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In vitro and Hydroponic Hairy Root Culture of *Urena lobata* to Obtain α-glucosidase Inhibitory Materials

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ABSTRACT
The number of people in the world with diabetes has increased dramatically over the recent year. The treatment of type II diabetes is complicated by several risk factors inherent to the disease. Elevated postprandial hyperglycemia (PPHG) is one of the risk factors. One important approach for the treatment of the disease is decreasing the PPHG. It is possible to inhibit certain carbohydrate hydrolyzing enzymes like α-glucosidase. U. lobata has been used as traditional medicine due its capability of decreasing blood glucose level. The objective of this study is to evaluate in vitro α-glucosidase inhibitory activity of the ethanol extract of U. lobata hairy root: in vitro and hydroponic culture hairy root for getting α-glucosidase inhibitory materials actively. This research demonstrated that the ethanol extract of hydroponic hairy root shows significantly stronger α-glucosidase inhibitory activity than stem, leaf and in vitro hairy root (6.76 µg/ml). It's noteworthy that, it has the same value of IC_{50} (4.88 µg/ml) as natural root (4.79 µg/ml). In in vitro culture, hairy root was formed by co-culturing with Agrobacterium rhizogenes ATTC 15834 after 7 days and their biomass get the highest weight on the 25th-day in liquid medium. In hydroponic culture, hairy root was induced at the hypocotyl as same as in vitro culture of 15-day-old plantlets and using aeroponic system instead of the classical one. In summary, we have demonstrated that α-glucosidase inhibition may possibly be the mechanism by which extract exerts antidiabetic activity. The success of hairy root culture method in our study shows a new potential to produce α-glucosidase inhibitory materials.
Keywords: *α-glucosidase, A. rhizogenes, hairy root culture, hydroponic, U. lobata*

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INTRODUCTION

U. lobata (Malvaceae) has been used as a traditional drug for treating flu or inflammation. Moreover, many recent studies reported that its root extract can reduce the blood glucose level in diabetic rats 22 or also reduce the body weight and fasting blood sugar of treated rabbits 4. However, there have not any previous report either presenting *in vitro* α-glucosidase inhibitory activity of *U. lobata* or using hairy root culture *U. lobata* to produce antidiabetic materials.

Diabetes mellitus is a chronic disorder of metabolism caused by an absolute or relative lack of insulin. It is characterized by hyperglycemia (high blood sugar) in postprandial and/or fasting state, and its severe form is accompanied by ketosis and protein wasting 5. It is also associated with a number of complications like retinopathy, neuropathy and peripheral vascular insufficiencies 7. Glucose homeostasis is the key for the treating diabetes. The treatment of type II diabetes is complicated by several risk factors inherent to the disease. Elevated postprandial hyperglycemia (PPHG) is one of the risk factors 12. PPHG is elevated by the action of α-glucosidase and α-amylase. Inhibition of these enzymes plays a major role in managing PPHG in diabetic patients. Inhibition of α-glucosidase enzyme activity leads to a reduction in disaccharide hydrolysis which has beneficial effects on glycemic index control in diabetic patients 213.

For 25 years, hairy root has been investigated as a biological system for the production of valuable compounds from medicinal plants. A better understanding of the molecular mechanism of hairy root development, which is based on the transfer T-DNA of *A. rhizogenes* into the genome of plant, has facilitated its increasing use in metabolic engineering 30. Hydroponic farming is becoming a favorable method to produce crops, plants and flowers all year around. Medicinal herbs cultivation is widespread and the interest for this kind of cultures is growing on fast pace. Harvesting the raw material from the wild can be very difficult due to the little control one can have dealing with problems like:

misidentification, genetic and phonotypical variability, active substances variability and toxic compounds. Cultivating the medicinal plants in a more controlled environment can overcome these difficulties. Hydroponic culture is getting very popular because of the results showed in recent practices. Production of medicinal herb and root crops in controlled environments provides opportunities for improving the quality, purity, consistency, bioactivity, and biomass production of the raw material. Producers of medicinal plants are also attracted to hydroponic systems for cultivation because recent studies proved that growing in soilless cultures in protected environments results in higher concentrations of active principles found in plants comparing with traditional soil cultivation because of the total control the grower can have 13. Due to challenges that are encountered in conventional soil-based agriculture the rapidly progressing technology may develop a trend to depend on this hydroponic technique for efficient utilization of natural resources including medicinal plants in the future 19.

