definitive explanation for the esotropia deviation. Despite their incidental nature, some of these findings may represent life-threatening conditions.

This study was conducted prior to the publication of the 2025 Asia-Pacific consensus statement (38) which advocates for selective brain MRI based on specific red flags such as nystagmus, superior oblique overaction, A-pattern deviation, neurological symptoms (for example, headache, ataxia, or papilledema), onset after 6 years of age, significant distance–near dissociation, recurrence, or atypical presentation in children under 4 years of age. Nonetheless, our findings underscore that abnormal neuroimaging results can be present in patients with AACE even in the absence of any risk factors or focal neurological deficits.

Consequently, the decision to pursue neuroimaging should follow a multifactorial approach that balances rigorous screening for neurological signs in accordance with current recommendations with the risk of missing potentially life-threatening incidental pathologies in otherwise asymptomatic individuals. Furthermore, the affordability and accessibility of MRI, the standard neuroimaging modality, remain significant barriers in many developing countries. The use of brain CT as an alternative diagnostic tool necessitates a careful recognition of its inherent diagnostic limitations.

Our study has several limitations. Notably, brain CT was the predominant imaging modality,

consistent with real-world practice and resource availability. However, CT is not the gold standard for neuroimaging in AACE and exhibits lower sensitivity for detecting lesions in the posterior fossa and subtle brainstem abnormalities, which are more reliably detected with MRI. Accordingly, our results may underestimate intracranial pathologies, leading to an underrepresentation of neuroimaging abnormalities in AACE. In addition, the small number of patients with abnormal neurological findings may have affected the statistical analysis, significantly limiting the reliability and validity of the regression analysis. Multivariable results should be interpreted cautiously due to possible overfitting and limited power. These findings are hypothesis-generating rather than definitive. Furthermore, missing or incomplete data due to the retrospective nature of the study represents a significant limitation. Larger prospective studies are needed for developing future guidelines regarding the role of neuroimaging in AACE patients of all age groups.

CONCLUSIONS

Although neurological abnormalities are infrequent in children with AACE, clinicians must remain aware of the possibility of underlying brain abnormalities despite the absence of neurological symptoms, focal signs, or evidence of increased intracranial pressure. While brain MRI unequivocally remains the diagnostic gold standard, the selection of a neuroimaging modality warrants consideration of multiple factors, including resource availability, and the specific risk-benefit profile of alternative modalities within different clinical settings.

ACKNOWLEDGMENTS

None

FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest to report.

AUTHOR CONTRIBUTIONS

T.J.: data collection, conceptualization, visualization, interpretation of data, writing — original draft, writing — review and editing; A.S.: data collection and interpretation of data; W.S.: data collection and interpretation of data; J.S.: data collection and interpretation of data; S.A.: data collection and interpretation of data; A.W.: conceptualization, visualization, interpretation of data, writing — review and editing, and final approval.

All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

According to the policy implemented during this study, the ethics committee does not permit the authors to share the data with other entities. The datasets used and analyzed for the current study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

This study was approved by the Ethics Committee of the Faculty of Medicine, Chiang Mai University (study code, OPT-2568-0377 and date of approval, June 10, 2025).

INFORMED CONSENT STATEMENT

Not applicable.

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Prediction of Low Birth Weight From Quadruple Screening in Sanpatong Hospital, Chiang Mai

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Received: July 10, 2025;

Revised: December 19, 2025;

Accepted: January 29, 2026

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ABSTRACT

OBJECTIVE This study aimed to develop a predictive model for low birth weight (LBW) using maternal history and the second trimester biochemical quadruple screening test in singleton pregnancies delivered at Sanpatong Hospital, Chiang Mai, Thailand.

METHODS: This retrospective cohort study collected data from medical records of 873 singleton pregnancy women who underwent the quadruple test at 14–16 weeks of gestation and delivered at Sanpatong Hospital between October 1, 2016, and September 30, 2023. Data collected included maternal characteristics, quadruple test results, obstetric complications, and birth weight. Multivariable logistic regression was used to analyze associations between various factors and LBW. Model performance was evaluated using receiver operating characteristic (ROC) curve analysis.

RESULTS: The incidence of LBW was 6.76% (59 out of 873 deliveries). Significant factors associated with LBW in multivariable analysis included maternal body mass index (BMI) (AOR = 0.924, $p = 0.018$), nulliparity (AOR = 0.524, $p = 0.021$), high-risk quadruple test for aneuploidy (AOR = 16.061, $p = 0.006$), preeclampsia (AOR = 3.573, $p = 0.007$), preterm labor (AOR = 36.526, $p < 0.001$), fetal growth restriction (AOR = 45.726, $p < 0.001$), MoM of AFP (AOR = 2.598, $p = 0.001$), and MoM of β -hCG (AOR = 1.352, $p = 0.003$). Four predictive models were developed: history-only (AUC = 0.673), biochemical-only (MoM of AFP and β -hCG; AUC = 0.604), a combined model (history + biochemical; AUC = 0.707), and a full combined model (history + biochemical + complication; AUC = 0.902). The full combined model demonstrated superior performance in predicting LBW.

CONCLUSIONS: The full combined model incorporating clinical history and quadruple test biochemical markers (AFP and β -hCG) effectively predicts LBW. These findings suggest the potential for early identification of at-risk pregnancies, enabling timely interventions to improve maternal and neonatal outcomes.

KEYWORDS low birth weight, quadruple test, prediction model, alpha-fetoprotein, β -hCG

INTRODUCTION

Low birth weight (LBW), defined as a birth weight less than 2,500 grams, remains a significant global public health concern, contributing substantially to neonatal mortality and morbidity (1). Infants born with LBW face increased risks of short-term complications such as respiratory distress syndrome, hypothermia, and infections, as well as long-term health issues including developmental delays, chronic diseases in adulthood, and neurodevelopmental impairments (2). Globally, the incidence of LBW is estimated to be around 15.00-20.00% (1), with Thailand reporting an incidence of 9.91% in 2022 (3).

Early identification of pregnancies at high risk for LBW is crucial for implementing timely interventions, optimizing prenatal care, and preparing for appropriate delivery management. Various maternal characteristics and obstetric complications have been identified as risk factors for LBW, including poor maternal nutritional status, infections, preeclampsia (PE), preterm labor (PTL), and fetal growth restriction (FGR) (4-7).

Maternal serum biochemical markers, routinely used in second-trimester aneuploidy screening (e.g., quadruple test), have also been explored for their potential role in predicting adverse pregnancy outcomes, including LBW. The quadruple test typically measures alpha-fetoprotein (AFP), human chorionic gonadotropin (hCG), unconjugated estriol (uE3), and inhibin-A (Inh-A) (4, 8). While primarily designed for chromosomal abnormalities, deviations in these marker levels may reflect placental dysfunction or fetal growth disturbances (9).

Despite the established role of clinical factors and the potential utility of biochemical markers, comprehensive predictive models integrating both aspects are essential for improved risk stratification, particularly in specific regional populations. Data on the predictive value of the quadruple test in relation to LBW in Thailand, especially in provincial hospitals like Sanpatong Hospital, is limited.

This study aimed to develop a predictive model for LBW using maternal history and quadruple test biochemical screening in singleton pregnancies delivered at Sanpatong Hospital, Chiang Mai.

METHODS

The study protocol was approved by the Sanpatong Hospital Ethical Committee (COE No.

18/2567, approved October 1, 2024). Patient confidentiality was maintained throughout the study. This was a retrospective cohort descriptive study conducted at Sanpatong Hospital, a community hospital in Chiang Mai Province, Thailand. Data were collected from the medical records of singleton pregnant women who received quadruple test screening at 14-16 weeks of gestation and delivered at Sanpatong Hospital between October 1, 2016, and September 30, 2023.

The sample size for this study was determined using the formula $n = (k \times \text{EPV})/P$, with a prevalence of LBW of 9.91%. The number of predictor variables (k) was set at 8, and the events per variable (EPV) was set at 10. This calculation yielded a required sample size of 808 participants. However, to enhance the robustness of the analysis, all eligible individuals who met the inclusion criteria were included, resulting in a total sample of 873 participants. Exclusion criteria included pregnant women with pre-existing medical conditions, pregnancies with known fetal chromosomal abnormalities, incomplete quadruple test data, and those who delivered at other hospitals.

Definitions used in this study are as follows: LBW was defined as a birth weight less than 2,500 grams. High risk for aneuploidy was determined by quadruple test screening results that indicated a risk exceeding the established cutoff of 1:250 for chromosomal abnormalities. Maternal obstetric complications were defined as PE (based on blood pressure of 140/90 mmHg or higher with proteinuria more than 300 mg in 24 hours), FGR (estimated fetal weight from ultrasound biometry less than 10th percentile for gestational age), abortion (fetal loss at less than 24 weeks), PTL (regular uterine contraction with cervical change before 37 complete weeks).

Data were extracted from medical records and included maternal characteristics (age, parity, body mass index at the time of the quadruple test, and gestational age at testing), quadruple test results (risk assessment for aneuploidy (high risk vs. low risk) and multiple of median values for AFP, β -hCG, Inh-A, and uE3, maternal obstetric complications (such as gestational diabetes, PE, PTL, and fetal growth restriction), and neonatal outcome (including infant sex, mode of delivery, gestational age at delivery, and birth weight).

Data were analyzed using SPSS software (version 16.0). Descriptive statistics were used to

summarize baseline characteristics: median and interquartile range (IQR) for continuous variables, and number and percentage (n, %) for categorical variables. Differences between the LBW and normal birth weight (NBW) groups were assessed using the Mann-Whitney U test for continuous variables and chi-square test or Fisher's exact test for categorical variables.

Multivariable logistic regression analysis was performed to identify independent factors associated with LBW. Variables with a $p < 0.05$ in univariate analysis and those considered clinically important were included in the multivariable model. The adjusted odds ratios (AOR) with 95% confidence intervals (CI) were calculated. The performance of predictive models for LBW was evaluated using the receiver operating characteristic (ROC) curve. The area under the curve (AUC) was used to assess the discriminative ability of the model.

RESULTS

A total of 873 singleton pregnant women were included in this study. Among them, 59 (6.76%) infants were classified as LBW, while 814 (93.24%) were of NBW.

The baseline characteristics of the study population, described in Table 1 included the median maternal age of 27 years (IQR: 24–31), with no significant difference between LBW and NBW

groups ($p = 0.220$). Maternal BMI at the quadruple test was significantly lower in the LBW group (median 21.2 kg/m², IQR: 19.1–23.8) compared to the NBW group (median 22.9 kg/m², IQR: 20.5–26.5) ($p = 0.003$). Nulliparity was significantly more frequent in the LBW group (64.4% vs 48.6%, $p = 0.019$). High-risk quadruple test for aneuploidy was also significantly higher in the LBW group (13.6% vs. 3.1%, $p < 0.001$).

Regarding maternal obstetric complications, PE (10.2% vs. 3.1%, $p = 0.004$), PTL (40.7% vs. 1.8%, $p < 0.001$), and FGR (22.0% vs. 0.6%, $p < 0.001$), were significantly more prevalent in mothers delivering LBW infants. The median gestational age at delivery was significantly lower in the LBW group (259 days, IQR: 250–269) compared to the NBW group (274 days, IQR: 269–279) ($p < 0.001$).

The distributions of the maternal serum quadruple-test markers—AFP, β -hCG, Inh-A, and uE3, expressed as multiples of the median (MoM)—by birth weight group is presented in Figure 1. The median MoM of AFP was significantly higher in the LBW group (1.03, IQR: 0.86–1.30) than in the NBW group (0.98, IQR: 0.77–1.21) ($p = 0.037$). Conversely, the median MoM of uE3 was significantly lower in the LBW group (1.02, IQR: 0.86–1.23) compared to the NBW group (1.12, IQR: 0.94–1.36) ($p = 0.039$). No significant differences were observed for MoM of β -hCG and MoM of Inh-A in the univariable analysis (Table 1).

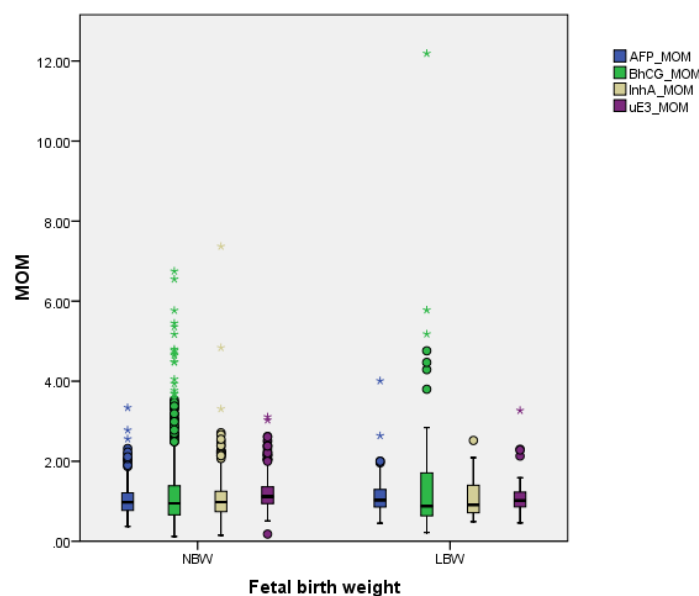


Figure 1. Box and whisker plots of maternal serum Quadruple test markers by fetal birth weight status (normal birth weight (NBW) and low birth weight (LBW))

AFP_MOM, alpha-fetoprotein multiple of the median; B-hCG_MOM, beta-human chorionic gonadotropin multiple of the median; InhA_MOM, inhibin A multiple of the median; uE3_MOM, unconjugated estriol multiple of the median

The multivariable logistic regression analysis (Table 2) identified several independent predictors for LBW, including maternal age (AOR = 0.937, 95%CI: 0.81-0.986, $p = 0.021$), nulliparity (AOR = 0.524, 95%CI: 0.302-0.908, $p = 0.021$), and BMI at the time of the quadruple test (AOR = 0.924, 95%CI: 0.866-0.987, $p = 0.018$), all of which were significantly associated with an increased risk of LBW.

Factors associated with LBW from multivariable analysis (Table 2) identified several independent predictors for LBW. Maternal age (AOR = 0.937, 95%CI: 0.891-0.986, $p = 0.021$), nulliparity (AOR = 0.524, 95%CI: 0.302-0.908, $p = 0.021$), and BMI at quadruple test (AOR = 0.924, 95%CI: 0.866

- 0.987, $p = 0.018$) were significantly associated with an increased risk of LBW.

