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The Effect of "*Teucrium polium L.*" Extracts on Insulin Release from *in situ* Isolated Perfused Rat Pancreas in a Newly Modified Isolation Method: the Role of Ca^{2+} and K^{+} Channels

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ABSTRACT

Background: "*Teucrium polium L.*" (TP) has been long recommended in Iranian folk medicine for its anti-diabetic activities. We attempt here to evaluate the effect of TP extract on insulin secretion in rat pancreas. **Methods**: Rat pancreas was isolated *in situ* and perfused with Krebs solution containing low glucose (LG, 2.8 mM) or high glucose (HG, 16.7 mM) as perfusate. The aqueous extract (Aq. E) and methanolic extract (Met. E) of TP aerial parts and two partition fractions of Met. E were added to perfusate to evaluate insulin release. Diazoxide (DZX) and verapamil (VPM) were also used for assessing the probable mechanism of the effects. In each experimental group, the peak and baseline of insulin levels in effluent samples were compared. The GC/MS analysis was carried out to detect active ingredients in the extracts. **Results:** Adding Met. E to the LG caused a significant increase ($P<0.05$) in insulin release from the basal level of 0.17 ± 0.05 µg/l to a peak value of 3.94 ± 1.29 µg/l. when Met. E was introduced to the HG, there was a further protracted stimulation of insulin release from 2.15 ± 1.35 µg/l to 6.16 ± 0.52 µg/l. Both DZX and VPM when added separately to the LG, led to inhibition of Met. E induced insulin secretion. The Aq. E and fractions had no significant effect on insulin secretion. Only in the Met. E, the component 5-hydroxy-4',7-dimethoxyflavone (apigenin-4',7-dimethylether) was detected. **Conclusion**: It can be concluded that the insulinotropic properties of TP extracts can be attributed to the presence of apigenin existing only in Met. E, but not in Aq. E and fractions. Moreover, certain types of K^{+} and Ca^{2+} channels take part in this effect. *Iran. Biomed. J. 14 (4): 178-185, 2010*

Keywords: Teucrium extract, Pancreas, Perfusion, Insulin

INTRODUCTION

The world prevalence of Diabetes mellitus (DM) among adults (aged 20–79 years) will reach 6.4%, affecting 285 million adults, in 2010 and will increase to 7.7% (439 million adults) by 2030. Between 2010 and 2030, there will be a 69% increase in numbers of adults with DM in developing countries and a 20% increase in developed countries. These predictions indicate a growing burden of DM, particularly in developing countries [1].

A noticeable percentage of DM patients are afflicted with a few complications of this disease [2-4]. The excess of morbidity and mortality associated with DM and its complications emphasize the importance of new or alternative therapeutic strategies in prevention and treatment of this chronic disorder.

Medicinal plants serve as excellent resources for new medications and today, we see an increasing interest in their use. The WHO has recommended medicinal plants to be used more effectively in the healthcare systems [5]. Medicinal herbs grow vastly

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in Iran and are widely used for natural treatment of diseases. One of these medicinal plants, which has been recommended in Iranian folk medicine for treatment of DM since centuries ago, is "*Teucrium polium L.*" (TP).

TP (family Lamiaceae) is one of the 300 species of the genus *Teucrium* and is found mainly in the Mediterranean and western Irano-Turanian sphere [6]. In Iran, this medicinal plant, also named as Calpoureh, is widely distributed and traditionally used by native inhabitants as herbal tea, a spice or a hypoglycemic agent that is recommended by herbalists.

Some biological and therapeutic effects have been reported for TP such as anti-oxidant [7-12], anti-inflammatory [13, 14], anti-nociceptive [15, 16], anti-pyretic [17, 18], anti-microbial [17, 18], hypolipidemic [19], hepatoprotective [20, 21], anti-gastric ulcer [22], cytotoxic and apoptotic effects [23].

Some studies have shown that oral, intravenous or intraperitoneal administration of a TP crude extract to STZ-induced diabetic rats can significantly decrease serum glucose values [24-26]. It has also been demonstrated that administration of a hydro-alcoholic extract of TP by gavage enhances serum insulin level in rats [25]. Other reports show that the TP crude extract has a dose-dependent stimulatory effect on insulin secretion from rat-isolated islets [25, 27].

