Tropical Journal of Pharmaceutical Research July 2024; 23 (7): 1055-1061
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v23i7.2

Original Research Article

Rosemary leaf extract alleviates testicular impairment, oxidative stress, and apoptosis in streptozotocin-induced diabetic rats

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Sent for review: 11 February 2024 *Revised accepted: 9 July 2024*

Abstract

Purpose: *To investigate the protective effect of ethanol extract of rosemary leaf (EERL) (Rosmarinus officinalis L., family Lamiaceae) against testicular damage in streptozotocin (STZ) induced diabetes rats.*
Methods: *The study involved four groups of rats (n = 6). Type 2 Diabetes (T2D) was induced by injection of 50 mg/kg of STZ. Group 1 served as the control (non-diabetic) group. Group 2 included T2D rats without treatment. Group 3 comprised T2D rats treated with EERL at 200 mg/kg. Group 4 consisted of T2D rats treated with EERL at a dose of 400 mg/kg. The extract was administered orally once a day for 4 weeks. Fasting blood glucose (FBG), serum testosterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and oxidative stress markers (superoxide dismutase (SOD), malondialdehyde (MDA), reduced glutathione (GSH), and glutathione peroxidase (GPx)) levels were assessed. Additionally, histopathological examination using H & E staining and immunohistochemical analysis was conducted to evaluate the expression of proliferating cell nuclear antigen (PCNA) and caspase-3 in the testes.*
Results: *Compared to the untreated T2D rats, EERL administration at 200 mg/kg and 400 mg/kg produced a significant decrease ($p < 0.05$) in FBG levels and elevated levels of serum testosterone, FSH, and LH hormones ($p < 0.05$). Additionally, EERL administration (both doses) compared to the untreated T2D group led to a significant ($p < 0.05$) reduction in testicular MDA content and significant ($p < 0.05$) enhancement of GSH, SOD, and GPx. Moreover, EERL exhibited potential inhibition of STZ-induced testicular damage and successfully averted apoptotic reactions by significantly reducing caspase-3 expression.*
Conclusion: *Ethanol extract of rosemary leaf (EERL) diminishes testicular damage, and counteracts oxidative burden and apoptosis in T2D rats. Ethanol extract from rosemary may be a source of molecules for development as a medicine to support sexual hormones in individuals with diabetes.*

Keywords: *Rosemary, Hyperglycemia, Reproductive hormones, Oxidative stress, Apoptosis*

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INTRODUCTION

Diabetes significantly disrupts sexual function and fertility in animal models and human patients, causing substantial challenges to reproductive health [1]. The testes, which are essential for male fertility are affected by diabetes. Because of this, treatments are desperately needed in order to maintain quality reproductive health. Precise mechanisms behind

diabetes-induced sexual dysfunction and fertility problems remain shrouded in mystery. It has been suggested the major pathway for diabetes-induced sexual dysfunction is the pituitary-testicular axis [2]. Gonadotropin-releasing hormone (GnRH) stimulates the pituitary gland, resulting in secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which are key players in reproductive and hormonal functions. High blood sugar interferes with the hypothalamus–pituitary-testicle communication system, making the pituitary gland less responsive to GnRH [3].

The perennial plant rosemary (*Rosmarinus officinalis* L.), a member of the Lamiaceae family, is native to the Mediterranean region. It is currently grown all over the world [4]. Rosemary leaves possess a wide range of pharmacological effects, such as antioxidant, anti-inflammatory, antidepressant, and antimicrobial effects. Moreover, rosemary may alleviate conditions such as atherosclerosis, liver damage, and high cholesterol [5]. Numerous bioactive components, including carnosic acid, carnosol, and rosmarinic acid are responsible for the pharmacologic and therapeutic potential of rosemary [6]. In rats with alloxan-induced diabetes, rosemary essential oil significantly reduced the risk of male sexual organ injury by protecting it from oxidative stress [7].

Rosmarinic acid increases testosterone levels in diabetic male rats, thereby improving libido and sexual behavior [8]. In mice with STZ-induced diabetes, rosemary diethyl ether fraction enhanced sexual behavior and reproductive function [4].

