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Mechanism of action of a bioassay-guided aqueous fraction of *Pterocarpus marsupium* Roxb hardwood on glucose-dependent insulin secretion

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Abstract

We previously demonstrated that a high molecular weight aqueous fraction of *Pterocarpus marsupium* Roxb. (PM) hardwood has insulinotrophic properties. However, the pharmacological mechanisms by which this fraction modulates insulin secretion remained unknown. The present study therefore isolated the insulinotrophic activity enriched fraction (AEF) from aqueous extract of PM and examined its pharmacological mechanisms. A bioassay method utilizinginsulin secretion from mouse pancreas *in vitro,*was used to fractionate the insulinotrophic activity of PM and to delineate its pharmacological mechanisms. In addition, the effect of AEF on glucose clearance in normoglycemic, non-diabetic sheep *in vivo* was examined. The AEF mimicked the effect of sulphonylureas on insulin secretory pathways and modulated insulin biosynthesis. However, unlike tolbutamide, AEF-induced insulin secretion is glucose-dependent. Furthermore, three daily intravenous administrations of AEF had prolonged effects on glucose responsiveness in non-diabetic normal sheep. The use of AEF to combat the adverse effects of hyperglycemia appears to be beneficial by enhancing and sustaining the glucose-dependent insulin secretion processes in pancreas. Of note, the insulinotrophic effect of AEF is prolonged by many hours to days, unlike the numerous conventional insulin secretagogus which over stimulate the β-cells or pose a risk of hypoglycemia.

Keywords: Pterocarpus marsupium; Glucose-dependent insulin release; Traditional medicine; Type 2 diabetes mellitus

Introduction

The increasing prevalence and incidence of type 2 diabetes mellitus (T2DM) and associated metabolic diseases [1] demands various novel therapeutic strategies for their treatment and management. Although pathogenesis of T2DM remains largely unknown, it has been well demonstrated that T2DM occurs when insulin secretion no longer compensates for insulin resistance which is often associated with obesity, aging and illness [2]. The goal of many current pharmacological treatments is the same irrespective of the cause of T2DM: namely, to normalise blood glucose. Importantly, insulin must be released from the pancreas in an exquisitely exact amount, at the correct time and in a correct pattern [3]. So far, no pharmacological agent can take over or restore this exquisite sensing capacity when pancreas is diseased, and no agent can restore the exact pattern of insulin kinetics. Consequently, scientists are now investigating a number of traditional medicinal plants to identify potentially useful compounds.

Ayurvedic physicians and the populations in central India have a traditional belief that the water stored overnight in a wooden tumbler made up of *Pterocarpus marsupium* Roxb. (PM) hardwood is a magical remedy to treat T2DM [4-6]. The antidiabetic activity of PM has been demonstrated in experimental animal models [7-13], and also in humans [14]. However, there is some controversy over the identity of the antidiabetic principle of PM [9, 13, 15-19]. Furthermore, the pharmacological process by which PM affects glucose homeostasis is still not completely understood. Hence, the present work fractionated the insulinotrophic activity of the aqueous extract of PM and examined the pharmacological and physiological mechanisms by which it elicits its effects on insulin secretion from mouse pancreas *in vitro* and on glucose clearance in sheep *in vivo*.

Materials and methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) and the antibiotic-antimycotic solution used (10,000 units/ml penicillin G sodium, 10,000 µg/ml streptomycin sulphate, 25 µg/ml amphotercin B, 0.85% saline) were purchased from Gibco, Invitrogen Australia Pty Limited, VIC, Australia. Bovine Serum Albumin (BSA), diazoxide, arginine, KCl and tolbutamide were purchased from Sigma Aldrich Pty Limited, NSW, Australia. Human insulin (Actrapid®) was purchased from Novo Nordisk Pharmaceutical Pty Limited, NSW, Australia. Tissue culture plates, Syringe filters (0.45 m and 0.22 m) were purchased from Sarstedt Australia Pty Limited, SA, Australia. All of the chemicals used were of analytical grade, unless otherwise specified.

Animal ethics

Animal experimentation using mice and sheep were approved by University of New England Animal Ethics Committee and are in accordance with NHMRC Guidelines for Animal Experimentations.

