

Kafirin microparticle encapsulated sorghum condensed tannins exhibit potential as an anti-hyperglycaemic agent in a small animal model

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Short title

Encapsulated sorghum condensed tannins as anti-hyperglycaemic agent

ABSTRACT

In vitro analysis has indicated that sorghum condensed tannins (SCT) survived simulated gastric digestion and inhibited digestive amylases when encapsulated in sorghum kafirin protein microparticles (SCT-KEMS). This study investigated SCT-KEMS as a potential anti-hyperglycaemic nutraceutical agent *in vivo*. Oral starch tolerance tests were performed on healthy rats. SCT-KEMS prevented a blood glucose spike and decreased the maximum blood glucose level by a mean of 11.8% compared to the water control, the same reduction as the acarbose standard. Neither SCT-KEMS nor acarbose elevated serum insulin levels. Further, the rats took the SCT-KEMS willingly, unlike the case with the unencapsulated SCTs. SCT-KEMS are potentially effective nutraceuticals for the management of hyperglycaemia because of the high affinity of SCT for the proline-rich kafirin and kafirin's slow digestibility, which enables SCT bitterness to be masked and delivered to the small intestine to inhibit carbohydrate hydrolysis, reducing glycaemic response.

Keywords: Encapsulation; hyperglycaemia; kafirin; oral starch tolerance test; sorghum tannins

1. Introduction

Functional foods, including plant-based nutraceuticals, can play a role in the treatment of Type 2 diabetes (TD2). Plant extracts have traditionally been used to reduce the extent of carbohydrate digestion by inhibition of starch hydrolysing enzymes (Etxeberria et al., 2012). With less reported side effects than conventional pharmaceuticals, coupled with their lower cost, natural medicinal products are being considered as alternatives (Tundis et al., 2010).

Using nutraceuticals from locally grown plants could be highly beneficial in sub-Saharan-Africa, where drug-based therapies have limited success due to weak healthcare systems. By 2035 some 41 million people in Africa will suffer from diabetes (IDF, 2013), with T2D being the most prevalent.

Condensed tannins isolated from sorghum, an indigenous African cereal (sorghum condensed tannins (SCT)), strongly inhibit both α -amylase and α -glucosidase *in vitro*, the latter at far lower concentration than acarbose (an α -glucosidase inhibitor used for diabetes treatment) (Links et al., 2015). When SCT preparations were encapsulated in microparticles made from kafirin (sorghum prolamin protein), (KEMS), they retained inhibitory activity against both α -amylase and α -glucosidase during simulated gastrointestinal digestion. Thus, SCT-KEMS may have potential as a nutraceutical to attenuate hyperglycaemia by inhibiting carbohydrate hydrolysing enzymes in the small intestine. The objective of this study was to prove, in principle, the anti-hyperglycaemic effects of SCT, by testing the efficacy of SCT-KEMS versus acarbose in regulating blood glucose levels and determining whether SCT-KEMS administration results in decreased insulin response in healthy, normo-glycaemic rats in an oral starch tolerance test (OSTT).

2. Materials and methods

2.1 Materials

SCT were extracted from bran of red type III (high-tannin) sorghum (cultivar PAN 3860) using methanol (Links et al., 2015). The extract was dried at ambient temperature and defatted with hexane. The SCT content of the dried preparation was 312 mg/100 mg (as is) catechin equivalents. The SCT preparation consisted of mainly proanthocyanidins and procyanidins (procyanidin B type (Svensson et al., 2010)) (condensed tannins), plus anthocyanins, 3-deoxanthocyanins, flavanones flavones and flavonols, and phenolic acids, as identified by liquid chromatography-mass spectrometry (Adetunji et al., 2015). The condensed tannins were present in an approx. proportion of polymers (DP >8) and oligomers (DP 2-8) of 130:44:1 relative to the non-tannin phenolics, as measured by normal phase HPLC. Total kafirin (82% protein as is basis) for KEMS preparation was extracted from non-tannin, white tan-plant sorghum (cultivar Orbit) as described by Emmambux, and Taylor (2003). Maltodextrin (419699, Dextrose Equivalent 13-17) and acarbose were obtained from Sigma-Aldrich (Johannesburg, South Africa). A solid phase-conjugated sandwich Enzyme-linked Immunosorbent Assay (ELISA) (EZRMI-13K) kit for rat/mouse serum insulin determination was obtained from Merck Millipore (Johannesburg, South Africa).

