Valorisation of the industrial waste of *Chukrasia tabularis* A.Juss.: Characterization of the leaves phenolic constituents and antidiabetic-like effects


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ABSTRACT

*Chukrasia tabularis* A.Juss. is a canopy tree widely distributed in Asia and commonly used for construction-grade timber. While the residues resulting from the timber exploration constitute the major source of waste, other parts of the plant remain underutilized. Therefore, aiming the valorisation of a major residues resulting from *C. tabularis* wood industry, the leaves were here investigated on their potential content in bioactive constituents, but also on their capacity to modulate mediators and enzymes engaged in metabolic disorders, particularly those involved on the development and progression of diabetes.

HPLC–DAD–ESI/MS and UPLC-ESI-QTOF-MS characterization of a methanol extract obtained from the leaves, allowed the identification of 25 phenolic constituents, quercetin-3-O-rhamnoside being identified as the main bioactive. The leaf extract and the major flavonoid (quercetin-3-O-rhamnoside) were investigated on their impact towards a series of targets involved in the physiopathology of diabetes. The extract displayed significant scavenging properties against nitric oxide and superoxide radicals, inhibiting also lipid peroxidation and aldose reductase activity. While no noteworthy effects were noted on pancreatic lipase and α-amylase activity, the extract strongly inhibited α-glucosidase (IC_{50} = 21.14 µg/mL) and proved to be ca. 5 times more effective than the benchmark drug, acarbose. Moreover, the leaf extract significantly inhibited also 5-lipoxygenase (5-LOX) (IC_{50} = 13.12 µg/mL). Kinetic studies on α-glucosidase and 5-LOX activity disclosed a mixed type inhibition. Furthermore, *C. tabularis* extract reduced LPS-induced overproduction of NO, L-citrulline and IL-6 in activated RAW 264.7 macrophages. When individually assayed, quercetin-3-O-rhamnoside significantly contributed to the antiradical properties and inhibitory effects of the extract upon the enzymatic targets, but other phenolic bioactives appear also to underlie the recorded anti-inflammatory effects.

Taken together, our results demonstrate that the leaves of *C. tabularis* are rich in phenolic constituents with a great potential to improve metabolic disorders. The evidenced bioactivity of this industrial product might feed R&D programs for the development of new drugs that might simultaneously improve glycaemic, oxidative and inflammatory benchmarks in diabetic patients.

Keywords: *Chukrasia tabularis*; HPLC–DAD–ESI/MS; Diabetes; Flavan-3-ols derivatives; Dimethylquercetin derivatives; Quercitrin.

Abbreviations: 5-LOX, 5-lipoxygenase; AR, aldose reductase; DPPH®, 1,1-Diphenyl-2-picrylhydrazyl; IL, interleukin; K_m, Michaelis constant; LPS, lipopolysaccharide; LTB_4, leukotriene B4; p-NPB, p-nitrophenyl butyrate; p-NPG, p-nitrophenyl glucopyranoside; SAR, structure-activity relationship; V_{max}, maximum velocity.
1. Introduction

Metabolic disorders, including obesity and diabetes, constitute a worldwide pandemic that continues to grow at an alarming level (Saklayen, 2018). Despite the current pharmacotherapeutic arsenal, several entities, namely the World Health Organization, made clear that the success of primary health care in upcoming years will be driven also by the development of herbal drugs and the discovery of new plant-derived constituents (WHO, 2019). In this regard, our group has been actively engaged on the identification and investigation of plants from Southeast Asia, that were undervalued on their antidiabetic and anti-inflammatory properties, and that now call for advanced preclinical studies (Andrade et al., 2020, 2019; Ferreres et al., 2021, 2017).

*Chukrasia tabularis* A.Juss. (family: Meliaceae) is a canopy tree commonly distributed in tropical Asia (Kaur and Arora, 2009), ethnomedicinal surveys indicating its use in traditional medicine (Kaur and Arora, 2009). However, the tree is mainly reputed due to the use of construction-grade timber (Kaur and Arora, 2009). Besides, the bark, containing high-valued commercial gums and tannins, is also used in tanning processes (Kaur and Arora, 2009). Previous studies demonstrate that *C. tabularis* is particularly rich in the triterpenoid class of limonoids (Luo et al., 2012b; Nakatani et al., 2004; Peng et al., 2016; Zhang et al., 2008, 2014), mainly occurring on the wood and bark, many of which being shown to have relevant biological/pharmacological properties (Luo et al., 2012a, 2012b; Peng et al., 2016). Indeed, most studies on *C. tabularis* deal with the structural diversity and biological properties of terpenes. Still, the occurrence of phenolic bioactives is rarely reported on the species, tabularin (Purushotaman et al., 1977), gallic acid, catechin, epicatechin, quercetin and 3-O-glycosylated derivatives, tannic acid and 7-hydroxycoumarin (Kaur et al., 2011; Kaur and Arora, 2009) being the only polyphenols reported until the date.

At the moment, while the timber of the tree is exhaustively cropped, the leaves remain an unexploited industrial waste. Therefore, bearing in mind the 3 Rs policy, the potential reutilization of *C. tabularis* by pharmaceutical and dietary supplements industries was here assessed, considering the anti-diabetic and anti-inflammatory properties of the leaves. Effects on enzymatic targets (α-glucosidase, α-amylase, pancreatic lipase, and aldose reductase) and radical species engaged in diabetes development and progression were investigated for the first time. Also, considering the inflammatory background underlying diabetes (Pollack et al., 2016), we have used cellular models to detail the mechanisms of the anti-inflammatory effects. Finally, and attempting to broaden knowledge on *C. tabularis* secondary metabolites, the polyphenolic fingerprint of the leaves was characterized by means of HPLC-DAD-ESI/MS² and UPLC-ESI-QTOF-MS².
2. Materials and methods

2.1. General chemicals and standards

RAW 264.7 macrophages were obtained from the ATCC®. Standards of epicatechin, isorhamnetin-3-O-glucoside, kaempferol-3-O-glucoside, luteolin-7-O-glucoside and quercetin-3-O-rhamnoside were obtained from Extrasynthese S.A. (Genay, France).

