


Anti-Inflammatory and Antinociceptive Effects of *Boesenbergia rotunda* Polyphenol Extract in Diabetic Peripheral Neuropathic Rats

Peng Wang¹, Chaoling Wen², Opeyemi Joshua Olatunji ³

¹Department of Pharmacy, Wuhu Second People's Hospital, Wuhu City, 241001, Anhui, People's Republic of China; ²Anhui Traditional Chinese Medicine College, Wuhu City, 241001, Anhui, People's Republic of China; ³Traditional Thai Medical Research and Innovation Center, Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, 90110, Thailand

Correspondence: Opeyemi Joshua Olatunji, Traditional Thai Medical Research and Innovation Center, Faculty of Thai Traditional Medicine, Prince of Songkla University, Hat Yai, 90110, Thailand, Email opeyemi.j@psu.ac.th

Introduction: Diabetic peripheral neuropathy (DPN) is still one of the most prevailing complication of diabetes and it affects a huge diabetic population. *Boesenbergia rotunda* is a ginger species that has both culinary and medicinal applications. Recent studies have revealed that *B. rotunda* has potential applications in diabetes, pain and inflammatory related disorders. As such this study investigated the potential of *B. rotunda* extract (EBR) in attenuating DPN in rats.

Methods: DPN was induced in male Sprague Dawley rats using a combination of 30% fructose solution and streptozotocin (40 mg/kg). Afterwards diabetic rats were treated with EBR (100 and 400 mg/kg) for 5 weeks. DPN was assessed using thermal hyperalgesia, cold and mechanical allodynia and rotarod test, while nociceptive responses were assessed by formalin and acetic acid test. In addition, serum proinflammatory cytokine levels were determined using ELISA kits.

Results: EBR displayed hypoglycemic effect by significantly reducing the blood glucose concentration of treated diabetic rats, while simultaneously alleviating the reduced body weight. Furthermore, EBR markedly alleviated thermal hyperalgesia, cold and mechanical allodynic responses as well as ameliorated motor coordination in the treated diabetic rats. In addition, EBR significantly reduced nociceptive responses in the formalin and acetic acid test, as well as decreased serum levels of proinflammatory cytokines (TNF- α and IL-1 β).

Conclusion: The results suggested that EBR exerted anti-inflammatory and anti-nociceptive effects, thus alleviating diabetic painful neuropathy.

Keywords: diabetic peripheral neuropathy, diabetes, *Boesenbergia rotunda*, polyphenols, anti-inflammatory

Introduction

Diabetes mellitus (DM) is a serious, lifelong and incurable metabolic disease with devastating consequences. Almost half a billion people were estimated to be living with DM in 2019 and an additional 200 million people have diabetes but undiagnosed.^{1,2} DM occurs due to abnormalities in the production of insulin from the pancreas or when the body becomes insensitive and unable to effectively utilize the insulin produced by the pancreas,³ resulting in hyperglycemia, hyperlipidemia as well as alteration in lipids, carbohydrates and protein metabolism.⁴ The increase in endogenous glucose production in DM accompanied by excessive oxidative stress has been identified as major culprits in several diabetes induced comorbidity including neuropathy, nephropathy, retinopathy, cardiovascular diseases and neurodegenerative diseases.⁵⁻⁷

Diabetic peripheral neuropathy (DPN) is a common symmetrical degenerative comorbidity in diabetic patients and it affects approximately 30–50% of DM patients.^{8,9} DPN is clinically characterised by sensory, autonomic and motor nerve function defects, which includes hyperalgesia, limb numbness, loss of reflexes and muscle atrophy. All these may

eventually lead to infections, foot ulcers and amputations.^{10,11} Although DPN on its own may not be life threatening, however it can significantly affect the quality of life of diabetic patients and create huge financial burden.^{12,13}