This research focus on deciphering the ability of *U. lobata* on treating diabetes type II via the inhibition of α-glucosidase activity and how to apply hairy root technique on getting antidiabetic materials actively: *in vitro* and hydroponic hairy root culture.

METHODOLOGY

Preparation of Ethanol Extract

The leaf, stem and root of *U. lobata* were used. The plants were locally collected in district 9, Ho Chi Minh city, Vietnam. The coarse material was weighed, labeled and submitted to Plant Biotechnology laboratory, district 5, HCM city, Vietnam for extraction. The coarse powder of leaf, stem and root of *U. lobata* was added to a 1 L bottom flask. To above settings, 500 ml of ethanol was added and the mixture was applied maceration technique. Finally the mixture was filtered and the extract was collected by using a reflux consenter at 65°C for about 1 h.

Chemicals

α-glucosidase and *p*-nitrophenyl-α-D-glucopyranoside (PNPG) were purchased from Sigma, America. Acarbose (positive control) was purchased from Glucobay Company. All other solvents and chemicals were of analytical grade.

α-glucosidase Inhibitory Activity of Ethanol Extract of U. lobata

The α-glucosidase inhibitory activity was assessed by the standard method as described by Know, Apostolidis, and Shetty 10, with slight modifications. Briefly, a volume of 50 µl of sample solution and 40 µl of 0.1 M phosphate buffer (pH 6.8) containing α-glucosidase solution (0.2 U/ml) was incubated in 96 well plates at 37°C for 20 min. After pre-incubation, 40 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) was added to each well and incubated at 37°C for another 20 min. Then the reaction was stopped by adding 130 µl of 0.2 M Na_2CO_3 into each well, and absorbance readings (A) were recorded at 405 nm by micro-plate reader and compared to a control which had 50 µl of buffer solution in place of the extract. For blank incubation (to allow for absorbance produced by the extract), enzyme solution was replaced by buffer solution and absorbance recorded. The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows:

$$\%\ \text{Inhibition} = \frac{Aco - At}{Aco} \times 100$$

Where, A_{co} is absorbance of the control and A_t is absorbance of the sample. The concentration of inhibitors required for inhibiting 50% of the α-glucosidase activity under the assay conditions was defined as the IC_{50} value.

Preparation of A. rhizogenes

A. rhizogenes ATTC 15834 was obtained from Microbial Type Culture Collection Centre (MTCC), Japan. *A. rhizogenes* strain ATCC 15834 was grown in nutrient broth medium (beef extract 3 g/l, peptone 5 g/l, pH 7.0) for 48 h with shaking (110 rpm) at 25 ± 1°C.

Plant Material

In vitro: seeds of *U. lobata* were washed with 70% alcohol for few seconds and rinsed three times with distilled water. Then the seeds were brought to surface sterilized with bleach solution (1 NaOCl : 2 distilled water) for 20 min and again rinsed with sterile distilled water for 3 to 5 times. Disinfected seeds were placed individually on tubes containing MS (Murashige and Skoog, 1962) basal medium. The basal medium consisted of salts and vitamins of MS medium, added 3% sucrose and solidified with 0.8% (w/v) agar. Before adding agar, the medium was adjusted to pH 5.8, and then sterilized by autoclaving at 121°C for 15 min. The seeds were germinated in growth chamber at 20-25°C under standard cool white fluorescent tubes with a 16 h photoperiod.