2.2. Plant material and extraction

The leaves of C. tabularis were collected in Kasetsart University, Bangkok, Thailand in January 2018, taxonomic identification being carried out by Prof. Dr. Srunya Vajrodaya (Department of Botany, Faculty of Science, Kasetsart University). The plant material was air-dried, powdered and sieved to fine powder (mean particle size ≤910 μm) (39.1 g). The extract was prepared through maceration with 1 L of methanol for seven days, at 28 °C. After this period, the resulting extract was filtered through Whatman® grade 1 filtration paper and concentrated to dryness in a Rotavapor® R-210 (Buchi, Mumbai, India). A total of 2.3 g of dry extract was obtained and stored at -20ºC, and protected from light until further analysis.

2.3. Phytochemical study

2.3.1. HPLC-DAD-ESI(Ion Trap)-MS® qualitative analysis

The chromatographic analyses were carried as described before (Ferreres et al., 2015), exclusively varying gradient conditions. A water-formic acid (1%) (A) and acetonitrile (B) system was employed as mobile phase, with a flow rate of 800 μL/mL. The mobile phase started with 5% B and used a gradient to obtain 25% B at 30 min and 70% B at 35 min. Chromatograms were recorded at 250, 280, 330 and 350 nm, and the full scan mass covered the range from m/z 100 up to 1500. MS data were acquired in the negative ionisation mode.

2.3.2. UPLC-ESI-QTOF-MS® qualitative analysis

Determination of the exact mass was performed with the same equipment as in Andrade et al. (2020). The mobile phase consisted in a mixture of acidified water (0.1 % formic acid) (A) and acidified acetonitrile (0.1 % formic acid) (B), and compounds were separated according to the gradient conditions: 0 min 5% B, obtaining 40% B at 12 min and 70% B at 15 min. For the electrospray interface analysis, the following conditions apply: gas temperature 280 °C, drying gas 11 L/min, nebulizer pressure 45 psi, sheath gas temperature 400 °C, sheath gas flow 12 L/min. The MS system operated in negative ion mode with the mass range set at m/z 100–1500 in a full scan resolution mode. Further conditions as previously described in Garcia et al. (2016).
2.3.3. **HPLC-DAD quantitative analysis**

Quantification of the phenolic constituents was performed by HPLC-DAD, using a Kinetex column (5 μm, C18, 100 A, 150×4.6 mm; Phenomenex, Macclesfield, UK), under the same chromatographic conditions outlined in 2.3.2., chromatograms being recorded at 280 and 350 nm.

Quantification was attained by graphical interpolation of the absorbance recorded with commercial standards. Compounds were quantified as follows: epicatechin (2), kaempferol-3-O-glucoside (13), quercetin-3-O-rhamnoside (16), luteolin-7-O-glucoside (18) and isorhamnetin-3-O-glucoside (24) were quantified against their own standard curves; proanthocyanidins (1, 3-12, 14, 15, 17, 19, 20 and 22) were quantified as epicatechin at 280 nm; compound 23 was quantified as kaempferol-3-O-glucoside at 350 nm; compounds 21 and 25 were quantified as isorhamnetin-3-O-glucoside at 350 nm. Standard curves were built and the limits of detection (LOD) and quantification (LOQ) were determined taking into account the residual standard deviation (σ) of the regression curves and the respective slopes (S), according to LOD = 3.3σ/S and LOQ = 10σ/S (Table S1, Supplementary data).

2.4. **Antiradical activity**

Scavenging activity upon 1,1-diphenyl-2-picrylhydrazyl (DPPH·), nitric oxide (NO) and superoxide (O₂⁻) radicals was determined as before described (Andrade et al., 2020). Quercetin and ascorbic acid were used as positive control.

2.5. **Inhibition of lipid peroxidation**

Effects on lipid peroxidation were determined by measuring the formation of conjugated dienes from linolenic acid at 233 nm (Ferreres et al., 2012). 3,5-di-tert-4-Butylhydroxytoluene (BHT) was used as positive control.

2.6. **Aldose reductase inhibition**

Aldose reductase inhibition was determined by measuring NADPH falloff at 340 nm, as described before (Andrade et al., 2020). Rutin was used as positive control.

2.7. **Carbohydrates metabolizing enzymes inhibition**

α-Amylase and α-glucosidase inhibition were assessed as previously described (Andrade et al., 2020). The antidiabetic drug acarbose was used as positive control.

Kinetic studies on α-glucosidase inhibition were performed with three concentrations of extract (5, 20 and 40 µg/mL), using increasing concentrations of p-nitrophenyl glucopyranoside (p-NPG) (0.017-1.67 mM). Results were fitted into a
Michaelis-Menten kinetic equation to obtain the kinetic parameters (maximum velocity, $V_{\text{max}}$ and Michaelis constant, $K_m$) and data were further analysed by the nonlinear regression fit models on GraphPad Prism 6.0 Software.

2.8. Pancreatic lipase inhibition

Porcine pancreatic lipase inhibition was determined by measuring the 4-nitrophenol release from $p$-nitrophenyl butyrate ($p$-NPB) at 410 nm as previously reported by Andrade et al. (2020). Orlistat was used as positive control.

2.9. 5-LOX inhibition

Inhibitory capacity was evaluated according to a previously published protocol (Andrade et al., 2019), quercetin being used as positive control. Kinetic studies on 5-LOX inhibition were performed with three concentrations of extract (2, 10 and 70 µg/mL), using increasing concentrations of linolenic acid (1-600 µM). Results were fitted into a Michaelis-Menten kinetic equation to obtain the kinetic parameters (maximum velocity, $V_{\text{max}}$ and Michaelis constant, $K_m$) and data were further analysed by the nonlinear regression fit models on GraphPad Prism 6.0 Software.

2.10. RAW 264.7 macrophages culture

RAW 264.7 cells derived from murine macrophages (passages 6 to 50) were cultured in DMEM + GlutaMAX, supplemented with 10% FBS and 1% penicillin/streptomycin, under humidified atmosphere (5% CO$_2$) at 37 °C. After reaching enough confluence, cells were scraped and subcultured for the cellular assays, as described below.