2.2 Methods

2.2.1 Preparation of kafirin microparticles

SCT and kafirin were independently dissolved in 70% (w/w) aqueous ethanol at 70°C. The SCT was then mixed with the kafirin solution with constant stirring. Water was used to

coacervate the SCT/KEMS (Links et al., 2015), which was washed with water to remove unbound SCT, air-dried and milled to a fine powder. The SCT-KEMS contained 29% bound SCT. This desirable high content of bound SCT is due to its very strong affinity to kafirin, as a consequence of kafirin's high proline content (Emmambux and Taylor, 2003). KEMs alone were prepared as described but without SCT addition.

2.2.2 *Animals*

Thirty healthy, adult (15 week) male Sprague Dawley rats (260-350 g) were used for the study. Male rats were chosen to avoid the possible effect of female sex hormones on blood glucose levels (Reichelt et al., 2013) and because they were easier to handle, thus lessening potential stress effects on the glucose response. The University of Pretoria Animal Ethics Committee (EC039-14) granted approval for all animal experimental procedures. The rodents were maintained at the University of Pretoria's Biomedical Research Centre, with *ad-lib* access to standard rodent chow (Epol, South Africa) and high-pure water, with a 12 h light/dark cycle at $22\pm 2^{\circ}\text{C}$ and relative humidity of $60\pm 10\%$. The rats were housed in pairs in Type 3 rat cages (1291H, Eurostandard) and were acclimatised for one week prior to the start of the study.

2.2.3 *Oral starch tolerance test (OSTT)*

OSTT was performed according to Wolf et al. (2002) and Ali et al. (2013) with some modifications. Rats were randomly assigned in five groups with six rats per group. Rats were fasted for 12 h prior to each OSTT. Treatments were SCT-KEMS (400 mg/kg body weight, containing 116 mg SCT/kg body weight), KEMS alone (284 mg/kg), SCT alone (116 mg/kg), acarbose (10 mg/kg) and distilled water (vehicle, negative control, 0.75 ml). All treatments were administered orally into the buccal pouch with a syringe. Fifteen min. post

treatment, rats were administered maltodextrin (4 g/kg) orally via a syringe. Blood was collected via tail venipuncture. Blood glucose was measured before (0 min) and at 15, 30, 45, 60, 75, 90, 105 and 120 min post maltodextrin administration using a OneTouch Select glucometer (Lifescan, Johannesburg, South Africa). Blood glucose data were used to determine the blood glucose spike 15 min after maltodextrin administration, maximum blood glucose reached over analysed period and area under blood glucose response curve (AUC). AUC was calculated using: $AUC_{\text{glucose}} = \text{mmol/L} \times \text{h}$ (Ali et al., 2013).

2.2.4 Serum insulin

Blood (0.2 ml) was collected from the tail vein for insulin measurement before (0 min) and 120 min after the OSTT to limit pain and stress. Blood was allowed to clot for approx. 30 min and then centrifuged at $2600 \times g$ for 15 min to obtain serum, which was frozen at -20°C until analysis. Insulin was determined using a solid phase-conjugated sandwich ELISA and analysed at least in triplicate (three replicates) per rat.

2.2.5 Statistical analysis

One-way analysis of variance (ANOVA) was used to analyse data from the five groups for blood glucose at each time interval, blood glucose at 15 min, maximum blood glucose, AUC and serum insulin using Statistica software version 12 (StatSoft, Tulsa, OK, USA).

Differences between groups were assessed by Fisher's Least Significant Difference test.

