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<th>Journal:</th>
<th>Songklanakarin Journal of Science and Technology</th>
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<td>Manuscript ID</td>
<td>SJST-2018-0092.R2</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Original Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>16-Aug-2018</td>
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<td>Complete List of Authors:</td>
<td>Falah, Syamsul; Institut Pertanian Bogor Fakultas Matematika dan Ilmu Pengetahuan Alam, Biochemistry Prabowo, Ahmad; Institut Pertanian Bogor Fakultas Matematika dan Ilmu Pengetahuan Alam, Biochemistry Ichsan, Sitha; Bogor Agricultural University, Biochemistry Suminto, Syaefudin; Institut Pertanian Bogor Fakultas Matematika dan Ilmu Pengetahuan Alam, Biochemistry</td>
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<td>Keyword:</td>
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Original Article

A Preliminary Study on α-Glucosidase Inhibitory and Antidiabetic Activity of Indonesia Toona sinensis Bark Extract in Alloxan-Induced Diabetic Rats

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A Preliminary Study on α-Glucosidase Inhibitory and Antidiabetic Activity of Indonesia Toona sinensis Bark Extract in Alloxan-Induced Diabetic Rats

Abstract

This study aimed to determine the antidiabetic activity of Toona sinensis bark extract. The inhibitory activity of α-glucosidase was measured using a spectrophotometer, and its antidiabetic activity was determined in vivo. Phytochemical analysis showed that ethanol and water extracts contained flavonoids, saponins, and phenolic hydroquinones. In addition, alkaloids and tannins were found in the ethanol extract. Inhibition of α-glucosidase activity showed that the half maximal inhibitory concentration value for the ethanol and water extract was 0.60 µg/ml and 3.60 µg/ml, respectively. Blood analysis revealed that a dose of 150 mg/kg body weight (BW) ethanol extract reduced blood glucose level by 70.8%. Meanwhile, glibenclamide (0.25 mg/kg BW) and 300 mg/kg BW ethanol extract decreased the level by 69% and 52%, respectively. We concluded that ethanol extract of T. sinensis is more potential as herbal remedy at a dose of 150 mg/kg BW than at 300 mg/kg BW.

Keywords: alloxan, antidiabetic, α-glucosidase, diabetic rats, Toona sinensis

1. Introduction

Diabetes mellitus (DM) is a chronic disease caused by inherited and/or acquired deficiency in insulin production by the pancreas, or by the ineffectiveness of the produced insulin (Nagappa, Thakurdesai, Rao, & Singh, 2003). The two main types of
diabetes are type 1 and type 2. Type 1 diabetes is characterized by an absolute
deficiency of insulin secretion, associated with auto-immune destruction of pancreatic
cells; it is more likely to occur in family members of affected patients (Bottini, Vang,
Cucca, & Mustelin, 2006). Type 2 diabetes, accounting for more than 90% of cases, is
cased by resistance to insulin's action combined with impaired insulin secretion
(Warren, 2004).

DM can be treated by anti-diabetic drugs, some of which are derived from plants
and spices that have antioxidant and antidiabetic activities (Minaiyan, Ghannadi,
Mohavedian, & Hakim-Elahi, 2014; Rates, 2001; Zolfaghari, Shokoohinia, Sadeghi,
Mahmoudzadeh, & Minaiyan, 2012). In Indonesia, alternative medications used locally
are usually derived from herbaceous plants. One of these plants that may have potential
for drug development is *T. sinensis* (Meliaceae), which is widely distributed in
Southeast Asia (Edmonds & Staniforth, 1998). All parts, including seeds, bark, root
bark, petioles and leaves, are claimed to have medicinal efficacy (Cho et al., 2003a,
2003b). *T. sinensis* leaves have been used to treat enteritis, dysentery, metabolic
diseases, general infections and itching (Perry, 1980). The bark is used as an astringent
and depurative agent, the powdered root is used as a corrective, and the fruits are used
to treat eye infections (Perry, 1980).