Boesenbergia rotunda (fingerroot) belongs to the family Zingiberaceae and it is a ginger species that is indigenous to many southeast Asian countries and notable for its culinary purposes. *B. rotunda* is traditionally used in the treatment of fever, rheumatism, muscle pain, peptic ulcer, stomach disorders and bacterial infections.^{14,15} Aside its folk use, *B. rotunda* have shown several bioactivities including biofilm formation inhibition, antiobesity, antioxidant, anticancer, antiviral, anti-inflammatory activities and in vitro antidiabetic activities.^{16–18} Furthermore, *B. rotunda* is particularly rich in polyphenols notably chalcones and flavonoids, which are majorly responsible for its in vitro antidiabetic effects of the plant.^{16,19,20} Consequently, we hypothesized that *B. rotunda* rhizome extract could exert ameliorative effects against diabetes and diabetic complications. However, there are no report on the effect of *B. rotunda* in in vivo antidiabetic models or its protective effects against diabetic induced comorbidity. This study investigated the effects of *B. rotunda* rhizome extract on diabetic peripheral neuropathy in high fructose/streptozotocin induced diabetic rats.

Materials and Methods

Chemicals and Reagents

Streptozotocin and fructose were purchased from Alfa Aesar (Massachusetts, United States) and Kemaus (New South Wales, Australia). The kit for measuring proinflammatory cytokines determination were purchased from Abcam (Cambridge, United Kingdom). All other reagents and solvents are of analytical grade and purchased from RCI Labscan.

Plant Material

The rhizomes of *B. rotunda* was purchased from a Traditional Medicinal Herbs Store in Hat Yai, Songkla, Thailand. Authentication of the plant material and deposition of voucher specimen (BR-TT-050) was done at the Faculty of Thai Traditional Medicine, Prince of Songkla University.

Preparation of Extract

The chopped and dried rhizomes of *B. rotunda* (1 kg) was pulverised with a mechanical grinder and the powdered samples was macerated exclusively with 5 L of 95% ethanol for 24 h. The pooled ethanolic extracts was filtered and dried using reduced pressure. The dried extract was resuspended in distilled water and extracted with n-hexane and ethyl acetate. The EtOAc soluble portion of *B. rotunda* (EBR) was dried and stored at 4°C until use.

Isolation of Compounds from EBR

The ethyl acetate fraction (EBR, 5 g) was loaded on sephadex LH-20 column and eluted with MeOH-CH₂Cl₂ (1:1) to obtain 40 collected fractions which was pulled together to obtain 8 (Fr 1–8) fractions based on their TLC profiles. Panduratin A (650 mg) was purified from sub fractions Fr 4 by recrystallization using a mixture of MeOH/CH₂Cl₂. Fraction Fr 5 was also subjected to column chromatography eluting with stepwise gradient of EtOAc/MeOH to afford 15 subfractions. The combinations of subfractions 8–12 was recrystallized with MeOH to afford pinostrobin (900 mg). Another portion of EBR (10 g) was separated on silica column chromatography using hexane/EtOAc and EtOAc/MeOH in a gradient elution manner to afford 20 fractions (F1–F20). Fractions 6–9 (485 mg) was chromatographed on Sephadex LH-20 (MeOH), silica column chromatography (hexane-CH₂Cl₂) and recrystallization (hexane-EtOAc) to afford panduratin A (480 mg), pinocembrin (150 mg) and cardamonin (25 mg). The combined fractions 10–11 was subjected to column chromatography on silica using gradient elution of CH₂Cl₂/EtOAc/MeOH and sephadex LH-20 (MeOH) and recrystallized in CHCl₃/MeOH mixture to afford alpinetin (58 mg) and boesenbergin A (76 mg).

Animals

Twenty four six weeks old male Sprague Dawley rats (160 ± 20 g) were randomly divided into four groups and housed in stainless steel cages under standard environmental and experimental animal husbandry conditions. The animals were acclimatized for 7 days with access to standard rat chows and normal water *ad libitum*. Animal maintenance procedures

was in conformity with the approved procedures of the Research Ethics Committee of Wuhu Second People's Hospital, Wuhu, China (Ethical protocol approval number: WuhueyLLWYH/2021/0928). In addition, the procedures of the National Institute of Health on experimental use of animals were strictly followed.

Animal Groupings, Induction of DM and Treatment

The rats were randomly allotted into four experimental groups (six rats each) as stated below:

Normal Healthy Control (NHC): treated with vehicle (5% DMSO),

Diabetic Control (DC): diabetic rats treated with vehicle,

DLEBR: diabetic rats treated with low dose (100 mg/kg) of EBR extract,

DHEBR: diabetic rats treated with high dose (400 mg/kg) of EBR extract.