Basal blood glucose data differed significantly ($p < 0.05$) between groups and therefore blood glucose data was analysed by normalising to percentage of the basal blood glucose. Outliers were removed from data sets using Dixon's Q-test (Dean & Dixon, 1951).

3. Results and discussion

3.1 Blood glucose response

The blood glucose response curves showed that SCT-KEMS, acarbose and KEMS alone were effective in preventing the blood glucose spike observed with the control and SCT alone (Figure 1A). The blood glucose response curves normalised to time 0 showed that only acarbose and SCT-KEMS did not cause a glucose spike (Figure 1B). Three different glucose responses could be discerned in the normalised data. Administration of water alone resulted in a glucose spike followed by a dip, minimum value at 45 min and then a subsequent rise and a levelling off after 75 min. SCT alone and KEMS alone resulted in a glucose spike at 15 min, followed by a small dip at 30 min before rising again to a level close to, or higher than the 15 min spike. SCT-KEMS and acarbose, as stated, did not cause a glucose spike, but rather the glucose level rose slowly to a high between 60 and 75 min. The glucose maxima for SCT-KEMS and acarbose were much lower than any blood glucose levels for SCT or KEMS alone. Post 75 min, the glucose response of SCT-KEMS and acarbose reduced further until at 120 min the SCT-KEMS had the lowest level of all the treatments. Using the actual data (Figure 1A), SCT-KEMS decreased ($p < 0.05$) the maximum blood glucose level by a mean of 11.8%, compared to the water control, whereas the reduction caused by acarbose was somewhat less (9.4%). When the data were normalised, there was no significant difference between the SCT-KEMS and acarbose treatments for the maximum blood glucose level (Figure 1B). The actual data also showed that SCT-KEMS had the lowest AUC (Figure 2A), significantly lower than the water control and SCT alone. The normalized data (Figure 2B) showed that the AUC of SCT-KEMS was lower than SCT alone and KEMS alone.

Condensed tannins inhibit the activity of digestive enzymes and thereby delay starch digestion (Kim et al., 2011). Hence, the SCT were probably slowly released from the kafirin

Figure 1: Blood glucose response curve after oral maltodextrin challenge. **A:** Acarbose, SCT-KEMS, KEMS alone, SCT alone and the control, water. **B:** Data normalised to time 0. Means \pm SD, n=6 rats (SCT-KEMS, n=5). Values with different letters differ significantly at each time point ($p < 0.05$).