Previous reports have demonstrated that *T. sinensis* leaf extracts have multiple
applications, including in anti-proliferation of human lung adenocarcinoma cells (A549)
(Chang, Hung, Huang, & Hsu, 2002), hypoglycemic effects (Chang et al., 2002; Fan et
al., 2007), treatment of diabetes-associated high blood pressure (Yang, Hwang, & Hong,
2003), augmenting uptake of glucose in 3T3-L1 adipocytes (Hseu et al., 2008; Hsu,
Yang, Hwang, & Hong, 2003), and antioxidant activities using different antioxidant
models (Hseu et al., 2008). The highest dose tested (5.0 g/kg BW) did not show an acute lethal effect in mice (Liao et al., 2007).

All previous studies report on plants cultivated in Taiwan or China. The potential of *T. sinensis* from Indonesia, especially of its bark as an antidiabetic agent, has not yet been studied. This is important because environmental conditions and geographic variations are known to affect the chemical composition of plants (Figueiredo, Barroso, Pedro, & Scheffer, 2008). Here, we report the phytochemical components, α-glucosidase inhibitory activity, and antidiabetic activity of Indonesian *T. sinensis* bark extract using alloxan-induced diabetic Sprague–Dawley rats as a bioassay.

2. Materials and Methods

2.1 Plant material

Bark was stripped from *T. sinensis* trunk, collected from Sumedang (6°51′35″S, 107°55′15″E; altitude 650 m), West Java, Indonesia in March 2011. The plant was identified and deposited by Department of Forest Engineering, School of Life Sciences and Technology, Institut Teknologi Bandung, Indonesia with voucher specimen number SF.03.2011. The chipped bark (5000 g) was dried at 50°C until the moisture content was <10%; then it was ground in a Wiley mill (Thomas Scientific, New Jersey, USA). The resultant meal was sieved through 40- and 80-mesh screens.

2.2 Extraction of *T. sinensis* bark

The bark meal (1.5 kg air dried) was macerated three times with 70% (v/v) ethanol (Sigma-Aldrich, Darmstadt, Germany) at room temperature for 48 h (Ningappa, Dinesha, & Srinivas, 2008). An aqueous extract was produced by heating a mixture of
bark powder and water (1:10) at 100°C for 4 h. The obtained extracts were filtered and concentrated using a rotary evaporator (Eyela N-1100, Tokyo, Japan) at 40°C. The crude extracts were used in our biological assays. Water and 70% (v/v) ethanol were used as solvents for safety purpose in the medical human application.

2.3 Qualitative phytochemical analyzes

The extracts were screened for the presence of secondary metabolites such as alkaloids, saponins, flavonoids, phenolic hydroquinones, triterpenoids, and tannins. All solvents used were analytical grade (Merck, Darmstadt, Germany). Phytochemical contents were detected qualitatively by using Harborne's procedures (1987) as follows.

Alkaloids. The extract (100 mg) was combined with 3 ml of chloroform and three drops (ca. 150 µl) of ammonia. The chloroform fraction was separated and acidified with 10 drops (500 µl) of H₂SO₄ (2.0 M). Three H₂SO₄ fraction samples were each combined with Dragendorff, Meyer, or Wagner reagents. The presence of alkaloid was indicated by formation of a white precipitate upon addition of the Meyer reagent, an orange precipitate with Dragendorff reagent, and a brown precipitate with Wagner reagent.

Saponins. Aliquots of 100 mg extract were added to 2 ml of H₂O and heated for 5 min. The mixtures were cooled and then stirred for >10 minutes. Appearance of foam in more than 30 minutes indicated the presence of saponins.

Flavonoids. Aliquots of 100 mg extract were soaked with 2 ml of 30% (v/v) methanol, heated, and then filtered. Filtrates were combined with 1 drop (50 µl) of concentrated H₂SO₄; a red color indicated the presence of flavonoids.

Phenolic hydroquinone. Aliquots of 100 mg extract were soaked with 2 ml of 30%
(v/v) methanol, heated, and then filtered. The filtrates were combined with 1 drop (ca. 50 µl) of NaOH 10% (w/v); formation of red color indicated the presence of phenolic hydroquinones.