As literature review shows, there is no study to assess the effect of TP on isolated perfused pancreas endocrine parameters and determine the mechanism of effects. To confirm and complete previous research findings, in the present study, the aqueous and methanolic crude extracts of TP aerial parts and two partition fractions of methanolic extract (Met. E) have been evaluated for their effects on insulin secretion from isolated *in situ* perfused rat pancreas. Furthermore, certain expected physiological pathways have been investigated for finding the probable mechanism of the effects.

MATERIALS AND METHODS

Animals. Male (locally produced) rats, 9-11 weeks old and 250-300 g, were housed in groups of five in an air-conditioned colony room on a 12 h light/dark cycle at a constant temperature (21 ± 0.5°C) and supplied with a standard pelleted diet and tap water ad libitum. Procedures involving animals and their care were conducted in accordance with Institutional Guidelines of Ethics Committee of Tehran University of Medical Sciences (Iran) and in conformity with the criteria outlined in the Guide for the Care and Use of Laboratory Animals (Copyright 1996, National Academy of Sciences, http://books.nap.edu/readingroom/books/labrats/).

Chemicals and reagents. NaCl, KCl, $CaCl_2$, $MgSO_4$, KH_2PO_4, $NaHCO_3$, extra-pure methanol (99.9 %) and BSA were obtained from Merck (Germany). The other chemicals, used in this study, consist of heparin sodium 5000 U (Caspian Tamin, Iran), DZX (Sigma, Germany), VPM hydrochloride (Lekoptin), ketamine hydrochloride 5% (Trittau, Germany), xylazine (Alfasan; Woerden Holland) and dimethyl sulfoxide (Carlo Ebra Reagenti, UN).

Plant material. The wild TP were collected from mountains in Tehran province (Iran) in spring 2007. A voucher herbarium specimen (no. 1696) was deposited in the herbarium of the Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The aerial parts were separated, cleaned, and protected from direct sunlight, air-dried at room temperature for 7 days, then kept in a closed container at 25°C. Suitable amounts of dried plant were coarsely grounded and used for extraction.

Preparation of extracts and fractions. To obtain the aqueous extract (Aq. E), 44 g of the ground plant material was macerated using 1500 ml of deionized water followed by 15 minutes of boiling under continuous stirring. In preparation of the Met. E, 25 g of the ground plant material was macerated with 250 ml of methanol (99.5 %) at room temperature for 48 hours during which it was occasionally mixed. The extracting was followed by rapid filtration through a crude cellulose filter and then by a more delicate filtration through Whatman no. 1 filter. The Aq. E was concentrated using steam bath whereas a rotary evaporator was used to remove methanol from the Met. E. The dry weight in the extract was evaluated in samples dried at 80°C until constant weight. The percentage yields based on the dried starting material was 21% for dried Aq. E and 13.7% for dried Met. E (w/w). Successively, the Met. E was subjected to the following fractions: 75 ml of petroleum ether was added to 11 g of Met. E and the mixture combined continuously for 48 hours in a closed container. Then, the soluble part

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was separated by filtration and in free contact with air the petroleum ether fraction (PtE) was earned. Chloroform (75 ml) was added to the insoluble residue (from previous phase), and similar to the PtE, chloroform fraction (Clf) was obtained. The concentrated extracts and fractions as stock were stored at -20°C in a desiccant until require. PtE and Clf were dissolved in polyoxyethylene sorbitan monolate (Twin 80, 2%) before being used in the experiment. The final concentration of Twin in all solutions adjusted to 9%. DZX was dissolved in DMSO (0.2% in all solutions) and suitable concentrations of the extracts, fractions, DZX and VPM were prepared by dilution of the stocks with standard Krebs solution.

Experimental procedure:

