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Syzygium cumini leaf extract inhibits LDL oxidation, but does not protect the liproprotein from glycation

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ABSTRACT

Etnopharmacological relevance: *Syzygium cumini* (L.) Skeels is a plant widely used in folk medicine to treat diabetes mellitus (DM). The tea from its leaves is frequently used by diabetics for lowering hyperglycemia. There is a close relationship between DM and atherosclerosis, a chronic immuno-inflammatory disease, were the early stages encompass oxidative and glycative modifications in the structure of low density lipoprotein (LDL).
Aim of this study: To investigate the potential protective effects of aqueous-leaf extract from *Syzygium cumini* (*S.c*Ext) against $CuSO_4$-induced oxidation and methylglyoxal (MG)-induced glycation of human LDL *in vitro*.
Materials and methods: LDL oxidative changes were evaluated by measuring conjugated dienes (CD) formation, thiobarbituric acid reactive substances (TBARS) levels, quenching of tryptophan (Trp) fluorescence and structural modifications in LDL particle. In LDL glycated by MG (glyLDL), we determined the levels of fluorescent advanced glycation end products (AGEs) and mobility by agarose gel electrophoresis.
Results: *S.c*Ext blocked oxidative events induced by $CuSO_4$ in human LDL, plasma and serum. Fourier transform infrared spectroscopy (FT-IR) revealed that specific regions of apoB100 were oxidized by $CuSO_4$ in human LDL and that S.cExt reduced these oxidations. Unlike, the increased AGEs levels and eletrophoretic mobility observed in LDL MG-glycated were not modified by *S.c*Ext.
Conclusion: The findings herein indicate that *S.c*Ext could be tested in atherogenesis models as potential protective agent against LDL oxidation.

1. Introduction

Atherosclerosis, a chronic inflammatory process characterized by formation of atherosclerotic plaques in large and medium-sized arteries, has been pointed as the underlying cause of coronary heart diseases, stroke and peripheral vascular disease (Steinberg and Witztum, 2002; Zha et al., 2012). Many components of the vascular, metabolic, and immune systems are involved in this process. However, the elevated levels of low density lipoprotein (LDL) in blood remain the most important risk factor for atherosclerosis (Steinberg and Witztum, 2002). Several studies have demonstrated that oxidative modifications that occur in LDL particle trapped in the arterial wall are key-initiating events in early atherogenesis (Chisolm and Steinberg, 2000; Nishi et al., 2002; Stocker and Keaney, 2004; Matsuura et al., 2006; Barcelos et al., 2011). The oxidized LDL (oxLDL) acts as an atherogenic particle by inducing endothelial cell activation, secretion of pro-inflammatory mediators and expression of adhesion molecules; phenomena that promote the recruitment of inflammatory cells into subendothelial layer of the artery wall (Witztum and Steinberg, 1991; Yoshida and Kisugi, 2010). oxLDL are recognized by the scavenger receptors on macrophages and, then internalized to form foam cells, an important hallmark of the atherosclerotic lesion (Enriksen et al., 1981).

Clinical and experimental studies have reported a close relationship between diabetes mellitus (DM) and atherosclerosis (Yoshida and

Abbreviations: AG, Aminoguanidine; AGEs, Advanced glycation end products; CD, Conjugated dienes; DM, Diabetes mellitus; EDTA, Ethylenediaminetetraacetic acid; FT-IR, Fourier transform infrared spectroscopy; glyLDL, Glycated LDL; GO, Glyoxal; LDL, Low density lipoproteins; MG, Methylglyoxal; oxLDL, Oxidized LDL; RAGE, Receptor of advanced glycation end products; S.cExt, Aqueous-leaf extract from *Syzygium cumini* (L.) Skeels; TBARS, Thiobarbituric acid reactive substances; Trp, tryptophan

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Kisugi, 2010; Rabbani et al., 2011). There is evidence that the risk for atherosclerosis development is ≅ three-fold higher in diabetic subjects (Lyons et al., 1986). Hyperglycemia favors protein glycation, where glucose or other reactive carbonyl compounds such as glyoxal (GO) and methylglyoxal (MG) react with protein amino groups, leading to the formation of advanced glycation products (AGEs) (Thornalley et al., 2001). AGEs, after binding to their receptor (RAGE), trigger pathological events such as oxidative stress, pro-inflammatory responses and cell death (Lee et al., 2006; Ott et al., 2014). In this scenario, MG-induced LDL glycation plays an important role in the pathogenesis of DM (Rabbani et al., 2011). Glycation of LDL by MG is directed to the arginine residues present in apoB-100 and it culminates in the formation of hydroimidazolone Nδ-(5-hydro-5-methyl −4-imidazolon-2-yl)-ornithine residues (MG-H1), which are considered the major advanced glycation product in the physiological systems (Thornalley, 2005).