Activity enriched fraction (AEF) preparation

Pterocarpus marsupiumRoxb hardwood was purchased from an Ayurvedic medical store, India. Aqueous extract of PM (PME) was prepared as previously described [13]. Briefly, 30 litres of PME (0.4 mg/ml) was eluted through 500 ml of Q Sepharose (QS) anionic ion exchange resin column (Amersham Biosciences, NJ, USA) that had been pre-equilibrated with 20 mMTris/HCl buffer, pH 8.0. The column had an internal diameter of 4.5 cm and a bed height of 15 cm. The flow rate was maintained at 10 ml/min throughout the run. The material that eluted through the QS column (QSE) was collected and stored at -20ºC for further investigations. The material retained on the QS column was then eluted using 1 litre of 1M NaCl in 20 mM Tri/HCl buffer, pH 8. The salt eluted material (QSR) from the QS column was then concentrated through 10 kDa cut-off ultrafiltration membrane (YM-10; DIAFLO, Amicon Scientific, Australia) to a final volume of 10 ml and washed with distilled water. The material that diffused through 10 kDa cut-off membrane (QSR/10KE) was collected and stored at -20ºC for further investigations. The material that was retained by the membrane (QSR/10KR) was further fractionated using a Sephadex G25 (Sx) column (PD-10; Amersham Biosciences, NJ, USA). This fractionation process yielded 10 fractions (Sx1, Sx2.....Sx10) each consisting of 10 ml. After every fractionation process, the fractions were tested for their effect on insulin secretion from mouse pancreas tissues in vitro. Bio-assay results indicated that the insulinotrophic activity of PM was found to be enriched in the following order: PME>QSR>QSR/10KR>Sx2 (data not shown). Based on the bio-assay results Sx2 was selected for further pharmacological examinations and this fraction was identified as activity-enriched fraction (AEF). This fraction was brown in color and its concentration was found to be 0.83 mg/ml. The yield from the raw material was 0.007%.

Insulin secretion *in vitro*

Insulin secretion from mouse pancreas was carried out as previously described [13, 20]. For the concentration response, pancreas tissues were incubated with treatments, PME or AEF of increasing final concentration, for 24 hours. To examine the glucose-dependent insulin secretion, tissues were incubated without or with treatments for 24 hours either in 5 mM (normoglycemic) or 12 mM (hyperglycemic) media glucose. To investigate the pharmacological mechanisms underlying AEF-induced insulin secretion, pancreatic tissues were incubated either in the absence or presence of AEF (1 10^{-3}µl; volume adjusted to 10 µl with phosphate buffer saline) without or with pharmacological agents including, tolbutamide (0.1 mM; a [K^+sub.ATP] channel blocker), diazoxide (0.5 mM; an established opener of [K^+sub.ATP] channel), arginine (10 mM; a cationic amino acid that depolarizes β-cells) and KCl (25 mM; a membrane depolarizer) for 24 hours in the media containing 12 mM glucose. To determine whether AEF affect insulin synthesis, mouse pancreatic tissues were incubated at 12 mM media glucose for 24 hours in the absence or presence of AEF and the media samples were collected at 12 and 24 hours. After this period, the tissues were allowed to recover in culture media containing 5 mM glucose for 24 hours. The tissues from the wells were then transferred and homogenized in tubes containing 1 ml radio immunoassay (RIA) buffer. The tissue and media samples from each well were then stored at –20ºC until analyzed for insulin content.

Intravenous glucose tolerance test (IVGTT)

To determine the effect of AEF on glucose clearance, IVGTT was conducted in normoglycemic, non-diabetic sheep. Sheep are a good model for studying glucose responsiveness because they are not exposed to dietary glucose, steady baseline glucose levels, being entirely reliant on gluconeogenesis, and are relatively insulin resistant [21]. Briefly, 12 sheep (Merino wethers) of 2 to 3 years of age were obtained from the Rural Properties at the University of New England (Armidale, NSW). They were acclimatised for 3 days in individual pens (12 m2 in area) and allowed ab lib access to feed (blend of white chaff and lucerne) and water. All the animals were weighed and randomly divided into control and treatment groups (6 animals per group). On the day prior to beginning experiment (day -4), jugular catheters were inserted and the animals remained untreated. The following morning (day -3) and also over the next two days (day -2 and -1), intravenous glucose was administrated (2.5 mg per kg body mass) via the catheter as a bolus over 1 min to stabilise the normal glucose clearance. On day 0, blood samples were collected via catheter to evaluate the basal glucose and insulin levels (0 min). Following this, intravenous glucose was administrated (2.5 mg per kg body mass) via catheter as a bolus over 1 min to initiate the glucose tolerance test (GTT). The blood samples (10 ml) were withdrawn at 15, 30, 45, 60, 90, 120 and 180

minutes. Following the day 0 IVGTT and over the next two days (days 1 & 2), the control and treatment groups, received a daily intravenous injections of saline (0.9% NaCl) or AEF (5 l; diluted in 10 ml saline), respectively. On day 3, an IVGTT was performed. After the day 3 IVGTT, catheters were removed and the animals were moved to an open paddock. On day 20, again all these animals were brought back to the pens and catheters were reinserted. The following morning (day 21), an IVGTT was performed. Over the next two days both these groups received an intravenous glucose (2.5 mg per kg body mass) via catheter as a bolus over 1 min. On day 25 IVGTT was performed. All blood samples were collected in heparinized tubes and centrifuged immediately at 3000 rpm for 20 minutes. The plasma was separated and stored at –20ºC until analyzed.