Among quadruple test results, high risk for aneuploidy significantly increased the risk of LBW (AOR = 16.061, 95%CI: 2.215-116.455, $p = 0.006$). Crucially, maternal obstetric complications such as PE (AOR = 3.573, 95%CI: 1.405-9.086, $p = 0.007$), PTL (AOR = 36.526, 95%CI: 17.628-75.681, $p < 0.001$), and FGR (AOR = 45.726, 95%CI: 15.632-133.754, $p < 0.001$) were very strong predictors.

Regarding quadruple test biochemical markers in the multivariable model, MoM of AFP (AOR = 2.598, 95%CI: 1.458-4.631, $p = 0.001$) and MoM of β -hCG (AOR = 1.352, 95%CI: 1.107-1.652, $p = 0.003$) were significantly associated with LBW. MoM of

Table 1. Baseline characteristics of maternal and infant

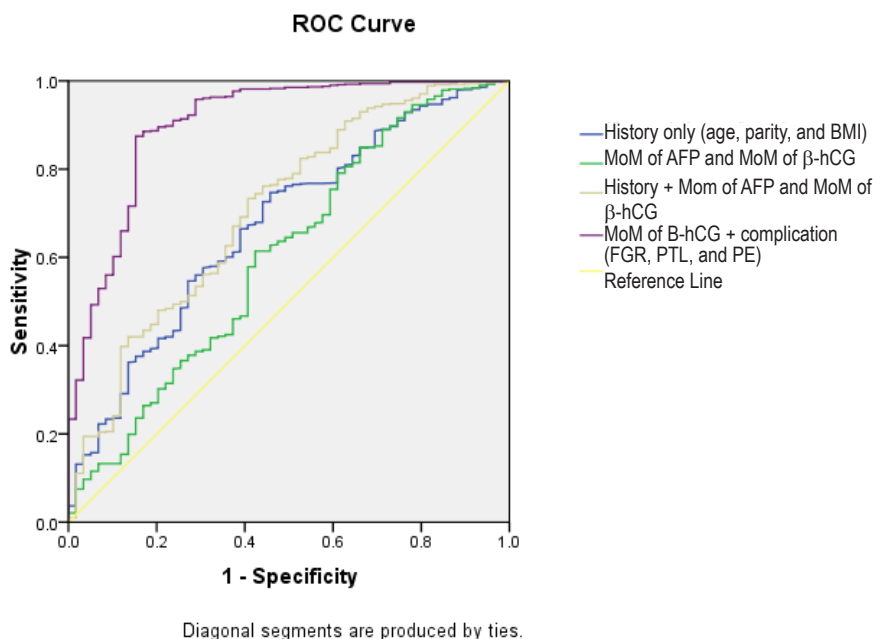
Characteristics	Total (N = 873)	LBW (N = 59)	NBW (N = 814)	p-value
Age, years, median (IQR)	27 (24-31)	25 (21-31)	27 (24-31)	0.220
Primigravida (G1), n (%)	385 (44.1)	33 (55.9)	352 (43.2)	0.058
Nulliparous (P0), n (%)	434 (49.7)	38 (64.4)	396 (48.6)	0.019*
BMI at Quad test, kg/m ² , median (IQR)	22.9 (20.4-26.4)	21.2 (19.1-23.8)	22.9 (20.5-26.5)	0.003*
GA at Quad test, days, median (IQR)	110 (106-115)	109 (106-115)	110 (106-115)	0.533
Past history, n (%)				
Have history of preterm birth	10 (1.1)	1 (1.7)	9 (1.1)	1.000
Have history of preeclampsia	1 (0.1)	0 (0.0)	1 (0.1)	0.505
Quad test result, n (%)				
Low risk	836 (95.8)	49 (83.1)	787 (96.7)	0.000*
HR for aneuploidy	33 (3.8)	8 (13.6)	25 (3.1)	
HR for NTD	4 (0.4)	2 (3.3)	2 (0.2)	
Maternal obstetric complication, n (%)				
Gestational diabetes	52 (6.0)	5 (8.5)	47 (5.8)	0.388
Preeclampsia	31 (3.6)	6 (10.2)	25 (3.1)	0.004*
Preterm labor	39 (4.5)	24 (40.7)	15 (1.8)	0.000*
Fetal growth restriction	18 (2.1)	13 (22.0)	5 (0.6)	0.000*
Infant sex, n (%)				
Male	474 (54.3)	30 (50.8)	444 (54.5)	0.582
Female	399 (45.7)	29 (49.2)	370 (45.5)	
Route of delivery, n (%)				
Vaginal delivery (ND, VE, and breech)	622 (71.2)	48 (81.4)	574 (70.5)	0.076
Cesarean section	251 (28.8)	11 (18.6)	240 (29.5)	
GA at delivery, days, median (IQR)	273 (268-278)	259 (250-269)	274 (269-279)	0.000*
Fetal birth weight, grams, median (IQR)	3115 (2830-3378)	2290 (2153-2415)	3150 (2895-3400)	0.000*
Quad test value, median (IQR)				
MoM of AFP	0.98 (0.77-1.23)	1.03 (0.86-1.30)	0.98 (0.77-1.21)	0.037*
MoM of β -hCG	0.95 (0.65-1.39)	0.88 (0.64-1.71)	0.95 (0.66-1.39)	0.720
MoM of Inh-A	0.97 (0.74-1.26)	0.91 (0.72-1.40)	0.95 (0.66-1.39)	0.892
MoM of uE3	1.12 (0.94-1.35)	1.02 (0.86-1.23)	1.12 (0.94-1.36)	0.039*

BMI, body mass index; HR, high risk; NTD, neural tube defect; ND, normal delivery; VE, vacuum extraction; GA, gestational age; MoM, multiple of median; AFP, alpha feto-protein; β -hCG, beta-human chorionic gonadotropin; Inh-A, inhibin-A; uE3, unconjugated estriol; *, $p < 0.05$ indicates statistic significance.

Table 2. Factors associated with low birth weight

Variable	Adjusted odds ratio	95%CI	p-value
Age groups	0.937	0.891-0.986	0.021*
Parity	0.524	0.302-0.908	0.021*
BMI at Quad test	0.924	0.866-0.987	0.018*
Quad test result			
HR for aneuploidy	16.061	2.215-116.455	0.006*
HR for NTD	3.125	0.377-25.918	0.291
Maternal obstetric complication			
Preeclampsia	3.573	1.405-9.086	0.007*
Preterm labor	36.526	17.628-75.681	0.000*
Fetal growth restriction	45.726	15.632-133.754	0.000*
Quad test value			
MoM of AFP	2.598	1.458-4.631	0.001*
MoM of β -hCG	1.352	1.107-1.652	0.003*
MoM of Inh-A	1.092	0.657-1.815	0.734
MoM of uE3	0.643	0.292-1.417	0.273

BMI, body mass index; HR, high risk; NTD, neural tube defect; ND, normal delivery; VE, vacuum extraction; GA, gestational age; MoM, multiple of median; AFP, alpha feto-protein; β -hCG, beta-human chorionic gonadotropin; Inh-A, inhibin-A; uE3, unconjugated estriol; *, $p < 0.05$ indicates statistic significance.

**Figure 2.** Receiver operating characteristic (ROC) curve for the prediction of low birth weight using maternal serum MoM of AFP and MoM of β -hCG

Inh-A and MoM of uE3 did not show significant associations in this model (Table 2).

Figure 2 illustrates the ROC curves for the four predictive models. The history-only model, which included maternal age, parity, and BMI, showed limited discriminative ability with an AUC of 0.673 (95%CI: 0.602-0.743, $p < 0.001$). In contrast, the biochemical-only model, using only

MoM of AFP and MoM of β -hCG, demonstrated a lower discriminative ability with an AUC of 0.604 (95%CI: 0.523-0.685, $p = 0.008$). The combined model, integrating history, MoM of AFP, and MoM of β -hCG, showed fair discriminative power with an AUC of 0.707 (95%CI: 0.635-0.779, $p < 0.001$). At its optimal cut-off, this model yielded a sensitivity of 73.3% and a specificity of 59.3%. The

full combined model, integrating history, MoM of AFP, MoM of β -hCG, and complication (FGR, PTL, and PE), achieved the highest discriminative power, with an excellent AUC of 0.902 (95%CI: 0.855-0.949, $p < 0.001$). At its optimal cut-off, this full model yielded a sensitivity of 88.1% and a specificity of 84.7%. This full combined model significantly outperformed the other models, highlighting the added value of integrating quadruple test markers with clinical factors for predicting LBW.

DISCUSSION

This study aimed to investigate the association between quadruple test biochemical screening results and LBW in singleton pregnancies at Sanpatong Hospital, Chiang Mai, and to develop a comprehensive predictive model. The findings reveal several significant factors associated with LBW, contributing valuable insights for clinical practice, particularly within a regional hospital setting.

The observed LBW incidence of 6.76% in our study population is lower than global estimates (15.0-20.0%) (1) and the national average for Thailand (9.9% in 2022) (3). This difference may be attributed to the specific demographic characteristics of a district hospital population, which might differ from urban centers or national averages. Furthermore, the stringent inclusion and exclusion criteria, such as the exclusion of women with certain pre-existing medical conditions or those delivering at other facilities, could have contributed to a healthier study cohort.

Our findings that lower maternal BMI, nulliparity, and high-risk quadruple test for aneuploidy are significantly associated with LBW are consistent with existing literature (7-9). Importantly, obstetric complications such as PE, PTL, and FGR were found to be very strong independent predictors of LBW. These results reinforce the well-established understanding that preterm birth and FGR are primary determinants of LBW, both of which are linked to short-term and long-term adverse health outcomes for the infant (2, 10). Maternal nutritional status and infections are also acknowledged contributors to LBW, indirectly reflected through factors like BMI and complications (7).

Regarding the role of quadruple test biochemical markers, our study found that higher MoM of AFP and lower MoM of uE3 were significantly associated with LBW in univariable analysis (Table 1). Furthermore, higher MoM of AFP remained a significant predictor in the multivariable model (Table 2). This aligns with research indicating a negative correlation between AFP levels and birth weight (11), and studies suggesting AFP as a key marker within the triple test associated with birth weight (11). Elevated AFP levels have also been linked to various adverse pregnancy outcomes including pregnancy-induced hypertension, spontaneous abortion, preterm birth, FGR, oligohydramnios, and placental abruption (12).

While univariable analysis showed no significant difference in MoM of β -hCG and Inh-A between LBW and NBW groups, the multivariable model revealed a significant association between higher MoM of β -hCG and increased risk of LBW. This discrepancy might be due to the confounding effects of other clinical variables in the univariable setting, which are controlled for in the multivariable analysis. Literature supports the association of elevated β -hCG with increased risk of LBW (13), PE, fetal demise, preterm delivery, and FGR (14). The observed differences in findings across studies, including ours, could stem from variations in analytical methodologies, population characteristics, or the specific set of co-factors considered in statistical models.

The predictive performance evaluation demonstrated the significant utility of the developed logistic regression models. The full combined model, integrating clinical history (age, parity, BMI), biochemical markers (MoM of AFP and β -hCG), and complications (FGR, PTL, and PE), achieved an excellent discriminative ability with an AUC of 0.902. At its optimal cut-off, this model achieved a sensitivity of 88.1% and a specificity of 84.7%. This indicates that the model correctly identified 52 out of 59 (88.1%) actual LBW cases in the study population, highlighting the substantial value of a comprehensive assessment approach that incorporates quadruple test data alongside maternal clinical factors. In contrast, the model relying solely on MoM of AFP and β -hCG exhibited considerably lower predictive performance (AUC = 0.604). The model integrating history and the two biochemical markers (AUC = 0.707) showed

better performance than the biochemical-only model, and the history-only model (AUC = 0.673) was still outperformed by the combined models. These results underscore the limitations of using biochemical markers alone for LBW prediction, yet emphasize their incremental benefit when combined with relevant clinical information.

A key strength of this study is its focus on a specific population within a district hospital in rural Thailand, providing valuable local data where information on LBW prediction models is often limited. The retrospective design allowed for a relatively large sample size ($N = 873$) and the use of real-world clinical data. However, the retrospective nature also presents limitations, including reliance on the completeness and accuracy of medical records and the potential for unmeasured confounding variables. The study was conducted in a single institution, which may limit the generalizability of findings to broader populations. Furthermore, while comprehensive, the predictive models did not include all potential risk factors for LBW that might be present in a prospective study design.

CONCLUSIONS

This study demonstrates that combining quadruple test markers (AFP and β -hCG) with key maternal clinical factors can effectively predict LBW risk. Early identification allows timely interventions and targeted care, potentially improving maternal and neonatal outcomes in settings similar to Sanpatong Hospital.

ACKNOWLEDGEMENTS

This research was successfully completed thanks to the great kindness of Thawith Kaewpre-sert, Director of Sanpatong Hospital, who has permitted and supported this research throughout. I would also like to thank the nurses and staff in the antenatal care unit and labor room units for their assistance in collecting the data.

FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest to report.

AUTHOR CONTRIBUTIONS

The author confirms sole responsibility for the study conception and design, data collection, statistical analysis and interpretation of results, and manuscript preparation.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are not publicly available due to patient confidentiality and institutional privacy policies of Sanpatong Hospital. However, they are available from the corresponding author upon reasonable request and with permission from the Sanpatong Hospital Ethical Committee (COE No. 18/2567).

INSTITUTIONAL REVIEW BOARD STATEMENT

The study protocol was approved by the Sanpatong Hospital Ethical Committee (COE No. 18/2567, approved October 1, 2024). Patient confidentiality was maintained throughout the study.

INFORMED CONSENT STATEMENT

Patient consent was waived due to the retrospective nature of the study, which utilized de-identified medical records. The study was conducted with the approval of the Sanpatong Hospital Ethical Committee, ensuring that all data were handled confidentially.

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Gout Through the Ages: Past, Present, and Future

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Received: February 22, 2026;

Revised: April 7, 2026;

Accepted: April 22, 2026

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ABSTRACT

Gout is a disease unique to humans, resulting from the evolutionary loss of functional uricase, the enzyme that converts uric acid to the more soluble allantoin. This loss causes elevation of serum uric acid (SUA). With sustained hyperuricemia, deposition of monosodium urate (MSU) crystals in and around joints and extra-articular tissues occurs. Clinically, gout manifests as recurrent episodes of acute inflammatory arthritis and, when untreated or undertreated, may progress to more frequent flares, tophus formation, and structural joint damage. Persistent hyperuricemia is also linked to nephrolithiasis, chronic kidney disease progression, and increased cardiometabolic burden. This review summarizes gout across the past, the present, and the future. Historical perspectives span early descriptions dating to the Egyptian period and extend through the period just prior to the discovery of light microscopy that led to enabling identification of MSU crystals. The modern era is reviewed through the development of classification criteria; mechanisms of MSU crystal-induced inflammation; genetic determinants of urate homeostasis; and the roles of renal and intestinal urate transport in hyperuricemia. Metabolomic signatures and gut microbiota are discussed alongside current approaches to urate-lowering therapy (ULT) and flare prevention. Future directions include precision, individualized medicine integrating clinical features with molecular and imaging data to improve risk stratification and guide therapy. They also include targeted anti-inflammatory strategies, newer ULT and uricosuric agents, and the application of metabolomics and microbiome science. Finally, the long-term possibility of uricase gene restoration as a transformative approach to reconstitute urate metabolism and potentially modify disease course is discussed.