T2DM was induced in the animals following previously described method.⁴ Briefly, the animals in the DC, DLEBR and DHEBR groups were administered with 30% fructose solution *ad libitum* for four weeks, while the NHC animals were simultaneously given normal water during the same period. After a 12 h overnight fast, streptozotocin (STZ, 40 mg/kg) dissolved in sodium citrate buffer (0.1 M; pH 4.5) was intraperitoneally injected to the animals in the DC, DLEBR and DHEBR groups, while the NHC rats received the same volume of citrate buffer. After three days, blood glucose concentration was measured in all the animals with a portable glucometer (Accu-Chek Guide) using blood obtained from the tail tip. T2DM was affirmed in rats with fasting blood glucose level above 250 mg/dL. Five days after confirmation of T2DM, the animals were administered with their respective treatment as indicated above on a daily basis by oral gavage for 5 weeks. Weekly fasting blood glucose level and body weight changes were determined during the treatment period. After the treatment, all the animals were subjected to series of pain behavioural experiments.

Evaluation of Hyperalgesia

Hot Plate and Tail Flip Test

Thermal hyperalgesia was evaluated in all the experimental animal groups using hot plate and tail flip test. Briefly, the hot plate apparatus used was set at a temperature of 55 ± 1 °C. The animals were individually placed on the apparatus and the time it takes for the rats to display the first sign of nociceptive response including jumping and paw licking (paw withdrawal latency, PWL) was adjudged as pain threshold and recorded. A cut-off time of 35s was used to avoid paw tissue damage.

For the tail flip test, the temperature of the hot water bath was set at 50 ± 1 °C and the tail of the rats was immersed into the hot water bath until the animals showed the first sign of struggle, tail flicking or withdrawal (tail withdrawal latency, TWL). A cut off time of 12s was set to avoid tissue damage. A decrease in the tail withdrawal time signifies hyperalgesia.

Cold Allodynia Test

The sensitivity of the rats hind paw to cold allodynia was measured by inserting the hind paw of the rats in cold water set at 4.5 ± 1 °C. The hind paw withdrawal latency (CPWL) was measured in seconds. A limit of 20 secs was set to avoid tissue damage.

Mechanical Allodynia

Mechanical allodynia was determined in the hind paws of all the rats with the aid of von Frey filaments. Briefly, the rats were individually positioned on top of a wired meshed surface and von Frey filaments with varying bending forces was applied on the plantar surface of the right hind paw for 5 s. The paw withdrawal threshold was evaluated by increasing the stimulus strength of the filament until paw withdrawal was displayed by the animals.

Motor Coordination Test

Motor coordination was evaluated using a rotarod apparatus. The rats were trained twice a day for three consecutive days, during this period the animals were positioned on a rotating rod accelerating from 0–25 rpm. The time it takes for the animals to dismount from the rotarod was recorded in seconds. Three trials was performed on the test day.

Acetic Acid-Induced Writhing

Acetic acid nociception was induced in the animals by intraperitoneal injection of 10 mL/kg of 1% acetic acid to induce characteristic abdominal writhing and stretching. The number of abdominal writhing and stretching was counted from 0–30 min after acetic acid injection.

Formalin-Induced Paw Licking Test

The formalin-induced paw licking test was performed in the rats after treatment by injecting 50 μ L of 2.5% formalin solution subcutaneously into the right hand paw of the animals. The time spent licking the injected paws was recorded during the neurogenic pain phase (0–5 mins after injection) and the inflammatory pain response (15–30 min after the injection).

Animal Sacrifice

Upon completion of the behavioural experiment, the rats were anesthetized using thiopental and bled through cardiac puncture. The blood collected blood was processed to obtain the serum. Proinflammatory cytokine levels (TNF- α and IL- β) were quantified in the serum using the ELISA kits.

Statistical Analysis

All data represents mean \pm SD (n=6). For comparisons among multiple groups, one-way ANOVA with Bonferroni post hoc analysis was used. $P < 0.05$ was defined as statistically significant. Data analysis was performed on GraphPad Prism (version 5.0; GraphPad Software, USA).

