

Bioactive Natural Products from *Piper betle* L. Leaves and their α -Glucosidase Inhibitory Potential

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Abstract: *Piper betle* L. can be a valuable proposal for the prevention and treatment of several disorders. In fact, its leaves are largely consumed as mouth freshener and masticator, being known for their biological properties. This material possesses several kinds of bioactive natural products. Considering that diabetes is a worldwide disease with strong impact on human health, this work intended to explore the *in vitro* α -glucosidase inhibitory capacity of *P. betle* leaves, as well as to improve the knowledge on their metabolic pattern. Thus, a targeted metabolite analysis of the aqueous and ethanolic extract was performed by GC-MS, a similar qualitative profile of both extracts being observed. Fourteen metabolites were determined in *P. betle* leaves, five of them for the first time. Alanine and β -sitosterol were the main amino acid and sterol, respectively. Stearic and palmitic acids were the predominant fatty acids. A strong capacity to inhibit α -glucosidase was noticed, ethanol extract being more active than the positive control (acarbose) tested under the same conditions. Taking into account the results obtained, this work further extends the potential application of this herbal medicine, suggesting that its consumption may have a positive impact in the prevention and treatment of diabetes disease.

Keywords: Antidiabetic; bioactive natural product; herbal medicine; *Piper betle* L. © 2016 ACG Publications. All rights reserved.

1. Plant Source

Medicinal species are important in traditional medicine and modern pharmaceutical drugs. In fact, the interest in the analysis of their bioactive compounds has increased in the last years. An example of an important and interesting medicinal plant is *Piper betle* L. (Piperaceae). The genus *Piper* includes approximately 1000 species of herbs, shrubs, small trees and hanging vines, some of them being

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worldwide used as spices and in traditional medicine. *Piper betle* L. (Piperaceae) is a tropical plant closely related to common pepper. Its leaves have a strong pungent aromatic flavour and are widely used as mouth freshener and masticatory in Asia [1].

Plant material was supplied and identified by Cristóvão Belo (Ph.D., Faculdade de Ciências da Educação, Universidade Nacional Timor Lorosa'e). Leaves were collected in March 2009, in Ossoala, Baucau district, East Timor. Samples were collected from different positions in the tree, placed in sterile plastic bags, transported to the laboratory in insulated sealed ice-boxes and dehydrated at 40°C for 5 days and kept dry and protected from light. The dried material was powdered and sifted (< 910 µm). A voucher specimen was deposited at Laboratório de Farmacognosia, Faculdade de Farmácia, Universidade do Porto (Pbtl-032009).

2. Previous Studies

Several studies have demonstrated that *P. betle* leaves have several pharmacological properties, including anti-inflammatory, anti-allergic, hepatoprotective, antioxidant, antimutagenic, anticarcinogenic, antifungic, antihelmintic, anti-hyperglycaemic, xanthine oxidase inhibition, chemopreventive and anticholinesterasic [2–10].

Diabetes mellitus is a chronic metabolic disorder caused by an improper balance of glucose homeostasis, which has a significant impact on health, life quality and life expectancy of patients, as well as on the health care system [11]. Diabetes is characterized by hyperglycaemia, which may result from a deficiency in insulin secretion, insulin action, or both [11]. Therefore, the main goal of diabetes mellitus treatment is to achieve blood glucose levels as close to normal as possible. α -Glucosidase is an enzyme that catalyses the final step in the digestive process of carbohydrates, and hence α -glucosidase inhibitors (e. g. acarbose, miglitol, and voglibose) could retard the use of dietary carbohydrates and suppress postprandial hyperglycaemia [12]. Nevertheless, some of these inhibitors present several side effects, such as abdominal distention and diarrhoea [11]. In this field, natural inhibitors from plants have revealed a strong activity against this enzyme, with minimal side effects [13]. As far as we know, there is no work reporting the effect of these leaves on α -glucosidase.