Hydroponic: both hydroponic experiments were obtained from March 1st to June 30th. Seeds were germinated on soil tray in the greenhouse for 15 days before transferring to hydroponic systems. In classical hydroponic culture, temperature was maintained between 25 and 28°C by a cooling system. The

plants grew under natural light conditions without additional light supply; 2 – 3 cm each particle clay was used as substrate. The plant density was 20×20 cm, put in closed container (50 cm height, 40 cm width, 60 cm length) with a total of 10 plants per container. Nutrient solution (pH 5.5 – 6, EC=Ms cm^{-l}, NO_3^-, SO_4^{2-}, $H_2PO_4^-$, Cl^-, K^+, Ca^{2+}, Mg^{2+}, NH_4^+, Na^+ and micronutrients) were supplied at the first day and diluted follow the ratio (0.8 L nutrient : 19.2 L water). Fungicide and insecticide treatments were applied when appropriate. In aeroponic culture, this technique was performed in the same greenhouse, plant density and containers. Water and nutritive elements were supplied with the same nutrition solution as in the hydroponic culture. For this purpose four fog nozzles with a spraying capacity of 1.8 L/min located at the bottom of the containers sprayed nutrient solution every 10 minutes for 30 s using a timer into the inner part of the box in order to keep the root wet. The same phytosanitary treatments were applied as in the aeroponic system.

Establishment of Hairy Root Cultures

In vitro: from 15-day-old aseptically germinated seedlings, the leaf, stem and root segments were excised and cut into small pieces (1 cm). These segments were soaked in the bacterial suspension (OD_{600} = 0.6) for 20 min for infection. Their segments were injured all over the surface to facilitate the infection process before treatment with *A. rhizogenes*. After five days of co-cultivation, the explants were transferred to hormone-free medium containing MS salt and vitamins, 30 g/l sucrose, added 250 mg/l cefotaxime to remove growth of *A. rhizogenes* on the surface of the explants, and maintained at 25°C under continuous darkness for hairy root induction. Within two weeks, numerous hairy roots had emerged from the wounded sites. After 30 days, the number of responsive explants and number of hairy roots/explant were recorded and the data were calculated. About 0.2 g fresh roots were inoculated into 250 ml Erlenmeyer flask containing 20 ml MS liquid medium, cultured in shaker at 80 rpm and 25 ± 1°C for 30 days.

Hydroponic: 15 days after sowing, *U. lobata* plants were cut off their roots, made injuries at hypocotyl and then soaked in the bacterial suspension (OD_{600} = 0.6) for 30 min for infection. After five days of co-cultivation, they were washed with distilled water added 250 mg/l cefotaxime to remove growth of *A. rhizogenes* on the surface of the hypocotyl and then were transferred into hydroponic systems.

After 30 days, both biomass of the *in vitro* and hydroponic hairy root were collected and extracted in ethanol by the process in paragraph 2.1.

Detecting the Rooting Genes (PCR Analysis)

The *A. rhizogenes* transferred genes *rolB* were used as targets for PCR analyses in *U. lobata* hairy roots. Genomic DNA was isolated from 100 mg of *U. lobata* hairy root cultures *in vitro* and hydroponic, 100 mg of 15 day-old *U. lobata* seedling root *in vitro* and hydroponic tissue using CTAB/PV method described by Stewart and Via (1993) 32. DNA was extracted from non-transformed roots as the negative control. Plant genomic DNA was visualized after electrophoresis in 1.5% agarose gels. Ri-plasmid of *A. rhizogenes* was isolated by method described by Curier and Nester (1976) 9 and Shoja 28 to do the positive control. Primers for *rolB* gene were F-*rolB* (5'- GCTCTTGCAGTGCTAGATTT-3') and R-*rolB* (5'-GAAGGTG CAAGCTACCTCTC-3'); The expected amplified fragment sizes were 423 bp for *rolB* gene. PCR reaction was carried out in total volume of 25 µl reaction mixtures: 1 µl plant genomic DNA (or Ri plasmid DNA), 1 µl dNTPs 2 mM, 2 µl DreamTaq DNA polymerase (0.5 unit/µl), 5 pmol of each primer, 2.5 µl DreamTaq buffer, adding enough water. Conditions for *rolB* amplification were as follow: initial denaturation at 95°C for 5 minutes, 35 cycles of amplification (95°C for 30 s, 54°C for 30 s and 72°C for 60 s) and 5 minutes extension at 72°C. PCR amplification was visualized after electrophoresis in 1.5% agarose gels.

