Effect of *Calendula officinalis* Extract Against Streptozotocin Induced Diabetes in Male Rats

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ABSTRACT

Diabetes is the most prevalent metabolic disease worldwide. Diabetes has been ranked as the fifth leading cause of global death. This study aimed to examine the antidiabetic effect of Calendula officinalis on male rats. Forty male Wistar rats weighing 200±10g were distributed into four equal groups (n=10). Group (1) was kept as control negative, while groups 2,3 and 4 were injected intraperitoneally (i.p.) with streptozotocin (STZ) to induce diabetes. Group (2) was kept as the control positive group, while groups (3) and (4) received Calendula officinalis in doses of two hundred and four hundred mg/kg/d; respectively. The experimental period was 6 weeks, ended by sacrificing all rat groups; blood collected for biochemical analysis and pancreas was taken for histopathological examination. The results of diabetic rats treated with Calendula officinalis showed a significant increase in insulin, GSH, SOD levels and serum inflammatory cytokines (TNF- and IL-1), while there were significant decreases in glucose levels and MDA compared to the control negative group. There was also an improvement in histopathological changes observed in the pancreases of diabetic rats. Therefore, the administration of Calendula officinalis extract had antioxidant and hypoglycemic effects on diabetic rats.

Key Words: Diabetes, Calendula Officinalis, Streptozotocin, Rats, Cytokines, Histopathological.

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INTRODUCTION

Diabetes is a noteworthy worldwide issue because of drastically expanding around the world [1]. Diabetes mellitus (DM) is common all over the world, nevertheless it is more widespread (especially Type 2) in the more developed countries [2]. The DM is a group of complex and chronic metabolic disorders with diverse multiple etiologies. It is characterized by hyperglycemia [3]. Diabetes is a known risk factor for developing cardiovascular diseases [4]. Long-term complications may affect the organs such as kidneys, eyes, nerves, heart and blood vessels, and in the absence of effective treatment, result in death [5]. Diabetes mellitus is among the top 10 causes of death whether globally along with the cancer, cardiovascular diseases and respiratory diseases [6]. It has been reported that, approximately 5 million deaths in 2017 worldwide were attributable to diabetes in age range of 20–99 years [7]. The World Health Organization (WHO) ranked the Kingdom of Saudi Arabia (KSA) as the second highest country in the Middle East, while it ranked this country world widely as the seventh regarding DM incidence [8, 9].

Medicinal plants have been proven to have significant benefits in therapeutic uses, because of their efficacy, economical efficiency, and safety without any serious health problems. Thus, finding new antidiabetic drugs made of natural plants has been always desired because there are substances in them that have been indicated to be used as alternative and safe drugs for DM [10].

Calendula officinalis. (Asteraceae) is an herb which grows annually; its flowers' color is yellow to orange, and it is commonly planted in the Mediterranean region. Its common name is marigold [11]. *Calendula officinalis* is rich in minerals and vitamins; it has been used to cure muscular pain and piles, and also as an antiseptic. The petals have been used as natural color instead of saffron for coloring and flavoring rice, soups and other food types [12].

Many researchers reported that *Calendula officinalis* is safe to be used as a medicine for the treatment of inflammation, anti-HIV, cytotoxic, hepatoprotective and many other diseases [13]. The plant contains many antioxidant compounds such as flavonoids, esquiterpenes

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glycosides, xanthophylls, saponins, triol triterpenes and volatile oils [14]. Therefore, this study aimed to investigate the effect of oral intake of *Calendula officinalis* extract in STZ-induced diabetic rats.

MATERIAL AND METHODS

Material:

Plant material:

Fruits of *Calendula officinalis* were obtained from the local market, Jeddah, Saudi Arabia.

Animals:

Adult male Wister albino rats, weighing 200±10g, were purchased from King Fahd Medical Research Center. They were kept in standard laboratory conditions, and fed on a standard AIN-93 diet [15]. They were kept according to the standard guide for the care and use of laboratory animals in King Fahd Medical Research Center.

Chemicals and kits:

Streptozotocin and kits for serum biochemical analysis were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Methods:

Extraction of Calendula officinalis flowers:

Calendula officinalis flower methanolic extract was prepared by soaking two hundred grams of *Calendula officinalis* powder in one liter of 90% ethyl alcohol with continuous shaking for five days, and then it was kept in a refrigerator. Ethanol was evaporated by a rotor evaporator (Büchi labortechnik AG, R-215, Switerland) attached to a vacuum pump. Twenty grams (semi solid) of both extracts were suspended with 2 ml Tween 80 (suspending agent) in 100 ml distilled water to prepare a 20% alcoholic extract [16].