2.10.1. Cell viability

Interference with the viability of RAW 264.7 cells was assessed as reported before (Andrade et al., 2019; Pereira et al., 2019). Cell viability is expressed in percentage, by comparing treated cells with the negative control (untreated cells).

2.10.2. Determination of NO and L-citrulline levels in LPS-stimulated RAW 264.7 macrophages

Effects on NO and L-citrulline levels were determined as reported by others (Andrade et al., 2019; Pereira et al., 2019). NO and L-citrulline levels are expressed in percentage, by comparing treated cells with the negative control (untreated cells). The iNOS inhibitor, $N$-methyl-$L$-arginine (25 µM), was used as positive control.
2.10.3. Determination of TNF-α and IL-6 levels in LPS-stimulated RAW 264.7 macrophages

Effects on TNF-α and IL-6 levels were determined according to a previous reported procedure, with minor modifications (Pereira et al., 2019). Cells were cultured in 96-well plates (35 000 cells/well), allowed to attach for 24 h and treated with active concentrations of extract (i.e., causing a significant reduction on NO levels) for 2 h. Thereafter, LPS (1 µg/mL, final concentration) was co-incubated with the extract for another 22h. Cellular supernatants, containing tumour necrosis factor α (TNF-α) and interleukin (IL)-6, were collected and frozen at -80 ºC until analysis. Attached cells were lysed with RIPA buffer, collected and frozen at -80 ºC. TNF-α and IL-6 levels in cell supernatants were determined using an ELISA kit, according to the manufacturer’s protocol. The Bradford method was utilized to determine the total protein content on cell lysates, TNF-α and IL-6 amounts being normalized to the protein content.

2.11. Statistical analysis

Statistical analysis was performed on GraphPad Prism 6.0 Software (San Diego, US). After excluding significant outliers pointed by the Grubbs' test, normality was checked through D’Agostino-Pearson normality test, One-way analysis of variance (ANOVA), with Dunnett as post hoc test, being used to compare each experimental condition with the respective control (untreated cells). Differences at \( p < 0.05 \) were considered significant. All the results are expressed as the mean ± SD of at least three independent experiments, performed in triplicate.

3. Results and discussion

3.1. Phenolic characterization of C. tabularis leaves

HPLC–DAD–ESI/MS\(^*\) analysis of the methanol extract obtained from \( C. \, tabularis \) leaves allowed the identification of 25 phenolic compounds (Table 1). Compounds 13, 16, 18, 21 and 23-25 display UV spectra of flavonoids. Peaks 1-12, 14, 15, 17, 19, 20 and 22, in particular, were detected in the UV chromatogram registered at 280 nm (Fig. 1) and exhibit spectral data of flavan-3-ols (280 nm) (Table 1). While 2 exhibits a deprotonated molecular ion at \( m/z \) 289 and co-chromatographically matches epicatechin, all other flavon-3-ols exhibit MS data indicating that they are dimers and trimers.
Fig. 1. UV Chromatogram (280 nm) of the methanol extract obtained from C. tabularis leaves. Identity of compounds 1–25 as in Table 1.

Compounds 1, 3-6 and 8, with the same deprotonated molecular ion at m/z ~561.1406 and a similar MS fragmentation, solely differ in the relative abundances of their ions. The ion at m/z 289 is the most abundant fragment being detected, oftentimes corresponding to the base peak (Table 1) (MS²QTOF(561): 289.0717, [C₁₅H₁₃O₆]⁺, pentahydroxyflavan) that coincides with the deprotonated ion of catechin/epicatechin. A series of flavan-3-ol derivatives complying with these characteristics (UV and molecular formula C₃₀H₂₆O₁₁) have been detected, most of them corresponding to dimers with a total of nine hydroxyls, including gambiriin C (epiafzelechin-(4-8)-catechin), fisetinidol-(4β-8)-catechin, fisetinidol-(4α-8)-catechin and catechin-(4α-8)-epiafzelechin. Therefore, current data suggests that constituents 1, 3-6 and 8 might correspond to dimers of catechin/epicatechin and epiafzelechin or fisetinidol.

Compounds 7, 9-12 and 15, sharing a deprotonated molecular ion at m/z ~833.2092, can be labelled as trimers with a total of thirteen hydroxyl groups. The ion at m/z 561 is very abundant, and sometimes the base peak, in their MS² fragmentations, displaying also the same fragmentations ions of the dimers 2-6 and 8 (Table 1). In the MS³(833→561) the base peak is the ion at m/z 289 (data not shown), the ion 289.0717 being also observed in MS²(QTOF). This indicates that these trimers derive from the previous ones, being characterized by an additional tetrahydroxyflavan moiety. In this matter, they might possibly correspond to those previously reported by Steynberg et al. (1997): epifisetinidol-(4β-8)-epicatechin-(6-4β)-epifisetinidol, ent-fisetinidol-(4β-8)-catechin-(6-4β)-ent-fisetinidol and ent-fisetinidol-(4β-8)-catechin-(6-4α)-ent-fisetinidol.

Compounds 14, 17, 19, 20 and 22, with a deprotonated molecular ion at m/z ~817.2135, must be trimers exhibiting one hydroxyl group less than the preceding ones, their base peak being the ion at m/z 561. Additional ions are the same as those observed in the MS fragmentation of 7, 9-12 and 15, except for some ions that are displaced by 16 amu. Likewise, the base peak in the MS³ (817→561) is the ion at m/z 289 (data not shown), while the ion 289.0717 is detected in
MS²(QTOF). Based on the above, the possible presence of catechin/epicatechin and the base peak at m/z 561 suggests that these compounds derive from the previously described dimers and exhibit an additional trihydroxyflavan, possibly guibourtinidol (3,7,4′-trihydroxyflavan).

Compounds 13, 16, 18, 21 and 23-25 have an UV spectrum of flavonoids and exhibit deprotonated molecular ions of monoglycosides (Table 1). Except for compound 21, the other flavonols detected in C. tabularis extract have a MS fragmentation that is characterized by the occurrence of a base peak ion corresponding to their deprotonated aglycons: 13 and 23 kaempferol, 16 quercetin, 18 luteolin and 24 isorhamnetin. Moreover, UV spectral data of the flavonol derivatives 13, 16 and 23 indicate that the glycosylation occurs in position 3, pointing that they correspond to kaempferol-3-O-hexoside (13), quercetin-3-O-rhamnoside (16) and kaempferol-3-O-rhamnoside (23). Comparison with authentic standards allowed to unequivocally label compounds 13, 18, 16 and 24 as kaempferol-3-O-glucoside, luteolin-7-O-glucoside, quercetin-3-O-rhamnoside and isorhamnetin-3-O-glucoside, respectively.