Data Processing and Statistical Analysis

Analyses of variance were performed using SAS, Proc. GLM (SAS Institute, 1989).

RESULT AND DISCUSSION

In vitro α-glucosidase Inhibition Assay

α-glucosidase is known as one of the key enzymes in the human digestive system to degrade starch to monosaccharide and cause the rise in blood glucose 15. To investigate the capability of reduction of diabetes risk of *U. lobata*, the ethanol extracts of root, stem and leaf were examined for α-glucosidase inhibitory activity. The root extract showed the highest activity (IC_{50} = 4.72^dµg/ml ± 0.12 µg/ml) and followed by the stem (IC_{50} = 56.47^cµg/ml ± 2.83 µg/ml), leaf extract (IC_{50} = 113.12^bµg/ml ± 18 µg/ml) comparing with the positive control acarbose (IC_{50} = 441.73^aµg/ml ± 148.75 µg/ml). The inhibition of α-glucosidase in different concentration is showed in Fig. 1. The results found that all of the studied samples showed inhibitory activity against α-glucosidase.

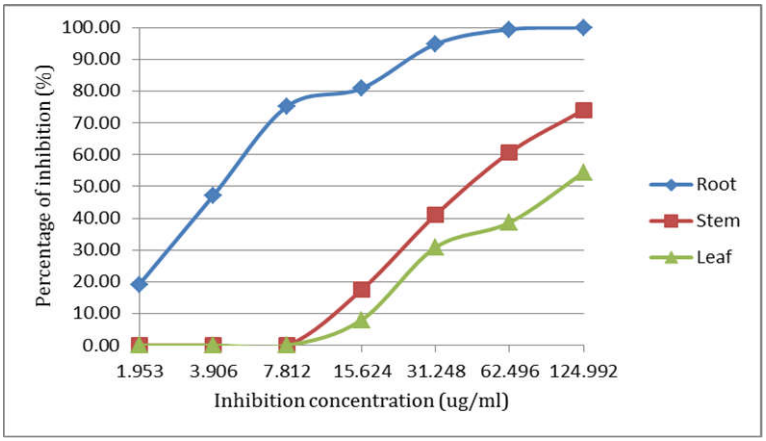

Fig. 1. α-glucosidase (0.2 U/ml) inhibitory of ethanol extract of root, stem and leaf in different concentrations.

There were similar studies of α-glucosidase inhibitory activity in different plants: the IC_{50} of *Centaurea calcitrapa* was 4.38 mg/ml 23, the leaf extract of *Belamcanda chinensis* was 800 µg/ml 8, the ethanol extract of *Andrographis paniculata* was 17.2 mg/ml 24. The IC_{50} of *U. lobata* is the lowest of these plants in previous studies. Root extract of *U. lobata* has been used as herbal medicine to treat such diverse ailment as colic, malaria, gonorrhea, fever, wounds, toothache, and rheumatism 11. Its water extract was found to reduce the blood glucose levels in diabetic rats 22, and reduced the body weight and fasting blood sugar of treated rabbits 11. These facts support the high α-glucosidase inhibition ability of the ethanol extract of *U. lobata*. Among the three, the root extract of *U. lobata* presented high potential for application as an antidiabetic medicine product.