This present study investigated the protective effect of ethanol rosemary extract (ERE) against STZ-induced testicular toxicity in rats, focusing on the underlying mechanisms, especially the pituitary-testicular axis, oxidative stress, and apoptosis.

EXPERIMENTAL

Preparation of ethanol rosemary extract (ERE)

Following the method of [9], 50 g of finely powdered rosemary leaves (authenticated by an expert taxonomist, CS-5-2019) were macerated for 12 h in 200 mL 70 % ethanol using a reflux extractor. The extract was filtered using Whatman filter paper (No. 31), and the ethanol was removed using a rotary evaporator. The extract was air-dried and stored at 4 °C in airtight containers.

Animals

A total of 24 adult male Wistar rats weighing between 200 - 250 g were used in this study. The rats were obtained from the King Fahad Research Centre (KFRC) at King Abdulaziz University (KAU), Jeddah, Kingdom of Saudi Arabia. The animals were allowed to acclimatize for 7 days. During this period, the animals were fed with normal feed and water. The study was conducted in a controlled laboratory environment with regulated temperature (22 ± 3 °C), humidity levels (50 - 55 %), and a 12 h light / dark cycle.

Ethical considerations

Ethical approval was obtained from the Ethical Research Committee in Biomedical Sciences at KAU (approval no. 346-19), and the study complied with the internationally accepted guidelines for the care and use of laboratory animals, as published by the US National Institutes of Health [10].

Induction of type 2 diabetes (T2D)

Rats were subjected to a lone intraperitoneal injection of freshly prepared STZ (St. Louis, MO, USA) at 50 mg/kg, dissolved in citrate buffer (0.1 M, pH 4.5), to induce diabetes [11]. Rats were considered diabetic if they had blood sugar levels exceeding 198 mg/dL [12].

Design and ERE administration

A total of 24 rats were randomized into 4 equal groups. Group 1 was control group (non-diabetic) rats and were administered distilled water. Group 2 comprised diabetic rats administered distilled water. Group 3 comprised diabetic rats administered ERE (200 mg/kg) [12]. Group 4 comprised diabetic rats administered ERE (400 mg/kg). Oral gavage was used to deliver distilled water and ERE once/day for 4 weeks.

Sample collection

Following the experiment, the final body weight (g) of the rats was measured. The rats that had been fasting were humanely sacrificed under diethyl ether anesthesia. Blood (1 mL) was withdrawn from the orbital sinus into a sterile 1.5 mL microcentrifuge tube, and after centrifugation at -4 °C, the serum was isolated and subsequently stored at -80 °C for subsequent analysis. Decapitation with a rodent guillotine was used for dissection, and the testes were isolated, rinsed with saline, weighed, and preserved in 10 % formalin solution.

Determination of fasting blood glucose level (BGL)

Blood samples were collected from the tail vein and glucose levels were measured digitally (Johnson & Johnson (USA) blood glucose meter) [13].

Assessment of serum testosterone level

Rat testosterone was quantified in serum samples using a Boster Biological Technology ELISA kit following the manufacturer's protocol.

Evaluation of serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels

Concentrations of LH and FSH in the serum were assessed at El-Safwa Laboratory in Tanta, Egypt, utilizing an automated chemiluminescence immunoassay system (ADVIA Centaur, Bayer HealthCare).

Determination of serum oxidative stress markers

Serum oxidative stress markers were determined using superoxide dismutase (SOD), malondialdehyde (MDA), reduced glutathione (GSH), and glutathione peroxidase (GPx) kits (Biodiagnostic, Egypt) following the manufacturer's protocol.

Histopathological examination of testes

Formalin-fixed testicular tissues underwent staining with hematoxylin and eosin (H & E), and specimens were inspected under a light microscope to detect changes in tissue histology.

Immunohistochemical determination of proliferating cell nuclear antigen (PCNA) and caspase-3

Antibodies specific to PCNA (an indicator of cell proliferation), and caspase-3 (an indicator associated with apoptosis), were used for staining of tissue sections. This immunoperoxidase technique, also known as the PAP (peroxidase/anti-peroxidase) method, amplifies the signal for easier visualization. A pathologist, blinded to the sample groups then examined the stained slides under a microscope and captured photomicrographs of the various sections.