In situ isolated perfused pancreas model. After overnight fasting (22 ± 2 hours) with free access to water, a tail blood sample was taken for measurement of fasting blood glucose using a glucometer (Accu-Chek, Roche Diagnostic, Germany). Only rats with fasting blood glucose between 75 to 100 mg/dl were used for experiments. Rats were then anesthetized with an intraperitoneal injection of rodent combination anesthesia (ketamine, 70 mg/kg; xylazine, 15 mg/kg and phosphate buffered saline, 0.7 ml/kg). The procedure for *in situ* surgical isolation and vascular perfusion of the rat pancreas was performed as described in the previous study [28] with several modifications. Briefly, after opening the abdominal cavity, the duodenum was transected distal to the sphincter of Oddi (5 cm from pyloric sphincter), and the proximal end was cannulated and tied securely around a cannula. This allowed the pancreatic and duodenal juice produced throughout the experiment to be drained and collected. The beginning of duodenum and the lower end of esophagus with nearby vessels were ligated. Two loose ligatures were applied around the portal vein and also the adjacent anatomical parts (common bile duct and hepatic artery). Abdominal aorta from the diaphragm up to near the left renal artery was exposed and the right renal artery and superior mesenteric artery were ligated adjacent to the aorta. Two loose ligatures around the aorta above the left renal artery were conducted. Afterward, the abdominal aorta above the celiac artery was ligated and immediately the aorta via loose ligatures was cannulated with a sterile IV cannula (yellow, G 24). Perfusion of the pancreas started so that the perfusate was administered into the celiac artery via abdominal aorta and run into the prepared pancreas by an open circuit 'non-recycling perfusion system'. The gland was perfused with Krebs solution containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 2.8 or 16.7 mM D-glucose to which BSA (5 g/l) was added. The perfusate was continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide and the resulting pH was close to 7.4. The temperature of perfusate adjacent to the pancreas was maintained constant at 37°C. The perfusate was directed to the pancreas using a peristaltic pump (Meredos GmbH, Bovenden, Germany). Flow rates through the pancreas were approximately 1.5 ml/min at perfusion pressures of about 34 mm Hg. The portal vein was then cannulated and the pancreatic effluent was continuously collected at 1 min intervals in ice-chilled tubes, and then stored at -70°C for insulin assay. At the end of perfusion, pancreas was perfused with methylene blue to confirm the true isolation of the pancreas.

Perfusion protocols. In all experimental groups, after isolation of the pancreas, the isolated organ was perfused with Krebs solution containing low glucose (LG, 2.8 mM) for 20 min as an equilibration period. Then, the substance/s was added to perfusate as discussed below. At the end of perfusion, the perfusate was switched back to the LG to study the post-intervention activity. Vehicles (Twin, DMSO) were added to LG with constant concentration as needed. This study was carried out in 6 groups (4 isolated tissues in each group): In groups 1, 2 and 3, Met. E, PtE or Clf were added to perfusate in minutes 20-30. In group 4, pancreases were perfused with Krebs solution containing high glucose (HG, 16.7 mM) in minutes 20-30 and Met. E was added in minutes 25-30. In groups 5 and 6, DZX (0.3 mM/l) or VPM (0.05 mM/l) were added to perfusate in minutes 20-35 and Met. E was added in minutes 25-35. Based on literature reviews [9-11, 25] and our pilot study, the extracts/fractions were used at a dose of 1 mg/ml.

Biochemical analysis. Insulin assays were performed with rat insulin ELISA kit (Mercodia, Uppsala, Sweden; Sensitivity: 0.07 µg/l) and ELISA reader (Sunrise Model, TECAN Co., Austria) according to the manufacturer's protocol.

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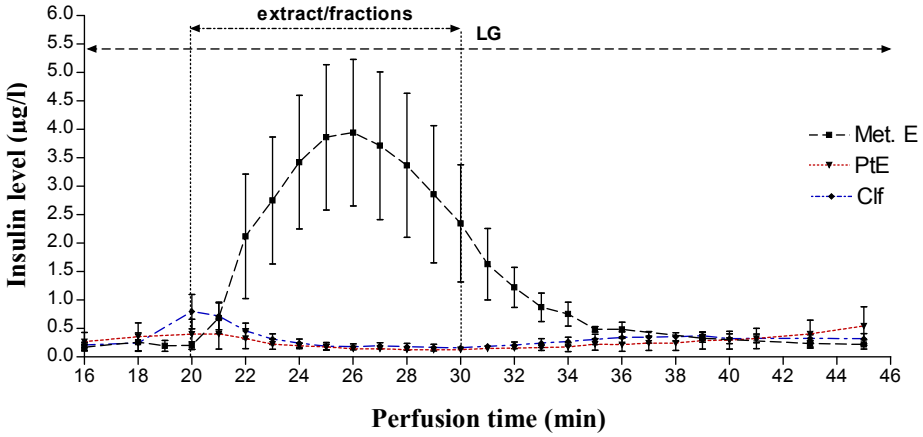

Fig. 1. Effects of TP extract/fractions on insulin release from *in situ* isolated perfused rat pancreas. Met. E caused a significant (P<0.05) increase in insulin release from the basal level at min 20 to a peak value at min 26, but PtE and Clf have no significant effect on insulin secretion. Data are presented as means ± SEM from four separate experiments. Krebs solution contained LG (2.8 mM). Met. E, methanol extract; PtE, petroleum ether fraction; Clf, chloroform fraction (1 mg/ml); LG, low glucose.