Phytochemical Screening of *Calendula officinalis* flowers Extract:

The phytochemical analysis of *Calendula officinalis* flowers' extract had been performed to find the presence of the major chemical constituents, including alkaloids, flavonoids, glycosides, Steroids, saponins and tannins using standard procedures of analysis [17].

Induction of diabetes and experimental design:

Forty male Wistar rats weighing 200±10 g (n=40) were kept under control conditions in a conventional animal house. They were fed a standard diet with access to water *ad libitum* [18]. The rats were divided into two main groups; the first (n-=10) served as control negative group, and the second group of DM rats (n=30) were injected intraperitoneally (i.p.) once by STZ (65 mg/kg) [19] to induce diabetes, after 12 h fasting. Since, the injection of STZ, the rats were given glucose solution (10%) in feeding bottles for 24 h to prevent hypoglycemia. Then, after 3 days fasting, blood sample was collected from tail vein of all the survived rats to analyze glucose level [20]. The diabetic rats selected for the study should have had blood glucose levels (>200 mg/dL) according to Dhanabal *et al.* [21].

Four groups of rats were divided as follows:

Group (1) included normal rats (control); Group (2) was DM control positive group; Group (3) was - DM + *Calendula officinalis* L (200 mg/kg/d); Group (4) included DM + *Calendula officinalis* L (400 mg/kg/d).

After 6 weeks of the experimental period, the treatments were stopped, and the rats in each group were sacrificed under light ether anesthesia. Blood samples were collected by heparinized capillary tubes, kept for couple of hours, and centrifuged at 3000 rpm for 15 min. Then the separated serum was stored at -20˚C for the subsequent analyses. The pancreas was removed for the histopathological studies.

Determination of serum biomarkers:

Blood Sugar and insulin were tested according to Mastan *et al*. [22]. Oxidative stress biomarkers including glutathione superoxide dismutase (SOD), reduced glutathione (GSH) and malondialdehyde (MDA) were tested using ELISA kits. The procedures were performed according to the manufacturer's protocols. Anti-inflammatory cytokines Interleukin– 1 (IL- 1) and tumor necrosis factor-α (TNF- α) were tested according to Piguet *et al*. [23] and Dinarello [24], respectively.

Histopathological examination:

The pancreas from all the experimental groups was excised of the body after sacrificing the rats. The tissues were fixed in 10% neutral formalin, dehydrated, and embedded in paraffin wax. Fixed tissues were cut at 5 µm sections, and then stained with hematoxylin-eosin by routine procedures [25].

Statistical analysis:

The analysis of the resulted data was done by SPSS software Version 22. The values were expressed as mean ± SD, and analyzed by one-way variance (ANOVA) followed by the comparison test (t-test). The results were considered as statistically significant at P≤0.05 according to Snedecor and Cochron [26].

RESULTS

The phytochemical screening of *Calendula officinalis* flowers' alcoholic extract revealed that it contained large amounts of flavonoids and glycosides; moderate amounts of alkaloids Steroids, and saponins and a few tannins as depicted in Table (1).

Table 1: Phytochemical screening of *Calendula officinalis* flowers extract

Phytochemical	Test results
Alkaloids	++
Flavonoids	+++
Glycosides	+++
Steroids	++
Saponins	++
Tannins	+

The following symbol indicated the intensity of active compounds: a small amount (+), a moderate amount (++), and large amount (+++).

Table (2) demonstrates the effect of *Calendula officinalis* extract on the blood glucose and insulin levels against STZ induced diabetes in male rats. Streptozotocin administration (control positive) caused a significant increase in the glucose level (211.76±3.95) (P<0.05) associated with the significant decrease (134.82±2.95) (P<0.05) in insulin level as compared with the control negative group (98.53±4.65 and 57.22±2.16) for blood glucose and insulin levels; respectively.

The oral administration of *Calendula officinalis* produced significant changes in the glucose and insulin levels (135.32±2.43, 106.46±2.13, 88.42±2.17 and 61.38±1.89); respectively (p<0.05) in a dose-dependent manner as compared with the control positive group (211.76±3.95 and 134.82±2.95) (p<0.05).

Table 2. Hypoglycemic effect of *Calendula officinalis* extract on blood glucose and insulin levels in diabetic rats.

Groups	BG (mg/dl)	Insulin (ng/ml)
Negative control (-ve)	98.53±4.65c	57.22±2.16c
Positive control (+ve)	211.76±3.95a	134.82±2.95a
Calendula officinalis (200 mg/kg/day)	135.32±2.43b	88.42±2.17b
Calendula officinalis (400 mg/kg/day)	106.46±2.13c	61.38±1.89c

- Values are presented as mean ±SD.
- Values with different superscript letters within a column are significantly different at P<0.05.

