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Understanding the mode-of-action of *Cassia auriculata via in silico* and *in vivo* studies towards validating it as a long term therapy for type II diabetes

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Epigallocatechin gallate (PubChem CID: 65064)
Ellagic acid (PubChem CID: 5281855)
Taxifolin (PubChem CID: 439533)
Quercetin-3-O-β-D-glucopyranoside (PubChem CID: 15959354)
Chrysin (PubChem CID: 5281607)
Kaempferol 7-O-rhamnoside-4′-O-glucoside (PubChem CID: 5280863)
Procyanidin B2 (PubChem CID: 122738)

ABSTRACT

Ethnopharmacological relevance: Cassia auriculata (CA) is used as an antidiabetic therapy in Ayurvedic and Siddha practice. This study aimed to understand the mode-of-action of CA *via* combined cheminformatics and *in vivo* biological analysis. In particular, the effect of 10 polyphenolic constituents of CA in modulating insulin and immunoprotective pathways were studied.

Materials and methods: In silico target prediction was first employed to predict the probability of the polyphenols interacting with key protein targets related to insulin signalling, based on a model trained on known bioactivity data and chemical similarity considerations. Next, CA was investigated in *in vivo* studies where induced type 2 diabetic rats were treated with CA for 28 days and the expression levels of genes regulating insulin signalling pathway, glucose transporters of hepatic (*GLUT2*) and muscular (*GLUT4*) tissue, insulin receptor substrate (*IRS*), phosphorylated insulin receptor (*AKT*), gluconeogenesis (*G6PC* and *PCK-1*), along with inflammatory mediators genes (*NF-κB*, *IL-6*, *IFN-γ* and *TNF-α*) and peroxisome proliferators-activated receptor gamma (*PPAR-γ*) were determined by qPCR.

Results: In silico analysis shows that several of the top 20 enriched targets predicted for the constituents of CA are involved in insulin signalling pathways *e.g.* PTPN1, PCK-α, AKT2, PI3K-γ. Some of the predictions were supported by scientific literature such as the prediction of PI3K for epigallocatechin gallate.

Based on the *in silico* and *in vivo* findings, we hypothesized that CA may enhance glucose uptake and glucose transporter expressions *via* the IRS signalling pathway. This is based on AKT2 and PI3K-γ being listed in the top 20 enriched targets. *In vivo* analysis shows significant increase in the expression of *IRS*, *AKT*, *GLUT2* and *GLUT4*.

CA may also affect the PPAR-γ signalling pathway. This is based on the CA-treated groups showing significant activation of PPAR-γ in the liver compared to control. PPAR-γ was predicted by the *in silico* target prediction with high normalisation rate although it was not in the top 20 most enriched targets.

CA may also be involved in the gluconeogenesis and glycogenolysis in the liver based on the downregulation of *G6PC* and *PCK-1* genes seen in CA-treated groups. In addition, CA-treated groups also showed decreased cholesterol, triglyceride, glucose, CRP and Hb1Ac levels, and increased insulin and C-peptide levels. These findings demonstrate the insulin secretagogue and sensitizer effect of CA.

Conclusion: Based on both an *in silico* and *in vivo* analysis, we propose here that CA mediates glucose/lipid metabolism *via* the PI3K signalling pathway, and influence AKT thereby causing insulin secretion and insulin sensitivity in peripheral tissues. CA enhances glucose uptake and expression of glucose

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transporters in particular *via* the upregulation of *GLUT2* and *GLUT4*. Thus, based on its ability to modulate immunometabolic pathways, CA appears as an attractive long term therapy for T2DM even at relatively low doses.

1. Introduction

Type 2 diabetes mellitus (T2DM) is characterised by: (a) insulin resistance, which is a decline in the response towards insulin-stimulated glucose uptake in the adipose tissue and liver, and (b) disruption of insulin secretion due to the deterioration of pancreatic β-cell (Vijan, 2010). The risk of T2DM is high in people with a genetic predisposition to diabetes and this risk greatly increases with lifestyle factors such as lack of physical activity, overweight and obesity (Bray, 2004). Several studies have explored the link between obesity and inflammation, as well as its effect on insulin resistance and secretion (Navarro-Gonzalez et al., 2011; Chen et al., 2013). It has been shown that adipose tissues release pro-inflammatory cytokines e.g. TNF-α, IL-1B, IL-6, and such cytokines are elevated in obese patients (Stein et al., 2013). These cytokines disrupt the insulin signalling pathway by inhibiting tyrosine phosphorylation of IRS-1 (Insulin receptor substrate 1). In addition, high free fatty acid (FFA) has been shown to stimulate the release of pro-inflammatory cytokines, disrupting the insulin signalling pathway through the same mechanism described previously (Shah, 2007; Boni-Schnetzler et al., 2009). FFA has also been linked to the activation of both pro-inflammatory cytokine transcription factor NF-κB and innate immune system, leading to inflammation and insulin resistance (Sakai et al., 2003; Lee et al., 2005; Daniele et al., 2014; Esser et al., 2014). Another inflammatory mediator, PPAR-γ has been reported to reduce insulin-stimulated glucose uptake in the liver and skeletal muscles through the disruption of PI3K-AKT signalling pathway (Ortega et al., 2014). High level of inflammatory marker, C-reactive protein (CRP) has been shown to be elevated in T2DM patients (Kramer et al., 2014). These findings underline the close relationship between immune system and metabolic pathways in the pathogenesis of T2DM. In fact, proteins involved in insulin signalling pathway (IGF-1R; Insulin growth factor 1 receptor) have been linked to the pathogenesis of inflammatory diseases such as asthma (Fernández et al., 2001; Lee et al., 2013). Although T2DM on its own causes improper inflammatory response, long term use of oral antidiabetic therapy such as sulfonylureas (glibenclamide) and biguanides (metformin) have been demonstrated to further exacerbate this situation (Mello et al., 2011). Hence, this underscores the pressing need for new long term drug therapies for T2DM that not only target insulin signalling pathway, but also modulate inflammatory pathways.