Results

Effects of EBR on Blood Glucose Concentration and Body Weight

There was a significant increase in the initial blood glucose concentration of rats in the DC, DLEBR and DHEBR groups compared with the HNC group (Figure 1A). Whereas, treatment of diabetic rats with EBR (100 or 400 mg/kg) resulted in a dose dependent decrease in blood glucose concentration at the end of the treatment, while the DC maintained a higher blood glucose concentration at the end of the treatment (Figure 1A).

In addition, as shown in Figure 1B, the body weight gain of the DC group was significantly lowered when compared to the HNC group, while treatment with EBR (100 or 400 mg/kg) significantly improved the body weight gain compared to the DC group (Figure 1B).

Effects of EBR on Thermal Hyperalgesia in Diabetic Rats

As shown in Figure 2A and B, there was marked increase in thermal hyperalgesia in the DC rats as observed by decrease in the pain latency of the DC group in the hot plate and tail flip tests compared to the HNC group (Figure 2A and B).

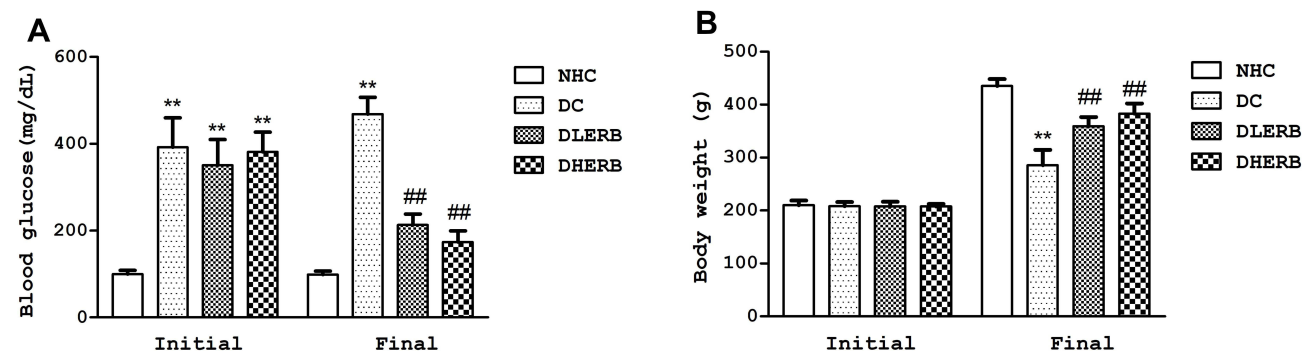


Figure 1 Effect of EBR on (A) fasting blood glucose concentration, (B) body weight gain. Data was presented as the mean \pm SD (n = 6). ** $P < 0.05$ vs HNC group, ## $P < 0.05$ vs DC group.