Regarding the chemical composition of *P. betle* leaves, some studies were performed to describe their phenylpropanoids and flavonoids [4, 9, 14 – 16], sesquiterpenes [17], as well as the presence of β -sitosterol, stearic and palmitic acids [18, 19]. Nonetheless, it is well known that, due to their rich metabolic diversity, plants provide a great challenge in metabolomics, which has proven to be a valuable tool for the comprehensive profiling of plant-derived samples, for the study of plant systems and natural products research [20].

3. Present Study

Standards and reagents

Reference compounds were purchased from various suppliers: alanine, valine, isoleucine, proline, norvaline (internal standard), cholesterol, cholestanol, stigmasterol, β -sitosterol, desmosterol (internal standard), methyl linolelaidate (internal standard), as well as palmitic, stearic, oleic, linoleic and linolenic acids were obtained from Sigma (St. Louis, MO, USA). α -Glucosidase (type I from baker's yeast), 4-nitrophenyl α -D-glucopyranoside (PNP-G) and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA)

were obtained from Sigma (St. Louis, MO, USA). Acarbose was from Bluepharma® Genéricos (Coimbra, Portugal). Potassium di-hydrogen phosphate and ethanol were from Merck (Darmstadt, Germany).

Plant extracts

In order to improve the knowledge on the chemical composition and on the α -glucosidase inhibitory activity of *P. betle* leaves, an aqueous lyophilized and an ethanol extract were prepared.

For the aqueous extract, 5 g of *P. betle* dried leaves were extracted with 300 mL of boiling water for 30 min, with subsequent filtration through a Büchner funnel. Afterwards, the resulting extract was lyophilized and kept in the dark until analysis.

The ethanol extract was prepared as follows: 3 g of *P. betle* dried leaves were mixed with ethanol (5 × 150 mL) under magnetic stirring (500 rpm), for 10 min, at 40°C. The resulting extract was filtered through a Büchner funnel, under vacuum, and then concentrated to dryness under reduced pressure (40°C).

Amino acids, fatty acids and sterols determination

Derivatization process

Trimethylsilylation procedure was performed as reported before [21]. Briefly, 0.2 g of each extract was transferred to a glass vial and 100 μ L of each internal standard (norvaline, methyl linolelaidate and desmosterol) were added. The solvent was evaporated under a nitrogen stream and 100 μ L of the derivatization reagent, MSTFA, was added to the residue. The vial was capped, vortexed and heated for 30 min in a dry block heater, maintained at 60°C.

GC-MS

GC-MS analysis was performed with a Varian CP-3800 gas chromatograph coupled to a Varian Saturn 4000 mass selective ion trap detector (USA) and a Saturn GC/MS workstation software version 6.8, with a VF-5 ms (30 m x 0.25 mm x 0.25 μ m) column (VARIAN) [21]. A CombiPAL autosampler (Varian, Palo Alto, CA) was used for all experiments. The injector port was heated to 250°C and the injections were performed in split mode, with a ratio of 1/40. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min. The Ion Trap was set as follows: transfer line, manifold and trap temperatures were 280, 50, and 180°C, respectively. The mass ranged from 50 to 600 m/z , with a scan rate of 6 scan/s. The emission current was 50 μ A and the electron multiplier was set in relative mode to an auto tune procedure. The maximum ionization time was 25.000 μ s, with an ionization storage level of 35 m/z . The injection volume was 1 μ L and the analysis was performed in Full Scan mode. The oven temperature was set at 100°C for 1 min, then increasing 20°C/min to 250°C, held for 2 min, 10°C/min to 300°C and held for 10 min. All mass spectra were acquired in the electron impact (EI) mode. Identification of compounds was achieved by comparison of their retention time and mass spectra with those from pure standards trimethylsilyl (TMS) derivatives analysed under the same conditions, and from NIST05 MS Library Database. For quantification purposes, each sample was injected in triplicate and the amount of metabolites was determined from the calibration curves of the respective standards. All compounds were quantified in Full Scan mode, with the exception of linoleic (m/z 262, 337 and 352), linolenic (m/z 191, 335 and 350) and oleic (m/z 264, 339 and 354) acids that were quantified by the area obtained from the re-processed chromatogram, using the characteristic m/z fragments.