KEYWORDS gout, hyperuricemia, uric acid, treatment, management

INTRODUCTION

Gout is a chronic inflammatory condition marked by episodes of sudden, painful arthritis caused by long-term high serum uric acid (SUA) levels that exceed solubility and form monosodium urate (MSU) crystals in body tissues, especially in and around joints. If left untreated, the crystal burden increases and can lead to more frequent flares, tophi, and progressive joint damage and deformity. Gout can also affect kidneys,

contributing to renal insufficiency and urinary stones. Hyperuricemia and gout have also been linked to cardiovascular diseases. Gout is essentially unique to humans because of the loss of functional uricase during evolution. Uric acid has both prooxidant and antioxidant properties, with its antioxidant role possibly offering protection against oxidative damage and supporting longevity under certain conditions (1, 2).

Gout is considered an ancient disease, having been described several thousand years ago. With advances in clinical medicine, substantial progress has been made in the diagnosis and management of gout. In addition, several emerging strategies are currently being developed to improve gout control in the future. This review focuses on the diagnosis and management of gout from antiquity to the future.

THE ANCIENT PERIOD

The oldest evidence of gout in humans was identified in the uric acid-containing urinary calculi in an Egyptian mummy from approximately 7000 BC (3) and by the presence of a tophus at the great toe of an Egyptian mummy (4). Unfortunately, nothing was known about the characteristics of the disease at that time.

The documented history of gout dates back to ancient Greece, when Hippocrates (460–370 BC), known as the ‘Father of Medicine,’ described gout as the ‘unwalkable disease’ and used the term “*podagra*” to refer to acute arthritis of the big toe. He believed that gout was the result of an excess of one of the human humors. He wrote 5 aphorisms on gout which remain valid until now. These aphorisms are 1) eunuchs do not get gout or become bald, 2) women do not get gout unless their menstrual periods cease, 3) young men do not get gout before they become sexually active, 4) gout-related inflammation usually subsides within 40 days, and 5) gout attacks are more common in spring and autumn (5). Aelius Galenus (129–216 AD) was another Greek physician, described as “the most accomplished of all medical researchers of antiquity,” who described the characteristics of gout and tophi. Galen believed that human diseases were caused by imbalance of the four human humors: 1) blood (sanguine), associated with the heart and air; 2) yellow bile (choleric), associated with the liver; 3) black bile (melancholic), associated with the spleen; and 4) phlegm (phlegmatic), associated with the brain and water (6).

Interestingly, despite the long duration of the Roman and Byzantine periods, there was not much information regarding gout. However, it was recognized that gout was more prevalent among the rich and was sometimes named the “disease of kings.” This likely reflected the very high consumption of wine among the wealthy. Unfortunately,

Roman wines were sometimes prepared with grape syrup to enhance the color, sweetness, and preservation, which was boiled down in a lead pot, resulting in contamination with lead. This could cause lead poisoning, renal toxicity, and gout (7). Whether this led to the end of the Roman empire is speculative (7, 8).

No specific treatment for gout was identified during the Egyptian period; however, there was evidence that the Egyptians treated several diseases by using plants, animal products, and minerals. In addition, they also used precious metals and stones to prevent disease (9). Hippocrates believed that diet could efficiently control and prevent gout. His treatment included using high-dose white hellebore to induce vomiting and diarrhea; however, this herb could be toxic to the cardiovascular and neurological systems (10). He also suggested scorching the veins contiguously with the affected joint. The first evidence of a pharmacologic approach to the treatment of gout probably started with Alexander of Tralles, a Greek physician during the 6th century (around 600 AD), who used autumn crocus (*Colchicum autumnale* - which contains colchicine) as a powerful purgative during that time. Due to the toxicity of autumn crocus, it was not used to treat gout for several centuries (11).

THE MODERN PERIOD

Diagnosis of gout

The modern period of gout might be considered to have started when Antoni van Leeuwenhoek (1632–1723 AD), a Dutch scientist, invented the microscope in 1677 to examine parasites and bacteria (12). With his microscope, he also observed a solid material resembling chalk in patients with gout but did not know its nature. Approximately 100 years later, in 1787, the British chemist William Hyde Wollaston identified the chemical structure of uric acid (13). During this period, Thomas Sydenham (1624–1689), an English physician who also suffered from gout, gave a very clear clinical description of an acute gout attack (5). During the 19th century, Alfred Baring Garrod, a British physician, emphasized the close link between gout and elevated uric acid. He described these deposits as crystalline and interstitial and proposed that urate deposition is a primary driver of the inflammatory process rather than a consequence of it. Unfortunately, many physicians

at that time did not accept his explanation (14). An association between gout and uric acid was clearly demonstrated by McCarty et al. in 1961 when he identified MSU crystals, using both ordinary and polarized light microscopy, in synovial fluid obtained from patients with acute gouty attacks (15). He performed an experiment in which injection of MSU crystals into himself and canine joints caused an acute gouty arthritis attack shortly after the injection (16).

Classification criteria

Since the discovery of MSU crystals, the knowledge of hyperuricemia and gout has expanded markedly. Several classification criteria have been developed, including Rome 1963 (17), New York 1966 (18), American Rheumatism Association (ARA) 1977 (19), Mexico 2010 (20), the Netherlands 2010 (21), and the most recent 2015 American College of Rheumatology (ACR)/European Alliance of Associations for Rheumatology (EULAR) classification criteria (22). All of these criteria have shown variable sensitivity and specificity, with overall acceptable performance (22–24). The advantage of the 2015 ACR/EULAR classification criteria is that the diagnosis can be made without identification of MSU crystals, and the criteria are based on a weighted scoring system. Those who have a score of ≥ 8 can be classified as gout. In addition to obtaining scores based on the location of joint involvement, the characteristics and time course of typical arthritis, and SUA levels, scores can also be obtained from advanced imaging modalities (e.g., ultrasound (US), dual-energy computed tomography (DECT), or magnetic resonance imaging (MRI)) that show specific imaging characteristics of gout. These instruments have shown high sensitivity and specificity for MSU deposits (25, 26). Use of these imaging techniques is not only for diagnosis of gout but also for monitoring dissolution of MSU crystals during follow-up (27). However, these classification criteria have some limitations. The clinical characteristics of gout, such as the characteristic episodic pattern of joint inflammation and the time course of gout flares, might vary among individuals, making it difficult to obtain these scores using the current classification criteria (22). Moreover, a limitation of these criteria is that individuals who meet the minimal score may not actually have gout, whereas

individuals with other diseases may exceed the minimal score required for gout classification (24).

Pathogenesis of gout

The pathogenic mechanism of hyperuricemia in gout was initially demonstrated in 1965 by Wyngarden, who found that patients with gout required SUA levels approximately 2–3 mg/dL higher than normouricemic controls to achieve the same level of uric acid excretion through the kidney, indicating that gout patients had a defect in urinary excretion of uric acid (28). In addition, during that same period, Lesch-Nyhan syndrome, a rare sex-linked recessive disease characterized by severe hyperuricemia and severe neurological deficits (cerebral palsy, mental retardation, movement disorder, and aggressive behavior), was identified and was later found to be due to deficiency of hypoxanthine-guanine-phosphoribosyltransferase (HGPRT) enzymes that convert hypoxanthine to inosine monophosphate in the purine and uric acid synthesis pathway (29, 30).

Pathogenesis of MSU crystal-induced inflammation

MSU crystal-induced inflammation became a central focus of gout research soon after MSU crystals were identified as the cause of acute gouty arthritis. Early work showed that shortly after neutrophils and mononuclear phagocytes ingest MSU crystals, crystal-containing phagosomes fuse with lysosomes; subsequent rupture of the phagolysosomal membrane causes cell injury or death and the release of inflammatory mediators (31). These mediators include prostaglandins, kinins, proteolytic enzymes, and reactive oxygen species, which amplify local inflammation and recruit additional leukocytes (32). Later studies showed that MSU crystals, when combined with lysosomes, can activate NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, leading to activation of caspase-1 and production of interleukin-1 β (IL-1 β), a key cytokine driving the acute gout flare (33–37). MSU crystals can also act as danger signals by priming and augmenting inflammatory signaling through Toll-like receptors and activating the MyD88 intracellular pathway, thereby promoting further IL-1 β production (37). Multiple cell types, including neutrophils, monocytes/macrophages, and lymphocytes, are involved

in this process (37). Activated neutrophils can generate neutrophil extracellular traps (NETs), which may both intensify inflammation and participate in subsequent containment of crystals (38).

The resolution of a gout flare involves coordinated counter-regulatory mechanisms such as crystal coating/opsonization; apoptosis and efferocytosis of recruited cells with increased transforming growth factor β (TGF- β); induction of anti-inflammatory cytokines, such as IL-10 and IL-37 (33, 37, 38); activation of transcriptional regulators such as PPAR γ (36); and engagement of specialized pro-resolving lipid mediators (e.g., lipoxins, resolvins, protectins, and maresins) that limit leukocyte recruitment and promote restoration of tissue homeostasis (38).

Uric acid transporters

Several decades later, researchers found that defect in renal excretion of uric acid was due to abnormalities in several tubular urate transporters that control uric acid excretion, including urate transporter 1 (URAT1), glucose transporter 9 (GLUT9), ATP-binding cassette sub-family G member 2 (ABCG2), and other organic anion transporters (OATs) (39). In addition, with advances in genetic studies, several genetic loci have been found to be associated with these urate transporter abnormalities, for example, SLC22A12 for URAT1, SLC2A9 for GLUT9, SLC22A11 for OAT4, and SLC22A13 for OAT10 transporters. These genetic abnormalities differ among ethnic groups, and several loci are commonly observed among individuals of Asian descent (SLC22A12, ABCG2, SLC2A9, and others minor alleles) (40–42).

Generally, hyperuricemia can be classified into 2 types, according to renal excretion. The under-excretion type (urine uric acid [UUA] < 600 mg/day on a purine-free diet), the major cause of hyperuricemia, is mainly caused by abnormality of URAT1 transporter in the renal tubules. However, a recent study found that abnormality in ABCG2 (both in the renal tubules and intestinal mucosa) also plays an important role in hyperuricemia. Defects in the ABCG2 transporter result in diminished intestinal uric acid excretion, and a shift toward increased renal uric acid excretion (or renal overload type) (43). However, selection of urate lowering therapy (ULT) is still based on whether the patient is classified as a uric acid un-

derexcretor (UUA < 600 mg/day) or an overexcretor (UUA $\geq$ 600 mg/day).

Gut microbiota

Recently, gut microbiota has been linked to many human diseases through its role in modulating immunity. Gut microbiota may contribute to both the development and prevention of hyperuricemia. Several *Lactobacillus* species can break down inosine and guanosine, thereby inhibiting uric acid biosynthesis and reducing intestinal purine absorption, which helps maintain lower uric acid levels. In contrast, other bacteria such as *Escherichia* and *Shigella* species can secrete xanthine deaminase, an enzyme that converts hypoxanthine and xanthine to uric acid, resulting in higher uric acid level. Furthermore, *Phascolarctobacterium* and *Bacteroides* species have been reported to be prominent and proposed as part of a “core microbiota” in gout patients. *Bacteroides* species have been associated with purine metabolism and may influence urate handling, including pathways related to conversion of uric acid to allantoin. Long-term consumption of diets enriched with fructose, purines, and fat can modify the composition of the gut microbiome, which can affect the intestinal role in purine and uric acid metabolism. On the other hand, elevation of SUA can induce chronic inflammation in the intestinal tract, potentially resulting in further changes to the composition of the gut microbiome. This supports a bidirectional relationship between the gut microbiome and uric acid (44–46).

Treatment of gout

Due to the toxicity of autumn crocus, this plant was not used for the treatment of gout until 1833, when Pierre-Joseph Pelletier and Joseph Bienaimé Caventou, two French chemists, isolated colchicine from the root of autumn crocus (47). Unfortunately, colchicine was not used in the treatment of gout until 1967, when Wallace performed a clinical study using high-dose colchicine for the treatment of gout and showed its therapeutic effectiveness, despite many patients developing diarrhea (48). This high-dose regimen of colchicine was used as a therapeutic diagnostic test for gout for several decades. In 2010, Terkeltaub et al. found that a loading regimen of low-dose colchicine was as effective as a high-dose regimen,

but with fewer adverse events (49). A recent study showed that a non-loading colchicine regimen was also as effective as a loading low-dose regimen (50).

Aspirin, the first non-steroidal anti-inflammatory drug (NSAID), was identified in 1897 and was synthesized and marketed two years later. Interestingly, aspirin at high dose has a uricosuric effect, whereas at low dose it causes hyperuricemia. However, aspirin was not recommended for the treatment of gout due to concern that it could interfere with SUA levels and aggravate acute gout attacks. The second NSAID, phenylbutazone, synthesized in 1949, was effective in controlling acute arthritis in gout (51). It also has a uricosuric effect. However, it was withdrawn from the market around 1980 due to its association with blood dyscrasia (52). Subsequently, several NSAIDs (such as indomethacin, ibuprofen, naproxen, diclofenac, etc.) were developed and remain widely used in the treatment of gout. However, gastrointestinal toxicity is recognized as a major adverse effect of these compounds. Around 1990, the COX-2 enzyme was identified as a major mediator of inflammation in the prostaglandin pathway. Subsequently, specific COX-2 inhibitors, such as celecoxib and etoricoxib were developed and have been shown to be as effective as traditional NSAIDs, with fewer gastrointestinal side effects (53, 54).