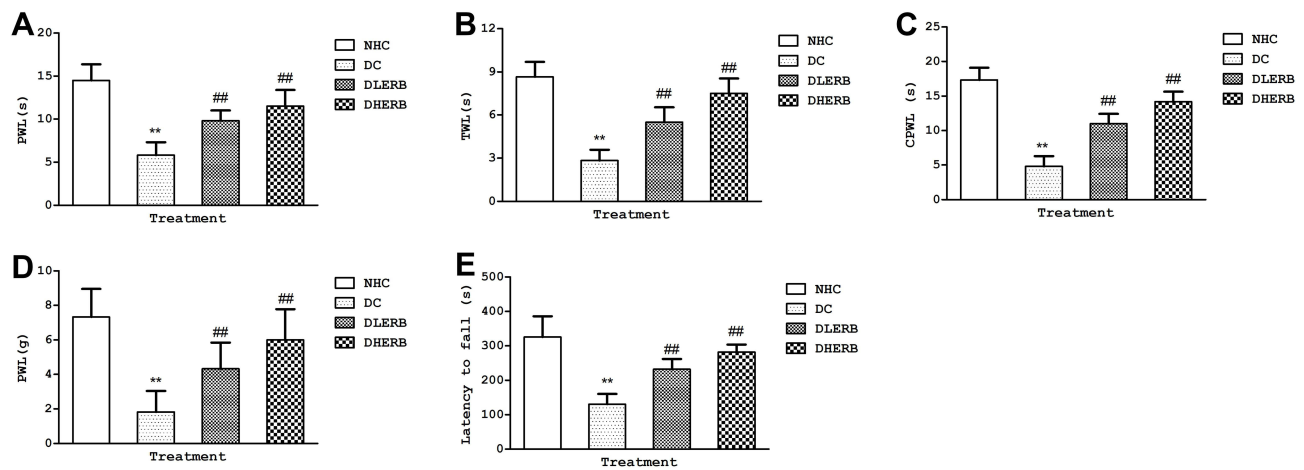


Figure 2 Effect of EBR on alleviating thermal hyperalgesia, cold, mechanical allodynia and improving motor coordination activity in DPN rats. (A) PWL of the hot plate test, (B) TWL of the tail flip test, (C) cold paw withdrawal latency (D) paw withdrawal latency measured by Von Frey filament (E) rotarod test. Data was presented as the mean \pm SD (n = 6). **P<0.05 vs HNC group, ##P<0.05 vs DC group.

However, in the diabetic rats treated with EBR (150 or 400 mg/kg), significant increase was observed in their pain latency response, indicating reduced thermal hyperalgesia compared to the untreated DC group (Figure 2A and B).

Effects of EBR on Cold and Mechanical Allodynia in Diabetic Rats

In the cold water test, the rats in the DC group exhibited significantly reduced paw withdrawal latency (CPWL) compared to normal control group (Figure 2C). In the EBR treated groups, the CPWL of the rats were increased significantly in comparison with DC group (Figure 2C). Furthermore, the effect of EBR on mechanical hyperalgesia in diabetic rats is as shown in Figure 2D. There was obvious decrease in mechanical threshold in the DC group as compared to HNC group. Whereas, treatment of diabetic rats with EBR significantly increased the mechanical pain threshold in the treated rat groups compared to the untreated DC group (Figure 2D).

Effects of EBR on Motor Coordination in Diabetic Rats

The DC rats showed signs correlated with motor coordination dysfunction as evidenced by shorter dismount latency time on the rotarod apparatus when compared to the HNC group that had longer dismount latency time (Figure 2E). Whereas, treatment of diabetic rats with EBR significantly increased the duration time of the treated rats on the rotarod apparatus when compared to the DC animals (Figure 2E).

Effects of EBR on Formalin Paw Licking in Diabetic Rats

As shown in Figure 3A, the response of the DC rats was observed to be significantly increased as observed by marked increase in the paw licking time in both phases (neurogenic and inflammatory phases) compared to the HNC group. Whereas, treatment of diabetic rats with EBR significantly decreased the neurogenic and inflammatory phases paw licking time when compared to the DC group (Figure 3A).

Effects of EBR on Acetic Acid-Induced Writhing in Diabetic Rats

As shown in Figure 3B, the number of abdominal writhes was markedly increased in the untreated DC group compared to HNC group. Compared to DC control group, EBR treatment (100 and 400 mg/kg) significantly decreased the number of abdominal writhing in the treated animals when compared to the untreated DC group (Figure 3B).