Glucose and Insulin assay

The glucose concentration of plasma samples were analysed with DADE clinical analyzer (DADE-XL, Dupont, USA). RIA was used to measure the insulin levels in pancreas culture media and plasma samples, as previously described [20].

Data analysis

From IVGTT data, area under curve (AUC) for insulin and glucose was calculated in Origin™ software (Version 7 – Microcal Software Inc., Northampton, MA, USA). Glucose tolerance (GluAUC (0-180 min); min.mmol/L) and absolute insulin secretion (InsAUC (0-60 min); min. IU/ml) was calculated as previously described [13]. Experimental data were analysed statistically, using the general linear model procedure in SAS statistical software (SAS Institute Inc. Cary, NC, USA). The data were evaluated using one-way ANOVA followed by Student-Newman Keulspost hoc test. Values were considered to be significantly different at $p<0.05$ and presented as mean ± standard error mean (SEM).

Results

Concentration-dependent effect of AEF and PME on insulin secretion from mouse pancreas is shown in Figure 1, indicating the enrichment of activity after purification using bioassay-guided fractionation method.

AEF modulate insulin secretion and synthesis *in vitro*

To uncover the pharmacological mechanisms responsible for the insulin releasing activity of AEF, we examined the effect of glucose on the insulin secretory properties of AEF (Figure 2A). Under normoglycemic condition, unlike tolbutamide (4 fold increase from control), AEF have shown no effect on insulin secretion. However, in the presence of high glucose, both AEF and tolbutamide stimulated the insulin release from pancreas (5 and 4 fold increase from control, respectively). Interestingly, tolbutamide ([K+sub.ATP] channel blocker) had no effect on AEF-induced insulin secretion and diazoxide ([K+sub.ATP] channel opener) inhibited the insulin releasing effect of both AEF and tolbutamide (Figure 2B). Both arginine (-cell depolarising agent) and KCl (membrane depolarizer) had potent insulin secreting activity (Figure 2C). However, arginine failed to augment the insulin secretory effect of AEF, whereas KCl significantly inhibited the insulin secreting activity of AEF. Next, we examined the effect of AEF on insulin synthesis Figure 3. The media insulin concentrations of AEF treated groups at both 12 hours (287.3 ± 12.8 IU/ml) and 24 hours (380.6 ± 2.4 IU/ml) incubation, under hyperglycemic culture conditions and followed by a 24 hours (141.7 ± 14.3 IU/ml) incubation under normoglycemic culture conditions were significantly greater than the control group. Interestingly, the insulin content in the tissues treated with of AEF (715 ± 11.2 IU/ml) was significantly higher than the control tissues.

Prolonged insulinotrophic effects of AEF *in vivo*

The effect of three daily intravenous administrations of AEF (5 l) on glucose clearance and insulin secretion in normal sheep were examined. The baseline parameters (day 0) including plasma glucose and insulin levels of both control and AEF treatment groups were similar (data not shown).

The plasma glucose levels of AEF treated group on day 3 at 60 and 120 minutes were significantly lower than the control (Figure 4A), whereas there were no changes observed at day 21 and 25. The glucose AUC (glucose tolerance; GluAUC (0-180 min); min mmol/L) of AEF treated group (40.3 % decrease from control) on day 3 was lower than the control (Figure 4B). However, the glucose AUC of AEF treated group on days 21 and 25 was not different from control.

The comparison of insulin levels on days 3, 21 and 25 at 0, 15 and 60 minutes of the control and AEF treatment are shown in Figure 5A. No significant difference in insulin level on days 21 and 25 at time 0 and 60 minutes were observed for either group, although insulin level was significantly decreased on day 3 at 60 minutes in the AEF group. Interestingly, at 15 minutes on days 3, 21 and 25, the plasma insulin level of the AEF treated group was significantly higher than the control group. The insulin AUC (absolute insulin secretion; InsAUC (0-60 min); min IU/ml) of the AEF treated group on days 3, 21 and 25 (298, 419 and 298 % increase from control, respectively) was significantly higher than the control group (Figure 5B).