Compounds 21 and 25, with a deprotonated molecular ion at m/z 491.1195 (molecular formula, C_{23}H_{24}O_{12}) and a deprotonated aglycon at m/z 329.0665, correspond to trihydroxy-dimethoxyflavon-hexoside isomers. The occurrence of 3-O-glycosylated derivatives of quercetin (16) and isorhamnetin (24) suggests that 21 and 25 can be labelled as dimethylquercetin (methylisorhamnetin) derivatives. In fact, considering the abundance of the ion (base peak) at m/z 476 [(M-H)-15]⁻ in compound 21 and its absence in 25, 21 can tentatively be identified as 7,3´-dimethyl-quercetin-3-O-hexoside and 25 as 3´,4´-dimethyl-quercetin-3-O-hexoside.

Quantitative analysis (Table 1) evidenced that proanthocyanidins (29 796.41 ± 1 441.45 mg/kg of dry extract) are the main structural class of polyphenols occurring in the extract obtained from C. tabularis leaves, corresponding to ca. 70 % of the total quantifiable phenolic content. Trimers (26 402.61 ± 1150.90 mg/kg of dry extract) represent ca. 90% of the total quantifiable content of proanthocyanidins, trimer 6 (15) being detected as the main constituent (12 839.08 ± 570.51 mg/kg of dry extract), followed by the flavonol quercetin-3-O-rhamnoside (quercitrin; 16) (10 021.22 ± 231.01 mg/kg of dry extract). Moreover, it worth to note that quercitrin (16) represented over 80 % of the flavonoids fraction and, consequently, its partial contribution for the activity of the extract was next investigated.

Our MS-based analysis evidenced that a series of phenolic constituents with biologic relevance can be easily extracted from the leaves of C. tabularis with methanol. In fact, this is the first time that HPLC–DAD–ESI/MS² and UPLC-ESI-QTOF-MS² methods are described to characterize the phenolic composition of the species. Even though some of the previously described constituents were not detected in our samples, the dietary flavan-3-ol, epicatechin (2) and the high valued proanthocyanidins were identified (Kaur et al., 2011; Kaur and Arora, 2009). Still, this is the first study allowing to know on the occurrence of phenolic bioactives in C. tabularis leaves.
3.2. Effects on carbohydrates and lipids absorption enzymatic systems

In order to investigate the antidiabetic properties of *C. tabularis* leaves, inhibitory effects were assessed against enzymatic systems involved on the intestinal absorption of dietary carbohydrates and lipids. The carbohydrates digestive enzymes α-glucosidase and α-amylase catalyse the hydrolysis of complex dietetic carbohydrates into low molecular weight monosaccharides, allowing their uptake by the enterocytes (Etsassala et al., 2019). On the other hand, pancreatic lipase is the main enzyme responsible for fat digestion, being involved on the hydrolysis of dietary triglycerides, allowing the absorption of free fatty acids (Liu et al., 2020). Inhibition of carbohydrates digestive enzymes and pancreatic lipase delays the intestinal absorption of sugars and free fatty acids, respectively, alleviating postprandial hyperglycaemia and hyperlipidaemia (Etsassala et al., 2019; Liu et al., 2020). Despite of the therapeutic efficacy of the antidiabetic drugs that are currently available and act through the inhibition of these enzymes, their side-effects frequently lead to treatment discontinuation (Etsassala et al., 2019; Liu et al., 2020). Therefore, there is an evident need of new pharmacological alternatives. Even though the predictive activity of the enzymes used in *in vitro* studies can easily vary according to their biological origin (e.g. yeast, human, rat) and obtaining processes, most high-throughput studies are usually performed with α-amylase and pancreatic lipase from mammalian origin (porcine) and α-glucosidase for yeast origin, reliable results being obtained in comparation with the positive controls (Andrade et al., 2020; Ferreres et al., 2021).

While no noteworthy α-amylase inhibition was observed (23.32 ± 5.14 % inhibition at 500 µg/mL), a significant and concentration-dependent inhibition of the α-glucosidase activity was recorded (IC\textsubscript{50} = 21.14 µg/mL; being ca. 5 times more active than the reference drug acarbose) (Fig. 2A). In fact, a strong and selective α-glucosidase inhibition, along with minor/mild effects on α-amylase activity, may overcome some of the adverse effects of the mainstay drugs, such as acarbose (Etsassala et al., 2019). Considering that the type of inhibition might also influence the pharmacological outcomes, we further studied the mode of inhibition elicited by the extract. As seen on Fig. 2C, a significant decrease on \( V_{\max} \) was recorded at all concentrations, followed by a \( K_m \) increase at 20 and 40 µg/mL, suggesting a mixed-model of inhibition. Consequently, contrary to acarbose, that binds to the substrate binding site on the α-glucosidase pocket (competitive inhibitor), *C. tabularis* leaf extract constituents bind to both enzyme and enzyme-substrate complex, having in this case, according to the recorded \( \alpha \) value (8.511) higher affinity to the enzyme itself. Thus, this inhibitory mechanism resembles to the competitive one displayed by acarbose (data not shown). Finally, worth to note that the leaf extract displayed neglectable effects on pancreatic lipase (17.38 ± 2.19 % at 1000 µg/mL), pointing to its selectivity for the alleviation of postprandial hyperglycaemia.
Fig. 2. Inhibition of α-glucosidase (A) and aldose reductase (B) upon treatment with C. tabularis leaf extract. Kinetics of α-glucosidase (C) activity in the absence and presence of increasing concentrations of C. tabularis leaf extract. Results represent the mean ± SD of three independent experiments, performed in triplicate. Statistical significance: ** p < 0.01 and **** p < 0.0001 (ANOVA, Tukey’s multiple comparison test).

