The Antioxidant and Hypoglycemic Properties and Phytochemical Profile of Clusia latipes Extracts

Ronald Silva-Rivas1, Natalia Bailon-Moscoso2, Luis Cartuche1, Juan Carlos Romero-Benavides1,*

ABSTRACT

Introduction: The prevalence of diabetes has increased more rapidly in low and middle-income countries than in high-income countries. Type 2 diabetes mellitus (T2DM), which is the most common form of diabetes, is caused by the inefficient use of insulin in the body and is characterized by disrupted insulin action or secretion. Also, oxidative stress plays an important role in the development of disease. The goal of this study is to identify the antioxidant and hypoglycemic properties of Clusia latipes, an endemic species of Central and South America.

Methods: The antioxidant and hypoglycemic capacity of the extracts (hexane, ethyl acetate, and methanol) of the leaves and stems of Clusia latipes were evaluated. From the most potent extract, the phytochemical study was carried out and fractionated. Antioxidant activity was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), while hypoglycemic capacity was measured by alpha-glucosidase inhibition.

Results: The extracts with the highest antioxidant capacity are the extracts with the highest alpha-glucosidase inhibition activity. Inhibitory activity increased in samples extracted with medium polar (ethyl acetate) and polar (methanol) solvents. Phytochemical screening of these extracts revealed the presence of alkaloids, carbohydrates, flavonoids/xanthones, quinones, saponins, and tannins. The highest alpha-glucosidase inhibitory activity was detected in the ethyl acetate fraction obtained from leaf methanol extract, with a half-maximal inhibitory concentration (IC50) value of 0.90 μg/ml. The major constituent isolated from the same fraction was isouquercitrin.

Key words: alpha-glucosidase inhibitory activity, DPPH, Phytochemical screening, Type 2 diabetes mellitus.

INTRODUCTION

Diabetes mellitus has become one of the primary threats to human health, with its rapidly increasing prevalence, and gravely debilitating clinical complications1. Over the past decade, diabetes prevalence has risen faster in low and middle-income countries than in high-income countries. The World Health Organization (WHO) has projected that DM will be the 7th leading cause of death in 2030. Diabetes is a chronic disease that occurs when the pancreas does not produce sufficient insulin or when the body cannot effectively use the insulin it produces. Insulin is a hormone that regulates blood sugar. Hyperglycemia or high blood sugar is a common effect of uncontrolled diabetes, which might lead to serious heart, blood vessel, eye, kidney, and nerve damage over time 2,3. Type 2 diabetes mellitus (T2DM), which is the most common form of diabetes, is produced by the ineffective use of insulin in the body and is characterized by disorders of insulin action and secretion. Either one of these mechanisms could be the predominant feature, but generally, when diagnosed, both are manifested4. In T2DM, particularly, it is believed that oxidative stress caused by overproduction of reactive oxygen species (ROS) is the cause of vascular complication development5. Hyperglycemic condition of DM patients will produce tissue damage via the formation of ROS through five major mechanisms that have been shown to be activated by mitochondrial ROS overproduction; increased flux of glucose and other sugars through the polyol pathway; increased formation of advanced glycation end products; increased expression of the receptor for advanced glycation end products (RAGE) and its activating ligands; activation of protein kinase C isofoms; and over-activity of the hexosamine pathway6-9.

Alpha-glucosidase is a critical enzyme that catalyzes the cleavage of absorbable monosaccharides starting with disaccharides and oligosaccharides6. In this manner, alpha-glucosidase inhibitors reduce postprandial hyperglycemia by slowing intestinal carbohydrate digestion10. Alpha-glucosidase inhibitors are capable of suppressing postprandial hyperglycemia; they are generally used to prevent or treat type II diabetes11. Plants play an important role in health care and are an important source of potentially bioactive substances12. The genus Clusia is widely distributed in the tropical and subtropical regions of Central and South America13. In the species of this genus, a great variety of biological activities have been found: broad-spectrum antimicrobial activity; chemopreventive cancer effects and antioxidant activity14; anti-inflammatory and anti-hepatoxic activity and inhibitory action of the human immunodeficiency virus (HIV)15-16; and cytotoxic
activity\textsuperscript{17,18}. In the present study, both the antioxidant and anti-glycemic activity of leaf and stem extracts of \textit{C. latipes} were evaluated.