The detrimental events elicited by oxidized and glycated LDL (glyLDL) have stimulated the search for agents with potential antioxidant and anti-glycative properties (Esterbauer et al., 1989a, 1989b; Belguendouz et al., 1997; de Bem et al., 2008; Barcelos et al., 2011). Of note, medicinal plants have attracted increasing attention of researches. *Syzygium cumini* (L.) Skeels (*S. cumini*, Myrtaceae syn: *Eugenia jambolana*), popularly known as "jambolão" or "black plum", is a plant used in Brazil and Asia as antidiabetic agent (Souza et al., 2014; Bhatia et al., 2014; Goyal, 2015; Esakkimuthu et al., 2016). Different parts of *S. cumini* (L.) Skeels have been shown to possess promising activity against DM and related abnormalities, such as hyperlipidemia (Ravi et al., 2005; Sharma et al., 2011; Maiti et al., 2013; Ulla et al., 2017). Nonetheless, most of studies on DM models have been carried out with fruits and seeds (Ayyanar et al., 2012; Nahid et al., 2017; Sharma et al., 2017; Priya et al., 2017), and there is less experimental scientific support about the *in vitro* and/or *in vivo* pharmacological properties of leaves from *S. cumini* (L.) Skeels (Baldissera et al., 2016; Poongunran et al., 2017). However, tea of *S. cumini* (L.) Skeels leaves is also widely used in folk medicine to lower hyperglycemia (Nadkarni, 1976; Jain, 1991; Oliveira et al., 2005; Ayyanar and Subash-Babu, 2012). Interestingly, literature findings have indicated the antioxidant, antifungal and antibacterial potential of leaf extracts of *S. cumini* (L.) Skeels (Braga et al., 2007; Mohamed et al., 2013; Ruan et al., 2008; Ecker et al., 2017).

Considering the hypoglycemic effects of *S. cumini* (L.) Skeels, the relationship established among LDL, atherosclerosis and hyperglycemia, as well as the importance of more scientific information about the biological activities of *S. cumini* (L.) Skeels leaves, we hypothesize that *S. cumini* (L.) Skeels leaf extract could reduce LDL susceptibility to oxidation and glycation. Then, the effects of aqueous-leaf extract from *S. cumini* (L.) Skeels (*S.c*Ext) on the susceptibility of LDL to oxidation induced by copper and glycation induced by MG were evaluated to test these assumptions.

2. Materials and methods

2.1. Chemicals

Methylglyoxal (MG) and $CuSO_4.5H_20$ were obtained from Sigma-Aldrich (St. Louis, MO). All the other chemicals were of analytical reagent grade and were purchased from Merck (Rio de Janeiro, Brazil).

2.2. Plant material

S. cumini (L.) Skeels leaves were collected from botanical garden of Universidade Federal de Santa Maria (UFSM). The leaves were dried (5% humidity, room temperature) and powdered, and then prepared as infusion at concentration of 30% (30 g of powder/ 100 ml of water) for 30 min. Afterwards, the infusion was filtrated and lyophilized. The yield of the extract obtained after lyophilization was ≅ 25%. The product was stored at –20 °C and dissolved in distilled water for use in the different assays.

2.3. HPLC of S.cExt

High performance liquid chromatography (HPLC-DAD) was performed using the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto Sampler (SIL-20A), equipped with Shimadzu LC-20AT reciprocating pumps connected to the degasser DGU 20 A5 with integrator CBM 20A, UV–VIS detector DAD (diode) SPD-M20A and Software LC solution 1.22 SP1. Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm × 250 mm) packed with 5 μm diameter particles. The mobile phase was water containing 2% acetic acid. The lyophilized aqueous extract from leaves of *S. cumini* (L.) Skeels was analyzed, dissolved in water at a concentration of 5 mg/ml. The presence of six phenolics compounds was investigated, namely: gallic, chlorogenic and caffeic acids and the flavonoids quercetin, rutin and kaempferol. The identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.5 ml/min, injection volume 40 μl and the wavelength were 254 nm for gallic acid, 325 nm for caffeic and chlorogenic acids, and 365 nm for quercetin, rutin and kaempferol. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–400 nm). Calibration curve for gallic acid: Y = 10237x + 1454.8 (r = 0.9998); caffeic acid: Y = 11372x + 1366.0 (r = 0.9996); rutin: Y = 11473x – 1375.7 (r = 0.9999); quercetin: Y = 12135x – 1392.6 (r = 0.9997) and kaempferol: Y = 129623x – 1343.9 (r = 0.9991). All chromatography operations were carried out at ambient temperature and in triplicate.

2.4. LDL isolation

This study was approved by the Ethics Committee of Universidade Federal de Santa Maria (n° 0089.0.243.000-07). LDL isolation was performed by discontinuous density-gradient ultracentrifugation using fresh human plasma as described by Silva et al. (1998), with minor modifications. Plasma of non-fasted healthy normolipidemic voluntary donors was collected and pooled with 1 mg/ml ethylenediaminetetraacetic acid (EDTA). Sucrose (final concentration of 0.5%) was added to prevent LDL aggregation and solid KBr (0.326 g/ml) to adjust the EDTA-plasma to a density of 1.22 g/ml. Five milliliters of this mixture (EDTA-plasma-KBr) was placed in a centrifuge tube and 5 ml of a sodium chloride solution (density 1.006 g/ml) was overlaid on the top of the mixture. Ultracentrifugation was run at 65.000 rpm for 2 h at 4 °C using a Himac CP80MX ultracentrifuge. LDL particles were collected by aspirating the yellow/orange band at the center of saline layer. Isolated LDL was submitted to exhaustive dialysis with 10 mM phosphate buffer (pH 7.4) for 24 h at 4 °C. After dialysis, isolated LDL was stored at −20 °C for less than 2 weeks. Protein quantification of the isolated LDL was performed according to the method described by Lowry et al. (1951).

2.5. LDL oxidation

As the incubation of LDL with copper culminates in lipid peroxidation, this parameter is commonly used as *in vitro* assays to verify LDL susceptibility to oxidation. Early and end products from lipid peroxidation chain reactions can be monitored by formation of conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS), respectively. Here we evaluated the effect of *S.c*Ext on CD formation under different oxidation conditions: in LDL, LDL isolated from human plasma treated with *S.c*Ext and in serum. TBARS levels were measured in LDL and plasma.

2.5.1. Conjugated dienes formation

2.5.1.1. LDL. LDL samples (50 μg protein/ml) were incubated in a