3.3. Effects on radical species

The intimate link between the development of metabolic disorders, as diabetes and obesity, and the overproduction of free radicals and reactive oxygen species (ROS) is deep-rooted and well-sustained (Panigrahy et al., 2016). Activation of the mitochondrial electron transport chain and exacerbation of the polyol pathway (through aldose reductase overactivity) are pointed as pivotal sources of oxidative stress in diabetes (Panigrahy et al., 2016). Reactive species activate cellular stress sensitive pathways, leading to β-cell dysfunction and insulin resistance, are also responsible for formation of macro and microvascular lesions, mostly on the retina, eye and kidney (Panigrahy et al., 2016). In fact, oxidative stress amelioration has demonstrated to have positive outcomes on diabetes progression (Guevara-cruz et al., 2021).

Initially, to provide new insights on the antiradical properties of C. tabularis, the scavenging capacity of the leaf extract was first checked against the synthetic radical DPPH•, significant antiradical effects being recorded (Fig. S1A, Supplementary data) (IC\textsubscript{50} = 24.23 µg/mL). While being able to scavenge both ‘NO and O\textsubscript{2}–’, the extract was particularly effective towards the latter, with an IC\textsubscript{50} value of 28.81 µg/mL being estimated (Fig. S1B and Fig. S1C, Supplementary
data). Relevantly, the extract reduced lipid peroxidation, known to be increased in diabetic and obese patients (Tangvarasittichai, 2015); an IC\textsubscript{50} value of 166.45 µg/mL was estimated (Fig. S1D., Supplementary data), proving to be more effective than BHT (IC\textsubscript{50} = 295.29 µg/mL), herein used as a positive control. Moreover, taking into account the active role of aldose reductase on the installation of metabolic oxidative stress (Panigrahy et al., 2016), and also considering its involvement on the development of diabetes-related complications (Devi et al., 2018), the impact upon the enzymatic activity was investigated. A concentration-dependent reduction of aldose reductase activity was observed upon exposure to the leaf extract (IC\textsubscript{50} = 20.98 µg/mL) (Fig. 2B), proving to be ca. 6 times more active than the positive control, rutin (IC\textsubscript{50} = 150.37 µg/mL). While this is the first study demonstrating the inhibitory capacity of \textit{C. tabularis} upon aldose reductase, the scavenging properties of the plant have been previously demonstrated by others. In agreement with our findings, Kaur and colleagues have found that a leaf extract inhibited lipid peroxidation (Kaur et al., 2009, 2008) and also scavenged O\textsuperscript{2-} radicals (Kaur et al., 2011).

3.4. Effects on inflammatory mediators

The low-grade of chronic inflammation is also a pathological hallmark of diabetes, being characterized by the production and secretion of several inflammatory mediators from activated adipocytes (Pollack et al., 2016). Indeed, there is an increasing evidence on the beneficial effects of immunomodulatory and anti-inflammatory treatments on glycaemic control and on the amelioration of β-cell dysfunction and insulin resistance (Pollack et al., 2016).

To screen the anti-inflammatory properties of the leaf extract, the ability to inhibit the main enzyme involved on the production of the pro-inflammatory leukotrienes was firstly assessed (Filgueiras et al., 2015). 5-LOX catalyses the oxygenation of arachidonic acid to 5-hydroxyeicosatetraenoic acid, initiating the biosynthesis of leukotrienes, namely LTB\textsubscript{4}, that increases reactive oxygen and nitrogen species production, contributing for β-cell destruction (Filgueiras et al., 2015). 5-LOX inhibition restrains leukotriene B\textsubscript{4} (LTB\textsubscript{4}) production and might alleviate the inflammatory events underlying metabolic conditions (Andrade et al., 2020, 2019; Ferreres et al., 2018; Filgueiras et al., 2015; Ribeiro et al., 2014). \textit{C. tabularis} leaf extract significantly inhibited 5-LOX activity with concentration-dependency (IC\textsubscript{50} = 13.12 µg/mL, Fig. 3A), though in less extent than the positive control quercetin, with an IC\textsubscript{50} value of 2.53 µg/mL. The reaction \(V_{\text{max}}\) significantly decreased at 2, 10 and 70 µg/mL, while no significant variations in \(K_{m}\) were observed, except at the highest tested concentration (70 µg/mL), where a significant (\(p < 0.05\)) \(K_{m}\) decrease was recorded (Fig. 3B). Therefore, the extract displays a mixed inhibitory mechanism, where its constituents can bind directly to the catalytic site of 5-LOX and also to the enzyme-linolenic acid complex. However, the mixed inhibitory mechanism resembles more to the uncompetitive type (\(\alpha = 0.2212\), Fig. 3B), meaning that, contrary to what is observed on α-glucosidase inhibition (Fig. 3C), the extract constituents have greater affinity to the enzyme-substrate complex. Commonly, in this type of inhibition,
the inhibitor does not structurally resemble to the substrate, thus the substrate addition does not reverse the inhibitory effect.

**Fig. 3.** (A) Inhibition of the activity of 5-LOX by the methanol extract obtained from *C. tabularis* leaves. (B) Kinetics of 5-LOX activity in the absence and presence of increasing concentrations of *C. tabularis* leaf extract. Results represent the mean ± SD of three independent experiments, performed in triplicate. Statistical significance: * p < 0.05, ** p < 0.01 and *** p < 0.001 (ANOVA, Tukey’s multiple comparison test) (ANOVA, Tukey’s multiple comparison test).