**MATERIALS AND METHODS**

**Plant material**

The leaves and stems of \textit{Clusia latipes} Planch. & Triana, \textit{Clusiaceae} were collected in Gonzanama-Quilanga in the Loja province of Ecuador, and species identification was made by Fani Tinitana, PhD. A voucher specimen (PPN-CI 002) was deposited at the Herbarium of the Universidad Técnica Particular de Loja, Ecuador.

**Extraction and partitions of \textit{Clusia latipes}**

For 7 days at 30°C, the plant material was dried in a tray dryer with airflow and then manually pulverized. Leaves and stems were processed separately. The pulverized leaves (1540 g) and stems (1608 g) were extracted by static maceration at ambient temperature with hexane (Hex), ethyl acetate (EtOAc), and methanol (MeOH) sequentially during three days with each solvent\textsuperscript{16}. The procedure was repeated three times and concentrated on a rotary evaporator (Buchi R210; Switzerland, Flawil) at 50 mbar and 35°C, to yield a total of six extracts: leaf extracts, 71.00 g (Hex-L), 28.52 g (EtOAc-L), and 283.87 g (MeOH-L); and stem extracts, 39.44 g (Hex-S), 44.23 g (EtOAc-S), and 117.23 g (MeOH-S).

The most active extracts were partitioned. A portion of the dried methanolic leaf (20 g) and stem (20 g) extracts were then dissolved in methanol:water 9:1 v/v and sequentially partitioned three times with 400 mL of each solvent ([Hexano (Hex), dichloromethane (DCM), and ethyl acetate (EtOAc)] using a separatory funnel at room temperature (Figure 1). The solvents were removed using a rotary evaporator (Buchi R210; Switzerland, Flawil) at 35°C under vacuum. From the Hex leaves fraction (F-Hex-L), 0.59 g was obtained. The Hex stems fraction (F-Hex-S) yielded 0.19 g, the DCM leaves fraction (F-DCM-L) 5.21 g, and the DCM stems fraction (F-DCM-S) 10.28 g, the EtOAc leaves fraction (F-EtOAc-L) 1.82 g, the EtOAc stems fraction (F-EtOAc-S) 2.02 g, the aqueous leaves fraction (F-Aq-L) 11.15 g, and the aqueous stems fraction (F-Aq-S) 6.37 g.

**Phytochemical screening**

Using the most active extracts and their partitions phytochemical tests were done. Phytochemical screening to test for the presence of secondary metabolites (alkaloids, flavonoids, quinones, saponins, tannins, and terpenoids-steroids), carbohydrates, and fats in the extracts and fractions was carried out using standard procedures. Phytochemical screening results from tests on extracts and fractions revealed the presence or absence of the main secondary metabolites and other phytochemicals based on the presence (+) or absence (−) of expected color changes. The tests performed were based on those reported in the literature\textsuperscript{19,20}.

**Isolation of secondary metabolites**

The most active sample, the EtOAc leaves fraction (1.82 g), was separated by column chromatography with C18-reversed-phase silica gel (40–63 μm; Merck) with extract:silica (g/g) proportion of 1:20, eluting with a gradient solvent system of MeOH-H\textsubscript{2}O in v/v ratios of 50:50, 60:40, 70:30, 80:20, 90:10, and 100:0 to obtain 200 eluates; according to the chromatographic profile in Thin-layer chromatography (TLC) analysis, 20 fractions were obtained (F1–F20). Fractions with a good chromatographic profile were separated by column chromatography with silica gel 60 (63-200 μm; Merck) in proportion de 1:100 (extract:silica, g/g), eluting with mixtures of three solvents EtOAc-MeOH-H\textsubscript{2}O in v/v ratios of 90:6:4, 85:10:5, 81:11:8, 77:13:10, and 74:16:10. Finally, these are purified on a column of Sephadex LH-20 (Merck) with MeOH-H\textsubscript{2}O 1:1 (v/v), obtaining a yellow amorphous solid, which was identified as isooricitrin.