Establishment of Hairy Root Culture

In vitro culture

In *in vitro* culture, hairy roots were induced from root, stem and leaf explants of *U. lobata* when infected with *A. rhizogenes*. The percentage of responsive explants and number of hairy root per explants were varied in different segments (Table 1). The hairy root from root, stem and leaf segments were thin with faster growth rate and they also have branches unlike normal roots. The negative control segments did not induce any root from injuries. The difference in root length and number of roots per kind of explants are showed in Fig. 2. The results indicated that the leaf of *U. lobata* was the best material for inducing hairy root with the most sensitive response and the highest number of roots per explant. All kinds of segments had hairy root induction over 50%, so we used them to culture in liquid medium.

Table 1: Percentage of Responsive Explants And Number Of Hairy Root/Explants Was Varied In Different Segments

Segments	Percentage of response ± IQR (%)	Number of roots per ex-plant ± IQR (root)
Root	21.67^{b} ± 2,89	2.00^{b} ± 1,00
Stem	39.33^{b} ± 1,15	4.33^{b} ± 1,15
Leaf	93.33^{a} ± 11,55	9.72^{a} ± 1,18

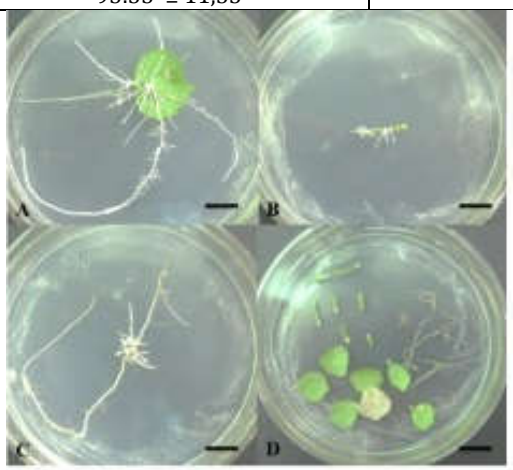

Fig. 2. The difference of root length and number of roots per kind of explants (A: leaf, B: stem, C: root, D: control).

Hairy root from root, stem and leaf were grown in 20 ml of MS liquid medium. During incubation, the hairy root changed gradually from white to brown, and some brown substance was secreted from them into the medium after 3 – 4 weeks of culture. The growth pattern of *U. lobata* hairy root revealed that it grew slowly on the first 10 days of culture (Fig. 3). However, the hairy root grew faster subsequently, and the fastest growth happened during days 15 – 25. After 25 days of culture, the growth rate began to slow down, but the biomass of the hairy root still increased until day 30th of culture.

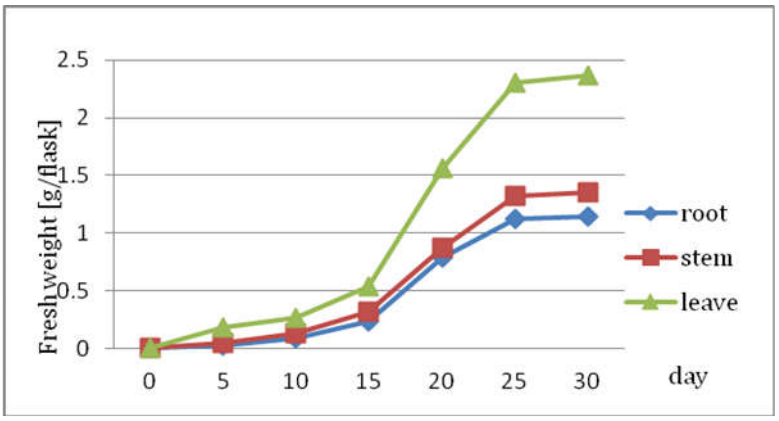

Fig. 3. Time course of the growth of *U. lobata* hairy root from root, stem and leaf.