Statistical analysis

The results were analyzed statistically using GraphPad Prism 5. Measurement data were presented as mean ± standard deviation (SD). Group comparison was done using one-way analysis of variance (ANOVA) and the Tukey-Kramer post hoc test was used for comparison. $P < 0.05$ was statistically significant.

RESULTS

Effect of ERE on final body and testicular weight

The untreated diabetic group showed a significant reduction in body and testicular weight compared to control group ($p < 0.05$). Neither 200 mg/kg nor 400 mg/kg ERE treatment groups showed any changes in body weight compared to untreated diabetic group. However, diabetes led to significantly lower testicular weight ($p < 0.05$) compared to control group which was significantly reversed at 400 mg/kg ERE ($p < 0.05$; Figure 1).

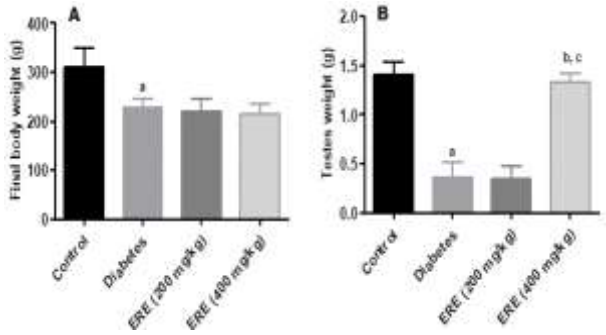

Figure 1: A: Final body weight (g) (B): Testicular weight (g) of the control, the untreated diabetic and the diabetic treated with ERE (200 and 400 mg/kg) groups. **Key:** Values are expressed as mean ± SD (n= 6) per group. $^{a}P < 0.05$ vs control group, $^{b}p < 0.05$ vs diabetes group, and $^{c}p < 0.05$ vs ERE group (200 mg/kg)

Effect of ERE on fasting blood glucose level (BGL)

Compared to control group, untreated diabetic rats had significantly higher BGL ($p < 0.05$). Upon treatment, diabetic rats with ERE at 200 and 400 mg/kg showed significantly reduced BGL ($p < 0.05$). However, ERE at 400 mg/kg significantly reversed the diabetes-induced increase in BGL to levels similar to those in healthy rats (Figure 2).

Effect of ERE on serum testosterone level

Compared to the control rats, untreated diabetic rats had significantly lower serum testosterone levels ($p < 0.05$). Treated diabetic rats with 200 and 400 mg/kg ERE significantly increased serum testosterone ($p < 0.05$). However, ERE at

400 mg/kg significantly reversed the diabetes-induced reduction in serum testosterone level compared to ERE at 200 mg/kg ($p < 0.05$; Figure 3).

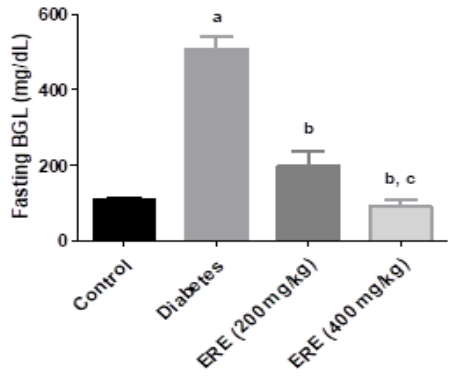

Figure 2: Fasting blood glucose level (BGL) of control, untreated diabetic and diabetic treated with ERE (200 and 400 mg/kg) groups. ***Key:*** Values were expressed as mean ± SD (n = 6) per group. [a]$P < 0.05$ vs control group, [b]$p < 0.05$ vs diabetes group, and [c]$p < 0.05$ vs ERE (200 mg/kg)

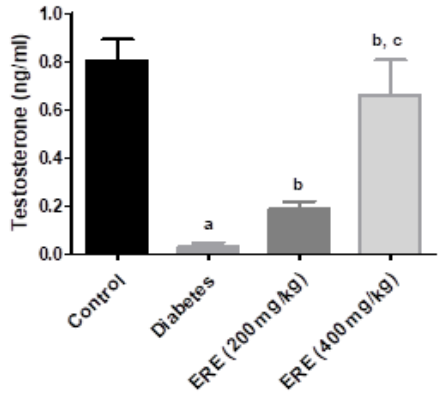

Figure 3: Serum testosterone level of control, untreated diabetic and diabetic treated with ERE (200 and 400 mg/kg) groups. ***Key:*** Values were expressed as mean ± SD (n = 6) per group. [a]$P < 0.05$ vs control group, [b]$p < 0.05$ vs diabetes group, [c]$p < 0.05$ vs ERE group (200 mg/kg)