α -Glucosidase inhibition

The evaluation of the ability to inhibit α -glucosidase was determined spectrophotometrically in a Multiskan Ascent plate reader (Thermo Electron Corporation), based on the reaction with PNP-G [22]. The absorbance was measured at 400 nm and three independent assays were performed in triplicate. Results were compared with that of acarbose (positive control), tested under the same conditions.

4. Discussion

The use of a method allowing the simultaneous determination of several classes of compounds in *P. betle* leaves may contribute to increase the knowledge on the metabolic composition of this herbal medicine. Therefore, in this study the aqueous and ethanol extracts of *P. betle* leaves were analysed by GC-MS after derivatization with N-methyl-N-(trimethylsilyl) trifluoroacetamide in order to explore their composition in amino acids, fatty acids and sterols.

Amino acids

Amino acid biosynthesis in young plants is regulated by a metabolic network that links nitrogen assimilation with carbon metabolism, being controlled by the metabolism of four central amino acids, namely glutamine, glutamate, aspartate and asparagine. These amino acids are then converted into all other amino acids in various biochemical processes. They also serve as major transport molecules of nitrogen, including transport from vegetative to reproductive tissues, and their metabolism is subjected to a concerted regulation by physiological, developmental, and hormonal signals [23].

The study of the free amino acids profile of *P. betle* leaves aqueous and ethanol extracts revealed the presence of two essential amino acids (valine and isoleucine) and two non-essential ones (alanine and proline) (Figure 1, Table 1), which were already reported in this species [24]. Quantitative differences were detected amongst both extracts, the aqueous extract being the one with higher amounts of these metabolites. This fact is not surprising, since amino acids are soluble in polar solvents; therefore, the use of water associated with high temperatures enables their extraction. Alanine was clearly the major compound in both extracts, accounting for more than 83% of the total of amino acids (Table 1). This compound inhibits the enzyme pyruvate kinase, regulates gluconeogenesis and glycolysis, ensuring the production of glucose in the human organism during periods in which there is no food intake [25]. The other amino acids, even though being present in lower amounts, also have important roles. For example, valine and isoleucine are substrates for the synthesis of glutamine, a non-essential amino acid that regulates the expression of genes with a role in the defence against oxidative stress [25].