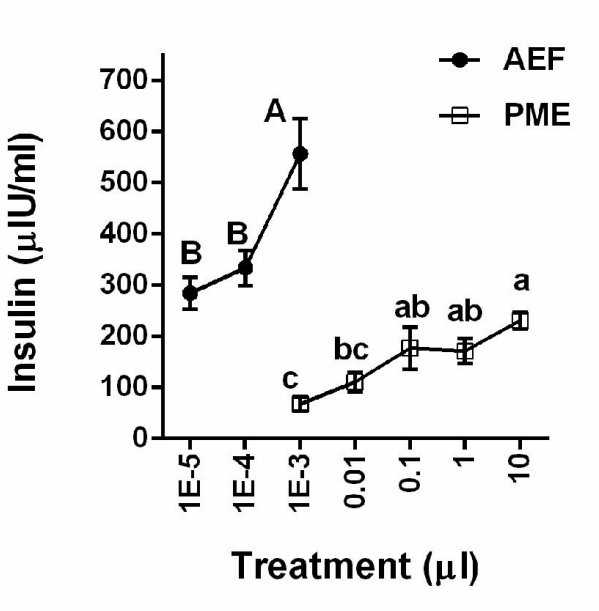
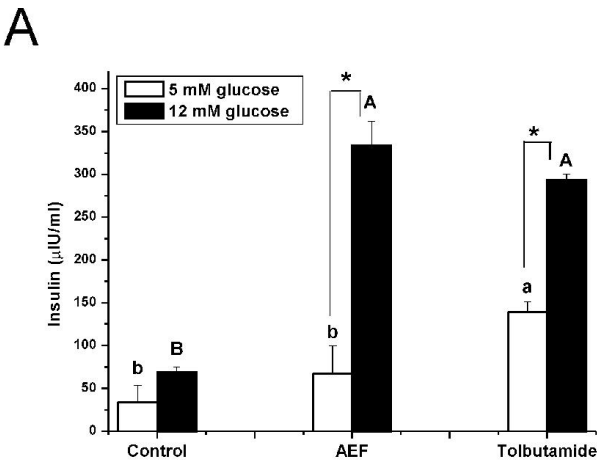
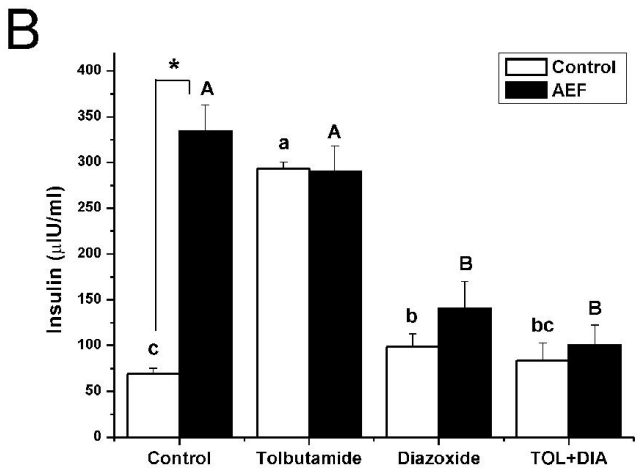


Figure 1 Effect of varying concentration of PME and AEF on insulin secretion from mouse pancreatic tissues over chronic incubation (24 hrs) under hyperglycemic (12 mM glucose) culture conditions. Values are expressed as mean ± SEM. Means indicated without a common letter are significantly different (p<0.05).





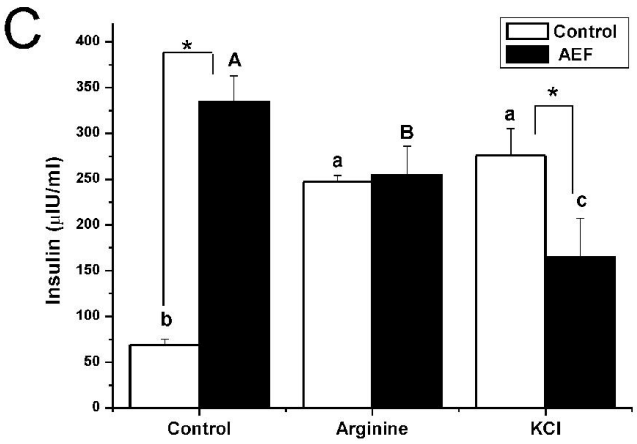


Figure 2. A Effect of glucose (5 and 12 mM) on insulin secreting activity of control (phosphate buffer saline), AEF (1 10^{-3}µl; volume adjusted to 10 µl with phosphate buffer saline) and tolbutamide (0.1 mM) from mouse pancreatic tissues *in vitro.* **B**. Effect of tolbutamide (0.1 mM) and diazoxide (0.5 mM) either alone or together (TOL+DIA) on control or AEF-induced insulin secretion from mouse pancreas for 24 hours. **C**. Effect of arginine (10 mM) or KCl (25 mM) on control or AEF-induced insulin secretion from mouse pancreas for 24 hours. Values are expressed as mean ± SEM. Means indicated without a common letter are significantly different relative to the control group (p<0.05) and means indicated with an asterisk (*) are significantly different relative to the treatment group (p<0.05).

