

Postprint: Study on In Vitro Hypoglycemic Activity of Different Polarity Extracts from *Pyrrosia*

Authors: Zhuangyuan Cup, Ling Meidi, Wei Aihong, Li Rongdi, Liu Xiaomin, Nie Hua, Zhang Shengyuan

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Abstract

Using different polar extracts of the 95% ethanol extract from *Pyrrosia lingua* as test materials, with acarbose as a positive control, the pNPG (p-nitrophenyl- α -D-glucopyranoside, pNPG) and DNS (3,5-dinitro salicylic acid, DNS) methods were employed to evaluate the α -glucosidase and α -amylase inhibitory activities of these extracts. Enzyme kinetic methods and Lineweaver-Burk plot analysis were further used to determine the inhibition type of the most active extract against α -glucosidase, aiming to provide a scientific basis for the further development and utilization of *Pyrrosia lingua*. The results showed that the aqueous extract exhibited the strongest inhibitory activity against both α -glucosidase and α -amylase, with IC₅₀ values of $(4.71 \pm 0.72) \text{ g} \cdot \text{mL}^{-1}$ and $(48.40 \pm 0.32) \text{ g} \cdot \text{mL}^{-1}$, respectively, and was significantly stronger than other extracts ($P < 0.05$). Its inhibitory effect on α -glucosidase was stronger than that of acarbose, while its effect on α -amylase was weaker. The IC₅₀ values of acarbose against α -glucosidase and α -amylase were $(2,857.36 \pm 1.35) \text{ g} \cdot \text{mL}^{-1}$ and $(16.41 \pm 0.63) \text{ g} \cdot \text{mL}^{-1}$, respectively. Enzyme kinetic studies revealed that the aqueous extract produced reversible inhibition against α -glucosidase, and Lineweaver-Burk plots indicated competitive inhibition. In summary, the aqueous extract of *Pyrrosia lingua* demonstrates potent α -glucosidase and α -amylase inhibitory activities, representing a natural source of α -glucosidase inhibitors with minimal gastrointestinal side effects.

Full Text

Preamble

Study on the In Vitro Hypoglycemic Activity of Different Polar Extracts from *Pyrrosia lingua*

ZHUANG Yuanbei¹², LING Meidi², WEI Aihong¹², LI Rongdi¹², LIU Xiaomin¹², NIE Hua¹², ZHANG Shengyuan^{12*}

- (1. Guangdong Provincial Key Laboratory of Conservation and Precision Utilization of Characteristic Agricultural Resources in Mountainous Areas, Jiaying University, Meizhou 514015, Guangdong, China;
2. Institute of Hakka Medicinal Bio-resources, Medical College, Jiaying University, Meizhou 514031, Guangdong, China)

Abstract

This study investigated the α -glucosidase and α -amylase inhibitory activities of different polar extracts from the 95% ethanol extract of *Pyrrosia lingua* using the pNPG (p-nitrophenyl- α -D-glucopyranoside) and DNS (3,5-dinitrosalicylic acid) methods, with acarbose as the positive control. The inhibition type of the most active extract against α -glucosidase was analyzed using enzyme kinetics and Lineweaver-Burk plots to provide a scientific basis for further development and utilization of *P. lingua*. The results showed that the water extract exhibited the strongest inhibitory effect on both α -glucosidase and α -amylase, with IC_{50} values of $(4.71 \pm 0.72) \text{ g} \cdot \text{mL}^{-1}$ and $(48.40 \pm 0.32) \text{ g} \cdot \text{mL}^{-1}$, respectively, which were significantly higher than those of other extracts ($P < 0.05$). The water extract's α -glucosidase inhibition was stronger than acarbose, while its α -amylase inhibition was weaker than acarbose. The IC_{50} values of acarbose against α -glucosidase and α -amylase were $(2,857.36 \pm 1.35) \text{ g} \cdot \text{mL}^{-1}$ and $(16.41 \pm 0.63) \text{ g} \cdot \text{mL}^{-1}$, respectively. Enzyme kinetic studies revealed that the water extract produced reversible inhibition of α -glucosidase, and Lineweaver-Burk analysis indicated competitive inhibition. These findings demonstrate that the water extract of *P. lingua* possesses potent α -glucosidase and α -amylase inhibitory activities, representing a natural source of α -glucosidase inhibitors with minimal gastrointestinal side effects.