GC/MS analysis. GC/MS analysis of Met. E and fractions was performed on a mass spectrometer connected to a gas chromatography system (GC/MSD) 5975 c series Agilent, which was equipped with a HP-5MS capillary column (30 m × 0.25 mm; film thickness of 0.25 µm). Helium was used as carrier gas at a rate flow of 1 ml/min. The GC oven temperature was programmed from 70°C to 270°C at a rate of 4°/min and kept at 270°C for 2 min. MS was performed at 1 scan s^{-1} with ionizing voltage of 70 V and ion source temp. of 250°C. Separated compounds were identified by comparing their retention times with those of authentic standards injected under the same chromatographic conditions and by comparison of their retention indices and their mass spectra of the unknown peaks with the MS Library.

Statistical analysis. The effect of stimulants on insulin release was calculated by comparison between peak and baseline (control) insulin level in the effluent samples. All data are presented as means ± SEM. Means of insulin concentrations before and after interventions were compared using paired student's *t*-test and significance level set at P<0.05.

RESULTS

In our pilot study, we found that the aqueous crude extract in different doses (0.01, 0.1, 0.6, 1 and 10 mg/ml) does not have any effect on insulin secretion from isolated pancreas.

Effects of extract/fractions of TP on insulin release. Met. E (1 mg/ml) caused a significant (P<0.05) increase in insulin release during a 10-minute perfusion, from the basal level of 0.17 ± 0.05 µg/l at min 20 to a peak value of 3.94 ± 1.29 µg/l at min 26, but PtE and Clf had no significant effect on insulin secretion (Fig. 1).

Effect of Met. E on glucose-stimulated release of insulin. A 5 minute perfusion of pancreas with HG caused a significant (P<0.05) steep rise in insulin release from the basal level of 0.11 ± 0.01 µg/l at min 20 to a peak value of 4.85 ± 1.19 µg/l at min 22 (Fig. 2). In the next 5 minutes of perfusion, when Met. E was introduced in HG, there was a further protracted stimulation of insulin release from 2.15 ± 1.35 µg/l at min 26 to 6.16 ± 0.52 µg/l at min 30, which reversed the falling insulin output observed in the continued presence of 16.7 mM glucose alone.

Effect of Met. E on insulin release in presence of DZX or VPM. The final study was performed to evaluate the possible mechanisms underlying the insulin secretory actions of TP. There was no significant change in insulin level when DZX or VPM was added to LG (Fig. 3). When Met. E was added to LG containing DZX/VPM, no significant increase was seen in insulin secretion from pancreas (DZX: basal level of 0.12 ± 0.01 µg/l in min 20, peak value of 0.53 ± 0.16 µg/l in min 34; VPM: basal level of 0.22 ± 0.06 µg/l in min 20, peak value of 0.63 ± 0.42 µg/l in min 30).