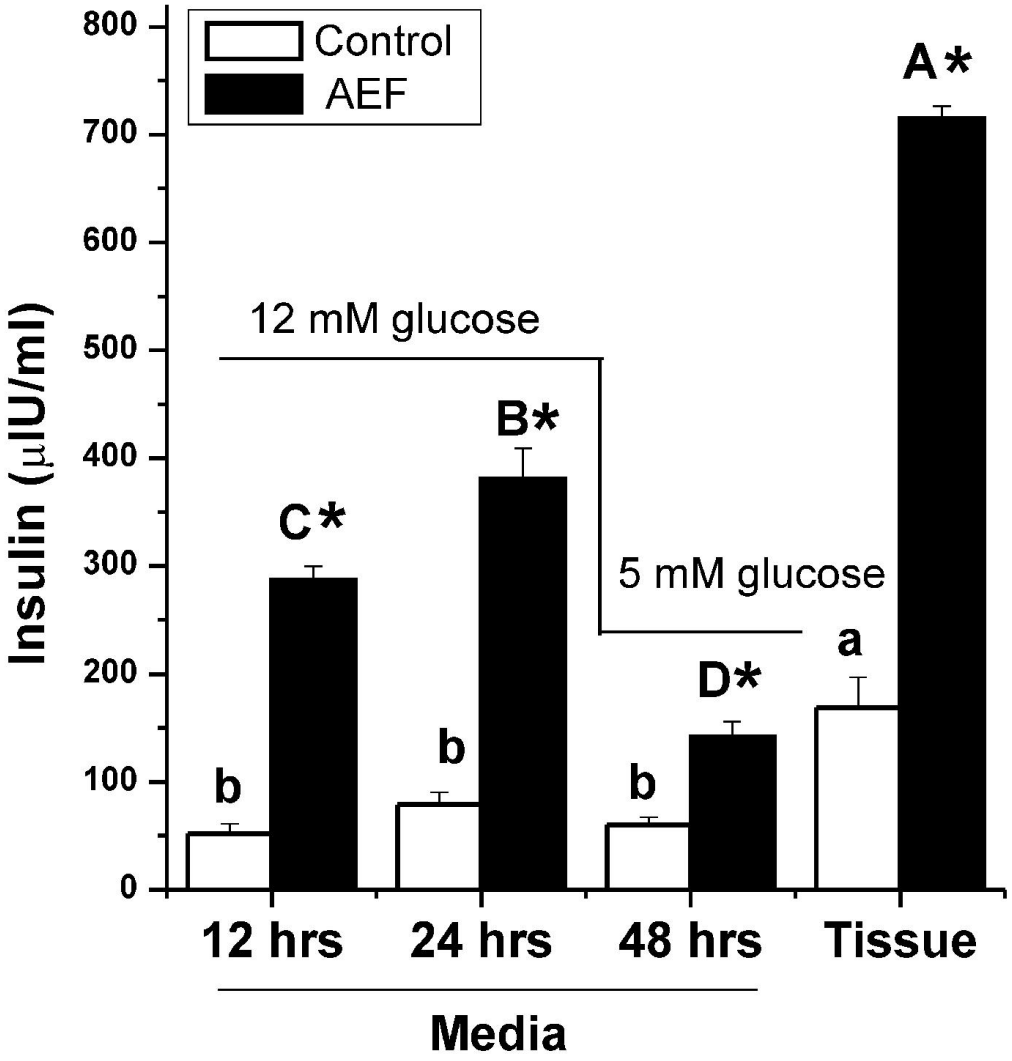


Figure 3 Effect of AEF (1 10^{-3}μl; volume adjusted to 10 μl with phosphate buffer saline) on insulin secretion and synthesis by mouse pancreatic tissues over 24 hours under hyperglycemic glucose (12 mM) and followed by 24 hours under basal glucose (5 mM). Media samples were withdrawn at 12, 24 and 48 hours. Tissues from respective treatments were homogenized and analyzed. Values are expressed as mean ± SEM Means indicated without a common letter are significantly different relative to the control group ($p<0.05$) and means indicated with an asterisk (*) are significantly different relative to the treatment group ($p<0.05$)