**Characterization and identification of secondary metabolites**

The melting point was determined using a Fisher John apparatus (Fisher Scientific Company, USA), and the temperature was not corrected. The H\textsuperscript{1} (400 MHz) and 13C NMR (100 MHz) spectra were recorded on Varian 400 MHz-Premium Scheled equipment (Agilent Technologies, USA). CD\textsubscript{2}OD was used as the solvent, and chemical shifts were expressed in parts per million (ppm). Coupling constants (J) were reported in Hz.

**Assays for antioxidant capacity**

All extracts and fractions were evaluated for antioxidant capacity using stable free radicals of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS). DPPH radical scavenging assay

DPPH free radical scavenging capacity was evaluated with a microplate analytical assay according to the literature\textsuperscript{23,24}, with slight modifications. A 30-μl aliquot of the different sample concentrations and standard were mixed with 270 μL of DPPH\textsuperscript{−} in methanol solution 100 μM (O.D. adjusted to 1.1 at 515 nm). After incubation at 20°C for 60 min, the absorbance of each solution was measured using a microplate reader (EPOCH 2 BioTek; USA, Vermont) at 515 nm. The percentage of scavenging activity was determined by following the equation described by Cheng et al. (2006):

$$\text{SC}_{\text{sample}} = \left(1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}}\right) \times 100$$

The SC\textsubscript{sample} value was obtained through interpolation from linear or logarithmic regression analyses according to the behavior of the data, and the sample concentrations required to scavenge 50% of the DPPH radical were determined. Trolox was used as the reference compound.

**ABTS radical scavenging assay**

For the ABTS assay, the procedure followed the method of Thaipong et al. (2006) with slight modifications. From the different concentrations, a 30-μl aliquot was taken, and a 270 μL of ABTS\textsuperscript{+} solution (ABTS 7.4 mM and 2.6 mM persulfate, 1:1 ratio) was added to a 96-well microplate assay. This was incubated at 20°C for 60 min, and the absorbance of each solution was recorded at 715 nm in a microplate reader (EPOCH 2 BioTek; USA, Vermont). The percentage of ABTS\textsuperscript{+} scavenging by the sample was calculated using the Eq. (1). Trolox was used as the reference compound.
α-glucosidase inhibitory activity

Ten milligrams of each sample was dissolved in 1 ml of methanol:H_{2}O (1:1 ratio). In cases of complete inhibition, dilutions of the sample solution were made in phosphate-buffered saline (PBS; SIGMA).

The α-glucosidase enzyme inhibitory effect was determined using a 96-well microtiter plate with p-nitrophenyl-α-D-glucopyranoside (pNPG; SIGMA) as substrate according to the methods described by Tao et al. (2013), with slight modifications. First, 5 μl of the sample was mixed with 75 μl of PBS (SIGMA) and 20 μl of enzyme solution (0.15 U/ml in PBS pH 7.4; SIGMA). This mixture was then pre-incubated at 37 °C for 5 min. After preincubation, 20 μl of pNPG (5 mM in phosphate buffer, pH 7.4) was added and then incubated at 37 °C. Acarbose (5 mg/ml) was used as a positive control. The amount of p-nitrophenol (p-NP) released was measured at 405 nm for 60 min, recording the absorbance every 5 min on a spectrophotometer microplate reader (EPOCH 2 BioTek; USA, Vermont). The results were expressed as percentage inhibition using the formula previously described:

\[
\text{Inhibition} \% = \left( \frac{A_o - A_s}{A_o} \right) \times 100
\]

where \(A_o\) is the absorbance recorded for the enzymatic activity without inhibitor (control) and \(A_s\) is the absorbance recorded for the enzymatic activity in the presence of the inhibitor (sample test). The IC\(_{50}\) was calculated using GraphPad Prism v 5.0 software.

RESULTS

Regarding antioxidant capacity, both in the DPPH free radical elimination activity test and ABTS free radical test, we found that the greater the polarity of the extracts, the greater the antioxidant capacity. Thus, leaf and stem methanol extracts had SC\(_{50}\) values of 6.44 μg/ml and 6.77 μg/ml, respectively. In the ABTS free radical test, the methanolic extracts present SC\(_{50}\) values of 5.43 μg/ml for leaves and 4.59 μg/ml for stems (Table 1).

