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Cytoprotective effect of *Coreopsis tinctoria* extracts and flavonoids on tBHP and cytokine-induced cell injury in pancreatic MIN6 cells

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ABSTRACT

Ethopharmacological relevance: Coreopsis tinctoria flowering tops infusion is traditionally used in Portugal for treating the symptoms of diabetes. Recent studies have revealed its antihyperglycemic activity when administered for 3 weeks to a STZ-induced glucose intolerance model in the rat and glucose tolerance regain was even clearer and pancreatic function recovery was achieved when administering *Coreopsis tinctoria* flavonoid-rich AcOEt fraction.

In this study we aimed to evaluate the protective effect of *Coreopsis tinctoria* flowering tops aqueous extract, AcOEt fraction and the pure compounds marein and flavanomarein, against beta-cell injury, in a mouse insulinoma cell line (MIN6) challenged with pro-oxidant tert-Butyl-Hydroperoxide (tBHP) or cytokines.

Materials and methods: The protective effects of *Coreopsis tinctoria* flowering tops extracts and pure compounds were evaluated through pre-incubating MIN6 cells with samples followed by treatment with tBHP (400 μM for 2 h) after which viability was determined through ATP measurements. In order to assess whether plant extracts were involved in decreasing reactive oxygen species, superoxide anion production was determined through a lucigenin-enhanced chemiluminescent method. Lastly, the direct influence of *Coreopsis tinctoria* extracts and main compounds on cell survival/apoptosis was determined measuring caspase 3 and 7 cleavage induced by cytokines.

Results: Coreopsis tinctoria flowering tops extracts (25–100 μg/mL) and pure compounds (200–400 μM), when pre-incubated with MIN6 cells did not present any cytotoxicity, instead they increased cell viability in a dose dependent manner when challenged with tBHP. Treatment with this pro-oxidant also showed a rise in superoxide radical anion formation in MIN6 cells. This increase was significantly reduced by treatment with superoxide dismutase enzyme (SOD) but not by pre-treatment with *Coreopsis tinctoria* flowering tops extracts. Caspase 3/7 activation measurements show that *Coreopsis tinctoria* flowering tops extracts, as well as marein and flavanomarein, significantly inhibit apoptosis.

Conclusions: Coreopsis tinctoria extracts and pure compounds show cytoprotection that seems to be due to inhibition of the apoptotic pathway, and not through a decrease on superoxide radical production.

1. Introduction

Chronic exposure to elevated blood glucose levels due to decreased insulin secretion or to insulin resistance is a defining feature of type 2 diabetes and is not only responsible for the development of micro and macrovascular complications but also has a toxic effect on pancreatic beta-cells, where oxidative stress has recently been given an important role (Bonora, 2008). Pancreatic beta-cells are known to be particularly vulnerable to oxidative stress when compared to other tissues, mainly because of their relatively low level of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase (Evans et al., 2002; Lapidot et al., 2002; Lenzen, 2008). Stimuli such as pro-inflammatory cytokines and/or chronic elevation of blood glucose combined with oxidative stress generation in pancreatic islets may provoke beta-cell death by apoptosis (Corbett et al., 1992; Tiedge et al., 1997; Amrani et al., 2000; Le May et al., 2006). Decrease in beta-cell mass, either through an increase in apoptosis or a decrease in proliferation, is one of the factors responsible for the progression of type 2 diabetes (Weir and Bonner-Weir, 2004). Currently available therapeutic agents lower blood glucose through multiple mechanisms, but do not directly reverse the decline in beta-cell mass. Therefore molecules that can also engage signalling pathways in the islet beta-cell which lead to stimulation

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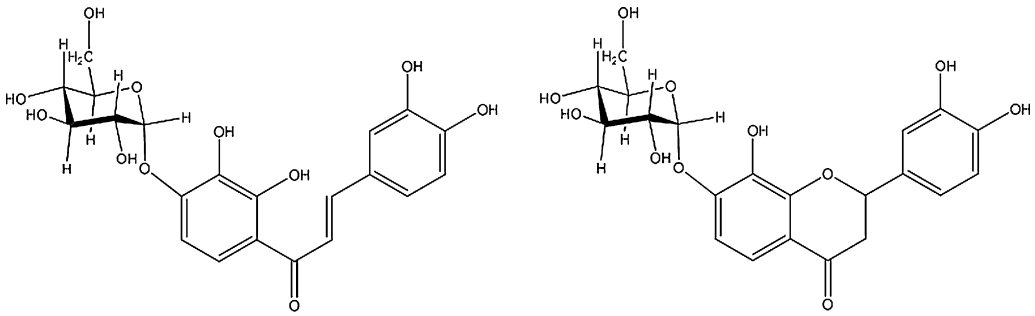

Fig. 1. *Coreopsis tinctoria* pure compounds tested in MIN6 cells; (A) Marein, a chalcone and (B) Flavanomarein, a flavanone.

of beta-cell replication and inhibition of beta-cell apoptosis are promising leads for a therapeutic strategy for the prevention of type 2 diabetes evolution. In that aspect, flavonoids are interesting molecules that occur normally in the diet and exhibit a variety of beneficial effects on health, namely in cardiovascular disease and diabetes (Garcia-Lafuente et al., 2009).