Triterpenoids. Aliquots of 100 mg extract were combined with 2 ml of 30% ethanol, heated and filtered. The filtrates were evaporated and then diethyl ether was added. Liebermann–Burchard reagent [3 drops (ca. 150 µl) of acetic acid anhydride and 1 drop (ca. 50 µl) of concentrated H₂SO₄] was added to the ether layer. A reddish-violet pigment indicated presence of triterpenoids.

Tannins. Aliquots of 100 mg extract were combined with 2 ml of H₂O and heated for 5 minutes. The mixtures were filtered and the filtrates combined with FeCl₃ 1% (b/v). The presence of tannins was indicated by the formation of dark-blue or greenish-black color.

2.4 α-Glucosidase inhibition assay

The α-glucosidase inhibition assay was performed as described previously (Sancheti, Sancheti, Bafna, & Seo, 2011) using an ELISA test kit (Bio-Rad, Singapore). Acarbose was used as a standard or positive control in a series of concentrations (2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 µg/ml). A standard solution, a blank, and the sample concentrations of 12.5, 6.2, 3.1, and 1.5 µg/ml were placed into 50 µl capacity microplate wells (96-well microplate type, Bio-Rad, Singapore). To each of the wells, 50 µl of 100 mM phosphate buffer (pH 7.0) was added. All chemical reagents were purchased from Merck (Darmstadt, Germany). A total of 25 µl of α-glucosidase at a concentration of 1.0 mg/ml in 100 mM phosphate buffer (pH 7.0) was placed into the microplate wells. The enzyme substrate that contained of 50 µl of 100 mM phosphate
buffer (pH 7.0) and 25 µl of 500 µM 4-nitrophenyl α-D-glucopyranoside (p-NPG) in 100 mM phosphate buffer (pH 7.0), was added to start the assay. All the treatments were incubated at 37°C for 30 min. The enzyme reaction was stopped by adding 100 µl of 200 mM Na$_2$CO$_3$. All tests were replicated three times. The reaction product was measured with a microplate reader (Bio-Rad, Singapore) at 400 nm. The percentage inhibition was then calculated to determine the half maximal inhibitory concentration value ($IC_{50}$) value, as follows:

\[
\% \text{ inhibition} = \left[1 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of positive control}}\right)\right] \times 100
\]

2.5 Tested animals

Male Sprague–Dawley rats (250–350 g) obtained from the Faculty of Veterinary Medicine, Bogor Agricultural University, Indonesia were fed with a standard laboratory diet and distilled water ad libitum for an acclimatization period of 2 months until the age of 3.5–4 months. All animal experiments were approved by the ethics committee of the animal laboratory, Department of Biochemistry, Bogor Agricultural University, and performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Body weight of the rats was measured on the day 4, 7, 10, and 14 before treatment with alloxan.

2.6 Experimental design

The animals were randomly divided into five groups with four rats in each group. Group A, comprising of normal rats, was intraperitoneally administered NaCl 0.9% (w/v) (Merck, Darmstadt, Germany) and orally administered distilled water 1.0 ml daily for 14 days. Group B, comprising of diabetic control rats, was orally administered distilled
water 1.0 ml daily for 14 days. Group C, comprising of diabetic rats, was orally administered glibenclamide (0.25 mg/kg) daily for 14 days. Group D comprised of diabetic rats that were orally administered ethanol extract (150 mg/kg) daily for 14 days. Group E included diabetic rats that were orally administered ethanol extract (300 mg/kg) daily for 14 days.

Alloxan (Sigma-Aldrich, Darmstadt, Germany) 150 mg/kg was injected intraperitoneally in rats from groups B–E on the first day. Treatment with the extracts and glibenclamide was started 48 h after alloxan injection. Blood samples were obtained from the tail vein of rats fasting for 16 h; blood glucose levels were measured using an Accu-Check® glucometer (Miles Inc, New York, USA). Fasting blood glucose and body weight were measured on days 0, 4, 7, 10, and 14 after induction (Cing, 2010).