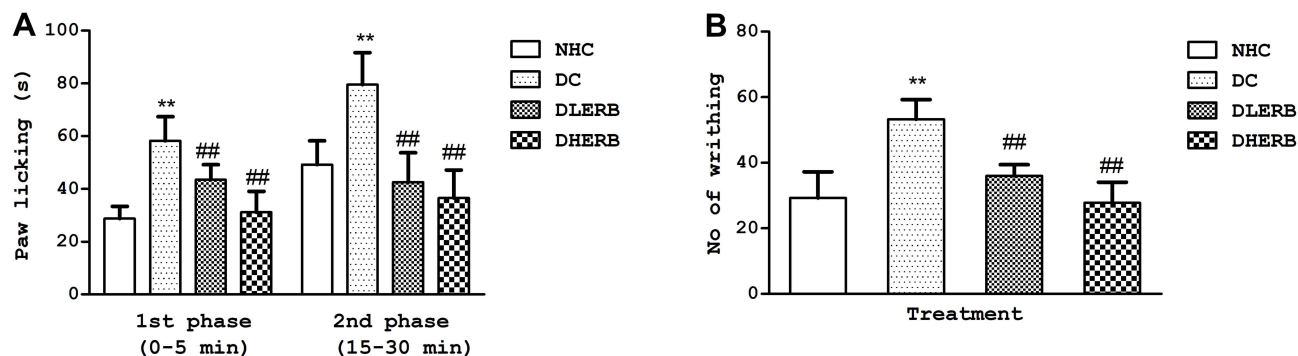


Figure 3 Effect of EBR on alleviating nociceptive responses in DPN rats. (A) Paw licking duration tested by formalin test (B) number of abdominal writhing tested by acetic acid test. Data was presented as the mean \pm SD ($n = 6$). ** $P < 0.05$ vs HNC group, ### $P < 0.05$ vs DC group.

Effects of EBR on Serum Proinflammatory Cytokines in Diabetic Rats

As shown in Figure 4, the DC rats showed significant increases in the serum concentration of TNF- α and IL-1 β compared to the normal control rats. However, treatment of diabetic rats with EBR significantly and dose dependently reduced serum levels of these proinflammatory cytokines when juxtaposed with the DC control group (Figure 4A and B)

Chemical Characterization of EBR

The phytochemical investigation of EBR using various chromatographic separation techniques and recrystallization afforded the isolation and identification of six compounds, all polyphenols including panduratin A, pinostrobin, pinocembrin, cardamonin, boesenbergin A and alpinetin (Figure 5). All the isolated compounds were identified via spectroscopic data analyses and comparison with previous literature data.

Discussion

Diabetes is a chronic metabolic disorder associated with increase in blood glucose concentration, paving the way for the development of multiple diabetic associated comorbidity such as cardiovascular diseases, neuropathy, neurodegenerative diseases and nephropathy.^{5,21} Unfortunately most of the synthetic antidiabetic drugs commonly used for treating diabetes have not shown efficacy in reversing diabetic complications, in addition to the reported side effects of these drugs.³ The development of DPN is largely influenced by low blood flow and hyperglycemia, as such maintaining strict glycaemic control have been proposed as a viable option in the prevention or treatment of DPN.^{10,22}

Several studies have highlighted the relevance of medicinal plants in the treatment of diabetic complications including DPN.²³ Polyphenols are one of the major bioactive components in medicinal plants and they have been reported as multitarget agents with powerful antidiabetic, antioxidant and anti-inflammatory activities.²⁴ Furthermore, polyphenols have been shown to be effective in the treatment of a variety of diseases which include diabetes and diabetes-associated

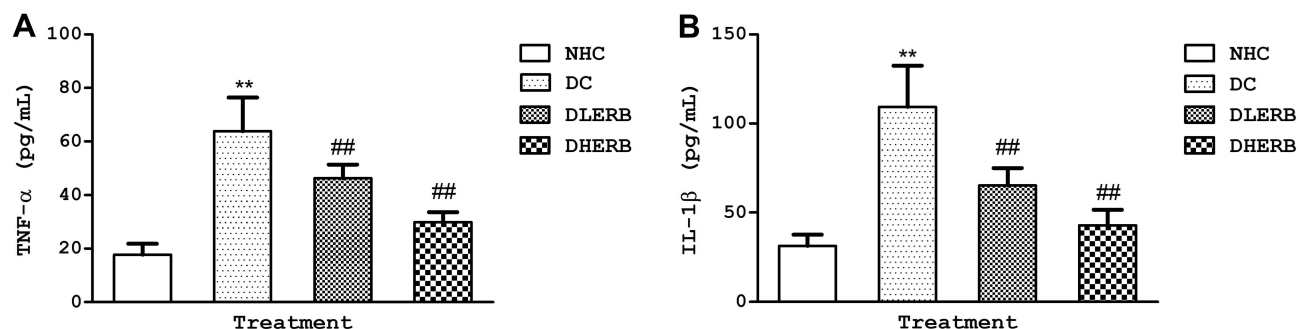


Figure 4 Effect of EBR on alleviating serum proinflammatory cytokines in DPN rats. (A) Tumor necrosis factor alpha (B) interleukin 1 beta. Data was presented as the mean \pm SD ($n = 6$). ** $P < 0.05$ vs HNC group, ### $P < 0.05$ vs DC group.