Previous studies have revealed that some flavonoids, namely flavonols, flavanols flavones, isoflavones and an aurone exhibit cytoprotective effects on injured beta-cells whether through direct antioxidant activity and/or beta cell mass preservation (Han, 2003; Hara et al., 2006; Kim et al., 2007a,b,c; Lee et al., 2010; Fu et al., 2010; Song et al., 2010). However not much have been done regarding chalcones and flavanones, the major constituents in *Coreopsis tinctoria* Nutt. flowering tops infusion (Dias et al., 2010a, b; Zhang et al., 2006), a traditionally used preparation for diabetes treatment in Portugal (D'Oliveira Feijão, 1973).

In our previous work (Dias et al., 2010b) *Coreopsis tinctoria* flavonoid-rich ethyl acetate fraction was administered to a STZ-glucose intolerant rat model for 3 weeks, resulting in glucose tolerance regain and pancreatic function recovery. Those results pointed either to a strong antioxidant-mediated protective activity against ROS-injury of pancreatic beta-cells, or to a mechanism that involved either an inhibition of apoptosis or promotion of proliferation. So, in the present study we decided to evaluate the cytoprotective effect of pre-treatment on cultured MIN6 cells with *Coreopsis tinctoria* extracts and the derived pure compounds marein and flavanomarein (Fig. 1) both through superoxide anion scavenging on tert-Butyl Hydroperoxide (tBHP) challenged cells and on cytokine-induced cell death through apoptosis.

tBHP is a membrane-permeant short chain analogue of lipid hydroperoxides widely used as a pro-oxidant agent to study oxidative stress *in vitro* (Sardão et al., 2007; Fernandes et al., 2010). Once inside the cells tBHP generates tert-butoxy radicals as well as other reactive oxygen species (ROS), which induce several physiological alterations such as lipid peroxidation, depletion of intracellular glutathione and DNA damage and, depending on the concentration, have been reported to cause apoptosis or necrosis in many cell types (Kweon et al., 2004). It is known that mitochondrial superoxide production is increased in chronic hyperglycemia and obesity (Patane et al., 2002) and superoxide production causes activation of uncoupling protein-2 (UCP2), a member of the mitochondrial anion carrier protein that regulates beta-cell membrane potential, which results in pancreatic beta-cell dysfunction (Krauss et al., 2003), revealing the relevance of superoxide measurement in pancreatic beta cells. Accordingly, antioxidant molecules that can inhibit production of ROS, including superoxide radical anion, can prevent cell death (Choi et al., 2003).

In the present work we used a chemiluminescent method in order to detect superoxide anion generation by MIN6 cells pre-incubated with *Coreopsis tinctoria* extracts prior to oxidant treatment. On exposure to superoxide, chemiluminescent probes release a photon, which can be detected by a scintillation counter or a luminometer. The lucigenin (Bis-N-methylacridinium nitrate)-based chemiluminescent method is a highly specific and widely used method for detecting superoxide anions either intra and extracellularly (Munzel et al., 2002; Bartosz, 2006).

In order to verify the capacity of *Coreopsis tinctoria* flowering tops extracts and pure compounds to protect MIN6 cells, preserving beta-cell mass through inhibition of apoptosis, we used pro-inflammatory cytokines; IL1-beta, TNF-alpha and IFN-gamma, a mixture already known to contribute to beta-cell apoptosis through nuclear factor-B (NF-B) activation and endoplasmatic reticulum (ER) stress (Kharroubi et al., 2004).

Overall, this study aimed to evaluate the potential of *Coreopsis tinctoria* flower extracts and their previously determined major constituents, marein and flavanomarein (Dias et al., 2010a,b), to prevent or delay the progression of beta-cell damage and in that way contribute to the better understanding of the implicated compounds and mechanisms involved in its previously reported antihyperglycemic activity.

2. Methods

2.1. Plant material and flavonoids

Commercially available dried flower tops of *Coreopsis tinctoria* Nutt. (Asteraceae) were purchased from herbal shops in Lisbon, Portugal, in 2006. Accordingly with the supplier, plant material was cultivated in Portugal, flower tops were collected in midsummer and dried in accordance with Guideline on Good Agricultural and Collection Practice for Starting Materials of Herbal Origin (EMEA/HMPC/246816/2005). Plant identity was confirmed and a voucher specimen (code CTD1) was lodged in Faculty of Pharmacy, University of Lisbon. The aqueous extract (30%, w/w dry plant material) as well as the ethyl acetate (AcOEt) fraction (5.3% w/w dry plant material) were prepared and chemically characterized by HPLC-UV and HPLC-DAD-MS/MS as previously described (Dias et al., 2010a,b). Marein and flavanomarein were acquired from Extrasynthése.

2.2. Cell culture and samples pre-incubation conditions

MIN6 cells (mouse insulinoma beta-pancreatic cells) were obtained from Professor J.-I. Miyazaki (University of Osaka, Japan) and, were cultured in DMEM 5921 (Sigma) supplemented with 25 mM D-Glucose, 10% Fetal calf serum, 2% Penicillin/Streptomycin, 2% L-Glutamine. Cells, passage 36 to 39, were plated in 96 well tissue culture plates at a density of 2×10^4/well, and left to adhere overnight and then pre-incubated in medium supplemented either with *Coreopsis tinctoria* extract, fraction or pure compounds for