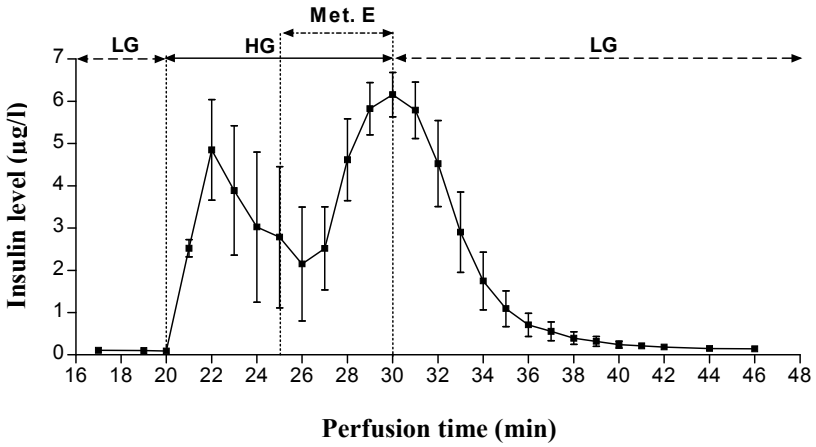

Fig. 2. Effect of TP methanolic extract (Met. E, 1 mg/ml) on glucose-stimulated release of insulin from *in situ* isolated perfused rat pancreas. Perfusion of pancreas with high glucose (HG) caused a significant (P<0.05) steep rise of insulin release from the basal level at min 20 to a peak value at min 22. When Met. E was introduced in HG, there was a further stimulation of insulin release from the basal level at min 26 to a peak value at min 30. Data are presented as means ± SEM from three separate experiments. Krebs solution contained LG (2.8 mM) and HG (16.7 mM). Met. E, methanol extract; LG, low glucose; HG, high glucose.

Identification of compounds in the extract. The only bioactive component that detected in the *Met. E* was 5-hydroxy-4', 7-dimethoxyflavone (apigenin-4', 7-dimethylether).

DISCUSSION

The *in situ* perfused pancreas method has been discussed by some researchers [29, 30]. We have introduced certain modifications in the previous procedure in order to better save the normal tissue of the pancreas. With an aortic approach, all legations on vessels and the GI were carried out without hemorrhage or severance of any of the abdominal organs. Our results show that the pancreatic tissue in all parts of the experiments were alive until the end of perfusion; therefore, this modified model can be effective and is recommended for studies on the pancreas where live tissue is needed.

Some researchers have reported hypoglycemic effects of Aq. E and alcoholic extract of TP [24-26]. On the basis of our findings, the Aq. E of TP does not have any effect on pancreas insulin secretion. Therefore, probably it reduces blood glucose via mechanisms such as enhancement of peripheral metabolism of glucose rather than an increase in insulin release.

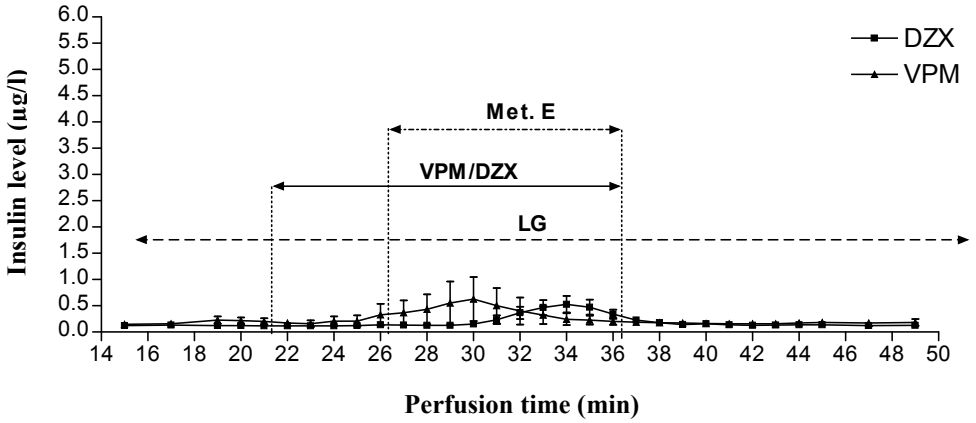

Fig. 3. Effect of TP Met. E (1 mg/ml) on insulin release from *in situ* isolated perfused rat pancreas in presence of DZX or VPM. When Met. E was added to low glucose containing DZX/VPM, no significant increase was seen in insulin secretion from pancreas. Data are presented as means ± SEM from four separate experiments. Krebs solution contained LG (2.8 mM). Met. E, methanol extract; LG, low glucose.

In the present study, it is clearly demonstrated that TP methanolic extract stimulates the basal insulin secretion, and is also able to potentiate glucose-stimulated release of insulin from *in situ* isolated perfused rat pancreas. Our results are in agreement with those reported by other groups of investigators using a variety of other methods (isolated islets, serum level insulin) in assessing the insulinotropic activity of TP extracts [25, 27]; both mentioned studies report the enhancement of insulin release by hydro-alcoholic TP crude extract. Our findings show that only alcoholic extracts of TP can increase insulin secretion from the pancreas.