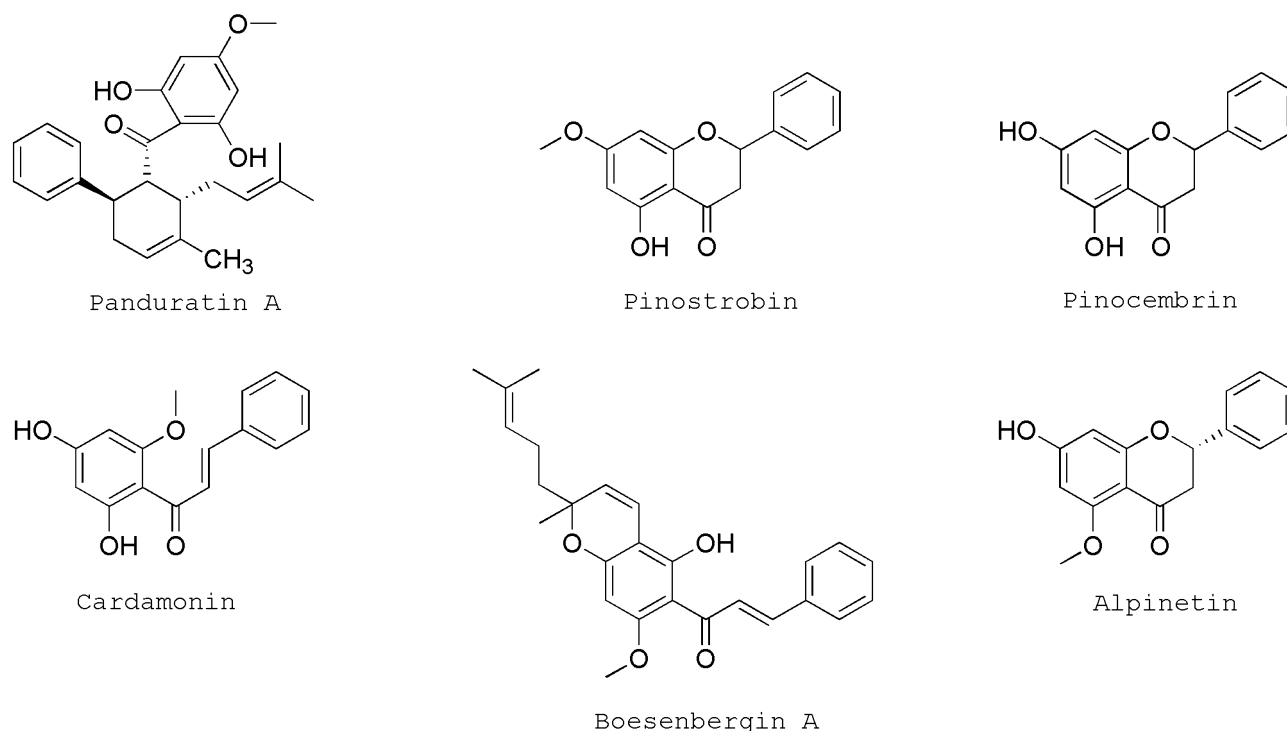


Figure 5 Chemical structure of the isolated polyphenolic compounds from EBR extract.

problems. In this regard, *B. rotunda* a culinary and medicinal plant rich in polyphenolic constituents is widely used as a remedy for the treatment diabetes, rheumatic and muscle pain.¹⁴ As such this study evaluated the ameliorative effects of *B rotunda* polyphenol extract against diabetes induced peripheral neuropathic pain.