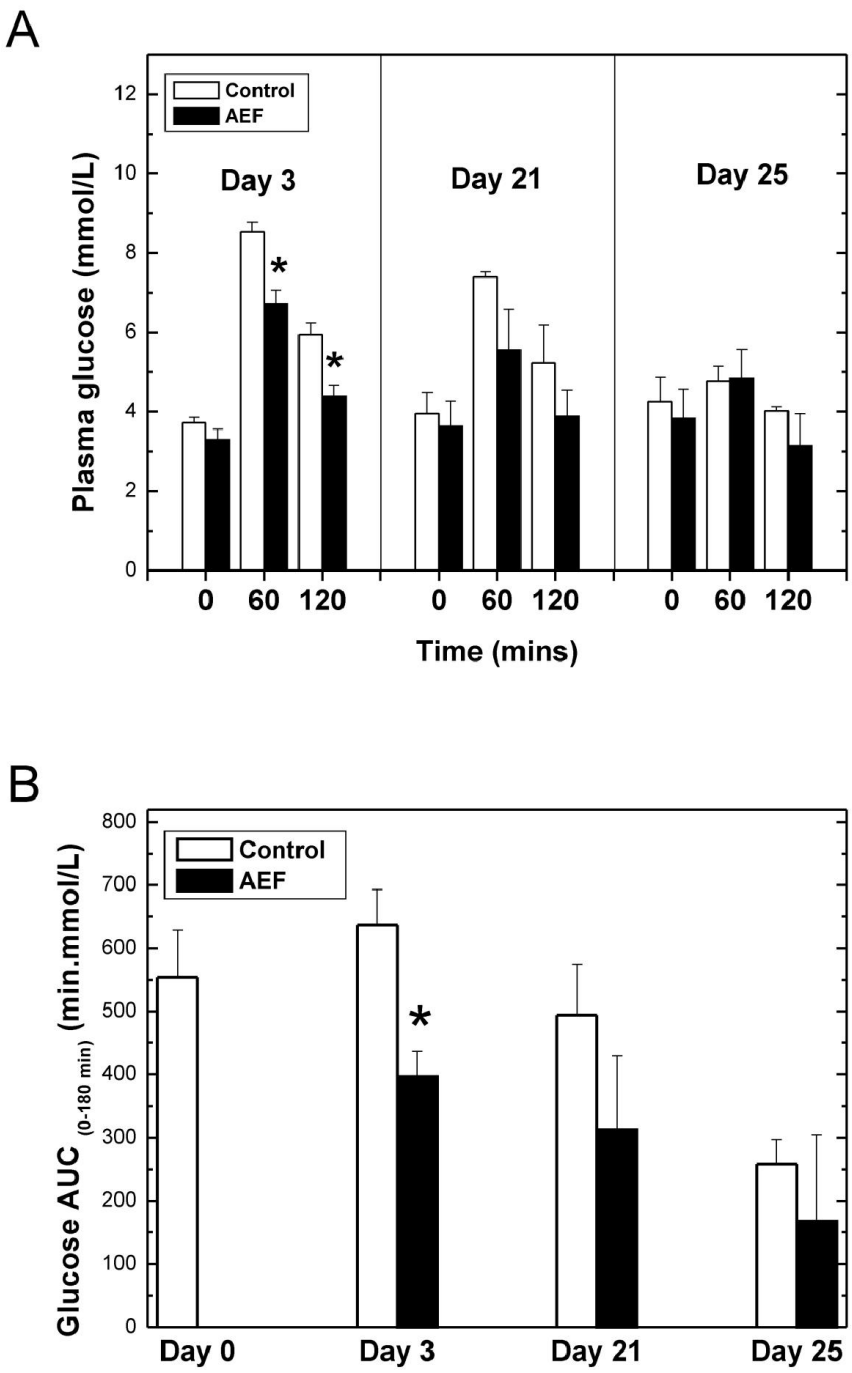


Figure 4 Effects of three daily intravenous administrations of AEF (5µl; diluted in 10 ml saline) on glucose clearance in normal sheep. **A**. Plasma glucose (mean ± SEM) of saline and AEF treated groups after an IVGTT conducted on days 3, 21 and 25 at 0, 60 and 120 minutes. **B**. Mean (± SEM) glucose AUC $_{(0\text{-}180\ min)}$ (min mmol/L) in sheep, after an IVGTT performed on days 3, 21 and 25. Means indicated with an asterisk (*) are significantly different relative to the control group ($P<0.05$); n=6.