Keywords: *Pyrrosia lingua*, α -glucosidase, α -amylase, acarbose, extract

Introduction

In 2019, approximately 463 million people aged 20–79 worldwide had diabetes, with about 116.4 million in China alone (International Diabetes Federation, 2019). Diabetes has become the third most serious disease threatening human health (Yang WY, 2018). Sustained postprandial hyperglycemia is a major cause of vascular endothelial damage and retinopathy complications in diabetic patients, and effective control of postprandial blood glucose elevation is crucial for preventing diabetes and its complications (Chinese Diabetes Society, 2018). Drugs targeting α -glucosidase and α -amylase, such as acarbose and voglibose, have become first-line clinical treatments for reducing postprandial glucose. However, studies have shown that long-term use of acarbose, due to its strong inhibition of α -amylase, often causes gastrointestinal side effects including flatulence and abdominal discomfort (Su Q, 2018; Yuan XH et al., 2016).

This has drawn medical researchers' attention to finding novel, highly effective α -amylase and α -glucosidase inhibitors with fewer side effects from traditional Chinese medicine and natural sources, yielding a series of research achievements in recent years (Fan L et al., 2016).

Pyrrosia lingua (Thunb.) Farwell, belonging to the family Polypodiaceae and genus *Pyrrosia*, is known by the aliases "Shi Jian" and "Shi Wei." It is mainly produced in Guangdong, Fujian, Taiwan, and other regions, and is traditionally used to treat urinary tract infections, hematuria, urinary stones, and nephritis (National Pharmacopoeia Commission, 2015). *P. lingua* is a commonly used folk herbal medicine for diabetes prevention and treatment (Zhou Y, 2016), and modern pharmacological studies have confirmed its effects on blood glucose and glucose tolerance in mice (Wang B and Huang CG, 2008) and its ability to improve diabetic nephropathy (Liu XL et al., 2018). Furthermore, our research group previously conducted preliminary screening for in vitro hypoglycemic activity of characteristic and advantageous medicinal plants in the Hakka region of Meizhou, finding that *P. lingua* exhibited promising in vitro hypoglycemic effects. To further clarify the inhibitory effects and mechanisms of *P. lingua* on α -glucosidase and α -amylase, this study used acarbose as a positive control and pNPG and starch as substrates to evaluate the inhibitory activities of different polar extracts from the 95% ethanol extract of *P. lingua* against α -glucosidase and α -amylase. The inhibition type against α -glucosidase by the most active extract was determined through enzyme kinetics and Lineweaver-Burk analysis, providing a scientific basis for further research on the chemical constituents and pharmacodynamics of *P. lingua*'s hypoglycemic effects.

Materials and Methods

Plant Material

Pyrrosia lingua was collected in April 2016 from Yinna Mountain Nature Reserve in Meizhou City, Guangdong Province, and identified by Associate Professor ZHANG Shengyuan from the Institute of Hakka Medicinal Bio-resources at Jiaying University Medical College as the perennial herb *Pyrrosia lingua* (Thunb.) Farwell belonging to Polypodiaceae family and *Pyrrosia* genus.

Preparation of Different Polar Extracts from *P. lingua*

Two kilograms of *P. lingua* were dried at 50 °C, pulverized, and passed through a 40-mesh sieve. The material was extracted with 2 volumes of 95% ethanol via ultrasonic extraction for 30 min (200 W, 40 kHz, 45 °C), repeated three times, and concentrated to obtain an extract (230 g). Two hundred grams of the extract were suspended in distilled water and sequentially extracted with different solvents to yield petroleum ether extract (25 g), ethyl acetate extract (66 g), n-butanol extract (53 g), and water extract (50 g).

α -Amylase Inhibitory Activity

The DNS method described by Ding HJ et al. (2020) was used to determine the α -amylase inhibitory activity of different polar extracts, with three replicates and acarbose as the positive control.

α -Glucosidase Inhibitory Activity

The pNPG method described by Zhang SY et al. (2018) was used to determine the α -glucosidase inhibitory activity of different polar extracts, with three replicates and acarbose as the positive control.