Furthermore, to provide additional clues on the anti-inflammatory effects of *C. tabularis* leaves, LPS-stimulated macrophages were used as an inflammatory cellular model; LPS triggers macrophages activation, causing the overexpression of pro-inflammatory cytokines (TNF-α, IL-6) and pro-inflammatory proteins (inducible nitric oxide synthase, iNOS) (Andrade et al., 2019; Pereira et al., 2014, 2019; Pieretti et al., 2022). As evidenced in **Fig. 4A**, *C. tabularis* leaf extract significantly reduced NO levels of LPS-stimulated RAW 264.7 macrophages to 62.79 ± 7.00 and 81.63 ± 7.87 %, after 24h treatment with 250 and 125 µg/mL, respectively. Moreover, a significant L-citrulline falloff was also recorded at 125 and 250 µg/mL, reducing levels down to 71.04 ± 10.21 % and 81.32 ± 7.11%, correspondingly. Once both NO and L-citrulline are produced by cellular iNOS in a stoichiometric way, the dual reduction of the pro-inflammatory mediators results from iNOS inhibition (expression and/or activity) (Andrade et al., 2019) (**Fig. 4A and D**). In agreement, the iNOS inhibitor N-methyl-L-arginine (25 µM) also reduced both NO and L-citrulline levels. At effective concentrations (125 and 250 µg/mL), the extract also enabled the reduction of IL-6 levels to 42.16 ± 8.09 and 38.20 ± 8.83 pg/mg protein, respectively, with no relevant alteration on basal TNF-α levels (**Fig. 4B**). Relevantly, as no inference
with the mitochondrial activity was detected (Fig. 4A and Fig. S2., Supplementary data), we can assure that the anti-inflammatory effects displayed by the extract (Fig. 4A and B) did not outcome from cell death. To sum up, a general mechanistic insight of the anti-inflammatory properties of *C. tabularis* leaf extract is schematized in Fig. 4D. In accordance with our findings, Peng and colleagues also reported the inhibitory effects of *C. tabularis* leaves on inflammatory biomarkers in LPS-stimulated RAW 264.7 macrophages (Patel et al., 2014). However, authors did not assess the effects upon IL-6 levels, which, along with the previously reported IL-1β inhibition, should be taken in consideration (Patel et al., 2014).

**Fig. 4.** (A) Effects resulting from the treatment with *C. tabularis* leaf extract on the cell viability, NO levels and L-citrulline levels in LPS-stimulated RAW 264.7 macrophages. (B) Effects on IL-6 and TNF-α levels in LPS-stimulated RAW 264.7 macrophages upon treatment with *C. tabularis* leaf extract. (C) Mechanistic insight of *C. tabularis* anti-inflammatory effects. Cells were pre-treated for 2 h with the extract, followed by 22 h co-treatment with LPS (1 µg/mL). Results represent the mean ± SD of four independent experiments, performed in triplicate/duplicate (L-citrulline and ELISA). Statistical significance: **** p < 0.0001 NO levels significantly different from the control (LPS-stimulated untreated macrophages); ### p < 0.001 and #### p < 0.0001 L-citrulline levels significantly different from the control.
3.5. Impact of the phenolic bioactives present in C. tabularis leaves on the biological effects

Until date, studies on the chemical profile of C. tabularis almost exclusively deal with the isolation and characterization of limonoids, including chuktabularins (Peng et al., 2016), tabularisins (Peng et al., 2016), tabulalides (Nakatani et al., 2004) and chuktabrins (Li et al., 2013; Zhang et al., 2008), a reduced number of polyphenolic constituents being identified (Kaur et al., 2011). However, our work demonstrates the occurrence of several phenolic compounds (4189.95 ± 1 768.87 mg/kg dry extract) (Table 1), namely flavonols (12 103.55 ± 327.42 mg/kg dry extract) and oligomeric flava-3-ols (29 796.41 ± 1 441.44 mg/kg dry extract). The structural scaffold of these phenolics are determinant to their multiple biological properties, including anti-inflammatory (Ribeiro et al., 2014), antioxidant (Amić et al., 2007) and antidiabetic (Proença et al., 2017).

Flavonoids have shown to exhibit inhibitory effects against α-glucosidase and structural features were highlighted by structure-activity relationship (SAR) studies, namely the 2,3-insaturation at the C ring and, the hydroxylation at C5 in the A ring and at C4’ in the B ring (Proença et al., 2017). Indeed, most flavonoids identified in C. tabularis extract comply with these structural requirements, and therefore their contribution to the inhibitory effects above discussed, is expected.

Relevantly, we observed that when tested at 10 µg/mL (corresponding to its content on the highest concentration of extract being tested), quercitrin (16) (10 021.22 ± 231.01 mg/kg dry extract, Table 1) inhibited 13.46 ± 4.69 % of α-glucosidase activity, pointing to its role on the inhibitory properties displayed by the extract (Fig. S3B, Supplementary data).

Moreover, other phenolic constituents present in the extract are also expected to contribute for the extract inhibitory activity since as shown by others, epicatechin (2) (Pujirahayu et al., 2019), kaempferol-3-O-glucoside (13) (Hong et al., 2013), luteolin-7-O-glucoside (18) (Borges et al., 2021) and kaempferol-3-O-rhamnoside (23) (Cao et al., 2021) also display significant α-glucosidase inhibitory effects (IC₅₀ values of 578 µg/mL, 71 µg/mL, 10 µg/mL and 0.41 µg/mL respectively). Hence, the synergic interaction between such bioactives can explain the strong α-glucosidase inhibitory activity displayed by the leaf extract.

Due to their privileged structural scaffolds, phenolic compounds can convert reactive oxygen and nitrogen species into relatively stable radicals, halting radical oxidative cascades (Amić et al., 2007). Particularly concerning proanthocyanidins (1, 3-12, 14, 15, 17, 19, 20 and 22), occurring in high amounts in C. tabularis leaf extract (29 796.41 ± 1 441.44 mg/kg dry extract, Table 1), studies suggest a positive correlation between its content and the antiradical activity (Monagas et al., 2009). In addition, the remaining flavonols (compounds 13, 16, 21 and 23-25) (11 605.05 ± 311.99 mg/kg dry extract) and the luteolin derivative 18 (498.50 ± 15.43 mg/kg dry extract) (Table 1) are also acknowledged on their radical scavenging properties (Amić et al., 2007). The double bond at C2-C3 in conjugation with