This study established a high fructose/STZ diabetic model characterized by insulin resistance and hyperglycemia. The combination of high fructose and streptozotocin induces insulin resistance and destroys pancreatic beta cells leading to decrease in insulin production causing excessive increase in circulating blood glucose concentration.^{3,4,25} The results from this study indicated that the administration of EBR significant reduced blood glucose level in the treated diabetic rats. Furthermore, our study observed a dramatic weight loss in the diabetic animals which was attenuated in the EBR treated rats. Previous literatures have reported that diabetes induces body weight loss, polyphagia and polydipsia.^{3,26}

DPN results in painful sensation, reduced motility and may eventually lead to amputation in extreme cases. Hyperglycemia induces oxidative stress in the neurons and sciatic nerves leading to nerve destruction.²⁷ Studies have shown that DPN reduces pain threshold and hypersensitivity to different pain stimulus which has been strongly associated with marred myelinated C fibres in diabetes resulting in hyperalgesia and pricking pain sensations.^{28,29} Pain related behavioral parameters including thermal and mechanical hyperalgesia have employed for evaluating DPN. Diabetic animals displayed significant hyperalgesia and increased sensitivity to pain stimulus in the hot plate, tail flick and von Frey test, which was consistent with previous studies.^{5,28,30} Administration of EBR to diabetic rats markedly improved thermal, cold and mechanical hyperalgesia in the treated diabetic rats.

Excessive hyperglycemia has been unequivocally shown to instigate progressive decline in motor coordination and nociceptive responses.^{5,31} In the current study, the diabetic rats exhibited significantly impairment in motor coordination on the rotarod test. In addition, in line with the results obtained from both the thermal and mechanical hyperalgesia, diabetes caused marked increase in nociception. Moreover, previous studies have shown that hyperglycemia through the action of ROS and oxidative stress leads to the release of inflammatory mediators including pro-inflammatory cytokines and prostaglandins from macrophages, Schwann cells and mast cells of the nociceptive neurons leading to increase in nociceptive responses.^{5,31} In line with earlier studies, diabetic rats showed amplified nociceptive responses in the acetic acid (abdominal stretching) and formalin tests (paw licking or biting behaviour).^{30,32} The result obtained from this study

demonstrated that ERB significantly attenuated acetic acid and formalin induced abdominal stretching and paw licking time, respectively in the treated diabetic rats.

The role of inflammation instigated by hyperglycemia induced ROS has been extensively reported in diabetic complications including neuropathy.^{10,33} Numerous studies have shown that inflammatory cytokines have a significant impact on the functioning of the nervous system. Proinflammatory cytokines including TNF- α and IL-1 β are critically involved in pain and hyperalgesia. In addition, the occurrence and severity of diabetic neuropathic pain is aggravated by increase in cytokines generation.^{34,35} Prolonged hyperglycemia can induce the glycosylation of nerve myelin protein, resulting in the activation of lymphocytes and macrophages leading to damages in peripheral nerve sheaths.^{34,36} In the present study it was observed that diabetic rats showed significantly increased levels of TNF- α and IL-1 β , whereas treatment with EBR alleviated the increased cytokine levels.

Understanding the phytochemical profile of *B. rotunda* is critical in the identification of bioactive constituents responsible for the observed antidiabetic, anti-inflammatory and analgesic effects. This study isolated and characterized six polyphenolic constituents as pinostrobin, pinocembrin, panduratin A, cardamomin, boesenbergin A and alpinetin. The findings of these polyphenols in EBR is in line with previous studies, as well as with their antidiabetic and anti-inflammatory properties. Pinocembrin and its O-methylated derivative pinostrobin have been demonstrated to have significant antinociceptive and anti-inflammatory effects.³⁷ Potipiranun et al also demonstrated that pinocembrin, panduratin A, cardamomin, and alpinetin displayed significant anti-glycation effect, while panduratin A and pinocembrin showed good α -glucosidase inhibition.^{16,20} Besides, pinostrobin and pinocembrin have both demonstrated extensive neuroprotective effects by in several disease models.^{38–40} These previous studies together with the results from our study suggests that these metabolites may play significant roles in the observed anti-neuropathic activity of EBR.

Conclusion

The results from this study suggested that EBR displayed ameliorative effects against hyperglycemia-induced DPN, through its anti-inflammatory and antinociceptive effects, thus minimizing pain hypersensitivity and exaggerated nociception. Further mechanistic studies is required.

Disclosure

The authors declare no conflicts of interest in this work.

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