Kinetic Studies of α -Glucosidase Inhibition

Determination of α -Glucosidase Activity and Reaction Time Following the method of Wen ZH et al. (2019), the initial reaction rates of $4.0 \text{ mmol} \cdot \text{L}^{-1}$ pNPG solution were measured at different enzyme activities (0.02, 0.04, 0.06, 0.08, 0.10, $0.12 \text{ U} \cdot \text{mL}^{-1}$). Time was plotted on the x-axis and pNP peak area on the y-axis.

Investigation of α -Glucosidase Inhibition Type Referring to methods by Lai XY et al. (2016) and Li B et al. (2015), the initial reaction rates were measured at sample concentrations of 0, 5, and $7 \text{ g} \cdot \text{mL}^{-1}$ with pNPG solution at $4.0 \text{ mmol} \cdot \text{L}^{-1}$ and α -glucosidase activities of 0.06, 0.10, 0.14, 0.18, and $0.22 \text{ U} \cdot \text{mL}^{-1}$. Enzyme activity was plotted on the x-axis and initial reaction rate on the y-axis to determine reversible or irreversible inhibition.

Reversible inhibition includes four types: competitive, non-competitive, uncompetitive, and mixed inhibition (Song F et al., 2019). The initial reaction rates were measured at sample concentrations of 0, 5, and $7 \text{ g} \cdot \text{mL}^{-1}$ with $0.1 \text{ U} \cdot \text{mL}^{-1}$ α -glucosidase and pNPG concentrations of 1, 2, 4, 8, and $16 \text{ mmol} \cdot \text{L}^{-1}$. The reciprocal of pNPG concentration was plotted on the x-axis and the reciprocal of initial reaction rate on the y-axis to generate Lineweaver-Burk plots for determining the specific inhibition type.

Statistical Analysis

All experiments were performed in triplicate. Data were processed using Origin 8.5 software, and results are expressed as mean \pm standard deviation ($\bar{x} \pm s$). The corresponding half-maximal inhibitory concentrations (IC_{50}) were calculated. One-way ANOVA and Duncan's test were used to examine significant differences among multiple groups ($P < 0.05$ or $P < 0.01$).

Results

α -Amylase Inhibitory Activity of *P. lingua* Extracts

As shown in Figure 1 [Figure 1: see original paper], different polar extracts of *P. lingua* and acarbose exhibited dose-dependent inhibitory effects on α -amylase, with inhibition rates increasing with concentration. Among the different polar extracts, the water extract showed the strongest α -amylase inhibitory activity, with an IC_{50} value of $(48.40 \pm 0.32) \text{ g} \cdot \text{mL}^{-1}$, which was significantly lower than that of n-butanol extract $(530.89 \pm 6.95) \text{ g} \cdot \text{mL}^{-1}$, ethyl acetate extract $(4,929.36 \pm 1.56) \text{ g} \cdot \text{mL}^{-1}$, and petroleum ether extract $(50,148.70 \pm 1.94) \text{ g} \cdot \text{mL}^{-1}$ ($P < 0.05$).

α -Glucosidase Inhibitory Activity of *P. lingua* Extracts

As shown in Figure 2 [Figure 2: see original paper], different polar extracts of *P. lingua* and acarbose demonstrated dose-dependent inhibitory effects on α -glucosidase, with inhibition rates increasing with concentration. Among the different polar extracts, the water extract exhibited the strongest α -glucosidase inhibitory activity, with an IC_{50} value of $(4.71 \pm 0.72) \text{ g} \cdot \text{mL}^{-1}$, which was significantly lower than that of n-butanol extract $(25.35 \pm 0.12) \text{ g} \cdot \text{mL}^{-1}$, ethyl acetate extract $(193.77 \pm 1.81) \text{ g} \cdot \text{mL}^{-1}$, petroleum ether extract $(388.48 \pm 4.71) \text{ g} \cdot \text{mL}^{-1}$, and acarbose $(2,857.36 \pm 1.35) \text{ g} \cdot \text{mL}^{-1}$ ($P < 0.05$).