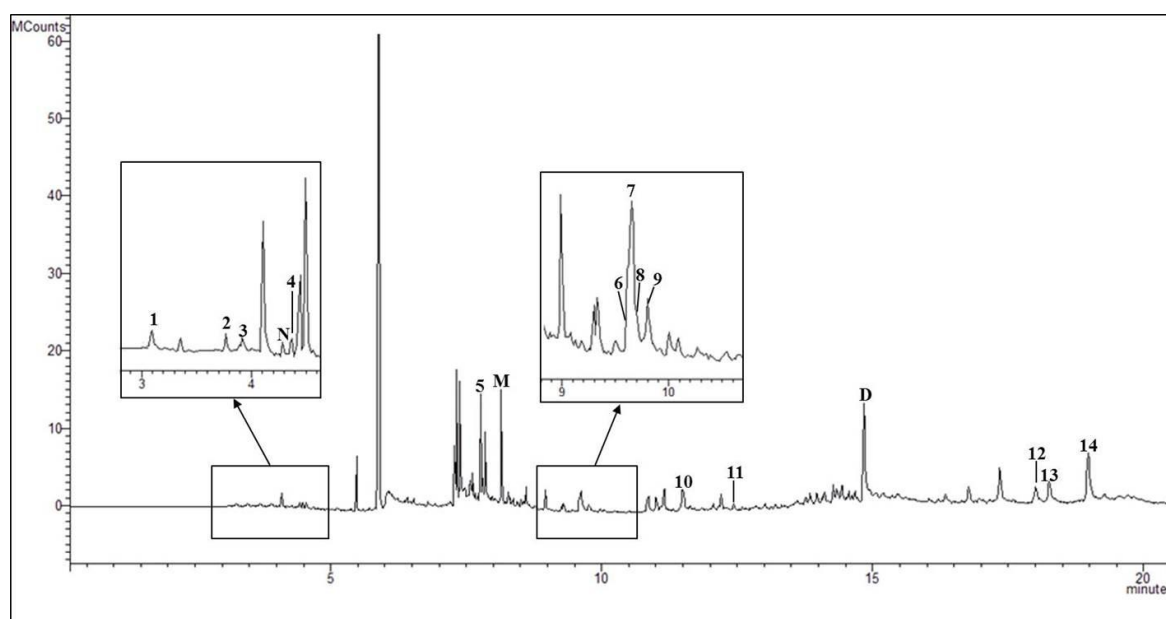


Figure 1. GC-MS profile of *P. betle* leaves ethanol extract. Peaks' identity as in Table 1. Internal standards: N, norvaline; M, methyl linolealdate; D, desmosterol

Fatty acids

The biosynthesis of fatty acids occurs in the cytosol and begins with the oxidation of a molecule of acetyl-coenzyme A (acetyl-CoA) to malonyl-coenzyme A, which reacts with another molecule of acetyl-CoA. The resulting compound suffers further condensation, reduction and dehydration reactions, originating palmitic (C16:0) or stearic acid (C18:0). These metabolites are thereafter desaturated to monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) [26].

Six fatty acids were determined in both extracts of *P. betle* leaves, five of them being fully identified. To the best of our knowledge, oleic, linoleic and linolenic acids are reported for the first time in this material. Fatty acids with monocarboxylic structures comprising C14:0 to C18:3 were found (Figure 1, Table 1). Quantitative differences were also observed and, unlike what happened with amino acids composition, the ethanol extract presented the higher fatty acid content (Table 1), which is not surprising due to the lipophilicity of these molecules. With the exception of palmitic acid, the other fatty acids were present in vestigial amounts in the aqueous extract. Both extracts are essentially constituted by SFA (ca. 91% of total fatty acids), followed by PUFA (ca. 6% of total fatty acids). Amongst SFA, palmitic acid was clearly the main compound, corresponding to more than 55% of total SFA. This compound is recognized by its antibacterial properties and by its capacity to cause apoptosis in neuroblastoma cells [27, 28].

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With respect to PUFA, linolenic acid was the main metabolite (ca. 79% of PUFA total contents) (Table 1). Due to its anti-inflammatory and anti-proliferative properties this compound has an important role in the prevention of several chronic diseases [29]. Linoleic acid, the other PUFA identified, is considered to be an essential fatty acid, as it cannot be synthesized by the human organism. This compound can be converted to hormone-like substances called eicosanoids, which affect physiological reactions ranging from blood clotting to immune response [30].

Regarding MUFA, oleic acid was the only metabolite identified in *P. betle* leaves (Table 1). This is an omega-9 fatty acid, not essential for humans, that is known for its capacity to reduce the incidence of cardiovascular diseases, as well as for its anti-diabetic and anti-inflammatory properties [31].

Sterols

Plant sterols are products of the isoprenoid biosynthetic pathway, where isopentenyl pyrophosphate serves as the fundamental building block for the biosynthesis of all sterols [32].

Four sterols were determined in both extracts of *P. betle* leaves, three of them being fully identified (Figure 1, Table 1). As far as we know, cholesterol and stigmasterol are described for the first time in this material. As it happened with fatty acids, the ethanol extract presented higher amounts of these metabolites (Table 1), which could be expected as these compounds are lipophilic molecules and have higher affinity for this solvent. β -Sitosterol and stigmasterol were the main compounds, corresponding to ca. 43 and 41% of total sterols, respectively (Table 1). These metabolites are the principal Δ^5 -sterols in several plant materials, being the most efficient compounds acting in membranes to restrict the motion of fatty acyl chains, due to their stereochemistry associated with the presence of an ethyl group at C-24 [33].

Furthermore, plant sterols are important health promoter components of the diet. In fact, due to their capacity to inhibit intestinal cholesterol absorption and effects in hepatic/intestinal cholesterol metabolism, the intake of these metabolites may cause a decrease in plasma cholesterol levels [34].