Table 3. Hypoglycemic effect of *Calendula officinalis* extract on serum MDA, GSH and SOD levels in diabetic rats.

Groups	MDA (nmol/g protein)	GSH (µg/mg protein)	SOD (U/mg)
Negative control (-ve)	12.35 ±1.54c	6.13 ±1.02a	58.29 ± 1.11a
Positive control (+ve)	24.98 ±1. 68a	3.54 ±0.17c	26. 46 ± 1.64c
Calendula officinalis (200 mg/kg/ day)	18.44 ±1.21b	4.86 ±0.67b	43.11 ± 1.41b
Calendula officinalis (400 mg/kg/ day)	13.73 ±1.02c	5.96±0.19a	55.54±0.23a

- Values are presented as mean ± SD.
- Values with different superscript letters within a column are significantly different at P<0.05.

The results revealed that STZ untreated group recorded significant increase (p<0.05) in MDA, and significant decrease in both GSH and SOD (p<0.05) compared with the control negative group after 6 weeks. The treatment with *Calendula officinalis* extract at the two doses used induced significant improvement (p<0.05) in MDA, GSH and SOD values compared with STZ untreated group. The high dose had better effect compared with the low dose in reducing the MDA and increasing GSH and SOD levels (Table 3).

The statistical analysis of the Anti-inflammatory cytokines was summarized in Table (4). The results revealed that, in the control positive group IL-1and TNF-α levels were significantly increased (p<0.05) more than in the control negative group .On the other hand, there was a significant decrease (p<0.05) of IL-1and TNF-α levels in STZ treated groups (200 and 400 mg/kg) when compared with STZ untreated group. The levels of IL-1and TNF-α returned to nearly normal range in the high dose treated group.

Table 4. Hypoglycemic effect of *Calendula officinalis* extracts on serum IL-1 and TNF-α level in diabetic rats.

Groups	IL-1 (pg/ml)	TNF-α (pg/ml)
Negative control (-ve)	22.33 ± 1.41c	11.42 ± 0.86c
Positive control (+ve)	57.78± 0.87a	25. 73 ± 0.76a
Calendula officinalis (200 mg/kg/ day)	49.26± 1.49b	16.17 ± 1.24b
Calendula officinalis (400 mg/kg/ day)	23.43± 0.34c	13.22±1.37c

- Values are presented as mean ± SD.
- Values with different superscript letters within a column are significantly different at P< 0.05.

Histopathlogical results

Pancreas sections of normal control rats showed normal pancreatic cell histopathology (Fig.1.A). Pancreas of the positive control group showed hypertrophy and hyperplasia of Langerhans beta-cell islets associated with nuclei pyknosis (Fig. 1. B & C). While the diabetic rats' pancreas treated with two hundred mg/kg b.wt. *Calendula officinalis* showed a slight pancreatic congestion (Fig.1. D & E). Sections of pancreas of the rats which received orally four hundred mg/kg b.wt. *Calendula officinalis* showed no histopathological changes (Fig.1. F).

DISCUSSION

This study investigated the effect of oral intake of *Calendula officinalis* extract in STZ-induced diabetes after six weeks of the experimental period. Streptozotocin is the most widely used substance for causing diabetes, and it has been indicated that it leads to oxidative stress by increasing ROS and intensifying the problems related to diabetes [27–29].

In this study, the results indicated that there was a significant increase in blood glucose, and a significant reduction in the level of insulin in the positive control group compared with the negative control group. These results might be due to the damage of pancreatic beta cells caused by STZ combined with free radicals which were generated by the oxidative stress production [30]. As oxidative stress is caused by hyperglycemia, people with diabetes encounter dysfunctions of tissue [27, 31]. The destruction of beta cells and reduction of insulin production lead to diabetes.