(LPS-stimulated untreated macrophages); ++ p < 0.01 and +++ p < 0.001 IL-6 levels significantly different from the control (LPS-stimulated untreated macrophages) (ANOVA, Tukey’s multiple comparison test).
a 4-oxo function in the C-ring, as observed in flavonoids 13, 16, 18, 21 and 23-25, enhances the flavonoid phenoxyl radical stabilization and, consequently, increases the radical scavenging activity (Amić et al., 2007). Besides, the catechol moiety in B-ring, as occurring in quercitrin (16) (10 021.22 ± 231.01 mg/kg dry extract) and luteolin-7-O-glucoside (18) (498.50 ± 15.43 mg/kg dry extract) (Table 1), also increases the antiradical effects, once it confers higher stability to the resulting radicals (Amić et al., 2007). Worth referring that, when tested at its corresponding amounts on the highest extract concentration, quercitrin (16) scavenged 38.29 ± 2.43 % and 15.58 ± 1.86 % of ‘NO and O₂⁻ radicals, respectively (Fig. S3B, Supplementary data), thus contributing for the antiradical effects elicited by the extract. Besides, at 4 µg/mL (its corresponding concentration on the highest extract concentration tested) 16 also reduced lipid peroxidation up until 14.38 ± 4.15 %, evidencing its contribution for the attenuation of oxidative degradation of lipids (Fig. S3B, Supplementary data).

Flavonoids also meet the structural requirements for aldose reductase inhibition, interacting at the enzyme hydrophobic pocket with the hydrophobic residues and with the hydrophilic anionic binding site (Stefek, 2011). The presence of a 7-hydroxyl group at the A ring, the catechol moiety at the B ring and the 2–3 double bond at C ring enhance the inhibitory activity and improve the flavonoids anchoring to the enzyme active site (Stefek, 2011). In fact, and as evidenced by others, the flavonoids epicatechin (2) (Murata et al., 1994), luteolin-7-O-glucoside (18) (Li et al., 2014), kaempferol-3-O-glucoside (13) and kaempferol-3-O-rhamnoside (23) inhibit aldose reductase activity with estimated IC₅₀ values of 23 µg/mL, 4 µg/mL, 0.85 µg/mL and 0.95 µg/mL, respectively. Consistently, the major flavonoid, quercitrin (16) (10 021.22 ± 231.01 mg/kg dry extract, Table 1), has been also reported to be a strong aldose reductase inhibitor (IC₅₀ = 67 µg/mL) (Matsuda et al., 2002). In agreement, we found that 16 inhibited 16.64 ± 5.38 % of aldose reductase activity at a concentration as low as 1.25 µg/mL, significantly contributing to the inhibitory effects of the extract (Fig. S3B, Supplementary data).

C. tabularis phenolic constituents also account to the recorded anti-inflammatory properties. Specifically concerning to epicatechin oligomers, Schewe and co-workers demonstrated that their 5-LOX inhibitory capacity was inversely related with the chain size, monomeric and dimeric procyanidins being ascribed as the most active (Schewe and Kühn, 2002). In this matter, it is worth to note the considerable amounts of dimeric (3393.79 ± 290.54 mg/kg dry extract) and trimeric (26 402.61 ± 1 150.90 mg/kg dry extract) flavon-3-ols occurring in C. tabularis leaf extract (Table 1) and that might underlie the extract’s effects as well. Flavonoids are also known to inhibit pro-oxidant enzymes, including lipoxygenases (Ribeiro et al., 2014). SAR studies indicate that the catechol moiety in A and B rings, along with the presence of the 2,3 instauration and the 4-oxo group in the C ring, are the predominant requirements for 5-LOX inhibition (Ribeiro et al., 2014). Indeed, several flavonoids detected in C. tabularis leaf extract meet most of these structural requisites. As demonstrated by us, the main flavonoid (16) (10 021.22 ± 231.01 mg/kg dry extract) (Table 1) led to 23.00 ± 6.92 % inhibition of 5-LOX activity when tested at 0.72 µg/mL (concentration equivalent to its occurrence on the highest extract concentration) (Fig.
Additionally, experimental studies sustain the inhibitory effects of epicatechin (2) (Schewe et al., 2002), luteolin-7-O-glucoside (18) (Jin et al., 2009) and kaempferol-3-O-rhamnoside (23) (Kim et al., 2006) towards 5-LOX, suggesting their additional contribution to the activity of the extract.

Regarding the effects on the cellular inflammatory model, even though Costa and colleagues have demonstrated that 13 and 16 were capable of reducing NO production in LPS-stimulated macrophages (IC_{50} values of 0.85 and 1.03 µg/mL, respectively) (Costa et al., 2015), we observed that 16 (when tested at a concentration equivalent to their occurrence on C. tabularis leaf extract, 2.5 µg/mL), had no significant effects on cellular NO and L-citrulline levels (Fig. S3C, Supplementary data). Therefore, such observations suggest that other phenolic and/or non-phenolic constituents might be responsible for the anti-inflammatory effects of C. tabularis leaves in RAW 264.7 macrophages.

Overall, data gathered herein evidence that the phenolic constituents identified on C. tabularis leaves contribute, in some extent, to the antiradical properties of C. tabularis, playing also a role on the inhibition of the enzymes involved on diabetes development and progression. Nonetheless, extracts are complex mixtures, and consequently the influence of other secondary metabolites that were not studied herein, ought not be excluded. In addition, eventual synergic or antagonic interactions between the extract components must also be considered.

4. Conclusions

The current work demonstrates that C. tabularis leaves can be valued by their content in polyphenolic bioactives and by its evidenced antidiabetic and anti-inflammatory properties. Twenty-five phenolic compounds were identified and quantified, 24 of which being reported for the first time on the genus. The scientific outcomes, relevance and novelty deal also with the significant impact recorded against enzymatic systems that are enrolled in the progression of diabetes, attenuation of the underling radical storm and mitigation of the inflammatory activation of RAW 264.7 macrophages. It is worth to highlight that besides the triad of pleiotropic effects (antidiabetic, anti-inflammatory and antiradical), the extract proved to be ca. 5 times more effective than acarbose on the inhibition of α-glucosidase. Quercetin-3-O-rhamnoside (quercitrin, 16) was found to contribute to the radical scavenging properties of the extract, as well as to the inhibitory effects upon α-glucosidase, aldose reductase and 5-LOX, but other bioactives appear to contribute to the recorded anti-inflammatory effects in the inflammatory cellular model.