These results were similar to those of previous studies on hairy root cultures of *P. phaseoloides* 27. In log – phase, the biomass of hairy root from leaf was the highest (Δ_m = 2.310 g FW), followed by that from stem (Δ_m = 1.324 g FW) and root (Δ_m = 1.128 g FW) (Fig. 4). At the same time, *U. lobata* hairy roots grew faster than *in vitro* root, so *in vitro* hairy root culture in liquid medium is a good technique for getting antidiabetic materials actively.

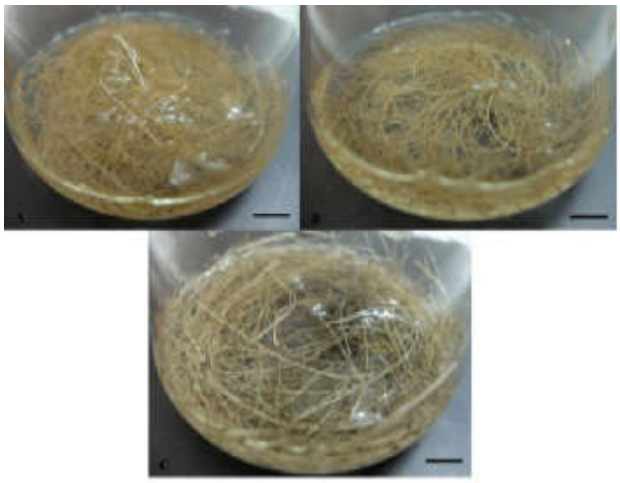

Fig. 4. Hairy root of leaf (A), root (B) and stem (C) after 30 days culture *in vitro*.

Hydroponic culture

10-, 15- and 30-day-old *U. lobata* were induced with *A. rhizogenes* to examine the dedifferentiation ability of plant. The percentage of responsive plants, days of forming roots, fresh weight (FW) and dry weight (DW) of root were varied in different ages (Table 2).

Table 2: Transgenic Ability and Root Biomass Of *U. Lobata*'s Different Ages after 30 Day Culture

Age (day)	Timing of rooting ± IQR (day)	Rooting percentage ± IQR (%)	FW ± IQR (g)	DW ± IQR (g)
10	3.000^{b} ± 0.000	100.000^{a} ± 0.000	69.120^{b} ± 3.330	2.193^{c} ± 0.213
15	3.000^{b} ± 0.000	100.000^{a} ± 0.000	95.079^{a} ± 3.611	4.261^{a} ± 0.275
30	4.666^{a} ± 0.577	73.333^{b} ± 5.774	90.629^{a} ± 2.195	3.800^{b} ± 0.060

The hairy root from 10- and 15-day-old plantlets was formed faster than the 30-day-old one. However, the root biomass of the 10-day-old plantlets was fewer than the 15- and 30-day old one. Moreover, the 15- was more productive in term of transgenic ability than the 30-day-old. The results indicated that 15-

day-old *U. lobata* has more potential in root biomass production than the others. Nin *et al.* (1997) have reported that specificity of *Agrobacterium* transformation is closely connected with the age and hormonal balance of the host plant 20.

15-day-old *U. lobata* after forming hairy root was also cultured in different hydroponic systems. The influence of the cultivation system was highly significant for all analyzed yield parameters (Fig. 5).

Fig. 5. Root biomass after 30 days culture in A: aeroponic and B: classical hydroponic.

Compared with the total product in classical hydroponic (43.607^{b} g FW and 1.989^{b} g DW), the total root biomass per container in the aeroponic system (111.198^{a} g FW and 11.311^{a} g DW) was over 2.5 fold higher in FW and over 5.6 fold higher in DW. Root grown in aeroponics showed an increased amount of biomass due to high humidity conditions in the system 25. Moreover, aeroponic technique optimizes root aeration which is certainly the major factor leading to an increasing yield compared with classical hydroponic systems 29. Soffer *et al.* (1988) indicated that dissolved O_2 is essential to root formation. Oxygen affected the timing of rooting, rooting percentage, number of roots, and root length 29. In the same result, characterization of plant growth and development showed that several species such as *Tanacetum parthenium*, *Achillea millefolium*, *Taraxacum officinale* and *Calendula officinalis* were well adapted to greenhouse hydroponic growing condition and provide a high quality product per square meter in a short time period 14.