Effect of ERE on serum LH and FSH levels

Compared to control group, untreated diabetic rats had significantly lower serum LH and FSH levels ($p < 0.05$). Furthermore, ERE at 200 and 400 mg/kg did not show any changes in serum LH levels compared to the untreated diabetic group. Following treatment with 200 and 400 mg/kg ERE, serum FSH levels were significantly increased ($p < 0.05$). However, ERE at 400 mg/kg significantly reversed the diabetes-induced decrease in serum FSH level compared to ERE at 200 mg/kg ($p < 0.05$; Table 1).

Table 1: Serum testosterone level of animal groups

Group	LH (mIU/mL)	FSH (mIU/mL)
Control	2.26±0.47	4.50±0.55
Diabetes	1.13±0.12[a]	1.33±0.41[a]
ERE (200 mg/kg)	1.23±0.24	3.17±0.68[b]
ERE (400 mg/kg)	1.23±0.10	4.33±0.82[b, c]

Values were expressed as mean ± SD (n = 6). [a]$P < 0.05$ vs. control group, [b]$p < 0.05$ vs. diabetes group, [c]$p < 0.05$ vs. ERE (200 mg/kg)

Effect of ERE on testicular histopathological alterations

Histo-architectural evaluations of testicular sections revealed the presence of normal seminiferous tubules (ST) with fully preserved germ cell layers containing mature sperm tails. Additionally, the interstitial cells and blood vessels appeared normal (Figure 4 A). Untreated diabetic testes sections showed marked deformity and shrinkage of the ST with degenerative changes, loss of germ cell layers, desquamation into lumen and absence of mature sperms. Moreover, interstitial cells looked degenerated with thickened blood vessels (Figure 4 B). Testes of rats treated with 200 mg/kg ERE showed moderate preservation of ST structures, numerous tubules showed degenerative loss of germ cell layers, and interstitial spaces showed homogenously stained oedematous fluid (Figure 4 C). However testicular sections of diabetics treated with EERL (400 mg/kg) exhibited germ cell layers of full thickness, with the majority containing mature sperm tails, few showed desquamated cells or absence of mature sperms, normal interstitial cells, and blood vessels (Figure 4 D).

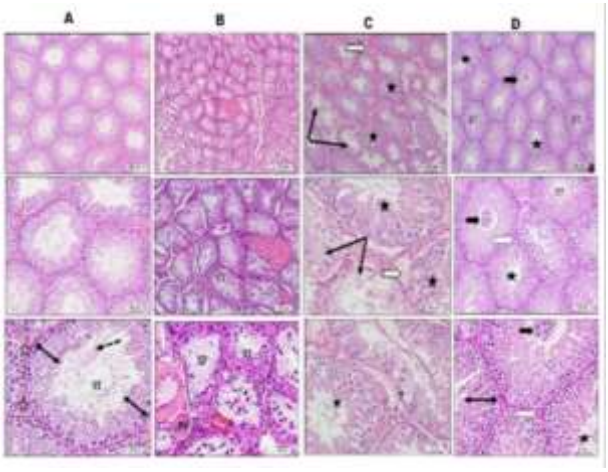

Figure 4: Sections from rat testes stained by H&E and photographed at x100 bar = 200 µm, x 200 bar = 100 µm, and x400 bar = 50 µm. A: Control group. B: Untreated Diabetic group. C: ERE (200 mg/kg) treated diabetic group. D: ERE (400 mg/kg) treated diabetic group

Table 2: Serum oxidative stress markers of animal groups

Group	MDA (nmol/L)	GSH (mg/dL)	SOD (U/mL)	GPx (mU/mL)
Control	6.44±1.62	9.02±1.12	1360±513	143.70±24.33
Diabetes	12.99±1.61[a]	4.90±0.49[a]	697±129[a]	69.67±11.34[a]
ERE (200 mg/kg)	5.72±1.92[b]	8.27±1.43[b]	1208±257[b]	117.70±15.55[b]
ERE (400 mg/kg)	6.37±1.81[b]	8.58±1.75[b]	1340±175[b]	141.2±39.05[b]