Urate lowering therapy

The fundamental principle of gout treatment is preventing recurrent flares and promoting MSU crystal dissolution. This goal of treatment is not only to control symptoms but also to bring the disease into remission by using ULT. Currently, three classes of ULT are available: xanthine oxidase inhibitors (XOIs; allopurinol, febuxostat, and topiroxostat), uricosuric agents (probenecid, benzbromarone, sulfinpyrazone, and dotinurad), and uricase-based therapy (pegloticase). The concept of treat-to-target (T2T) in gout began around 2012, when physicians realized that bringing SUA down below its saturation point, typically < 6.0 mg/dL can lead to dissolution of crystals, prevention of flares, and possibly bring gout into remission. In achieving this goal, patients' education and adherence to medications, as well as SUA monitoring, play a very important role in reaching

the target (55, 56). Currently, clinical remission is defined as no gout flares over 12 months, absence of subcutaneous tophi, and SUA < 0.36 mmol/L (< 6 mg/dL) over 12 months (57). However, this definition does not include criteria confirming complete resolution of MSU crystal deposition from the body.

Several international rheumatology organizations, including the ACR (58), the EULAR (59), and the Asia-Pacific League of Associations for Rheumatology (APLAR) (60), have recommended XOIs, particularly allopurinol, as first-line therapy for hyperuricemia in gout, and consider uricosuric agents when patients cannot tolerate allopurinol or XOIs, or have an inadequate response to them. Uricase-based therapy is reserved for those who fail to respond to XOIs or uricosurics. The major concern regarding XOIs, particularly allopurinol, is the development of severe cutaneous adverse reactions (SCAR) or allopurinol hypersensitivity syndrome (AHS). This life-threatening condition is strongly associated with the presence of HLA B*58:01, which is more prevalent in East and Southeast Asian populations (61, 62). Genetic screening is recommended prior to initiating allopurinol in individuals of East and Southeast Asian descent, including Han Chinese, Koreans, and Thais (58), and in countries or areas where the population has a gene frequency of > 5% (60, 62). However, routine HLA B*58:01 screening prior to allopurinol initiation is complicated in developing and least developed countries by population diversity, limited access to the test, and associated costs, thus hindering widespread implementation. The limitation to widespread implementation of HLA-B*58:01 screening is its relatively low positive predictive value, largely because AHS is rare; therefore, a positive result indicates increased risk rather than certainty of hypersensitivity. Cost-effectiveness analysis of HLA B*58:01 testing before initiating allopurinol in East and Southeast Asia, where the prevalence of HLA B*58:01 is high, has shown conflicting results (63–68).

Looking at the primary pathophysiology of hyperuricemia in gout, there is a defect in urate transporters, particularly URAT1 in renal tubules, leading to diminished renal excretion of uric acid; therefore, use of uricosuric agents that act on URAT1 should address the primary mechanism of hyperuricemia and gout. However, the presence

of urinary tract stones, advanced renal insufficiency, and possible drug–drug interactions with cytochrome P450–metabolized medications are major limitations to their use. Interestingly, a 2025 report from a gout registry from the Asia Pacific region found that only 3% of gout patients received uricosuric agents (69). The very low rate of uricosuric agent use in the Asia Pacific region might be related to the limited availability of uricosuric agents, physicians' lack of familiarity with their use, and the prioritization of XOIs as recommended by international guidelines. A negative history of passing stones or flank pain suggestive of urinary calculi, absence of red blood cells on urinalysis, and a spot urine uric acid-to-creatinine ratio < 0.2 , which suggests uric acid underexcretion, may increase clinicians' confidence in prescribing uricosuric agents (70).

THE FUTURE

Newer imaging modalities

In future clinical practice, gout may still be a clinical diagnosis, with synovial fluid examination reserved for situations where diagnostic uncertainty remains. However, among individuals at risk for gout, identifying MSU crystal deposition earlier, ideally before the first episode of acute arthritis, would be of interest, as this could enable timelier counselling and preventive management. In this context, US and DECT may play a greater role in detecting subclinical MSU crystal depositions prior to the onset of acute arthritis (71, 72). As these technologies continue to become smaller and more portable, their use in ambulatory settings may become more feasible. Nevertheless, their diagnostic yield, particularly for US, will remain operator-dependent and will require appropriate training; therefore, US and DECT are best framed as adjunctive modalities that can support decision-making in selected diagnostically challenging or high-risk cases, rather than as universal necessities for early diagnosis.

Genetic study

In the future, identification of genetic variants that predispose individuals to hyperuricemia may enable a more personalized approach to gout prevention and management (40–42). Genetic testing in children with a family history of gout could help identify individuals who are likely to develop

sustained hyperuricemia and gout, supporting earlier lifestyle counselling, closer monitoring of SUA, and probably initiation of ULT earlier in selected high-risk patients. Genetic profiling may also inform treatment selection by clarifying whether hyperuricemia is driven predominantly by reduced renal urate excretion, impaired extra-renal urate excretion, or both. It also could help predict variability in treatment response or prognosis. Ultimately, genetic markers could be integrated with clinical and imaging data to support precision prevention strategies and optimize long-term outcomes.

Metabolomics

Metabolomics is the comprehensive measurement of small molecules, or metabolites, in biological samples such as blood, urine, or synovial fluid. It provides a real-time assessment of biochemical function and activity that reflects genetic background, diet, comorbidities, the microbiome, and environmental exposures. Metabolomic profiling may support precision medicine by enabling molecular phenotyping, discovery of diagnostic and prognostic biomarkers, and identification of actionable metabolic pathways that could be targeted therapeutically (73). A study has shown that metabolomic profiles differ between the acute and intercritical periods of gout, and that specific metabolites may help identify patients at risk of imminent attacks (74). Therefore, this might help inform earlier or more individualized preventive strategies alongside clinical assessment and imaging.

Newer pharmacological therapy

Looking at the pathogenesis of acute gout, multiple inflammatory pathways are involved, such as NLRP3 inflammasome, the mitogen-activated protein (MAP) kinase family, the cysteine-dependent aspartate-directed protease (caspase) system, the mitochondrial pyruvate carrier (MPC) pathway, TGF- β , and the NET system. These pathways are being investigated as potential therapeutic targets for acute arthritis in gout (33, 75). Several ULTs, including uricosuric agents, XOIs, and uricase-based therapies, are currently being investigated in clinical trials (76). Among these three ULT classes, uricosuric agents are among the most extensively investigated. Many

of them target the URAT1 transporter. This trend indicates that treating gout by targeting the primary pathogenic mechanism of renal urate transport, particularly URAT1, is receiving more attention. As the ABCG2 has recently been found to be involved in urate excretion in the intestine and kidney, drugs targeting the ABCG2 transporter for the management of hyperuricemia will be of interest.

With the advances of nanotechnology, this system has come to play an important role in clinical medicine. It offers innovative solutions to limitations in traditional drug delivery systems. These nanoparticles, ranging from 1-100 nanometers in size, have unique properties, including a high surface area-to-volume ratio, enhanced solubility, and the ability to bind to specific tissues or cells, making them highly effective in delivering therapeutic agents (77). Several types of nanomaterials are used to develop nanoparticles, including polymeric nanoparticles, lipid-based nanoparticles, metallic nanoparticles, magnetic nanoparticles, and silica nanoparticles. Colchicine, allopurinol, and uricase formulations using nanotechnology have recently been under development (78). It might be possible that this technology will be applied to existing and novel therapies to treat gout in the future.

Modification of gut microbiota

Because alterations of the gut microbiome have been shown to be associated with hyperuricemia, manipulation of the gut microbiome is another potential means to decrease SUA (44). Plant-based components, such as ginsenosides, curcumin, and flavonoid compounds, have been shown to reduce SUA (45). Certain strains of *Lactobacillus* species can reduce uric acid production by uptaking and degrading inosine, guanosine, and adenosine in the intestine, thereby reducing the intestinal absorption of uric acid from the gastrointestinal tract (79). For example, a study found that patients with hyperuricemia or gout who took yogurt that contained *Lactobacillus gasseri* PA-33 demonstrated a significant reduction in SUA without serious side effects (80). Therefore, modification of gut microbiota may reduce SUA and could be applicable as a treatment, or as adjunctive treatment, for hyperuricemia and gout in the future.

Restoration of the uricase gene

The completion of the human gene sequence in the early 21st century led physicians to be able to identify areas of uricase gene deletion. Missense mutation at codon 119, and non-sense mutation at codon 33 in the human uricase gene, leading to loss of urate oxidase activity, and the sequence-specific oligonucleotides responsible for these mutations have been identified (81, 82). With advances in gene editing technology, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), scientists are able to edit genes (either insertion or deletion), edit single or multiple base pairs, and regulate gene expression (either activation or suppression), which can treat, prevent, or cure genetically related human diseases (83). A recent study using this technique inserted the deleted uricase gene into human liver cells and found that the liver cells could not only prevent hyperuricemia but also correct other metabolic abnormalities (84, 85). It may be possible to restore this gene in humans in the future.

Uricase restoration: potential benefit and risk

Prooxidant and antioxidant properties of uric acid have been well recognized (86, 87). Urate itself is an electron donor and can trigger several relevant reactive oxygen species (ROS), including superoxide anion, hydroxyl radical, and hydrogen peroxide. These ROS can further stimulate several intracellular signaling pathways related to inflammation. Conversely, uric acid is a major antioxidant in the body, accounting for approximately two-thirds of the body's antioxidant capacity, in part by stimulating superoxide dismutase, a major antioxidant enzyme. These antioxidant properties could prevent cellular destruction and probably help explain the expanding human lifespan.

Whether the higher brain function, particularly cognitive function and intelligence, in humans when compared with other primates, is related to higher SUA as a result of human evolution is not clearly understood. High SUA has been found to be associated with higher intellectual and cognitive function (88). A study in healthy individuals found that higher SUA levels were associated with higher intelligence quotient (IQ) scores (89-91). Another study found that elderly individuals with mild cognitive impairment had lower SUA than those who did not (92).

The effect of SUA on neurological diseases also shows conflicting results. Several meta-analyses, and systematic reviews, have reported that hyperuricemia was associated with an increased risk of stroke, cardiovascular events, and all-cause mortality (93, 94), and hyperuricemia treatment has also been reported to be associated with reduced all-cause mortality and improved cardiovascular outcomes (95). These findings support the prooxidant effects of hyperuricemia and its association with other comorbidities, particularly hypertension.

Regarding neurodegenerative diseases, such as Alzheimer disease (AD), Parkinson disease (PD), and amyotrophic lateral sclerosis (ALS), studies have shown that patients with these neurodegenerative diseases had lower SUA, and that lower SUA levels were associated with advancement and progression of disease (96, 97). These findings may support the antioxidant effect of uric acid in mitigating oxidative stress-induced neuron degeneration and/or its neuro-protective effect. It remains unclear whether lower SUA in neurodegenerative disease partly reflects reverse causality (e.g., disease-related weight loss, reduced caloric intake, inflammation, or frailty), rather than a direct causal role of SUA. However, there is also evidence that hyperuricemia can have detrimental effects on brain tissue. High SUA has been shown to be correlated with cognitive impairment, and patients with gout also had smaller brain volume and were vulnerable to the development of neurodegenerative disease (98, 99), as well as an increased risk of stroke (100). This appears to contrast with the studies above linking higher SUA with better cognitive performance; however, gout and hyperuricemia frequently coexist with cardiometabolic comorbidities and systemic inflammation, which may mediate vascular brain injury and cognitive decline. Therefore, the long-term balance between the benefits and risks of low SUA resulting from uricase restoration should be considered.

CONCLUSIONS

Gout results from the evolutionary loss of functional uricase in humans, which leads to hyperuricemia and subsequent MSU crystal deposition. This evolutionary change, which led to higher SUA levels in humans than in other primates, may

have been associated with enhanced intellectual and cognitive function. Although the clinical features and course of gout are well recognized and effective treatments are available, unmet needs in gout management remain. Advances in nanotechnology may enable newer and more effective drug-delivery strategies, improve efficiency and allow more targeted therapy. In addition, progress in human genome editing may eventually permit restoration of this ancient gene. However, evidence regarding the association between SUA and neurodegenerative disease and stroke remains conflicting. Therefore, any attempt to restore the uricase gene should be approached cautiously, with close attention to potential long-term neurological and vascular outcomes.

ACKNOWLEDGMENTS

None

FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

CONFLICTS OF INTEREST

The author declares no conflict of interest

AUTHOR CONTRIBUTION

W.L. was solely responsible for conceptualization, literature review, and writing (original draft and editing) of this manuscript.

DATA AVAILABILITY STATEMENT

Not applicable

INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable

INFORM CONSENT STATEMENT

Not applicable

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The Potential of Pomegranate as a Therapy for Thyroid Eye Disease: A Systematic Review

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Received: August 8, 2025;

Revised: October 4, 2025;

Accepted: November 26, 2025

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ABSTRACT

Pomegranate (*Punica granatum*), a natural antioxidant, may help reduce oxidative stress and inflammatory markers in thyroid eye disease (TED), an autoimmune disorder affecting approximately one-third of patients with thyroid disease. This systematic review evaluated its efficacy and safety in TED management. Following PRISMA 2020 guidelines, a comprehensive search of ScienceDirect, PubMed, and SagePub identified English-language full-text articles published from 2010 to 2024. Editorials and non-DOI reviews were excluded. From over 400 publications screened, eight met the inclusion criteria. Evidence suggests that quercetin, a key flavonoid in pomegranate, can neutralize superoxide anions, inhibit lipid peroxidation, and induce apoptosis in tumor cells. In-vitro studies further indicate that quercetin suppresses fibroblast proliferation and reduces hyaluronic acid secretion in TED. These findings support pomegranate's potential as an adjunct therapy for TED; however, further clinical trials are needed to establish optimal dosage, formulation, and bioavailability for therapeutic use.

KEYWORDS pomegranate, quercetin, thyroid eye disease, antioxidant, oxidative stress

INTRODUCTION

Thyroid eye disease (TED) is a complex immune-mediated inflammatory disease and the most common underlying factor in orbital disease globally (1, 2). The clinical presentation of TED remains consistent across various thyroid disorders and shows no significant differences between unilateral and bilateral cases (3). Patients with TED experience reduced quality of life (QoL) from visual impairment and psychosocial stress. While medical and surgical treatments can help, early diagnosis and timely management are essential to prevent progression to sight-threatening stages (4, 5). Therefore, ongoing research is focused on exploring therapeutic strategies to reduce the impact of this disease (6).

Oxidative stress contributes to TED and other endocrine autoimmune disorders. Normally reg-

ulated for cellular balance, its disruption causes cellular damage and inflammation that drive disease progression (7).

Increased markers of oxidative stress have been linked to the development of hyperthyroidism and TED, suggesting that oxidative stress may exacerbate the inflammatory process in orbital tissues (7, 8). This highlights antioxidants as potential therapies, as they can modulate oxidative stress and inflammation, making them valuable adjuncts in managing TED and related disorders.