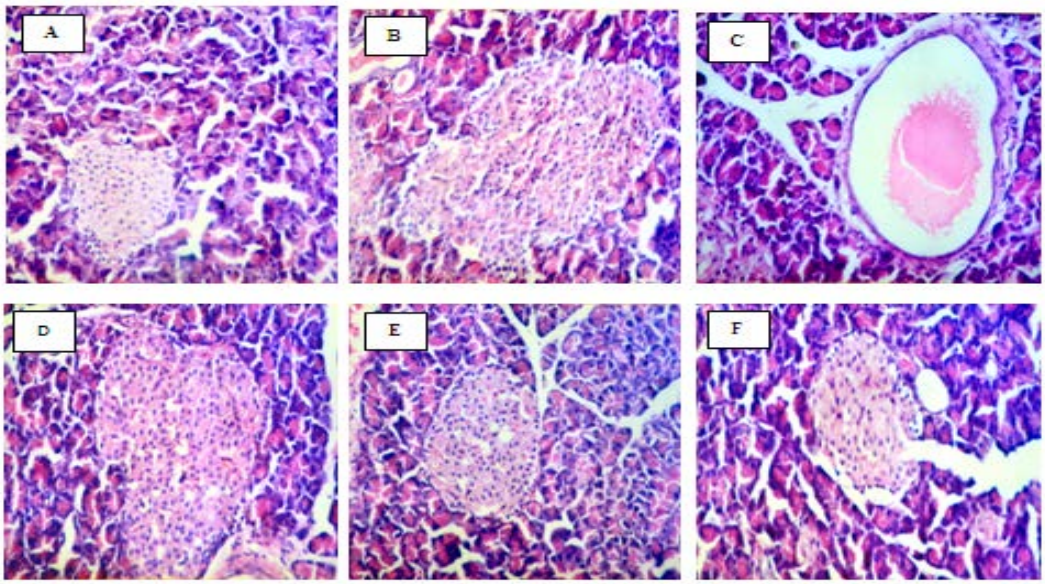

Fig. 1: **Photomicrography illustrating H&E-stained sections of pancreas in different groups. Pancreas of control negative rats showing no histopathological changes (A). In diabetic rats, pancreas sections showing hypertrophy and hyperplasia of beta cells islets of Langerhans associated with the pyknosis of their nuclei (B), with cystic dilatation of pancreatic duct (C). In diabetic rat pancreas treated with 200 mg/kg b.wt. *Calendula officinalis* showing vacuolation of β cells of islets of Langerhans (D), with slight congestion of pancreatic blood vessels (E) while pancreas rats received 400 mg/kg b.wt. *Calendula officinalis* orally, showing no histopathological changes (F). (H and E x 400)**

Oral administration of *Calendula officinalis* extract showed decrease in glucose level and increase in insulin level of both streptozotocin treated groups compared to the positive control group. This might be due to the antioxidants present in *Calendula officinalis* extract that was confirmed by the phytochemical screening which revealed that it had large amounts of flavonoids and glycosides; moderate amounts of alkaloids Steroids and saponins. These results were in the same line with Khalid *et al*. [32] who examined the administration of two hundred and six hundred mg/kg of the extract of marigold. The results indicated that there was a significant reduction in blood glucose, while insulin was significantly increased in glucose-loaded rats. Furthermore, it has been indicated that hydroalcoholic extract obtained from the leaves of marigold remarkably decreased blood glucose, cholesterol, and phospholipids in the diabetic rats with alloxan [30].

The injection of STZ resulted in inducing oxidative stress which was confirmed by an increase in MDA level, a decrease in serum GSH and SOD activities, and also there was an increase in pro-inflammatory cytokines IL-1 and TNF-α levels as compared to the non diabetic group. The present findings were in the same compatibility with the results reported previously by Adewoye and Adele [33], Gao *et al*. [34] and Miao *et al*. [35]. This negative effect can be attributed to the changes in gene expression, which led to myocardial cell death; it has also been observed that the main reason for changes was oxidant-antioxidant imbalance [36]. It can be suggested that increased oxidative stress by reducing plasma antioxidants and increasing peroxidation of lipids could increase the susceptibility of mesangial cells to free radicals injury.

Wolf and Ziyadeh [37] demonstrated that hyperglycemia had been reported to lead to increased formation of advanced glycation end-products (AGEs), oxidative stress, polyol activation and hexosamine flux, causing inflammation. A strong relationship has been found between the inflammatory processes, the subsequent β-cells dysfunction, and insulin signaling impairment [38]. TNF-α is a pleotropic peptide which is very important in several inflammatory and cytotoxic reactions [39].

The damage of tissue by oxidative stress could be reduced by lowering oxidative stress. Antioxidants act as a radical scavenger and inhibit lipid peroxidation and other free radical processes, protecting the human body against various diseases [40]. *Calendula officinalis* extract treatment reduces MDA significantly, and increases the GSH and SOD activities. These results may be due to the antioxidant activity of the extract of *Calendula officinalis*. This study results were consistent with Cordova *et al*. [41] and Victorrajmohan *et al*. [42] who stated that increased GSH levels play an important role in protecting the body from toxic chemicals.

CONCLUSION

All the above complications in diabetic rats can be related to the oxidative stress of increased glucose levels in the blood. Oxidative stress can cause oxidative damage to cell membranes and change in the structural and functional integrity of subcellular organelles, resulting in various complications of diabetic disease. Treatment with *Calendula officinalis* can decrease the risk of diabetes because of its antioxidant activity.

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