Values were expressed as mean ± SD (n = 6) per group. [a]$P < 0.05$ vs control group, [b]$p < 0.05$ vs diabetes group

Effect of ERE on serum oxidative stress markers

Compared to control group, untreated diabetic rats had significantly higher serum MDA levels ($p < 0.05$). Treating diabetic rats with ERE at 200 mg/kg and 400 mg/kg resulted in significantly lower serum MDA ($p < 0.05$; Table 2). Furthermore, untreated diabetic rats exhibited significantly lower levels of serum GSH, SOD, and GPx compared to control group ($p < 0.05$). Treatment with either 200 or 400 mg/kg ERE resulted in significantly higher serum GSH, SOD, and GPx levels ($p < 0.05$; Table 2).

Effect of ERE on testicular PCNA expression

Sections from rat testes of control group showed high positive PCNA immunoexpression in the nuclei of intact germ cells (Figure 5 A). Sections of testes of untreated diabetic rats showed a marked decrease in PCNA immunoexpression in degenerated germ cells (Figure 5 B). Sections of testes-treated animals (200 mg/kg ERE) showed preserved PCNA immunoexpression in ST with intact germ cells and decreased immunoexpression in damaged ST (Figure 5 C). Testes of diabetic animals treated with 400 mg/kg ERE showed significant preservation of PCNA immunoexpression compared to 200 mg/kg ERE untreated diabetic groups (Figure 5 D).

Effect of EERL on testicular caspase-3 expression

Sections from rat testes of control group showed weak caspase-3 immunoexpression. Sections from untreated diabetic rats showed germ cells marked by degenerative changes and increased caspase-3 immunoexpression compared to control group. Furthermore, testes of ERE (200 mg/kg) treated rats showed decreased caspase-3 immunoexpression whereas testes of ERE (400 mg/kg) treated diabetic rats showed a significant decrease in caspase-3 immuno-expression similar to control group (Figure 6).

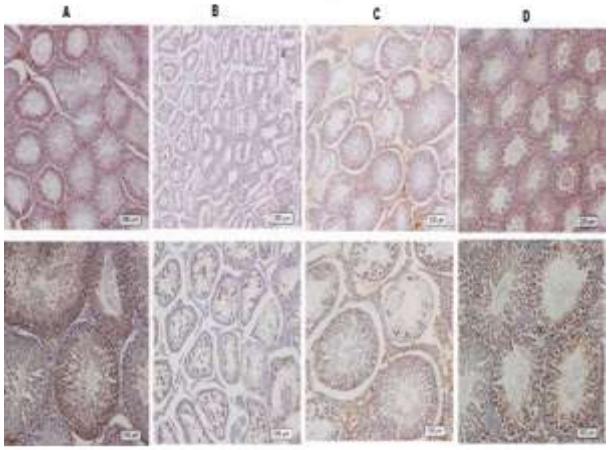

Figure 5: Sections from rat testes immunostained for PCNA expression photographed at x100 bar = 200µm and x 200 bar = 100µm. ***Key:*** A: Control group showed high positive PCNA immunoexpression in nuclei of intact germ cells. B: Untreated diabetic group showed a significantly decreased PCNA immunoexpression in degenerated germ cells. C: ERE (200 mg/kg) treated diabetic group showed preserved PCNA immunoexpression in seminiferous tubules with intact germ cells with decreased immunoexpression in damaged seminiferous tubules. D: ERE (400 mg/kg) treated diabetic group showed significant preservation of PCNA immunoexpression compared to ERE (200 mg/kg) and untreated diabetic groups