Pomegranate (*Punica granatum*), in the Punicaceae family, has long been used medicinally for its anti-inflammatory and astringent effects, especially in wound healing and gastrointestinal infections (9).

Pomegranate seeds, arils, and peel contain bioactive compounds, with the peel richest in

medicinal components such as minerals and polyphenols (10).

The strong antioxidant and anti-inflammatory properties of pomegranate have been studied in various clinical and preclinical trials, demonstrating its potential in managing diabetes, cardiovascular diseases, and cancer (10, 11).

Clinical evidence shows pomegranate reduces oxidative stress and inflammation in rheumatoid arthritis and, with long-term use, lowers blood pressure and carotid artery thickness in hypertension (12).

Although promising, research on pomegranate's effects in thyroid-related degeneration is limited. This study investigates its effectiveness and safety as an antioxidant in TED and explores its therapeutic potential in thyroid.

METHODS

Protocol

This study strictly adheres to the PRISMA 2020 guidelines to ensure methodological rigor and maintain the highest quality of research. By following these guidelines, the review process was designed to be transparent, reproducible, and grounded in strong scientific principles. Key procedures applied include comprehensive literature searches, accurate data extraction, and careful synthesis of findings to minimize bias. This stringent approach not only enhances the credibility of the study but also strengthens the reliability of the conclusions, providing a solid foundation for evidence-based research. This systematic review primarily examines the role of pomegranate in the management of TED and encompasses experimental studies, including human and animal studies.

Eligibility criteria

This study aimed to evaluate the role of pomegranate as an antioxidant through a systematic review and data synthesis of various studies. By identifying emerging patterns and trends in the literature, this research is expected to contribute to the development of more effective therapeutic approaches and patient care strategies. To maintain the reliability of the analysis, this study applied tight eligibility and exclusion standards, including only peer-reviewed research published from 2010 to 2024. Authenticity of each study

was verified using DOI, while non-research documents such as reviews, editorials, and duplicate entries were excluded to maintain the quality and focus of the dataset.

This careful selection approach ensures that the analysis is based on reliable and relevant sources, thereby enhancing the validity of the findings. With a meticulous methodology, this study is expected to deepen the understanding of pomegranate's potential as an antioxidant in TED cases. Ultimately, the findings of this study could contribute to the improvement of patient care strategies and enhance treatment outcomes for those affected by this disease.

Search strategy

This systematic review was conducted in accordance with the PRISMA guidelines to evaluate the potential of pomegranate as a therapy for thyroid eye disease. An extensive literature search was independently carried out by the authors across three electronic databases: PubMed, ScienceDirect, and Google Scholar. The search covered studies published from the inception of each database in 2010 to 2024. The search strategy combined relevant keywords, including "thyroid," "antioxidant," "*Punica granatum*," "flavonoid," and "oxidative stress." Boolean operators (AND, OR) were applied to refine the search and ensure the inclusion of all pertinent studies.

Risk of bias

The assessment of risk of bias for the included studies in this review was conducted using the OHAT Risk of Bias Tool (Table 1), which is specifically designed for mechanistic, in-vitro, and in-vivo experimental studies. This tool evaluates bias across key domains including selection of samples, potential confounding factors, consistency of interventions, validity of outcome measurements, completeness of data, selective reporting, and other potential sources of bias such as funding or conflicts of interest. Each study was independently evaluated within these domains and categorized as having a low risk of bias, some concerns, or a high risk of bias. Overall, the majority of studies demonstrated a low risk of bias in domains related to performance, detection, attrition, and reporting, reflecting methodological rigor in experimental execution and outcome

Table 1. Critical appraisal of studies with OHAT risk of bias (RoB) Tools

Domain	Yoon et al. (2012)	Lisi et al. (2011)
Selection bias	Tissue origin well described: 13 GO patients and normal blepharoplasty controls; minimized selection bias	Fibroblasts obtained from GO patients and controls, with clear methodological description; low selection bias
Confounding bias	Some concerns due to variability in patient-derived tissues; prior treatments may have influenced responses	Some concerns as donors differed in clinical history and some had prior therapy (e.g., corticosteroids, thyroidectomy)
Performance bias	Quercetin applied at graded doses with proper controls; culture conditions consistent	Flavonoids tested in multiple concentrations with appropriate controls under standardized conditions
Detection bias	Outcomes assessed with validated assays (Western blot, ELISA, RT-PCR, zymography, migration assay)	Outcomes measured with validated assays (MTT, Western blot, flow cytometry, cytokine/HA assays)
Attrition bias	Experiments repeated ≥ 3 times, no missing data reported	All experiments performed in triplicate, results consistently reported
Reporting bias	All prespecified outcomes (fibrotic markers, MMPs, TIMPs) were reported	Outcomes (proliferation, apoptosis, HA production, LDH, cell cycle) reported comprehensively
Other bias	Funded by public grants; no conflicts of interest declared	Government-funded; no conflicts declared
Overall assessment	Low-moderate risk of bias, with primary concerns related to confounding due to donor variability	Low-moderate risk of bias, mainly due to potential confounding from prior patient treatments

reporting. Some concerns were noted primarily in the domain of confounding bias, given the variability of human-derived samples and differences in prior clinical treatments that could influence fibroblast characteristics. Importantly, no study was judged to have a consistently high risk of bias across all domains. This systematic assessment enhanced transparency and strengthened the reliability of the review process, ensuring that conclusions were supported by evidence derived from studies with clearly defined and systematically appraised risk profiles. The SYRCLE tool revealed limitations primarily related to allocation concealment, blinding, and incomplete outcome data, which were inconsistently addressed across investigations. Overall, while several included studies demonstrated methodological robustness, variability in quality was evident across designs. These findings emphasize the need for greater methodological rigor and consistent reporting in future research to ensure the validity and reliability of conclusions regarding the therapeutic potential of pomegranate in TED. Table 2 presents the critical appraisal of the included studies using the SYRCLE's Risk of Bias tool.

PICOS framework

The PICOS framework, comprising population, intervention, comparison, outcome, and study design, serves as a pivotal tool for structuring systematic review questions and refining eligibility criteria. By breaking down the research question into these specific domains, PICOS ensures that study selection is aligned with the objectives of the review. This structured approach enhances both the coherence and methodological rigor of the synthesis. Moreover, by explicitly articulating inclusion and exclusion criteria, the PICOS framework improves the accuracy of literature searches, minimizes retrieval of irrelevant studies, and accelerates the screening of pertinent research across multiple databases.

In this systematic review, the PICOS framework delineated the principal criteria guiding the selection of studies investigating the therapeutic potential of pomegranate (*Punica granatum*) in TED. Table 3 outlines the PICOS framework, which defines the key criteria used to determine study inclusion in this systematic review examining the potential of pomegranate as a therapy for thyroid eye disease. The population of interest includes both human subjects clinically diagnosed

Table 2. Critical appraisal with SYRCLE risk of bias tools

SYRCLE domain	EJH - Noya (MSG+PPE)	JMH - Mahmoud (Mancozeb+PF)	Benha - Mousa (Semicarbazide +PP)	Kufa - Arrak (Lead+Ellagic)	Anatomical record - El-Din (Lithium+PPE)
1. Sequence generation (allocation sequence adequately generated?)	'Randomly divided' but no method described	Randomization stated, method not detailed	Split into groups 'at random' but no sequence method	'Randomly divided', no randomization method reported	'Randomly divided' reported, allocation method not specified
2. Baseline characteristics (groups similar at baseline?)	Baseline weight/age reported and groups appear balanced	Baseline weight/age reported; groups described and comparable	Baseline weights/ages reported and balanced	Baseline data provided (age, weight) and balanced	Baseline characteristics (age/weight) reported and comparable
3. Allocation concealment	Allocation concealment not reported	Not reported	Not reported	Not reported	Not reported
4. Random housing (animals randomly housed?)	Housing procedures described but random housing not stated	Housing conditions reported but no randomization of cage placement	Standard housing, no random housing statement	Housing conditions described; random housing not stated	Cages/conditions described; random housing not stated
5. Blinding of caregivers/investigators (performance bias)	No blinding of caregivers/investigators reported	Blinding not described; probable high risk	No blinding of caretakers reported	No blinding reported	No blinding reported
6. Random outcome assessment	Outcome sampling not described as randomized	Morphometry tests fields/sections but random selection not explicit	Morphometry described, selection randomness not stated	Morphometry methods described, random field selection not explicit	Measurement/fields described, not explicitly randomized
7. Blinding of outcome assessor (detection bias)	High - No blinding of assessors reported	IHC and image analysis described but blinding not reported	No outcome assessor blinding described	No blinding of histology/IHC readers reported	IHC and image analysis done; blinding not reported
8. Incomplete outcome data (attrition)	Low - All animals analyzed; attrition appears minimal	Numbers per group reported and analyzed; no dropouts described	All groups (n) reported; recovery group included; no dropouts	Sample numbers reported; no explicit dropouts	Group sizes and results reported; no dropouts
9. Selective outcome reporting	Prespecified hormonal, histological, IHC outcomes reported	Outcomes described in methods appear in results	Hormonal, histological, IHC outcomes reported as described	Main outcomes reported but no preregistration/protocol to confirm	Main prespecified outcomes (hormones, OS markers, histology) reported
10. Other bias (funding/conflicts/other problems)	Funding/conflict statement not detailed; methods standard	Funding/conflicts minimal; PFE product from industry source (possible COI)	Ethics mentioned; funding/conflicts not clearly described	Older paper (2010); limited conflicts/funding reporting	University grant funding, no conflicts noted

with TED irrespective of age, gender, or ethnicity, and animal models of the disease thereby ensuring broad applicability across diverse demographics and experimental settings.

The intervention centers of pomegranate and its derivatives include juice, peel, seeds, and phytochemical extracts with a particular focus on their antioxidant, anti-inflammatory, and immunomodulatory properties relevant to the pathogenesis of TED. Comparators encompass placebo, no treatment, and standard therapeutic approaches for TED, enabling meaningful evaluation of the efficacy of pomegranate relative to

conventional management.

The outcomes of interest emphasize disease-related biomarkers and clinical or experimental endpoints, with primary measures targeting oxidative stress, inflammation, immune modulation, and indicators of TED progression. Such outcomes are crucial in assessing the potential of pomegranate to alter disease mechanisms and clinical trajectory. Eligible study designs include original experimental studies—in-vivo, in-vitro, animal-based, or clinical—that generate direct evidence of the role of pomegranate in TED. Studies published prior to 2010, those not directly

Table 3. PICOS framework for included studies

PICOS component	Details	Inclusion criteria	Exclusion criteria
P (population)	Humans with thyroid eye disease (TED) regardless of age, gender, or ethnicity and animal models with TED	Humans with TED of any age, gender or ethnicity or animal subjects with TED	Studies published before 2010; studies of unrelated conditions without connection to thyroid disease or ocular manifestations
I (intervention)	Pomegranate (<i>Punica granatum</i>) or its extracts (juice, peel, seed, phytochemicals)	Studies examining potential therapeutic effects of pomegranate in TED. Studies investigating antioxidant, anti-inflammatory, or immunomodulatory effects relevant to TED pathogenesis	Studies not using pomegranate or unrelated interventions
C (comparison)	Placebo, no treatment, or other standard therapies for TED	Documented studies with comparator or baseline for TED-related outcomes	Studies with no outcomes related to TED
O (outcomes)	Clinical or experimental outcomes relevant to TED progression	Outcomes associated with oxidative stress, inflammation, immune modulation, or disease progression in TED	Studies not reporting outcomes directly related to TED
S (study design)	Experimental studies (in-vivo, in-vitro, animal, or clinical) with original data	Original experimental studies relevant to TED	Non-experimental or non-original research (editorials, reviews, commentaries, opinion pieces, non-English publications)

addressing TED outcomes, investigations unrelated to thyroid disease or ocular involvement, and non-experimental or non-original research (e.g., reviews, editorials, commentaries, and non-English publications) were excluded to maintain methodological rigor and the relevance of the evidence base.

Data retrieval

After conducting the initial search, the authors reviewed each article by assessing the title and abstract to determine relevance before performing more comprehensive assessment. Studies that aligned with research objectives and which met eligibility criteria were selected for subsequent evaluation, ensuring the detection of coherent and relevant patterns. To maintain consistency and comparability of findings, only complete articles written in English were considered. A thorough screening procedure was implemented to ensure all included studies truly supported the research objectives.

A comprehensive evaluation was carried out on the title, authors, publication date, research location, and methodology, ensuring that the final dataset remained focused, high-quality, and rele-

vant. This systematic approach enhances the reliability of the findings and provides a strong foundation for drawing credible conclusions that can be applied in further research contexts.

Assessment of quality and data compilation

The authors implemented a thorough initial screening process by evaluating each article's title and abstract which were reviewed to identify relevant studies for further review. Only studies that met the specified relevance criteria proceeded to the in-depth review stage. This stringent approach ensures that the selected studies are contextually relevant and aligned with the research objectives, thereby enhancing the overall quality and relevance of the review. The initial search of three electronic databases—PubMed (n = 4), ScienceDirect (n = 24), and Google scholar (n = 929)—identified a total of 957 records. After the removal of 20 duplicates, 937 records were screened by title and abstract. Of these, 201 articles were published prior to 2010, were not relevant to the objective (486 studies), were non-original research articles or non experimental studies (145 studies), did not have an available full-text pdf (80 studies), or were not published in the English language

(10 studies). A total of 15 full-text reports were sought for retrieval; however, nine could not be accessed. Ultimately, 6 studies fulfilled all inclusion criteria and were incorporated into the qualitative synthesis of this systematic review. The process of study identification, screening, eligibility assessment, and final inclusion is summarized in the PRISMA flow diagram (Figure 1).

RESULTS

The PRISMA flow diagram outlines the study selection process. Following removal of duplicates and screening of titles, abstracts, and full texts, six studies met the eligibility criteria. These comprised animal studies (pomegranate peel extract, PPE) and in-vitro/ex vivo studies in quercetin treatment in orbital fibroblasts and orbital fat tissues.

Characteristics of included studies

The included studies investigated the therapeutic effects of pomegranate on the thyroid. Six experimental studies fulfilled the eligibility criteria and were included in this review. Four were conducted on rodent models of chemically induced thyroid dysfunction, while two investi-

gated the effects of quercetin on primary human orbital fibroblasts obtained from patients with Graves' orbitopathy. The interventions comprised *Punica granatum* derivatives, including whole fruit extract, peel extract, and ellagic acid, as well as quercetin, a structurally related flavonoid. The comparators were either toxicant-exposed animals or untreated fibroblast cultures. Outcome measures across the studies included serum thyroid hormones (T3, T4, TSH), oxidative stress and antioxidant biomarkers (MDA, SOD, GSH), apoptotic indices (caspase-3, Bax), histological and ultrastructural analyses, as well as fibrotic markers (fibronectin, collagen I α , α -SMA) and matrix metalloproteinase (MMP) activity (13-16).