α -Glucosidase inhibitory activity

Ethanol and aqueous extracts from *P. betle* leaves showed strong capacity to inhibit α -glucosidase in a concentration-dependent way, with IC_{50} values of 0.069 and 0.257 mg/mL, respectively, the first being more effective (Figure 2). It was observed that this extract was more active than acarbose (reference compound tested under the same conditions), which showed an IC_{50} of 0.30 mg/mL. Furthermore, the extracts of *P. betle* leaves proved to be very promising in comparison with other plants analysed under the same conditions, some of them used in the treatment of this disease, as *Viscum album* L. (IC_{50} = 11.7 mg/mL) [35] and *Glycyrrhiza uralensis* Fisch. (IC_{50} = 20.1 mg/mL) [36] and *Spergularia rubra* (L.) Presl & J. C. Presl (IC_{50} = 2.55 mg/mL) [22]. According to the results found, the inhibition of α -glucosidase may be one of the mechanisms involved in the hypoglycemic effects of *P. betle*.

Table 1. Metabolites in *P. betle* leaves extracts (mg/kg of dry weight)^a.

	Primary metabolites				Secondary metabolites			
	Amino acids		Fatty acids		Sterols			
	Aqueous	Ethanol	Aqueous	Ethanol	Aqueous	Ethanol		
(1) Alanine	5244.19 ±118.45	735.39 ±25.09	(5)Palmitic acid (C16:0)	2401.92 ±79.89	3693.48±239.98	(11)Cholestanol derivative ^b	nq	157.05 ±6.19
(2) Valine	280.58 ±18.41	5.50 ±0.29	(6)Linoleic acid (C18:2)	nq	95.98 ±0.92	(12) Cholesterol	nq	95.50 ±0.01
(3) Isoleucine	413.77 ±12.95	38.54 ±2.62	(7)Linolenic acid (C18:3)	nq	338.41±4.99	(13) Stigmasterol	nq	639.34 ±1.54
(4) Proline	210.09 ±5.20	96.50 ±0.40	(8) Oleic acid (C18:1)	nq	231.31±5.64	(14) β -Sitosterol	94.56 ±9.22	681.09 ±9.82
			(9) Stearic acid (C18:0)	nq	826.84 ±9.14			
			(10)Palmitic acid derivative ^b	nq	2116.91±159.60			
Σ	6148.63	875.93	Σ	2401.92	7302.93	Σ	94.56	1572.98

^a Compounds determined as trimethylsilyl derivatives and quantification results from three determinations. Results are expressed as means \pm standard deviations. ^b Tentatively identified based on its mass fragmentation. Σ , sum of determined compounds; nq, not quantified.