In this study, we also assessed some fractions of TP methanolic extract (PtE and Clf) to find which part of the extract is responsible for its effect and the results show that they do not have any effect on insulin secretion. Referring to our results, it would be reasonable to propose that the insulinotropic properties of TP extracts can be attributed to the presence of certain bioactive components existing only in Met. E, found neither in PtE nor in Clf.

Phytochemical investigations on the TP aerial parts extracts have shown the presence of some flavonoids and flavons such as 4', 5-dihydroxy-6, 7-dimethoxyflavone (cirsimaritin); 3', 5 dihydroxy-4', 6, 7-trimethoxyflavone (eupatorin); 5-hydroxy-4', 7-dimethoxyflavone (apigenin-4', 7-dimethylether) and 4', 5, 3'-trihydroxy-6,7-dimethoxyflavone (cirsiliol) [7, 31-33]. The biological activity of some above compounds with respect to the hypoglycemic or insulinotropic effects of the plant has been reported in some researches.

In one study, the hypoglycemic activity of TP extracts was referred to the presence of flavonoids, sterols, and volatile oils as active components [13]. In another study, it was indicated that the major flavonoids from a TP methanol extract (rutin and apigenin) can protect pancreatic islets against exposure to STZ *in vitro* due to their anti-oxidant activity, thereby significantly increasing insulin release by almost 56 % compared with STZ-treated islets [7]. Previously it has been showed that rutin has a hypoglycemic effect probably via an extra pancreatic pathway (insulin-independent mechanism), possibly by stimulating glucose utilization in extra hepatic tissues [34]. Several studies have demonstrated the hypoglycemic effects of flavonoids (particularly apigenin), as well as their action on insulin secretion and glucose-induced insulin release [35-37]. In agreement with the above studies in the present findings, probably the apigenin-4', 7-dimethyl ether as the chief component of the TP methanol extract, is responsible for stimulating the insulin release from pancreas.

There is a well known physiologic pathway for stimulation of insulin secretion from B cells. The intracellular ATP formed by oxidative phosphorylation inhibits ATP-sensitive K^+ (K_{ATP}) channels, reducing K^+ efflux. This depolarizes the B cells, and Ca^{2+} influx is increased via the voltage-gated Ca^{2+} (Ca_V) channels. This Ca^{2+} influx (from intra-cellular or extra-cellular reservoirs) stimulates release of insulin by exocytosis of a readily releasable pool of insulin containing secretory granules, producing the spike of insulin secretion [38, 39]. The insulin stimulatory agents affect on at least one part of this pathway. The β-cell K_{ATP} and Ca_V channels have been considered a potential target for the development of novel agents for treatment of diabetes. At least six Ca_V channels have been discussed in the pancreatic B cells that all of them are involved in stimulus-secretion coupling [38]. Also, several types of K_{ATP} channels have been observed in these cells [40].

In the present study, DZX (a K_{ATP} channel opener) and VPM (a Ca_V channel blocker) were added separately to the perfusate to evaluate the changes in extract induced insulin release. As our results show, both DZX and VPM led to inhibition of TP induced insulin secretion. Therefore, these data indicate that the insulinotropic effect of TP extract could be mediated through some types of K_{ATP} and Ca_V channels (physiologic pathways). Previously it has been demonstrated that the compounds which block K_{ATP} channels stimulate insulin secretion at both LG and HG concentrations [41]. We have shown that the TP extract can stimulate insulin release at LG and HG concentrations so that it may be mediated via K_{ATP} channels. It is likely that the effective components of the extract stimulated insulin secretion via K_{ATP} channels, Ca_V channels or both of them.

More studies are needed to evaluate the effect of the major components in the "*TP*" plant extracts on insulin release from isolated pancreas, isolated islets or clonal beta cells for finding the active component and its mechanism of action. Our findings suggest that in the field of Therapeutic research on diabetes "*TP*" is highly noteworthy for particular investigation due to demonstrated central effects on the pancreas and peripheral hypoglycemic effects of this plant. Toxicology studies and clinical trials are required to determine if TP has the potential to be an effective and safe glucose lowering agent for diabetic patients.

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It can be concluded that the apigenin in the "*TP*" methanolic extract stimulates the basal and glucose-stimulated insulin secretion from *in situ* isolated perfused rat pancreas probably due to its effect on K_{ATP} and Ca_V channels in the pancreatic B cells.

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