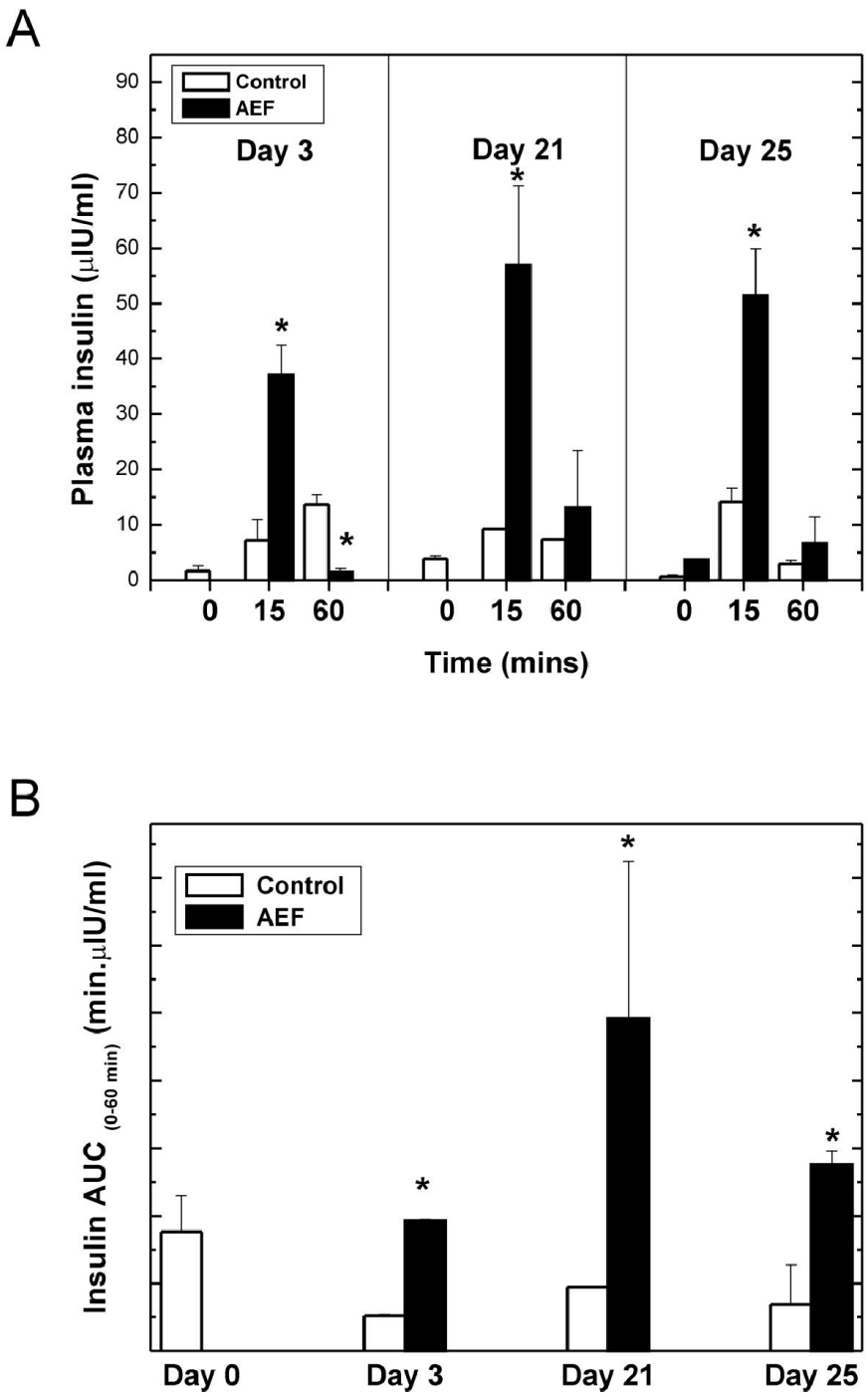


Figure 5 Effects of three daily intravenous administrations of AEF (5μl; diluted in 10 ml saline) on plasma insulin in normal sheep. **A**. Plasma insulin (mean ± SEM) of saline and AEF treated groups after an IVGTT conducted on days 3, 21 and 25 at 0, 60 and 120 minutes. **B**. Mean (± SEM) insulin AUC $_{(0\text{-}60\ min)}$ (min μIU/ml)) in sheep, after an IVGTT performed on days 3, 21 and 25. Means indicated with an asterisk (*) are significantly different relative to the control group ($P<0.05$); n=6.

Discussion

The present study demonstrates that the insulinotrophic properties of the bioassay-guided purified fraction of aqueous extract of PM hardwood (AEF) is prolonged and provides evidence for the pharmacological processes responsible of insulinotrophic effect of AEF.

In vitro

Insulin secretion by pancreatic -cells in response to glycemic control is maintained by nutrients, neurotransmitters and other hormones [22]. However, glucose is the primary regulator of insulin synthesis and secretion [23]. The potent insulin secretion stimulatory effect of AEF in response to elevated glucose concentration suggests that -cell metabolism is able to augment the insulin release stimulus. In addition, the high insulin content in pancreatic tissues treated with AEF indicates the possible role of AEF on insulin biosynthesis. Our observation that insulin-releasing activity of AEF increases gradually from 12 hours to 24 hours under hyperglycemic culture conditions followed by a decrease under basal conditions indicates AEF-induced insulin secretion is glucose-dependent.

Studies to evaluate the possible pharmacological mechanisms underlying the insulin-releasing action of AEF indicated a similarity to the sulphonylurea class of drug. Until recently, sulphonylureas were the only drugs used in the treatment of T2DM to stimulate insulin secretion. The sulphonylureas mimic the effect of glucose on the insulin secretory pathway. These agents stimulate insulin secretion by binding to sulphonylurea receptors in pancreatic -cells. Activation of this receptor enables the closure of plasma membrane [K+sub.ATP] channels and result in membrane depolarisation followed by opening of voltage dependent Ca2+ channels. The elevated level of intracellular Ca2+ triggers the release of insulin [24, 25]. Defective closure of [K+sub.ATP] channel in diabetic -cells may be expected to impair the production of a triggering signal by agents that depolarize by inward current. Diazoxide, a pharmacological inhibitor of sulphonylurea receptors, blocks insulin secretion by preventing closure of [K+sub.ATP] channel [26]. In this study, as expected, the insulin releasing effect of tolbutamide (first generation sulphonylurea) was inhibited by diazoxide. Interestingly, diazoxide also inhibited the insulin releasing effect of AEF, indicating that the involvement of [K+sub.ATP] channel closure in AEF-induced insulin secretion. Notably, tolbutamide failed to augment the effect of AEF on insulin secretion, suggesting a synergistic effect and that both tolbutamide and AEF are likely to use the same pathways to stimulate insulin secretion.