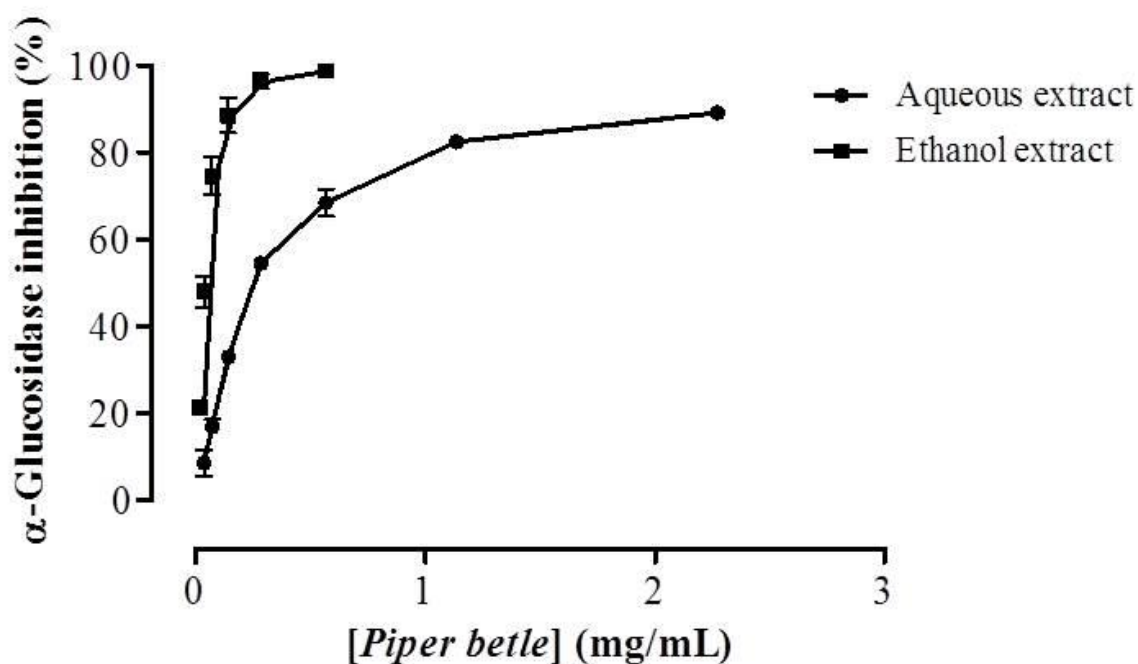


Figure 2. *In vitro* α -glucosidase inhibition of *P. betle* leaves aqueous and ethanol extracts. Values show mean \pm SEM of 3 independent experiments performed in triplicate.

The capacity of both *P. betle* leaves extracts could be related with presence of fatty acids. A study performed by Su and collaborators [37] demonstrated that unsaturated fatty acids, namely oleic, linoleic and α -linolenic acids were more active than the saturated ones (palmitic and stearic acids), the strongest activity noted with oleic acid, followed by linoleic acid. According to those authors, the presence of double bonds in the chemical structure plays a crucial role in the α -glucosidase inhibitory activity. In addition, this study also demonstrated that oleic and linoleic acids were more effective than acarbose, suggesting a competitive inhibition of α -glucosidase activity [37]. Other study performed by Artanti and colleagues [38] also demonstrated that oleic and linoleic acids were most active than the other identified fatty acids. In addition, these authors showed that the mixture of palmitic, stearic, oleic, linoleic and linolenic acids was less active than the individual effect of oleic and linoleic acids, which can suggest the existence of antagonistic effects [38].

In this context, the effect observed for *P. betle* aqueous extract could be correlated with the presence of palmitic acid (Table 1). Regarding *P. betle* ethanol extract, besides the contribution of palmitic acid, the inhibitory activity could be partially attributed to stearic, oleic, linolenic acids and, to a less extent, to linoleic acid too (Table 1).

Taking into account that extracts are complex mixtures, the existence of other metabolites non-determined in this work, namely the presence of *C*-glycosyl flavones, which were already described in *P. betle* aqueous and ethanol extracts [9], could also contribute to the observed activity. In fact, *C*-glycosyl flavones were described as α -glucosidase inhibitors, being more active than acarbose [39]. The inhibitory activity of these phenolic compounds is related with the presence of a double bond between C-2 and C-3 and with the hydroxyl group at C-5, C-3' and C-4'. Thus, α -glucosidase inhibition can be partially related

with the presence of 2"-*O*-hexosyl-6-*C*-hexosyl-luteolin, 2"-*O*-rhamnosyl-6-*C*-hexosyl-luteolin, 2"-*O*-hexosyl-8-*C*-hexosyl-apigenin and 2"-*O*-rhamnosyl-8-*C*-hexosyl-apigenin in both extracts of *P. betle* leaves [9].

In conclusion, this study provides insights about the potential of two extracts of *P. betle* leaves (aqueous and ethanol) to inhibit a key enzyme relevant to the treatment of diabetes mellitus, the ethanol one being more active than acarbose (positive control). Furthermore, this work provides qualitative and quantitative data of bioactive natural products, such as amino acids, fatty acids and sterols, giving a broader view of this herbal medicine metabolic profile. Qualitative differences were observed between the two analysed extracts. As far as we are aware, five compounds are described for the first time. Therefore, the work reported herein adds value to the species knowledge. From the above observations, it was possible to conclude that *P. betle* is a promising source of α -glucosidase inhibitory agents, which incites the use of this herbal medicine in human health applications, both in pharmaceuticals and food industries products.

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