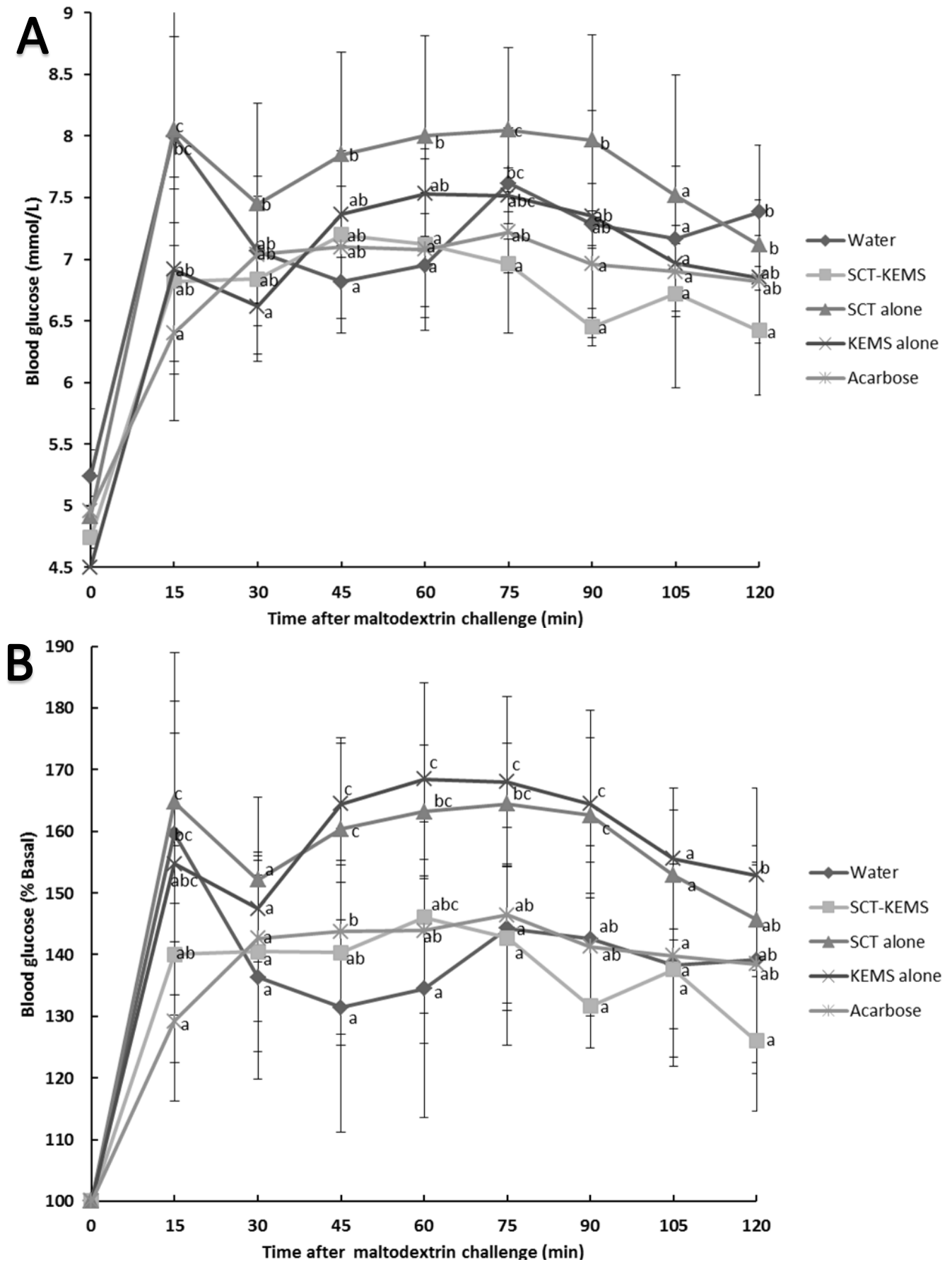
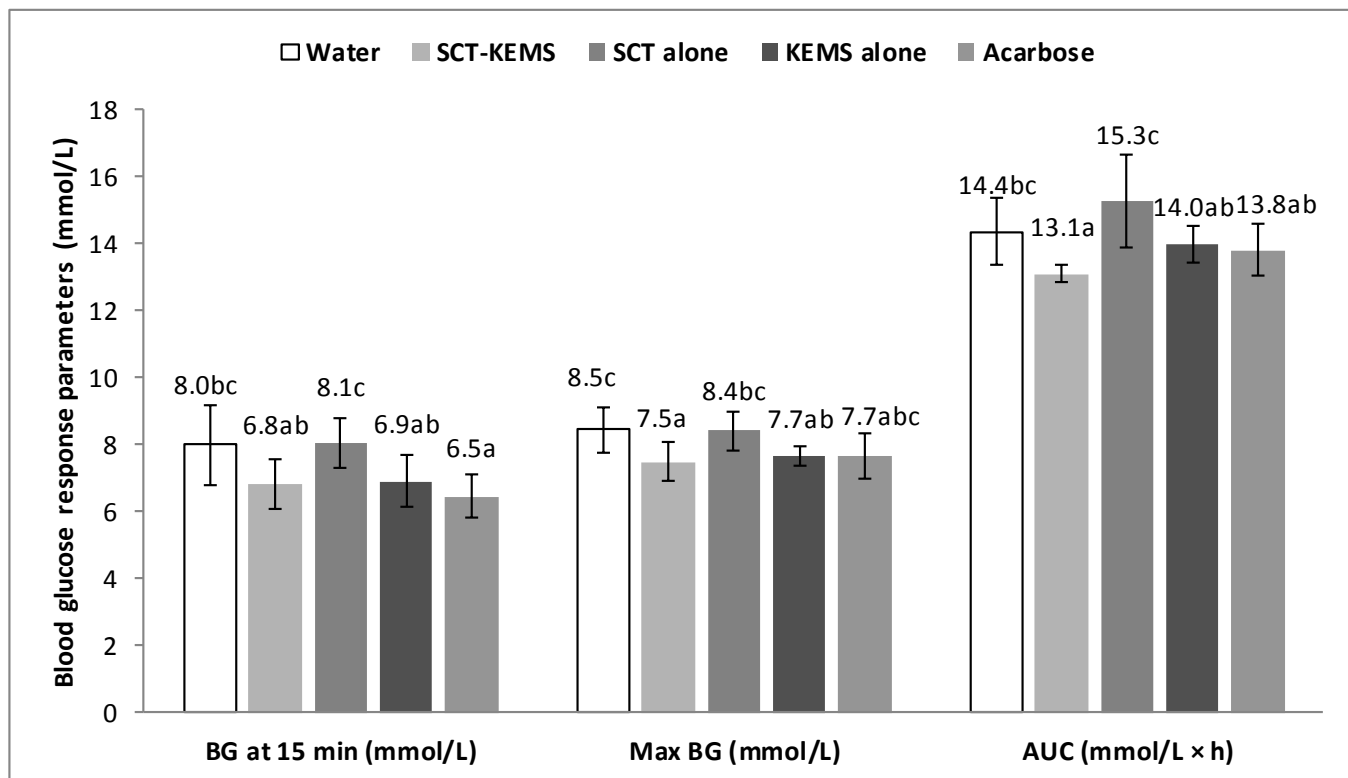