Alternatively, a number of secondary messenger pathways and secretory machinery in -cells ([K+sub.ATP] channel-independent pathways) have been proposed [24]. For example, arginine and other cationic amino acids depolarize -cells because of their entry as a positive charged form [27-29]. Consistent with this view, arginine, which promotes insulin secretion by depolarizing the pancreatic -cells, failed to affect AEF-induced insulin secretion. Likewise, AEF does not stimulate insulin secretion from chemically depolarized pancreatic tissues incubated with 25 mM of KCl. These observations suggest the role of other secondary messenger pathways in AEF-induced insulin secretion.

Importantly, hypoglycemia induced by excessive insulin secretion is a major complication of current pharmacological treatments of T2DM [24, 30]. The enhancement of insulin secretion only at hyperglycemic but not in normoglycemic conditions suggests that AEF would not provoke hypoglycemia under basal physiological condition.

In summary, the treatment of AEF on mouse pancreas tissues stimulates glucose-dependent insulin secretion by possibly acting on [K+sub.ATP] channel, like glucose and sulphonylureas. However unlike sulphonylureas, the null effects of AEF on basal insulin secretion, the lack of synergetic effects with tolbutamide and absence of potentiation of insulin secretion from chemically depolarized pancreatic tissues, prompted us to suggest that AEF may also act on other downstream secondary insulin secretory pathways like G-protein-coupled receptor pathways. This is particularly true of potentiators such as the incretin hormones that stimulate intracellular cAMP productions, activation of protein kinase A (PKA) and protein kinase C (PKC). The resultant activation of PKA and PKC, in turn, can phosphorylate and activate the [K+sub.ATP] channel and cause exocytosis of insulin [31]. Nonetheless, these results indicate AEF exhibits insulin releasing effects, partially by mimicking the effects of sulphonylureas on the insulin secretory pathway and partially by having additional actions on either pancreatic -cell nutrient metabolism or insulin biosynthesis.

In vivo

Physiologically, glucose tolerance involves a complex interaction among pancreatic -cell insulin secretion, action of insulin to increase glucose disappearance and decrease endogenous glucose production [32]. In sheep, AEF treatment induced a significant increase in plasma glucose clearance on day 3. However, this effect was no longer significant on day 21 and 25. This is possibly related to insulin resistance in young animals and not insulin deficiency as indicated by the increased insulin AUC during the glucose tolerance test. The impairment of glucose tolerance with aging from the young animal through puberty to the adult is common in the sheep [21]and rat [33], in contrast to humans, where glucose tolerance is maintained across this period by compensatory changes in insulin secretion [34, 35]. The acute plasma insulin secretion at 15 min in response to intravenous glucose bolus during IVGTT was markedly elevated on day 3, 21 and 25. Animals exhibiting high levels of insulin when there is minimal change in glucose levels in our studies is in parallel with a

previous study by Gupta [7], where PME was infused via stomach tube and reported that sensitization of -cells of the islets of the pancreas in albino rats resulted in secretion of large amounts of insulin in response to glucose. Moreover, the rise in plasma insulin secretion in response to glucose reveals that the insulin releasing effect of AEF is glucose-dependent.

Interestingly, similar to in vitro observations, AEF has no significant influence on basal glucose or insulin levels, indicating that the administration of AEF is relatively safe and would not provoke hypoglycemia unlike conventional insulinotrophic agents. In summary, the present in vivo experimental results indicate that three daily intravenous administrations of AEF have prolonged effects on insulin secretion as well as on glucose clearance in normal, non-diabetic sheep. Importantly, the potent effect of AEF on both insulin secretion and glucose tolerance supports our in vitro findings that, constituent(s) of AEF have direct effects on tissue involved in glucose homeostasis.

Conclusion

Our results from both in vitro and in vivo experiments provide convincing evidence that AEF has potent insulinotrophic properties and this effect is prolonged by many hours to days, unlike many conventional insulin secretagogues which exhaust -cells by overstimulation. Despite these findings, a great deal of research is still needed to characterise AEF (structural and chemical properties). Importantly, the effects of AEF on different stages of pathogenesis of T2DM, different animal models of diabetes and toxicological studies addressing the effects associated with the long term use would be required prior to being considered for clinical application.

Competing interests

Authors of this manuscript have no competing interests to declare.

Authors' contributions

SKM designed the study, performed all experiments, analysed the data and prepared the manuscript. JRM supervised all and reviewed the manuscript.

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