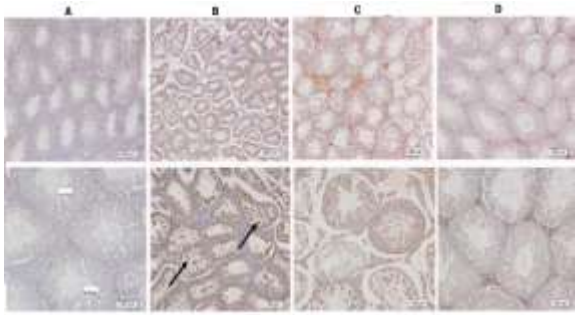

Figure 6: Sections from rat testes immunostained for caspase-3 expression photographed at x100 bar = 200µm and x 200 bar = 100µm. ***Key:*** A: Control group showed weak caspase-3 immunoexpression. B: Untreated diabetic group showed marked germ cell degenerative changes and increased caspase-3 immunoexpression (black arrows) compared to the control (white arrows). C: ERE (200 mg/kg) treated diabetic group showed decreased caspase-3 immunoexpression. D: ERE (400 mg/kg) treated diabetic group showed more decrease in caspase-3 immunoexpression which reached control group

DISCUSSION

This study showed the potential advantages of ethanol extract of rosemary leaf (EERL) in the biochemical, histological, and immunohisto-chemical changes associated with diabetic testicular damage. The outcomes of this study revealed that the administration of ethanol extract from rosemary leaves effectively reversed hyperglycemia observed in the diabetic group. These findings are consistent with prior studies demonstrating the hypoglycaemic properties of rosemary extracts and their several components [4,14]. Several mechanisms underlie the anti-hyperglycemic and insulin-like actions of ERE. Among them is its ability to improve the uptake and utilization of glucose, decrease glycogen levels, and speed up the glycolysis process [14].

Some studies have provided evidence indicating that diabetes mellitus induces oxidative stress, resulting in the generation of reactive oxygen species that affect various organs in the body. It has been reported that the phenolic compounds present in rosemary protect against oxidative stress-induced via various diseases such as diabetes mellitus [15]. The effectiveness of the extract of Rosemary leaves may be credited to the existence of flavonoids. Flavonoids have demonstrated various activities and have been shown to inhibit lipid peroxidation while raising concentrations of GPx. These actions play a protective role against oxidative damage in various tissues by neutralizing reactive oxygen species [16]. In this study, the administration of ERE to diabetic rats resulted in significantly higher testosterone levels compared to the untreated diabetic group. In another study, co-administration of rosemary essential oil with etoposide demonstrated an improvement in testosterone levels compared to treatment with etoposide alone [16]. The antioxidative properties are credited to the sulfuric compounds and elevated alpha-tocopherol levels. This capability enhances Leydig cell functionality, resulting in heightened testosterone production and enhanced spermatogenesis [17]. The presence of rosmarinic acid and rosemary flavonoid-rich fractions demonstrated the capability to enhance serum testosterone levels and improve sexual behavior in rats with diabetes induced by STZ [8,4]. In this present study, alterations in the pituitary-testicular axis were observed in STZ-induced diabetic rats and manifested as a reduction in FSH, LH, and testosterone secretion from Leydig cells. Histologically, rats treated with STZ to induce diabetes exhibited a significant reduction in testicular weight and increased apoptosis within the testes. Previous studies have revealed that diabetic animals exhibited reduced testicular weight and atrophy of ST [18]. This indicates that apoptosis plays a critical role in the impairment of testicular function during diabetes. Treatment of diabetic rats with ERE caused significant restoration of testicular weights. This implies that antioxidant therapy exerts a protective effect on the testes. Induction of diabetes using STZ leads to significant reduction in serum LH levels which explains the impaired function of Leydig cells. The decrease in insulin levels caused by diabetes indirectly lowers serum LH levels, likely through a mechanism associated with FSH. This ultimately impacts the functionality of Leydig cells. Treatment with aqueous rosemary extract caused a significant rise in LH and FSH levels [19].

CONCLUSION

The ethanol extract of rosemary leaves reverses diabetes-induced testicular damage, exerts strong antioxidant characteristics, and improves testicular function. These findings suggest that the extract is a potential lead in the isolation of molecules for the development of suitable medicines would enhance fertility, regulate sexual hormones and support sperm development in individuals with diabetes.

DECLARATIONS

Acknowledgements

The authors, gratefully acknowledge the Deanship of Scientific Research for their technical and financial support. This work was supported by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant no. D-184-130-1439.

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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