Altogether, our results demonstrate that C. tabularis leaves ought not to remain an industrial waste from the wood sector, and should be considered as a source of constituents with therapeutic and/or functional properties. Based on the content in bioactive ingredients, this devalued plant material might be included in R&D pipelines for the development of novel therapeutic approaches targeting the events underlying the physiopathology of diabetes.
Funding

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Credit authorship contribution statement

Conceptualization: Patrícia Valentão, Paula B. Andrade and David M. Pereira. Investigation; Catarina Andrade, Angel Gil-Izquierdo, Federico Ferreres; Data curation and formal analysis: Patrícia Valentão, Paula B. Andrade, David M. Pereira and Nelson G. M. Gomes; Resources: Paula B. Andrade and Sutsawat Duangsrisai; Supervision: Patrícia Valentão, Paula B. Andrade and David M. Pereira. Writing – original draft: Catarina Andrade. Writing – review & editing: Patrícia Valentão, Paula B. Andrade, David M. Pereira and Nelson G. M. Gomes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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https://doi.org/10.1016/j.bmcl.2014.06.069
Table 1. Rt, UV, molecular formula, [M-H]; MS[^\text{2}[M-H]] data and content of the phenolic compounds identified in the methanolic extract obtained from *C. tabularis* leaves.

<table>
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<th>Compounds[^a]</th>
<th>Rt (min)</th>
<th>UV (nm)</th>
<th>Formula</th>
<th>[M-H], m/z [b]</th>
<th>MS[^\text{2}[M-H]], m/z (c)</th>
<th>Extract (mg/kg dry extract)[d]</th>
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[^a] Main observed fragments. Other ions have been detected but were not significant. MS ions and their relative abundance were obtained by ESI(Ion Trap), except [M-H]; which was obtained by ESI-QTOF. Data from other MS[^\text{2}[M-H]] ions is presented in the main text.


[^c] Coelutes with other compounds and its UV spectrum could not be well observed.

[^d] Results correspond to mean ± SD (n = 3)

[^e] Sum: 41 899.95 ± 1 768.87
Supplementary Data

Valorisation of the industrial waste of *Chukrasia tabularis* A.Juss.: characterization of the leaves phenolic constituents and antidiabetic-like effects

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Table S1. Linear regression equation parameters, limit of detection (LOD) and limit of quantification (LOQ) for external standards used on the quantitative analysis.

Fig. S1. (A) DPPH, (B) O$_2^-$ and (C) NO scavenging activities of C. tabularis leaf extract. (D) Inhibition of lipid peroxidation upon exposure to C. tabularis leaf extract. Results represent the mean ± SD of three independent experiments, performed in triplicate (DPPH, NO and O$_2^-$) or duplicate (lipid peroxidation).

Fig. S2. Effects of the methanol extract obtained from the leaves of C. tabularis on the viability of RAW 264.7 macrophages.

Fig. S3. (A) Chemical structure of quercetin-3-O-rhamnoside (16). (B) Reduction/inhibitory activity displayed by quercitrin (16) against the selected in vitro targets in the cell-free assays. (C) Effects of quercitrin (2.5 µg/mL) on the cell viability, NO levels and L-citrulline levels in LPS-stimulated RAW 264.7 macrophages. Cells were pre-treated for 2 h with the extract, followed by 22 h co-treatment with LPS (1 µg/mL). Concentrations of quercitrin corresponding to those occurring at the highest concentration on C. tabularis leaf extract being tested in each assay. Results represent the mean ± SD of at least three independent experiments, performed in triplicate or duplicate (L-citrulline and lipid peroxidation).
Table S1. Linear regression equation parameters, limit of detection (LOD) and limit of quantification (LOQ), for external standards used on the quantitative analysis.

<table>
<thead>
<tr>
<th>Standards</th>
<th>Linear equation</th>
<th>Concentrations</th>
<th>LOD</th>
<th>LOQ</th>
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<tbody>
<tr>
<td></td>
<td>Slope (α)</td>
<td>Intercept (b)</td>
<td>R²</td>
<td>range (μg/mL)</td>
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<tr>
<td>Epicatechin</td>
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<td>14.13</td>
<td>0.9984</td>
<td>5-650</td>
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<tr>
<td>Luteolin-7-O-glucoside</td>
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<td>95.30</td>
<td>0.9997</td>
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<td>Ishoramnetin-3-O-glucoside</td>
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<td>45.34</td>
<td>0.9995</td>
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<tr>
<td>Kaempferol-3-O-glucoside</td>
<td>60.91</td>
<td>30.38</td>
<td>0.9994</td>
<td>4-60</td>
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<tr>
<td>Quercetin-3-O-rhamnoside</td>
<td>51.79</td>
<td>211.81</td>
<td>0.9999</td>
<td>16-250</td>
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</tbody>
</table>
Fig. S1. (A) DPPH•, (B) O$_2^•$ and (C) NO scavenging activities of *C. tabularis* leaf extract. (D) Inhibition of lipid peroxidation upon exposure to *C. tabularis* leaf extract. Results represent the mean ± SD of three independent experiments, performed in triplicate (DPPH•, NO and O$_2^•$) or duplicate (lipid peroxidation).
Fig. S2. Effects of the methanol extract obtained from the leaves of *C. tabularis* on the viability of RAW 264.7 macrophages. Cells were pre-treated for 24 h with the extract/medium. Results represent the mean ± SD of four independent experiments, performed in triplicate. Statistical significance: *** $p < 0.001$ significantly different from the control (untreated macrophages) (ANOVA, Tukey’s multiple comparison test).
Fig. S3. (A) Chemical structure of quercetin-3-O-rhamnoside (16). (B) Reduction/inhibitory activity displayed by quercitrin (16) against the selected *in vitro* targets in the cell-free assays. (C) Effects of quercitrin (2.5 µg/mL) on the cell viability, NO levels and L-citrulline levels in LPS-stimulated RAW 264.7 macrophages. Cells were pre-treated for 2 h with the extract, followed by 22 h co-treatment with LPS (1 µg/mL). Concentrations of quercitrin corresponding to those occurring at the highest concentration on *C. tabularis* leaf extract being tested in each assay. Results represent the mean ± SD of at least three independent experiments, performed in triplicate or duplicate (L-citrulline and lipid peroxidation).