Protective effects of pomegranate in chemically induced thyroid toxicity.

The majority of the animal studies consistently demonstrated that *Punica granatum* extracts exerted protective and restorative effects against diverse toxicological insults. Mahmoud et al. reported profound histo-architectural derangements in rats exposed to mancozeb, characterized by follicular shrinkage, reduced colloid content, and increased oxidative stress biomarkers Co-ad-

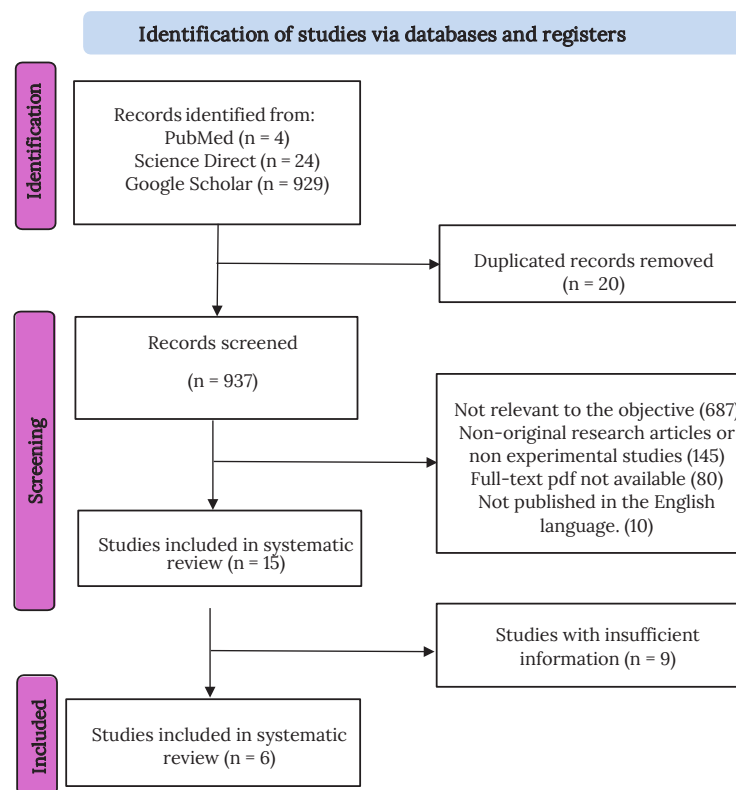


Figure 1. Literature search flow diagram

ministration of standardized pomegranate fruit extract significantly mitigated these alterations, restoring follicular integrity, normalizing thyroid hormones, and reducing PCNA immunoreactivity, thereby highlighting an antioxidative and anti-proliferative mechanism. That study reported pomegranate juice supplementation significantly improved oxidative stress indices in hypothyroid rats. Malondialdehyde (MDA) decreased by 37% ($p < 0.01$), catalase activity increased by 45% ($p < 0.05$), and thyroid weight normalized compared with hypothyroid controls. Serum TSH was reduced by 41% ($p < 0.05$), whereas T3 and T4 were increased by 32% and 36%, respectively (13). Mousa et al. evaluated ellagic acid as an adjuvant therapy in thyroid-disrupted models. Ellagic acid administration significantly reduced histopathological damage scores by 43% ($p < 0.01$) and improved antioxidant enzyme activity by 38% and 47%, respectively. Hormonal assays showed a 29% increase in serum T4 and a 22% reduction in TSH compared to untreated hypothyroid rats ($p < 0.05$). This study found that semicarbazide exposure disrupted the endocrine and structural integrity of the thyroid gland, resulting in decreased PAS staining, increased collagen deposition, and elevated caspase-3 expression. Pomegranate peel extract (PPE) co-treatment restored hormonal balance, reduced apoptosis, and preserved nearly normal thyroid histology, with a protective effect superior to that observed in the recovery-only group, suggesting a potent antioxidant-mediated cytoprotective role (14). Arrak, JK investigated ellagic acid, a polyphenolic constituent of pomegranate, ellagic acid (100-200 mg/kg), against lead acetate toxicity in adult rats. Lead exposure significantly decreased serum T3 and T4 levels by 35-40% at weeks 4 and 6 ($p < 0.01$), alongside reductions in parathyroid hormone (PTH) and calcium. Treatment with ellagic acid (200 mg/kg) restored thyroid hormones to near-control values, with T3 and T4 increasing by 28% and 31%, respectively, compared to the lead-only group ($p < 0.05$). PTH levels were significantly elevated by 24% at day 42, and serum calcium increased by 18% ($p < 0.05$), demonstrating a dose-dependent amelioration. Lead intoxication reduced T3, T4, and Ca^{2+} levels while suppressing PTH secretion. A therapeutic dose of ellagic acid produced significant hormonal restoration, in

contrast to the subtherapeutic protective dose, underscoring a dose-dependent curative potential (16). El-Din et al. examined lithium carbonate (LC) (30 mg/kg)-induced hypothyroidism in rats. That study found lithium exposure decreased FT3 and FT4 levels by 42% and 38%, respectively ($p < 0.001$), and increased TSH by 2.5-fold ($p < 0.001$). MDA was significantly elevated (+65%, $p < 0.01$), while superoxide dismutase (SOD) activity was reduced (-45%, $p < 0.01$). Co-administration of PPE (250 mg/kg) restored FT3/FT4 to 90% of control values and reduced TSH by 48% relative to the lithium-only group ($p < 0.01$). Oxidative stress markers normalized, with MDA reduced by 40% and SOD elevated by 52% ($p < 0.05$), accompanied by histological evidence of reduced follicular apoptosis. In conclusion, the study demonstrated that chronic lithium administration produced follicular involution, increased Bax expression, and reduced antioxidant capacity (SOD), accompanied by disorganized ultrastructural morphology. Administration of PPE attenuated these deleterious changes, reestablishing antioxidant defense, decreasing pro-apoptotic markers, and preserving ultrastructural features, suggesting a dual mechanism of antioxidative and antiapoptotic modulation (15). Taken together, these animal studies establish convergent evidence that *Punica granatum* derivatives are capable of ameliorating chemically induced thyroid dysfunctions through restoration of endocrine homeostasis, mitigation of oxidative stress, and inhibition of apoptosis.

Quercetin and thyroid-associated orbitopathy

Two in-vitro studies elucidated the potential therapeutic role of quercetin in modulating fibroblast behavior relevant to the pathogenesis of Graves' orbitopathy (GO). Lisi et al. assessed the impact of quercetin (30-150 μM) on orbital fibroblasts from patients with GO (17). Quercetin significantly reduced fibroblast proliferation beginning at 30 μM , with a 46% reduction at 100 μM compared to untreated controls ($p < 0.01$). Hyaluronic acid (HA) release was reduced by 35% ($p < 0.05$), whereas apoptosis was not significantly affected. Rutin and quercitrin showed no significant activity, indicating quercetin's specific efficacy. The study demonstrated that quercetin inhibited proliferation of orbital fibroblasts de-

rived from both GO patients and controls, beginning at a concentration of 30 μM . This effect was mediated via necrosis and cell cycle arrest, without influencing apoptosis, and was accompanied by a significant reduction in HA secretion, an early pathogenic hallmark of GO. Notably, glycosidic derivatives of quercetin (rutin and quercitrin) did not reproduce these effects, suggesting that the aglycone form is critical for bioactivity (17). Yoon et al. further confirmed the antifibrotic effects of quercetin in primary GO orbital fibroblasts and whole orbital tissue cultures. Quercetin (10–50 μM) dose-dependently suppressed TGF- β -induced fibronectin (–40%, $p < 0.01$) and collagen Ia expression (–35%, $p < 0.05$). IL-1 β -stimulated MMP-9 secretion decreased by 48% ($p < 0.01$), whereas MMP-2 activity was modestly enhanced at low doses (+15%, NS) but suppressed at higher doses (–30%, $p < 0.05$). In tissue explants, quercetin reduced collagen mRNA by 33% and MMP-9 secretion by 41% ($p < 0.01$), supporting its potential role in preventing fibrotic remodeling. The study extended these findings by showing that quercetin exerted dose-dependent antifibrotic effects in both primary orbital fibroblasts and whole orbital fat tissue cultures. Specifically, quercetin suppressed TGF- β -induced fibronectin and collagen Ia expression, modulated IL-1 β -stimulated MMP activity (downregulating MMP-9 while differentially affecting MMP-2), and inhibited α -SMA expression, thereby attenuating fibroblast-to-myofibroblast differentiation. These data support the hypothesis that quercetin interferes with extracellular matrix remodeling, a process central to fibrosis in GO (18).

Integrated evidence synthesis

Across experimental models, *Punica granatum* derivatives consistently demonstrated protective roles against chemically induced thyroid insults, mediated by antioxidative, anti-inflammatory, and antiapoptotic pathways. Conversely, quercetin targeted the fibroproliferative and fibrotic pathways of GO, exerting anti-proliferative and anti-fibrotic actions in-vitro. While the former highlights the ability of pomegranate to preserve endocrine organ function under toxic stress, the latter illustrates the capacity of flavonoids to modulate autoimmune-mediated orbital remodeling. Collectively, these findings underscore

the translational potential of polyphenolic compounds as adjunctive strategies in thyroid-related pathologies, although further in-vivo validation and clinical translation remain warranted.

DISCUSSION

TED is primarily caused by orbital inflammation resulting from the activation of fibroblasts in the orbital tissues (19). Over the years, various pathogenic mechanisms have been proposed, but recent research highlights the crucial role of CD34+ fibroblasts in the development of this disease. These fibroblasts respond to molecular signals by differentiating into adipocytes and myofibroblasts, contributing to the tissue remodeling process within the orbit. Additionally, CD34+ fibroblasts produce thyroglobulin, thyroid-stimulating hormone (TSH), and various other thyroid-related peptides, which interact with TSH receptors and the complex of insulin-like growth factor I (IGF-1). This signaling cascade triggers adipogenesis and increases the synthesis of HA by fibroblasts, leading to an increased accumulation of orbital fat tissue and excessive glycosaminoglycan buildup in the extraocular muscles (EOMs) (20). In clinical manifestation in patient with active TED, presenting with pronounced proptosis, retraction of both upper and lower eyelids, and associated conjunctival redness and swelling. Consequently, the expansion of soft tissue volume in the orbit causes intraorbital congestion and increased pressure, ultimately resulting in the characteristic pathological features of TED (21).

The antioxidant potential of pomegranate is mainly due to its capacity to neutralize reactive oxygen species (ROS) and suppress oxidative stress, two processes that play a crucial role in the development of TED. Studies have shown that pomegranate extract effectively neutralizes free superoxide anions and hydroxyl radicals, as well as preventing lipid peroxidation and oxidative damage in animal macrophages (22). The high polyphenol content in pomegranate peel is believed to be a major factor contributing to its antioxidant effects. Phytochemical analysis indicates that ellagitannins, particularly punicalagin and its derivatives, are the most abundant polyphenols in pomegranate peel and possess strong antioxidant potential (23, 24).

This systematic review synthesized evidence from six preclinical and in-vitro studies that evaluated the therapeutic potential of pomegranate-derived compounds (ellagic acid, peel and fruit extracts) and quercetin in the context of thyroid dysfunction and GO. Collectively, these studies provide converging evidence that polyphenolic compounds exert antioxidant, endocrine-modulating, and antifibrotic effects through distinct but complementary biological pathways. Four experimental studies conducted in rat models demonstrated that pomegranate extracts and ellagic acid confer significant protection against chemically induced thyroid toxicity. Arrak JK showed that ellagic acid (100-200 mg/kg) restored T3 and T4 levels by up to 31% relative to lead acetate-treated rats, while normalizing calcium and PTH levels of ellagic acid as presented in Table 4. This study demonstrates that ellagic acid extracted from *Punica granatum* (pomegranate) exerts a significant protective and therapeutic effect against lead acetate-induced thyroid and parathyroid toxicity in rats. Exposure to lead acetate resulted in a marked decrease in circulating thyroid hormones (T3 and T4), PTH, and serum calcium levels, accompanied by histological alterations in thyroid and parathyroid tissues. These changes are consistent with the established toxic effects of lead on endocrine organs, particularly through oxidative stress mechanisms and interference with hypothalamic-pituitary-thyroid (HPT) axis regulation (16).

Similarly, El-Din et al. reported that PPE attenuated lithium-induced thyroid dysfunction, restoring FT3 and FT4 to 90% of baseline and reducing TSH by nearly 50%, alongside significant reductions in oxidative stress biomarkers (15). In this study, rats that received LC concurrently with *Punica granatum* (PG) powder at a dose of 250 mg/kg body weight for eight weeks demonstrated marked biochemical and histological improvement compared with those administered LC alone as shown in Table 4. Administration of LC alone induced significant thyroid dysfunction, characterized by decreased serum T3 and T4 levels, elevated TSH, and degenerative changes in follicular epithelial cells with colloid depletion. These alterations are consistent with lithium's well-established toxic effects, mainly mediated through oxidative stress, mitochondrial

dysfunction, and inhibition of iodide uptake and hormone synthesis. However, co-administration of PG powder effectively mitigated these deleterious effects, highlighting its potent antioxidant and cytoprotective properties. The polyphenols, flavonoids, and ellagitannins contained in pomegranate act as strong free radical scavengers and inducers of endogenous antioxidant enzymes such as SOD, catalase (CAT), and glutathione peroxidase (GPx). This enzymatic upregulation reduces MDA levels, thereby minimizing lipid peroxidation and preserving the integrity of thyroid follicular cell membranes. Moreover, PG supplementation exhibited antiapoptotic and anti-inflammatory activities, evidenced by decreased caspase-3 expression, increased Bcl-2 expression, and reduced inflammatory cell infiltration within thyroid tissue. These effects contribute to the maintenance of cellular viability and the prevention of apoptosis induced by lithium-mediated oxidative stress. After eight weeks of treatment, the combined LC and PG group showed a significant recovery of thyroid hormonal homeostasis, with T3 and T4 levels approaching normal and TSH levels markedly reduced compared with the LC-only group. These results suggest that the protective effects of PG are both dose and time-dependent, as prolonged administration allows sustained antioxidant action and gradual tissue repair. Histopathological analysis supported the biochemical findings, revealing nearly normal thyroid architecture in the LC and PG groups, characterized by cuboidal follicular epithelium and homogeneous colloid. In contrast, the LC group displayed follicular distortion, epithelial vacuolation, and inflammatory infiltration. The preservation of follicular morphology indicates that PG not only prevents oxidative injury but may also facilitate tissue regeneration. In summary, oral administration of pomegranate powder at 250 mg/kg for eight weeks effectively alleviated lithium-induced thyroid toxicity through its antioxidant, antiapoptotic, and anti-inflammatory mechanisms. This nutraceutical intervention helps maintain redox balance, stabilize cellular membranes, and restore thyroid structural and hormonal integrity. In conclusion, PPE offers significant protection against lithium-induced thyroid damage by maintaining antioxidant capacity, inhibiting apoptosis, and attenuating inflammation.