Detecting the Rooting Genes (PCR Analysis)

Further confirmation of transgenic status of the tissue was done by the PCR amplification of the DNA isolated from the hairy roots using reverse primers of *rolB*. *A. rhizogenes* (colony PCR) served as the positive control and DNA from the non-transformed seedlings root served as the negative control. All transformants showed presence of the *rolB* (423 bp) in DNA amplified product (Fig. 6).

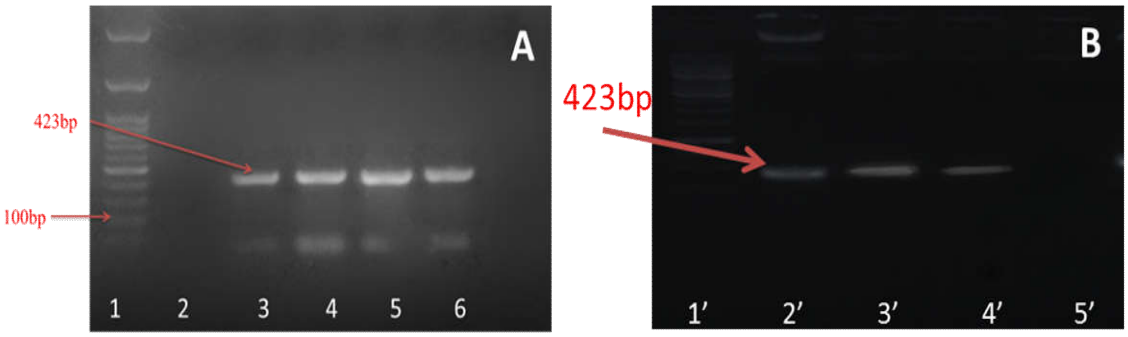

Fig. 6. PCR amplification of 423 bp fragment of the rolB gene. A: Lane 1 = molecular weight marker (100 bp ladder); lane 2 = DNA from in vitro non-transformed root (negative control); lane 3 = A. *rhizogenes* ATTC 15834 DNA (positive control); lane 4, 5, 6 were DNA transformed root from segments: root, stem and leaf in vitro, respectively. B: Lane 1'= molecular weight marker (100 bp ladder); lane 2'= A. *rhizogenes* ATTC 15834 DNA (positive control), lane 3', 4'= DNA transformed root from hydroponic hairy root; lane 5'= DNA from hydroponic non-transformed root (negative control).

There have not been many previous studies working on hairy root of *U. lobata*. However, Stephanie Moss *et al.* (2006) had demonstrated *Gossypium hirsutum* and *G. barbadense* (as same as family of *U. lobata*) were infected with *A. rhizogenes* ATTC 15834 produced hairy roots 31. The hairy root induced from root, stem and leaf segments *in vitro* and hairy root induced from hydroponic hypocotyl may be used for obtaining transgenic *U. lobata* plants.

In vitro α-glucosidase Inhibition Assay of Hairy Root

The ethanol extracts of *U. lobata* hairy root were examined for α-glucosidase inhibitory activity to investigate their properties on reduction of diabetes risk. The natural root (IC_{50} = 4.79^{b}μg/ml ± 0.11 μg/ml) and hydroponic hairy root (IC_{50} = 4.88^{b}μg/ml ± 0.04 μg/ml) extract showed higher activity than *in vitro* hairy root (IC_{50} = 6.76^{a}μg/ml ± 0.07 μg/ml). The results found that hairy root extract showed inhibitory activity against α-glucosidase (Table 3; Fig. 7).