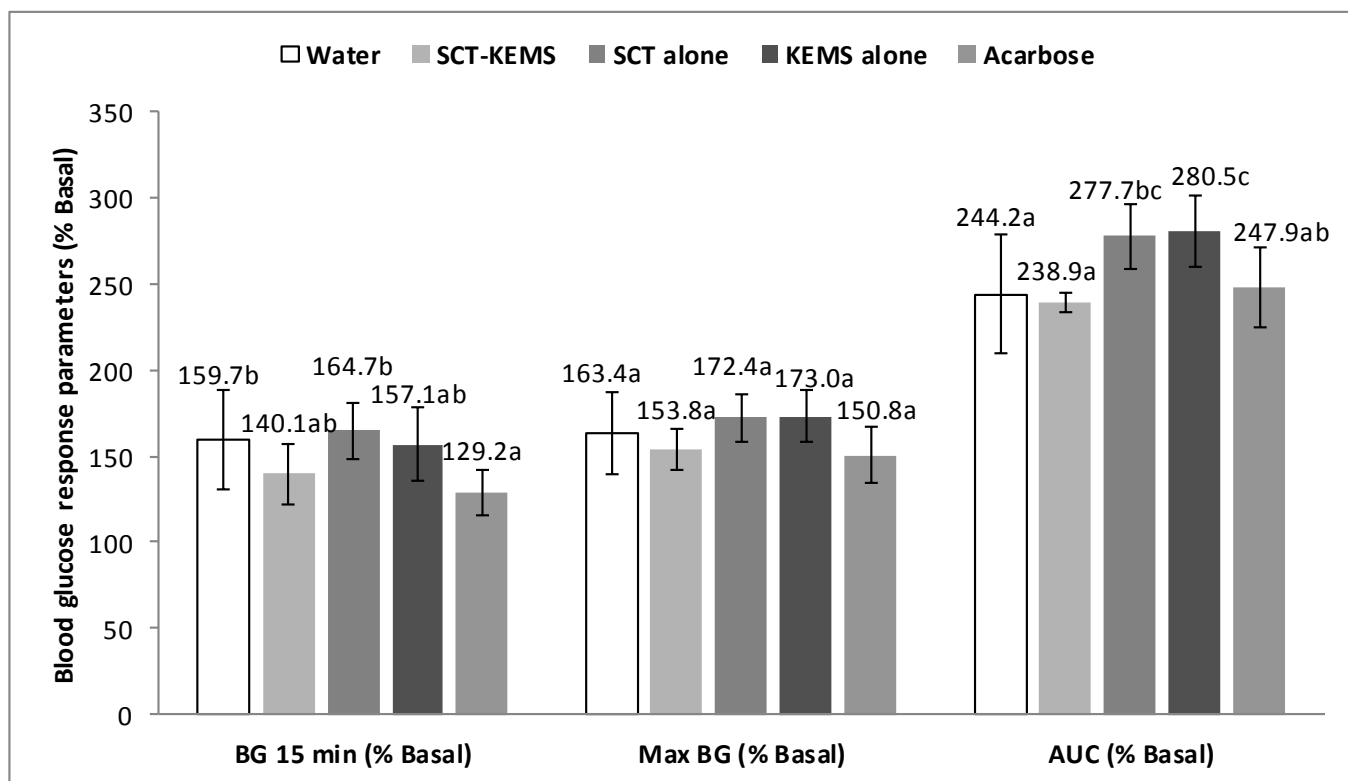
Fig. 2. Blood glucose (BG) response after maltodextrin challenge. **(A)** BG at 15 min, maximum BG over analysed time period and area under blood glucose curve (AUC) of rats treated with SCT-KEMS, SCT alone, KEMS alone, acarbose or the water control.