On the other hand, the α-glucosidase inhibitory activity was observed in vitro in all samples. The positive acarbose control had an IC\(_{50}\) of 377 μM (243.39 μg/ml), which was in good agreement with the results reported by Feng et al. (2011). Similar to the α-glucosidase inhibitory activity, the samples that showed high activities were of methanolic extracts of leaves and stems with IC\(_{50}\) values of 5.01 μg/ml and 2.30 μg/ml, respectively (Table 1).

Given the activity of the extracts, the most effective extracts were fractionated according to the scheme presented in Figure 1. The results obtained from the phytochemical examination revealed the presence of alkaloids, carbohydrates, flavonoids/xanthones, quinones, saponins, and tannins (Table 2).

The antioxidant capacity and α-glucosidase inhibitory activity were evaluated from the fractions of methanol extracts obtained from leaves and stem (Table 3). The antioxidant activities of the DCM, EtOAc, and aqueous leaf and stem fractions increased significantly relative to the Hex fractions. The fractions with the highest biological activity are those obtained in EtOAc (F-EtOAc), both in leaves and stems. The SC\(_{50}\) of F-EtOAc from leaves was 4.70 μg/ml, and for DPPH and 3.29 μg/ml for ABTS, thus exhibiting higher antioxidant activity for ABTS. The F-EtOAc stems had a DPPH antioxidant capacity of SC\(_{50}\): 3.58 μg/ml and ABTS of 2.27 μg/ml (Table 3).

Similar to the extracts, the fractions with the highest antioxidant capacity also have the highest α-glucosidase inhibitory activity. Thus, the F-EtOAc leaves and F-EtOAc stems exhibited potency, with IC\(_{50}\) values ranging from 0.90 to 3.88 μg/ml (Table 3).

Based on these results, the F-EtOAc leaf fraction was separated by column chromatography to obtain a flavonoid glycoside, isoquercitrin (7.3 mg); the structure is shown in Figure 2. The structural characterization of this compound was carried out by spectroscopic and spectrometric analyses and by comparison with published data.

Table 1: DPPH and ABTS free radical-scavenging activity and α-glucosidase inhibitory activity of Clusia latipes extracts.

<table>
<thead>
<tr>
<th>Morphological structure</th>
<th>Extracts</th>
<th>DPPH</th>
<th>ABTS</th>
<th>α-glucosidase inhibitory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SC(_{50}) (μg/ml)</td>
<td>IC(_{50}) (μg/ml)</td>
<td>SC(_{50}) (μg/ml)</td>
</tr>
<tr>
<td>LEAVES</td>
<td>Hex</td>
<td>&gt;100</td>
<td>64.80 ± 0.21</td>
<td>231.00 ± 0.75</td>
</tr>
<tr>
<td></td>
<td>EtOAc</td>
<td>85.53 ± 0.88</td>
<td>42.45 ± 0.52</td>
<td>33.96 ± 1.15</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>6.44 ± 0.52</td>
<td>5.43 ± 0.30</td>
<td>5.01 ± 0.75</td>
</tr>
<tr>
<td></td>
<td>Hex</td>
<td>&gt;100</td>
<td>52.06 ± 0.44</td>
<td>177.40 ± 0.48</td>
</tr>
<tr>
<td></td>
<td>EtOAc</td>
<td>46.93 ± 0.69</td>
<td>25.81 ± 0.45</td>
<td>5.80 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>6.77 ± 0.59</td>
<td>4.59 ± 0.34</td>
<td>1.30 ± 0.15</td>
</tr>
</tbody>
</table>

Hex = hexane; EtOAc = ethyl acetate; MeOH = methanol. All values were expressed as means ± standard error (n = 3).

Table 2: Phytochemical screening of methanolic extracts of C. latipes leaves and stems and their fractions.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>LEAVES</th>
<th>STEMS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MeOH</td>
<td>F-Hex</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fats</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids/ Xanthones</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Quinones</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = presence, - = absence
Table 3: DPPH and ABTS free radical-scavenging activity and α-glucosidase inhibitory activity of fractions obtained from methanolic extracts of C. latipes leaves and stems.