Table 3: α-Glucosidase Inhibitory Of Three Kind Of Root In Different Concentration

Inhibitory concentration (μg/ml)	Percentage of inhibition (%)			
	0.122	**1.953**	**3.906**	**7.813**
R	3.53	18.82	47.06	77.06
I	14.12	15.29	34.12	54.71
E	9.41	18.24	47.65	75.29

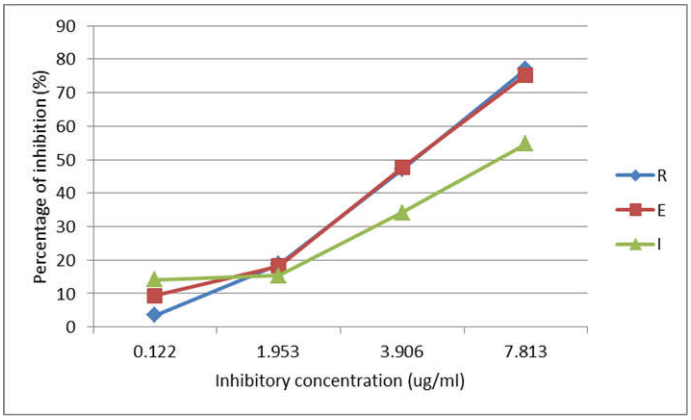

Fig. 7. α-glucosidase inhibitory (0.2 U/ml) of extract of natural root (R), hairy root *in vitro* (I) and hydroponic (E) in different concentrations.

Onoagbe *et al.* demonstrated water extracts of the roots of *U. lobata* significantly depressed blood glucose concentration in the diabetic rats throughout the duration of the investigation (1-6 weeks) relative to untreated diabetic rats and also phytochemical screening showed the presence of steroidal/triterpenoidal saponins 22. Moreover, there were many studies indicated that *U. lobata* had a lot of flavonoids such as: kaempferol, quercetin, tiliroside 2, in ethyl acetate portion and n-butanol portion of a 95% ethanol extract of branches and leaves found ten flavonoid compounds: kaempferol, rutin, quercetin, afzelin, astragalin, tiliroside, crenuloside 26. Some specific saponins and flavonoid were demonstrated for diabetes type II treatment that utilizes the ability to reduce the risk of postprandial hyperglycemia. Saponin presented in *Terminalia catappa* L. was demonstrated having strong α-glucosidase inhibitory activity 1, or quercetin (a flavonoid) was known as a α-glucosidase inhibitor with multi-targeted therapeutic potential in relation to diabetic complications 18. In previous studies, hairy root was able to produce more content of metabolite or new secondary metabolites so it can be a potential medicine resource. Another study reported that *Panax ginseng* hairy roots synthesized the same saponins, as those of the native root, up to about 2.4 times in the quantity, and up to about 2 times in comparison with that of the ordinary cultured root, on dry weight basis 33. Furthermore, Zheng *et al.* indicated that the growing conditions can be strictly controlled in greenhouse hydroponic culture to produce highly standardized plant material for the industrial extraction of bioactive compounds 34. Soilless cultivation also allows regulating the secondary metabolism involved in the accumulation of pharmacological active principles through appropriate manipulation of the nutrient solution fed to the plants 6. Therefore, bioactive compounds such as saponin or flavonoid may increase their content in *U. lobata* hairy root which is the potential α-glucosidase inhibitory material.

CONCLUSION

To the best of our knowledge, this work is the first report of the α-glucosidase inhibitory activity, the *in vitro* and hydroponic cultures of hairy root of *U. lobata*. These induced hairy roots may be served as an alternative source of anti-diabetic material with high α-glucosidase inhibitory activity and also by applying *in vitro* and hydroponic culture techniques more root biomass can be obtained in the short time period. By this method, α-glucosidase inhibitory materials can be produced initiatively.

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