(B) Data normalised to time 0. Means \pm SD, N = 6 (SCT-KEMS, N = 5). Values with different letters differ significantly ($p < 0.05$).

A



B

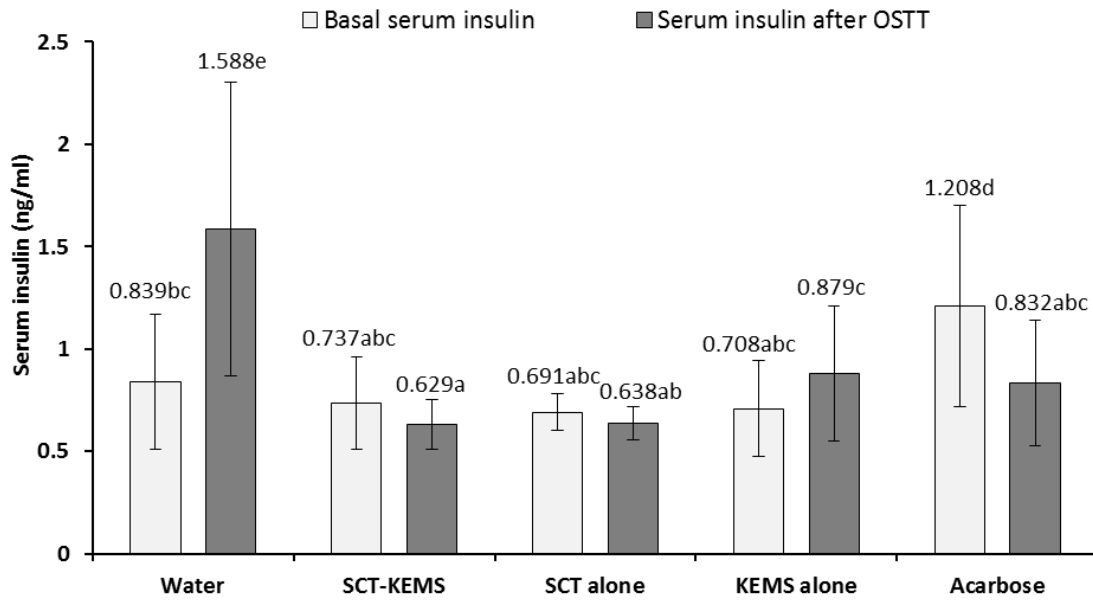


microparticles during digestion and thus able to inhibit intestinal amylases, decrease starch digestion and thereby reduced the amount of glucose available for absorption.