2.7 Histopathology

Necropsies were conducted at the Laboratory of Histopathology, Faculty of Veterinary Medicine, Bogor Agricultural University, Indonesia, whereas the results were analyzed at the Veterinary Research Institute (Balitvet), Bogor, Indonesia. All animals were sacrificed by cervical dislocation on day 14. Pancreases were excised, isolated, and subjected to histopathological studies and microscopy (Bansal et al., 2012). Pancreatic tissues were immediately removed and washed with ice-cooled saline, and then fixed in 10% (v/v) of neutral formalin (Merck, Darmstadt, Germany). Sections were stained in haemotoxylin (Sigma-Aldrich, Darmstadt, Germany) and eosin (Sigma-Aldrich, Darmstadt, Germany), mounted and observed under a microscope (CX-21 Halogen Olympus, Tokyo, Japan).
2.8 **Statistical analysis**

This study used a completely randomized design with five treatment groups and four replications. As a measure of inhibitory activity, the concentrations required for IC$_{50}$ of α-glucosidase's activity were determined. Values reported are the mean of five experiments ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA, PASW Statistics 18.0.0, Hong Kong). Duncan’s test was used for multiple comparisons. The values were considered to be significantly different when $p < 0.05$.

3. **Results**

3.1 **Phytochemical constituents**

Phytochemical assays of the ethanol extract of *T. sinensis* bark revealed the presence of flavonoids, tannins, phenolic hydroquinones, saponins, and alkaloids, whereas the hot water extract tested positive for the presence of flavonoids, phenolics hydroquinones, and saponins (Table 1). The average yields of the ethanol and hot water extracts of *T. sinensis* were 4.8 and 2.6% w/w, respectively.

3.2 **α-Glucosidase inhibition**

The effect of *T. sinensis* bark extracts against α-glucosidase was evaluated and the results, expressed as IC$_{50}$ values, indicated that ethanol extract possessed a high potency with an IC$_{50}$ value of 600 ng/ml. This value is higher than that of the hot water extract (3.60 µg/ml). Nevertheless, neither extract was better than acarbose (positive control), which gave a value of 80 ng/ml (Table 2).
3.3 Body weight and food intake

Generally, body weights of rats in all treatment groups increased in the adaptation period (day 14 to day 0) as shown in Figure 1. After fourteen days of induction (day 0 to day 14), the body weight decreased in all groups except in the normal group. However, the food intake (g/rat/day) of diabetic rats increased after treatment (data not shown).

3.4 Blood glucose level

Blood glucose level measurement was started on the day 4. Three days after treatment induction (day 7), blood glucose levels in groups B, C, D, and E had decreased by 16.6%, 30.4%, 32%, and 11%, respectively (Figure 2). Blood glucose levels were measured on day 10 or 14 after induction in groups B, C, D, and E had decreased by 56.2%, 69%, 71%, and 52%, respectively.

3.5 Histological studies

The histochemistry of different pancreases of each group is shown in Figure 3. In group A, the histological section showed whole cells and normal tissues. In the hyperglycemia group (B), hemorrhaging was observed in the islets of Langerhans. Acinar cell nuclei showed lysis, while in group C (glibenclamide group), acinar cells were still normal. Also in group C, Langerhans cells appeared normal in size but their nuclei were partially damaged. The sections of group D showed a more normal appearance, as indicated by the presence of normal blood vessels and acinar cells. However, at 300 mg/kg BW (group E) acinar cells were necrotic, and hemorrhaging occurred in the islets of Langerhans.
4. Discussion

Ethanolic and hot-water extracts of *T. sinensis* contained flavonoids, triterpenoids, alkaloids, tannins, and phenols, which are all known to be bioactive antidiabetic agents (Battu et al., 2007; Nagappa et al., 2003). Wang, Yang, and Zhang (2007) reported that the phenolic compounds present in *T. sinensis* are gallic acid and its derivatives, gallocatechins, and flavonoids (especially quercetin and rutin). Alkaloids, flavonoids, terpenes, and anthraquinones have all been found to have a role in inhibiting α-glucosidase activity (Chen, Luo, Cui, Zhen, & Liu, 2000; Kunyanga et al., 2011; Luo, Wu, Ma, & Wu, 2001). The phytochemical analysis also showed a slight difference in secondary metabolites of extracts from Indonesia and other countries. The difference lies primarily in the variety of secondary metabolites produced by the plant. This is probably due to variation in the environmental and geographic condition among the countries (Figueiredo et al., 2008).