Inhibition Type of *P. lingua* Water Extract on α -Glucosidase

Determination of α -Glucosidase Activity and Reaction Time As shown in Figure 3 [Figure 3: see original paper], within the reaction time range of 0-10 min, the enzymatic reaction progress curves for enzyme activities of $0.02\text{-}0.12 \text{ U} \cdot \text{mL}^{-1}$ showed good linear relationships (linear fitting of each curve from 0-10 min yielded R^2 values of 0.999), indicating a constant rate. Therefore, a sampling time point of 10 min was established for initial rate determination.

Determination of Reversible or Irreversible Inhibition Type As shown in Figure 4 [Figure 4: see original paper], the straight lines obtained at water extract concentrations of 0, 5, and $7 \text{ g} \cdot \text{mL}^{-1}$ all passed through the origin, with decreasing slopes as the water extract concentration increased, indicating reduced initial reaction rates. This pattern represents typical reversible inhibition characteristics, confirming that the water extract of *P. lingua* produces reversible inhibition of α -glucosidase.

Determination of Competitive, Non-competitive, Uncompetitive, or Mixed Inhibition Type As shown in Figure 5 [Figure 5: see original paper], the three lines intersected on the y-axis, with a constant maximum reaction rate (V_{\max}) of $0.0066 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$. The slope (Michaelis constant K_m)

increased with inhibitor concentration, representing typical competitive inhibition. This indicates that the water extract of *P. lingua* exerts competitive inhibition against α -glucosidase.

Discussion and Conclusion

Pyrrrosia lingua is a commonly used folk herbal medicine for diabetes prevention and treatment (Zhou Y, 2016). Modern pharmacological studies have demonstrated its effects on blood glucose and glucose tolerance in mice (Wang B and Huang CG, 2008) and its ability to improve diabetic nephropathy (Liu XL et al., 2018). However, no reports have investigated whether its mechanism involves inhibiting α -glucosidase and α -amylase. This study is the first to reveal that different solvent extracts from the 95% ethanol extract of *P. lingua* possess potent inhibitory activities against α -glucosidase and α -amylase, enriching the understanding of *P. lingua*'s hypoglycemic targets.

Currently, clinically used α -glucosidase inhibitors often cause gastrointestinal side effects due to excessive α -amylase inhibition (Li B et al., 2015), and research suggests that this inhibitory potency is related to the number of glucose molecules connected to the pseudodisaccharide structure (Gu JF and Chen ZJ, 2009). This study systematically evaluated the in vitro α -glucosidase and α -amylase inhibitory activities of different solvent extracts from *P. lingua* 95% ethanol extract. The results showed that the inhibitory potency of all extracts against both enzymes followed the order: water extract > n-butanol extract > ethyl acetate extract > petroleum ether extract ($P < 0.05$). The most active water extract exhibited competitive inhibition against α -glucosidase, identical to the positive control acarbose. This may be related to the presence of pseudodisaccharide structures in *P. lingua* that are necessary for inhibiting α -glucosidase and α -amylase, similar to acarbose. Such structures consist of cyclitols and amino sugars and are polar oligosaccharide analogs (Gu JF and Chen ZJ, 2009). However, whether *P. lingua*'s in vivo α -glucosidase inhibition mechanism is identical to that of acarbose requires further investigation. Additionally, the stronger α -glucosidase inhibition and weaker α -amylase inhibition observed with *P. lingua* extracts may be attributed to differences in the number of glucose molecules connected to the active compounds' structures compared to acarbose.

This study reveals that among different solvent extracts from *P. lingua* 95% ethanol extract, the water extract exhibits the strongest α -glucosidase and α -amylase inhibitory activities, with reversible competitive inhibition against α -glucosidase. These findings indicate that the high-polarity chemical fraction of *P. lingua* is rich in active components that inhibit α -glucosidase and α -amylase, representing a natural source of α -glucosidase inhibitors with minimal gastrointestinal side effects. However, the specific active constituents responsible for inhibiting α -glucosidase and α -amylase, their in vivo and in vitro mechanisms of action, and structure-activity relationships remain unclear. Future research should focus on systematic investigation of the chemical constituents, mecha-

nisms of action, and structure-activity relationships of the high-polarity fraction to elucidate the scientific basis of *P. lingua*'s hypoglycemic effects.

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