Table 4. Summary of evidence from included studies

Author (Year)	Model / design	Intervention	Outcomes measured	Key findings
Mahmoud et al. (2023) (13)	Rats, mancozeb-induced thyroid toxicity	Pomegranate fruit extract (150 mg/kg/day)	Serum T3, T4, TSH; oxidative stress biomarkers (SOD, MDA, GSH); histology; PCNA	PFE ameliorated mancozeb-induced thyroid damage, restored hormones, reduced oxidative stress, preserved follicular structure.
Mousa et al. (2025) (14)	Rats, semicarbazide-induced thyroid toxicity	Pomegranate peel extract (500 mg/kg/day)	Serum T3, T4, TSH; histology (PAS, collagen, follicular integrity); caspase-3	PPE improved hormonal balance, reduced apoptosis, restored near-normal histology, superior to recovery-only group.
Arrak (2010) (16)	Rats, lead acetate-induced thyroid & parathyroid toxicity	Ellagic acid (100-200 mg/kg) from pomegranate	Serum T3, T4, parathyroid hormone, Ca ²⁺ ; histology of thyroid and parathyroid	Therapeutic dose of ellagic acid restored thyroid/parathyroid function and Ca ²⁺ levels; protective dose less effective.
El-Din et al. (2022) (15)	Rats, lithium-induced thyroid dysfunction	Punica granatum (PG) powder (250 mg/kg/day)	Serum T3, T4, TSH; SOD, MDA; histology; Bax expression; ultrastructural features	PPE reduced lithium-induced follicular involution, oxidative stress, apoptosis; preserved ultrastructure.
Lisi et al. (2011) (17)	Primary orbital fibroblasts (Graves' orbitopathy)	Quercetin (30-150 µM) vs rutin/quercitrin	Fibroblast proliferation, necrosis, apoptosis, hyaluronic acid release	Quercetin inhibited fibroblast proliferation and HA secretion via necrosis and cell cycle blockade; rutin/quercitrin ineffective.
Yoon et al. (2012) (18)	Primary orbital fibroblasts & orbital fat tissue (Graves' orbitopathy)	Quercetin (10-50 µM)	Fibronectin, collagen Iα, α-SMA, MMP-2/9, TIMP; fibrosis markers	Quercetin suppressed fibrotic markers, modulated MMP activity, and inhibited fibroblast-to-myofibroblast differentiation.

These findings provide strong experimental evidence for the therapeutic potential of PPE in mitigating thyroid gland dysfunction associated with oxidative stress. Further investigations, including clinical studies, are recommended to explore the translational relevance of these results and to optimize dosage regimens for safe and effective human application (15).

These findings are also corroborated by Mahmoud et al. which reported that pomegranate fruit extract (150 mg/kg/day) significantly ameliorated mancozeb-induced thyroid damage in rats, as evidenced by normalized serum thyroid hormone levels (T3, T4, TSH), reduced oxidative stress markers (SOD, MDA, GSH), and preserved histological architecture as detailed in Table 4 (13). Administration of pomegranate fruit extract protected the thyroid gland from the toxic effects of Mancozeb in adult male rats. Rats treated with

Mancozeb alone showed clear signs of thyroid dysfunction, including a significant reduction in serum T3 and T4 levels, elevated TSH, and marked histological changes such as follicular atrophy, loss of colloid, and cytoplasmic vacuolations. These findings confirm the well-known thyroid-disrupting action of Mancozeb, mainly due to its ability to generate oxidative stress and inhibit thyroid peroxidase activity, which impairs hormone synthesis. In contrast, co-administration of pomegranate fruit extract markedly improved both hormonal and structural parameters of the thyroid gland. Treated rats showed near-normal levels of T3, T4, and TSH, with preserved follicular architecture and dense colloid content. These improvements can be attributed to the strong antioxidant and anti-inflammatory properties of pomegranate bioactive compounds such as ellagic acid and punicalagin which neutralize ROS,

reduce lipid peroxidation, and enhance endogenous antioxidant enzymes like SOD and catalase (13) (Table 4).

Mousa et al. showed that semicarbazide exposure caused significant thyroid dysfunction and tissue damage in adult male rats, while PPE provided a clear protective effect (14). Rats that received SEM alone had markedly decreased levels of T3 and T4, with elevated TSH, indicating hypothyroidism. Histological findings confirmed these results, showing degenerated and vacuolated follicular cells, disrupted follicles, loss of colloid, and inflammatory cell infiltration. These alterations are consistent with the toxic effects of SEM, mainly due to oxidative stress, increased free radical generation, and apoptosis within thyroid tissue. In contrast, rats treated with PPE (500 mg/kg/day) along with SEM showed significant improvement as summarized in Table 4. Thyroid hormone levels were restored toward normal values, and histological examination revealed almost normal follicular architecture with well-preserved colloid and minimal cellular damage. PPE also reduced collagen deposition and caspase-3 expression, indicating decreased fibrosis and apoptosis. These beneficial effects are attributed to the strong antioxidant and anti-inflammatory properties of PPE, which contains bioactive compounds such as ellagic acid and punicalagin that scavenge free radicals and protect thyroid cells from oxidative injury. In summary, PPE effectively protects the thyroid gland from semicarbazide-induced toxicity by reducing oxidative stress, inflammation, and apoptosis while restoring normal thyroid function. These results suggest that PPE could serve as a natural therapeutic agent for preventing thyroid dysfunction caused by chemical or environmental toxins. Further studies are needed to confirm these effects and to determine the optimal safe dose for clinical application (14) (Table 4).

Two in-vitro studies focused on quercetin's role in modulating fibroblast proliferation and extracellular matrix remodeling in GO. Lisi et al. reported that quercetin significantly suppressed orbital fibroblast proliferation by 46% at 100 μ M and reduced HA secretion by 35% without inducing apoptosis. When treated with quercetin at concentrations of 30-150 μ M, fibroblast growth and HA secretion were markedly reduced as shown in Table 4. The inhibitory effect was asso-

ciated with cell cycle arrest and necrosis, suggesting that quercetin interferes with fibroblast viability and extracellular matrix production. In contrast, other related flavonoids such as rutin and quercitrin did not show similar effects, indicating that the non-glycosylated (aglycone) form of quercetin is necessary for its biological activity. The reduction of fibroblast proliferation and HA release is particularly important in the context of GO, where excessive fibroblast activation contributes to orbital tissue expansion, inflammation, and fibrosis. By suppressing these processes, quercetin may help limit the pathological tissue remodeling that underlies the clinical manifestations of GO. The results also suggest that quercetin's effects are linked to its antioxidant and signaling-modulating properties, potentially involving inhibition of key proliferative pathways such as PI3K/Akt and MAPK. The lack of effect from rutin and quercitrin may be due to their lower cellular uptake and bioavailability compared with quercetin. In summary, quercetin acts as a potent inhibitor of orbital fibroblast proliferation and HA secretion via necrosis and cell cycle blockade, demonstrating potential as a natural therapeutic compound for controlling tissue remodeling in GO. Further studies are required to confirm these findings in-vivo and to evaluate its safety and clinical applicability (17).

Yoon et al. demonstrated that quercetin 10-50 μ M of quercetin increased MMP-2 expression and activity, but dose-dependently suppressed MMP-9 expression and activity as summarized in Table 4. In tissue cultures, quercetin dose-dependently inhibited MMP-2 and -9 activity and secretion, but 30 and 50 μ M of quercetin increased tissue MMP-2 mRNA. MMP-9 and collagen I α mRNA levels were dose-dependently suppressed. In conclusion, quercetin inhibited fibrotic markers and affected MMP-2 and MMP-9 activities in primary cell and orbital fat tissue cultures from GO at nontoxic concentrations. These results support the potential use of quercetin for active inflammation and treatment or prevention of chronic fibrosis in GO (18).

Integrative interpretation and translational relevance

The convergence of evidence from animal and cell-based studies suggests that pomegranate extracts and quercetin act on complementary

axes of thyroid pathology and pomegranate compounds primarily restore endocrine and redox balance while quercetin modulates fibrotic and inflammatory pathways in orbital fibroblasts. This dual action positions them as promising nutraceutical or adjuvant candidates in managing thyroid dysfunction and GO. Nevertheless, caution is warranted. All included studies were either animal models or in-vitro experiments, limiting direct clinical extrapolation. The heterogeneity in experimental design, differences in polyphenol type (fruit, peel, or isolated ellagic acid), dose (100–500 mg/kg in animals; 10–150 μ M in cells), and duration of exposure, further complicates direct comparison. Additionally, none of the studies conducted pharmacokinetic or bioavailability analyses, which remain critical for understanding whether effective concentrations can be achieved in humans.

Strengths, limitations, and future research

A strength of this evidence base lies in its consistency: across distinct models and toxins (lead acetate, lithium, mancozeb, semicarbazide), pomegranate extracts consistently restored thyroid hormone levels and oxidative balance. Similarly, quercetin reproducibly suppressed fibroblast proliferation and fibrosis-associated markers in GO cultures. However, limitations include small sample sizes, lack of randomized controlled human trials, and absence of long-term safety data. Future research should focus on well-designed translational studies, including dose-response clinical trials, pharmacodynamic assessments, and comparative studies against standard therapies such as levothyroxine for hypothyroidism or glucocorticoids for GO. Furthermore, combination strategies involving polyphenols with established drugs warrant exploration, as synergistic effects may enhance efficacy while minimizing adverse outcomes.

CONCLUSIONS

This systematic review highlights robust pre-clinical evidence supporting the therapeutic potential of pomegranate polyphenols and quercetin in thyroid dysfunction and Graves' orbitopathy. By targeting oxidative stress, endocrine disruption, fibroblast proliferation, and extracellular matrix remodeling, these compounds provide mech-

anistic plausibility for adjunctive use. Clinical translation remains premature, but the evidence provides a strong rationale for future trials to establish efficacy, safety, and optimal therapeutic regimens.

ACKNOWLEDGMENTS

The authors would like to express their gratitude to all researchers whose work was included in this systematic review. We also acknowledge the contributions of colleagues and academic mentors who provided constructive feedback during the preparation of this manuscript.

FUNDING

The authors declare that no funding was received for conducting this review or preparing this manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest related to this manuscript.

AUTHOR CONTRIBUTIONS

D.A.W., N.P., A.T., D.H.T: conceptualization, methodology, software, writing – review & editing; D.A., N.P., A.T.: data curation, writing – original draft preparation; visualization, investigation; N.P., A.T., D.H.: supervision, project administration, funding acquisition; D.A.W., N.P., A.T., D.H.H.: software, validation, formal analysis, resources.

All authors have read and agreed to the published version of the manuscript

DATA AVAILABILITY STATEMENT

No new data were generated in this study. All data supporting the findings of this review are derived from previously published articles, which are appropriately cited within the manuscript.

INSTITUTIONAL REVIEW BOARD STATEMENT

Ethical approval was not required for this study, as it is a systematic review of previously published research.

INFORMED CONSENT STATEMENT

Informed consent was not applicable, as this study did not involve the recruitment of human participants.

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***Pseudomonas Putida* Bacteraemia in a Haemodialysis Patient from Karnataka, India: A Rare Case Report**

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Received: August 13, 2025;

Revised: October 18, 2025;

Accepted: November 26, 2025

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ABSTRACT

Pseudomonas putida is a Gram-negative, non-fermenting bacillus traditionally considered a low-virulence environmental organism, but increasingly recognized as an emerging opportunistic pathogen in healthcare settings. Infections are most often reported in immunocompromised patients, particularly those with end-stage renal disease (ESRD), diabetes, malignancies, or indwelling medical devices. We report a case of *P. putida* bacteraemia in a 69-year-old female with ESRD on maintenance haemodialysis, type 2 diabetes mellitus, and hypertension, who presented with acute breathlessness, vomiting, diarrhea, and altered sensorium. Blood cultures performed using the BacT/Alert® system grew *P. putida*, confirmed by VITEK 2 Compact and MALDI-ToF. The isolate was resistant to trimethoprim/sulfamethoxazole but remained susceptible to ceftazidime, cefepime, carbapenems, aminoglycosides, and fluoroquinolones. Empiric meropenem therapy was continued upon identification, leading to marked clinical improvement within five days. Four cycles of haemodialysis were administered; however, the patient was discharged against medical advice due to financial constraints, and follow-up cultures could not be obtained. This case underscores the clinical significance of *P. putida* as an emerging nosocomial pathogen in patients with multiple comorbidities. Rapid identification, timely initiation of effective empiric therapy, and antimicrobial stewardship are essential to improve patient outcomes and mitigate complications, particularly as multidrug-resistant strains of *P. putida* are increasingly reported worldwide.

KEYWORDS bacteraemia, chronic kidney disease, haemodialysis, opportunistic infection, *Pseudomonas putida*

INTRODUCTION

Pseudomonas putida (*P. putida*) is a Gram-negative, oxidase-positive, non-fermentative bacillus that is widely distributed in environmental niches such as soil and fresh water (1). Its resilience in adverse conditions allows it to survive on damp hospital surfaces and within moist equipment, making it a potential contaminant in healthcare settings (2, 3). Such environmental persistence provides multiple possible routes for transmis-

sion within hospitals (4).

Although often regarded as having low inherent pathogenicity, *P. putida* may cause opportunistic infections in individuals with impaired immunity, including those with chronic kidney disease (CKD), malignancies, diabetes, or those receiving immunosuppressants or maintenance haemodialysis (1, 5). Only a small number of adult bloodstream infection cases have been documented, most of which have been associated with pneumonia,

catheter-related bloodstream infections (CRBSI), or infections involving the skin and soft tissues (6).

Its clinical relevance is further increased by its capacity for biofilm formation and the acquisition of diverse antimicrobial-resistance mechanisms, both of which can complicate treatment (7). Here, we describe a case of bloodstream infection due to *P. putida* in a haemodialysis recipient with multiple comorbidities, highlighting the diagnostic complexities and treatment considerations when dealing with uncommon opportunistic pathogens in high-risk patient groups.