<table>
<thead>
<tr>
<th>Morphological structure</th>
<th>Fractions of methanol extracts</th>
<th>DPPH SC_{50} μg/ml</th>
<th>ABTS SC_{50} μg/ml</th>
<th>α-glucosidase inhibitory IC_{50} μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEAVES</td>
<td>F-Hex</td>
<td>&gt;100</td>
<td>63.68 ± 0.77</td>
<td>102.80 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>F-DCM</td>
<td>7.27 ± 0.70</td>
<td>5.74 ± 0.38</td>
<td>174.96 ± 1.02</td>
</tr>
<tr>
<td></td>
<td>F-EtOAc</td>
<td>4.70 ± 0.80</td>
<td>3.03 ± 0.35</td>
<td>0.90 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>F-Aq</td>
<td>6.52 ± 0.53</td>
<td>5.03 ± 0.68</td>
<td>3.04 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>F-Hex</td>
<td>&gt;100</td>
<td>70.16 ± 0.57</td>
<td>128.98 ± 0.71</td>
</tr>
<tr>
<td>STEMS</td>
<td>F-DCM</td>
<td>6.86 ± 0.48</td>
<td>3.97 ± 0.51</td>
<td>110.85 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>F-EtOAc</td>
<td>3.58 ± 0.50</td>
<td>2.27 ± 0.51</td>
<td>3.88 ± 0.81</td>
</tr>
<tr>
<td></td>
<td>F-Aq</td>
<td>4.20 ± 0.43</td>
<td>2.75 ± 0.33</td>
<td>2.98 ± 0.13</td>
</tr>
</tbody>
</table>

All values were expressed as means ± standard error (n = 3).

CONCLUSION

The highest α-glucosidase inhibitory activity was detected in the ethyl acetate fraction obtained from leaf methanol extract, with a half-maximal inhibitory concentration (IC_{50}) value of 0.90 μg/ml. In this study, we observed an association between α-glucosidase content and the antioxidant activities of the C. latipes fractions.

We propose that the α-glucosidase inhibitory and antioxidant activities of C. latipes could be due to the presence of isoquercitrin.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENT

The authors would like to thank James Salcedo-Ramírez for the contribution to the development of the tests for biological evaluation. This work has been financed by Universidad Técnica Particular de Loja (Grant PROY_CCSAL_1266).

REFERENCES

Silva-Rivas, et al.: The Antioxidant and Hypoglycemic Properties and Phytochemical Profile of Clusia latipes Extracts

GRAPHICAL ABSTRACT

**SUMMARY**

All the data shown revealed the antioxidant and hypoglycemic capacity of the extracts of the leaves and stems of *Clusia latipes*. The highest α-glucosidase inhibitory activity was detected in the ethyl acetate fraction; with a half-maximal inhibitory concentration (IC₅₀) value of 0.90 μg/ml. Isoquercitrin was isolated from the fraction more active.

ABOUT AUTHORS

**Ronald Silva-Rivas**, He has a degree in Chemistry and Biology and also a master’s degree in Chemistry Applied by the Universidad Tecnica Particular de Loja.

**Natalia Bailon-Moscoso Ph.D.** She is currently a Professor of Genetic, Medical Genetic and Molecular Pathology and Researcher in the Department of Health at Universidad Tecnica Particular de Loja, Ecuador, since October 2007. She is the Head of the Biomedicine and Environmental. Her interests are focused on the study of various chronic diseases (cancer, inflammation, metabolic syndrome) and how natural products could contribute to their treatment.

**Dr. Luis Cartuche** is currently the Head of the Bioassay laboratory, of the Chemistry Department at Universidad Tecnica Particular de Loja, Ecuador. He has over 10 years of experience in teaching Biochemistry. He is specialized in chemistry of natural products with a special interest in secondary metabolites isolated from marine actinobacteria and also, he conducts a battery of in vitro bioassays to determine antimicrobial and enzyme inhibition properties from natural matrices. He has published more than 16 scientific articles.

**Juan Carlos Romero-Benavides Ph.D.** He is currently Professor of Organic Chemistry and Chemistry of Natural Products and Researcher in the Department of Chemistry at Universidad Tecnica Particular de Loja, Ecuador, since October 2002. He is the Head of the Natural Products: cancer and parasitism research group (PNCyP). His main research interests focus on natural products (biochemistry and chemistry), synthetic methodology and natural product derivatization with the goal of discovering leads from natural sources and optimization of novel anti-inflammatory, anticancer and anti-parasitic drugs.