The α-glucosidase inhibitory activity of *T. sinensis* extracts might be caused by the phytochemical constituents in the extracts. Yin, Zhang, Feng, Zhang, and Kang (2014) reported that flavonoids, terpenes, quinones, and phenols all have antidiabetic activity. Recent studies have determined that flavonoid compounds such as xanthones, flavanones, flavans, anthocyanins, and chalcones have α-glucosidase inhibitory activity (Ichiki et al., 2007; Jong-Anurakkun, Bhandari, Hong, & Kawabata, 2008; Kato et al., 2008; Lee, Lin, & Chen, 2008; Seo et al., 2007; Zhang & Yan, 2009). Another report on antidiabetic activity indicated that phenolic compounds such as stilbenoids had an α-glucosidase inhibitory potency (Lam, Chen, Kang, Chen, & Lee, 2008). According to Zhao, Zhou, Chen, and Wang (2009), the most effective compound from *T. sinensis* that can act as an antidiabetic agent is gallic acid, followed by procyanidin and catechin.
The ethanol extract had a smaller IC\textsubscript{50} than the hot water extract, implying that some secondary metabolites in this extract interacted with the $\alpha$-glucosidase. Since the enzyme mainly consist of protein, it is believed that tannins in the ethanol extract decrease enzymatic activity as a result of enzyme complexation. In addition, some studies also suggested that tannins could be acted as potential inhibitors (Adamczyk, Simon, Kitunen, Adamczyk, & Smolander, 2017). This result guided us to examine the ethanol extract in \textit{in vivo} experiment.

The animal experiment examined food intake, blood glucose level, and histopathology. The body weight increase during the adaptation period suggests that rats in all treatments were normal and healthy during the ongoing adaptation. In this study, treatments were conducted over a period of fourteen days because the effect of alloxan is best observed in the first two weeks after induction. After induction, although the body weight of rats decreased, their food intake remained the same. Further, blood glucose level increased after alloxan-induction treatment indicating that the rats were diabetic. Zajac, Shrestha, Patel, and Poretsky (2010) reported that the general characteristics of diabetes Type 1 were an increased food intake, a decreased body weight, and damage to the pancreas, suggesting that the diabetic condition in rats could improve the food efficiency ratio (weight gain/food intake) by reducing food intake and decreasing the body weight (Sheng et al., 2017).

The reduced blood glucose levels in the glibenclamide group showed the effect of glibenclamide treatment. Glibenclamide specifically acts on pancreatic $\beta$ cells, increasing insulin secretion. It binds to the transmembrane complex consisting of sulfonylurea receptors in the liver (SUR1) and ATP-sensitive potassium ion channels. This process will close the channels, causing membrane depolarization, opening of the
calcium channels, and an increase in the concentration of intracellular free calcium. Increased calcium levels trigger the activation of proteins regulating insulin secretion in the pancreas. Sufficient amounts of the insulin can lower blood sugar levels by inhibiting endogenous glucose production and increasing glucose uptake in insulin-sensitive tissues (Krentz & Bailey, 2005; Obici & Martins, 2010).

Blood glucose assays showed that the decreased blood glucose levels caused by treatment with ethanol extract at a dose of 150 mg/kg BW was greater than at a dose of 300 mg/kg BW. Administering the bark extract at a dose of 300 mg/kg BW was less optimal ($p < 0.05$). The presence of a pro-oxidant effect arising from administering large amounts of antioxidant is suspected to cause decreased blood glucose levels (Maddux et al., 2000). However, in the 150 mg/kg BW and glibenclamide 0.25 mg/kg BW groups the amount of decrease was the same. Accordingly, 150 mg/kg BW was concluded to be less effective than glibenclamide. This also suggested that only small compounds in the crude extract contribute to the antidiabetic activity. Here, quercetin from T. sinensis leaves exhibits significant antihyperglycemic and liver cell-protective effects in a high-carbohydrate/high-fat diet in an alloxan-induced mouse model of diabetes.