One such compound that targets both insulin and inflammatory pathway is *Cassia auriculata* (CA). The Ayurvedic name of CA is *Shibi kul*, and according to the Ayurvedic system, CA decreases aggravated *pitta* and *kabha* in *prameha* (diabetes) (Rajasekharan et al., 1982; Nanjaraj Urs et al., 2015). Many Ayurvedic formulations of CA e.g. *Avaarai panchaga chooranam* (Joy et al., 2012), a herbal formulation that reduces blood sugar level, were found to be beneficial (Pari et al., 2001; Babu et al., 2004) and has been widely used for treating diabetes. Several studies have reported on the beneficial effects of the long term usage of CA and the outcomes were favourable with minimal complications (Joshi et al., 2000; Latha et al., 2003). Other effects have also been reported for CA which include antibacterial and antifungal activity (Duraipandiyan and Ignacimuthu, 2007), analgesic and anti-inflammatory effects (Nsonde Ntandou et al., 2010) and antioxidant property (Manonmani et al., 2005). Abesundara et al. (2004) proposed that CA reduces blood sugar level by improving the utilization of glucose through increased glycolysis. Additionally, Latha and Pari (2003) demonstrated that CA prevents lipid peroxidation-induced membrane damage, suggesting the antiperoxidative role of CA in T2DM. The antidiabetic potential of CA flower extract on hepatic glycolytic and gluconeogenic enzymes has also been reported in streptozotocin diabetic rats (Latha and Pari, 2003). Meanwhile, CA leaves was shown to display antidiabetic effects in mild and severe diabetic rats (Gupta et al., 2009). In our previous study, we showed the involvement of CA in modulating the immune system (John et al., 2011). Hence, in this study we attempt to elucidate the mode-of-action (MOA) of CA in modulating the immune and metabolic pathways, which as described previously is beneficial in the treatment of T2DM. The diversity of the effects reported on CA suggests that the effects may not only be from interaction with specific protein targets but may also be due to the physicochemical properties of the polyphenols. Additionally, unlike synthetic compounds, these polyphenols do not seem to have a clearly identifiable protein targets that it may interact with. Herein, CA was subjected to *in silico* and *in vivo* studies, which also serves to assess its potential for long term therapy in T2DM. In this study, electron spray ionisation-mass spectrometry (ESI-MS) data of CA polyphenols obtained during our previous lab study (see Supplementary Information S1 for full detail) was used in both *in silico* and *in vivo* analyses.

In silico target prediction have been extensively used in MOA analysis where potential protein target(s) modulated by a novel compound can be predicted and subsequently its MOA can be hypothesized. One of the first *in silico* target prediction published was the PASS (Prediction of Activity Spectra for Substances) (Poroikov et al., 2007). The current version utilises more than 260,000 biologically active compounds exhibiting over 3500 types of biological activity obtained from databases and scientific journals as the training set (Poroikov et al., 2007). When a compound is subjected to testing in PASS, it will generate two different scores, P_a and P_i (Poroikov et al., 2007). The former is the probability of the compound being active and the latter is the probability of the compound being inactive. Since then, different implementations and applications of *in silico* target prediction have been published in scientific literature. One such example is the work of Mohd Fauzi et al. (2013) where twenty different medicinal classes from both traditional Chinese medicine and Ayurveda were analysed in their implementation of target prediction. It was found that the phenotypes of the 'tonifying and replenishing medicinal class' from TCM can be connected to the targets predicted (Mohd Fauzi et al., 2013). For example, the anti-hyperglycaemic activity (Zhao et al., 2005) of this class can be connected to the prediction of sodium glucose cotransporters 1, which is responsible for the uptake of glucose. Other recent works in this area include the work of Ravindranath et al. (2015) and Cortes-Ciriano et al. (2015).

Complementary to *in silico* target prediction, potential *in vivo* benefits were also evaluated by treating different doses of CA polyphenolic extract to experimental T2DM rats for 28 days. The effect of CA on insulin signalling and inflammatory pathways were analysed by measuring expression levels of genes regulating