SCT alone (i.e. without encapsulation) generally showed the highest blood glucose level amongst the treatments. This was expected as our previous research showed that un-encapsulated SCT lost most of their inhibitory action when subjected to simulated gastrointestinal digestion (Links et al., 2015). However, an increase in blood glucose caused by tannins has not been previously reported. This is possibly because in previous studies involving phenolic extracts from sorghum, administration was by gavage (Chung et al., 2011). Increase in blood glucose, at least in part, may be attributed to the resultant stress as SCT are strongly bitter and astringent (Kobue-Lekalake et al., 2007). Stress can lead to increased corticosterone secretion, which in turn can stimulate gluconeogenesis (Cawley, 2012). Additionally, stress can also increase salivary α -amylase secretion and result in digested starch (glucose) in the small intestine before pancreatic starch digestion (Nater & Rohleder, 2009).

The effect of encapsulating SCT in KEMS on the masking of their bitter and astringent taste was not specifically assessed. However, it was observed that the rats took in the SCT-KEMS much more willingly than the SCT alone. Also, the glucose response of the SCT-KEMS was similar to acarbose (Figure 1). Thus, it can be assumed that the encapsulation successfully masked the bitterness and astringency of SCT and thus fulfilled an important function of a polyphenol encapsulation agent.

Fig. 3. Insulin before (basal) and after maltodextrin challenge. Serum insulin of rats treated with SCT-KEMS, SCT alone, KEMS alone and acarbose. Means \pm SD, N = 6 (acarbose after OSTT, N = 5). Values with different letters differ significantly ($p < 0.05$).



3.2 Serum insulin

Basal insulin levels were not different between the water control and all treatments, with the exception of the acarbose group, which had significantly higher ($p < 0.05$) basal insulin levels (Figure 3). This higher basal insulin level for acarbose may be a consequence of the small group size. The water control showed significant increase ($p < 0.05$) (89%) in serum insulin after the OSTT. However, SCT-KEMS, SCT alone and acarbose treatments prevented elevation of insulin levels.

Surprisingly, SCT alone maintained high blood glucose levels without stimulating additional insulin secretion. This could have been because of the intracellular inhibition of glucose uptake by flavonoids (Nomura et al., 2008). Also, as mentioned, stress due the SCT bitterness could have triggered corticosterone release. Corticosterone inhibits pancreatic insulin release in rats (Barseghian & Levine, 1980).

Although the KEMS alone treatment reduced the blood glucose spike in comparison to the control (Figure 1), other than the control it was the only treatment to indicate increased insulin levels after the OSTT.

Insulin maintains glucose homeostasis by stimulating glucose utilisation by skeletal muscle and adipose tissue (Muniyappa et al., 2008). In healthy subjects, insulin secretion is stimulated in proportion to the blood glucose level (Wilcox, 2005). Insulin secretion was probably not stimulated after the SCT-KEMS, KEMS alone and acarbose treatments as the blood glucose level was not high enough to trigger an insulin response. Consequently, the insulin data indicate that the decreased blood glucose level observed after SCT-KEMS treatment was due to the inhibition of digestive amylases and not insulin-stimulated glucose clearance.

4. Conclusions

This preliminary work shows SCT encapsulated in kafirin microparticles can decrease blood glucose levels similar to acarbose, after ingestion of carbohydrate in healthy rats after an OSTT. SCT-KEMS can also prevent elevation of serum insulin. By encapsulation of SCT in kafirin microparticles, it seems to mask the bitterness and astringency of SCT and enables them to be delivered to the small intestine where they inhibit carbohydrate hydrolysis. These multiple effects exerted by SCT-KEMS are due to SCT's strong affinity for the proline-rich kafirin (Emmambux and Taylor, 2003) and kafirin's slow digestibility by intestinal proteinases as consequence of its hydrophobicity and disulphide-bonded cross-linking (Duodu et al., 2003). Thus, encapsulating SCT in kafirin microparticles has potential as a novel, affordable nutraceutical-type treatment for the management of hyperglycaemia.

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