According to Eliakim-Ikechukwu and Obri (2009), alloxan selectively destroys β cells in the islets of Langerhans, inducing type 1 DM. In the glibenclamide group, acinar and Langerhans cells appeared normal. However, the Langerhans nucleus was partially damaged. The presence of some β cells in this group indicated that glibenclamide has anti-hyperglycemic activity in alloxan-induced diabetic rats by stimulating insulin secretion (Rao, Sudarshan, Rajasekhar, Nagaraju, & Rao, 2003).
Histopathological sectioning also showed that the administration of bark extract at a
dose of 150 mg/kg BW provided the best treatment effect on the pancreas of rats
compared to the glibenclamide and the 300 mg/kg groups. Acinar cell necrosis and
hemorrhaging of the islets of Langerhans at 300 mg/kg BW might be due to damage
caused by the pro-oxidant activity. Yang et al. (2006) reported that \textit{T. sinensis} extracts
could generate reactive oxygen species, especially hydrogen peroxide, a potent pro-
oxidative agent.

5. Conclusion

In conclusion, this study revealed that both ethanol and hot water extracts of \textit{T.
sinesis} contain phytochemical substances related to reported antidiabetic agents. Our
findings also demonstrate that \textit{T. sinensis} bark has antidiabetic activity. Further
investigation is required to identify the bioactive compounds responsible for this
activity. In addition, a toxicological analysis is needed for further development.

References

and their complex interaction with different organic nitrogen compounds and
doi:10.1002/open.201700113

Bansal, P., Paul, P., Mudgal, J., Nayak, P. G., Pannakal, S. T., Priyadarsini, K. I., &
effects of the flavonoid rich fraction of \textit{Pilea microphylla} (L.) in high fat
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Figures

Figure 1. Body weight of rats before and after treatment with ethanolic extracts of *T. sinensis* bark. Data are presented as mean ± SEM. A, normal; B, hyperglycemia; C, glibenclamide (0.25 mg/kg BW); D, extract 150 mg/kg BW; E, extract 300 mg/kg BW. * p < 0.05 (ANOVA).
Figure 2. Blood glucose levels after 0–14 days of treatment with ethanolic extracts of *T. sinensis* bark. Data are presented as mean ± SEM. A, normal; B, hyperglycemia; C, glibenclamide (0.25 mg/kg BW); D, extract 150 mg/kg BW; E, extract 300 mg/kg BW.

* * p < 0.05 (ANOVA).
Figure 3. Histopathological sections of rats' pancreases after different treatments. Data are shown at a magnification of 40×. (A) Normal: (1) islets of Langerhans, (2) blood vessel. (B) Hyperglycemia: (2) blood vessel (congestion), (3) acinar cell (necrosis). (C) Glibenclamide: (4) infiltration of inflammatory cell. (D) Extract 150 mg/kg BW: (1)
islets of Langerhans, (2) blood vessel. (E) Extract 300 mg/kg BW: (1) islets of Langerhans (hemorrhage), (2) acinar cell (necrosis).
Table 1. Phytochemical constitutes of T. sinensis bark extracts.

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<td>Alkaloids</td>
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<td>−</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic hydroquinone</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>−</td>
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(+): Positive; (−): negative.

Table 2. Inhibitory effect of T. sinensis bark extracts and acarbose against α-glucosidase.

<table>
<thead>
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<th>IC_{50} (µg/ml)</th>
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<td>70% ethanol extract</td>
<td>0.60^{a}</td>
</tr>
<tr>
<td>hot water extract</td>
<td>3.60^{b}</td>
</tr>
<tr>
<td>acarbose</td>
<td>0.08^{c}</td>
</tr>
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</table>

^{a,b,c}: The different letter on the IC_{50} values indicates statistical significance (p > 0.05).