CASE PRESENTATION

Case history

A 69-year-old female with a known history of CKD stage V on maintenance haemodialysis, ischemic heart disease, type 2 diabetes mellitus (on oral hypoglycaemics), systemic hypertension (on antihypertensives), bilateral moderate non-proliferative diabetic retinopathy, and Grade I hypertensive retinopathy presented to the emergency department with sudden-onset breathlessness, accompanied by multiple episodes of vomiting and loose stools. She had also developed altered sensorium for one day. This was the patient's first admission to our hospital, and no prior medical records were available. She reported febrile episodes lasting approximately five days, one month prior to admission. Additionally, she experienced giddiness and had been on prolonged bed rest with decreased mobility for the past month.

On admission, the patient was pale. Her pulse rate was 86 beats per minute, blood pressure 140/80 mmHg, and oxygen saturation was 98% while receiving BiPAP support. The BiPAP settings included an inspiratory positive airway pressure (IPAP) of 12 cm H₂O, and an expiratory positive airway pressure (EPAP) of 6 cm H₂O. On auscultation, bilateral crepitations and rhonchi were noted. Her random blood glucose was found to be elevated. She was hemodynamically stable at presentation and did not meet criteria for septic shock. She was admitted to the Intensive Care Unit (ICU) for suspected metabolic acidosis.

Initial laboratory investigations demonstrated significant renal dysfunction, with markedly elevated blood urea (122 mg/dL) and serum creatinine (9.85 mg/dL) levels, which were consistent

with the patient's CKD status. Electrolyte analysis showed sodium at 137 mmol/L and mildly elevated potassium at 5.7 mmol/L. Uric acid was raised to 9.5 mg/dL. Glucose estimation indicated a random plasma glucose level of 352 mg/dL and an HbA1c of 6.9%, indicating suboptimal glycemic control. Hematological parameters showed anemia with a hemoglobin level of 6.9 g/dL and a total leukocyte count of 8,170 cells/ μ L. Peripheral smear testing suggested microcytic hypochromic anemia with neutrophilia (95.5%, reference range 40–80%), and serum ferritin was markedly elevated at 1,730 ng/mL. Urine microscopy showed 10–15 leukocytes per high power field, raising the possibility of a urinary tract infection or ongoing inflammation; however, urine culture showed no significant bacteriuria. A chest x-ray demonstrated bilateral heterogeneous opacities suggestive of pulmonary edema. Sputum culture grew normal oropharyngeal flora.

Microbiological evaluation

Blood specimens were obtained aseptically and inoculated in paired BACT/ALERT® Aerobic Culture Media bottles (two bottles per set, BioMérieux, Auvergne-Rhone-Alpes, France). Once the BacT/Alert system signalled growth, subcultures were grown on 5% sheep blood agar, MacConkey agar, chocolate agar, and nutrient agar (Himedia, Mumbai, India) using standard streak plate technique. These plates were incubated at 37 °C for 24–48 hours to evaluate colony appearance and growth patterns across media types.

On nutrient agar (Figure 1a), the colonies appeared moist and irregular without any pigment. Chocolate agar (Figure 1b) demonstrated similar non-pigmented, moist colonies without hemolysis or pigment spread. On 5% sheep blood agar (Figure 1c), the colonies were round, moist, and non-hemolytic, whereas MacConkey agar (Figure 1d) revealed pale, irregular, non-lactose-fermenting colonies. Gram stain examination showed gram-negative rod-shaped bacilli (Figure 2). Both catalase and oxidase reactions were positive. Biochemical profiling demonstrated negative arginine dihydrolysis as well as absence of lysine and ornithine decarboxylase activity.

The organism was identified as *P. putida* with 99% confidence using the VITEK® 2 Compact System (BioMérieux, Marcy l'Etoile, France) with

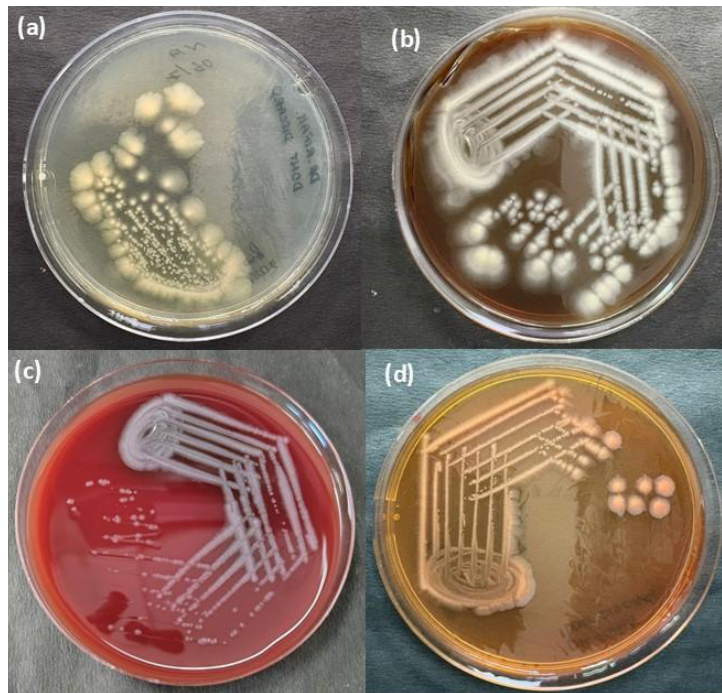


Figure 1. Growth characteristics of *Pseudomonas putida* isolated from a haemodialysis patient on different culture media. (a) The organism produced non-pigmented, moist, irregular colonies on nutrient agar; (b) greyish, moist colonies on chocolate agar; (c) round, moist, non-hemolytic colonies on blood agar; and (d) pale, non-lactose-fermenting colonies on MacConkey agar.

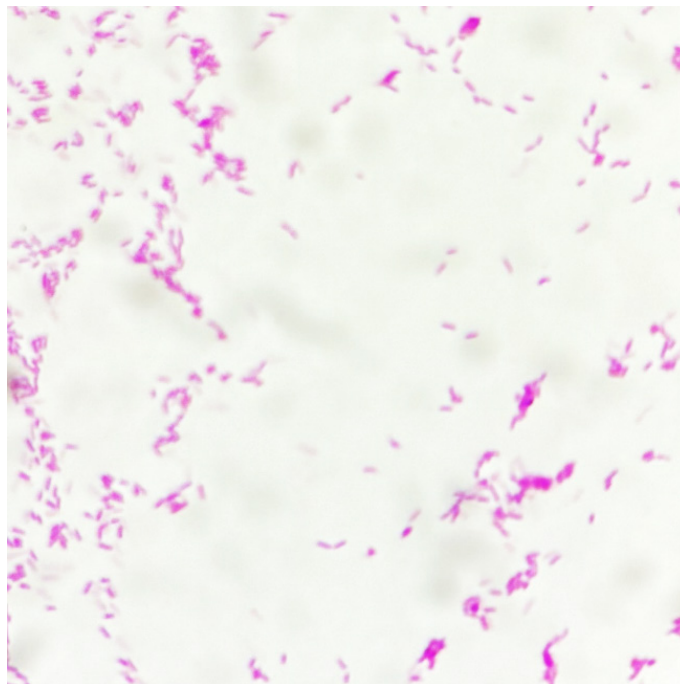


Figure 2. Gram stain from colonies grown on culture plates, confirming the Gram-negative bacillary morphology with uniform staining characteristics.

a Gram-negative identification card, and confirmation was achieved by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-ToF) mass spectrometry (BioMérieux, France).

Antimicrobial susceptibility was assessed on the VITEK® 2 platform using the automated tur-

bidimetric method. MIC values ($\mu\text{g/mL}$) were interpreted following Clinical and Laboratory Standards Institute (CLSI) criteria (8). The isolate exhibited resistance to trimethoprim/sulfamethoxazole ($\geq 320 \mu\text{g/mL}$), intermediate susceptibility to piperacillin/tazobactam ($32 \mu\text{g/mL}$) and cefo-

perazone/sulbactam (32 µg/mL), and susceptibility to ceftazidime (2 µg/mL), cefepime (2 µg/mL), imipenem (2 µg/mL), meropenem (2 µg/mL), gentamicin (≤ 1 µg/mL), ciprofloxacin (0.12 µg/mL), and levofloxacin (0.5 µg/mL).

Clinical course and management

Empiric antibiotic therapy was initiated with intravenous meropenem (1 g stat), followed by 500 mg once daily. This regimen was maintained after *P. putida* was isolated from the patient's blood culture. By day five, the patient demonstrated marked clinical improvement.

Following nephrology consultation, the patient was initiated on haemodialysis during the present admission. She underwent four cycles of dialysis, after which a Perm-cath was inserted to facilitate ongoing vascular access. She was advised to continue dialysis three times per week.

However, due to financial constraints, the patient's family declined further treatment. Consequently, repeat blood cultures could not be obtained, and the patient was discharged against medical advice after completing a total of 5 days of intravenous meropenem therapy. If antibiotic therapy had been continued, de-escalation from meropenem would have been considered according to the susceptibility results and the patient's clinical response, in accordance with antimicrobial stewardship principles.

DISCUSSION

Pseudomonas putida, classified within the fluorescent group of *Pseudomonas*, is an uncommon isolate in clinical microbiology and has long been considered an environmental bacterium with limited pathogenic potential. In recent years, its role as an opportunistic pathogen in healthcare settings has become more evident. Reported infections have frequently involved immunocompromised patients, especially those with indwelling vascular devices, exposure to contaminated medical fluids, recent invasive interventions, or chronic systemic illnesses (3, 5). Although the majority of cases occur in patients with underlying vulnerabilities, infections in immunocompetent individuals have also been documented. For example, Singh et al. (9) described a healthy adult who progressed rapidly to septic shock due to a *P. fluorescens/putida* complex infection, under-

scoring that significant pathogenicity is possible even in the absence of traditional risk factors.

In a four-year review by Yoshino et al., five cases of *P. putida* bacteremia were reported, four of which were linked to medical devices, yielding an overall institutional isolation rate of just 0.22% (10). In the present case, the coexistence of end-stage renal disease (ESRD), diabetes, and anemia likely contributed to the patient's susceptibility. The bacterium's propensity for biofilm development and its ability to acquire multidrug-resistance traits further reinforce the need for rigorous infection prevention strategies (11).

Comparable cases in the literature are limited. Rowe et al. described a 63-year-old patient with diabetes, advanced CKD, and hypertension who developed *P. putida* bacteremia secondary to cellulitis. The isolate was sensitive to multiple β -lactams, and the patient improved with targeted antimicrobial therapy (5). Similarly, Salabei et al. (12) reported bullous cellulitis caused by *P. putida* in an ESRD patient following exposure to contaminated water, highlighting environmental acquisition as a plausible risk factor in dialysis populations. While no direct soft-tissue focus or catheter-related source was identified in our case, transient bacteremia from a subclinical entry site, possibly linked to dialysis access, cannot be excluded.

Overall, the outlook for *P. putida* bloodstream infection is generally positive, with clinical improvement reported in approximately 93% of cases, provided management is guided by culture and susceptibility results, as is standard for Gram-negative bacteremia (13, 14). Nevertheless, increasing reports of carbapenem-resistant strains raise concerns (13, 15). Proposed resistance mechanisms include derepression of chromosomal AmpC β -lactamases, acquisition of plasmid- or integron-borne β -lactamases spanning multiple classes (e.g., carbenicillinases, class A extended-spectrum β -lactamases, class D oxacillinases, and class B metallo- β -lactamases), reduced permeability of the outer membrane, and overexpression of efflux pumps (14).

Beyond β -lactams, variable susceptibility to other antimicrobial classes has also been documented. Tan et al. reported resistance rates of up to 90% for trimethoprim/sulfamethoxazole, and > 50% for quinolones and cefoperazone/

sulbactam. In contrast, aminoglycosides such as amikacin often retain the highest activity, with susceptibility rates of 80–86% (4). Our isolate's gentamicin susceptibility aligns with these observations, although nephrotoxicity limits aminoglycoside use in ESRD patients. Sulfonamides, by comparison, show consistently poor efficacy against *P. putida*, with resistance rates exceeding 90% in multiple reports (4, 16), likely driven by widespread use in both clinical and agricultural settings. These patterns underscore the importance of individual susceptibility testing to guide therapy, as non- β -lactam options may still provide valuable alternatives or adjuncts in selected cases (4).

The presence of atypical resistance determinants can make treatment particularly challenging. A striking example is provided by Cortazzo et al., who described a pediatric oncology patient with multidrug-resistant *P. putida* bacteremia that required ceftolozane-tazobactam therapy; the child recovered after 60 days and was able to continue chemotherapy (17). In our patient, susceptibility to meropenem allowed effective monotherapy and rapid clinical recovery.

This report adds to the growing body of evidence that *P. putida*, while rare, should be recognized as a potential bloodstream pathogen in patients with ESRD and other forms of immunosuppression. Prompt recognition, early initiation of appropriate antimicrobial therapy, and close clinical monitoring were likely pivotal in the patient's favourable clinical improvement.

CONCLUSIONS

This case underscores *P. putida* as an emerging opportunistic pathogen in patients with significant comorbidities, including end-stage renal disease. Although rare, clinicians should maintain a high index of suspicion for *P. putida* in septic presentations, particularly among immunocompromised hosts. Prompt identification, early empiric broad-spectrum coverage, and subsequent therapy guided by susceptibility results remain essential for optimizing patient outcomes and reducing the risk of complications.

ACKNOWLEDGEMENTS

None

FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

AUTHOR CONTRIBUTIONS

V.K.K: conceptualization, methodology, validation, formal analysis, resources, writing – review & editing, visualization, supervision, project administration; K.V.S: conceptualization, methodology, formal analysis, investigation, resources, data curation, writing – original draft; A.A.A: conceptualization, methodology, formal analysis, investigation, writing – original draft, writing – review & editing, visualization; R.R: conceptualization, methodology, resources, data curation, investigation, writing – original draft.

INSTITUTIONAL REVIEW BOARD STATEMENT

The Institutional Ethics Committee of K S Hegde Medical Academy reviewed the case and concluded that a full IEC review was not required; hence, no Institutional Review Board Statement (IRB) approval number was issued.

INFORMED CONSENT STATEMENT

Written informed consent for publication was obtained from the patient. The preparation of this case report adhered to the ethical standards of K S Hegde Medical Academy, Nitte (Deemed to be University), Mangalore, India and the principles of the Declaration of Helsinki.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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